Abstract
This thesis aims at investigating novel and rational strategies to identify secondary metabolites from plant or microbial origin by targeted isolation; either guided by HPLC-bioactivity profiling or by metabolomics. Firstly, a microfractionation strategy was elaborated for mass-limited extracts that is compatible with the detection and identification of the purified compounds by microflow NMR as well as in vivo zebrafish bioassays. This approach revealed several flavanoids that explain the antiangiogenic activity of the Rhynchosia viscosa extract. Secondly, strategies were developed for the analysis of microorganism co-cultures. Microfractionation led to the isolation of a co-culture induced compound in the low microgram range from the co-culture of Acremonium strictum and Fusarium oxysporum. For the co-culture of Hohenbuehelia reniformis and Fusarium solani, multivariate data analysis highlighted metabolites responsible for the differences between pure culture and co-culture extracts. A two-step chromatographic purification permitted the targeted isolation of highlighted metabolites – u.a. several novel and [...]
Advanced Strategies for the Study of Bioactive Plant Constituents and Co-culture Induced Fungal Metabolites

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pour obtenir le grade de Docteur ès sciences, mention interdisciplinaire

par

Nadine Bohni

de Basel-Stadt (BS) et Zunzgen (BL)

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La Faculté des sciences, sur le préavis de Monsieur J.-L. WOLFENDER, professeur ordinaire et directeur de thèse (Section des sciences pharmaceutiques), Monsieur N. LANGE, professeur associé (Section des sciences pharmaceutiques), Madame K. GINDRO, docteure (Agroscope, Changins, Suisse), Madame D. TASDEMIR, professeur (National University of Ireland, Galway, Ireland) et Madame E. SCHMITT, docteure (Natural Products Unit, Novartis, Basel, Switzerland), autorise l’impression de la présente thèse, sans exprimer d’opinion sur les propositions qui y sont énoncées.

Genève, le 17 juin 2014

Thèse - 4695 -

Le Décanat

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".
“There are more things in heaven and earth, Horatio,
Than are dreamt of in your philosophy.”

Hamlet
Natural products remain an invaluable source for drug discovery since nature’s imagination on the structural diversity of chemical compounds is beyond reach. In addition, plant and microbial secondary metabolites have proven successful as drugs or drug leads for a number of diseases, e.g., against inflammation and cancer or against human and plant pathogens as fungi and yeasts.

The use of natural products for drug discovery is hampered by time-consuming purification processes for the isolation of bioactive molecules from complex natural matrices, as well as various drawbacks related to compatibility with up-to-date bioassays and high-throughput screening. Moreover, the bottleneck for identification or structure elucidation of the isolated compounds is detection by NMR that is limited by its intrinsic insensitivity compared to other detectors such as photo diode array or mass spectrometry. Recent advances in probe technologies, however, make NMR detection now accessible for low microgram amounts of sample. Such progress favorably impacts the scale at which bioactive natural products can be discovered and thus accelerates the drug discovery process. In this context, this thesis aims at investigating novel and rational strategies to identify secondary metabolites from plant or microbial origin. This is achieved by targeted isolation, either guided by HPLC-bioactivity profiling or by metabolomics.

In the first part of the thesis, a microfractionation strategy was elaborated for mass-limited extracts that is compatible with the detection and identification of the purified compounds by microflow NMR, and, at the same time, with modern in vivo zebrafish bioassays. The procedure was extended to include direct quantification by NMR with minimal sample handling for later dose-response analysis that is well adapted for microgram-amount samples. The practical application to the methanolic extract of the Tanzanian plant *Rhynchosia viscosa* revealed several known constituents and one novel compound that explain the anti-inflammatory and antiangiogenic activity of this extract.
In the second part of the thesis, the phytochemical methodologies were applied to the analysis of fungal extracts. In contrast to plant extracts, extract content from microbial sources depends largely on culture conditions. Under optimal culture conditions, microbes tend to activate only a fraction of their biosynthetic machinery. Microorganism co-culture using solid culture media has shown successful to stimulate the production of additional and bioactive secondary metabolites. Thus, a metabolomics approach was established that would highlight induced metabolites from fungal co-cultures using chemometrics. In a screening of more than 100 co-cultures of human and plant pathogenic fungal strains, chemometric results and morphological observations enabled the selection of co-cultures for in depth-analysis: *Acremonium strictum* with *Fusarium oxysporum* as well as *Hohenbuehelia reniformis* with *Fusarium solani*.

In a third part, the microfractionation strategy developed for plant extracts was successfully applied to the isolation of an induced compound in the low microgram range from the co-culture of two human pathogenic strains, *Acremonium strictum* and *Fusarium oxysporum*. Nevertheless, the boundaries of microfractionation were apparent as such small-scale cultures allowed NMR analysis of isolated compounds only for selected co-culture examples. The need for larger-scale cultures was evident due to the complex nature of most fungal extracts. These large-scale cultures would also permit the isolation of the milligram amounts of fungal constituents needed for extended biological testing.

Finally, for the identification of co-culture induced antifungal constituents, the co-culture of agricultural Basidiomycete *Hohenbuehelia reniformis* and human pathogenic *Fusarium solani* was studied. Multivariate data analysis highlighted metabolites that were responsible for the differences between pure culture and co-culture extracts. On the other hand, the observation of pigment formation upon co-culture was not revealed by MS-based metabolomics but by UHPLC-UV metabolite profiling only. For the isolation of these compounds, a large-scale co-culture was set up and a prefractionation strategy was developed that allowed the removal of sugars and apolar extract constituents. A two-step chromatographic purification permitted the targeted isolation of highlighted metabolites, among them several novel and anti-*Fusarium* quinones as well as seven pigments.
These various studies show the potential of advanced chromatographic procedures for the isolation of natural products from various matrices. Using efficient purification systems and sensitive NMR technology in combination with well-selected bioassays, milligram amounts of plant extract are now sufficient for activity profiling and decrease the time for complete phytochemical analysis substantially. As for the isolation of fungal constituents from solid medium co-culture experiments, the developed extraction, prefracionation and purification strategies make this source accessible for the analysis and isolation of secondary metabolites. This targeted isolation protocol in combination with statistical analysis of the co-culture experiments aids the evaluation of induced and bioactive constituents unreached by other means. Moreover, the characterization of such compounds gives an insight into microbial interaction.

These advanced strategies can help in tracking new and bioactive chemical entities to bring forward natural product research. The methodology of metabolite induction by co-culture might be applied to other systems to bring to light the presence and subsequent activation of cryptic biosynthetic pathways in microorganisms that are involved in the production of bioactive compounds. Cutting-edge technology such as metabolic engineering and synthetic biology can profit from this knowledge for the efficient production of next-generation’s drugs or drug leads.
**Résumé**

La nature représente une source inestimable de composés chimiques complexes et extrêmement variés développés au cours de l’évolution qui sont d’un grand intérêt pour la découverte de nouveaux médicaments. Ainsi, les métabolites secondaires issus de plantes ou de micro-organismes ont fait leurs preuves en tant que candidats médicaments ou médicaments en tant que tels pour le traitement de nombreuses maladies, par exemple, contre l'inflammation et le cancer ou contre les pathogènes humains et végétaux comme les champignons et les levures.

Le caractère fastidieux des procédés d'isolement de molécules bioactives à partir de matrice naturelle complexes, ainsi que les différents inconvénients liés à la compatibilité avec les méthodes actuelles d’essais biologiques et de criblage à haut débit, entrave l'utilisation des produits naturels pour la découverte de nouveaux médicaments. Une des difficultés principales est l'identification complète des composés isolés en faible quantité par RMN en raison du manque intrinsèque de sensibilité de cette technique spectroscopique par rapport à d'autres détecteurs, tels que la détection par barrette de diodes ou la spectrométrie de masse. Cependant, les progrès récents en technologie de sonde RMN notamment ont permis la détection de faibles quantités d’échantillon (de l’ordre du microgramme). Ce changement important de la limite de détection a un impact favorable sur l'échelle à laquelle les produits naturels bioactifs peuvent être découverts et par là même accélère le processus de découverte de nouveaux médicaments. Dans ce contexte, cette thèse vise à étudier des stratégies nouvelles et rationnelles pour identifier des métabolites secondaires de plantes ou d'origine microbienne. Dans ce but, des isolements ciblés, guidés par profilage HPLC, basés sur l’activité biologique ou par localisation de biomarqueurs en métabolomique, ont été mis en œuvre.

La première partie de la thèse présente une stratégie de micro-fractionnement élaborée pour les extraits de faible masse compatible avec la détection et l'identification des composés purifiés par RMN micro-fluidique, ainsi que des essais biologiques *in vivo* sur des poissons zèbres (*Danio rerio*). La procédure a été optimisée en incluant une quantification directe par RMN tout en réduisant au minimum les manipulations d’échantillon pour estimer correctement des réponses...
biologiques dose dépendantes pour des échantillons de l’ordre du microgramme. L’application pratique de cette stratégie à l’extrait méthanolique de la plante tanzanienne *Rhynchosis viscosa* a révélé plusieurs constituants connus et un nouveau composé responsables de l’activité anti-inflammatoire et anti-angiogénique de cet extrait.

Dans la deuxième partie de la thèse, des approches phytochimiques similaires ont été appliquées à l’analyse d’extraits fongiques. Contrairement aux extraits de plantes, la teneur en métabolites secondaires des extraits issus de sources microbiennes dépend largement des conditions de culture. Dans des conditions optimales, les microbes ont tendance à activer une fraction seulement de leur machinerie biosynthétique. La co-culture de micro-organisme en milieu solide s’est montrée efficace pour stimuler la production de métabolites secondaires bioactifs induits. Une approche métabolomique utilisant des méthodes chimiométriques a été développée afin de mettre en évidence les métabolites induits par co-culture de microorganismes fongiques. Grâce à un criblage sur plus de 100 co-cultures de souches pathogènes fongiques humaines et végétales, les résultats chimiométriques et observations morphologiques ont permis la sélection de co-cultures pour des analyses approfondies: *Acremonium strictum* avec *Fusarium oxysporum* ainsi que *Hohenbuehelia reniformis* avec *Fusarium solani*.

Dans la troisième partie de ce travail, la stratégie de micro-fractionnement développée pour des extraits de plantes a été appliquée avec succès à l’isolement d’un composé induit présent à l’échelle du microgramme à partir de la co-culture de deux souches pathogènes humaines, *Acremonium strictum* et *Fusarium oxysporum*. Néanmoins, les limites de cette méthodologie de micro-fractionnement ont été mises en évidence comme l’analyse par RMN des composés isolés était possible seulement pour des exemples sélectionnés de co-culture. En raison de la nature complexe de la plupart d’extraits fongiques, il a été nécessaire de développer des cultures à grande échelle. Celles-ci vont permettre d’isoler les constituants fongiques à l’échelle de milligramme, des quantités nécessaires pour les essais biologiques.

Enfin, pour l’identification de composés antifongiques induits, la co-culture du Basidiomycète agricole *Hohenbuehelia reniformis* et du pathogène humain *Fusarium solani* a été étudiée. Une analyse statistique
multivariée a permis de mettre en évidence les métabolites responsables des différences entre la culture pure et les co-cultures. D'autre part, l'observation de la formation de pigments lors d'une co-culture n'a pas été révélée par l'étude métabolomique basée sur la spectrométrie de masse mais seulement par le profilage UHPLC-UV. Pour l'isolement de ces composés, une co-culture à grande échelle a été mise en place et une stratégie de préfractionnement permettant l'élimination des sucres et des constituants apolaires de l'extrait a été développée. Une purification par chromatographie préparative en deux étapes a permis l'isolement ciblé de différents métabolites, dont plusieurs nouvelles quinones anti-\textit{Fusarium} ainsi que sept pigments.

Ces différentes études ont montré le potentiel de méthodes chromatographiques de pointe pour l'isolement de produits naturels provenant de diverses matrices. L'utilisation de systèmes de purification efficaces avec des technologies de RMN sensibles associées à des essais biologiques bien choisis, permettent de mener à bien un profilage sur quelques milligrammes d'extrait de plante et de diminuer considérablement le temps nécessaire à une investigation phytochimique complète. Les stratégies d'extraction, de préfractionnement et de purification mises au point ont rendu possible l'analyse et l'isolement de métabolites secondaires de champignons issus des expériences de co-culture. Ces protocoles d'isolement ciblé, en combinaison avec des analyses de données multivariées, permettent de localiser efficacement la présence de composés bioactifs induits par co-culture. La caractérisation de ces composés ont permis d'obtenir un bon aperçu des phénomènes biochimique liés à l'interaction microbienne.

Ces stratégies d’investigation chimique avancées permettent d’accélérer et rationaliser la recherche de nouvelles entités chimiques bioactives. La méthodologie d’induction de métabolites par co-culture peut être appliquée à d'autres types de microorganismes et permet de mettre en évidence ou d’activer des voies de biosynthèse cryptiques. Des technologies de pointe comme l'ingénierie métabolique et la biologie synthétique peuvent tirer profit de ces résultats pour la recherche de médicaments et de candidats médicaments de nouvelle génération.
## Outline

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Abbreviations

ANOVA  Analysis of variance
APCI  Atmospheric pressure chemical ionization
API  Atmospheric pressure ionization
ASE  Accelerated solvent extraction
BPI  Base peak ion
$^{13}$C  Carbon-13
CD  Circular dichroism
COSY  Correlation spectroscopy
1D  One-dimensional
2D  Two-dimensional
3D  Three-dimensional
DA  Discriminant analysis
DCM  Dichloromethane
DEM  Dichloromethane/ethyl acetate/methanol
DEPT  Distortionless enhancement by polarization transfer
DESI  Desorption electrospray ionization
DI-MS  Desorption ionization-MS
DMSO  Dimethylsulfoxide
DMW  Dichloromethane/methanol/water
DNMT  DNA methyltransferase
DNP  Dictionary of Natural Products (database)
DNP  Dynamic nuclear polarization (NMR method)
DON  Deoxynivalenol
dpf  Day post fertilization
DQF  Double quantum filtered
DSS  Sodium 3–(trimethylsilyl)–1–propanesulfonate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>ECD</td>
<td>Electronic circular dichroism</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EI-MS</td>
<td>Electron ionization-MS</td>
</tr>
<tr>
<td>ELSD</td>
<td>Evaporative light scattering detection</td>
</tr>
<tr>
<td>ERETIC</td>
<td>Electronic reference to access in vivo concentrations</td>
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<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
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<td>FID</td>
<td>Flame ionization detection (detection)</td>
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<td>FID</td>
<td>Free induction decay (NMR)</td>
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<tr>
<td>GC</td>
<td>Gas chromatography</td>
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<td>HCS</td>
<td>High-content screening</td>
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<td>HDAC</td>
<td>Histone deacetylase</td>
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<td>HIF</td>
<td>Hypoxia-inducible factor</td>
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<td>HMBC</td>
<td>Heteronuclear multiple-bond correlation spectroscopy</td>
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<td>^3H NMR</td>
<td>Proton NMR</td>
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<tr>
<td>HMOC</td>
<td>Heteronuclear multiple-quantum correlation spectroscopy</td>
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<tr>
<td>hpf</td>
<td>Hours post-fertilization</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>HR</td>
<td>High-resolution</td>
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<tr>
<td>HRMS</td>
<td>High-resolution mass spectrometry</td>
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<tr>
<td>HRS</td>
<td>High-resolution screening</td>
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<tr>
<td>HSQC</td>
<td>Heteronuclear single-quantum coherence spectroscopy</td>
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<tr>
<td>HTS</td>
<td>High-temperature superconducting (NMR coil material)</td>
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<tr>
<td>HTS</td>
<td>High-throughput screening (drug discovery)</td>
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<tr>
<td>IMS</td>
<td>Imaging MS</td>
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<tr>
<td>IR</td>
<td>Infrared</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>IS</td>
<td>Internal standard</td>
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<tr>
<td>ISV</td>
<td>Intersegmental vessels</td>
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<tr>
<td>LC</td>
<td>Liquid chromatography</td>
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<tr>
<td>LDPI</td>
<td>Laser desorption postionization</td>
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<td>LPLC</td>
<td>Low pressure liquid chromatography</td>
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<td>LPS</td>
<td>Lipopolysaccharides</td>
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<td>LR</td>
<td>Low-resolution</td>
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<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption ionization</td>
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<tr>
<td>MeOH</td>
<td>Methanol</td>
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<td>MeCN</td>
<td>Acetonitrile</td>
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<tr>
<td>MF</td>
<td>Molecular formula</td>
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<td>MIST</td>
<td>Metabolites in Safety Testing (regulations)</td>
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<td>MPLC</td>
<td>Medium pressure liquid chromatography</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
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<td>MS^n</td>
<td>Multistage mass spectrometry</td>
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<tr>
<td>MVDA</td>
<td>Multivariate data analysis</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
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<tr>
<td>NDF</td>
<td>Non-dermatophyte filamentous fungus</td>
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<tr>
<td>NI</td>
<td>Negative ionization</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>ttNMR</td>
<td>Tube transfer NMR</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>NOESY</td>
<td>Nuclear Overhauser enhancement spectroscopy</td>
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<tr>
<td>NPs</td>
<td>Natural products</td>
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<tr>
<td>NRPS</td>
<td>Non-ribosomal peptide synthases</td>
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<tr>
<td>OPLS</td>
<td>Orthogonal projection to latent structure</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>OSMAC</td>
<td>One strain-many compounds</td>
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<tr>
<td>$P$</td>
<td>Peak capacity</td>
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<tr>
<td>PAIN</td>
<td>Pan-assay interference</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PBST</td>
<td>Phosphate buffered saline + Tween</td>
</tr>
<tr>
<td>PC</td>
<td>Principal component</td>
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<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDB</td>
<td>Potato dextrose broth</td>
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<tr>
<td>PDA</td>
<td>Photo diode array (detection)</td>
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<tr>
<td>PDA</td>
<td>Potato dextrose agar (culture medium)</td>
</tr>
<tr>
<td>PI</td>
<td>Positive ionization</td>
</tr>
<tr>
<td>PKS</td>
<td>Polyketide synthase</td>
</tr>
<tr>
<td>PLE</td>
<td>Pressurized liquid extraction</td>
</tr>
<tr>
<td>PLS</td>
<td>Partial least-squares regression</td>
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<tr>
<td>POChEMon</td>
<td>Projected orthogonalized chemical encounter monitoring</td>
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<tr>
<td>PTU</td>
<td>1-Phenyl-2-thiourea</td>
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<tr>
<td>PULCON</td>
<td>Pulse length based concentration determination</td>
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<tr>
<td>Q</td>
<td>Quality factor (NMR probe technology)</td>
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<tr>
<td>QC</td>
<td>Quality-control</td>
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<tr>
<td>qNMR</td>
<td>Quantitative NMR</td>
</tr>
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<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<td>QS</td>
<td>Quorum sensing</td>
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<tr>
<td>QTOF MS</td>
<td>Quadrupole time of flight MS</td>
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<tr>
<td>QUANTAS</td>
<td>Quantification by artificial signal</td>
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<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
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<tr>
<td>RI</td>
<td>Refractive index</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>RP</td>
<td>Reversed phase</td>
</tr>
<tr>
<td>RLM</td>
<td>Relative leukocyte migration</td>
</tr>
<tr>
<td>RT</td>
<td>Retention time</td>
</tr>
<tr>
<td>RVO</td>
<td>Relative vascular outgrowth</td>
</tr>
<tr>
<td>SAHA</td>
<td>Suberoylanilide hydroxamic acid</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal-to-noise ratio</td>
</tr>
<tr>
<td>SIM</td>
<td>Selected-ion monitoring</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid phase microextraction</td>
</tr>
<tr>
<td>SSF</td>
<td>Solid-state fermentation</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin-related modifier</td>
</tr>
<tr>
<td>SUS plot</td>
<td>Share and unique plot</td>
</tr>
<tr>
<td>TDDFT</td>
<td>Time dependent density function theory</td>
</tr>
<tr>
<td>TIC</td>
<td>Total ion chromatograms</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TOCSY</td>
<td>Total correlation spectroscopy</td>
</tr>
<tr>
<td>TOFMS</td>
<td>Time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>TRFLP</td>
<td>Terminal restriction fragment length polymorphism</td>
</tr>
<tr>
<td>UAE</td>
<td>Ultra-sound assisted extraction</td>
</tr>
<tr>
<td>UHPLC</td>
<td>Ultra high performance liquid chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet (detection)</td>
</tr>
<tr>
<td>UV</td>
<td>Unit variance (statistics)</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLC</td>
<td>Vacuum liquid chromatography</td>
</tr>
<tr>
<td>WET</td>
<td>Water suppression enhanced through $T_1$ effects</td>
</tr>
<tr>
<td>XIC</td>
<td>Extracted ion chromatogram</td>
</tr>
<tr>
<td>YBCO</td>
<td>Yttrium barium copper oxide</td>
</tr>
<tr>
<td>ZON</td>
<td>Zearalenone</td>
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Aim and structure of the thesis

Microorganisms have a long track record as important sources of novel bioactive natural products, particularly in the field of drug discovery. Nevertheless, many microbial gene clusters seem silent under standard laboratory growth conditions and many more bioactive microbial metabolites could possibly be detected and identified. One way to activate the production of secondary metabolites from silent genes is through the application of biotic stress to the microorganisms, such as in microorganism co-culture. This has recently been used increasingly extensively to study natural interactions and discover new bioactive metabolites. Solid medium co-cultures mimic the natural growth of fungi, especially, and is, so far, rarely used for the chemical analysis and isolation of microbial constituents. These solid medium co-cultures are usually carried out on small Petri dishes and the amount of extract obtained from such cultures is only a few milligrams. Thus, strategies are needed that are compatible with this kind of mass-limited samples, in terms of isolation and (NMR) detection. This thesis aims at the development of solid medium co-culture for the induction, detection and identification of novel NPs. The strategy for the isolation of natural products from mass-limited samples was first developed on plant extracts and, thereafter, applied to solid medium co-cultures.

This manuscript is organized into three major sections. A general introduction that comprises an overview on natural product research in chapter I with a special aim at phytochemical aspects and two detailed parts on detection of natural products by NMR, miniaturized NMR probes (microNMR), quantitative NMR (qNMR) and uses and applications of the diverse forms of LC-NMR. The next part, chapter II, reviews the different ways of activation of silent genes in microorganisms and the role of microorganism co-culture in this field.

The second section with chapter III focuses on plant extract applications and includes the development of miniaturized strategies for the isolation of bioactive secondary metabolites from mass-limited plant extracts.

The third section focuses on fungal extracts. In chapter IV, the methodology for the detection of co-culture-induced fungal metabolites is described. In a second part, chapter V, the strategies developed for mass-limited plant extracts where applied to fungal extracts. In this selected case, this was achieved with success, but it was apparent that large-scale co-cultures were necessary for the majority of co-culture examples. With this, further strategies had to be developed to isolate fungal metabolites from these extracts, especially in terms of enrichment or prefractionation of extracts as detailed in chapter VI. In this last part, detection of co-culture-induced metabolites and large-scale isolation strategies were applied to the study of a co-culture with the aim of isolating antifungal compounds.
Most of the chapters of this thesis are based on research articles, reviews, and book chapters published or submitted during the thesis work. These articles were reformatted. All references are compiled in a common list at the end of the manuscript.
General introduction
I. Generalities on natural products and methodological development for their isolation and detection
1. **Natural products in drug discovery**

Natural products (NPs) is the umbrella term for any molecule that is produced by a live organism that is not strictly necessary for the survival of the organism [1]. These molecules, secondary metabolites, are not implicated in the basic cellular machinery and are mostly small molecules with a molecular weight less than 1500 Da. Many NPs have diverse biological functions, e.g., as antibiotic agents, attractants or repellents to others. Yet, the biological function of many NPs is not apparent and not known.

Nature has been an invaluable source of therapeutic agents [2,3], either in its use as traditional medicine or as inspiration for the pharmaceutical industry in the development of drugs. However, during the last two decades, many pharmaceutical companies have closed down their NP research units [3,4]. The decreased interest in NP research of the pharmaceutical industry is mainly attributed to the diverse difficulties that arise when working with living matter. NP research is hampered by, among others, access to primary material, identification and isolation of secondary metabolites in complex mixtures and by unreliable resupply. To speed up the time for research from a molecule to a lead structure, research programs in industry were shifted to high-throughput screening (HTS) of compound libraries created by combinatorial synthesis. Inclusion of natural extracts did not comply with the time frame allocated for these programs [5]. This approach was not yet successful in yielding more drug leads, mainly because these combinatorial compounds did not bear lead-like structures. Comparison of different physicochemical parameters of drugs on the market, natural products and combinatorial compounds [6] resulted in the subsequent improvement of screening libraries to include NP-like compounds or privileged scaffolds [7]. Overall, NPs exhibit more stereogenic centers and have more complex structures, contain more carbon, hydrogen and oxygen atoms and less nitrogen atoms than synthetic molecules, and have high polarities [8].

A lead compound is a bioactive compound whose chemical structure may be transformed by subsequent modification into a clinically useful drug.

Nevertheless, research on plant or microbial NPs continues to be of high interest as shown by the still increasing number of publications in the field (Figure I.1). Current advancements in genomics, chemical biology and analytical chemistry even anticipate an increasing importance of NP research in the search for new bioactives of potential use as future drugs or drug leads [9].

Medicinal plants, or plants in general, have been the object of studies to find bioactive NPs for more than 200 years [3]. Microbes, on the other hand, have gained interest as remarkable sources for bioactive compounds since the ‘golden age of antibiotics’ in 1950-1960 that was initiated by the mass-production of penicillin after the 2nd World War [10]. As for plant NPs,
the number of publications for NP research on bacteria and fungi is steadily increasing as well (Figure I.1).

![Figure I.1: Number of publications in the field of natural product research. Retrieved in Web of Knowledge (Thomson Reuters) using the following keywords: ‘natural products’ refine ‘plants’ (white bars) and ‘natural products’ refine ‘bacteria’ OR ‘fungi’ (black bars). Data collected on the 3rd of March, 2014.](image)

The number of reported NPs is estimated to be approx. one million in 2014 [4] and these molecules originate from plants, microbes or animals. Substantially more plant-derived NPs are known compared to NPs originating from bacteria and fungi (17% according to estimations from J. Bérdy in 2012 [10], Figure I.2). Reasons might include the larger genome size of plants compared to microbes and thus, the capacity to produce more secondary metabolites compared to microbes. In addition, plants have been studied for longer compared to microbes. However, the number of known microbial compounds might be biased as research was, for a long time, centered on industrial laboratories and thus, it is probable, that microbial compounds remained often unpublished. Interestingly, for bioactive NPs, the portion of microbial-derived NPs is substantially increased and this trend is even more striking for marketed microbial-derived drugs (Figure I.2, Figure I.3), depending on the source of information [10-12].
Recent advances in chemistry, biology and genomics should facilitate the compatibility of NPs with industry’s lead finding and HTS programs. Overall, the following strategies are important to pursue NP research in the future as stated by [10]:

1. Bioprospecting, access new sources that possibly biosynthesize bioactive molecules
2. Find more selective and efficient methods to identify and isolate NPs
3. Develop new methods to access full potential of known NP-producers, e.g. methods that activate the production of secondary metabolites from cryptic pathways

The first point will not be detailed in this thesis work. More information can be found, for example, in a recently published book that details bioprospecting as strategy to find new drugs [13]. During the course of this thesis, point 2 and 3 were addressed towards an advancement of NP research. On the one hand, strategies were developed for a more rapid isolation of natural products making use of newly developed methods in NP detection, notably in NMR spectroscopy (point 2). On the other hand, the method of microorganism co-culture was exploited for the identification and biological evaluation of induced NPs (point 3).
Figure I.3: Number of compounds that are known as approved drugs, clinical trial drugs or known bioactive compounds classified by the kingdom (Bacteria, Fungi or Viridiplantae) from which they were isolated.
The data is assembled on the basis of data from [11]. Compounds from the Metazoa kingdom were excluded. Note the different y-axes for approved drugs and clinical trial drugs (left axis) and for searchable known bioactive compounds (right axis).

In the following introductory chapters, a short overview of the typical workflow used for the isolation of small molecules from natural extracts is given (chapter I.A). Advances in analytical techniques that allow for more efficient identification of NPs, notably in nuclear magnetic resonance (NMR) detection, are covered in the two following chapters (I.B and I.C). Novel methods that permit access to the greater genomic potential of microorganisms are discussed in the chapter on microorganism co-culture (chapter II.A).
I.A Strategies for the rapid isolation of natural products from plant & microbial extracts
1. Natural products research

For thousands of years, traditional medicine has relied on bioactive constituents from nature, primarily from plants. The isolation of morphine in the 19th century from the cut seed pods of poppy (Papaver somniferum, Papaveraceae) set the stage for the isolation of bioactive molecules from natural sources [14].

Today, NP research aims at the assessment of compound diversity within given organisms (e.g., chemotaxonomy), the structural evaluation of a particular class of compounds, of compounds with a certain biological activity or of compounds with particular structural features. This includes, in most cases, obtaining pure compound through their isolation. As NPs are chemically diverse, isolation methods are, at best, universal and permit the isolation of molecules exhibiting a large range of different physicochemical properties.

Classical isolation strategies often imply a combination of different isolation strategies [15], using such techniques as counter-current chromatography, centrifugal partition chromatography, liquid-liquid extraction or sequential liquid chromatography. These procedures are complicated and time-demanding. Several comprehensive review articles and books assemble the mainly used methodologies in NP research [16-18]. In the need to facilitate the drug discovery process, new routes from natural extracts to the isolation of bioactive NPs have been developed using hyphenated techniques based on analytical methods [19]. These include liquid chromatography (LC) hyphenated to nuclear magnetic resonance (NMR) for on-line identification of extract constituents [20] or LC hyphenated to mass spectrometry for either on-line identification or subsequent at-line identification by other methods such as NMR [21]. Today, more rapid isolation procedures, at a smaller scale are more frequently applied with the aim of getting pure compounds faster or for the isolation of minor extract constituents [22]. Here, the main focus will be on these rapid isolation strategies with particular emphasis on microfractionation (see section 5.1 and Figure I.A.3).

Strategies towards compound isolation are diverse [18]. In general, the workflow consists of extraction from biological matrix, extract preparation for later purification, compound isolation and identification (Figure I.A.1). Ideally, several compounds are isolated within the same isolation step and methods are applicable to different extract types with minimal optimization. Furthermore, the isolation should not be restricted to a certain class of compounds and no prior knowledge of the targeted molecule should be required.

2. Extraction of natural products

After growth or culture of the biological material, extraction is the first step towards the isolation of NPs. The extraction method depends not only on the available biological material but also on the type of study that is planned. For studies that aim at the detection of active principles of traditionally used medicine, the traditional extraction should be taken into account. For example, alkaloid containing plants are often traditionally chewed which is equivalent to an acidic extraction that permits the solubilization of the bioactive alkaloid in an
aqueous solvent, respectively in the saliva of the patient. In this context, the biological material should be extracted in a way that matches the traditional preparation as closely as possible. On the other hand, in cases where no ethnopharmacological knowledge is available, the extraction method should aim at extracting, in the best case, the ensemble of secondary metabolites from the biological matrix. Different methodologies are described in a recently updated book [18]. As mentioned, the extraction methods may differ depending on the biological material and thus, the extraction of plant and fungal material is treated separately.

2.1 Extraction of dried plant material

Most often, plant material is soaked in a solvent and after an incubation time, the liquid is separated from the plant material through filtration and the solvent is then removed to obtain a crude extract. Methods include maceration, decoction, Soxhlet extraction, accelerated solvent extraction, ultrasonic or microwave assisted extraction [25]. The polarity of the solvent determines what kind of molecules are extracted. For example, water will extract mainly polar
constituents whereas hexane or dichloromethane extracts only apolar compounds. Extractions can be executed consecutively, e.g., by a first extraction with hexane – with the aim of removing fatty acids, followed by extractions with different organic solvents of increasing polarity. This permits some sort of enrichment of the different crude extracts. On the other hand, extraction with a solvent that permits the solubilization of molecules with a wide range of physicochemical properties, such as methanol, allows a complete crude extract to be obtained. This is preferred for the development of general approaches that aim to be applied to a wide range of different primary materials.

2.2 Extraction of fungal material

The matrix to obtain a fungal extract from is particular. In contrast to plants, the microorganisms under study are cultured on or in a growth medium from which it cannot be separated easily. Some metabolites are excreted into the culture medium and should possibly be captured in the extract as well. Microorganisms are cultured on either solid or liquid medium [26,27] and the extraction is, again, matrix-dependent.

2.2.1 Extraction of liquid cultured fungal material

Two main strategies are employed for the extraction of microorganisms that were cultivated in liquid medium (fermentation). The first method is the direct liquid-liquid extraction of the water-based culture medium with an organic solvent that is immiscible with water, such as ethyl acetate [28]. The limited solubility of ethyl acetate in water permits the separation of the (polar) culture medium from less polar secondary metabolites that have been secreted into the liquid medium. The second method implies a first step of centrifugation to separate the culture medium from the biomass. The biomass is then extracted with an organic solvent, e.g., acetone [29]. Both methods allow for a separation of the culture matrix from the crude extract, but the later method will not be able to capture secondary metabolites that had been released into the culture medium.

2.2.2 Extraction of solid-state cultured fungal material

Solid medium cultures (solid-state fermentations, SSFs) mimic the natural growth of fungi, but so far, are rarely used for the isolation of microbial constituents. Several methods are possible. The fungus can be scraped off the surface of the solid medium and subsequently extracted, similar to what is done on plant extracts (section 2.1). But secreted metabolites would not be extracted in this way. Then, extraction can either be done on the fresh medium directly or the medium can be lyophilized prior to extraction.

Extraction of the fresh medium has shown to be successful for the isolation of fungal constituents [30]. After addition of the extraction solvent and pounding of the medium, the mixture is let to macerate under agitation and then filtered. Because of the slimy nature of the culture medium, this filtration step is cumbersome as the filter paper is easily clogged. Its applicability to larger amounts of fungal culture or for the analysis of a high number of samples is very limited.
Alternatively, the fungal culture can be lyophilized prior to extraction, after quenching with liquid nitrogen [31]. This has the advantage that the filtration step becomes easier and that the water content of the extraction solvent can be controlled. Again, the physicochemical properties of the extraction solvent will determine which compound classes are extracted from the culture and fungal matrix. Commonly, ethyl acetate is used as extraction solvent of solid medium cultures [32].

Alternatively, microorganisms can be cultivated on other solid media as well, such as corn or rice, and the extraction method has to be adapted in consequence [33].

3. Sample preparation

For the success of the later purification step(s) and for the metabolite profiling of the extract at the analytical level (see section 4), the crude extracts might need to be processed additionally. This preparation step depends on what purification strategy is pursued later.

Liquid chromatography (LC) has proved its efficacy in allowing the isolation of natural products for tens of years already [16]. Often, reversed-phase (RP) chromatography is used for the exploratory purification of NP extracts (see section 5). The polarity range of molecules that can be analyzed by RP chromatography is between log \( P \) -1 and log \( P \) 8 [34]. Less polar compounds are not retained on the column. In consequence, the concentration of retained...
molecules is decreased when the extract contains many polar constituents and thus, minor constituents cannot be analyzed as efficiently (Figure I.A.2). For apolar compounds (high log P value), these will be almost irreversibly adsorbed onto the C_{18} derivatized silica gel and subsequently decrease column lifetime and deteriorate chromatographic performance of the column. Thus, an enrichment of the crude extract is desired to increase the efficiency of the following purification step. The removal of apolar constituents is highly recommended to protect and assure long usability of the columns used for the subsequent purification or analysis. The removal of polar constituents, on the other hand, may be welcome to increase the efficiency of the following isolation step.

Sample preparation can be done using solid phase extraction (SPE). Thereby, the sample is eluted over a solid phase with isocratic solvent conditions. Silica gel derivatized with C_{18} (reversed phase (RP) silica gel) is used with a single elution step of, for example, 85% aqueous methanol [35]. Nevertheless, other solvent mixtures or solid phases might be used, such as the polystyrene resins from Diaion or normal phase (NP) silica gels, or crude extracts might be separated into several enriched fractions by step-wise elution with solvent mixtures of different polarities [36].

4. **Compound detection, metabolite profiling and dereplication**

Secondary metabolites are organic molecules and diverse detection methods exist that are compatible for such compounds that can be used either for metabolite profiling of extracts, for monitoring the separation during microfractionation or for the analysis of pure constituents. Detectors can be grouped as non-destructive or destructive methods. Some detectors acquire spectral information during separation and thus allow for compound identification as well (on-line identification). On the other hand, some detectors are specific to certain elements or structural features within a molecule or are regulated to do so (e.g., selected-ion monitoring, SIM in MS). The use of such selective detectors demands prior knowledge of the sample content or limits analysis to specific analytes (targeted analysis). In the search of new chemical entities or when no prior knowledge on the sample is available, untargeted analyses are preferred.

4.1 **Detection of natural products**

4.1.1 **Ultraviolet detection**

In NP research, UV detection is most commonly employed, for both metabolite profiling and separation monitoring. The technique is non-destructive and sensitive. In addition, the UV spectrum that can be acquired with photo diode array (PDA) detectors allows for identification of compound class. On the downside, only compounds bearing a chromophore (conjugated double bonds or aromatic rings) are detected and the detector response is dependent on the detection wavelength and the absorptivity (molar absorption coefficient, $\varepsilon$) of the detected molecule. Compounds with strong chromophores, such as flavonoids or quinones, give a high detector response and are readily detected. On the other hand, fully saturated molecules, such as most saponins and certain steroids and terpenes, remain not detectable in practice.
because solvents absorb certain wavelengths (cutoff) or modifiers interfere with such weak chromophores.

### 4.1.2 Mass spectrometry

Another widely used detector is MS. The technique is destructive but very sensitive. MS detection can be used to monitor separation when a splitter is set up to deviate a small part of the eluent towards the detector and keep the main part for collection, such that sample loss is negligible. The MS detector is hyphenated to LC through an interface that permits ionization whereas atmospheric pressure ionization (API) sources, such as electrospray ionization (ESI), are commonly employed [37]. Flow-splitting has to comply with several requirements, for example, optimal flow rate for efficient ionization of analytes, deviation of sufficient amounts of sample to MS for sensitive detection of major and minor extract constituents and retention of the majority of the sample for collection (and subsequent at-line analysis). The ionization in ESI is concentration-dependent [38] and complications, such as clustering [39] and ion suppression or detector saturation, can occur at high concentrations.

Several types of MS detectors exist. Overall, detectors can be grouped into low resolution (LR) detectors that measure masses to unit mass resolution (e.g., ion trap MS), ideal for separation monitoring, and high resolution (HR) detectors, for metabolite profiling, that can achieve resolution between 5,000 up to 1,000,000 (e.g., time-of-flight mass spectrometry (TOFMS), quadrupole-TOFMS (Q-TOF) or Orbitrap-type instruments) and thus permit the separate detection of isobaric compounds [35]. Another important factor for MS instrumentation, particularly important for metabolite profiling, is mass accuracy, how close to the real value (accuracy) the exact mass of an analyte is measured. This is important for molecular formula determination and discussed in section 4.3 Dereplication. The detector response is dependent on the ionizability of the analytes, the intense detection of a compound is thus not necessarily related to a high amount of this compound present in the extract. In addition, ion suppression can occur through the so-called matrix effect, the intensity of the analyte ions is diminished through co-eluting sample constituents [40]. Thus, the quantity of the analyte is underestimated. In metabolite profiling, MS can be used for compound identification, either through the determination of the molecular formula (with instruments that provide high mass accuracy in the range of 1-5 ppm) or through the application of tandem MS (MS/MS) and the structure elucidation by analysis of fragment ions [41].

### 4.1.3 Evaporative light scattering detection

In recent years, a detector developed in the seventies has gained increasing interest for its use in NP research [42], the evaporative light scattering detector (ELSD). In this technique, the eluent is nebulized using a gas flow (compressed air or nitrogen) and the resulting aerosol is transported through a drift tube where the solvent is evaporated. The remaining solid particles are transported to the detection cell where the light of a laser beam is scatter and measured. The signal is proportional to the number of particles and thus, to the quantity of the chromatographic peak. The technique is destructive and less sensitive than UV detection,
but it can be used for isolation monitoring with a split [43], similar to MS detection, with only limited sample loss. Nevertheless, due to the semi-quantitative detector response and its universal applicability, ELS detection is a good option to aid NP isolation [44].

4.1.4 Nuclear magnetic resonance

Another detection method with a quantitative detector response is NMR. Whereas the technique is non-destructive, it is also the least sensitive of the described detection methods. A detailed description of NMR and its use in NP research is given in the following chapters I.B and I.C. Atomic nuclei with spin 1/2 (among biological nuclei: $^1$H, $^{13}$C, $^{15}$N, and $^{31}$P) are detectable by NMR and the sensitivity is highest for $^1$H NMR detection. Thus, this technique allows for universal and quantitative detection of almost all secondary metabolites, theoretically all those bearing protons.

4.1.5 Other detectors

Other detectors exist for monitoring compound isolation, e.g., chemiluminescence nitrogen detectors for nitrogen-bearing compounds or refractive index (RI) detector. Compound identification is possible with X-ray crystallography as well. Recently, the hyphenation of X-ray crystallography with HPLC has been reported (crystalline sponge method [45,46]) which pledges the X-ray analysis of LC peaks down to the nanogram scale. Its practical use in NP research remains to be proven.

4.2 Metabolite profiling and on-line identification

For the comprehensive analysis of chemical constituents in a complex sample, metabolite profiling based on high-resolution (HR) LC and MS has caught on in the analysis of plant and microbial extracts. Whereas high resolution on the LC side offers the separation of a maximum of extract constituents, high resolution on the MS dimensions permits detection of major and minor extract constituents and separation of co-eluting compounds [37]. This is an excellent tool for optimal dereplication (see next section).

**Metabolite profiling** terms an analysis aiming at detecting all compounds present in a sample to provide a complete picture of, at best, all sample constituents. In the context of this work, metabolite profiling is based on LC and implicates high resolution in both the separation and the detection dimension.

The amount of sample required for detection is dependent on the detection technique. Isolated compounds are generally characterized by nuclear magnetic resonance (NMR), mass spectrometry (MS), ultraviolet (UV) spectroscopy, or ultimately by X-ray crystallography to unveil their identity. In metabolite profiling and for on-line dereplication, different detectors must be used, depending on the structural class of the compound and depending on whether the compound has been previously described. Whereas known compounds are, in the
majority of cases [47,48], sufficiently characterized by UV and high-resolution MS (HRMS) and HR tandem MS (MS/MS) spectroscopy (dereplication), novel compounds often need to be analyzed by NMR spectroscopy for a so-called ‘de novo structure elucidation’.

Compounds that cannot be identified on-line or compounds undergoing biological evaluation must be isolated and analyzed at-line (see also chapter I.C, section 3). The amount of isolated compound that is needed for a full characterization is strongly dependent on the sensitivity of the detection method. UV and MS are very sensitive techniques and samples down to 10 pg/mL (≈ 10 ppm) or lower are commonly analyzable [49]. On the other hand, sample amount requirements for NMR analysis are several-fold higher. Conventional NMR systems, such as 5-mm room-temperature NMR probes in a 500 MHz magnet, require at least several hundreds of micrograms up to a few milligrams of sample for complete de novo structure elucidation with 2D NMR analysis. When the most sensitive NMR technologies are used (see chapter I.B and I.C), proton NMR (1H NMR) is possible with as little as 200 ng of sample [50], but at least microgram-amounts are necessary for 2D NMR analyses [51].

4.3 Dereplication

The isolation of secondary metabolites from complex natural extracts is a time-demanding process. Thus the importance of focusing forces on the isolation of new molecules. Therefore, one of the first steps in the analysis of chemical constituents in natural extracts is the identification of previously isolated molecules, a process called dereplication.

**Dereplication** is the process of detecting and identifying previously isolated and already known natural products within a mixture. Dereplication is important to focus the effort of isolation, structure elucidation and pharmacological assessment on novel compounds [52].

Strategies in dereplication normally include metabolite profiling based on the chromatographic analysis of the natural extract with PDA or MS detection. Comparison of retention time and the exact mass or a UV spectrum with authentic standards or (in-house) database allows for very efficient dereplication but is restricted to compounds that have been previously analyzed on a comparable LC-(PDA)-MS system with equal standardized experimental conditions [53-55]. Combination of LC with high resolution mass spectrometry (HRMS) [54], tandem mass spectrometry (MS/MS)[53], multistage MS (MS^n)[56], PDA [57] or NMR [58] detection generates data that afford information on molecular weight, structural fragments or compound class of the detected metabolites. This information is used to search in databases, such as the Dictionary of Natural Products (DNP) [59] which assembles over 226,000 NPs, mainly of plant origin, or MarinLit and Antibase [60,61] which are dedicated to marine and microbial NPs, with more than 29,000 marine and 42,000 microbial NPs, respectively. As a next step, possibly identified metabolites can be limited to those being isolated from taxonomically close species only. This is called dereplication based on
chemotaxonomic information. Chemotaxonomic discrimination has been shown very effective in the dereplication of plant extracts [59,62], but it is less efficient for microbial extracts. In the absence of in-house databases or previous experience on the studied organism, dereplication has to be based on literature search and publicly or commercially available databases and is thus restricted to the published research/information. Relatively few studies are published on the microbial metabolites in general (previous chapter, Figure I.2 and I.3) and studies are limited to a few microbial species, e.g., the Aspergilli or the Streptomyces [63].

The dereplication workflow usually starts at the MS information where possible molecular formulae (MF) are determined. The number of possible MF decreases with increasing spectrometer accuracy. At a given mass accuracy, however, confidence in MF assignment decreases with increasing molecular weight. A molecule of 300 Da will lead to 11 possible MF if analyzed on a MS instrument with a mass accuracy of 5 ppm (e.g., Micromass LCT Premier TOFMS), compared to 2 MF for a 1 ppm mass accuracy (e.g., Synapt G2 Q-TOF). On the other hand, a compound of 700 Da will lead to 538 MF at 5 ppm mass accuracy and 108 MF at 1 ppm [64]. Through the application of heuristic filtering [64,65], the number of plausible MF is further narrowed down. Filters include isotopic pattern matching (e.g., i-Fit in MassLynx software), elemental ratio probabilities and database search on MF from NPs [66].

5. Purification and compound isolation

As mentioned above, liquid chromatography is a predominant technique for the isolation of secondary metabolites from complex mixtures. In the context of this work, the focus will mainly be on methods that permit rapid isolation of pure compounds from natural extracts, in particular on the microfractionation strategy. Chromatographic conditions can be transferred among columns of different dimensions while retaining equal separation through a procedure called geometrical transfer [67]. This, in turn, facilitates upscale of chromatographic separations and permits the rapid development of isolation strategies, the basis of microfractionation (Figure I.A.3).

To obtain equal separation on two columns with different dimensions (length and inner diameter), LC conditions need to be transferred geometrically [67]. This permits easy comparison between two chromatographic runs and facilitates chromatographic upscale.

Loading capacity is the amount of sample (in mg) that can be injected (loaded) onto a column at which, in theory, the chromatographic resolution is maintained. Loading capacity is dependent on the column volume (dependent on column dimensions and stationary phase) whereas column i.d. is important as it contributes as square to the column volume.
For compound purification, it is important to obtain a high degree of solute purity and to produce a high amount of (pure) compound per unit time (throughput). The amount of extract that can be fractionated in one chromatographic run (loading capacity) is important as well and is linked to the two preceding factors [68]. In addition, the purification of several compounds in one chromatographic injection is desired as it allows the isolation process to be speeded up.

5.1 Microfractionation

As mentioned above, the sample demands depend on the applied spectroscope and thus, the purification step should yield sufficient amounts of (pure) compound to comply with the suitable spectroscopic method (e.g., MS or NMR). On the other hand, accelerating the isolation procedure could speed up the process of discovering novel bioactive compounds and the microfractionation strategy has brought in promising results [19,69]. In this line, RP LC offers the advantage of full compatibility at different scale, from UHPLC-based HR metabolite profiling to semipreparative HPLC or even medium pressure liquid chromatography (MPLC) with, e.g., MS-based separation monitoring. The consistent use of RP LC permits the continuous follow-up of extract constituents along the chromatographic separations at different scales.

**Microfractionation** terms an isolation strategy that allows for the purification of several extract constituents within one chromatographic run from few milligrams of extract. Often, samples are collected such that they are compatible with HTS, in a 96-, or even 1536-well plate format.

The use of microfractionation for the isolation and subsequent identification of small molecules was enabled through advances in NMR detection, such as the development of sensitive probes for on-line or at-line detection. This permits the phytochemical investigation of mass-limited samples [30,70] or the isolation of minor extract constituents [71]. Several aspects are of particular importance for microfractionation if pure compounds are to be obtained rapidly in one step for further NMR and/or bioactivity characterization. As a rule of thumb, the better the chromatographic resolution of the column used for microfractionation, the more extract constituents are obtained as pure compounds in shorter time. Important parameters are the selectivity of the stationary phase, the type of solvents used, the specifications of the gradient, the quality of the sample preparation, the loading capacity and the quality of the used solvents. Some columns leak out small amounts of column material that is concentrated in the collected microfractions and contaminates the isolated compounds. In addition, molecules that are incompatible with the chosen chromatographic phase, such as fatty acids or certain chlorophylls for RP-LC that stay adsorbed on the head of a column, might be gradually eluted from the column (see also above, 3. Sample preparation) and contaminate the collected microfractions.
Figure I.A.3: Overview of the microfractionation strategy for metabolite identification and bioactivity assessment.

A. High-resolution (HR) profiling of crude extracts by UHPLC-TOF-MS. B. Transfer of analytical conditions to the semipreparative level to keep the same selectivity of separation (BPI traces are displayed). C. Microfractions are collected into deep well plates (one fraction/minute) and dried by vacuum centrifugation. D. Aliquots of all fractions are taken and analyzed for their bioactivity. E. Microflow NMR analysis is performed on active microfractions to obtain structural and quantitative information. The molecular formula is extracted from the HR metabolite profiling (A). The concentration of the compound present in the microfraction can be estimated using quantitative NMR. The activity is then evaluated by a dose-response curve. Source: [72].

For microfractionation, the samples are preferably collected in containers that permit the uptake of the microfractions in a small volume of solvent. Vials of a volume of 1 to 2 mL, such as 96-deep well plates are commonly used [43]. These deep well plates are made from plastics, such as polypropylene or polystyrene, and contain phthalates that can leak from the container. Evaluation of these background contaminations and careful optimization and selection of column and collection material are essential to obtain clean NMR spectra for further successful structure identification.
The challenging part in the successful application of microfractionation lies in balancing column loading and chromatographic resolution. Increasing the amount of sample loaded onto the column (column loading) allows the collection of larger amounts of isolated compound and thus, the successful detection of minor constituents. On the other hand, overloading columns substantially deteriorates chromatographic resolution and in consequence, fewer extract constituents are collected as pure compounds. In addition, the limited solubility of natural extracts in aqueous-organic solvents often further limits column loading [73,74]. Advances in column material and column packing increased the loading capacity and allow for chromatographic resolution close to analytical HPLC. The loading capacity of columns used for microfractionation (semipreparative HPLC column, e.g., 250 × 10 mm i.d.) is given as 100 – 300 mg by manufacturers. However, for the purification of NPs with diverse physicochemical properties and for a chromatographic resolution of several hundreds of plates, and thus, comparable resolution to analytical HPLC, wants to be maintained, column loading should not exceed approx. 20% of the reported capacity.

6. Bioassays

Nature has been a source of therapeutical agents for thousands of years and an important number of modern drugs are derived from natural products [12,75]. In NP research, the bioactivity of isolated compounds is very important and needs to be assessed to further evaluate the interest for a given NP. For pharmacological assessment of isolated NPs, amount demands range from nanograms to grams, depending on the biological test assay or the study type. Tests can either be done on enzymes (enzymatic assay, e.g., acetylcholinesterase assay [76]), in vitro, on cells directly, e.g., NF-kB reporter assay [77], or in vivo, on small animals as Daphnia sp. or zebrafish (Danio rerio [78]) and bigger animals as rodents and mammals. The validity of the test for a comparable response in humans increases in that order. On the other hand, the amount of sample necessary for testing also increases in the same order.

High-content screening (HCS) is a method used in biological research and drug discovery that allows the identification of substances that alter the phenotype of a cell or an organism.

The classical discovery process for natural products from plant or microbial extracts has been, and still is, bioactivity-guided (or bioassay-guided) fractionation [18,79]. At every fractionation step, extracts or fractions are tested until the bioactive constituent is obtained as pure compound. This process is time-demanding, especially when multiple fractionation steps have to be employed. Used assays should be cheap and capable of analyzing many samples in short time (high-throughput screening, HTS). Unfortunately, the validity of most low-cost HTS assays is limited. Through the reduction of a biological test to one target, the interactions that happen within a live animal can not be assayed. This often leads to a bad performance of in vitro-active compounds in in vivo studies [80]. More expensive and time-demanding assays,
e.g., in vivo assays or chemical genetics [81], are mandatory for hit validation and to get in-depth information on the mode of action.

In the past twenty years, high content screening gained increasing interest as it allows for a hypothesis-independent analysis of pharmacological activities. This is also increasingly applied in the pharmaceutical industry [82]. Zebrafish offers a good model for a high content and in vivo assay while at the same time, only small sample amounts are necessary for testing. It is thus an ideal test format for the early bioactivity assessment of mass-limited samples. In addition, zebrafish have proven compatible for testing of complex plant or microbial extracts [78].
I.B Advanced spectroscopic detectors for identification and quantification: Nuclear magnetic resonance

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Contribution: Outline of book chapter, design and optimization of figures, writing of the chapter on quantitative NMR, proofreading of the article
Abstract

Nuclear magnetic resonance spectroscopy (NMR) provides a rich source of structural information which when combined with other spectroscopic data allows structural elucidation of complex compounds as well as a universal detection method for compounds of interest in complex mixtures. NMR is a nondestructive technique that facilitates its coupling with other spectroscopic methods such as mass spectrometry. The key to on-line NMR detection is the design of the flow probe. Suitable probes for continuous flow, stop flow, loop collection, solid-phase extraction, and microflow probes are described emphasizing their general applications and limitations. Sensitivity and probe volume are major limitations of NMR coupling to liquid chromatography. Solvent selection for separations needs to consider the unique characteristics of NMR, for example the use of deuterated solvents for proton detection, and methods available for solvent suppression. Practical applications of LC-NMR are presented to illustrate the use of this technique in different areas of analytical chemistry.
1. Introduction

HPLC is one of the most versatile techniques for the analysis of a large number of organic compounds that also includes a large majority of natural products (NPs). It can be used to directly profile complex mixtures, without derivatization steps or complex sample preparation procedures. In hyphenation with spectroscopic detectors (mainly photodiode array, PDA, and mass spectrometers, MS), spectra holding structural information (molecular weights, molecular formula, and characteristic UV absorption bands) can be recorded on-line after HPLC separation of the analytes of interest [83]. This approach provides an efficient and sensitive identification of known constituents (dereplication) provided that databases or standards are available.

For the rapid de novo identification of unknown analytes, advanced spectroscopic detectors—hyphenated either directly or indirectly to HPLC—are needed. In this respect, LC–NMR (LC–nuclear magnetic resonance) provides important complementary structural information to LC–PDA–MS, as de novo structural assignment on a given LC peak can be performed. Within a single analytical run, compounds are efficiently separated from a complex mixture; simultaneously, NMR spectra are recorded on each LC peak, and important data, such as chemical shifts, multiplicity, integrals, dynamic processes, and chemical exchange, are obtained. The interest of directly coupling HPLC with NMR resides not only in the fact that full structure elucidation and stereochemical information can be obtained (through two-dimensional NMR spectra) but also that it is a universal and nondestructive detection technique. LC–NMR can provide the comprehensive detection of any hydrogen-containing compound present in the HPLC eluate [84] and their absolute quantification. Additionally, since the method is nondestructive, it is possible to fully recover the sample. Coupling HPLC with NMR could thus be regarded as an ideal combination. However, the main bottleneck of this technology is its low intrinsic sensitivity compared to other HPLC detectors. To partly overcome this issue, many strategies that involve various operational modes in hyphenated systems or at-line coupling of the NMR to the HPLC with sample preconcentration methods, such as SPE–NMR (solid phase extraction–NMR) or tubeless technology (e.g., microflow NMR as CapNMR™) have again boosted the use of this powerful identification method. In this chapter, a brief overview of the methodology is provided and both on-line and at-line usage of sensitive NMR methods for LC peak identification and quantification are discussed.

2. Hyphenation of NMR with HPLC

In contrast to the hyphenation of HPLC with MS (LC-MS), the coupling of HPLC with NMR (LC-NMR) does not require specific interfaces. The heart of a LC–NMR system consists of a dedicated flow probe through which the eluent from the HPLC flows and delivers the separated analytes. Probes that can be used for flow injection and discussions of their inherent sensitivities are presented in the next section.

A typical LC-NMR setup consists of an HPLC system equipped with a sensitive UV or MS detector coupled to a NMR instrument (medium to high field strength: >400 MHz) equipped
with a flow probe. The flow cell of such a probe typically employs detection volumes between 40 and 120 μl, which are much larger than conventional UV detection volumes (8 μl) [85], because the NMR detector is much less sensitive than UV or MS detectors. As described later, smaller NMR detection volumes are available, but these are not routinely used for on-line HPLC detection. Large detection volumes for LC–NMR therefore represent a compromise between the needs of chromatography (small volume for good resolution) and those of NMR (large volume for line-shape quality and sensitivity).

Figure I.B.1: Summary of the different ways to hyphenate NMR with HPLC either on-line or at-line. (a) On-flow LC-NMR in a hyphenated system with PDA and MS detection; (b) LC–SPE–NMR with postcolumn addition of H$_2$O for trapping drying and reinjection in a LC–NMR flow cell; (c) CapNMR detection after HPLC microfractionation. Source: Figure reproduced with permission from CRC Press from [86].

In a typical LC–NMR setup, a valve is placed between the sensitive LC detector (UV or MS) and the NMR flow probe. This valve is triggered by the sensitive detector in order to stop the LC flow when the analyte of interest reaches the NMR flow probe after a calibrated delay time, Figure I.B.1 (a). This mode of operation, called stop-flow, is used when a high number of transients needs to be acquired to improve the signal-to-noise (S/N) ratio for a given analyte. In some setups, the stop-flow valve can be replaced by a loop collector that automatically collects the peaks of interest without stopping the LC flow. Off-line postchromatographic
analysis of the content of the loops is performed automatically in LC–NMR [87] and [88]. With high quantities of analyte (typically a few tens of μg per LC peak), the LC-NMR analysis can be performed in on-flow mode. In this case, the LC eluent flows directly through the probe and the NMR spectra are recorded in real time during a single analytical HPLC separation. However, this approach, while being simple, is rather limited in terms of sensitivity and achievable spectral quality.

3. **Advances in NMR sensitivity**

As just described, the major problem in LC–NMR is the intrinsic low sensitivity of the NMR detection. Currently, the successful use of NMR as a HPLC detector generally requires preconcentration of the chromatographic peak. Current state-of-the-art NMR can characterize low nanomole quantities of material [89]. Several factors, especially NMR probe design and the mode of operation, influence the ability to be able to make these measurements.

3.1 **Magnetic field**

The signal in an NMR experiment arises from the transition between low and high nuclear-spin energy states. In a spin 1/2 system (which includes $^1$H, $^{13}$C, $^{15}$N, and $^{31}$P, and so accounts for most biological nuclei) there are two possible energy levels, $\alpha$ and $\beta$, for a nuclear spin in a magnetic field. The resonant frequency, $\omega_0$, is given by the energy difference between the two energy levels and is linearly proportional to the applied magnetic field, $B_0$, according to the equation $\omega_0 = -\gamma B_0$, where $\gamma$ is the gyromagnetic ratio and is a fixed constant for a particular nucleus. The fundamental reason for low intrinsic sensitivity in NMR is the very small difference between energy levels. For example, a 14.1 T magnet produces a 600 MHz frequency for $^1$H. At room temperature, thermal energy is nearly five orders of magnitude larger than the energy associated with 600 MHz, and thus the two nuclear energy levels are almost equally populated, with the lowenergy nuclear state in excess of the upper (e.g., the polarization) by only 1 in 10,000 spins. The net result is that most of the potential NMR signal is canceled, and only the small excess is detected. Nuclear relaxation and detection efficiency complicate the relationship between S/N and magnetic field strength, but it is commonly accepted that the NMR S/N ratio increases between $B_0$ 1.5 and $B_0$ 1.75 [90]. Typical field strengths for LC–NMR are between 9.4 T (400 MHz) and 14.1 T (600 MHz). Shielded magnets, which allow for more compact installations with LC instrumentation, enable higher field NMR systems to be used.

To significantly improve the S/N in NMR, dynamic nuclear polarization (DNP) is an increasingly popular technique. The principle of DNP is to transfer the polarization of electrons to nuclei. Electrons are also spin 1/2, and they have much larger polarizations in a given magnetic field than nuclear spins. There are several approaches to DNP, but in all of them, the polarization of the electron is transferred to the nucleus, which results in a much larger nuclear polarization and S/N increases that can be more than 10,000-fold more than conventional NMR [91]. The major drawback to DNP is that the polarization lasts only as long as the T1
relaxation time of the polarized nucleus, and it takes several hours to build up the polarization. Therefore, with current technology, it is not possible to do DNP with on-line LC-NMR, but it is possible to use it for isolated LC peak identification and is enabling major advances in related applications.

Figure I.B.2: Demonstration of the benefits of smaller diameters and solenoid coils on NMR sensitivity.
Two-dimensional $^1$H–$^{15}$N HSQC data were collected under identical experimental conditions, using the same pulse sequences, the same number of scans, the same field strength ($14.1 \, T = 600 \, MHz \, ^1H$), and the same spectrometer. Moreover, both data sets have the same total amount of protein. (a) Spectrum collected with a home-built triple-resonance 2.5-mm solenoid coil with a sample volume of 60 μl and a protein concentration of 1 mM. (b) Spectrum collected using a commercial 5-mm standard saddle coil probe with a sample volume of 600 μl and a protein concentration of 0.1 mM. The traces are meant to give a better indication of S/N and were extracted at the $^{15}$N frequencies indicated. Complete details are in [92].
Source: Figure reproduced with permission from Elsevier from [92].

3.2 NMR probe design
Magnetic field strength has technical, economic, and practical upper limits, and DNP is currently accessible for only specialized applications. However, the probe is perhaps the most important component in the S/N and overall performance of an NMR experiment, and there
are three independent and relatively cost-effective ways that the probe can be optimized for S/N improvements in LC–NMR applications. First, the sample and coil diameter can be reduced; second, the coil and associated preamplifiers and other electrical components can be cryogenically cooled; third, the coils can be made from material that is capable of carrying a higher current density than traditional materials like copper. Each of these is discussed in more detail. Overall sensitivity in NMR is influenced by many factors, including the magnet, probe, spectrometer electronics, pulse sequence, optimization of parameters, and data processing. Therefore, it is difficult to report absolute numbers, but at the end of this section, an overall comparison with respect to choice of probes is provided.

3.1 Smaller is better

The sensitivity of an NMR experiment increases in inverse proportion to the diameter of the coil [92]. Thus, the smaller the coil, the better is the performance. However, this obviously comes at the cost of reduced volume, so it is useful in performance comparisons to normalize the sensitivity by the mass of the sample. An example of a mass-sensitivity comparison is shown in Figure I.B.2, which compares NMR spectra between a commercial 5-mm probe with a saddle coil (Figure I.B.3) and a custom designed and fabricated 2.5-mm solenoid probe [92]. Each spectrum has the same mass of protein, but the concentrations and volumes differ by a factor of 10. This nicely demonstrates that S/N is improved with a smaller, solenoid coil (Figure I.B.2). Solenoid coils have their own advantage relative to saddle coils and can provide up to two or three times greater sensitivity [90]. The differences in Figure I.B.2 are from both the diameter and the geometry of the coil.

For mass-limited samples such as HPLC peaks, the best sensitivity arises from samples that can be concentrated into a small volume and measured in a small diameter probe. Solenoid probes are naturally suited for LC–NMR, because samples need to be added by flow or by removing the probe and inserting a sealed glass tube, but this latter solution is impractical both in convenience and also by the fact that air bubbles are difficult to remove from small sealed capillary tubes. The CapNMR™ probe is a good example of a solenoid loaded by flow, which can be manual or interfaced to an LC system. One major advantage of solenoid probes is that the coil can be simultaneously tuned to multiple frequencies, allowing for near-optimal performance on different channels [92], and these are now available commercially from Protasis.

Small solenoid coils have another advantage: Multiple coils can be added to the same probe head, allowing for parallel detection [93]. This approach was initially developed by Webb and coworkers [94] for several applications, including monitoring continuous flow capillary electrophoresis [95] and a probe with eight coils for the simultaneous measurement of eight compounds [96]. A commercially available probe with two solenoid coils is available from Protasis [97].
I.B.1 NMR for identification and quantification

Figure I.B.3: Comparison of the several coil designs.
Samples for NMR analyses sit in the coils, which are located in the probe and tuned to deliver and detect radio frequencies necessary for NMR: (a) saddle coil, (b) solenoid, (c) Alderman–Grant coil, (d) high-temperature, superconducting (HTS) coil, with schematic of layout for an HTS triple resonance probe ($^1$H, $^{13}$C, $^{15}$N, and $^2$H lock frequencies). The upper coils (a, b, c) are made from copper-based materials. Saddle coils (a) are often used for $^1$H detection in top-loading probes and Alderman–Grant coils (c) are often used for decoupling coils. Coils a, c, and d are compatible with top-loading probes, while the solenoid (b) is most easily loaded with flow and thus is most naturally interfaced with liquid chromatography. The top-loading coils can also be used as LC detectors with an appropriate tube with a flow cell and interfaced with LC tubing. Flow cells are commercially available for larger coil sizes, such as 5-mm probes. More details on HTS coils can be found in Brey et al., 2006 [98].

Source: Jason Kitchen from the National High Magnetic Field Laboratory drew a-c.

More-conventional saddle coils with top-loading samples are also available in reduced coil sizes, including several choices in the 2–3-mm range as well as a 1-mm probe from Bruker. All these give improved performance for mass-limited samples when compared with a standard 5-mm probe. Most of these top-loading probes can also be equipped with a specialized flow cell that interfaces with tubing from an LC instrument, but as far as we are aware, the smallest diameter vertical flow cells (e.g., 1 mm) have not been made.

3.2 Cryogenic probes
Perhaps the most important technical advance in NMR probes has been cryogenic cooling of the coils and associated electronics [99]. By reducing the noise of the measurement, cryogenic
probes offer up to about four times greater S/N than their room-temperature counterparts. They are commercially available in many sizes and configurations, including 5-mm, smaller volumes, and with $^1$H, $^{13}$C, or $^{15}$N, or other nuclei optimized for detection. Cryogenic probes are considerably more expensive than room-temperature probes; they require more infrastructure and maintenance than conventional probes; and they are cumbersome to change, making them a poor choice for multiuser–multineed facilities. However, a 5-mm cryogenic probe with a flow cell is a very good approach for an NMR detector in a dedicated LC-NMR system. In principle, cryogenic cooling of solenoid coils add the same benefit as vertical coils while retaining the many advantages of solenoids, but this configuration is technically very challenging and, to our knowledge, has not been attempted or implemented.

3.3 High-temperature superconducting coils

All the probes discussed already are constructed with conventional copper-based conductors, Figure I.B.3(a–c). An alternate design is the use of a high-temperature, superconducting (HTS) material such as yttrium barium copper oxide, YBCO, Figure I.B.3(d). When cooled in a cryogenic system, these conductors have much better quality factors (Q) than copper wire probes, resulting in high current densities and large B1 fields. All this translates to increased sensitivity, and with the cryogenic cooling and reduced noise, HTS coils offer the best S/N available in commercially available probes. Commercial vendors are typically quite secretive about the coils used in a particular probe, but HTS coils are currently being used and offer the highest S/N in a particular configuration. As shown in Figure I.B.3(d), HTS probes are loaded from the top and are thus most often used with tubes and typically to characterize isolated LC peaks. Because of the geometric constraints of HTS construction, Figure I.B.3(d), HTS probes have relatively small diameters, but it is possible that larger-diameter probes will be available in the future with new technological advances. HTS probes could be used with flow-cell inserts, but this is difficult with small-diameter probes. The HTS probe advantages of the highest sensitivity are at least partially offset by high cost, the same cost and relative inflexibility as conventional cryogenic systems described previously, and difficulty in manufacturing and cost of repair and replacement of coils. The same principles already described about size of coils apply to HTS probes: The smallest coils have the highest mass sensitivity. The NHMFL group in collaboration with Bruker designed and built the smallest, a 1-mm HTS probe [98]; small-diameter HTS probes are available through both major NMR vendors.

3.4 Sample amounts typically analyzable according to the probes and the magnet field

As mentioned, NMR sensitivity depends on many factors, but some guidelines about what to expect can be given. A standard 5-mm room-temperature probe with a saddle coil serves as the least-sensitive baseline for comparison. Using this probe, one can expect good performance with 600 μl of a 1-mM solution. For a molecule with a molecular weight of 500 Da, this would be 300 μg of sample. In nonconductive solvents, a standard 5-mm cryogenic
probe provides up to four times greater S/N than its room-temperature counterpart, so only 75 μg is required for the same S/N as the room-temperature probe baseline. A 1-mm HTS cryogenic probe has approximately five times greater mass sensitivity than a 5-mm cryogenic probe [98], so this probe would provide the same S/N as our comparison on just 15 μg of sample, but this material needs to be dissolved in about 10 μl of solvent, in comparison to 600 μl of solvent for the 5-mm probes. Therefore, small volume coils are good for mass-limited but not concentration-limited samples.

Comparisons between a flow solenoid and top-loading tube coil are complicated by the volume requirement needed to get the sample into the coil. The 1-mm CapNMR™ solenoid probes have active sample volumes of approximately 1.5 μl but require about 5 μl for analysis [100]. In a comparison between the 1-mm HTS probe [98] and the CapNMR™ probe, it was found that the two probes had similar mass sensitivity in the active volume but that the 1-mm HTS probe with a tube had about three times greater mass sensitivity for the total amount of solvent required. In practice, both small-volume probes can deliver outstanding results if samples are not limited by solubility. For example, on a 600-MHz system, COSY NMR spectra can be acquired using the CapNMR™ system with less than 10 μg in about 1 hour, 13C–HSQC with 30 μg in 5 hours, and 70 μg for 13C–HMBC in about 15 hours. Direct observation of 13C–NMR data can already be obtained with 200 μg of NPs [43]. Using the 1-mm HTS probe, high-quality 13C–HMBC (at natural abundance) was obtained on 15 μg of a compound with a molecular weight of over 900 Da in 48 hours [89].

3.5 Strategies for obtaining NMR information from a given LC peak

As discussed, NMR sensitivity represents a key issue in a LC–NMR experiment. On a given instrument, different chromatographic strategies or mode of operation has to be used to place the highest possible number of NMR-active nuclei of an analyte in the center of the flow probe without compromising too much the chromatographic resolution while also keeping the analyte fully soluble in the flow cell. Thus, according to the needs, LC–NMR can be operated in direct or indirect hyphenation and the analytes may be concentrated prior to detection with approaches such as SPE–NMR or with other tubeless probes such as microflow NMR (Figure I.B.4).

4. Direct LC–NMR hyphenation (On-flow/stop-flow LC–NMR)

The on-flow mode is the simplest approach in LC–NMR analysis, Figure I.B.4(a), for the measurement of 1H–NMR spectra of the main compounds in a complex mixture. For this approach, analytical HPLC columns providing relatively high loading capacity are preferred, either large diameter (up to 10 mm) or long columns (e.g., 250 mm). In direct hyphenation with NMR, most separations are performed in the reversed-phase mode and, for facilitating solvent suppression (see later), H₂O is replaced by D₂O. The crude mixtures are dissolved in an appropriate deuterated solvent and relatively large volumes are injected. When working with complex mixtures, such as crude plant extracts, the amounts injected on column may reach up to 20 to 50 mg.
Figure I.B.4: Different strategies for obtaining NMR information from LC-peaks. 
(a) On-line LC–NMR requires overloaded HPLC conditions, a two-dimensional contour plot of NMR signals during chromatographic separation is obtained, and only the main compounds 1–4 are detected. (b) At-line NMR after multiple injections of mixture in standard analytical conditions, LC peaks are either trapped on SPE cartridges or collected in tubes, $^1$H NMR of minor constituents, such as 5, can be obtained and, depending on the S/N, two-dimensional NMR spectra can be generated. (c) At-line
NMR after single injection provides $^1$H NMR spectra of main compounds only with sensitive probes, only a poor $S/N$ ratio is obtained for minor constituents. (d) At-line NMR after semipreparative LC, the same selectivity for semipreparative LC and for analytical can be kept with calculated gradient transfer, and higher sample loading is provided. (e) Semipreparative LC with MS triggered collection of LC peaks provides a selective detection of partially coeluting minor constituent, and sensitive NMR probes are needed for obtaining the corresponding NMR spectra.

With such quantities of sample, LC separation conditions are not optimal from a chromatography viewpoint and resolution is affected; however, these separation conditions provide reasonable NMR detection of the most abundant constituents of a mixture. During the HPLC separation, the on-flow $^1$H–NMR spectra are continuously recorded as a set of scans (transients) in discrete increments. The separation is typically performed at 1 ml/min flow rate. To allow for more signal averaging and improved sensitivity, the separation may also be performed at lower flow rates [21]. In this latter case, more transients per increment can be recorded. In the on-flow operation mode, only single $^1$H–NMR information for the main constituents of a mixture can be recorded, although in principle, rapid two-dimensional data could be obtained on abundant compounds, using techniques such as Frydman’s ultrafast methods [101]. The quantity of analytes detected combined with the short residence time in the probe cell are the limitations that affect the sensitivity. In this mode of operation, access to $^{13}$C–NMR spectra (sensitivity of about four orders of magnitude lower than $^1$H) is not practically feasible, but other nuclei, such as $^{19}$F, can be efficiently monitored [102].

In direct LC–NMR hyphenation, with the exception of water, nondeuterated solvents are used and most separations are performed in the gradient mode. For NMR detection, this requires that the nondeuterated solvent signals, which are several orders of magnitude more intense than those of the analytes, Figure I.B.5(b), have to be suppressed. This is technically challenging, because the solvent chemical shifts change according to the change of solvent composition during the gradient, Figure I.B.5(a).

### 4.1 Direct LC–NMR hyphenation (On-flow/stop-flow LC–NMR)

With state-of-the-art NMR technology, solvent suppression of nondeuterated signals can be efficiently performed by several solvent suppression techniques such as presaturation (NOESY presaturation), soft-pulse multiple irradiation, or WET (water-suppression enhanced through $T_2$ effects). The WET sequence, in particular, consists of a combination of pulsed field gradients, shaped rf pulses, and shifted laminar pulses, which can be used with $^{13}$C decoupling for an efficient removal of the $^{13}$C satellites within the solvent [103], Figure I.B.5(d). These solvent suppression sequences require knowledge of the frequency of the solvent signals. In an HPLC gradient elution, these frequencies continuously change during the run, Figure I.B.5(a). To solve this problem, an initial single transient NMR spectrum is acquired automatically prior to solvent suppression, and the frequency for solvent suppression is automatically determined for each time point in the HPLC run.
Figure I.B.5: Principle of on-flow LC–NMR measurements with the analysis of the enriched fraction of Orophea enneandra (Annonaceae).

(a) Typical on-flow LC–NMR experiment without solvent suppression, which highlights the shift of the solvent signal during gradient elution. (b) Spectrum recorded in MeCN:D$_2$O without solvent suppression, the peaks of the analytes are hardly visible. (c) On-flow LC–$^1$H NMR contour plot of the O. enneandra extract (2 mg injected) obtained after solvent suppression (WET sequence). All analyte signals appeared as dots. The signal of HOD is negative and continually shifted during the LC gradient. HPLC conditions: Column Nova-Pak C18 (150 × 3.9 mm i.d., 4 μm); MeCN–D$_2$O gradient (20:80 to 95:5 in 50 min); 0.05% TFA; 1 ml/min. LC–NMR conditions: 24 scans/increment, flow cell (60 μL, 3 mm i.d.), 500 MHz. The UV on the side has been recorded with only 20 μg of extract injected. (d) On-flow LC–$^1$H- NMR spectrum of one of the main constituents (polycerasoidol) with regions where solvent suppression occurred highlighted.

Source: Figure reproduced with permission from CRC Press from [87].

The combination of highly loaded LC separations with automatically adapted solvent suppression enables the detection of the $^1$H–NMR resonances of all main analytes in a mixture. The results of this on-flow run are displayed in the form of a two-dimensional plot with NMR frequencies (in ppm) on one axis and time corresponding to the chromatographic separation on the other, Figures I.B.4(a) and I.B.5(c). This two-dimensional plot provides a good overview of the complexity of a mixture as well as preliminary information on the chemical nature of the constituents and their respective amounts. This is illustrated in Figure I.B.5(c) by the on-flow analysis of crude extract of the plant Orophea enneandra [104]. The $^1$H NMR spectra corresponding to the main LC peaks can be displayed, but the NMR signals occurring near to the solvent lines are not observable, as they are suppressed together with the solvent signal.
To obtain better sensitivity or additional structural information on a given LC peak, LC–NMR can be operated in the stop-flow mode [87]. In this case, as discussed previously, the flow is stopped when the peak of interest reaches the center of the flow cell. The residence time of the analytes can be controlled; this enables the acquisition of a high number of transients for minor constituents that are not detectable in the on-flow mode. Furthermore, two-dimensional NMR spectra of the most abundant constituents can be recorded as well, providing useful additional structural information. In contrast to on-flow approaches, all measurements are performed on a sample that is kept in the flow cell, and better line shape quality and more efficient solvent suppression can be obtained. However, diffusion problems may appear when the separation is performed under isocratic conditions. To avoid deterioration of the LC separation from multiple stops during elution, peaks can be automatically collected by loop storage during the LC separation and subsequently analyzed [87].

4.2 Indirect LC–NMR hyphenation (LC–SPE–NMR)

To overcome some major drawbacks of direct NMR hyphenation (use of expensive deuterated water for the separation, solvent suppression, overloaded LC columns), indirect hyphenation strategies were developed. In this context, the introduction of LC–SPE–NMR represented a major breakthrough [105] and [106].

The interface to hyphenate LC and NMR in LC–SPE–NMR, Figure I.B.1(b), consists of a solid-phase extraction system, while the flow probe used is similar to that of LC–NMR. An automated SPE fraction collector is connected to the flow probe via capillaries. After chromatographic separation, LC peaks triggered by UV or MS detection are trapped automatically on individual SPE cartridges, Figure I.B.1(b). Then, an adequate amount of water is added post column, and because of this change in the elution power of the eluent, the analytes of interest are retained on the SPE cartridges. To transfer the analytes to the NMR flow cell, the SPE cartridges are first dried with nitrogen then eluted with a suitable deuterated solvent that has sufficient elution power. LC–SPE–NMR has major advantages over conventional LC–NMR. Nondeuterated HPLC-grade solvents are used for the chromatographic separations. The transfer to the NMR flow cell requires only small volumes of about 300 μl of deuterated solvents, and no or weak solvent suppression is needed. The complete volume for sample elution, <30 μl, is close to the volume of the flow cell and this ensures that, for each LC peak, the whole amount of analyte is concentrated in the flow cell. For conventional LC–NMR operation, this is not always the case, since the elution volumes of LC peaks may surpass the detected NMR volume, resulting in sample dilution and thus compromise the sensitivity of the detection. Moreover, LC–SPE–NMR approaches can easily be used with tubes and small-volume, top-loading probes by using a robot to fill racks of tubes and introducing them to the instrument with an automated sample changer.

Multiple trapping of a given analyte on a specific SPE cartridge is another advantage of SPE–NMR, Figure I.B.4(b). Mixtures can be separated in optimal LC conditions without overloading,
Figure I.B.4(c), and the analytes are accumulated as a result of multiple separations of the same mixture and subsequent trapping. The analyte preconcentration provided by SPE–NMR as well as the possibility of multiple trapping substantially increase the NMR sensitivity, especially for minor compounds, and allow for multiple one- and two-dimensional NMR experiments to be acquired.

A limitation of LC–SPE–NMR is related to the need for a careful optimization of the trapping conditions on SPE cartridges. In this respect, the physicochemical properties of the analytes must be accounted for in selecting the best SPE trapping material and the nature and amount of the eluent. The transfer of the analyte as a sharp elution band from the SPE also depends strongly on the nature of the deuterated solvent used [107]. The whole LC–SPE–NMR operation can be automated in integrated state-of-the-art systems, which is extremely useful especially if multiple trapping is needed [108].

### 4.3 Microfractionation and at-line microNMR analysis

Another alternative for the NMR analysis of LC peaks by indirect hyphenation, Figure I.B.1(c), is to perform a postchromatographic analysis of the peaks collected from either a single analytical HPLC run, Figure I.B.4(c) major constituents, or a semipreparative separation with sensitive microflow NMR methods (CapNMR™) on the microfractionated and dried peaks, Figure I.B.4(d) minor constituents. For analyzing mixtures, the same HPLC procedure as described for LC–SPE–NMR can be used; however, in this case, the LC peaks are not trapped on SPE cartridges but simply collected into vials or in a microtiter plate. As with LC–SPE–NMR, a single or multiple collection of a given analyte is possible, but in this case, no optimization of the SPE trapping conditions is required, and all peaks are dried from the microtiter plate, usually by vacuum centrifugation, Figure I.B.4(c). Each sample can be manually or automatically dissolved in a volume slightly exceeding the volume of the flow cell (typically 6.5 μl for a microflow probe with a 5 μl flow-cell volume), filtered, injected into the probe, and parked in the center of the microcoil by pushing it with an adjusted push volume that depends on the length of the flow passing between the NMR and the injection system [43] and [109]. Alternatively, samples can be loaded into tubes for analysis in a top-loading probe. For automation, a liquid handler (similar to an HPLC autosampler hyphenated with high-precision micropumps) that delivers microliter flow rates of deuterated solvents can be used to precisely position microfractions in the center of the flow cell. The NMR spectrometer can then be triggered to perform the set of one- and two-dimensional NMR analysis required for each sample in the same microtiter plate. Thanks to the intrinsic sensitivity provided by the design of the microflow NMR probe (see previously) this represents a particularly attractive approach to enhancing NMR sensitivity for mass-limited sample uses [100]. In this respect, a thoroughly optimized automated droplet microfluidic NMR loading method (Microdroplet NMR) has been reported. It enables the performance of NMR off-line from LC–UV–MS; accommodates the disparity in sample mass and time requirements between MS and NMR, Figure I.B.4(e); and allows NMR spectra to be obtained retrospectively, after review of the LC–MS data [50]. With such an approach, interpretable one-dimensional NMR spectra were
obtained from analytes down to the 200 ng level in 1 h/well automated NMR data acquisitions. When applied to a cyanobacterial extract showing antibacterial activity, the platform recognized several previously known metabolites, down to the 1% level, in a single 30-μg injection, and highlighted one unknown for further study [50].

Figure I.B.6: Analysis of a series of adjacent microfractions (F43–F46) from the crude methanolic extract of Arabidopsis thaliana. 50 mg of the extract was separated on a C18 column (250 × 10.0 mm i.d.) with a linear MeCN:H2O gradient and microfractions were collected every 30 seconds. (a) Zoom into the 21 to 25 min microfractionation by LC-MS with display of the single ion trace ions of partially coeluting LC peaks. (b) 1H–CapNMR spectra recorded for the microfractions F43–F46. (c) HSQC CapNMR spectrum recorded on F46 containing a pure flavonoid triglycoside (about 50 μg on a 500 MHz NMR).

Figure I.B.6 shows an example of the use of CapNMR™ by the microfractionation of crude leaf extract of Arabidopsis thaliana. In this case, 50 mg of the crude methanolic extract of the aerial parts of the plant was separated on a large HPLC C18 column (250 × 10 mm i.d.) and the separation was monitored by MS. The CapNMR™ spectra were recorded for all dried microfractions collected every 30 seconds. The combination of the MS information obtained during the microfractionation, see the single ion trace in Figure I.B.6(a), and the CapNMR™ spectra, Figure I.B.6(b), enable the identification of the main flavonoid glycosides present. As an example, the two-dimensional NMR 1H–13C HSQC CapNMR™ spectra recorded for the
microfraction F46 is displayed, Figure I.B.6(c). In this case, the complete de novo structure identification could be performed based on a full set of one- and two-dimensional CapNMR™ spectra recorded on about 50 μg of the microfraction F46 in about 18 hours on a 500 MHz instrument.

The microflow NMR probe can also be used in direct LC–NMR hyphenation in the on-flow mode. In this case, since microflows are used, the whole separation can be performed in fully deuterated solvents. Such separation has been performed, for example, for the separation of essential bioactive isomers of the carotenoid bixin0020 [110]. In this case, the chromatographic separation utilized an isocratic elution method with mixtures of acetone-d₆ and D₂O (acetone-d₆/D₂O = 92:8 v/v) by injecting 500 nl of the sample on a capillary C30 column (150 mm × 250 μm). The method enabled the recording of high-quality stop-flow microflow ¹H NMR spectra with limited amounts of mixture. However, in this case, high peak concentrations similar to those obtained by postchromatographic analysis of dried microfractions could not be attained.

In most microflow NMR applications, the analysis of LC peaks are performed at-line by postchromatographic NMR analysis of the peaks transferred by microfluidics to the microflow probe [111]. In this respect, other sensitive microNMR methods, such as those involving microtubes, can also be used.

For at-line NMR analysis, the transfer from HPLC peak collection is therefore not necessarily made with fluidics. Microprobes using disposable 1-mm capillary sample tubes with a sample volume of 5 μl, similar to that of microflow probes, also represent an interesting approach for the analysis of limited amounts of analytes collected from HPLC separation [112]. In this case, the sample needs to be transferred from the dried collected fraction to the microtubes, and this part is less easy to automate than with tubeless approaches. However, the advantage of working with capillary microtubes is that the samples do not interact with the probe itself, and problems related to bad dissolution of samples as precipitation and clogging are avoided. This approach can be used with any probe with coils larger than the tube size, and in general, smaller sample volumes offer NMR sensitivity improvements.

An additional advantage of using microtubes is that the probe can be cryogenically cooled, and small-volume microprobes are commercially available (e.g., a 1.7-mm cryogenic probe from Bruker). With such a setup, high sensitivities can be attained, and the two-dimensional NMR of LC peaks obtained from a single conventional analytical HPLC run is practically feasible [113]. Essentially any probe with a diameter big enough to accommodate the microtubes can be used, including those optimized for ¹³C detection.

4.4 Practical considerations for NMR detection on microgram amounts of sample

When performing an HPLC separation prior to NMR detection, HPLC solvents have to be selected with great care; most HPLC-grade solvents are not NMR grade, and protonated
impurities might interfere. This becomes especially important with any high-sensitivity NMR probe. For at-line applications, according to the type of stationary phase used, tiny amounts of the packing material might bleed and generate signals visible in the $^1$H–NMR spectra. The intensity of these impurity signals are further strengthened by the concentration factor obtained either by SPE trapping or drying of microfractions. For this type of at-line measurement, drying of the samples, either on SPE or in microtiter plates, has to be very efficiently done, and hygroscopic deuterated solvents need to be stored under inert gas to avoid residual water. Contamination can also come from the vials or the microtiter plate used for collecting microfractions or the SPE for SPE–NMR. The quality and the background NMR signals generated by these devices has to be tested according to the conditions and solvents used for measurements, and control NMR spectra with just solvent blanks are always recommended.

5. **Integration with a multiple detection system (LC-NMR-MS)**

For *de novo* on-line or at-line structure determination, NMR data alone are often insufficient, and different spectroscopic information has to be gathered. In this respect, UV, IR and MS data can be recorded on-line with HPLC. However, the specific needs for all these detectors are different, and while a comprehensive hyphenation of all these spectroscopic detectors in addition to NMR is possible [114], it is usually not optimal if the highest spectral quality has to be obtained from each hyphenated method. The most robust systems, which have also evolved into completely automated systems, are those involving LC–NMR–MS [88]. In this case, the MS is used to trigger the trapping of the LC peak of interest for subsequent NMR measurements. With the advent of shielded magnets, this is now possible, because the MS instrument can be physically placed close to the center of the NMR magnet. The main problems for such platforms arise from sample overloading into the MS systems and a shift of the molecular ion species due to proton–deuterium exchange reactions. This, however, can be solved by using an efficient postcolumn splitter, which diverts a small portion of the flow into the mass spectrometer and enables proton–deuterium back exchange by dilution with an appropriate makeup flow. The great advantage of LC–NMR–MS is that the MS data can be directly correlated to the acquired NMR spectra for a given LC peak. Furthermore, the MS detection can be used to deconvolute coeluting components facilitating thus the interpretation of the data obtained in subsequent microfractions, see Figure I.B.1.

6. **Quantification capabilities**

NMR as a detector offers also the possibility to get an absolute quantitative result. The signal response—in contrast to the classical detectors of chromatography, UV, and mass spectrometry—is independent of the chemical nature of the analyte, and therefore, no authentic reference material is needed for quantification. Another considerable advantage is that, in addition to the quantitative information, the full qualitative information about the structure of the analytes is provided. This aspect can be important in direct LC–NMR
hyphenation but also in indirect hyphenation of NMR with HPLC to estimate the amount of an unknown compound without need of its purified form.

### 6.1 General considerations on quantitative NMR

A given NMR signal is proportional to the number of nuclei giving rise to it, as long as the experimental conditions are well defined. For reliable and accurate quantitative results, it is important that the nuclei under investigation are fully relaxed, and a pulse recycle delay (relaxation delay + acquisition time) of five times the spin lattice relaxation time ($5 \times T_1$) is usually recommended. Moreover, an adequate S/N ratio and a properly defined baseline are indispensable for reproducible integration, and differential saturation or NOE enhancements have to be avoided [115]. Finally, data processing window functions need to be chosen with care, and the first point needs to be equal to 1 to obtain reliable results; this can be achieved with exponential and cosine functions, but other common window functions, such as Gaussian, do not work.

Absolute sample quantities or concentrations cannot be determined by NMR directly. Therefore, the NMR response has to be related to a calibrant. Typically, an internal standard of known concentration is added to the sample. An adequate internal standard should be chemically stable and inert, inexpensive, nonhygroscopic, and nonvolatile, available in high purity; their signals should not interfere with the signals of the analyte and be soluble in the NMR solvent used [116]. Commonly, compounds with simple $^1$H–NMR spectra, preferably one singlet only, are used. The recommended standard for all biological samples is DSS (sodium 3–(trimethylsilyl)–1–propanesulfonate), because it is relatively insensitive to pH and temperature.

### 6.2 Methods for quantification in on-line and at-line LC–NMR

A given LC peak can be detected either by NMR immediately after the separation by on-flow LC–NMR or individual fractions can be collected/concentrated (either in tubes or on SPE cartridges) for at-line LC–NMR analysis.

Few applications of quantitative on-flow LC–NMR have been described. Dedicated NMR flow probes are used as described previously. The quantification is done internally and depends on whether gradient or isocratic LC is applied. In the isocratic mode, an internal standard—preferably eluting at the end of the chromatographic run—is added to the sample and the signal integrations are compared. In the gradient mode, the reference standard has to be added to the mobile phase to compensate for the changing solvent composition, which influences the resonance signals. In this case, the signal of the internal standard must not interfere with the signals of the analytes [117]. The relaxation delay has to be carefully chosen to allow for complete relaxation of the nuclei used for quantification.

In the case of at-line LC-NMR analysis, no restriction is placed on the type of probe to be used, and quantification can be performed with classical quantitative NMR (qNMR) methods. Three main types can be distinguished: internal or external standards or reference to an electronic
signal. In this last case, special hardware and software is needed for electronic referencing (ERETIC and QUANTAS [118] and [119]).

If internal standardization is chosen, the sample is spiked with the internal standard (IS) of known concentration. By comparison of the integrals of the NMR signals of the analyte and the IS, the concentration of the analyte can be calculated. An accuracy of as low as 0.5% can be obtained with this method [116], which is among the best that can be achieved with qNMR. A major drawback is that an exogenous compound is added to the sample, which might be unwanted if the sample is, for example, used for biological testing after NMR analysis. To overcome this issue, internal standardization using the NMR solvent signals recently were developed. Micro- and millimolar amounts of sample were quantified based on comparison with the residual solvent signal of DMSO-$d_5$ in DMSO-$d_6$ (deuterated dimethylsulfoxide) [120], while nanomolar amounts were estimated by referencing to the $^{13}$C satellites $^{13}$CHCl$_3$ in CDCl$_3$ in high-sensitivity NMR probes [89].

For applications where addition of an internal standard is undesired, external standardization is an alternative. A reference sample is measured separately under the same conditions as the analytes. The sample-dependent pulse width (360° pulse) is determined for the reference compound and the analytes, and the absolute intensities of the NMR signals are compared. The concentration of the analyte is calculated, taking into account differences in pulse width, temperature, and gain. Wider and Dreier [121] show that the values from the reference sample stay constant for several months and an accuracy lower than 2% can be achieved.

7. **Fields of application**

The unambiguous structural assignment of a given analyte in a mixture is essential in the large majority of applications that deal with HPLC profiling studies. This need is even more striking with the analytes that are not easily accessible by synthesis for confirmation of dereplication results obtained by sensitive methods, such as LC–MS or LC–MS/MS. Consequently, on-line or at-line LC–NMR have been used mainly to solve challenging issues for the rapid identification of natural products in various complex crude extracts or of metabolites in body fluids. On-flow LC–NMR is now used mainly for the characterization of products that are unstable in their pure form, while otherwise, most at-line methods are preferred for obtaining higher quality spectra. Some of the applications to these various fields are summarized in Table I.B.1. This, of course, is not exhaustive but a brief overview of the type of studies that can be performed.

7.1 **Dereplication and rapid *de novo* identification of natural products in complex extracts**

In natural products research, when searching for bioactive NPs, the crude extracts from plant or microorganism sources are classically first biologically screened and followed by activity-guided fractionation [122]. The chemical screening of the extracts constitutes an efficient and complementary approach for dereplication purposes and targeting the isolation of new promising bioactive constituents. This chemical screening is performed mainly by LC–PDA and
LC–MS or LC–MS/MS [123]. However, the lack of commercially available UV–PDA, LC–MS, or LC–MS/MS databases limits this procedure to be universal. In this respect, LC–NMR or at-line microNMR methods represent an important complement to unambiguously confirm the LC peak annotation for unusual NPs, mainly by \(^1\)H–NMR analysis. Furthermore, interpretation of a whole set of two-dimensional NMR experiments enables de novo structure elucidation of unknown compounds at the microgram scale.

Different reviews summarized the potential of LC–NMR and related techniques for the dereplication of plant metabolites [87], [109], [123] and [124]. Several applications related to the identification of NPs from marine sources at the nanomole scale have been achieved, mainly with low-volume tube probes and microflow probes [125]. Microflow NMR has been reported to be efficiently implemented in a high-throughput NP chemistry platform for the generation and analysis of large NP libraries [126]. In addition to crude extracts, other applications with limited biological samples have been reported that demonstrate the utility of NMR data for either dereplication or full identification of novel compounds. Some examples include the characterization of steroids from a few fireflies [127], induced plant defense hormones [71], identification of C. elegans mating pheromone [128], and stress-induced fungal metabolites at the Petri dish scale [30].

Since these mass-sensitive NMR applications provide detection at the microgram level, chromatographically isolated sample amounts are often below the limit of accurate weighing and were therefore quantified by qNMR. This, for example, was the case for fungal peptides from marine sources [61] and for macrolides isolated from a marine sponge [129]. Saponins from starfish [130] were directly quantified from on-flow LC–NMR experiments.

### 7.2 Analysis of unstable compounds

One advantage of on-flow LC–NMR is that it enables the structural analysis of a given LC peak without need of its physical isolation, which might cause degradation. The analysis of carotenoid stereoisomers from biological tissues, avoiding isomerization and oxidation was successfully performed on-flow either by conventional LC–NMR [131] or more recently by microflow on-flow methods [110]. In plant extracts, the study of unstable iridoid cinnamic esters, which undergo light-induced cis-trans isomerization of the cinnamic moieties and trans-esterification of the cinnamic moiety on their rhamnose unit, was demonstrated thanks to a combination of on-flow and stop-flow LC–NMR [132].
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$^1$ PLE-LC-SPE-NMR is pressurized liquid extraction-solid phase extraction coupled to LC-NMR.
7.3 Metabolite identification in metabolomics

Plant metabolomics is a new field in which sensitive NMR approaches are starting to play an important role. While conventional NMR methods have been widely used for the rapid and direct profiling of biological fluid analysis [148], microNMR methods have started to be applied with the advantage of being applicable to restricted amounts of samples [149]. At-line microflow NMR methods can also be an efficient complement to MS–metabolomics approaches for de novo identification of low-abundance biomarkers. Indeed, the unambiguous characterization of biomarkers highlighted by such approaches often represents a bottleneck to the whole process [150]. In this case, and provided that enough sample is available, an MS-triggered microisolation of the biomarkers of interest revealed by MS–metabolomics can be performed after transfer of the LC–MS conditions to the semipreparative level. With such an approach, low abundance wound-induced jasmonate derivatives targeted by a metabolomic study in Arabidopsis thaliana leaves were fully characterized de novo [71].

7.4 Metabolite identification in body fluids

LC–NMR has been primarily used for the identification of drug metabolites and in stability tests for the structure elucidation of metabolic products in the development of new pharmaceuticals. Drug metabolites have been analyzed using LC–NMR in blood or urine samples [150]. For example, fluorinated metabolites of the HIV-1 reverse transcriptase inhibitor 5-chloro-1-(2',3’ dideoxy-3'-fluoro-erythro-pentofuranosyl) uracil were identified using LC–19F–NMR and MS [102]. LC–MS–SPE–NMR was used in another study to confirm futile metabolic deacetylation that took place in human urine after administration of phenacetin-(CH₃)-H-2 [136].

With the new regulations on the safety testing of drug metabolites (MIST, Metabolites in Safety Testing) issued by the FDA, at-line LC–qNMR is applied to drug metabolites isolated from pooled liver microsome samples of preclinical species [151]. Recently, qNMR using ¹⁹F (or ¹H) has emerged as a tool to obtain mass balance in drug discovery (yield determination, reaction monitoring) and metabolism studies to replace traditional chromatographic techniques [152] and [153].

7.5 Identification of pharmaceutical impurities

Another field of application that requires high NMR sensitivity is the characterization of impurities in formulated pharmaceuticals [154]. In this respect, the identification of the degradation products of a cephalosporin antibiotic was successfully achieved by a combination of LC–NMR, LC–MS and LC–IR without complicated isolation or purification processes [155]. The chemical structures of three degradation products of loxoprofen sodium hydrate were elucidated by LC–MS and pressurized liquid extraction (PLE) coupled to SPE and LC–NMR (PLE–SPE–LC–NMR). With PLE, the low level of the degradation products in the formulation could be concentrated in less time than in conventional techniques [144], where
the loop storage procedure was used in LC–NMR analysis. $^1$H–NMR loxoprofen spectrum was compared with the spectra of the degradation products and their structures were elucidated.

In a commercial erythromycin formulation, pseudoerythromycin not previously reported in the literature was identified. The most characteristic signal and J coupling were observed in LC–NMR analysis. The whole structure was determined in a separate at-line NMR experiment using conventional one- and two-dimensional NMR spectra [143].

LC–NMR was also efficient in the identification of bulk drug impurities present in manufactured lots of a novel NK1 antagonist (vestipitant). The information obtained in LC–NMR analysis was crucial to the understanding of the synthetic chemical process route.
robustness and the developability of the drug. Information on the major unidentified impurities was obtained after LC–NMR analysis (Figure I.B.7) of the mother liquor sample enriched in impurities that are normally retained in very low amount in the final drug after crystallization. High-resolution $^1$H, $^{19}$F and $^{13}$C–NMR spectra were acquired in the stop-flow mode. Further structural confirmation was possible with $^1$H–$^{13}$C two-dimensional NMR as well as $^1$H–$^{15}$N two-dimensional NMR [156].

8. **Conclusion**

The remarkable increase in sensitivity related to the development of new probe technology and access to higher magnetic-field strengths have boosted the use of NMR for the characterization and quantification of analytes related to a given analytical HPLC peak. Developments in LC–NMR over the past 20 years have transformed the technique from its academic inception to integration as an important component of chemical and biochemical analysis. As discussed, depending on the problem, several strategies may be followed to obtain NMR information for a given LC peak. On-flow NMR analyses have resulted in a number of successful applications that rapidly provide NMR information on major LC peaks in a mixture. The number of applications with this direct hyphenation approach has decreased, since on-flow LC–NMR yields only one-dimensional $^1$H NMR spectra; moreover, compromises for solvent suppression and chemical shifts that are dependent of solvent mixtures composition are required. However, the techniques are very useful for the analysis of labile compounds. LC–NMR has thus evolved toward the efficient LC–SPE–NMR–MS platforms that solved most of the problems associated with the direct use of LC–NMR and also provided access to key two-dimensional NMR experiments via enrichment by multiple collections of given LC peaks. Progress in probe technology and especially the development of microflow probes considerably enhance the detection sensitivity. The use of such probes in relation to efficient LC–MS-triggered microfractionation and automated injection from 96-well plates is very useful for highly soluble analytes. For larger sample volumes, sensitive detection with cryogenic probes is optimal, but the cost associated with such technology has to be taken into account. The development of sensitive NMR approaches is still ongoing and the advent of HTS probes in this field and the implementation of efficient polarization transfer methods also might become a very interesting alternative for chromatographers who need to identify a given LC peak at high sensitivity.

To generate high-quality NMR data, HPLC high-resolution separations need to be further advanced. In this respect, the development of columns packed with sub-2-μm particles is of high interest. Such columns, however, are used at present for the separation of very limited quantities of sample; very-efficient LC columns that enable higher sample loading will significantly improve LC–NMR applications. For coeluting analytes or unfractionated mixtures, several approaches at the interface between metabolomics and traditional natural products chemistry are available, as recently reviewed [122].

It is safe to assume that NMR methods in relation with HPLC profiling of complex mixtures will continue to evolve and provide even more sensitivity and high resolution in both the
chromatographic and spectroscopic dimensions. The bottleneck of metabolite profiling, however, will continue to reside in the unambiguous identification of the analytes detected; and in this respect, NMR remains a critical technology for *de novo* identification of unknown LC peaks. It seems likely that future developments in the field of LC–NMR will come about as a result of continued increases in sensitivity and throughput.

9. **Acknowledgments**

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1.C On-line and at-line LC-NMR and related micro NMR methods in natural product analysis

This chapter was accepted as book chapter.


Contribution: Creation of the table on LC-NMR applications, design and layout of all figures, writing of the sections on quantitative NMR and bioactivity-profiling, proofreading of the article and preparation for submission
Abstract

Nuclear magnetic resonance spectroscopy (NMR) is essential for the *de novo* structure identification of natural products. This technique can be directly or indirectly hyphenated to high performance liquid chromatography (HPLC) leading to efficient LC-NMR platforms. NMR is, however, a very insensitive detection method compared to mass spectrometry and several strategies for its efficient hyphenation with HPLC have been developed. This includes on-line LC-NMR, SPE-NMR, or other at-line approaches involving microflow probes or microtubes in combination with cryoprobes. All of these various technologies and their potentials and limitations in the field of natural product research are described. Recent applications involving other complementary detection methods such as LC-MS or biological HPLC profiling illustrate its utilization.

**Keywords:** LC-NMR, SPE-NMR, microflow NMR, micro NMR, cryo NMR, natural products, phytochemical analysis, dereplication, metabolite profiling
1. Introduction

Metabolite profiling by different high performance liquid chromatography (HPLC) techniques, most notably HPLC-mass spectrometry (HPLC-MS) and HPLC-photo diode array (HPLC-PDA) detection or a combination of these techniques in HPLC-PDA-MS, are extremely useful tools for the early metabolite identification of natural products (NPs) on-line in very complex natural matrices. Recently, this process has been significantly improved by the increase in the use of high-resolution (HR) mass spectrometers, such as time-of-flight instruments (time-of-flight mass spectrometry, TOFMS) in the hyphenation with LC [157]. The high mass and spectral accuracies provided by LC-HRMS instruments offer now unambiguous molecular formula assignment when used with an adapted heuristic filtering procedure (Kind et al. 2007). This represents key steps for dereplication because NP research molecular formulae can be used in combination with chemotaxonomic information to generate putative structure attribution to a given LC peak [59].

Because of the lack of generic databases [especially LC-MS and LC-tandem mass spectrometry (LC-MS/MS) databases] that could provide efficient early metabolite identification of previously reported NPs, the identification of NPs in metabolite profiling studies based only on HRMS (high-resolution mass spectrometry) and MS/MS in combination with PDA spectra is not sufficient to unambiguously ascertain the structure of the analytes of interest. Recently, efforts regarding the prediction of retention time as support for dereplication [158] have also been made but none of these LC approaches can claim a complete on-line structural identification without access to standards or specific databases for confirmation of the metabolite identification.

In this respect, metabolite identification must rely on nuclear magnetic resonance (NMR), which is known to provide key structural information on atom connectivities that are complementary to those provided by MS, such as the molecular formula. NMR is thus known to be an essential tool for the de novo identification of NPs, whereas structure identification relies on the use of exhaustive one-dimensional (1D) and two-dimensional (2D) homo- and heteronuclear NMR measurements [159]. Over the past three decades, NMR hardware and pulse sequences [160] have evolved considerably, especially the development of 2D NMR experiments, which has provided an efficient way to fully characterized NPs according to established schemes of spectral interpretation [161]. One-dimensional proton NMR (1H NMR) spectra are characteristic for a given compound, and the comparison of such data with those of previously reported compounds is key for the rapid dereplication of NPs.

For all these reasons and also because it is a highly nonselective detection technique, (1H NMR spectroscopy will detect any hydrogen-containing compound present in the HPLC eluent in a sufficient amount, regardless of its structure), NMR can be considered the ideal detector for HPLC hyphenation [84,87]. Coupling HPLC with NMR spectroscopy (LC-NMR) represents one of the most powerful methods for the separation and structural elucidation...
of NPs in mixtures [85]. Since the mid-1990s, LC-NMR has been established as a very efficient method for the on-line identification of organic molecules, and it was readily applied to the structural identification of NPs in crude plant extracts. The on-line coupling of HPLC with NMR, however, suffers from the very low intrinsic sensitivity of NMR. Additionally, as discussed herein, various strategies to provide high quality NMR spectra in hyphenation with HPLC, either on-line or at-line, are required for both efficient LC peak identification and absolute quantification purposes. In light of the needs of phytochemical analysis and plant metabolomics for both on-line identification and dereplication purposes, the possibilities and limitations of these approaches are discussed in this chapter.

1.1 Sensitivity and NMR probe design

Among all detectors for HPLC, NMR is by far the least sensitive. The improvements in sensitivity and resolution that have been achieved in NMR methods in recent years have strongly accelerated the pace at which NPs are identified. NMR magnets have steadily evolved in field strength and consoles in electronics, and over the past few years, in the development of more sensitive probe heads, such as microcoils or cryogenically cooled probe heads. Furthermore, the application of pulsed field gradients has resulted in significant changes [162].

1.1.1 Probe and coil design of NMR flow cells

From a historical viewpoint, the interest in combining separation methods with \(^1\)H NMR spectroscopy arose in the late 1970s [163]. Because of the inherent lack of sensitivity of NMR instruments at that time, the use of LC-NMR to solve analytical problems was delayed by almost two decades [84].

The introduction of LC-NMR as a detector for HPLC has to firstly rely on the development of flow probes through which the eluent from the HPLC has to enter, which enables the introduction of the analytes of interest as they are separated by HPLC. These on-flow NMR probes (or continuous-flow probes) consisted of a U-type glass tube fixed in the dewar of the NMR probe body. This design (‘saddle’-shaped geometry) was breaking the central symmetry of the conventional tubes setup. Even if no rotation could be applied in such a flow cell, the first application showed excellent resolution, approaching those registered with rotation of the NMR tube [164].

For on-line applications of LC-NMR, the volume of the described flow cell represented a compromise between the needs of chromatography and those of NMR. Indeed, this type of cell employs detection volumes between 40 and 120 µL, which are much larger than conventional UV detection cells (8 µL). LC-NMR is a volume-sensitive detection technique that requires the maximization of NMR-active nuclei by extension of the detection volume that has to match, as closely as possible, that of the elution volume of the HPLC peaks. Furthermore, the line shape in on-flow mode is directly related to the residence time of the analytes in the flow cell, and the measured signal half width increases with increasing flow rate [85].
Figure I.C.1: Comparison of different NMR tubes and NMR probe designs for the understanding of NMR sensitivity.

(a) On-scale illustration of the inner diameter of different NMR tubes ranging from the conventional 5-mm tube to the smallest available 1-mm microtube. (b) Illustration of a microflow NMR probe with a solenoid coil that has a sample volume of 1.5 µL (A), a conventional 5-mm saddle-type coil NMR probe (B) and a 3-mm NMR flow probe with a saddle-type coil (C). (c) Approximate time line and qualitative comparison of recent milestone NMR probe innovations. Mass sensitivity ($S_m$, linear 'y' scale) for $^1$H NMR of a hypothetical fixed-mass sample as a function of probe fill volume ($V_f$, note the logarithmic 'x' scale) for room-temperature probes (lower line) and cryoprobes (upper line) at a fixed field, $B_0$. Source: Molinski, 2010. NMR of natural products at the 'nanomole-scale'. Nat Prod Rep 27: 321-329. (d) Mathematic relations of probe design parameters and NMR sensitivity expressed as S/N.
As will be shown, the development of such a flow probe has permitted the direct practical detection of HPLC peaks by NMR and many applications to NP analysis have demonstrated the usefulness of this type of hyphenation. However, the efficiency of detection of such on-flow probes is optimized when the sample can be pre-concentrated using solid phase extraction (SPE) and eluted in deuterated solvents in the LC-SPE-NMR mode with an optimized elution volume to match that of the flow probe.

The necessity for compromise between the volume of the flow cell and the volume of the LC peak and the necessity for solvent suppression and other issues described in the following section have accelerated the development of other probes for at-line NMR measurement of microquantities of analytes collected from HPLC.

In this respect, reduced-diameter solenoid NMR coils represent a particularly attractive approach to enhancing NMR sensitivity for small-volume, mass-limited sample uses (Figure I.C.1b) [100]. The sensitivity of an NMR experiment increases in inverse proportion to the diameter of the coil [90]. Thus, for mass-limited samples, such as HPLC peaks, the best sensitivity arises from samples that can be concentrated into a small volume and measured with a small-diameter probe. For such small-diameter probes, solenoidal coils exhibit a several-fold enhancement in NMR sensitivity, when compared to that of the Helmholtz coils (Figure I.C.1b) as discussed in section 3.

Solenoid probes are naturally suited for LC-NMR because samples need to be added by flow, which can be manual or interfaced to an LC system. This approach has resulted in a commercially available solenoid probe called the CapNMR™ probe [97], which can be simultaneously tuned to multiple frequencies, allowing for near-optimal performance on different channels [92]. These small solenoid coils can also be added as multiple coils in the same probe head, allowing parallel detection and thus a higher analyte throughput [93].

1.1.2 Detection issues

Other than the design of the flow probes, another issue with HPLC-NMR hyphenation is the compatibility of the solvent with NMR detection. In NMR, deuterated solvents are used to record the signal of the analytes present in much smaller amounts than those of the solvent. In HPLC, the analytes are typically separated in an acetonitrile and water mixtures because most separations are carried out by reversed-phase (RP) chromatography [83]. The use of nondeuterated solvents represents a challenging issue for NMR detection, because the signals related to these solvents will be several orders of magnitude more intense than those of the analytes of interest. This problem has been overcome by the development of fast, reliable and powerful solvent suppression techniques such as presaturation (nuclear Overhauser enhancement spectroscopy (NOESY) presaturation), soft pulse multiple irradiation, or WET (water suppression enhanced through $T_1$ effects). The WET sequence, in particular, consists of a combination of pulsed field gradients, shaped radio frequency (rf) pulses, and shifted laminar pulses that can be used with carbon-13 ($^{13}$C) decoupling for efficient removal of the $^{13}$C satellites within the solvent [103]. This type of solvent suppression provides good cancellation of the solvent signal and multiple lines can
be suppressed as shown for the analysis of ion-pair chromatography, where many lines must be removed [165]. This solvent suppression sequence provides a very selective and efficient suppression; therefore, the maximum receiver gain can be used to maximize the detection of the analyte signals. The analyte signals would otherwise not be detectable in the spectra without suppression because of the restricted dynamic range of the NMR.

However, one main issue is that NMR signals of analytes of interest resonating at the same frequency as acetonitrile (MeCN) or methanol (MeOH) will also be suppressed [87]. To minimize these effects and to obtain better quality spectra, the water is typically replaced by deuterated water in LC-NMR because it is not too costly. Some authors have also performed the entire LC-NMR separation using fully deuterated solvents. This can notably increase the cost of analysis when standard HPLC flow rates (1 mL min\(^{-1}\)) are used. However, the use of fully deuterated solvent in capillary HPLC application is perfectly compatible because of the low solvent consumption of such systems [166]. Furthermore, the continuous flow of sample in the detector coil complicates solvent suppression. To ensure satisfactory suppression, these pulse sequences require knowledge of the frequency of the solvent signals during HPLC gradient elution. And this requires that an initial single transient NMR spectrum is acquired automatically before solvent suppression and that the frequency for solvent suppression is automatically determined for each time point in the HPLC run. An example of the efficiency of solvent suppression is shown in Figure I.C.3a and b.

As discussed later, other ways of coupling NMR to HPLC, such as SPE-NMR or at-line micro NMR methods, exist that circumvent such problems and permit recording of spectra using fully deuterated solvents.

### 1.2 On-line versus at-line approaches for obtaining NMR spectra from an analyte of interest within a mixture

Because NMR sensitivity represents a key issue, different chromatographic strategies and/or modes of operation have been used to preconcentrate the maximum amount possible of an analyte for NMR detection (Figure I.C.2). The goal in such approaches is to place the highest possible number of NMR-active nuclei in the active volume of the detection probe. In direct hyphenation of LC-NMR, this can be performed by increasing the sample loading in HPLC; however, this might results in a loss of chromatographic resolution. This is acceptable only to a certain extent, depending on the chromatographic resolution required for the given analyses.

Therefore, in relation to the complexity of the mixture to be analyzed and the concentration of the LC peak required to be identified, LC-NMR can be operated in direct hyphenation or analytes may be preconcentrated before NMR detection with approaches such as SPE-NMR, microfractionation, drying and postchromatographic analysis in microflow NMR probes, or through the use of sensitive NMR probes with microtubes (Figure I.C.2). The postchromatographic analyses of microfractions after HPLC profiling...
require more automation or handling than direct hyphenation; however, the quality and the sensitivity of the NMR spectra can be better optimized.

<table>
<thead>
<tr>
<th>NMR type</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (A)</td>
<td>On-line information Good spectral quality</td>
<td>Poor sensitivity Solvent suppression</td>
</tr>
<tr>
<td>D (O + LC solvent)</td>
<td>On-line information Better spectral quality No flow stop during HPLC</td>
<td>Medium sensitivity Solvent suppression</td>
</tr>
<tr>
<td>E (O + LC solvent)</td>
<td>On-line information Better spectral quality No flow stop during HPLC</td>
<td>Medium sensitivity Solvent suppression</td>
</tr>
<tr>
<td>F (O + LC solvent)</td>
<td>Sample enrichment Good spectral quality Sample enrichment Good spectral quality</td>
<td>No direct automation Handling of micro-fractions Need for drying Possible clogging of the flow cell</td>
</tr>
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**Figure I.C.2: Modes of operation and advantages and disadvantages of the on-line and at-line LC-NMR approaches.**

Various modes of operation of liquid chromatography-nuclear magnetic resonance (LC-NMR) using direct (a-c) and indirect (d and e) hyphenation techniques. Indication of NMR probe type to be used with the different hyphenation approaches (A: microflow probe with solenoid coil; B: tube probe with saddle-type coil; and C: flow probe with saddle-type coil, see also Figure I.C.1). Advantages and disadvantages of the different LC-NMR techniques. Source: Wolfender, 2010. LC-NMR and related techniques for the rapid identification of plant metabolites, in High Performance Liquid Chromatography in Phytochemical Analysis, eds. M. Waksmdzka-Hajnos and J. Sherma, CRC Press, Boca Raton, pp. 287–329.
In this chapter, LC-NMR methods will be divided into two categories. The first type will group all strategies that provide spectra from NMR using direct hyphenation with HPLC with or without preconcentration. In the second category, applications of micro NMR methods to microfractions obtained after HPLC separation either by flow probes or by microtubes are discussed.

2. Direct hyphenation of HPLC with NMR

Direct hyphenation of HPLC and NMR allows the acquisition of NMR spectra either directly from the LC peaks as they elute from the HPLC column (on-flow mode) or by stopping the flow (stop-flow mode) using techniques such as collecting the peaks in loops (loop-storage mode) or trapping the peaks on SPE cartridges followed by further elution (SPE-NMR) (Figure I.C.2).

All of these modes of operation will be considered direct hyphenation of HPLC to NMR because an NMR instrument is physically connected to the HPLC even if the LC peaks are brought to the flow probe using automated enrichment procedures.

For all of these modes of operation, a typical LC-NMR setup consists of an HPLC system equipped with a sensitive UV or MS detector coupled to an NMR instrument (medium to high field strength: >400 MHz), which is equipped with a flow probe [167]. In this case, the LC-NMR flow probe of the saddle type employs detection volumes between 40 and 120 µL (Figure I.C.1). With such volumes, cryogenized flow probes are also available [168] that can provide better signal-to-noise (S/N) ratios. These large detection volumes are required for satisfactory line shape quality and sensitivity but may be detrimental to the quality of the chromatographic separation [85].

2.1 LC-NMR and LC-SPE-NMR

2.1.1 On-flow and stop-flow LC-NMR

Acquiring NMR spectra from eluting LC peaks in the on-flow mode is rather simple because no specific automation between the HPLC and NMR detector is required (Figure I.C.2a).

The separations are typically run using an RP gradient, and the water is replaced by deuterated water (D₂O) to enhance spectral quality. Because the organic modifier is not deuterated, solvent suppression is performed in situ, and the frequencies are calculated based on a single scan (scout scan), which precedes the set of scans that will be acquired to create an increment. Thus, ¹H NMR spectra are continuously recorded as a set of scans (transients) in discrete increments and generate the on-flow LC-NMR plot. Typically, and depending on the flow rate used (1 mL min⁻¹ down to 0.1 mL min⁻¹ [21]), scans will be accumulated as sets of 16-128 transients for most applications. Because a single scan will take roughly 1 s, the frequency of acquisition in on-flow operation will be between 20 s and 2-3 min.

In on-flow LC-NMR, these slow acquisition frequencies on the detector side will typically yield between one and four NMR spectra over a given LC-NMR peak. This will lead to very
low chromatographic resolution compared to LC-MS detection, where frequencies can reach 100 ms or less. The on-flow LC-NMR is not sensitive because of compromises related to the flow cell volume and the line shape, and the general spectral qualities are not optimum because of the short residence time in the probe cell and because of continuous changes in the solvent composition related to the use of chromatographic gradients.

This acquisition mode is, however, very useful for obtaining a rapid overview of the main constituents in an extract and for gaining a general idea of their quantities because NMR is an absolute quantification method.

The results of these on-flow runs are displayed in the form of a 2D plot with NMR frequencies (in parts per million, ppm) on one axis and the time corresponding to the chromatographic separation on the other. A typical LC-NMR 2D plot of a crude plant extract is displayed in Figure I.C.3a, where the dichloromethane extract of *Potamogeton lucens* L. (*Potamogetonaceae*) was analyzed. In this case, the LC-NMR analysis was performed at a rather low flow rate of 0.15 mL min\(^{-1}\), optimizing the number of scans per increment to increase sensitivity (number of transients 256). As shown on the 2D plot, several NMR regions of the main peaks could be highlighted, such as the signals of the furano moiety and the exocyclic methylene characteristics of the detected furano-ent-labdane (Figure I.C.3a). The corresponding LC \(^1\)H NMR spectrum of one of the main LC peaks is shown in Figure I.C.3b. This spectrum was acquired using the stop-flow mode to improve sensitivity and solvent suppression. On the 2D on-flow plot, at approximately 2 ppm, the residual signals of MeCN were still visible after solvent suppression, and the line corresponding to the HOD shifted with the gradient because of the change in solvent composition (Figure I.C.3a).

In this mode, the quality of the spectra obtained is not optimum, and only 1D \(^1\)H NMR spectra can be obtained using this technique; however, other nuclei, such as \(^{19}\)F, have been efficiently monitored by this technique [102]. This acquisition mode is the only one that provides true on-line detection, which has several advantages if unstable products have to be studied [132].

The sensitivity and the quality of spectra can be enhanced by simply stopping the flow rate when the analyte of interest reaches the center of the flow probe. This mode of operation is called stop-flow. In this case, long acquisitions can be performed, enhancing the S/N ratio and 2D NMR spectra, which allow mainly \(^1\)H-\(^1\)H correlation spectroscopy (COSY) or total correlation spectroscopy (TOCSY) to be recorded. To optimize the efficiency of the stop-flow measurement, a key aspect is to have a sensitive detector (UV or MS) before the NMR, which allows triggering of a valve (the stop valve) that will halt the HPLC eluent exactly when the analyte is passing through the NMR flow cell (Figure I.C.2b). This requires that a well-calibrated delay between the first detector and the NMR is determined. In the stop-flow mode, the quality of solvent suppression is generally superior to the on-flow mode because the eluent peak is measured in a fix composition. A typical stop-flow spectrum is shown in Figure I.C.3b. However, one drawback is that if many peaks in an extract have to
be analyzed during long 2D NMR experiments, the different stops will affect the quality of the LC separation especially if isocratic separations are performed.

To avoid this, the flow rate is stopped for the analysis of each LC peak during the stop-flow mode. It is also possible to collect the peak of interest in loops that have a volume that matches that of the LC-NMR flow cell (loop-storage mode) (Figure I.C.2c). In this case, a valve will trigger the collection of the LC peak in separate loops during the separation of the mixture. Each peak will then be analyzed post-chromatographically [88]. This mode is more practical than the stop-flow mode but requires more automation, whereas the sensitivities of the two techniques remain comparable [87].

Figure I.C.3: On-line LC-NMR versus at-line LC-NMR.
(a) On-flow LC-UV-\textsuperscript{1}H NMR analysis of the dichloromethane extract of Potamogeton lucens (10 mg injected). Flow rate: 0.15 mL min\textsuperscript{-1}, 256 scans increment\textsuperscript{-1}. (b) The \textsuperscript{1}H NMR spectrum of 1 was recorded in stop-flow mode with 512 scans (acquisition time 180 min). Source: Waridel et al., 2004. ent-Labdane glycosides from the aquatic plant Potamogeton lucens and analytical evaluation of the lipophilic extract constituents of various Potamogeton species. Phytochemistry 65: 945-954. (c) At-line LC-UV-NMR analysis of a mixture of 19 natural products of diverse polarity (50 mg injected). Flow rate: 4.7 mL min\textsuperscript{-1}. Fractions were collected every 20 s yielding 80 fractions in 30 min. (d) The \textsuperscript{1}H NMR spectrum of every fraction was recorded with 128 scans using automated injection from a 96-well plate with the OMNMR™ system. The spectrum of xanthotoxin is given as an example, the peak at 7.2 ppm belongs to the partially co-eluting naphthazarin, and the methoxy signals are from the partially co-eluting papaverine. Data and figures were kindly provided by Samuel Bertrand [169].
For all of these on-line approaches, analytical HPLC columns providing relatively high loading capacity are commonly used. These $C_{18}$ columns have a large diameter, up to 10 mm, a long length (e.g., 250 mm), or both [23]. In the RP mode, $H_2O$ is replaced by $D_2O$ to facilitate solvent suppression. The crude extracts or fractions are dissolved in an appropriate deuterated solvent ($DMSO-d_6$ or $CD_3OD$) and relatively large volumes are injected. When complex mixtures must be analyzed, 20-50 mg may be injected onto the column. Such high loadings may deteriorate the resolution of the LC separation when compared to standard analytical HPLC conditions; however, these separation conditions provide reasonable NMR detection of the most abundant constituents of a mixture [167].

### 2.1.2 LC-SPE-NMR

One of the major drawbacks of the direct hyphenation of HPLC to NMR is that the sensitivity is not optimum because the LC peaks are not very concentrated and elute in volumes that often exceed the volume of the flow cell. Furthermore, the chromatographic resolution is often compromised because of the need for high loading of the samples to achieve satisfactory sensitivity for the detection of the main compounds in the mixture. Another issue is that the spectra are recorded in a mixture of HPLC grade MeOH or MeCN with $D_2O$. This requires that efficient solvent suppression sequences are used, which might also suppress analyte signals. The NMR shifts also depend on the nature of the solvent and spectra recorded in the HPLC eluent of various compositions because the use of elution gradients will cause the comparisons of the chemical shifts with those of previously reported compounds in standard deuterated solvents to be difficult.

To overcome these problems, efficient methods that enable preconcentration of the analytes before detection in the LC-NMR flow cell and further elution in a fully deuterated solvent, have been developed. To date, the most used technique is LC-SPE-NMR [105,106,159] and the hyphenation of HPLC to NMR via SPE trapping is now fully automated through state-of-the-art NMR setups [88,159].

The development of LC-SPE-NMR resulted from the observation that an SPE cartridge could be used, post-column, as an analyte enrichment device [170]. To trap a given analyte separated by RP HPLC, an SPE cartridge installed postcolumn and preconditioned with a solvent of low elutotropic strength, such as $H_2O$ or $D_2O$, can be efficiently used. A sensitive detector used on-line (UV or MS) triggers the collection of a given analyte automatically on individual SPE cartridges. The cartridges can be used in a 96-well plate format for the collection of multiple peaks. Once the analyte flows through the SPE cartridge, it will be retained, provided enough water was added postcolumn for trapping. To transfer the analytes to the NMR flow cell, the SPE cartridges are first dried with nitrogen and then eluted with a suitable deuterated solvent that has sufficient elution power (Figures I.C.2d and Figure I.C.4b).
Figure I.C.4: An example of HPLC-SPE-NMR.
Workflow of HPLC-SPE-NMR exemplified by the analysis of isoflavonoids from Smirnowia iranica. (a) HPLC chromatogram of the ethanolic extract of S. iranica roots on a C18 column. The acetonitrile gradient profile in water is shown and the chromatogram shows average absorbance at 254 and 300 nm. (b) Principle of operation of the instrumentation used for HPLC-SPE-NMR. The separation is run at a flow rate of 0.8 mL min⁻¹ and water is added after UV detection for optimal trapping onto the SPE cartridge (C18 HD). The trapping of the individual peaks is triggered by UV detection. The cartridges are dried using a nitrogen gas flow for 30 min and eluted with 282 µL of deuterated acetonitrile for analysis in a 30-µL NMR flow probe. (c) One-dimensional spectra obtained in HPLC-SPE-NMR mode with peak 10 (glyasperin H) after one, three, and six trappings on the same SPE cartridge. (d) COSY spectrum obtained in HPLC-SPE-NMR mode after seven trappings with peak 10 (glyasperin H, total acquisition time 6 h 10 min). Source: Lambert et al., 2005b. Rapid extract dereplication using HPLC-SPE-NMR: Analysis of isoflavonoids from Smirnowia iranica. J Nat Prod 68: 1500-1509.

Such a process has different advantages. With this approach, the elution volumes of the LC peaks do not pose a problem compared to LC-NMR because the trapped peaks elute from the SPE cartridge in a fix volume of deuterated solvent. The elution volume of such SPE cartridges (10 x 2 mm i.d.) is 30-60 µL, which is close to the volume of a LC-NMR flow probe. This elution volume can even be reduced for mass-limited samples if smaller SPE cartridges...
are used (10 × 1 mm i.d.) and if detection is achieved using lower volume microflow NMR probes (5 µL flow cell) [134]. Owing to adequate matching between the elution and detection volumes, the NMR acquisition is performed on the entire amount of analyte in a given LC peak. Because the elution is performed in fully deuterated solvent, no solvent suppression is required (residual solvent signal resulting from an incomplete drying might require suppression). Contrary to LC-NMR, in LC-SPE-NMR, the entire mixture separation can be performed in standard HPLC grade solvents, and the transfer to the NMR flow cell requires only small volumes of deuterated solvents (ca. 300 µL). Instead of using LC-NMR flow probes, LC-SPE-NMR approaches can also be used with tubes and small-volume top-loading probes (Figure I.C.1a) using a robot to fill racks of tubes and introducing them into the instrument with an automated sample changer [167].

Typical LC-SPE-NMR analysis mixtures can be separated in optimal LC conditions without overloading, allowing for maximization of the HPLC resolution. This yields the trapping of only a few micrograms of the compound on the SPE cartridges; however, one of the advantages of SPE-NMR is that multiple trapping is possible. In this case, the HPLC separation of the same mixture is repeated several times, and a given analyte is accumulated using multiple subsequent trappings on a given SPE cartridge. The analyte preconcentration provided by SPE-NMR as well as the possibility of multiple trappings substantially increases the NMR sensitivity, especially for minor compounds, and allows for multiple 1D and 2D NMR experiments to be acquired [167].

An example of the effect of multiple trappings on sensitivity is illustrated by the analysis of isoflavonoids from *Smirnowia iranica* Sabeti (Fabaceae) shown in Figure I.C.4c. The $^1$H NMR spectra of glyasperin H (peak 10), a minor constituent in the HPLC-UV trace of the extracts, is shown in Figure I.C.4a and shows a significant enhancement in the S/N ratio after six trappings. As a result of this multiple trapping technique, 2D NMR spectra could be successfully recorded as shown by the COSY in Figure I.C.4d [171].

The main advantage of LC-SPE-NMR resides in the trapping of the analytes. To achieve good LC focusing by this method, the efficiency of SPE trapping has to be optimized so that the analyte elutes as a sharp band in the flow probe (Figure I.C.4b). This can be regarded as a limitation of LC-SPE-NMR because the trapping can be, to some extent, compound dependent. Thus, the physicochemical properties of the analytes must be accounted for when selecting the best SPE trapping material as well as the nature and amount of the eluent. Furthermore, the transfer of the analyte is also dependent on the nature of the deuterated solvent used [107]. For NP analysis, most of the trapping is performed on cartridges containing a divinylbenzene polymer or C$_{18}$ phase with 1-2 mL of H$_2$O as the LC-makeup flow. The large majority of neutral compounds can be efficiently trapped by this method; however, charged polar compounds, such as alkaloids or organic acids, might not be retained; therefore, other methods or materials, such as ion exchange or porous carbon, are required [159]. In multiple trapping experiments, the cartridges normally show a linear improvement in the S/N ratio up to approx. 100 µg. However, depending on the nature of
the analytes and the trapping material, the saturation of the SPE cartridge might also occur leading to poor S/N ratio [172].

The entire LC-SPE-NMR operation can be automated using integrated state-of-the-art systems that incorporate MS and UV detections, which are extremely useful, especially if multiple trapping is needed [108].

2.1.3 Multiple hyphenated systems integrating LC-NMR and automation

NMR represents an ideal detector for *de novo* on-line or at-line structure determination. The complete structure assignment of a given analyte requires gathering spectroscopic information using several different techniques, such as UV and MS spectra, and to some extent, infrared (IR) and circular dichroism (CD) spectra can also be recorded on-line during the LC-NMR experiment [173,174]. As a result of the introduction of shielded magnets, multiple hyphenation is efficient because the detectors can be physically placed close to the center of the NMR magnet. The multiple hyphenation of various spectroscopic detectors in addition to NMR is possible for online experiments [114]; however, it requires many compromises because of specific needs for detectors. The most efficient combination consists of the LC-NMR-MS platform [88], where the MS is used to trigger trapping of the LC peak of interest for subsequent NMR measurements and to generate complementary information for metabolite identification. The main issues with such platforms are sample overloading into the MS systems and a resulting shift of the molecular ion species because of proton-deuterium exchange reactions. However, this can be solved using an efficient postcolumn splitter, which diverts a small portion of the flow into the mass spectrometer and enables proton-deuterium back exchange by dilution with an appropriate make-up flow [167]. Efficient LC-SPE-NMR setups have also evolved toward advanced HPLC-PDA-MS-SPE-NMR platforms that can integrate CD measurements when on-line characterization of the chirality of the compounds is required [175].

Today, the combination of LC-MS in LC-SPE-NMR is completely integrated into the setup. The LC peaks are analyzed using MS before NMR detection, and the monitoring of the ions in MS during HPLC separation provides a precise MS trigger of the chromatographic peaks of interest [176]. The MS detection can be used to deconvolute co-eluting components, thus facilitating the interpretation of the obtained NMR data.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Compound class</th>
<th>NMR setup</th>
<th>NMR experiments</th>
<th>Solvent</th>
<th>Sample amounts</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angelica dahurica (Fisch. ex. Fisch. ex. Benth. Ex. Hook.)</td>
<td>Furanoacoumarins</td>
<td>500 MHz, cryo LC-NMR probe (60 µL flow cell)</td>
<td>1H NMR using on-flow, loco-tic conditions with a cryo probe, LC-NMR-MS, C30 column (250×4.6 mm, 3 µm).</td>
<td>D2O/CD3CN (0.5 mL min⁻¹)</td>
<td>2.5 mg of ethanolic extract (50 mg mL⁻¹, 50 µL injection)</td>
<td>[177]</td>
</tr>
<tr>
<td>Sophora flavescens Ait.</td>
<td>Flavonoids</td>
<td>500 MHz, cryo LC-NMR probe (60 µL flow cell)</td>
<td>1H NMR using on-flow, LC-NMR with a cryo probe, presaturation with WET.</td>
<td>D2O/CD3CN (0.5 mL min⁻¹)</td>
<td>1 mg of ethanolic extract (50 mg mL⁻¹, 20 µL injection)</td>
<td>[133]</td>
</tr>
<tr>
<td>Archangium cf. geephyra</td>
<td>Anthocyanins</td>
<td>600 MHz, cryo LC-NMR probe (120 µL flow cell)</td>
<td>1H NMR using stop-flow, LC-NMR with a cryo probe, no solvent suppression.</td>
<td>D2O/CD3CN (0.05% TFA)</td>
<td>Not specified</td>
<td>[142]</td>
</tr>
<tr>
<td>Cimicifuga heracleifolia</td>
<td>Caffeic acid derivatives</td>
<td>500 MHz, cryo LC-NMR probe (60 µL flow cell)</td>
<td>1H NMR using stop-flow and on-flow, presaturation during the relaxation delay, and mixing time at two frequencies.</td>
<td>D2O/CD3CN (+ 0.1% FA)</td>
<td>5 mg of buthanolic extract (100 mg mL⁻¹, 50 µL injection)</td>
<td>[179]</td>
</tr>
<tr>
<td>Petasites japonicus (Siebold &amp; Zucc.) Mami.</td>
<td>Flavonoids and quinic acids derivatives</td>
<td>500 MHz, cryo LC-NMR probe (60 µL flow cell)</td>
<td>1H NMR using stop-flow LC-NMR, presaturation with WET.</td>
<td>D2O/CD3CN (+ 0.25% FA)</td>
<td>2 mg of methanolic extract (100 mg mL⁻¹, 20 µL injection)</td>
<td>[180]</td>
</tr>
<tr>
<td>NMR setup</td>
<td>Sample</td>
<td>Compound class</td>
<td>NMR experiment</td>
<td>Elution volume and solvent</td>
<td>Sample amounts</td>
<td>References</td>
</tr>
<tr>
<td>-----------</td>
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</tr>
<tr>
<td><strong>600 MHz, LC-NMR probe (30 μL flow cell)</strong></td>
<td>Artemisia absinthium L. and commercial preparation of <em>A. absinthium</em></td>
<td>Sesquiterpene lactone, lignans and flavonoids</td>
<td>$^1$H and $^{13}$C NMR, COSY, HSQC, HMBC</td>
<td>CD$_3$CN (153 μL)</td>
<td>3x 0.3 mg of absinthin and degradation products (13 mg mL$^{-1}$, 20 μL injection)</td>
<td>[181]</td>
</tr>
<tr>
<td><strong>400 MHz, LC-NMR probe (30 μL flow cell)</strong></td>
<td><em>Artemisia absinthium</em> S. esquiterpene lactone, lignans and flavonoids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>600 MHz, cryo 3-mm LC-NMR probe (60 mL flow probe)</strong></td>
<td>Boronia megastigma (Nees)</td>
<td>Norisoprenoids, monoterpenoids and cucurbates</td>
<td>$^1$H and microNMR, COSY, TOCSY, NOESY, HSQC, HMBC</td>
<td>CD$_3$CN</td>
<td>Not specified</td>
<td>[183]</td>
</tr>
<tr>
<td><strong>600 MHz, cryo 5-mm NMR probe (cryofit 30 μL active volume)</strong></td>
<td><em>Myrica gale</em></td>
<td>C-methylated flavanones and dihydrochalcones</td>
<td>$^1$H NMR, COSY, HSQC, HMBC</td>
<td>CD$_3$CN</td>
<td>6x 1.2 mg of extract (200 mg mL$^{-1}$, 6 μL injection)</td>
<td>[184]</td>
</tr>
<tr>
<td><strong>500 MHz, cryo 5-mm NMR probe</strong></td>
<td><em>Anthriscus sylvestris</em> L. (Hoffm.)</td>
<td>Lignans</td>
<td>$^1$H NMR, NOE, COSY, NOESY, HSQC, HMBC</td>
<td>DMSO-$d_6$</td>
<td>Not specified (5 μL injection)</td>
<td>[185]</td>
</tr>
<tr>
<td><strong>400 MHz, LC-NMR probe (30 μL flow cell)</strong></td>
<td><em>Machilus philippinensis</em> Merr.</td>
<td>Glycosylated flavonoids</td>
<td>$^1$H and $^{13}$C NMR, COSY, NOESY, HSQC, HMBC</td>
<td>CD$_3$CN</td>
<td>3x 1 mg of enriched fraction (50 mg mL$^{-1}$, 20 μL injection)</td>
<td>[186]</td>
</tr>
<tr>
<td><strong>600 MHz, LC-NMR probe (30 μL flow cell)</strong></td>
<td><em>Eriobotrya japonica</em> (Thunb.) Lindl.</td>
<td>Flavonoids</td>
<td>$^1$H and $^{13}$C NMR, COSY, HSQC, HMBC</td>
<td>CD$_3$CN</td>
<td>Quantity not specified (20 μL injection), ten trappings</td>
<td>[187]</td>
</tr>
<tr>
<td><strong>600 MHz, cryo 5-mm NMR probe (cryofit 30 μL active volume)</strong></td>
<td><em>Solanum lycopersicum</em> L. (tomato mutant)</td>
<td>Flavonoids</td>
<td>$^1$H NMR</td>
<td>CD$_3$OD (227 μL)</td>
<td>Quantity not specified (20 μL injection)</td>
<td>[188]</td>
</tr>
<tr>
<td><strong>400 MHz, LC-NMR probe (30 μL flow cell)</strong></td>
<td><em>Litsea acuminata</em> (Blume) Kurata, <em>L. hypophaea</em> Hyata, <em>Neolitsea acuminatissima</em> (Hyata) Kaneh &amp; Sasaki, and <em>N. kanishi</em> (Hayata) Kaneshe &amp; Sasaki</td>
<td>Flavonoids</td>
<td>$^1$H NMR (SPE-NMR)</td>
<td>CD$_3$CN (SPE-NMR)</td>
<td>1 mg of enriched fraction (100 mg mL$^{-1}$, 10 mL injection)</td>
<td>[189]</td>
</tr>
<tr>
<td>Frequency</td>
<td>NMR Probe</td>
<td>Plant or Compound</td>
<td>Spectroscopy</td>
<td>Solvent</td>
<td>Enriched Fraction</td>
<td></td>
</tr>
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<td></td>
</tr>
<tr>
<td>400 MHz, LC-NMR probe (120 μL flow cell)</td>
<td>Phyllanthus myrtifolius</td>
<td>Lignans and stilbenoids</td>
<td>$^1$H NMR, COSY, NOESY</td>
<td>CD$_3$CN and CD$_3$OD</td>
<td>0.4 mg enriched fraction (40 mg mL$^{-1}$, 20 mL injection) and 0.4 mg enriched fraction (20.8 mg mL$^{-1}$, 20 mL injection)</td>
<td></td>
</tr>
<tr>
<td>600 MHz, cryo LC-NMR probe (60 μL flow cell)</td>
<td>Thymus vulgaris L.</td>
<td>Monoterpenes, flavonoids and phenyl propanoids</td>
<td>$^1$H NMR, COSY, HSQC, HMBC</td>
<td>CD$_3$CN</td>
<td>0.1 mg of defatted methanol-water extract (10 mg mL$^{-1}$, 10 mL injection) and 20 trappings</td>
<td></td>
</tr>
<tr>
<td>800 MHz, cryo 5-mm NMR probe with cryofit 60 μL active volume and with 2.5-mm tube</td>
<td>Origanum vulgare L.</td>
<td>Polyphenolic compounds</td>
<td>$^1$H and $^{13}$C NMR, COSY, TOCSY, NOESY, HSQC, HMBC</td>
<td>CD$_3$CN (200 mL)</td>
<td>0.4 mg of extract (20 mg mL$^{-1}$, 20 mL injection) and 10 trappings</td>
<td></td>
</tr>
<tr>
<td>500 MHz, 3-mm LC-NMR probe (60 μL flow cell)</td>
<td>Teucrium polium L.</td>
<td>Flavonoids</td>
<td>$^1$H NMR, COSY, TOCSY, HSQC, HMBC</td>
<td>CD$_3$CN</td>
<td>Quantity not specified (30 mL injection)</td>
<td></td>
</tr>
<tr>
<td>400 MHz, LC-NMR probe (30 μL flow cell)</td>
<td>Alnus formosana Burk</td>
<td>Diarylheptanoids</td>
<td>$^1$H and $^{13}$C NMR, COSY, NOESY, HSQC, HMBC</td>
<td>CD$_3$OD and pyridine-$d_5$</td>
<td>2 mg and 0.673 mg of enriched fraction (20 mL injection) and three trappings</td>
<td></td>
</tr>
<tr>
<td>500 MHz, cryo 5-mm NMR probe (cryofit 30 μL active volume)</td>
<td>Wachendorfia thyrsiflora L.</td>
<td>Phenylphenalenones</td>
<td>$^1$H NMR, COSY, HSQC, HMBC</td>
<td>CD$_3$CN</td>
<td>2.2 mg injected (110 mg mL$^{-1}$ in DMSO, 20 mL injection) and 4 mg injected (200 mg mL$^{-1}$ in DMSO, 20 mL injection) and six trappings</td>
<td></td>
</tr>
<tr>
<td>600 MHz, cryo 5-mm NMR probe (cryofit 30 μL active volume)</td>
<td>Camellia sinensis (black, green, and white tea extracts)</td>
<td>Flavonoids</td>
<td>$^1$H NMR, COSY</td>
<td>CD$_3$OD (227 μL)</td>
<td>Quantity not specified (40 μL injection)</td>
<td></td>
</tr>
</tbody>
</table>
2.2 Applications of LC-NMR in phytochemical analysis

2.2.1 LC-NMR applications

In phytochemical analysis, LC-NMR methods have been predominantly used for dereplication studies or targeted de novo identification of given bioactive metabolites in crude extract profiling studies. In Table I.C.1, a summary of the most recent applications (2010-2013) is presented, including the type of experiments and the probe used. For earlier applications, the reader can refer to other reviews [87,159,197].

Among the different studies published, recently, the on-line identification of the anti-inflammatory constituents of Angelica dahurica (Fisch. Ex hoffm.) Benth. Et Hook (Apiaceae) using LC-NMR is representative of the type of data that can be obtained [177]. An HPLC-based activity profiling approach was used to investigate the anti-inflammatory properties of the extract using a nitric oxide (NO) inhibitory activity bioassay. In the first step and to localize the compound responsible for the biological activity, the ethanol extract was fractionated by HPLC on an analytical C$_{30}$ RP column (250 × 4.6 mm, i.d.; 3 μm). Five fractions corresponding to the main peaks were collected and submitted to the NO inhibition assay and two LC peaks were found to be responsible for the activity. Then, an additional analysis was performed using the same conditions and replacing H$_2$O with D$_2$O for the LC-NMR-MS analysis. On the basis of these data, the active compounds were identified as known furanocoumarins — imperatorin and phellopterin.

Another recently developed method for the rapid identification of NPs having antimicrobial activities is through the direct coupling of a bacterial bioassay with chromatography [142]. This approach was based on a whole-cell bioluminescent reporter gene assay that was coupled with thin layer chromatography (TLC) for primary hit detection and LC-MS and LC-NMR for dereplication and structure elucidation of the active compounds. Using this strategy, a new gyrA promoter named inthomycin A was identified from a myxobacterial extract without an isolation step. Dereplication of the active constituents was performed directly in the crude myxobacterial extract by LC-HRMS and LC-NMR experiments. LC-NMR coupling experiments were run in the stop-flow mode utilizing similar chromatographic conditions as applied for the HPLC/bioluminescence measurement. The bioactive component was identified by stop-flow $^1$H-$^1$H TOCSY and COSY experiments. The total time of acquisition was 14.5 h and was performed with 22 μg μL$^{-1}$ of the crude extract. The MS and NMR data obtained were then compared to those in the Chapman & Hall/CRC Chemical Database, which enabled the dereplication of inthomycin A as the active compound.

2.2.2 On-line microflow NMR applications

While microflow NMR (e.g., CapNMR™) is mainly used at-line, it can also be used in direct LC-NMR hyphenation in the on-flow mode. In this case, the entire separation can be performed using fully deuterated solvents because of the small amount of deuterated solvent required. Such an approach was used, for example, during the separation of essential bioactive isomers of the carotenoid bixin. Here, the mixture profiling was performed on a Pronto-SIL C$_{30}$ (3 μm,
200 Å, 150 mm × 250 µm) in the isocratic mode (acetone-d₆/D₂O = 92:8 v/v). The sample was injected in a small volume of 500 nL. This method enables the recording of high quality stop-flow microflow ¹H NMR spectra and offers the possibility to acquire 2D ¹H-¹H COSY-NMR spectra with limited amounts of mixture. Finally, using a combination of LC-MS and LC-microflow NMR, it was possible to elucidate the structures of two stereoisomers of bixin [110].

2.2.3 LC-SPE-NMR applications

Because of the limitations of LC-NMR, especially in terms of sensitivity, over the past few years, HPLC-SPE-NMR has been used with growing frequency for the rapid dereplication of complex mixtures in NP research [168].

This technique has been used to investigate the phytochemical composition of aerial parts and roots of *Wachendorfia thyrsiflora* L. (Haemodoraceae). The separation of the extracts from aerial and roots plant parts was performed on a Purospher RP18e column (5 µm; 250 × 4.6 mm) with a flow rate of 1 mL min⁻¹ and a gradient of MeCN and H₂O. Furthermore, only 2 mg was injected onto the column, and the column was monitored by UV. Six cumulative trappings were performed for each LC-peak that was selected for the 1D and 2D NMR analyses, and MeCN-d₃ was used to transfer of the metabolite of interest. Identification was performed on 18 compounds including 11 phenylphenalenones in both the root and aerial extracts of this plant [195].

For *de novo* structure identification of NPs, detection and assignment of ¹³C resonances are often essential for unequivocal elucidation. However, the ¹³C acquisition is a challenging task because the gyromagnetic ratio of ¹³C is only one-fourth that of ¹H and because the natural abundance of ¹³C is only 1.1% of the total carbon nuclei. In addition, the overall receptivity of ¹³C is approximately 5700 times lower than that of ¹H at the same magnetic field strength [167]. ¹³C NMR data can be acquired indirectly by ¹H-detected one-bond (heteronuclear single-quantum coherence spectroscopy, HSQC, and heteronuclear multiple-quantum correlation spectroscopy, HMQC) and multiple-bond (heteronuclear multiple-bond correlation spectroscopy, HMBC) 2D ¹H-¹³C heteronuclear chemical shift correlation experiments. However, in some cases, carbon atoms and even hydrogen bearing carbon atoms can be difficult to detect because of the dependence on coupling constants, coupling path geometry, and substituent patterns.

Direct ¹³C measurement was demonstrated to be measurable on sample limited amounts using a combination of a miniaturized cryo NMR probe (30 µL) with an integrated cooled carbon channel preamplifier and several peak trappings on the HPLC-SPE-NMR system. Such an approach was applied for the identification of triterpenoids from a *Ganoderma lucidum* (Curtis ex Fr.) P. Karst (Ganodermataceae) extract [198]. First, the SPE trapping was optimized using three triterpenoid standards. A defatted methanol extract was fractionated using vacuum liquid chromatography (VLC). Using HRMS and ¹H NMR, a pooled triterpenoid fraction was selected and further fractionated using semipreparative RP HPLC. Then, one of the fractions that contained few chromatographic peaks was submitted to SPE-NMR. To achieve
the expected S/N ratios in the $^{13}$C NMR experiment, six trappings of 80 μL injections were necessary, which corresponded to approximately 560 μg. Using this procedure, an acquisition time of 13 h resulted in spectra with adequate S/N ratios for the detection of all $^{13}$C signals. Structural elucidation was performed based on combinations of 1D and 2D NMR experiments (double quantum filtered correlation spectroscopy, DQF-COSY, HSQC, and HMBC), allowing the identification of two minor ganoderic acids.

Although SPE-NMR is currently applied to a wide variety of organic compounds, certain classes of compounds have limited application. This is the case for the positively charged alkaloids that are often poorly trapped on the commonly used SPE at low pH. To overcome this problem two new approaches for efficient SPE trapping of such compounds have been developed [199]. The authors used a divinylbenzene polymer cartridge (GP resin) and a postcolumn dilution of a 0.1 M NaOH solution to enhance the trapping of alkaloids on the commonly used SPE. However, this procedure was incompatible with phenolic alkaloids. The authors also evaluated the trapping and elution efficacy on an SPE cartridge with a mixed-mode cation exchanger (sulfonyl groups on a polydivinyl-benzene backbone). First, this method was evaluated using 24 alkaloid standards that covered a wide range of $pK_a$ values, log $P$ values, aliphatic and aromatic skeletons, and a variety of functionalities, such as alcohol groups and carboxylic that could influence the trapping efficiency. Finally, this method was applied to the analysis of the plant extracts containing alkaloids such as Huperzia selago (L.) Bernh. ex Schrank & Mart. (Lycopodiaceae) and Triclisia patens Oliv. (Menispermaceae). The methodology proposed was efficient for the trapping and elution of complex aliphatic alkaloids and phenolic alkaloids as evidenced by the acquisition of 2D NMR data for all trapped compounds. In contrast, GP resin proved only viable for the H. selago alkaloids, and the trapping and elution of bisbenzylisoquinoline alkaloids was dubious.

3. Microflow NMR and micro NMR for sensitive at-line detection

The use of directly coupled LC-NMR applications has decreased substantially in the recent years because of the major drawbacks of this technique, such as the need for solvent suppression or chemical shifts because of solvent composition changes when used in the gradient mode LC. In addition, owing to the intrinsically low sensitivity of the NMR detection, only major mixture constituents are detectable. This has resulted in an increased use of LC-SPE-NMR [159], which has overcome most of the issues encountered in on-flow LC-NMR but at the price of extensive automation.

With the emergence of NMR systems with improved mass- and/or concentration-sensitivity, alternative methods to LC-SPE-NMR based on at-line NMR detection of LC peaks that can be collected by an independent and efficient microfractionation procedure have increased [109,200].
3.1 Design of micro NMR probes with enhanced sensitivity

Three strategies have been employed to enhance NMR sensitivity: the use of a different coil type compared to conventional NMR systems in microflow NMR, the reduction of noise during the acquisition through cryogenic cooling of the probe in micro-cryoprobes and the development of new coil material in high-temperature superconducting (HTS) probes. The influence of the coil diameter and the noise of the receiver on the NMR S/N ratio are illustrated in Figure I.C.1d. With these advances, the amount of sample needed for 1D and 2D NMR acquisition decreased to the nanomolar range [125], enabling NMR data acquisitions on small molecules (molecular mass <1000 Da) in the microgram range [51,127,201] or below for the direct $^{13}$C NMR detection of LC peaks [198].

3.1.1 At-line microflow NMR

As mentioned, the sensitivity of an NMR experiment increases in inverse proportion to the diameter of the coil [90] (Figure I.C.1d). Thus, flow probes with small coils (microflow probes) have been placed on the market for better performance [100]. These solenoid probes are naturally suited for LC-NMR because samples need to be introduced using flow. In this respect, the CapNMR™, a commercially available solenoid probe, represents an interesting option for mass-limited samples.

With such probes, the collection of LC peaks is not directly hyphenated to HPLC as is the case with LC-SPE-NMR but the LC peaks are microfractionated from independent HPLC profiling analyses (see in the following text) and the microfractions of interest are dried and analyzed postchromatographically in the microflow probe [202]. To achieve the best sensitivity, the dried analytes (typically a few micrograms) are dissolved in a volume of deuterated solvent that slightly exceeds the volume of the microflow cell (5 µL). The very concentrated samples are then parked into the center of the probe by injection and further pushed with a calibrated volume of deuterated solvent (push volume). The loading can be performed using manual injection with a syringe of a given analyte, or microfractions can be injected automatically from a 96-well plate [200]. These microtiter plates can be used for HPLC collection. The LC microfractions are dried and redissolved in a minimal amount of deuterated solvent. An LC autosampler system connected to a microliter pump providing the corrected deuterated solvent for pushing each individual sample into the microflow probe is then used to measure the NMR spectra of all of the microfractions automatically. Compared to a conventional NMR setup with 5-mm tubes, a sensitivity gain of up to five-fold can be obtained, and 2D NMR measurement becomes addressable with only a few tenths of micrograms of analyte [109,127]. An example of the spectra that can be obtained from a mixture of NPs analyzed overnight from consecutive automated injection from a 96-well plate is shown in Figure I.C.3c and d. Figure I.C.3c consists of a pseudo-LC 2D plot that was reconstituted by merging all of the CapNMR™ spectra recorded for the consecutive microfractions obtained from the LC separation. As shown, when compared to an on-flow LC-NMR 2D plot (Figure I.C.3a), this mode of acquisition does not require any solvent suppression because the spectra are measured in a deuterated solvent. Furthermore, because all of the microfractions are
analyzed in the same solvent, such as deuterated methanol, there is no shift as a result of the change of composition because of to the gradient elution mode (Figure I.C.3c). The spectrum of a furanocoumarin acquired under these conditions is shown in Figure I.C.3d. Compared with LC-NMR (Figure I.C.3b), a good quality spectrum (Figure I.C.3d) was obtained directly from the analysis of the corresponding microfraction without the requirement of solvent suppression.

As for LC-SPE-NMR, it has been shown that this type of microflow probe can also be integrated in microscale LC-MS-NMR platforms via a thoroughly optimized automated droplet microfluidic NMR loading method (microdrop NMR). With such an approach, by post chromatographic analysis of the analytes of interest, interpretable 1D NMR spectra were obtained from analytes down to the 200 ng level in 1 h per well using an automated NMR data acquisition. Such platforms were efficient in the dereplication of known cyanobacterial compounds and the prioritization of the isolated unknown using a single 30 μg injection of a crude extract [50].

Excellent line shape can be obtained with small solenoid probes and because of the minute amount of solvent used, the residual solvent line in the spectra is much less important than in conventional probes, which also helps to maximize gain values for enhanced sensitivity (Figure I.C.3d). Another advantage of solenoid probes is that the coil can be simultaneously tuned to multiple frequencies, allowing for near-optimal performance on different channels and thus the analysis of multiple samples at the same time [92]. These are now available commercially from Protasis [97].

### 3.1.2 1-mm and other microtubes

In line with the development of microflow probes, microprobes with optimized sensitivity using disposable 1-mm microtubes (Figure I.C.1a) with a sample volume of 5 μL, similar to that of the CapNMR™ probe, have also been brought to the market [112]. In this case, the automation is less easily achieved than with tubeless approaches, and the samples must be transferred from the dried collected fraction to the microtubes (Figure I.C.2e). One advantage of this approach compared to microflow NMR is that the microtubes do not interact with the probe itself, and problems related to the poor dissolution of samples, such as precipitation and clogging that might arise in microflow approaches, are avoided. Microtubes, such as Shigemi microtubes, can also be used to enhance the sensitivity of mass-limited samples in probes with larger diameters; however, the filling factor will not be optimized to the same extent as for dedicated microprobes, and the gains in sensitivity will be decreased [203].

### 3.1.3 Additional sensitivity with cryo NMR probes

An additional advantage of using microtubes is that the probe can be cryogenically cooled, and these small volume microprobes (e.g., a 1.7-mm cryo probe) are commercially available. The development of cryo NMR probes has brought NMR sensitivity to a new level. As the noise of the measurement is reduced by cooling the coil and the associated electronics with helium ($V_{\text{noise}}$ in Figure I.C.1d), the S/N ratio is improved by as much as four times compared to room-temperature probes [204]. Different probe configurations exist from all major NMR vendors,
from 5-mm tube probes to 1.7-mm microtube probes (MicroCryoProbe™). The cryogenized option is also available for LC-NMR flow probes (CryoFlowProbe). Such probes have boosted the sensitivity of NMR measurements of samples with limited amounts (Figure I.C.1c) as well as for compounds with relatively low solubility, such as proteins that require a relatively large volume to be solubilized [125].

Figure I.C.5: Comparison of $^1$H NMR spectra of 2 µg of natural product suaveolindole acquired on a microflow and a microcryo NMR probe.
The increased NMR sensitivity of the microflow NMR system compared to a conventional NMR system (5-mm tube probe, saddle-type coil) is because of its improved coil design (solenoid type), whereas the improved sensitivity of the microcryo system is both a result of the cryogenically cooled coil system and the smaller dimensions of the coil (saddle type). The NMR mass- and concentration-sensitivity of the
**microcryo NMR system is clearly superior to the sensitivity of the microflow NMR system.** (a) For NMR analysis on a 1.7-mm microcryo system (MicroCryoProbe™), 2 µg of the natural product suaveolindole was dissolved in 13 µL of deuterated methanol, placed in a 1-mm diameter tube, and then, the \(^1\)H NMR spectrum was acquired using 64 scans (approximate analysis time: 5 min). (b) For the analysis on the microflow NMR system (CapNMR™), 2 µg of the natural product suaveolindole was concentrated in 6 µL of deuterated methanol, injected into the probe with the aid of a syringe, and the \(^1\)H NMR spectrum was acquired using 1024 scans (approximate analysis time: 1 h 20 min). (c) An HSQC spectrum was acquired on a microcryo system using a 10-µg sample with an acquisition time of 1 h. (Spectra were kindly provided by Mark O’Neil-Johnson, Sequoia Sciences Inc.)

With such a setup, high sensitivities can be attained and 2D NMR measurements of LC peaks obtained from a single conventional analytical HPLC run are feasible [113].

An example of the advantages in terms of sensitivity that can be observed using microtubes with a cryo probe compared to a room-temperature microflow probe is shown by the analysis of the same amount of an indole alkaloid on both probes of the same 600 MHz NMR system (Figure I.C.5). As shown, the \(^1\)H spectra could be recorded with only 2 µg of the compound; however, a much better S/N ratio was obtained with the MicroCryoProbe™ using only 64 scans (Figure I.C.5a), whereas 1024 scans were required for the CapNMR™ probe (Figure I.C.5b). With 10 µg of the same compound in the MicroCryoProbe™, an HSQC spectrum could be recorded in 1 h (Figure I.C.5c).

To the best of our knowledge, probes with extremely small active volumes, such as the one holding 1-mm microtubes or the microflow solenoid probes, are not available as cryogenated probes most likely because of the difficulty in adapting the technology to such small volumes. The main drawback of this technology is the price of the cryogenated probes and that the maintenance costs associated with these probes are significantly higher than that of room-temperature probes.

Another way to increase sensitivity is to improve the quality of the coil material. For most NMR probes, the rf coils are constructed from copper-based conductors (Wolfender et al. 2013). The use of HTS materials such as yttrium barium copper oxide (YBCO), to build the rf coil in cryo probes results in higher sensitivity, and together with the other advantages (reduced noise) of cryo probes, gives HTS coils the best S/N ratio among commercially available NMR probes [98,167]. Owing to geometric restrictions in the construction of HTS coils, only probes with small-diameter coils and tube probes are currently commercially available. As for the other probes, a higher sensitivity is achieved with HTS coils of small diameters, and 1-mm probes using the materials described have been built [98].

### 3.2 Isolation and purification of NPs for sensitive NMR analysis

At-line microisolation procedures before sensitive NMR could be performed either by multiple collection of a given peak of interest using standard HPLC conditions (4 mm i.d. columns) or by semipreparative separations with columns of relatively small diameters (e.g., 10 mm i.d.). These latter types of HPLC columns are usually preferred because they allow relatively high loadings (few milligrams up to tens of milligrams of crude mixture), they can be operated at
flow rates compatible with collection in 96-deep well plates (2-3 mL min\(^{-1}\)) and the microfraction volume is relatively restricted (1-2 mL) [202]. Such volumes enable the collection directly in 96-deep well plates that can be evaporated in a single step. According to the complexity of the extracts or fractions studied, this microfractionation procedure typically yields microfractions that contain between a few micrograms to approximately hundreds of micrograms for the most abundant metabolites [71,202]. At-line microfractionation can also be performed on an LC-SPE-NMR platform, and in this case, the peak trapped on SPE can be eluted into microtubes of various volumes [198,205].

**Figure I.C.6: Two-step software-driven purification of natural products using microfractionation.**

(a) UHPLC-TOFMS analysis of crude extract performed with the two gradient slopes necessary for optimizing the separation of metabolites (2–5) through software modeling. (b) Targeted metabolite enrichment with simulated separation of these four metabolites and corresponding semipreparative LC-MS separation. (c) Final targeted purification of metabolite 4 with fingerprint of the enriched fraction containing 4 that was used for modeling, elution models of three different isocratic separation conditions for the final purification step, and corresponding final semipreparative LC-MS purification using the isocratic condition at 39% B. Source: Bertrand et al., 2013. De novo production of metabolites by fungal co-culture of Trichophyton rubrum and Bionectria ochroleuca. *J Nat Prod* 76: 1157-1165.

Efficient procedures exist for the scaling up of the high-resolution metabolite profiling of extracts obtained at the analytical level using LC-MS, HPLC, or UHPLC (ultra high performance liquid chromatography) and the further optimization of separating the analyte of interest at
the semipreparative scale [206]. For the optimization of the chromatographic resolution of a given analyte in a complex mixture, optimized elution conditions can be calculated using chromatographic software based on the comparison of the LC peak that is to be isolated in two linear gradient elutions of different slopes (Figure I.C.6a). On the basis of this, a simulated chromatogram can be generated to evaluate the efficiency of the separation obtained (Figure I.C.6b). To keep the same selectivity at the analytical and semipreparative scale, the analytical conditions have to be transferred to the semipreparative conditions using geometric transfer on the correctly characterized LC instruments (Figure I.C.6b) [67]. Such rules have been reported and applied successfully for the isolation of minor crude extract constituents for further microflow NMR characterizations [71,207].

As an example, the UHPLC-TOFMS profiling of the crude extract of a fungal co-culture extract is shown in Figure I.C.6a. The analysis was performed with the two gradient slopes necessary for optimizing the separation of the targeted metabolites through software modeling. On the basis of this information, a simulated separation could be computed (Figure I.C.6b), which was then used for the first enrichment step by semipreparative separation. A second simulation (Figure I.C.6c) aided in finding the isocratic conditions for a final isolation step, which yielded a few tens of micrograms of the desired biomarker for further microflow NMR analysis [207].

At-line microisolation procedures, before sensitive NMR measurement at the microgram scale, require careful optimization because when working with such small amounts of analytes, signals from solvent impurities or column bleeding may considerably deteriorate the quality of the NMR spectra. Indeed, most HPLC-grade solvents are not NMR-grade and protonated impurities might interfere. Moreover, according to the type of stationary phase used, small amounts of the packing material might bleed and generate signals visible in the $^1$H NMR spectrum that are further strengthened by the concentration of the fractions. When dealing with microgram amounts of samples, the drying procedures are also important, as residual water (Figure I.C.3d) will deteriorate the spectral quality and thus introduce unwanted solvent lines that might need to be suppressed. Therefore, efficient eluent evaporation procedures, such as vacuum centrifugation, must be used, and hygroscopic deuterated solvents need to be stored under inert gas to avoid the presence of residual water. Contamination can also come from the vials, the microtiter plate used for collecting microfractions or the SPE cartridge used for SPE-NMR. The quality and the background NMR signals generated by these devices must be tested according to the conditions and solvents used for each of the measurements, and control NMR spectra using only solvent blanks are recommended to compare NMR spectra and correctly discriminate impurity signals from those of the analytes [167].

3.3 Applications of at-line micro NMR in phytochemical analysis

At-line NMR applications that are considered herein are those where the NMR flow cells are not used in direct hyphenation with HPLC. This includes at-line microflow measurements after microfractionation and all experiments using microtubes as well as those involving SPE-NMR
with transfer to microtubes, which is a method that is frequently used in this field. All of these applications are summarized in Table I.C.2.

### 3.3.1 At-line microflow NMR applications

A significant number of recent applications (Table I.C.2) were performed using microflow NMR (CapNMR™). For example, the identification of the antimicrobial compounds from the rhizome of *Peucedanum ostruthium* W.D.J.Koch (Apiaceae) was performed using this method, following a classical bioguided isolation procedure based on assays with three pathogenic bacteria (*Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus*) [208]. The activity of the crude ethyl acetate extract and fractions obtained using different chromatographic methods were investigated by the agar disk diffusion method. All fractions were analyzed using an LC-UV-electrospray ionization-MS (LC-UV-ESI-MS) and a microflow NMR probe on a 600 MHz instrument. The constituents of the fraction with the highest antibacterial activity were rapidly identified as the coumarin derivatives, oxypeucedanin and oxypeucedanin hydrate. The $^1$H NMR analysis of oxypeucedanin was obtained using a 15-$\mu$g ($\sim 52$ nmol) sample, whereas the analysis of oxypeucedanin hydrate was achieved using a 22-$\mu$g ($\sim 72$ nmol) sample. The small amounts of these two compounds were sufficient to obtain unambiguous $^1$H NMR spectra in approximately 2 min and 20 s.

In the frame of a high-throughput phytochemical investigation based on the initial normal-phase flash chromatography of crude extracts by a purification step including a semipreparative RP HPLC, the constituents of the apolar root extract of *Greenwayodendron suaveolens* (Engl. & Diels) Verdc. (Annonaceae) were efficiently studied. This strategy afforded milligram amounts of the major compounds (39.8–194 μg) and micrograms of the minor constituents (710–980 μg) including three new and two known indole sesquiterpene alkaloids that were characterized by HRMS and 1D and 2D microflow NMR. One of the isolated compounds, named pentacyclindole, was determined to possess a new NP framework and showed interesting antibiotic activity against clinical isolates of *S. aureus* [209].
Table I.C.2: Recent applications (2010-2013) of microflow NMR (CapNMR™) and micro NMR (3.0 – 1.0-mm microtubes) for at-line metabolite identification.

### At-line microflow NMR (CapNMR™)

<table>
<thead>
<tr>
<th>NMR setup</th>
<th>Sample</th>
<th>Compound class</th>
<th>NMR experiments</th>
<th>Solvent</th>
<th>Sample amounts</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>600 MHz, microflow NMR (5 µL flow cell)</td>
<td><em>Radix imperatoriae</em> (Peucedanum ostruthium W.D.J. Koch)</td>
<td>Oxypeucedanin and corresponding hydrate (psoralene, coumarin)</td>
<td>1H NMR</td>
<td>CDCl₃</td>
<td>Injection of 52 and 72 nmol (150 and 220 µg in 15 µL)</td>
<td>[208]</td>
</tr>
<tr>
<td>600 MHz, microflow NMR (5 µL flow cell)</td>
<td><em>Oncidium</em> spp.</td>
<td>Stilbenoids</td>
<td>1H NMR, COSY, NOESY, HSQC, HMBC</td>
<td>CD₂OD and CDCl₃</td>
<td>50-480 µg in 6.5 µL</td>
<td>[113]</td>
</tr>
<tr>
<td>500 MHz, microflow NMR (5 µL flow cell)</td>
<td><em>Eschscholzia californica</em></td>
<td>Alkaloids</td>
<td>1H NMR</td>
<td>DMSO-δ₆</td>
<td>200 ng to 8 µg</td>
<td>[210]</td>
</tr>
</tbody>
</table>

### At-line microNMR (1.7- and 1-mm microtubes) after isolation or LC-SPE-NMR trapping

<table>
<thead>
<tr>
<th>NMR setup</th>
<th>Sample</th>
<th>Compound class</th>
<th>NMR experiments</th>
<th>Solvent</th>
<th>Sample amounts</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>600 MHz, cryo 1.7-mm NMR probe</td>
<td><em>Oncidium</em> spp.</td>
<td>Stilbenoids</td>
<td>1H NMR, COSY, NOESY, HSQC, HMBC</td>
<td>CD₂OD and CDCl₃</td>
<td>5-33 µg</td>
<td>[113]</td>
</tr>
<tr>
<td>600 MHz, cryo 1.7-mm NMR probe</td>
<td><em>Ganoderma lucidum</em> (Curtis ex Fr.) P. Karst</td>
<td>Triterpenoids</td>
<td>13C NMR (13h acquisition time and detection limit 0.5 µg)</td>
<td>CD₂OD</td>
<td>600 µg of enriched fraction, (7 mg mL⁻¹ and 80 µL injection), HPLC-SPE-NMR and transfer to NMR tube and six trappings</td>
<td>[198]</td>
</tr>
<tr>
<td>600 MHz, cryo 1.7-mm NMR probe and 5-mm NMR probe</td>
<td><em>Carthamus oxyacantha</em> M. Bieb.</td>
<td>Glycosylated spiranes, lignin glycoside and vanilic acid</td>
<td>1H NMR, COSY, NOESY, HSQC, HMBC</td>
<td>CD₁CN (30 µL) and CD₂OD</td>
<td>500 µg (20 mg mL⁻¹; 25 µL injection), HPLC-SPE-NMR and transfer to NMR tube and eight trappings</td>
<td>[211]</td>
</tr>
<tr>
<td>500 MHz, LC-NMR probe (60 µL flow cell) 600 MHz, cryo 1.7-mm NMR probe</td>
<td>24 alkaloid standards and extracts of <em>Huperzia selago</em> (L.) Bernth. ex Schrank &amp; Mart. (Lycopodiaceae) and <em>Triclisia patens</em> Oliv.</td>
<td>Alkaloids</td>
<td>1H NMR, COSY, NOESY, HSQC, HMBC</td>
<td>CD₂OD, CDCl₃, and CH₃OD + 5% NH₄OH</td>
<td>250 and 200 µg and of alkaloid residue (5 and 20 mg mL⁻¹, 50 and 10 µL injection); HPLC-SPE-NMR and transfer to NMR tube; 4, 6 or 10 trappings</td>
<td>[199]</td>
</tr>
<tr>
<td>NMR setup</td>
<td>Sample (plant)</td>
<td>Compound class</td>
<td>NMR experiments</td>
<td>Solvent</td>
<td>Sample amounts</td>
<td>References</td>
</tr>
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<tr>
<td>600 MHz, cryo 5-mm NMR probe with 1.7-mm tube</td>
<td><em>Cinnamomum subavenium</em> Miq.</td>
<td>Dibenzocycloheptanoids</td>
<td>$^1$H and $^{13}$C NMR, COSY, NOESY, HSQC, HMBC</td>
<td>CD$_2$OD</td>
<td>1 mg of enriched fraction (50 mg mL$^{-1}$, 20 µL injection), HPLC-SPE-NMR and transfer to NMR tube and three trappings</td>
<td>[212]</td>
</tr>
<tr>
<td>500 MHz, LC-NMR probe (60 µL flow cell) 600 MHz, cryo 1.7-mm NMR probe</td>
<td><em>Carthamus oxyacantha</em> M. Bieb. and <em>Penicillium namyslowski</em> K.M. Zalessky</td>
<td>Glycosylated spiranes, lignin glycosides and fatty acids</td>
<td>$^1$H NMR, COSY, NOESY, HMBC, HSQC</td>
<td>CD$_3$CN (30 µL)</td>
<td>0.6 mg (24 mg mL$^{-1}$, 25 µL injection) and 0.05 mg (5 mg mL$^{-1}$, 10 µL injection) and HPLC-SPE-NMR and transfer to NMR tube</td>
<td>[58]</td>
</tr>
<tr>
<td>500 MHz, 1-mm and 5-mm NMR probe</td>
<td><em>Artemisia persica</em> Boiss.</td>
<td>Bisaboloxide sesquiterpene</td>
<td>$^1$H NMR, COSY, NOESY, HSQC, HMBC (1-mm probe) $^{13}$C NMR (5-mm probe)</td>
<td>C$_6$D$_6$</td>
<td>Microgram and milligram scales</td>
<td>[213]</td>
</tr>
<tr>
<td>500 MHz, 1-mm and 5-mm NMR probe</td>
<td><em>Abras precatorius</em> ssp. <em>africanus</em></td>
<td>Isoflavan quinones</td>
<td>$^1$H and $^{13}$C NMR, COSY, NOESY, HSQC, HMBC</td>
<td>CDCl$_3$ and CD$_2$OD</td>
<td>Microgram and milligram scales</td>
<td>[214]</td>
</tr>
</tbody>
</table>

At-line microtube NMR (2.5- and 3-mm tubes) after LC-SPE-NMR trapping

<table>
<thead>
<tr>
<th>NMR setup</th>
<th>Sample (plant)</th>
<th>Compound class</th>
<th>NMR experiments</th>
<th>Solvent</th>
<th>Sample amounts</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>400 MHz, 3-mm and 5-mm NMR probe with 3-mm tube</td>
<td><em>Nauclea pobeguini</em> (Pobég.) E.M.A.Petit</td>
<td>Alkaloids</td>
<td>$^1$H and $^{13}$C NMR, HSQC, HMBC</td>
<td>CD$_3$CN</td>
<td>1 mg of enriched fraction (200 mg mL$^{-1}$, 5 µL injection) and HPLC-SPE-NMR and transfer to NMR tube</td>
<td>[215]</td>
</tr>
<tr>
<td>400 MHz, 3-mm and 5-mm NMR probe with 3-mm tube</td>
<td><em>Ormocarpum kirkii</em> S. Moore</td>
<td>Isoflavonoids</td>
<td>$^1$H and $^{13}$C NMR, HSQC, HMBC</td>
<td>CD$_3$OD</td>
<td>1 mg of enriched fraction (50 mg mL$^{-1}$, 20 µL injection) and HPLC-SPE-NMR and transfer to NMR tube</td>
<td>[216]</td>
</tr>
<tr>
<td>500 MHz, cryo NMR probe with 2.5-mm tubes</td>
<td><em>Styrchnos usambarensis</em> Gilg</td>
<td>Alkaloids</td>
<td>$^1$H and $^{13}$C NMR, COSY, HSQC, HMBC</td>
<td>CD$_3$CN</td>
<td>Quantity not specified, HPLC-SPE-NMR and transfer to NMR tube and three trappings</td>
<td>[217]</td>
</tr>
<tr>
<td>800 MHz, 5-mm cryo NMR probe with 2.5-mm tube</td>
<td>Apple peel (<em>Malus × domestica</em> Borkh.)</td>
<td>Flavonoids</td>
<td>$^1$H NMR</td>
<td>CD$_3$OD</td>
<td>200 µg (10 mg mL$^{-1}$, 20 µL injection) and HPLC-SPE-NMR and transfer to NMR tube</td>
<td>[205]</td>
</tr>
</tbody>
</table>
3.3.2 At-line micro NMR applications

An alternative to microflow NMR requires the use of 1-mm microtubes having approximately the same sample volume. Recently, this technique was used for the structure elucidation of five antiplasmodial bisabololoxide sesquiterpene diesters from the aerial parts of *Artemisia persica* Boiss. (Asteraceae) [213]. An HPLC-time-based activity profiling using 350 µg of extract enabled an efficient correlation of biological activity to a specific chromatogram region. LC-MS\(^n\) analysis together with at-line micro NMR analysis revealed the presence of several compounds in the active fraction. Targeted isolation of the active compounds was performed by a combination of normal-phase medium pressure liquid chromatography (MPLC) and semipreparative RP HPLC. Structure elucidation was achieved by 1D and 2D NMR experiments using a 1-mm NMR probe. Relative configurations of the isolated compounds were established on the basis of three \(^1\text{H} - ^1\text{H}\) coupling constants and nuclear overhauser enhancement (NOE) difference spectra. Finally, the relative and absolute configurations of the active compounds were determined by comparing the experimental electronic circular dichroism (ECD) spectroscopy with the simulated ECD data to find possible stereoisomers using time-dependent density function theory (TDDFT). Some of the isolated compounds exhibited *in vitro* antimalarial activity against *Plasmodium falciparum* in the low micromolar range.

The same strategy has been used for the identification of the active compounds in *Abrus precatorius* L. ssp. *africanus* (Fabaceae) against the protozoan parasite *Trypanosoma brucei rhodesiense*. An HPLC microfractionation of the extract in a 96-well plate, followed by biological activity, was used to localize the active peaks that were analyzed by 1D and 2D NMR in 1-mm microtubes. Using this approach, two isoflavan hydroquinones and three isoflavan quinones were characterized *de novo* [214].

3.3.3 MicroCryo probe applications

The use of MicroCryoprobes™ represents further improvement in NMR sensitivity. Such an approach was found to be key in the investigation of Orchidaceae metabolites that were isolated at the microgram scale. In this respect, the search for new anticancer agents in several species of the Orchidaceae family led to the identification of 15 stilbenoids, including a new phenantheraicinone and two new dihydrostilbenes from *Oncidium microchilum* Bateman ex Lindl., *Oncidium isthmi* Schltr., and *Myrmecophila humboldtii* Rolfe (Orchidaceae). The compounds were isolated using the combination of an initial normal-phase flash chromatography followed by a semipreparative HPLC separation with an RP-C\(_{18}\) stationary phase. Despite the small quantity obtained (between 5 and 480 mg per compound), high quality 2D NMR spectra were measured, which allowed structural elucidation of all of the compounds. Several of the compounds were found to inhibit the proliferation of NCI-H460 and M14 cancer cell lines [113].

The combination of SPE-NMR (to trap target compounds) and 1.7-mm microtubes (to enhance the NMR analysis sensitivity) has been used with success by several groups (Table I.C.2). This approach was used for the phytochemical investigation of the *Carthamus*
**General introduction**

oxyacantha M.Bieb. (Asteraceae) extract and was also compared to results from a traditional approach. The classical approach was time consuming and was used for a nontargeted fractionation of the extract, which led to the isolation of only a few compounds. However, HPLC-PDA-HRMS-SPE-NMR was used for efficient targeted isolation and identification of the selected constituents. This hyphenated approach, involving VLC and semipreparative HPLC, was found to be considerably faster and required less solvent and other consumables than the classical approach. The SPE-NMR system employed 1.7-mm tubes, and the NMR spectra were acquired using a cryogenically cooled probe. The elution of the SPE cartridges into the microtubes was performed with a robotic liquid handler, and the tubes were managed using an automated sample changer. For the preliminary analysis, 30 peaks were selected for adsorption onto SPE cartridges to obtain $^1$H NMR spectra. On the basis of the UV or MS data, two or three trappings were performed for each peak. Looking at their $^1$H NMR spectra, most of the peaks were found to correspond to saturated fatty acids and flavonoids. On the basis of these initial $^1$H NMR data, 11 peaks, presenting unusual NMR signals, were selected for SPE-NMR cumulative trappings for additional studies using 2D NMR. A total of 15 compounds, of which four were new spiro compounds, were finally characterized [211].

Recently, a similar approach involving LC-SPE-ttNMR (ttNMR, tube transfer nuclear magnetic resonance) was used to quickly screen α-glucosidase inhibitors in complex matrices [205]. Alpha-glucosidase inhibitors are oral antidiabetic drugs that prevent carbohydrate digestion and are used for the treatment of diabetes mellitus type 2 [218].

This approach of using LC-SPE-ttNMR was divided into three main steps: first, the active extract was fractionated using a chromatographic scout-separation in a 96-well plate for activity evaluation; second, the glucosidase inhibition of the analytes was assessed in the microtiter plates, which permitted the establishment of an HPLC biochromatogram for the localization of the active compounds; and finally, the LC-SPE-NMR analysis was targeted on the active peaks to elucidate their structures. This methodology was applied to the analysis of the ‘Pink Lady’ apple peel extract. The 180 fractions that were collected during the scout separation were assessed for α-glucosidase inhibitory activity after evaporation of the HPLC solvent. Analytes with α-glucosidase inhibitory activity were correlated with their corresponding peaks in the HPLC chromatogram. The information obtained was then used for the setup of the UV-threshold-based trapping of the active peaks for multiple trapping onto the SPE cartridges. Trapped compounds were automatically dried for 45 min using a nitrogen gas flow and subsequently eluted into 2.5-mm NMR tubes with 140 mL of methanol-$d_4$. This strategy allowed the identification of epicatechin, reynoutrin, and avicularin as the active compounds.

### 3.3.4 Quantification using at-line LC-NMR applications

Direct quantification by NMR (qNMR) after on-line or at-line LC-NMR has been attempted for a limited number of applications. For on-line LC-qNMR quantification can be achieved using an internal standard that is either added to the sample before chromatographic separation for the isocratic mode or added to the LC solvent in gradient mode to
compensate for the changing solvent composition [117]. For at-line LC-NMR approaches, isolated compounds are sometimes quantified using an internal standard [219], with the $^{13}$C satellite signals (which is adapted particularly for use with cryo probes) [89] or by comparison with the residual solvent signal [120]. An innovative approach was used in the field of environmental toxicology that involved using NMR to quantify cyanide in water, whereby the ions were derivatized with a fluorinated reagent; then, the reaction mixture was purified by LC-SPE-NMR; and finally, after trapping and using an internal standard that was added to the analyte with the NMR solvent, the compound was quantified by at-line $^{19}$F and $^1$H NMR [220]. Through the combination of different information, such as the retention time from the LC and the chemical shift of NMR spectroscopy, higher specificity for cyanide quantification was achieved compared to that previously observed with common analyses techniques such as gas chromatography.

3.4 Advantages of microfractionation for tracking complementary activity

As full structural characterization of NPs eluted in microgram amounts from an HPLC column has become feasible thanks to the improvement in NMR sensitivity, the direct analysis of the generated microfractions in a biological assay seems the logical next step in NP drug discovery. Several on-line biochemical assays have already been developed [221,222], whereby detection is achieved using either UV, fluorescence or MS spectrometry. Drawbacks of these methods (termed high-resolution screening, HRS) are, on the one hand, the limited validity of biochemical assay compared to in vivo or high-content assays and, on the other hand, the lack of determination of the potency of the detected activity as these detectors do not show a quantitative response to analytes in complex mixtures such as plant extracts. Microfractionation coupled to NMR offers the possibility to quantify the generated microfractions in one step with the analyte characterization and directly use the generated microgram amount samples for dose-response assessment.

This strategy was applied for the detection of antiangiogenic and anti-inflammatory compounds from the Tanzanian plant *Rhynchosia viscosa* (Roth) DC. (Fabaceae) [202] and is shown in Figure I.C.7. Here, the use of an internal standard for NMR quantification is not desired (so that the samples are not contaminated before biological testing) and an external standardization method (pulse length based concentration determination, PULCON [121]) was adopted. At first, the enriched methanolic extract was microfractionated using time-triggered collection (Figure I.C.7a and b). Then, all generated microfractions were screened in an in vivo zebrafish assay for their antiangiogenic activity (Figure I.C.7c). Bioactive microfractions were subsequently analyzed using $^{1}$H NMR (Figure I.C.7d) and their content was characterized (Figure I.C.7e). The same analyses permitted the quantification of the isolated compounds (Figure I.C.7f) and thus, the microfractions containing compounds in a pure form and at highest amount were taken to perform dilution series for concentration-response analysis directly on the microgram amount samples.
Figure 1.C.7: High-resolution screening of plant extract with direct estimation of potency of isolated microgram amount samples using quantitative NMR and microfractionation.

(a) Semipreparative HPLC chromatogram for the microfractionation of the enriched extract of Rhynchosia viscosa (Roth) DC. HPLC conditions: XBridge™ BEH C₁₈ column (250 × 10 mm i.d., 5 μm); A: 0.1 vol.% formic acid-H₂O, B: 0.1 vol.% formic acid-MeOH, 40-90% in 74.9'; 2.3 mL min⁻¹; ESI-MS detection in negative ionization mode. (b) Microfractions were collected every 30 s into 96-deep well plates. (c) All collected microfractions were tested for antiangiogenic activity on a zebrafish model. Several microfractions eluting after 30 min were inhibiting angiogenesis to 70-100% and were analyzed by ¹H NMR using a microflow NMR probe [CapNMR™], (d) and contained the isoflavone genistein (e). *: impurity. (f) The amount of genistein in the individual microfractions was quantified with an external standard using PULCON. (g) The microfraction with the highest amount of genistein (F1) was used to perform a dilution series for a concentration-response analysis of the antiangiogenic activity. Source: Bohni et al., 2013. Integration of microfractionation, qNMR and...

4. Conclusion

There is an important need for the rapid and efficient identification of NPs in complex biological mixtures, especially for NP-based drug discovery programs and for many aspects related to food supplements or phytopharmaceuticals. This need has been increased considerably by the rapid advancement of metabolomics where peak annotation represents a major bottleneck in the approach and structures of a large number of biomarkers need to be correctly assigned. Despite the considerable advances in metabolite profiling methods based on advanced LC-MS or LC-MS/MS methods and as a result of the lack of unified MS databases for small molecule, the unambiguous identification of NPs must rely mainly on NMR.

Over the past three decades, the sample amount required for de novo structure identification has decreased from the range 20-50 mg to less than 1 mg for conventional 1D and 2D NMR measurements. With access to higher magnetic field strengths and the development of dedicated micro NMR probes, sensitivity has greatly increased and $^1$H NMR spectra can be recorded in the sub-microgram level, whereas 2D NMR experiments can be obtained with only a few micrograms using state-of-the-art NMR technology. These amounts can now be readily obtained from the single HPLC metabolite profiling analysis of a crude natural extract.

The hyphenation of NMR to HPLC has evolved over the years to achieve rapid metabolite identification in crude natural extracts. Direct hyphenation of on-flow LC-NMR has been demonstrated to be practical and feasible at the expense of some compromises. To overcome most of the limitations associated with on-flow LC-NMR and to provide access to key 2D NMR experiments, efficient methods of analyte enrichment via multiple collections of given LC peaks have been integrated and have evolved toward automated and efficient LC-SPE-NMR-MS platforms providing high quality spectra, on-line in a flow cell, or at-line after transfer in microtubes.

To further improve sensitivities for limited amounts of sample, room-temperature microflow probes and small-diameter cryo probes have been developed. Such probes are no longer used on-line but are used at-line after HPLC separation. Their integration for efficient peak identification requires that metabolite profiling methods be scaled up, enabling the separation of milligram amounts of complex extracts with a good prediction of the accuracy. For efficient microfractionation of the extracts, LC-MS-triggered fractionation strategies can be used, which allow the precise collection of given LC peaks based on their extracted ion traces. This can be performed directly in 96-well plates, and subsequent NMR analysis of the wells can be automated to yield high quality 1D and 2D NMR spectra using only a few micrograms of NPs. However, the spectral quality is still strongly dependent on the quality of the chromatographic separation; therefore, the HPLC high-resolution separations at the semipreparative scale need to be further advanced, as
this has been the case at the analytical level with the introduction of columns packed with sub-2 µm particles used for UHPLC.

For applications involving unresolved LC peaks or the analysis of fractions still containing a mixture of a few NPs, multivariate data analysis methods for the NMR data sets and covariance analysis with orthogonal methods such as LC-MS may be useful to extract NMR information of pure constituents from simple mixtures [122].

The quality of metabolite profiling studies will continue to rely on NMR for unambiguous identification. With the development of HPLC biological profiling assays, NMR will also be used, with increasing frequency, for the absolute quantification of the amounts collected by microfractionation and for the assessment of the bioactive potency of given NPs.

The $^1$H NMR spectra that are generated by a well-defined deuterated solvent using sensitive at-line approaches have the advantage of being universal and comparable between instruments. In this respect, the creation of tools and NMR databases of NPs that are easily searchable and implementable should improve the efficiency of peak identification in HPLC. The high-throughput acquisition of NMR data obtained with these latest technologies and the access to NMR databases should very significantly boost NP research in the years to come.

5. Acknowledgments

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II. Microorganism co-culture
Microorganisms have proven to be promising candidates for the production of clinically useful drugs as well as novel chemical entities [4,223]. Among microorganisms, fungi are known to produce a wide array of secondary metabolites and several drugs of fungal origin have been developed already. Fungi are particularly interesting sources for antibiotics research, whereas important antibiotics include amphotericin B and penicillin. Furthermore, ciclosporin had been discovered as antifungal agent [224] and is an important immunosuppressive agent today.

Within the framework of this thesis work, fungi of the genus *Fusarium* were of particular interest. Fungal species of the genus *Fusarium* are ubiquitously found in nature, as common soil saprophytes or as plant pathogens. For instance, *Fusarium oxysporum* is the causal agent of vascular wilts in several hosts, such as peas, sugar beets and tomatoes. *Fusarium solani* is a pathogen of a large number of plant species and is associated with dry rot of potatoes and root diseases of peas. In medicine, *Fusarium* species, mostly *F. solani* and *F. oxysporum*, have not only emerged as major opportunistic fungi in patients with severe immunosuppression, but were also found to be infectious fungi in fungal nail infection (onychomycosis) [225]. Furthermore, the chemical diversity of the genus *Fusarium* is rich [226].

One important phenomenon leading to secondary metabolite production is the interaction between fungi. These compounds, called ‘mycoalexins’ [30], play an important role in defense, similar to phytoalexins in plants. Competition among fungi for nutrients and living space has led to enhanced enzymes and secondary metabolites production to secure their own successful growth [227,228].

The term *microbiome* designates the “ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share” the same confined space [229]. Accordingly, fungal communities are called *mycobiome*.

In various situations in plant pathology, such as esca of grapevine, fungal communities live in a confined space together in the host organism (i.e., fungi are restricted to a small space with few and scarce nutrient sources). The members of this mycobiome interact and may well adapt their secondary metabolite production in response to other community members. Analogously, nails can be considered as a confined space. Mixed infections (at least two fungal species) have also been clearly demonstrated to occur in onychomycosis and cause almost 15% of infections [225]. In some cases, it could be possible that a dermatophyte (fungi of the genus *Trichophyton*, the main pathogen of onychomycosis) has been eliminated by confrontation with a non-dermatophyte filamentous fungus (NDF) such as *Fusarium* sp. or *Acremonium* sp. that settled in a previously infected nail [230]. However, the interactions of different species of fungi in nail infections remain an open research area.

The artificial reconstitution of onychomycosis-derived fungi by cultivation in the same confined space, co-culture on a Petri dish on solid medium, mimics the nail mycobiome. This
approach can be used to study interactions at the molecular level and to evaluate if common traits between human and phytopathogenic species exist, for example. In addition, this approach can be useful to exploit NPs that are induced in fungus-fungus interactions to search for new antifungals. In the present study, substances active against *Fusarium* spp. were of particular interest.

The concept of microorganism co-culture is reviewed in the following chapter. The focus is laid on co-culture as method to increase the chemodiversity that fungi and bacteria are capable of. The chemical and biological background as well as methods for the chemical analysis of co-cultures are given.
II.A Metabolite induction via microorganism co-culture: a potential way to enhance chemical diversity for drug discovery

This chapter was published as research review article.


* These authors contributed equally to this work.

Contribution: Preparation of the parts of the table of applications, design and optimization of half of the figures, writing of the introductory chapters (1-3 and 5) and description of the co-culture applications, proofreading of the article
Abstract

Microorganisms have a long track record as important sources of novel bioactive natural products, particularly in the field of drug discovery. While microbes have been shown to biosynthesize a wide array of molecules, recent advances in genome sequencing have revealed that such organisms have the potential to yield even more structurally diverse secondary metabolites. Thus, many microbial gene clusters may be silent under standard laboratory growth conditions. In the last ten years, several methods have been developed to aid in the activation of these cryptic biosynthetic pathways. In addition to the techniques that demand prior knowledge of the genome sequences of the studied microorganisms, several genome sequence-independent tools have been developed. One of these approaches is microorganism co-culture, involving the cultivation of two or more microorganisms in the same confined environment. Microorganism co-culture is inspired by the natural microbe communities that are omnipresent in nature. Within these communities, microbes interact through signaling or defense molecules. Such compounds, produced dynamically, are of potential interest as new leads for drug discovery. Microorganism co-culture can be achieved in either solid or liquid media and has recently been used increasingly extensively to study natural interactions and discover new bioactive metabolites. Because of the complexity of microbial extracts, advanced analytical methods (e.g., mass spectrometry methods and metabolomics) are key for the successful detection and identification of co-culture-induced metabolites.

This review focuses on co-culture studies that aim to increase the diversity of metabolites obtained from microbes. The various strategies are summarized with a special emphasis on the multiple methods of performing co-culture experiments. The analytical approaches for studying these interaction phenomena are discussed, and the chemical diversity and biological activity observed among the induced metabolites are described.

**Keywords:** mixed fermentation, co-culture, natural products, interspecies communication, gene cluster activation, microorganisms, mass spectrometry, metabolomics, microbiome, antimicrobials
1. Introduction

Natural products (NPs) are important sources of novel bioactive compounds. Although many industries have ceased or significantly reduced their NP drug discovery programs, NPs continue to be of interest to pharmaceutical companies [75,231]. Indeed, nature provides a massive reservoir of organisms that produce potentially beneficial compounds to be discovered and explored (bioprospecting) [11]. The inventiveness of nature regarding the production of innovative and unusual molecular skeletons (chemodiversity) is unmatched.

Nevertheless, because NPs are obtained from living species—as opposed to via combinatorial synthesis—there are several aspects that make NP drug discovery more complicated. For example, the organisms from which NP extracts are to be obtained must be identified, and questions concerning re-sourcing and intellectual property rights must be addressed. The production of secondary metabolites (metabolic expression) in these living species depends on growth or culture conditions, which can be difficult to control. In addition, NP extracts are complex mixtures, which further complicates their assessment for bioactivity because the presence of overly toxic or PAIN (pan-assay interference) compounds may mask the effects of the sought-after compounds or active constituents are present in too small a quantity. NPs or NP extracts that are compatible with high-throughput assays can be obtained through the enrichment or isolation of desirable compounds, but these tasks can be labor-intensive and difficult. Furthermore, one of the major challenges in working with NPs is the rediscovery of previously identified molecules. Avoiding rediscovery demands accurate chemical analysis of the extracts and detailed databases of all known compounds (dereplication [53,54,61,123]). In addition, NPs are often complex molecules and exhibit numerous stereocenters, making the elucidation of their structures challenging. Overall, drug discovery from NPs is not considered to be highly compatible with high-throughput screening (HTS) techniques or the time frame allocated for the validation of a hit compound [5] demanded by industrial drug discovery programs. Thus, in the industry, drug discovery programs mainly operate on the basis of NP-like compounds produced through combinatorial synthesis [6,7].

However, in recent years, progress has been made on several fronts. These include improvements in chromatography techniques, which allow accelerated isolation of molecules from complex mixtures [126,202,232,233]; in analytical techniques, notably in nuclear magnetic resonance (NMR) detection, which now requires only microgram amounts of compounds for identification [127,129,234]; and in molecular biology, which provides for alternative routes to generate NPs [235]. Hence, these advances should facilitate the compatibility of NPs with industry drug discovery programs.

Among the established sources of NPs, microorganisms have proven to be promising candidates for the production of novel scaffolds as well as marketable drugs [4,12]. One particularly well-known example is provided by the β-lactam, penicillin. Other examples of important leads isolated from microorganisms (Figure II.A.1) include the macrolides used as antibiotics (erythromycin), antifungals (amphotericin B) and immunosuppressive drugs.
(cyclosporin) and depsipeptides with antibiotic activity (fusafungin). Furthermore, the cholesterol-lowering agents of the statin class (e.g., lovastatin) were the fourth bestselling group of pharmaceuticals in 2013 in the US [236] and were the bestselling NP-derived drugs. Moreover, microorganisms are also known to produce particularly innovative scaffolds, such as pleurotine [237], enediyne antibiotics [238] or diketopiperazines [239].

Bacteria and fungi are therefore important sources of drugs and lead compounds [10,12,240]. For example, 33% of all natural product-related antitumor drugs approved by the US Food and Drug Administration (FDA) by 2012 were of microbial origin or were derivatives of microbial metabolites [223]. Compounds from microorganisms and marine organisms constituted the

Figure II.A1: Microbial natural products. Molecules of pharmaceutical interest or exhibiting particular structural diversity from microbial sources.
largest group of promising anticancer drugs among the compounds evaluated in oncology clinical trials as of 2004 [241]. Compared to other natural resources, microbes are presently the most attractive source of NPs in drug discovery [5], mainly because of their ubiquitous occurrence [242-244], their extensive biodiversity and the large chemodiversity that can be found within a given species [11,242,245]. To further broaden the diversity of species to be studied, methods have been developed to utilize thus far uncultivable microorganisms [246,247].

Another key feature of these organisms is their ability to be cultured in the laboratory and the fact that the production of NPs can be optimized and scaled up as required. For microorganisms, culturing can be performed in liquid or solid media [32] or in fermenters [248]. The culturing conditions are substantially controlled by the composition of the culture medium [249,250].

With the development of molecular biology techniques, microorganisms can also be engineered to produce specific compounds. The desired molecules are then obtained through heterologous expression in a host organism to circumvent culturing problems or to access otherwise silent gene clusters [251,252]. Particular efforts were made to develop additional methods for the activation of silent gene clusters [235,253-257]. A method has been developed to screen bacterial DNA for preserved gene regions that code for different classes of polyketides, non-ribosomal peptides or diterpenes. This method is complementary to the other existing methods and allows for the selection and prioritization of promising producers [258]. Moreover, methods combining genomics and chemistry have been established that allow the simultaneous identification of novel bioactive compounds and their respective mechanisms of action [81]. All of these advances have led to a favorable situation, in both academia and the pharmaceutical industry, in which the use of NP libraries continues “to cataly[z]e innovation in biomedical research” [5,9,259,260].

Among these general considerations regarding the usefulness of microorganisms in NP research, a core area of interest is the possibility of generating new scaffolds to discover novel bioactive compounds. In addition to the bioprospecting of species not previously studied, which carries a high risk of redundancy, one strategy consists of exploiting biosynthetic pathways that are not triggered under normal laboratory growth conditions in known producers.

There are various methods for inducing chemodiversity in selected organisms at different levels, ranging from the genome to the metabolome (Figure II.A.2). In addition, the chemical-ecological relationships that occur in microorganism communities (interspecies interactions) can be exploited. In this respect, the novel approach in which microbes are grown together (co-culture or confrontation experiments) has received increasing interest related to the potential discovery of new leads and to understand the triggering of specific biosynthetic pathways, mainly related to defense.
This review assembles various historical examples of microbial co-culture as well as recent applications that highlight the use of such methods from the perspective of increasing microbial chemodiversity. A general introduction that summarizes ways of modeling chemodiversity is provided, with a particular focus on co-culture in solid and liquid media. The different analytical approaches that are used to study the microbial metabolome and to highlight novel metabolite induction in co-cultures are then described. Finally, the challenges and perspectives arising from this new field of research are discussed.

2. Strategies for increasing the chemodiversity of microbes

Based on the genome sequences of some fungi (e.g., [261]), it is estimated that microorganisms are capable of producing many more compounds than are observed in the analysis of conventional in vitro cultures [262]. Thus, many biosynthetic gene clusters are presumably silent under standard laboratory conditions [263]. Several methods have been developed to activate these cryptic gene clusters or, more generally, to augment the chemodiversity of which the microorganisms are capable (Table II.A.1). This can be achieved at the genome, transcriptome, proteome or metabolome levels (Figure II.A.2), for which the methods are briefly described below and have been extensively reviewed (e.g., [255,256,264-266]). Section 3 provides further details on methods that involve varying the culture conditions or co-culturing multiple organisms to modulate the chemical composition of microbial extracts.

2.1 Accessing and altering the genes

Since the genome sequences of several fungi and bacteria have been made available, influencing the biosynthesis of secondary metabolites through the manipulation of genome sequences has become possible [235,256,266]. The methods used for this purpose—summarized as metabolic engineering—include various methods for gene knockout, promoter exchange and overexpression of transcription factors [267,268]. NPs that are generated from these modified gene clusters are then obtained through either heterologous expression, where the gene cluster is inserted into a different microorganism in which culturing is straightforward, i.e., a host organism such as Escherichia coli, or homologous expression, where the gene cluster is back-inserted into the original microorganism. Alternatively, the microorganism’s genome can be altered to induce greater artificial chemodiversity [265] via mutasynthesis. In this technique, the genes responsible for the uptake of the starting substrate of a metabolite are disabled, and the starting block of choice is fed to the microorganism (combinatorial biosynthesis). Thus, an altered NP is generated using the starting block provided to the organism. Mutasynthesis demands substrate flexibility concerning these unnatural substrates. All of these molecular methods require that the biosynthetic gene clusters are sequenced and that putative functions are assigned (through whole-genome sequencing or via genome mining [267]). An alternative approach is random or ultraviolet (UV) mutagenesis, which has been successfully applied to generate antibacterial
compounds from *Aspergillus oryzae* [269] and a novel macrolide from a *Sphaeropsidales* sp. mutant [270].

**Figure II.A.2: Methods to influence secondary metabolite biosynthesis in microorganisms.**
Possible methods for influencing the production of secondary metabolites in microorganisms or augmenting their chemodiversity. The upper methods (blue and green backgrounds) demand prior knowledge of the genome sequence. Abbreviation: OSMAC, one strain-many compounds approach.

Another field that seems promising for increasing and accessing the chemodiversity of microorganisms is metagenomics [246]. Under this approach, DNA from complex microbial communities (e.g., soil or gut microbiomes) is extracted and cloned in laboratory hosts to create a DNA library. The DNA from these metagenomic libraries is then purified and fractionated for direct recombinant expression in a host. The generated clones are either tested directly for the production of bioactive compounds in a particular assay or the metabolites are extracted and tested through a HTS approach [246,247].

### 2.2 Interfering with transcription

Inside eukaryotic cells, the genome is packaged into chromatin with histone proteins, and this structure exerts profound control over gene transcription [271]. The chromatin structure is closely associated with the epigenetic state of histones, alkaline proteins that package and order the DNA into nucleosomes. Epigenetic histone modifications include the production of acetylated, methylated or ubiquitinated lysines, methylated arginines and phosphorylated serines. Together with DNA methylation, which also occurs in non-eukaryotes, the modulation
of chromatin structure has major effects on the overall expression of genes and thus subsequent metabolite biosynthesis.

Table II.A.1: Methods to increase chemodiversity in microorganisms through manipulation or activation of biosynthetic genes.

Several facets of the utility of the presented methods are given. The general prerequisites, whether the microorganisms must be cultivatable (cultivation-dependence) and whether the genome-sequence must be known (genetic information) are indicated. Another aspect is whether the method permits the activation of silent biosynthetic genes (activation of silent genes). Then, an estimate is given regarding whether the method potentially allows the generation of larger quantities of the compound (upscalability) and whether the method is capable of possibly yielding novel structures (generation of novel structures). Abbreviations: NP, natural product; OSMAC, one strain-many compounds; ☒, Yes; ☐, No.

<table>
<thead>
<tr>
<th>Method</th>
<th>Prerequisite</th>
<th>Activation of silent genes</th>
<th>Up-scalability</th>
<th>Generation of novel structures</th>
<th>References^a</th>
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<tr>
<td>Mutasynthesis</td>
<td>☒, ☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>[265, 272]</td>
</tr>
<tr>
<td>Heterologous expression</td>
<td>☐, ☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>[252, 255, 256]</td>
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<tr>
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<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>[255, 256, 272]</td>
</tr>
<tr>
<td>Enzyme inhibition</td>
<td>☒, ☒</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>[256, 273, 274]</td>
</tr>
<tr>
<td>Precursor-directed biosynthesis</td>
<td>☒, ☒</td>
<td>☐</td>
<td>☒</td>
<td>☐</td>
<td>[265]</td>
</tr>
<tr>
<td>Substrate feeding</td>
<td>☒, ☒</td>
<td>☐</td>
<td>☒</td>
<td>☐</td>
<td>[265, 272]</td>
</tr>
<tr>
<td>Co-culture, liquid medium^b</td>
<td>☒, ☐</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>[256, 275, 276]</td>
</tr>
<tr>
<td>Co-culture, solid medium^c</td>
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<td>☒</td>
<td>☐</td>
<td>☐</td>
<td>[31, 277]</td>
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<tr>
<td>OSMAC</td>
<td>☒, ☐</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>[248, 256, 278-280]</td>
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<tr>
<td>Epigenetic modification^d</td>
<td>☒, ☐</td>
<td>☒</td>
<td>☒</td>
<td>☒</td>
<td>[256, 264, 281]</td>
</tr>
</tbody>
</table>

^a Review articles describing the different methods from the viewpoint of NP and drug discovery (unless not available; then, original articles are given) / ^b Involved microorganisms must tolerate the same culture conditions / ^c A large-scale solid-state fermenter has been developed recently [32]. The applicability to solid-media co-cultures remains to be proven. / ^d Has not yet been tried on bacteria.

For example, a deficiency in the methylation of specific histones permits the induction of silent gene clusters in *Aspergillus nidulans* [281]. This gives rise to the possibility of using epigenetic modifiers to induce the transcription of otherwise silent gene clusters [282, 283]. Such epigenetic manipulation can be achieved using small molecules, such as 5-azacytidine, suberoylanilide hydroxamic acid (SAHA) or nicotinamide, that inhibit either DNA
methyltransferase (DNMT) or histone deacetylase (HDAC). This technique has been applied in several fungi [264].

Additionally, global manipulation of transcription factors allows the simultaneous targeting of the expression of several secondary metabolite gene clusters. This approach has been demonstrated in Aspergillus spp., in which the overexpression of laeA, a protein with homology to methyltransferase, activated or enhanced the production of several known bioactive compounds (nonribosomal peptides, polyketides, terpenes, etc.) [284].

Because epigenetic modulation affects the transcription of many different gene clusters, interference at this level can have negative effects on the survival of the targeted microorganism. Indeed, reduced growth has been observed under the influence of epigenetic modifiers, and such an approach can currently be considered untargeted with an unpredictable likelihood of success [282]. The diversity of epigenome-related targets among fungi is large, and not all fungal strains respond to currently known epigenetic modifiers [264].

2.3 Altering biosynthesis

The addition of enzyme inhibitors to the culture medium can block certain biosynthetic pathways, thus shifting secondary metabolite biosynthesis towards the production of other NPs from either silent or poorly expressed gene clusters [273,274]. Enzyme inhibition may cause both the elicitation and inhibition of NP biosynthesis. For example, tricyclazole inhibits the production of 1,8-dihydroxynaphthalene in Sphaeropsidales sp. and simultaneously induces the production of sphaerolone and dihydrosphaerolone [285,286].

One possible target for secondary metabolite induction is the SUMOylation of proteins (SUMO: small ubiquitin-related modifier), which is involved in transcriptional regulation by post-translational modification of the proteins. A study conducted in an Aspergillus nidulans mutant incapable of protein SUMOylation showed altered NP formation compared to the intact fungus [287]. Various inhibitors of SUMOylation have been discovered [288-290], but their utility in the induction of novel NPs remains to be proven.

As discussed previously (see section 2.1), metabolomic modifications can be performed upstream in biosynthetic pathways by feeding the microorganisms unnatural substrates (precursor-directed biosynthesis [265]). This approach requires substrate flexibility of the enzymes involved, and the corresponding biosynthetic origin of the targeted NP must be known. This method presents a high likelihood of leading to pharmaceutically relevant compounds because functionalities leading to drug-like properties can specifically be introduced into the modified molecules. This approach is useful for optimizing particular scaffolds to increase bioactivity or reduce toxicity.

Overall, the use of enzyme inhibitors/inducers, similar to the use of epigenetic modifiers, mainly relies on serendipity, except when the complete regulation of a gene cluster has previously been described.
2.4 Modifying metabolite structures in extracts

Enhancing the chemodiversity of metabolites is also possible through chemical modification of their structures. Classically, minor skeleton modifications are attained through various methods of derivatization, and major alterations can be achieved using semi-synthesis on isolated microbial metabolites [265,272]. Alternatively, chemical modifications can be performed directly at the metabolome level (on the crude extract), without prior isolation of individual compounds (chemodiversity enhancement). This strategy was successfully applied to modify β-caryophyllene and flavonoids [291,292].

Again, the chance of obtaining pharmaceutically relevant compounds is increased, as drug-like properties can be specifically introduced into the modified molecules to optimize the bioactivity and safety profile of an NP.

3. Genome sequence-independent approaches for inducing chemodiversity

The production of secondary metabolites by microorganisms is strongly dependent on environmental factors, such as growth conditions and biotic and abiotic stresses [248,278,280]. Thus, the selective variation of the culture conditions (mainly abiotic) for a given microbe [278,279] and/or stress induction through interaction with other competing microorganisms in a co-culture [275,276,293,294] represent interesting ways to generate enhanced chemodiversity. Such approaches can affect various levels of the cellular machinery (genome, transcriptome, proteome or metabolome).

Because genomic information is only available for a limited number of microorganisms, precluding the use of sophisticated molecular biology techniques, multiple other methods have been developed to overcome the difficulty of NP production under certain culture conditions. These approaches have been inspired by various examples in nature, where microbial communities (the so-called microbiome) are omnipresent (see section 4).

3.1 Variation of culture conditions

Culture conditions affect the metabolite profiles of microorganisms, which has led researchers to perform experiments with different culture media to optimize metabolite production. This method is known as the OSMAC (one strain-many compounds) approach [278], in which culture media and nutrients are systematically varied to simulate different environments and consequently induce the production of different metabolites. The parameters that are varied include light [295], pH, temperature and the oxygen supply. The media are varied in terms of carbon, nitrogen and phosphorus sources, inorganic salts or trace metal contents [280]. The culture medium can also be supplemented with other small molecules or heavy metals that function as growth inducers or suppressors (as detailed above, e.g., for HDAC or enzyme inhibitors) or through unknown mechanisms (e.g., when using the organic solvents dimethylsulfoxide and ethanol [296]). In some cases, these modifications influence transcription factors [297 and references therein,298] and can be considered a form of
epigenetic modulation. Applications of the OSMAC approach have proven to be successful for the production of drug-like compounds and novel metabolites [278,286,299].

3.2 Mixed fermentation and solid medium co-culture

Another way to stimulate the biosynthesis of secondary metabolites is to challenge a given microorganism with biotic stress, which can be induced through interaction with other microbial partners.

Indeed, in nature, microorganisms are often found in communities (see section 4), where they produce secondary metabolites related to the interaction type involved (sexualization, growth inhibition or stimulation), mainly in the context of defense or nutrient competition. Such situations can be reproduced artificially by culturing two or more microorganisms together [300]. This strategy has often been defined as ‘co-culture’ when solid media are used or ‘mixed fermentation’ when liquid media are involved. These interactions might either mimic naturally occurring communities (mainly in the framework of chemical ecology studies) or constitute an artificial community for studying NP induction specifically in confrontation zones (mainly to discover new bioactive compounds). All of these aspects will be discussed in the second part of this review.

Figure II.A.3: Number of publications per year in the field of microorganism co-culture.
Evolution of research efforts in the field of microorganism co-culture, as indicated by the number of publications per year from 1950 to 2013. Journal articles, reviews, conference proceedings and book chapters with the following keywords were searched in the Web of Knowledge (Thomson Reuters): microorganism co-culture, mixed fermentation, coculture and combined-culture.
The concept of co-culture has generated increasing interest from the scientific community, and as shown in Figure II.A.3, the number of applications of this strategy has increased greatly over the last decade. Although the morphology of microorganismal interactions has been of interest to microbiologists for years, the resurgence of interest in this field, especially concerning biochemical aspects, is most likely related to the technological advances in the analysis of such complex biological systems (see section 6).

The co-culture approach has been applied to study diverse issues, such as i) in the fundamental investigation of natural communities in an agricultural context (the rhizosphere or mycorrhizosphere) [301]; ii) to elucidate symbiosis phenomena (e.g., the antibiotic protection of a coral by its symbiont [302]); iii) to investigate human microbiome interactions (e.g., positive interactions leading to a ‘healthy situation’) [303-307]; iv) for the induction of pharmaceutically interesting secondary metabolites [207,308,309], such as gliotonitrin A [310]; and v) in targeted applications to improve the production yields of specific fermentation products (e.g., vitamin C synthesis [311]).

4. Microbial interactions in nature

As mentioned above, microbial interactions are ubiquitous and can be found in every biocoenosis. Soil [312] and aquatic or terrestrial organisms, such as plants [313-315], animals [316-318], fungi [319,320] and protists [321], generally host a set of microbes sharing specific ecological niches. These microorganisms include the Prokaryotes (bacteria, archaea) and Eukaryotes (protists, fungi) as well as viruses. They grow and survive in these environments as long as the nutrient sources are sufficient, either in mutualistic relationships or as antagonists. Classically, studies on the biodiversity of microorganisms in a given environment have only been possible in specific cultures using adapted culture media. However, the approach is limited to several groups of microorganisms, including some fungi and bacteria. Some microorganisms are impossible to cultivate artificially because of their dependence on microbe-microbe interactions for development [322]. Others are difficult to detect due to their lack of competitiveness, which leads to growth inhibition in the presence of another microorganism. The recent development of methods that are totally independent of classical cultures, such as polymerase chain reaction (PCR) [323,324], restriction fragment length polymorphism (RFLP) [325], pyrosequencing [326] and, more recently, metagenomics [318,327], metatranscriptomics [328] and other ‘meta’omics’ methods [329], represent new ways to study complex ecosystems and their interactions [330]. As a result, fungal and bacterial communities have been discovered in substrates that were previously considered to be nearly devoid of microorganisms, further emphasizing the importance of studying communities [331].

These complex fungal or bacterial communities have been defined as ‘microbiomes.’ This term includes all of the microbes (bacteria, archaea, viruses, protists and fungi) living in a particular environment or host [332], and this concept has received strong interest, especially in humans.
The coexistence of several microorganisms that share the same niche can affect the organisms’ growth, adaptation patterns, morphology and developmental patterns [333,334], as well as their ability to synthesize proteins and secondary metabolites. Thus, microbial communities have a major influence on their immediate environment or host. For example, in soy sauce production, the composition of the microbial community and the dynamic changes it undergoes during fermentation have been found to be crucial to the quality of the soy sauce [335].

Additionally, the level of microbiome organization (e.g., in biofilms) confers advantages to a community, e.g., by increasing resistance or virulence [333].

Historically, microbial communities have mainly been considered to study antagonistic growth-inhibiting interactions. These interactions have been widely detailed in wood-inhabiting fungal communities, bacterial soil communities [336,337] and specific bacterial-fungal interactions related to the synthesis of antibiotics [310]. The best-known example of such bacterial-fungal interactions is the accidental discovery of penicillin in an unintended co-culture (contamination) of *Staphylococcus* spp. with *Penicillium* sp. in 1928 by Sir Alexander Fleming [338]. Recent research has demonstrated that the interaction between each partner is, among other factors, mediated by the expression of small molecules [275].

### 4.1 Fungal communities

An important phenomenon in triggering the production of secondary metabolites is the interaction between fungi [297] or between fungi and bacteria [256]. The competition for nutrients and space leads fungi to synthesize enzymes and secondary metabolites to enhance their own growth. Fungal colonies develop as multicellular filaments (hyphae) that form an interconnecting network (mycelium) [339]. Two mycelia in close proximity to each other can interact in different ways, specifically in mutualistic, neutralistic or competitive interactions, and can even switch from one interaction type to another [340]. In confined spaces, the fungi are restricted to only one resource unit; i.e., both nutrients and space are very limited. There are two types of competitive mycelial interactions: either one fungus inhibits other individuals or one fungus uses a nutrient/space resource, which is consequently not available to another fungus, a process referred to as exploitation competition. These types of antagonistic interactions occur either at a distance or upon/after physical contact between individual hyphae or mycelial networks [341]. This implies that active recognition of the presence of other mycelia occurs and initiates a combative response. However, the biological mechanisms linked to this recognition are not clear [342]. Reactivity of mycelia at a distance would require the release and recognition of both water-soluble and volatile diffusible compounds, such as in the recognition of sexual partners with trisporic acids in the fungal order Mucorales [343]. However, in some cases, contact between individual hyphae must occur before a response is stimulated, sometimes followed by cell death at one or both mycelial fronts [344,345].
The changes that occur during interactions can include the production of extracellular secondary metabolites, notably phenolic and quinonic compounds [346]. A number of such metabolites have been identified during the past decade in interactions among the mycelia of wood-decaying fungi. In addition to changes in the metabolome, it has been shown that production of different enzymes can be induced during these mycelial interactions. For example, in wood, the production of enzymes such as phenoloxidases and peroxidases [228], including laccase [347] and manganese-dependent peroxidase [348], as well as different lignin-degrading enzymes [349], can be induced.

Wood also represents a good context in which to study fungus-fungus interactions in a confined space [350]. This highly complex substrate shelters a dynamic fungal community, including numerous fungal species occurring in succession [351-353]. Competitive mycelial interactions are very important in the overall development of fungal communities in wood [227,354].

It has been shown that the secondary metabolites of some wood-decaying fungi can act as total inhibitors or stimulators of the growth of other fungal protagonists [355]. In the case of competitive interactions, competing fungi can form zone lines (also defined as confrontation or barrage zones) that are morphologically different from the pure cultures [356]. The strong coloration of such zones denotes significant metabolic activity, which can potentially be exploited to search for novel metabolites [342,357]. A number of such metabolites involved in interactions between the mycelia of wood-decaying fungi (e.g., volatile compounds, such as acetone and 2-methyl-1-butanol, or quinones, such as podosporin A) have been identified during the past decade [358-361]. However, very little attention has been paid to competition between fungi to produce phytotoxic or antifungal substances. Similarly, few studies discuss the exploitation of such phenomena to detect disease markers. Recently, a study using the fungi implicated in esca, an apoplectic disease of grapes, demonstrated the complexity and richness of such zone lines. Transposition of parts of the fungal community onto an artificial medium permitted the identification of melleins with fungitoxic, phytotoxic and bactericidal activity [30]. Solid medium co-culture has been applied based on the approach detailed in section 5, which illustrated its potential for the discovery of new compounds with such activities.

Other ecological niches where fungi live in a confined space include human finger- and toenails, where opportunistic fungi may interact with the dermatophytes that are involved in superficial mycoses [225,362]. The presence of non-dermatophyte filamentous fungi (NDF) and dermatophytes that may act as the etiological agents of onychomycoses [230] has been recently demonstrated via PCR [362]. The process that governs these fungal interactions is not yet known, but artificial co-culturing of hospital isolates has revealed the induction of quinones and other secondary metabolites [357,363].

Other mycobiomes that are now well studied with respect to the progression of fungal or fungus-associated human pathologies [303,364] include those of the oral and nasal cavities,
and the lungs, gut or skin [304]. Cui et al. [303] demonstrated that specific mycobiomes are associated with different diseases and that these communities contribute to disease through interactions within fungi and those between the whole mycobiome and the host.

### 4.2 Bacterial communities

Bacteria often organize into multicellular populations, mainly as biofilms, to colonize an ecological niche [365]. One or several bacterial species interact closely and evolve in communities to exploit limited resources in a confined environment to ensure species survival and procure advantages such as access to nutrients, dynamic growth or increased antibiotic resistance [366]. Chemical communication among the bacterial population appears to be crucial for the integration of new species and the formation of the so-called ‘climax community’, where the protagonists co-exist in a balanced and stable manner as a function of the surrounding micro-environment. For example, the plasticity of cyanobacterial communities permits them to survive in a wide range of salinities [367], and conversely, environmental modifications evoke dynamic changes in the composition and ratio of bacteria within the communities [368]. In *Drosophila melanogaster*, it has been shown that the diet plays a crucial role in influencing the bacterial microbiome of the digestive tract [369]. The microbiome composition evolves in relation to host physiology or according to modification of the substrate over different time scales, as shown, for example, in human cadavers [370]. Similarly, changes in coral physiology due to environmental factors impact the diversity of the mucus-associated bacterial communities [371].

### 4.3 Bacterial-fungal communities

Mixed populations of bacteria and fungi occur in a wide variety of ecological niches and are conditioned by the access to nutrients and subsequent colonization of the substrates. The composition of such complex communities is directly linked to specific conditions, such as a particular health or disease state in the case of human microbiomes [372].

Rich microbial diversity is also observed in soil. One gram of soil can contain up to ten billion microorganisms, likely including between 1,000 and 10,000 species of unknown prokaryotes [373]. The interactive relationships and combinations of individuals and communities are therefore essentially infinite. In such communities, complex mutualistic interactions exist, including the use of fungal hyphae by bacterial cells for protection against soil modifications or for access to nutrients. Some of these interactions are key factors, as exemplified by the oxalate-carbonate pathway in soil, which implies a specific interaction of fungi and bacteria [374].

All these examples of specific natural microbial communities demonstrate the importance of the cohabitation abilities of the implicated species, allowing them to reach an equilibrium in a given ecological niche. These microbiomes are governed by complex multidimensional interactions, among which chemical communication and secondary metabolite induction represent promising sources of chemical diversity. These communities are worth being
studied in depth, both to understand the key regulation phenomena and to assess the bioactivity of the identified molecules.

5. From natural communities to chemodiversity enhancement

Numerous natural interactions between microbes can be exploited to mimic confrontations under artificial conditions. From a biochemical perspective, all of these interaction phenomena are characterized by the induction of bioactive defense metabolites or toxins, such as phytoalexins in plants [375] or mycoalexins in fungi [30]. Thus, accurate knowledge of the secondary metabolite composition of the organisms entering into the competition is required for an in-depth investigation of these interactions. Sophisticated analytical methods are often required to detect prominent as well as subtle but significant metabolome variations (detailed in section 6).

Biotechnology research has attempted to mimic culture conditions in vitro for selected microbes to generate natural microbiota, produce high-value compounds and increase the productivity of particular strains. The transfer of a species from its natural habitat to an artificial environment must be carried out via successive improvement steps, based on a good understanding of the growth mechanisms of the target microorganisms. This is important, for example, because the production of secondary metabolites is associated with the formation of the asexual reproduction organs in fungi [376]. The colonization of the medium is dependent on the type of cultured microorganism: filamentous fungi ensure their growth through the extension of hyphae producing lateral or apical branches, leading to the formation of macroscopic mycelia, whereas bacterial cells attach to a surface and can organize themselves as a biofilm, embedded in an extracellular matrix of polymeric substances (mainly polysaccharides) [377].

However, microorganismal morphology can be modified in relation to the substrate type and culture conditions. For example, in most cases, the development of a fungal species on a solid medium leads to circular colonies with characteristic growth margins, whereas in liquid medium, the same species could develop as plates, crusts or ‘pellets’ of mycelial aggregations. Therefore, upscaling microbial cultures for the biosynthesis of secondary metabolites is strongly dependent on the type of substrate involved and on several qualitative biological parameters, such as the carbohydrate and nitrogen sources and macro- and microelement availability, as well as engineering constraints (temperature, hygrometry, light, oxygen supply) [341]. Complex multidimensional interactions cannot be studied directly because the contributions from the different individuals are difficult to isolate. One strategy consists of studying the interaction between two representative partners under controlled conditions. For this purpose, microbes can be either cultivated on solid or in liquid media, but the culture conditions must be compatible for both partners.
5.1 Co-cultures on solid substrates

Fungi are well adapted to develop on solid media because they use hyphal growth to colonize unexplored regions containing nutrients [376]. The growing fungal tip is the center of intense metabolic activity, mainly to ensure hyphal extension.

Morphologically, the interaction of two fungi can lead to four major ‘interaction types’ (distance inhibition, zone lines, contact inhibition and overgrowth), as defined through the observation of numerous co-culture experiments performed on solid agar plates (Figure II.A.4) [31]. This type of behavior can also be observed in natural habitats, such as the colored zone lines in wood [227].

![Morphological interactions observed among two fungi in a Petri dish.](image)

*Figure II.A.4: Morphological interactions observed among two fungi in a Petri dish.*

Historically, the first *in vitro* co-cultures of fungi were performed on solid media. This culturing format has permitted the study of morphogenetic and metabolic changes that occur at the mycelial front [378] as well as interaction patterns [31,207,227,379]. Many additional studies have been accomplished based on the confrontation of fungi, bacteria and protists with each other [374,378,380-382], including studies of fungus-bacterium interactions [383] or fungus-fungus interactions [379,383-385]. To investigate the metabolic processes related to mycelial interaction and their relevance for industrial purposes, sets of various fungi could be selected. The relevant fungi can be chosen on the basis of taxonomic criteria, ecological data (sharing an ecological niche) [386], or growth rate behavior, or they can be selected randomly. Because more than 5 million fungal species could exist [63,387], there are incredible opportunities for developing an almost infinite number of interactions, which could lead to bioactive NPs. This is also true for other microorganisms, for which the biosynthetic potential of such groups has been greatly underexplored. Conducting species confrontations on solid media permits the morphology of the species involved in the interactions to be distinguished, and thus, the area of ‘chemical warfare’ between partners, where metabolite induction phenomena are likely to occur, can be accurately localized. However, solid medium cultures are usually performed at
the Petri dish scale, and only limited amounts of metabolites can be extracted from such culturing conditions [207]. This represents a drawback when specific metabolites need to be isolated for de novo identification or bioactivity studies.

5.2 Co-cultures in liquid substrates

The co-culture of different species of microorganisms in liquid nutrient media is referred to as mixed fermentation, as observed in the natural processes employed in wine fermentation. For instance, Capece et al. [388] were able to obtain 160 isolates of Saccharomyces cerevisiae associated with Sangiovese grapes, and the interactions of six representative strains were studied to understand the natural community that was important during wine making. In liquid media, it is not possible to exploit the modalities of the interactive behavior between microorganisms, but the induction of NPs can be monitored.

Fermentation is a well-established method to produce secondary metabolites from pure strains. Interesting metabolites that have traditionally been extracted from the fruiting bodies of medicinal mushrooms with high production costs are now obtained in liquid culture, which is an efficient way to produce increased amounts of these metabolites [389]. However, each parameter involved in cultivation within the bioreactor must be adapted to the developmental pattern and physiology of the fungus. This is important for the optimization of the rheological properties and metabolic activity during the development of the biomass [390]. Bacterial co-cultures are readily performed in liquid medium, and growth is easily monitored via turbidimetric methods.

Mixed fermentation systems have been developed among various microorganisms, including bacterium-fungus [391-394], bacterium-bacterium [395], bacterium-protist [396], archaea-fungus [381] and fungus-fungus [397] co-cultures, involving both budding and filamentous forms. As reported by Pettit [276], mixed fermentation is an effective way to increase NP libraries.

5.3 The induction of chemical diversity in microbes via co-culture

Several groups have shown that co-culturing can activate silent gene clusters, but the molecular mechanisms by which this is achieved often remain unknown. In fact, microbes can produce compounds that function as transcriptional regulators and epigenetic modifiers. In one study, a co-culture of Aspergillus fumigatus with a bacterium was demonstrated to activate the same silent pathways as the addition of an epigenetic modulator [308].

Co-cultivation of microbes can also result in gene mutation and subsequent expression of otherwise silent gene clusters [398] or even the exchange of whole gene fragments (horizontal gene transfer), which can result in the production of previously undetected chemical structures (1) [399].

Microbial co-cultures have also been used to explore the modulation of quorum sensing (QS) [400] in the search for new antibiotics because QS modifiers might function as this type of
drug. Indeed, QS molecules play effective regulatory roles within bacterial populations by adapting global growth to the level of nutrient availability. QS might also be of interest in other aspects of drug discovery; for instance, a QS lactone produced by *Pseudomonas aeruginosa* was shown to halt cell proliferation and induce cell apoptosis in human breast cancer cells [401].

### 5.4 Advantages and drawbacks of solid medium co-culture over mixed fermentation

Microbial co-culture could represent a challenging approach for inducing chemodiversity in microbes compared to epigenetic modulation or OSMAC, mainly because of reproducibility issues [282]. Nevertheless, there is evidence that in certain cases, the activation of silent pathways requires the physical presence of a second microbe (cell-cell interaction) and that metabolites alone (e.g., heat-killed cells, cell-free supernatants or extracts) are not always sufficient to induce the production of secondary metabolites. König et al. [308] showed that *Aspergillus fumigatus* produced fumicyclines (28, 29) only during mixed fermentation with *Streptomyces rapamycinicus* and not in the presence of the bacterial metabolites. In this particular case, the novel metabolite could be obtained through overexpression of the corresponding polyketide synthase (PKS) gene or the addition of the HDAC inhibitor SAHA. Similar results were observed in *A. nidulans* following direct physical contact with specific actinomycete strains. The physical contact caused the activation of gene clusters and resulted in the production of orsellinic acid [309], which was not produced during monoculture. In selected conditions, comparable molecular processes might be involved in co-culture as in epigenetic modification. This is supported by a study involving the co-culture of the plant pathogens *Eutypa lata* and *Botryosphaeria obtusa*, which demonstrated the upregulation of O-methylmellein [30], a compound that was upregulated in the plant pathogen *Stagonospora nodorum* through the addition of the epigenetic modifiers SAHA and nicotinamide [283].

The generation of secondary metabolites is also linked to the nutritional environment, and it has been shown that in some cases, antibiotic production and sporulation are increased in nutritionally poor media [298]. Similarly, in a co-culture experiment, the number of induced metabolites was found to be greater when a smaller volume of growth medium was used [363]. Furthermore, solid medium cultures have been found to yield a substantially greater number of metabolites compared with liquid medium cultures [402]. Solid medium co-culturing constitutes an easy, flexible and low-cost format in which to screen NP production. However, the large-scale production of co-cultures on solid media remains a rather complicated process. Several techniques have been developed to enable the upscaling of solid medium co-cultures [30,32,357]. Such approaches may generate sufficient material to allow the isolation of induced microbial metabolites in adequate amount for in-depth bioactivity studies. However, the use of pure strains and mixed fermentation remains critical if upscaling to industrial production is needed.
6. Monitoring co-culture metabolite induction

As discussed above, the co-culture of microorganisms leads to various morphological interaction patterns, and modifications might occur at different levels (from genome to metabolome, Figure II.A.2). It was clearly demonstrated that various silent genes are expressed in microbial strains that are confronted in co-cultures. This phenomenon highlights the potential for co-culture of microorganisms to induce secondary metabolite production, possibly leading to the discovery of new chemical entities.

To highlight significant changes in the microbial metabolome composition in either solid medium co-cultures or mixed fermentation, various analytical strategies, including simple targeted quantification, differential metabolite profiling and metabolomics or mass spectrometry imaging, must be applied.

In some cases, the observed metabolite induction is striking and can be easily monitored using simple methods, such as thin layer chromatography (TLC) or high performance liquid chromatography (HPLC). In other cases, when no significant modifications are observed, sensitive metabolomics approaches involving advanced data mining are necessary [403]. Alternatively, the induction of metabolites can be highly localized to the zone of confrontation, for which molecular imaging approaches are well adapted.

![Graph](image)

**Figure II.A.5: Analytical techniques that permit monitoring of microorganism co-culture.**

Prevalence of analytical techniques used to monitor chemical changes in microbial co-culture given in Supplementary Table II.A.S1. Abbreviations: TLC, thin layer chromatography; HPLC-UV, high performance liquid chromatography-ultra violet; HPLC-RI, HPLC-refractive index; GC-FID, gas chromatography-flame ionization detection; LC-MS, liquid chromatography-mass spectrometry; DI-MS, direct ionization-MS; DESI-MS, direct electrospray ionization-MS; MALDI-IMS, matrix-assisted laser desorption-ionization-imaging MS; LDPI-MS, laser desorption postionization-MS; NMR, nuclear magnetic resonance.

The various analytical techniques reported for studying the induction of microbial metabolites (Table II.A.2, Supplementary Table II.A.S1) are shown in Figure II.A.5, together with their frequency of use to date. Some methods only allow the detection of particular metabolites, whereas others also provide structural information to allow their identification in databases (mass spectrometry (MS), tandem mass spectrometry (MS/MS), UV) or de novo identification (NMR). The various analytical strategies applied will be discussed in the following sections.
6.1 Targeted and untargeted metabolite profiling

Targeted analyses (quantification of particular compounds, mainly toxins) were first used to demonstrate induction phenomena during microbial growth. This was achieved through simple differential comparison via HPLC-UV [404-408], gas chromatography-flame ionization detection (GC-FID) [405,409,410] or HPLC-MS [411,412], or through GC-MS [409]. In addition, bio-guided fractionation procedures have been employed to isolate particular compounds from microbial co-culture broths when a particular induction of bioactivity was observed [413-416]. This has generally led to the identification of the induced metabolites responsible for that particular activity. In a number of microbial co-culture experiments, over-production of pigments in the culture medium or at the confrontation zone has been observed [30,207,417-420]. In these cases, analytical methods based on color detection (UV/VIS), TLC [342] or HPLC-UV [308,416,421] were employed to demonstrate the induction and subsequent identification of the pigments.

6.2 Metabolite profiling and fingerprinting methods

In addition to these targeted analyses, untargeted metabolite profiling approaches have recently been applied. These generic methods aim to be as comprehensive as possible and provide data that can either be used for simple differential analyses or for qualitative comparisons. Alternatively, the fingerprints obtained in various biological replicates can be employed in metabolomic analyses following the application of the chemometric analysis.

The induction of primary microbial metabolites has been assessed through proton nuclear magnetic resonance (1H NMR) [381], HPLC-refractive index (RI) detection [422,423] or GC-MS [311,342,424,425]. For secondary metabolites, simple TLC [426] or HPLC-UV has been successfully employed [309,310,382,426-429]. However, for such generic fingerprinting, direct MS or hyphenated MS methods are much more powerful because of their sensitivity and the resolving power of such detectors [37,83]. Furthermore, MS and especially high-resolution MS (HRMS) can provide key structural information for the identification of the induced metabolites (see section 6.5).

Furthermore, direct analyses with minimal or no sample preparation have been efficiently applied, including direct ionization-MS (DI-MS) [430], desorption electrospray ionization-MS (nano-DESI-MS) [431-433], matrix-assisted laser desorption ionization-MS (MALDI-MS) [277,302,433,434] and laser desorption postionization-MS (LDPI-MS) [435]. Total MS spectra of microbial extracts or particular spots from solid medium cultures can be generated using all of these approaches.
Table II.A.2: Selected examples of solid and liquid medium co-cultures of two microorganisms.
Overview on recent and/or particularly interesting microorganism co-cultures including bacterium-bacterium, bacterium-fungus and fungus-fungus interactions. The structures of the induced compounds are presented in Figure II.A.7. A comprehensive list is given in Supplementary Table II.A.S1. Abbreviations: DESI, desorption electrospray ionization; DON, deoxynivalenol; GC, gas chromatography; HPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; IMS, imaging mass spectrometry; MS, mass spectrometry; MS/MS, tandem mass spectrometry; qRT-PCR, quantitative real-time polymerase chain reaction; SPME, solid phase microextraction; UHPLC, ultra high pressure liquid chromatography; UV, ultra violet; ZON, zearalenone.

<table>
<thead>
<tr>
<th>Microorganisms involved in the interaction</th>
<th>Culture medium</th>
<th>Detection of metabolite induction</th>
<th>Compounds induced by co-culturing</th>
<th>Compound class</th>
<th>Chemical novelty</th>
<th>Biological properties of induced compounds</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterium vs. Bacterium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces padanus</em> &amp; <em>Rhodococcus fascians</em></td>
<td>Liquid medium</td>
<td>Bioguided isolation (antibiotic activity), genome analysis</td>
<td>Rhodostreptomycin A and B (1) (horizontal gene transfer)</td>
<td>Aminoglycosides</td>
<td>Yes</td>
<td>Antimicrobial activity</td>
<td>[399]</td>
</tr>
<tr>
<td><em>Streptomyces cinnamirinus</em> &amp; <em>Alteromonas sp.</em></td>
<td>Liquid medium</td>
<td>HPLC-MS</td>
<td>Lobocompactol (2)</td>
<td>Diterpene</td>
<td>No</td>
<td>Antifouling activity, antioxidant and anticancer activity</td>
<td>[436]</td>
</tr>
<tr>
<td><em>Streptomyces coelicolor</em> &amp; <em>S. coelicolor</em> / <em>Amycolatopsis sp.</em> / <em>Streptomyces sp.</em> / <em>Streptomyces sp.</em> / <em>S. vinidochromogens</em></td>
<td>Solid medium</td>
<td>NanoDESI-MS and MALDI-IMS</td>
<td>Many detected compounds, four new acyl-desferrioxamines (3)</td>
<td>Hydroxamic acid</td>
<td>No</td>
<td>Antimicrobial activity</td>
<td>[433]</td>
</tr>
<tr>
<td><strong>Fungus vs. Fungus</strong></td>
<td></td>
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<tr>
<td><em>Gloeophyllum abietinum</em> &amp; <em>Heterobasidion annosum</em></td>
<td>Liquid and solid medium</td>
<td>HPLC</td>
<td>Oosponol (4), oospoglycol (5), melledonal A (6), melledonal C (7)</td>
<td>(4, 5): Polyketides, (6, 7): sesquiterpenes</td>
<td>Yes</td>
<td>Antimicrobial activity</td>
<td>[378,404]</td>
</tr>
<tr>
<td>Two unidentified Fungi</td>
<td>Liquid medium</td>
<td>Not reported</td>
<td>Marinamide (8), methyl marinamide (9)</td>
<td>Alkaloids</td>
<td>No</td>
<td>Antibacterial activity, cytotoxic against tumor cell lines (IC50 in the low nanomolar range)</td>
<td>[397,439]</td>
</tr>
<tr>
<td>Fungus</td>
<td>Bacterium</td>
<td>Medium</td>
<td>Technique</td>
<td>Product</td>
<td>Type</td>
<td>Activity</td>
<td>Notes</td>
</tr>
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<tr>
<td><em>Eutypa lata</em> &amp; <em>Botryosphaeria obtusa</em></td>
<td></td>
<td>Solid</td>
<td>UHPLC-MS</td>
<td>Hydroxylated O-methylmelleins (10)</td>
<td>Polyketide</td>
<td>No</td>
<td>Non-hydroxylated derivative: antifungal and phytotoxic activity, induced metabolites showed no activity [30]</td>
</tr>
<tr>
<td><em>Alternaria tenuissima</em> &amp; <em>Fusarium culmorum</em> / <em>Fusarium graminearum</em></td>
<td></td>
<td>Liquid (wheat kernel)</td>
<td>HPLC-MS/MS, quantification of six toxins</td>
<td>Deoxynivalenol (DON, 11), zearalenone (ZON, 12)</td>
<td>DON: trichothecene (sesquiterpene); ZON: polyketide</td>
<td>Yes</td>
<td>DON: Antimicrobial activity [440,441], ZON: cytochrome P450 3A4 inhibition reported [411]</td>
</tr>
<tr>
<td><em>Fusarium tricinctum</em> &amp; <em>Fusarium begoniae</em></td>
<td></td>
<td>Liquid</td>
<td>HPLC</td>
<td>Subenniatin A (13), B (14)</td>
<td>Depsipeptides</td>
<td>Yes</td>
<td>Inactive in cytotoxic and antibacterial bioassays [442]</td>
</tr>
<tr>
<td><em>Trichophyton rubrum</em> &amp; <em>Bionectria ochroleuca</em></td>
<td></td>
<td>Solid</td>
<td>UHPLC-MS</td>
<td>Hydroxysulfoxy-2,2''-dimethylthielavin P (15)</td>
<td>Polyketide</td>
<td>No</td>
<td>N/A [207]</td>
</tr>
<tr>
<td><em>Alternaria tenuissima</em> &amp; <em>Nigrospora sphaerica</em></td>
<td></td>
<td>Liquid and solid</td>
<td>HPLC-UV</td>
<td>Stemphyperylenol (16), alterperylenol (17)</td>
<td>Polyketides</td>
<td>Yes</td>
<td>Antifungal activity (stemphyperylenol) [428]</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em> &amp; <em>Serratia</em> sp. / <em>Achromobacter</em> sp.</td>
<td></td>
<td>Liquid</td>
<td>SPME-GC-MS</td>
<td>Isocaryophyllene (18), α-humulene (19), cyclocaryophyllan-4-ol (20)</td>
<td>Sesquiterpenes</td>
<td>Yes</td>
<td>Suppression of virulence in <em>F. oxysporum</em>, antimicrobial activity of isocaryophyllene [443]</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em> &amp; <em>Sphingomonas</em> sp.</td>
<td></td>
<td>Liquid</td>
<td>HPLC</td>
<td>Glionitrin A (21)</td>
<td>Diketopiperazine alkaloids</td>
<td>No</td>
<td>Antimicrobial activity and cytotoxicity [310]</td>
</tr>
<tr>
<td><em>Aspergillus nidulans</em> &amp; <em>Escherichia coli</em></td>
<td></td>
<td>Liquid</td>
<td>qRT-PCR of the polyketide gene, HPLC</td>
<td>Orsellinic acid (22), lecanoric acid (23), polyketides F-9775A (24) and F-9775B (25)</td>
<td>Polyketides</td>
<td>Yes</td>
<td>(22): Antimicrobial activity [444], (23): inhibits ATP synthesis and electron transfer, antimicrobial activity [444,445], (24-25): antiosteoporosis activity [309]</td>
</tr>
<tr>
<td>Organism Combination</td>
<td>Full Genome Microarray</td>
<td>HPLC-MS</td>
<td>HPLC-UV</td>
<td>HPLC</td>
<td>HPLC-MS</td>
<td>Counting of Oosposes</td>
<td>HPLC-MS</td>
</tr>
<tr>
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<td>---------</td>
</tr>
<tr>
<td>Aspergillus fumigatus &amp; Streptomyces <em>sp. aeruginosus</em></td>
<td>Liquid medium</td>
<td>Full genome microarray, HPLC-MS</td>
<td>HPLC-UV</td>
<td>HPLC</td>
<td>HPLC-MS</td>
<td>Counting of oospores, HPLC-MS</td>
<td>Diterpenoids</td>
</tr>
<tr>
<td>Aspergillus fumigatus &amp; Streptomyces <em>sp. aeruginosus</em></td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
</tr>
<tr>
<td>Fusarium tricinctum &amp; Bacillus subtilis</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
</tr>
<tr>
<td>Aspergillus fumigatus &amp; Streptomyces <em>sp. aeruginosus</em></td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
</tr>
<tr>
<td>Phytophthora nicotianae &amp; Phytophthora nicotianae</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
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</tr>
<tr>
<td>Others</td>
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<td>Liquid medium</td>
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<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
<td>Liquid medium</td>
</tr>
</tbody>
</table>

### Alkaloids

- **Aspergillus fumigatus** & **Streptomyces peucetius**
  - Liquid medium
  - HPLC-MS
  - Fumiformamide (26), N,N'-[(1Z,3Z)-1,4-bis(4-methoxyphenyl)1,3-dicyclohexadiene(27)] together with two known N-formyl derivatives and the xanthodiol analogue BU-4704.
  - Yes
  - Cytotoxic activity

- **Aspergillus fumigatus** & **Streptomyces rapamycinicus**
  - Liquid medium
  - Full genome microarray, HPLC-UV
  - Fumicyclines A (28) & B (29)
  - Meroterpenoids
  - No
  - Yes
  - Cytotoxic activity

- **Fusarium tricinctum** & **Bacillus subtilis**
  - Solid medium
  - HPLC-UV
  - Induction of 13 compounds, three novel compounds (racemaminoester batanin (20), 2-carboxyaminoester batanin (31) and bacteriolactam (32)), and several diketopiperazine alkaloids and other known compounds.
  - No
  - No activity of de novo-induced compounds.

- **Aspergillus fumigatus** & **Streptomyces bullii**
  - Liquid medium
  - HPLC-MS
  - 11-O-Methylpseurotin A (33), and several diketopiperazine alkaloids and other known compounds.
  - No
  - No activity of diketopiperazine alkaloids.

### Meroterpenoids

- **Phytophthora nicotianae** & **Phytophthora nicotianae**
  - Liquid medium
  - Counting of oospores, HPLC-MS
  - Mating hormone α1 (34), and α2 (35)
  - No
  - No activity of (α1 + α2) compounds.

### Polyketides

- **Fusarium tricinctum & Bacillus subtilis**
  - Liquid medium
  - HPLC-UV
  - Induction of 13 compounds, three novel compounds (racemaminoester batanin (20), 2-carboxyaminoester batanin (31) and bacteriolactam (32)), and several diketopiperazine alkaloids and other known compounds.
  - No
  - No activity of de novo-induced compounds.

### Others

- **Others**
  - Liquid medium
  - HPLC-MS
  - Counting of oospores, HPLC-MS
  - Mating hormone α1 (34), and α2 (35)
  - No
  - No activity of (α1 + α2) compounds.
DI-MS provides spectra that reflect the metabolite composition within an extract through simple infusion in an electrospray source. MALDI-MS [447] and LDPI-MS [448] are desorption methods that allow the ionization of metabolites directly from fungal or bacterial strains on solid medium. Using such methods, rastering over an agar plate provides a good method for recording the total MS spectra at defined positions, and the compilation of such data facilitates MS imaging of a particular microbial co-culture zone (see section 6.4). Nano-DESI allows the sampling of a microbial colony directly from a Petri dish, without sample preparation [431,433]. This apparatus performs nanoliter-scale liquid extraction of the biological surface, and the extract is directly infused into a mass spectrometer. Compared to desorption techniques such as MALDI, which efficiently analyze metabolites of high molecular weight as well as peptides and proteins, nano-DESI-based approaches involve an electrospray ionization process and therefore can also be efficiently used to analyze small molecules [449]. All of these techniques cause the direct ionization of the samples but may suffer from important matrix effects due to the large variety of compounds that are ionized and detected simultaneously.

Metabolite profiling strategies using hyphenated MS techniques such as LC-MS [30,31,207,310,391,392,436,442,446,450-453] or GC-MS [342,409,443] have often been employed. In such methods, MS spectra are acquired for microbial metabolites that are first separated through chromatography. Such approaches generally exhibit a lower throughput than direct MS methods and require more tedious sample preparation. However, they suffer less from ion suppression effects, which are likely to occur in MS. In addition, they provide a good means of separating isobaric structures, which are often found in NP extracts.

Gas chromatography generally generates HR profiles, and molecular identification is easily achieved through database searches based on electron ionization-MS (EI-MS) spectra and retention indices [342] when reference metabolites are present in the database. In comparison, the resolution of liquid chromatography is generally lower [454], but when combined with an HR mass spectrometer using soft ionization methods, such as ESI, this method provides a good separation of metabolome constituents in both the chromatographic and MS dimensions.

The resolution or throughput of LC can be greatly improved through ultra high performance liquid chromatography (UHPLC) using sub-2 μm silica beads [455] compared to standard HPLC. Using this type of UHPLC-HRMS approach, very rapid fingerprinting can be achieved over a gradient of 2-5 min [456-458]. Alternatively, very high resolution can be obtained using longer columns and gradient times, where peak capacities exceeding 1,000 can be achieved [455]. Fast UHPLC-MS is commonly employed to profile large numbers of biological replicates, which are usually necessary for metabolomics studies [37,459]. In LC-MS, HRMS spectra recorded with state-of-the-art instruments provide mass accuracies below 5 ppm. When combined with heuristic filtering, this level of accuracy usually enables the molecular formulas of the metabolites to be confirmed with good confidence [65]. This is often sufficient for peak annotation when the metabolites of interest have been previously reported, and further information can be generated through MS/MS (detailed in section 6.5).
6.3 Metabolomics

Once microbial strains have been chemically analyzed using any of the methods or combinations of the methods described above, the collected data must be analyzed. In co-culture experiments, the goal is to highlight secondary metabolites that are either produced \textit{de novo} or are up- or downregulated upon interspecies competition. A simple comparison of chromatographic traces may be sufficient to identify the compounds that are strongly induced. This approach is suitable for the analysis of chromatograms obtained via HPLC-UV [309,382,405,428,429,442], TLC [342] and GC-FID [405,409,410]. In most cases, this methodology allows the selection of compounds detected in the co-culture but not detected in cultures of the pure strains, referred to as \textit{de novo}-induced metabolites.

MS detection generates large datasets, and in addition to the major metabolites, minor constituents can be sensitively and selectively detected. Similar to the other fingerprinting methods, the induction of a strong ion can be directly observed through the comparison of total MS spectra or LC-MS chromatograms. This is possible when important fold changes in the intensities (sufficiently high induction rates [436]) are recorded for well-defined ions or for LC peaks that are well resolved in the total ion chromatograms (TIC) in LC-MS. This approach has been applied to the data generated by various techniques, such as DI-MS [430] or LC-MS [30,391,392,394,442,446,452].

A better overview of all of the metabolomic changes that occur in co-culture experiments can be obtained if fingerprinting data, especially those obtained through MS, are evaluated with newly developed chemometric tools, such as those used for metabolomics studies [37].

For this purpose, an automated data mining procedure must be used to analyze the large MS datasets generated. A first step is to filter the data (blank subtraction, denoising) and convert them into a data matrix (Figure II.A.6A) through alignment of the detected features generated via spectral binning, in the case of direct MS analysis [460], or through automatic peak detection (peak picking), in the case of the hyphenated techniques [461]. Various currently well-established software packages, including some freeware, can be used to perform this step [37,462]. In a subsequent step, the data matrix is analyzed using statistical tools, including either supervised or non-supervised methods, or elaborate machine-learning algorithms to highlight non-obvious information [462-464]. This ultimately provides a way to visualize the differentiation of the co-culture replicates from those of the pure strains (score plots) and, hence, highlights the corresponding regulated metabolites (loading plot, and their interrelation in a so-called biplot).
Figure II.A.6: Different metabolomics approaches employed to study metabolite induction during microorganism co-culture, illustrated for the case of solid medium co-culture.

The red and blue colors in the figure correspond to the cultures of the two pure strains, whereas the purple color indicates the co-culture, in which the information from the two microorganisms cannot be separated. (A) represents the metabolomics approach (exemplified by the treatment of LC-MS data); the extracts from cultures of the pure strains and the co-culture are first analyzed through LC-MS. The generated raw data are transformed into a data matrix through automatic peak picking. The data are then explored to highlight the induced metabolites. The first approach (I.) consists of specific searches for de novo induction via searching peak-by-peak features that are only detected in the co-culture. The second approach, represented in figure (II.), demonstrates a multivariate data analysis, in which the data are first presented through a PCA that indicates metabolite induction. In a second step, (O)PLS-DA analysis leads to selection of the induced metabolites. The third strategy (III.) is the POChEMon approach, which strategically constructs two complementary models, a ‘mixing model’ between the two pure strain culture data and another ‘competition model’ that specifically describes the information on features that are unique to the co-culture data. Finally, typical induction patterns selected through the three different data mining approaches are presented. (B) represents the imaging MS approach. The surface of an agar plate where both microorganisms are grown together is
sequentially rastered to acquire an MS spectrum for every location. Images are generated for each ion detected (for example, ions a-f) to highlight their location in the co-culture and, thus, to select those metabolites present in the confrontation zone of the co-culture (as represented by the induction pattern generated through imaging MS). Abbreviations: LC-MS, liquid chromatography-mass spectrometry; PCA, principal component analysis; PLS, partial least square; OPLS, orthogonal projection to lateral structure; DA, discriminant analysis; POChEMon, projected orthogonalized chemical encounter monitoring.

One potential data mining approach consists of automated searching for de novo-induced metabolites (Figure II.A.6A I.). In this case, only features (the detected metabolites, corresponding to the mass-to-charge ratio \( m/z \) at a particular retention time) that are detected in the co-culture extract and that are not found in the pure-strain cultures are considered. Simple scripts can be employed to compare the large datasets generated through MS detection and, thus, allow the detection of the induced minor compounds \([31,207]\). These scripts systematically compare the ‘detected’ status (peak height over a certain threshold) of every feature identified, selecting those that are uniquely ‘detected’ in the co-culture replicates. The highlighted features correspond to highly reproducible induced metabolites. De novo induction stricto sensu is not always detected in co-cultures, but other important metabolomic changes correspond to the up- or downregulation of some metabolites \([436]\). To demonstrate all of the types of microbial metabolomic changes that may occur due to fungal confrontation and to take into consideration the dynamic aspects related to fungal growth, more elaborate chemometric tools should be used.

A second data mining approach involves the application of conventional statistical tools to replicates of pure strains and co-cultures, as carried out in metabolomic analyses (Figure II.A.6A II.). For microbial co-culture experiments, various methods of mining the data have been employed, including principal component analysis (PCA) \([31,311,342,424,435]\), analysis of variance (ANOVA) \([411,450,451,453]\), partial least squares regression coupled with discriminant analysis (PLS-DA) \([31]\), orthogonal projections to latent structure-DA (OPLS-DA) \([357,465,466]\) and similar approaches \([425]\).

From a data mining perspective, the two states ‘control’ and ‘stressed’ are compared in traditional metabolomic analyses. However, the ‘induced’ state observed in the co-cultures should be compared with the two pure strains simultaneously, and none of the conventional methods accomplishes this.

As a first exploratory step, it is often informative to explore the data through an unsupervised approach, such as PCA \([467]\). PCA displays the relationships among the sample distribution, which may reveal groupings, trends or outliers \([468]\). In co-culture experiments, three well-differentiated clusters are often observed, confirming the differences in metabolite composition between the pure strains and the compared co-culture. When the co-culture cluster is located away from the barycenter of the two pure-strain clusters, it most likely indicates the existence of metabolic modifications that are not related to the mixing of the metabolomes of the two pure strains and suggests the presence of new metabolites that are specifically induced by the interaction (Figure II.A.6A II.). One approach for selecting the induced biomarkers consists of comparing the co-culture data with the pure-strain data using
univariate approaches [464,469]. In this case, the biomarkers of interest are selected through the exploration of each feature detected individually. The selection is based on the fold change in the peak area between the co-culture and the two pure-strain cultures and therefore requires an evaluation of statistical significance based on Student’s t-test (with only the most intense pure-strain peak area being considered). In comparison with the previously described de novo detection approach, this strategy focuses more on fold-change information, rather than the ‘detected’ status. Therefore, upregulated ions are also highlighted. A second approach consists of the construction of two (O)PLS-DA models through multivariate statistical analysis (comparison of the co-culture with each pure-strain culture). Each model permits the classification of features according to its capacity to separate two groups (the co-culture and one of the pure-strain cultures). Thus, features can be selected that are highly specific to the co-culture compared to a single pure-strain culture. Finally, shared and unique (SUS) plots are presented, providing a two-dimensional representation of the various features [470]. Both dimensions correspond to the relevance of the biomarkers in the two designed models. This approach may help to highlight the induced biomarkers in both models.

However, these last approaches are not well adapted for studying mixed microbial cultures [465,466]. PCA describes the metabolome variation among all samples but does not highlight co-culture-induced modifications specifically. Similarly, (O)PLS-DA constructs models that are highly focused on comparison of the co-culture with the individual pure-strain cultures but does not consider the co-culture as a mixture of the two microbial species and therefore does not describe the metabolic variation expected to occur in the co-culture experiment in an interpretable form. None of the previously described approaches takes advantage of the particular biological setup provided by the co-culture experiment, and therefore, their biological relevance is limited. Thus, these conventional statistical tools might not always highlight critical co-culture-specific information. In addition, the biological variations observed during microbial growth [282] complicate these tasks. Thus, there is a need for innovative data mining approaches to highlight significant upregulation phenomena when extracts obtained from solid medium co-cultures or mixed fermentation are analyzed.

To address this particular need, a novel approach was recently developed, referred to as projected orthogonalized chemical encounter monitoring (POChEMon, Figure II.A.6A III.) [465,466]. This data mining strategy is based on the intrinsic structure of the co-culture experiment. Initially, the co-culture data are mathematically re-constructed as a mixture of all pure-strain cultures in which metabolic variation can be explored. The first step is the creation of a mixed model from the two pure-strain metabolomes that represents the co-culture experiments. The position of the co-culture replicates is already informative: if they are positioned closer to one of the two pure strains, it indicates that this particular strain is the major metabolite producer in the particular co-culture. The residual information in the co-culture replicates is then likely associated with the interspecies competition, specifically when shared by multiple replicates. From this information, a second model is built. This latter model, referred to as the competition model, explores these residuals through PCA. All of the co-culture experiments are compared, and upregulated features are highlighted using a biplot.
This approach has been found to provide information about generally induced metabolites and about metabolites induced only in specific replicates [465,466]. Because specific replicated behaviors are taken into account, clear induction phenomena found only in a few replicates can still be highlighted. This approach might be superior in accounting for the high variability that is found in such complex systems. This approach can also highlight features that are downregulated in co-cultures and therefore help to delineate interspecies crosstalk in microbial models related to naturally co-habiting microorganisms.

All of these approaches provide a list of biomarkers with a particular m/z or an m/z at a particular retention time together with its specific intensity across all replicates. For key features, it is always important to verify the modifications in the raw MS data. Often, several features may be related to one specific metabolite (due to the detection of adducts), and relationships between features (correlated features) must be established to further validate the induction of a specific metabolite. Typical differences in metabolite behavior highlighted among the pure strains and co-cultures using these different data mining approaches are shown in Figure II.A.6A. The de novo induction detection approach enables the selection of de novo-induced metabolites corresponding to highly reproducible and highly induced compounds that are not detected in pure-strain cultures. The classical statistical approach (e.g., using PCA followed by (O)PLS) highlights highly reproducible upregulated metabolites. Among the induced compounds that are identified, de novo-induced metabolites are generally highlighted unless more significantly upregulated features are present in the data. Finally, the recently described POChEMon approach also indicates induced metabolites with high specific reproducibility in certain co-culture replicates. Thus, all of these approaches are complementary for the selection of compounds of interest in natural product drug development.

### 6.4 Imaging mass spectrometry

The comparison of extract profiles from pure-strain cultures and co-cultures allows the global metabolic changes taking place in solid medium co-cultures or in mixed fermentation to be highlighted. The induction of metabolites in co-cultures is usually localized at the zone of confrontation in solid media, and methods allowing precise spatial localization are particularly useful to highlight the induction in the specific zones of interaction and to understand which partner is producing a particular metabolite [277]. Recently developed imaging MS (IMS) technologies provide an efficient method to analyze the spatial distribution of molecules within solid agar media [472]. Various technologies have been used to explore the spatial distribution of metabolites via MS in co-cultures or in solid media, including MALDI and nano-DESI [432,434]. The sequential rastering across a surface while acquiring a mass spectrum from every location is necessary for IMS. From the obtained dataset, multiple images can be extracted that represent the spatial distribution of each ion detected with high spatial resolution, depending on the raster step size [473]. The analysis of these images (through mapping) allows the presence of some secondary metabolites to be visualized as a function of their location in the solid culture medium (Figure II.A.6B). The major limitation of this method lies in its spatial resolution, which is generally approximately 10 μm, although the most recent
advances allow IMS with a resolution of up to 1 μm to be performed [474]. However, most of the available methods exhibit a sufficient spatial resolution to localize induction in the confrontation zone between microorganisms.

The choice of ionization method has a strong influence on the type of metabolites detected. MALDI is better suited for large metabolites, peptides or proteins, while nano-DESI reveals smaller secondary metabolites.

The spatial distribution of metabolites in interacting microorganisms has been studied only using MALDI and nano-DESI [277,302,431-433]. In these analyses, the metabolites of interest were selected based on their spatial distribution, specifically, the metabolites located in the interaction zone where the two microorganisms are in contact. This approach provides interesting insight into microbial interaction mechanisms and may also help delineate interspecies crosstalk in microbial models. Three-dimensional (3D) visualization of the metabolite distribution was recently achieved via 3D IMS [475]. This technique takes advantage of traditional technology in serial cross-sections of microbial colonies grown on agar. The reconstructed 3D images of selected metabolites allow their spatial distributions within the microorganisms to be visualized.

Such methods are powerful but may suffer from ion suppression. A complete survey of all induction phenomena in the interacting microorganisms most likely requires a combination of imaging and metabolomics approaches.

6.5 Metabolite identification

In most studies addressing microbial co-cultures (>60% see Table II.A.2, Supplementary Table II.A.S1), the induction of previously isolated compounds has been reported. This information can be important if the co-culture is studied within the context of an ecological niche. However, if the goal is to generate new chemical diversity through the activation of silent biosynthetic pathways, it is important to identify the induced metabolites correctly. This can be achieved through the dereplication of MS and MS/MS data [37,53,54] prior to purifying the highlighted metabolites. The identification of the highlighted metabolites based only on the MS data represents the most challenging step [53]. Indeed, MS identification requires comparison with databases, and in many cases, information for the majority of microbial metabolites is not accessible. As a result, only putative assignments based on molecular formulae (MF) and microbial chemotaxonomy can be made. Furthermore, even when the appropriate databases are available, because chemical novelty is expected in the study of induced secondary microbial metabolites, de novo structure elucidation is required, which must involve NMR.

When GC-MS is employed for profiling, the identification workflow is partially based on the comparison of the EI-MS spectra with reference compounds and with database searches based on MS spectra and retention indexes [311,342,409,424,425,443,476]. When no clear identification is achieved, the evaluation of compounds is much more difficult. In such cases, preparative GC may be used for compound purification for further identification [477]. The
use of soft ionization techniques, such as chemical ionization, field emission and field desorption, can be employed in GC; these methods are less informative but may be employed to confirm the molecular weight of the metabolites of interest.

When DI-MS, nano-DESI-MS and LC-MS are used, the metabolites are ionized through soft ionization techniques, mostly involving ESI. However, atmospheric pressure chemical ionization (APCI) can also be employed. Such methods mainly produce molecular ion species that appear in the form of adducts, such as [M+H]+, [M+Na]+, [M+H+CH₃CN]+ if acetonitrile is used as solvent, [M+H-H₂O]+ in positive ion mode or [M–H]–, [M+HCO₂]–, or [M–H+CO₂]– in negative ion mode [54,476]. Furthermore, dimers, which complicate interpretation, may also be formed in the ion source. Therefore, prior to any dereplication, the correct molecular weight should be defined through adduct recognition [478]. In addition, comparison of the different ionization modes (positive or negative ionization (PI, NI)) may help to unambiguously reveal molecular ions [59]. The presence of these different ions complicates the dereplication process, but it is important to note that during the data mining step, several m/z features can be associated with a single induced metabolite. This must also be taken into account when analyzing the loading results from metabolomic datasets.

To improve identification, HRMS detectors are now generally employed. Using such instruments, the MF can be determined based on an accurate mass, along with a high spectral accuracy of particular molecular ions. However, the unambiguous determination of the MF is still difficult, even when mass accuracies below 1 ppm are obtained, particularly for compounds with a high molecular weight (>500 Da) [64]. To reduce the number of possible MF, different heuristic filters can be applied [65]: i) restrictions on the number of elements; ii) LEWIS and SENIOR chemical rules; iii) isotopic patterns; iv) hydrogen/carbon ratios; v) the elemental ratios of nitrogen, oxygen, phosphorus and sulfur versus carbon; vi) elemental ratio probabilities; and vii) in the case of GC-MS data, the presence of trimethylsilylated compounds. When high-resolution multistage MS (HRMSⁿ) spectra can be recorded, these spectra can be employed to reduce the number of MF based on the determination of fragment MF [479]. To speed up this time-consuming process, such workflows may be automated [480].

Based on the putative MF, the identification of metabolites is achieved using various NP databases. This process usually leads to multiple putative identities for each highlighted feature. To further reduce the number of possible structures and improve the accuracy of peak annotation, other filters based on retention time prediction [158,481] and microbial phylogeny [363] can be applied. When applied to co-cultures, these approaches have resulted in several putative identifications [31,207,363], with the differences in hit rates being greatly reduced in poorly studied microorganisms. A recent systematic study addressing various fungal co-culture experiments demonstrated that co-culture-induced metabolites display a lower database hit rate than the constitutively produced compounds [31]. This interesting result might indicate that induced metabolites exhibit a greater chance of being novel compared to constitutive microbial metabolites. It might also be that the detected metabolites correspond to constitutive metabolites that are biotransformed during the interaction (analogues) corresponding to unreported NPs. In any case, since dereplication of
such compounds cannot be performed based on MS only, complete de novo structure identification by NMR would be needed to further confirm the metabolites’ identity.

As a complementary approach, MS/MS spectra networking has been successfully applied for microbial compound identification [482]. This strategy is based on searching for similarities in MS/MS spectra among various microbial metabolites. In some cases, this procedure has led to the successful identification of a large number of structurally analogous metabolites based on similar fragmentation patterns [302,431]. For example, a large series of desferrioxamines (3) was identified in co-cultures in which Streptomyces coelicolor were combined with various other bacteria [433].

As co-culture-induced metabolites are usually unsuccessfully dereplicated, the purification and subsequent de novo identification of these metabolites through NMR is the only possible means of achieving unambiguous identification [16]. For this purpose, classical isolation strategies based on multiple fractionation steps have largely been employed [30,308,391,392,394,397,399,413,415,418,419,428,446,452,483,484]. Recently, LC-MS-targeted isolation based on software-driven protocol optimization was used to improve the purification procedures and rapidly isolate the highlighted compounds [71,207]. This approach takes advantage of the possibility of simulating metabolite elution [485] to optimize a purification protocol. This targeted strategy allows a reduction in the number of purification steps required to isolate a pure compound from a crude extract. Unfortunately, the purification of metabolites can sometimes be very challenging due to the small amount of material available and the small amount of the highlighted compound produced [207]. In addition, because biomarker detection is based on MS, the intensity of the peaks is not directly correlated with the amounts present, and an intense ion may represent an extremely small amount of a very efficiently ionized metabolite [83]. Thus, despite the high sensitivity of the most recent NMR instruments, the intrinsic differences in sensitivity between MS and NMR may lead to unsuccessful identification of some metabolites [207]. To solve such problems, large-scale co-culture experiments should be undertaken in both liquid and solid media to improve isolation efficiency [357]. The production of metabolites in solid media is now also possible at larger scales due to recently developed agar-supported solid-state fermenters that mimic what occurs in Petri dishes over an area of two square meters [32].

To address the incompatibilities between metabolomic approaches, which require a large number of replicates, and the identification of the metabolites following purification, a novel strategy was recently developed [363]. In this approach, the co-culture profiles obtained from 2-cm Petri dishes were compared with those obtained in 15-cm dishes. Metabolomic analysis was performed on the smaller dishes after four days of growth. Using this method, most of the de novo-induced metabolites (16/18) were detected in the extract obtained in the 15-cm Petri dishes after three weeks of growth. This finding clearly demonstrates that solid medium co-culture can be scaled up without loss of metabolome diversity.
7. Bioactivity of metabolites induced via co-culture

Figure II.A.7: Selected natural products induced by microorganism co-culture. The numbers of the molecules correspond to the numbers given in Table II.A.2.

Among the 58 examples of co-culture studies that focused on secondary metabolite induction summarized in Supplementary Table II.A.S1 (Supplementary information, selected examples...
are shown in Table II.A.2), the identified biomarkers were found to cover almost all classes of microbial metabolites. Some of the induced compounds were observed to show numerous activities. Naturally, many antimicrobials were identified because this type of activity is expected to arise from microbe-microbe interactions as a direct defense mechanism.

### 7.1 Type of induced metabolites

Among the classes of compounds observed in co-culture experiments (selected examples shown in Figure II.A.7), polyketides have often been reported. This result might be attributed to the high abundance of polyketide-producing organisms as well as the great structural diversity of this class of compounds [486].

The activation of silent PKS gene clusters through co-culturing has been demonstrated. This activation resulted in the production of a large variety of induced polyketides in various strains of *Aspergillus* sp. (e.g., *A. nidulans* [309] and in *A. fumigatus* [308]). In *A. nidulans*, the induced compounds were shown to be 2,4-dihydroxybenzoic acid derivatives and dimers of these compounds (22-25). A similar compound, consisting of a sulfonated trimer of 2,4-dihydroxybenzoic acid (15), was also identified in a co-culture of *Bionectria ochroleuca* and *Trichophyton rubrum*. When *A. fumigatus* was co-cultured with a bacterium, other polyketides (fumicyclines A (28) and B (29)) were identified. Furthermore, upregulation of the polyketide zearalenone (ZON), among other known mycotoxins, was observed in co-cultures of *Fusarium culmorum* and *Alternaria tenuissima* [411]. In addition, a large variety of polyketides with special structural features has been reported, including aromatic monocycles [382,411,415,429,487], linear skeletons [413,414,426,452] and multiple aromatic fused cycles [30,342,382,391,407,421,428,438,484].

The production of peptides through the activation of non-ribosomal peptide synthase (NRPS) gene clusters [488] has also been widely reported in the literature. Various types of peptides have been found, such as depsipeptides (13-14) [392,442], iturins [302] and lipoaminopeptides [430]. NRPS can also be involved in the production of hydroxamic acid compounds (e.g., 3) such as siderophores [489], and such compounds have been reported to be induced through co-culture [433,434,453]. These molecules are involved in the scavenging of iron, which is important for cell growth, from the environment.

Some of these metabolites may be generated through mixed biosynthetic pathways. This is the case, for example, for the cytochalasins observed to be induced during the co-culture of two *Aspergillus* spp. [405] as a result of the activation of hybrid NRPS-PKS gene clusters [490]. Another example is the tetramic acid-derived 11-O-methylpseurotin A2 (33) that was induced in a co-culture of *Aspergillus fumigatus* with *Streptomyces bullii* [394]. PKS-NRPS gene clusters have been shown to be required for the biosynthesis of the pseurotin compound class [491].

The induction of terpenes via activation of terpene synthase [492] has also been reported. This class includes compounds such as sesquiterpenes (11, 18-20) observed in mixed fermentations involving *Fusarium* spp. [411,443], diterpenes (2, 34, 35) produced in various settings [406,412,436] and triterpenes occurring in a mixed fermentation of *Aspergillus* sp.
with *Streptomyces* sp. [394]. Other mixed biosynthetic pathways based on terpene synthases leading to meroterpenoids have been reported as well [308,493]. Furthermore, various alkaloids were shown to be induced through co-culture. The reported alkaloids mostly corresponded to cyclic aromatic amines such as procyanines (monomers or dimers) [419,434], marinamides (8, 9) [397,439], pyrazines [494], pyrrols [418,421,495] and picolinic acid derivatives [453]. An unusual example is the N-formyl alkaloids (26, 27) produced in a co-culture of *A. fumigatus* with *S. peucetius* [446]. Additionally, the production of free fatty acids [409,451] and glycosides [399] has been reported in few cases.

Overall, no clear trend has yet been demonstrated regarding whether particular compound classes are more prone to be induced through microbial co-culture. This may be related to the small number of potential microbe interactions studied to date and to the relatively low diversity of the microorganism species involved. *Aspergillus* spp. and *Streptomyces* spp. have mainly been studied in this context. Nevertheless, in comparison with strategies based on gene activation for accessing novel molecules, co-culture appears to activate specific genes, but depending on the interaction type, metabolites resulting from various unrelated pathways may be induced. This contrasts with the molecular biological approaches, in which the PKS and NRPS gene clusters have been found to be the predominant pathways activated [235]. The limited knowledge of other gene clusters may explain why only a few studies using molecular biological approaches have observed the activation of other gene cluster families. Therefore, co-culture can be considered an untargeted method of gene activation that allows the production of very structurally different secondary metabolites.

### 7.2 Biological activity of the induced metabolites

During co-culture on solid media, clear long-distance growth inhibition is observed in some cases [207,233,416,420,428,446,451,496-498]. This observation is expected to be linked to the production of antimicrobial compounds as a defense mechanism by one of the two microorganisms being studied. The activity of the isolated metabolites induced by the co-culture has been evaluated in some instances, but this has not always led to the successful identification of the antimicrobial compounds [446,451]. A recent study that examined a large number of co-cultures indicated that only 5% of more than 600 co-cultures tested demonstrated long-distance growth inhibition and that such interactions are not linked to a particularly striking pattern of metabolite induction compared to other morphological patterns [207].

In general, various studies have observed an increase in the antimicrobial activity of extracts from co-cultures in comparison to those from corresponding pure-strain cultures [405]. Several targeted studies on known antimicrobial compounds have demonstrated that they can be induced by co-culturing [411,438]. For example, co-culturing *Gloeophyllum abietinum* with *Heterobasidion annosum* induced the production of the known antibiotics oosponol (4), oospoglycol (5), melledonal A (6) and melledonal C (7) [438]. This clearly highlights the potential for co-culture to induce antimicrobial compounds useful for drug discovery. Furthermore, various untargeted differential metabolite profiling strategies and metabolomic
approaches have been employed to identify promising molecules, which have, in some cases, led to the identification of antimicrobial compounds (see Supplementary Table II.A.S1). In a co-culture of *Pseudomonas aeruginosa* with *Aspergillus fumigatus*, IMS revealed local induction of iturins, which are molecules that exert antifungal activities, in the confrontation zone [302]. In addition, the production of iron (III)-scavenging metabolites (siderophores) in the confrontation zones between *P. aeruginosa* and *A. fumigatus* and between *Streptomyces coelicolor* and *Amycolatopsis* sp. [433,434] may highlight survival competition between the two microorganisms. This competition for ferric ions may lead to growth inhibition of the microorganisms that do not produce these metabolites.

In addition to antimicrobial activity, induced metabolites also show diverse other biological activities. Various compounds exhibit cytotoxic activity against different tumor cell lines as well as antiparasitic activity (against malaria, trypanosoma and leishmania, see Table II.A.2, Supplementary Table II.A.S1). For example, production of the anticancer agent taxol by *Paraconiothyrium* sp. was induced in presence of *Alternaria* sp. or *Phomopsis* sp., all three of which are endophytes of *Taxus* trees [412]. Some hydroxamic acid siderophores [433,434] have been reported to possess a wide range of biological activities [437,499]. In addition to compounds presenting activities of therapeutic interest, induced metabolites with antifouling activity (2) [436] have also been reported.

To further evaluate the potential of induced metabolites as leads for various drug targets, co-culture extracts can be subjected to biological HPLC profiling [500] for the rapid localization of bioactive metabolites in these microbial extracts. HPLC microfractionation is a valuable method that is compatible with the small amounts of extracts that are usually obtained from solid medium co-cultures (milligram amounts). These bioassays can be performed at-line, and this format is compatible with the evaluation of compounds collected in the microgram range (typically, tenths of micrograms). NMR information complementary to that provided by MS can now be obtained for such minute amounts of material using highly sensitive microNMR methods [89] to improve dereplication results, and this information can also be used to quantify the amounts collected for further assessment of the potency of microbial metabolites [202] in various assays. Regarding antimicrobial activity, bioassays can be performed at-line using bioautography [501] or microassays [502] or on-line, coupled directly to a separation device, such as LC [500]. Such micro-isolation procedures are well adapted to the amounts of material that are usually obtained from microbial metabolomics studies (e.g., by pooling replicates) and may lead to effective, very early identification of induced metabolites with high bioactive potential.

### 7.3 Chemical novelty among the induced metabolites

Among the compounds reported to be induced during microbial co-culture, approximately half of the identified molecules represent new natural products (see Table II.A.2, Supplementary Table II.A.S1). This is a high proportion and indicates that new structures are likely to be found when induced compounds are specifically targeted. This situation is even
more striking for metabolites that are induced *de novo* and are therefore not constitutively produced by any of the studied strains.

Dereplication based on MS data is a mandatory step to evaluate the possible novelty of induced metabolites [207,259,363]. The failure of peak annotation based on molecular formula assignment and chemotaxonomy cross-searches in databases of microbial metabolites indicates a high probability that a new metabolite has been detected. However, the success of dereplication is highly dependent on whether in-depth secondary metabolite analysis has been previously carried out on the studied microorganisms. Importantly, as noted above, induced metabolites exhibit lower hit rates in databases in comparison with constitutively expressed microbial metabolites, even in poorly studied strains. This demonstrates the great interest in co-culturing to induce interesting, chemodiverse metabolites. A general trend observed among the studied co-cultures is upregulation of the production of a series of analogues that share the same skeleton [30,391,394,429,433,434,446].

So far, microbial co-culturing has not yielded many examples of induced molecules possessing novel skeletons but instead has resulted in the identification of derivatives of known microbial metabolites. Similarly, the bioactivity potency of these compounds has not been found to be significantly different from other constitutive metabolites. However, several findings, such as the discovery of marinamides (8, 9), which have nanomolar anti-tumor activity [439], and glionitrin A (21), which shows submicromolar cytotoxic activity towards the DU145 human cancer cell line [310], indicate that this approach still has great potential to yield not only derivatives of known compounds but also novel skeletons or new lead structures.

8. Conclusion

Microbial genomic data produced in the last decade have revealed an incredible diversity of metabolic pathways for which no final product has yet been identified. It is now widely accepted that microorganisms harbor massive potential for the biosynthesis of compounds of high chemical diversity. However, many of these pathways are cryptic, and strategies to activate them are needed to explore the full chemical diversity of such organisms.

Accessing metabolites that are not expressed under standard growth conditions is challenging, but such resources are now attainable due to the recent progress made in the fields of biology and analytical chemistry as well as data mining and the possibility of applying these approaches in combination.

These developments encompass the stimulation of endogenous metabolite production through the so-called ‘cryptic pathways’ via various treatments or combining superior genetic engineering approaches with synthetic chemistry to generate chemical polymorphism. In this context, the various recent papers described in this review have shown that microorganisms can efficiently activate the *de novo* production or upregulation of a wide range of chemical agents to compete with other microorganisms for nutrient or space when they are co-cultured in artificial conditions. Despite some promising findings, further work needs to be carried out...
to verify whether microorganism co-culture preferentially yields new molecular skeletons or derivatives of known compounds.

The investigation of induction phenomena in interacting microbes is extremely challenging due to the complex nature and chemical diversity of their metabolomes. Furthermore, microbial metabolomes in co-cultures generate chemical variability that should be accurately assessed to highlight the compounds of interest resulting from such interactions. This requires sophisticated and sensitive analytical approaches, mainly based on high-resolution mass spectrometry for either extract profiling or direct MS imaging of the co-cultures. Relevant and reproducible biomarkers of microbial interactions are progressively being identified through advanced data mining methods that are capable of tracking subtle induction phenomena. As shown in this review, various approaches have made this task possible, and innovative strategies to efficiently perform such analyses are now at our disposal.

However, particular efforts should be made to identify biomarkers and characterize their bioactivity, even when an efficient dereplication protocol based on high-resolution MS or MS/MS is available. Nevertheless, the accuracy of structural assignment using such methods is strongly dependent on the availability of databases or standards for microbial metabolites. Depending on the strains examined, the quality of peak annotation and biomarker identification may vary greatly. Furthermore, the hit rate in NP databases is low for de novo-induced metabolites, indicating a potential to yield novel structures. To achieve the identification of induced metabolites, strategies involving LC-MS-targeted microfractionation and de novo structure assignment based on sensitive NMR approaches are therefore mandatory. Some of these approaches have been illustrated here and provide appropriate methods to assess the biological activity of the isolated compounds, which is critical to the process of understanding the relevance of the observed induction phenomena.

The study of complex microbial interactions has become feasible through combinations of the most recent biotechnological and analytical methods. This promising approach constitutes a strategic method to highlight novel structures and, possibly, new skeletons with relevant biological activity and potentially new modes of action in the field of drug discovery. These investigations are keys to understanding the chemical events that govern interactions between microorganisms in defined ecological niches from a fundamental perspective. Considering the exponential increase in the number of papers on this topic that have been published in the last several years, it is safe to conclude that the use of this approach will expand rapidly and yield important and fascinating discoveries.
<table>
<thead>
<tr>
<th>Microorganisms involved in the interaction</th>
<th>Culture medium</th>
<th>Detection for metabolite induction</th>
<th>Compounds induced by co-culturing</th>
<th>Compound class</th>
<th>Chemical novelty</th>
<th>Biological properties of induced compounds</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Bacillus subtilis &amp; Streptomyces coelicolor</td>
<td>Solid medium</td>
<td>Induction of colored compound</td>
<td>Undecylprodigiosin</td>
<td>Alkaloid (pyrrole)</td>
<td>No</td>
<td>Antibiotic activity, antimalaria activity [503]</td>
<td>[418,495]</td>
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<td>Lactobacillus spp. / Streptococcus thermophilus &amp; Propionibacterium freudenreichii</td>
<td>Liquid medium</td>
<td>HPLC-UV, GC-FID, dynamic headspace GC-MS</td>
<td>Free fatty acid</td>
<td>-</td>
<td>No</td>
<td>-</td>
<td>[409]</td>
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<td>Enterobacter &amp; Pseudomonas aeruginosa</td>
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<td>Induction of colored compound &amp; gene induction</td>
<td>Pyocyanin</td>
<td>Alkaloid</td>
<td>No</td>
<td>Co-culture extract shows moderate activity against E. coli and yeast</td>
<td>[419]</td>
</tr>
<tr>
<td>Streptomyces padanus &amp; Rhodococcus fascians</td>
<td>Liquid medium</td>
<td>Bioguided isolation (antibiotic activity), genome analysis</td>
<td>Rhodostreptomycin A and B [1] (horizontal gene transfer)</td>
<td>Aminoglycosides</td>
<td>Yes</td>
<td>Antimicrobial activity</td>
<td>[399]</td>
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<tr>
<td>Streptomyces cinnabarinus &amp; Alteromonas sp.</td>
<td>Liquid medium</td>
<td>HPLC-MS</td>
<td>Lobocompactol [2]</td>
<td>Diterpene</td>
<td>No</td>
<td>Antifouling activity, antioxidant and anticancer activity</td>
<td>[436]</td>
</tr>
<tr>
<td>Bacillus subtilis &amp; Streptomyces coelicolor</td>
<td>Solid medium</td>
<td>MALDI-TOFMS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[277]</td>
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<tr>
<td>Tsukamurella pulmonis &amp; Streptomyces lividans / Streptomyces endus</td>
<td>Solid and liquid media</td>
<td>HPLC-UV</td>
<td>Actinorhodin, undecylprodigiosin, alchivemycin A</td>
<td>Alkaloid (pyrrole), polyketide</td>
<td>Only for alchivemycin A</td>
<td>Antibiotic activity</td>
<td>[421]</td>
</tr>
<tr>
<td>Species Combination</td>
<td>Medium Type</td>
<td>Analytical Method(s)</td>
<td>Analyte(s)</td>
<td>Antimicrobial Activity</td>
<td>Reference</td>
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<td></td>
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<tr>
<td><em>Bacillus megaterium</em> &amp; <em>Ketogulonicigenium vulgare</em></td>
<td>Liquid medium</td>
<td>GC-TOFMS</td>
<td>Primary metabolism</td>
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<td>[424]</td>
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<td>GC-TOFMS</td>
<td>Primary metabolism</td>
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<td>[311]</td>
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<td><em>Streptococcus thermophilus</em> &amp; <em>Bifidobacterium lactis</em></td>
<td>Liquid medium (milk)</td>
<td>HPLC</td>
<td>Primary metabolism</td>
<td>-</td>
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<td><em>S. coelicolor</em> &amp; <em>B. subtilis</em></td>
<td>Solid medium</td>
<td>Nano-DESI-MS</td>
<td>Actinorhodin, SapB, SKF</td>
<td>No</td>
<td>[432]</td>
<td></td>
<td></td>
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<td><em>E. coli</em> &amp; <em>E. coli</em></td>
<td>Solid media</td>
<td>LDPI-MS</td>
<td>-</td>
<td>-</td>
<td>[435]</td>
<td></td>
<td></td>
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<tr>
<td><em>Streptomyces coelicolor</em> &amp; <em>S. coelicolor</em> / <em>Amycolatopas</em> sp. / <em>Streptomyces</em> sp. / <em>S. viridochromogenes</em></td>
<td>Solid medium</td>
<td>Nano-DESI-MS &amp; MALDI-IMS</td>
<td>Many detected compounds, four new acyl-desferroxamines (3)</td>
<td>Hydroxamic acid</td>
<td>No</td>
<td>Antimicrobial activity [437] [433]</td>
<td></td>
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<td><em>B. subtilis</em> &amp; <em>S. ondeidensis</em></td>
<td>Solid medium</td>
<td>Nano-DESI-MS</td>
<td>-</td>
<td>-</td>
<td>[431]</td>
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<tr>
<td><em>Lentinus edodes</em> &amp; <em>Trichoderma spp.</em></td>
<td>Liquid medium</td>
<td>TLC, HPLC-UV</td>
<td>Lentialexin</td>
<td>Alkyne</td>
<td>Yes</td>
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<td>[426]</td>
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<tr>
<td><em>Gloeophyllum abietinum</em> &amp; <em>Heterobasidion annosum</em></td>
<td>Liquid and solid medium</td>
<td>HPLC</td>
<td>Oosponol (4), oospoglycol (5), melledonal A (6), melledonal C (7)</td>
<td>4, 5: Polyketides, 6, 7: sesquiterpenes</td>
<td>Yes</td>
<td>Antimicrobial activity [378,404]</td>
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<tr>
<td><em>Monascus sp.</em> &amp; <em>Saccharomyces cerevisiae</em> / <em>Aspergillus oryzae</em></td>
<td>Liquid and solid medium</td>
<td>Pigment induction and morphological change</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[417]</td>
<td></td>
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<td><em>Acremonium sp.</em> &amp; <em>Mycogone rosea</em></td>
<td>Solid medium</td>
<td>HRMS/MS</td>
<td>Acremostatins A, B, C</td>
<td>Peptides (lipooaminopeptides)</td>
<td>Yes</td>
<td>-</td>
<td>[430]</td>
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**Fungus vs. Fungus**

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<th>Analyte(s)</th>
<th>Antimicrobial Activity</th>
<th>Reference</th>
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<tr>
<td><em>Gloeophyllum abietinum</em> &amp; <em>Heterobasidion annosum</em></td>
<td>Liquid and solid medium</td>
<td>HPLC</td>
<td>Oosponol (4), oospoglycol (5), melledonal A (6), melledonal C (7)</td>
<td>4, 5: Polyketides, 6, 7: sesquiterpenes</td>
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438

378, 404

431
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<th>Species/Strain</th>
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<th>Activity Assay</th>
<th>Main Metabolites</th>
<th>Compound Type</th>
<th>Activity Notes</th>
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<tbody>
<tr>
<td><em>Candida albicans</em> mutant &amp; <em>Chloridium</em> sp. / <em>Sporoschisma mirabile</em></td>
<td>Liquid</td>
<td>Anti-Candida activity</td>
<td>-</td>
<td>-</td>
<td>Antibacterial activity, cytotoxic against tumor cell lines (IC₅₀ at low nanomolar levels)</td>
</tr>
<tr>
<td>Two unidentified fungi</td>
<td>Liquid</td>
<td>Not reported</td>
<td>Marinamide (8), methyl marinamide (9)</td>
<td>Alkaloids</td>
<td>No</td>
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<tr>
<td></td>
<td>Liquid</td>
<td>Not reported</td>
<td>6-Methyl salicylic acid, cyclo-(phenylalanine-phenylalanine) dipeptide</td>
<td>Aromatic acids, diketopiperazine</td>
<td>No</td>
</tr>
<tr>
<td>Stereum hirsutum &amp; <em>Coprinus micaceus</em> / <em>Coprinus disseminatus</em></td>
<td>Solid</td>
<td>TLC, GC-TOFMS</td>
<td>Malic acid, 1,2-dihydroxyanthraquinone, and one unknown metabolite</td>
<td>Polyketides</td>
<td>No</td>
</tr>
<tr>
<td>Aspergillus clavatonanicus &amp; <em>Pythium ultimum</em></td>
<td>Solid</td>
<td>HPLC-UV</td>
<td>Clavatol, patulin</td>
<td>Polyketides</td>
<td>No</td>
</tr>
<tr>
<td><em>Eutypa lata</em> &amp; <em>Botryosphaeria obtusa</em></td>
<td>Solid</td>
<td>UHPLC-MS</td>
<td>Hydroxylated O-methylmelleins (10)</td>
<td>Polyketide</td>
<td>No</td>
</tr>
<tr>
<td>Aspergillus clavatus &amp; <em>Aspergillus fumigatus</em></td>
<td>Solid</td>
<td>HPLC-DAD, GC-FID</td>
<td>Cytochalasin E, cytochalasin K</td>
<td>Alkaloids (indoles)</td>
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<td>Two <em>Aspergillus</em> sp.</td>
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<td>Neoaspergillic acid</td>
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<td>Two unidentified fungi</td>
<td>Liquid</td>
<td>Not reported</td>
<td>8-Hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether</td>
<td>Polyketide</td>
<td>Yes</td>
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<td><em>Penicillium pinophilum</em> &amp; <em>Trichoderma harzianum</em></td>
<td>Liquid</td>
<td>HPLC-UV</td>
<td>Secopenicillide C, penicillide, MC-141, Stromemycin</td>
<td>Polyketides</td>
<td>Only for secopenicillide C</td>
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<td>Organism(s)</td>
<td>Medium Type</td>
<td>Technique</td>
<td>Metabolites</td>
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<tr>
<td>-------------------------------------------------</td>
<td>-------------</td>
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<tr>
<td><em>Fusarium verticillioides</em> &amp; <em>Ustilago maydis</em></td>
<td>Solid</td>
<td>UPLC-TOF MS</td>
<td>Up and downregulation of metabolites</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Paraconiothyrium variabile</em> &amp; <em>Fusarium oxysporum</em></td>
<td>Solid</td>
<td>LC-MS</td>
<td>13-Ox0-9,11-octadecadienoic acid, 13-hydroperoxy-9,11-octadecadienoic acid</td>
<td>Lipid No -</td>
<td></td>
</tr>
<tr>
<td><em>Ustilago maydis</em> &amp; <em>Fusarium verticillioides</em></td>
<td>Liquid</td>
<td>UHPLC-TOF MS, transcription analysis</td>
<td>Fusaric acid, siderophores</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Alternaria tenuissima</em> &amp; <em>Fusarium culmorum / Fusarium graminearum</em></td>
<td>Liquid</td>
<td>HPLC-MS/MS, quantification of six toxins</td>
<td>Deoxynivalenol (DON, 11), zearalenone (ZON, 12)</td>
<td>-</td>
<td></td>
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<tr>
<td><em>Fusarium tricinctum</em> &amp; <em>Fusarium beigoniae</em></td>
<td>Liquid</td>
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<td>Subenniatin A (13), B (14)</td>
<td>Depsipeptides Yes -</td>
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<tr>
<td><em>Trichophyton rubrum</em> &amp; <em>Bionectria ochroleuca</em></td>
<td>Solid</td>
<td>UHPLC-M5</td>
<td>Hydroxysulfoxy-2,2''-dimethylthielavin P (15)</td>
<td>Polyketide No N/A</td>
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<td><em>Alternaria tenuissima</em> &amp; <em>Nigrospora sphaerica</em></td>
<td>Liquid and solid</td>
<td>HPLC-UV</td>
<td>Stemphyperylenol (16), alterperylenol (17)</td>
<td>Polyketides Yes -</td>
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</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> &amp; <em>Spathaspora arborariae</em></td>
<td>Liquid</td>
<td>HPLC-UV-RI</td>
<td>Primary metabolism</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Penicillium</em> sp. &amp; <em>Trichoderma</em> sp.</td>
<td>Liquid</td>
<td>HPLC-UV-M5</td>
<td>(Z)-2-Ethylhex-2-enedioic acid, (E)-4-oxo-2-propylideneoct-7-enooic acid</td>
<td>Polyketides Yes -</td>
<td></td>
</tr>
<tr>
<td><em>Paraconiothyrium</em> sp. &amp; <em>Alternaria</em> sp. / <em>Phomopsis</em> sp.</td>
<td>Liquid</td>
<td>LC-MS</td>
<td>Taxol</td>
<td>Terpenes No -</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fungus vs. Bacterium</th>
<th>Solid and Liquid Media</th>
<th>Antibacterial Activity</th>
<th>Polyketide</th>
<th>Antibacterial Activity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frateruria sp. (former Gluconobacter sp.) &amp; Neurospora crassa / Aspergillus oryzae</td>
<td>Solid and liquid media</td>
<td>Enacyloxin II Polyketide</td>
<td>-</td>
<td>-</td>
<td>[413,414,496]</td>
</tr>
<tr>
<td>Pseudomonas fluorescens &amp; Fusarium oxysporum spp.</td>
<td>Liquid medium, and on artificial soil medium</td>
<td>HPLC-UV, transcriptional analysis</td>
<td>-</td>
<td>-</td>
<td>[427,487]</td>
</tr>
<tr>
<td>Pseudomonas fluorescens &amp; Pythium ultimum / Rhizoctonia solani</td>
<td>Solid medium</td>
<td>HPLC-UV</td>
<td>-</td>
<td>-</td>
<td>[416,498]</td>
</tr>
<tr>
<td>Pestalotia &amp; unidentified bacterium</td>
<td>Liquid medium Bioguided isolation</td>
<td>Pestalone Polyketide</td>
<td>Yes</td>
<td>Moderate in vitro cytotoxicity, potent antibacterial [509]</td>
<td>[415]</td>
</tr>
<tr>
<td>Libertella sp. &amp; unidentified bacterium</td>
<td>Liquid medium HPLC-MS</td>
<td>Libertellinenone A, B, C, D Diterpene</td>
<td>Yes</td>
<td>Weak activity against Candida albicans, libertellinenone D demonstrated potent cytotoxicity against HCT-116 human colon carcinoma cancer cell line</td>
<td>[391]</td>
</tr>
<tr>
<td>Emericella sp. &amp; Salinispora arenicola</td>
<td>Liquid medium LC-TOFMS</td>
<td>Emericellamides A and B Cyclic depsipeptides</td>
<td>Yes</td>
<td>Modest antibacterial activities</td>
<td>[392]</td>
</tr>
<tr>
<td>Fusarium oxysporum &amp; Serratia sp. / Achromobacter sp.</td>
<td>Liquid medium SPME-GC-MS</td>
<td>Isocaryophyllene (18), a-humulene (19), cyclocaryophyllan-4-ol (20) Sesquiterpenes</td>
<td>Yes</td>
<td>Supression of virulence in F. oxysporum, antimicrobial activity of isocaryophyllene</td>
<td>[443]</td>
</tr>
<tr>
<td>Aspergillus fumigatus &amp; Sphingomonas sp.</td>
<td>Liquid medium HPLC</td>
<td>Glionitrin A (21) Diketopiperazine alkaloids</td>
<td>No</td>
<td>Antimicrobial activity and cytotoxicity</td>
<td>[310]</td>
</tr>
<tr>
<td><strong>Aspergillus nidulans &amp; Escherichia coli</strong></td>
<td>Liquid medium</td>
<td>QRT-PCR of the polyketide gene, HPLC</td>
<td>Orsellinic acid (22), lecanoric acid (23), polyketides F-9775A (24) and F-9775B (25)</td>
<td>Polyketides</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa &amp; Aspergillus fumigatus</strong></td>
<td>Solid medium</td>
<td>MALDI-TOFMS</td>
<td>Triacetylfusarinine C, fusarinine C, 1-hydroxyphenazine, 1-methoxyphenazine, phenazine-1-sulfate, phenazine dimers</td>
<td>Hydroxamic acid, phenazine</td>
<td>Yes, but not for all</td>
</tr>
<tr>
<td><strong>Piromyces sp. &amp; Methanobrevibacter thaueri</strong></td>
<td>Liquid medium</td>
<td>¹H NMR</td>
<td>Primary metabolites</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Aspergillus fumigatus &amp; Streptomyces peucetius</strong></td>
<td>Liquid medium</td>
<td>HPLC-MS</td>
<td>Fumicyclines A (28), B (29)</td>
<td>Meroterpenoids</td>
<td>No</td>
</tr>
<tr>
<td><strong>Aspergillus fumigatus &amp; Streptomyces rapamycinicus</strong></td>
<td>Liquid medium</td>
<td>Full genome microarray, HPLC-UV</td>
<td>Fumicyclines A (28), B (29)</td>
<td>Meroterpenoids</td>
<td>No</td>
</tr>
<tr>
<td><strong>Bacillus amyloliquefaciens &amp; Aspergillus fumigatus &amp; Aspergillus niger</strong></td>
<td>Solid medium</td>
<td>MALDI-MS</td>
<td>Iturins</td>
<td>Peptides</td>
<td>No</td>
</tr>
<tr>
<td><strong>Fusarium tricinctum &amp; Bacillus subtilis</strong></td>
<td>Solid medium</td>
<td>HPLC-UV</td>
<td>Induction of 13 compounds, three novel compounds macrocarpon C (30), 2-((carboxymethylamino)benzoic acid (31), (−)-citreoisocoumarinol (32)</td>
<td>Polyketides</td>
<td>No</td>
</tr>
<tr>
<td><strong>Aspergillus fumigatus &amp; Streptomyces bulill</strong></td>
<td>Liquid medium</td>
<td>HPLC-MS</td>
<td>11-O-Methylpsenrotin A (33), and several diketopiperazine alkaloids and other known compounds</td>
<td>Lactam (pyrrolidone), diketopiperazine alkaloids</td>
<td>No</td>
</tr>
<tr>
<td>Organism</td>
<td>Medium Type</td>
<td>Method</td>
<td>Products</td>
<td>Activity</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------------------------</td>
<td>----------</td>
<td>-----------------------------------------</td>
<td>------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Saccharopolyspora erythraea &amp; Fusarium pallidoroseum</td>
<td>Liquid medium</td>
<td>HPLC-MS</td>
<td>Equisetin, ophiostin, N-demethyl-ophiostin, pallidorosetin A, pallidorosetin B</td>
<td>Lactam, Yes, but not for all</td>
<td>[512]</td>
</tr>
<tr>
<td>Others</td>
<td>Liquid medium</td>
<td>Counting of oospores, HPLC-MS</td>
<td>Mating hormones α1 [34] and α2 [35]</td>
<td>Diterpenoids, No</td>
<td>[406,408]</td>
</tr>
<tr>
<td>Dinoroseobacter shibae &amp; Thalassiosira pseudonana</td>
<td>Liquid medium (separation by 0.22μm membrane)</td>
<td>GC-MS</td>
<td>Primary metabolism</td>
<td>-</td>
<td>[425]</td>
</tr>
<tr>
<td>Ankistrodesmus sp. &amp; Rhizobium spp.</td>
<td>Liquid medium</td>
<td>Cell counting, gravimetric analysis of lipid, GC</td>
<td>Primary metabolism</td>
<td>No</td>
<td>[410]</td>
</tr>
</tbody>
</table>
9. Acknowledgments

This work was supported by the Swiss National Science Foundation Sinergia Grant CRSII3_127187 and Grant CR23I3_143733 / 1, which were awarded to J.-L.W. and K.G. We gratefully acknowledge the first nine *Grands Crus de Bordeaux* (Bordeaux, France) for their financial support for the salary of Dr. Sylvain Schnee: château Ausone, château Cheval blanc, château Haut-Brion, château Lafitte Rothschild, château Latour, château Margaux, château Mouton Rothschild, château Petrus and château d’Yquem. We also thank Pierre-Marie Allard for helpful discussion on epigenetic modification and Sarah Berndt, Nurhuda Manshoor, Jeroen J. Jansen and Mariam Mnatsakanyan for revising the manuscript.
Plant constituents
In view of the identification of novel fungal metabolites issued from solid medium co-cultures, and knowing of the limited sample amounts available from this particular sample type (few milligrams of crude extract), miniaturized methods that are compatible with metabolite identification by NMR and biological testing of pure compounds had to be established. As the phytochemistry & bioactive natural products research group at the University of Geneva has a long history of experience in the analysis of plant constituents, a model plant extract was selected to develop a miniaturized and generic isolation strategy based on HPLC microfractionation. Instrumental parameters as well as an efficient dereplication procedure were optimized using a root extract of the well-studied Fabaceae *Lupinus albus*. Furthermore, the efficiency of gradient transfer from UHPLC to analytical and semipreparative HPLC, a key element for efficient microfractionation, was studied with this model extract as well.

The Molecular Biodiscovery group in Leuven was trying to identify antiangiogenic and anti-inflammatory constituents from the Tanzanian plant *Rhynchosia viscosa* using a high-content zebrafish assay. The microfractionation strategy was found suitable to be combined with this *in vivo* assay. Towards the biological profiling of the *R. viscosa* extract, the devised quantitative NMR procedure, important to link the potency of individual extract constituents with assay response, was tested on the model extract as well. This preliminary work is described in chapter III.A. The study of the biological profiling of the extract of *R. viscosa* for the identification of antiangiogenic and anti-inflammatory is described in chapter III.B [202].
III. Microfractionation for the purification of mass-limited samples

III.A Optimization of microfractionation procedures for bioactivity profiling and dereplication using a model plant extract
1. Introduction

During the course of the project on the biological profiling of *Rhynchosia viscosa* (chapter III.B), a microfractionation strategies in combination with quantification of microfractions by NMR was optimized using a model extract from the plant *Lupinus albus* (Figure III.A.1). This plant was easily accessible and several plants were cultivated in the greenhouse of a collaborating research group. Several parameters had to be optimized and verified such as the dereplication procedure, the assurance of an efficient gradient transfer from UHPLC to analytical and semipreparative HPLC, as well as the accuracy of quantification by NMR.

*L. albus* has been well-studied phytochemically, and its extract composition was likely similar to *R. viscosa* (both from the *Fabaceae* family). The root extract of *L. albus* was thus selected for the optimization of instrumental parameters related to the gradient transfer. Furthermore, the presence of genistein in the extract of *R. viscosa* was known at this stage and *L. albus* was a known producer of genistein. It was estimated appropriate to verify at-line quantification by NMR for genistein with HPLC-UV on-line quantification within the extract for genistein.

2. Results & discussion

For the chemical investigation of secondary metabolites from *L. albus*, three methanolic extract of the dried roots, stems and flowers were prepared and analyzed by UHPLC-TOFMS. The presence of genistein, known through comparison with an authentic standard and the in-house database [59], was only revealed in the root extract. Thus, only the analysis of this extract was further pursued.

2.1 Metabolite profiling and dereplication

For the analysis of chemical constituents, extracts were analyzed using a long gradient by ultra high performance-photo diode array-time-of-flight mass spectrometry (UHPLC-PDA-TOFMS, metabolite profiling, Figure III.A.2). Advantages to this approach are shown by Eugster et al. [59].

Overall, more compounds were detected in negative ionization (NI) mode compared to positive ionization (PI) mode. MS permitted the detection of a far greater number of extract constituents compared to PDA. Manual inspection of the PDA spectra of the seven most intense peaks showed that several isoflavones (chromophore type A and B) were present among the extract constituents. The two chromophore types indicate a difference in hydroxylation at position 5 and 6 of the ring A. The chromophore type C of the more apolar constituents is not specific to be indicated for a specific compound class [513].
The presence of genistein in the extract was confirmed by comparison of exact mass \( m/z \) 269.0443 \([M-H]^-\) calc. \( m/z \) 269.0450, \( \Delta 2.6 \) ppm and retention time [59]. The corresponding PDA spectrum (chromophore type B) was consistent with hydroxylation at position 5 such as for the isoflavone genistein.

For the rapid identification of known natural products (NPs) from the \textit{L. albus} extract, a generic dereplication procedure was developed that permitted the cross-search of database entries from the Dictionary of Natural Products (DNP) with detected mass-to-charge ratios \( m/z \) in LC-HRMS datasets. This approach benefits from the automated peak picking procedure in the free data treatment software MZmine and automated search in custom NP databases. The development of this automated dereplication procedure was possible with the introduction of the ‘custom database search’ option in the data treatment software MZmine 2 in 2012 [514,515]. This procedure is based on the chemotaxonomic information that is given for database entries in the DNP. Therefore, in a first step, peak lists have to be generated from chromatograms, e.g., from metabolite profiling. The software MZmine has the advantage over other data treatment software that many parameters are adjustable and that the effect of individual parameters are directly visualized through the preview function. Parameters have to be adjusted based on the individual chromatogram (Table III.A.3), especially the parameters ‘noise level’ and retention time tolerance. The parameter ‘\( m/z \) tolerance’ is dependent on the performance of the used mass spectrometer. The procedure can be extended to identify possible adducts [54,516] in the peak lists.
Table III.A.3: Steps and parameters used during the automatic peak picking procedure by MZmine 2 for dereplication in metabolite profiling chromatograms.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Raw data methods -&gt; Filtering -&gt; Data set filtering</td>
<td>Filter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crop filter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Retention time (min)</td>
<td>0.5 to 30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 to 30</td>
</tr>
<tr>
<td>2) Raw data methods -&gt; Peak detection -&gt; Mass detection</td>
<td>Mass detector</td>
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</tr>
<tr>
<td></td>
<td>Centroid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Noise Level</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>MS level</td>
<td>1</td>
</tr>
<tr>
<td>3) Raw data methods -&gt; Peak detection -&gt; Chromatogram builder</td>
<td>Min time span (min)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Min height</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>m/z tolerance (ppm)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>4) Peak list methods -&gt; Peak detection -&gt; Chromatogram deconvolution</td>
<td>Algorithm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Local minimum search</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chromatographic threshold (%)</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Search minimum in RT range (min)</td>
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</tr>
<tr>
<td></td>
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<td>0.1</td>
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<tr>
<td></td>
<td>Minimum relative height (%)</td>
<td>5</td>
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<td></td>
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<td>5</td>
</tr>
<tr>
<td></td>
<td>Minimum absolute height</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Min ratio of peak top/edge</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Peak duration range (min)</td>
<td>0 to 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 to 10</td>
</tr>
<tr>
<td>5) Peak list methods -&gt; Isotopes -&gt; Isotopic peaks grouper</td>
<td>m/z tolerance (ppm)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Retention time tolerance (min)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Maximum charge</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Representative isotope</td>
<td>Most intense</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Most intense</td>
</tr>
<tr>
<td>6) Peak list methods -&gt; Identification -&gt; Complex search (optional)</td>
<td>Ionization method</td>
<td>[M-H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[M+H]+</td>
</tr>
<tr>
<td></td>
<td>RT tolerance</td>
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<tr>
<td></td>
<td>m/z tolerance (ppm)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
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<tr>
<td></td>
<td>Max complex peak height (%)</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>7) Peak list methods -&gt; Identification -&gt; Custom database search</td>
<td>m/z tolerance (ppm)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Retention time tolerance (absolute, min)</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
</tr>
</tbody>
</table>

In a second step, database entries associated to the biological source, in this case the genus *Lupinus*, are exported from the DNP. Therefrom, a custom database is generated in csv (comma-separated values) format that contains the following information: unique metabolite ID, accurate mass-to-charge ratio (m/z) in either negative ionization (NI) or positive ionization (PI), retention time, molecular formula and metabolite name. Here, the CRC code was used as unique metabolite ID as the integrality of DNP entries hold this code. Enzymes (and entries without known molecular formula) were excluded from the custom database. Two custom databases were created, one for identification in NI LC datasets and one for PI LC datasets. Therefore, as an approximation, the exact mass of a proton (1.007825 Da) was subtracted or added to the accurate mass (nominal mass) to get the accurate m/z. As the retention time was unknown for most metabolites, the value was set to 0. As a result, the retention time is not taken into account in the identification step.
Using this automated dereplication procedure, in the NI LC dataset, 85 features (detected \( m/z \) at a given retention time) were putatively assigned to known Lupinus metabolites. In the PI LC dataset, 24 features were putatively identified. In both dataset, several \( m/z \) were detected with different retention times indicating the prevalence of isomers. For example, a compound with \( m/z \) 417.134 (NI mode) was detected at six different retention times (16.5, 18.2, 18.4, 18.8, 19.4 and 21.4 min). These features were all putatively identified as lupindipyranoisoflavone A (CAS: 76754-30-8, Figure III.A.3). Two other regioisomers of this metabolite exist and 16 more constitutional isomers are reported in DNP. Unambiguous peak identification would only be possible in comparison with an authentic standard and a second orthogonal property, e.g., the retention time [47,48]. Ultimately, NMR detection can help differentiate isomers.

![Figure III.A.3: Structure of Lupindipyranoisoflavone A.](image)

The isoflavone genistein was not automatically annotated in the LC-MS dataset because the chemotaxonomic information is incomplete in the corresponding DNP database entry. The biological source is given as “widely distributed in the Leguminosae subf. Papilionoideae” which is an obsolete taxonomic description and not exploitable with the presented approach. This is one of the major drawbacks of this dereplication procedure. Nevertheless, this automated procedure still offers considerable gain of time compared to ‘manual’ dereplication (inspection of every detected peak for the determination of molecular formula and comparison with known metabolites in the species).

### 2.2 Optimization of the microfractionation procedure

For the targeted isolation of genistein from the extract, chromatographic conditions were optimized by analytical HPLC-PDA analysis, in a first step. Conditions were geometrically transferred to UHPLC-TOFMS for comparison and to semipreparative HPLC for subsequent isolation of genistein [67] (Figure III.A.4). The same stationary phase had been used for all three columns.

From Figure III.A.4, it can be seen that the transfer from analytical to semipreparative HPLC results in a very close match of retention behavior of polar as well as apolar extract constituents. This was mastered through the installation of a degasser unit for both solvent lines of the (semi)preparative LC-UV instrument. Optimal separation was monitored by verification of the pressure reading throughout the isolation run. Only upon installation of a degasser unit could a homogeneous pressure level be obtained throughout a chromatographic run.
On the other hand, the transfer from analytical to UHPLC does not result in matching retention behavior. More polar extract constituents tend to elute earlier in UHPLC compared to HPLC whereas more apolar compounds tend to elute later in UHPLC. This can be explained through additional diffusion effects related to the larger diameter in HPLC columns compared to UHPLC columns. These effects are currently not well mastered in geometrical transfer software yet. The software used here was developed for transfer among UHPLC columns only and yields only approximate results for transfers among columns of very different inner diameters.

![Figure III.A.4: Optimized chromatographic conditions for targeted isolation of genistein in L. albus root extract on UHPLC, analytical HPLC and semipreparative HPLC.](image)

Geometrical transfer [67] of chromatographic conditions from analytical HPLC (250 × 4.6 mm i.d., 5 μm column) to UHPLC (top panel, 150 × 2.1 mm i.d., 1.7 μm column) and to semipreparative HPLC (bottom panel, 250 × 10 mm i.d., 5 μm column). The transfer to the UHPLC level is not well-mastered, whereas the transfer to semipreparative HPLC matches very well.

### 2.3 Targeted isolation of genistein

For the targeted isolation of genistein, an enriched fraction of the *L. albus* root extract was purified by semipreparative HPLC. The analytical HPLC optimized gradient was transferred geometrically to a column with the same stationary phase but with a larger inner diameter with milligram amount loading capacity. It enabled the purification of microgram-amounts of pure compound within one purification run.
The injected amount of sample (9.75 mg of enriched extract) was well below the loading capacity of the column as specified by the manufacturer (approx. 100 mg) but assured to maintain a chromatographic resolution close to analytical HPLC [202]. The injected volume was also limited by the solubility of the sample in the dissolution solvent mixture (15 to 50% aqueous acetonitrile) compatible with HPLC.

Microfractions were collected every 30 s and genistein (retention time 18 min) was located based on the on-line UV monitoring during the purification run. The corresponding microfractions were dried on a vacuum centrifuge, concentrated into a 96-deepwell plate for storage. At a later time point, microfractions were concentrated into HPLC vial inserts for at-line quantitative NMR (qNMR) as well as quantitative HPLC-UV analysis.

2.3.1 At-line quantification by quantitative NMR

Obtained sample amounts from milligrams of extracts are in the range of a few micrograms. These amounts are too low to be accurately weighted on an analytical balance. Thus, for the quantification of isolated genistein, a qNMR method using an external calibration (pulse length based concentration determination, PULCON [121]) was used. Further information can be found in the Supporting information of chapter III.B [202]. Briefly, the relaxation delay $T_1$ was experimentally determined for all protons of genistein to choose the recycle delay for qNMR acquisition and to determine the $^1$H signals that are suitable for quantification. For the aromatic proton signal with chemical shift $\delta$ 7.40, a relaxation delay of 2 s was determined. As complete relaxation is reached at $5 \times T_1$, the recycle delay of 20 s does thus allow for complete relaxation of this proton signal that was chosen for quantification. The amount of isolated genistein was determined to be 110 µg. The enriched fraction of the L. albus root extract contained thus 11.3 µg of genistein per mg of sample.

2.3.2 At-line and on-line quantification by HPLC-UV

The genistein containing microfractions were recovered after qNMR analysis, dried down again and diluted in 1 mL for subsequent quantification by HPLC-UV. For this quantification, analysis at done at 254 nm. With this approach, the amount of isolated genistein was determined to be 119 µg. This results in a genistein content of 12.2 µg per mg of sample. This corresponds to a discrepancy of 8% between the two at-line quantification procedures.

As a last step, the amount of genistein was quantified on-line by HPLC-UV directly in the enriched fraction of the L. albus root extract. The amount of genistein within the fraction was determined to be 13 µg per mg of sample. Therefrom, it was concluded that almost 15% of the extract is lost during semipreparative isolation, either through losses during injection, through irreversible adsorption on the stationary phase or through general sample handling.

3. Conclusion

For the optimization of instrumental parameters of the microfractionation strategy, a methanolic extract of the roots of the Fabacea plant Lupinus albus was analyzed. In a first step, the chemical constituents of the extract were analyzed by metabolite profiling. Known
Lupinus compounds were searched in the extract using an automated dereplication procedure based on the Dictionary of Natural Products. It could be shown that databases used for dereplication have to be refined by chemotaxonomic information to reduce the number of false-positive hits in automated peak annotation. However, two major drawbacks have to be highlighted. Because of incomplete information on biological sources of NPs reported in DNP, known metabolites are sometimes not annotated. Furthermore, as the DNP does not contain a second orthogonal physicochemical property (e.g., retention times), differentiation of isomers is difficult. Nevertheless, this approach allows for a substantial gain of time for the peak annotation of extract constituents compared to ‘manual’ dereplication.

In a second step and towards the targeted isolation of genistein from the L. albus extract, the gradient transfer efficiency from analytical HPLC to UHPLC as well as from analytical to semipreparative HPLC was evaluated. It could be shown that the transfer from analytical to semipreparative HPLC works well and results in matching chromatographic separation.

In a third step, on-line and at-line quantification of genistein amounts in the extract by NMR and by HPLC-UV were compared. Quantification results from the two different approaches (qNMR and HPLC-UV) deviated less than 10%. The qNMR approach was thus considered a valuable alternative to HPLC-UV as it enables simultaneous structure identification. Moreover, qNMR can also be used for the absolute quantification of unknown constituents which is key when used in combination with at-line bioassays to estimate the potency of a given activity. Furthermore, comparison between quantification of isolated genistein with quantification in the enriched fraction showed that recovery yields from sample purifications by microfractionation were higher than 85%.

These studies were valuable for the fundamental development of the microfractionation strategy that was applied to the biological profiling of Rhynchosia viscosa for the evaluation of its antiangiogenic and anti-inflammatory constituents (chapter III.B and [202]).

4. Materials & methods

4.1 Plant material, extraction and sample preparation
Lupinus albus plants (Amiga F) were grown in the green house at Agroscope ACW (Nyon, Switzerland) until flowering (two to three months). Plant material was separated into flowers, roots and shoots, dried by lyophilization and ground. The powdery plant samples were exhaustively extracted with methanol (MeOH) by maceration. The dry methanolic extracts were obtained after removing the solvent by evaporation under reduced pressure.

For metabolite profiling, the crude methanolic extracts were solubilized in 85% aqueous MeOH and purified by SPE (SepPak® Vac, C18, 2g/6 mL, Waters) using 85% aqueous MeOH for elution. For the targeted isolation of genistein from the root extract, the crude methanolic extract was adsorbed onto Kieselgur (für Säulenchromatographie, Merck, also known as celite) for purification by SPE (SepPak® Vac, C18, 2g/6 mL, Waters). Polar extract constituents
were removed by elution with 100% water and the enriched extract was obtained by elution with 85% MeOH. In this enriched fraction, 27% of the extract mass were retained.

4.2 UHPLC-PDA-TOFMS analyses

UHPLC-PDA-TOFMS analyses were performed using an Acquity™ UPLC chromatograph and a Micromass-LCT Premier Time of Flight mass spectrometer equipped with an ESI interface (Waters). For the metabolite profiling, separation was performed on a 150 × 2.1 mm i.d., 1.7 μm, Acquity BEH C18 UPLC column (Waters) in the gradient mode at a flow rate of 0.46 mL/min with the following solvent system: A = 0.1 vol% formic acid (FA)-H2O, B = 0.1 vol% FA-acetonitrile (MeCN); 5–95% B in 30 min. The injected volume was 2 μL. Detection was performed in NI mode in the range m/z 100-1000 in centroid mode with a scan time of 0.3 s. ESI conditions were: capillary voltage 2800 V, cone voltage 40 V, MCP detector voltage 2400 V, source temperature 120 °C, desolvation temperature 300 °C, cone gas flow 20 L/h, and desolvation gas flow 700 L/h. The mass spectrometer was internally calibrated by infusion of a solution of leucine-enkephalin (2 μg/mL, Sigma-Aldrich) through the lockmass spray probe at a flow rate of 10 μL/min, using a second Shimadzu LC-10ADvp LC pump. The PDA traces were recorded from 210 to 460 nm. Samples were analyzed at a concentration of 0.5 mg/mL in 85% aqueous MeOH.

The chromatographic conditions for the targeted isolation of genistein were as follows: solvent system A = 0.1 vol% FA-H2O, B = 0.1 vol% MeCN; 15-40% B in 4.13 min, 40-90% in 4.08 min, 90-100% in 0.27 min. The other parameters were as described above.

4.3 Dereplication

Dereplication of UHPLC-TOFMS analyses based on chemotaxonomic information was done using an automated procedure as explained in section 2.1. Briefly, native LC-MS data were analyzed with data treatment software (MZmine 2, version 2.10 [515]) to generate peak lists of all chromatograms. Data were deisotoped and chromatograms aligned. Parameters were optimized and are given in Table III.A.3. Known compounds from plants of the genus Lupinus were retrieved from the DNP (version 22:1, CRC Press, Taylor & Francis) and searched within these peak lists.

4.4 Targeted isolation of genistein from L. albus root extract

4.4.1 HPLC-PDA analysis for optimization of chromatographic conditions

HPLC-PDA analyses were performed using a HP 1100 instrument equipped with an HP1100 PDA detector. Separation was performed on a 250 × 4.6 mm i.d., 5 μm, XBridge C18 HPLC column (Waters) in gradient mode at a flow rate of 1 mL/min with the following solvent system: A = 0.1 vol% FA-H2O, B = 0.1 vol% FA-MeCN; 15–40% B in 15 min, followed by 40–90% in 15 min. The injected volume was 10 μL. Samples were prepared in 50% aqueous MeCN with 0.1 vol% FA. The PDA traces were recorded from 200 to 600 nm and the following wavelengths were selected for recording: 254, 280 and 360 nm.
4.4.2 Semipreparative separation of the crude extract

Semipreparative HPLC-UV isolation was performed using a preparative LC-UV instrument (Spot Prep II 50, Armen Instrument). Isolation was performed on a 250 × 10 mm i.d., 5 μm, XBridge C18 HPLC column (Waters) in gradient mode at a flow rate of 4.7 mL/min with the following solvent system: A = 0.1 vol% FA-H₂O, B = 0.1 vol% FA-MeCN; 15–40% B in 14 min, followed by 40–90% in 15 min. Separation was monitored by UV at 254 and 270 nm. Chromatographic conditions had been transferred geometrically using HPLC Calculator 3.0 software (http://www.unige.ch/sciences/pharm/fanal/lcap/telechargement.htm) [67]. The injection volume was 250 μL and the sample was prepared in 50% aqueous MeCN with 0.1 vol% FA. Both solvent lines were equipped with a degasser (ERC-3415, Ercatech). Microfractions were collected every 30 s in glass tubes and dried in a vacuum centrifuge (HT-4X, Genevac). The microfractions were then transferred to a 96-deepwell plate. Genistein was located in the microfractions based on the UV monitoring during separation.

4.5 NMR and qNMR analyses

Analyses on microfractions were done on a 500 MHz NMR instrument (INOVA, Agilent) that was equipped with a microflow probe (CapNMR™). Probes were operated at 298 K. Quantitative NMR was employed as described in chapter III.B (Supplementary information). Briefly, the optimal pulse width at 90° was arrayed (at 360°) for every individual sample and lays between 4.1 and 4.2 μs. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased, baseline corrected using a 1st order polynomial function and calibrated to the residual methanol peak at 3.31 ppm using MestReNova (version 6.01, Mestrelab Research S.L.) The signals were integrated manually and the concentration was determined using PULCON [121]. Maleic acid was used as external standard.

4.6 HPLC-UV analysis

For the quantification of genistein in the L. albus root extract, four calibration points for genistein (synthetic, Acros) were made, prepared from a 1 mg/mL stock solution (1 to 30 μg/mL) in 50% aqueous MeCN with 0.1 vol% FA. Quantification was done with detection at 254 nm. For the quantification of genistein in microfractions, five calibration points were made (20 to 100 μg/mL). Microfractions were diluted in 1 mL of 50% aqueous MeCN with 0.1 vol% FA.

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III.B Integration of microfractionation, qNMR and zebrafish screening for the in vivo bioassay-guided isolation and quantitative bioactivity analysis of natural products

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*/** These authors contributed equally to this work.

Contribution: Design of experiments, metabolite profiling and isolation, development of qNMR workflow, interpretation of results and writing of the manuscript
Abstract

Natural products (NPs) are an attractive source of chemical diversity for small-molecule drug discovery. Several challenges nevertheless persist with respect to NP discovery, including the time and effort required for bioassay-guided isolation of bioactive NPs, and the limited biomedical relevance to date of in vitro bioassays used in this context. With regard to bioassays, zebrafish have recently emerged as an effective model system for chemical biology, allowing in vivo high-content screens that are compatible with microgram amounts of compound. For the deconvolution of the complex extracts into their individual constituents, recent progress has been achieved on several fronts as analytical techniques now enable the rapid microfractionation of extracts, and microflow NMR methods have developed to the point of allowing the identification of microgram amounts of NPs.

Here we combine advanced analytical methods with high-content screening in zebrafish to create an integrated platform for microgram-scale, in vivo NP discovery. We use this platform for the bioassay-guided fractionation of an East African medicinal plant, Rhynchosia viscosa, resulting in the identification of both known and novel isoflavone derivatives with antiangiogenic and anti-inflammatory activity. Quantitative microflow NMR is used both to determine the structure of bioactive compounds and to quantify them for direct dose-response experiments at the microgram scale. The key advantages of this approach are (1) the microgram scale at which both biological and analytical experiments can be performed, (2) the speed and the rationality of the bioassay-guided fractionation – generic for NP extracts of diverse origin – that requires only limited sample-specific optimization and (3) the use of microflow NMR for quantification, enabling the identification and dose-response experiments with only tens of micrograms of each compound. This study demonstrates that a complete in vivo bioassay-guided fractionation can be performed with only 20 mg of NP extract within a few days.

Keywords: zebrafish, qNMR, microfractionation, Rhynchosia viscosa, in vivo anti-inflammatory assay, in vivo antiangiogenic assay, UHPLC-TOFMS, microflow NMR, drug discovery, natural products, chromatography
1. **Introduction**

Natural products (NPs) are an important source of drug-like compounds for the discovery of new therapeutic candidates and over time their chemical diversity has contributed significantly to the development of drugs for a wide range of diseases. The majority of new drugs approved within the last thirty years are either natural products themselves or are derived from natural products [2,75,517].

Currently, most drug discovery programs are based on high-throughput screening (HTS) to rapidly query the bioactivity of large libraries of synthetic compounds. In contrast, the isolation and characterization of bioactive secondary metabolites present in complex NP extracts involves the application of several complementary methodologies that require considerably more time and effort [518,519]. In addition, there are several inherent caveats associated with testing NPs in HTS. Crude extracts from various species of plants, fungi, and bacteria, herein after called NP extracts, are complex mixtures of mostly uncharacterized compounds, some of which might have undesired effects. The chemical properties of certain secondary metabolites might hinder the test readout and interfering constituents present in the crude extract can either mask the biological activity [520] or cause toxic effects that lead to false positives, e.g. in enzymatic assays. Nevertheless, a considerable advantage of NPs is their chemical diversity. The chemical space occupied by NPs is different from the one occupied by synthetic compounds – often with far greater degrees of 3-dimensionality and structural complexity. NPs are a promising source of diverse molecular scaffolds for the discovery of novel lead compounds against original targets [231] and recently, combinatorial libraries with NP-like compounds have been used for HTS [75].

Bioassay-guided fractionation has proven successful as a well-established platform to isolate and characterize active constituents present in NP extracts, which are then suitable for HTS [521,522]. However, such an approach requires multiple chromatographic steps and large amounts of biological material. Recent technological improvements in the area of chromatographic separation methods have nevertheless provided new possibilities to accelerate the overall process of bioassay-guided fractionation. In particular, the development of microfractionation approaches based on advanced high performance liquid chromatography (HPLC) techniques is now enabling the systematic separation of complex plant extracts using more widely applicable protocols [68]. The increasing sophistication of such techniques by linking them directly (on-line) or indirectly by adding an additional step of sample concentration (at-line) with analytical assays allows the more rapid dereplication of extracts – identifying known NPs prior to thorough characterization – thereby focusing resources on novel molecules.

Although active constituents present in NP extracts can now be identified more quickly as less time is expended on the purification of inactive constituents, still appreciable amount of time is invested if the bioactive compounds need to be isolated for the determination of their structure and in-depth biological testing. This is the bottleneck of bioassay-guided isolation
since the *de novo* structure elucidation of small molecules relies on NMR spectroscopy, which has intrinsically low sensitivity. Nevertheless, with the emergence of microflow NMR [126] and cryo and microcryo NMR technologies [113, 129, 211] used routinely in NP drug discovery, the boundaries could be pushed to the low microgram scale of sample needed for the acquisition of $^1$H-$^{13}$C and $^{13}$C spectra.

When working with HPLC-based biological profiling, another issue is to quantify the potency of a given extract constituent in a given bioassay since the microgram quantities obtained by microfractionation have to be correctly estimated [151]. Weighing of the individual microfractions is not only impractical but also inaccurate at sub-milligram quantities. Furthermore, compound purity is not taken into account. Since NMR gives an absolute signal response, it can not only provide unambiguous compound identification but allows precise quantification even of unknown compounds and estimate ratios in fractions still containing mixtures. NMR quantification can be performed either with an internal standard, using the ERETIC (electronic reference to access *in vivo* concentrations) [523] method that demands specialized electronic equipment, or the PULCON (pulse length based concentration determination) [121] method with reference to an external standard.

The ultimate impact of these new methods on the field of NP discovery, however, will be determined by the bioassays with which they can be combined. The recent report of a microfractionation approach involving the coupling of microbore HPLC separation with an at-line 1536-well biochemical screening assay for protein kinase A activity assessment and with parallel QTOF MS (quadrupole time of flight MS) data acquisition for analyte identification is an excellent example of the potential of this technology [69].

Despite its utility for HTS of active compounds, the reliance of such strategies on enzymatic or *in vitro* cell-based assays to assess their biological activity limits the biomedical relevance of the active metabolites isolated in this manner. By combining high-resolution microfractionation with high-content assays, the activity of the separated constituents would be analyzed and validated to an appreciably higher degree.

In contrast with enzymatic or cell-based reporter assays, high-content bioassays (e.g. phenotypic assays using cells or organisms) allow the unbiased analysis of pharmacological activity. In particular, *in vivo* animal models offer the possibility to screen for biomedically relevant bioactivities in a target- and pathway-independent manner. Nevertheless, mammalian models such as rodents require larger amounts of compound (in the milligram range) for activity analysis, and are therefore not ideal *in vivo* platforms for rapid HPLC profiling and microfractionation strategies.

In this context, zebrafish bioassays represent an attractive alternative to determine the *in vivo* bioactivity of chromatographic fractions containing only microgram amounts of individual compounds. Zebrafish – *Danio rerio* – have recently emerged as a reliable *in vivo* vertebrate model system for functional genomics and drug discovery [524]. Beyond their many physiological and pharmacological similarities to mammals, zebrafish have important
advantages such as high fecundity (up to hundreds of offspring per day), the small size of embryos and larvae (0.5 to 5 mm depending on the developmental stage), optical transparency and rapid development ex utero. These features confirm zebrafish as a versatile in vivo experimental model compatible with HTS and microfractionation techniques in the field of NP discovery [78]. In this regard, the amenability of using zebrafish embryos and larvae in microtiter plates (96- and even 384-well design) allows early in vivo analysis of the activity of small-molecule compounds isolated by microfractionation approaches. Depending on the potency of these isolated compounds, the requirement of only microgram amounts to induce an initial biological response represents another excellent benefit of using zebrafish as a model organism over other higher vertebrates (e.g. rodents, in which the active dose requirements are usually a thousand-fold higher [43]).

This latter feature is key for NP discovery, as many high-resolution separation methods based on HPLC, particularly microfractionation, result in very limited amounts of samples that would otherwise be insufficient for the in vivo analysis of activity.

In this study we combine HPLC profiling with microfractionation and sensitive microflow NMR at-line detection with a high-content in vivo screen in zebrafish for the rapid identification of bioactive NPs in crude plant extracts as well as for the direct estimation of their biological activity and potency at the microgram level. We illustrate this approach by investigating both the anti-inflammatory and the antiangiogenic activity of a Fabaceae plant used in traditional Tanzanian medicine, Rhynchosia viscosa (Roth) DC. Optimization of the workflow with minimal amounts of extract was successfully achieved providing a generic approach that is adaptable for any other sample, even if extracts are only available in milligram amounts (e.g. because the phytochemical analysis is done on a herbarium sample, supply of the extract is difficult or the biological species under investigation is small in size).

2. Results & discussion

2.1 Anti-inflammatory and antiangiogenic activity of Rhynchosia viscosa in zebrafish

Using a zebrafish-based inflammation assay [525], we screened crude methanolic extracts from over 80 East African medicinal plants. The extract of Rhynchosia viscosa (Roth) DC. (Fabaceae) inhibited leukocyte migration in tail-transected four days post-fertilization (4 dpf) larvae in a concentration-dependent manner (Figure III.B.1). The anti-inflammatory effect of the crude extract of R. viscosa was evident at 50 µg/mL – a concentration at which a relative leukocyte migration (RLM) value of 0.39 was achieved (Figure III.B.1D), in comparison with an RLM of 0.24 achieved by 100 µM indomethacin as a positive control (Figure III.B.1E). Interestingly, the ethnomedicinal use of R. viscosa in Tanzania (local name: mfundofundo) includes the topical treatment of inflammatory skin disorders and insect bites (M. J. Moshi, personal communication), prompting us to perform follow-up studies for the identification of anti-inflammatory constituents of this plant.
**Figure III.B.1: Anti-inflammatory activity of the methanolic extract of Rhynchosia viscosa.**

Anti-inflammatory activity was determined in an acute inflammation assay based on tail transection and treatment with lipopolysaccharides (LPS). A to D, zebrafish larvae are 4 days post-fertilization (dpf) with anterior to the left, scale bar = 10 µm. After tail transection and LPS exposure, stained leukocytes appear as black-brown spots migrating to the injured area in the transected tails. Migrating leukocytes were counted on one side in the tail in the region to the right of the dashed red arc and migration values were expressed as relative leukocyte migration (RLM) (E). A, tail of an uncut larva; B, negative control (DMSO 1%); C, positive control (indomethacin 100 µM) D, crude extract of R. viscosa at 50 µg/mL; E, graph displaying the RLM of 4 dpf larvae (n=10) subjected to tail transection and incubation with R. viscosa. RLM ≤ 0.5 was established as cutoff for anti-inflammatory activity. * p < 0.05.

**Figure III.B.2: Antiangiogenic activity of the methanolic extract of Rhynchosia viscosa.**

Inhibition of vascular outgrowth was determined in fli-1:EGFP transgenic embryos. At 16 hours post-fertilization (hpf), embryos were incubated with different concentrations of the methanolic extract of the plant and angiogenic effects were assessed at 48 hpf. A to C, all embryos are 48 hpf, with anterior to the left, scale bar = 10 µm. A, untreated control (DMSO 1%); B, zoom of A (dashed box) showing normal outgrowth of intersegmental vessels (ISV) along the trunk of the larva (arrows); C, embryo treated with 50 µg/mL crude methanolic extract of R. viscosa. Inhibition or reduction of ISV growth is observed along the trunk (arrows); D, IC\textsubscript{50} curve and values showing the inhibitory activity of the methanolic extract of R. viscosa.

In parallel, we also screened the extracts of these East African medicinal plants for their capacity to inhibit angiogenesis, based on their ability to restrict vascular outgrowth in fli-1:EGFP transgenic zebrafish embryos [526], which exhibit vasculature-specific expression of
enhanced green fluorescent protein (EGFP) during embryonic and larval development. In addition to the identification of *Oxygonum sinuatum* (Meisn.) Dammer (Polygonaceae) and *Plectranthus barbatus* Andrews (Lamiaceae) as antiangiogenic extracts [527], we found that the methanolic extract of the aerial parts of *R. viscosa* inhibited intersegmental vessel (ISV) outgrowth in *fli-1*:EGFP embryos in a concentration-dependent manner (Figure III.B.2). In order to rapidly localize the compounds responsible for the bioactivity, high-resolution HPLC-based bioassay-guided fractionation of the extract was performed using the zebrafish vascular outgrowth assay given its higher throughput and lower assay volume compared to the lipopolysaccharide (LPS)-enhanced leukocyte migration assay.

2.2 **Generic chromatographic procedure for optimal one-step microfractionation of NP extracts for the rapid localization of bioactive constituents**

For the rapid isolation and identification of the bioactive constituents of *R. viscosa* we developed a generic chromatographic procedure which combines (1) ultra high pressure liquid chromatography – photo diode array – time of flight mass spectrometry (UHPLC-PDA-TOFMS) for extract profiling, (2) gradient transfer for one-step separation on semipreparative HPLC and (3) microfractionation for a rapid collection of all LC peaks for further bioactivity assessment (Figure III.B.3).

2.2.1 **UHPLC-PDA-TOFMS profiling and dereplication**

Initially, a metabolite profiling at the analytical scale was performed with microgram amounts of crude extract on UHPLC-PDA-TOFMS to evaluate the extract complexity. This method combines high-resolution separation on sub-2 µm particle columns with high-resolution MS detection, which provides molecular formula information for all analytes on-line [157]. For this generic profiling, the separation was achieved on an enriched extract with optimal conditions for maximal peak capacity [528] (Figure III.B.3A). The metabolite profiling revealed a large number of detected peaks to have PDA spectra corresponding to polyphenols with molecular weights ranging from 250 to 450 Da. Most of the PDA spectra were characteristic for either flavones or isoflavones, both known to be present in the Fabaceae family [529]. The high-resolution MS data gained from the UHPLC-PDA-TOFMS analysis provided molecular formula information for all detected LC peaks giving a first overview of the extract composition. This preliminary structural information was later used in combination with the bioassay results for the dereplication of the bioactive constituents.
Figure III.B.3: Generic procedure for the rapid identification of bioactive constituents from medium polar plant extracts.

A, Generic ultra high pressure liquid chromatography – photo diode array – time of flight mass spectrometry (UHPLC-PDA-TOFMS) chromatogram. UHPLC conditions: Acquity BEH C$_{18}$ column (150 × 2.1 mm i.d., 1.7 μm); A: 0.1 vol% formic acid (FA)-H$_2$O, B: 0.1 vol% FA-acetonitrile, 5-95% B in 30'; 0.46 mL/min; ESI-MS detection in negative ion (NI) mode; B, Optimized UHPLC-PDA-TOFMS chromatogram for methanolic extract of R. viscosa. UHPLC conditions: Acquity BEH C$_{18}$ column (100 × 2.1 i.d., 1.7 μm); A: 0.1 vol.% FA-H$_2$O, B: 0.1 vol% FA-methanol (MeOH), 40-90% in 11.4'; 0.306 mL/min, ESI-MS detection in NI mode; C, Semipreparative high performance liquid chromatography (HPLC) chromatogram for the...
microfractionation of the enriched extract of *R. viscosa*. HPLC conditions: XBridge™ BEH C18 column (250 × 10 mm i.d., 5 μm); A: 0.1 vol.% FA-H2O, B: 0.1 vol.% FA-MeOH, 40-90% in 74.9'; 2.3 mL/min; ESI-MS detection in NI mode. The chromatographic gradient is geometrically transferred using mathematical models to obtain a comparable elution of extract constituents. Fractions were collected every 30 s directly into 96-deepwell plates. The so generated microfractions were aliquoted for antiangiogenic screening (10% aliquot A), for fast LC-MS analysis (1%, aliquot B), and for NMR analysis (89%, aliquot C); D, Antiangiogenic screen on 180 microfractions generated by microfractionation. Positive bars show inhibition of angiogenesis of microfractions tested at high concentration; negative bars show inhibition of angiogenesis of selected microfractions at 25 μM. The concentration was determined by quantitative NMR (qNMR) (H); E, Determination of IC50 using the quantitative information obtained by qNMR (H); F, On-line PDA and high-resolution MS information from (A) for the dereplication of plant constituents; G, 1H NMR spectra using the CapNMR™ probe for structure confirmation of bioactive constituents; H, Integration of well resolved aromatic protons for quantification of bioactive constituents to establish the potency of the antiangiogenic and anti-inflammatory activity of the targeted compounds (D, E).

2.2.2 Determination of generic parameters for microfractionation

In order to rapidly determine which compounds were responsible for the bioactivity of the enriched extract, a microfractionation strategy was developed to enable the acquisition of fractions in 96-well plate format with sufficient quantities both for bioactivity testing (antiangiogenic assay) and for structural elucidation (high-resolution MS and 1H NMR analysis) of the bioactive compounds at the analytical level, starting with only a few milligrams of extract. According to the sensitivity of the zebrafish antiangiogenic assay which was deduced from several known antiangiogenic compounds with a range of in vivo potencies in zebrafish including SU5416 and emodin [527], it was estimated that the microfractionation procedure should yield at least 1 μg per well for an initial tracking of the antiangiogenic activity over the entire chromatogram. On the other hand, since compound identification was foreseen based on microflow 1H NMR, it was necessary to keep a minimum of 5 μg for further dereplication. In addition, as the bioassay is carried out in a 96-well plate format that includes controls, the number of fractions had ideally to be 90 or a multiple thereof.

In order to obtain 5-10 μg per microfraction, it was estimated that 1.5 mg of enriched extract would be required. The loading was multiplied by a factor of 10 to ensure that most of the activity could be assessed and minor bioactive constituents could be detected, factoring in the recovery of a given metabolite through microfractionation on reversed phase (RP) columns is ~70% (see Supplementary information Text S1). It was thus estimated that 20 mg of enriched extract would be sufficient for the entire microfractionation procedure and a column with an adapted loading capacity was selected. To minimize sample handling, fractions were collected directly into 96-deepwell plates, facilitating the subsequent drying of all samples at once by vacuum centrifugation, whereas a maximum volume of 1.2 mL of eluent per well had to be respected.

A column geometry of 250 × 10 mm was found to be a good compromise between loading capacity, HPLC resolution and microfraction volumes. In order to fill the deepwells with adequate eluent volumes and collect peaks with sufficient resolution, a fraction collection time of 30 sec and a flow rate of 2.3 mL/min were chosen.
Based on the gradient time constraints of the microfractionation procedure (90 min × 180 microfractions), corresponding gradient time and flow rate were calculated for the analytical UHPLC (gradient time 11.4 min, flow rate 306 µL/min). This was necessary to optimize the gradient for the separation of the NPs in a specific extract at the analytical scale. For a good predictability of the separation efficiency between UHPLC and semipreparative HPLC, the same phase chemistry and columns geometries with similar theoretical peak capacities [67] were chosen (see Materials & methods).

All of these steps are generic, as the procedure is adaptable for any medium-polar extract compatible with RP separation.

2.2.3 Separation optimization specific to *Rhynchosia viscosa* and microfractionation

Since all generic parameters were fixed by the requirements of the bioassay and the structure identification, only the solvent system and the gradient needed to be adapted for profiling. Therefore, the chromatographic gradient method for the microfractionation was optimized on UHPLC-PDA-TOFMS by adapting the generic profiling gradient to maximize mixture component resolution over the run time allowed by the collection. In the case of *R. viscosa*, a linear gradient from 40% to 90% methanol (MeOH) was optimal (Figure III.B.3B) (see Materials & methods). This gradient was directly transferred to the semipreparative system. The enriched extract (19.8 mg) was chromatographed in one step (Figure III.B.3C) and 180 microfractions were generated and collected into 96-deepwell plates. Each microfraction (1.15 mL total volume) was divided into three aliquots: for the zebrafish angiogenesis assay (115 µL, 10% of the total volume, aliquot A); for LC-MS analysis (11.5 µL, 1% of the total volume, aliquot B); and for microflow NMR analysis (ca. 1.12 mL, 89% of the total volume, aliquot C).

2.2.4 Antiangiogenic screen of microfractions

Microfractions were screened for antiangiogenic activity using the zebrafish-based vascular outgrowth assay described above. In an initial screen, 60% of each aliquot A (equivalent to 70 µL of the original 115 µL) was used. Inhibition was observed as the absence or reduction of vascular outgrowth. Microfractions inducing complete inhibition of vascular outgrowth or embryonic toxicity were tested at one third of this concentration (20% of each aliquot A, equivalent to 23 µL of the original 115 µL). This *in vivo* biological profiling revealed six main chromatographic zones containing antiangiogenic compounds at high concentration (30.0-33.0 min, 54.5-55.0 min, 56.5-57.0 min, 60.5-61.0 min, 66.5-68.5 min, 71.5-72.5 min and 79.5 min) (Figure III.B.3D). When testing at the lower concentration, only four zones (30.0-33.0 min, 54.5-55.0 min, 56.5-57.0 min and 60.5-61.0 min) were still active (data not shown). To rapidly identify the constituents responsible for the antiangiogenic activity and to estimate the amount tested in the corresponding microfractions, 1H NMR spectra were recorded using microflow NMR.
2.3 Rapid compound identification in bioactive microfractions

In the first active chromatographic zone, ten consecutive microfractions were found to inhibit angiogenesis (80-100% inhibition of vascular outgrowth at high concentration). The MS data recorded during microfractionation indicated a nominal mass of $m/z$ 269 [M-H]$^-$ for the main compound eluting in this region. The corresponding exact mass recorded during the UHPLC-PDA-TOFMS profiling of the extract was $m/z$ 269.0461 (compound a) indicative of the molecular formula $C_{15}H_{10}O_5$ (calc. $m/z$ 269.0450, Δ 4.1 ppm). This was also validated by application of heuristic filtering [59,65] (see Materials & methods). A cross search with this molecular formula and with chemotaxonomic information (Fabaceae, Leguminosae) in the Dictionary of Natural Products (DNP) [530] revealed that a could correspond to 7,3',4'-trihydroxyflavone or 5,7,4'-trihydroxyisoflavone (genistein). In addition, the PDA spectrum presented an absorption maximum ($\lambda_{\text{max}}$) at 260, 290 sh and 325 sh nm characteristic for isoflavones such as genistein. Compound a was easily confirmed to be genistein (Figure III.B.4) by the comparison of the $^1$H NMR spectrum of the corresponding microfraction obtained by microflow NMR (CapNMR™) with literature values [531]. In all the fractions collected in the 30.0-33.0 min region, the $^1$H signals of genistein were present confirming it to be responsible for the in vivo antiangiogenic activity observed (Figure III.B.3D).

![Figure III.B.4: Antiangiogenic constituents of methanolic extract of Rhynchosia viscosa. Compounds a and c exhibit antiangiogenic and anti-inflammatory activity.](image)

The last two microfractions in this first zone contained another constituent with $m/z$ 299.0549 (compound e) consistent with the molecular formula $C_{16}H_{12}O_7$, and possibly another isoflavone derivative based on the dereplication by TOFMS and PDA (calc. $m/z$ 299.0556, Δ 2.3 ppm, $\lambda_{\text{max}}$ 260, 290 sh, 340 sh nm). The identification of this isoflavone was based on interpretation of the corresponding additional $^1$H signals to those of genistein in this microfraction. The presence of a methoxy substituent ($\delta$ 3.90) was revealed and its position...
at C-3’ was confirmed by comparison with reported data [532]. The molecule was finally identified as 3’-O-methylorobol. Further bioactivity analyses were not undertaken for this constituent as the molecule was not isolated as a pure compound but only in a mixture with genistein.

In the second active zone of the chromatogram, the two microfractions contained one single constituent (compound b) with \( m/z \) 477.1195 ([M-H]⁻, \( C_{26}H_{22}O_{9} \), calc. \( m/z \) 477.1186, \( \Delta \) 1.9 ppm). A database search yielded six NPs with this molecular formula but none were isolated from Fabaceae species, nor were they consistent with the \(^1\)H NMR spectrum of b. The complete structure of this polyphenol could not be determined de novo only based on these data. The compound was named rhynchoviscin and its full structural identification is discussed below in the section “De novo identification of the novel compound b”.

In the third zone, the two microfractions contained another constituent (compound c) with \( m/z \) 351.0886 ([M-H]⁻, \( C_{20}H_{16}O_{6} \), calc. \( m/z \) 351.0869, \( \Delta \) 2.0 ppm) and with aromatic \(^1\)H signals typical of an isoflavone. This molecular formula matched with more than 100 possibilities in DNP and no hypothesis could be deduced. The \(^1\)H NMR spectrum in deuterated methanol (methanol-d₄) was consistent with the configurational isomers licoisoflavone B and sophoraisoflavone A. An additional experiment by re-dissolution of the microfraction in acetone-d₆ confirmed that it was sophoraisoflavone A (Figure III.B.4) by comparison of the \(^1\)H chemical shift of 5-OH (δ 13.07) [533].

In the fourth zone, two microfractions contained one major constituent (compound d) with \( m/z \) 353.1037 ([M-H]⁻, \( C_{20}H_{18}O_{6} \), calc. \( m/z \) 353.1025, \( \Delta \) 3.4 ppm) consistent with prenylated isoflavone derivatives. Beside the aromatic protons characteristic for isoflavones, \(^1\)H signals characteristic for a prenyl group were detected (two methyl signals (δ 1.66 and 1.77) correlating to a vinyl proton (δ 5.25) and further connected to a downfield-shifted methylene group (δ 3.38), as determined by 2D NMR). Comparison of chemical shifts with literature data [534] confirmed d to be licoisoflavone A (Figure III.B.4).

At the high concentration, three more active zones were detected for compounds eluting after 66 min (Figure III.B.3D). No exploitable NMR spectra could be recorded (no aromatic signals were detected) in the corresponding microfractions, and the activity was not seen when tested at the low concentration. These microfractions were not further studied.

### 2.4 Quantification of bioactive molecules and correlation with antiangiogenic activity

To rapidly evaluate the potency of the bioactivity measured, a reliable estimation of the concentration present in each tested microfraction was made. In order to be generic and not have to depend on standards, NMR was used for quantification. Microflow NMR was found to be well-suited for the limited sample amounts present in the microfractions.
2.4.1 Quantitative microflow NMR

For NMR quantification a strategy that does not alter the sample by addition of an internal standard was favored so that any interference with bioassays is avoided. In this respect, a quantitative NMR (qNMR) method using an external calibration (PULCON [121]) was used. Further information on PULCON and the validation of the qNMR method are given in the Supplementary information Text S2.

Overall, the microflow qNMR method (1) provides a universal detection, (2) provides accurate estimation of sample amount in the microgram range without need of any reference compounds, and (3) is compatible with in vivo bioassays enabling fast and reliable identification of bioactive NPs.

2.4.2 Quantification of bioactive constituents of *Rhynchosia viscosa*

The optimized qNMR parameters were used for the acquisition of the $^1$H NMR spectra of *R. viscosa* and thus, within the same experiment, both identification and quantitative information could be obtained for all microfractions displaying antiangiogenic activities. The proton signal chosen for quantification of all the polyphenols corresponded to an aromatic proton signal on cycle B well isolated from interfering signals (Figure III.B.3H). Quantifiable amounts were between 3 and 90 µg per microfraction. A maximum analysis time of 50 min (128 transients) was found to be a good compromise between throughput and detection limits.

For the bioactive compounds (a to d), the microfractions containing the greatest amounts were the following: a (32.5 min, 87 µg), b (54.5 min, 50 µg), c (56.5 min, 35 µg), d (61.0 min, 55 µg). These sub-milligram amounts could be readily converted into precise concentrations for determination of IC$_{50}$ values in the bioassays, since molecular weight in each case was known from the LC-MS results. Thus, even at this stage, a good estimation of the bioactive potency of the unknown compound b could be established.

2.4.3 Assessment of the purity of microfractions by fast UHPLC-PDA-TOFMS

Prior to bioassay analysis and in parallel to NMR analysis, the purity of the microfractions selected for IC$_{50}$ measurements was also determined using a fast UHPLC-PDA-TOFMS analysis using aliquot B kept from the microfractionation (see above). This revealed that the microfractionation generated always at least one microfraction containing only one constituent for compounds a to d. This also validates the reasoning to choose a collection strategy of 30 sec per microfraction.

This indicated that the strategy chosen was able to rapidly generate pure microfractions with well-defined quantities of compounds to be evaluated biologically in the low microgram range.
2.5 Antiangiogenic and anti-inflammatory activity of compounds a to d

In the initial screen of the microfractions, a rapid localization of the bioactive constituents in the extract could be efficiently established (Figure III.B.3D). This screen, however, provides information on how the initial activity of the extracts is distributed among its constituents based on their relative abundance in the extract. Now, since the purity and the amount of each compound in each microfraction is known from qNMR and MS analysis, a reliable evaluation of the potency of the activity could be performed for the determination of IC\textsubscript{50} values.

For this, aliquot C of each microfraction (89% of the original 1.15 ml, which was previously used for NMR analysis) was recovered and used to make a fixed-concentration solution in DMSO to perform a concentration-response analysis and determine IC\textsubscript{50} values for antiangiogenic activity for compounds a to d. Genistein (a) and licoisoflavone A (c) displayed similar levels of potency, with IC\textsubscript{50} values of 24.2 μM and 16.7 μM, respectively. Sophoraisoflavone A (d) and rhynchoviscin (b) were less potent but still clearly antiangiogenic, with IC\textsubscript{50} values of 50.7 μM and 41.3 μM, respectively (Figure III.B.5). All four compounds phenocopied the antiangiogenic effects of the R. viscosa extract in this assay (Figure III.B.2).

![Image of bioactive compounds](image-url)

**Figure III.B.5: Bioactive compounds of Rhynchosia viscosa in the vascular outgrowth assay.** IC\textsubscript{50} curves and values were determined for each of the bioactive constituents of the methanolic extract of R. viscosa. Each compound, at six different concentrations, was assessed for their effect in the inhibition of intersegmental vessel (ISV) growth. A to F, all embryos are 48 hours post-fertilization (hpf), with anterior to the left, scale bar = 10 μm. A, untreated control (DMSO 1%); B, zoom of A (dashed box) showing normal outgrowth of intersegmental vessels (ISV) along the trunk of the larva (arrows); C, embryo treated with 50 μM genistein; D, embryo treated with 100 μM rhynchoviscin; E, embryo treated with 50 μM licoisoflavone A; F, embryo treated with 50 μM sophoraisoflavone A; G, IC\textsubscript{50} curves and values (μM) for each of the bioactive compounds of R. viscosa.
Since the crude extract also exhibited anti-inflammatory activity, compounds \textit{a} to \textit{d} were also assessed using the LPS-enhanced leukocyte migration assay in zebrafish larvae. Moderate but significant inhibition of leukocyte migration was observed for genistein and sophoraisoflavone A at 12.5 and 25 µM (Figure III.B.6B-C). Intriguingly, no significant anti-inflammatory activity was observed for licoisoflavone A or rhynchoviscin, indicating some structure-dependent activity differences between these related compounds (data not shown).

Genistein, an isoflavone synthesized by \textit{Fabaceae} species and usually derived from soybeans, inhibits the tyrosine kinases EGFR (epidermal growth factor receptor), pp60\textsuperscript{v-src}, and pp110\textsuperscript{fag-fes} at pharmacological doses, with negligible effects against serine/threonine kinases such as protein kinase A, protein kinase C, and phosphodiesterase [535]. With regard to its role in inflammation, genistein inhibits LPS-induced nitrite production by cultured macrophages and protects against LPS-induced necrosis by reducing nitric oxide release via the downregulation of inducible nitric oxide synthase [536]. Genistein also inhibits leukocyte-endothelium interaction, thereby modulating vascular inflammation, and reduces reactive oxygen species (ROS) by attenuating the expression of ROS-producing enzymes [537].

\textbf{Figure III.B.6: Anti-inflammatory effect of genistein and sophoraisoflavone A.} 
\textit{A} to \textit{C}, zebrafish larvae are 4 dpf (days post-fertilization) with anterior to the left, scale bar = 10 µm. Migrating leukocytes were counted on one side in the tail in the region to the right of the dashed red arc and migration values were expressed as relative leukocyte migration (RLM) (C). \textit{A}, negative control (DMSO 1%); \textit{B}, genistein 25 µM; \textit{C}, sophoraisoflavone A 25 µM; \textit{D}, graph displaying the RLM in 4 dpf larvae (n=10) after treatment with genistein and sophoraisoflavone A. RLM ≤ 0.5 was established as cutoff for anti-inflammatory activity. * \( p < 0.05 \).

Regarding its role in angiogenesis, genistein as well as other isoflavones are known to inhibit mammalian endothelial cell proliferation and migration \textit{in vitro} [538,539]. \textit{In vivo}, genistein has been found to inhibit angiogenesis in mouse models of melanoma and breast cancer [540].
and to inhibit retinal neovascularization, as well as to downregulate vascular endothelial growth factor (VEGF) and hypoxia-inducible factor (HIF1α) expression, in a mouse model of oxygen-induced retinopathy [541].

To date, no antiangiogenic or anti-inflammatory activity has previously been reported licoisoflavone A and sophoraisoflavone A.

In the initial screen, the inhibition of angiogenesis was dependent on the original amount of each constituent in the extract. The qNMR results enable the correlation of compound amounts with bioactivity. For genistein, the analysis revealed the antiangiogenic activity of each microfraction to correlate well with its calculated amount and thus the bioactivity profile in the initial screen had a direct quantitative link with this compound. For compounds b, c and d, similar activities were observed in the primary screen for microfractions containing these pure compounds, and these results were consistent with the subsequent IC50 analysis for each molecule – indicating the ability of this in vivo approach to identify microgram-level quantities of NPs possessing only moderate levels of bioactivity.

2.6 De novo identification of the novel compound b

During the first phase of dereplication and microfractionation compound b could not be identified. Given that no phytochemical analysis has been reported for R. viscosa and that the antiangiogenic activity of b was moderate, large scale isolation using a MS-targeted fractionation yielded 420 μg of b. The NMR spectra of b obtained from the large scale isolation matched the ones obtained during the first microfractionation. A splitting of some of the NMR signals was indicative of the possible presence of two isomers. Attempts to separate these two isomers using high-resolution isocratic conditions were not fruitful and structure identification was thus performed on the mixture by extensive 2D and 13C NMR spectroscopy (Supplementary information Figure III.B.S3).

Proton and carbon signals were assigned with the help of 1H, COSY, HSQC, HMBC (short and long range) and APT experiments recorded in deuterated DMSO (DMSO-d6).

The 1H NMR spectrum showed signals of two 1H pairs of a 4-oxy-phenyl group at δH 7.20/7.24 (d, J = 8.6 Hz) and 6.74 (d, J = 8.6 Hz), a tetra-substituted phenyl ring with the two proton signals at δH 6.09 (d, J = 1.4 Hz) and 5.96 (d, J = 1.4 Hz), a penta-substituted aromatic ring with a proton at δH 5.94, a dihydrofuran ring substituted by two tertiary methyl and a secondary methyl group (δH 0.94/0.97 (3H, s), 1.18/1.21 (3H, s), 1.24/1.26 (3H, d, J = 6.4 Hz) and 4.40/4.47 (1H, q, J = 6.4 Hz)) and five hydroxyl groups (δH 6.19/6.23 (1H, s), 9.38 (1H, brs), 9.63 (2H, brs) and 12.09 (1H, brs)). These signals were consistent with the skeleton of a benzodihydrofuran fused to a benzodihydropyran with a phenyl ring attached to the junction between furan and pyran ring (Figure III.B.4). This skeleton has been found in biflavonoids from Daphne geraldii [542].

A long-range HMBC experiment showing a correlation between the carbon C-2" and the hydroxyl group 3-OH as well as H-6" and H-4" protons confirmed that the tetra-substituted
ring is linked to the dihydrofuran with the hydroxyl group 3-OH. On the other side, $^3J_{CH}$ HMBC correlations between carbon C-6 with H-8 and the tertiary methyl groups attached the dihydrofuran to the penta-substituted aromatic ring. Its linkage in position 6, 7 (instead of 5, 6) was confirmed by the downfield shift of the hydroxyl proton (5-OH) at $\delta_H$ 12.09 indicating a hydrogen bridge between 5-OH and the carbonyl C-4.

Several peaks (H-5'', H-4'', H-3'', H-2'', H-6'', 3-OH, H-2', H-6') were doubled and the carbon atoms affected were located on the methylated dihydrofuran ring (C-1'' – C-5''), the phenol moiety (C-1' – C-6') and the bridged carbon atoms between the dihydropyran and the dihydrofuran ring (C-2 and C-3). This could indicate that stereoisomerism is located at the bridge between the dihydropyran and the dihydrofuran rings as observed for similar biflavonoids where the structure was established by X-ray on the co-crystals of the stereoisomeric mixture [542]. Thus, b corresponds to a very rare skeleton and this new compound was named rhynchoviscin; its structure as well as the ones of a, c, d and e are given in Figure III.B.4.

3. Conclusion

The known anti-inflammatory and antiangiogenic activities of genistein provide an initial validation of our NP discovery approach. We used in vivo zebrafish-based assays to screen crude plant extracts and subsequently, perform UHPLC-PDA-TOFMS profiling and bioassay-guided microfractionation to isolate the bioactive constituents of R. viscosa. These were then structurally elucidated via high-resolution MS and microflow NMR.

Applying this generic miniaturized procedure, the phytochemical analysis and the generation of microfractions for biological evaluation of an NP extract and its individual constituents is feasible within one day. An initial evaluation of the biological profile of a given NP extract and its constituents is therefore achievable within approximately one week in high-content zebrafish-based bioassays.

This strategy represents a substantial acceleration of the NP-based drug discovery process and allows valuable resources required for the isolation of larger amounts of bioactive molecules for testing in mice to be dedicated only towards extracts having already demonstrated promising bioactivity in vivo at the microgram scale.

The key advantages of this approach are the microgram scale at which both biological and analytical experiments can be performed and the speed and the rationality of the bioassay-guided fractionation, which are generic for NP extracts of diverse origin, and require only limited sample-specific optimization [543]. Moreover, TOFMS and microflow NMR data enable dereplication early in the NP discovery process, and the systematic use of in vivo assays enables the identification of natural products with novel bioactivities that to date could not readily be determined through traditional assays.

In addition to genistein, bioactive constituents of R. viscosa included licoisoflavone A and sophoroisoflavone A – isoflavone derivatives that are structurally closely related. The novel
compound identified by this study, rhynchoviscin, indicates the potential of this integrated approach to also identify bioactive NPs that occur only in limiting quantities, and which have only moderate bioactivity. Overall, these initial results demonstrate the potential of zebrafish bioassay-guided microfractionation, in combination with high-resolution MS and sub-milligram NMR techniques, to rapidly identify bioactive NPs and to quantitatively determine their in vivo bioactivity.

4. Materials & methods

4.1 Ethics statement

Permission to collect *R. viscosa* was granted by the Muhimbili University of Health and Allied Sciences in Dar es Salaam, Tanzania. Permission by local or federal government authorities was not required to collect this species on public land. Furthermore, as *R. viscosa* is not a protected or endangered species, the collection of this species for any purpose, including for scientific research, is not regulated.

All animal procedures were performed in accordance with Belgian and European Laws, guidelines and policies for animal experimentation, housing and care (Belgian Royal Decree of 6 April 2010 and European Directive 2010/63/EU on the protection of animals used for scientific purposes of 20 October 2010). This project was approved by the Animal Ethics Committee of the University of Leuven (approval number P101/2010).

4.2 General experimental procedures

Molar extinction coefficients were determined on a Perkin Elmer UV/VIS Lambda 20 spectrometer and calculated based on the quantities determined by NMR.

4.3 Chemicals & compounds

Solvents used for sample preparation were MeOH from VWR (HiPerSolv CHROMANORM), ultrapure water (Direct-Q 3 UV water purification system, Millipore), and dichloromethane (DCM, VWR). For the HPLC isolation step, solvents were HPLC grade MeOH Chromanorm from VWR, formic acid (FA, 98%) from Fluka and ultrapure water (Millipore). ULC/MS grade MeOH, acetonitrile (MeCN), H₂O and FA (99%) from Biosolve was used for the UHPLC-PDA-TOFMS analyses. For the NMR experiments, methanol-\textit{d}_4 (99.8% atom deuterium), acetone-\textit{d}_6 and DMSO-\textit{d}_6 (99.9% atom deuterium) was obtained from Armar Chemicals and Cambridge Isotope Laboratories Inc. respectively. Genistein (99% pure) was obtained from Acros Organics and maleic acid (ReagentPlus® >99.0%) from Sigma-Aldrich. For the bioassays, 1-phenyl-2-thiourea (PTU) and tricaine (ethyl 3-aminobenzoate) were purchased from Sigma-Aldrich, DMSO from Acros Organics.

4.4 Plant material, extraction, prepurification

*Rhynchosia viscosa* (Roth) DC. was collected on public land in Tabora, Tanzania and a voucher specimen (number HOS 3119) was deposited at the Faculty of Pharmacy of the Muhimbili
University of Health and Allied Sciences (MUHAS), Dar es Salaam, Tanzania. The plant material was dried at room temperature and ground. The dry, powdery plant sample was exhaustively extracted with MeOH by maceration. The dry methanolic extract was obtained after removing the solvent by evaporation under reduced pressure. Prior to testing, an aliquot of the dry methanolic extract was suspended in 100% DMSO; this stock solution was then kept at -20 °C.

The crude methanolic extract of *R. viscosa* was dissolved in 80% aq. MeOH and purified by SPE (ZEOprep 60, C18, 40-63 μm, Zeochem AG) using 80% aq. MeOH. Then, the sample was solubilized in 95% aq. MeOH and eluted over a polyamide-filled cartridge with 95% aq. MeOH that was pre-conditioned with MeOH and 95% aq. MeOH [544] to remove tannins from the extract. The sample was evaporated to dryness under reduced pressure and a reddish solid as well as an orange oil was obtained. This sample was extracted with DCM for enrichment and the remaining part was used for microfractionation.

### 4.5 Microfractionation by semipreparative LC-MS

The enriched extract (19.8 mg) was redissolved in pure MeOH, filtered over a 0.45 μm Nylon 66 syringe filter (BGB Analytik AG) and fractionated by means of semipreparative HPLC. The gradient method was transferred using HPLC Calculator v3.0 [67]. The separation was accomplished on a Varian modular HPLC system with a Varian 9012 pump coupled through a Thermo Scientific electrospray ionization (ESI) interface to an ion trap mass spectrometer instrument (LCQ, Thermo Scientific) and a UV detector (at 254 nm, 2151 variable wavelength monitor, LKB Bromma) to monitor the separation. A splitter enabled 50 μL/min of the flow coming from the HPLC to enter the mass spectrometer. The following negative ionization-ESI (NI-ESI) conditions were used: capillary temperature, 200 °C; capillary voltage, -38 V; spray voltage, 3 kV; tube lens offset, -3 V. The acquisitions were performed in NI mode using a full scan mode over an *m/z* range of 150–1000. An in-source fragmentation energy of 5 V was applied. The separation was performed on a 250 × 10 mm i.d., 5 μm, XBridge™ BEH C18 column (Waters) in gradient mode at 2.3 mL/min with the following solvent system: A = 0.1 vol% FA-H2O, B = 0.1 vol% FA-MeOH; 40% B for 3.4 min and 40-90% B in 74.7 min and 90% B for 12 min. The injected volume was 500 μL. Fractions of 1.15 mL were collected every 30 s with a Gilson FC204 Fraction Collector directly into conical-bottom 96-deepwell plates (VWR). An aliquot of each microfraction (115 μL; 10% of the total microfraction volume, aliquot A) from the semipreparative isolation step was transferred to a 96-well plate (Nunc, V96, PP, 0.45 mL), dried in a vacuum centrifuge (Genevac HT-4X, Genevac Inc.) and used for bioactivity testing in zebrafish. Another aliquot of each microfraction (11.5 μL; 1% of the total microfraction volume, aliquot B) was transferred to a 96-well plate (Nunc, V96, PP, 0.45 mL), diluted to 200 μL with 85% aq. MeOH, sealed and stored at 5 °C for further purity check by UHPLC-PDA-TOFMS.

### 4.6 UHPLC-PDA-TOFMS experiments

UHPLC-PDA-TOFMS analyses were performed using an Acquity™ UPLC chromatograph and a Micromass-LCT Premier Time of Flight mass spectrometer equipped with an ESI interface.
(Waters). For the profiling of the crude extract, analyses on the generic gradient method were performed using a 150 × 2.1 mm i.d., 1.7 μm, Acquity BEH C_{18} UPLC column (Waters). For the optimized gradient method, a 100 × 2.1 mm i.d., 1.7 μm, Acquity BEH C_{18} UPLC column (Waters) was used and for the verification of the purity and identity of the microfractions, a short analysis was performed on a 50 × 2.1 mm i.d., 1.7 μm, Acquity BEH C_{18} UHPLC column (Waters). The analysis conditions are given in detail in the Supplementary information Text S1.

4.7 Dereplication procedure

The procedure published by Funari et al. [59] was used for the dereplication of compounds in the crude extract and identification of the isolated compounds. For the database search (DNP, SciFinder), hits were refined by searching for compounds isolated from Fabaceae species. More details on the dereplication procedure are given in the Supplementary information Text S1.

4.8 Quantitative microflow NMR measurements

NMR spectra of the microfractions were recorded on a Varian INOVA 500 MHz NMR instrument at 25 °C, equipped with a microflow NMR probe (CapNMR™) and an automated sample injection unit (One Minute-NMR™) from Protasis. Remaining amounts of microfractions (89% of the total microfraction volume, aliquot C) were diluted in 10 μL of methanol-\text{d}_4 whereof 8 μL were injected.

For the quantitative studies, the relaxation delay $T_1$ was experimentally determined for all protons of genistein to choose the recycle delay for qNMR acquisition and to determine the $^1$H signals suitable for quantification. The protons on cycle B ($T_1 = 2.0 - 2.3$ s) and C ($T_1 = 2.7$ s) were fully recovered (time > 5×$T_1$) within a recycle delay of less than 15 s, whereas the protons on cycle A were only fully recovered after 25 s. A recycle delay of 20 s was set for qNMR experiments and well resolved $^1$H signals on cycle B were chosen for quantification. The optimal pulse width at 90° was arrayed (at 360°) for every individual sample and lays between 4.1 and 4.2 μs. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased, baseline corrected using a 1st order polynomial function and calibrated to the residual methanol peak at 3.31 ppm using MestReNova (version 6.01, Mestrelab Research S.L.) The signals were integrated manually and the concentration was determined using PULCON [121]. Maleic acid was used as external standard.

4.9 Zebrafish

The transgenic line $fli-1$:EGFP [526] was obtained from the Zebrafish International Resource Center at the University of Oregon (Eugene, Oregon, USA). Zebrafish husbandry, embryo collection, and embryo and larva maintenance were performed as previously described [545,546]. For the leukocyte migration assay, zebrafish embryos at one day post fertilization (dpf) were exposed to PTU to suppress melanization (Supplementary information Text S1). For
this assay and for confocal imaging, larvae were anesthetized with tricaine (Supplementary information Text S1).

The leukocyte migration assay was performed in 24-well microtiter plates using ten 4 dpf larvae per well in 1 mL of Danieau’s medium (Supplementary information Text S1). The vascular outgrowth assay was performed in 96-well microtiter plates using five embryos at 16 hours post-fertilization (hpf) per well in 200 µL of Danieau’s medium. Extracts and compounds were solubilized in DMSO, and were added to the Danieau’s medium up to a maximum DMSO concentration of 1%.

4.10 Anti-inflammatory assay
Prior to assessment of the anti-inflammatory activity of *R. viscosa* and its derivatives, *in vivo* toxicological tests were performed to establish the maximum tolerated concentration of each sample (Supplementary information Text S1). Next, a LPS-enhanced leukocyte migration assay was performed. Briefly, larvae were pre-incubated (1 hour at 28 °C, ± 0.5) with specific concentrations of each sample. Negative controls, containing only vehicle (1% DMSO), and positive controls, indomethacin 50-100 µM, were processed in parallel. After pre-incubation, larvae were anesthetized and subjected to complete tail transection made 0.5 mm (± 0.2) from the tip of the tail of each larvae under microscopy light (Carl Zeiss Stemi 2000C) using a scalpel. Next, tail-cut larvae were briefly rinsed in Danieau’s medium without tricaine and incubated for seven hours with specific concentrations of each sample containing 10 µg/mL LPS (*Salmonella typhosa* ATCC 10749, Sigma-Aldrich). After this incubation, larvae were fixed in 4% paraformaldehyde and kept overnight at 4 °C. Fixed larvae were gently washed with PBST (PBS-1X phosphate buffered saline, Gibco + 0.1% Tween 20) and next subjected to incubation (15 minutes at room temperature) with 1 mL of freshly prepared staining solution (Leucognost® Pox, Merck). Evaluation of the migrating leukocytes to the injured region was done in one side of each larva under light microscopy and scoring of the migration was assessed according to a 5-point index of staining intensity. The average of these values for each experimental group were normalized against the average values of the control group (1% DMSO) and expressed as RLM, which for significant anti-inflammatory activity has a cutoff point of RLM ≤ 0.5. All experiments were performed in duplicate, with ten larvae per condition. Statistical analysis was done using GraphPad Prism 5 software using one-way analysis of variance (ANOVA).

4.11 Angiogenesis assay
Prior the initiation of ISV outgrowth, *fli-1:EGFP* embryos at 16 hpf were incubated (32 hours at 28°C, ± 0.5) with specific concentrations of extracts and compounds. Negative controls, containing only vehicle (1% DMSO) were processed in parallel. The microfraction samples for biological profiling (aliquot A of each microfraction) were dried, re-solubilized in 3 µL DMSO and diluted to 150 µL with Danieau’s medium, of which 90 µL were used for a first screen. Microfractions with 100% inhibitory activity or exhibiting toxicity were tested at a lower concentration (1/3 of the initial concentration).
Inhibition of vascular outgrowth along the trunk of every larva was evaluated under UV microscopy light (MZ10F Leica stereo microscope) at 48 hpf and scoring of antiangiogenic activity was done according to a 5-point index for vascular outgrowth. The average of the values for each experimental group was normalized against the average of the values of the control group (1% DMSO), yielding a relative vascular outgrowth (RVO) score that was then expressed as percentage of inhibitory activity. All experiments were performed in duplicate, with five larvae per condition. Statistical analysis and IC_{50} curves were done using GraphPad Prism 6 software using nonlinear regression to fit the data to the log (inhibitor) vs. response curve (variable slope). Representative embryos were subjected to confocal imaging (see below).

4.12 Confocal imaging

Confocal imaging (Figure III.B.2 and III.B.5) was carried out using a Nikon A1R confocal unit (Nikon) mounted on a Ti2000 inverted microscope (Nikon). For the imaging, 4x (0.2 N.A.) and 10x (0.45 N.A.) lenses were used. For detecting the fluorescence of the fish embryos, a 488 nm laser line (CVI Melles Griot) and detection filters for the range of 515-550 nm were used. Confocal stacks of the whole fish or the depicted regions were acquired and projections of the maximum intensity of the 3D volume shown. During imaging, zebrafish embryos were anesthetized using 0.1 mg/mL tricaine in Danieau’s medium.

4.13 Novel compound from *Rhynchosia viscosa* with antiangiogenic activity

Rhynchoviscin (b). Insufficient material was available to obtain an optical rotation value. Purity: 80% (determined by NMR). UV (MeOH) \( \lambda_{\text{max}} \text{ (log } \varepsilon) \) 304 nm (4.39); \(^1\)H NMR (DMSO-d\(_6\), 500 MHz, CapNMR™ probe, \( \delta_H \)): 0.94/0.97 (3H, s, H-5'''), 1.18/1.21 (3H, s, H-4'''), 1.24/1.26 (3H, d, J = 6.4 Hz, H-3'''), 4.40/4.47 (1H, q, J = 6.4 Hz, H-2'''), 5.90 (1H, d, J = 1.4 Hz, H-4''), 5.99 (1H, s, H-8), 6.03/6.04 (1H, d, J = 1.4 Hz, H-6''), 6.19/6.23 (1H, s, 3-OH), 6.74 (2H, d, J = 8.6 Hz, H-3'/H-5'), 7.20/7.24 (2H, d, J = 8.6 Hz, H-3'/H-5'), 9.38 (1H, brs, 4'-OH). \(^{13}\)C NMR (DMSO-d\(_6\), 500 MHz, CapNMR™ probe, \( \delta_C \)): 13.8/14.3 (C-3'''), 20.8 (C-5''), 24.5/25.4 (C-4''), 42.6 (C-1''), 80.3 (C-3), 90.4 (C-6''), 90.4/90.6 (C-2''), 91.4/91.5 (C-8), 97.3 (C-4''), 99.8 (C-4a), 105.6 (C-2''), 113.0 (C-6), 114.5 (C-3'/C-5'), 117.1 (C-2), 124.6 (C-1'), 127.9/128.1 (C-2'/C-6'), 155.5 (C-3''), 158.2 (C-4'), 161.0 (C-5''), 161.5 (C-1''), 163.9 (C-8a), 167.0 (C-7), 192.8 (C-4). ESI-MS (NI mode): m/z 477.1195 [M-H]⁻ (C\(_{26}\)H\(_{22}\)O\(_9\), calc. m/z 477.1186, Δ 1.9 ppm).

Detailed structure information on compound a, c, d and e can be found in the Supplementary information (section 5.1.4). NMR spectra for rhynchoviscin are given in Supplementary information (section 5.3).

5. Acknowledgments

We gratefully acknowledge Philippe J. Eugster for the acquisition of UHPLC-PDA-TOFMS data and for assistance on dereplication, and the Aquaculture Core Facility of the Biomedical
6. **Supporting information**

6.1 **Supplementary materials & methods**

6.1.1 **UHPLC-PDA-TOFMS experiments**

UHPLC-PDA-TOFMS analyses for the profiling of the crude extract were performed using an Acquity™ UPLC chromatograph and a Micromass-LCT Premier Time of Flight mass spectrometer equipped with an ESI interface (Waters). For the generic gradient method, detection was performed in positive ionization (PI) and NI mode in the range m/z 100-1000 in centroid mode with a scan time of 0.3 s. ESI conditions were: capillary voltage 2800 V, cone voltage 40 V, MCP detector voltage 2450 V, source temperature 120 °C, desolvation temperature 300 °C, cone gas flow 20 L/h, and desolvation gas flow 800 L/h. The mass spectrometer was internally calibrated by infusion of a solution of leucine-enkephalin (2 μg/mL, Sigma-Aldrich) through the lockmass spray probe at a flow rate of 10 μL/min, using a second Shimadzu LC-10ADvp LC pump. The separation was performed on a 150 × 2.1 mm i.d., 1.7 μm, Acquity BEH C18 UPLC column (Waters) in the gradient mode at a flow rate of 0.46 mL/min with the following solvent system: A = 0.1 vol% FA-H2O, B = 0.1 vol% FA-MeCN; 5–95% B in 30 min. The injected volume was 2 μL. The PDA traces were recorded from 210 to 450 nm.

For the optimized gradient method, detection was performed in negative ion (NI) mode as above. ESI conditions in NI mode were: capillary voltage 2800 V, cone voltage 40 V, MCP detector voltage 2400 V, source temperature 120 °C, desolvation temperature 300 °C, cone gas flow 20 L/h, and desolvation gas flow 700 L/h. The separation was performed on a 100 × 2.1 mm i.d., 1.7 μm, Acquity BEH C18 UPLC column (Waters) in the gradient mode at a flow rate of 0.306 mL/min with the following solvent system: A = 0.1 vol % FA-H2O, B = 0.1 vol % FA-MeOH; 40–90% B in 11.4 min. The injected volume was 1 μL.

For the verification of the identity and purity of the microfractions, a short analysis was performed on a 50 × 2.1 mm i.d., 1.7 μm, Acquity BEH C18 UHPLC column (Waters) in the gradient mode at a flow rate of 0.3 mL/min with the following solvent system: A = 0.1 vol% FA-H2O, B = 0.1 vol% FA-MeCN. The injected volume was 1 μL, 1% of the microfraction was taken and diluted to 200 μL with H2O:MeCN 1:1 + 0.1 vol% FA.

6.1.2 **Dereplication procedure**

The procedure published by Funari et al. [59] was used for the dereplication of compounds in the crude extract and identification of the isolated compounds. Briefly, possible molecular
Plant constituents

formulae were calculated in MassLynx™ (Waters) using a mass tolerance of 15 ppm, allowing atoms C, H, O and N with no restrictions. These formulae were refined by applying heuristic filtering (seven golden rules [65]) and only molecular formula reported from natural origin and isolated from plant sources were considered. Furthermore, database hits were refined by searching for compounds isolated from Fabaceae species. For molecules exhibiting the typical isoflavone PDA absorption, the isoflavone skeleton was used to restrict the database search (DNP, SciFinder).

6.1.3 Zebrafish procedures

Danieau’s medium

Danieau’s medium was used for maintenance of newly collected zebrafish embryos and for all experimental incubations. A stock solution (30X consisting of 1.74 M NaCl, 21 mM KCl, 150 mM Hepes buffer (pH 7.1-7.3), 18 mM Ca(NO₃)₂ and 12 mM MgSO₄) was prepared in advance and kept at room temperature. The working solution – 0.3X Danieau’s medium – prepared as a 1/100 dilution of the stock solution in ultrapure water, included methylene blue (0.03M) to avoid fungus and bacterial growth. This working medium was kept at room temperature.

Treatment with 1-phenyl-2-thiourea (PTU)

For inhibition of melanocytes that can interfere with visual assessment of the stained migrating leukocytes, zebrafish embryos were treated with PTU. A stock solution (2 mM PTU) was prepared in ultrapure water in advance and kept at room temperature until needed. At one day post-fertilization, 1/10 dilution of the stock solution in Danieau’s is used as a medium for embryos maintenance. To ensure proper inhibition of melanocytes, PTU was exchanged daily until the tail cut assay was performed at four days post-fertilization.

Treatment with ethyl 3-aminobenzoate (tricaine)

To reduce animal discomfort during experimental procedures, zebrafish larvae were anesthetized using tricaine. A stock solution (10 mg/mL tricaine) was prepared in ultrapure water in advance and kept at 4°C until needed. Larvae subjected to tail cut and to confocal imaging were immersed in 1/100 dilution of the stock solution in Danieau’s medium.

Toxicological evaluation

Prior to assessment of the anti-inflammatory activity, in vivo toxicological tests were performed to establish the maximum tolerated concentration of each sample. Zebrafish larvae at 4 dpf were treated with different concentrations of the extract and isolated compounds, and incubated at 28 °C (± 0.5). For the next eight hours, hourly microscopic examination of the larvae was done to determine signs of toxicity, e.g. cardiovascular defects (arrhythmia or decreased circulation), balance defects (loss of posture), locomotor defects (decreased touch response) or death. Concentrations inducing any of these effects were not considered for testing anti-inflammatory activity.
6.1.4 Isolated compounds from *Rhynchosia viscosa* with antiangiogenic activity

Genistein (a). $^1$H NMR (methanol-$d_4$, 500 MHz, CapNMR™ probe, $\delta_{HH}$): 6.23 (1H, d, $J = 2.1$ Hz, H-6), 6.34 (1H, d, $J = 2.1$ Hz, H-8), 6.85 (2H, d, $J = 8.8$ Hz, H-3'/H-4'), 7.37 (2H, d, $J = 8.8$ Hz, H-2'/H-5'), 8.05 (1H, s, H-2). ESI-MS (NI mode): $m/z$ 269.0461 [M-H]$^-$ (C$_{15}$H$_{10}$O$_5$, calc. $m/z$ 269.0450, $\Delta$ 4.1 ppm). These data were identical to literature values [531] and those obtained on a commercial sample.

Sophoraisoflavone A (c). UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$) 259 nm (4.78); $^1$H NMR (methanol-$d_4$, 500 MHz, CapNMR™ probe, $\delta_{HH}$): 1.38 (6H, s, H-5''/H-6''), 5.60 (1H, d, $J = 9.9$ Hz, H-3''), 6.23 (1H, d, $J = 2.1$ Hz, H-6), 6.36 (1H, d, $J = 2.1$ Hz, H-8), 6.40 (1H, d, $J = 8.3$ Hz, H-5'), 6.68 (1H, d, $J = 9.9$ Hz, H-2''), 6.95 (1H, d, $J = 8.3$ Hz, H-6''), 7.93 (1H, s, H-2). ESI-MS (NI mode): $m/z$ 351.0876 [M-H]$^-$ (C$_{20}$H$_{16}$O$_6$, calc. $m/z$ 351.0869, $\Delta$ 2.0 ppm).

Licoisoflavone A (d). $^1$H NMR (methanol-$d_4$, 500 MHz, CapNMR™ probe, $\delta_{HH}$): 1.66 (3H, s, H-4''), 1.77 (3H, s, H-5''), 3.38 (2H, s, H-1''), 5.25 (1H, m, H-2''), 6.27 (1H, d, $J = 2.1$ Hz, H-6), 6.39 (1H, d, $J = 2.1$ Hz, H-8), 6.43 (1H, d, $J = 8.3$ Hz, H-5''), 6.85 (1H, d, $J = 8.3$ Hz, H-6''), 8.05 (1H, s, H-2). $^{13}$C NMR (methanol-$d_4$, 125 MHz): $\delta$ 16.8 (C-5'''), 22.5 (C-1''), 24.8 (C-4'''), 93.7 (C-8), 99.3 (C-6), 107.6 (C-5''), 123.3 (C-2''), 128.1 (C-6''), 130.3 (C-3''), 154.5 (C-4''), 155.8 (C-2), 182.2 (C-4). ESI-MS (NI mode): $m/z$ 353.1037 [M-H]$^-$ (C$_{20}$H$_{18}$O$_6$, calc. $m/z$ 353.1025, $\Delta$ 3.4 ppm).

3’-O-Methylorobol (e). $^1$H NMR (methanol-$d_4$, 500 MHz, $\delta_{HH}$): 3.90 (3H, s, 3’-O-CH$_3$), 6.23 (1H, d, $J = 1.9$ Hz, H-6), 6.36 (1H, d, $J = 1.9$ Hz, H-8), 6.86 (1H, d, $J = 8.2$ Hz, H-5''), 6.97 (1H, dd, $J = 8.2$, 1.2 Hz, H-6''), 7.16 (1H, d, $J = 1.2$ Hz, H-2''), 8.10 (1H, s, H-2). ESI-MS (NI mode): $m/z$ 299.0549 [M-H]$^-$ (C$_{16}$H$_{12}$O$_7$, calc. $m/z$ 299.0556, $\Delta$ 2.3 ppm).

6.2 Supplementary information on quantitative microflow NMR

For NMR quantification, a strategy which does not alter the sample by addition of an internal standard was favored so that any interference with bioassays is avoided. In this respect, a quantitative NMR (qNMR) method using an external calibration (PULCON [121]) was used. PULCON correlates the absolute intensities of $^1$H signals in two spectra measured in different solution conditions. Therefore, the exact pulse length (360° radio frequency pulse) was determined for every sample to account for inter-sample differences. The microflow NMR setup that includes a sample injection module is particularly suited for qNMR by PULCON. Indeed, microflow probes are known to provide high stability of the field homogeneity as the geometry of the sample is fixed and the sample fills the radio frequency coil the same way each time [547] ensuring a stable pulse width from one sample to the other. Furthermore, the automated injection makes the loading of the sample more reproducible which increases the accuracy of the quantification. The longitudinal relaxation time ($T_1$) for the protons of the various polyphenols was estimated by an inversion recovery experiment on different types of aromatic protons on a model isoflavone to ensure reliable quantification results (see Materials & methods, 4.8).
To verify that the method was suitable for estimation of amounts collected by microfractionation, an extract of *Lupinus albus*, known to contain genistein, was selected and microfractionated. Genistein was collected in a single microfraction and quantification results obtained by qNMR on this fraction were compared to standard HPLC-UV quantification of the corresponding LC peak in the extract. The results obtained by qNMR were 30% lower than the amount injected on column which can be explained by the recovery yield of the microfractionation procedure. The estimation of the amount by PULCON compared to a conventional method using an internal standard were similar, the latter being slightly more precise.

6.3 NMR spectra (\(^1\)H, APT, HSQC, HMBC) of rhynchoviscin

![NMR Spectrum](image)

*Figure III.B.S1: \(^1\)H NMR spectrum of rhynchoviscin (b) in DMSO-d₆ (500 MHz).*
Figure III.B.S2: APT spectrum of rhynchoviscin (b) in DMSO-d$_6$ (500 MHz).
Figure III.B.S3: 2D NMR spectrum (HSQC) of rhynchoviscin (b) in DMSO-d$_6$ (500 MHz).

Figure III.B.S4: 2D NMR spectrum (gHMBC) of rhynchoviscin (b) in DMSO-d$_6$ (500 MHz).
Fungal metabolites
This second part of the thesis (chapter IV to VI) is dedicated to the detection, isolation and structural identification of fungal metabolites. In a first step, workflows for the sensitive detection of co-culture-induced metabolites were developed based on metabolomic approaches. A large number of co-cultures (138) were screened for their capacity to induce the production of additional secondary metabolites. This is described in chapter IV [31].

In chapter V, the microfractionation procedure developed for plant extracts (chapter III) was applied to the isolation of fungal metabolites. Induced metabolites were isolated from a co-culture involving the two onychomycosis-derived strains *Fusarium oxysporum* and *Acremonium strictum* (chapter V.A).

As a last study, induced metabolites from the co-culture of *Hohenbuehelia reniformis* and *Fusarium solani* were studied by metabolomics with various multivariate data analysis methods to highlight induction phenomena. The co-culture extract had antifungal activity against the clinical isolate *F. solani*. Therefore, milligram-amounts of pure co-culture constituents were isolated from a large-scale co-culture for the identification of the co-culture-induced metabolites and anti-*Fusarium* compounds (chapters VI.A, B and C).
IV. Detection of co-culture induced fungal metabolites

IV.A Detection of metabolite induction in fungal co-cultures on solid media by high-throughput differential ultra high pressure liquid chromatography–time-of-flight mass spectrometry fingerprinting

This chapter was published as research article.


* These authors contributed equally to this work.

In the article presented as chapter IV.A, the term mono-culture was used to describe pure cultures. This term is equivalent to pure culture that was used in the other chapters throughout the thesis manuscript.

Contribution: Design of experiments, execution of preliminary work, supervision of students that were working on this project
Abstract

Access to new biological sources is a key element of natural product research. A particularly large number of biologically active molecules have been found to originate from microorganisms. Very recently, the use of fungal co-culture to activate the silent genes involved in metabolite biosynthesis was found to be a successful method for the induction of new compounds. However, the detection and identification of the induced metabolites in the confrontation zone where fungi interact remain very challenging. To tackle this issue, a high-throughput UHPLC-TOFMS-based metabolomic approach has been developed for the screening of fungal co-cultures in solid media at the Petri dish level. The metabolites that were overexpressed because of fungal interactions were highlighted by comparing the LC-MS data obtained from the co-cultures and their corresponding mono-cultures. This comparison was achieved by subjecting automatically generated peak lists to statistical treatments. This strategy has been applied to more than 600 co-culture experiments that mainly involved fungal strains from the *Fusarium* genera, although experiments were also completed with a selection of several other filamentous fungi.

This strategy was found to provide satisfactory repeatability and was used to detect the biomarkers of fungal induction in a large panel of filamentous fungi. This study demonstrates that co-culture results in consistent induction of potentially new metabolites.

**Keywords:** fungal interactions, *Fusarium*, solid media, co-culture profiling, metabolomics, UHPLC-TOFMS
1. Introduction

Natural products (NPs) obtained from microorganisms are a historical source of lead compounds [75], but the attractiveness of such NPs is diminished because of the difficulties involved in working with complex mixtures [2] and because of the continual rediscovery of the same bioactive chemical structures in pharmacological screens despite the existence of dereplication processes [123]. Such continual rediscovery is inconsistent with the tremendous diversity of the cryptic biosynthetic pathways dedicated to the production of secondary metabolites, which have been revealed by genome sequencing programs [9,262,266]. Recently, a variety of strategies have been proposed for the activation of the production of these compounds. One such strategy is the co-cultivation of two microorganisms in a single environment, which is referred to as interspecies crosstalk [253,256,266,309].

Co-culturing exploits the fact that fungi have evolved highly specialised capacities to occupy environments, such as soil, rhizospheres, plants, mucosal membranes and guts, which also support dense populations of other microorganisms [548]. Interactions between microorganisms lead to the activation of complex regulation mechanisms, which results in the biosynthesis of highly diverse NPs [256], such as pheromones, defence molecules and metabolites that are involved in symbiotic associations [450]. Investigations into co-culture experiments with fungi have been limited, but co-culture has a high potential for the generation of enhanced chemical diversity, an advantage that has been demonstrated in both liquid [309,391,392,415,419,429,446,453,484,549] and solid co-cultures [30,342,383,424,450]. For example, emericellamide A and B were induced during a liquid co-culture of the marine-derived fungus *Emericella* sp. with the marine actinomycete *Salinispora arenicola* [392]. Such stress-induced molecules exhibit specific noteworthy bioactivities, and some of them have been found to have antimicrobial [30,392,415,484,549], anticancer [391,446] and phytotoxic activities [30].

For practical reasons, the large-scale production of microbial metabolites is usually accomplished in liquid reactors as they are amenable to easy control of temperature, pressure and atmospheric composition [550,551]. However, solid-media cultures are more similar to the environmental conditions in which fungi evolved. Furthermore, these cultures have been found to yield a substantially higher number of metabolites from diverse microorganisms [402] and references therein]. In addition, solid media cultures facilitate visualisation of the interaction zone between the fungi [30,342], which allows for growth rate estimation. This also allows the excision of well-defined parts of the agar media for further analysis of specific zones of the confrontation, an advantage for metabolite localisation studies. Recent developments in scaling up microbial cultures [32] have opened the door to the study of microbial interactions on solid media for the purpose of drug discovery.

To monitor stress-related metabolome modifications in microorganisms, hyphenated analytical methods, such as liquid or gas chromatography coupled to mass spectrometry (LC-MS and GC-MS), are well suited because they can separate metabolites from crude extracts.
and provide spectroscopic information for full or partial chemical identification [123]. Only a few profiling studies of fungal interactions have been performed to delineate interspecies crosstalk [30,342,424,450]. GC-MS was applied to study the up- and down-regulation of metabolites in co-culture involving Stereum and Coprinus species. This analytical technique enabled molecule identification through database searches based on fragmentation patterns and retention indexes [342]. Several LC-MS studies have applied direct comparisons of monocultures with their corresponding co-cultures [30,391,392]. LC-MS can analyse extracts from microorganisms with minimal sample preparation. Compared to GC-MS, however, the LC-MS dereplication process often generates putative peak annotations only. Nevertheless, the localisation of induced compounds in LC-MS chromatograms enables targeted micro-isolation of such compounds and subsequent de novo identification by NMR [71]. This method also has the potential to assess the bioactivity of a given up-regulated metabolite. Recently, direct MALDI-imaging mass spectrometry experiments have revealed the production of high molecular weight (MW) metabolites at the interaction zone between confronting bacteria [432,552].

For metabolite profiling of complex matrices, ultra high-pressure liquid chromatography coupled to electrospray ionisation and time-of-flight mass spectrometry (UHPLC-TOFMS) is the latest cutting-edge technology [457]. UHPLC-TOFMS combines high LC and MS resolutions, high throughput and good reproducibility of the LC-MS data sets, an advantage that is important for data comparison in metabolomics studies [459,553]. This analytical platform has demonstrated its utility for high-throughput fingerprinting of a large set (> 15’000) of microbial extracts [458].

In this work, UHPLC-TOFMS fingerprinting was used to screen a large number of co-culture experiments for any chemical induction of low MW metabolites occurring in fungal co-cultures grown on solid media. Multivariate statistical treatments were applied to the UHPLC-TOFMS profiles to highlight compounds that were specifically induced because of the interactions between colonies of filamentous fungi. The results of this screening indicate that a large majority of fungi produce new substances when confronted with other fungi on solid media. The significance of these findings in the context of natural product research is discussed below.

2. Experimental

2.1 Biological material

The strains of fungi used in this study were clinical, soil or plant-derived isolates. Fusarium strains isolated from the difficult-to-treat onychomycosis were progressively collected at the Centre Hospitalier Cantonal Vaudois (CHUV, Lausanne Switzerland) and stored in the database of Agroscope ACW in vials containing diluted potato dextrose broth (PDB) solution (1:4) at 4°C (Table IV.A.S1 and http://mycoscope.bcis.ch/). Other fungal species from the collection that were representative of contrasting ecological niches were also added to the list. Soil isolates utilised in this study included Aspergillus clavatus, which is known to produce toxins and
bioactive compounds [554], Cladosporium sp. and Hohenbuehelia reniformis. Bionectria ochroleuca and Eutypa lata were also included as representatives of plant endophytes with the capability to degrade wood. Both of these fungi are able to form dark-brown substances upon interaction with other fungi (unpublished data and [30]). Two non-Fusarium species that cohabit with Fusarium in human nails [362] and that were isolated from onychomycosis were also included in this study. The first of these was an Acremonium strain known for its capacity to produce uncommon chlorinated metabolic compounds [555], and the second was a dermatophytic Trichophyton rubrum strain.

2.2 Chemicals

Extractions were performed with methanol (HPLC grade), isopropanol (HPLC grade), t-butanol (PA grade), dichloromethane (HPLC grade), and nano-pure water (Millipore). UHPLC-TOFMS analyses were performed using ULC/MS-grade acetonitrile and a mixture of water and formic acid (FA) from Biosolve (Valkenswaard, The Netherlands).

2.3 Culture and co-culture conditions

For mono-cultures, a 5-mm agar plug of a fungal pre-culture was inoculated in the centre of a 9-cm Petri dish containing 30 mL of potato dextrose agar media (PDA, Difco, BD & Co, Le pont de Claix, France). The Petri dishes were incubated at 21 °C.

Similarly, co-culture experiments were inoculated with two 5-mm agar plugs of the appropriate fungal strains on opposite sides of a Petri dish containing agar media (PDA), and the Petri dishes were incubated at 21 °C.

2.4 Extraction procedure

The confrontation zones of fungal co-cultures were excised with a razor blade as 1x1 cm pieces of agar and then freeze-dried. The dry material was transferred into an extraction vessel with 20 mL of solvent per 300 mg of dry material. The extraction properties of the following solvents were compared: methanol, isopropanol, t-butanol, dichloromethane/methanol/water (64:36:8) and dichloromethane/ethyl acetate/methanol (2:3:1) with or without 1% (v/v) FA. The solvent mixtures were freshly prepared, and unless otherwise noted, extractions were performed in a water-bath sonicator (Ultrasonic Cleaver 5200, Branson Ultrasoundics Corporation) at room temperature for 20 min. The sonicated samples were filtered through glass cotton. Finally, the extracts were dried under vacuum using a centrifugal evaporator (Genevac HT-4, SP scientific, Ipswich, Suffolk, UK). Uninoculated plates and plates inoculated with one fungal strain (i.e., mono-cultures as opposed to co-cultures) were used as blanks and controls, respectively.

An accelerated solvent extraction (ASE, Dionex, Olten, Switzerland) method was also tested, and the results obtained using two different solvent systems (dichloromethane/methanol/water (64:36:8) and methanol) were compared to those of the sonication procedure.
2.5 Sample preparation

During method development sample enrichment by classical solid phase extraction (SPE) filtration was achieved using Sep Pak Vac SPE C$_{18}$ cartridge (1 cc, 100 mg, Waters, Milford, MA, USA) using methanol/water 85:15 v/v as eluent [30].

To facilitate screening a large number of samples, the SPE procedure was simplified as follows. The dry extracts were dissolved in a 4:1 methanol/water mixture at a concentration of 2 mg/mL. A 1 mL aliquot of this solution was transferred into a 1.5 mL microtube containing 200 mg of reversed-phase C$_{18}$ material (Silica gel ZEOprep 60 C$_{18}$, Zeochem AG, Uetikon, Switzerland) to remove the most apolar compounds, as these compounds would be incompatible with reversed-phase chromatography. The tubes were gently shaken (Rotation Mischcher 3300, Vaudaux-Eppendorf, Basel, Switzerland) for 2 min and then centrifuged (Mikroliter Zentrifugen, Hettich) for 5 min. The supernatants were filtered through 0.45 µm filters and diluted 2x with methanol. When the same solvent was used, this procedure was found to produce similar results to those of the classical SPE filtration procedure but was easier to implement for the preparation of a large number of samples.

2.6 UHPLC-TOFMS analysis

UHPLC-TOFMS analyses were performed on a Micromass-LCT Premier Time-of-Flight mass spectrometer from Waters (Milford, MA, USA), which had an electrospray (ESI) interface coupled to an Acquity UPLC system (Waters, Baden-Daettwil, Switzerland). In separate runs, detection was achieved in both the positive (PI) and negative ion (NI) modes. The m/z range was set to be 100-1000 in centroid mode with a scan time of 0.25 s and an inter-scan delay of 0.01 s. The ESI conditions in PI and NI modes were as follows: capillary voltage of 2800 V, cone voltage of 40 V, source temperature of 120 °C, desolvation temperature of 250 °C, cone-gas flow of 20 L/h, and desolvation-gas flow 600 L/h. For internal calibration, a 5 µg/mL solution of leucine-enkephalin from Sigma-Aldrich (Steinheim, Germany) was infused through the lock-mass probe at a flow rate of 5 µL/min using a second Shimadzu LC-10AD vp LC pump (Duisburg, Germany).

UHPLC-TOFMS profiles were collected with either a 150 mm × 2.1 mm i.d., 1.7 µm Acquity BEH C$_{18}$ UPLC column (Waters, Baden-Daettwil, Switzerland) or an Acquity BEH C4 UPLC column (Waters, Baden-Daettwil, Switzerland) in gradient mode at a flow rate of 0.46 mL/min with the following solvent system: (A) 0.1 vol% FA in water; (B) 0.1 vol% FA in acetonitrile. Analysis began with an isocratic step of 5% B for 3.60 min, which was followed by a gradient from 5% to 95% B over 34.50 min. The column was then washed for 6.93 min with 95% B, reconditioned with 5% B for 0.87 min and finally equilibrated with 5% B for 9.50 min. The temperature was maintained at 40 °C, and the injection volume was 2 µL.

UHPLC-TOFMS fingerprints were recorded with a 50 mm × 1 mm i.d., 1.7 µm Acquity BEH C$_{18}$ UPLC column (Waters, Baden-Daettwil, Switzerland) in gradient mode at a flow rate of 0.3 mL/min with the solvent system described above. The protocol used to establish the fingerprints included a direct geometric transfer of the metabolite profiling procedure.
calculated using a dedicated Excel spread sheet, ‘HPLC calculator’ [67,556]. The gradient was increased from 5% to 95% B in 4 min. The column was then washed for 0.8 min with 95% B, reconditioned with 5% B for 0.1 min and finally equilibrated with 5% B for 1.1 min. The temperature was maintained at 40 °C, and the injection volume was 1 μL. Analyses were performed randomly and included quality control and blank samples after every 10 sample runs. The sample list was randomly generated using an Excel macro (Supplementary Material).

2.7 Peak picking and data analysis

Native MassLynx data (Waters, Baden-Daettwil, Switzerland) were converted into nCDF (common data format) data using DataBridge software (Waters, Baden-Daettwil, Switzerland). Automatic feature detection was performed between 0.5 and 4.5 min with MZmine2 software [515] using parameters selected according to the TOFMS detector. Peaks with a width of at least 0.03 s and an intensity greater than either 20 counts (NI) or 50 counts (PI) were selected with a 5 ppm \( m/z \) tolerance and deconvoluted. Deisotope filtering was applied using the ‘isotopic peaks grouper’ module with tolerance parameters adjusted to 0.03 s and 5 ppm. Feature alignment and gap filling were achieved with an \( m/z \) tolerance of 15 ppm and a retention-time (RT) tolerance of 0.3 min. The features detected from blank samples and uninoculated agar samples were removed from the generated matrix. The full procedure for feature detection is presented in Table IV.A.S2. The exported feature lists were compared using Microsoft Excel, and multivariate analyses were performed using Unscrambler X software (CAMO Software AS, Norway). Principal component analysis (PCA) and partial least-squares regression (PLS-DA) applied after unit variance (UV) scaling were used for sample discrimination as well as the generation of biplots and the list of features (loadings).

The detection threshold for selection of the induced peaks was set to 1% of the mean peak area of the 10 most intense features in the dataset. Features that were retained were considered significantly induced and were filtered again according to increases in peak areas. The filtering results were verified for the main biomarkers in the corresponding raw UHPLC-TOFMS data.

3. Results and discussion

Solid media was preferred over liquid cultures for the screening of induction phenomena in fungal co-cultures for the following reasons. i) From a fungal ecology perspective, solid media better mimics the natural growth conditions of filamentous fungi in an aerobic environment and enables better development because it is a solid support. ii) From a practical perspective, solid media enables visualisation of the development of fungal colonies over the experimental time and allows excision of a part of the agar media for further analysis of the location of fungal confrontations.

Mono-cultures of filamentous fungi were produced via inoculation of an agar pre-culture plug in the middle of a Petri dish, while co-culture plates were inoculated on opposite sides of a single Petri dish of the type used for mono-cultures. The co-culture plates were incubated for
several weeks. Colony growth leading to interaction with the competing colony was monitored with the unaided eye. Chemical analyses were focused on the confrontation zone, and the results were compared to those obtained from mono-cultures.

The method of metabolic induction through fungal co-culture was evaluated by culturing all of the *Fusarium* strains included in the collection of clinical isolates with representatives of a fungal collection of phytopathogenic and environmentally derived fungal strains (http://mycoscope.bcis.ch/). As described below, more than 600 co-culture experiments were screened.

To highlight the metabolite modifications that are caused by fungal interactions, an LC-MS fingerprinting method was adapted to the analysis of agar solid media. The development of this adapted method included the optimisation of an extraction procedure to detect the largest possible number of metabolites over a wide range of polarities. The repeatability of the fingerprinting method, as well as the metabolite profiles of biological replicates, was verified to ensure that satisfactory statistical results were obtained even when screening a large number of samples.

### 3.1 UHPLC-TOFMS metabolite profiling of fungal cultures in solid media

To profile the largest possible number of fungal metabolites, a repeatable and generic extraction procedure was devised. Because different fungal strains may produce highly divergent metabolite patterns, preliminary analyses were used to select those fungal strains that produced metabolites with a large range of polarities. This representative strain set included two *Fusarium solani* strains, one *Aspergillus clavatus* and one *Fusarium oxysporum* strain (See Fig. IV.A.S1 for the UHPLC-TOFMS fingerprint of each). All of the strains were grown in standard solid-media conditions and were mixed to form homogenous materials. This pooled solid media was used to optimise the extraction procedure as well as the high-resolution metabolite profiling and rapid fingerprinting LC-MS methods.

#### 3.1.1 Extraction of fungal metabolites from solid media

To ensure good extraction repeatability, the solid culture media from the Petri dishes was frozen and lyophilised prior to extraction, a step taken to exclude interference from the water content. The metabolic activity and growth speed of some fungal strains from the collection cultivated on agar plates varied significantly according to species. These behavioural differences strongly impact the water content of solid cultures, a result that was observed in the first days after inoculation (data not shown).

Accelerated solvent extraction (ASE) and ultra-sound assisted extraction (UAE) were evaluated as potential extraction methods for this spongy material. The solvent systems used in these evaluations consisted of either methanol or a dichloromethane/methanol/water mixture. No major differences in yield or in chemical composition of the extracts were observed between these two methods (data not shown). Therefore, UAE was preferred for the screening of a
large number of samples because it was relatively easy to implement. Such a type of extraction technology was reported to give good extraction yields when applied to solid media [557].

The 4 selected fungal cultures mentioned above were lyophilised, homogenised and subjected to UAE for 20 minutes with a 70X excess of solvent when compared to the amount used for the dried media (v/m ratio). This high ratio of solvent was necessary to fully submerge the lyophilised material. Repeated extractions revealed that approximately 90% of the extractable material was obtained during the first extraction step. To maintain the simplicity of this procedure and cope with the large number of screened samples, one extraction step was considered to be sufficient. Within this procedure, the metabolite extraction capacities of 6 different solvents or solvent systems comprising a wide range of polarities, while still remaining compatible with reversed-phase HPLC, were compared.

To evaluate the metabolite diversity in the solid culture media extracts, a generic UHPLC-TOFMS profiling method was applied. The profiling was performed using an extended linear acetonitrile/water gradient on a 150 mm UHPLC column to ensure high peak capacity ($P = 408$ as calculated by HPLC calculator 3.0 [67,556]) and thus baseline separation of most of the metabolites present. The detection was achieved in both positive (PI) and negative ionisation (NI) modes, which allowed monitoring of the largest possible number of metabolites. A classical SPE sample preparation was performed to remove very lipophilic constituents (see below).

The UHPLC-TOFMS profiles obtained in NI mode for all 6 extraction solvent systems used are presented in Figure IV.A.1. In all cases, this analytical procedure yielded well-resolved UHPLC-TOFMS base peak intensity (BPI) chromatograms. When compared to the isopropanol and $t$-butanol extracts, the methanol extract was found to contain a larger number of polar compounds ($RT$ from 0 to 20 min). By contrast, the complement of less-polar metabolites ($RT$ from 20 to 35 min) in the methanol extract was found to be less diverse than those of the isopropanol and $t$-butanol extracts. To optimise the extraction of most metabolites over a large range of polarities, solvent mixtures were also investigated. Three previously reported solvent mixtures were assessed: dichloromethane/methanol/water (64:36:8) (DMW) [30] and dichloromethane/ethyl acetate/methanol (2:3:1) (DEM) (both with and without 1% (v/v) of FA) [557]. The addition of FA into the DEM solvent system led to reduced chemical diversity, as assessed by the drastic decrease in the number of detected peaks.

In the less-polar region of the chromatogram ($RT$ from 20 to 35 min), both the DMW and DEM extracts showed a higher number of peaks than the isopropanol and butanol extracts. Moreover, the chemical diversity in the apolar region was evenmore pronounced when DEM was used. In the polar region ($RT$ from 0 to 20 min), DEM performed similarly to methanol.

When compared to all of the previously tested extraction solvent systems, DMW was found to yield a substantially higher number of peaks in the polar region of the chromatogram. Because this solvent system was already found to be appropriate for the extraction of non-
polar constituents, it was considered to have a relatively broad extraction capacity and was the extraction solvent system chosen for the rest of this study.

Considering the large number of samples to be analysed, a SPE method prior to UHPLC-TOFMS analysis was established to prevent sample carryover and maximise the lifespan of the column. Thus, the highly lipophilic compounds were removed from the extracts using C\textsubscript{18} SPE [30,558]. To assess the number of metabolites lost during the SPE step, the extracts were profiled using a C\textsubscript{4} column that had a lower retention of lipophilic constituents. A comparison of the crude extract profiles with those of the SPE-purified extracts recorded using the C\textsubscript{4}.

**Figure IV.A.1:** A-F) High-resolution NI UHPLC-TOFMS metabolite profiles (BPI traces) from extracts obtained using 6 different solvent systems (the solvents used are indicated in the figures) from culture media pooled from four representative fungal strains (i.e., 2 Fusarium solani, 1 Fusarium oxysporum and 1 Aspergillus clavatus). UHPLC conditions: Acquity C\textsubscript{18} 150 × 2.1 mm I.D., 1.7 µm column, 460 µL/min, 40°C, 2.6% slope gradient. G) Fast NI UHPLC-TOFMS fingerprint obtained after gradient transfer using the DMW extract (F). UHPLC conditions: Acquity 50 × 1.0 mm I.D., 1.7 µm column, 300 µL/min, 40°C, 22.5% slope gradient. This high-throughput method was used to generate the ion maps for the screening of all the involved strains.
column revealed that, for DMW extracts, there was no significant difference in the detected lipophilic constituents (RT over 26 min). It was found, however, that the SPE procedure eliminated the most lipophilic constituents from the butanol extract (Figure IV.A.S2).

Because the SPE protocol reduced the sample weight by approximately 30% without affecting the reversed-phase profiles of DMW extracts, it was systematically applied to all of the screened samples in an effort to preserve experimental reproducibility. To facilitate screening a large number of samples (see below), the SPE procedure was simplified by mixing the samples with C18 particles prior to filtration. This modified procedure was found to produce results similar to those of direct SPE filtration.

In conclusion, extraction by UAE with DMW as the solvent system followed by SPE was found to be a highly suitable sample preparation method for generic reversed-phase profiling of fungal metabolites.

3.1.2 Development of the UHPLC fingerprinting methods

To chemically profile a large number of fungal confrontations as well as their respective pure strains (more than 400 samples in this study), a high-throughput UHPLC-TOFMS fingerprinting method was developed that was based on the high-resolution profiling analysis used to optimise the metabolite extraction. Development of this rapid fingerprinting method was necessary because the high-resolution LC-MS profiling procedure required more than 55 min to complete and was not compatible with the screening of a large number of samples.

To decrease the analysis time, the column length was reduced from 150 mm to 50 mm. This column geometry was found to yield higher peak capacities with gradient times of less than 10 min [528,559]. When a 50 mm column with a diameter of 2.1 mm was used, the optimal flow rate required to reach 90% of the maximum pressure allowed by the system (1000 bar, the optimum condition for high peak capacity) [455] was approximately 1.2 mL/min. This flow rate was not suitable for electrospray ionisation (ESI) [560], and rather than split the flow rate, a reduced-diameter column (1.0 mm) was selected. The optimal flow rate for this column was 0.3 mL/min. This flow rate was suitable for the ESI source of the TOFMS detector and considerably reduced the solvent consumption. The selectivity of both the profiling and fingerprinting methods were indistinguishable because of the application of a gradient transfer method [67,556]. By altering the column geometry and gradient conditions, the overall analysis time was reduced from 55 min to 6 min (Figure IV.A.1). However, while a tenfold increase in throughput was obtained, these changes resulted in a twofold decrease in theoretical peak capacity (from 408 to 190) [556]. Nevertheless, this analytical procedure was found to be compatible with the management of a reasonably timely analysis of a large series of samples without much loss of efficiency in the LC separation. Figure IV.A.1F and Figure IV.A.1G compare the UHPLC-TOFMS BPI profile and fingerprint obtained from the same DMW extract. Although the fingerprint required a shorter analysis time, the selectivity of this method was similar to that of the profile. This result was confirmed by comparing the extracted ion chromatograms (XICs) of the main LC peaks. The decrease in peak capacity was
not detrimental to the detection of features (m/z × RT) because the orthogonal TOFMS detection increased metabolite separation and the BPI profiling method did not reveal the presence of many isomeric structures. The UHPLC-TOFMS fingerprints were thus represented in the form of 2D ion maps, in which the metabolites were resolved in both the LC and MS dimensions. An example of such an ion map is included in Figure IV.A.2, which presents the PI and NI UHPLC-TOFMS fingerprints of a *Fusarium* pure strain extract with their corresponding BPIs. As shown, the PI and NI fingerprints were very complementary, and all of the features were well separated despite the use of fast LC separation (Figure IV.A.2).

TOFMS not only can contribute an additional separation dimension to all of the spectral features but also can allow the user to deduce molecular formulae from its high-mass-accuracy data, a process referred to as dereplication [561]. For example, one of the main peaks of Figure IV.A.2 was detected in both the PI and NI modes at an RT of 1.97 min for m/z values of 722.3989 Da and 720.3885 Da, respectively. The m/z difference of 2 Da between the two ionisation modes was the basis for the identification of the [M+H]+ and [M–H]− adducts. The high mass-accuracy of the TOFMS data (<5 ppm) permitted 3 molecular formulae to be deduced in both the NI and PI modes. Moreover, the comparison of theoretical and observed isotopic patterns (low normalised i-fit value) as well as the application of heuristic filtering [65] allowed for the determination of an unambiguous molecular formula (C₃₄H₅₉NO₁₅) [64]. A search for this molecular formula in natural product databases [57,530] matched with two possible isomers – fumonisin B₁ and isofumonisin B₁ – which are produced by *Fusarium* sp. [562,563]. These results indicate that the screening procedure developed in this work was able to detect known fungal metabolites efficiently and that high-resolution TOFMS data could be reliably used for the putative identification of features induced by fungal confrontation and the estimation of their novelty.

### 3.1.3 Repeatability of extraction and UHPLC–TOFMS fingerprinting analysis for a large set of microbial extracts

To assess the reproducibility of the fungal growth conditions, sample preparation methods and LC–MS analyses, a representative set of 5 pure strain mono-cultures and 6 co-cultures were selected. The 5 fungal strains used included two *F. solani*, one *T. rubrum*, one *Cladosporium* sp. and one *A. clavatus* strain. Five co-culture experiments, which involved both of the *F. solani* strains cultured with the 3 others fungal strain, were analysed in parallel to verify the consistency of the metabolite profiles when fungal interactions were present. Nine biological replicates of each sample were grown independently and submitted to UHPLC–TOFMS fingerprinting.
Figure IV.A.2: PI and NI 2D ion maps obtained from the UHPLC-TOFMS fingerprints of the DMW extract of Fusarium oxysporum (Sin106) (see conditions in Figure IV.A.1G). Peak annotations based on the high mass and high spectral accuracy of this technique are used for the assignment of molecular formulae.
The figure shows satisfactory resolution of most of the features in the ion maps. For example, the TOFMS data and literature search provided the identification of fumonisin B₁ or isofumonisin B₁ as the best putative structures associated to the two features with masses of 722.3989 (PI) and 720.3831 Da (NI).

9 Replicates of the uninoculated agar plates were subjected to the same treatment and served as control samples so that the agar background detected in the LC-MS analyses could be excluded. A quality-control sample (QC), which consisted of an equal mixture of all of the previously described samples (108 in total), was prepared to assess the stability of the system over the time required for analysis of the complete sample set.

The samples were analysed in random order. The blank samples (injection solvent only) and quality QC were analysed every 12 samples. In addition, 6 analyses of both blank samples and QC samples were performed before and after the analysis of the complete set of fungal samples. To facilitate the set-up of an appropriate sample list, an Excel Macro was developed (Supplementary Material). Both the PI and NI fingerprints were analysed independently to ensure high MS acquisition frequency and detection stability. Altogether, more than 300 injections were performed sequentially. This procedure was adapted from an established metabolomic protocol that has been applied to the analysis of body fluids [459].

All of the LC-MS fingerprints were automatically processed using open-source peak-picking software [461,515] to generate lists of features (RT, m/z value, and peak area) for the entire set of samples (the fully detailed procedure is explained in Table IV.A.S2). The system stability over the time required to obtain the whole dataset was evaluated via a principal component analysis (PCA, Unit Variance scaling) involving all the samples with a special focus on the QC samples. The relatively tight clustering of QC samples (data not shown) suggested that the fingerprints were suitable for further detailed analysis of fungal metabolite variations. In addition, the XICs of selected characteristic ions of the QC samples revealed a very good match amongst the RT data (approximately 1% coefficient variation) for more than 25 analytical replicates that were representative of the whole sample list. Blank samples were found to contain very few contaminants, and their patterns were very similar over the whole dataset, indicating no detectable carryover effects.

A PCA analysis of all of the pure strains also revealed satisfactory clustering of the biological replicates (Figure IV.A.S3). Within a given pure strain, more than 80% of all the PI- and NI-detected peaks exhibited biological variation of less than 30% (coefficient variation of the peak areas of given peaks for all replicates). When a variation of less than 50% was considered, the detection was 95%. Considering that the growth of fungal strains (growth speed, colony morphology) may be prone to significant variations over subsequent cultures, these results were determined to be very satisfactory (Table IV.A.S3 and IV.A.S4). For the 9 biological replicates of the 6 different co-cultures, the same repeatability was observed among replicates (Table IV.A.S3 IV.A.S4). This indicates that the metabolite fingerprinting method developed for fungi grown on solid media generates acceptable datasets that can be used for further metabolomic analyses.
3.1.4 Search for metabolite induction in fungal co-culture by data mining

Because agar plates are limited in space and contain restricted amounts of nutrients, the co-cultivation of fungal strains forces competitive interactions [227]. A co-cultivation experimental set-up was used here to stimulate the production of secondary compounds by interacting fungi that would not be released by mono-cultures grown in identical conditions. To evaluate the induction of novel compounds by the six representative fungal co-cultures described above, UHPLC-TOFMS fingerprints of the fungal mono-cultures and their corresponding co-cultures were compared.

As shown in Figure IV.A.3, a visual comparison of the fingerprints displayed as 2D ion maps (RT × m/z) of 2 mono-cultures and their corresponding co-culture was sufficient to detect the most obviously induced metabolites among several hundreds of spectral features. For example, when *Fusarium solani* was confronted with *Cladosporium* sp., two intense features (highlighted by a box frame on the UHPLC-TOFMS ion map in NI and PI mode) were only visible after confrontation and were not observed in spectra obtained from the corresponding mono-cultures. Comparison of the selected ion trace XICs of these features further confirmed that they were not present in detectable quantities in the mono-cultures but were clearly present in the interaction experiment. Observation of this induction phenomenon was consistent over the nine replicates.

To screen for all of the induced fungal metabolites and detect minor induced compounds as well, a statistical approach was established. Multivariate analysis [468] was applied to nine replicates of each of the five mono-cultures and six co-cultures, a total of 99 samples. For a given confrontation, all of the detected features with intensities greater than an appropriate threshold were selected by an automated peak-picking procedure (see Experimental). This procedure was applied to the nine replicates of the co-culture and mono-cultures of each pair of fungi (27 samples).

As a first step, an unsupervised PCA (UV scaling) was performed on these 27 samples for each interaction and *Cladosporium* sp.), a well-resolved clustering of the 27 samples in 3 groups was obtained (Figure IV.A.4A). This behaviour was observed for most of the interaction experiments. However, in some cases, PCA analysis resulted in poorly resolved clustering of the co-culture samples apart from their corresponding mono-cultures (Figure IV.A.S4 and IV.A.S5). The well-resolved sample clustering obtained showed that the co-culture fingerprints did not overlap with the two corresponding mono-culture clusters. This indicated that the dataset contained information that discriminated co-cultures from mono-cultures and that, in addition, some features that were only associated with the co-cultures were likely to be present. This was confirmed by further analysis, as features only associated with the co-cultures were found within the dataset of induced compounds (1 co-culture vs. 2 mono-cultures, see below). This indicates that fungal interactions modulate the biosynthetic pathways that produce secondary metabolites [309].
Fungal metabolites

IV.A Detection of metabolite induction in fungal co-cultures

Figure IV.A.3: General strategy to study metabolite induction by fungal co-cultivation on solid media in Petri dishes. In this example, Fusarium solani (Sin58) was co-cultivated with Cladosporium sp. (Sin137). The PI and NI 2D ion map fingerprints are shown. The round insets represent a schematic presentation of the Petri dishes of the two mono-cultures with the co-culture in the middle. Visual inspection of the 2D ion maps already enabled the observation of some of the features induced by co-culturing. The XICs of two features (228.1970 Da at 3.40 min and 414.3226 Da at 3.42 min) were detected in the PI and NI modes, respectively.

As a second step, supervised analysis of the data via PLS was used to detect overexpressed compounds in the co-cultures (Figure IV.A.4B). The loadings associated with the PLS separation of the co-cultures and mono-cultures were selected to determine the induced features. A biplot of the loadings and the score plot provided a good means for the ranking of the features responsible for discriminating between the co-cultures and the two corresponding mono-cultures on the PLS plot. For all of these selected features, the induction rate was estimated by comparing the mean peak area of given co-culture treatment, the most significant features (those with an induction rate higher than threefold) were selected. Finally, the XICs for significantly induced features were plotted to validate these results.

An example of the XICs that correspond to the induced features is given in Figure IV.A.4C and IV.A.4D. In these figures, two types of induction patterns are recorded: i) de novo biosynthesis in which there was no detection in mono-cultures of a compound that was clearly present in co-cultures (Figure IV.A.4C); and ii) up-regulation in co-cultures of compounds that were already present in mono-cultures, which is expressed as fold changes induction (Figure IV.A.4D).
In the 6 fungal interactions studied using this method, a few tens of features were detected in the PI and/or NI modes (Table IV.A.S5). When considering the fungal combinations, however, important variations were observed in the number of induced features, a value that ranged from 0 to 29. For example, the interaction between *F. solani* (Sin58) and *Cladosporium* sp. (Sin137) yielded 11 and 29 features in the NI and PI modes, respectively (Table IV.A.S5).

These results further supported the previously reported existence of chemical induction in fungal co-cultures [253,256,266].

**3.1.5 Estimation of chemical novelty generated by fungal interactions**

To evaluate whether the induced features described novel NPs, the high mass-accuracy of TOFMS detection was used to verify matches between the obtained high-accuracy MW and those recorded in the Dictionary of Natural Product (DNP) database [530]. This rapid procedure was preferred over determination of the molecular formula of each induced feature for the purpose of dereplication, as the latter method, described for the peak annotation of constitutive metabolites in Figure IV.A.2, is a more laborious process.

The determination of MWs was performed with a relatively large mass-accuracy tolerance (<15 ppm) to improve the chances of finding a matching candidate. The main suspected adducts ([-H and +HCOO in NI and +H and +Na in PI]) [54] were taken into account for these calculations. Rough evaluation of chemical novelty was assessed based on the existence of these MW values in the DNP.
Fungal metabolites

Interestingly, a majority of the peaks (around 80%) from mono-culture analysis matched at least one structure recorded in the DNP. This hit number was close to zero when the induced features were searched (Table IV.A.S5). Such an approach must be used with care, but it can be used for the rapid preliminary estimation of the chemical novelty of the induced compounds detected in filamentous fungi co-cultures.

3.2 Screening for chemical diversity in fungal interactions

The results obtained with the strategy described above, which was based on a limited set of fungal co-cultures, showed that fungal interactions on solid media yield chemical novelty. To further evaluate the potential for chemical induction in a larger variety of fungal interactions, 657 co-culture experiments were examined.

3.2.1 Morphological patterns of fungal interactions on solid media

This series of co-culture experiments was performed on agar-containing Petri dishes. The front zone of each interaction was observed over a period of 8 weeks. From these observations, four groups that displayed distinct morphological patterns of interaction (interaction types) were defined (Figure IV.A.5).

In the first type of interaction – referred to as distance-inhibition type – fungal growth stopped at a distance from the competing fungal colony. This pattern strongly suggested the release of an antifungal substance(s) into the medium that worked to inhibit the growth of both fungi. In the second type of interaction, which was only observed in two cases, the fungal colonies grew large enough to contact one another and produced a dark precipitate similar to the so-called ‘zone lines’ observed in wood-decaying fungi confrontation fronts [30]. In this type of interaction, the production of new substances is a confirmed observation of the dark precipitate itself. The third group of interactions included those in which the fungi grew large enough to contact one another but produced no obvious evidence of substance release. This type of interaction was referred to as the contact inhibition type. In the last group, interactions resulted in the complete invasion of one colony by the other. This type of interaction was referred to as overgrowth.

For the majority of co-culture experiments, the types of interactions observed evolved over time, which led to the observation of intermediate behaviours. Thus, for example, some interactions yielded colonies that would initially stop their development at a distance for a few weeks until one fungal colony would resume its expansion and produce an overgrowth-type interaction. In other cases, colonies would develop up to mycelium contact while the colony’s morphology would change substantially in the vicinity of the interaction front.

To verify whether the types of morphological interactions could be related to the number of induced metabolites at the interaction zones, only co-cultures displaying one of the 4 well-defined interaction types (Figure IV.A.5) were kept for LC-MS fingerprint screening.

For this reason, only 138 co-culture experiments (21% of the all interactions) and the mono-cultures of the 83 fungal strains involved in these confrontation experiments were analysed.
These selected interactions include 31 distance-inhibition types, 1 zone-line type, 45 contact-inhibition types and 61 overgrowth types (Table IV.A.S1 and IV.A.S6). In this part of the work, and in view of the large number of interactions to be examined, all samples were analysed without replicates to gain rough evaluations of whether a given interaction type could be related to a higher induction of small secondary metabolites.

### 3.2.2 Chemical induction and types of fungal interactions

To record the fingerprints of all of the 138 co-cultures and optimise the concentrations of those substances specifically released by fungal interactions, the front lines of the co-cultures were excised and processed as described above. By observing fungal interactions on solid media, access to the exact zones of fungal interactions was enabled.

![Colony morphologies of fungal co-cultures](image)

**Figure IV.A.5**: Colony morphologies of fungal co-cultures. Four ‘interaction types’ (distance-inhibition, zone-line, contact-inhibition and overgrowth) were defined from observations of 657 co-culture experiments performed on solid agar plates. Mean values of the numbers of induced compounds detected by NI or PI UHPLC-TOFMS are displayed below the corresponding interaction types. Only well-filtered de novo induced features were considered. A schematic of the interaction side-view is provided at the bottom of the figure.

The UHPLC-TOFMS fingerprints of these co-cultures were analysed in both NI and PI modes and were compared to their corresponding mono-cultures. For this dataset, only the number of de novo induced metabolites in the interaction zones was considered. Thus, only the features of sufficient intensity that were detected in the interaction zones but not in the corresponding mono-cultures were taken into consideration. Based on the criteria selected,
the number of features induced in each sample (a few tens) varied strongly according to the fungal combinations.

A mean value for the number of induced compounds detected in both PI and NI modes was calculated for each type of interaction (Figure IV.A.5). For all of the interaction types, the number of low-molecular-weight compounds induced de novo was not significantly different. Thus, even co-cultures that led to visible antimicrobial activity (distance inhibition) or to the visible formation of new products in the form of dark precipitates were not found to have produced more induced features. This result demonstrates that there is no correlation between the morphological aspects of the interacting colonies and the production of new low MW metabolites detectable with the method used.

Conversely, these results also demonstrate that the large majority of fungi grown on agar plates reacted to the presence of another microorganism by producing substances that were not produced in mono-cultures.

4. Conclusion

The strategy that was developed in this work regarding co-culture experiments of filamentous fungi grown on solid media and subsequent LC-MS fingerprinting has revealed induction phenomena in these microorganisms that may be linked to the activation of cryptic biosynthetic pathways.

The protocol was optimised for co-culturing of fungi on solid media and was found to efficiently generate samples in which metabolite-induction phenomena could be easily observed. Solid-media co-cultures were found to provide a good means of easily following the morphological development of fungal colonies and excise zones in which fungal confrontations occur. The study of more than 600 fungal strains subjected to such co-culturing revealed 4 main morphological types of fungal interactions.

To profile the low-molecular-weight metabolites produced by this large collection of fungi and their co-cultures, a high-throughput UHPLC-TOFMS method was devised. The sample preparation process developed for the analysis of a large series of samples achieved broad extraction of fungal metabolites from solid agar media with no major interferences from the culture media and no problematic carryover effects. UHPLC fingerprinting conditions were generic and were optimised using high-resolution metabolite profiling. This enabled the acquisition of 10 fungal fingerprints per hour. The high mass and spectral accuracy of the TOFMS detector was used for dereplication of the main fungal metabolites and for the rough estimation of the novelty of features induced by fungal interactions.

The method was found to be repeatable and generated acceptable datasets that were used for further metabolomic analysis. Furthermore, the advantage of using a profiling method based on UHPLC for this type of study is that this technique is amenable to the possibility of scaling up the separation for the isolation of biomarkers of interest for full identification by NMR and for further characterisation of their bioactivity profiles [30].
The application of this method to a large collection of co-cultures grown on solid media demonstrated that most fungal interactions resulted in the production of compounds that were not produced by the corresponding fungal mono-cultures grown under identical conditions. No correlation between the morphologies of the interacting colonies – defined as the interaction type – and the number of induced compounds could be made.

The screening results did indicate, however, that the compounds specifically produced at the fungal interaction zones in co-cultures seem to correspond to novel chemical compounds that are scarcely represented in natural products databases. These findings indicate that fungal interaction may be a new and promising source of lead compounds.

Finally, as these low MW compounds seem to be released as a response to microbial interactions, it can be speculated that they may possess antimicrobial activities, a speculation that has been previously reported [30,392,415,484,549]. In this respect, continuing work is under way to measure the differential bioactivity profiles [564] of a large number of co-cultures and their respective mono-cultures. The metabolomic methods presented here will be applied in combination with the bioactivity results to detect the induced compounds responsible for such antimicrobial activities.

5. Acknowledgments

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6. Supplementary information

The macro that was developed to randomise the sample set is available as Supplementary data from the publisher:


Figure IV.A.55: Preliminary NI UHPLC-TOFMS fingerprints of four single cultures: two *F. solani* (Sin5 & Sin58), one *A. clavatus* (Sin141) and one *F. oxysporum* (Sin 17) to assess chemical diversity of the four extracts used to develop the extraction and sample preparation method.
Dichloromethane/Methanol/Water Extract

Figure IV.A.S6: Impact of SPE procedure on fungal metabolite profiles.
UHPLC-TOFMS profiles of the mixed fungal extract (using butanol or DMW) using an Aquity BEH C4 (2.1 x 150, 1.7µm) column. The same LC parameters than for profiles displayed in Figure IV.A.1 were applied. Both extracts were enriched using SPE on either C4 or C18 solid phase. These profiles showed that in the butanol extract the C18 SPE process remove the more apolar compounds in comparison to C4 SPE. In the case of the extraction by the DMW the more apolar peaks are unchanged. This demonstrated that DMW is compatible for analysis C18 column.
Figure IV.A.57: PCA of UHPLC-TOFMS fingerprints data (PI and NI using UV scaling) from the extracts of five fungal strains mono-cultures (T. rubrum (Sin39), F. solani (Sin58 & Sin74), A. clavatus (Sin141) and Cladosporium sp. (Sin137)).
Figure IV.A.S8: PCA of NI UHPLC-TOFMS fingerprints. Each PCA plot shows the data produced by the analysis of one co-culture and the two corresponding mono-culture. PCA plots show non-overlapping clustering of the three groups. Moreover, except for Sin74xSin39, the co-culture cluster does not stand in the middle of the two mono-cultures.
Figure IV.A.S9: PCA of PI UHPLC-TOFMS fingerprints. Each PCA plot shows the data produced by the analysis of one co-culture and the two corresponding mono-culture. PCA plots show non-overlapping clustering of the three groups. Moreover, except for Sin74xSin39, the co-culture cluster does not stand in the middle of the two mono-cultures.
**Table IV.A.S2:** List of the 83 fungal strains isolated either from clinical patients, soil or plants stored in Agroscope ACW fungal collection [http://mycoscope.bcis.ch/teh](http://mycoscope.bcis.ch/teh).

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**Table IV.A.S3: Steps and parameters used during the automatic peak picking procedure by MZmine 2.**

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Table IV.A.S4: Repeatability over nine replicates of peak-area extracted by automated peak picking from NI UHPLC-TOFMS fingerprints using five mono-cultures and six co-cultures.

Repeatability of peak area over the nine replicates is evaluated for each detected feature using its coefficient of variation (cv). Each line of the table shows the percentage of detected features with a cv below the cv indicated shown on the left. As shown for most mono-cultures or co-cultures, more than 80% of the peak area features displayed cv below 30% (first framed line). This percentage is higher than 95% when cv’s below 50% are considered (second framed line).

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Number of features for all replicates: 176, 458, 848, 311, 479, 450, 624, 647, 599, 634, 677
Table IV.A.S5: Repeatability over 9 replicates of peak-area extracted by automated peak picking from PI UHPLC-TOFMS fingerprints using 5 mono-cultures and 6 co-cultures.
Repeatability of peak area over the 9 replicates is evaluated for each detected feature using its coefficient of variation (cv). Each line of the table shows the percentage of detected features with a cv below the cv range indicated on the left. As shown for most mono-cultures or co-cultures, more than 80% of the peak area features displayed cv below 30% (first framed line). This percentage is higher than 95% when cv's below 50% are considered (second framed line).

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Number of features for all replicates: 484 1141 1518 919 1024 964 1660 1280 1074 1360 1282
Table IV.A.S6: Peaks numbers (PI and NI) detected in co-cultures and in their two corresponding mono-cultures over 6 co-culture experiments are listed. For each experiment, peaks were selected according to intensity higher than 1% of the mean intensity of the 10 most intense peaks. Possible molecular formulae were automatically generated from the detected m/z values (less than 15 ppm of mass differences with +H and +Na adducts in PI and with –H and +HCOO adducts in NI). Number of peaks generating putative molecular formulae that yielded positive matches in the Dictionary of Natural Products (DNP, CRC press, December 2011) are listed in the last two columns of the table.

In this analysis, induction was considered when minimum of threefold increase of peak area was observed in co-cultures. Because peak detection was based on 1% of the mean intensity of the 10 most intense peaks, the detection threshold varied from one co-culture experiment to another. For this reason, the number of features detected in each of the three Sin58 mono-culture series, for example were not identical. The same explanation also holds true for the three Sin74 mono-culture series. This observation does not affect the conclusions drawn from this analysis.

<table>
<thead>
<tr>
<th>Fungal culture</th>
<th>Number of features</th>
<th>Number of features with positive matches</th>
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</tr>
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<tr>
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<td>22</td>
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<tr>
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<td>9</td>
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Table IV.A.S7: List of the 138 co-culture experiments used in the UHPLC-TOFMS screening.

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<th>Zone line</th>
<th>Contact inhibition</th>
<th>Invasion</th>
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V. Microfractionation for the purification of fungal metabolites

V.A Isolation of induced metabolites from fungal co-culture grown on solid media: Is one Petri dish enough?

This research chapter will be prepared for submission.

Co-authors are: K. Gindro, O. Schumpp, S. Bertrand, M. Monod, J.-L. Wolfender

Contribution: Performance of metabolite profiling, compound isolation and identification, analysis of results, discussion of results and writing of the article
Abstract

**Introduction** – Genomic analyses suggest that fungi are capable of biosynthesizing more secondary metabolites than have been discovered so far as it is assumed that many biosynthetic pathways are silent under common laboratory conditions. These cryptic pathways are sometimes activated through the application of biotic stress.

**Objectives** – Combined growth of two fungal (co-culture) on solid medium is used to induce the production of bioactive compounds. The isolation of induced compounds is attempted with the fewest possible number of Petri dishes that yields sufficient compound amount for NMR identification. Fungi used in this study were derived from patients suffering from onychomycosis, mainly *Fusarium* spp.

**Methodology** – Co-culture of two filamentous fungi is carried out in 9-cm Petri dishes and induced compounds are detected by UHPLC-TOFMS metabolite profiling. Induced compounds are isolated using MS-based microfractionation and compounds are identified using at-line microflow NMR analysis.

**Results** – In this study, the toxic compound fusaric acid was detected and isolated from the confrontation zone of the co-culture of *Acremonium strictum* and *Fusarium oxysporum*. Furthermore, the prevalence of fusaric acid in pure culture extracts of *Fusarium* spp. and the possible induction of this *Fusarium* metabolite in a panel of co-cultures involving *Fusarium* spp. was investigated.

**Conclusion** – Co-culture on solid medium in Petri dishes was successful in inducing the *de novo* production of bioactive compounds in the central confrontation zone. An experiment with only three 9-cm Petri dishes yielded sufficient sample amount for structure identification of the isolated compounds by microflow NMR spectroscopy.

**Keywords:** *Acremonium strictum*, *Fusarium oxysporum*, microorganism co-culture, solid medium, induction, fusaric acid, confrontation zone
1. Introduction

Fungi are the producers of several bioactive secondary metabolites that got important drugs and drug leads [3]. Moreover, genomic analyses of several fungal strains suggest that the potential to produce secondary metabolites is even larger than previously expected [565]. Thus, only a fraction of potential fungal metabolites have been detected so far as many biosynthetic genes, possibly leading to functional enzymes, are silent under standard laboratory conditions. Several studies have demonstrated the successful activation of such cryptic gene clusters through the application of abiotic [278] or biotic [309] stress.

Microorganisms are often organized in communities – called microbiomes, the assembly of all living microbes in a defined environment – and secondary metabolites are produced for defense or nutrient competition, often accompanied by morphological changes (sexualization, growth inhibition or stimulation). The artificial reproduction of microbiomes by culturing two or more microorganisms together constitutes a method for the application of biotic stress [566]. The simulation of given natural communities has shown to induce the production of secondary metabolites of potential biological importance. Such bioactive natural products (NPs) may result in the discovery of new drugs or drug leads [566].

The fungal biota in a particular environment is referred to as ‘fungal microbiome’ or ‘mycobiome’, in relation to the well-established concept of the microbiome. An unexpected environment of a mycobiome is the onychomycosis-infected human nail. The presence of several fungal strains on one affected nail could be made apparent [362]. Casual agents of onychomycoses are mainly dermatophytes as *Trichophyton* spp. but also non-dermatophyte filamentous fungi (NDF) as *Fusarium* spp., *Acremonium* spp. and *Aspergillus* spp. The combined growth of those human pathogenic fungi together in a co-culture experiment mimics the mycobiome of onychomycosis-affected nails and might stimulate the production of antifungal compounds involved in competitive interactions. This may lead to the discovery of novel compounds with potential use in onychomycosis treatment. Nevertheless, the de novo identification of such new metabolites is challenging as it requires structure elucidation by NMR.

In general, this requires important amounts of a given fungus to be cultured for the isolation of its secondary metabolites. Most reports on the isolation of microbial constituents include liquid culturing of several tens of liters of culture medium [567]. But fungi are well adapted to develop on solid media through hyphal growth to colonize unexplored regions in search of nutrients [376]. Studies on solid medium-cultured microorganisms include the assessment of morphological changes [417] or detection of metabolic changes by imaging mass spectrometry [277]. On the other hand, fungi are rarely cultured on solid media for the isolation of its secondary metabolites [30]. The extraction processes are tedious and large-scale culturing of microorganisms is more easily done with liquid media (fermentation). Nevertheless, microorganisms tend to produce different compounds depending on the
culture medium and method [278] and in general, extracts from solid medium-cultured fungi are more compound-rich than liquid culture extracts [26].

For metabolite profiling and dereplication by LC-HRMS, only a few micrograms of extract are needed since high quality MS and tandem MS (MS/MS) spectra can be acquired on-line with nano- to picogram-amounts of compound. De novo identification of unknown metabolites – or of metabolites that are not unambiguously identifiable – requires targeted isolation for further 1D and 2D NMR identification. This can be achieved at the microgram level with state-of-the-art microNMR methods [566]. Nevertheless, purification at this scale remains challenging.

The aim of this study was to investigate metabolome modifications in fungal strains that belong to the mycobiome of infected nails and to demonstrate induction phenomena through their artificial co-culture on solid media. Furthermore, the study is intended to showcase the targeted isolation of compounds derived from few Petri dishes of solid medium co-cultures that permit sufficient sample amount for NMR analysis and structure determination.

2. Experimental

2.1 Fungal material

The fungal strains were isolated from nails of patients suffering onychomycosis at the Centre Hospitalier Cantonal Vaudois (CHUV, Lausanne Switzerland) [362], from plants and soil. The strains were identified based on macromorphological traits and comparison of ITS sequence. All strains were stored in the fungal database of Agroscope in vials containing diluted PDB (Difco) solution (1:4) at 4 °C (http://mycoscope.bcis.ch/).

2.2 Growth conditions

For pure cultures, a 5-mm agar plug of a fungal preculture was inoculated in the center of a 9-cm Petri dish containing 30 mL of PDA (Difco) prepared with distilled water. The dishes were incubated at room temperature. Co-culture experiments were inoculated with two 5-mm agar plugs of the appropriate fungal strains on opposite sides of a Petri dish containing PDA, and the dishes were incubated at room temperature until physical contact of the two fungi, usually seven days.

2.3 Extraction procedure

Fungal material and solid medium were cut in small pieces with a scalpel, citric acid-disodium hydrogen phosphate buffer (C₆H₈O₇·H₂O, 0.1M Na₂HPO₄, pH 5.5) was added (twice the volume of buffer to fungal material, e.g., 20 mL of buffer solution for 10 g of fungal material) and everything mixed for 1 min (PRIMAX, Möller + Krempel AG, Bülach, CH). The mixture was transferred to Erlenmeyer flasks and macerated for 4 hours at 5 °C in the dark under agitation. The slurry was transferred to centrifugation bottles and macerated for 4 hours at 5 °C in the dark under agitation. The supernatant contains mainly peptides and
proteins and was not analyzed further. The pellet was washed with deionized water (centrifugation for 15 min at 4 °C with 4200 rpm) and the liquid discarded. The remaining pellet (metabolite fraction) was lyophilized.

The metabolite fraction was solubilized in a monophasic solvent mixture [chloroform:methanol:water, 64:36:8 (v/v), approx. 50 mL of solvent per 1 g of lyophilized metabolite fraction, enough to obtain a suspension] and macerated for 2 hours at room temperature under agitation. The extract was filtered over filter paper, washed with the solvent mixture and evaporated to dryness using a rotary evaporator.

2.4 Metabolite profiling by UHPLC-TOFMS

For high-resolution metabolite profiling, the fungal extracts were purified by solid phase extraction (SPE) on C18 silica gel (Sep-Pak® C18 1cc Vac cartridge, 105 μm particle size, Waters) using 80% methanol (HPLC grade, Sigma Aldrich) for elution to remove apolar constituents that are incompatible with reversed-phase chromatography (usually 30% of extract weight, data not shown).

The samples (two pure culture extracts, co-culture extract and blank sample from SPE) were diluted to 1 mg/mL with 80% methanol and analyzed using UHPLC-TOFMS in negative (NI) and positive ionization (PI) mode in the same experiment. The analyses were performed on a Micromass-LCT Premier time-of-flight mass spectrometer (Waters) equipped with an electrospray ionization (ESI) interface coupled to an Acquity UPLC system (Waters). The m/z range was set to 100−1000 in centroid mode with a scan time of 0.25 s and an interscan delay of 0.01 s. The ESI conditions in the PI and NI modes were as follows: capillary voltage of 2800 V, cone voltage of 40 V, source temperature of 120 °C, desolvation temperature of 250 °C, cone-gas flow of 20 L/h, and desolvation gas flow of 600 L/h. For internal calibration, a 2 μg/mL solution of leucine-enkephalin from Sigma-Aldrich (Steinheim, Germany) was infused through the lock-mass probe at a flow rate of 10 μL/min using a second Shimadzu LC-10ADvp LC pump.

The metabolite profiling was carried out on an Acquity BEH C18 150×2.1 mm 1.7 μm column equipped with a precolumn at a flow rate of 0.45 mL/min. The following solvent system was used. Solvent A, 0.1% formic acid (FA) in water, solvent B, 0.1% FA in acetonitrile, 7’ 5-40% B, 16’ 40-64% B, 1’ 64-100% B, 8’ 100% B. The temperature was maintained at 50 °C and the injection volume was 2 μL. Solvents were ULC/MS grade (Biosolve).

Base peak ion (BPI) chromatograms of the two pure culture and the co-culture analysis were used to assess metabolite induction. The molecular mass of compounds present in the co-culture only was searched in pure cultures using extracted ion chromatograms (XIC) to affirm metabolite induction.

2.5 Dereplication

For automated dereplication, native MassLynx data from UHPLC-TOFMS analyses were transferred to NetCDF (common data format) using Databridge (Waters). These files were then imported to MZmine 2 (version 2.10 [515]) for data processing and generation of peak
lists. The chromatograms were reduced to 0.5 – 30 min, and masses were detected in centroid mode with a noise level of 100. Then, peak lists were built with a minimum time span per peak of 0.1 min, a minimum intensity of 1000 and an $m/z$ tolerance of 30 ppm. The resulting peak lists were deconvoluted using the algorithm ‘local minimum search’ and deisotoped. All chromatograms were aligned using the function ‘join aligner’ and the combined list was filtered for duplicate peaks. The detailed parameters and the workflow are given in Supplementary Table V.A.5.

The detected masses within the peak lists were identified by a ‘custom database search’ using entries of Dictionary of Natural Products (DNP, version 22:1, CRC Press, Taylor & Francis). For this, entries with the biological source listed as *Fusarium*, *Gibberella* (name of teleomorph form of *Fusarium*) and *Acremonium* were exported and two CSV files were constructed that contained the CRC code as unique identifier, the compound name, the molecular formula and $m/z$ that was calculated from the exact mass (as given in DNP) by addition or subtraction of the proton mass (1.007276 Da) for the identification of compounds analyzed in PI and NI mode, respectively. Within MZmine 2, detected compounds are identified based on their exact mass with an $m/z$ tolerance of 30 ppm and by comparing isotopic patterns (heuristic filtering [65]) with a score of 50% or higher. The $m/z$ tolerance and the isotopic pattern match score were chosen large to compensate for $m/z$ errors due to alignment during data mining. Also, spectra in PI and NI mode were measured in the same chromatographic run and this diminishes the mass accuracy of the TOFMS analysis. In a second step, putatively identified compounds were searched in the original chromatograms with MassLynx (V4.1, Waters) and the molecular formula was verified using the in-built elemental composition tool (exact mass and isotopic pattern match).

2.6 **Semipreparative HPLC-MS purification of induced compound**

For the targeted isolation of the induced compound detected by metabolite profiling, the gradient used for metabolite profiling was geometrically transferred using HPLC Calculator 3.0 software (http://www.unige.ch/sciences/pharm/fanal/lcap/telechargement.htm) to a semipreparative column (XTerra® C18, 150 x 19 mm, 5 μm, Waters). The compound of interest eluted within the first gradient step of the chromatogram (5 – 40% B) and thus, the gradient was shortened to include only the first part of the gradient and the washing step.

The crude co-culture extract (12 mg) was solubilized in 80% methanol (0.23 mL) and filtered over a 0.45 μm Acrodisc® Nylon syringe filter (BGB Analytik, Böckten, CH).

The purification was performed on a modular HPLC system composed of a Varian 9012 pump (Palo Alto, CA, USA), a manual injection system (Rheodyne, IDEX Health & Science, Wertheim-Mondfeld Germany), a UV spectrometer set at 254 nm (2151 variable wavelength monitor, LKB Bromma, Pharmacia) equipped with a Pharmacia LKB-Rec 1 chart recorder, a fraction collector (FC204, Gilson, Middleton, WI, USA), an adjustable flow splitter, and an MS spectrometer (LCQ, Finnigan MAT, San Jose, CA, USA) with an ESI interface. Separations were performed using water with 0.1% FA as eluent A and acetonitrile with 0.1% FA as eluent B. A
fraction (1/170) of the flow was split to the MS detector for analysis. The following ESI conditions were used: capillary temperature, 180 °C; source voltage, 2.5 kV; and sheath gas nitrogen, 70 psi. The MS acquisitions were performed in PI mode using a full scan mode over an m/z range of 150–1000 and a scan time of 1 s.

Fractionation of the crude extract was performed on a 150 mm × 19 mm i.d., 5 μm, XTerra® Prep MS C18 ODB™ column (Waters) at 8.0 mL/min. The injection volume was 200 μL and the transferred conditions were a gradient increasing from 5% to 40% B in 32 min followed by an increase to 100% B in 0.1 min. The column was then washed for 17 min with 100% B. Fractions were collected in tubes every minute during the first 38 min and dried on a vacuum centrifuge (SpeedVac™).

2.7 Microflow NMR analysis of the isolated compound

Microflow NMR analyses were performed on a Varian Unity Inova 500 MHz NMR instrument (Palo Alto, CA, USA) equipped with a 5 μL microflow NMR probe (CapNMR™) from Protasis/MRM (Savoy, IL, USA) with an active volume of 1.5 μL. The samples were dissolved in 10 μL of CD3OD and injected manually using a microliter 25 μL syringe with a 22s gauge needle (Hamilton®). 1H NMR analysis was done with 128 transients at 298 K. Spectra were treated using MestReNova (version 8.0.2, Mestrelab Research S. L.) and chemical shifts were referenced to the residual protonated solvent signal (CD3OD, 3.31 ppm).

2.8 Targeted detection of fusaric acid

For automated and targeted analysis of fusaric acid prevalence in a previously recorded dataset of 229 chromatograms of pure cultures and co-cultures [31], MZmine 2 (version 2.10 [515]) and its ‘targeted peak detection’ function was used. Therefore, the native MassLynx files from UHPLC-TOFMS analyses were transferred to NetCDF using Databridge (Waters) and imported to MZmine 2. Only chromatograms in PI mode were processed to generate peak lists.

The chromatograms were reduced to 0.5 – 4.5 min, and masses were detected in centroid mode with a noise level of 50. The m/z value of fusaric acid was calculated using MassLynx molecular weight calculator from molecular formula and the ‘targeted peak detection’ function was applied with an m/z tolerance of 15 ppm (0.005 m/z). The resulting peak lists were deconvoluted using the Savitzky-Golay filter with a manually optimized derivative threshold level 90% (with the help of the preview function in MZmine 2) and deisotoped. All chromatograms were aligned using the function ‘join aligner’, gap filled using the function ‘same RT and m/z range gap filler’ (m/z tolerance range 15 ppm) and filtered for duplicate peaks (retention time tolerance 0.15 min). The detailed parameters are given in Supplementary Table V.AS2. The aligned chromatograms were exported to a .csv file and further analyzed in Excel. The graphs were created with SigmaPlot (12.0, Systat Software Inc.).
3. Results & discussion

As described below, several pairs of onychomycosis-derived fungi were cultured on Petri dishes and screened for morphological changes in fungal growth with the aim of finding de novo induced compounds. One co-culture was selected for in-depth analysis of its chemical constituents. Then, compound induction was monitored by UHPLC-TOFMS metabolite profiling. In a last step, induced metabolites were targeted for rapid isolation and identification by NMR by making use of recent advances in analytical chemistry (chromatography, NMR spectroscopy). Practical aspects related to the scale needed for such an isolation were also evaluated.

*Table V.A.1:* List of fast-growing fungi co-cultured with *Acremonium strictum* Sin29 in Petri dishes and visual assessment of the confrontation behavior ([31], overgrowth, contact inhibition, distance inhibition). Fungal growth was inhibited compared to growth as pure culture for all co-cultures with contact or distance inhibition.

<table>
<thead>
<tr>
<th>Overgrowth</th>
<th>Contact inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fusarium</em> sp. Sin10</td>
<td><em>F. oxysporum</em> Sin1</td>
</tr>
<tr>
<td><em>Fusarium</em> sp. Sin13</td>
<td><em>Fusarium</em> sp. Sin3</td>
</tr>
<tr>
<td><em>F. oxysporum</em> Sin14</td>
<td><em>Fusarium</em> sp. Sin4</td>
</tr>
<tr>
<td><em>Fusarium</em> sp. Sin20</td>
<td><em>F. oxysporum</em> Sin5</td>
</tr>
<tr>
<td><em>Fusarium</em> sp. Sin23</td>
<td><em>Fusarium</em> sp. Sin6</td>
</tr>
<tr>
<td><em>Fusarium</em> sp. Sin25</td>
<td><em>Fusarium</em> sp. Sin7</td>
</tr>
<tr>
<td><em>F. oxysporum</em> Sin26</td>
<td><em>Fusarium</em> sp. Sin8</td>
</tr>
<tr>
<td><em>Fusarium</em> sp. Sin27</td>
<td><em>Fusarium</em> sp. Sin9</td>
</tr>
<tr>
<td><em>F. oxysporum</em> Sin46</td>
<td><em>Fusarium</em> sp. Sin12</td>
</tr>
<tr>
<td><em>F. oxysporum</em> Sin48</td>
<td><em>F. oxysporum</em> Sin15</td>
</tr>
<tr>
<td><em>Fusarium</em> sp. Sin50</td>
<td><em>F. oxysporum</em> Sin16</td>
</tr>
<tr>
<td><em>F. oxysporum</em> Sin51</td>
<td><em>F. oxysporum</em> Sin17</td>
</tr>
<tr>
<td><em>Fusarium</em> sp. Sin52</td>
<td><em>Fusarium</em> sp. Sin19</td>
</tr>
<tr>
<td><em>Fusarium</em> sp. Sin53</td>
<td><em>F. solani</em> Sin54</td>
</tr>
<tr>
<td><em>F. solani</em> Sin56</td>
<td></td>
</tr>
<tr>
<td><em>F. oxysporum</em> Sin57</td>
<td></td>
</tr>
<tr>
<td><em>F. solani</em> Sin58</td>
<td></td>
</tr>
<tr>
<td><em>Fusarium</em> sp. Sin59</td>
<td></td>
</tr>
<tr>
<td><em>Fusarium</em> sp. Sin60</td>
<td></td>
</tr>
<tr>
<td><em>F. oxysporum</em> Sin61</td>
<td></td>
</tr>
<tr>
<td><em>F. oxysporum</em> Sin62</td>
<td></td>
</tr>
<tr>
<td><em>F. solani</em> Sin54</td>
<td></td>
</tr>
<tr>
<td><em>F. oxysporum</em> Sin2</td>
<td>not identified strain Sin22</td>
</tr>
<tr>
<td><em>Aspergillus</em> sp. Sin31</td>
<td></td>
</tr>
</tbody>
</table>

3.1 Choice of fungal co-culture for chemical analysis

Among the different fungal strains occurring in onychomycosis, isolates of *Trichophyton* spp. and NDF such as *Acremonium* spp. and *Fusarium* spp. have been identified [362]. Thus, a large number of human pathogenic NDF (mainly *Fusarium* sp.) were co-cultured with one partner. *Acremonium strictum* Sin29 was selected as a partner because of its diverse metabolite composition and fast growth. Indeed, constituent analysis of its pure culture (culture of one fungal strain in a separate Petri dish) revealed its capacity to produce chlorinated compounds based on the observed typical isotopic distribution pattern in the MS spectra (Δ 2 Da with relative intensity 3:1). This strain grows fast when cultured on the solid medium PDA, as it is able to cover a 9-cm diameter Petri dish within 1-2 weeks. Co-cultures were done on PDA in
Petri dishes and the confrontation behavior of the fungi was assessed visually. The observed confrontation types included overgrowth, contact inhibition, distance inhibition ([31], Table V.A.1). All co-cultured fungi for which a contact or distance inhibition could be observed, grew more slowly (growth inhibition) compared to their growth as pure culture. This observation might be explained by the production of volatile or water-diffusible antifungal compounds by one or both strains that, in consequence, results in growth inhibition.

The majority of tested fungi grew fast and were able to cover a 9-cm Petri dish within 1 to 2 weeks. Among these fast-growing fungi that were confronted with A. strictum Sin29, the following co-cultures were estimated to be of particular interest for further investigation based on their macroscopic appearance.

![Figure V.A.1: Picture of co-culture of Fusarium oxysporum Sin17 and Acremonium strictum Sin29 grown on solid medium in 9-cm Petri dishes.](image)

(A) shows the bottom view of the co-culture with F. oxysporum on the left (green dot) and A. strictum to the right (yellow dot). (B) shows the top view of the co-culture. The interaction zone that was excised to prepare the co-culture extract is indicated with a dashed line. (C) shows a close-up view of the altered mycelial growth in the interaction zone between the two fungi.

For three strains (Fusarium sp. Sin2, the unidentified strain Sin22 and Aspergillus sp. Sin31), a distance inhibition was observed. This confrontation pattern suggests the release of an antifungal substance into the medium that results in growth inhibition of both fungi. This ‘phenotype’ is thus intuitively interesting in the search of antifungal compounds [207]. Furthermore, five strains showed particularly clear contact inhibition: Fusarium sp. Sin1, Fusarium sp. Sin6, Fusarium sp. Sin12, F. oxysporum Sin17 and Fusarium sp. Sin54. Especially for the co-culture with F. oxysporum Sin17 (Figure V.A.1), it could be observed that hyphae of both strains were in contact but mycelial morphology was altered in the confrontation zone (Figure V.A.1C). This co-culture, A. strictum Sin29 with F. oxysporum Sin17, was chosen for an in-depth chemical analysis. Both strains were derived from nails of patients with onychomycosis that was resistant to standard treatment (azole drugs, such as terbinafine and itraconazole). The strains are pathogenic to humans.
3.2 Metabolite profiling of fungal pure culture and co-culture extracts

For the chemical investigation of secondary metabolites, one Petri dish of the co-culture of *A. strictum* with *F. oxysporum* was prepared. To get a detailed picture of the metabolite production in the interaction area, the central confrontation zone was carefully excised as shown in panel C of Figure V.A.2 to obtain the co-culture extract. One Petri dish yielded 4 mg of crude extract. Extracts of the two pure cultures and of the co-culture were analyzed in both PI and NI mode Figure V.A.2A and B).

*Figure V.A.2: UHPLC-TOFMS metabolite profiling of pure cultures of *F. oxysporum* and *A. strictum* and their co-culture.*

*Base peak ion (BPI) chromatograms (A) positive ionization and (B) negative ionization mode are shown. For the co-culture extract, the confrontation zone (C, excised zone indicated by dashed line) was cut to get an extract of the interaction zone.*
Overall, more compounds were detected in the NI mode, especially for the pure culture extracts. For the *A. strictum* pure culture extract, all detected compounds in PI were also detectable in NI but the extract constituents were better ionized in NI which led to the detection of additional compounds. Only few compounds were detected in the co-culture extract. This is in accordance with the fact that only the confrontation zone was extracted that comprises almost no fungal biomass but mainly fungal compounds that were released into the medium. From the visual inspection of the co-culture chromatograms, a compound in PI at 4 min [4, mass-to-charge ratio (m/z) 180 Da], generating an intense peak in the co-culture extract, was absent in both pure culture extracts. In addition, two other compounds with lower intensities were also detected in the co-culture extract only (2, m/z 196 at 1.5 min, 3, m/z 178 at 3.0 min). These compounds appeared *de novo* induced upon co-culture, this means, their production could thus not be observed in either of the pure cultures but only in the co-culture [31,207]. Based on the results from HRMS and the general dereplication procedure applied to theses fungal extracts (see below), the molecular formula of the most intense compound (4, m/z 180 Da) was C_{10}H_{13}NO_{2}. This information, cross searched with literature on *Fusarium* fungi metabolites, indicated that it could likely be fusaric acid. To further confirm this hypothesis, and at the same time, elaborate a strategy for the targeted isolation of induced compounds from solid medium co-cultures, 4 was targeted for isolation using semipreparative HPLC (see section “Isolation of induced compounds from solid medium co-culture extract”).

### 3.3 Automated dereplication of pure culture and co-culture extracts

In addition to the annotation of induced compounds, the obtained LC-MS profiles were also analyzed to assess the fungal metabolome by dereplication. The high-resolution chromatograms from UHPLC-TOFMS were dereplicated for known fungal metabolites reported in DNP (version 22:1, CRC Press, Taylor & Francis) based on an automated procedure using MZmine 2. As already apparent from the visual representation of the UHPLC-TOFMS chromatograms (Figure V.A.2), only a handful of compounds were detected in PI mode for all three extracts. The peak lists generated in MZmine 2 were cross-checked with *Fusarium* and *Acremonium* metabolites found in DNP. Extending the search to all microbial metabolites reported in DNP provides putative annotation for almost all detected peaks but the false positive rate can be very high. Thus, chemotaxonomic information was included in the dereplication procedure. In this way, seven peaks (Table V.A.2) could be putatively assigned based on the high mass accuracy and the spectral accuracy of the MS. In the pure culture extract of *A. strictum*, the compounds ilicicolin C (7) and acremofuranone A (6) and B (5) were annotated. Similarly, the compound HA 23 (1) was identified in the pure culture extract of *F. oxysporum*.

The three *de novo* induced compounds detected in the co-culture extract could be putatively assigned to fusarinolic acid (2), dehydrofusaric acid (3) and fusaric acid (4).
**Table V.A.2:** List of detected compounds in positive ionization (PI) UHPLC-TOFMS metabolite profiling in the extracts of *F. oxysporum* and *A. strictum* pure cultures and the co-culture that were putatively assigned to known fungal metabolites based on the Dictionary of Natural Products (version 22.1, CRC Press, Taylor & Francis). The derived molecular formulae are consecutively numbered and the position is shown in the chromatogram (Figure V.A.2).

<table>
<thead>
<tr>
<th>PI UHPLC-TOFMS metabolite profiling</th>
<th>m/z (Da)</th>
<th>rt (min)</th>
<th>Molecular formula</th>
<th>Exact mass (Da)</th>
<th>Δ (ppm)</th>
<th>CRC code</th>
<th>CAS</th>
<th>Putative identity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. oxysporum</em> pure culture</td>
<td>601.4001</td>
<td>24.2</td>
<td>C_{34}H_{52}N_{2}O_{7} (1)</td>
<td>601.3853</td>
<td>24.6</td>
<td>LQX93</td>
<td>n/a</td>
<td>HA 23</td>
</tr>
<tr>
<td>co-culture</td>
<td>196.0990</td>
<td>1.5</td>
<td>C_{10}H_{13}NO_{5} (2)</td>
<td>196.0974</td>
<td>8.2</td>
<td>FTS52</td>
<td>26108-30-5</td>
<td>Fusarinolic acid</td>
</tr>
<tr>
<td></td>
<td>178.0880</td>
<td>3.0</td>
<td>C_{10}H_{11}NO_{3} (3)</td>
<td>178.0868</td>
<td>6.7</td>
<td>FVX97</td>
<td>3626-76-4</td>
<td>Dehydrofusaric acid</td>
</tr>
<tr>
<td></td>
<td>180.1030</td>
<td>3.9</td>
<td>C_{10}H_{13}NO_{2} (4)</td>
<td>180.1025</td>
<td>2.8</td>
<td>FTS47</td>
<td>536-89-6</td>
<td>Fusaric acid</td>
</tr>
<tr>
<td><em>A. strictum</em> pure culture</td>
<td>403.1652</td>
<td>20.4</td>
<td>C_{23}H_{27}O_{3}Cl (5)</td>
<td>403.1676</td>
<td>6.0</td>
<td>PVP88</td>
<td>1114927-91-1</td>
<td>Acremofuranone B</td>
</tr>
<tr>
<td></td>
<td>421.1785</td>
<td>21.2</td>
<td>C_{23}H_{29}O_{3}Cl (6)</td>
<td>421.1782</td>
<td>0.7</td>
<td>PVP28</td>
<td>1114927-89-7</td>
<td>Acremofuranone A</td>
</tr>
<tr>
<td></td>
<td>407.1989</td>
<td>22.3</td>
<td>C_{23}H_{31}O_{3}Cl (7)</td>
<td>407.1899</td>
<td>0.0</td>
<td>HJB60</td>
<td>1202381-02-9</td>
<td>Ilicicolin C</td>
</tr>
</tbody>
</table>

In NI UHPLC-TOFMS, almost no metabolites were satisfactorily ionized in the *Fusarium* pure culture and in the co-culture extract. About 50 compounds were detected in the *Acremonium* pure culture extract whereas ilicicolin C (7), antibiotic LL-Z 1272ε (8), acremofuranone A (5) and B (6) could be putatively assigned (Table V.A.3) based on their accurate mass, isotopic pattern match and chemotaxonomic information. The structure of all these dereplicated compounds are shown in Supplementary Figure V.A.S1. Ilicicolin C and antibiotic LL-Z 1272ε are listed in DNP as *Fusarium* metabolites but both compounds were recently isolated from a sponge-derived *Acremonium* sp. as well as from fungi of other genera.

The extracted ion traces corresponding to acremofuranone A and ilicicolin C evidenced the presence of various isomers in the *A. strictum* pure culture extract. A search in DNP for the molecular formula of acremofuranone A (C_{23}H_{29}O_{3}Cl) returned three additional fungal metabolites [8′-hydroxyascochlorin (CRC code: OFL20, CAS: n/a), ascofuranone (CRC code: CKR68, CAS: 38462-04-3), chaetomugilin K (CRC code: PYM46, CAS: 1187848-03-8)]. For the molecular formula of ilicicolin C (C_{23}H_{31}O_{3}Cl), DNP search returned two additional fungal metabolites [ilicicolinic acid A (CRC code: MWZ28, CAS: 152607-06-2), LL-Z 1272α epoxide (CRC code: PZN75, CAS: 1202381-02-9)]. The detected compounds have not been automatically assigned to these other compounds as their biological source was unrelated to *Acremonium* spp. but these or derivatives thereof have been isolated from an *Acremonium* sp. [555]. Tandem MS (MS/MS) analysis might help in differentiating the compounds. Confident identification of the detected compounds would have necessitated comparison with authentic standards or subsequent isolation and NMR analysis (level 1 according to Metabolomics Standards Initiative [MSI]). Such MS-based dereplicated compounds were thus only annotated putatively (level 2 according to MSI). In this study, however, the interest was...
mainly related to the induced compounds and, in this context, the targeted isolation for confirmatory analysis by NMR was performed only on the co-culture extract.

Table V.A.3: List of detected compounds in negative ionization (NI) UHPLC-TOFMS metabolite profiling in the extracts of F. oxysporum and A. strictum pure cultures and the co-culture that were putatively assigned to known fungal metabolites based on the Dictionary of Natural Products (version 22:1, CRC Press, Taylor & Francis). The derived molecular formulae are consecutively numbered and the position is shown in the chromatogram (Figure V.A.2).

<table>
<thead>
<tr>
<th>m/z (Da)</th>
<th>rt (min)</th>
<th>Molecular formula</th>
<th>Exact mass (Da)</th>
<th>∆ (ppm)</th>
<th>CRC code</th>
<th>Putative identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>419.1608</td>
<td>15.7</td>
<td>C₂₃H₂₉O₅Cl (6)</td>
<td>419.1625</td>
<td>4.1</td>
<td>PVV28</td>
<td>Acremofuranone A</td>
</tr>
<tr>
<td>419.1607</td>
<td>17.0</td>
<td>C₂₃H₂₉O₅Cl (6)</td>
<td>419.1625</td>
<td>4.3</td>
<td>PVV28</td>
<td>Acremofuranone A</td>
</tr>
<tr>
<td>419.1636</td>
<td>17.9</td>
<td>C₂₃H₂₉O₅Cl (6)</td>
<td>419.1625</td>
<td>2.6</td>
<td>PVV28</td>
<td>Acremofuranone A</td>
</tr>
<tr>
<td>371.2205</td>
<td>18.5</td>
<td>C₂₃H₂₈O₄ (8)</td>
<td>371.2222</td>
<td>4.6</td>
<td>HJB61</td>
<td>Antibiotic LL-Z 1272e</td>
</tr>
<tr>
<td>401.1502</td>
<td>20.4</td>
<td>C₂₃H₂₉O₅Cl (5)</td>
<td>401.1520</td>
<td>4.5</td>
<td>HJB60</td>
<td>Acremofuranone B</td>
</tr>
<tr>
<td>405.1860</td>
<td>20.6</td>
<td>C₂₃H₃₁O₄Cl (7)</td>
<td>405.1833</td>
<td>6.7</td>
<td>HJB60</td>
<td>Ilicicolin C</td>
</tr>
<tr>
<td>419.1609</td>
<td>21.3</td>
<td>C₂₃H₂₉O₅Cl (6)</td>
<td>419.1625</td>
<td>3.8</td>
<td>PVV28</td>
<td>Acremofuranone A</td>
</tr>
<tr>
<td>405.1805</td>
<td>22.3</td>
<td>C₂₃H₃₁O₄Cl (7)</td>
<td>405.1833</td>
<td>6.9</td>
<td>HJB60</td>
<td>Ilicicolin C</td>
</tr>
<tr>
<td>405.1849</td>
<td>24.3</td>
<td>C₂₃H₃₁O₄Cl (7)</td>
<td>405.1833</td>
<td>3.9</td>
<td>HJB60</td>
<td>Ilicicolin C</td>
</tr>
</tbody>
</table>

3.4 Strategy for and isolation of induced compounds from solid medium co-culture extract

In order to ascertain the identity of the induced peaks and estimate their amounts, the targeted isolation was attempted on a limited number of Petri dishes. This was also used to estimate if an NMR spectrum could already be retrieved from compounds that are largely induced in the central confrontation zone of the co-culture. It was estimated that three Petri dishes would yield approx. 0.4 mg and thus sufficient sample amount for 1D and sensitive 2D NMR analyses, such as ¹H–¹H COSY (correlation spectroscopy).

For the targeted isolation of the de novo induced compounds (2, 3, 4), the SPE step was omitted to minimize sample handling and the crude extract was purified by semipreparative HPLC. The previously optimized gradient method used for UHPLC analysis was transferred geometrically [67] to an HPLC column with milligram amount loading capacity to enable the
purification of extract constituents within one purification run. The geometrical transfer assures obtaining the same chromatography (relative retention time, resolution) on the semipreparative scale as on the analytical scale. A non-optimal flow rate for the semipreparative purification had to be used (8 mL/min instead of the calculated 13 mL/min) to be consistent with pressure limitations of the HPLC instrumentation.

The injected amount of extract (10 mg) was well below the loading capacity of the column as specified by the manufacturer (approx. 250 mg) but assured to maintain a chromatographic resolution close to analytical HPLC [202]. In addition, the concentration of the injected extract depends on its solubility in the given injection volume and this is often the limiting factor for the purification of NP extracts.

The semipreparative isolation was monitored online by UV and the eluent was split such that a small part was diverted for direct PI MS analysis whereas the main part was directed to the fraction collector. The online MS monitoring permits the direct m/z assignment of every collected peak and allows the direct localization of the targeted compounds (Figure V.A.3). The de novo induced compound with m/z 180 (4) eluted at 19 min, the two minor de novo induced compounds at 16 min (3, m/z 178) and 9 min (2, m/z 196 Da). The collected microfractions were dried on a vacuum centrifuge and concentrated into HPLC vial inserts that permit maximum sample recovery within a small volume (10 μL) for later microflow NMR analysis.

![Figure V.A.3: PI MS chromatogram of semipreparative purification of A. strictum and F. oxysporum co-culture extract.](image)

The targeted induced compounds eluted at 9 (2), 16 (3) and 19 (4) min.
3.5 Identification of de novo induced metabolite by microflow NMR

The microfractions containing the targeted compounds were directly prepared for injection into the microflow NMR probe. The $^1$H NMR spectrum of (4) showed three aromatic protons and four signals consistent with a butyl chain (Figure V.A.4). The spectrum is well compatible with the structure of fusaric acid (Figure V.A.4). Comparison with a reference spectrum confirmed that fusaric acid was the targeted de novo induced compound. The particular configuration of the acidic carboxyl group in ortho-position to the pyridine moiety might explain the observation that the compound is not well ionized in Nl mode, as likely, the acidic proton is linked by a hydrogen bridge with the nitrogen atom and thus, inaccessible for deprotonation. The alkaline nature of the compound explains the poor chromatographic behavior (tailing) of this compound in LC.

![Figure V.A.4: $^1$H NMR spectrum of de novo induced compound (4) that corresponds to fusaric acid. Spectrum acquisitioned in CD$_3$OD on CapNMR™ probe (500 MHz spectrometer) at 25 °C with 128 transients. The structure of fusaric acid, the assignments and the integrals (normalized to resonance at 8.41 ppm) are given in the spectrum.](image)

The quantity of isolated compound was too low to be accurately weighed. As the peak area of the signals in $^1$H NMR is proportional to the amount of sample, an estimation of the amount can be made based on a comparison of the peak area of the residual protonated solvent signal to the peak area of a proton of the analyzed compound. The isolated quantity of fusaric acid could thus be estimated to be of approx. 100 μg. This would correspond to a concentration of almost 4 ppm in the confrontation zone. The toxicity of fusaric acid to some plant pathogenic microorganisms is in the same range [568].

It was possible to obtain a $^1$H-$^1$H COSY spectrum but only in an overnight experience (analysis time 10h30min, Supplementary Figure V.A.S2) and cross-peaks were visible for the alkyl chain, but not for the aromatic protons.
Fungal metabolites

The other two induced compounds (2, 3) were collected as well but their quantity was beyond the detection limit of our NMR instrumentation (estimated to 10 μg), even for the acquisition of a 1D $^1$H NMR spectrum.

Only three 9-cm Petri dishes were used for the isolation of the main induced compound (4). Thus, in selected cases, the isolation of the major induced fungal metabolites obtained from solid medium co-cultures is feasible at this small scale to obtain exploitable NMR spectra. The limitation of the approach, however, is strongly linked to the sensitivity of the NMR instrumentation used for spectral acquisition. In our case, 1D proton sensitivity was estimated to approx. 10 μg, but state-of-the-art systems can have lower detection limits and demanding $^1$H-$^{13}$C 2D NMR spectra can be exploited with less than 10 μg [569].

3.6 Production of fusaric acid by *Fusarium* spp.

Fusaric acid has first been reported in 1934 by Japanese researchers as a secondary metabolite of *Gibberella fujikuroi* (Sawada) Wollenw. (current name *Fusarium monoliforme*). Since then, it was reported to occur in several subspecies of the genus *Fusarium*. Studies by Bacon et al. [570] had shown that the amount of fusaric acid produced by agriculturally relevant *Fusarium* spp. was strain-dependent. In addition, the detection of fusaric acid in the fungal extract was dependent on the growth medium (corn or liquid medium). Whereas all tested *Fusarium* spp. produced fusaric acid when grown on corn, only few strains produced the compound when grown in liquid medium. Recently, it was found that trace amounts of metal (Cu$^{2+}$ and Zn$^{2+}$) suppress fusaric acid production when the fungus (here *Fusarium oxysporum* f. sp. *ciceri*) was cultured in liquid medium [571]. On the other hand, culture media that were complemented with the allelochemical $p$-hydroxybenzoic acid stimulated fusaric acid production in the plant-pathogenic *Fusarium oxysporum* f. sp. *niveum* up to 380% [572].

Accordingly, the producer of fusaric acid in the co-culture is undoubtedly *F. oxysporum*. It appears probable that the production of fusaric acid is not only inducible upon change of culture medium but also by co-culture with another fungus. Hence, this is the first example of the induction of the mycoalexin fusaric acid through microorganism co-culture.

In mammals, fusaric acid is implicated in neuromodulation and displays cytotoxicity to normal as well as cancer cell lines. For plants, fusaric acid is cytotoxic and causes necrosis [573]. Several studies suggest that fusaric acid is a virulence factor for fusariosis [571,574,575]. The upregulation of fusaric acid production in certain *Fusarium* spp. through co-inhabiting fungi might indicate that, on top of nutrient composition, the mycobiome plays a role in virulence of *Fusarium* spp. as well.

3.7 Prevalence of fusaric acid in fungal extracts

To cement the finding that the production of fusaric acid in *Fusarium* spp. is strain-dependent and its production can be induced by co-culture, this biomarker was systematically searched by extraction of its single ion trace ($m/z$ 180 at 1.4 min) in a large set of fungal strains. These
strains were isolated from nails of onychomycosis-patients, as plant pathogens or as environmental saprophytes [31].

From the 68 fungal pure-strain culture extracts, 58 were of the genus *Fusarium*. Thereof, 35 strains produced fusaric acid when grown in Petri dishes on PDA (Figure V.A.5). No clear correlation between species (i.e., *F. solani* and *F. oxysporum*) and production quantities of fusaric acid could yet be established. Work is ongoing to sequence five loci of these fungal strains and map the capacity of producing this phytotoxin on a detailed phylogenetic tree at the subspecies level (Hofstetter et al. unpublished results). Nevertheless, it is interesting to see that, under the given culture conditions, not only environmental but also human-derived *Fusarium* spp. produce fusaric acid. Whether it is produced within nails and the possible biological implications this might entail for onychomycosis remains to be studied.

![Figure V.A.5: Prevalence of fusaric acid in Fusarium pure culture extracts. Data are grouped according to the Fusarium subspecies (F. oxysporum, F. solani, F. proliferatum, F. coeruleum or not further specified). Peak area values beyond <50,000 (detection threshold) can be considered as noise.](image)

The upregulation of fusaric acid production (>2x) upon co-culture could be observed in 12 co-cultures (Figure V.A.6). More precisely, four fungi led to an upregulation in some *Fusarium* spp. from 2.8 to 18 times. For the majority of *Fusarium* spp., the production of fusaric acid was downregulated upon co-culture with a second fungus. Downregulation of around 0.5 (14 co-cultures) can be explained due to a dilution effect as two fungal metabolomes are present in the extract. Substantial downregulation (0.1 and less) was observed for 36 co-cultures. This can be explained by detoxification of fusaric acid [576].
Figure V.A.6: Upregulation of fusaric acid upon co-culture.
Targeted analysis of peak area of fusaric acid within chromatograms of metabolomics data of fungal co-cultures. Co-culture examples where the production of fusaric acid was upregulated are shown in comparison to fusaric acid production in the respective pure culture. Co-cultures where de novo induction was observed are marked in bold.

In four cases, the de novo induction of fusaric acid could be observed: F. oxysporum Sin57 with Cladosporium sp. Sin137, Fusarium sp. Sin30 with Trichophyton rubrum Sin38 and Cladosporium sp. Sin137, and Fusarium proliferatum Sin101 with Hohenbuehelia reniformis Sin138.

This study has proven the strong induction of fusaric acid as a mycoalexins for the co-culture of F. oxysporum Sin17 and Acremonium strictum Sin29. However, the monitoring of this compounds over a large number of strains indicated that the production of fusaric acid is strain-dependent and its induction is not always observed in co-culture. Furthermore, some
pure strains have shown a basal level of fusaric acid, whether in others, fusaric acid was not produced in the pure culture but the production was strongly inducible by interaction with a second strain. The exact mechanism that triggers such a metabolic response remains to be studied in more depth to understand if specific conditions are needed for the upregulation of this mycotoxin. This study also proves the feasibility to obtain exploitable NMR spectra of major induced metabolites from fungal co-cultures that are isolated from as few as three 9-cm Petri dishes.

4. Acknowledgments

Philippe J. Eugster and Trixie A. Bartholomeusz are acknowledged for providing UHPLC-TOFMS analyses and for assistance in microflow NMR analysis, respectively. This work was supported by the Swiss National Science Foundation Sinergia Grant CRSII3_127187, which was awarded to Jean-Luc Wolfender, Michel Monod and Katia Gindro. Mariam Mnatsakanyan and Trixie A. Bartholomeusz are thanked for helpful comments on structure and language of the manuscript.

5. Supplementary information

5.1 Chemical structures of dereplicated molecules

Figure V.A.S1: Structures of dereplicated molecules.
The numbers correspond to its molecular formula and its occurrence in the different chromatograms is given in Figure V.A.2 and Table V.A.2.
## 5.2 Code correspondence of fungal strains

*Table V.A.S1: Code correspondence of fungal strains used in the sinergia project CRSII3_127187 with CHUV number, newly assigned Agroscope number and species identity.*

<table>
<thead>
<tr>
<th>CHUV number</th>
<th>Agroscope number</th>
<th>species</th>
<th>CHUV number</th>
<th>Agroscope number</th>
<th>species</th>
</tr>
</thead>
<tbody>
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<td>2309</td>
<td>Sin33</td>
<td>Trichophyton rubrum</td>
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<tr>
<td>201</td>
<td>Sin2</td>
<td>Gibberella / F. oxysporum</td>
<td>2344</td>
<td>Sin34</td>
<td>T. rubrum</td>
</tr>
<tr>
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<td>Fusarium sp.</td>
<td>2348</td>
<td>Sin35</td>
<td>T. mentagrophytes</td>
</tr>
<tr>
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<td>2354</td>
<td>Sin36</td>
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</tr>
<tr>
<td>468</td>
<td>Sin5</td>
<td>Gibberella / F. oxysporum</td>
<td>2369</td>
<td>Sin37</td>
<td>Scopulariopsis brevicaulis</td>
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<tr>
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<tr>
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<td>Sin46</td>
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<td>3214</td>
<td>Sin56</td>
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<td>Sin57</td>
<td>Gibberella / F. oxysporum</td>
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<tr>
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<td>3428.2</td>
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<tr>
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<td>Sin32</td>
<td>Aspergillus sp.</td>
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<td></td>
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</table>
### 5.3 MZmine parameters

**Table V.A.S2:** Steps and parameters used during the automatic peak picking procedure by MZmine 2 for dereplication in metabolite profiling chromatograms. The same parameters were used for NI and PI UHPLC-TOFMS data.

<table>
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<th>Parameters</th>
<th>Value</th>
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</thead>
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<td>Crop filter</td>
</tr>
<tr>
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<td>Retention time (min)</td>
</tr>
<tr>
<td>2) Raw data methods -&gt; Peak detection -&gt; Mass detection</td>
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<td>Centroid</td>
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<td>Noise Level</td>
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<td></td>
<td>MS level</td>
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<tr>
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<tr>
<td></td>
<td>Min height</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>m/z tolerance (ppm)</td>
<td>30</td>
</tr>
<tr>
<td>4) Peak list methods -&gt; Peak detection -&gt; Chromatogram deconvolution</td>
<td>Algorithm</td>
<td>Local minimum search</td>
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<td></td>
<td>Chromatographic threshold (%)</td>
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<td></td>
<td>Search minimum in RT range (min)</td>
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<tr>
<td></td>
<td>Minimum relative height (%)</td>
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</tr>
<tr>
<td></td>
<td>Minimum absolute height</td>
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<td>Peak duration range (min)</td>
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</tr>
<tr>
<td></td>
<td>Retention time tolerance (min)</td>
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<tr>
<td></td>
<td>Maximum charge</td>
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<tr>
<td></td>
<td>Representative isotope</td>
<td>Most intense</td>
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</tr>
<tr>
<td></td>
<td>Weight for m/z</td>
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</tr>
<tr>
<td></td>
<td>Retention time tolerance (min)</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Weight for RT</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Compare isotope pattern</td>
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</tr>
<tr>
<td></td>
<td>Isotope m/z tolerance (ppm)</td>
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</tr>
<tr>
<td></td>
<td>Minimum absolute intensity</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Minimum score (%)</td>
<td>50</td>
</tr>
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<td>7) Peak list methods -&gt; Filtering -&gt; Duplicate peak filter</td>
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<td></td>
<td>RT tolerance</td>
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<tr>
<td>8) Peak list methods -&gt; Identification -&gt; Custom database search</td>
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<td></td>
<td>Retention time tolerance (absolute, min)</td>
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Table V.A.S3: Steps and parameters used during the automatic peak picking procedure by MZmine 2 for targeted analysis of fusaric acid in PI UHPLC-TOFMS fingerprinting analyses.

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<tr>
<th>Steps</th>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Raw data methods -&gt; Filtering -&gt; Data set filtering</td>
<td>Filter</td>
<td>Crop filter</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Retention time (min)</td>
</tr>
<tr>
<td>2) Raw data methods -&gt; Peak detection -&gt; Targeted peak detection</td>
<td>Intensity tolerance (%)</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>m/z tolerance (ppm)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Retention time tolerance (min)</td>
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</tr>
<tr>
<td>3) Peak list methods -&gt; Peak detection -&gt; Chromatogram deconvolution</td>
<td>Algorithm</td>
<td>Savitzky-Golay</td>
</tr>
<tr>
<td></td>
<td>Min peak height</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Peak duration range (min)</td>
<td>0 to 10</td>
</tr>
<tr>
<td></td>
<td>Derivative threshold level (%)</td>
<td>90</td>
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<tr>
<td>4) Peak list methods -&gt; Alignment -&gt; Join aligner</td>
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</tr>
<tr>
<td></td>
<td>Weight for m/z</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Retention time tolerance (min)</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>Weight for RT</td>
<td>10</td>
</tr>
<tr>
<td>5) Peak list methods -&gt; Gap filing -&gt; Same RT and m/z range gap filler</td>
<td>m/z tolerance (ppm)</td>
<td>15</td>
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<tr>
<td>6) Peak list methods -&gt; Filtering -&gt; Duplicate peak filter</td>
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<td>15</td>
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<td></td>
<td>RT tolerance</td>
<td>0.15</td>
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</table>
Figure V.A.S2: $^1$H-$^1$H COSY spectrum of de novo induced compound (4) that corresponds to fusaric acid.
Cross-peaks are visible for the alkyl-chain. The assignment corresponds to the numbers given in Figure V.A.4.
VI. Large-scale isolation strategies for co-culture extracts
In my opinion, and with the experience of this thesis on working with plant and fungal extracts (chapters III and VI), there are two reasons why classical isolation strategies that were primarily developed for plant extracts, are not easily applicable to the isolation of fungal metabolites. The first aspect is the high prevalence of secondary metabolites with strong chromophores (see also chapter III.A). Nevertheless, consistent use of ELSD-guided isolation, as now adopted by many NP research laboratories, helps to circumvent this problem. The second aspect is the high content of primary metabolites, e.g., sugars and fatty acids, in extracts of solid medium-cultured fungi. An adapted prefractionation strategy was adopted for these fungal extracts and this is outlined in chapter VI.A. The gained experience was applied to the chemical investigation of the co-culture of Basidiomycete *Hohenbuehelia reniformis* and the onychomycosis-derived and human pathogenic *Fusarium solani*. This project is described in chapter VI.B. Many practical aspects, particular to the work with solid medium co-cultures, were assessed during the work on these samples and thus, supplementary information is given in chapter VI.C.
VI.A Strategies towards handling of fungal extracts produced at larger scale: NMR analysis and prefractionation procedure
1. Introduction

Microorganism co-culture has shown successful in inducing the production of secondary metabolites (see chapter II.A and V.A). Nevertheless, the isolation of such induced metabolites is cumbersome and obtaining enough pure compound from solid medium co-cultures for bioactivity assessment was only successful for selected examples [30].

In consequence, further co-culture experiments were planned that include a much bigger number of Petri dishes to obtain several tens of grams of fungal extract to work with (chapter VI.B). The microfractionation strategy that has been developed (see chapter IV and V) was thus not compatible with these large-scale amounts anymore. Some aspects that are important for the compatibility of these fungal extracts with the analysis of the chemical constituents (metabolite profiling) and isolation of milligram-amounts of fungal metabolites are discussed here.

2. Results & discussion

Many of the fungal extracts were brightly colored which indicates that they contain many pigments. Such compounds are easily detectable because of their important chromophores, but their real abundances in the extract is in fact very low. In order to isolate such minor extract constituents, larger amounts of fungal extract were produced. In this respect, it is important to assure that the extract composition is not significantly altered when the fungi are grown in larger Petri dishes. This could be successfully achieved and verified by careful metabolite profiling and had been demonstrated by Bertrand et al. [577].

Semipreparative HPLC is not feasible for the rapid purification of gram-amounts of extracts, as only tens of milligrams can be loaded on the HPLC columns compatible with this approach. Preparative chromatographic methods, such as Flash chromatography or medium pressure liquid chromatography (MPLC), do not provide the same chromatographic resolution as semipreparative HPLC but have shown successful for the rapid isolation of plant metabolites in sufficient amounts for biological testing [578]. Based on such considerations, an optimized enrichment step was found mandatory prior to a rapid and efficient isolation of fungal constituents. Thus, in a first step, different crude fungal extracts were analyzed to get an overview on the most abundant compound classes present in the extracts to select appropriate enrichment methods. In a second step, different prefractionation strategies were tested and evaluated for their specificity and applicability. Then, the most suitable strategy was applied to the extract of a co-culture that was subsequently studied in detail for the isolation of its secondary metabolites (see section 2.3 and chapter VI.B).

2.1 Composition of solid medium fungal extract

Metabolite profiling by liquid chromatography (LC) with mass spectrometry (MS) detection is the established method for a first evaluation of the chemical composition of natural extracts [59,579]. Nevertheless, several intrinsic characteristics entail that LC-MS gives only a biased overview on the extract content. For LC-MS analysis, a simple sample preparation step of the
crude extract, such as solid phase extraction (SPE), is often necessary to avoid alteration of the chromatographic performance of the column or to prevent contamination of the MS. In addition, samples need to be solubilized in hydro-organic solvents for reversed-phase (RP) LC and filtered prior analysis to maintain optimized chromatographic resolution. In consequence, insoluble extract constituents are not analyzed. Furthermore, detection is restricted to ionizable compounds. Thus, LC-MS does not permit seeing the integrality of an extract composition. Furthermore, MS response is strongly compound dependent (see introduction I.A and [37]).

Figure VI.A.1: Composition of different extracts from fungi cultivated on solid medium assessed by $^1$H NMR.
Spectra of three pure culture extracts, measured in either deuterated dimethylsulfoxide (DMSO-$d_6$) or deuterated methanol (CD$_3$OD).

Analysis of samples by nuclear magnetic resonance spectroscopy (NMR), on the other hand, permits to see all organic (proton-bearing) extract constituents and can be regarded as universal from this viewpoint. Sample preparation is reduced to simple dissolution of the extract in a solvent that permits to solubilize, in the best case, all extract constituents. However, NMR is significantly less sensitive than MS. Advantages and drawbacks related to NMR detection in natural product research are discussed in chapter I.B and I.C [49,167].
After lyophilization of the culture medium and extraction, several fungal extracts were thus analyzed by $^1$H NMR to assess the extract composition (Figure VI.A.1) and to obtain preliminary unbiased quantitative information on their main constituents. As proton chemical shifts in NMR analysis depend on the neighboring functional groups, the observed chemical shift ranges can be, to some extent, associated to functional groups and thus to compound classes. All extracts showed predominant proton signals between 3 and 4 ppm, which is indicative for protons at secondary and tertiary carbons in sugar molecules. Depending on the fungus and, probably, the culture time, more or less glucose from the culture medium was found present in the extracts. For example, comparing the $^1$H NMR spectrum of uninoculated PDA with the co-culture of *H. reniformis* and *F. solani*, compounds dominating the composition of the culture medium, e.g., glucose, were found as main constituents of the co-culture extract (Figure VI.A.2). However, *Hohenbuehelia* spp. are known to produce a variety of polysaccharides [580] and signals associated to the polysaccharides produced by the fungus can not be distinguished from the culture medium constituents. For the pure culture of *T. rubrum*, almost no sugars were detected in the extract. This fungus was cultivated for eight weeks and had probably used up all the provided nutrients [577]. Many fungi are known to produce a variety of fatty acids and this is observable in the spectra of *F. solani* and *T. rubrum* as well where proton signals between 1 and 1.5 ppm are indicative for the alkyl chains of fatty acids.

Thus, fungal extracts mainly contain fatty acids and sugars. The secondary metabolites of interest represent only minor constituents compared to these matrix compounds or primary fungal metabolites. Therefore, an appropriate enrichment method should be effective in the removal of these very polar (sugars) and apolar (fatty acids) molecules. SPE is a widespread
technique at the analytical level and allows removing polar and apolar molecules in one operation. Different sorbent materials exist and selected materials were thus tested for optimal selectivity. The procedure was adapted for its applicability to gram-amounts of fungal extracts.

2.2 Prefractionation strategies for fungal extracts

For SPE in NP research, reversed phase sorbents [silicagel derivatized with C\textsubscript{18} alkyl chains (RP silicagel or C\textsubscript{18} silicagel)] is one of the most commonly used. Application of a step gradient on such a sorbent (stationary phase) would allow separating polar and apolar extract constituents. A dry loading of the extract was necessary, as extracts were only partially soluble in water and because the first elution step is with 100% water to elute the very polar compounds. Hence, the samples were adsorbed onto the filter medium kieselgur (known by the brand name celite) that allows for non-selective desorption with polar and apolar solvents and less irreversible adsorption of the sample. The elution steps for SPE were as follows: 100% water, 20%, 50% and 85% aqueous methanol (MeOH), followed by 100% MeOH and dichloromethane. The first step (100% water) would elute polar constituents such as sugars and salts. The following steps (20%, 50% and 85% MeOH) elute medium polar constituents that are well compatible with further analysis by RP LC. The later steps with pure MeOH and dichloromethane elute apolar constituents that are incompatible with RP LC. These compounds could however be collected but have to be further analyzed by normal phase LC.

![Figure VI.A.3: SPE prefractionation of three different fungal extracts using C\textsubscript{18} silicagel.](image)

Elution of fungal extract using different eluents. Eluents yielding prefractions that are compatible with reversed-phase liquid chromatography analysis are shaded grey.

Three different extracts from fungi cultivated on PDA were then subjected to C\textsubscript{18} SPE. The mass balance of the different prefractions obtained by C\textsubscript{18} SPE is given in Figure VI.A.3. Only the first prefraction (very polar constituents) and the prefractions yielding ‘chromatographable’ compounds (grey-shaded bars) are shown. The apolar prefractions are all summarized as ‘apolar fraction’. Depending on the individual fungal extract, the different prefractions yielded different amounts. The pure culture extract of \textit{T. rubrum} contained 8% of polar constituents whereas the pure culture extract of \textit{Acremonium strictum} contained 1%
and the co-culture extract more than 70%. This is in accordance with $^1$H NMR analyses (Figure VI.A.1).

The obtained prefractions were analyzed by $^1$H NMR and UHPLC-TOFMS. The example of the pure culture extract of *A. strictum* is shown in Figure VI.A.4. According to NMR and UHPLC analysis, the first three prefractions resemble each other as do the last two prefractions. The range of 4 to 8 ppm in $^1$H NMR is of particular interest for the detection of secondary metabolites as it includes protons belonging to, or in close proximity to, aromatic rings, unsaturations, alcohols and ketones as present in polyketides and terpenes that have shown interesting bioactivities [486]. In the example of *A. strictum* (Figure VI.A.4), those compounds were enriched in the 85% MeOH and pure MeOH fractions. This was coherent with UHPLC-TOFMS analysis, were more medium polar compounds with good ionizability were detected. Specifically for *A. strictum*, and in contrast to many other fungal extracts, the apolar extract constituents were not fatty acids but apolar polyketides as can be seen from the $^1$H NMR spectrum of the MeOH prefraction. In general and also in comparison with prefractions from other fungal and plant extracts (data not shown), the prefractionation on C$_{18}$SPE did not allow to obtain selective desorption for the three elution steps that yield chromatographable extracts. Thus, the 20% and 50% aqueous MeOH elution steps were omitted in subsequent applications.

![Figure VI.A.4: UHPLC-TOFMS and $^1$H NMR analysis on prefractions of pure culture extract of Acremonium strictum.](image)

Analyses of five prefractions are shown, elution with water, 20%, 50% and 85% aqueous MeOH and pure MeOH. The positive ionization mode (PI) UHPLC-TOFMS chromatograms are normalized to 5000 ion counts for the three polar prefractions and to 50,000 ion counts for the two more apolar prefractions. For the $^1$H NMR spectra, the two more polar prefractions were analyzed in deuterated water (D$_2$O), the remaining prefractions in deuterated methanol (CD$_3$OD).
A second sorbent was evaluated for its compatibility with fungal extracts, the polystyrene-based porous HP20SS [17]. This sorbent had been employed successfully for the fractionation of marine invertebrate extracts and had two distinct advantages over C$_{18}$ silicagel: a higher loading capacity and orthogonal chromatographic behavior to RP LC [36]. The prefractionation strategy was adapted from Bugni et al. [36] and applied to the co-culture extract of *H. reniformis* and *F. solani*. The mass balance of the obtained prefractions in comparison to C$_{18}$ SPE is shown in Figure VI.A.5. HP20SS SPE had the advantage of yielding two prefractions that are compatible with later RP LC analysis (elution with 25% aqueous 2-propanol (iPrOH) and 50% aqueous iPrOH), compared to C$_{18}$ SPE. In addition, a larger amount of extract was recovered for later purification (9% in HP20SS SPE vs. 6% in C$_{18}$ SPE). Prefractionation with both sorbents was effective in separating out the most polar extract constituents as well as the apolar compounds. $^1$H NMR analysis showed no characteristic signals for sugars and fatty acids in the two chromatographable prefractions (Figure VI.A.6). Furthermore, these two prefractions had clearly different composition (particularly striking in HPLC-ELSD analysis, Figure VI.A.6) which indicates that HP20SS SPE permits the selective desorption of secondary metabolites. The sorbent material HP20SS is thus well compatible for the enrichment of fungal extracts for the chemical analysis of secondary metabolites.

The amount of extract that can be purified per amount of sorbent (loading capacity) is higher for HP20SS than for C$_{18}$ silicagel. Approximately seven times more C$_{18}$ silicagel is needed for effective SPE (2 g C$_{18}$ silicagel per 300 mg crude extract), whereas for HP20SS, only twice the amount is needed (600 mg of HP20SS per 300 mg crude extract [36]). In the project on the co-culture of *H. reniformis* and *F. solani*, about 100 g of crude extract would need to be purified, which would necessitate 700 g of C$_{18}$ silicagel or 200 g of HP20SS. Because of the more selective desorption behavior and the higher loading capacity, SPE with the sorbent material HP20SS was privileged for this large-scale prefractionation.

![Figure VI.A.5: SPE prefractionation of a fungal co-culture extract using two different adsorbent material, C$_{18}$ silicagel and HP20SS. Elution of fungal extract using different eluents. Eluents yielding prefractions that are compatible with reversed-phase liquid chromatography analysis are shaded grey.](image-url)
**Figure VI.A.6: $^1$H NMR and HPLC-ELSD-MS analysis on prefractions of co-culture extract of H. reniformis and F. solani.**

Analyses of the two chromatographable prefractions are shown, elution with 25% and 50% aqueous iPrOH. For the $^1$H NMR spectra, the prefractions were analyzed in deuterated methanol (CD$_3$OD). The spectrum of the prefracion eluted with 100% water (blue trace) is shown for comparison. For PI HPLC-ELSD-MS, chromatograms are normalized to 1e8 ion counts for MS detection.

**2.3 Large-scale prefractionation using HP20SS SPE**

Large amounts of extract of the *H. reniformis* and *F. solani* co-culture were generated (see chapter VI.B for more information on this particular co-culture). Whereas SPE for smaller extract amounts had been done using a SPE manifold, prefractionation for tens of grams of extract had to be aided by a pump and columns had to be manually packed for this purpose.

The crude extract (in total approx. 115 g) of the co-culture was fractionated in two steps (35 and 80 g) using a low pressure preparative LC (LPLC) Flash instrument (Figure VI.A.7). Two columns were connected in succession as no sufficiently large column was available that would take up all sorbent material. Columns for fractionation were filled with an additional volume of sorbent to increase chromatographic efficiency. This was necessary as it has been observed that sugars were not completely eluted in the first elution steps with the sorbent amounts used in preliminary studies. Indeed, this resulted in ‘sugar-free’ chromatographable fractions (Figure VI.A.9). Compounds of deeply red color were concentrated in the two chromatographable prefractions (Figure VI.A.8) and the more apolar prefractions contained mainly fatty acids (Figure VI.A.9). In total, 2.7 g of material (dry weight) were obtained in the prefractions eluted with 50% iPrOH (P3, 2.3%) and 0.5 g in the prefracion eluted with 25% iPrOH (P2, 0.4%). The apolar prefractions contained a total of 7.1 g (P4 to P6, 6.1%). The more polar prefractions (P0 and P1) were not concentrated to dryness.
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3. Conclusion

In conclusion, extracts from fungi cultivated on solid media were successfully enriched in aromatic compounds and pigments using HP20SS SPE. The main extract constituents such as
sugars and fatty acids were selectively removed. Two enriched prefractions were generated that were compatible with further compound purification using RP LC. The prefractionation strategy was efficaciously applied to the fractionation of several tens of grams of crude extract in one operation using a LPLC Flash instrument. This prefractionation step should facilitate the later rapid isolation of microbial constituents since it provides an efficient enrichment in secondary metabolites as shown by the NMR and HPLC analyses performed. The procedure was integrated into a project on the isolation of antifungal compounds that were induced upon co-culture of two fungal strains (see chapter VI.B).

4. Materials & methods

4.1 NMR analyses

Analyses were done on a Varian INOVA 500 MHz instrument (Agilent) using a 5-mm tube probe. Crude extracts of the uninoculated agar, pure cultures and co-culture were measured in CD$_3$OD or DMSO-$d_6$, prefractions were analyzed in D$_2$O, CD$_3$OD or CDCl$_3$. Deuterated solvents were obtained from Armar Chemicals.

4.2 Extraction of solid medium-cultured fungi

The different fungi were cultured in Petri dishes on PDA. Prior extraction, the culture medium with the fungus was cut in 1-cm pieces, frozen with liquid nitrogen and lyophilized. The dried pieces were transferred to adapted glassware for extraction with either the monophasic solvent mixture dichloromethane-MeOH-water (64:36:8, v/v) or with dichloromethane (HPLC grade) or MeOH (technical grade). For the *T. rubrum* and the *A. strictum* sample (as all small-scale cultures), the material was extracted in a water-bath sonicator at room temperature for 20 min. For large-scale cultures, here the pure culture of *F. solani* and *H. reniformis* and the corresponding co-culture sample, the material was extracted twice by maceration for 24 h. The extracts were dried under reduced pressure.

4.3 Prefractionation

For the comparison of the prefractionation efficacy of SPE using C18 silicagel and HP20SS, different fungal extracts were used, pure culture extract of *T. rubrum* (Sin38) and *A. strictum* (Sin29, see chapter V.A for more information on this sample) and co-culture extract of the co-culture of *H. reniformis* and *F. solani* (*H. reniformis* Sin138 and *F. solani* Sin58, see chapter VI.B for more information on this particular co-culture). For C18 SPE, the extract (250 mg) was solubilized and mixed in a mortar with approx. four times more kieselgur (für Säulenchromatographie, Merck) than sample (w/w), thus 1g of kieselgur for 250 mg of sample. The suspension was dried at 50 °C in the oven and the dried mixture was ground to a fine powder. The adsorbed extract was added to a pre-packed C18 SPE cartridge (2 g, 12cc, Waters Sep-Pak C18). The extract was eluted with five column volumes (15 mL) of the following elution solvents: water, 85% aqueous MeOH, MeOH and dichloromethane. For HP20SS SPE, the extract (250 mg) was solubilized and mixed in a mortar with twice the amount of Diaion® HP20SS (Supelco) compared to the sample amount (w/w), thus 500 mg of HP20SS for 250 mg
of sample. The suspension was dried at 50 °C in the oven and the dried mixture was ground to a fine powder. The adsorbed extract was filled into an empty Flash cartridge (15 g solid loaders, Grace) and the extract eluted with five column volumes (15 mL) of the following eluents: water, with 25%, 50% and 75% aqueous iPrOH (for GC, Sigma-Aldrich) and MeOH. The obtained prefractions were dried under nitrogen flux. The SPE procedure was performed using a SPE manifold (Alltech).

For the large-scale prefractionation of the co-culture extract (H. reniformis and F. solani), the procedure was slightly adapted. The altered steps are detailed here. The suspension of extract and HP20SS was dried under reduced pressure (Rotavap). The SPE was performed using a Flash instrument (SpotFLASH, Armen) operated at a maximum pressure of 22 bar at 35 to 50 mL/min. For that, an additional volume of HP20SS was filled into two glass column coupled in succession (Sepacore C-690, 460 × 36 mm and 460 × 26 mm, Büchi), the adsorbed extract was added on top.

4.4 UHPLC-TOFMS analyses

UHPLC-TOFMS analyses were performed using an Acquity™ UPLC chromatograph and a Micromass-LCT Premier Time of Flight mass spectrometer equipped with an ESI interface (Waters). For the metabolite profiling, separation was performed on a 150 × 2.1 mm i.d., 1.7 μm, Acquity BEH C18 UPLC column (Waters) in the gradient mode at a flow rate of 0.46 mL/min with the following solvent system: A = 0.1 vol% formic acid (FA)-H2O, B = 0.1 vol% FA-acetonitrile (MeCN); 5–95% B in 30 min. The injected volume was 2 μL. Detection was performed in PI and NI mode in the range m/z 100-1500 in centroid mode with a scan time of 0.3 s. ESI conditions were: capillary voltage 2800 V, cone voltage 40 V, MCP detector voltage 2450 V, source temperature 120 °C, desolvation temperature 350 °C, cone gas flow 20 L/h, and desolvation gas flow 800 L/h. The mass spectrometer was internally calibrated by infusion of a solution of leucine-enkephalin (2 μg/mL, Sigma-Aldrich) through the lockmass spray probe at a flow rate of 10 μL/min, using a second Shimadzu LC-10ADvp LC pump.

For the analysis of prefractions of A. strictum, separation was performed on a 50 × 1 mm i.d., 1.7 μm, Acquity BEH C18 UPLC column (Waters) in the gradient mode at a flow rate of 0.3 mL/min with the following solvent system: A = 0.1 vol% FA-H2O, B = 0.1 vol% FA-MeCN; 5–95% B in 4.9 min. The injected volume was 1 μL. Samples were prepared at 0.2 mg/mL. Detection was performed in PI and NI mode in the range m/z 100-1000 in centroid mode with a scan time of 0.3 s. ESI conditions were: capillary voltage 2800 V, cone voltage 40 V, MCP detector voltage 2450 V, source temperature 120 °C, desolvation temperature 300 °C, cone gas flow 20 L/h, and desolvation gas flow 600 L/h.

4.5 HPLC-ELSD-MS analyses

HPLC-ELSD-MS analyses were performed on a HP1100 chromatograph, hyphenated to an ELS detector (Sedex 85 LT-ELSD, Sedere) and through a Thermo Scientific ESI interface to an ion trap MS instrument (LCQ, Thermo Scientific). Separation was performed on a 250 × 4.6 mm
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i.d., 5 μm, XBridge C₁₈ HPLC column (Waters) in gradient mode at a flow rate of 1 mL/min with the following solvent system: A = 0.1 vol% FA-H₂O, B = 0.1 vol% FA-MeCN; 5-95% B in 25 min. The injected volume was 20 μL and samples were prepared at 5 mg/mL in 85% aqueous MeOH. Detection was performed in PI mode in the range of m/z 150-1000 in centroid mode. An in-source fragmentation of 5 V was applied to reduce formation of adducts. The ELSD was operated at 2 bar of nitrogen gas, drift tube temperature 40 °C and gain 7. A splitter enabled 250 μL/min of the flow coming from the HPLC to go to the ELS detector and 150 μL/min to enter the MS.

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VI.B Induced pigments and anti-*Fusarium* compounds from the co-culture of *Hohenbuehelia reniformis* and *Fusarium solani*

This research chapter, together with chapter VI.C as supplementary information, will be prepared for submission as original research article.

Co-authors are: O. Schumpp, F. Mehl, S. Schnee, L. Marcourt, S. Bertrand, M. Monod, K. Gindro and J.-L. Wolfender

Contribution: Design of experiments, execution of extraction, compound isolation and metabolite profiling, development of prefractionation procedure, performance of dereplication, metabolomics study and metabolomics data treatment, performance of antifungal tests, interpretation of results and writing of the manuscript
Abstract

Microorganism co-culture (combined growth of two or more microorganisms) was chosen to search for possible anti-Fusarium compounds. For this, the Basidiomycete *Hohenbuehelia reniformis* was co-cultivated with a human pathogenic strain *Fusarium solani* isolated from patients suffering from onychomycosis.

The co-culture of both fungi in the same Petri dish strongly induced the release of red pigments in the growth medium and a distinct distance repulsion between the strains was clearly observed. An LC-HRMS-based metabolomics study proved to be efficient in detecting upregulation of several metabolites. On the other hand, the upregulation of pigment production could only be confirmed using a targeted UHPLC-UV analysis.

Several *Fusarium* pigments were isolated from the solid medium co-culture extract at large scale and their structure was confirmed by 1D and 2D NMR analysis. In addition, seven novel hydroquinones and quinones and one novel pyrone were isolated and their structure elucidated by extensive spectroscopic analysis. Hydroquinones exhibited moderate antifungal activity. These hydroquinones and quinones may be responsible for the repulsion observed when *H. reniformis* and *Fusarium solani* were co-cultivated.

This work discusses how to link results obtained from MS-based metabolomics (identification of biomarkers as MS features) and the targeted isolation of such biomarkers for de novo structure elucidation by NMR and assessment of biological activity. It also demonstrates that pigments, quinones and hydroquinones are strongly inducible in fungal co-cultures.

**Keywords:** co-culture, *Hohenbuehelia reniformis*, *Fusarium solani*, induction, solid culture medium, metabolomics, antifungals
1. Introduction

Microorganisms are promising candidates as producers of novel and bioactive metabolites. Well-known examples and important drug leads include the β-lactams (antibiotics, penicillin), macrolides with antibiotic (erythromycin), antifungal (amphotericin B) or immunosuppressant activity (cyclosporin) and statins (cholesterol-lowering agent, lovastatin). Compared to other natural resources, microbes are presently a very trendy source of natural products (NPs) in drug discovery [5]. There are several reasons for that. Firstly, microorganisms occur ubiquitously in various ecosystems, e.g., in the lithosphere [581,582], on organic substrates as in soil [312,393], as endophytes in close association with plants [243], or in the sea [244,583]. Moreover, technical advances now permit access to these habitats. Secondly, an extensive biodiversity is expected among fungi and bacteria [245,584]. Furthermore, an important chemodiversity is possible within given species [11]. Thirdly, newly developed molecular biology methods permit access to so far uncultivable microorganisms [246,247].

Genomic analyses of several fungal strains have shown a much larger number of biosynthetic gene clusters than what was expected from the number of isolated compounds from fungi [261]. It is thus expected that many gene clusters are silent under standard laboratory conditions [565]. Efforts are now undertaken to activate those pathways to access the chemical diversity that fungi might be capable of [256]. One way to stimulate the biosynthesis of secondary metabolite is to apply stress, either abiotic or biotic. Abiotic stress [278] is employed through starvation, application of UV or, alternatively, through culture medium supplementation with various inhibitors as epigenetic modifiers [282]. Biotic stress is applied, e.g., through the interaction with another microbial partner. This may lead to the potential discovery of new leads and might help to understand the triggering of specific biosynthetic pathways, mainly related to defense [585-587].

The application of biotic stress mimics the fact that microorganisms are often organized in communities (microbiome, the assembly of all living microbes in a defined environment). Within a microbiome, secondary metabolites are produced for defense or nutrient competition and NP production is often accompanied by morphological changes. These interaction experiments help entangle interspecies crosstalk, e.g., in the framework of chemical ecology studies [300], or the gained knowledge can be used to prospect new strains with the potential to produce bioactive compounds [294,588]. Otherwise, artificial communities can be constructed with the sole goal to discover new bioactive compounds.

Among the fungi, species of the genus *Fusarium* are ubiquitously found in nature as common soil saprophytes or as plant pathogens. Furthermore, the prevalence of *Fusarium* spp. as causative agent of fungal nail infection (onychomycosis) is rising [225] and *Fusarium* species, mostly *F. solani* and *F. oxysporum*, have emerged as major opportunistic fungi in patients with severe immunosuppression. Not all onychomycosis-causing fungi respond to current antifungal treatment (azoles, e.g., terbinafine, miconazole) and especially *Fusarium* spp. seem
insensitive to azole drugs [589]. Hence, new antifungal agents active against *Fusarium* spp. are needed.

The presence of a microbiome of several filamentous fungi in onychomycosis-infected human nails could be made apparent recently [362]. This fungal microbiome (the mycobiome [304]), a community of fungal strains, has recently raised considerable research interest [566]. The combined growth (co-culture) of onychomycosis-derived fungi has already shown to be successful in inducing the production of bioactive compounds ([590], chapter VA.). Similar morphological observations are made for co-cultures with fungal strains coming from different ecosystems [31]. In addition, the induction of novel metabolites could be shown for a co-culture of a onychomycosis-derived dermatophyte and a soil isolate [207] which constitutes an example for the establishment of an artificial community. With the help of such artificial communities, interactions and induced metabolites can be studied at the molecular level. Metabolites that are induced in the interaction likely play an important role in defense and may be exploited in the search of new antifungals.

For the detection of induced metabolites, metabolite profiling, the chromatographic analysis of extract constituents with both high resolution in the LC and the MS dimension [59], is useful [31]. The identification of known NPs in comparison with database entries or authentic standard samples is possible by LC-MS at the nano- or picogram level. Nevertheless, NMR spectroscopy is indispensable for the identification of unknown compounds or for the discrimination between isomers [47,55]. Recent advances in NMR probe design, with the commercialization of cryogenically cooled and miniaturized probes, such as the MicroCryoProbe™ that was introduced in 2007, the detection limit for $^1$H NMR spectra was pushed down to the low microgram range [569]. This opens up a whole range of possibilities for the analysis of metabolites that cannot be obtained in large quantities, such as very minor extract constituents or metabolites from mass-limited samples that were below NMR detection limits so far ([207] and chapter V.A). However, milligram-amounts of sample are still necessary for an extensive biological evaluation of isolated fungal metabolites.

The setup of co-cultures on solid media in Petri dishes has several considerable advantages compared to fermentation. Firstly, screening a large number of co-cultures for macromorphological changes is easier [31]. Growth of solid media mimics natural growth behavior of filamentous fungi [376] and, in general, more secondary metabolites are produced in solid than in liquid media [26]. Furthermore, metabolite production is dependent on the culture medium, thus similar conditions should be used to obtain comparable metabolite profiles. Unfortunately, solid medium cultures on the Petri dish level yield only low milligram amounts of extract. The amount of pure compounds obtained from such cultures are in the low microgram range which is limiting for biological testing. Thus, scale-up of fungal culture is necessary to respond to these needs.

The aim of this study was to demonstrate the induction of small molecules through microorganism co-culture by metabolomics, to scale-up co-culture production and to rapidly
isolate and identify induced metabolites from co-cultures grown on solid medium in sufficient amounts for biological testing. Therefore, a co-culture with the interaction type ‘distant inhibition’ [31] as well as an observed diffusion of red compounds into the culture medium was selected for the investigation of its metabolome and induction phenomena. This co-culture was also used as a case study to evaluate the overall strategy to identify and characterize induced bioactive constituents. The fungi involved in this interaction were an onychomycosis-derived Fusarium solani and the Basidiomycete Hohenbuehelia reniformis with the aim of identifying antifungal compounds that are active against Fusarium.

2. Results & discussion

In the search of new antifungals active against Fusarium spp., an onychomycosis strain of Fusarium solani was grown with the environmental Basidiomycete Hohenbuehelia reniformis (Basidiomycota, Pleurotaceae) on solid medium. To begin with, the interaction was observed on solid medium in a Petri dish. Macromorphologically, a distinct distance inhibition was witnessed (Figure VI.B.1). The two strains ceased growing a few millimeters apart and no hyphal growth was observed in the ‘confrontation zone’ between the two strains [31]. This might be explained by the release of either volatile or water-diffusible fungistatic or fungitoxic compounds. Additionally, the diffusion of red pigments into the culture medium could be observed.

2.1 Anti-Fusarium activity of fungal extract

The morphological observation of the Petri dish co-culture might indicate, as mentioned above, the diffusion of antifungal compounds into the culture medium that would explain the observed growth behavior of the two fungi. Therefore, methanolic extracts\(^2\) of all samples – the pure cultures of F. solani and H. reniformis as well as the co-culture extract – were tested.

\(^2\) Preliminary tests had shown that most features detected in CHCl\(_2\)-MeOH-H\(_2\)O extracts were also detectable in the methanolic extracts. Thus, methanolic extracts were used for the extraction of large-scale cultures.
on fungistatic and fungitoxic activity on the onychomycosis-derived clinical isolate *F. solani*. Whereas the pure culture extract of *F. solani* showed fungitoxic activity at 10 mg/mL, the extract of *H. reniformis* showed only fungistatic activity (growth inhibition) at the same concentration. However, the co-culture extract showed fungitoxic activity at 1 mg/mL already. Thus, it can be postulated that, the combined growth of the two fungi most likely stimulated the production of antifungal compounds.

### 2.2 Metabolite profiling and dereplication of fungal extracts

In order to detect if the long distance inhibition between the two strains was related to metabolite induction (morphologic macroscopic observation, Figure VI.B.1) and to identify the antifungal constituents, extracts of pure cultures and the co-culture were prepared. The profiling of secondary metabolites by UHPLC-TOFMS enabled the dereplication of known compounds.

![Figure VI.B.2](image)

*Figure VI.B.2: Metabolite profiling of pure culture extracts of *F. solani* and *H. reniformis* in comparison with the co-culture extract.*

Base peak ion (BPI) chromatograms in positive (left) and negative (right) ionization mode are shown.

Overall, LC peaks detected in the co-culture were of higher intensities compared to the two pure culture extracts when extracts were injected at the same concentration (Figure VI.B.2).
The higher intensities may be explained by a general higher consumption of sugars from the medium in the co-culture compared to the pure cultures which would result in a higher relative concentration of secondary metabolites. Increased sugar consumption during fungal growth has been observed in a study where fungi were grown in 12-well plates [577].

In general, more constituents were detected in the extract of *F. solani* and the negative ionization (NI) mode enabled the detection of a larger number of features. A feature is the detected mass-to-charge ratio \(m/z\) at a given retention time. With soft ionization techniques such as electrospray ionization (ESI), only a few features will correspond to a given metabolite [37]). A simple comparison of the base peak ion (BPI) profiles of the co-culture extract compared to the pure culture extracts did not reveal striking differences as this was the case for other co-cultures of naturally interacting fungal strains [30]. The prominent appearance of new compounds in the co-culture that were undetectable in the pure cultures, *de novo* metabolite induction, could not be highlighted. An MS-based differential metabolomic study [566] based on the comparison of 10 replicates was thus carried out to evidence more subtle changes in metabolite composition. This would permit the revelation of features that are induced *de novo* or significantly upregulated in the co-culture extracts (see section 2.3).

In order to get a better idea of the composition of the two pure cultures and the co-culture and since increased antifungal activity was revealed for the co-culture extract, a putative identification of the fungal constituents based on high-resolution MS (HRMS) was performed. Several compounds were dereplicated based on chemotaxonomic information from the three extracts and could be putatively assigned to known fungal constituents either reported in the same genus or in the same family (Table VI.C.18 and Table VI.C.19).

In the *F. solani* and the co-culture extracts, several naphthoquinones were putatively identified based on related molecular formulae that were retrieved from exact mass and MS spectral accuracy information. These compounds might be responsible for the red color that is apparent in the culture medium of the co-culture. As several constitutional isomers of these naphthoquinones exist, the exact identity of each detected peak couldn’t be definitely determined based on HRMS only. It would necessitate either the comparison with an authentic standard sample or the isolation and subsequent identification by an orthogonal method, e.g., NMR spectroscopy. One of the detected peaks, with \(m/z\) 289.023 eluting at 9.6 min, was assigned to \([M-H_2O+H]^+\) of the simultaneously detected \(m/z\) 307.083 \([M+H]^+,\) presumably fusarubin), after manual inspection of the raw data. The large peak eluting at 24 min (see Figure VI.B.2) was putatively assigned to the cyclic peptide cyclosporin that is known for its antifungal activity [591]. Comparison with an authentic standard confirmed the peak annotation.

On the other hand, among the *Hohenbuehelia* metabolites and the co-culture extracts, four peaks were observed exhibiting the same \(m/z\) but different retention time \((m/z\) 355.155 in NI mode eluting at 13.4 and 14.3 min, \(m/z\) 357.172 in PI mode eluting at 10.3, 12.0 and 14.3 min). These peaks were putatively identified as either leucopleurotin or dihydropleurotic acid, two
isomeric constitutive metabolites that were previously isolated from different *Hohenbuehelia* spp. The exact identity of each peak could not be unambiguously defined. In addition, apolar compounds eluting towards the end of the chromatographic run, were annotated as unspecific fatty acids.

The presence of novel compounds was likely as many peaks were detected and could not be dereplicated.

\[ {^1}H \text{ NMR (500 MHz, DMSO-}d_6) \]

*Figure VI.B.3: \[ {^1}H \text{ NMR spectrum of the three crude methanolic extracts of } F. \text{ solani, } H. \text{ reniformis and the co-culture solubilized in DMSO-}d_6 \text{ at a concentration of } 10 \text{ mg/0.75 mL (500 MHz, 30 °C, number of transients: 16).} \]

The three crude extracts were also analyzed by \[ {^1}H \text{ NMR (Figure VI.B.3) to get an overview of the most abundant extract constituents that is less biased than by LC-MS profiling [37]. Indeed, in reversed-phase (RP) LC-MS, solid phase extraction (SPE) is necessary that will remove apolar constituents. Furthermore, ionization is strongly compound dependent and main constituents might not be detected while minor, well-ionizable, constituents will lead to very intense peaks. For NMR analysis, the crude extracts were directly dissolved, without further sample preparation, in DMSO that allows the solubilization of most extract constituents. The } {^1}H \text{ NMR spectra show a high content in sugars in all extracts (δ}_H 3 \text{ to 4 ppm, 4.1 and 4.9 ppm). Fatty acids from the extract of } F. \text{ solani (δ}_H 1 \text{ to 1.5 ppm, } \text{CH}_2 \text{ of alkyl chains) and co-culture were observed as well. The spectrum of the co-culture looks like a combination of the two pure culture-spectra. The emergence of additional resonances is not apparent but might be hidden in the crowded regions of the spectrum.}*

2.3 **Metabolomics approach to identify possible antifungals in fungal co-cultures**

As the *de novo* induction of any compound was not apparent from neither UHPLC-TOFMS metabolite profiling nor \[ {^1}H \text{ NMR analysis that would explain the increased antifungal activity of the co-culture extract, a metabolomics approach was set. Therefore, ten Petri dishes of each pure culture and co-culture were grown and extracted independently to evaluate biological variations and to assure sufficient replicates for multivariate data analysis (MVDA).} \]
All samples were prepared for rapid UHPLC-TOFMS fingerprint analysis using a well characterized protocol (based on [31], section 4.5.1) to minimize variation due to sample handling and analysis.

2.3.1 *De novo* induction and upregulation

As this was the case for the HR metabolite profiling (Figure VI.B.2), the rapid fingerprinting of all extracts revealed that the secondary metabolite content of the two pure cultures was different. The BPI chromatogram of the co-culture extract mainly revealed a combination of both the pure strain metabolomes (Figure VI.B.4). The apparition of new peaks was not directly apparent and, in consequence, the chromatograms were analyzed using chemometrics. The statistical analysis after peak picking and alignment of the raw LC-MS data was primarily done using unsupervised MVDA by principal component analysis (PCA). The first two principal components (PCs) permitted the clear separation of the two pure cultures and the co-culture (Figure VI.C.96) which indicates that the three classes [pure culture *F. solani* (Fus), pure culture *H. reniformis* (Hr) and co-culture (HrxFus)] are clearly different, which is the expected result. The co-culture clustered more closely to the pure culture of *H. reniformis*. This was also apparent by visual examination of the chromatogram were the co-culture extract content was predominantly composed of *H. reniformis* constituents (Figure VI.B.4). The first PC separates the two pure cultures and the second PC permits the distinction of the co-

![UHPLC-TOFMS fingerprinting for metabolomics analysis](image-url)

*Figure VI.B.4: UHPLC-TOFMS fingerprinting for metabolomics analysis.*
culture. The co-culture doesn’t fall in the middle of the two pure cultures which is a clear indication for co-culture-induced metabolic modification [465].

The loadings of the PCA results, however, cannot be directly exploited to reveal induction phenomena in the co-culture at the level of individual features.

Figure VI.B.5: Volcano plot of detected features of both NI and PI UHPLC-TOFMS fingerprints on ten replicates each of pure culture of \( F.\ solani \) and \( H.\ reniformis \) and the co-culture. Each point on the scatter plot shows the average peak area difference of a feature in the co-culture compared to its presence in the pure culture (x-axis, fold-change) and the corresponding p-value (y-axis). Values were calculated by comparison of the co-culture data with one pure culture which correspond to the one with highest peak area. Features that exhibit a fold-change of more than two-fold and a significance of <0.05 are annotated with \( m/z \), retention time and ionization mode. Features that are mentioned in the text are highlighted in red.

The visualization of the different extract constituents (features) with respect to their content in the pure culture extract compared to the co-culture extract can be summarized in a volcano plot [592] which gives a better insight to induction phenomena (Figure VI.B.5). In such a plot, for every feature in the dataset, one mean value is calculated for every class, based on detected peak areas of the features. Then, mean values of a specific feature of the co-culture are compared to its mean value in both pure cultures. The fold-change is calculated compared to the pure culture that exhibits the higher mean value. In a next step, a t-test is done to evaluate if peak areas are significantly different between co-culture and pure culture and the p-value is retained. Features with fold-change >2 and p-value <0.05 were considered
interesting. These features were manually inspected for peak area based on their respective extracted ion chromatograms (XICs). Most of the highlighted features were of very low intensity, 31 had a peak area of >1’000 and only 4 had a peak area of >10’000 (25-30% of the main peaks in the BPI chromatogram in PI mode). Thus, the biggest changes were due to low abundance metabolites or compounds that were poorly ionized. (The following annotation will be used throughout the text to designate features: PI478.195@1.67 for a feature detected in PI mode with m/z 478.195 eluting at 1.67 min [577].) With the volcano plot visualization, the three most apparent features were PI478.195@1.67, NI387.038@0.84 and PI400.163@2.05. None of these compounds could be dereplicated (section 2.2 and Table VI.C.18 and Table VI.C.19) and might potentially be novel molecules.

In the metabolite profiling (section 2.2 and Figure VI.B.2), several compounds were dereplicated as naphthoquinones, possible suspects of the observed colorization of the co-culture (Figure VI.B.1). A systematic search for these compounds in the volcano plot revealed that only one feature was detected. The feature PI307.082@1.46 with an average peak area of 7’227 and a fold-change of 1.88 compared to the F. solani pure culture extract (p-value <0.002). The other naphthoquinones were not detected in the rapid fingerprinting. This might be due to possible ion suppression effects that are likely to occur since high-throughput constraints do not provide baseline separation of all constituents. It seems improbable that a two-fold increase of this compound makes up for the manifest colorization of the co-culture extract. The induction of pigments was thus independently evaluated by an orthogonal UHPLC-UV profiling.

### 2.3.2 UHPLC-UV analysis to visualize induction of pigments

Since fusarubin highlighted above as a possible marker has a UV spectrum with a specific band at 450 – 550 nm that is in relation to red color, a targeted analysis of the pure cultures and the co-culture by UHPLC-UV at 500 nm was undertaken.

![Figure VI.B.6: Chromatograms of UHPLC-UV analysis at 498 nm of pure culture extract of H. reniformis (left), F. solani (right) and the co-culture extract (middle).](image)

In this way, the de novo induction of two major and several minor pigments in the co-culture could be clearly revealed (Figure VI.B.6). The molecular formula C_{15}H_{14}O_{7} of isomeric pigments eluting at 9.7 min and at 14.6 min matched with several naphthoquinones such as fusarubin.
or novarubin. The first compound was also detected in pure cultures of \textit{F. solani} but clearly upregulated, the second compound was \textit{de novo} induced in the co-culture. An additional \textit{de novo} induced pigment (C$_{15}$H$_{11}$NO$_5$) at 13.0 min was detected and it could correspond to the naphthoquinone bostricoidin. Other metabolites detected in UV could not be annotated as corresponding peaks were not detected in the UHPLC-TOFMS profiling.

This finding indicates that \textit{F. solani} is reacting on the biotic stress with the release of naphthoquinones as has been shown for plant pathogens of the genus \textit{Fusarium} under abiotic stress [593].

2.3.3 Supervised statistical analysis for the detection of significant biomarkers

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{Results of OPLS-DA on NI UHPLC-TOFMS fingerprinting on ten replicates each of \textit{F. solani} and \textit{H. reniformis} pure culture and the corresponding co-culture. Score plots for both \textit{F. solani} (A, model M1) and \textit{H. reniformis} (B, model M2) samples with co-culture samples and the resulting share-and-unique (SUS) plot (C) are shown. Model M1 is placed on the vertical and model M2 on the horizontal axis. The most significant biomarkers are highlighted in red and blue in the SUS plot. Comparison of peak area of four selected features (D) shows the prevalence...}
\end{figure}
of different biomarkers among the pure cultures and the co-culture as well as their position in the SUS plot (green, red, yellow and orange frames). Feature NI305.087@1.46 describes an ion detected in NI mode with m/z 305.087 eluting at 1.46 min.

In order to further detect if significant biomarkers in addition to pigments can be highlighted by MS-based metabolomics, supervised MVDA methods, complementary to PCA and volcano plot, were also used. In this context, supervised methods such as OPLS-DA (orthogonal projections to latent structures discriminant analysis) in combination with share-and-unique (SUS) plot visualization [470] has shown useful in highlighting biomarkers when three situations are compared.

For both datasets analyzed in NI as well as PI mode, two OPLS-DA models were generated based on belonging to one of the pure cultures or to the co-culture. For the first model, *F. solani* pure culture samples were placed against the co-culture samples (*F. solani* × co-culture). For the second model, *H. reniformis* samples were placed against the co-culture samples (*H. reniformis* × co-culture). For the visualization on a share and unique (SUS) plot [470], the model of *F. solani* × co-culture was computed on the y-axis, the *H. reniformis* × co-culture on the x-axis. All four models had good predictability (*Q*² (cum) 0.786 – 0.852) and satisfactory separative capability (*R*² (cum) 0.807 – 0.885). The detailed values are given in Table VI.B.1.

**Table VI.B.1: Results of OPLS-DA models for differentiation between sample classes (pure culture, co-culture).**

<table>
<thead>
<tr>
<th>Models</th>
<th>Model size</th>
<th><em>R</em>² (cum)</th>
<th><em>Q</em>² (cum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NI UHPLC-TOFMS dataset</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>F. solani</em> × co-culture</td>
<td>1+0</td>
<td>0.867</td>
<td>0.852</td>
</tr>
<tr>
<td><em>H. reniformis</em> × co-culture</td>
<td>1+0</td>
<td>0.885</td>
<td>0.848</td>
</tr>
<tr>
<td>PI UHPLC-TOFMS dataset</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>F. solani</em> × co-culture</td>
<td>1+0</td>
<td>0.807</td>
<td>0.800</td>
</tr>
<tr>
<td><em>H. reniformis</em> × co-culture</td>
<td>1+0</td>
<td>0.832</td>
<td>0.786</td>
</tr>
</tbody>
</table>

In all datasets and in all models, samples were separated according to the first predictive latent variable already. The features with highest positive x- and y-value in the SUS plot (red and blue spots in Figure VI.B.7) correspond to shared features of the co-culture. Features with both x- and y-value >0.8 highlight mainly co-culture-induced compounds (red spots in Figure VI.B.7, induction in Table VI.C.21 and Table VI.C.22). Therefrom, 3 features were retained from the PI LC-MS dataset and 7 features from the NI dataset. Furthermore, features with an x-value >0.8 only highlight upregulated metabolites produced by *H. reniformis*, whereas features with a y-value >0.8 highlight upregulated metabolites produced by *F. solani*. Thereof, 7 features were retained as being upregulated *Fusarium* compounds and 11 features for *Hohenbuehelia* compounds. The list of selected features was extended to include other features with x- and y-value >0.5 (11 features in PI dataset, 15 features in NI dataset) which were interesting because of high intra-class variations (see also Figure VI.C.97 and Figure VI.C.99).
2.3.4 Identification of significant biomarkers by peak annotation

Some of the biomarkers highlighted by OPLS-DA had already been shown detected to be induced through either MS- and UV-based metabolite profiling (section 2.2 and section 2.3.2) or visualization on the volcano plot (section 2.3.1). Thus, the three methods provide not only redundant but also complementary information on the fungal interaction.

In total, 60 features were retained from OPLS-DA and volcano plot (Table VI.C.21 and Table VI.C.22) for further inspection since metabolites that are induced or upregulated in the co-culture have a greater chance of being responsible for the observed increased bioactivity of the co-culture extract. Among these features, 6 were found redundant due to detection in both ionization modes or detection as adducts. Possible molecular formulae were determined for all these features using heuristic filtering \[59,65\]. For 17 features, however, valid molecular formulae could not be determined reliably \[64\] because the isotopic pattern could not be retrieved, either due to low abundance (<100 ion counts) or due to coelution with isobaric metabolites. The corresponding molecules were thus either of low abundance in the extract or poorly ionized, or both.

Among the 37 features with valid molecular formula, 5 could match compounds either within the genus *Fusarium* or in the family Pleurotaceae. For example among *Fusarium* spp., for the feature NI307.082@1.46 (C_{15}H_{14}O_{7}), three naphthoquinones were found in the Dictionary of Natural Products (DNP), fusarubin, novarubin and 2,5-dihydroxy-6,8-dimethoxy-3-(2-oxopropyl)-1,4-naphthoquinone. The remaining 32 features did not match with any reported metabolites and can be new or at least new in these genera (Table VI.C.21 and Table VI.C.22).

This dereplication, however, is limited since isomers exist. In most cases, and especially when authentic standards are not available, the unambiguous identification of the fungal metabolites requires their isolation and subsequent analysis by NMR.

2.4 Isolation of significant biomarkers and pigments

The main goal of the study to identify antifungal compounds active against *Fusarium* spp. and to verify the hypothesis of a correlation with induction phenomena. Since NMR was needed for unambiguous identification of the biomarkers and they had to be obtained in a pure form for bioassays, their targeted isolation at the low milligram level was undertaken.

Based on the extraction yield evaluated from analytical samples and the semiquantitative results from NMR, it was estimated that 150 Petri dishes (15 cm inner diameter) were needed to obtain sufficient amounts of extract for the isolation process. This yielded 120 g of a crude large-scale extract that exhibited most of the features highlighted by metabolomics.

As revealed by NMR, the crude extract contained large amounts of sugars and fatty acids (Figure VI.B.3). Thus, a prefractionation procedure adapted from Bugni et al. [36] based on solid phase extraction (SPE) with the polystyrene resin Diaion® HP20SS was scaled-up accordingly and applied to obtain seven enriched prefractions (see chapter VI.A). All seven prefractions were tested for antifungal activity and prefraction P3 exhibited highest activity.
with fungitoxic activity at 500 μg/mL. All the other prefractions showed only moderate fungistatic activity with P4 and P5 being the most active in this case (500 μg/mL). The prefraction P3 was thus selected for further purification. Furthermore, most features detected by the metabolomic analysis could be found in P3.

To purify gram-amounts of sample in one chromatographic run, P3 was purified using medium pressure liquid chromatography (MPLC). Supplementary information on the MPLC purification procedure is given in chapter VI.C. The obtained MPLC fractions were further purified using semipreparative HPLC. All fractions (300) were individually analyzed by high-throughput UHPLC-UV and UHPLC-TOFMS. Based on the gathered data analyzed in the form of MPLC × UHPLC plots, fractions were grouped for semipreparative purification.

Pigments were purified by a UV-targeted isolation at 500 nm using semipreparative HPLC-UV. For the isolation of significant biomarkers, MPLC fractions were scouted for corresponding m/z and isolated. In both cases, scouting was aided by ELSD analysis to be aware of quantitative distribution of constituents within fractions since neither MS nor UV provided a quantitative estimation (dependence on ionizability and chromophore, respectively).

One limitation for the efficient purification of MPLC fractions was the problem of sample loading onto the semipreparative column due to limited sample solubility in the injection solvent that had to be kept compatible with HR separation. Since this required repeated injections of low concentration samples, semipreparative purification with a core-shell column that permits higher injection volumes was preferred to significantly increase the throughput.

2.5 *De novo* identification of isolated metabolites

The structure elucidation of all isolated compounds was performed based on 1D and 2D NMR and HRMS. Seven red and violet pigments (Figure VI.B.8), known *Fusarium* metabolites, were isolated and NMR data matched with literature values. With the exception of 1 and 3, only few micrograms of pure compound could be isolated. The structure confirmation of 6 was actually only possible through analyses on a MicroCryo NMR probe.

![Figure VI.B.8: Structures of isolated pigments from the co-culture of *F. solani* and *H. reniformis*.](image)

In addition, the labdane dysodensiol D (8), previously isolated from the plant *Dysoxylum densiflorum* [594] but also recently from the Basidiomycete *Trametes ostreiformis* [595], was
isolated from the co-culture extract. Two carboxylic acids (19, 20) were isolated and their structure was coherent with antibiotic 1233B described from an Ascomycete species [596]. A comparison of $^1$H NMR of 19 and 20 indicated configurational isomerism of the two hydroxyl groups. The configuration could not be established with nondestructive methods because of the structural flexibility of the side chain.

A small compound with nominal mass 280 Da was isolated and NMR did not match with previously reported molecules. The structure was established based on extensive spectroscopy analysis with NMR and HRMS (chapter VI.C, section 2.4). This new compound was also detected in pure $F. solani$ cultures and the name solanipyrone (9) is thus proposed for this compound. The configuration of the side chain could not be established with nondestructive methods because of the flexibility of the alkyl chain and limited sample amounts (<1 mg).

![Figure VI.B.9: Structures of isolated quinones and hydroquinones and other compounds from the co-culture of $F. solani$ and $H. reniformis$.](image)

Two different compounds with the same molecular mass 356 Da were isolated and NMR data matched with leucopleurotin (16) and dihydropleurotinic acid (17). In addition, seven compounds exhibited very similar $^1$H NMR spectra and were identified as novel congeners of either dihydropleurotinic acid, compound 18, or of leucopleurotin, compounds 10 to 15 (Figure VI.B.9). The structure elucidation of these congeners was made in comparison with 1D and 2D NMR spectra of dihydropleurotinic acid and leucopleurotin and using extensive
spectroscopic analyses. The in-depth description of these molecules is detailed in chapter VI.C, section 2.4.

2.6 Unambiguous identification of MS-metabolomics highlighted features

Based on the UHPLC-MS monitoring of all collected MPLC fractions, most of the MPLC fractions containing metabolomics-highlighted features highlighted were purified. Despite the very large scale used (120 g of crude extract), the amounts obtained for final purification by semipreparative HPLC enabled only the isolation of seven features at the low milligram scale. In other cases, the features were well detected in the MPLC fractions but did not generate ELSD signals, and thus, corresponding amounts precluded NMR analysis. The isolation of the different features also enabled the purification of other fungal metabolites present in the same MPLC fractions. Some of these compounds helped confirmation or disconfirmation of the peak annotations.

In order to verify that isolated compounds correspond to highlighted features, compounds were finally injected in the same conditions as for the metabolomic study. The occurrence of the metabolites with the same exact mass and retention time enabled their unambiguous identification.

Seven features could be linked to isolated compounds. The features NI305.067@1.46 [M-H]−, PI307.082@1.46 [M+H]+ and PI289.071@1.46 [M-H2O+H]+ were associated with fusarubin (1). This compound was equally detected in NI as in PI mode. The production of fusarubin was upregulated in the co-culture and this was highlighted by all three associated features (Figure VI.C.97, Figure VI.C.99). NI387.144@1.60 [M-H]− was identified as leucopleurotinic acid (10), a *Hohenbuehelia* metabolite upregulated in the co-culture (Figure VI.C.99). In PI mode, PI357.172@1.76 [M-H2O+H]+ and PI339.163@1.76 [M-2H2O+H]+ were linked to leucopleurotinic acid B (11). This *Hohenbuehelia* metabolite was also upregulated in the co-culture. Furthermore, PI373.169@2.20 [M+H]+ was identified as leucopleurotinic acid E (14). Evaluation of the XIC of this feature in all samples revealed that this biomarker was strongly induced in certain co-culture samples only (Figure VI.C.98). The prevalence of this compound in the co-cultures was highly variable.

While some features were putatively assigned based on molecular formula only, it could be proven based on the retention time of the isolated compounds that PI357.173@1.57 (molecular formula C21H24O5) does not correspond to leucopleurotin (16) nor to dihydropleurotinic acid (17) and PI291.078@0.83 (molecular formula C15H14O6) doesn’t correspond to be javanicin (7). These features probably correspond to other isomers that are not yet reported. Furthermore, among the newly isolated compounds, NI373.165@1.48 (molecular formula C21H26O6) is not leucopleurotinic acid B (11), NI371.042@0.95 (molecular formula C23H23O6) is not leucopleurotinic acid E (14) and PI387.181@2.63 (molecular formula C22H26O6) is not dihydropleurotinic acid B (18). These results highlight the limitation of MS-based dereplication and the need for comparison with authentic standards.
From the identified highlighted biomarkers, none was particularly strongly upregulated in the co-culture. For fusarubin (1), a two-fold change was observed, depending on ionization mode. The induction of this pigment was further confirmed by the UHPLC-UV analysis and was also seen by the color change observed macroscopically. In addition, other pigments that were found induced in UHPLC-UV analysis were also identified. These latter compounds, however, were difficult to detect in the short fingerprinting analysis and were not clearly highlighted by MVDA.

The three novel hydroquinones leucopleurotinic acid A (10), leucopleurotinic acid B (11) and leucopleurotinic acid E (14) show no clear trend on their specific up- or downregulation. Fold-changes, as seen on the volcano plot, were below two or even negative.

2.7 Inter-sample variation among fungal cultures

As discussed, some of the features that were found significantly upregulated, yielded low fold-changes. Nevertheless, it has to be brought to notice that high variations were observed among replicates (Figure VI.B.10, Figure VI.C.98 and Figure VI.C.100), especially when co-cultures were analyzed. Taking the example of 10, upregulation up to nine-fold were observed, depending on the individual sample that is compared [sample Hr_04 (4th replicate of \( H. \) reniformis pure culture) with HrxFus_03 (3rd replicate of co-culture)]. For other sample pairs, e.g., Hr_04 and HrxFus_05, production rates seem almost constant. This observation had been made for other co-culture systems already [465,566,577] and might be what is described as “capricious behavior of fungi to alter metabolite profiles when re-cultured” by Williams et al. [282]. It has to be stressed that these replicates had been started from the same pre-cultures and had been cultivated for the same number of days under controlled conditions (light cycle, humidity and temperature), the different replicates correspond to different Petri dishes.
dishes. Clearly, critical factors that influence fungal growth are not known and cannot be controlled to date in a setup as it is presented here.

2.8 Antifungal activity of isolated *H. reniformis* metabolites

Isolated quinones and hydroquinones were tested on their capacity to inhibit fungal growth or to kill the pathogen, fungistatic or fungitoxic activity, against a clinical *F. solani* in a microtiter plate assay [564]. Leucopleurotin (16) showed fungitoxic activity at 100 μM. Antimicrobial activity in the same range was reported for leucopleurotin against different molds [597]. The better-studied derivative pleurotin possesses antitumor activity [28] and is active against gram-positive bacteria, mycobacteria and dermatophytes [598].

Antifungal testing of the other isolated leucopleurotin congeners is ongoing. Leucopleurotinic acid B (11), E (14) and F (15) as well as dihydropleurotanic acid (17) could not be tested against *F. solani* because of insufficient compound purity (purity <80% according to 1H NMR).

Fusarubin (1) was also tested but, as expected, did not exhibit antifungal activity at the highest concentration tested (1 mM). The other *Fusarium* pigments were not tested as only trace amounts (<1 mg) could be isolated from the co-culture extract. Furthermore, these pigments were produced by this particular *F. solani* strain, the isomer fusarubin was not active as well and these quinones are not expected to show fungitoxic or fungistatic activity against this strain.

3. Conclusion

In this work, the co-culture of *Hohenbuehelia reniformis* and human pathogenic *Fusarium solani* grown on solid culture medium was studied for the identification of antifungal compounds against *Fusarium* sp. Anti-*Fusarium* activity was enhanced in the co-culture extract compared to the pure culture extracts indicating the induction of antifungal compounds. In addition, distance repulsion was observed as well as colorization of the culture medium suggesting the production of antifungal compounds and pigments, respectively. Induction phenomena were studied on ten replicates using two different MS-based metabolomics approaches, volcano plot and SUS plot visualization (OPLS-DA). The induction of pigments was evidenced by targeted UHPLC-UV analysis, but not by MS-based analyses due to poor ionizability.

For the isolation of co-culture-induced as well as antifungal metabolites, the large number of 150 Petri dishes (15-cm i.d.) was prepared. Therefrom, 20 compounds were isolated and identified using extensive spectroscopic analyses based on NMR and HRMS. Seven known pigments were isolated and it could be shown that their upregulation in the co-culture is responsible for the colorization of the culture medium. Among others, 7 novel congeners of leucopleurotin and dihydropleurotanic acids were isolated. Leucopleurotin exhibits moderate fungitoxic activity against *F. solani* which might explain the observed distance repulsion in the co-culture.
Detailed analysis of biomarker prevalence in different replicates of pure culture and co-culture samples points out the high variability in metabolite production between biological samples.

This work shows the potential of microorganism co-culture for the identification of antifungal compounds. Furthermore, the developed detection workflows permit the study of interacting fungi, and microorganisms in general, on the molecular level. This might help in gaining insight into the complex processes within microbiomes.

In addition, the isolation strategies of large-scale cultures based on solid culture media are applicable to cultures produced on even larger scale on modern solid-state fermenters [32].

4. Materials & methods

4.1 General experimental procedures

The purity of isolated compounds was estimated based on $^1$H NMR.

4.2 Chemicals & solvents

For extraction and MPLC, technical grade methanol (MeOH) was used. For sample preparation and all fractionation, deionized water (H$_2$O) was freshly prepared (Direct-Q 3 UV water purification system, Millipore). For the prefractionation, all organic solvents were HPLC grade. Isopropanol (iPrOH) was purchased from Acros Organics, MeOH was obtained from VWR (HiPerSolv CHROMANORM) and Sigma Aldrich (Chromasolv®) and dichloromethane (DCM) from VWR (HiPerSolv CHROMANORM). For purification, acetonitrile (MeCN) was purchased from Brunschwig (HPLC-S, gradient grade) and formic acid (FA, p.a., <98%) was obtained from Sigma Aldrich. ULC/MS grade MeCN, H$_2$O and FA (99%) from Biosolve was used for UHPLC analyses. For NMR analyses, deuterated MeOH and chloroform were obtained from Armar Chemicals; deuterated DMSO, MeCN and water were obtained from Cambridge Isotope Laboratories Inc.

4.3 Fungal strains

Fusarium solani Sin58 was isolated from onychomycosis collected at the Centre Hospitalier Cantonal Vaudois (CHUV, Lausanne Switzerland) [362]. Hohenbuehelia reniformis Sin138 was collected as soil isolate by Agroscope ACW. Both strains were stored in the database of Agroscope ACW in vials containing diluted PDB solution (1:4) at 4°C (http://mycoscope.bcis.ch/).

4.4 Fungal culture

The co-cultures conditions were similar to the previously described procedure [31]. For pure cultures, a 5-mm agar plug of a fungal pre-culture was inoculated in the center of a 9-cm Petri dish containing 30 mL of PDA (Difco, BD & Co, Le pont de Claix, France). The dishes were incubated at 21°C. Similarly, co-culture experiments were inoculated with two 5-mm agar plugs of the appropriate fungal strains on opposite sides of a 15-cm Petri dish containing 120 mL of PDA, and the dishes were incubated at 21°C for eight weeks. A total of 150 large Petri
dishes of co-culture were generated for the isolation of bioactive constituents. For the pure cultures, ten 9-cm Petri dishes were produced per strain.

4.5 Extraction of fungal material from solid media

The extraction procedure was different for the metabolomics samples as for isolation of fungal constituents.

4.5.1 Samples for metabolomics

For metabolomics, aliquots were taken from the large Petri dishes. Therefore, disks (ø 1 cm, corresponds to approx. 1 g of sample) were excised, placed in an Eppendorf tube and then freeze-dried. The ultrasound extraction (20 min) was performed using freshly prepared DCM/MeOH/H$_2$O (64:36:8) solvent mixture directly in the tube. The extract and the agar disk were transferred into a plastic syringe (3 mL, NORM-JECK, Henke Sass Wolf) and filtered into a new Eppendorf tube (1.7 mL, Axygen, Microtubes UltraClear), taking care to squeeze the agar disk with the plunger to retrieve a maximum volume of extract. The extracts were dried in a vacuum centrifuge (HT-4X, Genevac, SP scientific, Ipswich, Suffolk, UK). Then, the extracts were solubilized in 250 µL 85% aqueous MeOH and purified by SPE (SepPak® Vac 1cc (100 mg) C18, Waters) using 85% MeOH. The purified extracts were dried in a vacuum centrifuge. The purified extracts were diluted to 1 mg/mL in 85% MeOH for UHPLC-TOFMS analysis (fingerprinting). Uninoculated dishes and dishes inoculated with one fungal strain were used as blanks and controls, respectively. Ten replicates were prepared for each class of samples (both pure cultures, co-culture and uninoculated culture medium).

4.5.2 Samples for isolation of bioactive constituents

Fungal cultures were cut in pieces and freeze-dried in crystallizing dishes. The lyophilized fungal material was dampened with MeOH and transferred to Erlenmeyer flasks. Enough solvent was added to cover the fungal material. Two extractions were done using maceration for 12h, the extract was dried under reduced pressure at 40 °C and using lyophilization. A total of 120 g of crude extract was obtained.

4.6 UHPLC experiments

UHPLC-TOFMS analyses were performed using an Acquity™ UPLC chromatograph and a Micromass-LCT Premier Time of Flight mass spectrometer equipped with an ESI interface (Waters). For metabolite profiling, separation was performed on a 150 × 2.1 mm i.d., 1.7 µm, Acquity BEH C$_{18}$ UPLC column (Waters) in the gradient mode at a flow rate of 0.46 mL/min with the following solvent system: A = 0.1 vol% FA-H$_2$O, B = 0.1 vol% FA-MeCN; 5–95% B in 30 min. The injected volume was 2 µL. Detection was performed in PI and NI mode in the range m/z 100-1500 in centroid mode with a scan time of 0.3 s. ESI conditions were: capillary voltage 2800 V, cone voltage 40 V, MCP detector voltage 2450 V, source temperature 120 °C, desolvation temperature 350 °C, cone gas flow 20 L/h, and desolvation gas flow 800 L/h. The mass spectrometer was internally calibrated by infusion of a solution of leucine-enkephalin (2

294 VI.B Compounds from the co-culture *H. reniformis* and *F. solani*
$\mu$g/mL, Sigma-Aldrich) through the lockmass spray probe at a flow rate of 10 $\mu$L/min, using a second Shimadzu LC-10ADvp LC pump.

For the verification of the purity and identity of the MPLC fractions, pure compounds and metabolomics analysis, a short analysis (fingerprinting) was performed on a 50 x 2.1 mm i.d., 1.7 $\mu$m, Acquity BEH C$_{18}$ UHPLC column (Waters) in the gradient mode at a flow rate of 0.3 mL/min with the following solvent system: A = 0.1 vol% FA-H$_2$O, B = 0.1 vol% FA-MeCN. The injected volume was 1 $\mu$L. MS parameters were as above, except for desolvation temperature 300 °C and desolvation gas flow 600 L/h. For the analysis of the MPLC fractions, 2% of each fraction was stored in a 96-deepwell plate. For UHPLC-TOFMS analysis, 20 $\mu$L of each fraction was used to create pooled samples covering 10 MPLC fractions each.

UHPLC-UV analyses were performed using an Acquity™ UPLC chromatograph. All analyses were performed with the same parameters and columns as UHPLC-TOFMS. Injection volumes were double and PDA traces were recorded from 210 to 500 nm. Two UV traces were extracted: 280 nm and 498 nm.

4.7 Automated dereplication procedure

The procedure described in chapter V.A was used for the dereplication of compounds in the crude extract. Briefly, native MassLynx data from UHPLC-TOFMS were analyzed with data analysis software that allows for data processing of MS datasets (MZmine 2, version 2.10 [515]) to generate peak lists. The detected masses within these peak lists were identified by a custom database search using entries of DNP (version 22:1, CRC Press, Taylor & Francis). Search was restricted to entries with the biological source listed as the species Fusarium and the family Pleurotaceae. As only three compounds are listed with the biological source of the species Hohenbuehelia, thus the search had been extended to the family level.

4.8 Metabolomics data treatment

UHPLC-TOFMS data were converted to NetCDF datafiles using Databridge (MassLynxV4.1, Waters) and imported into MZmine 2 (version 2.10 [515]). The chromatograms were reduced to 0.5 – 4.5 min and mass lists were generated for every chromatogram using a noise level of 20 or 100 for negative or positive ionization spectra, respectively. A m/z tolerance of 0.005 Da or 15 ppm and a time span of 0.1 to 2.0 min was allowed for every detected peak. A peak is defined as a given mass (precision of 15 ppm or 0.005 Da) at a given retention time (retention time tolerance 0.1 min). Peak lists were generated using a minimum peak height of 20 or 100 for negative or positive ionization spectra, respectively. Peaks were filtered for shoulder peaks using a mass resolution of 60,000 and the lorentzian extended peak shape model. The peak lists were deconvoluted with a local minimum search using a chromatographic threshold of 35%, a minimum relative height of 5% and a minimum in retention time range of 0.1 min. Isotopic pattern were removed (deisotoping) and all peak lists were aligned using join aligner and the same weight for mass and retention time. The aligned peak list was gap filled with an intensity tolerance of 20%. Duplicate peaks were removed. Peaks showing an unnatural peak shape were manually deleted from the aligned peak list as well as peaks that appeared in all
culture medium control samples. Complexes and adducts [54] were identified but kept in the
dataset for later statistical analysis. The so aligned and filtered peak lists were exported as .csv
files for later multivariate data analysis.

4.8.1 Volcano plot
The aligned and filtered peak lists were imported into Microsoft Excel. For each feature (m/z
at a given retention time), the average sum (and standard deviation) was calculated among
pure culture and co-culture separately. Then, the fold-change and corresponding p-value (t-
test) was calculated by comparison of the average sum of the co-culture with each pure
culture. The values for whatever pure culture had a higher average sum was displayed. To
obtain a volcano plot [592], all features were plotted with its p-value on the y-axis (in
logarithmic scale) and the fold-change on the x-axis.

4.8.2 Multivariate data analysis
MVDA by PCA and OPLS-DA were performed with SIMCA-P+ 12© (Umetrics, Sweden). Data
were Pareto-scaled (and a seven-fold procedure, using a default option of the software for
cross-validation, was applied). PCA models were evaluated according to the percentage of
explained initial variance $R^2_X$. OPLS models were evaluated by the goodness of fit $R^2_Y$ and the
prediction ability $Q^2$.

4.9 Isolation of fungal constituents from solid-medium co-culture
Crude extracts were enriched using an optimized prefractionation procedure with the sorbent
Diaion® HP20SS (see chapter VI.A and section 4.9.1). The most active enriched prefraction was
subjected to MPLC (section 4.9.2). Selected MPLC fractions were further purified by HPLC
(section 4.9.3) to obtain pure compounds.

4.9.1 Prefractionation using Diaion HP20SS
For prefractionation, the crude extract was solubilized with MeOH and H$_2$O and adsorbed onto
the resin HP20SS (twice the quantity of extract, Diaion). The suspension was dried under
reduced pressure. An additional volume of HP20SS was filled into a glass column (Sepacore C-
690, 460 × 36 mm, Büchi) and completed with the adsorbed extract. The remaining extract
was filled into a second glass column (Sepacore C-690, 460 × 26 mm, Büchi). The
prefractionation was executed on a Flash instrument (SpotFLASH, Armen) operated at a
maximum pressure of 22 bar at 35 to 50 mL/min. The extract was eluted with five volumes of
the following solvent systems: water, 25% aqueous iPrOH (iPrOH$_{aq}$), 50% iPrOH$_{aq}$, 75%
iPrOH$_{aq}$, MeOH and dichloromethane yielding prefractions P1 to P6 [36]. The prefractions
were reduced under pressure at 40 °C and rendered to complete dryness using lyophilization.

4.9.2 Medium pressure liquid chromatography
The enriched prefraction P3 (2 g) was adsorbed onto RP silicagel (12 g, Zeoprep C18, 40-60
µm, Zeochem) and the powder was mixed with an equivalent amount of sand (12 g). The
sample was chromatographed on a MPLC system equipped with a a self-packed MPLC column
(Sepacore C-690, 460 × 49 mm; Zeoprep C18 15-25 μm 60 Å, irregular particles, Zeochem), a pump (Büchi pump manager C-615, Büchi pump module C-605), a UV detector (UV photometer C-640, Büchi) and a fraction collector (fraction collector C-660, Büchi), piloted by Sepacore Record software (Büchi). H₂O+0.1% FA and MeOH+0.1% FA was used as solvent system at a flow rate of 5 mL/min. The gradient system was as follows: 50% isocratic for 224 min, 189 min from 50 to 55%, isocratic at 55% for 1894 min and 1894 min from 55 to 100%. Detection was at 280 and 540 nm (0.1 AU/V, sampling rate 0.75 s, half width 4 nm) and fractions were collected every 50 or 100 mL, according to UV. The MPLC fractions were dried on a rotatory evaporator and under nitrogen flux; fraction amounts were between 1 and 37 mg per fraction (see chapter VI.C).

### 4.9.3 Purification by semipreparative HPLC

Selected fractions from MPLC were further purified using either a conventional fully porous C18 silicagel semipreparative column or a preparative C18 core-shell column. All fractions were scouted using HPLC-PDA-ELSD-MS to define the wavelength that would best reflect the semi quantitative ELSD trace. This led to the choice of 280 nm as the detection wavelength for the purification of most MPLC fractions. For the targeted isolation of red and violet pigments, 500 nm was used as detection wavelength.

For semipreparative HPLC purification with a fully porous C18 column, a 250 × 10 mm i.d., 5 μm, XBridge C₁₈ column equipped with a pre-column on a HPLC-PDA instrument (HP1100, Agilent) was used. The conditions were as follows: flowrate 3.7 mL/min; solvent system H₂O+0.1% FA (A) and MeCN+0.1% FA (B); relevant detection wavelengths 280 and 500 nm. Gradient systems were optimized using a 250 × 4.6 mm i.d., 5 μm, XBridge C₁₈ column equipped with a pre-column on the same HPLC-PDA instrument and geometrically transferred using HPLC Calculator 3.0 software [67]. MPLC fractions were solubilized in 20 – 50% aqueous MeOH and manually injected (injection loop 250 μL, Rheodyne 7725i 6-port injection valve).

For semipreparative HPLC purification with a core-shell column, a 250 × 21.2 i.d., 5 μm, Kinetex™ C18 100 Å, AXIA (Phenomenex) column equipped with a pre-column was used. Purifications were executed on a preparative LC-UV instrument (Spot Prep II 50, Armen Instrument) hyphenated to an ELSD instrument (Sedere 75, 40 °C, gas: compressed air; split enabled approx. 100 μL/min to go to ELSD). Conditions were as follows: flowrate 25 mL/min; solvent system H₂O (A) and MeCN (B); detection wavelengths 210, 280 or 500 nm. Gradient systems were optimized using a 100 × 3 mm i.d., 2.6 μm, Kinetex™ C₁₈ 100 Å column (Phenomenex) on a HPLC-PDA-ELSD-MS instrument and geometrically transferred [67]. MPLC fractions were solubilized in 20 – 50% aqueous MeCN and manually injected (injection volume 2 mL, injection loop 5 mL).

### 4.10 NMR analyses

Analyses were done on a Varian INOVA 500 MHz instrument (Agilent) using a 5-mm tube probe. Selected samples were analyzed on a 600 MHz Bruker Avance III system equipped with the cryogenically cooled TCI 1.7-mm MicroCryoProbe™. Crude extracts of the two pure
cultures and the co-culture were measured in DMSO-d$_6$, prefractions were analyzed in D$_2$O (P1), CD$_3$OD (P2, P3, P4, P5) or CDCl$_3$ (P6). NMR analyses included the following sequences: $^1$H, $^1$H-$^1$H COSY (or DQF-COSY), NOESY, HSQC, HMBC, APT and 1DNOESY.

4.11 Isolated molecules

**Fusarubin (1).** 3,5,10-Trihydroxy-7-methoxy-3-methyl-4-dihydro-1H-benzo[g]isochromene-6,9-dione. 10 mg. Purity >85%. $^1$H NMR (DMSO-d$_6$, 500 MHz, δ$_{H}$): 1.48 (3H, s, 3-CH$_3$), 2.59 (1H, d, $J = 17.9$ Hz, H-4”), 2.78 (1H, d, $J = 17.9$ Hz, H-4”), 3.91 (3H, s, 7-OCH$_3$), 4.69 (2H, m, 1-CH$_2$), 6.11 (1H, s, 3-CH$_3$), 6.46 (1H, s, H-8), 12.51 (1H, brs, OH), 13.00 (1H, brs, OH). $^{13}$C NMR (DMSO-d$_6$, 125 MHz, δ$_{C}$): 28.2 (3-CH$_3$), 32.6 (C-4), 56.8 (7-OCH$_3$), 57.1 (C-1), 92.8 (C-3), 106.9 (C-9a), 109.5 (C-8), 133.0 (C-4a), 136.0 (C-10a), 159.5 (C-5), 160.4 (C-7), 177.3 (C-6), 184.4 (C-9).

$^1$H NMR (CD$_3$CN, 500 MHz, δ$_{H}$): 1.54 (3H, s, 3-CH$_3$), 2.65 (1H, d, $J = 17.9$ Hz, H-4”), 2.88 (1H, d, $J = 17.9$ Hz, H-4”), 3.91 (3H, s, 7-OCH$_3$), 4.78 (2H, m, 1-CH$_2$), 6.30 (1H, s, H-8), 12.63 (1H, brs, OH), 13.01 (1H, brs, OH). ESI-MS (PI mode): m/z 307.0811 [M+H]$^+$ (C$_{15}$H$_{15}$O$_7$, calc. m/z 307.0818, Δ 2.3 ppm), 289.0708 (most intense) [M-H$_2$O+H]$^+$ (C$_{15}$H$_{13}$O$_6$, calc. m/z 289.0712, Δ 1.4 ppm). UV (MeCN/H$_2$O, λ (nm)): 304, 475 (sh), 499, 535 (sh).

**3-O-Methylfusarubin (2).** 5,10-Dihydroxy-3,7-dimethoxy-3-methyl-3,4-dihydro-1H-benzo[g]isochromene-6,9-dione. 0.4 mg. Purity >95%. $^1$H NMR (CD$_3$CN, 500 MHz, δ$_{H}$): 1.50 (3H, s, 3-CH$_3$), 2.66 (1H, d, $J = 18.2$ Hz, H-4”), 2.91 (1H, dd, $J = 18.2$, 2.2 Hz, H-4”), 3.26 (3H, s, 3-OCH$_3$), 3.90 (3H, s, 7-OCH$_3$), 4.52 (1H, dt, $J = 17.8$, 2.6 Hz, H-1”), 4.82 (1H, dd, $J = 17.8$, 1.8 Hz, H-1”), 6.29 (1H, s, H-8), 12.53 (1H, brs, OH), 13.01 (1H, brs, OH). ESI-MS (PI mode): m/z 321.0970 [M+H]$^+$ (C$_{16}$H$_{12}$O$_7$, calc. m/z 321.0974, Δ 1.2 ppm), m/z 307.0829 [M-CH$_2$+H]$^+$ (C$_{15}$H$_{13}$O$_7$, calc. m/z 307.0818, Δ 3.6 ppm), m/z 289.0722 (most intense) [M-CH$_2$OH+H]$^+$ (C$_{15}$H$_{12}$O$_6$, calc. m/z 289.0712, Δ 3.5 ppm). UV (MeCN/H$_2$O, λ (nm)): 303, 475 (sh), 498, 534 (sh).

**Solaniol (3).** 5,8-Dihydroxy-7-(2-hydroxypropyl)-2-methoxy-6-methylnapthalene-1,4-dione (PubChem). 3.6 mg. Purity 75%. $^1$H NMR (DMSO-d$_6$, 500 MHz, δ$_{H}$): 1.11 (3H, d, $J = 6.2$ Hz, 3-CH$_3$), 2.28 (3H, s, 1-H$_3$), 2.80 (2H, m, 4-H$_2$), 3.91 (3H, s, 7-OCH$_3$), 3.91 (1H, behind 7-OCH$_3$ signal, H-3), 4.67 (1H, d, $J = 4.7$ Hz, 3-OH), 6.49 (1H, s, H-8), 12.91 (1H, brs, OH), 13.41 (1H, brs, OH). $^{13}$C NMR (DMSO-d$_6$, 125 MHz, δ$_{C}$): 13.3 (C-1), 24.1 (3-CH$_3$), 36.5 (C-4), 57.5 (7-OCH$_3$), 66.2 (C-3), 109.9 (C-8). ESI-MS (PI mode): m/z 293.1017 [M+H]$^+$ (C$_{15}$H$_{17}$O$_6$, calc. m/z 293.1025, Δ 2.7 ppm). m/z 275.0917 [M-H$_2$O+H]$^+$ (C$_{15}$H$_{15}$O$_5$, calc. m/z 275.0919, Δ 0.7 ppm). Literature values: [α]$_{25}$S$_{899}$ + 122° (c, 0.053 in MeOH) [599]. $^1$H NMR in CDCl$_3$: [600].

**Anhydrofusarubin (4).** 5,10-Dihydroxy-7-methoxy-3-methyl-1H-benzo[g]isochromene-6,9-dione. <1 mg. Purity 90%. $^1$H NMR (CD$_3$CN, 500 MHz, δ$_{H}$): 2.00 (3H, d, $J = 1.0$, 3-CH$_3$), 3.90 (3H, s, 7-OCH$_3$), 5.21 (2H, s, 2-CH$_2$), 6.02 (1H, q, $J = 1.0$, H-4), 6.30 (1H, s, H-8). ESI-MS (PI mode): m/z 289.0729 [M+H]$^+$ (C$_{15}$H$_{12}$O$_6$, calc. m/z 289.0712, Δ 5.9 ppm). UV (MeCN/H$_2$O, λ (nm)): 290, 340 (sh), 545.
Anhydrofusarubin lactol (5). 1,5,10-Trihydroxy-7-methoxy-3-methyl-1H-benzo[g]isochromene-6,9-dione. <1 mg. Purity >80%. ¹H NMR (CD₃CN, 500 MHz, δH): 0.82 (3H, d, J = 6.9 Hz, 12-CH₃), 0.86 (3H, d, J = 6.9 Hz, 13-CH₃), 1.10 (1H, m, H-8’), 1.17 (3H, s, 14-CH₃), 1.32 (1H, m, H-7), 1.37 (1H, m, H-9’), 1.38 (1H, m, H-2’), 1.42 (1H, m, H-1), 1.42 (1H, m, H-8”), 1.47 (1H, m, H-9”), 1.84 (1H, pq, J = 6.9, 2.8 Hz, H-11), 1.98 (1H, m, H-3”), 2.01 (1H, m, H-2’), 2.21 (1H, m, H-6), 2.34 (1H, m, H-3”), 4.21 (1H, s, 10-OH), 6.94 (1H, d, J = 5.4 Hz, H-5), 12.08 (1H, brs, 15-COOH). ¹³C NMR (CDCl₃, 125 MHz, δC): 15.4 (C-12), 17.6 (C-2), 21.25 (C-13), 21.32 (C-8), 25.3 (C-3), 26.6 (C-11), 27.9 (C-14), 34.4 (C-9), 36.5 (C-6), 43.5 (C-7), 44.7 (C-1), 69.7 (C-10), 130.5 (C-4), 141.5 (C-5), 169.0 (C-15) (literature values in CDCl₃ [594]). ESI-MS (PI mode): m/z 291.0876 [M+H]+ (C₁₅H₁₁OB₃O₅, calc. m/z 291.0869, Δ 0.2 ppm).

Dysodensiol D (8). (1R,6R,7S,10R)-10-Hydroxy-7-isopropyl-10-methyl-1,2,3,5,6,7,8,9-octahydrodiphenalen-4-carboxylic acid. 2.9 mg. Purity >99%. ¹H NMR (DMSO-d₆, 500 MHz, δH): 0.82 (3H, d, J = 6.9 Hz, 12-CH₃), 0.86 (3H, d, J = 6.9 Hz, 13-CH₃), 1.10 (1H, m, H-8’), 1.17 (3H, s, 14-CH₃), 1.32 (1H, m, H-7), 1.37 (1H, m, H-9’), 1.38 (1H, m, H-2’), 1.42 (1H, m, H-1), 1.42 (1H, m, H-8”), 1.47 (1H, m, H-9”), 1.84 (1H, pq, J = 6.9, 2.8 Hz, H-11), 1.98 (1H, m, H-3”), 2.01 (1H, m, H-2’), 2.21 (1H, m, H-6), 2.34 (1H, m, H-3”), 4.21 (1H, s, 10-OH), 6.94 (1H, d, J = 5.4 Hz, H-5), 12.08 (1H, brs, 15-COOH). ¹³C NMR (DMSO-d₆, 125 MHz, δC): 15.4 (C-12), 17.6 (C-2), 21.25 (C-13), 21.32 (C-8), 25.3 (C-3), 26.6 (C-11), 27.9 (C-14), 34.4 (C-9), 36.5 (C-6), 43.5 (C-7), 44.7 (C-1), 69.7 (C-10), 130.5 (C-4), 141.5 (C-5), 169.0 (C-15) (literature values in CDCl₃ [594]). ESI-MS (PI mode): m/z 235.1696 [M+H]+ (C₁₁H₂₂O₃, calc. m/z 235.1698, Δ 0.9 ppm).

Solaniphyrine (9). (E)-6-(1’,3’-Dimethyl-5’-hydroxymethyl-hept-1’-enyl)-4-methoxy-pyran-2-one. 0.8 mg. Purity >90%. ¹H NMR (DMSO-d₆, 500 MHz, δH): 0.82 (3H, t, J = 7.2 Hz, H-7”), 0.98 (3H, d, J = 6.5 Hz, 3’-CH₃), 1.22 (2H, m, H-4’b, H-5’), 1.26 (2H, m, H-6’), 1.35 (1H, m, H-4’a), 1.86 (3H, s, 1’-CH₃), 2.69 (1H, m, H-3’), 3.26 (1H, behind solvent signal, 5’-CHHOH), 3.29 (1H, behind solvent signal, 5’-CHHOH), 3.88 (3H, s, 4-OCH₃), 4.32 (1H, brs, 5’-CH₂OH), 5.52 (1H, d, J = 1.8 Hz, H-3), 6.10 (1H, d, J = 1.8 Hz, 5-H), 6.14 (1H, dd, J = 9.7, 11.2 Hz). ¹³C NMR (DMSO-d₆, 125 MHz, δC): 13.7 (C-7”), 12.9 (1’-CH₃), 21.1 (3’-CH₃), 23.4 (C-6’), 30.7 (C-3’), 38.6 (C-4’), 39.9 (C-5’), 57.1 (4-OCH₃), 63.7 (5’-CH₂OH), 89.4 (C-3), 109.3 (C-5), 141.1 (C-2’), 167.9 (C-4), 180.9 (C-2). ESI-MS (PI mode): m/z 281.1765 [M+H]+ (C₁₆H₂₅O₄, calc. m/z 281.1753, Δ 4.3 ppm). UV (MeCN/H₂O, λ (nm)): 265 (sh), 274.

Fungal metabolites
Leucopleurotinic acid A (10). 0.7 mg. Purity >95%. $^1$H NMR (DMSO-d$_6$, 500 MHz, δ$_H$): 1.04 (3H, d, J = 6.38 Hz, H$_2$-21), 1.24 (1H, m, H-20'), 1.59 (1H, m, H-13''), 1.62 (1H, m, H-19''), 1.73 (1H, m, H-19'), 1.76 (1H, m, H-12''), 1.84 (1H, m, H-20'), 1.93 (1H, m, H-11), 1.94 (1H, m, H-10), 1.96 (1H, m, H-13'), 1.98 (1H, m, H-12'), 2.18 (1H, m, H-9), 2.32 (1H, td, J = 12.13, 3.78 Hz, H-14), 2.78 (1H, d, J = 11.90 Hz, H-14), 3.14 (1H, dd, J = 10.42, 6.80 Hz, H-8''), 3.40 (1H, behind solvent signal, H-8'), 4.21 (1H, brs, 8-OH), 7.35 (2H, s, H-3, H-4), 11.51 (1H, brs, 5-OH), 11.73 (1H, brs, 2-OH). $^1$H NMR (CD$_3$CN, 500 MHz, δ$_H$): 1.09 (3H, d, J = 6.4 Hz, H$_3$-21), 1.37 (1H, m, H-20''), 1.60 (1H, m, H-19''), 1.65 (1H, m, H-13''), 1.85 (1H, m, H-12''), 1.85 (1H, m, H-19''), 1.88 (1H, m, H-11), 1.88 (1H, m, H-20''), 1.99 (1H, m, H-10), 2.03 (1H, H-13''), 2.09 (1H, H-12''), 2.26 (1H, m, H-9), 2.49 (1H, td, J = 12.0, 3.6 Hz, H-14), 2.75 (1H, d, J = 12.0 Hz, H-17), 3.27 (1H, m, H-8''), 3.50 (1H, m, H-8'), 7.27 (2H, s, H-3, H-4), 11.56 (1H, s, 5-OH), 11.85 (1H, s, 2-OH). $^{13}$C NMR (DMSO-d$_6$, 125 MHz, δ$_C$): 18.3 (C-21), 23.2 (C-12), 28.3 (C-20), 29.6 (C-13), 35.7 (C-19), 37.2 (C-9), 41.2 (C-10), 46.7 (C-14), 49.2 (C-11), 59.4 (C-18), 60.2 (C-17), 65.2 (C-8), 112.5 (C-6), 113.0 (C-1), 128.0 (C-4), 128.4 (C-3), 153.8 (C-5), 154.6 (C-2), 173.6 (C-15), 203.0 (C-16), 205.4 (C-7). $^{13}$C NMR (CD$_3$CN, 125 MHz, δ$_C$): 17.7 (C-21), 23.1 (C-12), 28.3 (C-20), 29.9 (C-13), 36.4 (C-19), 37.3 (C-9), 41.6 (C-10), 46.1 (C-14), 49.9 (C-11), 59.5 (C-18), 60.8 (C-17), 66.0 (C-8), 112.4 (C-6), 113.3 (C-1), 128.0 (C-4), 128.5 (C-3), 154.7 (C-5), 156.5 (C-2), 173.2 (C-15), 203.1 (C-16), 205.9 (C-7). ESI-MS (NI mode): m/z 387.1450 [M-H]$^-$ (C$_{21}$H$_{23}$O$_7$, calc. m/z 387.1444, Δ 1.5 ppm), m/z 775.2966 [2M-H]$^-$ (C$_{22}$H$_{25}$O$_{10}$, calc. m/z 775.2966, Δ 5.7 ppm). ESI-MS (PI mode): m/z 389.1599 [M+H]$^+$ (C$_{21}$H$_{25}$O$_7$, calc. m/z 389.1600, Δ 0.3 ppm), m/z 371.1481 [M-H$_2$O+H]$^+$ (C$_{21}$H$_{23}$O$_6$, calc. m/z 371.1495, Δ 3.8 ppm). UV (MeCN/H$_2$O, λ (nm)): 261, 398.

Leucopleurotinic acid B (11). (16R)-16-Hydroxy-leucopleurotinic acid. 1.8 mg, isolated in a 1:2 mixture with leucopleurotin (16). $^1$H NMR (CD$_3$CN, 500 MHz, δ$_H$): 1.04 (3H, d, J = 7.3 Hz, H$_3$-21), 1.59 (1H, H-19''), 1.62 (1H, H-13''), 1.83 (1H, m), 1.85 (1H, H-19''), 2.03 (1H, m, H-17), 2.08 (1H, m, H-9), 2.10 (1H, H-13''), 4.49 (1H, s, H-16), 2.12 (1H, H-14), 4.94 (1H, s, H-7), 6.58 (1H, d, J = 8.5 Hz, H-4), 6.67 (1H, overlapping with signal from leucopleurotin (16), H-3), 8.50 (1H, brs, OH), 8.74 (2H, brs, OH). $^{13}$C NMR (CD$_3$CN, 125 MHz, δ$_C$): 21.3 (C-21), 31.8 (C-13), 34.2 (C-9), 35.1 (C-19), 43.8 (C-14), 45.0 (C-10), 46.4 (C-18), 52.6 (C-11), 52.8 (C-17), 67.0 (C-16), 77.6 (C-8), 83.3 (C-7), 116.7 (C-3), 117.3 (C-4), 120.9 (C-6), 124.9 (C-1), 149.5 (C-2). ESI-MS (PI mode): m/z 357.1689 [M-H$_2$O+H]$^+$ (C$_{21}$H$_{25}$O$_5$, calc. m/z 357.1702, Δ 3.6 ppm).

Leucopleurotinic acid C (12). 16-Methoxy-leucopleurotinic acid. 6.1 mg. Purity 75%. $^1$H NMR (CD$_3$CN, 500 MHz, δ$_H$): 1.04 (3H, d, J = 7.3 Hz, H$_3$-21), 1.49 (1H, m, H-19''), 1.64 (1H, m, H-13''), 1.71 (1H, m, H-20''), 1.82 (1H, m, H-19''), 1.84 (1H, m, H-11), 1.85 (1H, m, H-19''), 1.85 (1H, m, H-12''), 1.89 (1H, m, H-12''), 2.09 (1H, m, H-9), 2.10 (1H, m, H-14), 2.12 (1H, m, H-13''), 2.19 (1H, m, H-17), 2.23 (1H, m, H-10), 3.37 (3H, s, 16-OCH$_3$), 3.90 (1H, dd, J = 13.0, 2.2 Hz), 3.99 (1H, s, H-16), 4.07 (1H, dd, J = 13.0, 7.9 Hz, H-8''), 4.92 (1H, s, H-7), 6.60 (1H, d, J = 8.6 Hz, H-4), 6.68 (1H, d, J = 8.6 Hz, H-3), 8.44 (1H, brs, 15-OH). $^{13}$C NMR (CD$_3$CN, 125 MHz, δ$_C$): 21.3 (C-21), 22.7 (C-12), 26.3 (C-20), 31.8 (C-13), 34.1 (C-9), 34.9 (C-19), 43.0 (C-14), 45.1 (C-10), 46.5 (C-18), 47.0 (C-17), 52.5 (C-11), 57.0 (16-OCH$_3$), 75.5 (C-16), 77.5 (C-8), 83.0 (C-7), 116.8 (C-3), 117.5 (C-4), 121.2 (C-6), 123.3 (C-1), 149.9 (C-2), 152.2 (C-5), 177.3 (C-15). ESI-MS (NI mode):
m/z 387.1823 [M-H]⁻ (C₂₂H₂₇O₆, calc. m/z 387.1808, ∆ 3.9 ppm), m/z 775.3718 [2M-H]⁻ (C₄₄H₅₅O₁₂, calc. m/z 775.3694, ∆ 3.1 ppm), m/z 433.1849 [M+HCOO]⁻ (C₂₃H₂₅O₈, calc. m/z 433.1862, ∆ 3.0 ppm).

**Leucopleurotinic acid D (13).** 16-Hydroxy-15-O-methyl-leucopleurotinic acid. <0.2 mg. Purity 75%. ¹H NMR (CD₂CN, 500 MHz, δH): 1.02 (3H, d, J = 7.2 Hz, H₃-21), 1.39 (1H, m, H-19''), 1.60 (1H, m, H-13''), 1.75 (1H, m, H-20''), 1.82 (1H, m, H-12''), 1.82 (1H, m, H-11), 1.83 (1H, m, H-20''), 1.84 (1H, m, H-19''), 1.93 (1H, m, H-12''), 2.03 (1H, m, H-13''), 2.10 (1H, m, H-9), 2.25 (1H, m, H-10), 2.26 (1H, m, H-17), 2.28 (1H, m, H-14), 3.52 (3H, s, 15-OCH₃), 3.85 (1H, dd, J = 12.9, 2.7 Hz, H-8''), 4.09 (1H, dd, J = 12.9, 8.0 Hz, H-8''), 4.96 (1H, s, H-7), 5.29 (1H, d, J = 5.3 Hz, H-16), 6.60 (2H, s, H-3, H-4), 8.09 (1H, brs, 16-OH). ¹³C NMR (CD₂CN, 125 MHz, δC): 21.3 (C-21), 22.4 (C-12), 26.1 (C-20), 32.1 (C-13), 33.9 (C-9), 34.0 (C-19), 40.7 (C-14), 45.8 (C-10), 49.5 (C-18), 52.0 (15-OCH₃), 52.7 (C-11), 53.7 (C-17), 70.5 (C-16), 77.2 (C-8), 81.3 (C-7), 117.6 (C-4), 117.7 (C-3), 121.0 (C-6), 121.5 (C-1), 151.2 (C-2), 151.7 (C-5), 178.1 (C-15). ESI-MS (NI mode): m/z 387.1808 [M-H]⁻ (C₂₂H₂₇O₆, calc. m/z 387.1808, ∆ 4.4 ppm), m/z 775.3661 [2M-H]⁻ (C₄₄H₅₅O₁₂, calc. m/z 775.3694, ∆ 4.3 ppm), m/z 433.1848 [M+HCOO]⁻ (C₂₃H₂₅O₈, calc. m/z 433.1862, ∆ 3.2 ppm).

**Leucopleurotinic acid E (14).** 16-Oxo-15-O-methyl-leucopleurotinic acid. <0.2 mg. Purity 50%, contains 25% fusarubin (1) and 25% 3-O-methylfusarubin (2). ¹H NMR (CD₂CN, 500 MHz, δH): 1.06 (3H, d, J = 7.2 Hz, H₃-21), 1.29 (1H, m, H-19''), 1.71 (1H, m, H-13''), 1.75 (1H, m, H-20''), 1.81 (1H, m, H-19''), 1.81 (1H, m, H-11), 1.88 (1H, m, H-20'), 1.91 (2H, m, H-2, H-12), 2.10 (1H, behind HDO signal, H-9), 2.11 (1H, behind HDO signal, H-13'), 2.32 (1H, m, H-10), 2.50 (1H, d, J = 12.2 Hz, H-17), 2.59 (1H, td, J = 12.2, 4.0 Hz, H-14), 4.02 (1H, d, J = 13.2 Hz, H-8''), 4.15 (1H, dd, J = 13.2, 7.5 Hz, H-8''), 5.35 (1H, s, H-7), 6.82 (1H, dd, J = 9.0, 0.9 Hz, H-3), 7.05 (1H, d, J = 9.0 Hz, H-4), 8.67 (1H, brs, 15-OH), 12.03 (1H, s, 2-OH). ¹³C NMR (CD₂CN, 125 MHz, δC): 20.9 (C-21), 22.1 (C-12), 25.7 (C-20), 30.2 (C-13), 33.8 (C-19), 34.2 (C-9), 34.7 (C-14), 44.9 (C-10), 51.2 (C-11), 60.5 (C-17), 77.9 (C-8), 82.4 (C-7), 114.5 (C-1), 119.1 (C-3), 123.5 (C-6), 128.2 (C-4), 150.8 (C-5), 157.5 (C-2), 204.7 (C-16). ESI-MS (PI mode): m/z 373.1660 [M+H]+ (C₂₁H₂₅O₆, calc. m/z 373.1651, ∆ 2.4 ppm). ESI-MS (NI mode): m/z 371.1488 [M-H]⁻ (C₂₁H₂₃O₆, calc. m/z 371.1495, ∆ 1.9 ppm), m/z 743.3032 [2M-H]⁻ (C₄₂H₄₇O₁₂, calc. m/z 743.3068, ∆ 4.8 ppm), m/z 417.1552 [M+HCOO]⁻ (C₂₂H₂₅O₈, calc. m/z 417.1549, ∆ 0.7 ppm).

**Leucopleurotinic acid F (15).** 2,5-Dihydro-dihydropleurotinic acid, named leucopleurotinic acid. 1.4 mg. Purity 72%, contains 23% dihydropleurotinic acid (17) and 6% of another pleurotin congener. ¹H NMR (CD₂CN, 500 MHz, δH): 1.04 (3H, d, J = 7.2 Hz, H₃-21), 1.34 (1H, m, H-19''), 1.61 (1H, m, H-13''), 1.73 (1H, m, H-20''), 1.81 (1H, m, H-19'), 1.82 (1H, m, H-12''), 1.83 (1H, m, H-11), 1.85 (1H, m, H-20''), 1.91 (1H, m, H-12''), 1.92 (1H, m, H-17), 2.06 (1H, m, H-13''), 2.15 (1H, m, H-9), 2.23 (1H, m, H-14), 2.28 (1H, m, H-10), 2.54 (1H, d, J = 18.2 Hz, H-16''), 2.61 (1H, dd, J = 18.2, 5.7 Hz, H-16''), 3.89 (1H, dd, J = 12.9, 2.3 Hz, H-8''), 4.09 (1H, dd, J = 12.9, 7.9 Hz, H-8''), 4.96 (1H, s, H-7), 6.45 (1H, d, J = 8.54 Hz, H-4), 6.59 (1H, d, J = 8.54 Hz, H-3), 8.25 (1H, brs, 15-OH). ¹³C NMR (CD₂CN, 125 MHz, δC): 21.3 (C-21), 22.6 (C-12), 25.8 (C-16), 25.9 (C-20), 31.6 (C-13), 32.8 (C-9), 33.8 (C-19), 44.5 (C-14), 46.0 (C-10), 46.1 (C-17), 47.5 (C-18), 52.7 (C-
Leucopleurotin (16). 10.0 mg. Purity >95%. $^1$H NMR (CD$_3$CN, 500 MHz, $\delta_{\text{H}}$): 0.93 (3H, d, $J = 7.0$ Hz, H-21), 1.21 (1H, m, H-19’’), 1.41 (1H, m, H-13’’), 1.73 (1H, m, H-11), 1.76 (1H, m, H-19’), 1.81 (1H, m, H-20’’), 1.82 (1H, m, H-12’’), 1.89 (1H, m, H-20’), 1.91 (1H, m, H-12’), 2.15 (1H, m, H-13’), 2.16 (1H, m, H-9), 2.25 (1H, m, H-10), 2.43 (1H, m, H-14), 2.43 (1H, m, H-17), 3.91 (1H, dd, $J = 12.9$, 3.3 Hz, H-8’’), 4.07 (1H, dd, $J = 12.9$, 8.0 Hz, H-8’), 5.1 (1H, s, H-7), 5.59 (1H, d, $J = 6.8$ Hz, H-16), 6.67 (1H, d, $J = 8.7$ Hz, H-4), 6.73 (1H, d, $J = 8.7$ Hz, H-3). $^{13}$C NMR (CD$_3$CN, 125 MHz, $\delta_{C}$): 21.1 (C-21), 22.9 (C-12), 25.1 (C-13), 26.0 (C-20), 31.9 (C-19), 33.2 (C-9), 39.6 (C-14), 44.9 (C-10), 49.8 (C-18), 50.2 (C-17), 53.5 (C-11), 74.7 (C-16), 77.3 (C-8), 81.8 (C-7), 117.0 (C-3), 118.9 (C-4), 120.2 (C-6), 121.0 (C-1), 150.6 (C-2), 152.2 (C-5), 176.9 (C-15). ESI-MS (PI mode): $m/\text{z}$ 357.1710 [M+H]$^+$ (C$_{21}$H$_{25}$O$_5$S, calc. $m/\text{z}$ 357.1702, $\Delta$ 2.2 ppm). UV (MeCN/H$_2$O, $\lambda$ (nm)): 250, 314 (sh).

Dihydropleurotinic acid (17). 4.8 mg. Purity 99%. $^1$H NMR (CD$_3$CN, 500 MHz, $\delta_{\text{H}}$): 0.91 (3H, d, $J = 7.0$ Hz, H-21), 1.21 (1H, m, H-19’’), 1.54 (1H, qd, $J = 12.7$, 4.5 Hz, H-13’’), 1.66 (1H, m, H-11), 1.69 (1H, m, H-20’’), 1.72 (1H, m, H-12’’), 1.73 (1H, m, H-20’), 1.82 (1H, m, $J = 12.95$, 3.9 Hz, H-12’), 1.90 (1H, m, $J = 12.9$, 2.6 Hz, H-17), 2.01 (1H, m, H-19’), 2.02 (1H, m, H-13’), 2.09 (1H, m, H-14), 2.11 (1H, m, H-10), 2.15 (1H, m, H-9), 2.41 (2H, m, H$_2$-16), 3.35 (1H, dd, $J = 12.3$, 6.5 Hz, H-8’’), 3.91 (1H, dd, $J = 12.3$, 8.6 Hz, H-8’), 4.43 (1H, s, H-7), 6.70 (1H, d, $J = 10.1$ Hz, H-3), 6.67 (1H, d, $J = 10.1$ Hz, H-4), 8.98 (1H, brs, 15-OH). $^{13}$C NMR (CD$_3$CN, 125 MHz, $\delta_{C}$): 21.1 (C-21), 22.4 (C-12), 25.0 (C-16), 25.7 (C-20), 31.2 (C-13), 32.6 (C-9), 35.1 (C-19), 43.3 (C-14), 44.8 (C-17), 46.7 (C-10), 47.4 (C-18), 52.4 (C-11), 72.8 (C-7), 75.4 (C-8), 136.8 (C-3), 138.4 (C-4), 141.1 (C-6), 141.6 (C-1), 176.9 (C-15), 187.4 (C-5), 188.2 (C-2). In agreement with literature values [605]. ESI-MS (NI mode): $m/\text{z}$ 355.1564 [M-H]$^-$ (C$_{21}$H$_{23}$O$_5$S, calc. $m/\text{z}$ 355.1545, $\Delta$ 5.3 ppm), $m/\text{z}$ 311.1662 [M-CO$_2$-H]$^-$ (C$_{20}$H$_{23}$O$_3$, calc. $m/\text{z}$ 311.1647, $\Delta$ 4.8 ppm). UV (MeCN/H$_2$O, $\lambda$ (nm)): 250, 314 (sh), 338.

Dihydropleurotinic acid B (18). 16-Methoxy-dihydropleurotinic acid. 6.6 mg. Purity 97%. $^1$H NMR (CD$_3$CN, 500 MHz, $\delta_{\text{H}}$): 0.91 (3H, d, $J = 7.0$ Hz, H$_3$-21), 1.37 (1H, ddd, $J = 13.2$, 11.1, 6.8 Hz, H-19’’), 1.53 (1H, qd, $J = 12.5$, 4.8 Hz, H-13’’), 1.64 (1H, m, H-11), 1.65 (1H, m, H-20’’), 1.75 (1H, m, H-12’’), 1.70 (1H, m, H-20’), 1.78 (1H, m, H-12’), 1.89 (1H, td, $J = 12.5$, 4.2 Hz, H-14), 2.06 (1H, m, H-13’), 2.06 (1H, m, H-10), 2.08 (1H, m, H-19’), 2.13 (1H, s, H-17), 2.15 (1H, m, H-9), 3.35 (3H, s, 16-OCH$_3$), 3.35 (1H, s, H-8’’), 3.88 (1H, s, H-16), 3.90 (1H, dd, $J = 12.3$, 8.6 Hz, H-8’), 4.37 (1H, s, H-7), 6.73 (2H, s, H-3/H-4), 9.09 (1H, brs, 15-OH). $^{13}$C NMR (CD$_3$CN, 125 MHz, $\delta_{C}$): 21.2 (C-21), 22.5 (C-12), 26.0 (C-19), 31.2 (C-13), 32.5 (C-9), 36.5 (C-19), 42.7 (C-14), 45.9 (C-10), 46.07 (C-17), 46.12 (C-18), 52.3 (C-11), 58.1 (16-OCH$_3$), 73.33 (C-16), 73.26 (C-7), 75.5 (C-8), 137.0 (C-3), 138.3 (C-1), 138.6 (C-4), 142.4 (C-6), 177.0 (C-15), 187.4 (C-2), 187.8 (C-5). ESI-MS (NI mode): $m/\text{z}$ 385.1660 [M-H]$^-$ (C$_{22}$H$_{27}$O$_6$, calc. $m/\text{z}$ 385.1651, $\Delta$ 2.3 ppm), $m/\text{z}$ 771.3417 [2M-H]$^-$(C$_{44}$H$_{51}$O$_{12}$, calc. $m/\text{z}$ 771.3381, $\Delta$ 4.7 ppm).
Antibiotic 1233 (19). (10E,12E)-3-hydroxy-2-(hydroxymethyl)-8,10,12-trimethyltetradeca-10,12-dienedioic acid. 2.3 mg. Purity 97%. $^1$H NMR (DMSO-$d_6$, 500 MHz, $\delta_\text{H}$): 0.80 (3H, d, J = 6.6 Hz, H$_3$-16), 1.03 (1H, m, H-7’), 1.21 (1H, m, H-5’’), 1.22 (1H, m, H-6’’), 1.26 (1H, m, H-7’’), 1.29 (1H, m, H-4’’), 1.31 (1H, m, H-6’), 1.39 (1H, m, H-4’), 1.42 (1H, m, H-5’), 1.64 (1H, m, H-8), 1.75 (3H, s, H$_3$-17), 1.83 (1H, dd, J = 13.2, 7.9 Hz, H-9’’), 2.04 (1H, dd, J = 13.2, 6.7 Hz, H-9’), 2.14 (3H, s, H$_3$-18), 2.32 (1H, td, J = 8.4, 4.7 Hz, H-2), 3.50 (1H, dt, J = 8.4, 4.2 Hz, H-3), 3.61 (1H, m, H-15’’), 3.69 (1H, dd, J = 10.3, 4.7 Hz, H-15’), 5.60 (1H, s, H-13), 5.72 (1H, s, H-11). $^{13}$C NMR (DMSO-$d_6$, 125 MHz, $\delta_\text{C}$): 17.6 (C-17), 18.7 (C-18), 19.2 (C-16), 25.2 (C-5), 26.2 (C-6), 30.0 (C-8), 36.2 (C-4), 36.3 (C-7), 48.4 (C-9), 55.1 (C-2), 61.1 (C-15), 69.1 (C-3), 118.3 (C-13), 129.2 (C-11), 140.7 (C-10), 152.0 (C-12), 167.6 (C-14), 174.8 (C-1). ESI-MS (NI mode): m/z 341.1976 [M-H]$^-$(C$_{18}$H$_{29}$O$_6$, calc. m/z 341.1964, $\Delta$ 3.5 ppm).

Antibiotic 1233 (20). (10E,12E)-3-hydroxy-2-(hydroxymethyl)-8,10,12-trimethyltetradeca-10,12-dienedioic acid. 5.5 mg. Purity 96%. $^1$H NMR (DMSO-$d_6$, 500 MHz, $\delta_\text{H}$): 0.80 (3H, d, J = 6.6 Hz, H$_3$-16), 1.07 (1H, m, H-7’’), 1.26 (2H, m, H$_2$-4), 1.26 (1H, m, H-5’’), 1.26 (1H, m, H-7’’), 1.30 (1H, m, H-6’’), 1.38 (1H, m, H-6’), 1.38 (1H, m, H-5’), 1.64 (1H, m, H-8), 1.76 (3H, s, H$_3$-17), 1.83 (1H, dd, J = 13.0, 8.2 Hz, H-9’’), 2.06 (1H, dd, J = 13.0, 6.3 Hz, H-9’), 2.15 (3H, s, H$_3$-18), 2.38 (1H, dt, J = 8.2, 5.8 Hz, H-2), 3.52 (1H, m, H-15’’), 3.55 (1H, m, H-15’), 3.60 (1H, m, H-3), 5.58 (1H, s, H-13), 5.73 (1H, s, H-11). $^{13}$C NMR (DMSO-$d_6$, 125 MHz, $\delta_\text{C}$): 18.1 (C-17), 18.8 (C-16), 19.0 (C-18), 30.2 (C-8), 34.5 (C-6), 36.1 (C-7), 46.2 (C-4), 48.4 (C-9; 52.3 (C-5), 54.9 (C-2), 59.7 (C-15), 68.8 (C-3), 117.8 (C-13), 128.7 (C-11), 140.8 (C-10), 153.5 (C-12), 167.3 (C-14), 174.7 (C-1). ESI-MS (NI mode): m/z 341.1976 [M-H]$^-$(C$_{18}$H$_{29}$O$_6$, calc. m/z 341.1964, $\Delta$ 3.5 ppm).

4.12 Bioassay on F. solani Sin58

Crude extracts, prefractions and selected isolated compounds were tested on their capacity to suppress growth of F. solani Sin58 [564]. The test was executed in 96-well plates using solid medium (PDB:PDA 3:7) at a total volume per well of 200 µL. For inoculation with the filamentous fungus, mycelium was scrapped from the fungus cultivated on PDA and diluted in sterile water. Each well was inoculated with 10 µL of a 10$^6$ spores/mL solution. Plates were covered and incubated at 21 °C for a total of seven days and fungal growth was visually assessed after four and seven days.

The fungus was tested for its tolerance towards low percentages of organic solvent in the growth medium. MeOH and MeCN were tolerated even at 10% (v/v) whereas DMSO inhibited fungal growth from 7% (v/v) on. This was necessary as most samples were not completely soluble in aqueous solutions and had to be solubilized in organic solvent prior testing. Several known antifungal agents were assessed for their usefulness as positive controls against F. solani Sin58. Miconazole, fluconazole, tebuconazole, griseofulvin and amphotericin B as well as the formulated tebuconazole-containing fungicide Horizont® (250 g/L tebuconazole and >25% N,N-dimethyldecanamid) were evaluated. Griseofulvin was included because of its natural product scaffold whereas amphotericin B was tested because of recent evidence of efficacy of this ergosterol binder against onychomycoses caused by Fusarium spp. that are insensitive to itraconazole treatment [606]. Crude extracts were solubilized in DMSO and
tested at 10, 5, 1 mg/mL, 500, 100, 50, 10, 5, 1 µg/mL, 500 and 100 ng/mL. Enriched extracts were solubilized in H₂O, DMSO or MeCN and tested at 1 mg/mL, 500, 100, 50, 10, 5, 1 µg/mL, 500, 100, 50 and 10 ng/mL. The fungicides were tested at 10 mM down to 100 nM and the isolated compounds at 1 mM down to 10 nM. A selectivity for the differentazole fungicides was observed. The most active sample was the formulated product Horizont® that inhibited fungal growth at 1 µM whereas the pure compound tebuconazole was only active at 100 µM which might be due to a toxic effect of the formulating agent in Horizont®. Griseofulvin and fluconazole were not active at the highest concentration tested (10 mM). Thus, Horizont® was included as positive control (one row) and one column was left untreated as negative control on each assay plate. All test were executed in triplicates. Organic solvent compatibility testing and evaluation for positive control substances (except amphotericin B) was done in six repetitions.

5. Acknowledgments

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VI.C Supplementary information: Induced pigments and anti-*Fusarium* compounds from the co-culture of *Hohenbuehelia reniformis* and *Fusarium solani*
1. Introduction

Many practical aspects along the isolation of fungal metabolites from the co-culture of *Hohenbuehelia reniformis* and *Fusarium solani* were omitted in the previous chapter for reasons of clarity. In this additional chapter, analyses around the medium pressure liquid chromatography (purification), challenges that were encountered during the targeted isolation of induced biomarkers as well as the *de novo* structure elucidation of the hydroquinones 10 to 15 and the quinone 18 are described. This chapter also contains supplementary tables and figures concerning the dereplication and more detailed results of the MVDA.

2. Results & discussion

The co-culture extract had antifungal activity the clinical isolate *F. solani*. For the assessment of the biological activity of the pure antifungal compounds, milligram-amounts of pure compound had to be isolated. Therefore, a large scale culture was prepared to obtain 120 g of crude extract. After sample enrichment (see chapter VI.A), an aliquot of the most active enriched fraction (P3, 2 g) was purified by MPLC. The use of the microfractionation strategy was not feasible for the purification of gram-amounts of sample, as the loading capacity of corresponding semipreparative HPLC columns is lower than 100 mg (see chapter III.A, III.B and IV.A). In this respect, MPLC is adapted to the purification of extracts in the gram range and milligram-amounts of pure compounds can be obtained [578]. Furthermore, geometrical transfer of chromatographic conditions from analytical HPLC to MPLC are now possible [607].

2.1 Medium pressure liquid chromatography on fungal extract

For the targeted isolation of co-culture-induced metabolites (see also chapter VI.B, section 2.4), fractions obtained after MPLC purification were analyzed gravimetrically (Figure VI.C.1), by UHPLC-UV and by UHPLC-TOFMS (Figure VI.C.2).

![Figure VI.C.1: UV traces (280 and 540 nm) of MPLC separation and mass balance (blue bars) of collected MPLC fractions.](image)

The MPLC purification was monitored by UV at 280 and 540 nm. Monitoring at 280 nm reflected well the concentration distribution of the different extract constituents (gravimetric distribution of MPLC fraction amounts). Monitoring at 540 nm is specific to the detection of the naphthoquinone pigments present in the extract (see also chapter VI.B, section 2.3.2).
Figure VI.C.2: At-line UHPLC-TOFMS (top panels) and UHPLC-UV (bottom panels) analysis of MPLC separation. The evolution of the MPLC separation is shown through 2D LC plots (UHPLC×MPLC). The UHPLC separation is plotted on the x-axis, whereas the y-axis shows the MPLC separation (individual analyses on all MPLC fractions).

An overview on the distribution of sample constituents among the collected fractions was obtained through the visualization of MPLC fraction content in the form a 2D LC plots (Figure VI.C.2). All MPLC fractions were therefore analyzed at-line by UHPLC-TOFMS (negative and positive ionization mode) and by UHPLC-UV at two different wavelengths. Two-dimensional matrices were constructed from the different chromatograms of the MPLC fractions (UHPLC retention time in rows and MPLC fractions, corresponding to MPLC retention time, in columns). These matrices were represented as contour plots. TOFMS analysis showed that most compounds are concentrated in fractions #160 to #280. The analysis at 280 nm showed that three major peaks were separated along the MPLC fractions around sample #150, #180 and #250.

The UHPLC-TOFMS analyses permitted the localization of significant biomarkers in the different MPLC fractions. Only MPLC fractions containing significant biomarkers [based on corresponding mass-to-charge ratio (m/z) and retention time] were retained for semipreparative purification. Therefore, fractions could be grouped based on gathered information from the UHPLC-UV chromatograms.
The analysis at 498 nm shows that several pigments were present in the extract and that some were separated (e.g., compound with retention time 1.4 min in MPLC fraction #300) by MPLC. A pigment with retention time 1.5 min and $m/z$ 307 was observed in two parts of the MPLC chromatogram, around fraction #150 as well as around #330. Two representative fractions (#115 and #330) were analyzed by NMR (Figure VI.C.3) which revealed that, indeed, the same compound was eluted from the MPLC column at two different retention times. The NMR spectrum showed that both fractions contained the same two major compounds. One of these could be isolated in pure form and was later identified as fusarubin (see section 2.4.1). The second compound could not be obtained in pure form and was not successfully identified.

**Figure VI.C.3: Fusarubin prevalence in different MPLC fractions.**

Based on the individual chromatograms, some of the MPLC fractions (# 15-16, 109-110, 161, 185 and 330) were analyzed by NMR to get an unbiased overview on the purity of the collected MPLC fractions (Figure VI.C.4). It can be seen from all analyses that the separation was poor and that many compounds are still co-eluting. Therefore, MPLC fractions were further purified by semipreparative HPLC.
Figure VI.C.4: \(^{1}H\) NMR spectrum of five MPLC fractions (# 15-16, 109-110, 161, 185 and 330) in CDCl\(_3\). MPLC fractions were chosen based on purity as observed in UHPLC-UV analyses. NMR analysis showed that none of these MPLC fractions yielded pure compounds directly.

2.2 Development of strategy for the targeted isolation of highlighted biomarkers

For the isolation of pigments, MPLC fractions were purified by UV-targeted isolation at 500 nm using semipreparative HPLC-UV. Significant biomarkers (chapter VI.B, section 2.3) were located in the MPLC fractions based on information gathered from UHPLC-TOFMS fingerprinting analyses. The chromatographic separation was then optimized on analytical HPLC using PDA, evaporative light scattering (ELS) and MS detection. MS detection permitted the identification of significant biomarkers in the biomarkers whereas ELSD showed the quantitative distribution of constituents within the sample. PDA detection allowed the selection of the appropriate UV wavelength for monitoring of the semipreparative isolation. For most fractions, 280 nm reflected the quantitative distribution of sample constituents best (Figure VI.C.5). The chromatographic conditions were geometrically transferred [67] to semipreparative HPLC.

For the efficient purification of milligram-amounts of pure compounds from MPLC fractions using semipreparative HPLC, one limiting factor was the loading. Sample had to be dissolved in a solvent mixture close to the starting conditions to be compatible with high-resolution separation. However, samples were only sparingly soluble in these polar solvent mixtures. A higher proportion of organic solvent in the injection solvent increased the solubility but deteriorated chromatogram resolution, especially if injection volumes of more than 10% of the flow rate were employed. A compromise had to be found between injection volume and solvent composition. In consequence, sample loading onto the semipreparative column was
low and repeated injections of low concentration samples were necessary for the purification of bigger amounts of pure compound. The newly introduced semipreparative core-shell HPLC columns are operated at very high flow rate (25 mL/min compared to 3.7 mL/min for the fully porous semipreparative HPLC column) and enable the application of higher injection volumes (2 mL compared to 250 μL) and thus, permitted higher sample loading which significantly increased the throughput.

Figure VI.C.5: Optimization of semipreparative purification of MPLC fractions using analytical HPLC-PDA-ELSD-MS.
In a first step, chromatographic separation of MPLC fractions is optimized on a 100 × 3 mm i.d., 2.6 μm core-shell column (Kinetex) using PDA, ELS and MS detection. In a second step, chromatographic conditions are geometrically transferred [67] to semipreparative HPLC on a 250 × 21.1 mm i.d., 5 μm core-shell column (Axia).

For the final purification by semipreparative HPLC, both types of columns were used. The core-shell column enabled the purification of the hydroquinones and quinones. These compounds were insoluble in water.

2.3 Difficulties encountered during targeted isolation of highlighted biomarkers
Even though most of the features detected by MVDA (chapter VI.B, section 2.3) were well detected in the MPLC fractions, the identity and structure of only seven features could be confirmed through the isolation of corresponding compounds. Two difficulties could be
evaluated that complicated the targeted isolation of significant biomarkers. Some features were well-detected in the MPLC fractions but their concentration in the samples was very low. The compound amounts were largely overestimated by MS and effective amounts precluded NMR analysis. For other the compounds, the corresponding molecular ion was not detected in MS due to strong adduct formation under the employed instrumental conditions. These two cases are illustrated below.

2.3.1 Adduct formation

For the compound 15, the molecular ion m/z 400 was observed in PI ESI-MS analyses. Nevertheless, de novo structure elucidation by NMR analysis (section 2.4.4) suggested a molecular mass of 358 Da. The observed ion could correspond to the acetonitrile adduct of 15. Determination of the molecular formula by heuristic filtering based on HRMS analysis confirmed the adduct formation (Figure VI.C.6). The formation of this stable adduct has been observed by Nielsen et al. [54] for other fungal metabolites.

![Figure VI.C.6: Detection of adduct ion in MS for leucopleurotinic acid F (15). Only the acetonitrile adduct \([M+H+MeCN]^+\) was detected for 15 in PI ESI-MS analyses under the employed instrumental conditions. The formation of this stable adduct has been observed by Nielsen et al. [54]. NMR analysis confirmed the structure and thus, the molecular mass of the pure compound.]

2.3.2 Detection of low abundance but well-ionized biomarkers

The biomarkers with m/z 499 (NI ESI-MS) was detected in some of the MPLC fractions (Figure VI.C.7). Nevertheless, isolation of the corresponding pure compound was unsuccessful.
Inspection of the MS spectra indicated co-elution with another compound detected at m/z 373. Careful inspection of the extracted ion chromatograms (XIC) of the two ions indicated that two individual compounds are detected and that adduct formation or in-source fragmentation is precluded. For both ions, no compound could be isolated in pure form.

**Figure VI.C.7: Detection of feature with m/z 499 in NI ESI-MS.** The compound was co-eluting with another compound (m/z 373). Comparison of XIC of m/z 499 and 373 confirmed that these ions correspond to two individual compounds (no adduct formation or in-source fragmentation) as the two peak shapes are different. The ion detected at m/z 371 corresponds to leucopleurotinic acid E (14).

### 2.4 Structure elucidation of novel metabolites in the co-culture

Several compounds were isolated from the co-culture extract of *Hohenbuehelia reniformis* and *Fusarium solani* (chapter VI.B) and could not be assigned to any known structure based on dereplication (see section 2.2 of chapter VI.B). An overview of all isolated compounds is given in the previous chapter in Figure VI.B.8 and VI.B.9. In brief, seven naphthoquinones (1 to 7), one sesquiterpene (8), one pyrone (9), seven hydroquinones (10 to 16), two quinones (17 and 18) and two fatty acids (19 and 20) were isolated from the co-culture extract and their structure was elucidated using MS and NMR spectroscopy. Among these molecules, 9, 10 to 15 as well as 18 are novel compounds (Figure VI.C.8). The detailed spectroscopic data of all isolated compounds, including exact mass measurements, can be found in condensed form in the previous chapter VI.B, section 4.11. NMR chemical shifts in table form, NMR spectra and the interpretation of the various spectra obtained for de novo structure determination is discussed below.
314 VI.C Supplementary information: Induced pigments and anti-Fusarium compounds

Figure VI.C.8: Novel compounds isolated from the co-culture extract of *Fusarium solani* and *Hohenbuehelia reniformis*.

### 2.4.1 Naphthoquinones 1–7

The presence of the various naphthoquinones was detected in the extracts. Fusarubin (1) was dereplicated by HPLC-PDA-UV and HRMS and its structure was confirmed by 1D and 2D NMR analyses after isolation and by comparison with literature values [608]. This compound exhibited a particular UV spectrum with three adsorption bands: $\lambda_{\text{max}}$ 220, 304 and 499 nm with shoulders at 475 and 535 nm. This UV spectrum was observed for several other isolated compounds (2 to 7). All these compounds were of red or violet color and have been designated as ‘pigments’. Through NMR analysis, 3-0-methylfusarubin (2), solaniol (3), anhydrofusarubin (4), anhydrofusarubin lactol (5), bostrycoidin (6) and javanicin (7) were identified. All these molecules have been previously isolated from *Fusarium* spp. including *F. solani* [609]. Some of these compounds (2 to 5, as well as 7) were only detected in the co-culture extract. Their previous description in the genus *Fusarium* indicates that *F. solani* and not *H. reniformis* is the producer of these compounds.

### 2.4.2 Sesquiterpene 8

The labdane dysodensiol D (8), previously isolated from the plant *Dysoxylum densiflorum* [594] but also recently from the Basidiomycete *Trametes ostreiformis* [595] was isolated from the co-culture extract. The structure was confirmed by extensive spectroscopic analysis that matched with literature values.

Figure VI.C.9: Structure and numbering scheme for 8.
Table VI.C.2: Chemical shift assignment of 8.

<table>
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<tr>
<th>Position</th>
<th>Dysodensiol D (8) (DMSO-d$_6$)</th>
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</tr>
<tr>
<td>C-2</td>
<td>13C</td>
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<td>C-15</td>
<td>13C</td>
</tr>
</tbody>
</table>

Figure VI.C.10: APT spectrum of 8.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-d$_6$, 30 °C.
Figur VI.C.11: $^1$H NMR spectrum of 8.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-$_d_6$, 30 °C.
VI.C Supplementary information: Induced pigments and anti-Fusarium compounds

Figure VI.C.12: $^1$H-$^1$H DQF-COSY spectrum of 8.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-d$_6$, 30 °C.
Figure VI.C.13: \(^1\)H-\(^{13}\)C HSQC spectrum of 8.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-d\(_6\), 30 °C.
Figure VI.C.14: $^{1}H-^{13}C$ gradient HMBC spectrum of 8.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-d$_6$, 30 °C.
Figure VI.C.15: $^1$H-$^1$H noesy spectrum of 8.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-d$_6$, 30 °C.
2.4.3 Pyrone 9

For compound 9 with the nominal molecular mass 280 Da, the molecular formula was determined as C\textsubscript{16}H\textsubscript{24}O\textsubscript{4} based on HRMS measurement \([m/z \text{ 281.1765 \text{ [M+H]}^+} (C\textsubscript{16}H\textsubscript{25}O\textsubscript{4}, \text{ calc. } m/z \text{ 281.1753, } \Delta 4.3 \text{ ppm})].\] Structure elucidation was performed by extensive 2D NMR spectroscopy.

The \(^1\text{H} \) NMR spectrum showed a pair of aromatic proton signals in meta-position (\(\delta_H 6.10, 5.52; J = 1.8 \text{ Hz} \)). Through HMBC correlation of H-3 (\(\delta_H 5.52\)) to a carbonyl atom (\(\delta_C 180.9, \text{ C-2} \)) and a methoxy-substituted aromatic carbon (\(\delta_C 167.9, \text{ C-4} \)), as well as correlation of H-5 (\(\delta_H 5.52\)) to aromatic C-3 (\(\delta_C 89.4 \)) and C-6 (\(\delta_C 160.3 \)), the presence of a pyrone ring with a methoxy group at C-4 [4-OC\textsubscript{3}H\textsubscript{3} (\(\delta_H 3.88\)) / C-4 (\(\delta_C 167.9\))] was confirmed (Figure VI.C.16). HMBC of H-5 showed the presence of a conjugated double bond linked to C-6. This double bond is substituted with a methyl group as established based on HMBC correlations from 1'-CH\textsubscript{3} (\(\delta_H 1.86 \)). A second methyl group (\(\delta_H 0.98, \text{ d, } J = 7.0 \text{ Hz} \)) showed an HMBC with the sp\textsuperscript{2}-hybridized C-2' and a CH (\(\delta_C 30.7, \text{ C-3'} \)) and a CH\textsubscript{2} (\(\delta_C 38.6, \text{ C-4'} \)). The presence of a hydroxymethyl group was evidenced through \(^1\text{H} \) and HSQC experiments: two proton signals [seen in HSQC (\(\delta_H 3.29 \) and 3.26), hidden by solvent signals in \(^1\text{H} \) NMR spectrum] that are attached to the same carbon (\(\delta_C 63.7 \)) are downfield-shifted through the influence of an attached hydroxy group (\(\delta_H 4.32 \)).

The linkage of the hydroxymethylene group to C-5' was confirmed by the HMBC correlation from H-4' (\(\delta_H 1.35, \text{ m} \)) to the CH\textsubscript{2} (\(\delta_C 63.7, \text{ 5'-CH}_2\text{OH} \)). The remaining proton signals could be assembled to an ethyl-chain attached at C-5' according to COSY and HMBC. The configuration of the double bond was deduced to be trans based on a nuclear Overhauser effect (NOE) between 1'-CH\textsubscript{3} (\(\delta_H 1.86, \text{ s} \)) and H-3' (\(\delta_H 2.69, \text{ m} \)).

![Figure VI.C.16: Numbering scheme and key HMBC and COSY correlations of novel pyrone (9).](image)

This molecule was also detected in pure cultures of \(F.\ solani\). The name solanipyrone (9) is thus proposed for this compound. The configuration of the side chain (C-5', C-3') could not be established with nondestructive methods because of the flexibility of the alkyl chain and limited sample amounts (<1 mg).
Table VI.C.3: NMR shifts of novel pyrone (9).
Proton and carbon signals were assigned with the help of $^1$H, COSY, NOESY, HSQC and HMBC experiments recorded in deuterated DMSO (DMSO-d$_6$).

<table>
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<th>Position</th>
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<th>NOE</th>
</tr>
</thead>
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<tr>
<td>$^{13}$C</td>
<td>$^1$H</td>
<td>$^{13}$C $^1$H</td>
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</tr>
<tr>
<td>C-2</td>
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<td></td>
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<tr>
<td>C-3</td>
<td>H-3</td>
<td>5.52 (1H, d, $J = 1.8$ Hz)</td>
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<td>C-4</td>
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<td>4-OCH$_3$</td>
<td>3.88 (3H, s)</td>
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<td>6.10 (1H, d, $J = 1.8$ Hz)</td>
<td>3, 6, 1$'$</td>
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<tr>
<td>C-6</td>
<td>160.3</td>
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<tr>
<td>C-1$'$</td>
<td>124.8</td>
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<tr>
<td>1$'$-CH$_3$</td>
<td>1$'$-CH$_3$</td>
<td>1.86 (3H, s)</td>
<td>6, 1$'$, 2$'$</td>
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<tr>
<td>C-2$'$</td>
<td>H-2$'$</td>
<td>6.14 (1H, dd, $J = 9.67, 1.12$ Hz)</td>
<td>1$'$-CH$_3$</td>
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<td>H-3$'$</td>
<td>2.69 (1H, m)</td>
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<tr>
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<td>3$'$-CH$_3$</td>
<td>0.98 (3H, d, $J = 6.50$ Hz)</td>
<td>2$'$, 3$'$, 4$'$</td>
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<td>C-4$'$</td>
<td>H-4$'$</td>
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<tr>
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<td>H-5$'$</td>
<td>1.22 (1H, m)</td>
<td>5$'$, 6$'$</td>
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<tr>
<td>5$'$-CH$_2$OH</td>
<td>5$'$-CH$_2$OH</td>
<td>3.29 (1H)</td>
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<tr>
<td>C-6$'$</td>
<td>H-6$'$</td>
<td>1.26 (2H, m)</td>
<td></td>
</tr>
<tr>
<td>C-7$'$</td>
<td>H-7$'$</td>
<td>0.82 (3H, t, $J = 7.23$ Hz)</td>
<td>6$'$, 5$'$</td>
</tr>
</tbody>
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Figure VI.B.17: $^1$H NMR spectrum of 9.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-$d_6$, 30 °C.
Figure VI.C.18: $^1$H-$^1$H gradient COSY spectrum of 9.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-d$_6$, 30 °C.
Figure VI.B.19: NOESY spectrum of 9.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-d$_6$, 30 °C.
Figure VI.B.20: $^1$H-$^{13}$C HSQC spectrum of 9.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-d$_6$, 30 °C.
Figure VI.B.21: $^1$H-$^{13}$C gradient HMBC spectrum of 9.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-$d_6$, 30 °C.
2.4.4 Dihydroquinones 10 – 16

Two different compounds with the same nominal molecular mass 356 Da (molecular formula \( \text{C}_{21}\text{H}_{24}\text{O}_5 \)) were isolated and their NMR data matched with known leucopleurotin (16) and dihydropleurotinic acid (17) [605]. The presence of these two compounds in the co-culture extract was already revealed by dereplication (chapter VI.B, section 2.2). Several isolated compounds (11 to 15, Figure VI.C.8 and Figure VI.B.9) exhibited very similar UV spectra to 16. NMR spectra revealed that all these compounds, as well as 10, are new derivatives of 16.

Dihydroquinone 16: known leucopleurotin

For 16 (molecular mass 356 Da, \( \text{C}_{21}\text{H}_{24}\text{O}_5 \)), the UV spectrum shows one major band at \( \lambda_{\text{max}} \) 300 nm which is consistent with a hydroquinone chromophore (NIST Webbook entry of CAS: 123-31-9, http://webbook.nist.gov/cgi/cbook.cgi?ID=C123319). The structure was confirmed by extensive 1D and 2D NMR. A brief description of the observed correlations is given to set the basis for the structure elucidation of other isolated congeners 10 to 15.

The \(^1\text{H} \) NMR of 16 had signals for a tetra-substituted aromatic ring [two protons in ortho-position \( [\delta_\text{H} 6.73, 6.67; J = 8.7 \text{ Hz}] \)], two ether groups \( [\delta_\text{H} 5.1/\delta_\text{C} 81.8 \text{ and } \delta_\text{H} 4.07/\delta_\text{C} 77.3] \) and a lactone \( [\delta_\text{H} 5.59/\delta_\text{C} 74.7, 176.9] \). Several overlapping signals for \( \text{CH}_2 \) groups and an iso-methyl group \( [\delta_\text{H} 1.03; J = 7.02 \text{ Hz}/\delta_\text{C} 21.1] \) were also apparent. Carbon chemical shifts from the APT experiment and phase-sensitive HSQC indicated one quaternary and \( \text{sp}^3 \)-hybridized carbon \( [\delta_\text{C} 49.8] \). Furthermore, six tertiary carbons \( [\delta_\text{C} 39.6, 44.9, 50.2, 53.5, 74.4 \text{ and } 81.8] \), whereas two thereof are oxygenated \( [\delta_\text{C} 74.4 \text{ and } 81.8] \) and engaged in the lactone and ether group, respectively. HMBC and COSY correlations confirmed these groups (Figure VI.C.22) and established the connectivity of the fused six-ring system characteristic for the meroterpenoid leucopleurotin.

![Figure VI.C.22: Key HMBC and COSY correlations of leucopleurotin (16).](image)
Table VI.C.4: NMR shifts of leucopleurotin (16).
Proton chemical shifts (δ_H) are given in ppm and coupling constants (J) in Hz. Proton and carbon chemical shifts were assigned with the help of 1H, APT, COSY, NOESY, HSQC and HMBC experiments recorded in deuterated acetonitrile (CD₃CN).

<table>
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<tr>
<th>Position</th>
<th>Leucopleurotin (16) (CD₃CN)</th>
<th>HMBC correlations</th>
<th>NOE</th>
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</thead>
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<td>C-1</td>
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<tr>
<td>C-2</td>
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<td>H-3</td>
<td>6.73 (1H, d, J = 8.7 Hz)</td>
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<tr>
<td>C-4</td>
<td>H-4</td>
<td>6.67 (1H, d, J = 8.7 Hz)</td>
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<tr>
<td>C-5</td>
<td>152.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-6</td>
<td>120.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-7</td>
<td>H-7</td>
<td>5.11 (1H, s)</td>
<td>1, 5, 8, 11, 17, 18, 19, 8′, 12″, 14</td>
</tr>
<tr>
<td>C-8</td>
<td>H-8′</td>
<td>4.07 (1H, dd, J = 12.9, 8.0 Hz)</td>
<td>7, 12, 21, 7, 8″</td>
</tr>
<tr>
<td>C-9</td>
<td>H-9</td>
<td>3.91 (1H, dd, J = 12.9, 3.3 Hz)</td>
<td>7, 9, 10, 21, 8′</td>
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<tr>
<td>C-10</td>
<td>H-10</td>
<td>2.16 (1H, m)</td>
<td></td>
</tr>
<tr>
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<td>H-11</td>
<td>1.73 (1H, m)</td>
<td>7, 10, 12, 18, 17</td>
</tr>
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<td>C-12</td>
<td>H-12′</td>
<td>1.91 (1H, m)</td>
<td>9, 10</td>
</tr>
<tr>
<td>C-12″</td>
<td>H-12″</td>
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<td>–</td>
</tr>
<tr>
<td>C-13</td>
<td>H-13′</td>
<td>2.15 (1H, m)</td>
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</tr>
<tr>
<td>C-13″</td>
<td>H-13″</td>
<td>1.41 (1H, m)</td>
<td>12, 14, 17</td>
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<tr>
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<td>H-14</td>
<td>2.43 (1H, m)</td>
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<tr>
<td>C-15</td>
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</tr>
<tr>
<td>C-16</td>
<td>H-16</td>
<td>5.59 (1H, d, J = 6.8 Hz)</td>
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<td>H-17</td>
<td>2.43 (1H, m)</td>
<td>6, 7, 13, 14, 11, 16</td>
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<td></td>
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<td>H-19′</td>
<td>1.76 (1H, m)</td>
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<td>H-19″</td>
<td>1.28 (1H, m)</td>
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<td>H-20′</td>
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<td>H-20″</td>
<td>1.81 (1H, m)</td>
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<td>H₂-21</td>
<td>1.03 (3H, d, J = 7.2 Hz)</td>
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<td>5-OH</td>
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* not observed
Figure VI.C.23: $^1$H NMR spectrum of 16.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.24: $^1$H-$^1$H gradient COSY spectrum of 16.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.25: NOESY spectrum of 16.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.26: $^1$H-$^{13}$C HSQC spectrum of 16.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.27: $^1$H-$^{13}$C gradient HMBC spectrum of 16.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.28: APT spectrum of 16.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.

Figure VI.C.29: Three-dimensional structure of leucopleurotin (16) and nuclear Overhauser effect (NOE) of proton H$_3$-21 and H-7.
NOEs were in agreement with the stereochemistry determined by X-ray crystallography [610,611].

The configuration of leucopleurotin (16) was verified by a series of selective NOEs (Figure VI.C.25 and Figure VI.C.29) and matched the published configuration [611]. The configuration of the iso-methyl group was confirmed through the NOEs between H-7 (δ$_H$ 5.1) and H-12 (δ$_H$ 1.82), H-14 (δ$_H$ 2.43) and H-8′ (δ$_H$ 4.07), as well as all NOEs from H$_3$-21. An NOE between H-7 (δ$_H$ 5.1) and H-16 (δ$_H$ 5.59) was not observed which supports the deduction of their anti-position. This is in agreement with the proposed stereochemistry that was determined by X-ray crystallography (Figure VI.C.29, [610,611]).

The structure elucidation of the leucopleurotin congeners 10 to 15 was made in comparison with 1D and 2D NMR spectra of leucopleurotin (16) as described in more detail below.
Dihydroquinone 13

For 13, HRMS indicated a molecular formula of C_{22}H_{28}O_{6} (molecular mass 388 Da), consistent with 16 plus a methoxylate. The UV (λ_{max} around 300 nm) and the NMR spectra (comparison on Table VI.C.12 and Table VI.C.13) of 13 resembled that of 16 but with several differences. Most notably, the H-16 and C-16 signals were shifted upfield (δ_{H} 5.29, δ_{C} 121.5), the two aromatic protons (H-3, H-4) exhibited near identical shifts and appeared as one apparent singlet. Additional signals characteristic for a methoxy group were apparent from both ^{1}H and ^{13}C chemical shifts (δ_{H} 3.52 and δ_{C} 52.0). The signals corresponding to H-16/C-16 could be assigned based on a HMBC correlation (Figure VI.C.36) between H-16 and C-1 (δ_{H} 5.29, δ_{C} 121.5). The upfield shifted H-16 was consistent with a hydroxy group at C-16 in 13 instead of a lactone as this was the case for 16. This is also coherent with the observation of the overlapped aromatic signals appearing as singlet which indicates almost symmetrical substitution of surrounding atoms (hydroxy group at C-16, ether group at C-7). The signals corresponding to H-14/C-14 (δ_{H} 2.28, δ_{C} 40.7) were assigned based on a HMBC correlation between H-16 and C-14. Carbon chemical shift of C-14 was only marginaly downfield-shifted compared to 16 which is coherent with a methoxycarbonyl substitution at C-14. Moreover, the methoxy group (15-OCH_{3}), exhibited an HMBC correlation between 15-OCH_{3} and C-15, and this supports the methoxycarbonyl substitution. The COSY and HMBC spectra confirmed that the remaining connectivities of 13 were the same as in 16.
Table VI.C.5: NMR shifts of leucopleurotin congener leucopleurotinic acid D (13).
Proton chemical shifts ($\delta_H$) are given in ppm and coupling constants ($J$) in Hz. Proton and carbon signals were assigned with the help of $^1$H, apt, COSY, NOESY, HSQC and HMBC experiments recorded in deuterated acetonitrile (CD$_3$CN).

<table>
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<th>Position</th>
<th>Leucopleurotinic acid D (13) (CD$_3$CN)</th>
<th>HMBC correlations</th>
<th>NOE</th>
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<td>$^{13}$C</td>
<td>$^1$H</td>
<td><strong>C-1</strong></td>
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<tr>
<td>C-1</td>
<td>121.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-2</td>
<td>151.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-3</td>
<td>H-3 117.7</td>
<td>6.60 (2H, as)</td>
<td>5, 6, 8, 17, 18, 19 H-8', H-12', H-14</td>
</tr>
<tr>
<td>C-4</td>
<td>H-4 117.6</td>
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<td>2</td>
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<tr>
<td>C-5</td>
<td>151.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-6</td>
<td>121.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-7</td>
<td>H-7 81.3</td>
<td>4.96 (1H, s)</td>
<td>1, 5, 6, 8, 17, 18, 19 H-8', H-12', H-14</td>
</tr>
<tr>
<td>C-8</td>
<td>H-8' 77.2</td>
<td>4.09 (1H, dd, $J = 12.9, 8.0$ Hz)</td>
<td>7, 21 H-7, H-12'</td>
</tr>
<tr>
<td>C-9</td>
<td>33.9</td>
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<td></td>
</tr>
<tr>
<td>C-10</td>
<td>45.8</td>
<td>2.25 (1H, m)</td>
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<td>C-11</td>
<td>52.7</td>
<td>1.82 (1H, m)</td>
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</tr>
<tr>
<td>C-12</td>
<td>H-12' 22.4</td>
<td>1.93 (1H, m)</td>
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</tr>
<tr>
<td>C-13</td>
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<td>C-14</td>
<td>H-13' 40.7</td>
<td>2.03 (1H, m)</td>
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</tr>
<tr>
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<td>H-13'' 178.1</td>
<td>1.60 (1H, m)</td>
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</tr>
<tr>
<td>C-16</td>
<td>H-16 70.5</td>
<td>5.29 (1H, d, $J = 5.3$ Hz)</td>
<td>1, 14 H-17, H-19''</td>
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<td>C-17</td>
<td>H-17 53.7</td>
<td>2.26 (1H, m)</td>
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<td>C-18</td>
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<td>C-19</td>
<td>H-19' 34.0</td>
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<td>H-20' 26.1</td>
<td>1.83 (1H, m)</td>
<td>7, 11, 18, 20 H-16</td>
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<td>C-21</td>
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<td>1.02 (3H, d, $J = 7.2$ Hz)</td>
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</tr>
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<tr>
<td>15-OCH$_3$ 15-OCH$_3$ 52.0 3.52 (3H, s)</td>
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* not observed
Figure VI.C.30: $^1$H NMR spectrum of 13.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.31: $^1$H-$^1$H gradient COSY spectrum of 13.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.32: NOESY spectrum of 13.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.

Vi.C Supplementary information: Induced pigments and anti-Fusarium compounds
Figure VI.C.33: $^1$H-$^{13}$C HSQC spectrum of 13.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.34: $^1$H-$^{13}$C gradient HMBC spectrum of 13.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
The configuration of 13 was assumed to be identical to that of 16. The relative configuration of the OH at C-16 (anti to H-14) was deduced from a NOESY experiment. NOE and key HMBC correlations are shown in Figure VI.C.36. The NOE between H-16 and H-19 indicated that the OH at C-16 was in anti-position to H-14 (distance H-16 to H-17 is 2.4 Å for anti compared to 4.2 Å for syn). The dihedral angle between H-16 and H-17 was in agreement with the observed $J$-coupling ($\delta_{H-16}$ 5.29, d, 5.3 Hz). In addition, theoretical calculations based on MM2 minimization of the three-dimensional structures showed 30% less steric energy for the anti-compared to the syn-position which may strengthen the other deductions. The structure and proposed configuration of 13 is shown in Figure VI.C.36.
Hydroquinone 12

For compound 12, HRMS indicated the same molecular formula as for 13, C_{22}H_{28}O_{6}. The UV spectrum was again coherent with a hydroquinone structure. Compound 12 was isolated in a 3:1 mixture with 16 and the structure was determined in the mixture sample. NMR spectra resembled that of 13 with two distinct differences. The H-16 signal (δ_H 3.99) was shifted upfield whereas the C-16 (δ_C 75.5) signal was slightly shifted downfield compared to 13. Furthermore, the H-14 and C-14 signals (δ_H 6.68, 6.60; J = 8.6 Hz) were shifted upfield and the two aromatic protons (H-3, H-4) exhibited different chemical shifts (as in 16) and were apparent as two doublet signals (δ_H 6.68, 6.60; J = 8.6 Hz). As for 13, an additional proton signal was apparent compared to 16 which was consistent with a methoxylation of the molecule (δ_H 3.37/δ_C 52.0). HMBC correlation permitted the connection of this methoxy group to C-16. This is consistent with the altered chemical shifts mentioned above. A singlet signal was observed for H-16 which would be consistent with a dihedral angle of 90 °C between H-16 and H-17. Based on this reasoning, the relative configuration at C-16 was assumed to be syn for H-16 to H-14.

Table VI.C.6: NMR shifts of leucopleurotin congener leucopleurotinic acid C (12).
Proton chemical shifts (δ_H) are given in ppm and coupling constants (J) in Hz. Proton and carbon signals were assigned with the help of 1H, apt, COSY, HSQC and HMBC experiments recorded in deuterated acetonitrile (CD_{3}CN).

<table>
<thead>
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<th>Position</th>
<th>Leucopleurotinic acid C (12) (CD_{3}CN)</th>
<th>HMBC correlations</th>
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<td>11C</td>
<td>1H</td>
<td>12C</td>
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<td>C-2</td>
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<td>C-3</td>
<td>H-3</td>
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<td>6.68 (1H, d, J = 8.6 Hz)</td>
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<td>C-4</td>
<td>H-4</td>
<td>117.5</td>
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<td>6.60 (1H, d, J = 8.6 Hz)</td>
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<td>4.07 (1H, dd, J = 13.0, 7.9 Hz)</td>
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* not observed
**Figure VI.C.37: $^1$H NMR spectrum of 12.**
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.38: $^1$H-$^1$H gradient COSY spectrum of 12.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.39: $^1$H-$^{13}$C HSQC spectrum of 12.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.40: $^1$H-$^{13}$C gradient HMBC spectrum of 12.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Hydroquinone 11

For compound 11, HRMS indicated a molecular formula of C_{21}H_{26}O_{6} (molecular mass 374 Da), consistent with 16 plus a hydroxylate. Similarly to 13, the UV spectrum was coherent with 16 which indicated that this molecule holds the hydroquinone chromophore as well. Compound 11 was isolated in a 1:2 mixture with 16 and the structure was determined in the mixture sample. NMR spectra resembled that of 13 with several subtle differences. The H-16 signal (δ\text{H} 4.49) was considerably shifted upfield compared to 13 (and downfield compared to 12). Furthermore, the H-17 signal was also shifted upfield and the methoxy signal was missing. This indicated a difference in the area C-16/C-17/C-14. The observed chemical shifts and the molecular formula were consistent with a hydroxylation at C-16 and a free carboxylic acid at C-14. The co-elution of 11 with 16 suggests a hydrolysis of 16 during sample preparation of the isolated compound prior to NMR analysis. Nevertheless, the corresponding m/z was observed in the MPLC fraction from which the compound was isolated as well as in the crude co-culture extract. The absence of NOE with H-16 and the observation of a singlet signal for H-16 indicates syn-position of H-16 (compared to H-14), similar to 12. This favors the assumption that 11 wasn’t formed through hydrolysis of 16, which would result in retained configuration at C-16, but through a S\textsubscript{N}2-reaction at C-16. This compound could have formed during the extraction as the extraction solvent contained water and methanol (see also section 2.5).
Table VI.C.7: NMR shifts of leucopleurotin congener leucopleurotinic acid B (11).
Proton chemical shifts (δ_H) are given in ppm and coupling constants (J) in Hz. Proton and carbon signals were assigned with the help of ^1H, apt, COSY, NOESY, HSQC and HMBC experiments recorded in deuterated acetonitrile (CD_3CN).

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<th>NOE</th>
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<td>H-8'</td>
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<td>H-7</td>
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<tr>
<td></td>
<td>H-8''</td>
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<td></td>
</tr>
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<td>H-9 34.2 2.08 (1H, m)</td>
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<td>H-12'</td>
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<td>H-12''</td>
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<td>C-13</td>
<td>H-13' 31.8 2.10 (1H)</td>
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<td>C-14</td>
<td>H-13'' 1.62 (1H)</td>
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<tr>
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<td>H-16 67.0 4.49 (1H, s)</td>
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<td>C-17</td>
<td>H-17 52.8 2.03 (1H, m)</td>
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<td>C-18</td>
<td>46.4</td>
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<td>C-19</td>
<td>H-19' 35.1 1.85 (1H)</td>
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<td>H-19'' 1.59 (1H)</td>
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<td>16-OH *</td>
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* not observed
Figure VI.C.42: $^1$H NMR spectrum of 11 (red assignment, in mixture with 16, blue assignment). Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.43: $^1$H-$^1$H COSY spectrum of 11 (in mixture with 16).
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.

Figure VI.C.44: NOESY spectrum of 11 (in mixture with 16).
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.45: $^1$H-$^{13}$C gradient HSQC spectrum of 11 (in mixture with 16).
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.46: $^1$H-$^{13}$C gradient HMBC spectrum of 11 (in mixture with 16).
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Hydroquinone 14

For compound 14, HRMS indicated a molecular formula of C$_{21}$H$_{24}$O$_6$ (molecular mass 372 Da). Its UV spectrum was coherent with the hydroquinone chromophore. NMR spectra resembled that of 16 with several differences. Most notably, the aromatic signals (δ$_H$ 6.82, 7.05; J = 9.0 Hz) were shifted downfield, as were the proton signals at C-8 (δ$_H$ 4.15, 4.02; J = 13.2 Hz), at C-14 (δ$_H$ 2.59) and C-17 (δ$_H$ 2.50). The proton signal for H-16 was absent. Among the carbon chemical shifts, signals for C-2 (δ$_C$ 157.5) and C-17 (δ$_C$ 60.5) were shifted downfield whereas the signal for C-14 (δ$_C$ 34.7) was shifted upfield. Consistent with an electron withdrawing group at C-16, the compound bears a carbonyl group at C-16. HMBC and COSY confirmed that the connectivities of 14 and 16 were the same. HMBC correlation between H-17 (δ$_H$ 2.50) and C-160 (δ$_C$ 204.7) confirmed the carbonylation at C-16.
Table VI.C.8: NMR shifts of leucopleurotin congener leucopleurotinic acid E (14).
Proton chemical shifts (δH) are given in ppm and coupling constants (J) in Hz. Proton and carbon signals were assigned with the help of 1H, COSY, NOESY, HSQC and HMBC experiments recorded in deuterated acetonitrile (CD3CN).

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<td>150.8</td>
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<td>123.5</td>
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<td>H-7 82.4</td>
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* not observed
Figure VI.C.48: $^1$H NMR spectrum of 14.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
**Figure VI.C.49:** $^1$H-$^1$H gradient COSY spectrum of 14.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.

**Figure VI.C.50:** $^1$H-$^{13}$C HSQC spectrum of 14.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.51: $^1$H-$^{13}$C gradient HMBC spectrum of 14.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Hydroquinone 15

HRMS indicated a molecular formula of C_{21}H_{26}O_{5} (molecular mass 358 Da) for 15. The UV spectrum of 15 was coherent with the hydroquinone chromophore. NMR spectra resembled that of 16 with one distinct difference. Carbon chemical shift of C-16, identified through COSY and HMBC correlations, was substantially shifted upfield (\(\delta_{C} 25.8\)) and bearde two protons. The C-16 position was thus not oxygenated. The configuration at C-14 was assumed to be identical to 16, thus the carboxy group at C-14 would be in anti-position to H-17.

**Table VI.C.9: NMR shifts of leucopleurotin congener leucopleurotinic acid F (15).**
Proton chemical shifts (\(\delta_{H}\)) are given in ppm and coupling constants (J) in Hz. Proton and carbon signals were assigned with the help of \(^1H\), COSY, HSQC and HMBC experiments recorded in deuterated acetonitrile (CD\(_3\)CN).

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<tr>
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* not observed
Figure VI.C.52: 1H NMR spectrum of 15 (blue assignment, in mixture with 17, red assignment).

Varian ANOVA (Agilent), 500 MHz, 5 mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.53: $^1$H-$^1$H gradient COSY spectrum of 15 (blue assignment, in mixture with 17, red assignment). Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.54: $^1$H-$^{13}$C HSQC spectrum of 15 (blue assignment, in mixture with 17, red assignment). Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.55: $^{1}H$-$^{13}C$ gradient HMBC spectrum of 15.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
**Hydroquinone 10**

HRMS indicated a molecular formula of $C_{21}H_{24}O_7$ (molecular mass 388 Da) for 10. The UV spectrum showed a strong band at $\lambda_{max}$ 400 nm which indicated that the chromophore was different from the compounds 11 to 16.

The $^1$H NMR and HSQC spectrum in DMSO-$d_6$ (Table VI.C.10) of 10 had signals for a symmetrically substituted hydroquinone ring ($\delta^H$ 7.35), several secondary and tertiary carbon atoms and an iso-methyl group ($\delta^H$ 1.04; $J = 6.4$ Hz). COSY correlations established the connectivity of a hydroxylated iso-propyl group (C8/C9/C21/C10). Correlations from COSY and HMBC helped establish the five-membered ring (C-10/C-11/C-18/C-19/C-20). The connectivities of the six-membered ring fused to C-11/C-18 was ascertained by COSY and HMBC through correlations with the quaternary carbon C-18. HMBC to carbonyl C-16 ($\delta^C$ 203.0) from H-3 ($\delta^H$ 7.35) and H-17 ($\delta^H$ 2.78), as well as HMBC to a second carbonyl (C-7, $\delta^C$ 203.0) from H-4 ($\delta^H$ 7.35) and H-11 ($\delta^H$ 1.93) permitted the placement of a dihydroquinone ring fused to the hydroquinone (C-1 to C-6). This is consistent with the observation that carbon chemical shifts for C-1 and C-6 were shifted upfield while all other signals (C-2 to C-5) were shifted downfield compared to 16. Relevant COSY and HMBC correlations are shown in Figure VI.C.56. The structure of 10 is derived through a ring-opening of the ether ring known from leucopleurotin (16) and dihydropleurotinic acid (17). The relative configuration of 10 is assumed to be identical to that of 16. This was confirmed by NOESY, in particular by NOEs observed between H-17 ($\delta^H$ 2.78) and H-11 ($\delta^H$ 1.93), H-13'' ($\delta^H$ 1.59) and H-19'' ($\delta^H$ 1.62, Figure VI.C.57 and Figure VI.C.60).

![Figure VI.C.56: Key HMBC and COSY correlations of dihydropleurotinic acid (10).](image-url)
### Table VI.C.10: NMR shifts of leucopleurotin congener leucopleurotinic acid A (10).
Proton chemical shifts ($\delta_H$) are given in ppm and coupling constants ($J$) in Hz. Proton and carbon signals were assigned with the help of $^1$H, apt, COSY, TOCSY, NOESY, HSQC and HMBC experiments recorded in DMSO-$d_6$.

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<th>HMBC correlations</th>
<th>NOE</th>
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* not observed

*not observed

**Figure VI.C.57: Proposed configuration of leucopleurotinic acid A (10).**
Figure VI.C.58: $^1$H NMR spectrum of 10.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-d$_6$, 30 °C.
Figure VI.C.59: $^{1}H-^{1}H$ gradient COSY spectrum of 10.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-$d_{6}$, 30 °C.

Figure VI.C.60: NOESY spectrum of 10.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-$d_{6}$, 30 °C.
**Figure VI.C.61**: $^1$H-$^1$H TOCSY spectrum of 10.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-d$_6$, 30 °C.

**Figure VI.C.62**: $^1$H-$^{13}$C HSQC spectrum of 10.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-d$_6$, 30 °C.
Figure VI.C.63: $^1$H-$^{13}$C gradient HMBC spectrum of 10.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-d$_6$, 30 °C.
Fungal metabolites

VI.C Supplementary information: Induced pigments and anti-*Fusarium* compounds

**Figure VI.C.64: APT spectrum of 10.**
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-d$_6$, 30 °C.

**Table VI.C.11: NMR shifts of leucopleurotin congener leucopleurotinic acid A (10).**
Proton chemical shifts (δ$_H$) are given in ppm and coupling constants (J) in Hz. Proton and carbon signals were assigned with the help of $^1$H, apt, COSY, TOCSY, NOESY, HSQC and HMBC experiments recorded in deuterated acetonitrile (CD$_3$CN).

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* not observed
Figure VI.C.65: $^1$H NMR spectrum of 10.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
**Figure VI.C.66:** $^1$H-$^1$H gradient COSY spectrum of 10.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.

**Figure VI.C.67:** $^1$H-$^1$H NOESY spectrum of 10.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.68: $^1$H-$^{13}$C HSQC spectrum of 10.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.69: $^1$H-$^{13}$C gradient HMBC spectrum of 10.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
The compound 10 to 15 were detected in pure cultures of *H. reniformis* as well. The Basidiomycete is thus the producer of these molecules in the co-culture. All these molecules are derived from the free carboxylic acid form of the known molecule leucopleurotin and thus, the names leucopleurotonic acid A to F are proposed for these compounds. $^{13}$C and $^1$H NMR chemical shifts for 10 to 16 are summarized in Table VI.C.12 and Table VI.C.13.

### Table VI.C.12: $^{13}$C-NMR shifts of novel congeners 10 to 15 of leucopleurotin (16).

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* not observed
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H-16

2.75 (d, J = 12.0)
1.85 (m)
1.60 (m)
1.88 (m)
1.37 (m)
1.09 (d, J = 6.4)
11.85 (s)
11.56 (s)
*
*
─
─
─

3.50 ( m)
3.27 (m)
2.26 (m)
1.99 (m)
1.88 (m)
2.09
1.85 (m)
2.03
1.65 (m)
2.49 (td, J = 12.0, 3.6)

3.40
3.14 (dd, J = 10.4, 6.8)
2.18 (m)
1.94 (m)
1.93 (m)
1.98 (m)
1.76 (m)
1.96 (m)
1.59 (m)
2.32 (td, J = 12.1, 3.8)

H-17
2.78 (d, J = 11.9)
H-19‘
1.73 (m)
H-19‘‘
1.62 (m)
H-20‘
1.84 (m)
H-20‘‘
1.24 (m)
H3-21
1.04 (d, J = 6.4)
2-OH
11.73 (brs)
5-OH
11.51 (brs)
8-OH
4.21 (brs)
15-OH
*
16-OH
─
15-OCH3 ─
16-OCH3 ─
* not observed

7.27 (s)

7.35 (s)

10 (CD3CN)

H-3
H-4
H-7
H-8‘
H-8‘‘
H-9
H-10
H-11
H-12‘
H-12‘‘
H-13‘
H-13‘‘
H-14

10 (DMSO-d6)

3.99 (s)

12 (CD3CN)
6.68 (d, J = 8.6)
6.60 (d, J = 8.6)
4.92 (s)
4.07 (dd, J = 13.0, 7.9)
3.90 (dd, J = 13.0, 2.2)
2.09 (m)
2.23 (m)
1.84 (m)
1.89 (m)
1.85 (m)
2.12 (m)
1.64 (m)
2.10 (m)

2.19 (m)
1.85 (m)
1.49 (m)
1.82 (m)
*
1.71 (m)
1.04 (d, J = 7.3) 1.04 (d, J = 7.3)
*
*
*
*
−
−
*
8.44 (brs)
*
−
─
─
−
3.37 (s)

2.03 (m)
1.85 (m)
1.59 (m)

4.49 (s)

2.10
1.62
2.12 (m)

*

2.08 (m)
*
1.83 (m)

*

11 (CD3CN)
6.67
6.58 (d, J = 8.5)
4.94

Table VI.C.13: 1H-NMR shifts of novel congeners of leucopleurotin (16).

2.26 (m)
1.84 (m)
1.39 (m)
1.83 (m)
1.75 (m)
1.02 (d, J = 7.2)
*
*
−
−
8.09 (brs)
3.52 (s)
─

5.29 (d, J = 5.3)

4.96 (s)
4.09 (dd, J = 12.9, 8.0)
3.85 (dd, J = 12.9, 2.7)
2.10 (m)
2.25 (m)
1.82 (m)
1.93 (m)
1.82 (m)
2.03 (m)
1.60 (m)
2.28 (m)

6.60 (s)

13 (CD3CN)

2.50 (d, J = 12.2)
1.81 (m)
1.29 (m)
1.88 (m)
1.75 (m)
1.06 (d, J = 7.2)
12.03 (s)
*
−
8.76 (brs)
−
−
−

─

2.11
1.71 (m)
2.59 (td, J = 12.2, 4.0)

1.91 (m)

14 (CD3CN)
6.82 (dd, J = 9.0, 0.9)
7.05 (d, J = 9.0)
5.35 (s)
4.15 (dd, J = 13.2, 7.5)
4.02 (d, J = 13.2)
2.10
2.32 (m)
1.81 (m)

15 (CD3CN)
6.59 (d, J = 8.5)
6.45 (d, J = 8.5)
4.96 (s)
4.09 (dd, J = 12.9, 7.9)
3.89 (dd, J = 12.9, 2.3)
2.15 (m)
2.28 (m)
1.83 (m)
1.91 (m)
1.82 (m)
2.06 (m)
1.61 (m)
2.23 (m)
2.61 (dd, J = 18.2, 5.7)
2.54 (d, J = 18.2)
1.92 (m)
1.81 (m)
1.34 (m)
1.85 (m)
1.73 (m)
1.04 (d, J = 7.2)
*
*
−
8.25 (brs)
−
−
−
2.43 (m)
1.76 (m)
1.28 (m)
1.89 (m)
1.81 (m)
1.03 (d, J = 7.2)
*
*
─
−
−
−
−

5.59 (d, J = 6.8)

16 (CD3CN)
6.73 (d, J = 8.7)
6.67 (d, J = 8.7)
5.1 (s)
4.07 (dd, J = 12.9, 8.0)
3.91 (dd, J = 12.9, 3.3)
2.16 (m)
2.25 (m)
1.73 (m)
1.91 (m)
1.82 (m)
2.15 (m)
1.41 (m)
2.43 (m)


2.4.5 Quinone 17 and 18

For 17, the UV spectrum showed one major band at $\lambda_{\text{max}}$ 250 nm (shoulder bands at 300 and 350 nm) which is consistent with a p-benzoquinone chromophore (NIST Webbook entry of CAS: 106-51-4, http://webbook.nist.gov/cgi/cbook.cgi?Name=Quinone&Units=SI). HRMS indicated a molecular formula of C$_{21}$H$_{24}$O$_5$ and this compound was dereplicated as leucopleurotin or dihydropleurotinic acid. The NMR spectra did not match with leucopleurotin (16, section 2.4.4) but with literature values of dihydropleurotinic acid [605]. The structure was verified by extensive spectroscopic analyses based on 1D and 2D NMR. A brief description of the observed correlations is given to set the basis for the structure elucidation of the isolated congener 18.

Figure VI.C.70: Structure and configuration of dihydropleurotinic acid (17) and novel congener dihydropleurotinic acid B (18).

Quinone 17: dihydropleurotinic acid

The $^1$H NMR of 17 (Table VI.C.14 and Table VI.C.15) had signals for a tetrasubstituted aromatic ring [two superimposed doublet signals accounting for two protons in ortho-position ($\delta_H$ 6.70, 6.67; $J = 10.1$ Hz)] and an ether group ($\delta_H$ 4.43/$\delta_C$ 72.8 and $\delta_H$ 3.91, 3.35; $J = 12.3$ Hz/$\delta_C$ 75.4). Furthermore, several overlapping signals for CH$_2$ groups and an iso-methyl group ($\delta_H$ 0.91; $J = 7.0$ Hz/$\delta_C$ 21.1), similar to spectra from 10 to 16, were also apparent. Carbon chemical shifts indicated one quaternary sp$^3$-hybridized carbon ($\delta_C$ 47.4) and six tertiary carbons ($\delta_C$ 32.6, 43.3, 44.8, 46.7, 52.4 and 72.8). Thereof, one tertiary carbon was oxygenated ($\delta_C$ 72.8) and engaged in the ether group. HMBC and COSY correlations confirmed these groups (Table VI.C.14 and following spectra) and established the connectivities of the five ring systems.
Table VI.C.14: NMR shifts of dihydropleurotinic acid (17).
Proton chemical shifts ($\delta_H$) are given in ppm and coupling constants ($J$) in Hz. Proton and carbon chemical shifts were assigned with the help of $^1$H, APT, COSY, HSQC and HMBC experiments recorded in deuterated acetonitrile ($CD_3CN$).

<table>
<thead>
<tr>
<th>Position</th>
<th>Dihydropleurotinic acid (17) ($CD_3CN$)</th>
<th>HMBC correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{13}$C</td>
<td>$^{1}$H</td>
</tr>
<tr>
<td>C-1</td>
<td>141.6</td>
<td></td>
</tr>
<tr>
<td>C-2</td>
<td>188.2</td>
<td></td>
</tr>
<tr>
<td>C-3</td>
<td>H-3</td>
<td>136.8</td>
</tr>
<tr>
<td>C-4</td>
<td>H-4</td>
<td>138.4</td>
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<tr>
<td>C-5</td>
<td></td>
<td>187.4</td>
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<tr>
<td>C-6</td>
<td></td>
<td>141.1</td>
</tr>
<tr>
<td>C-7</td>
<td>H-7</td>
<td>72.8</td>
</tr>
<tr>
<td>C-8</td>
<td>H-8'</td>
<td>75.4</td>
</tr>
<tr>
<td></td>
<td>H-8''</td>
<td></td>
</tr>
<tr>
<td>C-9</td>
<td>H-9</td>
<td>32.6</td>
</tr>
<tr>
<td>C-10</td>
<td>H-10</td>
<td>46.7</td>
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<td>C-11</td>
<td>H-11</td>
<td>52.4</td>
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<tr>
<td>C-12</td>
<td>H-12'</td>
<td>22.4</td>
</tr>
<tr>
<td></td>
<td>H-12''</td>
<td></td>
</tr>
<tr>
<td>C-13</td>
<td>H-13'</td>
<td>31.2</td>
</tr>
<tr>
<td>C-14</td>
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<td></td>
</tr>
<tr>
<td>C-15</td>
<td>H-14</td>
<td>43.3</td>
</tr>
<tr>
<td>C-16</td>
<td>H-16'</td>
<td>25.0</td>
</tr>
<tr>
<td></td>
<td>H-16''</td>
<td></td>
</tr>
<tr>
<td>C-17</td>
<td>H-17</td>
<td>44.8</td>
</tr>
<tr>
<td>C-18</td>
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<tr>
<td>C-19</td>
<td>H-19'</td>
<td>35.1</td>
</tr>
<tr>
<td></td>
<td>H-19''</td>
<td></td>
</tr>
<tr>
<td>C-20</td>
<td>H-20'</td>
<td>25.7</td>
</tr>
<tr>
<td></td>
<td>H-20''</td>
<td></td>
</tr>
<tr>
<td>C-21</td>
<td>H$_2$-21</td>
<td>21.1</td>
</tr>
<tr>
<td></td>
<td>15-OH</td>
<td>8.98</td>
</tr>
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</table>
Figure VI.C.71: $^1$H NMR spectrum of 17.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
**Figure VI.C.72: $^1$H-$^1$H gradient COSY spectrum of 17.**

Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.

**Figure VI.C.73: APT spectrum of 17.**

Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.74: $^1$H-$^{13}$C gradient HSQC spectrum of 17.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.75: $^1\text{H}$-$^{13}\text{C}$ gradient HMBC spectrum of 17.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Quinone 18

For 18, the UV and the NMR spectra showed similarities with that of 17 (Table VI.C.15, Table VI.C.16 and following spectra). Three distinct differences in the NMR spectra of 18 compared to 17 could be observed. The signal for C-1 was shifted downfield, the signal for C-16, identified through COSY and HMBC, was substantially shifted downfield and the corresponding 1H signal integrated for only one proton indicating substitution at C-16. Furthermore, additional signals, characteristic for a methoxy group were apparent (δ_H 3.35 and δ_C 58.1). HMBC between the methoxy group (δ_H 3.35) and C-16 (δ_C 73.3) confirmed methoxylation at C-16. This is consistent with the observed downfield shift of C-1, C-16 and H-16.

Table VI.C.15: _13C and _1H NMR chemical shift of dihydropleurotinic acid (17) and its congener 18.

<table>
<thead>
<tr>
<th>Position</th>
<th>17 (CD_3CN)</th>
<th>18 (CD_3CN)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>_13C</td>
<td>_1H</td>
</tr>
<tr>
<td>C-1</td>
<td>141.6</td>
<td>138.3</td>
</tr>
<tr>
<td>C-2</td>
<td>188.2</td>
<td>187.4</td>
</tr>
<tr>
<td>C-3</td>
<td>H-3</td>
<td>136.8</td>
</tr>
<tr>
<td></td>
<td>H-4</td>
<td>138.4</td>
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<tr>
<td>C-5</td>
<td>187.4</td>
<td>187.8</td>
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<td>C-6</td>
<td>141.1</td>
<td>142.4</td>
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<tr>
<td>C-7</td>
<td>H-7</td>
<td>72.8</td>
</tr>
<tr>
<td></td>
<td>H-8'</td>
<td>75.4</td>
</tr>
<tr>
<td></td>
<td>H-8''</td>
<td>3.35 (1H, dd, J = 12.3, 6.5 Hz)</td>
</tr>
<tr>
<td>C-9</td>
<td>H-9</td>
<td>32.6</td>
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<td>C-10</td>
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<td>C-11</td>
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<td>52.4</td>
</tr>
<tr>
<td>C-12</td>
<td>H-12'</td>
<td>22.4</td>
</tr>
<tr>
<td></td>
<td>H-12''</td>
<td>1.72 (1H, m)</td>
</tr>
<tr>
<td>C-13</td>
<td>H-13'</td>
<td>31.2</td>
</tr>
<tr>
<td></td>
<td>H-13''</td>
<td>1.54 (1H, q, J = 12.7, 4.5 Hz)</td>
</tr>
<tr>
<td>C-14</td>
<td>H-14</td>
<td>43.3</td>
</tr>
<tr>
<td>C-15</td>
<td>176.9</td>
<td>177.0</td>
</tr>
<tr>
<td>C-16</td>
<td>H-16</td>
<td>25.0</td>
</tr>
<tr>
<td>C-17</td>
<td>H-17</td>
<td>44.8</td>
</tr>
<tr>
<td>C-18</td>
<td>47.4</td>
<td>46.1</td>
</tr>
<tr>
<td>C-19</td>
<td>H-19'</td>
<td>35.1</td>
</tr>
<tr>
<td></td>
<td>H-19''</td>
<td>1.21 (1H, m)</td>
</tr>
<tr>
<td>C-20</td>
<td>H-20'</td>
<td>25.7</td>
</tr>
<tr>
<td></td>
<td>H-20''</td>
<td>1.69 (1H, m)</td>
</tr>
<tr>
<td>C-21</td>
<td>H-21</td>
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<tr>
<td></td>
<td>2-OH</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5-OH</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>15-OH</td>
<td>8.98 (1H, brs)</td>
</tr>
<tr>
<td>16-OCH_3</td>
<td>16-OCH_3</td>
<td>-</td>
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</tbody>
</table>

The configuration of 18 was assumed to be the same as in dihydropleurotinic acid (17). The configuration of the methoxy group at C-16 was explored using selective NOE. A well-isolated signal in the _1H NMR spectrum (δ_H 1.89, H-14) was irradiated and NOEs were observed with H-7 and H-16. These NOEs confirmed that the overall configuration is identical to the one
reported [597]. Furthermore, these NOEs together with the observation of a singlet signal for H-16 indicates that H-16 is in syn-position to H-14, as for 12 (Figure VI.C.76).

Figure VI.C.76: Configuration of dihydropleurotinic acid B (18). Nuclear Overhauser effect (NOE) from proton H-14 are shown with arrows. The proton at C-16 is in syn-position to H-14.
Table VI.C.16: NMR shifts of dihydropleurotinic acid B (18). Proton chemical shifts ($\delta_H$) are given in ppm and coupling constants ($J$) in Hz. Proton and carbon chemical shifts were assigned with the help of $^1$H, APT, COSY, HSQC and HMBC experiments recorded in deuterated acetonitrile (CD$_3$CN).

<table>
<thead>
<tr>
<th>Position</th>
<th>Dihydropleurotinic acid B (18) (CD$_3$CN)</th>
<th>HMBC correlations</th>
<th>NOE</th>
</tr>
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<td>$^{13}$C</td>
<td>$^1$H</td>
<td>$^{13}$C</td>
<td>$^1$H</td>
</tr>
<tr>
<td>C-1</td>
<td>138.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-2</td>
<td>187.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-3</td>
<td>137.0</td>
<td>6.73 (2H, s)</td>
<td>1, 5</td>
</tr>
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<td>C-4</td>
<td>138.6</td>
<td></td>
<td>2, 6</td>
</tr>
<tr>
<td>C-5</td>
<td>187.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-6</td>
<td>142.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-7</td>
<td>73.26</td>
<td>4.37 (1H, s)</td>
<td>1, 5, 6, 8, 11, 17, 18, 19</td>
</tr>
<tr>
<td>C-8</td>
<td>75.5</td>
<td>3.90 (1H, dd, $J = 12.3, 8.6$ Hz)</td>
<td>7, 10, 21</td>
</tr>
<tr>
<td>C-9</td>
<td>32.5</td>
<td>2.15 (1H, m)</td>
<td>8, 9, 10, 21</td>
</tr>
<tr>
<td>C-10</td>
<td>45.9</td>
<td>2.06 (1H, m)</td>
<td>8, 9, 11, 12, 18, 19, 20, 21</td>
</tr>
<tr>
<td>C-11</td>
<td>52.3</td>
<td>1.64 (1H, m)</td>
<td>7, 12, 13, 17, 18, 19</td>
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<tr>
<td>C-12</td>
<td>H-12'</td>
<td>1.78 (1H, m)</td>
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</tr>
<tr>
<td>C-13</td>
<td>H-13'</td>
<td>1.75 (1H, m)</td>
<td></td>
</tr>
<tr>
<td>C-14</td>
<td>H-14</td>
<td>1.89 (1H, td, $J = 12.5, 4.2$ Hz)</td>
<td>12, 13, 15, 16, 17, 18 H-7, H-16, H-19</td>
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<tr>
<td>C-15</td>
<td>177.0</td>
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</tr>
<tr>
<td>C-16</td>
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<td>C-17</td>
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<td>2.13 (1H, s)</td>
<td>1, 15</td>
</tr>
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<td>C-18</td>
<td>46.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-19</td>
<td>H-19'</td>
<td>2.08 (1H, m)</td>
<td></td>
</tr>
<tr>
<td>C-20</td>
<td>H-20'</td>
<td>1.70 (1H, m)</td>
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<td>C-21</td>
<td>H$_2$-21 15-OH</td>
<td>0.91 (3H, d, $J = 7.0$ Hz)</td>
<td>8, 9, 10</td>
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<td>15-OH</td>
<td>9.09 (1H, brs)</td>
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</tr>
<tr>
<td>16-OCH$_3$</td>
<td>58.1</td>
<td>3.35 (3H, s)</td>
<td>16</td>
</tr>
</tbody>
</table>
Figure VI.C.77: $^1$H NMR spectrum of 18.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.78: $^1$H-$^1$H gradient COSY spectrum of 18.  
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.

Figure VI.C.79: $^1$H-$^1$H NOESY spectrum of 18.  
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
Figure VI.C.80: $^1$H-$^{13}$C gradient HSQC spectrum of 18. Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
VI.C Supplementary information: Induced pigments and anti-Fusarium compounds

Figure VI.C.81: $^1$H-$^13$C gradient HMBC spectrum of 18.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
**Figure VI.C.82: APT spectrum of 18.**
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.

**Figure VI.C.83: Selective 1D NOE spectrum of 18.**
Irradiation at 1.9 ppm (H-14). Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, CD$_3$CN, 25 °C.
2.4.6 Fatty acid 19 and 20

The two compounds 19 and 20 exhibited both an UV spectrum with \( \lambda_{\text{max}} \) 280 nm that could not be related to any particular chromophore. HRMS indicated a molecular formula of \( \text{C}_{18}\text{H}_{30}\text{O}_6 \) for both compounds and NMR chemical shifts and information from 2D NMR analyses for both 19 and 20 matched literature values from a compound named ‘antibiotic 1233B’ [596] (Figure VI.C.84). \(^1\)H NMR spectra of 19 and 20 were very similar (Figure VI.C.85 – Figure VI.C.93). Differences were observed for the \(^1\)H chemical shift of the hydroxylation sites of the molecule, H-2, H-3 and both protons at C-15. This indicates configurational isomerism of the two hydroxyl groups. The configuration could not be established with nondestructive methods because of the structural flexibility of the side chain.

\[
\begin{align*}
\text{OH} & \\
\text{O} & \\
\text{OH} & \\
\text{HO} & \\
\text{O} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\text{C} & \\
\end{align*}
\]

*Figure VI.C.84: Numbering scheme for 19 and 20.*

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<td>C-17</td>
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<tr>
<td>C-18</td>
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Figure VI.C.85: $^1$H NMR spectrum of 19.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-$d_6$, 30 °C.
**Figure VI.C.86:** APT spectrum of 19. Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-d$_6$ 30 °C.

**Figure VI.C.87:** $^1$H-$^{13}$C HSQC spectrum of 19. Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-d$_6$ 30 °C.
Figure VI.C.88: $^1$H-$^{13}$C HMBC spectrum of 19.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-$d_6$, 30 °C.
Figure VI.C.89: $^1$H NMR spectrum of 20.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-$d_6$, 30 °C.
**Figure VI.C.90: APT spectrum of 20.**
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-$d_6$, 30 °C.

**Figure VI.C.91: $^1$H-$^{13}$C HSQC spectrum of 20.**
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-$d_6$, 30 °C.
Figure VI.C.92: $^1$H-$^{13}$C HMBC spectrum of 20.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-$d_6$, 30 °C.
Figure VI.C.93: $^1$H-$^1$H TOCSY spectrum of 20.
Varian ANOVA (Agilent), 500 MHz, 5-mm tube probe, DMSO-$d_6$, 30 °C.
2.5 Stability of hydroquinones and quinones in dimethylsulfoxide and methanol

During the NMR analyses of prefractions and MPLC fractions, it was observed that solubility was best in DMSO. In consequence, this solvent was chosen for the analysis. However, degradation was observed in deuterated DMSO for the pleurotin quinone samples (compounds 10 – 18) over the short time frame of 28h – which corresponded to the time to analyze a complete set of 1D and 2D NMR analyses. When comparing $^1$H NMR spectra at $t=0$ and after 28h, several proton signals exhibited altered intensity differences and the disappearance of proton signals demonstrated the beginning rearrangement or degradation of the compounds (Figure VI.C.94).

![Figure VI.C.94: Rearrangement of pleurotin congeners in DMSO. Altered intensity differences and disappearance of proton signals (highlighted in dashed boxes) demonstrate the alteration of the initial compounds (black line) within 28h (red line).](image)

The purity of isolated compounds was verified by short UHPLC-TOFMS analysis. In this course, the isolation of the pleurotin hydroquinones and quinones as pure compounds was unsuccessful at first. The subsequent analysis of one of these samples immediately after semipreparative isolation and before solvent removal could demonstrate the degradation of these molecules in the presence of methanol. Whereas UHPLC chromatograms confirmed the purity of the isolated compound when analyzed immediately, the apparition of new peaks in the chromatogram after 5h demonstrated the beginning transformation (Figure VI.C.95).
Fungal metabolites

VI.C Supplementary information: Induced pigments and anti-Fusarium compounds

Figure VI.C.95: NI UHPLC-TOFMS chromatogram of 12 dissolved in MeOH/H₂O analyzed at t=0 and five hours later.

Base peak ion (BPI) trace is shown. The compound was isolated using MeOH and H₂O as elution solvents and was immediately analyzed after collection (< 30 min, without evaporation). The sample was stored at 10 °C between the two analyses. The gradual formation of 11 (374 Da) from 12 (388 Da) is already clearly observed after five hours. The sample is contaminated with small amounts of 20 (342 Da).

In consequence, methanol was avoided for subsequent isolation steps and replaced by acetonitrile. Consequently, NMR analyses were done in deuterated acetonitrile. After that, several of these pleurotin congeners were successfully isolated as pure compounds (chapter VI.B, section 4.11).

3. Materials & methods

3.1 NMR analyses

Analyses were done on a Varian INOVA 500 MHz instrument (Agilent) using a 5-mm tube probe. Crude extracts of the two pure culture and the co-culture were measured in DMSO-d₆, prefractions were analyzed in D₂O (P0, P0-1, P1), CD₃OD (P2, P3, P4, P5) or CDCl₃ (P6). MPLIC fractions were analyzed in DMSO-d₆ and CD₃CN. NMR analyses for de novo structure determination of pure compounds included the following sequences: ¹H, ¹H-¹H COSY (or DQF-COSY), NOESY, HSQC, HMBC, APT and 1DNOESY. Analyses were performed at 298 K (303 K for DMSO-d₆).

3.2 2D LC plots of MPLIC separation (UHPLC×MPLIC)

For UHPLC analyses of MPLIC fractions, an aliquot (2%) of each fraction was stored in a 96-deepwell plate. For UHPLC-TOFMS analyses, 20 μL of each fraction was used to create pooled samples covering 10 MPLIC fractions each. For UHPLC-UV analyses, 200 μL of each fraction was transferred to a 96-well plate.

UHPLC-TOFMS analyses were performed using an Acquity™ UPLC chromatograph and a Micromass-LCT Premier Time of Flight mass spectrometer equipped with an ESI interface (Waters). A short analysis was performed on a 50 × 2.1 mm i.d., 1.7 μm, Acquity BEH C₁₈ UHPLC column (Waters) in the gradient mode at a flow rate of 0.3 mL/min with the following solvent system: A = 0.1 vol% FA-H₂O, B = 0.1 vol% FA-MeCN. The injected volume was 1 μL. Detection was performed in PI and NI modes in the range m/z 100-1500 in centroid mode with a scan time of 0.3 s. ESI conditions were: capillary voltage 2800 V, cone voltage 40 V, MCP detector voltage 2450 V, source temperature 120 °C, desolvation temperature 300 °C, cone gas flow 20 L/h, and desolvation gas flow 600 L/h. The mass spectrometer was internally calibrated by
infusion of a solution of leucine-enkephalin (2 μg/mL, Sigma-Aldrich) through the lockmass spray probe at a flow rate of 10 μL/min, using a second Shimadzu LC-10ADvp LC pump.

UHPLC-UV analyses were performed using an Acquity™ UPLC chromatograph. All analyses were performed with the same parameters and columns as UHPLC-TOFMS. Injection volumes were doubled and PDA traces were recorded from 210 to 500 nm. Two UV traces were extracted: 280 nm and 498 nm.

Data from UHPLC-TOFMS were opened as base peak ion (BPI) traces in MassLynx software (Waters) and the chromatogram was exported into Excel to create a matrix of chromatographic traces of all MPLC fractions. Data from UHPLC-UV were exported from Empower software as .aia files into Excel to create the matrix. The matrices were imported into MATLAB (R2013a, Mathworks®) and plotted as filled 2-D contour plot. Contour levels were manually adjusted for every plot for optimal readability. Plots were transferred to vector graphics editor software for transformation into black-and-white figures.

3.3 Purification by semipreparative HPLC

The conditions for semipreparative HPLC are described in the Materials & methods section of chapter VI.B.
## 4. Supplementary tables and figures

Dereplication and list of features highlighted by MS-based metabolomics.

### 4.1 Dereplication of *Fusarium* and *Hohenbuehelia* metabolites

**Table VI.C.18: Dereplication of *Fusarium* and *Hohenbuehelia* metabolites in the three studied extracts analyzed in negative ionization mode.**

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<th>Δ (ppm)</th>
<th>Molecular formula</th>
<th>Identity</th>
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**Table VI.C.19: Dereplication of Fusarium and Hohenbuehelia metabolites in the three studied extracts analyzed in positive ionization mode.**

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### 4.2 Workflow and parameters for MZmine 2

**Table VI.C.20: Steps and parameters used during the automatic peak picking procedure by MZmine 2 for MS-based metabolomics of PI and NI UHPLC-TOFMS fingerprinting analyses.**

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<td>RT tolerance</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10) Manual removal of peaks with unnatural peak shape and peaks present in blank and Agar samples</td>
<td>Retention time tolerance (min)</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>m/z tolerance (ppm)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Max complex peak height (%)</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11) Peak list methods → Identification → Complex search</td>
<td>RT tolerance (min)</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12) Peak list methods → Identification → Adduct search</td>
<td>Custom list based on fragments reported by Nielsen et al. [54]</td>
<td></td>
</tr>
</tbody>
</table>
4.3 Principal component analysis of fungal co-culture

![Principal component analysis of fungal co-culture and pure culture of Fusarium solani and Hohenbuehelia reniformis analyzed by UHPLC-TOFMS fingerprinting.](image)

*Figure VI.C.96: Principal component analysis of fungal co-culture and pure culture of Fusarium solani and Hohenbuehelia reniformis analyzed by UHPLC-TOFMS fingerprinting.*
### 4.4 List of interesting features

**Table VI.C.21: List of features that are highlighted as significant biomarkers by OPLS-DA and by volcano plot from the positive ionization mode dataset.**

Molecular formulae were determined using heuristic filtering on HRMS data. Only ‘natural’ molecular formulae were taken into account [66].

<table>
<thead>
<tr>
<th>m/z (Da)</th>
<th>rt (min)</th>
<th>identity</th>
<th>Volcano plot*</th>
<th>Molecular formula (putative assignment)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>fold-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>induction</td>
<td></td>
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<td></td>
<td>p-value</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Induction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>404.1894</td>
<td>1.41</td>
<td></td>
<td>8.36</td>
<td>1.07E-06</td>
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<tr>
<td>476.1752</td>
<td>1.52</td>
<td></td>
<td>98.34</td>
<td>1.94E-04</td>
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<tr>
<td>387.1810</td>
<td>2.63</td>
<td></td>
<td>10.35</td>
<td>1.92E-03</td>
</tr>
<tr>
<td><strong>Upregulation from <em>F. solani</em></strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>307.0817</td>
<td>1.46</td>
<td>Fusarubin (1)</td>
<td>3.44</td>
<td>4.85E-05</td>
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<tr>
<td>291.0870</td>
<td>0.83</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>289.0711</td>
<td>1.46</td>
<td>Fusarubin [M-H_{2}O+H]^+ (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Upregulation from <em>H. reniformis</em></strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>513.1721</td>
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<td>2.32</td>
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<td>519.2172</td>
<td>1.47</td>
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<tr>
<td>357.1718</td>
<td>1.76</td>
<td>Leucopleurotinic acid B (11)</td>
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<td></td>
</tr>
<tr>
<td>339.1633</td>
<td>1.76</td>
<td>[M-H_{2}O+H]^+ (11)</td>
<td></td>
<td></td>
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<tr>
<td>357.1735</td>
<td>1.57</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>355.1604</td>
<td>1.64</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>2.05</td>
<td></td>
<td>2.54</td>
<td>8.59E-04</td>
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<tr>
<td><strong>Other interesting features</strong></td>
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<td></td>
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<td>3.61</td>
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</tr>
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<td>373.1688</td>
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<td></td>
</tr>
<tr>
<td>743.2857</td>
<td>2.84</td>
<td></td>
<td></td>
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<td>575.2173</td>
<td>1.77</td>
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<tr>
<td>505.2558</td>
<td>3.06</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>400.3434</td>
<td>3.86</td>
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<td></td>
<td></td>
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<tr>
<td>640.2480*</td>
<td>1.65</td>
<td></td>
<td>8.12</td>
<td>6.67E-03</td>
</tr>
<tr>
<td>365.2012*</td>
<td>1.69</td>
<td></td>
<td>3.12</td>
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<tr>
<td>698.2756*</td>
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<td>2.34</td>
<td>3.78E-02</td>
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<tr>
<td>697.2689*</td>
<td>1.63</td>
<td></td>
<td>2.27</td>
<td>3.36E-02</td>
</tr>
</tbody>
</table>

*Only features with significant fold-change of more than 2 (p-value <0.05) are considered.

*Feature not among significant biomarkers according to OPLS-DA.
Figure VI.C.97: Individual induction pattern of features that were highlighted by MS-based metabolomics among all replicates. Features from PI UHPLC-TOFMS and exhibiting clear induction or upregulation from one of the two pure strains. Every plot represents one feature with the corresponding peak area for all replicates of the pure culture and the co-culture.
Figure VI.C.98: Individual induction pattern of features that were highlighted by MS-based metabolomics among all replicates.
Features from PI UHPLC-TOFMS and exhibiting no clear induction or upregulation. Two features highlighted by Volcano plot only are shown as well. Every plot represents one feature with the corresponding peak area for all replicates of the pure culture and the co-culture. These features show large intra-class variation.
Table VI.C.22: List of features that are highlighted as significant biomarkers by OPLS-DA and by volcano plot from the negative ionization mode dataset.

Molecular formulae were determined using heuristic filtering on HRMS data. Only ‘natural’ molecular formulae were taken into account [66].

<table>
<thead>
<tr>
<th>m/z (Da)</th>
<th>rt (min)</th>
<th>identity</th>
<th>Volcano plota</th>
<th>Molecular formula and putative assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>fold-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>induction</td>
<td>p-value</td>
</tr>
</tbody>
</table>

**Induction**

| 749.2404 | 1.18 |                    | 222.40 | 2.70E-04 | no isotope match possible |
| 891.2789 | 1.37 |                    | 69.43  | 2.02E-03 | C₂₀H₂₄O₁₁                  |
| 873.2689 | 1.62 |                    |        |          | no natural found           |
| 747.2317 | 1.25 |                    | 8.53   | 2.33E-05 | no isotope match possible  |
| 371.0424 | 0.95 |                    |        |          | C₂₂H₂₅NaO₅, C₂₁H₂₆O₆     |
| 401.0528 | 1.10 |                    | 9.14   | 1.00E-02 | C₁₉H₁₄O₁₀                  |
| 401.0527 | 1.22 |                    | 2.07   | 7.56E-04 | C₁₉H₁₄O₁₀                  |

**Upregulation from F. solani**

| 305.0667 | 1.46 | Fusarubin (1)      | 2.87   | 1.57E-02 | C₁₉H₁₂O₇ (fusarubin)       |
| 387.0384 | 0.84 |                    |        |          | C₁₈H₁₂O₁₀                  |
| 455.0253 | 0.84 | M+HCOONa-H M: 387.0384 Da | 2.07   | 2.65E-03 |                                          |
| 323.1852 | 2.11 |                    | 4.20   | 1.94E-07 | C₁₈H₁₂O₆                  |

**Upregulation from H. reniformis**

| 511.1548 | 2.35 |                    | 4.20   | 1.94E-07 | no isotope match possible   |
| 343.1903 | 1.53 |                    |        |          | C₂₀H₁₉N₄                  |
| 529.1657 | 2.05 |                    | 3.34   | 1.58E-03 | C₂₁H₁₉N₄O₂, C₂₁H₂₀N₆O₁₂, C₂₂H₂₀NaO₁₀, C₂₃H₂₃O₆   |
| 387.1438 | 1.60 | Leucopleurotinic acid A (10) | 26.21  | 7.90E-06 | C₂₁H₁₂O₇                  |

**Other interesting features**

| 892.2852 | 1.32 |                    | 355.85 | 2.31E-05 | no isotope match possible   |
| 575.2056 | 1.76 |                    | 3.85   | 3.20E-03 | C₂₀H₁₂NaO₉, C₂₀H₁₆O₁₂      |
| 559.1756 | 2.13 |                    | 298.21 | 1.47E-05 | no isotope match possible   |
| 505.2427 | 1.31 |                    | 9.30   | 3.07E-06 | C₂₁H₁₆O₈, C₂₂H₂₄N₂O₂       |
| 373.1651 | 1.48 |                    | 6.10   | 1.30E-05 | C₂₁H₁₂O₆                  |
| 745.2181 | 1.31 |                    | 207.01 | 4.08E-05 | C₂₂H₂₁O₁₀                  |
| 741.1886 | 1.69 |                    | 257.96 | 1.06E-03 | C₂₁H₁₂O₂₀, C₂₃H₂₄O₁₂      |
| 533.1963 | 1.66 |                    | 3.89   | 1.22E-02 | C₂₂H₁₄O₁₁, C₂₃H₁₆O₆       |
| 759.1994 | 1.31 |                    | 142.14 | 1.98E-04 | no isotope match possible   |
| 543.1804 | 2.27 |                    | 5.37   | 4.29E-02 | C₂₂H₂₁NaO₁₀, C₂₃H₂₂O₁₁     |
| 499.1543 | 2.18 |                    | 2.11   | 1.25E-02 | no isotope match possible   |
| 729.2261 | 1.62 |                    | 165.46 | 3.03E-03 | no isotope match possible   |
| 747.3427 | 2.01 |                    | 12.08  | 9.93E-05 | no isotope match possible   |
| 402.1722 | 1.43 |                    | 5.56   | 4.21E-04 | C₂₂H₂₁NO₆, C₂₃H₂₂N₂O₅     |
| 518.1835 | 1.86 |                    | 3.58   | 7.25E-08 | C₂₀H₁₉NO₆, C₂₁H₂₁N₂O₅     |
| 448.1801b | 1.38 |                    | 13.48  | 9.41E-03 | C₂₂H₂₁NO₆                  |
| 638.2313b | 1.70 |                    | 13.43  | 2.44E-02 | C₂₃H₁₄N₂O₁₄                |

*Only features with significant fold-change of more than 2. (p-value < 0.05) are considered.

b Feature not among significant biomarkers according to OPLS-DA.
Fungal metabolites

Supplementary information: Induced pigments and anti-Fusarium compounds

**Figure VI.C.99:** Individual induction pattern of features that were highlighted by MS-based metabolomics among all replicates. Features from NI UHPLC-TOFMS and exhibiting clear induction or upregulation from one of the two pure strains. Every plot represents one feature with the corresponding peak area for all replicates of the pure culture and the co-culture.
Figure VI.C.100: Individual induction pattern of features that were highlighted by MS-based metabolomics among all replicates. Features from NI UHPLC-TOFMS and exhibiting no clear induction or upregulation. Two features highlighted by Volcano plot only are shown as well. Every plot represents one feature with the corresponding peak area for all replicates of the pure culture and the co-culture. These features show large intra-class variation.

5. Acknowledgments

Soura Challal is acknowledged for putting in place the MPLC system. Laurence Marcourt is thanked for her help with NMR analysis and structure elucidation and Philippe J. Eugster for performing UHPLC-TOFMS analyses. This work included preliminary experiments with special...
NMR pulse sequences and Dean Olson, Lingyang Zhu, Damien Jeannerat and Axelle Cotte are acknowledged for helpful discussions and NMR analyses.
Conclusion & outlook

1. Conclusion

Natural product research is traditionally focused on the phytochemical analysis of natural extracts by exploring the biodiversity of various habitats. In the search for new chemical entities, however, years of research have shown that biodiversity is not always correlated with chemodiversity.

Among NPs from all biological sources, fungal metabolites have shown to cover wide chemodiversity and serve as leads for some of the most important (e.g., cyclosporin and penicillin) and economically most successful drugs (e.g., statins). Genomic analyses of several fungi have revealed that far more biosynthetic genes, putatively assigned to secondary metabolite encoding genes, are present in these microorganisms than are expressed under standard laboratory conditions. Knowing that fungi often live in communities with other microorganisms in their natural habitat (microbiome), it is claimed that secondary metabolite production is controlled by interaction with the environment. The imitation of the natural growth conditions through the combined growth of fungi (co-culture) has shown to be successful in activating secondary metabolite gene clusters. This has led to the discovery of new chemical entities (chapter II).

The co-culture of fungi on solid media (compared to more commonly used liquid media) has the advantage that it mimics the natural growth of fungi (hyphal tip growth). In addition, a large number of different co-cultures can be rapidly and easily screened in Petri dishes. Furthermore, fungi tend to produce a greater diversity of secondary metabolites in solid media. On the downside, solid medium cultures yield only small amounts of extracts and scaling-up is not as straight-forward as for liquid cultures. Six month after the start of this thesis work, the concept of using imaging mass spectrometry (IMS) and co-culture on solid media for the study of interspecies interactions has been published by a research group at the University College San Diego [434]. This fortified our plans to pursue research in this direction.

In the analysis of chemical constituents from fungal co-cultures, as in natural product research in general, the repeated rediscovery of already known molecules is a recurrent challenge. The process from a natural extract to chemical and biological assessment of the pure compounds is often long and cumbersome. It is thus of utmost importance to identify already known compounds as early as possible, to focus research efforts on new structures. Nevertheless, reliable metabolite identification necessitates the match of at least two orthogonal properties obtained on an authentic chemical standard [47]. Unfortunately, authentic chemical standards are often unavailable and thus, NMR spectroscopy is necessary to unambiguously ascertain the metabolite identification.

In view of the identification of novel fungal metabolites issued from solid medium co-cultures, and knowing of the limited sample amounts available from this particular sample type (few milligrams of crude extract), miniaturized methods that are compatible with metabolite
identification by NMR and biological testing of pure compounds had to be established. The phytochemistry & bioactive natural products research group at the University of Geneva has a long history of experience in the phytochemical analysis of plant extracts. Therefore, in a first step, a miniaturized isolation strategy (microfractionation) was developed for a plant extract used in traditional medicine in Tanzania, Rhynchosia viscosa (chapter III). This isolation strategy was combined with an in vivo assay for angiogenesis and inflammation using the zebrafish model. We showed that sample amounts could be scaled such as to satisfy the needs for both NMR detection and biological assessment. This allowed the rapid biological profiling of extract constituents from only 20 mg of natural extract. Furthermore, the implementation of quantitative NMR permitted the levelling of assay response and thus, the biological profiling could be linked directly to the potency of the individual extract constituents. The microfractionation strategy is generic and applicable to other medium polar extracts with only limited sample-specific optimization.

All isolation processes used in this work are based on reversed-phase liquid chromatography. RP LC is compatible for analyses at different scales. UHPLC enables the rapid profiling of complex extracts with only micrograms of extract [157], the hyphenation of UHPLC with HRMS allows the determination of the molecular formula for every detected compound which permits dereplication [59]. Semipreparative HPLC permits the loading of several tens of milligrams of extract and, in consequence, the isolation of microgram-amounts of pure compounds. Via geometric transfer of chromatographic conditions, comparable metabolite separation is obtained for semipreparative HPLC as for UHPLC. This facilitates and speeds up the isolation procedures as the optimization of the separation process is limited to the UHPLC dimension. Going even one step further, MPLC is adapted for the separation of gram-amounts of extract to obtain milligram-amounts of pure compounds [578] and the transfer of chromatographic conditions from analytical HPLC are now possible as well ([607] and chapter VI.B and VI.C).

Within the framework of this thesis work, fungi of the genus Fusarium were of particular interest. Fungal species of the genus Fusarium are ubiquitously found in nature, as common soil saprophyles or as plant pathogens, but also in medicine, as opportunistic fungi in patients with severe immunosupression or as etiological agent in fungal nail infection (onychomycosis). Like many other fungal species, Fusarium spp. live in fungal communities (fungal microbiome, mycobiome) which has been evidenced for both Fusarium spp. in plant pathology (e.g., esca of grapevine) as well as in onychomycosis [225]. Furthermore, Fusarium spp. seem insensitive to current standard antifungal treatment (azole drugs) and new antifungal agents that are active against Fusarium spp. are needed. In this respect, co-cultures including Fusarium spp. were selected with the aim of identifying anti-Fusarium compounds.

The second part of the thesis (chapter IV to VI) was dedicated to the detection, isolation and structural identification of fungal metabolites. With regard to the later isolation of co-culture-induced metabolites, workflows for the sensitive detection of co-culture induced metabolites were developed first. The procedure for the extraction of secondary metabolites from solid medium cultures was adapted in view of the high-throughput screening of a large number of
fungal co-cultures (chapter IV). The induction of metabolites in co-cultures using a metabolomic approach based on rapid UHPLC-TOFMS fingerprinting analyses could be made apparent. This work showed that metabolite induction is not linked to specifically observed morphological interactions pattern but that co-culture consistently provides induction of potentially new metabolites. However, the induction phenomena are not always straightforward to evidence and chemometrics is often mandatory to highlight biomarkers. The screening enabled the selection of co-cultures for further in-depth analysis, such as the structural characterization of co-culture-induced metabolites and the evaluation of their biological activities.

In a second step, the microfractionation strategy that was developed with plant extracts (chapter III) was applied to a fungal co-culture extract (chapter V). One co-culture was selected from a panel of co-cultures with different onychomycosis-derived fungal strains based on the observed macromorphological interaction pattern. The fungi involved in this interaction were a *Fusarium oxysporum* and an *Acremonium strictum* strain. The de novo induction of three metabolites in the central interaction zone could be detected by metabolite profiling using UHPLC-TOFMS. Their identity was putatively assigned to known *Fusarium* metabolites. For the confirmation of the putative annotations, the targeted isolation of these induced metabolites was undertaken. The minimally required number of Petri dishes for structure identification of co-culture induced fungal metabolites by NMR was assessed with the aim of evaluating limit sample requirements. In this case, only three Petri dishes were sufficient for the isolation and subsequent NMR structure confirmation of the major de novo induced metabolite. On the other hand, obtained sample amounts would not have sufficed for de novo structure elucidation (on our NMR instrumentation) nor be enough for thorough biological activity assessments.

In a next step, a second co-culture was analyzed in the search of anti-*Fusarium* compounds (chapter VI). For this, a co-culture exhibiting distance inhibition was selected. Furthermore, diffusion of red pigments in the culture medium was observed. The fungal strains involved were an onychomycosis-derived *Fusarium solani* and the Basidiomycete *Hohenbuehelia reniformis*. Differences between pure cultures and co-culture were subtle but de novo induction of metabolites was highlighted by MVDA based on short UHPLC-TOFMS fingerprinting analyses. The induction of the pigments could be made apparent by targeted UHPLC-UV analysis but not using MS-based methods. Reasons might include the limited ionizability, the low content in the extract paired with high UV absorbance and ion suppression in short fingerprinting analyses. The co-culture extract showed antifungal activity against *Fusarium solani* and the isolation of milligram-amounts of pure fungal constituents was undertaken for later assessment of the biological activity. Therefore, the co-culture was scaled-up and biomarkers highlighted by MVDA were targeted for isolation using a combination of different chromatographic systems. Among all isolated molecules, only a fraction of the MVDA-highlighted biomarkers could be isolated and their structure identified. Many of these biomarkers were of low abundance in the extract; sample amounts did not reflect the (ion) response in the MS-based study. Nevertheless, some insight in the specific
interaction between *F. solani* and *H. reniformis* could be gained. Several *Fusarium* pigments were identified that were upregulated in the co-culture. On the other hand, constitutive *Hohenbuehelia* hydroquinones and quinones with anti- *Fusarium* activity might be responsible for the observed distance inhibition.

In the course of these studies, strategies were developed for the detection, isolation and structure identification of co-culture-induced metabolites issued from solid media-cultured fungi. Thereby, the potential of fungal co-culture for the induction of metabolite production could be clearly demonstrated. The isolation of induced metabolites for NMR structure identification was possible in selected cases using a rapid microfractionation strategy. However, the necessity for larger-scale cultures had to be recognized due to limited success in the isolation of fungal constituents at the microgram scale and insufficient sample amounts of pure constituents for bioassay evaluation. For these larger-scale cultures, adapted fractionation procedures had been developed. All these strategies together enabled the identification of anti- *Fusarium* constituents as well as their isolation from solid medium co-cultures in milligram-amounts.
2. Concluding reflections

During the course of this thesis work, and compared to an ideal workflow, limitations and hindrances were encountered at several fronts. These are summarized in three sections that are of particular relevance for this work and are discussed herein.

2.1 Detection and peak annotation of fungal metabolites

For an efficient peak annotation of detected peaks in metabolite profiling or metabolomics, access to information on known metabolites, ideally in the form of in-house databases, is essential. When working in an academic setting, comprehensive in-house databases are difficult to build. The different projects in the laboratory include a wide variety of different plant and fungal species, building such a database that covers all these natural sources would take years. Furthermore, especially in my case, in-house experience on fungal metabolites was scarce. To circumvent this problem, strategies for peak annotation would thus have to be as generic as possible and make use of external information available from the scientific literature.

This can be achieved through the use of commercially available or open public databases and through the exploitation of scientific publications. This is what has been done here (see chapter III.A or VI.A for automated dereplication) but constraints were evident due to missing orthogonal information (in particular, retention times). In the case of dereplication based on chemotaxonomic information, several peaks were could not be annotated due to incomplete information on the biological source of NPs in DNP. For example, the fungal metabolite ilicicolin C (see chapter IV.A) has the following biological sources associated with: *Nectria coccinea, Nectria galligena* and *Fusarium* spp. Nevertheless, this compound has been isolated from a sponge-derived *Acremonium* sp. and fungi from other genera as well and this information was missing in DNP. In the work described in chapter IV.A, this compound was putatively identified in an onychomycosis-derived *Acremonium strictum*. A workaround for the problem of missing biological source information is to include databases that are unrestricted by chemotaxonomic information in the automated dereplication process. To test this approach, peak annotation was tried on an artificial mixture of 18 plant-derived NPs (Figure I.C.3 in chapter I.C) using a database that included all plant metabolites in DNP. However, beside the detection of far more features than there were real peaks in the chromatogram, a well-known limitation in automated peak detection [37], every features had several (around 5 to 10, up to 60 for some features) possible annotations. All NPs were correctly annotated but with a very high rate of false-positives. Thus, the use of one-dimensional databases (databases that include no second orthogonal physicochemical parameter) without chemotaxonomic restriction is not feasible.

Therefore, it would be of great help to have complete databases or databases that include several physicochemical parameters for a more efficient automated dereplication. Solutions exist that implement, for example, PDA or MS/MS spectra [53,55,56,612]. Nevertheless, these solutions should ideally be generically used for all data formats, applicable to all compound classes and cover all studied natural extracts. Very interesting in this regard is one recent
development in which algorithmic methods for the prediction of retention times are included into dereplication procedures [158,481,613].

Whereas the presented generic and automated dereplication approach here was done by post-processing of the acquired data, some software packages offer now an on-line dereplication directly during analysis. For this, databases are integrated right into the data acquisition software. However, these solutions are restricted to analyses with that particular software (and thus, to analysis on the instruments of a particular manufacturer). The advantage of the post-processing for dereplication lies in its generic applicability. It can be applied to all LC-HRMS datasets as long as the native data format is accessible.

Advances have been made in recent years on the prediction of NP production from the genes present in an organism. This has been nicely showcased by the Hertweck group [267] and recently by Xie et al. [258] (genome mining). In the future, it would thus be nice to be able to link the information on predicted biosynthesis genes with information on possible metabolites. This might be a more efficient approach than relying on chemotaxonomy only. However, it is probably still difficult to predict from genome mining whether biosynthetic genes would be functional in a given organism.

2.2 Isolation of fungal metabolites for NMR detection

In the frame of this work, for the isolation of fungal metabolites and their structure identification by NMR, it was at first proceeded as with plant extracts. However, the application of methodologies developed for plant extracts on fungal extracts was difficult due to high contents in primary metabolites in this latter matrix. This necessitated the development of extract enrichment strategies. Furthermore, the extracts issued from the unwieldy agar matrix exhibited large differences from one fungal strain to the other. In this regard, NMR analysis of crude extracts and enriched fractions was of great help for the evaluation of the most efficient fractionations. $^1$H NMR detection provides a quantitative response on all proton-bearing molecules and thus yields a quantitative overview on the organic molecules in a sample. Due to the limited dynamic range of NMR (two to three orders of magnitude, depending on the electronics), only main constituents are visible. Nevertheless, some compound classes can be clearly distinguished from $^1$H NMR spectra, such as sugars (3 – 4 ppm and anomeric proton around 5 ppm), fatty acids (alkylic CH$_2$ groups at 1 – 2 ppm) and aromatic compounds (5 – 8 ppm). This information helps guiding the enrichment steps as the removal of the unwanted primary metabolites from the extract is easily observable. Furthermore, the enrichment in aromatic compounds, common among the desired secondary metabolites, is easily observable as well.

Another important aspect during the targeted isolation of fungal extracts was the combined use of HPLC with PDA, ELSD and MS detection. That way, the fungal extracts could be characterized before and during the targeted isolation of induced metabolites. For the targeted isolation of biomarkers from fungal extracts, none of the three detectors (PDA, ELSD and MS) alone provided a complete picture of the sample content. PDA is a very sensitive detector but detector response is distorted as the quantity of strongly absorbing molecules is
overestimated whereas the quantity of weakly absorbing molecules are underestimated. The ELS detector provides quantitative detector response but it does not provide any spectroscopic information on the analytes. As a last detector, MS provides valuable spectroscopic information. However, while being very sensitive, the detector response is compound-dependent and extrapolation on quantities based on peak area is impossible for unknown metabolites. Furthermore, MS conditions that yield optimal detection for all analytes in complex natural extracts are impossible to set. Thus, MS conditions are always a compromise when generic metabolite profiling is performed. In combination, the three detectors provide a better overview on the sample content. ELS shows the most concentrated constituents in the sample whereas PDA and MS provide spectroscopic information for identification or specific detection for targeted isolation of given biomarkers.

For separation monitoring, PDA detection is still the most practical option as it is a non-destructive method. The use of ELS detection during the optimization of separation conditions permits the choice of an optimal wavelength that reflects best the concentration-distribution in the sample.

The ideal detector for both metabolite profiling and separation monitoring is universal, quantitative, non-destructive and sensitive. NMR would comply with everything except sensitivity. However, due to its intrinsic insensitivity, its limited dynamic range as well as impracticability due to the imperative use of deuterated solvents makes its use unrealistic in generic strategies for metabolite profiling and separation monitoring.

Besides detection, the choice of an adapted chromatographic support and conditions is very important for the successful isolation of fungal metabolites, especially in the presence of closely related isomeric compounds. Therefore, different semipreparative HPLC columns, mainly based on fully porous spherical particles as stationary phase, had been used for the isolation of pure compounds.

In the last project of this thesis work, the use of a core-shell chromatographic column for the isolation of milligram-amounts of pure compounds was key. Suitable columns had only come on the market in 2013. The main limitation in the isolation of NPs had been the limited solubility of the sample. Core-shell columns exhibit higher peak capacity through the application of higher flow rates while all at the same, back pressure is substantially lower compared to columns packed with fully porous particles [456]. This enables the application of higher injection volumes which abates the solubility issues. Core-shell technology has already attracted reasonable interest for applications in chemical and pharmaceutical analysis [614], but it will very likely be more widely used for the (semipreparative) isolation of NPs in the future as well.

2.3 Taming metabolite production in fungal co-cultures

During the in-depth analysis of extract constituents in different fungal extracts, but also in discussion with peers working on fungal extracts, the challenge of inconsistent metabolite production within a given strain was omnipresent. The high variability in metabolite
production between biological replicates could be made apparent in the project described in chapter VI. Clearly, critical factors that influence metabolite production are not fully controllable yet, in solid as well as liquid medium cultures.

Knowing these factors would permit insight into the regulation of metabolite production and enable the activation of silent gene clusters for the production of possibly new chemical entities. The strategies developed in these studies might prove useful for monitoring metabolite production in the search of those regulatory mechanisms.

Other research groups study interaction phenomena of microorganisms grown on solid media using imaging mass spectrometry (IMS). Such an approach provides spatial detection of induced metabolites at the contact surface between microorganisms. However, it is not compatible with NMR detection and only permits identification of known molecules or analogues thereof based on MS/MS comparison. This might not be sufficient in all cases and it precludes identification of new chemical entities, the unambiguous identification of isomers [48] as well as biological testing of pure extract constituents. In this regard, the presented strategies complement current research efforts in providing methods for the understanding of microorganism interactions and in its ability to yield sufficient compound amounts for NMR detection as well as biological assessments.
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Acknowledgments

Une thèse est certes un travail personnel mais ne peut être menée à bien sans l’aide et le soutien de nombreuses personnes. J’aimerais donc remercier ici toutes les personnes qui m’ont donné des opportunités, enseignée, aidé, guidée, accompagnée et soutenue pendant ce temps à Genève afin que ce projet aboutisse.

Tout d’abord, je remercie mon ‘Doktorvater’ Prof. Jean-Luc Wolfender, de m’avoir acceptée en tant que doctorante dans l’équipe des produits naturels. Les nombreux projets que tu m’as suggérés m’ont permis de grandir et de devenir plus indépendante. Merci pour ta confiance et ton soutient scientifique pour mener à bien ce travail de thèse.

I’d also like to acknowledge Dr. Esther Schmitt, Prof. Deniz Tasdemir, Prof. Norbert Lange and Dr. Katia Gindro for generously accepting to join my Ph.D. committee. I am very grateful for all the constructive remarks I had gotten from you for the manuscript as well as during the defense. Thank you for that.

Le travail de thèse se fait dans une équipe, un merci donc aux présents membres et alumni des équipes de Phytochimie et Produits Naturels Bioactifs et de Pharmacognosie : Adeline, Adlin, Amina, Andreas, Antonio, Aude, Aymeric, Aziza, Bernadette, Caroline, Chantal, Charlotte, Claudia, Daphné, Davide, Elisabeth, Emerson, Florence, Fred, Frédéric, Gaétan, Guillaume, Hakim, Jean-Luc, Joëlle, Karin, Karine, Laurence, Lise, Mariam, Maria-Luiza, Marcos, Mark, Martine, Mélanie, Muriel, Natalie, Olivier, Peihong, Philippe E, Philippe C, Pierre-Marie, Quentin, Raimana, Sandra, Samuel, Sarah, Soura, Sylvain, Sylvian, Trixie, Vincent, Yildi, et les nombreuses stagiaires et étudiants, parmi eux : Alban, Carine, Cristiano, Emanuela, Helena, Ilaria, Mehdi, Nurhuda, Rodrigo and Shaza. Durant ces cinq années, j’ai également pu faire connaissance d’autres personnes au sein de l’université, en particulier : Johanna, Jonathan, Leonardo L et Lucie. Une mention spéciale pour certains entre eux : Claudia, un immense merci d’avoir accepté d’être ma mentor et d’avoir toujours ta porte ouverte pour des conseils, autant au niveau scientifique qu’au niveau développement de carrière. Tu es un modèle pour moi ! Philippe E, grand frère de thèse, d’avoir partagé des secrets de MS, de m’avoir initiée à la course à pied et de m’avoir montré les joies de la voile. Samuel, pour des milliers d’e-mails et des échanges scientifiques constructifs. Caroline, pour avoir souffert avec moi, autant dans l’expérience de la thèse que pendant de nombreuses activités sportives. Florence, pour croire en moi et me rassurer. Adeline, pour être toi, le meilleur pour ta suite. Philippe C, pour de nombreuses discussions et que ta porte était toujours ouverte. Mélanie, je suis contente de t’avoir rencontrée. Laurence, tu m’étais indispensable et j’ai pu beaucoup apprendre grâce à toi. Merci.

Une grande partie de ce travail de recherche était en collaboration avec ACW à Changins. Travailler dans un projet multidisciplinaire demande de l’apprentissage en communication. C’était une expérience enrichissante que je ne voulrais pas me passer. Donc, merci à l’équipe de Changins : Katia, tu étais toujours à l’écoute et une super cheffe de projet. Ta curiosité
continue est un exemple pour moi. Olivier, tu m’as appris beaucoup en biologie, tu étais toujours disponible et tu m’as fait remarquer des points essentiels par rapport à la recherche et la thèse. Sylvain, tu avais toujours un gentil mot pour moi. Je garde des bons souvenirs de pipetage autant que de CO.

I worked at lot on the Protasis NMR probe and on the automated injection system. I want to thank the people from this company for their support: Dean, for introducing me to fluidics and teach me the methodology of troubleshooting. Steve and Craig, for coming to Geneva. I felt honored to meet you all later in Missouri and Illinois where I was so warmly welcomed.

As part of the thesis work, I was given the opportunity to work in Saint Louis. This would not have been possible without Mark who accepted to let me be part of the company. I was lucky to work with Stephanie, Russell and Courtney who introduced me to their workflows and to meet Krista, Vanessa, Tami, Julie, Steven, Gary and Heidi. A big ‘thank you’ to these people and to everyone that helped making the time in Saint Louis an unforgettable experience. For the financial support, I would like to acknowledge the foundations Ernest Boninchi and Marc Birkigt as well as the Subsidy Tremplin I was awarded in 2010.

Les projets impliquants les champignons ont fait parti d’un projet Sinergia (CRSII3_127187) qui a été accordé à Jean-Luc Wolfender, Katia Gindro et Michel Monod. Je remercie le fond national suisse (SNF) d’avoir financé ce projet ce qui m’a permis de faire ma thèse sur ce sujet fascinant.

J’aimerais remercier les membres du LCAP de m’avoir accueillie à midi et en particulier Florence et Julien, pour de nombreuses non-réponses, des discussions très intéressantes et de la ‘calmitude’. Merci aussi à Davy d’avoir pris du temps pour m’expliquer les concepts de la chromatographie liquide.

Trixie, for being my friend. I am so happy that I can still have you around in the future.

Et un grand merci à mes lectrices et traductrices Trixie, Florence et Caroline pour vérifier l’anglais de cette thèse et de permettre d’avoir un niveau de français (écrit) correct dans les parties respectives.


Die Deutschschweiz war nie sehr weit weg und so hab ich mich immer gefreut auf schöne Momente mit Anna, Beni, Dorian, Eli, Pascal und Tina in den Bergen.

# Appendices

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Appendix I – Scientific communications & collaborations

Journal articles


Book chapters


Oral communications


N. Bohni, O. Schumpp, S. Schnee, S. Bertrand, K. Gindro, J.-L. Wolfender (2013) Targeted Isolation of Induced and Bioactive Metabolites from Fungal Co-cultures. 61th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, Münster, D.


Appendix

**Poster communications**

only posters presented as first author are listed below


N. Bohni, O. Schumpp, N. Bruderhofer, J.-L. Wolfender (2011) *In planta* Quantification of the Fusarium Toxin Fusaric Acid by LC-MS/MS and Assessment of its Defence Induction Potential in Grapevine. 27th International Symposium on the Chemistry of Natural Products (ISCPNP27), Brisbane, Australia.


**Collaborations**

Collaboration with MRM/Protasis to set up the automated injection system for the microflow NMR probe (One Minute NMR for CapNMR™ probe) in an academic lab with multiple user access. I installed the system with Dr. Trixie Ann Bartholomeusz under the guidance of Dr. Dean Olson from Protasis in our laboratory. The system was used for experiments that were published in PLoS One.

Collaboration with Alexander D. Crawford from KU Leuven to find the anti-inflammatory and antiangiogenic principles in plant extracts using zebrafish as test organisms. An article was published in PLoS One.

Collaboration with Dr. Christophe Boissard and Dr. François Paul from Alpaflor/DSM on the occasion of the Master thesis supervision of Carine Vernet for the phytochemical analysis of extracts from Alpine plants of cosmetic interest. This work is documented in a report to DSM.

Collaboration with Dr. Damien Jeannerat and Axelle Cotte on 2D NMR experiments on natural product isomer mixtures. This work could not be completed and included in this thesis. Effort to exploit the data and valorize experimental work is ongoing.

Thanks to the Tremplin Subsidy, I could do a three-month internship in the laboratory of the Sequoia Sciences Inc. in Saint Louis (Illinois), USA with Dr. Mark O’Neil-Johnson under the supervision of Dr. Russell B. Williams and Stephanie M. Rice. I was working on the purification of plant extracts that were provided from Sequoia Sciences Inc. for their in-house database.
and on fungal extracts within the framework of the Sinergia project. The work on fungal extracts could not be completed and is thus not included in this thesis.

**Earlier work**

**N. Bohni, V. Wennrich, B. Hattendorf, D. Günther (2006) The Use of Ostracod Shells as a Paleothermometer by Measuring Calcium Isotope Ratios and Trace Elements with ICPMS. Oral presentation, SCS Fall Meeting, University of Zurich.**
Appendix II — CV

Neunbrunnenstrasse 167  birth date: 30.08.1984
8050 Zürich  nationality: Swiss / marital status: single
nadine.bohni@alumni.ethz.ch  +41 76 499 36 87

EDUCATION

03/2009 – 05/2014  Doctoral studies, School of Pharmaceutical Sciences, University of Geneva
10/2005 – 06/2010  Certificate of Teaching Ability in Chemistry, ETH Zurich
10/2003 – 02/2008  BSc & MSc Chemistry ETH Zurich, ETH Zurich, focusing on analytical chemistry

WORKING EXPERIENCE

03/2009 – 05/2014  Phytochemistry and Bioactive Natural Products, School of Pharmaceutical Sciences, University of Geneva

Research and teaching assistant

- Metabolomics to target induced microbial metabolites
- Extraction, isolation & purification of natural products from plant and solid-culture fungal extracts
- Used techniques: HPLC (UV, ELSD and MS detection), NMR and microflow NMR, mass spectrometry, metabolomics, structure elucidation using 1D and 2D-NMR and MS
- Supervision of students, preparation of presentation skills training course for Master students

03/2011 – 06/2011  Sequoia Sciences, Inc., St. Louis, USA

Research intern

- Isolation of antimicrobial and anticancer constituents from plant and microbial sources (internship as laureate of Subsidy Tremplin)
- Used techniques: HPLC (UV and ELSD detection), microcryo NMR, structure elucidation using 1D and 2D-NMR

10/2008 – 01/2009  Bioanalytics, Chemistry Department, ETH Zurich

Research assistant

- Experimental setup and execution of test of a miniaturized pH-sensor (for BASF, Ludwigshafen)

02/2005 & 02/2004  Zeochem AG, Uetikon a. S.

Intern

- Development of spherical silica gel and assistance in production of derivatised silica gel

2001 (ten days)  Roche, Basel

Schweizer Jugend Forscht Intern

- Synthesis of a precursor for a substance database
LANGUAGES
German (mother tongue) English (proficient level)
French (proficient level) Italian (basic knowledge)

COMPUTER SKILLS
Scientific software
MassLynx, Xcalibur, Chemstation, Empower, MNova, ACD (ChemDraw), mzMine, Matlab, SciFinder, Pubmed

General
Office, HTML, LaTeX, InDesign, Illustrator

MANAGEMENT EXPERIENCE
2012
Member of the organizing committee for a one-day career event (Career Day, 21.5.2012) at the School of Pharmaceutical Sciences Geneva-Lausanne

2010
Member of the organizing committee for a one-day conference (PhD Day, 24.6.2010, Hermence) at the School of Pharmaceutical Sciences Geneva-Lausanne

April 07 – August 07
Member of the faculty recruiting committee for an assistant professorship in bioanalytics (student representative)

September 04 – February 07
Member of the executive board of the VCS (association of chemistry students at ETH), thereof 2.5 years editor of the students magazine

AWARDS
2013, SCNAT/SCS Chemistry Travel Award

2010, Subsidy Tremplin (University of Geneva), scholarship to carry out a project of choice to promote the academic career

14.04.2010, Phytochemical Analysis Prize, Trends in Natural Products Research 2010 at De Montfort University, Leicester, UK

13.10.2006, METTLER-TOLEDO prize for the best oral presentation in the section analytical chemistry, SCS Fall Meeting, University of Zurich, CH

PUBLICATION RECORD
Publications: 8 (+ 2 book chapters)
Oral communications: 6, Poster presentations: 4 (+ 5 as co-author)

SOFT SKILLS
Writing, Communication
- Report writing
- Literature survey
- Written submissions
- Oral presentations in German, French and English

Management
- Conference and career day organization
- Research project management

Teaching
- Practical work supervision (chromatography, organic chemistry)
- Organization of career event and conference

INTERESTS
mountaineering – skiing/skitouring – triathlon
Appendix III

Guerre Chimique entre Champignons: Un Arsenal de Molécules Bioactives

Stéphanie Schürch, Katia Gindro, Olivier Schumpp
Mycology group, Agroscope Changins ACW, Nyon, Switzerland

Michel Monod, Julie Verrier
Departement of Dermatology and Venereology, Laboratory of Mycology, CHUV, Lausanne, Switzerland

Nadine Bohni, Jean-Luc Wolfender
School of Pharmaceutical Sciences, University of Lausanne, University of Geneva, Geneva, Switzerland

Contribution: Provision of preliminary experiments, proofreading of the article
Des champignons du genre *Fusarium* sont de redoutables pathogènes pour les plantes et pour l’homme. Les infections qu’ils provoquent répondent mal aux traitements usuels. Dans une approche novatrice, la recherche envisage d’exploiter la guerre chimique que se livrent les champignons comme source de molécules antifongiques utilisables aussi bien en médecine qu’en agronomie. Soutenu par le Fond national suisse de la recherche scientifique (SNF), ce projet interdisciplinaire fédère trois institutions de l’Arc lémanique: la station de recherche Agroscope Changins-Wädenswil ACW, le CHUV et l’Université de Genève.
Les *Fusarium*: pathogènes des hommes et des plantes


**Défendre son territoire**
La découverte de nouveaux traitements antifongiques est donc un objectif central, en médecine comme en agronomie. D’une manière générale, les champignons constituent une source importante de composés naturels, par exemple pour l’industrie pharmaceutique (antibiotiques et immunosuppresseurs) ou agro-alimentaire (arômes et ferments). Ils génèrent aussi des molécules extrêmement toxiques, telles que l’amantidine, les aflatoxines ou les trichothécènes, toutes produites par des moisissures des denrées alimentaires. De plus, lorsque plusieurs espèces de champignons se rencontrent dans un même substrat, elles peuvent interagir et se battre mutuellement en synthétisant des toxines, les mycoalexines (Glauser et al. 2009). Ce phénomène est particulièrement visible dans un substrat tel que le bois, où le territoire de chaque champignon est délimité par une bande noire (fig. 1A). Ces zones d’interaction ont pu être recréées sur des milieux de culture artificiels en plaçant à une certaine distance deux souches de champignons dégradant le bois et en observant leur croissance (fig. 1B et C). De façon plus générale, les interactions entre deux colonies peuvent être classées en quatre types selon leur aspect morphologique et celui de leur zone de contact (Schumpp et al. 2010):
• **Inhibition à distance** : une zone exempte de mycélium subsiste entre les partenaires (fig. 2A).
• **Inhibition par contact** : la croissance est stoppée lorsque les hyphes se touchent (fig. 2B).
• **Chevauchement** : un mycélium croît par-dessus l’autre (fig. 2C).
• **Formation de «zones de barrage»** : au point de contact des colonies, une zone infranchissable brun foncé est formée (fig. 2D).

**Menace chimique : les mycoalexines**
Afin d’étudier la production de composés induits par ces interactions, les profils métaboliques des cultures pures et des zones de confrontation ont été analysés par chromatographie liquide à ultra-haute pression (UHPLC) couplée à la spectrométrie de masse à temps de vol (TOFMS). Des confrontations entre champignons dégradant le bois ont montré que de nombreux métabolites secondaires sont synthétisés de novo dans la zone d’interaction (Glauser et al. 2009). Le profilage métabolique par UHPLC-TOFMS permet de déterminer en ligne les formules moléculaires des mycoalexines. La structure chimique d’un des métabolites le plus fortement induits, la O-méthylmelléine, a été élucidée par résonance magnétique nucléaire capillaire (CapNMR). L’activité biologique de ce composé a ensuite été évaluée, en particulier ses propriétés fongicides, antibiotiques, phytotoxiques ou encore cytotoxiques (= anticancéreuses ; fig. 3). On voit ainsi que des modifications métaboliques importantes peuvent avoir lieu dans les zones de confrontation et constituer un réservoir de molécules bioactives. Actuellement, des confrontations de *Fusarium* issus d’onychomycoses sont en cours. L’identification des molécules nouvellement synthétisées (Wolfender et al. 2009) et l’évaluation de l’activité biologique de ces mycoalexines permettent d’espérer découvrir des antifongiques d’intérêt pharmaceutique et agronomique, une des grandes forces de ce projet étant de réunir des spécialistes d’horizons très différents.

*Figure 2* | Les quatre types d’interactions entre champignons : A: inhibition à distance. B: inhibition par contact. C: chevauchement. D: formation d’une zone de barrage.
Guerre chimique entre champignons: un arsenal de molécules bioactives

Figure 3 | Tests d’activité biologique réalisés sur les extraits bruts des zones de confrontation ainsi que sur O-méthylmelléine purifiée. A: activité fongicide illustrée par l’inhibition de la croissance de Botryosphaeria obtusa (zc: zone de croissance ; zi: zone d’inhibition). B: activité bactéricide mise en évidence par l’inhibition de la croissance bactérienne en présence de O-méthylmelléine. C: activité phytotoxique de la O-méthylmelléine qui, à forte concentration, inhibe la germination des graines de cresson et, à plus faible concentration, perturbe le développement des germes.

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Appendix IV

Advanced Methods for Natural Product Drug Discovery in the Field of Nutraceuticals

Chimia 2011. 65(6): 400-406.

Jean-Luc Wolfender, Philippe J. Eugster, Nadine Bohni, Muriel Cuendet

School of Pharmaceutical Sciences, University of Lausanne, University of Geneva, Geneva, Switzerland

Contribution: Preparation of figure 3, proofreading of the article
1. Introduction

Medicinal plants, vegetables and food products are rich sources of natural compounds that may play a role in health. It can be by maintaining health, preventing or curing diseases. In medicinal plants, these compounds are considered as bioactive natural products and may ultimately be developed as drugs. In food, they would be defined as phytonutrients without having therapeutic claims but with significant health benefits that can be used in disease prevention. Phytonutrients are often non-essential food components and are consumed in large amounts by individuals in their everyday lives. Over the past 15 years, research on these positive effects has developed considerably to provide evidence to support a role in the prevention of various health problems, such as cardiovascular diseases and cancers. The structural complexity of phytonutrients is extremely vast and goes from simple phenols and phenolic acids to complex high-molecular-weight compounds, such as tannins and proanthocyanidins. They all have specific physicochemical properties. Clinical evidences on disease-risk reduction have been shown for a few food products. They are mainly due to their content in polyphenols as this class of chemicals is by far the most documented.

Recently, the term nutraceuticals, a term combining the words ‘nutrition’ and ‘pharmaceutical’, has emerged. It is defined as food or food components that provide health and medical benefits, including the prevention and treatment of diseases. Nutraceuticals can be found in a wide range of foods, from conventional to fortified or enriched foods, and also as dietary supplements. A dietary supplement is a product containing nutrients derived from food products that are concentrated in liquid or capsule form. The ‘dietary ingredients’ in these products may include: vitamins, minerals, herbs or other botanicals, amino acids, and substances increasing the total dietary intake, such as enzymes and organ tissues. However, despite the growing interest in herbal products, there is still no unified legislation. Indeed, phytonutrients from herbs and botanicals can be sold under various forms which would result in different regulations. On one hand, they can be consumed as capsules, powders, or pills, and in that case, they would be regulated as dietary supplements. On the other hand, they can be ingested as additives in conventional foods (e.g. teas, juices, chips) and would be considered as such. Since the legislation regarding the use of phytopharmaceuticals and traditional ingredients varies considerably from country to country, issues regarding their quality control and standardisation requirements remain open. Furthermore, the differences between phytopharmaceuticals and nutraceuticals are not always well defined.

A striking example is that preparations containing Hypericum perforatum (Saint John’s wort) are available as natural antidepressants only in pharmacies in Switzerland because of severe problems related to drug interactions. Similar extracts have been added to chocolate bars in the United States and are considered as dietary supplements which do not have to be approved by the U.S. Food and Drug Administration (FDA) before marketing.

1.1 Relationship between Phytotherapy and Nutrition

In modern approaches to nutrition and nutraceuticals, the search for bioactive compounds and the explanation of the mode of action of the phytonutrients within complex food products, is very similar to approaches used in pharmacognosy for studying medicinal plants. There are even cases when plants or enriched extracts used in phytotherapy are recognised as food supplements or nutraceuticals. They are often called functional foods and are

**Abstract:** Advances in analytical methods and bioassay development have helped to push forward the research in natural products. In plant extracts and nutraceuticals, bioactive compounds are part of a complex mixture. The development of high-resolution methods related to HPLC for both chemical and biological profiling has significantly increased the efficiency of classical bioactivity-guided fractionation procedures. Furthermore, the level of sensitivity obtained by these methods give the possibility to work with few micrograms of compound. This represents a key advantage for rapid localisation of the biological activity and subsequent identification of the compounds of interest. The same methods are also used to study the extracts from a metabolomic point of view. The possibility to study them as a whole can highlight synergistic effects, which are likely to occur in plant extracts and nutraceuticals. In this paper, the main trends are summarised and the developments made in our laboratory on profiling crude extracts with UHPLC-TOF-MS, natural product identification at the microgram level using microflow NMR and integration of these methods with biological evaluation are highlighted.

**Keywords:** Biological activity · Metabolomics · Microflow NMR · Nutraceuticals · UHPLC-TOF-MS

**Advanced Methods for Natural Product Drug Discovery in the Field of Nutraceuticals**

Jean-Luc Wolfender*, Philippe J. Eugster, Nadine Bohni, and Muriel Cuendet

*Correspondence: Prof. J.-L. Wolfender, Phytocimie et Produits Naturels Bioactifs, Ecole de Pharmacie Genève-Lausanne, Section des Sciences Pharmaceutiques, Université de Genève, Quai Ansermet 30, 1211 Genève 4, Switzerland, Tel.: +41 22 379 33 85, Fax: +41 22 379 33 99, E-mail: jean-luc.wolfender@unige.ch
designed to allow consumers to eat enriched foods close to their natural state, rather than by taking dietary supplements manufactured in liquid or capsule form. In the case of broccoli, super broccoli and BroccoSprouts have been developed these past few years. They contain respectively 3.5 and 20 times more sulforaphane than usually produced by the vegetable.

Still several questions may be raised. Do certain kinds of food contain pharmacologically active substances in concentrations that are high enough to have drug-like effects when consumed? Are biologically active compounds in food indicative of therapeutic value? Is traditional drug development suitable for testing the merits of food? Is it ethical to test food as a drug on patients? Will dietary disease management remain a pipedream?

1.2 Cancer Chemoprevention Using Phytonutrients

Dietary consumption of foods and herbal medicines is a convenient method of administrating potentially beneficial phytochemicals in a cost-effective manner. The benefits are even bigger for the prevention of chronic diseases, such as cancer, which implies long-term non-toxic treatment. Cancer is considered the end stage of a chronic disease process characterised by abnormal cell and tissue differentiation. This process of carcinogenesis eventually leads to the final outcome of invasive and metastatic cancer. Recent advances in defining cellular and molecular levels of carcinogenesis, along with a growing body of experimental epidemiological, and clinical trial data, have led to the development of cancer chemoprevention, a relatively new strategy in preventing cancer.

Beside the control of causative exogenous factors, such as cigarette smoking, dietary factors, and specific microorganisms, cancer chemoprevention can play an integral role in the overall strategy geared toward reducing the incidence of cancer. Rational and successful implementation of chemopreventive strategies relies intrinsically on tests for efficacy and mechanistic assays, as well as availability of promising chemopreventive agents, reliable intermediate biomarkers, and appropriate clinical cohorts to discover safe and effective drugs for primary and secondary prevention of human cancers. Various foods have already shown promising results in this field.

1.2.1 Cruciferous Vegetables

Epidemiological evidence relating cancer risk reduction to the consumption of cruciferous vegetables such as broccoli, cauliflower, cabbage, kale, bok choy, Brussels sprouts, radish, or various mustards, has been summarised in several comprehensive reports. Highly significant cancer risk reduction with increasing crucifer intake was observed in cohorts that developed prostate, breast, bladder, and lung cancer, and non-Hodgkin’s lymphoma. Some compounds are remarkably potent, such as sulforaphane (Fig. 1) and phenethyl isothiocyanate, components of broccoli and watercress, respectively.

1.2.2 Green Tea

On a worldwide basis, the most popular chemopreventive drink is green tea. Green tea is the water extract of the dry leaves of Camellia sinensis. (−)-Epigallocatechin-3-gallate (EGCG, Fig. 1), the most abundant catechin in green tea, is credited with the majority of health benefits associated with green tea consumption. The organ sites where tea or tea constituents are found to be effective include the skin, lung, oral cavity, oesophagus, stomach, small intestine, colon, liver, prostate, and bladder. Despite large amounts of data, it is still not clear whether green tea has a real clinical efficacy. The possible complications of translating results obtained in cell culture studies to animals and humans may come from possible artefacts due to the auto-oxidation of EGCG. Also, activities observed in cell culture with high concentrations of EGCG may not be relevant because of the limited systemic bioavailability of EGCG.

1.2.3 Allium Vegetables

The large genus, Allium, includes the onion, garlic, chive, leek and shallot. Collectively, preclinical investigations demonstrate consistently that cancer chemoprevention by garlic and related sulfur compounds (Fig. 1) is clearly evident and appears to be independent of the organ site or the carcinogen employed. Some organoselenium compounds, also found in Allium spp, are superior to the corresponding sulfur analogs in cancer prevention.

1.2.4 Tomato

Epidemiological studies have provided evidence that high consumption of tomatoes effectively lowers the risk of reactive oxygen species (ROS)-mediated diseases such as cardiovascular disease and cancer by improving the antioxidant capacity. Tomatoes are rich sources of lycopene (Fig. 1), an antioxidant carotenoid reported to be a more stable and potent singlet oxygen quenching agent compared to other carotenoids. In addition to its antioxidant properties, lycopene showed an array of biological effects including cardioprotective, anti-inflammatory, antimutagenic and anti-carcinogenic activities. The anticancer activity of lycopene has been demonstrated both in vitro and in vivo tumour models.

1.2.5 Red Wine

Resveratrol (3,4′,5-trihydroxystilbene, Fig. 1) is a phytoalexin produced in large amounts in grapevine skin in response to infection by Botrytis cinerea. This production of resveratrol blocks the proliferation of the pathogen, thereby acting as a natural antibiotic. Numerous studies have reported interesting properties of trans-resveratrol as a preventive agent against various pathologies such as vascular diseases, cancers, viral infection or neurodegenerative processes. Several epidemiological studies have revealed that resveratrol is probably one of the main microcomponents of wine responsible for its health benefits. Resveratrol acts on the process of carcinogenesis by affecting the three phases: tumour initiation, promotion and progression phases and suppresses the final steps of carcino-
genesis, *i.e.* angiogenesis and metastasis. Moreover, concentrations of resveratrol in blood seem to be sufficient for anti-invasive activity. Interestingly, low doses of resveratrol can sensitize to low doses of cytotoxic drugs and provide a strategy to enhance the efficacy of anticancer therapy in various human cancers.

1.2.6 *Curcuma*

Curcumin (Fig. 1), a yellow colouring ingredient present in turmeric (*Curcuma longa*), has emerged as one of the most powerful chemopreventive and anticancer agents. This compound has been shown to exert anti-carcinogenic effects in a diverse array of animal and cell culture models. It can act as a chemopreventive agent in cancers of colon, stomach and skin by suppressing colonic aberrant crypt foci formation and DNA adduct formation.\[10\] Also, curcumin has been shown to down regulate NF-kB thereby suppressing proliferation and inducing apoptosis, and to possess anti-angiogenic properties. Cell lines that are resistant to certain apoptotic inducers and radiation become susceptible to apoptosis when treated in conjunction with curcumin.

All of these examples suggest that phytonutrients can be effective in the prevention of cancer or other diseases. Consequently, their specific bioactivity or their activity within complex matrices needs to be assessed in the same way as it is done with medicinal plants during the process of drug discovery. Thus, we are concentrating on the latest methodologies for profiling activity and chemical content in plant matrices to identify bioactive compounds. On the other hand, we apply the same tools to evaluate the biological effects of the whole mixture by taking into consideration their chemical complexity using metabolomic approaches.

2. Improved Methods for Profiling Complex Mixtures Containing Phytonutrients

As mentioned above, nutraceuticals and phytopharmaceuticals are closely related and a good understanding of their mode of action in terms of health benefit is needed. Unlike the case of herbal drugs or phytopharmaceuticals, the profiling of all constituents in food products is not required for their registration as nutraceuticals. Legislation varies considerably from country to country. However, for the understanding of the health benefits of such ingredients, many bioactive food components need to be analysed for complete identification or quantification in plant, fruit or vegetable matrices. This is also important for safety and compliance, especially when herbal additives are used in food products. Furthermore, the biological activity of the main ingredients has to be studied to support health claims and control the absence of toxicity. Thus, state-of-the-art analytical methods and bioassays need to be developed taking into account the complexity of the matrices to study.

### 2.1 Profiling Phytonutrients in Nutraceuticals

For the analysis and profiling of these natural products either in plant materials or in body fluids, various methods have been described. They include mainly chromatographic techniques (HPLC, UHPLC, GC, HPTLC) and spectroscopic methods (MS, NMR) used either alone (HPLC-UV, HPLC-ELSD, GC-FID) or in hyphenation (LC-MS, GC-MS, LC-NMR). These methods can be used for targeted analysis (quantification, quality control, standardisation) or for phytonutrient profiling in complex matrices.

Profiling of phytonutrients in vegetables or traditional ingredients has been performed mainly with chromatographic methods based on HPLC.\[11\] Various simple detectors such as UV or ELSD (Evaporative Light Scattering Detection) have been used, but mass spectrometry (MS), thanks to its sensitivity and selectivity, is undoubtedly the best choice for the detection of nearly all natural products.\[12\]

In this respect, we have been involved in many studies for the profiling and dereplication of natural products in complex matrices. Our latest applications imply the development of Ultra High Pressure Liquid Chromatography (UHPLC) methods for a high-resolution chromatographic separation of plant extract constituents combined to Time-Of-Flight Mass Spectrometry (TOF-MS) for sensitive detection and accurate mass determination. The recent introduction of sub-2 μm packing columns and UHPLC systems operating at very high pressures up to 1000 bars, led to a remarkable decrease in analysis time, higher peak capacity and increased sensitivity and reproducibility compared to conventional HPLC.\[13\] This technology gives a comprehensive characterisation of nutraceuticals by providing a very detailed high-resolution profiling (Fig. 2B & D). At the same time it is used for high-throughput LC-MS fingerprinting (Fig. 2C).\[14\] Such a methodology has recently enabled the profiling of the chemical constituents of various plant or vegetable matrices with high efficiency,\[15\] and is also applied for metabolomic studies in this field (see below).

By selecting adequately the column length in UHPLC, it is possible, from a theoretical point of view, to increase the throughput by a factor 9 compared to conventional HPLC. On the other hand, by keeping strictly identical column lengths in both HPLC and UHPLC, it is hypothetically possible to increase the plate count by a factor 3 between columns (5 μm vs 1.7 μm particles). However, it becomes difficult to work in optimal flow rate conditions because of the important backpressure generated by long columns packed with sub-2 μm particles. Some separations involving 150 mm or even longer UHPLC columns have been reported in the literature and show very elevated efficiency.\[15\]

An example of the level of details a profile obtained by UHPLC or high-throughput fingerprinting provides, is shown for Ginkgo biloba in Fig. 2.\[15\] This plant is a widely used medicinal herb found in phytopreparations to enhance memory and concentration. Preparations of this plant have also been recently registered for their positive effect on delaying pathological symptoms of Alzheimer’s disease. Most of the clinical studies supporting these health claims have been performed on standardised extracts. However, similar extracts are sold as nutraceuticals or are being part of food ingredients, such as chocolate bars called ‘Unforgettable Dark Chocolate Bar’ without rigorous control of safety and quality. Methods for the profiling of the composition of phytopharmaceuticals but also nutraceuticals are therefore required.

As shown in Fig. 2, a very detailed picture of the composition of the extract can be obtained. At the same time, the TOF-MS detection provides the molecular mass for each of the constituents of the mixture with high accuracy (<5 ppm) (Fig. 2E). This is extremely useful to determine the molecular formula of the various phytonutrients and to provide, together with chemotaxonomic information, a good way to fully or partially identify the metabolites of interest. This recent powerful high resolution profiling method in both chromatographic and mass spectrometric dimensions (Fig. 2D) is thus very useful for the characterisation of a given nutraceutical and can be applied to many vegetable matrices.

### 2.2 Profiling Bioactivity

To study nutraceuticals, it is important to obtain a detailed picture of their composition as described before, but also to obtain information on the biological activity of the various phytonutrients. Classically in pharmacognosy, such an approach has been made by bioactivity-guided fractionation.\[16\] The biological activity is assessed directly on a crude extract by simple assays and is then followed during a multiple step chromatographic fractionation procedure of this complex mixture until pure bioactive compounds are isolated and characterised. This strategy has enabled the
discovery of various bioactive compounds from medicinal plants, but also from food products, as described previously. In this strategy, the path leading from the plant to its pure constituents is long. It involves work which might last anything from weeks to years. In order to rationalise and improve the efficiency of the approach, new strategies both in biological and chemical screening have to be developed in order to obtain valid chemical and bioactive information prior to starting the isolation work.

Improved methods for the localisation and characterisation of bioactivity remain thus a cornerstone for the study of phytonutrients, as well as for natural product-based drug discovery. Over the last decade, a wealth of new technologies and conceptual approaches for bioactivity screening have emerged such as bioautography, HPLC-based activity profiling and on-flow bioassays, assays based on capillary electrophoresis, molecular imprinted polymers, MS- and NMR-based methods, biosensors, and chip-based technologies for affinity separation and expression profiling. Among MS-based methods, ultrafiltration LC-MS has proved to be a powerful tool for screening biologically active compounds from botanical extracts because of its high-throughput on-line screening ability, and its high sensitivity and selectivity needed for the characterisation of compounds present at low concentrations in highly complex matrices without time-consuming purification procedures.

The assays that can be directly performed after TLC separation (bioautography) can be applied to many extracts or fractions at the same time. They are simple and have been widely used for a rapid localisation of bioactive compounds in extracts. Antioxidant and acetylcholinesterase inhibition activities can easily be evaluated by this method. However, TLC suffers from a lack of chromatographic resolution and the limited structural information that can be obtained on the compounds separated. The development of HPTLC which improves the resolution and the possibility to hyphenate TLC with MS detection will certainly push forward bioautography.

The assays that can be performed in connection with HPLC (on-line or at-line) are of special interest to us since they can nicely complement the detailed chemical profiles obtained by UHPLC-TOF-MS on crude extracts. Such approaches are often referred to as high-resolution screening. An example is the development of HPLC on-line assays to test for antioxidant activity, which is of interest for nutraceuticals. This type of product often has health claims related to antioxidant properties since many chronic diseases (e.g., cancer, arteriosclerosis, Parkinson’s disease, arthritis, etc.) are at least in part related to oxidative stress. On-line antioxidant activity can be measured by the post-column addition of a relatively stable coloured radical such as DPPH or ABTS. Radical scavengers are then detected as negative peaks because they reduce the model radical to its reduced, non-coloured form in a reaction coil. When combined with LC-MS and LC-NMR, reliable identification of active constituents becomes possible without the need of ever isolating them in a classical sense. Enzyme assays are, however, often not compatible with HPLC because of the organic modifier concentrations commonly used. Furthermore, the reaction time is often long and requires lengthy coils.

For performing rapid bioassays on HPLC peaks, at-line methods can be used. This involves the microfractionation of extracts in 96 well plates, plate drying to remove the solvent not compatible with the bioassays and dilution of the samples in a suitable solution or culture medium for performing the assay. Several examples of HPLC-based activity profiling in search of anti-inflammatory agents, monoamine oxidase inhibitors or GABA(A) modulators have demonstrated the efficiency of this approach. One of the bottlenecks of these approaches is the difficulty to quantify the potency of activity since the amount of natural products fractionated is often not known. Nevertheless this approach is very useful for a rapid localisa-
tion of bioactive compounds in complex chromatograms, and for rationalising and improving the identification of active ingredients.

2.3 Rapid Identification of Phytonutrients

In complex food products, the characterisation of the phytonutrients and their metabolites also represents an important task. With HPLC or UHPLC mostly, MS or MS/MS spectra will be generated either with high or low resolution, depending on the instrumentation used. MS gives the possibility to generate nominal mass molecular ions or accurate mass measurements for the determination of empirical formulas.[23] Furthermore, the use of tandem or hybrid MS instruments provides in-depth structural information through fragmentation of the molecular species by collision-induced dissociation (CID) reactions.[12]

For on-line identification purposes, the determination of the molecular weight is of great importance. This, however, requires the comparison of MS data obtained with different detection conditions in order to differentiate protonated or deprotonated molecules from adducts or fragments.[24] The use of high-resolution instruments, such as a TOF-MS or FTICR-MS system, enables the direct determination of the molecular formula of crude mixtures. The accuracy of MS measurement, usually below 5 ppm, and the ratio of intensities of the isotopic patterns, together with the use of rules for heuristic filtering of molecular formulae[25] and chemotaxonomic information, allow an efficient mass-based putative metabolite identification and dereplication (Fig. 2E).

Complementary structural information can be generated by CID in LC-MS/MS or MS[2] experiments. The generated CID spectra are, however, not comparable to those recorded by electron ionisation (EI), and this hampers a direct use of the standard EI-MS libraries for dereplication purposes. For analysis of fully unknown constituents, this approach usually cannot provide enough information to ascertain the structure, so that the combination with other on-line information is mandatory. For this, NMR used on-line (LC-NMR) or at-line (LC-SPE-NMR, microflow NMR) may contribute to the missing structural information.[24,26] This method is, however, less sensitive and more time-consuming than MS, but is often mandatory to ascertain structure identification.

Considering all of these aspects, we have developed a strategy combining high-resolution metabolite profiling of crude plant or vegetable extracts on high peak capacity UHPLC columns as shown in Fig. 2 with subsequent microfractionation of the extracts. The UHPLC conditions are transferred to the semi-preparative level using identical column stationary phase chemistry to ensure the same selectivity of separation (Fig. 3A & B). LC-MS monitored microfractionation is done with milligrams of extracts or mixture (Fig. 3B & C). Complete structural determination of the unknown compounds is then based on at-line microflow NMR (CapNMR™) experiments[27] with detection at the microgram level (Fig. 3E). This approach provides high quality 1D and 2D NMR spectra on the main phytonutrients directly from a single separation of a crude extract, and minor constituents can be characterised after one further step of purification that can be optimised through software simulation and calculation.[28]

2.4 Metabolite Identification and Bioactivity Assessment at the Microgram Level

Crude extract profiling, chemical characterisation and bioactivity profiling can ideally be performed in an integrated manner. For this, we are developing a strategy that uses the microfractions described above for biological testing with various bioassays. Plants or nutraceutical extracts are microfractionated with amounts of material ranging from 10 to 100 mg. The microfractionation is directly performed in 96 deep well plates. The fractions containing typically 10 to 100’s of µg of pure or semi-pure natural products are aliquoted in other plates for bioassays while the main part of the microfractions is kept for further chemical characterisation. After mea-

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Fig. 3. Overview of the concept for metabolite identification and bioactivity assessment. A. High-resolution (HR) profiling of crude extracts by UHPLC-TOF-MS. B. Transfer of analytical conditions to the semi-preparative level to keep the same selectivity of separation (BPI traces are displayed). C. Microfractions are collected into deep well plates (one fraction/minute) and dried by vacuum centrifugation. D. Aliquots of all fractions are taken and analysed for their bioactivity. E. Microflow NMR analysis is performed on active microfractions to obtain structural and quantitative information. The molecular formula is extracted from the HR profiling (A) as shown in Fig. 2F. The concentration of the compound present in the microfraction can be estimated using quantitative NMR. The activity is then evaluated by a dose-response curve.
measurement of the bioactivity, the wells of interest are analysed from the mother deep well plate by microflow NMR to perform metabolite identification together with molecular formula information extracted from the metabolite profiling. At the same time the amount of compound present in the fraction tested (typically a few micrograms) can be estimated by quantitative microflow NMR measurements (Fig. 3F).

This enables the rapid localisation of bioactive compounds (Fig. 3C & D), an estimation of the potency of the activity (Fig. 3F) and a complete or partial structure determination (Fig. 3E).

This concept has been validated in our search for phytotoxic [29] and anti-inflammatory compounds using zebrafish assays in the 96 well format. [30] Zebrafish have recently emerged as an attractive in vitro system for functional genomics drug discovery and can be used to assess toxicity. [31] Because of their small size, rapid development, optical transparency, and high genetic, physiologic, and pharmacologic similarities with humans, zebrafish embryos and larvae are an ideal model for studying bioactivity of natural product and phytonutrients already at the microgram scale. In our group, these activities are screened in collaboration with the University of Leuven (P. De Witte and A. Crawford). The microfractionation platform is presently optimised for searching for cancer chemopreventive agents from edible plants and vegetables by using a battery of short-term in vitro bioassays developed to monitor inhibition of tumorigenesis at the various stages. For initiation, induction of NAD(P)H:quinone reductase (QR) activity [32] and antioxidant activity [33] are assayed. Inhibition of inflammation factors such as NF-κB and nitric oxide, as well as the epigenetic activity are measured to assess the promotion stage. Finally, for progression, the evaluation of angiogenesis is performed.

3. Reductionist versus Holistic Approaches to Study the Health Benefit of Phytonutrients

As described above, the miniaturisation of the classical bioactivity-guided fractionation enables a significant increase of speed for tracking bioactivity and performing natural product identification. This type of investigation, however, is a reductionist approach and represents the current day paradigm of drug development: ‘single target, single compound’. It involves thus mainly the testing of compounds at the molecular level in, for example, receptor binding assays. To study the mode of action or the health benefit of phytotherapy, nutraceuticals or food products, a more holistic approach using systems biology is probably much more suitable to prove efficacy, since all of these products represent complex mixtures of phytonutrients. [34] These natural products can be prodrugs, have synergistic effects or other interactions with the rest of the complex matrices that can modify their pharmacodynamic properties. Synergy, prodrugs, and novel targets might be detected by a systems biology approach, whereas the reductionist approach will only recognise activity on already known targets, and will not detect synergism or prodrugs. In this respect metabolomics, a science that has emerged less than a decade ago, will be a major tool in recognising compounds connected to activity in complex food or phytotherapeutical products, and will also be very useful in evaluating the effects on the target organisms. It can be the patient in case of clinical trials with well-established traditional medicines [34] or the consumer following a given diet or ingesting a food supplement for the study of nutraceuticals or food products.

In order to get a global view of all metabolites present in a plant, vegetable or body fluid after ingestion of these products, metabolomic studies can be performed either based on NMR or on MS fingerprinting. Today, the most advanced studies combine the advantages of MS and NMR spectroscopy. [35] NMR fingerprinting is used to analyse complex mixtures directly without the need of prior chromatographic separations. The method is indeed simple, has a high-throughput, does not require specific sample preparation, and provides detection of all protons in a quantitative manner. Standard protocols exist for the extraction and the analysis of various plant tissues, [36] as well as for the study of biofluids. [37] The methods are extremely useful when a comparison of the major constituents of a plant extract is needed. The main drawback is that it lacks sensitivity and the identification of single compounds in extracts may be hindered by overlapping signals. Identification of biomarkers relies mainly on the comparison of NMR shifts of plant metabolites acquired in the same solvent conditions. De novo identification can be partly based on the acquisition of complementary 2D NMR experiments in the mixture.

MS provides a sensitive detection and the ability to identify metabolites based on MS/MS spectra when libraries are available. Because of its ability to analyse multiple analytes with a high sensitivity, MS is playing an increasingly important role in the progression of proteomics and metabolomics. Non-hypenated MS methods enable the rapid and high-throughput screening of hundreds of samples, mainly for metabolite fingerprinting, but have limited quantification and metabolite identification capabilities. In hyphenation with HPLC or UHPLC, MS is extremely powerful in terms of detection, quantification and identification of a wide range of metabolites. It enables a bidimensional detection where each metabolite is resolved in both chromatographic (retention time) and mass spectrometric (m/z) dimensions, and high-throughput can be obtained with UHPLC-TOF-MS where extract fingerprinting can be generated typically in less than 10 minutes.

NMR or MS datasets are potential sources of information and in turn a source of knowledge. [38] However, to make the leap from one to the other requires considerable data processing and statistical analysis. [39] With the development of metabolomic methods for the analysis of complex biological systems, data on thousands of compounds are available in a single experiment and comparing samples has become a problem of high dimensionality. Extracting knowledge from these data, revealing patterns among samples and identifying critical or discriminatory variables is not a straightforward task. Because changes in metabolite levels may be drastic or subtle, important statistical processing is mandatory to determine the relevance of an observed change. Common chemometric tools, such as principal component analysis (PCA), used for projecting multivariate data to a low-dimensional plot, are generally proposed for display and exploratory analysis purposes. For further investigation, supervised approaches remain very attractive according to the strong impact of their use in human metabolomics. [39]

As an example of a MS based metabolomic approach on herbs and their effect on body fluid compositions, UHPLC-TOF-MS has recently enabled the profiling of the chemical constituents of pu-erh tea, black tea, and green tea, as well as those of pu-erh tea products of different ages. Differences in tea processing resulted in differences in the chemical constituents and the colour of tea infusions. Human biological responses to pu-erh tea ingestion were also studied by UHPLC-TOF-MS in conjunction with multivariate statistical techniques. Metabolic alterations during and after pu-erh tea ingestion were characterised by increased urinary excretion of 5-hydroxytryptophan, inositol, and 4-methoxyphenylacetic acid, along with a reduced excretion of 3-chlorotyrosine and creatinine. This study highlighted the potential of such a metabolomic approach to assess the effects of nutritional interventions containing phytonutrients on human metabolism. [40]

Similarly to this study, NMR-based metabolomic approaches have been reported for the classification of chamomile flowers.
from different geographic locations\[41\] and for the detection of metabolites present in human urine samples.\[42\]

In our group, such approaches have been applied to the detection of low-abundance plant stress defence hormones using data mining applied to rapid UHPLC-TOF-MS fingerprinting (Fig. 2C) and subsequent microfractionation of the biomarkers were highlighted and characterised by high throughput NMR as described above.\[43\]

The same analytical strategy is currently being applied to study the effect of various phytopharmaceuticals with proven clinical efficacy for which classical reductionist bioactivity-guided fractionation approaches did not lead to the identification of the active ingredients. For such investigations, metabolic data acquired on body fluids will be analysed from a holistic viewpoint after ingestion of the preparations and the bioactivity of metabolised phytonutrients will be measured.

4. Conclusions

As it is the case for bioactive natural products in medicinal plants, phytonutrients, as essential constituents of dietary supplements, play an increasingly important role in health issues. Thus the search for active ingredients in food products, nutraceuticals or medicinal plants is very similar and requires advanced methods for both chemical and bioactivity profiling. In this respect, recent evolution of LC-MS towards UHPLC-TOF-MS has given the possibility to investigate complex biological matrices with very high resolution and sensitivity, and to obtain a precise, but still incomplete, picture of the plant or vegetable metabolome. At the same time, development of NMR towards microNMR methods has boosted the sensitivity of this key technique for metabolite identification and allowed full phytochemical investigation at the microgram level. The integration of information rich bioassays at the same scale provides rapidly key information enabling the localisation and characterisation of bioactive constituents. The comprehension of the mode of action of each of these bioactive natural products is extremely complex and requires a thorough analysis of the constituents in their original matrices (fruits, vegetables, herbs or enriched extracts), as well as an assessment of their bioavailability. For this, metabolomics plays an increasingly important role. Recently, exciting research opportunities have appeared with the use of systems biology approaches to study the mode of action of medicinal plants or the health benefits of functional foods or plant ingredients.

With the tremendous advancement in analytics, bioassays and data treatment over the last few years, it is safe to assume that research in natural products will continue to accelerate the pace at which significant discoveries are made in various health issues. However, this progress should also rely on a good understanding of the biological role of the major metabolites. The knowledge that metabolites from different geographic locations\[41\] and for the detection of metabolites present in human urine samples.\[42\] and for the detection of metabolites present in human urine samples.\[42\]

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Appendix V

Zebrafish Bioassay-guided Microfractionation for the Rapid In Vivo Identification of Pharmacologically Active Natural Products


Soura Challal, Nadine Bohni, Jean-Luc Wolfender
School of Pharmaceutical Sciences, University of Lausanne, University of Geneva, Geneva, Switzerland

Olivia E. Buenafe, Camila V. Esguerra, Peter A. M. de Witte, Alexander D. Crawford
Laboratory for Molecular Biodiscovery, University of Leuven, Leuven, Belgium

Contribution: Design of experiments, execution of experiments and writing of larger parts of the article
Zebrafish Bioassay-guided Microfractionation for the Rapid in vivo Identification of Pharmacologically Active Natural Products

Soura Challal\textsuperscript{a}, Nadine Bohni\textsuperscript{a}, Olivia E. Buenafe\textsuperscript{bc}, Camila V. Esguerra\textsuperscript{a}, Peter A. M. de Witte\textsuperscript{b}, Jean-Luc Wolfender\textsuperscript{a}, and Alexander D. Crawford\textsuperscript{b}

\textsuperscript{a}SCS-DSM Award for best poster presentation

Abstract: The rapid acquisition of structural and bioactivity information on natural products (NPs) at the sub-milligram scale is key for performing efficient bioactivity-guided isolations. Zebrafish offer the possibility of rapid in vivo bioactivity analysis of small molecules at the microgram scale – an attractive feature when combined with high-resolution fractionation technologies and analytical methods such as UHPLC-TOF-MS and microflow NMR. Numerous biomedical relevant assays are now available in zebrafish, encompassing most indication areas. Zebrafish also provide the possibility to screen bioactive compounds for potential hepato-, cardio-, and neurotoxicities at a very early stage in the drug discovery process. Here we describe two strategies using zebrafish bioassays for the high-resolution in vivo bioactivity profiling of medicinal plants, using either a one-step or a two-step procedure for active compound isolation directly into 96-well plates. The analysis of the microfractions by microflow NMR in combination with UHPLC-TOF-MS of the extract enables the rapid dereplication of compounds and an estimation of their microgram quantities for zebrafish bioassays. Both the one-step and the two-step isolation procedures enable a rapid estimation of the bioactive potential of NPs directly from crude extracts. In summary, we present an in vivo, microgram-scale NP discovery platform combining zebrafish bioassays with microscale analytics to identify, isolate and evaluate pharmacologically active NPs.

Keywords: Microflow NMR · Microfractionation · Natural products · UHPLC-TOF-MS · Zebrafish

Introduction

Natural products (NP) are an important resource for drug discovery and development – as therapeutic agents, as drug leads, and as pharmacological tools for target discovery and validation. The chemical diversity of NPs has contributed significantly to the development of drugs for a wide range of diseases.\textsuperscript{[1]}

NP research and drug discovery often begins with the source itself – plants, fungi, and microbes, as well as marine flora and fauna. The investigation can be guided e.g. by a plant’s ethnopharmacological use, or by a molecule category known to provide a given activity (chemotaxonomic targeting). Numerous efforts to optimize screening paradigms are enabling more efficient drug discovery based on NPs – for example, the use of small-molecule libraries,\textsuperscript{[2]} to decrease the time for isolation and purification; and the improvement of analytical methods for structure elucidation and dereplication,\textsuperscript{[3]} thereby decreasing sample amounts needed for analysis.\textsuperscript{[4]} Bioactivity-guided fractionation facilitates the isolation and identification of active compounds as well as the assessment of the compound toxicities, which is a crucial factor for further investigations.\textsuperscript{[5]}

Analyzing an NP matrix is a challenging task in many respects. Particularly plant extracts are complex mixtures which can contain several closely related compounds eluting in a narrow chromatographic area in metabolite profiling analysis. In addition, main constituents can mask minor compounds responsible for the targeted bioactivity.

Classical bioactivity-guided fractionation has provided a good means to solve such issues. However, such an approach requires multiple chromatographic steps and large amounts of biological material.\textsuperscript{[5]} This process is therefore labor-intensive and is generally regarded to be too slow to cope with most high-throughput screening paradigms.\textsuperscript{[6]}

In an effort to partially solve some of these issues, we report here the novel combination of microfractionation procedures with an in vivo bioassay platform to enable the rapid generation of both structural and bioactivity information from limited amounts of biological material.

Zebrafish as an in vivo Model for Microgram-scale NP Discovery

In contrast to enzymatic or cell-based reporter assays, high-content bioassays (e.g. phenotypic assays using cells or organisms) allow the hypothesis-independent analysis of pharmacological activity.
Particularly in vivo models offer the possibility to screen for biomedically relevant bioactivities in a target- and pathway-independent manner. Nevertheless, mammalian models such as rodents require larger amounts of compound (in the milligram range) for activity analysis, and are therefore not ideal in vivo platforms for bioassay-guided fractionation.

The recent emergence of in vivo, microgram-scale, high-throughput assays based on zebrafish embryos and larvae represents a unique opportunity for the rapid identification of bioactive NPs. Embryos and larvae of the zebrafish (Danio rerio, a tropical freshwater teleost) have within the past two decades emerged as an ideal model system not just for developmental and biomedical genetics, but also for chemical genetics and drug discovery.\(^6\) Although zebrafish were first proposed as a model for small-molecule discovery in 1957,\(^7\) it has only been in the last ten years that compound screens carried out using zebrafish assays have identified a growing number of bioactive small molecules in a variety of indication areas.\(^6,8\) More recently, we and others have established zebrafish as an in vivo platform for NP discovery,\(^9,10\) both for bioassay-guided fractionation as well as for screening prefractionated NP libraries.

Zebrafish offer several advantages as model organisms – most importantly, they are a well-characterized vertebrate species with a fully-sequenced genome, and produce large numbers of rapidly and synchronously developing transparent embryos and larvae. The rapid development of zebrafish ensures that all biomedically relevant tissues and organs form and achieve functionality within the first few days after fertilization. Of equal importance, zebrafish embryos and larvae are small (1–4 mm, depending on the developmental stage) and therefore compatible with 96- and even 384-well plates, thereby reducing the amount of compound required for this in vivo assay to the level of micrograms (depending on the potency of the compound, as little as 1 microgram may be sufficient for an initial bioactivity screen in zebrafish, approximately 1000-fold less than what is required for a typical dose in mice). This latter feature is key for NP discovery, as many high-resolution separation methods based on HPLC, particularly microfractionation, result in very limited sample sizes that would otherwise be insufficient for the in vivo analysis of bioactivity.

Bioassays based on zebrafish embryos and larvae are now available for a wide range of indication areas (see recent reviews\(^6,9\)). Briefly, zebrafish models have been developed for the analysis of inflammation, cancer, angiogenesis, diabetes, epilepsy, and several other diseases.\(^11,12\) Many of these models are potentially useful as in vivo assays for high-throughput screens of small molecules including NPs. Additionally, recent developments include zebrafish assays in the areas of bacterial and viral infection, alcoholism, and behavioral analysis (Fig. 1).

The ability to carry out in vivo assays in zebrafish at the microgram scale presents a unique opportunity to take advantage of analytical and microfractionation methods that have recently been developed at this level as well. The effective combination of these platforms will enable the rapid identification of NPs based on their pharmacological activity in vivo – an attractive possibility to expand the current scope of NP discovery.

**Fig. 1.** Zebrafish-based screening of crude extracts, fractions, and pure NPs. A wide variety of zebrafish assays are now available, including behavioral, toxicological, physiological and developmental assays. Most of these bioassays are suitable for NP discovery at the microgram scale, and can be used to screen crude extracts, chromatographic fractions (including microfractions), and pure compounds. Depicted above are zebrafish assays for epilepsy (automated video tracking of locomotor activity and seizure behavior), hepatotoxicity (whole-mount in situ hybridization analysis of the liver-specific fatty acid binding protein fabp10a), angiogenesis (visualization of intersegmental vessel outgrowth in a fli-1:EGFP transgenic line with vasculature-specific fluorescence), inflammation (visualization of leukocyte migration after tail transection via myeloperoxidase staining), and developmental signaling pathways (morphological analysis).
rapidly estimate the secondary metabolite composition of these extracts, methods providing high chromatographic resolution and online spectroscopic information are required. In this respect, hyphenated techniques such as Ultra-High-Performance Liquid Chromatography (UHPLC) coupled with high-resolution Time-Of-Flight Mass Spectrometry (TOF-MS), represent a state-of-the-art analytical platform both for profiling/dereplication studies as well as metabolomics.\(^3\) UHPLC uses columns packed with sub 2 µm particles and high operating pressures (up to 1000 bar) compared to standard HPLC. This results in a decrease in analysis time (e.g. for high-throughput analysis of many microfractions) or an increase in peak capacity (for in-depth profiling studies).

TOF-MS detection on the other hand provides a sensitive and universal detection of most NPs and its high mass-accuracy and high resolution gives the possibility to assign molecular formula on-line, a key element for dereplication.\(^3\)

The combination of high-resolution UHPLC with TOF-MS is an ideal tool for profiling complex biological and microbial extracts, and all spectroscopic information is obtained with microgram amounts of crude extract while the detection of NPs is realized in the nano- or picogram range.\(^3\)

**Semi-preparative LC-MS for Microfractionation**

In order to profile activity in a crude plant extract, NPs have to be obtained and isolated in the microgram range. This range is compatible both with *in vivo* assays in small organisms such as those presented with zebrafish, as well as *de novo* structure identification and absolute quantification that can be performed with state-of-the-art microflow NMR methods (see below).

In order to rapidly obtain microfractions of such amounts, semi-preparative HPLC is well suited since it provides higher loading capacities than analytical HPLC and reasonably high-resolution separation. Chromatographic gradients can be transferred from the UHPLC analytical level to the semi-preparative level to obtain similar separation selectivity. Semi-preparative HPLC is thus a suitable method for an efficient rapid microfractionation of crude plant extracts with no or reduced sample preparation.\(^4\)

Semi-preparative HPLC combined to MS detection enables the separation of a wide variety of compound classes to be monitored and to correlate the information with that from the UHPLC-TOF-MS profiling for an efficient localization of the compounds of interest.

**Microfractionation Strategies**

With the aim of rapidly localizing and identifying bioactive compounds either a one-step or a two-step microfractionation strategy can be applied according to the complexity and the nature of a given crude extract. Several plant extracts have been studied in this way to identify either anti-angiogenic NPs based on a physiological zebrafish assay or anti-convulsant NPs based on a behavioral zebrafish assay.

For example, in an ongoing search for anti-angiogenic compounds from a Tanzanian medicinal plant (Fabaceae), we employed a one-step microfractionation strategy. Dereplication by UHPLC-TOF-MS revealed the presence of isoflavones and other metabolites widespread throughout the profile (Fig. 2A). In this case, a gradient transfer from UHPLC to semi-preparative HPLC-MS (100 × 2.1 mm to 250 × 10 mm C18 column) enabled the isolation of bioactive compounds from 20 mg of crude extract in one step. The collection was done directly into two 96-deep well plates. Aliquots were taken for testing the microfractions for inhibition of angiogenesis in zebrafish and for subsequent high-throughput UHPLC-TOF-MS analysis of the microfractions of interest (Fig. 2B). The major part of the microfractions was kept for microflow NMR analysis (Fig. 2C). Several microfractions showed...

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**Fig. 2.** A: UHPLC-TOF-MS profiling of 1 µg of the crude extract of a Fabaceae species and generation of microfractions using semi-preparative HPLC monitored by MS. B: Collection of microfractions into 96-deep well plates and characterization of compounds of interest by UHPLC-TOF-MS. C: Structure elucidation and quantification by microflow NMR. D: UHPLC-TOF-MS profiling of 1 µg of the crude extract of a Solanacea species for the gradient transfer onto the semi-preparative HPLC monitored by UV. E: Collection of fractions into tubes. F: Microfractionation of the active fraction by HPLC-MS using two analytical columns coupled in series. Separation optimized by calculation based on two generic UHPLC gradients of different slopes. G: Structure elucidation and quantification of the active microfraction containing two isomeric compound by UHPLC-TOF-MS and microflow NMR.
an inhibition of angiogenesis in zebrafish and contained only single compounds all bearing an isoflavone skeleton.[15]

A different strategy has been used for the identification and isolation of anti-convulsant compounds from a Philippine medicinal plant (Solanaceae). Compared to the Fabaceae extract discussed above, the UHPLC-TOF-MS profile was more complex and showed the presence of related isomers partially coeluting (Fig. 2D). Since in this case a high LC resolution was needed, the gradient elution was optimized by performing two generic gradients of a slope of 1 and 3/6 and these data were used to create an optimal gradient by software calculation.[16] A first enrichment was performed on a semi-preparative HPLC column of 150 × 19 mm generating nine fractions (Fig. 2E). The active fraction on the zebrafish anti-convulsant assay corresponded to a zone with no UV active peaks. The MS data of this region revealed the presence of different high molecular weight compounds (see inset 2D ion map plot, Fig. 2D). Based on the optimized gradient calculated from the two profiling gradients, a high-resolution separation of the active fraction was performed on two 250 × 4.6 mm columns coupled in series (500 × 4.6 mm) (Fig. 2F). As shown in Fig. 2F, a high-resolution profiling was obtained on 20 mg of the active fraction. All compounds eluting in this second separation were collected into four 96-deep well plates and the activity was concentrated in well-defined microfractions containing some of the high molecular weight compounds. Even with this very high resolution separation the active fraction was found to contain two isomeric structures related to steroid glycosides.[17]

With both microfractionation strategies, microflow NMR analysis was used to confirm the identity of the compound isolated and to quantify the amount present in each fraction at the microgram level (Fig. 2G).

Microflow NMR for Identification and Quantification

NMR spectroscopy is the method of choice for the identification and structure elucidation of most small molecules. In combination with high-resolution MS, an unambiguous assignment is often possible. But while mass spectrometric methods are highly sensitive and require only nanogram amounts of sample, NMR is an insensitive technique. However, considerable efforts have been made in the last twenty years to improve its sensitivity either by reducing the noise level generated by the probes (cryo-probes) or by optimizing the cell design for maximizing filling factor and diameter (microflow NMR probes).

Thus, these recent probe technologies in combination with high field magnets enable the de novo structure isolation of NPs with a few micrograms of sample.[18]

In the strategy presented, a microflow probe (CapNMR™) was used for structure elucidation of the compounds isolated in the bioactive microfractions. Here, all dried microfractions were solubilized in less than 10 μl of deuterated solvent and analyzed automatically by using an automated sample injection unit (One Minute-NMR™[19]). On the 500 MHz system used, this allows for 2D experiments and de novo structure elucidation on as little as 50–100 μg of sample.

Assessment of the bioactive potency of unknown NPs obtained by microfractionation is a challenging task, since their quantities have to be correctly estimated. Weighting the microfractions is not only impractical, but also inaccurate at sub-milligram quantities. Using NMR, sample identification and quantification[20] can be combined in one analysis step. NMR quantification can be performed either with an internal standard, using the ERETIC method, or the PULCON[21] method with reference to an external standard. PULCON correlates the absolute intensities of two spectra measured in different solution conditions and was used for the estimation of the amount of bioactive NPs collected.

By applying this method, the bioactivity measured could be accurately related to the microgram amount tested and the purity of the microfractions could be assessed (Fig. 2C). In the case of coeluting NPs found in the same microfraction (Fig. 2F), each compound was quantified and its relative amount was estimated.

NP Discovery Platform Combining in vivo Zebrafish Assays with Microscale Analytics

We have described herein the development of an integrated platform for the rapid acquisition of structural and in vivo bioactivity information on sub-milligram amounts of NPs from crude extracts. The combination of sensitive and rapid analytical techniques (UHPLC-TOF-MS and microflow NMR) with bioassays in small organisms (especially zebrafish) provides a powerful strategy for the rapid identification and quantification of NPs and the rapid analysis of their pharmacological activity in vivo. Zebrafish are an attractive in vivo assay for NP discovery because of their small size and their strong similarities with humans from a genetic, physiological and pharmacological point of view. These features enable the use of only microgram amounts of active compounds, fractions, and crude extracts for bioactivity analysis using a large and growing panel of biomedically relevant in vivo assays (morphological, physiological, and behavioral). Such strategies may improve drug discovery efforts based on NPs by enabling the more efficient identification of novel, bioactive lead molecules.

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Appendix VI

De Novo Production of Metabolites by Fungal Co-culture of Trichophyton rubrum and Bionectria ochroleuca


Samuel Bertrand, Nadine Bohni, Jean-Luc Wolfender
School of Pharmaceutical Sciences, University of Lausanne, University of Geneva, Geneva, Switzerland

Olivier Schumpp, Katia Gindro
Mycology group, Agroscope Changins ACW, Nyon, Switzerland

Michel Monod
Departement of Dermatology and Venereology, Laboratory of Mycology, CHUV, Lausanne, Switzerland

Contribution: Provision of expertise on isolation strategies, proofreading of the article
De Novo Production of Metabolites by Fungal Co-culture of Trichophyton rubrum and Bionectria ochroleuca

Samuel Bertrand,‡ Olivier Schumpp,‡ Nadine Bohni,‡ Michel Monod,§ Katia Gindro,‡ and Jean-Luc Wolfender*‡†

†School of Pharmaceutical Sciences, EPGL, University of Geneva, University of Lausanne, Quai Ernest-Ansermet 30, CH-1211 Geneva 4, Switzerland
‡Swiss Federal Research Station Agroscope Changins-Wädenswil, Route de Duillier, P.O. Box 1012, CH-1260 Nyon, Switzerland
§Department of Dermatology and Venereology, Laboratory of Mycology, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland

Supporting Information

ABSTRACT: The co-cultivation of fungi has recently been described as a promising strategy to induce the production of novel metabolites through possible gene activation. A large screening of fungal co-cultures in solid media has identified an unusual long-distance growth inhibition between Trichophyton rubrum and Bionectria ochroleuca. To study metabolite induction in this particular fungal interaction, differential LC-MS-based metabolomics was performed on pure strain cultures and on their co-cultures. The comparison of the resulting fingerprints highlighted five de novo induced compounds, which were purified using software-oriented semipreparative HPLC-MS. One metabolite was successfully identified as 4″-hydroxy sulfoxy-2,2″-dimethylthielavin P (a substituted trimer of 3,5-dimethyl orsellinic acid). The nonsulfated form, as well as three other related compounds, were found in the pure strain culture of B. ochroleuca.

Natural products (NPs) are a historical source of valuable lead medicinal compounds,¹ of which microbial compounds represent an important part. However, the attractiveness of NPs is diminished because of the difficulties involved in working with complex mixtures² and the continual rediscovery of the same bioactive chemical structures in pharmacological screening despite the existence of dereplication processes.³ To generate chemical diversity, several approaches such as nontargeted metabolic engineering, epigenetic modification or elicitation, and the production of unnatural—natural scaffolds have recently been developed.⁴⁻⁷ In the case of microorganisms the induction of novel metabolites can be achieved by the activation of cryptic biosynthetic pathways dedicated to the production of secondary metabolites.⁴⁻⁶,⁸,⁹ Such approaches take into account the tremendous metabolite diversity revealed by genome sequencing programs and aim to reduce the continual rediscovery of NPs. One of these strategies consists of the co-cultivation of two microorganisms in a single confined environment to induce the production of new natural products via possible interspecies crosstalk.⁶,⁸

Co-cultivation of bacteria or fungi exploits the fact that in their environment (such as soil, rhizospheres, plants, mucosal membranes, and guts) microorganisms are in constant interactions.¹⁰ These interactions lead to the activation of complex regulatory mechanisms, which results in the biosynthesis of highly diverse NPs such as pheromones, defense molecules, and metabolites involved in symbiotic associations.³⁻⁶,⁸,⁹,¹¹ Moreover, stress-induced molecules exhibited specific antimicrobial,¹²⁻¹⁷ anticancer,¹⁸,¹⁹ and phytotoxic activities.⁴

To detect the induction of metabolite biosynthesis in microorganism co-cultures within the complex fungal metabolome, sensitive high-resolution (HR) techniques mainly based on mass spectrometry are required. Recently induction phenomena of protein and chemical substances in solid culture were demonstrated in bacterial co-culture through state-of-the-art MALDI-Mg²⁺⁻²２ or nanoDESI-MS²³ imaging approaches. To further identify induced secondary metabolites and assess their bioactivity, analytical strategies based on LC were employed, which allow for targeted microisolation of selected NPs.²⁴ Therefore, metabolite-profiling technology, such as ultra-high-pressure liquid chromatography coupled to electrospray ionization and time-of-flight mass spectrometry (UHPLC-TOF-MS), provided a fast and efficient determination of metabolome modification in complex matrices such as microbial extracts.²⁵,²⁶ When applied to fungal co-cultures, this approach allowed an efficient comparison of pure strain cultures versus their corresponding co-culture metabolome fingerprints to highlight de novo induced biomarkers¹⁴,²⁷ and

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RESULTS AND DISCUSSION

The fungal interaction studied in this work consisted of the co-culture of two fungi isolated from entirely different environments. The dermatophyte *T. rubrum* is the most common cause of nail infection worldwide. 30,31 This strain of *T. rubrum* (Sin146) used was isolated from difficult-to-treat onychomycosis collected at the Centre Hospitalier Cantonal Vaudois (CHUV, Lausanne, Switzerland). 30 This particular strain was able to generate long-distance interaction with the filamentous fungus *B. ochroleuca* (Sin80), a pattern of interaction that could be reliably reproduced over several experiments using either rich or poor growth media. *B. ochroleuca* (Sin80) is both a plant endophyte and soil saprophyte. 32 Interestingly, *B. ochroleuca* was also reported to act as a parasite of other fungi. 33 Therefore, the interaction of *B. ochroleuca* with *T. rubrum* is of great interest for the study of fungal interactions.

As previously described in screened fungal co-cultures, 37 pure strain cultures of filamentous fungi were produced via inoculation of an agar plug in the center of a Petri dish, while co-culture plates were inoculated on opposite sides of a single Petri dish. The plates were incubated for several weeks. Colony growth leading to interaction with the competing colony was monitored by eye. For the study of the *T. rubrum* and *B. ochroleuca* co-culture, nine replicates of the Petri dish solid culture were generated to provide enough material for purification of the induced metabolites. The long-distance interaction morphological pattern was reproduced in all replicates.

Identification of Induced Metabolites. Because chemical induction was reported to occur mainly in the confrontation zone, the solid media of that specific zone was excised for further chemical analyses. The results were compared to those obtained from the cultures of the corresponding pure strains grown in parallel under the same conditions.

The excised pieces of agar medium were extracted by sonication using a dichloromethane-methanol-water mixture according to our previously reported protocol. 27 The extracts were then subjected to reverse-phase solid-phase extraction to remove highly nonpolar compounds and reduce carryover effects in LC-MS.

Each extract was subjected to UHPLC-TOF-MS fingerprinting. 27 This procedure took advantage of the high resolution and fast separation capacities of UHPLC, as well as the HR detection offered by the TOF-MS detector. 34 The UHPLC-TOF-MS fingerprints generated 2D ion maps (*m/z* × *m/z*) in which all features associated with fungal metabolites were resolved in both the LC and MS dimension with high repeatability. Figure 2A shows the negative ionization (NI) 2D ion maps obtained for the culture of the pure strains and the co-culture.

All features (*m/z* × *m/z*) detected in the fingerprints of all replicates were extracted using an automated peak picking procedure that was optimized to retrieve only relevant chromatogram ion traces with a relatively low signal-to-noise threshold to keep as much relevant information as possible. 35 Because the goal was to localize and identify *de novo* induced compounds, intensities of signals from metabolites present in the co-cultures were kept to compare with the intensities of the corresponding pure strains. Only the most intense peaks (peak area >2000 counts-min) that occurred at least eight times in the nine replicates were considered. All of these features were displayed in reconstituted 2D ion maps (Figure 2B). This representation of fingerprinting data enabled a clear distinction of features that were not present in the pure strain culture but were present only in the co-culture (red dot in Figure 2B). These features were verified directly in the raw data by the extraction of their corresponding selected ion traces (Figure 2C). On the basis of this data treatment, from more than 600 features recorded in the initial fingerprints, only five compounds that displayed intense MS signals and were induced *de novo* in the zone of interaction were highlighted and considered for further metabolite identification (Figure 2, Figure S1 and Table S1).

Dereplication of the Induced Metabolites. To obtain preliminary information about the selected metabolites, a full process of dereplication was undertaken. The Dictionary of Natural Products (DNP) was used to search for positive matches of each of the selected masses. 27 The high mass accuracy molecular weights were deduced by taking into account the possible presence of the most probable adducts in the LC-MS conditions used ([M − H]− and [M + HCO3]− in NI). 25 To improve the chances of finding a matching candidate, a relatively large mass-accuracy tolerance (<15 ppm) was used. This rapid procedure revealed that all five exact masses matched known fungal NPs in the database (Table S1). This preliminary search was refined through molecular formula determination of the selected metabolites based on exact mass...
For all compounds, only one or two molecular formulas matched the HR-MS data generated within the tolerance of the instrument (5 ppm m/z tolerance and lowest i-fit below 2). A search in the DNP and SciFinder (ACS) for fungal metabolites particularly related to *B. ochroleuca* and *T. rubrum* matching these molecular formulas did not reveal any possible metabolites (Table S1). However, compound 2 had 10 matches for metabolites of fungal origin, none of which were related to species phylogenetically close to *B. ochroleuca* or *T. rubrum*.

This indicated that the selected *de novo* induced biomarkers probably correspond to novel NPs based on the hypothesis that all molecular formulas of metabolites from fungal origin, even of undescribed species, are reported in the DNP. This is in agreement with our previous observation regarding the dereplication results on the screening of a large number of
Figure 3. Two-step software-driven purification of de novo induced metabolites. (A) UHPLC-TOF-MS analysis of the co-culture performed with two gradient slopes necessary for optimizing the separation of targeted metabolites through software modeling. (B) Targeted fractionation for metabolite enrichment with (i) localization of the five biomarkers of interest, (ii) simulated separation of these markers, and (iii) corresponding semipreparative LC-MS separation. (C) Final targeted purification of biomarker 4: (i) localization of the five biomarkers of interest with an inset of the fingerprint of the enriched fraction containing 4 used for ion selection in the modeling procedure, (ii) modeling of the three different isocratic separation conditions for the final purification step, and (iii) final semipreparative LC-MS purification using the isocratic condition at 39% B.
fungal co-cultures. However, the low scores determined in database matching during dereplication could be explained by the lack of information about the metabolites produced by *B. ochroleuca* and *T. rubrum*.

**Targeted LC-MS Microisolation of Selected Induced Metabolites.** To identify induced metabolites related to highlighted features, a strategy for their targeted isolation was developed for further *de novo* structural identification by microflow NMR. This was performed by adapting our previously reported microisolation strategy. A two-step semipreparative HPLC-MS strategy was devised based on software-driven optimization to efficiently purify the biomarkers.

NMR requires microgram amounts of NPs for structure identification. Therefore, the nine co-culture samples used for the metabolomic study were pooled to obtain 636 mg of crude extract. Solid-phase extraction removed highly nonpolar compounds, leading to 456 mg of enriched extract that was used for the LC-MS targeted isolation.

To achieve optimal semipreparative separation of the crude extract, a two-step strategy for biomarker enrichment followed by a high-resolution final purification step was devised as illustrated in Figure 3.

To generate elution models for the optimization of each step, chromatography modeling software was used. The separation was optimized based on the chromatographic behaviors of each biomarker and its surrounding LC peak in two analytical UHPLC gradients of different slopes corresponding to elution retention factors (*k*<sub>0</sub>) of 3 and 10 (i.e., gradient slopes equal to 3 and 1%/min, respectively; Figure 3i). The retention of each metabolite was modeled in this way, and the optimal separation of the *de novo* induced metabolites (1 to 5) was calculated. The final LC-MS semipreparative conditions on a C<sub>18</sub> column (150 mm × 19 mm i.d., 5 μm) were determined after gradient transfer from the UHPLC level (Figure 3ii).

To stay within column capacity, repeated injections of the extract using these semipreparative LC-MS conditions generated 30 fractions with high repeatability and good separation without column overload. The semipreparative MS monitoring indicated the presence of the different features detected at the analytical level in well-defined specific fractions. Mass balance of this fractionation (Figure S2) indicated that 66% of the mass was found in the injection peak, while the remaining part was equally spread over the other fractions with quantities between 3 and 10 mg. The fractions containing the biomarkers were further analyzed by UHPLC-TOF-MS and were found to generate 5–10 LC peaks (Figure 3iii). The microfractionation process could have been improved using a larger semipreparative column providing similar peak capacity. However, the strategy used based on repeated injections was found to be a good compromise between column loadability and accuracy of the MS-triggered fractionation.

On the basis of the LC peaks detected in the enriched fractions and their behavior already measured in the UHPLC profiles of the crude extract, high-resolution separation conditions were calculated on a semipreparative C<sub>18</sub> column (250 mm × 10 mm i.d., 5 μm) that provided higher peak capacity. Targeted MS monitoring enabled the collection of the pure biomarkers as exemplified for biomarker 4 in Figure 3.

The purification of the *de novo* induced metabolites 1 to 5 was performed using this optimized protocol. In most cases, the UHPLC-TOF-MS analysis revealed that pure biomarkers were obtained. However, microflow NMR analysis of these induced metabolites dissolved in a minimum amount of deuterated solvent corresponding to the volume of the microflow NMR probe (5 μL) gave interpretable spectra only for compounds 4 and 5. Thus, despite the high sensitivity of this microflow NMR approach, the intrinsic differences in sensitivity between MS and NMR did not allow the identification of all biomarkers isolated from the initial extract. This indicated that some of the induced compounds were well ionized in MS but only present in trace amounts in the confrontation zone.

**Identification of the De Novo Induced Compound 4.** Biomarker 4 (Figure 4) was identified by a complete series of 1D and 2D NMR experiments, including 1H, gCOSY, edited-gHSQC, gHMBC, and NOESY (Table 1, Figure S3), in combination with HR-MS analyses.

As recorded during the dereplication process, the HR-MS spectrum of 4 indicated a molecular formula of C<sub>32</sub>H<sub>35</sub>O<sub>13</sub>S (17 unsaturations, Table S1). The 1H NMR spectra of 4 revealed the presence of nine methyls (δ<sub>H</sub> from 2 to 3) and two methoxy groups (δ<sub>H</sub> approximately 3.8). According to the gHMBC correlations, all methyl and methoxy groups were directly linked to an aromatic ring. The 13C chemical shifts deduced from the gHMBC correlations indicated the presence of six O-substituted aromatic carbons with typical signals between δ<sub>C</sub> 145 and 160. A careful analysis of these correlations revealed the presence of three 2,4-dihydroxy-3,5,6-trimethylenethylphenyl (C<sub>16</sub>H<sub>14</sub>O<sub>2</sub>) units in 4. This information, combined with the molecular formula C<sub>32</sub>H<sub>35</sub>O<sub>13</sub>S noted that three carbonyls, two methoxy groups, and one sulfate moiety still had to be assigned. On the basis of this structural information and biosynthetic considerations, 4 was deduced to be composed of three 3,5-dimethylolellinic acid subunits, which is a common skeleton among fungal metabolites.

Two of these subunits were methoxylated in position 2 (4-hydroxy-2-methoxy-3,5,6-trimethylenbenzoic acid) according to gHMBC. The sequence of the three substituted orsellinic acid moieties was determined on the basis of the NOE correlations between 12′–3′, 12′–5′, 14′–9′, and 14′–11′. The complete structure assignment of 4 matched the spectroscopic data previously reported for the fungal metabolite of an *Acremonium* species closely related to *B. ochroleuca*, PS-990 (4-(4-(4′-hydroxy-2′-methoxy-3′,5′,6′-trimethylenbenzyloxy)-2′-hydroxy-3′,5′,6′-trimethylenbenzyloxy)-2′-methoxy-3,5,6-trimethylenbenzoic acid, also named 2,2′-dime-thylthieliavin P, Figure 4). However, differences were observed between 4 and PS-990 in the 13C NMR chemical shifts of C-15, C-15′, C-17, C-17′, and C-16. These differences were related to the presence of a sulfate group revealed by HR-MS. According to the structure, only two phenolic positions (C-8 and C-16) were possible for the hydroxysulfonyl substitution. The position of the sulfate group was clearly assigned to C-16 because the 13C NMR chemical shifts of 4 showed that C-15, C-15′, C-17 and C-17′ were upfield shifted.
Table 1. $^1$H and $^{13}$C NMR Spectral Data for Compounds 4 and PS-990 in CD$_3$OD (500 MHz for $^1$H and 125 MHz for $^{13}$C)

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$^a$ND: not detected.

and C-16 was downfield shifted in comparison to PS-990 and no difference was observed around C-8. These modifications in the $^{13}$C NMR chemical shifts also matched well with its predicted NMR spectrum. Compound 4 was identified as 4-(4′-(2′-methoxy-4′-hydroxy-3′,5′,6′-trimethylbenzoxyl)-2′-hydroxy-3′,5′,6′-trimethylbenzoxyl)-2-methoxy-3,5,6-trimethylbenzoic acid (4′-hydroxy-sulfoxy-3′,5′,6′-trimethylbenzoxyl)-2-methoxy-3,5,6-trimethylbenzoic acid (4′-hydroxy-sulfoxy-3′,5′,6′-dimethylthielavin P). To our knowledge, this compound is reported here for the first time.

The $^1$H NMR spectra of 5 and its HR-MS spectra (C$_{19}$H$_{24}$O$_{4}$) indicated that the second induced metabolite was also an orsellinic acid derivative. Unfortunately, the 1D and 2D NMR spectra of 5 did not permit a successful structure elucidation; the amount of material available and the sensitivity of the microflow NMR probe were not sufficient to highlight the necessary key gHMBC and NOE correlations. Unfortunately the amount isolated for compounds 1–3 was insufficient to obtain $^1$H NMR spectra, and no conclusions could be drawn based on their molecular formula alone.

Origin of Compound 4 in the Co-culture. The structure of 4 was related to orsellinic acid, which was reported to be overproduced by Aspergillus nidulans when co-cultivated with Streptomyces hygroscopicus as a consequence of polyketide synthase gene activation. No information was available regarding the occurrence of related compounds in T. rubrum or in B. ochroleuca. In this fungal co-culture experiment, 4 could have been produced by either of these two strains.

Because 4 was only found in the co-culture, the occurrence of its non-sulfated form, PS-990 (Figure 4), in both pure culture and in co-culture was investigated. The extracted ion chromatogram of the [M – H]$^-$ of PS-990 was compared in the different extracts. The corresponding ion ($fingerprint = 2.85$ min, $m/z = 579.222$ Da, molecular formula of C$_{49}$H$_{49}$O$_{10}$) was found in both the pure strain culture of B. ochroleuca and in the co-cultures. In contrast to 4, this compound was not highlighted to be produced de novo (Figure S4).

To confirm the presence of PS-990 in the extract and further strengthen the structure identification of 4, PS-990 was isolated from fractions 23 and 24 of the initial fractionation. The corresponding NMR data matched those reported for PS-990 (Table 1, Figure SS). This further confirmed the secretion of PS-990 by B. ochroleuca in pure strain and co-cultures with T. rubrum. To further investigate the biosynthetic origin of 4, the building blocks of 4 and PS-990 were searched for by extraction of their corresponding deprotonated ion traces in the UHPLC-TOF-MS fingerprints. The [M – H]$^-$ ions, which could correspond to 4-(4′-hydroxy-2′-hydroxy-3′,5′,6′-trimethylbenzoxyl)-2-methoxy-3,5,6-trimethylbenzoic acid (m/z = 387.144 Da at t$_R$ 2.82 min), 2-methoxy-3,5,6-trimethylbenzoic acid (m/z = 209.082 Da at 2.83 min), and 3,5-dimethyloctylic acid (m/z = 208.081 Da at t$_R$ 2.85 min), were detected only in the extracts of B. ochroleuca and in the co-culture. Moreover, the concentration of those metabolites was similar in both types of extracts (according to UHPLC-TOF-MS peak area). None of these ions were detected in the extracts from T. rubrum. Taken together, these data show that PS-990 is released in the medium by B. ochroleuca. These data also suggest that PS-990 is further sulfated during fungal interaction. However, the origin and identity of the enzyme responsible for this chemical modification remain to be determined.

In conclusion, this work demonstrated the induction of new sulfated analogues of known compounds from natural sources through fungal co-culture. This work also showed that small-scale software-oriented purification is efficient for purifying compounds present in low concentrations from complex fungal extracts. However, for minor compounds, scale-up strategies need to be developed to produce additional material. Once the structures are ascertained, organic synthesis may in some cases be used as an alternative source. Thus, in the case of B. ochroleuca and T. rubrum co-culture, the chemical synthesis of compound 4 is possible using previously published strategies. If greater quantities of compound 4 can be obtained, it will be easier to elucidate its role in long-distance fungal interactions.

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**EXPERIMENTAL SECTION**

**General Experimental Procedures.** Extractions were performed with methanol (HPLC grade), dichloromethane (HPLC grade), and nanopure water (Millipore). UHPLC-TOF-MS analyses were performed using ULC/MS-grade acetonitrile and a mixture of water and formic acid from Biosolve (Valkenswaard, The Netherlands).

**Fungal Material.** The two strains used were selected according to a preliminary screening of fungal co-cultures for their ability to generate long-distance interactions. *Trichophyton rubrum* Sin146 (accession number for ITS sequence is KF053599) was isolated from onychomycosis collected at the Centre Hospitalier Cantonal Vaudois (CHUV, Lausanne Switzerland). *Bionectria ochroleuca* Sin80...
High-resolution UHPLC-TOF-MS profiles were recorded with a 150 mm × 2.1 mm i.d., 1.7 μm Acquity BEH C18 UPLC column (Waters, Baden-Daettwil, Switzerland) in gradient mode at a flow rate of 0.4 mL/min with the solvent system described above. The gradient was increased from 5% to 95% B over 15 or 45 min for the 3% and 1% slope gradients, respectively. The column was then washed for 7.5 min with 95% B, reconditioned with 5% B in 0.5 min, and equilibrated with 5% B for 7 min. The temperature was maintained at 40 °C, and the injection volume was 2 μL. These two different profiles were used to simulate the elution of metabolites present in the extract using OSIRIS (Datalsy, Grenoble, France) software.

**Automatic Peak Picking.** Native MassLynx UHPLC-TOF-MS data (Waters, Baden-Daettwil, Switzerland) were converted into nCDF (common data format) data using DataBridge software (Waters, Baden-Daettwil, Switzerland). Automatic feature detection was performed between 0.5 and 4.5 min with MZmine 2 software using parameters selected according to the TOF-MS detector. Peaks with a width of at least 0.03 s and an intensity greater than 50 counts (NI) were selected with a 0.02 Da m/z tolerance and deconvoluted. Deisotope filtering was applied using the “isotopic peaks grouper” module with the tolerance parameters adjusted to 0.05 s and 0.02 Da. Feature alignment was achieved with an m/z tolerance of 0.05 Da and a retention-time tolerance of 0.15 min. The full procedure for feature detection is presented in Table S2. The exported feature lists were compared using Microsoft Excel. Selected features were validated by exploring the raw data using MassLynx.

**Extract Preparation for Purification.** The nine replicates of the fungal co-culture extracts were pooled, leading to 635.5 mg of crude extract. Prior to any HPLC purification, the nonpolar portion of the crude extract was removed by solid-phase extraction after scaling-up. In a flask, 6.3 g of conditioned reverse-phase C18 material (silica gel ZEOPrep 60 C18, Zeochem AG, Uetikon, Switzerland) was added to the dried crude extract dissolved in 50 mL of methanol–water (85:15) and gently shaken overnight. The mixture was filtered to remove the C18 material and further washed three times with 50 mL of methanol–water (85:15). All liquid filtrates were pooled. The solvent was removed under reduced pressure, leading to 455.9 mg of extract compatible with semipreparative HPLC-MS purification.

**Semipreparative HPLC-MS Purification of Induced Compounds.** Purifications were performed on a modular HPLC system composed of a Varian 9012 pump (Palo Alto, CA, USA), a manual injection system (Rhodeyne, IDEX Health & Science, Wertheim-Mondfeld Germany), a fraction collector (FC204, Gilson, Middleton, WI, USA), a column heater (CT-30, Eppendorf, Hamburg, Germany) controlled by a temperature controller (TC-50; Eppendorf, Hamburg, Germany), an adjustable flow splitter, and an MS spectrometer (LCQ Deca, Finnigan MAT, San Jose, CA, USA) with an ESI interface. Separations were performed using water with 0.1% formic acid as eluent A and acetonitrile with 0.1% formic acid as eluent B. The column temperature was set at 40 °C. A fraction (1/600) of the flow was split to the MS detector for analysis. The following ESI conditions were used: capillary temperature, 180 °C; source voltage, 4.5 kV; and sheath gas nitrogen, 45 psi. The MS acquisitions were performed in NI mode using a full scan mode over an m/z range of 100–1000 and a scan time of 1 s.

Fractionation of the crude extract into 31 fractions was performed on a 150 mm × 19 mm i.d., 5 μm XBridge BEH C18 column at 80 mL/min. The separation protocol was optimized for a maximal resolution (maximal Rsmin) in a short time (less than 30 min: k between 0.4 and 7.0). The optimized conditions were a gradient increasing from 19% to 82% B in 25.2 min followed by an increase to 100% B in 0.8 min. The column was then washed for 5 min with 100% B, reconditioned to 19% B, and finally equilibrated with 19% B for 9.0 min. Fractions were collected in tubes every 1 min starting from 1 min. The fractionation was performed 18 times by injecting 200 μL of the extract dissolved at 50 mg/mL in methanol (20% of the mass loading capacity of the column). The corresponding fractions of the repeated fractionation were pooled together to give 31 fractions, which were dried under reduced pressure. Compound 1 was isolated in fraction 3, compound 2 in fraction 9, compound 3 in fraction 11, compound 4 in
fractions 13 and 14, and compound 5 in fractions 23 and 24. Each of these fractions was analyzed by UHPLC-TOF-MS using the same fingerprinting procedure to confirm the presence of the compound of interest.

The final purifications were performed on a longer XBridge BEH C18 column (250 mm × 10 mm i.d., 5 μm) at a flow rate of 3.1 mL/min. Fractions were collected in tubes every 0.24 min starting from 3 min. Compound 4 (1.0 mg) was isolated from fractions 13 and 14 using 39% B isocratic conditions. Compound PS-990 (0.9 mg) was isolated from fractions 23 and 24 using 48% B isocratic conditions. Both compounds were dried under nitrogen flux before NMR and LC-MS analyses for their identification. Compound 5 was estimated to be isolated at about 50 μg based on its micro 1H NMR signal-to-noise ratio. According to UHPLC-TOF-MS analysis of the compounds, 1−3 were isolated. However, only weak 1H NMR signals were recorded for these compounds. It indicated that they were isolated only in a few microgram amounts.

NMR Spectra of the Isolated Compounds. Microflow NMR analyses were performed on a Varian Unity Inova 500 MHz NMR instrument (Palo Alto, CA, USA) equipped with a 5 μL microflow NMR probe from Protasis/MRM (CapSavoy, IL, USA) with an active volume of 1.5 μL. All samples were dissolved in 10 μL of CD3OD.

Predictions of the NMR spectra were performed on ChemBioDraw 13 (CambridgeSoft) and H/C NMR Predictor 8.09 (ACD LAB).

ASSOCIATED CONTENT

Supporting Information

Extracted ion chromatograms and dereplication information of compounds 1 to 5; steps and parameters used during the automatic peak piking procedure by MZmine2; mass balance of these compounds. It indicated that they were isolated only in a few microgram amounts.

AUTHOR INFORMATION

Corresponding Author

*Tel: +41 22 379 33 85. Fax: +41 22 379 33 99. E-mail: jean-luc.wolfender@unige.ch.

Notes

The authors declare no competing financial interest.

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Appendix VII

Multi-Well Fungal Co-Culture for De Novo Metabolite-Induction in Time-Series Studies Based on Untargeted Metabolomics


Samuel Bertrand, Antonio Azzollini, Nadine Bohni, Jean-Luc Wolfender
School of Pharmaceutical Sciences, University of Lausanne, University of Geneva, Geneva, Switzerland

Olivier Schumpp, Katia Gindro
Mycology group, Agroscope Changins ACW, Nyon, Switzerland

Jacques Schrenzel
Clinical Microbiology Laboratory, Service of Infectious Diseases, Geneva University Hospital, Geneva, Switzerland

Michel Monod
Departement of Dermatology and Venereology, Laboratory of Mycology, CHUV, Lausanne, Switzerland

Contribution: Provision of expertise on isolation strategies, discussion of results, proofreading of the article
Multi-well fungal co-culture for de novo metabolite-induction in time-series studies based on untargeted metabolomics†‡

Samuel Bertrand,‡a,b Antonio Azzollini,‡a Olivier Schumpp, c Nadine Bohni, a Jacques Schrenzel, a Michel Monod, e Katia Gindro c and Jean-Luc Wolfender* a

The induction of fungal metabolites by fungal co-cultures grown on solid media was explored using multi-well co-cultures in 2 cm diameter Petri dishes. Fungi were grown in 12-well plates to easily and rapidly obtain the large number of replicates necessary for employing metabolomic approaches. Fungal culture using such a format accelerated the production of metabolites by several weeks compared with using the large-format 9 cm Petri dishes. This strategy was applied to a co-culture of a Fusarium and an Aspergillus strain. The metabolite composition of the cultures was assessed using ultra-high pressure liquid chromatography coupled to electrospray ionisation and time-of-flight mass spectrometry, followed by automated data mining. The de novo production of metabolites was dramatically increased by nutrient reduction. A time-series study of the induction of the fungal metabolites of interest over nine days revealed that they exhibited various induction patterns. The concentrations of most of the de novo induced metabolites increased over time. However, interesting patterns were observed, such as with the presence of some compounds only at certain time points. This result indicates the complexity and dynamic nature of fungal metabolism. The large-scale production of the compounds of interest was verified by co-culture in 15 cm Petri dishes; most of the induced metabolites of interest (16/18) were found to be produced as effectively as on a small scale, although not in the same time frames. Large-scale production is a practical solution for the future production, identification and biological evaluation of these metabolites.

1. Introduction

Microorganisms are a historical source of natural bioactive lead compounds,1,2 however, the attractiveness of such natural products (NPs) is reduced due to the continual rediscovery of the same bioactive chemicals in pharmacological screens despite the existence of dereplication processes.3 Various approaches to increase NP chemodiversity have been recently developed, such as non-targeted metabolic engineering, epigenetic modification or elicitation and the production of unnatural–natural products.2,4–7 Among such approaches, the activation of cryptic biosynthetic pathways through microorganisms co-culture (growing two microorganisms in a single confined environment) has gained attention because co-culture has led to the production of new or rare secondary metabolites under standard laboratory conditions.4–6,8–11 Genomic sequencing has revealed a considerable diversity of secondary metabolite encoding biosynthetic genes, and such approaches may permit the elucidation of potential metabolite diversity of a given microorganism11 and reduce the continual rediscovery of NPs. Moreover, most stress-induced molecules exhibit interesting biological activities,11 such as antimicrobial,13–23
To study the induction of metabolite biosynthesis in fungal co-cultures, sensitive high-resolution (HR) techniques, mainly based on mass spectrometry, are required. Metabolite profiling based on ultra-high pressure liquid chromatography coupled to electrospray ionisation and time-of-flight mass spectrometry (UHPLC-TOFMS) provides a rapid and efficient overview of the metabolome of complex matrices, such as microbial extracts. This approach was successfully applied to fungal co-cultures and allowed an efficient comparison of the metabolomic profiles of pure-strain cultures (“pure-strain cultures” will be referred to as “pure cultures” in the manuscript) and their corresponding co-culture metabolomic profiles for the identification of de novo induced compounds (secondary metabolites present in the co-culture but not in the pure cultures). Various studies have explored the induction of fungal metabolites in fungal co-cultures grown on solid media in 9 cm Petri dishes using untargeted liquid chromatography-mass spectrometry (LC-MS) metabolomics. In these studies, numerous co-cultures were screened, and various phenomena were observed.

(1) The growth rate of different fungi may vary significantly. Therefore, many fungi cannot easily be grown with others. It might be necessary to inoculate one of the two fungi days before inoculating the second one.

(2) The duration of growth has a huge effect on the metabolites present. In addition, the period of growth required to obtain confronting fungal colonies on 9 cm Petri dishes may be weeks or even months. This period can be even longer when larger dishes are used.

(3) The use of 9 cm diameter Petri dishes leads to a low concentration of material such that the quantity of the purified compounds may not be sufficient for comprehensive identification of the induced metabolites.

In the present paper, an approach based on small-scale co-culture experiments in 2 cm diameter Petri dishes is presented. This approach partially overcomes the problems mentioned above and provides a rational way to highlight de novo induced metabolites and study the evolution of their accumulation over time. To develop this protocol, two model fungi, Aspergillus clavatus (SIN141) and Fusarium sp. (PS54743), were selected for use because of their respective differential growth rates in 9 cm Petri dishes. In addition, large-scale production of the de novo induced metabolites was evaluated using 15 cm Petri dishes to assess the feasibility of up-scaling the method for future isolation and structural elucidation of these metabolites.

2. Results and discussion

To address the issues related to sampling in a metabolomics study, a down-scaled 2 cm Petri dish procedure was developed for the study of the de novo induction of secondary metabolites by fungal co-culture. Surprisingly, cultivation in this small-scale system strongly stimulated fungal growth. The surface of the well was covered within less than a week using most of the tested fungal species, whereas reaching this level of coverage takes several weeks in standard 9 cm Petri dishes. The rare exceptions of species with slow growth characteristics required no more than two weeks to fully cover the well’s surface. Thus, compared to the traditionally utilised 9 cm Petri dish co-cultures, a larger number of experiments can be performed in a shorter period.

For practicality, 12-well plates were used instead of independent 2 cm Petri dishes because they correspond to twelve 2 cm Petri dishes. The use of this format enabled the rapid and efficient generation of a large number of replicates for each pure and co-culture. One 12-well plate (Fig. S1A, ESI†) allowed three replicates for each pure culture, the co-culture and the non-inoculated agar (blanks); one metabolomics study required at least two plates.

Volume optimisation of the culture medium

The volume of potato dextrose agar (PDA) medium added to the Petri dish was optimised to allow the rapid induction of de novo secondary metabolites. The four following volumes were tested: 1, 2, 3 and 4 mL, in wells with a capacity of approximately 5 mL. The two model fungi (A. clavatus and Fusarium sp.) used in this study were grown in triplicate for three days. Subsequently, the content of each well was lyophilised and extracted by sonication using a monophasic dichloromethane–methanol–water solvent mixture. In our previous studies, this solvent system was found to be optimal for the extraction of a broad range of fungal metabolites. The extracts were dried under vacuum and then subjected to reversed-phase solid-phase extraction (SPE) to remove highly non-polar compounds and reduce their carryover effects in LC-MS. Finally, the enriched extracts were solubilised and further analysis. Compared to when using standard Petri dishes, extracting these smaller culture volumes significantly accelerated the sample-preparation procedure and substantially simplified the manipulation of a large number of cultures.

The samples were finally analysed according to a previously established protocol, using UHPLC-TOFMS in both positive (PI) and negative ionisation (NI) modes. This procedure takes advantage of the high-resolution and rapid separation capabilities of UHPLC as well as the HR detection offered by TOFMS. The UHPLC-TOFMS fingerprints generated 2D ion maps (retention time (RT) × m/z), in which all of the features associated with the fungal metabolites were resolved in both the LC and MS dimensions with high repeatability. All of the features (RT × m/z × peak area) detected in the fingerprints of each replicate were deconvoluted using an automated peak-picking procedure that was optimised for the retrieval of only the relevant chromatographic ion traces. A low signal-to-noise threshold was set to maximise the retention of relevant information. The de novo induction of metabolites was systematically evaluated for each set of samples based on the volume of culture medium used (Fig. 1). Ions corresponding to the same compound but detected using both ionisation modes (NI and PI) were counted only once. In addition, the unusual formation of adducts related to co-eluting compounds was also verified as such phenomenon may be sometimes observed in ESI, and may lead to features highlighted as de novo induced features during the data analysis process which would then be false positive. Fig. 1 shows that after three
days of growth, more metabolites were detected when the smaller volumes of culture medium (1 and 2 mL) were used. The number of induced metabolites was consistent with the previously reported results obtained using 9 cm Petri dishes.\textsuperscript{29,30} In contrast, fewer metabolites were induced in co-culture experiments performed using the larger volumes of culture medium (3 and 4 mL). However, metabolite intensity on the base peak chromatogram was similar between the different extracts. This indicated that metabolite production was improved when larger culture media volume was used as metabolite/sugar ratio is unchanged. The differences of \textit{de novo} induction observed suggested that the reduction of nutrients increased the competition between the two microorganisms and led to an increased number of \textit{de novo} induced metabolites. Similar results were previously reported for pure cultures of \textit{Streptomyces}, in which antibiotic production was triggered by poor nutrient conditions.\textsuperscript{37}

The smallest colonies were observed when the fungi were grown using 1 mL of culture medium (Fig. S2, ESI\textsuperscript{†}). However, no clear difference in the colony size was observed in cultures grown using 2, 3 or 4 mL of culture medium. In addition, the standard deviation for the extract mass obtained was more than six times lower for 1 or 2 mL of culture medium compared to 3 or 4 mL (Fig. S3, ESI\textsuperscript{†}), indicating less variability under the former than under the latter conditions. Consequently, a volume of 2 mL was selected for use in the 2 cm diameter wells to ensure the release of largest number of metabolites with the maximal growth rate and satisfactory repeatability.

\textbf{Dynamics of \textit{de novo} compound induction in the fungal co-culture}

Using the optimised condition (2 mL cultures), the induction of compounds was assessed at four different time points (two, four, seven and nine days, which will be referred to as D2, D4, D7, D9) to determine the optimal growth period for significant \textit{de novo} induction in the 12-well plates. For that purpose, six replicates of the co-culture experiment were used (six pure cultures of \textit{A. clavatus} and \textit{Fusarium} sp. and six co-cultures) (Fig. S1, ESI\textsuperscript{†} shows the 12-well plate format and Fig. S4, ESI\textsuperscript{†} shows the colony morphologies).

After growth, the content of each well was lyophilised and extracted by sonication using a monophasic dichloromethane–methanol–water solvent mixture. The weight of each extract was compared for each time point. From D2 to D7, the extract mass decreased and then, it remained constant between D7 and D9 (Fig. 2A). Proton NMR (\textsuperscript{1}H-NMR) profiling of the co-culture extracts revealed that the main constituent was glucose (anomeric protons at $\delta_H$ 5.2 and 4.5 ppm), which is the main source of carbon in the culture medium (Fig. 2B). However, the presence of sugar drastically decreased from D0 (day zero) to D7 and then, only slightly reduced between D7 and D9 (Fig. S5, ESI\textsuperscript{†}). This decrease is logically related to the sugar consumption required for fungal growth. This result further comforted the choice for cultures in reduce volumes (2 mL) to lower the sugar quantity extracted from the medium. In terms of extract composition, smaller culture volumes favour a better secondary metabolite to
sugar ratio. Large quantities of sugar from the growth medium would significantly impede secondary metabolites analysis.

To evaluate the de novo induction of metabolites during fungal growth, the fungal extracts were enriched by SPE and analysed using UHPLC-TOFMS for untargeted metabolite profiling. Generic linear RP LC gradients and both PI and NI electrospray ionisation (ESI) modes were used to perform a comprehensive survey of the metabolite composition of the extracts (Fig. S6, ESI†). Comparison of the total metabolites detected in all of the profiles revealed that only a few metabolites were present in the co-culture at the early time points (up to D4), whereas the number of metabolites was greater at later time points (after D4). This trend was inversely related to the sugar content, as determined using NMR (Fig. 2B and Fig. S6, ESI†).

The de novo induced features were counted at each time point by an automatic search for ions that were detected in the co-culture but were not detected in either of the pure strain cultures (Fig. 3, Table S1, ESI†). As during the optimisation of the culture medium volume used for fungal growth, the unusual formation of adducts related to co-eluting compounds was also verified. At D2, the level of de novo induction was very low: only one metabolite was highlighted in profiles showing only few other secondary metabolites (Fig. S6, ESI†). At D4, more induced metabolites were observed, and at D7 and D9, the number of de novo induced metabolites detected had further increased. The number of secondary metabolites that were revealed was in the range of that previously reported for de novo induction after several weeks of culture in 9 cm Petri dishes.²⁹

Altogether, 18 features were highlighted to be induced in the co-culture at a minimum of one time point. To study the trend of production of each of these metabolites, their level at each time point was monitored.

Among the 18 features, nine were not detected at any time point in the pure cultures. These features were considered as de novo induced in stricto sensu. Therefore, the strain producing these metabolites could not be determined; in the remainder of the manuscript, only these features will be described as de novo induced. The other nine highlighted features were detected in at least one of the pure cultures at a minimum of one time point. This result indicated that their production was up-regulated due to co-culture; and these particular features will be discussed later in the manuscript. Consequently, the fungi which was producing these particular features was defined.

As expected, many of the de novo induced features accumulated in the co-cultures over time (Fig. 4). This was the case for seven of the nine highlighted features. Features PI481.032@1.44 (the notation corresponds to an ion detected using the PI mode that had an \( m/z \) of 481.032 Da at a RT of 1.44 min), NI205.087@1.44 and PI265.178@2.55 were produced from D4 to D9. Features NI427.103@1.44, NI525.071@0.80 and NI445.114@1.03 accumulating from D7, and feature NI581.131@1.15 was produced only from D9.

Interestingly, not all the de novo induced features simply accumulated as a function of the period of growth. Two features, NI387.038@0.77 and NI305.066@1.46, were produced at D4 and D7 and could not be detected at D9. This result indicates the complexity and dynamic nature of fungal metabolism.

**Dynamics of metabolite up-regulation in fungal co-cultures**

In addition to the previously discussed de novo induced features, other ions were highlighted (Fig. 5). These ions correspond to features that were induced at one time point and that were produced at some stage of the experimental period by either *Fusarium* sp. or *A. clavatus*. This allowed identifying which of the two fungi had produced the metabolite. Four of these metabolites were produced by *Fusarium* sp. and five were detected in the pure culture of *A. clavatus* (Fig. 5).

These results revealed other induction patterns resulting from fungal co-culture. One particular pattern was the up-regulation of the production of a given metabolite by fungal co-culture. Feature PI585.391@3.15 was produced by *A. clavatus* under standard pure culture conditions, however, its production was clearly up-regulated during co-culture. The production of two other features (PI289.069@1.46 and PI286.070@1.85) by *Fusarium* sp. was highly up-regulated under co-culture conditions at D4. At the later time points (D7 and D9), these two features were not detected in the pure culture samples but their production was still evident in the co-culture sample. Therefore, they represent two examples of features that were highly up-regulated and that were induced over a longer time span in the co-culture compared to in the pure culture. Another feature displayed similar characteristics. NI151.039@1.28 was present in the co-culture from D4 to D9 and was detected in similar quantities only at D4 in the pure culture of *Fusarium* sp. Two other features that were detected in the pure culture of *A. clavatus* followed a similar pattern. PI194.153@0.77 and PI44.162@2.55 were both present in the pure cultures at D4 and D7 and in contrast, were detected in the co-culture extract only at D9. Interestingly, whereas this feature was produced only at D9 in the co-culture, it was produced at a comparatively lower concentration at D4 and D7 in the pure culture of *A. clavatus*.
Another induction pattern, the early production of specific metabolites in response to co-culture, was displayed by some of the features of interest. NI175.060@0.60, for example, was not detected in the pure culture of *Fusarium* sp. before D9 and was relatively up-regulated at D7 in the co-culture. The induced expression of this feature had begun by D4 because it was detected at a very low concentration at that point. Similarly, *A. clavatus* began to produce NI588.353@3.18 at D4 in the co-culture and later, at D7, in the pure culture. In addition, feature NI476.277@2.69 was detected already at D2 in the co-culture and was not observed in the pure cultures of *A. clavatus*. However, its concentration at D4 to D9 in the co-culture was reduced compared with that attained in the pure culture.

These differences in the induction pattern evolution over time appear to be metabolite-dependant, highlighting the complexity of metabolomic studies of microbial interactions that require integration of these dynamic issues. Using the strategy developed in this study, it has been shown that the dynamic of the induction of given metabolites observed as features were reproducible (*N* = 6). However, key biochemical events may occur at different points in time and the induced features may be present only transiently. Significant variations in the metabolite levels within a short time frame may explain why fungal metabolomic studies focusing on metabolite induction are known to be prone to variations and difficulties in terms of reproducibility.

**Dereplication of the selected metabolites**

The identification of the metabolites described above was beyond the scope of the current study; however, preliminary information was obtained through dereplication analysis based on the obtained HR-MS spectra. Molecular formulae were deduced based on exact mass accuracy, isotopic pattern matching, heuristic filtering and searches of the fungal metabolites included in the Dictionary of Natural Products. Multiple commonly detected adducts were considered to identify the features. When multiple results were found, a cross search based on the genus of the studied fungi (*Fusarium* and *Aspergillus* species) was performed. Only compounds that could be associated with fungi of the same genus or family were considered as...
putatively identified. For compounds produced by a pure strain with up-regulated expression in the co-culture, only the strain of origin was considered for the dereplication analysis. This strategy allowed drastically reducing the number of putative identifications (Fig. S7, ESI†) to only a few possibilities for each considered feature. Finally, five compounds were putatively identified and four features were not identifiable due to a large number of possible compounds (Table S1, ESI†). Nine peaks were putatively unannotated.

In the co-culture, gibepyrone F (NI151.039@1.28)42 and bostrycoidin (PI286.070@1.85)43 were produced by Fusarium sp. Fumiquinazoline C or D (PI444.162@2.55)44 was produced by A. clavatus. Two other de novo induced compounds were putatively identified as mitorubrin (NI427.103@1.44)45 and brefeldin A (PI265.178@2.55);46 based on phylogenic information, these two compounds were produced by A. clavatus. It is important to note that all the putatively assigned metabolites possess antimicrobial activities.42–44,47,48

Feasibility of up-scaled production for targeted isolation of de novo induced metabolites

Whereas several selected metabolites produced by the pure strains were dereplicated, the large majority of the de novo induced metabolites of interest in the present study could not be identified. This result emphasises the high potential of fungal co-cultures to generate novel NPs, but at the same time, renders the identification of such metabolites very challenging. Thus, a comprehensive de novo structural identification of such fungal metabolites and characterisation of their biological activities can be attained only through their targeted purification from the crude co-culture extract.48 Because the multi-well system described here yielded an average of only 5 mg of crude
extract per well (and the extracts contain large amounts of sugar), production at a larger scale is necessary to obtain at least microgram amounts of pure constituents for further characterisation. The co-culture condition for scale-up were investigated using large Petri dishes (15 cm) containing 60 times more growth medium and 14 times more growth space. UHPLC-TOFMS metabolite profiles of such a culture were obtained and compared to those obtained using the 12-well plate method.

At such a large scale, the growth rate of both fungi species was different from that at the small scale; A. clavatus grew slowly compared to Fusarium sp. Consequently, it was decided to inoculate Fusarium sp. in the middle of the Petri dish and to induce competition with four inocula of the second fungus, which were placed equidistant at the edges of the Petri dish (Fig. S1B, ESI†). This technique permitted replicating the species’ confrontation at different locations, which was expected to increase the concentration of the induced metabolites. After 24 days, the plate was almost completely covered by the fungi and the area covered by each fungus was similar (Fig. S8, ESI†); therefore, the growth was stopped. The culture medium was extracted using the same protocol as for the 12-well plates with adaptation of the volumes. Approximately 350 mg of large-scale extract was obtained from one large Petri dishes. After extract enrichment, PI and NI mode UHPLC-TOFMS analysis was conducted, the features that were the focus of the small scale study were searched by extraction of their specific ion chromatograms.

From the 18 metabolites of interest, 16 were detected in the extract of the large-scale co-culture. Only NI305.066@1.46 and NI476.277@2.69 were not observed in the large-scale UHPLC-TOFMS profiles. The induction patterns of these two features in the 12-well plates showed that their concentrations reached a maximum at D7 and D2, respectively, before decreasing during subsequent fungal growth. Their production may have followed a similar pattern in the large-scale co-cultures, which would explain their absence in the extract.

These results indicated that scaling-up the co-culture might allow the large-scale production of the induced metabolites of interest for further purification (three large Petri dishes producing 1 g of crude extract) and the identification of potentially novel fungal metabolites.

3. Conclusions

A multi-well procedure was devised to study fungal co-cultures based on 12-well plates containing 2 mL of growth medium using two model fungi. This strategy allowed obtaining rapidly a large number of replicates even with fungi having different growth rates (data not shown). Access to a sufficient number of replicates at different growth times provided a good way to confirm de novo metabolite induction phenomena with good confidence. The study of the induction of selected fungal metabolites in time series in such conditions showed the complexity of fungal interactions and highlighted how metabolomics experiments can tackle such issues. In the studied model co-culture, 18 metabolites were found to be de novo induced at some stage of the co-culture. The five features that could be putatively identified by dereplication correspond to compounds that have been reported to possess antimicrobial activities. This indicates that induction of cryptic pathways through co-culture is able to generate new NPs with interesting bioactivities, most likely related to defence.

The proposed strategy is applicable to different types of microorganisms and can be employed to study naturally occurring microbial interactions.59 This may therefore provide valuable data for the interaction mechanisms triggered by small molecules.

The method was reliably up-scalable to a large-scale 15 cm Petri dish format. Large amounts of crude extract can be obtained from cultures grown in multiple large Petri dishes (15 cm in diameter).50 This aspect is crucial for isolation of the metabolites of interest and their full structural and biological characterisation. This result suggested that even larger metabolite production could be accomplished using semi-industrial agar-supported solid-state fermenters.51 Notably, the generic 12-well plate strategy is also compatible with HPLC microfractionation coupled to biological testing for the early-stage identification of antifungal compounds52 because the amount of material generated for the metabolomics study (50 mg) are sufficient for the identification of induced metabolites with a high bioactivity potential at a very early stage. This strategy will therefore be applied in future co-culture studies for the rapid identification of antimicrobial compounds.

4. Materials and methods

Chemicals

The extraction were performed using methanol (HPLC grade, Fisher Scientific), dichloromethane (HPLC grade, VWR International), and nano-pure water (Millipore). The UHPLC-TOFMS analyses were performed using ULC/MS-grade acetonitrile and a mixture of water and formic acid (FA) purchased from Biosolve. PDA (Difco) was used as the culture medium.

Biological materials

Two fungi of very different origins were selected as models for this study. Aspergillus clavatus (Sin141) was isolated from soil. In contrast, the Fusarium sp. (PS54743) utilised was recovered from a blood sample of an immuno-compromised patient who was diagnosed with invasive fusariosis at the Geneva University Hospital. The two fungal strains were stored in the Agroscope ACW bank in vials containing a diluted potato dextrose broth solution (1 : 4) at 4 °C (http://mycoscope.bcis.ch/).

Multi-well culture and co-culture conditions

For optimisation, 1, 2, 3 or 4 mL of PDA was placed in the wells of 12-well plates (wells with a 2 cm internal diameter); 2 mL of PDA was selected for further experiments.

The two pure cultures, Sin141 and PS54743, were inoculated by placing 2 mm agar plugs of fungal pre-cultures in the centre of position A1-3 and B1-3, respectively, of a 12-well plate (Fig. S1A, ESI†).
Similarly, the co-cultures were prepared by inoculating two 2 mm agar plugs of pre-cultures of the two different fungal strains on the opposite sides of well C1-3. Wells A4, B4 and C4 were not inoculated and were used for the blank samples. The cultures were incubated at 21 °C for 3 days for studies of the effect of the volume of culture medium and for 2, 4, 7 and 9 days for studying the evolution of de novo metabolite induction.

Large-scale co-culture conditions

Large (15 cm) Petri dishes were prepared in triplicate using 120 mL of PDA. This volume corresponded to a 60-fold increase in the amount of growth medium and a 14-fold increase in the growth surface compared to those of the 2 cm wells. The plates were inoculated by placing one 5 mm agar plug of a pre-culture of Fusarium sp. in the center. In addition, four 5 mm agar plugs of a pre-culture of A. clavatus were placed equidistant at the edges of the Petri dish (Fig. SSB, ESIF). The Petri dishes were incubated at 21 °C for 24 days.

Extraction procedure for the small-scale samples

Extraction was performed according to previously described procedures, with adaptations to account for the reduced volume of the material to be extracted. Briefly, each fungal pure culture, fungal co-culture or non-inoculated agar sample was transferred to a glass tube and freeze-dried using a centrifugal evaporator (Genevac HT-4, SP Scientific). The extraction solvent mixture, dichloromethane–methanol–water 64:36:8 (v/v), was added (20 mL per 30 mg of dry material). In the case of the selected 2 mL volume of PDA used in the well of the 12-well plates, 4 mL of the extraction solvent mixture was used. The extractions were performed by sonication directly in the tubes in a water-bath sonicator (Ultrasonic Cleaver 5200, Branson Ultrasonics Corporation) at room temperature for 20 min. The sonicated samples were filtered through glass wool. The three sonicated samples were pooled and were dried under vacuum. Approximately 350 mg of extract was obtained from one 15 cm Petri dish.

Extraction procedure for large-scale co-culture samples

The large-scale co-culture samples were cut into 1 × 1 cm agar pieces using a razor blade and were freeze-dried (Freeze Dryer Alpha 2-4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH). The dry material was extracted three times by sonication with 750 mL of dichloromethane–methanol–water (64:36:8 (v/v)) in a water-bath sonicator at room temperature for 20 min. The sonicated samples were filtered through glass wool. The three extracts were pooled and were dried under vacuum. Approximately 350 mg of extract was obtained from one 15 cm Petri dish.

NMR profiling of fungal extracts

The extract was entirely dissolved in 600 μL of CD3OD and analysed using a Varian Unity Inova 500 MHz NMR instrument (Agilent Technologies) at 25 °C. 1H-NMR analysis was conducted using 64 transients at 302 K (29 °C). The spectra obtained were analysed using MestReNova software (version 8.0, Mestrelab Research S. L.), and the chemical shifts were referenced to the residual protonated solvent signal (CD3OD, 3.31 ppm).

UHPLC-TOFMS analysis

SPE was conducted to remove the most lipophilic compounds, which would ensure good repeatability of reversed-phase chromatography over a long series. Sample enrichment by classical SPE filtration was achieved using Sep Pak Vac SPE C18 cartridges (1 cc, 100 mg, Waters). Briefly, 1 mg of extract was dissolved in 1 mL of nano-pure water and eluted through the C18 cartridge. The extract was then eluted using methanol–water 85:15 (v/v) and the solution was adjusted to 1 mL.

UHPLC-TOFMS analyses were performed using a Waters Micromass-LCT Premier Time-of-Flight mass spectrometer that had an ESI interface coupled to an Acquity UPLC system (Waters). In separate runs, detection was achieved using both the PI and NI mode. The m/z range was set to 100–1000 in centroid mode with a scan time of 0.25 s and an inter-scan delay of 0.01 s. The ESI conditions in the PI and NI modes were as follows: capillary voltage of 2800 V, cone voltage of 40 V, source temperature of 120 °C, desolvation temperature of 250 °C, cone-gas flow of 20 L h⁻¹, and desolvation-gas flow 600 L h⁻¹. For internal calibration, a 5 μg mL⁻¹ solution of leucine-enkephalin (Sigma-Aldrich) was infused through the lock-mass probe at a flow rate of 5 μL min⁻¹ using a second Shimadzu LC-10ADvp LC pump.

UHPLC-TOFMS fingerprints were recorded with a 50 mm × 1 mm i.d., 1.7 μm Acquity BEH C18 UPLC column (Waters) in gradient mode at a flow rate of 0.3 mL min⁻¹ with the following solvent system: (A) 0.1% v/v formic acid (FA) in water; (B) 0.1% v/v FA in acetonitrile. The gradient was increased from 5% to 95% B in 4 min. The column was then washed for 0.8 min with 95% B, reconditioned to 5% B in 0.1 min and finally equilibrated with 5% B in 1.1 min. The temperature was maintained at 40 °C, and the injection volume was 1 μL of a 1 mg mL⁻¹ solution based on the extract content prior to SPE. The analyses were performed randomly and included quality control and blank samples after every 10 sample runs. The sample list was randomly generated using a dedicated Excel macro.

Peak picking and data analysis

Native MassLynx data (Waters) were converted into netCDF (common data format) using DataBridge software (Waters). Automatic feature detection was performed between 0.4 and 4.5 min with MZmine 2 software using parameters selected according to the TOFMS detector. Peaks with a width of at least 0.03 s and an intensity greater than 30 counts (both NI and PI) were picked with a 5 ppm m/z tolerance and the generated peak lists were deconvoluted. Deisotope filtering was applied using the “isotopic peaks grouper” module with tolerance parameters adjusted to 0.03 s and 5 ppm. Feature alignment and gap filling were achieved with a m/z tolerance of 15 ppm and a RT tolerance of 0.2 min. The features detected from blank samples and non-inoculated agar samples were removed from the generated matrix. The full procedure for feature detection is presented in Table S2 (ESI†).

To select de novo induced features, the ‘detected’ status (peak height over a certain threshold) of every feature was used. The features uniquely ‘detected’ in the co-culture replicates...
were selected (at least 5 times out of 6). A simple excel scripts was employed to explore the large data set generated through MS detection.\textsuperscript{30} Selected features were validated by exploration of the raw data using MassLynx. A particular attention was paid to adduct formed by two co-eluting compounds by searching manually for two molecular ions which may explain the formation of the selected features in the MS spectrum.

The statistical significance of the highlighted features was calculated using student's test to compare each pure culture and the co-culture data point. Only the \( p \)-value that corresponded to the comparison of the co-culture with the pure culture having the higher mean peak area for that specific feature was considered.

**Dereplication of the selected metabolites**

The dereplication process was based on the high mass accuracy molecular weights of ions detected using the TOFMS system. Only the \([M - H]^+\), \([M + HCO_2]^+\), \([M + H]^+\) and \([M + Na]^+\) adducts were considered.\textsuperscript{27} The molecular formulae of the selected ions were determined based on the exact mass accuracy, isotopic pattern matching and heuristic filtering.\textsuperscript{39,40} Finally, the molecular weight was searched in the Dictionary of Natural Products\textsuperscript{11} for positive matches among the chosen metabolites that were produced by *Fusarium* and *Aspergillus* sp. or by phylogenetically related fungi (based on the Catalogue of Life, Species2000, http://www.catalogueoflife.org/).

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**References**

Appendix VIII

High-throughput analysis of novel metabolites from co-cultured human pathogenic fungi using HPLC-MS offline coupled to microflow-NMR

Poster presentation at Trends in Natural Products Research, 11 – 14 April 2010, Leicester, United Kingdom

Nadine Bohni, Philippe J. Eugster, Gaétan Glauser, Guillaume Marti, Jean-Luc Wolfender

School of Pharmaceutical Sciences, University of Lausanne, University of Geneva, Geneva, Switzerland

Katia Gindro, Oliver Schumpp

Mycology group, Agroscope Changins ACW, Nyon, Switzerland
High-throughput analysis of novel metabolites from co-cultured human pathogenic fungi using HPLC-MS offline coupled to microflow-NMR

Nadine Bohni, Katia Gindro, Philippe Eugster, Olivier Schumpp, Gaëtan Glauß, Guillaume Martin, Michel Monod, Jean-Luc Wolfender

School of Pharmaceutical Sciences EPFL, University of Geneva, University of Lausanne, 30 Quai Ernest-Ansermet, 1211 Geneva 4, Switzerland

Mycoalexins. The screening of induced fungal metabolites is performed on fungi in confrontation directly at the Petri dish level using sensitive UHPLC-TOFMS and microflow NMR methods.

Human pathogenic fungi

Acremonium strictum and Fusarium oxysporum are one of the rare examples of being pathogenic for plants and for humans. Besides being responsible for 15% of all fungal nail infections and one of the main pathogens in persistent onychomycoses, species of Fusarium sp. were found to be the major opportunistic fungi in patients with severe immunosuppression. In addition, Fusarium onychomycoses are especially difficult to cure.

Stimulating metabolite production

Because several pathogenic fungi share the same ecological niche, they are expected to develop community interactions. By co-culturing different pathogenic fungi, we expect to stimulate the production of more and/or different metabolites (mycoalexins) [2]. This approach constitutes a source of novel natural products as preliminary results suggest [unpublished].

Results

A fungal crude extract was obtained by maceration in a saline solution to separate from peptidic components, followed by maceration in CHCl3/MeOH/H2O (64:36:8). The extract was further purified by SPE (80% MeOH) for the UHPLC analysis. For the microfractionation, the sample was sotubilised in 80% MeOH and filtered over a filter disk (0.45 μm).

By injecting 10 mg of crude extract (corresponding to three Petri dishes (130mm diameter), ~100 μg of substance was isolated. H-NMR confirms that the induced molecule is fusaric acid (5-Butylpicolinic acid).

Conclusion

This study clearly demonstrates that a strong induction of mycoalexin occurs in zone of confrontation between fungi at the Petri dish level. The technological platform used (chemical screening by UHPLC-TOFMS and identification by microflow NMR) provides a convenient way to characterise the fungal metabolites of interest and enable the isolation of enough material for further assays.

References


Aknowledgments

Thanks to Valerie Hofstetter for the phylogenetic analysis of the fungi. This work is founded by the SNF as a SINERGIA project (CRSII5_127187) to Prof. Jean-Luc Wolfender.)
Appendix IX

Rapid Identification of Mycoalexins from Human Pathogenic Fungi Using HPLC-UV-MS Offline Coupled to Microflow NMR at the Petri Dish Level

Poster presentation at SCS Fall Meeting, 16 September 2010, ETH Zurich, Switzerland

Nadine Bohni, Philippe J. Eugster, Jean-Luc Wolfender
School of Pharmaceutical Sciences, University of Lausanne, University of Geneva, Geneva, Switzerland

Katia Gindro, Oliver Schumpp
Mycology group, Agroscope Changins ACW, Nyon, Switzerland

Michel Monod
Departement of Dermatology and Venereology, Laboratory of Mycology, CHUV, Lausanne, Switzerland
Rapid identification of mycoalexins from human pathogenic fungi using HPLC-UV-MS offline coupled to microflow NMR at the Petri dish level

Nadine Bohn1, Katia Gindro2, Philippe J. Eugster1, Olivier Schumpp3, Michel Monod4, Jean-Luc Wolfender1

1School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Quai Ernest-Ansermet 30, 1211 Geneva, Switzerland
2Myology group, Agroscope Changins ACt, Route de Duinier, 1260 Nyon, Switzerland
3Service for Dermatology and Venereology, Laboratory of Mycology, CHUV, 1011 Lausanne, Switzerland

Introduction

Onychomycosis is the most prevalent nail disease. Although dermatophytes are the main cause of onychomycosis, *Fusarium* spp. and various other non-dermatophyte filamentous fungi are often isolated from abnormal nails. Because fungi are well known to release a large number of antimicrobial substances, the aim of this study is to investigate the interactions of different fungal species isolated from nails or from environmental sources and search for fungal metabolites specifically induced by the interaction (mycoalexins) that might be used as new antifungals.

Mycoalexins from human pathogenic fungi

Onychomycosis affects 2-18% of the population. While this disease is no life-threatening condition, current standard treatments are long and unpleasant. *Fusarium* spp. are responsible for 7.5% of onychomycoses, with *F. oxysporum* being the most prevalent strain and difficult to cure. With more people being immunocompromised, fungal infections of inner organs (e.g. lungs) become more serious.

Natural product discovery platform

**UHPLC-TOFMS profiling**

Fungi together with growth media are extracted by maceration. The extract is purified by means of solid phase extraction using 80% methanol (MeOH) and analysed by UHPLC-TOFMS. Mycoalexins are identified by metabolites profiling as compounds exclusively present in the confrontation zone and not in the pure cultures of the corresponding fungi.

**Microfractionation**

With the help of HPLC Calculator™ [3], the method used for the UHPLC analysis is adapted for different columns of the same chemical phase. Considering particle size and column dimensions, the gradient is upscaled to get a comparable separation. Thereby, the mycoalexin targeted by the UHPLC analysis could be isolated within one separation.

**Structure elucidation using CapNMR™**

In microflow NMR (CapNMR™ [4]), the sample is placed into a 5 µL flow cell in the middle of the magnet. The injection of the sample is automated with a LC autosampler and a solvent pump. Capillary tubing connect the benchtop autosampler/pump assembly with the microflow probe.

Conclusion

This study clearly demonstrates that a strong induction of mycoalexin occurs in the zone of confrontation between two fungal human pathogens interacting on artificial media. The technological platform used (chemical screening by UHPLC-TOFMS and identification by microflow NMR) provide an efficient way to characterise the fungal metabolites of interest and enable the isolation of enough material for further assays. Different fungal confrontations are presently screened by this means for the search of new antifungal agents and a better understanding of the interaction existing between fungal communities.

References & Acknowledgements


Thanks to Valerie Hoftetter for the phylogenetic analysis of the fungi. This work is funded by the SNF as a SINERGIA project (CRSII3_127187 to Prof. Jean-Luc Wolfender).
Appendix X

*In Planta* Quantification of the Fusarium Toxin Fusaric Acid by LC-MS/MS and Assessment of its Defence Induction Potential in Grapevine

Poster presentation at 27th International Symposium on the Chemistry of Natural Products (ISCPN27), 10 – 15 July 2011, Brisbane, Australia

Nadine Bohni, Jean-Luc Wolfender

School of Pharmaceutical Sciences, University of Lausanne, University of Geneva, Geneva, Switzerland

Olivier Schumpp, Nadia Bruderhofer

Mycology group, Agroscope Changins ACW, Nyon, Switzerland
In planta Quantification of the Fusarium Toxin Fusaric Acid by LC-MS/MS and Assessment of its Defence Induction Potential in Grapevine

Nadine Bohni, Olivier Schumpp, Nadia Bruderhofer, Jean-Luc Wolfender

School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Geneva, Switzerland

Agronomy group, Agroscope Châtenis ACW, Nyon, Switzerland

Introduction

Fusarium spp. are omnipresent microorganisms and present as saprobes in various environmental compartments, but also as endophytes in numerous plant species. Pathogenic and non-pathogenic Fusarium species produce the mycotoxin fusaric acid (FA). It has been shown that this Fusarium toxin induces plant defence in tomato, date palm, chickpea and model plants. FA can thus be considered as an elicitor.

The defense compounds of grapevine (Vitis vinifera) - mainly stilbenes - are well known. The leaves of Vitis vinifera were used as a model organism to study the defense response to the mycotoxin FA. To monitor the concentration of FA in the leaves, we developed a method to quantify this mycotoxin in plant samples. The leaves were therefore infiltrated with FA.

Fusaric Acid Quantification by LC-MS/MS

Sample preparation

Three small disks were cut out of the leaves and extracted with 100 µL of methanol (MeOH). 10% of the extract were eluted over a C18 SPE cartridge to remove highly apolar compounds. The dried sample was diluted to either 0.2 or 1 mL with H2O/acetonitrile (ACN) 95/5. The solutions were spiked with 6-Methylcopicolic acid to account for instrument variations.

Column choice for amphoteric compound

The amphoteric nature of the analyze (Fig. 3) leads to severe tailing of the chromatographic peak on most reverse phase columns (Fig. 1), a known problem for basic compounds.

![Column choice for amphoteric compound](image)

Time-dependent in planta concentration of FA

Samples from infiltrated leaves were taken immediately after FA exposure and after 24, 48 and 72 h. The concentration declines gradually with time (Fig. 4).

Analysis of FA-infiltrated leaf samples

Three leaves from Vitis vinifera cv. Chasselas were collected and subsequently infiltrated with 10 mM magnesium chloride (MgCl2) control, or with MgCl2 and FA and transferred into a humid chamber. Infiltration with FA concentrations higher than 1 mM lead to quick leaf necrosis. Infiltration with 1 mM leads to an effective concentration of 236 mM in leave tissues (42 ng/mg FW). Leave disks were collected at 0, 24, 48 and 72 h for phytoalexin (stilbene) analysis according to Pezet et al. [2] (Fig. 5, 6).

Conclusion & Outlook

We present a highly sensitive method for the quantification of the phytotoxin fusaric acid in plant samples. The compound could be quantified down to 200 pg per mg of fresh plant material using only minimal sample preparation. With this LC-MS/MS method, a Fusarium infection can be monitored and this allows to link in planta concentration of FA with the induction of plant defense compounds.

At non-toxic concentration, FA did not act as an elicitor in grapevine. Given that Fusarium species are natural endophytes, further investigations are ongoing to quantify the production of FA in various plant models were Fusarium species adopt an endophytic or pathogenic lifestyle.

References & Acknowledgements


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Appendix XI

*Hohenbuehelia reniformis* Extract is Active Against *Fusarium solani*

Poster presentation at SCS Fall Meeting 2012, 13 September 2012, ETH Zurich, Switzerland

**Nadine Bohni, Samuel Bertrand, Jean-Luc Wolfender**

School of Pharmaceutical Sciences, University of Lausanne, University of Geneva, Geneva, Switzerland

**Olivier Schumpp, Katia Gindro**

Mycology group, Agroscope Changins ACW, Nyon, Switzerland

**Michel Monod**

Departement of Dermatology and Venereology, Laboratory of Mycology, CHUV, Lausanne, Switzerland
Hohenbuehelia reniformis extract is active against human pathogenic Fusarium solani

Nadine Bohni, Olivier Schumpp, Samuel Bertrand, Katia Gindro, Michel Monod, Jean-Luc Wolﬀender

1. Introduction

Fungal nail infections, so called onychomycoses, aﬀect 2 - 18% of the population. While this disease is no life-threatening condition for immunocompetent people, current standard treatments are long and unpleasant. The fungi of the genus Fusarium are responsible for 7.5% of onychomycoses, but the prevalence of Fusarium spp. as causative agent of onychomycoses is rising and Fusarium spp. as well as other non-dermatophyte fungi appear to be insensitive to systemic standard treatments [1]. Hence, new antifungal agents active against Fusarium spp. are needed.

Additionally, in the growing population of immunocompromised patients, Fusarium spp. as well as other non-dermatophyte fungi appear to be insensitive to systemic standard treatments [1]. Hence, new antifungal agents active against Fusarium spp. are needed. Here, we present a new source of anti-Fusarium molecules: the fungus Hohenbuehelia reniformis.

2. Precipitation on polystyrene resin

Precipitation with the polystyrene resin HP20SS (Di-aion) [4] was successfully applied to remove a large amount of saccharides from the secondary metabolites. The adsorbed extract (2 g) was eluted with water to obtain the precipitation P1 that contains mainly glucose (3.0 – 4.0 ppm, 4.2 ppm (α-Glucose)) and fatty acids (CH2, 1.0 – 1.5 ppm).

3. Anti-Fusarium activity of microbial extract

The concentrated precipitation P3 containing aromatic and conjugated molecules and fatty acids was highly active against human pathogenic F. solani in a 96-well plate agar test [3]. The sugar precipitation P1 making up 73% of the extract mass does not exhibit antifungal activity.

4. Fractionation monitored by ELSD

The microbial extract contains many highly UV-active compounds but the major constituents don’t bear strong chromophores. Therefore, evaporative light scattering detection (ELSD) was chosen as detector for the isolation of the fungal metabolites with potential anti-Fusarium activity.

5. Example of identiﬁcation of isolated constituents by HRMS and NMR: microfraction P3-C2

The amount of constituent isolated was inferior to 0.3 mg which corresponds to < 0.15% of total extract mass. For the acquisition of 1H and 1H-1H cosy spectra (Figure 9), the sample was measured in a 3 mm micro NMR sample tube (Figure 6) that can be used with a conventional 5 mm inverse probe. The sample was solubilized in 150 μL of deuterated methanol (CD3OD).

6. Conclusion and perspective

Starting from 2 g of microbial extract, the biologically active constituents could be separated from the saccharides and concentrated in two prefractions. Therefore, several constituents were isolated in microgram quantities by semi-preparative HPLC-UV/ELSD. The use of a micro NMR sample tube permitted the acquisition of 1H and 1H-1H cosy spectra from these mass-selected samples.

For the shown example, the amount of isolated sample (< 0.3 mg) seems suﬃcient for the identiﬁcation of the molecule. The NMR data treatment is in process.

7. References and acknowledgment


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Appendix XII

Targeted Isolation of Biomarkers Highlighted by MS-based Metabolomics in Fungal Co-cultures

Poster presentation at 8ème journées scientifiques du RFMF, 19-21 May 2014, Domaine scientifique de la Doua, Villeurbanne (Lyon), France

Nadine Bohni, Florence Mehl, Samuel Bertrand, Jean-Luc Wolfender
School of Pharmaceutical Sciences, University of Lausanne, University of Geneva, Geneva, Switzerland

Olivier Schumpp, Sylvain Schnee, Katia Gindro
Mycology group, Agroscope Changins ACW, Nyon, Switzerland

Michel Monod
Departement of Dermatology and Venereology, Laboratory of Mycology, CHUV, Lausanne, Switzerland
Targeted isolation of biomarkers highlighted by MS-based metabolomics in fungal co-cultures

Nadine Bohn1, Olivier Schumpf1, Florence Meh1, Sylvain Schnee1, Samuel Bertrand1, Michel Monod2, Katia Gindro3, Jean-Luc Wolfender1

1School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Geneva, Switzerland
2Mycology and Biotechnology group, Institute for Plant Production Sciences, Agroscope, Nyon, Switzerland
3Department of Dermatology and Venereology, Laboratory of Mycology, CHUV, Lausanne, Switzerland

The prevalence of Fusarium spp. as causative agent of onychomycoses is rising and Fusarium spp. as well as other non-dermatophyte fungi appear to be insensitive to systemic standard treatment [1]. Hence, new antifungal agents active against Fusarium spp. are needed. The concept of microorganism co-culture, the combined growth of two or more microorganisms [2], was chosen to uncover possible anti-Fusarium compounds from the Basidiomycete Hohenbuehelia reniformis cultured with human pathogenic Fusarium solani.

3. PCA and two OPLS-DA models: score plots

4. Share-and-unique (SUS) plot

5. Targeted UHPLC-UV metabolite profiling of red pigments

6. Targeted isolation of biomarkers

7. Conclusion and perspective

Twenty compounds were isolated from the co-culture extract and identified using extensive spectroscopic analyses based on NMR and HRMS. Seven known pigments were isolated and it could be shown that their upregulation in the co-culture is responsible for the colorization of the culture medium. Among others, 7 novel congeners of leucopelletroin and dihydropeletroin acids were isolated. Leucopelletroin exhibits moderate fungitoxic activity against F. solani which might explain the observed distance repulsion in the co-culture. In total, seven features were identified from the OPLS-DA. The developed detection workflows permit the study of interacting fungi, and microorganisms in general, on the molecular level. This might help in gaining insight into the complex processes within communities of fungal strains that share the same confined space (microbiomes).

8. References and acknowledgments


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