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Reference

DOI: 10.1016/S0045-6535(03)00296-0

Available at:
http://archive-ouverte.unige.ch/unige:3699

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Short Communication

On the sedimentary occurrence of chlorophyllone a

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Received 6 May 2002; received in revised form 10 March 2003; accepted 12 March 2003

Abstract

The 132,173-cyclopheophorbide a enol (CPP) is shown to convert mainly to a ~1:1 mixture of (132–R/S) chlorophyllones a (Chlone), when chromatographed over silica gel or alumina supports. 15′-hydroxychlorophyllonelactone a and some other chlorophyll a related compounds are also tentatively identified as minor transformation products of CPP. This raises the possibility that the chlorophyllones reported in recent sediments may be analytical artifacts from CPP. However, data for the surface sediments from Lake Motte as well as literature data for other contemporary sediments show that, (i) they are not artifacts, (ii) considering that CPP is the intermediate compound in the formation of chlorophyllones from chlorophyll a, the hydroxylation of CPP in the sedimentary environment involves an enzymatic process leading preferentially to 132–S chlorophyllone a.

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Keywords: Chlorophyll a diagenesis; Cyclopheophorbide a enol; Oxidation; Chlorins; Chlorophyllone a; HPLC; APCI-MS

1. Introduction

Tetrapyrrolic pigments are commonly used as biomarkers to determine the sources and transformation pathways of organic matter in the water column and sediments (Baker and Louda, 1983; Filby and Van Berkel, 1987; Callot et al., 1990). Pheopigments, chlorophyll degradation products including pheophytsins, pheophorbides and pyro-analogues, generally represent the dominant pool of tetrapyrrolic pigments in contemporary sediments (e.g. Keely and Brereton, 1986; Woolley et al., 1998; Chen et al., 2001). These compounds are produced by reactions involving demetallation, hydrolysis of phytol ester, and decarboxmethoxylation of chlorophylls. In recent years however, we have identified another type of chlorin as a major transformation product of chlorophyll a (Chl, Fig. 1, 1) in the surface sediments of an eutrophic lake, namely chlorophyllone a (Chlone, 2) (Chillier et al., 1993). This compound was subsequently found in many other lacustrine and marine sediments (Harris et al., 1995a; Cariou-Le Gall et al., 1998; Kowalewska et al., 1999; Villanueva and Hastings, 2000; Airs et al., 2001) and considered to be the link between chlorophyll a and the bicycloalkanoporphyrin (3) found in older sediments (Keely et al., 1994; Harris et al., 1995b).

Before it was found in sediments, Chlone a had been isolated from a marine clam (Sakata et al., 1990). Another compound with close structural resemblance to Chlone a, 132,173-cyclopheophorbide a enol (CPP, 4) had also been isolated and characterized from a marine sponge (Karuso et al., 1986). CPP, probably a metabolic intermediate of Chlone a, was recently proved to be widely distributed in sediments by three different groups (Goericke et al., 2000; Louda et al., 2000; Ocampo et al., 2000). However, all three reports pointed out the instability of CPP. Ocampo et al. (2000) reported that CPP was almost completely destroyed when chromatographed over normal phase silica gel or alumina. Degradation occurred even on RP-HPLC columns when mobile phase contained antioxidants or buffer solutions.
Goericke et al. (2000) observed that CPP was also degraded in organic solvents, more particularly in the presence of air. Main degradation products were the two chlorophyle a isomers (chlone a and chlone a‘ in approximately equal amounts) and 132-oxopyropheophorbide a (oxoPPde a, 5). Finally, Louda et al. (2000), taking into account these results, concluded that many of the chlorophyllones reported in the literature could be artifacts, if the analytical procedure includes a chromatographic step on silica gel.

In this note we shall present data to demonstrate that chlorophyllone a found in the sediments of Motte Lake is not an analytical artifact.

2. Experimental

2.1. Materials and methods

132,173–cyclopheophorbide a enol was prepared from pyropheophorbide a methyl ester (6, Sigma, 95%) according to published methods (Falk et al., 1975; Ma and Dolphin, 1996; Ocampo et al., 2000), using sodium bis(trimethylsilyl) amide (Fluka, 1 M in THF) and sodium phosphate (Aldrich, 98%).

Extraction and separation of sedimentary pigments were done according to published procedures (King and Repeta, 1991; Chillier et al., 1993). In brief, approximately 500 g of wet sediment (containing about 80% water) was extracted ultrasonically (5 min) with four portions of acetone (250 ml) followed by three portions of methylene chloride (250 ml). The combined extracts were concentrated to 200 ml and the same volume of a solution of hexane/diethyl ether (30:70 v/v) was added. After elimination of the aqueous phase, the organic extract was dried over Na2SO4 and the solvents were removed under a nitrogen stream, leaving about 100 mg of a dark brown residue. The crude extract was submitted to preparative normal phase TLC (Merck, silica gel, 60, 0.5 mm). The glass plates were previously washed with acetone and activated 2 h at 120 °C. Elution was done...
with 25:75 v/v acetone/hexane mixture, using pyropheophorbide a methyl ester (6) as reference compound. The band at \(0.16 \leq R_f \leq 0.47\) was collected, extracted with acetone and analyzed by liquid chromatography.

HPLC analyses were performed with a Merck Hitachi system equipped with a Rheodyne 7125 Injector fitted with a 20 \(\mu\)l Loop and an Alltech Adsorbosphere HS RP-18 (150 \(\times\) 4.6 mm, 3 \(\mu\)m) column at a flow rate of 1.5 ml/min. Solvents were (A) methanol containing 20% (v/v) 0.5 M aqueous ammonium acetate and (B) acetone:methanol (80:20 v/v). The gradient was (time, A%): 0 min, 80%; 27 min, 5%; 34 min, 5%; 36 min, 80%. Either a L-4000 UV–visible detector, or a L-4500 Diode Array detector (300–800 nm) was used for detection. All solvents (Merck) used for liquid chromatography were HPLC-grade.

HPLC/MS was carried out by coupling the Hitachi HPLC system to a SSQ 7000 Finnigan MAT via an APCI interface. The interface conditions were: sheath gas (nitrogen) pressure 40 psi (no auxiliary gas), vaporizer temperature 370 °C, ion transfer capillary temperature 200 °C, corona electrode 5 \(\mu\)A. Spectra were recorded in positive and negative ionization modes with the capillary voltage +5 and −3 kV respectively. CID offset was set off in both modes.

3. Results and discussion

As pointed out by Goericke et al. (2000), HPLC separation of CPP using ‘traditional’ methods applied to tetrapyrrolic pigments is not possible. For example, on a Merck RP-18 column (Lichrosphere, 250 \(\times\) 4 mm) using binary (acetone/methanol) or ternary (acetone/methanol/water) solvent systems as we use in our laboratory (Verzegnassi et al., 1999; Riffé-Chalard et al., 2000) CPP was almost undistinguishable from the baseline. With a shorter column (Alltech RP-18 Adsorbosphere, 150 \(\times\) 4.6 mm, 3 \(\mu\)m), and using a solvent system and gradient (see Section 2) similar to those reported by Goericke et al. (2000), we have been able to obtain a satisfactory peak shape.

The chromatogram of the synthetic CPP (eluting at 21.6 min) is shown in Fig. 2. The APCI-MS of CCP shows base peak at \(m/z\) 517 in positive mode and \(m/z\) 516 in negative mode corresponding to the protonated \([M + H]^+\) and the radical anion \(M^-\) species respectively. The electronic spectrum of CPP (Fig. 3) recorded on a DAD, is similar to the one reported by Louda et al. (2000) with maxima at 361, 414 and 668 nm.

Superimposed on the chromatogram of synthetic CPP, is the chromatogram of the extract obtained after its chromatography on silica gel (20 \(\times\) 0.6 cm; eluent: methanol:methylene chloride 75:25 (v/v)). Almost complete disappearance of CPP is accompanied by the appearance of several earlier eluting components. HPLC/MS analysis of this mixture combined with on-line UV/vis spectra allowed the identification of the major components as shown in Table 1.

In APCI negative ion mode all compounds lead to abundant molecular anions \(M^-\) and, as expected with free base chlorins, no significant fragment ions could be observed (Verzegnassi et al., 2000). In positive ion mode, however, besides the prominent protonated molecular ions \([M + H]^+\), some fragments originating from the loss of peripheral substituents are often observed (e.g. Harris et al., 1995a; Verzegnassi et al., 1999).

The two major components eluting at 8.5 and 9.1 min were identified as the two chlorophyllone isomers 2\((\mathcal{S})/2\mathcal{R}\), in approximately 1:1 ratio, on the basis of their...
The component eluting at 12.1 min is either 13α-oxopyropheophorbide a methyl ester (50) or chlorophyllonic acid a methyl ester (70). Its APCI(+)−MS exhibits, besides the protonated molecular ion at \( m/z = 563 \), a minor ion at \( m/z = 503 \) generated by the loss of the carbomethoxy group together with a hydrogen atom ([M+H]C2H4O2)þ, a common fragmentation pathway of −COOMe containing chlorins (Chillier et al., 1994; Harris et al., 1995a; Chi Jie et al., 2002). It should however be noted that pyropheophorbide α methyl ester (6), with the carbomethoxy group in an analogous position as in compound 50, does not show this loss in our MS conditions. Although this pleads for the structure 70, a conclusive identification cannot be made without comparison with authentic samples since it is also possible that the \( m/z = 503 \) ion is not associated with MHþ at \( m/z = 563 \).

The mass spectra of the compound eluting at 15.5 min show the [M + H]þ ion at \( m/z = 549 \) and M− at \( m/z = 548 \), suggesting incorporation of an oxygen atom in chlorophyllone a. A minor fragment ion at \( m/z = 517 \) [M + H − 32]þ is also present. The data could be compatible with the 13α-oxopyropheophorbide a (5) but 5 should elute around 9 min in the HPLC conditions we used (c.f. Goericke et al., 2000). On the basis of retention time and UV/vis spectrum, we tentatively identify this compound as 15α-hydroxychlorophyllone lactone (8) previously reported by Watanabe et al. (1993) in marine bivalves, together with 2(S or R), and other oxidized chlorophyll a derivatives such as chlorophyllonic acid 7 and purpurin 18 (9) as well as its methyl ester (9).

The component (10) eluting at 16.6 min is an unidentified chlorin of molecular weight 596 as suggested by its MS data.

The chromatography of CPP in similar conditions but using an alumina support yielded the same components. Although the relative amounts of the minor products were different, 2(R/S) in a ∼1:1 ratio was again the major transformation product. When we repeated the chromatography on silica gel using different solvent mixtures (MeOH/CH2Cl2/acetone) and flow-rates, we again observed the same results, i.e. same components with different relative amounts, but 2(R/S) major and ∼1:1 ratio.

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>MW</th>
<th>APCI(+)</th>
<th>APCI(−)</th>
<th>( λ_{\text{max}} ) (nm) (DAD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2(S)</td>
<td>8.5</td>
<td>532</td>
<td>533 (100)</td>
<td>515 (34)</td>
<td>532 (100) 409, 503, 668</td>
</tr>
<tr>
<td>2(R)</td>
<td>9.1</td>
<td>532</td>
<td>533 (100)</td>
<td>515 (38)</td>
<td>532 (100) 404, 512, 668</td>
</tr>
<tr>
<td>5′ or 7′</td>
<td>12.1</td>
<td>562</td>
<td>563 (100)</td>
<td>503 (10)</td>
<td>562 (100) 400, 512, 668</td>
</tr>
<tr>
<td>8</td>
<td>15.5</td>
<td>548</td>
<td>549 (100)</td>
<td>–</td>
<td>548 (100) 400, 521, 668</td>
</tr>
<tr>
<td>10</td>
<td>16.6</td>
<td>596</td>
<td>597 (100)</td>
<td>–</td>
<td>596 (100) 404, 512, 650</td>
</tr>
<tr>
<td>4</td>
<td>21.6</td>
<td>516</td>
<td>517 (100)</td>
<td>–</td>
<td>516 (100) 361, 424, 668</td>
</tr>
</tbody>
</table>
From these results it is obvious that abiotic transformation of CPP leads mainly to chlorophyllones $R/S$ in a $\sim 1:1$ ratio, although thermodynamic calculations using molecular mechanics (HyperChem MM+), semi-empirical (HyperChem AM1) or ab initio methods (Gaussian98-HF-STO-3G) indicate that the thermodynamic stability of $2S$ is higher than that of $2R$ by 16–24 kJ/mol. Therefore, the abiotic oxidation of CPP to chlorophyllones is under kinetic control rather than thermodynamic reaction control and no interconversion $2S/2R$ is operative. On the other hand, data on the occurrence of the chlorophyllones in marine bivalves (Watanabe et al., 1993) as well as in copepod pellets (Talbot et al., 1999, 2000) show that either one of the isomers is present or largely predominant due to the stereospecificity of the enzymatic reaction.

The HPLC chromatogram of the green pigment fraction isolated from a surface sediment sample of the Motte lake is reproduced in Fig. 4. This fraction was obtained using the procedure described in our original paper (Chillier et al., 1993), i.e., including a TLC separation step on silica gel. The large predominance of $2S$ over $2R$ clearly indicates that, if not all, at least the major part of the sedimentary chlorophyllone is not an analytical artifact.

It is noteworthy that, when available, the pigment chromatograms showing the presence of chlorophyllones ($S/R$) in sediments from other lacustrine and marine environments are also characterized by a high predominance of $2S$ over $2R$ (Cariou-Le Gall et al., 1998; Kowalewska et al., 1999; Villanueva and Hastings, 2000; Airs et al., 2001). Finally, as already mentioned, copepod pellets exhibit also $2S/2R$ pair with isomer $S$ much more abundant (Talbot et al., 1999, 2000). This, in parallel to the sedimentary distribution, suggests that the main origin of sedimentary chlorophyllone is an enzymatic process due to the zooplankton herbivory. However, we should also note that $2S$ has also been reported in a diatoms mixture (Watanabe et al., 1993). Moreover, Talbot et al. (2000) observed the appearance of $2S/2R$ in a dinoflagellate culture after 48 h even in the absence of grazing copepods, once again with a large predominance of $2S$. It is therefore possible that endogenous algal enzymes may also be responsible for the occurrence of sedimentary chlorophyllones.

4. Conclusion

Abiotic transformation of CPP on normal phase chromatography yields mainly Chlone $a$ as a $\sim 1:1$ mixture of two epimers at C-13$^2$. On the other hand, when chlorophyllone is produced by the enzymatic transformation of chlorophyll $a$ by living organisms, such as marine bivalves or zooplankton feeding on phytoplankton, only one of the isomers is present or largely predominant. Chlorophyllone in Motte lake sediments, with major $13^2(S)$ isomer, is therefore not an analytical artifact but originates from a biogenic input.

Acknowledgements

The authors acknowledge the constructive reviews of two anonymous referees. This work was supported by the Fonds National Suisse de la Recherche Scientifique (Grant nos. 20-57201.99 & 20-063779.00).
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