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Reference

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Cell-Penetrating Dynamic-Covalent Benzopolysulfane Networks

Yangyang Cheng, Lili Zong, Javier López-Andarias, Eline Bartolami, Yasunori Okamoto, Thomas R. Ward, Naomi Sakai, and Stefan Matile*

Abstract: Cyclic oligochalcogenides (COCs) are emerging as promising systems to penetrate cells. Clearly better than and different to the reported diselenolanes and epidithiodiketopiperazines, we introduce the benzopolysulfanes (BPS), which show efficient delivery, insensitivity to inhibitors of endocytosis, and compatibility with substrates as large as proteins. This high activity coincides with high reactivity, selectively toward thiols, exceeding exchange rates of disulfides under tension. The result is a dynamic-covalent network of extreme sulfur species, including cyclic oligomers, from dimers to heptamers, with up to nineteen sulfurs in the ring. Selection from this unfolding adaptive network then yields the reactivities and selectivities needed to access new uptake pathways. Contrary to other COCs, BPS show high retention on thiol affinity columns. The identification of new modes of cell penetration is important because they promise new solutions to challenges in delivery and beyond.

Benzopolysulfanes (BPS) are cyclic oligochalcogenides (COCs) characterized by large rings of sulfur atoms fused to a benzene ring. Dominant are pentasulfides as in 1-3, i.e., BPS₅, also referred to as pentathiepins (Figure 1). They occur as natural products—with the dopamine-derived varapines, we introduce the benzopolysulfanes (BPS), which show efficient delivery, insensitivity to inhibitors of endocytosis, and compatibility with substrates as large as proteins. This high activity coincides with high reactivity, selectively toward thiols, exceeding exchange rates of disulfides under tension. The result is a dynamic-covalent network of extreme sulfur species, including cyclic oligomers, from dimers to heptamers, with up to nineteen sulfurs in the ring. Selection from this unfolding adaptive network then yields the reactivities and selectivities needed to access new uptake pathways. Contrary to other COCs, BPS show high retention on thiol affinity columns. The identification of new modes of cell penetration is important because they promise new solutions to challenges in delivery and beyond.

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Biotinylated and TEGylated BPS, 2 and 3 were prepared correspondingly. ETP, 4 was prepared from ethylamine 12, which was converted into heterocycle 13 as described.\textsuperscript{11} Methanolation of thioesters followed by treatment with \(\text{S}_2\text{Cl}_2\)\textsuperscript{18} removal of the tert-butyl ester in 14, and reaction with FL–NH\(_2\) yielded ETP, 4. A close congener of ETP, \(\text{S}_2\text{Cl}_2\), ETP, 6 was newly prepared from amine 15, also to explore the advantages of a phenyl group during synthesis.

Cellular uptake of COCs into HeLa Kyoto cells was monitored by both flow cytometry and confocal laser scanning microscopy (CLSM). The flow cytometry data (Figure 1a) were evaluated considering the different degree of fluorescence quenching by intact or reduced COCs (Supporting Information, Figures S1, S2, S6, and Table S1).\textsuperscript{12} Independent of any corrections applied, the uptake of BPS, 1 exceeded all other COCs. With correction, BPS, 1 was approximately 10-times more active than the previous best in the sulfur series, ETP, \(\text{S}_2\text{Cl}_2\)\textsuperscript{11} and approximately 140-times more active than the best explored asparagus acid (AspA) derivative \(\text{S}_2\text{Cl}_2\).\textsuperscript{19} Tetrasulfide ETP, 4 showed less uptake than disulfide ETP, 5. This result demonstrated that simple oligomer effects in ring-expanded COCs fail to explain the power of BPS, 1. The approximately 2.4-times higher activity of new phenoxethyl ETP, 6 compared to ethyl ETP, 5 suggested that the known contributions of aromatic rings to cellular uptake\textsuperscript{19} might also apply to COC-mediated uptake. However, the inactivity of control 8 confirmed that contributions from such secondary ion–π interactions at the membrane–water interface\textsuperscript{19} to the activity of BPS, 1 are almost negligible.

CLSM images confirmed that BPS, 1 is more active than all other COCs (Figure 1b–g). Concentration and time dependence analysis revealed binding to the plasma membrane with efficient delivery to the cytosol and particularly nucleus within one hour (Supporting Information, Figures S4 and S5). Many interpretations are possible for reduced activity at 4°C, including hindered endocytosis, decelerated oligochalcogenide exchange kinetics, or membrane stiffening (Supporting Information, Figure S6). Insensitivities toward several inhibitors indicated the absence of uptake by clathrin-mediated endocytosis (chlorpromazine), caveolaemediated endocytosis (methyl-β-cyclodextrin), and macro-pinocytosis (wortmannin, cytochalasin B; Supporting Information, Figure S7).\textsuperscript{19,21} A drop in activity to 70% upon preincubation with 2 mM Ellman’s reagent (DTNB) supports contributions from thiol-mediated dynamic covalent oligochalcogenide exchange\textsuperscript{10–14} to the uptake of BPS, 1 (Supporting Information, Figure S8). According to the MTT assay, none of the tested COCs were cytotoxic under experimental conditions (Leibowitz, 24 h, concentrations up to 50 μM; Supporting Information, Figure S9).

Compared to other COCs, benzopolysulfanes offer different reactivity, culminating in ring contraction and expansion from trisulfides to nonasulfides, i.e., 1–3, 16–21.\textsuperscript{19} reminiscent of elemental sulfur \(\text{S}_n\), with pentasulfides 1–3 being clearly preferred, followed by trisulfides 16 (Figure 2a,c and Supporting Information, Figure S38).\textsuperscript{1–3} The mechanism of these reversible interconversions remains under debate, with transient ring opening by traces of nucleophilic impurities the most likely explanation.\textsuperscript{1–3,6,21} BPS chemistry further includes sulfur replacement, nucleophilic displacement and oxidation, radicals, metal coordination, and photochemistry\textsuperscript{1–3} presumably much influenced, if not determined by the strings of electrophilic \(\sigma\) holes next to nucleophilic lone pairs on the lined-up sulfur atoms.\textsuperscript{22} The low \(pK_a\) values of thiophenols and persulfides facilitate ring opening to give interconvertible reactive intermediates, like RI-1 and RI-2, with preserved reactivity even in slightly acidic water. With ETP, 5 and diselenolanes, such less basic thiolates and selenolates were thought to account for mobility, i.e., their hypothetical mode of action as molecular walkers, walking along transmembrane disulfide tracks in membrane proteins.\textsuperscript{10} In a neutral, deuterated phosphate buffer, the \(^1\text{H}\) NMR spectrum of BPS, 3 remained unchanged at least for two weeks (Figure 2b: BPS, have nearly identical spectra). In the HPLC, equilibration with BPS, 16 was detectable within hours (Figure 2e and Supporting Information, Figure S22 and S38). In the presence of thiol (dithiothreitol, DTT, and glutathione 22, GSH), BPS, 3 transformed rapidly into multicomponent mixtures, with \(^1\text{H}\) NMR signatures changing with the substrate, time, and pH (Figure 2c–d and Supporting Information, Figures S10–S17).

According to HPLC analysis combined with low- and high-resolution mass spectrometry (MS), the reaction of BPS, 1 with GSH affords a dynamic-covalent network that includes unprecedented cyclic oligomers 23, from dimers 23, to heptamers 23, with up to nineteen sulfur atoms in a macrocycle of thirty-three atoms, besides the expected di- and mono-GSH-BPS, 2 conjugates 24 and 25, and reduced BPS, 26 (Figure 2f and Supporting Information, Figures S24 and S39–S46). The identification of these large cyclic BPS oligomers was interesting because oligomer effects have been shown to account for thiol-mediated uptake with ordinary disulfides.\textsuperscript{14,23} In contrast, AspA 7 remained intact even with large excess of GSH (Supporting Information, Figure S36). This very important difference in reactivity was consistent with the weaker uptake activity of AspA, thus supporting that dynamic-covalent networks matter for the mode of action of BPS. In agreement with the dynamic nature, the composition of the product library from BPS, 1 and GSH was altered by the subsequent addition of disulfides (GSSG, Figure 2g and Supporting Information, Figure S27; lipoic acid, Supporting Information, Figures S29 and S48). Although less efficiently, BPS networks also formed with only disulfides (GSSG,
DTNB, and lipoic acid. Supporting Information, Figures S18–S21, S25–S26, and S28), probably catalyzed by trace amounts of thiol impurities. The selectivity of the adaptive dynamic-covalent BPS network was exemplified by the inability of amines to influence the situation (Supporting Information, Figures S30–S35).

The dynamic-covalent networks\textsuperscript{[16,17]} of extreme sulfur species obtained from BPS 5 caused strong retention on thiol exchange affinity columns (Figure 3c vs. 3a,b). Release after addition of DTT to the mobile phase exceeded initial elution by far and was unusually slow, continuing far beyond three hours (Figure 3c, solid). Also, the application of reducing conditions in the presence of DTT led to slow elution over more than three hours (Figure 3c, dashed). These complex chromatograms were in sharp contrast to the signatures of \( \text{AspA}^7 \) and \( \text{ETP}^4 \) (\( \text{Sn} = 2 \)). Some permanent retention of dithio-27 until clean release with DTT was in agreement with its dominant endosomal capture (Figures 3a and 1c)\textsuperscript{[10]} Negligible retention of ring expanded \( \text{ETP}^4 \) as well as \( \text{ETP}^2 \) and \( \text{ETP}^5 \)\textsuperscript{[12]} was in agreement with dynamic-covalent walking\textsuperscript{[13]} along thiol and disulfide tracks, through affinity columns and into the cytosol and nucleus (Figures 3b and 1d–f). The complementary behavior of tetrasulfide \( \text{ETP}^4 \) and pentasulfide \( \text{ETP}^1 \) on columns and in cells supported that the high activity of the latter is not a general property of oligosulfides but specific for the dynamic-covalent networks produced by BPS 5 (Figures 3c,b and 1f,g). Further supporting the importance of the dynamic-covalent BPS network for function, preliminary observations suggest that increasing concentration of thiols in the media increase, rather than decrease, BPS-mediated uptake.

BPS-mediated delivery of proteins was probed first by the bioorthogonal uncaging of rhodamine \( \text{AspA}^7 \) by artificial metalloenzyme \( \text{ETP}^4 \) within HeLa Kyoto cells (Figure 4 and Supporting Information, Figures S49 and S50).\textsuperscript{[13,24]} In this reaction, protein-activated organometallic ruthenium complexes as in 29 cleave the allylcarbonyl protecting groups in the non-fluorescent substrate \( \text{AspA}^7 \) and liberate the fluorescent amine \( \text{ETP}^4 \). The cell-penetrating deallocase \( \text{ETP}^4 \) was prepared by adding ruthenium complex 29 and biotinylated BPS 5 to a streptavidin tetramer. Incubation of HeLa Kyoto cells first with cell-penetrating metalloenzyme \( \text{ETP}^4 \) and then, after washing, with the more hydrophobic, freely diffusing substrate \( \text{ETP}^4 \) resulted in the intracellular emission from the intracellularly uncaged fluorophores \( \text{ETP}^4 \). Emission intensities increased with increasing concentration of \( \text{ETP}^4 \), while the BPS-free control enzyme \( \text{ETP}^4 \) did not cause fluorescence inside of...
As a second approach to deliver proteins, we designed a cell-penetrating streptavidin (CPS) 32 with all four biotin binding sites available for different substrates. Such constructs are desirable to fully exploit the streptavidin–biotin binding sites available for different substrates. Such constructs are desirable to fully exploit the streptavidin–biotin binding sites available for different substrates. Such constructs are desirable to fully exploit the streptavidin–biotin binding sites available for different substrates.

In summary, we report that benzopolysulfanes mediate uptake into cells, better than all COCs explored so far. This activity is shown to originate from their transformation into adaptive dynamic-covalent networks of extreme sulfur species, including cyclic oligomers with up to nineteen sulfurs in the macrocycles, for selection and possibly amplification of the best. These dynamic-covalent BPS networks show high reactivity, high selectivity, and strong retention by thiols. While dynamic-covalent chemistry has been explored for cellular uptake, including examples also with imines, hydrazones, and boronic esters, high-affinity adaptive networks evolving in situ represent a new concept for thiol-mediated uptake of COCs and beyond. This is of interest because conceptually new ways to enter into cells have the intrinsic potential to, by acting differently, provide solutions for uptake problems that are otherwise intractable. The unusual nature of the identified dynamic-covalent BPS network in particular could be worth considering also with regard to templated amplification for functions beyond cellular uptake that involve distance-sensitive multivalency.[27]

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Conflict of interest

The authors declare no conflict of interest.

Keywords: adaptive networks · cellular uptake · cyclic oligochalcogenides · dynamic-covalent chemistry · polysulfanes

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One ring to rule them all: Benzopolysulfanes are introduced as the “lord of the rings” with regard to cell-penetrating cyclic oligochalcogenides, acting through an intriguing, in situ generated dynamic-covalent network of extreme sulfur species, including oligomers with up to nineteen sulfur atoms in one macrocycle, that excels with very high reactivity, selectivity as well as affinity to thiols.