



Cell-Penetrating Dynamic-Covalent Benzopolysulfane Networks

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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.^[1–3] Dominant are pentasulfides as in **1–3**, i.e., BPS₅, also referred to as pentathiepins (Figure 1).^[1–3] They occur as natural products—with the dopamine-derived varacin from tunicates probably best known—, have attracted attention as a challenge in total synthesis,^[4] and appeared as top hits from the screening of large libraries for targets in the brain of living animals.^[5] Benzopolysulfane rings are not very strained (XSSX dihedral angles $\geq 72^\circ$),^[6] but excel with

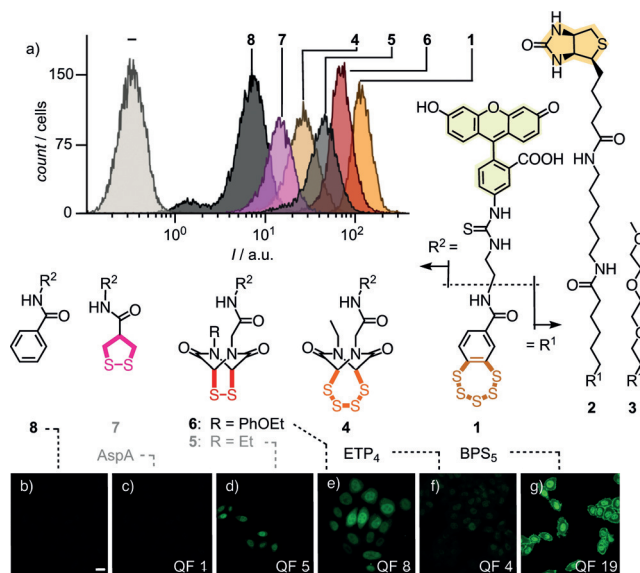


Figure 1. Cellular uptake of BPS₅ **1** into HeLa Kyoto cells compared to **4–8**. a) Selected original flow cytometry data (20 μ M, 45 min, Leibovitz's medium). The position of the compound numbers reflects the approximate fluorescence intensities after correction using quenching factors (QFs) of reduced COCs (note the log scale). b–g) Uncorrected CLSM images (10 μ M, scale bar: 10 μ m; laser power (LP): 15 %, with QFs of reduced COCs for correction if desired). Previously reported COCs are in gray.^[10,11]

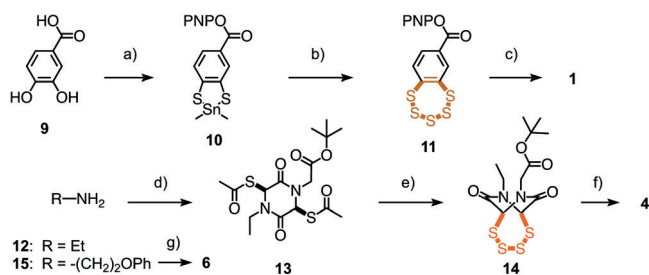
unique, complex chemistry.^[1–7] This extreme sulfur chemistry attracted our attention in our search for new ways to enter cells. From cell-penetrating peptides (CPPs)^[8] to cell-penetrating poly(disulfide)s (CPDs)^[9] and COCs,^[10–13] the cellular uptake via dynamic covalent dichalcogenide exchange chemistry has come to prominence, often referred to as thiol-mediated uptake, with exofacial thiols as initial targets and continuing on the way into the cell.^[10–15] Promising results with strained cyclic disulfides^[10,11] and diselenides^[12,13] called for a shift of attention to the extreme sulfur chemistry of higher oligochalcogenides.^[1] We here report that BPS₅, for example, **1**, outperforms all known COCs, and, most importantly, that BPS₅ penetrate cells in a new way, i.e., by in situ selection from adaptive dynamic-covalent networks^[16,17] of extreme sulfur species with high reactivity, high selectivity, and strong retention by thiols.

Target molecules **1–8** were accessible by substantial multistep synthesis (Figure 1, Scheme 1, and Supporting Information, Schemes S1–S3 and S5–S8). BPS₅ **1** was prepared from catechol **9** following the Greer procedure.^[3] Dithiastannole **10**, obtained in six steps from **9**, was treated with S₂Cl₂ to give **11**,^[3] which in turn was condensed with an amine equipped with fluorescein (FL–NH₂) to yield BPS₅ **1**.

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Scheme 1. a) 6 steps, Scheme S1;^[3] b) S_2Cl_2 , 0 °C to rt, 24 h, 20%;^[3] c) FL-NH₂, DMF, rt, 12 h, 14%; d) 6 steps, Scheme S5;^[11] e) 1. NH₃, MeOH, rt, 30 min, 2. S_2Cl_2 , CH₂Cl₂, 0 °C to rt, 2 h, 58%; f) 3 steps; g) 10 steps, Scheme S6. PNP = *p*-nitrophenyl.

Biotinylated and TEGylated BPS₅ **2** and **3** were prepared correspondingly. ETP₄ **4** was prepared from ethylamine **12**, which was converted into heterocycle **13** as described.^[11] Methanolysis of thioesters followed by treatment with S_2Cl_2 ,^[18] removal of the *tert*-butyl ester in **14**, and reaction with FL-NH₂ yielded ETP₄ **4**. A close congener of ETP₂ **5**,^[11] ETP₂ **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).^[12] Independent of any corrections applied, the uptake of BPS₅ **1** exceeded all other COCs. With correction, BPS₅ **1** was approximately 10-times more active than the previous best in the sulfur series, ETP₂ **5**,^[11] and approximately 140-times more active than the best explored asparagusic acid (AspA) derivative **7**.^[10] Tetrasulfide ETP₄ **4** showed less uptake than disulfide ETP₂ **5**. This result demonstrated that simple oligomer effects in ring-expanded COCs fail to explain the power of BPS₅ **1**. The approximately 2.4-times higher activity of new phenoxyethyl ETP₂ **6** compared to ethyl ETP₂ **5** suggested that the known contributions of aromatic rings to cellular uptake^[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^[19] to the activity of BPS₅ **1** are almost negligible.

CLSM images confirmed that BPS₅ **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). Insensitivity toward several inhibitors indicated the absence of uptake by clathrin-mediated endocytosis (chlorpromazine), caveolae-mediated endocytosis (methyl- β -cyclodextrin), and macropinocytosis (wortmannin, cytochalasin B; Supporting Information, Figure S7).^[9–15,20] A drop in activity to 70 % upon preincubation with 2 mM Ellman's reagent (DTNB) sup-

ported contributions from thiol-mediated dynamic covalent oligochalcogenide exchange^[9–14] to the uptake of BPS₅ **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 μ M; 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**,^[3] reminiscent of elemental sulfur S_n, with pentasulfides **1–3** being clearly preferred, followed by trisulfides **16** (Figure 2a,e and Supporting Information, Figure S38).^[1–3] The mechanism of these reversible interconversions remains under debate, with transient ring opening by traces of nucleophilic impurities the most likely explanation.^[1–3,6,21] BPS chemistry further includes sulfur replacement, nucleophilic displacement and oxidation, radicals, metal coordination, and photochemistry,^[1–3] presumably much influenced, if not determined by the strings of electrophilic σ holes next to nucleophilic lone pairs on the lined-up sulfur atoms.^[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 ETP₂ **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.^[13] In a neutral, deuterated phosphate buffer, the ¹H NMR spectrum of BPS₅ **3** remained unchanged at least for two weeks (Figure 2b; BPS_n have nearly identical spectra). In the HPLC, equilibration with BPS₃ **16** was detectable within hours (Figure 2e and Supporting Information, Figure S22 and S38). In the presence of thiols (dithiothreitol, DTT, and glutathione **22**, GSH), BPS₅ **3** transformed rapidly into multicomponent mixtures, with ¹H 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 BPS₅ **1** with GSH affords a dynamic-covalent network that includes unprecedented cyclic oligomers **23**, from dimers **23**₂ to heptamers **23**₇, with up to nineteen sulfur atoms in a macrocycle of thirty-three atoms, besides the expected di- and mono-GSH-BPS₂ conjugates **24** and **25**, and reduced BPS₂ **26** (Figure 2 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.^[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 BPS₅ **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,

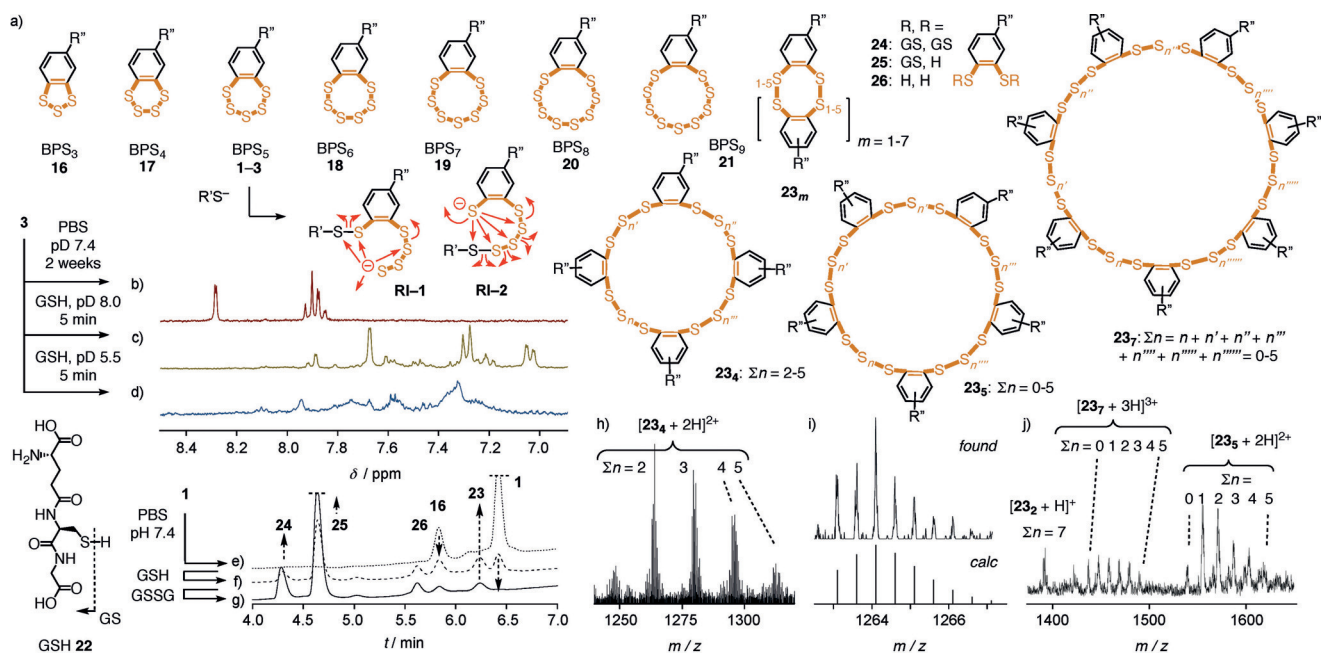


Figure 2. a) Possible ring expansion and contraction of BPS₅, interconvertible ring-opened intermediates RI, and selected products from intermolecular exchange. b–d) Diagnostic region of ¹H NMR spectra of **3** in deuterated PBS buffer, 2 weeks (b), and with GSH, 5 min, pD 8.0 (c), and pD 5.5 (d). e–g) HPLC traces of BPS₅ **1** in PBS buffer, pH 7.4, 30 min (e), and with 2 equiv GSH (f), followed by 100 equiv GSSG (g). h) Signal cluster of **23**₄ in UHPLC-TOF HRMS of **1** with **22** (2 equiv), with i) a zoom and a simulated spectrum for **23**₄ (Σn = 2), and j) signal clusters of **23**₂, **23**₅, and **23**₇ in LC-MS of **1** with **22** (2 equiv). R': See 1, 3, Figure 1; R': See 22.

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^[16,17] of extreme sulfur species obtained from BPS₅ 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 ETP₄ **4** and AspA **7**. Some permanent retention of dithio-

lane **7** until clean release with DTT was in agreement with its dominant endosomal capture (Figures 3a and 1c).^[10] Negligible retention of ring expanded ETP₄ **4** as well as ETP₂ **6** and ETP₅ **5**^[12] was in agreement with dynamic-covalent walking^[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 **4** and pentasulfide **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 BPS₅ (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 **27** by artificial metalloenzyme **28** within HeLa Kyoto cells (Figure 4 and Supporting Information, Figures S49 and S50).^[13,24] In this reaction, protein-activated organometallic ruthenium complexes as in **29** cleave the allylcarbonyl protecting groups in the non-fluorescent substrate **27** and liberate the fluorescent amine **30**.^[25] The cell-penetrating deallocase **28** was prepared by adding ruthenium complex **29** and biotinylated BPS₅ **2** to a streptavidin tetramer. Incubation of HeLa Kyoto cells first with cell-penetrating metalloallocase **28** and then, after washing, with the more hydrophobic, freely diffusing substrate **27** resulted in the intracellular emission from the intracellularly uncaged fluorophores **30**. Emission intensities increased with increasing concentration of **28**, while the BPS-free control enzyme **31** did not cause fluorescence inside of

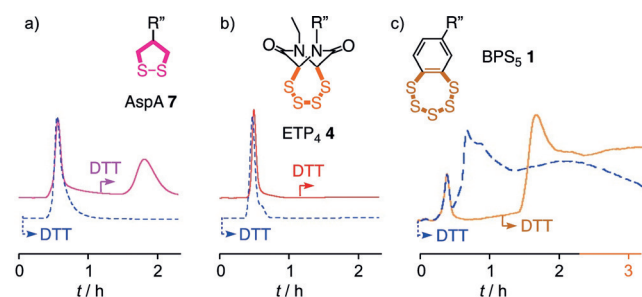


Figure 3. Thiol-exchange affinity column chromatograms of a) **7**,^[12] b) **4**, and c) **1** in 10 mM Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.5 with a 0–50 mM DTT gradient at t = 60–70 min (solid) and constant 50 mM DTT from t = 0 (dashed), R': See 1, 4, and 7, Figure 1.

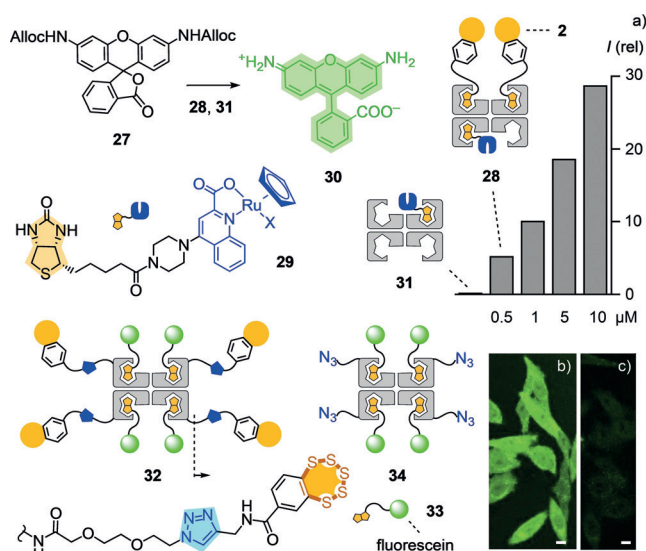


Figure 4. a) Fluorescence intensities in HeLa Kyoto cells treated with **31** (2.5 μM) or **28** (0.5–10 μM), then, after washing, with **27** (10 μM). CLSM images of HeLa Kyoto cells incubated with b) **32** and c) **34** (10 μM, 8 h; scale bars: 10 μm; LP: 30%).

the cells because it failed to penetrate (Figure 4a and Supporting Information, Figure S50). These results confirmed the central importance of delivery for bioorthogonal catalysis within cells.^[13]

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 technology. CPS **32** was prepared by covalently linking BPS₅ to a streptavidin via copper-catalyzed azide–alkyne cycloaddition reactions followed by loading the model substrate, a biotinylated fluorescein **33**. CLSM images of HeLa Kyoto cells incubated with CPS **32** revealed intense diffuse fluorescence in most parts of the cells except in the nucleoli, together with some endosome-like punctate dots (Figure 4b). In comparison, the uptake of control protein **34**, which lacks BPS₅, was negligible (Figure 4c). Ongoing studies on the biological applications of covalent BPS–streptavidin conjugates **32** include the targeted delivery of biotinylated anti-GFP nanobodies and biotinylated chloroalkanes together with biotinylated TAMRA fluorophores to mitochondria labelled with GFP and HaloTags, respectively.

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,^[16,26] 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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