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Effects of Sulfane Sulfur Content in Benzyl Polysulfides on Thiol-Triggered H₂S Release and Cell Proliferation

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Abstract

Investigations into hydrogen sulfide (H₂S) signaling pathways have demonstrated both the generation and importance of persulfides, which are reactive sulfur species that contain both reduced and oxidized sulfur. These observations have led researchers to suggest that oxidized sulfur species, including sulfane sulfur (S⁰), are responsible for many of the physiological phenomena initially attributed to H₂S. A common method of introducing S⁰ to biological systems is the administration of organic polysulfides, such as diallyl trisulfide (DATS). However, prior reports have demonstrated that commercially-available DATS often contains a mixture of polysulfides, and furthermore a lack of structure-activity relationships for organic polysulfides has limited our overall understanding of different polysulfides and their function in biological systems. Advancing our interests in the chemical biology of reactive sulfur species including H₂S and S⁰, we report our investigations into the rates and quantities of H₂S release from a series of synthetic, pure benzyl polysulfides, ranging from monosulfide to tetrasulfide. We demonstrate that H₂S is only released from the trisulfide and tetrasulfide, and that this release requires thiol-mediated reduction in the presence of cysteine or reduced glutathione. Additionally, we demonstrate the different effects of trisulfides and tetrasulfides on cell proliferation in murine epithelial bEnd.3 cells.

Graphical Abstract

1. Introduction

Sulfane sulfur (S^0) is formally defined as a sulfur atom that bears six valence electrons, no formal charge, can exist in a thiosulfoxide tautomer, and is bonded to two or more sulfur atoms or to a sulfur atom and an ionizable hydrogen.¹ This sulfur oxidation state is found in various sulfur-containing species including elemental sulfur, persulfides, and polysulfides.² Under physiological conditions, S^0 can be reduced in the presence of biological thiols, such as reduced glutathione (GSH) or cysteine (Cys), to generate the important signaling molecule hydrogen sulfide (H_2S) (Figure 1).³

H_2S is now commonly associated with the gasotransmitter family, which includes nitric oxide and carbon monoxide, because it is produced endogenously, can freely permeate cell membranes, and can act on specific cellular and/or molecular targets to exert physiological effects.⁴ Recent work has demonstrated H_2S -mediated signaling in processes including neurotransmission,⁵ vasodilation,⁶ and anti-inflammation.⁷ Towards efforts to provide exogenous H_2S as either a pharmacological tool or as a potential therapeutic,⁸ a number of groups have prepared small molecules capable of releasing H_2S under different conditions.⁹ Notably, a number of these developed donor motifs generate persulfides en route to H_2S release, which complicates whether the observed biological effects from such donors is due to H_2S release or S^0 . Recently, evaluation of H_2S -signaling mechanisms has revealed the formation of persulfides as a common intermediate leading to questions of whether persulfides and related S^0 -containing species are key signaling intermediates.¹⁰

When considering sulfur oxidation states, persulfides possess reduced sulfur and S^0 , implying potential significance of S^0 in sulfur biology.¹¹ Under physiological conditions, S^0 readily oxidizes free thiol residues to generate persulfides via a reaction mechanism known as persulfidation,¹² and this process has been shown to upregulate the activity of key enzymes such as xanthine oxidase.¹³ Additionally, the formation of persulfides under physiological conditions has been proposed to occur via the reaction of oxidized reactive sulfur species including disulfides, sulfenic acids, and *S*-nitrosothiols with H_2S .¹⁴ Interestingly, persulfidation of thiol-based antioxidants such as GSH leads to enhanced antioxidant activity when compared to corresponding thiols.¹⁵ Within the active sites of specific enzymes, persulfides can be stabilized and the H_2S release from an enzyme-bound persulfide has been shown in 3-mercaptopyruvate sulfurtransferase via thioredoxin-mediated reduction.¹⁶ Toward directly studying small molecule persulfide reactivity, recent work has demonstrated the ability to generate persulfides under physiological conditions¹⁷ for applications in protein persulfidation,¹⁸ reactive oxygen species scavenging,¹⁹ and H-atom transfer agents.²⁰ Previous work by our group demonstrated the ability to access discrete persulfides²¹ and study their reactivity under various conditions.²² A common, yet understudied pathway for accessing persulfides, hydropolysulfides, and other closely related reactive sulfur species is the thiol-mediated reduction of organic polysulfides.

Polysulfides are a class of organosulfur species commonly found in nature,²³ that have unique biological properties.²⁴ Readily isolated from alliums such as garlic,²⁵ diallyl trisulfide (**DATS**) is a simple, organic polysulfide which has been studied heavily in the field of biological sulfur chemistry. The commercial availability of **DATS** has led to its broad use

as an H₂S donor and source of S⁰. For example, the cytotoxicity of **DATS** has been reported in a wide array of human cancer cell lines,²⁶ although we note the cytotoxicity of **DATS** appears to be directly correlated to S⁰ content as the analogous monosulfide and disulfide demonstrate minimal cytotoxicity.²⁷ Upon examining the mechanism of action, **DATS** was found to suppress cell proliferation in human colon cancer cells (HCT-15) via *S*-allylation of Cys-12 β and Cys-354 β in β -tubulin rather than direct H₂S or persulfide release.²⁸ By contrast, the thiol-dependent release of H₂S from **DATS** in human red blood cells and the vasoactivity of garlic extract administration was found to be directly related to H₂S production.²⁹ In the presence of thiols, **DATS** releases H₂S upon nucleophilic attack at the α -sulfur to generate allyl persulfide, which further reacts with a second equivalent of thiol to generate H₂S.³⁰ Despite the accessibility of other organic polysulfides, studies have been primarily limited to **DATS**. Alternatively, researchers have relied heavily on the use of inorganic polysulfides,³¹ which spontaneously disproportionate under mild, aqueous conditions to yield complex mixtures.³² Recently, our group and others³³ have demonstrated the ability to access³⁴ and utilize organic polysulfides beyond **DATS** for thiol-triggered H₂S delivery.³⁵ Aligned with our interests in studying S⁰-containing reactive sulfur species, we sought to expand the toolbox of available organic polysulfides and study the effect of varying S⁰ content in a single series of polysulfides. Herein, we demonstrate the thiol-mediated release of H₂S from benzyl trisulfide and benzyl tetrasulfide respectively. Additionally, we demonstrate the ability of S⁰-containing benzyl polysulfides to suppress cell proliferation in bEnd.3 cells in a S⁰ content-dependent manner.

2. Materials and Methods.

Reagents were purchased from Sigma-Aldrich, Tokyo Chemical Industry (TCI), and/or Cayman Chemical Company and used directly as received. Deuterated solvents were purchased from Cambridge Isotope Laboratories and used as received. ¹H and ¹³C{¹H} NMR spectra were recorded on a Bruker 500 or 600 MHz instrument. Chemical shifts are reported relative to residual protic solvent resonances. Silica gel (SiliaFlash F60, Silicycle, 230-400 mesh) was used for column chromatography. All air-free manipulations were performed under an inert atmosphere using standard Schlenk technique or an Innovative Atmospheres N₂-filled glove box. UV-Vis spectra were acquired on an Agilent Cary 60 UV-Vis spectrophotometer equipped with a Quantum Northwest TC-1 temperature controller at 25 °C $\pm$ 0.05 °C.

2.1 Synthesis

2.1.1 SS-benzyl O-methyl carbonodithioperoxoate.—Methoxycarbonylsulfonyl chloride (2.6 mmol, 1.1 equiv.) was added to anhydrous CH₂Cl₂ (20 mL) and cooled to 0 °C in an ice bath under N₂. Once cooled, benzyl mercaptan (2.4 mmol, 1.0 equiv.) was added dropwise, and the reaction mixture was stirred for 1 h at 0 °C under N₂. After 1 h, the reaction was quenched with the addition of deionized H₂O (30 mL), and the organic layer was separated. The remaining aqueous layer was extracted with CH₂Cl₂ (2 $\times$ 30 mL), and the combined organic layers were washed with brine ($\times$ 1) and dried over MgSO₄. After filtration, the solvent was removed under reduced pressure, and the desired product purified by column chromatography (10% EtOAc in hexanes, *R_f* = 0.46). The product was isolated as

a clear liquid. Mass: 430 mg (83%) ^1H NMR (500 MHz, DMSO- d_6) δ : 7.38 – 7.26 (m, 5 H), 4.07 (s, 2 H), 3.79 (s, 3 H). $^{13}\text{C}\{^1\text{H}\}$ NMR (126 MHz, DMSO- d_6) δ : 168.35, 136.13, 129.44, 128.41, 127.60, 55.77, 42.07. TOF MS EI+ (m/z) $[\text{M} + \text{H}]^+$ calc'd for $\text{C}_9\text{H}_{10}\text{O}_2\text{S}_2$ 214.0122; found, 214.0120.

2.1.2 Benzyl trisulfide.—*SS*-benzyl *O*-methyl carbonodithioperoxoate (0.93 mmol, 1.0 equiv.) was added to THF (5 mL) and stirred briefly. Potassium *tert*-butoxide (0.46 mmol, 0.5 equiv.) was dissolved in 0.5 mL of deionized H_2O in an Eppendorf tube, and then added dropwise to the stirred reaction mixture at room temperature. After 15 h, the solvent was removed under reduced pressure, and the desired product purified by column chromatography (5% EtOAc in hexanes, R_f = 0.50). **Bn₂S₃** was isolated as a white solid. Mass: 47 mg (36%). ^1H NMR (500 MHz, CDCl_3) δ : 7.35 – 7.27 (m, 10 H), 4.03 (s, 4 H). $^{13}\text{C}\{^1\text{H}\}$ NMR (126 MHz, CDCl_3) δ : 136.69, 129.59, 128.76, 127.73, 43.32. HRMS-EI+ (m/z): $[\text{M} + \text{H}]^+$ calc'd for $\text{C}_{14}\text{H}_{14}\text{S}_3$, 278.02576; found, 278.02464.

2.1.3 Benzyl tetrasulfide.—Benzyl mercaptan (2.0 mmol, 1.0 equiv.) and pyridine (2.0 mmol, 1.0 equiv.) were added to anhydrous Et_2O (20 mL) and cooled to -78°C over 1 h under N_2 . Sulfur monochloride (1.2 mmol, 0.6 equiv.) was added dropwise, and the reaction mixture was stirred at -78°C for 2 h. The reaction was quenched with the addition of dH_2O (30 mL) and diluted with CH_2Cl_2 (30 mL). The organic layer was removed, and the aqueous layer was extracted with CH_2Cl_2 (20 mL $\times$ 2). The combined organic layers were washed organic layers with brine (20 mL $\times$ 1), dried over MgSO_4 , filtered, and evaporated under reduced pressure to yield the product as a yellow solid. Mass: 280 mg (89%). ^1H NMR (500 MHz, CDCl_3) δ : 7.37 – 7.27 (m, 10 H), 4.16 (s, 4 H). $^{13}\text{C}\{^1\text{H}\}$ NMR (126 MHz, CDCl_3) δ : 136.39, 129.64, 128.81, 127.87, 43.81. (HRMS experiments did not show the parent ion peak due to the weak internal S-S bond.)

2.1.4 S-Benzyl ethanethioate.—Benzyl mercaptan (1.6 mmol, 1.1 equiv.) and triethylamine (1.6 mmol, 1.1 equiv.) were added to anhydrous CH_2Cl_2 (30 mL) and cooled to 0°C in an ice bath under N_2 . Once cooled, acetyl chloride (1.4 mmol, 1.0 equiv.) was added dropwise, and the reaction was stirred at 0°C for 2 h under N_2 . The reaction was quenched with the addition of deionized H_2O (20 mL), and the organic layer was separated. The remaining aqueous layer was extracted with CH_2Cl_2 (2 $\times$ 20 mL) and the combined organic extractions were washed with brine (1 $\times$ 20 mL) and dried over MgSO_4 . After filtration, the solvent was removed under reduced pressure, and the desired product purified by column chromatography (20% CH_2Cl_2 in Hexanes, R_f = 0.25). The product was isolated as a clear liquid. Mass: 126 mg (52%). ^1H NMR (600 MHz, CDCl_3) δ : 7.31 – 7.27 (m, 4H), 7.27 – 7.23 (m, 1H), 4.13 (s, 2H), 2.35 (s, 3H). $^{13}\text{C}\{^1\text{H}\}$ NMR (151 MHz, CDCl_3) δ : 195.09, 137.56, 128.77, 128.60, 127.24, 33.42, 30.29. TOF MS (EI+) (m/z): $[\text{M} + \text{H}]^+$ calc'd for $\text{C}_9\text{H}_{10}\text{OS}$ 166.0452; found 166.0454.

2.2. H₂S Measurement Materials and Methods.

Phosphate buffered saline (PBS) tablets (1 $\times$, CalBioChem) were used to prepare buffered solutions (140 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4) in deionized water. Buffer solutions were sparged with N_2 to remove dissolved oxygen and stored in an N_2 -filled

glovebox. Donor stock solutions (in DMSO) were freshly prepared inside an N₂-filled glovebox prior to an experiment. Thiol (cysteine or GSH) stock solutions (in PBS) were freshly prepared in an N₂-filled glovebox immediately before use.

2.3. General Procedure for Measuring H₂S Release via Methylene Blue Assay (MBA).

Scintillation vials containing 20 mL of PBS were prepared in an N₂-filled glovebox. To these solutions, 20 µL of 500 mM analyte stock solution (in PBS) was added for a final concentration of 500 µM. The solutions were allowed to thermally equilibrate while stirring in a heating block at the desired temperature for approximately 20-30 min. Immediately prior to donor addition, 0.5 mL solution of the methylene blue cocktail were prepared in disposable 1.5 mL cuvettes. The methylene blue cocktail solution contained: 200 µL of 30 mM FeCl₃ in 1.2 M HCl, 200 µL of 20 mM *N,N*-dimethyl-*p*-phenylene diamine in 7.2 M HCl, and 100 µL of 1% (w/v) Zn(OAc)₂. To begin an experiment, 20 µL of 25 mM donor stock solution (in DMSO) was added for a final concentration of 25 µM. At set time points after the addition of donor, 500 µL reaction aliquots were added to the methylene blue cocktail solutions and incubated for 1 h at room temperature shielded from light. Absorbance values were measured at 670 nm 1 h after addition of reaction aliquot. Each experiment was performed in quadruplicate unless stated otherwise.

2.4 MBA Calibration Curve.

Solutions containing 0.5 mL of the methylene blue cocktail and 0.5 mL PBS containing 500 µM thiol (cysteine or GSH) were freshly prepared in disposable cuvettes (1.5 mL). Under inert conditions, a 10 mM stock solution of NaSH (Strem Chemicals) in PBS was prepared and diluted to 1 mM. Immediately after dilution, 1 mM NaSH was added to 1.0 mL solutions for final concentrations of 10, 20, 30, 40, and 50 µM. Solutions were mixed thoroughly, incubated at room temperature for 1 h, and shielded from light. Absorbance values at 670 nm were measured after 1 h.

2.5 Cell Culture.

The murine brain endothelial cell line bEnd.3 was obtained by ATCC (CRL-2299) and cultured in phenol red DMEM containing 10% premium grade fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) (10,000 units/mL penicillin and 10,000 µg/mL streptomycin). Cells were incubated at 37°C under 5% CO₂.

2.6 Cell Proliferation Assay.

bEnd.3 cells were seeded in Nunc® 96 well plates at 20,000 cells/well in 10% FBS, 1% PS DMEM and grown overnight. The next day, media was aspirated off and cells were rinsed in 1% PS-containing DMEM containing no FBS or phenol red. Media was replaced with the FBS-free DMEM containing either 0.5% DMSO (vehicle) or the tested compounds and cells were incubated under these conditions for 24 hours before treatment with Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies). No turbidity of the tested compounds was observed in DMEM up to the concentrations tested.

2.7 Statistical Analysis.

Cell proliferation data is represented as a percentage viability compared to the vehicle (Veh). Data for each treatment condition were averaged from several trials using a pooled average. Error was pooled from the standard deviations for each treatment condition found in each of these trials. A two-tailed Student's t-test was then performed comparing each treatment condition to the vehicle.

3. Results and Discussion

To investigate the impacts of sulfur content in polysulfides, we chose to study benzyl polysulfides ranging from benzyl sulfide (**Bn₂S**) up to and including benzyl tetrasulfide (**Bn₂S₄**). Using previously reported conditions,³⁶ we synthesized **Bn₂S₄** in 89% yield via treatment of benzyl mercaptan with sulfur monochloride (S₂Cl₂) in the presence of pyridine at -78 °C (Figure 2a). To access benzyl trisulfide (**Bn₂S₃**), we evaluated prior reports of symmetrical trisulfide synthesis. Most reports cite the use of sulfur dichloride (SCl₂), an unstable sulfur chloride reagent that exists in equilibrium with S₂Cl₂. We reasoned that using this reagent would provide an inseparable mixture of tri- and tetra-sulfides due to the SCl₂/S₂Cl₂ equilibrium. To avoid the use of SCl₂, we were drawn to a report by Harpp and co-workers that used alkoxide-mediated decomposition of sulfonylthiocarbonates to access trisulfides.³⁷ Although treatment of methoxycarbonylsulfonyl chloride with benzyl mercaptan readily afforded the desired precursor in 83% yield, we found that further treatment with potassium *tert*-butoxide (KO^tBu) in methanol provided an inseparable mixture of benzyl disulfide (**Bn₂S₂**) and **Bn₂S₃**. The proposed reaction mechanism by Harpp and co-workers involves an initial nucleophilic attack by the *tert*-butoxide anion at the carbonyl to form a carbonate and release benzyl persulfide. Based on previous work from our group, treatment of BnSSH with different bases resulted in disproportionation to yield various benzyl polysulfides, which supports the formation of benzyl persulfide under the current reaction conditions.²¹ Due to steric congestion and poor nucleophilicity, we viewed that nucleophilic attack by KO^tBu was unlikely and hypothesized that the active nucleophile in this reaction was hydroxide, which can be generated upon deprotonation of residual water in methanol by the *tert*-butoxide anion.

To test this hypothesis, we treated the benzyl sulfonylthiocarbonate precursor with ^tBuOK in a mixture of tetrahydrofuran and water. Consistent with our hypothesis, we were able to isolate **Bn₂S₃** in 36% yield (Figure 2b). Considering hydroxide as the active nucleophile, we have proposed a new reaction mechanism for the synthesis of symmetrical trisulfides via hydroxide-mediated decomposition of sulfonylthiocarbonates (see Supporting Information). In comparison to current methods of mild trisulfide synthesis, we note the use of hydroxide likely prevents the formation of symmetrical trisulfides containing base-sensitive functional groups. Using ¹H NMR spectroscopy, benzyl polysulfides can be identified by their respective peaks corresponding to benzylic protons which are unique to each polysulfide ranging from **Bn₂S₂** to benzyl pentasulfide in deuterated chloroform allowing for ease of characterization (Figure 2c).³⁸

With a series of benzyl polysulfides in hand, we next investigated the release of H₂S in the presence of cysteine and GSH. We anticipated that only **Bn₂S₃** and **Bn₂S₄** would release

H₂S and that **Bn₂S₄** would release twice as much H₂S relative to **Bn₂S₃**. To test our hypothesis, dibenzyl polysulfides (25 μM) were treated with an excess of cysteine or GSH (500 μM, 20 equiv.) in PBS (10 mM, pH 7.4) at 25 °C and H₂S release was measured via the spectrophotometric methylene blue assay (Figure 3).

As expected, we did not observe H₂S release from **Bn₂S** and **Bn₂S₂** in the presence of cysteine or GSH, consistent with a lack of S⁰ content. Additionally, we note that the lack of H₂S release from **Bn₂S₂** suggests that nucleophilic attack by Cys or GSH at the benzylic carbon to generate a H₂S-releasing persulfide intermediate does not occur.³⁹ For both GSH and Cys, we observed approximately twice as much H₂S released from **Bn₂S₄** than from **Bn₂S₃**, which is consistent with the higher S⁰ content of tetrasulfides when compared to trisulfides. In the presence of cysteine (500 μM, 20 equiv.), **Bn₂S₃** and **Bn₂S₄** released 8.6 (34% releasing efficiency) and 17.5 μM (35% releasing efficiency) H₂S respectively after 90 min. Similarly, we observed 4.3 μM (17% releasing efficiency) and 16 μM (32% releasing efficiency) H₂S released from **Bn₂S₃** and **Bn₂S₄** respectively in the presence of GSH (500 μM, 20 equiv.) after 90 min. To compare the release of H₂S from benzyl polysulfides to other commonly-used organic polysulfides, we measured the H₂S release from **DATS** under the reported conditions. By comparison, **DATS** is a faster H₂S donor than the benzyl polysulfides tested and yields approximately the same quantity of H₂S after 110 minutes as **Bn₂S₄**. To rationalize this observation, we compared the stability of the intermediate persulfides for each donor system (i.e. benzyl persulfide vs. allyl persulfide). To the best of our knowledge, allyl persulfide has never been isolated and is simply observed as a fleeting intermediate, which suggests that it may be significantly less stable than benzyl persulfide, which has been isolated, characterized, and studied extensively.²² Additionally, a comparison of the H₂S releasing curves demonstrates a brief induction period for **Bn₂S₃** and **Bn₂S₄** which we attribute to a buildup of benzyl persulfide in solution. Taken together, these results demonstrate the significance of pendant alkyl groups in organic polysulfides and their overall effect on the chemical reactivity of downstream reactive sulfur species including persulfides. Relative to other synthetic H₂S donors, we note that organic polysulfides provide lower H₂S releasing efficiencies, which is likely a result of higher lipophilicity. We anticipate the design of water-soluble polysulfides should provide more efficient H₂S donors.

Further advancing our studies, we sought to examine the effect of benzyl polysulfides on cell proliferation in murine epithelial bEnd.3 cells to determine whether the different releasing efficiencies for tri- and tetra-sulfides in solution translated into cellular environments. Previously, studies have shown administration of sodium hydrosulfide (NaSH) led a pro-proliferative effect in bEnd.3 cells.⁴⁰ The reported organic polysulfide library provides us a unique opportunity to directly examine the effects of S⁰ within a single series of polysulfides. With these compounds in hand, we sought to determine the impact of increasing S⁰ delivery on cell proliferation following 24 h treatments (Figure 4).

Use of synthetic H₂S donors such as AP39⁴¹ and direct administration of H₂S via NaSH⁴² has been shown to have cytoprotective effects on bEnd.3 cells. To the best of our knowledge, the effect of polysulfide administration has not been studied in this specific cell line. Consistent with a lack of S⁰ content, 24 h incubation with **Bn₂S** and **Bn₂S₂** (up to 200 μM)

did not reduce cell viability. We note the slight cytotoxicity of **Bn₂S₂** at concentrations over 100 μ M is likely due to perturbation of redox homeostasis arising from thiol-disulfide exchange between biological thiols and **Bn₂S₂**. Interestingly, S⁰-containing **Bn₂S₃** and **Bn₂S₄** at concentrations of 100 μ M and higher demonstrated considerable cytotoxicity. Notably, **Bn₂S₄** appears to be significantly more cytotoxic than **Bn₂S₃**, which is consistent with the increased S⁰ content in **Bn₂S₄** relative to **Bn₂S₃**.

Because biological thiols react with the tri- and tetrasulfides to generate two equivalents of benzyl mercaptan as a byproduct, we also tested the cytotoxicity of benzyl mercaptan to confirm that the observed cytotoxicity was due to the S⁰ content rather than the organic byproducts of the reaction. We elected to use *S*-benzyl ethanethioate (**BnSAc**) as a source of benzyl mercaptan to enhance cell permeability. We anticipated hydrolysis of this ester under physiological conditions would generate benzyl mercaptan and allow us to directly probe the effect of this thiol on cytotoxicity. Using treatment up to 400 μ M, we did not observe significant cytotoxicity from **BnSAc** suggesting the observed cytotoxicity for benzyl polysulfides is a direct reflection of S⁰ content, rather than organic byproducts of the reaction (Figure S8). To compare our results with a well-known organic polysulfide, we examined the effect of **DATS** administration on cell proliferation in bEnd.3 cells under the same conditions. Interestingly, treatment of bEnd.3 cells with increasing concentrations of **DATS** did not demonstrate significant cytotoxicity and suggests pendant alkyl groups in organic polysulfides can directly alter biological activities. Taken together, these results demonstrate the effect of increasing S⁰ content on cell proliferation in bEnd.3 cells and suggests organic polysulfides can afford varying biological activities independent of S⁰ content.

4. Conclusion

The identification of key intermediates in sulfur biology responsible for physiological changes such as vasodilation and anti-inflammation is of utmost importance. Recent work has begun to suggest the importance of S⁰ over H₂S in biology and modern synthetic techniques readily allow for access to simple organic polysulfides beyond **DATS**. Towards advancing our knowledge of reactive sulfur species in biology, we report our studies on examining S⁰ content in a single series of organic polysulfides ranging from monosulfide up to and including tetrasulfide. In the presence of biological thiols including cysteine and GSH, we demonstrate the release of H₂S from **Bn₂S₃** and **Bn₂S₄** respectively under physiological conditions. Additionally, we demonstrate the unique ability of **Bn₂S₃** and **Bn₂S₄** to suppress cell proliferation, whereas **DATS** has no effect in bEnd.3 cells. The results of this study warrant future investigations into the effects of various organic polysulfides and supports the significance of S⁰ in sulfur biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A series of benzyl (poly)sulfides with 1-4 sulfur atoms is prepared
- Rates of H₂S release after treatment with Cys and GSH are reported
- H₂S is only released from tri- and tetra-sulfides, but not mono- or di-sulfides
- The effects of trisulfides and tetrasulfides on cell proliferation in bEnd.3 cells are reported

Figure 1.
Common S^0 -containing small molecule species release H_2S upon reaction with biological thiols.

Figure 2.

(a) Synthesis of **Bn₂S₄**. (b) Synthesis of **Bn₂S₃**. (c) Comparison of benzylic ¹H NMR (500 MHz) signals between **Bn₂S₂**, **Bn₂S₃**, and **Bn₂S₄** in CDCl₃.

Figure 3.

(a) Reaction conditions for thiol-triggered release of H_2S from Bn_2S_n ($n = 1, 2, 3$, or 4). (b) Release of H_2S in the presence of cysteine. (c) Release of H_2S in the presence of GSH.

Figure 4. bEnd3 cell viability after 24-hour treatments of a series of organic polysulfides: (a) **Bn₂S**, (b) **Bn₂S₂**, (c) **Bn₂S₃**, (d) **Bn₂S₄**, and (e) **DATS**. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$