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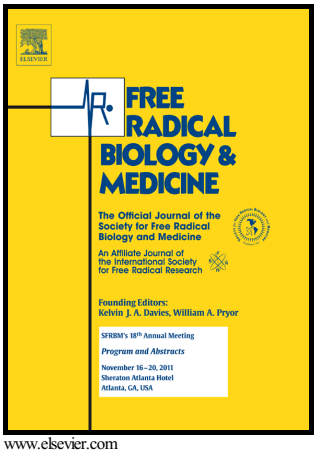


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# Effects of Sulfane Sulfur Content in Benzyl Polysulfides on Thiol-Triggered $H_2S$ Release and Cell Proliferation

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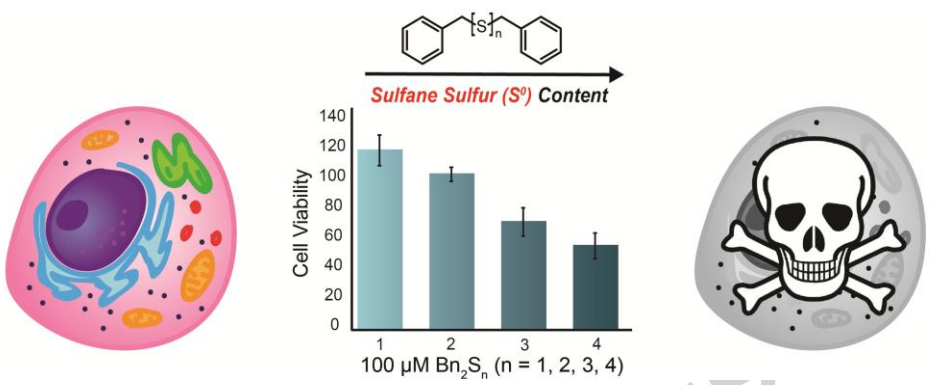
## Abstract

Investigations into hydrogen sulfide ($H_2S$) 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 ($S^0$), are responsible for many of the physiological phenomena initially attributed to $H_2S$. A common method of introducing $S^0$ 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 $H_2S$ and $S^0$, we report our investigations into the rates and quantities of $H_2S$ release from a series of synthetic, pure benzyl polysulfides, ranging from monosulfide to tetrasulfide. We demonstrate that $H_2S$ 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

[1] These authors contributed equally.

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]

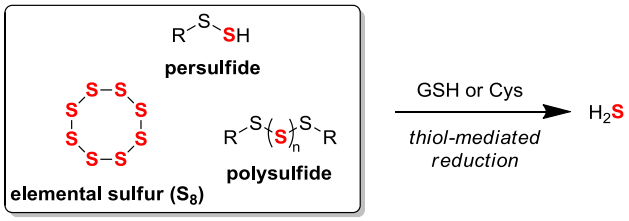


**Figure 1**. Common $S^0$-containing small molecule species release $H_2S$ upon reaction with biological thiols.

$H_2S$ 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 conditions[17] 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 persulfides[21] 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 $H_2S$ donor and source of $S^0$. 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 $S^0$ 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-12β and Cys-354β in β-tubulin rather than direct $H_2S$ or persulfide release.[28] By contrast, the thiol-dependent release of $H_2S$ from DATS in human red blood cells and the vasoactivity of garlic extract administration was found to be directly related to $H_2S$ production.[29] In the presence of thiols, **DATS** releases $H_2S$ upon nucleophilic attack at the α-sulfur to generate allyl persulfide, which further reacts with a second equivalent of thiol to generate $H_2S$.[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 others[33] have demonstrated the ability to access[34] and utilize organic polysulfides beyond **DATS** for thiol-triggered $H_2S$ delivery.[35] Aligned with our interests in studying $S^0$-containing reactive sulfur species, we sought to expand the toolbox of available organic polysulfides and study the effect of varying $S^0$ content in a single series of polysulfides. Herein, we demonstrate the thiol-mediated release of $H_2S$ from benzyl trisulfide and benzyl tetrasulfide respectively. Additionally, we demonstrate the ability of $S^0$-containing benzyl polysulfides to suppress cell proliferation in bEnd.3 cells in a $S^0$ 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 $^{13}C\{^1H\}$ 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 $N_2$-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 ± 0.05 °C.

### *2.1 Synthesis*

2.1.1 *SS-benzyl O-methyl carbono(dithioperoxoate)*. Methoxycarbonylsulfenyl chloride (2.6 mmol, 1.1 equiv.) was added to anhydrous $CH_2Cl_2$ (20 mL) and cooled to 0 °C in an ice bath under $N_2$. 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 $N_2$. After 1 h, the reaction was quenched with the addition of deionized $H_2O$ (30 mL), and the organic layer was separated. The remaining aqueous layer was extracted with $CH_2Cl_2$ (2 x 30 mL), and the combined organic layers were washed with brine (x1) and dried over $MgSO_4$. 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%) $^1$H NMR (500 MHz, DMSO-d$^6$) δ: 7.38 – 7.26 (m, 5 H), 4.07 (s, 2 H), 3.79 (s, 3 H). $^{13}$C{$^1$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*) $[M + H]^+$ calc'd for $C_9H_{10}O_2S_2$ 214.0122; found, 214.0120.

*2.1.2 Benzyl trisulfide*. *SS*-benzyl *O*-methyl carbono(dithioperoxoate) (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). **$Bn_2S_3$** was isolated as a white solid. Mass: 47 mg (36%). $^1$H NMR (500 MHz, $CDCl_3$) δ: 7.35 – 7.27 (m, 10 H), 4.03 (s, 4 H). $^{13}$C{$^1$H} NMR (126 MHz, $CDCl_3$) δ: 136.69, 129.59, 128.76, 127.73, 43.32. HRMS-EI+ (*m/z*): $[M + H]^+$ calc'd for $C_{14}H_{14}S_3$, 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 x 2). The combined organic layers were washed organic layers with brine (20 mL x 1), dried over $MgSO_4$, filtered, and evaporated under reduced pressure to yield the product as a yellow solid. Mass: 280 mg (89%). $^1$H NMR (500 MHz, $CDCl_3$) δ 7.37 – 7.27 (m, 10 H), 4.16 (s, 4 H). $^{13}C\{^1H\}$ 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 x 20 mL) and the combined organic extractions were washed with brine (1 x 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%). $^1$H 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). $^{13}C\{^1H\}$ 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*): $[M + H]^+$ calc'd for $C_9H_{10}OS$ 166.0452; found 166.0454.

*2.2 $H_2S$ Measurement Materials and Methods.* Phosphate buffered saline (PBS) tablets (1X, 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 $N_2$-filled glovebox prior to an experiment. Thiol (cysteine or GSH) stock solutions (in PBS) were freshly prepared in an $N_2$-filled glovebox immediately before use.

*2.3. General Procedure for Measuring $H_2S$ Release via Methylene Blue Assay (MBA).* Scintillation vials containing 20 mL of PBS were prepared in an $N_2$-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 $FeCl_3$ 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% $CO_2$.

*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 (**$Bn_2S$**) up to and including benzyl tetrasulfide (**$Bn_2S_4$**). Using previously reported conditions,[36] we synthesized **$Bn_2S_4$** in 89% yield via treatment of

benzyl mercaptan with sulfur monochloride ($S_2Cl_2$) in the presence of pyridine at -78 °C (Figure 2a). To access benzyl trisulfide (**$Bn_2S_3$**), we evaluated prior reports of symmetrical trisulfide synthesis. Most reports cite the use of sulfur dichloride ($SCl_2$), an unstable sulfur chloride reagent that exists in equilibrium with $S_2Cl_2$. We reasoned that using this reagent would provide an inseparable mixture of tri- and tetra-sulfides due to the $SCl_2/S_2Cl_2$ equilibrium. To avoid the use of $SCl_2$, we were drawn to a report by Harpp and co-workers that used alkoxide-mediated decomposition of sulfenylthiocarbonates to access trisulfides.[37] Although treatment of methoxycarbonylsulfenyl 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 (**$Bn_2S_2$**) and **$Bn_2S_3$**. 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.

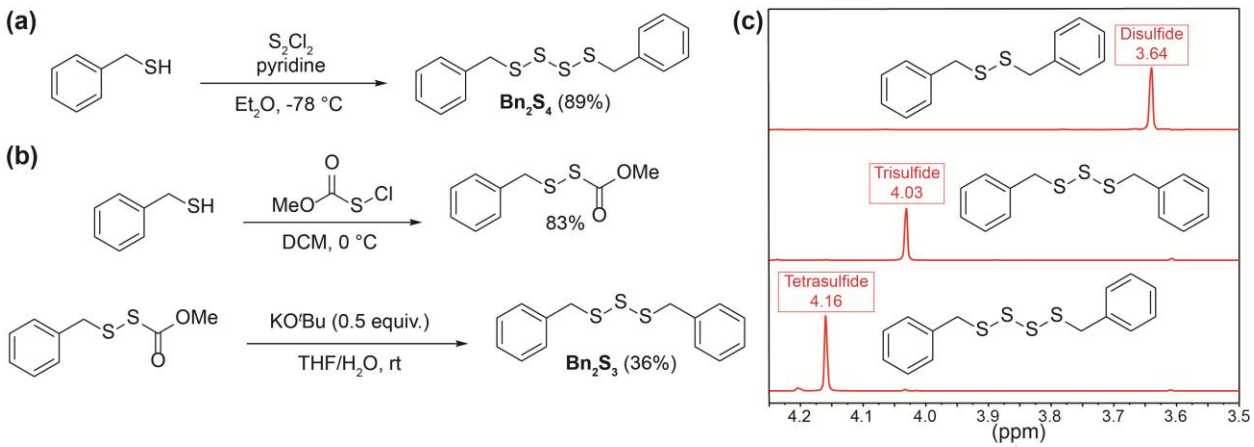



**Figure 2**. (a) Synthesis of **$Bn_2S_4$**. (b) Synthesis of **$Bn_2S_3$**. (c) Comparison of benzylic $^1$H NMR (500 MHz) signals between **$Bn_2S_2$**, **$Bn_2S_3$**, and **$Bn_2S_4$** in $CDCl_3$.

To test this hypothesis, we treated the benzyl sulfenylthiocarbonate precursor with $^t$BuOK in a mixture of tetrahydrofuran and water. Consistent with our hypothesis, we were able to isolate **$Bn_2S_3$** 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 sulfenylthiocarbonates (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 $^1$H NMR spectroscopy, benzyl polysulfides can be identified by their respective peaks corresponding to benzylic protons which are unique to each polysulfide ranging from **$Bn_2S_2$** 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 $H_2S$ in the presence of cysteine and GSH. We anticipated that only **$Bn_2S_3$** and **$Bn_2S_4$** would release $H_2S$ and that **$Bn_2S_4$** would release twice as much $H_2S$ relative to **$Bn_2S_3$**. 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 $H_2S$ release was measured via the spectrophotometric methylene blue assay (Figure 3).

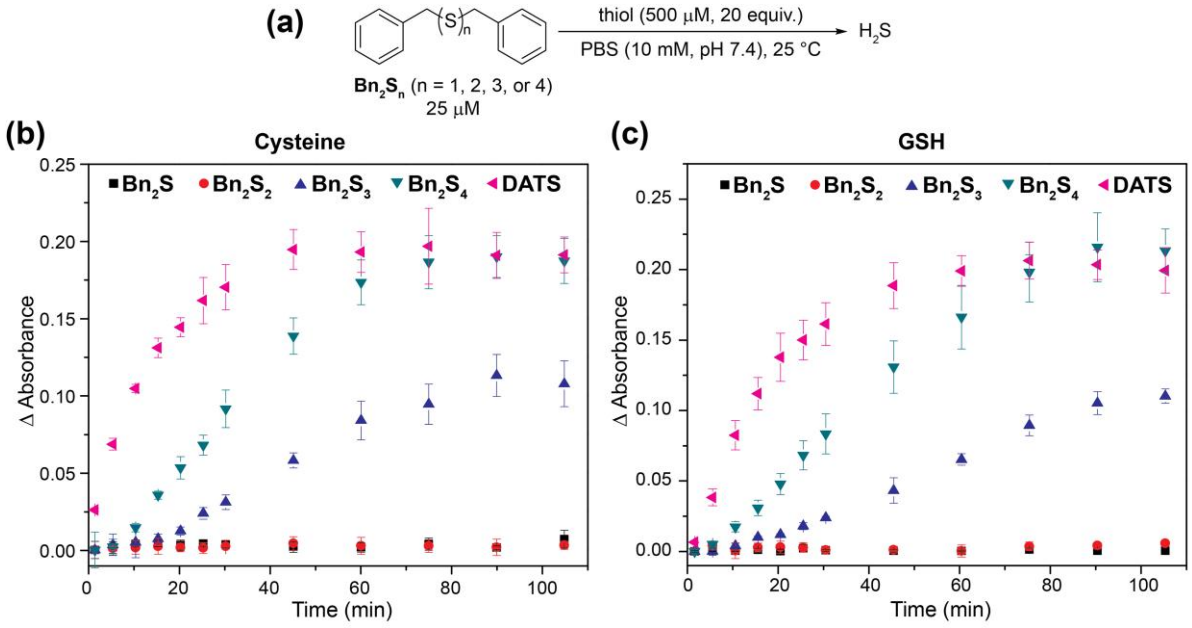



**Figure 3**. (a) Reaction conditions for thiol-triggered release of $H_2S$ from $\mathbf{Bn_2S_n}$ (n = 1, 2, 3, or 4). (b) Release of $H_2S$ in the presence of cysteine. (c) Release of $H_2S$ in the presence of GSH.

As expected, we did not observe $H_2S$ release from $\mathbf{Bn_2S}$ and $\mathbf{Bn_2S_2}$ in the presence of cysteine or GSH, consistent with a lack of $S^0$ content. Additionally, we note that the lack of $H_2S$ release from $\mathbf{Bn_2S_2}$ suggests that nucleophilic attack by Cys or GSH at the benzylic carbon to generate a $H_2S$-releasing persulfide intermediate does not occur.[39] For both GSH and Cys, we observed approximately twice as much $H_2S$ released from $\mathbf{Bn_2S_4}$ than from $\mathbf{Bn_2S_3}$, which is consistent with the higher $S^0$ content of tetrasulfides when compared to trisulfides. In the presence of cysteine (500 μM, 20 equiv.), $\mathbf{Bn_2S_3}$ and $\mathbf{Bn_2S_4}$ released 8.6 (34% releasing efficiency) and 17.5 μM (35% releasing efficiency) $H_2S$ respectively after 90 min. Similarly, we observed 4.3 μM (17% releasing efficiency) and 16 μM (32% releasing efficiency) $H_2S$ released

from **$Bn_2S_3$** and **$Bn_2S_4$** respectively in the presence of GSH (500 μM, 20 equiv.) after 90 min. To compare the release of $H_2S$ from benzyl polysulfides to other commonly-used organic polysulfides, we measured the $H_2S$ release from **DATS** under the reported conditions. By comparison, **DATS** is a faster $H_2S$ donor than the benzyl polysulfides tested and yields approximately the same quantity of $H_2S$ after 110 minutes as **$Bn_2S_4$**. 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 is may be significantly less stable than benzyl persulfide, which has been isolated, characterized, and studied extensively.[22] Additionally, a comparison of the $H_2S$ releasing curves demonstrates a brief induction period for **$Bn_2S_3$** and **$Bn_2S_4$** 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 $H_2S$ donors, we note that organic polysulfides provide lower $H_2S$ releasing efficiencies, which is likely a result of higher lipophilicity. We anticipate the design of water-soluble polysulfides should provide more efficient $H_2S$ 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 $S^0$ within a single series of polysulfides.

With these compounds in hand, we sought to determine the impact of increasing $S^0$ delivery on cell proliferation following 24 h treatments (Figure 4).

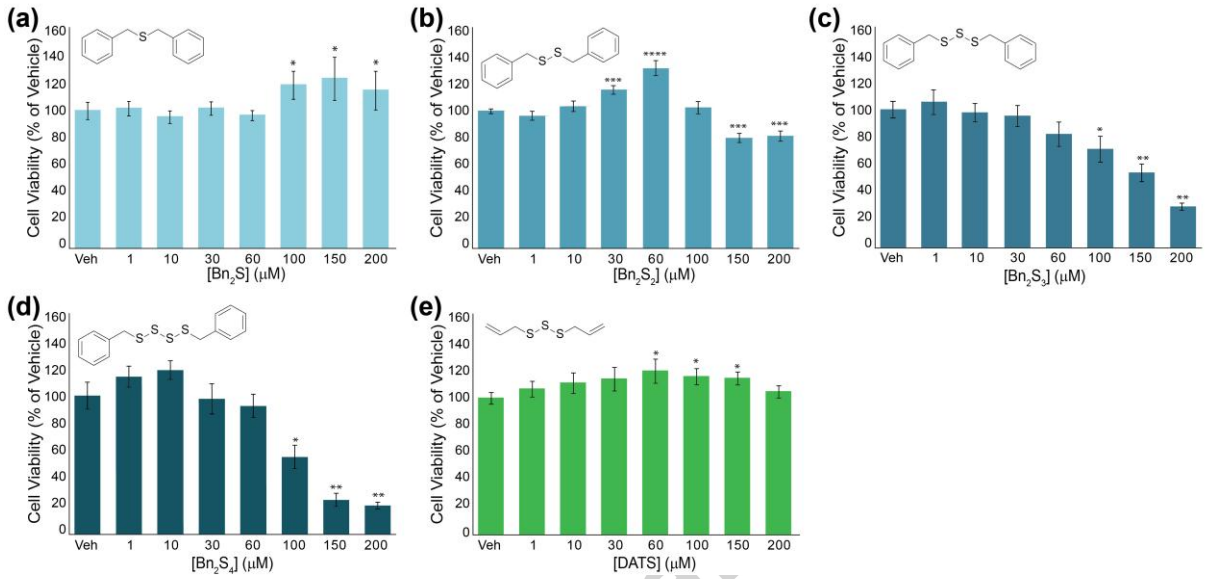



**Figure 4.** bEnd3 cell viability after 24-hour treatments of a series of organic polysulfides: (a) **$Bn_2S$**, (b) **$Bn_2S_2$**, (c) **$Bn_2S_3$**, (d) **$Bn_2S_4$**, and (e) **DATS**. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Use of synthetic $H_2S$ donors such as AP39[41] and direct administration of $H_2S$ via NaSH[42] 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 $S^0$ content, 24 h incubation with **$Bn_2S$** and **$Bn_2S_2$** (up to 200 μM) did not reduce cell viability. We note the slight cytotoxicity of **$Bn_2S_2$** at concentrations over 100 μM is likely due to perturbation of redox homeostasis arising from thiol-disulfide exchange between biological thiols and **$Bn_2S_2$**. Interestingly, $S^0$-containing **$Bn_2S_3$** and **$Bn_2S_4$** at concentrations of 100 μM and higher demonstrated considerable cytotoxicity. Notably, **$Bn_2S_4$** appears to be significantly more

cytotoxic than **$Bn_2S_3$**, which is consistent with the increased $S^0$ content in **$Bn_2S_4$** relative to **$Bn_2S_3$**.

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 $S^0$ 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 μM, we did not observe significant cytotoxicity from **BnSAc** suggesting the observed cytotoxicity for benzyl polysulfides is a direct reflection of $S^0$ 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 $S^0$ content on cell proliferation in bEnd.3 cells and suggests organic polysulfides can afford varying biological activities independent of $S^0$ 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 $S^0$ over $H_2S$ 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 $S^0$ 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 $H_2S$ from **$Bn_2S_3$** and **$Bn_2S_4$** respectively under physiological conditions. Additionally, we demonstrate the unique ability of **$Bn_2S_3$** and **$Bn_2S_4$** 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 $S^0$ in sulfur biology.

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## Highlights

- A series of benzyl (poly)sulfides with 1-4 sulfur atoms is prepared
- Rates of $H_2S$ release after treatment with Cys and GSH are reported
- $H_2S$ 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