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Thioglucose-derived tetrasulfide, a unique polysulfide model compound

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ABSTRACT

Polysulfides have received increased interest in redox biology due to their role as the precursors of H_2S and persulfides. However, the compounds that are suitable for biological investigations are limited to cysteine- and glutathione-derived polysulfides. In this work, we report the preparation and evaluation of a novel polysulfide derived from thioglucose, which represents the first carbohydrate-based polysulfide. This compound, thioglucose tetrasulfide (TGS4), showed excellent stability and water solubility. H_2S and persulfide production from TGS4, as well as its associated antioxidative property were also demonstrated. Additionally, TGS4 was demonstrated to significantly induce cellular sulfane sulfur level increase, in particular for the formation of hydropersulfides/trisulfides. These results suggest that TGS4 is a useful tool for polysulfide research.

1. Introduction

Polysulfides (RSS_nSR, $n \geq 1$) are naturally existing compounds that have been used for millennia for their antioxidant, antibacterial, and insect-repelling properties [1]. For instance, diallyl polysulfides are commonly isolated from garlic and other members of the *Allium* genus and are at least partially responsible for their beneficial properties. Interest in polysulfides has risen in recent years due to their connection with an important signaling molecule hydrogen sulfide (H_2S) [2]. Polysulfides can serve as the precursors of H_2S and be the metabolic products of H_2S. The presence of polysulfides with H_2S can lead to the formation of persulfides (RSSH), another highly reactive sulfur species and important regulators in redox biology [3]. Polysulfides are now being found in low millimolar and high micromolar concentrations in various human bodily fluids, which suggests that polysulfides have a greater importance within biological systems than traditionally believed [4].

Biologically relevant polysulfides are normally cysteine or glutathione derivatives. These compounds can be easily prepared and have good stability. They, together with diallyl polysulfides (shown in Scheme 1), are the most popular polysulfides used in many biological and biomedical studies [5]. Researchers often use them as H_2S donors or persulfide equivalents, as the reaction between polysulfides and cellular thiols (RSH) would produce H_2S or RSSH rapidly. As such, polysulfide compounds have exhibited a number of activities such as antioxidation and anti-inflammation.

Although cysteine- and glutathione-based polysulfides have proven to be useful tools for biological studies, exploring novel polysulfide molecular entities may generate new and unexplored biological activities. For this reason, we envisioned that carbohydrate-based polysulfides would assist in diversifying the currently limited scope of polysulfides used by researchers. With carbohydrates being the most abundant organic substance on earth, the potential to expand polysulfide chemistry into the realm of carbohydrates may be advantageous due to the vastly different metabolic and transport pathways used in carbohydrate metabolism and transportation compared to amino acids-derived polysulfides. In this article, we report the preparation and characterization of thioglucose tetrasulfide (TGS4) as a new carbohydrate-based polysulfide model compound that can be used in a variety of applications including H_2S donation, persulfide formation, protein persulfidation, and antioxidation.

2. Materials and methods

2.1. Materials and general methods

All reagents and solvents were of the highest quality available and purchased from Sigma-Aldrich, Cayman Chemical, Oakwood Chemical,

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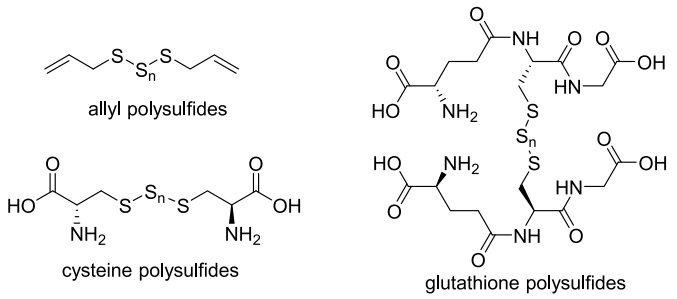


Scheme 1. The most popular polysulfides used for biological studies (n ≥ 1).

and Dojindo. Chemical reactions were magnetically stirred and monitored by thin-layer chromatography (TLC) with 0.25 mm pre-coated silica gel plates which were visualized with UV or with *p*-anisaldehyde stain. Flash chromatography was performed with silica gel 60 (particle size 0.040–0.062 mm). Both ^{1}H and ^{13}C NMR spectra were obtained from Bruker 400 and 600 MHz NMR spectrometers. H_2S concentrations were determined using a Unisense SULF-NP-211002 microsensor. UV–vis spectra were measured by a Thermo Scientific Evolution 350 UV–Vis Spectrophotometer using a 1 cm quartz cuvette. Fluorescence and absorbance spectra were recorded by a Molecular Devices SpectraMax iD3 Multi-Mode Microplate Reader when using a 96-well black/clear flat-bottomed plate.

2.2. Synthesis

2.2.1. Synthesis of TGS4

A suspension of 1-thio-*β*-D-glucose sodium salt (961 mg, 4.40 mmol, 1 equiv.) in freshly distilled ethanol (33 mL) was chilled to −78 °C under an argon atmosphere. After cooling the suspension for 10 min, S_2Cl_2 (0.21 mL, 360 mg, 0.6 equiv.) was added dropwise. The reaction mixture was allowed to stir until reaction completion was observed by TLC (2 h). Solid $NaHCO_3$ was then added to quench the reaction. The mixture was filtered to remove solids, and the filtrate was added directly to a silica gel column for purification using EtOAc and MeOH mixtures as eluants. Fractions containing pure product were combined and removed of solvents to afford TGS4 (50% yield) as a white solid product. ^{1}H NMR (600 MHz, CD_3OD) δ: 4.76 (d, 2H, $J = 9.2$ Hz), 3.92 (d, 2H, $J = 11.9$ Hz), 3.73 (dd, 2H, $J = 11.9$, 5.2 Hz), 3.51–3.43 (m, 4H), 3.42–3.38 (m, 4H); ^{13}C NMR (600 MHz, CD_3OD) δ: 91.85, 82.57, 79.53, 73.60, 71.24, 62.83; ESI-MS *m/z*: [M + H^+] calcd for $C_{12}H_{23}O_{10}S_4$ 455.0174; Found 455.0204.

2.2.2. Synthesis of OAc-TGS4

To a stirring solution of 1-thio-*β*-D-glucose tetraacetate (500 mg, 1.37 mmol, 1 equiv.) in CH_2Cl_2 (10 mL), pyridine (0.11 mL, 110 mg, 1.0 equiv.) was added under an argon atmosphere. The reaction was cooled to −78 °C and stirred for 1 h before adding S_2Cl_2 (0.065 mL, 110 mg, 0.6 equiv.) dropwise to the reaction mixture. The reaction was allowed to continue stirring at −78 °C until reaction completion was observed by TLC (2 h). The reaction was then quenched with saturated $NaHCO_3$ (2 mL) before washing the organic phase with H_2O (2 x 10 mL) and brine (2 x 10 mL). The organic phase was then dried over anhydrous $MgSO_4$ and filtered of solids. The solvent was removed *in vacuo* before purifying the crude residue by silica gel chromatography. Fractions containing pure product were then combined and removed of solvents to afford OAc-TGS4 (466 mg, 86% yield) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 5.27 (dd, 2H, $J = 19.7$, 10.1 Hz), 5.17 (dt, 4H, $J = 23.2$, 10.2), 4.74 (d, 2 H, $J = 9.8$ Hz), 4.29 (dd, 2H, $J = 12.4$, 3.4 Hz), 4.20 (dd, 2H, $J = 12.4$, 2.4 Hz), 3.82–3.77 (m, 2H), 2.11 (s, 6H), 2.07–1.99 (m, 18H); ^{13}C NMR (600 MHz, $CDCl_3$) δ: 170.81, 170.28, 169.46, 169.26, 88.22, 77.5, 73.86, 69.74, 68.10, 62.02, 20.94, 20.83, 20.73, 20.71; ESI-MS *m/z*: [M + Na^+] calcd for $C_{28}H_{38}O_{18}S_4Na$ 813.0833; Found 813.0845.

2.3. H_2S release measurements

2.3.1. Concentration-dependent H_2S release from TGS4 triggered by GSH

GSH and TGS4 stock solutions (10 mM) were freshly prepared in pure water. The GSH stock (0, 80, 200 or 400 μL) followed by the TGS4 stock (80 μL) was added to PBS (50 mM, pH 7.4) for a total volume of 8 mL in a sealable vial equipped with a magnetic stir bar. This resulted in a consistent final TGS4 concentration of 100 μM and GSH concentrations of 0, 100, 250, and 500 μM, respectively. The vial was immediately sealed, and a freshly calibrated Unisense H_2S microsensor was inserted into the vial. H_2S concentrations were monitored in real-time while the reaction vial stirred at room temperature for 1 h or 24 h.

2.3.2. Comparing H_2S release from TGS4 triggered by Cys, Hcy, or NAC

Each thiol was added to PBS (50 mM, pH 7.4) in a sealable 8 mL vial equipped with a magnetic stir bar so that the final concentration of thiol was 500 μM and the total volume was 7.92 mL. 80 μL of freshly prepared TGS4 stock (10 mM in water) was added to each solution for a final concentration of 100 μM. The vial was immediately sealed and allowed to stir for 55 min before inserting a freshly calibrated Unisense H_2S microsensor into the vial. The probe was allowed to equilibrate for 5 min before determining H_2S concentrations. This was repeated in triplicate.

2.3.3. H_2S liberation enhancement with GOx and GSH with TGS4

Stock solutions (10 mM) of GSH and TGS4 (or TGS2 as control) were prepared in ultrapure water. A 2 mg/mL GOx stock solution aliquot was freshly thawed before use. To an 8 mL sealable vial equipped with a magnetic stir bar, GSH (500 μL), GOx (40 μL), and TGS4 (100 μL) were added sequentially to PBS (7.48 mL). This resulted in final concentrations for GSH, GOx, and TGS4 to be 500 μM, 1 μg/mL, and 100 μM, respectively. The vial was immediately sealed, and a freshly calibrated Unisense H_2S microsensor was inserted into the vial. H_2S concentrations were monitored in real-time while the reaction vial stirred at room temperature for 1 h.

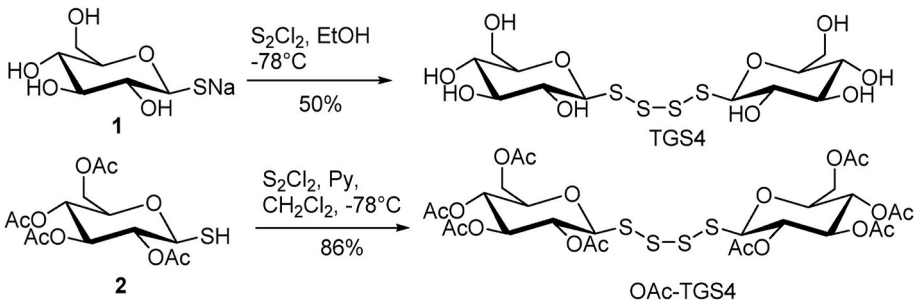
2.4. Autooxidation inhibition by TGS4

2.4.1. Autooxidation inhibition of fluorescein by TGS4

The reaction of fluorescein and peroxyl radicals was measured by the decay in absorbance at 494 nm as previously reported [6]. Briefly, Na_2S (10 mM), AAPH (400 mM), and TGS4 (1, 1.5, 2, 2.5, and 3 mM) stock solutions were freshly prepared in pure water. Fluorescein (10 mM) was also freshly prepared in DMSO. Fluorescein (10 μL) and Na_2S (150 μL) were added to a Falcon tube containing PBS (50 mM, pH 7.4) for a total volume of 10 mL and final fluorescein and Na_2S concentrations of 10 μM and 150 μM, respectively. To a 96 well plate, 186 μL aliquots of this fluorescein/Na_2S mixture was added followed by 4 μL of an appropriate TGS4 stock solution for final TGS4 concentrations of 20–60 μM. Lastly, the 10 μL of the AAPH stock solution was added to each well for a final AAPH concentration of 20 mM. The plate was mixed and immediately placed into a microplate reader (SpectraMax iD3) maintained at 37 °C, and UV–Vis absorbance was measured throughout the duration of the experiment.

2.4.2. Autooxidation inhibition of human plasma by TGS4

The measurement of lipid peroxidation was observed by measuring the increase in fluorescence of DPPP oxide as previously described [6]. Briefly, Na_2S (10 mM), AAPH (400 mM), and TGS4 (1, 1.5, 2, 2.5, and 3 mM) stock solutions were freshly prepared in pure water. DPPP (25 mM) was also freshly prepared in DMSO. DPPP (10 μL) was added to PBS (50 mM, pH 7.4) for a total volume of 9 mL. Human plasma (1 mL) was then added to the DPPP/PBS mixture, inverted to mix, and 186 μL aliquots of the resulting mixture were added a 96-well plate. This was followed by adding 4 μL of an appropriate TGS4 stock solution for final TGS4 concentrations of 20–60 μM. Lastly, the 10 μL of the AAPH stock solution was added to each well for a final AAPH concentration of 20 mM. The



Scheme 2. Preparation of TGS4 and OAc-TGS4.

plate was mixed and immediately placed into a microplate reader (SpectraMax iD3) maintained at 37 °C. DPPP oxide fluorescent measurements were observed using excitation and emission wavelengths of 345 and 385 nm, respectively. A single batch of a 10-person pooled human plasma (Innovative Research, #IPLANAC10ML) was used throughout this experiment, which was maintained at −20 °C until needed.

2.5. Cell imaging

HeLa cells were seeded in the inner wells of a 96-well black clear flat-bottomed TC-treated plate (ThermoFisher Scientific, #165305) in DMEM/F-12, HEPES medium supplemented with 10% fetal bovine serum (FBS) at 37 °C, 5% CO_2 overnight. Culturing media was then aspirated from all wells, and cells were washed twice with 1X PBS. TGS4 and TGS2 were prepared at 10 mM in Fluorobrite DMEM. Na_2S_2 was prepared at 10 mM in PBS. Compounds were diluted to 30 μM in Fluorobrite DMEM and added to cells (100 μL/well). Cells were incubated for 30 min at 37 °C, 5% CO_2 for 30 min. Media was then aspirated. Cells were washed 3X with PBS and treated with Fluorobrite DMEM containing SSP4 (5 μM, 0.08% DMSO) and CTAB (100 μM, 0.02% EtOH) or in only Fluorobrite DMEM. Cells were incubated for 30 min at 37 °C, 5% CO_2 before being washed 2X with PBS and suspended in Fluorobrite DMEM. Imaging occurred using the Keyence All-in-One Fluorescence Microscope (BZ-X810) (excitation: 470/40 nm; emission: 525/50 nm).

2.6. Cellular sulfur metabolomics

Sulfur metabolites in cells were determined by means of tandem mass spectrometry with use of thiol alkylating agent, β-(4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM) as previously reported [7,8]. The cells, including THP-1-differentiated macrophages and J774.1 cells, were treated with polysulfides at 250 μM for 30 min. Cells were then washed once with phosphate buffered saline (PBS) and were harvested with a methanol solution containing 5 mM HPE-IAM, followed by homogenization using a Bioruptor (Cosmo Bio, Tokyo, Japan). After incubation for 15 min at 37 °C, supernatants were separated via centrifugation and were diluted with 0.1% formic acid containing known amounts of isotope-labeled standards. Mixtures were then subjected to liquid chromatography-electrospray ionization-mass spectrometry with the Agilent 6460 Triple Quadrupole LC-MS/MS system (Agilent Technologies, Santa Clara, CA, USA). Precipitates were resuspended with 1% SDS in PBS and were then homogenized with a Bioruptor for 10 min, after which samples were used for quantifying protein concentrations with the BCA Protein Assay Kit (FUJIFILM Wako Pure Chemical Corporation). LC-MS/MS conditions were as follows: column, YMC-Triart C18 Plus column (2.1 × 50 mm) (YMC Co. Ltd., Kyoto, Japan); column temperature, 45 °C; injection volume, 10 μL; mobile phases: A, 0.1% formic acid, and B, acetonitrile; gradient (B concentration), 0 min–1%, 10 min–80%, 10.1 min–1%, 15 min–1%; and flow rate, 0.2 mL/min. The general conditions for ESI-MS were nebulizer gas, nitrogen, delivered at 50 psi; nebulizer gas temperature, 250 °C; capillary voltage, 3500 V; collision gas, and G1 grade, nitrogen (Taiyo Nippon Sanso Corporation, Tokyo, Japan). Table S6 provides details of the multiple reaction monitoring (MRM) parameters that we used in this study.

3. Results and discussion

To the best of our knowledge, carbohydrate-based polysulfides have not been reported. However, thiosugars such as thioglucose are known. Thioglucose has been used as a ligand to form metal complex nanoparticles [9] where the glucose moiety could help deliver nanoparticles through cell membranes, taking advantage of glucose transporters on the membrane [10]. Our recent studies even revealed that thioglucose could serve as a unique H_2S donor under catalysis by glucose oxidase (GOx) [11]. Starting from commercially available β-1-thioglucose and its O-acylated analog, the corresponding tetrasulfides (TGS4 and OAc-TGS4) could be prepared in one step (Scheme 2). Briefly, the –SH starting materials were treated with 0.6 equivalents of sulfur monochloride (S_2Cl_2) under low temperature (−78 °C) and offered the tetrasulfides in good yields (50% and 86%, respectively). Both compounds could be obtained on the gram scale. It is worth noting that β-1-thioglucose is more stable than its α-anomer and this stereochemistry was maintained in the reaction so TGS4 and OAc-TGS4 were also obtained as the β-isomer.

With these two tetrasulfides in hand, we first studied their stability, both in the solid form and in solutions. Like other polysulfides, their degradation should provide the corresponding disulfides and elemental sulfur (S_8). H NMR was used to monitor the degradation because of the distinct H NMR signals between tetrasulfides and disulfides. Both compounds appeared stable as solids and could be stored at room temperature for days without any appreciable degradation. OAc-TGS4 showed good solubility in organic solvents and exhibited excellent stability in solutions. For instance, no obvious degradation of OAc-TGS4 was observed in chloroform at ambient temperatures over several days. TGS4, on the other hand, showed excellent solubility in water but decreased stability in aqueous solutions. The solution of TGS4 in D_2O did not give a significant change in H NMR over 2 h while ~10% degradation was observed after 24 h (Fig. S1). Nevertheless, we believed that TGS4 possessed adequate stability as a polysulfide model compound.

A recent study by Akaike et al. found that some biologically relevant polysulfides are susceptible to hydrolysis-driven degradation in the presence of –SH labeling reagents such as iodoacetamide (IAM) and *N*-ethylmaleimide (NEM) [12]. To check the susceptibility of TGS4 to these chemicals, we monitored the changes of TGS4 in the presence of either IAM or NEM in D_2O and PBS buffer. Interestingly, we did not observe any changes over 4 h (Figs. S2 and S3). This enhanced stability of TGS4 toward –SH labeling reagents as compared to that of other polysulfides (such as cysteine- or GSH-derived ones) may be due to the multiple –OH groups in TGS4, as suggested by Akaike et al. [13] However, further studies are still needed to clarify the mechanism.

To better understand the structure of polysulfides, we pursued single-crystal X-ray diffraction (SC-XRD) measurements (Tables S1–S4).

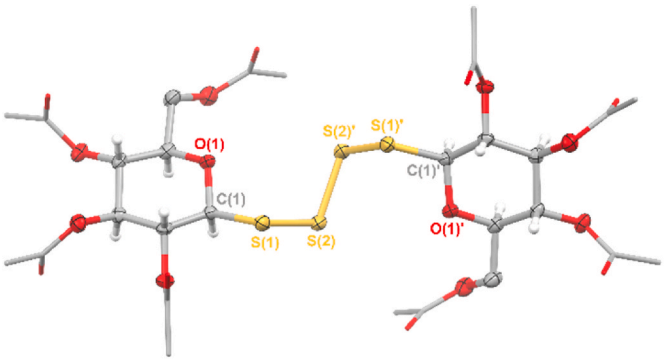


Fig. 1. Thermal ellipsoid plot (50 % probability) of **OAc-TGS4**. Acetate fragment displayed as capped sticks and non-glucosyl H-atoms removed for clarity.

Although we were unable to obtain crystals suitable for SC-XRD with TGS4, suitable crystals of OAc-TGS4 were obtained by vapor diffusing hexanes into OAc-TGS4 saturated THF in a sealed vial. As shown in Fig. 1, OAc-TGS4 crystallized in the monoclinic space group, C_2, with half of the tetrasulfide molecule present in the asymmetric unit. Consistent with other linear tetrasulfides [14], the central S–S fragment features a longer distance and smaller dihedral angle [i.e., sulfane–sulfane; S(2)–S(2)': 2.0516(12) Å, S(1)–S(2)–S(2)'–S(1)': 82.5(1)°] than the proximal S–S fragment [i.e., sulfane–sulfide; S(1)–S(2): 2.0346 (10) Å, C(1)–S(1)–S(2)–S(2)': 91.6(1)°]. Similarly, the central S–S fragment of OAc-TGS4 is ~0.02 Å longer than that of the corresponding acetate-protected glucosyl disulfide, OAc-TGS2 (Fig. S5, CCDC: LOJKUR and SUPFIS) [15]. The longer S–S bond distance found for the sulfane–sulfane linkage suggests a weaker S–S bond and heightened reactivity for OAc-TGS4 and TGS4 compared to OAc-TGS2 & TGS2.

As polysulfides are commonly considered as thiol-triggered H_2S donors, we next measured TGS4's H_2S release ability in the presence of GSH in PBS buffers. A Unisense H_2S microsensor was used in this study. As shown in Fig. 2A, an increased GSH concentration resulted in a more efficient liberation of H_2S from TGS4. Stoichiometrically, TGS4 could release up to 2 equivalents of H_2S and at least 4 equivalents of GSH are needed to achieve this goal. However, we measured only ~10% H_2S release when 1 eq. of GSH was used, and slightly more than 1 eq. of H_2S was released when 5 eq. of GSH was used. Both were under a 1 h incubation. We also tested the long period (24 h) release of H_2S from TGS4 under 5 eq. of GSH. As shown in Fig. 2C, H_2S concentration gradually increased in the first 2 h and then started to drop during further incubation (likely due to the escape of H_2S gas from the solutions). This releasing curve is typical as other thiol-triggered H_2S donors have shown similar curves [16]. These results indicate that H_2S generation from TGS4 can be maintained for hours. Since the actual cellular GSH concentration is estimated to be at mM levels, TGS4 should be an efficient H_2S donor in cellular environments.

Although GSH is the most dominant cellular thiol, we also tested three other thiols – cysteine (Cys), homocysteine (Hcy), and *N*-acetyl cysteine (NAC) for comparison (Fig. 2B). Cys and Hcy displayed similar H_2S release profiles as GSH while NAC led to significantly less H_2S release than the other thiols. This may be attributed to the lack of a free amine group in NAC. A recent work by Brown et al. suggests that the presence of free amines could accelerate the degradation of polysulfides [17].

It is clear now that polysulfides are effective H_2S donors in the presence of small molecule thiols. However, it is still unclear if –SH containing proteins could also promote H_2S release from polysulfides, and this has never been studied. Albumin is the most abundant protein in mammalian plasma, thus we tested TGS4's response with bovine serum albumin (BSA). It is known that BSA has one free –SH (C34) and 17 disulfide bonds. We determined the free thiol content in our BSA sample, which was freshly reduced by mercaptoethanol, and found it to be ~5 –SH per BSA. This suggested that two disulfide bonds were reduced. Then, TGS4 (100 μM) was treated with BSA or GSH under the same total thiol concentration (500 μM), and H_2S release was recorded. As shown in Fig. 3A, BSA was found to promote H_2S release from TGS4 more effectively than GSH. We proposed that the free thiol (-SH) residues of BSA could rapidly extract the sulfane sulfur atom of the

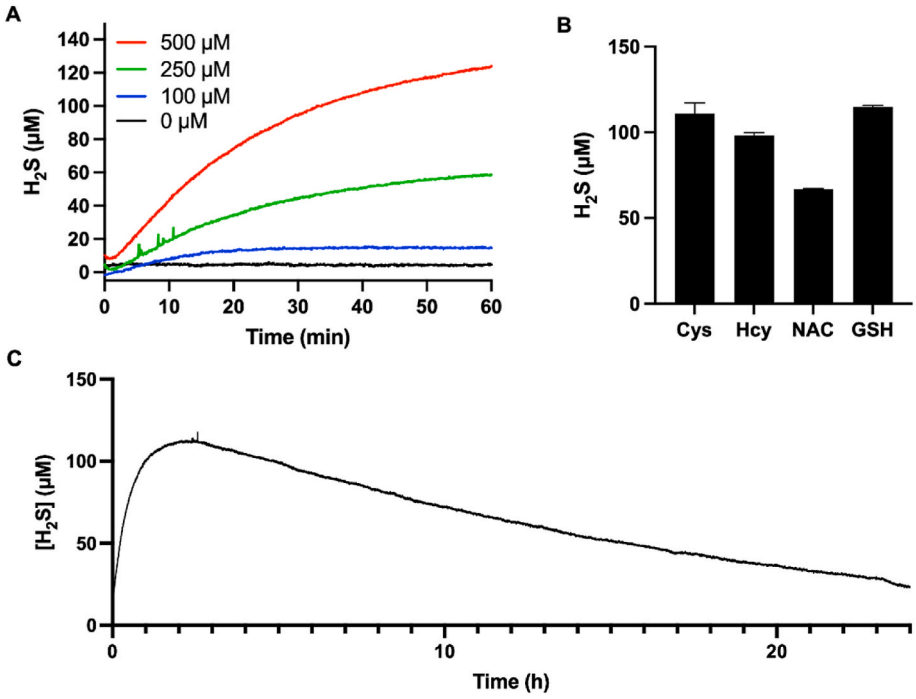


Fig. 2. (A) Concentration-dependent H_2S release from TGS4 (100 μM) and GSH (0–500 μM) in PBS (pH 7.4, 50 mM). (B) H_2S release from TGS4 (100 μM) after 60 min incubation with different thiols (500 μM) in PBS (pH 7.4, 50 mM). Error bars represent the mean ± SEM ($n = 3$). (C) Time-dependent H_2S release from TGS4 (100 μM) and GSH (500 μM) in PBS (50 mM, pH 7.4) over 24 h.

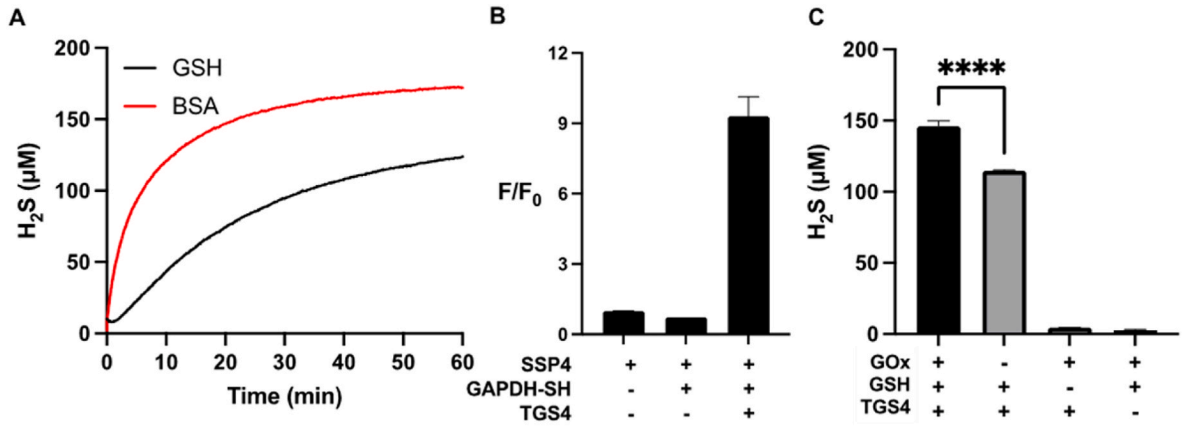


Fig. 3. (A) H_2S release from TGS4 (100 μM) with reduced BSA (100 μM) or with GSH (500 μM) in PBS (pH 7.4, 50 mM). (B) Fluorescence enhancement of SSP4 (5 μM) in the presence of GAPDH (5 μM) persulfidated by TGS4. Results are represented as the mean ± SEM ($n = 3$). (C) H_2S release after 60 min with or without GOx (1 μg/mL), GSH (500 μM), and TGS4 (100 μM) in PBS (pH 7.4, 50 mM). Error bars represent the mean ± SEM ($n = 3$). ****, $P < 0.0001$.

polysulfide TGS4 to form persulfides (-SSH), which is known as protein *S*-persulfidation. The persulfides would subsequently react with another –SH in close proximity to form the natural disulfide bonds in BSA and liberate H_2S. This process may be more efficient than the reaction with small molecule thiols like GSH.

To further demonstrate that TGS4 could induce protein *S*-persulfidation, we tested it with glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a commonly used *S*-persulfidation model. Briefly, reduced GAPDH (55 μM) was mixed with TGS4 (10 equiv.) in PBS (50 mM, pH 7.4) for 30 min. The modified protein was then purified by Zeba 7K MWCO column to remove the excess small molecule reagent. The level of persulfidation on GAPDH was validated by SSP4, a fluorescent sensor for persulfides, using our established protocol [18]. The signal was compared to that of untreated protein sample under the same concentration. As shown in Fig. 3B, TGS4 treatment led to a significant fluorescence increase as compared to that of the untreated protein under the same concentration. These results suggest that TGS4 is effective at protein *S*-persulfidation.

When TGS4 reacts with excess GSH, the final product should be thioglucose (GlcSH), in addition to H_2S production. In our previous research, we have demonstrated that glucose oxidase (GOx) could convert GlcSH to H_2S [11]. We thus wondered if the combination of GSH and GOx could further enhance H_2S production from TGS4. As shown in Fig. 3C, when GOx (1 μg/mL) was applied into the mixture of GSH (500 μM) and TGS4 (100 μM), H_2S production was increased by 21% compared to the reaction without GOx. This significant difference in H_2S release confirmed that GlcSH was formed from TGS4, and GOx activity was not affected by the polysulfide.

NADH is a universal electron donor and reductant in biology. It was hypothesized that NADH might react with polysulfides to produce H_2S. Our previous study investigated the reactions between a cysteine polysulfide with two NADH model compounds-Hantzsch ester and 1-benzyl-1,4-dihydronicotinamide (BNAH) [19]. It was found that H_2S was indeed generated. However, in those studies, the concentrations of substrates were quite high (50 mM of BNAH and 100 mM of cysteine tetrasulfide) and the reactions were done in pure organic solvents (EtOH and acetonitrile). We revisited this reaction using TGS4 and two other polysulfides-N-acetyl cysteine tetrasulfide (NACS2) and diallyl trisulfide (DATS). When these polysulfides (100 mM) were treated with BNAH (50 mM) in MeOH at 37 °C, a clean albeit slow conversion of BNAH to BNA^+ was observed over 5 h (monitored by NMR), with 83%–95% yields (see Table S5). These results indicate that polysulfides in general are reactive toward NADH model compounds under these conditions. Since TGS4 is highly water soluble, it was then used in pure aqueous buffers to test if polysulfides could directly react with the NADH coenzyme to release H_2S. An H_2S gas trapping experiment was employed for this study. Briefly, a 2 mL solution of TGS4 (20 mM) and NADH (10 mM) in Tris buffer (pH 7.4, 100 mM) was placed in a 20 mL scintillation vial. A separate 1.5 mL Eppendorf tube containing 0.5 mL of 10% $Zn(OAc)_2$ was then placed into the scintillation vial, which was then sealed and kept at 37 °C for 16 h in the dark. After this reaction, the trapping solution in the Eppendorf tube was added to a methylene blue cocktail

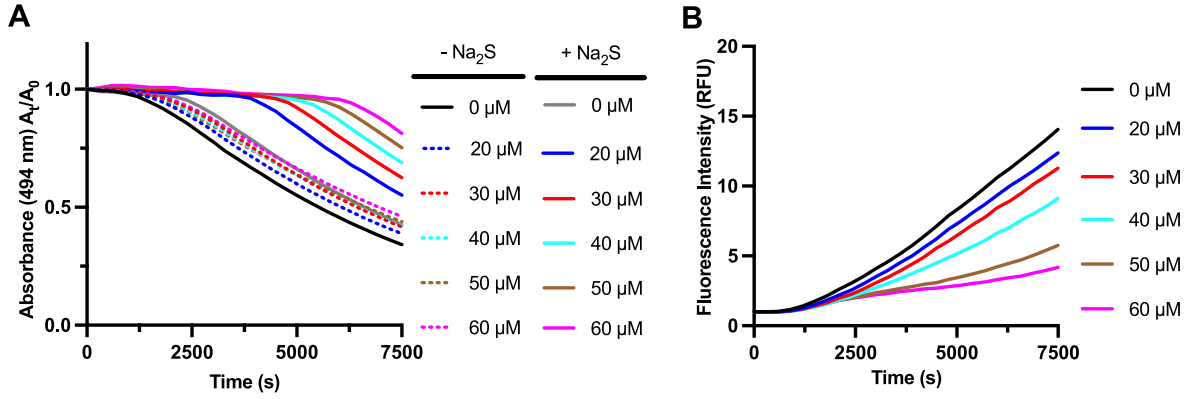


Fig. 4. (A) Concentration-dependent antioxidant function of TGS4 with or without the addition of Na_2S (150 μM) as observed by the delay of fluorescence decrease resulting from the autoxidation of fluorescein (10 μM) induced by AAPH (20 mM) in PBS (pH 7.4, 50 mM). (B) Concentration-dependent antioxidant function of TGS4 as observed by the increase of fluorescence resulting from the oxidation of DPPP (25 μM) induced by the autooxidation of human plasma (10%) by AAPH (20 mM) in PBS (pH 7.4, 50 mM).

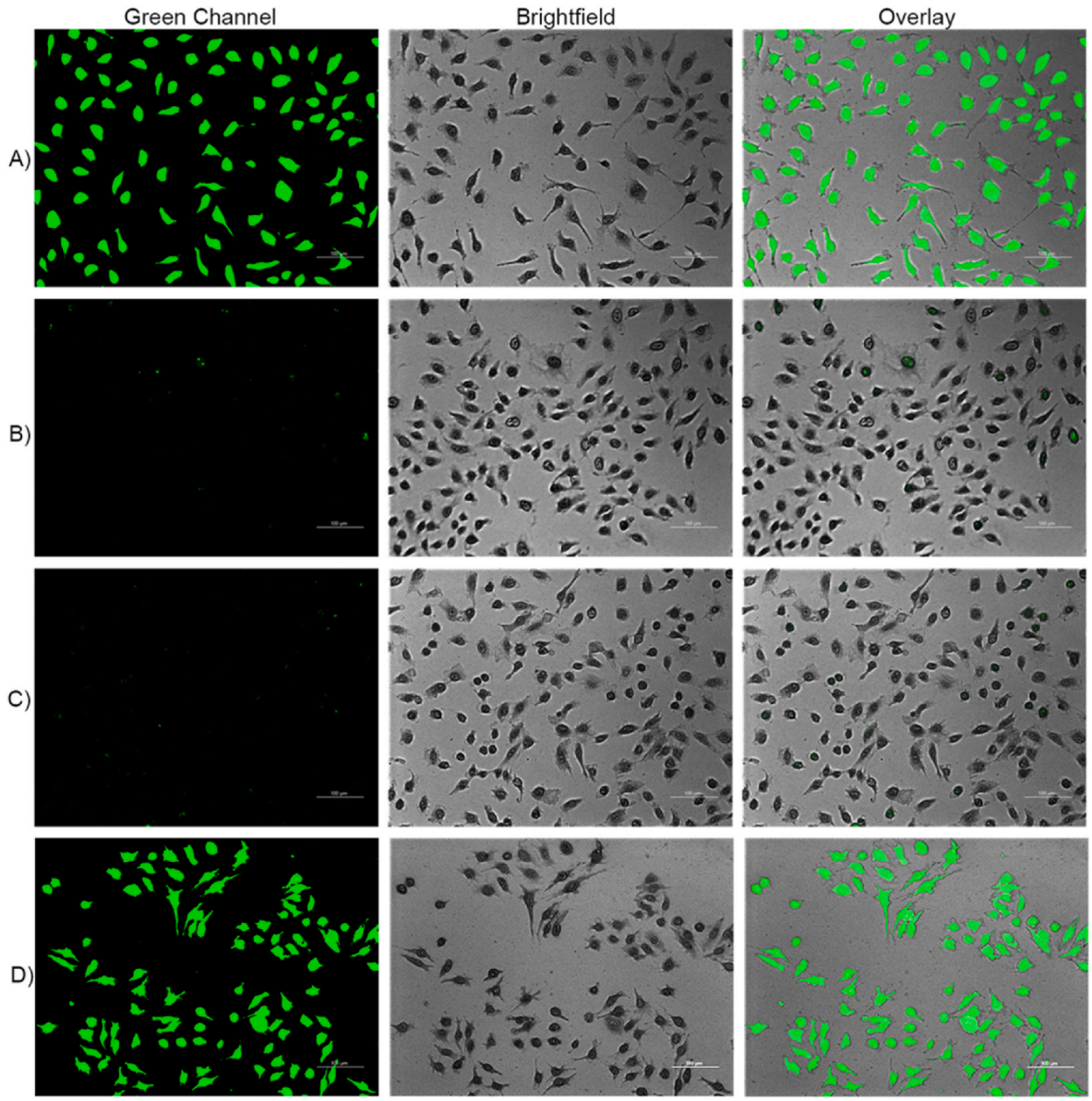


Fig. 5. Representative fluorescence images of HeLa cells treated with 30 μM of (A) TGS4, (B) TGS2, (C) no treatment, or (D) Na_2S_2 and incubated for 30 min at 37 °C, 5% CO_2 for 30 min. They were then washed 3X with PBS, treated with SSP4 (5 μM) and CTAB (100 μM), and incubated for 30 min at 37 °C, 5% CO_2. Cells were then washed 2X with PBS and suspended in Fluorobrite DMEM before imaging. Scale bars, 100 μm.

containing 0.5 mL each of $FeCl_3$ (33 mM in 1.2 M HCl) and *N, N*-dimethyl-1,4-phenylenediamine sulfate (20 mM in 7 M HCl). In the presence of H_2S, this solution will turn a blue color that has an absorbance measured at 670 nm. As predicted, H_2S was found to be liberated from TGS4 under these conditions. However, when a lower concentration of TGS4/NADH (1 mM: 0.5 mM) was used, no detectable H_2S was noted. As cellular NADH concentrations are estimated to be at the hundreds micromolar levels, we conclude that NADH-promoted H_2S release from polysulfides is not a significant source of H_2S in physiological contexts.

Polysulfides are often used as persulfide donors, and they can react with cellular thiols to *in situ* generate persulfides and execute persulfide-like activities [20]. The antioxidant activity of persulfides has been well documented and is attributed to perthiyl radical formation. We expected TGS4 to also show antioxidant activity, and two experiments were carried out to prove this property. The first one was to evaluate TGS4's inhibition toward the autooxidation of fluorescein with 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) [6]. As shown in Fig. 4A, the autooxidation of fluorescein would result in the loss of absorbance at 494 nm. When this system was treated with TGS4 only (20–60 μM), very minor antioxidation was observed. However, if H_2S (150 μM) was also present, a concentration-dependent delay in oxidation was noted (e.g. increased lag time before a loss of absorbance was observed). The second method was to test TGS4's inhibition of AAPH-induced oxidation on a fluorescent probe diphenyl-1-pyrenylphosphine (DPPP) in plasma-containing solutions. The oxidation of lipids in human plasma would result in the fluorescent turn-on of DPPP by its conversion into DPPP oxide. In this experiment, the addition of H_2S was unnecessary, as there were already sufficient free thiols present in the plasma to interact with TGS4. Again, we observed a concentration-dependent antioxidant function of TGS4 (Fig. 4B).

To showcase the ability of TGS4 to be used in cell studies, we performed a cell viability assay (CCK-8) using HeLa cells after a 24 h incubation with TGS4, its degradation product (TGS2), and its final reduction product thioglucose (all up to 100 μM). We observed very minor loss of cell viability (<20%, Fig. S8). We then carried out cell

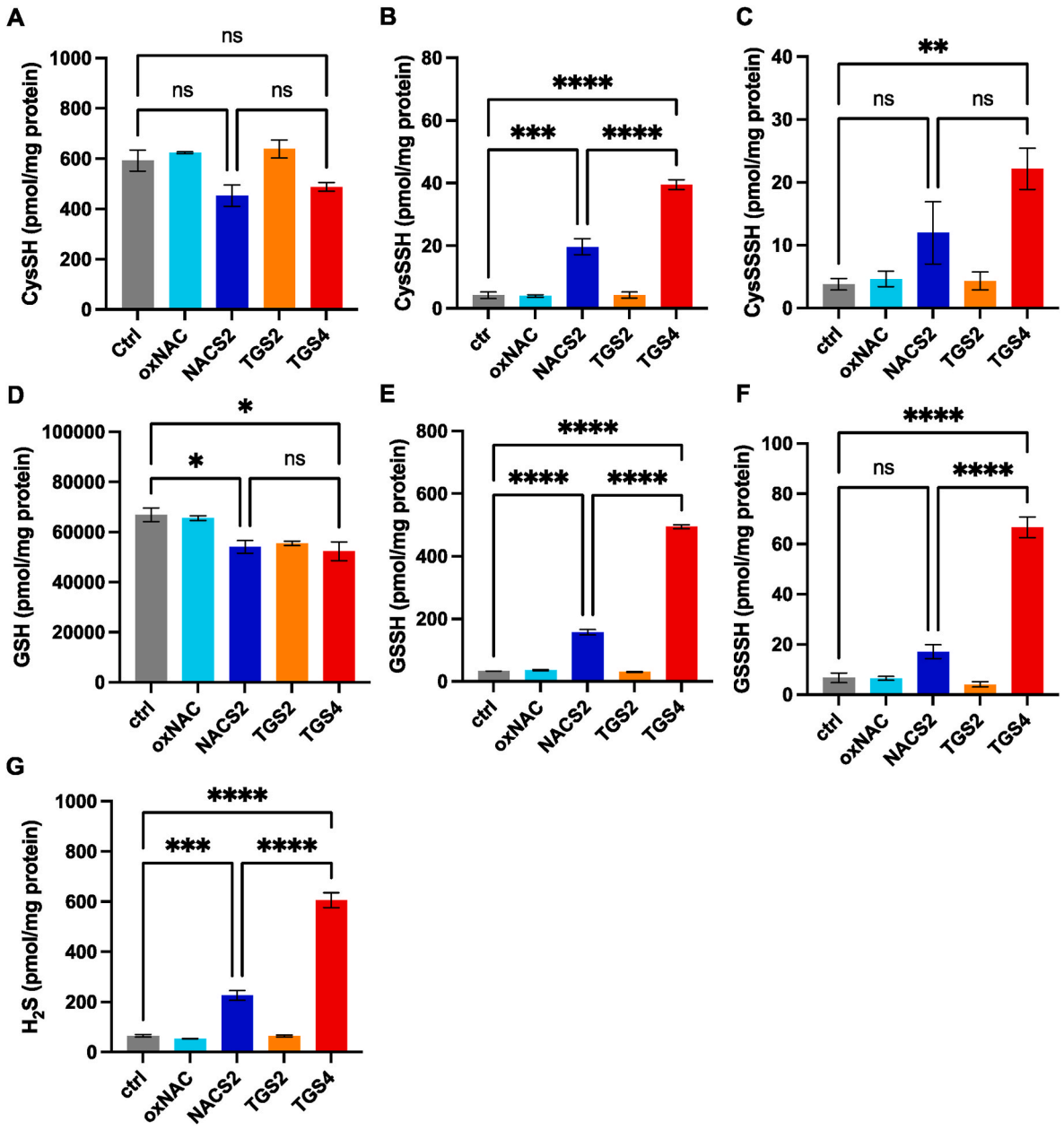


Fig. 6. Increase of cellular persulfides, polysulfides, and H_2S upon treatments with NACS2 and TGS4. Mouse macrophage J744.1 cells were treated with the indicated compounds at 250 μM for 3 h. Intracellular sulfur metabolites were determined by means of tandem mass spectrometry with use of the alkylating agent HPE-IAM. Sulfur species analyzed were (A) CysSH, (B) CysSSH, (C) CysSSSH, (D) GSH, (E) GSSH, (F) GSSSH, and (G) H_2S. Data are represented as mean ± SEM ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

imaging studies to see if TGS4 could deliver polysulfides into cells. Briefly, TGS4 (30 μM) was incubated with HeLa cells for 30 min before washing the cells thoroughly with PBS to remove extracellular TGS4. This was followed by applying SSP4 to the cells to monitor sulfane sulfur levels in the cells. As shown in Fig. 5, a significant increase in fluorescence was observed in cells treated with TGS4 compared to cells treated with TGS2 or cells with no treatment. These results demonstrate the ability of TGS4 to pass through the cell membrane, likely due to the assistance by glucose transporters on the cell membrane. This makes TGS4 a suitable polysulfide for biological study as it displays both low toxicity and good cellular uptake, which is vital to any compound used in these systems.

Recent studies by Sawa et al. suggested polysulfides such as *N*-acetylcysteine tetrasulfide (NACS2) are efficient at sulfur transfer between the polysulfide and naturally occurring endogenous thiols [8]. As a result, the concentrations of the endogenous low molecular weight persulfides and polysulfides in cells would increase if the cells were treated with NACS2. This can be determined by using the alkylating agent HPE-IAM to trap these persulfide species in cells, followed by LC-MS/MS analysis. We wondered if TGS4 could behave similarly to NACS2 in these studies and thus measured cellular persulfide contents in TGS4-treated cell models. Briefly, mouse macrophage J744.1 cells were treated with TGS4 (250 μM) for 3 h. Intracellular sulfur metabolites were then determined by trapping with HPE-IAM followed by tandem mass spectrometry analysis using known protocols. The experiments with NACS2 and the corresponding disulfides (oxNAC and TGS2) were also performed for comparison. The results are shown in Fig. 6. It was found that the treatment of TGS4 and NACS2 did not significantly change cellular thiol levels (Cys and GSH). However, cellular hydropersulfides and hydrotrisulfides (CysSSH, CysSSSH, GSSH, GSSSH) were

significantly increased. H_2S levels were also increased. Similar studies were also done with human THP-1 cells differentiated to macrophages, and the same results were observed (Fig. S9). Our results also showed that TGS4 was more potent than NACS2 in causing those changes, suggesting TSG4 was an efficient hydropersulfide donor for cell studies.

4. Conclusion

Polysulfides can enhance cellular H_2S and persulfide production and have seen increased use in the research of sulfur-related redox biology. However, currently available polysulfides for researchers are still limited, mainly cysteine and glutathione-based polysulfides. Here, we report the preparation and evaluation of a novel polysulfide model compound-thioglucose tetrasulfide (TGS4). This compound can be easily prepared in one step from commercially available starting materials. It displays good stability and solubility in water. Like other polysulfides, TGS4 can release H_2S when exposed to various biological thiols. TGS4 can also be used as a persulfide precursor in biological settings, which accounts for its antioxidation activity. Furthermore, TGS4 can induce the persulfidation of proteins and easily enter cells while also displaying low cytotoxicity. The persulfidation of endogenous thiols by TGS4 was also observed. All these results suggest TGS4 is a useful polysulfide model for further exploration of polysulfide functions.

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CRediT authorship contribution statement

Stephen Lindahl: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Meg Shieh:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Tianli Zhang:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Chunyu Guo:** Methodology, Investigation, Formal analysis, Data curation. **Jerome R. Robinson:** Writing – original draft, Formal analysis, Data curation. **Tomohiro Sawa:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Formal analysis, Data curation. **Ming Xian:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2024.103045.

Abbreviations

TGS4	thioglucose tetrasulfide
TGS2	thioglucose disulfide
OAc-TGS4	O-acylated thioglucose tetrasulfide
IAM	iodoacetamide
NEM	*N*-ethylmaleimide
GSH	glutathione
Cys	cysteine
Hcy	homocysteine
NAC	*N*-acetyl cysteine
GOx	glucose oxidase
NAC-S2	*N*-acetylcysteine tetrasulfide
DATS	diallyl trisulfide
NADH	dihydronicotinamide adenine dinucleotide
PBS	phosphate buffered saline
AAPH	azobis(2-amidinopropane) dihydrochloride
DPPP	diphenyl-1-pyrenylphosphine
SSP4	sulfane sulfur probe 4
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GlcSH	thioglucose

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