DEVELOPING AND UTILIZING CHEMICAL TOOLS TO PROBE THE NATURE OF BIOLOGICAL SULFUR SPECIES

by

SARAH BOLTON

A DISSERTATION

Presented to the Department of Chemistry and Biochemistry
and the Division of Graduate Studies of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

September 2021

DISSERTATION APPROVAL PAGE

Student: Sarah Bolton

Title: Developing and Utilizing Chemical Tools to Probe the Nature of Biological Sulfur Species

This dissertation has been accepted and approved in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Chemistry and Biochemistry by:

Bradley Nolen	Chairperson
Michael Pluth	Advisor
Victoria DeRose	Core Member
Bruce Branchaud	Core Member
Scott Hansen	Core Member
Anne Zemper	Institutional Representative

and

Andrew Karduna	Interim Vice Provost for Graduate Studies

Original approval signatures are on file with the University of Oregon Division of Graduate Studies.

Degree awarded September 2021

DISSERTATION ABSTRACT

Sarah Bolton

Doctor of Philosophy

Department of Chemistry and Biochemistry

September 2021

Title: Developing and Utilizing Chemical Tools to Probe the Nature of Biological Sulfur Species

Life on Earth has always been shaped by sulfur. Over 2 billion years ago, before our atmosphere became rich in oxygen, volcanic activity was more prevalent. As a result, sulfur-containing gasses were much more prominent in the early Earth atmosphere, causing it to be weakly reducing as opposed to than the oxidizing conditions today. It was this low-oxygen, high-sulfur atmosphere that allowed the creation of the earliest biomolecules, and life forms, on Earth. We see the legacy of this sulfur-based origin in our aerobic, eukaryotic cells today, which can metabolize reduced sulfur species into ATP.

One prominent biological sulfur species today is hydrogen sulfide (H_2S), produced in cysteine metabolism and a member of the gasotransmitter family of small, gaseous signaling molecules along with carbon monoxide (CO) and nitric oxide (NO). H_2S has gained increasing notice for its abilities to induce vasodilation and angiogenesis and reduce inflammation and oxidative stress, among others. This has motivated the development and use of triggerable H_2S donor molecules that more accurately mimic endogenous H_2S production than inorganic SH^- salts. Recent studies have found that the chemical biology attributed to H_2S may be caused by the downstream production of

oxidized sulfur species containing sulfane sulfur (S^0). S^0 is found in polysulfides and can both release H_2S in the presence of biological thiols and react with free cysteine residues on proteins to form persulfides (-SSH) in a process known as persulfidation. The ability of S^0 to persulfidate proteins has now been implicated in many of the observed effects of H_2S treatment, as persulfidation alters structure and function of proteins and serves as a regulatory switch. It is desirable to find ways of understanding and harnessing the power of protein persulfidation, and this necessitates the development of S^0 donor molecules.

We have developed systems to achieve this, including libraries of N-acetylcysteine (NAC)- and benzyl-based polysulfides. We also developed a method of solvating elemental sulfur (S_8), the only source of pure S^0. These systems are compatible with both cell and enzymatic assays and open the door to new S^0 discoveries.

This dissertation includes previously published coauthored material.

CURRICULUM VITAE

NAME OF AUTHOR: Sarah Bolton

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene
California Polytechnic State University, San Luis Obispo
Cuesta College, San Luis Obispo, California

DEGREES AWARDED:

Doctor of Philosophy, Chemistry, 2021, University of Oregon
Bachelor of Science, Biochemistry, 2016, California Polytechnic State University, San Luis Obispo

AREAS OF SPECIAL INTEREST:

Biochemistry and Chemical Biology
Biological Reagent Development

PROFESSIONAL EXPERIENCE:

Graduate Employee, University of Oregon, 2016-2021]
Learning Assistant, California Polytechnic State University, San Luis Obispo, 2015-2016]

GRANTS, AWARDS, AND HONORS:

National Institutes of Health Training Grant, University of Oregon, 2017-2020
Young Investigator Award, Society for Redox Biology and Medicine, 2020

PUBLICATIONS:

Lovell, T.C.; Bolton, S.G.; Kenison, J.P.; Shangguan, J.; Otteson, C.E.; Civitci, F.; Nan, X.; Pluth, M.D.; Jasti, R., Subcellular Targeted Nanohoop for One- and Two-Photon Live Cell Imaging. *ACS Nano* **2021**, Accepted for Publication.

Ismail, I.; Chen, Z.; Sun, L.; Ji, X.; Ye, H.; Kang, X.; Huang, H.; Song, H.; Bolton, S.G.; Xi, Z.; Pluth, M.D.; Long, Y., Highly Efficient H_2S Scavengers via Thiolysis of Positively-Charged NBD Amines. *Chemical Science* **2020**, *11*, 7823-7828.

Bolton, S.G.; Pluth, M.D., Modified Cyclodextrins Solubilize Elemental Sulfur in Water and Enable Biological Sulfane Sulfur Delivery. *Chemical Science* **2020**, *11*, 11777-11784.

Bolton, S.G.;† Cerda, M.M.;† Gilbert, A.K.; Pluth, M.D., Effects of Sulfane Sulfur Content in Benzyl Polysulfides on Thiol-Triggered H_2S Release and Cell Proliferation. *Free Radical Biology & Medicine* **2019**, *131*, 393-398.

Zhao, Y.; Bolton, S.G.; Pluth, M.D., Light-Activated COS/H_2S Donation from Photocaged Thiocarbamates. *Organic Letters*, **2017** *19* (9), 2278-2281.

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Michael Pluth for his mentorship and his guidance of this research, and I thank each of my committee members. I also want to express appreciation for my lab-mates, past and present, who provided support, materials, and insight – particularly Drs. Matthew Cerda and Andrea Steiger. I would like to thank the Institute of Molecular Biology for generously providing their facilities and the University of Oregon for providing a supportive work and research environment. This research was funded by National Institutes of Health Training Grant T32 GM007759 and National Science Foundation Grant CHE-2004150.

To Ryan, Andy, and Kaia

TABLE OF CONTENTS

Chapter Page

Chapter Page

Chapter Page

Chapter Page

Chapter Page

LIST OF FIGURES

Figure Page

Figure Page

LIST OF TABLES

Table Page

LIST OF SCHEMES

Scheme Page

CHAPTER I

MORE IS MORE: POLYSULFIDES, PERSULFIDES AND THE BIOLOGICAL ROLES OF SULFANE SULFUR

Portions of this dissertation are coauthored or based on collaborative projects. Chapter II was published in *Free Radical Biology & Medicine* in 2019 with Matthew Cerda and Annie Gilbert. Chapters III and V contain compounds synthesized by Matthew Cerda. Chapter VI was published in *Organic Letters* in 2017 with Yu Zhao. Chapter IV was published in *Chemical Science* in 2020 and was not coauthored nor collaborative, along with Chapters I and VI.

1.1. Introduction

Redox chemistry lies at the heart of all biological systems. The flow of electrons through the different subunits of the electron transport chain (ETC) in a series of oxidation-reduction reactions provide the energy needed to drive the enzyme ATP synthase and power the cells of aerobic organisms.[1] Given the reactivity of redox-active species, it is critical that the redox environment of a cell remains balanced. Oxidative stress (OS), where cellular systems suffer from excess levels of oxidant species or a shortage of antioxidants, has wide-ranging, damaging effects including oxidation of lipids, proteins, and DNA.[2, 3] Complex and sophisticated systems stabilize and regulate the relative amounts of redox-active species present throughout the cell through enzymatic activity and altered gene expression.[4, 5] Familiar redox active species include reactive oxygen species (ROS) and reactive nitrogen species (RNS), but another element

that is getting increasing recognition for its significance in the biological redox environment is sulfur. Sulfur is capable of accessing a wide range of redox states, from -2 to +6 in biologically relevant reactive sulfur species (RSS).[6] This range of redox states makes RSS uniquely versatile in cellular environments, and capable of influencing the redox environment.

One prominent RSS is hydrogen sulfide (H_2S), a member of the family of small, gaseous signaling molecules known as the gasotransmitters, which also includes carbon monoxide (CO) and nitric oxide (NO).[7-10] H_2S is endogenously produced from L-cysteine by the enzymes cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3MST).[11] The sulfur atom in H_2S has a redox state of -2, and H_2S is a fairly good reducing agent, with a two-electron reduction potential, $E^{\circ\prime}(HS^{2-}, H^{+}/2HS^{-})$, of −0.23 V.[6, 12] H_2S is also a known antioxidant agent in cells.[13] However, the primary antioxidative power of H_2S comes not from direct reaction with oxidants, but from the upregulation of more powerful reducing agents like glutathione (GSH).[14, 15] This upregulation is accomplished by the activation of Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which translocates to the nucleus and upregulates a suite of antioxidant genes.[16] H_2S also acts alongside NO in upregulating cellular cGMP levels, which in turn promotes angiogenesis, smooth muscle dilation, reduction of inflammation, and cardioprotection.[17-19]

The chemistry of H_2S reflects the versatility of sulfur. H_2S can be oxidized in cells into polysulfides, chains of oxidized and reduced sulfur species.[20] Polysulfides have unique chemistry and cellular interactions separate from those of H_2S. A diet rich in sulfur has been shown to promote longevity, and the presence of supercentenarians is

strongly correlated to sulfur-rich environments.[21] The sulfur we consume generally comes in the form of polysulfides and is especially prevalent in alliums. The best-studied natural organic polysulfide is diallyl trisulfide (DATS), found in garlic and produced in the decomposition of allicin.[22] DATS has been shown to offer a wide range of benefits to cells including reductions in OS[23, 24] and inflammation.[25, 26] Additionally, it shows promise as a potential cancer therapeutic through its ability to inhibit cancer cell metastasis[27] and promote cancer cell apoptosis.[28, 29]

Polysulfides are superior reducing agents to H_2S,[30] and demonstrate greater neuroprotective effects.[31] Like H_2S, polysulfides exert cell-protective antioxidant effects through the induction of Nrf2 translocation to the nucleus and the subsequent upregulation of antioxidant species synthesis.[32] Additionally, polysulfides induced maximal response of transient receptor potential (TRP)A1 channels in rat astrocytes at only 1/320 the concentration of H_2S. To understand these striking differences requires taking a closer look at polysulfides, particularly those containing S^0 atoms. S^0, also known as sulfane sulfur, is a sulfur atom that is either bound only to other sulfur atoms or is bound to a sulfur atom and an ionizable hydrogen (as in a persulfide) (Figure 1.1).

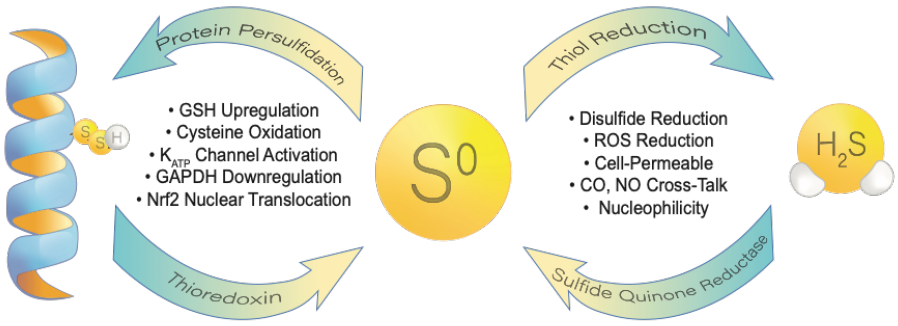


Figure 1.1. An overview of some of the roles of sulfane sulfur (S^0). Biological sulfur species can change from oxidized to reduced states to suit various cellular conditions or to activate different pathways.

S^0 can react with biological thiols such as cysteine or GSH to form H_2S.[33] Does this mean that the effects we see of polysulfide treatments in cells must simply be caused by "stored" H_2S? H_2S has only one sulfur atom in a -2 redox state, can reduce protein disulfide bonds, but not oxidize cysteine residues.[34] Conversely, the oxidized sulfane sulfur atom is able to react with reduced cysteine residues and form persulfides on proteins[35] in a process known as persulfidation.[36] Since H_2S can become oxidized in cells, researchers are now delving deeper into the biological roles of polysulfides and suspect that many of the observed effects of H_2S treatment could be due to the downstream production of polysulfide species.[37] This introductory chapter explores the current understanding of biologically relevant polysulfides and the process of protein persulfidation (Figure 1.2).

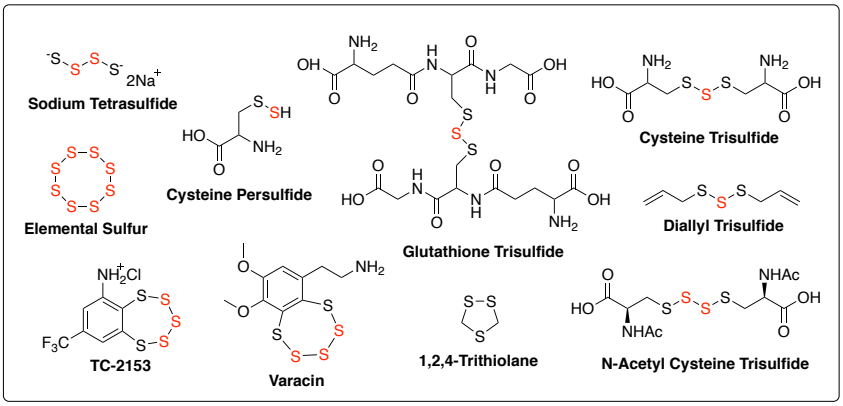


Figure 1.2. A selection of biologically relevant RSS containing oxidized sulfur. Sulfane sulfur atoms, which can react with thiols to form H_2S and enable persulfidation of proteins, are highlighted in red.

1.2. Types of Sulfane Sulfur Donors

1.2.1. Persulfides

Persulfides are compounds with the form R-SSH. The sulfur atom that is bound to both sulfur and the ionizable hydrogen atoms is sulfane sulfur, thus these compounds can

participate in the unique chemistry of polysulfides including H_2S release, enhanced antioxidative action, and the ability to persulfidate proteins.[38] Persulfides are formed through the oxidation of a reduced thiol.[33] One particularly unique feature of persulfide chemistry is the ability of persulfides to react with thiols to create another persulfide (transpersulfidation), or even to react with themselves to create larger polysulfides of varying lengths.[38] Persulfides are acutely unstable molecules and are generally transient in biological settings. The enhanced reactivity of persulfides compared with thiols is explained by a phenomenon known as the alpha effect. Namely, that an atom will display increased reactivity if there are unshared pairs of electrons on the adjacent atom.[39] Further, persulfides are primarily found in their deprotonated anionic form at physiological pH –the pK_a of GSSH is 3.49 units below that of GSH[40]— so these factors make them good nucleophiles. Persulfides can be produced naturally in cells by enzymes. For example, GSSH, which both inhibits cytochrome *c* and scavenges H_2O_2 more effectively than GSH, is produced by sulfide quinone oxidoreductase (SQR) and H_2S.[20, 41] Levels of GSSH and CySSH in mice range from 50-100 μM in the liver and 1-5 μM in the heart.[30] Stable intermediate persulfides can exist in the active site of some enzymes. The H_2S-releasing enzyme 3MST produces H_2S using 3-mercaptopyruvate (3MP), which is produced from L-cysteine by cysteine aminotransferase (CAT), as a substrate. The sulfur atom from 3MP reacts with a cysteine residue in the active site of 3MST and forms a persulfide which will release H_2S in the presence of thioredoxin or the reducing agent dithiothreitol (DTT).[42]

Given the powerful antioxidant effects of persulfides, the creation of organic persulfide donor compounds is desirable but highly constrained by their inherent

instability. Despite these limitations, several groups have produced persulfide donors and demonstrated their effects in cells. In 2018, Yuan et al. produced a stable GSSH donor which released GSSH in the presence of esterases.[43] This was done by caging the sulfane sulfur atom in GSSH as a thioester containing an esterase-sensitive trigger. Removal of this protecting group induces lactonization and the release of GSSH into the medium. The compound was shown to confer greater protection to H9c2 cells treated with H_2O_2 than GSH or Na_2S. Chaudhuri et al. published a photoactivated CySSH donor that utilizes an *o*-nitrobenzyl trigger to release the persulfide after irradiation with 380 nm light,[44] and Dillon et al. produced an N-acteyl cysteine persulfide (NAC-SSH) activated by bacterial nitroreductase.[45] Persulfide donors are still relatively rare, especially compared to the well-established field of H_2S donors, but progress is being made with these innovative approaches.

1.2.2. Inorganic Polysulfides

Inorganic polysulfides have the formula S_n^{2-}. They are anionic species containing no R group and are available as salts, such as Na_2S_2 or Na_2S_4. Despite being charged species, the interior sulfur atoms are still sulfane sulfur, so they share the reactive capabilities of other polysulfides. Like GSSH and CySSH, they are produced endogenously by 3MST and show enhanced cellular potency over H_2S.[46] In an early report on the significance of the downstream production of sulfane sulfur after H_2S treatment, Greiner et al. found that inorganic polysulfides derived from H_2S regulate the tumor suppressor protein phosphate and tensin homolog (PTEN) via the introduction of sulfane sulfur into the active site cysteine.[37, 47]

Inorganic polysulfides are the most commonly used polysulfides in biological polysulfide studies. This is due to the fact that they are inexpensive, readily available, and have good water solubility. Unfortunately, inorganic polysulfide salts are reactive and unstable, disproportionating into a mixture of S_n^{2-}, HS^-, S_n and HS_n^- in solution.[47] This makes the study of polysulfides challenging, as any treatment with inorganic polysulfides will contain both polysulfides and H_2S, making it difficult to assign observations to either species.

1.2.3. Organic Polysulfides

Organic polysulfides take the form R_2S_n (where n>2). This class of compounds includes polysulfides such as GSS_nH and $CySS_nH$ and are less reactive than persulfides. Organic polysulfides are a versatile platform from which to study the biology of polysulfides due to their tunability. In 2017, our group synthesized a library of phenyl- and N-acetyl cysteine-based tetrasulfides that demonstrated effective H_2S release in the presence of biological thiols.[48] Apart from their ability to release H_2S, one valuable function of the synthesis of organic polysulfides for cellular study is to elucidate the roles of sulfane sulfur. However, this reactivity is complicated by the fact that inorganic polysulfides are unstable and contain non-S^0 sulfur atoms, and organic polysulfides contain pendant alkyl groups. Both factors obfuscate the role of the sulfane sulfur atoms. In order to develop a method of studying sulfane sulfur, which is critical to understanding the biology of polysulfides, we then synthesized a library of benzyl polysulfides (Bn_2S_n, n=1-4).[33] This series of compounds differed only in their sulfur content, so this allowed us to control for the impacts of the pendant benzyl groups. After treatment of bEnd3 murine epithelial cells with the benzyl polysulfides (1-200 μM) for 24 hours, we found

no cytotoxicity in the mono- and disulfide, both containing no sulfane sulfur atoms. However, the tri- and tetrasulfide compounds, containing one and two sulfane sulfur atoms respectively, caused a steep decline in cell viability. Strikingly, the tetrasulfide was roughly twice as cytotoxic as the trisulfide, along with having twice the sulfane sulfur content. This was a clear indication that sulfane sulfur specifically was acting on the cells, though the exact mechanisms are not known.

Interestingly, when we treated the cells with identical concentrations of DATS, which contains one sulfane sulfur atom, we saw no toxicity whatsoever up to 200 μM. This indicates unique cellular chemistry of DATS, and that the pendant alkyl groups on an organic polysulfide can strongly influence the impact of sulfane sulfur on a system. These differences may be due to the different reactivities of the two alkyl groups. Allyl persulfides are much less stable than benzyl persulfides, which have been isolated and studied.[49] It is possible that a longer-lived benzyl persulfide intermediate species causes more cellular toxicity than a fleeting allyl persulfide due to the general volatility of persulfides. Regardless, DATS interacts with cells in particularly unique ways because it is derived from garlic, which humans and animals have been consuming for thousands of years. As plants evolved, they cultivated new defenses against predators by constantly developing natural products that targeted predator physiologies, i.e., poisons.[50] However, predators evolved in turn and adapted to consuming smaller amounts of these plants. This behavior enhances health because it leads to the induction of protective metabolic pathways. In the case of DATS allium phytochemicals like allicin, which DATS is derived from, are responsible for the strong smell and taste of garlic and are meant to dissuade consumption by predators.[51] Subsequent consumption of garlic leads to phase II

detoxification enzymes as protective pathways kick in to prevent poisoning, leading to protective cellular effects.[52]

There are few naturally-occurring slow-release H_2S compounds, but one is found in the seeds of *Parkia speciosa*, the "stinky bean". In 2017, Liang et al. isolated 1,2,4-trithiolane (TTL) from *P. speciosa*, a cyclic polysulfide that reacts slowly with GSH to increase cellular H_2S levels 6.23 times more effectively than DATS.[53] Analysis of the stinky bean essential oil revealed the presence of a suite of different cyclic organopolysulfides ranging in size from five-membered to eight-membered rings. Headspace analysis of crushed beans revealed a composition of over 40% H_2S, and that TTL was the most common of the detected polysulfide species.[54] Naturally-occurring cyclic polysulfides have also been isolated from animals. Some members of the sea squirt genus *Lissoclinum* from the Fiji Islands are the source of a cyclic polysulfide known as varacin, which demonstrates anticancer effects,[55] and Lissoclinotoxins A and B, which have antifungal properties.[56]

Synthetic cyclic organopolysulfides have also been developed and have already shown therapeutic potential. In 2012, Kulikov et al. synthesized a novel analogue of varacin known as 8-(trifluoromethyl)-1,2,3,4,5-benzopentathiepin-6-amine hydrochloride (TC-2153) which was patented in 2018 as an antidepressant.[21, 57] When administered to mice, the compound significantly decreased instances of catalepsy. The mechanism of action of TC-2153 was determined to be mediated by its effects on the brains seratoninergic system and brain-derived neurotrophic factor (NTF). Two years later, Xu et al. revisited TC-2153 and applied it to an Alzheimer's treatment model.[58] They sought to identify inhibitors of the enzyme striatal-enriched protein tyrosine phosphatase

(STEP), which is found to be overactive in several neurodegenerative disorders which include Alzheimer's.[59, 60] After a high-throughput screen of 150,000 commercially available compounds, eight candidates were selected which all contained sulfur atoms. Surprisingly, after being freshly resynthesized for subsequent study, none of the eight compounds retained any STEP-inhibitory activity. After analysis of one of the commercial preparations by HPLC, elemental sulfur (S_8) was found to be the contaminant responsible for the observed inhibition of STEP. The researchers elected not to pursue S_8 as a therapeutic pathway since it has virtually no water solubility.[61] They thus turned to TC-2153 due to its similar structure and found its STEP inhibition capacity to be comparable to that of S_8. Crucially, they identified the mechanism of inhibition as the modification of the active site cysteine, consistent with the ability of polysulfides to exert biological effects by persulfidating proteins at cysteine residues.

1.2.4. Elemental Sulfur

S_8 is a chalky yellow solid with a solubility of only (26.5 nM) at 25 ˚C.[61] This low solubility is the main factor in why S_8 has not been widely utilized as a therapeutic or H_2S prodrug. Because it is a cyclic polysulfide that contains only sulfur atoms, every atom in S_8 is a sulfane sulfur atom. This gives S_8 enormous potential not only as an H_2S donor, but also as an antioxidant or anti-inflammatory treatment, and even as an optimal system for understanding sulfane sulfur. Progress has been made in finding methods to utilize S_8 in biological contexts. One example, developed by SulfaGENIX, is the H_2S prodrug SG1002, an inorganic mixture of S_8, Na_2SO_4, $Na_2S_2O_3$, $Na_2S_3O_6$, $Na_2S_4O_6$, and $Na_2S_5O_6$.[62] The presence of these ionic materials helps to enhance the solubility of the otherwise insoluble S_8 molecule and make it available to cells.[21] In a Phase I clinical trial,

SG1002 was administered to heart failure patients, who have chronically low levels of circulating H_2S.[63] It was found that SG1002 treatment not only elevated concentrations of H_2S in plasma, but it also elevated levels of NO metabolites, demonstrating another pathway containing H_2S-NO crosstalk.

In 2020, our lab developed a novel and economical method of solvating S_8 through the use of supramolecular cyclodextrins.[64] Cyclodextrins are cyclic oligo polysaccharides naturally produced by some species of bacteria.[65] In recent decades, they have gained some prominence in the drug development industry due to the fact that they can solubilize relatively insoluble compounds. The molecules have a roughly cone-shaped structure, with a hydrophilic exterior and a hydrophobic interior.[66] Hydrophobic molecules are encapsulated noncovalently within the structure, allowing the combined cyclodextrin-drug complex to be solvated in water. Once in solution, the drug readily dissociates and is in equilibrium with the bound complex. Using 2-hydroxypropyl β-cyclodextrin (2HPβ), we managed to solvate S_8 at concentrations of up to 2.1 mM in water at physiological pH.[64] The 2HPβ/S_8 complex was stable both in solution and as a precipitated solid and it could efficiently release H_2S in solution at physiological pH. Treatment of cells with 2HPβ/S_8 was shown to confer anti-inflammatory effects and to increase intracellular S^0 levels. Due to the lack of any alkyl groups, these S_8 treatment strategies could greatly improve our understanding of S^0. The inexpensive nature of S_8 and relatively low toxicity of the cyclodextrins makes this an attractive route for future exploration of polysulfides and H_2S prodrugs.

1.3. Protein Persulfidation

Persulfidation (also known as S-sulfhydration, S-persulfidation, or sulfuration) is a posttranslational modification of a protein at a cysteine residue.[67, 68] Reduced cysteine residues react with oxidized sulfur atoms, leading to the appending of one or more sulfur atoms onto the cysteine, forming a persulfide. This modification changes both the structure and function of the affected protein and is therefore an important regulatory mechanism in biological systems. Persulfidation is a widespread phenomenon throughout the proteome, it was found that 10-25% of proteins in mouse liver lysate including actin and tubulin are persulfidated in physiological conditions.[68, 69] At this time, persulfidation is still poorly understood. It was long believed that H_2S was responsible for persulfidating proteins. However, both the sulfur atom in H_2S and the sulfur atom in a thiol such as cysteine are in the -2 redox state.[20] Atoms in the same oxidation state cannot react with each other, therefore it must be oxidized sulfur species that are responsible for persulfidation. This information calls into question much of what we thought we knew about the chemistry of H_2S, as it is likely that much of what we have seen should actually be attributed to polysulfides.

One of the best-known and most significant interactions H_2S has with cells is the induction of the translocation of Nrf2 to the nucleus and subsequent upregulation of cell-protective antioxidant genes.[32] In 2013, Yang et al. reported that the mechanism of H_2S on Nrf2 is the persulfidation of its regulatory protein Kelch Like ECH Associated Protein 1 (Keap1) at cysteine-151.[16] This causes Nrf2 to dissociate from Keap1 and translocate to the nucleus, where it then activates antioxidant genes by binding to the antioxidant response element (ARE) sites on the gene promoters.[70] An additional example of a

fundamental piece of H_2S biochemistry is the activation of KATP channels by persulfidation.[71] This is the mechanism by which H_2S engages in crosstalk pathways with NO to enhance smooth muscle vasorelaxation. Another piece of this pathway is the inhibition of phosphodiesterase (PDE) 5A, which hydrolyzes intracellular cGMP, through persulfidation.[72] In endothelial and smooth muscle cells, NO and H_2S work in tandem to raise cGMP levels by respectively activating soluble guanylyl cyclase (creating cGMP) and inhibiting PDE 5A (preventing the hydrolyzing of cGMP).[17] The end result is the enhancement of vasorelaxation and angiogenesis. H_2S itself was implicated in the observed cysteine modifications and persulfidation in these studies and others due to the fact that the studies are typically done by treating subjects with H_2S donors, or by knocking out H_2S-producing enzymes, and observing the effects. However, H_2S is readily oxidized in cells by enzymes such as sulfide quinone oxidoreductase,[73] and so these effects and likely others currently attributed to H_2S must be the result of the downstream production of oxidized persulfides and polysulfides from H_2S.

In addition to forming persulfides on the cysteine residues of proteins, oxidized sulfur species can also form polysulfdes and even protein dimers. In one interesting case, researchers studying copper-zinc superoxide dismutase (SOD1) were surprised to find the emergence of monomers and dimers with higher-than-expected masses in their mass spectrometry experiments.[74] These outlying species were crystalized, and the discrepancy was found to be caused by intruding sulfur. Specifically, protein dimers were bound together at cysteine-111 by a heptasulfide bridge, which could be ablated by GSH treatment. Nothing in the enzyme preparation or reagents was found to be the source of this oxidized sulfur, and it was eventually discovered that the overnight dialysis

treatments to remove ammonium sulfate was the culprit. Dialysis tubes were cleaned by boiling in EDTA, which produced a “strong sulfurous odor”. The persulfidation of SOD1 could be replicated by incubating the enzyme with solid S_8, indicating the interloping sulfur originated from the oxidized sulfur present in vulcanized rubber.

1.4. Conclusions

The field of polysulfides is still relatively new compared to the study of H_2S. Despite this, our relationship as a species with polysulfides goes back to the dawn of life. H_2S, in its role as a gasotransmitter, fills many roles that polysulfides cannot. However, the chemistry of polysulfides is much more versatile and wide-ranging. Evidence continues to emerge of the importance of persulfides and polysulfides in our cellular redox environment. The capacity that these oxidized species have to persulfidate proteins and directly modify their structure and function makes them hugely important and an overlooked component of epigenetics. There is still much ground to cover in the development of persulfide donors and of pure, stable sulfane sulfur donors that will enable us to learn more about the chemistry of these oxidized sulfur atoms. Most of what we know of the process of persulfidation is based on the formation of persulfides on cysteine residues. However, it is exciting to think of the possibility of other, naturally occurring protein dimerizations or the appending of longer polysulfides onto proteins that could be made possible through the process of persulfidation.

Our lab has developed many different chemical tools for the purpose of studying sulfur in biological settings. These tools must be water soluble, bioorthogonal, and non-cytotoxic. The ability to append triggering groups, such as with the persulfide donor

compounds noted earlier, is a critical component to mimicking natural sulfur sources so that we may learn more about how these species behave in cellular environments.

CHAPTER II

EFFECTS OF SULFANE SULFUR CONTENT IN BENZYL POLYSULFIDES ON THIOL-TRIGGERED H_2S RELEASE AND CELL PROLIFERATION

Bolton, S.G.; † Cerda, M.M.;† Gilbert, A.K.; Pluth, M.D., Effects of Sulfane Sulfur Content in Benzyl Polysulfides on Thiol-Triggered H_2S Release and Cell Proliferation. *Free Radical Biology & Medicine* **2019**, *131*, 393-398.

2.1. 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 2.1).[3]

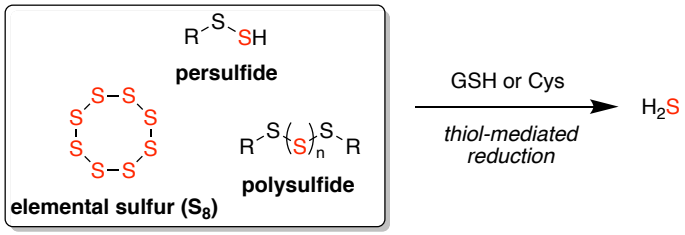


Figure 2.1. Common S^0-containing small molecule species release H_2S upon reaction with biological thiols.

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] Toward 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.[9-12] 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.[13]

When considering sulfur oxidation states, persulfides possess reduced sulfur and S^0, implying potential significance of S^0 in sulfur biology.[14] Under physiological conditions, S^0 readily oxidizes free thiol residues to generate persulfides via a reaction mechanism known as persulfidation,[15] and this process has been shown to upregulate the activity of key enzymes such as xanthine oxidase.[16] 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.[17] Interestingly, persulfidation of thiol-based antioxidants such as GSH leads to enhanced antioxidant activity when compared to corresponding thiols.[18] 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.[19] Toward directly studying small molecule persulfide reactivity, recent work has demonstrated the ability to generate persulfides under physiological conditions[20] for applications in protein persulfidation,[21]

reactive oxygen species scavenging,[22] and H-atom transfer agents.[23] Previous work by our group demonstrated the ability to access discrete persulfides[24] and study their reactivity under various conditions.[25] 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,[26, 27] that have unique biological properties.[28] Readily isolated from alliums such as garlic,[29] 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,[30-34] 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.[35] 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.[36] 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.[37] 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.[38] 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,[39] which spontaneously disproportionate under mild,

aqueous conditions to yield complex mixtures.[40] Recently, our group and others[41] have demonstrated the ability to access[42] and utilize organic polysulfides beyond DATS for thiol-triggered H_2S delivery.[43] 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.

2.2. Synthesis and Mechanism

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,[44] 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 2.2a). 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 a 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.[45] 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^{t}Bu) 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.[24] Due to steric congestion and poor nucleophilicity, we viewed that nucleophilic attack by KO^{t}Bu 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.

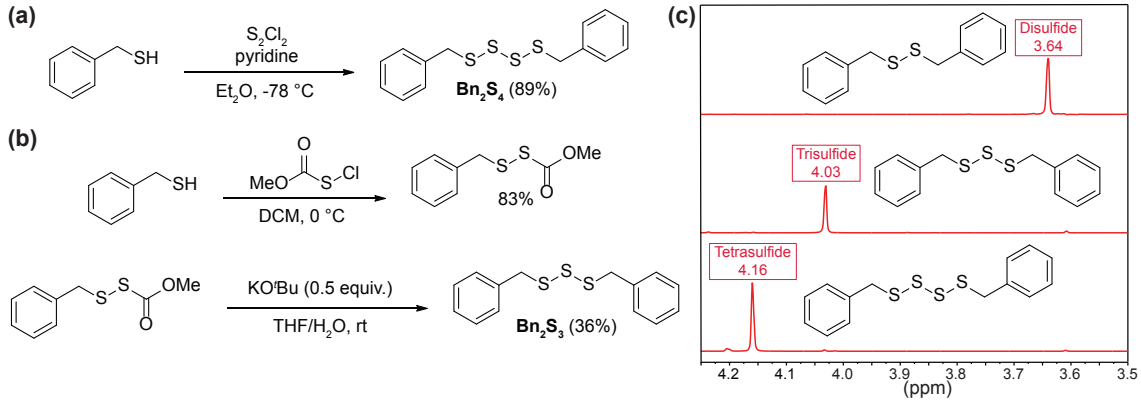


Figure 2.2. (a) Synthesis of Bn_2S_4. (b) Synthesis of Bn_2S_3. (c) Comparison of benzylic ^{1}H NMR (500 MHz) signals between Bn_2S_2, Bn_2S_3, and Bn_2S_4 in $CDCl_3$.

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 2.2b). 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 (Scheme A.1). 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 2.2c).[46]

2.3. Profiling H_2S Release

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 (MB) assay (Figure 2.3). Calibration curves were made in the presence of both 500 μM cysteine and 500 μM GSH (Figures A.1, A.2 respectively).

As expected, we did not observe H_2S release from **Bn_2S** and **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 **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.[47] For both GSH and Cys, we observed approximately twice as much H_2S released from **Bn_2S_4** than from **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.), **Bn_2S_3** and **Bn_2S_4** released 8.6 (34% releasing efficiency) and 17.5 μM

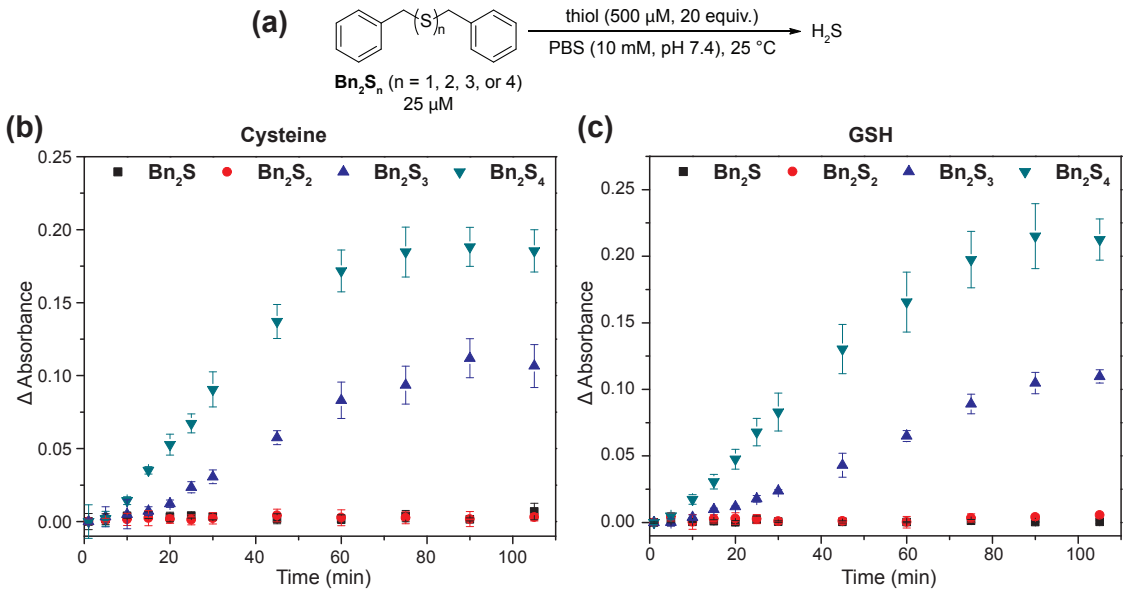


Figure 2.3. (a) Reaction conditions for thiol-triggered release of H_2S from Bn_2S_n (n = 1, 2, 3, or 4). (b) Release of H_2S in the presence of cysteine. (c) Release of H_2S in the presence of GSH.

(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. By comparison to other synthetic, small molecule H_2S donors, we attribute the relatively low H_2S releasing efficiencies to the high lipophilicity of organic polysulfides and we anticipate the design of water-soluble polysulfides should provide more efficient H_2S donors.

2.4. Cellular Proliferation Studies

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.[48] 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 2.4). Individual graphs may be found in Appendix A (Figures A.4-A.8).

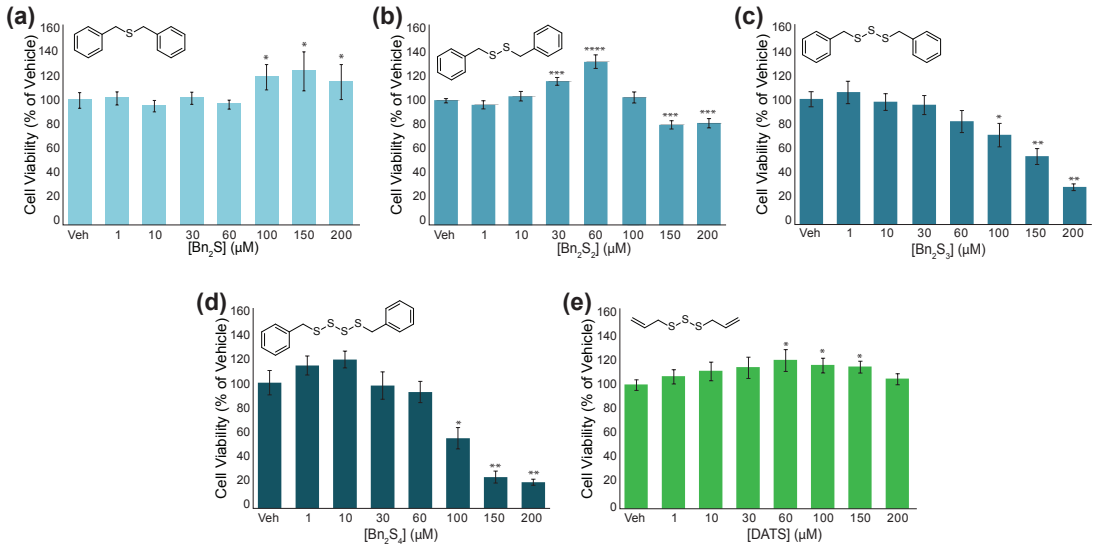


Figure 2.4. bEnd.3 cell viability after 24-hour treatments of a series of organic polysulfides: (a) Bn_2S, (b) Bn_2S_2, (c) Bn_2S_3, (d) Bn_2S_4, and (e) DATS. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Use of synthetic H_2S donors such as AP39[49] and direct administration of H_2S via NaSH[50] 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 A.3). 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.

2.5. Conclusions

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 benzyl trisulfide and benzyl tetrasulfide respectively under physiological conditions. Additionally, we demonstrate the unique ability of benzyl trisulfide and tetrasulfide to suppress cell proliferation, whereas DATS has no effect in murine endothelial (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.

2.6. Materials and Methods

2.6.1. Materials

Reagents were purchased from Sigma-Aldrich and/or Tokyo Chemical Industry (TCI) 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.

2.6.2. Synthesis

2.6.2.1. 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.

2.6.2.2. 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.

2.6.2.3. 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%). ^1H 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.)

2.6.2.4. 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.

2.6.3. H_2S Measurement by Methylene Blue Assay

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.

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.

2.6.4. Methylene Blue Assay 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.

2.6.5. Cell Culture

The mouse brain endothelial cell line bEnd.3 was obtained by ATCC (CRL-2299) and cultured in phenol red DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin (10,000 units/mL penicillin and 10,000 μg/mL streptomycin). Cells were incubated at 37˚C under 5% CO_2.

2.6.6. Cellular Proliferation Assays

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).

CHAPTER III

N-ACETYLCYSTEINE-BASED TETRASULFIDES EXERT DIFFERENTIAL INFLUENCE ON CELL PROLIFERATION AND MIGRATION PATHWAYS

Matthew Cerda synthesized the compounds described, and all assays were performed by Sarah Bolton

3.1. Introduction

Sulfur is an element that is uniquely versatile in biological environments due to its wide range of available redox states, from -2 to +6.[1] One particularly important reactive sulfur species (RSS) is hydrogen sulfide, or H_2S: a member of a family of small, gaseous signaling molecules known as the gasotransmitters that also includes carbon monoxide (CO) and nitric oxide (NO).[2-5] H_2S is produced in the metabolism of cysteine by cystathionine beta-synthase (CBS), cystathionine gamma-lyase (CSE), and 3-Mercaptopyruvate sulfurtransferase (3-MST).[6] The sulfur atom in H_2S, which is found as SH^- at a 3:1 SH^-:H_2S ratio at biological pH, is in the reduced -2 state.[7] This reduced sulfur atom is able to directly react with biological molecules like hemes. H_2S treatment can induce a suspended animation state via the reversible inhibition of cytochrome *c* oxidase by reduction of the heme motif.[7]

Like the other gasotransmitters, H_2S has been shown to play important roles in a variety of different metabolic pathways.[8] H_2S has been shown to promote antioxidant,[9-12] anti-inflammatory,[13-16] and vasodilating[17-19] effects in tissues. Notably, H_2S plays a prominent role in the processes of angiogenesis and wound healing.[20] Mice implanted

with Matrigel plugs and treated with zofenoprilat, an ACE inhibitor shown to induce CSE expression , displayed increased growth of new capillaries.[21] H_2S treatment has been shown to accelerate the wound closure rate in rats,[22] and endogenous angiogenic processes are directed by vascular endothelial growth factor (VEGF) upregulating H_2S production.[18, 23]

In addition to H_2S, oxidized sulfur species like polysulfides and persulfides are gaining increased attention as biological mediators. One major reason is the fact that oxidized sulfur, particularly sulfane sulfur (S^0) is capable of modifying proteins at cysteine residues in a process known as persulfidation.[24-27] Reduced cysteines react with oxidized S^0 present in polysulfides and take the form -SSH. Persulfidation was long attributed to H_2S itself, but the sulfur atom in H_2S shares the -2 redox state with the sulfur atom in reduced cysteine, so these species cannot react with each-other.[28] However, H_2S can induce the downstream production of oxidized sulfur species so it is therefore very likely that much of what we know of H_2S biochemistry should actually be attributed to S^0-containing species.[29] One example is the activation of K_{ATP} channels, a well-known effect of H_2S treatment that is now known to be caused by persulfidation.[30] However, S^0 can also react with biological thiols to produce H_2S, further clouding the picture.[31] More work is needed to fully disentangle the biological roles of H_2S and oxidized S^0 species.

Here, we utilize organic tetrasulfides based on N-acetylcysteine (NAC) published by our lab in 2017 to explore how S^0 impacts cellular proliferation and wound healing.[32] In 2019, we synthesized a series of benzyl polysulfides containing varying amounts of S^0 and a fixed pendant alkyl group as a system to study S^0 in cells.

However, we found very different impacts on cellular proliferation between our benzyl trisulfide and the naturally occurring diallyl trisulfide (DATS). NAC is a precursor to glutathione (GSH) synthesis and a well-known cell protective agent, giving this compound more biological relevance than the benzyl tetrasulfide.[33] With this in mind, we utilized NAC tetrasulfide (NAC_2S_4) to further probe the effects of the pendant alkyl group on the behavior of S^0.

3.2. Cellular Proliferation Assays

3.2.1. N-Acetylcysteine Tetrasulfide

We sought to evaluate the effect of NAC_2S_4 on cellular proliferation in murine epithelial (bEnd.3) cells. Our previous work with a series of benzyl polysulfides, up to benzyl tetrasulfide, showed increasing cytotoxicity with increasing levels of S^0.[31] NAC_2S_4, with two S^0 atoms, could therefore share a similarly high level of toxicity as the benzyl tetrasulfide. The toxicity would likely depend on the stability of any intermediate persulfide compounds made in the process of thiol reduction of NAC_2S_4. Persulfides are highly reactive species, and a longer-lived specimen would have the opportunity to cause increased amounts of cellular damage.

To evaluate the effects of NAC_2S_4 on cellular health, we incubated bEnd.3 cells with varying levels of NAC_2S_4 for 24 hours (Figure 3.1a). NAC_2S_4 has a pro-proliferative effect on bEnd.3 cells up to 200 μM, displaying no cytotoxicity at any of the concentrations studied, which may indicate reduced toxicity of the NAC persulfide compared to those generated in benzyl polysulfides.[31] To see if the pro-proliferative effect could be enhanced with a more lipophilic tetrasulfide species, we further treated cells with an NAC methyl ester tetrasulfide ($NACOMe_2S_4$). The methyl ester functional

groups on $NACOMe_2S_4$ reduce its polarity compared to the free acid NAC_2S_4 species. 24-Hour incubation with $NACOMe_2S_4$ had a strikingly different result (Figure 3.1b). Instead of a sustained pro-proliferative effect up to 200 µM, we found a sharp decline in cell viability starting at 150 µM. Interestingly, there is an initial pro-proliferative effect on bEnd.3 cells from $NACOMe_2S_4$ treatment, peaking at a 57% enhancement in viability at 30 µM $NACOMe_2S_4$ (corresponding to 60 µM S^0). While the 200 µM treatment of NAC_2S_4 brought about a 38% increase in cell viability, 200 µM of $NACOMe_2S_4$ reduced viability to only 15% of the vehicle. This level of toxicity is similar to that of the benzyl tetrasulfide in our previous work. It is likely that this toxicity has as similar cause: overloading cells with S^0 species, particularly those that can create more stable persulfides, can disturb the redox environment and be destructive to cells. Further, the enhanced lipophilicity of the $NACOMe_2S_4$ species more closely likens it to the similarly nonpolar benzyl tetrasulfide. It is likely that these species show such enhanced toxicity over NAC_2S_4 due to this enhanced cell permeability, and the NAC_2S_4 cannot cross the cell membrane efficiently enough to exert a similar effect.

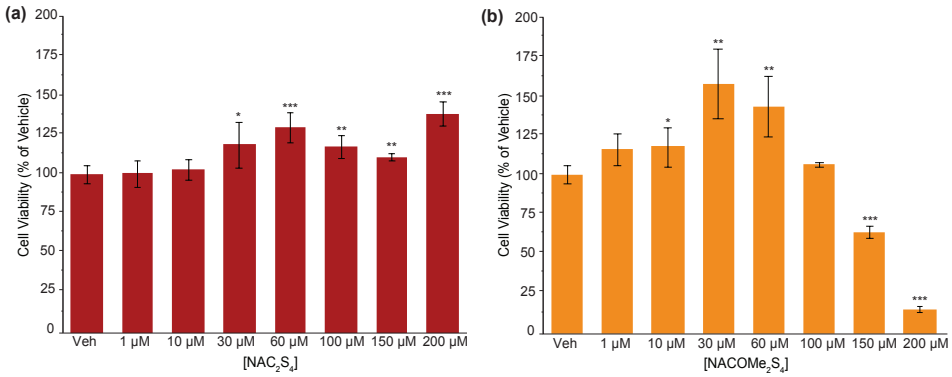


Figure 3.1. Effects of 24-hour treatments of (a) NAC_2S_4, and (b) $NACOMe_2S_4$ show a strong divergence in their effects on bEnd.3 cells. NAC_2S_4 enhances cell proliferation up to 200 µM and shows no cytotoxic effects. $NACOMe_2S_4$ has a similarly pro-proliferative effect peaking at 30 µM, above which viability begins to decline. Vehicle = 0.5% DMSO

3.2.2. N-Acetylcysteine, Cysteine, and N-Acetylcysteine Methyl Ester

Since the presence and identity of these pendant alkyl groups greatly influences the outcome of S^0 treatment, discerning the biochemistry of S^0 is challenging. However, organic polysulfides such as diallyl trisulfide in garlic are analogous to what cells encounter *in vivo*, so understanding the interaction of these pendant groups with S^0 is of interest. To study the effects of the pendant NAC groups on NAC_2S_4, we performed cellular proliferation assays on bEnd.3 cells at concentrations up to 200 µM for 24 hours with related alkyl species (Figure 3.2). NAC, a known antioxidant in medical use since the 1960s,[33] demonstrates no pro- or antiproliferative effect in bEnd.3 cells (Figure 3.2a). Likewise, cysteine shows little effect, with very small increases in cellular proliferation at low concentrations (Figure 3.2b). Interestingly treatment with N-acetylcysteine methyl ester, the more lipophilic species appended to the more toxic $NACOMe_2S_4$, demonstrated only a pro-proliferative influence on bEnd.3 cells (Figure 3.2c). This surprising result indicates that the effects of cell treatments of NAC_2S_4 and $NACOMe_2S_4$ must be due to S^0, and how S^0 interacts with these pendant alkyl groups.

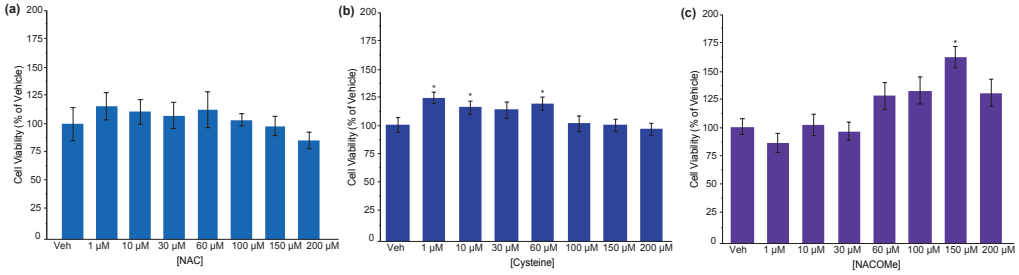


Figure 3.2. Cellular proliferation assays of bEnd.3 cells with (a) NAC, (b) cysteine, and (c) NACOMe after 24-hour treatments. All show mild or no pro-proliferative influence on bEnd.3 cells. Vehicle = 0.5% DMSO

3.3. Cellular Migration Studies

We then sought to explore the effects of S^0 on cell migration, which is directly applicable to wound healing and angiogenesis. Previous studies on bEnd.3 cells determined that H_2S treatment enhances endothelial cells' rate of migration after a confluent monolayer is scratched, simulating a wound.[18] The Scratch Assay is used to measure rates of cellular migration to close the "wound". The assay is performed by growing a confluent layer of endothelial cells, and then scratching this layer with a pipet tip, or similar object. The cells are then imaged from t=0, when the scratch is made, up to the chosen endpoint.[34] The difference in scratch area, which can be measured using various pieces of software, between the final and initial time points indicates the rate of migration of the cells.

Despite the wealth of literature on H_2S and wound healing, the possible roles of S^0 have not yet been studied. Given the number of biological roles previously attributed to H_2S but now known to be caused via persulfidation by S^0, we thought that this area merited more study. We performed the Scratch Assay on bEnd.3 cells treated with NAC_2S_4 for 24 hours, imaged once per hour on an incubated stage (Figure 3.3). Scratch closure rates were calculated by quantifying the scratch area in t=0 and t=24-hour images and determining the percentage of scratch area closure for each imaged site (Figure 3.3a). The results were surprising, even though NAC_2S_4 demonstrated pro-proliferative effects in bEnd.3 cells in cellular proliferation assays up to 200 μM, in cellular migration assays this concentration of NAC_2S_4 completely eliminated any activity (Figure 3.3b). Cells treated with 200 μM were not dead but could not migrate and initiated no scratch closure. The 100 μM treatment similarly inhibited cellular

mobility, but did not ablate it completely, with a 45% reduction in scratch area closed. Further, lower concentrations of NAC_2S_4 were shown to slightly enhance the rate of cellular migration, peaking at around about a 50% enhancement in the 10 µM treatment. These results indicate that S^0 is acting on cells in parallel pathways, influencing both cellular proliferation and cellular migration independently.

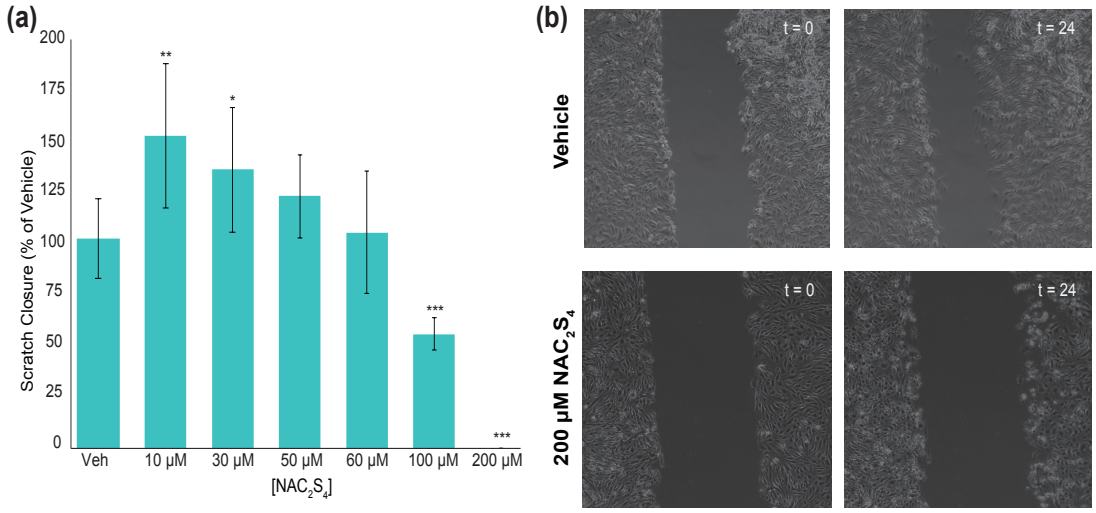


Figure 3.3. (a) Cellular migration rates in Scratch Assay after treatment with NAC_2S_4. (b) bEnd.3 cell monolayers were treated with NAC_2S_4 for 24 hours after being scratched with a pipet tip. At high concentrations of the tetrasulfide, migration is completely ablated without cell death. Vehicle = 0.5% DMSO

3.4. Conclusions

Sulfane sulfur offers an exciting new window into H_2S and sulfur biology. Given that so much of what we know of H_2S really involves the production of S^0 species capable of persulfidation, the availability of S^0 compounds warrants a second look at some documented H_2S pathways. Here we utilized organic polysulfides containing two S^0 atoms to study the effects of S^0 on cellular proliferation and wound healing. We find that large concentrations of a S^0-containing species capable of crossing the cell membrane are cytotoxic, but similar concentrations of a more polar S^0 species that can only partially cross the membrane have pro-proliferative effects. Further, we

find that large concentrations of this same polar tetrasulfide, NAC_2S_4, completely shut down cell migration pathways at these same high concentrations that enhance cellular proliferation. However, previous literature demonstrates that H_2S treatment enhances migration rates in the bEnd.3 cell line.[18] This discrepancy is likely explained by the fact that H_2S treatment leads to the production of S^0 species only downstream of its application to cells. It is likely that a large influx of oxidized sulfur disturbs the redox states of the many membrane proteins involved in cellular migration and adhesion that would be exposed to these high concentrations of extracellular NAC_2S_4. The influence of possible persulfidation of these proteins that are involved in cellular migration has implications in angiogenesis, wound healing, and likely processes such as cancer metastasis, and deserves further study. Further, these results support the need for further study and development in methods of studying cellular S^0 more directly.

3.5. Materials and Methods

3.5.1. Materials

Reagents were purchased from Sigma-Aldrich and/or Tokyo Chemical Industry (TCI), deuterated solvents were purchased from Cambridge Isotope Laboratories. NAC_2S_4 and $NACOMe_2S_4$ were synthesized as previously described.[32] The plate reader used in the cellular proliferation assays was a BioTek Synergy 2. All cellular images and videos were taken on a Leica DMi6 widefield microscope using phase contrast settings with an attached incubator (PeCon)

3.5.2. Cell Culture

bEnd.3 murine epithelial cells were obtained from ATCC (CRL-2299). Cells were cultured in DMEM containing phenol red, 10% premium-grade fetal bovine serum

(FBS) from VWR, and 1% penicillin-streptomycin (PS) containing 10,000 units/mL penicillin and 10,000 µg/mL streptomycin. (Gibco) Cells were maintained at 37˚C under 5% CO_2.

3.5.3. Cellular Proliferation Assay

bEnd.3 cells were seeded in Nunc 96 well Nunclon Delta plates at 20,000 cells/well under normal culturing conditions. The next day, media was aspirated off and replaced with FBS-free DMEM containing test compounds in 0.5% DMSO (vehicle). Cells were with test compounds for 24 hours before treatment with Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies). Data was normalized to vehicle, and pooled.

3.5.4. Scratch Assay

bEnd.3 cells were seeded on plastic 12 well plates (Corning) and grown until fully confluent under normal culturing conditions. When cells were confluent media was aspirated off, cells were scratched down the middle of each well with a 200 µL pipet tip, and scratch debris were rinsed off with PBS. PBS was aspirated off and replaced with serum-free, 1% PS DMEM (no phenol red) containing the test compound (O.5% DMSO vehicle). Cells were then put on microscope stage within an attached incubator and were maintained at 37˚C under 5% CO_2. In the microscope software (LasX), two points per well were identified as observation sites and their locations were recorded. A time experiment was set for 24 hours, with images taken of each site once per hour by the microscope. Scratch area was quantified using the MRI Wound Healing Tool macro in ImageJ for t=0 and t=24-hour images. t=0 scratches that were more than two standard deviations from the average t=0 scratch sizes were discarded. Data was

normalized to vehicle, and significance was calculated using Microsoft Excel's two-sample t-test (assuming equal variances) data analysis tool.

CHAPTER IV

MODIFIED CYCLODEXTRINS SOLUBILIZE ELEMENTAL SULFUR IN WATER AND ENABLE BIOLOGICAL SULFANE SULFUR DELIVERY

Bolton, S.G.; Pluth, M.D., Modified Cyclodextrins Solubilize Elemental Sulfur in Water and Enable Biological Sulfane Sulfur Delivery. *Chemical Science* **2020**, *11*, 11777-11784.

4.1. Introduction

Sulfur is a long-standing critical component to life on Earth. Prior to the Great Oxidation Event approximately 2.4 billion years ago, during which time the Earth's atmosphere became rich in O_2, the atmosphere on Earth was weakly reducing.[1] Volcanic activity was an abundant source of sulfur-containing species, and gases including sulfur dioxide (SO_2) and hydrogen sulfide (H_2S) were released and dissolved in pools or lakes.[2] Spark discharge experiments using a simulated atmosphere containing H_2S and a mixture of reducing gases thought to be present in the early Earth atmosphere have demonstrated abiotic synthesis of diverse organic compounds including amino acids.[3] In conditions reflecting those near deep-sea vents rich in iron-sulfur compounds, both H_2 and organosulfur compounds were generated from a mixture of H_2S and FeS under a N_2/CO_2 atmosphere in acidic conditions.[4] Sulfane sulfur (S^0), which is most commonly found as elemental octasulfur (S_8), is also found in these deep-sea environments and is another important source of biologically available sulfur.

Recently, interest in biological and synthetic S^0 sources has increased significantly due to the connection between such species and the small biological signaling molecule H_2S. H_2S is produced endogenously from cysteine metabolism and

serves signaling roles in diverse pathways. Along with carbon monoxide (CO) and nitric oxide (NO), H_2S is now recognized as member of the family of small molecules often referred to as gasotransmitters, which are produced enzymatically and act upon specific molecular targets within cellular environments.[5-7] One unique feature that distinguishes H_2S from CO and NO is that sulfur has biologically-accessible oxidation states ranging from -2 to +6 and participates in a complex redox cellular landscape.[8] In many eukaryotic organisms, H_2S serves as a source of biologically available sulfur and is intrinsically tied to both organic and inorganic S^0-containing species, including persulfides and related polysulfides/polysulfanes, in the S^0 pool. This redox labile pool can generate H_2S upon reduction or participate in transpersulfidation reactions to transfer S^0 moieties to cysteine residues.[9] The biological activity of H_2S is inextricably linked to the downstream production of S^0-containing species, and understanding the generation, action, and translocation of S^0 is critical to understanding the intertwined chemistry of reactive sulfur species in biology.

Despite this broad importance in both contemporary and evolutionary chemistry and biology, investigations into S^0 activity in aqueous systems is challenging due to the complex reactivity of available S^0 sources. Organic polysulfides, such as diallyl trisulfide (DATS) found in alliums including garlic, or other synthetic organic and inorganic polysulfides can act as sources of biologically available S^0. These systems present divergent reactivity based on the polysulfide chain length and pendant alkyl group.[10] Inorganic polysulfides in particular are unstable in aqueous conditions and quickly equilibrate to different polysulfide mixtures.[11] Despite these fundamental challenges, available S^0 sources demonstrate significant promise in different systems,

ranging from anticancer properties in several human cell lines[12-15] to enhanced antioxidative activity.[18] In all of these cases, however, the production of byproducts obfuscates the role of S^0.

An attractive approach to investigate the chemical biology of S^0 is to use the most common and simplest form of sulfane sulfur: S_8. However, use of S_8 directly is hindered by its low water solubility of 6.4 µg/L (19(6) nM) at 25 ˚C.[16] Despite this low solubility, S^0 found in volcanic deep-sea environments can be readily metabolized by thermophilic Archaean organisms found in these sulfur-rich habitats.[5] As one example, species of the order *Sulfolobales* can derive energy from the metabolism of S^0 via both aerobic and anaerobic pathways.[17, 18] Similarly, *Acidianus ambivalens* can utilize S^0 as both an electron donor and acceptor. Taken together, such organisms may provide clues into possible mechanisms of stabilizing bioavailable S^0 in an aqueous environment.

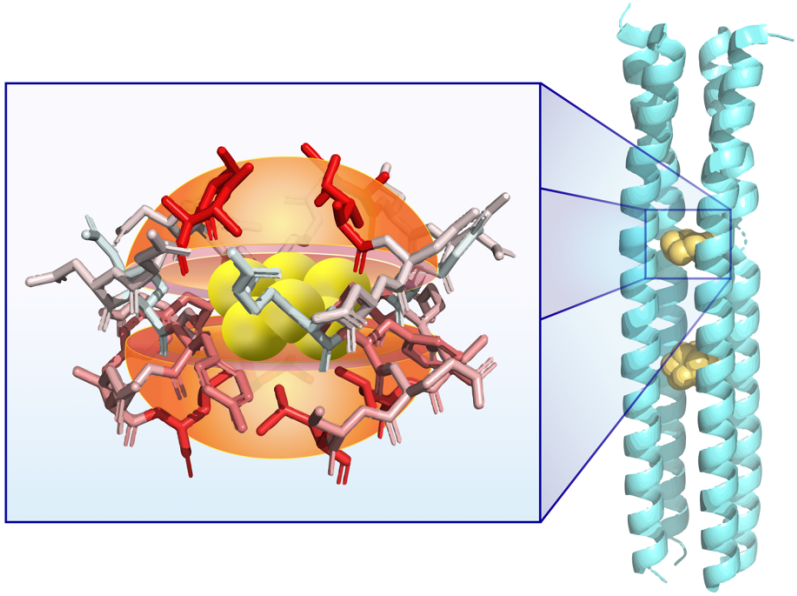
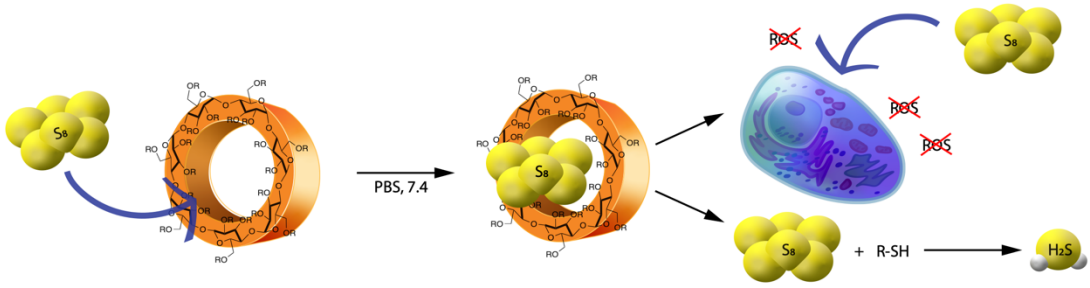


Figure 4.1. Structure of the tetrameric right-handed coiled-coil component of the tetrabrachion of the archaeon *S. marinus* (PDB: 5JR5) demonstrating two hydrophobic pockets in the core capable of encapsulating S_8. Highlighted residues are colored according to their hydrophobicity, where red indicates stronger hydrophobicity.

One potential strategy for S_8 solubilization and activation can be gleaned from the archaeon *Staphylothermus marinus*, a strict sulfur reducing anaerobe that requires S^0 as its terminal electron acceptor. Found near hot deep-sea vents, *S. marinus* is coated in thermostable filamentous glycoprotein structure called tetrabrachion that protrude from its surface. The tetrabrachion of *S. marinus* is composed of a four-stranded parallel coiled-coil structure with a hydrophobic core that is particularly stable.[19] The 24 kDa right-handed coiled-coil structure of the tetrabrachion contains hydrophobic cavities that have been found to encapsulate two S_8 molecules (PDB: 5JR5). Closer inspection of these S_8-binding cavities revealed that the sulfur motifs were held in place by van der Waals forces with aliphatic amino acid side chains leucine and isoleucine (Figure 4.1).[20] Additional computational investigations have investigated the transit pathways for exchange of water and S_8 within these structures.[21] Further supporting this observation that hydrophobic motifs can increase S_8 solubility, Steudel and Holdt demonstrated that the solubility of S_8 in water can be increased using surfactants, with S_8 concentrations reaching up to 0.1 mM in saturated hexadecyl(trimethyl)ammonium bromide (CTAB) solutions, although reactivity studies were not reported.[22] Taken together, these prior observations support that this approach to solubilize S_8 in aqueous environments may be more general and could also lead to new approaches to enable chemical accessibility of S^0-containing species in biological environments.

Understanding the intrinsic strategies for stabilizing simple S^0-containing sources in solution remains a key unmet need that could have significant impacts in broad fields ranging from contemporary chemical biology of reactive sulfur species to greener synthetic methods for sulfur-containing compounds. Here we report that

hydrophobic interactions within cavity-containing molecules, such as cyclodextrins (CDs), can be used to significantly solubilize S_8 in aqueous solutions, and that this solubilized S^0 is both chemically and biologically accessible. Specifically, we use 2-hydroxypropyl β-cyclodextrin (2HPβ) to generate 2HPβ/S_8 solutions that are stable and quantifiable, react with thiols to generate H_2S, exert antioxidant activities in cell models of oxidative stress, and increase intracellular S^0 levels (Scheme 4.1).



Scheme 4.1. Solubilization and chemical accessibility of S_8 in 2-hydroxypropyl β-cyclodextrin (2HPβ).

4.2. Selecting a Host Molecule

The simplest form of S^0, S_8, is readily available in high purity as a sublimed chalky yellow solid. Unfortunately, its use in biological applications is severely hampered by its hydrophobicity and low water solubility. The solubility of S_8 has been calculated to be only 1.9(6) x 10^{-8} mol/kg (or 1.5(2) x 10^{-7} M S^0), which is multiple orders of magnitude below biologically relevant S^0 concentrations.[16] Despite this low solubility, solid S_8 has been demonstrated to be biologically accessible by erythrocytes to produce H_2S,[23] which highlights the potential for increasing the bioavailability of S_8 in different systems. Moreover, these prior results suggest that biological pathways for sulfane sulfur activation from

elemental sulfur may be accessible if S_8 could be solubilized in aqueous environments.

Motivated by the binding of S_8 to the hydrophobic pockets of the archaeon *S. marinus* and increased solubility in hydrophobic environments, we envisioned that water-soluble compounds with hydrophobic interiors could enable similar S_8 binding. We also note that the 2008 Russian patent RU2321598C1 hypothesizes S_8 binding to cyclodextrin. Building from this hypothesis, we utilized CDs, which are cyclic oligosaccharides that contain a hydrophobic interior and that are widely utilized to bind and solubilize hydrophobic compounds.[24] This solubilization is due, in part, to the hydrophobic interior of CDs, which promotes encapsulation and binding of a non-polar guest, whereas the hydrophilic exterior enables water solubility. CDs are available in different sizes/volumes, and naturally produced CDs include α-CD, β-CD, and γ-CD, which contain 6, 7, and 8 glucose units, respectively. The choice of CD depends upon the size of the nonpolar compound to be solvated and the properties of the system being studied. Although natural CDs have limited water solubility, modification of the ring periphery with hydroxypropyl groups results in significant increases in solubility. In particular, 2-hydroxypropyl β-CD (2HPβ) and 2-hydroxypropyl γ-CD (2HPγ) have been used extensively, including in drug formulations to enable delivery of otherwise hydrophobic and insoluble compounds.[25]

4.3. Solubilizing Elemental Sulfur with 2-Hydroxypropyl β-CD

To test our general hypothesis, we treated aqueous solutions of 2HPβ with a 10-fold excess of solid S_8. We chose to start our investigations with the β-CD structure because the cavity volume (262 $Å^3$) is an ideal match for S_8 (~149 $Å^3$; 57% cavity occupancy), based general preference for encapsulated guests to occupy ~55% of host

volume.[26] After stirring as solution of 2HPβ with S_8 in water for several days and subsequent filtration to remove residual insoluble S_8, we observed a strong absorbance at 263 nm in the UV-vis spectrum, which is a characteristic absorbance of S_8.[27] By contrast, stirring S_8 in water under identical conditions but in the absence of 2HPβ failed to produce a significant S_8 absorbance (Figure 4.2). To test the stability of the solubilized 2HPβ/S_8, we next assessed whether the solution could be precipitated, filtered, and re-dissolved without loss of S_8. We precipitated the 2HPβ/S_8 complex with acetone, isolated the solid, and re-dissolved the resultant solid in buffer (Figure B.1). In these experiments, we observed that the same absorbance from the original and re-dissolved solutions, confirming the stability of the solubilized system in both the liquid and solid state

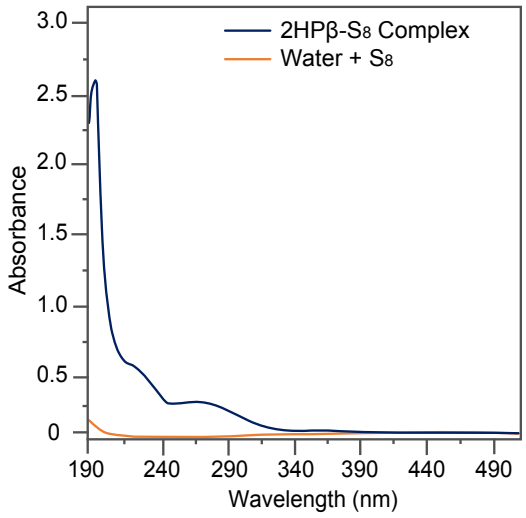


Figure 4.2. Comparison of the UV-vis spectra of S8 in water with S8 in aqueous solutions containing 2HPβ. Conditions: 4 mg of S8 in either 5 mL of PBS 7.4 PBS or 5 mL of 25% w/w 2HPβ in pH 7.4 PBS. Solutions were stirred for one day and then filtered prior to absorbance measurement.

4.4. Evaluating Other Solvating Agents for Elemental Sulfur

4.4.1. Non-Cyclic Sugars

To determine which components of the 2HPβ complex were responsible for S_8 solubilization, we next evaluated S_8 solvation in the presence of glucose and hydroxypropyl cellulose (HPC) as models for the sugar units of the 2HPβ macrocycle and the hydroxypropyl motif, respectively. After stirring an excess of S_8 to solutions of each saccharide in pH 7.4 phosphate buffered saline (PBS) buffer, the solutions were filtered, and UV-vis spectra were recorded (Figure 4.3). As shown in Figure 4.2, the characteristic absorbance at 263 nm corresponding to S_8 was significantly larger for 2HPβ (orange, 687 μM in this solution) than for glucose (aqua) or HPC (yellow). These data suggest that the cyclic structure and cavity of the CD are key components required for S_8 solvation. We next investigated the importance of the 2HPβ hydroxypropyl groups by testing S_8 solubilization with β-CD (lacking the 2-HP groups). We treated β-CD in pH 7.4 PBS buffer with excess S_8 and stirred for one month (Figure B.2). After filtering the solution, we failed to observe any solubilized S_8 by UV-vis spectroscopy, which suggests that the hydroxypropyl groups are required for S_8 solubilization.

4.4.2. 2-Hydroxypropyl γ-CD

To further investigate the requirement of the cyclic structure and presence of a cavity for S_8 solubilization, we also investigated whether 2HPγ (cavity volume: 427 $Å^3$) could solubilize S_8. We compared the S_8 solubilization in 25% w/w solutions of 2HPβ and 2HPγ in both pH. 7.4 PBS buffer and in water and observed significantly less S_8 solubilization from 2HPγ than from 2HPβ (Figure B.3). Taken together, these experiments strongly support that both the cyclic / cavity-containing structure and the

presence of the hydrophobic core and hydrophilic exterior are critical for the observed S_8 solvation by 2HPβ.

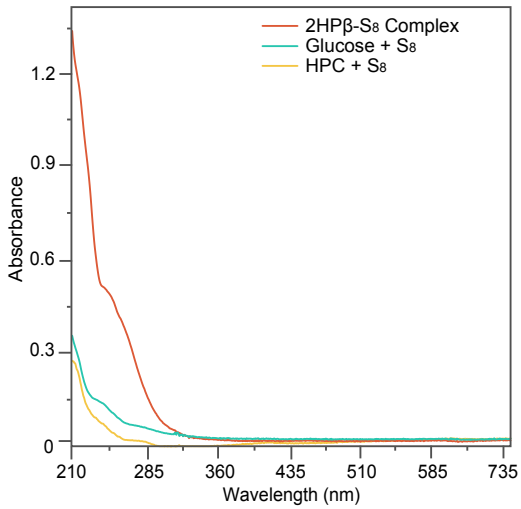


Figure 4.3. UV vis spectra of 640 mg S_8 in 10 mL pH 7.4 aqueous solutions containing 365 mg of 2HPβ, HPC, or glucose. 2HPβ demonstrates superior S_8 solvating ability to both non-cyclic saccharides.

4.5. Quantification of Solvated Elemental Sulfur

Building from these investigations, our next goal was to determine how much S_8 was solubilized in 2HPβ solutions. However, due to the low solubility of S_8 in water only the extinction coefficient for S_8 in methanol, 6730 $M^{-1}cm^{-1}$ at 263 nm, has been reported.[27] To confirm that the extinction coefficient for S_8 in methanol could be used to measure S_8 concentrations in the 2HPβ/S_8 complex, we first prepared a solution of S_8 in MeOH at a known concentration (278 μM). We then used the known S_8/methanol extinction coefficient to measure the concentration of S_8 in an existing 2HPβ/S_8 solution. We then diluted this solution to match the calculated value of [S_8] in the methanol stock solution and compared the resulting absorbance traces. If the extinction coefficient remains approximately constant between 2HPβ/S_8 and S_8 in methanol, then

the calculated [S_8] for the 2HPβ/S_8 stock solution should be correct, and dilution from this value to the S_8 concentration in the methanol stock solution should yield an identical concentration and thus an identical absorbances.

The resultant curves (Figure B.4) are similar with the 2HPβ/S_8 solution containing an S_8 concentration of 250 μM, which is ~12% different between the methanol/S_8 and 2HPβ/S_8 solutions. This observation confirms that the extinction coefficient does not change appreciably between these two systems. Based on these comparisons, we have used the molar extinction coefficient of S_8 in MeOH to quantify S_8 in the 2HPβ. These quantified concentrations are further supported by the quantitative S_8 conversion to H_2S by thiols (*vide infra*), which provides additional support for the value of the reported S_8 concentrations in the 2HPβ system. Applying this extinction coefficient to the 50% w/w 2HPβ solution, provides an S_8 concentration of 2.0 ± 0.2 mM (16 mM S^0) in water. When compared to the background solubility of S_8 in water, this constitutes ~10^5-fold enhancement in S_8 solubility.

4.6. Binding Stoichiometry of Elemental Sulfur to Cyclodextrin

Having established that S_8 is readily solubilized in aqueous solution of 2HPβ we next sought to investigate the stoichiometry and magnitude of the interaction between S_8 and 2HPβ. To probe these interactions, we monitored the observed [S_8] in solution as a function of increasing [2HPβ] (from 0-45% w/w) that were prepared with a 10-fold molar excess of solid S_8 in solution. Under these conditions, the activity of S_8 in solution remains constant, and increasing the [2HPβ] should result in a concomitant increase in [S_8] in solution. After stirring each solution for several days to ensure equilibrium, the solutions were filtered, and the S_8 concentration were measured by UV-

vis spectrophotometry (Figure 4.4a). As expected, the measured [S_8] increased linearly with increasing [2HPβ], which further supports a direct interaction between S_8 and the 2HPβ complex (Table B.1). The above constant activity data can be used to determine the binding stoichiometry and affinity between 2HPβ and S_8 through equation (1).[28] Under these conditions, the total S_8 content is defined as S_t in equation (1), and the concentration of unbound S_8, held constant throughout experiments to ensure constant activity, is defined as s_0 (1.9(6) x 10^{-8} mol/kg).[16] The constant *n* represents the binding stoichiometry.

$$(1)\ \log\frac{S_t - s_0}{s_0} = \log K_a + n * \log[2HP\beta]$$

Generating a log-log plot with the parameters of equation (1) demonstrates that the relationship between 2HPβ and S_8 is consistent with 1:1 binding (Figure 4.4b). Upon performing linear regression analysis, we obtained a K_a value of 3.4 ± 0.05 x 10^5 M^{-1} for the 2HPβ/S_8 complex, which is much stronger than the typical 10^2-10^3 M^{-1} binding affinities observed in βCD systems.[29] Although the above analyses are supportive of a 1:1 binding stoichiometry, we cannot definitively exclude equimolar higher order interactions from our data.

We repeated these binding stoichiometry experiments with 2HPγ and 2-hydroxypropyl α-CD (2HPα) (Figure B.5). Our expectation was that these differently sized CD hosts would not solubilize or bind S_8 as efficiently as 2HPβ For example, if S_8 were bound within 2HPα or 2HPγ, cavity occupancies of 86% and 32% would be observed, respectively, which is outside of the range of most host-guest interactions.[30]

Consistent with these expectations, 2HPγ solvated less significantly less S_8 and exhibited less linear binding behavior when compared to 2HPβ (Figure B.5a,b). Similarly, 2HPα solubilized very little S_8 with significant deviations from a well-defined binding relationship (Figure B.5c,d). These experiments further support that the size complementarity between S_8 and 2HPβ is an important factor in solubilization.

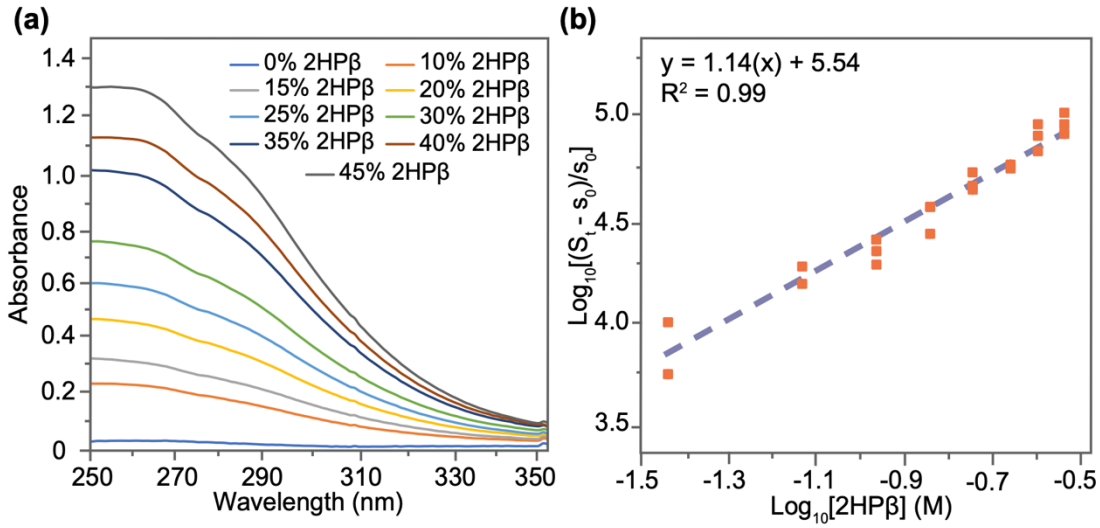


Figure 4.4. (a) UV-vis spectra of increasing concentrations of 2HPβ with a 10-fold excess of S8. (b) Log-log plot of quantified [S8] as a function of increasing [2HPβ]. Trials were performed in triplicate.

4.7. Solubilized Elemental Sulfur Is Chemically Accessible

Building from our data supporting S_8 solubilization in the 2HPβ/S_8 complex, we next sought to determine whether the solubilized S_8 is chemically accessible. To investigate this question, we first determined whether *tris*(2-carboxyethyl)phosphine (TCEP), which reacts with S^0 to generate an oxidized phosphine-sulfide product, could access the solubilized S_8 and generate the characteristic P=S and P=O products upon phosphine-mediated reduction and subsequent hydrolysis. Conveniently, this conversion can readily be monitored qualitatively by ^{31}P NMR spectroscopy with TCEP

(δ = 15.2-15.8 ppm), the resultant TCEP sulfide (δ = 51.5 ppm), and the associated TCEP oxide (δ = 53.0 ppm), all having characteristic NMR resonances. Prior to TCEP addition, the ^{31}P NMR spectrum of S_8 in a 25% w/w 2HPβ solution in pH 7.4 buffer only shows a peak at δ = 0 ppm from the PBS (Figure 4.5a). After TCEP addition and incubation overnight at room temperature, however, the ^{31}P NMR spectrum showed new peaks corresponding to unreacted TCEP, as well as the phosphine sulfide and oxide peaks at δ = 53.0 and 51.5 ppm, respectively. To confirm that the peaks were from oxidized TCEP products, we repeated the TCEP incubation with TCEP and S_8 in MeOD (Figure B.6a) and K_2S_5 in D_2O (Figure B.6b). In both cases, we observed the same peaks in the ^{31}P NMR spectrum at δ = 53.0 and 51.5 ppm, confirming product formation. In the absence of the 2HPβ/S_8, only the TCEP peak is observed, which confirms that TCEP oxide is not formed from adventitious oxidation. Similarly, addition of TCEP to a solution of 2HPβ did not generate TCEP oxide (Figure 4.5b). Taken together, these results demonstrate that the solubilized S_8 in the 2HPβ/S_8 solution is chemically accessible and can react directly with reductants.

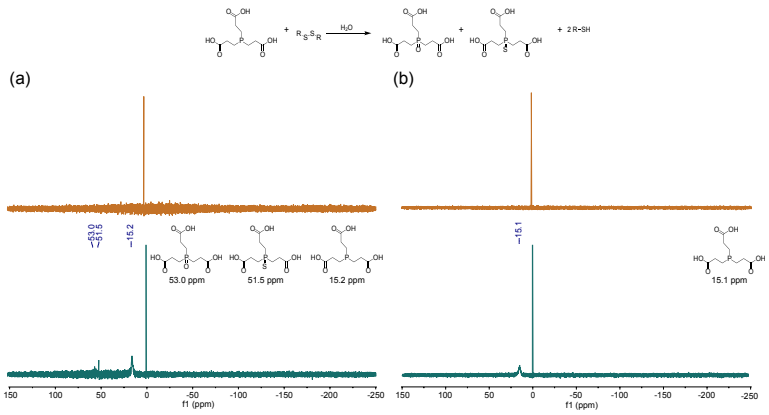


Figure 4.5. ^{31}P spectra in 2HPβ/S_8 or 2HPβ solutions incubated with TCEP. (a) 25% w/w solution of 2HPβ with 500 mg S_8 before (top) and after (bottom) addition of 10 mg TCEP with overnight incubation. (b) 25% w/w 2HPβ without sulfur in PBS before (top) and after (bottom) TCEP addition and incubation; no oxidized product peaks are observed.

4.8. Solubilized Elemental Sulfur Can Release H_2S in Solution

To further the potential biological relevance of the solubilized S_8 in the 2HPβ/S_8 complex, we next determined whether the solubilized S_8 could be reduced by thiols to release H_2S. One important feature of S^0 that contributes to its role in biology is its ability to release H_2S upon reduction by thiols. Polysulfides, such as DATS, are well established to release H_2S after reaction with thiols and are used broadly as exogenous sources of S^0. Although prior studies have investigated how different functional groups on polysulfide motifs impacts H_2S release, one limitation of these systems is that all of these compounds also generate organic byproducts upon H_2S release.[10, 30] As expected, a higher S^0 content in polysulfides leads to greater H_2S release, although tetrasulfides appear to be the largest synthetically-accessible and consistently-stable polysulfides. Using a similar logic of trying to maximize the S^0 content per donor motif, we envisioned that the solubilized 2HPβ/S_8 complex could also function as an entirely new approach to deliver S^0 and/or H_2S. Importantly, since S_8 is comprised entirely of S^0 it should be an effective donor, with the only byproduct being 2HPβ.

To determine whether the S_8 solubilized in the 2HPβ/S_8 system is accessible to thiols, and to quantify resulting H_2S release, we treated a 2HPβ/S_8 solution (25 μM S_8, 200 μM S^0 in 50% w/w 2HPβ) with 1 mM (5 equiv. with respect to S^0 atoms) of cysteine or reduced glutathione (GSH) under air-free conditions in pH 7.4 PBS. We measured H_2S release at different time points during the reaction by using the methylene blue assay, a colorimetric assay that measures H_2S production through the formation of the methylene blue dye (Figure 4.6). Calculated efficiency values assume all S^0 atoms can react to form H_2S. After 45 minutes, we observed 160 ± 5 μM H_2S

release (80% efficiency) from the 2HPβ/S_8 in the presence of cysteine (green). Under identical conditions, treatment of the complex with GSH (red), the most abundant biological thiol, yielded 220 ± 7 μM H_2S release after 45 minutes, corresponding to stoichiometric reduction of each S^0 atom. In the absence of S_8 with only 2HPβ and 500 μM cysteine, no H_2S was observed from the methylene blue assay (yellow), which confirms that 2HPβ and thiols alone do not spontaneously generate H_2S or result in methylene blue formation. Similarly, in the absence of 2HPβ we did not observe any H_2S release from S_8 (50 μM if fully soluble) and cysteine (500 μM, blue), confirming the importance of 2HPβ to the accessibility of S_8. These results show that S_8 is made chemically accessible in water by solubilization with 2HPβ, and that this sulfur can be reduced to H_2S with biologically relevant thiols.

In addition to the above experiments, we also determined whether S_8 needed to be pre-solubilized with 2HPβ prior to reaction with thiols, or whether 2HPβ could act as a catalyst for S_8 conversion to H_2S by thiols in water. To answer this question, we added 224 mg S_8 solid and 500 μM cysteine to 180 mg 2HPβ in 40 mL pH 7.4 PBS buffer and monitored H_2S generation using the methylene blue method. Under these conditions, we observed a faster peaking time of 15 minutes, but also a slightly lower overall efficiency of 147 μM H_2S release (74% efficient) (Figure B.6). These data indicate that S_8 does not need to be pre-solvated to the 2HPβ complex prior to thiol addition to facilitate reaction with thiols and subsequent H_2S release. These data may also support the role of 2HPβ as a phase transfer catalyst in these environments and that the rate of thiol-mediated reduction is faster than the rate of S_8 encapsulation. Expanding from the present system, these results suggest that hydrophobic motifs in more complex systems

may enable chemical accessibility of transiently formed S_8 from different redox processes.

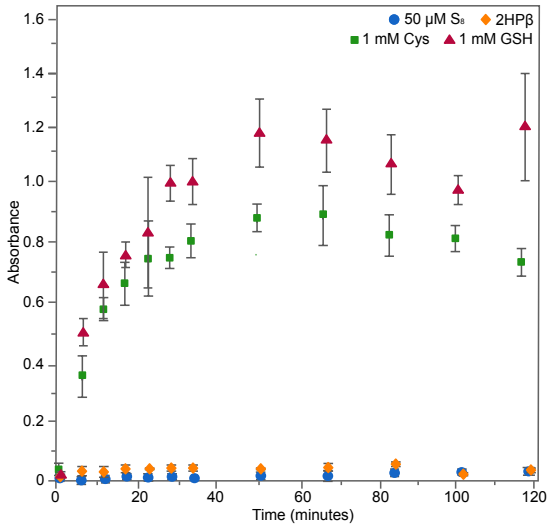


Figure 4.6. Release of H_2S from S_8 solvated in 2HPβ in the presence of cysteine (green) or GSH (red). 2HPβ alone (yellow) does not release H_2S in the presence of cysteine, and the amount of S_8 in solution at 50 μM without 2HPβ (blue) is not high enough for appreciable release. All data points collected in triplicate, and the error determined via standard deviation.

4.9. Sulfane Sulfur Is Taken Up by Cells

The accessibility of the solvated S_8 to biological thiols prompted us to investigate whether the solvated S_8 could be taken up into cells. Increasing intracellular levels of S^0 induces a cytoprotective effect by reducing oxidative stress, making direct S^0 donation desirable.[31] To this end, we treated HeLa cells with either 10 μM S_8 as 2HPβ/S_8 or an equivalent amount of 2HPβ alone for 24 hours. We then treated cells with the sulfane-sulfur selective fluorescent probe SSP4.[32] We observed that cells treated with 2HPβ/S_8 showed a significant increase in fluorescence when compared with those treated with 2HPβ alone, which is consistent with the bioavailability of the solubilized S_8 (Figure 4.7).[33] As a positive control, we repeated these experiments with

HeLa cells that were treated with the inorganic polysulfide K_2S_5 as a source of S^0, which also showed a significant SSP4 fluorescence response. These results support that the 2HPβ/S_8 system can cause significant increases in intracellular S^0 levels, though the efficiency of this uptake is not yet known.

4.10. Elemental Sulfur Confers Cell-Protective Effects Against Oxidative Stress

Finally, we sought to determine whether the bioavailable solubilized S_8 could be used to access protective effects associated with S^0/H_2S. Both H_2S and S^0 species play important antioxidant and anti-inflammatory roles throughout biology and are effective reducing agents able to neutralize damaging oxidants and free radical species.[34, 35] Polysulfides and persulfides containing S^0 have demonstrated potent antioxidative properties greater than those attributed to H_2S or thiols alone.[36] The well-studied antioxidant *N*-acetyl cysteine has also been shown to enhance the production of S^0.[31] As a whole, a common theme is that polysulfides, and their role as both H_2S and persulfide donor motifs, facilitates their protection against oxidative stress. Building from this observation, we reasoned that the high S^0 content of the 2HPβ/S_8 system should therefore make it an effective antioxidant in a cellular environment. With this in mind, we sought to determine whether the 2HPβ/S_8 system provided antioxidant potential in cellular environments.

We used the colorimetric Griess reagent to track relative levels of NO_x metabolites in RAW 264.7 macrophage cells pretreated with either 2HPβ/S_8 or 2HPβ, and then lipopolysaccharide (LPS). In the presence of proinflammatory cytokines such as LPS, RAW 264.7 cells produce NO from inducible nitric oxide synthase (iNOS).[37, 38] When formed, NO is rapidly oxidized to downstream NO_x species, and NO_2^- can be

quantified directly using the Griess assay, creating a colored product when cells are experiencing inflammation. Importantly, H_2S-releasing compounds have been previously demonstrated to significantly reduce NO_2^- formation in RAW 264.7 cells.[39, 40] To investigate the activity of the 2HPβ/S_8 in this system, we plated RAW 264.7 macrophage cells on 24 well plates and the following day treated with the delivery vehicle for 2HPβ/S_8 (PBS), different concentrations of 2HPβ/S_8, or equivalent concentrations of 2HPβ alone for 24 hours. The cells were then washed and treated with

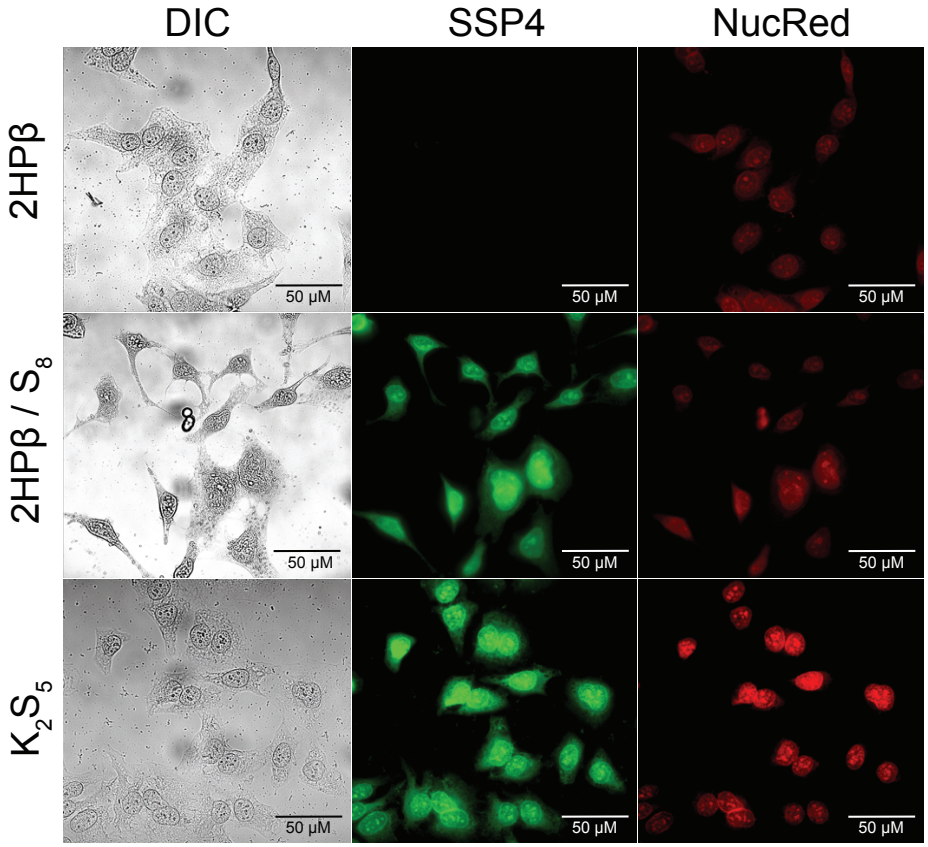


Figure 4.7. Fluorescent images of HeLa cells receiving 10 µM S_8 (top) the equivalent amount of 2HPβ alone (middle) or the inorganic polysulfide K_2S_5 (bottom) and imaged with the SSP4 probe for S^0. Scale bar = 50 µm.

1 µg/mL LPS for another 24 hours. After incubation, the media was collected from each well and the amount of NO_2^- was quantified using the Griess assay. The absorbance values of each treatment condition were normalized to the vehicle, which received no anti-inflammatory treatment. As shown in Figure 4.8, addition of the 2HPβ/S_8 complex results in a significant and decrease in NO_2^- formation across a range of concentrations, as evidenced by the decrease of absorbance of the Griess product. These data are consistent with H_2S and S^0 release. The 2HPβ complex alone also results in a reduction in NO_2^- levels, but to a much lower extent than the 2HPβ/S_8 system. It is possible that both H_2S and sulfane sulfur species generated from the solubilized S_8 both play roles in the reduction of NO_2^- levels in this assay, which wen taken together supports prior work in the field demonstrating that both H_2S and sulfane sulfur provide protective effects toward models of oxidative stress.

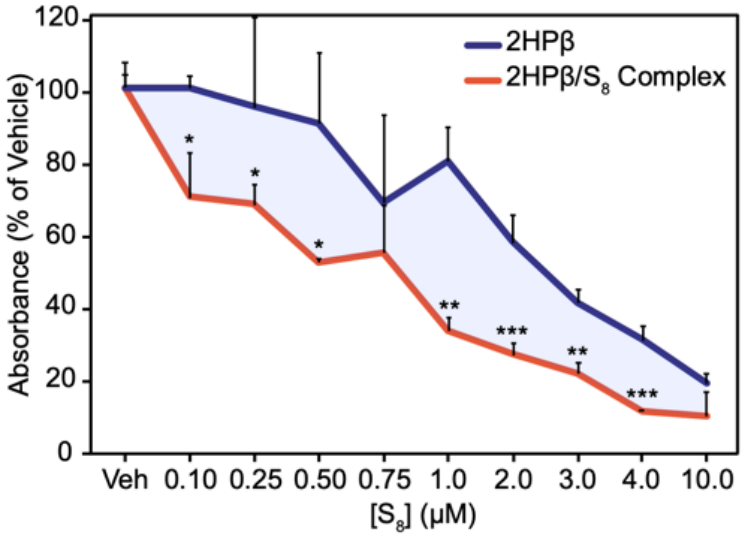


Figure 4.8. Relative levels of NO_2^- in RAW 264.7 macrophage cells treated with vehicle (PBS) and different concentrations of 2HPβ/S_8 (orange) or equivalent concentrations of 2HPβ alone (blue). Trials were performed in quadruplicate, and error determined by standard deviation.

4.11. Conclusions

We have demonstrated that hydrophobic cyclodextrins can facilitate solubilization and chemical activity of S_8 in water. In addition to providing a new and significant approach to delivering S^0/sulfane sulfur to aqueous and biological environments, these results provide fundamentally new insights that impact S_8 bioavailability. Building from the solubilization of S_8 by the 2HPβ system, these results may suggest that pools of oxidized sulfur can stably exist in biological hydrophobic structures such as proteins. Moreover, the demonstration that the 2HPβ system can effectively catalyze S_8 reduction to H_2S by thiols in water also highlights this approach as a method to limit S_8 accumulation. We anticipate that this and related systems currently under investigation in our lab will not only find utility as H_2S and sulfane sulfur delivery systems, but also in expanding investigations into how S^0 is managed in more complex biological systems.

4.12. Materials and Methods

4.12.1. Materials

Reagents were purchased from Fisher, Oakwood, and Tokyo Chemical Industry (TCI) and used as received. Deuterated solvents were purchased from Cambridge Isotope Laboratories. ^{31}P NMR spectra were recorded on a Bruker 500 MHz instrument. UV-vis spectra were recorded on an Agilent Cary 100 UV-vis spectrophotometer under standard conditions. Air-free techniques were performed under an inert N_2 atmosphere using Schlenk technique or in an Innovative Atmospheres glove box.

4.12.2. 2HPβ/S_8 Complex Formation

Different weight percentages (0-45%) of 2-hydroxypropyl β cyclodextrin (2HPβ) were added to pH 7.4 PBS buffer prepared from Millipore tablets in nanopure water in a stirred scintillation vial. A 10x molar equivalent of solid elemental sulfur (S_8) (Fisher) was added to this solution and shaken by hand until mixed. The vial was then stirred for several days at room temperature. After stirring, the solutions were taken up in plastic syringes and filtered through syringe filters (0.45 or 0.2 μm) to yield a clear solution that is colorless at low sulfur/cyclodextrin (CD) concentrations and yellow at higher concentrations. The S_8 concentration of each solution was quantified by UV-vis spectrophotometry in short-path quartz cuvettes using the molar extinction coefficient of S_8 in methanol (λ_{max} = 263 nm, ε = 6730 $M^{-1}cm^{-1}$).

4.12.3. S_8 Absorbance in MeOH versus buffer

To measure the absorbance of S_8 in methanol, 1.0 mg of S_8 was added to 7.0 mL of MeOH and shaken vigorously. The solution was then filtered into a quartz cuvette and the S_8 concentration was measured using the UV-vis absorbance at 263 nm. A stock solution of 2HPβ/S_8 in water was diluted to the same S_8 concentration and measured by UV-vis. Both spectra were then overlaid to determine the percent difference of the S_8 concentration value.

4.12.4. Comparison of S_8 Solvation Between 2HPβ and Other Saccharides

Scintillation vials were charged with 640 mg S_8 followed by 10 mL of pH 7.4 PBS buffer or 10 mL of pH 7.4 PBS buffer containing 365 mg of either 2HPβ, hydroxypropyl cellulose (HPC), or glucose. The vials were sealed and stirred for three days, after which they were filtered and analyzed by UV-vis. Similar experiments were

also performed with a 1.56% w/w solution of the parent non-functionalized β-CD in 8 mL pH 7.4 PBS buffer and 100 mg S_8. The resultant heterogenous solution was stirred for one month, filtered, and measured as described above. To evaluate S_8 solubilization in 2-hydroxypropyl γ-CD (2HPγ), a 25% w/w solution of 2HPγ or 2HPβ was prepared in 7.5 mL water or pH 7.4 PBS buffer. To each solution, 200 mg S_8 was added and solutions were stirred for two weeks, after which they were filtered and analyzed as described above.

4.12.5. Measurement of Binding Affinity of Elemental Sulfur to 2HPβ

Solutions of 2HPβ or other CDs of increasing weight percentage from 0 to 45% in pH 7.4 PBS were prepared from a 50% w/w stock solution of 2HPβ. To each of these vials were added 10-fold molar equivalents of S_8, and the resultant solutions were stirred for several days. After stirring, the solutions were filtered and the S_8 content quantified as described above. The measured S_8 concentrations from each sample were plotted against the 2HPβ concentration in a log-log plot described by equation (1). The apparent K_a value was obtained by linear regression analysis using Microsoft Excel's LINEST function. These experiments were performed in triplicate.

4.12.6. ^{31}P NMR Spectroscopy of Trapped Sulfur

NMR tubes were charged with 25% w/w 2HPβ/S_8 solutions in pH 7.4 PBS buffer and ^{31}P NMR spectra were recorded, after which a 30-fold molar excess of TCEP was added. The NMR tubes were incubated overnight at room temperature, after which additional ^{31}P NMR spectra were recorded. The presence of reductant-labile sulfur was characterized by the formation of TCEP oxidation products, including TCEP sulfide or TCEP oxide formed by hydrolysis. The parent TCEP peak is at δ = 15.2-15.8 ppm, and

the oxidized oxygen-containing and sulfur-containing product peaks are at δ = 53.0 (P=O) and 51.5 (P=S) ppm, respectively. To ensure that 2HPβ was not causing TCEP oxidation directly, the above experiments were performed with a solution containing only 25% w/w 2HPβ in pH 7.4 PBS and no oxidized TCEP products were observed.

4.12.7. Evaluating Sulfur Content of Liquid and Precipitated 2HPβ/S_8 Complex

Two scintillation vials were charged with 25% w/w 2HPβ and a 10-fold excess of S_8 in 10 mL pH 7.4 PBS buffer and stirred overnight. One solution was filtered and evaluated as described above. The other solution was filtered into a recrystallization dish filled with an excess of acetone, which results in formation of a white precipitate. The solvent was evaporated, and the resultant solid was collected and used to make a new 25% w/w solution, which was analyzed by UV-vis spectroscopy as described above.

4.12.8. Measurement of H_2S Release from 2HPβ/S_8 Complex in the Presence of Biological Thiols

A round bottom flask was charged with 40 mL of degassed pH 7.4 PBS buffer, a stir bar, and either 1 mM L-cysteine or reduced GSH in an N_2-filled glove box. The solutions were removed from the glovebox and 500 µL of 2.0 mM S_8 in 50% w/w 2HPβ (for a final S_8 concentration of 25 µM) was added by syringe. For each time point, a 500 µL aliquot was removed by syringe and added to 500 µL of the methylene blue cocktail in a 1.5 mL plastic cuvette. (Methylene blue cocktail contains: 200 µL 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) $Zn(OAc)_2$). The methylene blue reactions were incubated at room temperature for 1 hour, after which the absorbance at 670 nm was measured. For control

experiments, the above procedure was repeated but with either 0.5 mg S_8 (50 μM if fully soluble) or 0.9% w/w 2HPβ. All time points were collected in triplicate.

4.12.9. Calibration Curve for Sulfide Measurement

Solutions containing 500 μL of the methylene blue cocktail and 0.5 mL PBS containing 0.9% w/w 2HPβ were prepared in plastic cuvettes. A 100 mM stock solution of NaSH was prepared in 1.0 mL PBS under inert atmosphere. Solution was then diluted to 1 mM and added to the 1.0 mL solutions for final concentrations of 10, 20, 30, 40, 50, and 70 μM. The cuvettes were incubated at room temperature for 1 hour and absorbance at 670 nm was measured. All data points were measured in triplicate.

4.12.10 Fluorescent S^0 Imaging

HeLa human cervical cancer cells (ATCC CCL-2) were cultured in DMEM containing phenol red, 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 maintained at 37 ˚C under 5% CO_2. Cells were seeded in glass-bottomed 2 mL imaging dishes (MatTek) in DMEM containing 10% FBS, 1% PS and incubated overnight. The next day dishes were rinsed twice with FBS-free DMEM and incubated with test substances (solvated in PBS) in FBS-free DMEM for 24 hours. 2HPβ and 2HPβ/S_8 stock solutions both contained 50% w/w 2HPβ, with equal volumes added to control and test dishes respectively. K_2S_5 was solvated in a ~1 mg/mL stock solution in water, and 10 μL were added to positive control dish. After treatment, dishes were again rinsed twice with FBS-free DMEM and incubated with NucRed and 10 μM SSP4 (Dojindo) separately in FBS-free DMEM according to manufacturer instructions for 15 minutes each. The NucRed (Invitrogen) concentration was two drops per 10 mL

FBS-free DMEM. Dishes were rinsed twice with FluoroBrite DMEM (Thermo Fisher), and then imaged in this media. Image workup done with FIJI. 2HPβ/S_8 SSP4 image normalized to 2HPβ only image, where this image's intensity was turned down to background, and these identical settings applied to the 2HPβ/S_8 image.

4.12.11 Griess Assay

RAW 264.7 murine macrophage cells (ATCC TIB-71) were cultured in DMEM containing phenol red, 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 maintained at 37 ˚C under 5% CO_2. For the Griess assay, RAW 264.7 cells were seeded in sterile 12 well culture plates in DMEM containing 10% FBS, 1% PS and incubated overnight. The next day, the media was removed, and the cells were washed with PBS. The media was replaced with media containing either the 2HPβ/S_8 complex or an equivalent w/w percentage of 2HPβ in FBS-free, phenol red-free DMEM. After a 24-hour incubation, cells are again rinsed and media is replaced with FBS-free, phenol red-free DMEM containing 1 µg/mL lipopolysaccharide (LPS). After another 24-hour incubation, 150 µL of media from each well was pipetted into a 96 well plate containing 20 µL mixed Griess reagent (Invitrogen) and then mixed with 150 µL DI water. After incubation at room temperature for 30 minutes, plates were analyzed on a plate reader (BioTek Synergy 2).

CHAPTER V

INHIBITION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE BY ELEMENTAL SULFUR VALIDATES ROLE OF SULFANE SULFUR IN PROTEIN PERSULFIDATION BY POLYSULFIDES

N-acetylcysteine tetrasulfide was synthesized by Matthew Cerda. 2-Hydroxypropyl β-cyclodextrin/S_8 complex formation and all assays shown were performed by Sarah Bolton.

5.1. Introduction

The evolution of life on Earth has been shaped by hydrogen sulfide (H_2S). 2.4 Billion years ago, Earth's atmosphere was converted from a reducing to an oxidizing environment that is rich in O_2.[1] This crucial event lead to the evolution of multicellular life on Earth, because the reduction of oxygen delivers among the largest free energy release per electron transfer of all elements.[2] It is this feature, along with the fact that O_2 is a relatively stable gas species, that enabled the development of the specialized and compartmentalized features of more complex life. Prior to this point, the reducing atmosphere contained relatively high concentrations of H_2S, much higher than what is found today.[3, 4] It is under these conditions that early single-celled life began, H_2S and CO_2 were generated by sulfur-reducing microbes using the oxidized sulfur that was abundant in volcanic environments as electron acceptors.[5] Meanwhile, sulfur-oxidizing species used H_2S as an electron donor for anoxygenic photosynthesis.[6] Today, the influence of H_2S on Earth's earliest lifeforms is reflected in the very fabric of eukaryotic, multicellular life billions of years after it was displaced by oxygen as a primary energy source. It is theorized that the bacterial species that gave rise to

mitochondria was a sulfide-oxidizing α-protobacteria that developed a symbiotic relationship with an archaeon host.[7] This union may have been what enabled anaerobic species to survive the transition to an aerobic atmosphere, or it may have been due to the fact that the mitochondrian symbiote (and even mitochondria today,[8] through sulfide:quinone oxidoreductase), could produce ATP from H_2S.[9] The mementos of our ascension from the primordial H_2S world are reflected in mitochondria, and also in cellular responses to hypoxia,[10] and our complex cellular sulfur signaling systems.[8]

Today H_2S, carbon monoxide (CO), and nitric oxide (NO) are members of a family of endogenously produced signaling molecules known as the gasotransmitters.[11-14] These small, cell-permeable gasses act in numerous pathways throughout cells, sometimes acting in concert to achieve objectives such as angiogenesis and vasodilation.[15] Of the three gasotransmitters, H_2S is thought to have had the greatest influence on the naissance of eukaryotic cells.[7, 9]

Sulfur can be found with oxidation states ranging from -2 to +6,[16] and this versatility is largely what made it a critical component in the origin of life. H_2S is oxidized in cells to form polysulfides, which contain sulfane sulfur (S^0).[17] Years of study on the effects of H_2S treatment in cells have revealed benefits such as reduction of oxidative stress,[18-20] prevention of gastric ulcers,[21] and enhanced rates of wound healing.[22-24] Recently, evidence has emerged that indicates S^0 species are responsible for much of what we have observed after H_2S treatment.[25] This is due to a type of post-translational modification known as persulfidation, or sulfhydration, in which free cysteine residues react with S^0 to form persulfides (-SSH).[26-30] Since both cysteine and the sulfur atom of H_2S share a -2 redox state, H_2S cannot directly persulfidate proteins

unless they have been previously oxidized. By modifying cysteine residues, persulfidation changes both the structure and function of proteins and can serve as a regulatory, redox-sensitive enzyme "switch". A prominent example of a cellular process regulated by persulfidation is vasodilation, which is mediated by the persulfidation of Cys_{43} in the Kir 6.1 subunit of K_{ATP} channels. The persulfidation induces hyperpolarization and vasorelaxation of endothelial cells.[31]

Given the wide influence that persulfidation has over sulfur biology, it is critical to understand the process and find ways to harness this versatile system. Our lab has developed synthetic tools to aid in the study of S^0, including a series of benzyl polysulfides (Bn_2S_n, n = 1 – 4) that differs only in S content, allowing a more precise view of S^0 activity.[32] However, we found that treatment of cells with these compounds versus the allium-derived polysulfide diallyl trisulfide (DATS) had drastically differing results on cellular proliferation, likely due to the inevitably differing interactions between S^0 and the pendant alkyl groups of organic polysulfides. With this in mind, we developed a system for the solvation of elemental sulfur (S_8), a stable species composed entirely of S^0 atoms, using 2-hydroxypropyl β-cyclodextrin (2HPβ), to remove any ambiguity and confounding factors about the roles of S^0 itself.[33] We found that the solvated S_8 was capable of releasing H_2S after reaction with thiols, could enter cells, and could confer protection against oxidative stress in macrophages.

We then sought to evaluate the effectiveness of our 2HPβ/S_8 system in studying persulfidation. We built off previous literature by Jarosz *et al.* in 2015 which studied the persulfidation and subsequent inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with an inorganic polysulfide. Further, they found that H_2S treatment could

not replicate this inhibition and this, along with mass spec data demonstrating the persulfidation of Cys_{156} and Cys_{247} after polysulfide treatment, confirms that persulfidation is responsible for the observed phenomenon.[34] We thus find that the GAPDH system provides a useful opportunity of comparing the persulfidating capabilities of pure S^0 to other polysulfides, which will give us better insight into the roles of S^0 in persulfidation.

5.2. Obtaining the K_M and V_{max} of GAPDH, and the Saturating G3P Concentration

We began by determining the K_M and V_{max} of purified, lyophilized GAPDH to determine ideal concentrations of the substrate glyceraldehyde-3-phosphate (G3P) for our inhibition assays. Briefly, initial rates of the reduction of NAD^+ by GAPDH at constant NAD^+ concentration were plotted against different G3P concentrations ranging from 0-2 mM using the method described previously.[34] The K_M of our system was calculated as 0.59 mM, and the V_{max} was 0.16 µM NADH/sec (Figure 5.1). Enzyme velocity was calculated using a calibration curve for NADH concentration (Figure C.1). With this data in hand, we settled on a G3P concentration of 1 mM for our GAPDH inhibition assays.

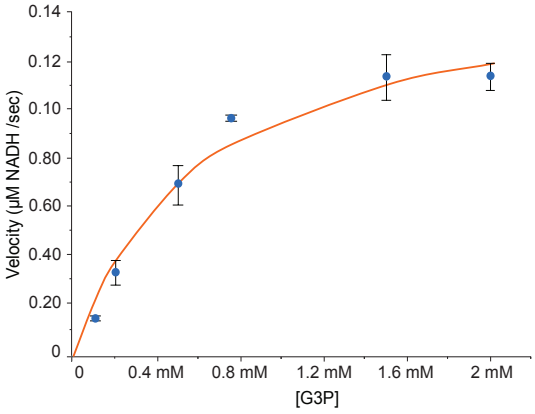


Figure 5.1. Michaelis-Menten curve for the kinetic relationship between GAPDH and G3P concentration. The K_M was calculated as 0.59 mM G3P, and the V_{max} as 0.16 µM NADH/sec .

5.3. Polysulfides Reduce GAPDH Activity, H_2S Has No Effect

We first sought to reproduce the results found in previous work[34] studying the interaction of GAPDH with oxidized sulfur species. H_2S and potassium polysulfide ("K_2S_x") were evaluated in their ability to inhibit GAPDH activity under various redox conditions. H_2S is a strongly reducing species, with a sulfur atom in the -2 state, and should not react or modify reduced GAPDH. Conversely, polysulfides containing S^0 can modify proteins at free cysteine residues through persulfidation. Jarosz *et al.* found that treatment of the reduced enzyme with NaSH, a soluble source of H_2S, had no impact on GAPDH function. However, K_2S_x treatment reduced enzyme activity by 60%, contradicting previous reports of an enhancement of GAPDH activity after sulfide treatment.[28]

To gain a basis of comparison for our pure S^0 system, we repeated these experiments on reduced GAPDH (Figure 5.2). Our results closely matched what was previously observed by Jarosz *et al*. We found that NaSH treatment produced no change in reduced GAPDH activity at 1 mM G3P (Figure 5.2a). This result is expected because NaSH cannot persulfidate reduced proteins, though it can persulfidate oxidized cysteine residues such as Cys-SOH or Cys-SNO.[26] This distinction is likely a source of past confusion on the protein-modifying abilities of H_2S, as proteins *in vitro* and *in cellulo* could be modified with these oxidized residues unless measures are taken to give the free cysteine residues a uniform redox state. Treatment of reduced GAPDH with K_2S_x also produced a result consistent with the work of Jarosz *et al.*, with GAPDH activity being reduced to 30% of vehicle (Figure 5.2b).

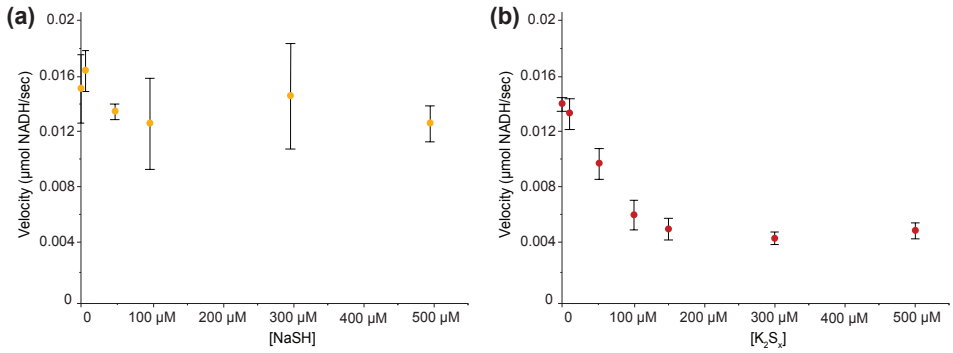


Figure 5.2. Reduced GAPDH inhibition by (a) NaSH and (b) K_2S_x. The reduced sulfur atom in NaSH cannot persulfidate a reduced protein unless it has oxidized cysteine residues present. However, the S^0-containing inorganic polysulfide K_2S_x can modify proteins by persulfidation, and here it inhibits GAPDH by 70%. All data points gathered in triplicate.

However, one limitation of this approach is the use of K_2S_x as a template for the study of polysulfides. Potassium polysulfide is an inorganic polysulfide, which makes it useful in biological studies because of its solubility but also unstable and prone to disproportionation into different polysulfide or other sulfur-containing species.[35] K_2S_x is not one specific polysulfide species but rather is a mixture, therefore an accurate molar mass cannot be obtained. For the purposes of this study, we assumed the molar mass of K_2S_5, which matches the elemental makeup of this inorganic salt. Due to this uncertainty, the exact S^0 present in each treatment level in this experiment is approximate rather than exact. Further, Jarosz *et al.* found that K_2S_x treatment of GAPDH that had been oxidized with glutathione disulfide (GSSG) partially rescued enzyme activity, which was also achieved with NaSH and dithiothreitol (DTT) treatments. The fact that the oxidized polysulfide K_2S_x would have the same effect on an oxidized enzyme as the two reducing species NaSH and DTT indicates that there was likely contamination of SH^- or another reduced species in the disproportionated mixture of inorganic polysulfide. This presents a valuable opportunity to evaluate our 2HPβ/S_8

system, which contains only S^0 atoms. This should remove any ambiguity of the redox state of the sulfur species being studied.

5.4. Elemental Sulfur Strongly Inhibits GAPDH Activity

We then repeated the inhibition assay using solubilized S_8. GAPDH incubated with concentrations of S_8 ranging from 0-100 μM (800 μM S^0) was strongly inhibited, with a complete shutdown in activity at 100 μM (Figure 5.3). The concentration of S_8 required to achieve the 60% reduction in activity seen with 200 μM K_2S_x treatment was less than 20 μM (160 μM S^0). This highlights the ambiguity of the S^0 content of K_2S_x and other inorganic polysulfides, as many non-S^0 atoms are present in the mixture that will not persulfidate proteins. One striking difference between treatment of GAPDH with K_2S_x and S_8 is the fact that GAPDH inhibition eventually leveled out at high concentrations of K_2S_x treatment, whereas S_8 increased inhibition steadily until complete shutdown at 100 μM. It is unclear why this happens, but likely involves the unstable nature of K_2S_x. It is possible that at very high concentrations, inorganic polysulfides shift in equilibrium away from S^0-rich species and persulfidation levels out. It is also possible that at these high concentrations the polysulfides begin precipitating and crashing out of solution, giving a cap to the possible amount of persulfidation that can be induced. These limitations strengthen the potential of solubilized S_8 as a means of studying S^0 and persulfidation under more ideal conditions and with less ambiguity.

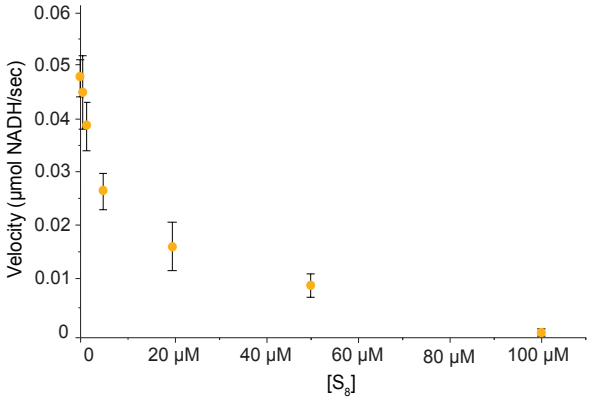


Figure 5.3. Inhibition of GAPDH with S_8, total shutdown at 100 µM makes S_8 a more efficient GAPDH inhibitor and persulfidation agent than K_2S_x.

5.5. Polysulfides Do Not Rescue Oxidized GAPDH Function

We next wanted to evaluate how S_8 affects the activity of oxidized GAPDH. Persulfidation inhibits GAPDH, and the process of persulfidation is an oxidative process, indicating that oxidizing GAPDH causes its inhibition. However, Jarosz *et al.* found that both K_2S_x and NasH treatments on GAPDH that had been oxidized with glutathione disulfide (GSSG) partially rescued GAPDH function.[34] This should not be possible if the polysulfide being studied contains no reducing sulfur atoms. The unstable nature of inorganic polysulfides is well known, and disproportionated K_2S_x samples will likely contain some reducing byproducts.[35] This indicates that the rescue of function seen is not due to a polysulfide, but to a reducing contaminant species. S_8, being entirely S^0 atoms, should not rescue oxidized GAPDH function, which would confirm the obfuscating nature of contaminant reducing species. To assess this, we incubated GAPDH with GSSG, while also incubating reduced GAPDH with tris buffer only, for two hours at room temperature. After two hours, both samples were run through PD-10 desalting columns packed with Sephadex G-25 resin for buffer exchange. The enzyme activity assay was then performed on both oxidized and reduced

GAPDH samples with S_8 (Figure 5.4). As expected, oxidized GAPDH displayed no rescue of function with S_8 treatment. The reduced GAPDH sample again showed a sharp decline in activity with increasing concentrations of S_8. This indicates that S^0 and polysulfides should not rescue oxidized GAPDH, because rescue requires reducing the enzyme back to its native form. This also highlights the benefits of the S_8 solvation system because the observed effects can more reliably be attributed to S^0 and not a disproportionated species.

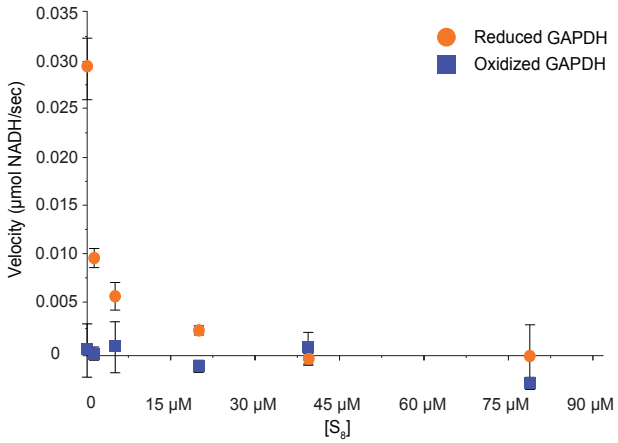


Figure 5.4. Activity of either native (orange) or oxidized (blue) GAPDH after S_8 treatment. S_8 strongly inhibits native GAPDH by oxidation and cannot rescue the function of oxidized GAPDH.

5.6. Organic Polysulfides Inhibit GAPDH, But Extent Varies

While S_8 enables the direct study of S^0 in cells and enzymes, in natural conditions our S^0 sources are organic polysulfides like DATS.[36-40] Because of this, it is important to understand how these species persulfidate proteins, and what the subsequent impacts of that persulfidation are, as it has been established that differing pendant alkyl groups radically change the impact of organic polysulfide treatment in cells.[32] To evaluate this, we looked to the naturally occurring polysulfide DATS and the synthetic N-acetylcysteine tetrasulfide (NAC_2S_4), which our lab synthesized with a

library of other organic tetrasulfides in 2017 and has studied in cells.[41] In a previous study, we found that treatment of bEnd.3 murine epithelial cells with a synthetic benzyl trisulfide was highly cytotoxic, while DATS treatment up to 200 μM caused no cytotoxicity.[32]

Could the toxicity have something to do with persulfidation, and could DATS simply be more inefficient at modifying enzyme activity, resulting in less biochemical change? When we applied DATS to GAPDH, we found a very similar result to treatment with K_2S_x (Figure 5.5a). Treatment of DATS up to 500 μM (500 μM S^0) reduced GAPDH activity by half, and a similar leveling-out behavior was seen here which began at 300 μM DATS treatment. Atom-for-atom, DATS is a less efficient persulfidation agent than S_8, which achieved a 50% reduction in GAPDH activity between 5 – 10 μM (40 – 80 μM S^0). This indicates that persulfidation under natural conditions with organic polysulfides is not solely based on the presence of S^0 atoms, and there is an interplay between S^0 and the pendant alkyl groups of the polysulfides. This is especially visible in the performance of NAC_2S_4 (Figure 5.5b), which sharply curtailed almost all enzyme activity at only 5 μM (10 μM S^0) and shut GAPDH down completely between 25 – 50 μM (50 – 100 μM S^0). This makes NAC_2S_4 an even more efficient inhibitor of GAPDH than S_8, which is purely S^0. These results indicate that the pendant alkyl groups may influence the extent and efficiency of protein persulfidation by S^0, or that the presence of these alkyl groups makes the effects of that persulfidation more, or less, pronounced. More study is needed to tease apart the many different factors of this complex regulatory system and having a viable source of pure S^0 goes a long way in alleviating some of the confounding factors.

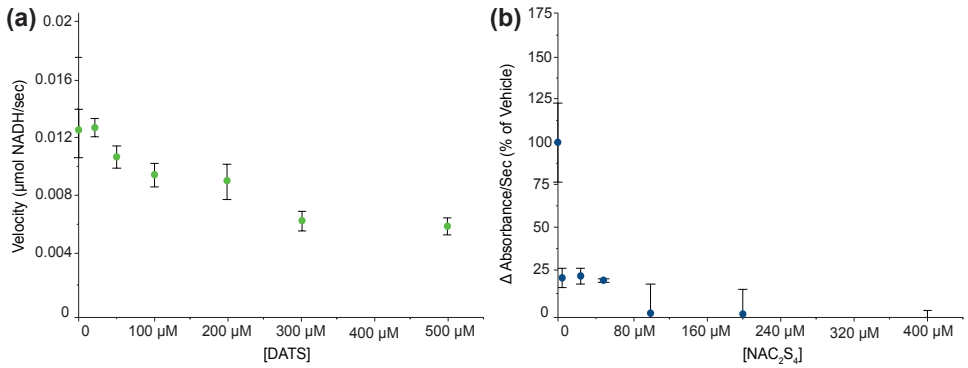


Figure 5.5. Inhibition of GAPDH is seen after the treatment of organic polysulfides (a) DATS and (b) NAC_2S_4, but extent of the inhibition differs greatly between the two. NAC_2S_4, though derived from the natural product N-acetylcysteine, completely shuts down GAPDH. DATS displays similar behavior to what was seen in the inorganic polysulfide K_2S_x, where inhibition levels out near the 50% mark.

5.7. Conclusions

It is becoming clear that understanding the biological roles of H_2S hinges on understanding the process of persulfidation. Far from being a simple reducing agent conferring cell-protective effects by direct reduction of reactive oxygen species, H_2S acts as a source for the downstream production of oxidized S^0, which can modify the structure and function of proteins.[17] A powerful example of this regulatory function by S^0 is the Nrf2/Keap1 system of protection against oxidative stress.[42] H_2S has long been recognized for its ability to reduce oxidative stress and inflammation. It is now known that the Nrf2/Keap1 system operates through persulfidation of Keap1, which frees Nrf2 to translocate to the nucleus and upregulate the production of GSH and other protective species.[43, 44] The benefits seen from H_2S treatments therefore cannot be separated from the indirect production of other cell-protective compounds. H_2S treatment increases intracellular S^0 concentrations, and S^0 goes on to act as a switch controlling cell-protective functions when needed.

To discern which cellular activities are modulated by H_2S (or other sulfur-containing species such as thiols) directly and which are ultimately caused by persulfidation by S^0, it is vital to have a system that reliably provides pure, stable S^0 for study. Historically, inorganic polysulfides have been the obvious choice due to their high solubility. However, their unstable nature means that exact treatments with S^0 are not possible, and these disproportionated mixtures may even contain reduced sulfur species that can hinder persulfidation. Organic polysulfides can be stable, but they cannot be separated from the influence of their pendant alkyl groups, which can greatly modulate the extent of persulfidation and the subsequent effects in cells.

Our 2HPβ/S_8 system provides a simple and reliable method for the study of S^0 and persulfidation. Here, we demonstrated the superior inhibitory action of solubilized S_8 versus inorganic polysulfides that have previously been studied. Further, we compare this with the effects of treatment from two organic polysulfides, DATS and NAC_2S_4. The strongly differing results between S_8, DATS, and NAC_2S_4 confirm that the identity of the pendant alkyl group determines the extent of enzyme modification. S^0 is the agent responsible for the act of persulfidation, but alkyl group identity determines its actual cellular outcomes. The use of pure S^0 in biological studies does much to untangle the complicated chemical pathways of reactive sulfur species. Controllable H_2S donor molecules have become essential to elucidating the functions of biological sulfur. The development of triggerable S^0 donor compounds would be similarly useful and provides a promising area of future study.

5.8. Materials and Methods

5.8.1. Materials

Reagents were purchased from Sigma-Aldrich, Cayman Chemical Company, Fisher Scientific, Oakwood Chemical, and Strem Chemicals. 2HPβ/S_8[33] and NAC_2S_4[41] were prepared as previously described. Lyophilized glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit muscle was purchased from Sigma and used in all experiments. Enzyme kinetic data was recorded on a Cary 60 UV-vis spectrophotometer.

5.8.2. Calibration Curve for Enzyme Velocity

NADH was solvated in 20 mM pH 7.8 tris buffer and added to 500 µL assay buffer (20 mM tris pH 7.8, 100 mM NaCl, 1 mM oxidized nicotinamide adenine dinucleotide (NAD^+), 10 mM sodium pyrophosphate, 20 mM sodium arsenate, 0.1 mg/mL bovine serum albumin (BSA)) for a total volume of 1 mL in plastic cuvettes. Each cuvette also contained 1 mM G3P. Cuvettes were read on Cary 100 Uv-vis at 340 nm and plotted on Microsoft Excel.

5.8.3. Determination of Saturating Glyceraldehyde-3-Phosphate Concentration

GAPDH was solvated in 20 mM pH 7.8 tris buffer to 1 mg/mL. 10 µL of this solution was added to 10 µL tris buffer aliquoted into microfuge tubes, and these were incubated at 37 °C for 30 minutes. After incubation, 1 µL of solution was removed from each tube and added to 500 µL assay buffer aliquoted into plastic 1.5 mL cuvettes. Cuvettes were placed in Cary 60 UV-vis spectrophotometer, and the reaction was initiated by addition of 500 µL glyceraldehyde-3-phosphate (G3P) (Sigma) in pH 7.8 tris for final reaction concentrations of 0 – 2 mM G3P. Kinetic scan taken at 37 °C for

two minutes at 340 nm with each reaction performed in triplicate. Data was analyzed by subtracting the initial absorbance value from the final value and dividing by the total seconds per reaction. K_M was calculated with the GRG Nonlinear solving method in Microsoft Excel.

5.8.4. GAPDH Inhibition Assays

We followed the reduced GAPDH enzyme assay as previously described.[34] Lyophilized GAPDH was solvated in 20 mM pH 7.8 tris buffer to 1 mg/mL. 10 µL of this solution was added to 10 µL tris buffer aliquoted into microfuge tubes containing the test compounds. Vehicles were pH 7.4 PBS buffer for NaSH and K_2S_x, 5% 2-hydroxypropyl β -cyclodextrin (2HPβ) for S_8, pH 7.8 tris buffer for diallyl trisulfide (DATS), and 2.5% DMSO for N-acetylcysteine tetrasulfide (NAC_2S_4). Experiment performed as described above. All values collected in triplicate and adjusted to the vehicle.

CHAPTER VI

LIGHT-ACTIVATED COS/H_2S DONATION FROM PHOTOCAGED THIOCARBAMATES

Zhao, Y.; Bolton, S.G.; Pluth, M.D., Light-Activated COS/H_2S Donation from Photocaged Thiocarbamates. *Organic Letters* **2017**, *19* (9), 2278-2281.

6.1. Introduction

Hydrogen sulfide (H_2S) is an important biological gaseous molecule that exhibits potent protective activities in mammals and which is recognized as the third member of the gasotransmitter family, along with nitric oxide (NO) and carbon monoxide (CO).[1-3] Endogenous H_2S is synthesized primarily from cysteine and homocysteine by enzymes such as cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and cysteine aminotransferase (CAT)/3-mercaptopyruvate sulfur transferase (3-MST).[4, 5] Both endogenous H_2S synthesis and exogenous H_2S administration exhibit promising protections in different stages of diseases.[6, 7] For example, H_2S can relax blood vessels by activating K_{ATP} channels[8, 9] and can rescue cardiomyocytes from oxidative stress damage by scavenging cellular reactive oxygen species (ROS).[10, 11] Furthermore, H_2S-releasing nonsteroidal anti-inflammation drugs (H_2S− NSAIDs), which couple NSAIDs with H_2S-releasing molecules, have been shown to significantly diminish NSAID-induced GI damage while maintaining potent anti-inflammation activities.[12-14] All of these findings illustrate the positive roles of H_2S in human health and also highlight the therapeutic potential of synthetic H_2S-releasing molecules against different types of damage and diseases.

To modulate biological levels of H_2S, researchers often use H_2S-releasing molecules ("H_2S donors") rather than inorganic sulfide salts, such as sodium sulfide (Na_2S) and sodium hydrogen sulfide (NaSH), due to the rapid and uncontrollable release of H_2S from these inorganic precursors.[15-21] Because of these limitations, synthetic H_2S donors have attracted significant attention in the past several years (Figure 6.1),[18, 22, 23] although numerous limitations remain. Aligned with the need to develop new methods of H_2S release, our laboratory recently reported a new class of H_2S donors by engineering the release of carbonyl sulfide (COS) from caged-thiocarbamate molecules.[24] The released COS is quickly hydrolyzed to H_2S by

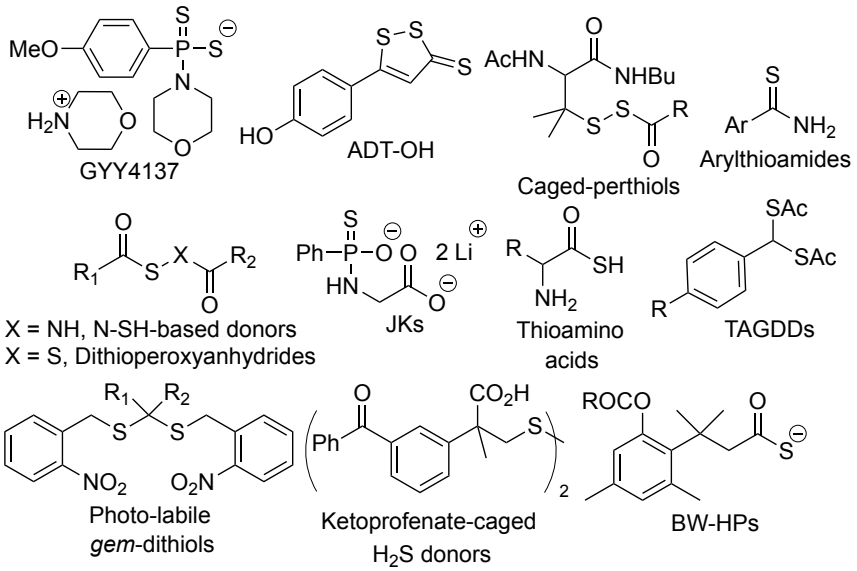
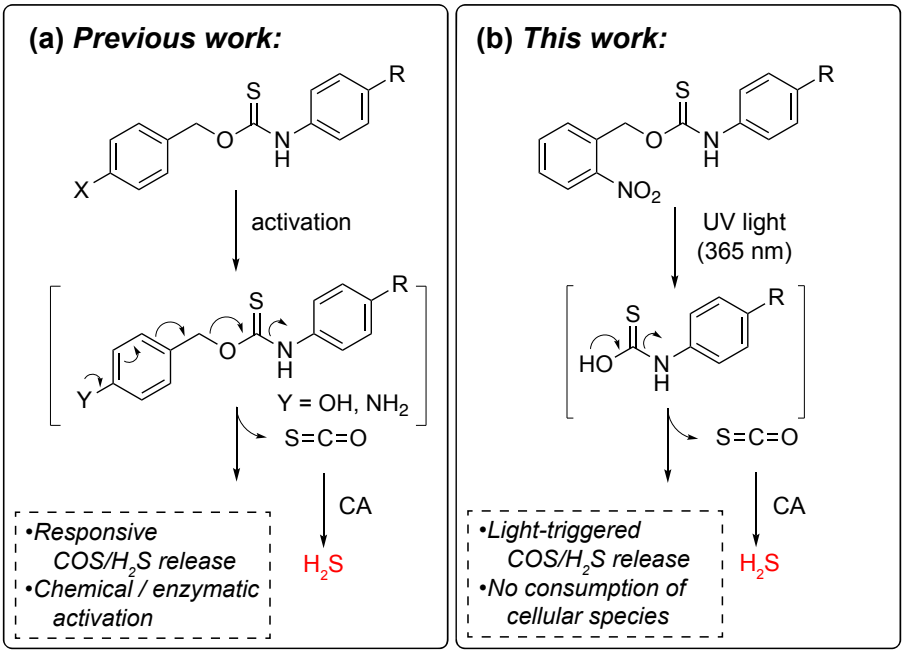


Figure 6.1. Examples of selected synthetic H_2S donors that release H_2S directly.

the ubiquitous enzyme carbonic anhydrase (CA) (Scheme 6.1a). We have used this general COS release strategy to develop platforms activated by endogenous triggers including reactive oxygen species (ROS)[25] as well as bioorthogonal triggers such as the cyclooctene/tetrazine click reaction.[26] Further demonstrating the utility of intermediate COS release to access H_2S donors, similar approaches have also been used

by other researchers to access donor motifs triggered by biological nucleophiles[27] and esterases.[28]

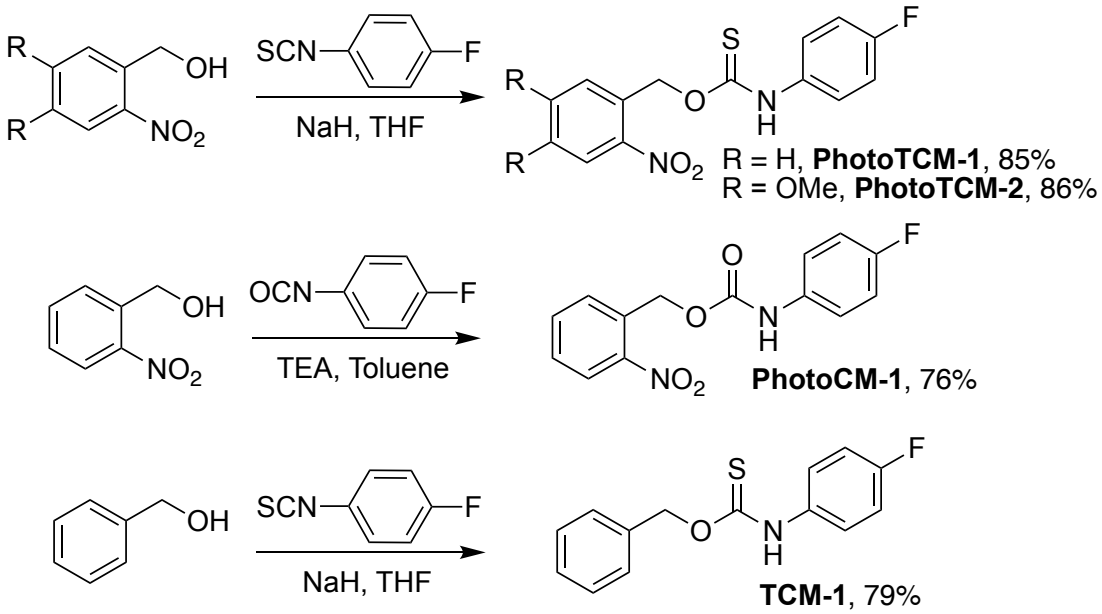
To expand the basic chemistry of triggered COS/H_2S release, we envisioned that caged-thiocarbamate compounds with photoactivatable triggering groups could serve as a new tunable H_2S release platform that responds to specific biorthogonal stimuli. Photoactivatable triggering groups have been widely used in chemistry and biology and offer advantages of high modularity and rapid triggering by an external stimulus.[29] For example, photoactivated donors that release CO,[30, 31] nitroxyl (HNO),[32-34] NO,[35-37] and H_2S[38-40] have all been reported and provide powerful tools for delivering these small molecule payloads in diverse applications. Herein, we report the first class of photolabile COS releasing molecules by employing the photoactivated *o*-nitrobenzyl (ONB) protecting group, (Scheme 6.1b) and demonstrate the compatibility of caged thiocarbamates with ONB photocleavage mechanisms.



Scheme 6.1. (a) Caged Thiocarbamates as Tunable H_2S Donors; (b) ONB-Caged Thiocarbamates as Photolabile H_2S Donors

6.2. Synthesis

To test our hypothesis that ONB-functionalized thiocarbamates can function as photolabile COS/H_2S donors, we prepared two donors (PhotoThioCarbaMate: **PhotoTCM-1** and **PhotoTCM-2**), the parent carbamate molecule (PhotoCarbaMate: **PhotoCM-1**), which releases CO_2/H_2O instead of COS/H_2S, and a thiocarbamate (TCM-1)[25] lacking the ONB photoactivated trigger (Scheme 6.2). **PhotoTCM-1** and **PhotoTCM-2** were prepared by treatment of *o*-nitrobenzyl alcohols with *p*-fluorophenyl isothiocyanate in the presence of sodium hydride in good yield. **PhotoCM-1** was synthesized from the reaction between *o*-nitrobenzyl alcohol and *p*-fluorophenyl isocyanate in the presence of triethylamine.



Scheme 6.2. Synthesis of Photolabile H_2S Donors and Photolabile Carbamate

6.3. Profiling H_2S Release

With these compounds in hand, we next tested their H_2S releasing profiles in the presence of CA. Common H_2S-responsive electrodes contain photosensitive

components and produce a photocurrent under UV light, making their use impractical for these investigations. Therefore, we chose to use the methylene blue assay to monitor H_2S release from **PhotoTCM-1** (50 μM) and **PhotoTCM-2** (50 μM) upon irradiation with an LED UV light (365 nm) in PBS buffer (pH 7.4, 10 mM) containing CA (25 μg/mL). Consistent with our hypothesis, we observed a time-dependent H_2S release from both **PhotoTCM-1** and **PhotoTCM-2** with a peaking time for H_2S release of ~10 min (Figure 6.2). Released H_2S was quantified using a calibration curve (Figure D.1).

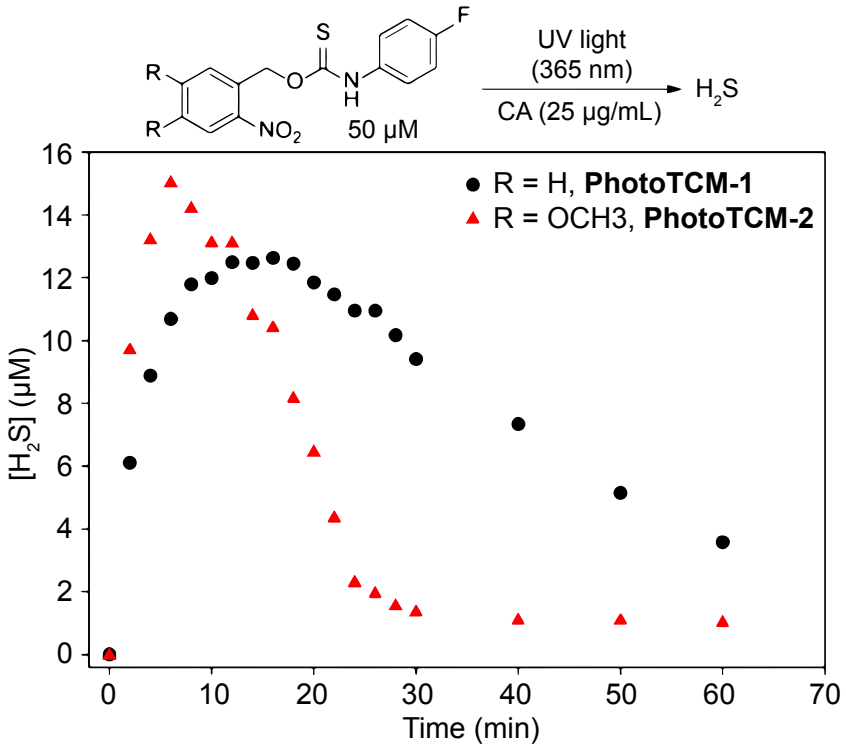


Figure 6.2. H_2S release from PhotoTCM-1 and PhotoTCM-2 in aqueous buffer at pH 7.4.

To confirm that the observed COS/H_2S release was due to photocleavage of the ONB protecting group, [38, 41, 42] a series of control experiments were performed. As shown in Figure 6.3a, UV irradiation of a PBS solution containing CA does not generate H_2S. Similarly, **PhotoTCM-1** does not generate H_2S in the absence of UV

irradiation. Upon UV irradiation of **PhotoTCM-1** in the absence of CA (bar 4 in Figure 6.3a), we observed low levels of H_2S, which is consistent with the slow background hydrolysis of COS to H_2S in the absence of CA. As expected, TCM-1, which lacks the ONB group, does not release H_2S upon the treatment of UV light, showing that the thiocarbamate linkage is not photodegraded to generate H_2S. Similarly, no H_2S release was observed from the carbamate control compound **PhotoCM-1**. As expected, incubation of **PhotoTCM-1** with CA under UV exposure results in a significant enhancement of H_2S concentration, indicating UV irradiation is essential to trigger H_2S release. Moreover, the measured H_2S is significantly reduced in the presence of acetazolamide (AAA), a well-known CA inhibitor. In addition, incubation of **PhotoTCM-1** with cellular nucleophiles, such as cysteine, homocysteine, reduced glutathione, oxidized glutathione, serine, and lysine, in the presence of CA does not generate H_2S, demonstrating **PhotoTCM-1** is stable toward cellular nucleophiles (Figure 6.3b). We note that **PhotoTCM-1** was incubated with the nucleophiles for 10 min to match the peaking time for the COS/H_2S release experiments. We did not investigate longer incubation times in this preliminary report, although it is possible that some side reactivity could occur at extended times or with more potent biological nucleophiles. Taken together, these studies demonstrate that ONB-functionalized thiocarbamates provide a functional platform to access photolabile H_2S donors and that H_2S production can be triggered and tuned by UV irradiation.

6.4. Mechanism of Action

The proposed COS/H_2S release mechanism from the PhotoTCM donors is shown in Scheme 3. UV-mediated cleavage of an ONB protecting group is well-

established in the literature and is known to proceed through a Norrish type II mechanism.[41, 42] Our results show that this cleavage mechanism is compatible with a thiocarbamate linking group, resulting in generation of a free thiocarbamate intermediate, which decomposes to produce COS and 4-fluoroaniline (Scheme 6.3).

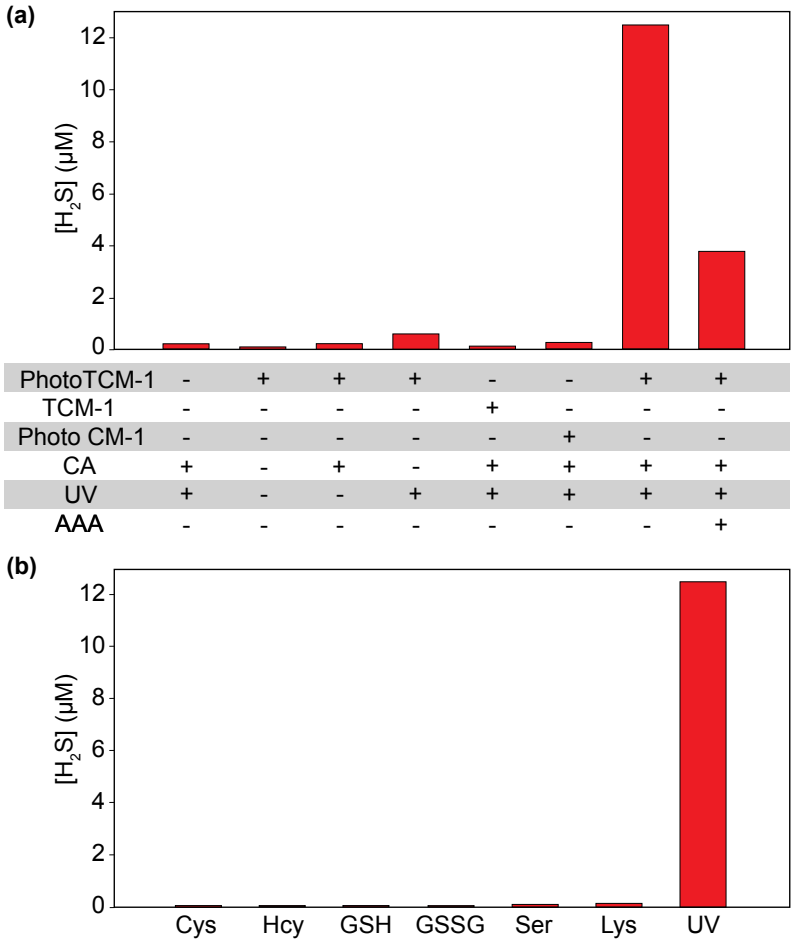
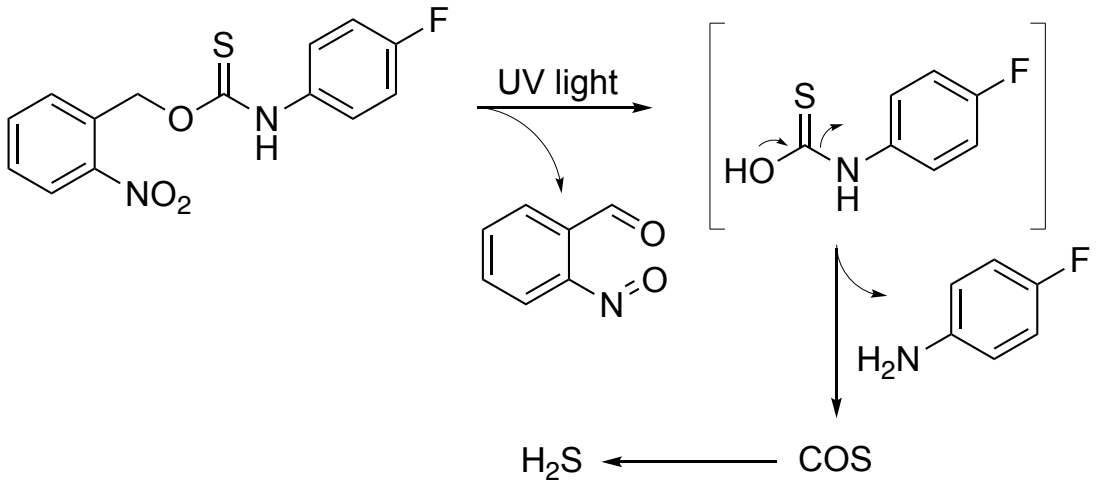


Figure 6.3. (a) H_2S release from PhotoTCM-1, TCM-1, and PhotoCM-1 under various conditions. (b) Effects of amino acids (500 μM, 5 mM for GSH) on H_2S release from PhotoTCM-1. Absorbance measurements were acquired after PhotoTCM-1 was incubated with amino acids in PBS for 10 min.

Consistent with this reaction mechanism, mass spectrometric analysis of the reaction mixture after UV irradiation clearly shows formation of 4-fluoroaniline (m/z 106.1) and 2- nitrosobenzylaldehyde (m/z 136.0) (Figure D.2). Additionally, we note that this COS/H_2S release does not proceed through an electrophilic quinone methide

intermediate often required in self-immolative cascade reactions, thus increasing the biological compatibility of this bioorthogonal delivery approach.



Scheme 6.3. Proposed H_2S-Releasing Mechanism

6.5. Conclusion

In summary, our investigations into two photolabile COS/ H_2S donors demonstrate that photolysis of the ONB protecting group is compatible with thiocarbamate linkages, thus providing a broad platform for further expansion. The time dependent H_2S release after UV activation suggests that these compounds can be used as new efficacious photolabile COS/H_2S donors. Further applications of these as well as other COS-releasing constructs triggered by different mechanisms are ongoing in our laboratory. As our understanding of H_2S in biology has expanded, our focus is also beginning to shift from H_2S donors alone to sulfane sulfur and polysulfides. Elucidating the chemistry of both oxidized and reduced sulfur species is critical to our overall understanding of sulfur biology and reacetive sulfur species.

6.6. Materials and Methods

6.6.1. Materials

Reagents were purchased from Sigma-Aldrich, Tokyo Chemical Industry (TCI), Fisher Scientific, or VWR and used directly as received. Silica gel (SiliaFlash F60, Silicycle, 230−400 mesh) was used for column chromatography. Deuterated solvents were purchased from Cambridge Isotope Laboratories (Tewksbury, Massachusetts, USA). ^{1}H and $^{13}C\{^{1}H\}$ NMR spectra were recorded on Bruker 500 MHz or Bruker 600 MHz NMR instruments at the indicated frequencies. Chemical shifts are reported in ppm relative to residual protic solvent resonances. UV light (365 nm) was generated by using a high-power LED bulb (800 mW, Mouser Electronics) situated 1 cm from a Pyrex round bottom flask. Methylene blue (MB) absorbances were measured using an Agilent Cary 60 UV-Vis spectrometer.

6.6.2. Synthesis

6.6.2.1. General Procedure for the Synthesis of Thiocarbamate Compounds

The *o*-nitrobenzyl alcohol species (1.0 equiv.) was combined with *p*-fluorophenyl isothiocyanate (1.0 equiv.) in anhydrous THF (15 mL) at 0 °C, followed by the addition of NaH (60% in paraffin liquid, 1.25 equiv.). The resultant mixture was stirred at 0°C for 20 min, after which the ice bath was removed, and the reaction mixture was stirred at room temperature until the completion of the reaction indicated by TLC. The reaction was quenched by adding brine (30 mL), and the aqueous solution was extracted with ethyl acetate (3 ×15 mL). The organic layers were combined, dried over $MgSO_4$, and evaporated under vacuum. The crude product was purified by column chromatography. NMR spectra were obtained at 60°C because thiocarbamates show

two sets of NMR resonances at room temperature due to slow rotation around the thiocarbamate functional group at room temperature.

6.6.2.2. PhotoTCM-1

PhotoTCM-1 was prepared from *o*-nitrobenzyl alcohol and *p*-fluorophenyl isothiocyanate using the general synthetic procedure described above (520 mg, 85% yield). ^{1}H NMR (500 MHz, DMSO-d6 at 60°C) δ (ppm): 11.20 (s, 1H), 8.13 (d, J = 5.0 Hz, 1H), 7.64 (m, 5H), 7.18 (t, J = 10.0 Hz, 2H), 5.89 (s, 2H). ^{19}F NMR (470 MHz, DMSO-d6 at 60°C) δ (ppm): - 116.9. ^{13}C{^{1}H} NMR (150 MHz, DMSO-d6 at 60°C) δ (ppm): 187.8, 160.7, 159.1, 148.0, 134.5, 132.0, 130.0, 129.8, 125.4, 125.2, 115.8, 67.5. IR (cm^{-1}): 3170, 3007, 1521, 1501, 1332, 1207, 1185, 1154, 1057, 1046, 791, 727, 561. HRMS m/z $[M + H]^+$ calcd. for $[C_{14}H_{12}FN_2O_3S]^+$ 307.0553; found 307.0558. M. P. (°C): 120 – 122.

6.6.2.3. PhotoTCM-2

PhotoTCM-2 was prepared from 4,5-dimethoxy-2-nitrobenzyl alcohol and *p*-fluorophenyl isothiocyanate using the general synthetic procedure described above (626 mg, 86% yield). ^{1}H NMR (500 MHz, DMSO-d6 at 60°C) δ (ppm): 11.6 (s, 1H), 7.72 (s, 1H), 7.56 (m, 2H), 7.18 (t, J = 10.0 Hz, 3H), 5.83 (s, 2H), 3.90 (s, 6H). ^{19}F NMR (470 MHz, DMSO-d6 at 60°C) δ (ppm): -116.9. ^{13}C{^{1}H} NMR (150 MHz, DMSO-d6 at 60°C) δ (ppm): 188.0, 160.7, 159.1, 153.8, 148.7, 140.4, 134.9, 125.6, 115.9, 112.3, 109.0, 69.4, 68.0, 56.7. IR (cm^{-1}): 3170, 3007, 1521, 1502, 1406, 1333, 1235, 1208, 1185, 1154, 1056, 1046, 791, 778, 726, 703, 681. HRMS m/z $[M + H]^+$ calcd. for $[C_{16}H_{16}FN_2O_5S]^+$ 367.0764; found 367.0755. M. P. (°C): 156 – 158.

6.6.2.4. PhotoCM-1

PhotoCM-1 was prepared from *o*-Nitrobenzyl alcohol (153 mg, 1.0 mmol, 1.0 equiv.) and *p*-fluorophenyl isocyanate (137 mg, 1.0 mmol, 1.0 equiv.) in anhydrous toluene (30 mL) at room temperature, followed by the addition of triethylamine (101 mg, 1.0 mmol, 1.0 equiv.). The resultant mixture was stirred at 110°C for 20 h, after which the oil bath was removed, and the reaction solution was cooled to room temperature. The reaction mixture was quenched by adding brine (30 mL), and the aqueous solution was extracted with ethyl acetate (3 × 15 mL). The organic layers were combined, dried over $MgSO_4$, and evaporated under vacuum. The crude product was purified by using column chromatography to afford pure **PhotoCM-1** as white solid (220 mg, 76%). ^{1}H NMR (500 MHz, CDCl3) δ (ppm): 9.95 (s, 1H), 8.14 (d, J = 10.0 Hz, 1H), 7.83 (t, J = 5.0 Hz, 1H), 7.75 (t, J = 10.0 Hz, 1H), 7.64 (t, J = 5.0 Hz, 1H), 7.49 (m, 2H), 7.14 (t, J = 10.0 Hz, 2H), 5.50 (s, 2H). ^{19}F NMR (470 MHz, $CDCl_3$) δ (ppm): -120.6. ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$) δ (ppm): 159.2, 157.3, 153.5, 147.8, 135.7, 134.6, 132.7, 129.8, 125.3, 120.4, 115.9, 63.0. IR (cm^{-1}): 3170, 3007, 1521, 1502, 1406, 1333, 1235, 1208, 1185, 1154, 1056, 846, 791, 778, 726, 703, 681, 561. HRMS m/z [M + H]$^{+}$ calcd. for $[C_{14}H_{12}FN_2O_4]^{+}$ 291.0781; found 291.0786. M. P. (°C): 146 – 148.

6.6.3. H_2S Release Assays

A **PhotoTCM** stock solution (50 µL, 20 mM in DMSO) was added to 20 mL of PBS (pH 7.4, 10 mM) containing CA (25 µg/mL) in a 25-mL Pyrex round bottom flask. One high power LED bulb (800 mW) was placed 1 cm away from the flask. After stirring the reaction solution at room temperature for 2 min, the LED bulb was turned on and 0.5 mL of the reaction aliquots were taken to UV cuvettes containing 0.5 mL of

MB cocktail (0.1 mL zinc acetate (1% w/v), 0.2 mL $FeCl_3$ (30 mM in 1.2 M HCl), and 0.2 mL N,N-dimethyl-p-phenylene diamine (20 mM in 7.2 M HCl)) periodically. The absorbance at 670 nm was then measured after 1 hour and was converted to H_2S concentration by using the H_2S calibration curve as shown in Figure D.1.

To study the effects of different combinations of CA, UV, and AAA on H_2S release on the three TCM compounds seen in Fig. 6.3a, the **PhotoTCM-1**, **TCM-1** or **PhotoCM-1** stock solution (25 μL, 20 mM in DMSO) was added to 10 mL of PBS (pH 7.4, 10 mM) with or without CA (25 μg/mL) and with or without AAA (10 μM) in a 25-mL Pyrex round bottom flask. The solution was irradiated or not irradiated by UV light for 10 min. Then, 0.5 mL of the reaction aliquot was taken to a UV cuvette containing 0.5 mL of MB cocktail. The absorbance at 670 nm was then measured after 1 hour by the MB assay.

To study the effects of amino acids on H_2S release from **PhotoTCM-1** (Figure 6.3b) A **PhotoTCM-1** stock solution (25 μL, 20 mM in DMSO) was added to 10 mL of PBS (pH 7.4, 10 mM) containing CA (25 μg/mL) in a 25-mL Pyrex round bottom flask. After stirring at room temperature for 5 minutes, the amino acid stock solution was added to reach the final concentration of 500 μM (5 mM for GSH). The resultant solution was irradiated under UV light for 10 min and the absorbance at 670 nm was then measured after 1 hour by the MB assay

6.6.4. Mechanism of Action Study

PhotoTCM-1 (15 mg) was dissolved in 4 mL of DMSO, followed by the addition of 2 mL of PBS (pH 7.4). The resultant solution was irradiated under UV light at 365 nm for 2 hours. H_2O (15 mL) was then added, and the aqueous solution was extracted with

ethyl acetate (15 mL x3). The organic layers were combined, washed with brine (15 mL x3), dried over $MgSO_4$, and removed under vacuum. The solid residue was dissolved in DMSO and injected into the Mass Spectrometer for product analysis (Figure D.2).

CHAPTER VII

CONCLUDING STATEMENTS

Sulfur is not the most obvious element to look toward to understand cell biology. Multicellular life is dominated by aerobes that utilize oxygen as a terminal electron acceptor in cellular respiration.[1] This dependence is due to the fact that the reduction of O_2 provides more free energy release per electron transfer than any element, save fluorine and chlorine.[2] It is this feature that allowed multicellular life to begin over two billion years ago, after the release of oxygen by cyanobacteria initiated the Great Oxidation Event (GOE).[3] Prior to that point, Earth's atmosphere was weakly reducing and contained larger concentrations of sulfur-containing gasses.[4] The landmark Miller-Urey experiment studied the effects of simulated Earth atmospheres on the creation of organic compounds from inorganic precursors, and a 2011 reexamination of preserved experimental samples from 1958 found that atmospheres rich in hydrogen sulfide (H_2S) produced significantly greater amino acid yields than other conditions.[5] This is the atmosphere in which life began. The sulfur-containing gasses originated from volcanic activity and were metabolized by early archaeans and bacteria in lieu of oxygen.[6-8]

Two billion years hence, cellular biology of aerobic eukaryotes still carries some relics that reveal ties to the distant, sulfur-based past. Mitochondria originated from sulfide-oxidizing α-protobacteria that invaded sulfide-reducing archaeans and maintained a symbiotic relationship. Eukaryotes also have the capacity to oxidize H_2S for ATP generation through the use of sulfide:quinone oxidoreductase (SQR).[9, 10] This

fundamental relationship between sulfur and the foundations of life motivates our studies into reactive sulfur species (RSS). Eukaryote cells most commonly encounter H_2S in its role as a gasotransmitter, a family of small, gaseous signaling molecules that includes carbon monoxide (CO) and nitric oxide (NO).[11] All three are produced endogenously, with H_2S being produced during the metabolism of cysteine.[12] Much study has been done on the outcomes and benefits of H_2S treatment in cells,[13] but attention is beginning to shift to oxidized sulfur species containing sulfane sulfur (S^0).[14] This is primarily due to the ability of S^0 to modify cysteine residues by persulfidation, a type of regulatory posttranslational modification.[15-17] Persulfidated proteins may upregulate production of cell-protective factors due to certain stimuli,[18-21] or inhibit them.[22] The study of protein persulfidation is complicated by the fact that there are no pure sources of S^0 other than the insoluble elemental sulfur (S_8), and developing tools to address this and learn more about S^0 and H_2S is highly desirable.

It is important to understand this powerful method of cellular regulation, which influences many diverse cell processes, and we have sought to develop methods of studying H_2S and S^0 to achieve this goal. Our lab has developed several triggerable H_2S donors that display increased biological relevance over the traditional H_2S source NaSH due to their controllable H_2S output.[23-27] Development of an organic benzyl polysulfide series differing only in sulfur content made strides towards extricating the observations of S^0 behavior from the obfuscation of differing non-S^0 groups.[28] It also revealed the large influence of differing pendant alkyl groups on the impact of S^0 in cells, which was further illustrated with our work on N-acetylcysteine-based tetrasulfides. We developed a novel method of studying pure S^0 in cells, the optimum way of observing S^0

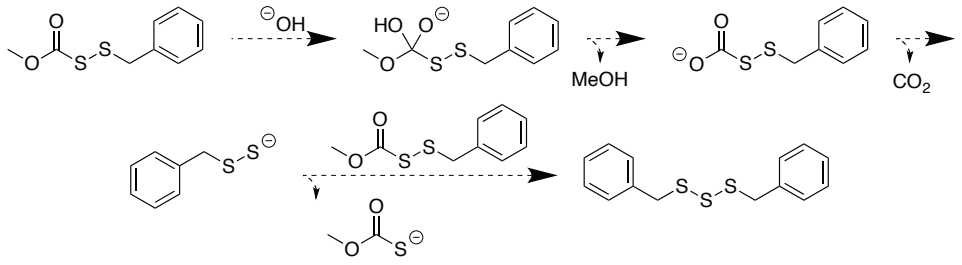
chemistry, using FDA-approved cyclodextrins.[29] We then demonstrated the use of this system on the persulfidation and inhibition of glyceraldehyde-3-phosphate (GAPDH), proving it to be a useful and straightforward method of studying protein persulfidation by S^0.

These efforts go a long way towards revealing the nature and mechanics of protein persulfidation and RSS. Given the wide influences of persulfidation in cells, a promising area of future study would be targeted, controllable S^0 donors. These could potentially be targeted to specific therapeutical target proteins, as it has already been shown that S^0 treatment has beneficial effects.[30] Efforts toward controllable S^0 donors have been made in the form of persulfide donors,[31-33] but the unstable nature of persulfides may limit their applications.[34]

APPENDIX A

CHAPTER II SUPPLEMENTARY MATERIALS

A.1. Supplementary Schemes



Scheme A.1. Proposed reaction mechanism for trisulfide synthesis via hydroxide-mediated decomposition of sulfenyl thiocarbonates.

A.2. Supplementary Figures

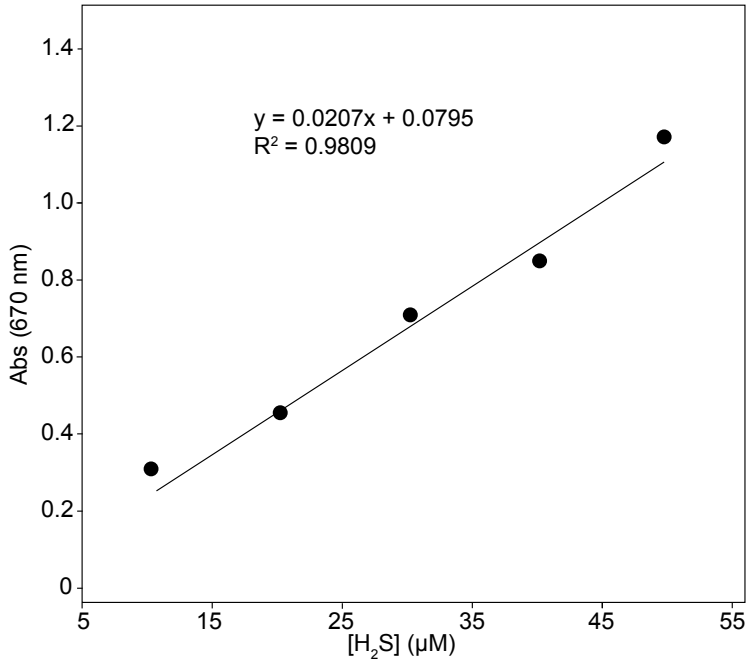


Figure A.1. MB calibration curve generated using known concentrations of NaSH (H_2S) in the presence of 500 μM cysteine.

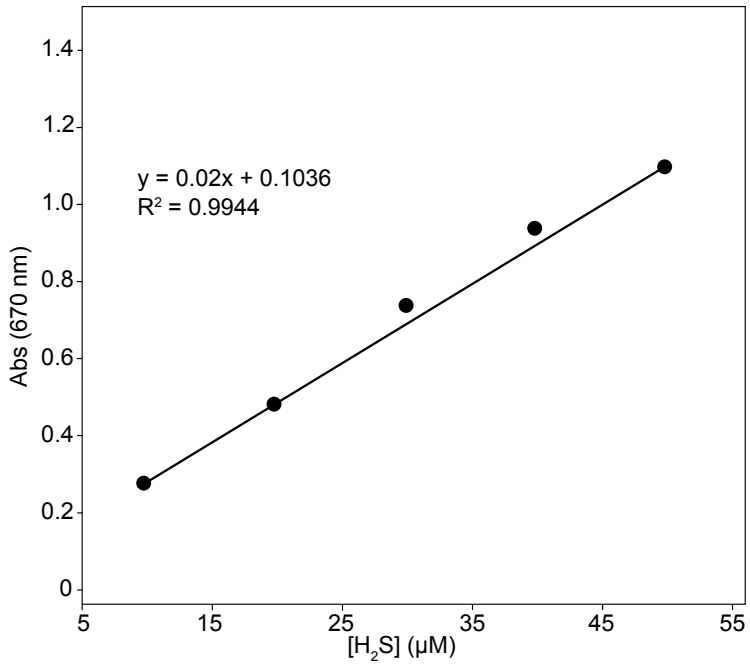


Figure A.2. MB calibration curve generated using known concentrations of NaSH in the presence of 500 μM GSH.

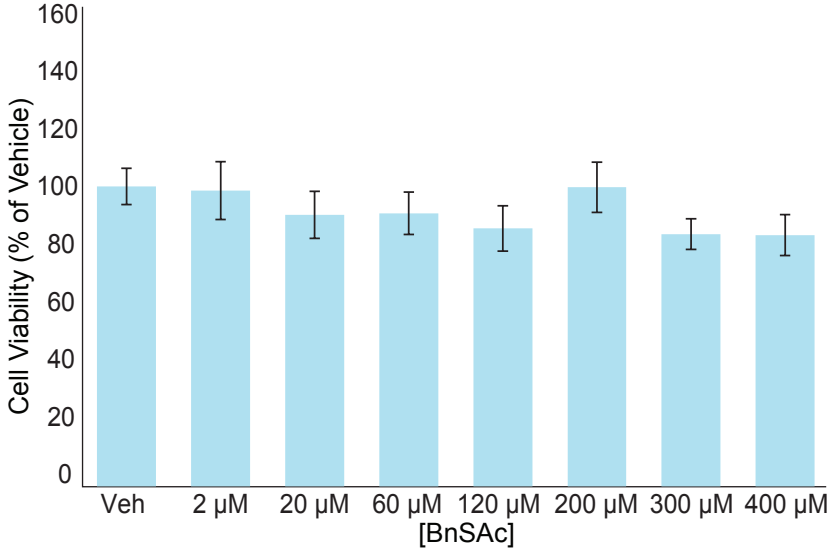


Figure A.3. Pooled average of cell proliferation data under BnSAc treatment.

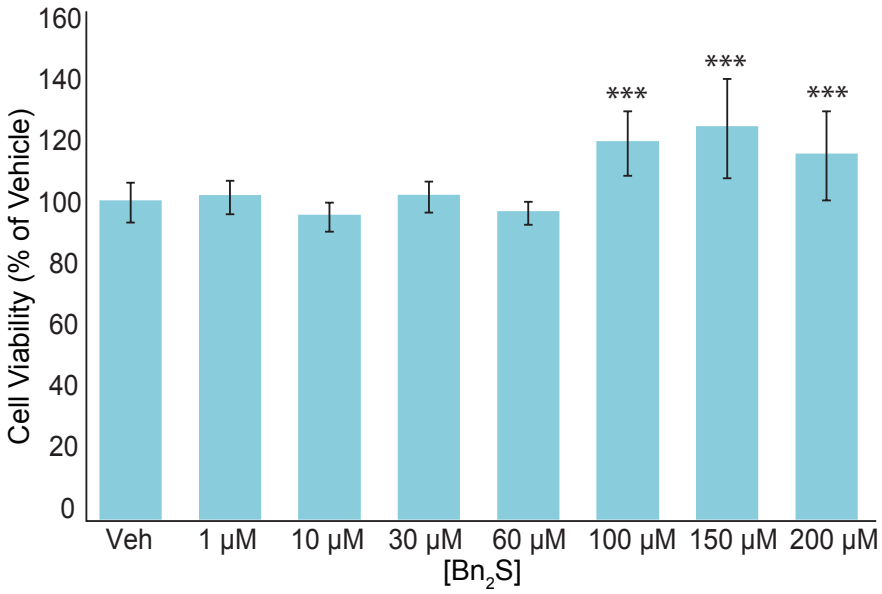


Figure A.4. Pooled average of cell proliferation data under Bn_2S treatment.

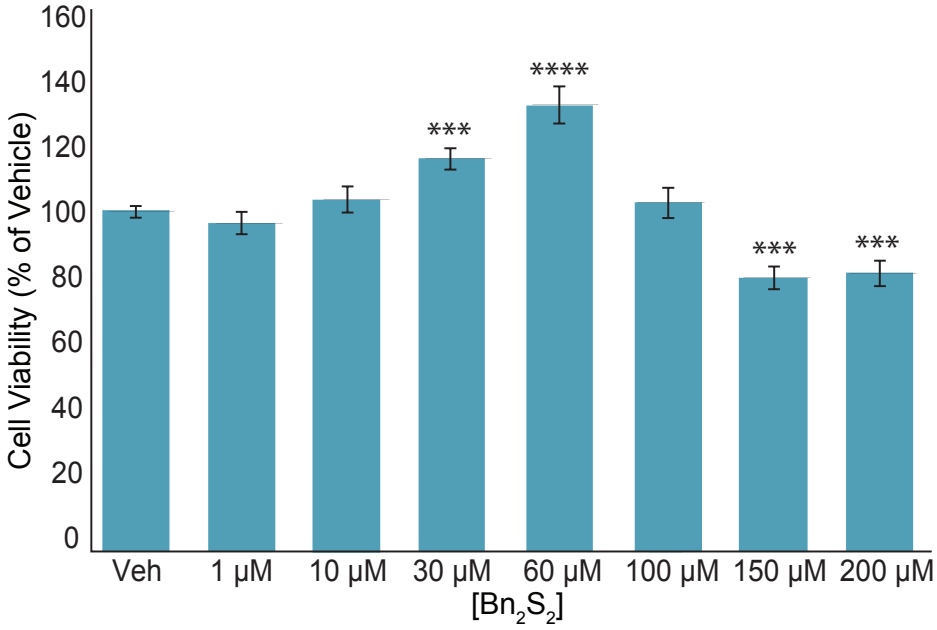


Figure A.5. Pooled average of cell proliferation data under Bn_2S_2 treatment.

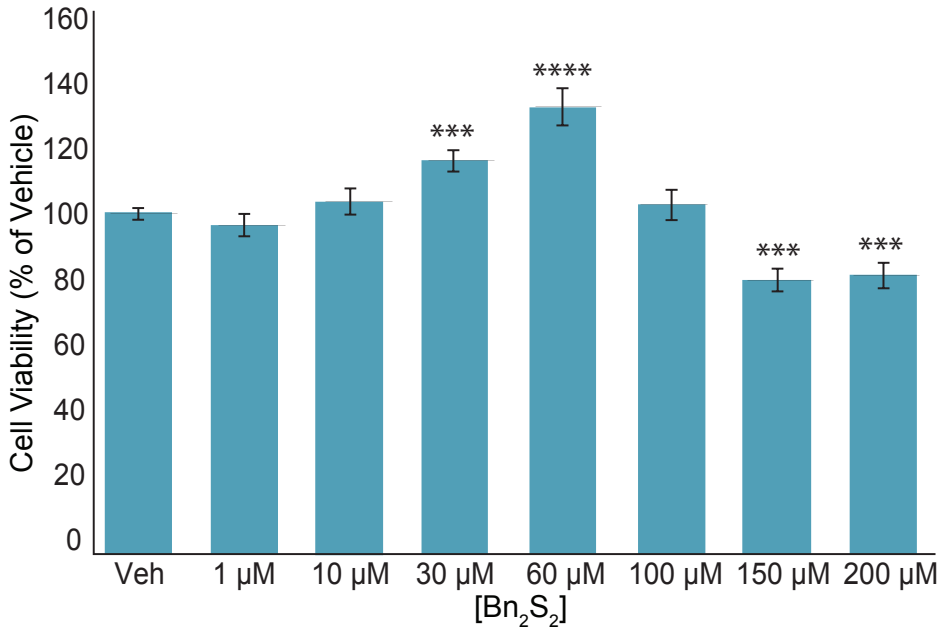


Figure A.6. Pooled average of cell proliferation data under Bn_2S_3 treatment.

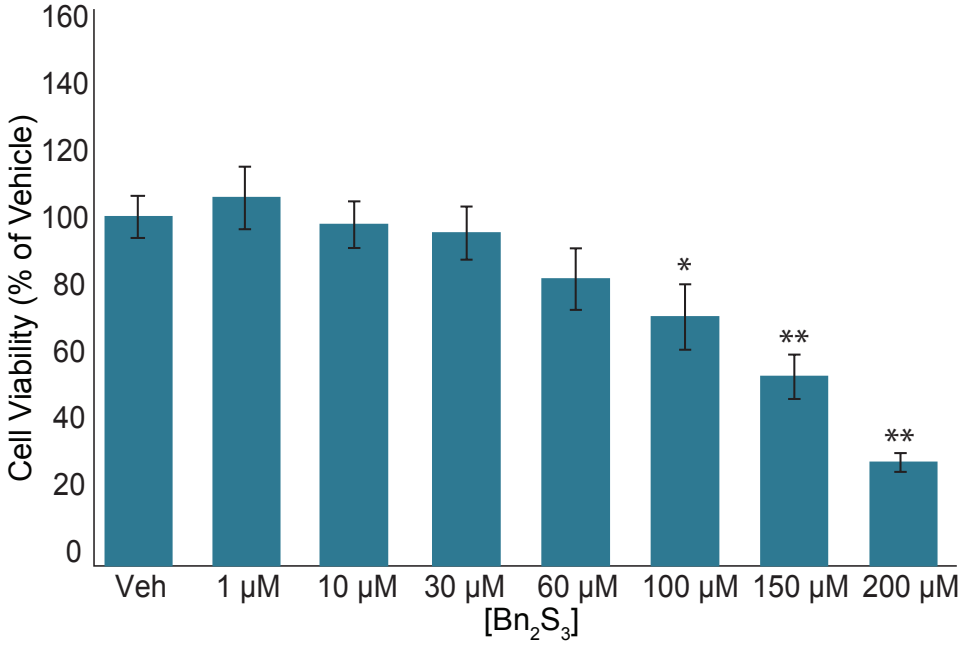


Figure A.7. Pooled average of cell proliferation data under Bn_2S_4 treatment.

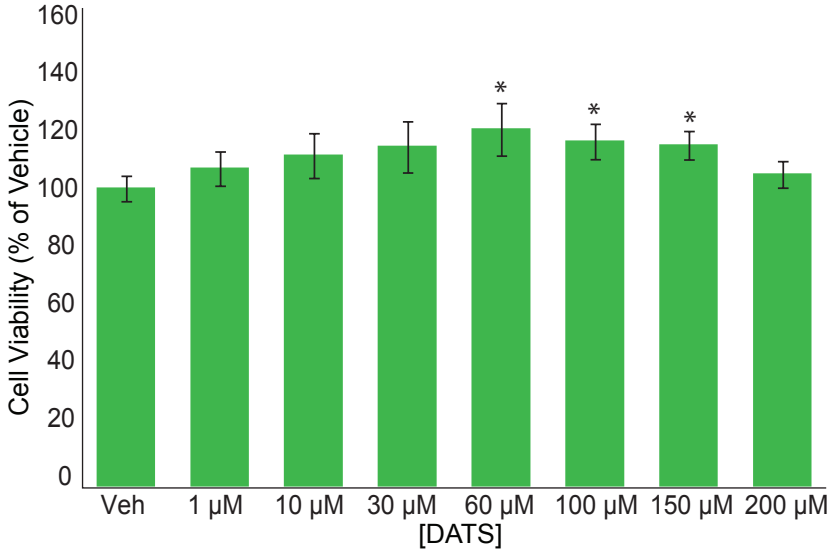


Figure A.8. Pooled average of cell proliferation data under DATS treatment.

APPENDIX B

CHAPTER IV SUPPLEMENTARY MATERIALS

B.1. Supplementary Figures

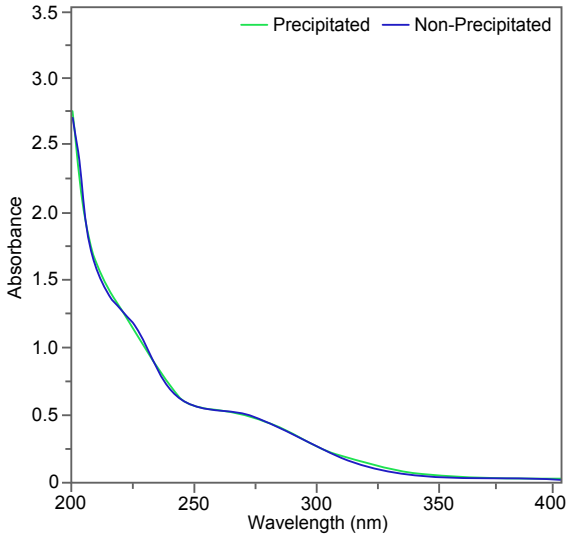


Figure B.1. UV-vis spectra of 2HPβ/S_8 solutions in pH 7.4 PBS that have been precipitated (cyan) or not precipitated (blue) before scanning. When precipitated solutions are reconstituted to the same w/w percentage, the [S_8] does not change.

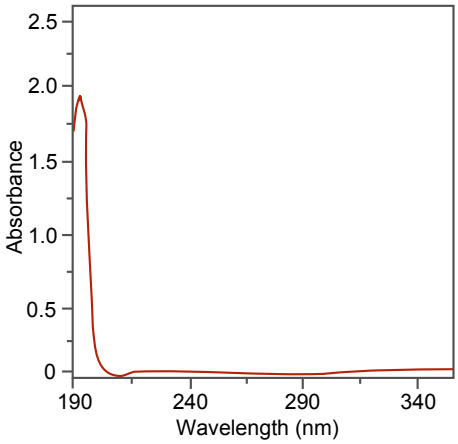


Figure B.2. UV-vis spectrum of βCD treated with S_8 in pH 7.4 PBS. No S_8 was observed in solution based on the lack of an absorbance peak at 263 nm.

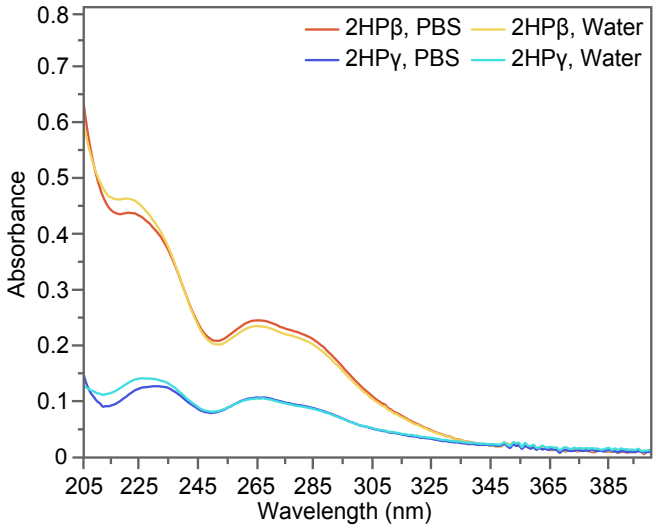


Figure B.3. UV-vis spectra of S_8 with 2HPβ (yellow, orange) and 2HPγ (cyan, blue) in water and PBS. 2HPβ demonstrates superior S_8 solvation ability over 2HPγ.

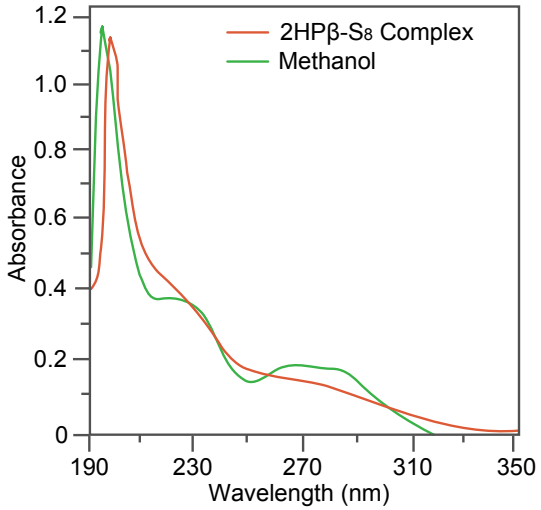


Figure B.4. UV-vis spectra of equimolar concentrations of S_8 solvated in methanol (green) and 2HPβ in pH 7.4 PBS (orange). There is a 12% difference in the absorbance at 263 nm, suggesting that at most the extinction coefficient differs in these two solvents by 12%, and that the MeOH extinction coefficient for S_8 in MeOH can be used to approximate the amount of S_8 in the 2HPβ systems.

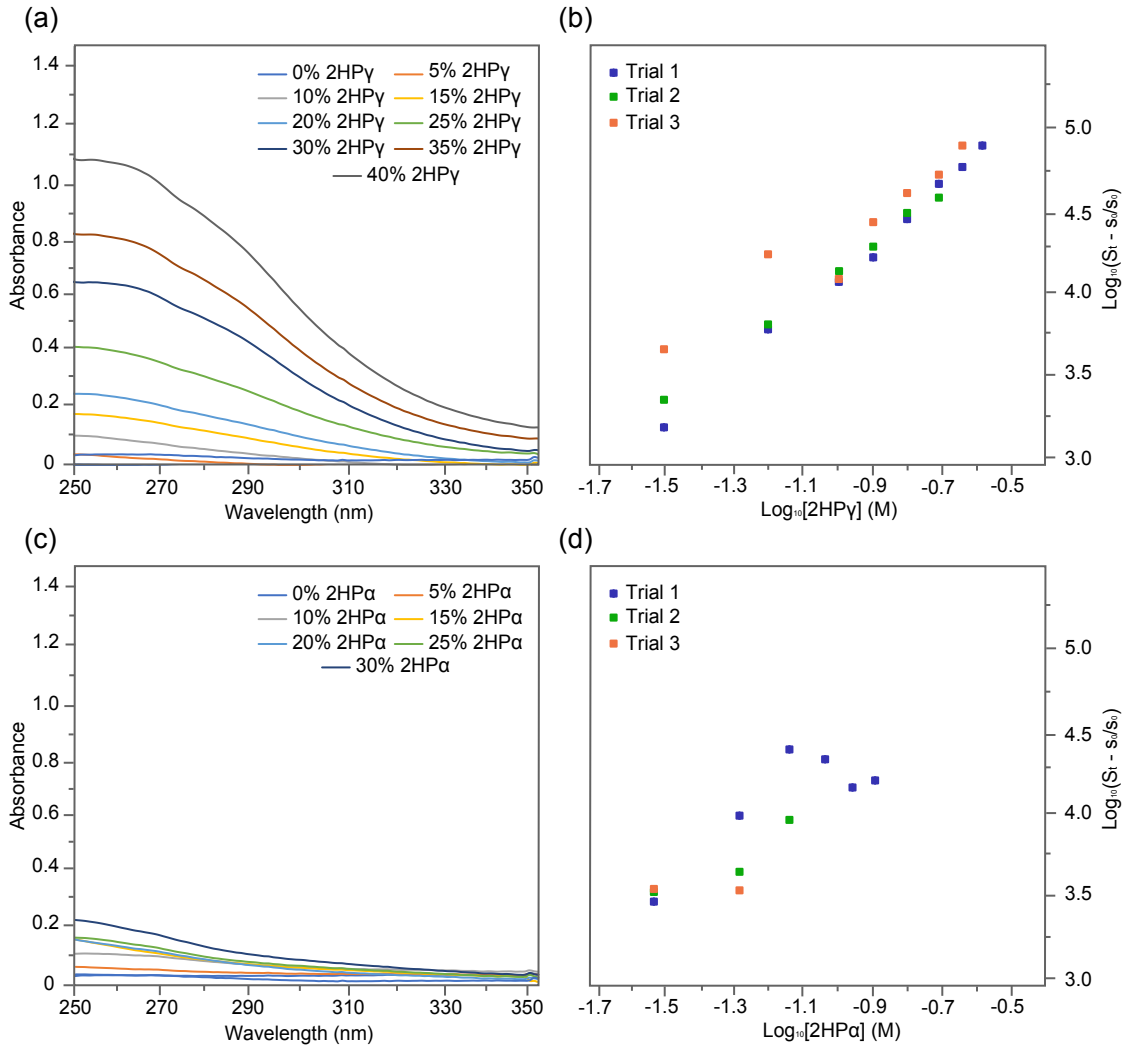


Figure B.5. UV-vis spectra of S_8 binding to (a) 2HPγ and (c) 2HPα, log-log plots (b) and (d) demonstrate the binding relationship of 2HPγ and 2HPα respectively. By comparison to 2HPβ, less S_8 is solubilized in 2HPγ and 2HPα. Similarly, the plots in (b) and (d) are significantly less linear than the corresponding plot for 2HPβ (Figure 4b), suggesting that a more complex binding relationship is present.

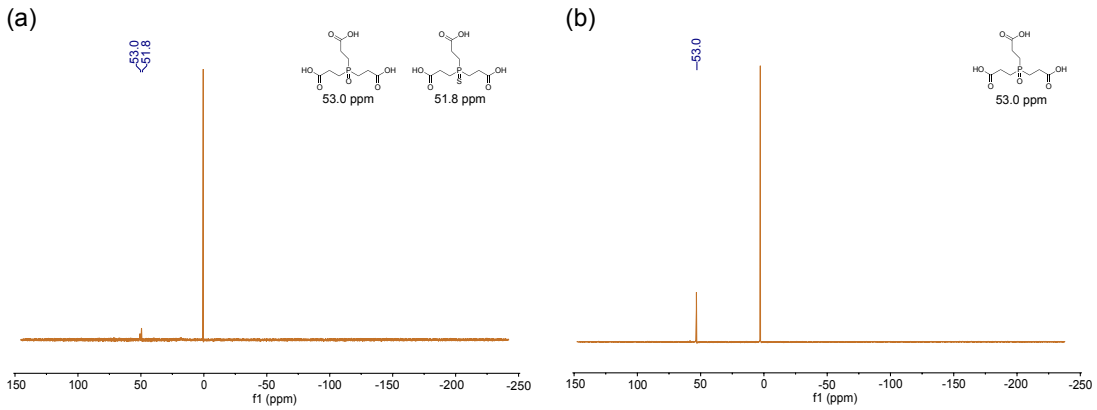


Figure B.6. $^{31}P\{^{1}H\}$ NMR spectra of (a) S_8 in MeOD and (b) K_2S_5 in D_2O demonstrating the formation of oxidized TCEP product peaks.

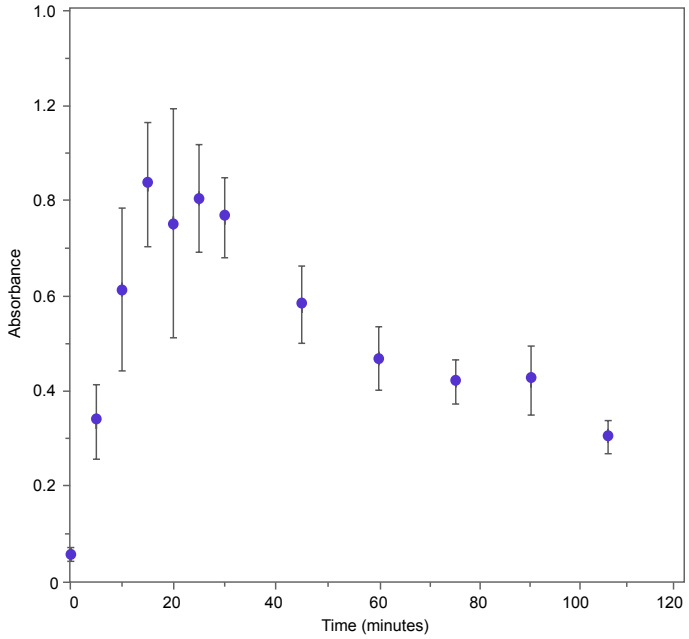


Figure B.7. Methylene blue curve of H_2S release from cysteine and solid 2HPβ and S_8 added separately rather than pre-solvated in a 2HPβ- S_8 complex. These data demonstrate that the 2HPβ- S_8 complex does not need to be pre-formed for efficient reduction of S_8 to H_2S by cysteine.

B.2. Supplementary Tables

% CD	Absorbance (263 nm)	[S_8] (mM)
0	0.012	0.01
5	0.106	0.16
10	0.212	0.32
15	0.282	0.42
20	0.422	0.62
25	0.605	0.90
30	0.717	1.06
35	0.991	1.47
40	1.140	1.69
45	1.286	1.91

Table B.1. Tabulated UV-vis spectral data for solubilized S_8 with increasing concentrations of 2HPβ with a 10-fold excess of S_8. UV-vis spectra are shown in Figure 4.4a.

APPENDIX C

CHAPTER V SUPPLEMENTARY MATERIALS

C.1. Supplementary Figures

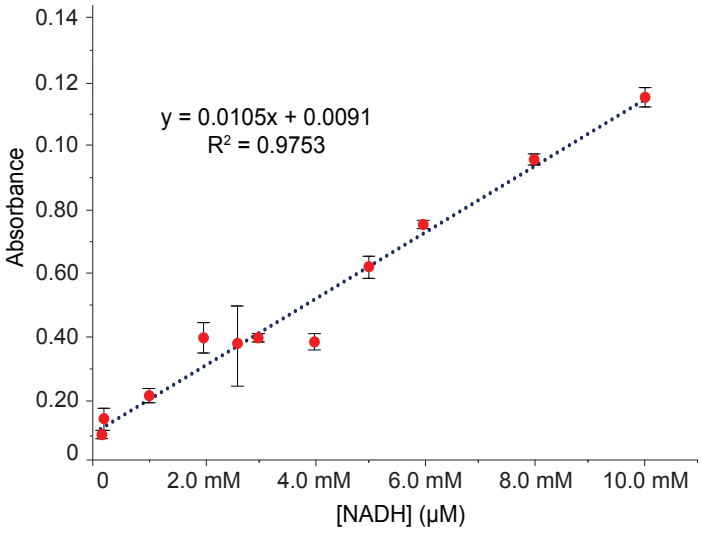


Figure C.1. Calibration curve for NADH concentrations (µM) versus absorbance.

APPENDIX D

CHAPTER VI SUPPLEMENTARY MATERIALS

D.1 Supplementary Figures

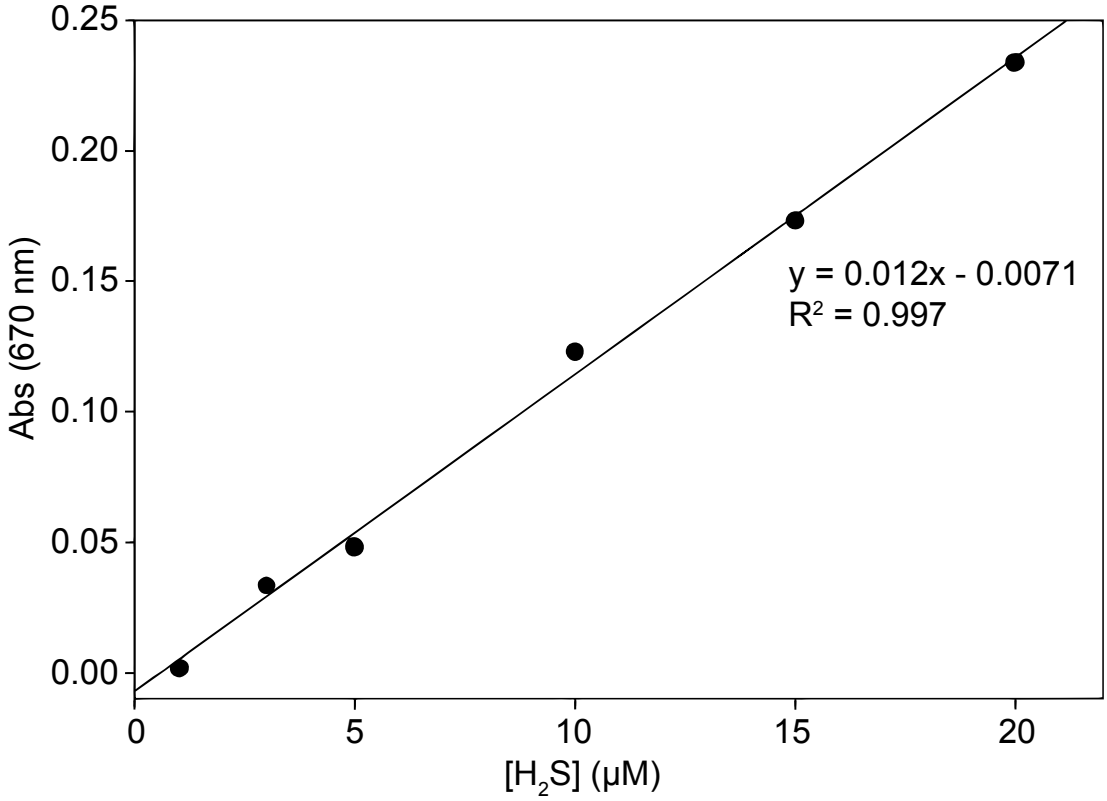


Figure D.1. Methylene blue (MB) H_2S release assay calibration curve. To 1.5 mL-UV cuvettes were added 0.5 mL of MB cocktail (vide infra) and 0.5 mL PBS buffer (pH 7.4, 10 mM). The resultant solution was mixed thoroughly, followed by the addition of an NaSH stock solution (1 mM) to make the final H_2S concentrations of 1, 3, 5, 10, 15, and 20 μM. The MB solution was allowed to react with H_2S for 1 hour before measuring the absorbance at 670 nm.

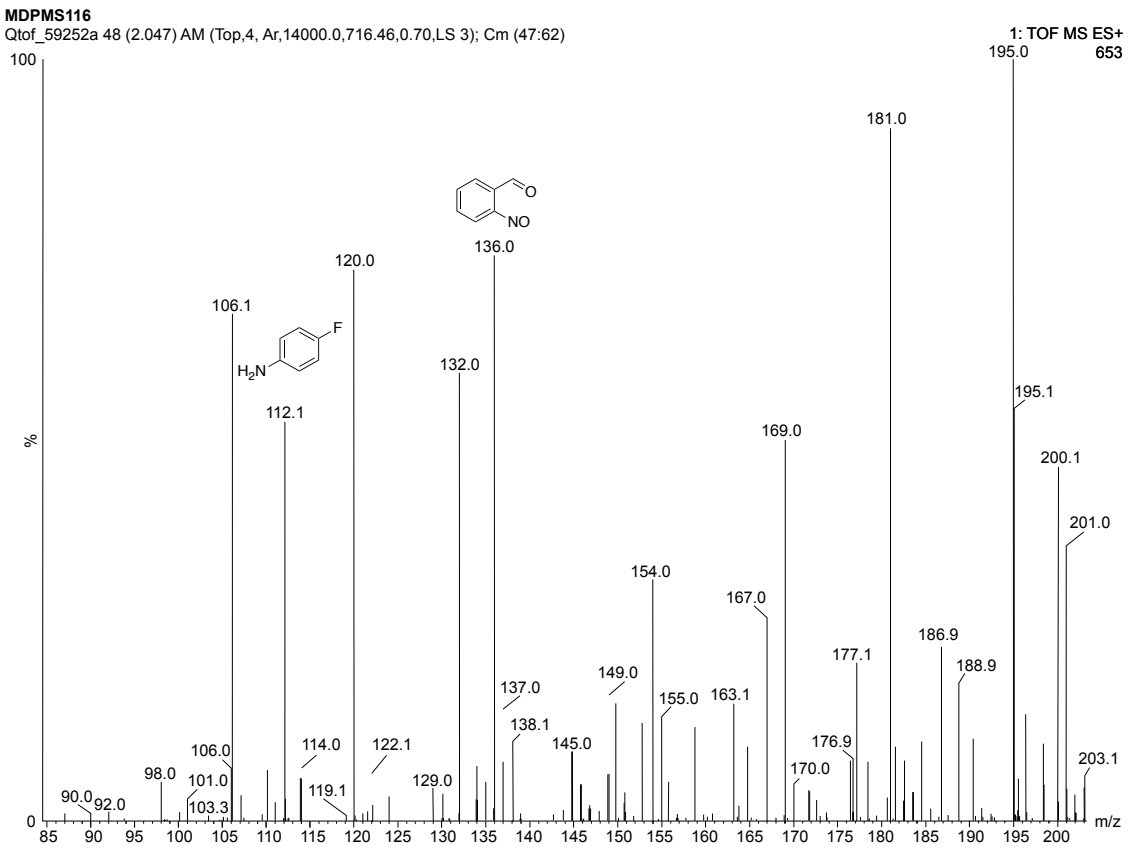


Figure D.2. Product analysis of photo-activated COS release from **PhotoTCM-1**.

REFERENCES CITED

CHAPTER I

1. Anraku, Y., Bacterial Electron Transport Chains. *Annual Review of Biochemistry* **1988,** *57* (1), 101-132.

2. Pizzino, G.; Irrera, N.; Cucinotta, M.; Pallio, G.; Mannino, F.; Arcoraci, V.; Squadrito, F.; Altavilla, D.; Bitto, A., Oxidative Stress: Harms and Benefits for Human Health. *Oxidative Medicine and Cellular Longevity* **2017,** *2017*, 8416763.

3. Finaud, J.; Lac, G.; Filaire, E., Oxidative Stress: Relationship with Exercise and Training. *Sports Medicine* **2006,** *36* (4), 327-358.

4. Kalyanaraman, B., Teaching the Basics of Redox Biology to Medical and Graduate Students: Oxidants, Antioxidants and Disease Mechanisms. *Redox Biology* **2013,** *1* (1), 244-257.

5. Tonelli, C.; Chio, I. I. C.; Tuveson, D. A., Transcriptional Regulation by Nrf2. *Antioxidants & Redox Signaling* **2018,** *29* (17), 1727-1745.

6. Lau, N.; Pluth, M. D., Reactive Sulfur Species (RSS): Persulfides, Polysulfides, Potential, and Problems. *Current Opinion in Chemical Biology* **2019,** *49*, 1-8.

7. Hartle, M. D.; Pluth, M. D., A Practical Guide to Working with H_2S at the Interface of Chemistry and Biology. *Chemical Society Reviews* **2016,** *45* (22), 6108-6117.

8. Mustafa, A. K.; Gadalla, M. M.; Snyder, S. H., Signaling by Gasotransmitters. *Science Signaling* **2009,** *2* (68), re2.

9. Ibrahim, H.; Serag, A.; Farag, M. A., Emerging Analytical Tools for the Detection of the Third Gasotransmitter H_2S, a Comprehensive Review. *Journal of Advanced Research* **2021,** *27*, 137-153.

10. Mancardi, D.; Penna, C.; Merlino, A.; Del Soldato, P.; Wink, D. A.; Pagliaro, P., Physiological and Pharmacological Features of the Novel Gasotransmitter: Hydrogen Sulfide. *Biochimica et Biophysica Acta* **2009,** *1787* (7), 864-872.

11. Baskar, R.; Bian, J., Hydrogen Sulfide Gas has Cell Growth Regulatory Role. *European Journal of Pharmacology* **2011,** *656* (1-3), 5-9.

12. Filipovic, M. R.; Zivanovic, J.; Alvarez, B.; Banerjee, R., Chemical Biology of H_2S Signaling through Persulfidation. *Chemical Reviews* **2018,** *118* (3), 1253-1337.

13. Chauhan, P.; Jos, S.; Chakrapani, H., Reactive Oxygen Species-Triggered Tunable Hydrogen Sulfide Release. *Organic Letters* **2018,** *20* (13), 3766-3770.

14. Kimura, Y.; Kimura, H., Hydrogen Sulfide Protects Neurons from Oxidative Stress. *The FASEB Journal* **2004,** *18* (10), 1165-1167.

15. Parsanathan, R.; Jain, S. K., Hydrogen Sulfide Increases Glutathione Biosynthesis, and Glucose Uptake and Utilisation in C2C12 Mouse Myotubes. *Free Radical Research* **2018,** *52* (2), 288-303.

16. Yang, G.; Zhao, K.; Ju, Y.; Mani, S.; Cao, Q.; Puukila, S.; Khaper, N.; Wu, L.; Wang, R., Hydrogen Sulfide Protects Against Cellular Senescence via S-Sulfhydration of Keap1 and Activation of Nrf2. *Antioxidants & Redox Signaling* **2013,** *18* (15), 196-1919.

17. Coletta, C.; Papapetropoulos, A.; Erdelyi, K.; Olah, G.; Modis, K.; Panopoulos, P.; Asimakopoulou, A.; Geroe, D.; Sharina, I.; Martin, E.; Szabo, C., Hydrogen Sulfide and Nitric Oxide are Mutually Dependent in the Regulation of Angiogenesis and Endothelium-Dependent Vasorelaxation. *Proceedings of the National Academy of Sciences* **2012,** *109* (23), 9161-9166.

18. Fonseca, M. D.; Cunha, F. Q.; Kashfi, K.; Cunha, T. M., NOSH-aspirin (NBS-1120), a }Dual Nitric Oxide and Hydrogen Sulfide-Releasing Hybrid, Reduces Inflammatory Pain. *Pharmacology Research & Perspectives* **2015,** *3* (3), e00133.

19. Chatzianastasiou, A.; Bibli, S.-I.; Andreadou, I.; Efentakis, P.; Kaludercic, N.; Wood, M. E.; Whiteman, M.; Di Lisa, F.; Daiber, A.; Manolopoulos, V. G.; Szabo, C.; Papapetropoulos, A., Cardioprotection by H_2S Donors: Nitric Oxide-Dependent and -Independent Mechanisms. *The Journal of Pharmacology and Experimental Therapeutics* **2016,** *358* (3), 431-440.

20. Kimura, H., Hydrogen Sulfide and Polysulfides as Biological Mediators. *Molecules* **2014,** *19* (10), 16146-16157.

21. Gojon, G.; Morales, G. A., SG1002 and Catenated Divalent Organic Sulfur Compounds as Promising Hydrogen Sulfide Prodrugs. *Antioxidants & Redox Signaling* **2020,** *33* (14), 1010-1045.

22. Benavides, G. A.; Squadrito, G. L.; Mills, R. W.; Patel, H. D.; Isbell, T. S.; Patel, R. P.; Darley-Usmar, V. M.; Doeller, J. E.; Kraus, D. W., Hydrogen Sulfide Mediates the Vasoactivity of Garlic. *Proceedings of the National Academy of Sciences* **2007,** *104* (46), 17977-17982.

23. Zeng, T.; Zhang, C.-L.; Zhu, Z.-P.; Yu, L.-H.; Zhao, X.-L.; Xie, K.-Q., Diallyl trisulfide (DATS) Effectively Attenuated Oxidative Stress-Mediated Liver Injury and Hepatic Mitochondrial Dysfunction in Acute Ethanol-Exposed Mice. *Toxicology* **2008,** *252* (1), 86-91.

24. Kuo, W.-W.; Wang, W.-J.; Tsai, C.-Y.; Way, C.-L.; Hsu, H.-H.; Chen, L.-M., Diallyl Trisufide Suppresses High Glucose-Induced Cardiomyocyte Apoptosis by Inhibiting JNK/NF[kappa]B Signaling via Attenuating ROS Generation. *International Journal of Cardiology* **2013,** *168* (1), 270.

25. Leung, W.-S.; Kuo, W.-W.; Ju, D.-T.; Wang, T.-D.; Shao-Tsu Chen, W.; Ho, T.-J.; Lin, Y. M.; Mahalakshmi, B.; Lin, J.-Y.; Huang, C.-Y., Protective Effects of Diallyl Trisulfide (DATS) Against Doxorubicin-Induced Inflammation and Oxidative Stress in the Brain of Rats. *Free Radical Biology & Medicine* **2020,** *160*, 141-148.

26. Sumedha, N. C.; Miltonprabu, S., Diallyl Trisulfide Ameliorates Arsenic-Induced Hepatotoxicity by Abrogation of Oxidative Stress, Inflammation, and Apoptosis in Rats. *Human & Experimental Toxicology* **2015,** *34* (5), 506-525.

27. Liu, Y.; Zhu, P.; Wang, Y.; Wei, Z.; Tao, L.; Zhu, Z.; Sheng, X.; Wang, S.; Ruan, J.; Liu, Z.; Cao, Y.; Shan, Y.; Sun, L.; Wang, A.; Chen, W.; Lu, Y., Antimetastatic Therapies of the Polysulfide Diallyl Trisulfide against Triple-Negative Breast Cancer via Suppressing MMP2/9 by Blocking NF-[kappa]B and ERK/MAPK Signaling Pathways. *PloS one* **2015,** *10* (4), e0123781.

28. Xiao, D.; Choi, S.; Johnson, D. E.; Vogel, V. G.; Johnson, C. S.; Trump, D. L.; Lee, Y. J.; Singh, S. V., Diallyl Trisulfide-Induced Apoptosis in Human Prostate Cancer Cells Involves c-Jun N-terminal Kinase and Extracellular-Signal Regulated Kinase-Mediated Phosphorylation of Bcl-2. *Oncogene* **2004,** *23* (33), 5594-5606.

29. Xiao, D.; Lew, K. L.; Kim, Y.-A.; Zeng, Y.; Hahm, E.-R.; Dhir, R.; Singh, S. V., Diallyl Trisulfide Suppresses Growth of PC-3 Human Prostate Cancer Xenograft In vivo in Association with Bax and Bak Induction. *Clinical Cancer Research* **2006,** *12* (22), 6836-6843.

30. Ida, T.; Sawa, T.; Ihara, H.; Tsuchiya, Y.; Watanabe, Y.; Kumagai, Y.; Suematsu, M.; Motohashi, H.; Fujii, S.; Matsunaga, T.; Yamamoto, M.; Ono, K.; Devarie-Baez, N. O.; Xian, M.; Fukuto, J. M.; Akaike, T., Reactive Cysteine Persulfides and S-Polythiolation Regulate Oxidative Stress and Redox Signaling. *Proceedings of the National Academy of Sciences* **2014,** *111* (21), 7606-7611.

31. Hisatsune, A.; Kurauchi, Y.; Takahashi, S.; Seki, T.; Katsuki, H., Polysulfide Protects Midbrain Dopaminergic Neurons from MPP^{+}-Induced Degeneration via Enhancement of Glutathione Biosynthesis. *Journal of Pharmacological Sciences* **2018,** *137* (1), 47.

32. Koike, S.; Ogasawara, Y.; Shibuya, N.; Kimura, H.; Ishii, K., Polysulfide Exerts a Protective Effect Against Cytotoxicity Caused by t-Buthylhydroperoxide Through Nrf2 Signaling in Neuroblastoma Cells. *FEBS letters* **2013,** *587* (21), 3548-3555.

33. Bolton, S. G.; Cerda, M. M.; Gilbert, A. K.; Pluth, M. D., Effects of Sulfane Sulfur Content in Benzyl Polysulfides on Thiol-Triggered H_2S Release and Cell Proliferation. *Free Radical Biology & Medicine* **2019,** *131*, 393-398.

34. Kimura, H., Signalling by Hydrogen Sulfide and Polysulfides via Protein S-sulfuration. *British Journal of Pharmacology* **2020,** *177* (4), 720-733.

35. Kimura, H., Signaling Molecules: Hydrogen Sulfide and Polysulfide. *Antioxidants & Redox Signaling* **2015,** *22* (5), 362-376.

36. Park, C.-M.; Weerasinghe, L.; Day, J. J.; Fukuto, J. M.; Xian, M., Persulfides: Current Knowledge and Challenges in Chemistry and Chemical Biology. *Molecular BioSystems* **2015,** *11* (7), 1775-1785.

37. Greiner, R.; Pálinkás, Z.; Bäsell, K.; Becher, D.; Antelmann, H.; Nagy, P.; Dick, T. P., Polysulfides Link H_2S to Protein Thiol Oxidation. *Antioxidants & Redox Signaling* **2013,** *19* (15), 1749-1765.

38. Dillon, K. M.; Matson, J. B., A Review of Chemical Tools for Studying Small Molecule Persulfides: Detection and Delivery. *ACS Chemical Biology* **2021,** *16* (7), 1128-1141.

39. Benchoam, D.; Cuevasanta, E.; Moller, M. N.; Alvarez, B., Hydrogen Sulfide and Persulfides Oxidation by Biologically Relevant Oxidizing Species. *Antioxidants* **2019,** *8* (2), 48.

40. Benchoam, D.; Semelak, J. A.; Cuevasanta, E.; Mastrogiovanni, M.; Grassano, J. S.; Ferrer-Sueta, G.; Zeida, A.; Trujillo, M.; Möller, M. N.; Estrin, D. A.; Alvarez, B., Acidity and Nucleophilic Reactivity of Glutathione Persulfide. *The Journal of Biological Chemistry* **2020,** *295* (46), 15466-15481.

41. Francoleon, N. E.; Carrington, S. J.; Fukuto, J. M., The Reaction of H_2S with Oxidized Thiols: Generation of Persulfides and Implications to H_2S Biology. *Archives of Biochemistry and Biophysics* **2011,** *516* (2), 146-153.

42. Mikami, Y.; Shibuya, N.; Kimura, Y.; Nagahara, N.; Ogasawara, Y.; Kimura, H., Thioredoxin and Dihydrolipoic Acid are Required for 3-Mercaptopyruvate Sulfurtransferase to Produce Hydrogen Sulfide. *Biochemical Journal* **2011,** *439* (3), 479-485.

43. Yuan, Z.; Zheng, Y.; Yu, B.; Wang, S.; Yang, X.; Wang, B., Esterase-Sensitive Glutathione Persulfide Donor. *Organic Letters* **2018,** *20* (20), 6364-6367.

44. Chaudhuri, A.; Venkatesh, Y.; Das, J.; Gangopadhyay, M.; Maiti, T. K.; Singh, N. D. P., One- and Two-Photon-Activated Cysteine Persulfide Donors for Biological Targeting. *Journal of Organic Chemistry* **2019,** *84* (18), 11441-11449.

45. Dillon, K. M.; Morrison, H. A.; Powell, C. R.; Carrazzone, R. J.; Ringel-Scaia, V. M.; Winckler, E. W.; Council-Troche, R. M.; Allen, I. C.; Matson, J. B., Targeted Delivery of Persulfides to the Gut: Effects on the Microbiome. *Angewandte Chemie International Edition* **2021,** *60* (11), 6061-6067.

46. Kimura, Y.; Toyofuku, Y.; Koike, S.; Shibuya, N.; Nagahara, N.; Lefer, D.; Ogasawara, Y.; Kimura, H., Identification of H_2S_3 and H_2S Produced by 3-Mercaptopyruvate Sulfurtransferase in the Brain. *Scientific Reports* **2015,** *5* (1), 14774.

47. Liu, H.; Radford, M. N.; Yang, C. T.; Chen, W.; Xian, M., Inorganic Hydrogen Polysulfides: Chemistry, Chemical Biology and Detection. *British Journal of Pharmacology* **2018,** *176* (4), 616-627.

48. Cerda, M. M.; Hammers, M. D.; Earp, M. S.; Zakharov, L. N.; Pluth, M. D., Applications of Synthetic Organic Tetrasulfides as H_2S Donors. *Organic Letters* **2017,** *19* (9), 2314-2317.

49. Bailey, T. S.; Zakharov, L. N.; Pluth, M. D., Understanding Hydrogen Sulfide Storage: Probing Conditions for Sulfide Release from Hydrodisulfides. *Journal of the American Chemical Society* **2014,** *136* (30), 10573-10576.

50. Murugaiyah, V.; Mattson, M. P., Neurohormetic Phytochemicals: An Evolutionary–Bioenergetic Perspective. *Neurochemistry International* **2015,** *89*, 271-280.

51. Mattson, M. P.; Cheng, A., Neurohormetic Phytochemicals: Low-Dose Toxins that Induce Adaptive Neuronal Stress Responses. *Trends in Neurosciences* **2006,** *29* (11), 632-639.

52. Tsai, C. W.; Yang, J. J.; Chen, H. W.; Sheen, L. Y.; Lii, C. K., Garlic Organosulfur Compounds Upregulate the Expression of the Pi Class of Glutathione S-Transferase in Rat Primary Hepatocytes. *The Journal of Nutrition* **2005,** *135* (11), 2560-2565.

53. Liang, D.; Bian, J.; Deng, L.-W.; Huang, D., Cyclic Polysulphide 1,2,4-Trithiolane from Stinky Bean (Parkia speciosa Seeds) is a Slow Releasing Hydrogen Sulphide (H_2S) Donor. *Journal of Functional Foods* **2017,** *35*, 197-204.

54. Miyazawa, M.; Osman, F., Headspace Constituents of Parkia speciosa Seeds. *Natural Product Letters* **2001,** *15* (3), 171-176.

55. Davidson, B. S.; Molinski, T. F.; Barrows, L. R.; Ireland, C. M., Varacin: a Novel Benzopentathiepin from Lissoclinum vareau that is Cytotoxic Toward a Human Colon Tumor. *Journal of the American Chemical Society* **1991,** *113* (12), 4709-4710.

56. Searle, P. A.; Molinski, T. F., Five new Alkaloids from the Tropical Ascidian, Lissoclinum sp. lissoclinotoxin A is Chiral. *Journal of Organic Chemistry* **1994,** *59* (22), 6600-6605.

57. Kulikov, A. V.; Tikhonova, M. A.; Kulikova, E. A.; Volcho, K. P.; Khomenko, T. M.; Salakhutdinov, N. F.; Popova, N. K., A new Synthetic Varacin Analogue, 8-(Trifluoromethyl)-1,2,3,4,5-Benzopentathiepin-6-amine Hydrochloride (TC-2153), Decreased Hereditary Catalepsy and Increased the BDNF Gene Expression in the Hippocampus in Mice. *Psychopharmacology* **2012,** *221* (3), 469-478.

58. Xu, J.; Chatterjee, M.; Baguley, T. D.; Brouillette, J.; Kurup, P.; Ghosh, D.; Kanyo, J.; Zhang, Y.; Seyb, K.; Ononenyi, C.; Foscue, E.; Anderson, G. M.; Gresack, J.; Cuny, G. D.; Glicksman, M. A.; Greengard, P.; Lam, T. T.; Tautz, L.; Nairn, A. C.; Ellman, J. A.; Lombroso, P. J., Inhibitor of the Tyrosine Phosphatase STEP Reverses Cognitive Deficits in a Mouse Model of Alzheimer's Disease. *PLoS Biology* **2014,** *12* (8), e1001923.

59. Goebel-Goody, S. M.; Wilson-Wallis, E. D.; Royston, S.; Tagliatela, S. M.; Naegele, J. R.; Lombroso, P. J., Genetic Manipulation of STEP Reverses Behavioral Abnormalities in a Fragile X Syndrome Mouse Model. *Genes, Brain and Behavior* **2012,** *11* (5), 586-600.

60. Kurup, P.; Zhang, Y.; Venkitaramani, D. V.; Xu, J.; Lombroso, P. J., The role of STEP in Alzheimer's disease. *Channels* **2010,** *4* (5), 347-350.

61. Boulegue, J., Solubility of Elemental Sulfur in Water at 298 K. *Phosphorus and Sulfur and the Related Elements* **1978,** *5* (1), 127-128.

62. Yang, C.-t.; Chen, L.; Xu, S.; Day, J. J.; Li, X.; Xian, M., Recent Development of Hydrogen Sulfide Releasing/ Stimulating Reagents and Their Potential Applications in Cancer and Glycometabolic Disorders. *Frontiers in Pharmacology* **2017,** *8*, 664-664.

63. Polhemus, D. J.; Li, Z.; Pattillo, C. B.; Gojon, G.; Giordano, T.; Krum, H., A Novel Hydrogen Sulfide Prodrug, SG 1002, Promotes Hydrogen Sulfide and Nitric Oxide Bioavailability in Heart Failure Patients. *Cardiovascular Therapeutics* **2015,** *33* (4), 216-226.

64. Bolton, S. G.; Pluth, M. D., Modified Cyclodextrins Solubilize Elemental Sulfur in Water and Enable Biological Sulfane Sulfur Delivery. *Chemical Science* **2020,** *11* (43), 11777-11784.

65. Szejtli, J., Introduction and General Overview of Cyclodextrin Chemistry. *Chemical Reviews* **1998,** *98* (5), 1743-1754.

66. Tiwari, G.; Tiwari, R.; Rai, A. K., Cyclodextrins in Delivery Systems: Applications. *Journal of Pharmacy & Bioallied Science* **2010,** *2* (2), 72-79.

67. Filipovic, M. R., Persulfidation (S-Sulfhydration) and H_2S. *Handbook of Experimental Pharmacology* **2015,** *230*, 29-59.

68. Yang, C.-t.; Devarie-Baez, N. O.; Hamsath, A.; Fu, X.-d.; Xian, M., S-Persulfidation: Chemistry, Chemical Biology, and Significance in Health and Disease. *Antioxidants & Redox Signaling* **2020,** *33* (15), 192-1114.

69. Mustafa, A. K.; Gadalla, M. M.; Sen, N.; Kim, S.; Mu, W.; Gazi, S. K.; Barrow, R. K.; Yang, G.; Wang, R.; Snyder, S. H., H_2S Signals Through Protein S-Sulfhydration. *Science Signaling* **2009,** *2* (96), ra72.

70. Corsello, T.; Komaravelli, N.; Casola, A., Role of Hydrogen Sulfide in NRF2- and Sirtuin-Dependent Maintenance of Cellular Redox Balance. *Antioxidants* **2018,** *7* (10), 129.

71. Mustafa, A. K.; Sikka, G.; Gazi, S. K.; Steppan, J.; Jung, S. M.; Bhunia, A. K.; Barodka, V. M.; Gazi, F. K.; Barrow, R. K.; Wang, R.; Amzel, L. M.; Berkowitz, D. E.; Snyder, S. H., Hydrogen Sulfide as Endothelium-Derived Hyperpolarizing Factor Sulfhydrates Potassium Channels. *Circulation Research* **2011,** *109* (11), 1259-1268.

72. Sun, Y.; Huang, Y.; Yu, W.; Chen, S.; Yao, Q.; Zhang, C.; Bu, D.; Tang, C.; Du, J.; Jin, H., Sulfhydration-Associated Phosphodiesterase 5A Dimerization Mediates Vasorelaxant Effect of Hydrogen Sulfide. *Oncotarget* **2017,** *8* (19), 31888-31900.

73. Lagoutte, E.; Mimoun, S.; Andriamihaja, M.; Chaumontet, C.; Blachier, F.; Bouillaud, F., Oxidation of Hydrogen Sulfide Remains a Priority in Mammalian Cells and Causes Reverse Electron Transfer in Colonocytes. *Biochimica et Biophysica Acta* **2010,** *1797* (8), 1500-1511.

74. You, Z.; Cao, X.; Taylor, A. B.; Hart, P. J.; Levine, R. L., Characterization of a Covalent Polysulfane Bridge in Copper−Zinc Superoxide Dismutase. *Biochemistry* **2010,** *49* (6), 1191-1198.

CHAPTER II

1. Toohey, J. I., Sulfur Signaling: Is the Agent Sulfide or Sulfane? *Analytical Biochemistry* **2011,** *413* (1), 1-7.

2. Toohey, J. I., Sulphane Sulphur in Biological Systems: a Possible Regulatory Role. *Biochemical Journal* **1989,** *264* (3), 625-632.

3. Wang, R., Physiological Implications of Hydrogen Sulfide: a Whiff Exploration that Blossomed. *Physiological Reviews* **2012,** *92* (2), 791-896.

4. Wang, R., Two's Company, Three's a Crowd: Can H_2S be the Third Endogenous Gaseous Transmitter? *The FASEB Journal* **2002,** *16* (13), 1792-1798.

5. Kimura, H.; Nagai, Y.; Umemura, K.; Kimura, Y., Physiological roles of hydrogen sulfide: synaptic modulation, neuroprotection, and smooth muscle relaxation. *Antioxidants & Redox Signaling* **2005,** *7* (5-6), 795-803.

6. Yang, G.; Wu, L.; Jiang, B.; Yang, W.; Qi, J.; Cao, K.; Meng, Q.; Mustafa, A. K.; Mu, W.; Zhang, S.; Snyder, S. H.; Wang, R., H_2S as a Physiologic Vasorelaxant: Hypertension in Mice with Deletion of Cystathionine Gamma-Lyase. *Science* **2008,** *322* (5901), 587-590.

7. Whiteman, M.; Li, L.; Rose, P.; Tan, C. H.; Parkinson, D. B.; Moore, P. K., The Effect of Hydrogen Sulfide Donors on Lipopolysaccharide-Induced Formation of Inflammatory Mediators in Macrophages. *Antioxidants & Redox Signaling* **2010,** *12* (10), 1147-1154.

8. Wallace, J. L.; Wang, R., Hydrogen Sulfide-Based Therapeutics: Exploiting a Unique but Ubiquitous Gasotransmitter. *Nature Reviews Drug Discovery* **2015,** *14* (5), 329-345.

9. Szabo, C.; Papapetropoulos, A., International Union of Basic and Clinical Pharmacology. CII: Pharmacological Modulation of H_2S Levels: H_2S Donors and H_2S Biosynthesis Inhibitors. *Pharmacological Reviews* **2017,** *69* (4), 497-564.

10. Powell, C. R.; Dillon, K. M.; Matson, J. B., A Review of Hydrogen Sulfide (H_2S) Donors: Chemistry and Potential Therapeutic Applications. *Biochemical Pharmacology* **2018,** *149*, 110-123.

11. Zhao, Y.; Biggs, T. D.; Xian, M., Hydrogen Sulfide (H_2S) Releasing Agents: Chemistry and Biological Applications. *Chemical Communications* **2014,** *50* (80), 11788-11805.

12. Bora, P.; Chauhan, P.; Pardeshi, K. A.; Chakrapani, H., Small Molecule Generators of Biologically Reactive Sulfur Species. *RSC Advances* **2018,** *8* (48), 27359-27374.

13. Filipovic, M. R.; Zivanovic, J.; Alvarez, B.; Banerjee, R., Chemical Biology of H_2S Signaling through Persulfidation. *Chemical Reviews* **2018,** *118* (3), 1253-1337.

14. Lau, N.; Pluth, M. D., Reactive Sulfur Species (RSS): Persulfides, Polysulfides, Potential, and Problems. *Current Opinion in Chemical Biology* **2018,** *49*, 1-8.

15. Kimura, H., Hydrogen Sulfide and Polysulfides as Biological Mediators. *Molecules* **2014,** *19* (10), 16146-16157.

16. Iciek, M.; Wlodek, L., Biosynthesis and Biological Properties of Compounds Containing Highly Reactive, Reduced Sulfane Sulfur. *Polish Journal of Pharmacology* **2001,** *53* (3), 215-225.

17. Mishanina, T. V.; Libiad, M.; Banerjee, R., Biogenesis of Reactive Sulfur Species for Signaling by Hydrogen Sulfide Oxidation Pathways. *Nature Chemical Biology* **2015,** *11* (7), 457-464.

18. Ida, T.; Sawa, T.; Ihara, H.; Tsuchiya, Y.; Watanabe, Y.; Kumagai, Y.; Suematsu, M.; Motohashi, H.; Fujii, S.; Matsunaga, T.; Yamamoto, M.; Ono, K.; Devarie-Baez, N. O.; Xian, M.; Fukuto, J. M.; Akaike, T., Reactive Cysteine Persulfides and S-Polythiolation Regulate Oxidative Stress and Redox Signaling. *Proceedings of the National Academy of Science* **2014,** *111* (21), 7606-7611.

19. Mikami, Y.; Shibuya, N.; Kimura, Y.; Nagahara, N.; Ogasawara, Y.; Kimura, H., Thioredoxin and Dihydrolipoic Acid are Required for 3-Mercaptopyruvate Sulfurtransferase to Produce Hydrogen Sulfide. *Biochemical Journal* **2011,** *439* (3), 479-485.

20. Artaud, I.; Galardon, E., A Persulfide Analogue of the Nitrosothiol SNAP: Formation, Characterization and Reactivity. *ChemBioChem* **2014,** *15* (16), 2361-2364.

21. Yu, B.; Zheng, Y.; Yuan, Z.; Li, S.; Zhu, H.; De La Cruz, L. K.; Zhang, J.; Ji, K.; Wang, S.; Wang, B., Toward Direct Protein S-Persulfidation: A Prodrug Approach That Directly Delivers Hydrogen Persulfide. *Journal of the American Chemical Society* **2018,** *140* (1), 30-33.

22. Powell, C. R.; Dillon, K. M.; Wang, Y.; Carrazzone, R. J.; Matson, J. B., A Persulfide Donor Responsive to Reactive Oxygen Species: Insights into Reactivity and Therapeutic Potential. *Angewandte Chemie International Edition* **2018,** *57* (21), 6324-6328.

23. Chauvin, J. R.; Griesser, M.; Pratt, D. A., Hydropersulfides: H-Atom Transfer Agents Par Excellence. *Journal of the American Chemical Society* **2017,** *139* (18), 6484-6493.

24. Bailey, T. S.; Pluth, M. D., Reactions of Isolated Persulfides Provide Insights into the Interplay Between H_2S and Persulfide Reactivity. *Free Radical Biology & Medicine* **2015,** *89*, 662-667.

25. Bailey, T. S.; Zakharov, L. N.; Pluth, M. D., Understanding Hydrogen Sulfide Storage: Probing Conditions for Sulfide Release from Hydrodisulfides. *Journal of the American Chemical Society* **2014,** *136* (30), 10573-10576.

26. Pluth, M.; Bailey, T.; Hammers, M.; Hartle, M.; Henthorn, H.; Steiger, A., Natural Products Containing Hydrogen Sulfide Releasing Moieties. *Synlett* **2015,** *26* (19), 2633-2643.

27. Jiang, C. S.; Muller, W. E.; Schroder, H. C.; Guo, Y. W., Disulfide- and Multisulfide-Containing Metabolites from Marine Organisms. *Chemical Reviews* **2012,** *112* (4), 2179-2207.

28. Steudel, R., The Chemistry of Organic Polysulfanes $R{-}S_n{-}R$ (n> 2). *Chemical Reviews* **2002,** *102* (11), 3905-3946.

29. Block, E., The Organosulfur Chemistry of the Genus Allium - Implications for the Organic Chemistry of Sulfur. *Angewandte Chemie International Edition* **1992,** *31* (9), 1135-1178.

30. Powolny, A. A.; Singh, S. V., Multitargeted Prevention and Therapy of Cancer by Diallyl Trisulfide and Related Allium Vegetable-Derived Organosulfur Compounds. *Cancer Letters* **2008,** *269* (2), 305-314.

31. Li, Y.; Zhang, J.; Zhang, L.; Si, M.; Yin, H.; Li, J., Diallyl Trisulfide Inhibits Proliferation, Invasion and Angiogenesis of Osteosarcoma Cells by Switching on Suppressor microRNAs and Inactivating of Notch-1 Signaling. *Carcinogenesis* **2013,** *34* (7), 1601-1610.

32. Xiao, D.; Lew, K. L.; Kim, Y. A.; Zeng, Y.; Hahm, E. R.; Dhir, R.; Singh, S. V., Diallyl Trisulfide Suppresses Growth of PC-3 Human Prostate Cancer Xenograft in Vivo in Association with Bax and Bak Induction. *Clinical Cancer Research* **2006,** *12* (22), 6836-6843.

33. Li, W.; Tian, H.; Li, L.; Li, S.; Yue, W.; Chen, Z.; Qi, L.; Hu, W.; Zhu, Y.; Hao, B.; Gao, C.; Si, L.; Gao, F., Diallyl Trisulfide Induces Apoptosis and Inhibits Proliferation of A549 Cells In Vitro and In Vivo. *Acta Biochimica et Biophysica Sinica* **2012,** *44* (7), 577-583.

34. Wang, Y. B.; Qin, J.; Zheng, X. Y.; Bai, Y.; Yang, K.; Xie, L. P., Diallyl Trisulfide Induces Bcl-2 and Caspase-3-Dependent Apoptosis via Downregulation of Akt Phosphorylation in Human T24 Bladder Cancer Cells. *Phytomedicine* **2010,** *17* (5), 363-368.

35. Antosiewicz, J.; Herman-Antosiewicz, A.; Marynowski, S. W.; Singh, S. V., c-Jun NH(2)-Terminal Kinase Signaling Axis Regulates Diallyl Trisulfide-Induced Generation of Reactive Oxygen Species and Cell Cycle Arrest in Human Prostate Cancer Cells. *Cancer Research* **2006,** *66* (10), 5379-5386.

36. Hosono, T.; Fukao, T.; Ogihara, J.; Ito, Y.; Shiba, H.; Seki, T.; Ariga, T., Diallyl Trisulfide Suppresses the Proliferation and Induces Apoptosis of Human Colon Cancer Cells Through Oxidative Modification of Beta-Tubulin. *Journal of Biological Chemistry* **2005,** *280* (50), 41487-41493.

37. Benavides, G. A.; Squadrito, G. L.; Mills, R. W.; Patel, H. D.; Isbell, T. S.; Patel, R. P.; Darley-Usmar, V. M.; Doeller, J. E.; Kraus, D. W., Hydrogen Sulfide Mediates the Vasoactivity of Garlic. *Proceedings of the National Academy of Sciences* **2007,** *104* (46), 17977-17982.

38. Liang, D.; Wu, H.; Wong, M. W.; Huang, D., Diallyl Trisulfide Is a Fast H_2S Donor, but Diallyl Disulfide Is a Slow One: The Reaction Pathways and Intermediates of Glutathione with Polysulfides. *Organic Letters* **2015,** *17* (17), 4196-4199.

39. Liu, H.; Radford, M. N.; Yang, C. T.; Chen, W.; Xian, M., Inorganic Hydrogen Polysulfides: Chemistry, Chemical Biology and Detection. *British Journal of Pharmacology* **2018,** *176* (4), 616-627.

40. Licht, S.; Davis, J., Disproportionation of Aqueous Sulfur and Sulfide: Kinetics of Polysulfide Decomposition. *The Journal of Physical Chemistry B* **1997,** *101* (14), 2540-2545.

41. Ercole, F.; Whittaker, M. R.; Halls, M. L.; Boyd, B. J.; Davis, T. P.; Quinn, J. F., Garlic-Inspired Trisulfide Linkers for Thiol-Stimulated H_2S release. *Chemical Communications* **2017,** *53* (57), 8030-8033.

42. Xu, S.; Wang, Y.; Radford, M. N.; Ferrell, A. J.; Xian, M., Synthesis of Unsymmetric Trisulfides from 9-Fluorenylmethyl Disulfides. *Organic Letters* **2018,** *20* (2), 465-468.

43. Cerda, M. M.; Hammers, M. D.; Earp, M. S.; Zakharov, L. N.; Pluth, M. D., Applications of Synthetic Organic Tetrasulfides as H_2S Donors. *Organic Letters* **2017,** *19* (9), 2314-2317.

44. Zysman-Colman, E.; Harpp, D. N., Optimization of the Synthesis of Symmetric Aromatic Tri- and Tetrasulfides. *Journal of Organic Chemistry* **2003,** *68* (6), 2487-2489.

45. Harpp, D. N.; Granata, A., Organic Sulfur Chemistry. XXI. Trisulfide Formation by Alkoxide Decomposition of Sulfenylthiocarbonates. *Tetrahedron Letters* **1976,** *17* (35), 3001-3004.

46. Ahrika, A.; Robert, J.; Anouti, M.; Paris, J.; Jiang, Z.-H.; Yan, S.-P.; Wang, G.-L.; Yao, X.-K.; Wang, H.-G.; Tuchagues, J. P.; Ögren, M., Nucleophilic Substitution of Alkyl Halides by Electrogenerated Polysulfide Ions in N,N-dimethylacetamide. *Acta Chemica Scandinavica* **1999,** *53*, 513-520.

47. Cai, Y. R.; Hu, C. H., Computational Study of H_2S Release in Reactions of Diallyl Polysulfides with Thiols. *Journal of Physical Chemistry B* **2017,** *121* (26), 6359-6366.

48. Coletta, C.; Papapetropoulos, A.; Erdelyi, K.; Olah, G.; Modis, K.; Panopoulos, P.; Asimakopoulou, A.; Gero, D.; Sharina, I.; Martin, E.; Szabo, C., Hydrogen Sulfide and Nitric Oxide are Mutually Dependent in the Regulation of Angiogenesis and Endothelium-Dependent Vasorelaxation. *Proceedings of the National Academy of Sciences* **2012,** *109* (23), 9161-9166.

49. Szczesny, B.; Modis, K.; Yanagi, K.; Coletta, C.; Le Trionnaire, S.; Perry, A.; Wood, M. E.; Whiteman, M.; Szabo, C., AP39, a Novel Mitochondria-Targeted Hydrogen Sulfide Donor, Stimulates Cellular Bioenergetics, Exerts Cytoprotective Effects and Protects Against the Loss of Mitochondrial DNA Integrity in Oxidatively Stressed Endothelial Cells In Vitro. *Nitric Oxide* **2014,** *41*, 120-130.

50. Yuan, S.; Shen, X.; Kevil, C. G., Beyond a Gasotransmitter: Hydrogen Sulfide and Polysulfide in Cardiovascular Health and Immune Response. *Antioxidants & Redox Signaling* **2017,** *27* (10), 634-653.

CHAPTER III

1. Lau, N.; Pluth, M. D., Reactive Sulfur Species (RSS): Persulfides, Polysulfides, Potential, and Problems. *Current Opinion in Chemical Biology* **2019,** *49*, 1-8.

2. Baskar, R.; Bian, J., Hydrogen Sulfide Gas has Cell Growth Regulatory Role. *European Journal of Pharmacology* **2011,** *656* (1-3), 5-9.

3. Polhemus, D. J.; Lefer, D. J., Emergence of Hydrogen Sulfide as an Endogenous Gaseous Signaling Molecule in Cardiovascular Disease. *Circulation Research* **2014,** *114* (4), 730-737.

4. Wallace, J. L.; Wang, R., Hydrogen Sulfide-Based Therapeutics: Exploiting a Unique but Ubiquitous Gasotransmitter. *Nature Reviews Drug Discovery* **2015,** *14* (5), 329-345.

5. Wang, R., Physiological Implications of Hydrogen Sulfide: a Whiff Exploration that Blossomed. *Physiological Reviews* **2012,** *92* (2), 791-896.

6. Kabil, O.; Banerjee, R., Enzymology of H_2S Biogenesis, Decay and Signaling. *Antioxidants & Redox Signaling* **2014,** *20* (5), 77-782.

7. Kabil, O.; Banerjee, R., Redox Biochemistry of Hydrogen Sulfide. *The Journal of Biological Chemistry* **2010,** *285* (29), 21903-21907.

8. Giuffrè, A.; Vicente, J. B., Hydrogen Sulfide Biochemistry and Interplay with Other Gaseous Mediators in Mammalian Physiology. *Oxidative Medicine and Cellular Longevity* **2018,** *2018*, 6290931.

9. Corsello, T.; Komaravelli, N.; Casola, A., Role of Hydrogen Sulfide in NRF2- and Sirtuin-Dependent Maintenance of Cellular Redox Balance. *Antioxidants* **2018,** *7* (10), 129.

10. Kimura, Y.; Kimura, H., Hydrogen Sulfide Protects Neurons from Oxidative Stress. *The FASEB Journal* **2004,** *18* (10), 1165-1167.

11. Mancardi, D.; Penna, C.; Merlino, A.; Del Soldato, P.; Wink, D. A.; Pagliaro, P., Physiological and Pharmacological Features of the Novel Gasotransmitter: Hydrogen Sulfide. *Biochimica et Biophysica Acta* **2009,** *1787* (7), 864-872.

12. Szczesny, B.; Modis, K.; Yanagi, K.; Coletta, C.; Le Trionnaire, S.; Perry, A.; Wood, M. E.; Whiteman, M.; Szabo, C., AP39, a Novel Mitochondria-Targeted Hydrogen Sulfide Donor, Stimulates Cellular Bioenergetics, Exerts Cytoprotective Effects and Protects Against the Loss of Mitochondrial DNA Integrity in Oxidatively Stressed Endothelial Cells In Vitro. *Nitric Oxide* **2014,** *41*, 120-130.

13. Calvert, J. W.; Coetzee, W. A.; Lefer, D. J., Novel Insights into Hydrogen Sulfide-Mediated Cytoprotection. *Antioxidants & Redox Signaling* **2010,** *12* (10), 1203-1217.

14. Fonseca, M. D.; Cunha, F. Q.; Kashfi, K.; Cunha, T. M., NOSH-Aspirin (NBS-1120), a Dual Nitric Oxide and Hydrogen Sulfide-Releasing Hybrid, Reduces Inflammatory Pain. *Pharmacology Research & Perspectives* **2015,** *3* (3), e00133.

15. Whiteman, M.; Li, L.; Rose, P.; Tan, C.-H.; Parkinson, D. B.; Moore, P. K., The Effect of Hydrogen Sulfide Donors on Lipopolysaccharide-Induced Formation of Inflammatory Mediators in Macrophages. *Antioxidants & Redox Signaling* **2010,** *12* (10), 1147-1154.

16. Zanardo, R. C. O.; Brancaleone, V.; Distrutti, E.; Fiorucci, S.; Cirino, G.; Wallace, J. L., Hydrogen Sulfide is an Endogenous Modulator of Leukocyte-Mediated Inflammation. *The FASEB Journal* **2006,** *20* (12), 2118-2120.

17. Benavides, G. A.; Squadrito, G. L.; Mills, R. W.; Patel, H. D.; Isbell, T. S.; Patel, R. P.; Darley-Usmar, V. M.; Doeller, J. E.; Kraus, D. W., Hydrogen Sulfide Mediates the Vasoactivity of Garlic. *Proceedings of the National Academy of Sciences* **2007,** *104* (46), 17977-17982.

18. Coletta, C.; Papapetropoulos, A.; Erdelyi, K.; Olah, G.; Modis, K.; Panopoulos, P.; Asimakopoulou, A.; Geroe, D.; Sharina, I.; Martin, E.; Szabo, C., Hydrogen Sulfide and Nitric Oxide are Mutually Dependent in the Regulation of Angiogenesis and Endothelium-Dependent Vasorelaxation. *Proceedings of the National Academy of Sciences* **2012,** *109* (23), 9161-9166.

19. Yang, G.; Wu, L.; Jiang, B.; Yang, W.; Qi, J.; Cao, K.; Meng, Q.; Mustafa, A. K.; Mu, W.; Zhang, S.; Snyder, S. H.; Wang, R., H_2S as a Physiologic Vasorelaxant: Hypertension in Mice with Deletion of Cystathionine γ-Lyase. *Science* **2008,** *322* (5901), 587-590.

20. Köhn, C.; Dubrovska, G.; Huang, Y.; Gollasch, M., Hydrogen Sulfide: Potent Regulator of Vascular Tone and Stimulator of Angiogenesis. *International Journal of Biomedical Science* **2012,** *8* (2), 81-86.

21. Terzuoli, E.; Monti, M.; Vellecco, V.; Bucci, M.; Cirino, G.; Ziche, M.; Morbidelli, L., Characterization of Zofenoprilat as an Inducer of Functional Angiogenesis through Increased H_2S Availability. *British Journal of Pharmacology* **2015,** *172* (12), 2961-2973.

22. Szabó, C.; Papapetropoulos, A., Hydrogen Sulphide and Angiogenesis: Mechanisms and Applications. *British Journal of Pharmacology* **2011,** *164* (3), 853-865.

23. Papapetropoulos, A.; Pyriochou, A.; Altaany, Z.; Yang, G.; Marazioti, A.; Zhou, Z.; Jeschke, M. G.; Branski, L. K.; Herndon, D. N.; Wang, R.; Szabo, C., Hydrogen Sulfide Is an Endogenous Stimulator of Angiogenesis. *Proceedings of the National Academy of Sciences* **2009,** *106* (51), 21972-21977.

24. Filipovic, M. R., Persulfidation (S-Sulfhydration) and H_2S. *Handbook of Experimental Pharmacology* **2015,** *230*, 29-59.

25. Yang, C.-t.; Devarie-Baez, N. O.; Hamsath, A.; Fu, X.-d.; Xian, M., S-Persulfidation: Chemistry, Chemical Biology, and Significance in Health and Disease. *Antioxidants & Redox Signaling* **2020,** *33* (15), 192-1114.

26. Filipovic, M. R.; Zivanovic, J.; Alvarez, B.; Banerjee, R., Chemical Biology of H_2S Signaling through Persulfidation. *Chemical Reviews* **2018,** *118* (3), 1253-1337.

27. Paul, B. D.; Snyder, S. H., H_2S Signalling Through Protein Sulfhydration and Beyond. *Nature Reviews Molecular Cell Biology* **2012,** *13* (8), 499-507.

28. Kimura, H., Hydrogen Sulfide and Polysulfides as Biological Mediators. *Molecules* **2014,** *19* (10), 16146-16157.

29. Lagoutte, E.; Mimoun, S.; Andriamihaja, M.; Chaumontet, C.; Blachier, F.; Bouillaud, F., Oxidation of Hydrogen Sulfide Remains a Priority in Mammalian Cells and Causes Reverse Electron Transfer in Colonocytes. *Biochimica et Biophysica Acta* **2010,** *1797* (8), 1500-1511.

30. Mustafa, A. K.; Sikka, G.; Gazi, S. K.; Steppan, J.; Jung, S. M.; Bhunia, A. K.; Barodka, V. M.; Gazi, F. K.; Barrow, R. K.; Wang, R.; Amzel, L. M.; Berkowitz, D. E.; Snyder, S. H., Hydrogen Sulfide as Endothelium-Derived Hyperpolarizing Factor Sulfhydrates Potassium Channels. *Circulation Research* **2011,** *109* (11), 1259-1268.

31. Bolton, S. G.; Cerda, M. M.; Gilbert, A. K.; Pluth, M. D., Effects of Sulfane Sulfur Content in Benzyl Polysulfides on Thiol-Triggered H_2S Release and Cell Proliferation. *Free Radical Biology & Medicine* **2019,** *131*, 393-398.

32. Cerda, M. M.; Hammers, M. D.; Earp, M. S.; Zakharov, L. N.; Pluth, M. D., Applications of Synthetic Organic Tetrasulfides as H_2S Donors. *Organic Letters* **2017,** *19* (9), 2314-2317.

33. Aldini, G.; Altomare, A.; Baron, G.; Vistoli, G.; Carini, M.; Borsani, L.; Sergio, F., N-Acetylcysteine as an Antioxidant and Disulphide Breaking Agent: The Reasons Why. *Free Radical Research* **2018,** *52* (7), 751-762.

34. Park, A. Y.; Guan, J.-L.; Liang, C.-C., In Vitro Scratch Assay: A Convenient and Inexpensive Method for Analysis of Cell Migration In Vitro. *Nature Protocols* **2007,** *2* (2), 329-333.

CHAPTER IV

1. Zahnle, K.; Schaefer, L.; Fegley, B., Earth's Earliest Atmospheres. *Cold Spring Harbor Perspectives in Biology* **2010,** *2* (10), a004895.

2. Ranjan, S.; Todd, Z. R.; Sutherland, J. D.; Sasselov, D. D., Sulfidic Anion Concentrations on Early Earth for Surficial Origins-of-Life Chemistry. *Astrobiology* **2018,** *18* (8), 1023-1040.

3. 55Parker, E. T.; Cleaves, H. J.; Dworkin, J. P.; Glavin, D. P.; Callahan, M.; Aubrey, A.; Lazcano, A.; Bada, J. L., Primordial Synthesis of Amines and Amino Acids in a 1958 Miller H_2S-Rich Spark Discharge Experiment. *Proceedings of the National Academy of Sciences* **2011,** *108* (14), 5526-5531.

4. Heinen, W.; Lauwers, A. M., Organic Sulfur Compounds Resulting From the Interaction of Iron Sulfide, Hydrogen Sulfide and Carbon Dioxide in an Anaerobic Aqueous Environment. *Origins of Life and Evolution of Biospheres* **1996,** *26* (2), 131-150.

5. Vandiver, M.; Snyder, S. H., Hydrogen Sulfide: A Gasotransmitter of Clinical Relevance. *Journal of Molecular Medicine* **2012,** *90* (3), 255-263.

6. Wang, R., Hydrogen Sulfide: the Third Gasotransmitter in Biology and Medicine. *Antioxidants & Redox Signaling* **2010,** *12* (9), 1061-1064.

7. Mustafa, A. K.; Gadalla, M. M.; Snyder, S. H., Signaling by Gasotransmitters. *Science Signaling* **2009,** *2* (68), re2.

8. Lau, N.; Pluth, M. D., Reactive Sulfur Species (RSS): Persulfides, Polysulfides, Potential, and Problems. *Current Opinion in Chemical Biology* **2019,** *49*, 1-8.

9. Kimura, H., Signaling of Hydrogen Sulfide and Polysulfides. *Antioxidants & Redox Signaling* **2015,** *22* (5), 347-349.

10. Bolton, S. G.; Cerda, M. M.; Gilbert, A. K.; Pluth, M. D., Effects of Sulfane Sulfur Content in Benzyl Polysulfides on Thiol-Triggered H_2S Release and Cell Proliferation. *Free Radical Biology & Medicine* **2019,** *131*, 393-398.

11. Liu, H.; Radford, M. N.; Yang, C. T.; Chen, W.; Xian, M., Inorganic Hydrogen Polysulfides: Chemistry, Chemical Biology and Detection. *British Journal of Pharmacology* **2018,** *176* (4), 616-627.

12. Hosono, T.; Fukao, T.; Ogihara, J.; Ito, Y.; Shiba, H.; Seki, T.; Ariga, T., Diallyl Trisulfide Suppresses the Proliferation and Induces Apoptosis of Human Colon Cancer Cells through Oxidative Modification of Beta-Tubulin. *Journal of Biological Chemistry* **2005,** *280* (50), 41487-41493.

13. Bat-Chen, W.; Golan, T.; Peri, I.; Ludmer, Z.; Schwartz, B., Allicin Purified from Fresh Garlic Cloves Induces Apoptosis in Colon Cancer Cells Via Nrf2. *Nutrition and Cancer* **2010,** *62* (7), 947-957.

14. Xiao, D.; Singh, S. V., Diallyl Trisulfide, a Constituent of Processed Garlic, Inactivates Akt to Trigger Mitochondrial Translocation of BAD and Caspase-Mediated Apoptosis in Human Prostate Cancer Cells. *Carcinogenesis* **2006,** *27* (3), 533-540.

15. Murai, M.; Inoue, T.; Suzuki-Karasaki, M.; Ochiai, T.; Ra, C.; Nishida, S.; Suzuki-Karasaki, Y., Diallyl Trisulfide Sensitizes Human Melanoma Cells to TRAIL-Induced Cell Death by Promoting Endoplasmic Reticulum-Mediated Apoptosis. *International Journal of Oncology* **2012,** *41* (6), 2029-2037.

16. Boulegue, J., Solubility of Elemental Sulfur in Water at 298 K. *Phosphorus and Sulfur and the Related Elements* **1978,** *5* (1), 127-128.

17. Liu, Y.; Beer, L. L.; Whitman, W. B., Sulfur Metabolism in Archaea Reveals Novel Processes. *Environmental Microbiology* **2012,** *14* (10), 2632-2644.

18. Kletzin, A.; Urich, T.; Muller, F.; Bandeiras, T. M.; Gomes, C. M., Dissimilatory Oxidation and Reduction of Elemental Sulfur in Thermophilic Archaea. *Journal of Bioenergetics and Biomembranes* **2004,** *36* (1), 77-91.

19. Peters, J.; Baumeister, W.; Lupas, A., Hyperthermostable Surface Layer Protein Tetrabrachion from the Archaebacterium Staphylothermus marinus: Evidence for the Presence of a Right-Handed Coiled Coil Derived from the Primary Structure. *Journal of Molecular Biology* **1996,** *257* (5), 1031-1041.

20. McDougall, M.; Francisco, O.; Harder-Viddal, C.; Roshko, R.; Meier, M.; Stetefeld, J., Archaea S-Layer Nanotube from a "Black Smoker" in Complex with Cyclo-Octasulfur (S_8) Rings. *Proteins* **2017,** *85* (12), 2209-2216.

21. Harder-Viddal, C.; McDougall, M.; Roshko, R. M.; Stetefeld, J., Energetics of Storage and Diffusion of Water and Cyclo-Octasulfur for a Nonpolar Cavity of RHCC Tetrabrachion by Molecular Dynamics Simulations. *Computational and Structural Biotechnology Journal* **2019,** *17*, 675-683.

22. Steudel, R.; Holdt, G., Solubilization of Elemental Sulfur in Water by Cationic and Anionic Surfactants. *Angewandte Chemie International Edition* **1988,** *27* (10), 1358-1359.

23. Searcy, D. G.; Lee, S. H., Sulfur Reduction by Human Erythrocytes. *Journal of Experimental Zoology* **1998,** *282* (3), 310-322.

24. Szejtli, J., Introduction and General Overview of Cyclodextrin Chemistry. *Chemical Reviews* **1998,** *98* (5), 1743-1754.

25. Loftsson, T.; Jarho, P.; Masson, M.; Jarvinen, T., Cyclodextrins in Drug Delivery. *Expert Opinion on Drug Delivery* **2005,** *2* (2), 335-351.

26. Mecozzi, S.; Rebek, J., The 55% Solution: A Formula for Molecular Recognition in the Liquid State. *Chemistry-A European Journal* **1998,** *4* (6), 1016-1022.

27. Steudel, R.; Jensen, D.; Gobel, P.; Hugo, P., Optical Absorption Spectra of the Homocyclic Sulfur Molecules S_n (n = 6, 7, 8, 9, 10, 12, 15, 20) in Solution. *Berichte der Bunsengesellschaft für Physikalische Chemie* **1988,** *92* (2), 118-122.

28. Conners, K. A., *Binding Constants, The Measurement of Molecular Complex Stability*. Wiley: New York, 1987.

29. Connors, K. A., Population Characteristics of Cyclodextrin Complex Stabilities in Aqueous-Solution. *Journal of Pharmaceutical Sciences* **1995,** *84* (7), 843-848.

30. Cerda, M. M.; Hammers, M. D.; Earp, M. S.; Zakharov, L. N.; Pluth, M. D., Applications of Synthetic Organic Tetrasulfides as H_2S Donors. *Organic Letters* **2017,** *19* (9), 2314-2317.

31. Ezerina, D.; Takano, Y.; Hanaoka, K.; Urano, Y.; Dick, T. P., N-Acetyl Cysteine Functions as a Fast-Acting Antioxidant by Triggering Intracellular H_2S and Sulfane Sulfur Production. *Cell Chemical Biology* **2018,** *25* (4), 447-459.

32. Chen, W.; Liu, C. R.; Peng, B.; Zhao, Y.; Pacheco, A.; Xian, M., New Fluorescent Probes for Sulfane Sulfurs and the Application in Bioimaging. *Chemical Science* **2013,** *4* (7), 2892-2896.

33. Olson, K. R.; Gao, Y.; Steiger, A. K.; Pluth, M. D.; Tessier, C. R.; Markel, T. A.; Boone, D.; Stahelin, R.; Batinic-Haberle, I.; Straubg, K. D., Effects of Manganese Porphyrins on Cellular Sulfur Metabolism. *Molecules* **2020,** *25* (4), 980.

34. Magierowski, M.; Magierowska, K.; Hubalewska-Mazgaj, M.; Surmiak, M.; Sliwowski, Z.; Wierdak, M.; Kwiecien, S.; Chmura, A.; Brzozowski, T., Cross-Talk Between Hydrogen Sulfide and Carbon Monoxide in the Mechanism of Experimental Gastric Ulcers Healing, Regulation of Gastric Blood Flow and Accompanying Inflammation. *Biochemical Pharmacology* **2018,** *149*, 131-142.

35. Nicolau, L. A.; Silva, R. O.; Damasceno, S. R.; Carvalho, N. S.; Costa, N. R.; Aragao, K. S.; Barbosa, A. L.; Soares, P. M.; Souza, M. H.; Medeiros, J. V., The Hydrogen Sulfide Donor, Lawesson's Reagent, Prevents Alendronate-Induced Gastric Damage in Rats. *Brazilian Journal of Medical and Biological Research* **2013,** *46* (8), 708-714.

36. Ida, T.; Sawa, T.; Ihara, H.; Tsuchiya, Y.; Watanabe, Y.; Kumagai, Y.; Suematsu, M.; Motohashi, H.; Fujii, S.; Matsunaga, T.; Yamamoto, M.; Ono, K.; Devarie-Baez, N. O.; Xian, M.; Fukuto, J. M.; Akaike, T., Reactive Cysteine Persulfides and S-Polythiolation Regulate Oxidative Stress and Redox Signaling. *Proceedings of the National Academy of Sciences* **2014,** *111* (21), 7606-7611.

37. Coletta, C.; Papapetropoulos, A.; Erdelyi, K.; Olah, G.; Modis, K.; Panopoulos, P.; Asimakopoulou, A.; Gero, D.; Sharina, I.; Martin, E.; Szabo, C., Hydrogen Sulfide and Nitric Oxide are Mutually Dependent in the Regulation of Angiogenesis and Endothelium-Dependent Vasorelaxation. *Proceedings of the National Academy of Sciences* **2012,** *109* (23), 9161-9166.

38. Aktan, F., iNOS-Mediated Nitric Oxide Production and its Regulation. *Life Sciences* **2004,** *75* (6), 639-653.

39. Whiteman, M.; Li, L.; Rose, P.; Tan, C. H.; Parkinson, D. B.; Moore, P. K., The Effect of Hydrogen Sulfide Donors on Lipopolysaccharide-Induced Formation of Inflammatory Mediators in Macrophages. *Antioxidants & Redox Signaling* **2010,** *12* (10), 1147-1154.

40. Zhao, Y.; Cerda, M. M.; Pluth, M. D., Fluorogenic Hydrogen Sulfide (H_2S) Donors Based on Sulfenyl Thiocarbonates Enable H_2S Tracking and Quantification. *Chemical Science* **2019,** *10* (6), 1873-1878.

CHAPTER V

1. Zahnle, K.; Schaefer, L.; Fegley, B., Earth's Earliest Atmospheres. *Cold Spring Harbor Perspectives in Biology* **2010,** *2* (10), a004895.

2. Catling, D. C.; Glein, C. R.; Zahnle, K. J.; McKay, C. P., Why O_2 is Required by Complex Life on Habitable Planets and the Concept of Planetary "Oxygenation Time". *Astrobiology* **2005,** *5* (3), 415-438.

3. Ranjan, S.; Todd, Z. R.; Sutherland, J. D.; Sasselov, D. D., Sulfidic Anion Concentrations on Early Earth for Surficial Origins-of-Life Chemistry. *Astrobiology* **2018,** *18* (8), 123-1040.

4. Tabibzadeh, S., Nature Creates, Adapts, Protects and Sustains Life Using Hydrogen Sulfide. *Frontiers in Bioscience* **2016,** *21* (3), 528-560.

5. Aroca, A.; Zhang, J.; Xie, Y.; Romero, L. C.; Gotor, C., Hydrogen Sulfide Signaling in Plant Adaptations to Adverse Conditions: Molecular Mechanisms. *Journal of Experimental Botany* **2021**, [online early access].

6. Johnston, D. T.; Wolfe-Simon, F.; Pearson, A.; Knoll, A. H., Anoxygenic Photosynthesis Modulated Proterozoic Oxygen and Sustained Earth's Middle Age. *Proceedings of the National Academy of Sciences* **2009,** *106* (40), 16925-16929.

7. Olson, K. R.; Donald, J. A.; Dombkowski, R. A.; Perry, S. F., Evolutionary and Comparative Aspects of Nitric Oxide, Carbon Monoxide and Hydrogen Sulfide. *Respiratory Physiology & Neurobiology* **2012,** *184* (2), 117-129.

8. Olson, K. R.; Straub, K. D., The Role of Hydrogen Sulfide in Evolution and the Evolution of Hydrogen Sulfide in Metabolism and Signaling. *Physiology* **2016,** *31* (1), 60-72.

9. Olson, K. R., Mitochondrial Adaptations to Utilize Hydrogen Sulfide for Energy and Signaling. *Journal of Comparative Physiology B* **2012,** *182* (7), 881-897.

10. Dombkowski, R. A.; Doellman, M. M.; Head, S. K.; Olson, K. R., Hydrogen Sulfide Mediates Hypoxia-Induced Relaxation of Trout Urinary Bladder Smooth Muscle. *Journal of Experimental Biology* **2006,** *209* (16), 3234-3240.

11. Mancardi, D.; Penna, C.; Merlino, A.; Del Soldato, P.; Wink, D. A.; Pagliaro, P., Physiological and Pharmacological Features of the Novel Gasotransmitter: Hydrogen Sulfide. *Biochimica et Biophysica Acta* **2009,** *1787* (7), 864-872.

12. Polhemus, D. J.; Lefer, D. J., Emergence of Hydrogen Sulfide as an Endogenous Gaseous Signaling Molecule in Cardiovascular Disease. *Circulation Research* **2014,** *114* (4), 730-737.

13. Wang, R., Two's Company, Three's a Crowd: can H_2S be the Third Endogenous Gaseous Transmitter? *The FASEB Journal* **2002,** *16* (13), 1792-1798.

14. Wang, R., Physiological Implications of Hydrogen Sulfide: a Whiff Exploration that Blossomed. *Physiological Reviews* **2012,** *92* (2), 791-896.

15. Coletta, C.; Papapetropoulos, A.; Erdelyi, K.; Olah, G.; Modis, K.; Panopoulos, P.; Asimakopoulou, A.; Geroe, D.; Sharina, I.; Martin, E.; Szabo, C., Hydrogen Sulfide and Nitric Oxide are Mutually Dependent in the Regulation of Angiogenesis and Endothelium-Dependent Vasorelaxation. *Proceedings of the National Academy of Sciences* **2012,** *109* (23), 9161-9166.

16. Lau, N.; Pluth, M. D., Reactive Sulfur Species (RSS): Persulfides, Polysulfides, Potential, and Problems. *Current Opinion in Chemical Biology* **2019,** *49*, 1-8.

17. Kimura, H., Hydrogen Sulfide and Polysulfides as Biological Mediators. *Molecules* **2014,** *19* (10), 16146-16157.

18. Kimura, Y.; Kimura, H., Hydrogen Sulfide Protects Neurons from Oxidative Stress. *The FASEB Journal* **2004,** *18* (10), 1165-1167.

19. Szczesny, B.; Modis, K.; Yanagi, K.; Coletta, C.; Le Trionnaire, S.; Perry, A.; Wood, M. E.; Whiteman, M.; Szabo, C., AP39, a Novel Mitochondria-Targeted Hydrogen Sulfide Donor, Stimulates Cellular Bioenergetics, Exerts Cytoprotective Effects and Protects Against the Loss of Mitochondrial DNA Integrity in Oxidatively Stressed Endothelial Cells In Vitro. *Nitric Oxide* **2014,** *41*, 120-130

20. Zhao, Y.; Pluth, M. D., Hydrogen Sulfide Donors Activated by Reactive Oxygen Species. *Angewandte Chemie International Edition* **2016,** *55* (47), 14638-14642.

21. Magierowski, M.; Magierowska, K.; Szmyd, J.; Surmiak, M.; Sliwowski, Z.; Kwiecien, S.; Brzozowski, T., Hydrogen Sulfide and Carbon Monoxide Protect Gastric Mucosa Compromised by Mild Stress Against Alendronate Injury. *Digestive Diseases and Sciences* **2016,** *61* (11), 3176-3189.

22. Wallace, J. L.; Dicay, M.; McKnight, W.; Martin, G. R., Hydrogen Sulfide Enhances Ulcer Healing in Rats. *The FASEB Journal* **2007,** *21* (14), 4070-4076.

23. Papapetropoulos, A.; Pyriochou, A.; Altaany, Z.; Yang, G.; Marazioti, A.; Zhou, Z.; Jeschke, M. G.; Branski, L. K.; Herndon, D. N.; Wang, R.; Szabo, C., Hydrogen Sulfide Is an Endogenous Stimulator of Angiogenesis. *Proceedings of the National Academy of Sciences* **2009,** *106* (51), 21972-21977.

24. Köhn, C.; Dubrovska, G.; Huang, Y.; Gollasch, M., Hydrogen Sulfide: Potent Regulator of Vascular Tone and Stimulator of Angiogenesis. *International Journal of Biomedical Science* **2012,** *8* (2), 81-86.

25. Greiner, R.; Pálinkás, Z.; Bäsell, K.; Becher, D.; Antelmann, H.; Nagy, P.; Dick, T. P., Polysulfides Link H_2S to Protein Thiol Oxidation. *Antioxidants & Redox Signaling* **2013,** *19* (15), 1749-1765.

26. Kimura, H., Signalling by Hydrogen Sulfide and Polysulfides Via Protein S-Sulfuration. *British Journal of Pharmacology* **2020,** *177* (4), 720-733.

27. Filipovic, M. R., Persulfidation (S-Sulfhydration) and H_2S. *Handbook of Experimental Pharmacology* **2015,** *230*, 29-59.

28. Mustafa, A. K.; Gadalla, M. M.; Sen, N.; Kim, S.; Mu, W.; Gazi, S. K.; Barrow, R. K.; Yang, G.; Wang, R.; Snyder, S. H., H_2S Signals Through Protein S-Sulfhydration. *Science Signaling* **2009,** *2* (96), ra72.

29. Paul, B. D.; Snyder, S. H., H_2S Signalling Through Protein Sulfhydration and Beyond. *Nature Reviews Molecular Cell Biology* **2012,** *13* (8), 499-507.

30. Filipovic, M. R.; Zivanovic, J.; Alvarez, B.; Banerjee, R., Chemical Biology of H_2S Signaling through Persulfidation. *Chemical Reviews* **2018,** *118* (3), 1253-1337.

31. Mustafa, A. K.; Sikka, G.; Gazi, S. K.; Steppan, J.; Jung, S. M.; Bhunia, A. K.; Barodka, V. M.; Gazi, F. K.; Barrow, R. K.; Wang, R.; Amzel, L. M.; Berkowitz, D. E.; Snyder, S. H., Hydrogen Sulfide as Endothelium-Derived Hyperpolarizing Factor Sulfhydrates Potassium Channels. *Circulation Research* **2011,** *109* (11), 1259-1268.

32. Bolton, S. G.; Cerda, M. M.; Gilbert, A. K.; Pluth, M. D., Effects of Sulfane Sulfur Content in Benzyl Polysulfides on Thiol-Triggered H_2S Release and Cell Proliferation. *Free Radical Biology & Medicine* **2019,** *131*, 393-398.

33. Bolton, S. G.; Pluth, M. D., Modified Cyclodextrins Solubilize Elemental Sulfur in Water and Enable Biological Sulfane Sulfur Delivery. *Chemical science* **2020,** *11* (43), 11777-11784.

34. Jarosz, A. P.; Wei, W.; Gauld, J. W.; Auld, J.; Özcan, F.; Aslan, M.; Mutus, B., Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) is Inactivated by S-Dulfuration In Vitro. *Free Radical Biology & Medicine* **2015,** *89*, 512-521.

35. Liu, H.; Radford, M. N.; Yang, C. T.; Chen, W.; Xian, M., Inorganic Hydrogen Polysulfides: Chemistry, Chemical Biology and Detection. *British Journal of Pharmacology* **2018,** *176* (4), 616-627.

36. Cai, Y.-R.; Hu, C.-H., Computational Study of H_2S Release in Reactions of Diallyl Polysulfides with Thiols. *The Journal of Physical Chemistry B* **2017,** *121* (26), 6359-6366.

37. Ercole, F.; Whittaker, M. R.; Halls, M. L.; Boyd, B. J.; Davis, T. P.; Quinn, J. F., Garlic-Inspired Trisulfide Linkers for Thiol-Stimulated H_2S Release. *Chemical Communications* **2017,** *53* (57), 8030-8033.

38. Leung, W.-S.; Kuo, W.-W.; Ju, D.-T.; Wang, T.-D.; Shao-Tsu Chen, W.; Ho, T.-J.; Lin, Y. M.; Mahalakshmi, B.; Lin, J.-Y.; Huang, C.-Y., Protective Effects of Diallyl Trisulfide (DATS) Against Doxorubicin-Induced Inflammation and Oxidative Stress in the Brain of Rats. *Free Radical Biology & Medicine* **2020,** *160*, 141-148.

39. Tsai, C. W.; Yang, J. J.; Chen, H. W.; Sheen, L. Y.; Lii, C. K., Garlic Organosulfur Compounds Upregulate the Expression of the Pi Class of Glutathione S-Transferase in Rat Primary Hepatocytes. *The Journal of Nutrition* **2005,** *135* (11), 2560-2565.

40. Zeng, T.; Zhang, C.-L.; Zhu, Z.-P.; Yu, L.-H.; Zhao, X.-L.; Xie, K.-Q., Diallyl Trisulfide (DATS) Effectively Attenuated Oxidative Stress-Mediated Liver Injury and Hepatic Mitochondrial Dysfunction in Acute Ethanol-Exposed Mice. *Toxicology* **2008,** *252* (1), 86-91.

41. Cerda, M. M.; Hammers, M. D.; Earp, M. S.; Zakharov, L. N.; Pluth, M. D., Applications of Synthetic Organic Tetrasulfides as H_2S Donors. *Organic Letters* **2017,** *19* (9), 2314-2317.

42. Tonelli, C.; Chio, I. I. C.; Tuveson, D. A., Transcriptional Regulation by Nrf2. *Antioxidants & Redox Signaling* **2018,** *29* (17), 1727-1745.

43. Koike, S.; Ogasawara, Y.; Shibuya, N.; Kimura, H.; Ishii, K., Polysulfide Exerts a Protective Effect Against Cytotoxicity Caused by t-Buthylhydroperoxide through Nrf2 Signaling in Neuroblastoma Cells. *FEBS Letters* **2013,** *587* (21), 3548-3555.

44. Yang, G.; Zhao, K.; Ju, Y.; Mani, S.; Cao, Q.; Puukila, S.; Khaper, N.; Wu, L.; Wang, R., Hydrogen Sulfide Protects Against Cellular Senescence via S-Sulfhydration of Keap1 and Activation of Nrf2. *Antioxidants & Redox Signaling* **2013,** *18* (15), 196-1919.

CHAPTER VI

1. Elrod, J. W.; Calvert, J. W.; Morrison, J.; Doeller, J. E.; Kraus, D. W.; Tao, L.; Jiao, X.; Scalia, R.; Kiss, L.; Szabo, C.; Kimura, H.; Chow, C.-W.; Lefer, D. J., Hydrogen Sulfide Attenuates Myocardial Ischemia-Reperfusion Injury by Preservation of Mitochondrial Function. *Proceedings of the National Academy of Sciences* **2007,** *104* (39), 15560-15565.

2. Ling, L. I.; Rose, P.; Moore, P. K., Hydrogen Sulfide and Cell Signaling. *Annual Review of Pharmacology and Toxicology* **2011,** *51* (1), 169-187.

3. Wang, R., Physiological Implications of Hydrogen Sulfide: a Whiff Exploration that Blossomed. *Physiological Reviews* **2012,** *92* (2), 791-896.

4. Paul, B. D.; Snyder, S. H., H_2S Signalling through Protein Sulfhydration and Beyond. *Nature Reviews Molecular Cell Biology* **2012,** *13* (8), 499-507.

5. Kabil, O.; Banerjee, R., Enzymology of H_2S Biogenesis, Decay and Signaling. *Antioxidants & Redox Signaling* **2014,** *20* (5), 77-782.

6. Polhemus, D. J.; Lefer, D. J., Emergence of Hydrogen Sulfide as an Endogenous Gaseous Signaling Molecule in Cardiovascular Disease. *Circulation Research* **2014,** *114* (4), 730-737.

7. Wallace, J. L.; Wang, R., Hydrogen Sulfide-Based Therapeutics: Exploiting a Unique but Ubiquitous Gasotransmitter. *Nature Reviews Drug Discovery* **2015,** *14* (5), 329-345.

8. Wallace, J. L.; Vong, L.; McKnight, W.; Dicay, M.; Martin, G. R., Endogenous and Exogenous Hydrogen Sulfide Promotes Resolution of Colitis in Rats. *Gastroenterology* **2009,** *137* (2), 569-578.

9. Zhao, W.; Zhang, J.; Lu, Y.; Wang, R., The Vasorelaxant Effect of H_2S as a Novel Endogenous Gaseous KATP Channel Opener. *The EMBO Journal* **2001,** *20* (21), 6008-6016.

10. Calvert, J. W.; Coetzee, W. A.; Lefer, D. J., Novel Insights into Hydrogen Sulfide-Mediated Cytoprotection. *Antioxidants & Redox Signaling* **2010,** *12* (10), 1203-1217.

11. Predmore, B. L.; Lefer, D. J., Hydrogen Sulfide-Mediated Myocardial Pre- and Post-Conditioning. *Expert Review of Clinical Pharmacology* **2011,** *4* (1), 83-96.

12. Gargallo, C. J.; Lanas, A., Is NSAIDs-Related Gastrointestinal Damage Preventable? *Journal of Digestive Diseases* **2013,** *14* (2), 55-61.

13. Sparatore, A.; Santus, G.; Giustarini, D.; Rossi, R.; Del Soldato, P., Therapeutic Potential of New Hydrogen Sulfide-Releasing Hybrids. *Expert Review of Clinical Pharmacology* **2011,** *4* (1), 109-121.

14. Kashfi, K., Anti-Cancer Activity of New Designer Hydrogen Sulfide-Donating Hybrids. *Antioxidants & Redox Signaling* **2014,** *20* (5), 831-846.

15. Tamizhselvi, R.; Moore, P. K.; Bhatia, M., Inhibition of Hydrogen Sulfide Synthesis Attenuates Chemokine Production and Protects Mice Against Acute Pancreatitis and Associated Lung Injury. *Pancreas* **2008,** *36* (4), e24-e31.

16. Zanardo, R. C. O.; Brancaleone, V.; Distrutti, E.; Fiorucci, S.; Cirino, G.; Wallace, J. L., Hydrogen Sulfide is an Endogenous Modulator of Leukocyte-Mediated Inflammation. *The FASEB Journal* **2006,** *20* (12), 2118-2120.

17. Pluth, M. D.; Bailey, T. S.; Hammers, M. D.; Hartle, M. D.; Henthorn, H. A.; Steiger, A. K., Natural Products Containing Hydrogen Sulfide Releasing Moieties. *Synlett* **2015,** *26* (19), 2633-2643.

18. Zhao, Y.; Biggs, T. D.; Xian, M., Hydrogen Sulfide (H_2S) Releasing Agents: Chemistry and Biological Applications. *Chemical Communications* **2014,** *5* (8), 11788-1185.

19. Zhao, Y.; Pacheco, A.; Xian, M., Medicinal Chemistry: Insights into the Development of Novel H_2S Donors. *Handbook of Experimental Pharmacology* **2015,** *230*, 365-388.

20. Kashfi, K.; Olson, K. R., Biology and Therapeutic Potential of Hydrogen Sulfide and Hydrogen Sulfide-Releasing Chimeras. *Biochemical Pharmacology* **2013,** *85* (5), 689-703.

21. Hartle, M. D.; Pluth, M. D., A practical Guide to Working with H_2S at the Interface of Chemistry and Biology. *Chemical Society Reviews* **2016,** *45* (22), 6108-6117.

22. Kang, J.; Li, Z.; Organ, C. L.; Park, C.-M.; Yang, C.-t.; Pacheco, A.; Wang, D.; Lefer, D. J.; Xian, M., pH-Controlled Hydrogen Sulfide Release for Myocardial Ischemia-Reperfusion Injury. *Journal of the American Chemical Society* **2016,** *138* (20), 6336-6339.

23. Zheng, Y.; Yu, B.; Ji, K.; Pan, Z.; Chittavong, V.; Wang, B., Esterase-Sensitive Prodrugs with Tunable Release Rates and Direct Generation of Hydrogen Sulfide. *Angewandte Chemie International Edition* **2016,** *55* (14), 4514-4518.

24. Steiger, A. K.; Pardue, S.; Kevil, C. G.; Pluth, M. D., Self-Immolative Thiocarbamates Provide Access to Triggered H_2S Donors and Analyte Replacement Fluorescent Probes. *Journal of the American Chemical Society* **2016,** *138* (23), 7256-7259.

25. Zhao, Y.; Pluth, M. D., Hydrogen Sulfide Donors Activated by Reactive Oxygen Species. *Angewandte Chemie International Edition* **2016,** *55* (47), 14638-14642.

26. Steiger, A. K.; Yang, Y.; Royzen, M.; Pluth, M. D., Bio-orthogonal "Click-and-Release" Donation of Caged Carbonyl Sulfide (COS) and Hydrogen Sulfide (H_2S). *Chemical communications* **2017,** *53* (8), 1378-1380.

27. Powell, C. R.; Foster, J. C.; Okyere, B.; Theus, M. H.; Matson, J. B., Therapeutic Delivery of H_2S via COS: Small Molecule and Polymeric Donors with Benign Byproducts. *Journal of the American Chemical Society* **2016,** *138* (41), 13477-13480.

28. Chauhan, P.; Bora, P.; Ravikumar, G.; Jos, S.; Chakrapani, H., Esterase Activated Carbonyl Sulfide/Hydrogen Sulfide (H_2S) Donors. *Organic Letters* **2017,** *19* (1), 62-65.

29. Klán, P.; Šolomek, T. s.; Bochet, C. G.; Blanc, A. l.; Givens, R.; Rubina, M.; Popik, V.; Kostikov, A.; Wirz, J., Photoremovable Protecting Groups in Chemistry and Biology: Reaction Mechanisms and Efficacy. *Chemical Reviews* **2013,** *113* (1), 119-191.

30. Chakraborty, I.; Carrington, S. J.; Hauser, J.; Oliver, S. R. J.; Mascharak, P. K., Rapid Eradication of Human Breast Cancer Cells through Trackable Light-Triggered CO Delivery by Mesoporous Silica Nanoparticles Packed with a Designed photoCORM. *Chemistry of Materials* **2015,** *27* (24), 8387-8397.

31. Chakraborty, I.; Carrington, S. J.; Mascharak, P. K., Design Strategies To Improve the Sensitivity of Photoactive Metal Carbonyl Complexes (photoCORMs) to Visible Light and Their Potential as CO-Donors to Biological Targets. *Accounts of Chemical Research* **2014,** *47* (8), 2603-2611.

32. Zhou, Y.; Cink, R. B.; Dassanayake, R. S.; Seed, A. J.; Brasch, N. E.; Sampson, P., Rapid Photoactivated Generation of Nitroxyl (HNO) under Neutral pH Conditions. *Angewandte Chemie International Edition* **2016,** *55* (42), 13229-13232.

33. Adachi, Y.; Nakagawa, H.; Matsuo, K.; Suzuki, T.; Miyata, N., Photoactivatable HNO-Releasing Compounds Using the Retro-Diels-Alder Reaction. *Chemical Communications* **2008,** (41), 5149-5151.

34. Matsuo, K.; Nakagawa, H.; Adachi, Y.; Kameda, E.; Tsumoto, H.; Suzuki, T.; Miyata, N., Alternative Photoinduced Release of HNO or NO from an Acyl Nitroso Compound, Depending on Environmental Polarity. *Chemical Communications* **2010,** *46* (21), 3788-3790.

35. Hishikawa, K.; Nakagawa, H.; Furuta, T.; Fukuhara, K.; Tsumoto, H.; Suzuki, T.; Miyata, N., Photoinduced Nitric Oxide Release from a Hindered Nitrobenzene Derivative by Two-Photon Excitation. *Journal of the American Chemical Society* **2009,** *131* (22), 7488-7489.

36. Fraix, A.; Sortino, S., Photoactivable Platforms for Nitric Oxide Delivery with Fluorescence Imaging. *Chemistry, an Asian Journal* **2015,** *10* (5), 1116-1125.

37. Nakagawa, H., Photocontrollable Nitric Oxide (NO) and Nitroxyl (HNO) Donors and their Release Mechanisms. *Nitric Oxide* **2011,** *25* (2), 195-200.

38. Devarie-Baez, N. O.; Bagdon, P. E.; Peng, B.; Zhao, Y.; Park, C.-M.; Xian, M., Light-Induced Hydrogen Sulfide Release from "Caged" gem-Dithiols. *Organic Letters* **2013,** *15* (11), 2786-2789.

39. Fukushima, N.; Ieda, N.; Sasakura, K.; Nagano, T.; Hanaoka, K.; Suzuki, T.; Miyata, N.; Nakagawa, H., Synthesis of a Photocontrollable Hydrogen Sulfide Donor using Ketoprofenate Photocages. *Chemical Communications* **2014,** *50* (5), 587-589.

40. Chen, W.; Chen, M.; Zang, Q.; Wang, L.; Tang, F.; Han, Y.; Yang, C.; Deng, L.; Liu, Y.-N., NIR Light Controlled Release of Caged Hydrogen Sulfide Based on Upconversion Nanoparticles. *Chemical Communications* **2015,** *51* (44), 9193-9196.

41. Dormán, G.; Prestwich, G. D., Using Photolabile Ligands in Drug Discovery and Development. *Trends in Biotechnology* **2000,** *18* (2), 64-77.

42. Edler, M.; Mayrbrugger, S.; Fian, A.; Trimmel, G.; Radl, S.; Kern, W.; Griesser, T., Wavelength Selective Refractive Index Modulation in a ROMP Derived Polymer Bearing Phenyl- and ortho-Nitrobenzyl Ester Groups. *Journal of Materials Chemistry C* **2013,** *1* (25), 3931.

CHAPTER VII

1. Zhao, R. Z.; Jiang, S.; Zhang, L.; Yu, Z. B., Mitochondrial Electron Transport Chain, ROS Generation and Uncoupling. *International Journal of Molecular Medicine* **2019,** *44* (1), 3-15.

2. Catling, D. C.; Glein, C. R.; Zahnle, K. J.; McKay, C. P., Why O_2 is Required by Complex Life on Habitable Planets and the Concept of Planetary "Oxygenation Time". *Astrobiology* **2005,** *5* (3), 415-438.

3. Schirrmeister, B. E.; de Vos, J. M.; Antonelli, A.; Bagheri, H. C., Evolution of Multicellularity Coincided with Increased Diversification of Cyanobacteria and the Great Oxidation Event. *Proceedings of the National Academy of Sciences* **2013,** *110* (5), 1791-1796.

4. Zahnle, K.; Schaefer, L.; Fegley, B., Earth's Earliest Atmospheres. *Cold Spring Harbor Perspectives in Biology* **2010,** *2* (10), a004895.

5. Parker, E. T.; Cleaves, H. J.; Dworkin, J. P.; Glavin, D. P.; Callahan, M.; Aubrey, A.; Lazcano, A.; Bada, J. L., Primordial Synthesis of Amines and Amino Acids in a 1958 Miller H_2S-Rich Spark Discharge Experiment. *Proceedings of the National Academy of Sciences* **2011,** *108* (14), 5526-5531.

6. Ranjan, S.; Todd, Z. R.; Sutherland, J. D.; Sasselov, D. D., Sulfidic Anion Concentrations on Early Earth for Surficial Origins-of-Life Chemistry. *Astrobiology* **2018,** *18* (8), 123-1040.

7. Aroca, A.; Zhang, J.; Xie, Y.; Romero, L. C.; Gotor, C., Hydrogen Sulfide Signaling in Plant Adaptations to Adverse Conditions: Molecular Mechanisms. *Journal of Experimental Botany* **2021**.

8. Johnston, D. T.; Wolfe-Simon, F.; Pearson, A.; Knoll, A. H., Anoxygenic Photosynthesis Modulated Proterozoic Oxygen and Sustained Earth's Middle Age. *Proceedings of the National Academy of Sciences* **2009,** *106* (40), 16925-16929.

9. Olson, K. R.; Donald, J. A.; Dombkowski, R. A.; Perry, S. F., Evolutionary and Comparative Aspects of Nitric Oxide, Carbon Monoxide and Hydrogen Sulfide. *Respiratory Physiology & Neurobiology* **2012,** *184* (2), 117-129.

10. Hildebrandt, T. M.; Grieshaber, M. K., Three Enzymatic Activities Catalyze the Oxidation of Sulfide to Thiosulfate in Mammalian and Invertebrate Mitochondria. *The FEBS Journal* **2008,** *275* (13), 3352-3361.

11. Mustafa, A. K.; Gadalla, M. M.; Snyder, S. H., Signaling by Gasotransmitters. *Science Signaling* **2009,** *2* (68), re2.

12. Hartle, M. D.; Pluth, M. D., A Practical Guide to Working with H_2S at the Interface of Chemistry and Biology. *Chemical Society Reviews* **2016,** *45* (22), 6108-6117.

13. Wang, R., Physiological Implications of Hydrogen Sulfide: a Whiff Exploration that Blossomed. *Physiological Reviews* **2012,** *92* (2), 791-896.

14. Toohey, J. I., Sulfur Signaling: Is the Agent Sulfide or Sulfane? *Analytical Biochemistry* **2011,** *413* (1), 1-7.

15. Filipovic, M. R., Persulfidation (S-Sulfhydration) and H_2S. *Handbook of Experimental Pharmacology* **2015,** *230*, 29-59.

16. Filipovic, M. R.; Zivanovic, J.; Alvarez, B.; Banerjee, R., Chemical Biology of H_2S Signaling through Persulfidation. *Chemical Reviews* **2018,** *118* (3), 1253-1337.

17. Yang, C.-t.; Devarie-Baez, N. O.; Hamsath, A.; Fu, X.-d.; Xian, M., S-Persulfidation: Chemistry, Chemical Biology, and Significance in Health and Disease. *Antioxidants & Redox Signaling* **2020,** *33* (15), 192-1114.

18. Corsello, T.; Komaravelli, N.; Casola, A., Role of Hydrogen Sulfide in NRF2- and Sirtuin-Dependent Maintenance of Cellular Redox Balance. *Antioxidants* **2018,** *7* (10), 129.

19. Koike, S.; Ogasawara, Y.; Shibuya, N.; Kimura, H.; Ishii, K., Polysulfide Exerts a Protective Effect Against Cytotoxicity Caused by t-Buthylhydroperoxide through Nrf2 Signaling in Neuroblastoma Cells. *FEBS letters* **2013,** *587* (21), 3548-3555.

20. Tonelli, C.; Chio, I. I. C.; Tuveson, D. A., Transcriptional Regulation by Nrf2. *Antioxidants & Redox Signaling* **2018,** *29* (17), 1727-1745.

21. Yang, G.; Zhao, K.; Ju, Y.; Mani, S.; Cao, Q.; Puukila, S.; Khaper, N.; Wu, L.; Wang, R., Hydrogen Sulfide Protects Against Cellular Senescence via S-Sulfhydration of Keap1 and Activation of Nrf2. *Antioxidants & Redox Signaling* **2013,** *18* (15), 196-1919.

22. Jarosz, A. P.; Wei, W.; Gauld, J. W.; Auld, J.; Özcan, F.; Aslan, M.; Mutus, B., Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) is inactivated by S-Sulfuration In Vitro. *Free Radical Biology & Medicine* **2015,** *89*, 512-521.

23. Zhao, Y.; Pluth, M. D., Hydrogen Sulfide Donors Activated by Reactive Oxygen Species. *Angewandte Chemie International Edition* **2016,** *55* (47), 14638-14642.

24. Zhao, Y.; Bolton, S. G.; Pluth, M. D., Light-Activated COS/H_2S Donation from Photocaged Thiocarbamates. *Organic Letters* **2017,** *19* (9), 2278-2281.

25. Steiger, A. K.; Yang, Y.; Royzen, M.; Pluth, M. D., Bio-Orthogonal "Click-and-Release" Donation of Caged Carbonyl Sulfide (COS) and Hydrogen Sulfide (H_2S). *Chemical Communications* **2017,** *53* (8), 1378-1380.

26. Steiger, A. K.; Marcatti, M.; Szabo, C.; Szczesny, B.; Pluth, M. D., Inhibition of Mitochondrial Bioenergetics by Esterase-Triggered COS/H_2S Donors. *ACS Chemical Biology* **2017,** *12* (8), 2117-2123.

27. Levinn, C. M.; Cerda, M. M.; Pluth, M. D., Activatable Small-Molecule Hydrogen Sulfide Donors. *Antioxidants & Redox Signaling* **2020,** *32* (2), 96-109.

28. Bolton, S. G.; Cerda, M. M.; Gilbert, A. K.; Pluth, M. D., Effects of Sulfane Sulfur Content in Benzyl Polysulfides on Thiol-Triggered H_2S Release and Cell Proliferation. *Free Radical Biology & Medicine* **2019,** *131*, 393-398.

29. Bolton, S. G.; Pluth, M. D., Modified Cyclodextrins Solubilize Elemental Sulfur in Water and Enable Biological Sulfane Sulfur Delivery. *Chemical Science* **2020,** *11* (43), 11777-11784.

30. Gojon, G.; Morales, G. A., SG1002 and Catenated Divalent Organic Sulfur Compounds as Promising Hydrogen Sulfide Prodrugs. *Antioxidants & Redox Signaling* **2020,** *33* (14), 11-1045.

31. Dillon, K. M.; Morrison, H. A.; Powell, C. R.; Carrazzone, R. J.; Ringel-Scaia, V. M.; Winckler, E. W.; Council-Troche, R. M.; Allen, I. C.; Matson, J. B., Targeted Delivery of Persulfides to the Gut: Effects on the Microbiome. *Angewandte Chemie International Edition* **2021,** *60* (11), 6061-6067.

32. Chaudhuri, A.; Venkatesh, Y.; Das, J.; Gangopadhyay, M.; Maiti, T. K.; Singh, N. D. P., One- and Two-Photon-Activated Cysteine Persulfide Donors for Biological Targeting. *Journal of Organic Chemistry* **2019,** *84* (18), 11441-11449.

33. Yu, B.; Zheng, Y.; Yuan, Z.; Li, S.; Zhu, H.; De La Cruz, L. K.; Zhang, J.; Ji, K.; Wang, S.; Wang, B., Toward Direct Protein S-Persulfidation: A Prodrug Approach That Directly Delivers Hydrogen Persulfide. *Journal of the American Chemical Society* **2018,** *140* (1), 30-33.

34. Benchoam, D.; Cuevasanta, E.; Moller, M. N.; Alvarez, B., Hydrogen Sulfide and Persulfides Oxidation by Biologically Relevant Oxidizing Species. *Antioxidants* **2019,** *8* (2), 48.