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A Review of Hydrogen Sulfide (H₂S) Donors: Chemistry and Potential Therapeutic Applications

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Abstract

Hydrogen sulfide (H₂S) is a ubiquitous small gaseous signaling molecule, playing an important role in many physiological processes and joining nitric oxide and carbon monoxide in the group of signaling agents termed gasotransmitters. Endogenous concentrations of H₂S are generally low, making it difficult to discern precise biological functions. As such, probing the physiological roles of H₂S is aided by exogenous delivery of the gas in cell and animal studies. This need for an exogenous source of H₂S provides a unique challenge for chemists to develop chemical tools that facilitate the study of H₂S under biological conditions. Compounds that degrade in response to a specific trigger to release H₂S, termed H₂S donors, include a wide variety of functional groups and delivery systems, some of which mimic the tightly controlled endogenous production in response to specific, biologically relevant conditions. This review examines a variety of H₂S donor systems classified by their H₂S-releasing trigger as well as their H₂S release profiles, byproducts, and potential therapeutic applications.

Graphical abstract

Keywords

Gasotransmitter; self-immolation; carbonyl sulfide; perthiols; cell signaling

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1. Introduction

Hydrogen sulfide (H_2S) has long been known as a foul smelling, toxic gas. Much of the early literature on H_2S focused on its removal from petroleum and pulp products, concerns due in large part to its noxious odor. The presence of H_2S in mammalian tissue has been known for decades, but it was not until the landmark paper by Abe and Kimura in 1996 that the endogenous production and signaling capacity of H_2S was elucidated.[1] Clarification of the cellular signaling mechanisms and biodistribution of its constitutive enzymes led to the induction of H_2S into a family of small signaling molecules known as gasotransmitters. The term gasotransmitter, coined by Wang in 2002, refers to the gaseous nature of these compounds at standard temperature and pressure in the bulk.[2] There are currently three accepted gasotransmitters: carbon monoxide (CO), nitric oxide (NO), and, most recently, H_2S . For a molecule to be considered a gasotransmitter, specific criteria must be met, including: regulated endogenous production, the ability to freely permeate cell membranes, and specific signaling function with specific cellular and molecular targets.[2] The acknowledgement of H_2S as a gasotransmitter has led to a renewed interest in this gas over the past two decades, with a focus on creating chemical tools to probe H_2S physiology, determining its signaling roles in various organs and systems across the plant and animal kingdoms, and exploiting its biological signaling capacity for therapeutic benefits.

1.1. Physical and Chemical Properties of H_2S

H_2S is a colorless, pungent gas with a boiling point of -60°C . [3] A saturated aqueous solution of H_2S has a concentration of 0.11 M at room temperature and pH of ~ 4.0 owing to the acidic nature of H_2S ($\text{p}K_{\text{a}1} = 6.98$). In an unsaturated aqueous H_2S solution at body temperature (37°C), the $\text{p}K_{\text{a}1}$ is 6.76, meaning that roughly 40% of sulfide species in the body exist as H_2S with the remaining population as hydrosulfide anion (HS^-). A very small and likely negligible amount exists as S^{2-} . Unlike water, H_2S does not form hydrogen bonds and is lipophilic, allowing it to pass through biological membranes and act as a paracrine signaling molecule. [4] The hydrosulfide anion can undergo oxidation in the presence of O_2 , forming oxidized sulfide species such as sulfite (SO_3^{2-}), sulfate (SO_4^{2-}), thiosulfate ($\text{S}_2\text{O}_3^{2-}$), polythionates (SnO_{n+2}^-), and polysulfides (S_x^{2-}), as well as other oxidized polysulfide species. [5] It is unclear whether it is H_2S , HS^- , or both that contribute to observed biological activity. [6] To eliminate uncertainty and confusion, in any reference to H_2S hereafter we acknowledge the appreciable presence of both species in biologically relevant media.

2. Endogenous Production of H_2S

Endogenous production of H_2S is a result of direct enzymatic desulfhydration of cysteine, catalyzed by cystathionine- γ -lyase (CSE) and cystathionine- β -synthase (CBS), and indirect desulfhydration catalyzed by 3-mercapto-sulfurtransferase (3-MST) in the presence of reductants.[7] CBS is present mostly in the central nervous system and the liver, while CSE is primarily responsible for H_2S production in the cardiovascular system. 3-MST is located predominantly in the mitochondria and produces H_2S in concert with cysteine

aminotransferase (CAT). [8-12] The pathways for endogenous production of H₂S are outlined in Figure 1.

2.1. CBS

CBS typically catalyzes a pyridoxyl 5'-phosphate (PLP) dependent reaction in which L-homocysteine and L-serine are condensed to form L-cystathionine with the release of water. [10] In the presence of L-cysteine, CBS performs the same β -replacement reaction but releases H₂S instead of water. CBS has very complex roles in the mammalian brain, acting as a powerful neuromodulator. [13, 14] CBS-produced H₂S selectively enhances N-methyl-D-aspartate (NMDA) receptor responses and appears to alter long-term potentiation in the hippocampus. [15] CBS is regulated by testosterone, S-adenosyl-L-methionine, and calmodulin/Ca²⁺-mediated pathways and may be a selective target for novel cancer therapies.

2.2. CSE

CSE catalyzes the PLP-dependent reaction of cysteine and homocysteine to pyruvate and α -ketoglutarate, respectively, resulting in the release of ammonia (NH₃) and H₂S. CSE is located solely in the cytoplasm of cells and is sequestered mostly in the cardiovascular system. Thus, this enzyme is under scrutiny as a possible drug target to treat the millions affected by heart disease. Modulating CSE activity has a profound impact in treating animal models of atherosclerosis and other cardiovascular conditions. [16] Additionally, recent studies suggest that CSE-produced H₂S exerts numerous cytoprotective effects ranging from alleviating ischemia-reperfusion (I/R) injury and attenuating cardiac arrhythmia to reducing myocardial infarction. [17-19] In addition to activity in the cardiovascular system, CSE also contributes to cellular signaling and homeostasis in other organs. For example, Lefer showed that inhibition of CSE attenuated D-galactosamine- and lipopolysaccharide (LPS)-induced liver injury. [20] This attenuation results from increased cellular levels of thiosulfate and homocysteine, upregulation of NF-E2 p45 factor 2 (Nrf2) and antioxidant proteins, among other complex factors.

2.3. 3-MST

Of the three H₂S-generating enzymes, 3-MST has been the least studied thus far. 3-MST catalyzes the conversion of cysteine to pyruvate with the assistance of CAT in a two-step reaction. CAT first catalyzes a transamination reaction to convert L-cysteine and α -ketoglutarate into 3-mercaptopyruvate and L-glutamate, respectively. 3-MST then converts 3-mercaptopyruvate into H₂S and pyruvate using L-cysteine or other biologically relevant thiols. [12] In addition to producing H₂S, 3-MST catalyzes formation of various sulfur oxides (SO_x) in perthiol redox cycles. 3-MST has complex, interconnecting roles with CBS in the brain. [21] Consequently, mutations in 3-MST causing reduced levels of H₂S and SO_x in the brain correlate to behavioral abnormalities and increased anxiety. [22]

2.4. H₂S Signaling Mechanisms

There are three main routes by which H₂S exerts its biological effects: metal center interactions, [23] reactive oxygen species (ROS)/reactive nitrogen species (RNS) scavenging,

[24] and S-persulfidation.[8] Although the first two routes have significance, S-persulfidation is accepted as the key process by which H₂S acts in a signaling capacity. S-Persulfidation (more commonly but less accurately called S-sulfhydration) is the process in which a thiol (R–SH) is converted into a perthiol (R–SSH, also called a perthiol). S-Persulfidation modulates the biological activity of proteins due to the decrease in p*K*_a and increase in nucleophilicity of perthiols with respect to thiols. [25] For example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme whose main functions are in the glycolysis and gluconeogenesis pathways, changes function upon S-persulfidation to inhibit cell apoptosis. [26] Similarly, S-persulfidation in K_{ATP} channels contributes to H₂S-induced vasodilation. [26] Due to the pronounced changes S-persulfidation enacts on proteins and small molecules, there is currently much investigation into the biological roles of perthiols and polysulfides. [27]

3. Chemical Tools for Studying H₂S

As interest in the physiological roles of H₂S has grown, the need for chemical tools used for studying H₂S has also increased. These tools include H₂S probes, which are molecules capable of responding to H₂S typically by changing their spectroscopic properties, CSE and CBS inhibitors, which reduce or eliminate endogenous H₂S production, and H₂S donors, which are molecules designed to release H₂S under specific conditions. For a review on compounds and methods used for detection of H₂S, we refer the reader to recent reviews on these topics.[28-30] We also refer the reader to reports on recent efforts to increase the selectivity and potency of CBS and CSE inhibitors.[31, 32] Here we focus solely on H₂S donors. To study H₂S in a physiologically relevant manner, donors with variable release rates and triggers are needed. This review is not a comprehensive look at H₂S donors, but rather a critical examination of recent developments in H₂S donor chemistry, highlighting specific examples of donors and discussing important considerations in designing and choosing donors for biological studies.

4. Direct Delivery of H₂S

4.1. Inhalation

When considering exogenous delivery of gasotransmitters, the most conspicuous method of delivery is inhalation of the gas directly. Inhalation offers the possibility to tune total payload delivery by carefully modulating pressure over time, and it circumvents some of the issues with intravenous delivery such as oxidation and volatilization of dissolved H₂S. Despite the difficulties in working with toxic and flammable H₂S gas directly, this delivery mode has been tested. For example, exogenous systemic delivery of H₂S via inhalation at 80 ppm for 3 h or greater in mice showed a reversible decrease in motor activity and body temperature with a corresponding increase in blood sulfide concentration.[33, 34] These studies led to the use of inhaled H₂S in hypothermic and normothermic mice as a method of inhibiting pulmonary and systemic inflammatory responses during physiological stress.[35, 36] However, H₂S inhalation has some inherent shortcomings due the toxicity and flammability of the gas, particularly gas storage, safe administration, and targeting. These

technical difficulties limit the overall impact of inhalation as a viable method of H₂S delivery, and few researchers are currently actively examining this delivery strategy.

4.2. Sulfide Salts

The most common class of H₂S donors employed in biological studies are the sulfide salts, sodium hydrosulfide (NaSH) and sodium sulfide (Na₂S). Although commonly referred to as donors, sulfide salts are simply solid analogs of the gas, providing direct, instantaneous access to the biologically relevant forms of sulfide (H₂S and HS⁻). Use of sulfide salts has been integral in the establishment of H₂S as a gasotransmitter, and these salts have been widely used to evaluate the therapeutic potential of exogenous H₂S delivery.

One of the early studies on exogenous H₂S delivery by Wang et al. employed aqueous NaSH solutions in evaluating rat aortic ring response in vitro.[37] NaSH delivery led to a 60% greater relaxation over controls, showing the vasorelaxant properties of H₂S. In a separate study by Du et al., delivery of NaSH solution via intravenous injection to rats with oleic acid-induced acute lung injury (ALI) alleviated the degree of ALI by decreasing IL-6 and IL-8 levels while simultaneously increasing IL-10 levels in the plasma and lung tissue.[38] This study also verified the hypothesis that down-regulation of endogenous H₂S levels in the cardiovascular system is involved in ALI pathogenesis.

Sulfide salts as H₂S donors have also exhibited efficacy in limiting cell damage from ROS in brain cells. Delivery of NaSH was tested as an antioxidant in an in vitro study where oxidative stress was induced in human neuroblastoma cells using hypochlorous acid (HOCl) to mimic HOCl overproduction from myeloperoxidase in the brain of patients with Alzheimer's disease. Exogenous NaSH inhibited protein oxidation, lipid peroxidation, and overall cytotoxicity.[39] In a hepatic I/R injury model, delivery of Na₂S (up to 1.0 mg/kg) prior to reperfusion inhibited lipid peroxidation and preserved a healthy balance of reduced glutathione (GSH), overall providing protection against I/R injuries.[40] Aqueous solutions of sulfide salts have also demonstrated efficacy in promoting ulcer healing,[41, 42] as well as quenching RNS.[43, 44]

While sulfide salts are a popular choice amongst biologists interested in elucidating endogenous roles and therapeutic prospects of H₂S, there are drawbacks to these H₂S donors both as chemical tools for studying H₂S biology and as potential therapeutics. Sulfide salts hydrolyze immediately upon dissolution in water, instantaneously establishing the equilibrium between H₂S, HS⁻, and S²⁻ species. Once this equilibrium is established, volatilization of H₂S occurs, lowering the overall concentration of sulfur species in solution. Additionally, air oxidation of HS⁻ catalyzed by trace metals in water further reduces the actual concentration of H₂S in solution.[6] These competing processes make it challenging to deliver a reproducible amount of H₂S via sulfide salts. In addition, sulfide salts lack targeting capabilities and thus are only of utility in systemic delivery. Finally, studies on H₂S biology using sulfide salts frequently require administration of high doses, causing H₂S blood and tissue concentrations to surge to supraphysiological levels and then drop rapidly. This delivery method lies in stark contrast to the endogenous production of H₂S, in which levels are tightly regulated. These shortcomings have led to the search for H₂S donors that give researchers the ability to control the dose, duration, timing, and location of release.

5. Measuring H₂S Release Kinetics

Aside from the direct sources of H₂S, all other donors must undergo some type of chemical reaction to release H₂S. Release may be triggered by water, light, a nucleophile such as a thiol, the action of an enzyme, or other stimuli. Measuring the kinetics of these reactions can be difficult and has been handled in several different ways by the community of researchers developing H₂S donors. Only in a few cases have rate constants been determined, making it difficult to compare release rates across different donor classes. Instead, most researchers compare different compounds within a specific class to assess structure-property relationships.

One common method of assessing release kinetics is to use an H₂S-selective electrochemical probe. These instruments provide a real-time analysis of H₂S concentration in solution, with release data taking the form of a curve approximating an inverted, extended U-shape. Most authors report both peaking time and peak concentration, marking the top of the inverted U-shape, or a peaking half-time, marking the time it takes to reach half of the peaking concentration. It is important to realize that this type of analysis does not enable calculation of a rate constant because the rates of H₂S loss due to volatilization, oxidation, and other reactions are unknown, and all of these reactions contribute to the shape of the curve. Additionally, peaking times and concentrations depend on the concentrations of the reactants. As a result, these types of peaking time/concentration data can only be used to compare across different donors that have been analyzed under the same conditions. These data may not reflect how donors would react in the bloodstream, where rapid dilution would alter their release profile.

Another strategy used to assess release kinetics is the methylene blue method, first reported by Fischer in 1883 and expounded upon by Siegel in 1965.[45, 46] Reaction of sulfide species in an acidic aqueous solution of *N,N*-dimethylphenylenediamine and iron (III) chloride (FeCl₃) leads to the formation of methylene blue. Siegel reported that methylene blue formation, monitored by readout of the absorbance at 650 nm, showed a linear relationship to concentration of Na₂S in solution from 0–80 μM. Methylene blue is a commonly employed assay in determining H₂S release from synthetic donors, allowing for the determination of release kinetics because cumulative release can be measured.

Close examination of the methylene blue method under the aforementioned conditions shows that methylene blue does not obey Beer's Law at concentrations greater than 1 μM due to the formation of dimer and trimer species.[6, 47] Additionally, formation of methylene blue occurs in the presence of a variety of sulfur species, including thiols, a common trigger of synthetic H₂S donors, further complicating analysis.[46] Therefore, control experiments must be conducted to properly account for methylene blue derived from the H₂S donor of interest compared with other potential sources. H₂S release half-lives determined by the methylene blue assay may not be comparable between various H₂S donor systems, but this method still has utility in direct comparisons between similar donors under identical experimental conditions.

H₂S-selective fluorescent probes are also routinely used in the detection and quantification of H₂S release kinetics. Many of these probes rely on the selective reduction of aryl or sulfonyl azides to amines by HS⁻ in a “turn on” fluorescence mechanism. One of the first examples of a probe based on the reduction of an aryl azide was from Chang and coworkers, who demonstrated the use of an aryl azide in imaging H₂S in cells.[48] Similarly, Wang and coworkers reported the synthesis of a reduction-based probe based on the prevalent dansyl fluorophore building block, a popular option amongst chemists for its relative ease of synthesis.[49] Probes that rely on the nucleophilicity of H₂S have also been reported in the literature and are typically used for quantification of sulfide in solution.[50-52] For a more comprehensive discussion on fluorometric determination of sulfide in solution, the reader is referred to a recent review on the topic.[53] Lastly, researchers have derivatized various forms of sulfide in solution using monobromobimane, which appears to give a more accurate quantification of sulfide in solution compared to the previously discussed methods.[54]

6. Naturally Occurring Donors

Consumption of garlic and onions is recognized as beneficial for prevention or treatment of cardiovascular disease, hypertension, thrombosis, and diabetes.[55] While there is still debate over which ingredients in garlic contribute to the overall health benefits attributed to consumption, H₂S-releasing compounds from garlic extract appear to be important components. The isolated H₂S-releasing compounds from garlic are byproducts of the breakdown of thiosulfonates (R-SO₂-SR).[56] Allicin, the most common of the thiosulfonates, decomposes into diallyl disulfide (DADS), diallyl sulfide (DAS), and diallyl trisulfide (DATS).[57] Subsequent research by Kraus et al. on DADS, DAS, and DATS revealed that human red blood cells converted these compounds into H₂S in the presence of free thiols and that treatment of aortic rings with these compounds led to vasorelaxation, similar to experiments using sulfide salts.[58] Linear and cyclic di-, tri-, and higher order polysulfide species have also been isolated from a variety of other plants but have not been as extensively studied as the aforementioned garlic-derived donors.[59]

Many of the studies concerning the garlic-derived H₂S donors have focused on the mechanisms by which the donors release H₂S in the presence of thiols. Of the three allicin-derived H₂S donors, Kraus showed that DATS, the compound with the most sulfur atoms, led to substantial H₂S generation upon reaction with naturally occurring thiols, including GSH, cysteine, homocysteine, and *N*-acetylcysteine.[58] Little H₂S was released from DADS and DAS, consistent with recent computational results indicating that DADS is a much poorer H₂S donor than DATS. [60] Of the thiols tested, treatment with GSH resulted in the greatest amount of H₂S release from all three allicin-derived species. Mechanistically, H₂S release may occur upon nucleophilic addition of a thiol to one of the alpha carbons of DATS, DADS, or DAS, as well as a sulfur atom within the polysulfide species, leading to the conclusion that the allyl substituent may influence rate of release. Treatment of rat aortic rings with the garlic-derived donors (100 μM) showed an increase in relaxation over addition of Na₂S (20 μM).

Naturally occurring H₂S donors may be attractive options for biologists carrying out in vivo studies with an eye toward clinical relevancy. This class of donors does not have some of the

toxicity concerns that accompany many synthetic donors, and some natural H₂S donors are commercially available. The limitations to the use of DATS, DADS, and DAS are that they are not structurally amenable to chemical transformations, have poor water solubility, and generate various byproducts after H₂S release. Additionally, isolation of these compounds from the milieu of garlic may reduce the desired physiological effects upon administration. These drawbacks make it challenging to employ these compounds in vitro and in vivo to improve our understanding of the complicated interplay between H₂S and other signaling compounds. Synthetic donors that provide tunable H₂S release rates via structural modification with discrete byproducts allow for a more in-depth analysis of the physiological roles of H₂S and, optimistically, clinically relevant H₂S-releasing prodrugs.

7. Synthetic H₂S Donors

7.1. Considerations for Synthetic Donors

It is useful to consider important characteristics in H₂S donors for use both as biological tools and as potential therapeutics. Of course, there is not a single ideal donor; for example, there are situations where a fast donor may be required and others where slow release is best. Sometimes release immediately upon dissolution in water may be useful, while in other cases release in response to a specific external or internal stimulus would be ideal. Despite these variations, a few characteristics are broadly desirable. H₂S donors should be water-soluble, stable under storage conditions, and generate only innocuous (if any) byproducts. They should also have a specific and well-defined release mechanism (i.e., release only in response to a specific nucleophile, a specific wavelength of light, or a specific enzyme). Many of the H₂S donors in the literature achieve a portion of these criteria but are lacking in some aspect. Nevertheless, there are H₂S donors that have been, or are currently, under evaluation in clinical trials. A few notable examples include the naproxen-based H₂S donor ATB-346 (Antibe Therapeutics), which exhibits anti-inflammatory effects; [61] GIC-1001 (Gicare Pharma Inc.), an orally administered trimebutine maleate salt H₂S donor used as an alternative to sedatives in colonoscopies; [62] and SG-1002 (SulfaGENIX), which is >90 % α -sulfur with the remainder being oxidized sulfur species, and was evaluated in an investigation aimed at increasing circulating H₂S and NO levels after heart failure.

As the field of H₂S donor chemistry continues to grow, more opportunities will ultimately arise for clinical examination of these compounds in various disease indications. In the remainder of this review, we aim to critically examine the current literature of synthetic H₂S donors. At the beginning of this decade, this would have been a very short list, but this field has grown rapidly in recent years. Here we discuss reported H₂S donors, categorized by their class of triggering mechanisms, as well as offer insight into potential future directions of the field.

7.2. Hydrolysis Triggered Donors

7.2.1. Lawesson's Reagent and Derivatives—Lawesson's reagent (LR) is a popular reagent for the thionation of ketones, esters, amides, and alcohols to the corresponding sulfur analogs. [63] LR is commercially available, making it another popular choice for groups studying H₂S physiology. LR releases H₂S in aqueous media over a much longer

period than sulfide salts, although reports on detailed release kinetics are sparse. In a study by Medeiros and coworkers, oral administration of LR prior to alendronate-induced gastric damage limited subsequent gastric impairment compared to controls.[64] The authors noted an increase in GSH levels in rats treated with LR, which they attributed to the relief of oxidative stress and inhibition of neutrophil infiltration. In another in vivo study employing LR as an H₂S donor, Cunha et al. demonstrated that exogenous H₂S delivery improved leukocyte adhesion and neutrophil migration to the site where sepsis was induced in mice, improving the overall survival probability of the infected mice.[65] The authors also noted that reducing endogenous H₂S production using a CSE inhibitor increased the mortality rate of the mice over the duration of the experiment. Despite these successes, LR has not been widely employed as an H₂S donor in large part due to its lack of water solubility. It also suffers from a release mechanism that is not well understood and an apparently very slow H₂S release rate.

GY4137 is a water-soluble derivative of LR, which also releases H₂S via hydrolysis.[66] Originally prepared as a vulcanization agent for rubber in 1957 (U.S. Pat. No. 2,954,379, filed 1957), GY4137 is readily accessed by stirring LR with morpholine at room temperature. In a direct amperometric comparison of H₂S release rates from GY4137 and NaSH, GY4137 released H₂S with a peaking time of ~10 min versus ~10 seconds for NaSH in phosphate buffer pH 7.4.[66] However, the peaking concentration for GY4137 was ~40-fold lower than for NaSH even with a 10-fold higher concentration of GY4137 used in this study. Incubation of GY4137 in phosphate buffer (pH 7.4 or 8.5) showed a sustained release for over an hour as determined spectrophotometrically, with an increase in release rate at lower pH. Deng et al. later noted that GY4137 maintained H₂S levels in cell culture above baseline for over 7 days,[67] but these results were obtained using the methylene blue assay, which cannot specifically provide evidence for H₂S over other sulfur-containing species and can provide spurious results in assays conducted in vitro and in vivo. [6] Based on these and other results, GY4137 is generally regarded as a slow-releasing H₂S donor. Intravenous or intraperitoneal injection (ip) of aqueous solutions of GY4137 (133 μmol/kg) in rats showed an increase in plasma sulfide levels after 30 min, which was sustained out to 2 h.[66] However, in this study sulfide levels were again measured by the methylene blue assay. GY4137 (200 μM) treatment of isolated rat aortic rings showed a later onset, but more sustained relaxation of the aortic ring compared to NaSH (300 μM). Similarly, treatment of anesthetized rats with both GY4137 and NaSH showed similar effects on blood pressure. However, GY4137 caused a slower onset but a more prolonged decrease in blood pressure while treatment with NaSH elicited only a transient decrease.

Due to its commercial availability and ease of handling, GY4137 is the most widely studied H₂S donor aside from sulfide itself. In addition to its cardiovascular effects, GY4137 has also shown efficacy in killing several types of cancer cells (HeLa, HCT-116, Hep G2, MCF-7, U2OS) in vitro, with selectivity over normal (non-cancerous) cells.[67] However, very high doses (800 μM) were used in these studies, and a 5-day incubation period was used due to the slow-releasing nature of this donor. In the same cell lines, NaSH and a non-H₂S releasing GY4137 control compound show diminished and no efficacy in cancer cell killing, respectively. The authors attribute the efficacy of GY4137 over NaSH in inducing cancer cell death to the sustained H₂S release profile.

Xian and coworkers developed a GYY4137 analog via substitution of the P–C bond in GYY4137 with a P–O bond.[69] These *O*-substituted phosphorodithioates were synthesized in four steps, generating the *O*-aryl and alkyl substituted 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) salts. H₂S release kinetics, as measured by a dansyl azide “turn-on” fluorescent probe, showed comparable kinetics to GYY4137 for the *O*-aryl phosphorodithioates with less than 1 % of total H₂S released after 3 h, while the *O*-alkyl phosphorodithioates did not show any H₂S release within this time frame. Initial studies on H9c2 cardiomyocyte viability under H₂O₂-induced oxidative stress after incubation with the *O*-aryl phosphorodithioate donors (50 and 100 μM) showed enhanced viability compared to untreated controls, although the difference was not statistically significant over GYY4137. When tested on B16BL6 mouse melanoma cell lines, the *O*-aryl phosphorodithioate donors showed decreased cell viability over untreated controls (~60 % relative to control) after 4 days. GYY4137 showed a >90 % killing of these cells relative to controls over the same time scale, indicating that the potency of GYY4137 may lie in the P–C bond that was removed in the *O*-aryl phosphorodithioates. These results provide evidence that factors beyond H₂S release may cause the biological effects attributed to GYY4137, perhaps stemming from some of the issues with GYY4137 discussed above.

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proline ring in the donor, which was corroborated by density functional theory calculations. The JK donors (25 and 50 μM) showed efficacy in reducing cellular damage resulting from anoxia/reoxygenation (A/R) treatment with H_2O_2 in vitro. The donors were also successful in reducing infarct size per area-at-risk via intracardiac injection in mice in an I/R model. Further investigation of phosphorodithioate donors in vivo involved doping poly(caprolactone) (PCL) fibers with the small molecule donor and examining the effects on a cutaneous wound model in mice.[71] The doped PCL fibers showed an extended H_2S release profile over the small molecule in solution, which is expected for a hydrolysis-triggered donor, and showed enhanced wound healing times over the non-doped PCL fiber control. From an application standpoint, these donors may be useful in treating diseases such as cancer, where pH differences exist between healthy and diseased tissue.

7.2.2. Dithiolthiones—1,2-Dithiole-3-thiones (DTTs) are a class of compounds also commonly considered to be in the family of hydrolysis-triggered H_2S donors. Made by the reaction of anethole with elemental sulfur, DTTs are easy to synthesize and can be readily attached to other molecules to make drug-DTT conjugates. However, this class of H_2S donors has two problems that must be considered when interpreting biological data. First, whether DTTs release significant amounts of H_2S under physiological conditions is unclear. Williams and coworkers showed that a substituted DTT hydrolyzed cleanly, with the thione species being converted into a carbonyl.[72] However, complete hydrolysis required 48 h at 120 $^{\circ}\text{C}$ in a DMSO/ H_2O mixture. The authors noted that hydrolysis under physiological conditions was very slow and did not present any data at 37 $^{\circ}\text{C}$. However, they did observe activity of several DTTs as COX-1 and COX-2 inhibitors, with less potency noted for the hydrolyzed DTTs against both targets. Second, it is also important to mention that anethole trithione (ADT), a DTT compound that is frequently derivatized and attached to other drugs to make H_2S -donating versions of these drugs, itself has biological activity. ADT is an FDA-approved bile secretion-stimulating drug that restores salivation and relieves dry mouth in chemotherapy-induced xerostomia.[73] The mechanism of action is unknown. Several groups have studied ADT and other DTTs and observed potent biological activity that they attribute to the H_2S -donating ability of these drugs. For example, the phenol derivative of ADT, commonly denoted as ADT-OH, reduces cell viability via inhibition of histone deacetylase[74, 75] and NF- κB activation.[76] Interestingly, a study by van der Vlies and coworkers demonstrated that conjugation of ADT-OH to poly(ethylene glycol) altered the cellular uptake mechanism and largely eliminated cytotoxicity at concentrations below 200 μM . [77] The reader is cautioned that in many papers reliable measurements of H_2S generation were not reported, and that often no control experiments were reported to rule out the possibility that the observed effects were due to the biological activity of DTTs themselves.

The DTT moiety has been appended to non-steroidal anti-inflammatory drugs (NSAIDs) and studied rather extensively. The first example of a DTT-NSAID was a diclofenac derivative published in 2007 by Moore and coworkers, termed *S*-diclofenac.[78] The DTT moiety in *S*-diclofenac was linked to the NSAID through an ester bond that released diclofenac and DTT upon esterase-catalyzed hydrolysis. H_2S release experiments in PBS buffer at room temperature showed no H_2S release, consistent with results noted above. Addition of rat

liver homogenates showed a minimal amount of H₂S release from both ADT-OH itself and *S*-diclofenac, determined to about 5 % of the theoretical maximum H₂S release for both donors after 30 min, as measured by an electrochemical probe. When examining plasma H₂S concentrations in live rats 6 h after ip injection, *S*-diclofenac-treated animals had a higher plasma H₂S concentration than the sulfide salt control, measured to be ~35 μM. As this value is well beyond currently accepted ranges for plasma H₂S concentration (below 1 μM), it is likely that the method used here generated H₂S from many sources that were erroneously attributed to free H₂S in plasma. Although the amount of H₂S generated in vivo from *S*-diclofenac is unclear, it is clear that it showed efficacy in several animal models, including models showing dose dependent responses towards inflammation[79, 80] and ulcerative colitis.[81]

More recently, a DTT derivative with a mitochondria-targeting triphenylphosphonium bromide tail was reported and named AP-39.[82] H₂S release from AP-39 in mouse brain endothelial cells was monitored using an azide-based fluorescent H₂S probe, with increased probe fluorescence localized to the mitochondria. Very little if any increase in fluorescence was observed from ADT-OH alone. These results indicate that AP-39 does indeed target the mitochondria, resulting in an increase in H₂S production. Whether H₂S is generated directly from DTT itself or as a result of increased enzymatic H₂S production is unclear. Regardless of the mechanism of action, AP-39 exhibited antioxidative activity, helping to maintain cell homeostasis by suppressing the initiation of mitochondrial cell death pathways.

All of the DTT compounds noted above linked the DTT unit to the drug using an ester bond. Pluth and coworkers recently synthesized DTT-NSAID conjugates via an amide linkage, which is more stable to hydrolysis than the corresponding ester.[83] The authors demonstrated the enhanced hydrolytic stability of the amide linkage via HPLC and UV-vis spectroscopy; however, no studies have been completed in a biological context to date. The authors also did not report on H₂S release from these compounds. It is possible that the hydrolytically robust amide linkage would increase the stability of H₂S-donating NSAIDs in the acidic environment of the stomach.

A notable compound in the DTT-NSAID class of donors is “NOSH aspirin.” NOSH aspirin is an aspirin derivative with a DTT moiety as well as a nitrate group capable of releasing NO.[84] Remarkably, NOSH aspirin showed >100,000 times the potency of aspirin alone after 48 h and approximately 250,000 times the potency of aspirin alone after 72 h in a human colon cancer cell line, with an IC₅₀ value of 50 nM. Interestingly, when examining cell growth inhibition in the presence of the three individual components of NOSH aspirin (ADT-OH, a small molecule NO donor, and aspirin), the cocktail had an IC₅₀ of 450 μM, a 9,000-fold difference compared with intact NOSH-aspirin. These results indicate that cancer cell growth inhibition is influenced by more than simply delivering DTT and NO concurrently with aspirin, but the reasons for this synergy remain unknown. NOSH aspirin has also been studied in vivo, with a mean reduction of 85 % in tumor size after daily treatment for 18 days (100 mg/kg) compared to untreated controls. Additional in vitro studies on NOSH-aspirin in SH-SY5Y human neuroblastoma cells showed enhanced anti-inflammatory capabilities over both an H₂S-releasing aspirin derivative and an NO-releasing aspirin derivative, suggesting that NOSH-aspirin may be an effective treatment in brain

injury.[85] NOSH-aspirin represents a promising area in conjugating NSAIDs to H₂S donor moieties in an effort to generate a concerted effect through combined delivery via clever chemistry.

7.3. Thiol-Triggered Donors

7.3.1. N-Benzoylthiobenzamides—Thiol-triggered H₂S donors were some of the first synthetic donors to be reported and are the most common class of non-hydrolysis-triggered synthetic donors. Thiol triggered donors are advantageous in that free thiols are relatively abundant nucleophiles in mammals and offer a platform from which thiol exchange can be used to accomplish H₂S release after nucleophilic addition. A series of *N*-(benzoylthio)benzamides reported by Xian et al. were among the first nucleophile-triggered H₂S donors.[86] Synthesized from substituted thiobenzoic acids, several *N*-benzoylthiobenzamides were evaluated for H₂S release, with a variety of release rates observed. The hypothesized thiol-triggered mechanism of release was confirmed with the formation of cystine, *N*-acetylcysteine, and benzamide in high yields. In cell studies, a selected *N*-(benzoylthio)benzamide protected human keratinocytes against methylglyoxal (MGO)-induced cell damage and dysfunction, a prevalent issue in diabetics.[87] These donors have also been evaluated in animal models of myocardial I/R injury, displaying a reduction in infarct size over controls in murine models, indicating a cardioprotective effect.[88]

In addition to small molecule applications, the therapeutic effects of these donors were also evaluated in polymer templates for tissue engineering applications. Wang et al. accomplished this by electrospinning PCL solutions containing various *N*-(benzoylthio)benzamide donors to make fibrous polymeric scaffolds doped with the small molecule donor.[89] Donor-loaded PCL fibers led to increased expression of collagen type I and III in wound healing models in mice, demonstrating the potential for H₂S-releasing materials in treating chronic wounds.

7.3.2. Acyl Perthiols, Dithioperoxyanhydrides, and Polysulfides—Another class of thiol-triggered donors, which ultimately require disulfide exchange to promote H₂S release, are the acyl perthiol donors. Acyl perthiol donors (RC(O)–S–SR') were first synthesized by Xian, where the R group is derived from penicillamine.[90] The donors were prepared from thiobenzoic acid derivatives and *N*-benzoyl cysteine methyl ester in two steps. These donors showed tunable release rates by varying the aromatic R substituent. H₂S release depended on both steric and electronic factors, with electron-withdrawing substituents accelerating release rates, and bulky substituents on the aromatic ring retarding release rates. These donors released H₂S over the course of minutes to hours under the conditions tested.

Analogous to Xian's acyl perthiol donors are the dithioperoxyanhydride class of donors (RC(O)–S–SC(O)R') reported by Galardon et al.[91] Both alkyl and aromatic dithioperoxyanhydride donors were readily prepared in one reaction step involving thiobenzoic acid and methoxycarbonylsulfonyl chloride (CH₃OC(O)SOCl) with fair overall yields. The donors showed similar levels of H₂S released when triggered with both cysteine

and GSH and slower release rates when treated with GSH, measured amperometrically. The compounds were non-cytotoxic at concentrations up to 200 μM in human fibroblasts and induced vasorelaxation in rat aortic ring models.

The final class of donors based on the breaking of an S–S bond are the tetrasulfide donors developed by Pluth et al.[92] This relatively simple design involves the treatment of a range of thiols with sulfur monochloride (S_2Cl_2) to generate the corresponding tetrasulfides ($\text{R}-(\text{S})_4-\text{R}$). Aromatic tetrasulfides released H_2S faster than their alkyl counterparts when triggered by GSH, likely as a result of the change in electrophilicity of the α/β sulfur. Interestingly, a trend in H_2S release rate was not observed when altering ring electronics, suggesting that other factors influence H_2S release from this system. Analogous to the observations of the amount of H_2S released for the natural donors DADS and DATS, the tetrasulfide donors released more H_2S than DATS, which was used in this study as a model donor.

7.3.3. Arylthioamides—Arylthioamides ($\text{ArC}(\text{S})-\text{NH}_2$) are a versatile class of donors that was first reported by Calderone and coworkers.[93] In this work, a series of twelve arylthioamides were synthesized either from substituted arylcyano compounds in two steps or via the direct thionation of select aryl amides using LR. All donors released H_2S in response to cysteine as measured amperometrically. Release studies were conducted at high concentrations of donor and thiol (1 mM and 4 mM, respectively), leading to rapid peak in release profile for all donors. The arylthioamides released only small amounts of H_2S , exhibiting maximum concentrations (C_{max}) between 3–21 μM . The fast rise to a steady state concentration for all the donors tested make these donors appear to be fast-releasing compounds; however, this quick rise to C_{max} in solution is misleading as the max H_2S concentration is a small fraction of the total available H_2S from the donors. The arylthioamides showed similar H_2S release profiles to DADS and GYY4137, two slow-releasing donors, when measured under the same experimental conditions. Interestingly, some of the donors released H_2S in the absence of a thiol trigger, indicating that they may also be hydrolysis-triggered. Alterations in ring electronics modulated release rates, but not in a predictable pattern. One donor, *p*-hydroxybenzothioamide, was evaluated in vitro in a rat aortic ring contraction study and completely abolished vasoconstriction at 1 mM in the presence of noradrenaline (NA) without the addition of exogenous cysteine; suggesting that biologically relevant levels of cysteine triggered sufficient H_2S release, at least at this high donor concentration. This donor was also evaluated for its effect on hyperpolarization in human vascular smooth muscle cells (HASMCS), showing a similar ability to hyperpolarize HASMCs at 300 μM compared with 1 mM NaSH, suggesting that slow, sustained levels of H_2S from *p*-hydroxybenzothioamide may be more effective than high instantaneous concentrations of H_2S .

Due to the observed slow, sustained H_2S release profile of *p*-hydroxybenzothioamide and ease of conjugation to other compounds, other researchers have combined this donor with polymers and other drugs as conjugates.[61] For example, Bowden et al.[94] synthesized statistical copolymers containing L-lactide and a *p*-hydroxybenzothioamide lactide derivative and showed release of the donor in the timescale of days to weeks, although no studies of H_2S release were included in this report.[94] In another example, the development

of a naproxen-hydroxybenzothioamide conjugate, ATB-346, was described.[61] In the initial report, its efficacy as an anticancer drug was investigated, revealing that it induced apoptosis in human melanoma cells in animal studies. ATB-346 has also shown efficacy in reducing gastrointestinal tract injury while maintaining utility as a chemopreventative agent against colorectal cancer when compared to naproxen.[95] Further studies on ATB-346 are underway by Antibe Therapeutics, where a phase II study was completed in 2016 for pain associated with osteoarthritis.

7.3.4. S-Aroylthiooximes—*S*-Aroylthiooximes (SATOs) are a class of thiol-triggered donors developed by Matson and coworkers.[96] SATOs (ArC(O)-S-N=CR_2) are synthesized by condensation of an aryl aldehyde or ketone and an *S*-aroylthiohydroxylamine (SATHA, ArC(O)-S-NH_2) in the presence of catalytic acid, analogous to oxime formation. A variety of substituted small molecule SATOs were synthesized, varying both the substituent on the SATHA component and the aldehyde or ketone. SATOs released H_2S in the presence of cysteine and other thiols but did not show release in the presence of amines or water alone. H_2S release was measured amperometrically as well as with the methylene blue method. A definitive electronics trend correlating the substituent on the SATHA ring with H_2S release was observed by fitting release half-lives as measured by methylene blue to a Hammett plot. Under the conditions tested, H_2S release half-lives ranged over an order of magnitude, from minutes to hours.

The modularity of the SATO formation reaction has allowed for SATOs to be extended to macromolecular systems.[97] Poly(SATOs) were synthesized via post-polymerization modification of functionalized polymethacrylates containing pendant aryl aldehydes with substituted SATHAs. H_2S release rates were slower in the polymer system than in analogous small molecules, and electronic effects were consistent with those observed in the initial small molecule SATO study. Extending the poly(SATO) system further, amphiphilic block copolymers containing SATO functional groups as the hydrophobic block were synthesized for studies on self-assembled polymer micelles for H_2S delivery.[98] The SATO micelles released H_2S over the course of several hours, approximately an order of magnitude slower than analogous small molecule SATOs under the same conditions. Treatment of HCT116 colon cancer cells with SATO micelles ($[\text{SATO}] = 250 \mu\text{M}$) reduced colon cancer cell viability to a greater degree than Na_2S , GYY4137, and a small molecule SATO at the same concentration, contributing further evidence suggesting that kinetics of H_2S release affects therapeutic potency.

In another example of the modularity of SATO chemistry, an amphiphilic peptide with the sequence IAVEEE was modified by appending an aryl aldehyde to the N-terminus.[99] The unsubstituted SATHA was conjugated to the modified peptide to form a SATO-based aromatic peptide amphiphile. The SATO aromatic peptide amphiphiles self-assembled in aqueous media to form nanofibers that gelled in the presence of calcium to afford a hydrogel using just 1 wt.% peptide. These peptide-based H_2S donors exhibited sustained H_2S release in the gel state with a peaking time of ~ 120 min and detectable H_2S out to 15 h in PBS buffer in the presence of cysteine, as measured amperometrically. In vitro studies using mouse brain endothelial cells showed minimal toxicity of the gels, a promising result for future in vivo studies as these gels show great potential for localized H_2S delivery.

7.4. Light Triggered Donors

7.4.1. Geminal-dithiols—Light-triggered prodrugs are useful tools for studies in vitro and hold promise as potential therapeutic candidates due to the bioorthogonality of visible light as a trigger. Light has an advantage over other triggers because it can affect H₂S release without perturbing any native biochemical processes, albeit only in areas of the body where sufficient light penetration is possible. Light-triggered prodrugs are ideal in applications where tissue-specific delivery is required. After prodrug administration, light of a particular wavelength can trigger release at the site of interest, minimizing off-target effects through direct spatial and temporal control over release.

One of the first examples of light-triggered H₂S donors was reported by Xian et al. in the form of geminal dithiols (ArCH₂–S–C(CH₃)₂–S–CH₂Ar). Gem-dithiols were prepared by treating *ortho*-nitrobenzylthiol or related derivatives with acetone and catalytic amounts of titanium(IV) chloride (TiCl₄) to bridge two thiols together via a thioacetal linkage.[100] The *ortho*-nitro group underwent cleavage when irradiated with UV light (365 nm), which has been demonstrated in a variety of systems,[101] to produce a geminal dithiol intermediate. This intermediate hydrolyzed to yield H₂S relatively rapidly. All of the donors released their full payload within ~30 min. Control experiments showed that no H₂S was released in the absence of UV light. Because hydrolysis of gem-dithiols is acid-catalyzed, H₂S release was accelerated at low pHs and retarded at higher pHs. A variety of other gem-dithiol derivatives were also prepared by varying the bridging group between thiols. In general, alkyl derivatives released H₂S with profiles similar to the model compound, but aryl variants exhibited much slower release by decreasing the rate of hydrolysis, most likely due to changes in both sterics and electronics as a result of adding an aromatic ring to the bridging group.

7.4.2. Ketoprofenate Photocages—Another example of a light-triggered H₂S prodrug is the ketoprofenate donor reported by Nakagawa et al.[102] Synthesis of this donor was accomplished in three synthetic steps. Upon irradiation by UV light, the donor released two equiv of 2-propenylbenzophenone and carbon dioxide (CO₂), along with one equiv of H₂S. To determine H₂S release behavior in a biological system, this donor was examined in a solution containing fetal bovine serum. No H₂S was detected without UV irradiation; however, 30 μM H₂S was detected from 500 μM donor after irradiation for 10 min, as measured by the methylene blue assay.

7.4.3. α-Thioetherketones—In addition to small molecules, polymeric light-triggered H₂S donors have been reported. Connal and coworkers developed a prodrug that incorporated a UV-responsive α-thioetherketone linkage, which decomposed into a thioaldehyde species and benzophenone, a byproduct that has been approved as safe by the FDA. [103] In the presence of an amine, the thioaldehyde generated H₂S, and an imine byproduct. The authors incorporated this donor into polymeric systems using α-thioetherketone-modified styrene along with water-soluble comonomers. These polymers were then used to prepare hydrogels. Growth of 3T3 fibroblasts was observed on the hydrogels, and an H₂S-selective fluorescent probe was used to visualize H₂S in the fibroblasts after UV irradiation. The polymeric α-thioetherketone donors highlight

promising potential applications for a variety of polymeric H₂S prodrug systems, particularly in applications that require localized delivery where gels and films may be advantageous.

Light-triggered H₂S donors provide feasible options for H₂S release under unique conditions, enabling a more specific triggering mechanism than nucleophiles typically offer. Using light as a trigger also provides the opportunity for spatiotemporal control of H₂S release. This donor class is most likely to find utility as chemical tools for studying H₂S biology in vitro due to the limited penetration depth of UV and visible light in mammalian tissue. However, the development of near-IR light-triggered donors may enable in vivo studies due to the ability of near-IR light to penetrate tissue at a greater depth than UV and visible light with little risk to surrounding tissue.

7.5. Enzyme-Triggered Donors

Utilizing enzymes as triggers for H₂S release offers several advantages over the other triggers mentioned. Enzymes are native to living organisms and often exhibit substrate and tissue specificity. Combined, these factors allow for specific drug targeting to a tissue of interest. Additionally, enzyme overexpression is a common cause of many diseases, offering potential targets to treat such diseases with the implementation of enzyme-triggered prodrugs. Although only a handful of enzyme-triggered H₂S donors have thus far been reported, these types of donors are poised to make a substantial impact in the coming years.

The first enzyme-triggered H₂S donors were a series of esterase-responsive compounds developed by Wang and coworkers. [104] These donors rely on a lactonization reaction popularly termed “trimethyl lock” (TML), which has been used to promote the release of a variety of drugs.[105] The TML system requires cleavage of a phenolic ester by an esterase, after which steric repulsion of three methyl groups triggers lactonization (via the Thorpe-Ingold effect), releasing a drug from a neighboring carbonyl group. In the case of the esterase-triggered H₂S donors, the authors included a thioester, which reacts in the lactonization step to release H₂S. The authors prepared a number of derivatives in this study through variation of the phenolic ester moiety as well as addition or removal of the methyl substituents on the aromatic ring. Because specifically placed methyl groups are required to drive cyclization after ester cleavage, it was expected that removing them would offer an avenue of slowing H₂S release. Indeed, derivatives lacking aryl methyl groups exhibited longer times to reach 50 % of the peaking concentration, ranging from 45–99 min, whereas prodrug containing aryl methyl groups reached 50 % of peaking concentration in 13–29 min, as measured by an H₂S electrode probe. In addition, several NSAID-TML hybrids were synthesized and evaluated for their efficacy as anti-inflammatory agents, successfully inhibiting TNF- α secretion. Finally, one TML derivative in this study (BW-HP-102) reduced losses in myocardial tissue in a mouse I/R injury model.[106] The authors are currently investigating the pharmacokinetics, precise mechanism of action, and safety profile of BW-HP-102 in an effort to bring this compound to clinical trials.

In another example enzyme-triggered H₂S donors, Chakrapani and coworkers merged the concepts of enzyme-specific cleavable functionalities with the protected geminal dithiol as an H₂S releasing moiety.[107] Rather than employing a photocleavable functionality, as seen

in Xian's work, the authors used a *para*-nitro benzyl thioether as the geminal dithiol protecting group. The nitro group on the benzene ring underwent selective reduction to an amine in the presence of nitroreductase, an enzyme found in bacteria. Reduction by nitroreductase led to an unstable intermediate, which underwent self-immolation to release the deprotected geminal dithiol, which in turn decomposed to generate H₂S via hydrolysis. The donors (50 μM) showed sustained H₂S release out to 45 min using a fluorescent BODIPY probe, with peak instantaneous H₂S concentrations of 30 μM in the presence of nitroreductase. In vitro studies using *E. coli* strains showed that the donor rescued the bacteria from oxidative stress resulting from treatment with common antibiotics, indicating that H₂S production in bacteria may be a mechanism leading to antibiotic resistance.

7.6. Dual Carbonyl Sulfide / H₂S Donors

A recent innovation in H₂S donor design is the synthesis of compounds that release carbonyl sulfide (COS), a compound that is an intermediate to H₂S generation and may itself be a gasotransmitter. Two studies in 1979 and 1980 first demonstrated that COS metabolism is linked to the generation of H₂S in vivo through the action of the ubiquitous enzyme carbonic anhydrase (CA).[108, 109] Since these seminal papers on COS physiology, the literature had been quiet on the topic until recently. For a more detailed account of the potential roles of COS in mammalian biology the reader is referred to a recent review on the topic. [110] The link between COS and H₂S via CA opens a new avenue of H₂S research through the synthesis of compounds that release COS. COS donors act as H₂S donors in the presence of CA, but also provide the opportunity to study COS as a potential gasotransmitter.

7.6.1. *N*-Thiocarboxyanhydrides—*N*-Thiocarboxyanhydrides (NTAs) are a class of COS releasing compounds first reported in 1971 as monomers for the synthesis of polypeptides. [111] Matson and coworkers saw these molecules as a potential nucleophile-responsive COS/H₂S donor platform with the advantage of releasing only COS and innocuous peptide byproducts. The sarcosine NTA derivative (R=CH₃, NTA1) was synthesized in three steps from sarcosine (*N*-methyl glycine).[112] The generation of COS, triggered by opening of the NTA with glycine, and the resulting dipeptide byproduct were confirmed by GCMS and LCMS, respectively. In the presence of glycine and CA, the conversion of COS into H₂S was confirmed via the methylene blue assay. A polymeric NTA donor (polyNTA1) was also synthesized via conjugation of NTA1 to a norbornene moiety and subsequent polymerization by ring-opening metathesis polymerization (ROMP). Comparison of the H₂S release kinetics of the NTA1 and polyNTA1 using a fluorescent H₂S sensor showed a 4-fold increase in release half-life for the polymer compared with NTA1, with half-lives in the range of hours for both donor types under the testing conditions. In vitro studies with mouse brain endothelial cells showed an enhancement in endothelial cell proliferation after treatment with NTA1 (100 μM) over controls.

7.6.2. Self-Immolative Thiocarbamates—A separate class of COS releasing prodrugs is based on a self-immolative reaction mechanism whereby COS release is the result of the decomposition of a thiocarbamate-containing compound in the presence of a specific trigger (Figure 2). The first self-immolative COS/H₂S donors were reported by Pluth et al. in the form of analyte replacement probes.[113] These thiocarbamate-based donors were prepared

by conjugation of an aryl azide to a pro-fluorophore via a thiocarbamate ($R-O(S)-NHR$) linkage. H_2S initiated self-immolation by reducing the aromatic azide to an amine, resulting in a cascade-like decomposition of the molecule to release COS, a quinone methide species, and the fluorophore. A 65-fold increase in fluorescence response to H_2S was observed over other reactive sulfur, nitrogen, and/or oxygen species (RSONS), demonstrating the trigger specificity of this self-reporting system.

Building off of their analyte replacement probes, Pluth and coworkers aimed to synthesize a similar class of self-immolative COS donors with RSONS-sensitive triggers.[114] These donors consist of an RSONS-active boron pinacol ester connected to *para*-substituted phenyl derivatives by way of the aforementioned thiocarbamate linkage. These donors were non-toxic and released H_2S in the presence of CA in response to H_2O_2 , superoxide (O_2^-), and peroxynitrite ($ONOO^-$), with H_2O_2 resulting in the greatest amount of H_2S release, as measured amperometrically. Using the phorbol 12-myristate 13-acetate (PMA) assay, a well-established method to induce production of H_2O_2 in macrophages,[115] the authors demonstrated that endogenously produced ROS also triggered H_2S release in these donors. Additionally, these donors exhibited cytoprotective effects—the presence of the prodrug showed a significant dose-dependent increase in cell viability (10-50 μM) after treatment with H_2O_2 (100 μM) compared to controls. These data suggest that this prodrug was not only capable of quenching RSONS as a result of the rapid reaction between arylboronates and RSONS in vivo, but also of reducing cellular damage in an oxidative environment through H_2S release.

Pluth et al. have also reported on the synthesis of enzyme-triggered self-immolative COS prodrugs, with the same general thiocarbamate-based structure as those discussed previously.[116] These COS donors contain a *para*-pivaloyl group, which is preferentially cleaved in the presence of esterases. These donors were stable in aqueous media, exhibiting no H_2S release in the presence of physiologically relevant levels of CA in the absence of esterase. However, when the authors introduced porcine liver esterase a relatively rapid release of COS/ H_2S resulted under the same conditions. Interestingly, these thiocarbamate donors exhibited higher levels of cytotoxicity than equivalent levels of Na_2S and GYY4137 in BEAS 2B human lung epithelial cells and in HeLa cells. The authors attribute the cytotoxicity to reduced cellular respiration and ATP synthesis, which is in line with the well-known ability of H_2S to inhibit cytochrome c oxidase. [117] The authors suggest that COS itself may have physiological functions different from those of H_2S in certain biological environments, leading to these unexpected observations.

Another example of enzyme triggered self-immolative COS prodrugs was reported by Chakrapani et al.[118] In this work the authors examined the use of pivaloyloxymethyl carbonothioates ($ArCH_2S-C(O)-OR$) and carbamothioates ($ArCH_2S-C(O)-NHR$) as esterase-triggered COS donors. These prodrugs consist of a *para*-pivaloyloxymethyl linkage that blocked self-immolation until triggered. After cleavage of this linkage, a carbamothioate ($-S-C(O)-NHR$) or carbonothioate ($-S-C(O)-OR$) anion formed, allowing for self-immolation and tandem release of COS and either an ester or amine. H_2S release did not depend on ring electronics, and minimal differences in release profiles were observed between carbonothioate and carbamothioate donors. The authors concluded that rapid

cleavage of the pivaloyloxymethyl group occurred, followed by self-immolation of a short lived intermediate to release COS and eventually H₂S in the presence of CA.

Another type of self-immolative H₂S prodrug comes in the form of “click-and-release” thiocarbamates.[119] This system utilized the inverse-electron demand Diels Alder reaction to click together tetrazine and thiocarbamate-containing *trans*-cyclooctene to selectively release COS. This strategy is different from other COS/H₂S donor systems because the triggering reaction is completely bio-orthogonal. In other words, release can theoretically be triggered by the researcher upon addition of tetrazine to cells or an animal. From this platform, Pluth and coworkers showed that H₂S release was dose-dependent on tetrazine, that the prodrug was non-toxic, and that CA was required to observe any measurable quantities of H₂S. However, the authors were unable to expand this strategy to a cellular environment due to incompatibility of the click-and-release reaction with the fluorescent probes currently used to image H₂S. The authors attributed this limitation to the rapid scavenging of H₂S by tetrazine via a known reduction mechanism.

Bio-orthogonal, light-activated thiocarbamates have also been reported by Pluth et al.[120] This system utilized an *o*-nitrobenzyl functionality that underwent UV-triggered cleavage. UV light exclusively initiated the release of H₂S, as amino acid nucleophiles produced no response after incubation in PBS for 10 min. Additionally, this system exhibited tunable release, with 4,5-dimethoxy substituted derivatives reaching peak instantaneous H₂S concentrations twice as fast as the unsubstituted donor, likely a result of the increased light absorption of the dimethoxy derivative. These donors have yet to be evaluated in biological systems.

The advent of COS donors enables the study of COS itself, including the intriguing possibility that COS may enact biological effects that differ from H₂S. While complete inhibition of the ubiquitous enzyme CA may be difficult due to its many isoforms, clever methods to study COS will continue to be developed. With the persistent production of dual H₂S/COS donors by chemists, the physiological interactions of COS may eventually be fully elucidated, affording a greater understanding of how small sulfur species signal cells and interact in the body.

8. Conclusions

Remarkable progress has been made in the field of H₂S donor chemistry in the short amount of time since the therapeutic potential of H₂S was discovered. Continued innovation from synthetic chemists will be a major factor in driving H₂S research forward in the coming years, with an eye toward building H₂S donors that enable biological studies on the (patho)physiological roles of this gas and have potential as clinically relevant H₂S-releasing therapeutics. Important questions remain unanswered in the H₂S field at large, which will require joint effort from chemists, pharmacologists, and biologists. One chief concern is determining the therapeutic window of H₂S for a given target as well as the active species giving the desired effect once the donor is delivered. An increased molecular and biological understanding of donor triggers and byproducts, species interchange, signaling mechanisms, and redox chemistry of H₂S donors will help elucidate biological activity and species

involved. The chemistry of H₂S donors is often complicated by the release of reactive byproducts, including unidentified sulfur species, a topic that needs further attention. As the field of H₂S research continues to grow, it is imperative that these key issues be addressed so that the field as a whole can move forward. Persistent innovation in synthetic donors and increased understanding of H₂S physiology may eventually enable a pathway to the clinic for H₂S therapeutics.

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Figure 1.
Graphical overview of endogenous H₂S production in mammalian cells.

Figure 2.

A) Schematic illustration depicting the concept of a self-immolative releasing its payload. B) An example mechanism depicting COS release from a generalized thiocarbamate.

Table 1Hydrolysis triggered H₂S donors.

H ₂ S donor	General Structure	Bioactivity	References
Sulfide salts	NaHS, Na ₂ S	Anti-inflammation Cardioprotective effects Diabetes amelioration	38-45
Lawesson's reagent		Anti-inflammation Vasodilation Anti-cancer	64-65
GY4137		Ion channel modulation Anti-inflammation	66-68
Phosphorodithioates		Anti-oxidant properties Anti-inflammation	69
Dithiolthiones		Anti-cancer proliferation Anti-inflammation	72-85

Table 2Thiol-triggered H₂S donors.

H ₂ S Donor	General Structure	Bioactivity	References
<i>N</i> -Benzoylthiobenzamides		Cardioprotection	86-89
Acyl perthiols		Cardioprotection	90
Dithioperoxyanhydrides		Vasodilation	91
Polysulfides		None reported	92
Arylthioamides		Vasodilation	61, 93-95
<i>S</i> -Aroylthiooximes		Anti-cancer proliferation	96-99

Table 3Light and enzyme triggered H₂S donors.

H ₂ S Donor	General Structure	Bioactivity	References
Geminal-dithiols		Restores AMR	100-101, 107
Ketoprofenate photocages		None reported	102
Trimethyl lock		Anti-inflammation	104, 106-107

Table 4Dual COS/H₂S donor systems.

H ₂ S Donor	General Structure	Bioactivity	References
<i>N</i> -Thiocarboxyanhydrides		Angiogenesis	110
Arylboronate thiocarbamates		Cardioprotection	113
Esterase-triggered thiocarbamates		Reduction in cellular respiration	115-117
“Click and release” thiocarbamates		None reported	118
<i>o</i> -Nitrobenzyl thiocarbamates		None reported	119