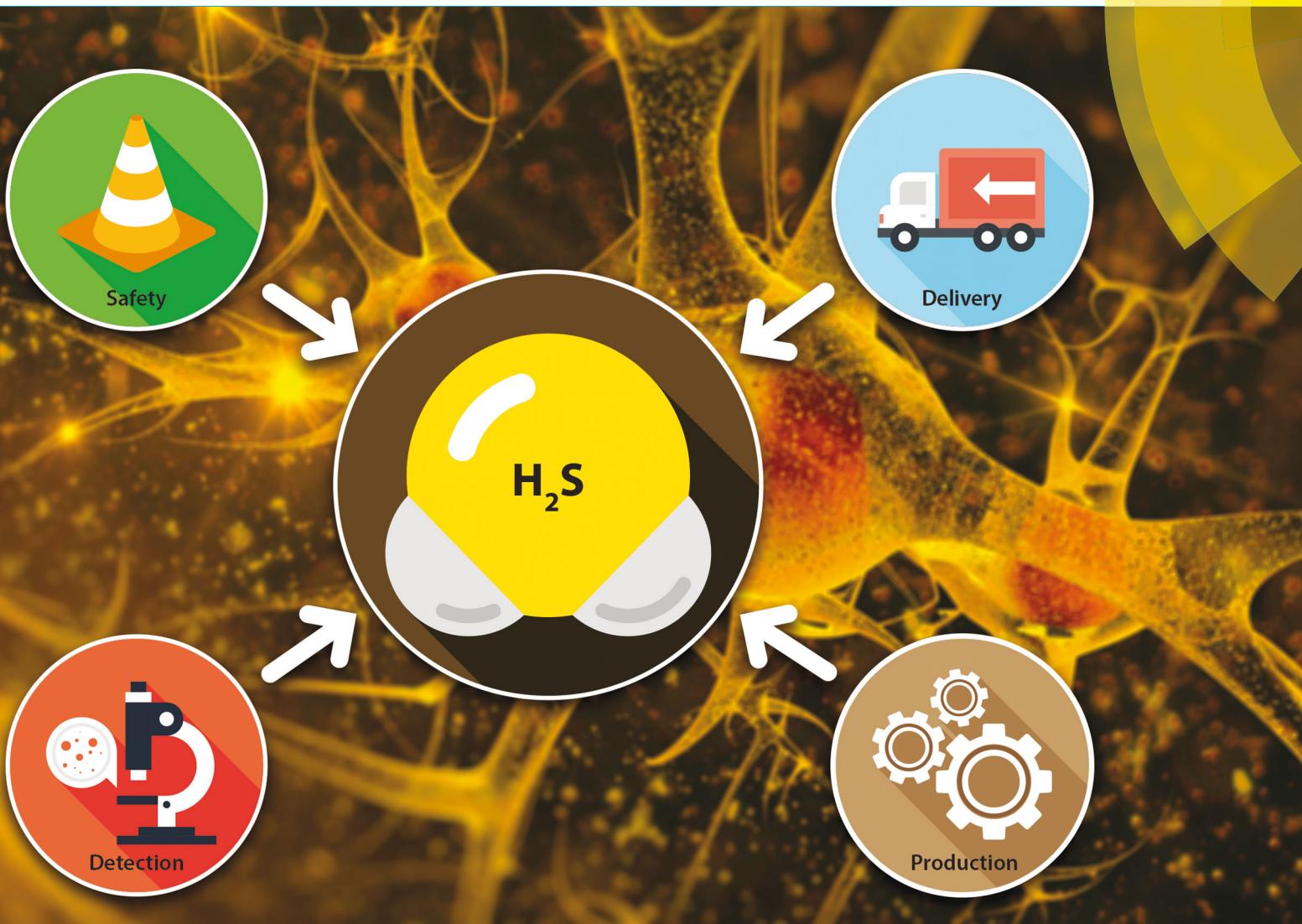


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TUTORIAL REVIEW

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A practical guide to working with H_2S at the interface of chemistry and biology

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A practical guide to working with H₂S at the interface of chemistry and biology

Matthew D. Hartle and Michael D. Pluth*

Hydrogen sulfide (H₂S) is the most recently accepted endogenously produced gasotransmitter and is now implicated in a variety of physiological functions. In this tutorial review, our goal is to provide researchers new to the field of H₂S chemical biology with practical considerations, pitfalls, and best practices to enable smooth entry into investigations focused on biological H₂S. We present practical handling and safety considerations for working with this reactive biomolecule, and cover basic roles of H₂S biogenesis and action. Experimental methods for modulating H₂S levels, including enzymatic knockout, RNA silencing, enzymatic inhibition, and use of small molecule H₂S donors are highlighted. Complementing H₂S modulation techniques, we also highlight current strategies for H₂S detection and quantification.

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Key learning points

- Basic properties of biological hydrogen sulfide (H₂S)
- The role of H₂S in the landscape of reactive sulfur, oxygen, and nitrogen species (RSONS)
- Practical safety considerations for handling H₂S and H₂S donors
- Methods for modulating biological H₂S: enzymatic knockout, RNA silencing, inhibition, stimulation, small molecule donors
- Methods for detecting and quantifying biological H₂S

Introduction

Hydrogen sulfide (H₂S) has emerged as an important biological signaling molecule that plays diverse roles in human health and physiology. Despite its long history as a toxic gas and environmental pollutant, H₂S now joins nitric oxide (NO) and carbon monoxide (CO) as a gasotransmitter – a small, enzymatically generated, gaseous molecule with a tightly regulated metabolism that impacts physiological functions.^{1–3} The innate chemical properties of H₂S, including its redox activity, acidity, and high nucleophilicity, allow for reaction with different cellular targets as part of its signaling capacity. By comparison, disentangling this chemistry is in many ways more complicated than that of NO and CO.⁴ Misregulation of endogenous H₂S is implicated in diverse physiological processes, including blood pressure regulation, immune response, and long term potentiation, as well as various diseases in the neuronal, gastrointestinal, circulatory, and endocrine systems.² Aligned with our rapidly expanding understanding of biological H₂S, new and

impactful investigative tools for elucidating its genesis, translocation, and action have emerged and continue to be an active and fruitful research area.

By contrast to biological studies focused on NO and CO, H₂S-focused investigations are often faced with unique experimental challenges – some of which are due to the recent emergence of H₂S chemical biology and others from the innate physical properties of H₂S. For example, although CO, NO, and H₂S are all gasses, common gas detection techniques, such as gas chromatography (GC) and chemiluminescence detectors provide robust methods for detection and quantification for CO and NO, respectively, whereas similar techniques have not been widely developed for H₂S. The different protonation states of H₂S, and its interaction with the redox-active biological sulfur pool, make analysis by GC or other common analytical methods challenging. Similarly, reaction-based imaging techniques, as well as synthetic donor scaffolds, are often more complicated than similar systems for NO or CO. Despite these challenges, recent studies have suggested the potential interplay between the biological chemistry of H₂S and NO⁵ as well as CO,³ thus highlighting the interconnectivity of these three gaseous molecules. These interactions, as well as those with H₂S and reactive sulfur, oxygen, and nitrogen species (RSONS) highlight the

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complexity of the biological landscape in which H_2S is involved, and the synergistic relationship of H_2S to other important and reactive small molecules in biology.

In this tutorial review, we highlight key points regarding H_2S chemical biology and provide a brief survey of accessible tools and methods for H_2S research at the interface of chemistry and biology. Our goal is not to provide a comprehensive overview of all investigative tools for H_2S research, but rather to highlight currently available classes of tools and to provide important considerations to help guide new researchers into the rapidly developing field of H_2S chemical biology.

Properties and enzymatic production of H_2S

H_2S is a weak acid with a first $\text{p}K_{\text{a}}$ of 7.0, and a second $\text{p}K_{\text{a}}$, corresponding to the deprotonation of hydrosulfide anion (HS^-) of >14 .¹ Under physiological conditions, HS^- constitutes about 80% of the speciation, neutral H_2S about 20%, and dianionic S^{2-} less than 1%.¹ The HS^- and H_2S protonation states provide water-solubility and lipophilicity, respectively, and HS^- is a potent nucleophile that can react with different electrophilic cellular targets including RSONS. Additionally, the different protonation states afford different metal-ligation and redox properties. The complex redox landscape of biological sulfur, with oxidation states ranging from -2 in H_2S to $+6$ in SO_4^{2-} , means that $\text{H}_2\text{S}/\text{HS}^-$ can be readily oxidized to other biologically-relevant reactive sulfur species. Indeed, the redox-labile/sulfane-sulfur pool may provide an important method of sulfide storage and transport, but also may provide new signaling pathways distinct from those associated with H_2S alone (*vide infra*).⁶

Enzymatic H_2S biosynthesis stems primarily from cystathionine γ -lyase (CSE), cystathionine- β -synthase (CBS), and 3-mercaptopropionate sulfurtransferase (3-MST)/cysteine aminotransferase

(CAT). In each of these pathways, the sulfur atom incorporated into H_2S is derived from the sulphydryl group of either homocysteine (Hcy) or l -cysteine (l -Cys). Details of these pathways have been recently reviewed in significant detail and are summarized briefly here (Fig. 1).^{1–3} CSE catalyzes the formation of H_2S and homolanthionine from 2 equiv. of Hcy, but can also work in concert with CBS to convert Hcy and Cys to H_2S and cystathionine. CBS can also catalyze the condensation of Hcy with serine (Ser) to generate cystathionine and H_2O . Upon reaction with CSE, cystathionine is converted back to l -Cys with concomitant formation of homoserine. This generated Cys can participate in the above pathways, can be hydrolyzed to Ser and H_2S by CBS or CSE, or can be condensed with a second equivalent of Cys by the same two enzymes to form lanthionine and H_2S . Cysteine is also a viable substrate for CAT, which generates 3-mercaptopropionate (3-MP), which is then converted to pyruvate, NH_3 , and H_2S by 3-MST. The 3-MST pathway also accounts for H_2S formed from D -Cys, which is first converted to 3-mercaptopropionate by DAO.

Practical handling and safety considerations

Hydrogen sulfide is a highly toxic gas, and adequate care must be given when handling H_2S gas, sulfide salts, and synthetic H_2S donors. Although the characteristic rotten egg odor is detectable as low as 0.01 ppm in air, odor alone should never be used as the primary method of safety monitoring. Rapid olfactory fatigue results upon exposure to concentrations greater than 100 ppm but also after continuous exposure to significantly lower levels. H_2S concentrations as low as 20 ppm cause eye and lung irritation, 300–500 ppm levels result in serious eye damage, and 700 ppm or higher levels can result in unconsciousness, respiratory failure, and death.⁷ The combination



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bio(in)organic models to investigate the chemical mechanisms of H_2S binding and reaction with heme and other metal- and non-metal-containing biologically relevant scaffolds.

Matthew D. Hartle earned his BS in chemistry with an area of special interest in space and planetary science and engineering from the Colorado School of Mines in 2012, where he conducted undergraduate research with Prof. Stephen Boyes. He is currently working towards his PhD under the direction of Prof. Michael Pluth in the Department of Chemistry & Biochemistry at the University of Oregon. His research interests focus on using



Michael D. Pluth

Oregon as an Assistant Professor. Research in the Pluth lab is focused on different aspects of molecular recognition, with particular emphasis in developing chemical tools for H_2S quantification and donation, as well as using bio(in)organic models to investigate modes of H_2S action.



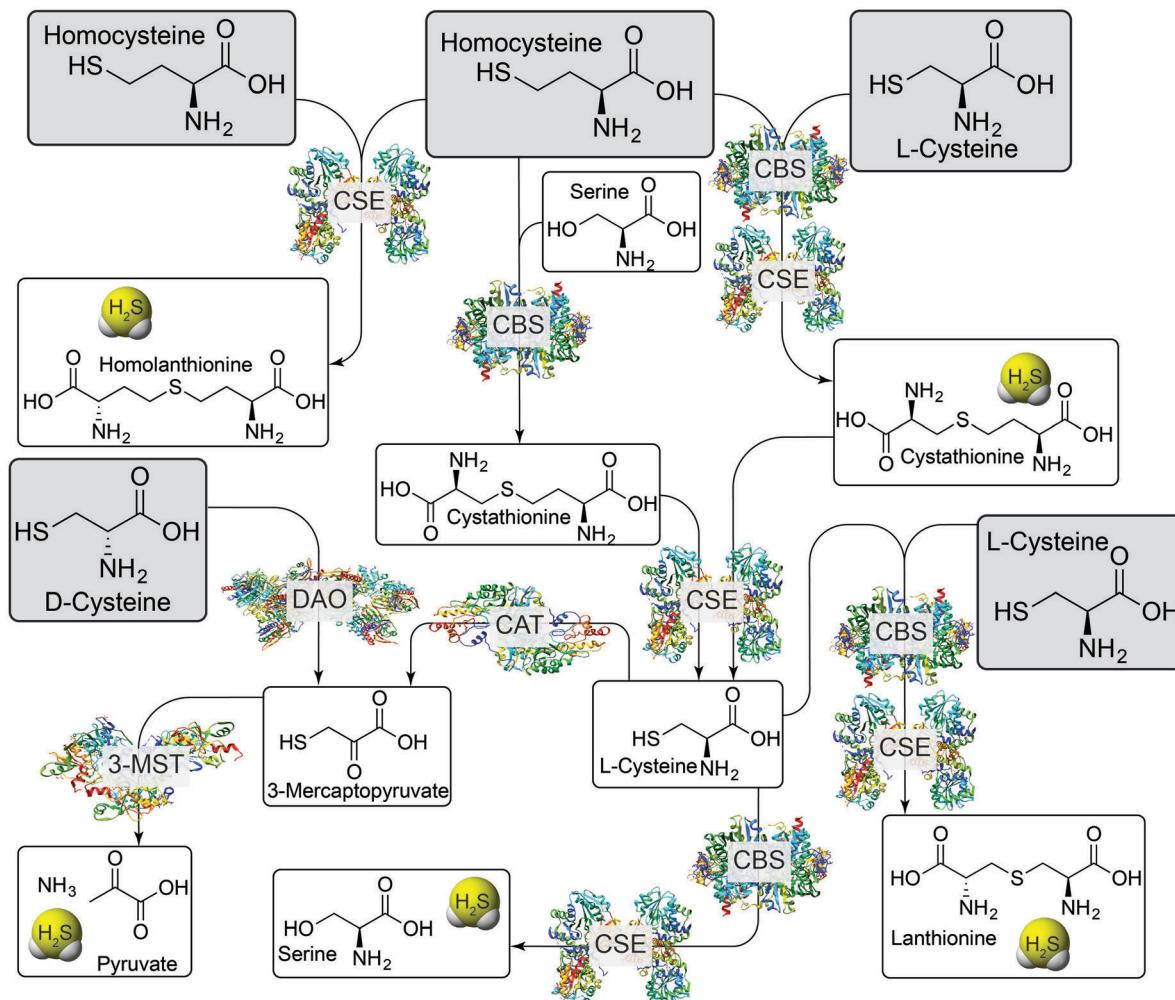


Fig. 1 Enzymatic H₂S production pathways. CSE: cystathionine γ -lyase; CBS: cystathionine β -synthase; CAT: cysteine aminotransferase; DAO: D-aminoacid oxidase; 3-MST: 3-mercaptopypyruvate sulfurtransferase. Grey boxes indicate common substrates for H₂S-producing enzymes.

of high toxicity with rapid olfactory fatigue necessitates handling in a well ventilated fume hood equipped with a commercially-available H₂S alarm, which detects H₂S gas and typically reports in the 5–10 ppm range, especially when using H₂S gas directly or quantities of sulfide salts sufficient to provide a potentially toxic response. It is also practical, both from a safety and odor-mediation standpoint, to quench any H₂S-containing solutions after use. Such quenching can be readily accomplished by preparation and use of a Zn²⁺ quenching solution, which rapidly reacts with free sulfide to generate insoluble ZnS. This quenching slurry can be prepared by adding 30 g Zn(OAc)₂, 9 g sodium citrate, and 12 g NaOH to 1 L of H₂O.⁷

For experiments involving aqueous sulfide solutions, it is often more practical to use sulfide salts, such as NaSH or Na₂S, rather than H₂S gas directly. Despite their convenience, the commercial purity of NaSH or Na₂S is often poor, and many samples contain significant quantities of elemental sulfur or polysulfides.^{4,8,9} In general, sulfide salts should be free-flowing white powders – any inclusion of yellow or other highly colored impurities should signal to the user that the sample does not

have the appropriate purity and should be discarded. Because of the propensity of H₂S/HS[–] to oxidize, especially in the presence of trace metal ions and oxygen, H₂S solutions should be prepared in anaerobic buffer under a blanket of nitrogen or argon in clean, metal-free, glass- or plastic-ware. Such solutions should also be prepared in septum-sealed vials to prevent significant loss from H₂S volatilization and also to enable easy transfer of sulfide solutions *via* gas-tight syringe. Because of this volatility and potential for oxidation, it is most practical to prepare sulfide-containing solutions immediately prior to use rather than to re-use solutions from previous experiments.

Interactions with the sulfane-sulfur pool

Complementing the signaling roles of biological H₂S, significant evidence suggests that sulfane-sulfur compounds, such as polysulfides, persulfides, and other sources of reductant-labile sulfur, play important biochemical roles.^{6,8,9} The term “sulfane

sulfur" refers to a sulfur atom with formally six valence electrons and no charge (S^0), which is bound to one or more sulfur atoms.¹⁰ Upon reaction with cellular reductants or thiols, sulfane-sulfur compounds can release sulfide, thus providing a convenient source of H_2S storage. Additionally, sulfane-sulfur species have distinct chemical reactivities that may contribute to additional modes of action. For example, persulfides (RSSH) are more nucleophilic than the corresponding thiols, and *S*-persulfidation of nucleophilic Cys residues in enzymes is known to modify enzymatic activity.¹ As completely inorganic species, hydropolsulfides (HSS_xH) are also important sulfane-sulfur species, as evidenced by a recent report demonstrating that 3-MST not only generates H_2S , but also H_2S_3 .⁸ The use of small molecule persulfides and persulfide-releasing motifs as H_2S donors (*vide infra*) and as model systems is quickly providing more information on persulfide reactivity and its role in the intricate chemistry of RONS.¹¹ Although isolated persulfides are typically unstable in solution and have not yet found utility in biochemical investigations, caged persulfides, which release persulfides upon hydrolysis or reaction with nucleophiles, or persulfides generated *in situ* from reaction of GSSG with HS^- , have been used to investigate different roles of persulfides in reactive sulfur species regulation and action.¹²

One of the most basic methods for sulfane-sulfur detection and quantification is the cold cyanolysis assay, which utilizes the reaction of sulfane sulfur atoms with CN^- at basic pH to form thiocyanate (SCN^-). Addition of excess ferric iron results in the formation of $[Fe(SCN)(H_2O)_5]^{2+}$, which can be readily detected and quantified by the characteristic absorbance at 460 nm (Scheme 1a).¹³ In practice, the cold cyanolysis method is usually used for purified proteins or samples in simple matrices and cannot differentiate between individual components, such as persulfides and polysulfides, in the sulfane-sulfur pool.

One significant limitation of the cold cyanolysis method is that persulfides are not differentiated from polysulfides or other sulfane-sulfur sources. As a step toward addressing this challenge, a tag-switch method to detect and assay persulfides was recently reported (Scheme 1b).¹⁴ In this method, all sulphydryl groups, both from thiols ($-SH$) and persulfides ($-SSH$) are labelled with an electrophile, such as methylsulfonyl benzothiazole (MSBT), to generate the corresponding benzothiazole thioether and disulfide,

respectively. Importantly, the resultant benzothiazole disulfide remains reactive toward specific carbon-based nucleophiles, allowing for conjugation with biotin tags. The biotinylated proteins can then be subjected to streptavidin pulldown and detection by standard Western blot or mass spectrometric techniques.¹⁴ Complementing these strategies, selective reaction-based fluorescent probes for polysulfides and sulfane sulfur have been reported and are the subject of a recent review.¹⁵ These, as well as other emerging tools, are poised to help differentiate between the genesis of H_2S and other reactive polysulfides.

Methods for modulating H_2S levels

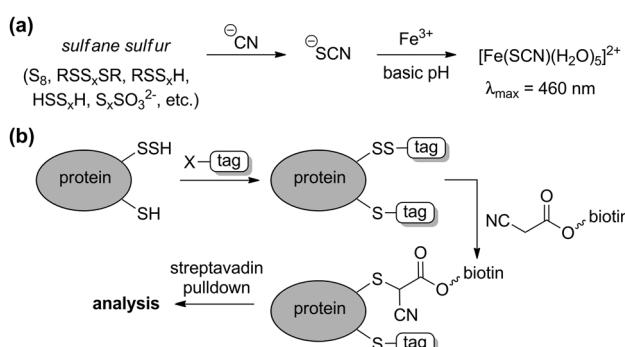
The ability to modulate cellular H_2S levels provides a cornerstone for investigating the actions of H_2S in biology. Such control can be achieved by selective knockout and/or knockdown of H_2S -producing enzymes, use of small-molecule competitive inhibitors or stimulators, or by administration of synthetic H_2S donors. In model cell lines and organisms, supplementation with exogenous H_2S can often provide protection (or rescue) from various (patho)-physiological disease states associated with abnormal H_2S biosynthesis. These insights suggest that synthetic H_2S donors may not only provide important chemical tools for understanding biological H_2S , but may also offer viable therapeutic potential for diseases associated with H_2S misregulation.

KO mouse models

Selective knockout (KO) of CSE, CBS, or 3-MST in different cell lines has been utilized in specific investigations, but the development of KO mouse models has provided a more broad platform on which to study the impacts of reduced enzymatic H_2S generation.¹⁶ Homozygous CBS^{-/-} KO in mice results in severe developmental growth problems, and few of the mice live past 4 weeks of age.¹⁷ The heterozygous CBS^{+/−} KOs exhibit better viability and present hyperhomocysteinemia (high levels of homocysteine in the blood) but have not been used extensively in H_2S -related investigations.¹⁷ By contrast, the CSE^{−/−} mouse model¹⁸ has found significantly more utility in H_2S -related investigations, including studies on the role of H_2S in blood pressure regulation, angiogenesis, and neurodegeneration, as well as many other studies. A second CSE^{−/−} mouse model exhibiting somewhat different phenotypes was also subsequently developed and used as an animal model for cystathioneemia.¹⁹ Homozygous 3-MST^{−/−} mice have been developed more recently and may also provide a useful animal model for H_2S -related investigations.²⁰ As a whole, the availability of KO mouse models provide a key tool in studying the impacts of H_2S in contextually-rich biological environments and overcome some of the limitations of small molecule inhibitors or sulfide donors (*vide infra*).

RNA knockdown methods

In addition to enzyme KO methods, gene knockdown by RNA interference (RNAi) methods has been used to silence CSE, CBS, and 3-MST. Importantly, these methods often provide a



Scheme 1 (a) Cold cyanolysis detection/quantification of sulfane sulfur. (b) General strategy for tag-switch labelling of protein persulfides.



more simple approach to overcome the limitation of animal models or small-molecule inhibitors, and also offer a complementary approach for other enzyme modulation experiments. For example, small interfering RNA (siRNA) methods have been used to silence CSE and CBS.²¹ Unlike small-molecule inhibitors for 3-MST (*vide infra*), siRNA methods aimed at this enzyme have proven efficacious in reducing H₂S production, leading to important insights into mitochondrial electron transport and cellular bioenergetics.²²

Small-molecule inhibitors

Small-molecule competitive inhibitors of H₂S-producing enzymes are commonly used to decrease H₂S synthesis in studies using isolated enzymes or cell culture models. On the basis of this widespread use, significant efforts have been made to discover or develop potent enzymatic inhibitors for CBS, CSE, and 3-MST. Although these studies have provided a useful suite of compounds for partial inhibition of H₂S-producing enzymes, identifying inhibitors with specificity for one enzyme over another and low inhibitory constant (K_i) values remains a significant challenge.

Commonly-used CSE inhibitors with $IC_{50} < 1$ mM are L-aminoethoxyvinylglycine (AVG, 1 μ M), β -cyano-L-alanine (BCA, 14 μ M), and propargylglycine (PAG, 40 μ M) (Fig. 2).^{16,23} Each of these inhibitors also affect other proximal phosphate (PLP)-dependent enzymes at mM concentrations, making it likely that off-target inhibition is observed at practical concentrations. Inhibition of CBS has proven significantly more challenging, with fewer inhibitors with $IC_{50} < 1$ mM identified. Hydroxylamine (HA, 278 μ M), aminoxyacetic acid (AOAA, 8.52 μ M), and trifluoroalanine (66 μ M) are among the most commonly used competitive inhibitors for CBS,²³ but none of these show marked selectivity for CBS over CSE. Recent studies have used high-throughput screening methods, often in combination with H₂S fluorescent probes (*vide infra*), to identify CBS inhibitors. Such screens have identified new lead compounds with good IC_{50} values in the low micromolar range, but many hits have not been compared directly with commonly-used inhibitors and their selectivity for CBS over CSE remains to be fully elucidated. Similar to the challenges with developing selective CBS inhibitors, selective inhibition of 3-MST has been unsuccessful to date.¹⁶

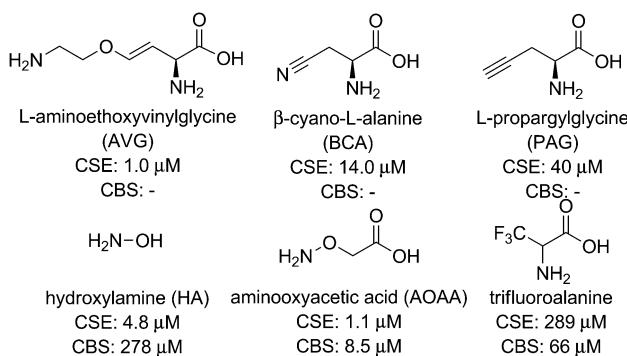


Fig. 2 Selected common CSE and CBS inhibitors with associated IC₅₀ values.

Given the overall difficulty in selectively inhibiting individual H₂S producing enzymes, the identification and development of new inhibitors for H₂S-producing enzymes is needed. Such inhibitors, if selective and functional at pharmacologically-reasonable concentrations, would provide important research tools for modulating cellular H₂S levels.

Enzymatic stimulators

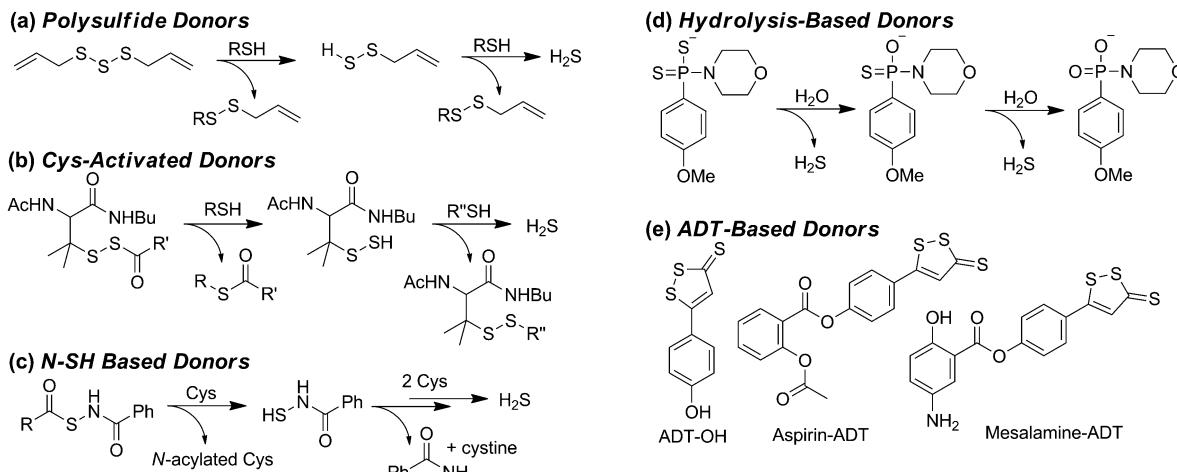
Complementing methods to reduce H₂S synthesis using small-molecule inhibitors, H₂S synthesis can also be increased by use of different enzymatic stimulators. The most common of such methods is use of S-adenosylmethionine (AdoMet), an allosteric activator of CBS, to increase enzymatic H₂S production.^{24,25} In addition to CBS, CSE stimulation has also been demonstrated using cytokines and endotoxins, such as tumor necrosis factor α (TNF- α) or lipopolysaccharides (LPS), involved in immune response.^{26,27} By contrast to CBS and CSE stimulation, common stimulators of 3-MST are not readily available and remain an active area of research.

Small-molecule H₂S donors

Complementing methods to modulate endogenous H₂S synthesis, the ability to increase endogenous H₂S levels using exogenous sulfide sources provides an important research and pharmacological tool for studying the roles of biological H₂S. Commonly-used sources of exogenous H₂S include inorganic salts, such as NaSH and Na₂S, as well as small-molecule synthetic donors. Although NaSH and Na₂S provide convenient sources of sulfide, the large dose of H₂S released upon addition to buffer does not match the slower, continuous, enzymatic production characteristic of endogenous H₂S synthesis. Additionally, the large bolus of H₂S often results in a toxicological response and is quickly oxidized/metabolized by the cellular environment. Motivated by these limitations, researchers have developed slow-release sulfide donors that better mimic the gradual H₂S release of enzymatic synthesis. As our understanding of sulfide biology continues to evolve, one common observation is that slow-releasing H₂S donors often elicit different cellular responses than inorganic sulfide salts.²⁸ For example, sulfide salts and slow-releasing donors have been shown to be pro- and anti-inflammatory, respectively, in different models of inflammation, including sepsis.²⁸ These differences highlight the importance of experimental design when using different sulfide sources, but also highlight the potential pharmacological importance of such slow-releasing donors. Here we provide a brief overview of the most commonly-used donor motifs along with their sulfide release mechanisms when known. We also refer interested readers to recent reviews on this topic.^{12,29}

Some of the most simple sulfide-donating motifs are organic polysulfides, which are often found in natural products. For example, diallyltrisulfide (DATS), isolated from garlic and other alliums, is a commonly used donor molecule, and other organic polysulfides such as varacin have also been implicated in H₂S release.³⁰ Because polysulfides are electrophilic, attack by GSH or other thiols results in formation of an intermediate persulfide, which after a second reaction with GSH releases H₂S (Scheme 2a).³¹





Scheme 2 Common motifs for small-molecule H_2S donors. (a) Organic polysulfides, (b) cysteine-activated H_2S donors, (c) cysteine-activated H_2S donors with N-SH motifs, (d) hydrolysis-based donors, and (e) anethole 1,2-dithiole-3-thione (ADT) derived donors.

Although DATS is the most commonly-used polysulfide donor, it is likely that other small molecule polysulfides are also potential platforms for H_2S release.

Inspired by disulfide exchange chemistry, chemists have also developed H_2S donors that are activated by nucleophilic attack by endogenous thiols.³² For example, protection of the thiol in penicillamine derivatives with an acyldisulfide results in persulfide formation after nucleophilic attack by Cys (Scheme 2b).³³ Once the persulfide is released, reaction with a second thiol generates a stable disulfide and extrudes H_2S . Application of such donors to the treatment of myocardial ischemia/reperfusion (MI/R) injury in murine models results in reduced circulating levels of MI/R biomarkers suggesting these and similar donor motifs exhibit cardiac protection and may have potential therapeutic applications.^{33,34}

Operating by similar Cys-activated H_2S release mechanisms, donors containing N -mercapto (N-SH) motifs also generate a persulfide intermediate en route to H_2S release (Scheme 2c).³⁵ In these donors, thiol exchange between Cys and the donor generates an S -acylated Cys intermediate, which undergoes native chemical ligation to rearrange to N -acylated Cys and an N -mercaptopbenzamide intermediate. This N-SH compound reacts with Cys to generate Cys persulfide, which reacts with a second equivalent of thiol to release H_2S . The rate of H_2S release from these scaffolds can be tuned by the addition of electron donating or withdrawing groups on the scaffold. Additionally, new H_2S -donating materials activated by thiol activation are also emerging, indicating the potential viability of this strategy for incorporation into therapeutics such as wound dressing to encourage angiogenesis.³⁶

In addition to donor activation by nucleophilic attack of thiols, other common donor constructs are activated by hydrolysis. For example, one of the most commonly used synthetic donors, GYY4137,³⁷ is a phosphino-dithioate derived from Lawson's reagent. GYY4137 undergoes slow hydrolysis in water to release H_2S (Scheme 2d), although the efficiency of sulfide release remains low.³⁸ The rate of hydrolysis is pH dependent and slow at physiological pH, contrasting the rapid release of H_2S by inorganic salts.

For example, treatment of precontracted aortic rings with NaSH results in relaxation in 20 to 30 seconds, whereas GYY4137 treatment requires nearly 10 minutes to achieve similar relaxation.³⁹

Another common class of H_2S donors is based on 1,2-dithiole-3-thiones, with the most often used being 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione (ADT-OH) (Scheme 2e).⁴⁰ Although the mechanism of H_2S release from these scaffolds remains to be fully elucidated, one benefit of this donor motif is the ease with which it can be tethered to different molecules through ester or hydrolytically-stable amide linkages.⁴¹ For example, linkage of a triphenylphosphonium cation imparts mitochondrial targeting for AP39, whereas ligation to common non-steroidal anti-inflammatory drugs (NSAIDs) has resulted in significant reduction in GI damage scores.⁴⁰ The use of ADT derivatives in NSAID applications highlight the potential therapeutic action of H_2S donors;⁴⁰ however, insights into the H_2S release mechanism from such donors is poised to make significant impacts into our understanding of the therapeutic action of these donor motifs.

All of the above donor motifs provide sources of sulfide in aqueous solution, but researchers interested in $\text{H}_2\text{S}/\text{HS}^-$ using biomimetic compounds in organic solvents also have access to organic-soluble sources of sulfide. In practice, H_2S gas is sufficiently soluble in organic solutions for most investigations; however, NaSH or Na_2S are generally insoluble in organic solvents. To aid in such investigations, we recently reported a simple method to prepare analytically-pure NBu_4SH , which provides access to an organic-soluble form of HS^- .⁴² Such compounds offer a convenient source of HS^- in organic solution, enabling the separation of HS^- from H_2S in different bio(in)organic model studies, which is otherwise not possible in aqueous solution.

H_2S detection and quantification

Coincident with the increased biological importance of H_2S , new methods for H_2S quantification and detection are rapidly

emerging. Ranging from binary presence/absence tests based on the formation of PbS from Pb^{II} salts to complex molecular architectures aimed at real-time H_2S detection, analytical methods of H_2S determinations comprise an important area of H_2S research. We highlight here general classes of analytical tools for H_2S research and describe their general benefits and pitfalls. On the basis of the rapid expansion of new methods for H_2S detection and quantification, as well as revisions and refinements of current methods, we refer the interested reader to a number of recent reviews on these topics.^{15,43}

Quantification methods

Commonly used sulfide quantification methods typically require sample homogeneity, which is straightforward for biological fluids such as blood or serum, but significantly more complicated for naturally heterogeneous samples such as those from cell or tissue culture experiments. Spectrophotometric methods such as the methylene blue (MB) assay have constituted one of the classical methods of H_2S quantification. This method leverages the FeCl_3 -catalyzed electrophilic aromatic substitution of *p*-dimethylamino aniline with sulfide to form MB, which has a characteristic absorbance at 670 nm and allows for sulfide quantification (Scheme 3a). Despite its widespread use, recent studies have demonstrated that the detection limit of the MB assay for sulfide is only 2 μM rather than previously-reported lower values, making it insufficiently sensitive to differentiate between sulfide levels in normal *versus* $\text{CSE}^{-/-}$ mice.⁴⁴ Furthermore, the MB method is performed under highly acidic conditions, which can result in sulfur extraction from other biological sources and diminish the accuracy of sulfide levels measured with this method. Researchers new to the field of sulfide quantification will notice the large number of past studies using the MB method but are cautioned when comparing H_2S levels measured with this method, especially when more accurate contemporary methods are available. For example, the monobromobimane (mBB) method, which utilizes the reaction of two equivalents of mBB with H_2S under basic conditions to generate fluorescent sulfide dibimane (SdB), enables quantification of H_2S by fluorescence HPLC with a detection limit of 2 nM (Scheme 3b).⁴⁴ Because mBB reacts with both H_2S and thiols, separation of the fluorescent signals by HPLC is required for quantification. Despite this limitation, a significant benefit of this method is that workflows have been developed to

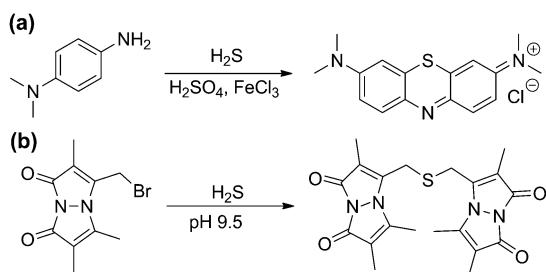
analytically separate the free, reductant-labile/sulfane-sulfur, and acid-labile sulfide pools, enabling more detailed investigations across distributions in complex systems.⁴⁴ Additionally, the mBB method has been used more recently to also detect and quantify different poly- and persulfides, providing analytical information about these important reactive sulfur species.⁴⁵

Complementing spectrophotometric H_2S quantification methods are other analytical tools, such as sulfide selective electrodes. These tools can provide both the ability to quantify H_2S , based on a calibration curve, and provide real-time H_2S detection in solution. Readers interested in the differences in available sulfide electrodes are referred to a recent review focused on this topic.⁴³ In general, sulfide electrodes employ an ion-selective membrane that allows H_2S permeability for solution-based measurements. In the solution phase of the electrode, a strongly basic environment allows the dianionic sulfide ion to reduce $\text{Fe}(\text{CN})_6^{3-}$ to $\text{Fe}(\text{CN})_6^{4-}$, which is subsequently re-oxidized at the platinum electrode to produce a current relative to H_2S concentration. One advantage of sulfide electrodes is that they can be used directly in mammalian tissues;⁴³ however, these electrodes cannot provide sub-cellular resolution of sulfide generation, storage, or transport. Even so, sulfide electrodes provide a convenient, time-resolved method for sulfide quantification or detection.⁴³

Detection methods

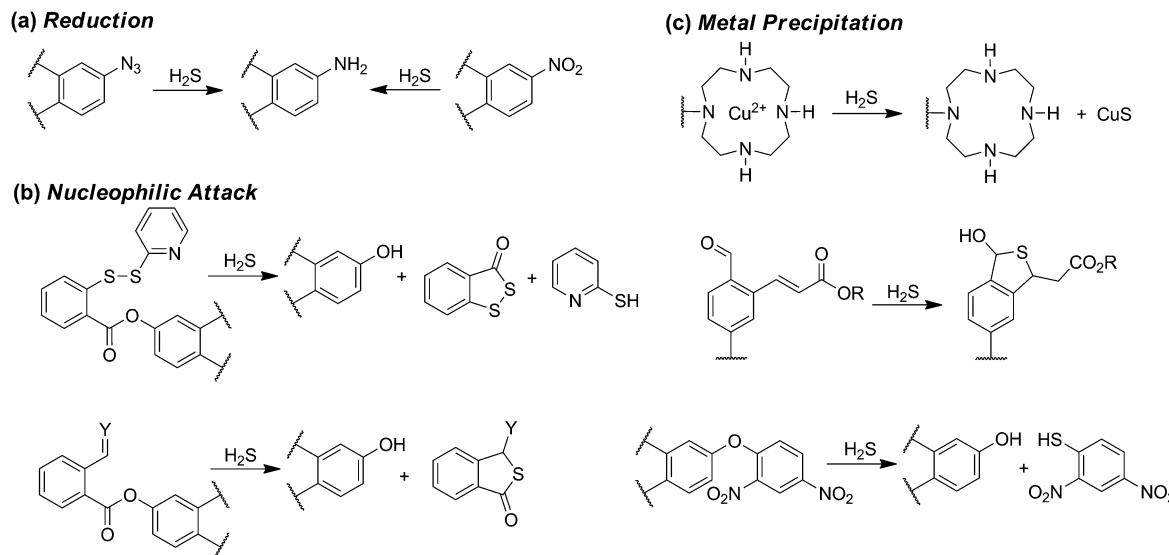
The development, refinement, and application of reaction-based probes for H_2S detection has expanded rapidly in the last few years. Such scaffolds typically couple various H_2S -selective reactions with different chromophores to generate a colorimetric or fluorescence response. Because of this design strategy, such methods are irreversible and the resultant methods measure accumulated probe activation rather than real-time H_2S dynamics. Despite these limitations, such constructs offer the potential to provide significantly higher spatiotemporal resolution than current H_2S quantification methods and also provide access to live cell and tissue imaging experiments. The predominant strategy for developing reaction-based probes for H_2S has been to utilize a fluorescence-quenching group on a fluorophore that can be modified or removed selectively by H_2S . As is common with most small molecule fluorescent probes, different detection strategies exhibit somewhat different selectivity patterns and each strategy may be better suited for certain types of investigations. The interested reader is referred to recent reviews on different aspects of reaction-based H_2S probe development.¹⁵

The most common reaction-based detection strategy to date is H_2S -mediated azide ($\text{R}-\text{N}_3$) reduction (Scheme 4a).¹⁵ Based on the ease of appending azides onto fluorogenic scaffolds, over 70 papers in the last few years described H_2S -detecting scaffolds using an azide to generate turn-on fluorescent probes (Scheme 4a).⁴⁶⁻⁴⁸ Reduction of other oxidized nitrogen functional groups, such as nitro groups, has also been utilized for H_2S detection.⁴⁸ For most fluorophores, the azide moiety quenches fluorescence by a push-pull energy transfer mechanism although other quenching mechanisms are also possible. Although the selectivity of azides for reduction by H_2S rather than biological thiols



Scheme 3 (a) Methylene blue (MB) and (b) monobromobimane (mBB) methods for H_2S quantification.





Scheme 4 Common motifs in reaction-based probes for H_2S , including reactions based on (a) reduction, (b) nucleophilic attack, and (c) metal precipitation.

is predominantly empirical, most azide-based probes provide good to excellent selectivity for H_2S over other RSONs. A recent mechanistic investigation established that HS^- is the active species involved in H_2S -mediated azide reduction, and that sulfide is oxidized to sulfane sulfur, such as HSS^- , during probe activation.⁴⁹

Further leveraging the high nucleophilicity of HS^- , a variety of H_2S sensing strategies have been developed in which sulfide attacks an electrophilic, fluorescence-quenching group appended on a fluorophore. Such scaffolds generally rely on the ability of H_2S to participate in two sequential nucleophilic reactions to remove or modify the protecting group. One benefit of this strategy is that it imparts inherent selectivity because thiols, which can only participate in one nucleophilic attack, are unable to remove the protecting group.¹⁵ One consequence of this selectivity, however, is that in many cases thiols can react with the electrophilic scaffolds, thus effectively consuming the probe prior to reaction with H_2S . This general strategy for H_2S sensing has been used in activated disulfides, α - β -unsaturated ketones, and other activated electrophiles (Scheme 4b).⁵⁰⁻⁵²

In addition to exploiting the high nucleophilicity of HS^- for sensing, the high metallophilic nature of sulfur has also been exploited.⁵³ For example, ligation of a paramagnetic metal ion, such as Cu^{2+} , to a fluorescent molecule results in fluorescence quenching. Subsequent reaction with H_2S results in CuS formation and precipitation, thus releasing the fluorophore and producing a turn-on response. This strategy is inherently selective for sulfide over other biological thiols, but depending on the ligand characteristics may also be prone to metal reduction and release by cellular reductants or thiols, or exchange of the quenching paramagnetic metal ion with more prevalent diamagnetic ions like Zn^{2+} .

Beyond H_2S , polysulfides and persulfides are quickly becoming reactive sulfur species of interest. In addition to contributing to the biological chemistry associated with H_2S , it is likely that

these sulfane-sulfur species provide signaling pathways distinct to those associated with sulfide. Aligned with this importance, reaction-based probes for polysulfides and other sulfane-sulfur sources are now beginning to emerge.^{54,55} The development and refinement of these, as well as other, tools appear poised to provide complementary information on the different pools of biological sulfide and further inform on the role of H_2S and sulfane-sulfur in the RSONs landscape.

Conclusions and outlook

Hydrogen sulfide is an increasingly important biological molecule joining NO and CO as a key signaling agent and adding a new level of complexity to the RSONs landscape. Importantly, many of the unanswered questions in the field lay at the interface of chemistry and biology, thus requiring collaborative investigations and innovation to develop, refine, and apply new investigative tools in this rapidly expanding area of research. Key challenges include developing selective inhibitors of H_2S producing enzymes, generating new classes of sulfide donors that do not consume cellular thiols for activation or that can be programmed to release H_2S in response to certain stimuli, and refining H_2S imaging methods so that they do not irreversibly consume sulfide. As researchers progress toward these goals, the toolbox of available methods for studying biological H_2S will continue to grow and become more accessible to researchers in adjacent fields (such as NO or CO biochemistry, redox biology, and pharmacology), thus broadening the potential impact of H_2S -related investigations in these diverse scientific communities.

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