Prodrugs of hydrogen sulfide and related sulfur species: recent development

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[ABSTRACT] Hydrogen sulfide (H₂S) is commonly referred to as the third gasotransmitter with firmly established physiological roles. Prodrug approaches have been broadly applied to deliver H₂S for various applications and mechanistic studies. Since S-persulfidation and glutathionylation are known to be important in cellular signaling by sulfur species, there have been interests in developing donors of persulfide and glutathione persulfide as well. In this review, we discuss the recent development in area of prodrugs for various sulfur species.

[KEY WORDS] H₂S; S-persulfidation; Light-sensitive H₂S prodrugs


Introduction

Hydrogen sulfide (H₂S) is widely known as a gas with a bad smell and toxicity. Less known is the fact that H₂S is a naturally occurring small signaling molecule in mammal. H₂S is often referred to as the third gasotransmitter alongside nitric oxide (NO) [1] and carbon monoxide (CO) [2], though some suggest that they be best termed as small signaling molecules [3]. Among the trinity of gasotransmitters, H₂S has the unique property of capable of existing in multiple forms including gas, solid (as a salt), or being dissolved in solution in either free or an ionized form under physiological conditions. It is also interesting to note that there is a good number of natural products, such as garlic-derived organosulfur compounds, which produce various sulfur species under certain biological conditions [4-6]. Several studies indicate that the pharmacologic effects from garlic-rich diet are related to H₂S generation [5-6]. The reported therapeutic effects of H₂S include anti-inflammation, anti-tumor, anti-oxidation, and ion channel regulation, among others [7]. There have been much effort in demonstrating the tremendous therapeutic potential of H₂S as has been covered in some recent comprehensive reviews [5-7, 14]. Therefore, this review will avoid duplicating what has already been covered in recent reviews and instead focus on the chemistry of sulfide donors, broadly define. Specifically, much of the studies of sulfide signaling depends on the availability of appropriate tools for the delivery of desired sulfur species to aid the elucidation of mechanisms. In discussing the field of sulfide donors, there are three important issues. First, sometimes sulfide-related signaling actually requires a species at a higher oxidation state than sulfide: e.g. persulfide [15-16]. Therefore, there is the question of whether there is a need to deliver a sulfur species at the desired oxidation state. Second, some sulfur species, such as hydrogen sulfide, hydrogen persulfide, and perthiol can undergo rapid exchange/scrambling, depending on the redox state of the solution or cellular environment [17]. Then a donor of “hydrogen sulfide” could be to increase the “sulfur pool,” instead of donating hydrogen sulfide itself per se. Third, the release mechanism and kinetics from each donor can make a difference in terms of the end results, especially in terms of the “effective concentration” of the desired sulfur species. We intend to cover new developments in this area by including information on reaction kinetics and oxidation state so that readers can examine these above issues should they be interested. In 2017, we reviewed reported H₂S donors up to that point and discussed several critical remaining issues in developing H₂S-based therapeutics [18]. This article will update recent developments by covering select examples since then.

Chemical Properties of H₂S

H₂S is known to be a colorless toxic gas with a strong rotten egg smell. With the first pKa of 6.88 and second pKa of 19, H₂S largely exists in the mono-ionized form under physiological conditions (pH = 7.4, 37 °C). H₂S and its ionized forms are easily oxidized in aqueous solution. The oxidation of H₂S is complicated, producing various sulfur species including elemental sulfur, persulfide, polysulfide, sulfite,
sulfate, thiosulfate etc \[^{15, 19}\]. In addition, some of these redox reactions are readily reversible. Another important property of the various sulfur species is their nucleophilicity. The large number of reactions that H\(_2\)S can undergo leads to challenges in studying its mechanism of action including difficulties in the real-time determination of the exact concentration of H\(_2\)S in biological systems and distribution of species at various oxidation states \[^{20-21}\]. Below we discuss the various sulfide donors in the context of these issues.

**S-persulfidation**

Post-translational modification (PTM) of protein is important in cellular signaling. In terms of sulfur-related PTM, a cysteine residue of a protein can undergo glutathionylation \[^{22-23}\], S-sulfenylation \[^{26-27}\], and S-persulfidation \[^{20-21}\] under different biochemical and redox environments. Up to now, more than 50 proteins have been reported as undergoing S-persulfidation as part of PTM, which is one mechanism through which H\(_2\)S is involved in signaling \[^{12-13}\]. Because subsequent discussions heavily involves donors for persulfidation, this section gives a brief description of persulfidation chemistry.

From a chemistry perspective, persulfide can be formed either from the oxidation of thiol species or reduction of polysulfide or other species at a higher oxidation state. Others and we have reported that H\(_2\)S can independently induce S-persulfidation on GAPDH \[^{32, 34-35}\]. In the presence of ROS, thiol group can be oxidized to sulfenic acid or further into sulfonic acids (Fig. 1) \[^{30}\]. As a simple reducing agent, H\(_2\)S itself can directly react with oxidative species to quench the above oxidations. Small polysulfide molecules can also be reduced by H\(_2\)S to produce persulfide. More importantly, several studies demonstrated that H\(_2\)S can reduce sulfenic acid to persulfide as a mechanism of reducing oxidative stress since further oxidation to the stage of sulfonic or sulfonic acid would lead to irreversible changes (Fig. 1) \[^{38}\]. In the cellular environment, the level of H\(_2\)S and persulfidated proteins are strongly correlated with introduction of oxidative species such as H\(_2\)O\(_2\).

In studying protein persulfidation, it is important to be able to selectively capture or identify persulfidation site(s). Along this line, several methods have been developed. Methyl methanethiosulfonate (MMTS) was previously reported to specifically react with thiols rather than persulfides, however, several studies have shown that persulfides can also react with MMTS \[^{15, 17}\]. Later it was found that this approach is not selective for persulfidation. Another strategy to detect persulfidation first uses iodoacetic acid (IAA) to react with both of thiol and persulfide residues. Only the disulfide residues formed with IAA can be reduced by dithiothreitol (DTT) to yield the free thiol group, but not the product from thiol and IAA. This two-step process allows for the labeling of the thiol product from DTT reduction, and thus unequivocally identifies the persulfidation site. In 2014, Xian and coworkers developed a two-steps tag-switch method for detection of protein S-persulfidation (Fig. 2A) \[^{38}\]. In the first step, a thiol-blocking reagent, methylsulfonyl benzothiazole (MSBT) can be used to block thiol and persulfide residues. Then a cleverly designed carbon-based nucleophile, methyl cyanoacetate (MCA) is used to selectively tag the disulfide intermediate by removing the MSBT moiety. In solution studies, the yield of MCA and a derivative of cysteine disulf-
Prodrugs

Historically, among all the reports in designing donors of various sulfur species, work in designing donors of H$_2$S is the most active. In recent years, this continues to be a very active field as one would understand. Recent activities have gravitated toward donors with triggered release using different mechanisms including light, pH, esterases, bioorthogonal reactions and thiols. Carbonyl sulfide (COS) can be hydrolyzed to H$_2$S by carbonic anhydrase (CA) in mammalian cells and has proven to be an important class of donors. In terms of linker chemistry used to tether the “trigger” with the sulfide donor moiety, the 1, 6-elimination strategy was the most widely explored for developing COS prodrugs (See below). Below we give an overview of recent developments.

Light-sensitive H$_2$S Prodrugs

In the first example of photo-sensitive prodrugs, H$_2$S was caged in the form of thioaldehyde, which can release H$_2$S in the presence of amines. Specifically, Connal and coworkers protected the thiobenzaldehyde by a photolabile group. Upon irradiation at 355 nm, the photocleavage reaction triggers the formation of the acetophenone byproduct and thioaldehyde $1$, which further reacts with an amine to release H$_2$S (Fig. 3A).

Both the irradiation time and the amount of amine affect H$_2$S release from the donor $^{[39]}$ Upon irradiation for 10 min, 5 min and 2 min, 133 μmol·L$^{-1}$ of the donor was able to give a sustained concentration of 60 μmol·L$^{-1}$, 30 μmol·L$^{-1}$ and 15 μmol·L$^{-1}$, respectively, within 30 min. When irradiation time was fixed at 10 min, 1 equivalent of glycine gave around 10 μmol·L$^{-1}$ H$_2$S from 133 μmol·L$^{-1}$ prodrug, while 20 equivalents of glycine triggered the release of 36 μmol·L$^{-1}$ of H$_2$S in PBS/ACN solution. Based on these small-molecule H$_2$S donors, three water-soluble polymer-based H$_2$S releasing materials were prepared. All of these polymeric prodrugs showed a more gradual H$_2$S release rate than the corresponding small-molecule donors. In 30 mins under UV light, 0.27 mg mL$^{-1}$ of water-soluble polymer-based thioaldehyde gradually released H$_2$S to a peak concentration of 60 μmol·L$^{-1}$, while 2.7 mg mL$^{-1}$ the corresponding hydrophobic polystyrene film-based donor reached a peak concentration of 20 μmol·L$^{-1}$ in PBS buffer. These polymeric materials were also studied for their ability to deliver H$_2$S to 3T3 fibroblast cells by using a green fluorescent H$_2$S probe, WSP-1. Upon UV irradiation for 1 to 5 min, fluorescence increase was observed and was interpreted as validation of the existence of H$_2$S in cells. Then thioaldehyde loaded into water-soluble polymer was used to study for its effect on inhibition of platelet aggregation. In an adenosine diphosphate (ADP)-stimulated platelet aggregation assay, it was found that 3 mg mL$^{-1}$ water-soluble polymeric H$_2$S prodrug was able to reduce the expression of P-selectin, which functions as a cell-adhesion molecule to mediate platelet aggregation.

In addition to using direct photo-triggered release, there are also H$_2$S donors that respond to photosensitizers. You and coworkers utilized a singlet oxygen (O$_2$) photosensitizer (PS) to initialize H$_2$S release from 1,3-diarylisobenzothiophene (DPBT) $^{[40]}$. Under visible light irradiation, the PS reacts with oxygen to produce O$_2$, leading to reaction between O$_2$ and DPBT to yield an unstable endoperoxide $5$ (Fig. 3B), which undergoes fragmentation to give H$_2$S and a 2-benzoylbenzophenone byproduct. The reaction rate between DPBT and O$_2$ was determined to be $7.4 \times 10^7$ mol·L$^{-1}$·s$^{-1}$. The formation of 2-benzoylbenzophenone was detected by HPLC. The same approach was used to develop a polymeric vesicle-based system to deliver H$_2$S by combining PS, DPBT and a fluorescent H$_2$S probe (SF4) in the Pluronic F-127 polymer. Understandably, the PS (PtOEP or IrOMe) makes a difference in the H$_2$S release rate and peak concentration. When 0.2 wt% DPBT and 0.01 wt% PS were used in the polymeric vesicle

![Fig. 3 Light-sensitive H$_2$S prodrugs](image-url)
system, H$_2$S concentration reached 4 μmol·L$^{-1}$ after 2 h with PtOEP as the PS, while 8 μmol·L$^{-1}$ of H$_2$S was achieved after 8 h irradiation when IrOMe was used. This rate difference was explained by a higher photon absorption of PtOEP ($ε = 6000$ mol·L$^{-1}$·cm$^{-1}$ at 532 nm) than IrOMe ($ε = 1900$ mol·L$^{-1}$·cm$^{-1}$ at 432 nm). In a study in HeLa cells, pretreatment with this H$_2$S prodrug (polymersomes doped with 0.2wt% DPBT and 0.01 wt% PtOEP) reduced acute apoptosis triggered by H$_2$O$_2$. However, no quantitative data was presented.

Singh presented a UV-Vis activated H$_2$S prodrug based on the Excited-State Intramolecular Proton Transfer (ESIPT) mechanism [41]. Upon irradiation, the donor molecule 7 was proposed to undergo ESIPT between the para hydroxyl group and benzothiazole moiety to yield an unstable intermediate, leading to intersystem crossing followed by a photo-Favorskii rearrangement to release H$_2$S and a blue fluorescent spirodiketone byproduct 8 (Fig. 3C). H$_2$S release kinetics was studied by using HPLC and the methylene blue test for H$_2$S. In pH 7.4 acetonitrile/PBS buffer (3 : 7), upon irradiation at ≥ 410 nm, the first-order rate constant for the decomposition of the donor was determined to be 1.74 × 10$^{-5}$ s$^{-1}$ when starting material disappearance was used for reaction monitoring. 100 μmol·L$^{-1}$ of the donor was able to release around 40 μmol·L$^{-1}$ of H$_2$S in 20 min, and the pseudo-first-order rate constant for the release was determined to be 1.32 × 10$^{-5}$ s$^{-1}$. The donor was also tested for delivering H$_2$S to HeLa cells by monitoring the fluorescence changes from green to blue fluorescence upon light irradiation, suggesting intracellular release of H$_2$S from the donor. No clear cytotoxicity towards HeLa cells was observed at up to 20 μmol·L$^{-1}$ dosage.

Pluth and coworkers masked a thiocarbamates with a photolabile ortho-nitrobenzyl protecting group to obtain UV-sensitive H$_2$S prodrugs [42]. Under UV light (365 nm), the protecting group is removed, releasing thiocarbamate intermediate 10, which decomposes to release carbonyl sulfide (Fig. 3D). Two prodrugs were synthesized with different substitutions on the masking ortho-nitrobenzyl group. In the presence of 25 μmol·L$^{-1}$ carbonic anhydrase, 50 μmol·L$^{-1}$ of compound 9 gave peak concentrations 12 μmol·L$^{-1}$ at around 10 min. No biological data was reported in the original literature.

These photo-sensitive prodrugs are very useful when spatio-temporal control is needed.

**pH-sensitive H$_2$S Prodrugs**

pH-sensitive prodrugs have been used in drug delivery for a long time. They can be used for selective activation depending on pH differences. For example, in the gastrointestinal system, the pH increases from the stomach to the colon. Further, the environment surrounding solid tumor is often more acidic than normal tissue and endosome and lysosome also have much lower pH than under physiological conditions. These pH differences can all be explored for targeted drug delivery.

Xian and co-workers reported ammonium tetrathiomolybdate (TTM) as a pH-dependent water soluble H$_2$S prodrug [43]. It was found that at pH 5, 500 μmol·L$^{-1}$ of TTM led to the generation of around 110 μmol·L$^{-1}$ of H$_2$S while only 30 μmol·L$^{-1}$ of H$_2$S was generated under otherwise identical conditions except for being at pH 7.4. The prodrug at 50 to 200 μmol·L$^{-1}$ showed protective effects on HaCaT cells against H$_2$O$_2$ along with decreased release of lactate dehydrogenase (LDH) and reduced mitochondrial membrane potential loss. Xian’s lab also reported a class of phosphonamidothioate-based H$_2$S prodrugs [44]. Under neutral or acidic conditions, the phosphorothioils 13 are formed by protonation. The subsequent cyclization by a nucleophilic carbonylate can drive the release of H$_2$S in PBS (Fig. 4A). At pH 7.4, 40 μmol·L$^{-1}$ of H$_2$S was detected from 100 μmol·L$^{-1}$ of the donor 11 at 90 min, while at pH 5.0, near 90 μmol·L$^{-1}$ of H$_2$S was detected within 10 min. A H$_2$S fluorescent probe, WSP-5, was used to affirm the delivery of H$_2$S into HeLa cells. The prodrug’s protective effect was assessed in a murine model of myocardial ischemia-reperfusion injury. Bolus injections of prodrug 11 at a dose of 50 or 100 μg·kg$^{-1}$ reduced the infarct size per area-at-risk (INF/AAR) by 55% and 56%, respectively.

Pluth and coworkers reported a γ-ketothiocarbamate compound as a base-sensitive COS prodrug [45]. In PBS, prodrug 15 can be converted to an enol form 16, which undergoes β-elimination to generate COS, methyl vinyl ketone and p-nitroaniline, which has a yellow color (Fig. 4B). Upon incubation of the donor in PBS containing carbonic anhydrase, the formation of H$_2$S was detected by the MB assay. The rate constant ($K_{obs}$) was determined to be 0.38 × 10$^{-3}$ s$^{-1}$ and 12.6 × 10$^{-3}$ s$^{-1}$ at pH 6.0 and 8.0, respectively. Prodrug 15 at 25 μmol·L$^{-1}$ was found to reduce LPS-induced NO$^+$ formation by around 25% when compared to the vehicle group. However, it was interesting to see that the control compound without the ability to release COS also led to a decrease of around 40% in LPS-induced NO$^+$ formation. The effect of the control was attributed to the byproduct, methyl vinyl ketone.

**Thiol-sensitive H$_2$S Prodrugs**

Thiol groups in the form of cysteine, glutathione, and hy-
Hydrogen sulfide are present throughout the body. Further, some thiol species are enriched at certain sites. For example, glutathione has a much higher concentration intracellularly (mmol·L⁻¹) than in the blood (μmol·L⁻¹); hepatocytes tend to have a higher concentration than other cell types; the lining fluid of the lung tends to have elevated concentrations of glutathione; stem cells may require high concentrations of glutathione; cancer and redox stress result in variations of intracellular glutathione concentrations; intracellular distribution among different compartments is heterogeneous; and there may exist a total glutathione gradient across the ER membrane. Therefore, thiol-activated hydrogen sulfide prodrugs are useful tools for various applications.

Martelli reported several derivatives of iminothioether as cysteine-sensitive H₂S prodrugs (Fig. 5A). In the presence of 4 mmol·L⁻¹ of cysteine (Cys), the half-lives of this series of prodrugs ranged from 0.2 to 11.9 min. At 1 mmol·L⁻¹ of the prodrug concentration, the maximal concentrations of H₂S achieved within 30 min of incubation ranged from 0.31 to 19.0 μmol·L⁻¹. Prodrugs 19 and 20 with half-lives 0.28 and 5.7 min, respectively, were used in biological assays. In a rat model of L-NAME-induced hypertension, it was found that prodrug 20 decreased the basal systolic pressure by 39 ± 5%. In contrast, the fast-release prodrug 19 did not show any effect. Such results suggest the need to examine the effect of duration and sustained concentration of H₂S.

Xu and coworkers reported the application of modified thioester compounds as slow-releasing H₂S donors. It was proposed that thioester 21 can be cleaved by Cys to yield allyl mercaptan 23. Subsequent oxidation and proposed thiol-mediated substitution would generate an unstable persulfide intermediate 27, which decomposes to release H₂S (Fig. 5B). In the presence of 1 mmol·L⁻¹ of cysteine, 15 to 52 μmol·L⁻¹ peak concentrations of H₂S were detected from 100 μmol·L⁻¹ of the various prodrugs in 3 to 8 hours. The cardioprotective effects of prodrug 21 was studied in a mouse model of myocardial infarction. A reduction of the infarct size to 75% or 64% of the control was observed after administration of the prodrug at 15 mg·kg⁻¹·day⁻¹ and 30 mg·kg⁻¹·day⁻¹, respectively. In this case, because H₂S is generated from the decomposition of persulfide, it is hard to totally attribute the biological effects to H₂S per se.

Caliendo and coworkers presented a strategy to use 1,2,4-thiadiazolidin-3,5-diones as thiol-sensitive H₂S donors. The thiol group on cysteine could initiate the nucleophilic substitution and thiol-disulfide exchange to form an unstable thiol acid intermediate 31, which can be hydrolyzed to release H₂S (Fig. 5C). In the presence of 4 mmol·L⁻¹ cysteine, 1 mmol·L⁻¹ of the prodrug exhibited a slow release profile, and the maximal concentrations of H₂S ranged from 2 to 65 μmol·L⁻¹. All analogous compounds were examined in a vasorelaxation experiment using isolated aortic rings, among which prodrug 29 exhibited the most significant effect with an EC₅₀ of 1.7 μmol·L⁻¹. It should be noted that the release process leads to the formation of more than one sulfur species. Therefore, the attribution of the observed vasorelaxation effect might need additional work to clarify.

Pluth reported caged-sulfonyl thiocarbonate derivatives of fluorescein as thiol-sensitive COS prodrugs with fluorescent output. In the presence of thiol species, the sulfonyl thiocarbonates can be cleaved to generate thiocarbonate intermediates, which further decompose to release COS and turn on the fluorescence of fluorescein (Fig. 5D). In the presence of 100 μmol·L⁻¹ cysteine and 25 μg·ml⁻¹ CA, 10 μmol·L⁻¹ of the prodrug 34 reached a fluorescent plateau within 20 min with 96% of a H₂S release yield. Further anti-inflammatory studies showed that 5−25 μmol·L⁻¹ of the donor 33 was able

Fig. 5 Thiol-sensitive H₂S prodrugs
to inhibit the NO\textsuperscript{2-} accumulation in RAW 264.7 cells induced by LPS. The same lab also reported cyclic sulfinyl thiocarbamates as thiol-sensitive COS prodrugs [60]. In the presence of a thiol species, the disulfide bond can be cleaved followed by dethioacboxylation to release COS (Fig. 5E). It was found that the prodrugs were responsive to cellular thiol species including GSH, Cys, Hey, NAC and penicillamine with various release rates. For example, in the presence of 1 mmol·L\textsuperscript{-1} of GSH, 25 μmol·L\textsuperscript{-1} of donor 37 released almost stoichiometric amount of H\textsubscript{2}S in PBS at room temperature. Further work involved the conjugation of the cyclic sulfinyl thiocarbamates to a non-steroidal anti-inflammatory agent (NSAID), naproxen. H\textsubscript{2}S delivery from the donor to HeLa cells was detected within 30 min by using a H\textsubscript{2}S fluorescent probe, SF7-AM. The biological activities of the drug and the H\textsubscript{2}S donor were not reported. In the presence of cysteine, thionoesters were also used as H\textsubscript{2}S prodrugs [60]. The thiol on cysteine can first attack the electrophilic carbon on prodrug 38 to form intermediate 39, which further generates compound 40 by releasing a phenol molecule (Fig. 5F). The subsequent intramolecular cyclization reaction initiated by the amino group on cysteine leads to the formation of intermediate 41, leading to the release of H\textsubscript{2}S. In the presence of 500 μmol·L\textsuperscript{-1} of cysteine, 25 μmol·L\textsuperscript{-1} of the donor 38 released around 20 μmol·L\textsuperscript{-1} H\textsubscript{2}S in PBS. The second order rate constant was determined to be 9.1 ± 0.3 mol·L\textsuperscript{-1}·s\textsuperscript{-1}.

It is important to note that some of the donors have persulfide structures. Thus, it is possible that some of the observed biological effects can be attributed to the persulfide, instead of hydrogen sulfide.

Esterase-sensitive H\textsubscript{2}S Prodrugs

Esterases are ubiquitous and have long been used for activation of prodrugs for various applications [88]. Naturally, the same strategy has been used in hydrogen sulfide prodrugs [18, 59]. As a recent example, Park and coworkers reported 1,3,5-trithiane installed with an esterase-sensitive trigger as a H\textsubscript{2}S donor [40]. The authors proposed a sophisticated mechanism to show that in the presence of an esterase, the prodrugs 44 and 50 can be hydrolyzed to form unstable intermediates 45 and 51, which undergo a cascade of reactions to release H\textsubscript{2}S (Fig. 6). Intermediates 47 and 48 were detected by GC-MS. In the absence of porcine liver esterase (PLE), 200 μmol·L\textsuperscript{-1} of donors 44 and 50 were able to generate around 5 to 10 μmol·L\textsuperscript{-1} of H\textsubscript{2}S in PBS within 4 h, while with the introduction of 1 unit of PLE, the concentration of H\textsubscript{2}S increased by one-fold. Cell viability assays did not show significant of toxicity of the donor at up to 200 μmol·L\textsuperscript{-1} in H9c2 cells.

Bio-orthogonal Reactions-based H\textsubscript{2}S Prodrugs

Recent years have seen much effort in using bioorthogonal reactions for prodrug activation including sulfide prodrugs [60]. Pluth reported a click-release strategy to release COS by using an inverse-electron demand Diels-Alder (IEDDA) reaction [62]. Specifically, a thiocarbamate is tethered to a trans-cyclooctene, which can undergo a facile reaction with tetrazine, leading to-functionalized dihydropridazine 55, which can undergo tautomerization, deprotonation, and re-aromatization to release COS (Fig. 7). In PBS buffer, 50 μmol·L\textsuperscript{-1} of donor 54 in the presence of 25 equivalents of tetrazine was able to release 12 μmol·L\textsuperscript{-1} of H\textsubscript{2}S as measured by a selective H\textsubscript{2}S electrode. Similar release was achieved in a mixture of sheep blood/plasma and PBS 1 : 1, though the release rate was slower than in buffer. However, in cell imaging studies, it was found that the donor was unable to turn on H\textsubscript{2}S fluorescent probes such as HSN\textsubscript{2}, WSP-5 and SF7-Am. Possible reasons for this was thought to in-

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**Fig. 6** Esterase-sensitive H\textsubscript{2}S Prodrugs
include quenching by tetrazine or the slow and ineffective release of H\(_2\)S from the donor.

**H\(_2\)S-drug Conjugates**

Because of the known anti-inflammatory effects of H\(_2\)S, it has been widely used to conjugate with other NSAIDs to reduce the side effects of the parent drug and also to create synergy. Qian and coworkers developed a series of hydrogen sulfide releasing capsaicin derivatives \[60\]. Capsaicin, derived from chili peppers, shows many types of therapeutic activities including analgesic, anti-inflammatory, cardioprotective and antioxidative effects. However, it can cause irritation after ingestion or inhalation. Based on the parent structure of capsaicin, several H\(_2\)S donors, ADT-OH, 4-OH-TBZ or 4-carbamothioylbenzoic were conjugated to the free phenolic OH at the C4 position of capsaicin. Totally 14 analogs of capsaicin and dihydrocapsaicin were synthesized via conjugation with various H\(_2\)S-releasing moieties. A fluorescent H\(_2\)S probe, DNZ-Az was used to study the H\(_2\)S release from conjugate 59 to 62 (Fig. 8A). In DMEM cell culture media, prodrug 60 showed the highest release ratio, which was 20% after 24 h incubation. The anti-nociceptive effects of the conjugates were evaluated by the capsaicin test, abdominal constriction test and tail flick test. It was found that analogs 59 to 62 exhibited better overall performance than the other compounds. For example, in an abdominal constriction test on mice, upon addition of 60 mg kg\(^{-1}\) of conjugate 60, the number of acid-induced writhes is five-fold lower than that of capsaicin-treated group. The antiproliferative activity of these compounds was also assessed on K562, Hela and MCF-7 cell lines. Conjugate 60 showed \(IC_{50}\) value of 4, 4 and 2 \(\mu\)mol L\(^{-1}\) on K562, Hela and MCF-7 cells respectively, which are much lower than that of the parent drug. To compare the gastric mucosal irritation response from parent drug and the H\(_2\)S conjugates, 30 mg kg\(^{-1}\) of prodrugs 59 to 62 p.o. were able to reduce the ulcerogenic scores by around 1-fold compared with CAP when studied in a rat model of peptic ulcer.

Li and coworkers reported several derivatives of the conjugates between enmein-type diterpenoids and H\(_2\)S prodrugs, thioctic acid or ADT-OH \[65\]. H\(_2\)S releasing ability of the prodrugs was measured by the MB assay. It showed that all tested conjugates exhibited a relative fast release with half-life under 5 min. One of the analogs, compound 63 (Fig. 8B) showed the most potent cytotoxicity against cancerous cell lines with \(IC_{50}\) of about 2 \(\mu\)mol L\(^{-1}\) on Bel-7402 cells, while the \(IC_{50}\) of the parent drug is about 23 \(\mu\)mol L\(^{-1}\). Compared to the parent drug, the conjugated prodrug 63 also has improved selectivity against cancer cells than normal cells. Between Bel-7402 cells and L-02 cells (normal cells), the selectivity of prodrug 63 is six-fold higher than the parent drug. Further mechanistic studies of the anti-proliferative effects of prodrug 63 led to the observation of G1 phase cell cycle arrest and mitochondrial membrane potential collapse upon the prodrug treatment.

**1,6-Elimination based COS (H\(_2\)S) Prodrugs**

In designing prodrug linkers, a 1, 6-elimination system is often used to tether the drug with a triggering moiety. Examples are included in Fig. 9. Most of them rely on the easy formation of a quinoid or a quinone methide structure if there is a benzylic leaving group or a protected amino or hydroxyl group. The same strategies have been applied to preparing COS prodrugs.

**Photo-sensitive COS (H\(_2\)S) Prodrugs**

Chapkrapani and coworkers reported a visible light-sensitive COS donor. In such a design, the masking group was changed to a BODIPY-based photolabile moiety (Fig. 9A) \[66\].

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**Fig. 7** Bio-orthogonal reactions-based H\(_2\)S prodrugs

**Fig. 8** H\(_2\)S-drug conjugates
A short oligo-ethylene glycol group was incorporated in the masking BODIPY part to increase aqueous solubility of the donor. Upon exposure to 470 nm light in MeOH, donor 64 decomposition and BODIPY solvolysis rate constants were determined to be 0.16 min⁻¹ and 0.20 min⁻¹ respectively. However, in pH 7.4 buffer, the decomposition rate constant decreased to 0.02 min⁻¹. H₂S generation was examined by the methylene blue assay and a H₂S-sensitive electrode. The donor was first irradiated at 470 nm followed by incubation with carbonic anhydrase in pH 7.4 buffer. The rate constant for H₂S release from the light activated-mixture was measured to be 0.03 min⁻¹, and the H₂S yield was found to be in the range from 30% to 40%.

**Reactive Oxygen Species (ROS)-sensitive COS (H₂S) Prodrugs**

Pluth and coworkers used an arylboronate moiety as an ROS-sensitive group to protect the phenol hydroxyl group (Fig. 9B) [66]. This design takes advantage of the reactivity of a boronate group with ROS, leading to the formation of a hydroxyl group. In the presence of 250 μmol·L⁻¹ or 500 μmol·L⁻¹ H₂O₂ and 25 μg mL⁻¹ CA in PBS, the yields of H₂S from 50 μmol·L⁻¹ of the prodrug 66 were 80% and 60%. The decreased yield of H₂S at a higher H₂O₂ concentration is probably due to H₂S scavenging by H₂O₂. The apparent second-order rate constant was measured to be 1.44 mol L⁻¹·s⁻¹ by using a H₂S selective electrode. To examine selectivity in prodrug activation, the effects of H₂O₂, O²⁻, ONOO⁻, ClO⁻, HO²⁻, singlet oxygen, cysteine, glutathione, GSNO, nitrite, sulfate, and NO were studied. It was found that only H₂O₂, O²⁻, ONOO⁻ were able to trigger H₂S release. Prodrug 66 showed no cytotoxicity on HeLa cells at up to 100 μmol·L⁻¹ concentration. To see the effect of endogenous H₂O₂ on the prodrug, RAW 264.7 cells were pre-incubated with 500 nmol·L⁻¹ phorbol 12-myristate 12-acetate (PMA) for 3 h before addition of the prodrug 66 and HSN₂. Compared to the control group, only the prodrug group turned on HSN₂ fluorescence in the presence of PMA. Further, the H₂O₂-sensitive donor (66) was tested for its ability to attenuate cytotoxicity induced by H₂O₂ in HeLa cells. Specifically, 50 μmol·L⁻¹ of the prodrug was able to rescue cells from 30% viability to around 70%. The control compound with only the H₂O₂ sensing part was only able to rescue cells to around 40%, suggesting the protecting effects being from COS or H₂S as well as from the boronate moiety. It is worth mentioning that H₂O₂ seemed to be able to directly convert COS to H₂S; however, detailed mechanism was not discussed in the publication.

By utilizing a similar 1, 6-elimination system, Chakrapani and coworkers also reported a series of ROS-sensitive COS prodrugs (Fig. 9B) [67]. Instead of using thiocarbamates, the Chakrapani study designed carbamothioates as the COS core. By tuning the pKa of the leaving group (amino group) from 5.34 to 10.53 (Fig. 9B), the release half-lives changed from 23.9 to 203 min.

**Esterase-sensitive COS (H₂S) Prodrugs**

Chakrapani and coworkers reported pivaloyloxymethyl-based carbonothioates and carbamothioates as esterase-sensitive CO prodrugs [68]. Upon addition of PLE, the removal of the pivaloyloxymethyl group would initiate the 1,6-elimination reaction to release COS (Fig. 9C). The kinetics were studied by a H₂S-fluorescent probe, Dn-Az. In the presence of 1 U PLE, the first-order rate constants of the prodrugs ranged from 0.01 to 0.04 min⁻¹.

Pluth reported a series of esterase-triggered thiocarbamates-based COS prodrugs [69]. Upon removal of the ester group by an esterase, the donor would undergo self-immolation to release COS (Fig. 9C). By tuning the steroid hindrance of the ester group, the kobs of the release rate can be tuned from 1.6 × 10⁻⁴ to 2.7 × 10⁻³ s⁻¹. In cytotoxicity studies, it was found that the smaller the hindrance of the ester protecting group is, the more cytotoxic effect from the donor was observed. For example, prodrug 72 has the smallest ester

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Fig. 9  1,6-Elimination based COS (H₂S) prodrugs
Cysteine-sensitive COS (H₂S) Prodrugs

Pluth and coworkers also reported a cysteine-triggered COS prodrug approach based on a self-immolative thioacarbamate \[^7\]. The design takes advantage of the presence of a Michael acceptor for capturing a thiol group through Michael addition. Then the thioether conjugate can undergo an intramolecular cyclization to cleave the phenolic intermediate, leading to a 1, 6-elimination reaction to release COS (Fig. 9D). In the presence of 500 \(\mu\)mol L\(^{-1}\) of cysteine, 50 \(\mu\)mol L\(^{-1}\) of the donor 74 led to 14 \(\mu\)mol L\(^{-1}\) of H₂S at around 2 h. For the selectivity studies, other biological nucleophiles such as serine, GSH, and lysine were also used; it was found that only cysteine was able to trigger the formation of H₂S in the presence of 25 \(\mu\)g mL\(^{-1}\) CA. H₂S formation was also confirmed with a fluorescent probe, SF7-AM, in bEnd.3 cells.

As summarized above, H₂S prodrugs with triggered release using various stimuli have been developed in recent years. These prodrugs should expand the toolbox available for future investigations into sulfide-mediated signaling.

H₂S₂ and Persulfide Prodrugs

It is well known that one of the mechanisms through which sulfur signaling happens is S-persulfidation or sulfhydration \[^36, 34, 36\]. It is very important to note that persulfidation cannot happen with hydrogen sulfide \(\text{per se}\). It has to be converted to a species at a higher oxidation state, e.g. persulfide. It is believed that increased persulfidation in the presence of hydrogen sulfide is at least partially the result of increased “sulfur pool” and subsequent oxidation to a higher oxidation state, which allows for persulfidation to happen. Alternatively, cysteine thiol can be oxidized to sulfenic acid, which can be converted to persulfidation product by reaction with H₂S. Then there is the question of whether delivering sulfur species at the persulfide state would offer more efficient persulfidation in solution, \textit{in vitro}, and \textit{in vivo}. With such thinking in mind, there have been efforts in making prodrugs and donors or hydrogen persulfide and perthiol.

A series of esterase-sensitive persulfide prodrugs was developed in our lab (Fig. 10A) by taking advantages of an unstable "hydroxymethyl disulfide" intermediate \[^7\]. After the removal of the ester masking group from the donor, the persulfide is released from the decomposition of the "hemiacetal" intermediate. By varying the hindrance on the masking ester group, we were able to tune the half-lives of the prodrugs from 12 to 145 s in the presence of 1 U PLE. For analysis of the release yield, persulfide was directly trapped by an electrophile, dinitrofluorobenzene (DNFB), to give 82% to 92% yield. Models of heart myocardial infarction reperfusion (MI/R) injury are commonly used to assess the effectiveness of sulfur donors in reducing damage size as measured by infarct size per area-at-risk ratio. In assessing the effectiveness of the persulfide donor described, it was found that treatment with 50–100 \(\mu\)g kg\(^{-1}\) of the prodrug 77 was able to significantly decrease on infarct size per area-at-risk.
Along a similar line, we have also designed prodrugs of hydrogen persulfide and glutathione persulfide using a "trimethyl lock" assisted lactonization reaction (Fig. 10A). Specifically, a "trimethyl lock"-accelerated lactonization system had been applied to deliver hydrogen sulfide to biological systems [39]. The same strategy was used to develop prodrugs of hydrogen persulfide and glutathione persulfide (GSSH) [34, 71]. As shown in Fig. 10A, esterase-mediated hydrolysis of the ester group should yield an unstable phenol hydroxyl intermediate, which is poised to undergo lactonization to generate hydrogen persulfide and a byproduct, lactone.

With the least bulky ester moiety, the half-life of the hydrogen persulfide donor 80 was determined to be around 24 min in PBS (2 U PLE), while in the case of the bulky ester bearing a cyclopentyl group, the half-life of persulfide release increased by seven-fold. The hydrogen persulfide generated from donor 80 was detected by trapping with monobromobimane (mBB), and the total yield was around 68%, comparable to the yield from pure sodium disulfide salt. When the same strategy was used to release GSSH, the trapping yield from DNFB was only about 10%. In order to probe the issue of low trapping yield for GSSH, mass spectrometric studies were conducted. It was found that under near physiological conditions (PBS buffer, pH 7.4, 37 °C), GSSH underwent quick disproportionation to generate GSSGG and H₂S. To study the ability for the H₂S prodrug to induce protein S-persulfidation, we used GAPDH as a model, which was known to be inhibited by persulfidation [32, 59]. It was found that the H₂S prodrug was able to induce GAPDH persulfidation, while H₂S was only able to do so in the presence of H₂O₂, as expected. On the subject of GSSH, glutathionylation is known to be important in cellular signaling [23-25, 72]. One of the functions of GSSH was its strong antioxidation effect. Therefore, we studied the ability for the GSSH prodrug 82 to protect H9c2 cells against H₂O₂-induced toxicity. It was found that 150 μmol·L⁻¹ of the prodrug was able to rescue cells from 40% viability to 60% under H₂O₂-induced oxidative stress. The same concentration of Na₂S, GSH and hybproduct lactone did not show any protective effect, which might suggest a unique anti-oxidative property from GSSH.

Xian and coworkers also utilized the unstable “hydroxy methyl” disulfide intermediate to release persulfides (Fig. 10B) [73]. Specifically, TMS and TES protecting groups were used as acid- or fluoride-sensitive moieties. In DMF/PBS buffer (4 : 1), at pH 7.4, for prodrug 83, the total decomposition requires 10 h, while at pH 2.5, it required 2.5 h to reach the total consumption. Persulfide trapping with iodoacetamide was studied in pure methanol. In the presence of KF, the trapping yields for benzyl and protected cysteine persulfides from donor 85 and 86 were 94% and 38% respectively. Toscano presented a precursor of modified cysteine persulfide based on S-substituted-thioureas [74]. The terminal sulfhydryl moiety of the persulfide was protected in a S-alkylthioisothiourea form. Under physiological conditions, the precursor 87 was designated to initiate deprotonation followed by an elimination reaction to produce persulfide and thiourea derivative (Fig. 10B). However, the persulfide seemed to prefer undergoing a trapping reaction with itself instead of the trapping reagent, N-ethylmaleimide (NEM). In this case, a gem-dimethyl moiety was installed next to the inner sulfur atom to prevent the self-perturbation of persulfide species. The persulfide trapping yield of produg 88 improved to 96% with 20 mmol·L⁻¹ NEM in pH 7.4 PBS buffer containing 10% D₂O.

Several ROS-sensitive persulfide prodrugs have also been reported. Matson and coworkers used an 1,6-elimination system to release N-acetyl cysteine (NAC) persulfide (Fig. 10C) [75]. Again, an aryl boronic ester was used as a latent hydroxyl group, generation of which is dependent on reaction with H₂O₂. Further, the formation of the hydroxyl group initiates self-immolation, leading to NAC persulfide and p-hydroxy-benzyl alcohol. The NAC persulfide was directly detected by LC-MS. 200 μmol·L⁻¹ of the donor 89 was shown to improve cell survival of H₂O₂-induced death from 30% to 100%. It should be noted that the control compound, which only contains aryl boronic ester group, was also able to increase the cell viability to around 70%. Chakrapani and coworkers also reported a ROS-sensitive benzylpersulfide donor using the boronate approach. Upon the removal of the vinyl boronate ester by H₂O₂, the unstable enolate intermediate could undergo a 1,4-O,S-relay to generate benzyl persulfide and byproduct cinnamaldehyde (Fig. 10C) [76]. In the presence of 10 equiv of H₂O₂, the decomposition rate constant of donor 91 was determined to be 5.3 × 10⁻⁴ min⁻¹. However, it was suggested that byproduct cinnamaldehyde could also react with benzyl persulfide, complicating the interpretation of the results somewhat. The donor (91) was also examined on its protective effects toward DLD-1 cells under oxidative stress. In the presence of 50 μmol·L⁻¹ of an oxidative stress inducer, menadione, 100 μmol·L⁻¹ of 51 increased the cell viability from 30% to 70%. The control compound, which only bears the aryl boronic ester group, did not show any protective effect.

Singh and coworkers reported a strategy to develop photo-sensitive persulfide prodrugs by using a well-known ortho-nitrobenzyl (ONB) phototrigger [77]. As shown in Fig. 10D, the terminal sulfhydryl group on NAC persulfide was protected by ONB. Upon light irradiation (λ ≥ 365 nm), the unstable enolate intermediate of an oxidant sensitive donor using the boronate approach. Upon the removal of the vinyl boronate ester by H₂O₂, the unstable enolate intermediate could undergo a 1,4-O,S-relay to generate benzyl persulfide and byproduct cinnamaldehyde (Fig. 10C) [76]. In the presence of 10 equiv of H₂O₂, the decomposition rate constant of donor 91 was determined to be 5.3 × 10⁻⁴ min⁻¹. However, it was suggested that byproduct cinnamaldehyde could also react with benzyl persulfide, complicating the interpretation of the results somewhat. The donor (91) was also examined on its protective effects toward DLD-1 cells under oxidative stress. In the presence of 50 μmol·L⁻¹ of an oxidative stress inducer, menadione, 100 μmol·L⁻¹ of 51 increased the cell viability from 30% to 70%. The control compound, which only bears the aryl boronic ester group, did not show any protective effect.
of them have been examined for their anti-oxidation effect at the cellular level, suggesting improved protective effects over that of the corresponding thiol. It would be interested to systematically compare other biological activities by using these prodrugs as tools. Evaluation of these prodrugs on regulating protein S-persulfidation will also be needed to guide future applications.

Conclusions

This review updates an earlier publication on the same subject on sulfide donors, broadly defined. There have been two trends in the current field: 1) developing new prodrugs triggered by different stimuli, especially for COS based prodrugs; 2) developing prodrugs of reactive sulfur species (RSS) at the persulfide oxidation state. Protein S-persulfidation and glutathionylation studies will especially benefit from the availability of donors of persulfide and glutathione persulfide of different properties.

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References


