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Therapeutics

FORUM REVIEW ARTICLE

Activatable Small-Molecule Hydrogen Sulfide Donors

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Abstract

Significance: Hydrogen sulfide (H_2S) is an important biological signaling molecule involved in many physiological processes. These diverse roles have led researchers to develop contemporary methods to deliver H_2S under physiologically relevant conditions and in response to various stimuli.
Recent Advances: Different small-molecule donors have been developed that release H_2S under various conditions. Key examples include donors activated in response to hydrolysis, to endogenous species, such as thiols, reactive oxygen species, and enzymes, and to external stimuli, such as photoactivation and bioorthogonal chemistry. In addition, an alternative approach to release H_2S has utilized the catalyzed hydrolysis of carbonyl sulfide (COS) by carbonic anhydrase to generate libraries of activatable COS-based H_2S donors.
Critical Issues: Small-molecule H_2S donors provide important research and pharmacological tools to perturb H_2S levels. Key needs, both in the development and in the use of such donors, include access to new donors that respond to specific stimuli as well as donors with well-defined control compounds that allow for clear delineation of the impact of H_2S delivery from other donor byproducts.
Future Directions: The abundance of reported small-molecule H_2S donors provides biologists and physiologists with a chemical toolbox to ask key biological questions and to develop H_2S-related therapeutic interventions. Further investigation into different releasing efficiencies in biological contexts and a clear understanding of biological responses to donors that release H_2S gradually (*e.g.*, hours to days) *versus* donors that generate H_2S quickly (*e.g.*, seconds to minutes) is needed. *Antioxid. Redox Signal.* 32, 96–109.

Keywords: hydrogen sulfide, reactive sulfur species, small molecule donors, carbonyl sulfide

Introduction

Hydrogen sulfide (H_2S), historically dismissed as a toxic and malodorous gas, has emerged in the scientific community as an important biological signaling molecule (1, 50, 90). The physicochemical properties of H_2S have been studied extensively, and we refer the interested reader to recent reviews that cover this area in depth (22, 38, 52, 59, 81, 103). Since its initial recognition as a relevant biomolecule, diverse scientific communities ranging from chemists to physiologists have focused on investigating the role of H_2S in various biosystems.

H_2S is produced endogenously in mammals predominantly from cysteine through the action of three main enzymes. Cystathionine-β-synthase is primarily localized in the nervous system, brain, and liver; cystathionine-γ-lyase produces H_2S primarily in the cardiovascular system; and 3-mercaptopyruvate sulfurtransferase is localized in the mitochondria (1, 11). Investigations into the biological roles of H_2S have established its critical roles in different disease states and pathologies in almost every human organ system (Fig. 1) (13, 28, 42, 45). As brief examples, H_2S plays important roles in the central nervous system, participates in neurotransmission, and has been shown to have neuroprotective effects, specifically in mouse models of Parkinson's disease (12, 60, 74). In addition, H_2S upregulates glutathione (GSH) production in the brain during periods of high oxidative stress and contributes to regulating key sodium channels in neuronal cells (74). In the respiratory system, H_2S plays roles in different conditions, including chronic obstructive pulmonary disease, pulmonary fibrosis,

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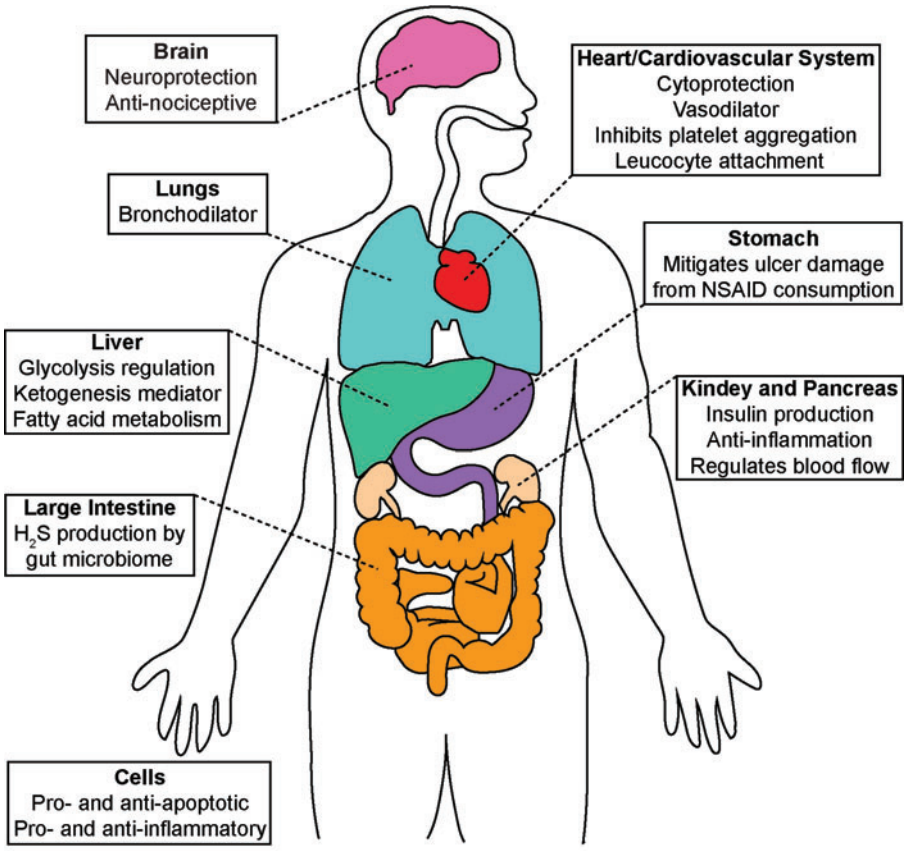

FIG. 1. Selected roles of H_2S in major organ systems. H_2S, hydrogen sulfide; NSAID, nonsteroidal anti-inflammatory drug. Color images are available online.

and hypoxia-induced pulmonary hypertension (21). In the cardiovascular system, H_2S mitigates oxidative stress and reduces myocardial injury related to ischemia–reperfusion events (27, 95, 100). Moreover, lower circulating H_2S levels are found in experimental models of heart failure, and CSE-deficient mice exhibit greater cardiac dysfunction after transverse aortic constriction, both of which suggest additional roles of H_2S in heart failure (46).

More broadly, H_2S interacts through several signaling pathways, such as the K_{ATP} channels, and promotes angiogenesis by the protein kinase B and phosphatidylinositol 3-kinase pathways (44). Low levels of H_2S have been demonstrated to promote cell proliferation and migration (88, 113). Importantly, the activity of administered H_2S often shows a stark concentration dependence, with low *versus* high concentrations frequently producing contrasting effects. More recently, a study of psoriasis patients demonstrated lower serum H_2S levels than healthy patients, underscoring the potential role that H_2S plays in skin protection and repair (3). As a whole, the established pathophysiological targets of H_2S are incredibly diverse, and they include activities as an established antiapoptotic (97), anti-inflammatory (35, 86, 87), and antioxidative agent (93), as well as contributing to many other processes.

With such a broad range of biological targets and activities, significant effort has focused on investigating and understanding the direct effects of H_2S on specific systems with a long-term goal of leveraging these insights to deliver H_2S-related therapeutic interventions. Much of the preliminary work in this area relied on direct inhalation of H_2S or administration of inorganic sulfide salts, such as sodium hydrosulfide and sodium sulfide (Na_2S). Although highly efficient, these systems often release an instantaneous bolus of H_2S and fail to mimic the more gradual rates or distributions of endogenous H_2S production (83, 101). This discrepancy, as well as other significant limitations, has driven the development of small-molecule ''donors'' that are capable of releasing H_2S under physiologically relevant conditions and in response to specific stimuli.

Many reported H_2S donor systems respond to specific exogenous or biocompatible stimuli to release H_2S (7, 26, 38, 61, 67, 76, 82, 94, 103). Such activation profiles allow for donor activities to be tuned to respond to specific activators and stimuli present in a given system. Although there is no single universal ''ideal donor,'' certain donor classes provide distinct advantages and useful properties. For example, donors should have readily accessible control compounds that can be used to clearly delineate observed biological activities and outcomes associated with H_2S from those of donor byproducts. Similarly, donors that respond to specific stimuli enable experiments in which H_2S delivery can be controlled or triggered by specific activators. Coincident with these primary needs, significant advances in the development of activatable H_2S donors have occurred in the past 5 years, with key examples including donors activated by light, various pH regimes, enzymes, biological thiols, and hydrogen peroxide (H_2O_2).

In developing activatable H_2S-releasing donors, a number of primary strategies have emerged, which are summarized briefly in this section but are expanded on in various sections of this review (Fig. 2). The first commonly used strategy is to

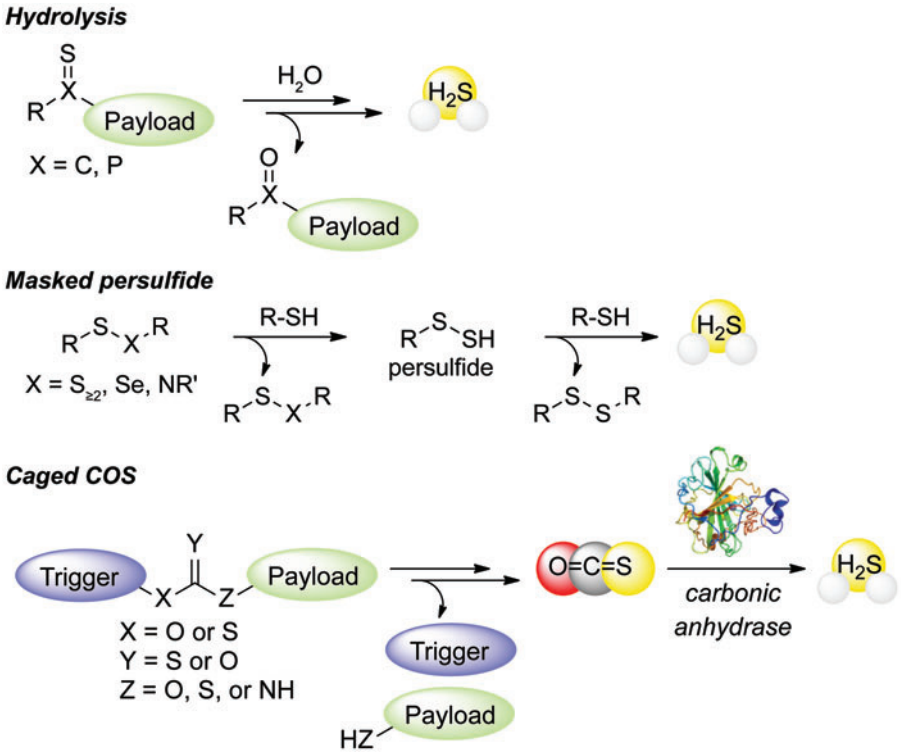

FIG. 2. General classes of H_2S release from small-molecule donors. Color images are available online.

replace an oxygen atom in a molecule with a sulfur, such that hydrolysis releases H_2S. A second common strategy is to develop systems that generate an intermediate persulfide, which can be subsequently cleaved by thiols, such as GSH or cysteine, to generate H_2S and a disulfide. A third strategy is to develop systems that release carbonyl sulfide (COS) as an intermediate, which can be quickly converted to H_2S, the ubiquitous mammalian enzyme carbonic anhydrase (CA) (79). These three general approaches are summarized in Figure 2 and will be discussed briefly throughout the review. Rather than focus on the mechanistic details of each structure in this review, we refer interested readers to the Supplementary Appendix, which includes the activation mechanism of different donor platforms.

Enzyme-Activated Donors

Because H_2S has myriad biological targets, disentangling the effects of sulfide delivery in specific environments can be challenging. One approach to overcome this difficulty is to incorporate an enzymatically cleaved trigger on a sulfide donor. This approach allows for donors to be developed that are stable until the activating group is cleaved or modified by the target enzyme to release H_2S or an H_2S equivalent, such as COS. This strategy has the benefit of being readily tuned to specific triggering groups and enzyme pairs. In addition, utilizing an enzyme to carry out the donor activation event does not consume cellular nucleophiles or thiols, which could otherwise perturb the redox balance of related reactive sulfur species—an inherent challenge with many thiol-triggered H_2S donors.

The first enzyme-triggered H_2S donor, HP-101, was reported by Zheng *et al.* (114) (Fig. 3a). In this system, esterase-mediated cleavage of an acyl-protecting group on the donor motif was used to generate an unstable phenolic intermediate that subsequently underwent an intramolecular lactonization with a pendant thioacid to release H_2S (114). Esterases are expressed in most tissue types and are involved in the activation or metabolism of ~10% of drugs (Fig. 3b) (32). One benefit of this design is that the rate of H_2S release could be tuned by varying the identity of the ester triggering group or by modifying the geminal-dimethyl substitution in the ''trimethyl lock'' backbone to facilitate lactonization. Notably, the authors were able to conjugate this sulfide-donating scaffold to the nonsteroidal anti-inflammatory drug naproxen, forming an activatable H_2S-drug hybrid.

In 2017, both Chauhan *et al.* (17) and our group (78) independently reported esterase-activated donors that functioned through the intermediacy of COS release. In these approaches, self-immolative thiocarbamates or thiocarbonates functionalized with ester motifs were enzymatically activated to release COS, which is rapidly metabolized to H_2S by CA, rather than H_2S directly. In this context, ''self-immolation'' refers to the spontaneous cascade reaction of a molecular linker after a chemical triggering event that results in release of a desired payload.

Chauhan *et al.* (17) utilized a *tert*-butyl ester trigger with an extended methylenedioxy linker to connect the donor motif to the core amine scaffold. These donors, such as Esterase-TCM-SA, encompassed both aryl- and benzylamine terminating *S*-alkyl thiocarbamates, as well as *S*-alkyl thiocarbonates (Fig. 3a). The different scaffolds exhibited different release rates based on the identity of the parent amine, and different toxicity profiles toward MCF-7 (human breast cancer) cells. Our group also reported *tert*-butyl ester triggered motifs, including the donor compound Esterase-TCM-OA, as well as the analogous triggerless and sulfide-depleted carbamate control compounds (Fig. 3a) (78). The COS/H_2S donors showed significant cytotoxicity in BEAS 2B human lung epithelial cells when compared with the carbamate and triggerless control compounds or

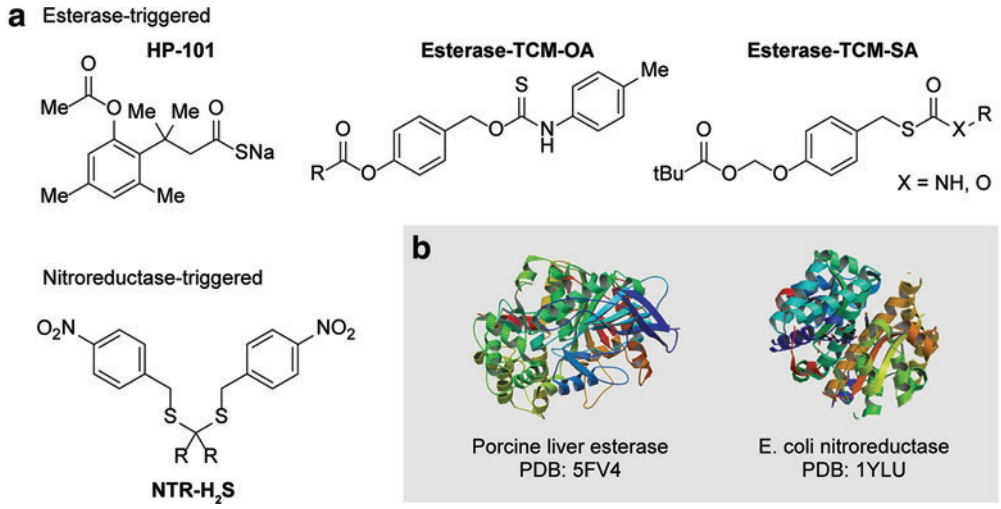

FIG. 3. Enzyme-activated donors and associated enzymes. (a) Structures of enzymatically triggered COS and H_2S donors. **(b)** Structures of PLE and *Escherichia coli* NTR. COS, carbonyl sulfide; NTR, nitroreductase; PLE, porcine liver esterase. Color images are available online.

with equivalent concentrations of the H_2S sources/donors Na_2S, AP39, or GYY4137.

Further investigations using bioenergetics assays revealed that the esterase-activated thiocarbamate donors inhibited mitochondrial respiration, whereas exogenous H_2S or the triggerless and sulfur-depleted control compounds did not. Building from the hypothesis that the observed cytotoxicity could be due to a buildup of COS in the mitochondria, due to faster rates of ester cleavage and self-immolation than COS hydrolysis by CA, we prepared a suite of esterase-triggered self-immolative thiocarbamates with esters of varying steric bulk. These esters displayed different rates of COS release, which correlated inversely with cytotoxicity in HeLa (human cervical cancer) cells. Again, the triggerless control and sulfur-depleted control compounds failed to show significant cytotoxicity, showing the utility of having readily accessible control compounds. These results support the hypothesis that COS may function as more than a simple H_2S shuttle in certain circumstances; however, these observations do not account for differences in subcellular localization of different donors or differential activities of various CA isoforms toward COS (49). We do note that as a whole, most developed COS-releasing compounds appear to function as H_2S donors, with activities directly attributable to the release of H_2S.

Shukla *et al*. (75) further expanded work on enzyme-triggered donor platforms to develop donors activated by bacterial nitroreductase (NTR) (Fig. 3b). NTRs are frequently found in bacteria and are also upregulated under hypoxic conditions in different cell types (8, 91). The NTR-mediated reduction of the electron-withdrawing nitro groups on NTR-H_2S to the corresponding aniline, with the nitrogen lone pair now free to resonate through to release the iminoquinone methide, was used to reveal a geminal-dithiol intermediate that hydrolyzes in buffer to generate H_2S (Fig. 3a). H_2S release was confirmed and measured in these systems by using monobromobimane and fluorescence assays. These donors have been used to study the role of H_2S in the intracellular redox balance and the development of antibiotic resistance in bacteria, specifically *Escherichia coli* (75).

Reactive Oxygen Species-Activated Donors

H_2S exhibits anti-inflammatory activities and protective effects against reactive oxygen species (ROS), which has motivated a number of groups to develop H_2S donors that are activated in the presence of ROS, such as H_2O_2. The cellular localization and levels of these ROS can vary in response to different stress states. In 2016, our group reported a caged thiocarbamate equipped with a pinacol boronate ester, Peroxy-TCM-OA, that self-immolates on exposure to H_2O_2 to release COS (Fig. 4) (108). These thiocarbamate donors are stable toward aqueous hydrolysis, but respond to H_2O_2, and to a lesser extent to superoxide and peroxynitrite, to release H_2S. In these studies, H_2S release was measured by using an H_2S-responsive electrode, and fluorescence imaging in HeLa cells confirmed that the thiocarbamate donor could be activated in a biological environment with either exogenous H_2O_2 or endogenous ROS. Cytotoxicity experiments showed that the donor provided cytoprotection against exogenous H_2O_2 treatment. The sulfur-depleted control compounds showed modest cytoprotection, due to H_2O_2 consumption by the boronate moiety, whereas the triggerless control compounds failed to provide protection against H_2O_2 as expected. These experiments underscore the importance of having high fidelity control compounds to fully understand the mechanism of action of donor molecules.

Expanding from this initial report, we reported experimental and computational investigations of all the COS-releasing isomers of boronate-functionalized thiocarbamates and thiocarbonates (106). We found that *S*-alkyl thiocarbamates, Peroxy-TCM-SA, released H_2S slower than the analogous *S*-alkyl thiocarbonates, and that *S*-alkyl dithiocarbonate released H_2S faster and more efficiently than the other *S*-alkyl derivatives. Further contributing to the understanding of COS/H_2S release profiles, Chauhan *et al.* (18) employed related *S*-alkyl boronate-functionalized thiocarbamates to demonstrate that the rate of H_2O_2-triggered COS release can additionally be tuned by the basicity of the amine payload. These investigations also demonstrated that alkyl amine payload, such as

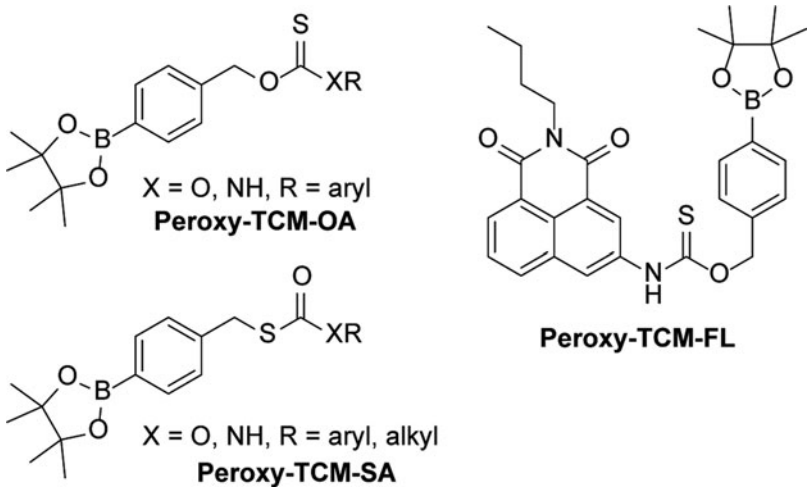

FIG. 4. H_2O_2-triggered COS donor scaffolds. H_2O_2, hydrogen peroxide.

a propylamine, significantly reduced the rate of H_2S release when compared with aryl amine payloads.

More recently, Hu *et al.* (39) further leveraged the boronate-functionalized self-immolative thiocarbamates to access the turn-on fluorescent H_2S donor Peroxy-FL (Fig. 4). Exchanging the amine payload with the 3-amino-*N*-butyl-1, 8-naphthalimide fluorophore allowed for the fluorescence of this system that is modulated by cleavage of the thiocarbamate motif to generate the parent aryl amine on the naphthalimide fluorophore. H_2S generation was confirmed by using the methylene blue assay, and the fluorescence response was demonstrated in both HeLa and RAW 264.17 murine macrophage cells. These donors released H_2S in response to both exogenous and endogenous H_2O_2, as demonstrated in cell culture experiments.

Hydrolysis-Based and pH-Sensitive Donors

Numerous H_2S donors are activated by hydrolysis mechanisms, and most of these respond through acid-mediated pathways. Figure 5 shows the structures of these donors and the pH value or ranges at which the compounds have been reported to release H_2S. This class of H_2S donors provides the unique opportunity to target specific diseases, cells, and/or organelles in which acidic microenvironments are present. As a simple example, thioacetamide can function as a pH-activated H_2S donor in extremely acidic environments (pH 1.0) and was initially used for the precipitation of dissolved metals as metal sulfides from acidic solutions for qualitative analysis (48). We note that the inherent toxicity of thioacetamide has severely limited the use of this H_2S donor in biological studies.

As interest in the chemical biology of H_2S has grown, the use of related thioamides as H_2S donors has expanded to include various aryl thioamides, which are highlighted in a separate Forum Review in this issue (58). Other simple small molecules have also been reported as pH-activated H_2S donors. For example, both thioglycine and thiovaline release H_2S in the presence of bicarbonate (HCO_3^-) at physiological pH (115). Both of these thioamino acids were demonstrated to increase intracellular cyclic guanosine monophosphate levels and promote vasorelaxation in mouse aortic rings, with both being more efficacious and potent than GYY4137.

One of the most commonly used, GYY4137, is a water-soluble H_2S donor that draws inspiration from Lawesson's Reagent (47), which is traditionally used in organic synthesis to prepare various organosulfur compounds (64). The release of H_2S from GYY4137 occurs slowly at physiological pH, but it can be accelerated under acidic conditions (pH <3.0) (51). Relative to other pH-sensitive H_2S donors, the biological activities of GYY4137 have been studied extensively and are highlighted in a separate review in this Forum. To tune the rate of pH-dependent H_2S release from P = S motifs related to GYY4137, Park *et al.* (63) investigated the use of analogous phosphorodithioates as H_2S donors. The inclusion of phenolic groups was found to enhance the rates of H_2S release at physiological pH, whereas alkyl alcohols decreased the efficiency of H_2S production consistent with the enhanced leaving group ability of phenols relative to alkyl alcohols. Moreover, pretreatment of H9c2 mouse cardiomyocytes with these H_2S donors provided significant cytoprotection against H_2O_2-induced oxidative damage. Interestingly, analogous experiments with GYY4137 failed to provide similar results due to the inherent cytotoxicity of this donor at higher concentrations.

In a follow-up study of phosphorodithioate-based H_2S donors by Feng *et al.* (30), they prepared a library of derivative compounds and examined the H_2S-releasing properties of these compounds. The cyclized derivative FW1256 displayed relatively high levels of H_2S release and potent cytotoxicity against MCF7 breast cancer cells. In 2016, this concept was revisited by Kang *et al.* (41), leading to the design of GYY4137 derivatives, including JK-2, that bear a pendant nucleophile that can participate in an intramolecular cyclization to generate H_2S (41). H_2S release was demonstrated within a range of pH 5.0 to 8.0, with significant enhancements in releasing efficiency more than GYY4137. Moreover, treatment with JK-2 resulted in significant reductions in infarction size in a myocardial ischemia–reperfusion injury mouse model.

In an alternative approach, our group recently reported pH-sensitive γ-ketothiocarbamate donors, including γ-KetoTCM-1, that function through intermediate COS release. This system was inspired by the use of 4-hydroxy-2-butanone esters to prepare self-immolative carbamate polymers that undergo β-elimination to generate carbon dioxide as a

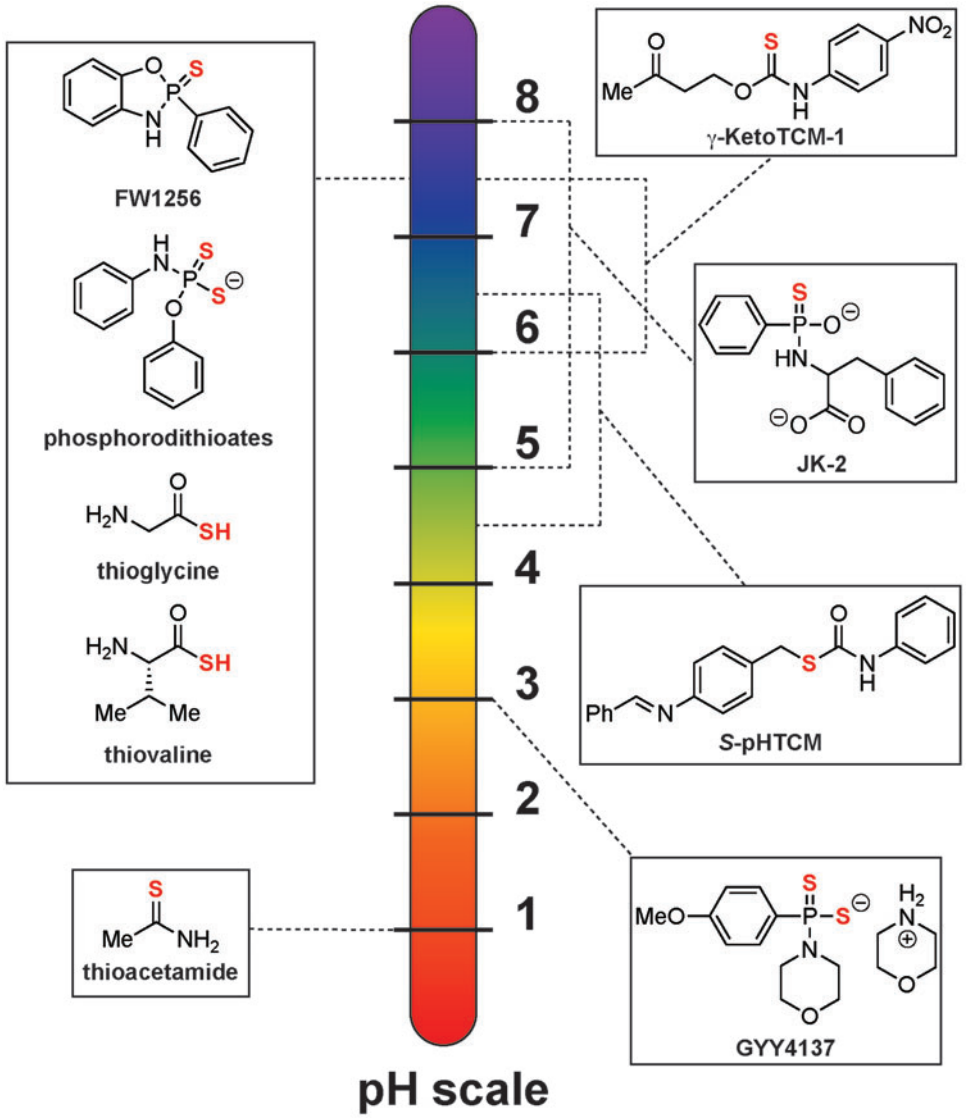

FIG. 5. Hydrolysis-activated H_2S donors. The pH windows shown represent specific pH values or pH windows in which H_2S release was reported or in which H_2S release was reported to be optimal. Color images are available online.

function of pH (71). The release of H_2S from γ-KetoTCM-1 was measured over a range of pH values (6.0–8.0), with increasing rates in more basic solution. The H_2S release half-life could be modified by structural tuning, and the donors provided anti-inflammatory activity in RAW 264.7 cells (109). More recently, we reported a self-immolative thiocarbamate (*S*-pHTCM) with a pendant aryl imine trigger as a pH-sensitive donor that releases COS/H_2S. (36) Notably, this triggering motif was designed to be activated within a specific acidic pH window and showed optimal cleavage rates between pH 4.3 and 7.3.

Thiol-Activated Donors

Compounds activated by biological thiols, including cysteine and reduced GSH, represent the largest class of small-molecule H_2S donors (Fig. 6a, b). The activation of many of these compounds proceed through persulfide intermediates, although others function through poorly understood mechanisms. The fundamental role and abundance of biological thiols, especially GSH, allows researchers to use these nucleophiles to probe the effects of H_2S donor administration. Expanding from thioacetamide, many aryl thioamides have been reported as H_2S donors. These compounds are stable at physiological pH and exhibit a cysteine-dependent H_2S release, yet the mechanism of H_2S release is unclear (58). Despite the low H_2S-releasing efficiencies, such compounds possess unique pharmacological activities, which are covered in a separate review in this Forum. The use of structurally related iminothioethers as cysteine-activated H_2S donors was reported by Barresi *et al.* (4). H_2S release from these donors was evaluated in buffer containing 4 mM cysteine, and releasing efficiencies were dependent on donor derivatization. In isolated rat hearts, two donors were demonstrated to reverse the effects of angiotensin II induced reduction in basal coronary flow, and studies on human aortic smooth muscle cells showed that these donors exhibited membrane hyperpolarizing effects. The mechanism of cysteine-mediated H_2S release from iminothioethers remains unclear.

Aryl isothiocyanates were first reported as cysteine-activated H_2S donors in 2014 by Martelli *et al.* (57). Although release efficiency from these compounds was relatively low and required millimolar levels of cysteine for release, the

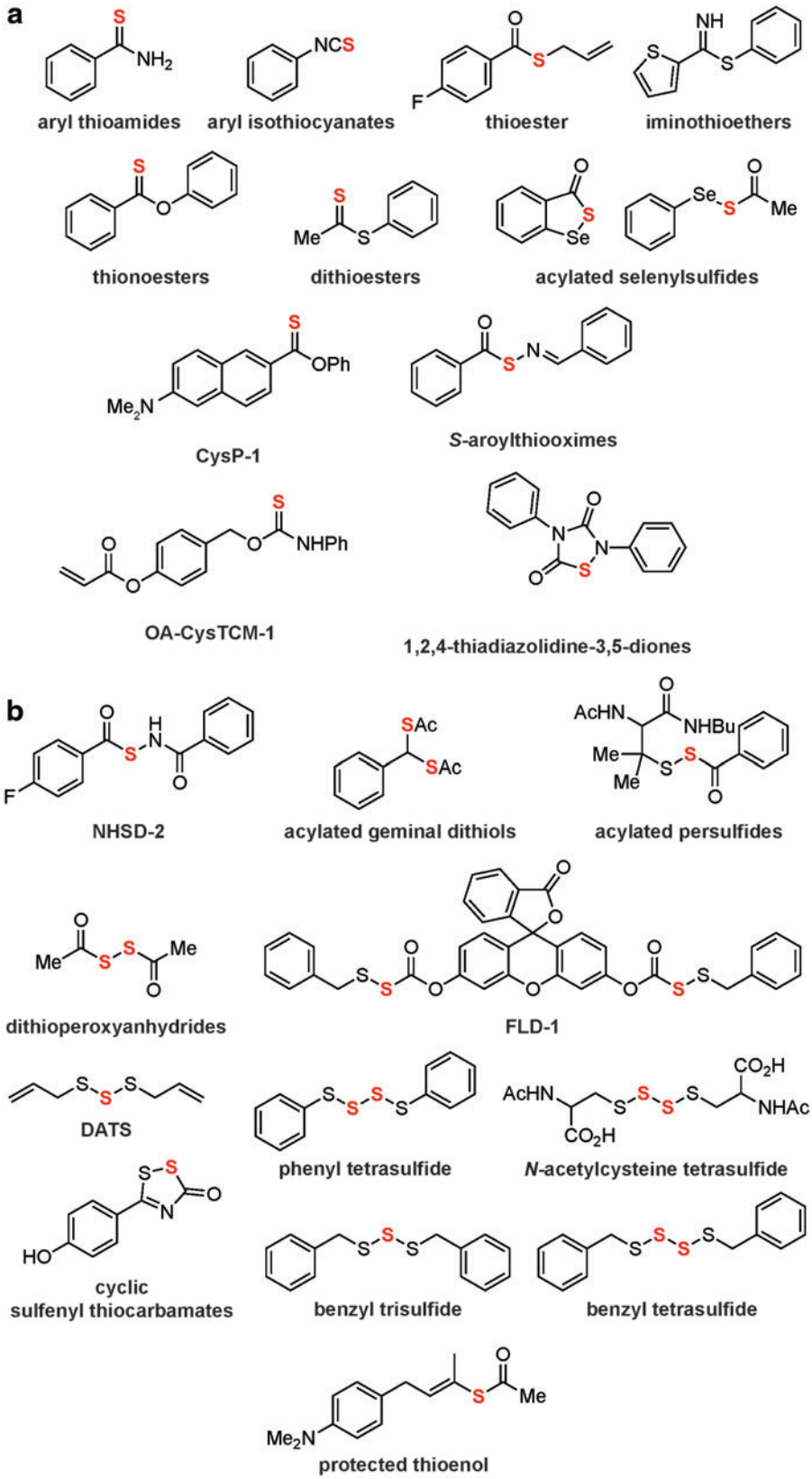

FIG. 6. Structures of donor compounds activated in the presence of biological thiols. **(a)** H_2S donors activated in the presence of cysteine. **(b)** H_2S donors activated in the presence of cysteine and GSH. GSH, glutathione. Color images are available online.

isothiocyanates were found to cause membrane hyperpolarization of vascular smooth muscle cells and vasorelaxation in coronary arteries, both of which are consistent with H_2S release. In 2019, Lin *et al.* (55) investigated the mechanism of H_2S release from aryl isothiocyanates, and their data suggest that H_2S release proceeds through a native chemical ligation-type mechanism after initial attack on the isothiocyanate by the cysteine sulfhydryl group.

Also building from a native chemical ligation mechanistic approach (24), our group reported in 2018 that thionoesters, which are structural isomers of thioesters commonly used in native chemical ligation, respond selectively to cysteine over other thiols to release H_2S with >80% efficiency (16). Mechanistic investigations demonstrated that the N-to-S acyl transfer step was the rate-limiting step of this reaction. We later expanded this approach to demonstrate that dithioesters, which are more synthetically diversifiable than thionoesters, release H_2S selectively in the presence of cysteine (15). This approach has also been extended to provide fluorescent H_2S donors activated in the presence of cysteine (CysP-1) (43). Proceeding through a key N-to-S acyl transfer step, Zhao *et al.* (107) showed that acyl-protected geminal dithiols react selectively to release H_2S in the presence of cysteine, through generation of an unstable geminal dithiol intermediate. Expanding to bioactive thioketone derivatives, Zhu *et al.* (116) demonstrated during an investigation into the metabolism of clopidogrel (Plavix) that an intermediate metabolite containing a thioenol motif releases H_2S efficiently at physiological pH, suggesting possible future application of these and related compounds as H_2S donor motifs.

Inspired by the unique reactivity of S–N motifs present in *S*-nitrosothiols, Zhao *et al.* (111) developed a series of compounds termed ''*N*-mercapto donors'' and demonstrated H_2S release in the presence of cysteine. The mechanism of H_2S release from these donors proceeds through the *N*-acylation of cysteine and generation of cysteine persulfide as the key H_2S-releasing intermediate. This class of donors was later expanded on in the development of NHSD-2, which exhibited significant cardioprotective effects in a murine model of myocardial–ischemia reperfusion injury (112). Also leveraging the generation of intermediate persulfide motifs, Zhao *et al.* (102) investigated the use of protected cysteine and penicillamine persulfide derivatives as H_2S donors in the presence of cysteine and GSH. These compounds function by initial attack on the donor by cysteine to generate a cysteine persulfide, which undergoes subsequent reaction with thiols to generate H_2S. These donors also provided H_2S-related protection against *in vivo* murine models of myocardial ischemia–reperfusion injury.

In a related approach, Foster *et al.* (31) reported the related *S*-aroylthiooxime class of compounds, which release H_2S in the presence of cysteine and can be tuned predictably by simple electronic modulation. H_2S release from these compounds likely proceeds by initial attack by cysteine on the donor to release an iminothiol intermediate, which further reacts with cysteine to generate a cysteine persulfide intermediate en route to H_2S release. More recently, the intermediate persulfide generation from S–X hybrid systems was further leveraged by Kang *et al.* (40) to develop a series of cyclic sulfur–selenium compounds and by Hamsath *et al.* (37) to develop acyclic sulfur–selenium compounds, which generate H_2S-releasing persulfides and analogous selenylsulfides in the presence of cysteine. In an alternative approach, Roger *et al.* (70) reported that dithioperoxyanhydrides also function as H_2S donors through the intermediate generation of persulfides in the presence of GSH and cysteine. These compounds were demonstrated to induce vasorelaxation in isolated rat aortic rings. In general, the use of persulfides as H_2S-releasing species has been of particular interest because a number of H_2S signaling mechanisms involve persulfidation of cysteine residues. In parallel to these developments, different methods of direct persulfide generation in water are of significant interest and advances in this area will be highlighted elsewhere in this Forum (62).

An often overlooked yet uncontrolled method of generating H_2S from persulfide intermediates is by treatment of organic polysulfides with thiols (66). The most widely used organic polysulfide, diallyl trisulfide (DATS), is a simple organosulfur compound readily isolated from alliums, including garlic (5). In the presence of thiols, DATS is reduced to generate allyl persulfide, which is further reduced by thiols to generate H_2S. We note that although diallyl disulfide (DADS) is often used in the literature as an H_2S donor, its apparent H_2S-releasing activity has been demonstrated to be a result of a DATS impurities (54). Both experimental and computational results support the lack of direct H_2S release from DADS in the presence of thiols, except for a minor, slow pathway involving attack at the α-carbon by a thiol to generate an allyl persulfide intermediate (10). Consistent with this slow release, the generation of H_2S from thioester donors reported by Yao *et al.* (98) is likely due to the intermediate release of allyl thiol and subsequent oxidation to form DADS, which results in slow H_2S donation.

Expanding investigations into H_2S from organic polysulfides, our group recently reported *bis*(aryl)- and *bis*(alkyl)-tetrasulfides as H_2S donors and demonstrated that tetrasulfides release more H_2S than trisulfides, as expected (14). In comparing a series of benzyl di-, tri-, and tetra-sulfides (6), we confirmed cysteine and GSH-mediated H_2S release occurs exclusively from dibenzyl trisulfide and dibenzyl tetrasulfide, which is consistent with the presence of sulfane sulfur (84). A related study by Ercole *et al.* (29) highlighted the efficient release of H_2S from polysulfide-based donors built around polyethylene glycol/trisulfide/cholesterol conjugates that assemble into supramolecular macrostructures.

COS-based H_2S donors that are activated by thiols have also been reported. In 2018, we reported a small library of sulfenyl thiocarbonate motifs, including FLD-1 that undergoes thiol-mediated decomposition to generate COS (105). By using a fluorophore as a payload that is released upon COS/H_2S donation, these donors provide a fluorescent response that correlates linearly with COS/H_2S release and allows for spatiotemporal resolution of cellular COS/H_2S release in live cell imaging. We also reported cysteine-selective COS-based H_2S donors, such as OA-CysTCM-1, which utilized a cysteine-mediated cyclization to activate a self-immolative donor motif (110). A large library of 1,2,4-thiadiazolidine-3,5-diones was reported by Severino *et al.* (72) that was demonstrated to release H_2S in the presence of cysteine. We note that the proposed mechanism of donor activation in this system proceeds through an anionic thiocarbamate intermediate, which likely results in the direct release of COS with concomitant hydrolysis to H_2S.

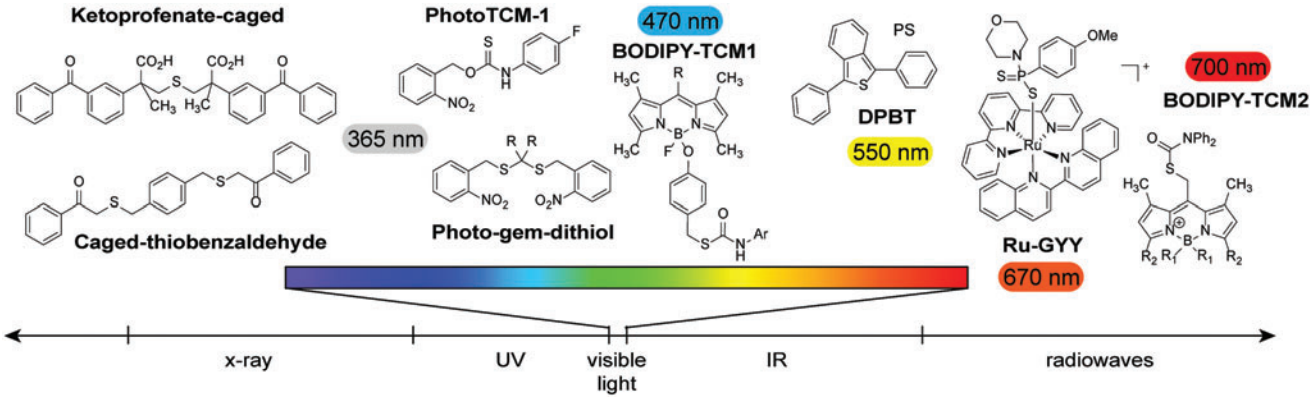

FIG. 7. Structures and excitation wavelengths of selected photoactivable H_2S donors. PS indicates photosensitizer. IR, infrared; UV, ultraviolet. Color images are available online.

Photoactivated Donors

The ability to control H_2S donation using external bioorthogonal stimuli that selectively activate the desired compound in the presence of diverse biological functional groups is a powerful method that has garnered significant interest. Of such strategies, photoactivatable donors offer the potential for high spatiotemporal control of H_2S release (Fig. 7). Photocaged species react on exposure to specific wavelengths of light to cleave a protecting group and reveal, in these cases, an H_2S-releasing moiety. This approach allows for noninvasive triggering of H_2S release in cells *in vitro* and the potential for photo-triggering on skin or at shallow sub-cutaneous levels (2, 19). The first photoactivated H_2S donor Photo-gem-dithiol was reported by Devarie-Baez *et al.* (25), in which a *bis*-orthonitrobenzyl protected geminal-dithiol undergoes a Norrish type II rearrangement on irradiation ($\lambda_{irr} = 365$ nm) to unmask an unstable gem-dithiol intermediate that is subsequently hydrolyzed to release H_2S. Sulfide release was confirmed by using the methylene blue assay, as well as through fluorescence imaging with HeLa cells. Moreover, these donors displayed pH-dependent H_2S release, consistent with other donors involving hydrolysis of gem-dithiols, an acid-mediated process. Similar photocleavable gem-dithiol scaffolds ($\lambda_{irr} = 365$ nm) have since been incorporated into water-soluble polymers and block copolymer nanoparticles (53, 89), as well as upconverting nanoparticles, which absorb low-energy near-infrared light ($\lambda_{irr} = 980$ nm) and emit ultraviolet to visible light, that can trigger H_2S release (20).

An alternative photocontrollable H_2S donor was reported by Fukushima *et al.* (34), which centers around a functionalized thioether that releases H_2S directly after photocleavage of the protecting groups, rather than relying on a subsequent hydrolysis step. Initially employing 2-nitrobenzyl photoactivatable groups, this approach was expanded to incorporate ketoprofenate photocages to avoid the production of the potentially deleterious 2-nitrosobenzaldehyde byproduct. These donors were further adapted to function by photoexcitation ($\lambda_{irr} = 325$–385 nm) of xanthone chromophores (33). Photocaged thiobenzaldehydes have also been used as light-activated H_2S donors. In these systems, irradiation ($\lambda_{irr} = 355$ nm) reveals a thiobenzaldehyde intermediate that requires a subsequent nucleophilic attack by an amine to liberate the H_2S (96). Such donors have been incorporated into both water-soluble H_2S releasing polymers and hydrogels. One benefit of this approach is that the byproduct of

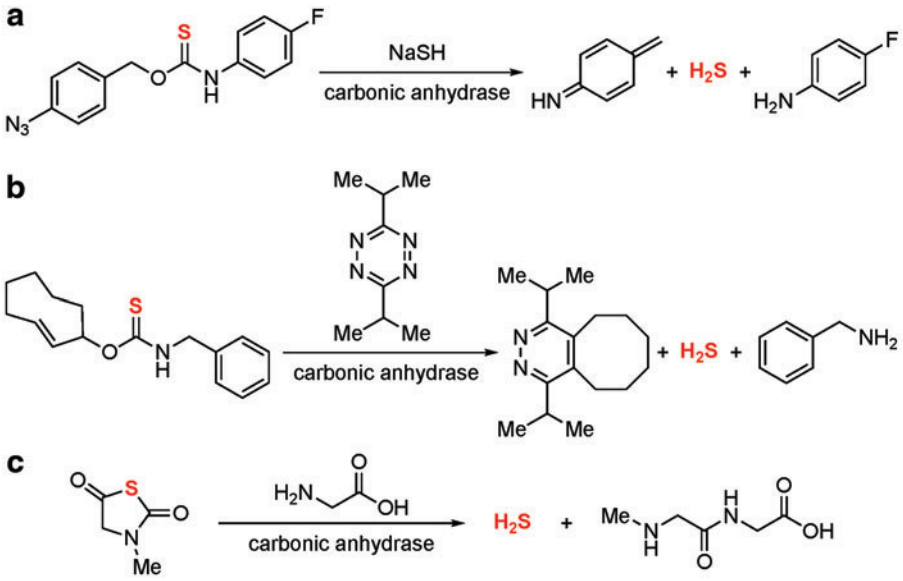

FIG. 8. Structures of COS-based donor compounds activated by miscellaneous activators. (a) H_2S-activated analyte replacement probe. **(b)** "Click and release" bioorthogonal COS-based H_2S donor. **(c)** Activation of *N*-thiocarboxyanhydride by glycine to generate H_2S. Color images are available online.

photolysis is simply acetophenone, which is a benign and Food and Drug Administration-approved excipient.

Expanding to visible light photoexcitation, Yi *et al.* (99) harnessed the photogeneration of singlet oxygen to trigger H_2S release. Photoirradiation ($\lambda_{irr} = 500–550$ nm) of a photosensitizer in the presence of ambient oxygen and 1,3-diphenylisobenzothiophene generated an unstable endoperoxide intermediate, which undergoes rapid fragmentation to generate 2-benzoylbenzophenone and H_2S (99). This system was incorporated into artificial vesicles or polymersomes, which enabled H_2S generation in water. An interesting advantage of this system is that the photoirradiation wavelengths are dictated by the choice of photosensitizer rather than the donor itself, which allows for a broad range of wavelengths to be used ($\lambda_{ex} = 380–550$ nm demonstrated).

A number of photoactivatable COS-based H_2S donors have also been reported. In 2017, our group reported the first light-activated COS-based H_2S donor, PhotoTCM-1, in which an *o*-nitrobenzyl protecting group masked a caged thiocarbamate, which was cleaved on irradiation ($\lambda_{irr} = 365$ nm) to release COS (104). PhotoTCM-1 was also shown through a series of selectivity studies to be stable to relevant biological thiols and nucleophiles, releasing COS only on photoirradiation. This strategy was later expanded by Sharma *et al.* (73) with a BODIPY-based photolabile group protecting an *S*-alkyl thiocarbamate that releases COS under irradiation at a more biocompatible wavelength ($\lambda_{irr} = 470$ nm) (BODIPY-TCM-1). More recently, efforts in this area have been focused on developing light-activated donors that function within a ''tissue-transparent'' window, the bounds of which are set by the absorbance of hemoglobin below 600 nm and of water above 900 nm (23, 65). This goal was accomplished by Stacko *et al.* (77) using a COS-based delivery approach coupled to a modified BODIPY core protecting an S-alkyl thiocarbamate. COS release from BODIPY-TCM-2 was accomplished with longer wavelength irradiation ($\lambda_{irr} = 700$ nm), which is a significant improvement over prior COS donors.

In a hybrid system, Woods *et al.* (92) reported a red-light activated complex of GYY-4137 and a common ruthenium photocage (Ru-GYY) that releases H_2S on irradiation ($\lambda_{irr} = 626$ nm). Interestingly, although GYY-4137 is known to spontaneously hydrolyze in aqueous systems, complexation to the ruthenium metal center suppresses this H_2S release. The authors were thus able to demonstrate controlled H_2S release from this donor, as well as its activity against a model of ischemia–reperfusion injury in H9c2 heart myoblast cells. The Singh lab recently reported a novel optical-readout-based phototriggered H_2S donor. Harnessing excited-state intramolecular proton transfer, which had been previously applied to monitoring nitric oxide donation (56) among other analytes, they developed a *p*-hydroxyphenacyl triggered donor that releases H_2S under irradiation ($\lambda_{irr} = 410$ nm), while simultaneously shifting the fluorescence of the donor (85). This change in fluorescence allowed for H_2S release to be monitored, and it was demonstrated to function in HeLa cells.

Miscellaneous Activatable Donors

In addition to the compounds described earlier in this review, a number of H_2S donors that are triggered by specific stimuli have been reported and do not correspond to the categories outlined earlier (Fig. 8). For example, our group reported the initial demonstration of leveraging intermediate COS release to access H_2S donors by developing self-immolative thiocarbamates, which we incorporated into analyte replacement fluorescent probes (Fig. 8a) (79). This donor serves as an analyte-replacement probe, as it regenerates the consumed H_2S during the mechanism of release.

In a bio-orthogonal delivery approach, we also demonstrated that self-immolative thiocarbamates bearing a *trans*-cyclooctene moiety can be utilized as ''click and release'' COS-based H_2S donors in the presence of tetrazines undergoing an inverse-electron demand Diels–Alder reaction (Fig. 8b) (80). Such bio-orthogonal approaches leverage the bimolecular reaction between two specific components to form a chemical bond while remaining inert to common reactive functional groups present in the surrounding biological milieu (9). The development of *N*-thiocarboxyanhydrides (NTA) by Powell *et al.* (68) provides an alternative to self-immolative thiocarbamates (Fig. 8c). This donor scaffold was demonstrated to release COS and generate a peptide byproduct in the presence of glycine and has recently been leveraged to develop a number of macromolecular H_2S donors (69), which is the topic of a related review in this Forum. One benefit of the NTA-based approach is that donor activation does not release electrophilic byproducts such as a para-quinone methide, which are often found in other self-immolative COS-based donors.

Conclusions and Outlook

The development of activatable small-molecule H_2S donors has been one of the most significant advances in the field of H_2S chemical biology over the past 5 to 10 years. This palette of triggerable H_2S donors provides researchers with a toolbox to better probe the biological activities of H_2S. The range of activators for controlled H_2S release has grown significantly in the past 5 years, and it is poised to enable new types of biological investigations that are not feasible with simple sulfide salts. Key needs include broader comparisons of different classes of donors in specific biological contexts to better delineate the bioavailability and localized release of H_2S from different donor constructs. Moreover, H_2S-releasing dynamics measured *in vitro* are likely to be perturbed in a biological system and may significantly alter the biological viability of these donors. As the chemistry that enables triggered release of H_2S and related reactive sulfur species from synthetic donors continues to evolve, the development of donors that respond to molecular stimuli upregulated during specific disease states is likely to provide new tools to harness the therapeutic potential of H_2S, alongside more finely tuned organelle or cell-type specific targeted donors. Overall, the rapid expansion of chemistry that enables small-molecule H_2S donors is poised to advance the field and help elucidate the inherent complexities of reactive sulfur species in biology.

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Supplementary Material

Supplementary Appendix

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Abbreviations Used

CA = carbonic anhydrase
COS = carbonyl sulfide
DADS = diallyl disulfide
DATS = diallyl trisulfide
GSH = glutathione
H_2O_2 = hydrogen peroxide
H_2S = hydrogen sulfide
Na_2S = sodium sulfide
NTR = nitroreductase
ROS = reactive oxygen species