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Discoveries

FORUM REVIEW ARTICLE

Development of Hydropersulfide Donors to Study Their Chemical Biology

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Abstract

Significance: Hydropersulfides (RSSH) are ubiquitous in prokaryotes, eukaryotic cells, and mammalian tissues. The unique chemical properties and prevalent nature of these species suggest a crucial role of RSSH in cell regulatory processes, yet little is known about their physiological functions.
Recent Advances: Examining the biological roles of RSSH species is challenging because of their inherent instability. In recent years, researchers have developed a number of small-molecule donors that efficiently release RSSH in response to various stimuli, including pH, thiols, reactive oxygen species, enzymes, and light. These RSSH donors have provided researchers with chemical tools to uncover the potential function and role of RSSH as physiological signaling and/or protecting agents.
Critical Issues: Because RSSH, hydrogen sulfide (H_2S), and higher order polysulfides are related to each other and can be present simultaneously in biological systems, distinguishing among the activities due to each of these species is difficult. Discerning this activity is critical to elucidate the chemical biology and physiology of RSSH. Moreover, although RSSH donors have been shown to confer cytoprotection against oxidative and electrophilic stress, their biological targets remain to be elucidated.
Future Directions: The development of RSSH donors with optimal drug-like properties and selectivity toward specific tissues/pathologies represents a promising approach. Further investigation of releasing efficiencies *in vivo* and a clear understanding of RSSH biological responses remain targets for future investigation. *Antioxid. Redox Signal*. 36, 309–326.

Keywords: hydropersulfide, hydropersulfide donor, persulfidation, cytoprotective agent, oxidative stress, antioxidant

Introduction

In the last decade, hydrogen sulfide (H_2S) has emerged as an important cell signaling molecule, joining nitric oxide (NO) and carbon monoxide (CO) as ''gasotransmitters'' that are produced in biological systems (68). In mammals, H_2S is produced enzymatically mainly by three enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (13). H_2S can influence a myriad of physiological functions, including neuronal transmission, vascular tone, cytoprotection, vasorelaxation, inhibition of myocardial ischemia/reperfusion injury, and slowing the development of atherosclerosis (6, 47, 48). Despite the beneficial effects of H_2S, underlying mechanisms remain poorly characterized. H_2S-mediated post-translational modifications of redox-active cysteines (Cys-SH) into cysteine hydropersulfide (Cys-SSH) have been proposed as a critical component of H_2S signaling (28). However, the direct reaction of H_2S with protein cysteine residues is not likely because H_2S is not expected to react with thiols in their reduced state. Rather, the reaction to form hydropersulfides (RSSH) occurs *via* the reaction of H_2S with oxidized thiols, for example, disulfides (RSSR) or sulfenic acids (RSOH).

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Recently, there has been intense interest in the physiological functions of cytosolic Cys-SSH and glutathione hydropersulfide (GSSH), as well as the persulfidation of protein cysteine residues, that is, conversion of a protein thiol (P-SH) to a protein hydropersulfide (P-SSH). Accumulating evidence suggests that RSSH could be fundamental to cell proliferation, growth, and survival (31, 56, 63, 86). This review focuses on the unique chemistry associated with this species and the recent progress that has been made developing precursor molecules to interrogate RSSH biological functions.

RSSH prevalence in biological systems

Recent advances in analytical methods for the quantification of RSSH in biological samples have led to identification of several low-molecular-weight RSSH in prokaryotes, eukaryotes, and mammals. For instance, Cys-SSH and Cys-SSH-derived hydropersulfides have been identified in a variety of cell types, including human lung adenocarcinoma A549 cells, neuroblastoma SH-SY5Y cells, cervical cancer HeLa cells, embryonic kidney HEK293 cells, bronchial epithelial cells, and mouse macrophage-like Raw264.7 cells (1, 39, 55, 80). High levels of GSSH (ca. 150 μM) have been found in mouse brains and about 50 μM is also detected in other organs including the heart and liver (39). In addition, homocysteine hydropersulfide (HCys-SSH) is also observed in mice organs. Akaike and coworkers have detected high levels of Cys-SSH, HCys-SSH, GSSH, cystine tetrasulfide (Cys-SSSS-Cys), cysteine pentasulfide (Cys-SSSSS-Cys), and glutathione trisulfide (GSSSG) in human plasma and tissues. Numerous enzymes and proteins have RSSH modification at cysteine residues. For instance, Snyder and coworkers have reported that in liver lysates treated with sodium hydrogen sulfide about 10%–25% of liver proteins, including actin, tubulin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are persulfidated under physiological conditions (65). Utilizing a highly specific protein hydropersulfide detection protocol, Nagy and coworkers have revealed that about 0.1%–1% proteins of the total protein pool in human embryonic kidney 293 cells and mouse liver are persulfidated (23) (Fig. 1).

RSSH species are also prevalent in bacteria. Giedroc and coworkers reported the presence of RSSH derived from bacillithiol, cysteine, and coenzyme A in *Staphylococcus aureus* (59). Importantly, they have demonstrated that these low-molecular-weight RSSH play an important role in regulating the global virulence of *S. aureus* by modulating the activity of the regulator MgrA *via* protein persulfidation. In addition, Takagi and Ohtsu have detected RSSH species, including Cys-SSH and GSSH in gram-negative bacterium *Escherichia coli* (66).

RSSH biosynthesis

Cys-SSH biosynthesis is catalyzed by mainly three enzymes: CBS, CSE, and cysteinyl-tRNA (transfer RNA) synthetases (CARSs). *In vitro* experiments with recombinant enzymes have revealed that both CBS and CSE can catalyze Cys-SSH synthesis utilizing cystine as a substrate (Fig. 2a) (39). Furthermore, Akaike and coworkers reported that overexpression of CBS and CSE significantly increased the cellular levels of Cys-SSH, GSSH, and P-SSH, indicating the involvement of these enzymes in RSSH biosynthesis (39). However, based on their endogenous levels, the kinetics of these enzymes, and physiological substrate concentrations, Cys-SSH biosynthesis by these enzymes has been reported to be negligible in the liver (72). In addition, the reducing environment in cells can result in higher levels of cysteine over cystine, which is a better substrate for H_2S production. However, if cystine concentrations increase under cellular stress conditions (*e.g.*, oxidative stress), the CSE-dependent Cys-SSH generation pathway can be major.

More recently, Akaike *et al*. made the exciting discovery that CARSs are primary Cys-SSH synthases utilizing cysteine as a substrate (1). CARSs also incorporate Cys-SSH directly into proteins during translation (Fig. 2b). It has been proposed that CARSs catalyze cysteinyl-tRNA production *via* a two-step mechanism (63). First, Cys-SSH are activated in the presence of adenosine triphosphate to form an enzyme-bound cysteinyl hydropersulfide adenylate, and in the second step, the activated Cys-SSH are transferred to the ribose hydroxyl group at the 3′-terminus of the cysteinyl-tRNA molecule. In mammals, two different CARSs are present—cytosolic CARS1 and mitochondrial CARS2 (34), both of which are efficient producers of Cys-SSH. Importantly, CARS2-dependent Cys-SSH generated in the mitochondria play a critical role in mitochondrial biogenesis and bioenergetics (1).

RSSH can be produced by the reaction of deprotonated H_2S (HS^-) with RSSR (29, 67). However, the physiological relevance of this reaction remains to be established because of the low concentration of disulfides in the reducing environment of cells and the relatively low reactivity of HS^- toward disulfides. For example, the rate of H_2S reaction with cystine is reported to be 0.6 $M^{-1}\cdot s^{-1}$ at pH 7.4, 25°C (16). However, the rate of HS^- reaction with disulfides within a protein environment can be accelerated (Fig. 3). For instance, the mitochondrial enzyme, sulfide quinone oxidoreductase (SQR), catalyzes H_2S oxidation *via* reaction with its critical disulfide to produce P-SSH (40, 52). Furthermore, the sulfane sulfur (a sulfur atom with six valence electrons and no charge) of a P-SSH can be transferred to acceptors such as biological thiols to produce the corresponding RSSH, for example, GSSH (Fig. 3) (79).

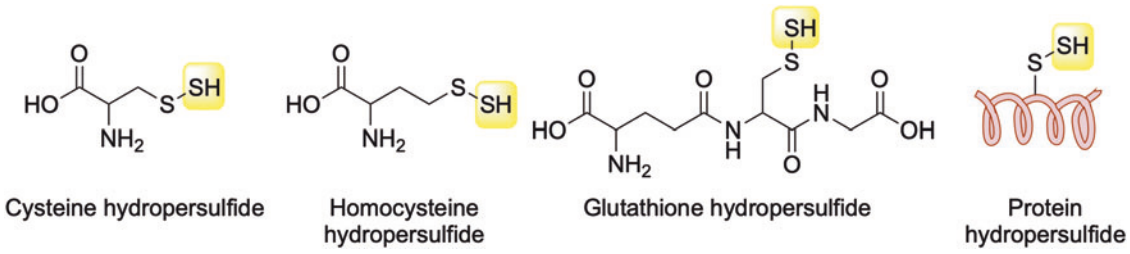

FIG. 1. Structures of biologically prevalent hydropersulfides. Color images are available online.

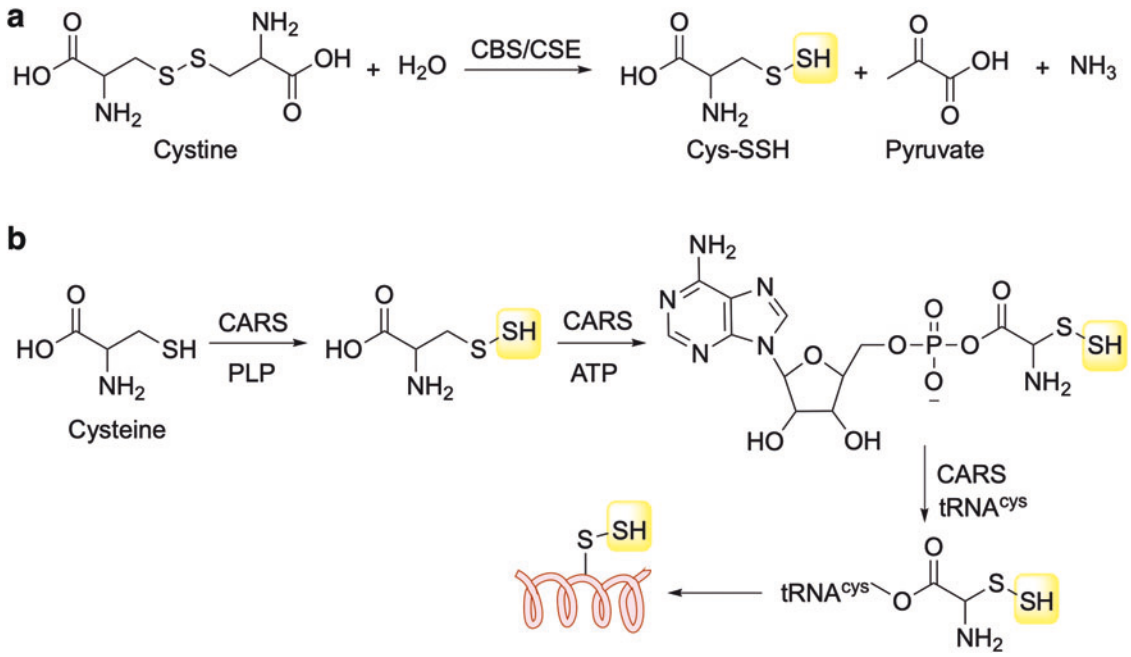

FIG. 2. Biosynthesis of cysteine hydropersulfide RSSH and protein hydropersulfides: (a) Cys-SSH biosynthesis catalyzed by CBS, CSE, and **(b)** CARS-mediated Cys-SSH biosynthesis and subsequent incorporation into proteins to produce protein hydropersulfides. CARS, cysteinyl-tRNA synthetase; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; Cys-SSH, cysteine hydropersulfide; tRNA, transfer RNA. Color images are available online.

HS^- can also react with other oxidized cysteine residues such as sulfenic acids in proteins (P-SOH) to form P-SSH (16). However, a caveat here is that healthy cells contain high concentrations of reduced thiols, which will compete with HS^- for reaction with sulfenic acids. Hence, only relatively stable and sterically constrained sulfenic acids have been proposed to react with H_2S/HS^- to produce P-SSH. Cuevasanta *et al.* recently reported that peroxiredoxin *Mycobacterium tuberculosis* alkyl hydroperoxide reductase E (*Mt*AhpE-SH) reacts rapidly with hydroperoxides to form a stable sulfenic acid (*M. tuberculosis* alkyl hydroperoxide reductase E sulfenic acid [*Mt*AhpE-SOH]) (18). This protein sulfenic acid subsequently reacts with H_2S ($1.4 \pm 0.2 \times 10^3$ $M^{-1} \cdot s^{-1}$, pH 7.4, 25°C) to give the hydropersulfide (*M. tuberculosis* alkyl hydroperoxide reductase-E hydropersulfide [*Mt*AhpE-SSH]). Alternatively, protein cysteine residues can react with compounds containing sulfane sulfur, including RSSH, polysulfides (RS_nSH or RSS_nSR, $n \geq 1$), inorganic polysulfides (HSS_nH, $n \geq 1$), thiosulfate ($S_2O_3^{2-}$), to produce P-SSH (Fig. 3) (10, 26, 33).

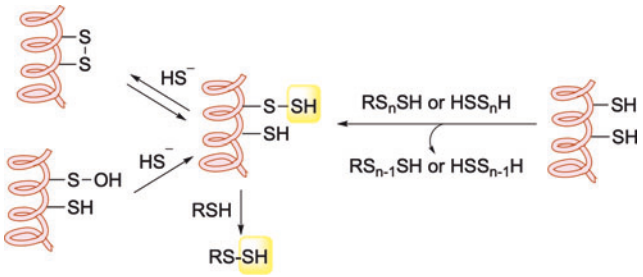

FIG. 3. Nonenzymatic pathways of RSSH generation. RSSH, hydropersulfides. Color images are available online.

Physicochemical properties of RSSH

The RSSH species possess unique chemical properties that are distinct from other physiologically relevant sulfur species, and this might help to explain why these species are prevalent in biological systems. We briefly compare the chemical properties of RSSH with those of RSH.

Acidity. The acidity of RSSH is predicted to be higher compared with RSH, presumably due to the stable nature of conjugate base RSS^- compared with thiolates. Everett *et al.* estimated the pK_a of 2-[(3-aminopropyl)amino]ethane RSSH to be 6.2, which is lower than the corresponding thiol ($pK_a = 7.6$) (24). Similarly, Pratt and coworkers proposed the pK_a of cumyl RSSH to be 7 (15), while the pK_a of cumyl thiol is likely to be higher than 10. Recently, the GSSH pK_a has been estimated to be 5.45, which is 3.49 units lower than that of glutathione (GSH) (9). Moreover, a computational study predicted the pK_a of Cys-SSH to be 4.3, which is nearly 4 units lower than that of Cys-SH ($pK_a = 8.29$) (16). These results indicate that RSSH will be substantially ionized to RSS^- under physiological conditions and the $[RSS^-]/[RSSH]$ ratio is likely much higher than the corresponding $[RS^-]/[RSH]$ ratio (Fig. 4).

Reductive capacity and H-atom donating ability. RSSH/RSS^- are superior reductants compared with RSH/RS^-(25). RSSH are also excellent H-atom donors to alkyl, alkoxyl, peroxyl, and thiyl radicals ($RS^\bullet$), compared with RSH (11, 15, 25). The enhanced H-atom transfer capacity of RSSH is mainly based on thermodynamic factors including the weak RSS-H bond dissociation enthalpy (ca. 70 kcal/mol) and the high stability of the RSSH radical (perthiyl radical [$RSS^\bullet$]). Moreover, $RSS^\bullet$ is a relatively weak oxidant and mainly

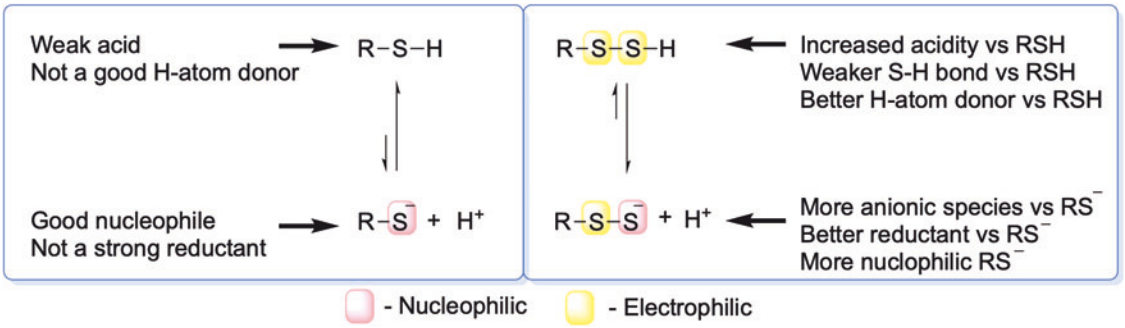

FIG. 4. Differences in chemical properties between RSH and RSSH. Color images are available online.

dimerizes dialkyl tetrasulfide (RSSSSR) (11). RS$^{\bullet}$, on the contrary, are reactive and capable of initiating radical chain reactions.

Nucleophilicity. RSSH are more nucleophilic than thiols with a similar pK_a, likely due to the alpha effect, that is, the increased nucleophilicity of an atom due to the presence of an adjacent atom with lone pair electrons (32). In addition, the increased acidity of RSSH relative to RSH results in a much greater concentration of the anionic state at physiological pH, further boosting their relative nucleophilicity compared with that of analogous thiols (Fig. 4). Reactivity studies by Paris and coworkers demonstrate that the rate of aryl RSSH reaction with alkyl halides in organic solvent is much higher than that of the corresponding aryl thiolates (7). Enhanced reactivity is also observed in the case of human serum albumin hydropersulfide (HSA-SSH) toward 4,4′-dithiodipyridine and peroxynitrite in comparison with human serum albumin (HSA-SH) (16). Likewise, the *Mt*AhpE-SSH reacts 43 times faster than *Mt*AhpE-SH with 4,4′-dithiodipyridine (18). Alvarez and coworkers recently reported that GSSH reacts 1200, 97, and 22 times more rapidly than GSH with the electrophiles, monobromobimane, peroxynitrite, and hydrogen peroxide (H_2O_2), respectively (9). Recently, we also demonstrated enhanced nucleophilicity of RSSH toward nitroxyl (HNO) compared with the corresponding RSH (78). The high nucleophilicity of RSSH can result in efficient scavenging of reactive oxygen species (ROS) and deleterious electrophiles generated *via* oxidative pathways. Consistent with this notion, Fukuto and coworkers have recently reported that increased levels of intracellular RSSH exhibit enhanced resistance to electrophilic stress in HEK293T cells (10). In addition, Akaike and coworkers demonstrated that CSE overexpression leads to increased intracellular levels of RSSH species in A549 cells, which confers resistance toward H_2O_2-mediated oxidative stress (39).

Electrophilicity. Interestingly, and unlike RSH and H_2S, RSSH in the neutral state are electrophilic and may react with nucleophiles, including P-SH, which can result in the generation of (Fig. 4). This transformation has been proposed to protect protein thiols from irreversible modification during oxidative and/or electrophilic insult (62). When P-SH is electrophilically modified (PS-alkyl) or oxidized to the RSOH, sulfinic acid [RS(O)OH], or sulfonic acid [$RS(O)_2OH$], it is generally considered to be irreversibly modified and permanently damaged. However, when P-SSH undergoes the equivalent modifications [generation of PSS-alkyl, P-SSOH, P-SS(O)OH, or P-$SS(O)_2OH$], each of these species can be reductively reversed back P-SH in biological systems *via* reactions with thiols. Nagy and coworkers have recently reported that persulfidation protects Cys-SH residue in proteins, for example, albumin, peroxiredoxin-2 (Prx2), and protein tyrosine phosphatase-1B (PTP1B), from irreversible oxidative loss of function by the formation of Cys-$SSO_{1\text{-}3}$H derivatives that can subsequently be reduced back by the thioredoxin system to the corresponding thiols (22). In addition, one of the most crucial differences between RSSH and other antioxidants is that RSSH may activate the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) by interacting with and oxidizing the thiol groups on the Nrf2 sequestering protein kelch-like ECH-associated protein 1 (KEAP1), leading to cellular protection *via* expression of antioxidant response element (ARE)-dependent genes (30). Thus, the RSSH species are chemically and biochemically distinct from other reductants and thiols, and therefore can have enhanced protective effects on stressed cells (Fig. 4).

Methods of protein persulfidation

Recent studies have suggested that protein persulfidation plays an important role in the biological signaling of H_2S and related species (28). However, the mechanism(s) by which persulfidation takes place and its impact on cellular functions are not fully understood. To understand the biochemistry of protein persulfidation, several methods have been developed for P-SSH generation. One strategy is the reaction of activated disulfides with equimolar amounts of H_2S. For example, a protein with a reactive cysteine is first treated with 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), to form a mixed disulfide, which is then further treated with H_2S to generate P-SSH (Fig. 5a) (30, 57). Another method involves the reaction of a protein sulfenic acid (*e.g.*, generated by cysteine oxidation with H_2O_2) with H_2S (Fig. 5b) (79). Incubation of protein thiols with oxidized H_2S species such as polysulfides has also been used to generate P-SSH (Fig. 5c) (23). However, these methods suffer from the involvement of relatively unstable and highly reactive species, potentially resulting in nonselective reactions. Recently, Wang and coworkers reported a cleaner method for protein persulfidation utilizing hydrogen disulfide (H_2S_2), which was produced *in situ* from a *bis*-protected disulfide derivative triggered by esterase (or phosphatase) (Fig. 5d) (76). Alternatively, a protein thiolate can be treated with 9-fluorenylmethyl disulfide to form a

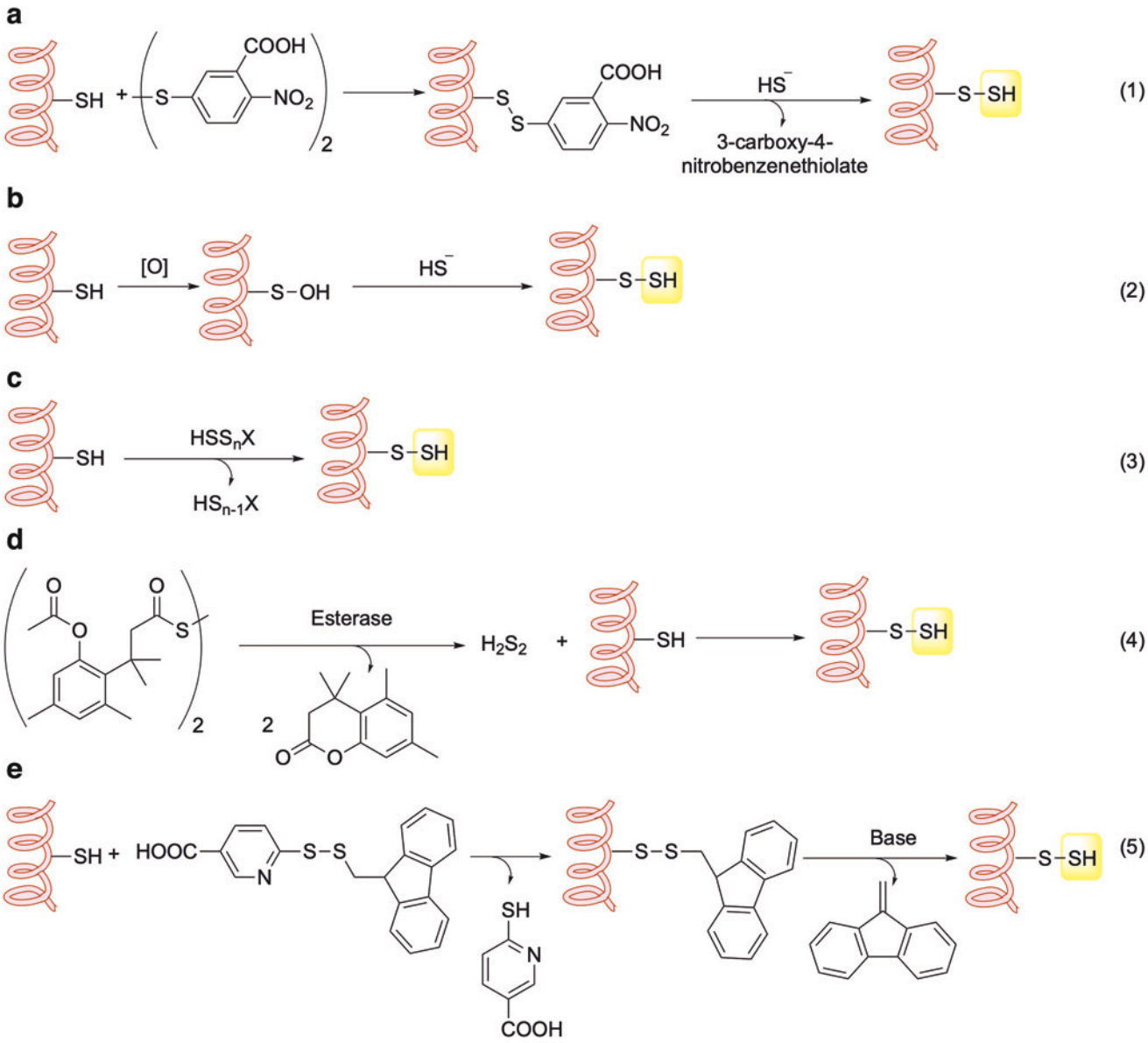

FIG. 5. Methods for the preparation of protein hydropersulfides: (a) the reaction of H_2S with protein thiol-TNB disulfide, **(b)** protein sulfenic acid reaction with H_2S, **(c)** protein thiol reaction with hydrogen polysulfide, **(d)** protein thiol reaction with hydrogen disulfide produced from the bis-protected disulfide derivative in the presence of esterase, **(e)** base-mediated deprotection of protein thiol-9-fluorenylmethyl disulfide. Color images are available online.

mixed disulfide, which can then be deprotected under basic conditions to generate P-SSH (Fig. 5e) (58), although alkaline conditions could lead to protein denaturation.

Detection of RSSH

Persulfidation is an endogenous and key post-translational modification, but RSSH are unstable, complicating their characterization in proteins. Initial methods relied on spectrophotometric analysis of P-SSH, however, this method is of limited utility as the RSSH ultraviolet (UV)-visible signature lies between 335 and 340 nm with a weak extinction coefficient (ca. 300 $M^{-1}\cdot cm^{-1}$) (17, 70). Alternatively, P-SSH could be labeled selectively and subsequently characterized, but a major bottleneck of such labeling is discriminating RSSH over thiols, sulfenic acids, and polysulfides. Fortunately, RSSH possess enhanced nucleophilicity as well as electrophilic properties, and as a result, differentiating RSSH from other reactive sulfur species is possible.

The first reported method for P-SSH detection was cyanolysis (70). The cold cyanolysis method involves cyanide ion (CN^-) attack on the outer sulfur of P-SSH to form a thiocyanate ion (SCN^-), which subsequently reacts with a ferric agent giving rise to a ferric thiocyanate complex ($\lambda_{max} = 460$ nm), allowing for the determination of P-SSH concentrations spectroscopically (Fig. 6). However, due to the reactivity of CN^- toward other sulfane sulfur containing species, cold cyanolysis is only viable for use on purified proteins and is not compatible for the detection of RSSH in biological samples.

Sawahata and Neal exploited the enhanced nucleophilicity of RSSH over RSH as well as the formation of disulfides

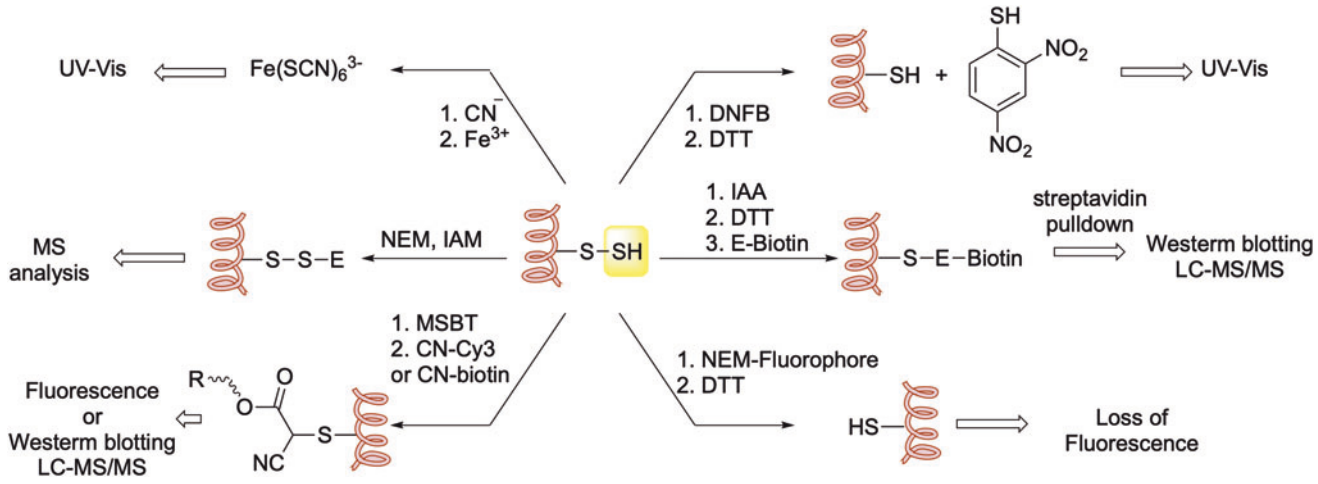

FIG. 6. Methods for RSSH detection. Color images are available online.

rather than thioethers when reacting with Sanger's reagent (1-fluoro-2,4-dinitrobenzene, DNFB) (64). They reported a rhodanese hydropersulfide reaction with DNFB to form a mixed disulfide, which was subsequently reacted with dithiothreitol (DTT) to release 2,4-dinitrobenezenthiol (λ_{max} 408 nm with an extinction coefficient of 13,800 $M^{-1} \cdot cm^{-1}$). DTT selectively reacts with disulfides over thioethers and thus provides specificity of P-SSH over protein thiols.

While direct detection of RSSH is possible using mass spectrometry (MS), this technique has some limitations, namely instability of the RSSH moiety and mass increases due to the addition of one sulfur atom and two oxygen atoms being very similar. Thus, direct detection is limited to small peptides. To bypass these issues, conventional thiol electrophiles such as *N*-ethylmaleimide (NEM), iodoacetamide (IAM), and others have been implemented to detect the persulfidated peptides (29, 57) (Fig. 6).

For the detection of RSSH in cellular environments, fluorescent probes, the modified biotin-switch, and the tag-switch method have been utilized. The tag-switch method was first reported by Snyder and coworkers utilizing *S*-methyl methanethiosulfonate (MMTS) as the thiol blocking reagent (53). MMTS was proposed to block thiols selectively over RSSH leaving the free RSSH to react with the biotinylating agent, biotin-HPDP (*N*-[(6-biotinamido) hexyl]-3-(2-pyridyldithio) propionamide). However, this method is controversial as RSSH have been reported to react with MMTS and also with an MMTS derivative, *S*-4-bromobenzyl methanethiosulfonate (57). Iterations of the tag-switch methods with iodoacetic acid (IAA) and derivatized NEM have been implemented (25, 49, 51, 69). The IAA method used analysis *via* Western blotting followed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis, whereas the derivatized NEM was detected *via* loss of fluorescent signal (Fig. 6). Both methods involve initial reactions with the respective electrophile followed by reduction of the newly formed disulfide with DTT. The NEM method is directly analyzed after this step for loss of fluorescence, whereas the IAA method requires an additional step involving a biotinylated IAM derivative that will react with the newly formed thiol for further analysis. Xian and coworkers further iterated on the tag-switch method by exploiting the formation of disulfides from RSSH reaction with MSBT (methylsulfonyl benzothiazole) as opposed to thioether formation from reaction of MSBT with thiol (79). First, MSBT is initially added to block both protein thiols and RSSH. The disulfide bond of the blocked RSSH is more reactive toward carbon nucleophiles and can react with cyanoactetae derivatives containing a reporter molecule such as CN-Cy_3 or CN-biotin in the next step (69), resulting in facile detection either *via* fluorescence or Western blotting (Fig. 6). Nagy and coworkers have developed a convenient, reliable, and highly specific protein persulfide detection method (23). Previous reviews have provided a more comprehensive evaluation of P-SSH detection methods (28, 73) (Fig. 6).

Historical Perspective on RSSH Generation and the Need for RSSH Donors

The first synthetic reports of RSSH date back to 1954. Böhme and Zinner reported the preparation of alkyl- and aryl-hydropersulfides (11a). The key intermediates, acyl disulfides, were prepared from the reaction of acetyl sulfenyl chloride with alkyl and aryl mercaptans (Fig. 7a). Although acid-mediated hydrolysis of acyl disulfides provided RSSH, their characterization was performed following iodine oxidation to more stable tetrasulfides. Methoxycarbonyl disulfides have also been utilized to form RSSH under strongly alkaline conditions (Fig. 7b) (43). These strategies have set the stage for researchers to expand the scope of characterizing and understanding the chemical properties of RSSH. However, the harsh reaction conditions (strongly acidic or basic) that have been used in these strategies to prepare RSSH are not amenable to biological systems.

Another approach used to study low-molecular-weight RSSH uses the reaction between H_2S and disulfides [Fig. 7c, Eq. (1)]. While this method has been applied in biological studies, it has several drawbacks since the reaction mixture does not contain RSSH as the only component. Instead, a mixture of polysulfides varying in length, thiol, and H_2S are present in solution [Fig. 7c, Eq. (2)], thus making it difficult to analyze the reactivity of RSSH directly.

More recently, researchers have synthesized sterically hindered and isolable RSSH, including trityl (54, 66a), *t*-butyl (4), penicillamine (36), and adamantyl (27)

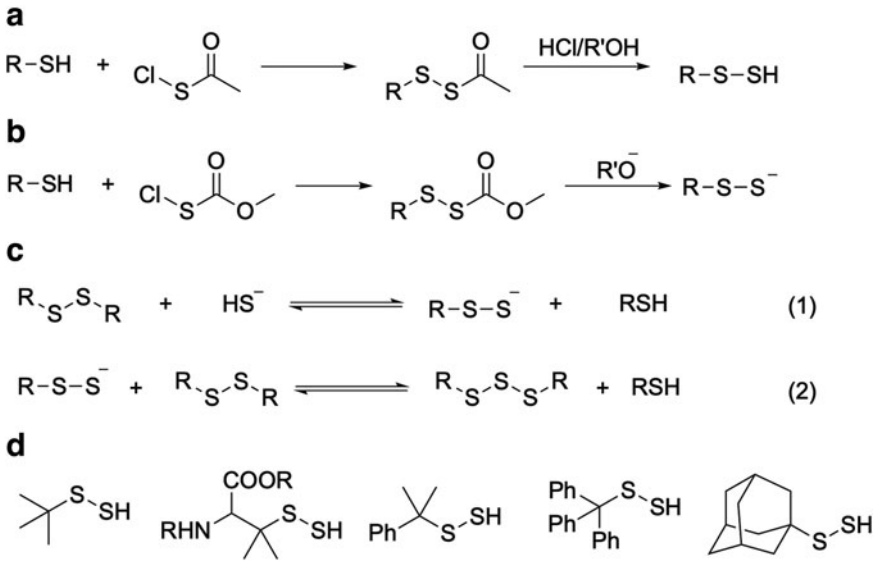

FIG. 7. Various methods of RSSH generation: (a) acid-mediated hydrolysis of acyl disulfides, **(b)** base-mediated decomposition methoxycarbonyl disulfides, **(c)** H_2S reaction with disulfides, and **(d)** structures of some isolable RSSH.

derivatives (Fig. 7d). Pluth and coworkers have synthesized some of these precursors to investigate the chemical properties of RSSH (5). Because these precursors still suffer from poor solubility and stability issues in aqueous buffers, these reactivity studies were conducted mostly in apolar organic solvents.

Because of their nucleophilic and electrophilic character, the RSSH species can self-react to give a variety of products, including polysulfides (RSS_nSR), inorganic polysulfides (HSS_nSH), H_2S, and S_8. Thus, with the exception of sterically hindered RSSH in nonaqueous solvent, RSSH cannot be isolated. As a result, one of the most crucial requirements to examine the potential functions and roles of RSSH as physiological signaling and/or protecting agents is the development of biologically useful donors (75). In recent years, a number of prodrug strategies for RSSH release have been developed, and the primary focus of this review is to summarize the variety of strategies that have been applied to RSSH generation in biological studies.

RSSH Donors Amenable for Studies in Biological Systems

pH-activated RSSH precursors

To address the intrinsic instability of RSSH, the terminal sulfhydryl group can be masked with a suitable protecting group to be cleaved by some stimuli. Galardon and coworkers have developed the *S*-methoxycarbonyl penicillamine disulfide (MCPD) that releases the RSSH MCP-SSH at pH 7.4 by intermolecular acyl transfer from the *S* to *N* atom (Fig. 8a) (3). RSSH generation from this precursor is pH-dependent and increases with the alkalinity of the solution as measured by the DTNB assay. RSSH generation was also confirmed using NEM as a trap to produce the MCP-SS-NEM adduct. This precursor possesses several advantageous properties, including ease of synthesis, solubility in aqueous solution, and stability at room temperature for months. Importantly, MCPD has been used as research tool to investigate the chemical properties of RSSH. For example, Galardon and coworkers have demonstrated the utility of this precursor in biologically relevant studies such as the RSSH-mediated conversion of 8-NO_2-cGMP to 8-SH-cGMP and the fast reduction of ferric cytochrome c to ferrous cytochrome c (3). Together with Fukuto and colleagues, our group has also used MCPD to demonstrate that RSSH can be easily oxidized by weak oxidants to generate the $RSS^{\bullet}$, which mainly dimerizes to RSSSSR (11). We also recently used MCPD to show that RSSH is a potent reductant that not only reduces ferric myoglobin ($Fe^{III}Mb$), but also reacts with oxymyoglobin ($O_2Fe^{II}Mb$) to produce a fleeting $Fe^{III}Mb$ intermediate, which is rapidly reduced to the ferrous form by RSSH (2). In

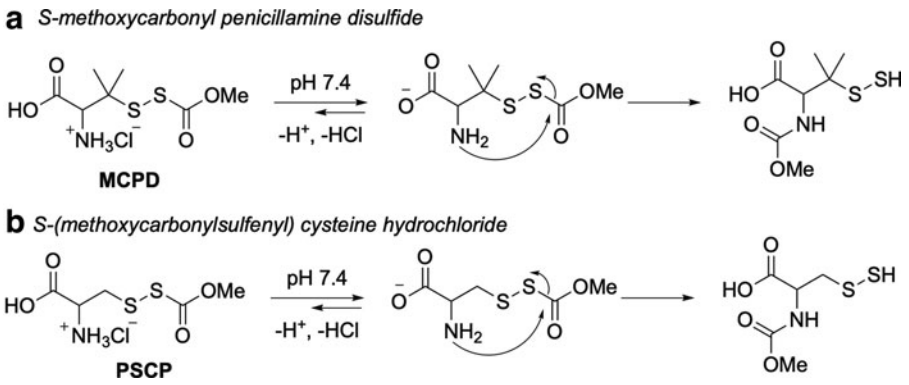

FIG. 8. RSSH generation from (a) MCPD and (b) PSCP. MCPD, *S*-methoxycarbonyl penicillamine disulfide; PSCP, persulfidated cysteine precursor.

addition, Lin *et al.* utilized MCPD as a precursor to study the RSSH-mediated reduction of water-soluble tetrazolium 8 (WST-8), a water-soluble salt utilized for assessing cellular metabolic activity (51).

Yang and coworkers have reported a similar strategy for Cys-SSH generation *via S*-(methoxycarbonylsulfenyl) cysteine hydrochloride, which they name as a persulfidated cysteine precursor (PSCP) (Fig. 8b) (81). At physiological pH, PSCP undergoes an intramolecular acyl transfer reaction to release Cys-SSH that eventually degrades to the corresponding tri- and tetrasulfides. Cell imaging assays show that PSCP increases intracellular sulfane sulfur levels in liver cancer SNU 398 cells, analyzed by the SSP4 flourescent probe (81). Interestingly, PSCP exerts anticancer effects in SNU398 and SNU 387 cancer cells; however, anticancer effects were not observed in other tumor cells, including lung cancer A549 cells, prostate cancer PC3 cells, cervical cancer HeLa cells, and MDA-MB-231 breast cancer cells. Notably, in normal cells such as Raw-blue macrophages, H9c2 cardiomyocytes, and normal liver cells, PSCP did not show severe toxicity. These results suggest that PSCP can inhibit tumor cell viability in a liver cancer-specific manner.

Xian and colleagues have reported a general scaffold containing *O*-silyl protected unsymmetrical disulfides (Fig. 9a) (42). These precursors undergo pH- or fluoride-mediated desilylation to release hemithioacetal intermediates, which rapidly undergo hydrolysis to release RSSH and aldehyde as a by-product. To examine the effect of the *O*-silyl

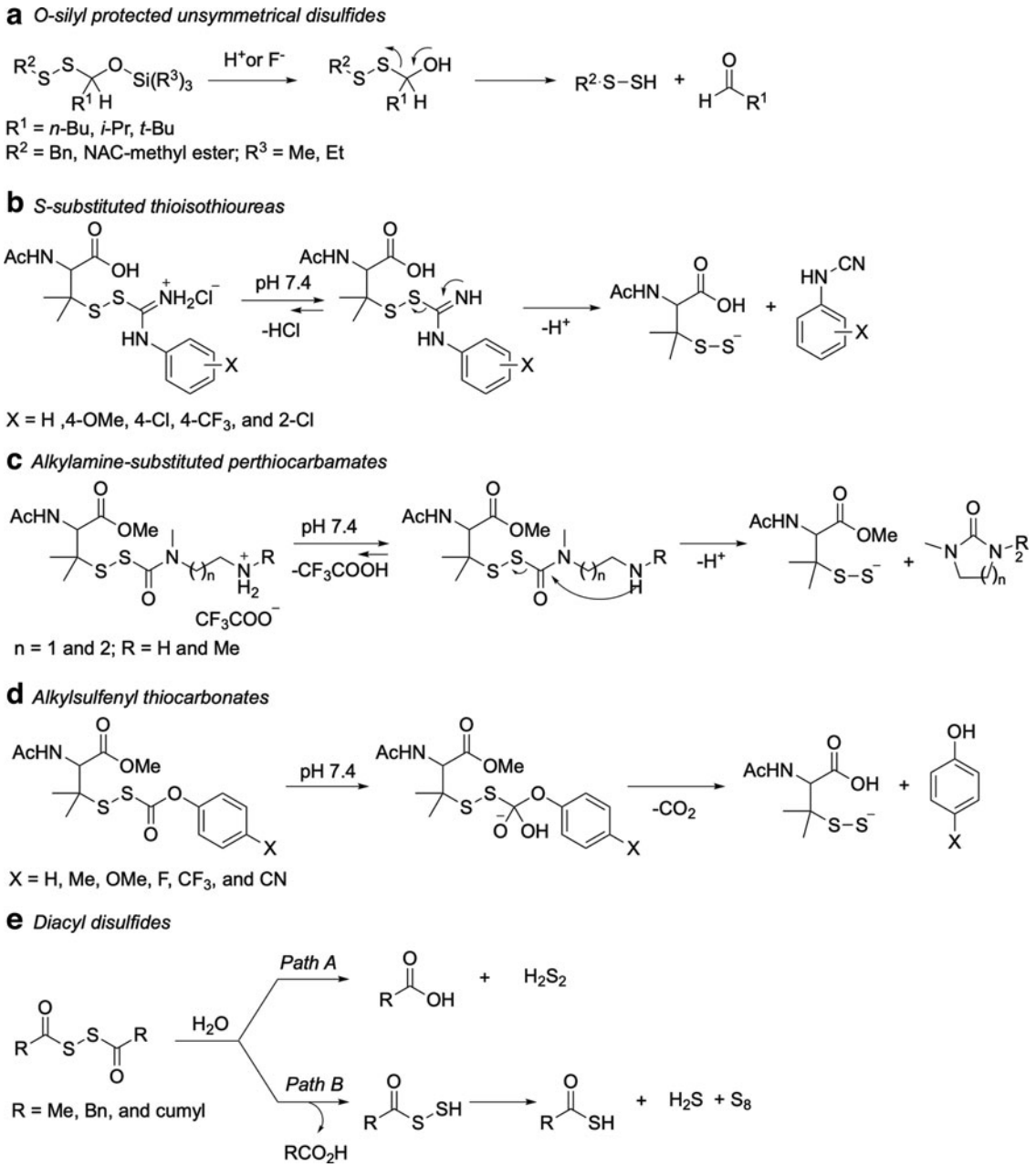

FIG. 9. RSSH generation using pH as a trigger: (a) *O*-silyl protected unsymmetrical disulfides, **(b)** *S*-substituted-thioisothioureas, **(c)** alkylamine-substituted perthiocarbamates, **(d)** alkylsulfenyl thiocarbonates, and **(e)** diacyl disulfides.

and R groups on the kinetics of RSSH release, several precursors were synthesized. Interestingly, precursors containing the *O*-trimethylsilyl (OTMS) group showed efficient RSSH release over a wide range of pH (5.0, 6.0, and 7.4), whereas *O*-triethylsilyl (OTES) group-containing precursors released RSSH at a much slower rate. As anticipated, RSSH generation in the absence of trapping agents resulted in the formation of a mixture of di- and trisulfides. These results are consistent with the unstable nature of RSSH under aqueous conditions. However, RSSH was trapped in the presence of IAM to form RSS-IAM. Based on this activation strategy, these precursors may find use for RSSH delivery to cellular environments that are acidic.

Our group has designed and synthesized *S*-substituted-thioisothioureas as efficient RSSH precursors (Fig. 9b) (46). Under physiological conditions, neutralization of *S*-substituted-thioisothioureas occurs to provide a neutral species that undergoes an elimination reaction to give RSSH and aryl cyanamide as a by-product. RSSH release from these precursors was measured by trapping with NEM. RSSH generation rates were tuned by structural modifications. For example, electron withdrawing substituents on the phenyl ring slowed the RSSH release rate.

We also reported an additional class of RSSH precursors in which the terminal sulfhydryl group is protected in the form of perthiocarbamate containing a terminal non-nucleophilic quaternary ammonium salt (Fig. 9c) (45). Neutralization of the quaternary ammonium salt under physiological conditions forms an active amine nucleophile that subsequently undergoes an intramolecular cyclization to release RSSH and cyclic urea. The kinetics of RSSH generation from these precursors was measured by trapping with β-(4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM). By varying the substituent on the trigger nitrogen and changing the length of the methylene spacer, we showed that the rate of cyclization can be tuned, thereby varying RSSH release rates with half-lives ranging from 1.4 to 484 min. In the presence of thiol, some precursors (*i.e.*, those with longer half-lives) were found to generate carbonyl sulfide (COS) as an additional product along with RSSH generation. COS can be hydrolyzed to H_2S by carbonic anhydrase in biological systems. Importantly, the potential therapeutic benefit of these precursors was demonstrated in the context of oxidative stress. A medium-lived precursor with a 16.7 min half-life was chosen for biological studies, and cytotoxicity assays revealed no toxicity toward H9c2 cardiomyocytes up to 150 μM. Interestingly, pretreating H9c2 cells with this RSSH precursor for 2 h showed a dose-dependent attenuation of H_2O_2-induced toxicity. Furthermore, protection against myocardial ischemia/reperfusion (MI/R) injury in *ex vivo* mouse hearts was also examined. Following 20 min of global ischemia, infusion of this RSSH precursor significantly attenuated the observed myocardial infarct size (16%) compared with the untreated group (42%).

More recently, we reported another strategy for RSSH generation based on the hydrolysis of alkylsulfenyl thiocarbonates under physiologically relevant conditions (Fig. 9d) (44). Here, the kinetics of RSSH release was measured by trapping with MMTS. We observe RSSH release rates that are tunable through modification of the thiocarbonate carbonyl group's electrophilicity, with half-lives ranging from 28 to 147 min. These precursors also react with thiols to release RSSH and a minor amount of COS. Remarkably, pretreatment of H9c2 cardiomyocytes with only 5 μM of an alkylsulfenyl thiocarbonate RSSH precursor (Fig. 9d, X = H) shows substantial protection against 200 μM of H_2O_2. Under similar conditions, the phenol by-product shows no protective effects, indicating that the protection is due to RSSH. We also demonstrated increased intracellular RSSH levels from this precursor utilizing the SSP4 probe. Taken together, these results demonstrate the potential therapeutic benefit of RSSH donors in the context of oxidative stress.

More recently, Xian and coworkers have developed diacyl disulfides as H_2S_2 donors (71). Under aqueous conditions (pH 7.4), either both acyl groups of the diacyl disulfide can be hydrolyzed to release H_2S_2 (Fig. 9e, Path A) or only one acyl group is hydrolyzed to form an acylated RSSH intermediate (Fig. 9e, Path B). This intermediate can further degrade to form other sulfur species. Incubation of these precursors in phosphate-buffered saline produces predominantly sulfane sulfur. The authors indicated that the formation of sulfane sulfur from these precursors could be attributed to both pathways, that is, either the decomposition of the acylated RSSH intermediate, produced from Path B, or H_2S_2, generated from Path A.

Thiol-activated RSSH donors

Polysulfides that raise hydropersulfide/polysulfide levels in cells by donating sulfur atoms to endogenous thiols have become important chemical tools for understanding the physiological and pathological roles of reactive sulfur species. Allicin, the active ingredient in garlic, undergoes decomposition to produce organosulfur compounds such as diallyl disulfide (DADS) and diallyl trisulfide (DATS). These polysulfides produce H_2S in the presence of biological thiols (Figs. 10a and 10b). In 2007, Kraus and coworkers demonstrated H_2S production from garlic in human red blood cells and the relaxation of rat aorta rings (8). Previously, both DADS and DATS were reported to produce H_2S in the presence of GSH through a thiol–disulfide exchange reaction followed by GSH reduction of allyl perthiol. However, Huang and coworkers have demonstrated that DADS releases only a minute amount of H_2S *via* a slow reaction with GSH through an α-carbon nucleophilic substitution pathway (Fig. 10a) (50), whereas DATS reacts rapidly with GSH to release H_2S in high yield. Although the pharmacological effects of garlic-derived polysulfides have been ascribed to H_2S generation, recent studies have suggested that RSSH (*e.g.*, allyl-SSH and GSSH) may be linked to their biological activity. For example, Kowalczyk-Pachel and coworkers have demonstrated that DADS and DATS increase total sulfane sulfur levels and CSE activity in normal mouse kidneys (38).

Fukuto and coworkers have utilized cysteine trisulfide (Cys-SSS-Cys) to increase intracellular RSSH levels (*e.g.*, Cys-SSH and GSSH) in many cell types. Importantly, Cys-SSS-Cys confers protection against oxidative (H_2O_2) and electrophilic (NEM) stress (51). Furthermore, Lin and coworkers have recently reported that Cys-SSS-Cys provides protection against NEM-induced cell death in *E. coli*, presumably through scavenging of NEM by Cys-SSH (37). In addition, they tested the effect of NEM-induced stress on

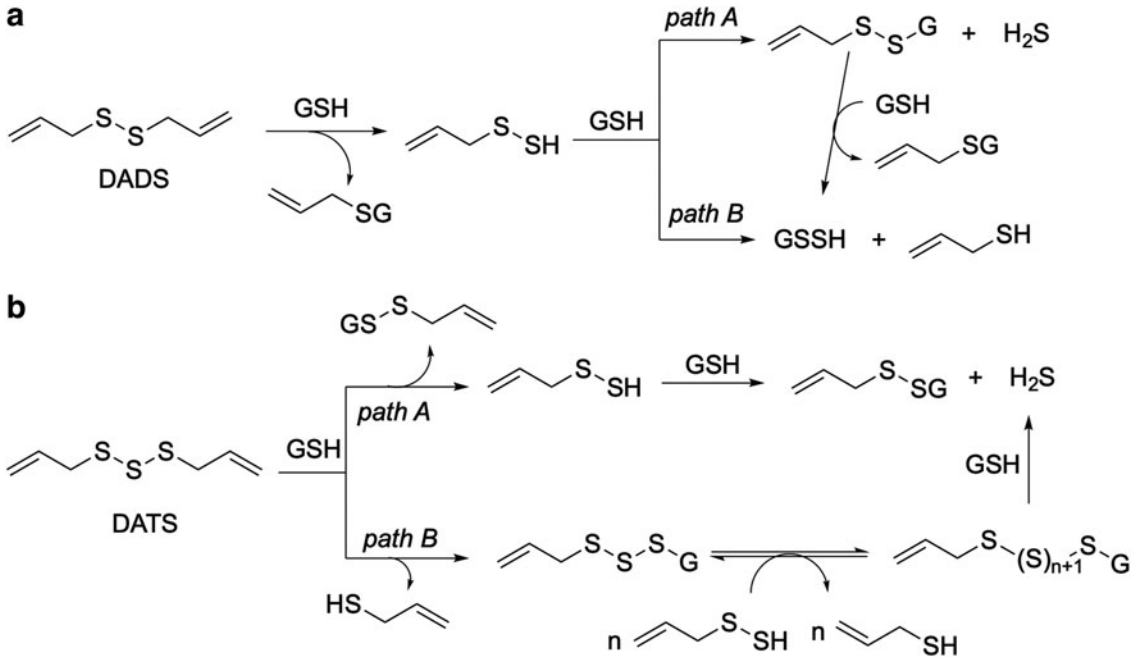

FIG. 10. Reaction of GSH with (a) diallyl disulfide and (b) diallyltrisulfide to produce RSSH and H_2S. GSH, glutathione; H_2S, hydrogen sulfide.

CD148 phosphatase activity and found that pretreatment with Cys-SSS-Cys leads to protection, again presumably *via* scavenging of NEM by Cys-SSH and GSSH.

Sawa and coworkers also synthesized *N*-acetylcysteine (NAC) polysulfides as thiol-activable RSSH donors (80). Treatment of RAW264.7 cells with ^{34}S-labeled NAC polysulfides was found to increase intracellular hydropersulfide/polysulfide levels and resulted in marked inhibition of lipopolysaccharide (LPS)-induced macrophage activation. Furthermore, NAC polysulfide treatment was demonstrated to inhibit the production of both tumor necrosis factor (TNF)-α and interferon-β in the mouse macrophage cell line Raw264.7. Moreover, administration of polysulfide donors protects mice from lethal endotoxin shock. These data indicate that cellular polysulfides negatively regulate TLR4-mediated proinflammatory signaling.

Due to the high concentration of thiols in cellular environments, small-molecule prodrugs that release RSSH upon reaction with thiol have been developed. For instance, Xian and coworkers reported *N*-(benzoylthio)benzamides as thiol-activated H_2S donors (Fig. 11a) (83). Initial thiol attack on the carbonyl carbon of the acyl thioamide leads to formation of a transient Cys-thiocarbonate that undergoes an S to N acyl transfer to give *N*-benzoyl-cysteine as a major by-product along with a sulfenamide intermediate. The sulfenamide further reacts with excess Cys-SH to give rise to Cys-SSH and releases benzamide. The Cys-SSH further reacts with Cys-SH ultimately giving cystine and H_2S. Although these compounds were originally developed as H_2S donors, Cys-SSH is produced as an intermediate from these compounds in the presence of thiols.

Xian and coworkers expanded the scope of thiol-triggered H_2S donors to cysteine perthiol-based H_2S donors where the acyl disulfide precursor undergoes reaction with thiol at the carbonyl carbon to release RSSH and the corresponding thiocarbonate as a by-product (Fig. 11b) (82). These perthiol-based H_2S donors have been shown to release H_2S in cells using the H_2S selective fluorescent probe WSP-1. Moreover, these donors display cardioprotective effects in an MI/R injury model. Administration of the acetyl-perthiol and benzoyl-perthiol donors at concentrations of 500 μg/kg and 1 mg/kg, respectively, resulted in reduction of infarct size. In addition, administration of these donors in mice after 22.5 min of ischemia shows reduction in cardiac troponin-I (a marker for acute myocardial infarction) levels. Although the cardioprotective effects of these compounds were ascribed to H_2S production, RSSH generation may also play a role.

Similarly, Galardon and coworkers have showed that acyl-protected disulfides generate H_2S *via* RSSH when reacted with thiols such as Cys-SH and GSH (Fig. 11c) (61). The potential of these donors under biological conditions was demonstrated by H_2S generation in HeLa cell lysates and Chinese hamster ovary (CHO) cells. An acetyl disulfide compound was found to induce endothelium-dependent vasorelaxation of isolated rat aortic rings.

Xian and coworkers have also developed cyclic acyl disulfides and selenylsulfides (RSeSH) as precursors to RSSH and RSeSH, respectively (19). These compounds produce RSSH or RSeSH upon reaction with nucleophiles such as thiols and amines. Two possible mechanisms were proposed for RSSH generation from these precursors. First, thiol (*n*-BuSH) can attack at the sulfur atom adjacent to the carbonyl group to form an *N*-acetyl penicillamine acyl disulfide intermediate [Fig. 11d, Eq. (1)], which can then undergo intramolecular cyclization to form a four-membered thiolactone and release *n*-BuSSH. Alternatively, thiol can attack the carbonyl group to form a thioester-linked tertiary RSSH [Fig. 11d, Eq. (2)]. Theoretical calculations indicate that the first step in both pathways is slightly exothermic. However, the step forming a four-membered thiolactone is quite endothermic; hence its formation is energetically less favorable and attack at the carbonyl dominates. Furthermore, the

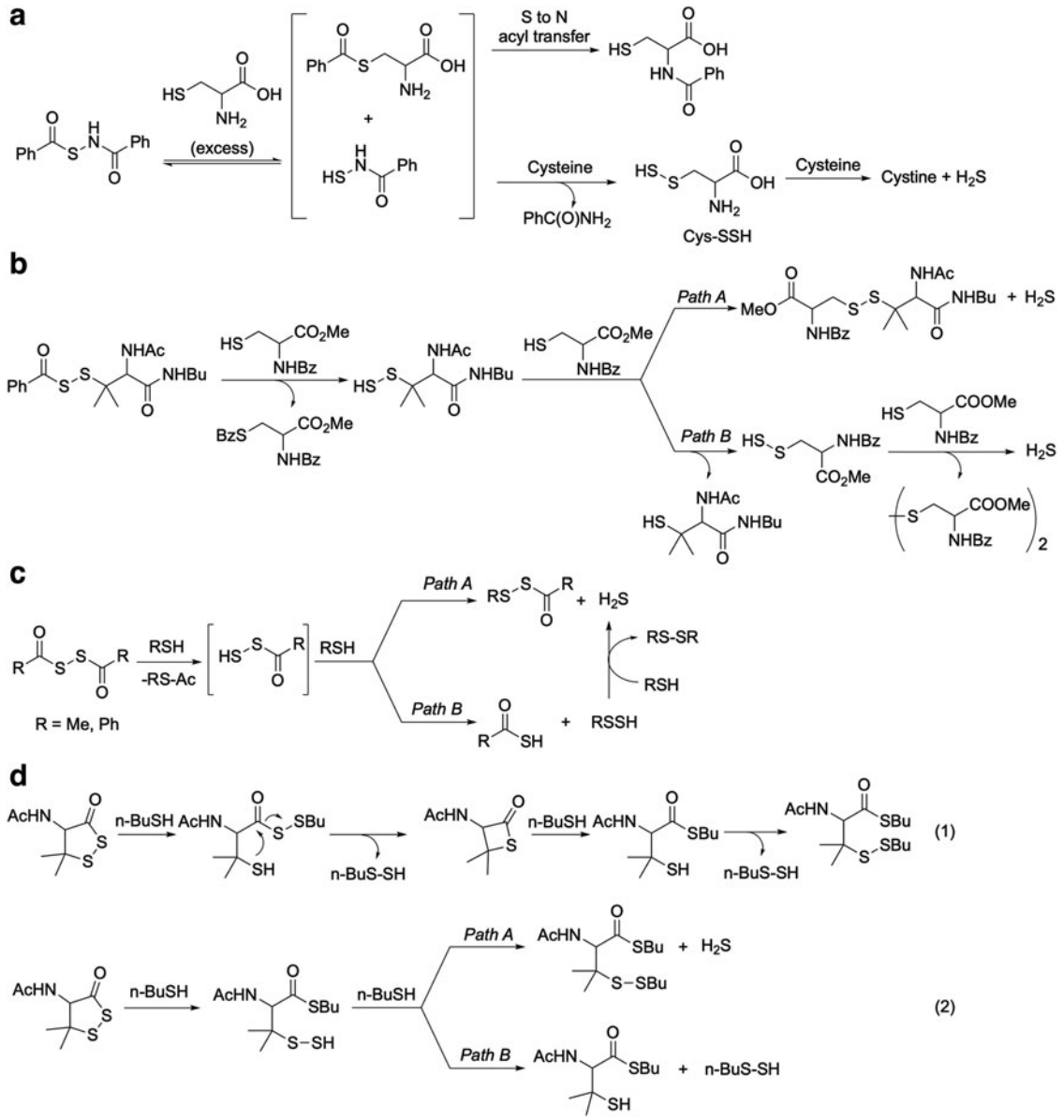

FIG. 11. Thiol-activated prodrugs: (a) *N*-(benzoylthio)benzamides, **(b)** *S*-acyl disulfides, **(c)** dithioperoxyanhydrides, and **(d)** cyclic acyl disulfides that release H_2S *via* an RSSH intermediate.

tertiary RSSH can react with *n*-BuSH at either the internal sulfur to give an unsymmetrical disulfide and H_2S [Fig. 11d, Eq. (2), Path A] or the terminal sulfur to produce thiol and *n*-BuSSH [Fig. 11d, Eq. (2), Path B]. These compounds were further tested for H_2S release with the methylene blue assay, and it was found that the acyl disulfide gives rise to ca. 50% H_2S in the presence of five equivalents of Cys-SH or GSH.

ROS-activated RSSH donors

ROS-responsive prodrugs have been developed for a variety of applications to take advantage of payload release targeted to cells or tissues that overproduce ROS. This strategy has been applied to release RSSH *via* triggering by a boronate ester scaffold following reaction with ROS. Matson and coworkers have implemented this strategy with the precursor Bpin-disulfide prodrug-*N*-acetyl cysteine (BDP-NAC), which is selectively activated by H_2O_2 over other potential oxidative or nucleophilic triggers to release NAC-RSSH (Fig. 12a) (60). BDP-NAC shows sustained RSSH release over the course of 2 h with protection of H9c2 cardiomyocytes against H_2O_2-induced oxidative stress. Furthermore, BDP-NAC shows superior protection when compared with commonly used H_2S donors such as Na_2S and GYY4137.

Chakrapani and coworkers have further expanded the scope of this approach with a vinyl boronate ester prodrug, which upon reaction with H_2O_2, triggers a 1,4-O-S relay release of RSSH (Fig. 12b) (12). The major by-product of this reaction is nontoxic cinnamaldehyde, which is utilized in the food industry as an additive. The released RSSH was characterized by DNFB trapping along with concomitant formation of the cinnamaldehyde by-product. Moreover, the authors demonstrated the ability of this RSSH precursor to mitigate oxidative stress in colon carcinoma DLD-1 cells. This precursor was tested

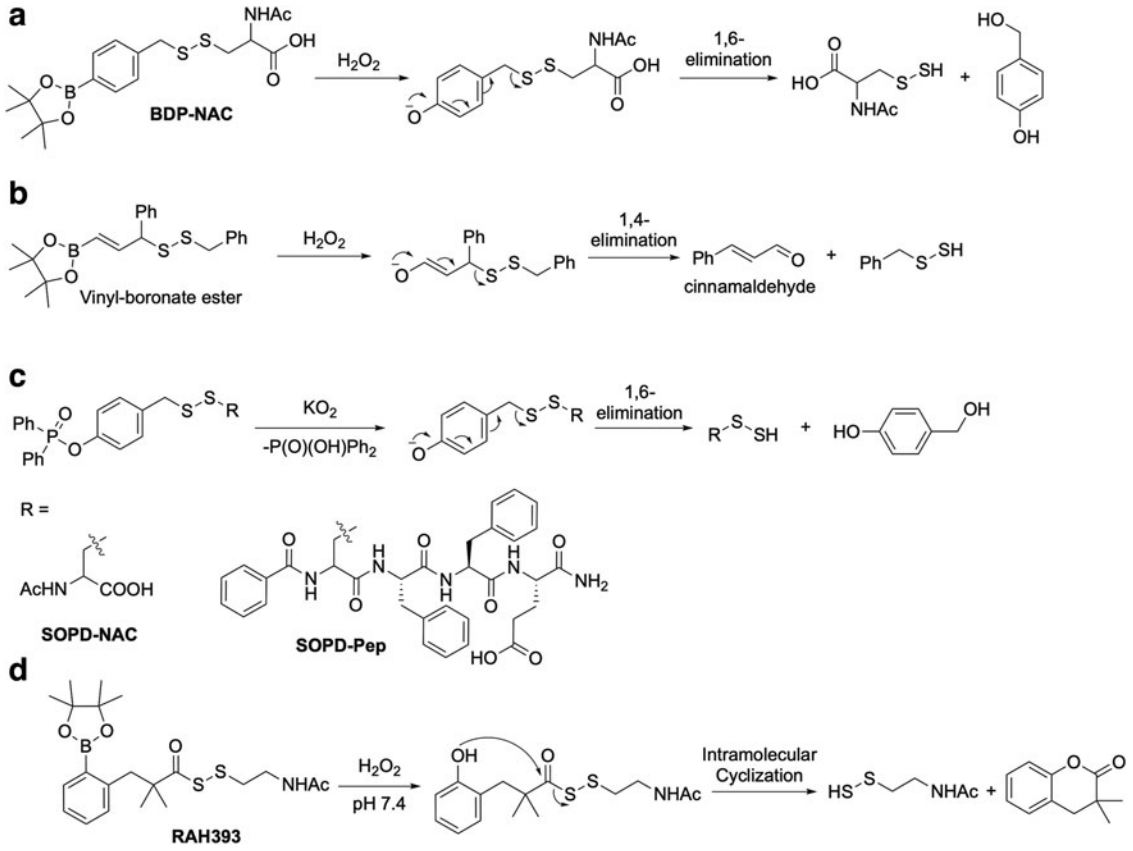

FIG. 12. RSSH generation from ROS-triggered donors: (a) BDP-NAC, **(b)** vinyl boronate ester, **(c)** SOPD-NAC/Pep, and **(d)** RAH393. ROS, reactive oxygen species.

against menadione, a known redox cycling agent and inducer of oxidative stress, with an observed increase in cell viability from 30% to 70% relative to control. An increase in viability from 40% to 80% against JCHD, a derivative of the compound juglone and a known ROS inducer, was also observed.

Matson and colleagues also reported a prodrug strategy utilizing superoxide ($O_2^{\bullet-}$) as trigger to release RSSH (68a). $O_2^{\bullet-}$ is a primary cellular ROS and its overproduction is implicated in various oxidative stress-related human diseases. NAC was conjugated to a thiol containing a diphenylphosphinate group that is selectively cleaved by $O_2^{\bullet-}$. This RSSH donor, termed SOPD-NAC ($O_2^{\bullet-}$-triggered RSSH donor), reacts specifically with $O_2^{\bullet-}$ to trigger 1,6-elimination to release NAC-SSH (Fig. 12c). To overcome SOPD-NAC's limited solubility and bioavailability, the precursor was modified to SOPD-Pep, which includes a short self-assembling tetrapeptide Bz-Cys-Phe-Phe-Glu (Bz-CFFE; Bz = benzoyl). The formation of RSSH was examined by measuring H_2S generation produced by RSSH reaction with Cys-SH using the H_2S selective fluorescent probe WSP-2. Upon treatment with $O_2^{\bullet-}$ followed by Cys-SH, SOPD-NAC and SOPD-Pep showed a fluorescence increase of 16- and 12-fold, respectively, relative to the control groups. Screening against other reactive sulfur, oxygen, and nitrogen species did not show a discernible rise in fluorescence, indicating selective RSSH release from these donors. Importantly, H_2O_2 failed to show a rise in H_2S levels, further validating the specificity of SOPD-NAC and SOPD-Pep to $O_2^{\bullet-}$. The biological application of SOPD-NAC and SOPD-Pep was also assessed in H9c2 cardiomyocytes, where both compounds were nontoxic at concentrations up to 200 μM. Oxidative stress was induced with *L*-buthionine-(*S*,*R*)-sulfoximine (BSO), an inhibitor of GSH biosynthesis that induces $O_2^{\bullet-}$ formation *in vitro*, and H_2S release was monitored with the H_2S-selective probe WSP-5. Interestingly, SOPD-Pep showed a significant increase in fluorescence relative to SOPD-NAC when cotreated with BSO. The higher fluorescence observed with SOPD-Pep was attributed to its ability to self-assemble into nanoribbons in cells, which enhances its bioavailability. The ability of these precursors to quench ROS was tested utilizing the ROS-sensing fluorescent probe dihydroethidium (DHE). Again, SOPD-Pep showed superior ROS quenching ability relative to SOPD-NAC. Matson and colleagues also explored the anti-inflammatory activities of these precursors on RAW 264.7 macrophages, which have been reported to generate significant amounts of $O_2^{\bullet-}$ when incubated with phorbol 12-myristate 13-acetate (PMA). Interestingly, when SOPD-Pep was coincubated with PMA in RAW 264.7 macrophages, cell viability increased from 51% to 81%. This result was validated by testing against the known H_2S donors, GYY4137, which releases H_2S in a slow sustained manner, and Na_2S, which releases H_2S in a bolus amount. GYY4137 showed little to no increase in viability and Na_2S showed an increase to about 60% viability, suggesting that RSSH derived from SOPD-Pep is superior in protecting RAW 264.7 macrophages against inflammation.

Lukesh and coworkers further iterated on the boronate ester strategy with placement of the boronate ester in the

ortho position (RAH393, Fig. 12d) (35). Upon introduction of H_2O_2, the boronate ester is converted to an alcohol that undergoes an intramolecular cyclization reaction to release an innocuous lactone by-product and *N*-acetylcysteamine hydropersulfide. Interestingly, when RAH393 is pre-incubated with HeLa cells before addition of H_2O_2, cell viability was increased from 33% to 84% and 100% for 100 and 200 μM RAH393, respectively. This shows the utility of RAH393 as a potential therapeutic against oxidative stress.

Enzyme-activated RSSH donors

Enzyme-sensitive prodrugs have been widely used in drug delivery. Similarly, RSSH prodrugs that are activated by enzymes have been used for the delivery of RSSH in biological systems. Under physiological conditions, these prodrugs are stable, but in the presence of enzymes release RSSH. For instance, Wang and coworkers have synthesized a novel set of RSSH prodrugs activated by pig liver esterase (PLE) *via* 1,2 elimination (Fig. 13a) (84). The addition of PLE under physiological conditions with various donors leads to the release of a carboxylic acid and an unstable hydroxylmethyl disulfide intermediate that subsequently collapses to release RSSH and form an aldehyde as a by-product. RSSH generation from these prodrugs was confirmed by trapping with the electrophilic trap, DNFB. The RSSH release rate was tuned by varying the acyl moiety, with the half-lives ranging from 12 to 145 s. Importantly, the *in vivo* effects of these prodrugs were demonstrated against MI/R injury in a murine mouse model. Hydropersulfide donor P2 (Fig. 13a, $R^1 = Me$, $R^2 = Et$), which releases ethyl hydropersulfide, was selected as the model compound due to its relatively good aqueous solubility and low toxicity of its by-product (acetaldehyde). This precursor reduced observed infarct sizes at concentrations from 50 to 100 μg/kg. Interestingly, P2 did not show protective effects at lower or higher doses, suggesting a bell-shaped RSSH therapeutic profile. Moreover, cardiac troponin-I (a marker for acute myocardial infarction) levels reflected the protective effects, with lower levels of troponin-I observed when the prodrug was administered.

Wang and coworkers have further expanded the scope of esterase-triggered precursors utilizing a trimethyl lock strategy to deliver GSSH (77). The GSSH precursor BW-GP-401, when activated by PLE, releases GSSH and a lactone by-product (Fig. 13b). The released GSSH was trapped with DNFB, although in low yield. The low trapping yield was attributed to GSSH disproportionation to GSSSG. PLE-activated BW-GP-401 also releases H_2S on the pathway to form GSSSG and in the presence of thiols such as NAC. The application of this prodrug to persulfidate proteins was demonstrated by modification of GAPDH. In addition, greater protection than Na_2S and GSH against H_2O_2-induced oxidative stress in H9c2 cardiomyocytes was also observed.

Matson and coworkers have also taken advantage of the esterase strategy by implementing a 1,6 elimination reaction to release RSSH with both a small-molecule and a polymeric NAC donor (Fig. 13c) (20). These donors were termed ester disulfide-prodrug (EDP)-NAC and poly(EDP-NAC) for the small-molecule and polymer-based RSSH donors, respectively. The half-life of EDP-NAC was found to be 1.6 h, whereas the half-life of poly(EDP-NAC) was estimated to be 36 h, demonstrating the utility of the polymeric platform for

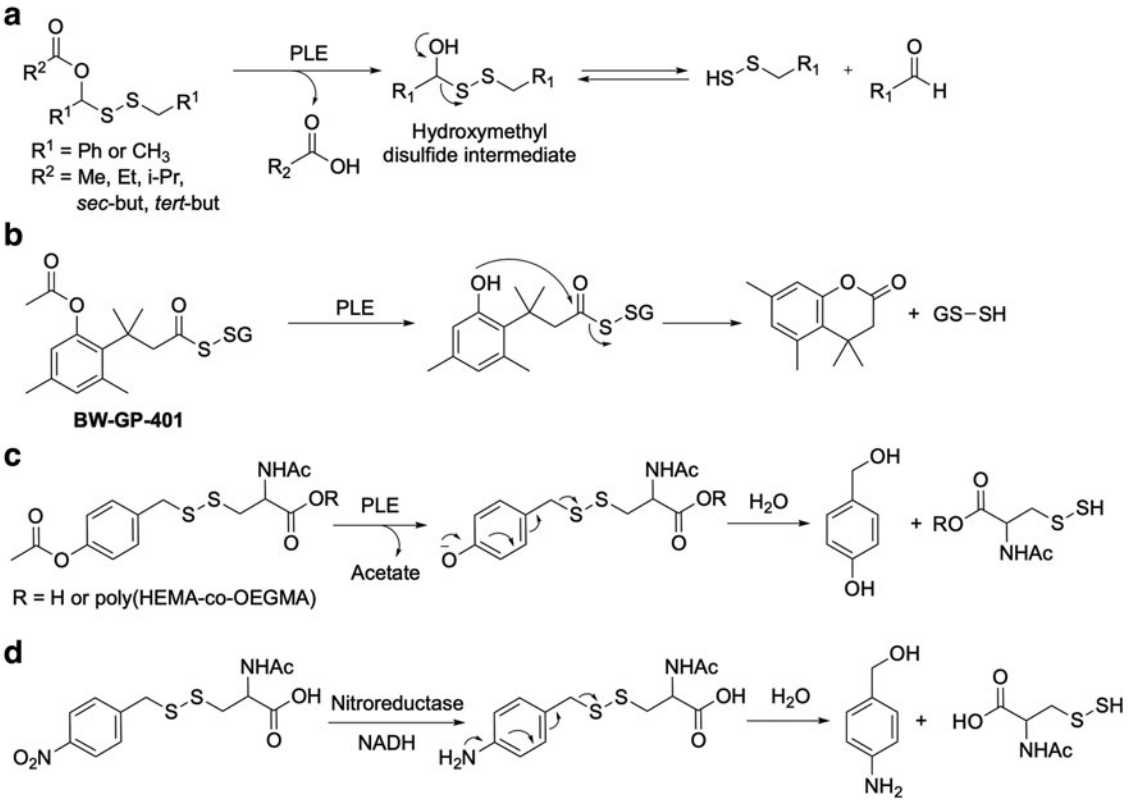

FIG. 13. RSSH donors activated by (a) esterase *via* 1,2 elimination to produce a hemithioacetal-like intermediate, (b) esterase *via* the trimethyl lock cyclization strategy, (c) esterase *via* 1,6 elimination, and (d) nitroreductase *via* 1,6 elimination.

sustained RSSH release over a long period. Utilizing H_2O_2 as an immediate ROS inducer, EDP-NAC rescued H9c2 cardiomyocytes from acute ROS stress, but poly(EDP-NAC) failed to protect cells from H_2O_2 toxicity, likely due to the slow RSSH release. In contrast, when H9c2 cardiomyocytes treated with 5-fluorouracil (5-FU), a chemotherapeutic known to generate ROS over longer periods of time, poly(EDP-NAC) showed better protection relative to that of EDP-NAC. RSSH-mediated protection in cancer cells was also examined to shed some light on the utility of these donors in conjunction with cancer therapeutics. In experiments utilizing 5-FU in MCF-7 breast cancer cells, no increase in viability was observed for cotreatment with either EDP-NAC or poly(EDP-NAC), demonstrating the utility of these donors as potential therapeutics to combat the known cardiotoxicity effects of 5-FU.

Using a similar strategy, Yang and coworkers have reported donors that were proposed to release protected Cys-SSH or penicillamine hydropersulfide upon reaction with intracellular esterase *via* 1,6-elimination (81). Control compounds that release protected cysteine/penicillamine were also prepared. Treatment with these donors significantly inhibited SNU398 cell viability, while no obvious inhibitory effects were observed for the control compounds that release the corresponding protected thiols. Although these results were promising, a control compound that lacks the esterase trigger and therefore cannot release RSSH also showed reduced cell viability, suggesting that the inhibitory effects found with these donors may not be due entirely to sulfane sulfur. Furthermore, these precursors did not show generation of sulfane sulfur within cells.

Matson and coworkers have expanded the scope of the enzyme-triggered 1,6 elimination strategy through the utilization of nitroreductase (NR) activation to release RSSH (21). The RSSH donor, nitroreductase disulfide prodrug–*N*-acetylcysteine (NDP-NAC), releases NAC-SSH upon activation by NRs (Fig. 13d), which are present in bacteria, located mostly in the mouth, skin, and intestines in mammals. In the presence of NR and NADH, NDP-NAC efficiently releases NAC-SSH, which was characterized *via* DNFB trapping, with a half-life of 1.5 h determined by ^{1}H NMR spectroscopy. Moreover, NDP-NAC was found to be nontoxic in H9c2 cardiomyocytes up to 400 μM and in *E. coli* up to 100 μg/mL. This prodrug was used to test the effect of exogenous administration of RSSH on the microbiome. Mice treated with control compounds lacking the *p*-nitro functional group or H_2S only gives rise to *Bacillus* species, which are prevalent in the healthy gut microbiome. In contrast, NDP-NAC gives rise to eight non-*Bacillus* species, which are common probiotic bacteria found in commercial supplements. In addition, NDP-NAC-treated mice show an increase in *Turicibacter sanguinis*, an important bacterium that positively impacts multiple biochemical pathways such as lipid and steroid metabolism. Furthermore, NDP-NAC-treated mice also show decreases in *Synergistales* bacteria, gram-negative opportunistic pathogens that are implicated in the formation and progression of cysts, abscesses, periodontal disease, and gastrointestinal infections. This study suggests that controlled delivery of RSSH to the gut may be useful for modulating the gastrointestinal microbiome during disease states.

Light-activated RSSH precursors

The photochemical release of biomolecules from light-sensitive precursors has been a longstanding strategy in biological research as it offers both spatial and temporal control over the administration of an effector species. Singh and colleagues have reported one- and two-photon-activated *o*-nitrobenzyl protected photoprecursors, offering spatiotemporal control over the release of NAC-SSH (Fig. 14) (14). Moreover, these precursors were stable in the dark, but release RSSH in the presence of UV light. Two-photon uncaging provides deeper tissue penetration with less phototoxicity. RSSH photorelease was characterized *via* trapping with DNFB and monobromobimane. Moreover, the biological application of these precursors was demonstrated in HeLa cells where both photoprecursors were found to be nontoxic up to a concentration of 200 μM before and after photolysis. These photoprecursors protected HeLa cells from H_2O_2-induced oxidative stress upon irradiation at 365 nm, confirming the cytoprotective effects of RSSH under oxidative environments.

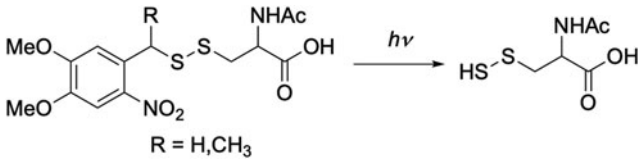

FIG. 14. Light-triggered RSSH generation from *O*-nitrobenzyl protected photoprecursors.

Conclusions and Future Directions

The development of RSSH donors for use in biological applications is expanding. Their application against various stressors (*e.g.*, oxidative or electrophilic) has shown promising results. However, the mechanisms leading to the observed protective effects are still unclear. There is ample evidence of RSSH protection against H_2O_2 and ROS-producing compounds in cell culture experiments. Interestingly, in several cases, RSSH donors are protective at lower concentrations relative to the stressor concentration, indicating there is more than direct ROS scavenging attributing to higher cell viability. Cellular signaling pathways may be activated by RSSH, leading, for example, to an increase in expression of ARE genes. The NRF2-KEAP1 signaling pathway, which may be activated by persulfidation of KEAP1, results in nuclear translocation of Nrf2 to upregulate ARE proteins such as CSE, CBS, GPX4, and others. In addition, RSSH may persulfidate specific proteins, protecting active site Cys-SH residues from irreversible oxidation. Many questions still need to be answered about the specific physiological roles that RSSH play in protection against oxidative insult. Having RSSH donors as research tools to study their discrete biological effects compared with other sulfane sulfur species is paramount to understanding their true impact. A range of chemically accessible and clean RSSH donors will hopefully shed light on unanswered questions regarding reactive sulfur species and their involvement in physiological and pathophysiological processes.

Authors' Contributions

V.S.K., S.C.A., and J.P.T. conceptualized the article and prepared the original draft, edits, and revisions; A.E., E.B., and S.P. contributed to the writing of the article.

Author Disclosure Statement

The authors declare no conflicts of interest.

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Abbreviations Used

5-FU = 5-fluorouracil
ARE = antioxidant response element
(BDP-NAC) = Bpin-disulfide prodrug-*N*-acetyl cysteine
BSO = *L*-buthionine-(*S,R*)-sulfoximine
CARSs = cysteinyl-tRNA synthetases
CBS = cystathionine β-synthase
CN^- = cyanide ion
COS = carbonyl sulfide
CSE = cystathionine γ-lyase
Cys-SH = cysteine
Cys-SSH = cysteine hydropersulfide
Cys-SSS-Cys = cysteine trisulfide
DADS = diallyl disulfide
DATS = diallyl trisulfide
DNFB = 1-fluoro-2,4-dinitrobenzene
DTNB = 5,5-dithiobis-(2-nitrobenzoic acid)
DTT = dithiothreitol
EDP = ester disulfide-prodrug
$Fe^{III}Mb$ = ferric myoglobin
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
GSH = glutathione
GSSH = glutathione hydropersulfide
GSSSG = glutathione trisulfide
H_2O_2 = hydrogen peroxide
H_2S = hydrogen sulfide
H_2S_2 = hydrogen disulfide
HCys-SSH = homocysteine hydropersulfide
IAA = iodoacetic acid
IAM = iodoacetamide
KEAP1 = kelch-like ECH-associated protein 1
MCPD = *S*-methoxycarbonyl penicillamine disulfide
MI/R = myocardial ischemia/reperfusion
MMTS = *S*-methyl methanethiosulfonate
MSBT = methylsulfonyl benzothiazole
*Mt*AhpE–SH = *Mycobacterium tuberculosis* alkyl hydroperoxide reductase E
*Mt*AhpE–SSH = *Mycobacterium tuberculosis* alkyl hydroperoxide reductase-E hydropersulfide
NAC = *N*-acetylcysteine
NDP-NAC = nitroreductase disulfide prodrug–*N*-acetylcysteine
NEM = *N*-ethylmaleimide
NR = nitroreductase
Nrf2 = nuclear factor erythroid 2-related factor 2
$O_2^{\bullet -}$ = superoxide
PLE = pig liver esterase
PMA = phorbol 12-myristate 13-acetate
PSCP = persulfidated cysteine precursor
P-SH = protein thiol
P-SSH = protein hydropersulfide
ROS = reactive oxygen species
$RS^{\bullet}$ = thiyl radical
RSeSH = selenylsulfides
RS_nSH or RSS_nSR = polysulfides
RSOH = sulfenic acid
RSS = reactive sulfur species
$RSS^{\bullet}$ = perthiyl radical
RSSH = hydropersulfides
RSSR = disulfide
RSSSSR = dialkyl tetrasulfide
tRNA = transfer RNA
UV = ultraviolet