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Chemical probes for molecular imaging and detection of hydrogen sulfide and reactive sulfur species in biological systems

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Hydrogen sulfide (H₂S), a gaseous species produced by both bacteria and higher eukaryotic organisms, including mammalian vertebrates, has attracted attention in recent years for its contributions to human health and disease. H₂S has been proposed as a cytoprotectant and gasotransmitter in many tissue types, including mediating vascular tone in blood vessels as well as neuromodulation in the brain. The molecular mechanisms dictating how H₂S affects cellular signaling and other physiological events remain insufficiently understood. Furthermore, the involvement of H₂S in metal-binding interactions and formation of related RSS such as sulfane sulfur may contribute to other distinct signaling pathways. Owing to its widespread biological roles and unique chemical properties, H₂S is an appealing target for chemical biology approaches to elucidate its production, trafficking, and downstream function. In this context, reaction-based fluorescent probes offer a versatile set of screening tools to visualize H₂S pools in living systems. Three main strategies used in molecular probe development for H₂S detection include azide and nitro group reduction, nucleophilic attack, and CuS precipitation. Each of these approaches exploits the strong nucleophilicity and reducing potency of H₂S to achieve selectivity over other biothiols. In addition, a variety of methods have been developed for the detection of other reactive sulfur species (RSS), including sulfite and bisulfite, as well as sulfane sulfur species and related modifications such as S-nitrosothiols. Access to this growing chemical toolbox of new molecular probes for H₂S and related RSS sets the stage for applying these developing technologies to probe reactive sulfur biology in living systems.

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1. Introduction

Biothiols are essential molecules in the cell, playing critical roles as antioxidants during injury and oxidative stress, as chelators for binding and interacting with metals, and as signaling agents. Hydrogen sulfide (H₂S), the simplest biothiol,



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is produced endogenously in humans through both enzymatic and non-enzymatic processes. H_2S can exist as a foul-smelling gas or dissolved in aqueous solution, primarily as the mono-anionic form, HS^- . H_2S and other reactive sulfur species (RSS) contribute to a broad array of physiological responses to maintain cellular health. RSS are active as antioxidants and signaling agents in a variety of tissue types,¹ including the liver,² gastrointestinal system,^{3–5} pancreas,⁶ brain,⁷ and circulatory system^{8–10} (Fig. 1). On the other hand, studies have established that unregulated, abnormal levels of H_2S may contribute to disease, as observed in models of Huntington's,¹¹ Parkinson's,¹² and Alzheimer's¹³ diseases. Despite the many physiological effects of H_2S in cellular and whole animal studies, precise molecular targets of H_2S are still being unraveled and remain an important goal.

At the molecular level, H_2S exhibits unique chemical characteristics, acting as both a good reducing agent and a good nucleophile. These nucleophilic properties may help elucidate its signaling capabilities, with the recent identification of potential electrophilic targets such as 8-nitro-cGMP.¹⁴ H_2S can also react with oxidized thiols, generating reactive persulfides,¹⁵ as well as interact reversibly with metal centers.^{16,17} Because of this potent chemical reactivity, the signaling roles of H_2S are diverse,^{18–21} ranging from oxygen sensing²² to modulation of phosphorylation events.²³ In particular, research by several laboratories, including those of the Snyder and Tonks groups, has shown that reversible sulfhydrylation of cysteine residues has the potential to alter the activities of some enzymes,^{23,24} including protein tyrosine phosphatases²⁵ and ATP-sensitive potassium channels.¹⁰ In this context, H_2S alone may not be responsible for all of the

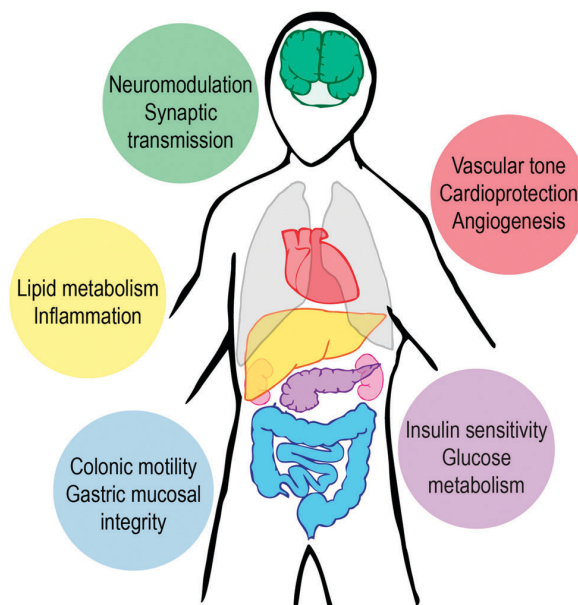


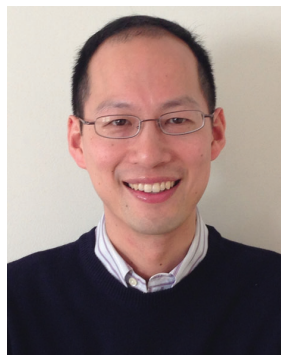
Fig. 1 Selected roles of H_2S in human physiology. H_2S produced throughout the human body modulates signaling processes in a variety of tissues, including the brain, cardiovascular system, liver, endocrine system, and gastrointestinal system.

observed downstream physiological effects. Indeed, emerging studies suggest that related RSS such as sulfane sulfur, as well as the metabolism of H_2S to other species, may help explain the diverse roles for this simple thiol.²⁶



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with Dan Nocera. After postdoctoral studies with Steve Lippard, Chris began his independent career at UC Berkeley in 2004. Research in the Chang lab is focused on chemical biology and inorganic chemistry, with particular interests in molecular imaging and catalysis applied to neuroscience and sustainable energy. His group's work has been honored by awards from the Dreyfus, Beckman, Sloan, and Packard Foundations, Amgen, Astra Zeneca, and Novartis, Technology Review, ACS (Cope Scholar, Eli Lilly, Nobel Laureate Signature, Baekeland), RSC (Transition Metal Chemistry), and SBIC.

Considering the challenges of measuring this volatile, reactive small molecule in living systems, the biologically relevant concentrations of H_2S have been debated.²⁷ As such, the development of new technologies to better study H_2S and related RSS in live cells, tissues, and whole animals is critical to gaining a more holistic understanding of how these transient chemical species contribute to physiology and pathology. Traditional analytical techniques such as colorimetric assays and gas chromatography have provided useful bulk measurements of biologically-produced H_2S , but the selective and sensitive detection of H_2S generation within intact, living cells and tissues has proven more difficult. To meet this challenge, we and others in the field have pursued a synthetic organic methods approach based on a fundamental understanding of molecular structure and function to develop reaction-based probes for sensitive, selective, and biocompatible fluorescence detection of H_2S in living systems.²⁸ In this review, we summarize the rapid progress in fluorescent H_2S probe development, focusing on the past 4 years and highlighting a variety of inventive strategies to achieve good reactivity and selectivity for H_2S over other biothiols. Organelle-targeted, cell-trappable, two-photon, and ratiometric probes have been reported, spanning a wide spectrum of emission colors. Finally, the advent of selective reporters that can differentiate between H_2S and higher RSS provides new opportunities for probing the complex interplay between these species in biological systems.

1.1. Reactive sulfur species (RSS) biochemistry

The production and metabolism of H_2S in mammalian cells are regulated by enzymes that are distributed throughout virtually every tissue type. The enzymes of the transsulfuration pathway, cystathionine gamma lyase (CSE) and cystathionine beta synthase (CBS), ensure that appropriate levels of cysteine and homocysteine are maintained in the cell.²⁹ The interconversion of these sulfur-containing substrates can lead to H_2S production by several linked pathways (Fig. 2).³⁰ Besides CSE and CBS, the combined action of two other enzymes, cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3-MST), can also generate H_2S .³¹ Mice deficient in CSE display elevated blood pressure,⁸ and CBS knockout mice exhibit retarded growth, cardiovascular problems, and poor survival.^{32,33}

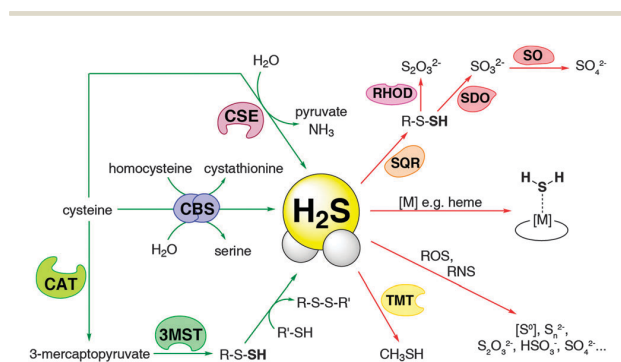


Fig. 2 Selected biochemical pathways for the production and metabolism of H_2S in mammalian systems.

Given its potent reactivity, organisms have developed efficient means for regulation of H_2S levels in tissue.^{34,35} At sufficient levels, H_2S is highly toxic, binding to hemeoproteins such as cytochrome *c* oxidase and inhibiting the electron transport chain.^{36,37} In the body, major detoxification occurs in the gut, which is populated by H_2S -producing enterobacteria.^{5,38} At the cellular level, mitochondrial detoxification of H_2S by sulfur quinone reductase (SQR), rhodanese (RHOD), and sulfur dioxygenase (SDO) converts H_2S into sulfite and thiosulfate. Subsequent action by sulfite oxidase (SO) produces sulfate as a final product. Other metabolic pathways include methylation of H_2S to methanethiol (CH_3SH) via thiol S-methyltransferase (TMT) and oxidation pathways. Non-enzymatic reactions involving ROS and RNS may produce many different oxidized sulfur species such as elemental sulfur and bisulfite,³⁹ while metal centers may act as both sources and sinks for H_2S owing to reversible binding interactions.⁴⁰ The effects of H_2S on metalloproteins containing iron^{41,42} and zinc⁴³ have been explored in terms of sulfide toxicity, transport, and redox signaling.

1.2. Scope of the review

This review will begin with an examination of current fluorescent probes used for molecular imaging of H_2S in living systems. Owing to space limitations, the assortment of highly sensitive colorimetric^{44,45} and HPLC-based probes such as monobromobimane^{27,46} that have been developed to improve upon existing classical H_2S detection methods such as the methylene blue assay⁴⁷ will not be covered in this review. In addition, fluorescent H_2S probes that have not yet been applied to cellular systems and probes that display a decrease in fluorescence upon treatment with H_2S are also generally not described in this review due to the difficulty in detecting changes in the analyte when such turn-off probes are applied to cells. This review places emphasis on probes that have been applied to the detection of RSS in living systems (Fig. 3). Literature reviews of other techniques for H_2S and sulfide detection, including electrode-based sensors⁴⁸ and chromatographic methods, are recommended.^{49–51} On account of the rapid growth of the field, we also direct the readers to other recent reviews on fluorescent probes for H_2S detection.^{52–55}

The second part of this review will focus on fluorescent probes for higher reactive sulfur species, including sulfane sulfur species, hydrogen polysulfides, and metabolites of H_2S like sulfite and bisulfite. We also survey chemical tools that have been developed for probing RSS-mediated modifications to proteins, such as sulfenic acids, persulfides, and S-nitrosothiols.

2. H_2S probes

As the smallest thiol, H_2S can act as a simple reductant as well as a good nucleophile for a variety of transformations in organic chemistry. Indeed, H_2S , sodium hydrosulfide, and sodium sulfide have been used as reducing agents for synthesis since the 1800s.^{56–58} These RSS have also been used as nucleophilic sulfur reagents to install sulfur functionalities onto various molecular scaffolds.^{59–61} The pK_a of H_2S is 7, and as

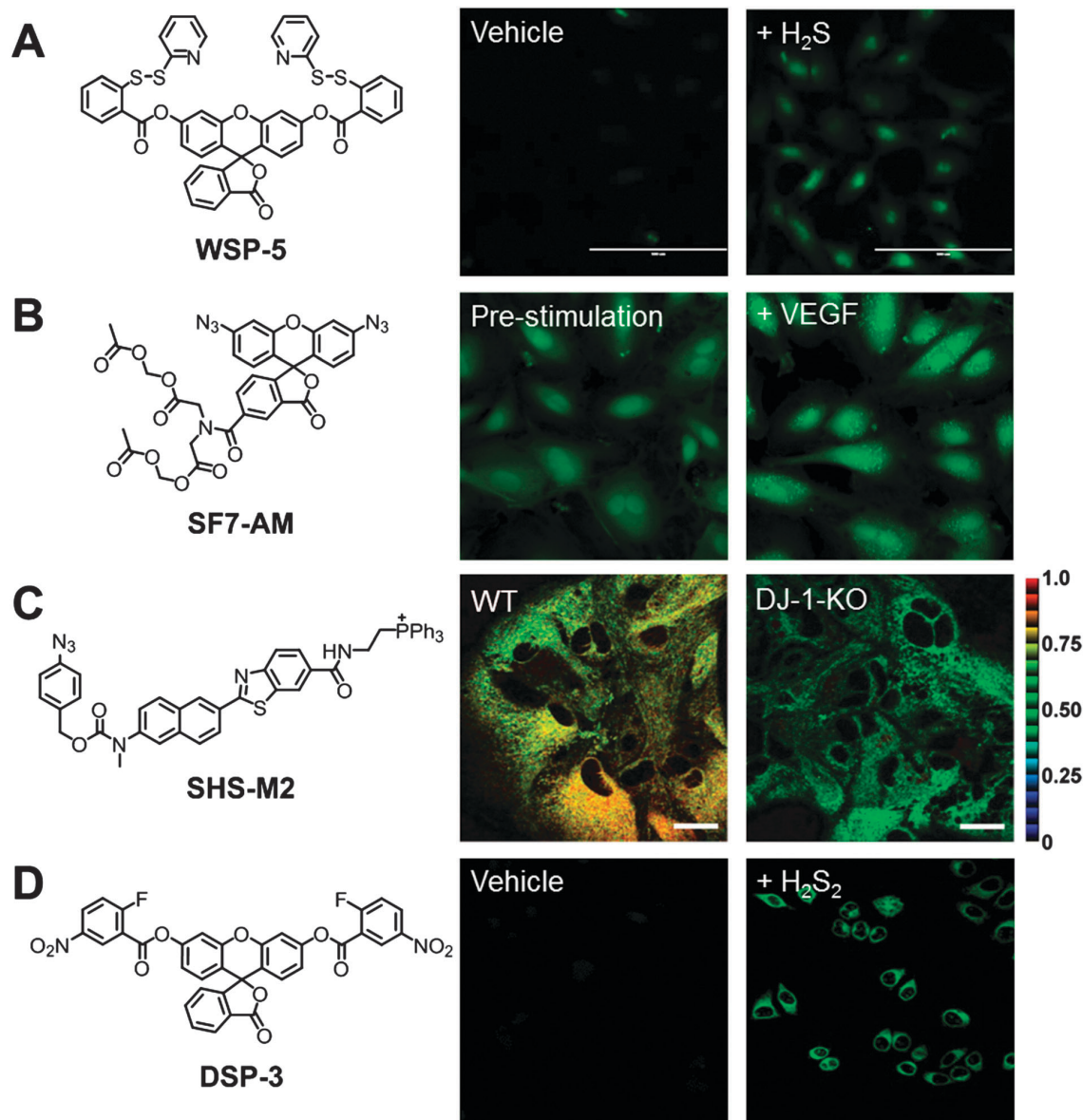


Fig. 3 Selected examples of live-cell imaging using chemoselective fluorescent H₂S and sulfane sulfur probes. (A) Detection of H₂S in HeLa cells loaded with WSP-5 and treated with 100 μ M NaHS. Reprinted (adapted) with permission from ref. 127, Copyright 2014 Wiley-VCH. (B) Detection of endogenous H₂S production in human umbilical vein endothelial cells upon stimulation with vascular endothelial growth factor (VEGF) using SF7-AM. Reprinted (adapted) with permission from ref. 68, Copyright 2013 National Academy of Sciences. (C) Visualizing attenuated H₂S production using the ratiometric probe SHS-M2 in DJ-1 knockout astrocytes, a model of Parkinson's disease. Reprinted (adapted) with permission from ref. 93, Copyright 2013 American Chemical Society. (D) Detection of 100 μ M H₂S₂ in HeLa cells by DSP-3. Reprinted (adapted) with permission from ref. 152, Copyright 2014 American Chemical Society.

such an estimated two-thirds of this RSS will exist as the anionic HS[−], an excellent nucleophile, in aqueous solution at physiological pH. Additionally, analytical chemists have employed hydrogen sulfide for centuries in the gravimetric analysis of metals, such as copper.^{62,63} These facets of H₂S in analytical and organic chemistry provide a starting point for the design of fluorescent probes that respond selectively and sensitively toward this RSS. The ever-expanding toolbox of

reaction-based probes employ three primary strategies for H₂S detection: reduction of azides to amines, nucleophilic reaction, and copper sulfide precipitation.

2.1. Azide reduction

Organic azides have been used extensively in chemical biology as a bioorthogonal functional group.⁶⁴ Their compatibility with living cellular systems combined with the relative synthetic

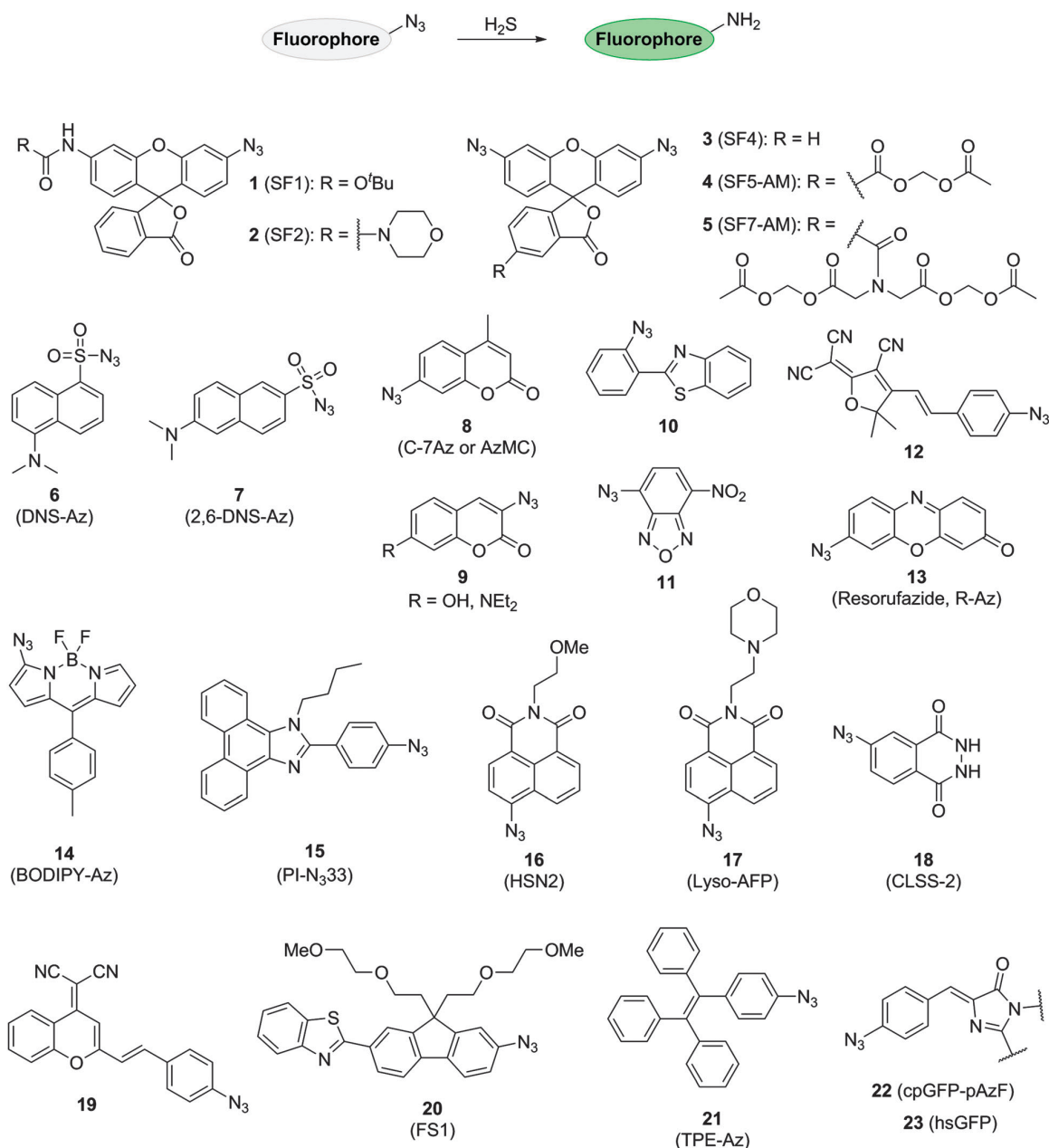


Fig. 4 Structures of fluorescent H₂S probes based on the chemoselective reduction of aryl azides to amines.

ease of installing azide moieties onto a wide array of amine-containing fluorophores has led to the rapid preparation of a wide variety of fluorescent azide-based probes (Fig. 4). H₂S detection occurs *via* the chemoselective reduction of azides to amines by this RSS. The first concomitant reports of azide-based fluorescent probes that could be used for H₂S detection in live cells were rhodamine-based sulfidefluor (SF) probes 1–2 functionalized with aryl azides by Lippert and Chang⁶⁵ and dansyl probe 6 featuring a sulfonyl azide by Wang *et al.*⁶⁶ The Wang group has since developed a 2,6-dansyl azide structural isomer of the original

probe, which exhibits improved solubility and selectivity.⁶⁷ Chang and Lippert have also expanded upon the SF series of probes, synthesizing bis-azido rhodamines 3–5 for a lowered background signal and appending acetoxymethyl (AM) ester trapping groups for enhanced cellular retention and trappability.⁶⁸ Similar bisazido carboxyrhodamine derivatives have also been prepared by Sun's group.⁶⁹ Using these second-generation H₂S probes, endogenous H₂S production in a model system of angiogenesis was visualized.

Incorporation of the azide trigger onto a variety of other fluorophore scaffolds has provided access to probes in a range

of colors. Reporters for H₂S based on coumarin (8–9),^{70–72} 2-(2-aminophenyl)benzothiazole (10),⁷³ 7-nitrobenz-2-oxa-1,3-diazole (11),⁷⁴ dicyanomethylenedihydrofuran (12),⁷⁵ resorufamine (13),⁷⁶ BODIPY (14),⁷⁷ phenanthroimidazole (15),⁷⁸ and the 1,8-naphthalimide scaffold (16)⁷⁹ have been described, including a lysosomally-targeted derivative (17).⁸⁰ The Pluth laboratory has also developed the first chemiluminescent H₂S probe 18 based on an azido derivative of luminol, which can be used to detect enzymatically-produced H₂S *in vitro*.⁸¹ A near-infrared (near-IR) probe 19 based on dicyanomethylene-4H-chromene that can also be used for two-photon imaging was reported simultaneously by Xu and Peng and their co-workers.^{82,83} Two-photon probes such as 20 have been synthesized,⁸⁴ as well as a tunable, aggregation induced emission (AIE) probe 21 based on tetraphenylethene developed by the Tang group.⁸⁵ The azide moiety has even been extended to genetically-encoded reporters 22–23, as the Ai laboratory used site-specific artificial amino acid incorporation to install a *p*-azidophenylalanine residue at Tyr66 in cpGFP.⁸⁶ An improved version of this probe, hsGFP, was also recently reported.⁸⁷ Using a new superfolder GFP template, Chen and Ai obtained a new genetically-encoded H₂S reporter with a larger dynamic range, improved selectivity, and more effective formation of the mature chromophore.

Several ratiometric probes have been reported that exploit the electronic differences between the azide and amino functionalities to achieve a shift in fluorescence emission (Fig. 5). Ratiometric imaging methods rely upon fluorescent probes that display fluorescence emission or excitation at two different wavelengths; in response to varying levels of analyte, these fluorescence peaks change in direct proportion to one another, independent of dye concentration. The ratiometric character of these probes can correct for differences in dye loading, photobleaching, and other factors that may complicate imaging results, thereby enabling acquisition of semi-quantitative data. Ratiometric probes are therefore of great interest to the probe development community given these special spectroscopic properties. The Han group utilized an azide-functionalized cyanine dye in probe 24 where the parent probe fluoresces at 710 nm, but upon its reduction to the amine the emission maximum shifts to 750 nm.⁸⁸ A related ratiometric cresyl violet probe 25 displayed a bathochromic shift in emission from 566 to 620 nm and was applied to both cells and zebrafish to detect exogenous H₂S using NaHS as the sulfide source.⁸⁹ A third ratiometric probe 26 was reported using an 4-azido-1,8-naphthalimide dye as a FRET acceptor conjugated to a

carbon dot.⁹⁰ The carbon dot emits at 425 nm and serves as the FRET donor; once the aryl azide on the fluorophore is reduced by H₂S to an aniline, the probe emits at 526 nm.

2.1.1. Self-immolative linkers. The selective reduction of azides to amines by H₂S has also been adapted to reaction-based imaging probes possessing self-immolative linkers, which are triggered upon the formation of an amino group (Fig. 6). A coumarin-based probe 27 operates by H₂S-mediated reduction of a benzylic azide, which generates a primary amine that can subsequently perform intramolecular attack on the ester carbonyl to release 8-hydroxycoumarin.⁹¹ Other probes have utilized the azide to mask dyes for 1,6-elimination, a strategy that has been previously explored for prodrug delivery.⁹² Reduction of the aryl azide to an aniline is followed by azaquinone methide formation and release of the fluorophore. Using this approach, Cho and co-workers reported the development of two-photon, ratiometric, mitochondrially-targeted probes 28–29, which were successfully applied to live cells and used to detect decreases in endogenous H₂S in astrocytes lacking DJ-1, a gene involved in Parkinson's disease.⁹³ Incorporation of the *p*-azidobenzyl group onto 2-(2'-hydroxyphenyl)benzothiazole (HBT) affords an alternative probe 30 using a similar strategy.⁹⁴ A series of ratiometric FRET probes (31, FRET-P1 through 5) developed by the Tang laboratory employs fluorescein and [4-(*N,N*-dimethylamino)phenylazo]benzoyl (DABCYL) as a FRET pair, tethered by a thiourea linkage.⁹⁵ Incorporation of an electron-donating methoxy group *ortho* to the azide increases the kinetics of the 1,6-elimination for azaquinone methides as previously demonstrated by the Phillips group.⁹⁶ The ratiometric probes 32 and 33 feature a *p*-azidobenzyl group attached to the 1,8-naphthalimide fluorophore *via* a carbamate linkage, which then undergoes 1,6-elimination to release an azaquinone methide^{97,98} with the resulting change in electronics giving rise to a shift in fluorescence emission. Such probes are useful for biological inquiry, but precautions must be taken to account for the release of azaquinone methide, which may potentially have strong alkylating abilities in biological systems.⁹⁹

2.1.2. Nitro group reduction. Like azide reduction, reduction of nitro groups generates amino products, which display significantly different electronic properties. Installation of nitro groups onto fluorophores can thus be used as a strategy for achieving H₂S-selective probes (Fig. 7). The first of these probes by the Pluth group utilized the 1,8-naphthalimide scaffold to afford a fluorescent turn-on probe 34.⁷⁹ The nitro group has been incorporated into cyanine dyes (35)¹⁰⁰ and coumarins (36)¹⁰¹

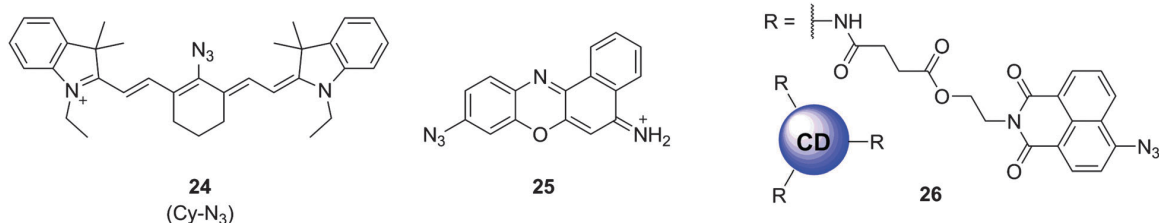


Fig. 5 Structures of ratiometric H₂S probes utilizing azide reduction to shift fluorescence emission.

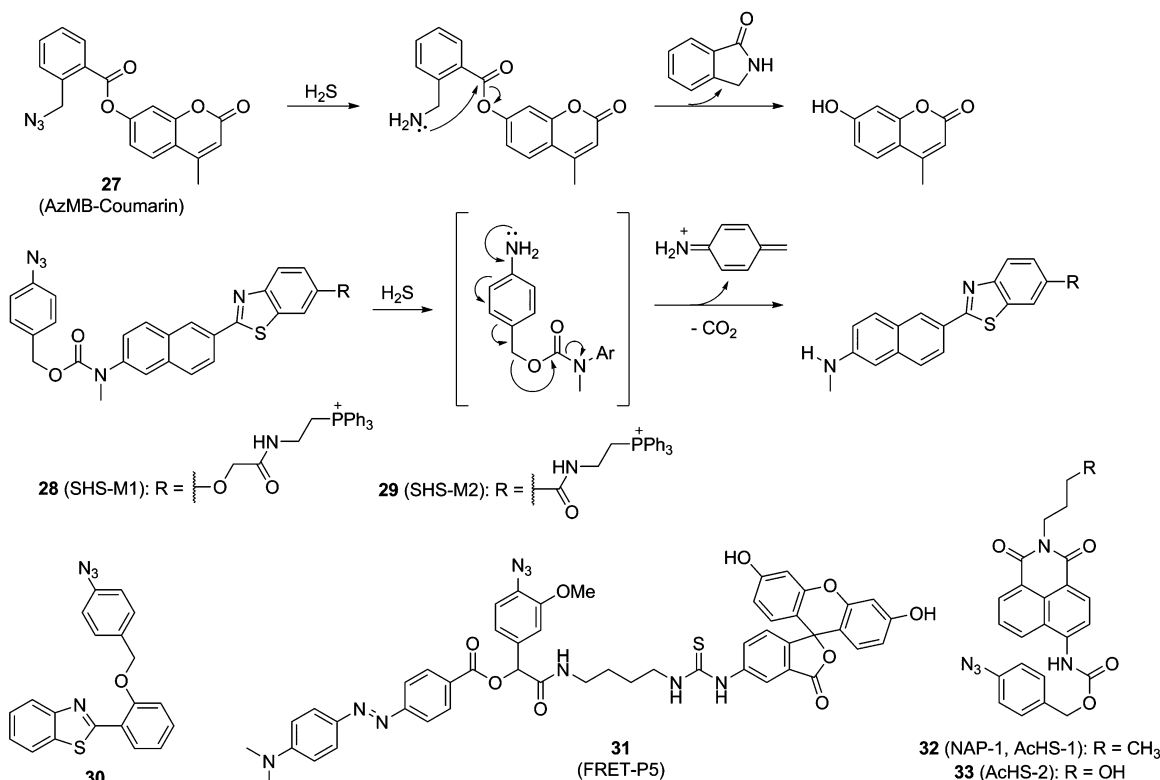


Fig. 6 Structures of fluorescent H_2S probes based on reduction of azide groups to amines, followed by self-immolative cleavage.

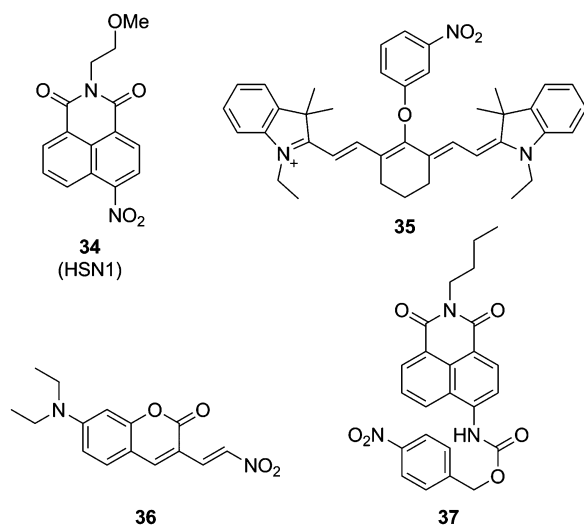


Fig. 7 Structures of fluorescent H_2S probes based on reduction of nitro groups to amines.

to yield fluorescent H_2S probes in a variety of colors. Self-immolative linkers, similar to those described in the previous section, have been explored as well (37).¹⁰² Due to the potential sensitivity of nitro groups toward endogenous reductases,^{103,104} appropriate controls must accompany the use of these probes

in live cell systems to confirm that H_2S production is responsible for the observed response.

2.2. Nucleophilic attack strategies

H_2S is an excellent nucleophile, existing predominantly as HS^- at physiological pH and therefore displaying higher nucleophilicity compared to many other thiols found in the cell. As such, several types of platforms have been applied to take advantage of this nucleophilic reactivity. These probe designs typically incorporate electrophilic functionalities that can be transformed in the presence of H_2S . Two strategies, the thiolysis of aryl nitro groups and the disruption of conjugated systems, exploit the selective addition of HS^- to a single electrophilic position on the probe scaffold. A major challenge associated with the detection of H_2S is achieving selective reactivity over other thiols present in the cellular environment, specifically glutathione and cysteine, which are typically present in millimolar concentrations. The nucleophilic properties of H_2S , particularly its ability to perform two consecutive nucleophilic reactions, have provided inspiration for probes that react robustly toward H_2S and display very limited response in the presence of other biothiols. These reporters, broadly categorized by their use of a disulfide exchange mechanism or tandem Michael addition strategy, incorporate two electrophilic centers into their design.

2.2.1. Aryl nitro thiolysis. The 2,4-dinitrophenyl (DNP) ether moiety has been employed extensively as a protecting group for

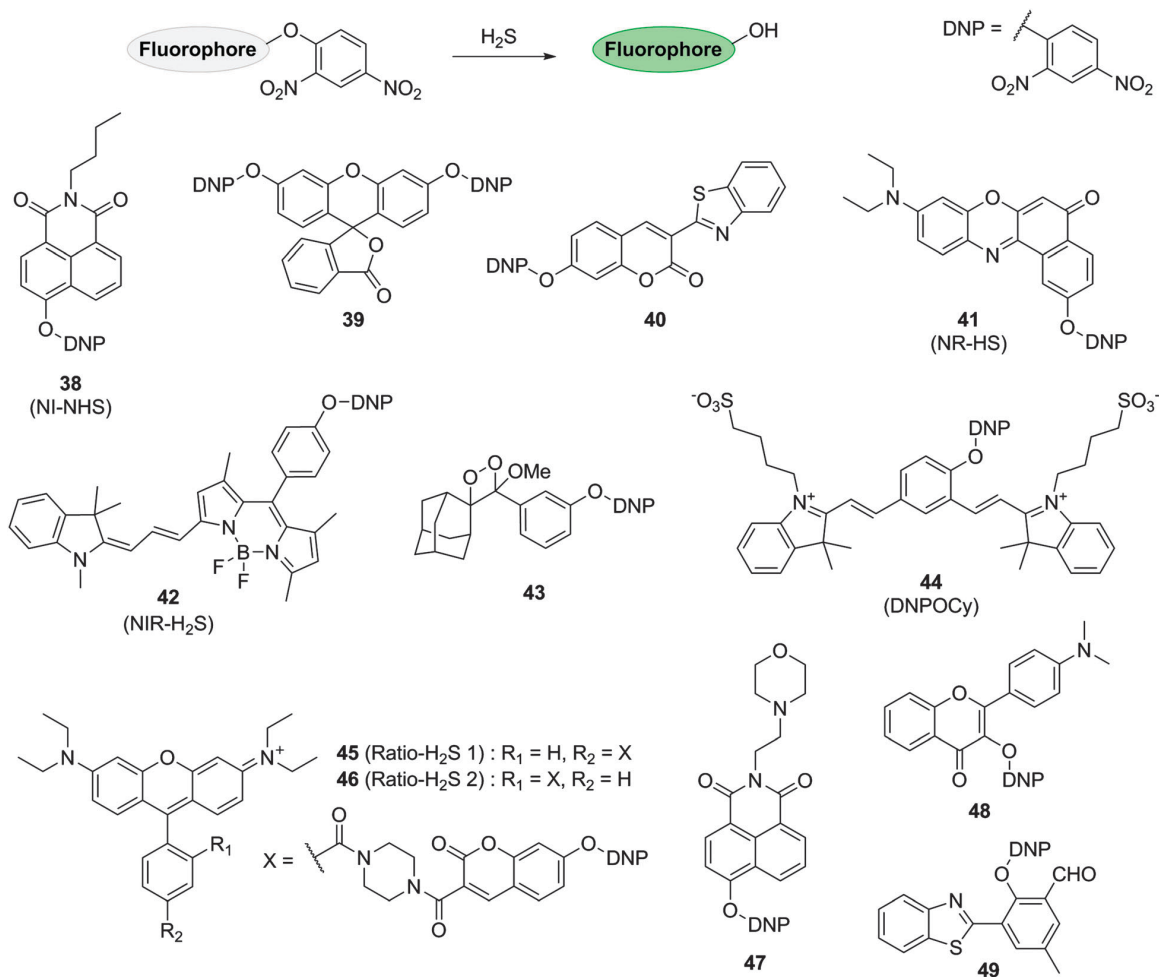


Fig. 8 Structures of fluorescent H₂S probes based on thiolysis of dinitrophenyl ethers.

tyrosines in peptide synthesis, with thiolytic cleavage achieved by treatment with 2-mercaptoethanol.^{105,106} Incorporation of the DNP functional group onto 1,8-naphthalimide (**38**),¹⁰⁷ fluorescein (**39**),¹⁰⁸ coumarin (**40**),¹⁰⁹ Nile Red (**41**),¹¹⁰ near-IR BODIPY (**42**),¹¹¹ and other fluorophore scaffolds has yielded H₂S probes in a wide range of emission colors (Fig. 8). A chemiluminescent probe **43** has also been reported, consisting of a stable adamantyl dioxetane that decomposes upon H₂S-mediated cleavage of the DNP ether.¹¹² A near-IR H₂S indicator **44** based on a cyanine dye was prepared and successfully applied to living cells, displaying ratiometric emission at 555 and 695 nm.¹¹³ A pair of H₂S probes **45–46** with ratiometric excitation was also reported, featuring a DNP-capped hydroxycoumarin linked to tetraethylrhodamine by a piperazine group.¹¹⁴ A lysosomal H₂S reporter **47** utilizing an *N*-alkyl morpholino group for targeting,¹¹⁵ as well as an excited state intramolecular proton transfer (ESIPT) probe **48**, have appeared in recent years.¹¹⁶ A particularly interesting design by Huang and co-workers involves incorporation of an aldehyde group onto an ESIPT fluorophore to give probe **49**. The aldehyde

undergoes fast reaction with H₂S followed by subsequent thiolysis of the proximal DNP ether.¹¹⁷

In addition to DNP, other nitro aryl groups such as nitrobenzofuran (NBD) have been examined for their ability to undergo thiolysis by H₂S via a nucleophilic aromatic substitution (S_NAr) mechanism (Fig. 9). A coumarin based probe **50** attached to NBD via a piperazine linkage was first reported by Wei *et al.* and used to image exogenous H₂S in cells.¹¹⁸

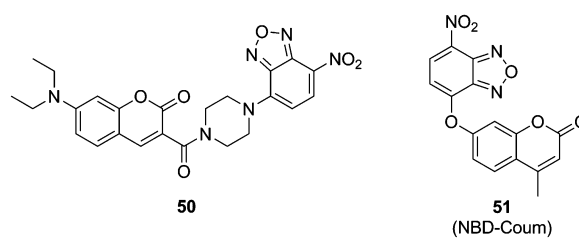


Fig. 9 Structures of fluorescent H₂S probes based on nucleophilic aromatic substitution of nitrobenzofuran.

The Pluth group has previously reported selective probes for colorimetric assays using the NBD scaffold.⁴⁴ Recently, Pluth and coworkers have expanded upon their mechanistic studies to develop a fluorescent NBD-coumarin probe **51** that would display different emission profiles in the presence of H₂S or biothiols, allowing for spectral differentiation of these species.¹¹⁹

2.2.2. Nucleophilic addition to conjugated systems. As a good nucleophile, HS[−] can undergo addition to electrophilic centers in fluorescent molecules such as cyanine dyes. This strategy has been harnessed for the development of ratiometric H₂S probes **52** and **53**, which relies on the disruption of an extended pi system to shift the fluorescence emission (Fig. 10).^{120,121} These ratiometric indicators have been successfully utilized in live cells, including applications in MCF-7 and HeLa cells, with probe **52** localizing to mitochondria. Treating live cells with NaHS produced changes in the fluorescence ratios for these probes, demonstrating successful detection of exogenous H₂S. The effective use of this strategy must account for possible cross-reactivity with other nucleophilic thiols, as related probes have been used previously for detection of biothiols.^{122,123} For these ratiometric H₂S probes, it has been suggested that the H₂S selectivity may be influenced by unfavorable electrostatic interactions between the positively-charged probe and the ammonium groups of zwitterionic cysteine and glutathione.¹²¹ Ratiometric H₂S probes are powerful tools for biological imaging experiments, and their continued development will enable further elucidation of H₂S biology in complex models.

2.2.3. Disulfide exchange. After nucleophilic attack of H₂S on electrophilic carbon, nitrogen, and sulfur centers, the resulting intermediate is also a thiol species, which can potentially undergo a second nucleophilic addition to a different position within a given probe molecule. This double nucleophilic character is unique to H₂S over other biologically relevant thiols. As such, several groups have designed probes that take advantage of this special reactivity and therefore offer excellent selectivity for H₂S over other RSS (Fig. 11). Moreover, the formation of mixed disulfides is well-precedented *in vitro* and

in the cell.^{124,125} The Xian group has harnessed disulfide exchange for the development of the first Washington State Probe, WSP-1 (**54**).¹²⁶ Displacement of the 2-thiopyridine affords a reactive, nucleophilic hydropersulfide, which readily undergoes intramolecular cyclization to release a disulfide product and 3-*O*-methylfluorescein as the fluorophore. The extension of this strategy to a series of probes (**55–58**) in a variety of colors demonstrates the versatility of this trigger and its ready application to coumarin, resorufin, and fluorescein-based scaffolds.¹²⁷ These probes are stable to esterases and display excellent selectivity for H₂S over other biothiols, as mixed disulfides formed between these probes and intracellular glutathione are reversible, meaning that the resulting disulfide cannot undergo cyclization. A related ratiometric disulfide exchange ESIPT probe **59** was developed based on HBT.¹²⁸

2.2.4. Tandem Michael addition. Another approach that has exploited the double nucleophilic ability of H₂S is the tandem Michael addition strategy, first reported by He, Zhao, and co-workers. These sulfide-selective fluorescent probes (**60–61**) are based on the 1,3,5-triaryl-2-pyrazoline and BODIPY scaffolds (Fig. 12).¹²⁹ Related BODIPY derivatives **62–63** have been reported.^{130,131} These H₂S probes feature an aldehyde functionality which serves as the site of the initial nucleophilic attack. The resulting hemithioacetal then undergoes nucleophilic 1,4-addition to the α,β-unsaturated ester, cyclizing and producing a turn-on fluorescence response. Like the disulfide exchange strategy, these probes are very selective for H₂S over other biothiols found in the cell due to the inability of other thiols to perform the second nucleophilic attack required for cyclization. These probes allow for ready imaging of H₂S in live cellular systems. The Xian laboratory also showed that this strategy could be modified to cleave the thioacetal cyclization product from the fluorophore in the last step (Fig. 13). Two probes **64–65** based on fluorescein have been designed using this strategy, incorporating two different Michael acceptors.¹³² A third probe **66** that uses a cyanine scaffold for a ratiometric response has also been described.¹³³

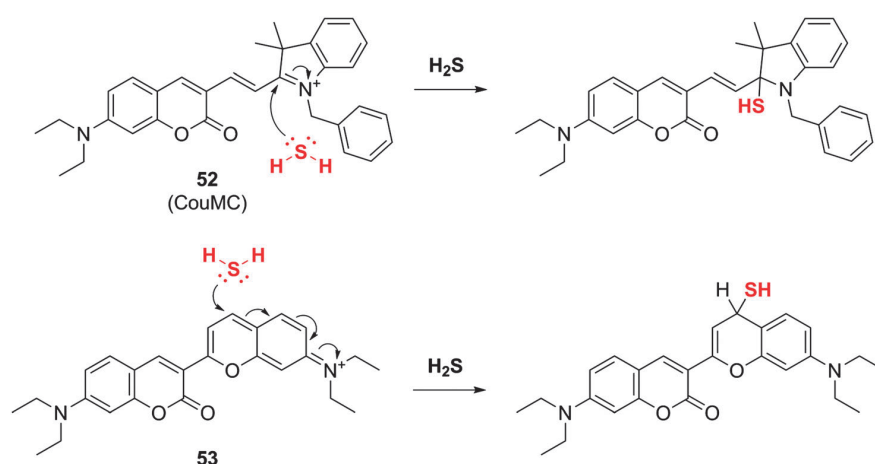


Fig. 10 Structures of ratiometric H₂S probes based on disruption of the conjugated π-system in a fluorophore and mechanism of their reaction with H₂S.

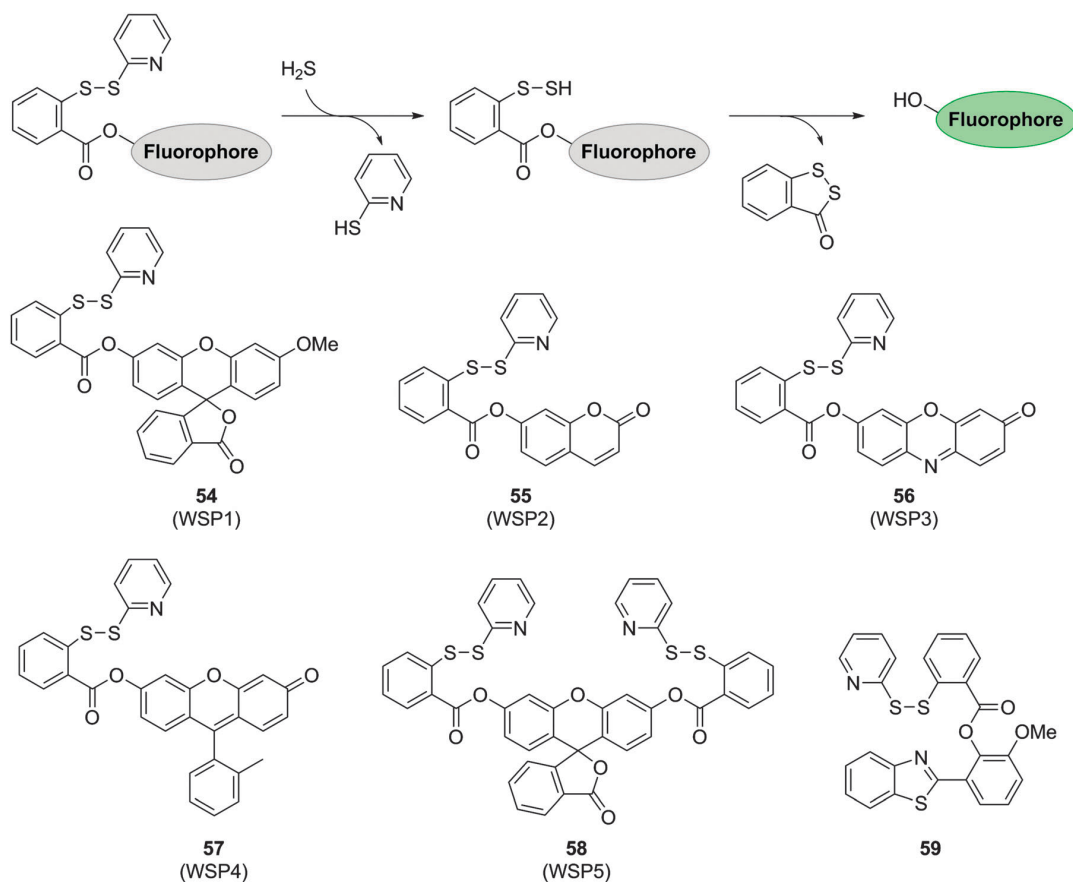


Fig. 11 Structures of H₂S probes utilizing the disulfide exchange mechanism to form a reactive hydropersulfide that undergoes nucleophilic addition to release the fluorophore.

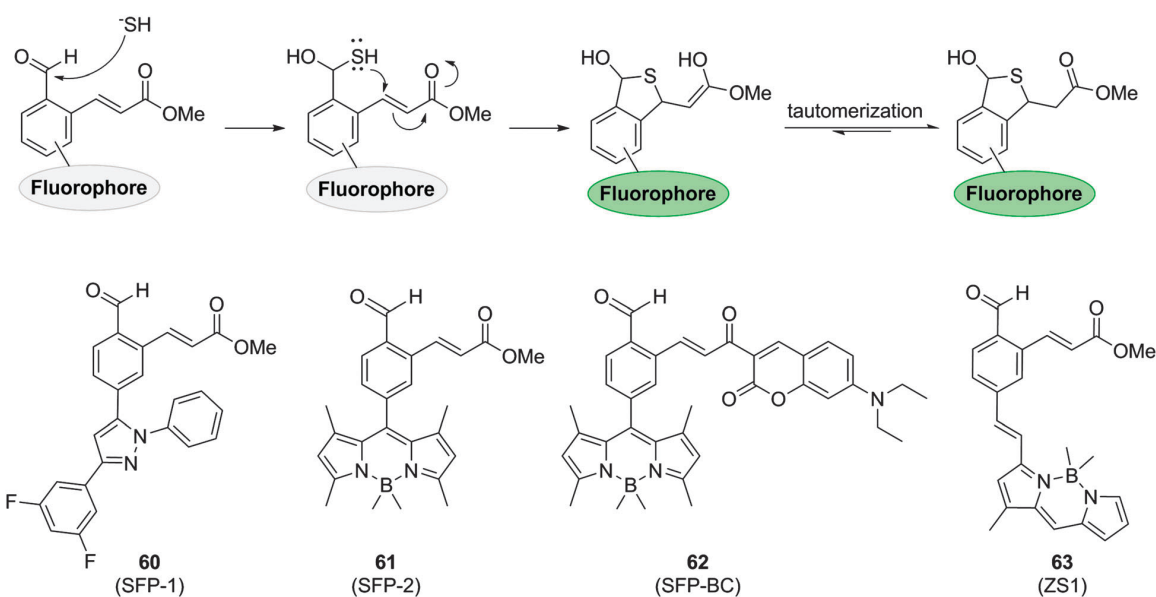


Fig. 12 Structures of fluorogenic probes containing α,β -unsaturated esters which undergo nucleophilic attack at two proximal electrophilic centers to trap H₂S in the product.

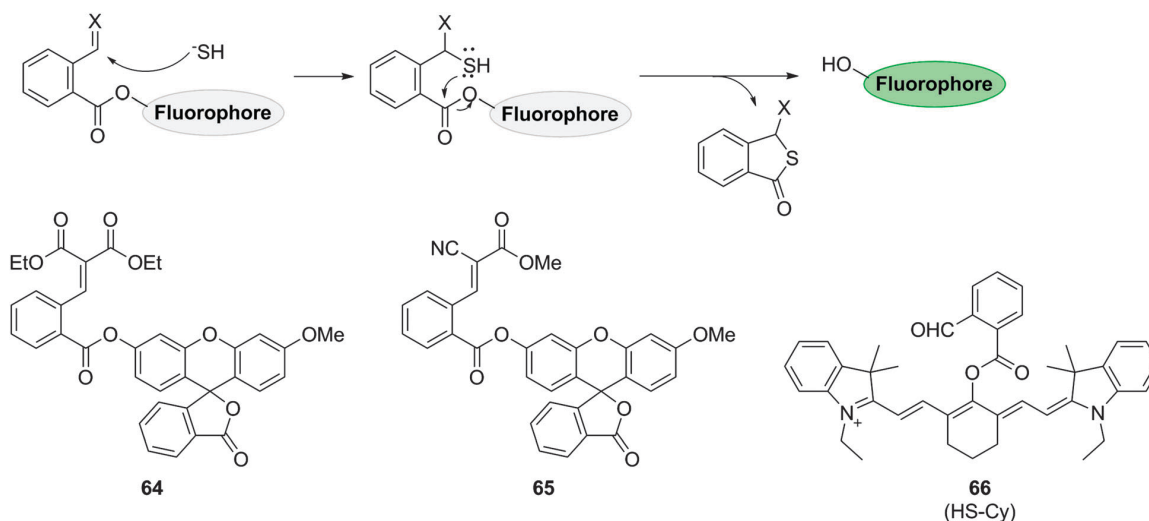


Fig. 13 Structures of fluorogenic probes containing α,β -unsaturated esters which undergo nucleophilic attack at two proximal electrophilic centers, with elimination of a fluorescent molecule in the final step.

2.3. CuS precipitation

Precipitation of copper sulfide (CuS), a classical method for quantitative analysis of the sulfide content, has been successfully adapted for fluorogenic H_2S detection *via* the incorporation of copper-binding moieties onto fluorophores (Fig. 14).¹³⁴ Nagano and co-workers applied this strategy to prepare copper-based dyes such as probe **67** for detection of H_2S in living cells.¹³⁵ When paramagnetic Cu^{2+} is bound to the cyclen azamacrocycle, fluorescence is quenched. Reaction of the copper ion with H_2S causes precipitation of CuS, releasing the dye in a fluorescent, unbound state. Nagano's initial report astutely noted that the choice of azamacrocycle potentially affects the reactivity of the probe, particularly in terms of its selectivity toward H_2S over other thiol species.¹³⁵ Thus, screening of probe performance in the presence of other RSS is especially important, since alternate combinations of fluorophores and receptors exhibit different properties. Additional probes utilizing the CuS precipitation strategy have been reported for fluorescein (**68–69**),^{136,137} anthracene (**70**),¹³⁸ and dimeric phenanthrene-fused dipyrromethene (**71**).¹³⁹ A two-photon probe **72** consisting of a carbon nanodot functionalized with *N*-(2-aminoethyl)-*N,N,N'*-tris(pyridin-2-ylmethyl)ethane-1,2-diamine (TPEA), a ligand used for copper binding, was used to image exogenous H_2S in model cellular systems and lung cancer tissue.¹⁴⁰

3. Fluorescent probes for higher RSS

3.1. Sulfane sulfur species

In addition to H_2S , increasing attention is being paid to the detection of higher-order RSS, as these reactive species are also of biological importance. In this context, sulfane sulfurs are common RSS of interest. Sulfane sulfur refers to a sulfur atom with six valence electrons but no charge (known as S^0). A number of sulfane sulfur molecules exist in biological systems, including persulfides (R-S-SH), polysulfides ($\text{R-S-S}_n\text{-S-R}$, $n > 1$),

hydrogen polysulfides (H_2S_n , $n > 1$), and protein-bound elemental sulfur.^{49,141–144} These RSS have unique biological activities such as modulating enzyme functions and participating in the synthesis of sulfur-containing vitamins and cofactors.¹⁴⁵ More recent studies on H_2S redox biology even suggest that sulfane sulfurs are the primary RSS signaling molecules downstream of H_2S .^{15,145–149}

Despite an increasing recognition of the importance of sulfane sulfurs in biological settings, their detection in living samples remains a challenge. Sulfane sulfurs possess a unique electrophilic character that can react with certain nucleophiles. For example, the traditional method for sulfane sulfur detection, *i.e.* cyanolysis, is based on the reaction with cyanide ion (CN^-) to form thiocyanate (SCN^-), which can be measured (by UV absorbance) as ferric thiocyanate (Fig. 15).¹⁵⁰ Taking advantage of this electrophilicity, several reaction-based fluorescent probes for sulfane sulfurs were developed recently by Xian and co-authors,¹⁵¹ employing a thiophenol that reacts with sulfane sulfurs to form an Ar-S-SH intermediate, which subsequently undergoes an intramolecular cyclization to release the pendant fluorophore. Probes such as SSP2 (**73**) showed fast and highly selective turn-on fluorescence responses to sulfane sulfurs (including polysulfides and elemental sulfur) over other RSS such as cysteine, glutathione, and H_2S with nM detection limits, and were effective in detecting both exogenous and endogenous sulfane sulfurs in cell imaging experiments.²⁶ We note that although these probes can effectively identify sulfane sulfurs from other reactive sulfur species, they cannot differentiate each member in the sulfane sulfur family, for example polysulfides (the oxidized form of sulfane sulfurs) *vs.* persulfides (the reduced form of sulfane sulfurs).

3.2. Fluorescent probes for hydrogen polysulfides

Hydrogen polysulfides (H_2S_n , $n > 1$) are a particularly interesting class of sulfane sulfurs that merit special mention, as they can be

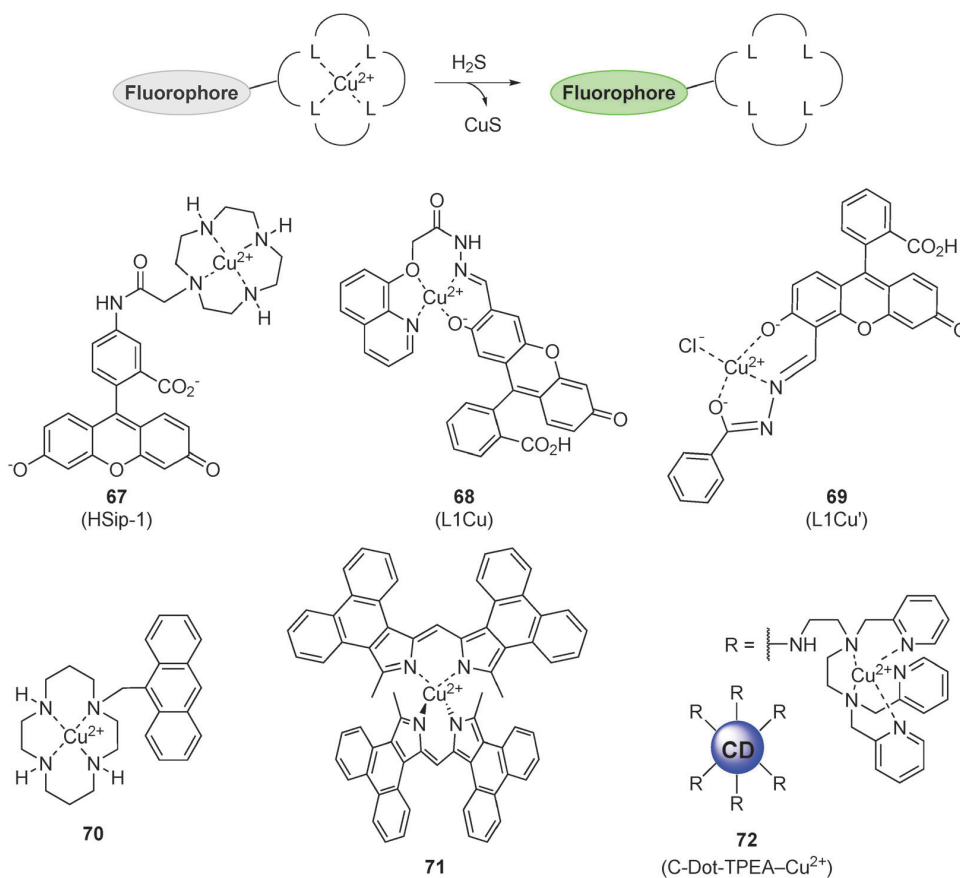
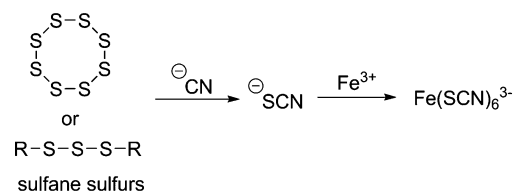


Fig. 14 Structures of fluorescent probes using copper sulfide precipitation to afford a turn-on response.

Cyanolysis



Fluorescent probes for sulfane sulfurs

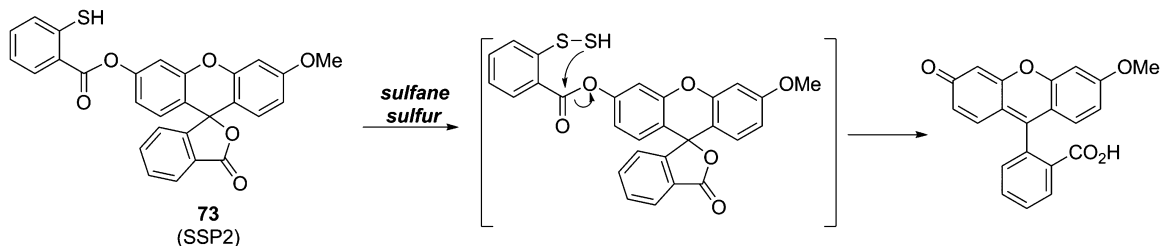


Fig. 15 Structures of fluorescent probes for sulfane sulfur.

considered to be oxidized forms or redox partners of H₂S. It is likely that H₂S_n and H₂S co-exist in biological systems and work collectively to regulate sulfur redox balance. From the

chemistry point-of-view, H₂S_n should be more reactive than H₂S owing in part to the α effect. For example, recent *in vitro* studies have revealed that H₂S_n are much more potent in protein

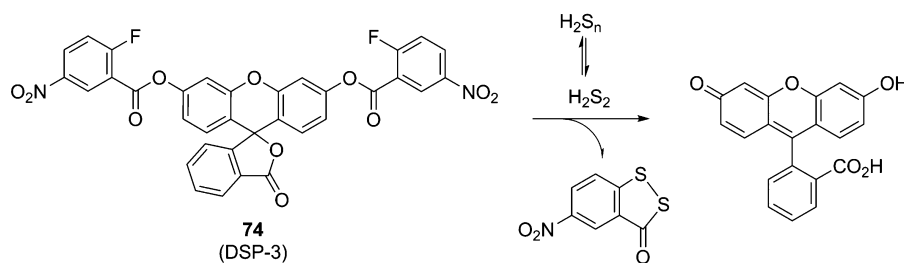


Fig. 16 Structures of fluorescent probes for hydrogen polysulfides.

S-sulfhydration reactions compared to $\text{H}_2\text{S}^{147-149}$ and suggest that some biological mechanisms that were originally attributed to H_2S may proceed through H_2S_n intermediates. H_2S_n is a combination of hydrogen polysulfides in which hydrogen disulfide (H_2S_2) is the smallest and likely the most reactive species in dynamic equilibrium with other H_2S_n .¹⁴⁷ Xian and co-workers exploited the two $-\text{SH}$ groups in H_2S_2 to make **74**,¹⁵² a probe where a 2-fluoro-5-nitro-benzoic ester can rapidly trap H_2S_2 and promote an intramolecular cyclization to release fluorescein (Fig. 16). This probe can also react with biothiols to form thioether products, but these compounds do not undergo cyclization and therefore do not yield an increase in the fluorescence signal. More interestingly, such thioether products can further react with H_2S_n (via the $\text{S}_\text{N}2\text{Ar}$ reaction) to give a turn-on fluorescence response, which confirms its selectivity. The detection limit of **74** was determined to be 71 nM.

3.3. Sulfite and bisulfite

Sulfur dioxide (SO_2) is a well-known air pollutant and has been studied extensively in toxicology. However, more recent work has shown that this molecule is generated endogenously, mainly from sulfur-containing amino acids through biosynthetic pathways¹⁵³⁻¹⁵⁵ such as transamination by aspartate aminotransferase (AAT).¹⁵⁶ SO_2 exhibits unique bioactivities such as vasodilatation and lowering of blood pressure.¹⁵⁷⁻¹⁵⁹ These observations have led to speculation that SO_2 can be considered a gasotransmitter,^{156,160,161} and thus the detection of SO_2 and its hydrated derivatives (sulfite and bisulfite) have attracted attention. SO_2 is readily soluble in water (40 L SO_2 [g] in 1 L H_2O) and forms a very stable hydrated SO_2 complex ($\text{SO}_2 \cdot \text{H}_2\text{O}$). As such, in aqueous environments the major chemical state of SO_2 is molecular SO_2 , not H_2SO_3 , the precursor of bisulfite (HSO_3^-) and sulfite (SO_3^{2-}).¹⁶² Hydrated SO_2 and bisulfite/sulfite are distinct molecular species that exhibit distinct chemical and biological functions,^{163,164} but both hydrated SO_2 and bisulfite/sulfite have been used in exploring the biological contributions of SO_2 ; hydrated SO_2 typically shows more potent activity than bisulfite/sulfite.^{161,163,164} Nevertheless, many studies on SO_2 fluorescent probes are centered on the detection of bisulfite/sulfite, and not the molecular SO_2 .

Exploiting the nucleophilicity of SO_3^{2-} , Chang and colleagues reported the first fluorescent probe for its detection (Fig. 17).¹⁶⁵ SO_3^{2-} is proposed to react with the ketone group of **75** to form the intermediate X, which then undergoes fast cyclization to

release the fluorophore. Both chromogenic and fluorogenic responses to SO_3^{2-} in HEPES buffer (pH 7.0, 10 mM) containing 2% acetonitrile were observed with a detection limit of 49 μM , and this strategy was expanded to develop a family of SO_3^{2-} reporters (**76–80**) that show good selectivity over other anions and RSS, including H_2S .¹⁶⁶⁻¹⁷⁰

Aldehyde triggers for $\text{SO}_3^{2-}/\text{HSO}_3^-$ have also been used for detection of these RSS (Fig. 18). Reactions with $\text{SO}_3^{2-}/\text{HSO}_3^-$ result in a change in the electron acceptor strength and the concomitant charge transfer efficiency. For example, based on the difference of the intramolecular charge transfer (ICT), the ratiometric probe **81** was found to be selective for HSO_3^- in pH 4.6 NaAc-HAc buffer with a detection limit of 0.4 μM ,¹⁷¹ and related ICT-based probes (**82–83**) were effective for HSO_3^- detection in pH 5 NaH_2PO_4 /citric buffer and in HeLa cells.^{172,173} The fluorescent indicator **84** for SO_3^{2-} based on the modulation of photoinduced electron transfer (PeT) was also reported; this probe was used to determine concentrations of SO_3^{2-} in food or beverages.¹⁷⁴

More recently, Wu and co-workers developed a water-soluble polylysine-based probe **85** for SO_3^{2-} that induced charge generation and aggregation induced emission (AIE) effects (Fig. 19).¹⁷⁵ The probe consisted of a modified polylysine with aldehyde groups and positively charged fluorophores (TPE-2N^+). After the aldehyde groups on polylysine chains reacted with SO_3^{2-} (in pH 7.0 HEPES buffer), the resulting negatively charged groups formed complexes with TPE-2N^+ and led to a fluorescence enhancement due to the AIE effects.

The reaction between glyoxal mono-hydrazone and HSO_3^- was also used in the design of fluorescent HSO_3^- probes (Fig. 20). In one example (probe **86**), formation of the addition product led to spirolactam opening of rhodamine to turn on fluorescence in Na_2HPO_4 -citric acid buffer (20 mM, pH 4.8) containing 10% ethanol with a detection limit of 0.89 μM .¹⁷⁶ In another example, probe **87** was weakly fluorescent owing to $\text{C}=\text{N}$ isomerization-induced fluorescence quenching,¹⁷⁷ but reaction with HSO_3^- formed an intramolecular hydrogen bond which inhibited isomerization and led to a fluorescence turn-on in acidic media (DMSO-acetate buffer, 100 mM, pH 5.0, 1 : 1, v/v).

As described above, many aldehyde/ketone based probes require acidic medium for detection of the analyte, as in neutral buffers (or in organic solvents) these probes showed strong responses to biothiols (for example, probes **82**, **83**, **86**, **87**).¹⁷⁸⁻¹⁸¹ In order to address this issue, fluorescent indicators based on

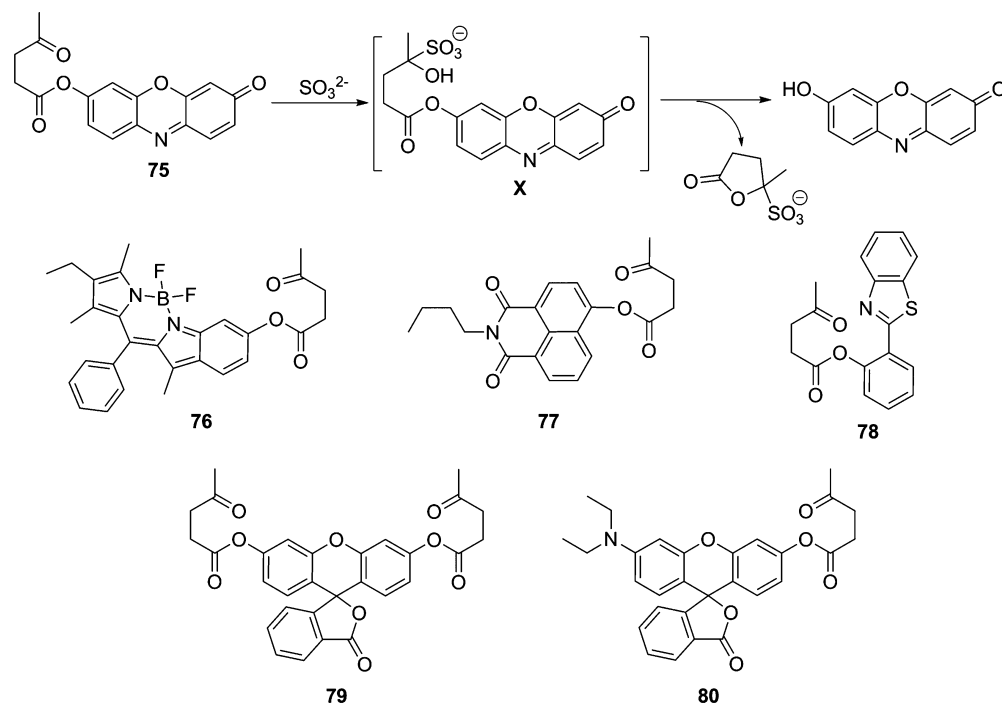


Fig. 17 Structures of fluorescent probes for $\text{SO}_3^{2-}/\text{HSO}_3^-$ featuring γ -ketoester triggers.

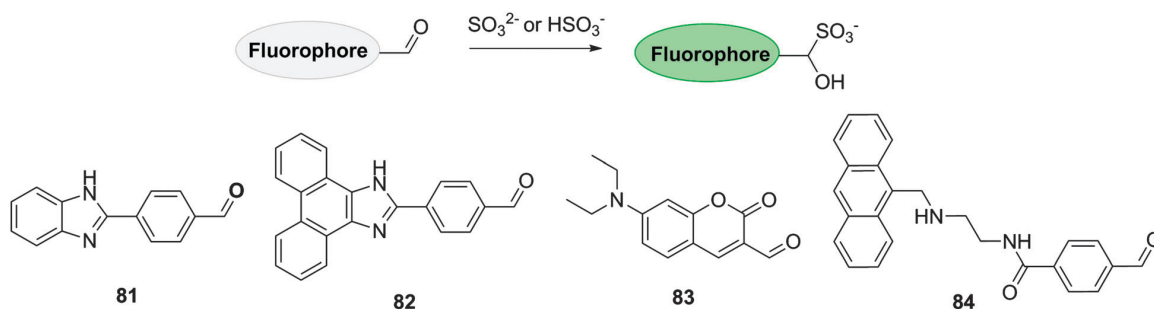


Fig. 18 Structures of fluorescent probes for $\text{SO}_3^{2-}/\text{HSO}_3^-$ utilizing aldehyde triggers.

nucleophilic addition to other double bonds (such as $-\text{N}=\text{N}-$, $-\text{C}=\text{N}-$, $-\text{C}=\text{C}-$) have been explored. For example, Chao and colleagues prepared a non-emissive dinuclear iridium(III) complex (**88**) bridged *via* an azo group (Fig. 21).¹⁸² The electron-withdrawing azo group can effectively quench the luminescence efficiency of **88** based on trapping electrons in the metal-to-ligand charge-transfer (MLCT) excited state. When **88** reacted with $\text{SO}_3^{2-}/\text{HSO}_3^-$ in HEPES buffer (10 mM, pH 7.5) containing 30% DMSO, a dramatic increase in the phosphorescence intensity at 600 nm was observed due to the addition-induced changes in the electronic and luminescence properties of the metal complex. The probe showed a highly selective off-on response to $\text{SO}_3^{2-}/\text{HSO}_3^-$ over other RSS with a detection limit of 0.24 μM for SO_3^{2-} and 0.14 μM for HSO_3^- . Moreover, **88** was applied to the detection of both exogenous and endogenous $\text{SO}_3^{2-}/\text{HSO}_3^-$ in living cells.

Martínez-Mañez and co-workers reported a chromofluorogenic nanoprobe for SO_3^{2-} in food and environmental samples by using hydrophobic hybrid organic–inorganic silica nanoparticles loaded with probe **89**, which can readily react with SO_3^{2-} *via* 1,6-conjugated addition (Fig. 22) in HEPES buffer (30 mM, pH 7.5), causing a color change from blue to pale yellow with a detection limit of 0.32 ppm.¹⁸³ The selectivity for SO_3^{2-} was ascribed to the preferential inclusion of SO_3^{2-} into the hydrophobic pockets in nanoparticles.

Guo's laboratory reported a colorimetric and ratiometric fluorescent probe **90** for HSO_3^- based on an addition–rearrangement cascade reaction (Fig. 23).¹⁸⁴ In PBS buffers (10 mM, pH 7.4, containing 30% DMF), the reaction between **90** and HSO_3^- caused a gradual decrease in fluorescence intensity at 633 nm, accompanied by the formation of a new emission peak at 478 nm and a distinct color change from violet to colorless.

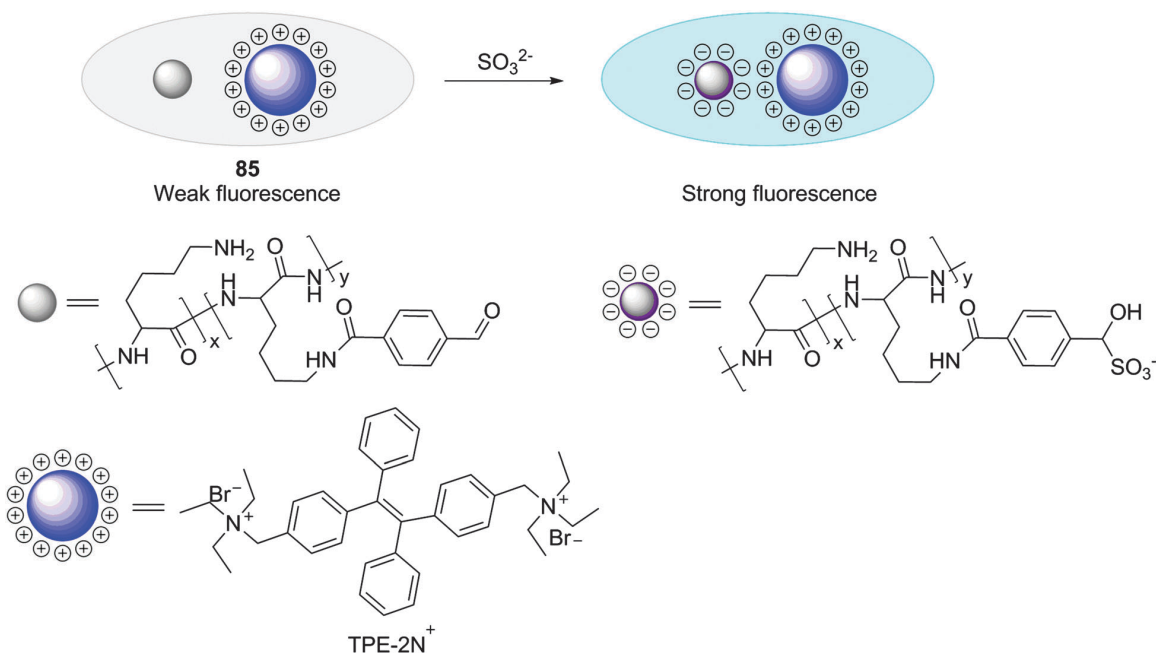


Fig. 19 Structure of a fluorescent probe for $\text{SO}_3^{2-}/\text{HSO}_3^-$ exploiting an aggregation induced emission strategy.

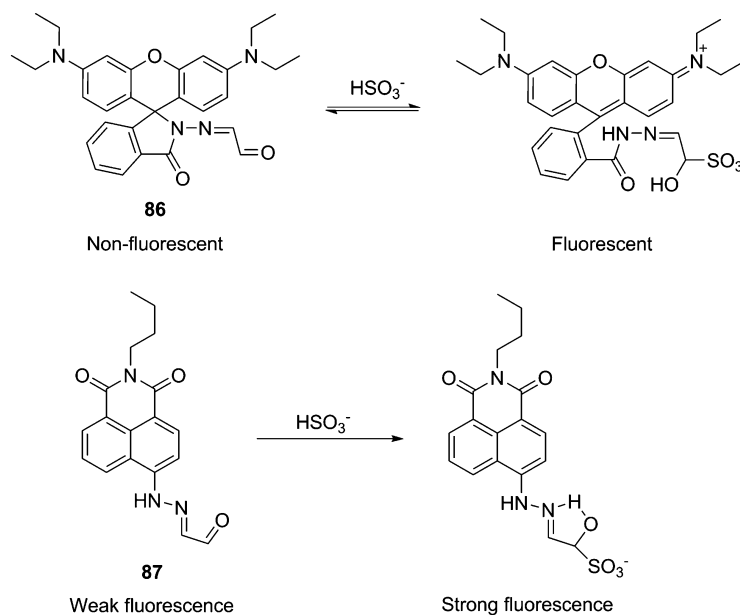


Fig. 20 Structure of fluorescent probes for $\text{SO}_3^{2-}/\text{HSO}_3^-$ featuring glyoxal mono-hydrazone moieties.

These optical changes were attributed to nucleophilic addition of HSO_3^- to the $\text{C}=\text{C}$ bond, interrupting the π -conjugation. The indicator did not respond to other RSS, including glutathione and H_2S . It was applied to detect SO_2 release in HeLa cells. Similar ratiometric probes (**91–92**) based on this approach were also reported,^{185,186} and more recent work by Yu and co-workers showed that the rearrangement step is dependent

on the reaction media, as they observed that SO_3^{2-} addition occurs primarily to the coumarin ring of probe **93** in pure aqueous solutions (HEPES buffer, 20 mM, pH 7.4).¹⁸⁷ Probe **93** is a water-soluble near-IR fluorescent probe with good selectivity for SO_3^{2-} and a 0.27 nM detection limit.

Finally, α,β -unsaturated systems have also been used in the design of SO_3^{2-} probes (Fig. 24). For example, 1,4-addition of

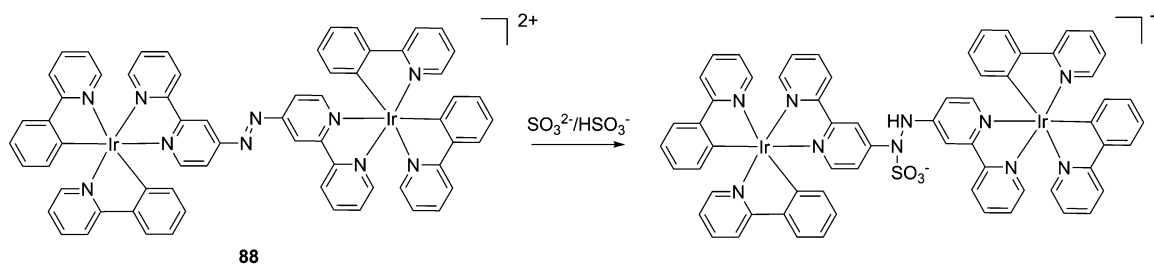


Fig. 21 Structure of a fluorescent probe for $\text{SO}_3^{2-}/\text{HSO}_3^-$ based on a dinuclear iridium(III) complex.

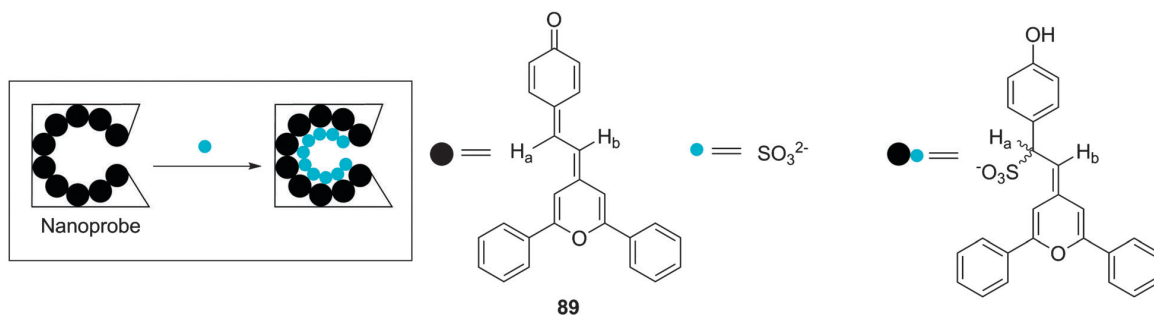


Fig. 22 Structure of a fluorescent nanoprobe for $\text{SO}_3^{2-}/\text{HSO}_3^-$ based on hybrid organic-inorganic silica nanoparticles.

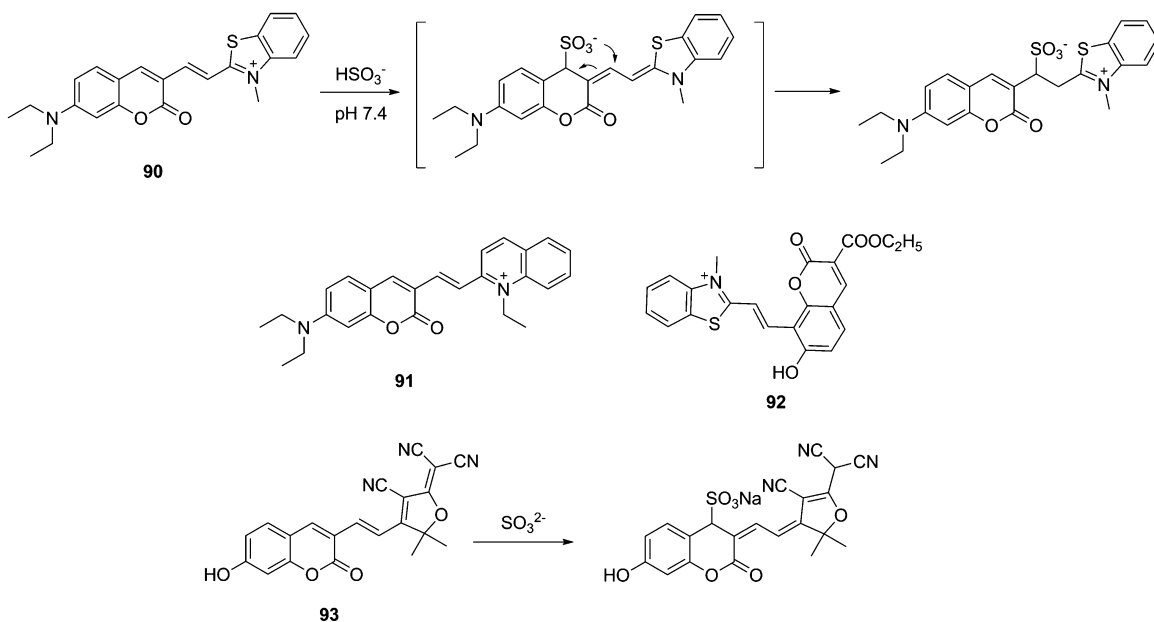


Fig. 23 Structures of fluorescent probes for $\text{SO}_3^{2-}/\text{HSO}_3^-$ which undergo nucleophilic addition followed by rearrangement to give a turn-on response.

SO_3^{2-} to **94** and **95** shortened π -conjugation of the probes, which induced a remarkable hypsochromic shift in the maximal emission intensity.^{188,189} The reactions of HS^- or GSH led to much less fluorescence enhancement for **94**. As for **95**, upon addition of HS^- , the maximal emission intensity shifted from 590 nm to 564 nm, owing to the reduction of the

azido group. However, after reaction with SO_3^{2-} , the maximal emission intensity shifted from 590 nm to 460 nm. Therefore, **95** may be used to differentiate HS^- and SO_3^{2-} . Indicators **96** and **97** were developed using a similar reaction, but it was found that HS^- could lead to obvious fluorescence enhancements.^{190,191}

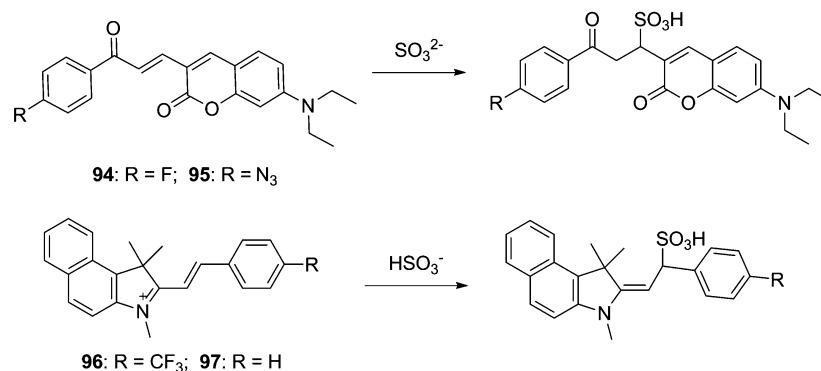


Fig. 24 Structures of fluorescent probes for $\text{SO}_3^{2-}/\text{HSO}_3^-$ incorporating α,β -unsaturated functionalities for reactivity.

3.4. S-nitrosothiols

RSS also include modified cysteine derivatives, both in protein systems and small molecules such as glutathione. Important members in this category are sulfenic acids (RSOH), persulfides (RS-SH), and S-nitrosothiols (RSNO). These adducts are generated endogenously by corresponding reactive oxygen/nitrogen/sulfur species: H_2O_2 , H_2S , and NO, playing important roles in redox signaling as these modifications often regulate protein function.¹⁹² A number of detection methods for these species have been reported,^{192–195} most of which focus on labeling or enriching protein cysteine modifications (for identifying specific proteins or proteomics analysis). To date, specific fluorogenic probes for RSOH have not been reported. RSSH belongs to the sulfane sulfur family so the probes for sulfane sulfurs, such as SSP2 (73), should also be useful for RSSH detection, although this has not yet been validated.

As for RSNO, two types of fluorescent probes (**98**, **99**) have been reported (Fig. 25).^{196,197} In one strategy, it was found that RSNO can react with two equivalents of phosphines to form phosphine oxide **A** and thio-azaylide **B**. If an ester group is presented, **A** can further react with the ester to form sulfenamide product **C**. This reductive ligation of RSNO is mechanistically similar to the Staudinger ligation developed in Bertozzi's laboratory.¹⁹⁸ Based on this reaction, Xian's laboratory reported an indicator based on RSNO mediated phosphine oxidation. Probe **98** is a triarylphosphine coumarin derivative where the phosphorus electron lone pair can quench the excited state of the coumarin fluorophore. Upon reaction with RSNO, oxidation of phosphine eliminates quenching effects and activates fluorescence. One drawback of this first-generation reagent is that other reactive oxidants such as H_2O_2 may also activate the probe. To solve this issue, Xian's group turned to a reductive ligation strategy, and the new reporter SNOPI **99** can rapidly react with RSNO to release free fluorescein and show strong fluorescence enhancement at 37 °C in Tris-HCl buffer (50 mM, pH = 7.4) containing 1% DMSO. This next-generation indicator showed good selectivity for various small molecule RSNOs, including S-nitroglutathione (GSNO), the predominant endogenous RSNO compound, but it is unclear if the probe can respond to protein SNO substrates. Notably, a variety of other

RSS or ROS such as thiols, disulfides, H_2S , H_2O_2 , and HClO did not yield a fluorescence response. HNO also gives a high response, which is not surprising,^{199,200} but within biological contexts HNO is a very short-lived species compared to RSNO. Even if HNO co-exists with RSNO in a given sample, HNO will likely decompose quickly (when its production is terminated) and not trigger false positives.

4. Chemical probes for RSS-mediated post-translational modifications

S-Sulfhydration (forming –S-SH adducts from cysteine –SH residues) is a newly recognized oxidative post-translational modification caused by RSS,¹⁹² which is receiving growing attention in the context of H_2S - and RSS-mediated signaling pathways. Although S-sulfhydration (also known as S-thiolation) is considered to be an emerging mechanism in the field of redox biology, it has been long known that formation of protein persulfides (P-S-SH) can occur in biological systems and they have been identified as intermediates in multiple biosynthetic pathways related to sulfur metabolism,¹⁴³ such as in the production of sulfur-containing vitamins and other biomolecules.^{25,143} Interestingly, recent proteomics data showing that a relatively large number of proteins are the targets of S-sulfhydration suggest that this post-translational modification is a broad, general phenomenon that regulates the function of numerous systems within cells.^{26,201} Precise mechanisms that regulate S-sulfhydration are still under investigation, but it is clear that H_2S cannot directly react with protein cysteine (–SH) to form persulfides without accompanying redox events and it is also unlikely that H_2S reacts directly with protein disulfides to give sulfhydration, as H_2S is a weak reductant compared to GSH.^{202,203} In contrast, H_2S can plausibly convert some reactive S-oxidized forms such as sulfenic acids (SOH) or nitrosothiols (SNO) for S-sulfhydration, and protein cysteines (SH) may react with oxidized H_2S derivatives, such as sulfane sulfurs or hydrogen polysulfides, to form persulfides. Indeed, recent work shows that polysulfides are much more potent than H_2S for inducing sulfhydration in proteins such as Keap1, TRPA1 channels, roGFP2, and PTEN.^{147–149}

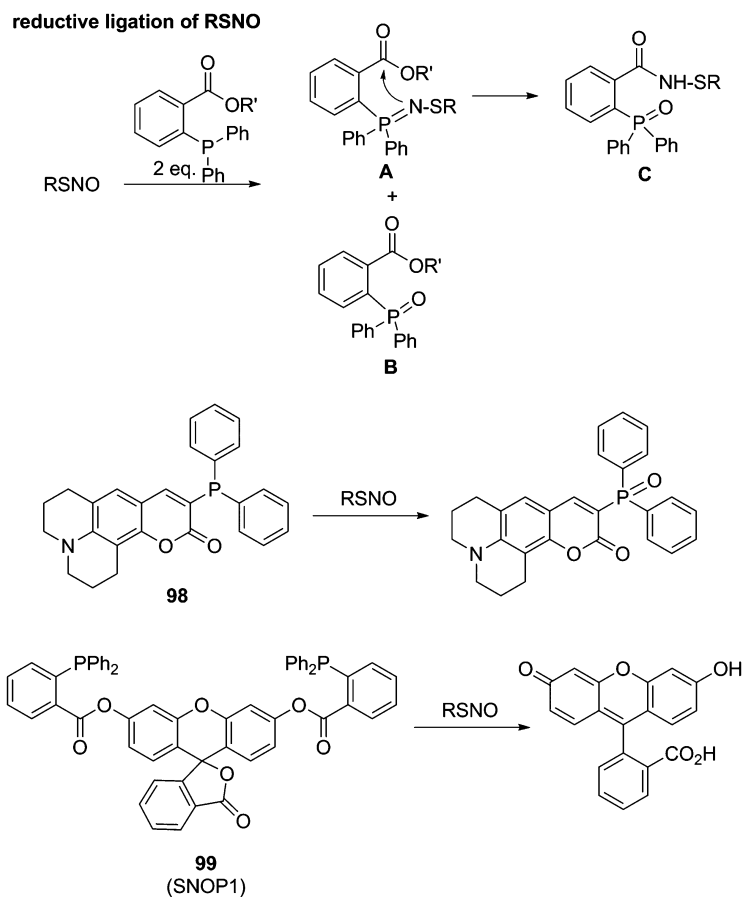


Fig. 25 Structures of probes for S-nitrosothiols.

In this context, several methods have been reported for the detection of S-sulphydration. Snyder and colleagues described a modified biotin switch technique (BST),²² which employs an alkylating agent S-methyl methanethiosulfonate (MMTS) to differentiate thiols and persulfides (Fig. 26). In this protocol,

protein thiols (–SH) are first blocked by MMTS. Persulfides (–S–SH) are believed to remain unreacted and be available for subsequent conjugation to biotin-HPDP. Using this method, a large number of proteins were identified as targets for S-sulphydration. However the underlying mechanism for the

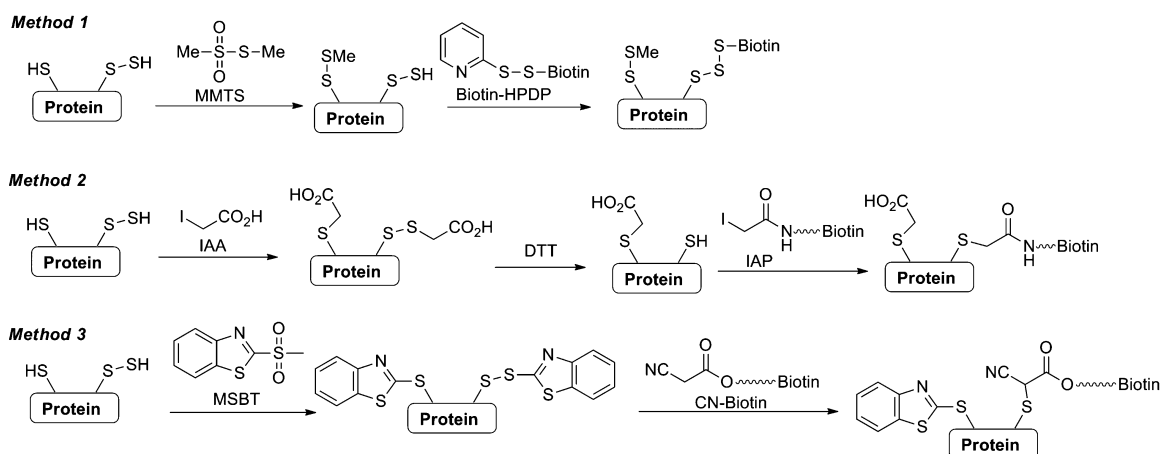


Fig. 26 Strategies for the detection of RSS-mediated post-translational modifications.

selectivity of MMTS for thiol vs. persulfide is unclear. Recent work in Carroll's laboratory has demonstrated that persulfides and thiols should have similar reactivities toward electrophiles such as MMTS.¹⁴⁴ Tonks and co-workers reported a S-sulphydration method where both –SH and –SSH could be blocked by alkylating reagents like iodoacetic acid (IAA), then the persulfide adducts (*i.e.* disulfides) could be reduced by DTT to form free –SH and subsequently be labeled with iodoacetamide-linked biotin (IAP).²³ The application of this method may be limited as DTT reduction may not be able to distinguish persulfide modifications from other DTT-reducible residues, such as normal protein disulfides, sulfenic acids, and S-nitrosothiols. Very recently a tag-switch method was reported as a selective method for protein S-sulphydration.²⁰⁴ This method requires two reagents to label protein persulfides in two-step reactions. The first step is to block –SH and –SSH by methylsulfonyl benzothiazole (MSBT). Like other thiol-blocking reagents, MSBT can effectively react with both groups to give –S-BT (benzothiazole) or –S-S-BT, respectively. These two adducts have very different reactivities towards nucleophiles. Protein–S-BT adducts are thioethers, which are unreactive to nucleophiles. In contrast, protein–S-S-BT are heterocycle-conjugated disulfides which are highly reactive to some carbon-based nucleophiles. When cyanoacetate-based reagents like CN-biotin are treated, stable thioether linkages will be formed. This tag-switch method appeared to be selective for protein persulfides and was used for identifying a number of S-sulphydrated proteins in cell lysates.^{26,204}

5. Concluding remarks

The emerging dynamic roles of H₂S and related RSS in physiology and pathology have spurred the rapid development of chemical probes to detect and differentiate these species in living systems. These reagents, available in a growing spectrum of colors and featuring a variety of cellular and subcellular targeting functionalities, are being used in developing technologies that will enable further exploration of RSS biology. In particular, key opportunities to be focused on include the development of more sensitive probes that enable the detection of endogenous levels of H₂S. Ratiometric probes that will allow for semi-quantitative measurements are also a priority, as well as probes that can be used in live animal models, allowing for a broader application of these chemical tools in biomedical research. Application of such emerging chemical technologies will help elucidate the mechanisms by which these reactive species modulate signaling pathways, maintain cellular function, and impact aging and disease states. In addition, trapable or targetable molecular and genetically-encoded probes offer the possibility of monitoring RSS in individual cells to better understand the intracellular movement of these species, especially between different cell and tissue types. Furthermore, the interactions of RSS with other reactive species like nitric oxide, hydrogen peroxide, carbon monoxide, and higher order reactive nitrogen, oxygen, and carbon species may also provide insight into how such structurally simple molecules are able to

elicit such a broad range of effects. This goal could be achieved using a combination of spectrally distinct chemoselective probes for dual imaging of synergistic species, such as H₂S and NO. The continued development of new chemistry to be used at the frontier of this exciting area promises to identify sources and targets of RSS biology.

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