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Chemical tools for the study of hydrogen sulfide (H_2S) and sulfane sulfur and their applications to biological studies

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Hydrogen sulfide (H_2S) functions in many physiological processes, including relaxation of vascular smooth muscles, mediation of neurotransmission, inhibition of insulin signaling, and regulation of inflammation. On the other hand, sulfane sulfur, which is a sulfur atom with six valence electrons but no charge, has the unique ability to bind reversibly to other sulfur atoms to form hydropersulfides (R-S-SH) and polysulfides (-S-S_n-S-). H_2S and sulfane sulfur always coexist, and recent work suggests that sulfane sulfur species may be the actual signaling molecules in at least some biological phenomena. For example, one of the mechanisms of activity regulation of proteins by H_2S is the *S*-sulfhydration of cysteine residues (protein Cys-SSH). In this review, we summarize recent progress on chemical tools for the study of H_2S and sulfane sulfur, covering fluorescence probes utilizing various design strategies, H_2S caged compounds, inhibitors of physiological H_2S-producing enzymes (cystathionine γ-lyase, cystathionine β-synthase and 3-mercaptopyruvate sulfurtransferase), and labeling reagents. Fluorescence probes offer particular advantages as chemical tools to study physiological functions of biomolecules, including ease of use and real-time, nondestructive visualization of biological processes in live cells and tissues.

Key Words: **hydrogen sulfide, sulfane sulfur, fluorescence probe, caged compound, enzyme inhibitor**

Hydrogen sulfide (H_2S), a toxic gas smelling of rotten eggs, plays key roles in many physiological processes, including relaxation of vascular smooth muscles,[1,2] mediation of neurotransmission,[3,4] inhibition of insulin signaling,[5] and regulation of inflammation.[6,7] The most commonly used detection methods for H_2S are the methylene blue method,[8] the electrode method[9] and the monobromobimane method.[10,11] However, these methods require destructive sampling, e.g., homogenization of biological samples. On the other hand, fluorescence detection has been widely used in biological studies to study the physiological roles of H_2S, because this technology is easy to use, and enables real-time, nondestructive detection in living cells and tissues. Many selective fluorescence probes for H_2S have been reported.[12–14] On the other hand, sulfane sulfur species consist of sulfur atom(s) with six valence electrons but no charge bound to other sulfur atom(s), as in hydropersulfides (R-S-SH) and polysulfides (-S-S_n-S-). They are attracting increasing interest, because it is reported that one of the mechanisms of activity regulation of proteins by H_2S is *S*-sulfhydration of cysteine residues (SH→SSH).[2,15] Since H_2S and polysulfide are redox partners, they would coexist in biological systems, and polysulfide seems likely to be much more effective than H_2S in *S*-sulfhydration from a reactivity point of view.[16] Therefore, sulfane sulfur is considered to mediate at least some of the biological activities of H_2S.

In this review, we summarize recent work on chemical tools (especially fluorescence probes) for the study of H_2S and sulfane sulfur, and we briefly review their applications to biological studies.

Chemical Characteristics of H_2S

H_2S is highly water-soluble, with pK_{a1} of 6.8 and pK_{a2} of approximately 14;[17] consequently, 80% of H_2S exists as strongly nucleophilic HS^- at pH 7.4. In addition, H_2S itself shows reducing ability, changing the oxidation state of sulfur from –2 (H_2S) to 0 (S); for example, it can reduce azide and nitro groups.[18,19] These properties have been utilized to develop off/on-type fluorescence probes for H_2S.

Development of Fluorescence Probes for H_2S

Fluorescence probes based on reduction of azide or nitro group to amino group. The first fluorescence probes utilizing azide reduction were SF1 and SF2, reported by Lippert *et al.*[20] (Fig. 1a). Reduction of the azide group of the xanthene moiety to amine leads to opening of the intramolecular spirocycle of the rhodamine scaffold; this restores the conjugated system of the xanthene moiety, leading to strong fluorescence (SF1 and SF2, Φ_{FI} = 0.50 and 0.60, respectively). SF1 and SF2 showed 7- and 9-fold fluorescence increases, respectively, within 1 h after addition of 100 μM NaHS. The same authors subsequently developed improved probes (SF4-7) with enhanced sensitivity and cellular retention, using the same strategy (Fig. 1a).[21] Moreover, H_2S fluorescence probes targeting specific organelles have also been developed. For example, Bae *et al.*[22] reported two fluorescence probes, SHS-M1 and SHS-M2, which incoporate a triphenylphosphonium group as a mitochondrial targeting moiety (Fig. 1b). Like the azide group, the nitro group can be reduced by H_2S; Montoya *et al.*[23] reported a fluorescence probe based on this design concept (HSN1; Fig. 1c left), while Wu *et al.*[24] reported colorimetric and

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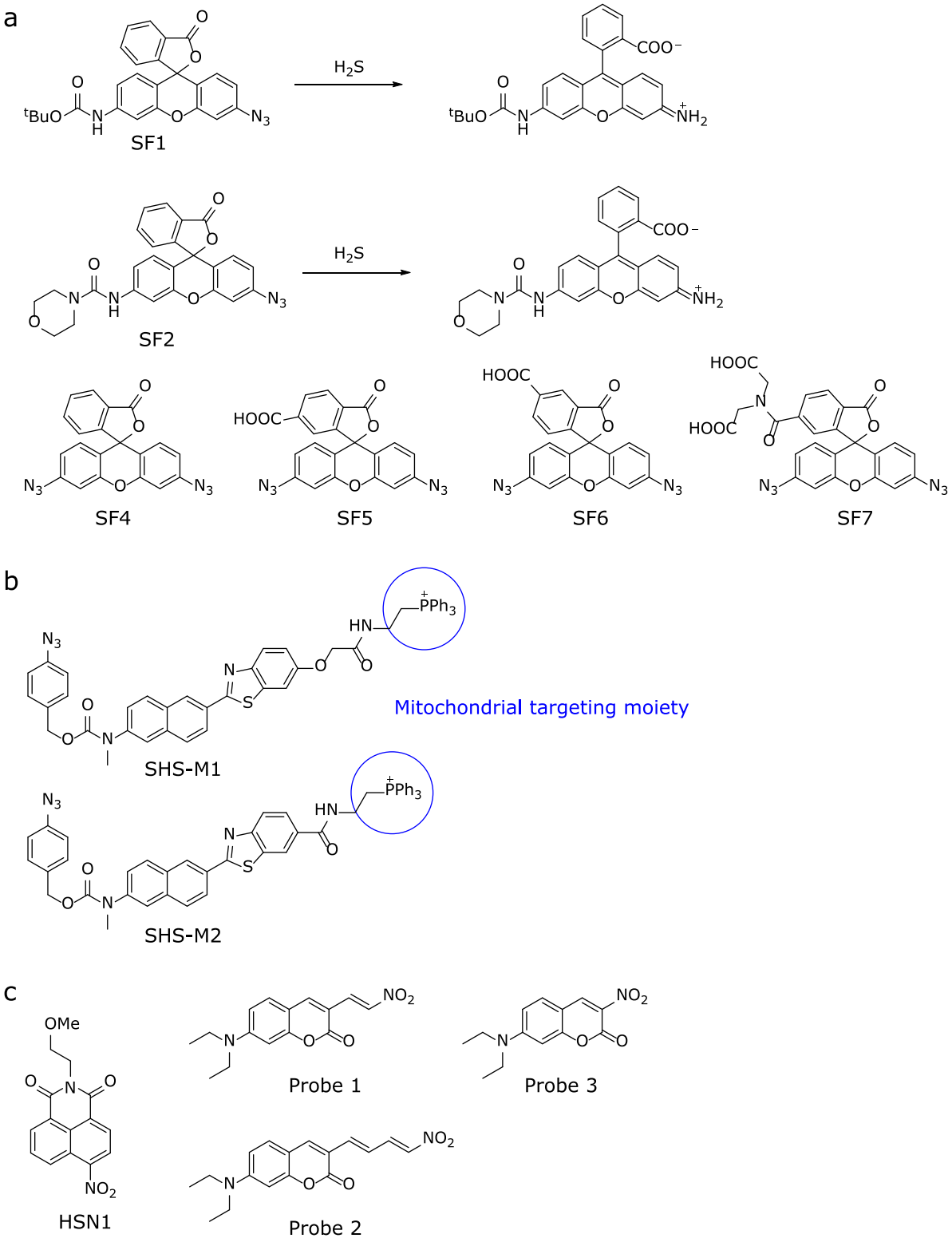

Fig. 1. Fluorescence probes for H_2S: (a) Probes utilizing reduction of an azide group. (b) Mitochondrially targeted probes utilizing reduction of azide. (c) Probes based on reduction of a nitro group.

ratiometric probes (Fig. 1c middle and right). Thus, several types of H_2S fluorescence probes utilizing azide or nitro group reduction have been reported.[12–14]

Fluorescence probes based on the nucleophilicity of HS^-. H_2S (HS^-) has strong nucleophilicity, which was utilized by Qian *et al.*[25] to design fluorescence probes SFP-1 and SFP-2 (Fig. 2a). In these probes, fluorescence off/on switching occurs via HS^-

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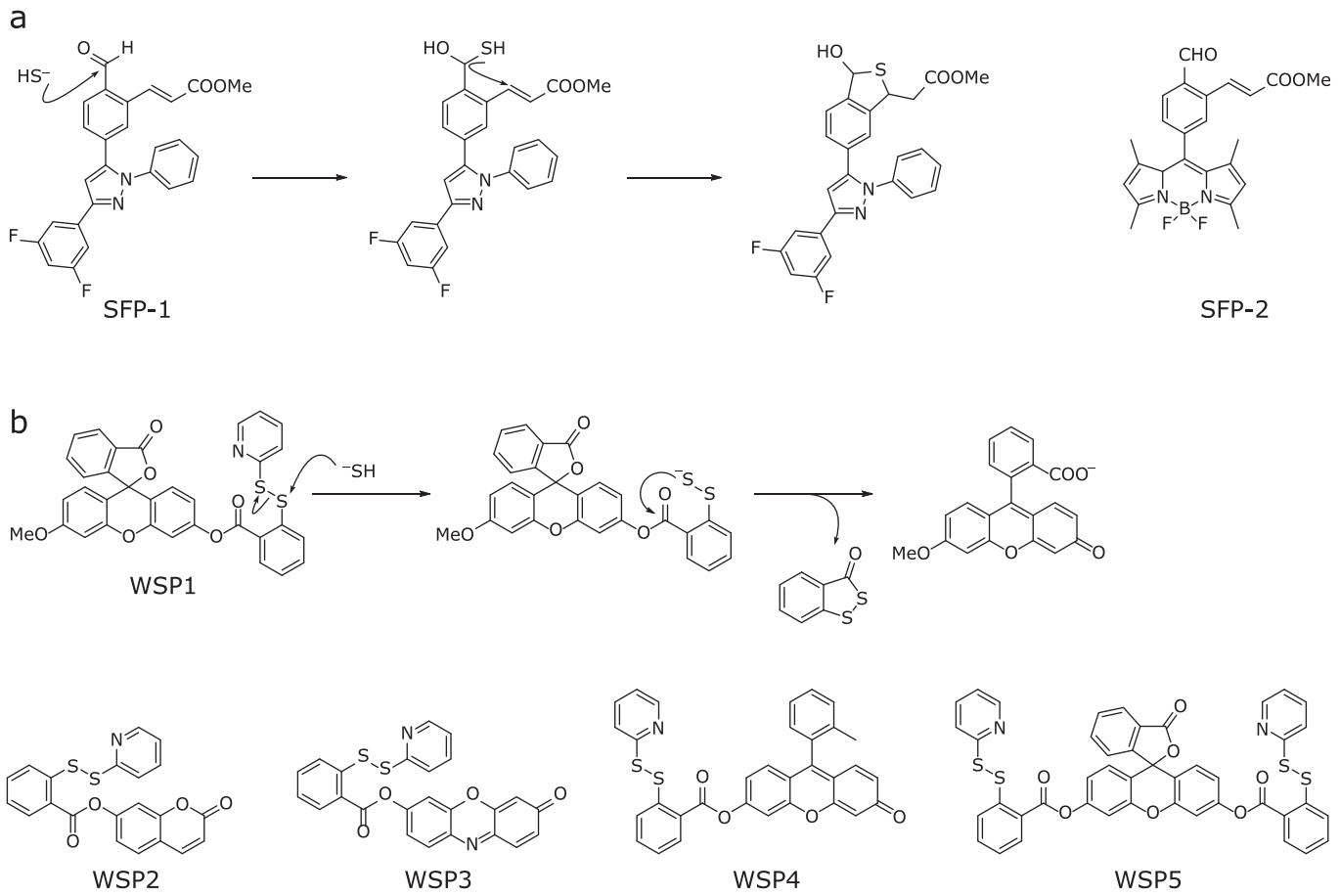

Fig. 2. Fluorescence probes for H_2S utilizing nucleophilic reaction of HS^-.

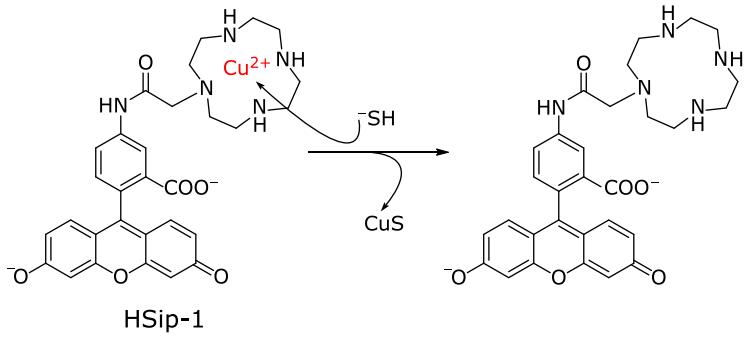

Fig. 3. Fluorescence probes based on the fluorescence quenching effect of Cu^{2+}.

addition to aldehyde, followed by Michael addition of the resulting intermediate to unsaturated methyl acrylate to form a thiohemiacetal under physiological conditions. The resulting stable tetrahydrothiophene shows strong fluorescence. Liu *et al.*[26] and Peng *et al.*[27] reported a fluorescein-based probe, WSP1-5 (Fig. 2b), in which the disulfide bond is cleaved by H_2S followed by intramolecular nucleophilic attack of the persulfide group on the ester moiety; this releases the fluorophore, resulting in a large fluorescence increase.

Fluorescence probes based on the quenching effect of copper ion (Cu^{2+}). It is well known that heavy metal ions such as iron (III) ion (Fe^{3+}) and Cu^{2+} quench the fluorescence of a nearby fluorophore,[28] and precipitation of CuS has been adapted for H_2S detection by incorporating a Cu^{2+} complex moiety in the fluorophore. Choi *et al.*[29] reported that a dipicolylamine (DPA)–fluorescein complex with Cu^{2+} showed a turn-on fluorescence response to H_2S, but without selectivity over other biothiols. Our group then designed and synthesized a H_2S fluorescence probe, HSip-1, in which Cu^{2+} is complexed with an azamacrocyclic ring, which forms stable complex with Cu^{2+} (Fig. 3).[30] We expected that Cu^{2+} would be released from the azamacrocyclic ring when H_2S binds to the Cu^{2+} center, resulting in a large fluorescence enhancement with selectivity against other biothiols. Indeed, HSip-1 showed a large and fast fluorescence increase after addition of 100 μM Na_2S. It also has extremely high water-solubility, so that DMSO (dimethylsulfoxide) or detergent is not

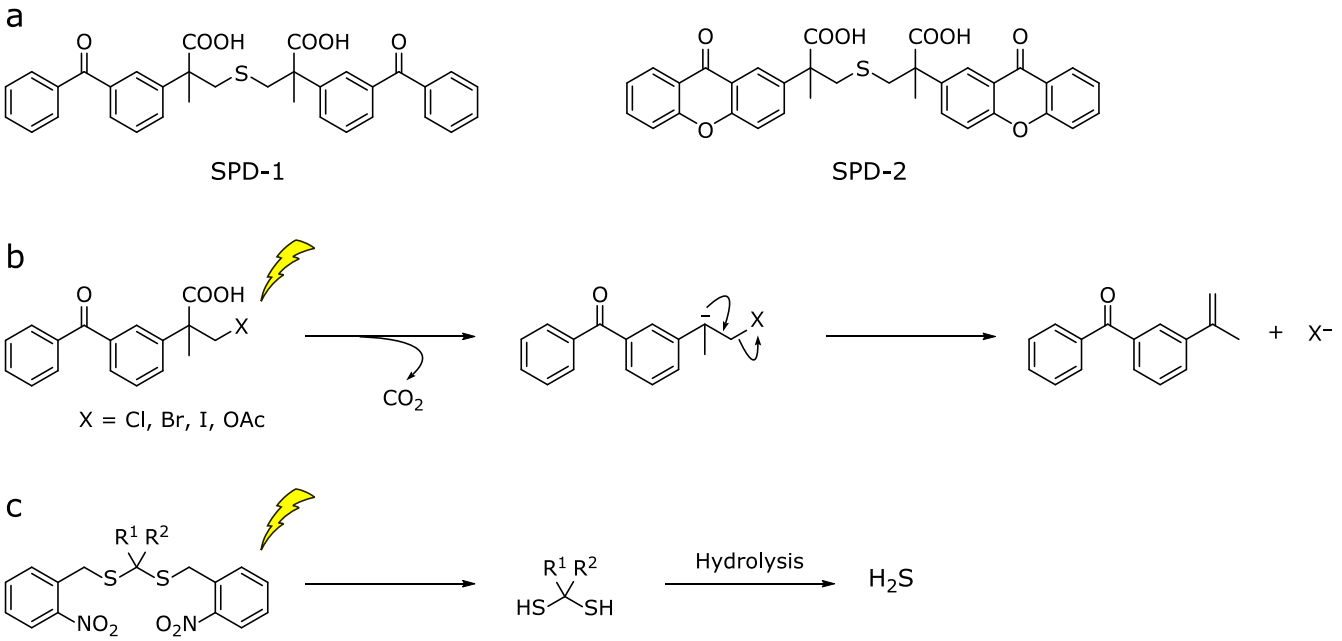

Fig. 4. (a) Chemical structures of SPD-1 and SPD-2. (b) Photoreaction mechanism of ketoprofenate-based caged compounds. (c) Reaction mechanism of caged *gem*-dithiol.

needed as a cosolvent. Further, although probes that release CuS may show cytotoxicity, HSip-1 was not toxic to living cells.[30] These properties make HSip-1 particularly suitable for biological studies both *in vitro* and *in vivo*.

Development of Photocontrollable Hydrogen Sulfide Donors

In biological research on H_2S, inorganic sulfide salts have been widely used as H_2S donors. However, H_2S generation from these salts is rapid and does not mimic endogenous H_2S release. Instead, controllable H_2S donors are required for detailed investigation of the physiological functions of H_2S. Fukushima *et al.*[31] reported a photolysis-induced H_2S donor, SPD-1, which enables precise control of the location, timing and dosage of H_2S release by means of light irradiation (Fig. 4a). SPD-1 is a caged compound, in which H_2S is directly protected by ketoprofenate, and generates H_2S proportionally to the irradiation time and light intensity, with simultaneous release of a photoproduct, 2-propenylbenzophenone (Fig. 4b).[32] They subsequently developed an improved H_2S donor, SPD-2, in which xanthone is used as a photolabile protecting group (Fig. 4a).[33] SPD-2 has longer absorbance wavelength than SPD-1, and the generation of H_2S can be precisely controlled by irradiation in the UVA range (325–385 nm). SPD-2 shows more efficient H_2S production than SPD-1, presumably because of its absorption wavelength range. Devarie-Baez *et al.*[34] also reported a caged H_2S donor based on the structure of geminal-dithiols (Fig. 4c), which are unstable in aqueous solutions, releasing H_2S. They protected the free SH of *gem*-dithiol with 2-nitrobenzyl, a widely used type of caging chromophore, to obtain a stable *gem*-dithiol-based H_2S donor. The free *gem*-dithiol intermediate is produced upon light irradiaton and hydrolysis of this intermediate affords H_2S.

Physiological Functions of Sulfane Sulfur

H_2S has been suggested to be an endogenous signaling molecule, and one of its regulatory mechanisms is thought to be *S*-sulfhydration of protein cysteine residues (SH→SSH).[2,15] However,

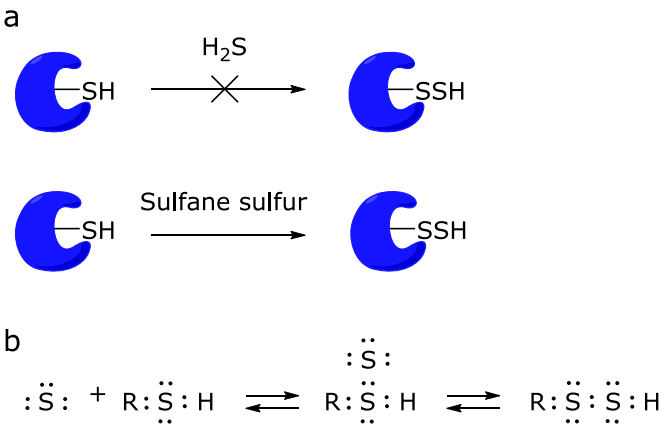

Fig. 5. *S*-sulfhydration reaction: (a) Sulfane sulfur is much more effective for the illustrated reaction than H_2S. (b) Proposed mechanism of *S*-sulfhydration reaction mediated by sulfane sulfur and tautomerization of hydropersulfide.

H_2S is a fully reduced sulfur species and is basically a reductant, whereas *S*-sulfhydration of cysteine by H_2S is an oxidation reaction. Further, thiols and H_2S are formally at the same oxidation state (2–), and would not react with each other.[17] Therefore, it is thought that *S*-sulfhydration is not mediated by H_2S directly (Fig. 5a). On the other hand, sulfane sulfur is a form of sulfur with six valence electrons and no charge (S^0), which has the unique ability to reversibly bind to other sulfur atoms, as seen in elemental sulfur (S_8), persulfides (R-S-SH) and polysulfides (-S-S_n-S-). Thus, it is considered that proposed *S*-sulfhydration reactions involving H_2S may actually be mediated by sulfane sulfur as shown in Fig. 5a and b. It has long been known that some sulfane sulfur species exist endogenously in biological systems,[35–37] but in general, their occurrence and function in cells and tissues remain unclear. Recently, Ida *et al.*[38] demonstrated that the enzymes cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) are capable of directly generating cysteine persulfide, Cys-

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SSH from cystine. Moreover some researchers explain that polysulfides with various numbers of sulfur atoms may be generated via oxidized H_2S[39,40] and it has also been shown that polysulfides induce Ca^{2+} influx by activating transient receptor potential (TRP)A1 channels in rat astrocytes much more efficiently than H_2S.[41] Thus, sulfane sulfur is a potentially important signaling/effector species, and much of the reported biological activity associated with H_2S may actually be due to sulfane sulfur. It has also been proposed that H_2S can be generated by degradation of persulfide, i.e., sulfane sulfur may be a precursor to biological H_2S in the presence of thiols.[38,42]

Development of Fluorescence Probes for Sulfane Sulfur

The increasing recognition of the importance of sulfane sulfur in biological systems has led to the development of fluorescence probes for sulfane sulfur. Hydropersulfide (R-SSH) has significantly different chemical properties from structurally related thiols (R-SH). The pK_a values of hydropersulfides are lower than that of H_2S, so hydropersulfides should be stronger and more reactive nucleophiles than thiols. In addition, sulfane sulfur is electrophilic, and can react with nucleophiles. Taking advantage of these properties, several off/on type fluorescence probes for sulfane sulfur have been designed and developed.

Fluorescence probes based on sulfane sulfur attachment to thiol. Chen *et al.*[43] reported the SSP series of fluorescence probes for sulfane sulfur (Fig. 6a). The design strategy for these probes is as follows. First, sulfane sulfur reacts with the sulfur atom of a thiol group of the probe, affording persulfide (R-SS^-). Then, intramolecular nucleophilic attack of persulfide (R-SS^-) on the ester moiety occurs, releasing the fluorophore (Fig. 6a). Thus, the probe shows a large fluorescence increase upon reaction with sulfane sulfur.

Fluorescence probes based on the nucleophilicity of H_2S_2/H_2S_n. Liu *et al.*[44] reported a fluorescence probe for hydrogen polysulfides (H_2S_n, n>1), which can be considered as oxidized forms of H_2S (Fig. 6b). In this molecular design, 2-fluoro-5-nitrobenzoate (the H_2S_2/H_2S_n recognition moiety) and fluorescein (the fluorophore) are linked together. As shown in Fig. 6b, nucleophilic aromatic substitution of H_2S_2/H_2S_n with the 2-fluoro-5-nitrobenzoate moiety of the probe affords the persulfide intermediate, and then intramolecular nucleophilic attack of the persulfide (R-SS^-) on the ester moiety releases the fluorophore. This probe can also react with biothiols to form thioether products, but these compounds do not undergo intramolecular cyclization, and therefore the probe shows high specificity for H_2S_n or end-free persulfide (^-S-S_n-S^-, n≥0). This approach can also be applied to other fluorophores: for example, Zeng *et al.*[45] reported a two-photon excited fluorescence probe for H_2S_2/H_2S_n, QS_n, using 2-benzothiazol-2-yl-quinolin-6-ol as a two-photon fluorophore and 2-fluoro-5-nitrobenzoate as a H_2S_2/H_2S_n recognition moiety (Fig. 6c). They successfully visualized both exogenous and endogenous H_2S_2/H_2S_n in living cells and zebrafish embryo. Moreover, Gao *et al.*[46] developed a near-infrared (NIR) fluorescence probe, Mito-ss, which has aza-BODIPY as the NIR fluorophore, 2-fluoro-5-nitrobenzoate as the H_2S_2/H_2S_n recognition moiety and the triphenylphosphonium group as a mitochondrial targeting moiety (Fig. 6c). Chen *et al.*[47] also reported a fluorescence probe, AP, based on an aziridine ring-opening reaction of H_2S_n (Fig. 6c).

Other Chemical Tools Including Enzyme Inhibitors and Labeling Reagents

Physiological H_2S is enzymatically produced by CSE, CBS and 3-mercaptopyruvate sulfurtransferase (3MST), and inhibitors of these enzymes have been widely used in biological studies. D,L-Propargylglycine (PAG) and β-cyano-L-alanine (BCA) are specific inhibitors of CSE, and aminooxyacetic acid (AOAA) inhibits both CSE and CBS (Fig. 7a).[48,49] However, these inhibitors do not have sufficiently high selectivity for the target enzyme, and no inhibitor for 3MST has been reported. So, selective and sensitive inhibitors for these enzymes are still required. Inhibitor screening assay based on fluorescence detection has great advantages in terms of speed and convenience, and can be applied to high-throughput screening (HTS) of large chemical libraries. Thorson *et al.*[50] performed CBS inhibitor screening of 1,900 chemical compounds by using 7-azide-4-methylcoumarin (AzMC) (Fig. 7b), and successfully identified several novel CBS inhibitors. Our group also performed HTS of a chemical library containing about 160,000 compounds for inhibitors of CSE and 3MST by using HSip-1.[51] In the assay, purified 3MST or CSE and the corresponding substrate [3-mercaptopyruvate (3-MP) or cysteine] were used, and HSip-1 reacted with H_2S released by the enzymatic reaction. Compounds that suppressed the fluorescence increase of HSip-1 in response to H_2S were selected as candidate inhibitors (Fig. 7c). After further examination of the selectivity of the hit compounds, we identified both 3MST- and CSE-selective inhibitors. We are currently investigating the possible utility of these inhibitors for biological studies.

In order to study the role of *S*-sulfhydration (SH→SSH) as an oxidative post-translational modification, Zhang *et al.*[52] reported a selective tag-switch method that can be used to label protein *S*-sulfhydrated residues. Since thiols and persulfides in proteins have similar reactivity, it is difficult to achieve selective detection of *S*-sulfhydrated residues among protein residues. They resolved this problem by using two reagents, one nucleophilic reagent and one reporter, which selectively label protein persulfides (protein Cys-SSH) in a two-step reaction as shown in Fig. 7d. The first step is to block both -SH and -SSH with methylsulfonyl benzothiazole (MSBT).[53] The corresponding adducts, -S-benzothiazole and -S-S-benzothiazole, have different reactivities towards nucleophiles; specifically, only the -S-S-benzothiazole moiety is highly reactive with carbon-based nucleophile, which was also developed by Zhang *et al.*[53] By using a carbon-based nucleophile conjugated with biotin, only protein persulfide residues can be labeled.

Biological Applications of Developed Chemical Tools for H_2S and Sulfane Sulfur

Next, we introduce some recent reports on the biological applications of these chemical tools. Ida *et al.* [38] used a fluorescence probe for sulfane sulfur, SSP2 (Fig. 6a), to study CBS and CSE activities inside cells, as well as a MS-based quantification of sulfane sulfur. They first used SSP2 to evaluate increased total sulfane sulfur level inside living A549 cells in which CSE/CBS was overexpressed, and then they assessed individual sulfane sulfur species in these cells by means of LC-MS/MS. Overexpression of CSE or CBS led to a dramatic increase of Cys–SSH and Cys–SSSH, and CBS knockdown caused a decrease of Cys–SSH. They proposed that facile sulfur atom transfers from low-molecular-weight species such as Cys–SSH to protein thiols result in protein Cys-*S*-polythiolation. In order to further examine this hypothesis in cells, they also used a tag-switch assay, in which protein Cys-SSH is selectively detected with a biotin-CN-labeled probe (a carbon-based nucleophile). They found that protein polysulfide residues were greatly increased after overexpression of CBS and CSE, and proteomic analysis identified several Cys-polythiolated proteins.

The role of sulfane sulfur in detoxification pathways is also a topic of increasing interest. For instance, Abiko *et al.*[54] reported that the electrophile methylmercury (MeHg) was metabolized to bismethylmercury sulfide $(MeHg)_2S$ by endogenous persulfides such as GSH persulfides and protein cysteine persulfides in biological systems. They used liver and heart cytosolic fractions prepared from WT and CSE-knockout mice, and detected $(MeHg)_2$ only in the fractions of WT mice. In another paper, they

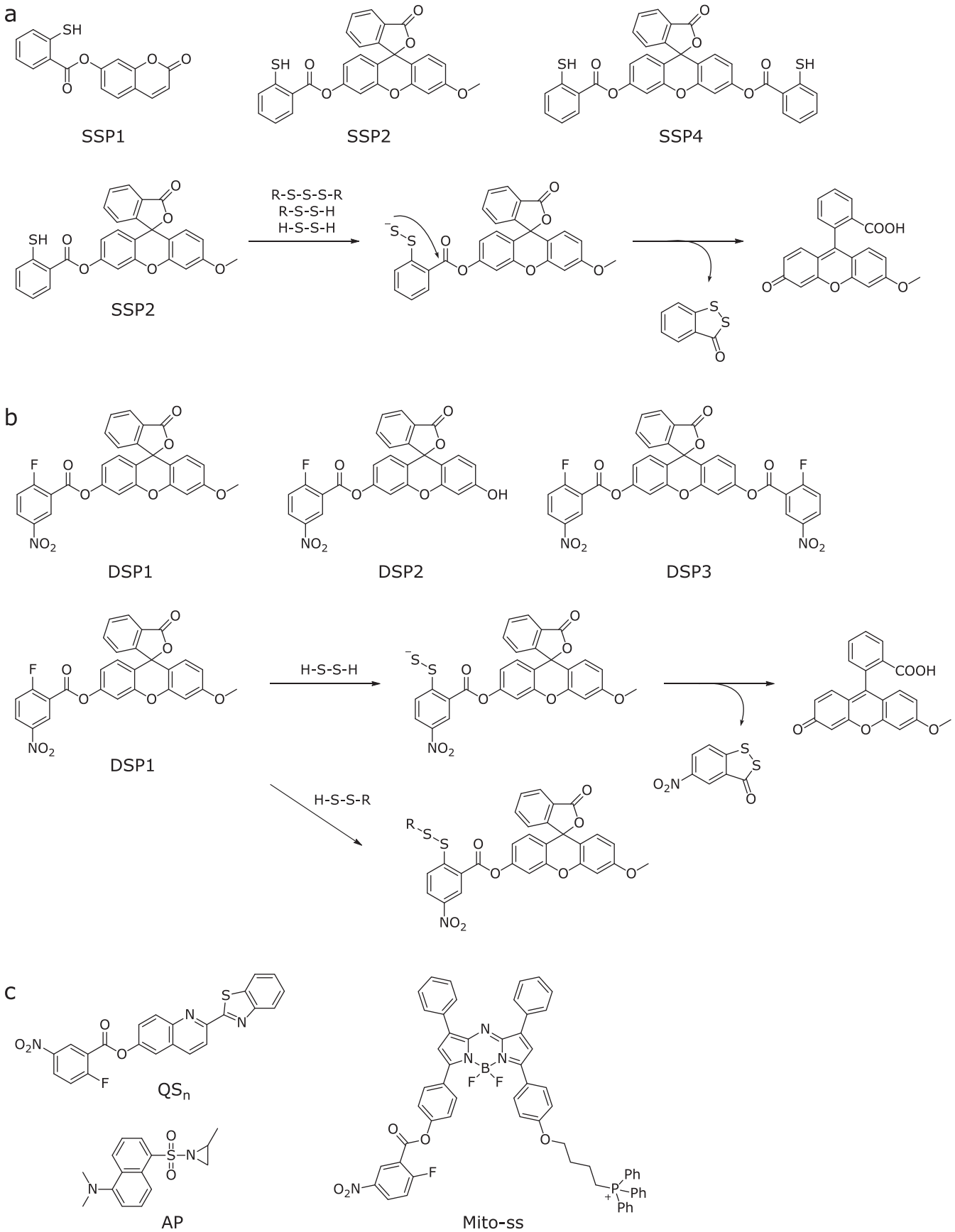

Fig. 6. Chemical structures and reaction mechanisms of (a) SSPs and (b) DSPs with sulfane sulfur and hydrogen persulfide. (c) Two-photon excitation and NIR fluorescence probes for hydrogen persulfide utilizing 2-fluoro-5-nitrobenzoate as the H_2S_2/H_2S_n recognition moiety, and a fluorescence probe for hydrogen persulfide utilizing an aziridine ring as a H_2S_2/H_2S_n recognition moiety.

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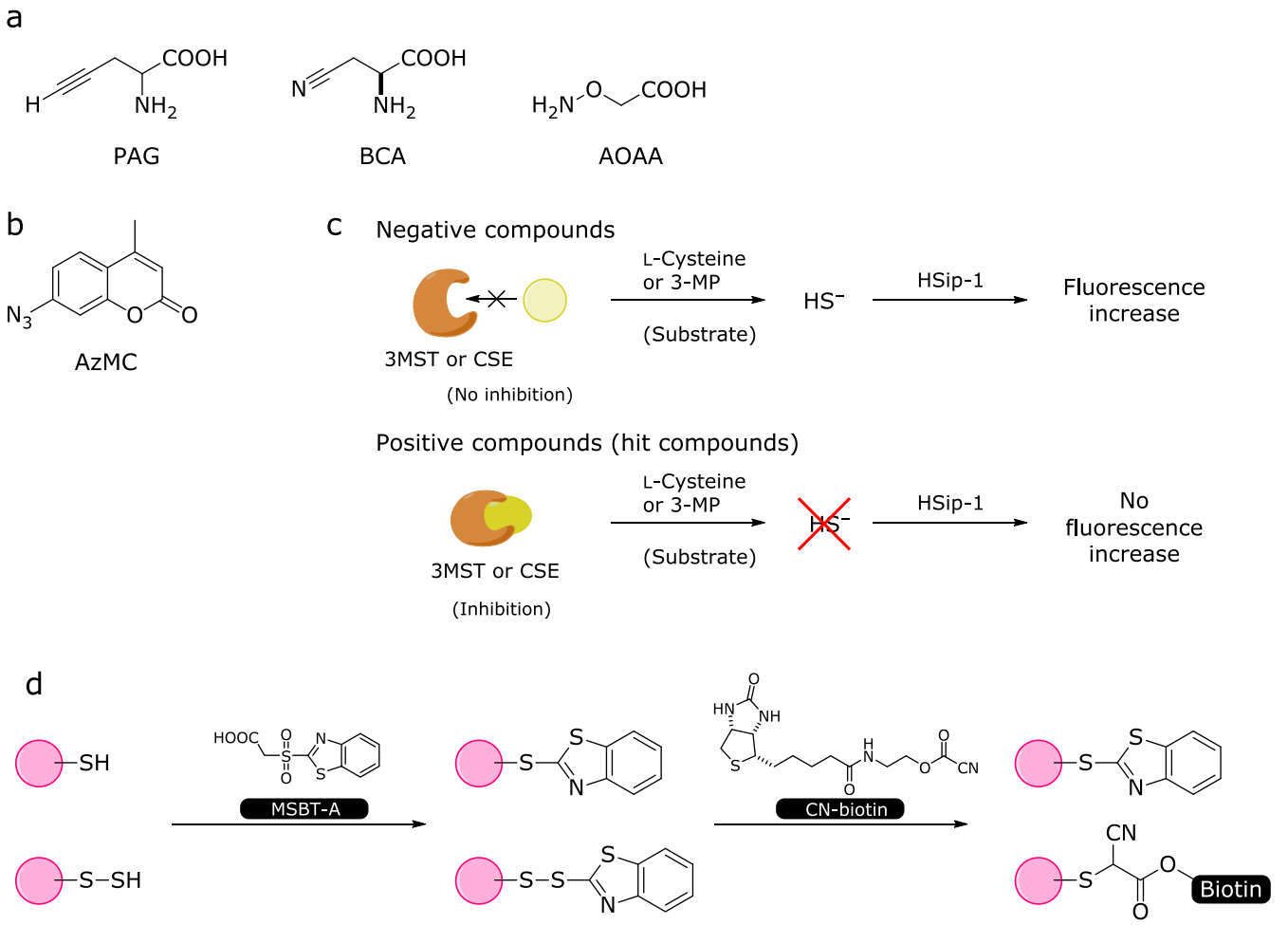

Fig. 7. (a) Chemical structures of inhibitors of CSE and CBS. (b) Chemical structure of AzMC. (c) Screening schemes for 3MST and CSE inhibitors. (d) Tag-switching technique for detecting protein Cys *S*-sulfhydration.

showed that the Keap1-Nrf2 pathway is activated by 1,2-dihydroxynaphthalene-4-sulfenic acid (1,2-NQH$_2$-SOH), generated by reaction of 1,2-NQ (1,2-naphthoquinone) with sulfane sulfur under oxidative stress, through the modification of Cys171 of Keap1.[55] Activation of the transcriptional factor Nrf2 causes up-regulation of downstream genes, such as glutamate-cysteine ligase and multidrug resistance-associated proteins (MRPs), facilitating the excretion of polar metabolites into the extracellular space. By using fluorescence probes, Marutani *et al.*[56] established that H_2S-donor compounds increased intracellular sulfane sulfur levels and had a cytoprotective effect. They designed unique hybrid molecules bearing a H_2S-releasing moiety as well as a *N*-methyl-D-aspartate receptor antagonist moiety. These hybrid molecules exhibited protective effects against oxygen and glucose deprivation-induced death of primary cortical neurons. They were also cytoprotective against cell death induced by 1-methyl-4-phenylpyridinium [MMP^+, a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)]. MPTP induces neurodegeneration of dopaminergic neurons in mammalian midbrain, which leads to Parkinson's disease-like symptoms.[57] To dissect the cytoprotective effect of the hybrid molecule, they separately assessed H_2S and sulfane sulfur levels in SH-SY5Y cells and in the culture medium without cells upon addition of the hybrid molecule by utilizing their specific fluorescence probes, HSip-1 and SSP4 (Fig. 3 and 6a). Interestingly, the results suggested that the cytoprotective effect of the hybrid molecule against MPP^+-induced toxicity may correlate with the ability to produce intracellular sulfane sulfur rather than H_2S, albeit this molecule releases H_2S but not sulfane sulfur. Although it remains unclear how such H_2S-releasing hybrid molecules affect the intracellular level of sulfane sulfur, compounds that increase intracellular sulfane sulfur levels may be potentially useful as neuroprotective agents to treat neurodegenerative diseases.

Conclusions and Prospects

Here, we have reviewed fluorescence probes for H_2S and sulfane sulfur based on a variety of design strategies, caged compounds of H_2S, inhibitors of CSE, CBS and 3MST, and labeling reagents for protein *S*-sulfhydrated residues, and their biological applications. It is well established that H_2S acts as a signaling and effector molecule, but its chemical mechanisms of action largely remain to be established. However, studies with recently developed fluorescence probes and detection methods for H_2S and sulfane sulfur have indicated that sulfane sulfur may be the actual signaling molecule mediating at least some of the physiological functions of H_2S. Further development and application of chemical tools for H_2S and sulfane sulfur studies, especially to detect endogenous H_2S or sulfane sulfur *in vivo*, are expected to throw light on the role of these mediators in the control mechanisms of various important physiological functions.

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