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New fluorescent probes for sulfane sulfurs and the application in bioimaging

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Abstract

A sulfane sulfur mediated benzodithiolone formation was developed. Based on this reaction, two fluorescent probes (**SSP1** and **SSP2**) for the detection of sulfane sulfur species (persulfide, polysulfide, and elemental sulfur) were prepared and evaluated. The probes showed high selectivity and sensitivity to sulfane sulfurs. Moreover, **SSP2** was successfully applied for bioimaging sulfane sulfurs in living cells.

Introduction

Reactive sulfur species (RSS) are a family of sulfurcontaining molecules found in biological systems. These molecules include thiols, S-modified cysteine adducts such as *S*-nitrosothiols and sulfenic acids, hydrogen sulfide, persulfide, polysulfide, as well as inorganic sulfur derivatives. RSS have attracted increasing attention in biomedical research because these molecules show a variety of physiological functions.[1-3] For example, hydrogen sulfide (H_2S) has been recently recognized as a new gaseous transmitter. The production of endogenous H_2S and exogenous administration of H_2S have been demonstrated to exert protective effects in many pathologies.[4-6] Sulfane sulfur compounds are another type of important RSS.[7-10] Sulfane sulfur refers to sulfur atom with six valence electrons but no charge (represented as S^0). Biologically important sulfane sulfur compounds include persulfides (R-S-SH), hydrogen persulfide (H_2S_2), polysulfides (R-S-S_n-S-R), and protein-bound elemental sulfur (S_8). Sulfane sulfur has unique reactivity to attach reversibly to other sulfur atoms and exhibit regulatory effects in diverse biological systems. These functions include posttranscriptional modification of transfer RNA, synthesis of sulfur-containing cofactors and vitamins, activation or inhibition of enzymes.[11] H_2S and sulfane sulfur always coexist and recent work suggests that sulfane sulfur species, derived from H_2S, may be the actual signaling molecules.[11-14]

Despite the rising interest in sulfane sulfur research, many fundamental questions regarding their production and mechanism of actions remain to be clarified. It is important, therefore, to understand the chemistry and properties of sulfane sulfur species. Accurate and reliable measurement of sulfane sulfur concentrations in biological samples is needed and can provide useful information to understand their functions. Currently the only method for sulfane sulfur detection is based on the reaction with cyanide ion to form thiocyanate, which can then be measured as ferric thiocyanate.[15] However this method requires post-mortem processing and destruction of tissues or cell lysates. Therefore it cannot be applied in real-time detection in biological samples. Fluorescence assays could be very useful in this field due to the high sensitivity and convenience. Although much progress have been made in the

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development of fluorescent probes for H_2S[16-18] and biological thiols,[19-21] fluorescent probes for sulfane sulfur detection are still unavailable. Here we report a first reaction-based fluorescent turn-on strategy for the detection of sulfane sulfurs.

Results and discussion

Sulfane sulfur compounds are reactive and labile. It is known that thiosulfoxide tautomers commonly exist.[22] As illustrated in Scheme 1, this property provides highly reactive intermediates such as **I** in sulfane sulfur species from which the sulfur atom can be readily removed by nucleophilc groups such as cyanide ion (CN^-). We envisioned that this reactivity could be used in the design of reaction based fluorescent probes for sulfane sulfurs. As shown in Scheme 1, sulfane sulfurs are likely to react with the nucleophilc component of a fluorescent probe like **A** to form a SH containing intermediate **A1**. If a *pseudo*-fluorescent group (like the ester shown in **A1**) is present as a potential electrophile at suitable position, the SH may undergo a spontaneous and fast cyclization to release the fluorophore and form product **B**. This strategy will allow us to visualize sulfane sulfur-related signal via convenient, sensitive, and non-destructive fluorescence measurement. We expected that substrate **A** should not react with other reactive sulfur species such as H_2S, cysteine, glutathione, etc., and therefore, the fluorescent signal should be selective only for sulfane sulfurs.

To prove this hypothesis, we prepared two probes (**SSP1** and **SSP2**, Scheme 2). Their synthesis and structure characterization were provided in experimental section and the supporting information. The fluorescence property of these probes was tested in aqueous PBS buffer solution (pH 7.4). We expected that the SH group would act as the nucleophile to trap the reactive sulfur atoms in sulfane sulfurs. The resulted intermediates, i.e. –S-SH adducts, should undergo a fast intramolecular cyclization[23] to release strong fluorescent molecules (7-hydroxylcoumarin and fluorescein), as well as benzodithiolone **1**.

With these two probes in hand, we first tested their fluorescence properties. Both compounds showed low fluorescence quantum yields ($\Phi = 0.06$ for **SSP1**; $\Phi = 0.05$ for **SSP2**) and exhibited no absorption features in the visible region. These compounds are quite stable as no considerable change in fluorescence spectrum was observed after they were stored at 4 °C for two weeks. And then we investigated their fluorescence responses to a model sulfane sulfur compound hydrogen persulfide (H_2S_2), using Na_2S_2 as the equivalent. As shown in Fig. 1, the fluorescence intensity of both probes increased dramatically (25 folds for **SSP1** and 50 folds for **SSP2**) if H_2S_2 was presented in the solution. In addition, the maximum intensity was reached in 5 min, which suggested the fluorescence turn-on reaction was fast. We also studied the effects of pH. Both probes worked effectively under neutral to weak basic pHs (7~8) (Figure S1 in supporting information). We observed small amount of hydrolysis of **SSP1** above pH 8 while **SSP2** was stable below pH 9.

To demonstrate the efficiency of the probes in determining sulfane sulfur concentration, varying concentrations of Na_2S_2 (0 ~ 50 μM) were tested with **SSP1** or **SSP2** (5 μM). For the purpose of reproducibility, a reaction time of 10 min was employed in these experiments. The fluorescence intensity increased linearly with the concentrations of Na_2S_2 changed up to 25 μM, and, thereafter, reached a steady state (Fig. 2). The detection limits[24] were found to be 73 nM (for **SSP1**) and 32 nM (for **SSP2**) suggesting the probes were highly sensitive and could be suitable for detecting sulfane sulfurs in living systems (Figure S2 in supporting information).

We next examined the selectivity of the probes for sulfane sulfurs over other reactive sulfur species (Fig. 3). Three representative sulfane sulfur compounds, i.e. H_2S_2, cysteine

polysulfide (we used a 1:1 mixture of cysteine-trisulfide and tetrasulfide), and elemental sulfur (S_8) were tested. All these sulfane sulfurs (at 25 μM) showed excellent fluorescence responses towards the probes (26 ~ 33 folds for **SSP1** and 50 ~ 60 folds for **SSP2**). In contrast, other biologically relevant sulfur species, including cysteine (Cys), glutathione (GSH), homocysteine (Hcy), oxidized glutathione (GSSG), H_2S (using Na_2S as the equivalent), thiosulfate, sulfite, and sulfate, did not show significant fluorescence enhancement even under much higher concentrations (up to mM). In addition, aldehyde species, which are potential electrophiles, did not show responses at biologically relevant concentrations. These results demonstrated good selectivity of the probes for sulfane sulfurs.

Since persulfide, polysulfide, and elemental sulfur showed good reactivities toward the probes, we conducted a model reaction to explore the fluorescence turn-on mechanism (Scheme 3). As such, probe model compound **2** was treated separately with persulfide, polysulfide, and S_8 in a 1:1 CH_3CN and PBS buffer mixtures. As expected, benzodithiolone **1** and phenol were isolated with good yields in all three reactions. These results confirmed the intramolecular cyclization mechanism proposed in Scheme 1.

As **SSP2** was identified as a better probe than **SSP1**, due to higher sensitivity and lower background fluorescence, this probe was selected for further evaluation. We investigated the specificity of **SSP2** for sulfane sulfurs in the presence of other RSS or aldehydes (i.e. interference experiments). We found that **SSP2** could give significant fluorescence enhancement for sulfane sulfurs even in the presence of other sulfur species or aldehydes (Figure S3 in supporting information). These results suggested that **SSP2** has the potential to be used for monitoring sulfane sulfur in complicate biological systems.

Finally we tested **SSP2** in imaging sulfane sulfurs in cells. Both H9c2 and HeLa cells were used in this study. Briefly, cells were incubated with compound **SSP2** for 20 min and then washed with PBS buffer. We did not observe significant fluorescent cells (Fig. 4a-d). However, strong fluorescence in the cells was observed after treatment with Na_2S_2 for 30 min. Cells treated with 100 μM Na_2S_2 showed obviously stronger fluorescence than cells treated with 50 μM Na_2S_2. Thus we conclude that probe **SSP2** can be used for the detection of sulfane sulfurs in cultured cells.

Conclusions

In summary, we report in this study a sulfane sulfurmediated benzodithiolone formation under mild conditions. This reaction proved to be selective for sulfane sulfur species including persulfide, polysulfide, and elemental sulfur. The reaction did not proceed with other biologically relevant sulfur species such as cysteine, glutathione (both reduced and oxidized forms), H_2S, thiosulfate, sulfite, and sulfate. Based on this reaction, two fluorescent probes **SSP1** and **SSP2** were developed for the detection of sulfane sulfurs. The efficiency of these probes was demonstrated in aqueous buffers and in cell imaging. Using this strategy, the concentration of sulfane sulfur can not only be measured by the fluorescence signal, but also be assessed from the analysis of the benzodithiolone product. To the best of our knowledge, this is the first example of fluorescent probes for the detection of sulfane sulfurs. We are now actively exploring more sensitive and responsive analogues for fluorescence imaging of sulfane sulfurs in living cells, tissues and animals. We are also utilizing these probes to study sulfane sulfur's contributions to physiological and pathological processes.

Experimental section

General methods and materials

All solvents were reagent grade. Reactions were magnetically stirred and monitored by thin layer chromatography (TLC) with 0.25 mm pre-coated silica gel plates. Flash chromatography was performed with silica gel 60 (particle size 0.040-0.062mm). Yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. Proton and carbon-13 NMR spectra were recorded on a 300 MHz spectrometer. Chemical shifts are reported relative to chloroform (δ 7.26) for ^{1}H NMR and chloroform (δ 77.0) for ^{13}C NMR. Absorption spectra were recorded on a Lambda 20 UV/VIS spectophotometer using 1 cm quartz cells. Fluorescence excitation and emission spectra were measured on Cary Eclipse fluorescence spectrophotometer.

Chemical synthesis

SSP2: to a solution of the S-pyridium disulfide precursor[23] (110.0 mg, 0.186 mmol) in THF/H_2O (6.0 mL/3.0 mL) was added PPh_3 (121.9 mg, 0.47 mmol) slowly at 0 °C. The mixture was allowed to warmed to r.t. and stirred for 0.5 h. THF was removed under reduced pressure and 10 mL of HCl (1N) was added to acidify the solution. Then the mixture was extracted with CH_2Cl_2 (20 mL). The organic layer was seperated and washed with brine. After dried by $MgSO_4$, the solvent was removed under reduced pressure and the resulted residue was purified by falsh column chromatography. **SSP2** was obtained as a white solid (72.6 mg, 81 % yield). ^{1}H NMR (300 MHz, CD3Cl) δ 3.89 (s, 3H), 4.62 (s, 1 H), 6.61-6.91 (m, 5 H), 7.40-7.71 (m, 5 H), 7.66 (m, 2 H), 8.03 (dd, *J* = 6.3, 0.9 Hz, 1 H), 8.24 (d, *J* = 7.8 Hz, 1 H) ; ^{13}C NMR (75 MHz, CD_3Cl) δ 169.6, 164.9, 161.7, 153.3, 152.5, 152.2, 152.0, 140.0, 135.5, 133.7, 132.5, 131.4, 130.1, 129.4, 129.3, 126.7, 125.3, 125.2, 124.7, 124.3, 117.8, 117.2, 112.2, 111.1, 110.8, 101.1, 82.7, 55.8; MS (ESI^+) m/z 505.0 ($M+Na^+$); IR 3063, 2945, 2551, 1761, 1610, 1496, 1462, 1418. mp 115-116 °C.

SSP1 was prepared using the same method as for **SSP2**. ^{1}H NMR (300 MHz, CD_3Cl) δ 4.61 (s, 1H), 6.44 (d, *J* = 9.6 Hz, 1 H), 7.17-7.28 (m, 3 H), 7.41 (m, 2 H), 7.56 (d, *J* = 8.4 Hz, 1 H), 7.73 (d, *J* = 9.6 Hz, 1 H), 8.26 (d, *J* = 8.4, 1 H). ^{13}C NMR (75 MHz, CD_3Cl) δ 164.4, 160.2, 154.6, 153.0, 142.8, 139.9, 133.5, 132.2, 131.2, 128.6, 124.9, 124.2, 118.6, 116.8, 116.1, 110.6; MS (ESI^+) m/z 321.0 ($M+Na^+$); IR 3094, 2922, 2530, 1731, 1618, 1583, 1461, 1395. mp 141-142 °C.

Cell culture and fluorescence imaging

H9c2 cells and HeLa cells were grown on glass-bottom culture dishes (Corning Inc.) in DMEM supplemented with 10% (v/v) FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C under a humidified atmosphere containing 5% CO_2. Before use, the adherent cells were washed one time with FBS-free DMEM. For intracellular H_2S_2 imaging, the cells were incubated with 50 μM **SSP2** in FBS-free DMEM (containing 200 μM CTAB) at 37 °C for 20 min. After removal of excess probe and washed with PBS (pH 7.4), the cells were incubated with 50 or 100 μM Na_2S_2 for 30 min in PBS buffer (pH 7.4, containing 500 μM CTAB). Cell imaging was carried out after washing the cells three times with PBS (pH 7.4). Images were taken on an EVOS fl fluorescence microscope from Advanced Microscopy Group (AMG).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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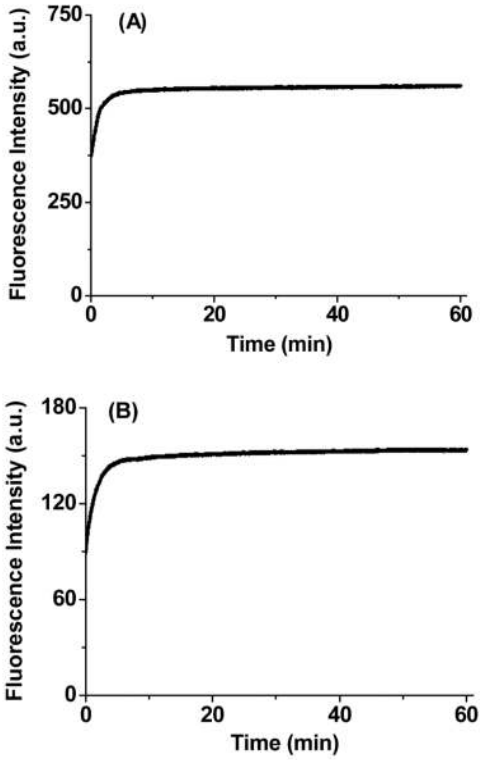


Fig. 1.

Time-dependent responses of the probes (5.0 μM) to Na_2S_2 (25 μM) in 50 mM PBS buffer (pH 7.4) with 1 mM CTAB at room temperature. Data were acquired at 458 nm and excited at 380 nm for **SSP1** (A); at 518 nm and excited at 482 nm for **SSP2** (B).

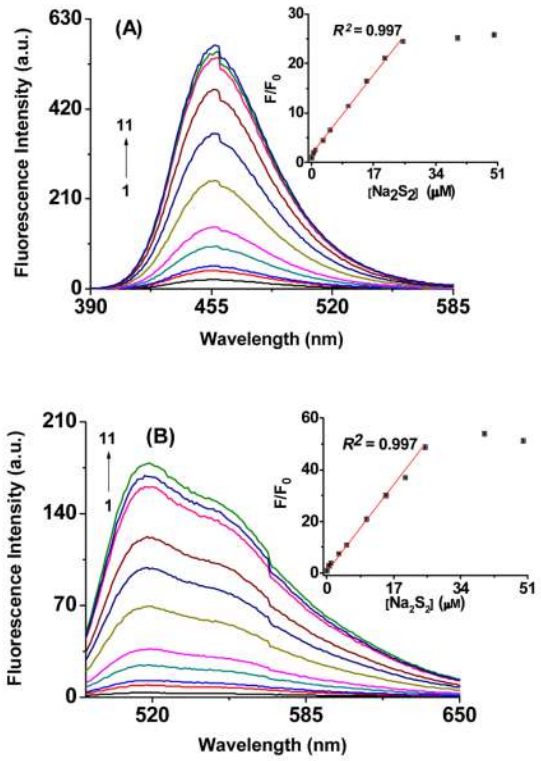


Fig. 2.

Fluorescence emission spectra of the probes (5 μM) with varied concentrations of Na_2S_2 (0, 0.5, 1, 3, 5, 10, 15, 20, 25, 40, 50 μM for curves 1-11, respectively). (A) **SSP1**, λ_{ex} = 380 nm. (B) **SSP2**, λ_{ex} = 482 nm. The reactions were carried out for 10 min at room temperature in PBS buffer (50 mM, pH 7.4) with 1 mM CTAB.

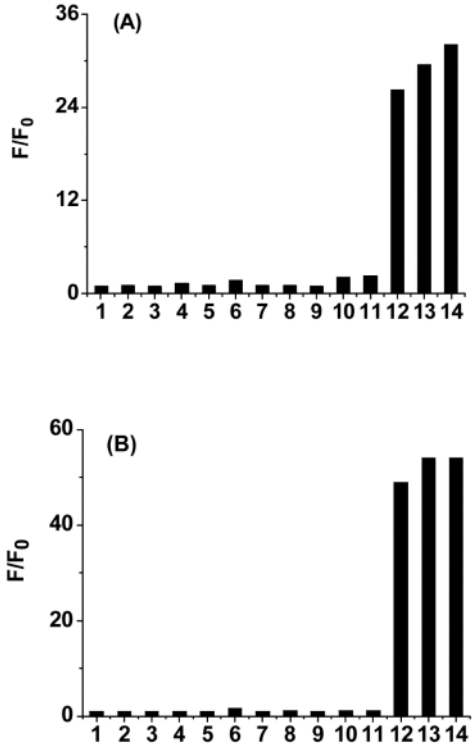


Fig. 3.

Fluorescence enhancement (F/F_0) of 5.0 μM probes in the presence of various RSS or aldehydes. (A) data of **SSP1** (Ex/Em = 380/458 nm). (B) data of **SSP2** (Ex/Em = 482/518 nm). The reactions were carried out for 10 min at room temperature in PBS buffer (50 mM, pH 7.4) with 1 mM CTAB. (1) probe alone; (2) 1 mM Cys; (3) 1 mM GSH; (4) 1 mM Hcy; (5) 100 μM GSSG; (6) 100 μM Na_2S; (7) 100 μM $Na_2S_2O_3$; (8) 100 μM Na_2SO_3; (9) 100 μM Na_2SO_4; (10) 100 μM formaldehyde; (11) 100 μM acetaldehyde; (12) 25 μM Na_2S_2; (13) 25 μM Cys-polysulfide; (14) 25 μM S_8.

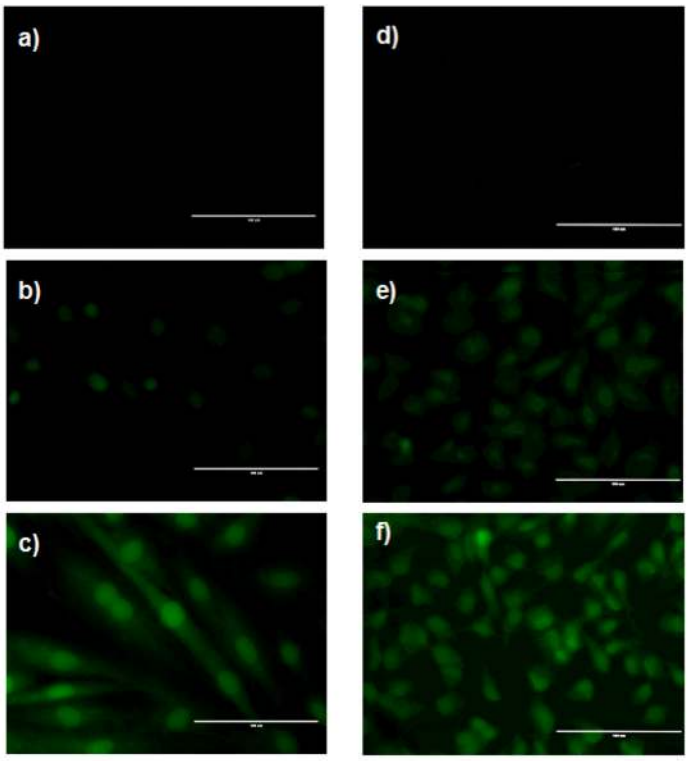
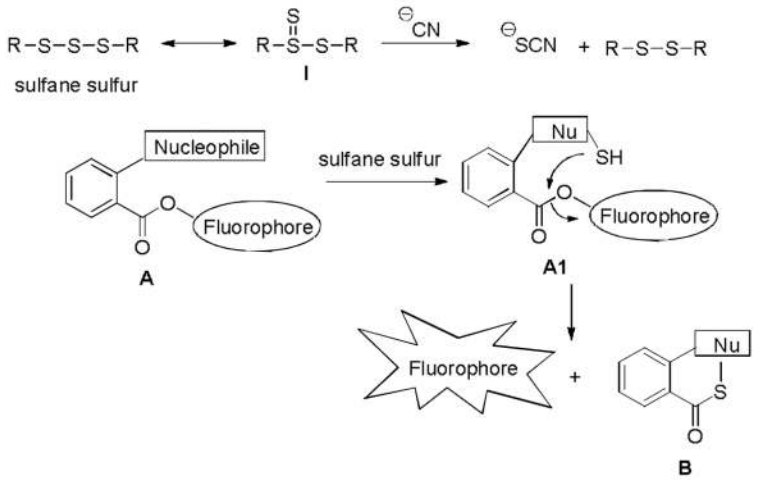
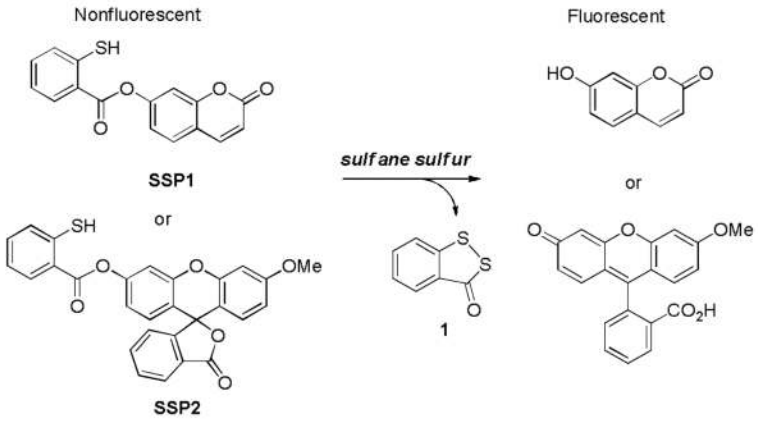


Fig. 4.

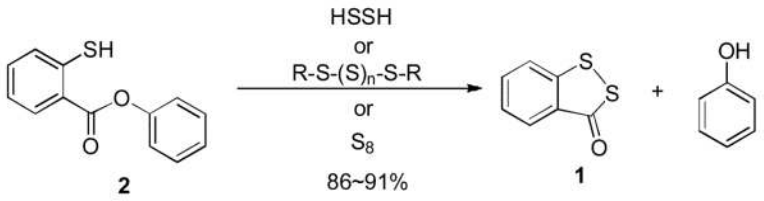
Fluorescence images of H_2S_2 in H9c2 cells (a, b, c) and HeLa cells (d, e, f). Cells on glass coverslips were incubated with **SSP2** (50 μM) for 20 min, then washed and subjected to different treatments. (a & d) controls (no Na_2S_2 was added); (b & e) treated with 50 μM Na_2S_2; (c & f) treated with 100 μM Na_2S_2.



Scheme 1.
Idea of the reaction-based fluorescent probes for sulfane sulfur detection.



Scheme 2.
Structures and proposed reactions of the probes.



Scheme 3.
Model reactions of the probe with sulfane sulfurs.