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Capture and Visualization of Hydrogen Sulfide via A Fluorescent Probe**

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Hydrogen sulfide (H₂S) has been known as a toxic pollutant for years. However, this molecule has been recently recognized as the third gaseous transmitter (the other two are nitric oxide and carbon monoxide).^[1–3] The production of H₂S in mammalian systems has been attributed to at least three endogenous enzymes:^[4–7] cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfur-transferase (MPST). These enzymes use cysteine or cysteine derivatives as substrates and convert them into H₂S within different organs and tissues. In addition to these enzymatic pathways, there are also a range of comparably simple chemical events which may liberate H₂S from the intracellular pool of 'labile' sulfur, for instance from the 'sulfane sulfur' pool (compounds containing sulfur atoms bound only to other sulfur atoms).^[8] The production of endogenous H₂S and exogenous administration of H₂S have been demonstrated to exert protective effects in many pathologies. For example, H₂S has been shown to relax vascular smooth muscle, induce vasodilation of isolated blood vessels, and reduce blood pressure. H₂S can also inhibit leukocyte adherence in mesenteric microcirculation during vascular inflammation in rats, suggesting H₂S is a potent anti-inflammatory molecule. Additionally, it has become evident that H₂S is a potent antioxidant and, under chronic conditions, can up-regulate antioxidant defense. Despite the rising interest in H₂S research, fundamental questions regarding regulation of its production, its mechanism of action, and its destruction remain. A critical debate in the field involves the biologically relevant levels of H₂S as current reports varying over 10⁵-fold concentration range.^[9–12] Obviously, accurate and reliable measurement of H₂S concentrations in biological samples is needed and can provide useful information to understand the function of H₂S. Currently the major methods for H₂S detection are colorimetric and electrochemical assays, gas chromatography, and sulfide precipitation.^[12–16] These methods often require complicate sample processing. Given the high reactivity of H₂S, these methods can yield variable results.^[9–12] Fluorescence based assays could be useful in this field due to the high sensitivity and convenience. However, fluorescence method for H₂S detection, especially for real-time detection in biological

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samples, is still very limited so far.^[17–19] Here, we report a reaction-based fluorescent turn-on strategy for the detection of H₂S.

We envisioned that H₂S is a reactive nucleophile in biological systems which can participate in nucleophilic substitution. In order to selectively detect H₂S, the key is to differentiate H₂S from other biological nucleophiles, especially thiols such as cysteine and glutathione. Theoretically, H₂S can be considered as a non-substituted thiol. It can undergo nucleophilic reaction two times, while other thiols like cysteine are mono-substituted thiols which can only undergo nucleophilic reaction one time. Based on this property, we expected that compounds containing bis-electrophilic centers could be useful reagents for H₂S detection. As shown in Scheme 1, H₂S should react with the most electrophilic component of a fluorescent probe like **A** to form a free SH containing intermediate **A1**. If another electrophile is presented at suitable position, like the ester group shown in **A1**, the SH group should undergo a spontaneous cyclization to release the fluorophore and form product **B**. This strategy not only can capture H₂S as a stable and analyzable product **B**, but also will allow us to visualize H₂S-related signal via convenient and sensitive fluorescence measurement. We envisioned that substrate **A** could also react with biological thiols like cysteine. However, the product **A2** should not undergo the cyclization to release the fluorophore. Therefore, the fluorescent signal should be selective only for H₂S.

With this idea in mind, we designed a reactive disulfide-containing probe (compound **1**). This compound was prepared from thiosalicylic acid **2** in two steps using the procedure shown in Scheme 2. The fluorescence property of this probe was tested in aqueous PBS buffer solution (pH 7.4). Compound **1** (fluorescence quantum yield: $\Phi = 0.003$) adopted a closed lactone conformation and exhibited no absorption features in the visible region (supporting information). We found that probe **1** reacted rapidly with H₂S to generate fluorophore **6** ($\Phi = 0.392$) and benzodithiolone **7** in good yields (Scheme 3). In these experiments, NaHS was used as the equivalent of H₂S. It is known that in aqueous state under the physiological pH of 7.4, the major form of H₂S exists as HS⁻; the ratio of HS⁻/H₂S is ~3:1.^[9]

As shown in Figure 1, the reaction of **1** with H₂S yielded significant fluorescence signal. Control experiments using cysteine or glutathione did not lead to any fluorescence increase. As expected, when H₂S and thiols like GSH co-existed, we still observed strong fluorescence. These results demonstrated that **1** was a selective fluorescent probe for H₂S.

The turn-on responses of **1** to H₂S and other biological thiols were also measured by a spectrofluorometer. As indicated in Figure 2, the fluorescence intensity of **1** increased dramatically (50~60 fold) if H₂S was presented in the solution (even when H₂S and other thiols were presented together). In addition, the maximum intensity was reached in 1 hour, which suggested the reaction was fast.

To demonstrate the efficiency of probe **1** in the measurement of H₂S concentration, **1** was treated with H₂S under a series of different concentrations in order to obtain a standard curve of emission intensity versus H₂S concentration. The concentration of compound **1** was maintained at 100 μ M, while the concentrations of NaHS varied from 0 to 10 μ M. As shown in figure 3, the fluorescent signal was indeed linearly related to the concentration of NaHS in such concentration range. These results demonstrated that probe **1** could detect H₂S both qualitatively and quantitatively.

Next, we used plasma to investigate the potential of probe **1** for use in the detection of H₂S in complex systems. Bovine plasma containing NaHS at different concentrations (0, 50, 100, and 500 μ M) were prepared first. These concentrations were within the range of those which

have been used to elicit physiological responses of H₂S (10–600 μM).^[19–22] These plasma solutions were then diluted and incubated with probe **1**. After 1 hour, the mixture was diluted again with PBS buffer and fluorescence signals were measured. As expected, strong fluorescence was observed in plasma solutions in the presence of NaHS (Figure 4). We noticed that the fluorescence intensity response to certain H₂S concentration obtained in plasma was lower than the signal obtained in pure buffer solutions. This is likely due to the fact that H₂S can be quickly scavenged by proteins present in plasma.^[18] Nevertheless we conclude that probe **1** can be used for the selective detection of H₂S in complex biological systems like plasma.

We also used cultured COS7 cells to investigate the potential of **1** for use in the detection of H₂S in cells. As shown in Figure 5, COS7 cells were incubated with compound **1** (100 μM) for 30 min and we did not observe any fluorescent cells. Strong fluorescence in the cells was induced after treatment with sodium sulfide (250 μM). Thus we conclude that probe **1** can be used for the detection of H₂S in cultured cells.

In summary, we reported in this study a H₂S-mediated benzodithiolone formation under mild conditions. This reaction proved to be selective for H₂S and it did not proceed with other biological thiols such as cysteine and glutathione. Based on this reaction, a fluorescent probe, i.e. compound **1**, was developed for the detection of H₂S. The efficiency of this probe was demonstrated in aqueous buffers and plasma, as well as in cells. Using this strategy, the concentration of H₂S can not only be measured by the fluorescence signal, but also be assessed from the analysis of the benzodithiolone product. We are now actively pursuing more specific H₂S fluorescent probes based on this new benzodithiolone formation and related reactions.

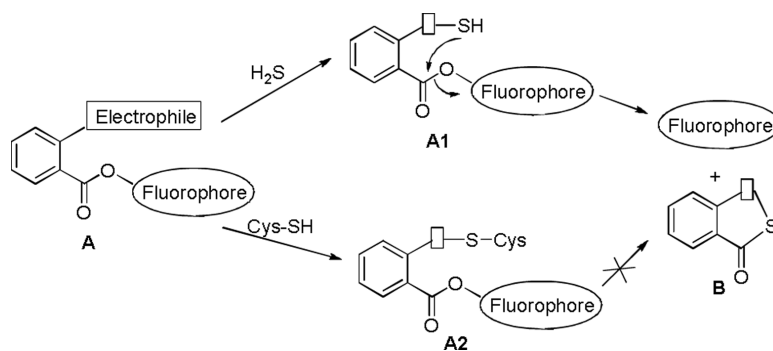
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

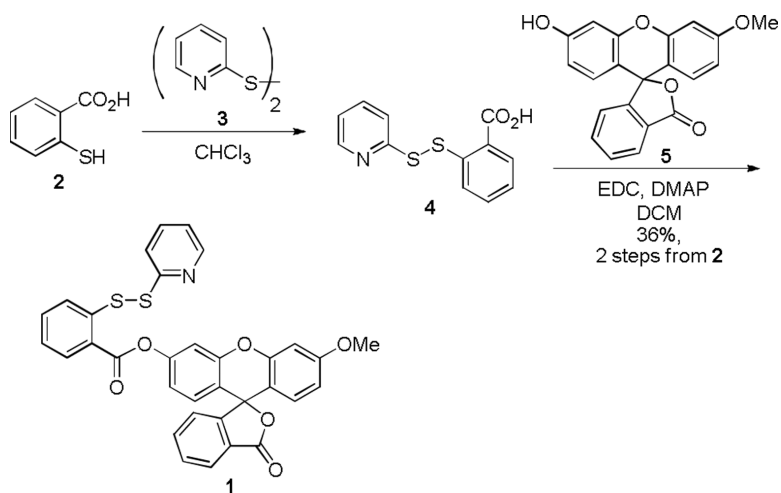
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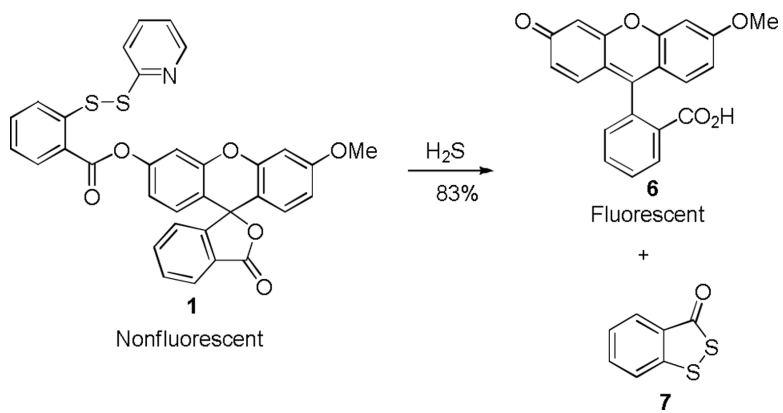
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Scheme 1.
Proposed fluorescent turn-on strategy



Scheme 2.
Synthesis of fluorescent probe **1**



Scheme 3.
Fluorescent probes and reaction with H₂S

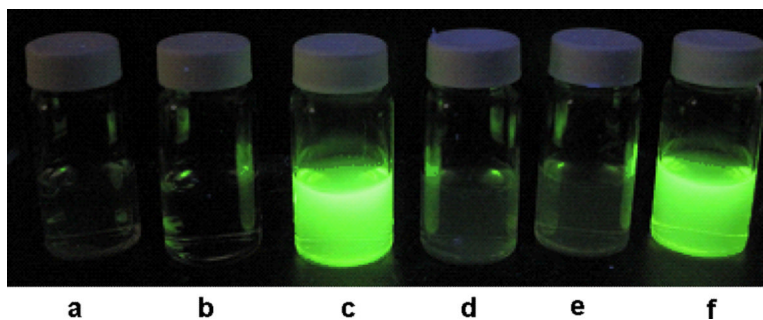


Figure 1

. Fluorescent images of probe **1**: a) **1** only (100 μM), b) NaHS only (50 μM), c) **1** (100 μM) + NaHS (50 μM); d) **1** (100 μM) + cysteine (50 μM); e) **1** (100 μM) + glutathione (50 μM), f) **1** (100 μM) + glutathione (50 μM) + NaHS (50 μM), in a mixture of PBS buffer (pH 7.4 mM) and CH_3CN (9:1).

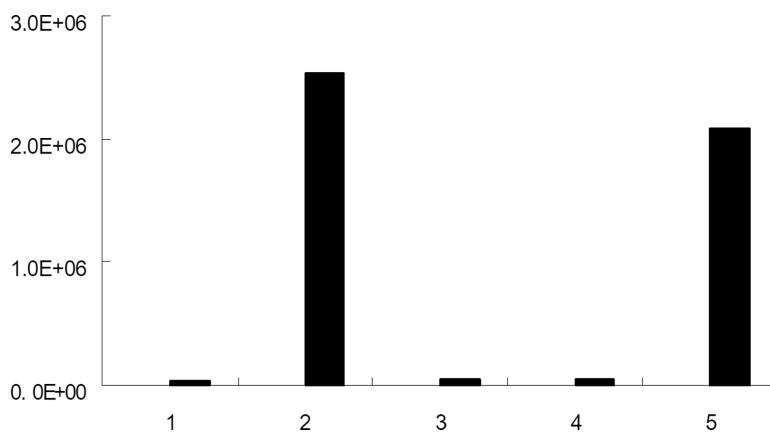


Figure 2. Fluorescence response of probe **1** toward H₂S and other thiols. 1) **1** only (100 μM), 2) **1** (100 μM) + NaHS (50 μM); 3) **1** (100 μM) + cysteine (50 μM); 4) **1** (100 μM) + glutathione (50 μM), 5) **1** (100 μM) + glutathione (50 μM) + NaHS (50 μM); measured in a mixture of PBS buffer (pH 7.4) and CH₃CN (9:1), λ_{ex} 465 nm, 25 °C.

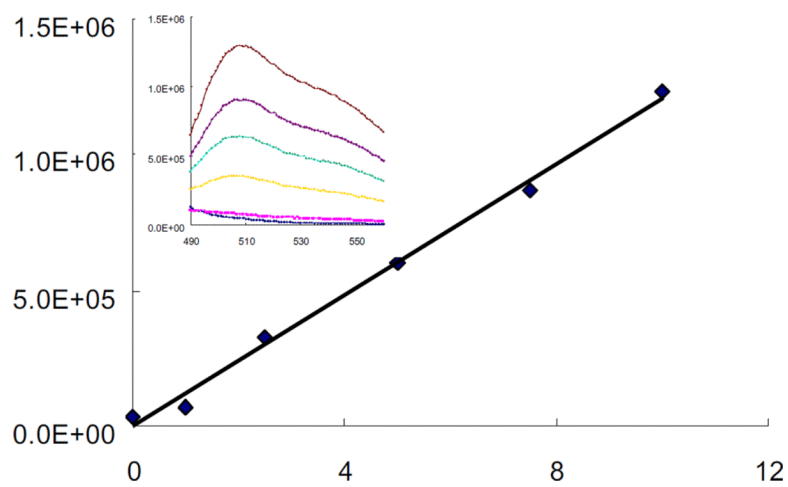


Figure 3. Linear correlation of fluorescent intensity toward H₂S concentration. NaHS concentration: 0, 1, 2.5, 5, 7.5, 10 μM.

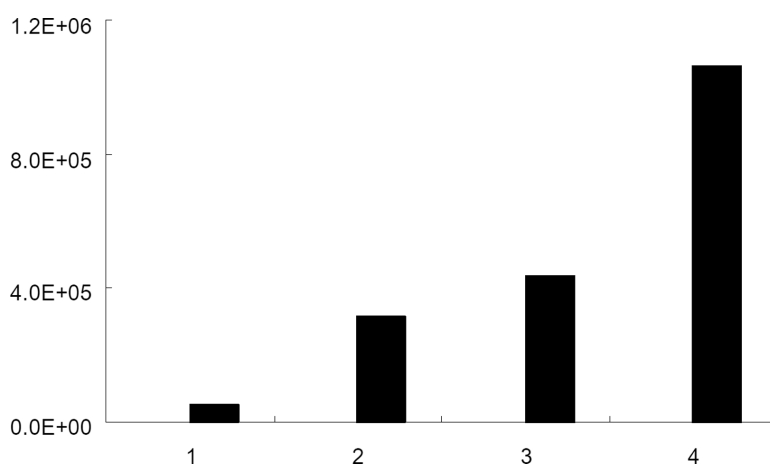


Figure 4. Fluorescence response of probe **1** to H₂S in plasma. 1) probe **1** only, 2) probe **1** + NaHS (50 μM^a, 2 μM^b), 3) probe **1** + NaHS (100 μM^a, 4 μM^b), 4) probe **1** + NaHS (500 μM^a, 21 μM^b). ^aoriginal concentration in plasma, ^bdiluted concentration when fluorescence was recorded.

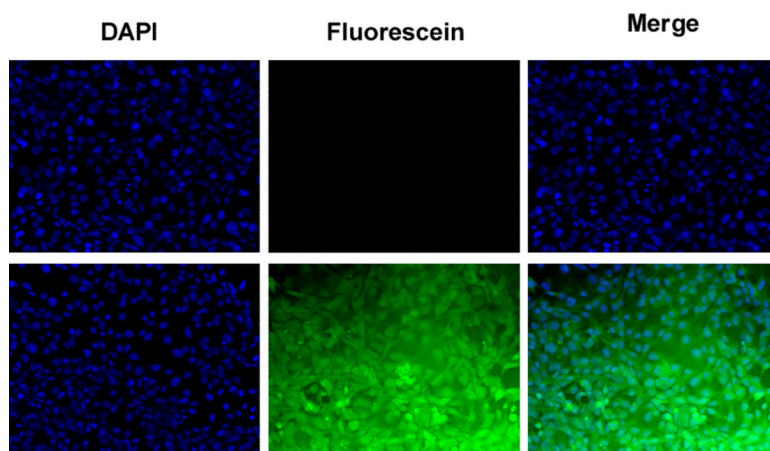


Figure 5. Fluorescence images of H₂S detection in COS7 cells using probe **1**. COS7 cells on glass coverslips were incubated with **1** (100 μM) for 30 min, and then subjected to different treatments. Top row was control (no sodium sulfide was added); bottom row was treated with sodium sulfide (250 μM).