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Reaction Based Fluorescent Probes for Hydrogen Sulfide

Chunrong Liu,[†] Bo Peng,[†] Sheng Li,[‡] Chung-Min Park,[†] A. Richard Whorton,[‡] and Ming Xian*[,†]

Department of Chemistry, Washington State University, Pullman, Washington 99164, United States, and Department of Pharmacology & Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710, United States

mxian@wsu.edu

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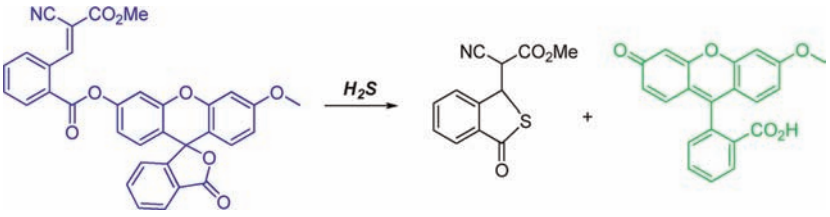
ABSTRACT



A reaction based fluorescence turn-on strategy for hydrogen sulfide (H_2S) was developed. This strategy was based on a H_2S-specific Michael addition−cyclization sequence. Other biological thiols such as cysteine and glutathione did not pursue the reaction and therefore did not turn on the fluorescence/consume the substrates. The probes showed good selectivity and sensitivity for hydrogen sulfide.

Hydrogen sulfide (H_2S) has traditionally been considered a highly toxic gas. However, recent studies have demonstrated that H_2S is an endogenously generated gaseous signaling molecule with very potent cytoprotective properties that are on par with those of two other well-known endogenous gaseotransmitters: nitric oxide (NO) and carbon monoxide (CO).[1] To date, hydrogen sulfide's exact mechanisms of action are still under active investigation. Some chemical and biochemical catabolic reactions of H_2S have been reported. For example, H_2S can react readily with methemoglobin to form sulfhemoglobin, which might act as the metabolic sink for H_2S. As a potential reductant, H_2S is likely to be consumed by endogenous oxidant species such as hydrogen peroxide, superoxide, peroxynitrite, etc. H_2S can also cause protein *S*-sulfhydration (i.e., to form −S−SH).[2] This process is potentially significant because it provides a possible mechanism by which H_2S alters the functions of a wide range of cellular proteins and enzymes. It is likely that many more important reactions of H_2S are to be discovered. Nevertheless, the production of endogenous H_2S and the exogenous administration of H_2S elicit a wide range of protective actions including vasodilation, anti-inflammatory, antioxidant, antiapoptotic, and down regulation of cellular metabolism during stressful states.[1] These results strongly suggest that modulation of H_2S levels could have potential therapeutic values for a number of diseases. It is important, therefore, to understand the chemistry and properties of H_2S and to appreciate the limitation and errors that may be generated when measuring H_2S in biological samples.

Currently a critical debate in the field is about the biologically relevant concentrations of H_2S as reports vary over a 10^5-fold range.[3] Traditional H_2S detection methods

[†] Washington State University.
[‡] Duke University Medical Center.

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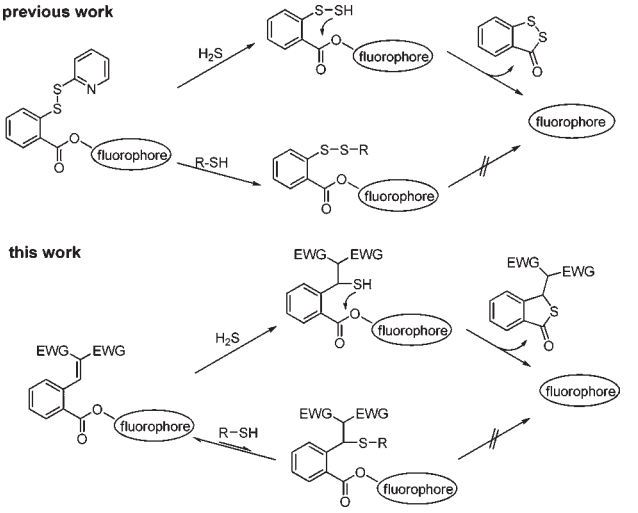
(colorimetric/electrochemical assays, gas chromatography, and sulfide precipitation)[4] often require complicated post-mortem processing and/or destruction of tissues or cell lysates. Given the high reactivity of H_2S in biological environments, these methods could yield inconsistent results. Fluorescence based assays could be useful in this field due to the high sensitivity and convenience. Fluorescence methods are suitable for nondestructive detection of biotargets in live cells or tissues with readily available instruments. Since 2011, several fluorescent probes which can potentially be used for H_2S detection in living systems have been reported.[5] The fluorescence turn-on strategies of these probes were based on several different H_2S-specific reactions.[5] Among these methods, the strategy developed by our group utilized the unique dual nucleophilicity of H_2S.[5b] In our first generation probes (Scheme 1), 2-pyridinyl disulfide was employed as the H_2S trap to initiate the tandem reaction to release the fluorophore. Such probes showed high selectivity for H_2S and potential applications in monitoring H_2S in living cells.[5b] However, the 2-pyridinyl disulfide could also react with biological thiols. Although the reaction will not lead to fluorescence turn-on, a relatively high probe loading may be necessary. To solve this problem, here we report a new generation of probes which only react with H_2S while not interfering with other thiols.

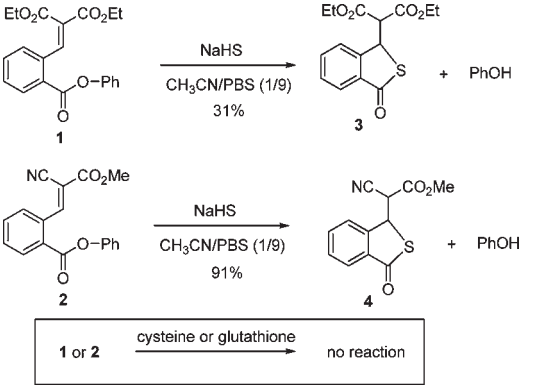
The design of the second generation probes is shown in Scheme 1. Experiments by Holmes et al. revealed that simple thiols reacted readily with some Michael acceptors at physiological pH, but the products could not be obtained or identified.[6] This is possibly due to the fact that the Michael addition was a rapid equilibrium process and no stable covalent product was formed. Based on Holmes' results, we envisioned that certain Michael acceptors might be useful to differentiate H_2S from biological thiols. It is also known that H_2S in aqueous solution has a pK_a of 7.0 while thiols have higher pK_a values of ~8.5. So, H_2S should be a better nucleophile than thiols in physiological media. If Michael acceptors are employed in the probes, they should be able to react with H_2S and promote the intramolecular cyclization to release the fluorophore. The reactions between Michael acceptors and biological thiols such as cysteine and glutathione, however, should be reversible and therefore should not consume the probes.

Scheme 1. Reaction-Based Fluorescence Turn-on Strategies



With this idea in mind, two model compounds **1** and **2** were prepared (see Supporting Information (SI) for the synthesis). When **1** was treated with H_2S (using NaHS as the equivalent) for 1 h at rt, the desired cyclization product **3** was obtained in 31% yield (Scheme 2). The remaining material was unreacted **1**. Compound **2** proved to be more reactive in this reaction. Under the same conditions, cyclization product **4** was obtained in 91% yield. As expected, when both compounds were treated with cysteine or glutathione, no Michael addition products were isolated. Only the starting materials were recovered. In addition, **1** and **2** showed no reaction toward primary amines and ammonia (see SI). These results confirmed our hypothesis that certain Michael acceptors can be used to selectively trap H_2S and they are not consumed by thiols.

Scheme 2. Model Reactions



We then prepared two Michael acceptor-based fluorescent probes (**5** and **6**, Figure 1) and tested their fluorescence

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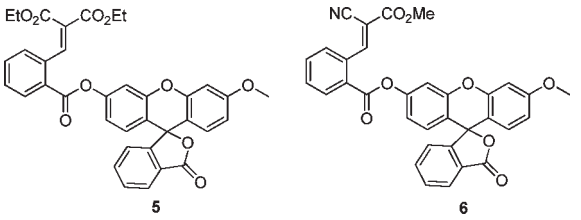

Figure 1. Structures of Michael acceptor-based fluorescent probes.

properties in aqueous buffers. Both compounds exhibited no absorption features in the visible region (fluorescence quantum yield of **5**: $\Phi = 0.0002$; **6**: $\Phi = 0.0008$). As shown in the images in Figure 2, the reactions of **5** and **6** with H_2S (using NaHS as the equivalent) yielded significant fluorescence signals. Control experiments using cysteine or glutathione did not lead to any fluorescence increase. As expected, when H_2S and thiols coexisted, we still observed a strong fluorescence change. In addition, we noticed that probe **6** apparently showed much better responses than probe **5**. This was consistent with the fact observed in Scheme 2 that the cyanoacrylate derivative had better reactivity with H_2S than the benzylidenemalonate derivative.

The turn-on responses of the probes were also measured by a spectrofluorometer. As shown in Figure 3, upon treatment of 5 μM probes **5** or **6** with 100 μM NaHS, significant increases in fluorescence intensity were observed. Within a 30 min reaction time under these conditions, the benzylidenemalonate probe **5** produced an 11-fold turn-on response. Again, the cyanoacrylate probe **6** proved to be more sensitive to H_2S, and it led to a 160-fold turn-on response (Figure 3). The analysis of reaction products confirmed the production of the corresponding fluorescein dye. When the probes were treated with abundant biologically relevant thiols, including 1 mM cysteine and 1 mM glutathione, no significant fluorescence increase was observed. Moreover, when H_2S (100 μM) and thiols (1 mM) coexisted, we still observed strong fluorescence enhancements using both probes. These results demonstrated that the turn-on responses of probes **5** and **6** were selective for H_2S and the probes could be used for the detection of H_2S in the presence of a high concentration of biological thiols.

To demonstrate the efficiency of the probes in the measurement of H_2S concentration, varying concentrations of NaHS (1−100 μM) were added to the solutions of **5** and **6** (5 μM). The fluorescence intensities were indeed linearly related to the concentrations of NaHS in such concentration ranges (Figures 4 and S2 in SI). The detection limit for H_2S using these probes was found to be ~1 μM.

We recognized that probes **5** and **6** contained ester groups which could potentially be hydrolyzed by cellular esterase. We then tested the stability of probes in the presence of esterase. As shown in Figure 5, the

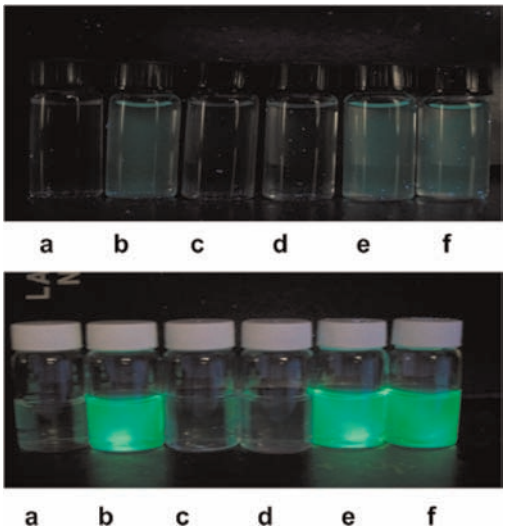

Figure 2. Fluorescence images of probe **5** (top row) and probe **6** (bottom row) in pH 7.4 phosphate buffer. (a) Probe only (5 μM); (b) probe + NaHS (100 μM); (c) probe + cysteine (1 mM); (d) probe + glutathione (1 mM), (e) probe + cysteine (1 mM) + NaHS (100 μM), (f) probe + glutathione (1 mM) + NaHS (100 μM).

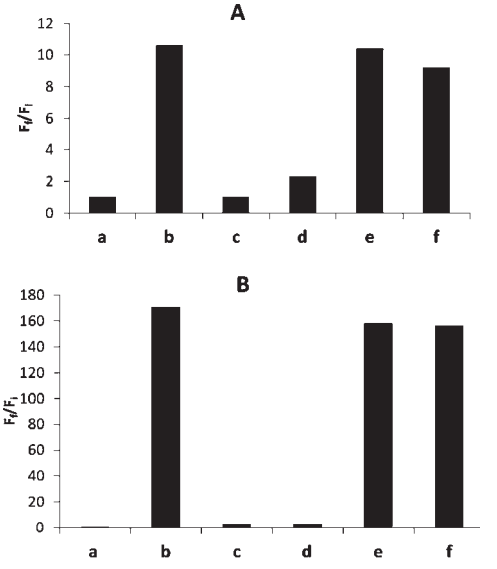

Figure 3. Fluorescence responses of (A) 5 μM probe **5** and (B) 5 μM probe **6** to H_2S and biologically relevant thiols: (a) probe only, (b) probe + NaHS (100 μM); (c) probe + cysteine (1 mM); (d) probe + glutathione (1 mM), (e) probe + cysteine (1 mM) + NaHS (100 μM), (f) probe + glutathione (1 mM) + NaHS (100 μM). Data were acquired in phosphate buffer (pH 7.4, 10 mM) with excitation at λ_{ex} 465 nm, 25 °C.

fluorescene intensity of both probes did not show a significant increase when they were treated with esterase for 2 h. After that, NaHS was added and a fluorescence increase was immediately observed. These results

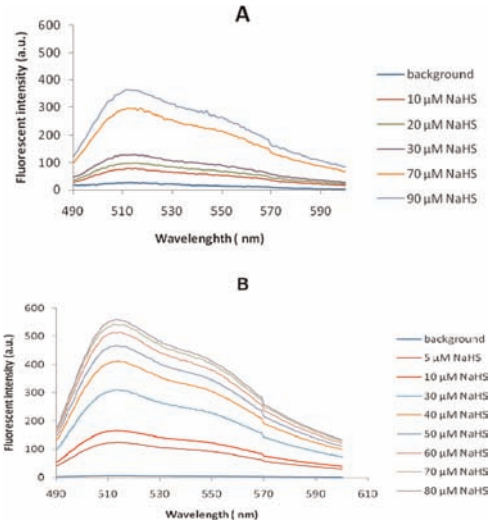

Figure 4. Fluorescence spectra of probe **5** (A) and probe **6** (B) in phosphate buffer (pH 7.4, 10 mM). Probe concentration was 5 μM. Spectra were recorded after incubation with different concentrations of NaHS for 30 min.

suggested that the probes were quite stable toward esterase.

Since probe **6** was identified as a better probe than compound **5**, due to its higher sensitivity and faster reaction with H_2S, this probe was selected for monitoring H_2S in living cells. Briefly, COS7 cells were incubated with compound **6** for 10 min and then washed twice with phosphate buffer (Figure 6). We did not observe any significant fluorescent cells. However, strong fluorescence in the cells was observed after treatment with sodium sulfide for 10 min. Thus we conclude that probe **6** can be used for the detection of H_2S in cultured cells.

In summary, we reported in this study an improved fluorescent probe for H_2S detection. The design was based on a Michael addition of H_2S followed by an intramolecular cyclization to release the fluorophore. This process proved to be selective for H_2S. Other biological thiols reacted with the Michael acceptors in a reversible way and did not lead to covalent products. Therefore, thiols could not turn on the fluorescence and did not consume the probe. Compared to other reaction based H_2S probes,[5] our design has some advantages. Our probes contain three structural subunits: a H_2S trapper, linker, and fluorophore. Many chemical modifications can be carried out to improve the efficiency of the probes. We believe this strategy will be useful for the design of more sensitive and selective probes for the detection of H_2S in biological systems.

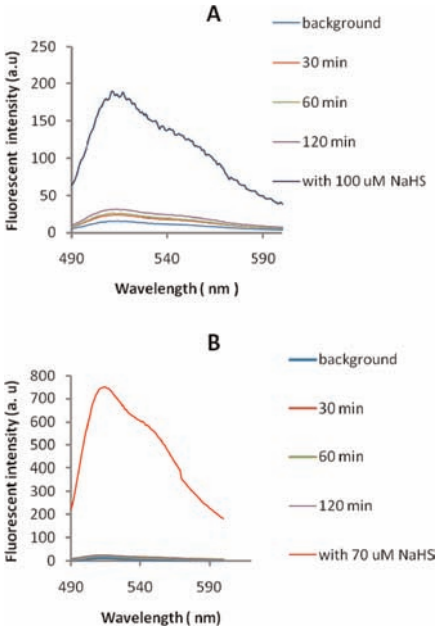

Figure 5. Fluorescence spectra of probe **5** (A) and probe **6** (B) in the presence of esterase. Probe concentration: 5 μM. Esterase concentration: 0.025 g/mL. Spectra were recorded after incubation for different times.

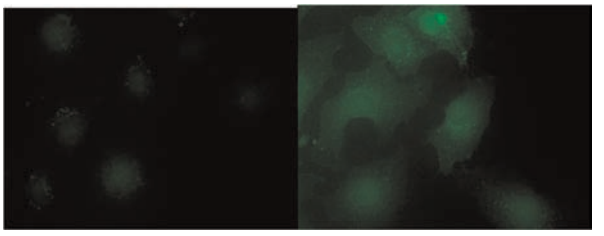

Figure 6. Fluorescence images of H_2S detection in COS7 cells using probe **6**. COS7 cells on glass coverslips were incubated with **6** (100 μM) for 10 min, washed, and then subjected to different treatments. Left was control (no sodium sulfide was added); right was treated with sodium sulfide (200 μM).

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Supporting Information Available. Spectroscopic and analytical data and selected experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org

The authors declare no competing financial interest.