JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
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Rational Design and Bioimaging Applications of Highly Selective Fluorescence Probes for Hydrogen Polysulfides

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Supporting Information

ABSTRACT: Reactive sulfur species have received considerable attention due to their various biological functions. Among these molecules, hydrogen polysulfides (H_2S_n, $n > 1$) are recently suggested to be the actual signaling molecules derived from hydrogen sulfide (H_2S). Hydrogen polysulfides may also have their own biosynthetic pathways. The research on H_2S_n is rapidly growing. However, the detection of H_2S_n is still challenging. In this work we report a H_2S_n-mediated benzodithiolone formation under mild conditions. Based on this reaction, specific fluorescent probes for H_2S_n are prepared and evaluated. The probe **DSP-3** shows good selectivity and sensitivity for H_2S_n.

Reactive sulfur species (RSS) are a family of sulfur-containing molecules found in biological systems. These molecules include thiols, hydrogen sulfide, persulfides, polysulfides, and S-modified cysteine adducts such as S-nitrosothiols and sulfenic acids. So far many RSS have been demonstrated to exert interesting biological functions.[1−3] Among those, hydrogen sulfide (H_2S) is probably most attractive as this gaseous molecule has been recently known as a critical cell signaling molecule, much like nitric oxide. Literature published in the past several years increasingly suggests that H_2S is a mediator of many physiological and/or pathological processes, especially in cardiovascular systems.[4−7] In contrast, hydrogen polysulfides (H_2S_n, $n > 1$) have received much less attention. These species can be considered as oxidized forms of H_2S and belong to sulfane sulfur in RSS family. From a chemistry perspective, H_2S and H_2S_n are redox partners and therefore very likely coexist in biological systems. On the other hand, H_2S_n may have their own biosynthetic pathways or can be generated from H_2S. H_2S_n could also be the precursors of H_2S through their degradation.

Because of these properties, some biological mechanisms that were originally attributed to H_2S may actually be mediated by H_2S_n. For instance, one of the most interesting reactions of H_2S is S-sulfhydration, i.e., converting protein cysteines (-SH) to persulfides (-S-SH). This reaction is significant because it provides a possible mechanism by which H_2S alters the functions of a wide range of cellular proteins and enzymes.[8−11] However, how this reaction proceeds is still unclear. Theoretically H_2S itself can hardly react with protein cysteine residues or disulfides to form S-sulfhydration. It is possible that H_2S reacts with modified cysteines such as S-nitrosothiols (SNO) or S-sulfenic acids (SOH) to form S-sulfhydration.[12,13] Recently the possibility that the reaction is caused by H_2S_n has been revealed.[14−17] From a reactivity point-of-view, H_2S_n should be much more effective in S-sulfhydration than H_2S. Kimura found that H_2S_n were indeed hundreds times more potent than H_2S in inducing Ca^{2+} influx in astrocytes via S-sulfhydration on TRPA1 channels.[18] He also found that H_2S_n were very effective in S-sulfhydration on Keap1, the key protein regulating Nrf2 signaling.[19] In another report by Dick and Nagy et al., H_2S_n were found to efficiently sulfhydrate proteins such as roGFP2 and PTEN, while H_2S could not cause sulfhydration in the presence of potassium cyanide, an H_2S_n scavenging reagent.[20]

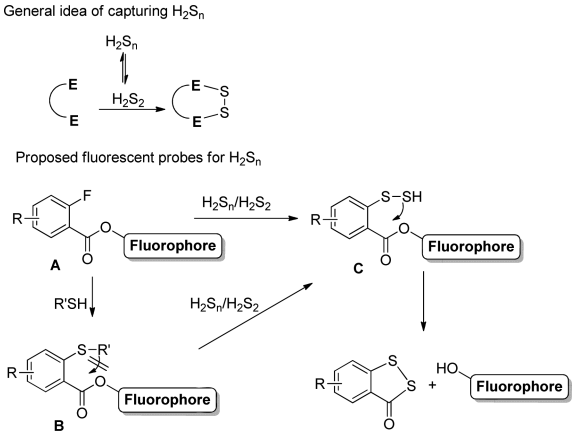
In order to better understand the roles of H_2S_n and differentiate H_2S_n from H_2S, it is important to study the fundamental chemistry/reactivity of H_2S_n and develop new methods for their detection. The traditional method for detecting H_2S_n is to measure UV absorption peaks at 290−300 and 370 nm, which is not sensitive and applicable for biological detections.[20] In this respect, fluorescence assays may be useful because of their high sensitivity and spatiotemporal resolution capability. Unfortunately, there is no report on such fluorescent probes for H_2S_n so far. To this end, we have initiated a program to study new reactions of H_2S_n, aiming at developing new fluorescent probes based on these reactions. Herein we report this attempt.

H_2S_n is a combination of polysulfide species. The dissolution of any polysulfide salts should result in similar distribution of these species (this will depend on the relative ratios of sulfide vs the oxidizing equivalents and the applied pH).[14] Hydrogen disulfide (H_2S_2) may be an active species of H_2S_n, and there should be a dynamic equilibrium between H_2S_2 and other H_2S_n.[18] Therefore, our focus has been put on the chemistry of H_2S_2. Taking the advantage of two -SH groups in H_2S_2, we envisioned that compounds containing bis-electrophilc groups should be able to selectively capture H_2S_2 (Scheme 1). If one of the electrophilic groups is a latent fluorophore and can be released under nucleophilic reactions (such as **A** in Scheme 1), the strategy may be suitable in the development of fluorescent probes for H_2S_2. It is possible that biothiols, i.e., cysteine (Cys) and glutathione (GSH), may compete with H_2S_2 in reacting with probe **A**. However, product **B** should not turn-on the fluorescence. Moreover, upon manipulating electronic properties of the probe, H_2S_n/H_2S_2 may further react with **B** (via the S_N2Ar

Received: March 28, 2014
Published: May 8, 2014

dx.doi.org/10.1021/ja502968x | J. Am. Chem. Soc. 2014, 136, 7257−7260

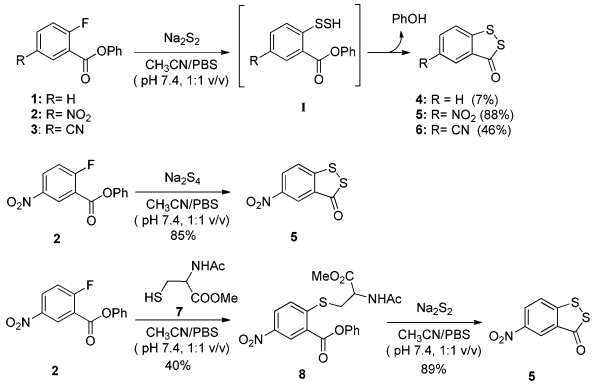
Scheme 1. Proposed Strategy for Capturing and Visualizing H_2S_n



reaction) to switch the thioether and turn on the fluorescence (*vide infra*).

With this idea in mind, three 2-fluorobenzoate derivatives (**1**–**3**) were prepared and studied in the reactions of H_2S_2 (Scheme 2). In this study H_2S_2 was always used as the primary model

Scheme 2. Model Reactions of the Probes with H_2S_n

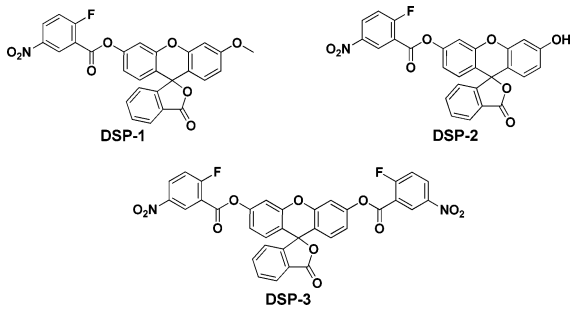


compound of H_2S_n. We expected that the activated fluorobenzoates should undergo nucleophilic aromatic substitution with H_2S_2 to form the corresponding persulfide intermediates **I**, which in turn undergoes a cyclization to form benzodithiolone products and release phenol. In these experiments freshly prepared solutions of Na_2S_2 were used as the equivalent of H_2S_2. The reactions were carried out in a mixed solution of CH_3CN/PBS (pH 7.4, 1:1 v/v). The products were analyzed after 1 h at room temperature. As expected, when the parent compound **1** was treated with H_2S_2, the desired cyclization product **4** was obtained in low yield (7%). The substrates with electron-withdrawing groups (-CN and -NO_2) showed much improved reactivity, and the corresponding cyclization products (**5** and **6**) were obtained in modest to good yields. As the nitro-substitution (compound **2**) was found to be most effective, this compound was selected for further studies.

We then tested the reaction between **2** and another hydrogen polysulfide model compound (Na_2S_4). The reaction worked well, and the desired cyclization product was obtained in good yield (85%). This result confirms that H_2S_2 may be the major component of H_2S_n or that there is a fast equilibrium between H_2S_n and H_2S_2.[18] It therefore suggests that compounds like **2** are suitable for capturing H_2S_n. Another concern is that biothiols may also react with the probes that are designed for trapping H_2S_n, leading to the consumption of the probes. To address this concern, we tested the reaction of **2** with a biothiol model **7**. The substitution product **8** was obtained in 40% yield under the same conditions. This suggests that biothiols are less reactive (than H_2S_n) toward the substrate. Moreover, compound **8** was able to further react with H_2S_n to give the cyclization product **5**. These results indicate that biothiols would not interfere with the detection of H_2S_n.

The reaction shown in Scheme 2 provides a possible application in developing fluorescent probes for H_2S_n. It is known that hydroxyl (-OH) protection (e.g., acylation or alkylation) of many fluorophores can result in fluorescence quenching, and deprotection can restore the fluorescence.[21−28] If -OH sensitive fluorophores are introduced to the benzoate of **2**, the resultant compounds would be specific probes for H_2S_n as they may react with H_2S_n to release the fluorophores. Based on this strategy, three probes (**DSP-1**, **DSP-2**, and **DSP-3**) are synthesized (Scheme 3). Detailed synthetic protocols and structure characterizations are provided in the Supporting Information.

Scheme 3. Structures of New H_2S_n Fluorescent Probes



Next we tested the probes' fluorescence properties and responses to H_2S_n. We first studied the detection conditions and found that PBS buffer (50 mM, pH 7.4) containing 25 μM cetrimonium bromide (CTAB) was the optimum system (Figure S1). **DSP-1** and **DSP-3** showed almost no fluorescence emission at 515 nm due to the protection of the two hydroxyl groups of fluorescein, but **DSP-2** showed some background fluorescence due to the protection of only one hydroxyl group of fluorescein. Upon treatment with Na_2S_2, both **DSP-1** and **DSP-3** gave significant fluorescence enhancements (Figure 1), whereas **DSP-2** did not, which may be attributed to its strong background

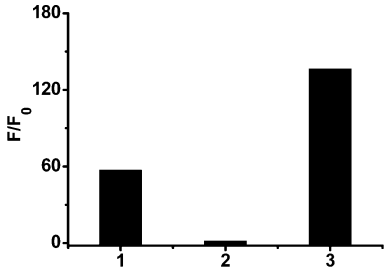


Figure 1. Fluorescence enhancements (F/F_0) of probe (10 μM) (1) **DSP-1**; (2) **DSP-2**; and (3) **DSP-3** with Na_2S_2 (50 μM) in PBS buffer (50 mM, pH 7.4) containing 25 μM CTAB. Reactions were carried out for 20 min at room temperature. Data were acquired at 515 nm with excitation at 490 nm.

dx.doi.org/10.1021/ja502968x | *J. Am. Chem. Soc.* 2014, 136, 7257−7260

fluorescence. As **DSP-3** exhibited a much stronger fluorescence response than **DSP-1** (137 vs 57 fold), this probe was selected for further evaluation.

Figure 2 shows the time-dependent fluorescence changes of **DSP-3** (10 μM) in the presence of Na_2S_2 (50 μM). The

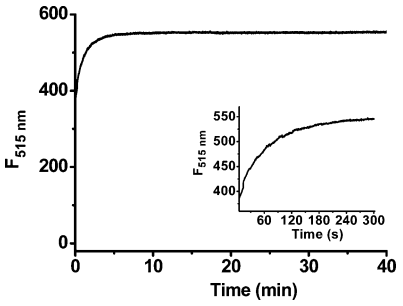


Figure 2. Time-dependent fluorescence intensity changes of **DSP-3** (10 μM) in the presence of Na_2S_2 (50 μM). Reactions were monitored for 40 min at room temperature.

maximum emission intensity at 515 nm was reached within 5 min, indicating a fast reaction. For the purpose of reproducibility, a reaction time of 20 min was employed in all other experiments. The effects of pH in this reaction were also investigated, and **DSP-3** was found to work effectively in neutral to weak basic pH range of 7−8 (Figure S2).

To test the selectivity of the probe for H_2S_n, **DSP-3** was treated with a series of RSS including GSH, Cys, Hcy, GSSG, H_2S, SO_3^{2-}, $S_2O_3^{2-}$, CH_3SSSCH_3, and S_8. As shown in Figure 3A, no

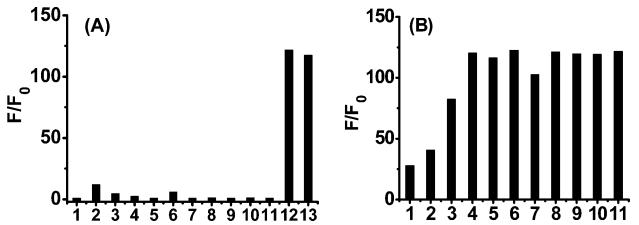


Figure 3. (A) Fluorescence enhancements (F/F_0) of **DSP-3** (10 μM) in the presence of various RSS. (1) probe alone; (2) 8 mM GSH; (3) 500 μM Cys; (4) 100 μM Hcy; (5) 100 μM GSSG; (6) 100 μM Na_2S; (7) 100 μM $Na_2S_2O_3$; (8) 100 μM Na_2SO_3; (9) 100 μM Na_2SO_4; (10) 100 μM CH_3SSSCH_3; (11) 100 μM S_8; (12) 50 μM Na_2S_2; (13) 50 μM Na_2S_4. (B) Fluorescence enhancements (F/F_0) of **DSP-3** (10 μM) to the mixture of various RSS with 50 μM Na_2S_2. (1) 1 mM GSH; (2) 500 μM Cys; (3) 100 μM Hcy; (4) 100 μM GSSG; (5) 100 μM Na_2S; (6) 100 μM $Na_2S_2O_3$; (7) 100 μM Na_2SO_3; (8) 100 μM Na_2SO_4; (9) 100 μM CH_3SSSCH_3; (10) 100 μM S_8; (11) 50 μM Na_2S_2.

significant fluorescence increase was observed for any of these compounds (columns 2−11). Only Na_2S_2 and Na_2S_4 gave strong fluorescence increase (columns 12 and 13). We also tested the responses of **DSP-3** to other representative amino acids and found no responses (Figure S3). Moreover, when Na_2S_2 (50 μM) and other RSS coexisted, we still observed obvious fluorescence enhancements (Figure 3B). Compared to the results of Na_2S_2 only, almost the same levels of fluorescence turn-on responses (without any loss) were observed for most of these compounds. GSH, Cys, and Hcy did cause some fluorescence decrease, presumably due to the reaction between H_2S_2 and thiols, leading to the decreased concentrations of H_2S_2 in solutions.[18−20,29] These results demonstrate good selectivity of **DSP-3** for H_2S_2 and hydrogen polysulfides, suggesting that **DSP-3** may be useful for monitoring of H_2S_n in biological systems.

To demonstrate the efficiency of this probe in the measurement of H_2S_n, varying concentrations of Na_2S_2 (0.5−50 μM) were added to the solutions of **DSP-3** (10 μM). The fluorescence intensities were linearly related to the concentrations of Na_2S_2 in the range of 0.5−15 μM (Figure 4). The detection limit[30,31] was calculated to be around 71 nM, indicating a high sensitivity.

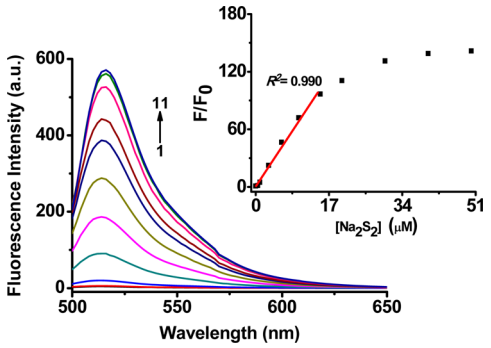


Figure 4. Fluorescence emission spectra of **DSP-3** (10 μM) with varied concentrations of Na_2S_2 (0, 0.5, 1, 3, 6, 10, 15, 20, 30, 40, 50 μM for curves 1−11, respectively). Reactions were carried out for 20 min at room temperature.

It should be noted that the biosynthetic pathways of H_2S_n are still unclear. Recent studies suggested that they may come from H_2S in the presence of reactive oxygen species (ROS).[12,14,15,17,18,20,32] We then applied **DSP-3** in detecting *in situ* generated H_2S_n from H_2S and ROS. As shown in Figure 5, the

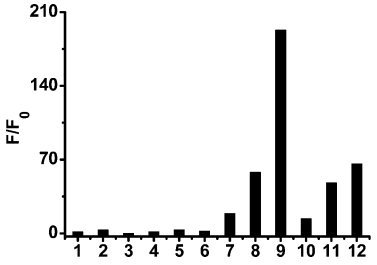


Figure 5. Fluorescence enhancements (F/F_0) of **DSP-3** (10 μM) in the presence of various reactive oxygen species (with or without H_2S). Reactions were carried out for 20 min at room temperature. (1) 50 μM H_2O_2; (2) 200 μM H_2O_2; (3) 50 μM ClO^-; (4) 50 μM O_2^-; (5) 50 μM ·OH; (6) 50 μM $^1O_2$; (7) 50 μM H_2O_2 + 50 μM Na_2S; (8) 200 μM H_2O_2 + 50 μM Na_2S; (9) 50 μM ClO^- + 50 μM Na_2S; (10) 50 μM O_2^- + 50 μM Na_2S; (11) 50 μM •OH + 50 μM Na_2S; (12) 50 μM $^1O_2$ + 50 μM Na_2S.

probe did not give any response to commonly existing ROS including hydrogen peroxide (H_2O_2), hypochlorite (ClO^-), superoxide (O_2^-), hydroxyl radical (•OH), and singlet oxygen ($^1O_2$) (columns 1−6). However, when H_2S was premixed with ROS (columns 7−12), significant fluorescence signals were obtained, suggesting the formation of H_2S_n in these systems. Apparently H_2S together with ClO^- gave the strongest signals (column 9), indicating that ClO^- is the most effective ROS converting H_2S to H_2S_n in our *in vitro* testing systems. This result confirms the discovery by Nagy et al. that hypochlorous acid can rapidly react with H_2S to form hydrogen polysulfides.[32]

Finally the application of **DSP-3** in monitoring H_2S_n in cultured cells was tested. As shown in Figure 6, HeLa cells were

dx.doi.org/10.1021/ja502968x | J. Am. Chem. Soc. 2014, 136, 7257−7260

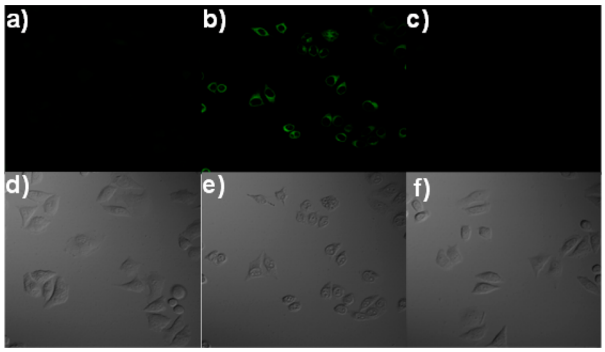


Figure 6. Confocal fluorescence images of H_2S_n in HeLa cells. Cells on glass coverslips were incubated with **DSP-3** (10 μM) for 20 min, then washed, and subjected to different treatments. (a) control (no Na_2S_2); cells treated with (b) 100 μM Na_2S_2 and (c) 100 μM Na_2S. Second row shows the corresponding differential interference contrast images for the first row.

first incubated with **DSP-3** (10 μM) for 20 min, and no fluorescence was observed. Strong fluorescence in the cells was induced after treating with Na_2S_2 (100 μM). In comparison, cells treated with H_2S (using 100 μM Na_2S) did not show obvious fluorescence. In addition, the cell viability assay demonstrated that **DSP-3** has almost no cytotoxicity (Figure S4). These results suggest that **DSP-3** is cell permeable and can be used in detecting H_2S_n (not H_2S) in cells.

In summary, we report in this study a H_2S_n/H_2S_2-mediated benzodithiolone formation under mild conditions. This reaction proves to be specific for H_2S_n/H_2S_2 over other RSS such as biothiols and H_2S. Based on this reaction, a fluorescent probe, **DSP-3**, was developed for sensitive and selective detection of H_2S_n/H_2S_2 in aqueous buffers as well as in cells. With probe **DSP-3**, we also confirm the possibility of H_2S_n formation from the reaction of H_2S with ROS such as ClO^-. We are now utilizing these probes to study the contributions of hydrogen polysulfides to physiological and pathological processes. It should be noted that more sensitive fluorescent probes for endogenous hydrogen polysulfides may be needed, and our present design approach should lead to the development of such probes by using more strongly luminescent fluorochrome.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work is supported by NIH (R01HL116571) and the ACS-Teva U.S.A. Scholar Grant.

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