Trapping Hydrogen Sulfide (H₂S) with Diselenides: The Application in the Design of Fluorescent Probes

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S *Supporting Information*
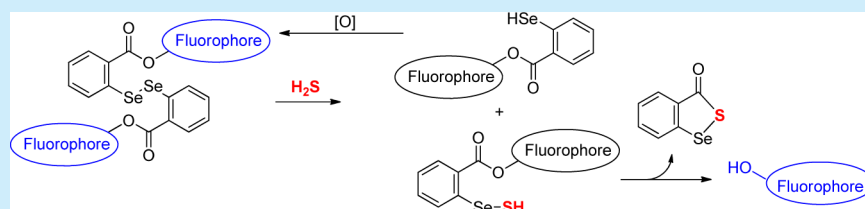
ABSTRACT: Here we report a unique reaction between phenyl diselenide-ester substrates and H₂S to form 1,2-benzothiaselenol-3-one. This reaction proceeded rapidly under mild conditions. Thiols could also react with the diselenide substrates. However, the resulted S−Se intermediate retained high reactivity toward H₂S and eventually led to the same cyclized product 1,2-benzothiaselenol-3-one. Based on this reaction two fluorescent probes were developed and showed high selectivity and sensitivity for H₂S. The presence of thiols was found not to interfere with the detection process.

Hydrogen sulfide (H₂S), traditionally known as a venomous gas with a stinky smell, has been recently recognized as an important signaling molecule.[1−5] H₂S is generated in mammalian cells by enzymes including cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST), and cysteine and derivatives serve as the substrates.[4−8] Recent studies have revealed a variety of functions of H₂S in physiological and pathological processes.[9−11] However, the mechanisms behind those roles are still poorly understood. H₂S is a highly reactive molecule that can react with many biological targets such as hemoglobin and phosphodiesterase.[12] These reactions are responsible for the biological functions of H₂S. On the other hand, these complicated reactions also make the detection of H₂S in biological systems very difficult. Early reports claim physiological H₂S concentrations can be as high as 20−80 μM in plasma. However, it is believed now that the concentrations of free H₂S are low, at submicromolar or nanomolar levels.[12b]

In the past several years fluorescence based assays have received considerable attention in this field and a number of fluorescent probes for H₂S have been reported.[13] All of these probes utilize reaction-based fluorescence change strategies, i.e. using certain H₂S-specific reactions to convert nonfluorescent substrates to materials with strong fluorescence or to change the fluorescent properties of the probes (for ratiometric probes). Currently three types of reactions are normally used in the design of probes: (1) H₂S-mediated reductions, often using azide (−N₃) substrates;[14] (2) H₂S-mediated nucleophilic reactions;[15,16] and (3) H₂S-mediated metal−sulfide formation.[17] In 2011 our laboratory developed the first nucleophilic reaction based strategy for the design of probes (i.e., **WSP** series).[15] The strategy is described in Scheme 1. The probe (**WSP**) contains two electrophilic centers. H₂S reacts with the pyridyl disulfide first to give a persulfide intermediate **1**, which precedes a fast cyclization to release the fluorophore and 1,2-benzodithiol-3-one **2**. It is anticipated that the probe can also react with biothiols to form **3**. Theoretically **3** could further react with H₂S to form **1** and then turn on the fluorescence. However, this reaction is somewhat slow, especially when the concentration of H₂S is low (under physiological conditions[18]). In addition, H₂S may react with both sulfurs of the disulfide of **3**, which makes this pathway not productive. It should be noted that the reaction between **WSP** and biothiols cannot turn the fluorescence on; therefore, the selectivity of **WSP** is found to be excellent. We were also able to optimize the detection conditions and found fluorescence "turn-on" could be achieved in a few minutes. Such a fast reaction allows effective detection of H₂S even when high concentrations of biothiols are presented, albeit the fluorescence signals are significantly decreased.[15b]

**Scheme 1. Design of the Fluorescent Probes for H$_2$S Detection**
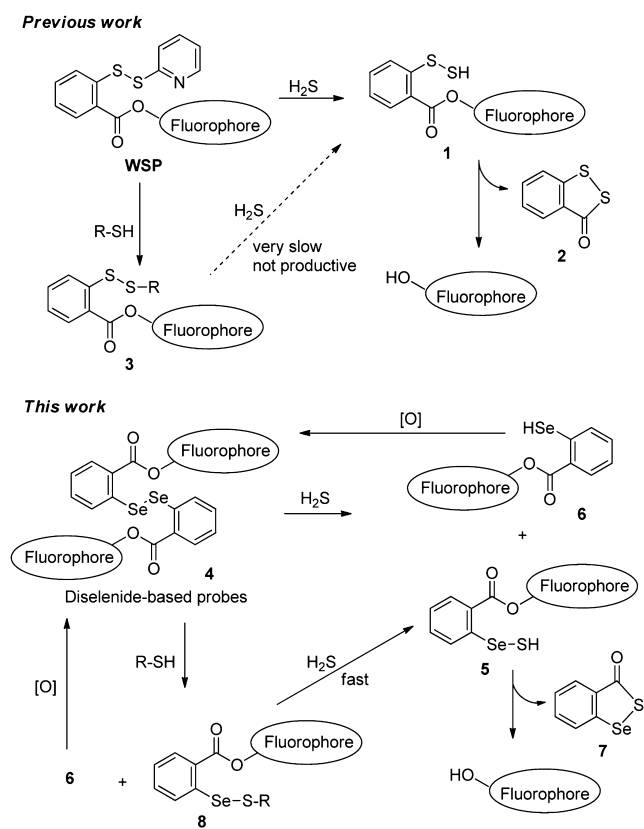
As described above, the possible consumption of **WSP** probes by biothiols is a weakness. To solve this problem two strategies may be applied: (1) a H$_2$S-specific "trapper" should be used to replace the pyridyl-disulfide. Such a trapper should only react with H$_2$S, not react with biothiols. This might be difficult as intracellular concentrations of biothiols are much higher than H$_2$S; (2) a nonconsumptive trapper with biothiols should be identified and used. Such a trapper may react with biothiols, but the reaction should not lead to the release of the fluorophore. Moreover, ideally the reaction product or intermediate should maintain high reactivity toward H$_2$S which therefore can lead to fluorescence "turn-on" by H$_2$S. Herein we report our progress in pursuing the latter strategy. Diselenide-based substrates were found to be suitable for this goal.

Our design of the diselenide-based strategy for the selective trapping of H$_2$S (not consumed by biothiols) is shown in Scheme 1. It is known that diselenide bonds can be cleaved by sulfur-based nucleophiles very effectively (about 5 orders of magnitude faster than disulfide bonds).[19,20] It is also known that diselenides can facilitate disulfide formation from thiols.[21] H$_2$S (pK_a 6.8) is a stronger nucleophile than common biothiols such as Cys and GSH. Therefore, we expect diselenide-based reagents such as **4** should react with H$_2$S very effectively. As such two products should be formed: a thio-benzeneselenol derivative **5** and a benzeneselenol derivative **6**. As an extremely unstable intermediate, benzeneselenol **6** should be rapidly oxidized to reform the probe **4**.[22] In contrast, **5** should undergo a fast and spontaneous cyclization to form 1,2-benzothia-selenol-3-one **7** and release the fluorophore. We also expected the diselenide bond should be quite reactive to biothiols

(RSH). Such reactions should lead to two products: **6** and a S–Se conjugate **8**. Again, **6** should be oxidized to regenerate the probe. As for **8**, previous studies revealed that nucleophilic attack by thiols at selenium is both kinetically much faster and thermodynamically more favorable.[19] As such, the reaction between **8** and other biothiols should not change the probe's structural template (i.e., maintaining the −S−Se− conjugation). However, if H$_2$S is present, the reaction should lead to intermediate **5**, and the following cyclization should produce **7** and the fluorophore. Overall we expected the probe would specifically react with H$_2$S to release the fluorophore and the presence of biothiols should not interfere with the process.

With this idea in mind, a model compound **9** was prepared and tested. As shown in Scheme 2, the reaction between

Scheme 2. Model Reactions between Compound 9 and H$_2$S
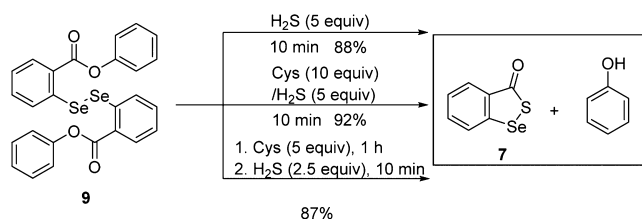
compound **9** and H$_2$S (5 equiv, using Na$_2$S as the equivalent) was found to be fast, which completed in 10 min. The desired products **7** and phenol were obtained in excellent yields. The yield of **7** was calculated based on two selenium moieties in the starting material. We did not observe the formation of the benzeneselenol product in the reaction. When Cys (10 equiv) coexisted with H$_2$S (5 equiv) in the reaction, we obtained the same products in high yields. More interesting, even if Cys (5 equiv) was treated with **9** first for 1 h, the addition of H$_2$S (2.5 equiv) still provided the desired products in similar yields. These results supported our hypothesis that diselenide-based substrates could selectively and effectively react with H$_2$S to form the cyclized product **7** and this reaction was not affected by thiols.

Based on this unique reaction we synthesized two fluorescent probes **SeP1** and **SeP2** (shown in Figure 1). 7-Hydroxycou-
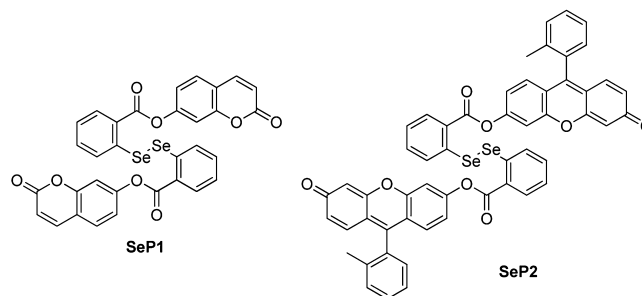

Figure 1. Structures of diselenide-based probes.

marin and 2-methyl TokyoGreen were selected as the fluorophores, as they have excellent fluorescence properties and their fluorescence can be easily quenched upon acylation on hydroxy groups.

With these two probes in hand, we tested their fluorescent properties. Both probes exhibited very weak fluorescence with low quantum yields (Φ < 0.1), due to esterification of the fluorophores. We then tested the probes' fluorescence

responses to H$_2$S in different solvent systems, and a mixed CH$_3$CN/PBS buffer (10 mM, pH 7.4, 1:1) solution was found to be the optimum system for the measurement. In this system the fluorescence intensity of the probe (10 μM) could reach the maximum in less than 2 min upon treatment of H$_2$S (using 50 μM Na$_2$S) as shown in Figure 2, demonstrating this was a fast
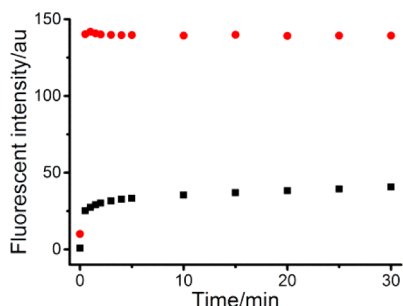

Figure 2. Time-dependent fluorescence changes of the probes (10 μM) in the presence of Na$_2$S (50 μM). The reactions were carried out for 30 min at room temperature in CH$_3$CN/PBS buffer (10 mM, pH 7.4, 1:1, v/v). Data were acquired at 455 nm with excitation at 340 nm for **SeP1** (■) and at 521 nm with excitation at 498 nm for **SeP2** (●).

process. Fluorescence increases were also found to be significant. Intensities increased 35- and 14-fold for probe **SeP1** and **SeP2** respectively. When a series of different concentrations of Na$_2$S were treated with the probes we observed fluorescence intensities increased in almost a linear fashion in the range 0−15 μM. Data obtained with **SeP2** are shown in Figure 3. The detection limit of **SeP2** was calculated to be 0.06 μM. Data of **SeP1** are shown in Figure S1 in the Supporting Information.
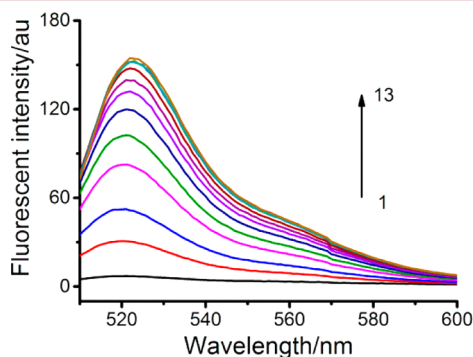
Figure 3. Fluorescence emission spectra of **SeP2** (10 μM) in the presence of varied concentrations of Na$_2$S (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 μM). The reactions were carried out for 5 min at room temperature in CH$_3$CN/PBS buffer (10 mM, pH 7.4, 1:1, v/v). Data were acquired with excitation at 498 nm for **SeP2**.

We also examined the selectivity of the probes for H$_2$S over other reactive sulfur species, including cysteine (Cys), glutathione (GSH), sulfite (SO$_3^{2-}$), sulfate (SO$_4^{2-}$), and thiosulfate (S$_2$O$_3^{2-}$). As shown in Figure 4, all of these species did not show a significant fluorescence increase even under much higher concentrations (up to mM). In addition, when H$_2$S (50 μM) coexisted with Cys or GSH (1 mM), we observed strong fluorescence responses that were comparable (at ∼90% levels) to the signals obtained with H$_2$S only. This was a significant improvement from our pyridyl disulfide based probes, which gave much decreased fluorescence when high
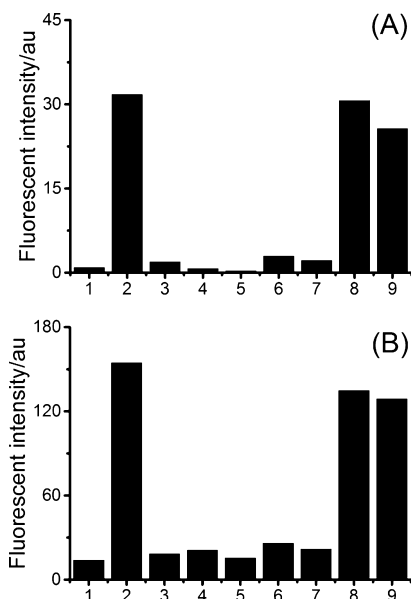

Figure 4. Fluorescence intensity of the probes (10 μM) in the presence of various reactive sulfur species: (1) control; (2) 50 μM Na$_2$S; (3) 200 μM Na$_2$SO$_3$; (4) 200 μM Na$_2$S$_2$O$_3$; (5) 200 μM Na$_2$SO$_4$; (6) 1 mM Cys; (7) 1 mM GSH; (8) 50 μM Na$_2$S + 1 mM Cys; (9) 50 μM Na$_2$S + 1 mM GSH. **SeP1** (A), **SeP2** (B).

concentrations of biothiols were present (at 25−55% levels of the signal of H$_2$S only).[15b]

Next we tested **SeP2** in imaging H$_2$S in cells. Freshly cultured HeLa cells were first incubated with **SeP2** (50 μM) for 30 min and then washed with DMEM to remove excess probe. We did not observe significant fluorescent cells (Figure 5). However, strong fluorescence in the cells was observed after treating with Na$_2$S (100 μM) for 30 min. These results demonstrated that **SeP2** could be used for cell imaging.

Finally we wondered if **SeP2** could be used to measure endogenous H$_2$S concentrations changes. To this end, human neuroblastoma cells (SH-SY5Y) were separately treated with L- and D-cysteine (which are H$_2$S biosynthestic substrates), S-
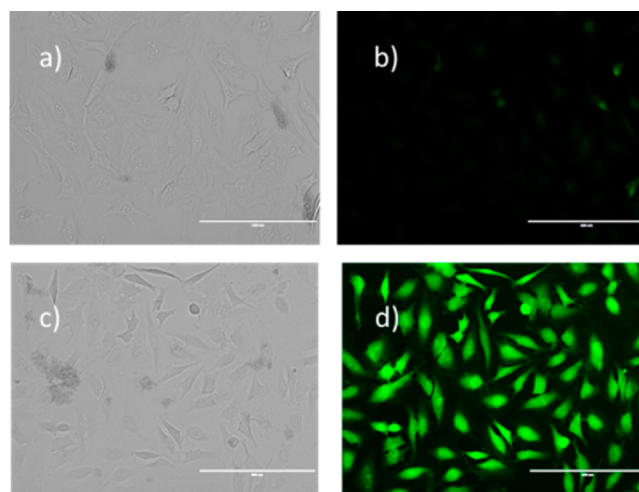
Figure 5. Fluorescence images of H$_2$S in HeLa cells using **SeP2**. Cells were incubated with the probe (50 μM) for 30 min, then washed, and subjected to different treatments. (a and b) Control (no Na$_2$S was added); (c and d) treated with 100 μM Na$_2$S (scale bar: 100 nm).

adenosylmethyonine (SAM, a CBS activator), and amino-oxyacetic acid (AOAA, a CBS inhibitor). **SeP2** was loaded into each experiment before or after treatments (see detailed protocols in the Supporting Information). Fluorescence intensities were measured by a plate reader and compared to both negative and positive controls. As shown in Figure 6, cells
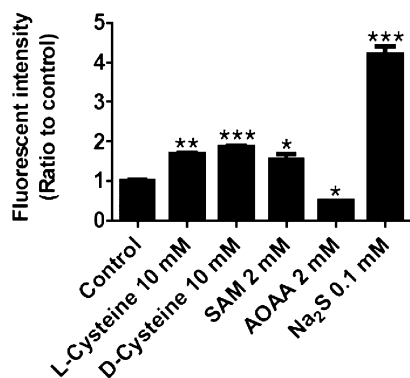
Figure 6. Fluorescent detection of in situ generated H_2S in human neuroblastoma cells ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs control; $N = 5$ each).

treated with H_2S substrates or CBS activator showed clearly enhanced fluorescence while cells treated with CBS inhibitor showed decreased fluorescence. Not surprisingly cells treated with Na_2S showed the strongest fluorescence. These results suggest that **SeP2** can be used in determining endogenous H_2S changes.

In conclusion, we reported herein a unique reaction between phenyl diselenide and H_2S to form 1,2-benzothiaselenol-3-one. The presence of thiols did not affect this process. Based on this reaction two fluorescent probes for the detection of H_2S were prepared and evaluated. The probes showed excellent sensitivity and selectivity.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed synthetic procedures, characteristic data, and experimental procedures. 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

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