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Development of Dual-Reactivity Based Fluorescence Probes for Visualizing Intracellular Hydrogen Polysulfides Signaling

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Abstract

Endogenous hydrogen polysulfides (H_2S_n , $n > 1$) have been recognized as important regulators in sulfur-related redox biology. H_2S_n can activate tumor suppressors, ion channels, and transcription factors with higher potency than H_2S . While H_2S_n are drawing increasing attention, their exact mechanisms of actions are still poorly understood. A major hurdle in this field is the lack of reliable and convenient methods for H_2S_n detection. In this work we report a H_2S_n -mediated benzodithiolone formation under mild conditions. This reaction takes advantage of the unique dual-reactivity of H_2S_n as both a nucleophile and an electrophile. It opens a door for better understanding H_2S_n 's chemical biology. Based on this reaction, three fluorescent probes (**PSP-1~3**) were synthesized and evaluated. Among the probes prepared, **PSP-3** showed a desirable off-on fluorescence response to H_2S_n and high specificity. It was successfully applied in visualizing intracellular H_2S_n .

Keywords

hydrogen polysulfides; imaging agents; fluorescence; cyclization; fluorescent probes

Reactive sulfur species (RSS) are a group of sulfur-containing molecules that play regulatory roles in biological systems. Important RSS include biothiols and S-modified protein cysteine adducts. RSS also include hydrogen sulfide (H_2S) and sulfane sulfurs (such as cysteine persulfides ($R-S-SH$) and polysulfides ($R-S-S_n-S-R'$, $n > 0$)). Understanding RSS's mechanisms of actions has become a very active research area in modern chemical biology, particularly from the methodological point of view.^[1] Among various RSS, hydrogen polysulfides (H_2S_n , $n > 1$) have attracted particular attention, mainly due to their involvement in H_2S -related redox biology.^[2] H_2S_n can be generated from endogenous H_2S upon reacting with reactive oxygen species (ROS).^[3] H_2S can also react with other sulfane sulfurs such as S_8 to form H_2S_n .^[2d,3c,4] Moreover, H_2S_n may have their own biosynthetic pathways. For example, while cystathionine γ -lyase (CSE)-mediated cystine metabolism can produce cysteine persulfide,^[5] it may also catalyze direct generation of H_2S_n from cystine.

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A series of recent works suggested that H_2S_n might be a new group of signaling molecules.^[2,3c,4] It was found that H_2S_n can activate tumor suppressors, ion channels, and transcription factors with higher potency than H_2S .^[4] Some physiological activities that were originally assigned to H_2S may actually be mediated by H_2S_n . One such example is S-sulfhydration.^[1a,5,6] This post-translational modification was previously thought to be the result of H_2S . However, recent results have demonstrated that H_2S_n are more effective than H_2S in S-sulfhydration.^[2,3b-c]

Although H_2S_n have now been recognized as potent physiological mediators, a number of issues remain to be clarified, such as the production and degradation pathways of H_2S_n , their regulatory mechanisms, and potential physiological stimuli that induce those regulatory mechanisms. To address these issues, it is critical to develop effective methods for H_2S_n detection. The traditional method is to measure UV absorption peaks at 290–300 nm and 370 nm, which has low sensitivity and is not applicable for biological detections. Fluorescence assays could be useful because of their high sensitivity and spatiotemporal resolution capability. While fluorescent probes for H_2S have been extensively studied,^[7] the probes for H_2S_n are still underdeveloped due to limited known chemistry of H_2S_n .

Our laboratory has initiated a program to study new chemistry/reactions of H_2S_n and develop reaction-based fluorescent probes for H_2S_n . In 2014, we reported the first H_2S_n -specific probes (**DSP**, Scheme 1) which employed a 2-fluoro-5-nitro-benzoic ester template to trap H_2S_n and promote an intramolecular cyclization to release a fluorophore.^[8] **DSP** probes, such as **DSP-3**, showed satisfactory sensitivity and selectivity for H_2S_n . Several other groups have adopted the same template to develop H_2S_n probes with interesting properties.^[9] However, a drawback of **DSP** is that 2-fluoro-5-nitro-benzoic esters can also react with biothiols. Although such reactions do not turn on fluorescence, the consumption of the probes is a problem. In theory, the reaction product, i.e. thioether **1**, can further react with H_2S_n to switch on fluorescence. We found that the reaction was somewhat slow and non-productive when H_2S_n concentration was low (at μM levels). Therefore, high loading of **DSP** probes may be needed for biological detections. Very recently our lab discovered another H_2S_n -specific aziridine opening reaction and developed a probe (**AP**) based on this reaction.^[10] **AP** showed excellent selectivity for H_2S_n , it did not react with biothiols or H_2S in physiological concentrations. However, due to limited options for aziridine-based fluorophores, the sensitivity of **AP** for H_2S_n was not optimal and attempts to use **AP** for imaging H_2S_n in cells were not successful. Obviously further improvement of the probes is needed. Herein we report the design, synthesis, and evaluation of a series of new fluorescent probes (**PSP1-3**) that showed high sensitivity and selectivity for H_2S_n and can be used for endogenous H_2S_n imaging.

The key of developing optimum probes is to identify a highly specific H_2S_n recognition unit. Ideally, such a recognition unit should only react with H_2S_n and not react with other sulfur species, especially biothiols. From a chemistry perspective, H_2S_n are quite different from thiols or H_2S . The estimated $\text{p}K_a$ values of H_2S_n are in the range of 3 to 5.^[11] For comparison, the $\text{p}K_a$ values of H_2S and biothiols are in the range of 7 to 9.2. Under physiological pH, H_2S_n are expected to be weak acids, and stronger and more reactive nucleophiles than biothiols and H_2S due to alpha-effects. In addition, H_2S_n belong to the

sulfane sulfur family. A character of sulfane sulfurs is that they can function as electrophiles and react with certain nucleophiles.^[12] Overall, H_2S_n have a unique dual-reactivity and can act as both nucleophiles and electrophiles. Taking advantage of this property, we envisioned template **2** might be specific for H_2S_n (Scheme 2). In **2**, a thioester was employed to trap the nucleophilicity of H_2S_n . One might worry that the thioester group could also react with biothiols as the reactions between thioesters and thiols are known, for example in the well-known Native Chemical Ligation.^[13] However, those reactions are mostly for synthetic purposes with high concentrations of reactants and special solvent conditions. The relatively low concentrations of biothiols in biological systems and the mild, neutral, and aqueous environments may make the reactions slow and non-productive. In addition, we expected the manipulation of R groups (steric and electronic effects) in **2** could significantly differentiate its reaction rates toward thiols and H_2S_n . If H_2S_n could selectively react with an appropriate thioester group, the product, i.e. **3**, should further react with H_2S_n (now serving as an electrophile) to form **4**. The following spontaneous cyclization should release the fluorophore. Overall, this process would be specific for H_2S_n .

Based on this idea, we synthesized three probes (**PSP-1**, **PSP-2**, and **PSP-3**, Scheme 3). Three different R groups (Me, tBu, and Ph) were used in order to explore the effects of acyl groups on the reactivity of thioesters toward biothiols. We first tested their fluorescence properties and responses to H_2S_n in PBS buffer. Freshly prepared Na_2S_2 solutions were used as the equivalents of H_2S_n . All three probes showed almost no fluorescence emission ($\Phi_{\text{PSP-1}} = \Phi_{\text{PSP-2}} = 0.02$; $\Phi_{\text{PSP-3}} = 0.01$) due to the protection of the two hydroxyl groups of fluorescein. Upon the treatment of Na_2S_2 (5 eq) for 30 min, the probes gave significant fluorescence enhancements (Figure 1A). **PSP-1** and **PSP-3** exhibited stronger fluorescence increases than **PSP-2**. Presumably the bulky t-butyl group of **PSP-2** decreased the reactivity toward H_2S_n . We also tested time-dependent fluorescence changes of the probes in the presence of Na_2S_2 (Figure 1B). The maximum emission intensities of **PSP-1** and **PSP-3** were reached within 2 min and 10 min respectively, indicating fluorescence turn-on was fast. The fluorescence turn-on of **PSP-2** was slower (~20 min), again suggesting the steric effect played a role in its reactivity. For the purpose of reproducibility, a reaction time of 30 min was employed in all of the following experiments.

Next we studied the stability of the probes in the presence of biothiols. As shown in Scheme 4, if the probes undergo thioester exchange with biothiols, the resultant product **5** should be sensitive to sulfane sulfurs like elemental sulfur (S_8) and produce strong fluorescence. Therefore, measuring fluorescence of the probes in a mixture of thiols and S_8 should be suitable for evaluating the stability of the probes to thiols.

We then tested fluorescence responses of the probes (10 μM) to Cys (1 mM) and GSH (5 mM) in the absence or presence of S_8 (50 μM). As shown in Figure 2, Cys, GSH, or S_8 alone did not induce any responses. The mixtures of biothiols (GSH or Cys) and S_8 produced strong fluorescence for **PSP-1**. The same mixtures caused almost no fluorescence increase for **PSP-2** and **PSP-3**. As a positive control, the thioester exchange product **5** gave strong fluorescence for S_8 under the same conditions. These results confirmed our hypothesis that the acyl group of thioester could affect its reactivity towards thiols. **PSP-1**

appeared to be labile to biothiols while **PSP-2** and **PSP-3** were stable. Therefore, the use of **PSP-2** and **PSP-3** should not be affected by cellular biothiols.

Having demonstrated the excellent reactivity and stability of **PSP-3**, this probe was selected for further studies. We next validated its selectivity for $\text{H}_2\text{S}_\text{n}$ over other common RSS including Hcy, GSSG, H_2S , SO_3^{2-} , $\text{S}_2\text{O}_3^{2-}$, SO_4^{2-} , and $\text{CH}_3\text{SSSCH}_3$. As shown in Figure 3A, no fluorescence increase was observed for these RSS (columns 2–8). Only Na_2S_2 and Na_2S_4 triggered strong fluorescence enhancements (columns 9 and 10). Additionally, It is known that $\text{H}_2\text{S}_\text{n}$ could be generated from the reactions between H_2S and ROS. Therefore, we wondered if the probe could sense *in situ* $\text{H}_2\text{S}_\text{n}$ formation from H_2S and ROS. As shown in Figure 3B, the responses of **PSP-3** to a series of ROS including hydrogen peroxide (H_2O_2), hypochlorite (ClO^-), superoxide (O_2^-), hydroxyl radical ($\bullet\text{OH}$), and singlet oxygen ($^1\text{O}_2$) were first tested. These ROS did not induce any fluorescence enhancements (columns 2–6). When H_2S was added to these ROS, a strong fluorescence signal was observed from ClO^- (column 9), but not from other ROS. These results agreed with previous discoveries that $\text{H}_2\text{S}_\text{n}$ can be efficiently derived from H_2S and ClO^- , but other ROS are not effective for $\text{H}_2\text{S}_\text{n}$ generation under these concentrations.^[3,8–10] The responses of **PSP-3** in the presence of other representative amino acids were also evaluated and no responses were observed (Figure S1).

To further evaluate the sensitivity of **PSP-3** for $\text{H}_2\text{S}_\text{n}$, a series of varied concentrations of Na_2S_2 (0 ~ 50 μM) were tested. The fluorescence intensities were linearly related to the concentrations of Na_2S_2 in the range of 0 ~ 20 μM (Figure S2). The detection limit was calculated to be 3 nM. This is the most sensitive probe for $\text{H}_2\text{S}_\text{n}$ reported so far. To clarify fluorescence turn-on mechanism, we studied the reactions between the probes and Na_2S_2 . All of the reactions gave fluorescein and benzodithiolone as the isolated products with good yields (80% ~ 90%) (Figure S3). As such, we believe the fluorescence turn-on is initiated by $\text{H}_2\text{S}_\text{n}$ -mediated thioester cleavage. The resulting thiolate further reacts with $\text{H}_2\text{S}_\text{n}$ and promotes cyclization to form benzodithiolone and release the fluorophore.

Next we wondered if **PSP-3** could be used for imaging $\text{H}_2\text{S}_\text{n}$ in cells. We first validated the capability of **PSP-3** in visualizing exogenous $\text{H}_2\text{S}_\text{n}$. As shown in Figure 4, HeLa, RAW264.7, and Vero cells were respectively incubated with **PSP-3** (5 μM) for 25 min. Extracellular probe was washed off, and no significant fluorescence was observed in cells. However, when cells were treated with Na_2S_2 or Na_2S_4 (30 μM) for 20 min, strong fluorescence was observed. These results demonstrated that **PSP-3** has good cell permeability and could be used to monitor $\text{H}_2\text{S}_\text{n}$ in cells. In addition, cell viability assay demonstrated that **PSP-3** has no cytotoxicity to cells (Figure S4).

$\text{H}_2\text{S}_\text{n}$ are short-lived species and can readily decompose in buffers. In this regard, *in-situ* generation of $\text{H}_2\text{S}_\text{n}$ from H_2S and ClO^- provides a more reliable and sustainable system for $\text{H}_2\text{S}_\text{n}$ production. Herein **PSP-3** was used to monitor *in-situ* generation of $\text{H}_2\text{S}_\text{n}$ in cells. As shown in Figure S5, neither ClO^- nor H_2S gave noticeable fluorescence responses while the mixture of $\text{H}_2\text{S}/\text{ClO}^-$ led to a strong fluorescence enhancement, which was even brighter than those obtained with exogenous $\text{H}_2\text{S}_\text{n}$. These results confirmed that *in-situ* generation of

H₂S_n by H₂S and ClO⁻ is a more effective system than simply using Na₂S₂ (or Na₂S₄) to maintain H₂S_n levels in cells.

Having demonstrated the capability of **PSP-3** in detecting exogenous H₂S_n in cells, we then sought to use **PSP-3** to monitor endogenous H₂S_n formation. Our recent studies found that CSE-overexpression causes significant elevation of persulfide and polysulfide levels in cells.^[5] Therefore, CSE over-expressed cells provide a suitable system for endogenous H₂S_n production. As shown in Figure 5a–d, normal and CSE-overexpressed^[5] COS-7 cells were separately treated with **PSP-3** for 30 min. As expected, CSE-overexpressed cells induced much-enhanced fluorescent signals as compared to normal cells. In fact, appreciable amount of H₂S_n was directly generated from a recombinant CSE in a cell-free reaction mixture containing cystine as a substrate (Figure S6). These results demonstrate that **PSP-3** is suitable for detecting endogenous H₂S_n. Additionally, Kimura et al recently described a new H₂S biosynthetic pathway from D-Cys in mammalian cells, which occurs predominantly in the kidney and the cerebellum.^[14] This process seems to be regulated by 3-mercaptopyruvate sulfurtransferase (3MST) and D-amino acid oxidase (DAO). It was suggested that H₂S produced from D-Cys might be stored as bound sulfane sulfur and the levels of sulfane sulfur can be significantly enhanced by D-Cys. In theory, H₂S_n could be produced from the reactions between H₂S and sulfane sulfurs. Therefore D-Cys stimulation in Vero cells may be a useful system of endogenous H₂S_n generation and this system was used to further validate the probe's sensitivity for endogenous H₂S_n. As shown in Figure 6e–h, the cells were incubated with **PSP-3** (5 μM) for 25 min at 37 °C. The control (Figure 5e) showed almost no fluorescence. When cells were treated with 30 μM D-Cys, a modest but obvious fluorescence was observed (Figure 5f). When the concentration of D-Cys was increased to 1 mM, we observed strong fluorescence (Figure 5g). Moreover, when the cells were first treated with sodium benzoate, a DAO inhibitor, the stimulation by D-Cys (1 mM) led to significantly weakened fluorescence. Taken together, these results indicate that D-Cys and DAO may be responsible for cellular H₂S_n production.

In summary, we report here the development of three new fluorescent probes (**PSP-1**, **PSP-2**, **PSP-3**) based on the unique dual-reactivity of H₂S_n. Among the probes prepared, **PSP-3** showed desirable off–on fluorescence response to H₂S_n and high specificity. **PSP-3** was also successfully applied in visualizing both exogenous and endogenous H₂S_n in cells. This novel probe overcomes the major limitations of previously reported H₂S_n probes, and it is expected to serve as a useful tool in understanding the physiological functions of H₂S_n.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1. Previous designs of H_2S_n -specific fluorescent probes.

Scheme 2.
The design of dual-reactivity based probes.

Scheme 3.
The structures of **PSP** probes.

Scheme 4.

Possible reactions of **PSP** probes with biothiols and S₈.

Figure 1.

(A) Fluorescence intensity increases (ΔF) of **PSP** (10 μ M) with Na_2S_2 (50 μ M): (1) **PSP-1**; (2) **PSP-2**; (3) **PSP-3**. (B) Time-dependent fluorescence intensity changes of **PSP** (10 μ M) in the presence of Na_2S_2 (50 μ M).

Figure 2.

Fluorescence increase value (ΔF) of each probe (10 μ M) in the presence of biothiols and S_8 : (a) Probe + 5 mM GSH; (b) Probe + 1 mM Cys; (c) Probe + 50 μ M S_8 ; (d) Probe + 5 mM GSH + 50 μ M S_8 ; (e) Probe + 1 mM Cys + 50 μ M S_8 ; (f) 10 μ M Compound **5** + 50 μ M S_8 .

Figure 3.

(A) Fluorescence intensity of 10 μM **PSP-3** in the presence of various RSS: (1) probe alone; (2) 100 μM Hcy; (3) 100 μM GSSG; (4) 200 μM Na_2S ; (5) 100 μM $\text{Na}_2\text{S}_2\text{O}_3$; (6) 100 μM Na_2SO_3 ; (7) 100 μM Na_2SO_4 ; (8) 100 μM $\text{CH}_3\text{SSSCH}_3$; (9) 50 μM Na_2S_2 ; (10) 50 μM Na_2S_4 . (B) Fluorescence intensity of **PSP-3** (10 μM) in the presence of ROS (with or without H_2S). (1) probe alone; (2) 200 μM H_2O_2 ; (3) 50 μM ClO^- ; (4) 50 μM O_2^- ; (5) 50 μM $\bullet\text{OH}$; (6) 50 μM $^1\text{O}_2$; (7) 200 μM H_2O_2 + 50 μM Na_2S ; (8) 200 μM H_2O_2 + 100 μM Na_2S ; (9) 50 μM ClO^- + 100 μM Na_2S ; (10) 50 μM O_2^- + 100 μM Na_2S ; (11) 50 μM $\bullet\text{OH}$ + 100 μM Na_2S ; (12) 50 μM $^1\text{O}_2$ + 100 μM Na_2S .

Figure 4. Fluorescence images of exogenous H_2S_n in HeLa (a–c), RAW264.7 (d–f), and Vero (g–i). Cells were incubated with **PSP-3** (5 μM) for 25 min, then washed and subjected to different treatments. (a, d, and g) controls (no Na_2S_2 or Na_2S_4); (b, e, and h) treated with 30 μM Na_2S_2 ; (c, f, and i) treated with 30 μM Na_2S_4 .

Figure 5.

Fluorescence images of endogenous H_2S_n in COS-7 (a–d) and Vero (e–h). (a) Cells not treated with **PSP-3**; (b) Normal cells treated with **PSP-3**; (c) CSE-overexpressed cells treated with **PSP-3**; (d) Cells treated with 20 μM Na_2S_2 . (e–g) Cells were incubated with **PSP-3** (5 μM) for 25 min, washed, and subjected to different treatments. (e) cells were incubated with FBS-free media for 30 min; (f) cells were incubated with 30 μM D-Cys for 30 min; (g) cells were incubated with 1 mM D-Cys for 30 min; (h) cells were pre-treated with sodium benzoate (250 μM) for 30 min, then washed and incubated with **PSP-3** (5 μM) for 25 min, and then washed and incubated with 1 mM D-Cys for 30 min.