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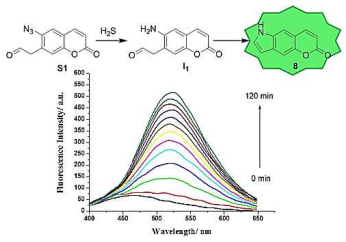
Assembly of indole fluorophore in situ for hydrogen sulfide signalling through substrate triggered intramolecular reduction/cyclization cascade: A sensitive and selective in aqueous solution

Ji Zhou, Yuanyuan Luo, Qiang Li, Jiaoning Shen, Rui Wang, Yufang Xu* and Xuhong Qian*



The fluorescence enhancement is due to the formation of the indole fluorophore through reduction by H_2S and cyclization.

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ARTICLE TYPE

Assembly of indole fluorophore in situ for hydrogen sulfide signaling through substrate triggered intramolecular reduction/cyclization cascade: A sensitive and selective probe in aqueous solution

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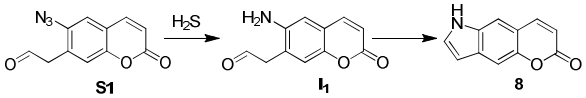
A novel probe of azide-coumarin-aldehyde triad (S1) for hydrogen sulfide (H_2S) was developed. The fluorophore for signaling is assembled in situ through H_2S triggered intramolecular reduction/cyclization cascade, and has a concomitant 10-fold enhancement of the fluorescent intensity at 520 nm. Fluorescence imaging of H_2S in living cells proved its potential for biological application.

Hydrogen sulfide (H_2S), an important member of endogenous gaseous signaling family[1], plays pivotal roles in a wide range of physiological processes, such as oxygen sensing[2], vasodilation[3], inflammation[4] and cardiovascular protection[5]. The concentration of H_2S ranges from 10 to 100 μM in blood[4, 6], and may reach 600 μM in the brains of rat, bovine and human[7]. The abnormal H_2S levels in human bodies have been associated with various diseases, such as Alzheimer's disease[8], Down's syndrome[9], chronic kidney disease[10] and liver cirrhosis[11]. It was also reported that H_2S can act as a scavenger for reactive oxygen species (ROS) and up-regulate brain antioxidant level[12]. Therefore, reliable methods for H_2S monitoring or detection can greatly promote the related biomedical research, have implications in disease diagnosis and therefore have been actively sought after. [1]

Methods based on metal-induced sulfide precipitation,[13] electrochemistry[14], colorimetry[15] and gas chromatography[16] are robust, though limited with low through-put. Fluorescence-based methods have attracted much attention because of the paramount sensitivity, selectivity and instrumental versatility. In addition, they are particularly suitable for in vitro and in vivo studies[17]. Existing fluorescent probes for H_2S are based on either its capability of reducing the electron withdrawing azido[18], nitro[19] or hydroxyamino[20] groups into electron donating amine, or its high nucleophilicity towards soft electrophiles such as various Michael acceptors[21], disulfide[22], aryl halide[23] and more[7d, 24]. The common theme of the existing H_2S probes is that H_2S triggers a transformation, which regenerates the push-pull scaffold of the pertinent fluorophores and hence mediates the signal transduction. Most fluorescent probes based on the dual nucleophilicity of H_2S often involve the initial nucleophilic attack of H_2S, the cyclization resulting from a second nucleophilic attack as well as the release of the fluorophore in the end[21d, 22a, 23b, 24a]. Herein, we report a novel H_2S probe, which, upon detection, exhibits an expansion in the conjugated system of a fluorophore and a desired fluorescence increase. Inspired by the work of Sames et al in developing probes for monoamine oxidases,[25] we design the assembly of indole fluorophore in situ[26] for hydrogen sulfide signaling through substrate triggered intramolecular reduction/cyclization cascade, which results in conjugation-expansion and showed a favorable fluorescence enhancement. Besides a red-shift can also be observed which is advantageous compared to a simple "turn-on" or "turn-off" signal since a false positive is less likely. Sames reported the "*in situ assembly*" probe design based on an oxidative deamination-cyclization sequence, whereas our work expands the scope of the substrates of this design and should be meaningful to the field of molecular probe community.

In this report, we designed and synthesized the probe **S1** (Scheme 1), which has an azido group and a formylmethyl group in close proximity. Reduction of the azido group by H_2S gives 7-formylmethyl-6-aminecoumarin (**I_1**), which spontaneously condensates to yield a conjugation-expanded fluorophore (**8**).



Scheme 1 The mechanism for H_2S detection.

The probe was prepared in seven steps (Scheme 2). Compounds **1-6** were synthesized according to the reported literature[25, 27] (Scheme S1, ESI†). Compound **6** could undergo diazotization reaction to afford compound **7**. The target molecule **S1** was obtained by oxidation of compound **7** with PCC. The reaction product **8** was prepared by hydrogenation of compound **4** using PtO_2 (Scheme S2, ESI†). Their structures were confirmed by ^1H NMR, ^{13}C NMR, and HRMS spectra (Section 4, ESI†).

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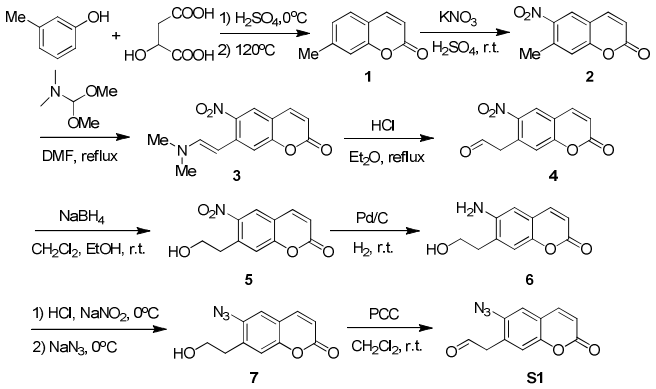

Scheme 2 The synthesis of the probe **S1**

First we investigated the spectroscopic properties of **S1** in the absence and presence of NaSH. Data were acquired at 37°C in water containing 5% CH_3CN as co-solvent. Free probe **S1** displayed a fluorescence band at 460 nm with the excitation maximum at 340 nm. The probe solution (15 μM in pure H_2O) was treated with 100 μM of NaHS solution (a common H_2S donor) and the emission was recorded. To our delight, an obvious fluorescence increase was observed at 520 nm with a 10-fold enhancement (Fig. 1a), which was in agreement with the spectroscopic data of independently synthesized compound **8**. Formation of **8** upon H_2S is also confirmed by ESI-MS experiment (Section 3, ESI†), with a peak at 186.1, which is $[\mathbf{8}+H]^+$. This supports our proposed detection mechanism (Scheme 1).

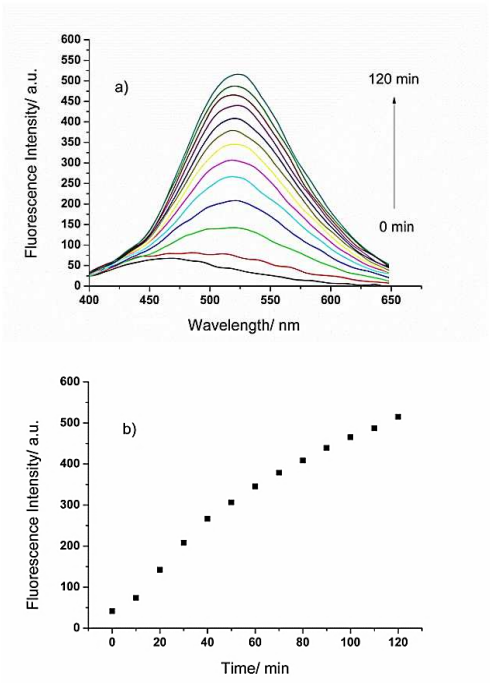

Fig. 1 (a) The emission spectra of S1 (15 μM) with H_2S (100 μM) in the water with 5% CH_3CN as co-solvent at 25°C. The data were recorded every 10 min. (b) Fluorescence responses ($I_{520\,nm}$) of S1 (15 μM) to H_2S (100 μM). Excitation wavelength was 340 nm. Slit: 10 nm, 10 nm.

Next we investigated the sensitivity of probe **S1** toward H_2S. Reaction of **S1** with H_2S at an increasing concentration results in a gradual enhancement in fluorescent intensity. Then 15 μM concentration of the probe was used to determine the sensitivity of the probe. A good linearity between the fluorescent intensity at 520 nm and the H_2S concentration in the range of 10-80 μM was observed, as shown in Fig.2b. The detection limit of the probe was calculated to be 0.22 μM (S/N=3) according to the method of the previous procedure[24c], indicating that the probe is sensitive and may be potentially useful in the detection of the physiological relevant H_2S whose concentration ranges from nano- to millimolar levels[18j, 24a].

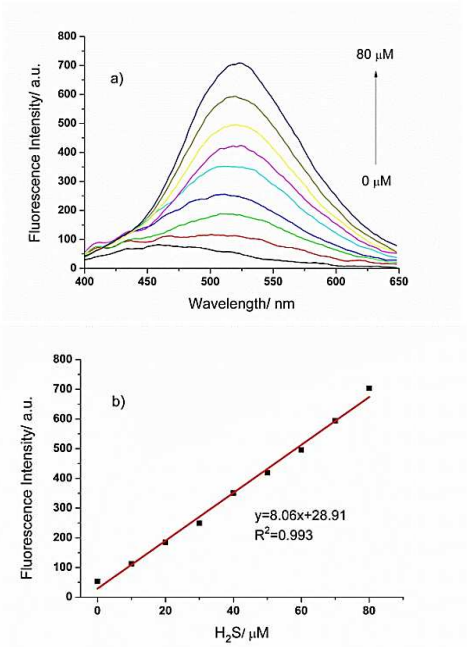

Fig. 2 (a) The emission spectra of the probe **S1** (15 μM) upon addition of H_2S (10 μM-80 μM) in the water (containing 5% CH_3CN as cosolvent). The data were recorded after 2.5 h. Excitation wavelength was 340 nm. Slit: 10 nm, 10 nm. (b) A liner calibration graph of the fluorescence responses ($I_{520\,nm}$) of S1 (15 μM) to H_2S (10 μM-80 μM).

To examine the selectivity of the reaction for H_2S, the probe S1 was treated with various species in the water, including Cl^-, F^-, Br^-, I^-, SO_3^{2-}, NO_3^-, HCO_3^-, H_2O_2, $S_2O_3^{2-}$, glutathione, cysteine and homocysteine (Fig. 3). Addition of these species did not give rise to the fluorescence enhancement at 520 nm, whereas 150 μM H_2S could lead to a 13-fold fluorescence enhancement, which indicated that S1 had a high selectivity for H_2S over other species.

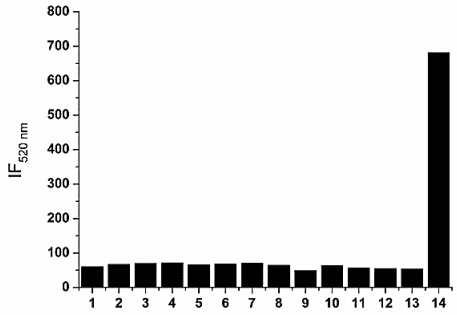

Fig. 3 Fluorescence responses of the probe **S1** (15 μM) to various relevant species in the water (with 5% CH_3CN) at room temperature. Excitation wavelength was 340 nm. Slit: 10 nm, 10 nm. Bars represent the final fluorescence intensity of **S1** with the following analytes over the original probe. (1) blank (2) Cl^- (2 mM) (3) F^- (2 mM) (4) Br^- (2 mM) (5) I^- (2 mM) (6) SO_3^{2-} (2 mM) (7) NO_3^- (2 mM) (8) HCO_3^- (2 mM) (9) H_2O_2 (2 mM) (10) $Na_2S_2O_3$ (2

mM) (11) GSH (2 mM) (12) Hcy (2 mM) (13) Cys (2 mM) (14) NaSH (150 μM)

We also investigated whether our probe can sense H_2S in the living cells. HeLa cells were incubated with the probe **S1** (15 μM) in culture medium for 20 min at 37 °C and washed with phosphate buffer (pH 7.4). Then the cells were treated with NaHS (250 μM) for another 40 min at 37 °C and washed three times with phosphate buffer (pH 7.4). As shown in Fig. 4b and 4d, HeLa cells without NaHS showed very weak fluorescence, whereas the cells treated with NaHS showed strong fluorescence in green channels (535-600 nm). This result indicates that the probe **S1** has the potential for biological application.

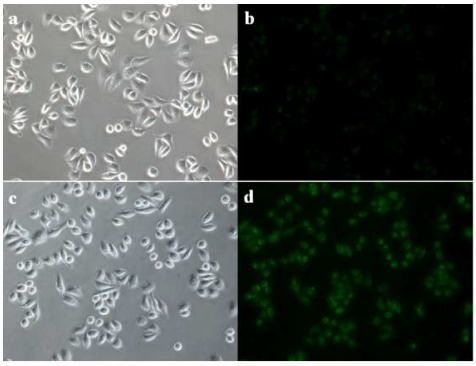

Fig. 4 Fluorescence and bright-field images of HeLa cells. (a) Bright-field image of HeLa cells incubated with **S1** (15 μM) for 20 min. (b) Fluorescence image of HeLa cells incubated with **S1** (15 μM) for 20 min. (c) Bright-field image of HeLa cells first incubated with **S1** for 20 min and then with NaHS (250 μM) added for another 40 min. (d) Fluorescence image of HeLa cells first incubated with **S1** for 20 min and then with NaHS (250 μM) added for another 40 min.The figures at the top right and the bottom right were collected at 535-600 nm (green channel).

In summary, we have developed a fluorescent probe for the detection of H_2S based on a conjugation expansion mechanism with good sensitivity and selectivity. The indole fluorophore for signaling is assembled in situ through substrate triggered intramolecular reduction/cyclization cascade. Moreover, the detection limit of the probe was calculated to be 0.22 μM. Fluorescence imaging of HeLa cells showed that the probe **S1** can be used in living cell imaging.

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28. Experimental section: (1) **Synthesis of 7-hydroxyethyl-6-azidecoumarin (compound 7)** To a 0 ^{o}C solution of compound 6 (400 mg, 1.95 mmol) in EtOAc (1 mL) and water (1 mL) was added concentrated hydrochloric acid (2 mL). The mixture was stirred for 15 min. To this solution was added a solution of sodium nitrite (245 mg, 3.51 mmol) in water (0.74 mL) over 5 min. After completion of the addition, the reaction was stirred for an additional 1.5 h. A solution of sodium azide (230 mg, 3.51 mmol) in water (0.7 mL) was added over 5 min. After 1 h, the reaction mixture was diluted with water (100 mL), extracted with ethyl acetate (2×50 mL). The combined organic layer was washed with dilute sodium hydroxide solution, then with brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel flash chromatography with 1:30 EtOAc/ CH_2Cl_2 to give the title compound (225 mg, 50%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.07 (d, J = 9.6 Hz, 1H), 7.67 (s, 1H), 7.32 (s, 1H), 6.50 (d, J = 9.6 Hz, 1H), 4.73 (t, J = 5.2 Hz, 1H), 3.61 (q, J = 6.4 Hz, 2H), 2.77 (t, J = 6.8 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.32, 150.89, 143.75, 135.91, 134.90, 118.99, 118.40, 117.77, 116.78, 60.70, 34.81. HRMS (EI): Calcd for $C_{11}H_9N_3O_3$ 231.0644; Found, 231.0646. (2) **Synthesis of 7-formylmethyl-6-azidecoumarin (compound S1)** To a solution of compound 7 (231 mg, 1 mmol) in anhydrous CH_2Cl_2 (30 mL) was added PCC (323 mg, 1.5 mmol). Then the mixture was stirred at room temperature for 4 h and filtered on diatomite. Then the diatomite cake was washed with CH_2Cl_2. To the filtrate was added water (100 mL). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel flash chromatography with 1:2 PE/ CH_2Cl_2 to give the title compound (45 mg, 20%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.78 (s, 1H), 7.70 (d, J = 9.6 Hz, 1H), 7.27 (s, 1H), 7.20 (s, 1H), 6.50 (d, J = 9.6 Hz, 1H), 3.81 (br, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 196.90, 160.04, 150.96, 141.93, 135.55, 128.71, 120.09, 119.06, 117.86, 116.19, 46.11. HRMS (EI): Calcd for $C_{11}H_7N_3O_3$ 229.0487; Found, 229.0486.