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ORIGINAL PAPER

Coumarin-based Fluorescent Probes for H_2S Detection

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Abstract Although hydrogen sulfide (H_2S) has been known as a toxic gas with unpleasant rotten egg smell, the correlation between H_2S and physiological processes has attracted scientists to develop brand new methods to monitor such a gaseous molecule in vitro and in vivo. Herein, we described a couple of coumarin-based fluorescent probes (**1a** and **1b**) that can be activated by reduction of azide to amine in the presence of H_2S. It should be emphasized that probe **1b** demonstrated high selectivity and sensitivity for H_2S over other relevant reactive sulfur species in vitro, as well as identified exogenous H_2S in living cells.

Keywords Coumarin · Fluorescent probe · H_2S · Cell imaging

Introduction

Recent years have witnessed that hydrogen sulfide (H_2S), well known as a undesired gas in life sciences, is a very meaningful endogenous gas compound for moderating a broad range of physiological processes, including—but not limited to—regulation of inflammation [1, 2], relaxation of vascular systems,[3] intervention of neurotransmission,[4] antioxidant,[5] mediation of insulin signaling,[6] and inhibition of apoptosis.[7, 8] The concentration of H_2S can vary from nano- to micro-molar level depending on physiological and pathological states.[9] It should be noted that this gas molecule has been considered as the third gaseous transmitter as well,[10–12] in addition to nitric oxide (NO)[13] and carbon monoxide (CO).[14] Obviously, it is very indispensable to trace and measure this small molecule for the studies within biological specimens. However, current major methods for H_2S detection, such as colorimetric[15, 16] and electrochemical assays,[17, 18] sulfide precipitation[19] and gas chromatography,[20] often require complicated sample processing and destruction of cells or tissues, which would lead to predicament in the accurate analysis of this important molecule.

Fortunately, the rapid progress drives the emergence of the assay for H_2S detection, which possesses the biotarget sensitivity and synthetic convenience. Up to date, a variety of fluorescent probes for tracking the H_2S in biological samples are available as reflecting diverse state-of-the-art OFF/ON fluorescent mechanism, including release of fluorophore triggered by undergoing nucleophilic reaction twice, [21] control of fluorescence based on azamacrocyclic Cu^{2+} complex chemistry, [22] as well as reduction of azide to amine.[23, 24]

In the current research, coumarin, a well-known fluorophore along with high quantum yield, small size, high permeability and easy synthesis handling, was employed as the fluorescent scaffold. It's pretty recognized that the substitution at the 3- and/or 7-positions of coumarin provides an indicative influence on its fluorescence property. Therefore, the coumarin analogues **1a** and **1b** were designed to be caged by the 3-azido group with no or weak fluorescence. Once reduction of azide to amine was triggered by hydrogen sulfide, these coumarin analogues would shed high fluorescence because of the distinction of electronegativity between the azido and amino groups (Scheme 1). [25]

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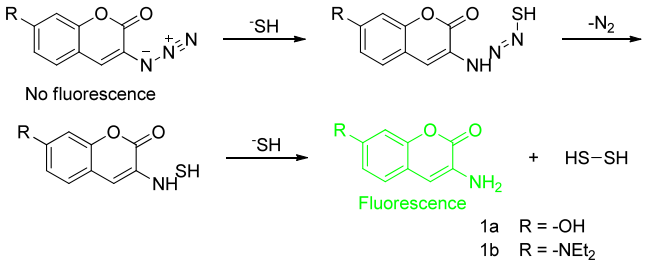


Scheme 1 Possible H_2S activation mechanism of fluorescent probes **1a** and **1b**

Experimental Section

Synthesis [26]

Materials and Instruments Chemicals used in the syntheses were purchased from Acros and Aldrich. Milli-Q water was used throughout all experiments. ^{1}H NMR and ^{13}C NMR were recorded on a Bruker 300 NMR spectrometer. Mass spectra were performed by the analytical and the mass spectrometry facilities at Shandong University.

Synthesis of Probe **1a**

2,4-dihydroxy benzaldehyde (2.76 g, 20 mmol), *N*-acetylglycine (2.34 g, 20 mmol), and anhydrous sodium acetate (4.92 g, 60 mmol) were dissolved in acetic anhydride (85 ml). The reaction mixture was refluxed for 5 h, and then poured onto ice to give a yellow precipitate. After filtration, the precipitate was washed by ice water and dried. A yellow solid (2.03 g) was obtained as **2a** (yield 38.9 %) in Scheme 2.

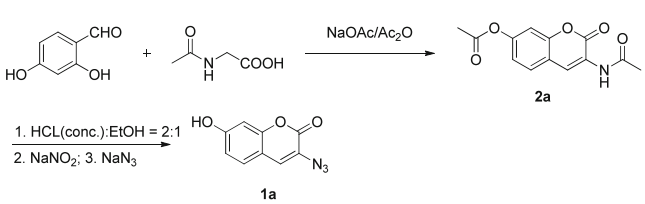
The yellow solid **2a** (0.3 g, 1.15 mmol) was refluxed in a solution of conc. HCl and ethanol (2:1, 15 mL) for 1 h, then ice water was added to dilute the solution. The solution was cooled in an ice bath and $NaNO_2$ (3.48 mmol) was added. After stirring for 10–15 mins, NaN_3 (5.23 mmol) was added in portions. The solution was stirred for another 1 h, the precipitate was filtered off, washed with ice water, and dried under reduced pressure to afford a brown solid; The solid was further purified with silica gel chromatography, elution with petroleum ether/ethyl acetate (10:1) afforded 0.2 g of pure **1a** (yield 52.2 %).^{1}H-NMR (DMSO-d_6, 300 MHz) δ 6.759 (d, J=2.1 Hz, 1 H), 6.810 (dd, J_1=8.4 Hz, J_2=2.1 Hz, 1 H), 7.478 (d, J=8.4 Hz, 1 H), 7.593 (s, 1 H), 10.538 (s, 1 H); ^{13}C NMR (DMSO-d_6, 300 MHz): δ 102.00, 111.29, 113.75, 121.10, 127.82, 129.04, 152.73, 157.25, 160.25; ESI-MS: m/z 226.2 $[M+Na]^+$; IR (KBr, cm^{-1}): 3301 (Ar-OH), 2121(N_3).

Synthesis of Probe **1b**

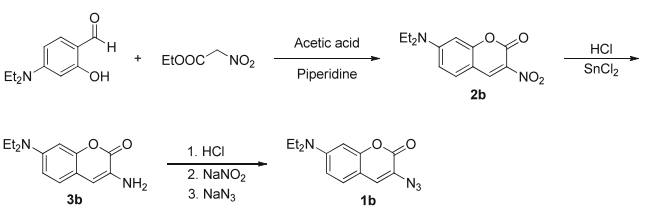
A mixture of 4-diethylamino salicylaldehyde (1.41 g, 7.2 mmol), ethyl nitroacetate (0.82 mL, 7.2 mmol), piperidine (0.1 mL), acetic acid (0.2 mL) and molecular sieves 4 Å in n-butanol (20 mL) was refluxed for a period of 24 h. Upon cooling to room temperature, a bright yellow solid formed, which was collected and dissolved in DMF (20 mL) at 80 °C. It was filtered again to remove the molecular sieves. The filtrate, upon addition to 100 ml of ice-cold water, yielded 3-nitro-7-diethylamino coumarin (**2b**) as a bright yellow solid: 1.30 g, 68.8 % (Scheme 3).

A suspension containing 37.4 % HCl (15 mL) and stannous chloride dihydrate (4.65 g, 20.625 mmol) was stirred at room temperature. **2b** (0.72 g, 2.75 mmol) was added in small portion. Stirring was continued for 5 h before the solution was poured onto 40 g of ice and made alkaline using sodium hydroxide solution (5 M) in an ice bath. The resulting suspension was then extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate and concentrated. A pale yellow solid of 0.58 g as 3-amino-7-diethylaminocoumarin **3b** was obtained (yield 91 %).

3b (310 mg, 1.3 mmol) was dissolved slowly in HCl aq. (18 %, 12 mL) at room temperature. The solution was cooled in an ice bath and a 5 mL solution of $NaNO_2$ (135 mg, 1.95 mmol) was added, the reaction mixture was stirred for 80 mins in an ice bath. This was followed by the addition of potassium acetate to adjust the pH of the resulting solution to 4. Then, NaN_3 (254 mg, 3.9 mmol) was added in portions. And the mixture was stirred at 0–5 °C for another 4 hours. The precipitated product was rapidly filtered, washed with ice-cold water and recrystallized by a mixed solvent of petroleum ether and ethyl acetate. A yellow needle crystal was obtained ^{1}H-NMR (DMSO-d_6, 300 MHz) δ 1.206 (t, J=7.2 Hz, 6 H), 3.407 (q, J=7.2 Hz, 4 H), 6.513 (d, J=2.4 Hz, 1 H), 6.595 (dd, J_1=8.7 Hz, J_2=2.4 Hz, 1 H), 7.100 (s, 1 H), 7.192 (d, J=8.7 Hz, 1 H); ^{13}C NMR (DMSO-d_6, 300 MHz): δ 11.92 (2 C), 44.29 (2 C), 96.91, 107.54, 108.96, 119.10, 127.06, 127.63, 149.34, 153.67, 157.86. ESI-MS: m/z 259.1189 $[M+H]^+$; IR (KBr, cm^{-1}): 2119(N_3).



Scheme 2 Synthesis of probes **1a**



Scheme 3 Synthesis of probes **1b**

Fluorometric Analysis

Buffer reagents were purchased from Aldrich and Acros and were used without purification. Water used for the fluorescence studies was doubly distilled and further purified with a Mill-Q filtration system. Nanodrop ND3300 Fluorospectrometer was used for all fluorescent studies.

Fluorescence Responses to H_2S

Methods 100 μL of 20 μM solution of **1a** or **1b** was prepared in fresh degassed 100 mM phosphate buffer at pH 7.4 (0.05 % DMF). And 100 μL of 200 μM solution of NaSH in fresh degassed 100 mM phosphate buffer at pH 7.4 was added for a final concentration of 10 μM **1a** or **1b**. Then, the fluorescent spectra were measured by Nanodrop ND3300 Fluorospectrometer at room temperature. Emission spectra were collected between 400 nm and 700 nm with λ_{ex}=390 nm (**1a**) or 380 nm (**1b**). Time points represent 0, 5, 15, 30, 45, and 60 min after addition of NaSH. The blank spectrum (black) was acquired from a 10 μM solution of **1a** or **1b** without the addition of NaSH.

Results and Discussion The probes have no or very weak fluorescence in 100 mM sodium phosphate buffer (pH 7.4, 0.05 % DMF). However, upon treatment of 10 μM **1a** or **1b** with 100 μM NaSH (a regular H_2S donor), they can emit a strong fluorescence enhancement as proposed. Within 1 h of reaction with NaSH, **1a** and **1b** generated 4.5- and 40-fold fluorescence enhancement, respectively (Fig. 1). Moreover, the fluorescence quantum yield of **1a** and **1b** was (0.16±0.013) % and (0.58±0.02) % in the absence of NaSH, respectively; in the meanwhile after 1 h reaction with NaSH, the quantum yields significantly increased to (0.66±0.015) % and (10.93±0.15) %, respectively. In view of the high quantum yield and fast reactivity of molecule **1b**, it could be a reasonable fluorescent toolkit for the forthcoming analysis.

Selectivity Tests

Methods Selectivity for probes **1a** or **1b** was measured by fluorescence responses at 0,5, 15, 30, 45, and 60 min with λ_{ex}=390 nm and λ_{em}=483 nm for **1a** or λ_{ex}=380 nm and λ_{em}=515 nm for **1b**, respectively. The final concentration of **1a** or **1b** was 10 μM while various reactive sulfur species

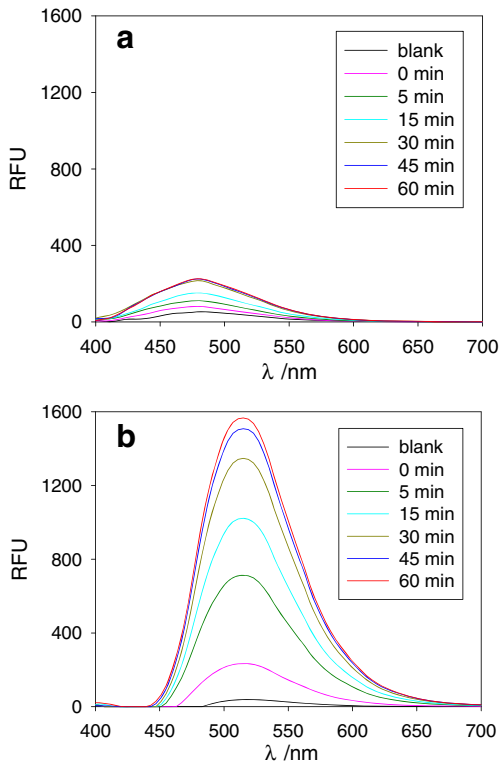


Fig. 1 Fluorescence responses of (**a**) 10 μM **1a** and (**b**) 10 μM **1b** to 100 μM NaSH. Data were acquired at 25 °C in 100 mM PBS buffer (pH7.4, 0.05 % DMF) with excitation at λ_{ex}=390 nm (**1a**) and 380 nm (**1b**). Emission was collected between 400 and 700 nm. Time points represent 0, 5, 15, 30, 45, and 60 min after addition of 100 μM NaSH. The black lines represent without addition of NaSH

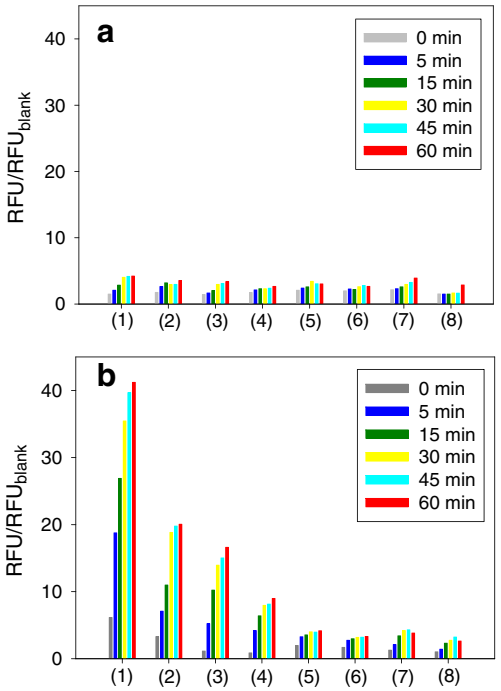


Fig. 2 Fluorescence responses of (**a**) 10 μM **1a** and (**b**) 10 μM **1b** to biologically relevant RSS. Bars represent relative responses at 483 nm (**1a**) and 515 nm (**1b**) at 0, 5, 15, 30, 45, and 60 min after addition of 100 μM RSS. Data were acquired in 100 mM PBS buffer (pH7.4, 0.05 % DMF) with excitation at λ_{ex}=390 nm (**1a**) and 380 nm (**1b**), respectively. RFU_{blank} means the relative fluorescent intensity of **1a** and **1b** without RSS. Legend: (1) NaSH; (2) cysteine; (3) glutathione; (4) thiophenol; (5) 4-chlorophenyl thiophenol; (6) 2-amino thiophenol; (7) NaSCN; (8) $NaHSO_3$

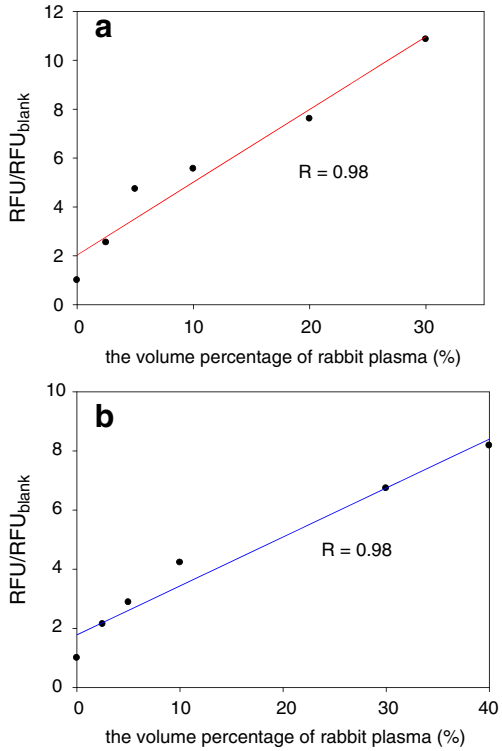


Fig. 3 Titration curves of (**a**) 10 μM **1a** and (**b**) 10 μM **1b** after treated with different volume percentage of commercial rabbit plasma (from 0 to 40 %) at room temperature. RFU_{blank} represents the relative fluorescent intensity without addition of rabbit plasma

were 100 μM. All assays were performed in 100 mM PBS buffer (pH7.4, 0.05 % DMF) at room temperature except the 4-chlorophenyl thiophenol and 2-amino thiophenol in 100 mM PBS buffer with 0.2 % ethanol.

Results and Discussion Afterwards, to examine the selectivity of these probes, various relevant reactive sulfur species (RSS) were added to the 10 μM **1a** or **1b** in 100 mM phosphate buffer (pH 7.4) with 0.05 % DMF. The read-out results manifestly showcased that both molecules present plausible selectivity for RSS. It should be emphasized that **1b** possesses higher sensitivity and selectivity for H_2S than **1a** assuming a greater fluorescence response to H_2S and higher RFU/RFU_{blank} versus cysteine and glutathione (2-fold), thiophenol (4-fold), and other relevant RSS (8-fold) (Fig. 2). It's well recognized that the electron-donating ability of substituent at the 7-position of 3-azidocoumarins can strengthen the intramolecular charge transfer (ICT) effect, thus manifesting enhancement of the fluorescence intensity and monopolizing the emission color [26, 27]. That may be the reason that probe **1b** has higher fluorescence response and better selectivity than **1a**.

Sulfide Detection of Rabbit Blood Plasma

In addition, considering the high fluorescent response for thiols of these probes, their abilities to detect the thiol in commercial rabbit plasma were evaluated as well.10 μL of 200 μM solution of **1a** or **1b** was prepared in fresh degassed 100 mM phosphate buffer at pH7.4 (0.05 % DMF). Next, different volumes of the rabbit blood plasma aliquots (0, 5, 10, 20, 40, 60 or 80 μL) and corresponding phosphate buffer (pH=7.4) aliquots (190, 185, 180, 170, 150, 130 or 110 μL) were added directly to 10 μL of 200 μM **1a** or **1b** solution with a final

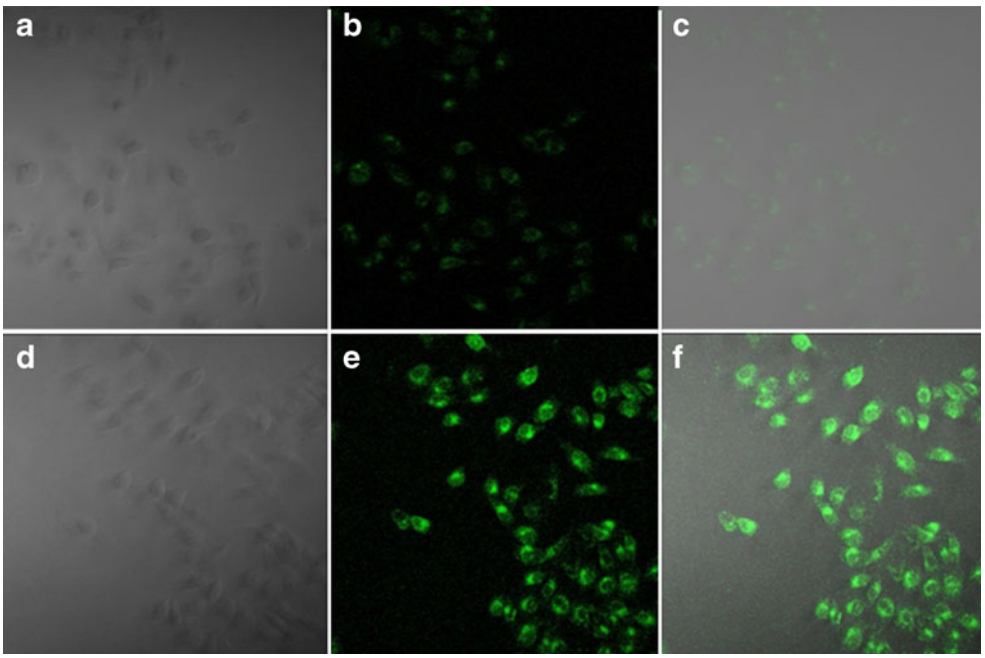


Fig. 4 Confocal microscopy images of H_2S detection in living PC-3 cells using **1b**. **a** is the bright field images of cells incubated with probe **1b** (20 μM), and **b** is the corresponding fluorescence images of (**a**); **c** is the overlap of (**a**) and (**b**); **d** represents the bright field images of cells incubated with probe **1b** (20 μM), after preincubation with 100 μM NaSH for 15 min at 37 °C; **e** represents the corresponding fluorescence images of (**e**); **f** represents the overlap of (**d**) and (**e**)

concentration of 10 μM. The details were following in Table s1. The mixture was shaken well. After 1 h, the relative fluorescent intensity (RFU) at 483 nm (**1a**) or 515 nm (**1b**) was recorded. The excellent linear relationship of RFU/RFU_{blank} against different volume percentage of rabbit plasma was obtained with R-value=0.98 (Fig. 3). In brief, these interesting results proposed that both **1a** and **1b** could be the promising tool for measuring thiols in biological samples.

Confocal Imaging Experiments

For probe **1b**, its well-behaved fluorescent property and selectivity for H_2S seemed that such molecule could be measured for the detection of H_2S in cultured cells. In this case, living PC-3 cells were used to investigate the fluorescence imaging by confocal microscopy. PC-3 cells were cultured in 24 well cell culture plate (Costar) as a monolayer in RPMI 1640 (Hyclone) supplemented with 10 % fetal bovine serum (FBS, Hyclone) at 37 °C in 5 % CO_2 overnight. And these cells were treated with 100 μM NaSH in 100 mM PBS buffer (pH 7.4, 0.05 % DMF) for 15 min at 37 °C room temperature and then incubated with 20 μM probe **1b** in RPMI 1640 for an additional 30 min at room temperature. And for the blank control, cells were incubated with 20 μM probe **1b** for 30 min without treated with NaSH. Images were collected by fluorescent confocal microscopy (OLYMPUS DP72).

As depicted in Fig. 4, PC-3 cells incubated with probe **1b** (20 μM) for 30 min without treated with NaSH provide weak fluorescent cells, which may be caused by the reaction between thiols and **1b** as described above. After PC-3 cells were treated with exogenous 100 μM NaSH for 15 min at 37 °C and then incubated with probe **1b** for an additional 30 min at room temperature, they could emit strong fluorescence imaging in the cells. These thought-provoking results confirmed again that probe **1b** could be employed for tracking H_2S in living cells.

Conclusion

In the current paper, two azido-caged coumarin analogues **1a** and **1b**, which can be applied to measure the reactive sulfur species in physiological environment, were well designed and synthesized herein. Probe **1b** displayed high sensitivity and excellent selectivity for H_2S over other relevant reactive sulfur species, and also could be used for both detecting H_2S in rabbit plasma and for real-time fluorescence imaging of H_2S in living cells. Notably, such a fast, proficient and cost-effective approach for H_2S detection would be very applicable in the modern emerging area of hydrogen sulfide.

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