Research article

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A fluorescent probe for biothiols based on the conjugate addition of thiols to α,β-unsaturated ester

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ABSTRACT: A sensitive fluorogenic probe 1 for biothiols was developed based on the Michael addition reaction. The probe 1 was readily synthesized via the reaction of 2-(4′-hydroxyphenyl) benzimidazole (HPBI) with acryloyl chloride and shows weak fluorescence emission. Upon mixing with biothiols, the fluorescence of 1 is significantly enhanced due to the conjugate addition of thiols to the α,β-unsaturated carbonyl moiety, thus eliminating the photoinduced electron transfer (PET) quenching of the fluorophore by the intramolecular carbon–carbon double bond. Cysteine (Cys) was selected as the representative thiol in the spectral experiment. A good linear relationship was obtained from 1.0 to 30.0 μmol L^{-1} for Cys and the detection limit was 0.17 μmol L^{-1}. Furthermore, probe 1 was highly selective for biothiols without the interference of some biologically relevant analytes and has been applied to detecting biothiols in human urine samples. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: biothiols; benzimidazole; fluorescent probe; Michael addition

Introduction

Low-molecular-weight aminothiols such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) continue to be of interest because the low/high level of aminothiols in the biological fluid is involved in various clinical disorders such as AIDS, heart disease, Alzheimer's and cardiovascular disease (1–3). Thus, the rapid, sensitive and selective detection of thiols is of considerable importance and significant interest. Among the various reported methods, fluorescent chemosensors and probes are more desirable since they are simple, sensitive and efficient. In the past few years, various fluorescent probes for thiols based on different mechanisms have been exploited, including Michael addition (4–9), cyclization reaction with aldehyde (10–18), cleavage reaction by thiols (19–27), and others (28–32). However, most of the fluorescent thiol probes developed so far are associated with some limitations, such as limited pH range (9), high fluorescence background (11), long response time (13), etc. Therefore, the development of fluorescent probes specifically recognizing biothiols under physiological conditions is of current interest.

More recently, the conjugate addition of thiols to α,β-unsaturated carbonyl compounds has been utilized for the developed fluorescent thiol probes (4–7). Among them, the maleimide-based probes have been widely reported (4,6,7), based on the attachment of a maleimide group to dye fragments, resulting in quenching of the fluorophore due to the intramolecular PET process, and the fluorescence quenching is relieved when the π-system of the maleimide is disrupted. However, maleimide synthesis can be challenging in some cases, and the deactivation maleimides by water (giving ring-opened amic acids) can compete significantly with thiol modification, particularly above pH 8.0 (33,34). For these reasons, the development of selective thiol-reactive acceptors is a subject of much current interest, especially systems that provide an optical signal upon conjugation.

On the other hand, it was reported that the conjugate 1,4-addition of thiols to α,β-unsaturated carbonyl compounds to form the β-sulfido carbonyls can performed smoothly in aqueous media (35). These results stimulate us to explore whether α,β-unsaturated esters could be employed as a platform for the construction of fluorescent thiol probes that may be potentially useful for thiol detection at physiological pH conditions.

Herein, we developed a new fluorescent thiol probe based on the conjugate addition of thiols to α,β-unsaturated esters. The probe **1** was synthesized via the one-step reaction of HPBI with acryloyl chloride and it displayed high selectivity toward thiols in neutral buffer solutions. In probe **1**, the α,β-unsaturated carbonyl moiety acted as a quencher of fluorophores' fluorescence. The chemosensing of fluorogenic probe **1** involved the conjugate addition of thiols to α,β-unsaturated carbonyl moiety to generate the corresponding β-sulfido carbonyl adduct, thus eliminating the photoinduced electron transfer (PET) quenching of the fluorophore by the intramolecular carbon–carbon double bond and leading to a dramatic increase in fluorescence intensity simultaneously (Scheme 1). The proposed method proves to be

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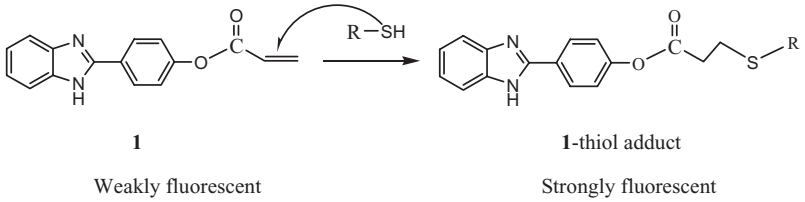
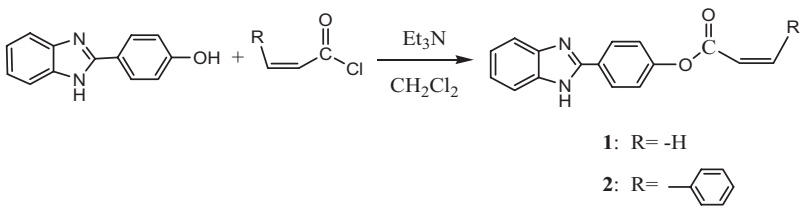


Scheme 1. Reaction of probe **1** with thiol-containing compound.



Scheme 2. Synthesis of **1** and **2** from HPBI

sensitive and selective and has been successfully used to detect biothiols in human urine samples.

Experimental

Apparatus

The fluorescence spectra and relative fluorescence intensity were performed on a Sanco CRT-970 spectrofluorimeter (Shanghai, China) with a 10 mm quartz cuvette. Absorption spectra were determined with a Shimadzu UV-1700 spectrophometer (Tokyo, Japan). Infrared spectra (IR) were taken in KBr pellets on a Bruker Tensor 27 IR spectrometer. Mass spectra were obtained with a Shimadzu GCMS-QP2010 system without separation. ^{1}H NMR spectra were recorded on a Varian Unity INOVA-400 spectrometer with tetramethylsilane (TMS) as the internal standard. All of the measurements were operated at room temperature at about 298 K.

Reagents

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents were purified and dried by standard methods prior to use. Twice-distilled water was used throughout the experiments. L-Cys, GSH (reduced form), 4-hydroxybenzaldehyde and cinnamoyl chloride were bought from Shanghai Chemical Reagent Company (Shanghai, China); DL-Hcy was purchased from Fluka; *o*-phenylenediamine was obtained from Xi'an Chemical Reagent Co.(Xi'an, China).

Synthesis of compound 1

Compound **1** was synthesized in two steps as shown in Scheme 2.

Synthesis of HPBI. HPBI was synthesized following the method described in the literature (36). Briefly, 1.2 g (10 mmol) of 4-hydroxybenzaldehyde was dissolved in 10 mL of ethanol. To this, 2.0 g of sodium bisulfite dissolved in 7 mL of water was added and the above mixture was stirred at room temperature for 4 h. After that, 1.08 g (10 mmol) of *o*-phenylenediamine dissolved in 5 mL DMF was added and the mixtures were refluxed for 5 h. After cooling, the mixtures were poured into ice–water. The precipitate was filtered off, washed with water several times and dried to give the desired product as white solids (1.6 g, 70% yield). ^{1}H NMR (400 MHz, DMSO), δ 9.97 (s, 1H), 7.99 (d, 2H, J = 8.4 Hz), 7.54 (d, 2H, J = 2.4 Hz), 7.16 (m, 2H) 6.91 (d, 2H, J = 3.2 Hz).

Compound 1. Acryloyl chloride was prepared following the method described previously (37). Then, compound **1** was prepared by a simple one-step reaction of HPBI with acryloyl chloride. To a stirred solution of HPBI (1.4 mmol, dissolved in 7 mL of dichloromethane), acryloyl chloride (0.1 mL, 1.5 mmol) and triethylamine (0.5 mL) were added dropwise. The mixture was stirred at room temperature for 4 h. After that, the organic layer was dried *in vacuo* and the crude product was then purified by silica gel (200–300 mesh) column chromatography eluted with ethyl acetate–petroleum ether (1:3, v/v) to give the desired product as a white solid (0.15 g, 31.5% yield). MS: m/z = 264.0 (M^+); M^+ calculated 264.26; ^{1}H NMR (400 MHz, $CDCl_3$), δ8.04 (d, 2H, J = 8.8 Hz), 7.58 (q, 2H, J = 3.2 Hz), 7.22–7.13 (m, 4H), 6.63 (d,1H, J = 16 Hz), 6.33 (m,1H), 6.05(d,1H, J = 10.4 Hz); IR (KBr, cm^{-1}): 3442, 3056, 2365, 1738, 1608, 1498, 1404, 1295, 1205, 1168, 1020, 981, 903, 850, 748.

Compound 2. Compound **2** was prepared by HPBI and cinnamoyl chloride by the same method as that used to obtain compound **1**. MS: m/z = 339.95 (M^+); M^+ calculated 340.16; IR (KBr, cm^{-1}): IR (KBr, cm^{-1}), 3480, 3058, 1768, 1712, 1632, 1496, 1477, 1449, 1394, 1274, 1248, 1167, 1134, 1078, 968, 929, 860, 764, 749, 703, 678; ^{1}H NMR (400 MHz, $CDCl_3$), δ 8.08 (d,1H, J = 8.8 Hz), 7.89 (dd,1H, J = 3.6 Hz), 7.74 (d,1H, J = 8.4Hz), 7.62 (m,3H), 7.44 (m, 4H), 7.27–7.31(m, 3H,), 7.17(s,1H) δ 6.64 (m,1H).

Procedure

Typically, to a 10 mL test tube containing 1.0 mL of NaH_2PO_4–Na_2HPO_4 buffer (0.2 mol L^{-1}, pH = 7.4), 2.0 mL of ethanol and 1.0 mL of compound **1** (0.2 mmol L^{-1}), an appropriate aliquot of

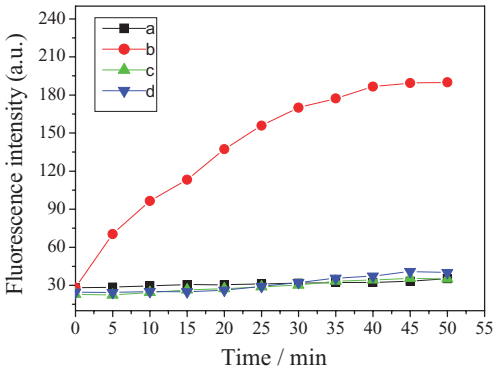


Figure 1. Time dependent fluorescence intensity changes observed during reaction of **1** (or **2**) (20.0 μmol L^{-1}) with Cys (6.0 μmol L^{-1}) in ethanol–water (20:80,v/v) at pH 7.4 phosphate buffer solution. (a) **1** only; (b), **1** + Cys; (c), **2** only; (d), **2** + Cys.

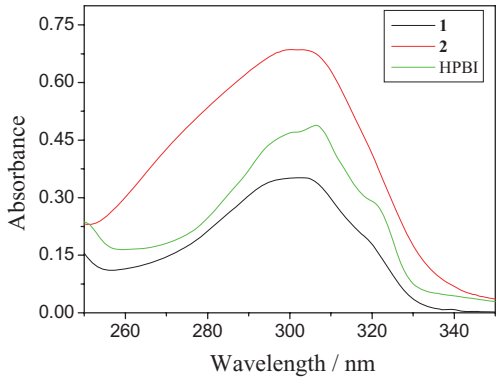


Figure 2. Absorption spectra of compounds **1** and **2** and HBPI in ethanol–water (20:80, v/v) at pH 7.4 phosphate buffer solution. The concentrations of **1**, **2** and HBPI are all 20.0 μmol L^{-1}.

Cys was added and the reaction mixture was diluted to 10.0 mL with water. The resulting solution was allowed to stand at room temperature (298 K) for 30 min, and then the absorption or emission spectra were recorded. For fluorescence intensity measurements, the excitation and emission wavelengths were at 306 and 348 nm, respectively.

Results and discussion

Effect of substituent on the fluorescence response of the probe

In this study, compounds **1** and **2** were synthesized and the reactivity of the probe toward biothiols were investigated by mixing them with Cys at 20% (v/v) buffered (phosphate buffer, pH 7.4) water–ethanol solution, respectively. As can be seen from Fig. 1, the fluorescence intensity of solution **1** increased dramatically upon mixing with Cys (the observed rate constant k_{obsd} at 20°C was found to be 58.4 M^{-1} s^{-1}) (38), whereas the fluorescence intensity of compound **2** remained almost unchanged under identical conditions. The significant difference between them lies in the substituent on the C=C bond of the probe. As for compound **2**, the addition of thiol to α,β-unsaturated carbonyl moiety of the probe is relatively difficult because of the steric hindrance effect, which provides us with some empirical rules on developing thiol-selective probes. Moreover, it was observed that the rate of conjugate addition to the α,β-unsaturated ester was relatively slower compared with that of α,β-unsaturated ketones. This can be explained by the β-carbon atom of α,β-unsaturated ketone being more electrophilic than the corresponding ester derivatives (35).

Spectra characteristics of compounds 1 and 2

The absorption spectra of compounds **1** and **2** and HBPI in a mixture of ethanol and phosphate buffer solution at pH 7.4 (20 mmol L^{-1}) were recorded and are shown in Fig. 2. It was observed that maximum absorption of compounds **1** and **2** and HPBI were both centered at about 304 nm. However, the absorbance followed the order **2** > HPBI > **1**. Furthermore, the fluorescence emission spectra of **1**, **2** and HPBI were also compared (Fig. 3). As expected, the introduction of α,β-unsaturated carbonyl group into HPBI led to almost nonfluorescent compounds **1** and **2**. The weakly fluorescence emission of **1** and **2** can be explained by PET quenching of fluorophore by the intramolecular C=C bond.

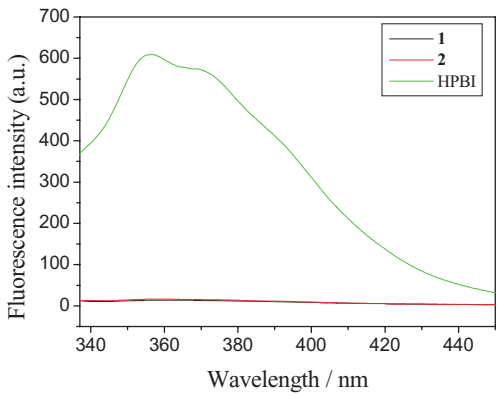


Figure 3. Fluorescence spectra (excitation at 304 nm) of compounds **1** and **2** and HPBI in ethanol–water (20:80, v/v) at pH 7.4 phosphate buffer. The concentrations of **1**, **2** and HPBI are all 8.0 μmol L^{-1}.

Sensing response of probe 1 toward biothiols

The sensing response of probe **1** toward the representative biological thiol (Cys) was examined with absorption and fluorescence spectroscopy. Addition of 30 μmol L^{-1} of Cys to a solution of probe **1** (20 μmol L^{-1}) in ethanol–water (20:80, v/v) at pH 7.4 phosphate buffer exhibited a slight increase in the absorbance at 304 nm (Fig. 4). Furthermore, the changes in the fluorescence spectra of probe **1** in the presence of increasing concentrations of Cys are shown in Fig. 5. The probe **1** itself was weakly fluorescent in 20% ethanol–phosphate buffer solution with a quantum yield (Φ) of 0.020 [using 2-(2′-hydroxyphenyl) benzimidazole as a standard] (39); however, the introduction of Cys caused a dramatic change in the fluorescence emission. The fluorescence emission at 348 nm increased linearly with increasing Cys concentration. The similar experimental phenomena were also observed when Hcy or GSH was added to the solution of **1** (see Supporting Information, Figs S1 and S2). Based on the previous studies (4,5,35), the fluorescence increase is most likely due to the formation of strongly fluorescent **1**-thiol adducts ($\Phi_{\mathbf{1}\text{-Cys}}$ = 0.49). Here, we provide some further evidence to confirm it. Firstly, upon addition of Cys to the solution of **1**, TLC data of the reaction mixture indicated that a new low R_f substance was formed [$R_{f(\mathbf{1})}$ = 0.52, $R_{f(\mathbf{1}\text{-Cys})} \approx 0$, ethyl acetate–petroleum ether (v/v) = 1:1], indicating the interaction of an α,β-unsaturated carbonyl group with Cys. Secondly, the reaction product was separated by TLC and its mass

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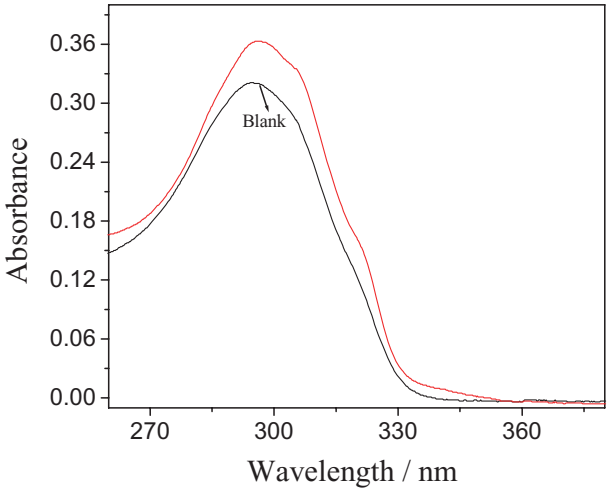

Figure 4. The absorption changes of **1** (20.0 μmol L^{-1}) in ethanol–water (20/80, v/v) at pH 7.4 phosphate buffer solution after addition of Cys (30 μmol L^{-1}) for 30 min.

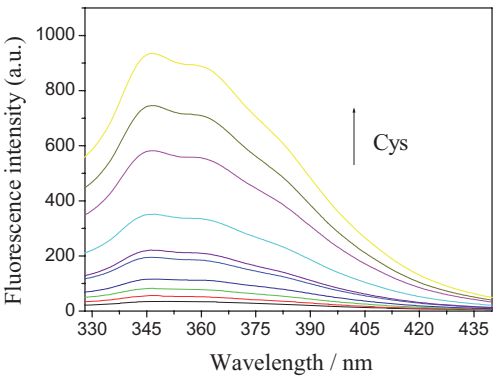

Figure 5. Fluorescence emission spectra (excitation at 306 nm) of **1** (20.0 μmol L^{-1}) upon addition of increasing concentration of Cys (0–20 equiv) in ethanol–water (20:80,v/v) buffered at pH 7.4. Each spectrum was obtained after addition of Cys for 30 min.

spectrum displayed peaks at *m/z* 386.1 [M^+ + H], indicating that **1**-Cys adduct is generated. Finally, the ^{1}H NMR spectra of probe **1** in the absence or presence of Cys were recorded under the identical conditions. As observed from Fig. 6, the resonance signal at δ 6.0–6.7 ppm, corresponding to the C=C bond proton disappeared, which is consistent with the formation of **1**-Cys adduct. Based on the aforementioned experimental results, we conclude that the fluorescence increase of the present system is due to the formation of **1**-Cys adduct.

Effect of pH

The effect of pH on the fluorescence spectrum of probe **1** was studied and the results are shown in Figure 3S (Supporting Information). It can be observed that both fluorescence spectra of **1** and **1**-Cys adduct were red-shifted with increasing pH, indicating that fluorescence spectra of **1** and **1**-Cys adduct are pH dependent. Furthermore, the influence of pH on the fluorogenic reaction was studied in the range 4.9–9.2, and the results are shown in Fig. 7. It can be seen that the fluorescence response of probe **1**

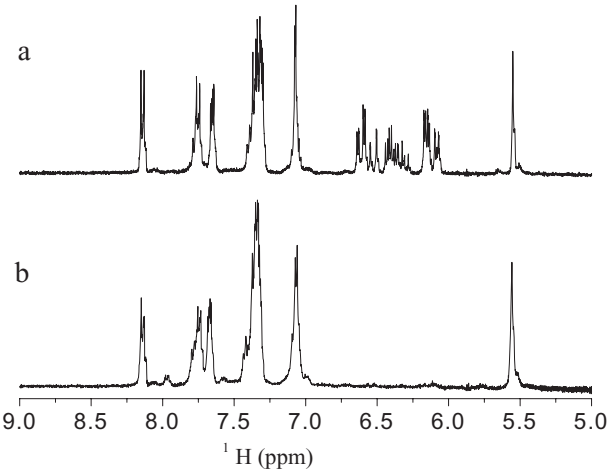

Figure 6. ^{1}H NMR spectra of probe **1** (7.6 mmol L^{-1}) in the absence (a) and presence of 4 equiv. of Cys (b) in D_2O–CD_3CN (1:1) solution. The spectrum b was measured after the addition of Cys for 40 min.

toward Cys was also pH-dependent and the fluorescence signal of probe **1** in the absence or presence of Cys both increased with increasing pH. The F/F_0 obtained the maximum value at pH 7.4 (Fig. 7, insert curve). Therefore, pH 7.4 phosphate buffer was selected for the proposed fluorogenic reaction in the subsequent experiments.

Optimization of the conditions

The effect of reaction time on the fluorescence signal of the system was studied and the results are shown in Fig. 1. It can be seen that, upon mixing with Cys, the fluorescence signal of the system increased gradually with increasing the reaction time. Although the sensitivity of the present method could be increased with increasing the incubation time, a 30 min reaction time was selected as a compromise of sensitivity and analytical frequency.

In the present study, the organic solvent–water solution was selected as the fluorogenic reaction medium. The effect of ethanol concentration on the fluorescence emission of the system was further studied, and the results are shown in Fig. 8. It can be seen that fluorescence increased with increasing the concentration of ethanol from 0 to 20%, and remained stable when ethanol concentration was above 20%. Therefore, 20% ethanol–water solution was selected in the subsequent experiment.

Selectivity studies

For an excellent fluorescent probe, high selectivity is a matter of necessity. The selectivity of probe **1** for thiols was investigated by adding some biologically relevant analytes under the same conditions. As shown in Fig. 9, probe **1** shows a 'turn-on' fluorescence response to biothiols (Cys, Hcy and GSH). In contrast, no significant changes in fluorescent emission were observed upon addition of other amino acids (Glu, Asp, Met, Leu, Ala, Tyr, Gly, His, Val, Thr, Arg, Iso, Phe, Lys), metal ions (K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}), hydrogen peroxide or ascorbic acid, indicating that the selectivity of **1** toward biothiols is remarkably high. To

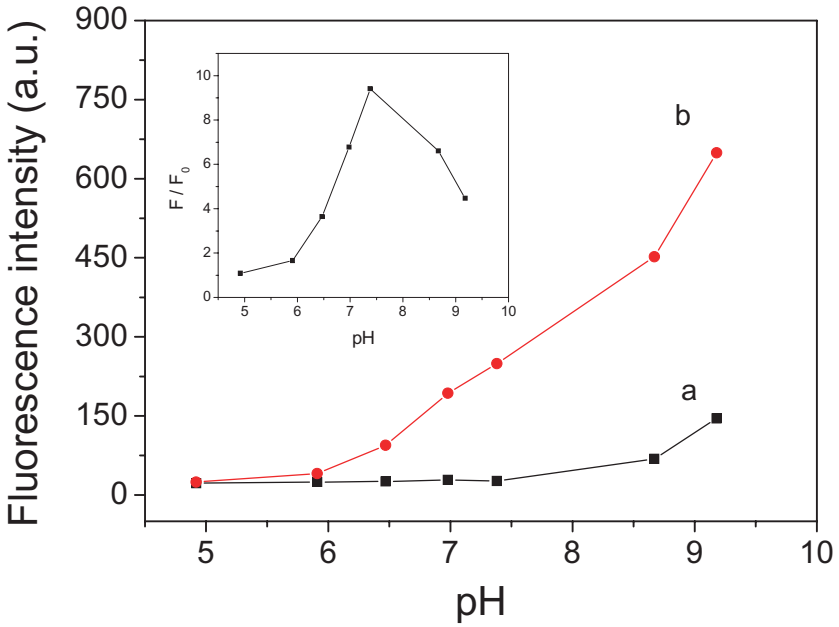

Figure 7. Effect of pH on fluorescence intensity of the Cys-induced conjugated addition of **1** (20.0 μmol L^{-1}). (a) **1** only; (b) **1** + Cys (10.0 μmol L^{-1}). Insert curve, the F/F_0 at different pH values, where F_0 and F are fluorescence intensity of probe **1** in the absence and presence of Cys, respectively.

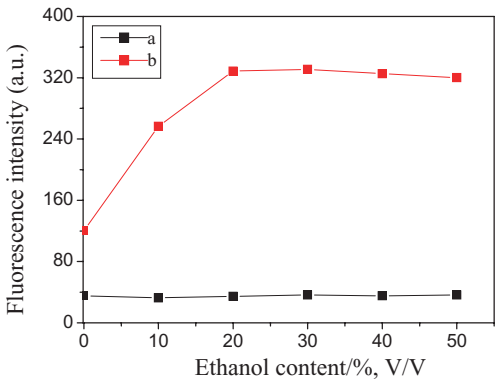

Figure 8. Effect of ethanol concentration on fluorescence intensity of the Cys-induced conjugated addition of **1** (20.0 μmol L^{-1}). (a) **1** only; (b) **1** + Cys (10.0 μmol L^{-1}). Other reaction and detection conditions are the same as those described in the procedure.

further confirm the high selectivity of the probe to biothiols, competition experiments were also carried out to test biothiols selectivity response in the presence of other competitors such as amino acids or metal ions. As displayed in Fig. 10, all of the biologically relevant analytes tested have virtually no obvious interference on the fluorescence detection of Cys. These facts proved that probe **1** merits a high selectivity of for sensing biothiols at physiological pH conditions, even with the involvement of some biologically relevant analytes.

Analytical characteristics of 1 for biothiols

Under the selected conditions given above, the fluorescence increment showed a linear relationship with the concentration of Cys in the range 1.0–30.0 μmol L^{-1} (r = 0.9925, Figure S4). The detection limit was 0.17 μmol L^{-1} (3δ). The relative standard deviation was 3.1% (n = 7) for 10.0 μmol L^{-1} of Cys.

Sample analysis

In order to evaluate the applicability of the proposed probe, a standard addition method with Cys as the standard (9) was employed to estimate the unknown concentration of thiols in a human urine sample from a healthy volunteer. The raw urine was collected from a healthy male volunteer. A 1.0 mL aliquotof this solution was transferred into a 10 mL volumetric flask and diluted to volume with water. Meanwhile, different concentrations of standard Cys solution were added to the aliquot of diluted urine samples. Then, the above samples were determined according to the procedure described above. The total content of biothiols in the urine sample was analyzed to be 25.0 ± 0.48 μmol L^{-1} (Table 1), which was within the concentration range corresponding to normal levels of thiols in urine samples (40).

Conclusion

In conclusion, we have developed a highly sensitive and selective fluorescent probe **1** for biothiols under physiological pH conditions. The fluorescence enhancement was attributed to the Michael addition of thiols to α,β-unsaturated carbonyl moiety in probe **1** to form the corresponding β-sulfido carbonyl adduct. Owing to the simplicity and sensitivity of the analysis, we believe that this probe will find many applications in a variety of settings.

Supporting information

Supporting information can be found in the online version of this article.

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Table 1. Determination of thiols concentrations in urine samples

Sample	Cys added (μmol L^{-1})	Total thiols found (μmol L^{-1})	Total thiols in urine (μmol L^{-1})	Recovery (%)	RSD (%) (n = 3)
Urine	0.0	2.5	25.0±0.48	—	1.9
	2.0	4.6	—	103.5	1.0
	4.0	6.5	—	97.6	5.5

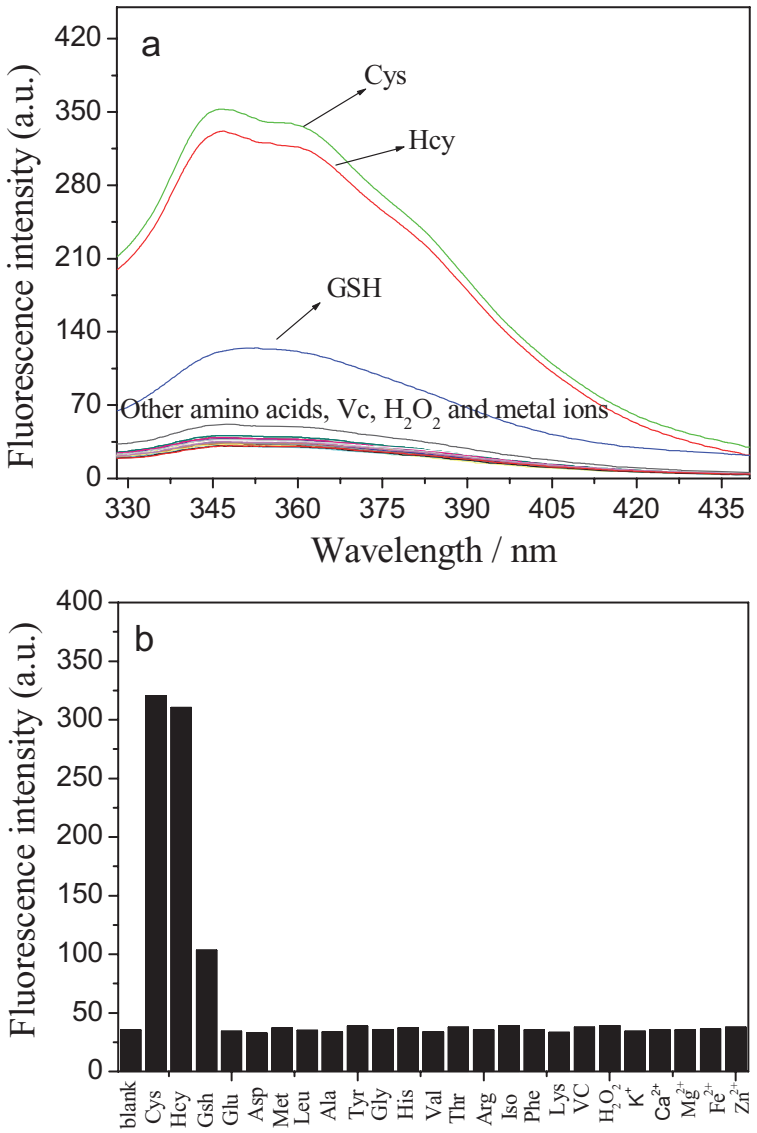

Figure 9. (a) Fluorescence spectra (excitation at 304 nm) of **1** (20.0 μmol L^{-1}) with various biologically relevant analytes (10 μmol L^{-1}) in ethanol–water (20:80, v/v) buffered at pH 7.4. (b) Fluorescence intensity changes of **1** (20.0 μmol L^{-1}) after addition of (10 μmol L^{-1}) of various biologically relevant analytes in ethanol–water (20:80, v/v) at pH 7.4 phosphate buffer solution. From left to right: **1** only (blank), Cys, Hcy, GSH, Glu, Asp, Met, Leu, Ala, Tyr, Gly, His, Val, Thr, Arg, Iso, Phe, Lys, ascorbic acid, H_2O_2, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}.

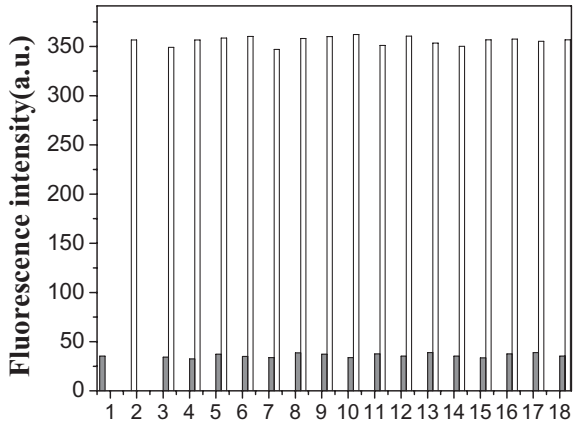

Figure 10. Fluorescence intensity changes of **1** (20.0 μmol L^{-1}) after addition of different analytes (100 μmol L^{-1}) in the presence of Cys (10 μmol L^{-1}) in ethanol–water (20:80, v/v) at pH 7.4 phosphate buffer solution. Grey bars represent the addition of the appropriate amino acids, metal ions or H_2O_2 to the solution of **1**. White bars represent the subsequent addition of Cys to the solution: (1) **1** only; (2) Cys; (3) Arg; (4) Glu; (5) His; (6) Val; (7) Asp; (8) Gly; (9) Met; (10) Tyr; (11) Thr; (12) Leu; (13) Lys; (14)K^+; (15) Ca^{2+}; (16) Mg^{2+}; (17) Zn^{2+}; (18) H_2O_2.

Acknowledgments

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