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A Sensitive and Selective Fluorescent Thiol Probe in Water Based on the Conjugate 1,4-Addition of Thiols to α,β-Unsaturated Ketones

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Abstract: Compound **1** was designed and synthesized as a new fluorescent thiol probe. Probe **1** was constructed on the basis of the conjugate 1,4-addition of thiols to α,β-unsaturated ketones. Notably, probe **1** has suitable water solubility, which allows the sensing assay to be performed in water. Probe **1** is highly sensitive for thiols with a 211-fold fluorescence dynamic range and a low detection limit of 9.25×10^{-7} M. The major features of probe **1** also include a high selectivity for thiols over other relevant biological species, excitation and emission in the visible region, rapid functioning at pH 7.4, and a good linear relationship between the fluorescence signal and the thiol concentration. Accordingly, these desirable characteristics may render probe **1** as potentially useful for biological applications.

Keywords: charge transfer · fluorescence spectroscopy · fluorescent probes · ketones · thiols

Introduction

Small-molecular-weight thiols, including cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), play a key role in maintaining the biological redox homeostasis through the equilibrium of free thiols and oxidized disulfides in biological systems.[1] However, abnormal levels of Cys, Hcy, and GSH are implicated in a variety of diseases, such as liver damage, skin lesions, slowed growth, edema,[2] dementia, Alzheimer's disease, coronary heart disease, carotid therosclerosis, dystonia, psoriasis, clinical stroke,[1c,d,3] lung damage, Parkinson's disease, and asthma.[1b,4] Therefore, it is of importance to detect thiols in biological samples.

Several analytical techniques, including high-performance liquid chromatography (HPLC),[5] capillary electrophoresis (CE),[6] electrochemical assay,[7] UV/Vis spectroscopy,[8] Fourier transform infrared (FTIR) spectroscopy,[9] mass spectrometry,[10] and fluorescence spectroscopy,[11] have been devoted to the detection of thiols. Among them, fluorescence sensing is most attractive, because it offers apparent advantages in terms of simplicity of implementation and high sensitivity. However, most of the fluorescent thiol probes developed are associated with some limitations, including the use of an organic or organic/water medium, a high-pH aqueous solution, a limited pH range, high background fluorescence, a long response time, excitation and emission in the UV region; these limitations necessitate tedious washing and isolation steps, and so forth. For example, Li and co-workers reported a fluorescent thiol probe that was employed in a mixture of aqueous methanol (7:3);[11a] Ajayaghosh and co-workers described a fluorescent thiol probe that worked at a high pH value (pH 9.6) in acetonitrile/water (1:1);[11c] Barbas and co-workers developed a fluorescent thiol probe that was excited with UV light and required a long response time;[11b] and Hong and co-workers devised a water-soluble fluorescent thiol probe that was excited with UV light and needs as many as 500 equivalents of thiol to induce a fluorescence response.[11g] Therefore, it is still necessary to construct fluorescent thiol probes that can be used for thiol detection with a rapid response under neutral aqueous conditions, preferably with excitation and emission in the visible region.

The conjugate addition of thiols to maleimides has been exploited in fluorescent thiol probe development.[11h–k] However, the maleimide-based probes can only be used within a limited pH range, preferably at pH < 7, because maleimides tend to hydrolyze at pH values greater than 7.[11j,k,12] On the other hand, the conjugate 1,4-addition of thiols to α,β-unsaturated ketones to form thioethers is a key reaction in organic synthesis (Scheme 1).[13] The reaction is normally performed under drastic conditions, such as with bases[14] or Lewis acids[15] as catalysts, at elevated temperature, or with

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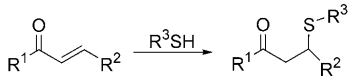


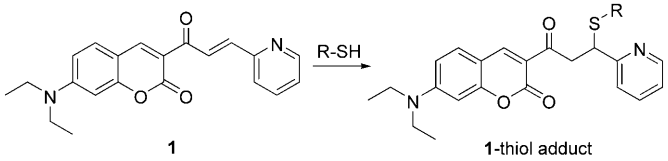
Scheme 1. Reaction of α,β-unsaturated ketones with thiols.

the use of organic solvents. However, Chakraborti and coworkers recently reported that this 1,4-addition could be conducted in water at room temperature without the use of any catalyst.[16] Encouraged by these biologically compatible conditions, we envisioned that the 1,4-addition of thiols to α,β-unsaturated ketones could be employed as a platform for the construction of fluorescent thiol probes that may be potentially useful for thiol detection at physiological pH values.

Herein, we describe a novel, selective and sensitive fluorescent thiol probe in water based on the conjugate addition of thiols to α,β-unsaturated ketones. The probe features a fast response, excitation and emission in the visible region, and a stable fluorescence signal over a wide pH range in aqueous solution. Furthermore, notably, the appropriate water solubility of the new probe allows the sensing to be performed in water. Thus, this new probe possesses several desirable attributes for potential use in the rapid sensing of thiols in biological fluids without difficult sample preparation.

Results and Discussion

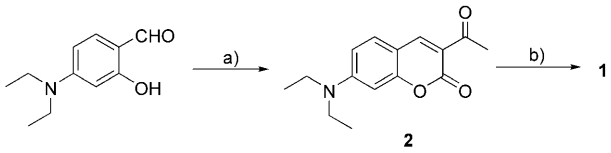
Design and synthesis of probe 1: To obtain a fluorescent thiol probe that is excited and that emits in the visible region in aqueous solution for potential biological applications, diethylaminocoumarin (Scheme 2) was selected as the



Scheme 2. The design concept of fluorescent turn-on probe **1** for thiols.

fluorophore, because of its high photostability and its excitation and emission in the visible region.[17] Furthermore, probe **1** also contains an α,β-unsaturated ketone moiety as a potential reaction site for thiols. To improve the water solubility, water-soluble pyridine is linked to the coumarin dye through the α,β-unsaturated ketone unit. We further hypothesized that the incorporation of the pyridine moiety may elicit intramolecular charge transfer (ICT) from the electron-rich diethylamino group at the 7-position of coumarin to the electron-poor pyridine moiety through the extended conjugation system.[18] In contrast, in the **1**–thiol adduct, the pyridine moiety is not conjugated to the coumarin dye; thus, the ICT process from the electron-rich diethylamino group to the electron-poor pyridine moiety no longer occurs. We may reasonably expect to observe distinct spectral properties in the probe before and after treatment with a thiol. This could serve as the basis for the thiol sensing of probe **1**.

The synthesis of compound **1** is depicted in Scheme 3. The intermediate, ketocoumarin **2**, was readily prepared from



Scheme 3. Synthetic route to probe **1**. Reagents and conditions: a) $CH_3COCH_2COOC_2H_5$, methanol, reflux, 10 h; b) 2-pyridinecarboxaldehyde, ethanol/acetonitrile, reflux, 12 h.

the reaction of 4-(diethylamino)salicylaldehyde with ethyl acetoacetate in 53% yield.[19] Compound **1** was then synthesized by an aldol condensation between compound **2** and 2-pyridinecarboxaldehyde in a moderate yield. NMR spectroscopy, ESIMS, and elemental analysis were employed to characterize the structures of the intermediate and the final product.

Optical properties of compounds 1 and 2: The absorption and emission spectra of probe **1** in phosphate buffer solution (25 mM phosphate buffer, pH 7.4) and of reference compound **2** in phosphate buffer/CH_3CN (99:1) are shown in Figure 1. Probe **1** exhibited an absorption peak with maximum at 466 nm, which has a 25 nm redshift in comparison with that of compound **2**. This redshift behavior can seemingly be ascribed to the ICT from the electron-donating diethylamino group at the 7-position of the coumarin to the electron-deficit pyridine moiety. Free probe **1** displayed almost no fluorescence in the phosphate buffer solution with a quantum yield (Φ) of 0.0002 (with quinine sulfate as

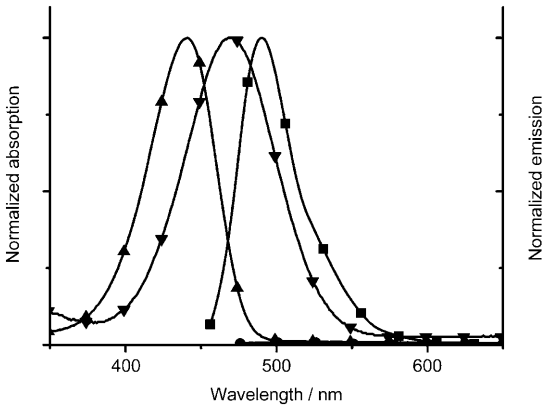


Figure 1. The normalized absorption spectra of **1** (▼) and **2** (▲) and the normalized emission spectra of **1** (●) and **2** (■), with the emission intensity ratios between **1** and **2** kept unchanged. Conditions: Probe **1** and reference **2** were in phosphate buffer solution and phosphate buffer/CH_3CN (99:1), respectively.

a standard),[20] in sharp contrast to the much higher fluorescence of reference **2** ($\Phi = 0.03$ in phosphate buffer/CH_3CN (99:1)). The low fluorescence emission of probe **1** may be explained by the formation of the nonfluorescent ICT state,[18] in good agreement with the ICT behavior observed in the absorption spectrum of probe **1**. The drastic difference in the emission intensity between compound **1** and reference **2** suggests that compound **1** may be used as a fluorescent turn-on probe for thiols, because the fluorophore of the **1**–thiol adduct (Scheme 2) is essentially the same as that of compound **2** (Scheme 3).

Sensing response of probe 1 to Cys: We first examined the sensing response of probe **1** to the typical biological thiol, Cys, with absorption and fluorescence spectroscopy. Addition of an increasing amount of Cys (0–20 equiv) to a solution of probe **1** (7 μM) in the buffer solution (25 mM phosphate buffer, pH 7.4) elicited a gradual decrease of the absorption peak at 466 nm and a progressive increase of a new absorption band centered at around 444 nm (Figure 2a). A well-defined isosbestic point was noted at 453 nm, which may indicate the formation of a new species upon treatment of probe **1** with Cys. Furthermore, the hypsochromic shift in absorption suggests the formation of the **1**–Cys adduct, as the aforementioned ICT is turned off in the adduct. This is also consistent with the observation that the absorption spectrum of the probe titrated with Cys closely resembles that of reference compound **2** (Figure S1 in the Supporting Information).

The changes in the fluorescence emission spectra of probe **1** in the absence or presence of Cys in the buffer solution (25 mM phosphate buffer, pH 7.4) are displayed in Figure 2b and Figure S2 in the Supporting Information. The free probe is essentially nonfluorescent; however, the introduction of Cys caused a dramatic change in the fluorescence spectra. A strong new emission peak at 496 nm was formed, and an enhancement in the fluorescence intensity of up to 211-fold was observed (Figure S3 in the Supporting Information). The detection limit for Cys was determined as 9.25×10^{-7} M under the experimental conditions (Figure S4 in the Supporting Information).[21] The combination of the low detection limit and the large fluorescence dynamic range indicates that the probe is highly sensitive to Cys. Furthermore, the addition of Cys immediately turned the visual emission color of the probe **1** solution from dark to green (Figure S5 in the Supporting Information), which provides further evidence for a fluorescence turn-on response. The fluorescence intensities at 496 nm were plotted as a function of the Cys concentration to obtain a calibration graph, which shows an excellent linear relationship, with the coefficient $R = 0.99447$ (Figure 3). This implies that probe **1** is potentially useful for the quantitative determination of thiol concentrations.

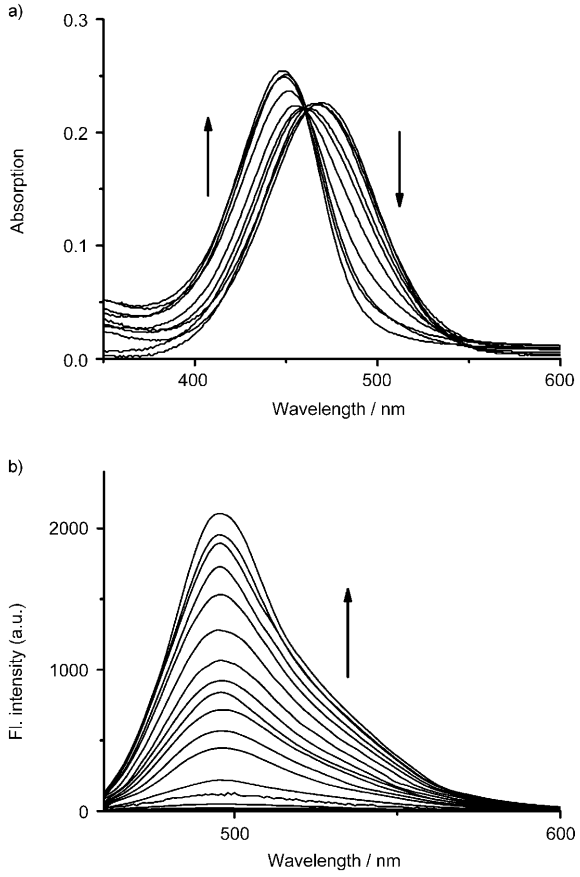


Figure 2. a) Absorption and b) emission spectra for probe **1** (7 μM) in buffer solution (25 mM sodium phosphate, pH 7.4) in the presence of Cys (0–20 equiv). The excitation wavelength was 444 nm.

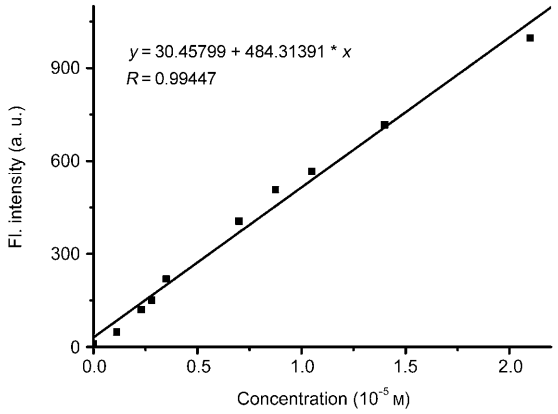


Figure 3. Plot of the fluorescence (Fl.) intensity at 496 nm as a function of Cys concentration.

Although the above spectral studies have indicated that, as designed, the sensing response of the probe to Cys is most likely due to the formation of the **1**–Cys adduct, we provided several further lines of evidence to corroborate this. Firstly, the absorption, emission and excitation spectra (Figures S1 and S6 in the Supporting Information) of the **1**–Cys adduct were very similar to those of reference compound **2**. This is in accordance with the fact that the **1**–Cys adduct and reference compound **2** have almost the same coumarin fluorophore. Secondly, we conducted ^{1}H NMR

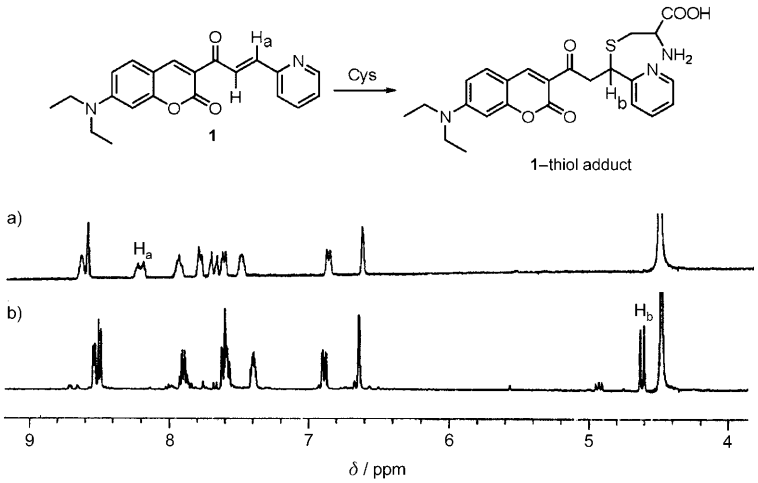


Figure 4. ^{1}H NMR spectral changes for probe **1** upon addition of Cys. a) Probe **1** only; b) probe **1** and Cys (3 equiv). [Probe **1**] = 6 mM in D_2O/CD_3CN (1:1) at room temperature.

spectroscopy studies of the probe in the absence or presence of Cys. As shown in Figure 4, the resonance signal at around $\delta = 8.20$ ppm, corresponding to the double-bond proton H_a, disappeared; concurrently, a new peak, assigned to the thioether methine proton H_b, emerged at $\delta = 4.62$ ppm, a result that is consistent with the formation of the **1**–Cys adduct. Finally, mass spectrometry analysis of the probe titrated with Cys in buffer solution (25 mM phosphate buffer, pH 7.4) confirms the formation of the **1**–Cys adduct. The mass spectrum displayed peaks at m/z 470.1 [M^++H] and 492.1 [M^++Na] (Figure S7 in the Supporting Information). The strong peak at m/z 452.1 [M^+−17] is typical for Cys derivatives.[10c]

Kinetic studies: The time course of the fluorescence of probe **1** (7 μM) in the absence or presence of Cys, Hcy, or GSH (20 equiv) in buffer solution (25 mM phosphate buffer, pH 7.4) is displayed in Figure 5. Free probe **1** exhibited no noticeable changes in emission intensity at 496 nm in buffer solution. However, upon introduction of Cys, the emission intensity essentially reached a maximum in less than 10 min. Thus, probe **1** is much faster than the Cys probes previously reported,[11a,b] and the probe may be useful for real-time sensing of Cys. A similar fluorescence increase was also observed for Hcy, although it took up to 20 min for the maximal intensity to be reached in this case. The probe exhibited much lower reactivity toward GSH. This finding is in good agreement with a previous report.[11a] The reactivity in the order Cys > Hcy > GSH can be rationalized on the basis of steric-hindrance effects on the thiol 1,4-addition reaction.[14a] In this work, an assay time of 20 min was selected in the evaluation of the selectivity and sensitivity of probe **1** toward thiols.

Effect of pH: To be useful in biological applications, it is necessary for a probe to function over a suitable pH range and particularly at physiological pH values. Thus, we set out to examine the influence of the pH value on the fluorescence intensity of probe **1** in the absence or presence of Cys in water. In the absence of Cys, almost no change in fluorescence intensity was observed for the free probe over a wide pH range of 1–12 (Figure 6). However, the fluorescence response of the probe toward Cys was pH dependent, and the maximal signal was observed in the pH range of 7–11. This indicates that probe **1** can be employed to detect thiols under neutral and basic conditions. Thus, probe **1** functions over a much wider pH range than the maleimide-based fluorescent probes. Furthermore, the observation that probe **1** can respond to Cys at a pH value lower than the pK_a value of the thiol group of Cys (pK_a = 10–11),[11f,22] that is, under

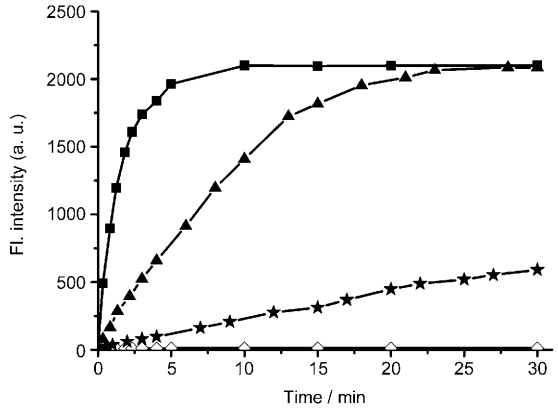


Figure 5. Reaction–time profile for probe **1** (7 μM) in the absence (△) and presence of 20 equivalents of Cys (■), Hcy (▲), or GSH (★) in buffer solution (25 mM phosphate buffer, pH 7.4). Kinetic studies were performed at room temperature. The fluorescence intensities at 496 nm were monitored at time intervals.

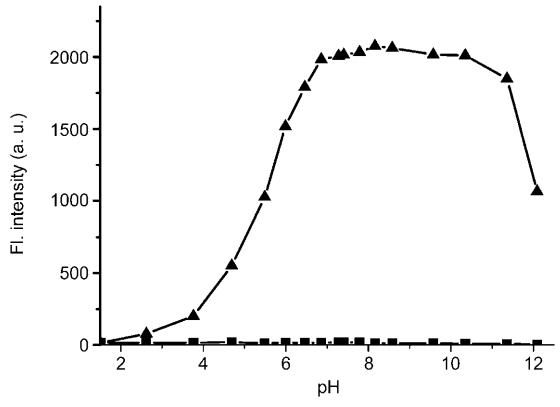


Figure 6. The fluorescence intensities at 496 nm for probe **1** (7 μM) in the absence (■) or presence (▲) of Cys (20 equiv) at various pH values. The excitation wavelength was 444 nm.

neutral conditions, indicates that deprotonation of the thiol group is not necessary for the fluorescence sensing. This is consistent with the report that conjugate addition of thiols to α,β-unsaturated carbonyl compounds can be carried out under neutral conditions.[16] The observation that probe **1** has a maximal sensing response at physiological pH values suggests that the probe is promising for biological applications.

Selectivity studies: To investigate its selectivity, probe **1** (7 μM) was treated with various biologically relevant analytes (such as representative amino acids, glucose, metal ions, reactive oxygen species, reducing agents, nucleosides, and small-molecule thiols) in buffer solution (25 mM phosphate buffer, pH 7.4) and monitored by absorption and emission spectroscopy. As exhibited in Figure 7a, introduction of Cys/Hcy/GSH to a solution of probe **1** resulted in a decrease of the absorption band centered at around 466 nm and formation of a new blueshifted absorption peak with a maximum at 444 nm. However, no noticeable changes in the absorption were observed upon addition of amino acids (Arg, Glu, Ala, Val, Ser, Leu, and Lys), metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, and Zn^{2+}), a reactive oxygen species (hydrogen peroxide), a reducing agent (nicotinamide adenine dinucleotide (NADH)), nucleosides (cytosine, thymine, adenine, and guanine), or glucose, which indicates that the α,β-unsaturated carbonyl compound **1** specifically reacted with the thiol-containing molecules in the buffer solution at pH 7.4. In good agreement with the variations in the absorption profile, the thiol-containing molecules induced a significant increase in the fluorescence intensity, with 211-, 180-, and 35-fold enhancements for Cys, Hcy, and GSH, respectively (Figure 7b and S3 in the Supporting Information). In contrast, the other biologically relevant analytes tested elicited no visible changes in emission. These data demonstrate that probe **1** has a high selectivity for thiol-containing molecules.

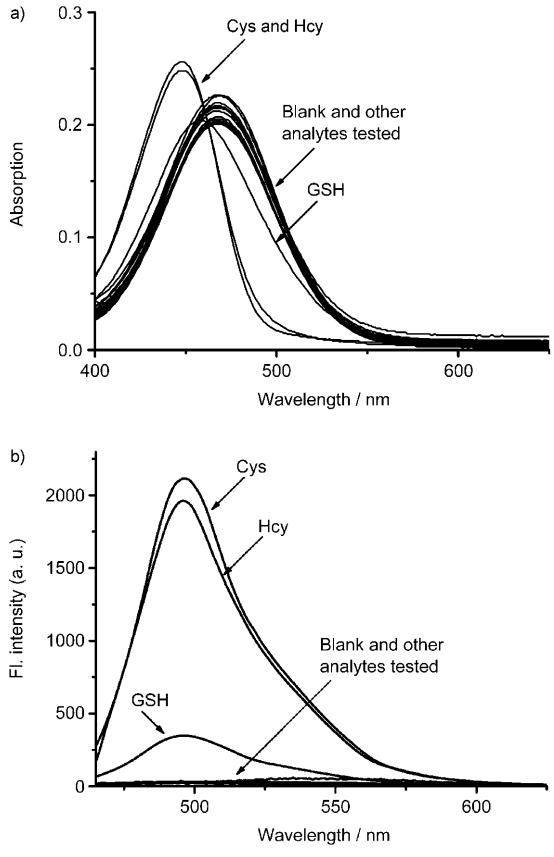


Figure 7. a) Absorption and b) emission spectra for probe **1** (7 μM) with or without various biologically relevant analytes (20 equiv), including representative amino acids, metal ions, small-molecule thiols, reactive oxygen species, reducing agents, nucleosides, and glucose in buffer solution (25 mM phosphate buffer, pH 7.4). The excitation wavelength was 444 nm.

To examine whether probe **1** could still retain its sensing response to the typical thiol (Cys) with potential competition from biologically relevant analytes, the probe (7 μM) was treated with Cys (20 equiv) in the presence of biologically relevant analytes (20 equiv) in buffer solution (25 mM phosphate buffer, pH 7.4). As displayed in Figure 8, all of the biologically relevant analytes tested have virtually no influence on the fluorescence detection of Cys. Thus, probe **1** seems to be useful for selectively sensing thiols at physiological pH values, even with the involvement of these biologically relevant analytes.

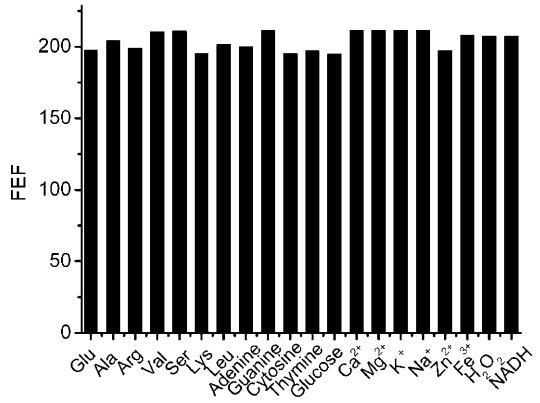


Figure 8. Fluorescence-enhancement factor (FEF) of probe **1** (7 μM) toward Cys (20 equiv) in the presence of different competing analytes (20 equiv) in the buffer solution (25 mM phosphate buffer, pH 7.4). Excitation at 444 nm. Emission at 496 nm.

Biological applications of probe 1 to newborn-calf serum and human urine: We next examined the usefulness of the probe for fluorescence sensing of thiols in a newborn-calf serum sample. The conversion of disulfides in the plasma sample to free thiols was conducted by treatment of the plasma sample with a reducing agent, triphenylphosphine, according to a literature procedure.[6a,8c] Different amounts (0, 5, 7, 10, 15, or 20 μL) of the newborn-calf serum after reduction were then directly added to a solution of probe **1** (7 μM) in buffer solution (3 mL; 25 mM phosphate buffer,

pH 7.4) at ambient temperature. As shown in Figure 9, the increases in the amount of newborn-calf serum induced a linear enhancement in the fluorescence intensity; this demonstrates that the probe is capable of sensing thiols in the

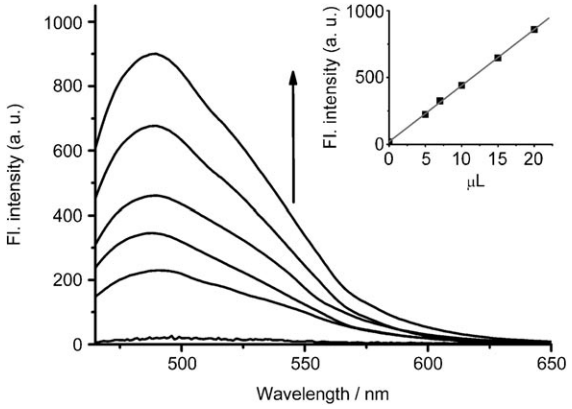


Figure 9. Emission spectra of probe **1** (7 μM) upon addition of different amounts (0, 5, 7, 10, 15, or 20 μL) of reduced newborn-calf serum solution to the buffer solution (25 mM phosphate buffer, pH 7.4). The inset shows a linear relationship between the fluorescence intensity and the volume of reduced newborn-calf serum added. Excitation at 444 nm.

plasma sample. Notably, the control experiment shows that the reduced plasma did not show any fluorescence enhancement when excited at 444 nm in the absence of probe **1** (Figure 10). Furthermore, probe **1** treated with triphenylphosphine in the absence of the plasma induced no visible fluorescence augmentation (Figure 10). Importantly, previous reports of thiol sensing in plasmas by fluorescent/colorimetric probes have usually required tedious sample preparation, including protein precipitation by acids, centrifugation, and filtration, mostly due to the use of organic solvents[8c,11c] or alkaline conditions[8b,11c] in the sensing assay. In contrast, probe **1** has suitable water solubility and functions well under neutral conditions, which allows the sensing assay to

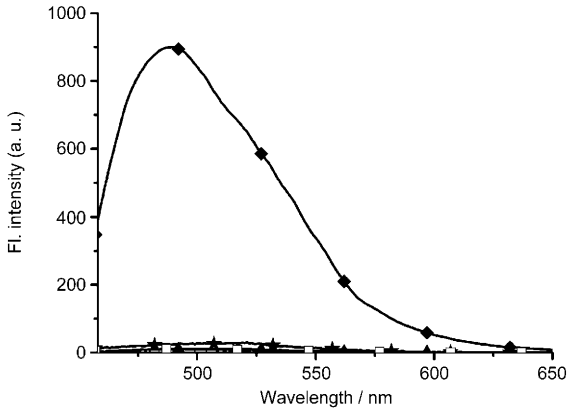


Figure 10. Emission spectra of probe **1** (7 μM) only (▲), the reduced newborn calf serum solution (20 μL) only (★), probe **1** (7 μM) with the addition of $P(Ph)_3$ (□), and probe **1** (7 μM) with the addition of reduced newborn calf serum solution (20 μL) (◆) in buffer solution (25 mM phosphate buffer, pH 7.4). Excitation at 444 nm.

be "directly" performed without complicated sample preparation. Thus, the detection of biological thiols by probe **1** is much more rapid, convenient, and probably more reliable, because the assay conditions are compatible with the biological conditions. Furthermore, for a quantitative application, a standard addition method with Cys as the standard[11c] was employed to estimate the unknown concentration of thiols in a human urine sample from a healthy volunteer. The total content of thiols in the urine sample was analyzed to be (25 ± 2) μM, which is well within the reported thiol concentration range for urine samples from healthy individuals.[6c,23]

Conclusions

Compound **1** was constructed as a novel fluorescent turn-on probe for thiols on the basis of conjugate addition of thiols to α,β-unsaturated ketones. Importantly, probe **1** operates well in a water medium. In addition, the probe is not only highly selective and sensitive for thiol-containing molecules at physiological pH values, but is also excited and emits in the visible region. Furthermore, the probe also features a fast signal response and good linearity. As fluorescent thiol probes that have these desirable attributes for bioassays are very sparse and highly sought, we expect that the new probe may be useful in potential biological applications, including the determination of thiol levels in biological fluids, fluorescence labeling of proteins, and the assaying of enzymes with a thiol as a product for enzyme-inhibitor screening. Because probe **1** is effective under neutral and basic conditions and maleimide-based probes are normally used at $pH < 7$, the probe developed herein should be a useful complement to maleimide-based fluorescent probes. The relatively small fluorescence quantum yield of the ketocoumarin fluorophore in water may hamper the use of probe **1** in bioimaging; however, this limitation may be alleviated by replacing the coumarin fluorophore with a dye (such as rhodamine, fluorescein, or boron–dipyrromethane (BODIPY)) that has a high fluorescence quantum yield in water. Efforts toward this end are in progress in our laboratory.

Experimental Section

General information and materials: 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 in all experiments. Melting points were determined with a Beijing Taike XT-4 microscope and are uncorrected. ESIMS analyses were performed by using a Waters Micromass ZQ-4000 spectrometer. Electronic absorption spectra were recorded with a Shimadzu UV-2450 spectrometer. The emission spectra were measured on a Hitachi F4500 fluorescence spectrophotometer. ^{1}H NMR spectra were measured on an Inova-400 spectrometer with tetramethylsilane (TMS) as an internal standard. Elemental analysis data were obtained with a Vario El III elemental analyzer. TLC analyses were performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from Qingdao Ocean Chemicals.

Preparation of probe 1: 3-Acetyl-7-diethylaminocoumarin (**2**; 200.0 mg, 0.77 mmol) and 2-pyridinecarboxaldehyde (85.8 mg, 0.80 mmol) were dissolved in ethanol/acetonitrile (5 mL; 1:1), and then three drops of piperidine were added as a catalyst. The mixture was heated to reflux for 12 h, and the solvent was removed under reduced pressure. The resulting residue was then purified by chromatography on silica gel with acetone/petroleum ether (1:5) as the eluent to give **1** as a brick-red solid (114.3 mg, 0.328 mmol, 42.6%). M.p. 144–146°C; ^{1}H NMR (400 MHz, [D$_1$]CDCl$_3$, TMS): $\delta = 1.26$ (t, 6H), 3.47 (q, 4H), 6.50 (d, $J = 2.4$ Hz, 1H), 6.62–6.65 (dd, $J_1 = 8.8$ Hz, $J_2 = 2.8$ Hz, 1H), 7.30 (t, 1H), 7.44 (d, $J = 8.8$ Hz, 1H), 7.66 (d, $J = 8.8$ Hz, 1H) , 7.52–7.79 (t, 1H), 7.81–7.85 (d, $J = 15.2$ Hz, 1H), 8.44–8.48 (d, $J = 15.6$ Hz,1H), 8.56 (s, 1H), 8.70 ppm (d, $J = 4.0$ Hz, 1H); UV/Vis (water): λ_{max} (log ε) = 466 nm (4.49); ESIMS: m/z (%): 349.2 (100) [M^++H], 371.1 (23) [M^++Na]; elemental analysis calcd (%) for $C_{21}H_{20}N_2O_3$: C 72.40, H 5.79, N 8.04; found: C 72.77, H 5.90, N 7.68.

Spectral measurements: The amino acids (Cys, Hcy, Arg, Glu, Ala, Val, Ser, Leu, and Lys), GSH, metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, and Zn^{2+}), reactive oxygen species (hydrogen peroxide), reducing agent (NADH), nucleosides (cytosine, thymine, adenine, and guanine), and glucose stock solutions were prepared in twice-distilled water. Probe **1** was dissolved in buffer solution (25 mM phosphate buffer, pH 7.4) at room temperature to afford the probe stock solution (14 μM). Test solutions were prepared by placing probe stock solution (1.5 mL) and an appropriate aliquot of each analyte stock into a flask and then diluting the solution to a volume of 3 mL with buffer solution. The resulting solution was shaken well before the absorption and emission spectra were recorded. Unless otherwise noted, for all measurements, the excitation wavelength was 444 nm and the excitation and emission slit widths were 5 nm. The thiol assay in biological fluids typically requires the reduction of disulfides to free thiols. This can be accomplished by using triphenylphosphine.[6a,8c] Thus, a newborn-calf serum solution obtained from a commercial source or a human urine sample from a healthy volunteer was treated with excess triphenylphosphine. Aliquots of the newborn-calf serum solution or the human urine sample after reduction were then added directly to probe **1** (7 μM) in buffer solution (3 mL; 25 mM phosphate buffer, pH 7.4), and the emission at 496 nm was recorded. The unknown amount of thiols in the humane urine sample was estimated by using the standard addition method with Cys as the standard.[11c]

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