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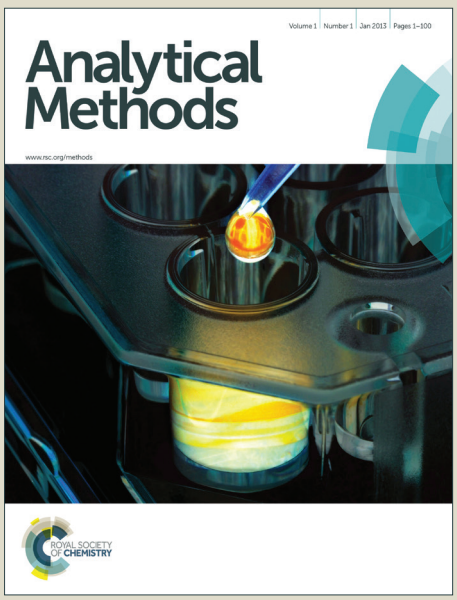

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A Simple and New Fluorescent and Colorimetric Probe Based on NBD-Maleimide for Detecting Thiols in Living Cells

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Abstract

A simple and new fluorescent and colorimetric probe bearing 7-nitrobenz-2-oxa-1,3-diazole and 4-maleimidophenol fragment for biothiols was designed and synthesized. The probe itself showed almost no background fluorescence ($\Phi_f < 1\times10^{-4}$) and displayed fluorescence turn-on response with selectivity for thiols over other relevant biological species in aqueous solutions. In addition, the probe exhibited 110 nm red-shifted absorption spectra accompanying with the color changes which could be obvious distinguished by naked-eye. Furthermore, the probe showed high sensitivity towards thiols with a detection limit of 1.2 $\times10^{-7}$ M (S / N = 3) and the mechanism regarding the optical responses of the probe to thiols was explained by density function theory (DFT) calculations. Finally, the probe has been successfully used to image thiols in Hela cells.

Keywords: Biothiol; Fluorescent probe; 7-nitrobenz-2-oxa-1,3-diazole;

4-Maleimidophenol

1 Introduction

Biological thiols such as glutathione (GSH), cysteine (Cys), and homocysteine (Hcy) play crucial roles in living organisms.[1] They are involved in many biological processes, including redox homeostasis, gene regulation, detoxify-cation of xenobioltics and cellular growth.[2] Abnormal levels of thiols in biological systems can cause several health problems, such as liver damage, slow growth, loss of muscle and fat, Alzheimer's diseases.[3] Thus, the detection of biological thiols in biological systems is important. Recently, several detection methods for thiols have been developed, such as mass spectrometry,[4] high performance liquid chromatography (HPLC),[5] capillary electrophoresis,[6] UV-Vis spectroscopy,[7] and fluorimetric sensors.[8] Compared with these methods, fluorimetric sensors have been the focus of attention because of its high sensitivity, inherent simplicity, instrument manipulability and live cell imaging.[9]

Fluorescent probe for thiols sensors are mainly based on mechanisms such as cleavage reaction by thiols,[10] Michael addition,[11] cyclization reaction with aldehyde,[12] and other mechanism.[13] Among these reaction mentioned above, Michael addition probes for thiols have received considerable attention. However, the disadvantages of reported sensors sometimes are high background fluorescence, long response time, and working at high pH aqueous solution.[14] Hence, the selective detection of thiols in living cells is still a challenge base on Michael addition reaction.

7-Nitrobenz-2-oxa-1,3-diazole (NBD) derivatives have been extensively used in fluorescent labeling reagents because they are good spectral properties and cell permeability.[15] In recent years, NBD have been used as fluorescent chemosensor for metal cations[16] and H_2S.[17] Moreover, very recently some reported fluorescent sensors have developed for detection of biothiols based on NBD derivatives.[18] Maleimide is a typical Michael receptor in organic reaction and it has been used in designing new fluorescent sensors for detection of biothiols. [19] However, to the best of our knowledge, this concept for attachment of Maleimide to NBD has not been developed. Especially, there are few studies and applications of colorimetric and fluorescent sensor with a large absorption shift based on NBD for highly sensitive detection of biothiols up to now. The colorimetric and fluorescence method belonged to spectra field is more efficient and convenient for thiols detecting. As a result, visual and fluorometric sensor for thiols has become a particularly important research field and is attracting growing interest.[20] Herein, design and synthesis of colorimetric and fluorescent chemosensor is significant for application in biological fields.

It is known that the NBD moiety is a fluorophore, and when NBD is conjugated with some strong electron-withdrawing groups, the fluorescence of the NBD derivative is weak [17a]. In addition, due to the electron-withdrawing ability of maleimide group, the sulphur anion (RS-) as a nucleophilic center can react with maleimide group by Michael addition. For this, we begin to consider once reacting the electron-withdrawing maleimide group with thiols, whether

the intermolecular charge-transfer (ICT) process will occur between maleimide group and the NBD moiety, and the fluorescence will be turned on, resulting a great change in fluorescence intensity of the probe. With this knowledge in hand, we decided to incorporate the maleimide moiety into the NBD core via a simple substitution reaction, obtaining a novel probe for detecting thiols. As shown in Figure **1**, compound **1** contains a NBD dye as a fluorophore and maleimide moiety in the 4-position of NBD as a Michael acceptor for thiols. Fortunately, compound **1** itself is non-fluorescent, whereas it displayed fluorescence turn-on response to thiols by Michael addition with maleimide in aqueous media. Moreover, compound **1** showed a large absorption shift to thiols and can serve as a naked-eye indicator for thiols by color change. In addition, compound **1** could be successfully applied for biothiol detection in living cells. Notably, this novel and simple NBD- maleimide based visual and turn on fluorescent probe for highly selective and sensitive detection of biological thiols with a low detection limit and low background fluorescent will fit the preference of further application.

2 Experimental

2.1 Reagents and chemicals

4-maleimidophenol and 4-chloro-7-nitro-benzoxadiazole (NBD-Cl) were purchased from Sigma-Aldrich Company. arginine, aspartic acid, glutathione, cysteine, histidine, proline, homocysteine, glutamic acid and p-methy thiophenol were obtained from Aladin Ltd. (Shanghai, China). All other chemicals were of analytical grade and

were used without further purification. Silica gel (200-300 mesh) was used for column chromatography.

2.2 Instrumentation

Fluorescence spectra were carried out using a Hitachi F-4500 spectrophotometer. UV-Vis spectra were measured on a Shimadzu UV2450 spectrophotometer. Fluorescence imaging of Hela cells was carried out using fluorescence microscopy (NIKON Eclipse Ti-S, Japan). ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker AVB-500 MHz NMR spectrometer (Bruker biospin, Switzerland). Mass spectra were obtained using a Bruker Daltonics BioTOF-Q mass spectrometer in the positive mode. The pH measurements were performed with a Sartorius basic pH-meter PB-10.

2.3 Synthesis of probe 1

(***Preferred position for scheme 1***)

As illustrated in Scheme 1, compound **1** was readily prepared in one step method. Briefly, to a solution of 4-chloro-7-nitro-benzoxadiazole (NBD-Cl) (0.2 g, 1 mmol) in 5 mL DMF, 4-maleimidophenol (0.28 g, 1 mmol) and potassium carbonate (0.17 g, 1.2 mmol) were added. The mixture was stirred at 80℃ for 8 h under N_2 atmosphere. Then, a rude mixture was filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (petroleum ether / ethyl acetate, 2:1, *V*/*V*) to give product **1** in 81% yield (0.26 g). ^{1}H NMR ($CDCl_3$, 500 MHz) δ(ppm): 10.06 (s, 1H), 8.67 (d, J = 8.3 Hz, 1H), 8.12 (d, J = 8.5 Hz, 2H), 7.63 (d, J = 8.5 Hz, 2H), 6.96 (d, J = 8.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ(ppm): 163.4,

154.1, 145.8, 144.8, 137.9, 136.1, 135.3, 130.5, 129.1, 121.9, 121.7, 109.6; ESI-MS m/z: 353.16, $[M+H]^+$.

2.4 Measurement procedure

A stock solution of compound **1** was prepared by dissolving compound **1** in acetonitrile. Fluorescence and absorption spectra were recorded in acetonitrile-water solution (1: 9 v/v, 50 mM HEPES buffer solution at pH 7.0). The amino acid and anion were dissolved in deionized water to prepare the stock solutions. The solution of p-methy thiophenol was prepared using absolute ethanol. The fluorescence intensity was recorded with a fluorescence spectrometer (λ_{ex} = 490 nm, λ_{em} = 554 nm, slit: 10 nm / 10 nm).

2.5 Cell culture

The Hela cells were incubated on 96-well plate for 24 h at 37℃ and were washed three times with PBS. The Hela cells were treated with compound **1** (20 μM, 1:99 DMSO / PBS, *V*/*V*) for 40 min at 37℃. In a control experiment, the cells were pre-treated with 0.5 mM N-methylmalermide in DMSO-PBS solution (1:99, *V*/*V*) for 120 min at 37℃. After washing with times with PBS buffer, the cells were incubated in the presence of compound **1** (20 μM, 1:99 DMSO / PBS, *V*/*V*) for 40 minute at 37 ℃. The fluorescence images were obtained using an inverted fluorescence microscopy (NIKON Eclipse Ti-S, Japan) with a 20 x objective lens.

2.6 Determination of fluorescence quantum yield

The fluorescence quantum yield was determined using the solutions of fluorescein (**Φ_F** = 0.90 in 0.1N NaOH [21]**)** as reference. The quantum yield was calculated using the following equation:

$$\Phi_{F(s)} = \Phi_{F(r)} (F_s A_r / F_r A_s)(n_s / n_r)^2$$

Where Φ_F is the fluorescence quantum yield, F is the area under the corrected emission curve, A is the absorbance at the excitation wavelength and n is the refractive index of the solvents used. Subscripts refer to the reference (r) or sample (s) compound.

3 Results and discussion

3.1 Spectra properties of compound 1

(***Preferred position for Fig. 1***)

The fluorescence spectra of compound **1** by different amounts of cysteine were examined at room temperature. As expected, the compound **1** exhibited almost negligible fluorescence without cysteine ($\Phi_f < 1\times10^{-4}$, Fig. 1). Upon addition of an increasing amount of cysteine to a solution of compound **1**, the fluorescence intensity at 554 nm is gradually increased (Fig. 1a). Meanwhile, about 70 μM of cysteine makes the fluorescence intensities reach a maximum (Φ_f = 2.4%). These observations imply the ICT transition from the maleimide group to the benzoxadiazole moiety in the compound **1**, which constituted the basis for the determination of concentrations of cysteine with compound **1**. Moreover, the fluorescence intensity of compound **1** at 554 nm showed linear response to cysteine in the range of 0 to 70 μM (Fig. 1b). The detection limit for cysteine is measured to

be 1.2×10^{-7} M ($S / N = 3$). The low detection limit shown that compound **1** is highly sensitive to cysteine (Table 1). The fluorescence response of compound **1** to cysteine was further indicated by the visual fluorescence color change of compound **1** solution from dark to green (Fig. 1a, inset).

To further get insight into the reaction of cysteine with compound **1**, the UV spectrum of compound **1** in the absence of cysteine and in the presence of cysteine were investigated in Fig. 2. As depicted in Fig. 2, compound **1** displayed a maximum absorption at 380 nm. When cysteine was added, the absorption peak at 380 nm decreased, and a new absorption band centered at 490 nm appeared. These results further provided a proof for the reaction of compound **1** with cysteine. The bathochromic-shift (110 nm) of the absorption wavelength is reflected in a change in the color of the solution from colourless to yellow (Fig. 2 inset), which is easily detectable by naked eye under visible light.

(***Preferred position for Fig. 2***)

3.2 Effects of reaction time on sensing cysteine

Reaction time is an important factor for reaction-based probes, and the time-dependent fluorescence spectra of compound **1** in the presence of cysteine were investigated. As shown in Fig. 3, in the absence of cysteine, the fluorescence intensity of compound **1** at 554 nm shows no obvious variations, which proves that compound **1** is very stable in such a sensing system. Upon the addition of 70 μM of cysteine, the fluorescence intensities enhanced with the increasing reaction time at 554 nm, and the fluorescence intensity reached the equilibrium within 15 minute. Therefore, a 15

minute reaction time was selected for the following experiments.

(***Preferred position for Fig. 3***)

3.3 Influence of pH value

The effects of pH on the fluorescence response of compound **1** to cysteine were investigated. These experiments were carried out at a pH range from 1.0 to 12.0, with the concentration of compound **1** fixed at 5 μM and of cysteine at 70 μM, respectively (Fig. 4). Fig.4 showed the fluorescence intensity of probe is very feeblish at pH 1.0-12.0. However, when the solution pH is between 5.0 and 7.0 or in the range of 7.0-9.0, a small fluorescence increase was induced in the presence of cysteine. At pH 7.0, upon addition of cysteine, the strong fluorescence was detectable. This indicated that compound **1** is pH-dependence in the detection of cysteine, and it could be used to detect cysteine around biological pH condition.

(***Preferred position for Fig. 4***)

3.4 Selectivity of compound 1

The selectivity towards the thiol group is one of the most important criteria for probe design. To evaluate the compound **1** as a highly selective chemosensor for thiols, we investigated the responses of compound **1** to various interfering species including a series of amino acids (alanine, spartic acid, proline, glutamic acid, arginine, histidine, glutathione, cysteine and homocysteine), metal ions (Fe^{3+}, Zn^{2+}, Hg^{2+} and Mg^{2+}), p-methly thiophenol and NaHS under the same conditions. As displayed in Fig. 5, the fluorescence of compound **1** at 554 nm was a significant

increase with cysteine, homocysteine and glutathione, no fluorescence response occurred for the other analytes. These results indicate that compound **1** has good selectivity toward thiols.

(***Preferred position for Fig. 5***)

3.5 Investigation of sensing mechanism

(***Preferred position for scheme 2***)

According to the chemistry of Michael addition reaction and the optical changes of compound **1** with thiols, a plausible reaction mechanism is proposed in Scheme 2. The fluorescence of the compound **1** was greatly weaken because of attachment of the maleimide ring. When the electron-deficient maleimide group becomes saturated by Michael addition of thiol, the parent benzoxadiazole backbone fluorescence turn on. To investigate the mechanism, a Job plot of compound **1** reaction with cysteine was examined, and the result showed compound **1** reacted with cysteine in a 1:1 molar ration (Fig.6). The mass spectrum of the reaction of compound **1** with cysteine showed one new peak with m/z of 496.45, which can be assigned as $[\mathbf{1}\text{-Cys+Na}]^+$. This result infers that compound **1** with m/z of 353.16 for $[\mathbf{1}\text{+H}]^+$ can be converted into **1**-Cys in the presence of cysteine (Fig.S4 in the supporting information). Thus, these studies confirmed the proposed reaction mechanism.

(***Preferred position for Fig. 6***)

3.6 Principle of operation

To get insight into the mechanism of emission changes in the presence of cysteine, compound **1** and 1-Cys were examined by density function theory (DFT) calculations at the B3LYP/6-31G* level using Gaussian 09 program. The calculated frontier molecular orbitals of **1** and **1**-Cys are shown in Figure 7. For compound **1,** both the HOMO and LUMO are localized on the entire benzoxadiazole backbone. Obviously, upon excitation, almost no electron transfer process will take place on compound **1,** thus rendering dark fluorescence. However, in the **1** + Cys adduct, the HOMO is distributed primarily on the maleimide adduct, whereas the LUMO is delocalized over the benzoxadiazole. Upon excitation, the ICT process will occur from the maleimide adduct to the benzoxadiazole moiety, and therefore inducing the fluorescence enhancement of compound **1** upon addition of cysteine.

(***Preferred position for scheme Fig.7***)

3.7 Biological imaging studies

In order to further explore the potential biological application of this sensor, in vitro detection of cysteine in Hela cells was evaluated. The Hela cells was first treated with compound **1** (20 μM) for 40 minute at 37℃, and then incubated for another 30 min after removal of the remaining compound **1**. The enhancement of fluorescence was observed (Fig.8). The results clearly demonstrated that compound **1** can penetrate the cell membrane and can be used for imaging of cysteine in living cells.

(***Preferred position for scheme Fig.8***)

4 Conclusion

In summary, a novel probe for biological thiols was designed and synthesized based on NBD dye with a maleimide group as functional trigger moiety. The favourable features of probe include low background fluorescence, working well at physiological pH values, high sensitivity and selectivity and a large absorption shift. Moreover, the fluorescence intensities of the compound **1** increase with increasing cysteine concentration, which is attributed to the ICT process from the maleimide group to the benzoxadiazole moiety in the compound **1.** Furthermore, the reaction mechanism of compound **1** with cysteine is investigated, revealing Michael addition reaction of cysteine to the maleimide group. In addition, compound **1** was successfully applied for the bioimaging for thiols. This strategy may pave a simple way to fabricate a chemical sensor in other applications.

Acknowledgments

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Figure captions:

Scheme 1 Synthetic route to compound **1**.

Fig.1 (a) Fluorescence spectra of compound **1** (5 μM) in acetonitrile-water solution (1: 9 *V*/*V*, 50 mM PBS buffer solution at pH 7.0) at various concentrations of cysteine (0-70 μM); (b) the right figure displays the fluorescent intensity vs the cysteine concentration.

Fig.2 UV-vis spectra of 20 μM compound **1** in the absence of cysteine (black line) and in the presence of cysteine (red line). Inset: the photographic images of compound **1** in the presence of cysteine under ultraviolet lamp irradiation.

Fig. 3 Time-dependent fluorescence intensity changes of compound **1** (5 μM) in the absence (a) and the presence of 70 μM Cys (b) in acetonitrile-water solution (1: 9 *V*/*V*, 50 mM PBS buffer solution at pH 7.0).

Fig. 4 Fluorescence intensities of 5 μM compound **1** in acetonitrile-water solution (1: 9 *V*/*V*, 50 mM PBS buffer) at various pH with 70 μM cysteine (red) and without cysteine (purple).

Fig. 5 Fluorescence emission spectra of 5 μM compound **1** in acetonitrile-water solution (1: 9 v/v, 50 mM PBS buffer solution at pH 7.0) with various thiols (70 μM).

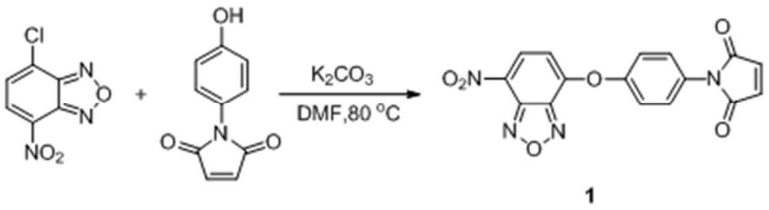
Scheme 2 Proposed mechanism for the response of compound **1** to cysteine.

Fig.6 Job's plot diagram of compound **1** for cysteine.

Fig.7 HOMO and LUMO distributions of compound **1** and **1- Cys**.

Fig. 8 Fluorescence microscopy images of Hela cells. (b) Images of cells pretreated

with N-methylmaleimide (0.5 mM) for 40 minute at 37℃and then incubated with compound **1** (20 μM) overnight at 37℃. (d) Images of cells incubated with compound 1 (20 μM) overnight at 37℃. (A) and (C) represent the bright-field images of (B) and (D), respectively.



Scheme 1 Synthetic route to compound **1**

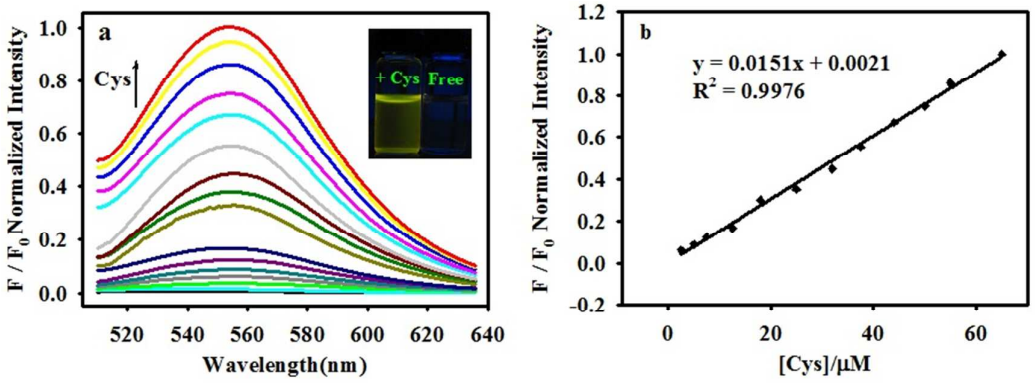


Fig.1 (a) Fluorescence spectra of compound **1** (5 μM) in acetonitrile-water solution (1: 9 *V*/*V*, 50 mM PBS buffer solution at pH 7.0) at various concentrations of cysteine (0-70 μM); (b) the right figure displays the fluorescent intensity vs the cysteine concentration.

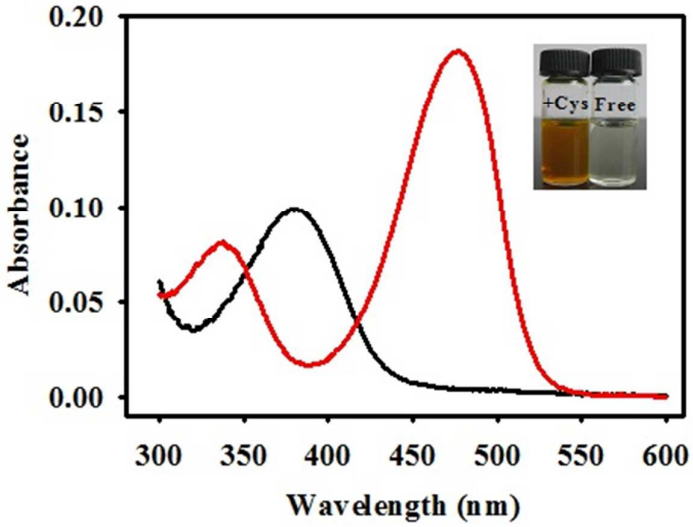


Fig.2 UV-vis spectra of 5 μM compound **1** in the absence of cysteine (black line) and in the presence of cysteine (red line). Inset: the photographic images of compound **1** in the presence of cysteine under ultraviolet lamp irradiation.

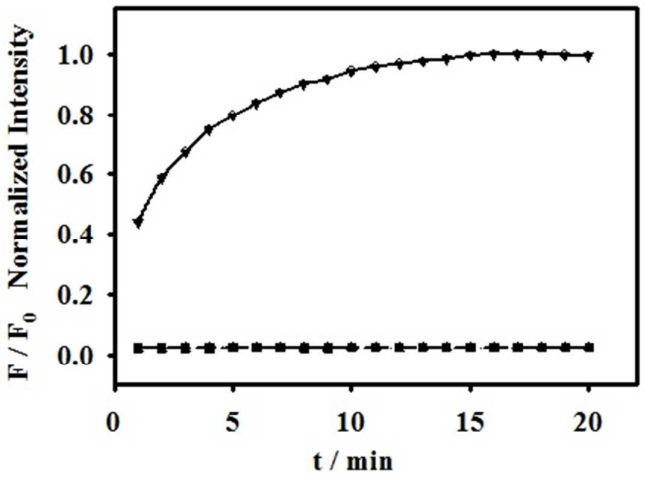


Fig. 3 Time-dependent fluorescence intensity changes of compound **1** (5 μM) in the absence (a) and the presence of 70 μM cysteine (b) in acetonitrile-water solution (1: 9 *V*/*V*, 50 mM PBS buffer solution at pH 7.0).

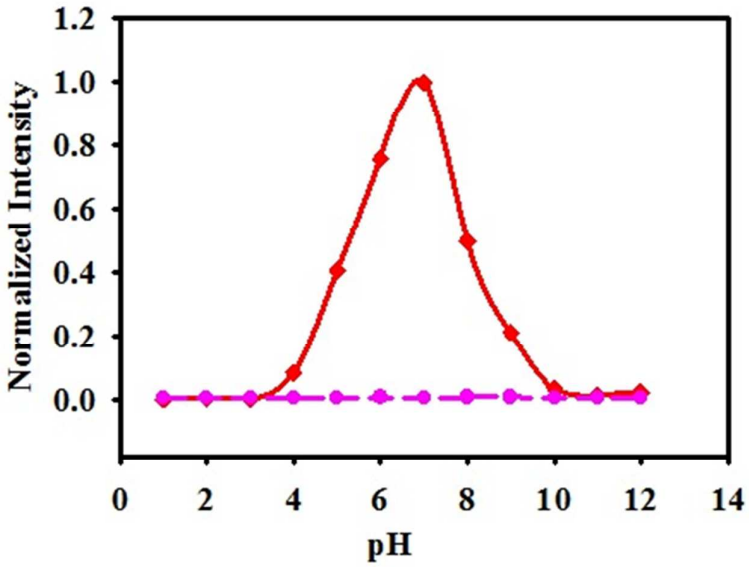


Fig.4 Fluorescence intensities of 5 μM compound **1** in acetonitrile-water solution (1: 9 V/V, 50 mM PBS buffer) at various pH with 70 μM cysteine (red) and without cysteine (purple).

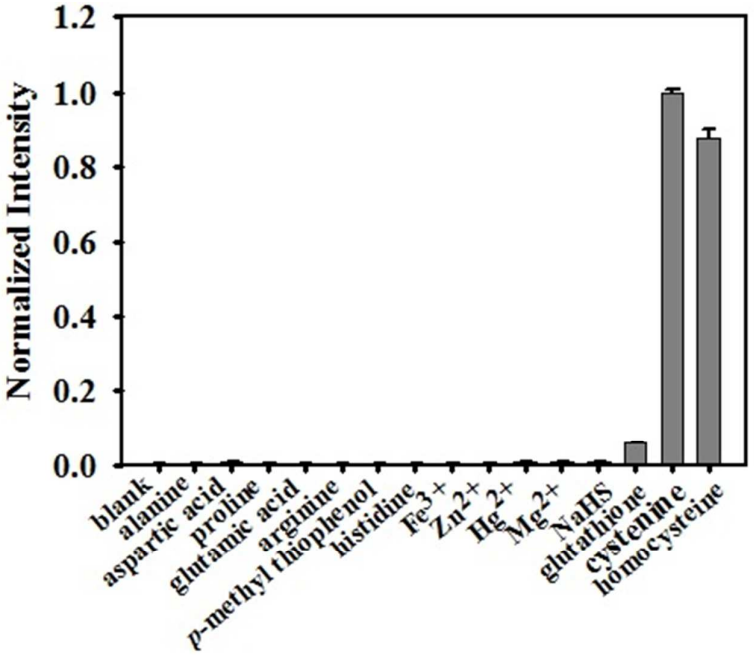
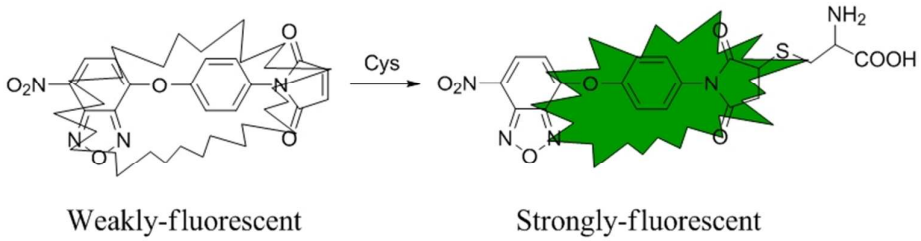


Fig. 5 Fluorescence emission spectra of 5 μM compound **1** in acetonitrile-water solution (1: 9 v/v, 50 mM PBS buffer solution at pH 7.0) with various thiols (70 μM).



Scheme 2 Proposed mechanism for the response of compound **1** to cysteine

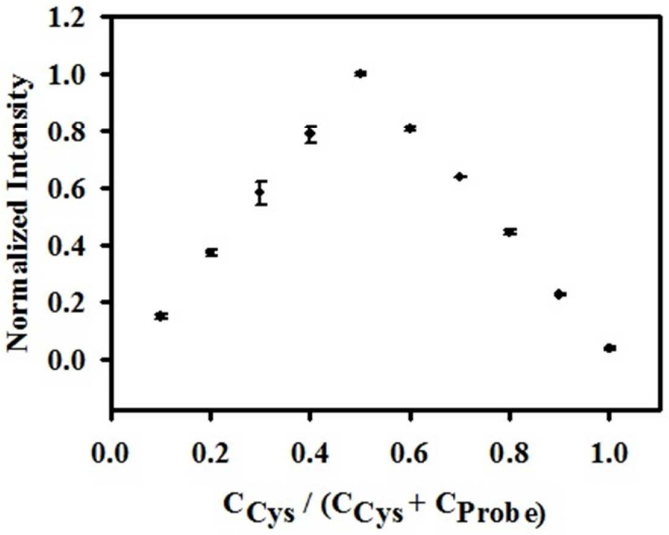


Fig.6 Job's plot diagram of compound **1** for cysteine

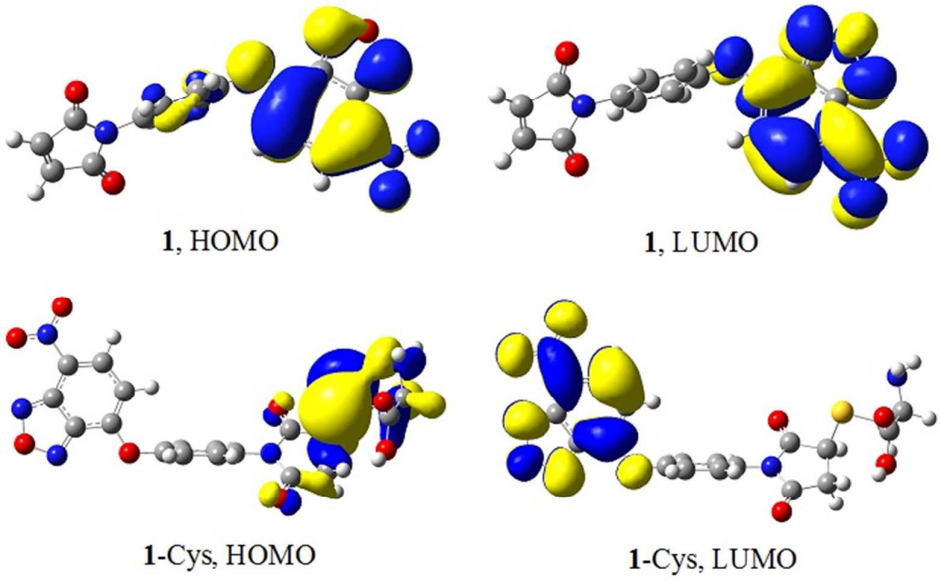


Fig.7 HOMO and LUMO distributions of compound **1** and **1- Cys**

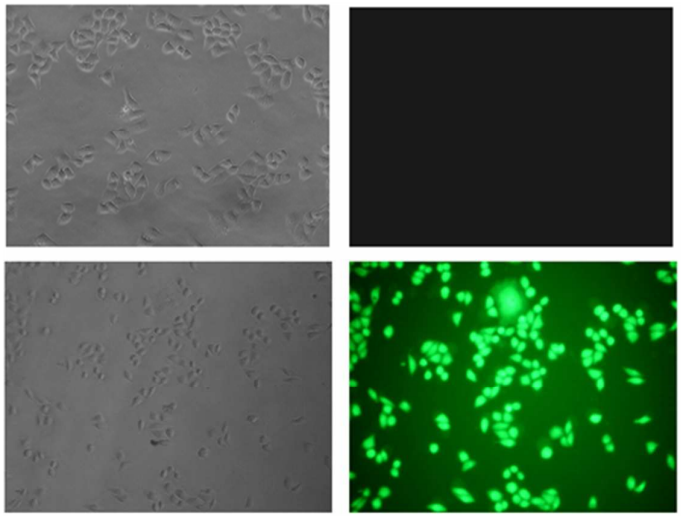


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Table **1** A comparison table about the detection limits for cysteine

Method	Testing media	Detection limit (μM)	Practical application
Ref.[22]	EtOH / phosphate buffer	0.11	Human plasma
Ref.[23]	Triton X-100 / PBS	0.3	Cell imaging
Ref.[24]	Methanol / PBS	0.13	Cell imaging
Ref.[25]	DMSO / phosphate buffer	0.22	Cell imaging
Ref.[26]	CH_3CN / HEPES	0.41	Cell imaging
This work	CH_3CN / PBS	0.12	Cell imaging