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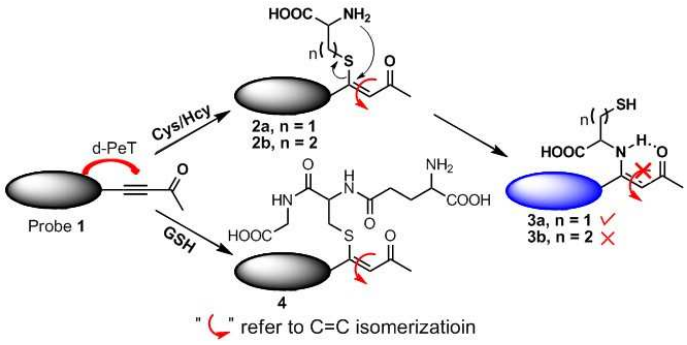
Graphical Abstract

Constructing fluorescent probe for specific detection of cysteine over homocysteine and glutathione based on a novel cysteine-binding group but-3-yn-2-one

Yawei Liu, Song Zhang, Xin Lv, Yuan-Qiang Sun, Jing Liu, and Wei Guo*

Based on a novel Cys-binding group “but-3-yn-2-one”, a new fluorescent probe was exploited, which could specifically detect Cys over Hcy/GSH in pure PBS buffer and cells, creating opportunity for studying the Cys-related physiological processes and diseases in biological systems.



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ARTICLE TYPE

Constructing fluorescent probe for specific detection of cysteine over homocysteine and glutathione based on a novel cysteine-binding group but-3-yn-2-one

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A but-3-yn-2-one-based 7-diethylaminocoumarin dye was exploited as fluorescent probe to specifically detect Cys over Hcy/GSH in pure PBS buffer. The probe itself is nonfluorescent due to the donor-excited photoinduced electron transfer (*d*-PET) process. The Cys-induced Michael addition-rearrangement cascade reaction leads to an amino-substituted product **3a** with strong fluorescent due to inhibiting C=C isomerization induced fluorescence quenching by a produced intramolecular N-H···O hydrogen bond. The Hcy (or GSH)-induced Michael addition reaction leads to a thiol-substituted product **2b** (or **4**), which lacks any intramolecular hydrogen-bonding interaction, and thus displays very poor fluorescence due to the efficient C=C isomerization induced fluorescence quenching. Even in the presence of Hcy (or GSH), the probe could also detect Cys with the obvious fluorescence enhancement. Assisted by laser scanning confocal microscope, we demonstrated that the probe could selectively image Cys in the human renal cell carcinoma 786-0 cells.

Introduction

Since the fluorescent indicators for calcium ion were reported by Tsien in the early 1980s,[1] fluorescent probes have been recognized as the efficient molecular tools that can help monitor and visualize trace amounts of samples in live cells or tissues because of its high sensitivity and high spatiotemporal resolution.[2] Given that biothiols, such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), play crucial roles in many physiological processes, and are closely related to many diseases,[3] a large number of biothiol fluorescent probes have been developed in recent years.[4] The corresponding design strategies are generally based on the strong nucleophilicity of the thiol group combined with various mechanisms such as Michael addition, cyclization, displacement of coordination, and cleavage reactions. Although these probes can distinguish Cys/Hcy/GSH from other amino acids, most of them cannot distinguish these biothiols from each other due to the similar structures and reactivity of Cys/Hcy/GSH. Because Cys, Hcy, and GSH levels are related with different physiological processes and diseases, the development of fluorescent probes that could discriminate between them is highly valuable for the better understanding of their respective molecular mechanism of action.

Based on the cyclization of Cys/Hcy with aldehydes (Fig. 1A)[5a] or acrylates (Fig. 1B),[5b] pioneered by Strongin group, the selective detection of Cys/Hcy over GSH could be realized.[6] Further, some specific probes for Cys were developed based on either the extended version of the two strategies[7,8] or Michael addition combined with steric and electrostatic interactions[9] or the Cys-induced S_NAr substitution-rearrangement reaction.[10] In fact, from a design point of view, it appears to be relatively easy to preclude the interference of GSH with these strategies. However, the discrimination between Cys and Hcy still remains a hit-or-miss proposition, often a matter of luck due to the very similar structures and reactivity of them. Moreover, when considering the practical application in biological system, some limitations still exist, such as use of organic solvent or surfactant, relatively poor selectivity, and the low sensitivity. Thus, it is highly desired to develop a novel molecular platform that could not only discriminate between Cys and Hcy, but also overcome the above-mentioned limitations.

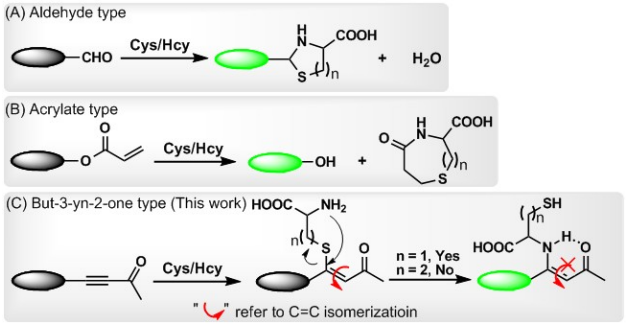


Fig. 1 Aldehyde (A), acrylate (B), and but-3-yn-2-one (C, this work) based fluorescent probes for Cys and Hcy.

We recently employed nitroolefin as a thiol binding group to construct a nitroolefin-based 7-diethylaminocoumarin fluorescent probe for biothiols.[11] Although the probe exhibited high selectivity toward Cys/Hcy/GSH over other amino acids, it failed

to distinguish these biothiols from each other because the probe followed the same reaction and fluorescence modulated mechanism for the three biothiols. In this paper, we present a new design strategy for highly selectively sensing of Cys over Hcy/GSH by employing a novel Cys binding group "but-3-yn-2-one" combined with the inhibiting C=C isomerization induced fluorescence quenching by a produced intramolecular N-H···O hydrogen bond (Fig. 1C). The results obtained indicated that the but-3-yn-2-one based probe was more specific toward Cys over Hcy/GSH than most of aldehyde or acrylate-based fluorescent probes, thus creating opportunity for studying the Cys-related physiological processes and diseases in biological systems.

Results and Discussion

Design rationale

As a proof of concept, we designed a fluorescent probe 7-diethylaminocoumarin–but-3-yn-2-one (**1**) by incorporation of but-3-yn-2-one group into 7-diethlyaminocoumarin fluorophore. The design rationale is depicted in Fig. 2 and illustrated as follows. We speculated that the initial Michael addition reaction between the thiol group of Cys (or Hcy) and the but-3-yn-2-one group would produce intermediate **2a** (or **2b**),[12] and the following intramolecular N,S-intramolecular rearrangement would lead to an amino-substituted product **3a** (or **3b**) *via* a five (or six)-membered cyclic transition state.[10,13] However, for the reaction of **1** with GSH, a thiol-substituted product **4** was highly expected because it is difficult for **4** to carry out an intramolecular N,S-intramolecular rearrangement to produce its amino-substituted product due to the unstable twelve-membered macrocyclic transition state.[10,13]

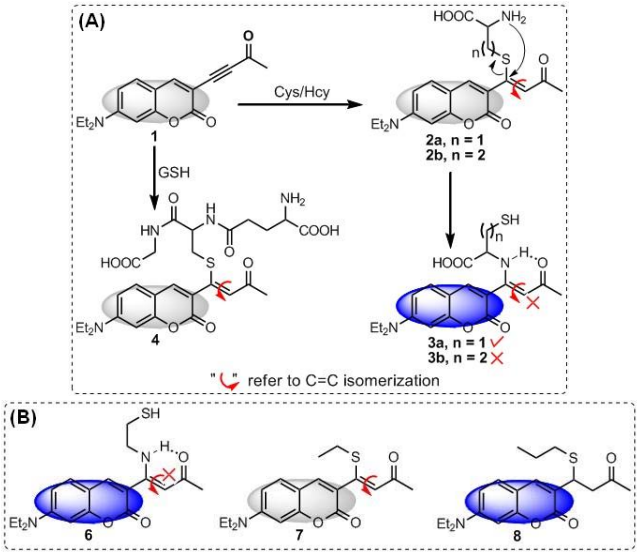
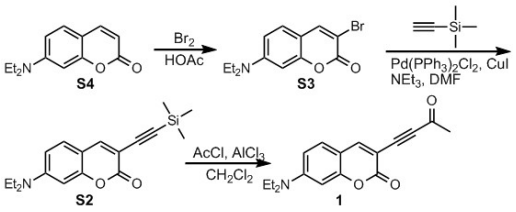


Fig. 2 (A) Proposed sensing mechanisms of probe **1** with Cys, Hcy, and GSH, respectively. (B) The control compounds **6**, **7**, and **8**.

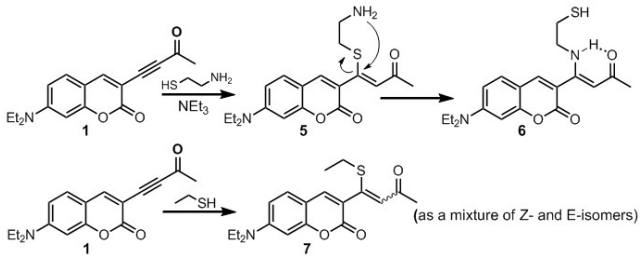
In addition, we speculated that probe **1** is weakly fluorescent due to the donor-excited photoinduced electron transfer (*d*-PET)[14] from the excited coumarin core to the electron-deficient but-3-yn-2-one group, as the case of nitroolefin-based 7-diethylaminocoumarin.[11] Moreover, compounds **2a** (or **2b**) and **4** were also expected to be weakly fluorescent due to the C=C isomerization induced fluorescence quenching just like *p*-hydroxybenzylideneimidazolone, a chromophore in green fluorescent protein.[15] However, compound **3a** (or **3b**) was expected to be fluorescent because the produced intramolecular N-H···O hydrogen bond, which has been observed in this type of compounds,[16] would inhibit the C=C bond isomerization process, just as the hydrogen bond-inhibited C=N bond isomerization observed in our previous study.[17] Based on the speculations, it appears to be possible for probe **1** to discriminate Cys/Hcy from GSH. But then again, if the N,S-rearrangement of **2b** is difficult, the reaction of **1** with Hcy would stay at the stage of weakly fluorescent **2b** rather than fluorescent **3b**. If so, we would reach the goal of discriminating Cys from Hcy/GSH.

Synthesis

To test the above speculations, we synthesized probe **1** from 7-diethlyaminocoumarin (**S4**) via the bromination, Sonogashira coupling of 7-diethylamino-3-bromo-coumarin (**S3**) and trimethylsilylethyne, followed by treatment of 7-diethylamino-3-trimethylsilanylethynyl-coumarin (**S2**) with AcCl/$AlCl_3$ (Scheme 1). In addition, we also synthesized two control compounds **6** and **7** (Scheme 2) in order to support our proposed mechanism. Their structures were confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS spectra (ESI†).



Scheme 1 Synthesis of probe **1**.



Scheme 2 Synthesis of control compounds **6** and **7**.

UV–vis Spectra Studies

With the probe in hand, we first examined the reactivity of **1** towards Cys, Hcy, and GSH through UV–vis spectra in PBS buffer (20 mM, pH 7.4) at 37 °C. As shown in Fig. 3 (details in Fig. S1, ESI†), the UV-vis spectra of free **1** showed a main absorption at 460 nm; however, addition of 20 equiv of Cys and GSH separately to the solution of **1** resulted in two blue-shifted absorption peaks after 60 min with the absorption maximums at 410 nm and 430 nm, respectively. The distinct absorption maximum suggested that the two reactions result in the different products. According to our proposed reaction mechanism, the former could be assigned to **3a** and the latter could be assigned to **4**. Our efforts to separate the two compounds to confirm the assignments were unsuccessful. However, the assignments could be supported by either the HRMS experiments, where the

corresponding molecular ion peaks could clearly be observed (Fig. S2, ESI†), or the corresponding control compounds **6** and **7**, whose absorption maximums matched well with those of **3a** and **4**, respectively (Fig. S3, ESI†). In addition, the speculation that the first-step reaction of **1** with Cys is the thiol-induced Michael addition that results in **2a** but not the direct amino-induced Michael addition that results in **3a** could be supported by the fact that probe **1** is inert to various thiol-free amino acids (see below, Fig. 6).

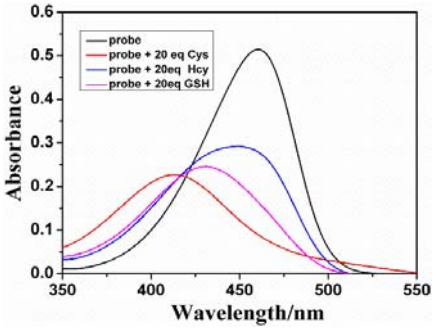


Fig. 3 Absorption spectra of **1** (10 μM) treated with 20 equiv of Cys, Hcy, and GSH in PBS buffer (20 mM, pH 7.4) at 37 °C after 60 min.

Notably, upon addition of 20 equiv of Hcy to the solution of **1**, an uncompleted chemical transformation could be observed after 60 min. Obviously, Hcy displayed the slower reaction rate than GSH. This appears to be abnormal due to the big steric hinderance of GSH. However, considering that the thiol group of Hcy has the higher pK_a value (10.00) than that of GSH (9.20),[9a] the thiol group of GSH should be a better nucleophile than that of Hcy regardless of the steric factor. This may be the main reason for the above observation. However, when the higher concentration of Hcy (1 mM) was used, the reaction could be completed within 60 min, and the resulting absorption spectra was more similar to that of GSH (Fig. S4, ESI†), supporting that the reaction of **1** with Hcy mainly stays at the stage of **2b**. Indeed, the result is important in view of the very similar reactivity of Cys and Hcy, and also constitutes the basis for but-3-yn-2-one based fluorescent probe to discriminate between Cys and Hcy.

In addition, we also performed the absorption titration assays with the high concentration of Cys or GSH (1.0 mM for each) to test if the product **3a** or **4** could further react with these biothiols. The spectra obtained (Fig. S4, ESI†) indeed matched well with those shown in Fig. 3, indicating that these products are stable, and could not further react with these biothiols.

Fluorescence Spectra Studies

Encouraged by the above results, we examined emission behaves of **1** upon addition of 30 equiv of Cys, Hcy, and GSH, respectively, in the same condition (Fig. 4). The free probe **1** showed a very poor fluorescence at 550 nm due to the *d*-PeT process.[14] Upon treatment with Cys, a new emission peak appeared at 492 nm and gradually reached equilibrium within 60 min (Fig. 4A), and in this case an approximate 60-fold increase in fluorescence intensity could be observed, indicating the **1**–Cys adduct **3a** is strong fluorescent due to inhibiting the C=C bond isomerization by a produced intramolecular N-H···O hydrogen bond. In fact, this could be supported by control compound **6**, which also displays strong emission with the same emission maximum in the condition (Fig. S5A, ESI†). By contrast, Hcy and GSH hardly elicited any significant fluorescence change of **1** at the same time scale (Figs. 4B and 4C), revealing that the **1**–GSH adduct **4** (or the intermediate **2**) is almost nonfluorescent due to the C=C bond isomerization-induced fluorescence quenching. This could indeed be supported by the control compound **7**, which is also weakly fluorescent (Fig. S5B, ESI†) due to lacking such N-H···O hydrogen-bond interaction, as well as the control compound **8**, which is strong fluorescent[18] due to lacking the C=C double bond in its molecular structure. In addition, the N-H···O hydrogen bond-inhibited C=C bond isomerization in **3a** could also be indicated by a comparison of ^{1}H NMR spectra between control compounds **6** and **7**. For example, due to the intramolecular N-H···O hydrogen-bonding interaction, compound **6** exists as a single isomer with a set of ^{1}H NMR signal (Fig. S6, ESI†); however, due to the absence of such intramolecular hydrogen-bonding interaction, compound **7** exists as a mixture of *Z*- and *E*-isomers with two sets of discernable ^{1}H NMR signals (Fig. S7, ESI†). Taken together, the above results are in very good agreement with our original assumption, and also confirm the potential of the but-3-yn-2-one-based fluorescent probe to discriminate Cys from Hcy/GSH (Fig. 4D).

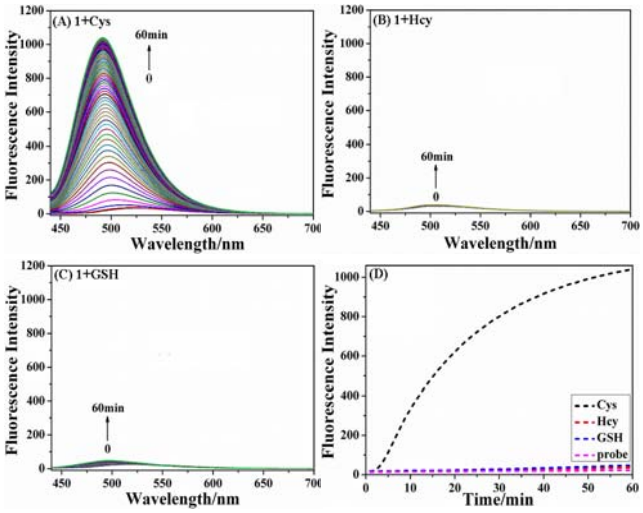


Fig. 4 Fluorescence spectra of **1** (5 μM) after addition of 30 equiv of Cys (A), Hcy (B), and GSH (C) as well as the corresponding time-dependent fluorescence intensity changes (D). Conditions: PBS buffer (20 mM, pH 7.4) at 37 °C; λ_{ex} = 420 nm, λ_{em} = 492 nm; slits: 5/10 nm, voltage: 550 V.

Although probe **1** displays high selectivity for Cys over Hcy and GSH, the response time seems to be long (60 min), which is probably due to the slow Michael addition of the thiol group of Cys with the alkyne group of but-3-yn-2-one. In fact, in the reaction of **1** with GSH or Hcy, the similar or slower reaction rate was also observed (Fig. S1), supporting the above speculation. Although for any fluorescent probe the fast response is desired, compared with the selectivity, the slow response is not a practical problem sometimes. For example, some reported fluorescent probes that show the longer response time (>2hr) could successfully be applied to image biothiols in cells.[6a,19]

Subsequently, we performed the fluorescence titration studies of **1** towards Cys. As shown in Fig. 5, a series of spectra of the solution of **1** with 0 to 180 μM Cys were recorded after 60 min. Upon treatment with the increasing concentrations of Cys, the

fluorescence intensity of **1** at 492 nm gradually increased, and reached saturation when the amount of Cys was more than 160 μM (Fig. 5A). A linear relationship with the Cys concentration from 0 to 30 μM could be obtained (Fig. 5B), and the detection limit for Cys was estimated to be 0.9 μM based on *S/N* = 3, which is indeed sensitive enough to detect and image Cys in cells (intracellular concentration for Cys: 30–200 μM).[20]

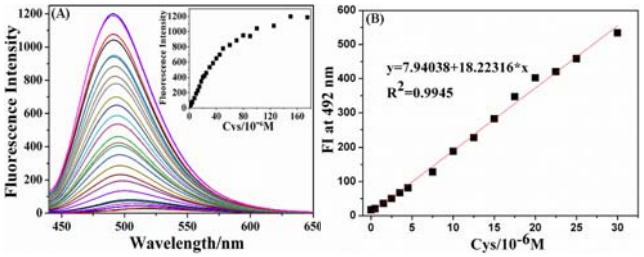


Fig. 5 Fluorescence spectra of **1** (5 μM) upon addition of Cys (0 – 180 μM in PBS buffer (20 mM, pH 7.4) (A) and the corresponding linear relationship between the fluorescent intensity and Cys concentration (B). Spectra were recorded after incubation with different concentrations of Cys for 60 min at 37 °C. λ_{ex} = 420 nm, λ_{em} = 492 nm; slits: 5/10 nm, voltage: 550 V.

Selectivity Studies

To further evaluate the specific nature of **1** for Cys, we also examined the fluorescence enhancement of **1** incubated with various natural amino acids (30 equiv) including Hcy, GSH, His, Glu, Asp, Val, Phe, Tyr, Ala, Ser, Leu, Arg, Pro, Thr, Glu, Trp, Ile, and Lys in PBS buffer (20 mM, pH 7.4). As can be seen in Fig. 6, these natural amino acids did not lead to any significant fluorescence changes of **1**, and only Cys elicited a dramatic increase in the fluorescence intensity. Moreover, the mixture of Cys and any amino acid also resulted in the almost same fluorescence turn-on of **1**, confirming the high selectivity of probe **1** towards Cys. Noteworthy is that even in the presence of Hcy or GSH (30 equiv), probe **1** also displayed the obvious response for Cys only with a slight decrease of fluorescent intensity. This may be because the initial thiol-induced addition reaction of Cys with **1** is faster than that of Hcy or GSH due to its stronger nucleophilicity as observed in most of the reported Michael addition-type probes. In view of the abundant intracellular GSH concentrations (1–10 mM),[21] we further test the fluorescence responses of **1** (5 μM) upon treatment with the mixture of GSH (1 mM)/Cys (0–350 μM) (Fig. S8). Even in the cases, we could still observe the obvious Cys-induced fluorescence enhancement, indicating the potential of probe **1** for biological application. In addition, the high selectivity of probe **1** for Cys could also be observed by naked eyes. When the solution of **1** was excited at 365 nm using UV lamp in the presence of 30 equiv of various amino acids, only Cys caused a strong fluorescence (Fig. 6).

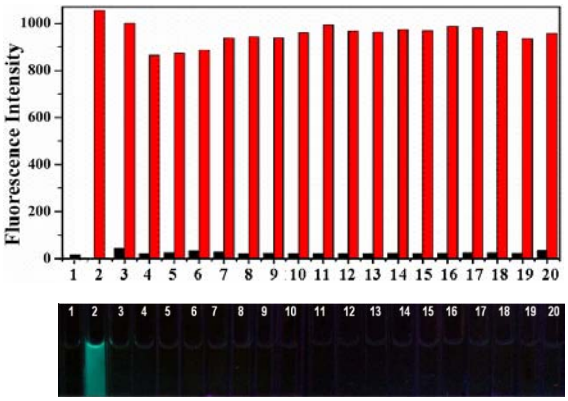


Fig. 6 (Top) Fluorescence intensity of **1** (5 μM) at 492 nm to 30 equiv of various amino acids and its competition graph with Cys. (1) probe **1** only, (2) Cys, (3) Hcy, (4) GSH, (5) His, (6) Gln, (7) Asp, (8) Val, (9) Phe, (10) Tyr, (11) Ala, (12) Ser, (13) Leu, (14) Arg, (15) Pro, (16) Thr, (17) L-Glu, (18) Trp, (19) Ile, (20) Lys. For 3–20, Black bar: **1** + amino acid; Red bar: **1** + amino acid + Cys. Each data was recorded at 60 min after addition of Cys. T = 37 °C; λ_{ex} = 420 nm λ_{em} = 492 nm; slits: 5/10 nm, voltage: 550 V. (Below) Images of **1** (5 μM) to 30 equiv of various amino acids excited by UV light of 365 nm.

The effect of pH

We also tested the effect of pH on the fluorescence response of probe **1** to Cys in B-R buffer measured with and without 30 equiv. of Cys (Fig. 7). It was found that probe **1** was almost nonfluorescent over a wide pH range of 2–12, and displayed the obvious response for Cys in the region of 7–9. The higher pH environment could result in the decomposition of coumarin lactone unit, and thus the decreased fluorescence signal. Thus, probe **1** could function properly at physiological pH.

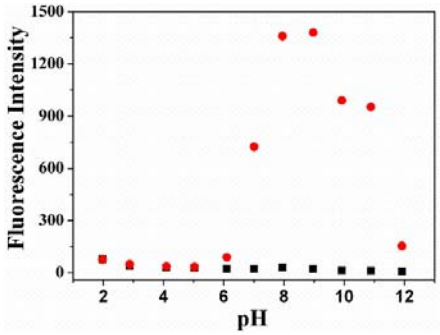


Fig.7 Changes in fluorescence intensity of **1** (5 μM) in B-R buffer measured with and without 30 equiv. of Cys as a function of pH. λ_{ex} = 420 nm, λ_{em} = 492 nm. Slits: 5/10 nm, voltage: 550 V.

Cell Imaging

Subsequently, we evaluated the capability of **1** to selectively image Cys in biological system (Fig. 8). The human renal cell carcinoma 786-0 cells were found to have almost no fluorescence (Fig. 8A) when excited at 405 nm. However, when 786-0 cells are incubated with **1** (10 μM), they gave obvious fluorescence (Fig. 8B), suggesting that **1** is responsive to intracellular Cys. When 786-0 cells were pre-treated with 0.2 mM Cys and then incubated with 10 μM **1**, they gave the stronger fluorescence (Fig. 8C). When 786-0 cells were pre-treated with 0.2 mM Hcy (or GSH), and then incubated with 10 μM **1**, they did not give fluorescence enhancement (Fig. 8D or 8E) when compared with the image shown in Fig. 8B. When 786-0 cells were pretreated

with N-ethylmaleimide (NEM, 1.0 mM, a trapping reagent of thiol species) and then incubated with 10 μM **1**, a remarkable decrease in fluorescence intensity was observed (Fig. 8F). These results suggest that probe **1** could specifically sense intracellular Cys without the interference of Hcy or GSH.

Conclusions

In summary, a reaction-type fluorescent probe **1** was constructed by incorporation a novel Cys binding group but-3-yn-2-one into 7-diethylaminocoumarin fluorophore. The probe could specifically detect Cys over Hcy and GSH as well as other amino acids in aqueous solution based on the Michael addition–rearrangement cascade reaction coupled with the inhibiting C=C isomerization-induced fluorescence quenching by a produced intramolecular N-H···O hydrogen bond. Preliminary fluorescence imaging experiments in cells indicate its potential to probe Cys chemistry in biological systems. Given that the fluorescence of many dyes could be quenched by C=C isomerization, we speculate that the hydrogen bond-inhibited C=C isomerization-induced fluorescence quenching promoted by Cys could also extend to other dyes, especially those near-infrared dyes, to facilitate various biological research.

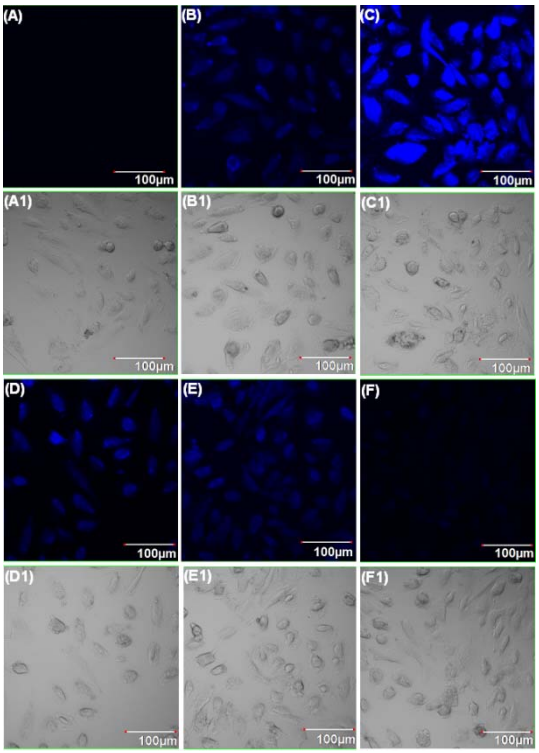


Fig. 8 Fluorescence Imaging of Cys in human renal cell carcinoma 786-0 cells using probe **1**. (A) 786-0 cells only. (B) 786-0 cells incubated with 10 μM of **1**. (C) 786-0 cells pre-incubated with 0.2 mM of Cys, and then treated with 10 μM of **1**. (D) 786-0 cells pre-incubated with 0.2 mM of Hcy, and then treated with 10 μM of **1**. (E) 786-0 cells pre-incubated with 0.2 mM of GSH, and then treated with 10 μM of **1**. (F) 786-0 cells pre-incubated with 1.0 mM of NEM, and then treated with 10 μM of **1**. (A1–F1) The corresponding brightfield images. Emission was collected at 425–525 nm (excited at 405 nm). Scale bar: 100 μm.

Experimental Section

General information and methods

All reagents and solvents were purchased from commercial sources and were of the highest grade. Solvents were dried according to standard procedures. All reactions were magnetically stirred and monitored by thin-layer chromatography (TLC). Flash chromatography (FC) was performed using silica gel 60 (200–300 mesh). Absorption spectra were taken on a Varian Carry 4000 spectrophotometer. Fluorescence spectra were taken on Hitachi F-7000 fluorescence spectrometer. The ^{1}H NMR and ^{13}C NMR spectra were recorded at 600 and 150 MHz, respectively. The following abbreviations were used to explain the multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad. High resolution mass spectra were obtained on a Varian QFT-ESI mass spectrometer.

Synthesis

Compound **S2** was synthesized according to the reported procedures.[22]

Probe 1

AcCl (70 μL, 1.0 mmol) was added to a solution of **S2** (313 mg, 1.0 mmol) in CH_2Cl_2 (15 mL) at 0 °C, followed by addition of $AlCl_3$ (666 mg, 5.0 mmol). After 15 min, the reaction was quenched with water and extracted with EtOAc. After the organic layer was dried over Na_2SO_4, the solvent was removed, and the residue was purified by column chromatography on silica gel (CH_2Cl_2) to yield probe **1** (134 mg, 47%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.91 (s, 1H), 7.27 (d, J = 9.0 Hz, 1H), 6.63 (dd, J_1 = 9.0 Hz, J_2 = 2.4 Hz, 1H), 6.48 (d, J = 2.4 Hz, 1H), 3.47 (q, J = 7.2 Hz, 4H), 2.46 (s, 3H), 1.25 (t, J = 7.2 Hz, 6H); ^{13}C NMR ($CDCl_3$, 150 MHz):187.16, 163.10, 160.22, 155.37, 153.02, 132.92, 112.61, 111.15, 103.39, 100.11, 95.14, 89.75, 48.04, 35.48, 15.34; HRMS for $[M+H]^+$: calcd 284.1281, found 284.1284.

Control compound 6

Probe **1** (100 mg, 0.35 mmol) and 2-aminoethanethiol (270 mg, 3.5 mmol) was dissolved in 15 mL acetonitrile, and one drop of triethylamine was added. The reaction mixture was stirred at 37 °C for 2 h, and then evaporated. The crude product was purified through column chromatography over silica (eluent: CH_2Cl_2/EtoAc/petroleum ether = 10/0.1/2) to give **6** as yellow solid (27 mg, yield : 21%). ^{1}H NMR (600 MHz, CDCl3): δ 7.67 (s, 1H), 7.32 (d, J = 9 Hz, 1H), 6.63 (dd, J_1 = 9 Hz, J_2 = 2.4 Hz, 1H), 6.50 (d, J = 1.8 Hz, 1H), 5.09 (s, 1H), 3.45 (q, J = 7.2 Hz, 4H), 3.38 (q, J = 6.6 Hz, 2H), 2.71 (q, J = 6.6 Hz, 2H), 2.32 (s, 1H), 2.09 (s, 3H), 1.48 (t, J = 8.4 Hz, 1H), 1.24 (t, J = 7.2 Hz, 6H). ^{13}C NMR (600 MHz, CDCl3): δ 199.37, 162.52, 161.97, 160.09, 154.52, 146.68, 132.51, 132.20, 112.27, 111.89, 110.60, 100.02, 50.77, 47.88, 32.18, 28.13, 15.32. HRMS: calcd for $[M+H]^+$ 361.1586, found 361.1580.

Control compound 7

Probe **1** (70 mg, 0.247 mmol) and ethanethiol (178 μL, 2,47 mmol) was dissolved in 10 mL acetonitrile and 5 mL ethyl alcohol, and triethylamine (344 μL, 2.47 mmol) was added. The reaction mixture was stirred at room temperature for 4 h, and then evaporated. The crude product was purified through column chromatography over silica (eluent: CH_2Cl_2/EtoAc = 10/0.5) to give **7** as a *Z*- and *E*-isomer mixture (32 mg, yield: 37%). ^{1}H NMR (600 MHz, CDCl3): 7.56 and 7.54 (S, 1H), 7.31 and 7.24 (d, J = 9 Hz, 1H), 6.63 and 6.61 (q, J_1 = 9 Hz, J_2 = 2.4 Hz, 1H), 6.57 and 6.55 (q, J_1 = 9 Hz, J_2 = 3 Hz, 1H), 6.43 and 6.16 (s,1H), 3.39–3.45 (m, 4H), 2.88 and 2.69 (q, J_1 = 15 Hz, J_2 = 7.2 Hz, 2H),

2.26 and 2.18 (s, 3H), 1.17–1.38 (m, 9H). ^{13}C NMR (600 MHz, CDCl3): δ 196.83, 162.22, 159.40, 153.91, 145.42, 144.29, 132.06, 123.65, 112.24, 111.82, 100.33, 100.05, 47.77, 33.56, 29.54, 16.78, 15.33. HRMS for $[M+H]^+$: calcd 346.1477, found 346.1469.

Procedures for biothiols sensing

The solutions of amino acids and reduced glutathione (GSH) were prepared in deionized water. A stock solution of **1** (2 mM) was prepared in CH_3CN. The stock solution of **1** was then diluted to the corresponding concentration (10 μM or 5 μM) with PBS buffer (20 mM, pH 7.4). Spectra data were recorded in an indicated time after the addition of amino acids.

Cell culture and fluorescence imaging

The human renal cell carcinoma 786-0 cells were provided by Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education (China). Cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % FBS (Fetal Bovine Serum) and 1% antibiotics at 37 ºC in humidified environment of 5% CO_2. Cells were plated on 6-well plate and allowed to adhere for 12 hours. Fluorescence imaging was performed with a Olympus FluoView FV1000 confocal microscope. Before the experiments, cells were washed with PBS 3 times. Then, the cells were incubated with **1** (10 μM), or pretreated with Cys (0.2 mM, 30 min) or Cys (0.2 mM, 30 min) or GSH (0.2 mM, 30 min) in DMEM medium at 37 °C. After each treatment, the cells were washed with PBS 3 times. Emission was collected at 425–525 nm (excited at 405 nm).

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