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A Naphthalimide-Based Glyoxal Hydrazone for Selective Fluorescence Turn-On Sensing of Cys and Hcy

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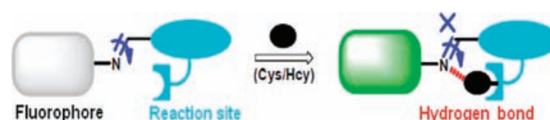
ABSTRACT



A fluorescent turn-on probe for Cys/Hcy based on inhibiting the C=N isomerization quenching process by an intramolecular hydrogen bond was reported. The probe exhibited higher selectivity toward Cys/Hcy over other amino acids as well as thiol-containing compounds.

Cysteine (Cys) and homocysteine (Hcy) play crucial roles in many physiological processes. Cys deficiency is implicated in many syndromes such as slow growth in children, skin lesions, hair depigmentation, liver damage, and edema.[1] Hcy has also been linked to an increased risk of Alzheimer's disease,[2] cardiovascular diseases (CVD),[3] inflammatory bowel disease, and osteoporosis.[4] Thus, it is important to develop efficient methods for the detection and quantification of Cys/Hcy in physiological media for academic research and clinic applications.[5] A variety of colorimetric and fluorescent probes for Cys/Hcy have been constructed by exploiting the high nucleophilic reactivity or transition metal affinity of the thiol group,[6] which involve specific reactions between probes and thiols, such as Michael addition,[7] cleavage reactions by thiols,[8] metal coordination,[9] cyclization with aldehydes,[10] and others.[11]

Fluorescence spectroscopy has become a powerful tool for sensing and imaging trace amounts of samples because of its simplicity, sensitivity, fast response times, and its application for not only in vitro assays but also in vivo

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imaging studies.[12] Many sensing mechanisms including photoinduced electron transfer (PET), excimer/exciplex formation, intramolecular charge transfer (ICT), metal–ligand charge transfer (MLCT), fluorescence resonance energy transfer (FRET),[12] aggregation-induced emission (AIE),[13] and excited-state intramolecular proton transfer (ESIPT) have been used in the design of fluorescent probes.[14] Accordingly, a variety of fluorescent probes for the detection of Cys/Hcy based on these conventional sensing mechanisms have also been reported in literature.[6–10]

Among the various sensing mechanisms, C═N isomerization has been actively developed in recent years.[14] It was found that C═N isomerization is the predominant decay process of excited states in compounds; thus those compounds are often nonfluorescent. However, C═N isomerization may be inhibited by either the complexation of metal cations to the group[15] or the simple removal of the C═N bond,[16] thereby leading to strong fluorescence emission. Following this strategy, a number of excellent C═N isomerization-based probes have been exploited for sensing various metal cations (e.g., Zn^{2+}, Cd^{2+}, Mg^{2+}, Cu^{2+})[15] as well as ClO^-.[16] However, to the best of our knowledge, the attractive mechanism has not been previously exploited to design fluorescent probes for the detection of biothiols,[6,14] presumably due to the absence of the appropriate interaction induced by these species to prevent the C═N bond isomerization-induced quenching.

Herein, we hope to report a naphthalimide-based glyoxal hydrazone (**1**) for the fluorescence turn-on detection of Cys/Hcy based on inhibiting the C═N isomerization-induced quenching process by an intramolecular hydrogen bond. The rationale is depicted in Figure 1 and illustrated as follows: (1) probe **1** will show weak fluorescence due to the rapid C═N isomerization in the excited state;[15] (2) the cyclization of aldehyde with Cys/Hcy will lead to the formation of thiazolidines/thiazinanes **1**-Cys/**1**-Hcy; (3) it is expected that the five-membered cyclic intramolecular hydrogen bond[17] between thiazolidine/thizaimane NH and the imine N atom in **1**-Cys/**1**-Hcy will prevent the C═N bond isomerization-induced quenching, thereby leading to a fluorescence enhancement of the system.

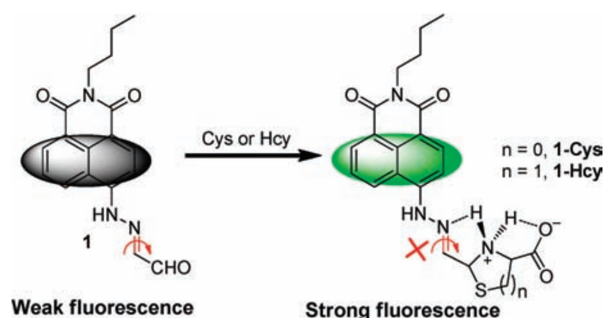

Figure 1. A plausible mechanism of response of **1** to Cys/Hcy.

Probe **1** was synthesized from the reaction between naphthalimide hydrazine **3**[18] and glyoxal **2** (Scheme S1). Its structural identification was confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS spectroscopy (Supporting Information (SI)). In fact, probe **1** displays almost the same spectral responses for Cys and Hcy in our conditions. Therefore, in the subsequent experiments, Cys was mainly used to evaluate the performance of probe **1**.

First, the fluorescence properties of probe **1** were tested in various solvents (Figure S1). It was found that the free **1** showed very weak fluorescence in aprotic solvents, such as DMSO, THF, and CH_3CN. By contrast, an obvious fluorescence enhancement of **1** could be observed in protic solvents, such as methanol (16-fold) and ethanol (11-fold). We attributed the weak fluorescence of **1** in aprotic solvents to the C═N isomerization-induced quenching. However, the strong fluorescence of **1** in protic solvents probably results from the intermolecular hydrogen bond interaction between the solvent molecules and the —N═CH—CHO moiety of **1** that partly inhibits the C═N isomerization-induced quenching process. Encouraged by the results, we envisioned that **1**-Cys would exhibit the strong emission in aprotic solvents, such as DMSO, because the quenching process will probably be inhibited by the intramolecular hydrogen bonds in **1**-Cys as shown in Figure 1.

Subsequently, to gain insight into the proposed mechanism, the fluorescence property of probe **1** in the absence or presence of Cys in DMSO was investigated. As shown in Figure 2, probe **1** (5 μM) showed a very weak fluorescence at 524 nm (Φ_F = 0.012). However, upon addition of Cys, the fluorescence intensity increased remarkably. The changes of the emission intensities nearly became constant when the amount of Cys added reached 400 equiv (Φ_F = 0.625 for **1**-Cys), and an approximately 52-fold fluorescence enhancement could be observed. The more marked

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fluorescence enhancement than that of **1** in protic solvents as well as the virtually unchanged emission shift of **1** upon addition Cys strongly suggests the C=N isomerization-induced quenching is inhibited in the **1**-Cys adduct due to the efficient intramolecular hydrogen bond interaction.

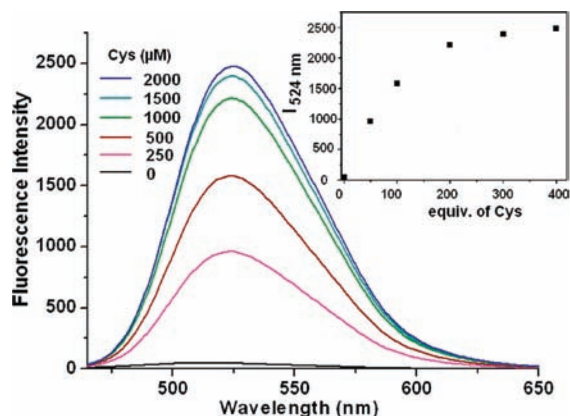

Figure 2. Fluorescence spectral changes of **1** (5 μM) upon addition of Cys (0−400 equiv) (λ_{ex} = 439 nm, λ_{em} = 524 nm; slit, 5 nm/10 nm) in DMSO. Each spectrum was recorded after 60 min (Figure S4).

In addition, the large fluorescence changes of **1** induced by Cys/Hcy can clearly be perceived by the naked eye. When probe **1** was excited at 365 nm using a UV lamp in the presence of 100 equiv of various amino acids as well as other thiol-containing compounds, such as mercaptopropionic acid (MPA), ethyl 2-mercaptoacetate (EMA), and reduced glutathione (GSH), only Cys and Hcy caused a strong green fluorescence (Figure 3), further supporting the proposed mechanism.

Figure 3. Visual fluorescence emissions of probe **1** (5 μM) to various amino acids and thio-containing compounds (100 equiv) in DMSO on excitation at 365 nm using UV lamp at rt.

To gain clear insight into its seletivity, probe **1** (5 μM) in DMSO was treated with 100 equiv of various amino acids as well as MPA, EMA, and GSH and monitored by fluorescence spectroscopy. In fact, these competitive species did not induce any significant fluorescence changes of **1**, and only Cys/Hcy elicited a dramatic increase in the fluorescence intensity (Figure 4). Moreover, in the presence of these competitive species, the Cys still resulted in similar fluorescence changes, suggesting that the probe is highly selective to Cys. A similar case was also observed for Hcy.

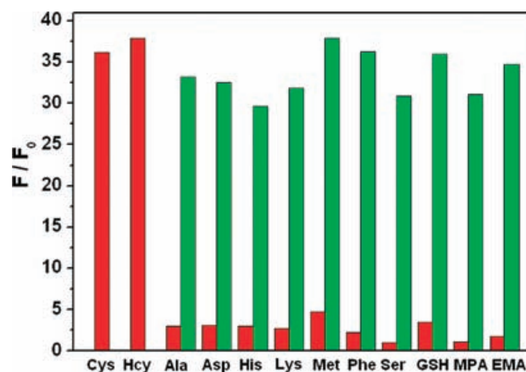

Figure 4. Fluorescence ratio of **1** (5 μM) at 524 nm in DMSO to various species, and its competition graph with Cys. Red bar: **1** + various species. Green bar: **1** + various species + Cys. $\lambda_{ex}/\lambda_{em}$ = 439 nm/524 nm. Slit, 5 nm/10 nm.

To confirm the formation of **1-**Cys, probe **1** was treated with Cys, and the reaction product was isolated (SI). The partial ^{1}H NMR spectra of **1** and the isolated **1**-Cys are shown in Figure 5. The resonance signal corresponding to the aldehyde proton at 9.59 ppm disappeared; however, concomitantly, two new peaks at 5.17 and 5.46 ppm assigned to the methine proton of the thiazolidine diastereomer emerged, consistent with the previous reports.[10d,h] This formation was further characterized by mass spectrometry analysis, in which the peaks at *m/z* 425.1287 (calcd = 425.1289) corresponding to [**1-Cys** $-H^+$] was clearly observed (Figure S2).

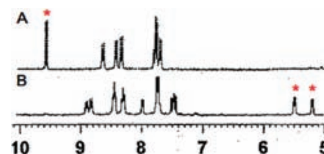

Figure 5. Partial ^{1}H NMR spectra of **1** (A) and **1**-Cys (B) in DMSO.

To explore the practical application of probe **1**, we also evaluated the effect of water on the fluorescence response of probe **1** to Cys. To this end, we repeated the above fluorescence experiments in DMSO−HEPES buffer solution (100 mM, pH = 7.4, 1:1, v/v). In these conditions, as shown in Figure 6, **1** still displayed obvious fluorescence enhancement upon gradual addition of Cys, but less effectively than that in DMSO. In fact, for many fluorescence dyes, the relatively weak fluorescence in water solution could usually be observed due to the efficient quenching of highly polar water molecules. However, the high selectivity (Figure S3) as well as a good linear relationship with the concentration of Cys between 0−500 μM (Figure 6) could still be obtained in these conditions.

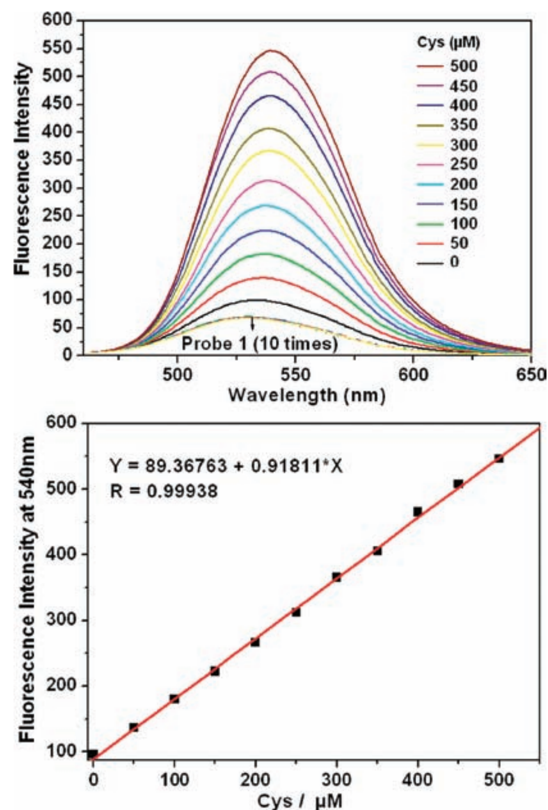

Figure 6. (Top) Fluorescence response of **1** (5 μM) to Cys in DMSO–HEPES buffer (100 mM, pH = 7.4, 1:1, v/v) ($\lambda_{ex}/\lambda_{em}$ = 439/540 nm. Slit: 5 nm/10 nm). (Bottom) Fluorescence response at 540 nm of **1** to Cys (0–500 μM). Each spectrum was recorded after 60 min (Figure S4).

To test the capability of **1** to image thiols in living cells,[19] *Tetrahymena thermophila* cells were incubated with **1** (10 μM) in PBS buffer containing 1/500 DMSO for 30 min at 30 °C.[20] Probe **1** was found to be cell-permeable and to react with intracellular thiols, resulting in green fluorescence emission, which could clearly be observed by confocal fluorescence microscopy (Figure 7b). In a control experiment, the cells were pretreated with *N*-ethylmaleimide (NEM, a trapping reagent of thiol species)[21] and further incubated with the probe for 30 min, providing very weak fluorescence (Figure 7c), in line with the emission profile of the free probe. Further, when cells were pretreated with 100 μM Cys and further incubated with the probe for 30 min, the cells showed the much stronger fluorescence (Figure 7d) than that in Figure 7b, indicating that the green fluorescence emission in Figure 7b is due to the formation of the **1**-Cys/Hcy adduct. The above results reveal that the mechanism of inhibiting the C=N isomerization-induced quenching by an intramolecular hydrogen bond in **1**-Cys still works well in living cells.

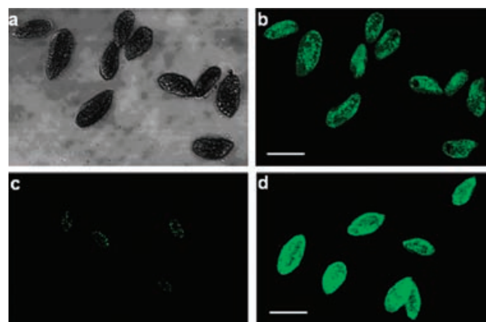

Figure 7. Confocal fluorescence images of *Tetrahymena thermophila* cells. The brightfield images (a) and their fluorescence images (b) in the presence of 10 μM of **1**. *Tetrahymena thermophila* cells were preincubated with 20 μM NEM (c) or 100 μM Cys (d) for 30 min and then treated with 10 μM of **1** for 30 min. Scale bar = 50 μm. Staining medium: DMSO–PBS (1:500, v/v, pH 7.4).

In conclusion, we have demonstrated a fluorescent turn-on probe **1** for Cys/Hcy based on inhibiting the C=N isomerization-induced quenching process by an intramolecular hydrogen bond. Probe **1** displays a highly selective fluorescent enhancement toward Cys/Hcy with an obvious fluorescent color change from dark to green. The probe was also applied to the biological imaging of Cys or Hcy inside living cells. We hope the results presented here may contribute to the development of novel C=N isomerization-based probes for the detection of biological small molecules.

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Supporting Information Available. Experimental procedures, supplemental data, and ^{1}H, ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

(19) The free intracellular Cys concentrations are on the order of 30–200 μM. Chung, T. K.; Funk, M. A.; Baker, D. H. *J. Nutr.* **1990**, *120*, 158–165.

(20) The cells incubated with 10 μM of **1** were still alive after 2 h, indicating the low toxicity of **1** to cells.

(21) Reviewer suggested a wise strategy to demonstrate the high selectivity of **1** toward Cys in cell imaging experiment by using a simple analog of **1** (phenyl-based glyoxal hydrazone) instead of NEM. However, the preliminary experiment based on the analog did not provide a positive result, presumably due to its low reactivity.