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Design and synthesis of NBD-S-dye dyads for fluorescently discriminative detection of biothiols and Cys/Hcy

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ABSTRACT

Based on thiolysis of NBD (7-nitro-1,2,3-benzoxadiazole) thioether, we designed and synthesized fast-response fluorescent probes for discriminative detection of biothiols and Cys/Hcy. Probes treated with GSH/H_2S could only release one emission; while reactions with Cys/Hcy generated two. Compared with probe **1**, probe **2** exhibited good stability and high sensitivity toward Cys/Hcy in PBS buffer. Moreover, **2** could eliminate the effect of probe consumption by GSH/H_2S when used to selectively detect Cys/Hcy and was applied for selective bioimaging of Cys in living mammalian cells successfully.

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1. Introduction

Biothiols play important roles in biological systems, which are recognized as antioxidants during injury and oxidative stress, also as chelators interacting with metals, and as essential signalling molecules. For instance, glutathione (GSH) serves many cellular functions, including maintenance of intracellular redox activities, xenobiotic metabolism, intracellular signal transduction and gene regulation.[1] Malfunction of biothiol levels are implicated with a number of diseases. Abnormal levels of cysteine (Cys) is linked to liver damage, slow growth, edema, *etc*.[2] High levels of homocysteine (Hcy) are associated with cardiovascular disease and Alzheimer's disease.[3] Endogenous H_2S was excessive in colorectal cancer cells.[4] Moreover, altered levels of thiols in various physiological media have been linked to specific pathological conditions. Owing to their significant roles, it is highly important to develop rapid, facile, and reliable methods for discriminative detection of different biothiols.

Because of high resolution and sensitivity, fluorescence imaging has attracted great attention in fields related to biomedicine. Recently, great efforts have been focused on developing fluorescent probes for biothiols, including cyclization reactions between aldehydes and aminothiols,[5] Michael addition,[6] cleavage reactions of 2,4-dinitrobenzenesulfonyl (DNBS) with thiols,[7] nucleophilic substitution reactions,[8] disulfide exchange reactions[9] and others.[10] However, the discriminative detection of different thiols is still a tough challenge due to their similar structures and reaction activities. Despite the challenge, several fluorescent probes have been developed to specially detect one or two biothiols.[11,12,8a,8b] Sun et al. reported a fluorescent probe for discrimination of Cys, Hcy, GSH, separately.[11e] Yoon et al. reported that a NBD thioether could be employed to detect Cys and Hcy selectively under weak acid condition.[12d] However, some probes suffered from long reaction time and consumption of probes by unwanted analytes. Although these reports greatly promoted the study of fluorescent probes on biothiols, innovative probes with good stability, fast response, high sensitivity and high selectivity toward Cys/Hcy without consumption of probes by undesirable analytes are still needed.

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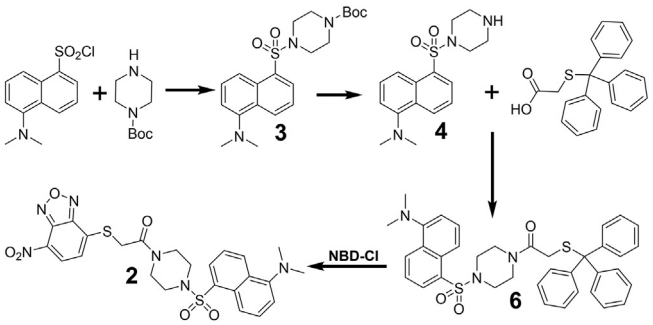
We have been interested in development of molecular probes for H_2S and biothiols for some time.[6a,13-15] One major challenge in development of biothiols probes is the discovery of a chemical reaction to effectively separate the reactivity of different biothiols including H_2S and other biospecies in physiological condition. To address this challenge, we discovered such a reaction of thiolysis of NBD amine in 2013, and successfully developed a selective fluorescent probe for H_2S.[14a] Pluth group discovered that thiolysis of NBD ether (thioether) could be used to discriminative detection of Cys and H_2S.[12,13] Therefore, we proposed that NBD-S-dye dyad could also be employed for detection and discrimination of biothiols (Scheme 1).

In this work, we employed a new strategy based on the thiolysis of NBD thioether to develop fluorescent probes for biothiols (Scheme 1). We chose dansyl or coumarin as fluorophore and NBD thioether as the reactive moiety. The NBD-based probes showed ignored fluorescence due to the quenching effect of fluorescence resonance energy transfer (FRET). After reaction with biothiols, fluorescence of the fluorophore was released and NBD part was transferred to a new NBD thioether. In the case of Cys/Hcy, the NBD thioether could transfer into NBD amine, which could give yellow fluorescence of NBD dyes. As a result, the NBD-S-dye dyad could give single emission for GSH/H_2S and two kind of emissions for Cys/Hcy. To our delight, probe **2** could eliminate the effect of probe consuption by GSH/H_2S when used to detect Cys/Hcy, superior to previous NBD-*O*-based fluorescent probes.

Synthesis route of probes were shown in Scheme 2 and Fig. S1. Dansyl chloride was reacted with *tert*-butyl piperazine-1-carboxylate using DIPEA to afford **3**, which was treated by TFA to generate **4**. Compound **6** was synthesized from coupling of **4** and **5**. **6** underwent a deprotection reaction using TFA and Et_3SiH, and then treated with NBD-Cl in CH_2Cl_2 to yield **2**. Similar synthesis route was used to yield **1**. The syntheses of probes are simple and quite straightforward, which is important for the wide application. The structures of probes were confirmed by ^1H NMR, ^{13}C NMR and high resolution mass spectra (HRMS).

2. Results and discussion

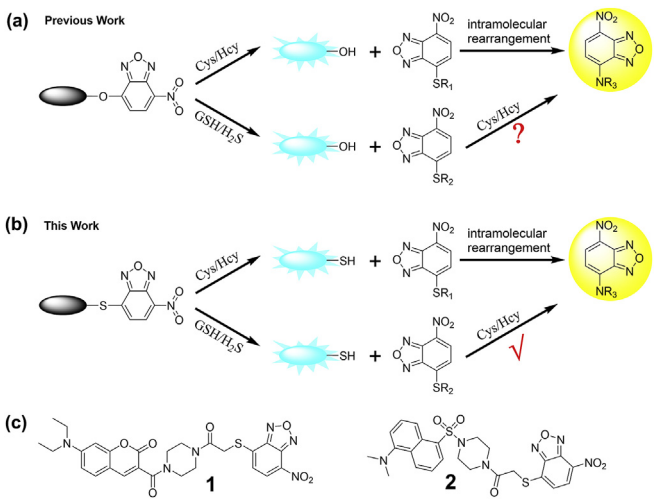
With probes in our hand, we firstly tested the time-dependent absorption spectra of **1** (5 μM) toward Cys (50 μM) in PBS buffer (pH 7.4, 50 mM, containing 10% DMSO). **1** displayed maximum absorbance at 418 nm, which slightly decreased with a new peak at 475 nm appearing after treatment with Cys (Fig. S2). We next investigated the fluorescent response of **1** with Cys (Fig. 1a) in PBS buffer, **1** showed weak fluorescence and Cys could triger 6-fold fluorescent enhancement. Unfortunately, **1** displayed undesirable stability in PBS buffer, which limited its further application (Fig. 1c).



Scheme 2. The synthesis route for probe **2**.

Subsequently, we examined the time-dependent absorbance and fluorescence spectra of probe **2** with biothiols in PBS buffer. As shown in Fig. 1 and Fig. S3, **2** exhibited a fast response in fluorescence and in absorbance with biothiols. In absorbance, new peak appeared at around 335 nm corresponding to dansyl, while the new peak at 475 nm could be due to the formation of NBD-Cys addition amine, which was identified by HRMS (see ESI). The fluorescent tests of probe **2** toward Cys was carried out in PBS buffer with excitation at 345 nm firstly (Fig. 1b and d). The spectra indicated that the increase in fluorescence at 550 nm was ca. 81 fold. Compared with **1**, **2** was quite stable in PBS buffer with no obvious fluorescent enhancement within 15 min. Therefore, we only employed probe **2** in following studies.

Absorption response of **2** toward Hcy, GSH and H_2S were also studied (Fig. 2a, Fig. S3). After reacting with biothiols, new absorbance dansyl peak appeared at around 335 nm. Particularly, new peaks at 475 nm were found for Cys/Hcy; while new peaks at 420 nm and 535 nm were responsible for GSH and H_2S, due to the formation of NBD-GSH and NBD-SH,[12] respectively. Under



Scheme 1. (a) Schematic illustration of NBD-*O*-based fluorescent probes for detection of biothiols; (b) NBD-S-based probes could be applied for discriminative detection of Cys/Hcy in the presence of GSH/H_2S; (c) chemical structures of probes **1** and **2**.

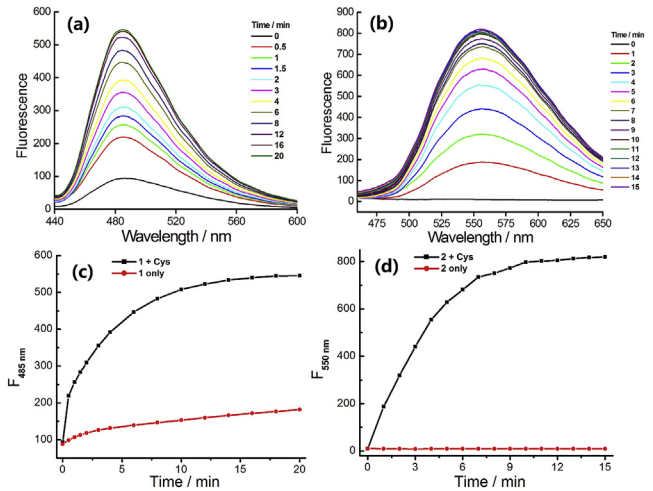


Fig. 1. (a) Time-dependent fluorescence spectra of **1** (1 μM) toward Cys (10 μM); (b) time-dependent fluorescent spectra of **2** (5 μM) toward Cys (100 μM); (c) time-dependent emission of **1** (1 μM) at 485 nm with or without Cys (10 μM); (d) time-dependent emission of **2** (5 μM) at 550 nm with or without Cys (100 μM). For **1**, excitation: 400 nm; for **2**, excitation: 345 nm.

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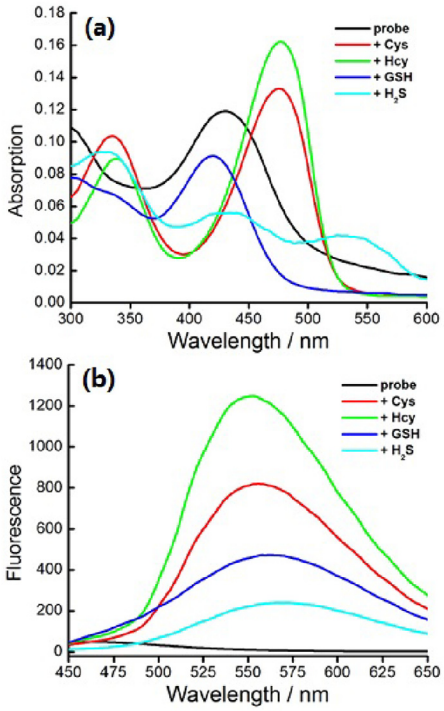

Fig. 2. (a) Absorption spectra of **2** (10 μM) with biothiols (200 μM); (b) Fluorescent spectra of **2** (5 μM) with biothiols (100 μM). Excitation: 345 nm.

excitation of 345 nm, the fluorescent enhancement was 118, 49 and 26 fold for Hcy, GSH and H_2S, respectively (Fig. 2b, Fig. S4). These results demonstrated that all biothiols could cause the thiolysis of NBD thioether to release the dansyl group. However, fluorescent properties after the thiolysis reaction were different, which should be due to the fluorescence contribution from the resulted NBD parts. Significant discrimination of the four biothiols could not be achieved under such a condition in fluorescence.

Then we studied the fluorescent properties of **2** with excitation at 470 nm in PBS buffer. As expected, Cys and Hcy could trigger obvious fluorescence response (Fig. 3 and Fig. S5), while GSH and H_2S could not. Probe **2** showed weak fluorescence (quantum yield ϕ, 0.08%) and the emissions at 550 nm were 116 and 189 fold for Cys (ϕ, 2.5%) and Hcy (ϕ, 3.0%), respectively. The reaction rate for Cys was slightly faster than that of Hcy, implying that the intramolcular rearrangement for Cys (five-member cycle) is easier.[12d]

To further study the sensitivity of **2** toward Cys/Hcy, the fluorescent intensity changes upon 470 nm excitation were closely monitored by addition of various concentrations of Cys/Hcy into **2** (Fig. S6). The fluorescence intensity at 550 nm was linearly related to the concentrations of Cys/Hcy from 0 to 25 μM (Fig. 4), and the detection limit was calculated using the 3σ/k method.[16] The detection limit was 35 nM and 26 nM for Cys and Hcy, respectively, implying that probe **2** was highly sensitive toward Cys/Hcy in PBS buffer.

To gain detailed information about the selectivity, **2** was incubated with various amino acids and biothiols. When excited at 345 nm, only four biothiols showed obvious fluorescent enhancement (Fig. 5a), which indicated that **2** could selectively detect biothiols over millimolar amino acids. While when excited at 470 nm, only Cys and Hcy triggered obvious fluorescence response and other analysts including millimolar GSH and H_2S exhibited ignored fluorescence (Fig. 5b). These experimental results implied that **2** could be used for selective detection of biothiols and for highly discriminative detection of Cys/Hcy over H_2S and GSH.

In order to clarify the influence on detection of Cys/Hcy in the presence of GSH, **2** (5 μM) was firstly treated with 40 eq. GSH for 10 min, then 40 eq. Cys or Hcy was added. As shown in Fig. 6, there was no obvious fluorescent response when **2** was incubated with GSH only, while the addition of Cys/Hcy triggered strong fluorescence. When **2** was reacted with 40 eq. GSH and 40 eq. Cys/Hcy simultaneously, obviuos fluorescent response was observed, implying that the existence of GSH did not interfere with the detection of Cys/Hcy. Similarly, experiments about H_2S were also carried out (Fig. S7), the results indicated that the presence of H_2S had no obvious influence on detecting Cys/Hcy.

Considering the resulted NBD thioethers from **2** and GSH/H_2S, we supposed that the thioethers could further react with Cys/Hcy to form the final fluorescent NBD amine compounds (Scheme 1), which were confirmed by HRMS (Fig. S10). To further clarify the reaction mechanism, 2-iodoacetamide was added subsequently after the incubation of **2** with Cys, and the corresponding products were alao confirmed by HRMS (Fig. S11). Therefore, we demonstrated that probe **2** could eliminate the effect of probe consumption by GSH/H_2S when used for discriminative detection of Cys/Hcy.

To test the biological applicability of probe **2**, we firstly examined whether **2** can be used to detect intracellular Cys in living cells. HeLa cells were pretreated with the thiol blocking reagent *N*-ethylmaleimide (NEM, 1 mM) for 30 min, and then incubated with **2** (2 μM) for 30 min and subsequently imaged using a confocal fluorescence microscopy. As shown in Fig. 7a, no obvious green fluorescence was observed. However, addition of 1 mM Cys into the NEM-treated cells triggered strong green fluorescence (Fig. 7b). Merge images showed that cells retained good morphology after incubation with **2**. These results demonstrated that **2** is cell-permeable and can react with intracellular Cys efficiently.

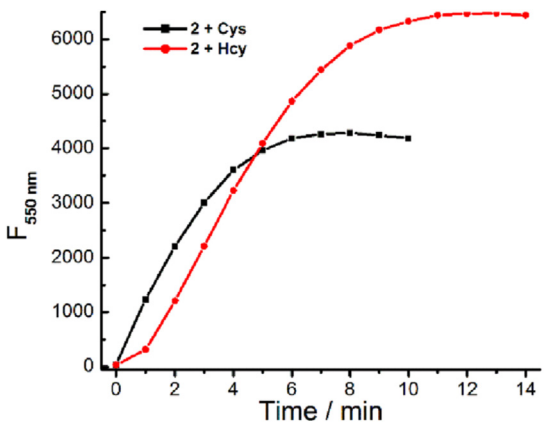

Fig. 3. Time-dependent emission of **2** (5 μM) at 550 nm toward Cys (100 μM) or Hcy (100 μM). Excitation: 470 nm.

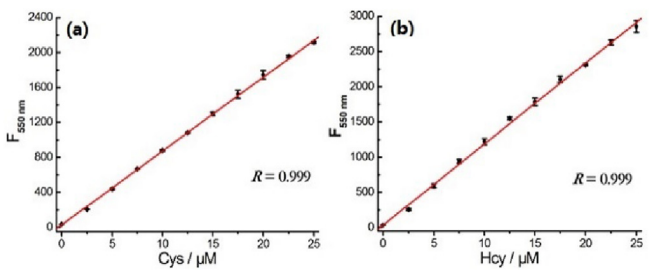

Fig. 4. Linear correlation between emissions at 550 nm with concentrations of Cys (a) or Hcy (b). Excitation: 470 nm.

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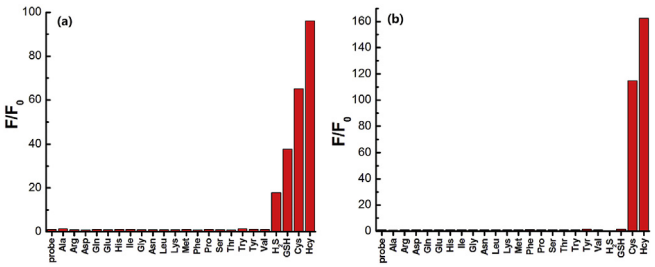

Fig. 5. Relative emissions of **2** (5 μM) at 550 nm toward different species in PBS buffer (pH 7.4, 50 mM, containing 10% DMSO). Excitation for (a) 345 nm; (b) 470 nm. All reactions were performed for 15 min.

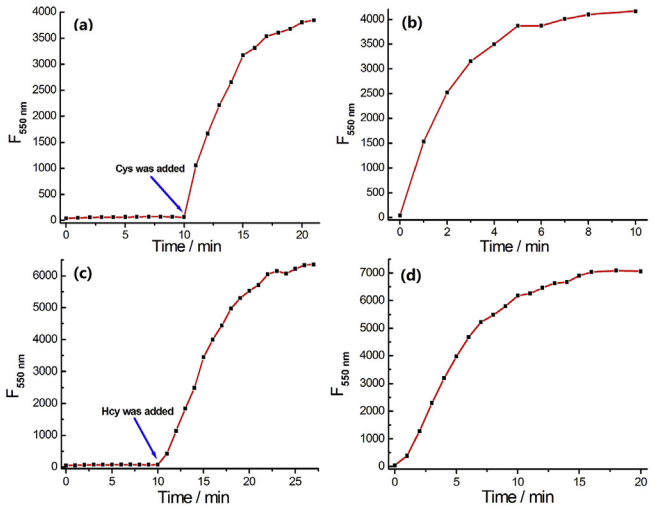

Fig. 6. Detection of Cys/Hcy in the presence of GSH in PBS buffer. **2** (5 μM) was incubated with GSH (200 μM) only for 10 min, then Cys (a, 200 μM) or Hcy (c, 200 μM) was added; (b) **2** (5 μM) was treated with GSH (200 μM) and Cys (200 μM) simultaneously; (d) **2** (5 μM) was treated with GSH (200 μM) and Hcy (200 μM) simultaneously. Excitation: 470 nm.

To examine whether **2** could detect intracellular Cys in the presence of GSH, NEM-treated cells were firstly incubated with GSH (1 mM) for 30 min and then **2** (2 μM) for 30 min as control experiment. The addition of GSH resulted in very weak fluorescence (Fig. 8a). However, strong fluorescence enhancement was observed when NEM-treated cells were incubated with GSH and Cys one by one or treated GSH and Cys simultaneously (Fig. 8b and c). The average fluorescent intensity (Fig. 8d) of Cys-treated cells was calculated to be about 9-fold higher than that of GSH-treated cells. These results implied that **2** could be used for bioimaging of intracellular Cys even in the presence of GSH in living cells.

3. Conclusion

In summary, we have designed and synthesized fast-response fluorescent probes based on thiolysis of NBD thioether. After reaction between our probes and biothiols, GSH/H_2S could only trigger one emission, while Cys/Hcy could trigger two. Probe **2** exhibited good stability, high selectivity and high sensitivity with detection limit of 35 and 26 nM for Cys and Hcy, respectively. Moreover, **2** could eliminate the effect of probe consumption by GSH/H_2S when used to detect Cys/Hcy in the presence of GSH/H_2S in PBS buffer. The bioimaging experiments indicated that **2** was cell-permeable and could be used for live-cell imaging of intracellular Cys even in the presence of GSH. These results make the

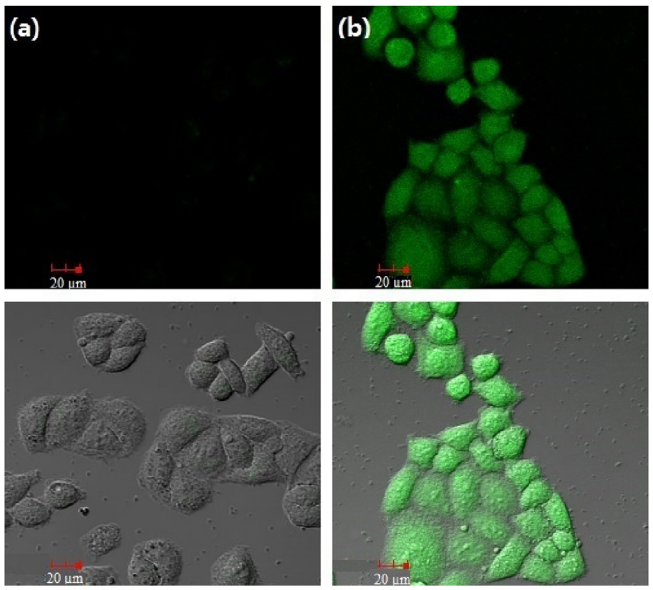

Fig. 7. Confocal microscopy images of probe **2** using for detection of intracellular Cys in living cells. HeLa cells were incubated with (a) NEM and then **2**; (b) NEM, then Cys and then **2**. **2** was 2 μM, NEM and Cys were 1 mM. The incubation time was 30 min respectively. The merge images of fluorescence and bright-field are shown below. Emission was collected at green channel (500–600 nm) with 488 nm excitation. Scale bar, 20 μm.

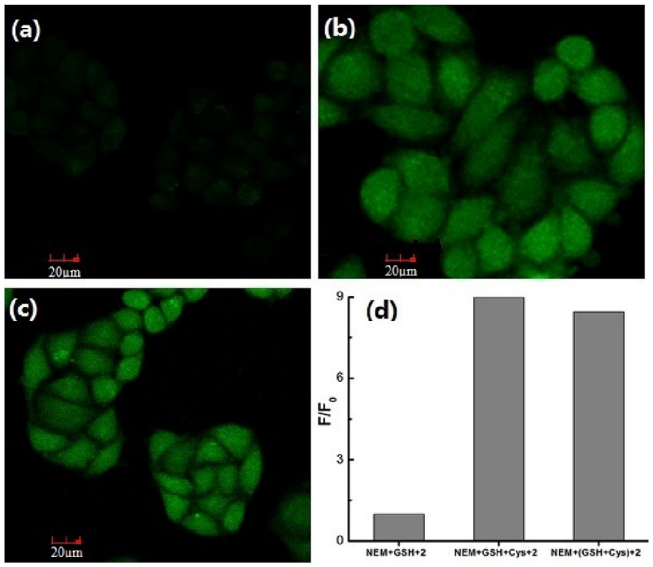

Fig. 8. Confocal microscopy images of probe **2** using for detection of intracellular Cys in the presence of GSH in living cells. HeLa cells were incubated with (a) NEM, then GSH and then **2**; (b) NEM, GSH, Cys and **2** one by one; (c) NEM, then GSH and Cys simultaneously and then **2**; (d) The relative mean fluorescence of images a-c. **2** was 2 μM, NEM, GSH and Cys were 1 mM. The incubation time was 30 min respectively. Emission was collected at green channel (500–600 nm) with 488 nm excitation. Scale bar, 20 μm.

molecular probes based on thiolysis of NBD thioether as promising tools for potential applications in discriminative detection of biothiols.

4. Experimental section

All chemicals and solvents used for synthesis were purchased

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from commercial suppliers and applied directly in the experiments without further purification. Merck silica gel 60 (100–200 mesh) was used for general column chromatography purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 spectrometer. Chemical shifts are reported in parts per million relative to internal standard tetramethylsilane ($Si(CH_3)_4$ = 0.00 ppm) or residual solvent peaks ($CDCl_3$ = 7.26 ppm; DMSO-d_6 = 2.50 ppm). ^{1}H NMR coupling constants (J) are reported in Hertz (Hz), and multiplicity is indicated as the following: s (singlet), d (doublet), t (triplet), dd (doublet of doublets), m (multiple). High-resolution mass spectra (HRMS) were obtained on an Agilent 6540 UHD Accurate-Mass Q-TOFLC/MS. The UV–visible spectra were recorded on a UV-3600 UV-VIS-NIR spectrophotometer (SHIMADZU, Japan). Fluorescence studies were carried out using F-280 spectrophotometer (Tianjin Gangdong Sci & Tech., Development. Co., Ltd).

4.1. Synthesis

4.1.1. Synthesis of **3**

To a CH_2Cl_2 solution (25 ml) of dansyl chloride (405 mg, 1.5 mmol) and *tert*-butyl piperazine-1-carboxylate (419 mg, 2.25 mmol), DIPEA (1.5 mmol) was added drop-by-drop at 0 °C. The mixture was stirred at room temperature for 2 h. After that, the reaction solution was washed with water and brine. The organic phase was dried by Na_2SO_4. Then, organic solvent was evaporated under reduced pressure. The resulted residue was purified by silica gel column chromatography with CH_2Cl_2/MeOH = 100/1 to get a yellow power **3** (284 mg, 45%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59 (d, J = 8.5 Hz, 1H), 8.40 (d, J = 8.7 Hz, 1H), 8.20 (dd, J = 7.3, 1.1 Hz, 1H), 7.55 (dd, J = 12.0, 4.4 Hz, 2H), 7.19 (d, J = 7.5 Hz, 1H), 3.48–3.41 (m, 4H), 3.19–3.11 (m, 4H), 2.89 (s, 6H), 1.39 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.3, 132.7, 131.1, 130.9, 130.5, 130.2, 128.3, 123.4, 119.8, 115.5, 80.5, 77.5, 77.2, 76.8, 45.6, 45.6, 28.4.

4.1.2. Synthesis of **6**

Compound **3** (120 mg, 0.29 mmol) was dissolved by a solution consisted of CH_2Cl_2 (2 ml), TFA (2 ml) and H_2O (20 μl), and the resulted mixture was stirred at room temperature for 40 min. After that, the reaction solution was removed under reduced pressure to get product **4**, which was used in following synthesis directly. Compound **5** (52 mg, 0.16 mmol) was dissolved by 4 ml DMF and then HATU (77 mg, 0.20 mmol) and DMAP (50 mg, 0.40 mmol) were added to the solution. After stirring for 5min, compound **4** (45 mg, 0.14 mmol) was added. The mixture was stirred at room temperature overnight, and then poured into 80 ml H_2O to give yellow-green precipitate by centrifugation. The precipitate was dried and then purified by silica gel column chromatography with CH_2Cl_2/MeOH = 1000/5 to get a yellow-green solid **6** (50 mg, 58%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.61 (d, J = 8.5 Hz, 1H), 8.35 (d, J = 8.7 Hz, 1H), 8.20 (d, J = 7.2 Hz, 1H), 7.56 (t, J = 8.0 Hz, 2H), 7.43 (d, J = 7.7 Hz, 6H), 7.29 (t, J = 7.5 Hz, 6H), 7.22 (t, J = 7.1 Hz, 4H), 3.59–3.53 (m, 2H), 3.17–3.10 (m, 2H), 3.06 (s, 4H), 2.92 (s, 6H), 2.88 (s, 2H).

4.1.3. Synthesis of probe **2**

To a solution of **6** (20 mg, 0.03 mmol) in 0.8 ml CH_2Cl_2 were added Et_3SiH (100 μl) and then TFA (200 μl) under nitrogen gas, and the mixture was stirred at room temperature for 1 h. After evaporation of the organic solvent, the residue was dissolved in CH_2Cl_2 (5 ml), NBD-Cl (30 mg, 0.15 mmol) and Et_3N (200 μl) were added under nitrogen gas at 0 °C. After stirring overnight, the mixture was evaporated under reduced pressure. The resulted residue was purified by silica gel column chromatography with CH_2Cl_2/MeOH = 1000/5 to get a yellow solid **2** (8 mg, 42%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.60 (d, J = 8.4 Hz, 1H), 8.34 (d, J = 7.7 Hz, 2H), 8.21 (d, J = 7.1 Hz, 1H), 7.55 (t, J = 7.7 Hz, 3H), 7.19 (d, J = 7.5 Hz, 1H), 4.14 (s, 2H), 3.65 (dd, J = 11.0, 5.6 Hz, 4H), 3.32 (s, 2H), 3.19 (t, J = 4.8 Hz, 2H), 2.89 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.8, 152.1, 149.3, 142.6, 138.3, 133.7, 132.2, 131.4, 131.1, 130.7, 130.4, 130.2, 128.5, 123.6, 123.3, 119.3, 115.6, 46.3, 45.6, 45.6, 45.4, 42.1, 34.4. HRMS calculated for $(M + H)^+$ $C_{24}H_{25}N_6O_6S_2^+$: 557.1272, found 557.1223.

4.2. General procedure for spectroscopic studies

Measurements were performed in degassed phosphate-buffered saline buffer (PBS, 50 mM, pH 7.4, containing 10% DMSO). Probes **1** and **2** were dissolved in DMSO to prepare 5 mM stock solutions. Probes were diluted in PBS buffer to afford the final concentrations of 1–25 μM. The quantum yield tests were carried out in PBS buffer using fluorescein (ϕ, 0.95) as reference. For the selectivity experiment, different bio-relevant molecules (100 mM or 25 mM) were prepared as stock solutions in PBS buffer. Appropriate amount of bio-relevant species were added to separate portions of the probe solution and mixed thoroughly. The reaction mixture was shaken uniformly before emission spectra were measured. All measurements were performed in a 3 ml corvette with 2 ml solution.

4.3. Cell cultures and fluorescence imaging

HeLa cells were cultured at 37 °C, 5% CO_2 in DMEM/HIGH GLUCOSE (GIBCO) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 4 mM L-glutamine. The cells were maintained in exponential growth, and then seeded in glass-bottom 35 mm plate at the density about 2 × 104/well. Cells were passaged every 2–3 days and used between passages 3 and 10. Cells were imaged on a confocal microscope (Olympus FV1000 UPLSAPO40X) with a 40 × objective lens. Emission was collected at green channel (500–600 nm) with 488 nm excitation. All images were analyzed with Olympus FV1000-ASW.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.tet.2017.10.020.

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