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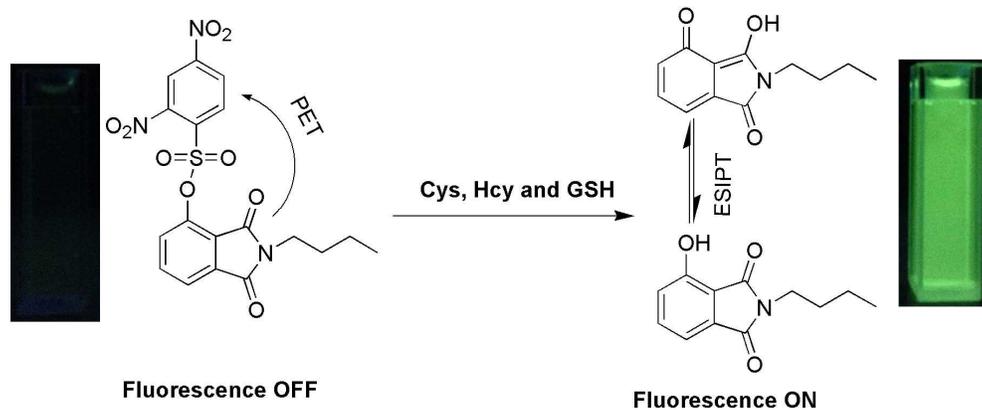


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A phthalimide-based fluorescent probe with a large Stokes shift (161 nm) was developed for the detection of thiols via the combination of PET and ESIPT mechanisms. This probe displayed good selectivity and high sensitivity toward thiols with a low detection limit (0.8 nM). Imaging intracellular thiols was successfully achieved in living SH-SY5Y cells.

ARTICLE

A phthalimide-based fluorescent probe for thiol detection with a large Stokes shift

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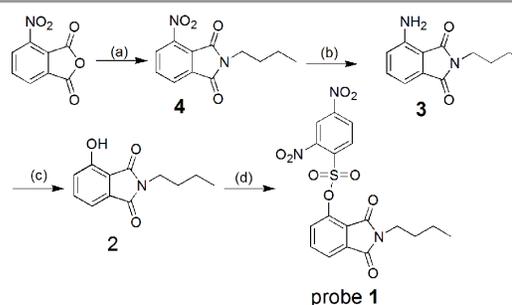
A phthalimide-based dye, probe **1**, was developed as a novel fluorescent probe for thiol detection with excellent selectivity and high sensitivity based on the combination of photo induced electron transfer (PET) and excited state intramolecular proton transfer (ESIPT) mechanisms. The probe can detect thiols quantitatively and selectively with a large Stokes shift (161 nm) and the detection limit (S/N = 3) is as low as 0.8 nM. Furthermore, this probe was successfully used for imaging thiols in living SH-SY5Y cells.

Introduction

Thio-containing amino acids play important physiological roles in a number of biological systems.¹ For example, cysteine (Cys) is an essential amino acid involving in protein folding by virtue of its ability to form inter- and intra-chain disulfide bonds with other cysteine residues. Cys deficiency can lead to many health problems such as skin lesions, hair depigmentation, liver damage, slow growth in children, and liver damage.² High levels of homocysteine (Hcy) are harmful and cause great risk of heart diseases, stroke and complications of diabetes.³ Similarly, glutathione (GSH) is an important endogenous antioxidant and detoxifying agent which can protect the cells from damage by reactive oxygen species, electrophiles and xenobiotics.⁴ Therefore, it is of great importance to develop methods for the rapid, sensitive and selective detection of thiols. Due to its high sensitivity, good selectivity, easy-to-operation and non-invasiveness, fluorescent probes are the powerful tools for imaging thiols in living cells.⁵ In the past several years, many fluorescent probes have been developed for the thiol detection based on various sensing mechanisms including photo induced electron transfer (PET),⁶ intramolecular charge transfer (ICT),⁷ excited state intramolecular proton transfer (ESIPT),⁸ forster resonance energy transfer (FRET),⁹ and so on. Although fluorescent probes employing one sensing mechanism can qualitatively and quantitatively detect thiols, the combination of two or more mechanisms in one fluorescent probe can bring more advantages such as higher sensitivity and better selectivity.¹⁰ For example, Jiang et al. reported a probe to distinguish Cd²⁺ from Zn²⁺ based on the combination of PET and ICT mechanisms, which was very difficult to achieve via a single sensing mechanism.¹¹ In our earlier work, we developed a red-emitting fluorescent probe based on PET and ESIPT

mechanisms which has a good sensitivity and selectivity for thiol detection.¹²

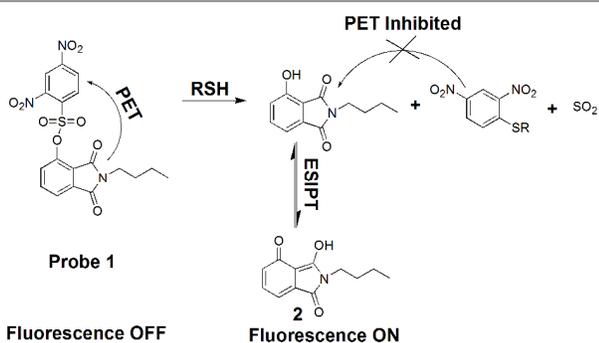
3-Hydroxyphthalimide is a small fluorophore possessing many favorable optical properties such as emission in green region, good photo stability, relatively high fluorescent quantum yield and easy synthesis. Importantly, upon excited, 3-hydroxyphthalimide displays an excited state intramolecular proton transfer (ESIPT) process in which the acidic hydroxyl proton transfers to the imide carbonyl oxygen, resulting in a large Stokes shift. These above traits suggest that 3-hydroxyphthalimide might be a good scaffold for the design of fluorescent probes.¹³ So far, there is no fluorescent probe reported on the basis of phthalimide derivatives.



Scheme 1. Synthetic route of probe **1**. (a) Butylamine, acetic acid, 120 °C, 2.5 h, yield 85.7%. (b) Palladium/C, H₂, CH₃OH, 65 °C, 12 h, 71.5%. (c) (1) NaNO₂, 50% H₂SO₄, 0 °C, 0.5 h; (2) 90 °C, 1 h, yield 85.4%. (d) 2,4-Dinitrobenzenesulfonyl chloride, Et₃N, 0 °C, 2 h, yield 73.6%.

In this work, 2,4-dinitrobenzenesulfonate of 3-hydroxyphthalimide, probe **1**, was developed as a fluorescent probe for the detection of biological thiols. The synthetic route of probe **1** is shown in Scheme 1. Probe **1** is non-fluorescent due to an effective PET process from phthalimide moiety to

2,4-dinitrobenzenesulfonate moiety.¹⁴ We expected that probe **1** could be converted into N-butyl-3-hydroxyphthalimide, reference dye **2**, through selective cleavage by thiols, which emits strong green fluorescence upon excited via an ESIPT process, as shown in Scheme 2.



Scheme 2. Schematic illustration of the reaction mechanism of probe **1** with thiols.

Results and discussion

The spectral measurements of probe **1** were conducted in HEPES buffer solution (10.0 mM, pH = 7.4) containing 20% CH₃CN. Reference dye **2** is strongly fluorescent ($\Phi_f = 0.27$) with a maximum at 516 nm and a large Stokes shift (161 nm) which is desirable for fluorescence microscopy studies wherein clearly separate excitation and emission bands can minimize the background noise (shown in Fig. S1). In contrast, probe **1** exhibits negligible fluorescence ($\Phi_f < 0.01$) because of the PET process and prohibitions of ESIPT process produced by the 2,4-dinitrobenzenesulfonate moiety, as seen in Scheme 2. In order to investigate the ability to detect thiols, titration experiment with various concentrations of Cys was performed on the solution of probe **1** (10.0 μ M) (shown in Fig. 1). The addition of Cys to the solution of probe **1** resulted in a strong green fluorescence ($\lambda_{em,max} = 516$ nm), indicating that Cys could effectively cleave dinitrobenzenesulfonate moiety. The fluorescence intensity grows with increasing the concentration of Cys and the enhancement can be up to 75 folds. A good

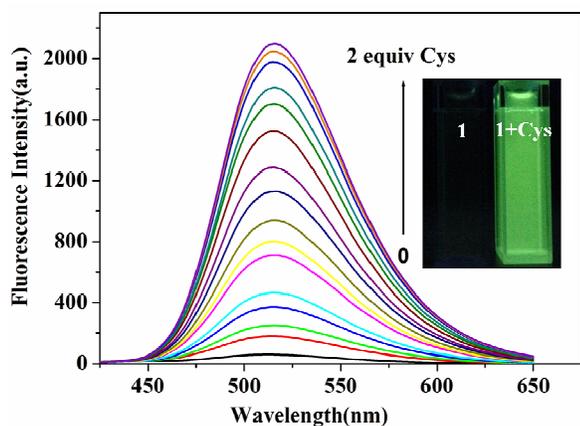


Fig. 1 Fluorescence spectra of probe **1** (10.0 μ M) in response to various amounts of Cys in HEPES buffer (10.0 mM, pH = 7.4) containing 20% CH₃CN. Excitation

wavelength at 358 nm. Inset: photographs the solution of probe **1** (10.0 μ M) before and after addition of Cys (2.0 equiv.) under a 365 nm UV lamp.

linear relationship between the fluorescence intensity at 516 nm and the concentration of Cys (0.0 - 9.0 μ M) was obtained with $R = 0.99379$. The calculated detection limit was 0.8 nM, which was sufficiently low for the detection of Cys in biological samples, as shown in Fig. 2. Similar results were obtained when we repeated the above fluorescence experiments using Hcy and GSH in replace of Cys, as shown in Fig. S2, S3, S4, S5, S6 and S7. The detection limits for Hcy and GSH are 1.1 nM and 0.6 nM, respectively. These results suggest that probe **1** can quantitatively detect thiols.

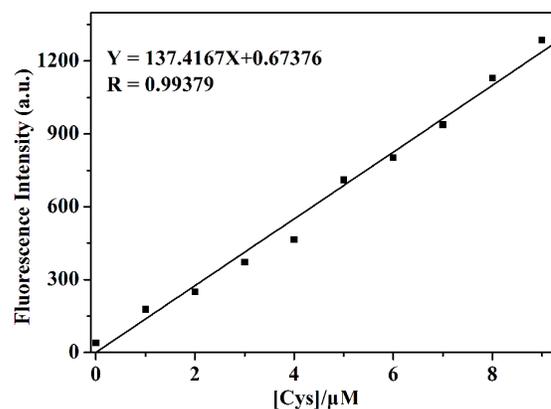


Fig. 2 The linear relationship between the emission intensity of probe **1** (10.0 μ M) at 516 nm and the concentration of Cys (0.0 - 9.0 μ M) in HEPES buffer (10.0 mM, pH = 7.4) containing 20% CH₃CN.

To further confirm the sensing mechanism, ¹H NMR, mass spectral analysis and High Performance Liquid Chromatography (HPLC) analysis were performed on probe **1** with Cys. As shown in Fig. 3, the reaction product of probe **1** with Cys and reference dye **2** had the very similar ¹H NMR spectra. Compared with the ¹H NMR spectrum of probe **1**, the protons at 8.71, 8.64 and 8.59 ppm, assigned to 2,4-dinitrobenzenesulfonyl group in probe **1**, disappeared in the ¹H NMR spectrum of the isolated reaction product. The mass spectra of probe **1** displayed a peak at $m/z = 450.0597$. However, the reaction product of probe **1** with Cys displayed a

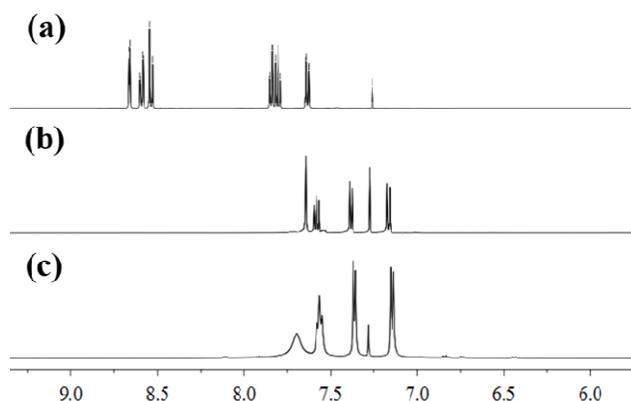


Fig. 3 Partial ^1H NMR spectra of probe **1** (a), the isolated product of probe **1** with Cys (b) and reference dye **2** (c) in CDCl_3 .

new peak at $m/z = 219.0890$ which was identical to the molar weight of reference dye **2** (Fig. S16 and S17). HPLC is an effective method widely used for the separation and analysis of mixtures of organic compounds. Thus, we conducted HPLC analysis on reference dye **2**, probe **1** and the reaction product of probe **1** with Cys (shown in Fig. 4). Probe **1** displayed a single peak with a retention time at 10.266 min while reference dye **2** produced a single peak with a retention time at 6.278 min. Upon the addition of Cys to the solution of probe **1**, the peak at 10.266 min disappeared with the appearance of a new peak at 6.281 min. These results proved that thiols could selectively cleave the 2,4-dinitrobenzenesulfonate moiety in probe **1** and generate reference dye **2**.

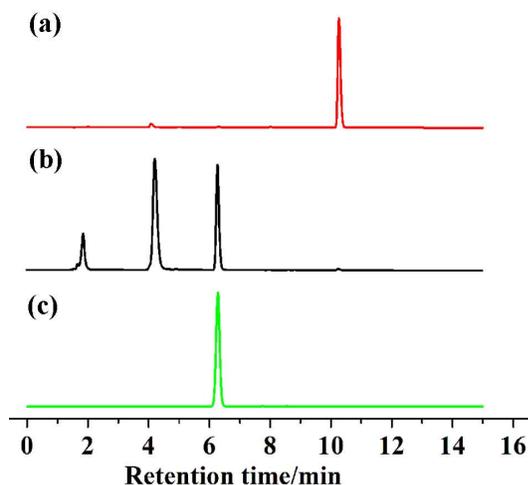


Fig. 4 The HPLC chromatograms: (a) probe **1** (100.0 μM); (b) probe **1** (100.0 μM) with 2.0 equiv. of Cys incubated for 2 h in HEPES buffer (10.0 mM, pH = 7.4) containing 20% CH_3CN ; (c) reference dye **2** (100.0 μM). Condition: eluent, H_2O containing 0.4% acetic acid/ CH_3CN ; gradient, 40-90% CH_3CN (0-10 min), 90% CH_3CN (10-15 min), flow rate, 0.8 mL/min; temperature, 25 $^\circ\text{C}$; detection wavelength, 310 nm; injection volume, 20.0 μL .

In addition, time-dependent fluorescence response of probe **1** with Cys, Hcy and GSH were also investigated respectively. As depicted in Fig. 5, the reaction of probe **1** (10.0 μM) with thiols

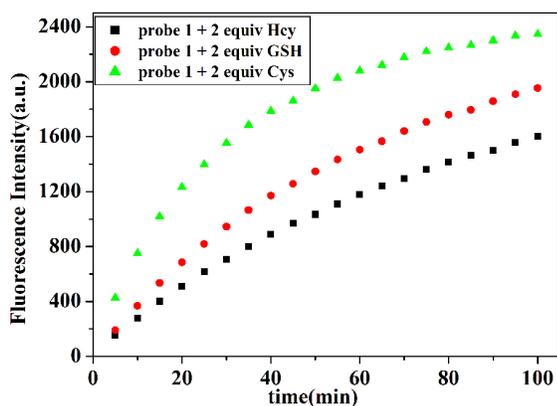


Fig. 5 Time-dependent fluorescence intensity of probe **1** (10.0 μM) upon the addition of 20.0 μM of Cys (green), Hcy (black) and GSH (red) in HEPES buffer (10.0 mM, pH = 7.4) containing 20% CH_3CN . Excitation wavelength at 358 nm.

is almost finished within 100 min. Within 5 min, fluorescence signals can be detected when the concentration of Cys is equal or above 20.0 μM . The reaction rates of probe **1** with GSH and Hcy are much slower than that for Cys, which may be attributed to the steric hindrance of Hcy and GSH.

To evaluate the selectivity performance of probe **1** for the detection of thiols, we investigated the fluorescence behavior of probe **1** toward other physiologically relevant amino acids including Asp, Ala, Val, Phe, His, Leu, Ser, Ile, Trp, Lys, Arg, Pro, Gly, Met, Tyr, Glu and Thr at identical conditions. As shown in Fig. 6, only Hcy, GSH and Cys could produce a prominent fluorescence enhancement. In contrast, other amino acid resulted in negligible fluorescence signal. Moreover, competitive assays indicated that the co-existence of typical thiol-free amino acids has no impact on the detection of thiols for probe **1** (shown in Fig. 7), suggesting that this probe exhibited a good selectivity toward thiols.

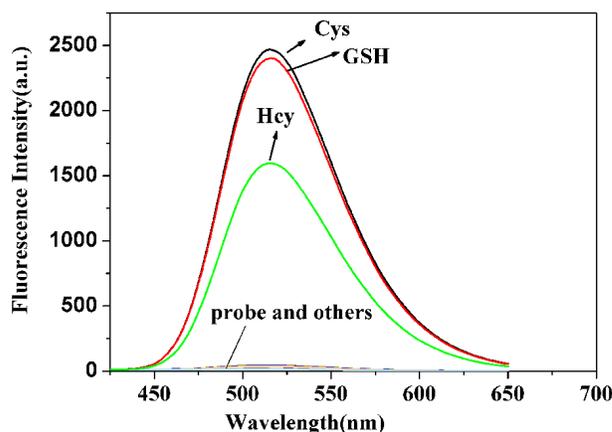


Fig. 6 Fluorescence spectra of probe **1** (10.0 μM) upon the treatment of various amino acids (2.0 equiv.) in HEPES buffer (10.0 mM, pH = 7.4) containing 20% CH_3CN . The amino acids include Asp, Ala, Val, Phe, His, Leu, Ser, Ile, Trp, Lys, Arg, Pro, Gly, Met, Tyr, Glu, Thr, Hcy, GSH and Cys.

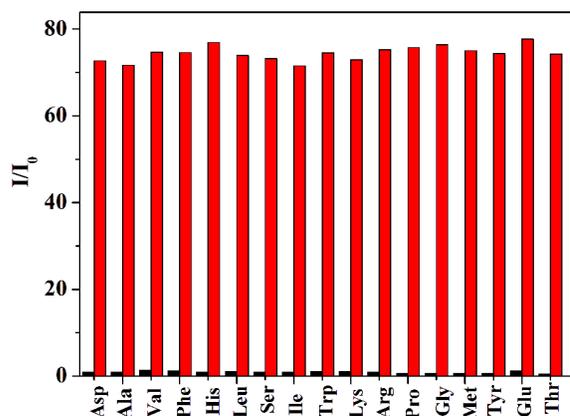


Fig. 7 Fluorescence enhancement (I/I_0) of probe **1** (10.0 μM) in response to the relevant amino acids (20.0 μM) (black bars) and Cys with the co-existence of relevant amino acids (red bars) in HEPES buffer (10.0 mM, pH = 7.4) containing 20% CH_3CN . Excitation wavelength at 358 nm. I_0 represents the emission

intensity of probe **1** at 516 nm. I represents the emission intensity at 516 nm of probe **1** with corresponding amino acids (20.0 μM).

We further investigated the pH influence on fluorescence behavior of probe **1** for the detection of Hcy, GSH and Cys. It can be seen in Fig. 8 that probe **1** was essentially non-fluorescent within a wide pH value from 2.0 to 11.0, indicating the good stability of probe **1**. Enormous fluorescence enhancement was observed upon addition of Hcy, GSH and Cys within a pH range from 5.0 to 10.0, which implied that probe **1** had the ability to detect thiols in physiological conditions.

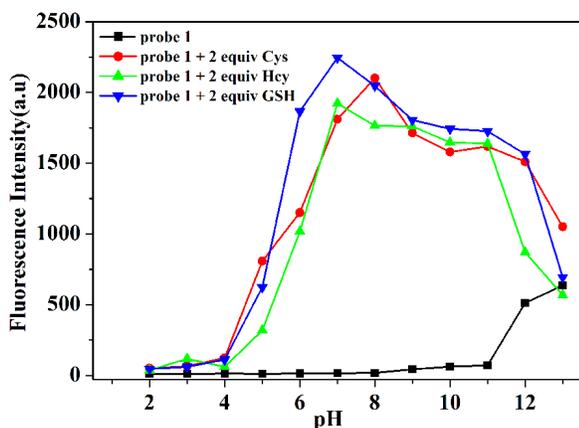


Fig. 8 Fluorescence intensity at 516 nm of probe **1** (10.0 μM) (black line) and probe **1** (10.0 μM) with Cys (red line), Hcy (green line) and GSH (blue line) at pH values from 2.0 to 13.0.

In order to explore the practicability of probe **1**, intracellular thiol imaging experiment was performed on this probe in living SH-SY5Y cells, as shown in Fig. 9. When the cells were incubated with probe **1** (10.0 μM) at 37 $^{\circ}\text{C}$ for 30 min, strong green fluorescence signal inside the cells was observed. In contrast, the cells pretreated with a thiol-blocking reagent (N-ethylmaleimide, 1.0 mM) and further incubated with probe **1** produced no fluorescence signal. These results suggested that probe **1** could permeate cell membrane and react with thiols to give easily distinguishable fluorescent signal.

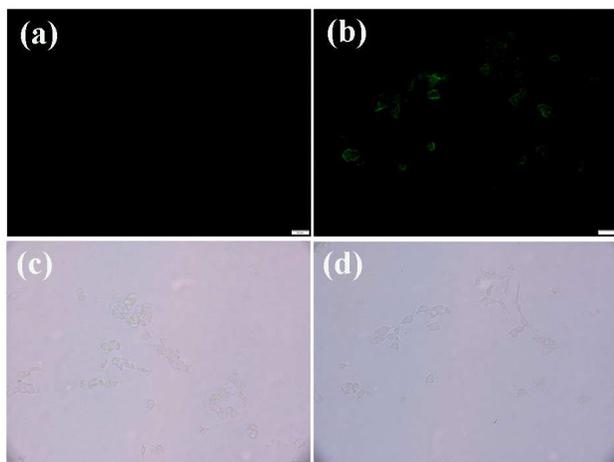


Fig. 9 (a) Fluorescence and (c) bright-field images of living SH-SY5Y cells pretreated with 1.0 mM NEM and incubated with probe **1** (10.0 μM) for 30 min at 37 $^{\circ}\text{C}$; (b) Fluorescence and (d) bright-field images of living SH-SY5Y cells incubated with probe **1** (10.0 μM) for 30 min at 37 $^{\circ}\text{C}$.

Experimental Section

Instruments

Mass spectra were obtained on MICROTOF-Q II mass spectrometer (Bruker Daltonics, Germany). ^1H and ^{13}C NMR spectra were recorded on a Bruker 500 NMR spectrometer. Chemical shifts were reported in parts per million using tetramethylsilane (TMS) as the internal standard.

All spectral characterizations were carried out in HPLC-grade solvents at 20 $^{\circ}\text{C}$ within a 10 mm quartz cell. UV-vis absorption spectroscopy was measured with a UV-2450 spectrophotometer, and fluorescence spectroscopy was determined on a Hitachi F-7000 spectrometer. The fluorescence quantum yields were measured at 20 $^{\circ}\text{C}$ with coumarin 151 as the reference ($\Phi_{\text{fl}} = 0.53$ in ethanol).¹⁵ Fluorescence imaging was performed with an Olympus IX83 inverted microscope. The pH measurement was carried out on a Leici PHS-3C meter. TLC silica gel plates and silica gel (mesh 200-300) for column chromatography were purchased from Qingdao Ocean Chemicals, China.

Materials and reagents

All chemicals and solvents were obtained from commercial suppliers and used as received. The probe **1** was prepared according to the synthetic route shown in Scheme 1.

Synthesis of compound 4

To a solution of 3-nitrophthalic anhydride (1.9361 g, 10 mmol) in acetic acid (20 mL) was slowly added butylamine (1.0951 g, 15 mmol) within 5 min. After stirring at room temperature for 10 min, the resulting mixture was refluxed at 120 $^{\circ}\text{C}$ for 2.5 h. After cooling to room temperature, the reaction mixture was poured into cold water (50 mL). The precipitated solid was collected by filtration and washed with water (3 mL \times 3) to give compound **4** as a white solid (2.1252 g, 85.7% yield). ^1H NMR (500 MHz, $\text{DMSO}-d_6$, TMS) δ_{H} 8.27 (d, $J = 8.1$ Hz, 1H), 8.16 (d, $J = 7.5$ Hz, 1H), 8.05 (t, $J = 7.8$ Hz, 1H), 3.57 (t, 2H), 1.63-1.51 (m, 2H), 1.38-1.24 (m, 2H), 0.90 (t, 3H). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ_{C} 166.5, 163.9, 144.6, 136.5, 134.1, 128.6, 127.1, 123.5, 38.1, 30.2, 19.9, 13.9.

Synthesis of compound 3

Compound **4** (0.4964 g, 2 mmol) was hydrogenated in methanol (15 mL) under reflux at 65 $^{\circ}\text{C}$ for 12 h with 10% Pd/C (0.0496 g, 5 mol%) as a catalyst. Then, the reaction mixture was filtered through celite to remove the catalyst. Next, the filtrate was concentrated in vacuum to give a residue. Pure compound **3** (0.6240 g, 71.5% yield) was obtained by silica gel column chromatography using petroleum ether/dichloromethane (1:1) as eluent. ^1H NMR (500 MHz, CDCl_3 ,

(TMS) δ_{H} 7.42 (dd, $J = 8.3, 7.1$ Hz, 1H), 7.16 (d, $J = 7.6$ Hz, 1H), 6.86 (d, $J = 8.8$ Hz, 1H), 5.13 (s, 1H), 3.65 (t, 2H), 1.63-1.70 (m, 2H), 1.35-1.42 (m, 2H). ^{13}C NMR (125 MHz, CDCl_3) δ_{C} 170.4, 168.8, 145.2, 135.0, 132.9, 121.0, 112.6, 111.4, 37.4, 30.8, 20.1, 13.7.

Synthesis of compound 2

To a solution of compound **3** (0.2180 g, 1 mmol) in 50% sulfuric acid (10 mL) at 0 °C was added the solution of NaNO_2 (0.0692 g, 1 mmol) in 2 mL water dropwisely. After stirring for 30 min at 0 °C, the mixture was heated to 90 °C and stirred for 1 h. The reaction mixture was diluted with 15 mL water, then extracted with ethyl acetate (20 mL \times 3). The organic layer was combined, washed with brine and dried over anhydrous Na_2SO_4 . After removal of the solvent, the solid was purified by silica gel flash chromatography using petroleum ether/dichloromethane (1:1) as eluent to afford compound **2** (0.1862 g, 85.4% yield). ^1H NMR (500 MHz, CDCl_3 , TMS) δ_{H} 7.70 (s, 1H), 7.56 (t, $J = 7.6$ Hz, 1H), 7.36 (d, $J = 7.2$ Hz, 1H), 7.15 (d, $J = 8.4$ Hz, 1H), 3.65 (t, 2H), 1.68-1.62 (m, 2H), 1.40-1.32 (m, 2H), 0.95 (t, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ_{C} 170.5, 167.9, 154.6, 136.1, 132.1, 122.5, 115.8, 114.6, 37.5, 30.6, 19.9, 13.4.

Synthesis of probe 1

To a solution of compound **2** (0.1096 g, 0.50 mmol) and Et_3N (0.1066 g, 1.5 mmol) in dichloromethane (10 mL) was added 2,4-dinitrobenzenesulfonylchloride (0.1650 g, 0.62 mmol) in dichloromethane (3 mL) dropwisely at 0 °C. While keeping the temperature at 0 °C, the resulting mixture was stirred for 2 h. The reaction mixture was washed with water and the organic layer was dried over anhydrous Na_2SO_4 . After removal of the solvent under reduced pressure, the resulting residue was further purified by silica gel flash chromatography using ether/dichloromethane (1:1) as eluent to give probe **1** (0.1605 g, 73.6% yield). HRMS (EI) m/z : calcd for $\text{C}_{18}\text{H}_{15}\text{N}_3\text{O}_9\text{S}$ [$M + \text{H}$], 450.0529; found, 450.0597. ^1H NMR (500 MHz, CDCl_3 , TMS) δ_{H} 8.71 (d, $J = 2.1$ Hz, 1H), 8.64 (dd, $J = 8.7, 2.2$ Hz, 1H), 8.58 (d, $J = 8.7$ Hz, 1H), 7.92-7.80 (m, 2H), 7.67 (d, $J = 8.0$ Hz, 1H), 3.56 (d, 2H), 1.65 - 1.46 (m, 2H), 1.23-1.29 (m, 2H), 0.91 (t, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ_{C} 166.6, 164.8, 150.9, 148.8, 143.3, 136.2, 134.4, 133.9, 129.5, 126.8, 123.3, 122.9, 120.3, 38.0, 30.4, 20.0, 13.6.

Synthesis of the reaction product of probe 1 with Cys

To a solution of probe **1** (0.0502 g, 0.1 mmol) in acetonitrile (5 mL) was added Cys (0.0242 g, 0.2 mmol) in distilled water (5 mL) at room temperature. After stirring for 4 h. the reaction mixture was extracted with dichloromethane (20 mL \times 3). The organic layer was combined and dried over anhydrous Na_2SO_4 . Remove the solvent by rotary evaporation, the obtained residue was purified by silica gel column chromatography using petroleum ether/dichloromethane (1:2) as eluent to give the target product (0.0142 g, 64.8% yield). ^1H NMR (500 MHz, CDCl_3 , TMS) δ_{H} 7.66 (s, 1H), 7.62-7.57 (m, 1H), 7.39 (d, $J = 7.2$ Hz, 1H), 7.17 (d, $J = 8.4$ Hz, 1H), 3.67 (t, 2H), 1.64-1.70 (m, 2H), 1.35-1.43 (m, 1H), 0.97 (t, 3H).

Imaging of SH-SY5Y cells

SH-SY5Y cells were seeded in a 6-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin. The cells were incubated under an atmosphere of 5% CO_2 and 95% air at 37 °C for 24 h. After washing with PBS buffer three times, the cells were incubated with probe **1** (10.0 μM) at 37 °C for 30 min in PBS buffer. Cell images were obtained after washing with PBS buffer three times. For a control experiment, SH-SY5Y cells were pretreated with 1.0 mM of *N*-ethylmaleimide (NEM) for 30 min at 37°C and then used.

Conclusions

In conclusion, 2,4-dinitrobenzenesulfonate of *N*-butyl-3-hydroxyphthalimide, probe **1**, was developed as a turn-on fluorescent probe toward thiols with excellent selectivity and high sensitivity. Upon the treatment with thiols, this probe produced a strong green fluorescence with a 75-fold enhancement and 161 nm Stokes shift. The sensing strategy was based on the combination of PET and ESIPT mechanisms. Preliminary biological experiments have indicated this probe has the potential to detect and image thiols in biological systems.

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Notes and references

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