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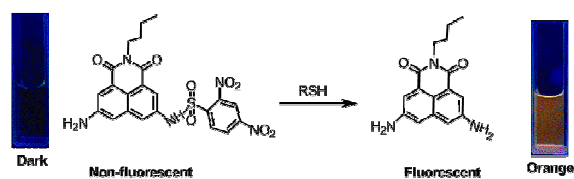
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Graphic Abstract

A novel 3, 6-diamino-1, 8-naphthalimide derivative as a highly selective fluorescent “turn-on” probe for thiols

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A novel 3, 6-diamino-1, 8-naphthalimide-based fluorescent “turn-on” probe exhibited high selectivity and low detection limit toward Cys.

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Communication

A novel 3, 6-diamino-1, 8-naphthalimide derivative as a highly selective fluorescent “turn-on” probe for thiols

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A novel 1, 8-naphthalimide derivative bearing 3-amino and 6-(2, 4-dinitrobenzenesulfonamido) groups was synthesized and found to exhibit fluorescent “turn-on” response to biothiols, including Cys, Hcy and GSH. This compound exhibited high selectivity and low detection limit, in particular toward Cys. The sensing mechanism involved the cleavage of the 2, 4-dinitrobenzenesulfonamido group and the formation of an electron-donating amino group. In addition, its properties for cellular imaging were also briefly discussed.

Biologically important thiol-containing molecules, such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH), play an essential role in many biological processes. Abnormal levels of these species are closely related to certain diseases, such as liver damage, skin lesions, Alzheimer’s disease, cardiovascular disease, heart disease and AIDS.¹ This biological and practical significance has spurred great efforts to develop efficient approaches to detect and quantify biothiols in physiological media. Remarkable among them are synthetic molecular sensors that can change optical properties predominantly in response to the presence of the analytes of interest.

In this regard, 1, 8-naphthalimide has served as an attractive fluorophore. Owing to its intense fluorescence, high sensitivity and photostability,^{2,3} 1, 8-naphthalimide has been widely used to synthesize various probes for the colorimetric and fluorescent detection of chemical and biological species from inorganic metal ions,⁴ anions,⁵ H₂S,^{6,7} biothiols,⁸⁻¹³ reactive oxygen species,¹⁴ nucleobases to cancer cells.¹⁵⁻¹⁷ These fluorescent probes have been largely confined to 4-substituted 1, 8-naphthalimide derivatives,^{2-7,13-17} for example 4-amino-1, 8-naphthalimides.^{6,7,13,15,16,18-20} As a matter of facts, all the amino-substituted 1, 8-naphthalimides, including 4-amino, 3-amino and 3, 6-diamino-substituted derivatives, emit strong fluorescence through a typical intramolecular charge transfer (ICT) process and in principle can be used in the field of fluorescence sensing.^{21,22,§} However, few molecular sensors based on 3-mono or 3, 6-bis-substituted 1, 8-naphthalimides have been reported to date.²¹

In the work reported herein, we designed a new 1, 8-naphthalimide derivative as a fluorescent “turn-on” probe for thiols, that is, compound **1** (Scheme 1). This compound bears 3-amino and reactive 6-(2, 4-dinitrobenzenesulfonamido) (DNs) groups. Because of this structural feature, compound **1** may be readily converted into strongly fluorescent 3, 6-diamino-1, 8-naphthalimide derivative **2** (Scheme 1), leading to immediate fluorescence regeneration and enabling the efficiency of fluorescence activation to be optimized.²³⁻²⁵ Thus, rapid and sensitive detection of targeting biothiol molecules is feasible by use of compound **1** rather than its corresponding analog having two DN groups. In addition, the left free amino group may

adjust the cellular permeability of the molecule itself.²⁵ Herein we report the synthesis of compound **1** and its sensing properties towards biothiols. In addition, its properties for cellular imaging are also briefly discussed.

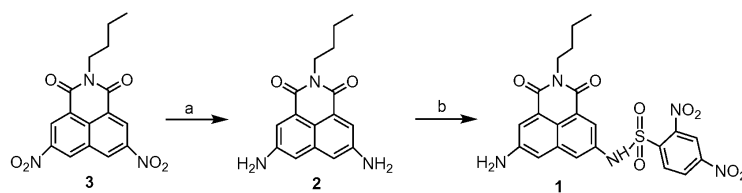
⁵⁵ Compound **1** was prepared according to the synthetic route in Scheme 1. Thus, palladium/carbon-catalyzed reduction of *N*-butyl-3, 6-dinitro-1, 8-naphthalimide **3** gave *N*-butyl-3, 6-diamino-1, 8-naphthalimide **2** in an 85% yield. Mono-acylation of compound **2** with 2, 4-dinitrobenzenesulfonyl chloride in the presence of 2, 6-dimethylpyridine as a base afforded compound **1** in a 28% yield. Compound **3** was prepared according to the reported protocols.²⁶ The structure of compound **1** was confirmed by NMR (¹H and ¹³C) and ESI-MS (LR and HR) (supporting information).^{§§}

⁶⁵ Fig. 1 shows the fluorescence changes that compound **1** undergoes upon the addition of various amino acids, including Cys, GSH, Hcy, Ala, Arg, Gln, Glu, Gly, His, Lys, Met, Phe, Ser, Ile, Val, Leu, Asp, Thr, Asn, Pro, Trp and Tyr, in 1 : 1 DMSO-PBS buffer (0.01 M, pH 7.4).^{§§§} It can be seen that compound **1** was essentially non-fluorescent, and addition of amino acids without thiol groups induced negligible changes in the fluorescence intensity. However, addition of thiol-containing Cys, Hcy and GSH caused up to 83-fold fluorescence enhancements with new strong emission at 560 nm. Of those three biothiols, Cys caused the highest increase in the fluorescence intensity, with a dramatic fluorescent color change from dark to orange (Fig. 1b). This result suggests that compound **1** exhibited fluorescent “turn-on” response toward Cys with much higher selectivity than toward the other amino acids.

⁸⁰ In order to ensure the accurate detection of Cys, the sensing behavior of compound **1** should not be interfered by other chemical species. Given the complexity of intracellular environments, we studied the response of compound **1** toward a wide range of common species, including reactive sulfur species (HS⁻, S²⁻ and HSO₃⁻), reactive oxygen species (H₂O₂, nucleophiles (KI) and metal ions (K⁺, Ca²⁺, Na⁺, Mg²⁺, Al³⁺, Zn²⁺, Fe³⁺, Cu²⁺, Mn²⁺ and Cd²⁺). As shown in Fig. 2, compound **1**

triggered no fluorescent feedback in the presence of NaHSO_3 , KI , H_2O_2 or metal ions. NaHS and Na_2S led to a slight fluorescence

increase. These results suggest that compound **1** exhibited fluorescent response to Cys with remarkably high selectivity.



Scheme 1. Synthesis of compound **1**. Reagents and conditions: (a) $\text{H}_2/\text{Pd/C}$, EtOH ; (b) **2**, 4-dinitrobenzenesulfonyl chloride (5.0 equiv), 2, 6-dimethylpyridine (5.0 equiv), anhydrous CH_2Cl_2 , rt, 45 h.

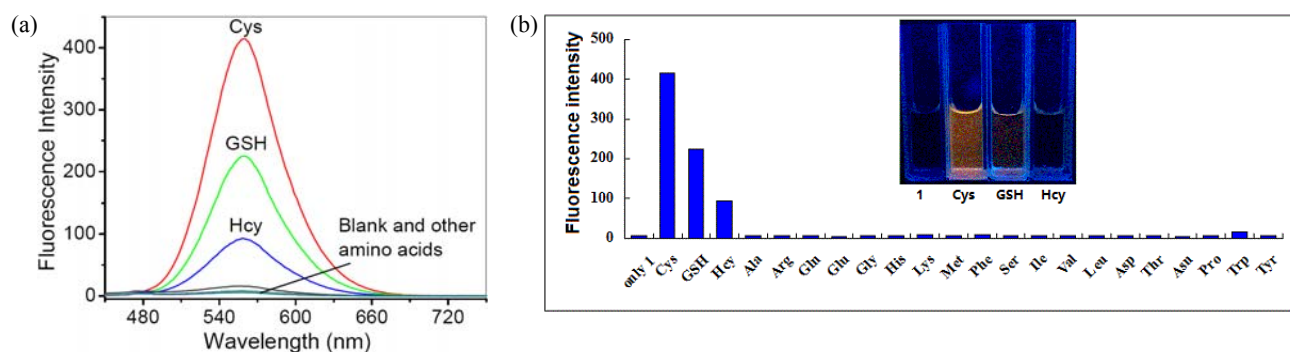


Fig. 1 (a) Fluorescence spectra and (b) fluorescence intensity at 560 nm of compound **1** (25 μM) in the presence of various amino acids (250 μM), including Cys, GSH, Hcy, Ala, Arg, Gln, Glu, Gly, His, Lys, Met, Ser, Ile, Val, Leu, Arg, Thr, Asn, Pro, Trp and Tyr (250 μM), in 1 : 1 DMSO-PBS buffer (0.01 M, pH 7.4). The inset in (b) denotes the color change of compound **1** in the presence of Cys, GSH and Hcy ($\lambda_{\text{ex}} = 365 \text{ nm}$).

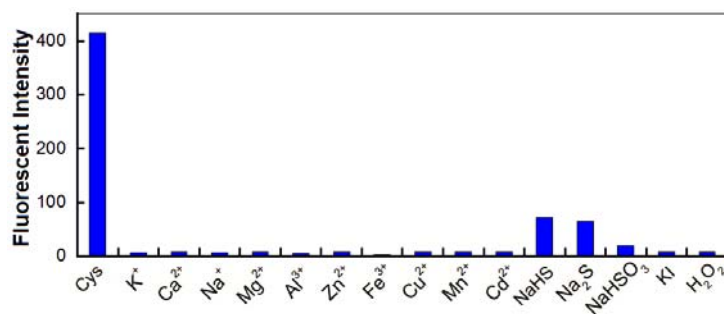


Fig. 2 Fluorescence response of compound **1** (25 μM) to a wide range of bioanalytes (250 μM) in 1 : 1 DMSO-PBS buffer (0.01 M, pH 7.4), including Cys, K^+ , Ca^{2+} , Na^+ , Mg^{2+} , Al^{3+} , Zn^{2+} , Fe^{3+} , Cu^{2+} , Mn^{2+} , Cd^{2+} , NaHS , Na_2S , NaHSO_3 , KI and H_2O_2 ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 415/560 \text{ nm}$).

To ensure practical application, a sensor should have low detection limit and reasonably short response time. Therefore we firstly measured the detection limit of compound **1** toward Cys, GSH and Hcy by means of spectrofluorimetric titrations. As shown in Fig. 3a, the fluorescent intensity increased with the increase in the concentrations of Cys until saturation was observed. The stoichiometric ratio of Cys to compound **1** was found to be 9 : 1, and the detection limit was calculated to be $2.0 \times 10^{-7} \text{ M}$ based on $\text{S/N} = 3$ (Fig. S10).²⁷ Under the same conditions, compound **1** exhibited response to GSH and Hcy with the detection limits being $4.3 \times 10^{-7} \text{ M}$ and $1.2 \times 10^{-6} \text{ M}$, respectively (Fig. S11 and S12).

Then we studied the time course of the response of compound **1** to Cys, GSH and Hcy under *pseudo*-first-order conditions and at

pH 7.4 and 25 $^\circ\text{C}$. (Fig. 3b, Fig. S13 and S14). Non-linear fitting of the initial concentration changes according to a *pseudo*-first-order kinetics equation afforded the rate constants of $3.0 \times 10^{-2} \text{ min}^{-1}$ for Cys, $9.5 \times 10^{-3} \text{ min}^{-1}$ for GSH and $5.9 \times 10^{-3} \text{ min}^{-1}$ for Hcy, respectively. Thus, the *pseudo*-first-order rate constant of Cys was 3- and 5-fold higher than those of GSH and Hcy, respectively.²⁸ These data underlie the selectivity of compound **1** to small-molecular-weight biological thiols in the order of Cys > GSH > Hcy.

The above-observed new strong emission peak at 560 nm is thought to be due to the cleavage of the DNs group to produce an electron-donating amino group. In other words, the sensing mechanism involves the nucleophilic attack of thiols on the DNs group of compound **1** to give highly fluorescent *N*-butyl-3, 6-

diamino-1, 8-naphthalimide **2**, leading to immediate fluorescence regeneration.^{23,24} To verify this, the response of compound **1** to Cys was investigated by ¹H NMR spectra. Upon the addition of Cys, the resonances at 7.82 ppm (H_a), 7.81 ppm (H_d), 7.66 ppm (H_e) and 7.19 ppm (H_b) of compound **1** disappeared. Meanwhile, two new resonances appeared at 7.61 ppm corresponding to H_a'

and H_d' and 7.08 ppm corresponding to H_b' and H_c' of compound **2**, respectively (Fig. 4b-c and Fig. S6). This result is indicative of the cleavage of the DNs group and the production of compound **2**. This was further supported by ESI-MS spectra, in which a peak at *m/z* 284.2 corresponding to [2+H]⁺ (calcd = 284.2) was clearly observed (Fig. S7 and S8).

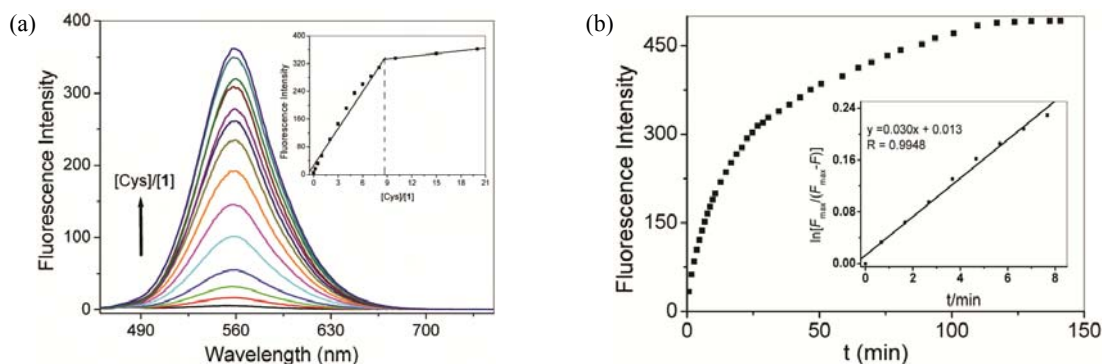
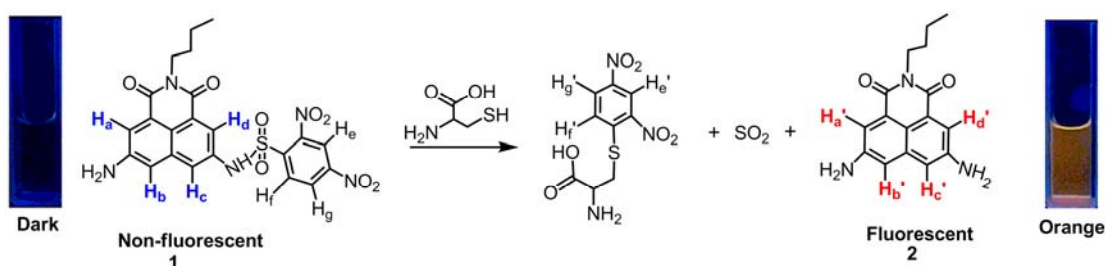


Fig. 3 (a) Spectrofluorimetric titration of compound **1** (25 μM) in 1 : 1 DMSO-PBS buffer (0.01 M, pH 7.4) upon the addition of Cys of varying concentrations from 0 to 500 μM. Inset: fluorescence change against [Cys]/[**1**] ratios. (b) Kinetic study of the response of compound **1** (25 μM) to Cys (500 μM) at 25 °C under pseudo-first-order condition. $\lambda_{\text{ex}}/\lambda_{\text{em}} = 415/560$ nm.



Scheme 2. Plausible mechanism of response of compound **1** to Cys.

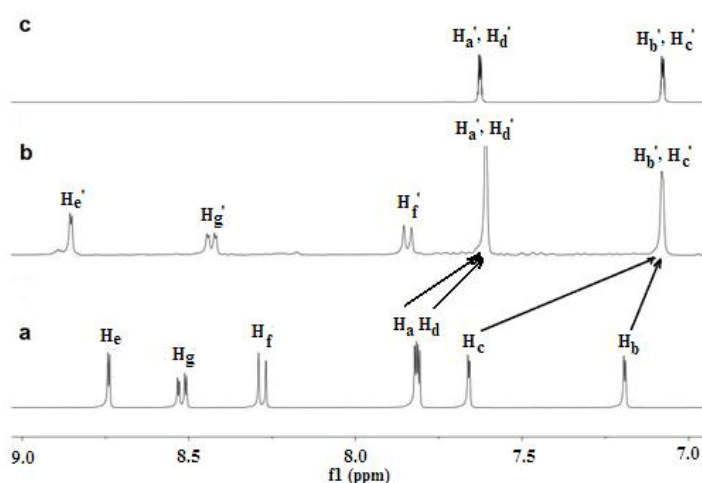


Fig. 4 Partial ¹H NMR spectra of compound **1** before the addition (a) and 72 h after the addition (b) of Cys, and of compound **2** (c) in *d*₆-DMSO/D₂O (5/2, v/v).

To evaluate whether compound **1** has sufficient cellular permeability and can be activated by intracellular biothiols in living cells, its permeability and subsequent monitoring of thiols in living cells were investigated.²⁹ Thus, human hepatocarcinoma HepG2 cells were incubated with compound **1** (5 μM) for 2 h. Strong red-fluorescence was exhibited inside the cells (Fig. 5d). In a control experiment, when cells were pretreated with *N*-ethylmaleimide (NEM, 20 μM , 0.5 h, a trapping reagent of thiol species), a remarkable decrease in fluorescence intensity was observed (Fig. 5g), indicating the specific detection of thiols by compound **1**. These results suggest that compound **1** could enter cells and make fluorescence labeling.

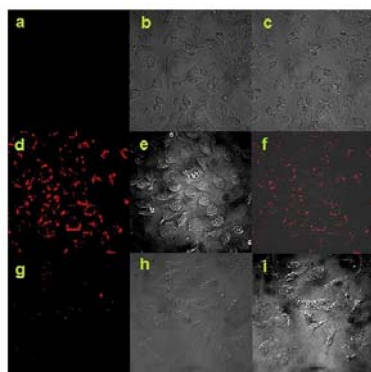


Fig. 5 Fluorescence images of HepG2 cells ($\lambda_{\text{ex}} = 488 \text{ nm}$) (a) in the absence of compound **1**; (d) incubated with compound **1** (5 μM) for 2 h; (g) pretreated with *N*-methylmaleimide (20 μM) for 30 min and then incubated with compound **1** (5 μM) for 2 h. (b, e, h) corresponding bright field images of (a, d, g); (c, f, i) overlays of the fluorescent and bright field images.

In summary, we have successfully developed a novel 3, 6-diamino-1, 8-naphthalimide-based fluorescent “turn-on” probe for biothiols. It functions *via* the cleavage of the DN₃ group and the formation of an electron-donating amino group. This compound exhibits high selectivity toward biothiols over the other natural amino acids and a wide range of common species, with an obvious fluorescent color change from dark to orange. This compound may also be applied in the biological imaging of biothiols in living cells.

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Notes and references

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[§] 3-Amino-1, 8-naphthalimides have lower fluorescence quantum yields, but larger Stokes shift than the 4-amino analogue. For example, *N*-butyl-4-amino-1, 8-naphthalimide exhibits maximum absorption at 430 nm ($\epsilon = 11600 \text{ M}^{-1}\text{cm}^{-1}$) and emission at 541 nm ($\Phi = 0.13$), whereas compound **2** exhibits maximum absorption at 418 nm ($\epsilon = 8300 \text{ M}^{-1}\text{cm}^{-1}$) and emission at 560 nm ($\Phi = 0.0594$). See reference 22 and supporting information for more details.

^{§§} Structural data for compound **1**: ¹H NMR (400 MHz, *d*₆-DMSO) δ 8.89 (d, *J* = 2.0 Hz, 1H), 8.58 (m, 1H), 8.30 (d, *J* = 8.4 Hz, 1H), 7.87 (d, *J* = 2.4 Hz, 1H), 7.86 (d, *J* = 2.4 Hz, 1H), 7.14 (d, *J* = 2.0 Hz, 1H), 3.97 (t, *J* = 7.2 Hz, 2H), 1.56 (m, 2H), 1.32 (m, 2H), 0.91 (t, *J* = 7.2 Hz, 3H); ¹³C NMR (100 MHz, *d*₆-DMSO) δ 163.7, 163.5, 150.6, 149.0, 148.3, 136.4, 135.4, 134.6, 132.1, 127.7, 123.6, 123.0, 121.6, 120.8, 120.6, 119.4, 118.5, 111.2, 30.0, 20.2, 14.1; ESI-MS *m/z*: 514.2 ($[M+H]^+$) and HR-ESI-MS for C₂₂H₁₉N₃O₈S ($[M+H]^+$) *m/z*: calcd 514.1033, found 514.1029.

^{§§§} We studied the effect of pH on the sensing properties of compound **1** toward Cys, and found that the emission intensity increased with the increase of pH from 3.0 to 7.5 and kept constant from pH 7.5 to 9.0 (Fig. S9). Therefore we chose pH 7.4 as the operational pH under which the highest sensitivity can be achieved.

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