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Bioorganic & Medicinal Chemistry Letters 18 (2008) 2246–2249
Bioorganic & Medicinal Chemistry Letters

Rhodamine-based fluorogenic probe for imaging biological thiol

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Received 8 January 2008; revised 3 March 2008; accepted 6 March 2008
Available online 10 March 2008

Abstract—We have developed a new fluorescent probe for biological thiol. The probe was synthesized by the modification of the 2,4-dinitrobenzenesulfonyl group with rhodamine 110. The selective detection of thiol species such as cysteine or glutathione was achieved in biological conditions. Moreover, the probe was successfully applied to the imaging of thiol species in living human cells.
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Cellular thiols play important roles in biological systems.[1–3] Changes in their levels are linked to oxidative stress associated with toxic agents and disease. Glutathione is the most abundant cellular thiol.[1] It acts as a scavenger nucleophile or reducing agent and protects cells against many carcinogens. Homocysteine is a risk factor for disorders including cardiovascular diseases and Alzheimer's disease.[2] The thiol group in cysteine (Cys) residues is involved in three-dimensional structures of proteins through disulfide bond formation.[3] Cys deficiency can cause several health problems. Thus, the detection of intracellular thiols is very important for investigating cellular function.

Ellman's reagent is the most widely used reagent for the determination of thiol groups.[4] However, this reagent can be used only in vitro because its assay is based on absorption change. Several thiol detection methods, including sensitive fluorescent probes, have been reported.[5–7] Fluorescein derivatives protected by 2,4-dinitrobenzenesulfonyl ester have been reported.[6] The probe reacts with biological thiol and offers high fluorescent intensity with short reaction times. However, the probe may yield undesired background fluorescence because the sulfonyl ester is hydrolyzed in biological aqueous solutions.

Recently, fluorescent probes with the 2,4-dinitrobenzenesulfonamide (DNB) group have been reported.[7] The sulfonamide group resists hydrolysis and yields no undesired background fluorescence. In this paper, we add a new fluorescent probe derived from Rhodamine (Rh) (Scheme 1). Rh dyes are highly fluorescent and resistant to photo-bleaching.[8a] Therefore, they are the most widely used fluorescent reagents for labeling biomolecules.[8] There is a series of Rh derivatives with a wide range of emissions, from 450 to 700 nm. Our design can be applied to many Rh derivatives and can create multiple color probes for the detection of biological thiol.

The chemistry of fluorogenic probes involves the nucleophilic attack of the thiol group on the DNB group, resulting in the cleavage of the sulfone-amide bond, and then Rh in its open lactone form emits a fluorescence signal (Scheme 1). The probe was synthesized in one step from commercially available Rh 110. The starting material was treated with 2,4-dinitrobenzenesulfonyl chloride in the presence of KO*t*-Bu in DMF for 16 h and the desired probe was obtained in 10% yield.[9] Mono-DNB-protected Rh was synthesized as a reference.[10]

The probe and mono-DNB Rh show very low fluorescence quantum yields, 0.0007 and 0.003, respectively, in contrast with the high yield of Rh, 0.645 (Table 1). This result indicates that the observed fluorescence signal comes from the Rh after the deprotection of the bis-DNB group in the probe when sensing biological thiol.

First, to test the fluorescence response, the probe was incubated in solution with or without Cys in Tris–HCl buffer (50 mM, pH 7.4). The resulting absorption and fluorescence spectra are shown in Figure 1. The solution

Keywords: Thiol; Imaging; Fluorescence; Probe; Synthesis.
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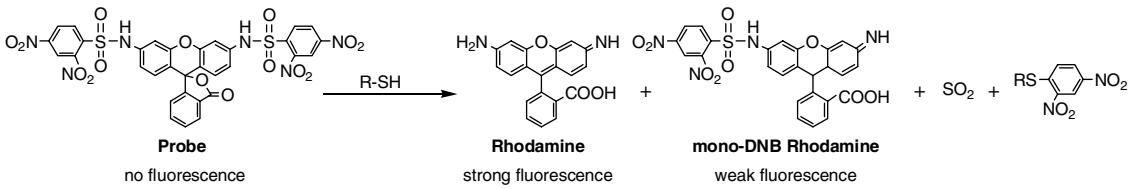
doi:10.1016/j.bmcl.2008.03.014



Scheme 1. The reaction mechanism of fluorogenic probe. The DNB group is cleaved through the reaction with thiol and the fluorescence signal appeared.

Table 1. The quantum yields of the compounds[a]

Compound	Probe	Mono-DNB Rh	Rh
Φ_F[b]	0.0007	0.003	0.645

[a] All measurements were done in sodium phosphate buffer (100 mM, pH 7.4). Compounds were excited at 490 nm.

[b] Quantum yields are determined by using fluorescein (0.85, 0.1 M NaOH) as a standard.

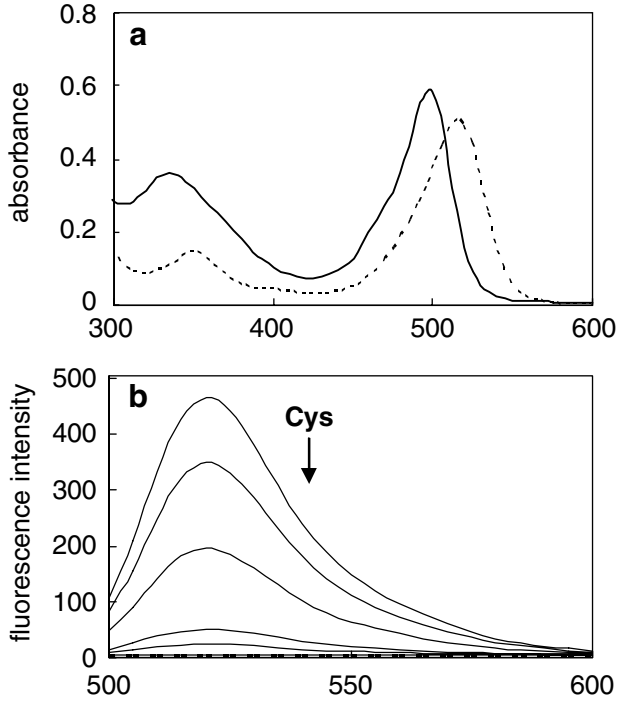


Figure 1. (a) The absorption spectra of 20 μM probe with (solid line) and without (dash line) 20 mM Cys in 50 mM Tris–HCl (pH 7.4). (b) Fluorescence spectra of 100 nM probe with (solid line) and without (dash line) 10, 5, 2.5, 1, 0.5, 0.1 mM Cys after incubation at 37 °C for 30 min in 50 mM Tris–HCl (pH 7.4). The fluorescence emission was monitored at the excitation of 490 nm.

without Cys exhibited maximum absorption at 516 nm, which is derived from the DNB group. When Cys was added to the probe solution, the maximum absorption peak was shifted to the shorter wavelength of 498 nm, resulting from the cleavage of the DNB group (Fig. 1a). Fluorescence properties were also examined. No significant fluorescence with excitation at 490 nm was observed for the probe without Cys. However, after the addition of Cys (10 mM) to the solution, a strong emission appeared around 520 nm and the emission was enhanced about 5800-fold (Fig. 1b). To test the sensitivity, fluorescence spectra were measured in various concentrations of Cys. The concentration limit for the detection of Cys was 100 μM with 100 nM of probe, where the signal to background ratio reached 12 times. To determine the chemical yield in the reaction of the probe with Cys, the reaction mixture was analyzed by HPLC (Fig. S1). The treatment of 1 mM Cys gave mono-DNB Rh and Rh in 14% and 41% yields, respectively.

Selectivity is an important issue in applying the probe to the detection of biological thiol. Probes should specifically respond to the thiol and yield no unexpected signal to other biological substances. To determine the selectivity, we treated the probe with various biological substances and measured the fluorescence signals. The selectivity of the probe for thiol was proved by monitoring the fluorescence emission at 522 nm with biorelevant analytes under physiological conditions. As shown in Figure 2, significant fluorescence intensity enhancement was observed for Cys, phosphorothioate, dithiothreitol, and glutathione (50- to 200-fold). However, the reaction with 2-mercaptoethanol (ME) showed only a small increase in fluorescence (11-fold). The pK_a value of ME shows a value of 9.5, higher than that of other thiols; therefore, the reaction with the DNB group should be slow.[11] HPLC analysis showed that ME treatment gave unreacted probe and mono-DNB Rh in 85% and 15% yields, respectively. As expected, the probe was inactive to other biological substances such as glycine, ascorbic acid, or hydrogen peroxide. Likewise, basic amino acid series such as Lys or Arg showed no signal. The probe was very stable in biological environments. For example, no increase in the fluorescence signal was detected after the probe was heated for 24 h at 55 °C in 50 mM Tris buffer (pH 7.4). Thus, we confirmed that the new probe offers good selectivity to biological thiol and no undesired fluorescence signals.

The most important application for this probe is the monitoring of thiol in living cells. To test this capability, we tried to image biological thiol in living HeLa cells. The pictures of brightfield images and fluorescence images were taken by fluorescence microscope (Fig. 3). When incubated with the probe (25 μM) for 15 min, cells showed significant fluorescence signals (Fig. 3d). The strong signal was localized to cytoplasm. On the other hand, nuclei showed weak fluorescence signals. In contrast, when cells were pretreated with the thiol-blocking reagent *N*-methylmaleimide[12] and then incubated with the probe in a similar manner, no fluores-

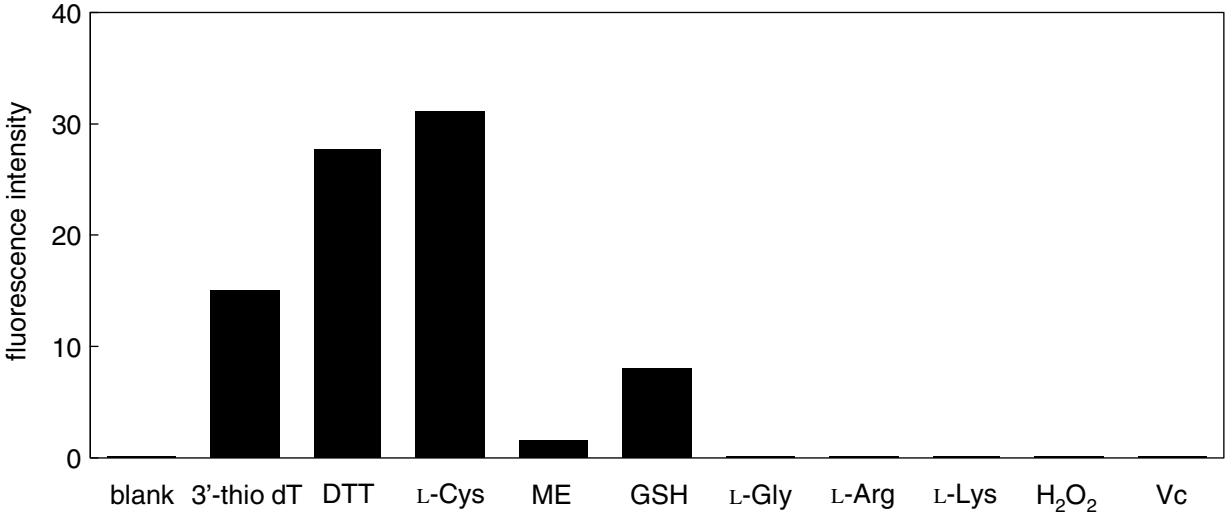


Figure 2. The fluorescence intensity of 1 μM probe with 1 mM thiols in 50 mM Tris–HCl (pH 7.4). After the addition of thiol, the solution was incubated at 37 °C for 30 min. The fluorescence emission at 522 nm was monitored by excitation at 490 nm. 3′-Thio dT, 3′-phosphorothioate-2′-deoxythymidine; DTT, dithiothreitol; GSH, glutathione; L-Gly, L-glysine; L-Arg, L-arginine; L-Lys, L-lysine; Vc, ascorbic acid.

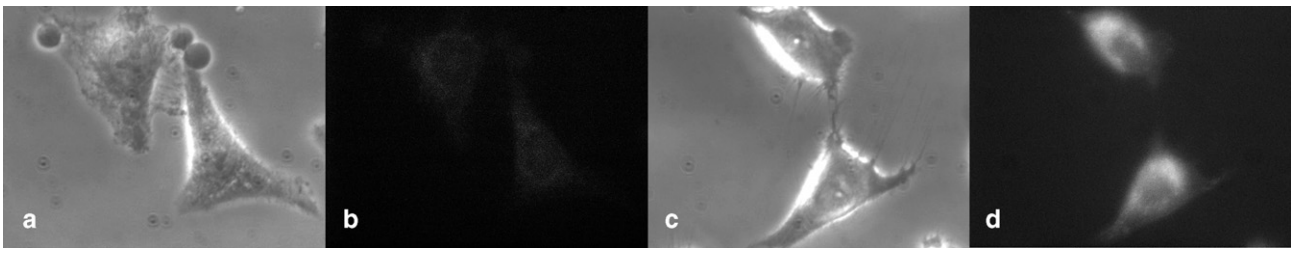


Figure 3. The fluorescence image of HeLa cells: (a and b) the control image of cells pretreated with *N*-methylmaleimide (1 mM) for 60 min at 37 °C and then incubated with probe (25 μM) for 15 min at 37 °C; (c and d) the image of cells incubated with probe (25 μM) for 15 min at 37 °C; (a and c) brightfield image; (b and d) fluorescence image. Microscope settings were as follows: excitation: 470/40 bandpass filter; emission: 525/50 bandpass filter; exposure time 300 ms.

cence signal was observed (Fig. 3b). The results demonstrate that the probe penetrates cell membranes and images the changes in thiol levels of living cells.

In conclusion, we developed a novel fluorescent probe that yields a signal responsive to thiols. The probe was successfully applied to the imaging of biological thiol in living cells.

Acknowledgments

H.A. was financially supported by the Riken Directors' Fund, MEXT (Ministry of Education, Culture, Sports, Science, and Technology), and NEDO (New Energy and Industrial Technology Development Organization).

Supplementary data

Synthetic procedure, analysis data, protocols for biochemical assay, and cell-based assay. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.03.014.

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9. Probe; ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$): δ 8.47–8.46 (d, 2H, $J = 2.2$), 8.37–8.34 (dd, 2H, $J = 2.2$, 11.0), 8.19–8.17 (d, 2H, $J = 8.8$), 7.91–7.89 (d, 1H, $J = 7.1$), 7.61–7.53 (m, 2H), 7.04–7.01 (m, 3H), 6.74–6.72 (dd, 2H, $J = 2.2$, 11.0), 6.53–6.51 (d, 2H, $J = 8.5$); ^{13}C NMR (99.5 MHz, $CDCl_3/CD_3OD$) δ 168.97, 151.38, 149.84, 148.06, 137.38, 135.28, 133.07, 130.11, 129.16, 127.12, 126.62, 125.99, 125.10, 123.58, 120.31, 116.52, 115.68, 108.70; HRMS (ESI) *m/z* calcd for $C_{32}H_{17}N_6O_{15}S_2$ (M–H) 789.0193; found 789.0176.
10. Mono-DNB Rh; ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$): δ 8.48–8.47 (d, 1H, $J = 2.2$), 8.43–8.40 (dd, 1H, $J = 2.2$, 6.6), 8.31–8.29 (d, 1H, $J = 8.8$), 8.05–8.02 (m, 1H), 7.64–7.61 (m, 2H), 7.15–7.13 (m, 3H), 6.85–6.82 (dd, 2H, $J = 2.2$, 6.8), 6.78–6.72 (m, 2H), 6.57–6.56 (d, 1H, $J = 2.2$), 6.49–6.46 (dd, 1H, $J = 2.2$, 6.6); ^{13}C NMR (99.5 MHz, $CDCl_3/CD_3OD$) δ 170.41, 154.34, 153.64, 152.49, 148.70, 148.01, 140.82, 132.86, 131.95, 129.87, 129.45, 129.11, 126.49, 125.87, 125.59, 119.33, 119.19, 114.26, 113.31, 107.06, 99.63; HRMS (ESI) *m/z* calcd for $C_{26}H_{15}N_4O_9S$ (M–H) 559.0560; found 559.0569.
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