

A Thiol-Specific Fluorescent Probe and Its Application for Bioimaging†

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Experimental Section

General methods. Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. Chromatography was carried out on silica gel 60 (230-400 mesh ASTM; Merck). Thin layer chromatography (TLC) was carried out using Merck 60 F₂₅₄ plates with a thickness of 0.25 mm. Preparative TLC was performed using Merck 60 F₂₅₄ plates with a thickness of 1 mm. ¹H NMR and ¹³C NMR spectra were recorded using Bruker 250. Mass spectra were obtained using a Jeol JMS 700 high resolution mass spectrometer. UV absorption spectra were obtained on UVIKON 933 Double Beam UV/VIS Spectrometer. Fluorescence emission spectra were obtained using RF-5301/PC Spectrofluorophotometer (Shimadzu).

Preparation of amino acids solutions for fluorescent study

Stock solutions (10 mM) of amino acids and other anylates Cys, Hcy, GSH, Gly, Phe, Ser, Glu, Lys, Arg, His, Ala, Gln, Met, Tyr and cystine in distilled water were prepared. Stock solutions of sensor **1** (1 mM) was also prepared in acetonitrile. In a typical experiment, test solutions were prepared by placing 30 μ L of the probe stock solution into a test tube, diluting the solution to 3 mL with 0.01 M HEPES (pH 7.4), and adding an appropriate buffer of each analytes stock. Normally, excitation was at 485 nm. Both the excitation and emission slit widths were 1.5nm/1.5 nm. Fluorescence spectra were measured after addition of analyte for 5 min. For low concentration titration of thiols, fluorescence spectra were measured after addition of thiols for 5 min, and the excitation and emission slit widths were either 3 nm and 3 nm, respectively.

Imaging of mammalian cells

Murine P19 carcinoma embryonic cells were cultured in culture media (DMEM supplemented with 10% FBS, 50 unit/mL of penicillin, and 50 μ g/mL of streptomycin) at 37 °C in a humidified incubator, and culture media were replaced with fresh media every day. The cells were seeded in a 6-well plate at a density of 10⁴ cells per mL in culture media. After 24 h, the cells were treated without or with 50 μ M NMM in culture media for 20 min at 37 °C. After washing with phosphate buffered saline (PBS) to remove the remaining NMM, the cells were further incubated with 20 μ M of **1** in culture media for 30 min at 37 °C. The cells were imaged by fluorescence microscopy (Nikon Eclipse TE2000; excitation filter; 450-490 nm, emission filter; 520 nm).

Imaging of zebrafish

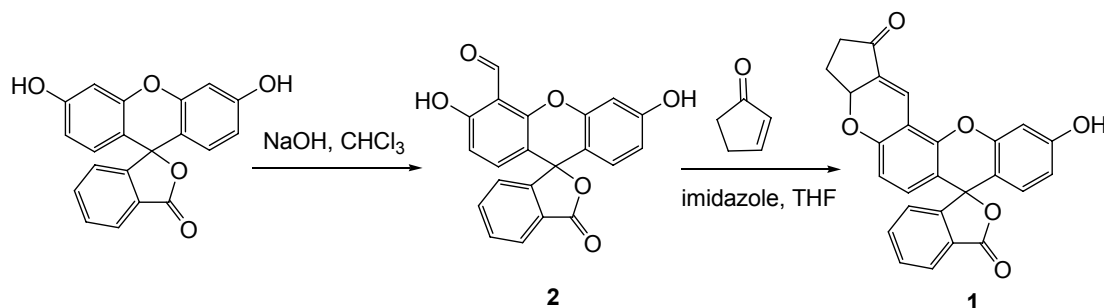
Zebrafish was kept at 28 °C and maintained at optimal breeding conditions. For mating, male and female zebrafish was maintained in one tank at 28 °C on a 12 h light/12 h dark cycle and then the spawning of eggs were triggered by giving light stimulation in the morning (Yang et al. *Nat. Protocols* **2007**, 2, 1740-1745). Almost all the eggs were fertilized immediately. The 3-day old zebrafish was maintained in E3 embryo media (15 mM NaCl, 0.5 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 0.15 mM

KH_2PO_4 , 0.05 mM Na_2HPO_4 , 0.7 mM NaHCO_3 , $10^{-5}\%$ methylene blue; pH 7.5). The zebrafish was incubated with or without 40 μM NMM for 20 min in E3 media for 30 min at 28 °C. After washing with E3 media to remove the remaining NMM, the zebrafish was further incubated with 20 μM of **1** in E3 media for 30 min at 28 °C. After washing with E3 media, the zebrafish was imaged by fluorescence microscopy.

Detection of thiol in zebrafish organs

Adult zebrafish (3 month old with identifiable organs) was exposed to 5 μM of **1** in E3 media for 5 min and 60 min at 28 °C. After washing with E3 media to remove the remaining probe, the zebrafish was dissected to isolate tissues and organs that were imaged by fluorescence microscopy and dissecting microscope (Stemi 2000-C, ZAISS, Germany).

Synthesis



Compound 2

Fluorescein-monoaldehyde **2** was synthesized according to the previous report (Reference: J. Am. Chem. Soc. 2005, 127, 15949-15958). Fluorescein (4 g, 12 mmol), 10 mL CHCl_3 , 6 mL MeOH and 0.06 g 15-crown-5 are placed in a 100 mL flask. Then 20 g 50% NaOH solution (50%) are carefully added while the reaction temperature is maintained at 55 °C. The mixture is stirred at this temperature for 5 h. After cooling, the mixture is acidified with 10 M H_2SO_4 . The precipitates is collected and dried in vacuo. Chromatography on silica gel (15:85 EtOAc:DCM) and followed recrystallization in CH_3OH afford 1.13 g product as pale yellow solid (yield 26%). ^1H NMR (CDCl_3 containing a little CD_3OD , 250 MHz) δ (ppm): 10.59 (1H, s), 7.95 (1H, d, $J = 7.5$ Hz), 7.69-7.56 (2H, m), 7.12 (1H, d, $J = 7.5$ Hz), 6.82 (1H, d, $J = 9.0$ Hz), 6.71 (1H, s), 6.55 (1H, d, $J = 9.0$ Hz), 6.54 (2H, s).

Compound 1

A solution of fluorescein-monoaldehyde **2** (360 mg, 1 mmol), 2-cyclopenten-1-one (164 mg, 2 mmol) and imidazole (68 mg, 1 mmol) in tetrahydrofuran (THF, 10 ml) was

mixed with deionized water (10 ml). The mixture was stirred at ambient temperature for 72 h at room temperature. After evaporation of under reduced pressure, the resulting mixture was extracted with ethyl acetate (3×15 ml). The organic layer was concentrated under reduced pressure. Chromatography of the crude product on silica gel using ethyl acetate and hexane (60:40) as eluent, followed by crystallization using CH₃OH/H₂O (50:50, v/v) gave yellow probe **1** 90 mg with a yield of 21%. ¹H NMR (DMSO-*d*₆, 250 MHz) δ (ppm): 10.31 (1H, s), 8.75 (1H, d, *J* = 7.5 Hz), 7.88-7.78 (2H, m), 7.69 (1H, s), 7.37 (1H, t, *J* = 7.5 Hz), 6.79 (1H, d, *J* = 8.25 Hz), 6.80-6.76 (2H, m), 6.67-6.65 (2H, m), 5.46 (1H, t, *J* = 8 Hz), 2.79-2.65 (2H, m), 2.20-2.00 (2H, m). ¹³C NMR (DMSO-*d*₆, 62.5 MHz) δ (ppm): 200.7, 168.4, 159.6, 156.2, 151.7, 151.4, 151.1, 149.1, 135.7, 131.8, 131.7, 131.4, 130.3, 129.0, 126.2, 124.7, 124.1, 120.0, 113.2, 112.6, 112.5, 110.4, 109.3, 109.0, 102.7, 82.3, 81.9, 75.6, 36.7, 27.4. FAB MS *m/z* = 425.1026 [M + H]⁺, calc. for C₂₆H₁₇O₆ = 425.1025.

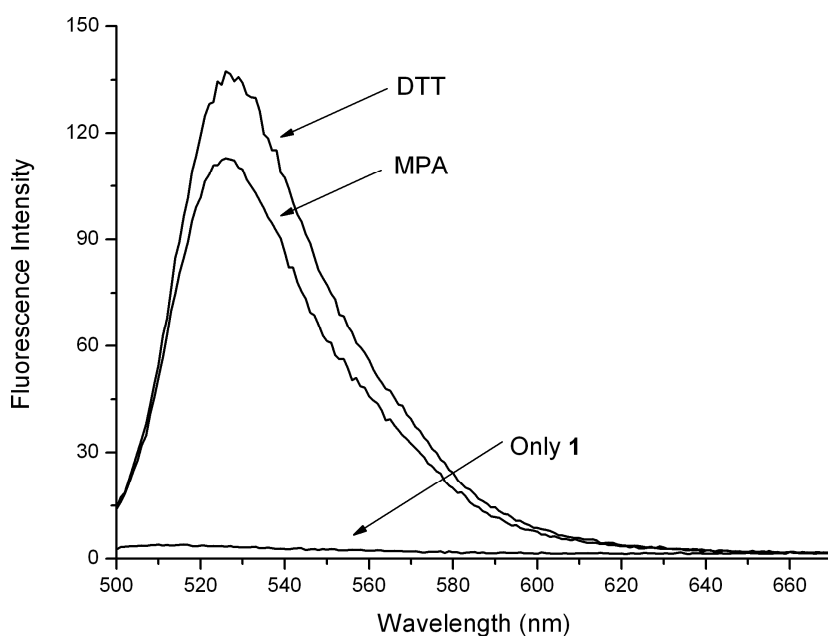


Fig. S1. Fluorescence spectra of **1** (10 μM) with MPA (2-Mercaptoethanol) and DTT (Dithiothreitol) (100 μM) in HEPES buffer (20 mM, pH 7.4, 1% CH₃CN) (λ_{ex} = 485 nm, slit: 1.5 nm/1.5 nm).

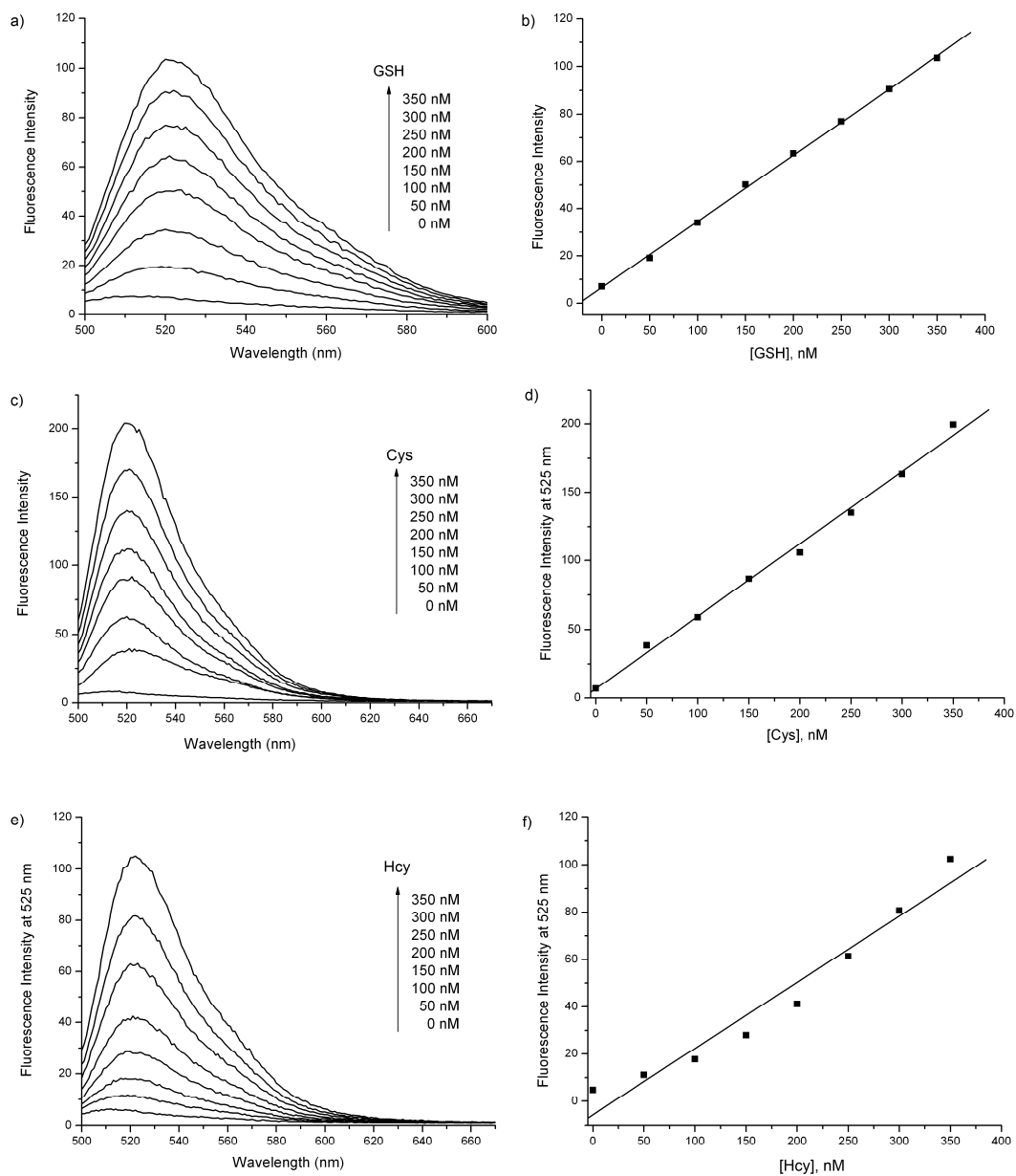


Fig. S2. (Left) Fluorescent titrations of **1** (1 μM) in response to the addition of low concentrations of thiols in CH_3CN -HEPES buffer (0.02 M, pH 7.4) (1:99, v/v) (excitation at 485 nm, slit: 3 nm/3 nm). (Right) The change in the fluorescence intensity of **1** (1 μM) at 520 nm against varied concentrations of thiols from 0 to 350 nM in CH_3CN -HEPES buffer (0.02 M, pH 7.4) (1:99, v/v).

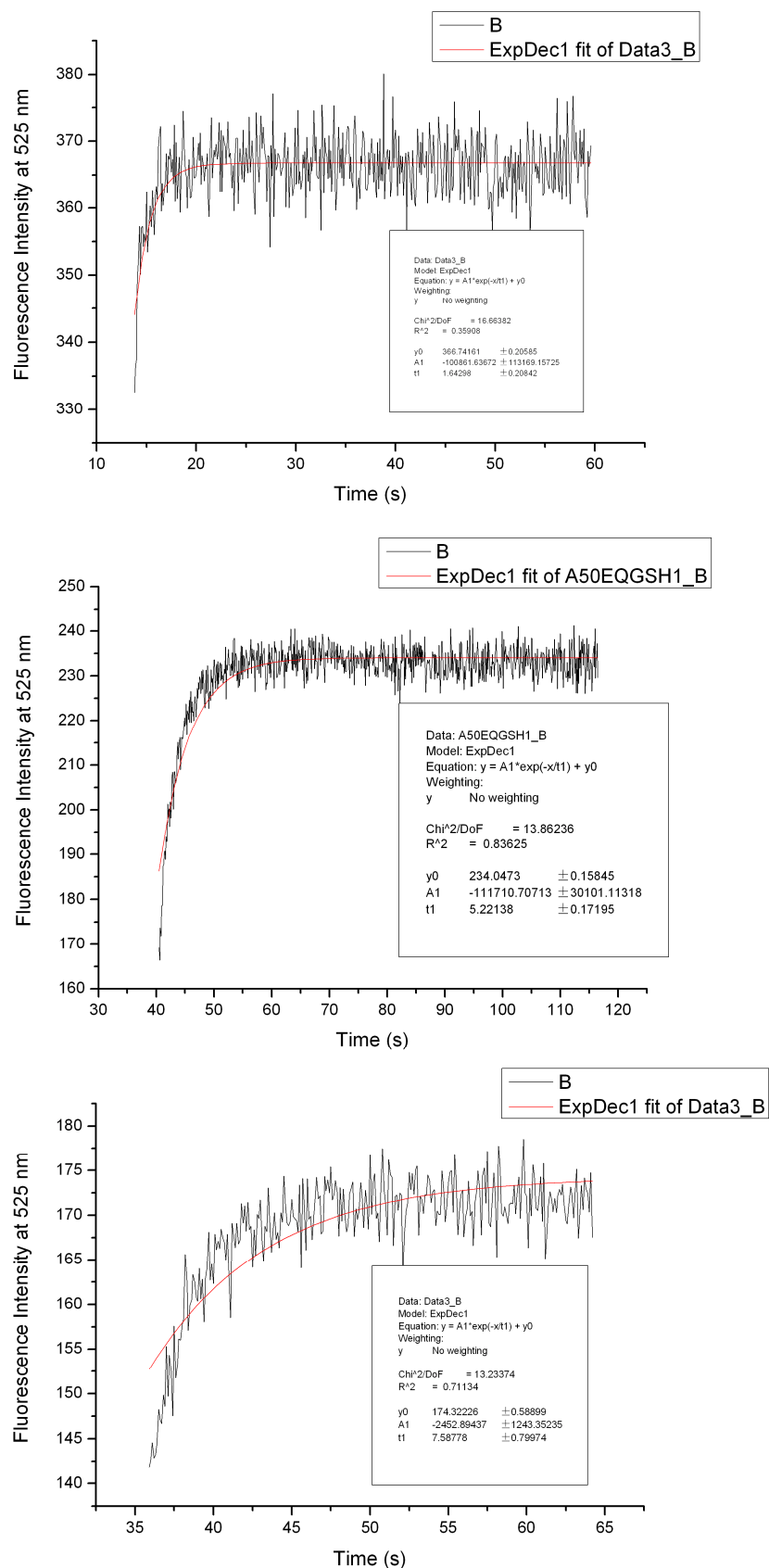


Fig. S3. Time-dependent fluorescence intensity of **1** at 525 nm in the presence of 50 μ M Cys (top), GSH (middle) and Hcy (bottom).

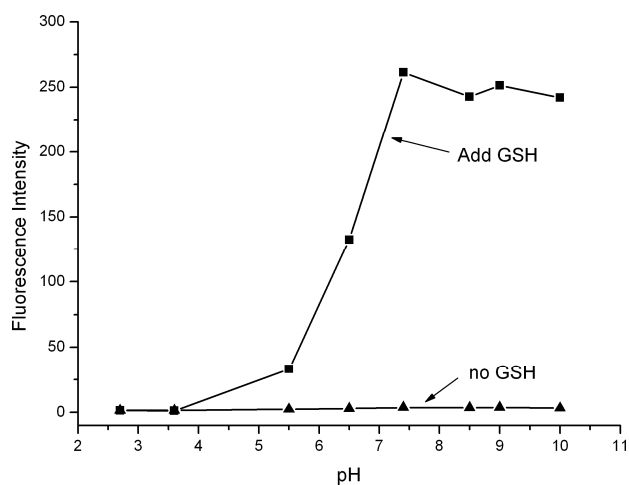


Fig. S4. The fluorescence intensity of **1** at 520 nm in the presence and absence of GSH under different pH (10 μ M sensor **1** in $\text{CH}_3\text{CN-H}_2\text{O}$ (1:99, v/v) system; $\lambda_{\text{ex}} = 485$ nm; Slit: 1.5 nm/1.5 nm). (Acetic acid was used for the buffers with pH value of 2.7 and 3.6, KH_2PO_4 was used for pH 5.5 buffer, MOPS for pH 6.5 buffer, HEPES for pH 7.4 buffer, Tris-HCl was used for pH 8.5 buffer, CHES was used for pH 9.0 buffer and CAPS was used for pH 10.0 buffer)

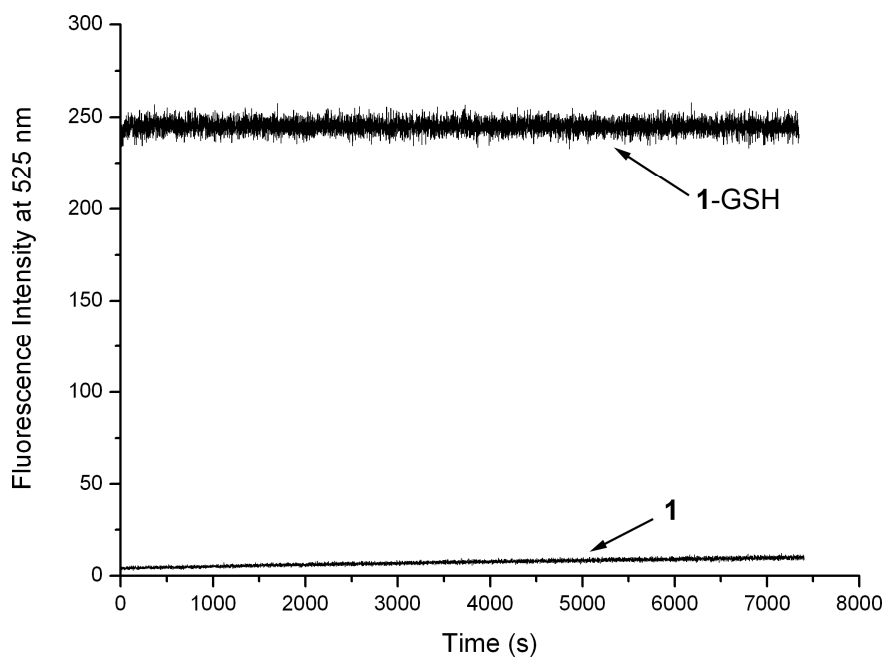


Fig. S5. Time-dependent fluorescence intensity of **1** and **1-GSH** at 525 nm.

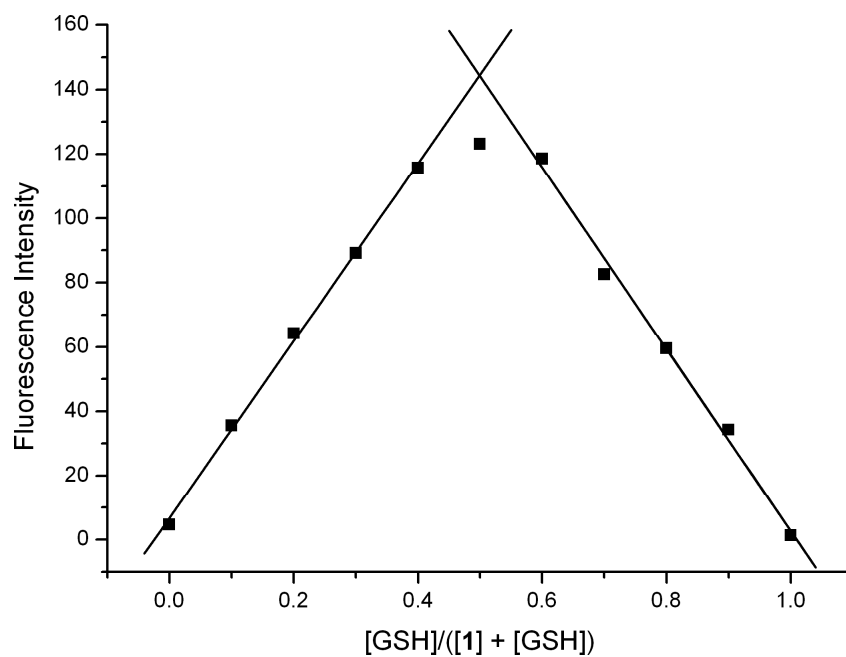


Fig. S6. Job's plot of the reaction between **1** and GSH in $CH_3CN/HEPES$ (20 mM) (1:99, v/v) solutions at pH 7.4. Total concentration of **1** and GSH was kept constant at 10.0 μM .

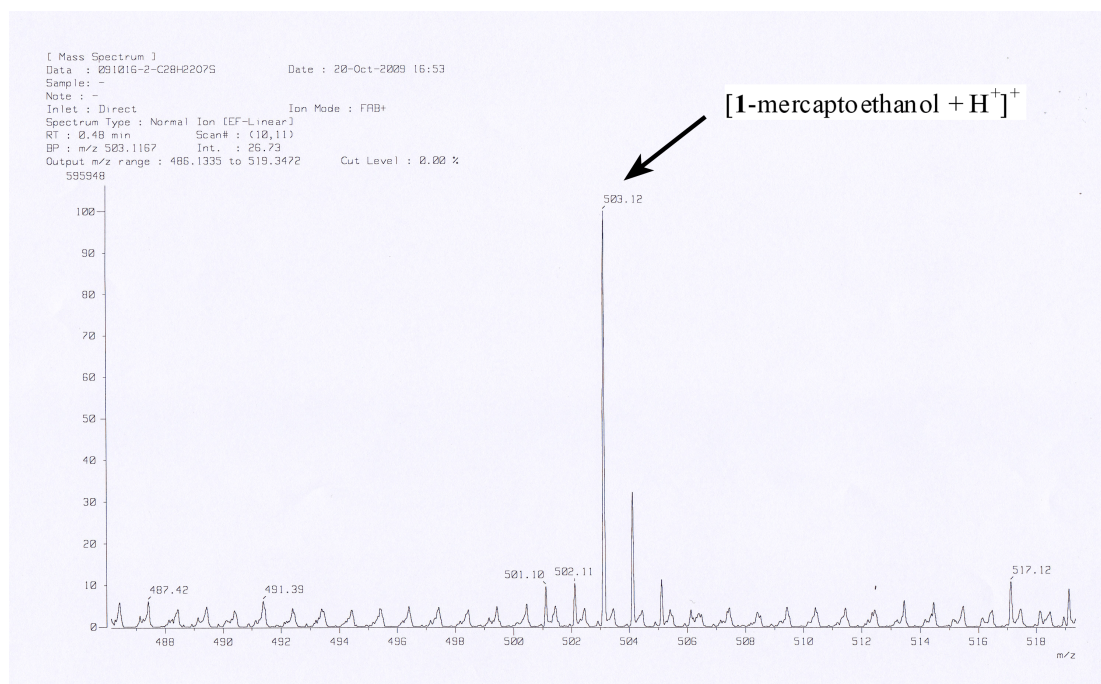


Fig. S7. The FAB mass of product obtained by mixing sensor **1** and 4 equiv 2-mercaptoethanol.

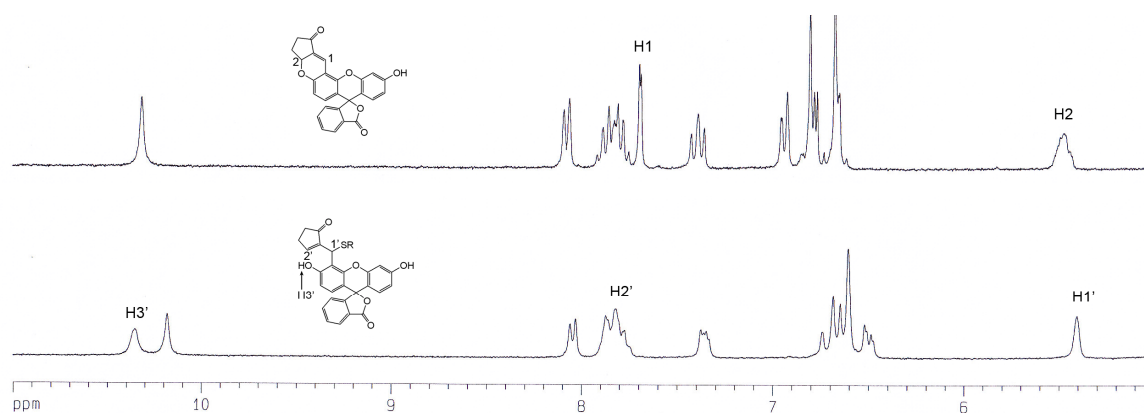


Fig. S8. ^1H NMR spectra of sensor **1** in the absence (top) and in the presence (bottom) of 4 equiv 2-mercaptoethanol in $\text{DMSO-}d_6$.

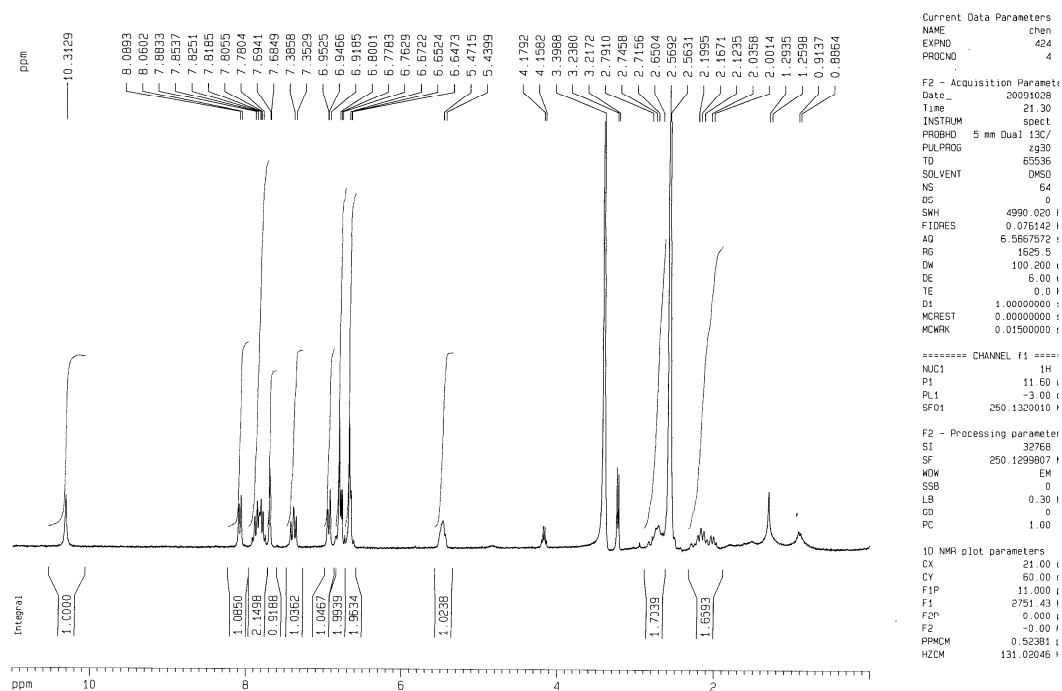


Fig. S9. ^1H NMR spectrum of **1**.

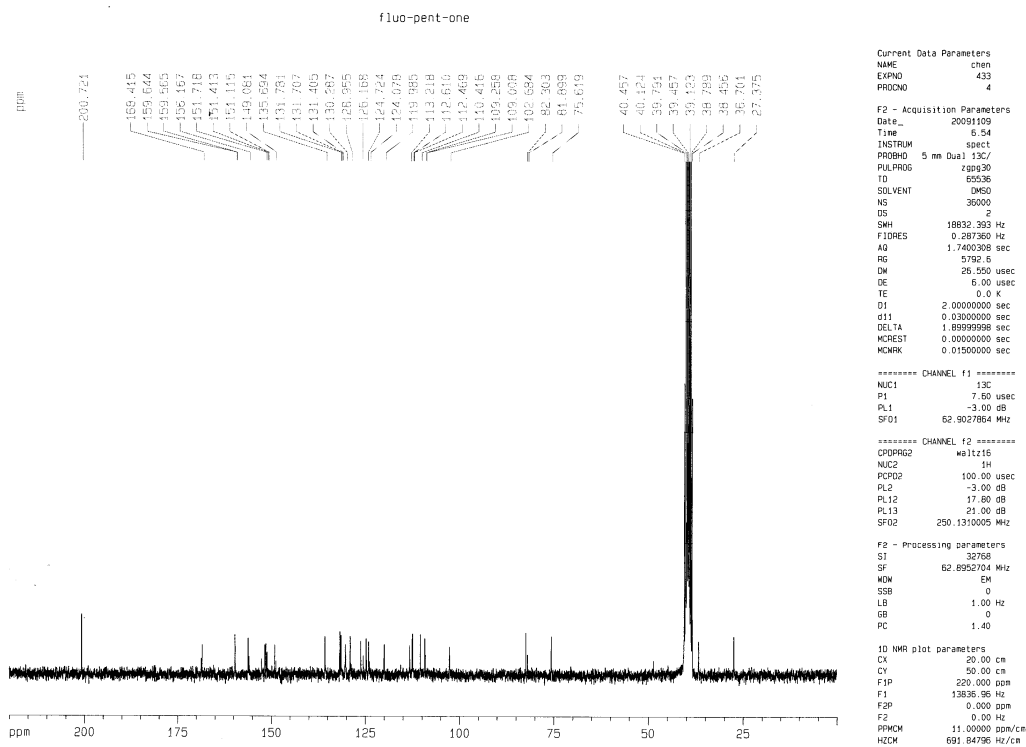


Fig. S10. ¹³C NMR spectrum of 1.

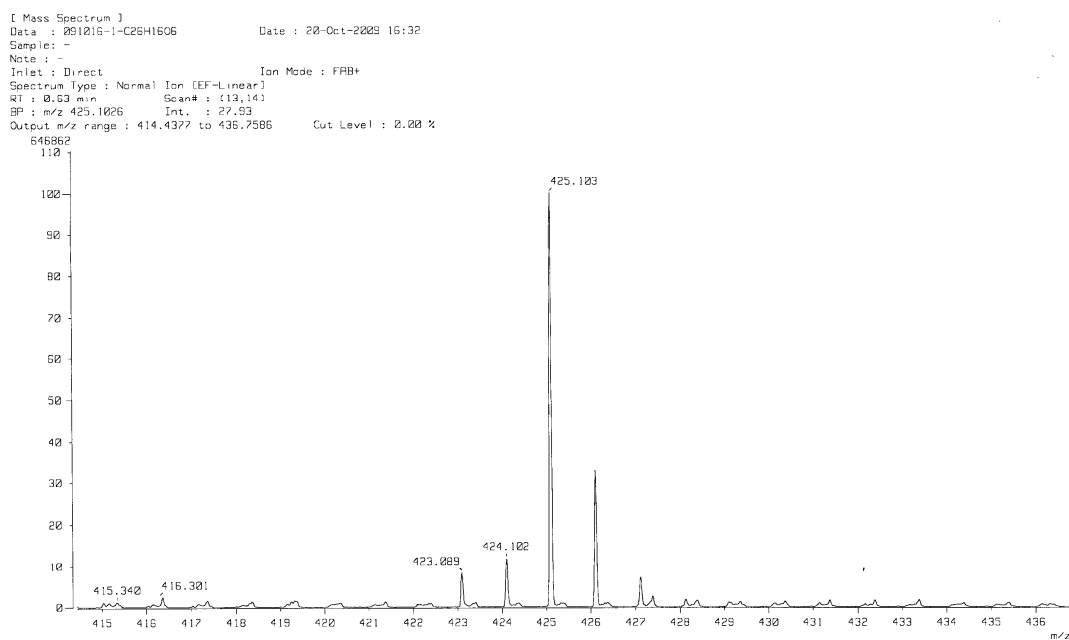


Fig. S11. FAB mass spectrum of 1.