Supplementary Information

A Red Fluorescent Probe for Thiols Based on 3-Hydroxyflavone and Its Application in Living Cell Imaging

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Materials and instruments:

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. NMR spectra were recorded on a BRUKER 400 spectrometer, using TMS as an internal standard. ESI-MS spectrum was measured on a Bruker Daltonics 6000 spectrometer. UV-Vis absorption spectra were measured using a Shimadzu UV-2450 spectrophotometer. Emission spectra were recorded at room temperature with a HITACHI F4600 fluorescence spectrophotometer with both the excitation and emission slit widths set at 5.0 nm. Cell imaging was performed with a Nikon C1si inverted microscope. TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from Qingdao Ocean Chemicals.

Preparation of test solutions:

Stock solutions of probe **1** were prepared at 1 mM in CH_3CN. The solutions of various control testing species including Asp, Ala, Val, Phe, His, Leu, Ser, Ile, Trp, Lys, Arg, Pro, Gly, Met, Tyr, Asn, Glu, Thr, and K^+, Na^+, Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+} metal ions were prepared in twice-distilled water. Solutions of probe **1** (10 μM) in 3 mL 35 mM HEPES buffer (pH 7.4) were prepared by placing 0.6 mL stock solution of the probe **1** (10 μM) and 0.3 mL CH_3CN in 2.1 mL of the aqueous buffer. The resulting solutions were mixed by shaking and incubated with appropriate testing species for 20 min at room temperature before recording spectra. Unless otherwise noted, the excitation wavelength was 444 nm.

Cell culture and fluorescence imaging:

HeLa cells were seeded in a 12-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 ℃ for 24 h. Immediately before the experiments, the cells were washed with PBS buffer. HeLa cells were then incubated with the probe (5 μM) at 37 °C for 30 min. For control experiments, HeLa cells were pretreated with *N*-methylmaleimide (1000 μM) for at 37 °C for 1 hour. After washing the cells three times with PBS buffer, pre-treated cells were incubated in the presence of probe **1** (5 μM) at 37 °C for another 30 min. Fluorescence imaging was performed after washing the cells three times with PBS buffer.

Synthesis of compound 3.

To a round-bottom flask (100 mL) equipped with a magnetic stirrer were added 4-(dimethylamino)cinnamaldehyde (0.35 g, 2mmol), 2-hydroxyacetophenone (0.27 g, 2mmol), sodium hydroxide (0.2 g, 5mmol) in 20 mL methanol. The resulting yellow mixture was refluxed (about 5 h) whereupon the color was dark red. The reaction mixture was cooled to room temperature, poured into 250 mL cold water and acidified with 6 M HCl to pH =2. The obtained solid was isolate by filtration, washed with100 mL 10% $NaHCO_3$ aqueous solution, and recrystallized from ethanol to give **3** as a pure compound (382 mg, 65%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (dd, *J* = 8.1, 1.3 Hz, 1H), 7.74 (dd, *J* = 14.5, 11.2 Hz, 1H), 7.48 – 7.39 (m, 3H), 7.11 (d, *J* = 14.5 Hz, 1H), 7.04 – 6.97 (m, 2H), 6.94 – 6.83 (m, 2H), 6.70 (d, *J* = 7.7 Hz, 2H), 3.03 (s, 6H).

Synthesis of compound 2.

To a 50 mL round-bottom flask was added compound **3** (293 mg, 1mmol) in a solution of 2 mL 20% NaOH and 20 mL methanol. The flask was cooled using an ice-water bath. A 1ml aliquot of 30% H_2O_2 solution was slowly added to the mixture. The resulting mixture was refluxed for 3 h while stirring. After cooling the reaction mixture to room temperature, the precipitated solid was collected by filtration and air dried. The crude product was determined to be pure by NMR analysis and used in the next step without further purification (175 mg, 57 %).

^{1}H NMR (400 MHz, DMSO-D_6) δ 7.95 (d, *J* = 8.4 Hz, 1H), 7.43 (dd, *J* = 17.0, 9.1 Hz, 3H), 7.34 (d, *J* = 8.8 Hz, 2H), 7.17 (ddd, *J* = 7.9, 5.0, 2.9 Hz, 1H), 6.97 (d, *J* = 16.2 Hz, 1H), 6.66 (d, *J* = 8.8 Hz, 2H), 2.88 (s, 6H).

Synthesis of probe 1.

This reaction was carried out under an argon atmosphere. To a stirred, room temperature mixture of compound **2** (307 mg, 1.0 mmol) and NaH (31 mg, 1.3 mmol) in 10 mL dry THF was added 2,4-dinitrobenzenesulfonyl chloride (347 mg, 1.3 mmol). The resulting reaction mixture was allowed to stir at room temperature for 2 h and subsequently quenched with 50 mL of water. The resulting solution was extracted twice with 50 mL dichloromethane. After drying the mixture over anhydrous Na_2SO_4, the drying agent was removed by filtration and the solvent was removed by distillation. The obtained residue was purified by silica gel column chromatography (CH_2Cl_2) to yield the desired product **1** (392 mg, 73%).
^{1}H NMR (400 MHz, DMSO-D_6) δ 9.09 (s, 1H), 8.54 (d, *J* = 26.9 Hz, 2H), 8.00 (d, *J* = 5.2 Hz, 1H), 7.76 (dd, *J* = 55.8, 23.5 Hz, 3H), 7.51 (s, 1H), 7.35 (d, *J* = 6.4 Hz, 2H), 6.73 (d, *J* = 6.8 Hz, 2H), 6.57 (d, *J* = 14.7 Hz, 1H), 3.02 (s, 6H). MS (ESI): found: m/z = 538.1 $(M+1)^+$, calcd. for $C_{25}H_{19}N_3O_9S$ = 537.1.

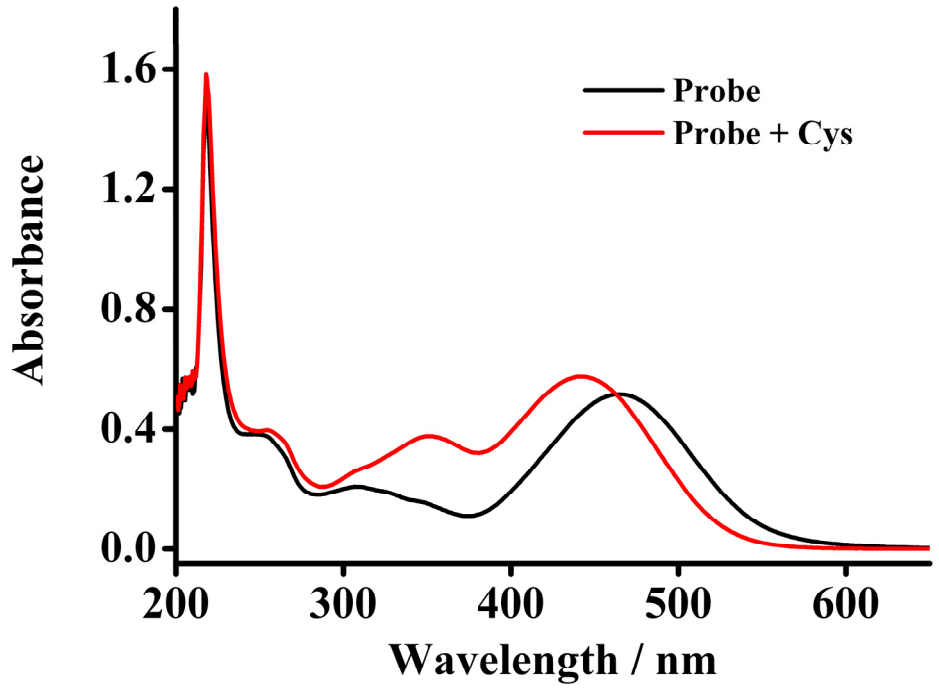

Figure S1. UV-vis absorption spectra of probe **1** (10 μM) (blank) in the absence and presence of Cys (20 equiv.) (red line)in HEPES buffer with 30% acetonitrile.

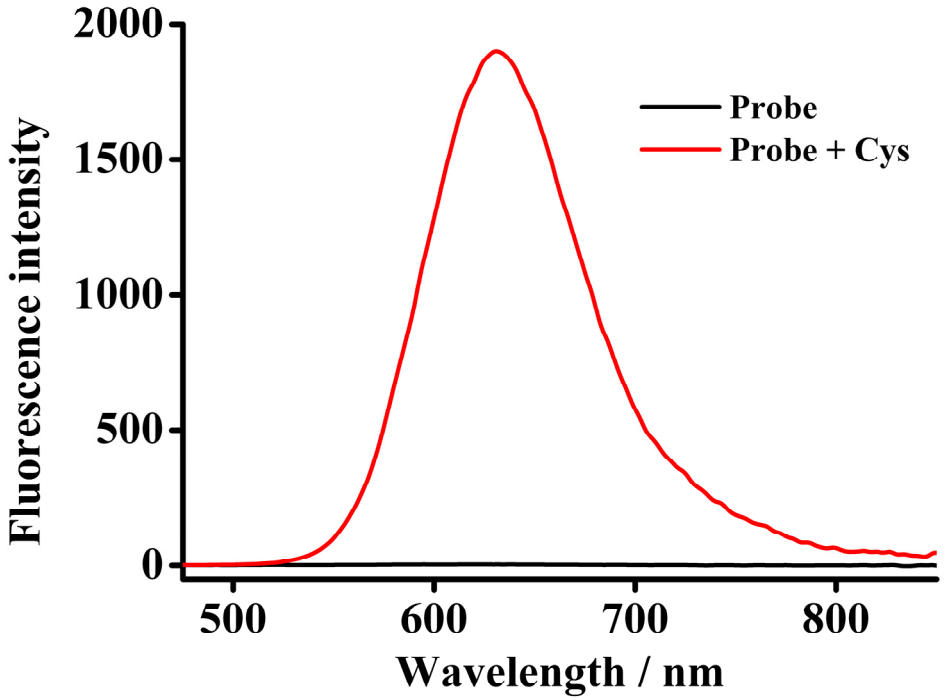

Figure S2. Fluorescence spectra of probe **1** (10 μM) in the absence and presence of Cys (20 equiv.) in HEPES buffer with 30% acetonitrile.

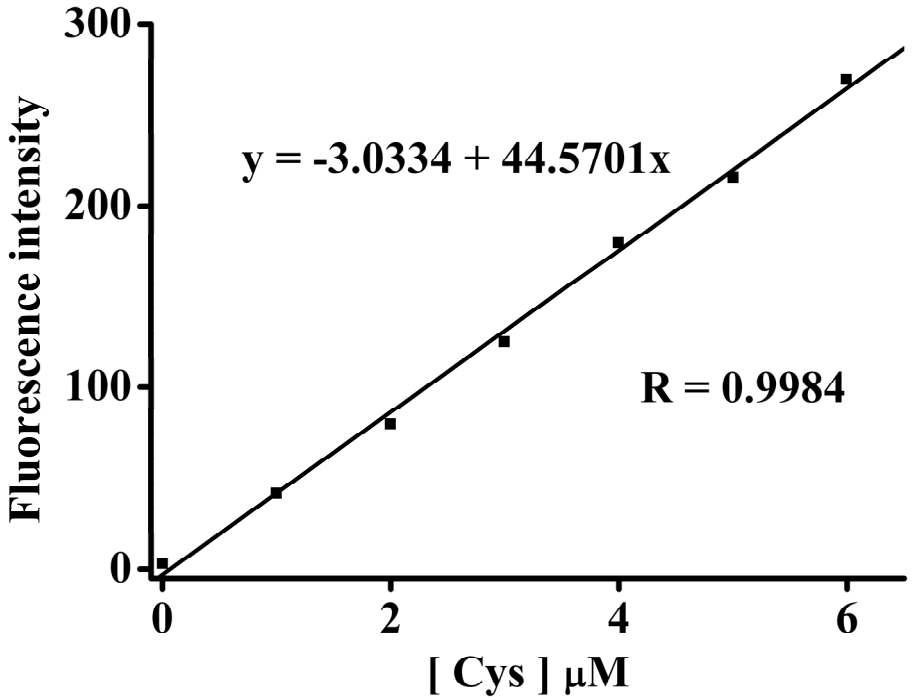

Figure S3. The linear relationship between fluorescence intensity and Cys concentration.

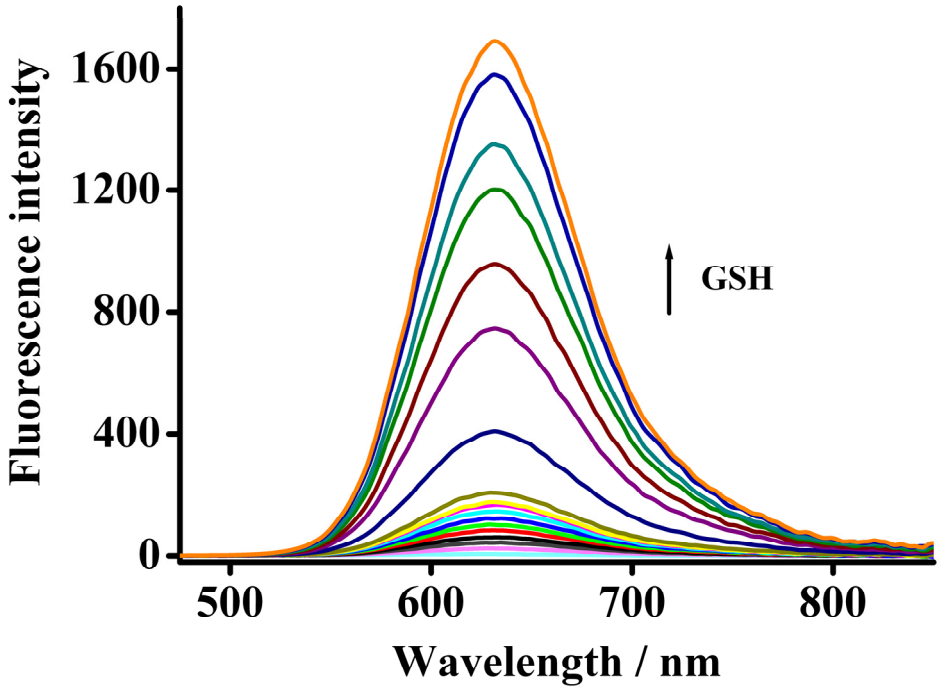

Figure S4. The fluorescence spectra (λ_{ex} = 444 nm) of probe **1** (10 μM) 20 minutes after the addition of GSH (0-200 μM) in HEPES buffer with 30% acetonitrile.

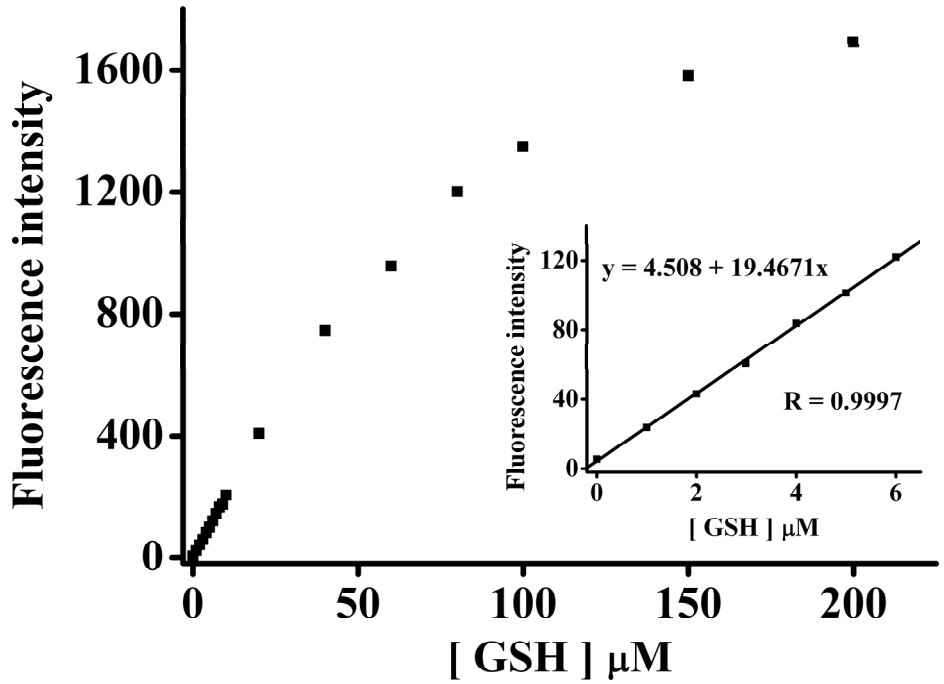

Figure S5. The fluorescence intensity of probe **1** (10 μM) with different amounts of GSH. Inset: the linear relationship between fluorescence intensity and GSH at low concentrations.

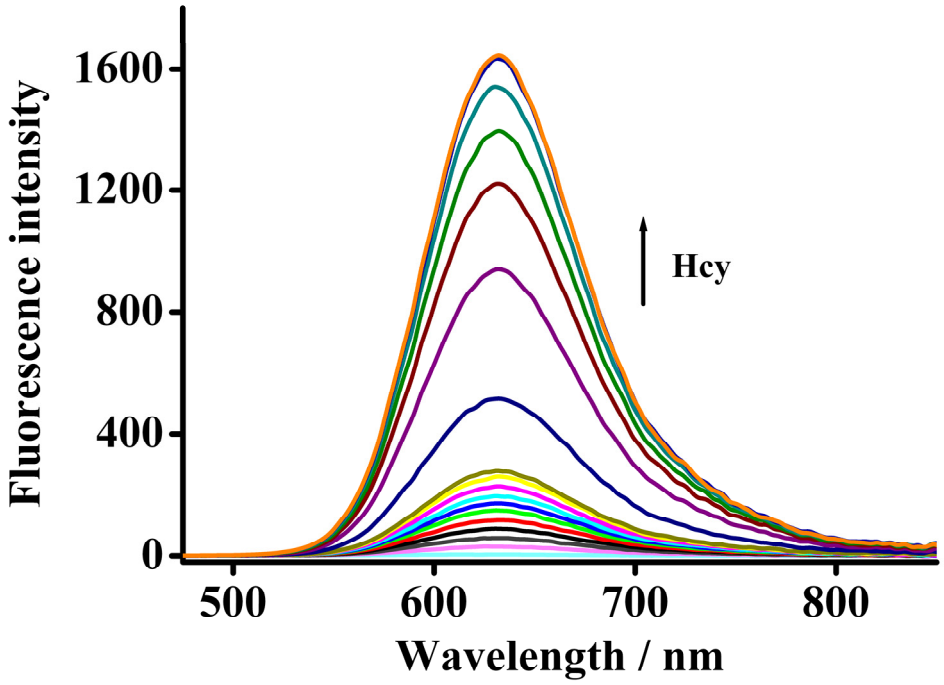

Figure S6. The fluorescence spectra (λ_{ex} = 444 nm) of probe **1** (10 μM) upon addition Hcy (0-200 μM) in HEPES buffer with 30% acetonitrile.

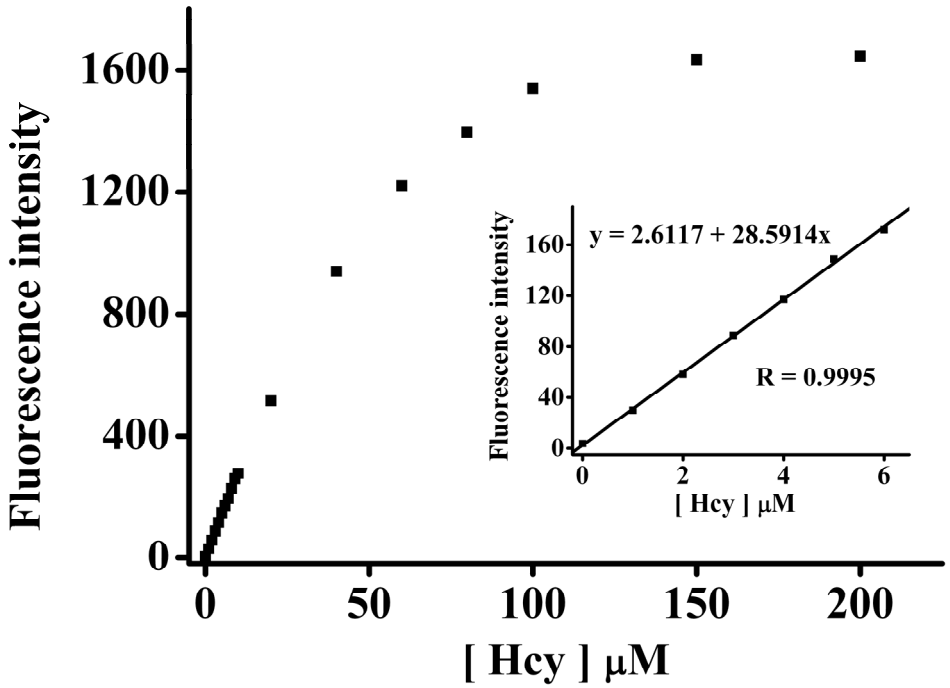

Figure S7. The fluorescence intensity of probe **1** (10 μM) with gradual addition of Hcy. Inset: the linear relationship between fluorescence intensity and Hcy at low concentration.

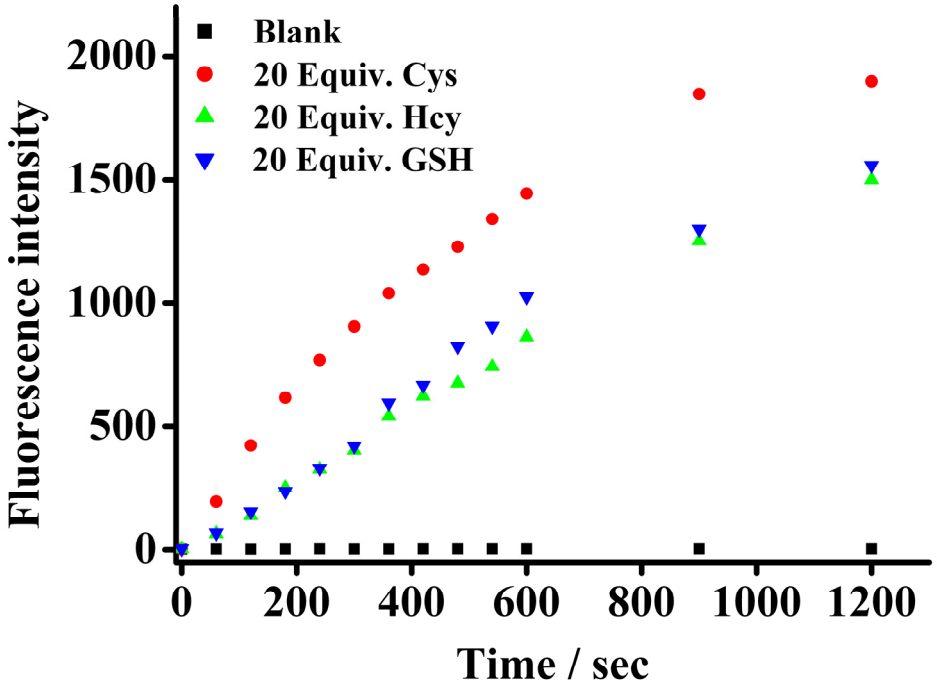

Figure S8. Kinetics of fluorescence enhancement rate (632 nm) for probe **1** (10 μM) with 20 equiv. of different RSH (Cys, Hcy, GSH) in HEPES buffer with 30% acetonitrile.

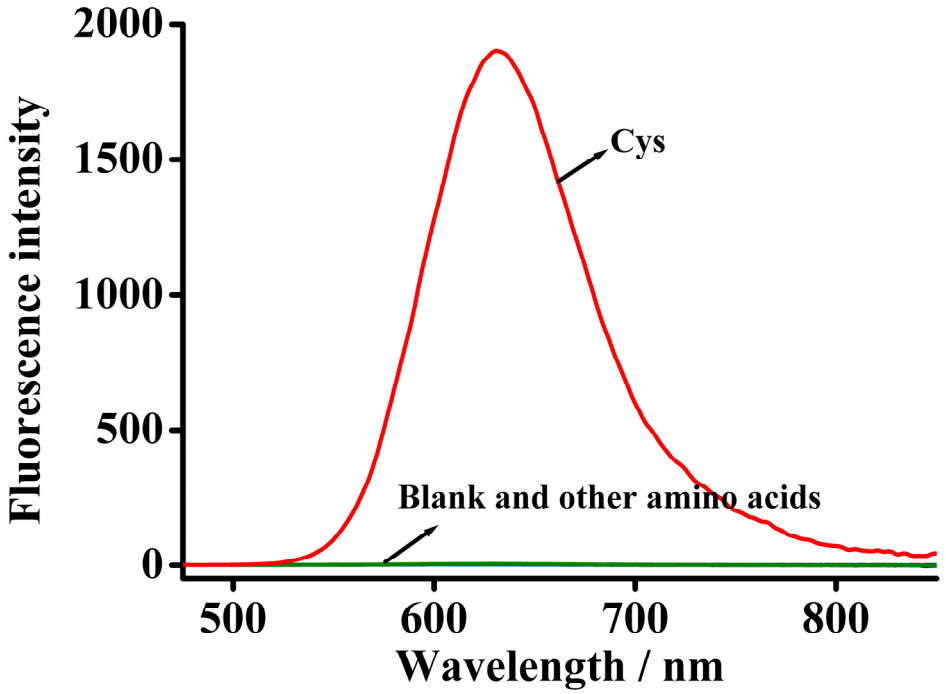

Figure S9. Fluorescence spectrum changes of probe **1** (10 μM) with Cys (20 equiv.) and other thiol-free amino acids in HEPES buffer with 30% acetonitrile.

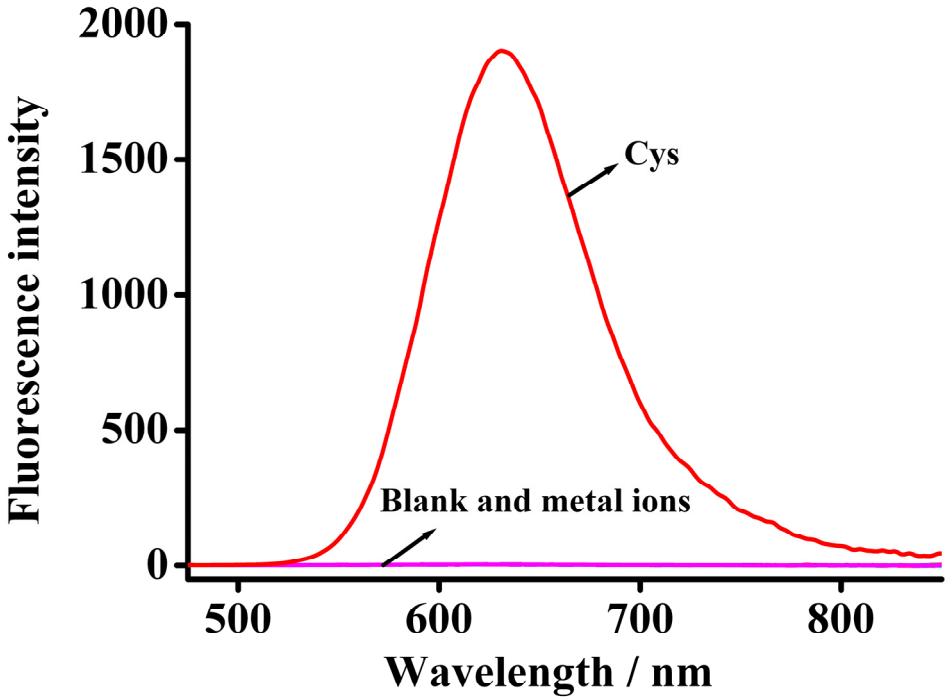

Figure S10. Fluorescence spectrum changes of probe **1** (10 μM) with Cys (20 equiv.) and metal ions (20 equiv.) in HEPES buffer with 30% acetonitrile.

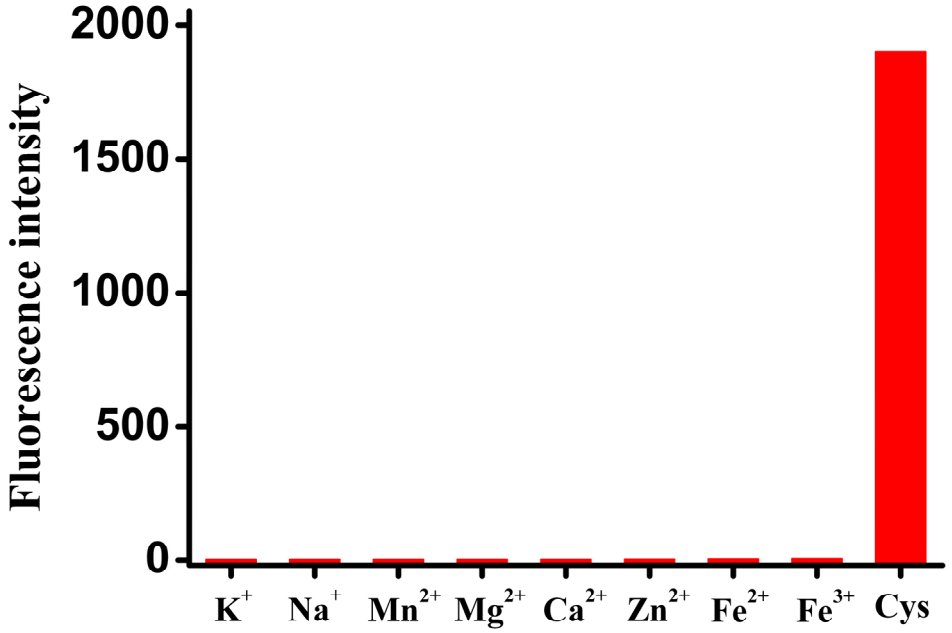

Figure S11. Fluorescence responses of probe **1** (10 μM) to Cys (20 equiv.) and metal ions (20 equiv.) in HEPES buffer with 30% acetonitrile.

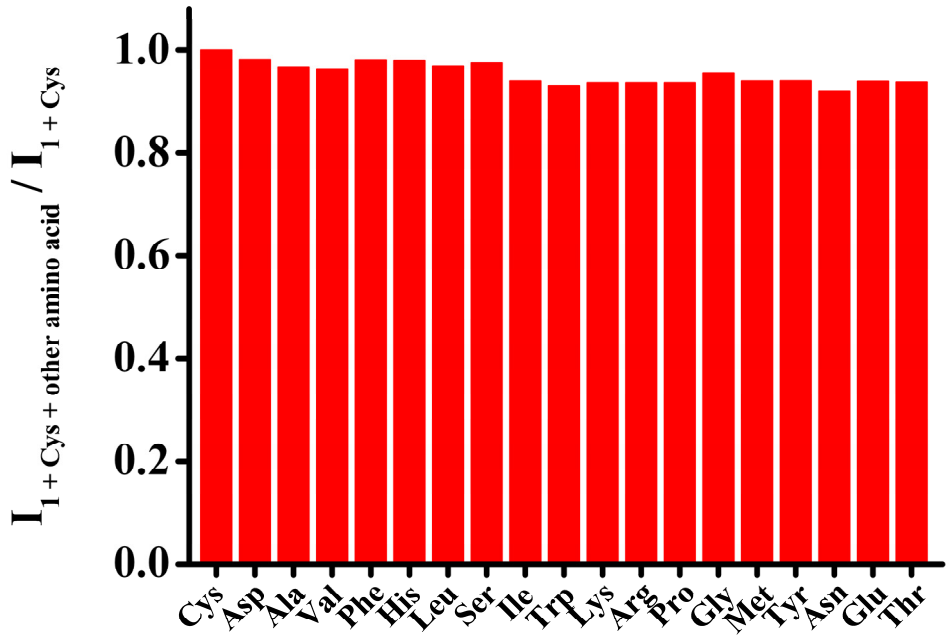

Figure S12. Interfering effect of various amino acids on the fluorescence intensity of probe **1** (10 μM) in the presence of Cys (20 equiv.) in HEPES buffer with 30% acetonitrile.

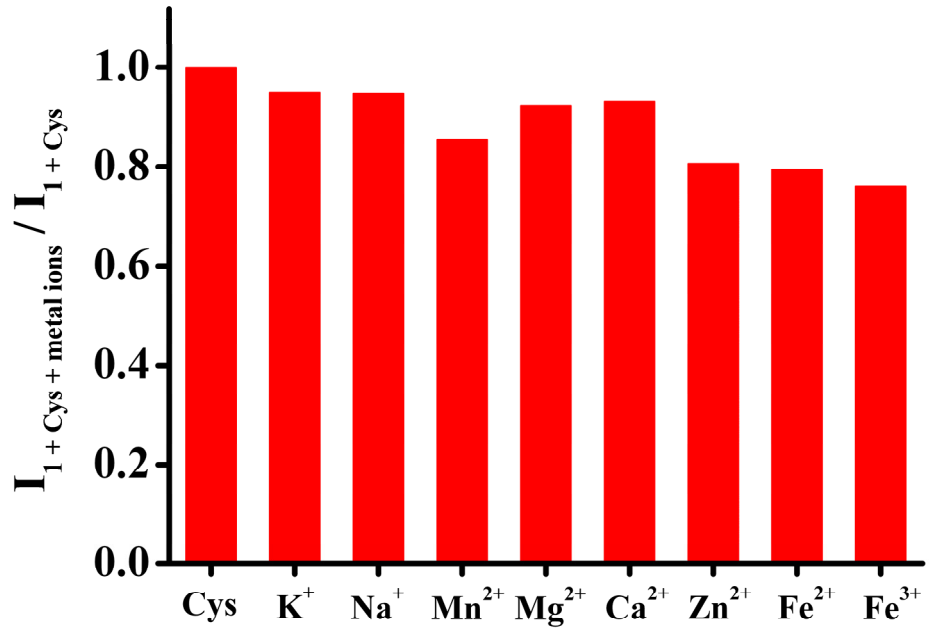

Figure S13. Interfering effect of metal ions (20 equiv.) on the fluorescence intensity of probe **1** (10 μM) in the presence of Cys (20 equiv.) in HEPES buffer with 30% acetonitrile.

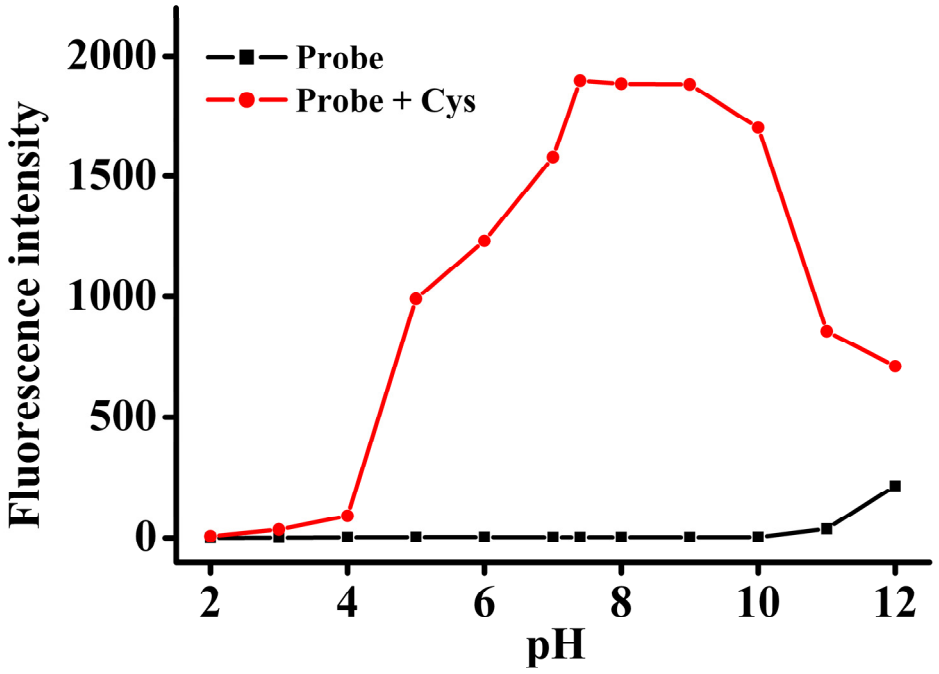

Figure S14. pH effect on the fluorescence intensity (632 nm) of probe **1** (10 μM) and probe **1** (10 μM) with Cys (20 equiv.).

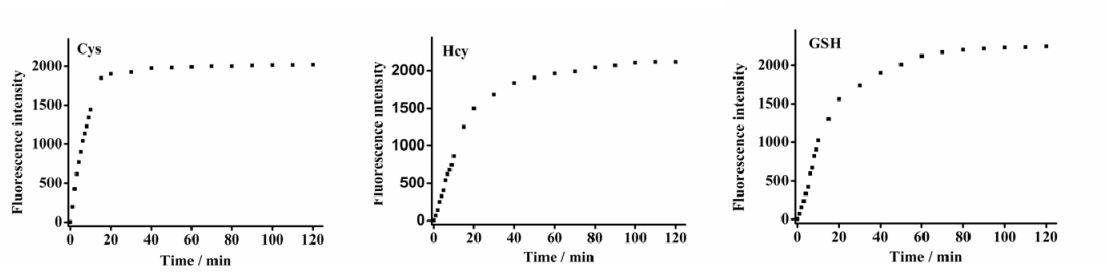

Figure S15. The full time–dependent fluorescence enhancement of probe 1 (10 μM) to 20 equiv. with different RSH (Cys, Hcy, GSH) in HEPES buffer with 30% acetonitrile.

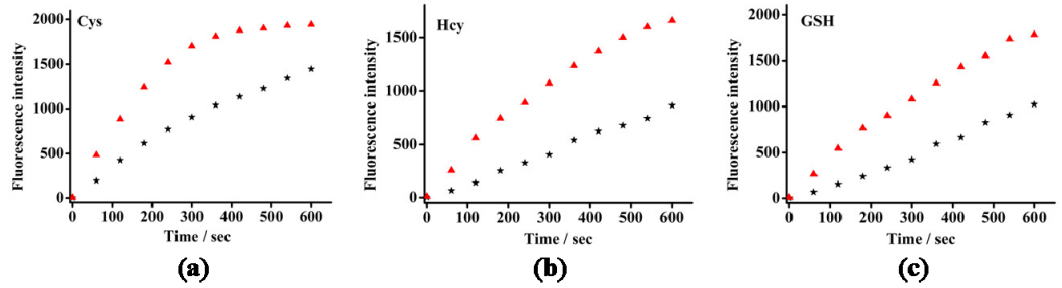

Figure S16. Kinetics of fluorescence enhancement rate (632 nm) for probe **1** (10 μM) with 20 equiv. of different RSH (Cys, Hcy, GSH) in HEPES buffer with 30% acetonitrile at different temperatures. (black ★: 25℃; red △: 37℃)

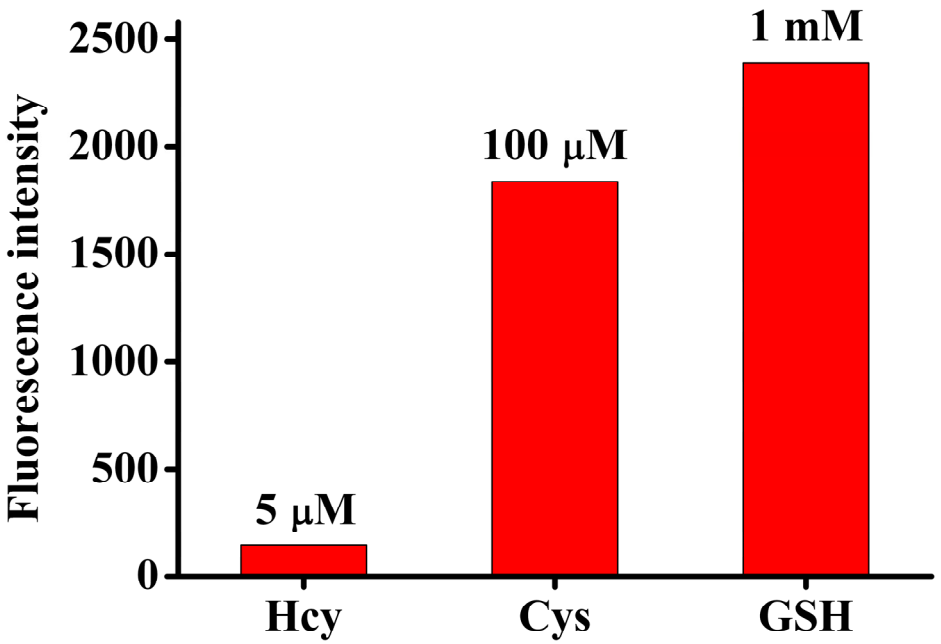

Figure S17. The fluorescence intensity enhancement of probe 1(10 μM) to Cys, Hcy, GSH at their physiological concentration in HEPES buffer with 30% acetonitrile.

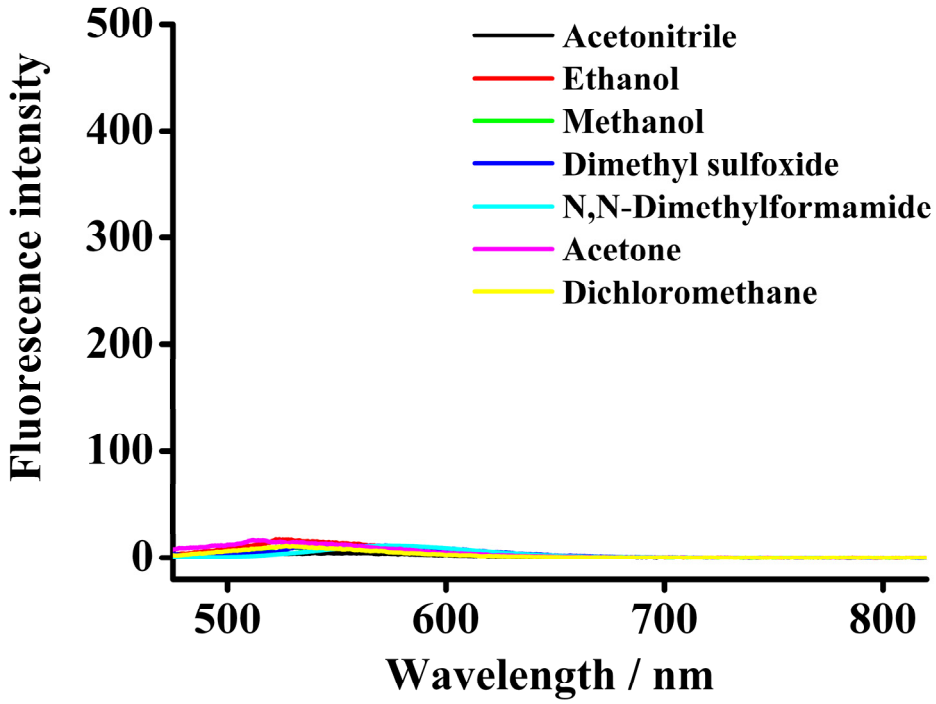

Figure S18. The fluorescence performance of probe 1 (10 μM) in different solvents.

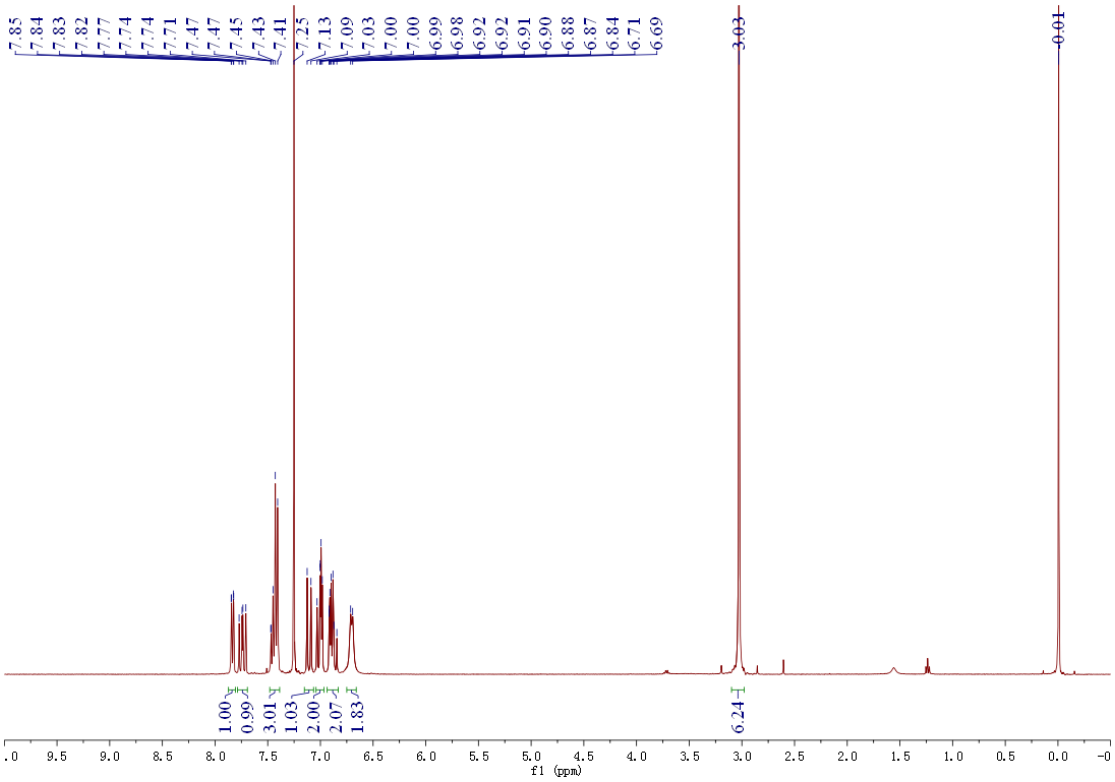

Figure S19. ^{1}H NMR spectrum of compound **3** in $CDCl_3$.

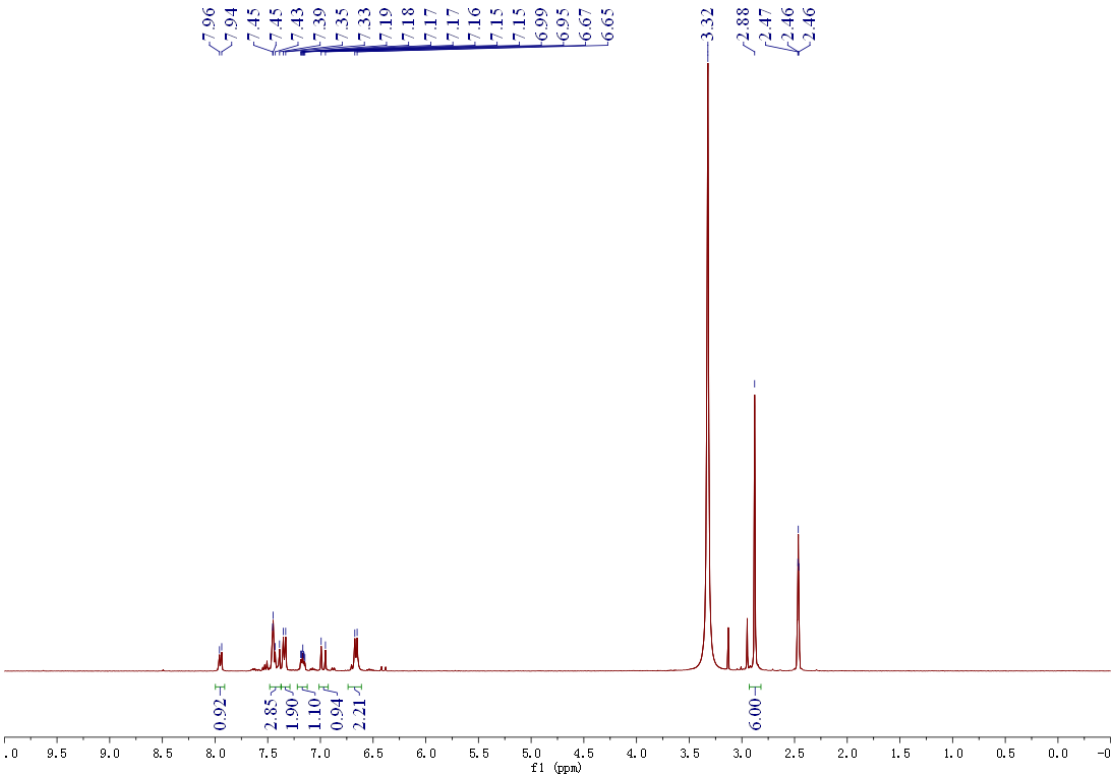

Figure S20. ^{1}H NMR spectrum of compound **2** in DMSO-d_6.

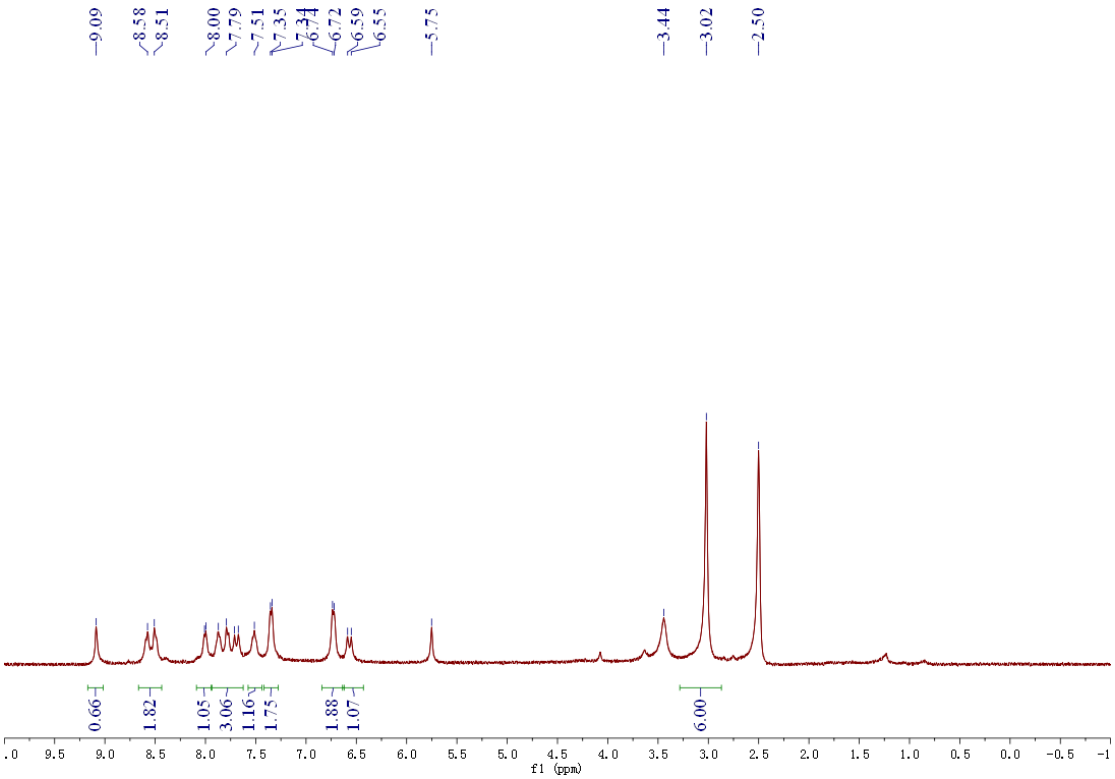

Figure S21. ^{1}H NMR spectrum of compound **1** in DMSO-d_6.

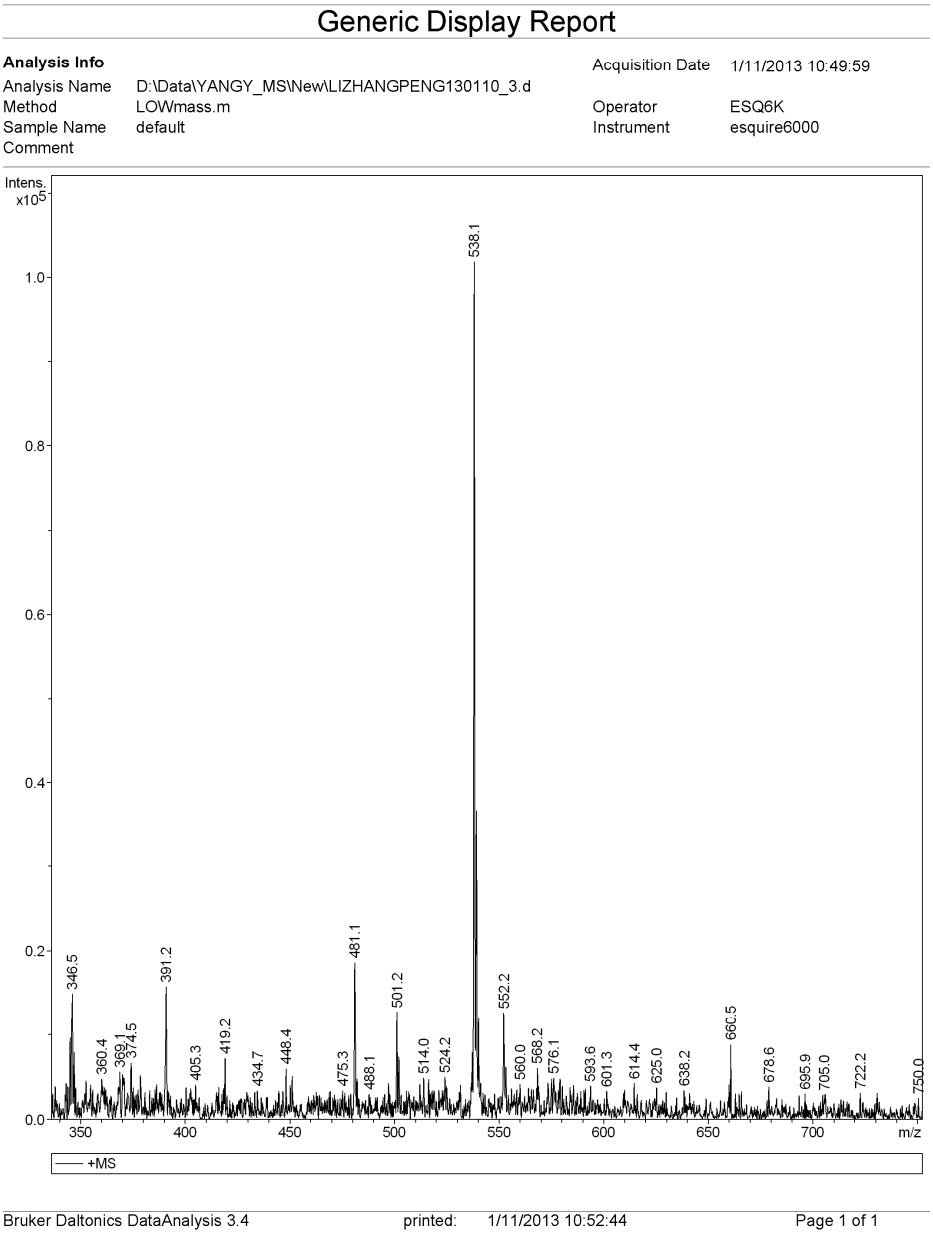

Figure S22. Mass spectrum of compound **1**.