Supporting Information

Simultaneous Fluorescent Imaging of Cys/Hcy and GSH from Different Emission Channels

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Experimental Section

1. General information and methods. All reagents and solvents were purchased from commercial sources and were of the highest grade. Solvents were dried according to standard procedures. All reactions were magnetically stirred and monitored by thin-layer chromatography (TLC). Flash chromatography (FC) was performed using silica gel 60 (200–300 mesh). Absorption spectra were taken on an Varian Carry 4000 spectrophotometer. Fluorescence spectra were taken on Hitachi F-7000 fluorescence spectrometer. The ^{1}H NMR and ^{13}C NMR spectra were recorded at 600 and 150 MHz, respectively. The following abbreviations were used to explain the multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad. High resolution mass spectra were obtained on a Varian QFT-ESI mass spectrometer.

2. Procedures for biothiols sensing

The solutions of amino acids, reduced glutathione (GSH) and various anions were

prepared in deionized water. A stock solution of **1** (1 mM) was prepared in CH_3CN. The stock solution of **1** was then diluted to the corresponding concentration (4 μM) with PB buffer (10 mM, pH 7.4). Spectra data were recorded in an indicated time after the addition of amino acids. All solutions of the anions were prepared from their sodium salts in deionized water. Superoxide solution was prepared by adding KO_2 (1 mg) to dry dimethyl sulfoxide (1 mL) and stirring vigorously for 10 min. Hydroxyl radical (·OH) was generated through the Fenton reaction of $Fe(ClO_4)_2$ and H_2O_2. Singlet oxygen ($^1O_2$) was generated by the reaction of NaClO with H_2O_2. NO was generated from DEA/NO (NO donor): DEA/NO was dissolved in 0.01 M NaOH as a stock solution (500 mM), stored at 0 °C, and prepared daily.

3. Cell culture and fluorescence imaging

The B16 cell line was provided by Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education (China). Cells were grown in RPMI 1640 medium supplemented with 10 % FBS (Fetal Bovine Serum) and 1% antibiotics at 37 ºC in humidified environment of 5% CO_2. Cells were plated on 6-well plate and allowed to adhere for 12 hours. Fluorescence imaging was performed with a LEICA TCS-SP5 Laser Scanning Confocal Microscope. Before the experiments, cells were washed with PBS 3 times. Then, the cells were incubated with **1** (2 μM), or pretreated with Cys (0.5 mM, 30 min), or GSH (1.0 mM, 30 min) in RPMI 1640 medium at 37 °C. After each treatment, the cells were washed with PBS 3 times. Emission was collected at 500–580 nm for green channel (excited at 458 nm), and at 610–720 nm for red channel (excited at 543 nm).

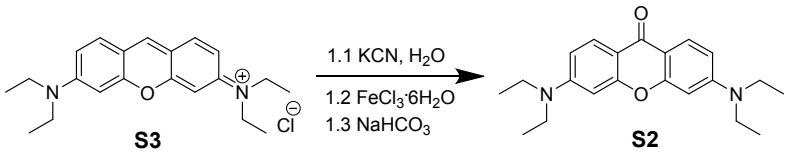
4. Cyotoxicity assays.

B16 Cells were grown in RPMI 1640 medium supplemented with 10 % FBS (Fetal Bovine Serum) and 1% antibiotics at 37 ºC in humidified environment of 5% CO_2. Immediately before the experiment, the cells well placed in a 96-well plate, followed by addition of increasing concentrations of probe **1**. The final concentrations of the probe were kept from 0 to 10 μM. The cells were then incubated at 37 ºC in an

atmosphere of 5% CO_2 and 95% air for 8 h, followed by MTT assays (n= 6). Untreated assay with RPMI 1640 medium (n = 6) was also conducted under the same conditions.

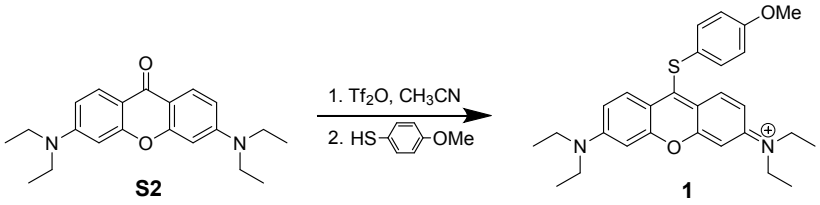
5. Synthesis

5.1 Synthesis of **S2**



A mixture of **S3** (0.358 g, 1.0 mmol), potassium cyanide (0.195 g, 3.0 mmol), and water (10 mL) was refluxed for 18 h, then cooled to room temperature, filtered, and washed with water. The resulting residue was suspended in 2.0 M aq HCl (100 mL). The suspension was poured dropwise onto a solution of $FeCl_3{\cdot}6H_2O$ (0.807 g, 3.0 mmol) in 2.0 M aq HCl (10 mL). The reaction mixture was heated at 90 °C for 12 h, then cooled to room temperature, filtrated, and washed well with water. The residue was suspended in saturated aq $NaHCO_3$ (50 mL), refluxed for 3 days, cooled to room temperature. The precipitate was collected by filter and then was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 50:1 v/v) to afford a light yellow solid (0.134 g, 39.7% yield). ^{1}H NMR (600 Hz, $CDCl_3$) δ 8.10 (d, J = 9.0 Hz, 2H), 6.65 (dd, J_1 = 1.8 Hz, J_2= 9.0 Hz, 2H), 6.69 (s, 2H), 3.46 (q, J = 7.2 Hz, 8H), 1.25(t, J = 7.2 Hz, 12H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.0, 161.402, 154.9, 130.7, 114.5, 111.5, 99.2, 47.6, 15.4; ESI-MS: $[M+H]^+$ calcd for 339.2066, Found 339.2067.

5.2 Synthesis of **1**



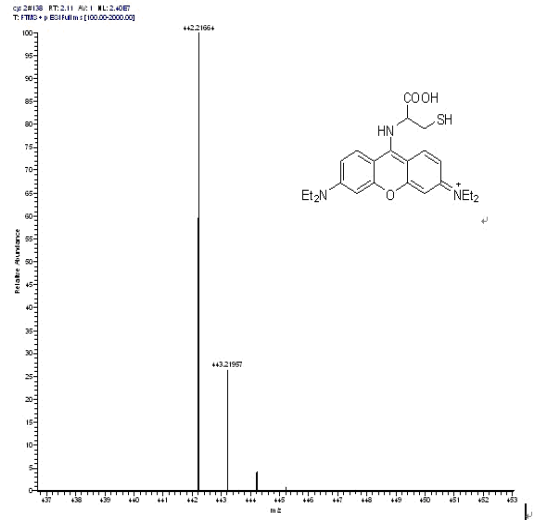
The mixture of **S2** (0.1 g, 0.3 mmol) in CH_3CN (10 mL) was stirred at 0 °C under N_2 for 10 min then Tf_2O (200 μL, 1.2 mmol) was added dropwise over 1 min.

The reaction mixture was stirred for 10 min then 4-methoxythiophenol (0.42 g, 3 mmol) was added. The mixture was stirred overnight at 25 ^{o}C (If the reaction was incomplete, another portion of Tf_2O and nucleophile was added). The solvents were removed under reduced pressure and the residue was purified by flash chromatography ($MeOH/CH_2Cl_2$ = 20/1) to afford the pure product **1** (80 mg, 44.3% yield). ^{1}H NMR (600 Hz, CD_3CN) δ 8.08 (dd, J_1 = 3.0 Hz, J_2= 9.6 Hz, 2H), 7.40(d, J = 8.4 Hz, 2H), 6.98 (d, J = 9.6 Hz, 2H), 6.92 (d, J = 8.4 Hz, 2H), 6.70 (s, 2H), 3.77 (s, 3H), 3.63(q, J = 7.2 Hz, 4H), 1.27(t, J = 7.2 Hz, 6H)； ^{13}C NMR (150 MHz, CD_3CN) δ 163.2, 159.9, 158.7, 158.4, 135.8, 133.8, 127.0, 118.5, 117.9, 117.2, 98.7, 58.1, 48.6, 14.8; ESI-MS: $[M]^+$ calcd for 461.2257, Found 461.2256.

6. Supplementary Spectra

Sample No.	Measured Mass	Exact Mass	Delta (m/z)	Delta (ppm)	Description
3a	442.21664	442.21589	0.00075	1.69600	

Sample No.	Measured Mass	Exact Mass	Delta (m/z)	Delta (ppm)	Description
3b	456.23083	456.23154	-0.00071	-1.55623	



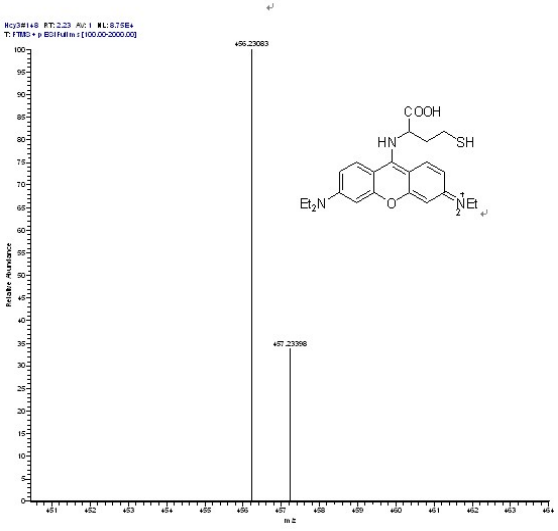


Figure S1 HRMS charts of **3a** and **3b**.

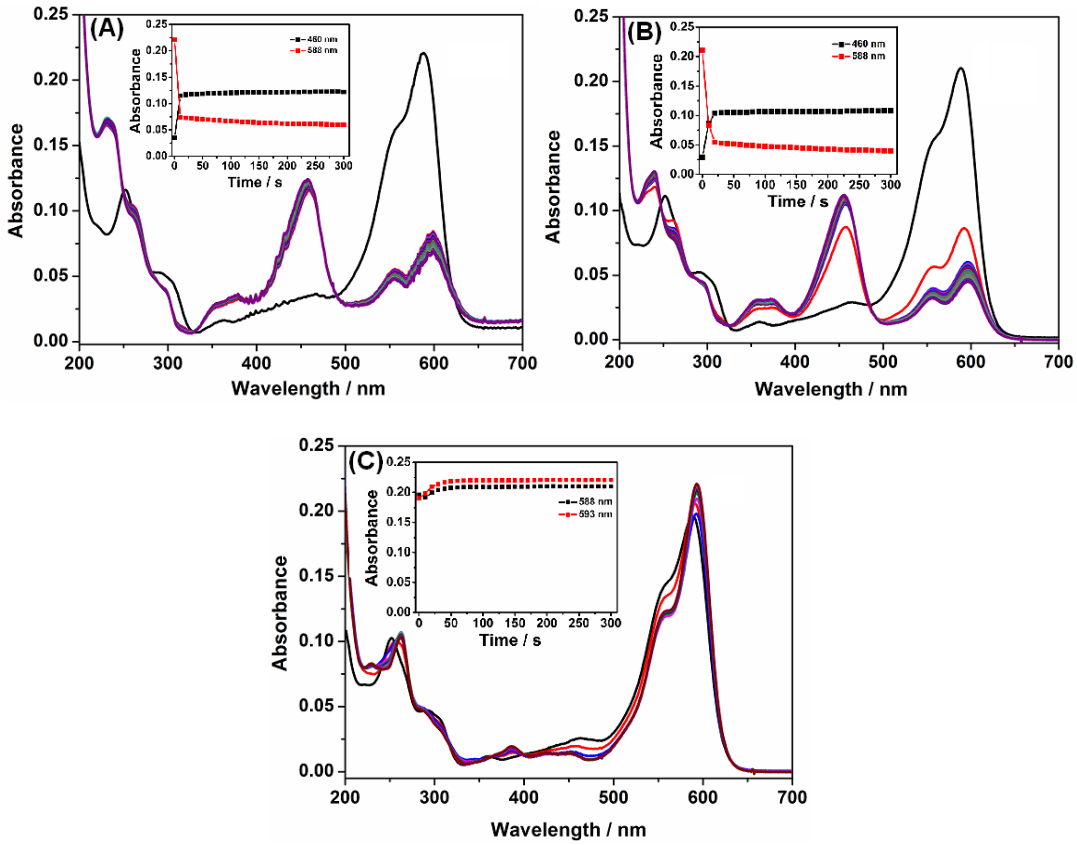


Figure S2 The time-dependent absorption spectra changes of **1** (4 μM) upon addition of 15 equiv of Cys (A), Hcy (B), and GSH (C) in PB buffer (10 mM, pH 7.4) at 25 °C.

Sample No.	Measured Mass	Exact Mass	Delta (m/z)	Delta (ppm)	Description
5a	762.40686	762.40475	0.00211	2.76756	

Sample No.	Measured Mass	Exact Mass	Delta (m/z)	Delta (ppm)	Description
5b	776.4205	776.4204			

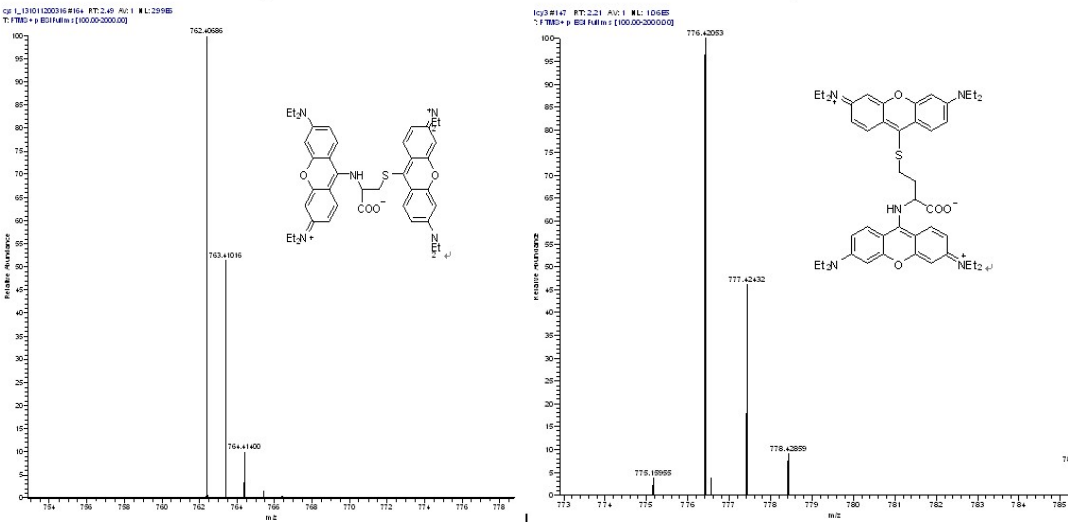


Figure S3 HRMS charts of **5a** and **5b**.

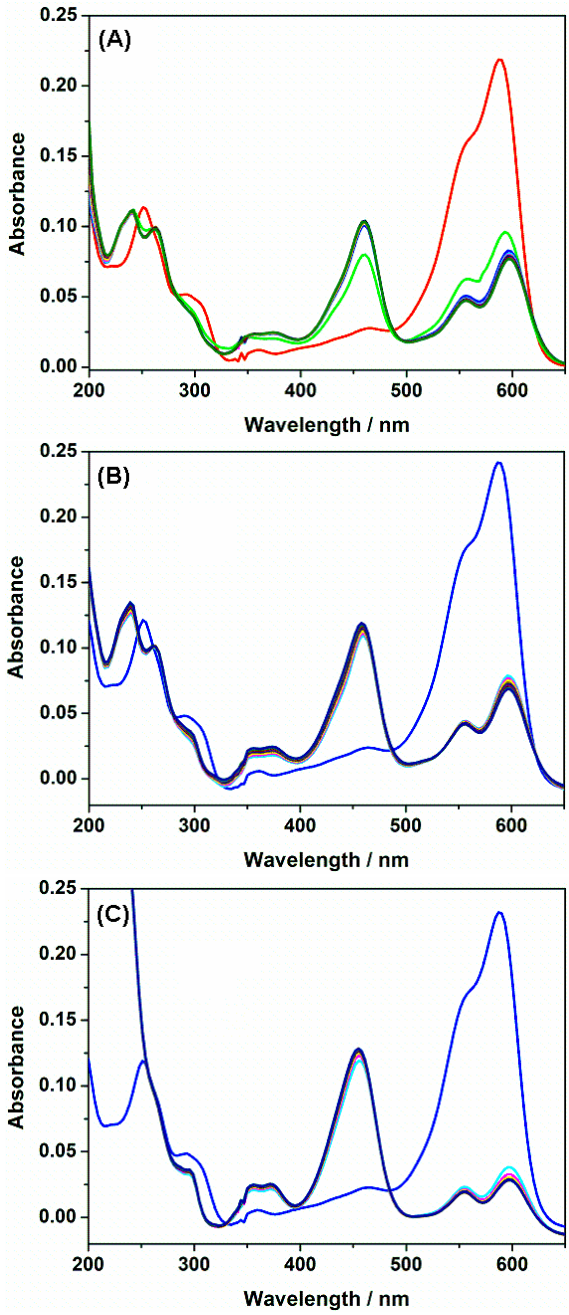


Figure S4 The time-dependent absorption spectra changes of **1** (4 μM) upon addition of 1 equiv (A), 10 equiv (B), and 100 equiv (C) of Cys, respectively, in PB buffer (10 mM, pH 7.4). Time range: 0–300 s.

Sample No.	Measured Mass	Exact Mass	Delta (m/z)	Delta (ppm)	Description
4	628.27875	628.27995	-0.00120	-1.90998	

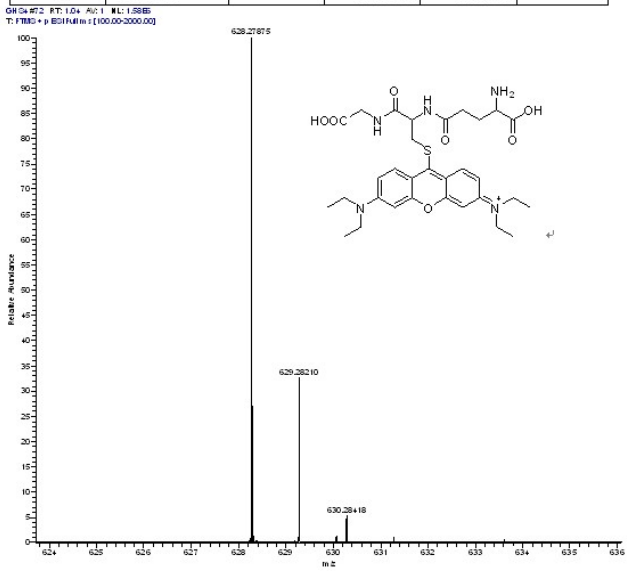


Figure S5 HRMS chart of **4**.

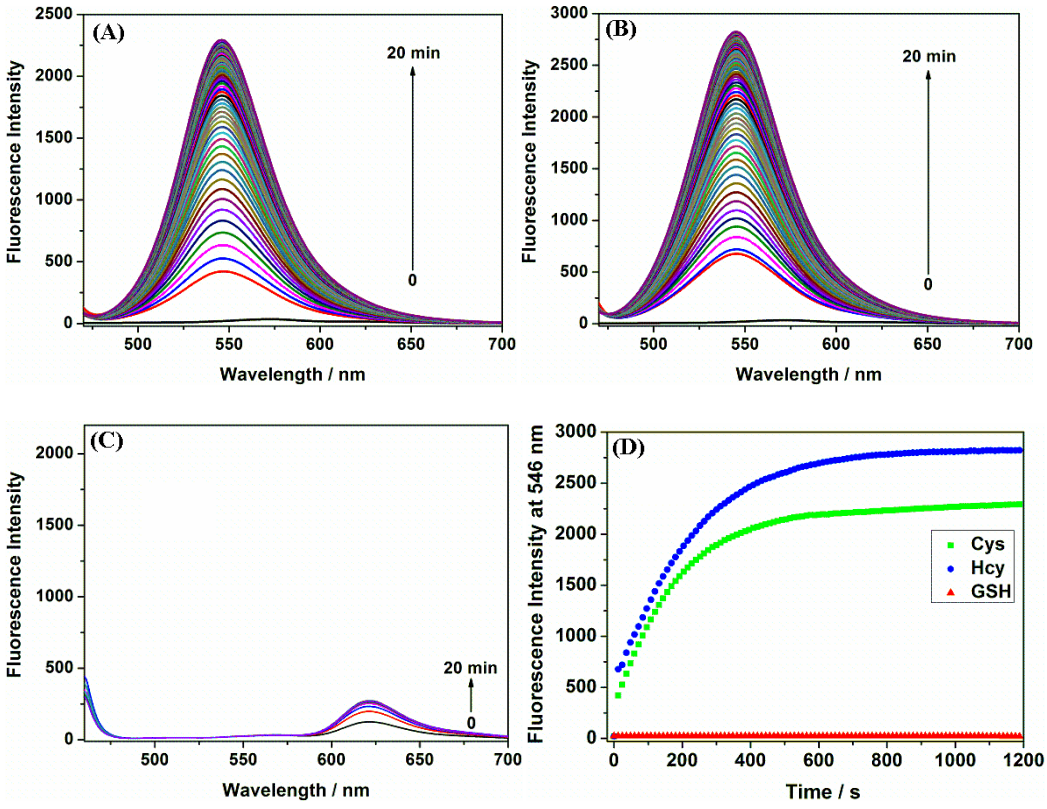


Figure S6 Time-dependent fluorescence spectra of probe **1** (4 μM) in the presence of 15 equiv of Cys (A), Hcy (B), and GSH (C), and the corresponding time-dependent fluorescence intensity changes (D). Conditions: PB buffer (10 mM, pH 7.4) at 25 °C; Slits: 5/5 nm; λ_{ex} = **455 nm**.

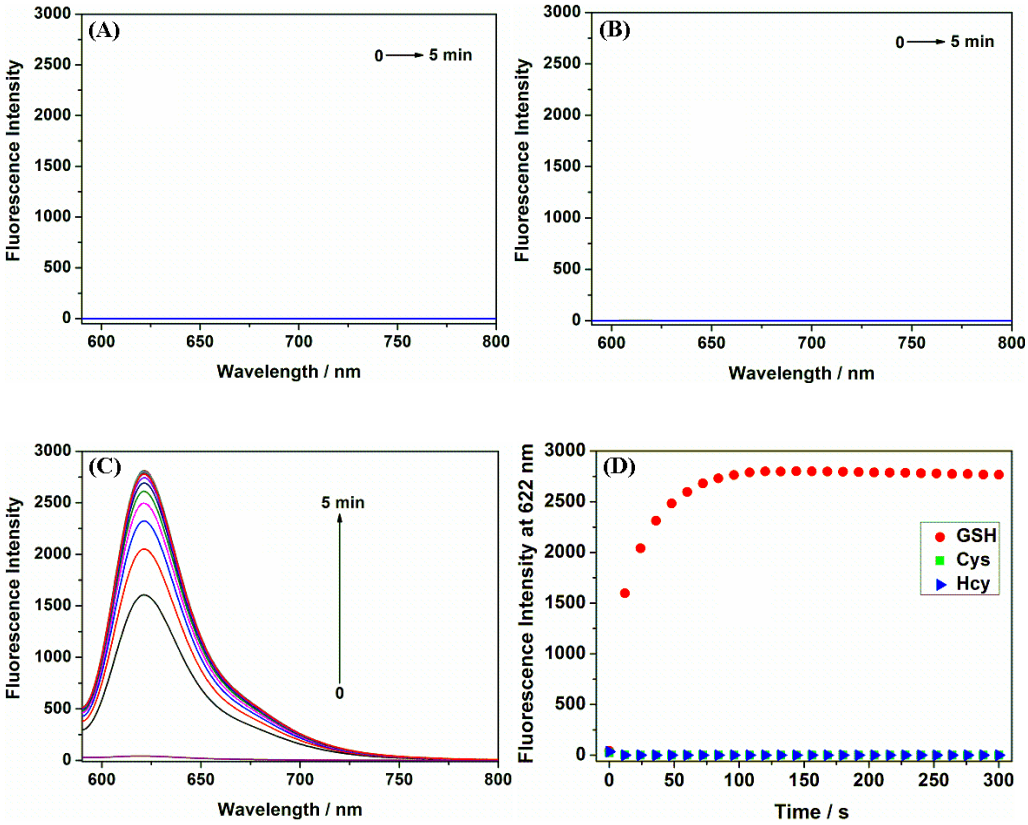


Figure S7 Time-dependent fluorescence spectra of probe **1** (4 μM) in the presence of 15 equiv of Cys (A), Hcy (B), and GSH (C), and the corresponding time-dependent fluorescence intensity changes (D). Conditions: PB buffer (10 mM, pH 7.4) at 25 °C; Slits: 5/5 nm; λ_{ex} = **580 nm**.

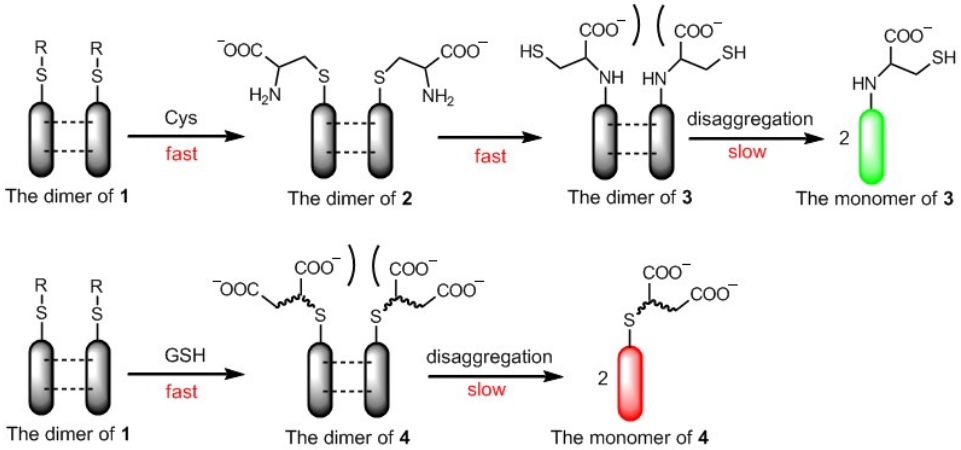


Figure S8 The proposed reaction of the dimer of **1** with Cys or GSH.

The kinetic studies revealed that the fluorescence responses [for Cys/Hcy: 10 min; for

GSH: ~2 min (Fig. S6 and S7)] obviously lag behind the absorption responses (Fig. S2). Given that xanthene dyes, such as pyronin and rhodamine, tends to form nonfluorescent dimer in solution (*J. Phys. Chem. B*, 2003, **107**, 13803), we speculated that the relatively slow fluorescence response is probably a result of the slow disaggregation of the nonfluorescent dimer of **3** (or **4**), formed by the fast reaction of the dimer of **1** with these biothiols, to release the fluorescent monomer **3** (or **4**) due to the electrostatic repulsion of the $-COO^-$ groups.

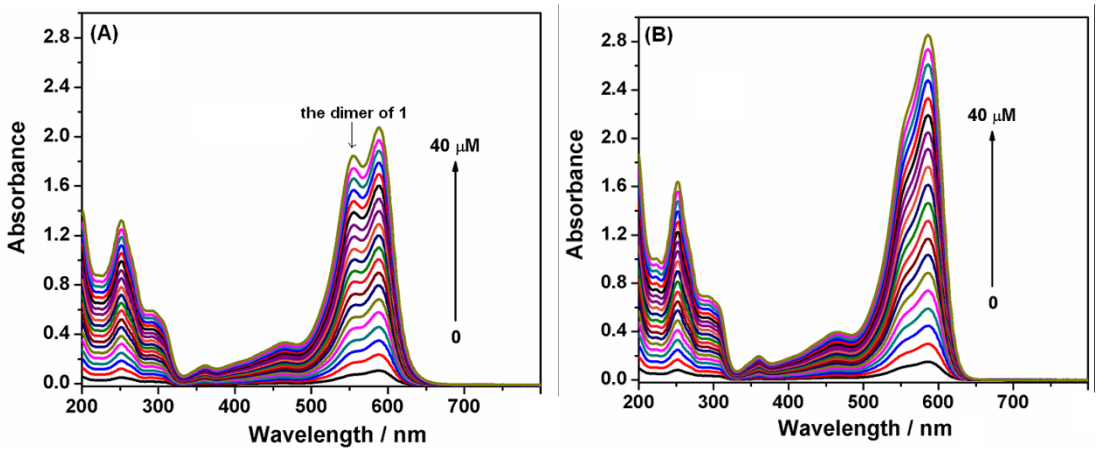


Figure S9 The concentration-dependent absorption spectra changes in PB buffer (10 mM, pH 7.4) (A) and CH_3CN (B), respectively. Due to the limited solubility of xanthene dyes in water (generally lower than 5×10^{-3} M), the dimers are the major aggregates in 10^{-6} to $1–2 \times 10^{-3}$ M aqueous solutions (*J. Phys. Chem. B*, 2003, **107**, 13803). Thus, in (A), we could clearly observe the blue-shifted absorption peaks, which characterize the formation of the dimers. However, due to the good solubility of probe **1** in CH_3CN, the blue-shifted peaks are less obvious (B), suggesting almost no dimer appears.

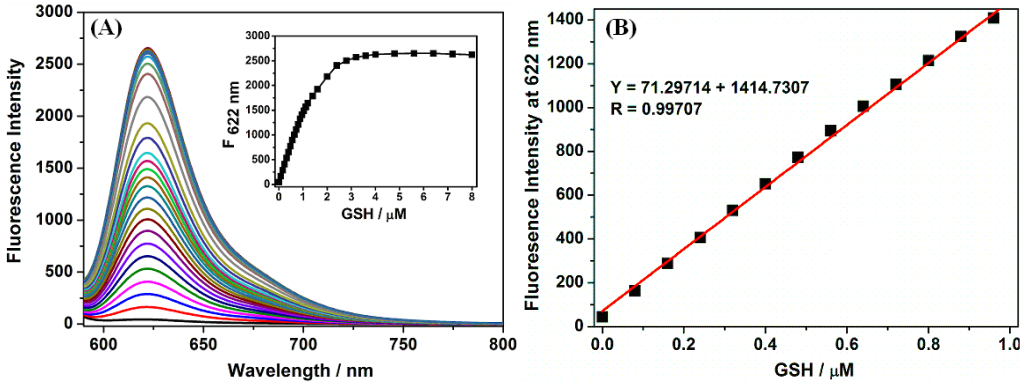


Figure S10 (A) Fluorescence spectra changes of **1** (4 μM) upon addition 0 to 2 equiv of GSH in PB buffer (10 mM, pH 7.4). (B) Fluorescence intensities of **1** at 622 nm to GSH concentration. Each spectrum was recorded after 5 min. Slits: 5/5 nm. λ_{ex} = 580 nm.

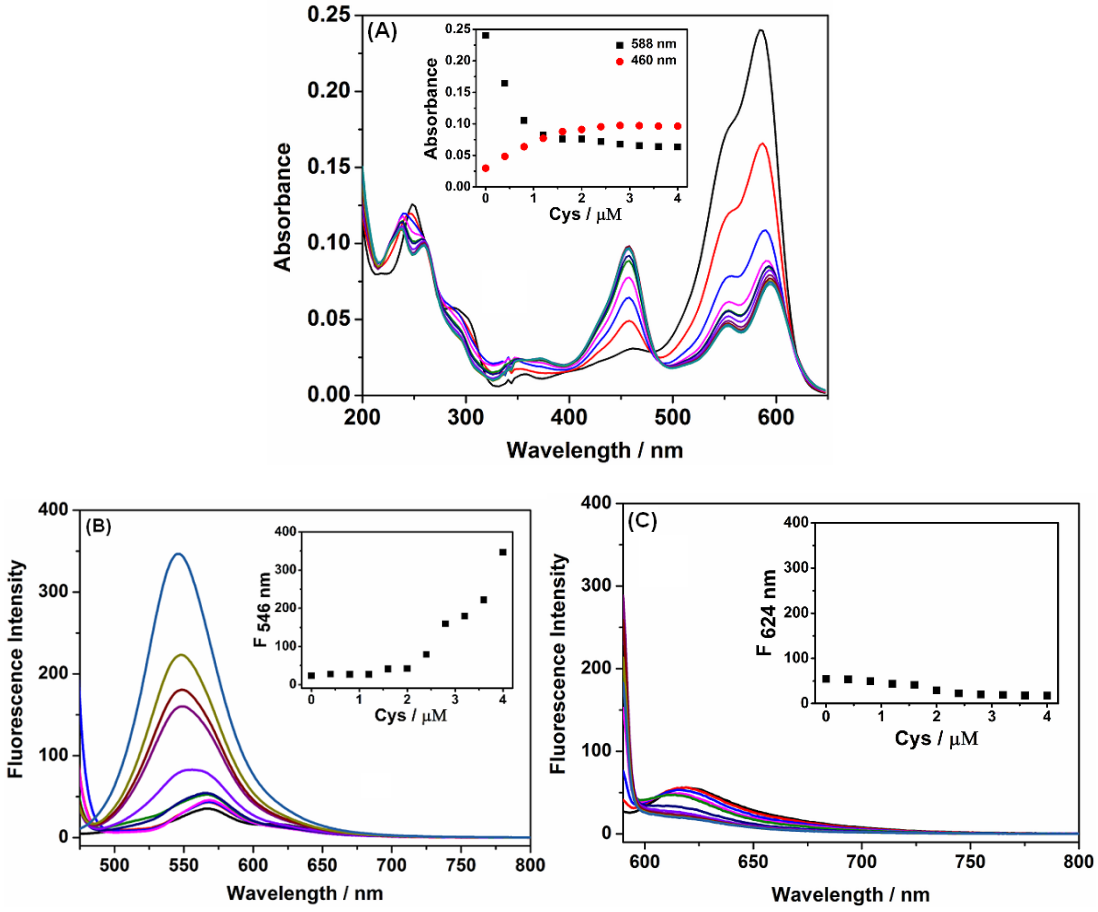


Figure S11 Absorption (A) and fluorescence (B,C) spectra of **1** (4 μM) upon addition of 0–1 equiv Cys in PB buffer (10 mM, pH 7.4) at 25 °C. Each spectrum was recorded after 20 min. λ_{ex} = 455 nm for (B). λ_{ex} = 580 nm for (C). Slits: 5/5 nm.

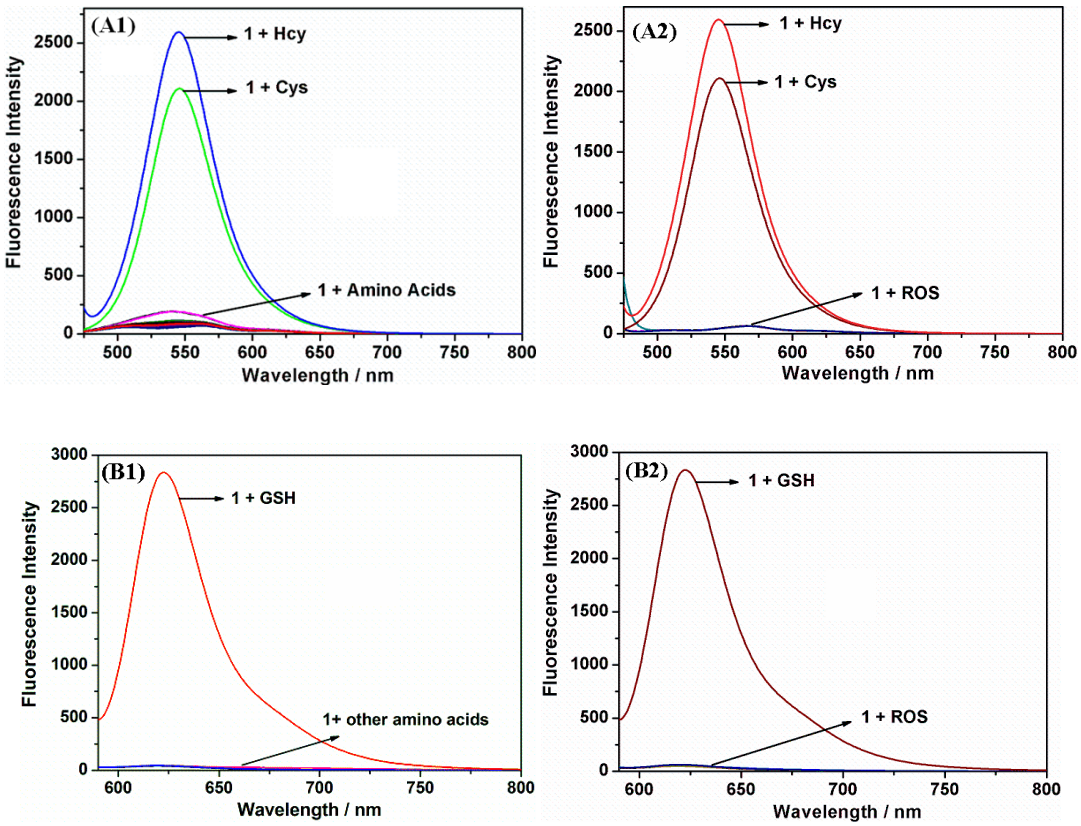


Figure S12 Fluorescence spectra of **1** (4 μM) upon addition of 15 equiv of various amino acids (A1 and B1) and biologically related ROS (A2 and B2) after 20 min in PB buffer (10 mM, pH 7.4). Amino acids: His, Glu, Asp, Val, Phe, Tyr, Ala, Ser, Leu, Arg, Pro, Thr, Gln, Try, Ile, Lys. ROS: H_2O_2, NaOCl, KO_2, $OH^{\bullet}$, $^1O_2$, NO, $NaNO_2$. **(A1 and A2) λ_{ex} = 455 nm. (B1 and B2) λ_{ex} = 580 nm.**

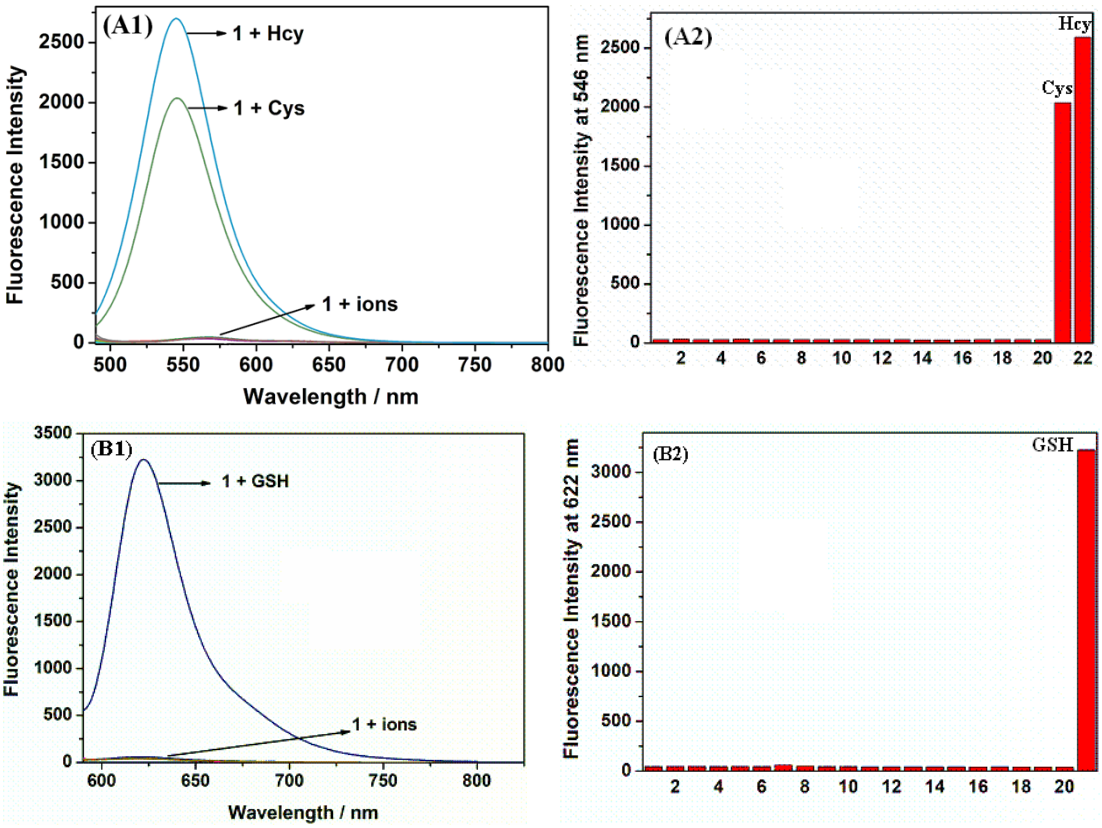


Figure S13 Fluorescence spectra (A1 and B1) and the corresponding intensities (A2 and B2) of **1** (4 μM) upon addition of 15 equiv of Cys/Hcy/GSH and 100 equiv of anions and cations after 20 min in PB buffer (10 mM, pH 7.4). (1) F^-, (2) Cl^-, (3) Br^-, (4) I^-, (5) CO_3^{2-}, (6) NO_3^-, (7) SO_4^{2-}, (8) HPO_4^{2-}, (9) $H_2PO_4^-$, (10) CH_3COO^-, (11) CN^-, (12) PPi, (13) K^+, (14) Ca^{2+}, (15) Na^+, (16) Mg^{2+}, (17) Al^{3+}, (18) Zn^{2+}, (19) Fe^{3+}, (20) Cu^{2+}. **(A1 andA2) λ_{ex} = 455 nm. (B1 and B2) λ_{ex} = 580 nm**.

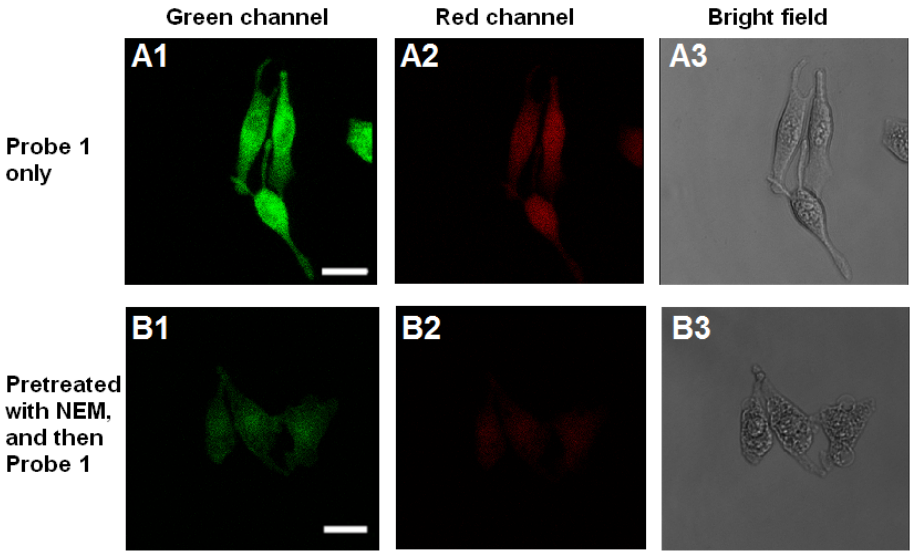


Figure S14 Imaging Cys and GSH in B16 cells using probe **1** from different emission channels. (A1–A3) B16 cells incubated with 4 μM of **1** in PBS buffer. (B1–B3) B16 cells pre-incubated with 1 mM of NEM in PBS buffer, and then treated with 4 μM of **1**. Emission was collected at 500–580 nm for green channel (excited at 458 nm), and at 610–720 nm for red channel (excited at 543 nm). Scale bar: 20 μm.

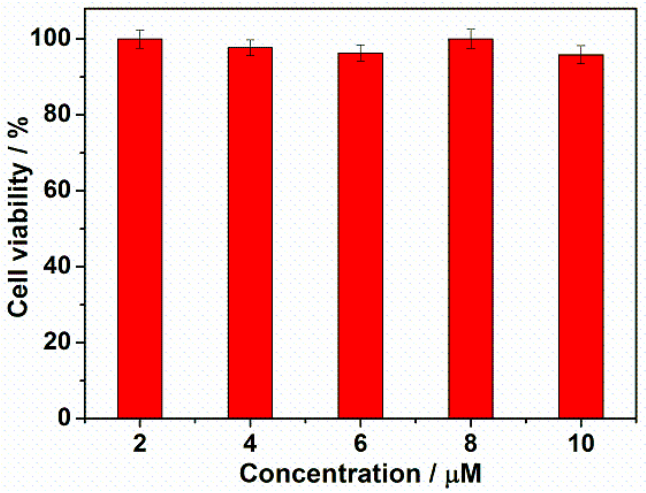


Figure S15 Percentage of viable B16 cells after treatment with indicated concentrations of **1** after 8 hours.

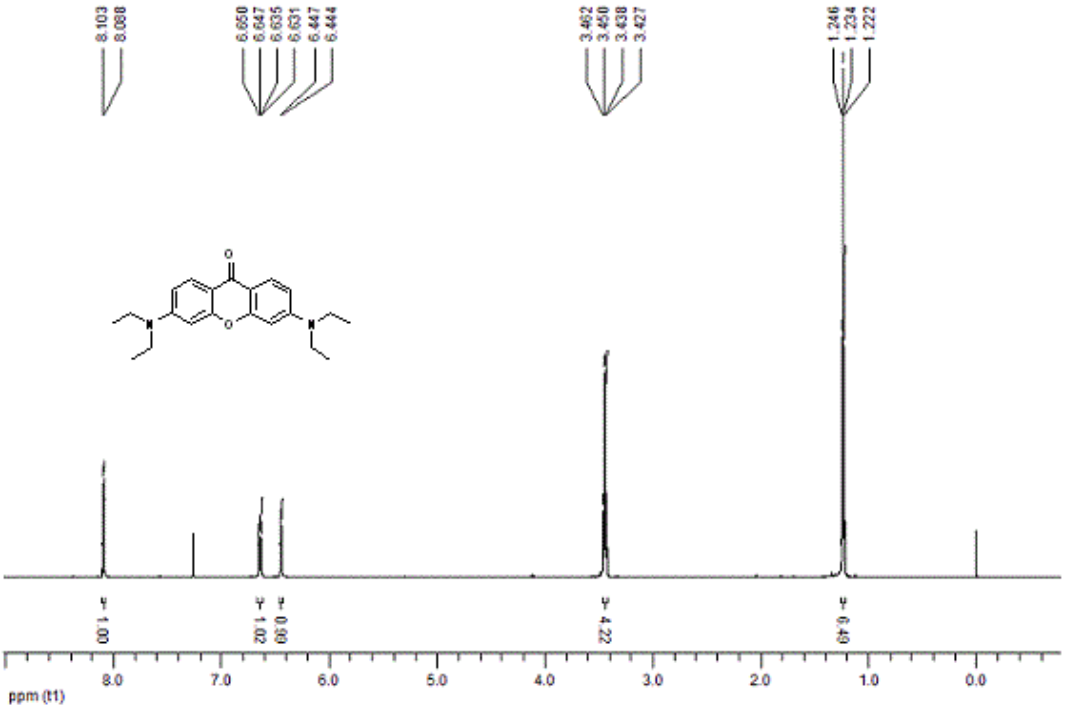


Figure S16 ^{1}H NMR chart of compound **S2** ($CDCl_3$, 600 MHz).

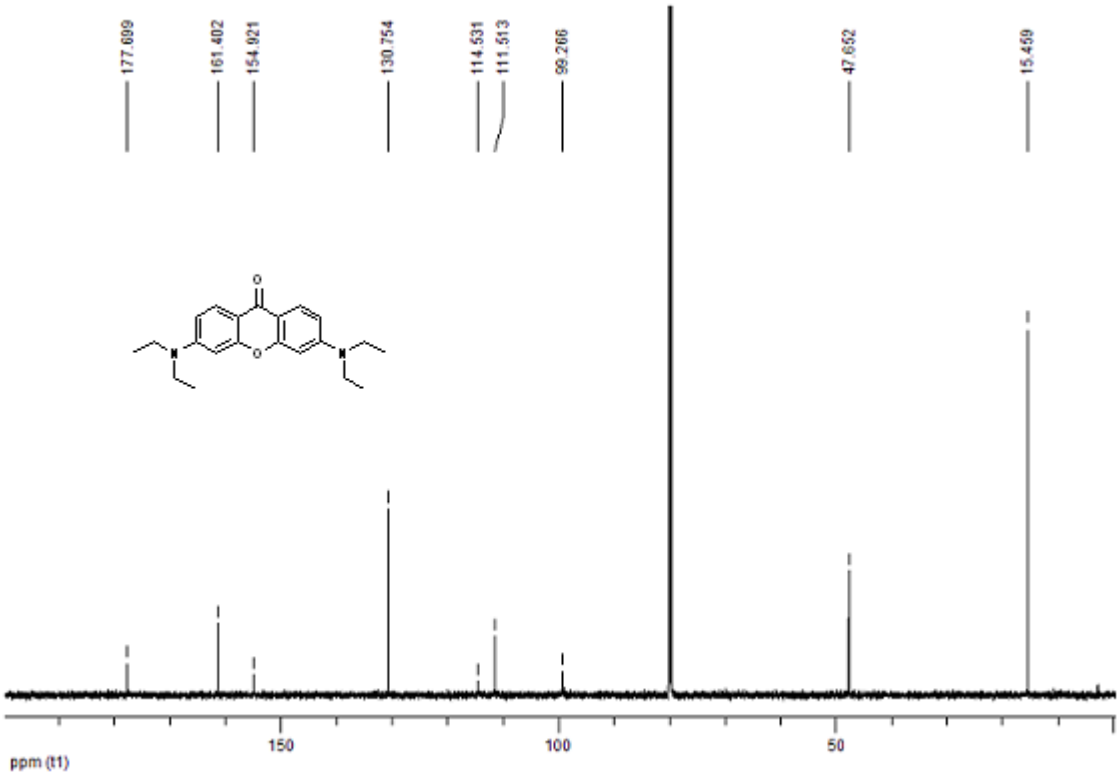


Figure S17 ^{13}C NMR chart of compound **S2** ($CDCl_3$, 150 MHz).

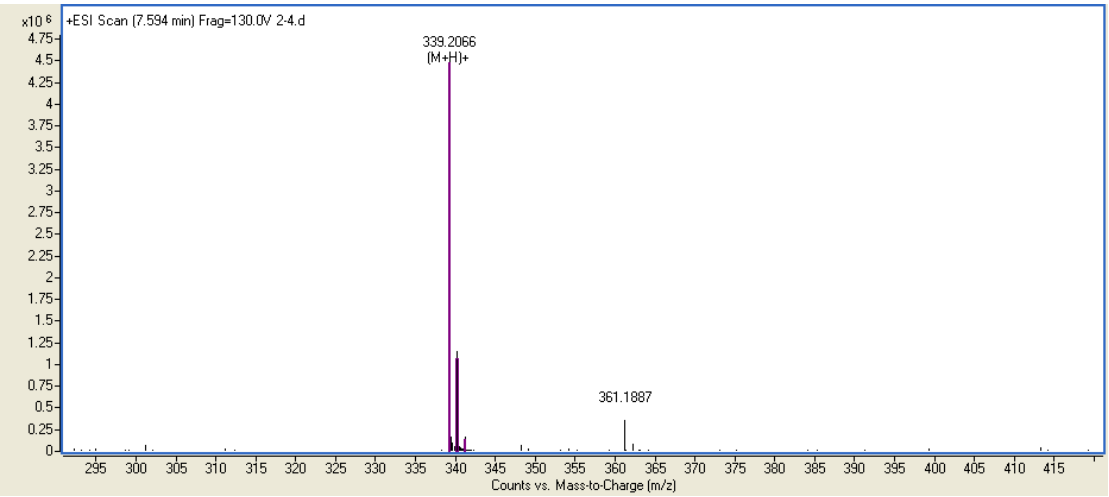


Figure S18 HRMS chart of the **S2**.

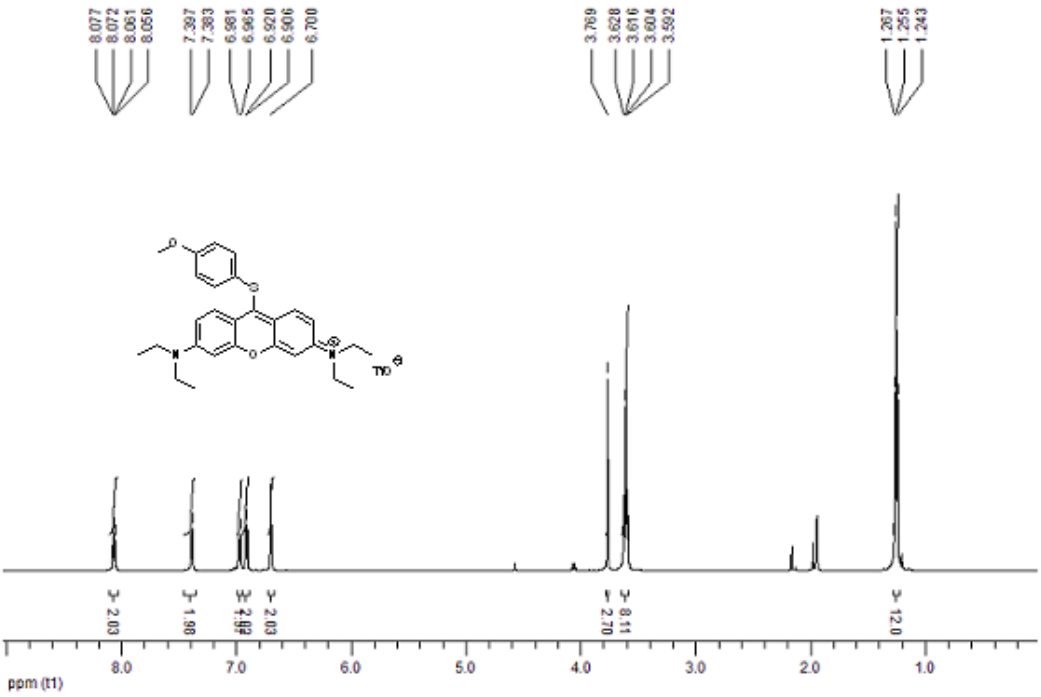


Figure S19 ^{1}H NMR chart of compound **1** (CD_3CN, 600 MHz).

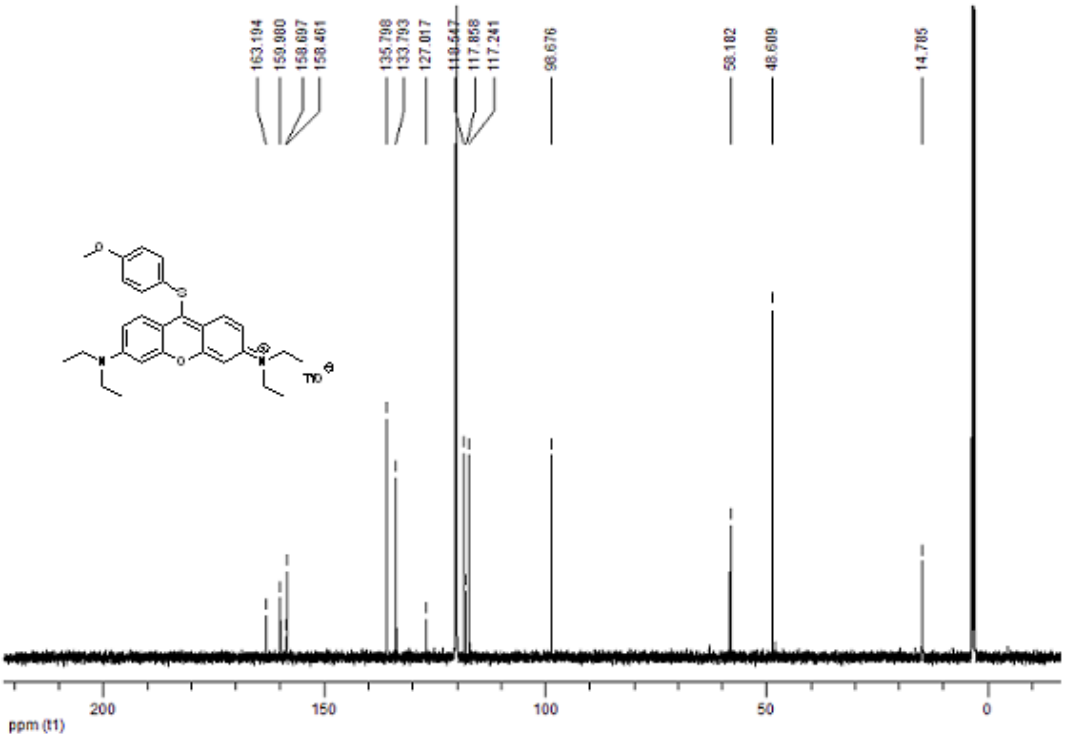


Figure S20 ^{13}C NMR chart of compound **1** (CD_3CN, 150 MHz).

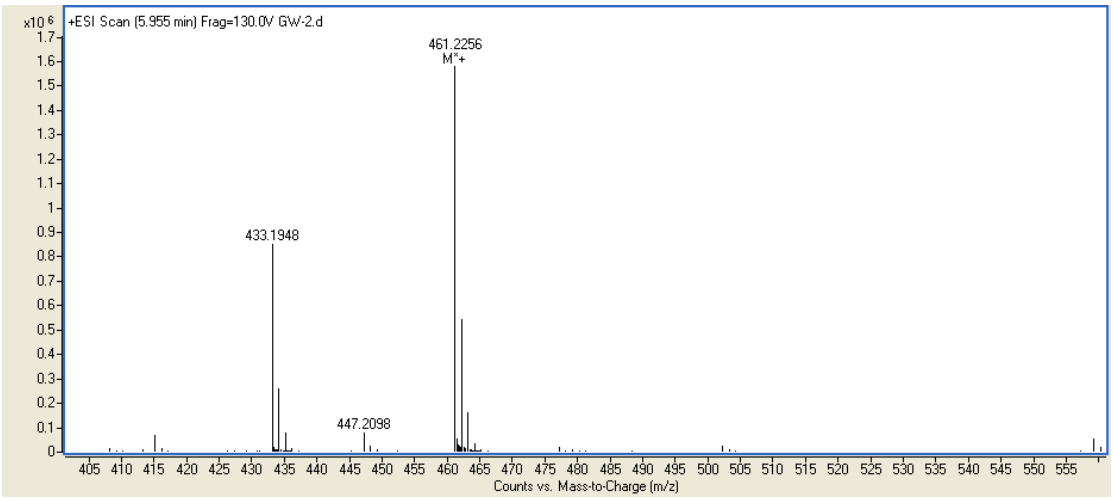


Figure S21 HRMS chart of the **1**.