Supporting Information

A Mitochondria-Targetable Fluorescent Probe for Peroxynitrite: Fast Response and High Selectivity

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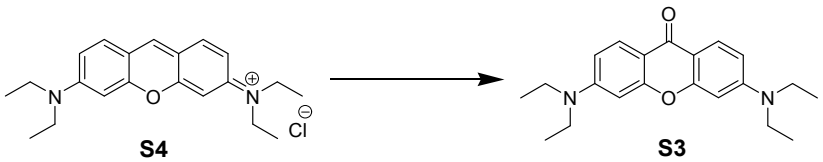
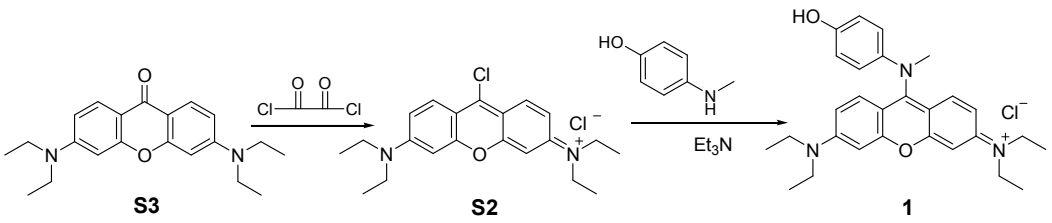
1. General methods

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 was performed using silica gel 60 (200–300 mesh). Absorption spectra were taken on a 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. Synthesis



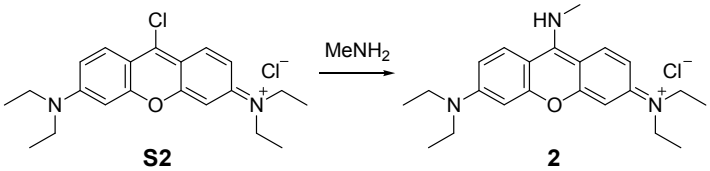
Compound S3: A mixture of **S4** (0.358 g, 1.0 mmol), potassium cyanide (0.195 g, 3.0 mmol), and water (10 mL) was refluxed for 18 h, then cooled to room temperature, filtered, and washed with water. The resulting residue was suspended in 2.0 M aq HCl (100 mL).The suspension was poured dropwise onto 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, 1H), 6.65 (dd, J_1 = 1.8 Hz, J_2= 9.0 Hz, 1H), 6.69 (s, 1H), 3.46 (q, J = 7.2 Hz, 4H), 1.25(t, J = 7.2 Hz, 6H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 175.2, 161.3, 158.5, 157.0, 155.1, 145.5, 142.6, 132.8, 132.0, 131.7, 131.0, 127.5, 120.1, 117.7, 115.1, 113.0, 110.8, 99.7, 58.2, 48.2, 39.5,15.8; ESI-MS:calcd for 339.2066, Found 339.2067.



Compound 1: Oxalyl chloride (1.2 eq.) was added to a solution of **S3** (0.338 g, 1 mmol) in dry CH_3CN (10 mL). The reaction mixture was stirred for 2 h at r.t.. After the reaction was completed, the solution was evaporated. The crude product was recrystallized from CH_3CN/ether to give the chloride species **S2** (0.152 g, Yield 38.8%). ^{1}H NMR (600 Hz, CD_3CN) δ 7.96 (s, 2H), 7.16 (d, J = 9.6 Hz, 2H), 6.73 (d, J = 2.4 Hz, 2H), 3.66 (q, J = 7.2 Hz, 8H), 1.27(t, J = 7.2 Hz, 12H).

The mixture of 4-methylaminophenol sulfate (0.344 g, 1 mmol) and Et_3N (0.242 g, 2.4 mmol) in CH_3CN (10 mL) was stirred for 30 min, then S**2** (0.196 g, 0.5 mmol) was added. The reaction mixture was further stirred for 5 min. The solvents were removed under reduced pressure and the residue was purified by flash

chromatography (CH_2Cl_2/MeOH = 15/1) to afford the pure product **1** (0.118 g, Yield 49.3%). ^{1}H NMR (600 Hz, $CDCl_3$) δ 10.41 (s, 1H), 7.46 (d, J = 9.6 Hz, 2H), 7.28 (d, J = 8.4 Hz, 2H), 7.02 (d, J = 9.0 Hz, 2H), 6.70 (dd, J_1 = 2.4 Hz, J_2= 9.6 Hz, 2H), 6.52 (d, J = 2.4 Hz, 2H), 3.95 (s, 3H), 3.52 (q, J = 7.2 Hz, 8H), 1.27(t, J = 7.2 Hz, 12H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 162.0, 161.4, 160.9, 156.1, 140.5, 133.0, 127.3, 121.2, 114.2, 110.0, 98.9, 51.7, 48.2, 15.5; ESI-MS: calcd for 444.2646, Found 444.2641.



S2 **2**

Compound 2: The mixture of S**2** (0.17 g, 0.41 mmol) in CH_3CN (10 mL) was stirred at 0 °C under N_2, then 40% methylamine solution (400 μL, 4.1 mmol) was added dropwise over 1 min. The reaction mixture was stirred for 20 min. After the solvent was evaporated, the residue was purified by silica gel column chromatography with CH_2Cl_2/MeOH = 15:1 as the eluent to give **2** (0.14 g, Yield 88.6%). ^{1}H NMR (600 Hz, DMSO-d_6) δ 10.43 (s, 1H), 8.58 (d, J = 9.0 Hz, 1H), 8.27 (d, J = 9.0 Hz, 1H), 6.89 (dd, J_1 = 2.4 Hz, J_2= 9.6 Hz, 2H), 6.65 (s, 1H), 6.54 (s, 1H), 3.58 (s, 1H), 3.52 (q, J = 7.2 Hz, 8H), 1.17(t, J = 7.2 Hz, 12H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.6, 158.2, 157.1, 155.9, 133.5, 130.1, 113.9, 113.4, 105.8, 104.4, 99.8, 98.9, 47.7, 38.3, 15.7; ESI-MS: calcd for 352.2383, Found 352.2389.

3. Preparation of the test solution

Deionized water and spectroscopic grade MeCN were used for spectroscopic studies. Superoxide solution (O_2^-) 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 *in situ* by the Fenton reaction (to generate OH•, Fe^{2+} was added in the presence of 10 eq of H_2O_2). Singlet oxygen ($^1O_2$) was generated in situ by addition of the HClO stock into a solution containing 10 eq of H_2O_2. Hypochlorite and hydrogen peroxide solution were prepared by dilution of commercial NaClO solution and H_2O_2 solution in deionized water. Nitric oxide (·NO) was generated from Sodium

Nitroferricyanide(III) Dihydrate (SNP), which was added into degassed deionized water under N_2 then stirred for 30 min at 25 °C. The aqueous solutions of $NaNO_2$ and $NaBO_3$ were freshly prepared and used as nitrite and perborate (NO_2^- and BO_3^-), respectively. Peroxynitrite solution was synthesized as reported.[1] The peroxynitrite concentration was estimated by using an extinction coefficient of 1670 $M^{-1}cm^{-1}$ at 302 nm. $C_{ONOO^-} = Abs_{302\ nm}/1.67$ (mM).

4. Cell culture and fluorescence imaging

Raw 264.7 macrophage cell line and bladder cancer cell line were 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.

Imaging exogenously added $ONOO^-$ in Raw 264.7 macrophage cells. Before the experiments, Raw 264.7 macrophage cells were washed with PBS 3 times. Then, the cells were pretreated with 5 μM **1** for 30 min, and then incubated with the $ONOO^-$ donor SIN-1 for 30 min in DMEM medium at 37 °C. To check whether probe **1** is affected by ClO^- and H_2O_2, Raw 264.7 macrophage cells were loaded with 5 μM **1** for 30 min, washed, and treated with 50 μM NaClO and H_2O_2, respectively, in DMEM medium at 37 °C.

Imaging endogenously generated $ONOO^-$ in Raw 264.7 macrophage cells. Before the experiments, the cells were washed with PBS 3 times. The cells were pretreated with 1 μg/mL LPS and 50 ng/mL INF-γ for 4 h and then incubated with probe **1** (5 μM) for 30 min at 37 °C. After that, the cells were washed with PBS 3 times. For inhibition assays, the cells were activated with LPS (1 μg/mL) and INF-γ (50 ng/mL) in the presence of AG (5 mM) or TEMPO (300 μM) for 4 h and then loaded with 5

μM probe **1** for 30 min. Emission was collected at 500–600 nm for green channel (excited at 405 nm).

*Fluorescence confocal images of bladder cancer cells costained by **1**, $ONOO^-$ donor SIN-1, and then MitoTracker Red FM or LysoTracker Red DND-99 or ER-Tracker Red.* To confirm that the probe could specifically stain the mitochondria, bladder cancer cells were incubated in a sequence with **1** (2 μM, 20 min), SIN-1 (100 μM, 20 min), and then MitoTracker Red FM (0.2 μM, 30 min) or LysoTracker Red DND-99 (50 nM, 30 min) or ER-Tracker Red (1.0 μM, 30 min) in DMEM medium at 37 °C. After each treatment, the cells were washed with PBS 3 times.

5. Cytotoxicity assays

Raw 264.7 macrophage 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 **1**. The final concentrations of **1** was kept from 0 to 8 μM. The cells were then incubated at 37 ºC in an atmosphere of 5% CO_2 and 95% air for 9 h, followed by MTT assays (n = 6). Untreated assay with RPMI 1640 medium (n = 6) was also conducted under the same conditions.

6. Supplementary Data

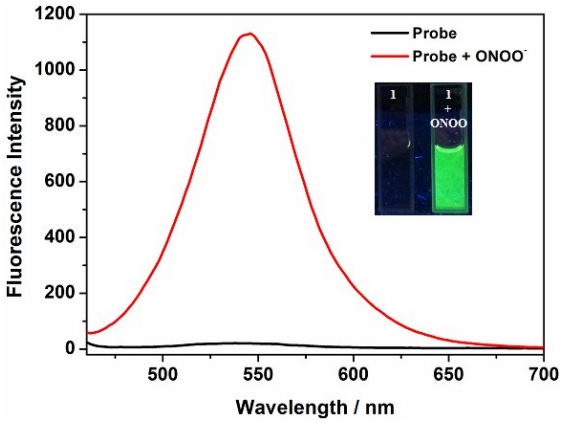


Figure S1. Fluorescence spectra of **1** in the absence and presence of 8 μM $ONOO^-$ in PB buffer (100 mM, pH 7.4) at 25 °C.

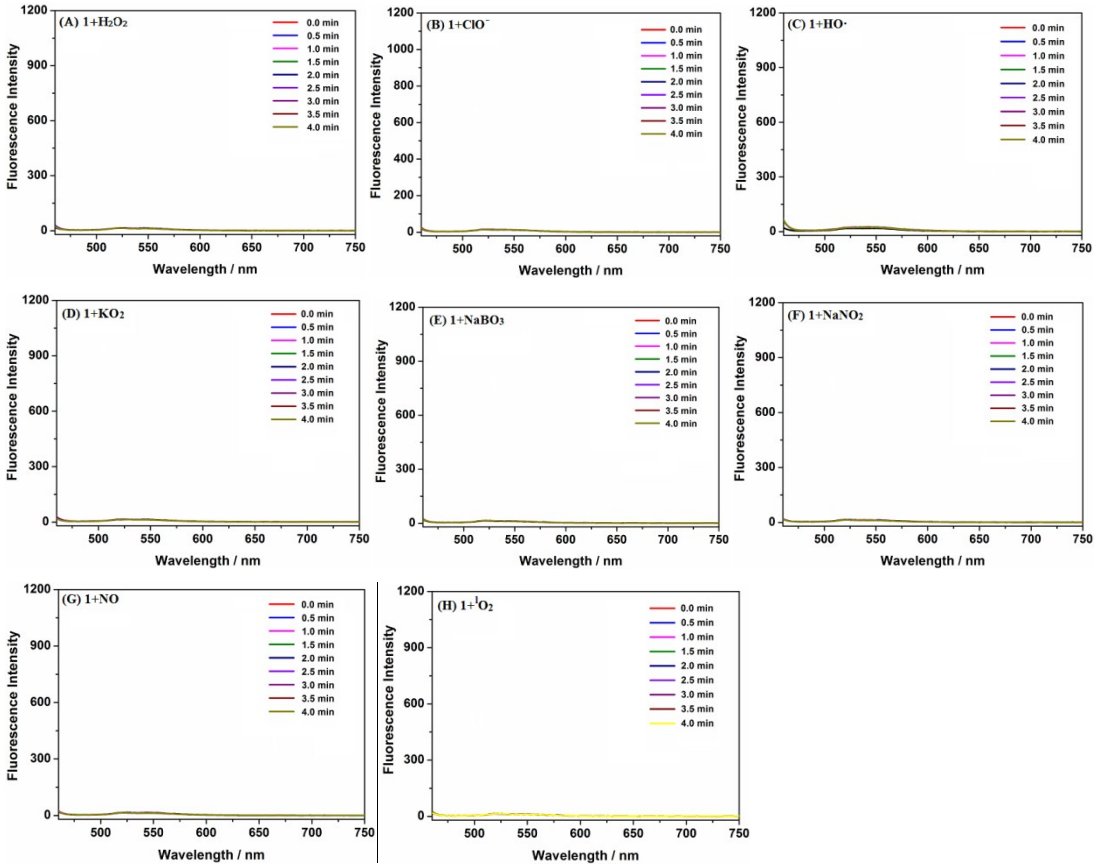


Figure S2. The time-dependent fluorescence spectra changes of **1** (1 μM) upon treatment with **10** equiv of various ROS, including (A) H_2O_2; (B) ClO^-; (C) •OH; (D)

$O_2^{\bullet-}$; (E) BO_3^-; (F) NO_2; (G) NO, and (H) $^1O_2$, in PB buffer (100 mM, pH 7.4) at 25 ^oC. λ_{ex} = 440 nm. Slits: 5/10 nm, voltage: 600 V.

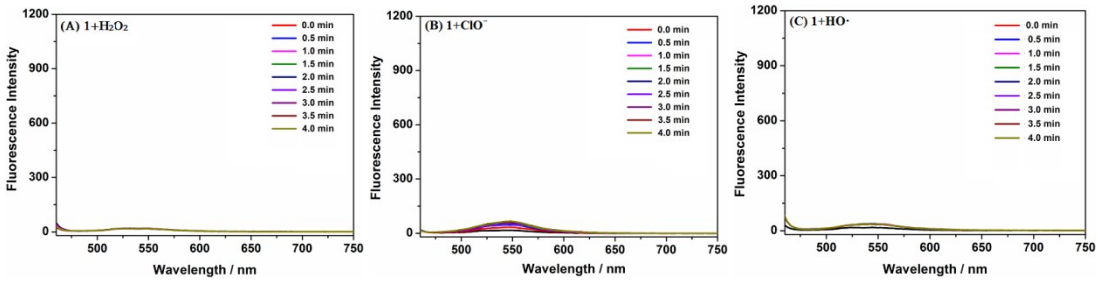


Figure S3. The time-dependent fluorescence spectra changes of **1** (1 μM) upon treatment with **100** equiv of various ROS, including (A) H_2O_2; (B) ClO^-; and (C) $^{\bullet}OH$, in PB buffer (100 mM, pH 7.4) at 25 ^oC. λ_{ex} = 440 nm. Slits: 5/10 nm, voltage: 600 V.

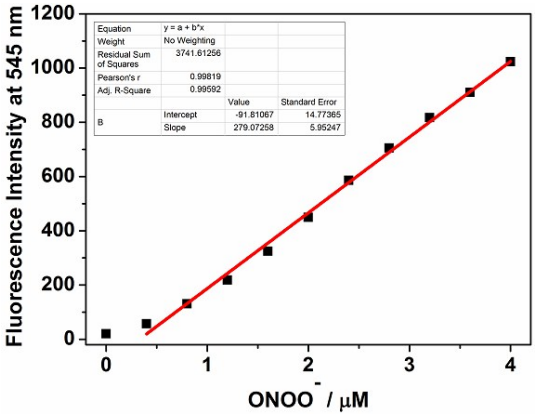


Figure S4. The corresponding linear relationship between the fluorescent intensity and $ONOO^-$ concentrations in PB buffer (100 mM, pH 7.4) at 25 ^oC.

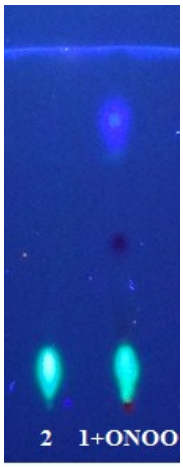


Figure S5. TLC analyst of probe **1** treated with $ONOO^-$ vs that of product **2** (CH_2Cl_2:EtoAc:MeOH = 5:5:1).

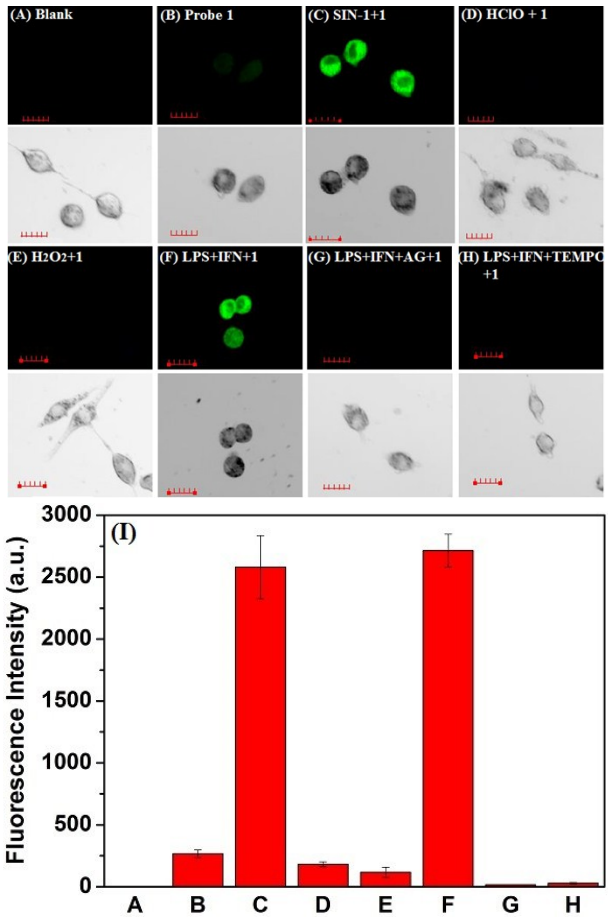


Figure S6. Fluorescence images of **1**-loaded RAW264.7 murine macrophages under different conditions. (A) Blank; (B) cells were treated with **1** (5 μM, 30 min) and then imaged; (C) **1**-loaded cells were treated with SIN-1 (1 mM, 30 min) and then imaged; (D) **1**-loaded cells were treated with H_2O_2 (50 μM, 30 min) and then imaged; (E) **1**-loaded cells were treated with NaClO (50 μM, 30 min) and then imaged; (F) cells were stimulated with LPS (1 μg/mL) and IFN-γ (50 ng/mL) for 4 h and then with **1** (5 μM, 30 min); (G) NOS inhibitor AG (5 mM) was co-incubated during LPS (1 μg/mL)/IFN-γ (50 ng/mL) stimulation and then with **1** (5 μM, 30 min); (H) superoxide inhibitor TEMPO (300 μM) was co-incubated during LPS/IFN-γ stimulation and then with **1** (5 μM, 30 min); (I) Quantification of fluorescence intensities of confocal microscopy images of RAW264.7 murine macrophages obtained under conditions A–H. Values are the mean intensities from the intensity

from six randomly selected fields. Image from band path of 500-600 nm upon excitation of **1** at 405 nm. Scale bar: 20 μm.

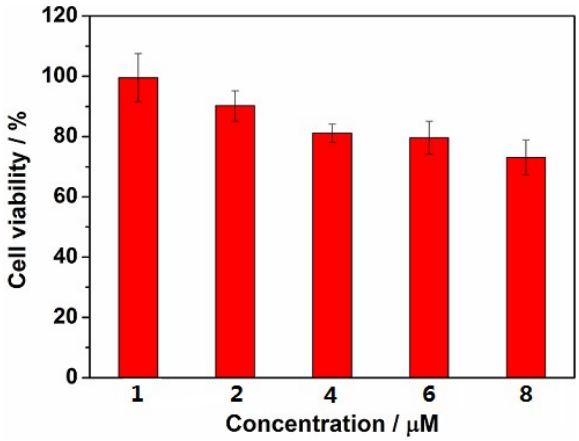


Figure S7. Percentage of viable Raw 264.7 macrophage cells after treatment with indicated concentrations of **1** after 9 hours.

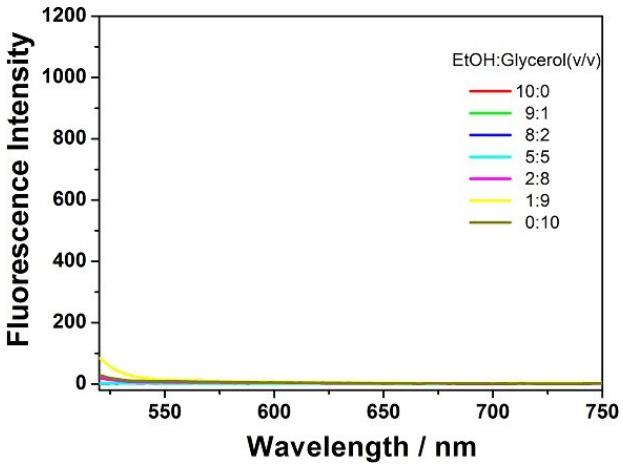


Figure S8. Changes of fluorescent spectra of probe **1** (1 μM) with the variation of solution viscosity (EtOH-glycerol system).

7. ^{1}H NMR, ^{13}C NMR, and HRMS charts of 1 and 2.

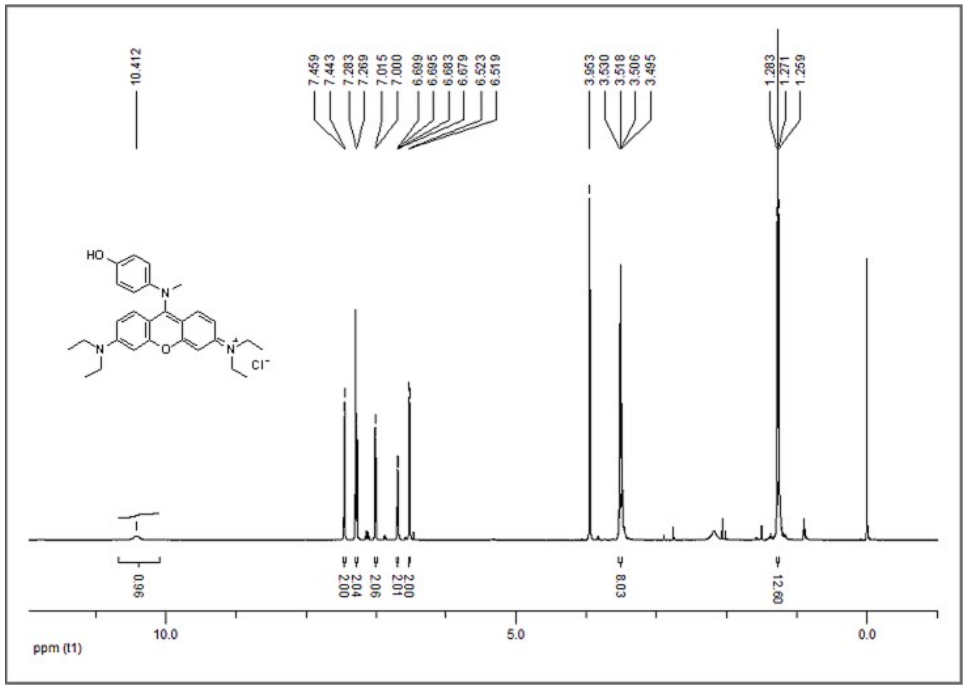


Figure S9 ^{1}H NMR chart of compound **1** (600 MHz, $CDCl_3$).

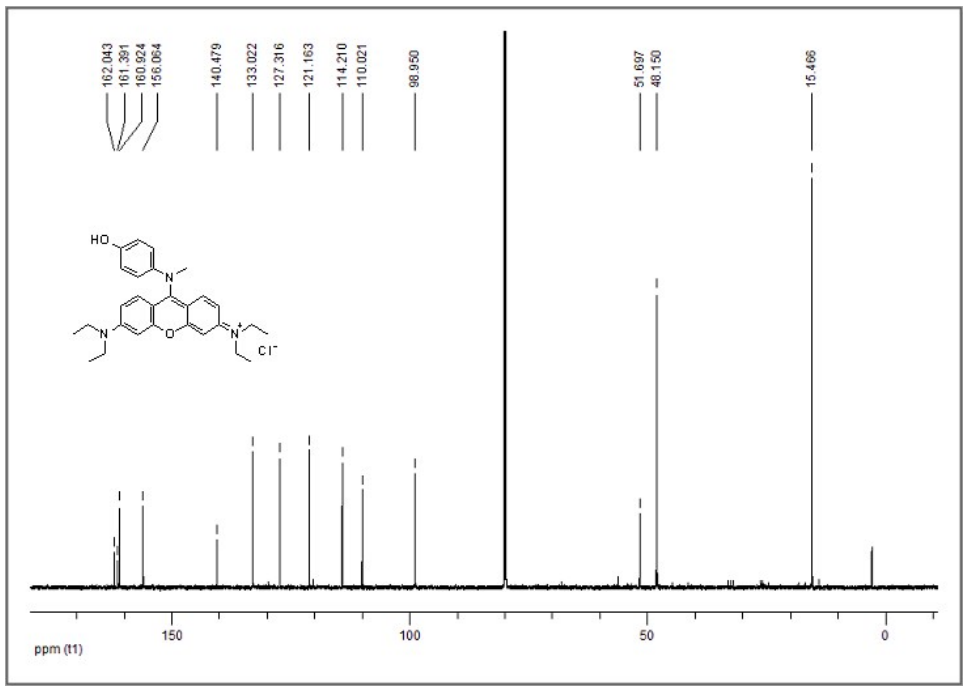


Figure S10 ^{13}C NMR chart ofcompound **1** (150MHz, $CDCl_3$).

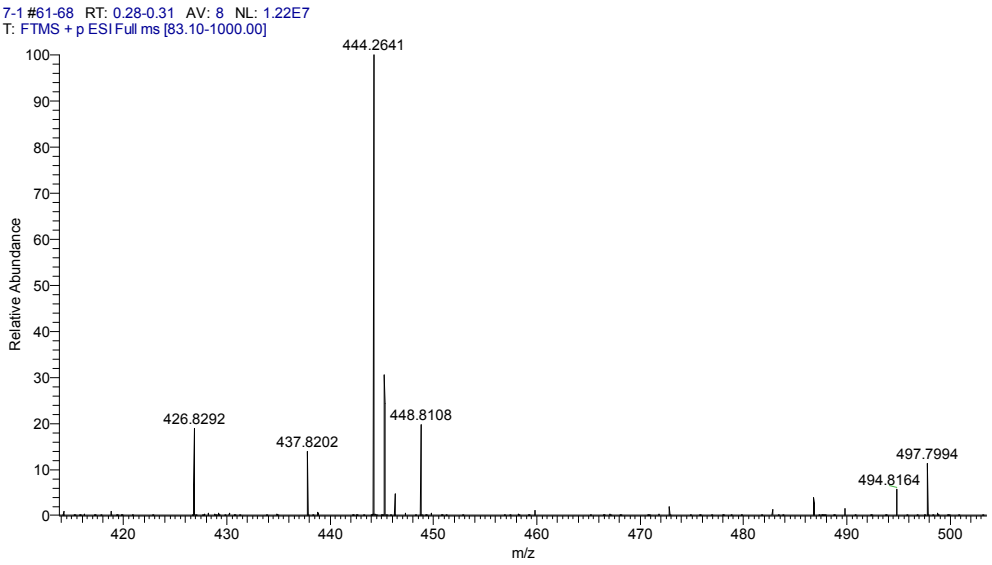


Figure S11 HRMS chart of compound **1**.

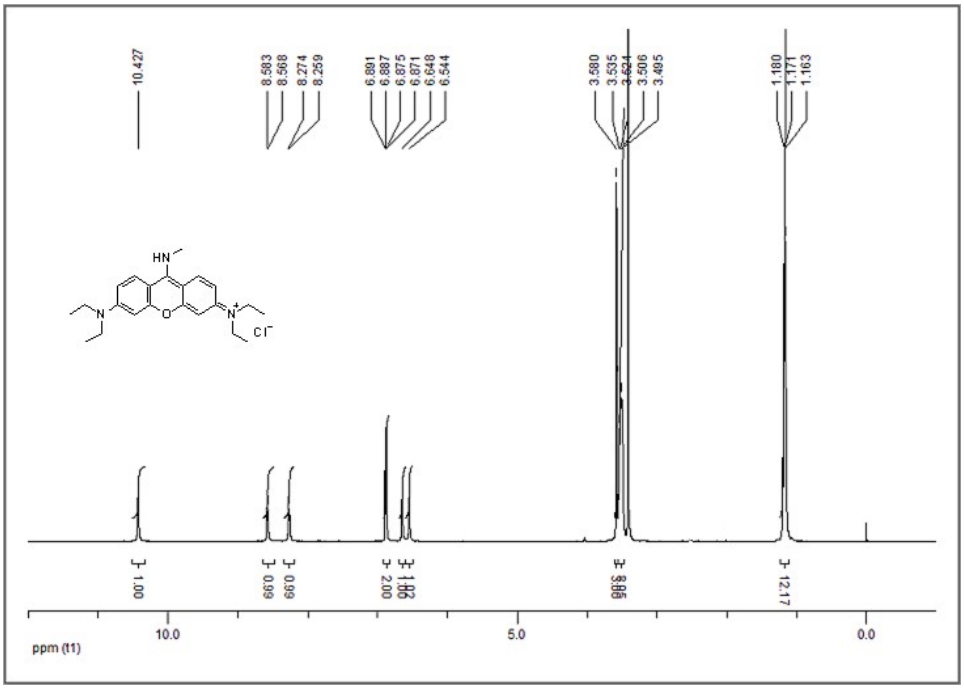


Figure S12 ^{1}H NMR chart of compound **2** (600 MHz, DMSO-d_6).

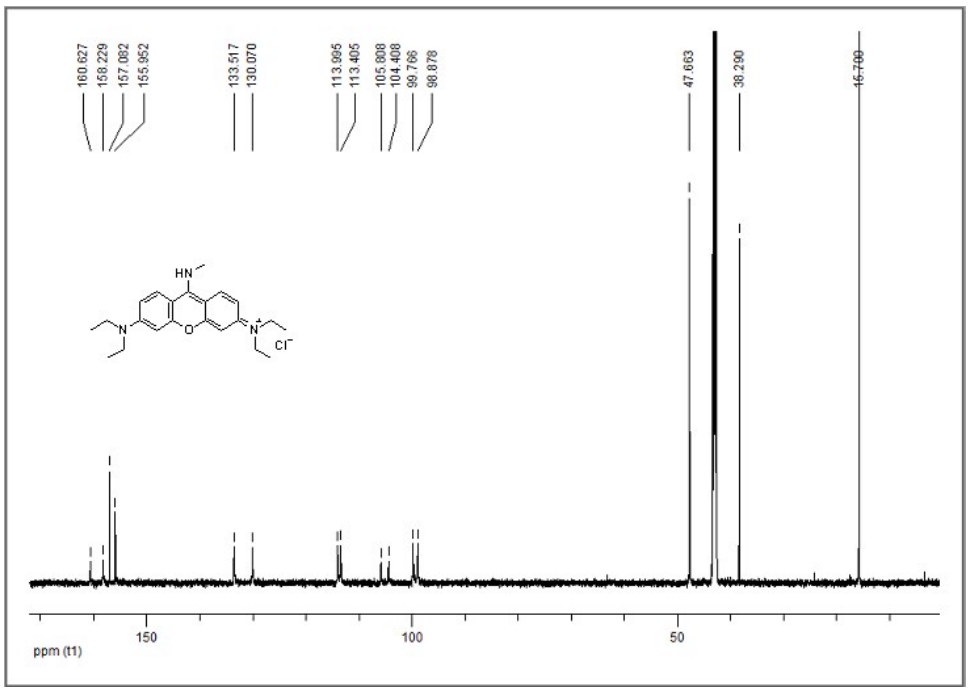


Figure S13 ^{13}C NMR chart ofcompound **2** (150MHz, DMSO-d_6).

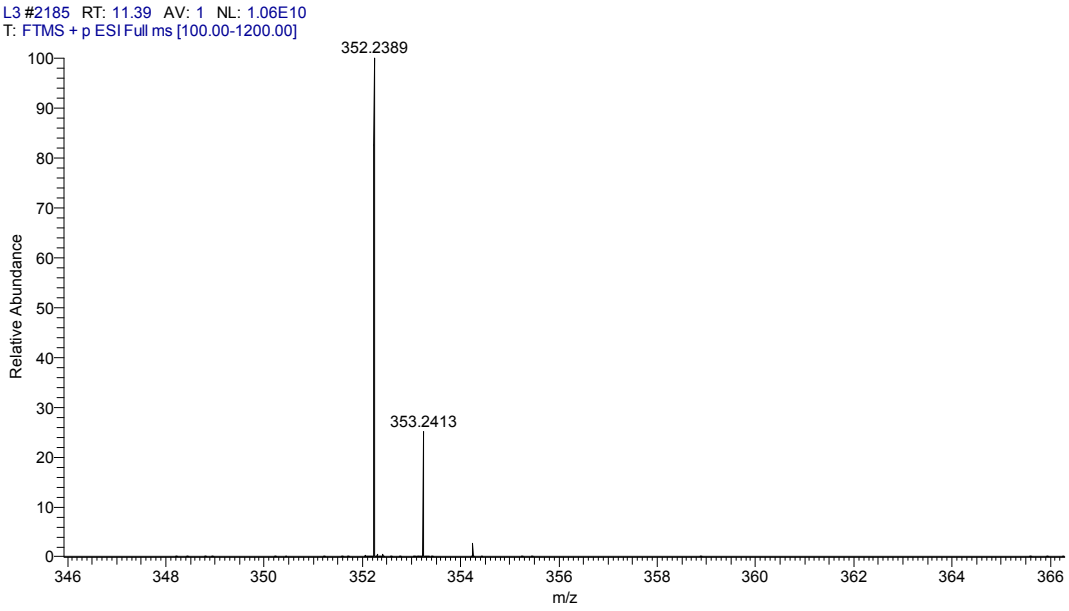


Figure S14 HRMS chart of compound **2**.

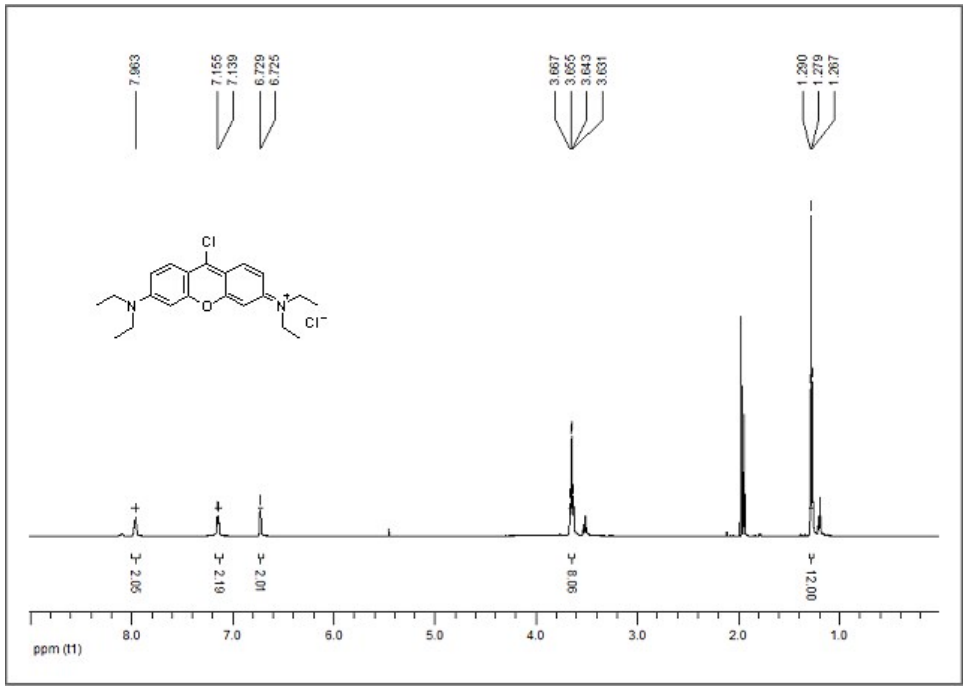


Figure S15 ^{1}H NMR chart of **S2** (600 MHz, CD_3CN).

8. Reference

1. R. M. Uppu, W. A. Pryor, *Anal. Biochem.*, 1996, **236**, 242.