Supporting Information

Dual-Channel Fluorescence Diagnosis of Cancer Cells/Tissues Assisted by OATP Transporters and Cysteine/Glutathione

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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 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. Live cell and tissue fluorescence images were acquired by Zeiss LSM 880+Airyscan Laser Scanning Confocal Microscope with a 60×oil–immersion objective lens.

2. Preparation of the test solution for evaluating the spectra properties of POP for Cys and GSH. A stock solution of **POP** (2 mM) was prepared in CH_3CN. The stock solution of **POP** was then diluted to the corresponding concentrations (2 μM or

4 μM) with PBS (10 mM, pH 7.4). The solutions of cysteine (Cys), reduced glutathione (GSH), DTT, Vc, NaHS, and various amino acids were prepared in deionized water. $ONOO^-$ was synthesized according to a reported procedure,[1] and its concentration was determined using an extinction coefficient of 1670 $M^{-1}cm^{-1}$ at 302 nm. $O_2^{\bullet-}$ was prepared by adding KO_2 (7.1 mg) and 18-Crown-6 (1 equiv) to dry dimethyl sulfoxide (5 mL) and stirring vigorously for 10 min. $^{\bullet}OH$ was generated in situ by the Fenton reaction (Fe^{2+} + 10 equiv H_2O_2), and its concentration was equal to the Fe^{2+} concentration. $^1O_2$ was generated in situ by adding NaClO solution into H_2O_2 solution (10 equiv), and its concentration was equal to the NaClO concentration. NO was generated from a commercially available NO donor NOC-9 (dissolved in 0.1 M NaOH solution). Nitroxyl (HNO) was generated from Angeli's salt. H_2O_2 solution was prepared by dilution of commercial H_2O_2 solution in deionized water, and its concentration was determined by using an extinction coefficient of 43.6 $M^{-1}cm^{-1}$ at 240 nm. ClO^- solution was prepared by the dilution of commercial NaClO solution in deionized water, and its concentration was determined using an extinction coefficient of 350 $M^{-1}cm^{-1}$ at 292 nm. The aqueous solutions of metal ions were freshly prepared from their chloride salts. For spectra studies, various analytes, except $^{\bullet}OH$ and $^1O_2$, were directly added to the solution of **POP** (2 μM) in PBS (10 mM, pH 7.4) at 37 °C, and then fluorescence spectra were recorded in the indicated time points. For $^{\bullet}OH$ or $^1O_2$, **POP** and H_2O_2 were premixed, and Fe^{2+} or NaClO were then added to the mixture, respectively.

3. Cell culture and fluorescence imaging. All of cell lines were kindly provided by Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education (China). HeLa Cells, A549 Cells, HepG2 Cells, HUVECs Cells, RASMC Cells, SHEE Cells, MSC Cells, and COS-7 Cells were grown in DMEM medium, and MCF-7 Cells, SMMC-7721 Cells, and BRL-3A Cells were grown in RPMI 1640 medium, both of which were supplemented with 10 % FBS (Fetal Bovine Serum) and 1% antibiotics at 37 ºC in humidified environment of 5% CO_2. Cells were plated on glass bottom cell culture dish (30 mm) and allowed to adhere for 12 hours. Before

experiments, cells were washed with PBS 3 times. Then, the cells were treated with **POP** (4 μM) in PBS for 30 min. After washed with PBS 3 times, the fluorescence imaging assays were performed.

4. Imaging cancer and normal tissue cryosections. Tumor-bearing mice were prepared by subcutaneous injection of A549 or HepG2 cells into the left axillae of the nude mice. After 14 days of tumor inoculation, the tumor-bearing nude mice were sacrificed after anesthesia, and various tissues, including tumor, heart, liver, spleen, lung, and kidney, were cryo-sectioned as 20 μm thickness. The harvested surgical specimens of patients, including human lung, thyroid, and ovarian cancer tissues as well as human ovarian and cervical normal tissues determined by doctors, were cryo-sectioned as 5 μm thickness. After washed with PBS three times, these slices were treated with **POP** (4 μM) for 20 min in PBS, followed by imaging under confocal microscopy. Note that, before **POP** treatment, these slices must be washed three times with PBS; otherwise, some background fluorescence caused by Cys/GSH in blood was also observed in normal tissues.

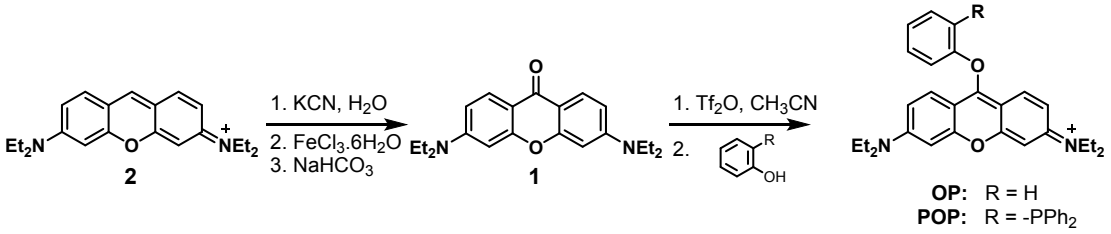
5. Cyotoxicity assays. The CCK-8 cell proliferation assay was applied to determine the viabilities of the cells treated with **POP**. HeLa cell suspensions with a density of 2.0×10^3 cells/well in 100 μL cell culture medium were added to 96-well plates. After cell attachment, the cells were treated by **POP** with different concentrations (0–10 μM). After another 24 h incubation, cells were washed twice with PBS and incubated with fresh medium containing 10 μL CCK-8. The absorbance at 570/492 nm was measured by *Synergy™ Mx Multi-Mode Microplate Reader*. Cell viability (%) = $(A_{\text{with probe}} - A_{\text{blank}} / A_{\text{control}} - A_{\text{blank}}) \times 100\%$.

6. Quantum yield determination. Fluorescence quantum yields of **POP**, **NP** (**POP** + 100 equiv of Cys), and **SP** (**POP** + 10 equiv of GSH) in PBS were determined with rhodamine B (Φ = 0.7, MeOH), coumarin 6 (Φ = 0.78, EtOH), and Gresyl violet perchlorate (Φ = 0.58, EtOH) as references, respectively. The quantum yield was calculated using Eq.1:

$$\Phi_u = [(A_s F A_u \eta^2)/ (A_u F A_s \eta_0^2)]\Phi s. \qquad (Eq.1)$$

Where A_s and A_u are the absorbance of the reference and sample solution at the reference excitation wavelength, FA_s and FA_u are the corresponding integrated fluorescence intensity, and η and η_0 are the solvent refractive indexes of sample and reference, respectively. Absorbance of sample and reference at their respective excitation wavelengths was controlled to be lower than 0.05. In this case, fluorescence quantum yields of **POP**, **NP**, and **SP** were determined to be 0.094 (λ_{ex} = 520 nm), 0.111 (λ_{ex} = 450 nm), and 0.299 (λ_{ex} = 580 nm), respectively.

7. Synthesis of POP



A mixture of pyronin **2** (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 intermediate xanthone **1** as 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 177.099, 161.402, 154.921, 130.754, 114.531, 111.513, 99.266, 47.652, 15.459; ESI-MS: $[M+H]^+$ calcd for 339.2066, Found 339.2067.

The mixture of **1** (0.1 g, 0.3 mmol) in CH_3CN (10 mL) was stirred at 0 °C under N_2 for 10 min, and then Tf_2O (200 μL, 1.2 mmol) was added dropwise over 1 min. The reaction mixture was stirred for 10 min, and then phenol derivative (3 mmol) was added. The mixture was further stirred at 25 °C for 2 h. The solvents were removed under reduced pressure, and the residue was purified by flash chromatography ($MeOH/CH_2Cl_2$ = 20:1) to afford the desired product **OP** or **POP**.

OP: 73.2% yield. ^{1}H NMR (600 Hz, $CDCl_3$) δ 7.57 (d, J = 9.0 Hz, 2H), 7.27 (d, J = 8.4 Hz, 2H), 7.22 (d, J = 8.4 Hz, 2H), 6.91 (dd, J1 = 2.4 Hz, J2= 9.6 Hz, 3H), 6.77 (d, J = 2.4 Hz, 2H), 3.64 (q, J = 7.2 Hz, 8H), 1.35(t, J = 7.2 Hz, 12H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.5, 159.4, 158.2, 158.1, 155.4, 155.3, 132.9, 131.4, 121.6, 116.6, 113.7, 113.3, 96.1, 95.8, 45.9, 12.6; ESI-MS: [M^+] calcd for 415.2380, Found 415.2379.

POP: 64.4% yield. ^{1}H NMR (600 Hz, $CDCl_3$) δ 7.69 (m, 1H), 7.59 (m, 1H), 7.49 (m, 2H), 7.42(m, 8H), 7.34 (t, J = 7.2 Hz, 1H), 7.19 (t, J = 7.2 Hz, 1H), 7.15 (d, J = 9.6 Hz, 1H), 7.00 (m, 1H), 6.76 (s, 1H), 6.65 (m, 2H), 3.59 (q, J = 7.2 Hz, 8H), 1.27 (t, J = 7.2 Hz, 12H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 162.6, 158.3, 137.4, 137.2, 137.0, 136.9, 135.4, 134.9, 134.8, 134.1, 132.4, 131.8, 131.7, 131.6, 131.5, 130.6, 128.7, 122.0, 121.9, 121.5, 121.4, 121.4, 119.2, 116.3, 109.4, 99.5, 48.9, 15.4; ESI-MS: [M^+] calcd for 599.2822, Found 599.2822.

8. Supplementary Spectra and Imaging data

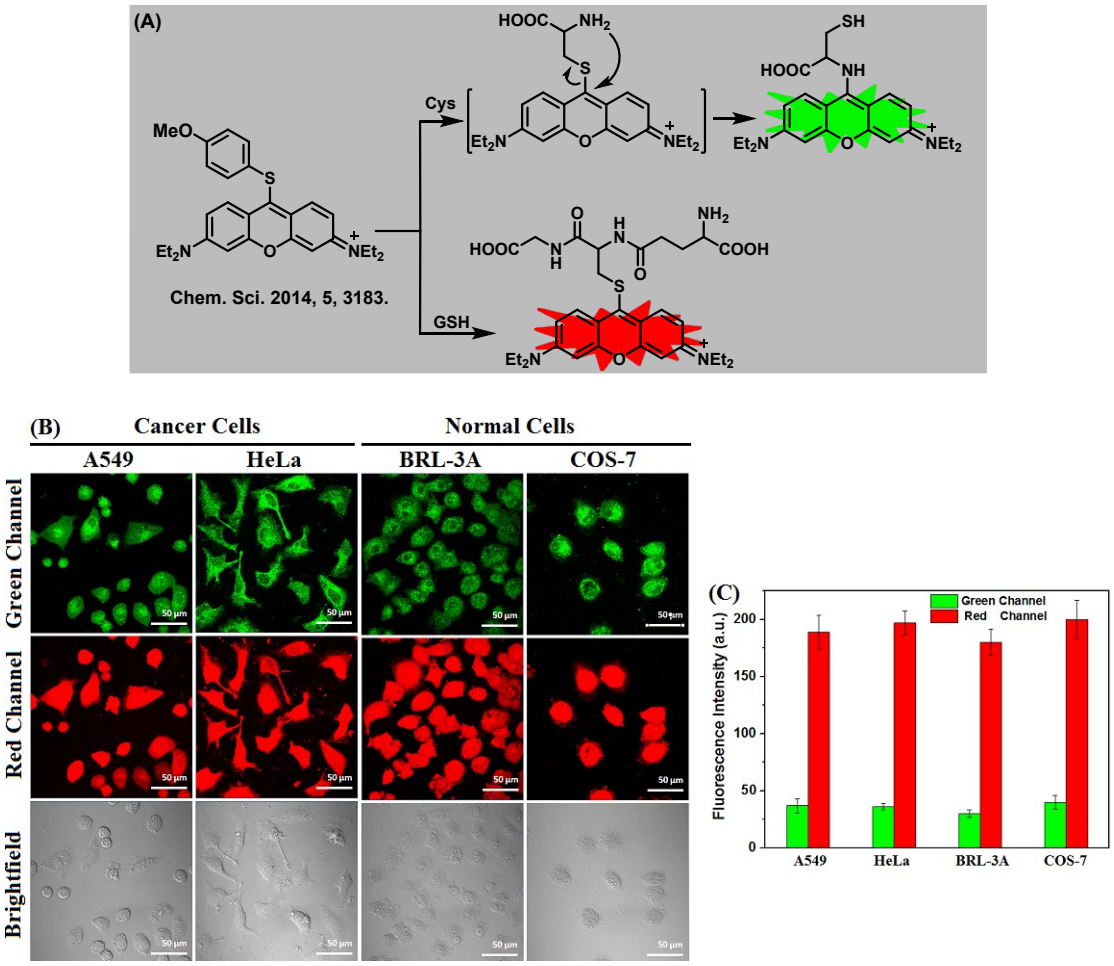


Figure S1. (A) Sensing Mechanisms of the 4-methoxythiophenol-functionalized pyronin dye for Cys and GSH, respectively, reported by us previously.[2] (B) Confocal fluorescence images of the cancer cells and normal cells treated with the pyronin dye (4 μM, 30 min) in PBS. (C) Average fluorescence intensity from images of (B). The results indicate that the dye does not differentiate cancer cells and normal cells due to its good permeability toward both cancer cells and normal cells as well as its high reactivity toward Cys and GSH. For green channel, the emission was collected at 500–540 nm (λ_{ex} = 488 nm); for red channel, the emission was collected at 650–700 nm (λ_{ex}: 561 nm). Scale bar: 50 μm.

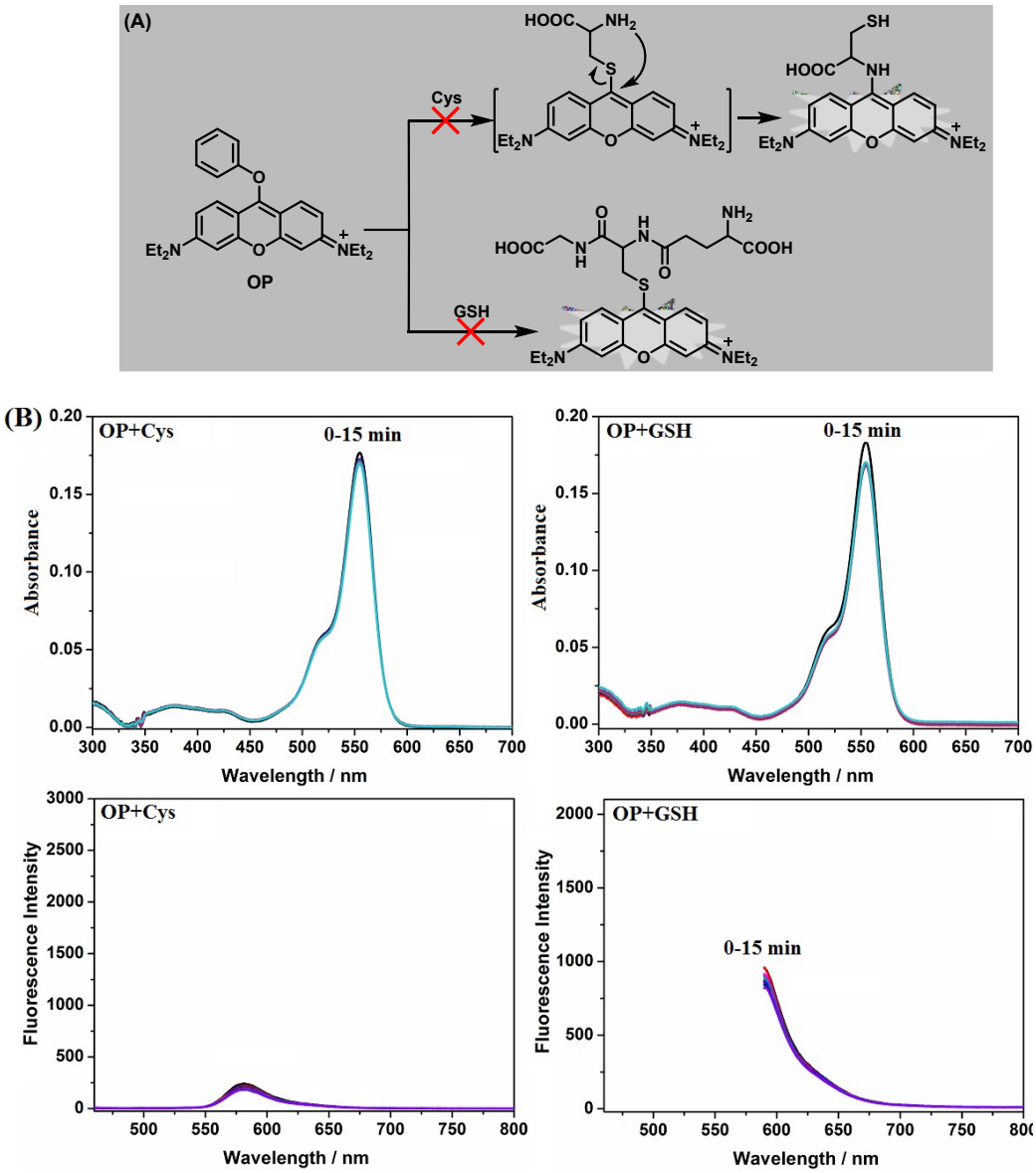


Figure S2. The phenol-functionalized pyronin **OP** does not undergo the Cys-induced substitution–rearrangement reaction and GSH-induced substitution reaction (A), as indicated by the time-dependent absorption and fluorescence spectra of **OP** (2 μM) treated with Cys (500 μM) and GSH (500 μM), respectively, in PBS (B) at 37 °C. λ_{ex} = 450 nm for **OP** treated with Cys; λ_{ex} = 580 nm for **OP** treated with GSH. Slits: 5/5 nm.

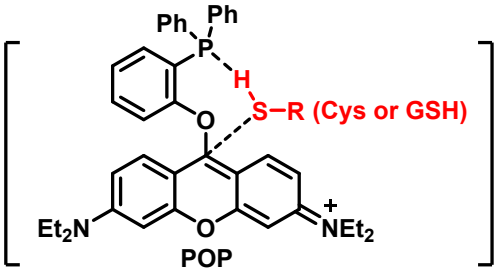


Figure S3. Schematic illustration of the poorly basic triphenylphosphine group-promoted S_NAr substitution reaction of **POP** with Cys or GSH by the synergetic deprotonation of their –SH group in the proposed reaction transition state.

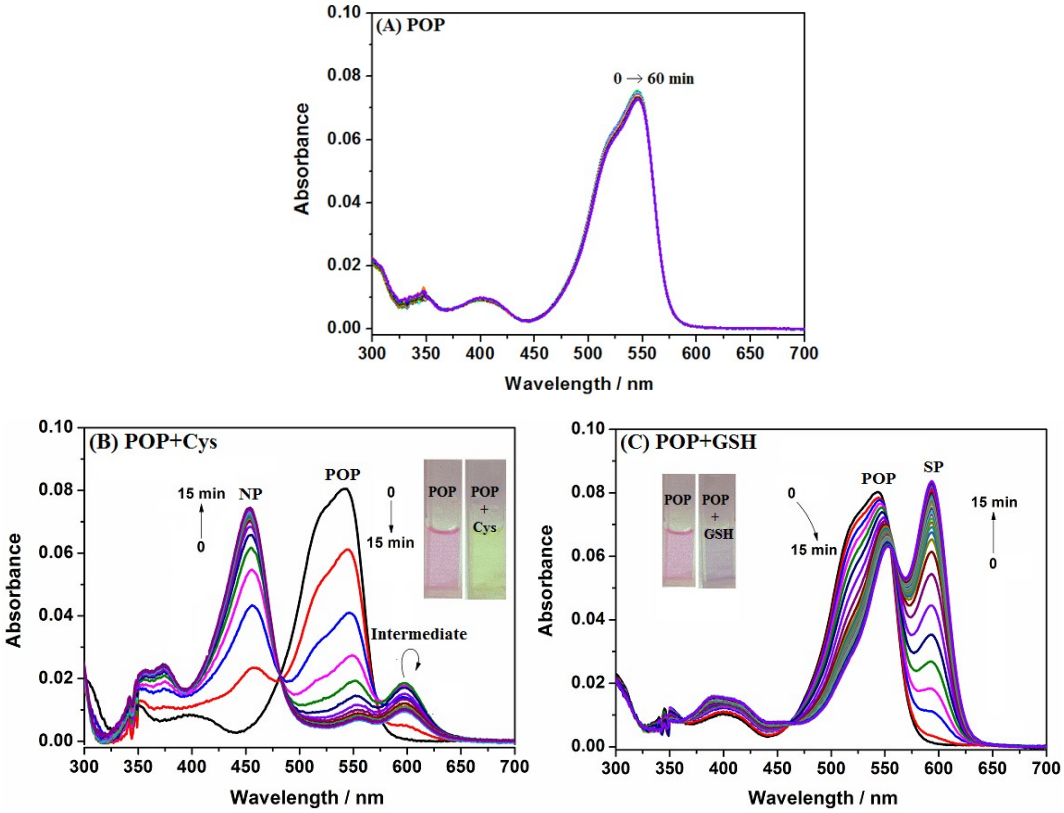


Figure S4. Time-dependent absorption spectra changes of **POP** (2 μM) (A) and **POP** (2 μM) treated with Cys (100 equiv) (B) and GSH (10 equiv) (C), respectively, in PBS (10 mM, pH 7.4) at 37 °C. Note that the addition of Cys to the solution of **POP** also resulted in a red-shift absorption peak at ca. 590 nm (B), which initially increased, then decreased, strongly indicating that the reaction of **POP** with Cys contains a thiolpyronin intermediate (Scheme 1).

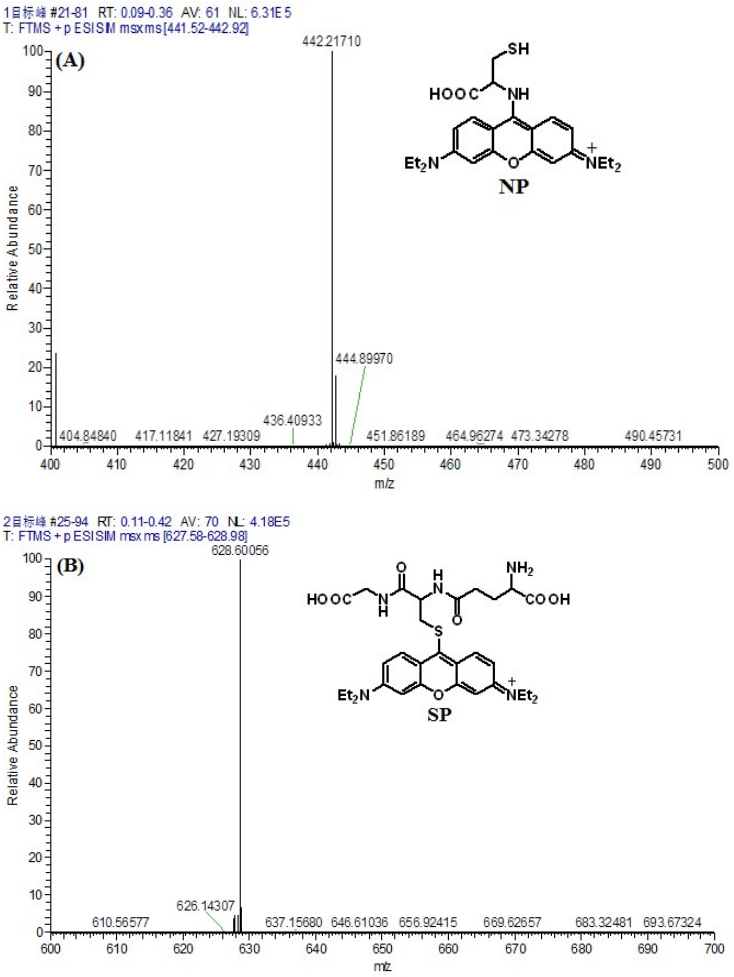


Figure S5. HRMS charts of **POP** treated with Cys (A) and GSH (B), respectively.

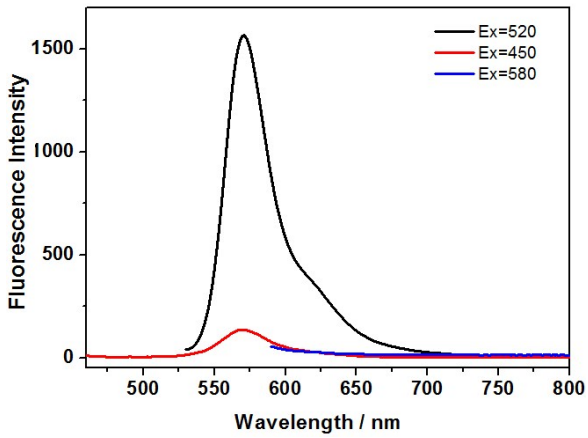


Figure S6. Fluorescence spectra of **POP** (2 μM) when excited at 520 nm, 450 nm, 580 nm, respectively, in PBS (10 mM, pH = 7.4) at 37 °C. The excitation wavelengths are close to the absorption maxima of **POP** (Abs_{max} = 545 nm), **NP** (Abs_{max} = 455 nm), and **SP** (Abs_{max} = 593 nm), respectively.

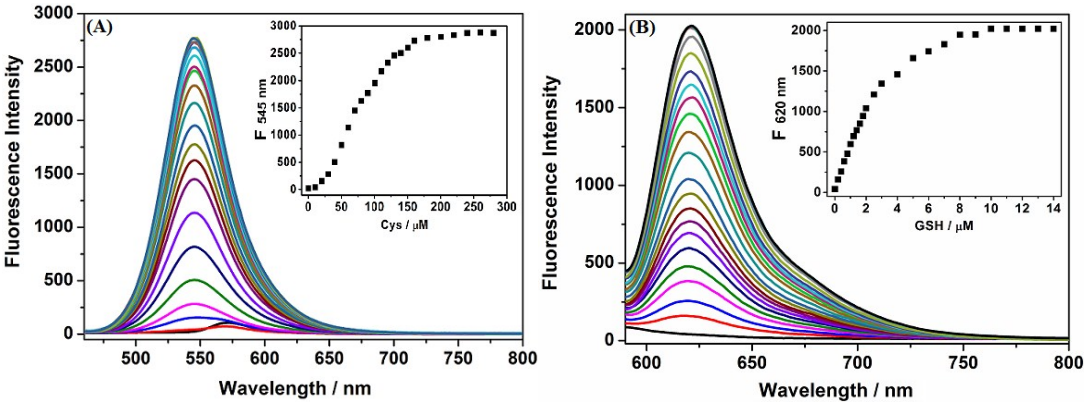


Figure S7. Fluorescence titration of **POP** (2 μM) toward Cys (0–300 μM) (A) and GSH (0–14 μM) (B), respectively, in PBS (10 mM, pH = 7.4) at 37 °C. Each spectrum was recorded after 10 min upon addition of Cys or GSH. For (A), λ_{ex} = 450 nm; For (B), λ_{ex} = 580 nm. Slits: 5/5 nm.

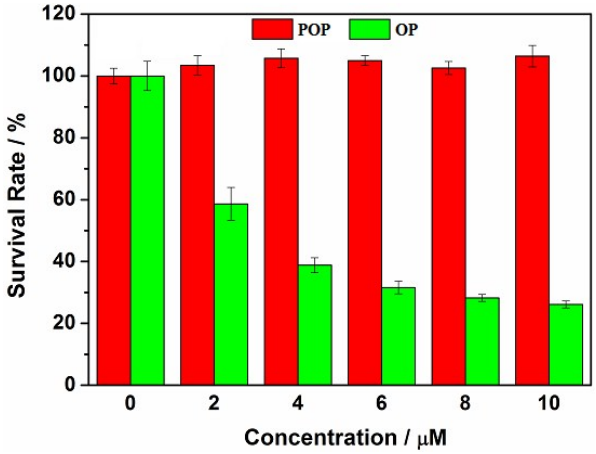


Figure S8. Cytotoxicity of **POP** and **OP** in cultured HeLa cells. Cell viability measured by CCK8 assays was reported as percentage relative to the untreated cells (mean±SD). The results indicate that the big steric hindrance of $-PPh_2$ group makes **POP** less cytotoxic relative to **OP**.

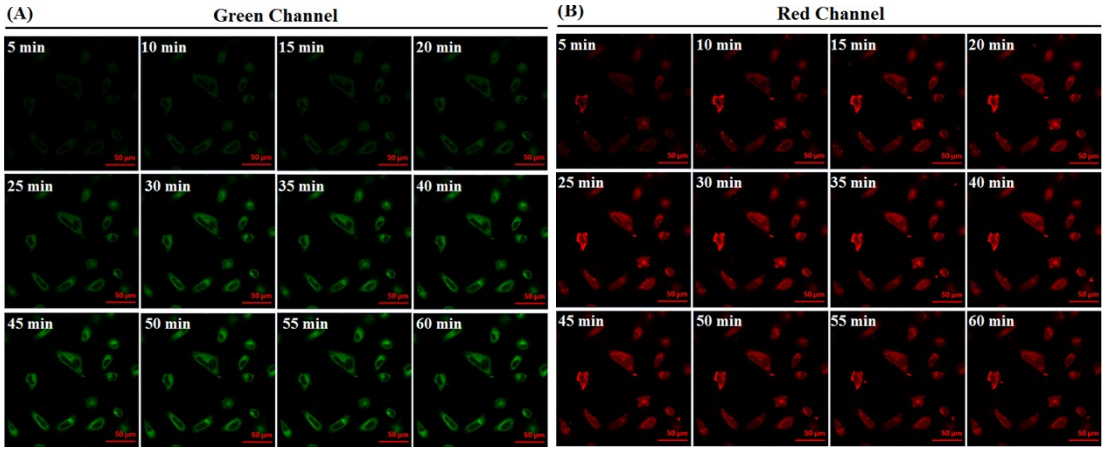


Figure S9. Confocal fluorescence images of HeLa cells stained with **POP** (4 μM) from green (A) and red (B) emission channels, respectively, which were obtained at the indicated time points. The results indicate that a 30 min incubation time is enough for obtaining the clear intracellular fluorescence images. For green channel, the emission was collected at 500–540 nm (λ_{ex} = 488 nm); for red channel, the emission was collected at 650–700 nm (λ_{ex} = 561 nm). Scale bar: 50 μm.

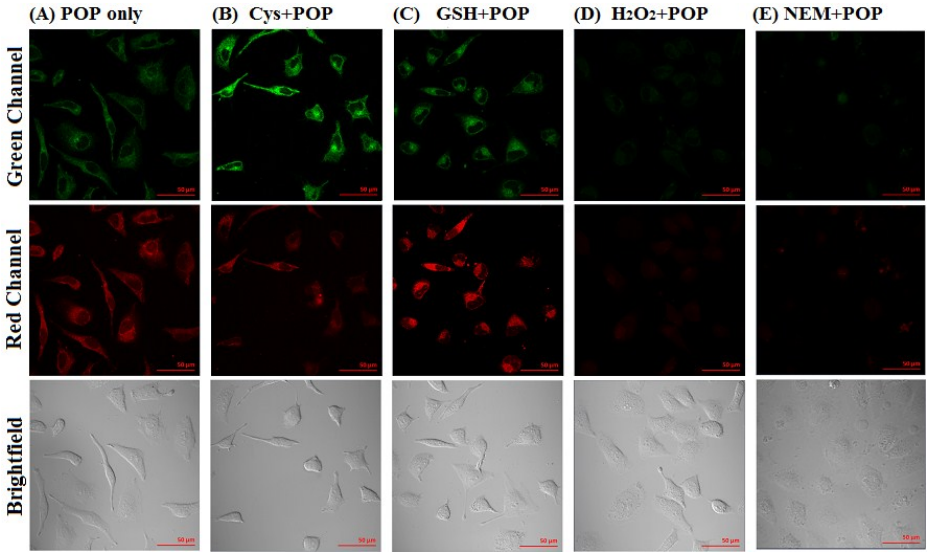


Figure S10. Confocal fluorescence images of HeLa cells treated with **POP** (4 μM, 30 min) (A), pretreated with Cys (500 μM, 30 min) and then treated with **POP** (4 μM, 30 min) (B), pretreated with GSH (500 μM, 30 min) and then treated with **POP** (4 μM, 30 min) (C), pretreated with H_2O_2 (500 μM, 30 min) and then treated with **POP** (4 μM, 30 min) (D), and pretreated with NEM (1 mM, 60 min) and then treated with

POP (4 μM, 30 min) (E) in PBS, respectively. For green channel, the emission was collected at 500–540 nm (λ_{ex} = 488 nm); for red channel, the emission was collected at 650–700 nm (λ_{ex} = 561 nm). Scale bar: 50 μm.

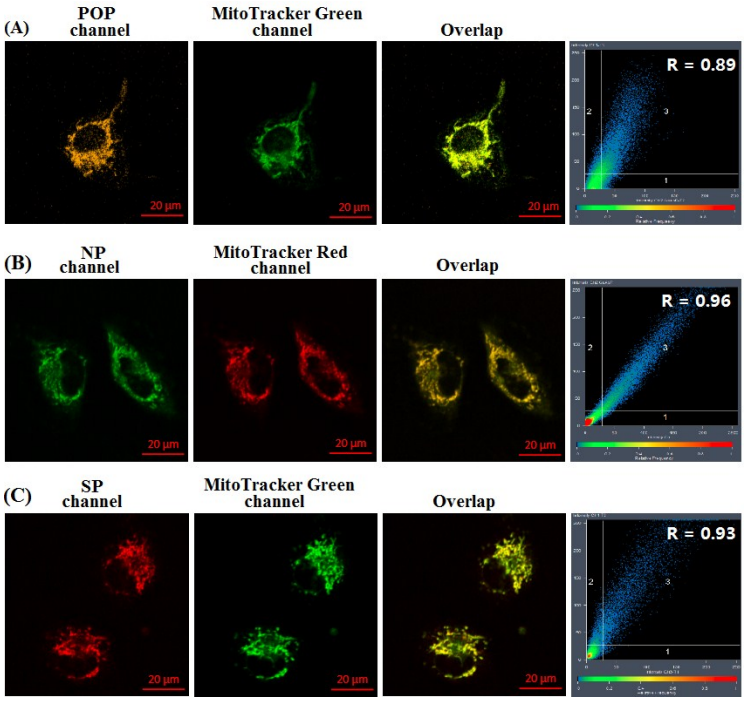


Figure S11. Confocal fluorescence images of HeLa cells costained with **POP** (2 μM, 30 min)/MitoTracker Green FM (0.5 μM, 15 min) (A), **POP** (2 μM, 30 min)/MitoTracker Red (0.5 μM, 15 min) (B), and **POP** (2 μM, 30 min)/MitoTracker Green FM (0.5 μM, 15 min) (A), respectively, in PBS. R refers to Pearson's co-localization coefficients. In (A), for **POP**, emission was collected at 575–650 nm (λ_{ex}: 543 nm); for MitoTracker Green, emission was collected at 500–540 nm (λ_{ex}: 488 nm). In (B), for **NP**, emission was collected at 500–600 nm (λ_{ex}: 488 nm); for MitoTracker Red, emission was collected at 650–750 nm (λ_{ex}: 633 nm). In (C), for **SP**, emission was collected at 600–700 nm (λ_{ex}: 561 nm); for MitoTracker Green, emission was collected at 500–600 nm (λ_{ex}: 488 nm). Scale bar: 20 μm.

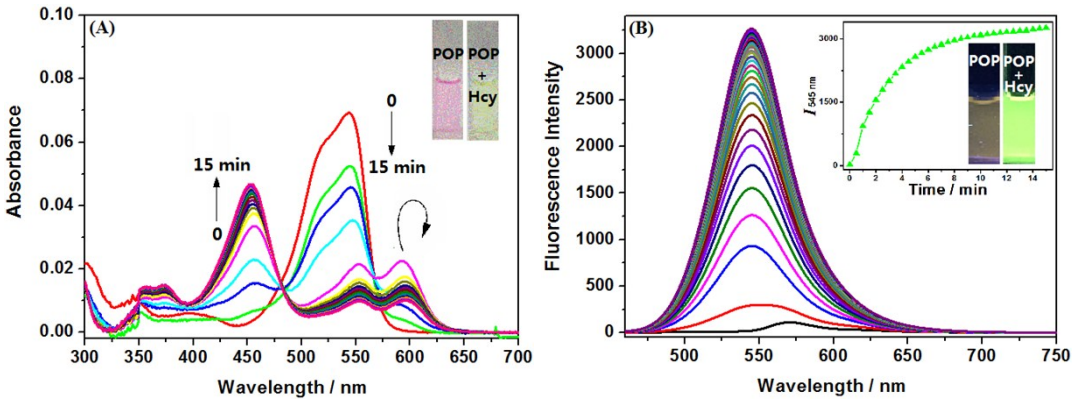


Figure S12. Time-dependent absorption (A) and fluorescence (B) spectra changes of **POP** (2 μM) treated with Hcy (100 equiv) in PBS (10 mM, pH 7.4) at 37 °C. For (B), λ_{ex} = 450 nm. Slits: 5/5 nm.

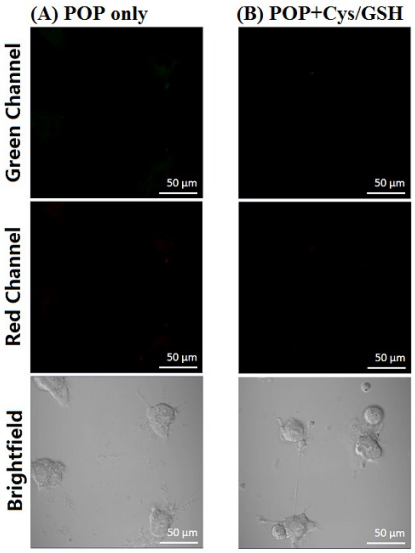


Figure S13. (A) Confocal fluorescence images of normal COS-7 cells treated with **POP** (4 μM, 30 min) in PBS. (B) Confocal fluorescence images of normal COS-7 cells pretreated with **POP** (4 μM, 30 min), and, after washed with PBS three times, then treated with Cys (500 μM)/GSH (500 μM) in PBS for another 30 min. For green channel, the emission was collected at 500–540 nm (λ_{ex} = 488 nm); for red channel, the emission was collected at 650–700 nm (λ_{ex} = 561 nm). Scale bar: 50 μm.

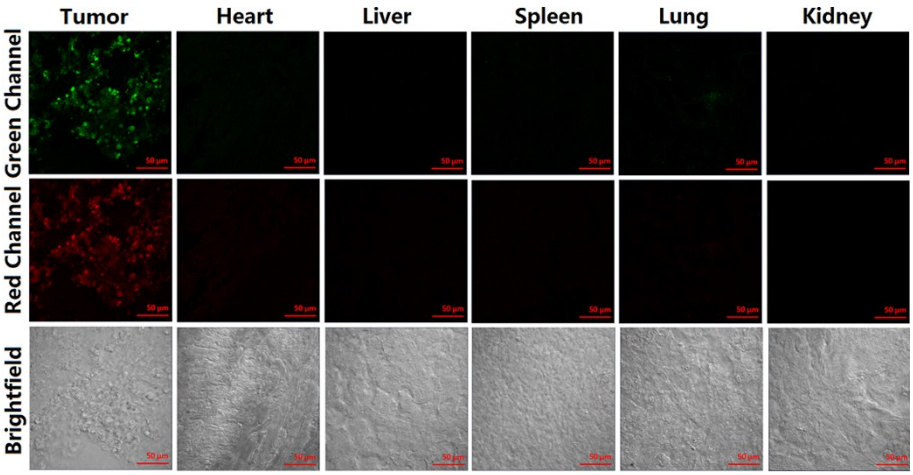


Figure S14. Confocal fluorescence images of **POP** (4 μM, 30 min) stained cancer and normal tissue cryosections from a HepG2 xenograft model of nude mouse. Scale bar: 50 μm.

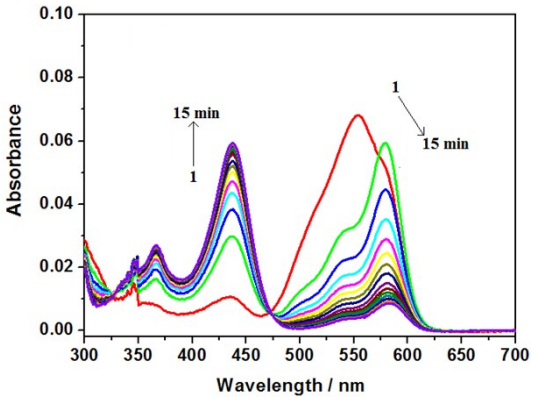


Figure S15. Time-dependent absorption spectra changes of **POP** (2 μM) in PBS (10 mM, pH = 7.4, **containing 10% fetal bovine serum (FBS)**) at 37 °C. Due to the abundant Cys existing in fetal bovine serum (FBS) (ca. 300 μM), the probe in PBS (10 mM, pH = 7.4, containing 10% FBS) showed a similar absorption spectra change to that treated with Cys in pure PBS (10 mM, pH = 7.4) (Fig. S4B).

9. ^{1}H NMR, ^{13}C NMR, and HRMS data of xanthone **1**, OP, and POP

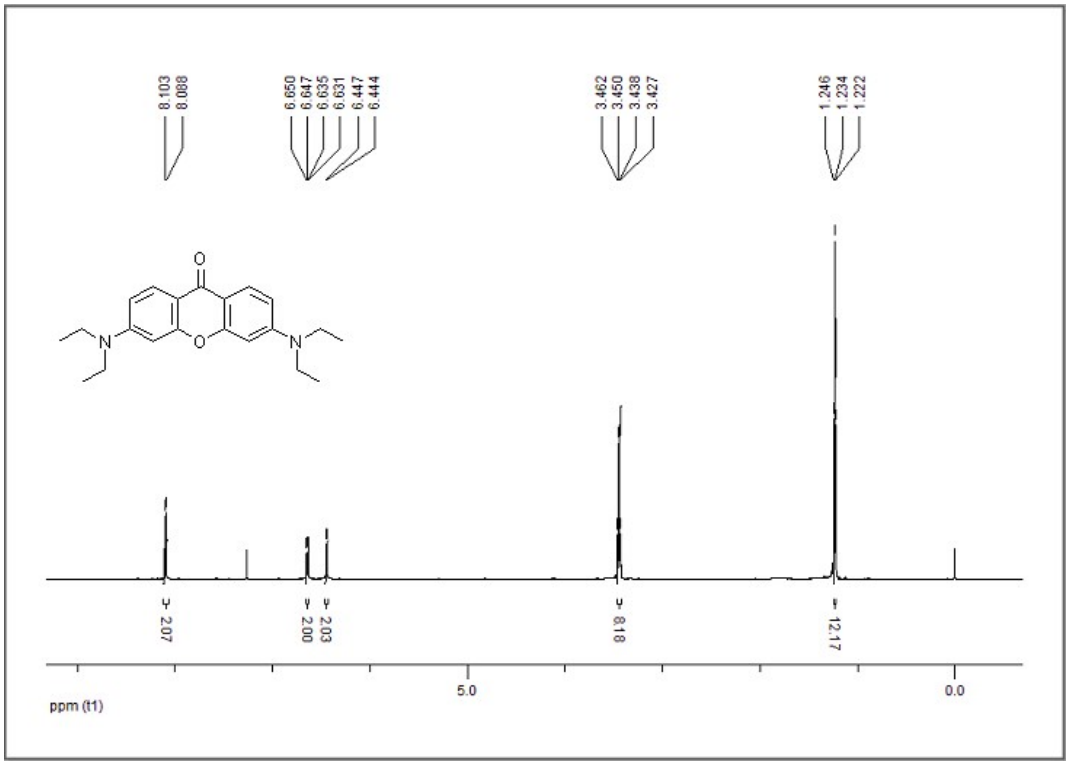


Figure S16. ^{1}H NMR chart of xanthone **1** ($CDCl_3$, 600 MHz).

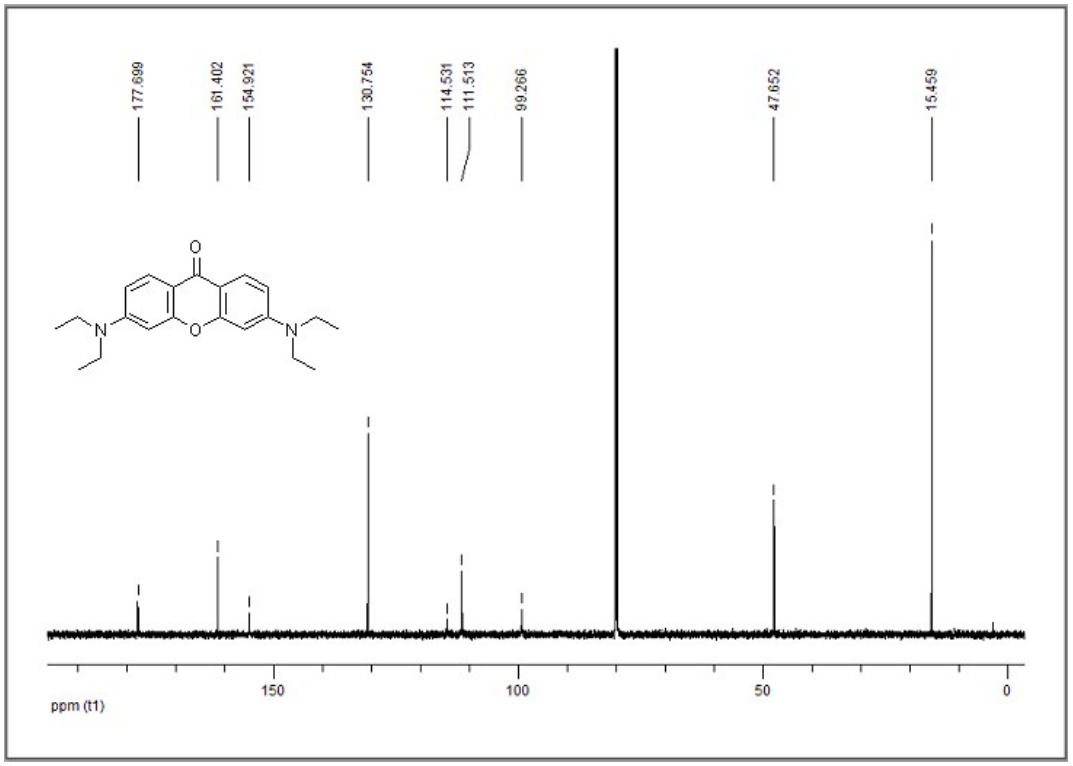


Figure S17. ^{13}C NMR chart of xanthone **1** ($CDCl_3$, 150 MHz).

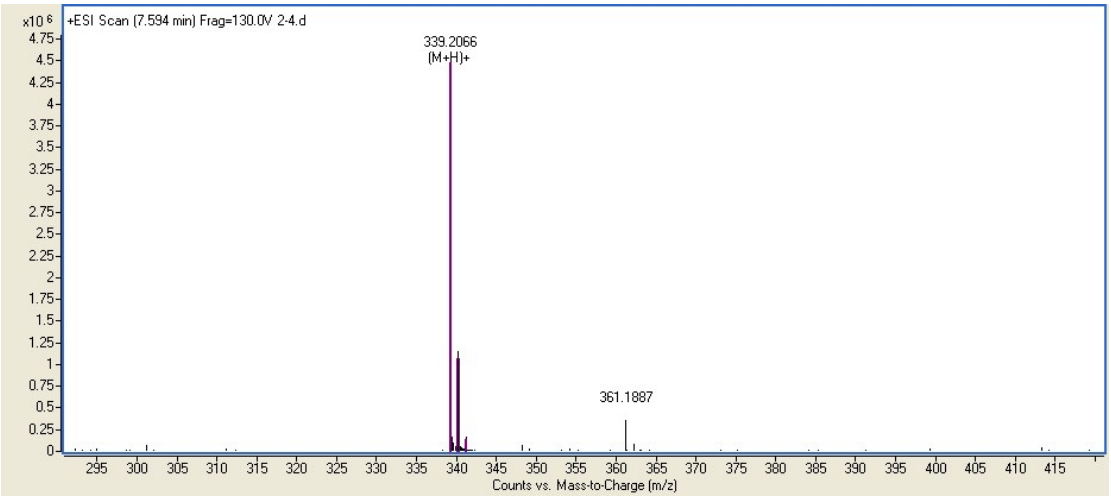


Figure S18. HRMS chart of xanthone **1**.

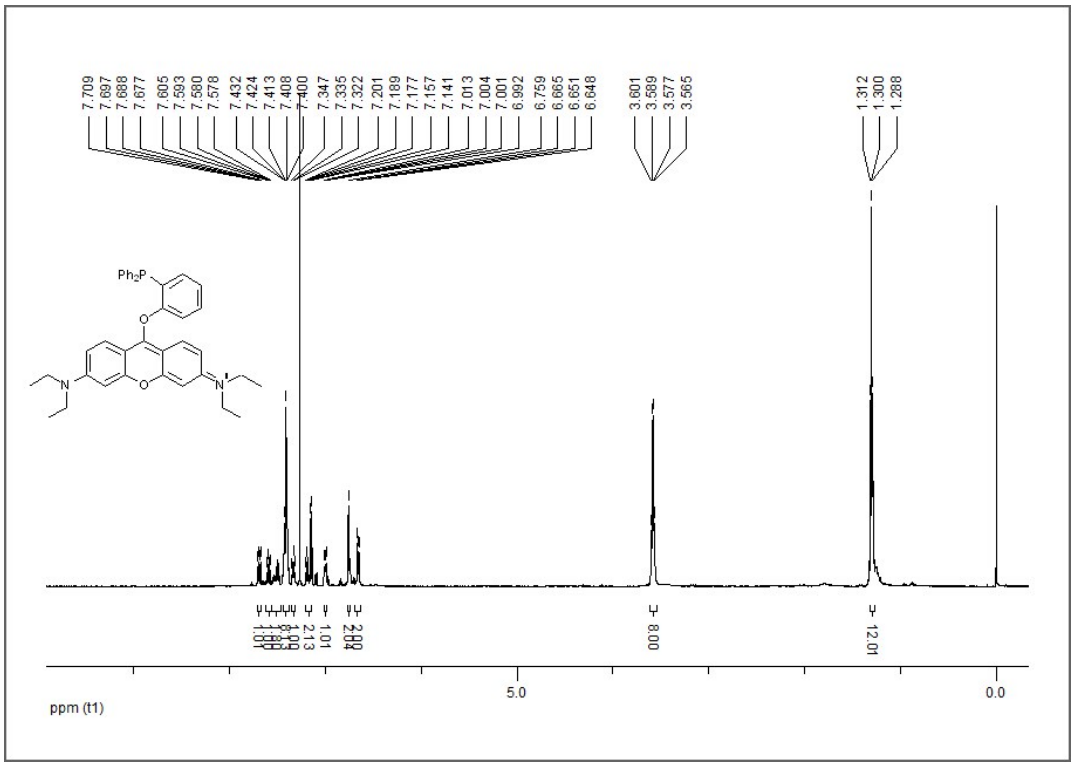


Figure S19. ^{1}H NMR chart of **POP** ($CDCl_3$, 600 MHz).

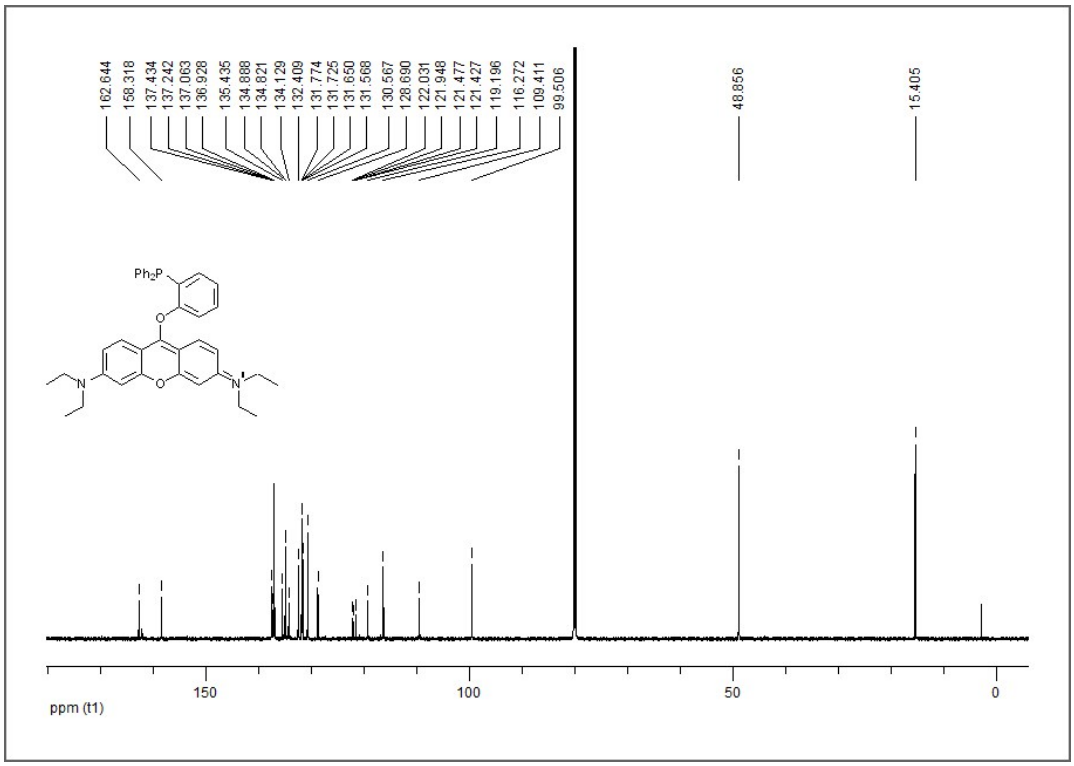


Figure S20. ^{13}C NMR chart of **POP** ($CDCl_3$, 150 MHz).

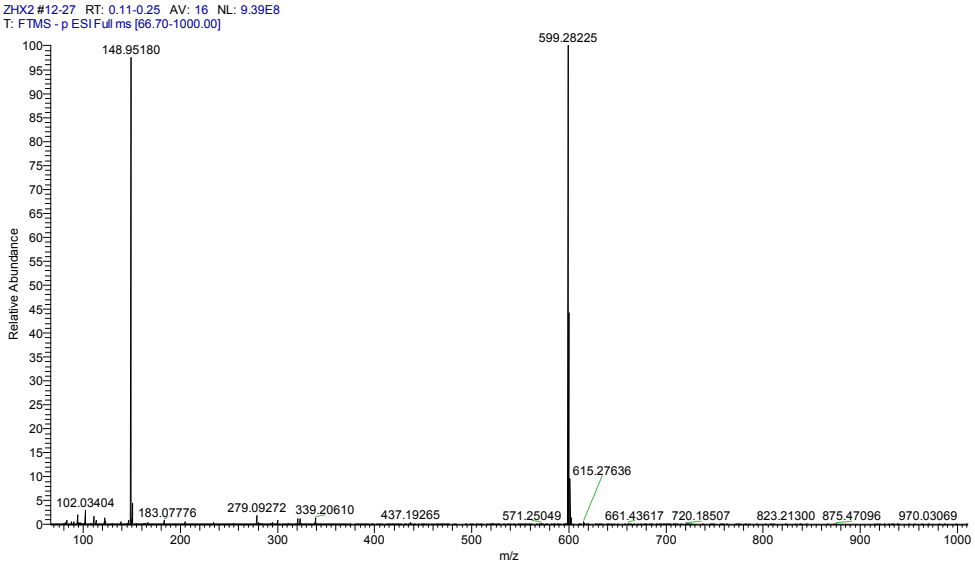


Figure S21. HRMS chart of **POP**.

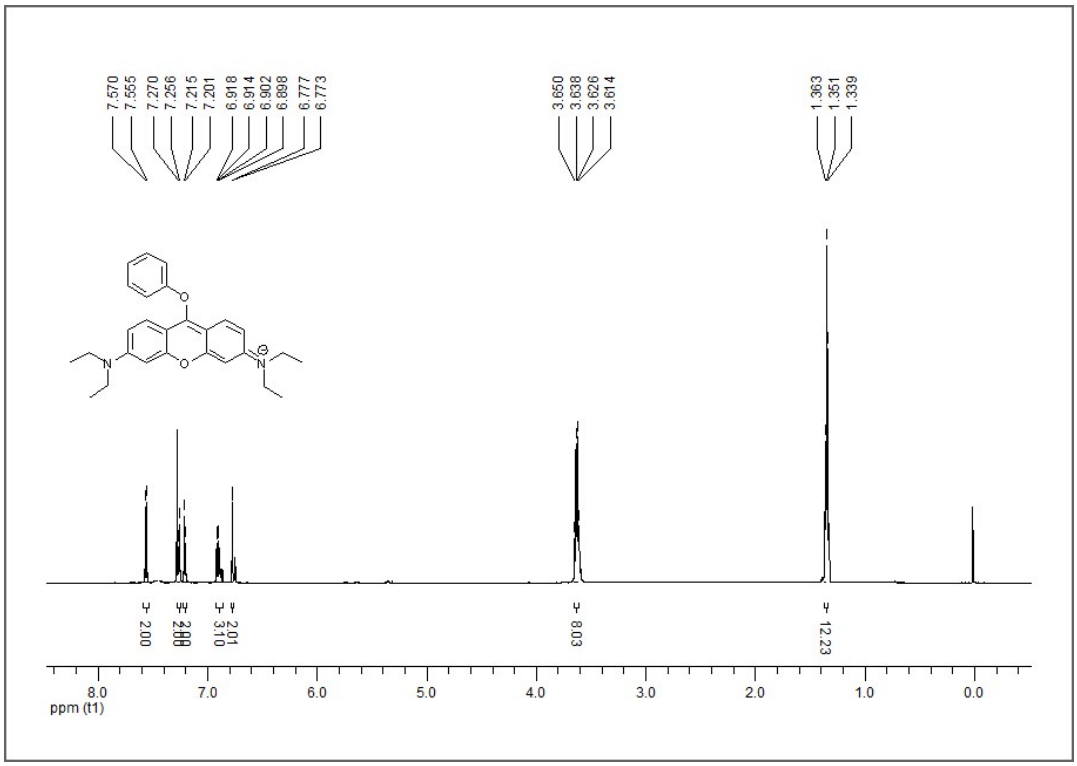


Figure S22. ^{1}H NMR chart of **OP** ($CDCl_3$, 600 MHz).

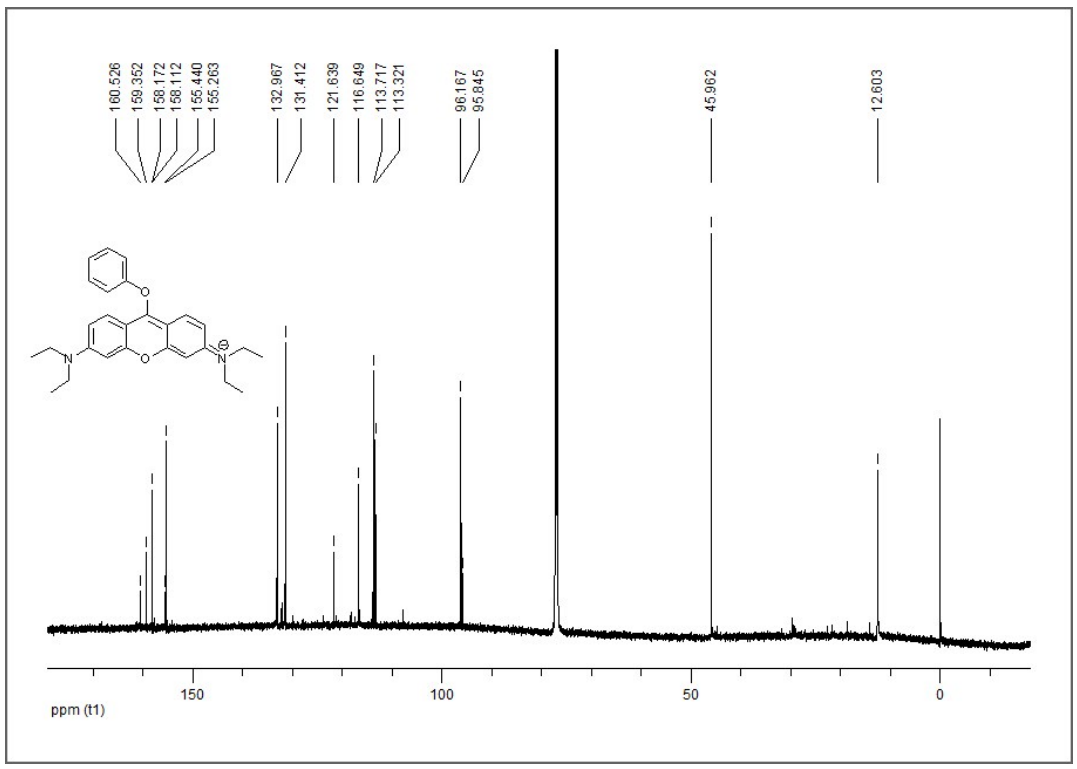


Figure S23. ^{13}C NMR chart of **OP** ($CDCl_3$, 150 MHz).

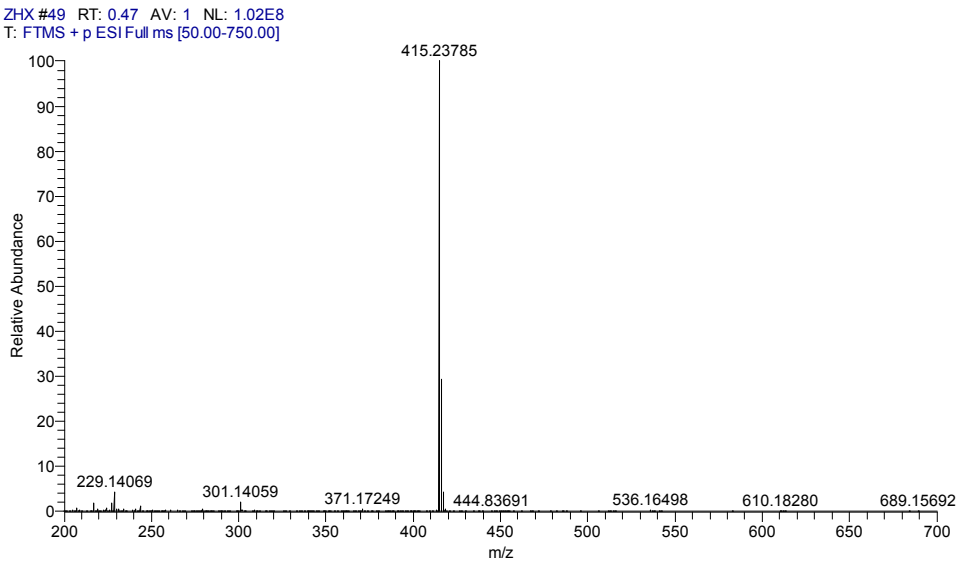


Figure S24. HRMS chart of **OP**.

10. References

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