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Electronic Supplementary Information (ESI)

A highly selective water-soluble optical probe for endogenous peroxynitrite

Ji-Ting Hou,[a] Jin Yang,[a] Kun Li,*[a,b] Ye-Xin Liao,[a] Kang-Kang Yu,[a] Yong-Mei Xie[b] and Xiao-Qi Yu*[a]

[a] *Key Laboratory of Green Chemistry and Technology, Ministry of Education, College of Chemistry, Sichuan University, Chengdu, 610064, P. R. China. E-mail: kli@scu.edu.cn, xqyu@scu.edu.cn; Fax: +86-28-85415886*

[b] *State Key Laboratory of Biotherapy, West China Hospital, West China Medical School, Sichuan University, Chengdu 610041, China.*

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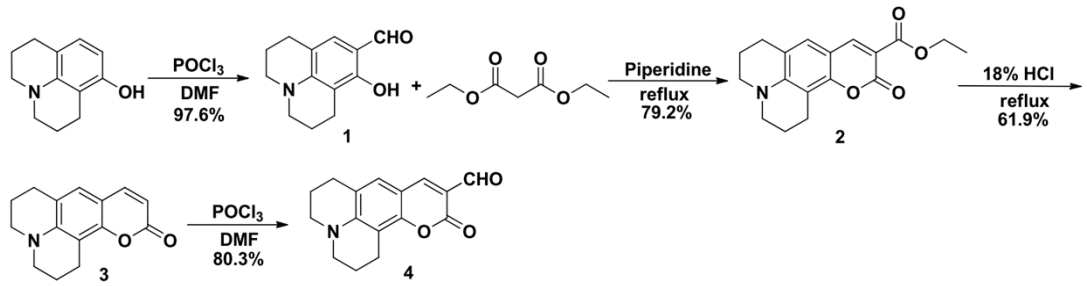
Experimental Section

General remarks for experimental

^{1}H NMR, ^{13}C NMR spectra were measured on a Bruker AM400 NMR spectrometer. Proton Chemical shifts of NMR spectra were given in ppm relative to internals reference TMS (1H, 0.00 ppm). ESI-MS and HRMS spectral data were recorded on a Finnigan LCQ^{DECA} and a BrukerDaltonics Bio TOF mass spectrometer, respectively. All pH measurements were performed with a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode. Fluorescence emission spectra were obtained using FluoroMax-4 Spectrofluorophotometer (HORIBA JobinYvon) at 298 K. Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. All the solvents were dried according to the standard methods prior to use. All of the solvents were either HPLC or spectroscopic grade in the optical spectroscopic studies.

Cyclic voltammetry. Cyclic voltammetry was performed on a CHI660D electrochemical analyzer (CH Instruments Inc., Shanghai). A three-electrode arrangement in a single cell was used for the measurements: a Pt wire was used as the auxiliary electrode, a Pt electrode and a GC electrode as the working electrode, and a Ag/AgCl (sat. NaCl) electrode as the reference electrode. Sample solutions contained 1 mM of each sample and 0.1 M sodium chloride as a supporting electrolyte.

Imaging of cells. Hela cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% Antibiotic-Antimycotic at 37^{o}C in a 5% CO_2/95% air incubator. For fluorescence imaging, cells (4×10^{3}/well) were passed on a 6-well plate and incubated for 24 h. Immediately before the staining experiment, cells were washed twice with PBS, incubated with 5 μM **Rh-TPP** or **Rh-Py** for 30 min at 37 ^{o}C. Then confocal fluorescent images were captured with an excitation light at 543 nm. Then, 100 μM NaClO was added and incubated for another 10 min and was imaged.



Preparation and Characterization of 1

5 mL of dry DMF was added dropwise to 1.33 mL of $POCl_3$ at 0 °C. The mixture is stirred for 15 min at 0°C under N_2 atmosphere. A suspension of 8-Hydroxyjulolidine (2.5 g, 13.2 mmol) in 3 mL of dry DMF was then added dropwise, followed by stirring at room temperature for 30 min. After that, the mixture was heated to 80 °C for another 30 min and was cooled to room temperature. A 25-mL portion of water was added with stirring and a green solid formed slowly. The precipitate was filtered, washed with water, and dried in vacuum overnight. The solid was dissolved in CH_2Cl_2 and was purified by column chromatography over silica gel to afford **1** (2.8 g, 12.9 mmol) as a yellowish solid. Yield: 97.6%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.83 (s, 1H), 9.39 (s, 1H), 6.86 (s, 1H), 3.30 (t, J = 8 Hz, 4H), 2.70 (t, J = 6.4 Hz, 4H), 1.95 (m, 4H). ESI-MS: m/z 218.16 $[M + H]^+$: (calcd 218.12).

Preparation and Characterization of 2

A mixture of **2** (2.17g, 10 mmol), diethylmalonate (3.2 mL, 20 mmol), and piperidine (1 mL) in anhydrous ethanol (20 mL) was refluxed for 6 h. The mixture was cooled, and the solvent was evaporated. The product was extracted with CH_2Cl_2, washed with brine, and purified by column chromatography over silica gel to afford **2** (2.48 g, 7.9 mmol). Yield: 79.2%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.33 (s, 1H), 6.95 (s, 1H), 4.37 (q, J = 7.1 Hz, 2H), 3.34 (dd, J = 11.8, 6.6 Hz, 4H), 2.89 (t, J = 6.4 Hz, 2H), 2.77 (t, J = 8 Hz, 2H), 2.03 – 1.93 (m, 4H), 1.39 (t, J = 7.1 Hz, 3H). ESI-MS: m/z 314.21 $[M + H]^+$: (calcd 314.14).

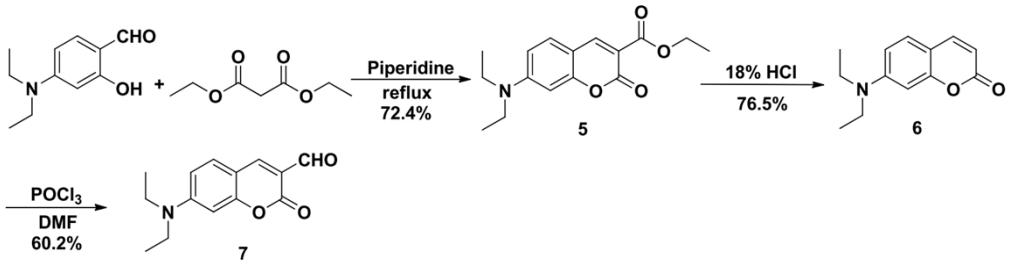
Preparation and Characterization of 3

Compound **3** (2.4g, 7.7 mmol) in 60 mL of 18% strength hydrochloric acid are heated to reflux for 7 h. After cooling, the pH value was adjusted to 4-5 with 45% strength sodium hydroxide. The crystalline precipitate was filtered off, thoroughly washed with water and dried in vacuum at 50 °C, which was further purified by column chromatography over silica gel to afford **3** (1.14g, 4.8 mmol) as an orange solid. Yield: 61.9%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (d, J = 9.2 Hz, 1H), 6.86 (s, 1H), 6.01 (d, J = 9.2 Hz, 1H), 3.28 (dd, J = 11.5, 5.8 Hz, 4H), 2.90 (t, J = 6.5 Hz, 2H), 2.78 (t, J = 6.3 Hz, 2H), 2.02-1.95 (m, 4H). ESI-MS: m/z 242.07 $[M + H]^+$: (calcd 242.12).

Preparation and Characterization of 4

3 mL of dry DMF was added dropwise to 0.3 mL of $POCl_3$ at 0 °C. The mixture is stirred for 15 min at 0°C under N_2 atmosphere. A suspension of **3** (602 mg, 2.5 mmol) in 3 mL of dry DMF was then added dropwise, followed by stirring at room temperature for 30 min. After that, the mixture was heated to 70 °C for another 2 h and was cooled to room temperature. A 25-mL portion of

water was added with stirring and a red solid formed slowly. The precipitate was filtered, washed with water, and dried in vacuum overnight, affording **4** (540 mg, 2.0 mmol) as an orange solid. Yield: 80.3%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.12 (s, 1H), 8.14 (s, 1H), 6.99 (s, 1H), 3.41 -3.37 (m, 4H), 2.90 (t, *J* = 6.4 Hz, 2H), 2.78 (t, *J* = 6.4 Hz, 2H), 2.06 – 1.92 (m, 4H). ESI-MS: m/z 270.18 $[M + H]^+$: (calcd 270.11).



Preparation and Characterization of 5

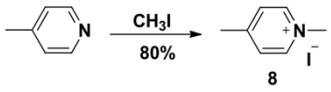
A mixture of 4-diethylamino salicyldehyde (3.86 g, 20 mmol), diethylmalonate (6.4 mL, 40 mmol), and piperidine (1.5 mL) in anhydrous ethanol (30 mL) was refluxed for 12 h. The mixture was cooled, and the solvent was evaporated. The product was extracted with CH_2Cl_2, washed with brine, and purified by column chromatography. It was further purified by recrystallization from petroleum ether/ethyl acetate to afford a yellow crystalline solid (4.2 g, 14.5 mmol). Yield: 72.4%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (s, 1H), 7.36 (d, *J* = 8.9 Hz, 1H), 6.61 (dd, *J* = 8.9, 2.3 Hz, 1H), 6.46 (d, *J* = 2.2 Hz, 1H), 4.38 (q, *J* = 7.1 Hz, 2H), 3.45 (q, *J* = 7.1 Hz, 4H), 1.39 (t, *J* = 7.1 Hz, 3H), 1.24 (t, *J* = 7.1 Hz, 6H). ESI-MS: m/z 457.21 $[M + H]^+$: (calcd 457.26). ESI-MS: m/z 290.11 $[M + H]^+$: (calcd 290.14).

Preparation and Characterization of 6

Compound **5** (2.9g, 10 mmol) in 60 mL of 18% strength hydrochloric acid was heated to reflux for 7 h. After cooling, the pH value was adjusted to 4-5 with 45% strength sodium hydroxide. The crystalline precipitate was filtered off, thoroughly washed with water and dried in vacuum at 50 °C, which was further purified by column chromatography over silica gel to afford **6** (1.67g, 7.7 mmol) as a light yellow solid. Yield: 76.5%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.54 (d, *J* = 9.3 Hz, 1H), 7.24 (d, *J* = 8.8 Hz, 1H), 6.56 (dd, *J* = 8.8, 2.5 Hz, 1H), 6.49 (s, 1H), 6.03 (d, *J* = 9.3 Hz, 1H), 3.41 (q, *J* = 7.1 Hz, 4H), 1.21 (t, *J* = 7.1 Hz, 6H). ESI-MS: m/z 218.16 457.21 $[M + H]^+$: (calcd 218.12).

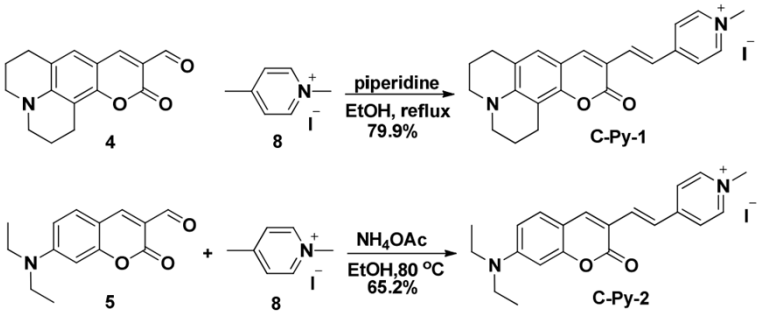
Preparation and Characterization of 7

4 mL of dry DMF was added dropwise to 0.4 mL of $POCl_3$ at 20-50 °C. The mixture was stirred for 45 min at 50 °C under N_2 atmosphere. A suspension of **6** (500 mg, 2.3 mmol) in 3 mL of dry DMF was then added, the mixture was warmed to 60 °C for 2 h and poured out onto ice water, and the whole is stirred for 2 h. The crystalline precipitate was filtered off, thoroughly washed with water and dried in vacuum at 50 ºC, which afforded an orange **7** (340 mg, 1.39 mmol) as an orange solid. Yield: 60.2%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.12 (s, 1H), 8.25 (s, 1H), 7.42 (d, *J* = 9.0 Hz, 1H), 6.64 (dd, *J* = 9.0, 2.4 Hz, 1H), 6.49 (s, 1H), 3.48 (q, *J* = 7.1 Hz, 4H), 1.26 (t, *J* = 7.1 Hz, 6H). ESI-MS: m/z 246.07 $[M + H]^+$: (calcd 246.11).



Preparation and Characterization of 8

4-picoline (4.65g, 50 mmol) and methyl iodide (10.8 g, 55 mmol) were mixed in toluene. The solution was stirred at room temperature for 4 h and then refluxed for 30 min. After cooling, the solution was filtered. The solid was washed with ethyl ether and was further dried under vacuum to afford **8** (9.64 g, 41 mmol) as a light yellow solid. Yield: 82%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.82 (d, J = 6.0 Hz, 2H), 7.96 (d, J = 6.0 Hz, 2H), 4.28 (s, 3H), 2.60 (s, 3H). ESI-MS: m/z 108.12 $[M]^+$: (calcd 108.08).



Preparation and Characterization of C-Py-1

Compound **4** (107 mg, 0.4 mmol), **8** (117 mg, 0.5 mmol) and 0.5 mL piperidine were mixed in 10 mL of anhydrous ethanol. The mixture was heated to reflux for 3.5 h and was then cooled to room temperature. The precipitate was filtered off, washed by cold ethanol and was further dried under vacuum to afford **C-Py-1** (155 mg, 3.2 mmol) as a purple solid. Yield: 79.9%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.75 (d, J = 6.9 Hz, 2H), 8.14 (d, J = 6.9 Hz, 2H), 8.10 (s, 1H), 7.83 (d, J = 16.0 Hz, 1H), 7.63 (d, J = 16.0 Hz, 1H), 7.14 (s, 1H), 4.21 (s, 3H), 3.35 (s, 4H), 2.78-2.68 (m, 4H), 1.91 (t, J = 9.8 Hz, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.2, 153.5, 151.6, 148.1, 145.9, 145.1, 137.6, 126.9, 123.3, 122.3, 119.9, 112.8, 108.6, 105.5, 56.5, 55.4, 50.1, 49.5, 47.1, 27.2, 21.1, 20.1, 19.0. $C_{23}H_{23}N_2O_2^+$ $[M]^+$: 359.1754, found: 359.1726.

Preparation and Characterization of C-Py-2

Compound **7** (74 mg, 0.3 mmol), **8** (106 mg, 0.45 mmol) and NH_4OAc (77 mg, 1.0 mmol) were mixed in 5 mL of anhydrous ethanol. The mixture was heated to reflux for 3 h and was then cooled in a refrigerator overnight. The precipitate was filtered off, washed by cold ethanol and was further dried under vacuum to afford **C-Py-2** (90 mg, 1.9 mmol) as an orange solid. Yield: 65.2%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.78 (d, J = 6.7 Hz, 2H), 8.24 (s, 1H), 8.16 (d, J = 6.8 Hz, 2H), 7.83 (d, J = 16.0 Hz, 1H), 7.68 (d, J = 16.0 Hz, 1H), 7.56 (d, J = 9.0 Hz, 1H), 6.81 (d, J = 6.7 Hz, 1H), 6.61 (s, 1H), 4.23 (s, 3H), 3.50 (q, J = 7.0 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.1, 156.8, 153.4, 152.4, 145.8, 145.3, 137.2, 131.2, 123.5, 123.1, 114.1, 110.5, 108.8, 96.7, 54.3, 53.6, 47.2, 44.8, 12.9. $C_{21}H_{23}N_2O_2^+$ $[M]^+$: 335.1754, found: 335.1729.

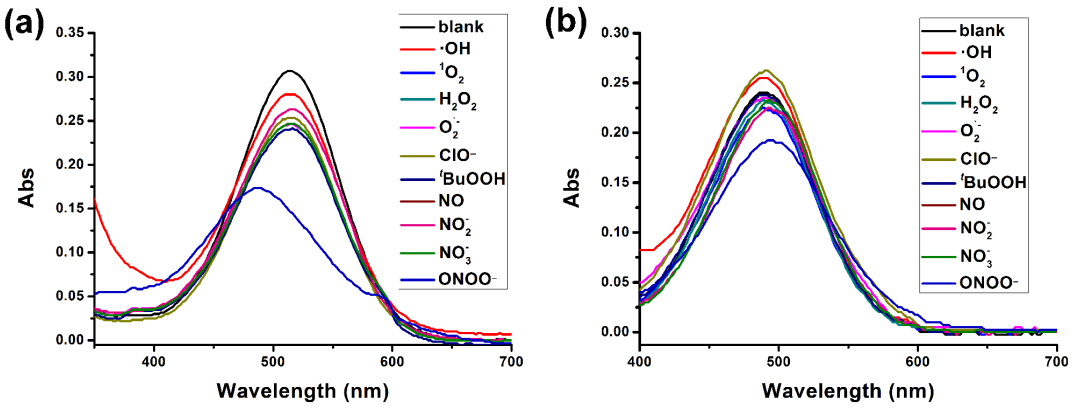


Figure S1. Absorption spectra of (a) **C-Py-1** and (b) **C-Py-2** before and after reaction with various ROS and RNS in PBS (pH 7.4, 10 mM). [**C-Py-1**] = [**C-Py-2**] = 5 μM. $ONOO^-$: $ONOO^-$ (final 50 μM) was added and the mixture was stirred at 20 °C. ClO^-: NaClO (final 100 μM) was added and the mixture was stirred at 20 °C. ·OH: ferrous perchlorate (100 μM) and H_2O_2 (0.2 mM) were added at room temperature. $O_2^{\bullet-}$: KO_2 was dissovled in the anhydrous DMSO and then the appropriate aliquot was added (final 100 μM). H_2O_2: H_2O_2 (final 100 μM) was added and the mixture was stirred at 20 °C. ^{t}BuOOH: ^{t}BuOOH (final 100 μM) was added and the mixture was stirred at 20 °C. $^1O_2$: ClO^- (100 μM) and H_2O_2 (0.2 mM) were added at room temperature; NO: NOC-5 (an NO donor) was added and the mixture was stirred at 20 °C (final 100 μM). NO_2^-: $NaNO_2$ (final 100 μM) was added and the mixture was stirred at 20 °C. NO_3^-: $NaNO_3$ (final 100 μM) was added and the mixture was stirred at 20 °C

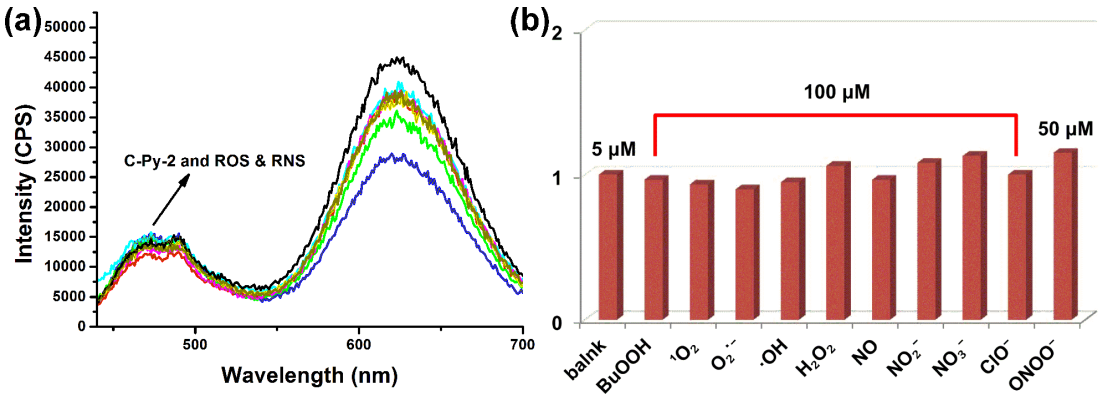


Figure S2. (a) Fluorescence spectra and (b) Fluorescence intensity of **C-Py-2** before and after addition of various ROS and RNS in PBS (pH 7.4, 10 mM). [**C-Py-2**] = 5 μM, [$ONOO^-$] = 50 μM. [other ROS and RNS] = 100 μM. λ_{ex} = 420 nm, slit: 3 nm/3 nm.

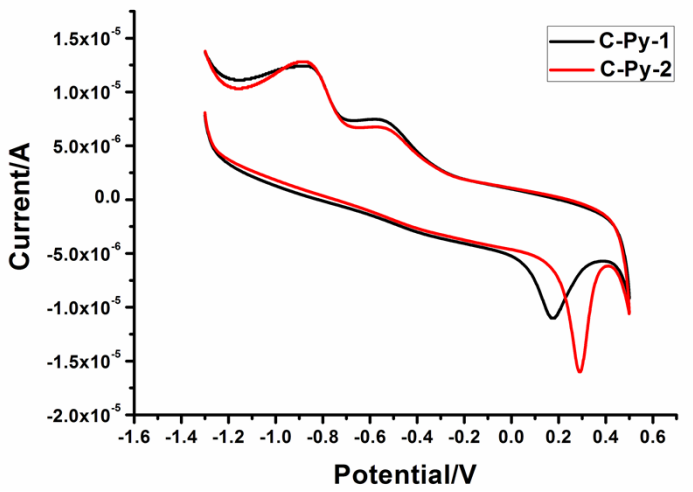


Figure S3. Cyclic voltammograms obtained at a glassy carbon electrode (1 mm diameter) for oxidation of **C-Py-1** and **C-Py-2** in water (containing 0.1 M NaCl as a supporting electrolyte). Potentials are versus Ag/AgCl (sat. NaCl).

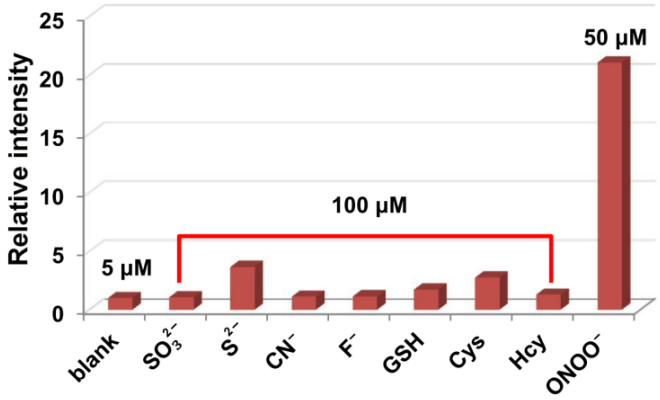


Figure S4. Fluorescence intensity of **C-Py-1** (5 μM) at 493 nm in the absence or prescence of various nucleophiles.

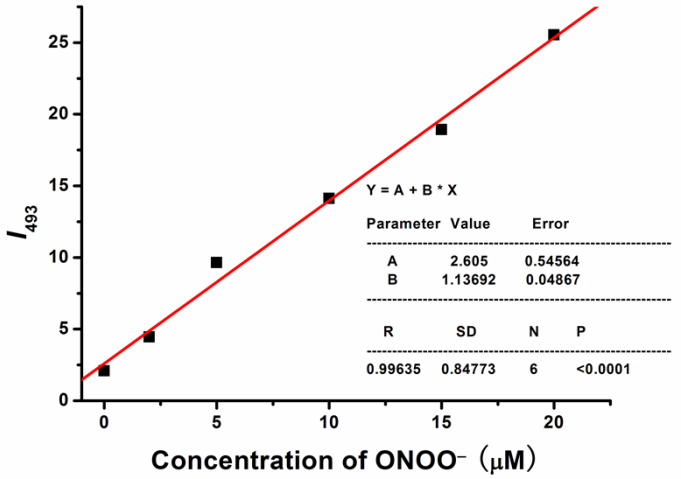


Figure S5. The titration curve plotted with the fluorescnece intensity of **C-Py-1** (5 μM) at 493 nm as a function of $ONOO^-$ concentration in range of 0-20 μM.

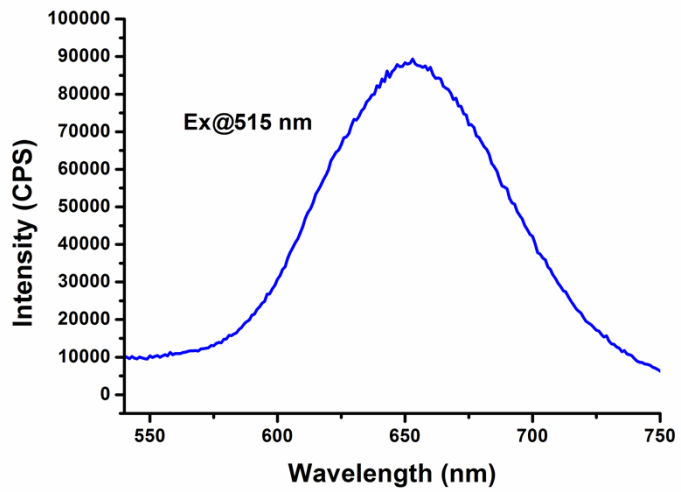


Figure S6. The fluorescence spectrum of **C-Py-1** (5 μM) in H_2O. Slit: 5 nm/5nm

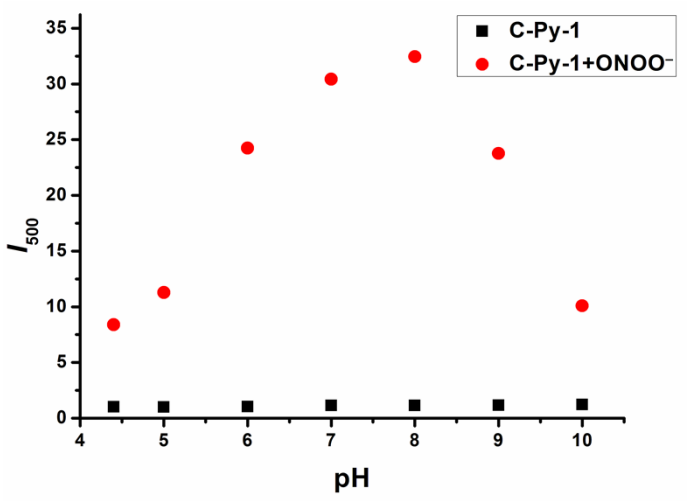


Figure S7. The effect of pH on the fluorescence intensity of **C-Py-1** (5 μM) in the absence or presenceof $ONOO^-$ (50 μM). λ_{ex} = 425 nm, slit: 3 nm/3 nm.

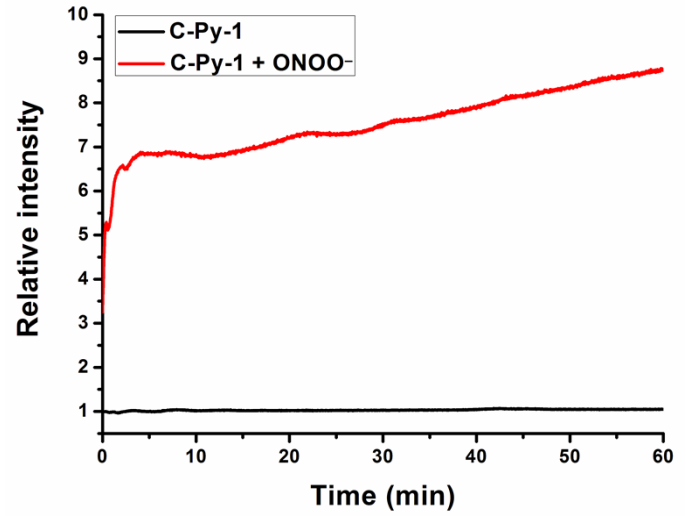


Figure S8. Time-dependent change of fluorescence intensity of **C-Py-1** in the absence or presence of $ONOO^-$ (25 μM). [**C-Py-1**] = 5 μM, λ_{ex} = 425 nm, slit: 3 nm/3 nm.

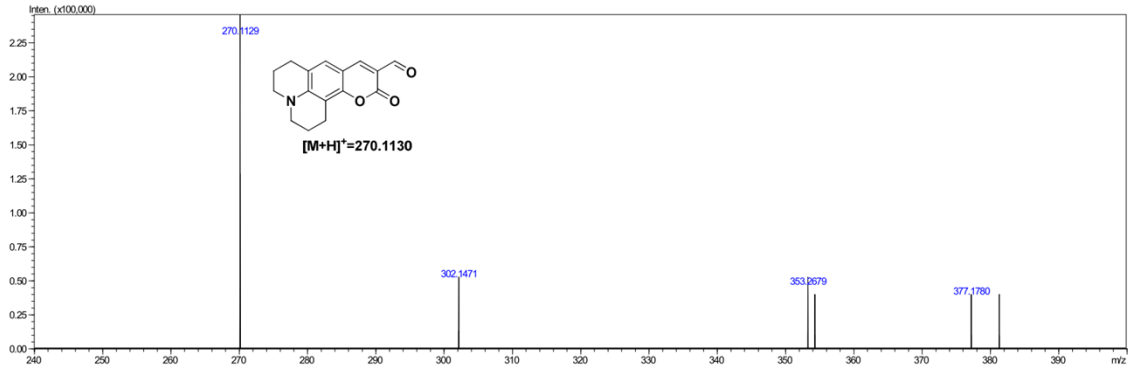


Figure S9. ESI spectra of **C-Py-1** upon addition of 10 equiv $ONOO^-$.

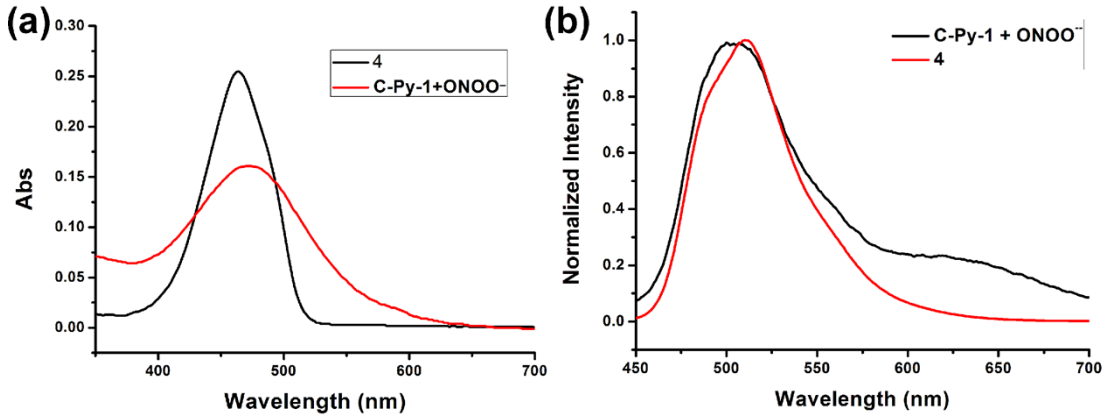


Figure S10. (a) Absorption spectra and (b) normalized fluorescence spectra of **C-Py-1** in the presence of $ONOO^-$ compared with that of aldehyde **4**.

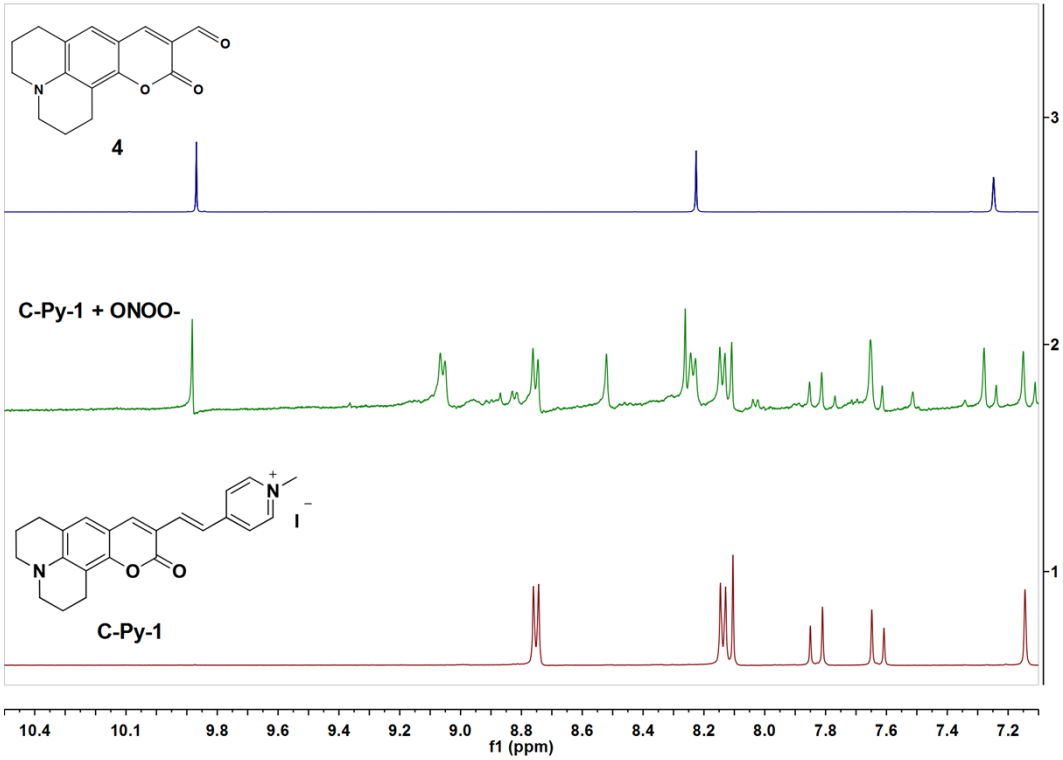


Figure S11. The ^{1}H NMR analysis of the mixture of C-Py-1 and $ONOO^{-}$ in DMSO-d_6.

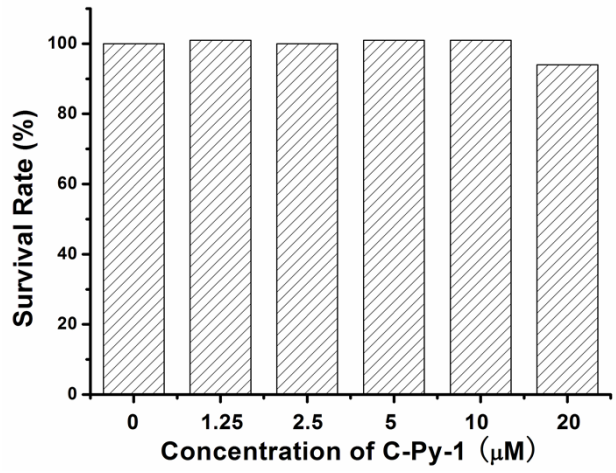
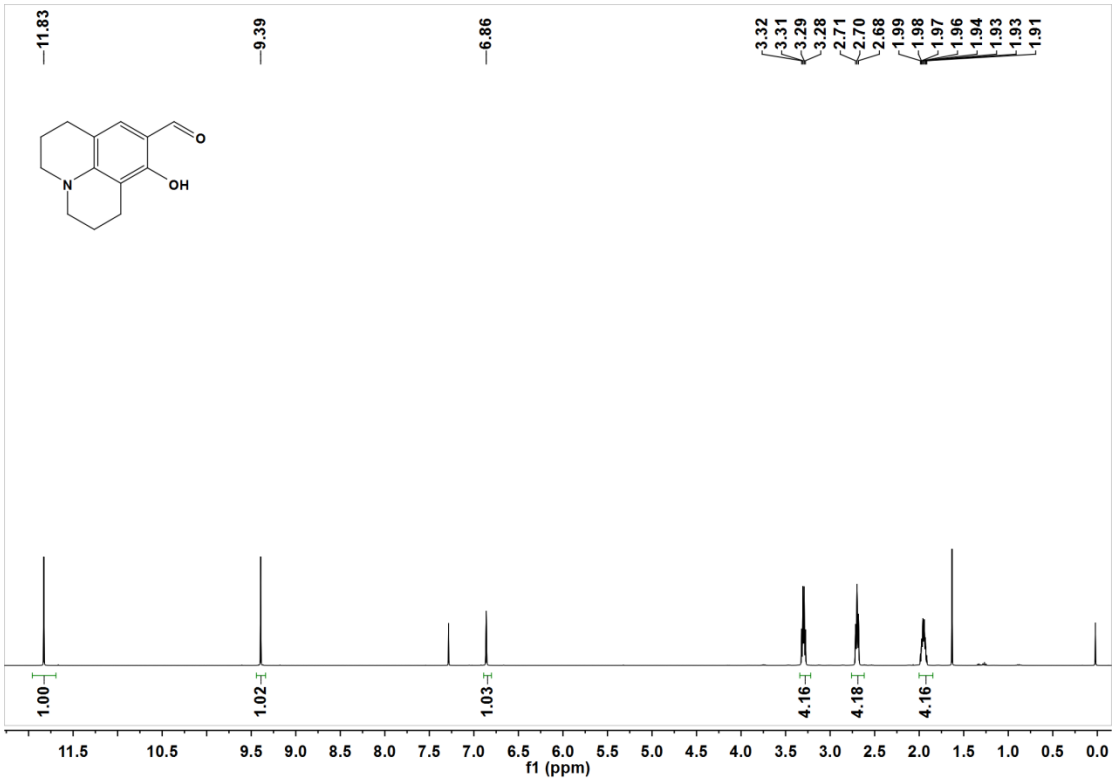
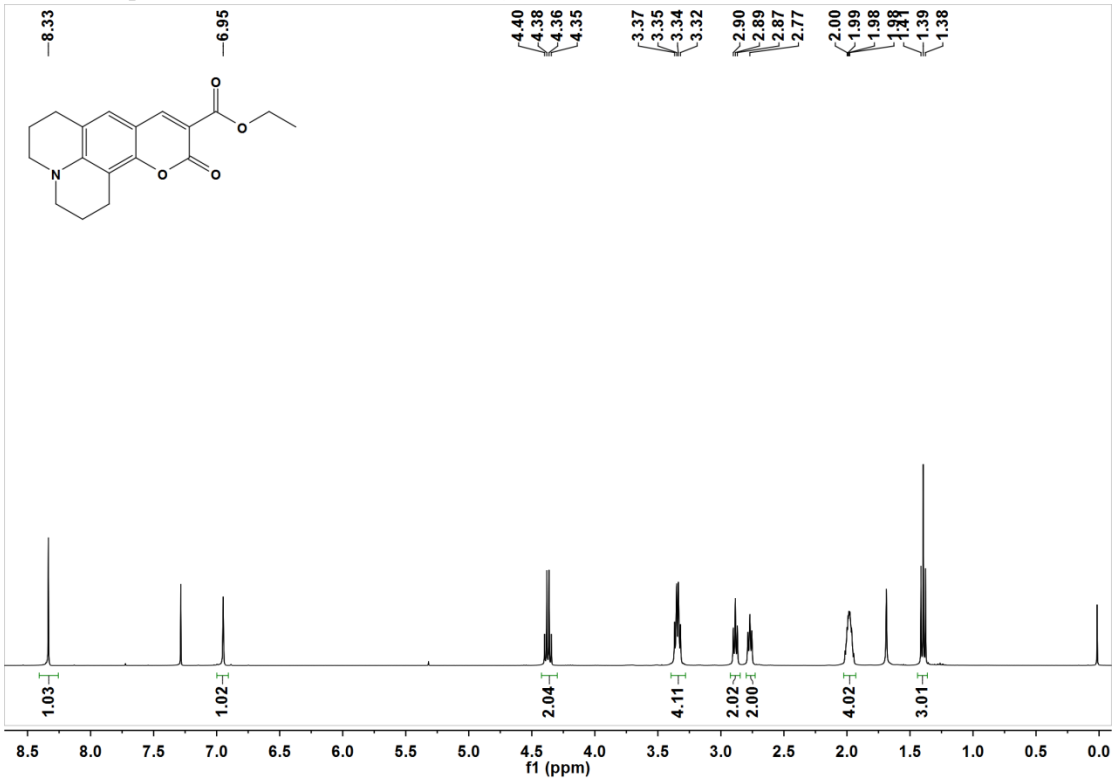


Figure S12. Effects of **C-Py-1** at varied concentrations on the viability of HeLa cells. The results are the mean standard deviation of three separate measurements.

^{1}H-NMR Spectrum of **1** in $CDCl_3$ (400 MHz):

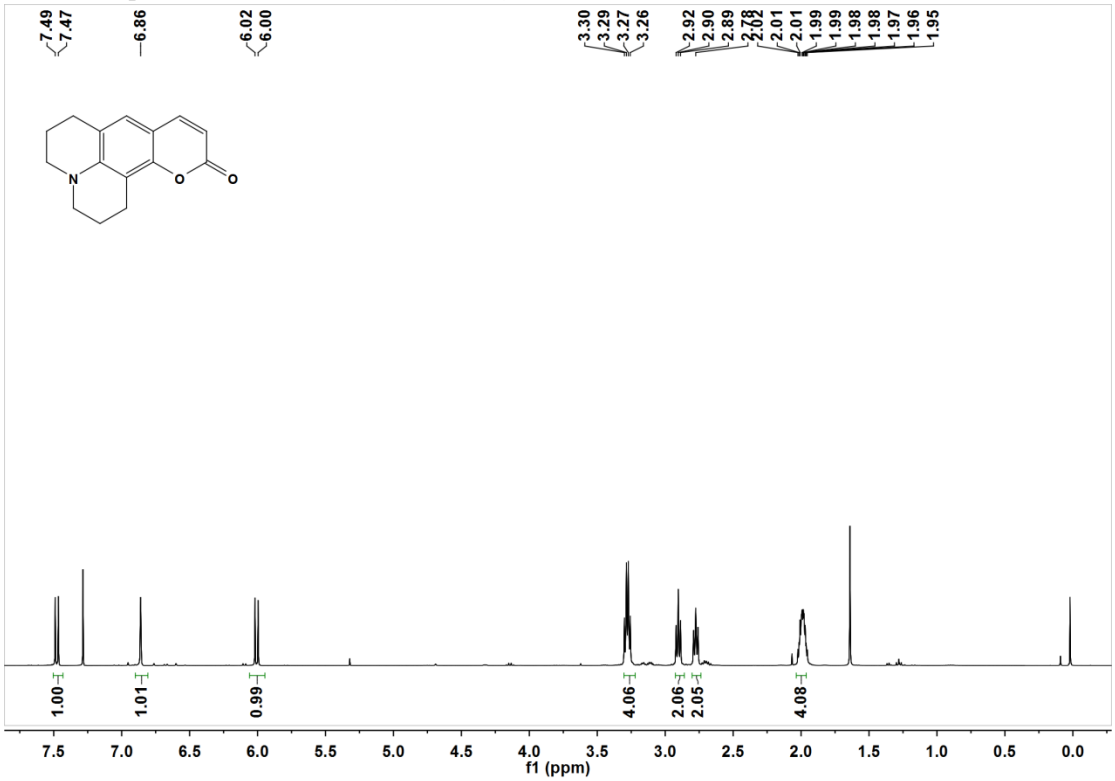


^{1}H-NMR Spectrum of **2** in $CDCl_3$ (400 MHz):

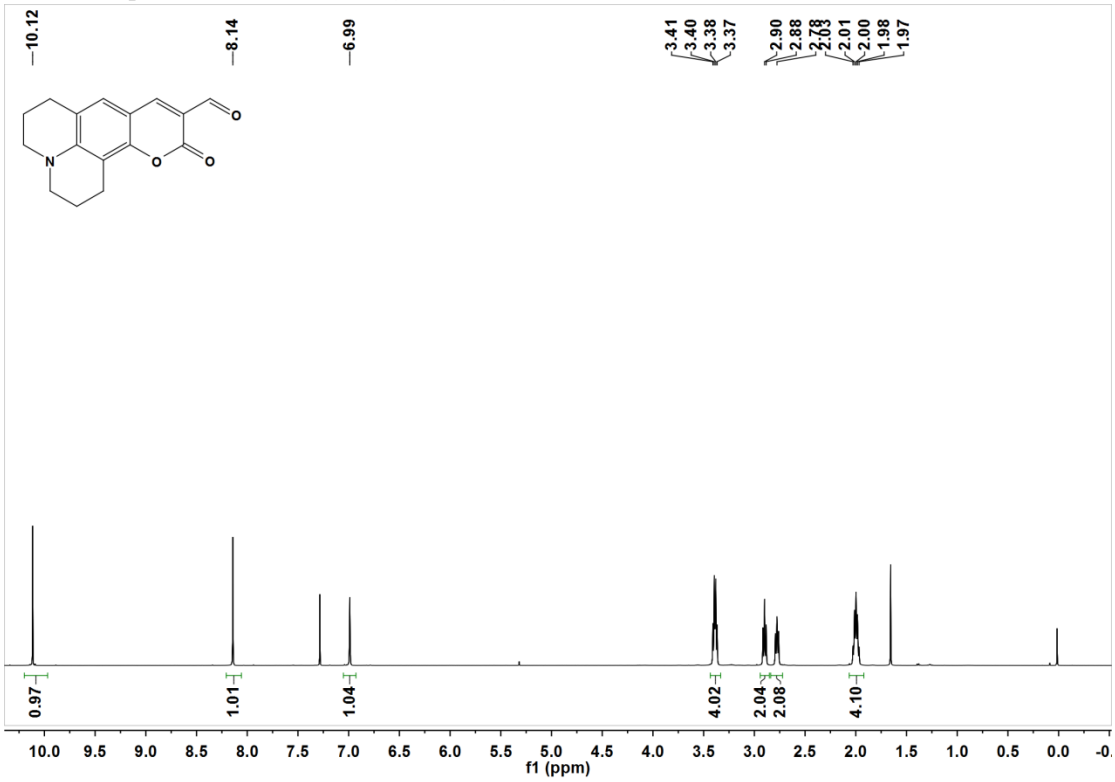


1

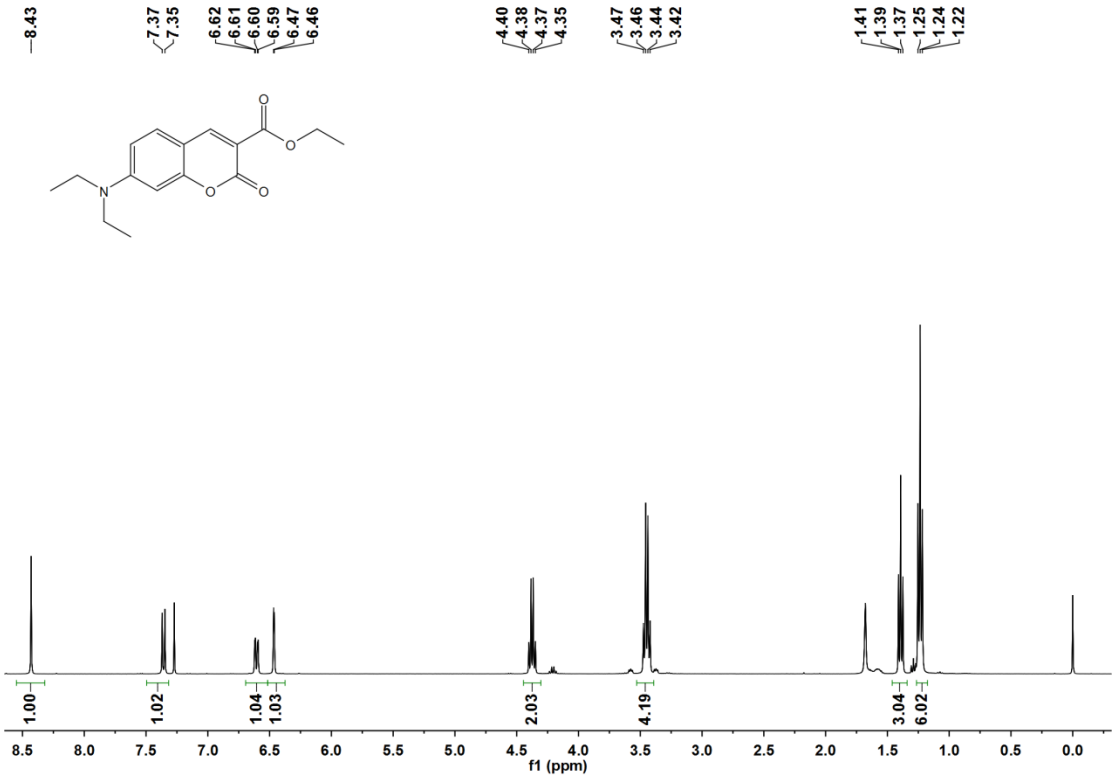
^{1}H-NMR Spectrum of **3** in $CDCl_3$ (400 MHz):



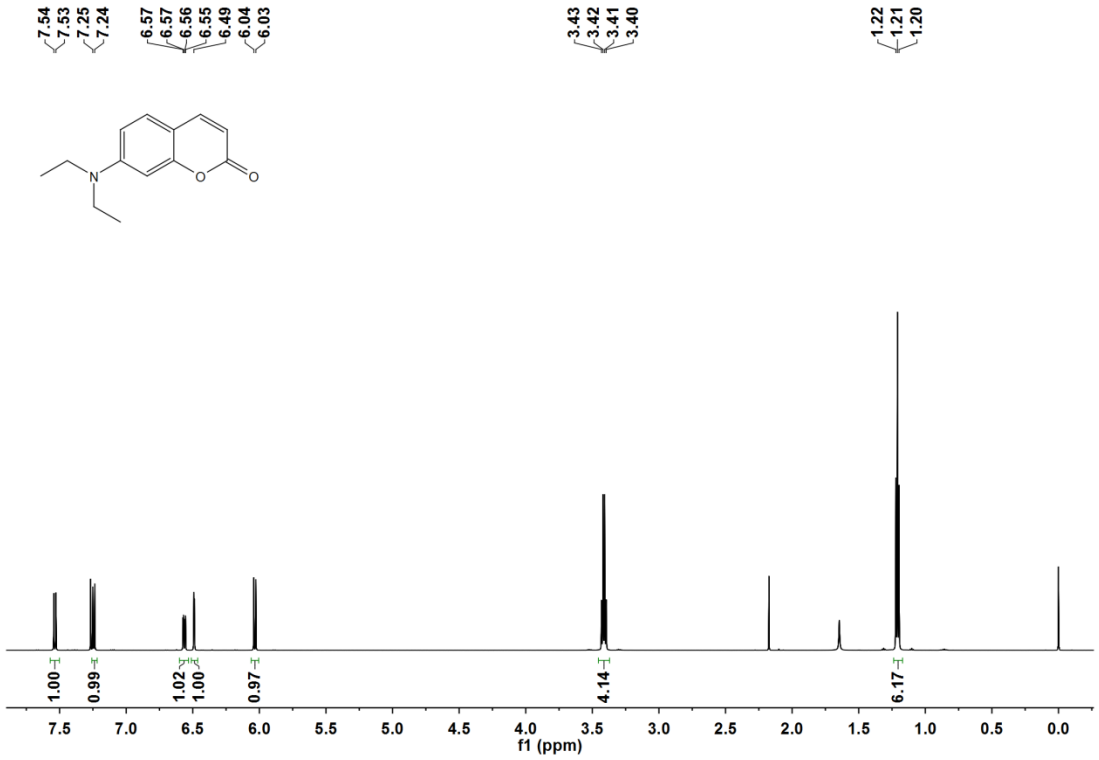
^{1}H-NMR Spectrum of **4** in $CDCl_3$ (400 MHz):



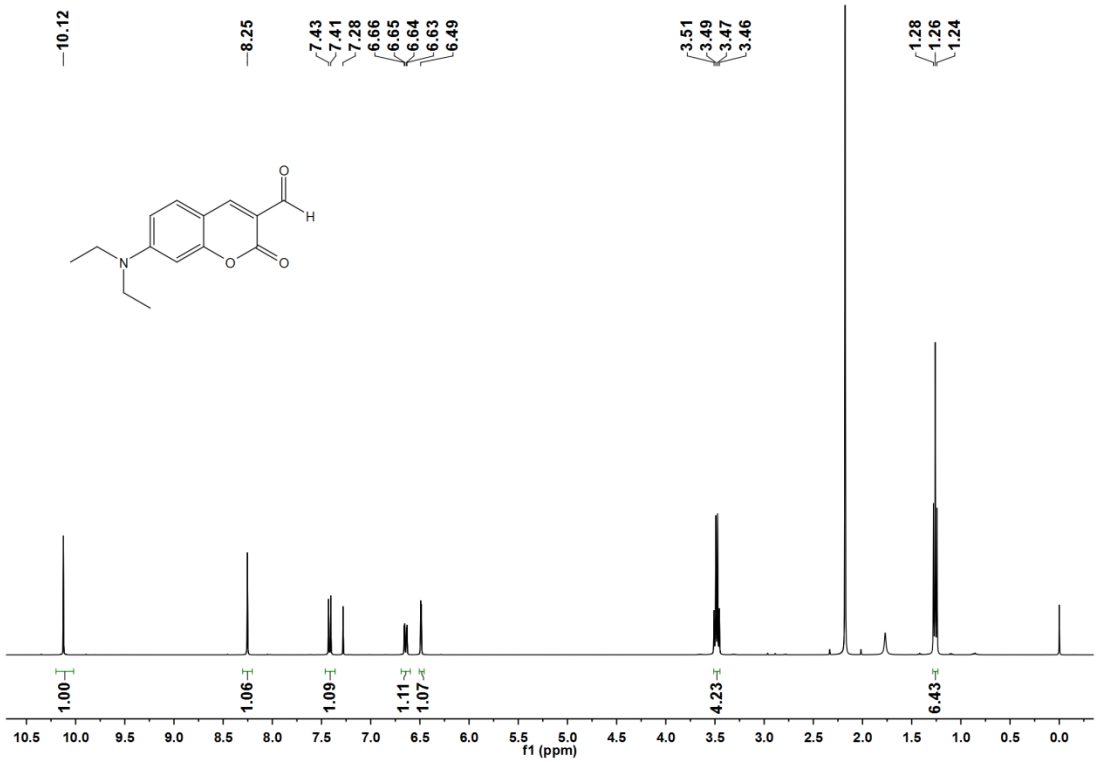
^{1}H-NMR Spectrum of **5** in $CDCl_3$ (400 MHz):



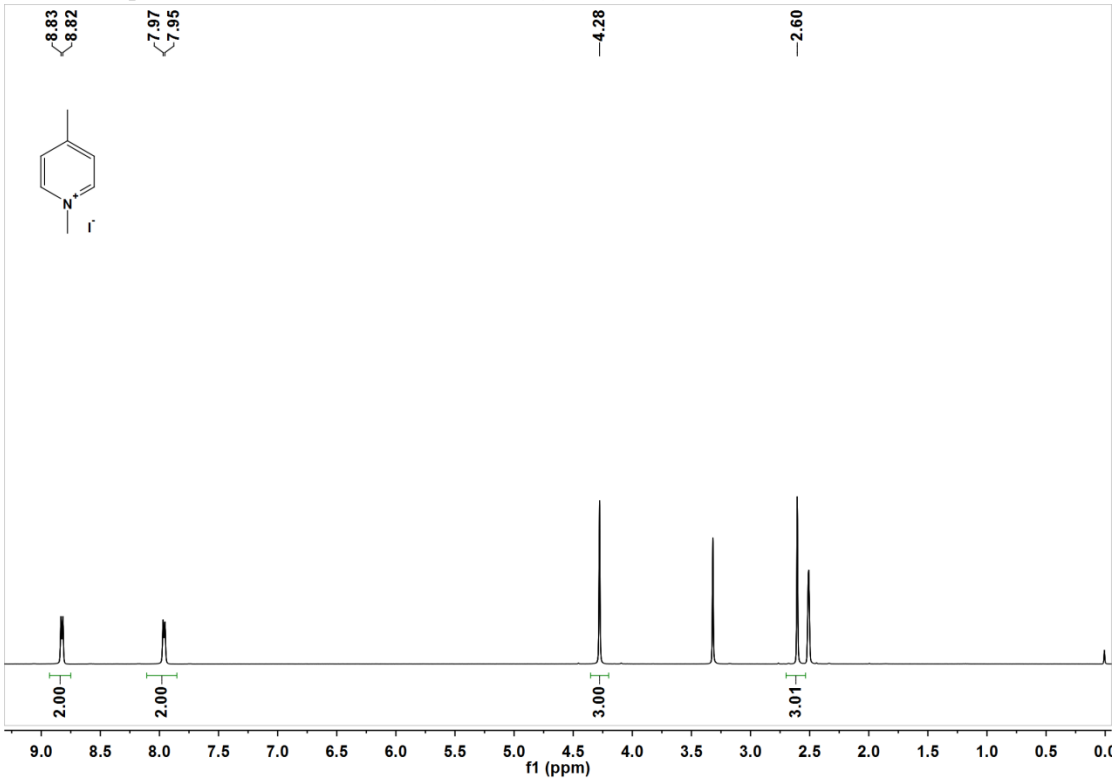
^{1}H-NMR Spectrum of **6** in $CDCl_3$ (600 MHz):



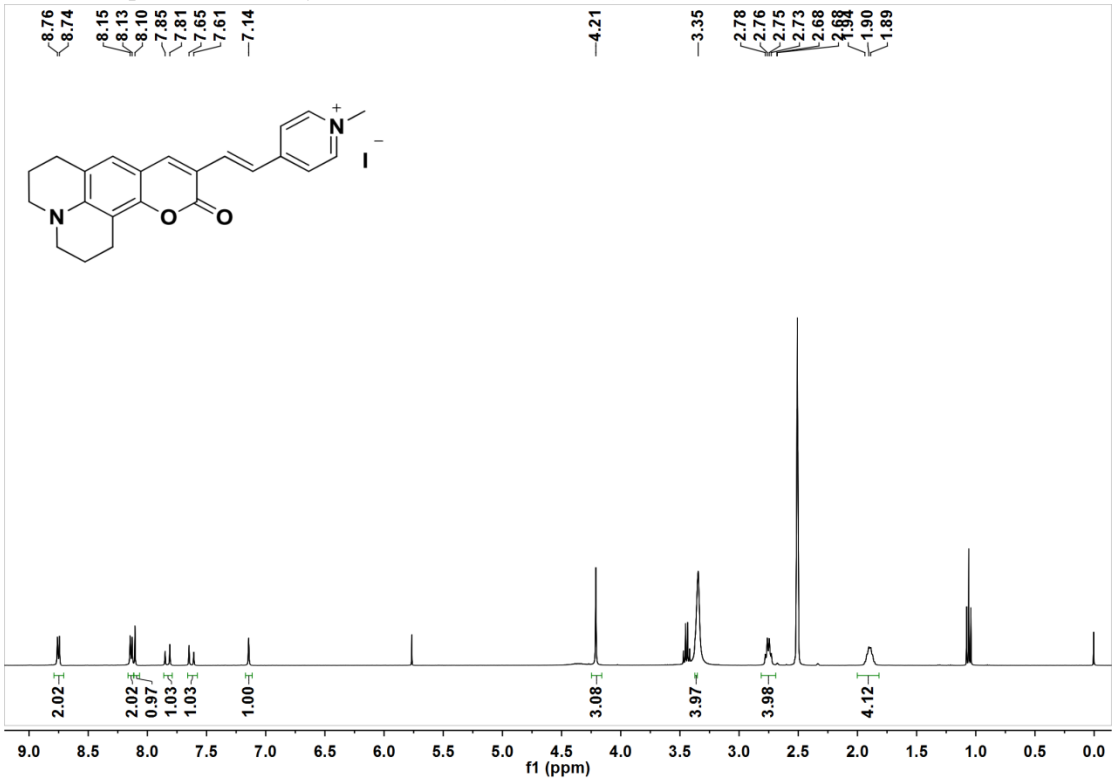
^{1}H-NMR Spectrum of **7** in $CDCl_3$ (400 MHz):



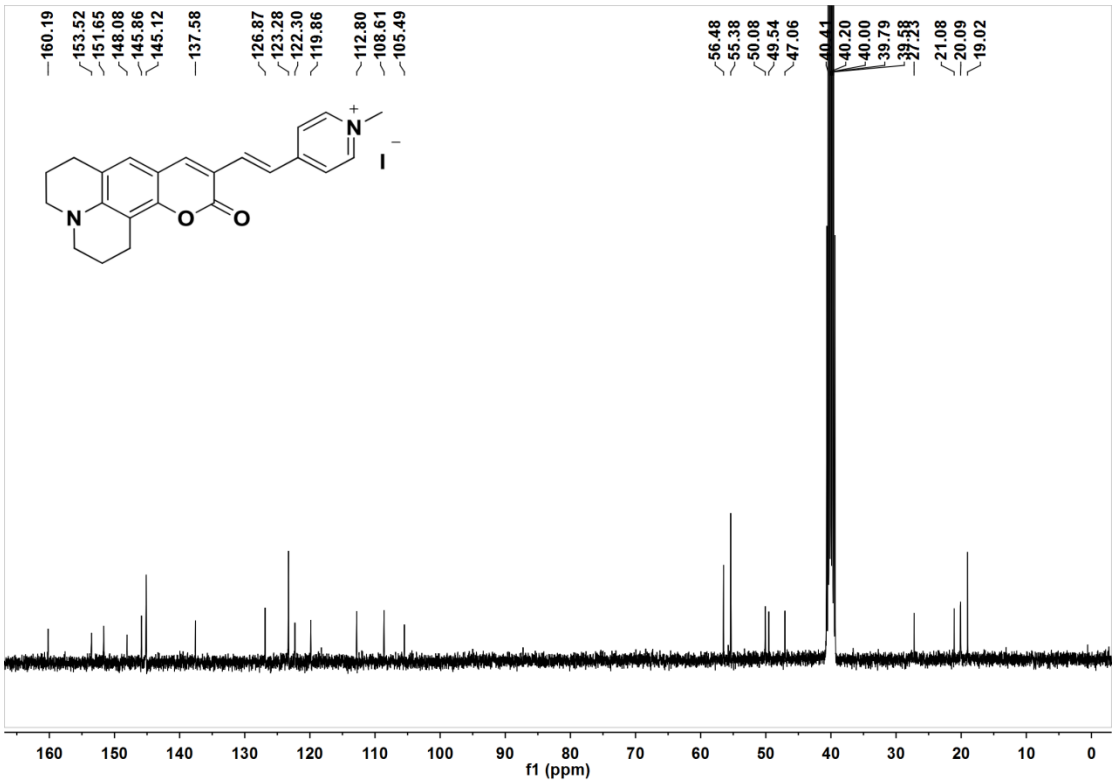
^{1}H-NMR Spectrum of **8** in DMSO-d_6 (400 MHz):



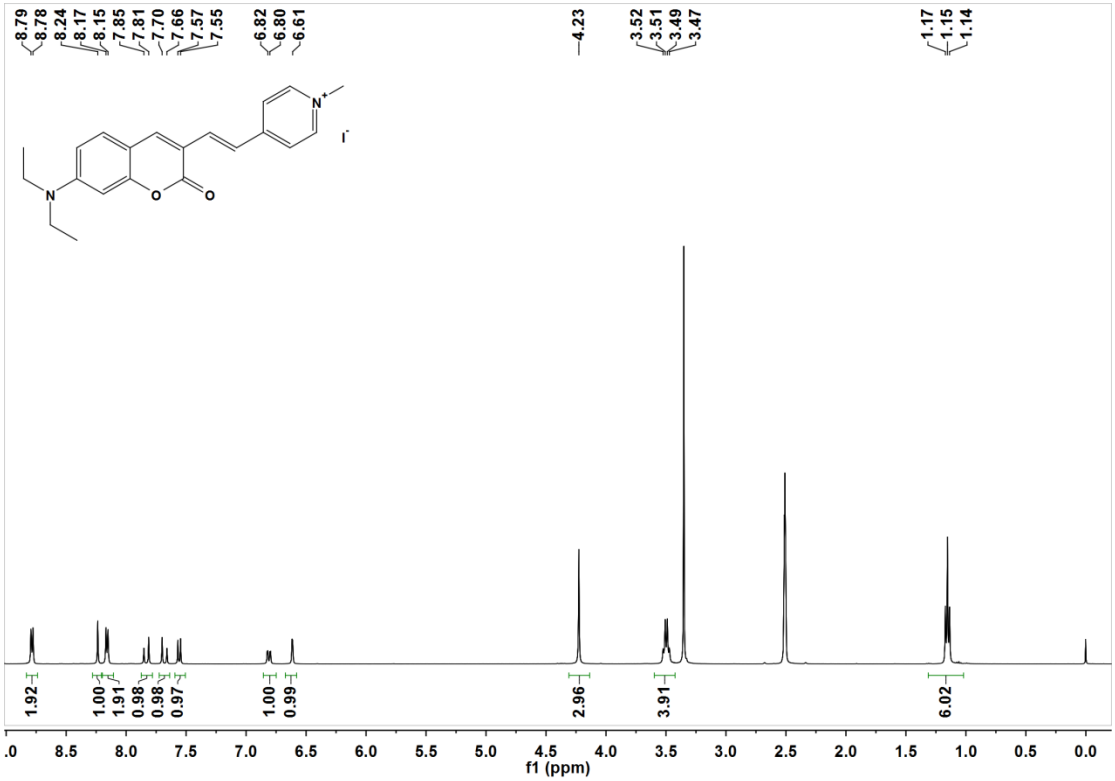
^{1}H-NMR Spectrum of **C-Py-1** in DMSO-d_6 (400 MHz):



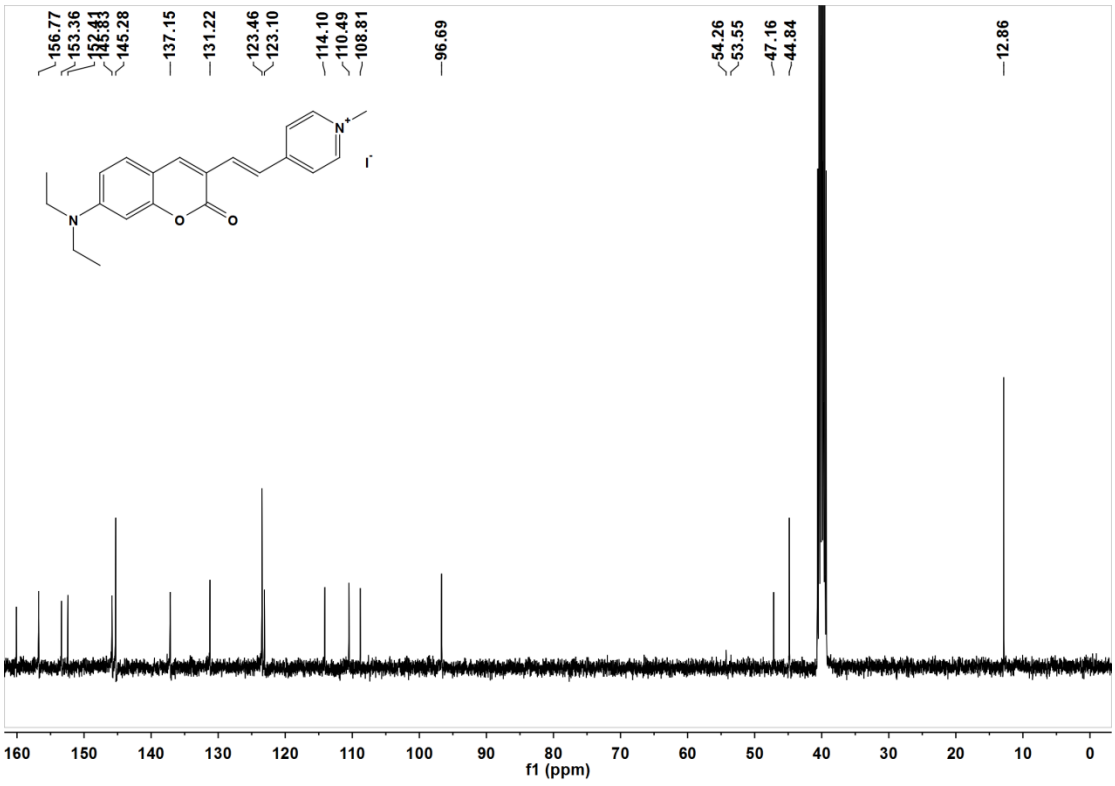
^{13}C-NMR Spectrum of **C-Py-1** in DMSO-d_6 (100 MHz)



^{1}H-NMR Spectrum of **C-Py-2** in DMSO-d_6 (400 MHz):

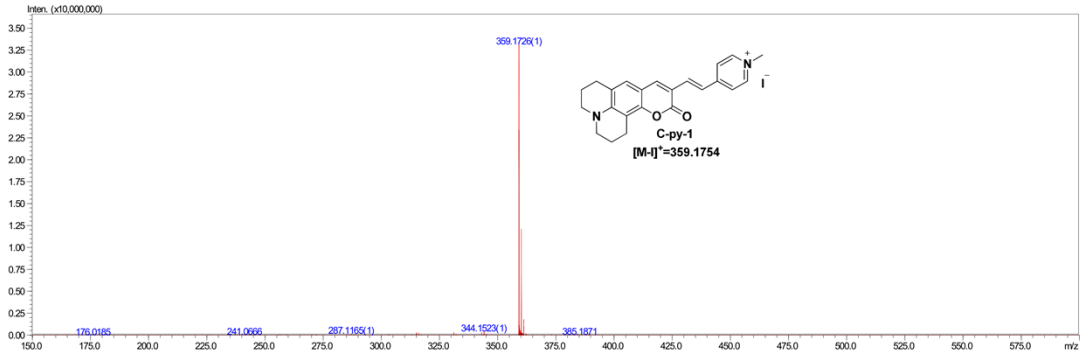


^{13}C-NMR Spectrum of **C-Py-2** in DMSO-d_6 (100 MHz)



HRMS spectra of **C-Py-1**:

Event#: 1 MS(E+) Ret. Time : 0.970 - 0.020 -> 0.302 Scan# : 195 - 5 -> 61



HRMS spectra of **C-Py-2**:

Event#: 1 MS(E+) Ret. Time : 0.850 - 0.160 -> 0.310 Scan# : 171 - 33 -> 63