Activity-based Two-photon Fluorescent Probe for Real-time and Reversible Imaging of Oxidative Stress in Rat Brain

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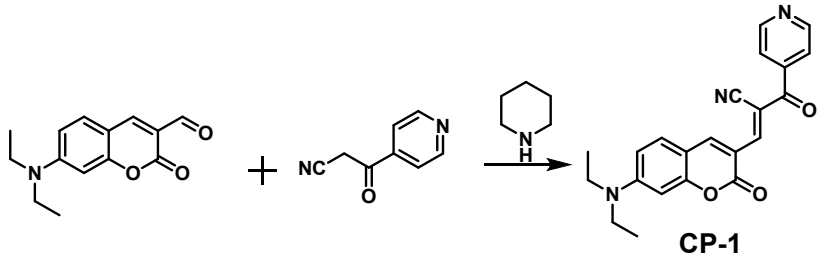
General

All reagents used for chemical synthesis were purchased from MedChemExpress(USA), Sigma-Aldrich (USA), or Heowns (China) and used as received. ^{1}H NMR spectra was recorded on an AVANCE III 400 HD spectrometer at room temperature. All absorption and fluorescence measurements were performed in Dulbecco's phosphate-buffered saline (DPBS) (pH = 7.4) buffer. Flow cytometry was performed on Beckman Coulter CytoFLEX. HeLa and SH-SY5Y cells were obtained from National Infrastructure of Cell Line Resources (Beijing). Cells were maintained in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Life Technologies) at 37 ^{o}C in the presence of 5% CO_2. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committees (IACUC) at National Center for Nanoscience and Technology of China (NCNST).

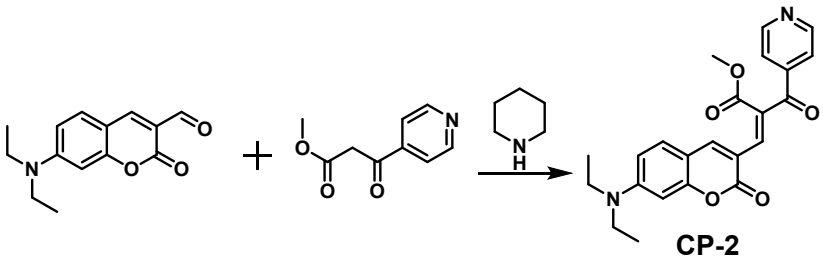
Synthesis of 3-(7-(diethylamino)-2-oxo-2H-chromen-3-yl)-2-isonicotinoylacryloyl cyanide (CP-1).



CP-1

Diethylaminocoumarin-3-aldehyde[1] (0.2 mM), 3-oxo-3-(pyridin-4-yl)propanenitrile (0.2 mM), and piperidine (0.2 mM) were mixed in ethanol and stirred for 10 h at room temperature. The crude product was purified by flash chromatography on a silica gel column using petroleum ether/ethyl acetate (1/1) as the eluent. Yield: 40%. ^{1}H NMR (400 MHz, DMSO-d_6): 8.82 (s, 1H,), 8.81 (d, 2H), 8.07 (s, 1H), 7.65 (m, 3H), 6.87 (d, 1H), 6.69 (s, 1H), 3.95 (q, 4H), 1.11 (t, 6H). ^{13}C NMR (400 MHz, DMSO-d_6): δ 12.9, 45.3, 97.3, 105.2, 109.0, 109.7, 111.8, 117.3, 122.5, 133.4, 144.1, 145.1, 150.3, 154.7, 158.4, 160.6, 189.7. ESI-MS m/z: calculated for **CP-1** ($C_{22}H_{19}N_3O_3$) $[M+H]^+$ 374.1, found 374.1, calculated $[M+Na]^+$ 396.1, found 396.1.

Synthesis of methyl -3-(7-(diethylamino)-2-oxo-2H-chromen-3-yl)-2-isonicotinoylacrylate (CP-2).

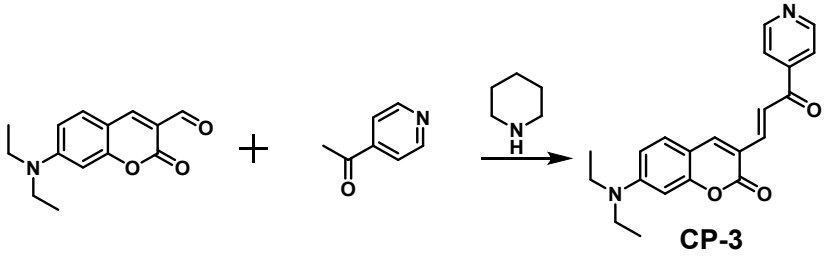


CP-2

Diethylaminocoumarin-3-aldehyde (0.2 mM), methyl 3-oxo-3-(pyridin-4-yl)propanoate (0.2 mM), and

piperidine (0.2 mM) were mixed in ethanol and stirred for 10 h at room temperature. The crude product was purified by flash chromatography on a silica gel column using petroleum ether/ethyl acetate (1/1) as the eluent. ^{1}H NMR (400 MHz, DMSO-d_6): 8.77 (d, 2H,), 8.10 (s, 1H), 7.81(s, 1H), 7.69 (d, 2H), 7.43(d, 1H), 6.76 (d, 1H), 6.48 (s, 1H), 3.66 (s, 3H), 3.43 (q, 4H), 1.11 (t, 6H). ^{13}C NMR (400 MHz, DMSO-d_6): δ 12.1, 44.85, 52.89, 96.61, 108.3, 110.6, 111.6, 121.9, 127.3, 131.6, 139.1, 143.7, 148.1, 151.1, 152.9, 157.2, 159.7, 165.7, 192.9. ESI-MS m/z: calculated for **CP-2** ($C_{23}H_{22}N_2O_5$) $[M+H]^+$ 407.1, found 407.1, calculated $[M+Na]^+$ 429.1, found 429.1.

Synthesis of 7-(diethylamino)-3-(3-oxo-3-(pyridin-4-yl)prop-1-en-1-yl)-2H-chromen-2-one (CP-3).



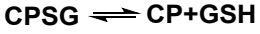
Diethylaminocoumarin-3-aldehyde (0.2 mM), 1-(pyridin-4-yl)ethan-1-one (0.2 mM), and piperidine (0.2 mM) were mixed in ethanol and stirred for 10 h at room temperature. The crude product was purified by flash chromatography on a silica gel column using petroleum ether/ethyl acetate (1/1) as the eluent. ^{1}H NMR (400 MHz, DMSO-d_6): 8.84 (d, 2H), 8.51(s, 1H), 7.95 (d, 1H), 7.86 (d, 2H), 7.72 (d, 1H), 7.52 (d, 1H), 3.50 (s, 3H), 3.33 (q, 4H), 1.15 (t, 6H). ^{13}C NMR (400 MHz, DMSO-d_6): δ 12.8, 44.8, 96.7, 108.9, 110.6, 113.2, 120.7, 121.8, 129.1, 131.4, 141.7, 144.6, 146.8, 151.2, 152.7, 157.1, 189.5. ESI-MS m/z: calculated for **CP-3** ($C_{21}H_{20}N_2O_3$) $[M+H]^+$ 349.1, found 349.2, calculated $[M+Na]^+$ 371.1, found 371.1.

Chemical and photo-physical study of CPs in the presence of GSH

To characterize the absorption and fluorescence change of different **CP** probes in the presence of GSH, and its potential for selective GSH detection, the different **CP** probes (40 μM in DPBS) were mixed with varied concentrations of GSH, increasing from 0 to 6 mM at room temperature. The absorption and fluorescence spectra of above mixtures were recorded after 30 min. of incubation.

To determine the reaction kinetics of **CP** probes with GSH, the fluorescence spectra of the mixture of 40 μM **CP** probes and 4 mM GSH were monitored and compared at different time (every 30 sec. for **CP-1**, every one min. for **CP-2**, and every 5 min. for **CP-3**).

Determination of K_d for GSH and CPs adduct



$$K_d = \frac{[CP]\,[GSH]}{[CPSG]} \quad \text{[Eq. 1]}$$

$$[CPSG] = [CP]_0 - [CP] \quad \text{[Eq. 2]}$$

$$[GSH] = [GSH]_0 - [CPSG] \quad \text{[Eq. 3]}$$

To calculate the dissociation equilibrium constant, the concentration of **CP**s in the reaction mixtures was determined using Beer-Lambert's Law, the concentration of **CPSG** and GSH in the reaction mixtures was calculated according to Eq. 2 and Eq. 3, respectively. The dissociation equilibrium constant was calculated according to Eq. 1.

Measuring the concentrations of CPs and CPSGs

10 mM **CP**s were diluted with DPBS to different concentrations, followed by absorption spectra measurement to generate a standard curve. In order to measure the concentration of **CP**s in a reaction mixture of **CP**s and GSH, the absorption spectra of 40 μM **CP**s and 4 mM GSH mixture was recorded, the concentration of **CP**s in the mixture was calculated according to the Beer-Lambert's Law. The concentration of **CPSG**s was calculated by subtracting remained **CP**s from 40 μM of total **CP**s before GSH addition. The dissociation equilibrium constant was then calculated according to Eq. 1.

Reversible and Selective GSH detection using CP-2

In order to study the reversibility of **CP-2** for GSH detection, **CP-2** (40 μM in DPBS) was first incubated with 4 mM GSH, and then added with 8 mM NEM. The absorption and fluorescence spectra of above mixture were recorded at indicated time until a plateau was observed.

To demonstrate the selectivity of **CP-2** for GSH detection, 40 μM **CP-2** was incubated with 1 mM GSH, 100 μM other biological thiols, 100 μM amino acids, 100 μM reactive oxygen or nitrogen species at 37 ^{o}C for 30 min., The fluorescence emission of above mixture was measured and compared to that of **CP-2** alone.

Cytotoxicity study of CP-2

HeLa cells were cultured and sub-seeded in 48-well plate at a density of 50,000 cells per well a day before the experiment. The cells were treated with different concentrations of **CP-2** for 10 h, followed by the cell viability measurement using Alamar Blue assay.

Intracellular GSH imaging using CP-2

HeLa cells were cultured and sub-seeded in a 35 mm glass-bottomed dish a day before the experiment. Cells were pre-treated with 1 mM NEM or 150 μM GSH-OEt supplied in DMEM for 30 min., and subsequently incubated with **CP-2** (20 μM) for 15 min. before CLSM imaging. The CLSM images were captured simultaneously at excitation of 405 nm and 488 nm with emission at channel 1 (500 - 550 nm) and channel 2 (620 - 660 nm) respectively.

To quantify the fluorescence change and intracellular GSH fluctuation, HeLa cells were cultured and sub-seeded in 48-well plate at a density of 50,000 cells per well a day before the experiment. Cells were pre-treated with 0.5 mM NEM or 150 μM GSH-OEt for 30 min., and then incubated with **CP-2** (20 μM) for 15 min. before flow cytometry analysis. For all flow cytometry studies, 6000 cells were counted and analyzed.

Real-time and reversible imaging of intracellular GSH in living cells

To demonstrate the use of **CP-2** for intracellular GSH imaging in real-time, HeLa cells were seeded in 35 mm glass-bottomed dish a day before the experiment. The cells were pre-treated with 20 μM **CP-2** for 15 min, washed with DPBS, and treated with 0.5 mM NEM before CLSM imaging at indicated time. The fluorescence intensity measured at indicated time was normalized to that of cells without NEM treatment.

To study the reversible GSH imaging using CP-2, HeLa cells were pre-incubated with 20 μM **CP-2** for 15 min., followed by the treatment of different concentrations of NEM for 30 min. The fluorescence image and intensity of treated cells were determined at excitation of 405 nm and 488 nm with emission at channel 1 (500 - 550 nm) and channel 2 (620 - 660 nm) respectively. To quantify intracellular GSH fluctuation, the fluorescence intensity ratio (F_{530}/F_{650}) of NEM treated cells was calculated, and compared to cells without any treatment.

Fluorescent imaging of neural cell GSH

For the intracellular imaging of GSH in neural cells, SH-SY5Y cells were cultured and sub-seeded on 35 mm glass-bottomed dish a day before the experiment. Cells were pre-treated with 1 mM NEM or 150 μM GSH-OEt supplied in DMEM for 30 min., and then incubated with **CP-2** (20 μM) for 15 min. before CLSM imaging. The CLSM images were captured simultaneously at excitation of 405 nm and 488 nm with emission at channel 1 (500 - 550 nm) and channel 2 (620 - 660 nm) respectively.

To study the effect of neurotoxin exposure on the change of intracellular GSH and oxidative stress, SH-SY5Y cells were treated with different concentrations of MPTP for 24 h, and then incubated with **CP-2** (20 μM) for 15 min. The CLSM images were collected as described above.

Two-photon microscopy (TPM) imaging of GSH using CP-2

To demonstrate the use of **CP-2** for TPM imaging of GSH in living cells, HeLa cells were pre-treated with 1 mM NEM or 150 μM GSH-OEt supplied in DMEM for 30 min., and subsequently incubated with **CP-2** (20 μM) for 15 min. before TPM imaging. HeLa cells without pre-treatment and incubated with same concentration **CP-2** were used as a control. The TPM images were collected at on Olympus FV1200MPE, with an excitation of 860 nm through a 20x Acroplan water-immersion objective lens.

GSH imaging in live rat brain using CP-2

Rat brain slices were prepared from 2-week old SD rat according to an approved experimental protocol at NCNST. Brain slices of 300 μm-thick were prepared using a vibrating-blade microtome in artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 10 mM D-glucose, 2.4 mM $CaCl_2$, and 1.3 mM $MgSO_4$). To study the effect of thiol scavenger on the effect of GSH fluctuation, brain slices were pre-treated with or without 100 μM NEM for 40 min, and then incubated 10 μM **CP-2** in aCSF with 95% O_2 and 5% CO_2 at 37 °C for 1 h. The brain slices were then imaged according to the procedure as described above.

References

1. K. Renault, P. Y. Renard, C. Sabot, *European J. Org. Chem.,* 2018, **2018**, 6494-6498.

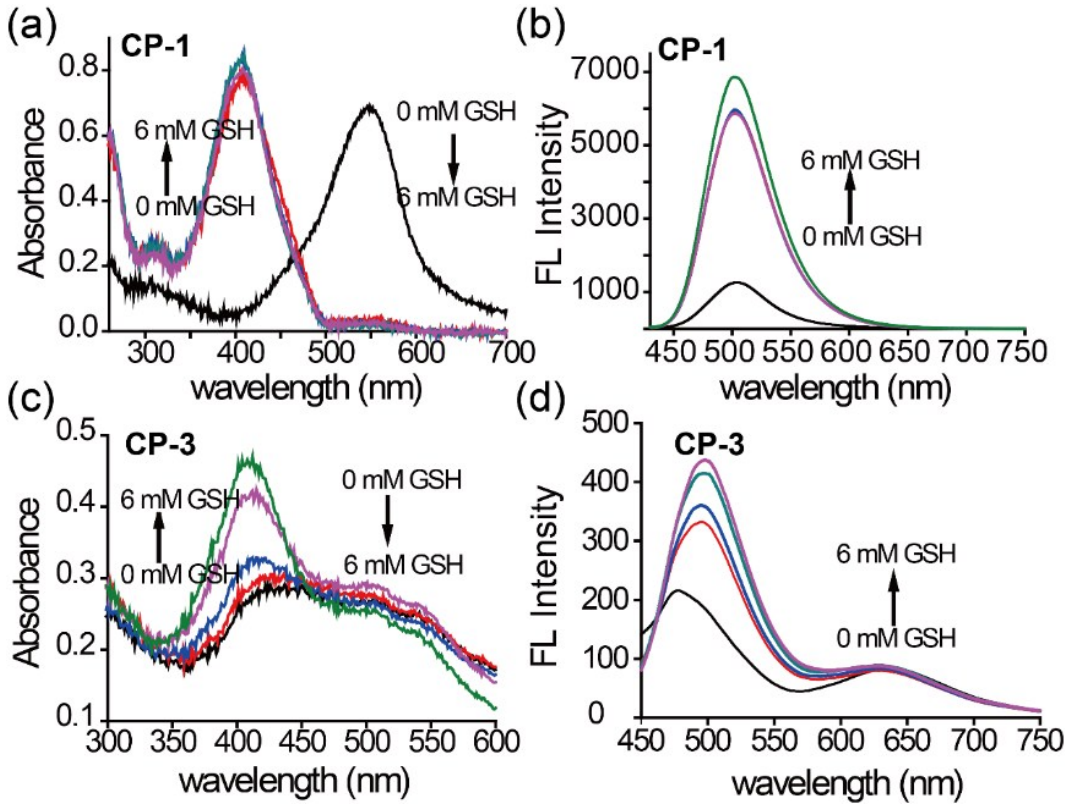


Figure S1. Absorption and fluorescence spectra of **CP-1** and **CP-3** with the addition of increased concentrations of GSH. Absorption spectra of **CP-1** (40 μM) (a) and **CP-3** (c), fluorescence spectra of **CP-1** (b) and **CP-3** (d) after GSH addition (0-6 mM) in DPBS buffer (pH = 7.4). For fluorescence measurement, the mixtures were excited at 450 nm.

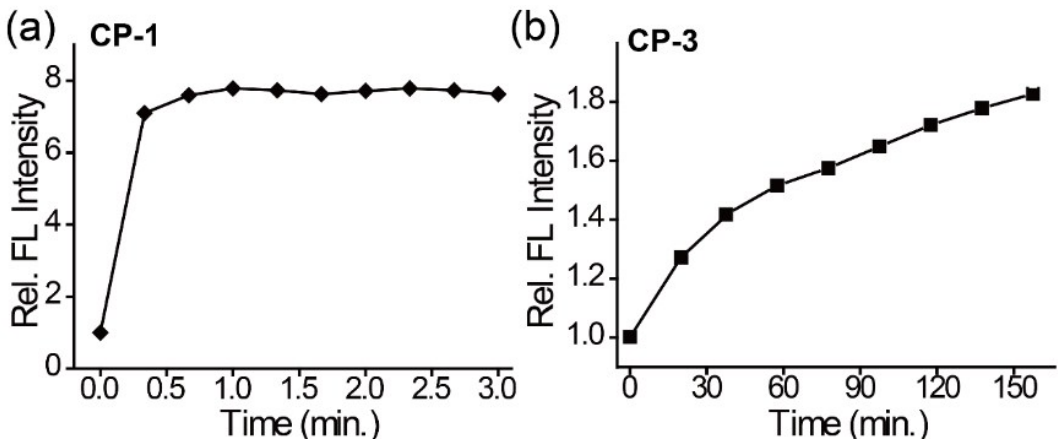


Figure S2. Time-dependent fluorescence intensity change of **CP-1** (a) and **CP-3** (b) in the presence of GSH. The normalized fluorescence intensity ratio of **CP-1** (40 μM, F_{510}/F_{650}) or **CP-3** (40 μM, F_{500}/F_{630}) after GSH (4 mM) addition.

Table S1. Comparison of the reaction kinetic parameters of **CP**s and GSH

Probe	Reaction rate constant k	Dissociation equilibrium constant $K_{d, GSH}$
CP-1	24.6 [$M^{-1}S^{-1}$]	81.8 μM
CP-2	1.01 [$M^{-1}S^{-1}$]	1.70 mM
CP-3	0.02 [$M^{-1}S^{-1}$]	3.98 mM

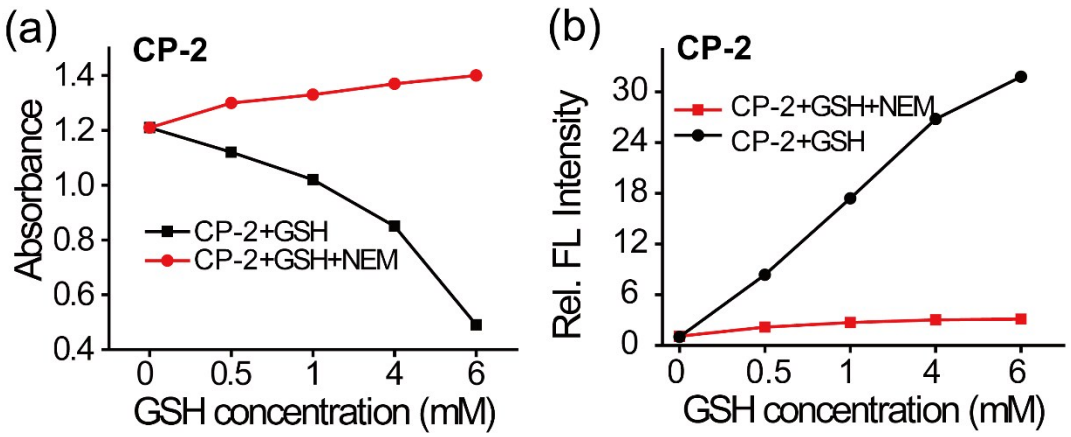


Figure S3. Absorption and fluorescence change of **CP-2** and GSH mixture with and without NEM treatment. (a) absorption (A_{470}) and (b) fluorescence intensity ratio (F_{530}/F_{650}) change of **CP-2** (40 μM) and varied concentration of GSH (0 – 6 mM) mixture with and without NEM addition (8 mM).

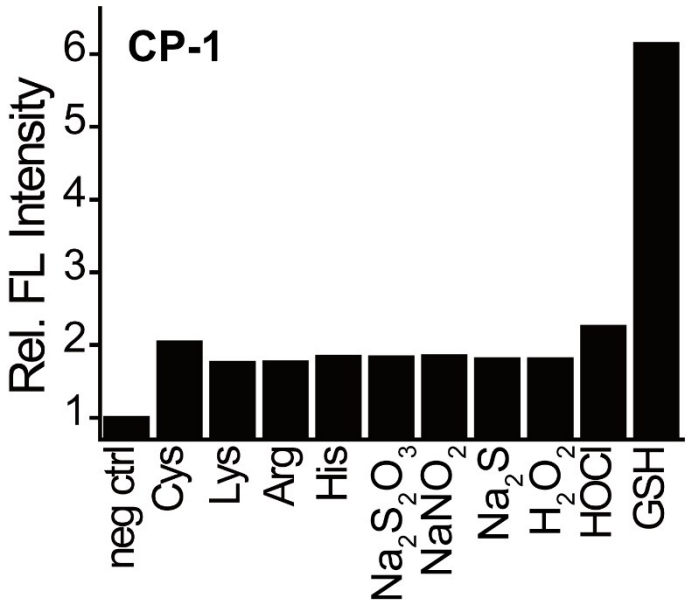


Figure S4. Selective GSH detection using **CP-1**, as evidenced by the fluorescence change of **CP-1** (F_{510}/F_{650}) in the presence of other important physiological species under their physiological conditions.

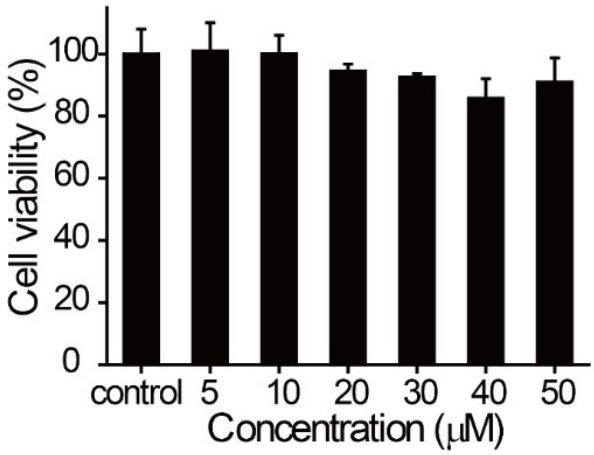


Figure S5. Cytotoxicity study of **CP-2**. HeLa cells were treated with **CP-2** at indicated concentrations for 10 h, the cell viability was then determined by Alamar Blue assay. HeLa cells without any pre-treatment were used as a control. The data was presented as mean ± SD.

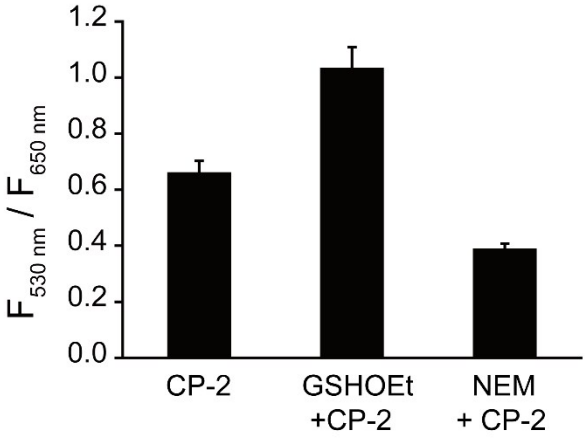


Figure S6. Cellular fluorescence intensity ratio (F_{530}/F_{650}) of HeLa cells pre-treated with 0.5 mM NEM or 125 μM GSH-OEt using **CP-2**. Scale bars, 10 μm. The data was presented as mean ± SD.

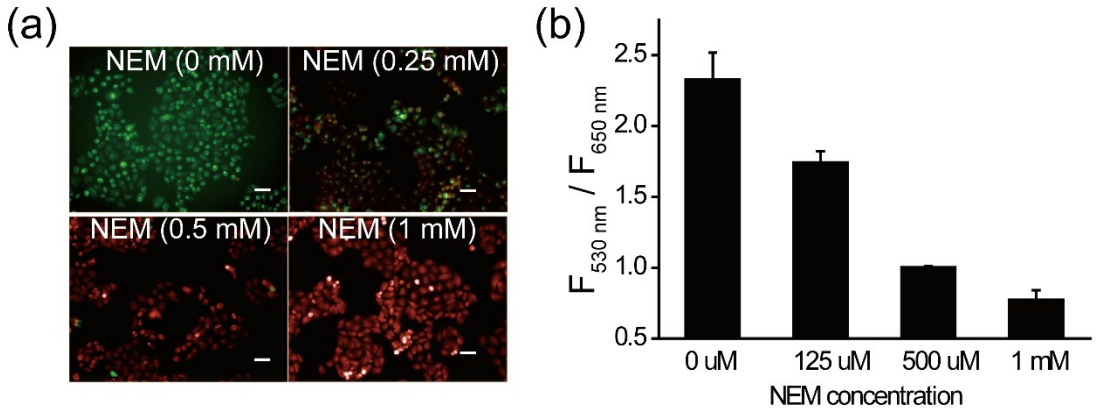


Figure S7. Overlay images of **CP-2** emission and **CPSG-2** emission (a), and fluorescent intensity ratio (F_{530}/F_{650}) (b) of HeLa cells pre-treated with **CP-2** (20 μM) in the presence of different concentrations of NEM. Scale bars, 50 μm. The data was presented as mean ± SD.

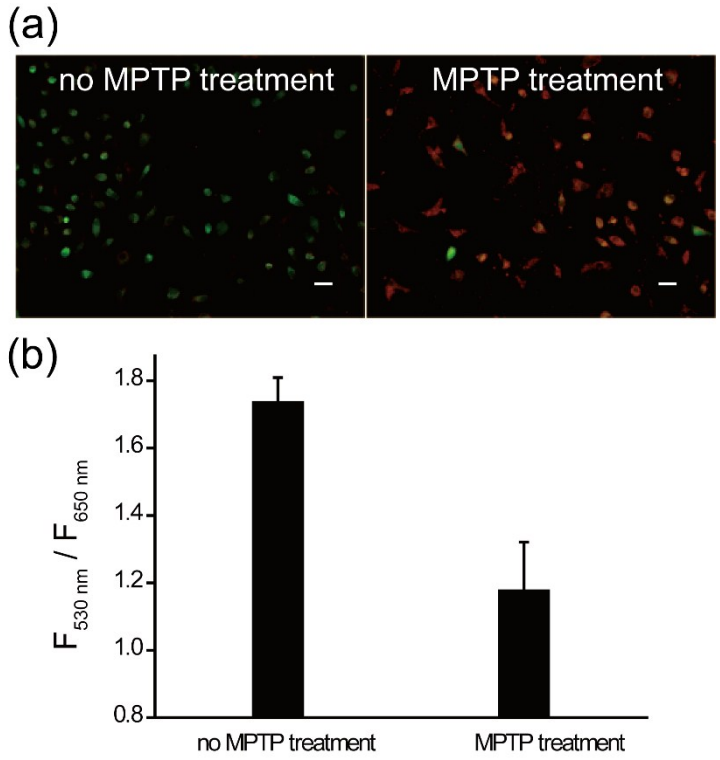


Figure S8. GSH change of SH-SY5Y cells with and without MPTP treatment, as measured by **CP-2** imaging. Overlay CLSM images of **CP-2** emission and **CPSG-2** emission (a) and fluorescent intensity ratio (F_{530}/F_{650}) (b) of SH-SY5Y cells pre-treated with and without MPTP (400 μM) before fluorescence imaging using **CP-2** (20 μM). Scale bars, 50 μm. The data was presented as mean ± SD.

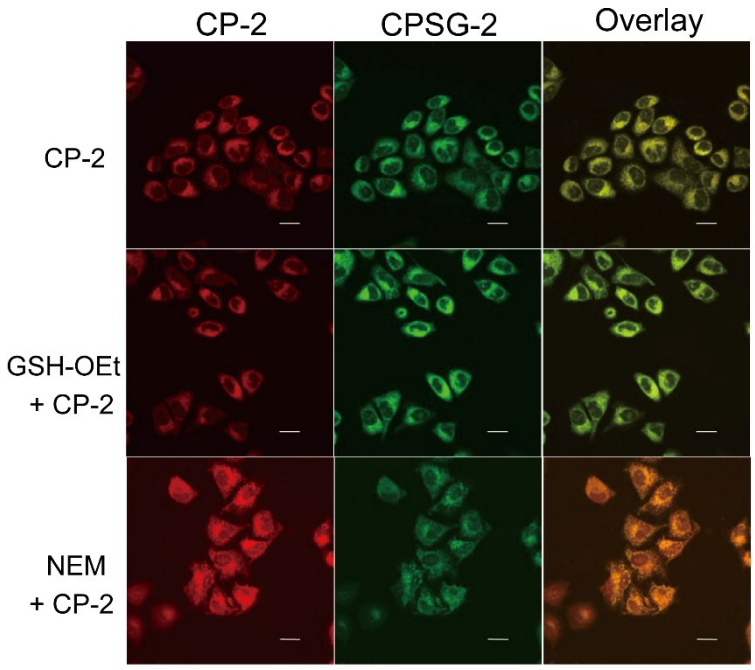


Figure S9. TPM images of intracellular GSH using **CP-2**. HeLa cells treated with either GSH-OEt (125 μM) or NEM (0.5 mM) were incubated with 20 μM **CP-2** before TPM fluorescence imaging. Scale bars, 10 μm.

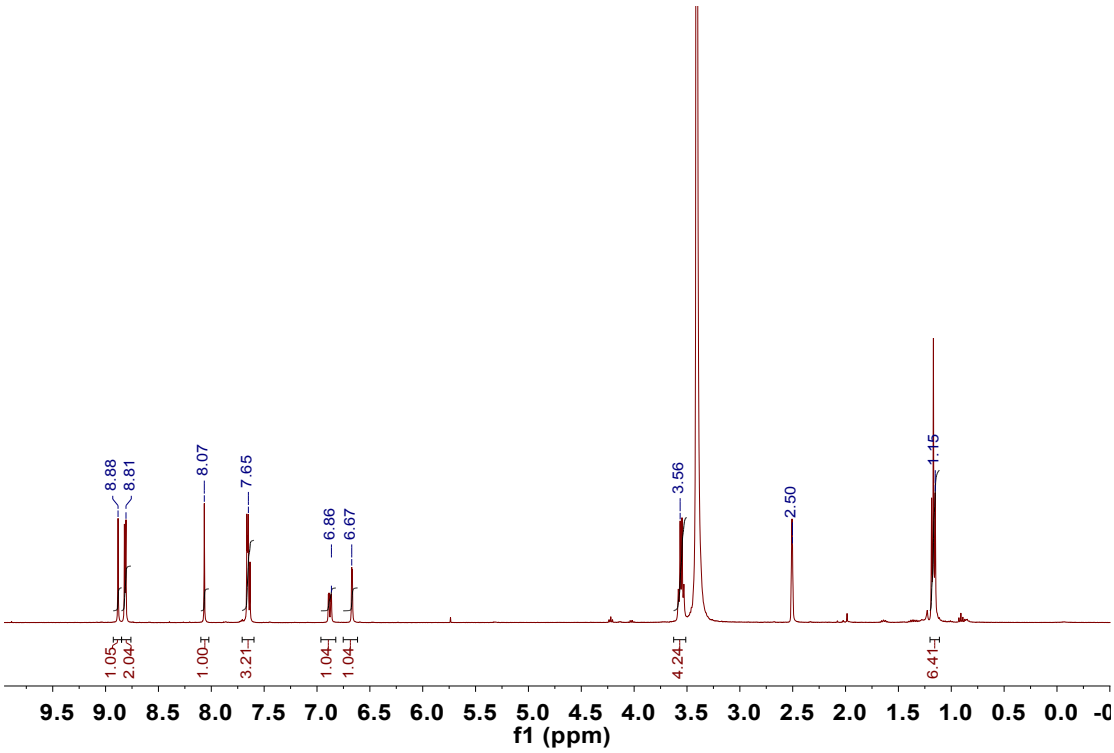


Figure S10. ^{1}H NMR spectrum of **CP-1** in DMSO-d_6 at room temperature.

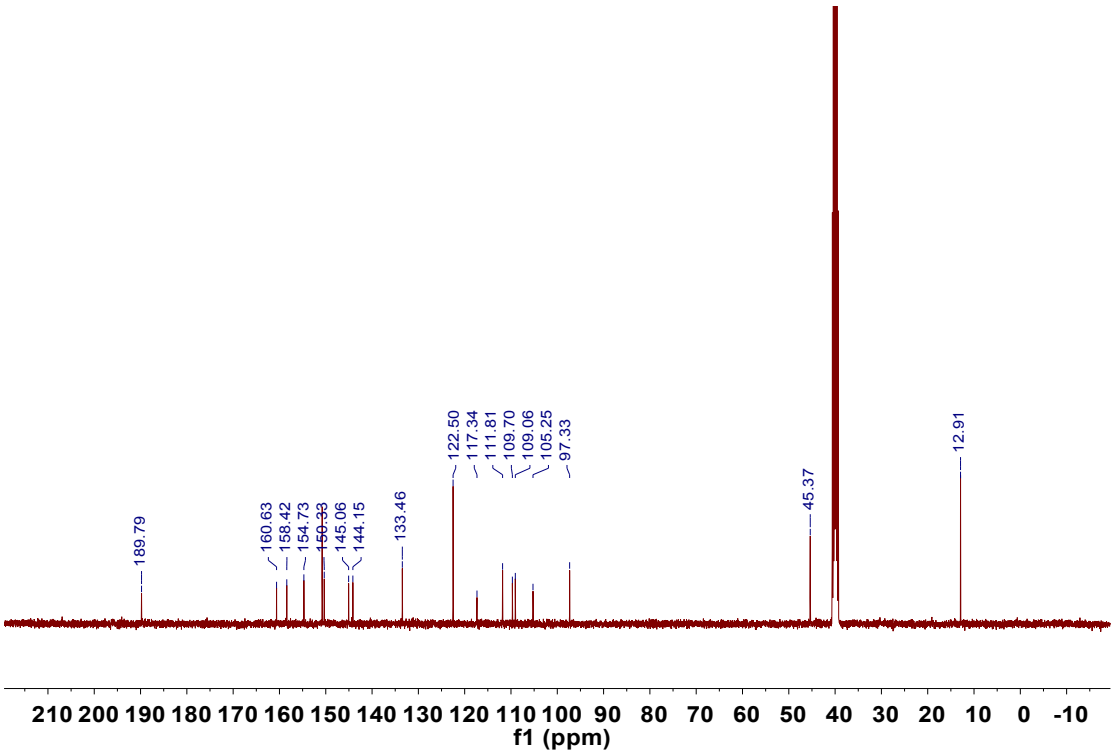


Figure S11. ^{13}C NMR spectrum of **CP-1** in DMSO-d_6 at room temperature.

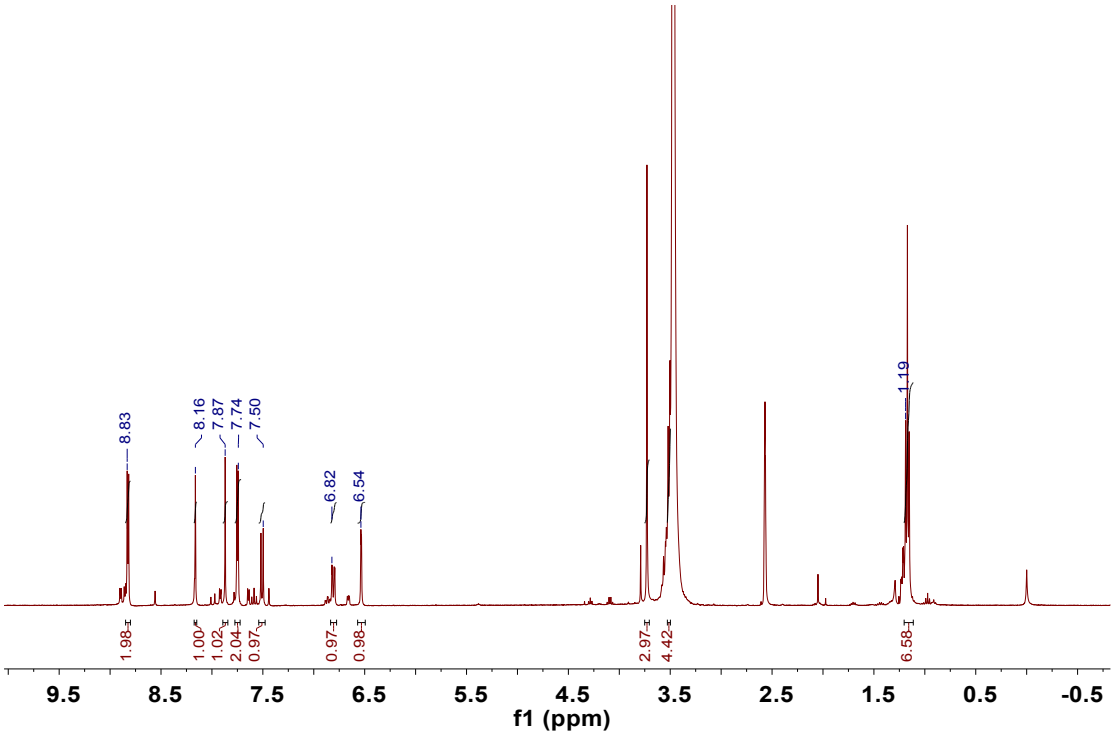


Figure S12. ^{1}H NMR spectrum of **CP-2** in DMSO-d_6 at room temperature.

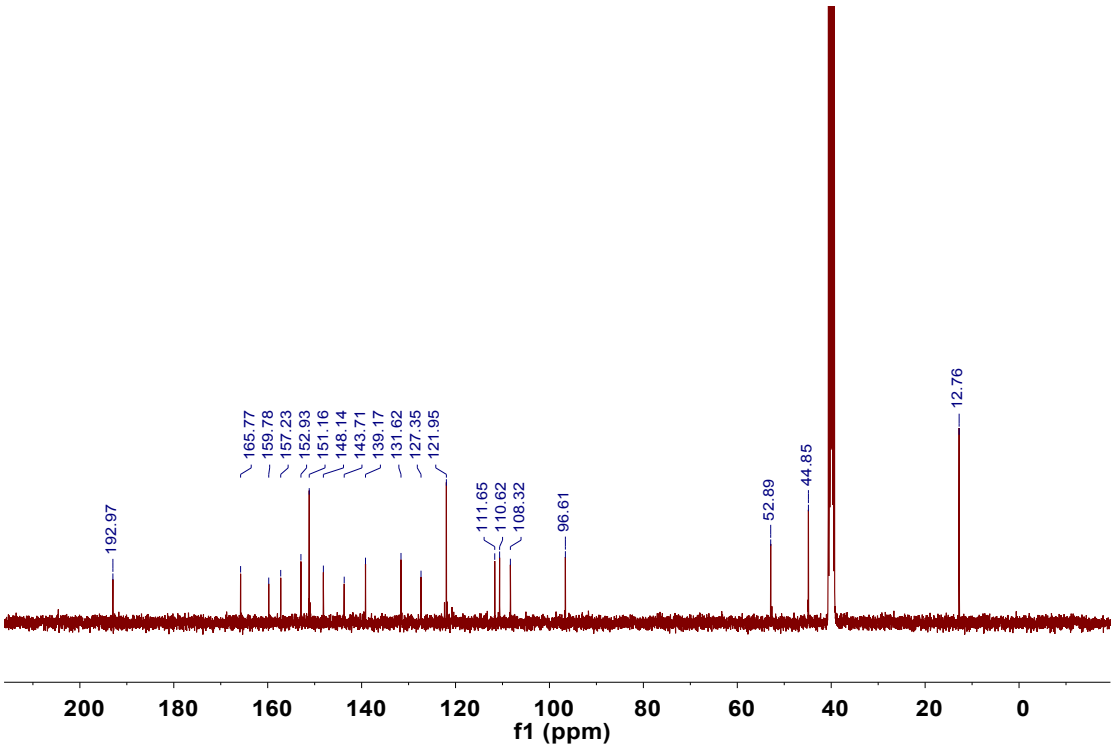


Figure S13. ^{13}C NMR spectrum of **CP-2** in DMSO-d_6 at room temperature.

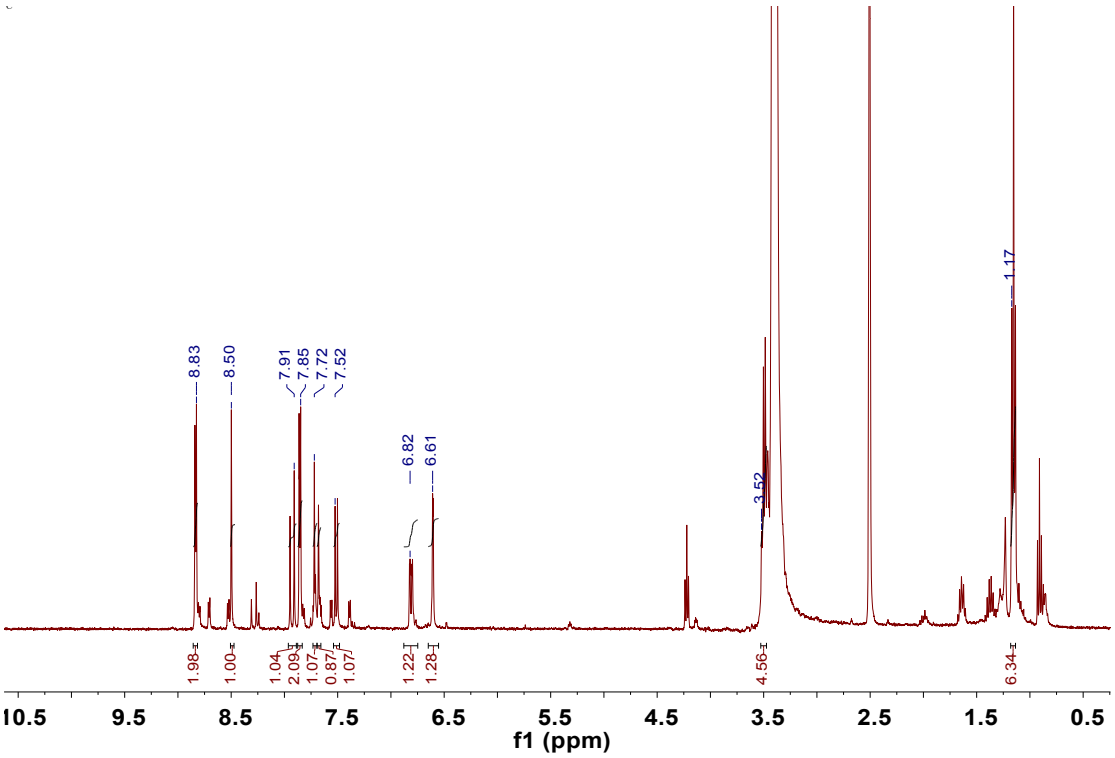


Figure S14. ^{1}H NMR spectrum of **CP-3** in DMSO-d_6 at room temperature.

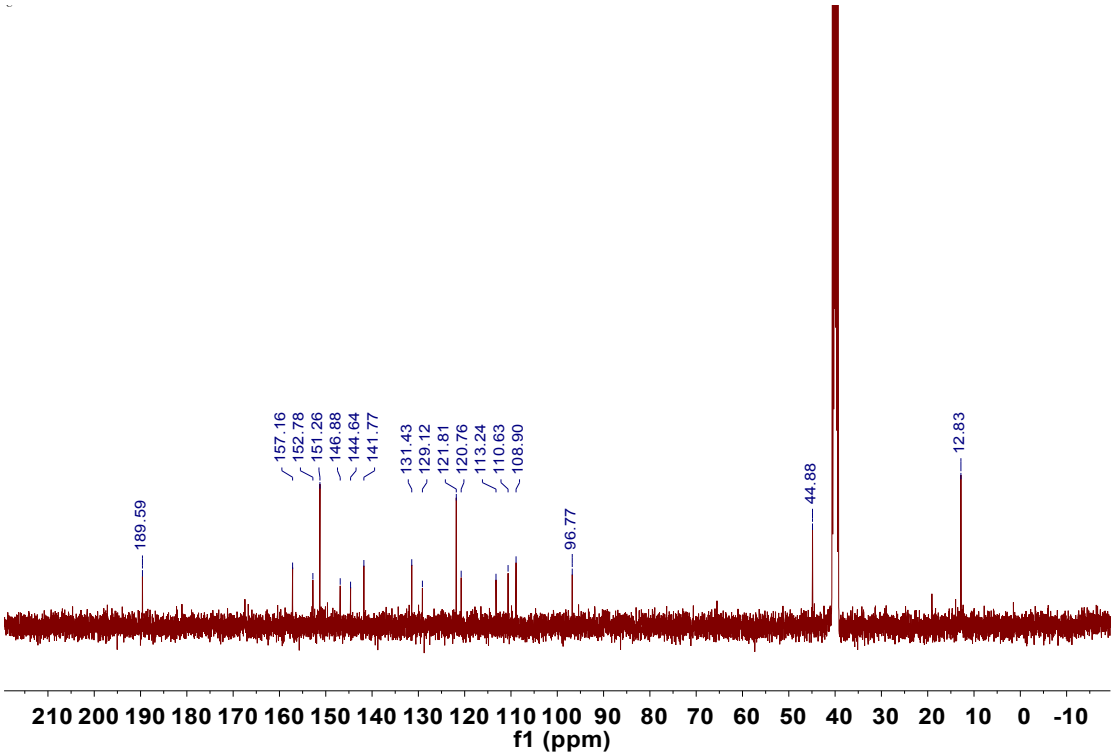


Figure S15. ^{13}C NMR spectrum of **CP-3** in DMSO-d_6 at room temperature.

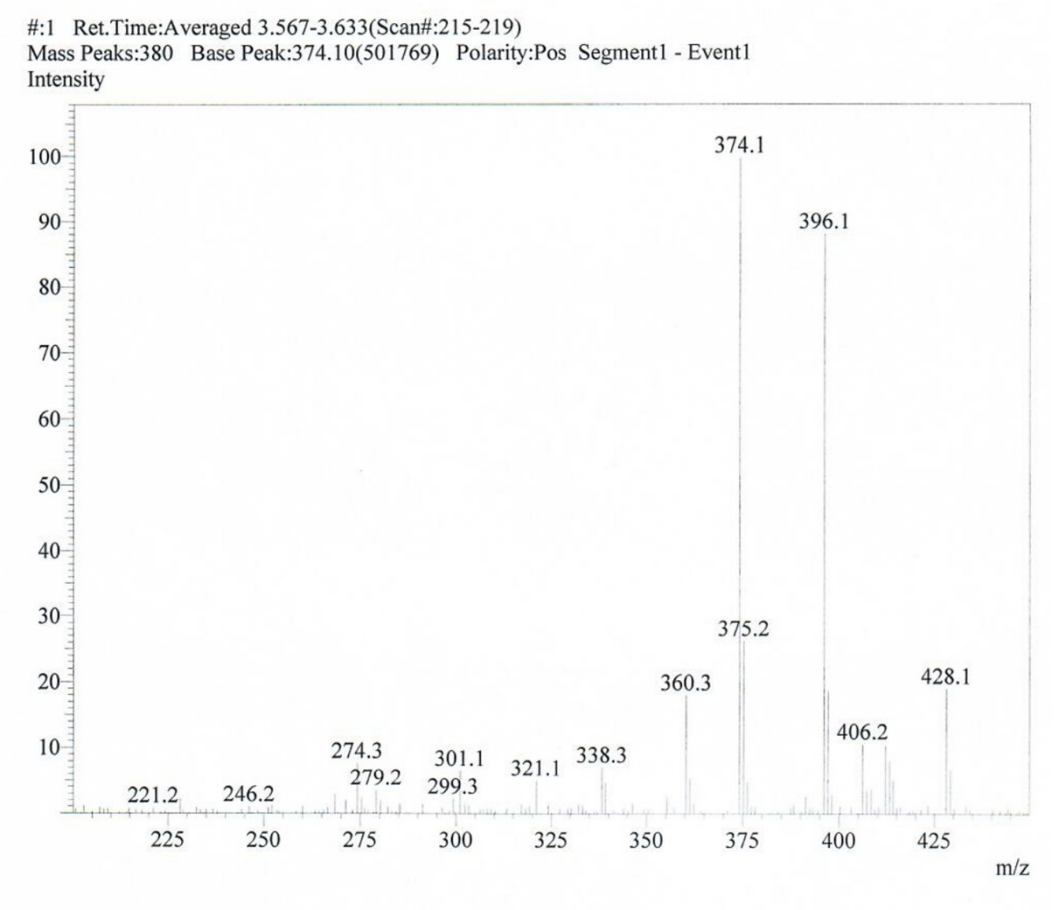


Figure S16. ESI mass spectrum of **CP-1** (positive mode).

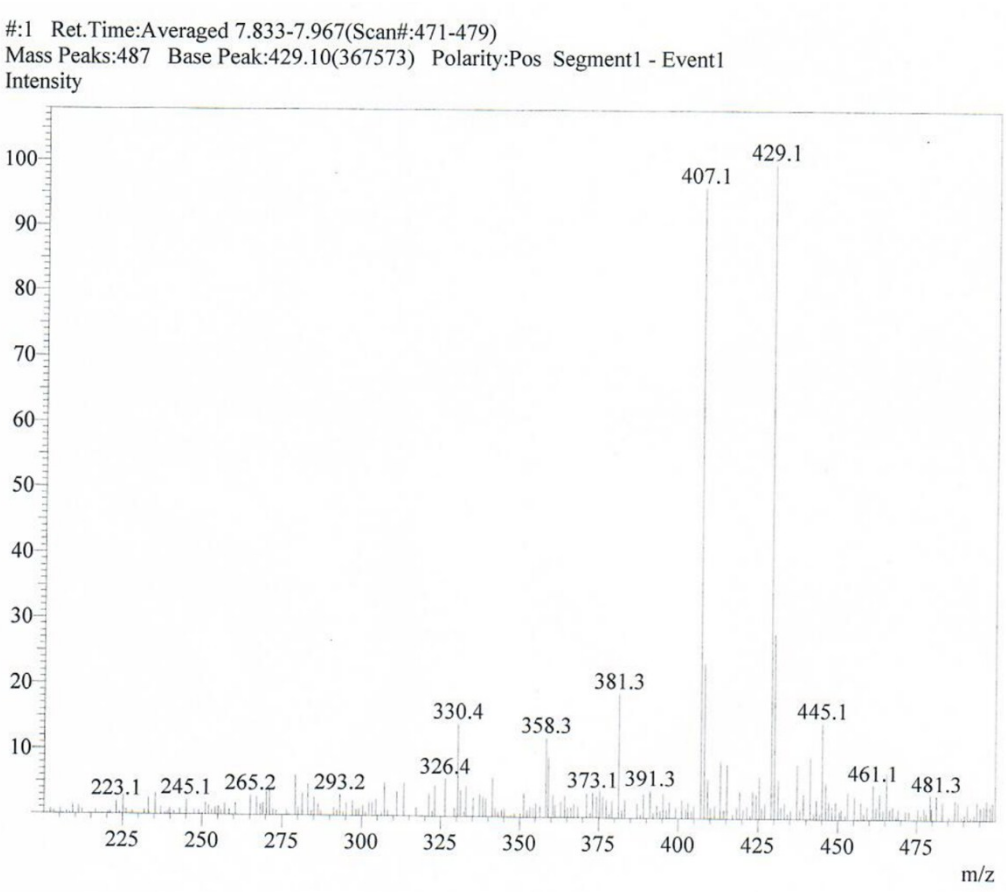


Figure S17. ESI mass spectrum of **CP-2** (positive mode).

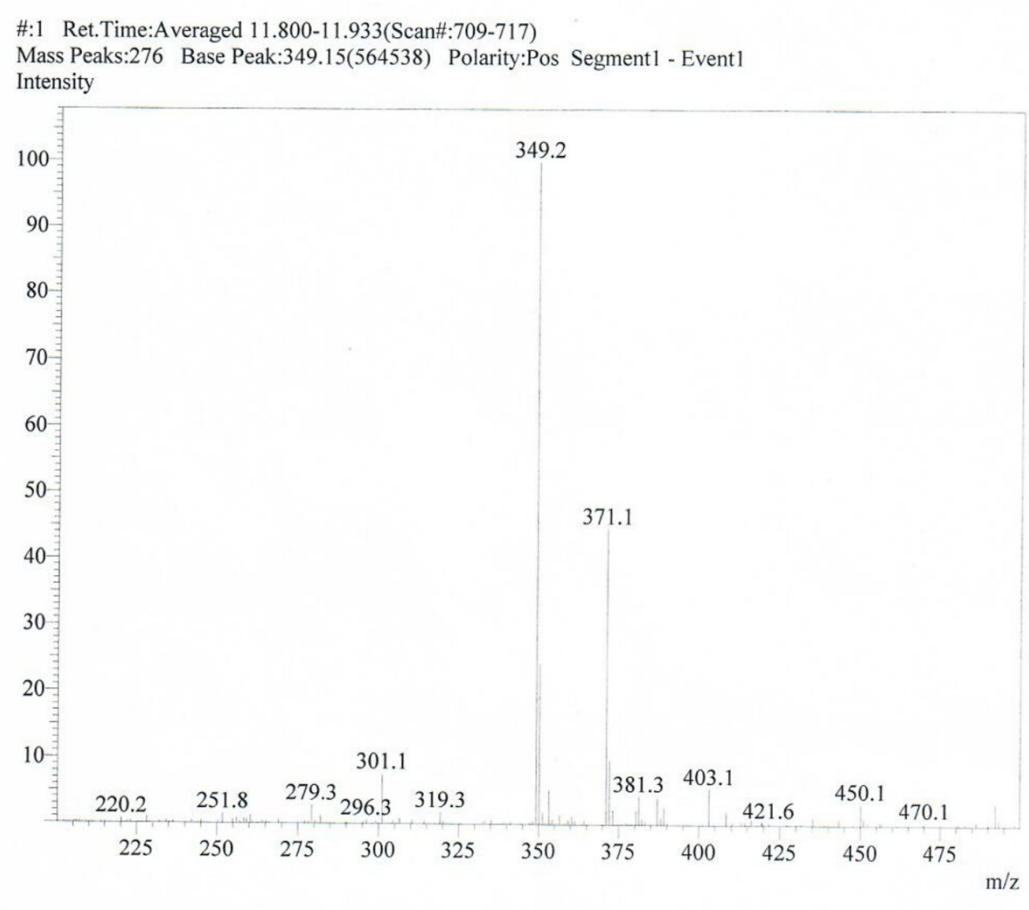


Figure S18. ESI mass spectrum of **CP-3** (positive mode).