Supporting Information

A multi-signal fluorescent probe for discrimination of cysteine/homocysteine, and glutathione and the application in living cells and zebrafish

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Determination of the detection limit

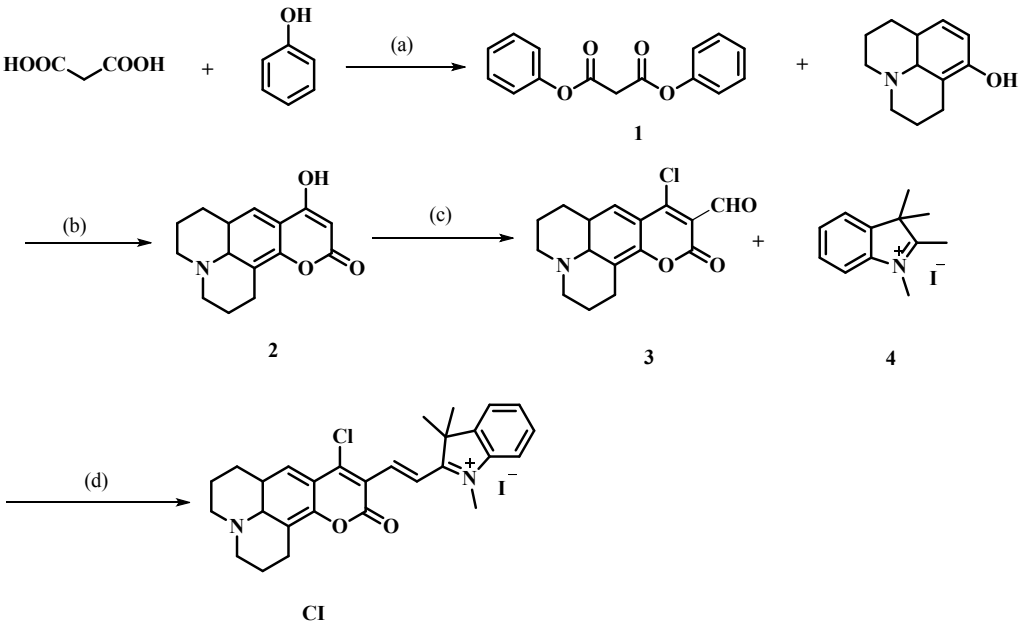
The detection limit was determined from the fluorescence titration data based on a reported method. **CI** (5.0 μM) was titrated with different concentrations of biological thiols for 30 min. The linear relationship and the concentration of biological thiols were fitted based on the fluorescence titration [1].

$$\text{Detection limit} = 3\sigma/k$$

Where σ is the standard deviation of the blank sample and k is the slope of the linear regression equation.

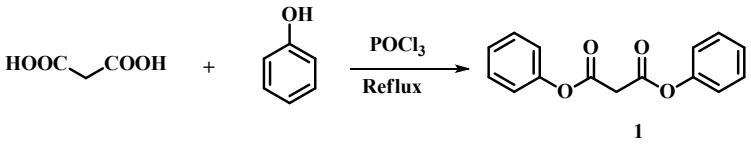
Synthetic procedures

Scheme S1. Synthesis of the fluorescent probe the **CI**



Reagents and conditions: (a) $POCl_3$, reflux, 1.5h; (b) toluene, reflux, 7h; (c) $POCl_3$, DMF, 60 °C, 12h; (d) ethanol, piperidine, 80 °C, reflux ,8h.

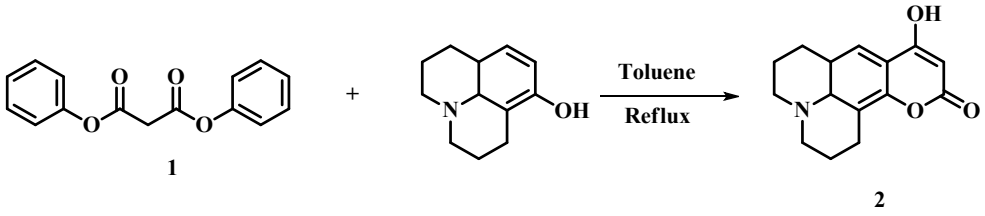
Synthesis of compound **1**.



1

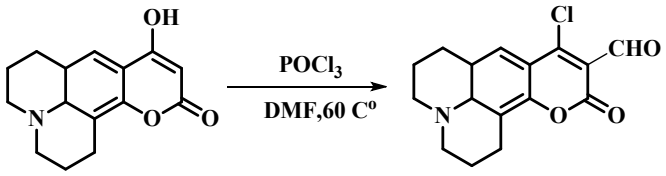
To a mixture of malonic acid (104 mg, 1 mmol) and phenol (188 mg, 2 mmol) was slowly added $POCl_3$ (108 μL, 1.2 mmol) at 0°C. The mixture was heated at 115 °C for 90 min. After cooled to room temperature, the upper layer was poured into 10 mL of water and extracted with EtOAc three times followed by the usual work. A pale brown oil diphenyl malonate (194 mg, 98 % yield) was got and pure enough to be used in the next step without further purification.

Synthesis of compound **2.**



Compound **1** (12.8 g, 50 mmol) and 8-Hydroxyjulolidine (9.56g, 50 mmol) were mixed in toluene (50 mL). The reaction mixture was refluxed for 9 hours. After cooled to room temperature, the precipitated solid was filtered and washed with petroleum ether and dried under vacuum to give compound **2** of 11.27 g. ^{1}HNMR (400 MHz, DMSO-d_6): δ 11.76 (s, 1H), 7.15(d, J = 8.0 Hz ,1H), 5.23 (s, 1H), 3.22 (q, J = 8.0 Hz,4H), 2.69 (d, J = 4.0 Hz, 4H), 1.87-1.96 (m, 4H); ^{13}CNMR (100 MHz, DMSO-d_6) δ 167.04, 163.29, 151.46, 146.44, 120.33, 117.77, 105.82, 103.56, 86.28, 49.68, 49.15, 27.42, 21.48, 20.61, 20.57; HR-MS calculated for $C_{15}H_{15}NO_3$ $[M+H]^+$ m/z 258.1125, found $[M+H]^+$ m/z 258.1128.

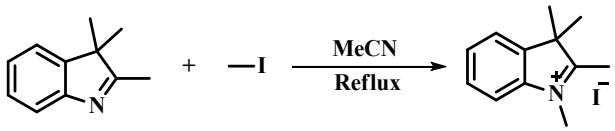
Synthesis of compound **3**.



2 **3**

Under nitrogen, 2.8 mL DMF was added dropwise to 2.8 mL $POCl_3$ at room temperature and stirred for 30 minutes to yield a red solution. Then 2.59 g compound 2 (10 mmol, dissolved in 13.2 mL DMF) was dropwise added to the above solution and get a scarlet suspension. Then the mixture solution was stirred at 60 °C for overnight and then poured into 100 mL of ice water. NaOH solution (20 %) was added to adjust the pH of the mixture to obtain a large amount of precipitate. The precipitated solid was filtered, dried and purified by column chromatography with dichloromethane /methanol (40:1) to give the compound **3** of 2.68 g. ^{1}HNMR (400 MHz, $CDCl_3$): δ 10.3 (s, 1H), 7.47 (s, 1H), 7.28 (s, 1H), 2.85-2.94 (m, 2H), 2.74 (d, J = 8.0 Hz, 2H), 2.00 (d, J = 4.0 Hz, 4H), 1.65 (s, 2H), 1.27(s, 1H); ^{13}CNMR (100 MHz, $CDCl_3$) δ 187.28, 160.30, 153.68, 151.47, 149.61, 124.94, 120.34, 109.75, 107.38, 105.61, 50.43, 49.99, 27.57, 20.99, 20.10, 20.02; HR-MS calculated for $C_{16}H_{14}ClNO_3$ $[M+H]^+$ m/z 318.0894, found $[M+H]^+$ m/z 318.0892.

Synthesis of compound **4**.



4

2,3,3-Trimethyl-3H-indole (3.20 g, 20 mmol), iodomethane (5.68 g, 40 mmol) were mixed and dissolved in acetonitrile (10 mL). Then the mixture was refluxed for 11 h under nitrogen atmosphere. The mixture was cooled down to room temperature to afford a light pink precipitate. After filtered, the filter cake

was washed with ethyl acetate (10 mL) for 3 times and dried in vacuum to give a light pink solid 4 (5.8 g, 96.0%). Compound **4** was not purified and used directly for the next reaction.

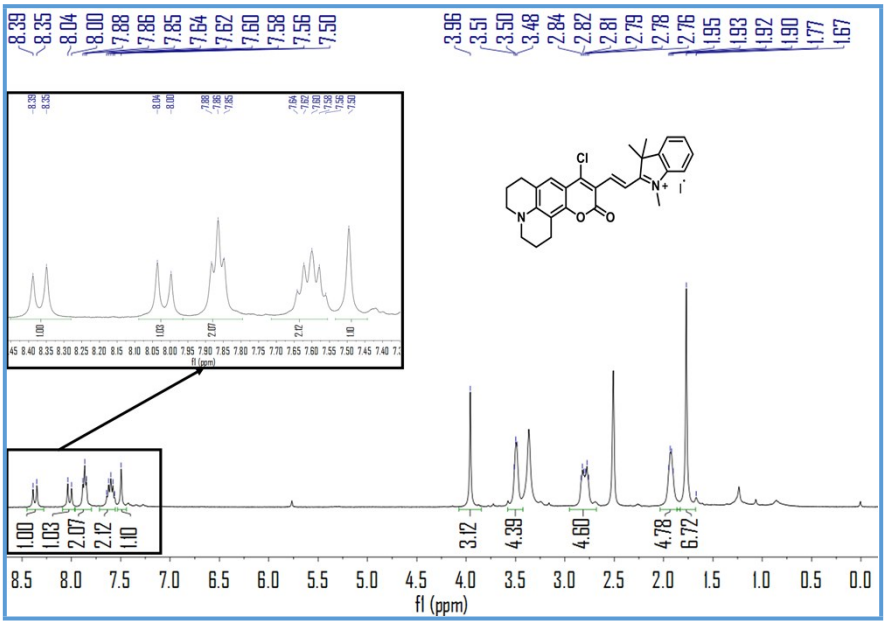


Fig. S1. ^{1}H NMR spectrum of **CI** in DMSO-d_6.

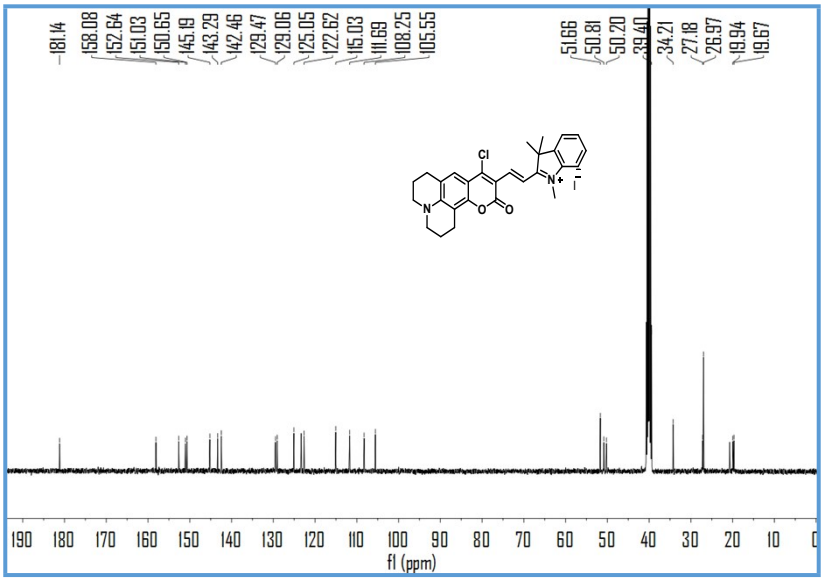


Fig. S2. ^{13}C NMR spectrum of **CI** in DMSO-d_6.

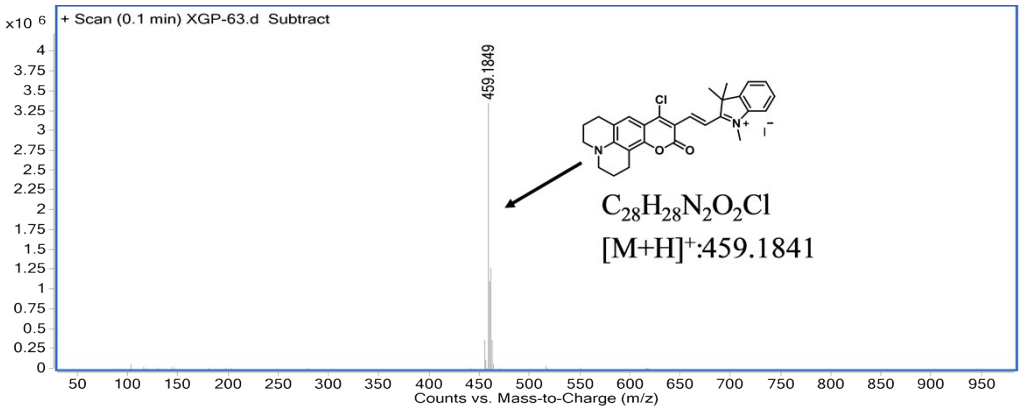


Fig. S3. HR-MS spectrum of **CI**.

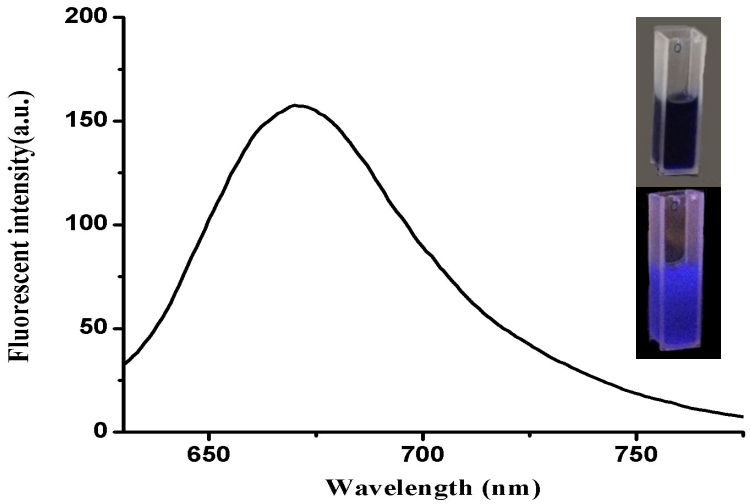


Fig. S4. The fluorescence intensity of **CI** (5 μM) with excitation at 614 nm. Insets: the color of the probe **CI** under the naked eye and the color of the probe **CI** under a 365 nm UV irradiation.

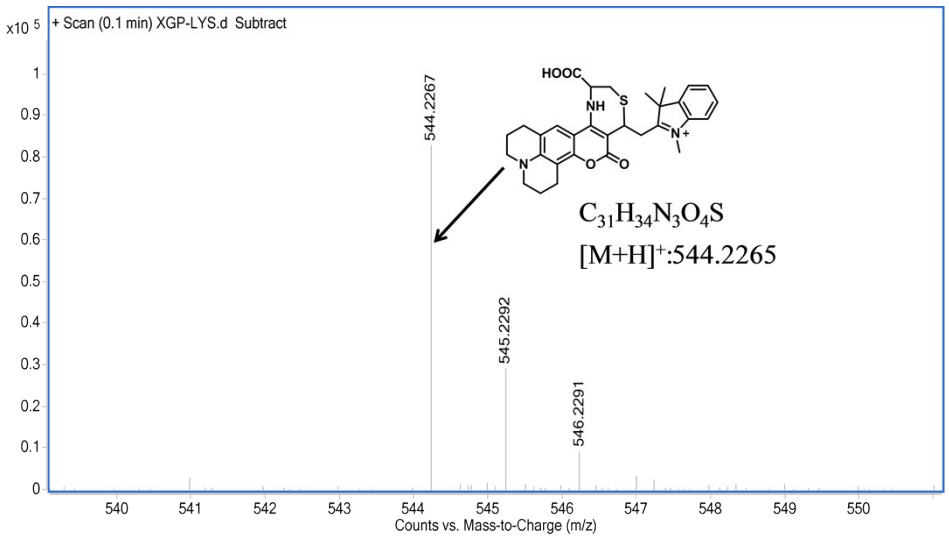


Fig. S5. HR-MS spectrum of 5 μM **CI** in the 40 μM Cys in 10 mM PBS buffer (pH 7.4, contain 1% DMSO as co-solvent).

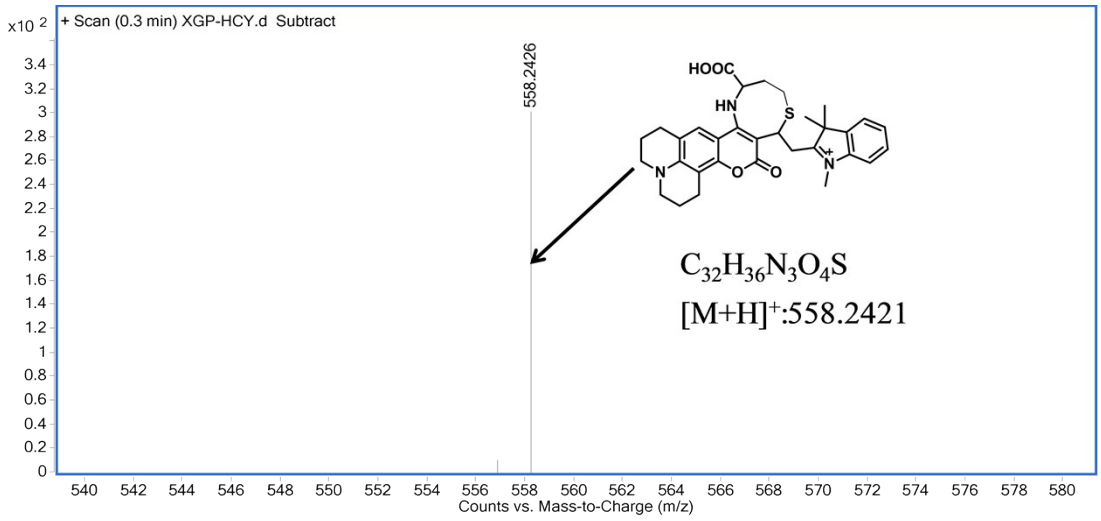


Fig. S6. HR-MS spectrum of 5 μM **CI** in the 40 μM Hcy in 10 mM PBS buffer (pH 7.4, contain 1% DMSO as co-solvent).

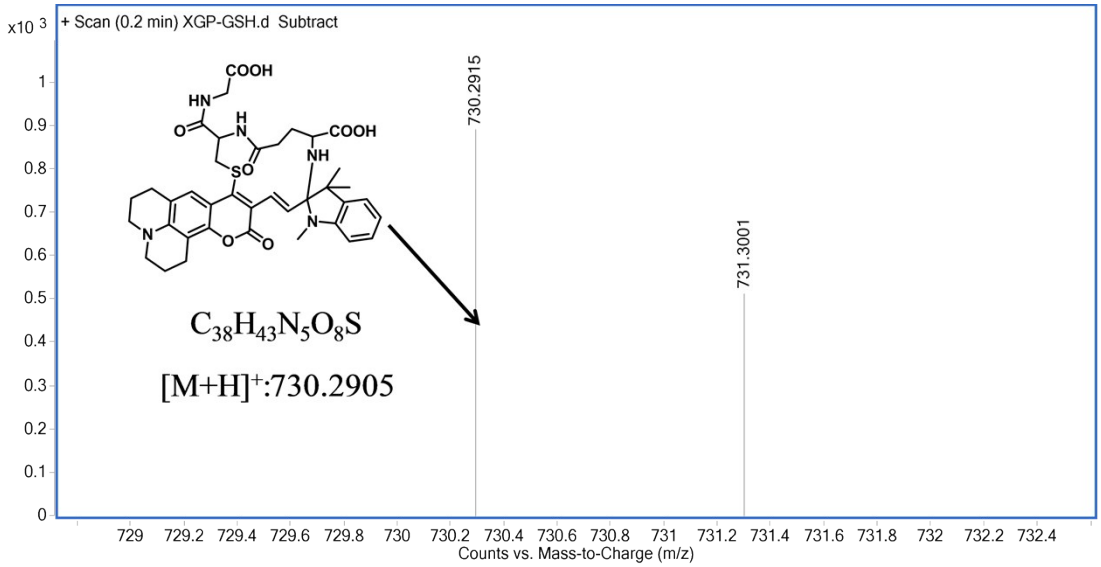


Fig. S7. HR-MS spectrum of 5 μM **CI** in the 40 μM GSH in 10 mM PBS buffer (pH 7.4, contain 1% DMSO as co-solvent).

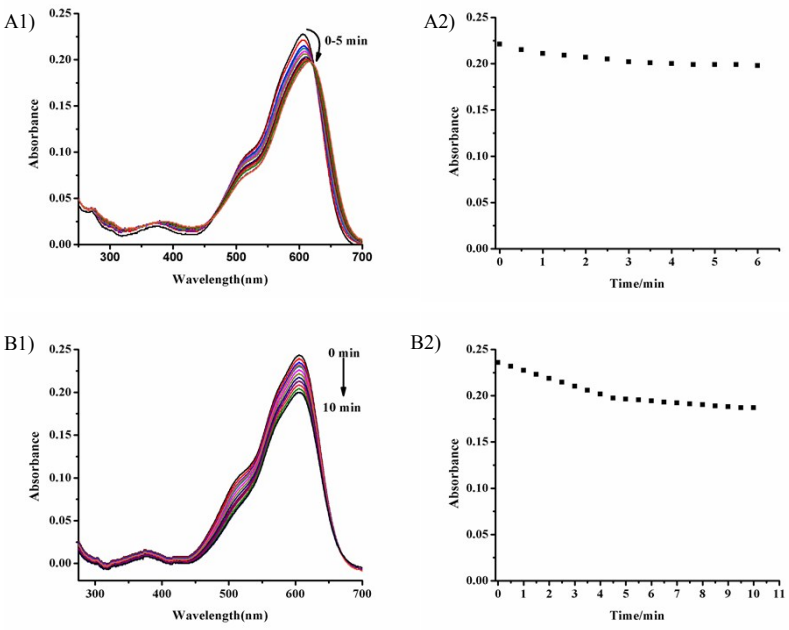


Fig. S8. Time-dependent absorption spectra of **CI** (5 μM) in the presence of 8 eq NAC (A1, A2) and 8 eq DL-2-Aminobutyricacid (B1, B2).

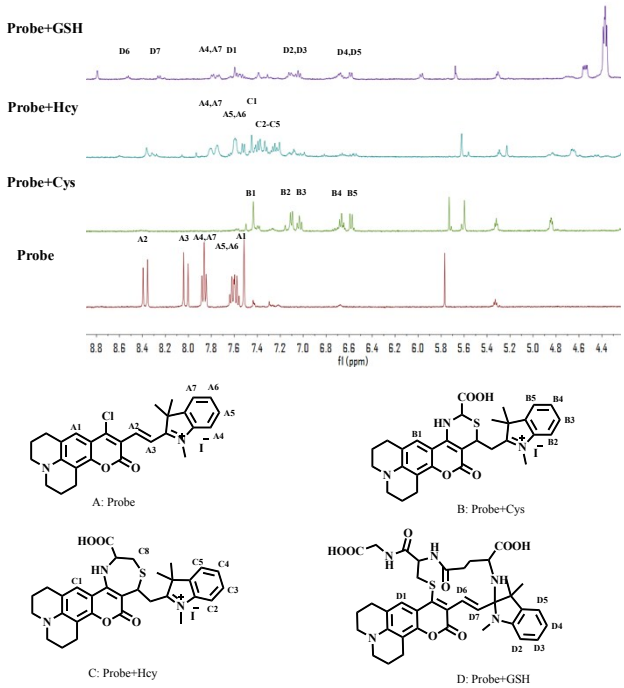


Fig. S9. Partial [1]HNMR spectra comparison of probe **CI**, Probe+Cys, Probe+Hcy, and Probe+GSH. The [1]HNMR spectra were obtained in DMSO-d_6–D_2O (2:1, v/v).

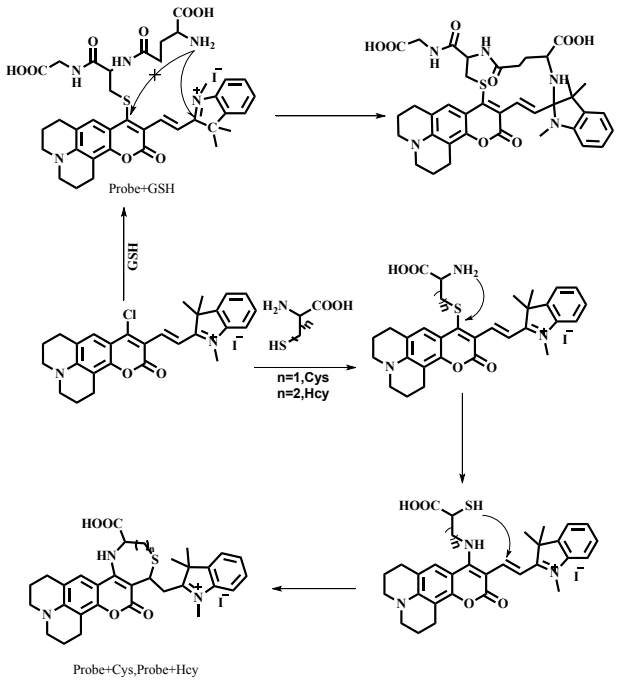


Fig. S10. Reaction mechanisms of probe **CI** with Cys, Hcy, GSH.

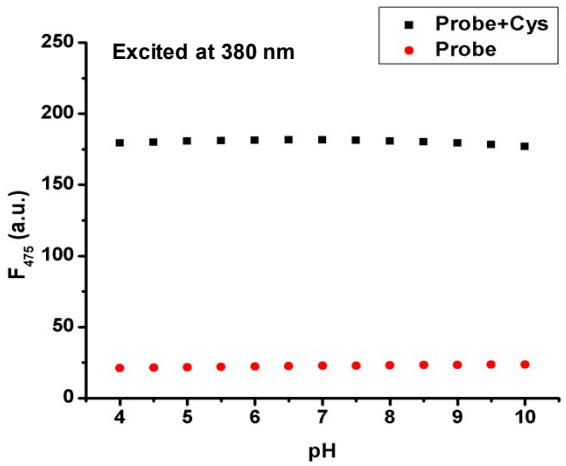


Fig. S11. The fluorescence intensity of **CI** (5 μM) in the presence or absence of Cys (40 μM) in various pH ranging from 4.0 to 10.0.

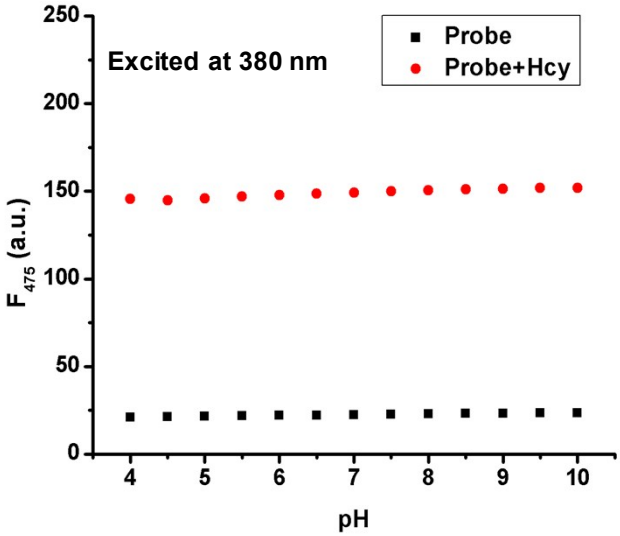


Fig. S12. The fluorescence intensity of **CI** (5 μM) in the presence or absence of Hcy (40 μM) in various pH ranging from 4.0 to 10.0.

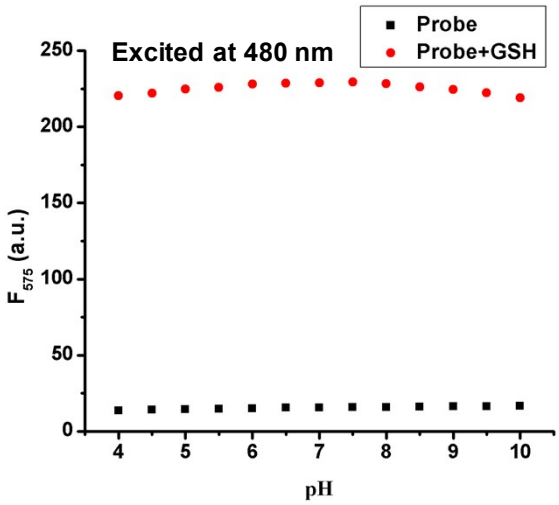


Fig. S13. The fluorescence intensity of **CI** (5 μM) in the presence or absence of GSH (40 μM) in various pH ranging from 4.0 to 10.0.

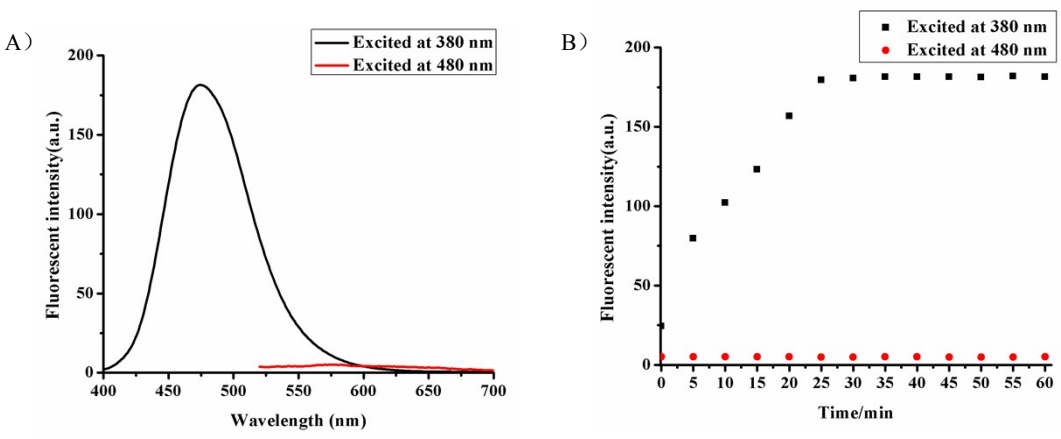


Fig. S14. The photophysical responses of the probe with the coexist of Cys and GSH. A) The fluorescent spectra of the probe with the coexist of Cys and GSH (Cys:GSH=8 eq:8 eq). B) The response rate experiments of the probe with the coexist of Cys and GSH (Cys:GSH=8 eq:8 eq), (■): the emission wavelength was 475 nm, (●): the emission wavelength was 575 nm.

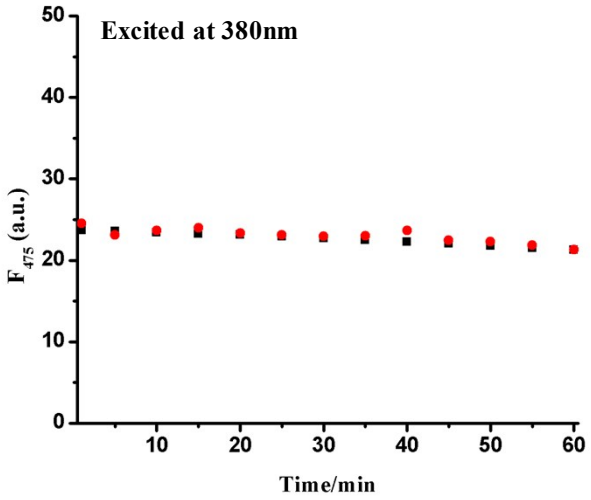


Fig. S15. Photostability profiles of **CI** (5.0 μM) in the absence [●] or presence of UV-irradiated [■] (365 nm).The fluorescence intensities at 475 nm were continuously monitored at time intervals in PBS (10 mM, pH 7.4, 1% DMSO). Time points represent 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min.

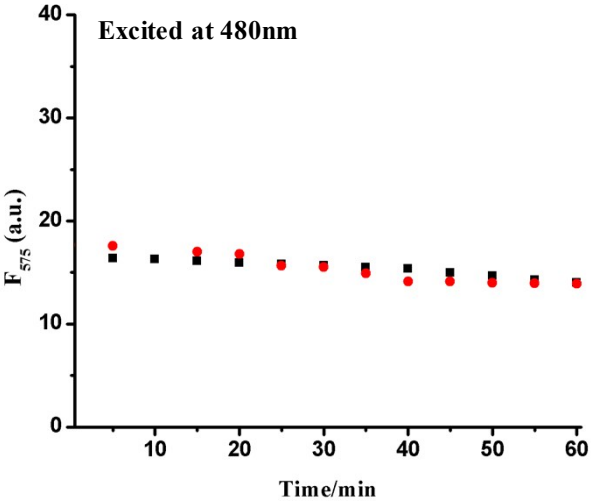


Fig. S16. Photostability profiles of **CI** (5.0 μM) in the absence [●] or presence of UV-irradiated [■] (365 nm). The fluorescence intensities at 575 nm were continuously monitored at time intervals in PBS (10 mM, pH 7.4, 1% DMSO). Time points represent 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min.

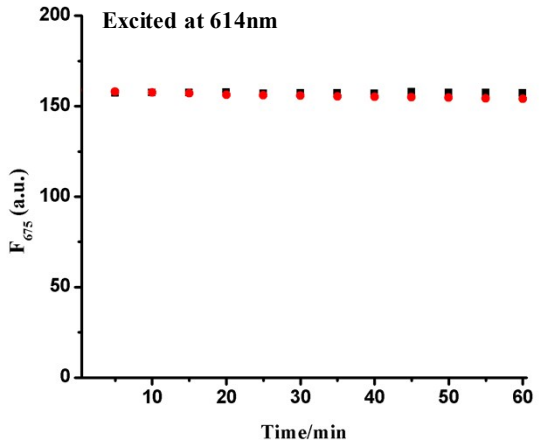


Fig. S17. Photostability profiles of **CI** (5.0 μM) in the absence [●] or presence of UV-irradiated [■] (365 nm). The fluorescence intensities at 675 nm were continuously monitored at time intervals in PBS (10 mM, pH 7.4, 1% DMSO). Time points represent 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min.

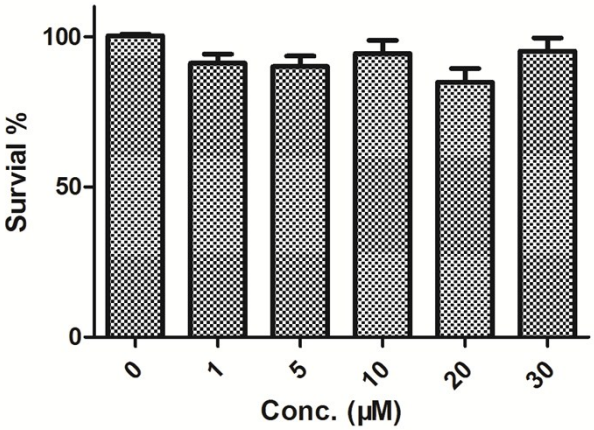


Fig. S18. Cell viability of HeLa cells incubated with probe **CI** of different concentration (0-30 μM) for **24** h.

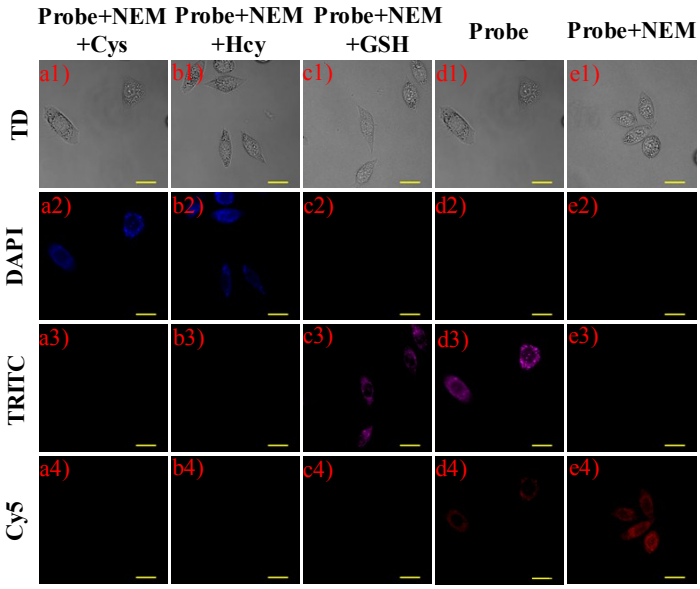


Fig. S19 One-photon fluorescence imaging of probe **CI** (5 μM) responding to respective biological thiols in cells. a-c) cells were incubated with NEM (500 μM) for 30 min, followed by addition of Cys (200 μM), Hcy (200 μM) and GSH (200 μM) respectively for 15 min, then incubated with **CI** (5 μM) for 30 min and imaged; d) the cells were incubated with **CI** (5 μM) for 30 min, then imaged; e) cells were incubated with NEM (500 μM) for 30 min, followed by addition of **CI** (5 μM) for 30 min and imaged. Scale bar = 20 μm.

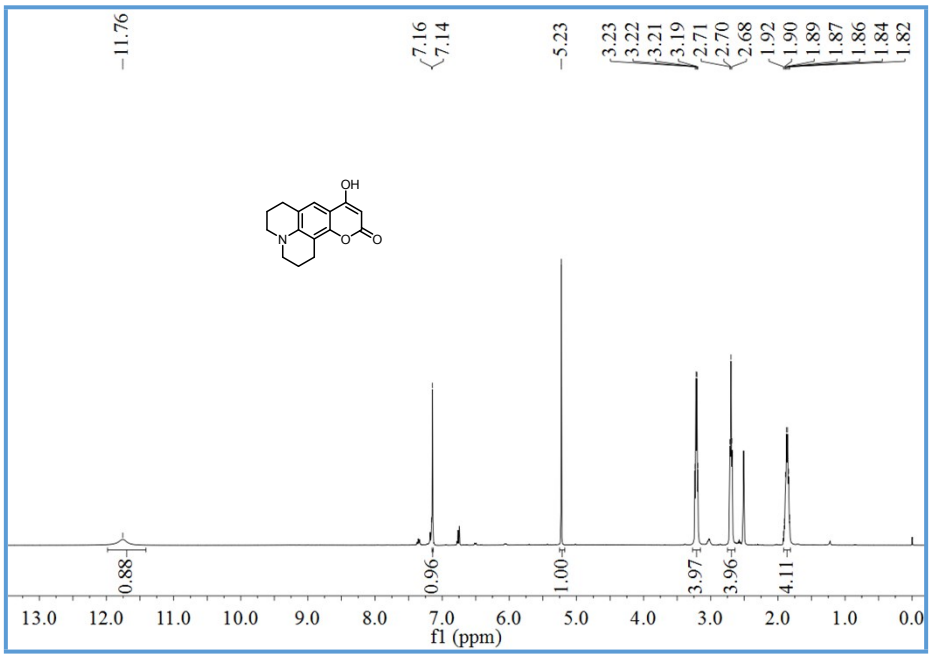


Fig. S20. ^{1}H NMR spectrum of **2** in DMSO-d_6.

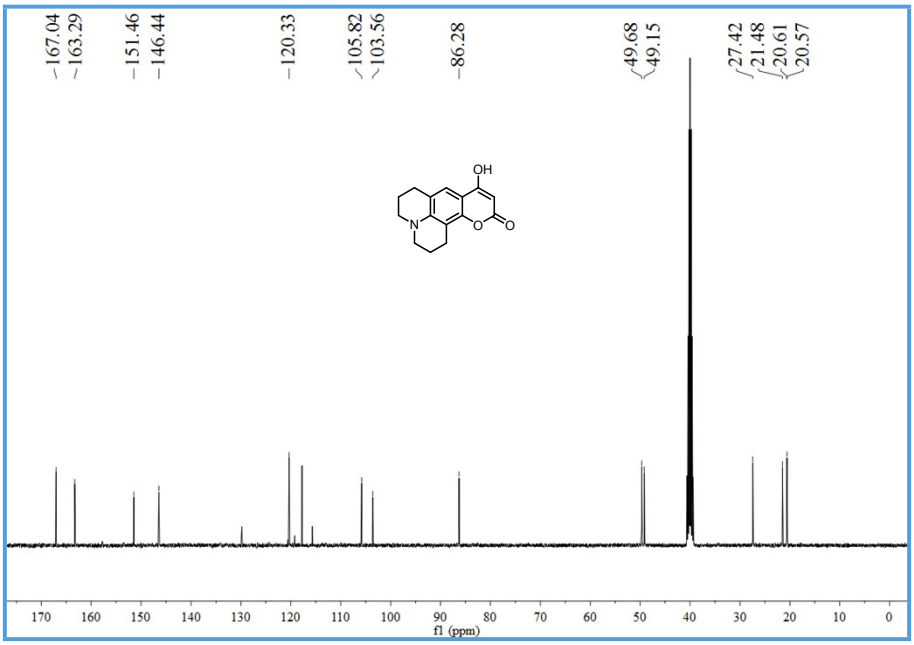


Fig. S21. ^{13}C NMR spectrum of **2** in DMSO-d_6.

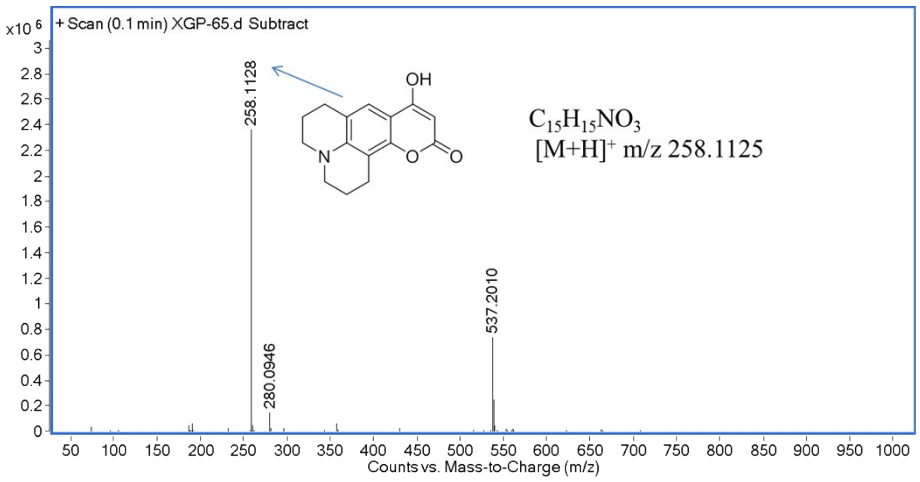


Fig. S22. HR-MS spectrum of **2**.

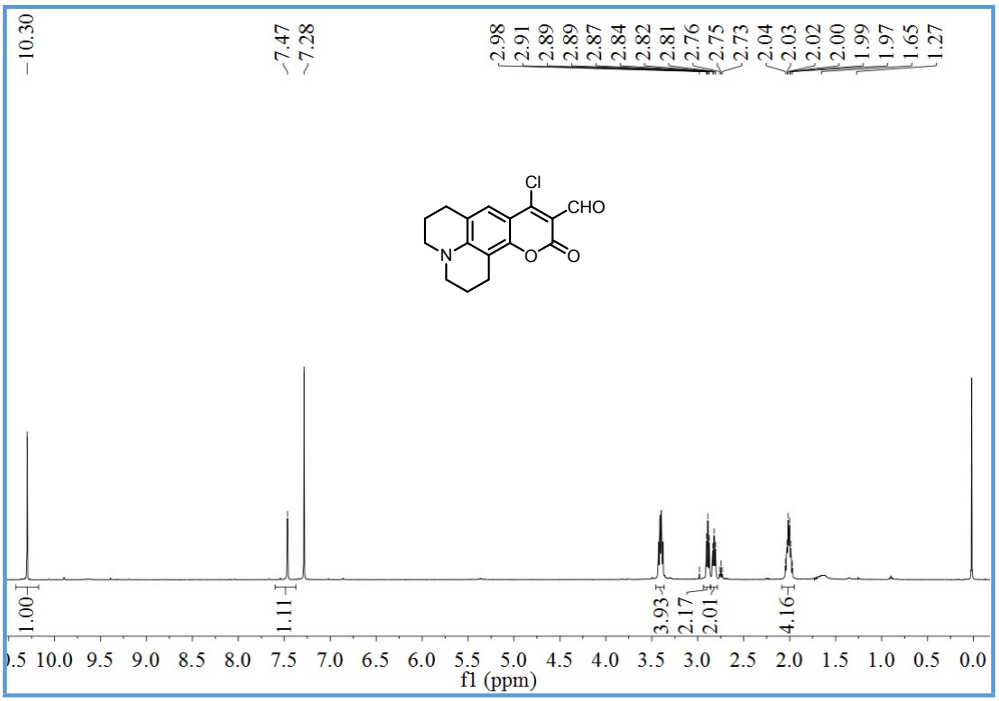


Fig. S23. ^{1}H NMR spectrum of **3** in $CDCl_3$.

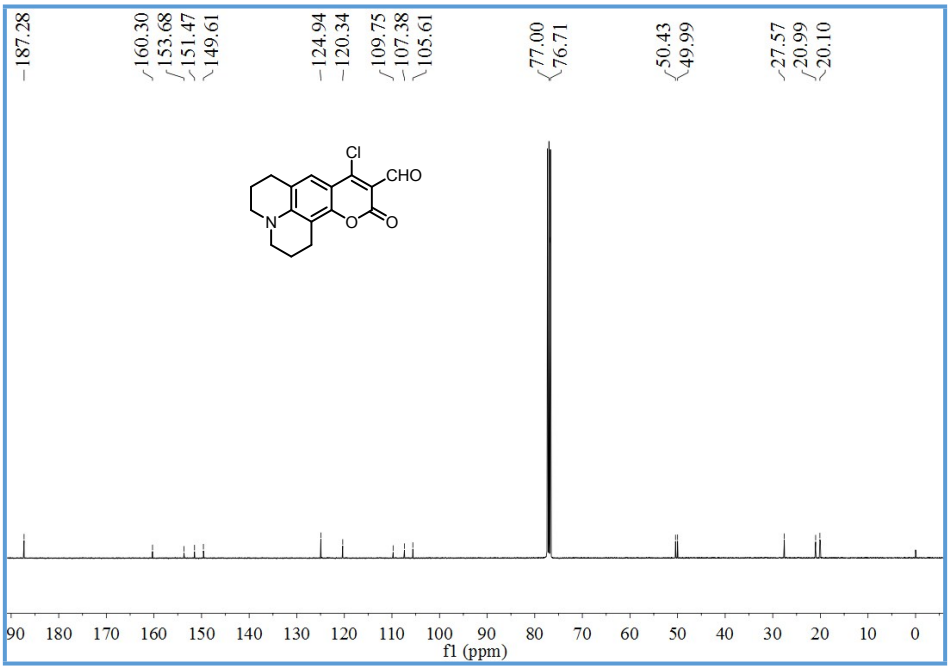


Fig. S24. ^{13}C NMR spectrum of **3** in $CDCl_3$.

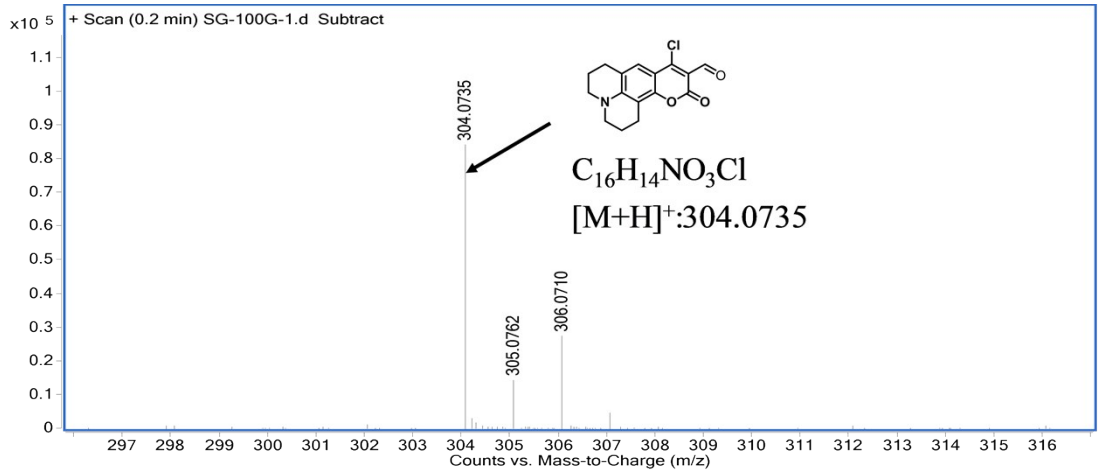


Fig. S25. HR-MS spectrum of **3**.

Reference:

[1] Y. Qian, J. Karpus, O. Kabil, S. Zhang, H. Zhu, J. Zhao and C. He. *Nat. Commun,* 2011, **2**, 495-498.