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Electronic Supplementary Information

Rapid detection of intracellular Cys over Hcy and GSH by a novel two-photon coumarinocoumarin-based colorimetric and fluorescent probe

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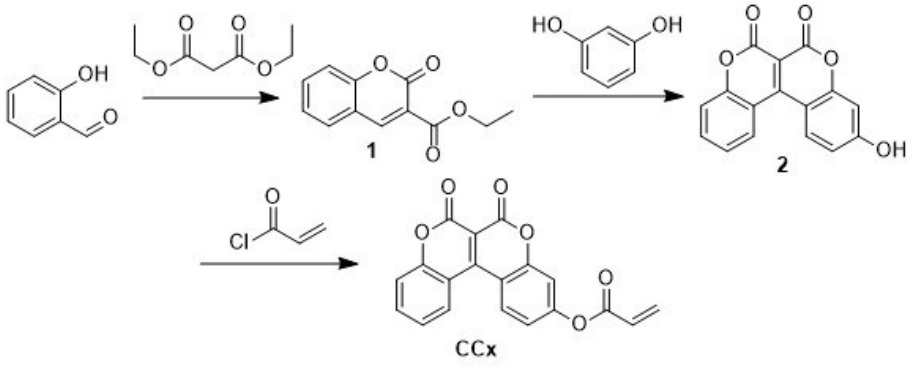
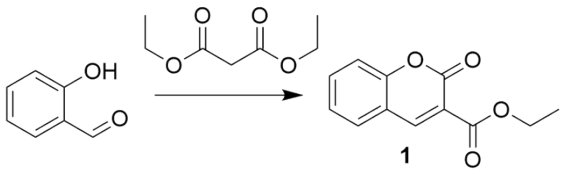
1. Synthesis



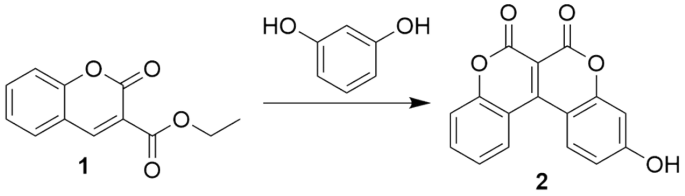
Scheme S1. Synthesis of **CCx**

1.1 Synthesis of ethyl coumarin-3-carboxylate (1)



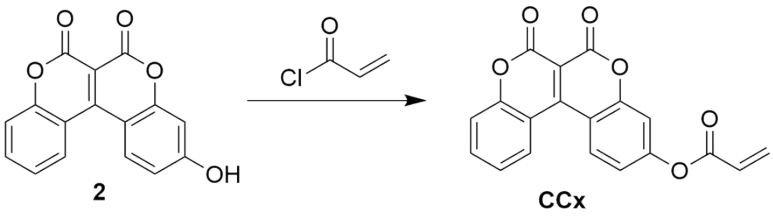
To a solution of salicylaldehyde (1.83 g, 15 mmol) in 30 mL of ethanol was added diethyl malonate (2.88 g, 18 mmol), 2 drops of acetic acid and piperidine (0.15 mL). The reaction mixture was stirred under reflux for 8 h, diluted with ice water (50 mL). After completion of the reaction, the cake was filtered and washed with water. The product was dried under vacuum to give a white solid (3.07 g, 94% yield).

1.2 Synthesis of 3-hydroxy-6H, 7H-chromeno [3, 4-c] chromene-6, 7-dione (2)



A mixture of **1** (2.18 g, 10 mmol) and m-dihydroxybenzene (0.55 g, 5 mmol) was heated at 140 °C under argon for 6 h. The resulting mixture was cooled, and 10 mL of ethanol was added. Next, the mixture was treated with ultrasound by 10 min, then let it stand until obtain sufficient precipitation. A yellow precipitate was formed, which was filtered off and dried at room temperature. The yield of product is 63%, and it's pure enough for the next step without recrystallization. MS (m/z, ESI) Calculated for $C_{16}H_9O_5$ m/z = 281.0444 [M + H]. Found m/z = 281.0444 (Fig. S24). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.29 (s, 1H), 8.33 (d, *J* = 8.0 Hz, 1H), 8.25 (d, *J* = 9.1 Hz, 1H), 7.84 – 7.75 (m, 1H), 7.51 – 7.42 (m, 2H), 6.93 (dd, *J* = 9.1, 2.5 Hz, 1H), 6.78 (d, *J* = 2.5 Hz, 1H).

1.3 Synthesis of 6, 7-dioxo-6H, 7H-chromeno [3,4-c]chromen-3-yl acrylate (CCx)



The dried **2** (0.28 g, 1 mmol) and trimethylamine (3 ml) were totally dissolved in plenty of anhydrous dichloromethane, and acryloyl chloride (0.1 ml) was added. The fluorescence of solution changed from green to colorless. After that, the solution was extracted with water (100 ml x 3) and saturated brine (100 ml), dried over Na_2SO_4. Then concentrated to afford a residue, which was chromatographed over silica gel (EA: PE= 1: 1, then DCM) to obtain **CCx** (yield= 91%).

2. Detection limit

The detection limit was calculated based on the fluorescence titration. To determine the S/N ratio, the emission intensity of **CCx** without thiols was measured by 30 times and the standard deviation of blank measurements was determined. Three independent duplication measurements of emission intensity were performed in the presence of Cys and each average value of the intensities was plotted as a concentration of Cys for determining the slope. The detection limit was then calculated with the following equation.

$$\text{Detection limit} = \frac{3\sigma_{bi}}{m}$$

Where σbi is the standard deviation of blank measurements, m is the slope between fluorescence intensity versus

sample concentration. The detection limit of the probe for Cys was determined to be 0.388 μM.

3. Two-photon fluorescence spectra

Two-photon fluorescence spectra were measured using an Edinburgh FLSP920. The excitation and emission slit widths were 2.0 nm and 2.0 nm, respectively. Two-photon absorption cross-sections were measured using the two-photon-induced fluorescence measurement technique. The two-photon absorption cross-sections (d) were determined by comparing their two-photon excitation fluorescence (TPEF) to that of fluorescein in different solvents, according to the following equation:

$$\delta = \delta_{ref} \cdot \frac{n_{ref}}{n} \cdot \frac{\Phi_{ref}}{\Phi} \cdot \frac{c_{ref}}{c} \cdot \frac{F}{F_{ref}}$$

In the equation, the subscript ref stands for the reference molecule. d is the two-photon adsorption cross-section value, n is the refractive index of the solution, F is the fluorescence quantum yield, c is the concentration of solution, F is the TPEF integral intensities of the solution emitted at the exciting wavelength. The d ref value of reference was taken from the literature.

4. One-photon fluorescence spectra

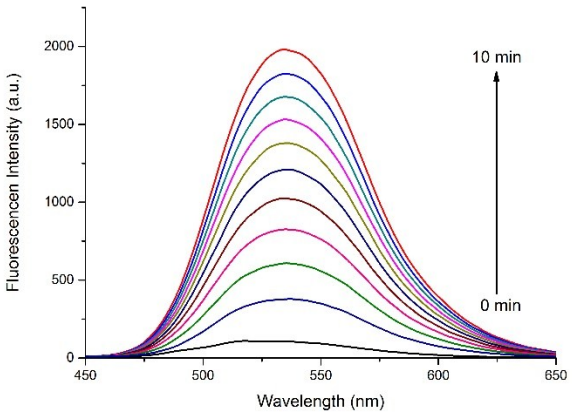


Figure S1. Fluorescence emission spectra of **CCx** (2 μM) with Cys (40 μM) under different reaction time in PBS buffer.

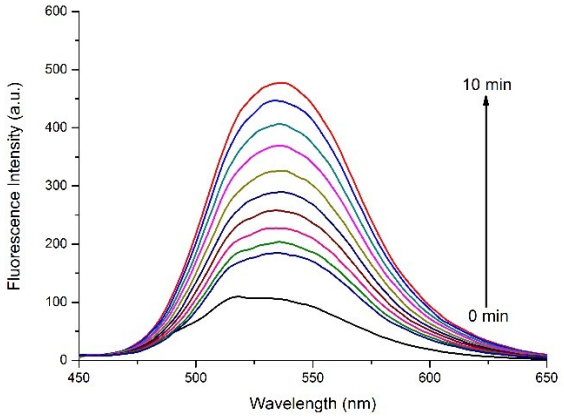


Figure S2. Fluorescence emission spectra of **CCx** (2 μM) with Hcy (40 μM) under different reaction time in PBS buffer.

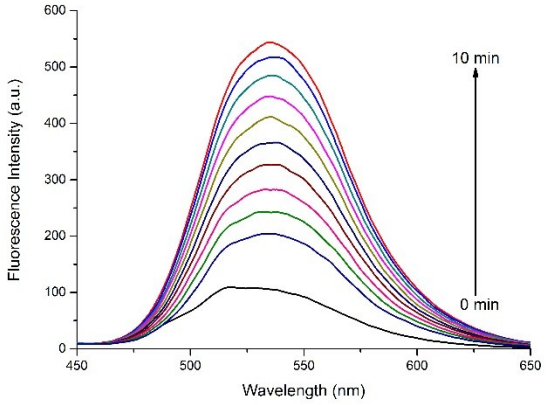


Figure S3. Fluorescence emission spectra of **CCx** (2 μM) with GSH (40 μM) under different reaction time in PBS buffer.

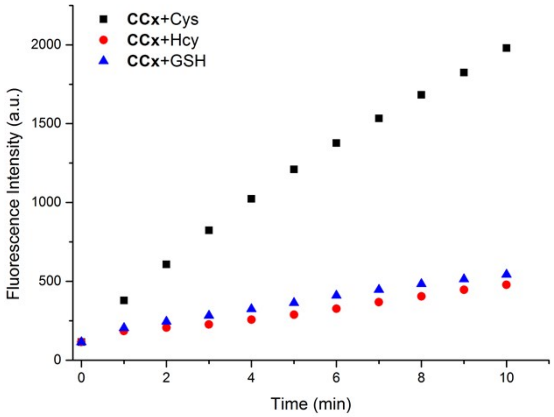


Figure S4. Time-dependent fluorescence intensity of **CCx** at 535 nm in the presence of 40 μM Cys, Hcy or GSH.

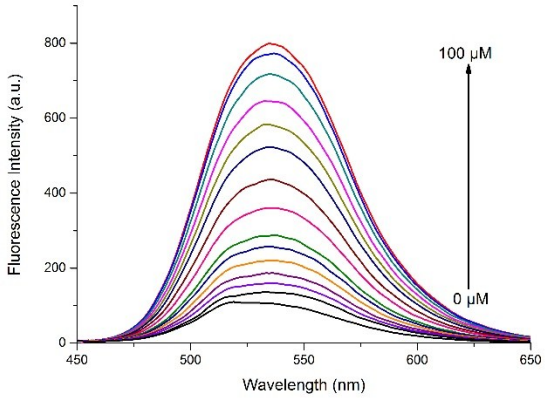


Figure S5. Fluorescence emission spectra of **CCx** (2 μM) in the presence of gradually varied concentration of Hcy (0–100 μM) in PBS buffer.

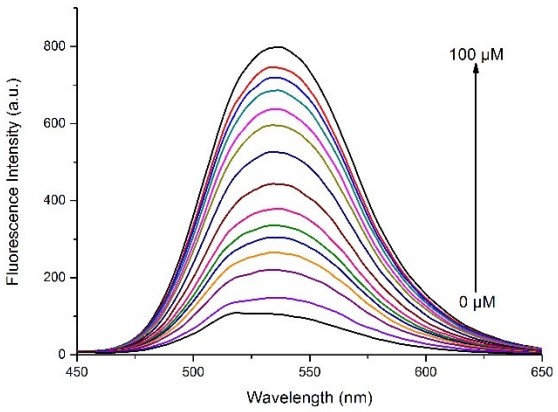


Figure S6. Fluorescence emission spectra of **CCx** (2 μM) in the presence of gradually varied concentration of GSH (0–100 μM) in PBS buffer.

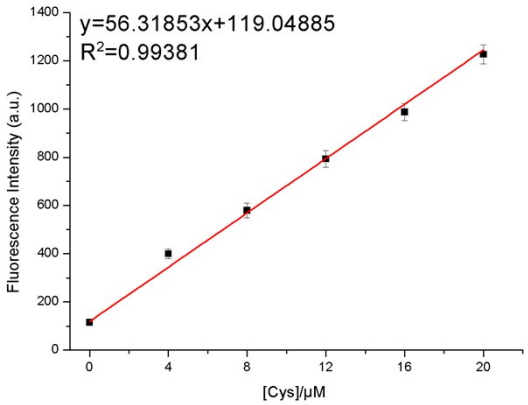


Figure S7. The change in the fluorescence intensity of **CCx** (2 μM) at 535 nm against varied concentration of Cys from 0 to 20 μM in PBS buffer.

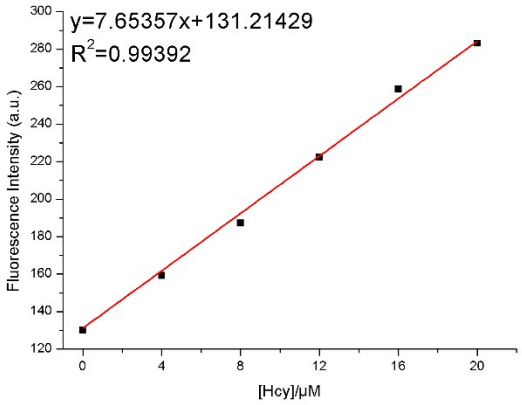


Figure S8. The change in the fluorescence intensity of **CCx** (2 μM) at 535 nm against varied concentration of Hcy from 0 to 20 μM in PBS buffer.

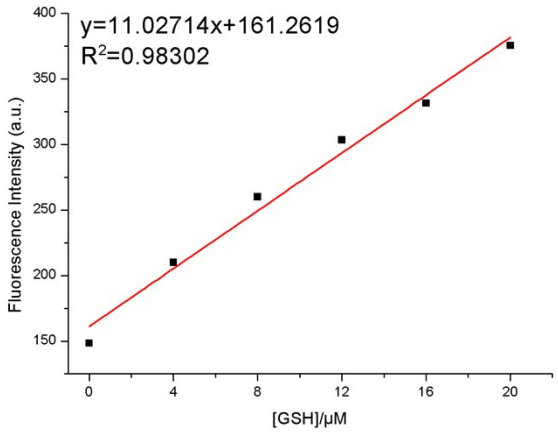


Figure S9. The change in the fluorescence intensity of **CCx** (2 μM) at 535 nm against varied concentration of GSH from 0 to 20 μM in PBS buffer.

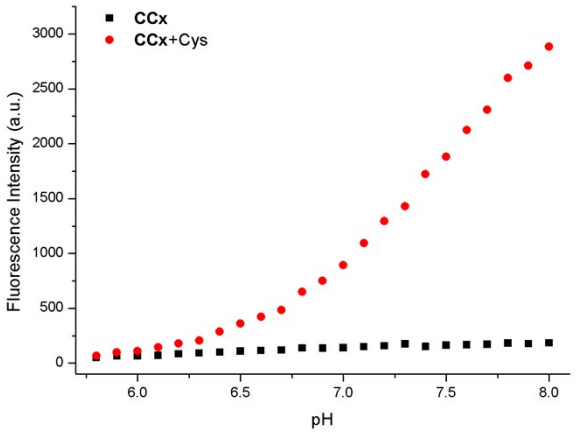


Figure S10. Fluorescence intensity of **CCx** (2 μM) in the absence and presence of 40 μM Cys under different pH from 5.8 to 8.0 in PBS buffer.

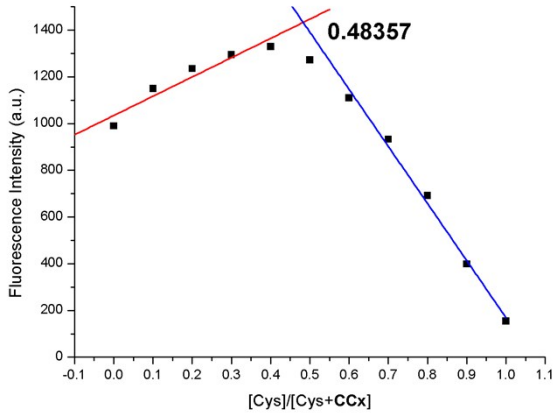


Figure S11. Job's plot of the reaction between **CCx** and Cys in PBS buffer. Total concentration of **CCx** and Cys was

kept constant at 10 μM.

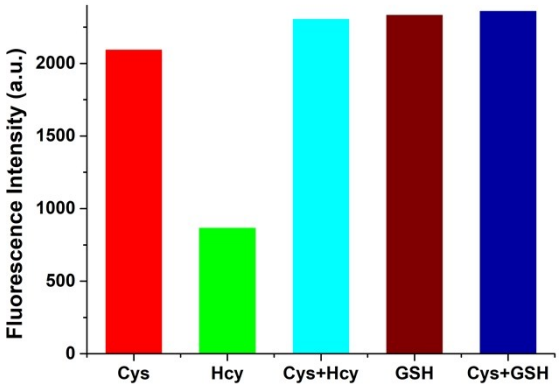


Figure S12. The fluorescence intensity of 2 μM **CCx** with Cys (40 μM), Hcy (100 μM), Cys (40 μM) + Hcy (100 μM), GSH (1 mM), Cys (40 μM) + GSH (1 mM).

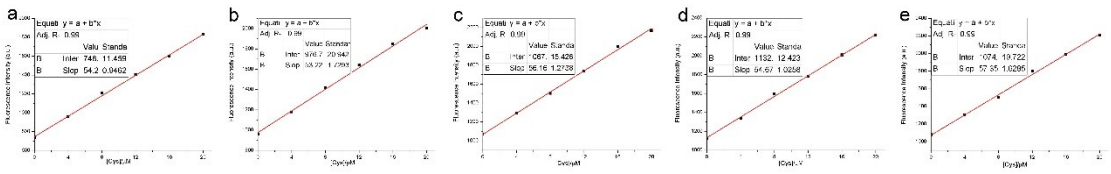


Figure S13. The titration curve of Chicken (a), Horse (b), Sheep (c), Cow (d) and Goat (e). Inter of each curve is value B, the concentration of Cys in serum = 10*(value B – value A)/slop.

5. OP fluorescent imaging

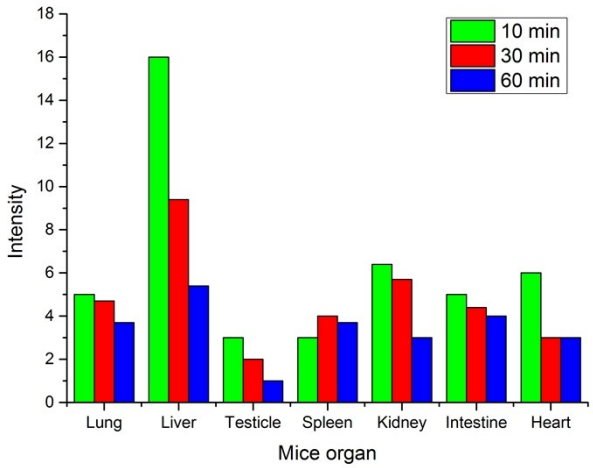


Figure S14. The bar chart of enrichment and metabolism experiment.

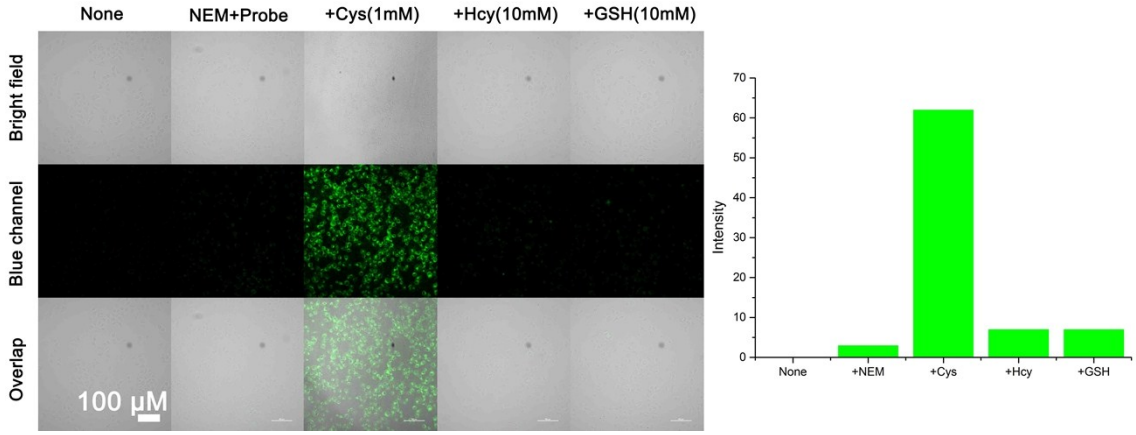


Figure S15. Images of HeLa cells treated with difference methods and the bar chart of intensity.

6. Study on TP fluorescence

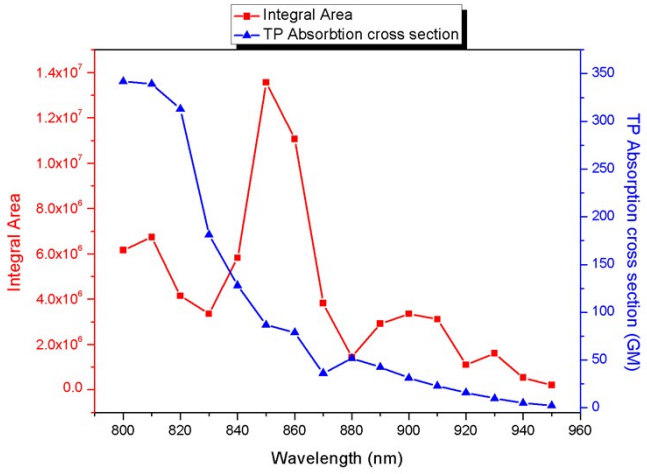


Figure S16. The integral area and TP absorption cross section of compound **2**.

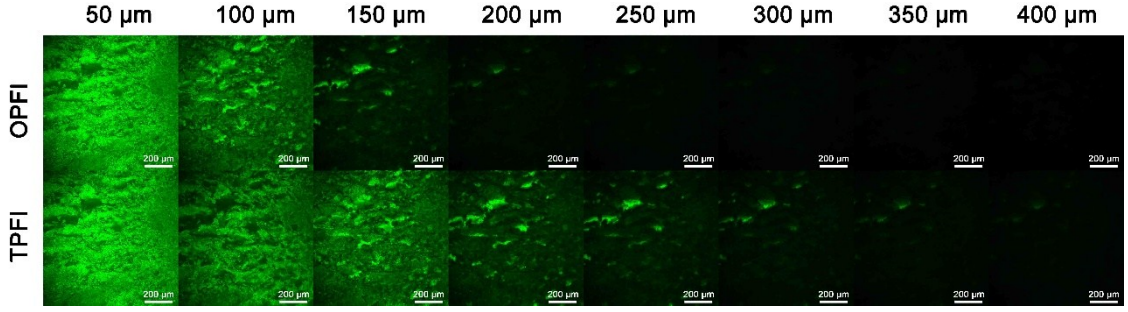


Figure S17. The Z-scan TPE (TPFI) and OPE (OPFI) confocal fluorescence imaging of thick mice tumor tissue slice stained by **CCx** at different penetration depths, the scale bar is 200 μm.

7. NMR and Mass spectra

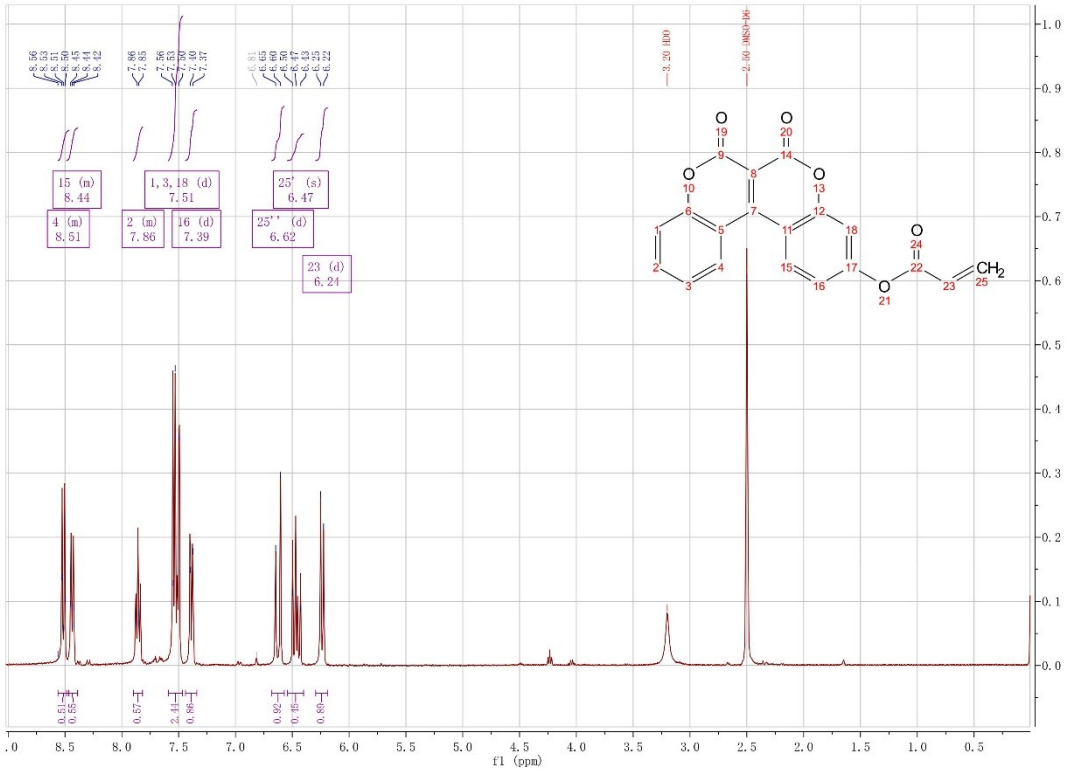


Figure S18. The ^{1}H NMR spectra of **CCx** in DMSO-d_6.

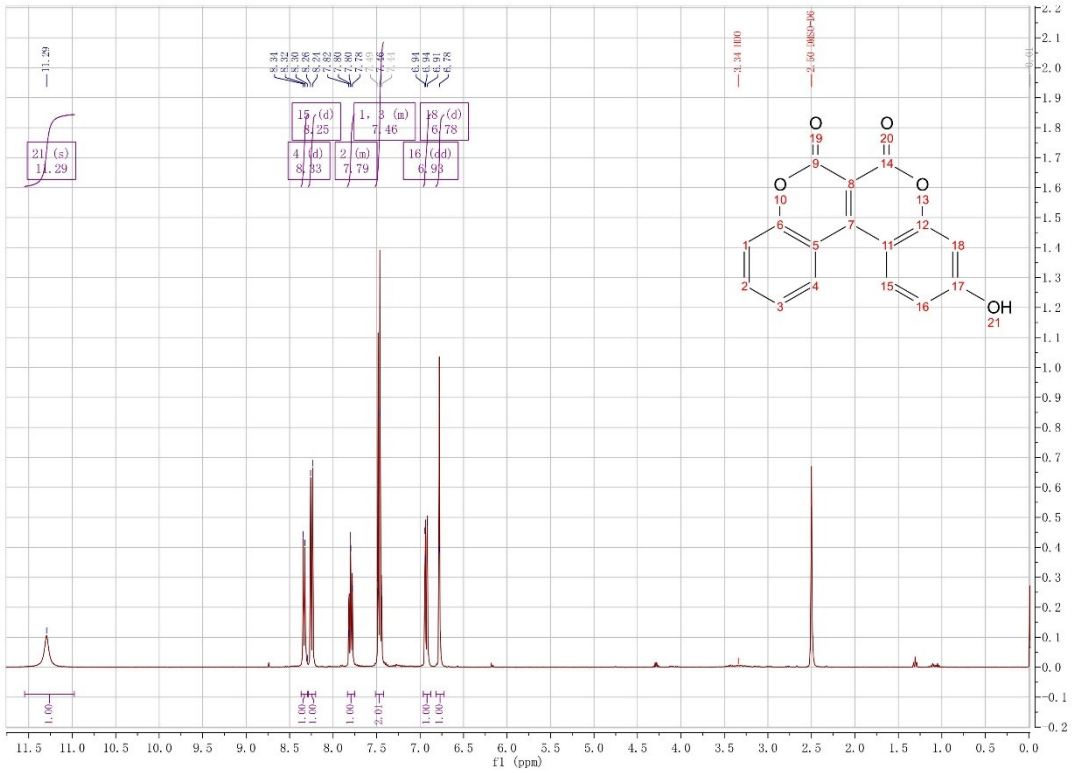


Figure S19. The ^{1}H NMR spectra of **2** in DMSO-d_6.

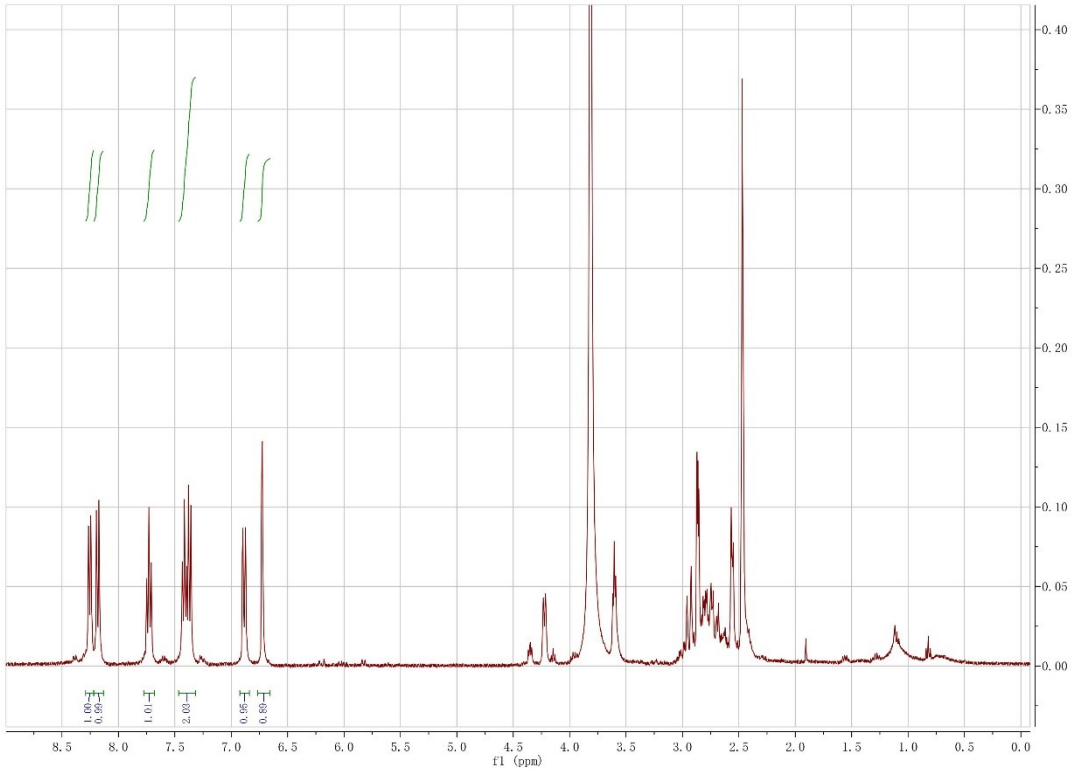


Figure S20. The ^{1}H NMR spectra of **CCx**-Cys in DMSO-d_6.

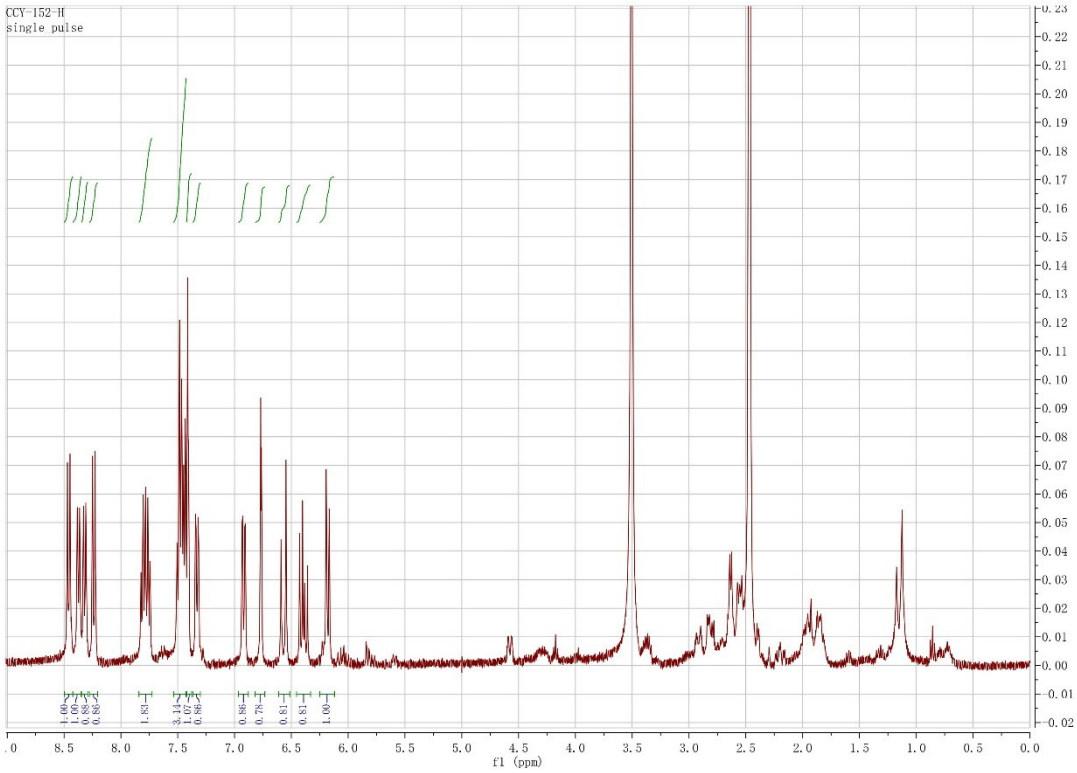


Figure S21. The ^{1}H NMR spectra of **CCx**-Hcy in DMSO-d_6.

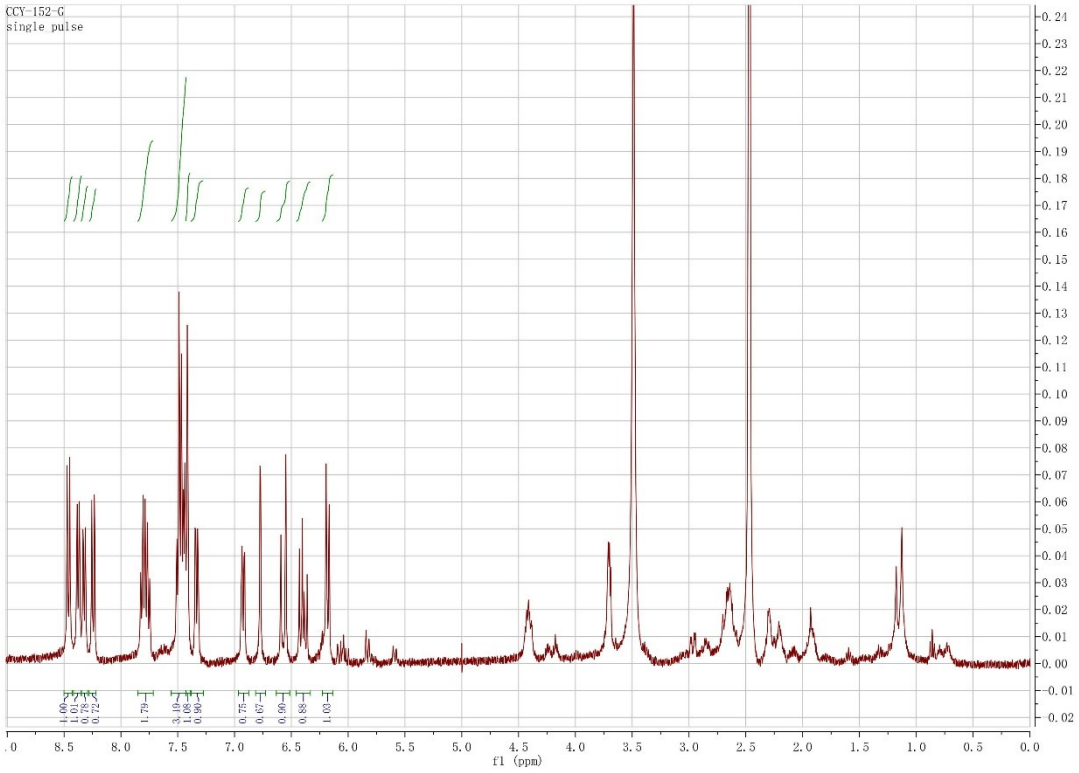


Figure S22. The ^{1}H NMR spectra of **CCx**-GSH in DMSO-d_6.

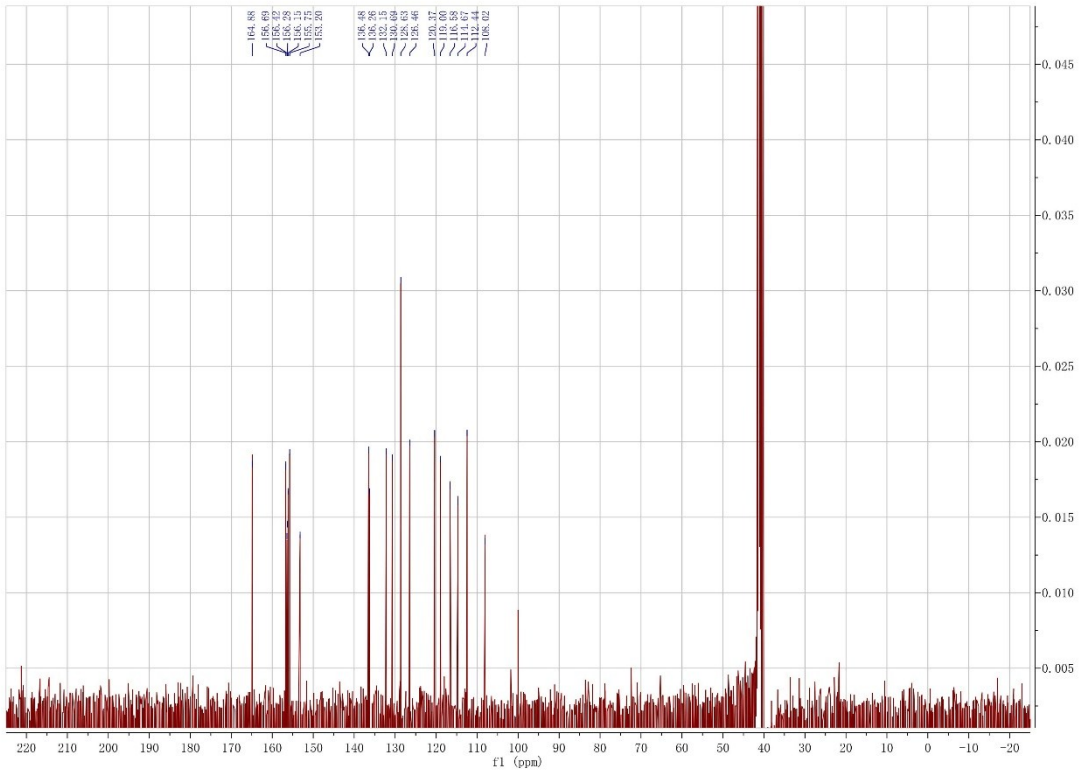


Figure S23. The ^{13}C NMR spectra of **CCx** in DMSO-d_6.

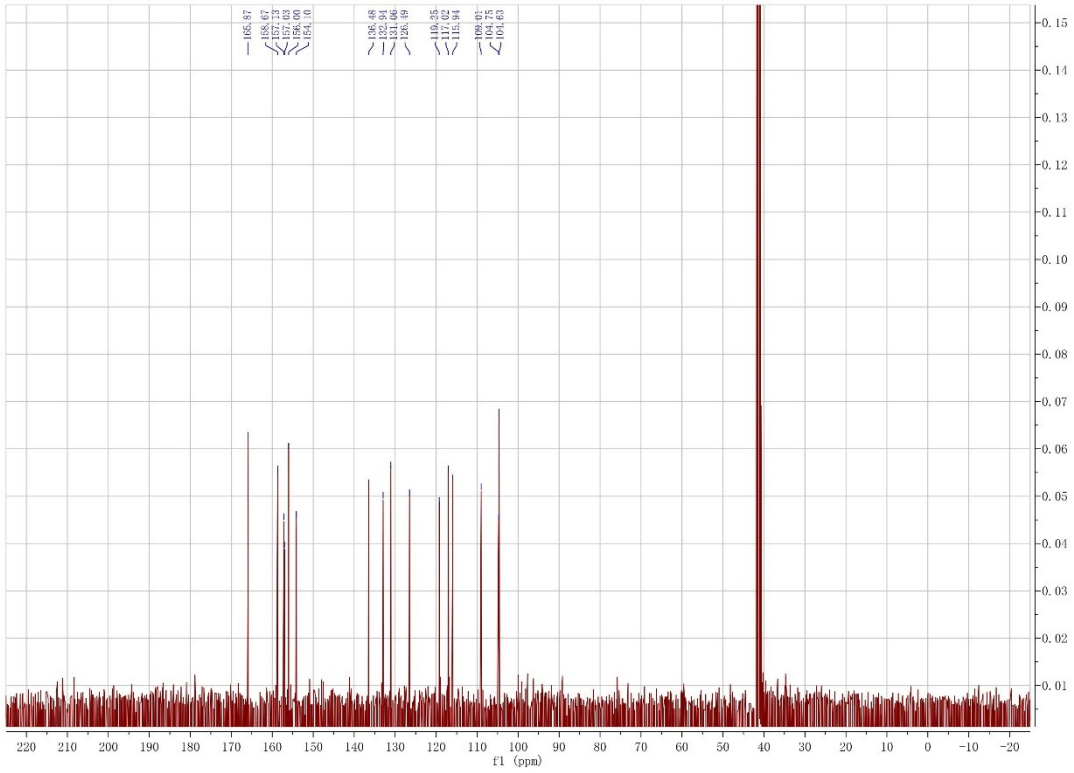


Figure S24. The ^{13}C NMR spectra of **2** in DMSO-d_6.

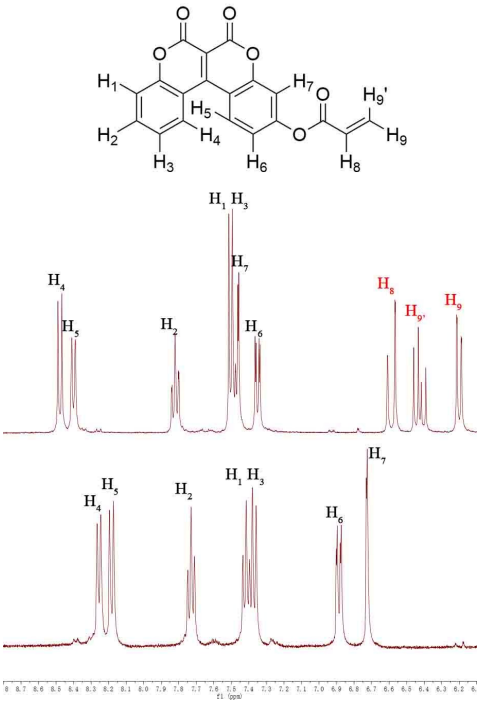


Figure S25. The change of ^{1}H NMR spectra of **CCx** after reacted with Cys.

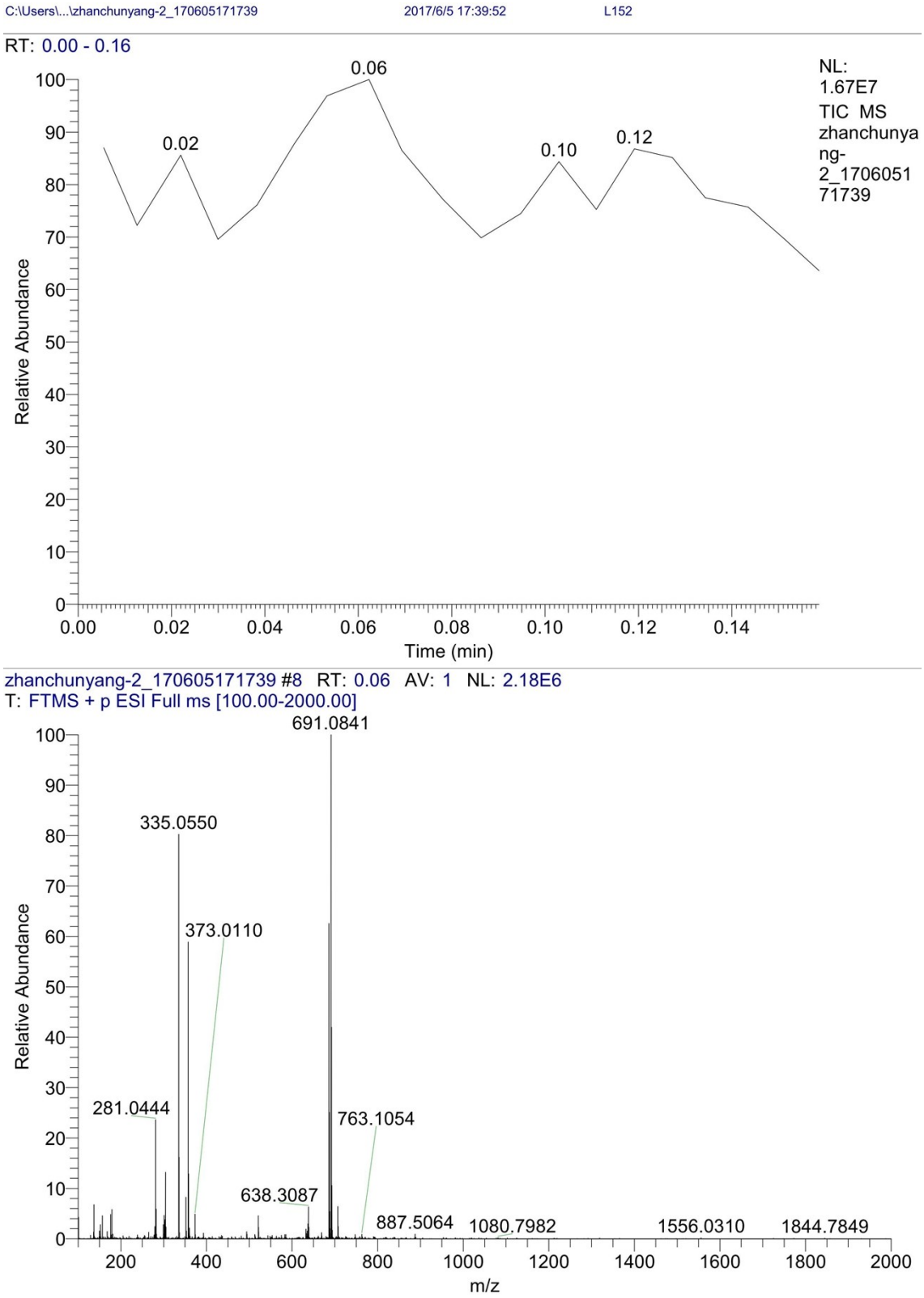


Figure S26. The Mass spectra of **CCx**.

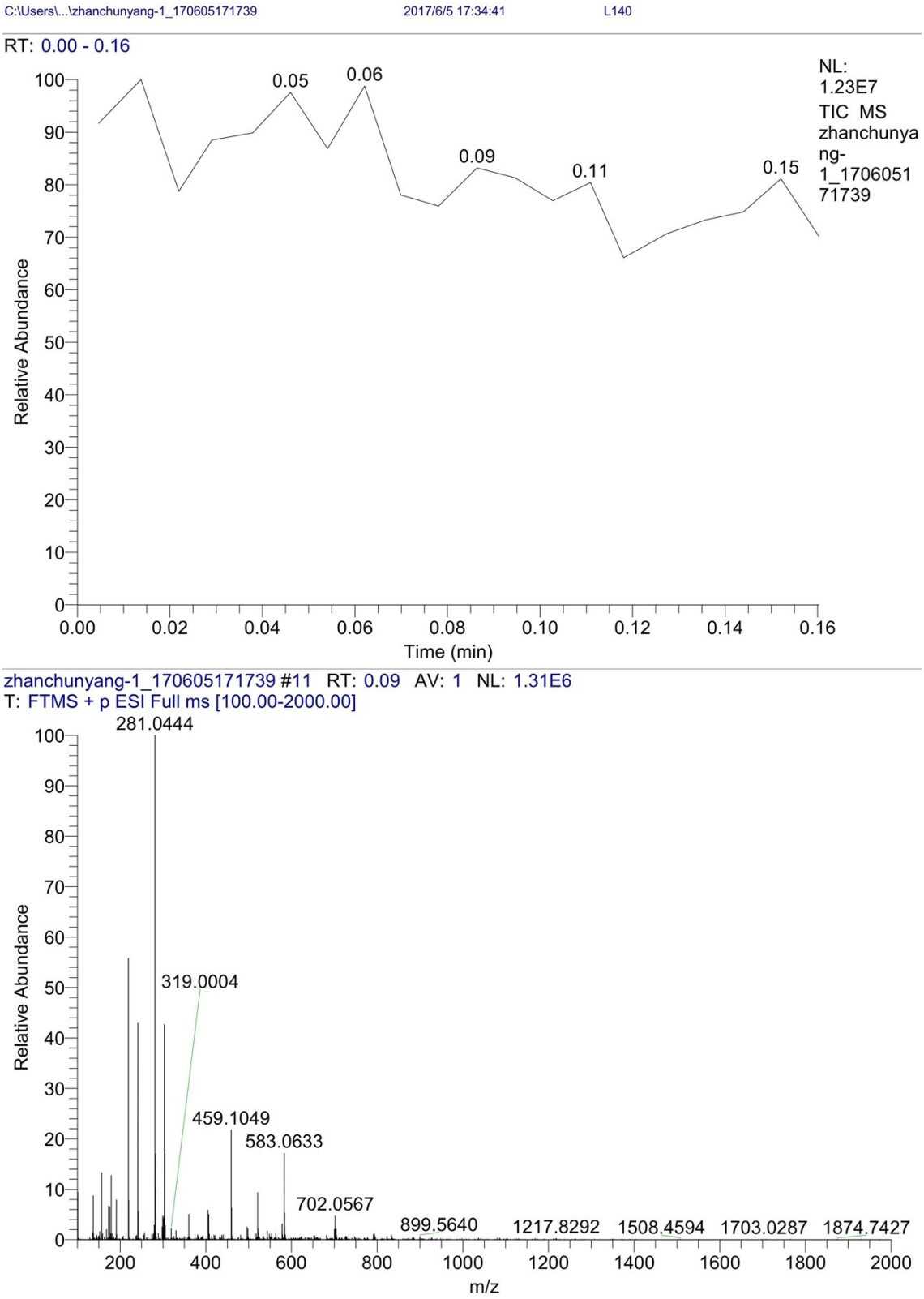


Figure S27. The Mass spectra of **2**.