Supporting Information for

Development of a two-photon turn-on fluorescent probe for cysteine and its bio-imaging applications in living cells, tissues, and zebrafish

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Materials and instruments

All of the reagents used are commercially available and purchased from commercial suppliers and used directly without further purification. All chemical reagents mentioned have potential danger and harm to health, they should be manipulated carefully and recovered, and should not be discard for environmental protection. The solvents were dried with sodium or calcium hydride and purified by distillation before use. Double distilled water was used throughout all experiments and test procedure. TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals. The ^{1}H NMR and ^{13}C NMR data was collected on AVANCE III 400 MHz Digital NMR Spectrometer and tetramethylsilane (TMS) was used as internal standard. Proton chemical shifts are reported in parts per million downfield form TMS. High resolution electrospray mass (HR-MS) were recorded on Apex-Ultra. Electronic absorption spectra were recorded on Shimadzu UV-2700 UV–vis spectrophotometer, fluorescent spectra were measured by Hitachi F4600 Fluorescent Spectrophotometer. Cell imaging was performed on Nikon A1MP Ti-e Fluorescence Microscopy. The pH value was determined on Mettler-Toledo pH Meter.

General procedure for the spectrum measurement

Unless otherwise noted, all the measurements were made according to the following procedure. The concentration of the probe stock solution was 1.0 mM in DMSO, and the analytes stock solutions were prepared in the ultrapure water at the appropriate concentration. In 5 mL volumetric flask, the test solution was prepared by placing 25 μL probe stock solution and 225 μL DMSO, requisite amount of analyte stock solution, then adjusted the final volume to 2 mL with PBS buffer. The spectrum tests were recorded with a 1 cm standard quartz cell at room temperature. The absorption spectra were obtained on a Shimadzu UV-2700 Power spectrometer. The photoluminescent spectra were recorded with a HITACHI F4600 fluorescence

spectrophotometer. The excitation wavelength was 400 nm, the excitation slit widths were 5 nm, and the emission slit widths were 5 nm.

Cells culture

The HeLa cells were cultured in 35 mm glass-bottom culture dishes with DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (Fetal Bovine Serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C for 24 h. Before the imaging experiments, the cells were washed with PBS for 2-3 times.

Fluorescence imaging of Cys in living cells

The living cells were divided into three groups for the exogenous Cys imaging experiments: the first group of cells were blank; the second group of cells were cultured with 10 μM **Co-Cys** for 30 min; the third group of cells were pre-cultured with 100 μM Cys for 30 min, then the cells cultured with 10 μM **Co-Cys** for another 30 min.

Cytotoxicity assays

HeLa cells line were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS and 100 U/mL of penicillin and 100 μg/mL streptomycin in an atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were then seeded into 96-well plates, and then 0-50.0 μM of **Co-Cys** (99.9% DMEM and 0.1% DMSO) was added respectively. Subsequently, the cells were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% air for 24 h. Finally, the absorbance of the solution was acquired by using the microplate reader at 570 nm. The toxicity of **Co-Cys** was calculated by the following formula.

$$\text{The cell viability (\%)} = (OD_s\text{-}OD_b)/(OD_c\text{-}OD_b) \times 100\%.$$

OD_s denotes the cells incubated with various concentrations of the probe, OD_c

denotes the cells without the probe, OD_b denotes the wells containing only the culture medium.

Quantum yields

The fluorescence quantum yields can be calculated by means of eqn (1):

$$\emptyset_s = \emptyset_r\left(\frac{A_r(\lambda_r)}{A_s(\lambda_s)}\right)\left(\frac{{n_s}^2}{{n_r}^2}\right)\frac{F_s}{F_r} \quad (1)$$

Where the subscripts s and r refer to the sample and reference, respectively. Φ is the quantum yield, F is the integrated emission intensity, A stand for the absorbance, and n is refractive index.

Fluorescence imaging in living zebrafish

Wild type zebrafish were purchased from the Nanjing EzeRinka Biotechnology Co. Ltd. All procedures for this study were approved by the Animal Ethical Experimentation Committee of Shandong University according to the requirements of the National Act on the use of experimental animals (China). The zebrafish were kept at 28 °C and optimal breeding conditions. For the fluorescence imaging experiments, 3-day-old zebrafish were transferred into a 30 mm glass culture dishes using a disposable sterilized dropper. 10 μM probe **Co-Cys** and 100 μM Cys was added for incubated for 30 min, followed by washing away gently. After that, the zebrafish were transferred into new glass bottom dishes for imaging. Prior to the imaging, we adopted 1% agarose gel for immobilization of zebrafish, and put zebrafish onto agarose with a little media to ready imaging. The imaging experiments were recorded through a Nikon A1MP confocal microscopy inverted fluorescence microscopy equipped with a cooled CCD camera. The fluorescence emission was collected at FITC channel (500-550 nm) upon excitation at 405 nm.

Mice liver tissue slices preparation and fluorescent imaging

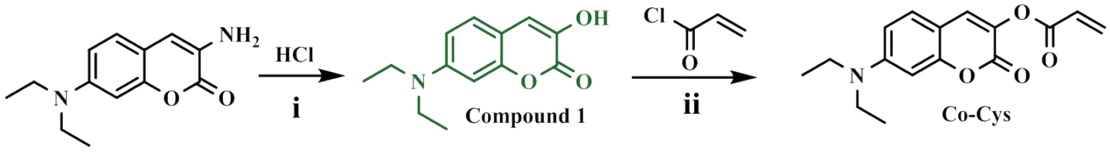
4 weeks old Kunming mice were purchased from Shandong University Laboratory Animal Centre (Shandong, China). Unless otherwise noted, all procedures for this study were approved by the Animal Ethical Experimentation Committee of Shandong University according to the requirements of the National Act on the use of experimental animals (China). The mice were killed by cervical vertebra dislocation, the liver tissues were cut into about 500 μm in size. For the control group, the liver tissue slices treated with **Co-Cys** (10 μM) for 30 min. For the experimental group, the liver tissue slices pretreated with Cys (100 μM) for 30 min, and then treated with **Co-Cys** (10 μM) for another 30 min. Then the imaging experiments were carried out.

The fluorescence images were acquired by a Nikon A1MP Ti-e confocal microscopy. The OP and TP fluorescence emission were obtained at 500-550 nm under excitation of 405 nm and 760 nm, respectively.

Synthesis

The synthetic route of the probe **Co-Cys** was shown in Scheme S1. The **Co-Cys** were synthesized according to the previous literature[1].

Scheme S1. Synthesis of the probe **Co-Cys**.



Reagents and conditions: (i) In the 100 mL round bottomed flask, 7-diethylamino-3-amino coumarin 1 mmol was added to the hydrochloric acid solution to be heated to 100 ℃, and the reaction was filtered, and the filter cake was 7-diethylamino-3-hydroxy coumarin (compound **1**). The concentration of hydrochloric acid solution is 1 ~ 1.5 mol/L, and the reaction time is 3 ~ 4 h. Yield: 91%. The product is not purified directly to the next step. (ii) The compound **1** and acroleyl chloride were stirred in dichloromethane, the reaction temperature was 25 °C the reaction time was 12 h, the vacuum distillation and vacuum drying were purified by column chromatography. Yield: 67%

[1]. Y. Zhang, J.H Wang W.J. Zheng, T.F. Chen and Q.X. Tong, *J. Mater Chem. B,* 2014, **2**, 4159-4166.

Synthesis of compound 1

In the 100 mL round bottomed flask, 7-diethylamino-3-amino coumarin (2 mmol, 464 mg) was added to the hydrochloric acid solution (30 mL) to be heated to 100 °C, and the reaction was filtered, and the filter cake was 7-diethylamino-3-hydroxy coumarin (compound **1**). The concentration of hydrochloric acid solution was 1 ~ 1.5 mol/L, and the reaction time is 3 ~ 4 h. Yield: 91%. The product is not purified directly to the next step ^{1}HNMR (400 MHz, DMSO-d_6) δ 9.51 (s, 1H), 7.29 (d, J=8,8 Hz, 1H), 7.02 (s, 1H), 6.65 (dd, J_1=8.8 Hz, J_2=2.3 Hz, 1H), 6.51 (d, J=2.3 Hz, 1H), 3.38 (dd, J_1=14.4 Hz, J_2=7.4 Hz, 5H), 7.62 (t, J=7.0 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 159.50, 152.02, 148.10, 137.47, 127.55, 117.53, 109.67, 108.86, 97.41, 44.33, 12.79. HR-MS m/z calcd for $C_{13}H_{15}NO_3$ [M+H] $^{+}$: 234.1125; found 234.1125.

Synthesis of the compound Co-Cys

The compound **1** (1 mmol, 233 mg) and acroleyl chloride (2 mmol, 180 mg) were stirred in dichloromethane, the reaction temperature was 25 °C the reaction time was 12 h, the vacuum distillation and vacuum drying were purified by column chromatography, and the leaching agent was dichloromethane / petroleum ether (V/V=5: 1), and the compound 7-diethylamino-3-acrylate coumarin (**Co-Cys)**. Yield: 67%. ^{1}HNMR (400 MHz, DMSO-d_6) δ = 7.93 (s, 1H), 7.45 (d, J=8.9 Hz, 1H), 6.77 (dd, J_1=8.9 Hz, J_2=2.3 Hz, 1H), 6.61 (m, 1H), 5.99 (m, 1H), 5.42 (dd, J_1=17.2 Hz, J_2=1.4 Hz, 1H), 5.32 (dd, J_1=10.5 Hz, J_2=1.0 Hz，1H), 4.75 (m, 2H), 3.44 (q, J=7.0 Hz, 4H), 1.12 (m, 6H). ^{13}CNMR (100 MHz, DMSO-d_6) δ = 157.35, 154.94, 152.61, 150.84, 133.00, 131.95, 130.40, 129.91, 119.46, 110.09, 106.45, 97.13, 69.63, 44.53, 40.48, 40.31, 40.15, 39.98, 39.81, 39.64, 39.48, 12.72. HR-MS m/z calcd for $C_{16}H_{17}NO_4$ [M+H] $^{+}$: 288.1230; found 288.1235.

Detection limit

The detection limit was determined from the fluorescence titration data based on a reported method.[2] The probe **Co-Cys** (5 μM) was titrated with Cys (0-55 μM) for 50 min. The fluorescent intensity data at 496 nm were normalized between the minimum intensity and the maximum intensity. A linear regression curve was then fitted to the normalized fluorescent intensity data and the point at which this line crossed the axis (Fig. S1) was considered as the detection limit (0.1080 μM).

[2] M.Shortreed, R.Kopelman, M. Kuhn and B. Hoyland. *Anal. Chem.*, 1996, **68**, 1414-1418.

Table. S1. The photophysical properties of **Co-Cys** in various solvents.

Probe	Solvents	λ^a/nm	λ^b/nm	Φ^c/%
Co-Cys	DMF	405	496	0.30
	DMSO	406	498	0.35
	MeCN	405	502	0.12
	THF	402	497	0.16
	PBS	405	488	0.10
	H_2O	405	482	0.09

λ^a and λ^b are maximum absorption and maximum fluorescent emission peak, respectively; Φ^c is fluorescence quantum yield determined by using (Φ=0.95) in water as the standard.

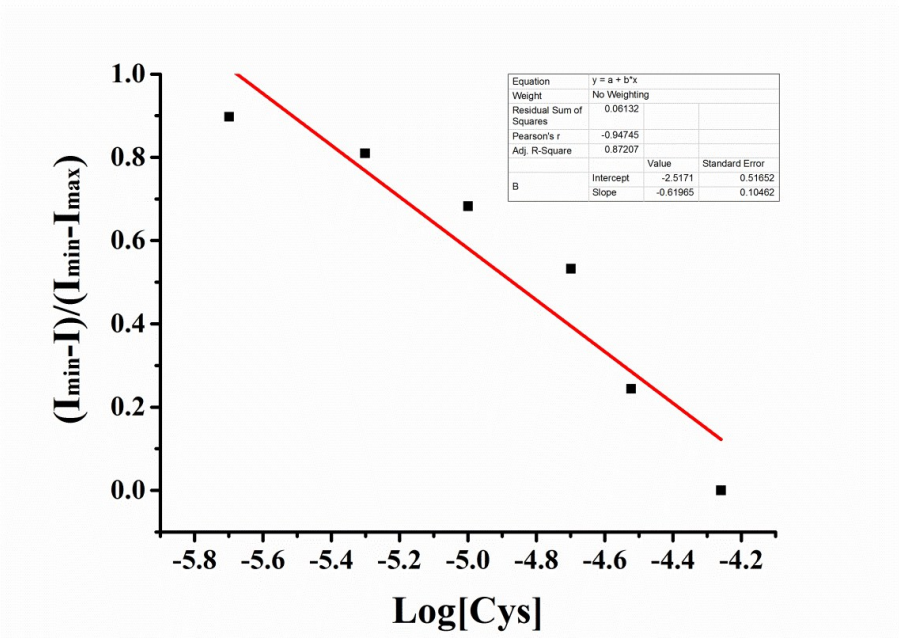


Fig. S1. Normalized response of the fluorescence signal by changing the concentration of Cys.

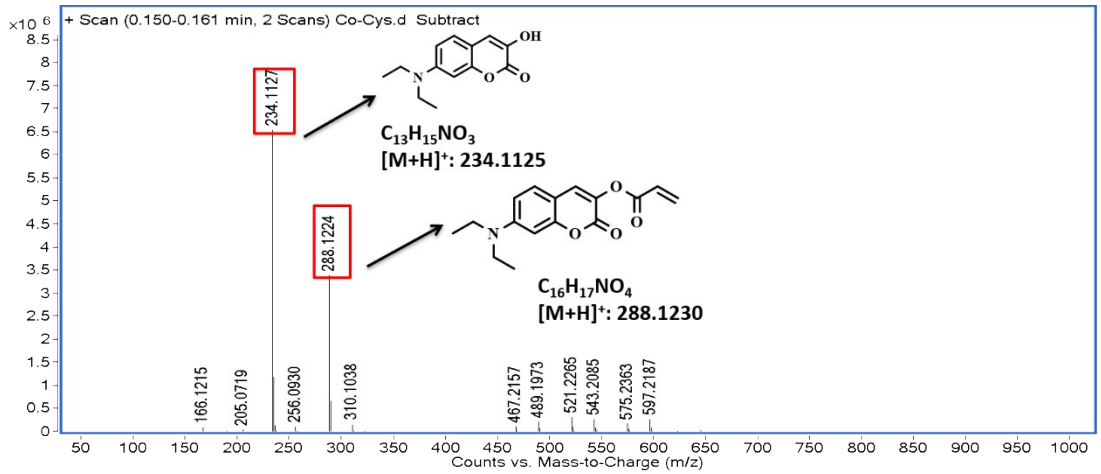


Fig. S2. HR-MS spectrum of 10 μM **Co-Cys** treated with 55 μM Cys in 10 mM PBS buffer (pH 7.4, 10% DMSO).

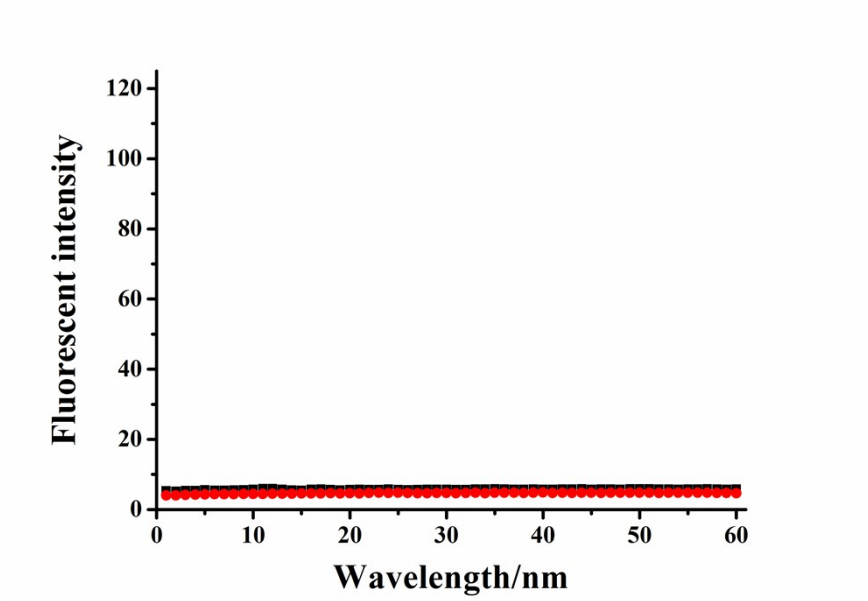


Fig. S3. Photostability profiles of **Co-Cys** (5 μM) in the absence or presence of UV-irradiated (365 nm). The fluorescence intensities at 496 nm were continuously monitored at time intervals in PBS (10 mM, pH 7.4, 10% DMSO). [ ]light; [ ]●light

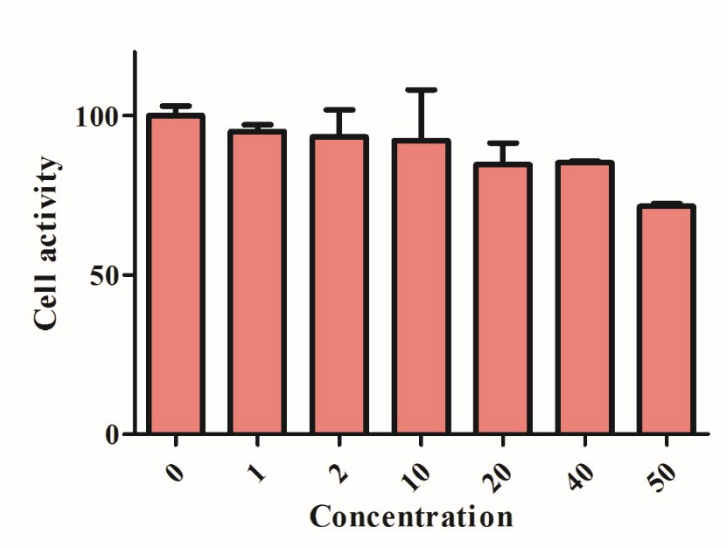


Fig. S4. Effects of the probe **Co-Cys** with varied concentrations (0-50 μM) on the viability of the Hela cells. The probe with varied concentrations was incubated with the cells for 24 h. The viability of the cells in the absence of the probe is defined as 100%, and the data are the mean standard deviation of five separate measurements.

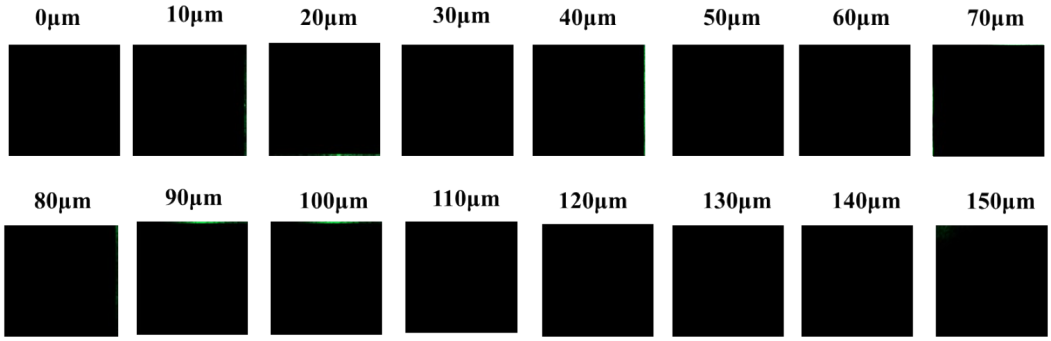


Fig. S5. Two-photon fluorescence imaging of the liver slides treated with **Co-Cys**. Fluorescence images of the liver slides incubated with **Co-Cys** (10 μM). Excitation was at 760 nm by the femtosecond laser and the emission collection was from 500-550 nm.

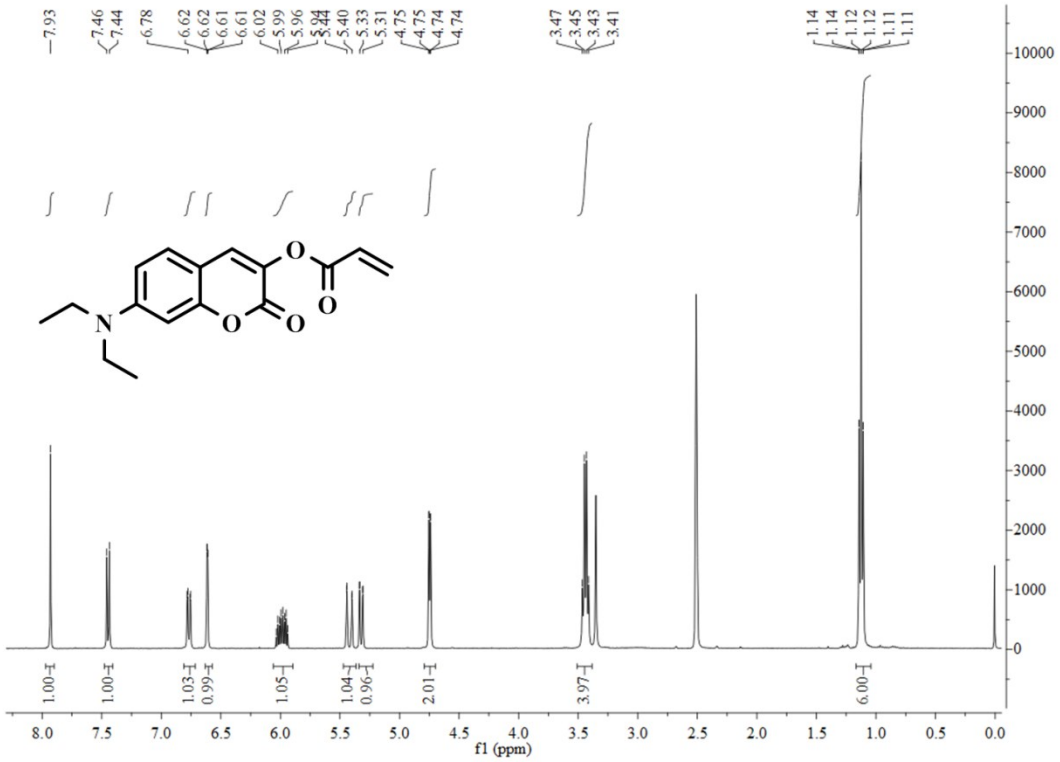


Fig. S6. ^{1}H NMR spectrum of the probe **Co-Cys**.

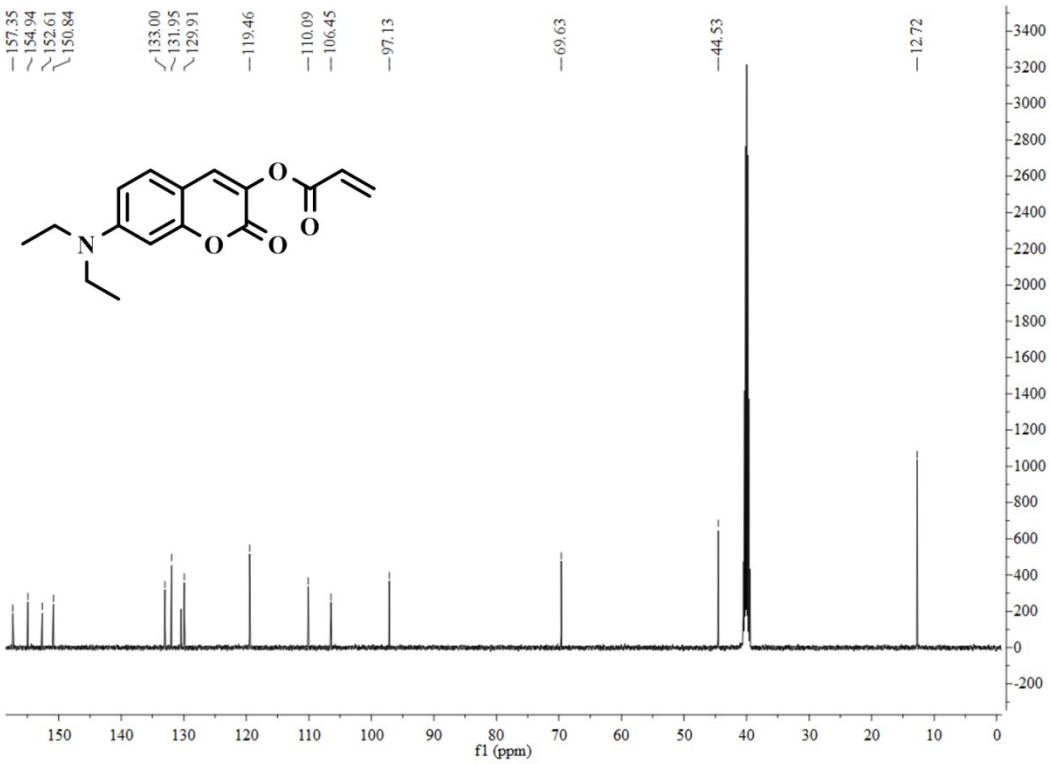


Fig. S7. ^{13}C NMR spectrum of the probe **Co-Cys**.

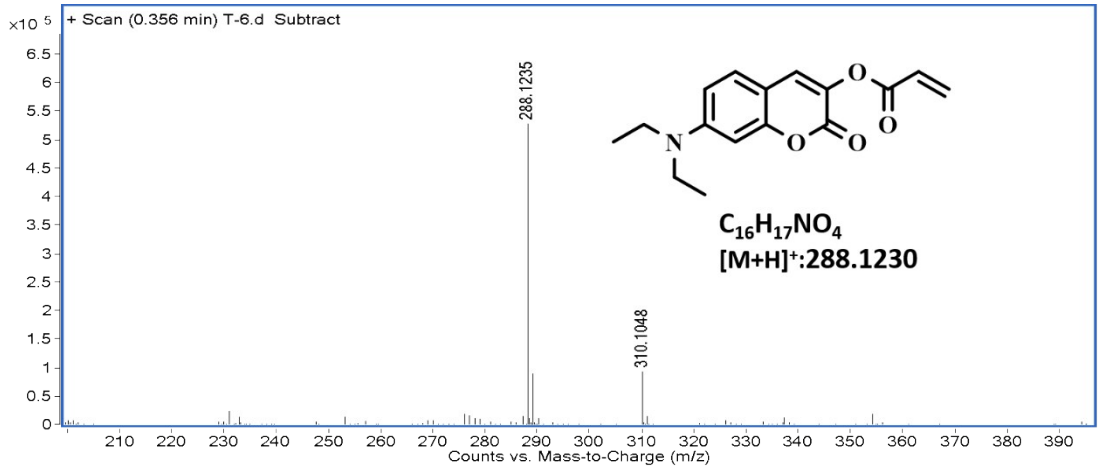


Fig. S8. HR-MS spectrum of the probe **Co-Cys**.

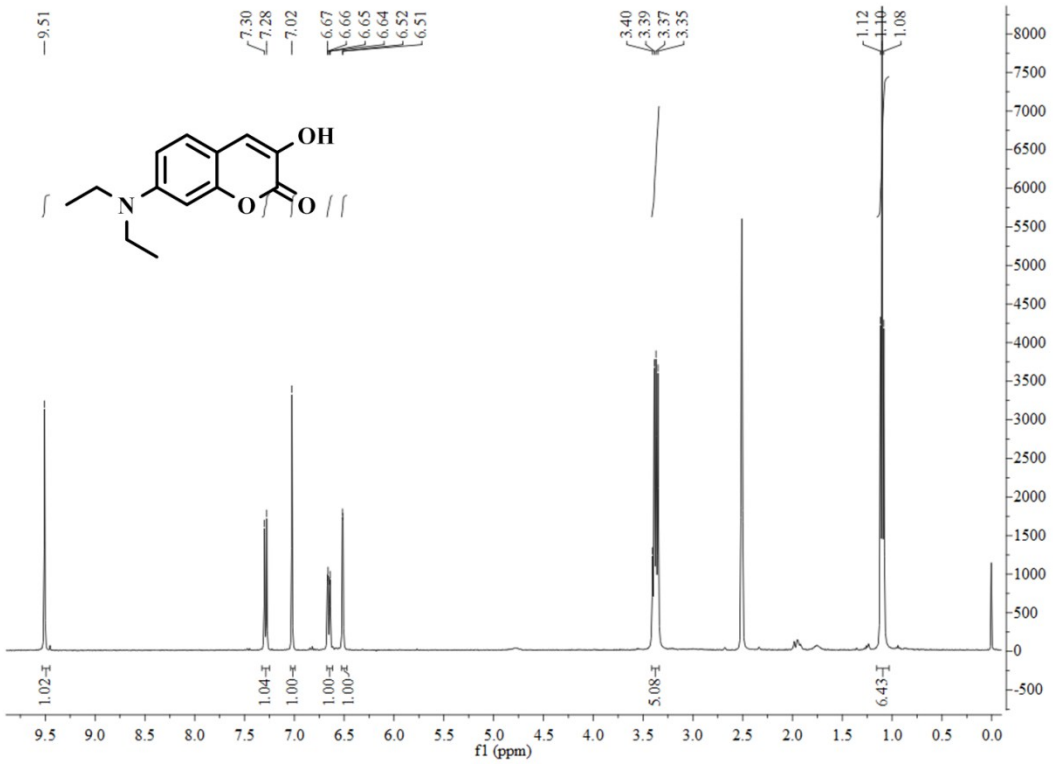


Fig. S9. [1]H NMR spectrum of the compound **1**

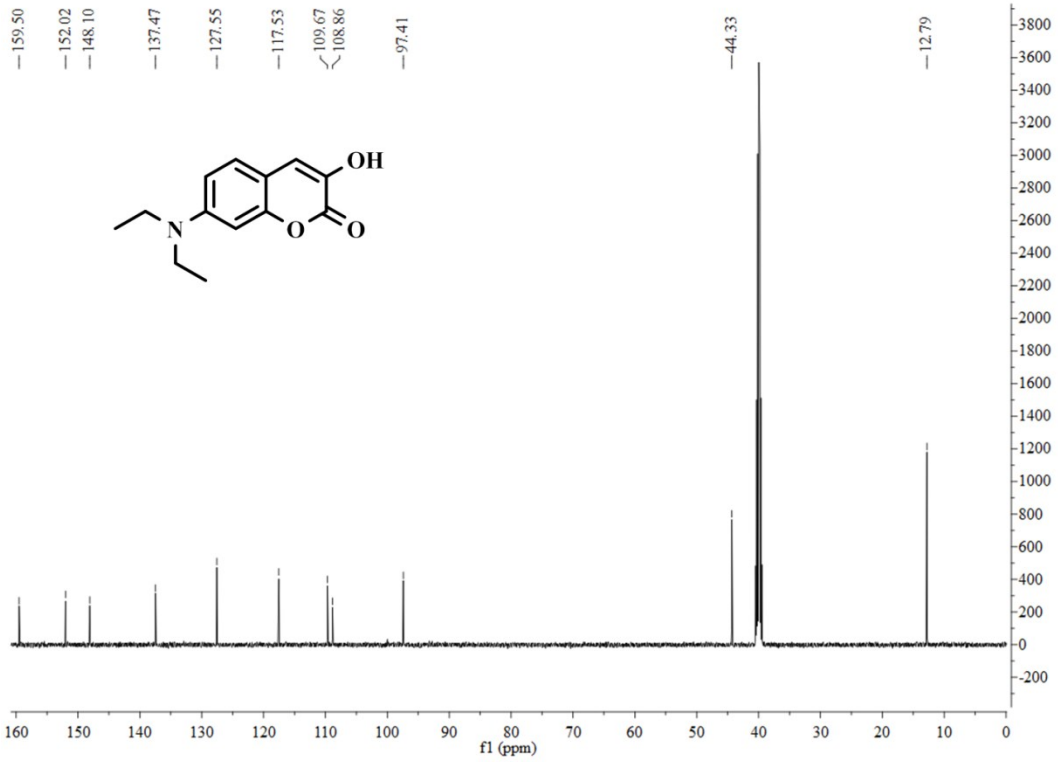


Fig. S10. [13]CNMR spectrum of the compound **1**.

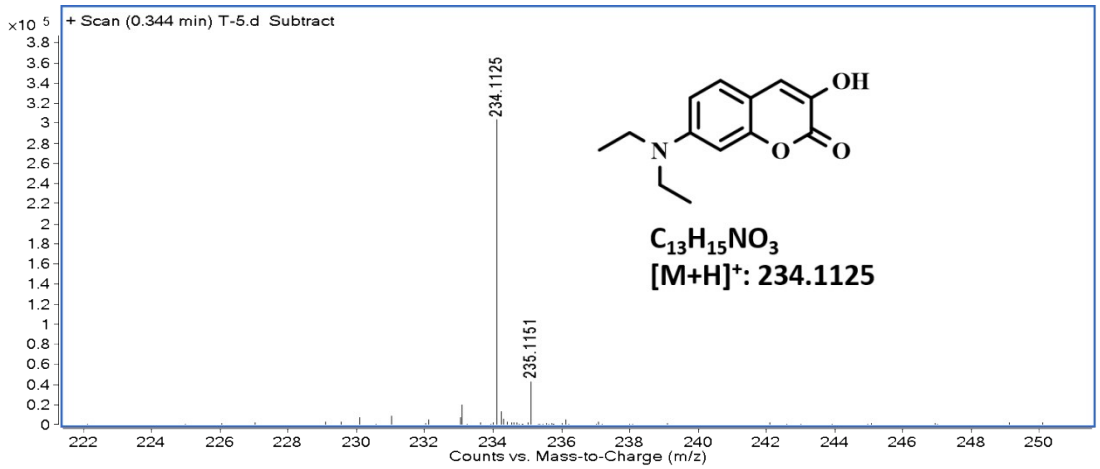


Fig. S11. HR-MS spectrum of the probe compound **1**.