Supporting Information for

A coumarin-based fluorescent probe for biological thiols and its application for living cell imaging

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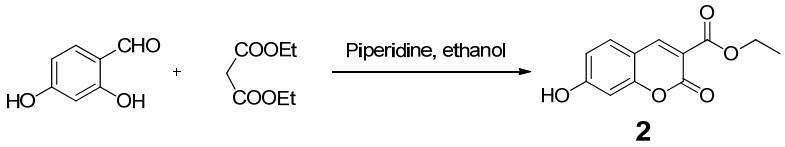
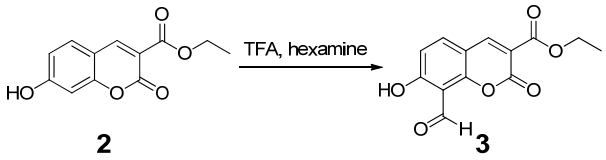
Materials and instruments: Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. Melting points of compounds were measured on a Beijing Taike XT-4 microscopy melting point apparatus, and all melting points were uncorrected. Mass spectra were recorded on a LXQ Spectrometer (Thermo Scientific) operating on ESI. ^{1}H NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz and 100 MHz respectively. Elemental (C, H, N) analysis were carried out using Flash EA 1112 analyzer. The Crystallographic data were collected on a Saturn 724^{+} CCD X-ray diffractometer by using graphite monochromated Mo $K\alpha$ (λ = 0.71070 Å). Electronic absorption spectra were obtained on a SHIMADZU UV-2450 spectrometer. Fluorescence spectra were measured on a CaryEclipse fluorescence spectrophotometer with 2.5 nm excitation and emission slit widths. Cells imaging were performed with an inverted fluorescence microscope (Carl Zeiss, Axio Observer A1). All pH measurements were performed with a pH-3c digital pH-meter (Shanghai ShengCi Device Works, Shanghai, China) with a combined glass-calomel electrode. 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.



2

Synthesis of ethyl 7-hydroxy-coumarin-3-carboxylate (2):

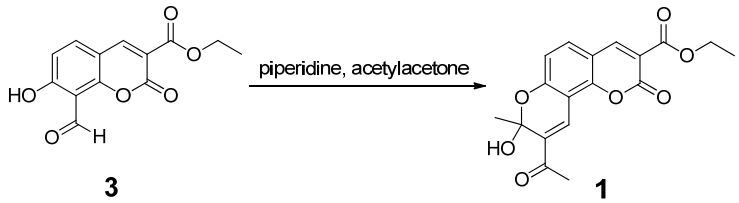
The compound **2** was synthesized according to a reported procedure.[1] Diethyl malonate (6.96 g, 43.45 mM), 2,4-dihydroxybenzaldehyde (6.0 g, 43.44 mM) and piperidine (1 mL) were dissolved in ethanol (75 mL), and then the solution was heated under reflux for 4 hours. After cooling, the precipitate was collected by filtration. The crude product was recrystallized in ethanol to afford the compound **2** as white solid (7.73 g, yield 76%). mp: 170.5-171.8 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 11.08 (s, 1H), 8.87 (s, 1H), 7.75 (d, J = 8.8 Hz, 1H), 6.84 (dd, J_1 = 8.8 Hz, J_2 = 2.4 Hz, 1H), 6.72 (d, J = 2.4 Hz, 1H), 4.26 (q, J = 7.2 Hz, 2H), 1.30 (t, J = 7.2 Hz, 3H). MS (*m/z*):235.2 $[M+H]^+$.



2 **3**

Synthesis of ethyl 7-hydroxy-8-formyl-coumarin-3-carboxylate (3):

A solution of compound **2** (2.0 g, 8.54 mM) and hexamine (1.2 g, 8.56 mM) in TFA (7 mL) was heated under reflux for 20 h, and then 60 mL water was added. The solution was further stirred for 30 min at 60^{o}C. After cooling, the precipitate was collected by filtration. The crude product was purified by chromatography on silica gel (dichloromethane: petroleum ether: ethanol = 100:100:1, v/v) to give the yellow solid compound **3** (1.41 g, yield 63%). mp: 189.7-190.0 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 12.51 (s, 1H), 10.61 (d, J = 0.4 Hz, 1H), 8.53 (s, 1H), 7.73 (d, J = 8.8 Hz, 1H), 6.96 (dd, J_1 = 8.8 Hz, J_2 = 0.4 Hz, 1H), 4.43 (q, J = 7.2 Hz, 2H), 1.43 (t, J = 7.2 Hz, 3H); MS (*m/z*):261.09 $[M-H]^-$.



3 **1**

Synthesis of ethyl 8-(2-acetyl-3-oxobut-1-en-1-yl)-7-hydroxy-2-oxo-2H-chromene -3-carboxylate (1):

A solution of compound **3** (190 mg, 0.724 mM), acetylacetone (362.7mg, 3.62 mM), and piperidine (15 μL) in chloroform (8 mL) was heated under reflux for 4 h. After cooling to room temperature, the precipitate was collected by filtration. The crude product was purified by chromatography on silica gel (dichloromethane: petroleum ether: ethanol = 50:50:1, v/v) to give compound **1** as white solid (197 mg, yield 79%). mp: 202.1-203.7 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.77 (s, 1H), 7.91 (d, J = 8.4 Hz, 1H), 7.78 (s, 1H), 7.55 (s, 1H), 7.01 (d, J = 8.4 Hz, 1H), 4.30 (q, J = 7.2 Hz, 2H),

2.50 (s, 3H), 1.88 (s, 3H), 1.32 (t, J = 7.2 Hz, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz), δ (ppm): 196.6, 163.1, 158.3, 156.0, 152.8, 150.0, 134.6, 133.9, 125.5, 114.6, 114.1, 112.0, 107.5, 100.2, 61.5, 28.0, 27.5, 14.6; MS (*m/z*):343.27 $[M-H]^-$.

Preparation of the test solution: The stock solution of probe **1** (2.5×10^{-4} M) was prepared in DMSO, and the stock solution of various biologically relevant testing species (1×10^{-3} M) was prepared by dissolving an appropriate amount of testing species in water. The test solution of the probe (5 μM) in aqueous solution (10 mM potassium phosphate buffer, pH 7.4, 2% DMSO as co-solvent) was prepared by placing 0.1 mL of the probe **1** stock solution and an appropriate aliquot of each testing species stock into a 5 mL volumetric flask, and then diluting the solution to 5 mL with 10 mM potassium phosphate buffer (pH 7.4). The resulting solution was shaken well and incubated at room temperature for 6 min before recording the spectra.

Determination of fluorescence quantum yield: Fluorescence quantum yield was determined using the solutions of Quinine Sulfate (Φ_F = 0.546 in 1N H_2SO_4) [2] as a standard. The quantum yield was calculated using the following equation: [3-5]

$$\Phi_{F(X)} = \Phi_{F(S)} (A_S F_X / A_X F_S) (n_X / n_S)^2$$

Where Φ_F is the fluorescence quantum yield, A is the absorbance at the excitation wavelength, F is the area under the corrected emission curve, and n is the refractive index of the solvents used. Subscripts S and X refer to the standard and to the unknown, respectively.

Determination of the detection limit: The detection limit was calculated according to the method used in the previous literature.[6] The fluorescence emission spectrum of probe **1** was measured by five times and the standard deviation of blank measurement was achieved. The fluorescence emission intensity (455 nm) was plotted as a concentration of Cys. The detection limit was calculated with the following equation:

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

Where σ is the standard deviation of blank measurement, k is the slop between the fluorescence emission intensity versus Cys concentration.

Determination of thiols in human blood serum: For determination of the biological thiols in human blood serum, the serum was firstly treated with a reducing agent, triphenylphosphine, to reduce all the oxidized disulfide to free thiols.[7] Briefly, 2 mL human blood serum sample was diluted with 1 mL distilled water, then treated with 1 mL triphenylphosphine solution in CH_3CN (1.5×10^{-3} M) for 30 min at room temperature. After filtration, aliquots of the reduced serum sample (50, 100, 150, 200, 250, 300 μL) were added directly to a solution of probe **1** (5 μM, the total volume was 3 mL) in aqueous solution (10 mM potassium phosphate buffer, pH 7.4, containing 2%

DMSO as co-solvent). After the resulting solution incubated at room temperature for 6 min, the fluorescence emsision spectra (λ_{ex}= 412 nm) were recorded.

Cell culture and fluorescence imaging: Pancreatic cancer cells were seeded in a 24-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum for 24 h. Pancreatic cancer cells were then incubated with probe **1** (5 μM) in the culture medium for 10 min at 37°C. After washing with PBS three times to remove the remaining probe, the fluorescence images were acquired with a fluorescent microscope (Carl Zeiss, Axio Observer A1). Excitation wavelength of laser was 365 nm, and emissions were centered at 445 ± 10 nm. For the N-ethylmaleimide control experiment, the cells were pre-treated with N-ethylmaleimide (1 mM and 5 mM) for 30 min at 37°C followed by washing with PBS three times. And then, the cells were incubated with probe **1** (5 μM) for 10 min at 37°C. Fluorescence imaging was carried out after washing the cells with PBS buffer three times.

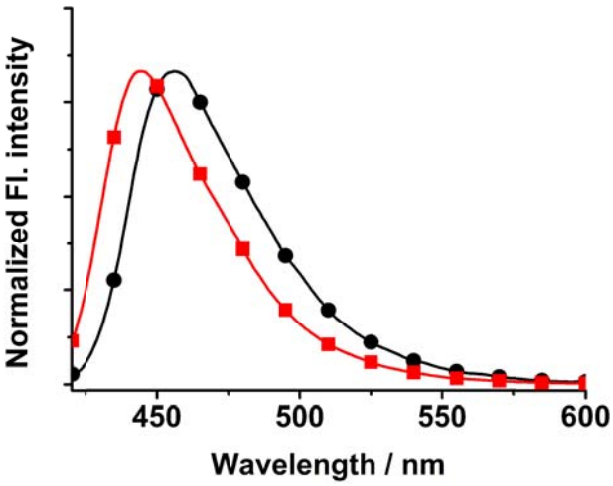


Figure S1. Normalized fluorescence emission spectra of probe **1** + Cys (350 μM) (●) and the reference compound **2** (■).

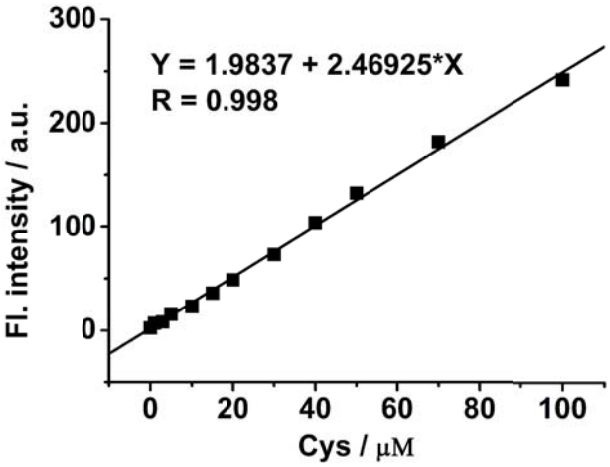


Figure S2. Changes in fluorescence intensity (455 nm) of probe **1** (5 μM) with various amount of Cys (0-100 μM), spectra were obtained with excitation at 412 nm.

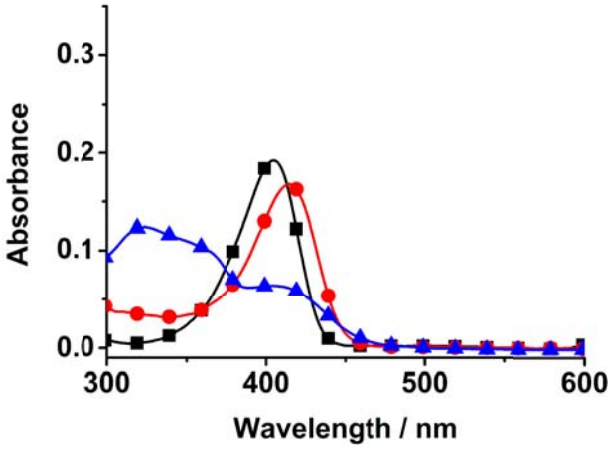


Figure S3. The absorption spectra of probe **1** (▲), probe **1** + Cys (350 μM) (●) and the reference compound **2** (■).

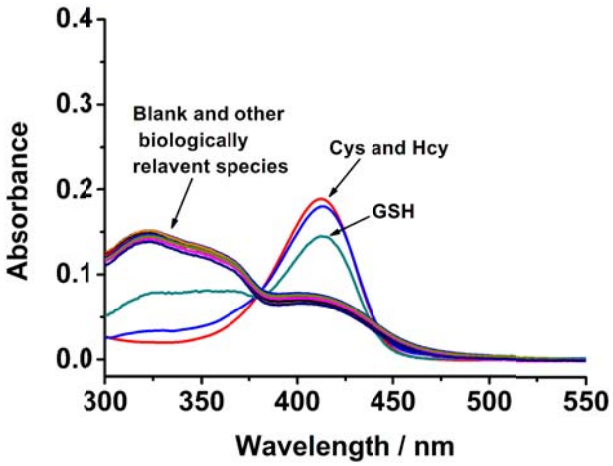


Figure S4. Absorption spectra of probe **1** (5 μM) in aqueous conditions (10 mM potassium phosphate buffer, pH 7.4, 2% DMSO as co-solvent) in the presence of various species (350 μM).

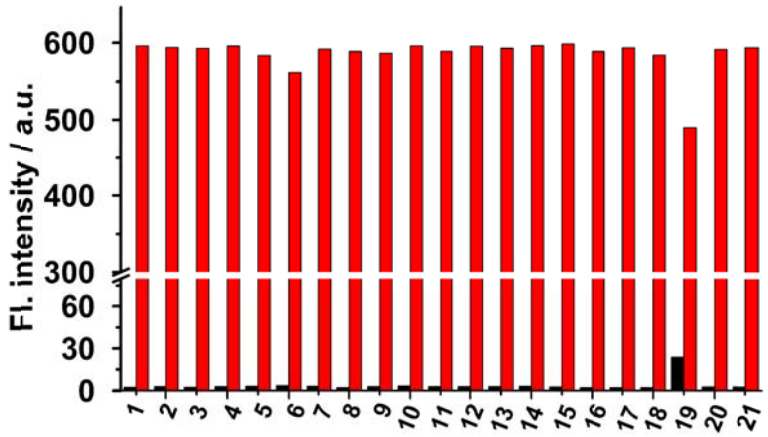


Figure S5. Fluorescence intensity (at 455 nm) response of probe **1** (5 μM) to various species (350 μM) in the absence (blank bar) and presence (red bar) of Cys (350 μM) in aqueous conditions (10 mM potassium phosphate buffer, pH 7.4, containing 2% DMSO as co-solvent). 1)blank; 2) Phe; 3) Ala; 4) Gly; 5) Glu; 6) Arg; 7) Lys; 8) Tys; 9) Leu; 10) Ser; 11) Val; 12) Gln; 13) Na^{+}; 14) K^{+}; 15) Mg^{2+}; 16) Zn^{2+}; 17) glucose; 18) H_2O_2; 19) H_2O_2 + Human Serum Albumin; 20) Benzenesulfinic acid sodium salt; 21) *p*-Toluenesulfonic acid sodium salt. Excitation wavelength was 412 nm.

Kinetic Studies: The reaction rate constant of probe **1** with Cys, Hcy, and GSH was estimated under *pseudo*-first-order kinetic conditions (5 μM probe **1** and 350 μM thiol). The reaction of probe **1** with thiols in aqueous conditions (10 mM potassium phosphate buffer, pH 7.4, containing 2% DMSO as co-solvent) was monitored by using the fluorescence intensity at 455 nm. The *pseudo*-first-order rate constant was determined by fitting the fluorescence intensities of probe **1** to the *pseudo*-first-order equation (1):[8-9]

$$\ln[(F_{max} - F_t) / F_{max}] = -k't \qquad (1)$$

where F_t and F_{max} are the fluorescence intensities at 455 nm at time t and the maximum value obtained after the reaction was completed. k' is the *pseudo*-first-order rate constant. Figure S6, Figure S7, and Figure S8 are the *pseudo*-first-order plot for the reaction of probe **1** with Cys, Hcy and GSH, respectively. The negative slope of the line provides *pseudo*-first-order rate constant: *k*'.

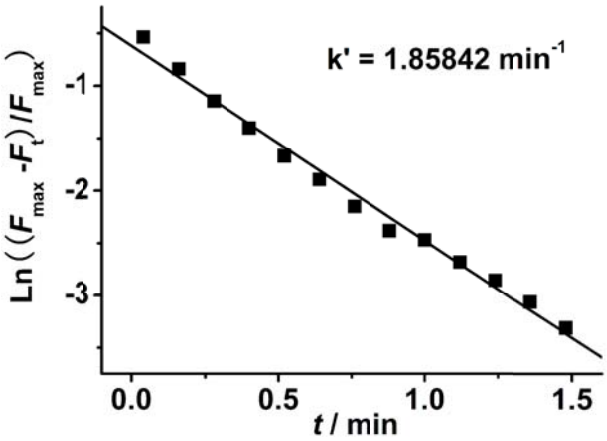


Figure S6. *Pseudo*-first-order kinetic plot of the reaction of probe **1** (5 μM) incubated with Cys (350 μM) in aqueous conditions (10 mM potassium phosphate buffer, pH 7.4, containing 2% DMSO as co-solvent). Slope= -1.85842 min^{-1}.

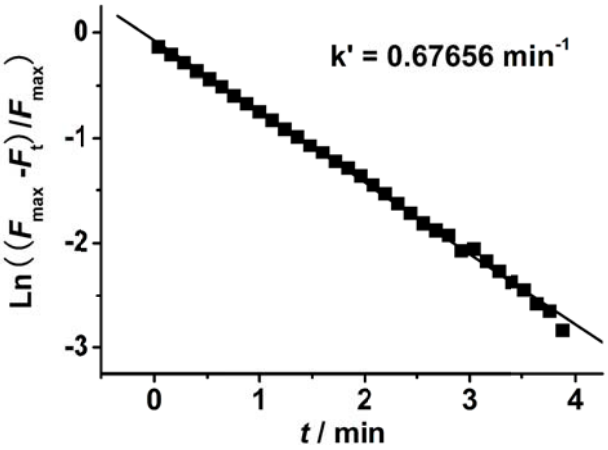


Figure S7. *Pseudo*-first-order kinetic plot of the reaction of probe **1** (5 μM) incubated with Hcy (350 μM) in aqueous conditions (10 mM potassium phosphate buffer, pH 7.4, containing 2% DMSO as co-solvent). Slope= -0.67656 min^{-1}.

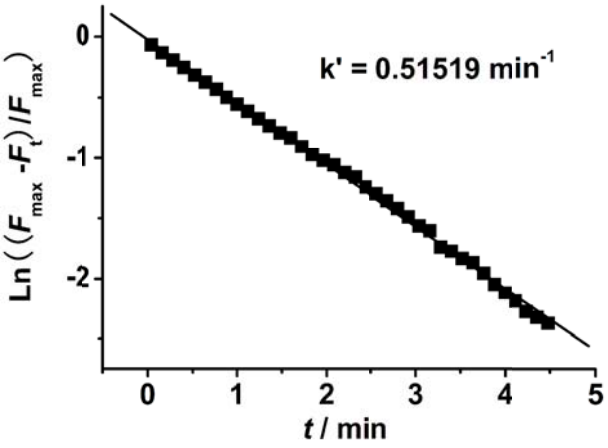


Figure S8. *Pseudo*-first-order kinetic plot of the reaction of probe **1** (5 μM) incubated with GSH (350 μM) in aqueous conditions (10 mM potassium phosphate buffer, pH 7.4, containing 2% DMSO as co-solvent). Slope= -0.51519 min^{-1}.

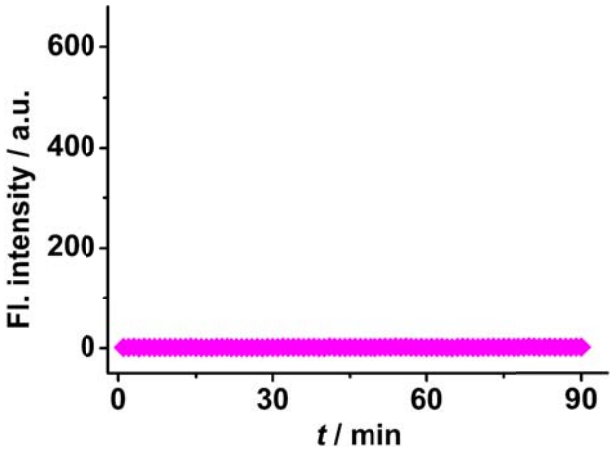


Figure S9. Fluorescence intensity at 455 nm for the probe **1** in aqueous conditions (10 mM potassium phosphate buffer, pH 7.4, containing 2% DMSO as co-solvent) after excitation at 412 nm for 0-90 minutes.

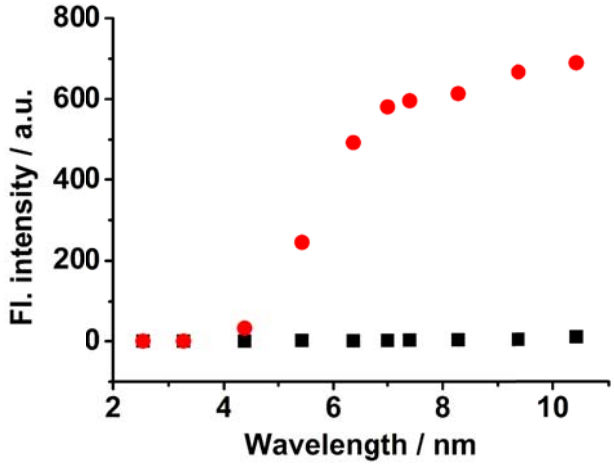


Figure S10. The variations of fluorescence intensity at 455 nm of probe **1** (5 μM) in the presence (●) or absence (■) of Cys (350 μM) as a function of pH. Excitation wavelength was 412 nm.

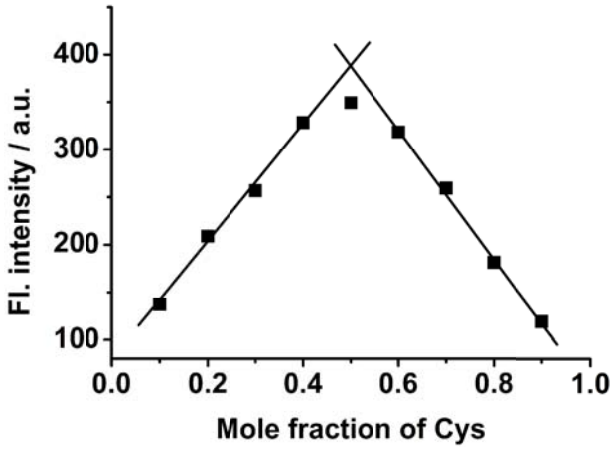


Figure S11. Job plot of the reaction between **1** and Cys in aqueous conditions (10 mM potassium phosphate buffer, pH 7.4, containing 2% DMSO as co-solvent). The variation of the emission at 455 nm was plotted as a function of the mole fraction of Cys. Total concentration of **1** and Cys was kept constant at 50.0 μM.

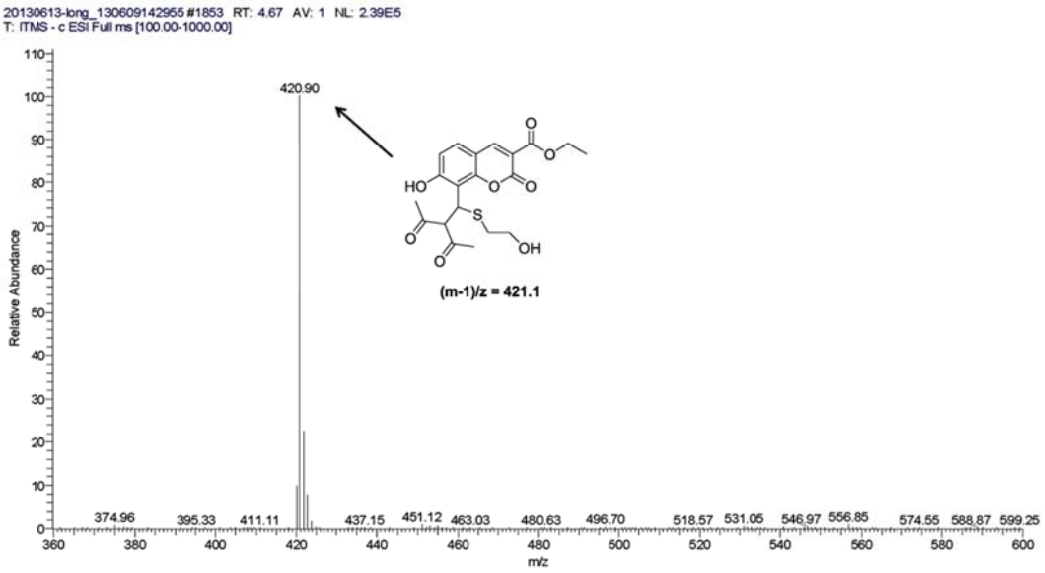


Figure S12. The ESI-Ms spectra of product obtained by mixing probe **1** and 2-mercaptoethanol.

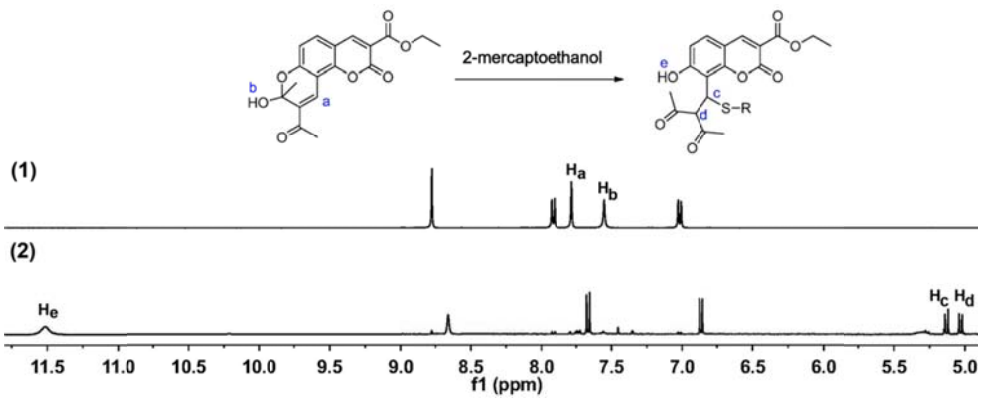


Figure S13. Partial ^{1}H NMR (400 MHz) spectra of: (1) probe **1**, and (2) probe **1** + 2-mercaptoethanol (excess) in DMSO-d_6.

Computational details: The ground state structures of the probe were optimized using density functional theory (DFT) with B3LYP functional and 6-31+G** basis set (considered as gas phase). The vertical excitation energies were carried out with the time dependent DFT (TD-DFT), based on the optimized structure of the ground state. All these calculations were performed with Gaussian 09 program.

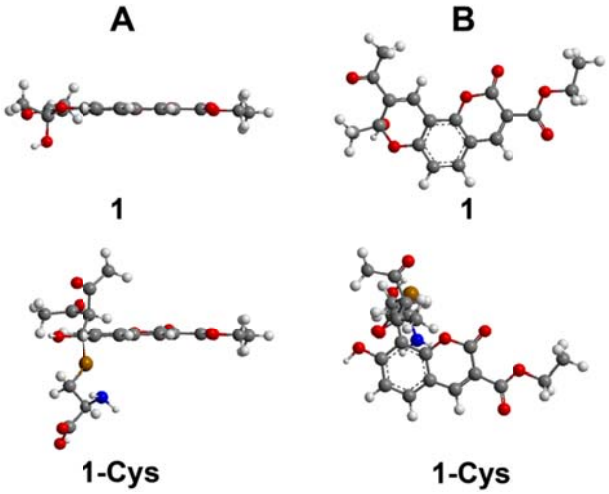


Figure S14. Optimized ground-state geometries of the probe **1** and **1**-Cys adduct. A) The side-view; B) The top-view.

Table S1. Selected electronic excitation energies (eV), oscillator strengths (*f*), main configurations, and CI coefficients of the low-lying excited states of the probe **1** and **1**-Cys. The data were calculated by TDDFT//B3LYP/6-31G** based on the optimized ground state geometries.

compound	Electronic Transition	TDDFT//B3LYP/6-31G**			
		Excitation Energy	*f*[a]	Composition[b]	CI[c]
1	$S_0 \rightarrow S_1$	3.35 eV	0.0003	H-2→L	0.47789
				H-2→L+1	0.48701
	$S_0 \rightarrow S_2$	3.50 eV	0.0104	H→L+1	0.47325
	$S_0 \rightarrow S_3$	3.72 eV	0.0850	H→L	0.62504
	$S_0 \rightarrow S_4$	3.79 eV	0.5122	H→L+1	0.49205
	$S_0 \rightarrow S_5$	4.13 eV	0.0161	H-2→L	0.50322
1-Cys	$S_0 \rightarrow S_1$	3.34 eV	0.0039	H→L	0.68820
	$S_0 \rightarrow S_2$	3.79 eV	0.3399	H-1→L	0.61131
	$S_0 \rightarrow S_3$	3.93 eV	0.0029	H-2→L	0.55713
	$S_0 \rightarrow S_4$	3.94 eV	0.0949	H-3→L	0.57114
	$S_0 \rightarrow S_5$	4.00 eV	0.0050	H-2→L+1	0.56400

[a] Oscillator strengths.

[b] H stands for HOMO and L stands for LUMO.

[c] The CI coefficients are in absolute values.

Table S2. Crystallographic parameters for probe **1**

Compound	probe **1**
Chemical formula	$C_{18}H_{16}O_7$
formula weight	344.31
Wavelength (Å)	0.71073
Crystal system	Monoclinic
Space group	Cc
T (K)	296(2)
a (Å)	8.320(10)
b (Å)	28.09(3)
c (Å)	7.621(9)
α(°)	90.00
β(°)	115.891(12)
γ(°)	90.00
V(Å^3)	1602(3)
Z	4
D (mg/m^{-3})	1.427
F(000)	720
μ(Mo *K*a)(mm^{-1})	0.111
θ range (º)	2.82 ~ 25.01
Goodness of fit on F^2	1.056
R_1, wR_2 [$I > 2\sigma(I)$]	0.0308, 0.0747
Reflections collected / unique	4420 / 2676 [R(int) = 0.0160]
R indices (all data)	0.0336, 0.0768

$$R = \Sigma||F_0| - |F_c||/\Sigma|F_0|,\ wR_2 = \{\Sigma[w(F_0^2 - F_c^2)^2]/\Sigma[w(F_0^2)^2]\}^{1/2}$$

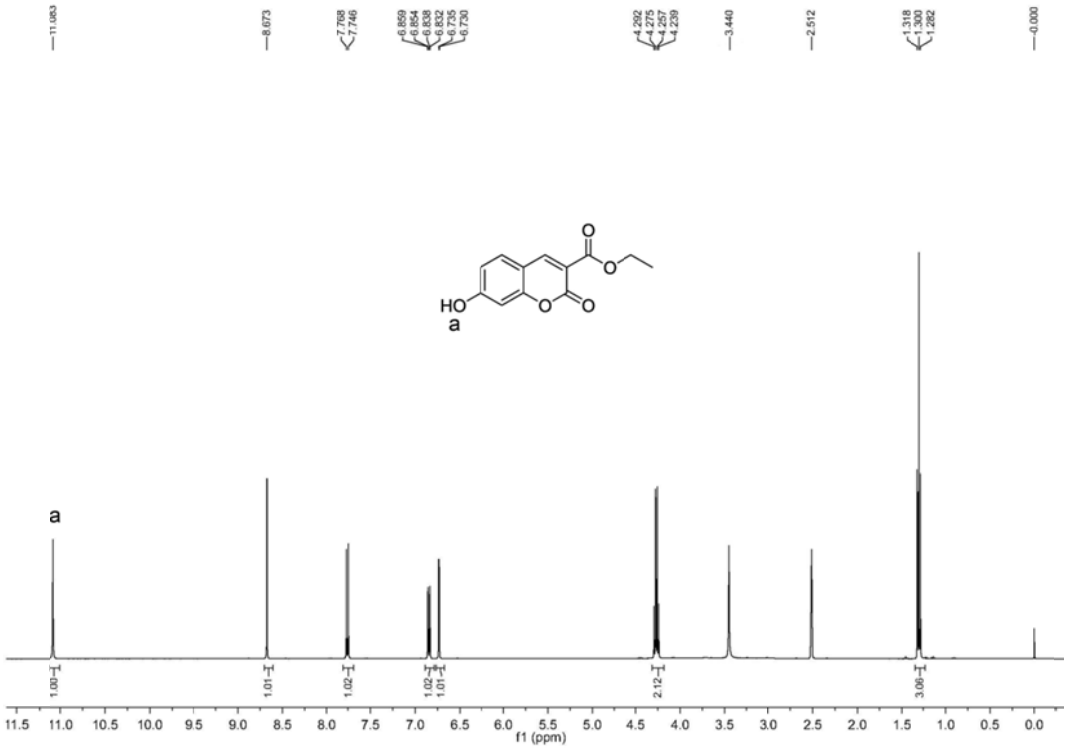


Figure S15. [1] H NMR spectra of compound **2** in DMSO-d_6.

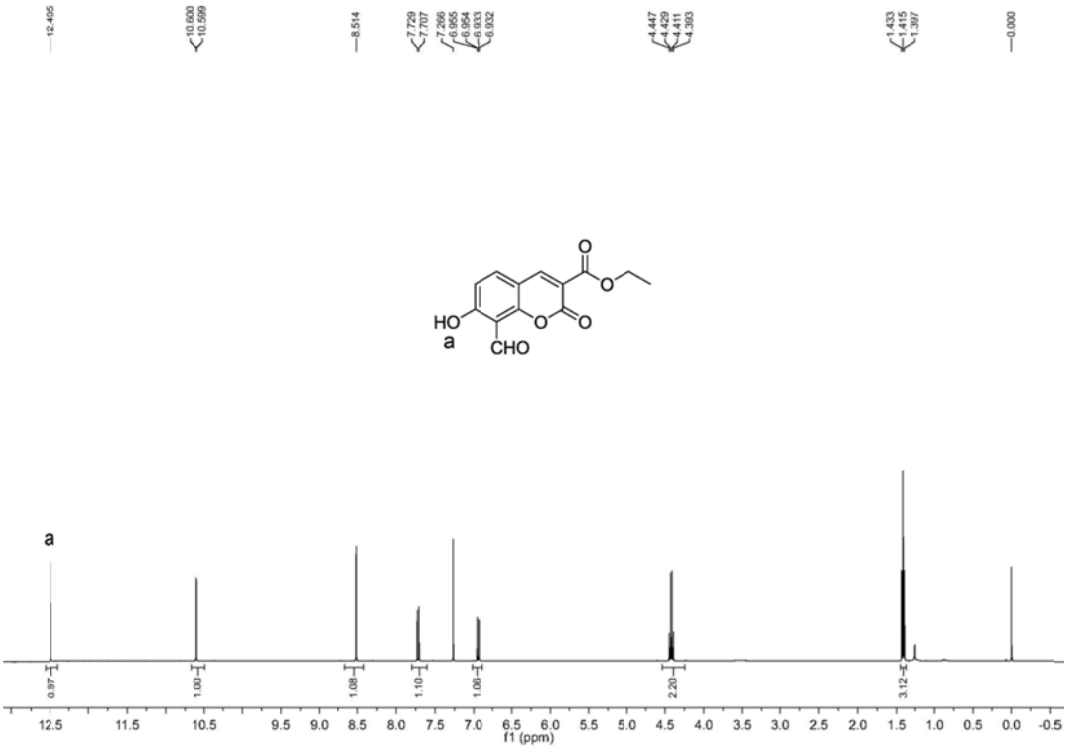


Figure S16. [1] H NMR spectra of compound **3** in $CDCl_3$.

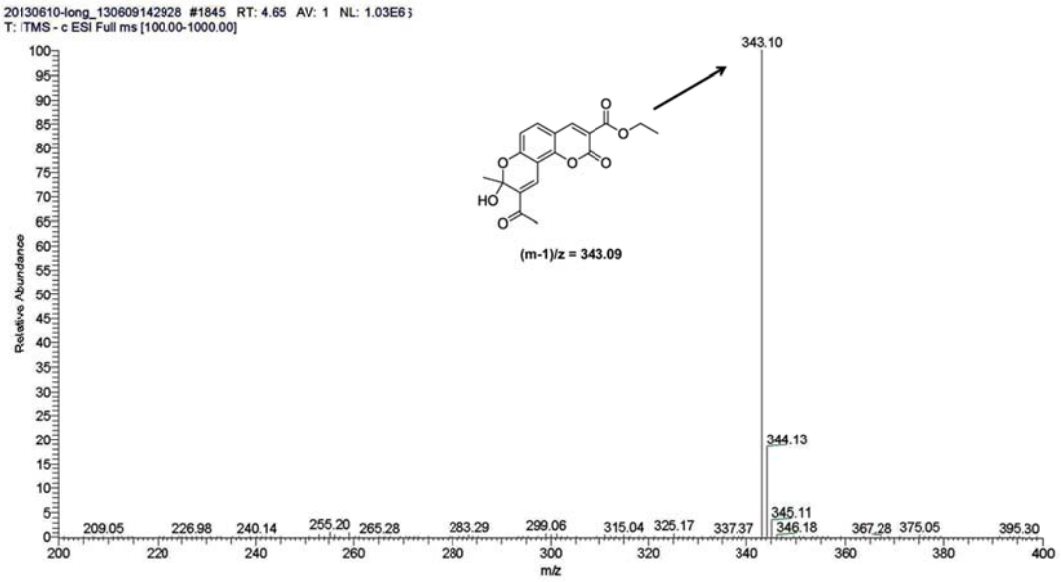


Figure S17. The ESI-Ms spectra of probe **1**.

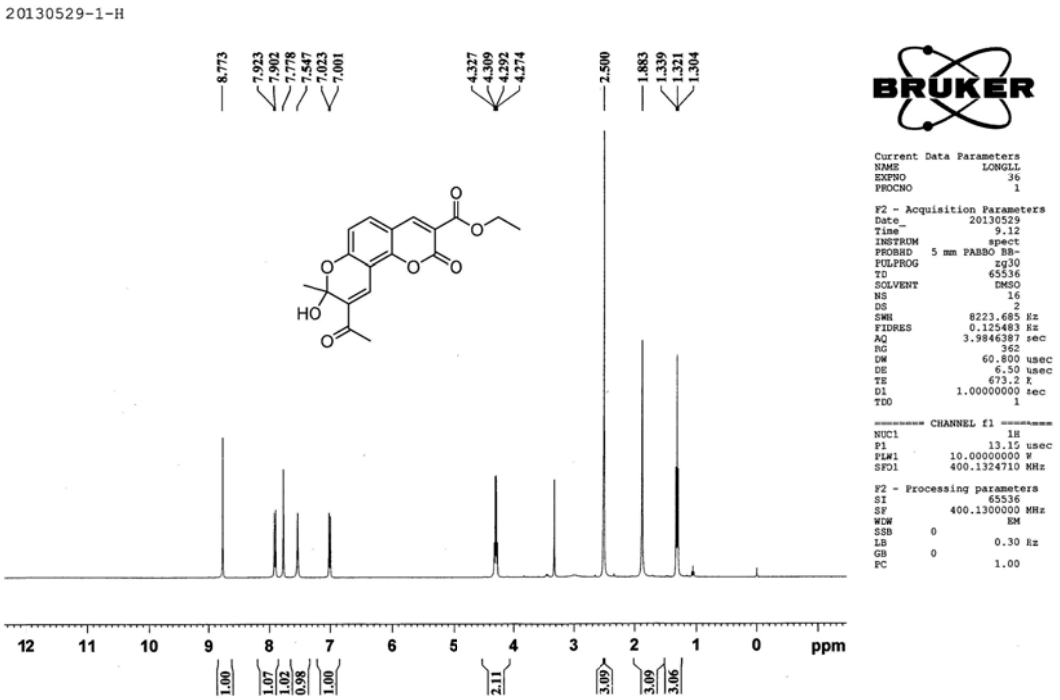


Figure S18. [1] H NMR spectra of probe **1** in DMSO-d_6.

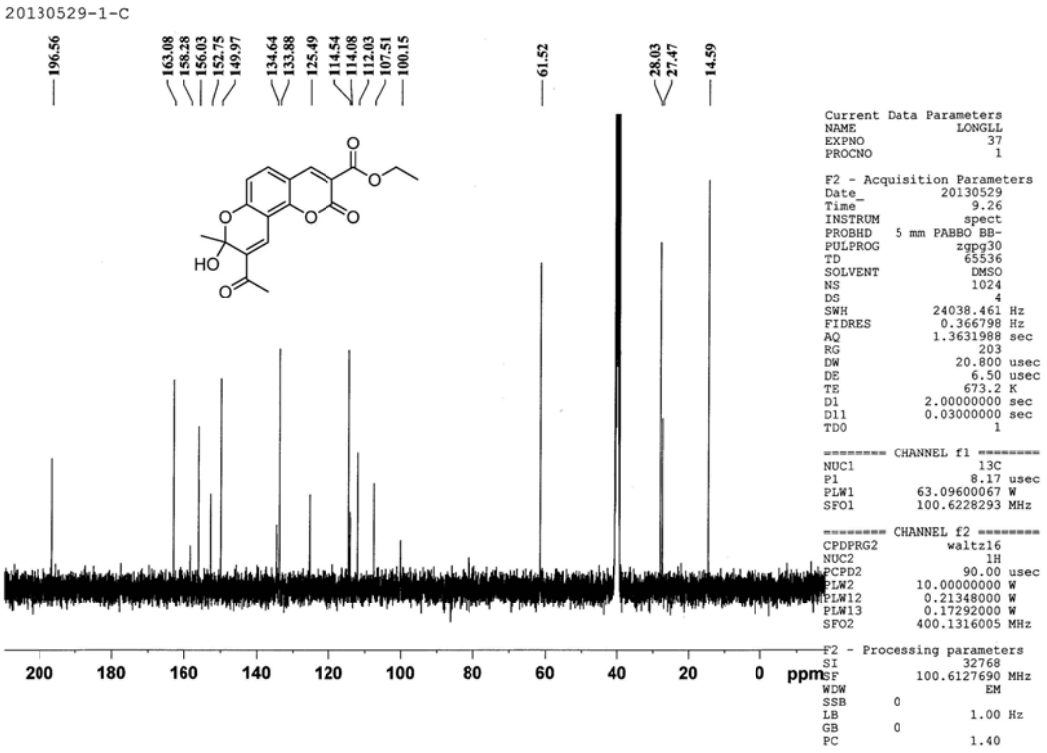


Figure S19. ^{13}C NMR spectra of probe **1** in DMSO-d_6.

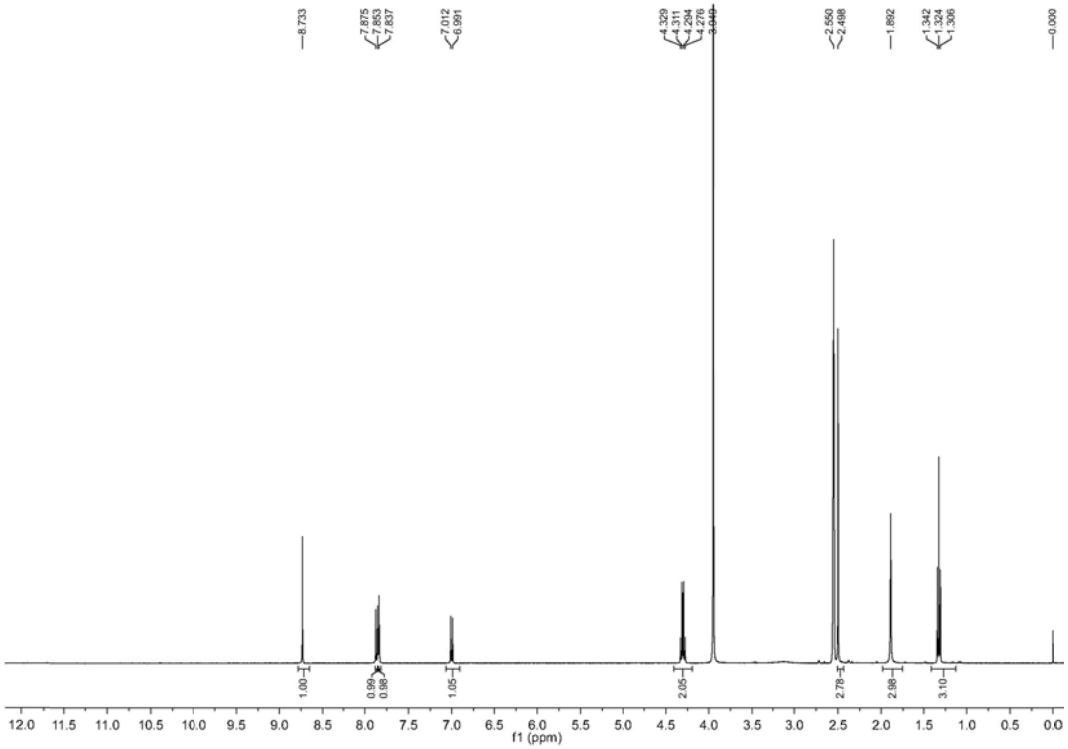


Figure S20. ^{1}H NMR spectra of probe **1** in D_2O / DMSO-d_6 (3:7, v/v).

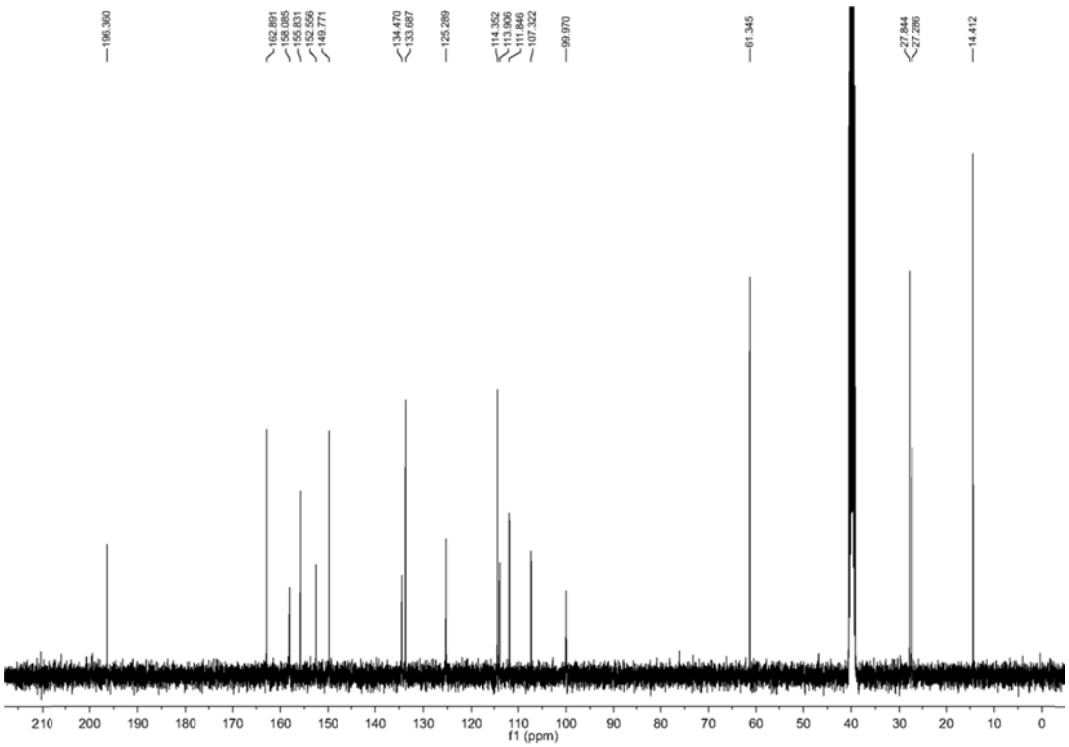


Figure S21. ^{13}C NMR spectra of probe **1** in D_2O / DMSO-d_6 (3:7, v/v).

References

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