Supporting Information

Heavy Atom Quenched Coumarin Probes for Sensitive and Selective Detection of Biothiols in Living Cells

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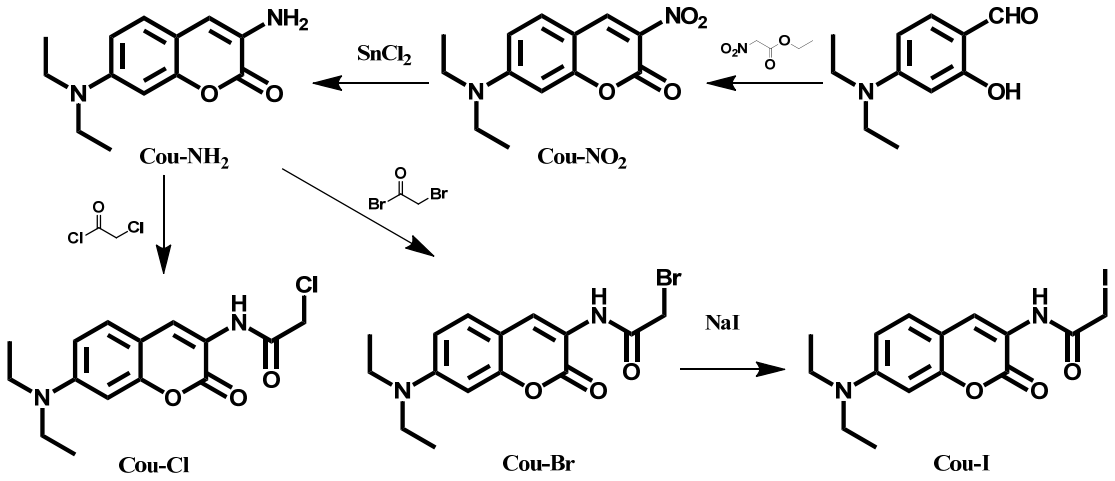
Materials and Instrument

Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AVANCE III 400 spectrometer (400 MHz) using TMS as internal standard. Steady-state emission spectra were recorded at room temperature on Varian Cary Eclipse Fluorescence Spectrophotometer and UV/Vis spectra were recorded on Beckman Coulter DU-800 UV/VIS Spectrophotometer. Cytotoxicity experiments were performed using SRB methods. Cell imaging was obtained from a High Content Analyzer (HCA).

Double distilled water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in water and acetonitrile (V/V = 9:1) at room temperature. All pH measurements were made with a Mettler Toledo FE20 – FiveEasy Plus^{TM}. Samples for absorption and fluorescence measurements were contained in microscale quartz cuvettes (10 mm×2 mm, 700 μL volume).

Synthesis of coumarin chemosensors



Scheme S1 Synthesis route of Cou-Cl, Cou-Br, Cou-I

Synthesis of Cou-NO_2 and Cou-NH_2 are referred to the previous literature[1].

4-diethylaminosalicylaldehyde (14.3 mmol, 2.8 g), ethyl nitroacetate (15.9 mmol, 1.6 mL), acetic acid (0.4 mL), a catalytic amount of piperidine (2-3 drops) were added to n-butanol (30 mL). The solution was refluxed for 12 h, and then cooled to room temperature. The precipitate was filtrated and washed with n-butanol to obtain the bright red solid Cou-NO_2 (2.0 g, 53%) after vacuum drying. The solid was used directly in the next step.

$SnCl_2$•H_2O (4.52 g, 20 mmol) and concentrated hydrochloric acid (20 mL) were added to 30 mL water, then compound Cou-NO_2 was added in batches. The mixture was stirred for 12 h at room temperature. The reaction solution was then carefully adjusted to alkalescence with 15% NaOH solution in an ice bath. After extraction by ethyl acetate, the combined organic phase was washed

with water. The resulting solution was dried with anhydrous sodium sulfate. After removal of the solvent, a brown-yellow solid was obtained. The resulting solid residue was purified by column chromatography on silica gel to obtain Cou-NH_2 as a yellow amorphous solid (1.57 g, 88%). ^{1}H NMR (400 MHz, DMSO-d_6, TMS) δ 7.19 (d, J = 8.7 Hz, 1H), 6.70 (s, 1H), 6.59 (dd, J = 8.8, 2.2 Hz, 1H), 6.49 (d, J = 2.0 Hz, 1H), 5.02 (S, 2H), 3.34 (q, J = 7.0,7.0 Hz, 4H),1.08 (t, J=7.0 Hz, 6H)., ^{13}C-NMR(101 MHz, DMSO-d_6) δ 159.80, 150.94, 146.84, 129.26, 126.20, 111.64, 110.36, 109.73, 97.76, 44.30, 12.81. MS (ESI^+) for $C_{13}H_{16}N_2O_2$, $[M+H]^+$ calculated 233.13, found 233.12

Synthesis of Cou-Cl

Under nitrogen, Cou-NH_2 (0.166 g, 0.5 mmol) and 1.5 mL triethylamine was dissolved in 20 mL dichloromethane (DCM). The mixture was stirred under the ice bath for 10~20 min, and chloroacetyl chloride (0.03ml, 1.1mmol, in 10 mL DCM) was then added to the above mixture. The solution was warmed to r.t and continued stirring for another 2~3 hours. After the completion of reaction, the mixtue was poured to deionized water, extracted by DCM. The combined organic phase was washed with 1M HCl, saturated $NaHCO_3$, and water successively, followed by dryness with anhydrous sodium sulfate. Upon removal of solvents, The resulting solid was purified by column chromatography on silica gel (DCM:PE=2:1 to 1:1) to obtain Cou-Cl as a bright yellow flocculent solid (0.120 g, 74%).^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 8.94 (s, 1H), 8.57 (s, 1H), 7.30 (d, J = 8.8, Hz, 1H), 6.64 (dd, J = 6.7, 2.0 Hz, 1H), 6.52 (S, 1H), 4.20 (s, 2H), 3.42 (q, J = 7.0,7.0 Hz, 4H),1.22(t, J=7.0 Hz, 6H). ^{13}C-NMR(101 MHz, $CDCl_3$) δ 164.55, 159.24, 153,03, 149.74, 128.91, 126.34, 118.07, 109.68, 107.87, 97.43, 44.81, 42.77, 12.45. MS (ESI^+) for $C_{15}H_{17}ClN_2O_3$, $[M+H]^+$ calculated 309.10, found 309.19 and 311.15.

Synthesis of Cou-Br

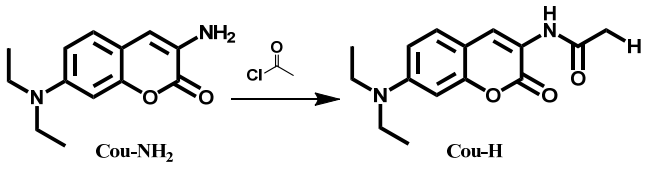
Under nitrogen, Cou-NH_2 (0.232 g, 1 mmol) and 0.2 g DMAP were dissolved in 4 mL DCM. The mixture was stirred under the ice bath for 10~20 min, and bromoacetyl bromide (0.08 mL, 1.1 mmol, in 4 mL DCM) was then added to the above mixture. The mixture was warmed to room temperature and continued stirring for another 2~3 hours. After the completion of reaction, the mixture was poured to deionized water, and extracted by DCM. The combined organic phase was washed with 1M HCl, saturated $NaHCO_3$, and water successively, followed by dryness with

anhydrous sodium sulfate. After removal of the solvent, the resulting solid residue was purified by column chromatography on silica gel (DCM:PE=2:1 to 1:1) to obtain Cou-Br as a bright yellow flocculent solid (0.224 g, 64%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 8.80 (s, 1H), 8.57 (s, 1H), 7.32 (d, J = 8.8, Hz, 1H), 6.65 (dd, J = 6.7, 2.0 Hz, 1H), 6.54 (S, 1H), 4.03(s, 2H), 3.43(q, J = 7.0,7.0 Hz, 4H),1.23(t, J=7.0 Hz, 6H). ^{13}C-NMR(101 MHz, $CDCl_3$) δ 164.22, 159.27, 153,00, 149.72, 128.93, 126.27, 118.31, 109.72, 107.94, 97.46, 44.82, 28.95, 12.44. MS (ESI^+) for $C_{15}H_{17}BrN_2O_3$, $[M+H]^+$ calculated 353.05, found 353.14 and 355.10

Synthesis of Cou-I

Cou-Br (0.117 g, 0.3 mmol) and 0.43 g NaI were dissolved in acetone, and the mixture was stirred in 50 ℃ for 5 hours. After the completion of reaction, the reaction solution was concentrated and then diluted by DCM. The DCM solution was then washed with brine and water successively, followed by dryness with anhydrous sodium sulfate. Upon removal of the solvent, the resulting solid residue was purified by column chromatography on silica gel (DCM:PE=2:1 to 1:1) to obtain Cou-I as a bright yellow flocculent solid (0.102 g, 85%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 8.57 (s, 1H), 8.81 (s, 1H), 7.31 (d, J = 8.8, Hz, 1H), 6.64 (dd, J = 7.0, 1.8 Hz, 1H), 6.53 (S, 1H), 3.90 (s, 2H), 3.43 (q, J = 7.0,7.0 Hz, 4H),1.23(t, J=7.0 Hz, 6H). ^{13}C-NMR(101 MHz, $CDCl_3$) δ 165.91, 159.40, 152.88, 149.66, 128.91, 126.31, 118.55, 109.74, 104.90, 97.40, 44.83, 27,82, 12.46. MS (ESI^+) for $C_{15}H_{17}IN_2O_3$, $[M+H]^+$ calculated 401.04, found 401.17

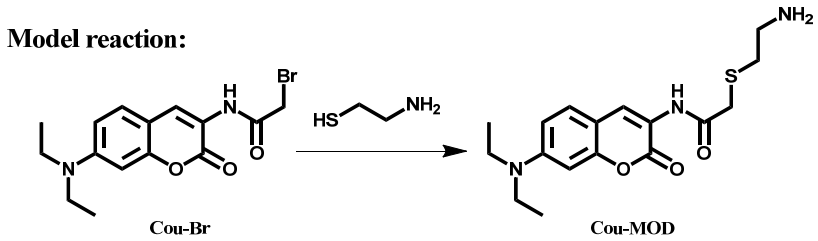
Synthesis of control probe (Cou-H) and reaction model (Cou-Mod)



Scheme S2 Synthesis of Cou-H

Under nitrogen, Cou-NH_2 (0.166 g, 0.5 mmol) and 1.5 mL triethylamine were dissolved in 20 mL DCM. The mixture was stirred under the ice bath for 10~20 min, and acetyl chloride (0.08 mL, 1.1

mmol, in 10 mL DCM) was then added to the above mixture. The mixture was warmed to room temperature and continued reaction for another 2~3 hours. After the completion of reaction, the mixture was poured to deionized water, and extracted by ethyl acetate. The combined organic phase was washed with 1M HCl, saturated $NaHCO_3$, and water successively, followed by anhydrous sodium sulfate. After filtration and removal of the solvent, the solid residue was purified by column chromatography on silica gel (DCM:PE=2:1 to 1:1) to obtain Cou-H as a red flocculent crystal (0.120 g, 74%).^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 8.59 (s, 1H), 7.92 (s, 1H), 7.31 (d, J = 8.8, Hz, 1H), 6.64 (dd, J = 7.0, 1.8 Hz, 1H), 6.52 (S, 1H), 3.42 (q, J = 7.0,7.0 Hz, 4H), 2.22(s,3H), 1.23(t, J=7.0 Hz, 6H). ^{13}C-NMR(101 MHz, $CDCl_3$) δ 168.95, 159.61, 152.61, 149.36, 128.67, 125.60, 119.00, 109.64, 108.28, 97.38, 44.75, 24.64, 12.45. MS (ESI^+) for $C_{15}H_{18}N_2O_3$, $[M+H]^+$ calculated 275.05, found 275.21



Scheme S3 model reaction

Cou-Br (0.035 g, 0.1 mmol) and 2-aminoethanethiol (0.77 g, 10 mmol) were dissolved in 50 mL PBS (ph=7.4) and 50 mL acetonitrile. The mixture was stirred at room temperature overnight. After the completion of reaction, the mixture was dilute and extract by DCM. The combined organic phase was washed with water, followed by dryness with anhydrous sodium sulfate. After filtration and evaporation of solvent, the solid residue was purified by column chromatography on silica gel (EA:PE=10:1) to obtain Cou-MOD as a pale yellow solid (0.032 g, 91%). ^{1}H NMR (400 MHz, DMSO-d6, TMS) δ 8.45 (s, 1H), 7.44 (d, J = 8.8 Hz, 1H), 6.70 (dd, J = 6.8, 2.0 Hz, 1H), 6.55 (d, J=1.8 Hz, 1H), 3.41 (q, J = 7.0,7.0 Hz, 4H), 2.76(t, J=6.6 Hz, 2H), 2.76(t, J=6.4 Hz, 2H), 1.12 (t, J=7.0 Hz, 6H). ^{13}C-NMR(101 MHz, DMSO-d6) δ 169.60, 158.72, 153.09, 149.70, 129.30, 127.37, 119.03, 109.97, 107.84, 97.11, 44.45, 41.47, 35.39, 35.51, 12.79. MS (ESI^+) for $C_{15}H_{18}N_2O_3$, calculated $[M+H]^+$ 350.16, found 350.31

Absorption spectra

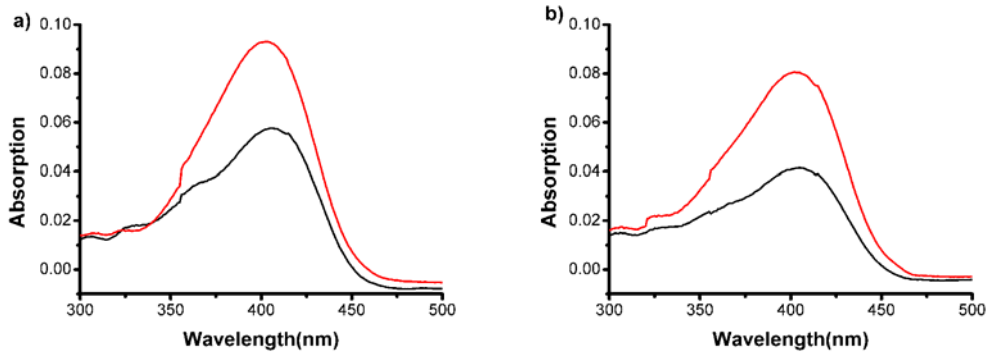


Fig. S1 Absorption spectra of Cou-Br (a), Cou-I (b) (5 μM) with (red line) or without (dark line) 500 μM Cys in degassed PBS (25 mM , pH =7.4, containing 10% CH_3CN).

Fluorescence spectra

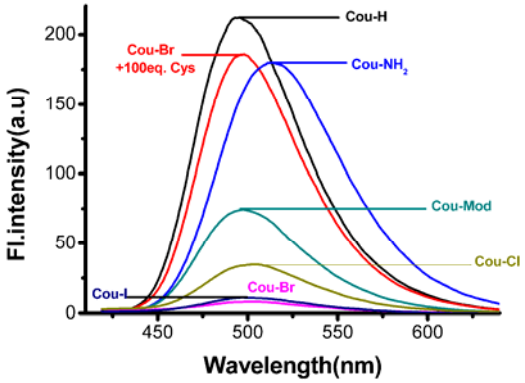


Fig. S2. Fluorescence spectra of 5 μM of Cou-NH_2, Cou-Cl, Cou-Br, Cou-I, Cou-H, Cou-Mod, respectively and Cou-Br (5 μM) after reaction with 100 equiv. Cys for 40 min in degassed PBS (25 mM, pH 7.4) containing 10% CH_3CN, λ_{ex} = 400 nm.

Quantum yield

The quantum yield of compounds Cou-Cl, Cou-Br, Cou-I, Cou-NH_2 and Cou-H were determined according to the literature.

$$\phi_S = \frac{\phi_R I_S A_R \lambda_{exR} \eta_S}{I_R A_S \lambda_{exS} \eta_R}$$

S and R represent sample and reference standard, respectively

Where Φ is quantum yield; I is integrated area under the corrected emission spectra; A is absorbance at the excitation wavelength; λ_{ex} is the excitation wavelength; η is the refractive index of the solution; the subscripts S and R refer to the unknown sample and the reference standard, respectively. We chose 9,10-diphenylanthracene in Cyclohexane solution (which quantum yield was 0.90) as reference standard[2]. The quantum yields of the compounds mentioned above were calculated and listed in Table S1, respectively.

Table S1 relative quantum yield of Cou-Cl, Cou-Br, Cou-I, Cou-NH_2, Cou-H

Compds.	*λabs (nm)*	*ε[$M^{-1}cm^{-1}$] [a]*	*Φ_f[b]*
Cou-Cl	374	11880	0.15
Cou-Br	374	14320	0.066
Cou-I	374	10920	0.073
Cou-NH_2	374	12080	0.66
Cou-H	374	17960	0.63

[a]: The average value of molar absorptivity

[b]: L. Joseph R. Principles of Fluorescence Spectroscopy, Springer Maryland, 3rd edn., 2006, pp. 9-12; L. Cui, Z. Peng, C. Ji, J. Huang, D. Huang, J. Ma, S. Zhang, X. Qian and Y. Xu, *Chem. Commun.* 2014, 50, 1485.

Dynamic studies of chemosensors with biothiols

pseudo-first-order rate constant[3]

The rate constant was determined according to the following equation:

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

Where F_t and F_{max} are the fluorescence intensity at time t, and the time after completion, respectively. The constant k' abtained as shown in Fig. S3. The absolute values of slope of the line for Cou-Br and Cou-I yielded a pseudo-first-order rate constants 8.11×10^{-2} min^{-1} and 10.3×10^{-2} min^{-1}, respectively.

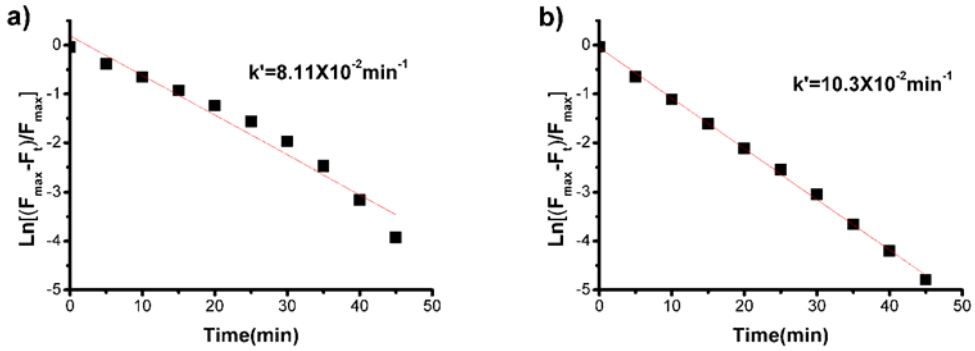
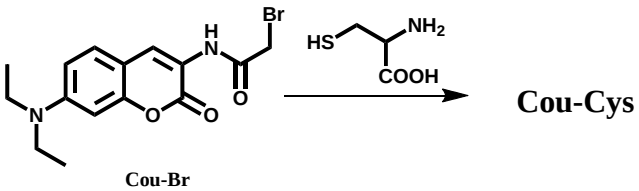


Fig. S3. pseudo-first-order rate figure of 5 μM Cou-Br (a) or Cou-I (b) reaction with 500 μM Cys in degassed PBS (25 mM, pH 7.4) containing 10% CH_3CN, λ_{ex} = 400 nm, λ_{em} = 495 nm.

Model reaction and mechanism



Scheme S4 reaction of Cou-Br with Cys

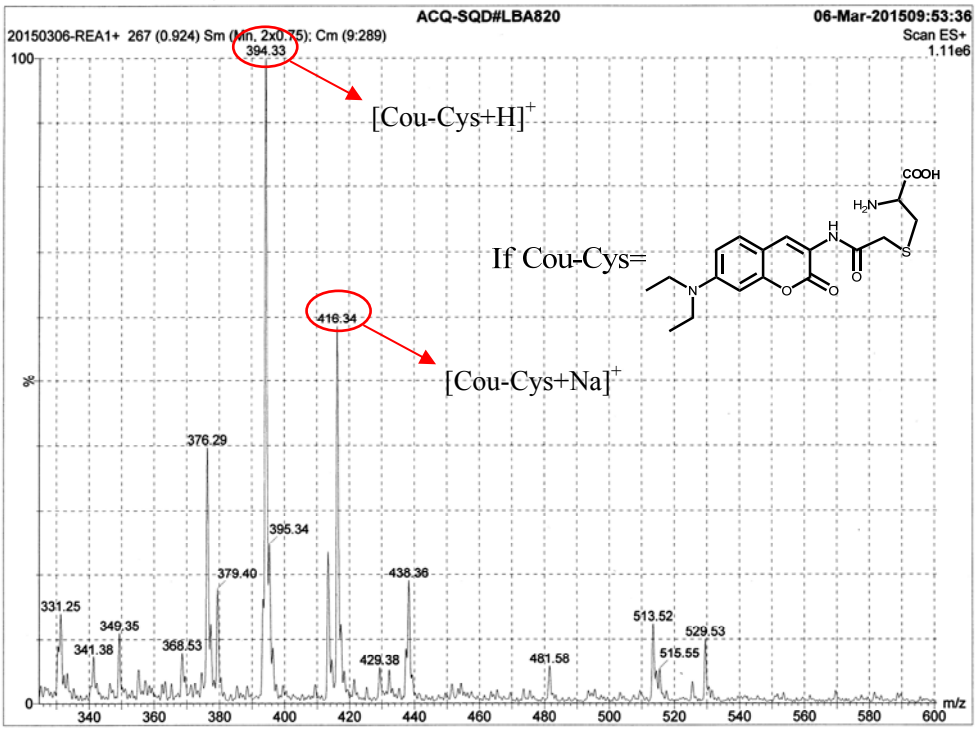
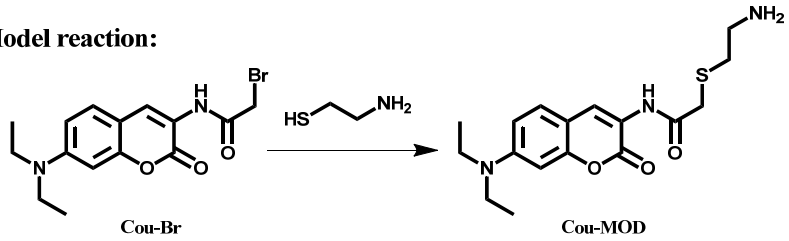


Fig. S4 MS (ESI^+) of Cou-Br after reaction with 100 equiv Cys for 50 min in degassed PBS , The PBS buffer was 25 mM PBS (pH 7.4) containing 10% CH_3CN. $[Cou\text{-}Cys+H]^+$ 394.14, found 394.33

Model reaction:



Scheme S3 model reaction

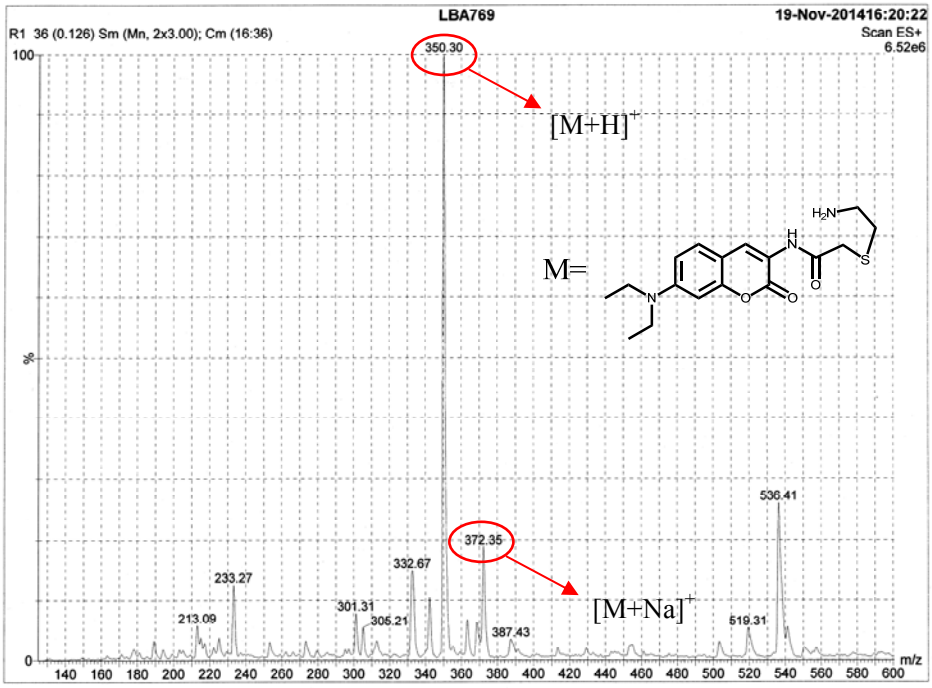
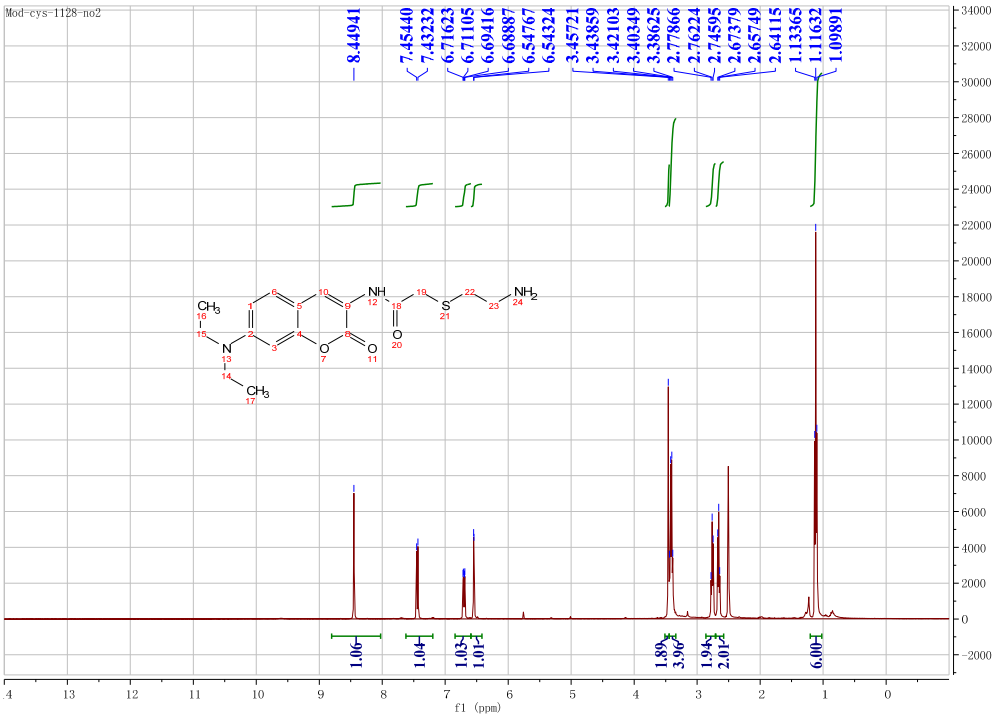


Fig. S5 MS (ESI^+) of Cou-Br after reaction with 100 equiv cysteamine for 50 min in degassed PBS. The PBS buffer was 25mM PBS (pH 7.4) containing 10% CH_3CN. $[M+H]^+$ calculated 350.15, found 350.30

^{1}H-NMR and ^{13}C-NMR of Cou-Mod



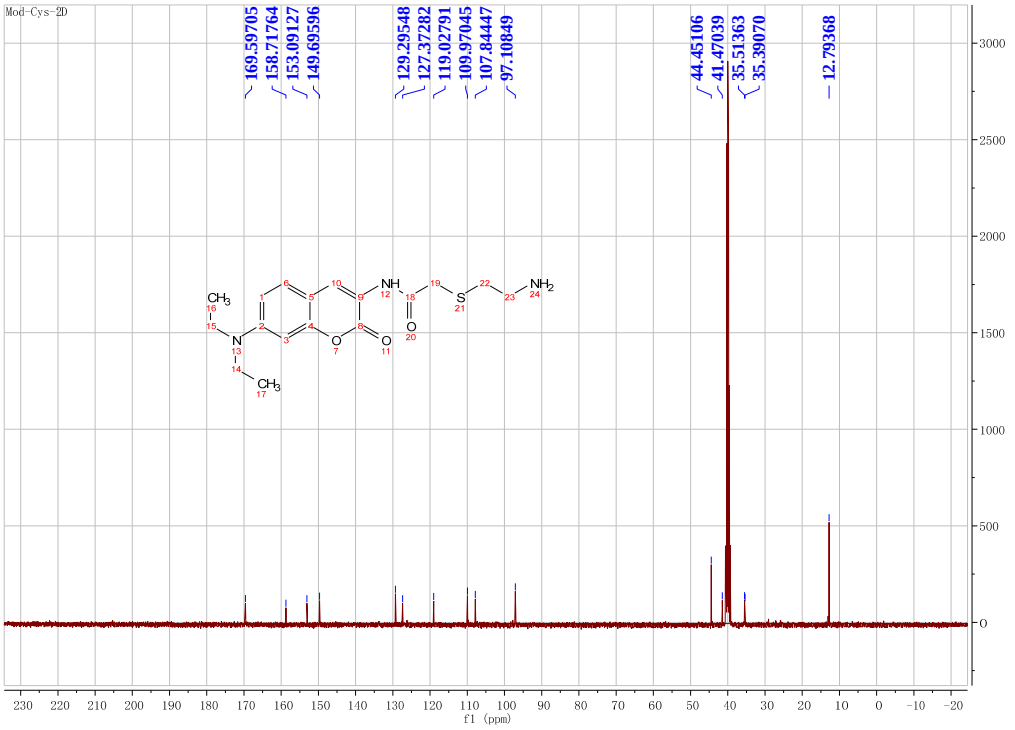


Fig. S6 NMR of Cou-Br after reaction with 100 equiv cysteamine for 50 min in degassed PBS. The PBS buffer was 25mM PBS (pH 7.4) containing 10% CH_3CN.

HMBC of Cou-Mod

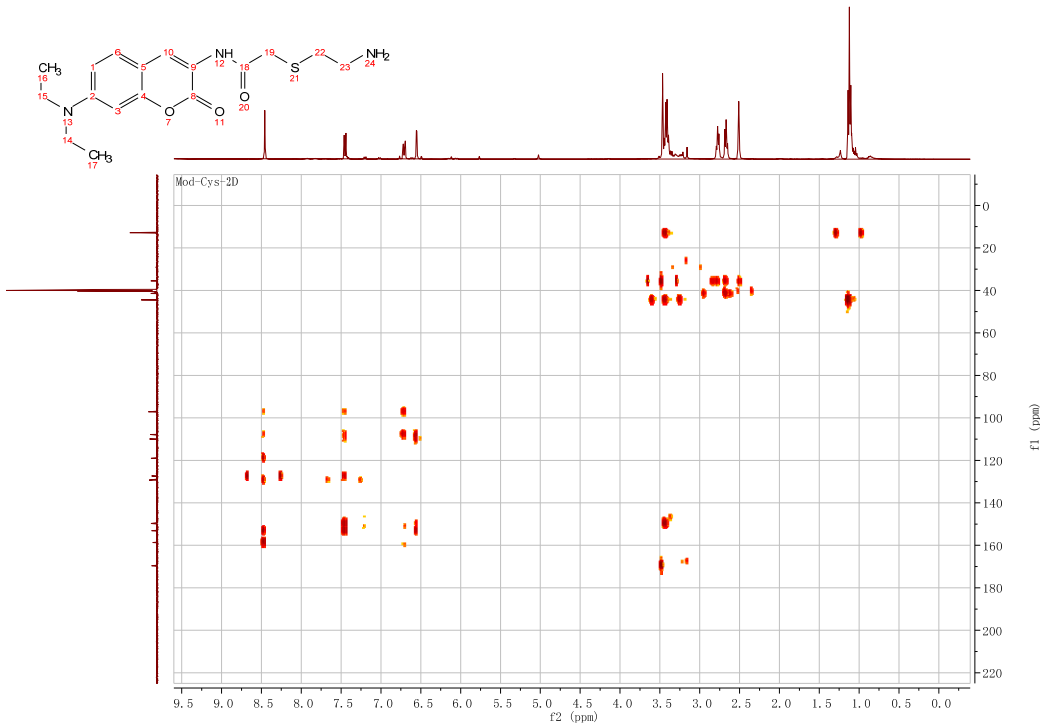


Fig. S7 **HMBC** of Cou-Br after reaction with 100 equiv cysteamine for 50 min in degassed PBS. The PBS buffer was 25mM PBS (pH 7.4) containing 10% CH_3CN.

Titration reaction of Cou-I

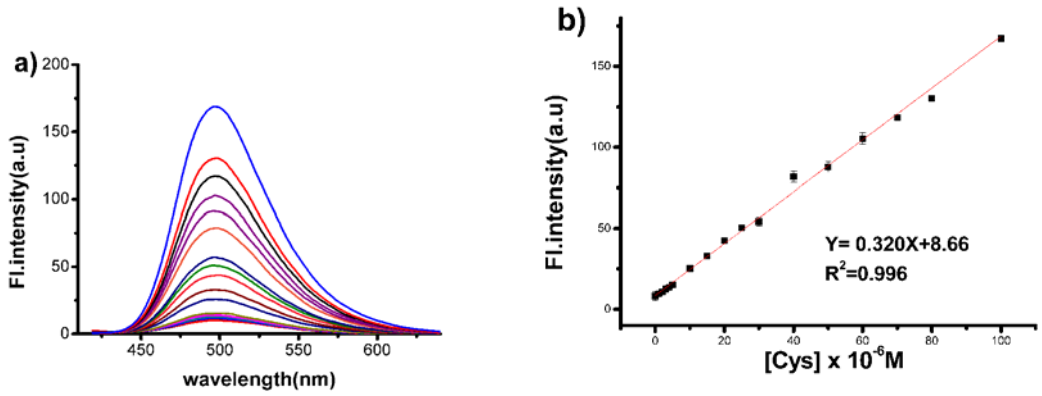


Fig. S8. (a) Fluorescence spectra of Cou-I (5 μM) upon addition of varied concentrations (0-500 μM) of Cys in degassed PBS buffer in 45 min. (b) The linear relationship between the fluorescence intensity of 495 nm of Cou-I and the concentration of Cys. The degassed PBS buffer was 25 mM PBS (pH 7.4) containing 10% CH_3CN. λ_{ex} = 400 nm, λ_{em} = 495 nm.

pseudo-secondary-order rate constant of Cou-Br[3]

The relationship between pseudo-first-order rate constant and pseudo-secondary-order rate constant accords with:

$$k' = K[M]$$

Where M is the concentration of Cys (or Hcy, GSH), the secondary-order rate constant is the slope of first-order rate constant along with the change of concentration (75 equiv., 100 equiv., 125 equiv., 150 equiv., respectively in this essay) of Cys (or Hcy, GSH). The constant K abtained was shown in Fig. S9. The pseudo-secondary-order rate constants of Cou-Br to Cys, Hcy, GSH were $K=7.8\times10^{-4}\ M^{-1}min^{-1}$, $K=1.5\times10^{-4}\ M^{-1}min^{-1}$, $K=2.4\times10^{-4}\ M^{-1}min^{-1}$.

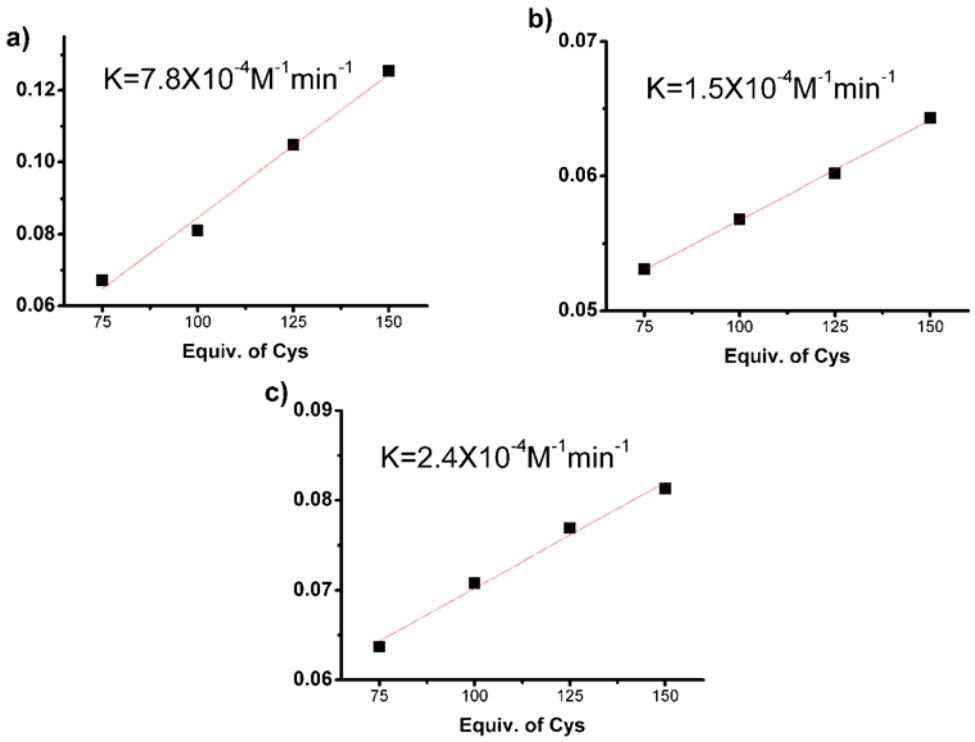


Fig. S9 Pseudo-secendary-order rates of 5 μM Cou-Br upon the reaction with varied concentrations Cys (a), Hcy (b), GSH (c) in degassed PBS. The PBS buffer was 25 mM PBS (pH =7.4) containing 10% CH_3CN. λ_{ex} = 400 nm, λ_{em} = 495 nm.

Table S2 Rate constants for Cou-Br to Cys, Hcy, GSH.

Compds.	*k'(min^{-1})*[a]	*k'Cys/k'*[b]	*K (M^{-1}min^{-1})*[c]	*k'Cys/k'*[d]
Cys	8.1 min^{-1}	1.0	7.8×10^{-4}M^{-1}min^{-1}	1.0
Hcy	5.6 min^{-1}	0.69	1.5×10^{-4}M^{-1}min^{-1}	0.19
GSH	7.1 min^{-1}	0.87	2.4×10^{-4}M^{-1}min^{-1}	0.30

a The pseudo-first-order rate constant of Cou-Br to 100 equiv. Cys, Hcy and GSH;
b The ratios of the pseudo-first-order rate constant of Cou-Br to 100 equiv. Cys;
c The pseudo-secondary-order rate constants of Cou-Br to Cys, Hcy and GSH;
d The ratios of the pseudo-secondary-order rate constant of Cou-Br to Cys.

Selectivity and competition studies

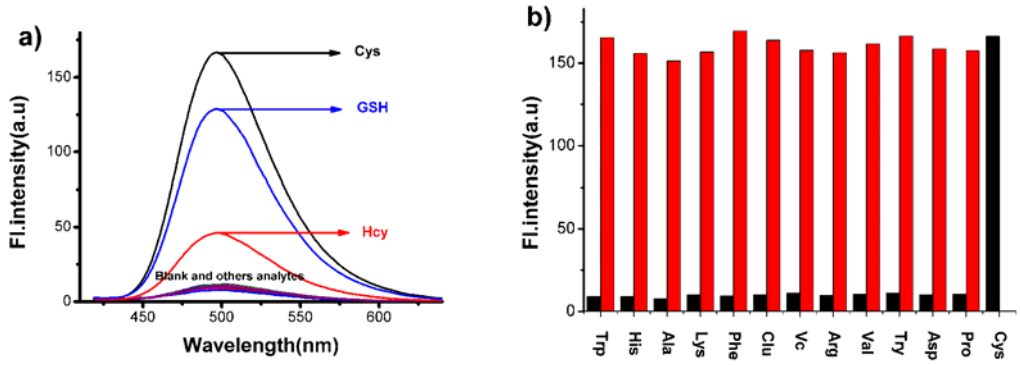


Fig. S10 (a) Fluorescence spectra of Cou-I (5 μM) to Cys (500 μM) and other analytes (500 μM Hcy and GSH, and 2 mM other amino acids and Vc) in PBS buffer (b) Fluorescence responses of Cou-I to other analytes (2 mM amino acids and Vc) without (black column) or with (red column) 500 μM Cys. The degassed PBS buffer was 25 mM PBS (pH 7.4) containing 10% CH_3CN. λ_{ex} = 400 nm, λ_{em} = 495 nm.

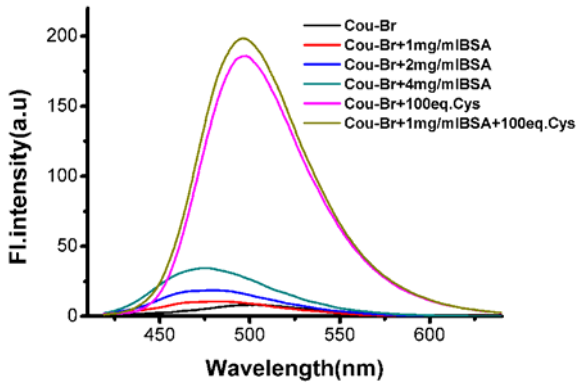


Fig. S11 Fluorescence spectra of Cou-Br (5 μM) upon addition of varied concentrations (mg/mL) of BSA, and 100 eq.Cys with or without 1mg/ml BSA in degassed PBS buffer after 45 min. The degassed PBS buffer was 25 mM PBS (pH 7.4) containing 10% CH_3CN. λ_{ex} = 400 nm.

Stability of probes

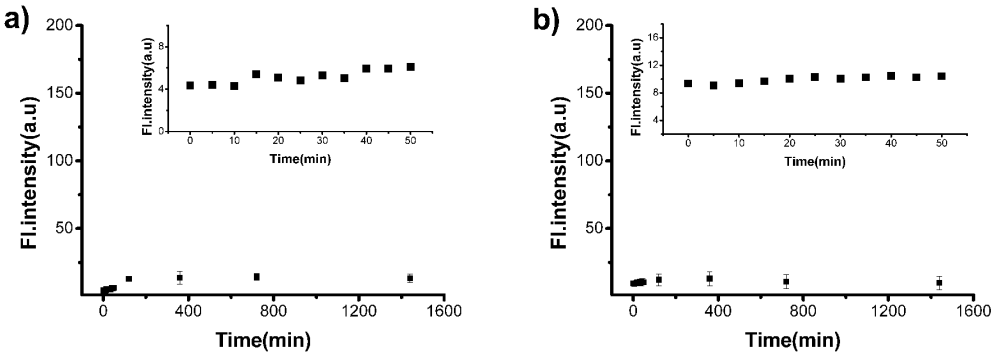


Fig. S12 Fluorescence responses of Cou-Br (a), Cou-I (b) (5 μM) within 24 hours in degassed PBS buffer. Inset: Fluorescence responses of Cou-Br (a), Cou-I (b) (5 μM) in 50 min in degassed PBS buffer. The PBS buffer was 25 mM PBS (pH 7.4) containing 10% CH_3CN. λ_{ex} = 400 nm, λ_{em} = 495 nm.

Response to BSA in the presence of a reducing agent

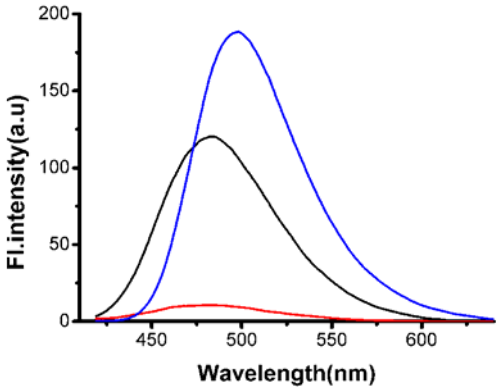


Fig. S13 Fluorescence spectra of Cou-Br (5 μM) upon the addition of 1 mg/ml of BSA(—), 1 mg/ml BSA in reduction state (—) and 100 eq. Cys(—) in PBS buffer after 45 min. The PBS buffer was 25 mM PBS (pH 7.4) containing 10% CH_3CN. λ_{ex} = 400 nm.

As we known, bovine serum albumin contain 583 amino acid residues which includes 17 disulfide bond formed by 35 cysteines. In addition, it has a free thiol at the 34th position of the peptide chain. The thiol moiety of BSA in natural physiological conditions generally exists in the form of disulfide bond. Therefore, all disulfide bond of BSA was fully reduced by the additoin of excess TCEP (Tris(2-carboxyethyl)phosphine. The fully reduced BSA was then quickly purified by

NAP column, followed by the quantification of protein concentration. The protein solution was then immediately used for the reaction with Cou-Br. As is shown in Fig. S15, fully reduced BSA leads to a certain amount of fluorescence increase (dark line) at 483 nm that is blue shifted in comparison to Cys-triggered fluorescence emission (495 nm) of Cou-Br.

pH sensitivity

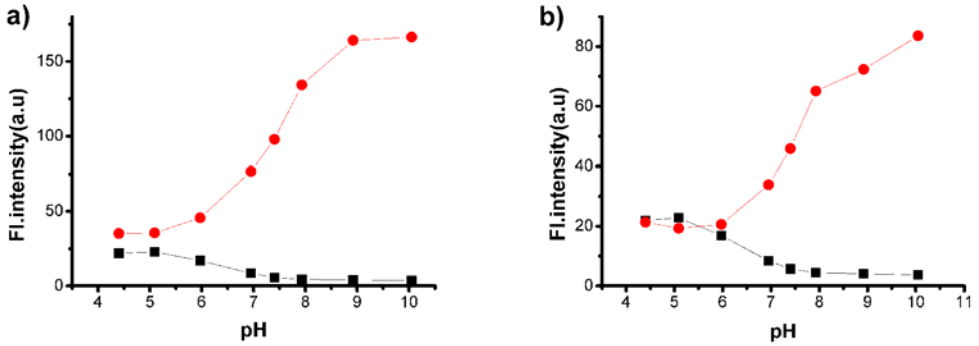


Fig. S14. Fluorescence responses of Cou-Br (5 μM) with or without 500 μM Hcy (a) and GSH (b) in different pH degassed PBS (4.4, 5.0, 6.0, 7.0, 7.4, 7.9, 8.9, 10.0) containing 10% CH_3CN after 45 min. λ_{ex} = 400 nm, λ_{em} = 495 nm.

Cell experiment[4]

Cytotoxic experiment

Cytotoxicity study of **Cou-Br** was explored by SRB method. HeLa cells were cultured in 96-well plate for 24 hours. Various concentrations of **Cou-Br** in DMEM were then added to HeLa cells. The plate was incubated for another 24 hours at 37 ^{o}C under humidified atmosphere and 5% CO_2, then 100 μL of 10% trichloroacetic acid was added to every well, and the cells continued incubation for another 1 hour at 4 ^{o}C. After washing with deionized water four times and drying, 100 μL of SRB solution (4 mg/ mL) was added to every well to stain the cells for 30 min. After removal of the SRB solution in everywell, the plate was washed for several times with 1% acetic acid. 100 μL of tris base (10 mM, pH 10.5) was then added to each well, and optical density of each well at 540 nm was read using FlexStation 3 Benchtop Multi-Mode Microplate Reader.

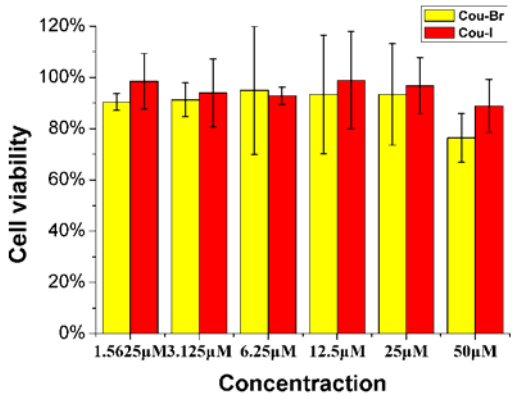


Fig. S15 Cell viability by a standard SRB method, the experiments were repeated three times and the data were shown as mean value (±S.D.).

Cell Imaging

HeLa cells were cultured in DMEM supplemented with 10 % fetal bovine serum and 5% CO_2 at 37 °C on a 96-well plates. After 24 hours, the cells were rinsed slightly 3 times with PBS to remove the media and then cultured in a new dose DMEM for later use. **Cou-Br** was added to above cells with final concentration of 5 μM and the cells were incubated for 50 min to detect intracellular thiol. On the other hand, NEM pre-treated HeLa cells (1 mM NEM for 30min) were incubated with Cou-Br for 50 min . In addition, NEM pre-treated HeLa cells were first incubated with 100 μM Cys for 30 min. Then the cells were washed and incubated with Cou-Br (5 μM) for another 50 min. All these cells were then imaged.

References

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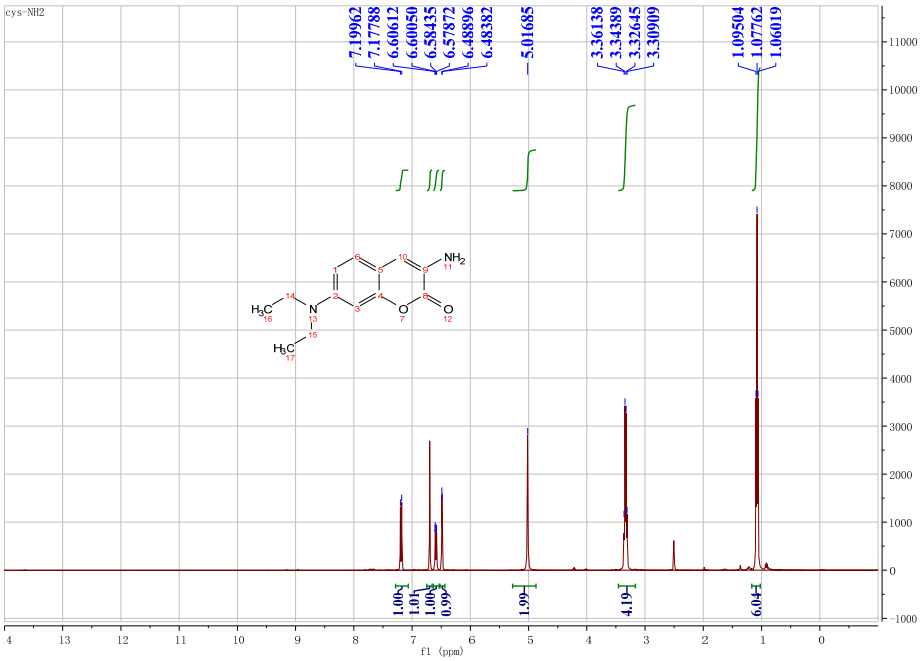
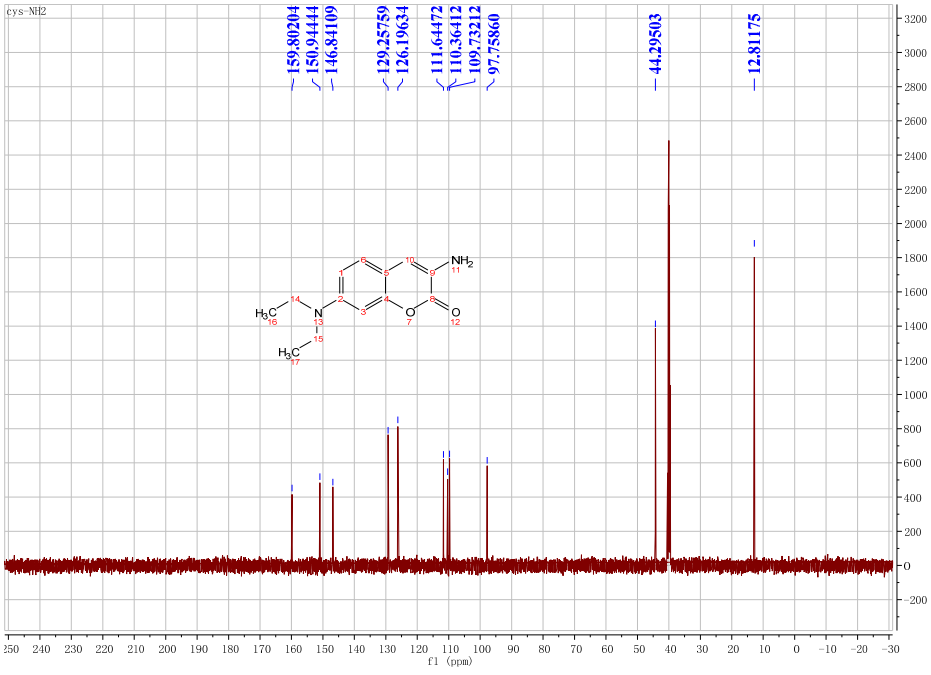
2.S. Hamai and F. Hirayama, *J. Phys. Chem.*, 1983, 87, 83–89.
3.T. J. Dale and J. Rebek, Jr., *J. Am. Chem. Soc.*, 2006, 128, 4500-4501.
4.L. Li, Y. Ji and X. Tang, *Anal Chem*, 2014, 86, 10006-10009.

NMR and MS spectra of all probes and intermediates

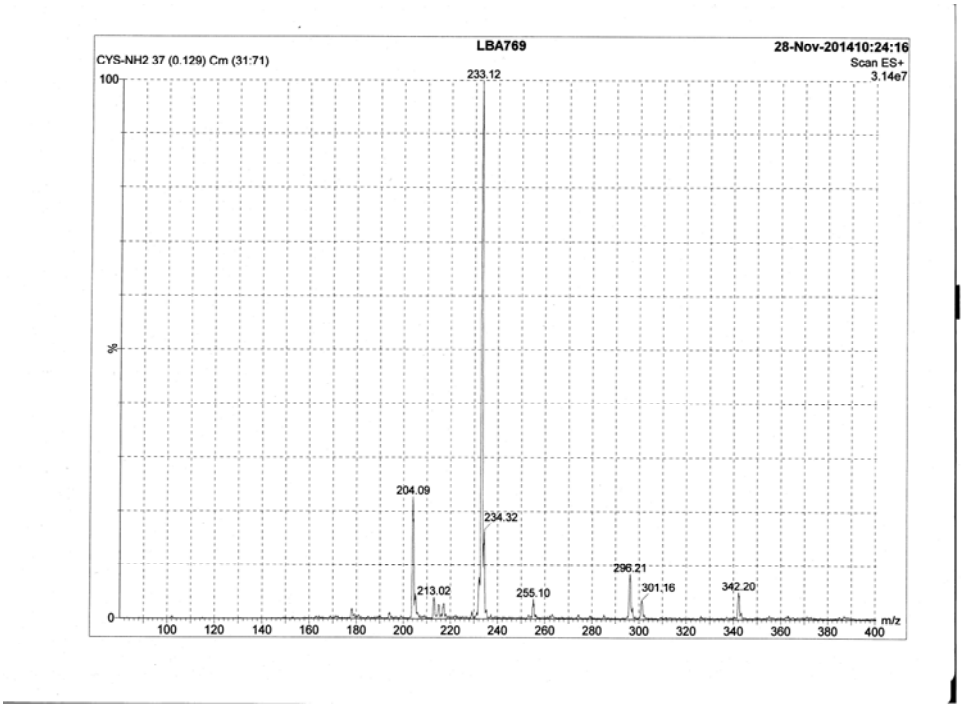
^{1}H-NMR spectra of Cou-NH_2



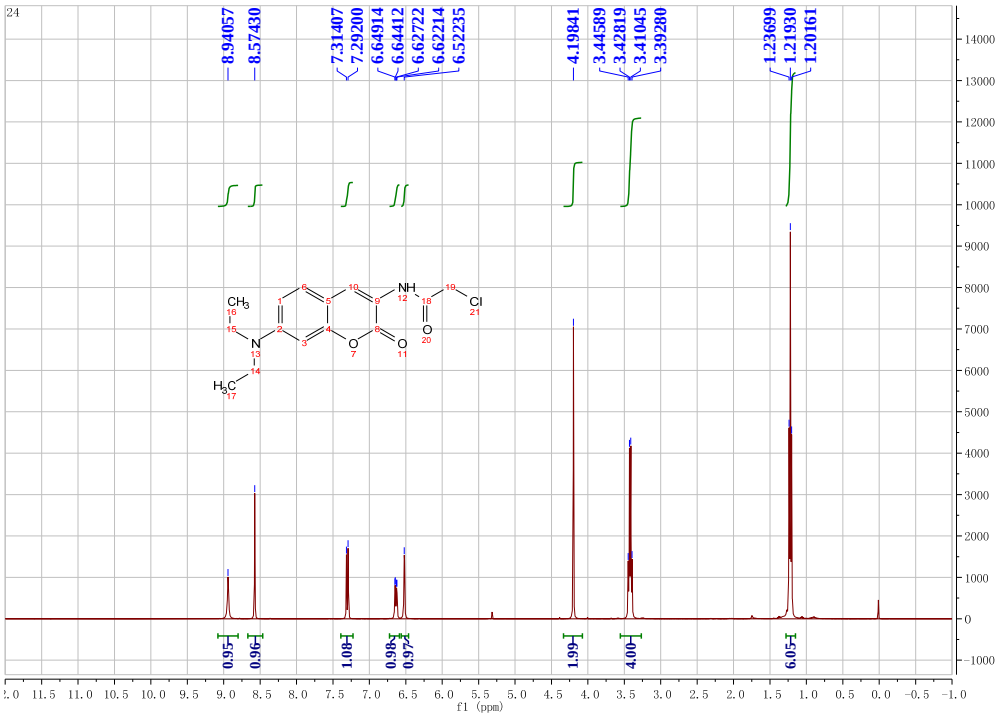
^{13}C-NMR spectra of Cou-NH_2



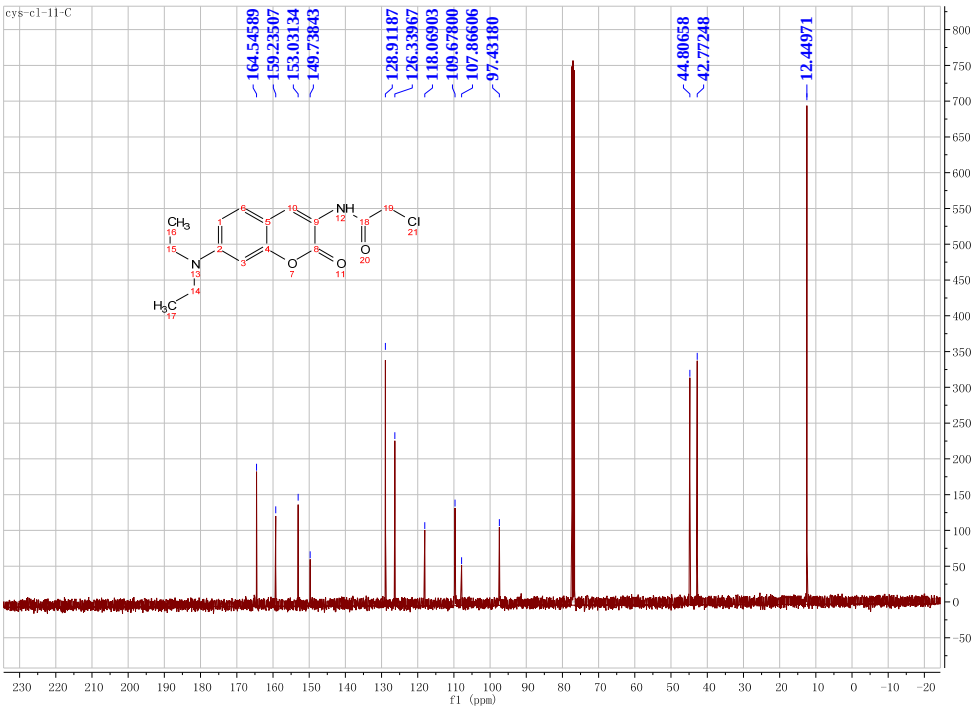
MS (ESI^{+}) of Cou-NH_2



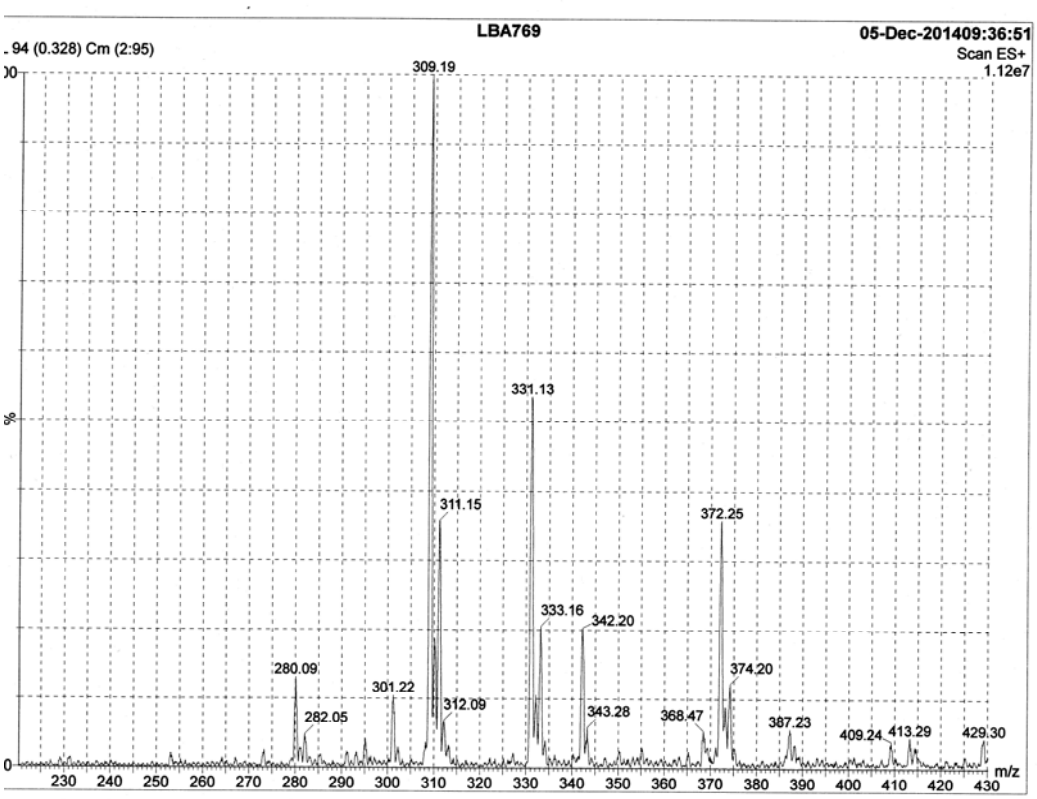
^{1}H-NMR spectra of Cou-Cl



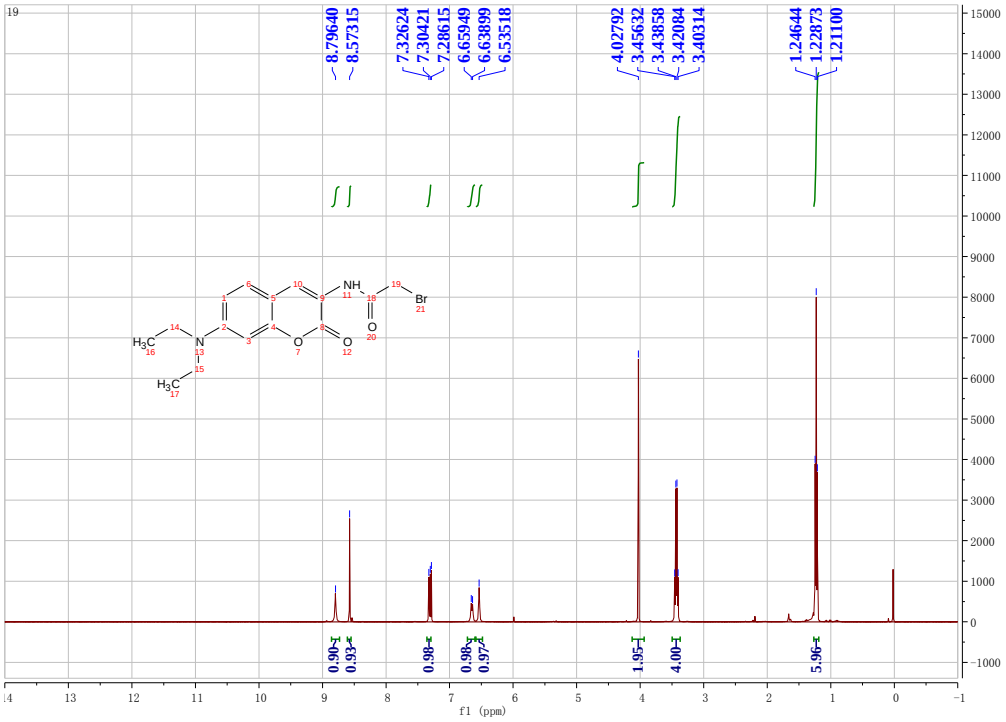
^{13}C-NMR spectra of Cou-Cl



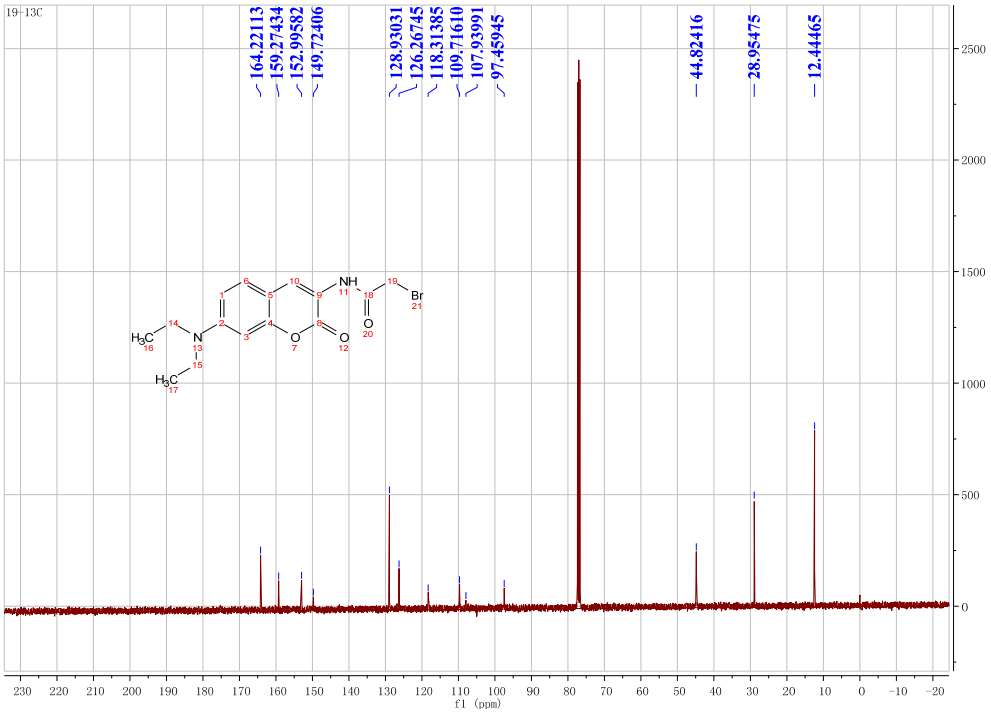
MS(ESI$^+$) of Cou-Cl



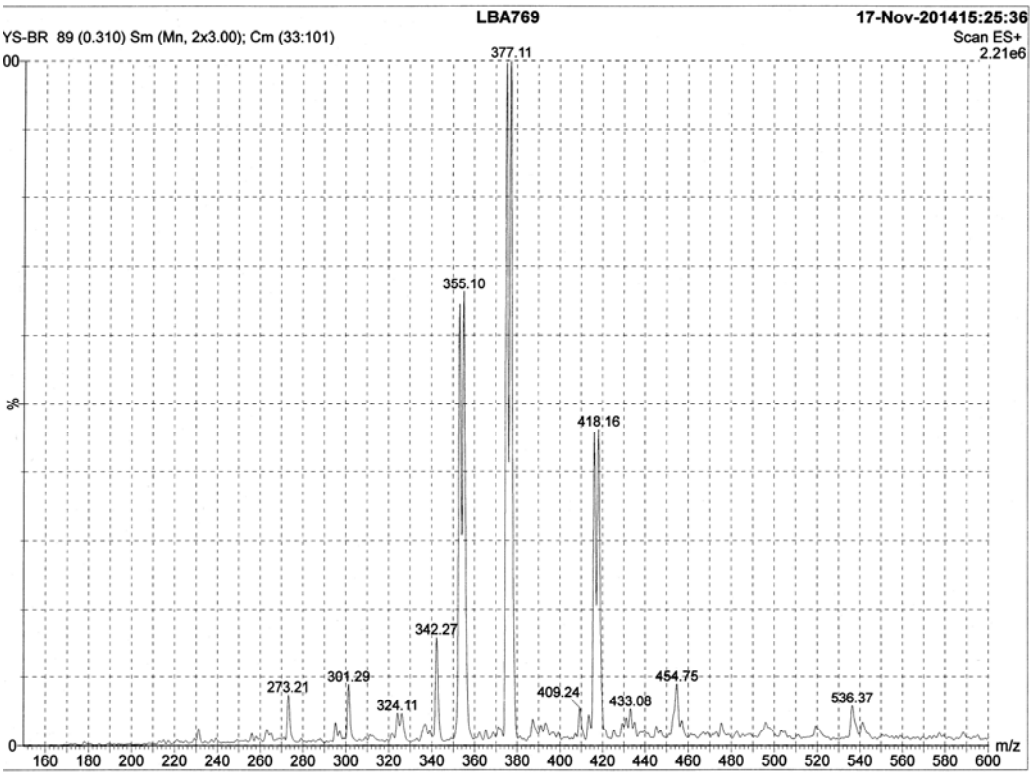
^{1}H-NMR spectra of Cou-Br



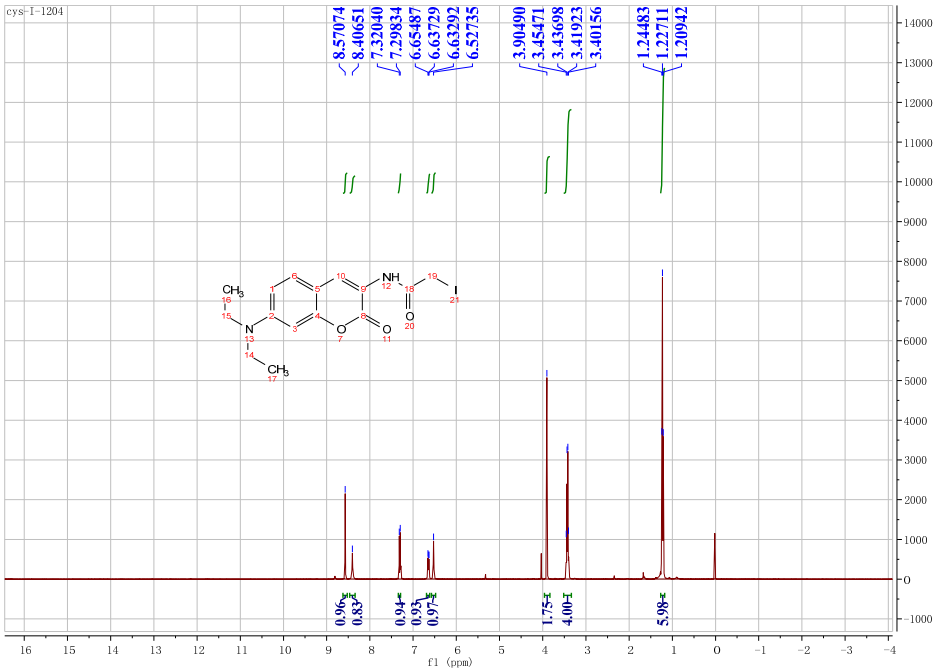
^{13}C-NMR spectra of Cou-Br



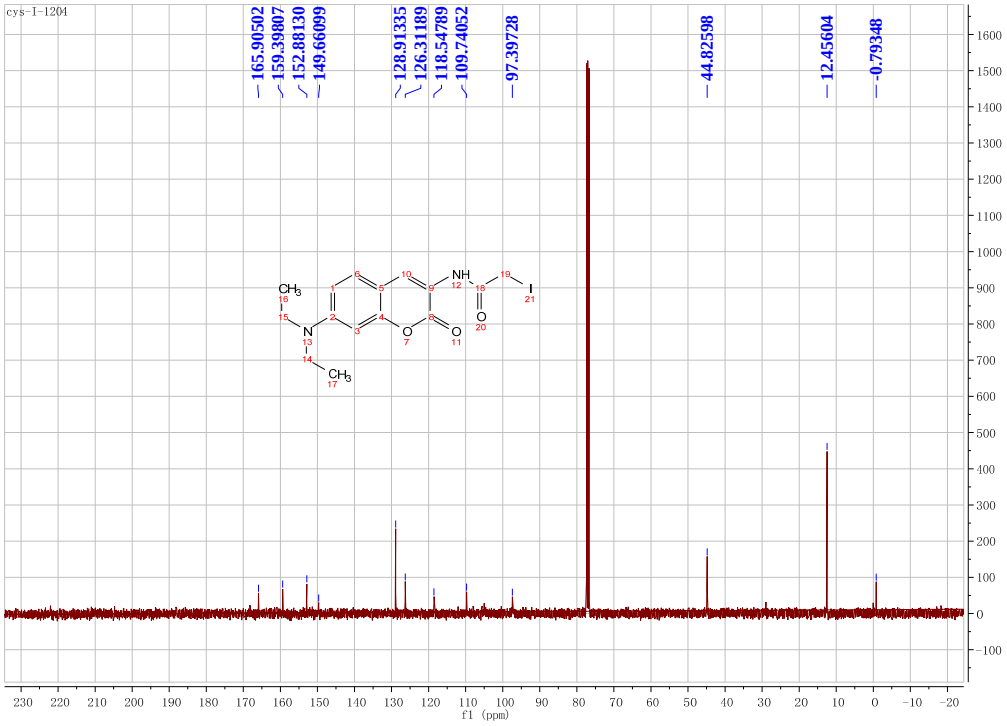
MS (ESI$^+$) of Cou-Br



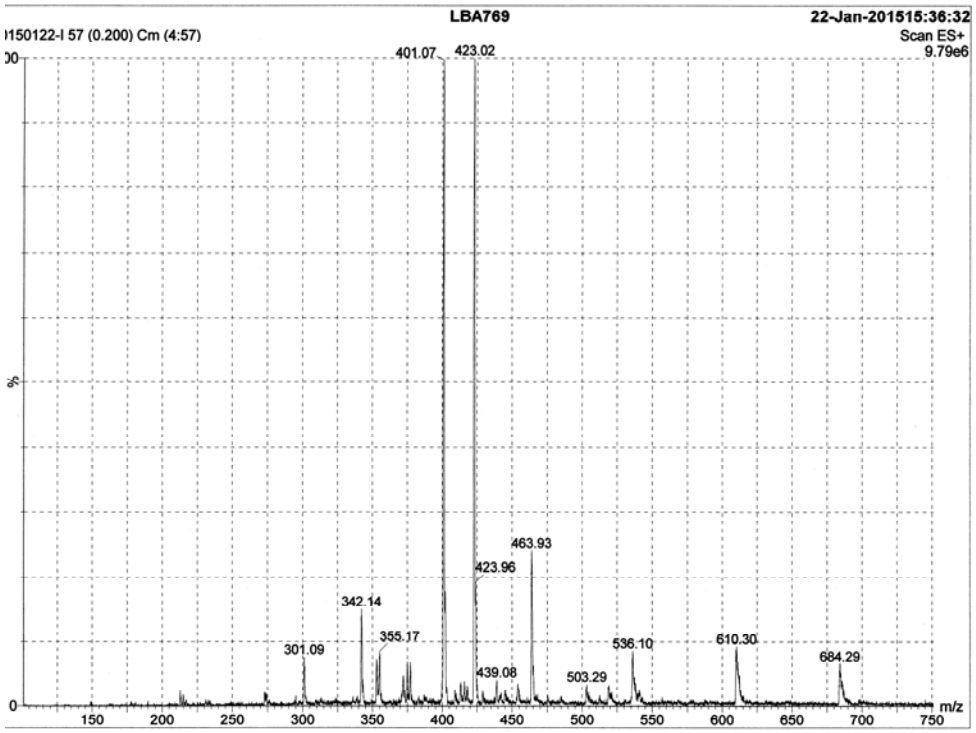
^{1}H-NMR spectra of Cou-I



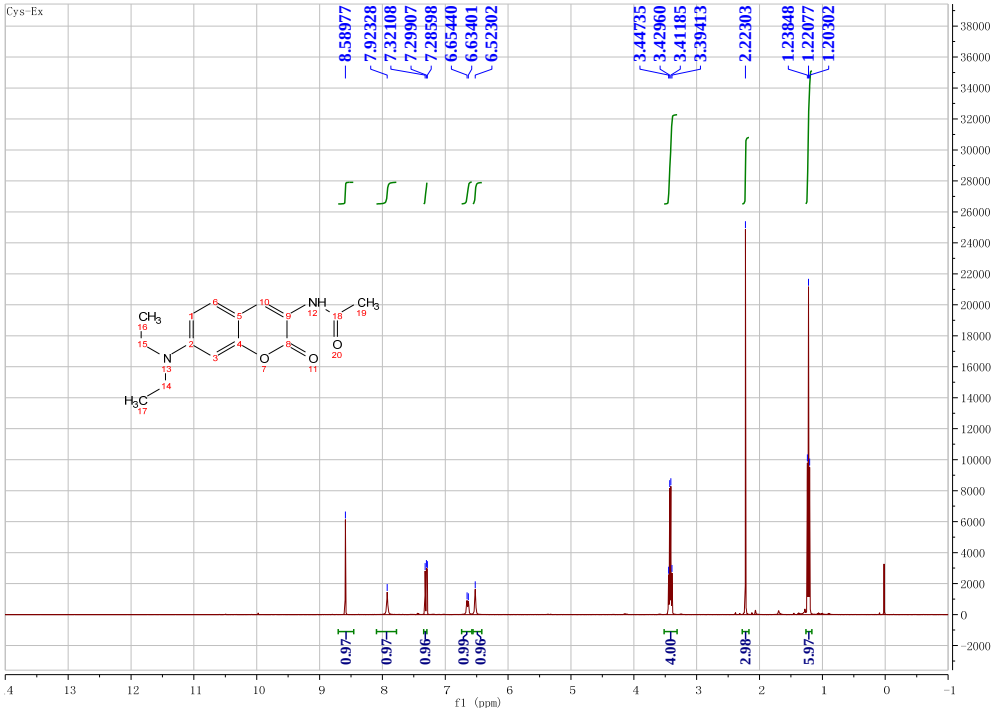
^{13}C-NMR spectra of Cou-I



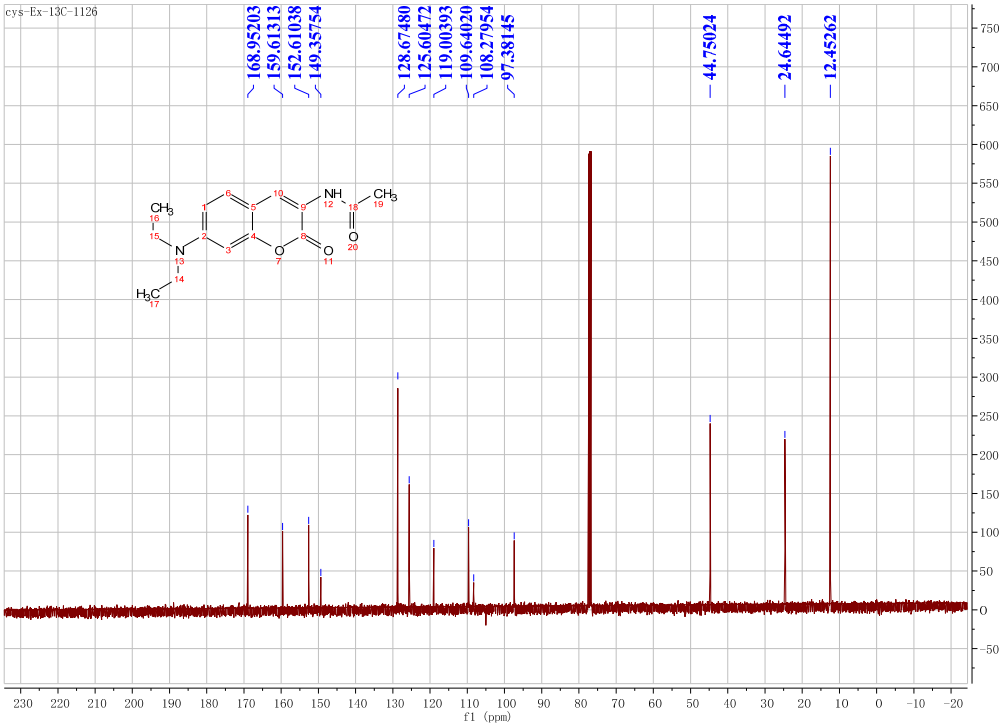
MS (ESI^+) of Cou-I



^{1}H-NMR spectra of Cou-H



^{13}C-NMR spectra of Cou-H



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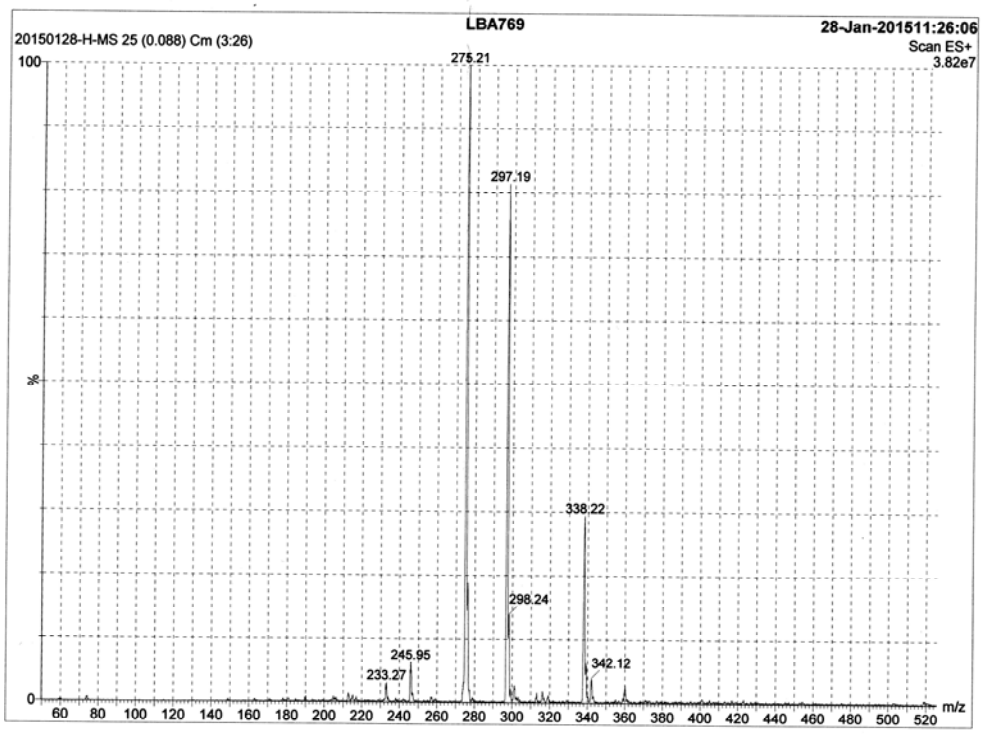

MS(ESI^+) of Cou-Mod