Electronic Supplementary Information

A Highly Selective and Reversible Fluorescent Cu^{2+} and S^{2-} Probe in Physiological Conditions and in Live Cells

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Materials and instruments

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified and dried by standard methods prior to use. Twice-distilled water was used throughout all experiments. The solutions of metal ions and anion were prepared from $CoCl_2$, $CuCl_2 \cdot 2H_2O$, $NiCl_2 \cdot 6H_2O$, $MgSO_4 \cdot 7H_2O$, KCl, NaCl, $BaCl_2$, $HgCl_2$, $CdCl_2 \cdot 2.5H_2O$, $MnSO_4 \cdot H_2O$, $Fe(NO_3)_3 \cdot 9H_2O$, $AgNO_3$, $Cu(ClO_4)_2$ and $FeCl_2$, $AuCl_3 \cdot HCl \cdot 4H_2O$，KBr, KCN, NaF, KI, NaClO, KSCN, $KBrO_3$, $NaBO_2$, Na_2S respectively. Melting points were determined with a Beijing taike XT-4 microscopy and were uncorrected. ESI-MS analyses were performed using a Waters Micromass ZQ-4000 spectrometer. Electronic absorption spectra were obtained on a LabTech UV Power spectrometer; Photoluminescent spectra were recorded with a HITACHI F4600 fluorescence spectrophotometer. UV-Vis absorption spectra were recorded with a Shimadzu UV-2450 spectrophotometer. Fluorescence images of HeLa cells were obtained using an Olympus FV1000 laser confocal microscope (Japan)，The MTT assay was obtained in a Benchmark Plus (Bio-Rad Instruments Inc., Japan). NMR spectra were recorded on a Bruker DRX-400 spectrometer using TMS as an internal standard. TLC analyses were 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.

Experimental Section

Synthesis of 5-tert-butyl-2-hydroxybenzene-1,3-dialdehyde (3): 3 was prepared by the reaction between 4-tert-butylphenol (1) (10.3 g; 68.4 mmol) and hexamethylenetetramine (2) (19.2 g; 137 mmol) in refluxing TFA (120 mL) under dry nitrogen for 24 h. Thus obtained reaction mixture was treated with 4 M aqueous HCl (400 mL). By extraction with CH_2Cl_2 (150 mL × 4 times) and column chromatography on silica gel with petroleum ether/ethyl acetate (20/1) as eluent, the product was isolated as pale yellow powder (2.03 g; 58%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.50 (s, 1H, OH), 10.25 (s, 2H, CHO), 7.99 (s, 2H, Ph), 1.36 (s, 9H, CH_3); ^{13}C NMR (100MHz, $CDCl_3$): δ 192.65, 161.73, 143.14, 122.65, 34.34, 34.12.

Synthesis of N-Methyl-2,3,3-trimethylindolenine(5): Under N_2, a mixture of 2,3,3-tri -methylindolenine (**4**) (0.79 g, 5.0 mmol) and iodomethane (0.85 g, 0.61 mmol) was heated to 50 °C for 2 h. After cooling the solution to room temperature, yellow crystals were collected by filtration. The solid was washed with ethanol and diethyl ether successively. The solvent was removed under vacuum, and the residue was recrystalized from absolute ethanol to give **5** in 70% yield. ^{1}HNMR (400 MHz, $CDCl_3$): δ 7.92 (m, 1H, Ph), 7.83 (m, 1H, Ph), 7.61 (m, 2H, Ph), 3.99 (s, 3H, CH_3), 2.79 (s, 3H, CH_3), 1.54 (s, 6H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 196.51, 142.63, 142.11, 129.82, 129.35, 123.87, 115.68, 54.47, 22.24, 14.93.

Synthesis of Spiropyran 6: Compound 5 (0.722 g, 2.0 mmol) in absolute ethanol (30 mL) was heated to reflux. Piperidine (0.3 mL, 3.0 mmol) was added to the solution. After the resulting mixture was completely mixed, 5-tert-butyl-2-hydroxybenzene-1,3-dialdehyde (0.412 g, 2.0 mmol) in absolute ethanol(9.0 mL) was introduced. The mixture was refluxed for 5 h. The solvent was removed by evaporation under reduced pressure. The crude residue was purified by silica gel

column chromatography using ethyl acetate/petroleum ether (1:20, v/v) as the eluant to afford 1 as a pink solid in74% yields.^{1}H NMR (400 MHz, $CDCl_3$): δ 10.13 (s, 1H, CHO), 7.65 (m, 1H), 7.24, (m, 1H), 7.05 (m, 1H), 6.88 (m, 1H), 6.84 (m, 1H), 6.52 (m, 1H), 5.79 (m, 1H), 2.75 (s, 3H), 1.33 (s, 3H), 1.30 (s, 9H), 1.18 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 188.18, 154.44, 146.77, 141.77, 135.31, 126.62, 120.38, 119.11, 118.57, 118.47, 105.89, 104.49, 50.95, 33.15, 30.47, 30.22, 30.09, 27.97, 24.75, 19.44.

Synthesis of compound (8): Rhodamine B hydrazide was prepared following a literature method. Rhodamine B hydrazide (456 mg, 1 mmol) and Spiropyran 6 (361 mg, 1 mmol) were dissolved in 20 mL of methanol. To this was added approximately 2 drops of acetic acid,and the resulting solution was refluxed for 10 h. The solvent was removed by evaporation under reduced pressure. The crude residue was purified by silica gel column chromatography using ethyl acetate\petroleum ether (1:5, v/v) as the eluant to afford 1 as a pink solid in 50%. ESI-Ms: m/z: 457.3 $[M+H]^+$

Synthesis of compound RB-SP1(9): Rhodamine 6G hydrazide (compound 2) was synthesized according to a reported method for the preparation of Rhodamine B hydrazide. AcOH (two drops) was added to a solution of Rhodamine 6G hydrazide (compound2) (37.0mg,0.086 mmol) and Spirobenzopyran1 (20.0 mg, 0.057 mmol) in EtOH (3 mL). Then the reaction mixture was stirred at 50 ℃ for 1 hour. The solvent of the mixture was removed under reduced pressure, and the resulting residue was purified on a silica gel column (petroleum ether / acetone = 3: 1) to afford compound 3 as a yellow powder (40. mg, isolated yield: 88%). IR(KBr): ν = 2971, 2870, 1695, 1611, 1510, 1455, 1362, 1298, 1260, 1223, 1131, 1029, 917, 827, 743 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.93 (s, 1H); 7.81 (d, *J* = 6.8 Hz, 1H; ArH), 7.34 (t, *J* = 7.0 Hz, 2H; ArH), 7.11 (t, *J* = 7.4 Hz, 1H; ArH), 7.04 (d, *J* = 7.4 Hz, 1H; ArH), 6.96 (d, *J* = 7.2 Hz, 1H; ArH), 6.86 (d, *J* = 2.4 Hz, 1H; ArH), 6.78 (t, *J* = 7.0 Hz, 1H; ArH), 6.61 (d, *J* = 10.4 Hz, 1H; $=CH_2$), 6.37 (t, *J* = 8.4 Hz, 3H), 6.25 (dd, *J* = 12.0 Hz, 2.4 Hz, 2H; $=CH_2$), 6.11 (d, *J* = 8.8 Hz, 1H), 5.45 (d, *J* = 10.4 Hz, 1H; CH=N), 3.24-3.18 (m, 8H, CH_2), 1.19 (s, 9H, CH_3), 1.08-1.04 (m, 12H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, TMS): δ = 164.24, 153.76, 153.65, 150.64, 150.58, 148.64, 148.61, 147.74, 143.31, 142.31, 136.46, 132.89, 130.64, 128.59, 128.23, 127.81, 127.55, 125.26, 123.88, 123.37, 123.20, 121.62, 121.20, 119.87, 118.78, 118.39, 107.63, 107.15, 104.28, 98.38, 98.32, 66.44, 51.24, 44.27, 34.13, 31.37, 29.73, 28.68, 25.54, 21.70, 12.69; ESI-Ms: m/z: 800.5 $[M+H]^+$.

Synthesis of compound RB-SP 2(10): Zinc powder (11.1 mg, 1.7 mmol) and two drops of acetic acid were added to compound RB-SP 1 (801 mg, 0.001 mmol) in dichloromethane (4 mL). The reaction mixture was stirred at 0 ℃ for 0.5 h. The solvent was removed under reduced pressure, and the resulting crude product was purified by column chromatography on silicapetroleum ether / acetone (3: 1, V/V) to afford compound 1 in 60% yields as a yellow solid. IR(KBr): ν = 3452, 2962, 2878, 1704, 1611, 1510, 1438, 1359, 1307, 1270, 1223, 1112, 1029, 983, 817, 789, 752 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 10.87 (s, 1H), 9.27 (s, 1H), 7.53 (t, *J* = 6.4 Hz, 2H; ArH), 7.39 (d, *J* = 6.0 Hz, 2H; ArH), 7.20 (d, *J* = 5.6 Hz, 1H; ArH), 7.16-7.12 (m, 3H; ArH), 7.00 (s, 1H; ArH), 6.59 (d, *J* = 6.8 Hz, 1H; ArH), 6.50-6.46 (m, 3H; ArH), 6.31 (d, *J* = 2.4 Hz, 1H; ArH), 6.26 (d, *J* = 6.0 Hz, 2H), 4.79 (d, *J* = 6.8 Hz, 1H), 4.52 (t, *J* = 6.4 Hz, 1H), 3.37-3.29 (m, 8H; ArH), 2.45 (s, 3H, CH_3), 1.54 (s, 3H, CH_3), 1.42 (s, 3H, CH_3), 1.17 (t, *J* = 6.8 Hz, 6H), 1.13 (t, *J* = 6.8 Hz, 6H), 1.01 (s, 9H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, TMS): δ = 164.12, 155.58, 153.70, 153.66, 153.43, 150.61, 149.03, 148.92, 145.63, 140.13, 139.93, 133.40, 130.24, 128.13, 127.75, 127.23, 125.58, 124.17, 123.81, 123.27, 121.55, 117.61, 113.94, 108.11, 105.38, 98.03, 97.97,

75.30, 66.05, 49.65, 44.38, 39.12, 33.76, 31.08, 26.52, 24.50, 12.59, 12.48; ESI-Ms: m/z: 803.1 $[M+H]^+$.

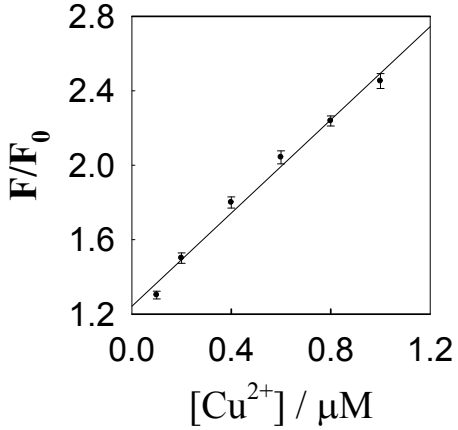


Figure S1. The liner equation of ratio of fluorescence and Cu^{2+} concentration.

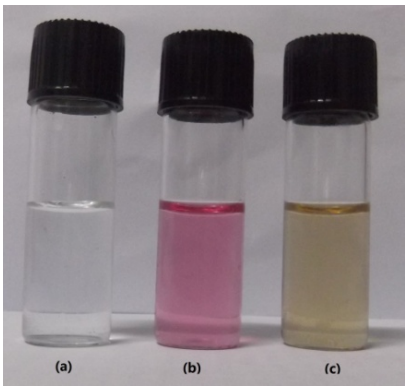


Figure S2: The visible color changes of the solution; (a) **RB-SP2** (10 μM); (b) **RB-SP2** (10 μM) + Cu^{2+} (10μM); (c) **RB-SP2** (10 μM) + Cu^{2+} (10μM) + S^{2-}(excess amount).

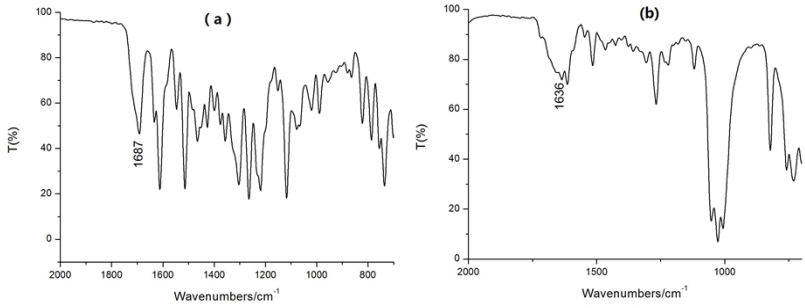


Figure S3: Infrared spectra of RB-SP2 (a), and RB-SP2 with Cu^{2+}

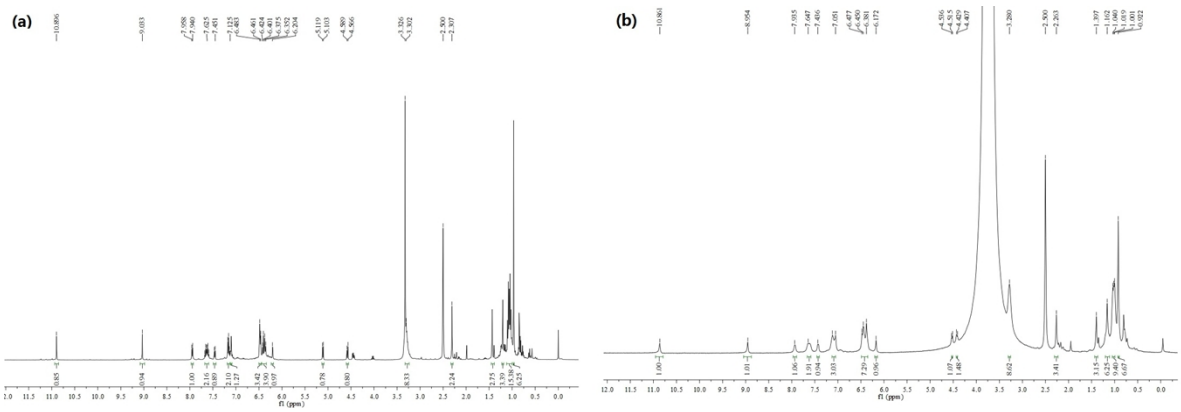


Figure S4: (a)^{1}H NMR of **RB-SP2** in d_6-DMSO; (b) ^{1}H NMR of **RB-SP2** in d_6-DMSO with $CuCl_2$ (2 equiv) in H_2O

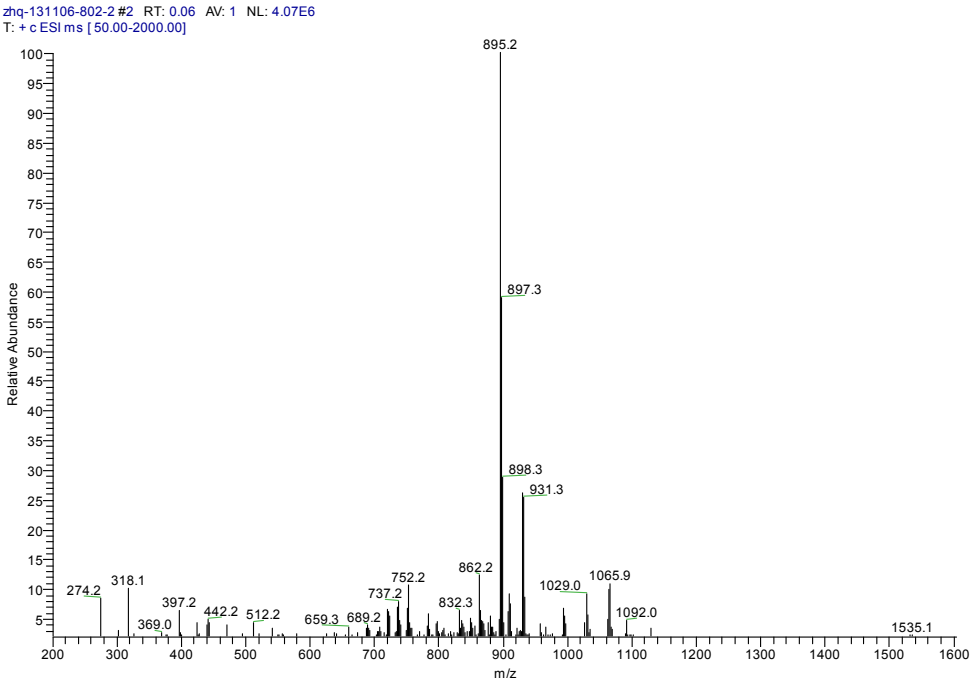


Figure S5: Mass of RB-SP2-Cu^{2+} complex

Calculation of Association Constant[11]

The association constant was determined from the fluorescence titration data according to a reported method [1] for a 1:1 metal-ligand binding mode. If a 1:1 metal-ligand complex is formed between a metal ion and a ligand, one can describe the equilibrium as follows:

$$M + Ligand \overset{K}{\rightleftharpoons} M\,(Ligand) \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where M and M(Ligand) denote a metal ion and its complex, respectively. The corresponding association constant, *K*, can be expressed as follows:

$$K = \frac{[M\,(Ligand)]}{[Ligand][M]} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

A response function for M is given below following the mass law:

$$\frac{\alpha}{1-\alpha} = \frac{1}{KC_T[M]} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

where C_T denotes the total concentration of the ligand in the system, α defined as the ratio between the free ligand concentration ([C]) and the total concentration of ligand C_T:

$$\alpha = \frac{[C]}{C_T} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

α can be determined from the emission changes in the presence of different concentrations of M:

$$\alpha = \frac{F - F_{max}}{F_{min} - F_{max}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (5)$$

where Fmax and Fmin are the limiting emission values for α = 1 (in the absence of M) and α = 0 (the ligand is completely complexed with M), respectively.

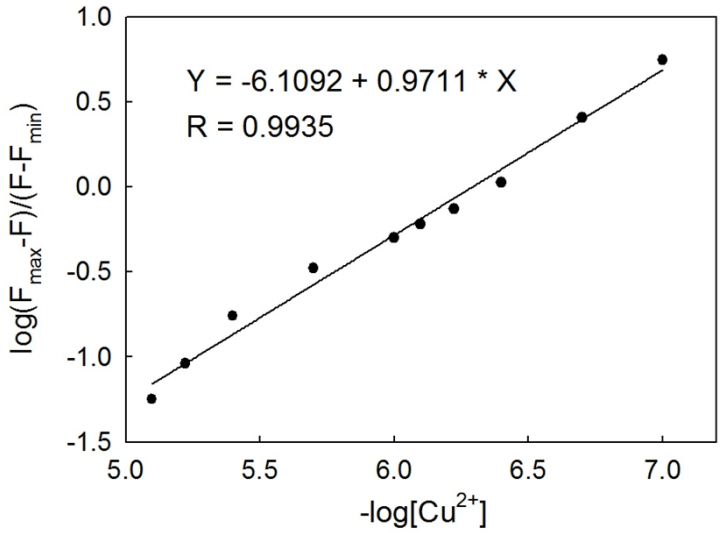


Figure S6. The binding constant of sensor **RB-SP2** and Cu^{2+} in C_2H_5OH/ Aqueous PBS (1 mM, pH 7.4; 4:6 v/v;1% DMSO). K=1.26 × 10^6 LogK=6.11 .

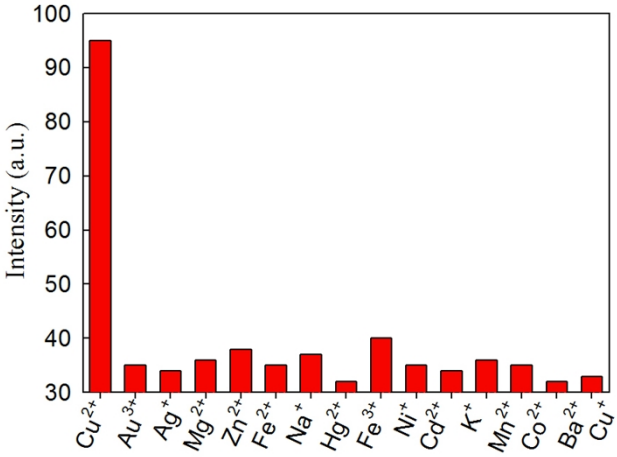


Figure S7. Fluorescence intensity changes of sensor **RB-SP2** (10 μM) in response to various metal species (10 μM) in C_2H_5OH/Aqueous PBS (1 mM, pH 7.4; 4:6 v/v; 1% DMSO). Excitation at 500 nm; emission at 587 nm.

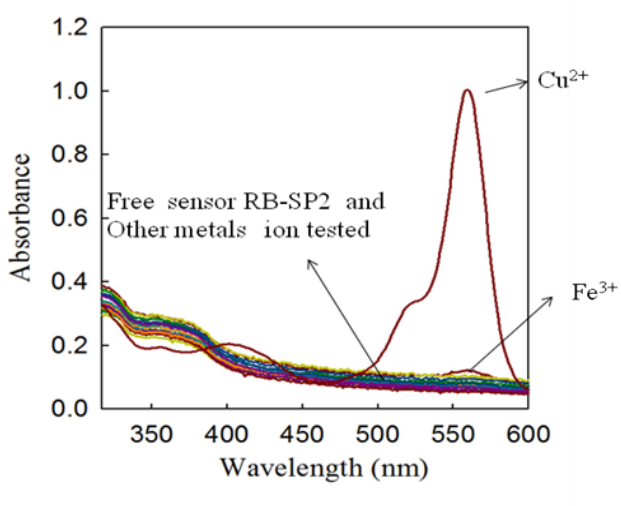


Figure S8: Changes in the UV-vis absorption spectra of sensor **RB-SP2** (10 μM) with metal ions (10 μM) in C_2H_5OH/ Aqueous PBS (1 mM, pH 7.4; 4:6 v/v; 1% DMSO). The metal species include Cu^{2+} ,Au^{3+}, K^{+}, Mg^{2+}, Hg^{2+}, Fe^{2+}, Ni^{2+}, Cd^{2+}, Ba^{2+}, Co^{2+}, Mn^{2+}, Na^{+}, Fe^{3+}, Zn^{2+} and Ag^{+}.

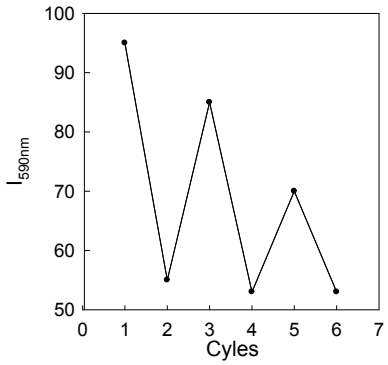


Figure S9: Fluorescent intensity of RB-SP2- Cu^{2+} (10 µM) in C_2H_5OH/Aqueous PBS (1 mM, pH 7.4; 4:6 v/v; 1% DMSO) upon the alternate addition of Na_2S/$CuCl_2$ with several concentrations (0:0, 10:0, 10:15, 20:15, 20:30 µM,

respectively). Excitation at 500 nm.

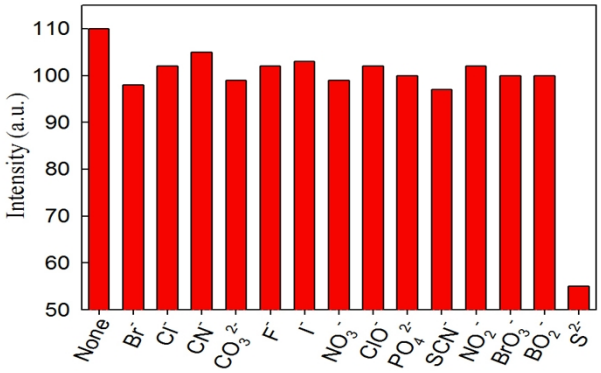


Figure S10: Fluorescence intensity changes of sensor RB-SP2 (10 μM) in C_2H_5OH/Aqueous PBS (1 mM, pH 7.4; 4:6 v/v;1% DMSO) containing 10μM Cu^{2+} (**RB-SP2-Cu^{2+}**) in response to 10μM various anions species Excitation at 500 nm; emission at 587 nm.

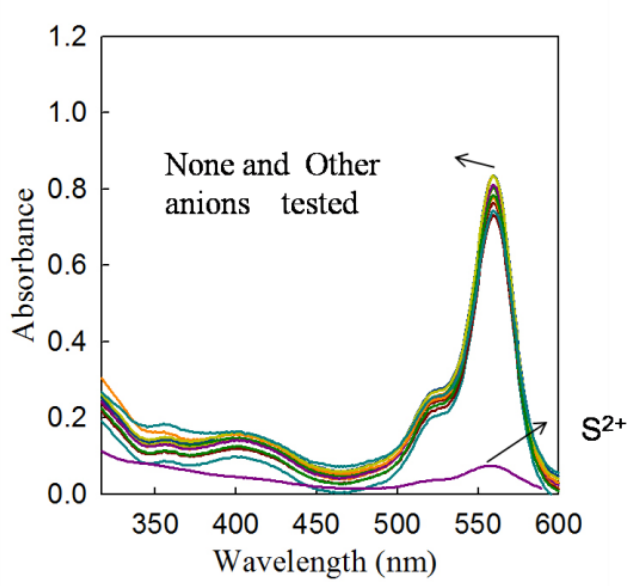


Figure S11: Changes in the absorption spectra of **RB-SP2** (10μM) in C_2H_5OH/Aqueous PBS (1 mM, pH 7.4; 4:6 v/v;1% DMSO) containing 10μM Cu^{2+} (**RB-SP2-Cu^{2+}**) is titrated in presence of 20 μM different anions S^{2-}, F^-, Cl^-, Br^-, I^-, CN^-, $H_2PO_4^-$, NO_3^-,ClO^-, ClO_4^-, SO_4^{2-} and HSO_3^-.

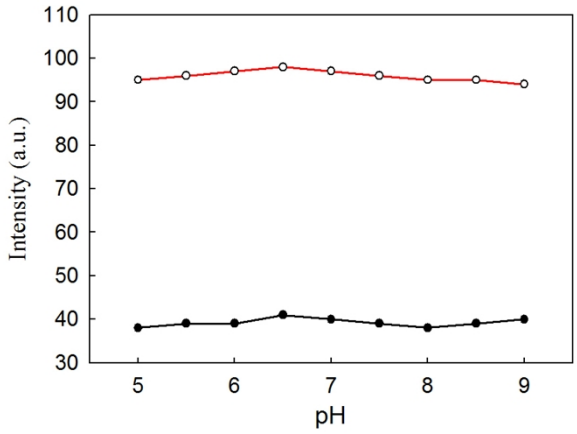


Figure S12: The fluorescence responses (at 587 nm) of free sensor **RB-SP2** (10 μM) (●) and sensor **RB-SP2** (10 μM) + 10 equiv Cu^{2+} (○) in C_2H_5OH/Aqueous PBS (1 mM, pH 7.4; 4:6 v/v; 1% DMSO).

Living Cell Imaging and Cytotoxicity Study.

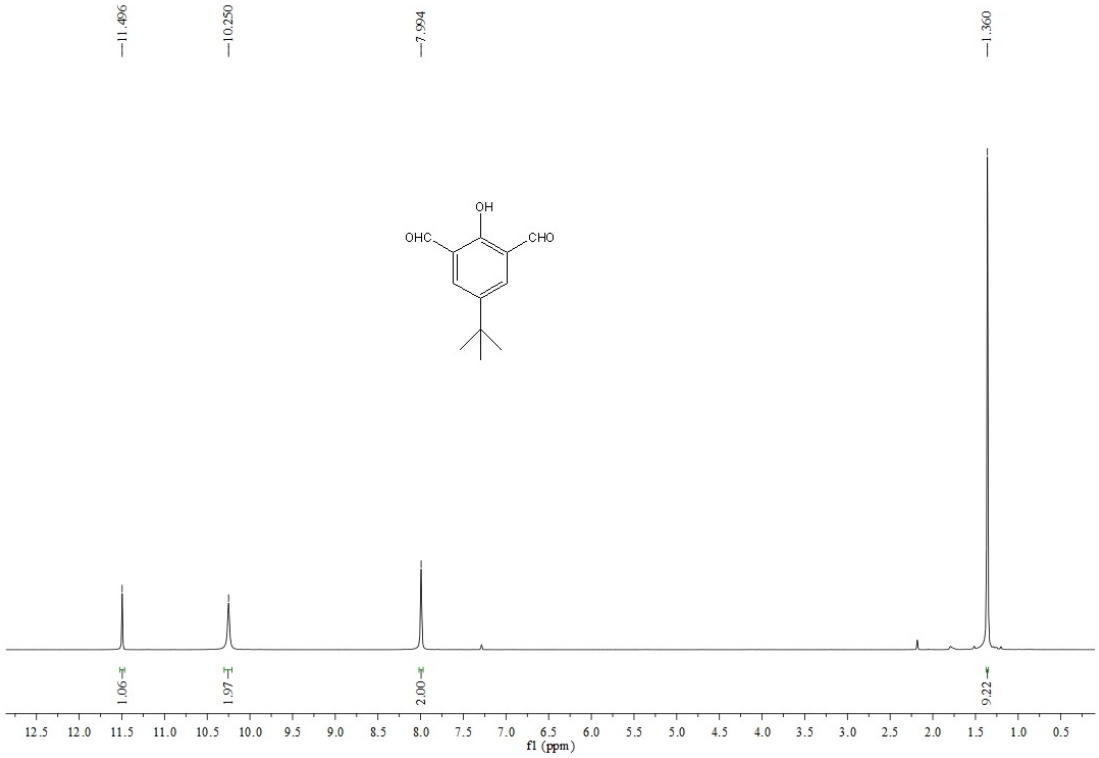
The cytotoxic effect of compound **RB-SP2** and **RB-SP2**−Cu complex was determined by an MTT assay following the manufacturer instruction (Sigma-Aldrich, MO). HeLa cells were initially propagated in a 25 cm^2 tissue culture flask in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 µg/mL), and streptomycin (100 μg/mL) in a CO_2 incubator. For cytotoxicity assay, cells were seeded into 96-well plates (approximately 10^4 cells per well), and various concentrations of compound **RB-SP2** and **RB-SP2−Cu** complex (15, 25, 50, 75, and 100 μM) made in DMEM were added to the cells and incubated for 24 h. Solvent control samples (cells treated with DMSO alone) and cells treated with $Cu(ClO_4)_2$ alone were also included in parallel sets. Following incubation, the growth media was removed, and fresh DMEM containing MTT solution was added. The plate was incubated for 3−4 h at 37 °C. Subsequently, the supernatant was removed, the insoluble colored formazan product was solubilized in DMSO, and its absorbance was measured by Benchmark Plus (Bio-Rad Instruments Inc., Japan) at 550 nm. The assay was performed in six sets for each concentration of compound **RB-SP2** and **RB-SP2−Cu** complex. Data analysis and calculation of standard deviation was performed with Microsoft Excel 2010 (Microsoft Corporation). For statistical analysis, a one way analysis of variance (ANOVA) was performed using Sigma plot.

Cell Culture and Imaging Studies: HeLa cells were obtained from the biomedical engineering center of Hunan University (Changsha, China). The cells were propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 µg/mL), and streptomycin (100 μg/mL). Cells were maintained under a humidified atmosphere of 5% CO_2 and at 37 °C incubator as mentioned before. For cell imaging studies, cells were seeded into a Confocal dish and incubated at 37 °C in a CO_2 incubator for one day. After one day cells were washed three times with phosphate buffered saline (pH 7.4) and incubated with 10 µM **RB-SP2** in DMEM at 37 °C for 1 h in a CO_2 incubator and observed under Olympus FV1000 laser confocal microscope. The cells were again washed thrice with PBS (pH 7.4) to remove the free L1, and then incubated in phosphate buffered saline with 10 μM $Cu(ClO_4)_2$ for 1 h. Again, images were

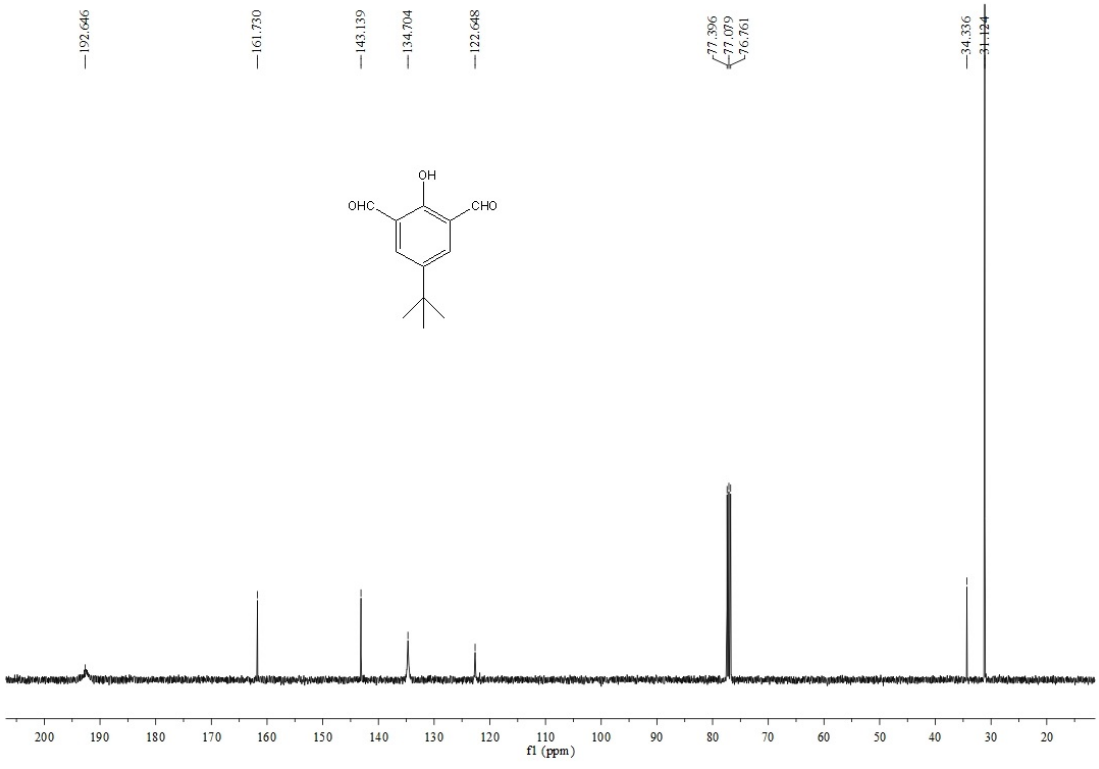
taken using confocal microscope. The cells were then treated with 20 μM of Na_2S solution, after incubation for 1 h; the cells were washed with PBS three times to remove free compound and ions before analysis. Then, fluorescence microscopic images were acquired.

References

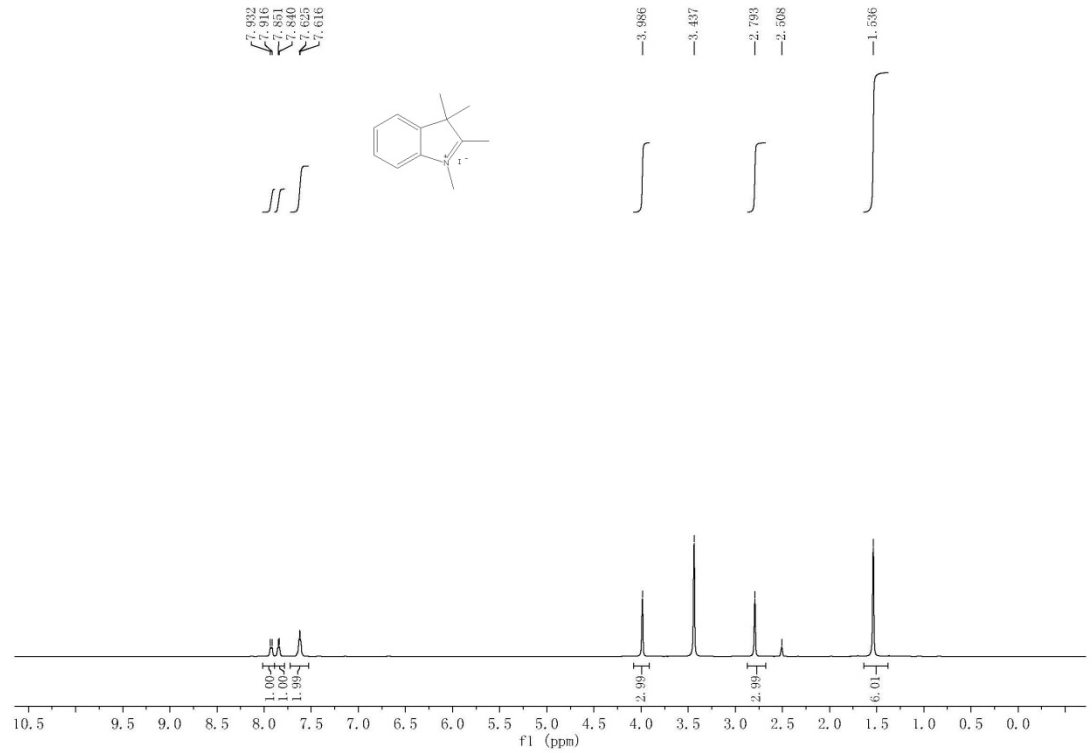
[1] R. Yang, K. Li, K. Wang, F. Zhao, N. Li, F. Liu, *Anal. Chem.* 2003, *75*, 612-621.



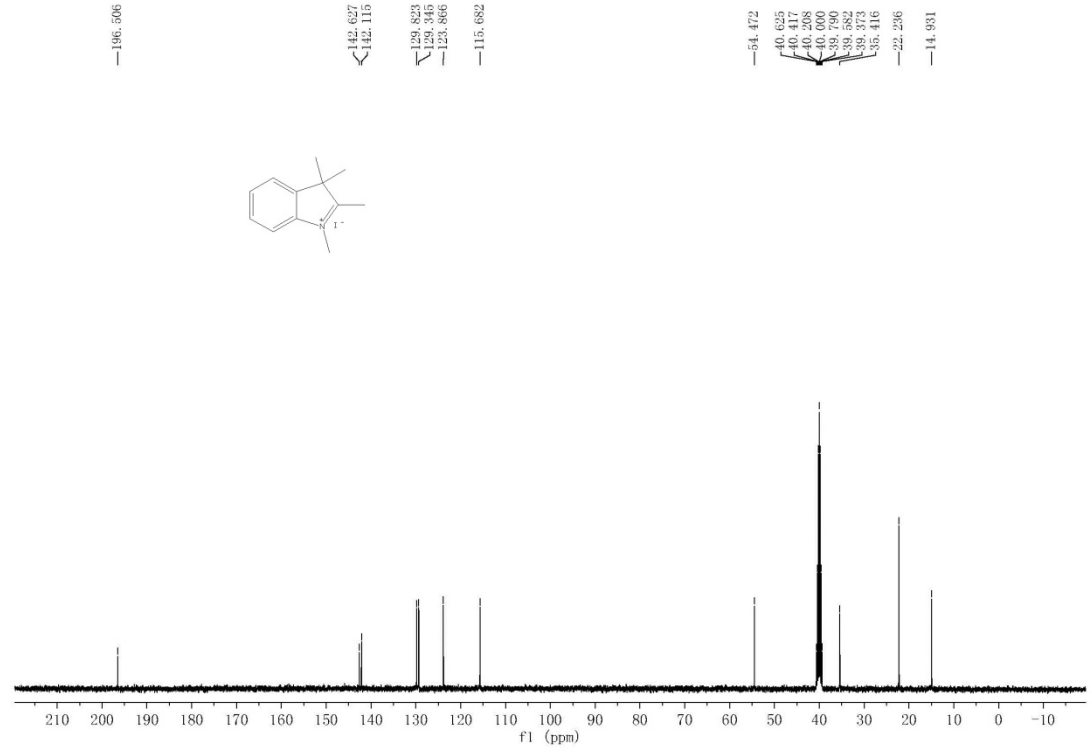
^{1}H NMR of **3** in $CDCl_3$



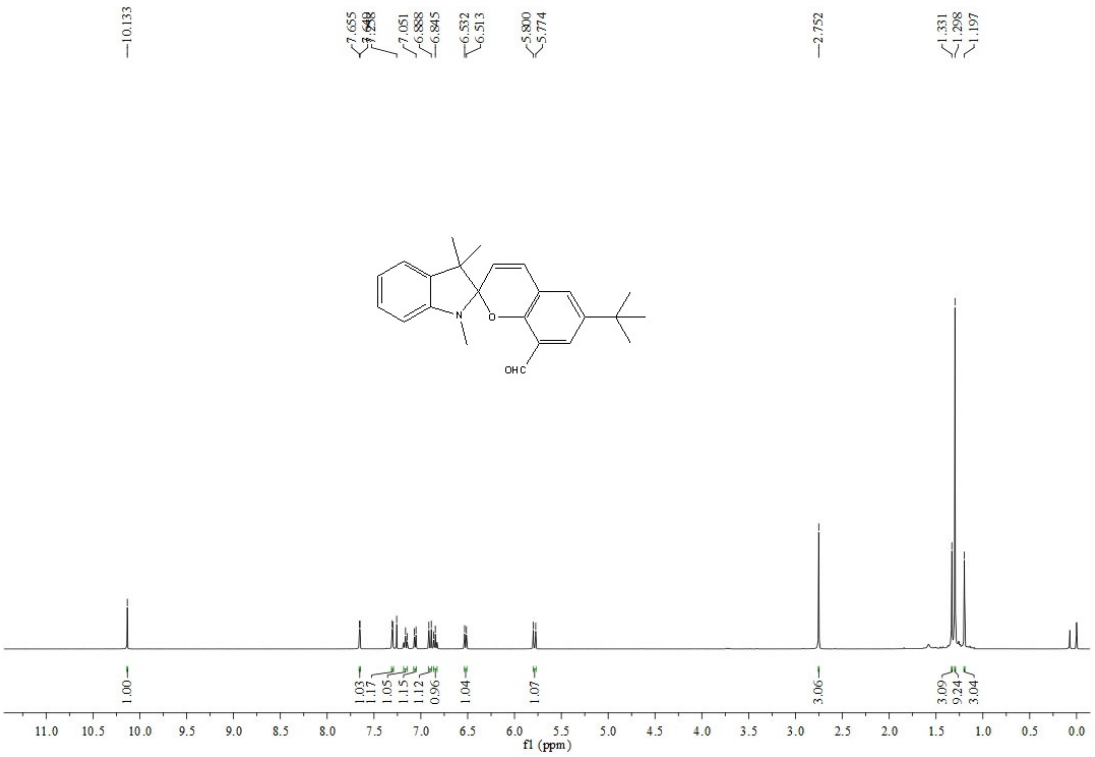
^{13}C NMR of **3** in $CDCl_3$



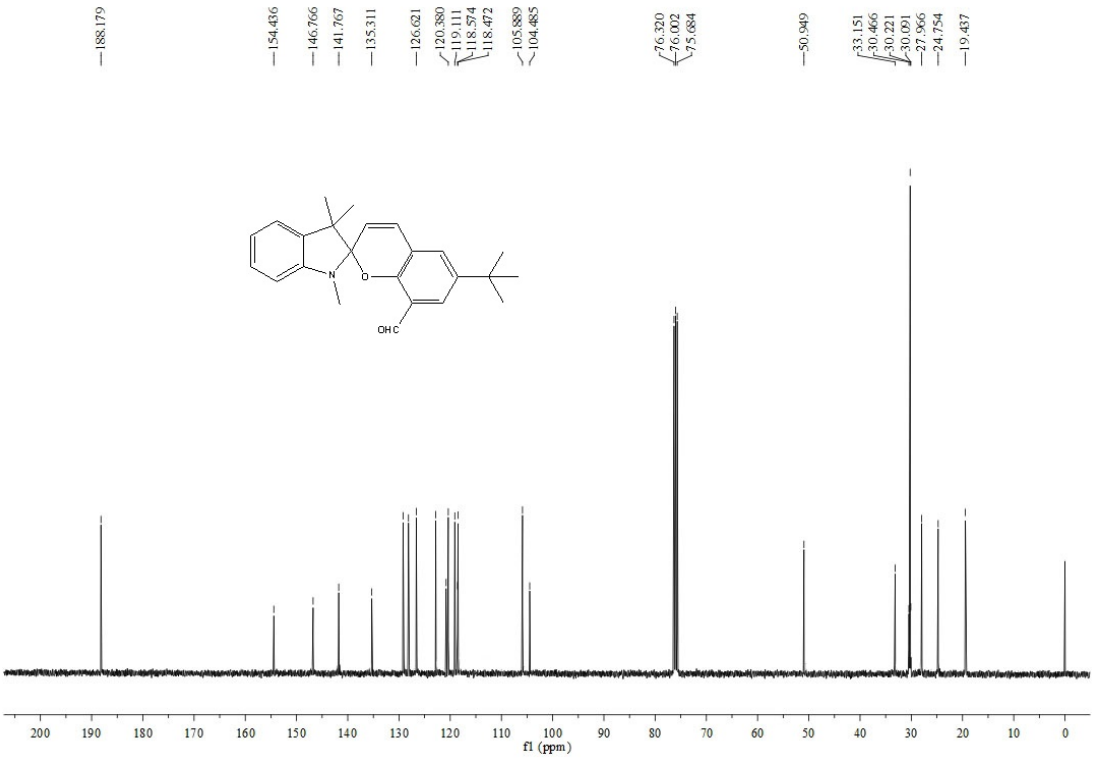
^{1}H NMR of **5** in $CDCl_3$



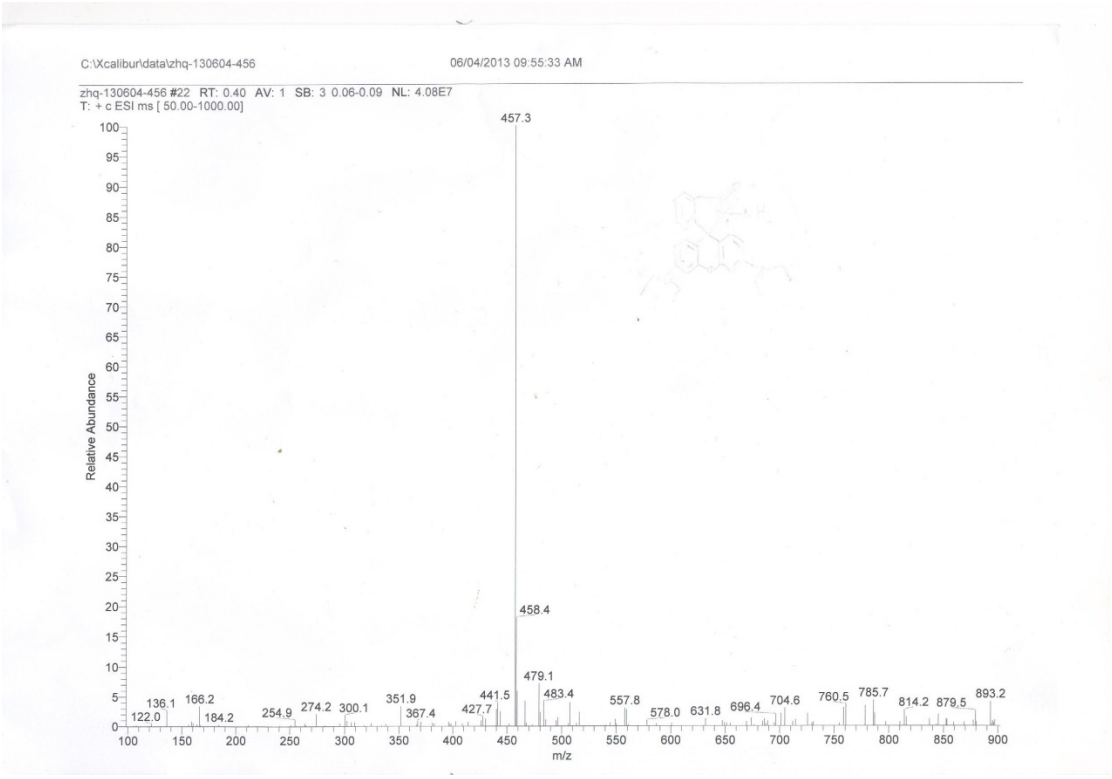
^{13}C NMR of **5** in $CDCl_3$



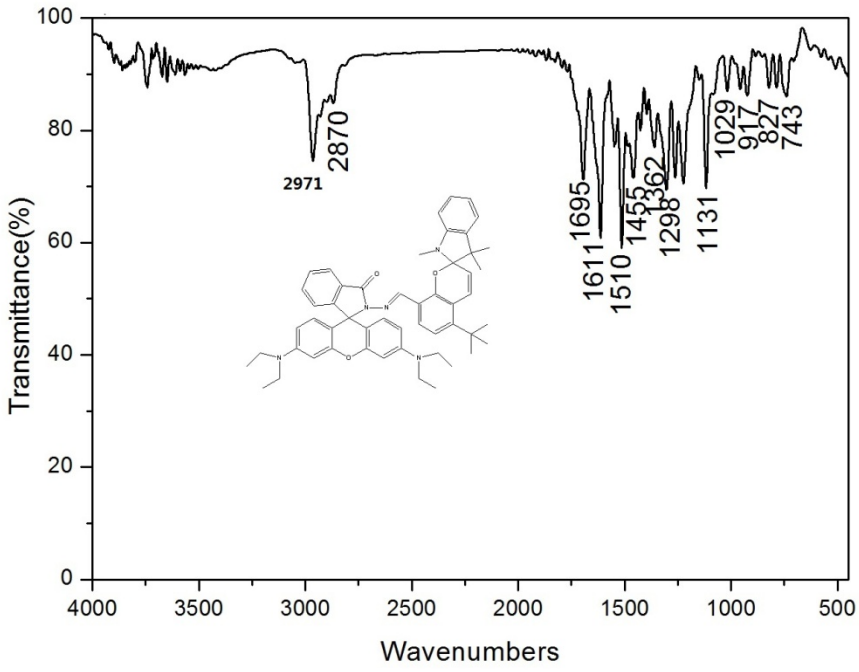
^{1}H NMR of **6** in $CDCl_3$



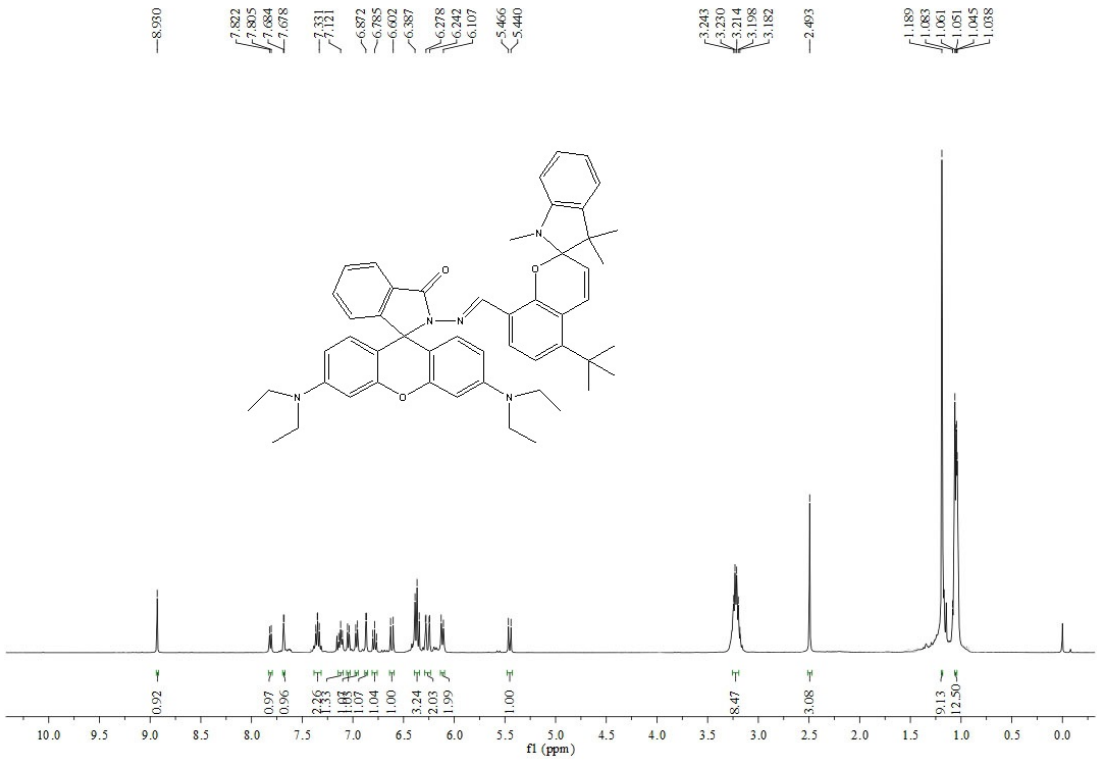
^{13}C NMR of **6** in $CDCl_3$



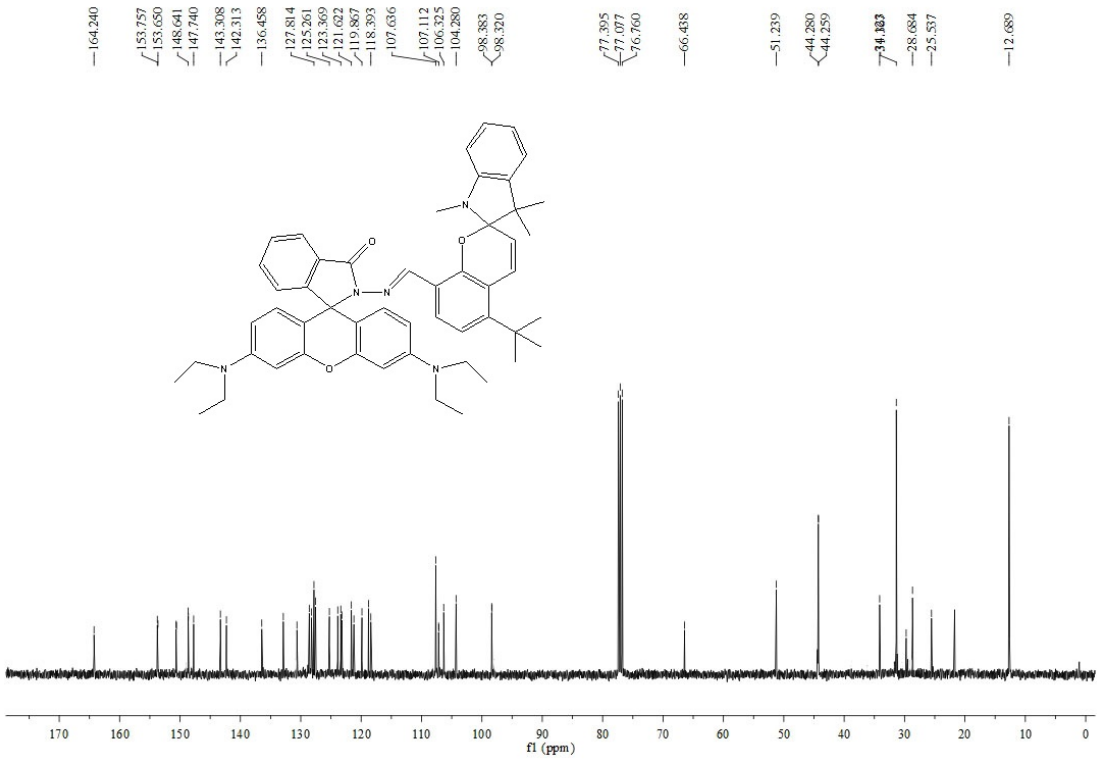
Ms of compound 8



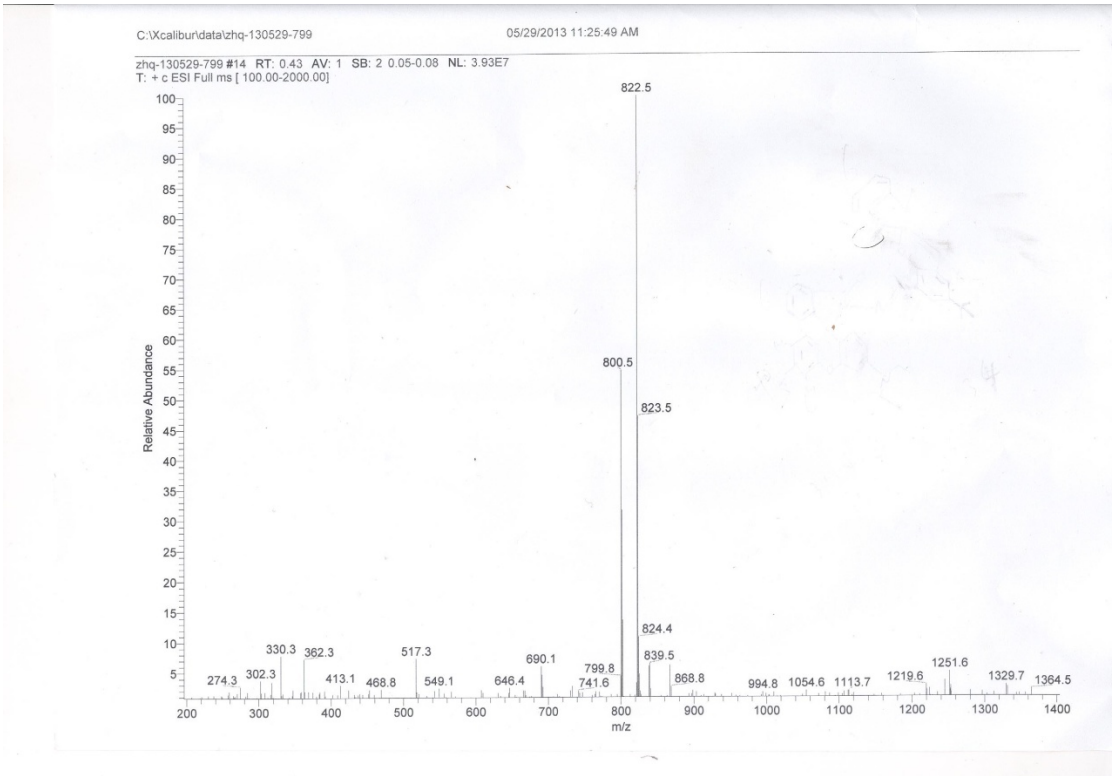
IR of compound 9 (*RB-SP1*)



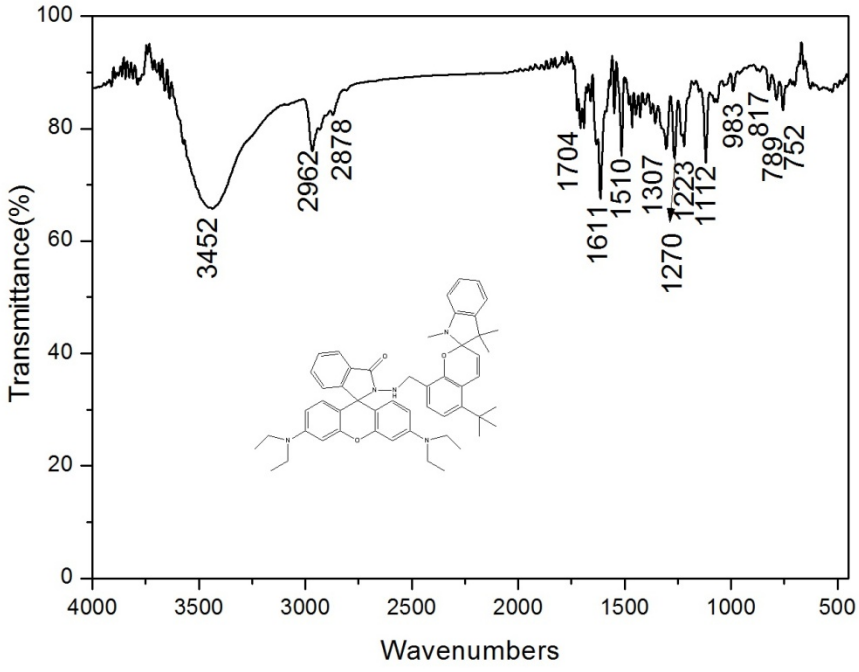
^{1}H NMR of **9** in $CDCl_3$



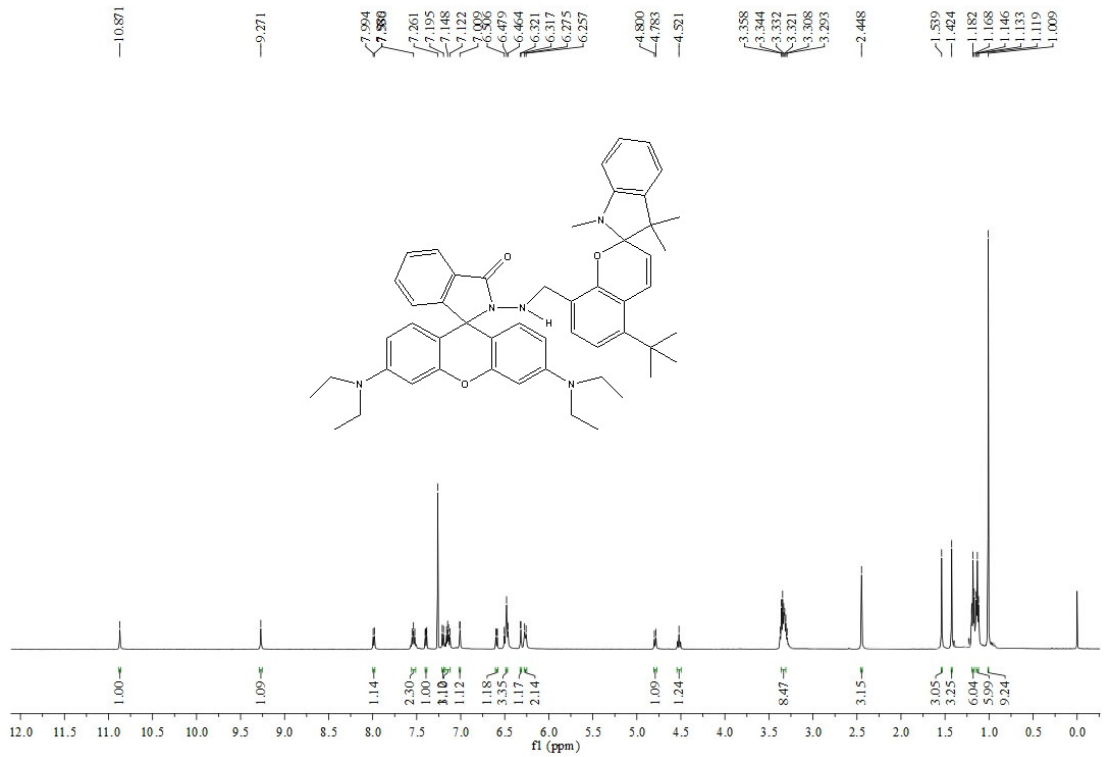
^{13}C NMR of **9** in $CDCl_3$



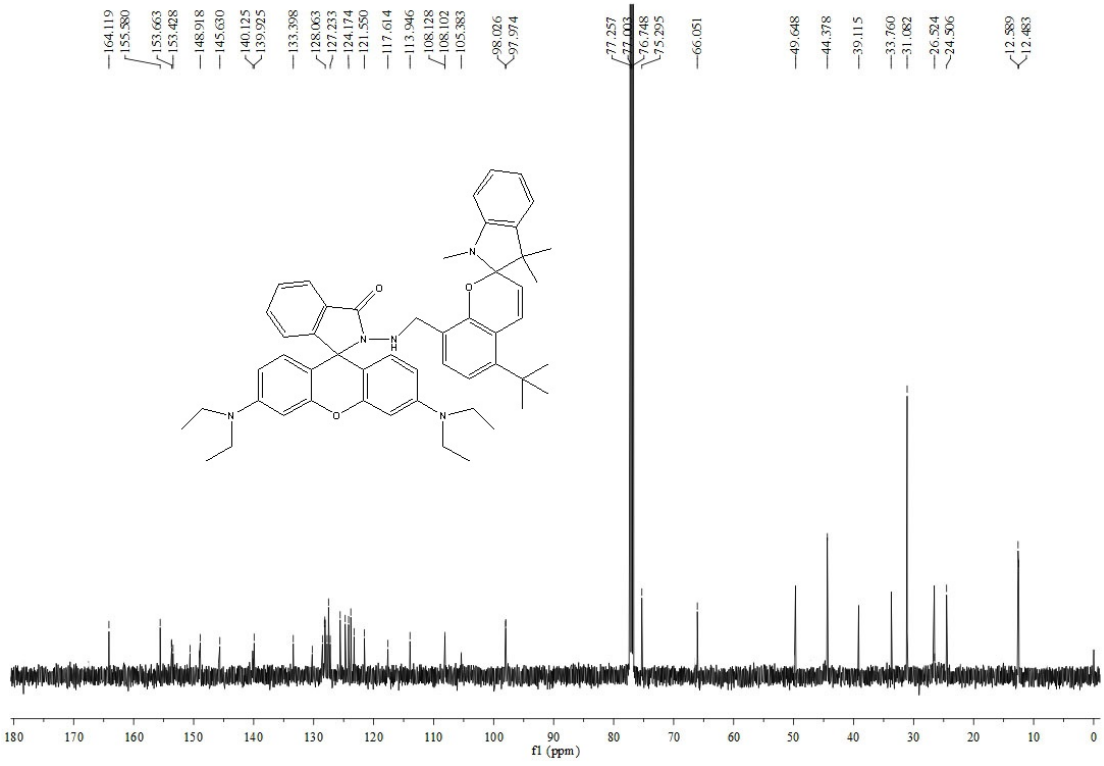
Ms of compound 9 (*RB-SP1*)



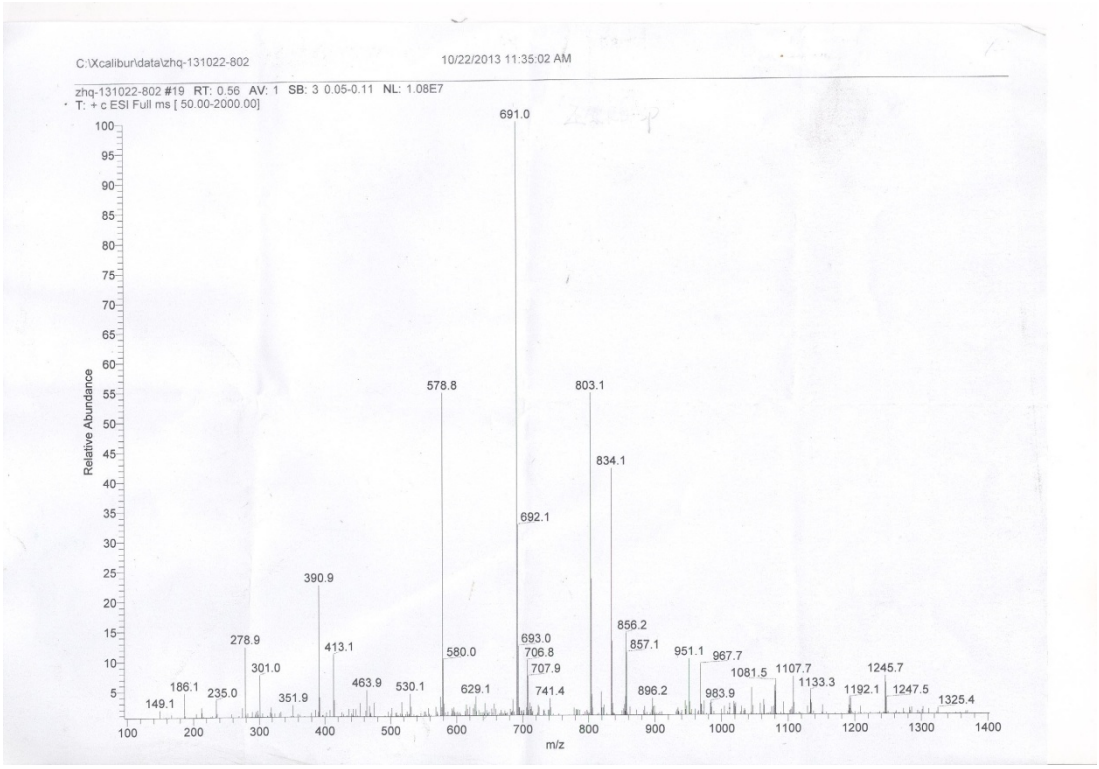
IR of compound 10 (*RB-SP2*)



^{1}H NMR of **10** in $CDCl_3$



^{13}C NMR of **10** in $CDCl_3$



Ms of compound 10 (*RB-SP2*)