Supporting Information

A FRET-ICT dual-quenching fluorescent probe with large *off-on* response for H_2S: synthesis, spectra and bioimaging

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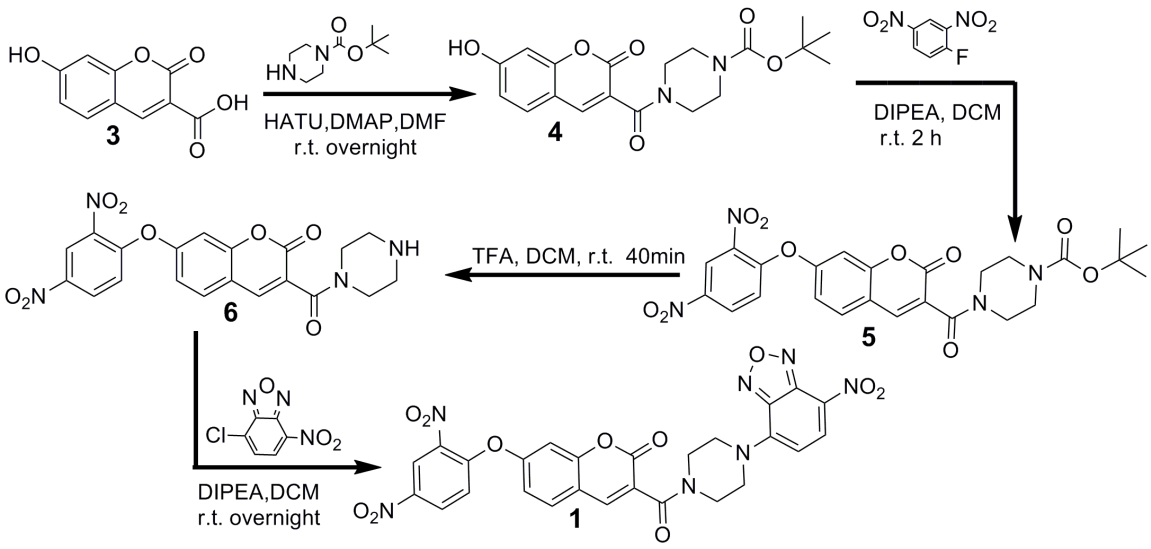
1. Reagents and instruments

All chemicals and solvents used for synthesis were purchased from commercial suppliers and applied directly in the experiment without further purification. The progress of the reaction was monitored by TLC on pre-coated silica plates (Merck 60F-254, 250 μm in thickness), and spots were visualized by basic $KMnO_4$, UV light or iodine. Merck silica gel 60 (70-200 mesh) was used for general column chromatography purification. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 (400 MHz ^1H; 100 MHz ^{13}C) spectrometer at room temperature. Chemical shifts are reported in parts per million

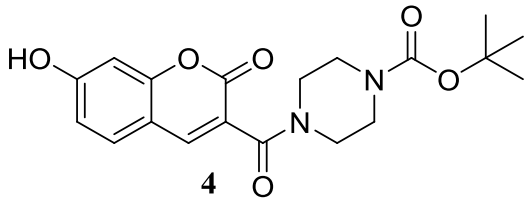
relative to internal standard tetramethylsilane ($Si(CH_3)_4$ = 0.00 ppm) or residual solvent peaks ($CDCl_3$ = 7.26 ppm). ^1H NMR coupling constants (*J*) are reported in Hertz (Hz), and multiplicity is indicated as the following: *s* (singlet), *d* (doublet), t (triplet), *q* (quartet), *bs* (board singlet), *m* (multiple). High-resolution mass spectra (HRMS) were obtained on a XEVO-G2QTOF (ESI) (Waters, USA) or Varian 7.0 T FTICR-MS.

2. Synthesis of probes



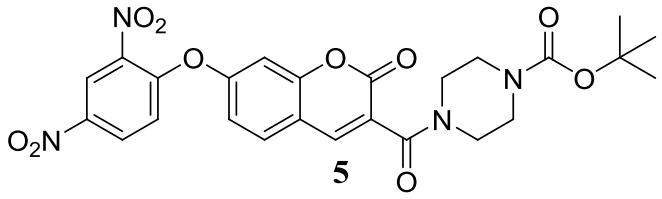
Scheme S1. Synthesis route of the probe **1**.

Synthesis of tert-butyl 4-(7-hydroxy-2-oxo-2H-chromene-3-carbonyl)piperazine-1-carboxylate (4):



Coumarin acid **3** (412 mg, 2 mmol) was dissolved by 8 ml DMF and then HATU (1.14 g, 3 mmol) and DMAP (733 mg, 6 mmol) were added to the solution. After stirring for 5 min, tert-butyl piperazine-1-carboxylate (558 mg, 3 mmol) was added to the solution. The mixture was stirred at room temperature overnight, and then DMF was removed under reduced pressure. The resulted residue was purified by silica gel column chromatography to give a light yellow solid **4** (160 mg, 21%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.11 (s, 1H), 7.82 (s, 1H), 7.29 (d, J = 8.6 Hz, 1H), 6. 79 (dd, J = 8.5, 2.2 Hz, 1H), 6.74 (d, J = 2.1 Hz, 1H), 3.75 (s, 2H), 3.57-3.50 (m, 4H), 3.39 (s, 2H), 1.47 (s, 9H).

Synthesis of tert-butyl 4-(7-(2,4-dinitrophenoxy)-2-oxo-2H-chromene-3-carbonyl) piperazine-1-carboxylate (5):



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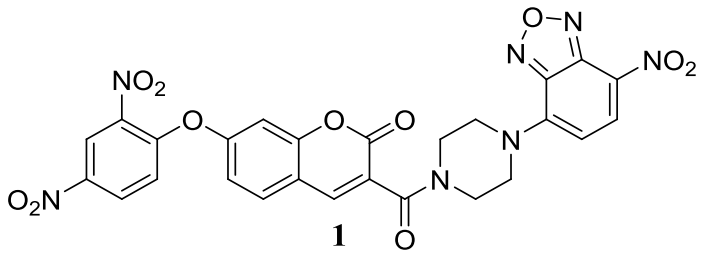
To a DCM solution (25 ml) of compound **4** (160 mg, 0.43 mmol) and 1-fluoro-2,4-dinitrobenzene (80 μl, 0.64 mmol), DIPEA was added in drop-by-drop at ice bath. The mixture was stirred at room temperature for 2 hours. After that, the reaction solution was washed with water and brine. The organic phase was dried by Na_2SO_4. Then, solvent was evaporated under reduced pressure. The resulted residue was purified by silica gel column chromatography with DCM/MeOH = 100/1 to give a yellow powder **5** (199 mg, 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.88 (d, J = 2.7 Hz, 1H), 8.45 (dd, J = 9.1, 2.7 Hz, 1H), 7.94 (s, 1H), 7.63 (d, J = 8.3 Hz, 1H), 7.28 (d, J = 4.3 Hz, 1H), 7.06 (m, 2H), 3.72 (s, 2H), 3.55–3.46 (m, 4H), 3.34 (s, 2H), 1.46 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.3,

158.1, 157.3, 155.6, 154.5, 153.6, 143.3, 142.8, 140.8, 130.8, 129.3, 124.4, 122.4, 121.4, 116.3, 116.0, 107.4, 80.5, 47.1, 42.2, 28.4.

Synthesis of probe 1:



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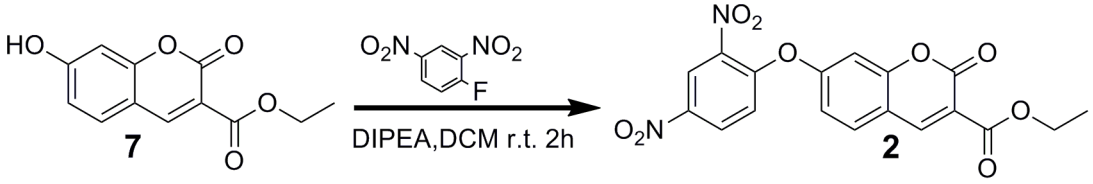
Compound **5** (140 mg, 0.26 mmol) was dissolved by a solution consisted of DCM (2 ml), TFA (2 ml) and H_2O (20 μl), and then the mixture was stirred at room temperature for 40 min. After that, the reaction solution was removed under reduced pressure to get product **6**, which was used in following synthesis directly. To a DCM solution (20 ml) of **6** and NBD-Cl (78 mg, 0.39 mmol), DIPEA was added in drop-by-drop at ice bath. The mixture was stirred at room temperature overnight. After removing the solvent under reduced pressure, the resulting residue was purified by silica gel column chromatography with DCM/MeOH = 1000/7 to get a red solid **1** (148 mg, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.92 (dd, J = 7.1, 2.7 Hz, 1H), 8.49 (dd, J = 9.1, 2.7 Hz, 1H), 8.44 (dd, J = 8.9, 4.4 Hz, 1H), 8.08 (s, 1H), 7.67 (d, J = 8.5 Hz, 1H), 7.31 (d, J = 9.1 Hz, 1H), 7.11–7.06 (m, 2H), 6.35 (d, J = 8.9 Hz, 1H), 4.27–4.17 (m, 4H), 4.06 (t, 2H), 3.72 (t, J = 4.7 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.6, 158.8, 157.6, 155.9, 153.4, 145.0, 144.9, 144.7, 144.3, 143.6, 134.9, 131.1, 129.4, 125.7, 123.7, 122.5, 122.4, 121.9, 116.3, 115.8, 107.3, 103.3, 49.3, 48.6, 46.4, 42.0. HRMS(ESI): m/z 604.1049 $[M+H]^+$ (calcd for $C_{18}H_{13}N_2O_9$, 604.1059).

Synthesis of probe 2:



To a DCM solution (20 ml) of compound **7** (117 mg, 0.5 mmol) and 1-fluoro-2,4-dinitrobenzene (129 μl, 0.75 mmol), the DIPEA was added in drop-by-drop at ice bath. The mixture was stirred at room temperature for 2 hours. After that, the reaction solution was washed with water and brine. The organic phase was dried by Na_2SO_4. And then the solvent was removed under reduced pressure. The resulting residue was subjected to column chromatography on silica with DCM/MeOH = 1000/8 to get a light yellow solid powder (160 mg, 84%). ^{1}H NMR (400 MHz, DMSO) δ 8.95 (d, J = 2.7 Hz, 1H), 8.81 (s, 1H), 8.54 (dd, J = 9.2, 2.8 Hz, 1H), 8.05 (d, J = 8.6 Hz, 1H), 7.53 (d, J = 9.2 Hz, 1H), 7.36 (d, J = 2.1 Hz, 1H), 7.28 (dd, J = 8.6, 2.3 Hz, 1H), 4.30 (q, J = 7.1 Hz, 2H), 1.31 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 162.5, 159.2, 156.1, 155.7, 152.8, 148.3, 143.0, 140.5, 132.6, 129.9, 122.3, 122.1, 116.4, 116.0, 115.3, 106.6, 61.2, 14.1. HRMS (ESI): m/z 401.0606 $[M+H]^+$ (calcd for $C_{18}H_{13}N_2O_9$, 401.0616).

3. Spectroscopic analysis of the probe

Spectroscopic measurements were performed in PBS (50 mM, pH 7.4) buffer. Compounds were dissolved into DMF to prepare the stock solutions with a concentration of 10.0 to 1.0 mM. The UV-visible spectra were recorded on a UV-3600 UV-VIS-NIR-spectrophotometer (SHIMADZU, Japan). Fluorescence study was carried out using F-

7000 fluorescence spectrophotometer (HITACHI, Japan). All reaction mixture was shaken uniformly before emission spectra measurement.

The detection limit was calculated with the following equation:

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

Where σ is the standard deviation of fluorescence intensity of **1**; k is the slop between the fluorescence intensity versus H_2S concentration (k = 1.338, Fig. S5). The fluorescence emission spectrum of probe **1** was measured by six times and the standard deviation of blank measurement was found to be 0.127. The detection limit is 0.28 μM.

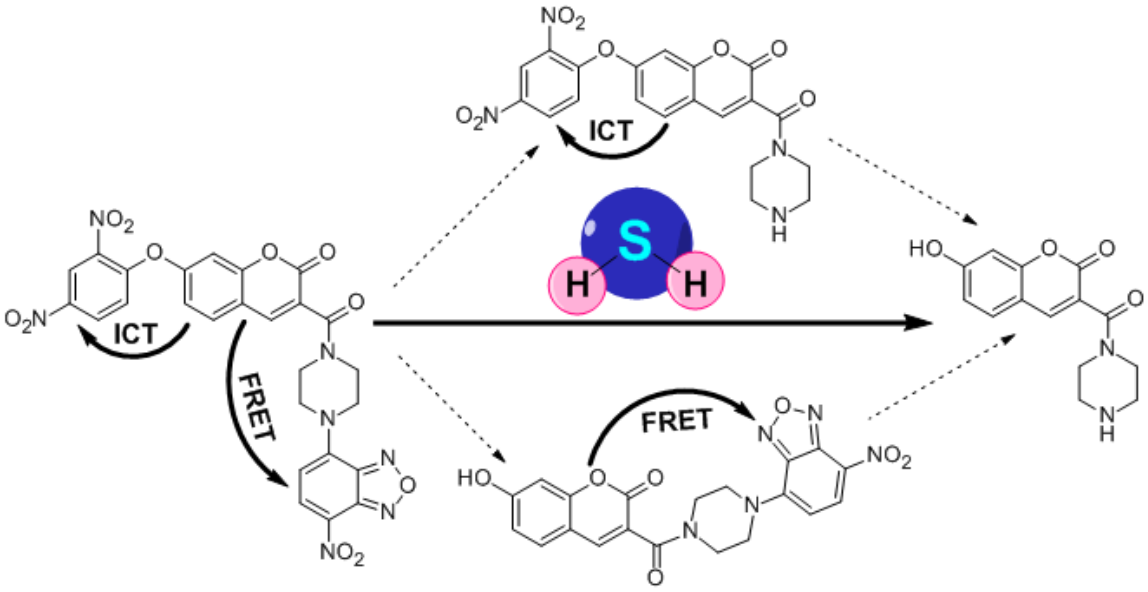
4. MTT assay

HEK-293 cells were cultured at 37 ℃, 5% CO_2 in DMEM/HIGH GLUCOSE (GIBICO) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 4 mM L-glutamine. The cells were maintained in exponential growth, then seeded in 96 well plate at the density about 5000/well. There was also a background group with only medium in each well but no cells. When the confluence reached 90%, cells were treated with probe **1** at the final concentration from 0.5 μM to 16 μM, control groups were only treated with the same amount of DMSO. After incubation at 37 ℃, 5% CO_2 for 16 h, each well of cells were treated with 20 μl MTT solution (5 mg/ml), and incubated for another 4 h. Then each well was treated with 150 μl DMSO at 37 ℃ for 10 min, and then the OD_{470} for each well were recorded on Safire 2. Cell viability was calculated by using the formula: (experiment group - background) / (blank group - background)*100%.

5. Fluorescence microscope experiments

HEK293A and HeLa cells were cultured as description in the above part. The cells were maintained in exponential growth, and then seeded in glass-bottom 35 mm plate at the density about 2×10^4 / well. Cells were passaged every 2-3 days and used between passages 3 and 10. For exogenous H_2S imaging, cells were first treated with **1** (2 μM) at 37 ℃ for 30 min and then incubated with Na_2S (50 and 200 μM) for another 30 min. Control cells were only treated with probe at 37 ℃ for 30 min. Cells were imaged on a confocal microscope (Olympus FV1000 UPLSAPO40X) with a 40 × objective lens. Emission was collected at blue channel (425-475 nm) with 405 nm excitation. All images were analyzed with Olympus FV1000-ASW. For endogenous H_2S imaging, cells were incubated with thiols (L-Cysteine, 200 μM) for 30 min at 37 ℃ and 5% CO_2, PBS washed, and then incubated with **1** (2 μM) for another 30 min. The media was replaced by PBS and cells were imaged immediately after media exchange.

5. Supplementary figures



Scheme S2. Reaction mechanism of probe **1** toward thiol H_2S.

Fig. S1. Photographs of probe **1** (10 μM) before and after reacting with Na_2S (1mM) for 1 h under UV lamp (365 nm).

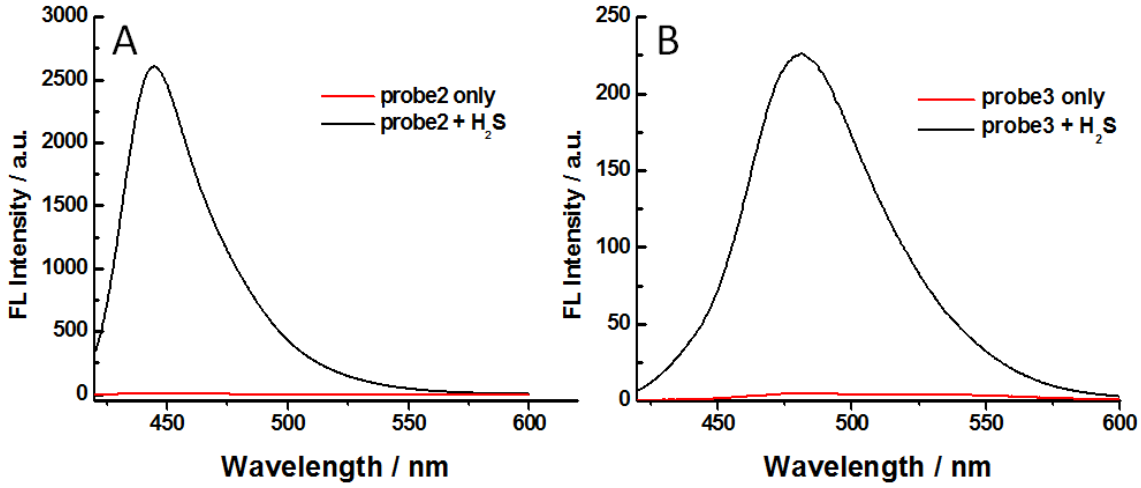


Fig. S2. Fluorescence response of 1 μM **2** (A) and 1 μM **3** (B) toward H_2S (100 μM) overnight. Slits: 2.5/5 nm. The *off-on* response for **2** and **3** are 340 and 48.5 folds, respectively. Slits: 2.5/5 nm.

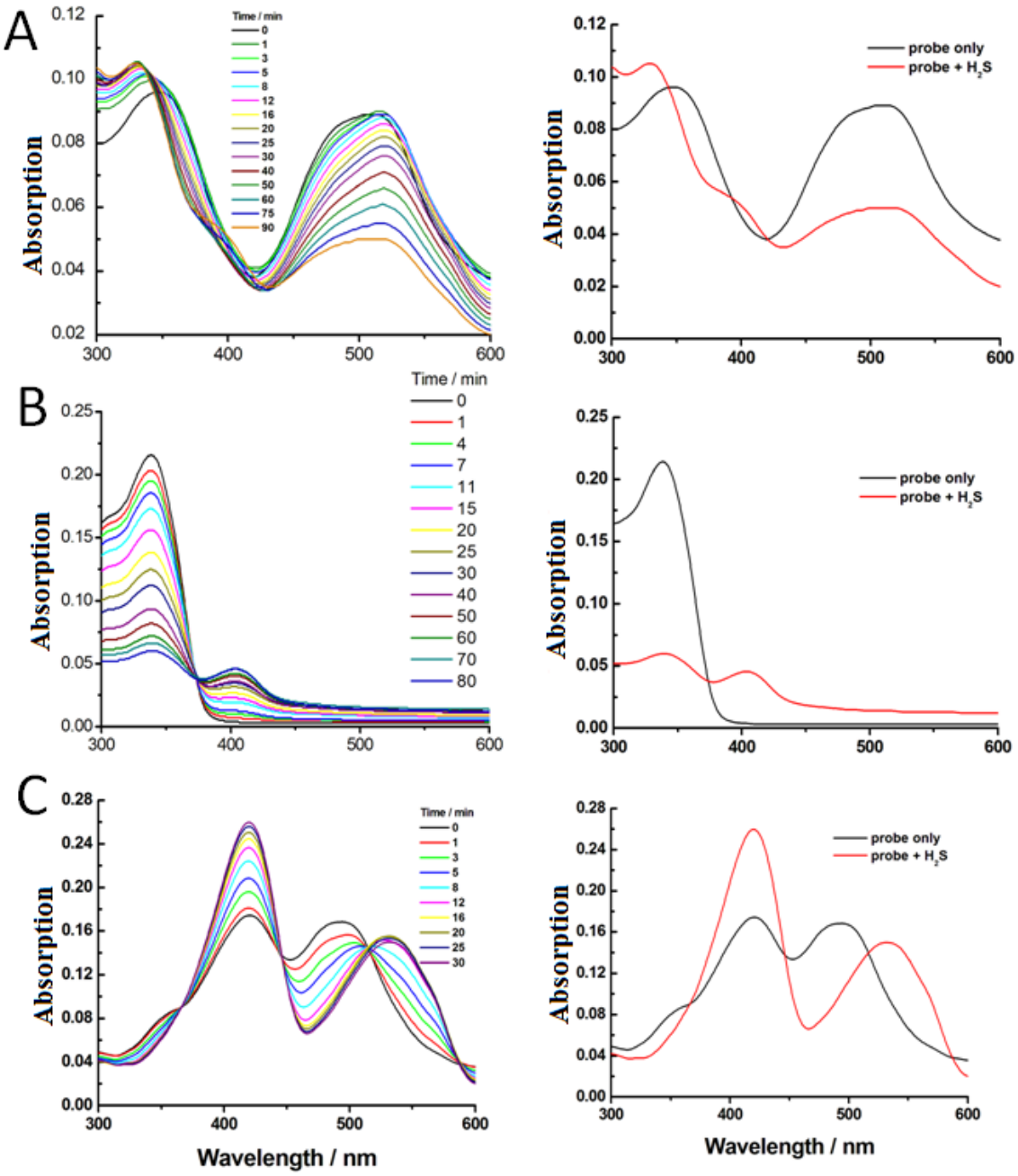


Fig. S3. The time-dependent absorption spectra of 10 μM probes **1-3** for (A-C), respectively, upon treatment with Na_2S (1 mM) in PBS buffer containing 10% DMSO.

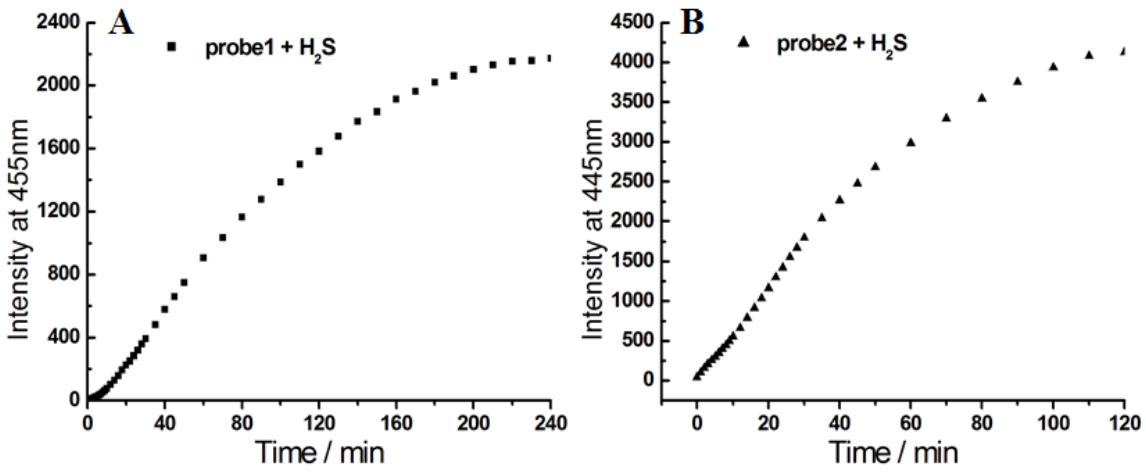


Fig. S4. Time-course experiments of 1 μM **1** (A) or **2** (B) with H_2S (500 μM) at 25 ℃ in PBS buffer (50 mM, pH 7.4). Slits: 2.5/5 nm.

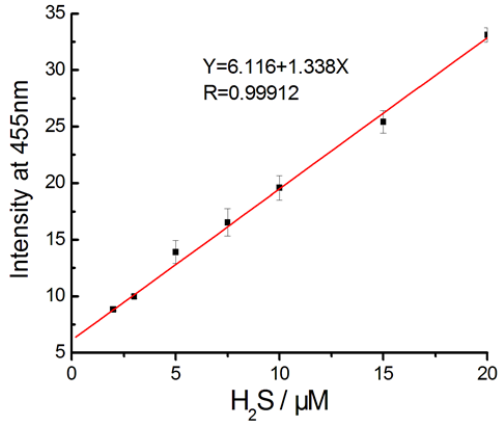


Fig. S5. Linear relationship between fluorescence intensity of 1 μM **1** in the presence of different concentrations of H_2S at 25 ℃ in PBS buffer (50 mM, pH 7.4) after 30 min incubation. Slits: 2.5/5 nm. The detection limit was calculated as 0.28 μM.

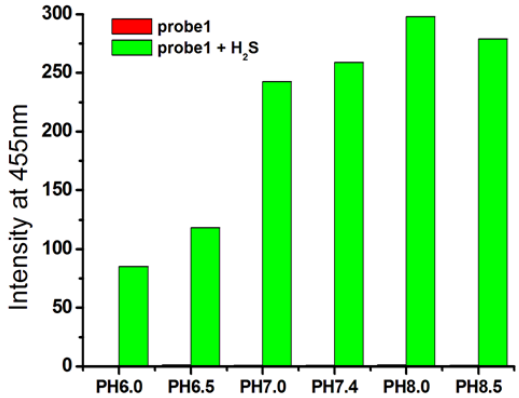


Fig. S6. Emission intensity of pH-dependent experiments for probe **1**. The reaction of probe **1 (**1 μM**)** and H_2S (200 μM) was performed with PBS buffers at different pH values at 37 ℃ for 1 h.

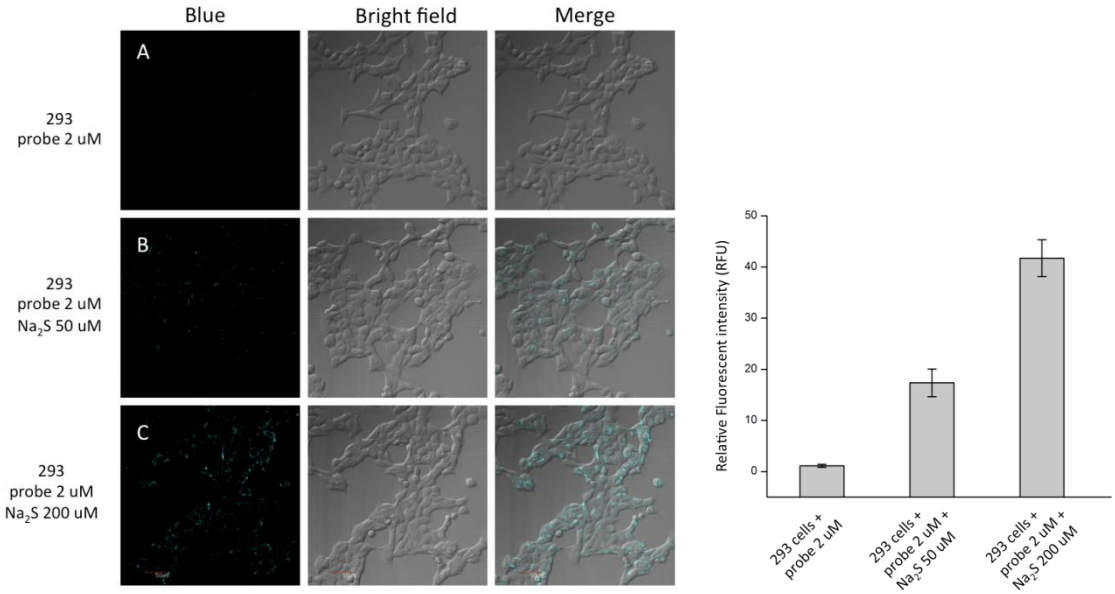


Fig. S7. Visualization of exogenous H_2S with probe **1** in HEK293A cells using laser scanning confocal microscopy images. HEK293A cells with (A) **1** (2 μM), (B) **1** (2 μM) and then Na_2S (50 μM), (C) **1** (2 μM) and then Na_2S (200 μM). Scale bar, 50 μm.

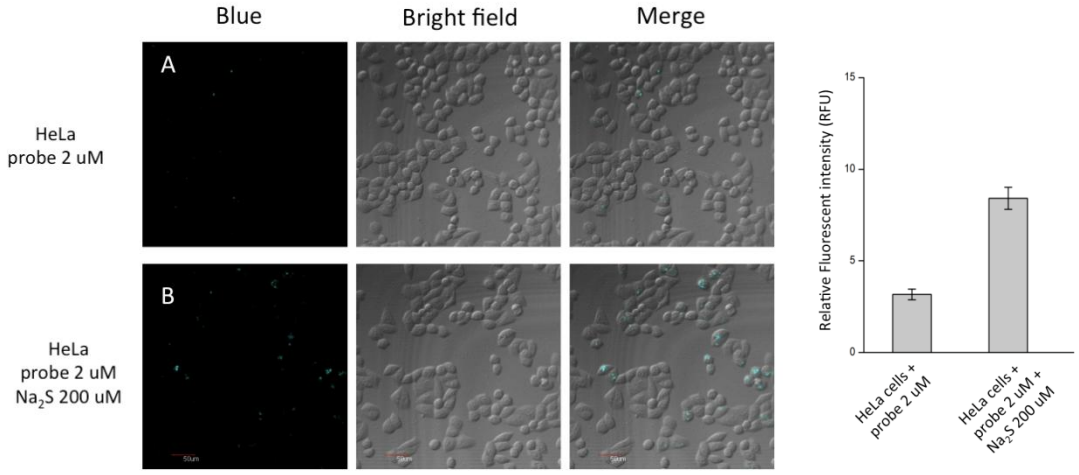


Fig. S8. Visualization of exogenous H_2S with probe **1** in HeLa cells using laser scanning confocal microscopy images. HeLa cells with (A) **1** (2 μM), (B) **1** (2 μM) and then Na_2S (200 μM).

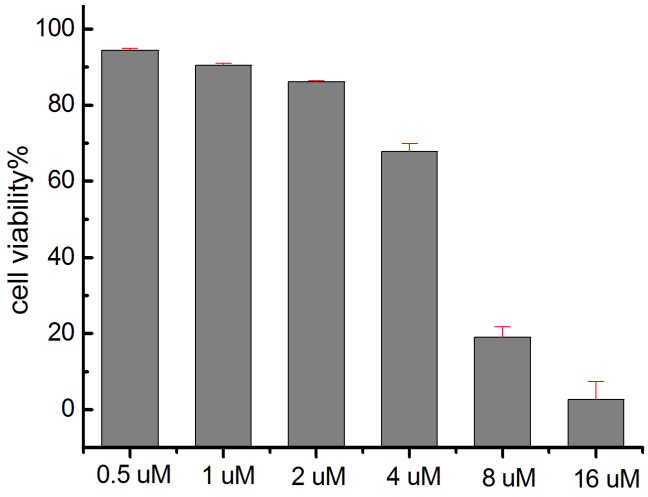


Fig. S9. The cytotoxicity of the probe **1** evaluated by the MTT assay.

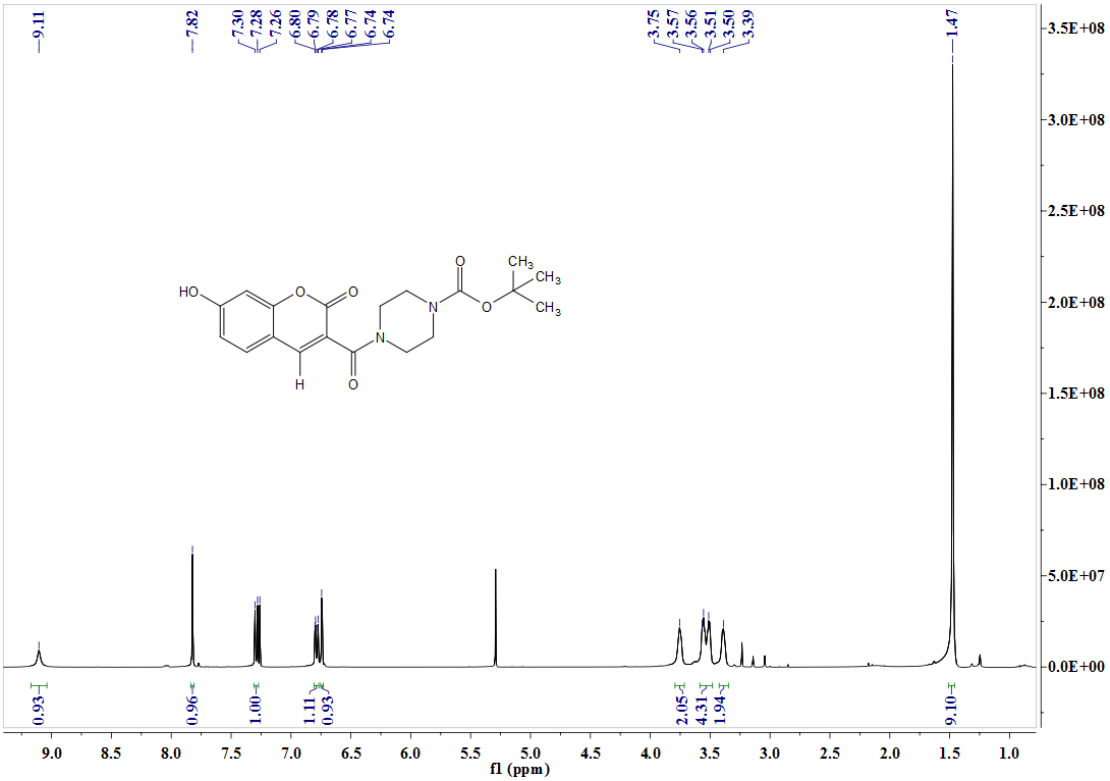


Fig. S10. ^{1}H-NMR of compound **4**.

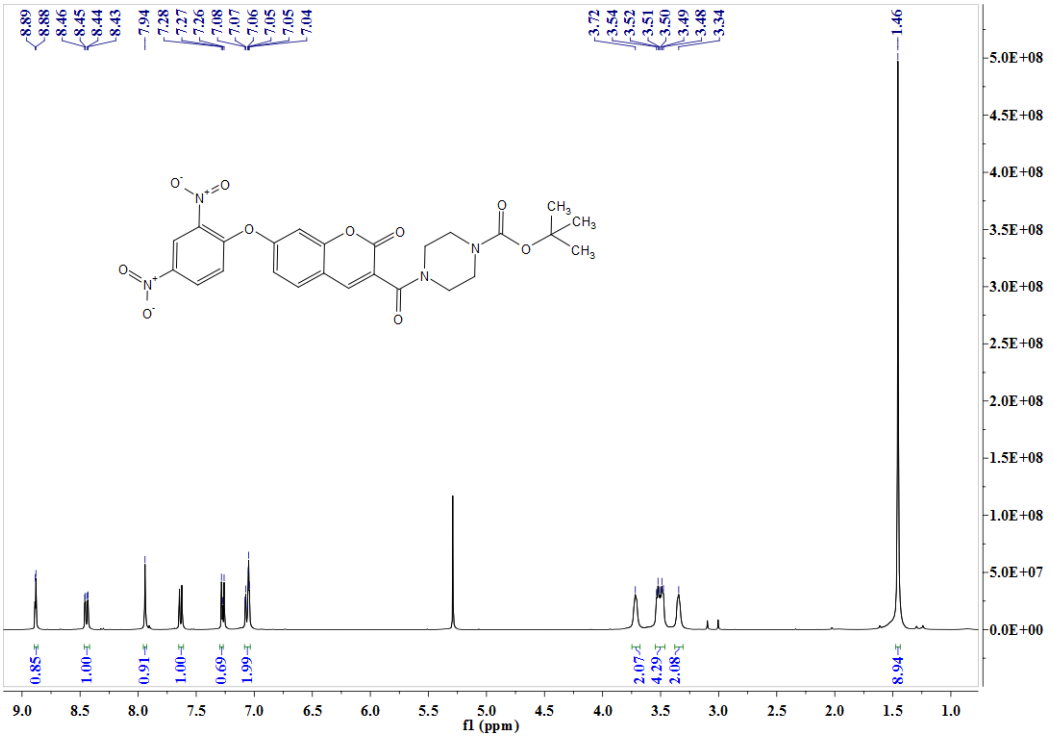


Fig. S11. ^{1}H-NMR of compound **5**.

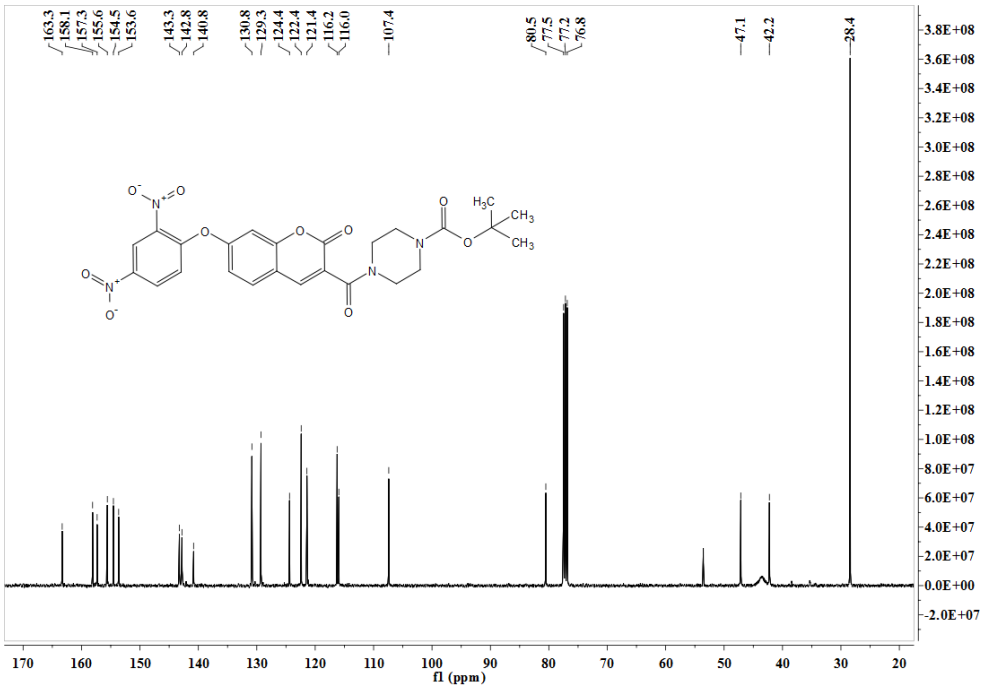


Fig. S12. ^{13}C-NMR of compound **5**.

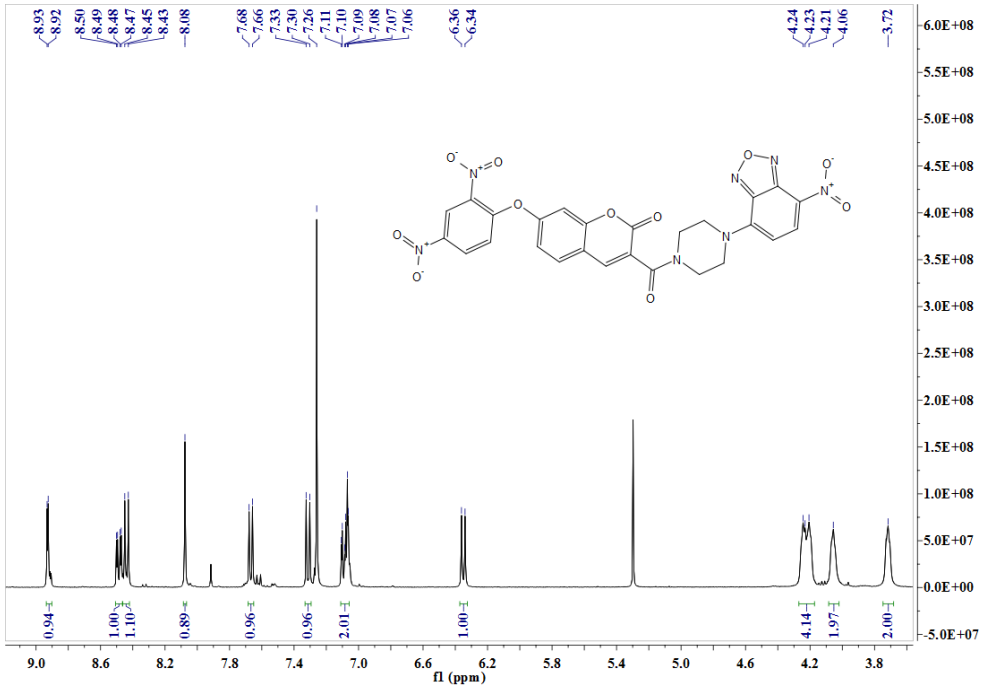


Fig. S13. ^{1}H-NMR of compound **1**.

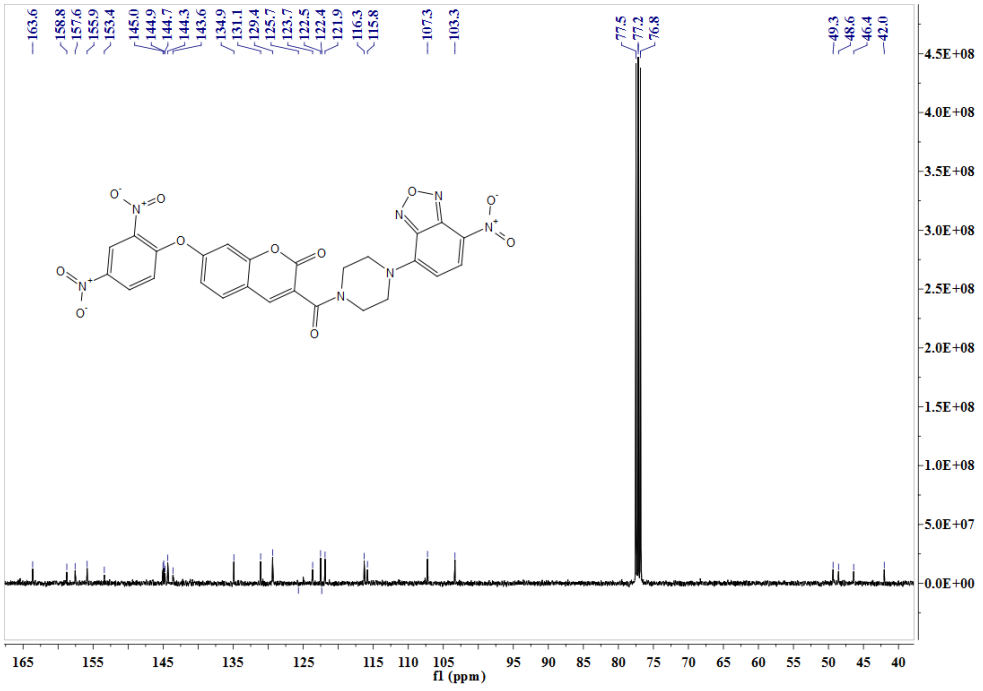


Fig. S14. ^{13}C-NMR of compound **1**.

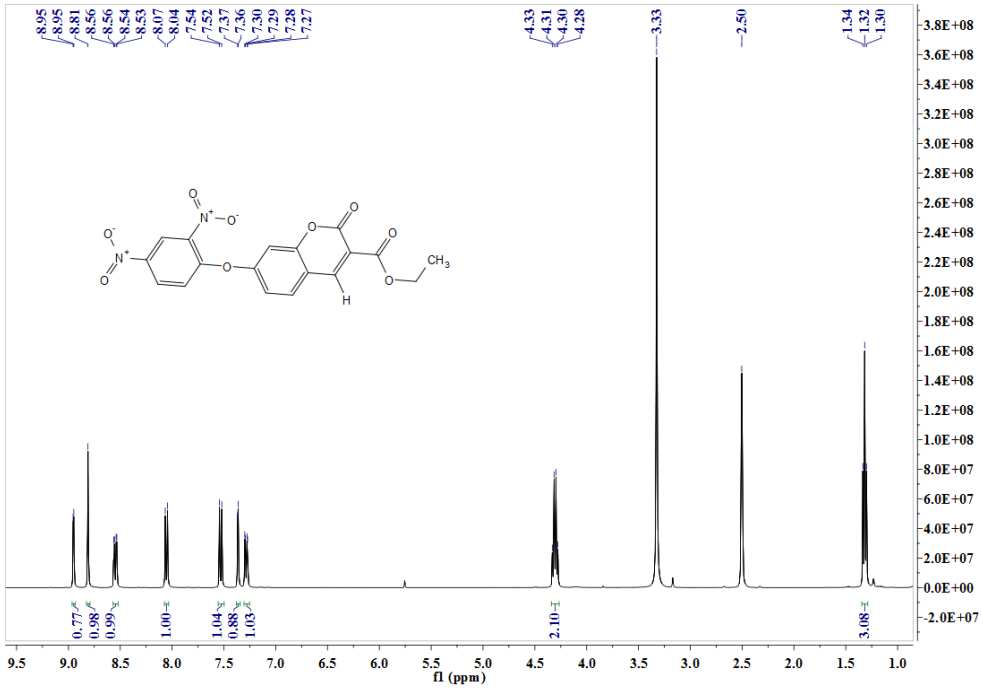


Fig. S15. ^{1}H-NMR of compound **2**.

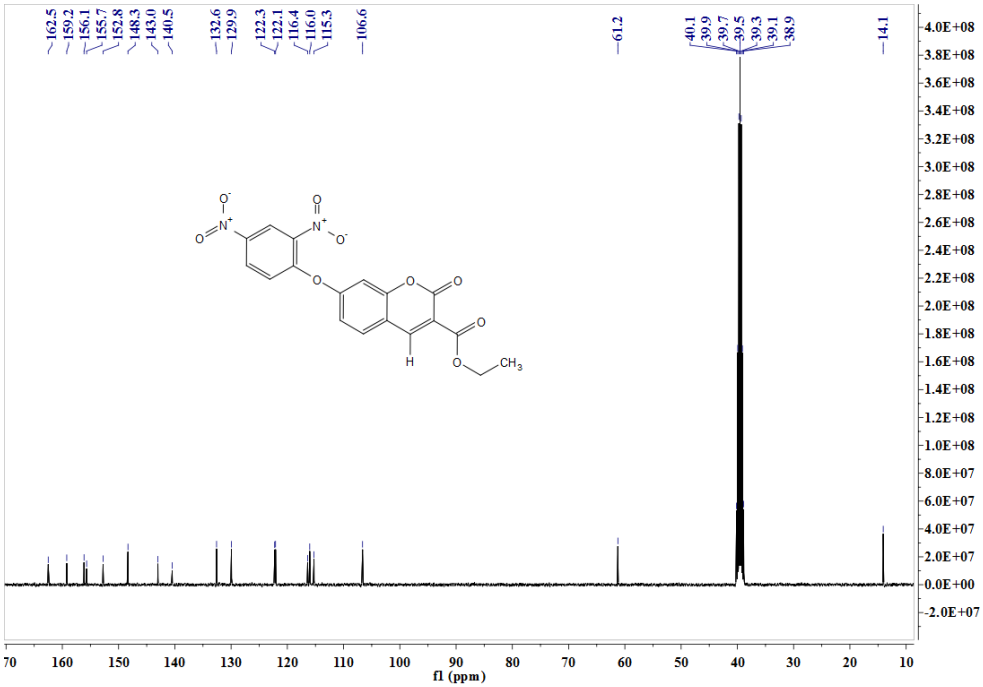


Fig. S16. ^{13}C-NMR of compound **2**.

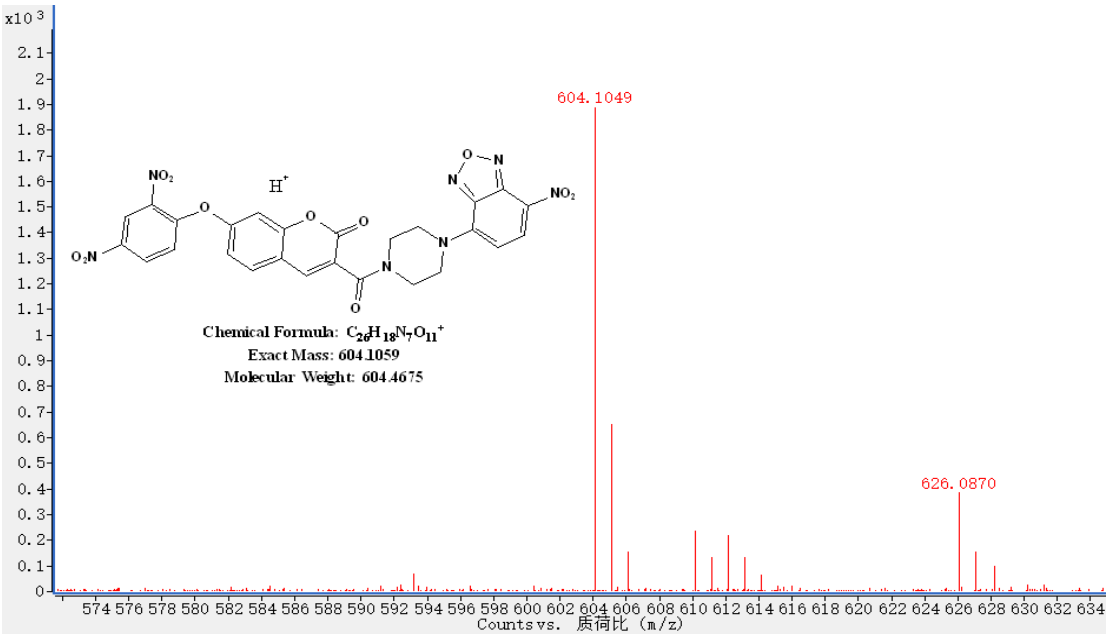


Fig. S17. HRMS of compound **1**.

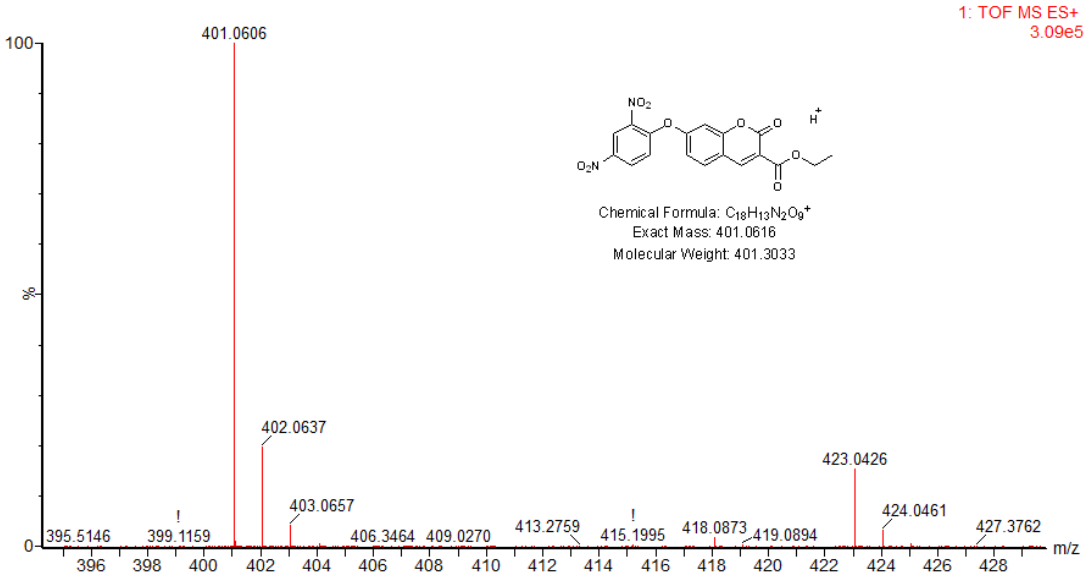


Fig. S18. HRMS of compound **2**.