Supporting Information

Rational design of a dual-reactive probe for imaging the biogenesis of both H_2S and GSH from L-Cys rather than D-Cys in live cells

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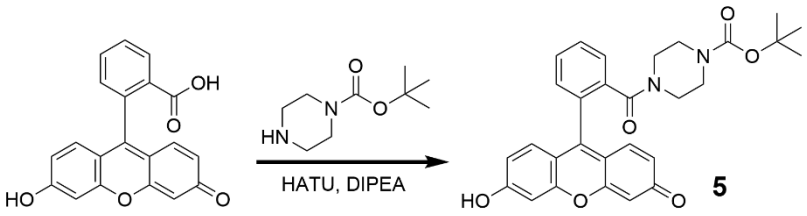
1. Reagents and instruments

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 UV light. Merck silica gel 60 (70-200 mesh) was used for general column chromatography purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 spectrometer. Chemical shifts are reported in parts per million relative to internal standard tetramethylsilane ($Si(CH_3)_4$ = 0.00 ppm) or residual solvent peaks (DMSO-d_6 = 2.50 ppm). ^{1}H NMR coupling constants (J) are reported in Hertz (Hz), and multiplicity is indicated as the following: s (singlet), d (doublet), m (multiplet). High-resolution mass spectrum (HRMS) was obtained on an Agilent 6540 UHD Accurate-Mass Q-TOFLC/MS. The UV-visible spectra were recorded on a UV-6000 UV-VIS-NIR-spectrophotometer (METASH, China). Fluorescence studies were carried out using F-280 spectrophotometer (Tianjin Gangdong Sci & Tech., Development. Co., Ltd, China).

2. Synthetic procedure of probes

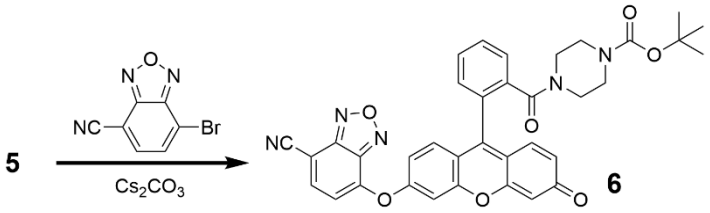
Synthesis of 5



Fluorescein (166 mg, 0.50 mmol) was dissolved in anhydrous DMF (10 mL), and then HATU (570 mg, 1.5 mmol) and DIPEA (524 μL, 3.0 mmol) were added into the DMF solution. After stirring for 10 min, 1-boc-piperazine (112 mg, 0.60 mmol) was added. The mixture was stirred at room temperature overnight, and then DMF was removed under reduced pressure. The residue was purified by silica gel column chromatography to give **5** (150 mg, 60%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.73–7.67 (m, 2H), 7.66–7.58 (m, 1H), 7.53–7.45 (m, 1H), 6.95 (d, J = 9.2 Hz, 2H), 6.84–

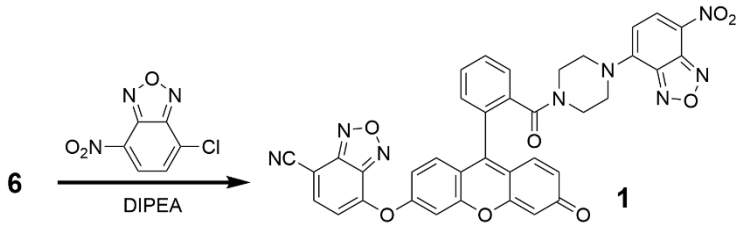
6.42 (m, 4H), 3.27–3.21 (m, 4H), 3.17–3.06 (m, 4H), 1.37 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.8, 153.6, 148.3, 143.6, 135.4, 131.1, 130.9, 130.4, 129.6, 129.3, 127.3, 79.3, 48.6, 46.8, 41.0, 28.0. HRMS (ESI): m/z $[M+H]^+$ calculated for $C_{29}H_{29}N_2O_6^+$: 501.2020; found: 501.2021.

Synthesis of 6



CBD-Br was synthesized by a similar method according to the reported literature.[1] Compound **5** (135 mg, 0.27 mmol) and CBD-Br (90 mg, 0.41 mmol) were dissolved in anhydrous DMF (5 mL), and then Cs_2CO_3 (176 mg, 0.54 mmol) was added to the solution. The resulted mixture was stirred at 70 ℃ for 12 h. The solvent was removed under reduced pressure, and the residue was purified by column chromatography to give a yellow solid **6** (100 mg, 56%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.35 (d, J = 6.8 Hz, 1H), 7.77–7.63 (m, 3H), 7.62–7.50 (m, 2H), 7.30 (d, J = 7.0 Hz, 1H), 7.24–7.16 (m, 2H), 7.01 (d, J = 9.2 Hz, 1H), 6.46 (d, J = 9.2 Hz, 1H), 6.19 (s, 1H), 3.37–3.18 (m, 4H), 3.18–3.08 (m, 4H), 1.37 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 184.7, 167.2, 158.2, 158.1, 154.1, 153.1, 150.2, 149.0, 147.2, 144.9, 143.8, 135.9, 131.7, 131.3, 130.9, 130.4, 130.0, 127.9, 120.0, 118.8, 116.8, 116.0, 114.8, 108.0, 105.6, 95.1, 79.7, 47.2, 41.5, 28.4. HRMS (ESI): m/z $[M+H]^+$ calculated for $C_{36}H_{30}N_5O_7^+$: 644.2140; found: 644.2147.

Synthesis of probe 1



To a solution of **6** (64 mg, 0.10 mmol) in CH_2Cl_2 (2 mL) was added trifluoroacetic acid (TFA, 1 mL), and then the mixture was stirred for 2 h at room temperature. The

solvent and TFA were removed under reduced pressure. The residue was redissolved in anhydrous DMF (5 mL), and NBD-Cl (40 mg, 0.20 mmol) and DIPEA (87 μL, 0.50 mmol) were added into the solution. After stirring overnight, the solvent was removed under reduced pressure, and the residue was subjected to silica gel column chromatography to give red solid **1** (45 mg, 64%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.46 (d, J = 9.0 Hz, 1H), 8.31 (d, J = 7.7 Hz, 1H), 7.83–7.72 (m, 3H), 7.67–7.53 (m, 2H), 7.33–7.15 (m, 3H), 7.06 (d, J = 9.7 Hz, 1H), 6.53 (d, J = 9.1 Hz, 1H), 6.45 (d, J = 9.7 Hz, 1H), 6.19 (s, 1H), 4.15–3.92 (m, 4H), 3.81–3.69 (m, 2H), 3.69–3.53 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 184.2, 166.9, 157.8, 157.6, 152.7, 149.7, 148.5, 146.8, 145.2, 144.7, 144.4, 143.4, 136.2, 135.2, 131.2, 130.7, 130.1, 129.6, 127.6, 121.5, 119.6, 118.4, 116.4, 115.5, 114.3, 107.6, 105.1, 103.4, 94.6, 49.1, 48.1, 45.7, 41.0. HRMS (ESI): m/z $[M+H]^+$ calculated for $C_{37}H_{23}N_8O_8^+$: 707.1633; found: 707.1639.

3. Solubility Tests

The water solubility of probe **1** was checked by concentration-dependent absorbance spectra of the probe in phosphate buffer (PBS, 50 mM, pH = 7.4). For all cases, the final concentration of DMSO in buffer was maintained to be 0.5%. The plots of absorbance intensities at 360 nm against the dye concentrations were linear at low concentrations and showed downward curvature at higher concentrations. The maximum concentration in the linear region was considered as the solubility.[2]

4. Spectroscopic studies

All measurements were performed in degassed PBS (50 mM, pH 7.4, containing 10% DMSO). The probe was dissolved into DMSO to prepare the stock solutions with concentrations of 1-10 mM. Various stock solutions (100 mM) of different analytes were prepared in PBS buffer. Different bio-relevant species were added to separate portions of the probe solution and mixed thoroughly before spectra tests. All

measurements were performed in a 3 mL sealed corvette with 2 mL solution at room temperature, and all fluorescence spectra were obtained by excitation at 469 nm. For the selectivity experiments, fluorescence spectra of probe **1** (1 μM) toward different species in the presence of H_2S (100 μM) or GSH (5 mM) in PBS buffer were monitored. All tested species were 100 μM, and the reactions were incubated for 1 h at room temperature.

5. Quantum yields

The quantum yields of probe **1** and its dual-reactive product **4** were measured in PBS (50 mM, pH = 7.4, including 2% DMSO) with fluorescein in 0.1 M NaOH solution as the standard ($\Phi = 0.925$).[3] The excitation wavelength was at 470 nm, and the emission range was 500-600 nm. The quantum yield was calculated using the following equation: $\Phi = \Phi_S \times (F/F_S) \times (A_S/A) \times (n^2/n_S^2)$, where Φ is the 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. Subscript S refers to the standard.

6. HPLC measurements

For the HPLC analysis, a mixture of probe **1** (0.2 mM), H_2S (1 mM) and GSH (5 mM) in PBS (50 mM, pH = 7.4, 30% DMSO) at 37 ℃ was analyzed by HPLC at different reaction times. Conditions: ANGELA TECHNOLOGIES HPLC LC-10F; C_{18} column with 4.6 mm x 250 mm; detection wavelength: 500 nm. Buffer A: 0.1% (v/v) trifluoroacetic acid in water; buffer B: acetonitrile; flow: 1 mL/min. The elution conditions were: 0-3 min, buffer B: 5-40%; 3-23 min, buffer B: 40-90%; 23-25 min, buffer B: 90-5%.

7. Cell culture and MTT assay

HeLa (human cervical cancer cells) cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 medium with 10% fetal bovine serum and 1% penicillin/streptomycin under standard cell culture conditions at 37 °C in a humidified CO_2 incubator. The cytotoxicity of probe **1** was determined via a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay by using the HeLa cells. Briefly, the HeLa cells were transferred to the 96-well plate and cultured for 24 h before tests. After that, the culture medium was replaced with a fresh one and the cells were incubated with different concentrations of probe **1** (0, 5, 10, 20, 30, 40 and 50 μM) for 24 h. Then, 5 mg/mL MTT in PBS (20 μL) was added to each well and incubated for another 4 h. Finally, the medium was replaced with 150 μL of DMSO to dissolve the purple formazan crystals. The absorbance intensity in each well was detected at 490 nm by a microplate spectrophotometer (SpectraMax M2E (Molecular Device, Inc.)).

8. Cellular imaging

The feasibility of probe **1** to detect intracellular H_2S and GSH was evaluated by confocal fluorescence imaging. Briefly, glass bottom dishes were added into a 12-well plate before cells were seeded. Then, the HeLa cells were transferred to the 12-well plate and cultured for 12 h before experiments. After that, the culture medium was replaced with the fresh one and the cells were treated with the tested reagents before fluorescence imaging. For bioimaging in the presence of exogenous H_2S and endogenous GSH, HeLa cells were pre-incubated with 100 μM Na_2S for 60 min, then washed and incubated with 10 μM probe **1** for another 60 min. For bioimaging in the presence of L-Cys or D-Cys, cells were pre-treated with NEM (1 mM) for 30 min and washed, and then co-incubated with 500 μM D-Cys or L-Cys and 10 μM probe **1** for another 60 min. For the control bioimaging, cells were pre-treated with NEM (1 mM) for 30 min and washed, and then incubated with the H_2S-biogenesis inhibitor[4] AOAA

(200 μM) for 30 min followed by with 10 μM probe **1** and 500 μM L-Cys for 60 min. After the incubation, all the cells were quickly washed with PBS, and then fixed with 4% paraformaldehyde solution for 10 min. After that, the cells were further washed with PBS and treated with DAPI (2 μg/mL) for 10 min. Finally, the cells were washed using PBS and imaged using a confocal microscope (Olympus FV1000) with a 40 × objective lens. The emission of the probe was collected at the green channel (500-550 nm) with 488 nm excitation. DAPI stained cells were observed through the blue channel (450-500 nm) with 405 nm excitation.

9. References

1. A. M. Thooft, K. Cassaidy and B. VanVeller, *J. Org. Chem.*, 2017, **82**, 8842-8847.

2. S. K. Bae, C. H. Heo, D. J. Choi, D. Sen, E.-H. Joe, B. R. Cho and H. M. Kim, *J. Am. Chem. Soc.*, 2013, **135**, 9915-9923.

3. L. Qiao, H. Nie, Y. Wu, F. Xin, C. Gao, J. Jing and X. Zhang, *J. Mater. Chem. B*, 2017, **5**, 525-530.

4. M. R. Filipovic, J. Zivanovic, B. Alvarez and R. Banerjee, *Chem. Rev.*, 2018, **118**, 1253-1337.

10. Supplementary figures

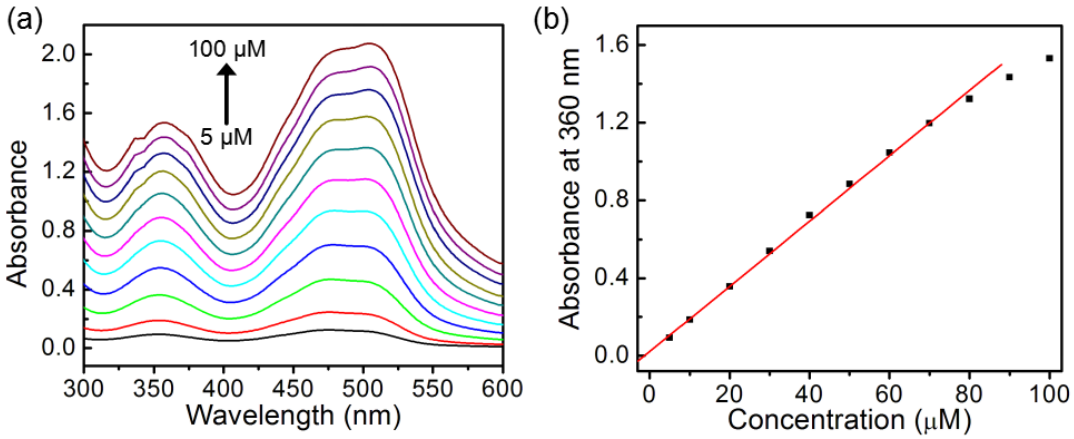


Fig. S1 (a) The UV-vis spectra of probe **1** at different concentrations in PBS buffer (50 mM, pH = 7.4, containing 0.5% DMSO). (b) The linear relationship between absorbance at 360 nm and the concentration of probe **1**.

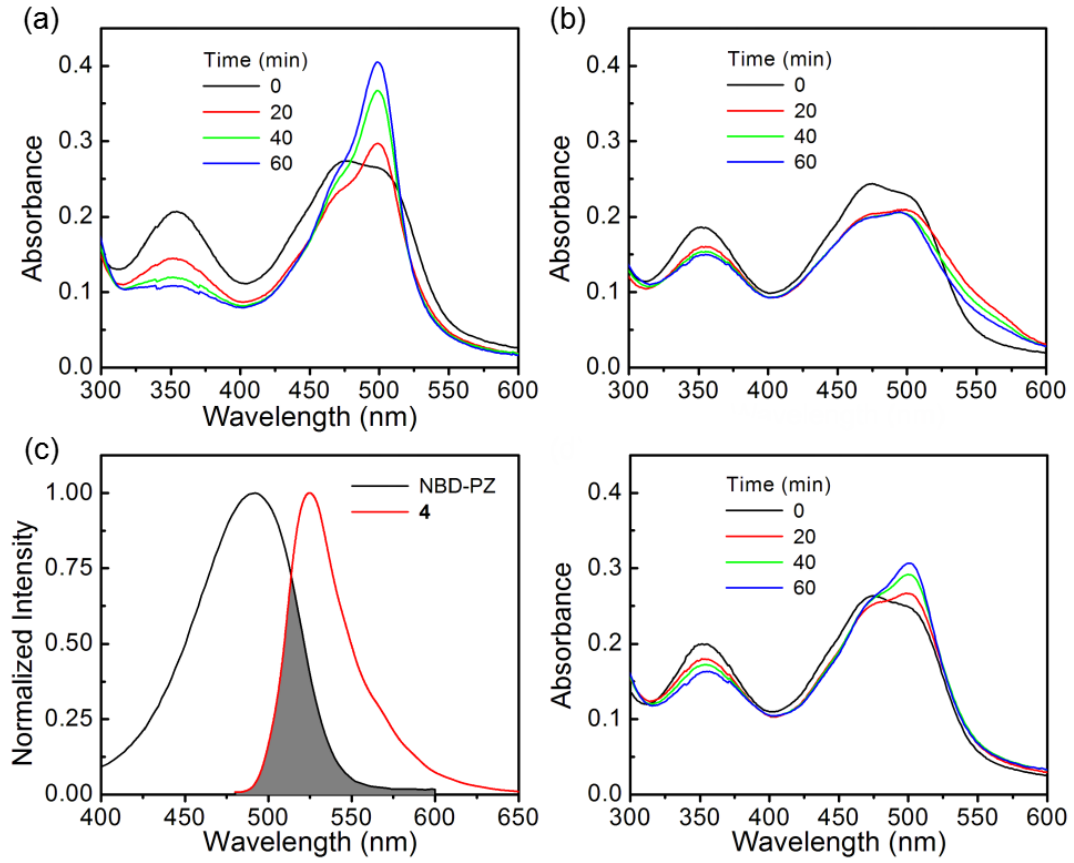


Fig. S2 Time-dependent spectra of probe **1** (10 μM) in the presence of H_2S (1 mM) and/or GSH (10 mM) in PBS buffer (pH 7.4, containing 10% DMSO). (a) Time-dependent absorbance spectra of **1** with both H_2S and GSH. (b) Time-dependent absorbance spectra of **1** with H_2S alone. (c) Normalized absorption spectrum of NBD-PZ and the emission of **4**. The shaded area represents the overlap of two curves. (d) Time-dependent absorbance spectra of **1** with GSH alone.

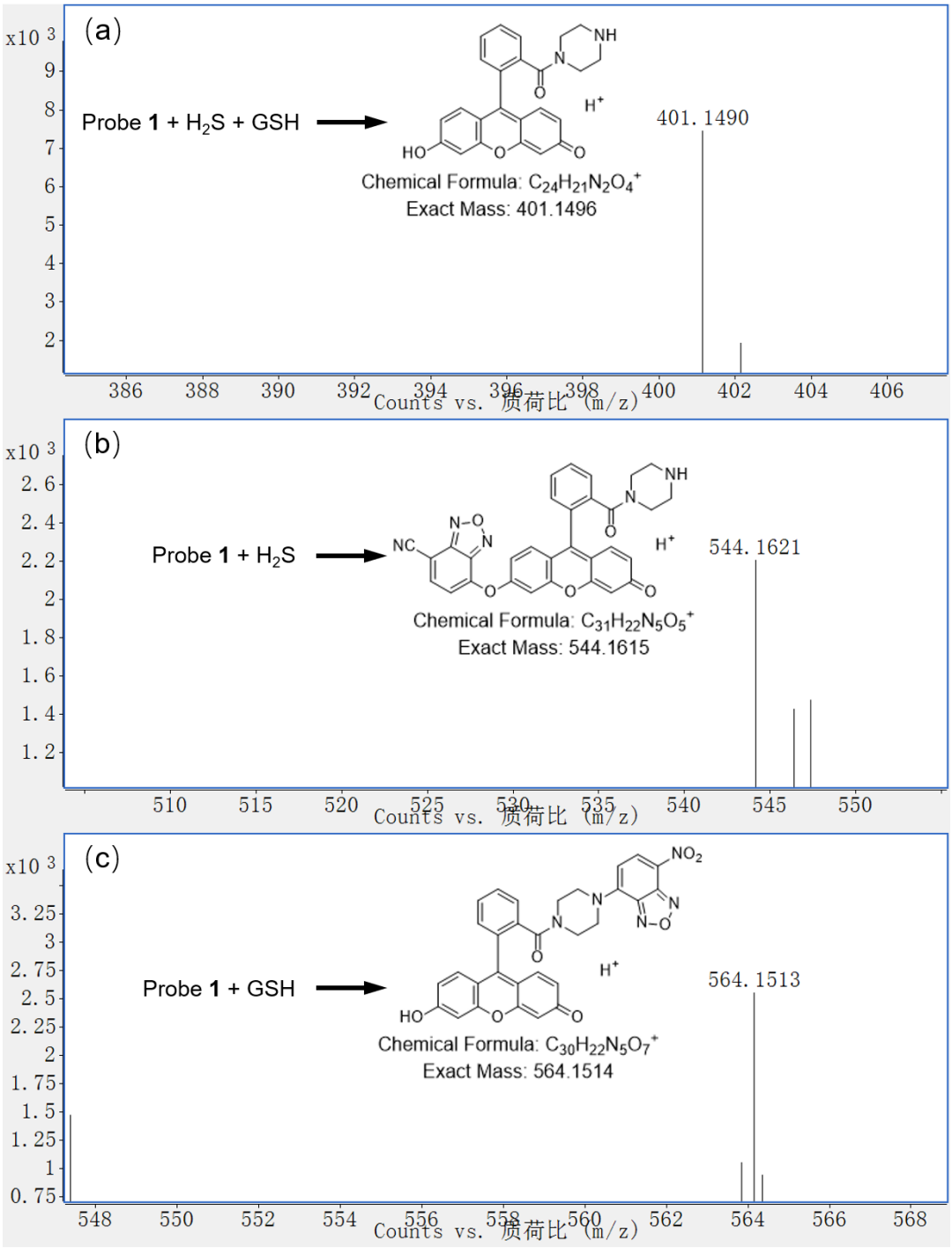


Fig. S3 HRMS spectra of the reactions of probe **1** incubated with H_2S and GSH simultaneously (a) or separately (b, H_2S; c, GSH) in PBS buffer (50 mM, pH 7.4 containing 30% DMSO).

Reaction time (min)	Turn-on fold of probe **1**		
	H_2S + GSH	H_2S	GSH
10 min	64.7	5.5	7.9
30 min	252	15.1	23.0
60 min	412	28.6	43.6

Table S1. Time-dependent fluorescent turn-on folds of probe **1** (1 μM) toward H_2S (100 μM) and/or GSH (2 mM) from Figure 1.

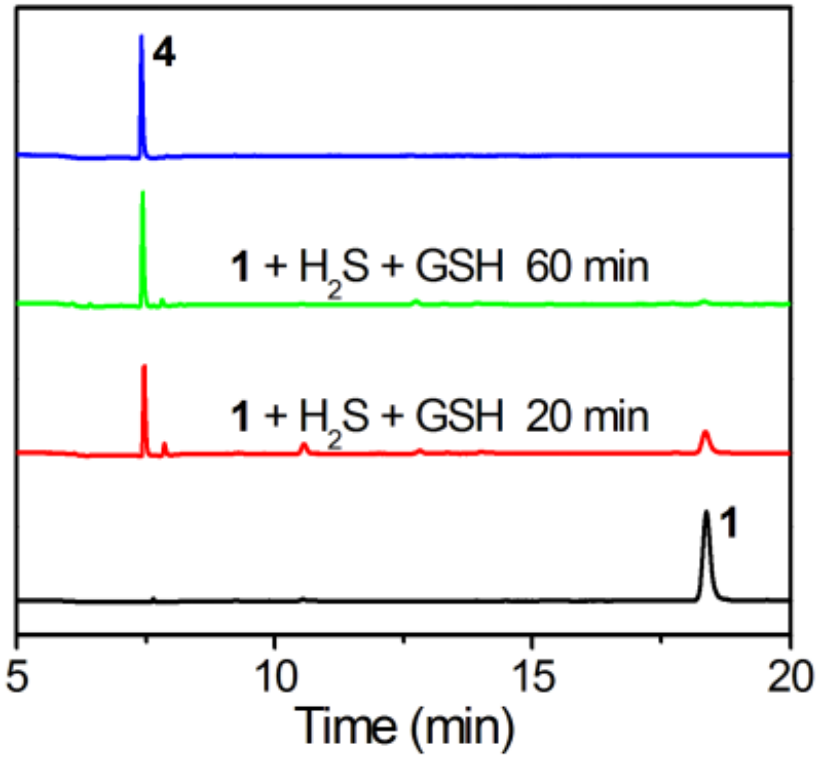


Fig. S4 Time-dependent HPLC analyses of probe **1** (0.2 mM) in the presence of H_2S (1 mM) and GSH (5 mM) in PBS buffer (50 mM, pH 7.4, containing 30% DMSO) at 37 ℃. The incubation times were indicated inset for different traces. Detection wavelength: 500 nm.

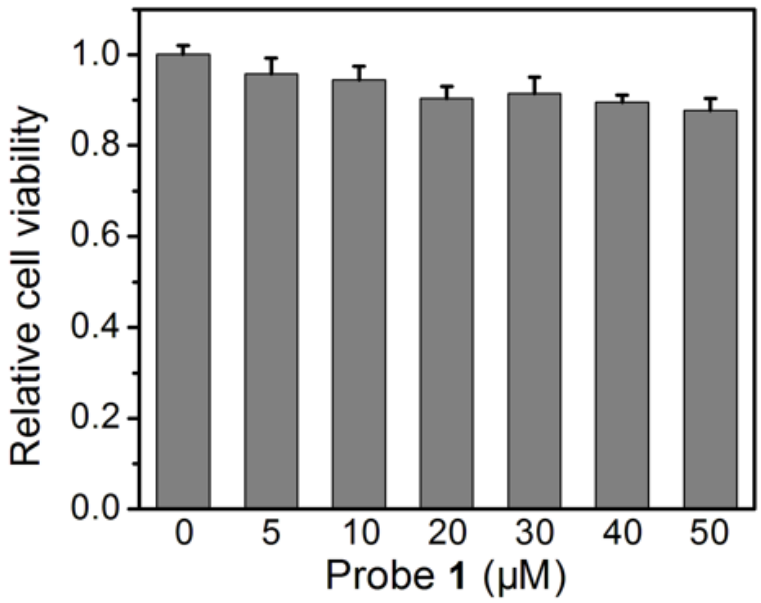
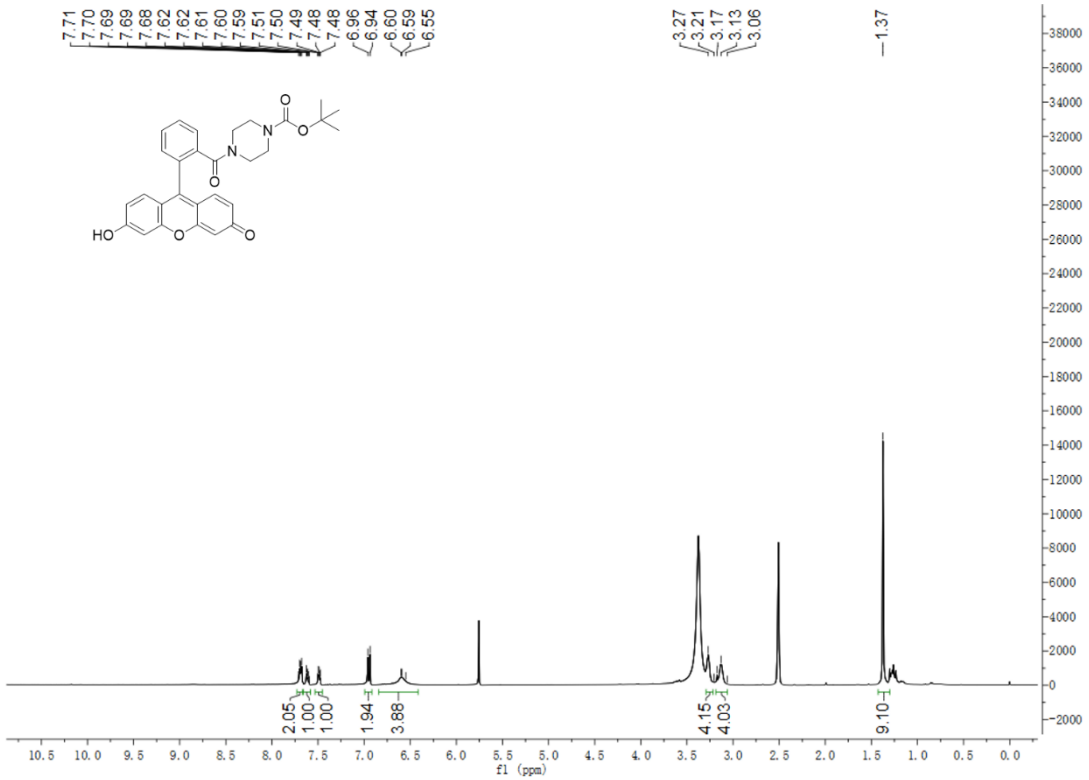
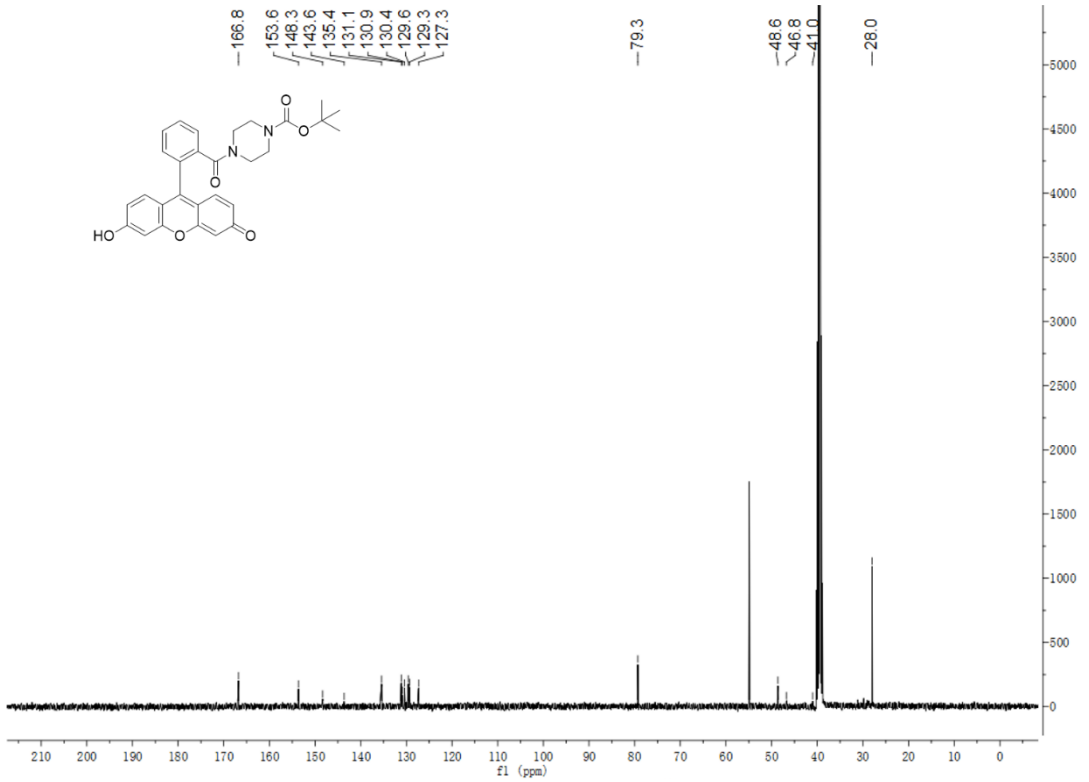
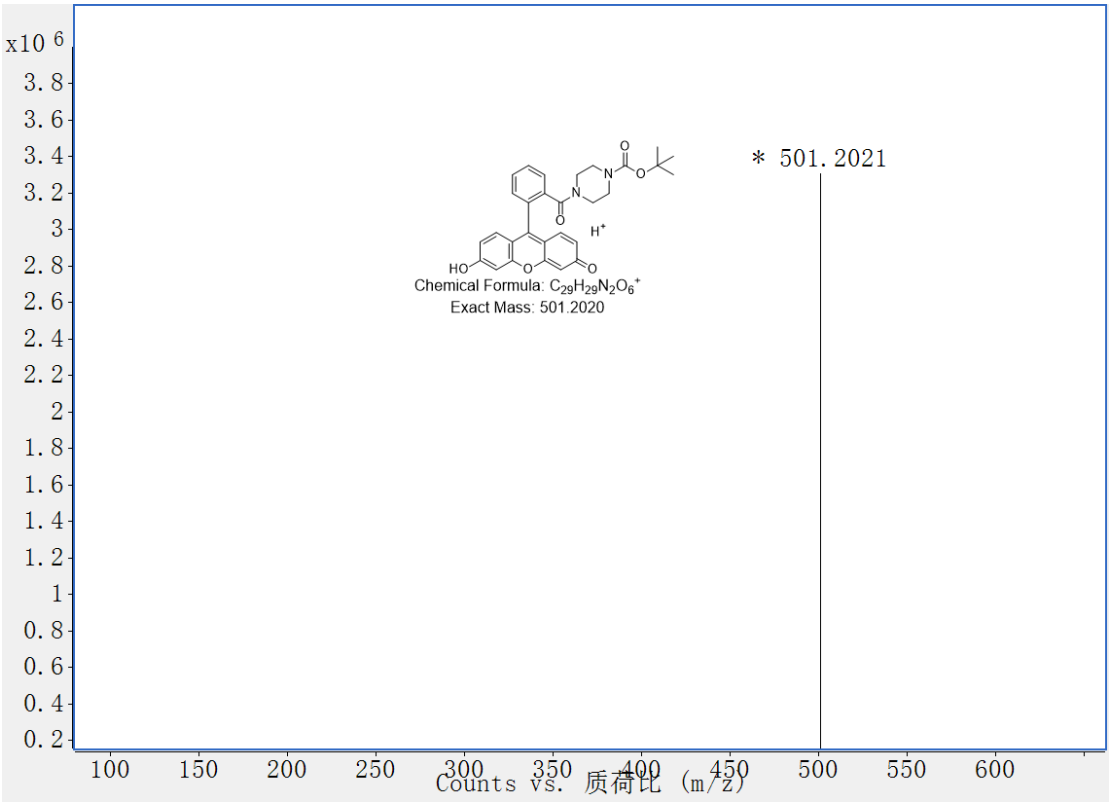


Fig. S5 Relative cell viability of HeLa cells in the presence of different concentrations of probe **1** for 24 h incubation. The results are expressed as mean ± S.D. (n = 3).

11. Supplementary NMR and MS spectra



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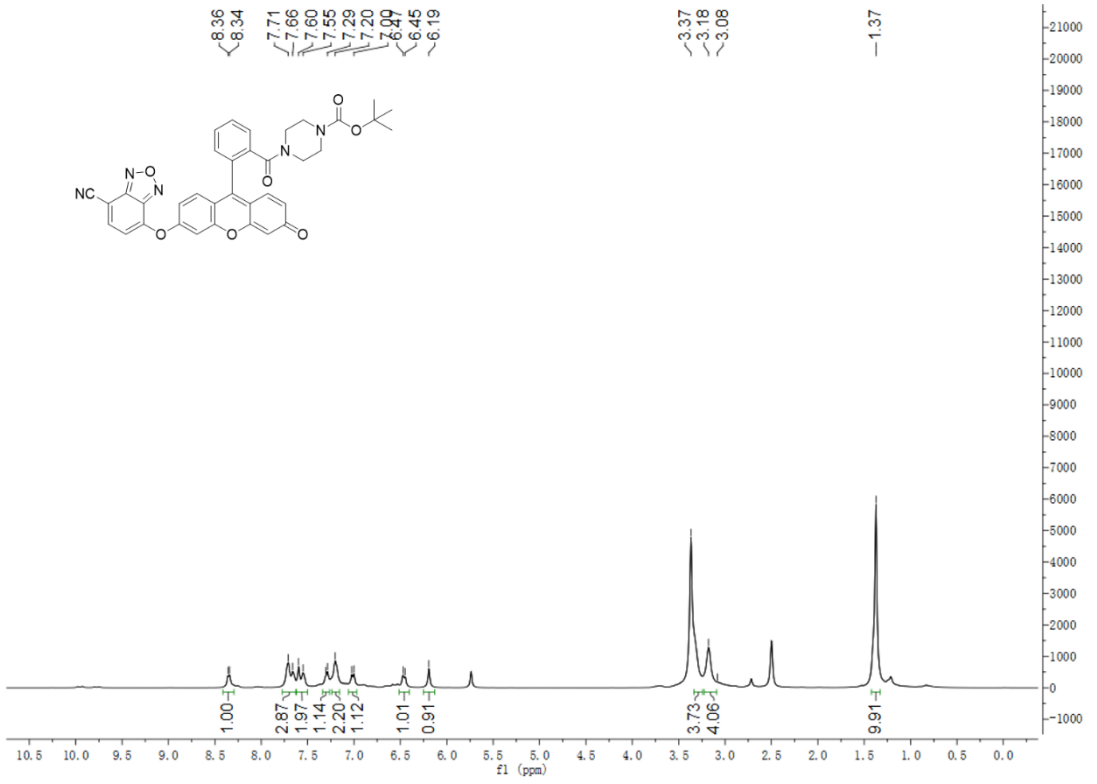
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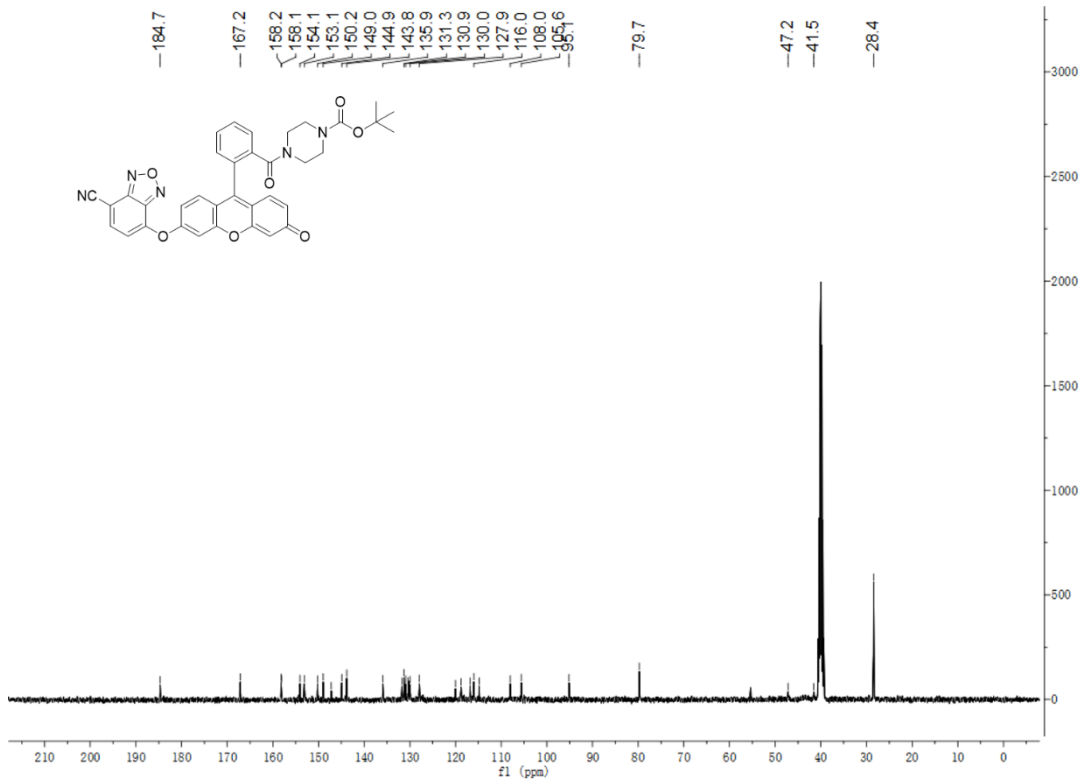
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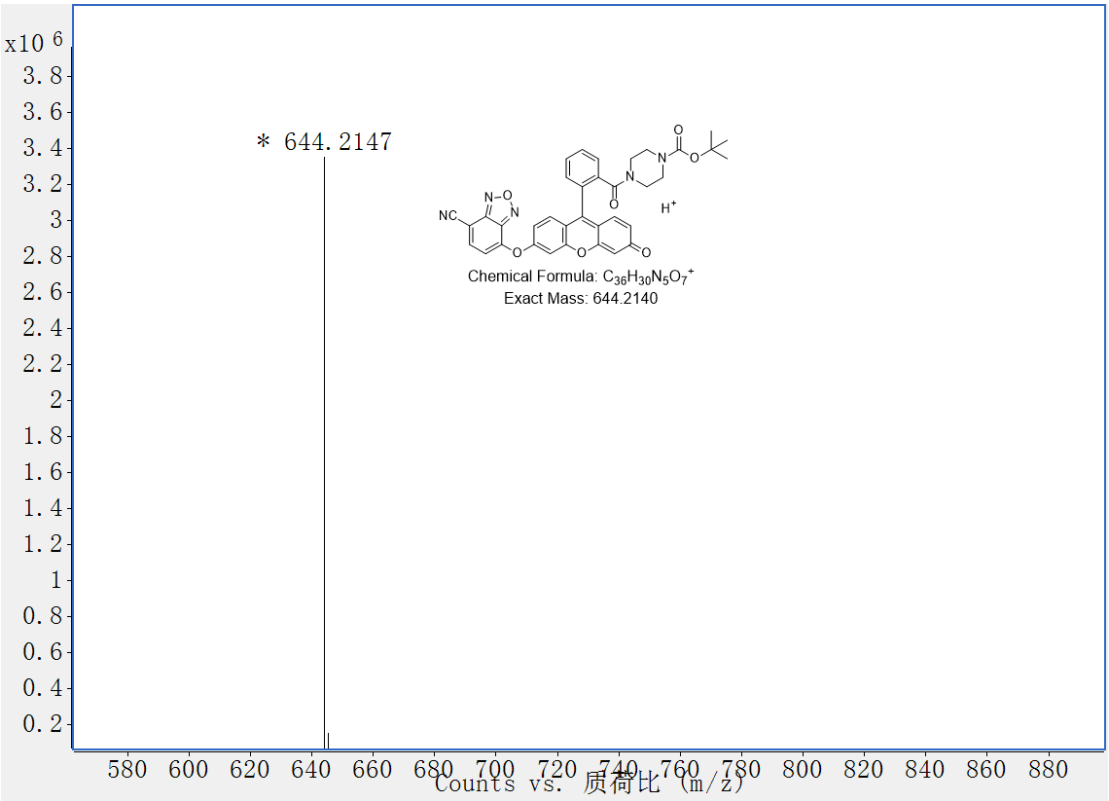
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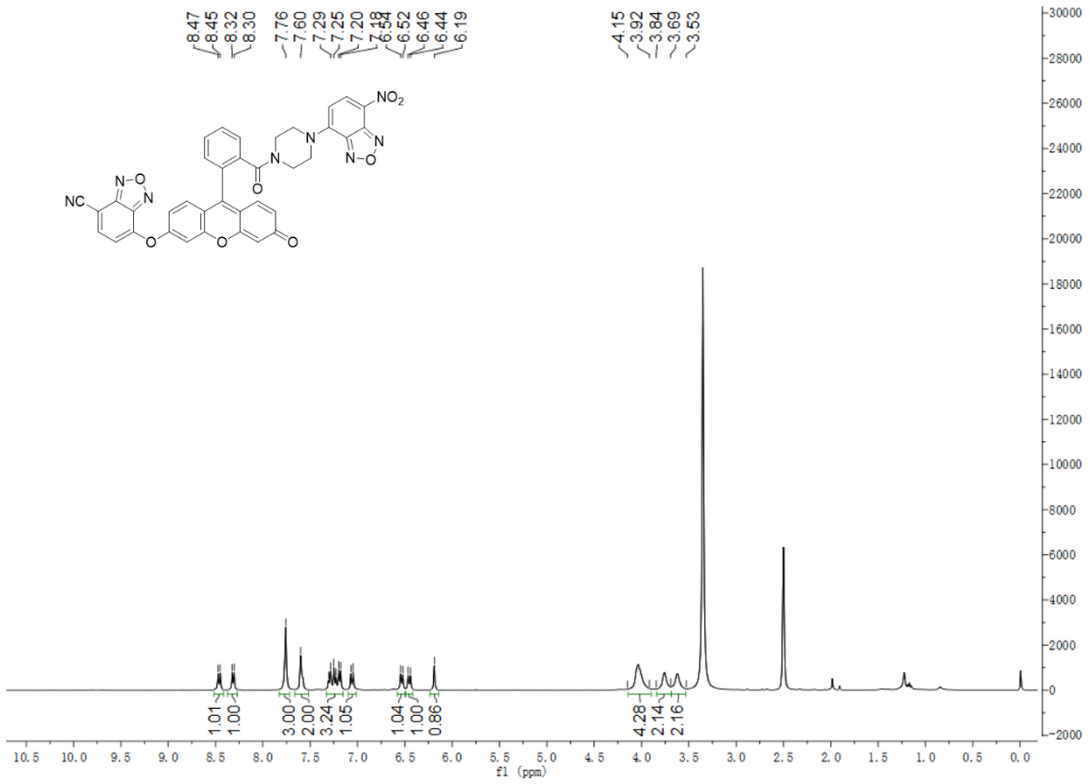
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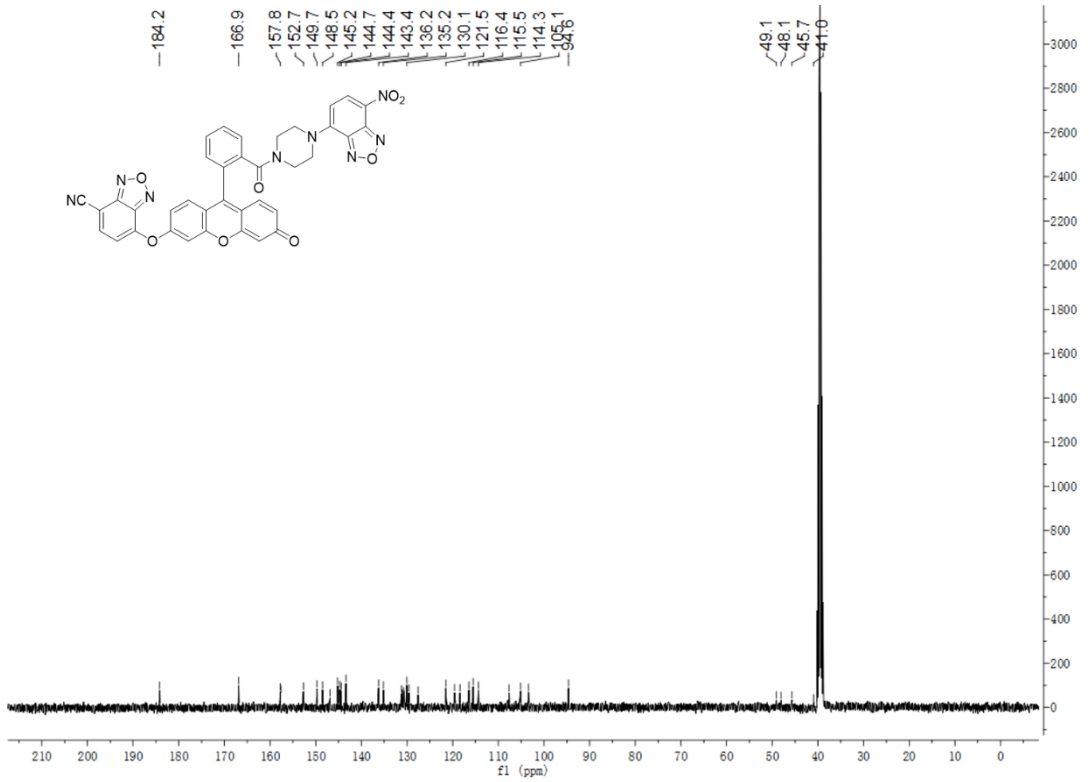
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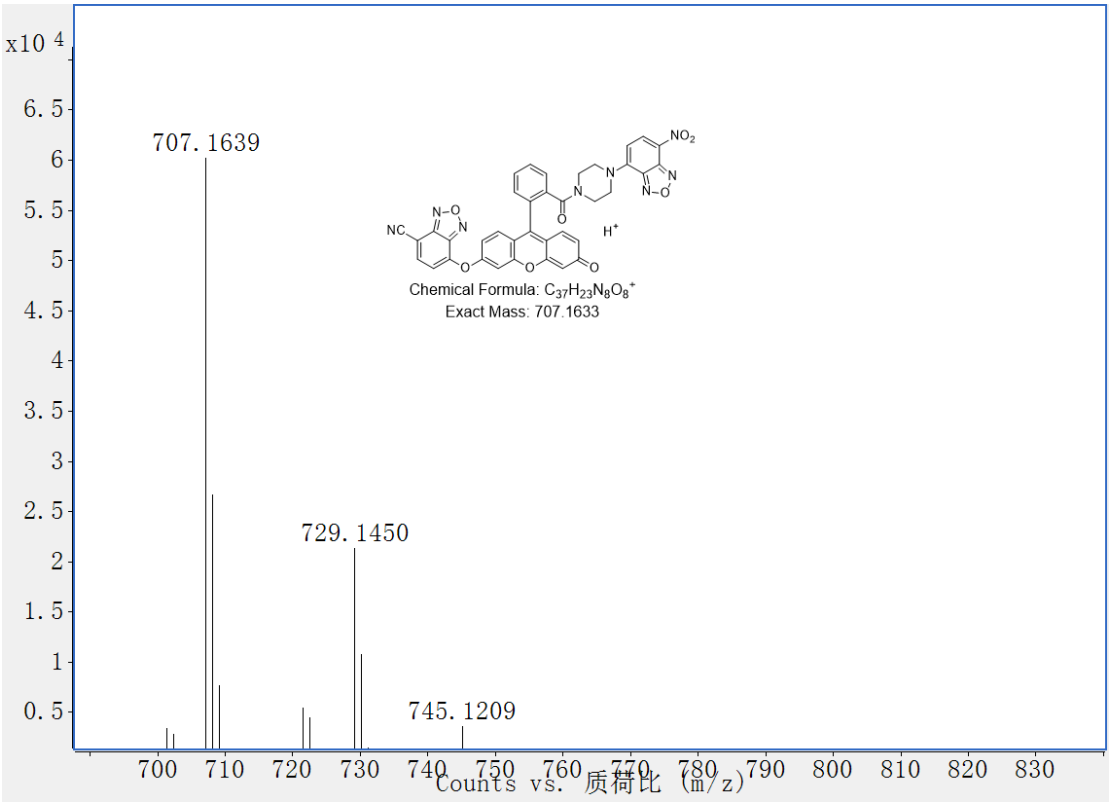
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