Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Thiolysis of CBD arylethers for development of highly GSH-selective fluorescent probes

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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 ($CDCl_3$ = 7.26 ppm, 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), dd (doublet doublet), t (triplet), 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).

2. Synthetic procedure of probes

Synthesis of compound NBD-Coum

NBD-Coum was synthesized based on a reported work.[1] ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.67 (d, J = 8.3 Hz, 1H), 7.98 (d, J = 8.7 Hz, 1H), 7.55 (d, J = 2.1 Hz, 1H), 7.44 (dd, J = 8.5, 2.1 Hz, 1H), 7.00 (d, J = 8.3 Hz, 1H), 6.46 (s, 1H), 2.49 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 159.4, 155.6, 154.2, 152.9, 151.8, 145.4, 144.5, 135.1, 131.2, 127.8, 118.1, 116.7, 114.0, 111.8, 108.7, 18.2.

Synthesis of probe 1

CBD-Br was synthesized by a similar method according to the reported literature.[2] ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.29 (d, J = 7.3 Hz, 1H), 8.14 (d, J = 7.3 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 149.8, 148.2, 142.2, 135.1, 115.7, 114.6, 99.9. 3-

carboxylate-7-hydroxy coumarin (40 mg, 0.17mmol) and CBD-Br (30 mg, 0.14 mmol) were dissolved in anhydrous DMF (5 mL) and Cs_2CO_3 (138 mg, 0.42mmol) was added to the mixture. The reaction was heated to 50 ℃ and stirred for 5 h. The solvent was removed under reduced pressure and purified by column chromatography to obtain a yellow solid **1** (24 mg, 47.1%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 8.83 (s, 1H), 8.38 (d, *J* = 7.7 Hz, 1H), 8.09 (d, *J* = 2.9 Hz, 1H), 7.51 (d, *J* = 2.3 Hz, 1H), 7.42 (dd, *J* = 8.6, 2.3 Hz, 1H), 7.20 (d, *J* = 7.7 Hz, 1H), 4.31 (q, *J* = 7.1 Hz, 2H), 1.32 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 162.5, 158.5, 156.1, 155.7, 149.8, 148.5, 148.3, 144.5, 143.5, 132.5, 129.5, 116.6, 115.6, 115.6, 114.4, 107.3, 94.7, 61.3, 14.1. HRMS (ESI): m/z $[M+H]^+$ calculated for $C_{19}H_{12}N_3O_6^+$: 378.0721; found: 378.0713.

Synthesis of probe 2

Compound **Nap-OH** was prepared according to the previous literature.[3] ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 11.86 (s, 1H), 8.51 (d, *J* = 8.3 Hz, 1H), 8.44 (d, *J* = 7.2 Hz, 1H), 8.33 (d, *J* = 8.2 Hz, 1H), 7.74 (t, *J* = 7.8 Hz, 1H), 7.15 (d, *J* = 8.2 Hz, 1H), 4.06 – 3.96 (m, 2H), 1.63 – 1.53 (m, 2H), 1.33 (dd, *J* = 15.0, 7.4 Hz, 2H), 0.91 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 163.7, 163.0, 160.3, 133.5, 131.1, 129.2, 128.9, 125.6, 122.4, 121.8, 112.6, 110.0, 40.2, 29.8, 19.8, 13.7. Compound **Nap-OH** (40 mg, 0.15 mmol) was dissolved in anhydrous DMF (2 mL) and NaH (11 mg, 0.45 mmol) was added to the mixture slowly. After the mixture stirred 10 min, **CBD-Br** (33 mg, 0.15 mmol) dissolved in DMF (1 mL) was slowly added to the reaction system. The reaction was heated to 50 ℃ and stirred for 10 h. Then the reaction mixture was dissolved in ethyl acetate, washed with water (30 mL x 2), brine (30 mL x 3), dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography to give the pure desired product **2** as a pale yellow solid (37 mg, 60.0%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.67 (dd, *J* = 7.2, 0.7 Hz, 1H), 8.62 (d, *J* = 8.0 Hz, 1H), 8.39 (d, *J* = 8.4 Hz, 1H), 7.83 (d, *J* = 2.9 Hz, 1H), 7.81 (d, *J* = 2.6 Hz, 1H), 7.48 (d, *J* = 8.0 Hz, 1H), 6.74 (d, *J* = 7.7 Hz, 1H), 4.21 – 4.16 (m, 2H), 1.72 (t, *J* = 7.6 Hz, 2H), 1.46 (dt, *J* = 15.1, 7.4 Hz, 2H), 0.98 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.8, 163.2, 154.3, 150.5, 149.4, 144.1, 140.8, 132.5, 131.9, 123.0, 128.1,

127.4, 124.5, 123.4, 121.2, 117.0, 113.6, 112.5, 96.5, 40.5, 30.3, 20.5, 14.0. HRMS (ESI): m/z $[M+H]^+$ calculated for $C_{23}H_{17}N_4O_4^+$: 413.1244; found: 413.1145.

3. Stability measurement of the probes

The stability of the probes was tested in degassed phosphate buffer (PBS, 50 mM). Briefly, 10 μM **1** or **NBD-Coum** was incubated in PBS containing 2% DMSO at room temperature for 0-60 min, and the fluorescence intensity of the solution was obtained by excitation at 404 nm and 350 nm, respectively. Similarly, 5 μM **2** was incubated in PBS containing 10% DMSO at room temperature for 0-60 min, and the stability data was obtained by monitoring the fluorescence intensity of the resulted solution by excitation at 469 nm.

4. General procedure for spectroscopic studies

All measurements were performed in degassed phosphate buffer (PBS, 50 mM, pH 7.4, containing 2% DMSO to **1**, **NBD-Coum** and 10% DMSO to **2**). Compounds were dissolved into DMSO to prepare the stock solutions with concentrations of 1-10 mM. Various stock solutions (20 mM) of different analytes were prepared in PBS buffer. Appropriate amount of bio-relevant species was added to separate portions of the probe solution and mixed thoroughly. The reaction mixture was shaken uniformly before spectra were measured. All measurements were performed in a 3 mL corvette with 2 mL solution at room temperature and all the fluorescence spectra were obtained by excitation at 404 nm (probe **1**), 350 nm (probe **NBD-Coum**) or 469 nm (probe **2**).

For quantum yields, probe **1** in the absence and presence of GSH were tested in PBS buffer (50 mM, pH = 7.4, 2% DMSO) using 7-amino-4-methylcoumarin ($\Phi = 0.88$) as the reference fluorophore. Probe **2** in the absence and presence of GSH were measured in PBS (50 mM, pH = 7.4, 10% DMSO) with fluorescein in 0.1 M NaOH solution as the reference fluorophore ($\Phi = 0.925$). 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 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. Subscript S refers to the standard.

For the selectivity experiment, fluorescence spectra of probe **2** (5 μM) toward different species with or without GSH in PBS buffer were monitored. All reactions were incubated for 1 h at room temperature. GSH was 5 mM and all other species were 100 μM.

For the determination of the detection limit, probe **1** (10 μM) or **2** (5 μM) was incubated with various concentrations of GSH (0.1-1.0 mM) for 1 h before recording the emission profiles. The detection limit was calculated with the following equation:[4]

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

Construction of fitting curves and calculation of parameters including dissociation constant K_d were done with the following equation. Fluorescence intensity at the fluorescence maximum (denoted as F in the following formula) was plotted against GSH concentration. F_{free}, F_{bound} and K_d denote fluorescence intensity of the GSH-free and GSH-bound states and the dissociation constant with GSH, respectively.[5]

$$F = F_{\text{bloud}} + \frac{F_{\text{free}} - F_{\text{bloud}}}{1 + \frac{[\text{GSH}]}{K_d}}$$

5. HPLC Measurements

For the HPLC studies, a mixture of probe **2** (200 μM) and GSH (10 mM) in PBS (50 mM, pH = 7.4, 30% DMSO) was analyzed by HPLC at different reaction time. Conditions: ANGELA TECHNOLOGIES HPLC LC-10F; C_{18} column with 4.6 mm x 250 mm; detection wavelength: 360 nm. Buffer A: 0.1% (v/v) trifluoroacetic acid in water; buffer B: methanol; flow: 1 mL/min.

The elution conditions were: 0-3 min, buffer B: 5-80%; 3-16 min, buffer B: 80-95%; 16-18 min, buffer B: 95-5%; 18-20 min, buffer B: 5-5%.

6. Cell culture and MTT assay

HeLa cell (human cervical cancer cells) line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HeLa (human cervical cancer

cells) was cultured with DMEM (GIBICO) supplemented with FBS (10%), penicillin (100 U/mL), streptomycin (100 U/mL), and L-glutamine (4 mM) medium under standard cell culture conditions at 37 °C in a humidified CO_2 incubator. The cytotoxicity of probe **2** 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 experiments. After that, the culture medium was replaced with a fresh one and the HeLa cells were incubated with different concentrations of probe **2** (0, 5, 10, 20, 30 and 40 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.)).

7. Imaging in living cell

The feasibility of probe **2** to detect intracellular GSH was evaluated by fluorescence imaging. Briefly, glass bottom dishes were added into a 24-well plate before cells were seeded. Then, the HeLa cells were transferred to the 24-well plate and cultured for 12 h before experiments. After that, the culture medium was replaced with the fresh one. In the experimental group, HeLa cells were co-incubated with probe **2** for 2 h, while the control group cells were pre-treated with the thiol blocking reagent N-ethylmaleimide (NEM, 1 mM) for 30 min, and then incubated with probe **2** for another 2 h. After incubation, the cells were quickly washed with PBS, and then fixed with 4% paraformaldehyde solution for 10 min. After that, the cells were washed with PBS and then treated with DAPI (1 μg/ml) for 10 min. Finally, the HeLa cells were washed using PBS and imaged using a confocal microscope (Olympus FV1000) with a 40 × objective lens. The emission of probe was collected at the green channel (500-550 nm) with 488 nm excitation. DAPI stained HeLa cells were observed through the blue channel (450-500 nm) with 405 nm excitation.

8. Reference

1. M. D. Hammers and M. D. Pluth, *Anal. Chem.,* 2014, **86**, 7135-7140.

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

3. H. C. Zhu, H. M. Zhang, C. X. Liang, C. Y. Liu, P. Jia, Z. L. Li, Y. M. Yu, X. Zhang, B. C. Zhu and W. L. Sheng, *Analyst,* 2019, **144**, 7010-7016.

4. J. Radford-Knoery and G. A. Cutter, *Anal. Chem.*, 1993, **65**, 976-982.

5. K. Umezawa, M. Yoshida, M. Kamiya, T. Yamasoba and Y. Urano, *Nat. Chem.*, 2017, **9**, 279-286.

9. Supplementary figures

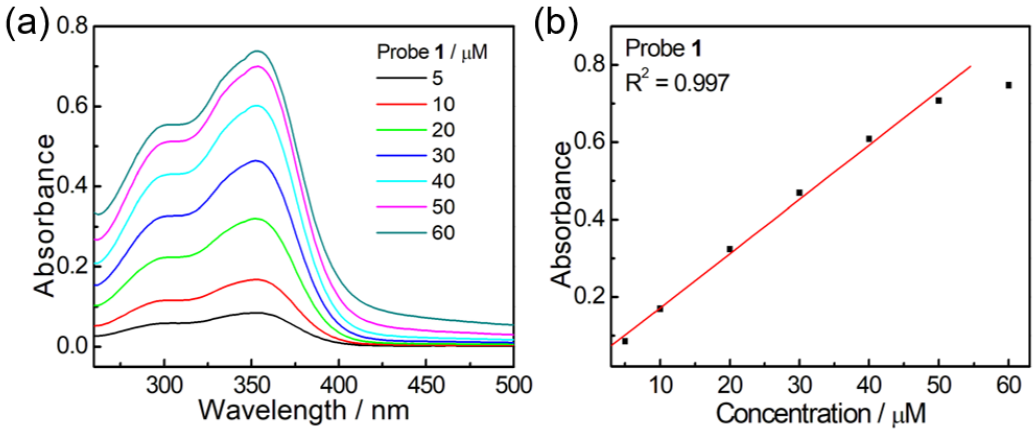


Fig. S1 (a) The UV-vis spectra of probe **1** at different concentrations in PBS buffer (50 mM, pH = 7.4, containing 2% DMSO). (b) The linear relationship between absorbance at 354 nm and the concentration of probe **1**.

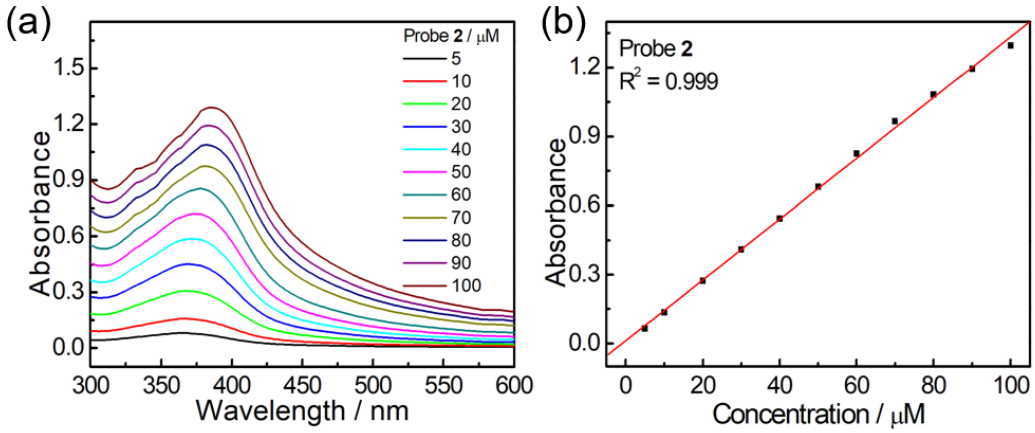


Fig. S2 (a) The UV-vis spectra of probe **2** at different concentrations in PBS buffer (50 mM, pH = 7.4, containing 10% DMSO). (b) The linear relationship between absorbance at 364 nm and the concentration of probe **2**.

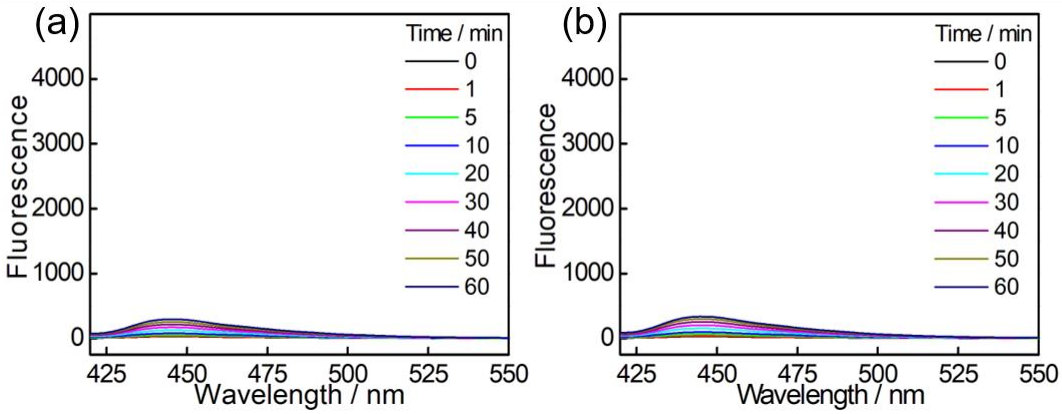


Fig. S3 Fluorescent spectra of 10 μM probe **1** toward 100 μM Cys (a) or 100 μM Hcy (b) for 1h (Ex. 404 nm).

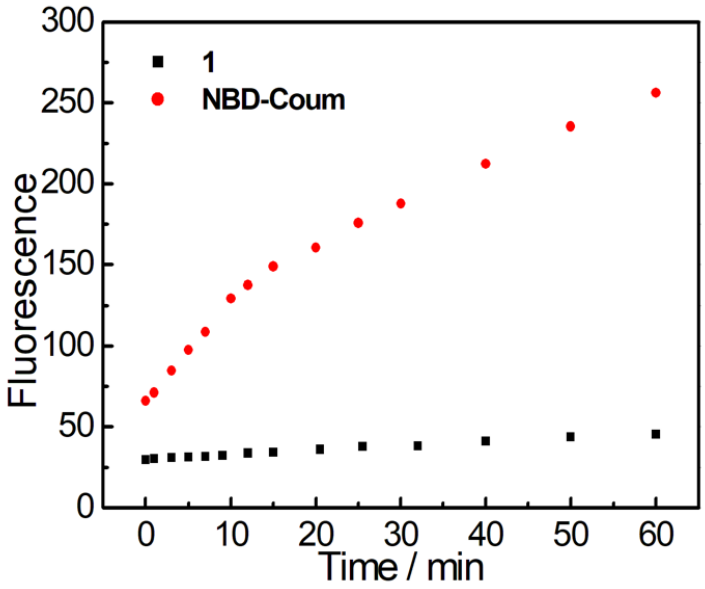


Fig. S4 The fluorescence changes of probe **1** (10 μM) and **NBD-Coum** (10 μM) in PBS buffer (Em. 445 nm for **1** and 450 nm for **NBD-Coum**).

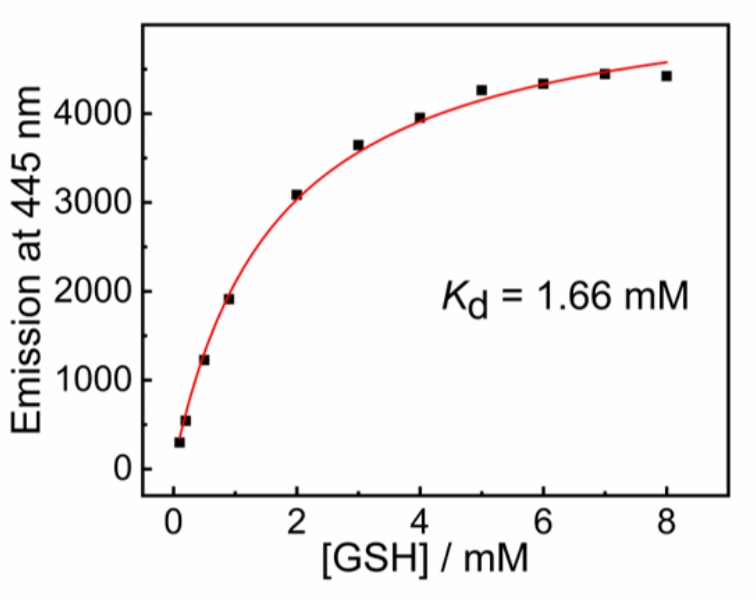


Fig. S5 The fluorescence changes of probe **1** (10 μM) in the presence of various concentrations of GSH (Ex. 404 nm). The dissociation constant K_d was calculated to be 1.66 mM.

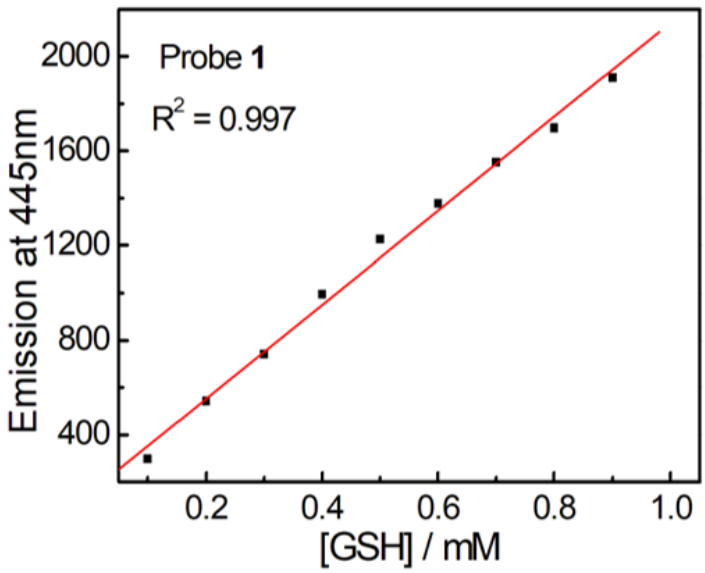


Fig. S6 The fluorescence response of probe **2** (5 μM) upon addition of GSH (0–8 mM) in PBS buffer (Ex. 404 nm). The detection limit was calculated to be 0.74 μM.

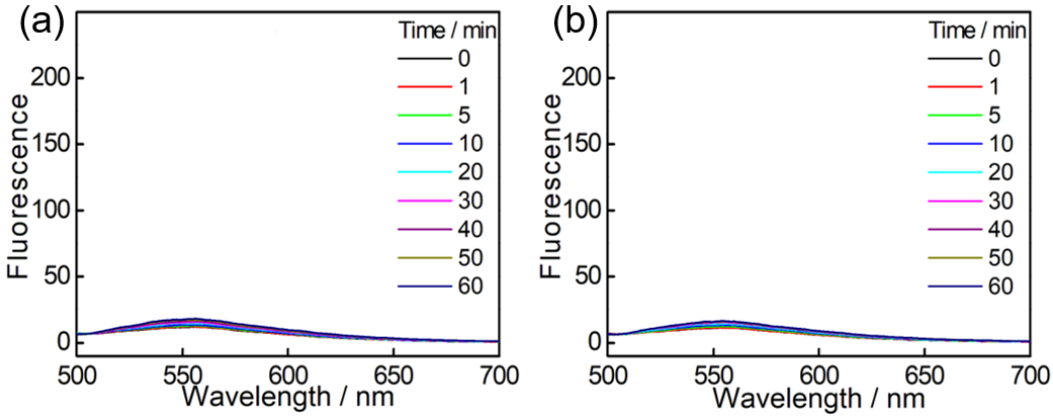


Fig. S7 Fluorescent spectra of 5 μM probe **2** toward 100 μM Cys (a) or 100 μM Hcy (b) for 1h (Ex. 469 nm).

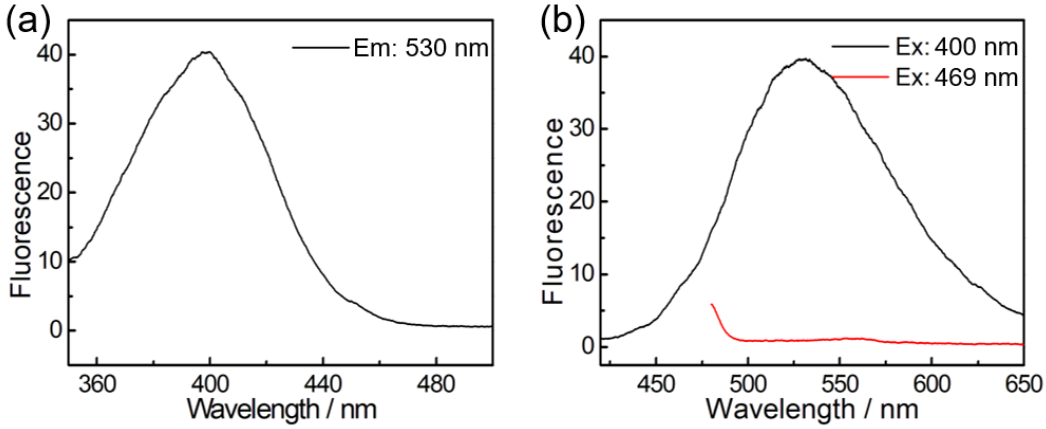


Fig. S8 (a) The excitation spectrum (emission at 530 nm) of 10 μM **CBD-Br** in the presence of 10 mM GSH after 3 h incubation in PBS (pH 7.4). (b) The emission spectra of 10 μM **CBD-Br** in the presence of 10 mM GSH with different excitation wavelengths.

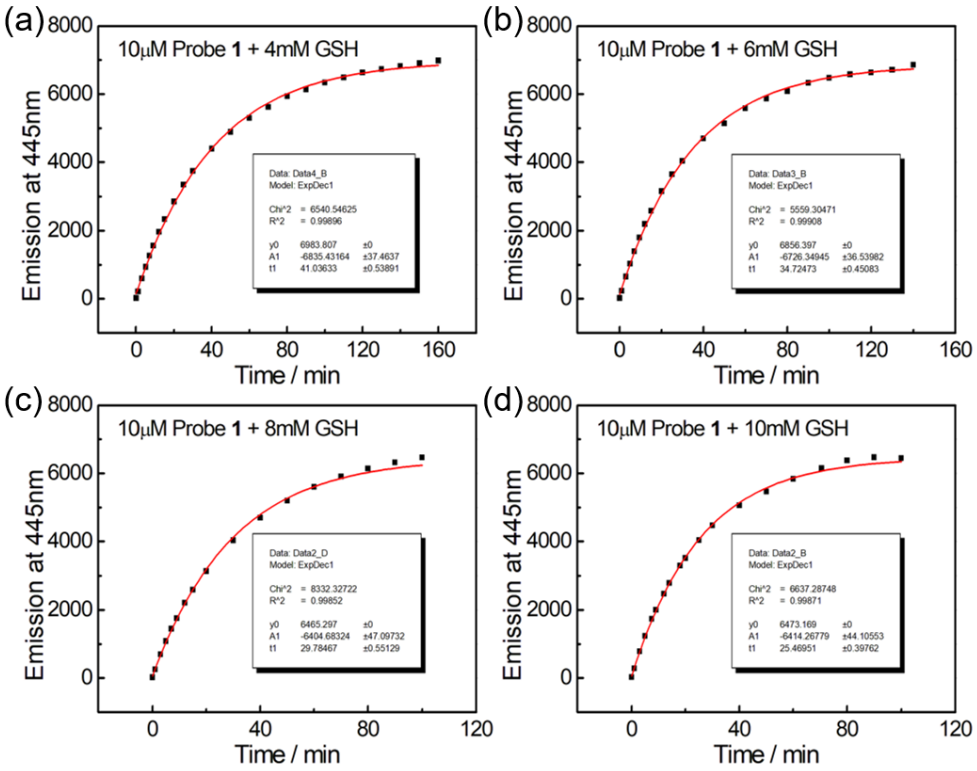


Fig. S9 Time-dependent fluorescence intensities of probe **1** (10 μM) in the presence of different concentrations of GSH.

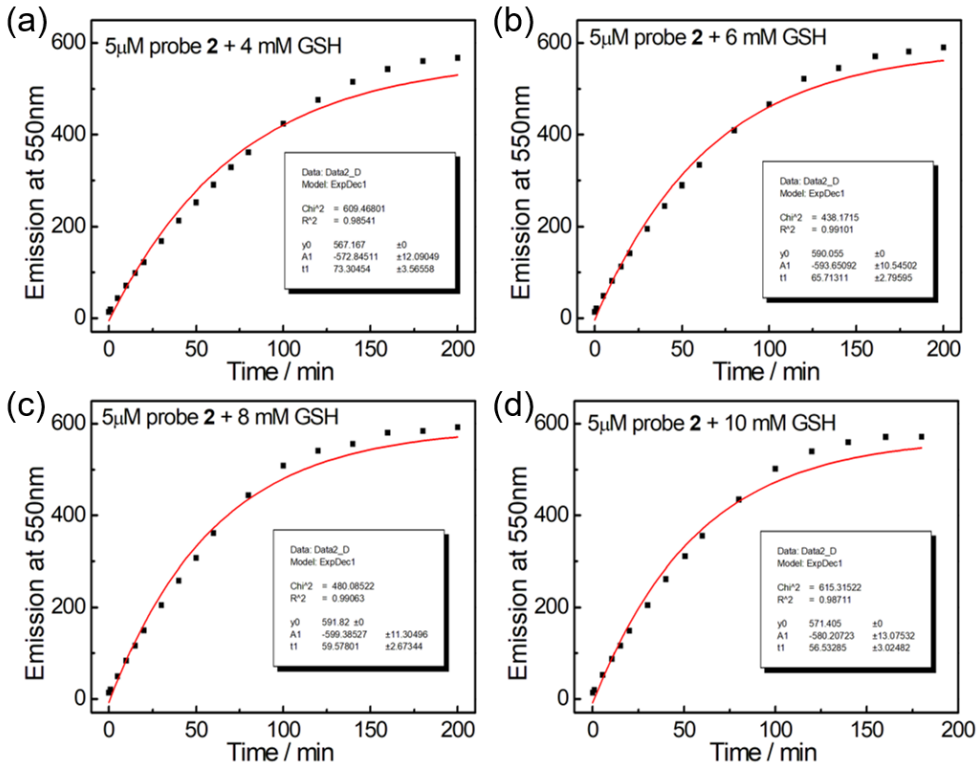


Fig. S10 Time-dependent fluorescence intensities of probe **2** (5 μM) in the presence of different concentrations of GSH.

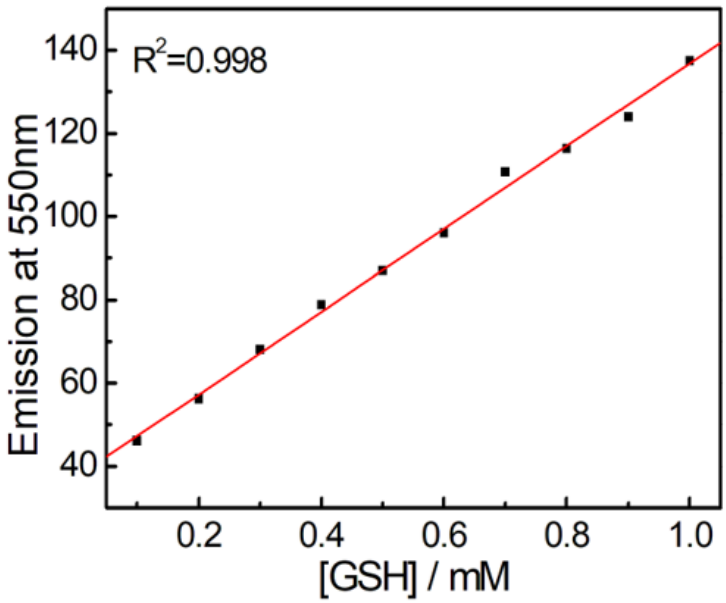


Fig. S11 The fluorescence changes of probe **2** (5 μM) in the presence of various concentrations of GSH (Ex. 469 nm). The detection limit was calculated to be 24.1 μM.

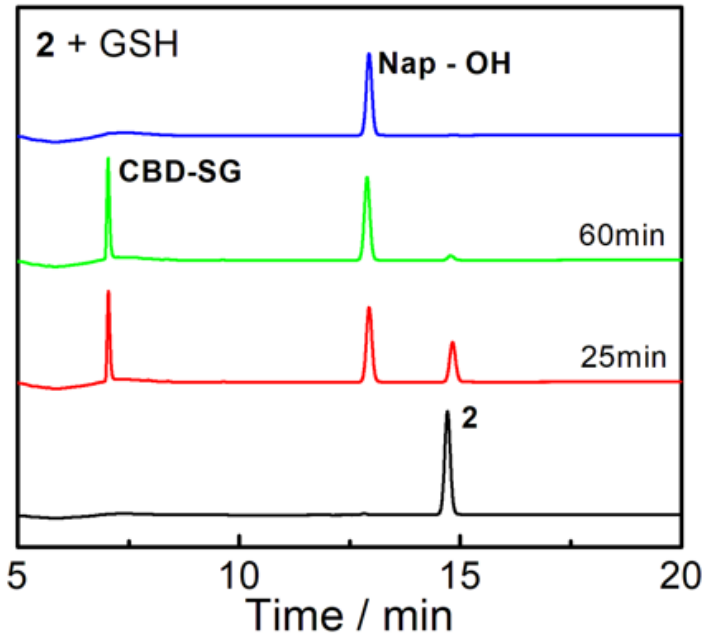


Fig. S12 Time-dependent HPLC traces of **2** (200 μM) treated with GSH (10 mM) in PBS buffer (50 mM, pH 7.4, containing 30% DMSO).

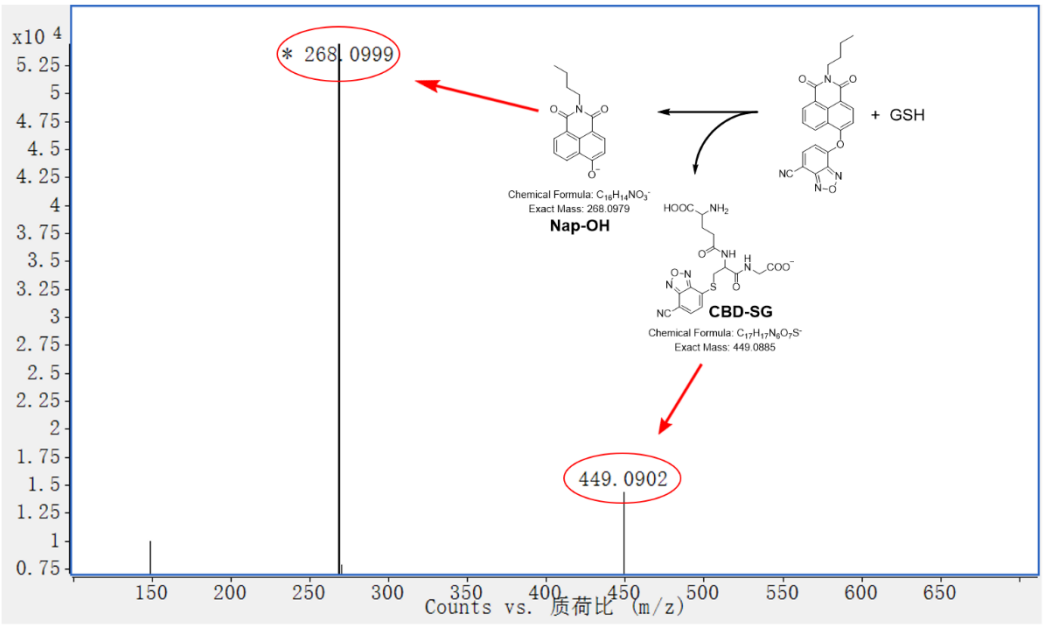


Fig. S13 HRMS spectrum of probe **2** (1 mM) incubated with GSH (2 mM) in PBS buffer (50 mM, pH 7.4, containing 50% DMSO) for 5 h.

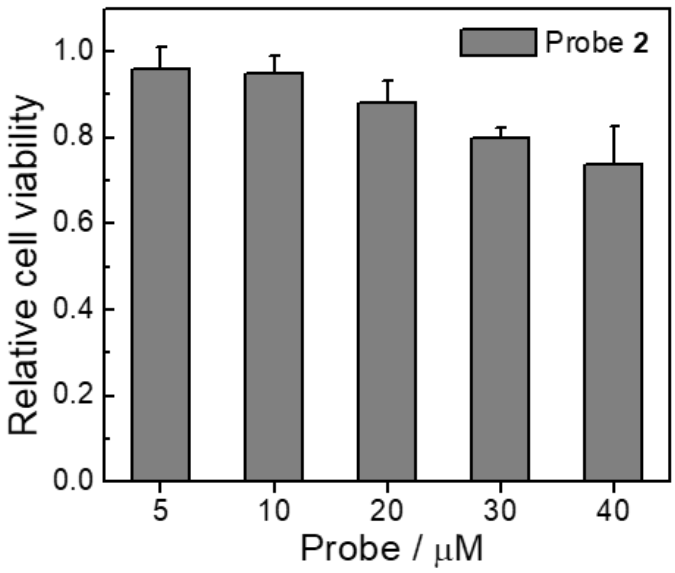
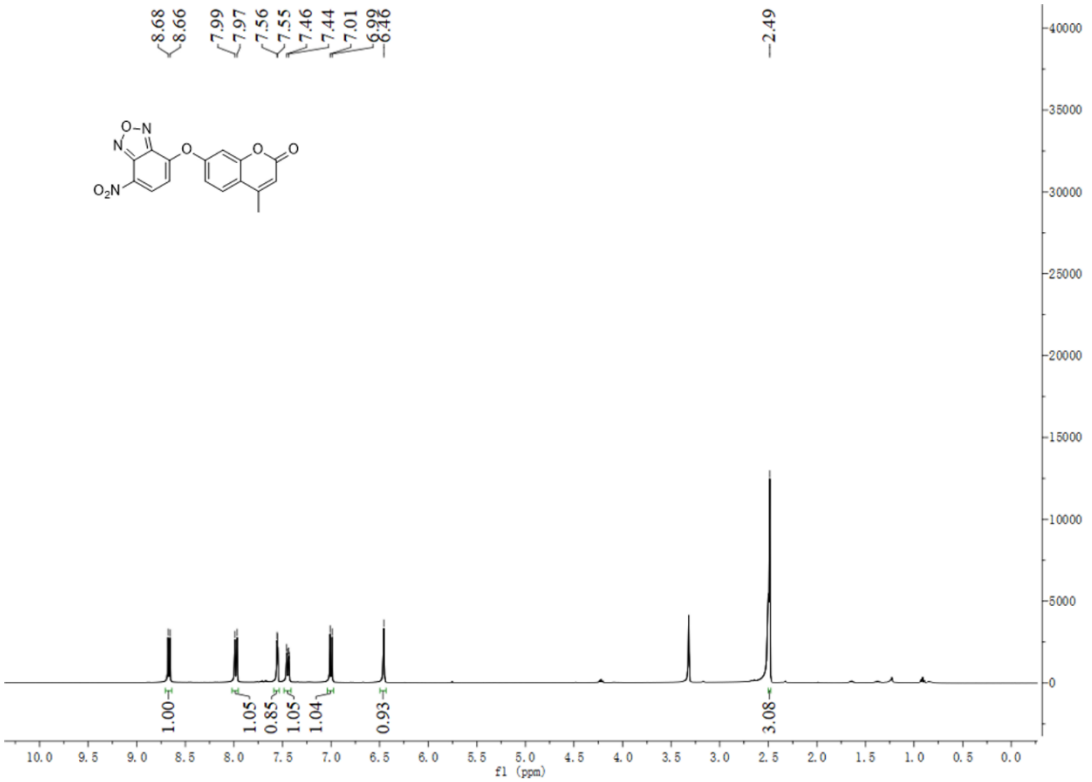
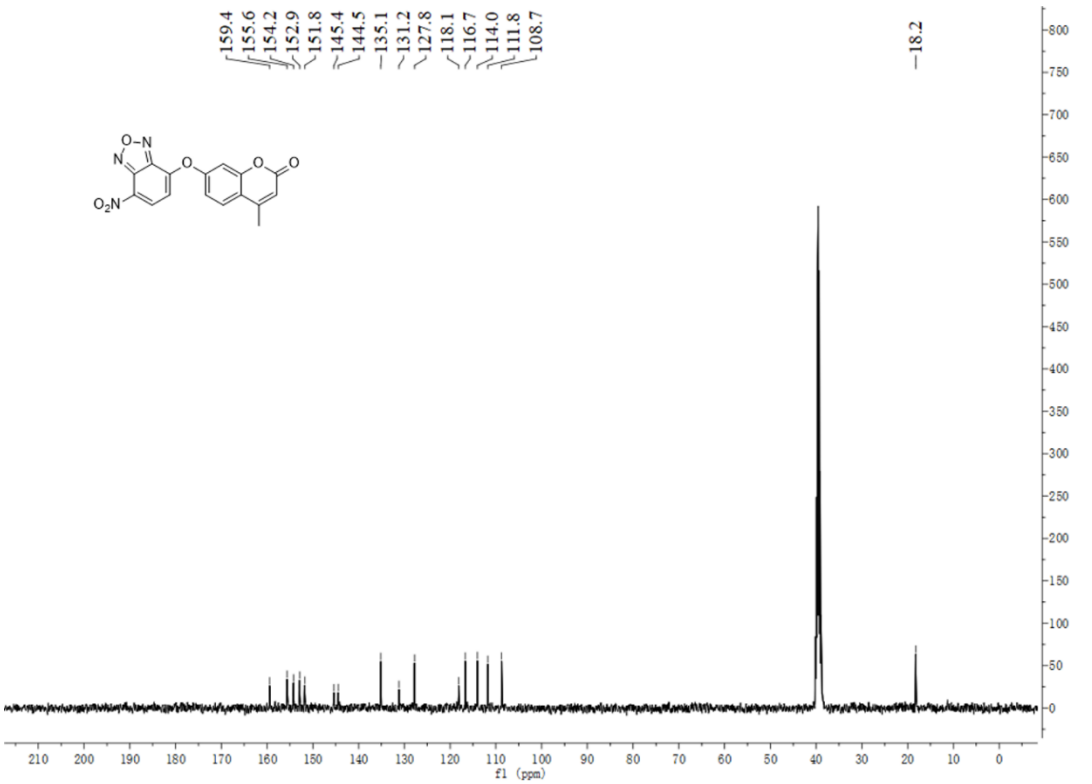
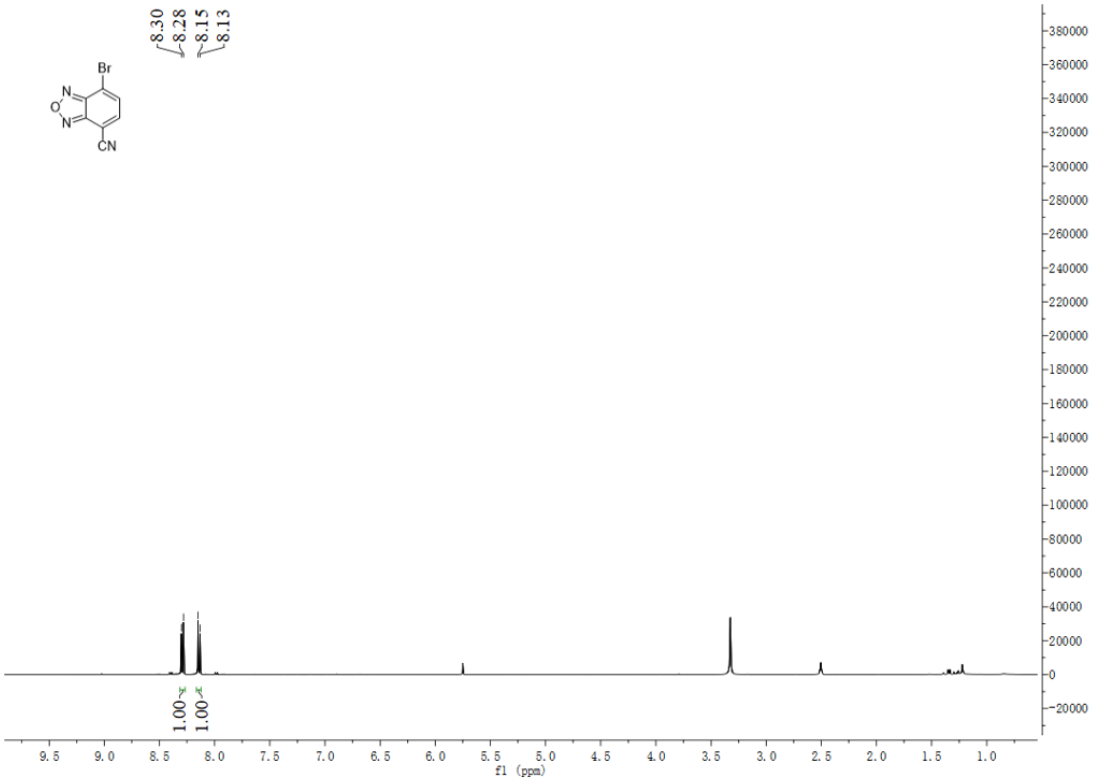


Fig. S14 Relative cell viability of HeLa cells after treatment for 24 h with probe **2** by MTT assay.

10. Supplementary NMR and HR-MS spectra



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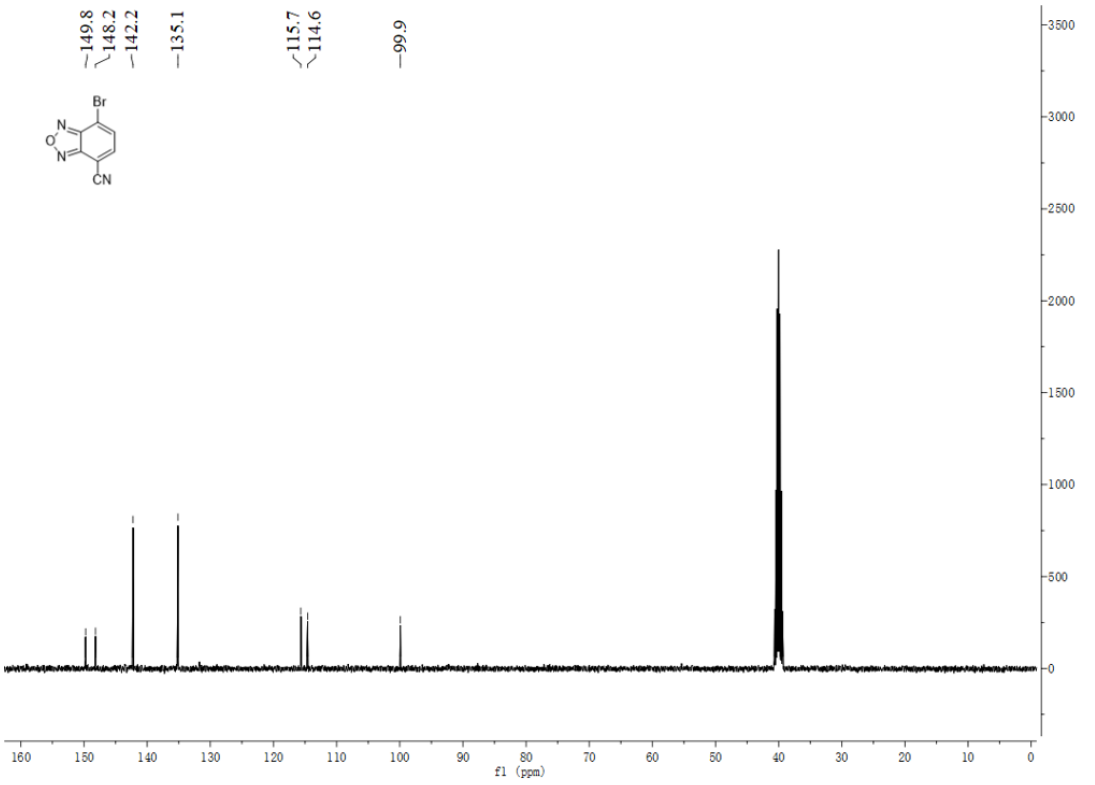
O
N
CN
1.00
1.00
f1 (ppm)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142.2
135.1
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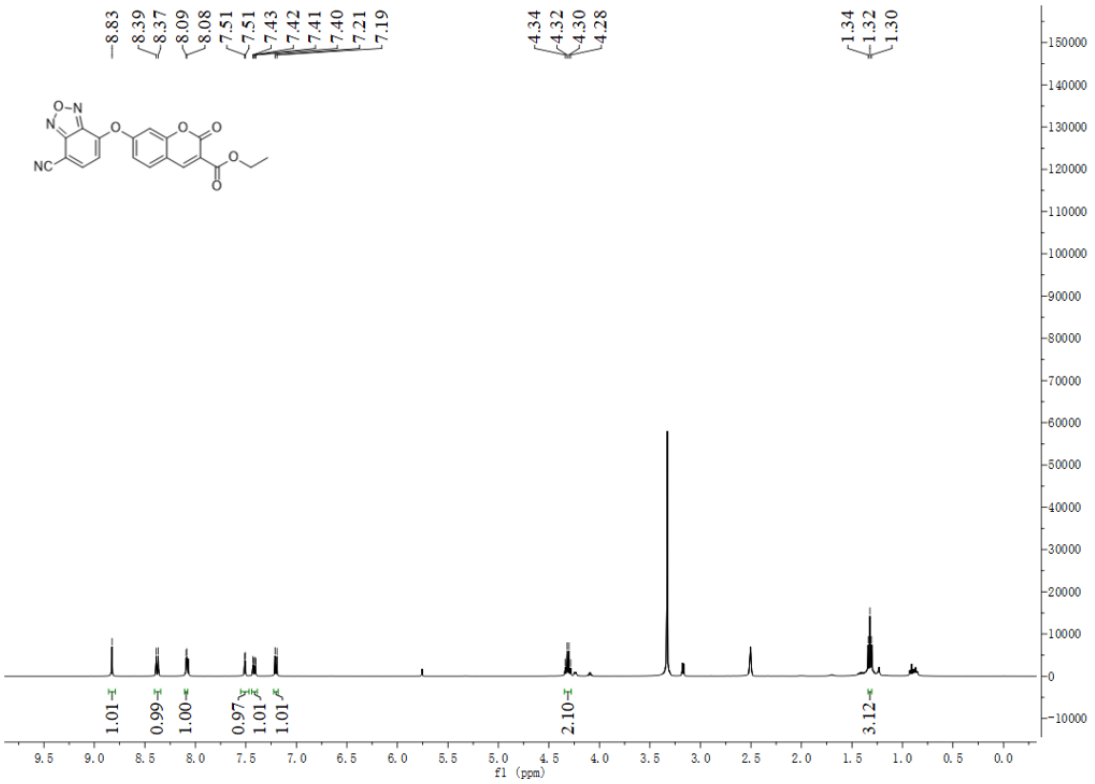
114.6
99.9
Br
N
O
N
CN
f1 (ppm)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7.41
7.40
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7.19
4.34
4.32
4.30
4.28
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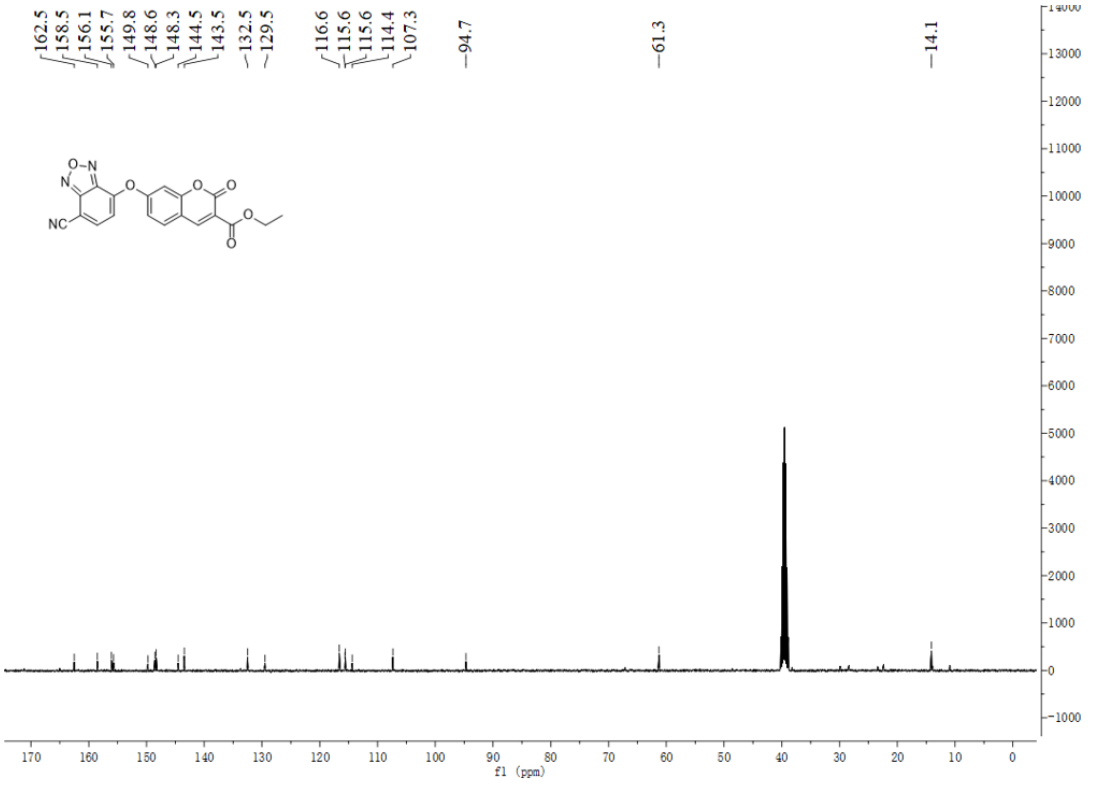
1.00
0.97
1.01
1.01
2.10
3.12
f1 (ppm)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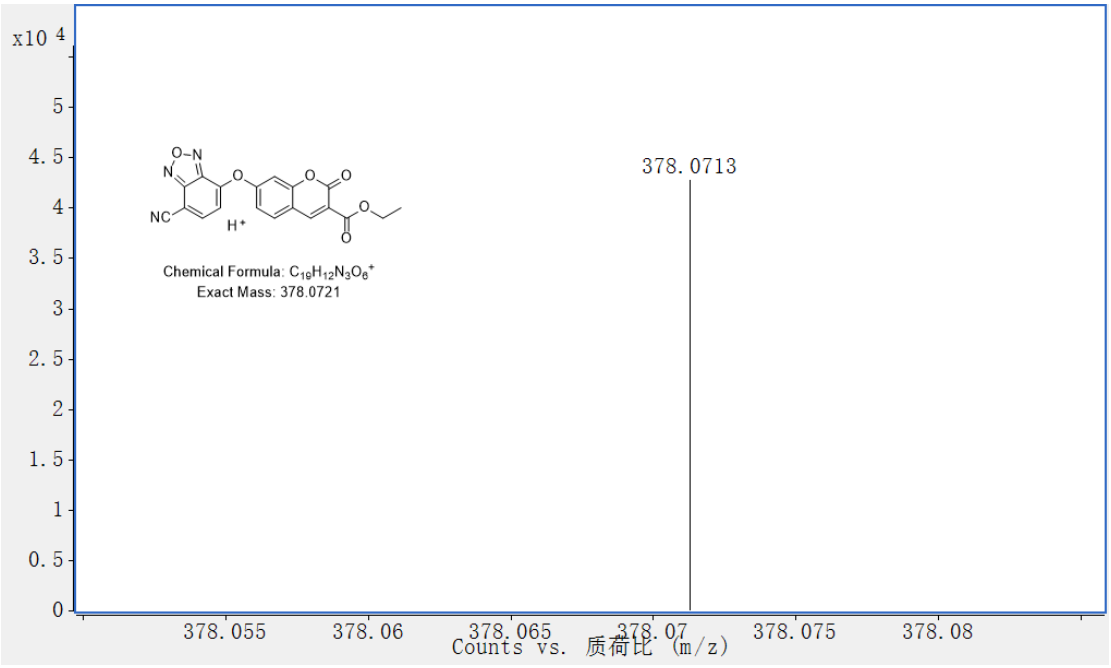
14.1
f1 (ppm)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3
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2
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1
0.5
0
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Exact Mass: 378.0721
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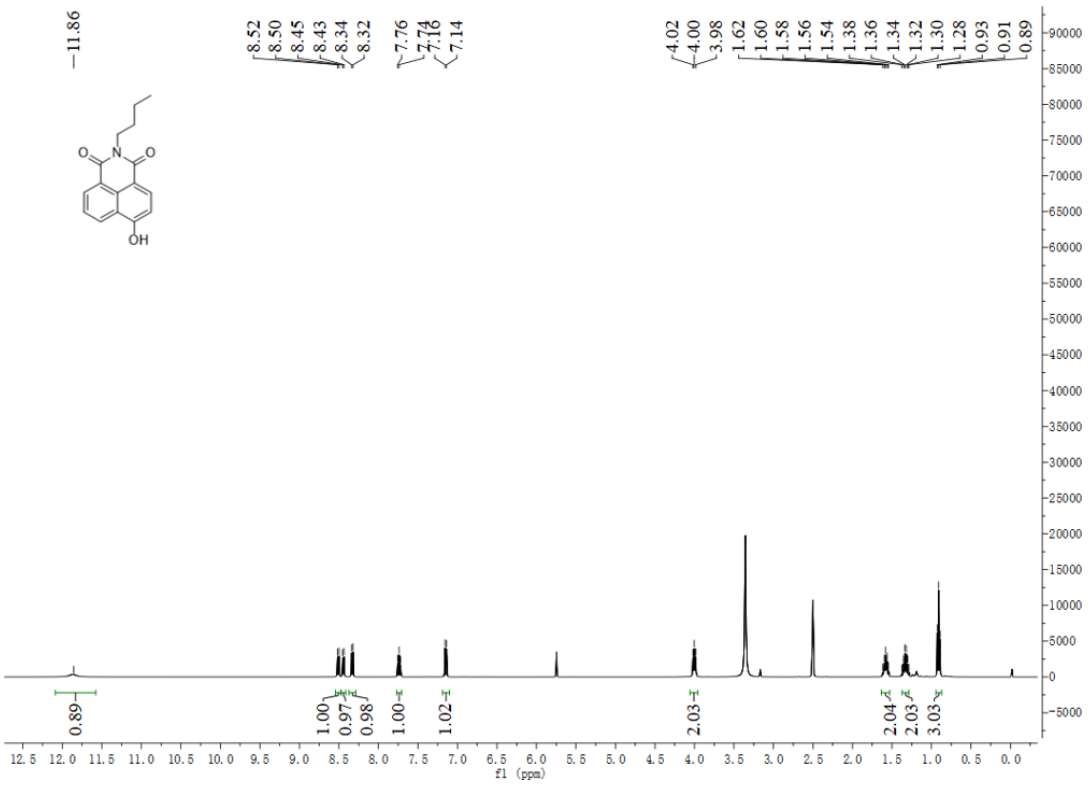
378.055
378.06
378.065
378.07
378.075
378.08
Counts vs. 质荷比 (m/z)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8.43
8.34
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7.14
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1.56
1.54
1.38
1.36
1.34
1.32
1.30
1.28
0.93
0.91
0.89
OH
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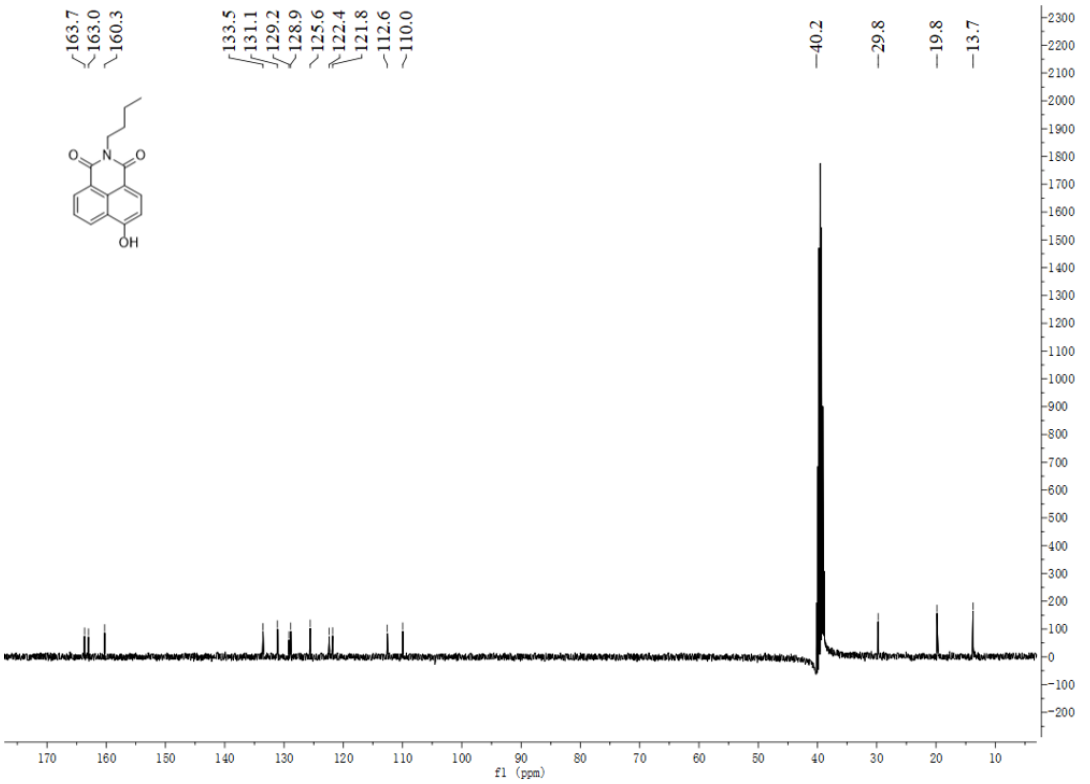
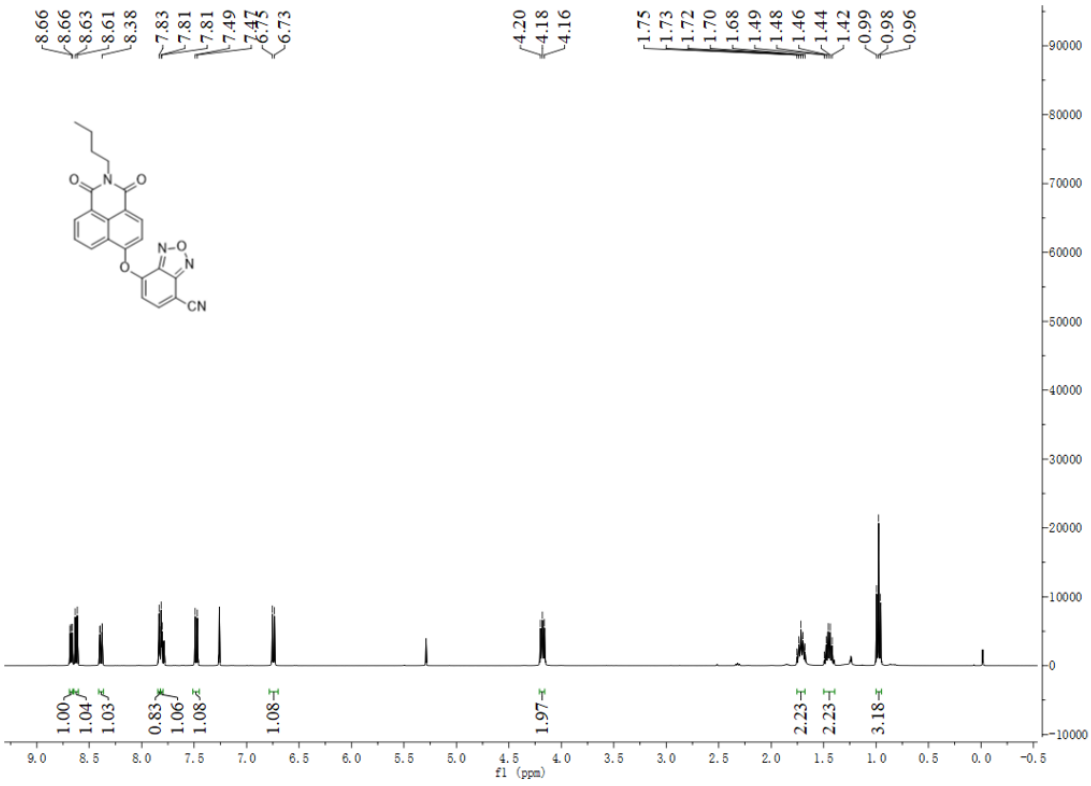
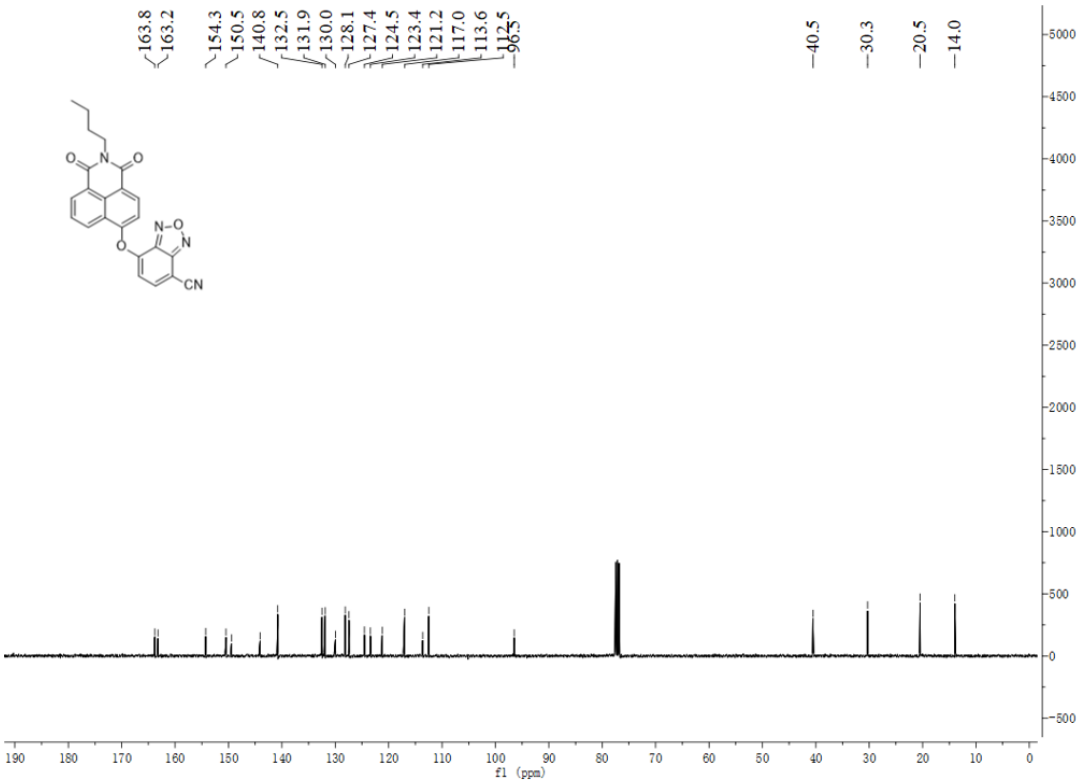
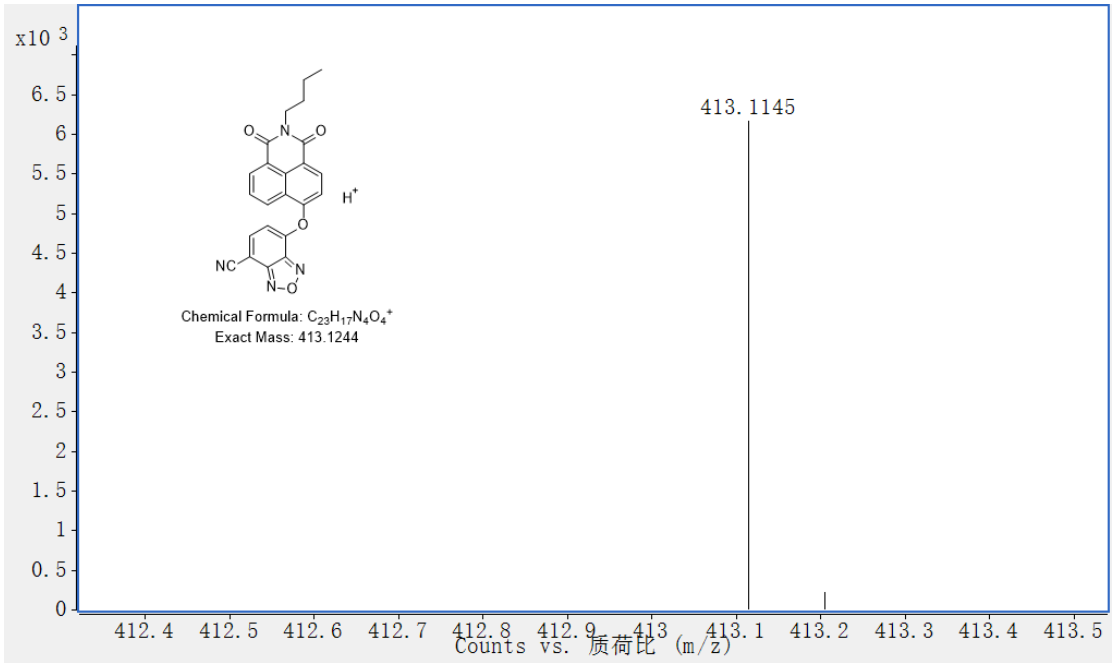
0.97
0.98
1.00
1.02
2.03
2.04
2.03
3.03
f1 (ppm)


163.7
163.0
160.3
133.5
131.1
129.2
128.9
125.6
122.4
121.8
112.6
110.0
40.2
29.8
19.8
13.7
OH
f1 (ppm)


8.66
8.66
8.63
8.61
8.38
7.83
7.81
7.81
7.49
7.47
7.45
6.73
4.20
4.18
4.16
1.75
1.73
1.72
1.70
1.68
1.49
1.48
1.46
1.44
1.42
0.99
0.98
0.96
CN
1.00
1.04
1.03
0.83
1.06
1.08
1.08
1.97
2.23
2.23
3.18
f1 (ppm)


163.8
163.2
154.3
150.5
140.8
132.5
131.9
130.0
128.1
127.4
124.5
123.4
121.2
117.0
113.6
112.5
96.5
40.5
30.3
20.5
14.0
CN
f1 (ppm)


x10 3
413.1145
H+
NC
Chemical Formula: C23H17N4O4+
Exact Mass: 413.1244
Counts vs. 质荷比 (m/z)