Electronic Supplementary Material

Novel lysosome-targeted anticancer fluorescent agents used in zebrafish and nude mouse tumour imaging

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Content

1. The experimental materials and equipment

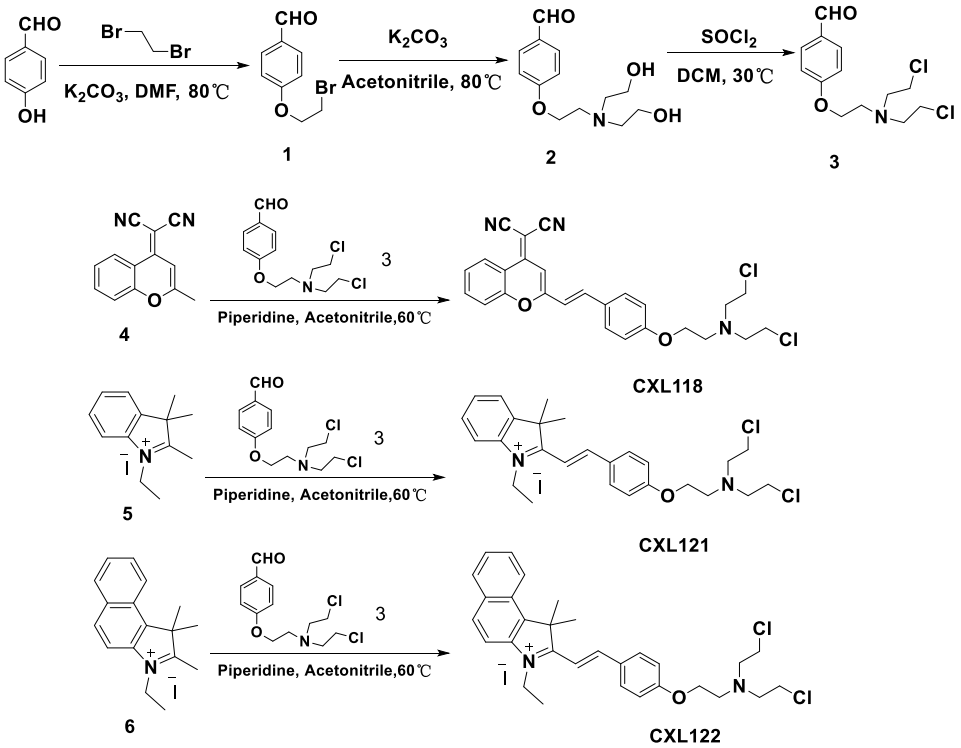
All reagents are purchased with no special note. All of the solvents were dried according to the standard methods and were spectroscopic grade in the optical spectroscopic studies. NMR spectra were measured on a Bruker AM-400. The ^{1}H NMR (400 MHz) and ^{13}C NMR (101 MHz) chemical shifts were given using $CDCl_3$ and DMSO-d_6 as the internal standard. The ^{1}H NMR (400 MHz) chemical shifts were given in ppm relative to the internal reference TMS. ESI-MS and HRMS spectral data were recorded on a Finnigan LCQDECA and a Bruker Daltonics Bio TOF mass spectrometer, respectively. JUNYI Power Supply of JY300C was used for DNA agarose gel electrophoresis, data recorded in the Molecular Imager of Gel Doc TM XR+ with Image Lab TM Software of BIO-RAD. Inhibition rate is calculated by software of Graph Pad Prism 5.01 (Graph Pad Software, USA).

2. Synthesis and characterization of compounds CXL118, CXL121, and CXL122

The synthetic procedures of probes **CXL118**, **CXL121**, and **CXL122** were depicted in Scheme S1. All the designed compounds contain two parts that are chromophoric units and *N*-mustards "warhead". Initially, the intermediates of chromophoric units **4**, **5**, and **6** and *N*-mustards "warhead" including 4-(2-(bis(2-chloroethyl)amino)ethoxy)benzaldehyde (**3**) were synthesized.

(E)-2-(2-(4-(2-(bis(2-chloroethyl)amino)ethoxy)styryl)-4H-chromen-4-yliden e)malononitrile (CXL118): 2-(2-methyl-4H-chromen-4-ylidene)malononitrile **(4)** (208 mg, 1 mmol) and 4-(2-(bis(2-chloroethyl)amino)ethoxy)benzaldehyde **(3)** (289

mg, 1 mmol) were dissolved in dry acetonitrile (100 mL for 14mmol) under argon. Piperidine (91 μL, 1 mmol) was added and the solution was stirred at 60 ℃ for 8 h. The red solution was concentrated and the product was purified by crystallization or column chromatography. Brick red solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.93 (t, J = 12.4 Hz, 1H, Ar-H), 7.75 (t, J = 8.2 Hz, 1H, Ar-H), 7.58-7.56 (m, 4H, Ar-H), 7.45 (t, J =4.2 Hz, 1H, Ar-H), 6.96(d, J = 8.4 Hz, 2H, -C*H*=C*H*-), 6.85 (s, 1H, Ar-H), 6.69(d, J = 8.6 Hz, 1H, Ar-H), 4.10(t, J = 6.4 Hz, 2H, -O-CH_2-CH_2-), 3.56 (t, J = 6.2 Hz, 4H, N(CH_2)$_2$), 3.10-3.03(m, 4H, -CH_2Cl), 2.02 (m, 2H, -O-CH_2-CH_2-). ^{13}C NMR (101 MHz, DMSO) δ 159.08, 153.37, 152.50, 139.13, 135.81, 130.56, 126.57, 125.10, 119.49, 117.56, 116.43, 115.60, 106.54, 60.00, 56.58, 53.03. MS m/z (ESI): calcd for $C_{26}H_{24}Cl_2N_3O_2^+$: 480.12; found 480.13.



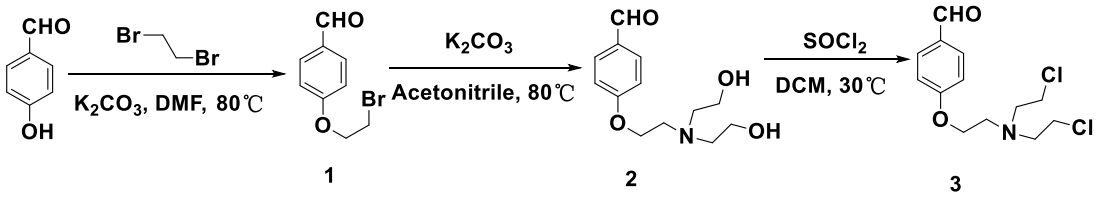
Scheme S1. Synthetic routes of compounds **CXL118**, **CXL121**, and **CXL122.**

(E)-2-(4-(2-(bis(2-chloroethyl)amino)ethoxy)styryl)-1-ethyl-3,3-dimethyl-3H-indol-1-ium (CXL121): The specific synthesis method is the same as that of the compound **CXL118**, and the reaction mixture gives the compound **CXL121** as a dark green solid. Yield 23%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.30 (t, J = 8.6 Hz, 2H, Ar-H), 8.22 (d, J = 8.2 Hz, 1H, Ar-H), 7.56 (d, J = 10.4 Hz, 1H, Ar-H), 7.57-7.50 (m, 4H, Ar-H), 7.06 (t, J = 10.6 Hz, 2H, -C*H*=C*H*-), 5.05-5.00 (q, J = 14.4 Hz, 7.2 Hz, 2H, -NCH_2CH$_3$), 4.15 (t, J = 6.4 Hz, 2H, -O-CH_2-CH$_2$-), 3.52 (t, J = 8.6 Hz, 3H, -CH_2Cl), 3.25-3.19(m, 1H, -CH_2Cl), 3.18-2.99 (m, 6H, N(CH_2)$_3$), 1.84 (s, 6H, -(CH_3)$_2$), 1.62(t, J = 6.8 Hz, 3H, -CH$_2$-CH_3). ^{13}C NMR (101 MHz, DMSO) δ 181.70, 162.16, 154.21, 153.84, 144.26, 140.90, 133.67, 130.10, 129.56, 128.61, 123.57, 116.22, 115.41, 110.95, 55.91, 52.58, 51.75, 28.81, 26.23, 22.53, 14.15. MS m/z (ESI): calcd for $C_{26}H_{33}Cl_2N_2O^+$: 459.19; found 459.19.

(E)-2-(4-(2-(bis(2-chloroethyl)amino)ethoxy)styryl)-3-ethyl-1,1-dimethyl-1H-benzo[e]indol-3-ium (CXL122): The specific synthesis method is the same as that of the compound **CXL118**, and the reaction mixture gives the compound **CXL122** as a dark green solid. Yield 19%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32-8.19 (m, 4H, Ar-H), 8.12 (d, J = 8.6 Hz, 1H, Ar-H), 8.07 (t, J = 10.6 Hz, 1H, Ar-H), 7.84 (t, J = 8.4 Hz, 1H, Ar-H), 7.77-7.71 (m, 2H, Ar-H), 7.67(t, J = 8.0 Hz, 1H, Ar-H), 7.09(dd, J = 12.4 Hz, 8.2 Hz, 2H, -C*H*=C*H*-), 5.17(q, J = 10.6 Hz, 8.4 Hz, 2H, -NCH_2CH$_3$),4.16(t, J = 8.8 Hz, 2H, -O-CH_2-CH$_2$-), 3.56(t, J = 4.8 Hz, 3H, -CH_2Cl), 3.19(t, J = 12.0 Hz, 1H，-CH_2Cl), 3.12-3.01(m, 6H, N(CH_2)$_3$), 2.10(s, 6H, -(CH_3)$_2$), 1.69(t, J = 8.2 Hz , 3H, -CH$_2$-CH_3). ^{13}C NMR (101 MHz, DMSO) δ 182.32, 163.12, 157.92, 153.89, 138.68,

133.71, 131.51, 131.14, 130.06, 128.82, 127.03, 123.51, 115.89, 115.46, 113.53, 109.74, 59.39, 57.46, 54.09, 53.74, 26.11, 14.34. MS m/z (ESI): calcd for $C_{30}H_{35}Cl_2N2O^+$: 509.2121; found 509.22.

3. Synthesis of intermediate compounds

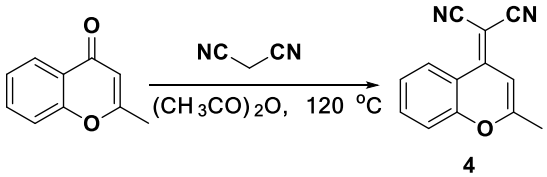


4-(2-bromoethoxy)benzaldehyde (1): p-Hydroxybenzaldehyde (0.02 mol, 2.44 g), dibromoethane (0.04 mol, 15.04 g) and K_2CO_3 (0.04 mol, 5.52 g) were dissolved in 30 mL DMF solution and reacted at 80 °C for 6 hours. TLC monitoring showed that after the reaction was completed, K_2CO_3 solid was filtered by heat, diluted by water, extracted by ethyl acetate, washed by saturated $NaHCO_3$ three times, and then dried by spinning. The colorless oily liquid was obtained by crossing the column with PE: DCM=1:1. Under the condition of 0 °C, solid was precipitated and then filtered to obtain 2.9 g white solid with a yield of 63.88%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.89 (s, 1H), 7.85 (d, J =8.6 Hz, 2H), 7.02 (d, J = 12.4 Hz, 2H), 4.38 (t, J = 6.8 Hz, 2H), 3.67(t, J = 8.4 Hz, 2H). MS m/z (ESI): calcd for $C_9H_{10}BrO_2^+$: 228.10; found 228.09.

4-(2-(bis(2-hydroxyethyl)amino)ethoxy)benzaldehyde (2)： Compound **1** (1.0 eq, 10 mmol, 2.27 g), diethanolamine (5.0 eq, 50 mmol, 5.25 g, 5.2 mL) and K_2CO_3 (4.0 eq, 40 mmol, 5.42 g) were dissolved in 5 mL acetonitrile solution and reacted at 80 °C for 10 hours. A colorless oily liquid of 1.09 g was obtained by filtering K_2CO_3 solids while hot, drying and passing through the column with a yield of 43.08%. ^{1}H

NMR (400 MHz, DMSO-d_6) δ 9.87 (s, 1H), 7.86 (d, J =8.4 Hz, 2H), 7.12 (d, J = 10.6 Hz, 2H), 4.36 (t, J = 6.8 Hz, 2H), 4.14 (t, J = 6.2 Hz, 2H), 3.45(q, J = 10.6 Hz, 6.4 Hz, 4H),2.93 (t, J = 6.4 Hz, 2H),2.65 (t, J = 8.4 Hz, 4H). MS m/z (ESI): calcd for $C_{13}H_{19}NO_4^+$: 254.14; found 254.13.

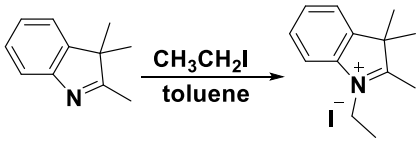
4-(2-(bis(2-chloroethyl)amino)ethoxy)benzaldehyde (3): The 253 mg compound **2** obtained from the previous step was dissolved in anhydrous DCM. Under the protection of nitrogen, $SOCl_2$ of 1 mL was dripped, N, N-dimethylformamide of catalytic amount was added, stirred for 1 hour at room temperature, and then reacted at 40 °C for 2-4 hours. TLC monitoring showed that 200 mg colorless oily liquid was obtained by drying the reaction liquid and crossing the column with EA: PE=1:4. The yield was 68.97%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.89 (s, 1H), 7.84 (d, J =8.6 Hz, 2H), 7.00 (d, J = 8.6 Hz, 2H), 4.12 (t, J = 8.4 Hz, 2H), 3.54 (t, J = 6.4 Hz, 4H), 3.09(t, J=6.4 Hz, 2H), 3.00 (t, J = 6.8 Hz, 4H). MS m/z (ESI): calcd for $C_{13}H_{17}Cl_2NO_2^+$: 290.07; found 290.08.



4

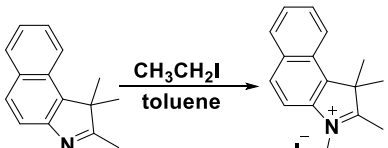
2-(2-methyl-4H-chromen-4-ylidene)malononitrile (4): To a solution of 2-methyl-4H-chromen-4-one (2.20g, 0.0403 mol) and malononitrile (1.54g, 0.0483 mol) in acetic anhydride, the reaction mixture was stirred at 120 °C for 10 h. Add 10 mL of H_2O to reflow 30 min, vacuum concentration to solid, reoccupy MeOH recrystallization, a red crystal was formed, and the crystal was filtered and washed by hexane, yield: 40.3%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.92 (d, J = 8.8 Hz, 1H), 7.72 (t,

J = 7.3 Hz, 1H), 7.49 – 7.43 (m, 2H), 6.72 (s, 1H), 2.44 (s, 3H). MS m/z (ESI): calcd for $C_{13}H_{19}N_2O^+$: 209.07; found 209.16.



5

1-ethyl-2, 3, 3-trimethyl-3H-indol-1-ium (5): A solution of 2,3,3-trimethyl-3H-indole (1.59 g, 0.010 mmol) and methyl iodide (1.69 g, 0.012 mmol) was dissolved in 100 mL of toluene and slowly heated to reflux for 10 hours. The reaction progress was monitored by TLC. After completion of the reaction, the solution in the reaction system was spin-dried under a vacuum pump and solidified by the addition of petroleum ether. The product was used directly in the next step reaction. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.17 (d, *J* = 8.6 Hz, 1H), 7.76 (d, *J* = 8.4 Hz, 1H), 7.12-6.72 (m, 2H), 3.53 (dd, *J* =8.2 Hz, 4.7 Hz, 2H), 2.34 (s, 3H), 1.77 (s, 6H), 1.51 (t, J = 8.6 Hz, 3H). MS m/z (ESI): calcd for $C_{13}H_{18}N^+$: 188.14; found 188.16.



6

3-ethyl-1, 1, 2-trimethyl-1H-benzoindol-3-ium (6): The method is the same as compound **5**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.38 (d, *J* = 8.8 Hz, 1H), 8.31 (d, *J* = 12.2 Hz, 1H), 8.23 (d, *J* = 8.0 Hz, 1H), 8.16 (d, *J* = 8.6 Hz, 1H), 7.82-7.72 (m, 2H), 4.63 (dd, *J* =12.2 Hz, 4.6 Hz, 2H), 2.94 (s, 3H), 1.77 (s, 6H), 1.16 (t, *J* = 8.6 Hz, 3H). MS m/z (ESI): calcd for $C_{17}H_{20}N^+$ 238.15, found 238.05.

4. Spectrometric determination of other compounds

Compounds **CXL118**, **CXL121**, and **CXL122** were respectively dissolved in DMSO, the concentration of solution is 1 mM. When measuring absorption and emission wavelength, these compounds were diluted into assay solution (10 μM, 1mL) from the original solution (1 mM, 1 μL) by adding DMSO and PBS solution. The emission wavelength and absorption wavelength were measured by measuring instrument.

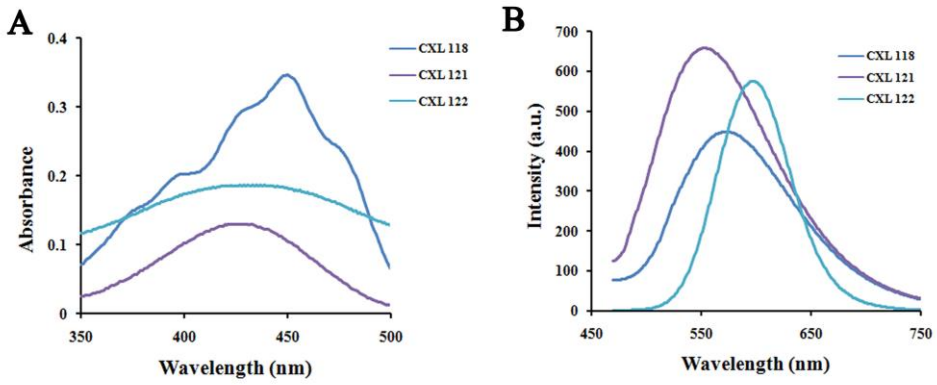


Figure S1. **Absorption and emission spectra of compounds CXL118, CXL121, and CXL122 in DMSO solution (10 μM). A**: The absorption spectrum of compounds. **B**: The emission spectra of compounds.

Table S1 The excitation wavelength and emission wavelength of these compounds.

Compound	Ex(nm)	Em(nm)
CXL118	450	573
CXL121	427	553
CXL122	433	597

5. Cell apoptosis analysis by flow cytometer (FCM)

All cell lines used were obtained from American Type Culture Collection (ATCC). These cell lines were cultured in culture medium supplemented with 10% PBS (Phosphate Buffer Solution). All cell lines were maintained at 37 °C in incubator with 5% CO_2. Annexin V-FITC/PI Apoptosis Detection Kit (BDFACSCalibur) was used as described before. In brief, cells (1~2×10^5 cells/well) were seeded in 6-well plates and treated with compound for 72 h. Following the manufacturer's instructions, data were collected by FCM and analyzed with Flow Jo7.6.1 software.

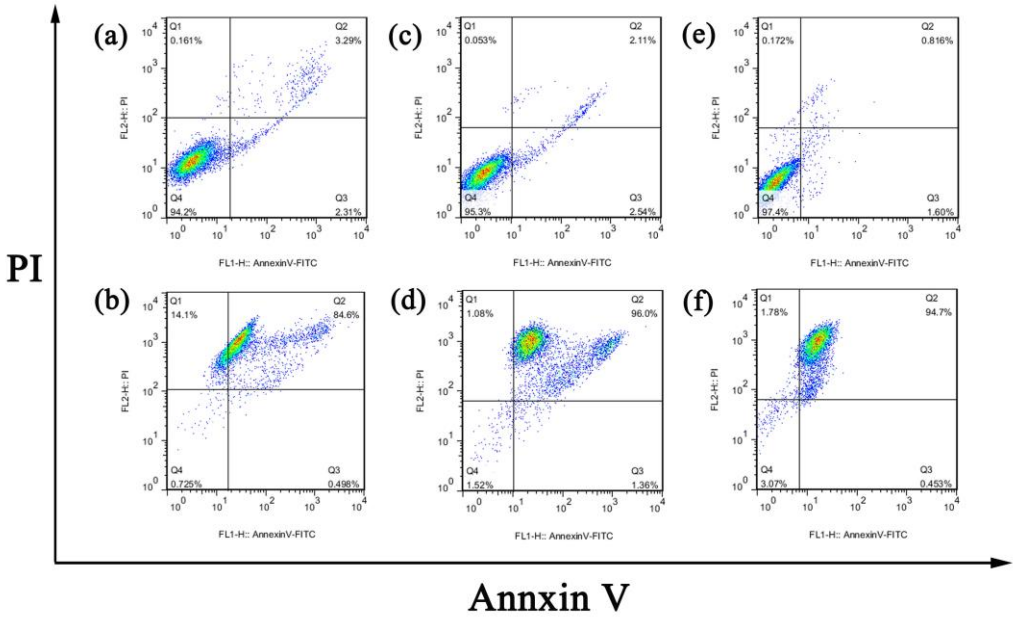


Figure S2: The cell apoptosis induced by **CXL122** was shown. (a, c, and e): control, only cells and medium, without probes; (b): With the compounds, and added Annexin V and PI, respectively; (d): Added compounds, with Annexin V, respectively; (f): No dyeing, only add the compounds, respectively.

6. The cell toxicity test of these compounds

All cell lines used were obtained from American Type Culture Collection (ATCC). These cell lines were cultured in culture medium supplemented with 10%

FBS (Fetal Bovine Serum) at 37 °C in incubator with 5% CO_2. A preliminary MTT assay was performed on cultured A549 (human non-small cell lung cancer cells), DU145 (human prostate cancer cells) and HeLa (human cervical cancer cells), respectively. These compounds include **CXL118**, **CXL121**, and **CXL122**. The Figure S3 shows a summary of the inhibitory rates of different concentrations of different compounds under different cells in three different cancer cells.

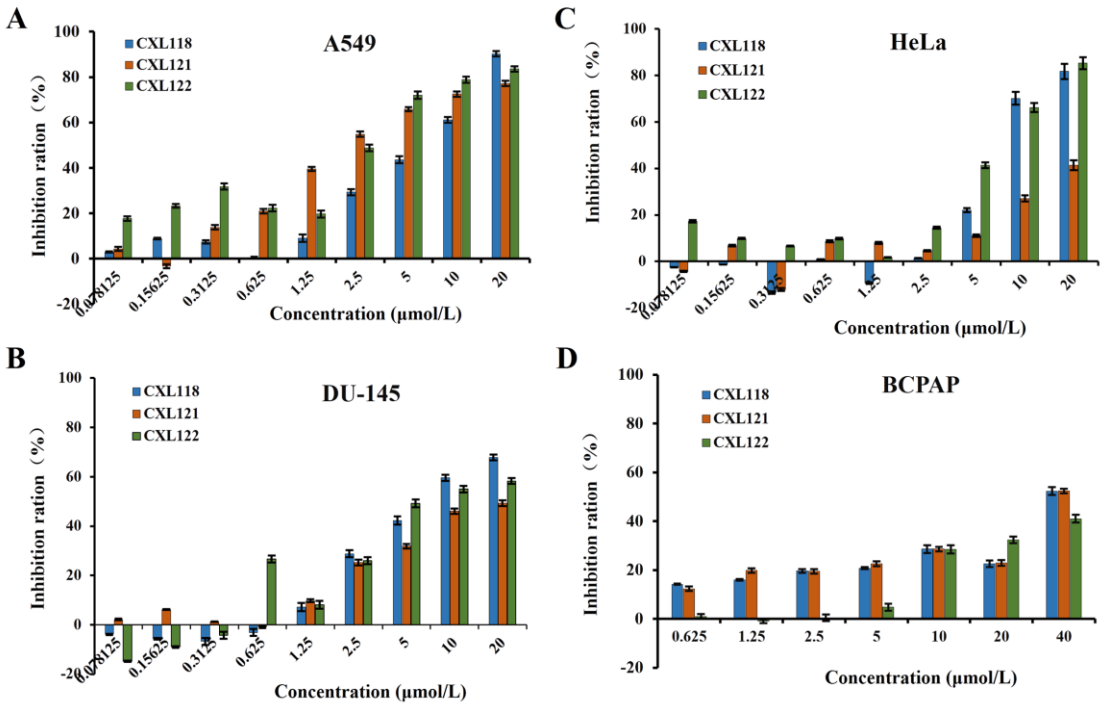
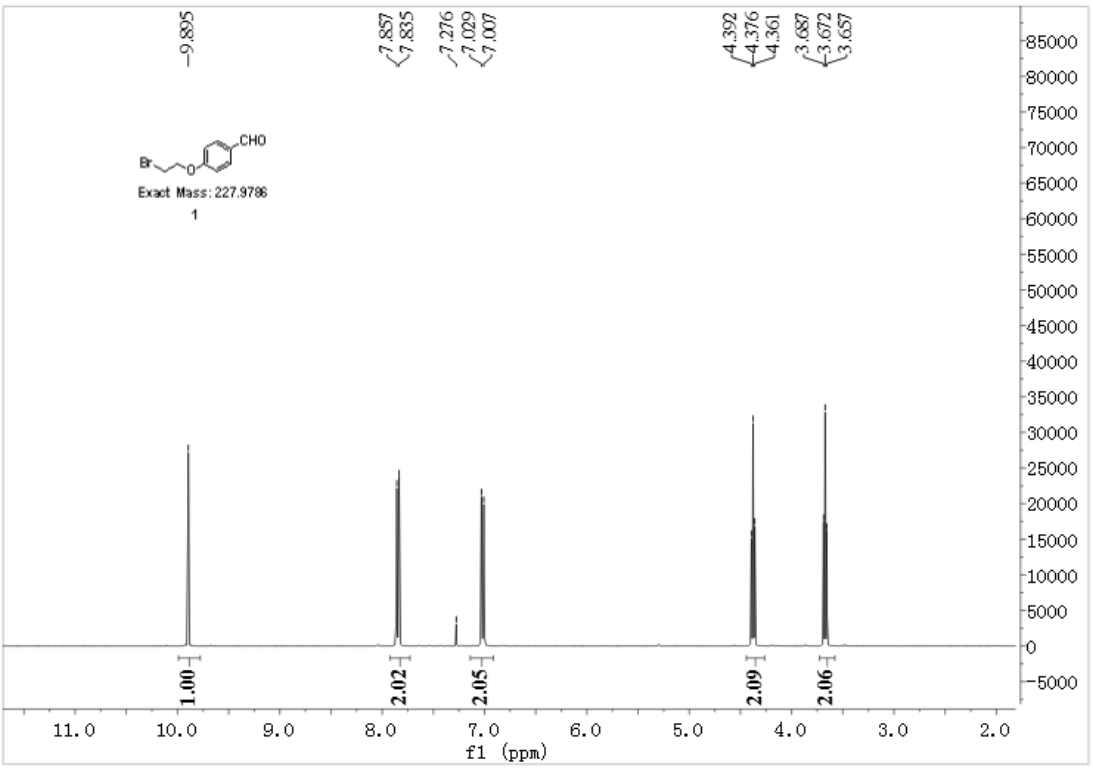
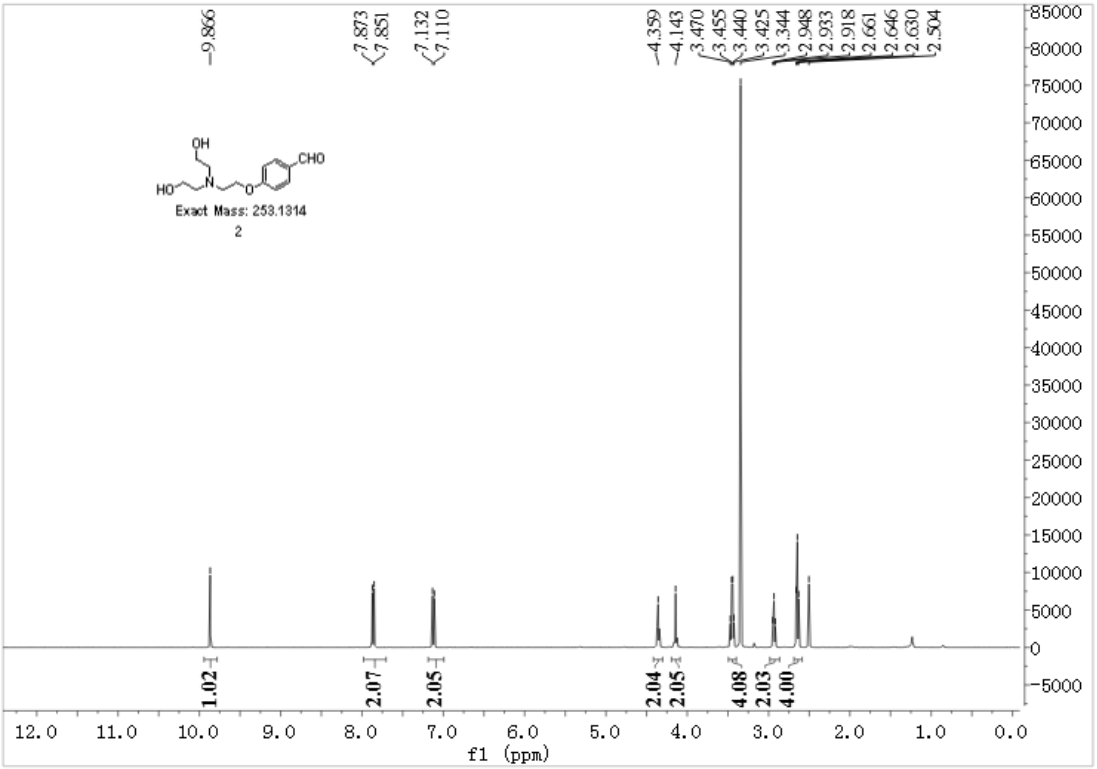
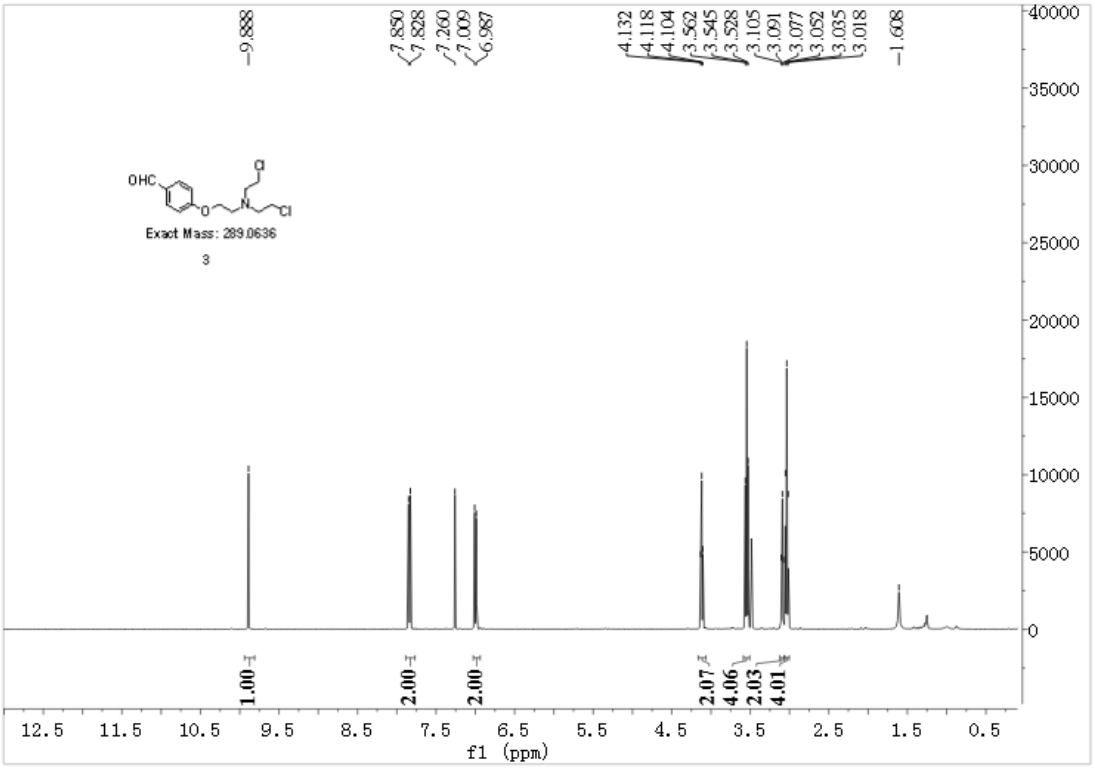


Figure S3: MTT assay on cultured HeLa (human cervical cancer cells), DU145 (human prostate cancer cells) and A549 (human non-small cell lung cancer cells) with different compounds**.**

7. NMR spectrum



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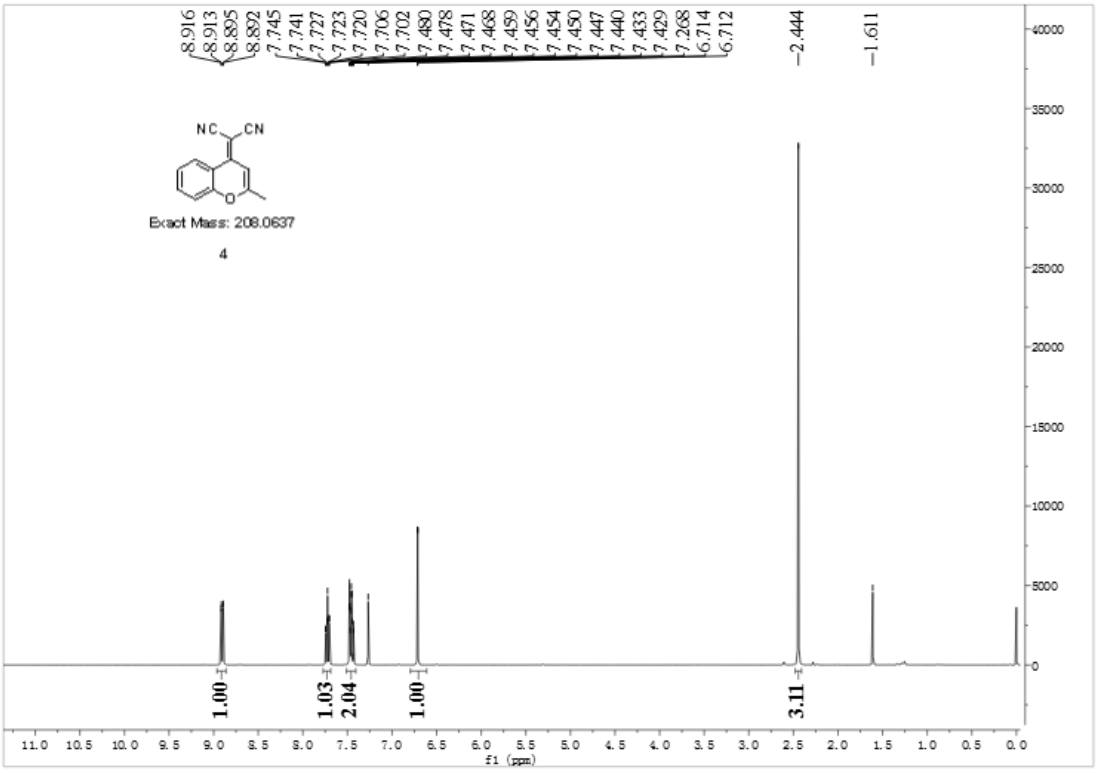
f1 (ppm)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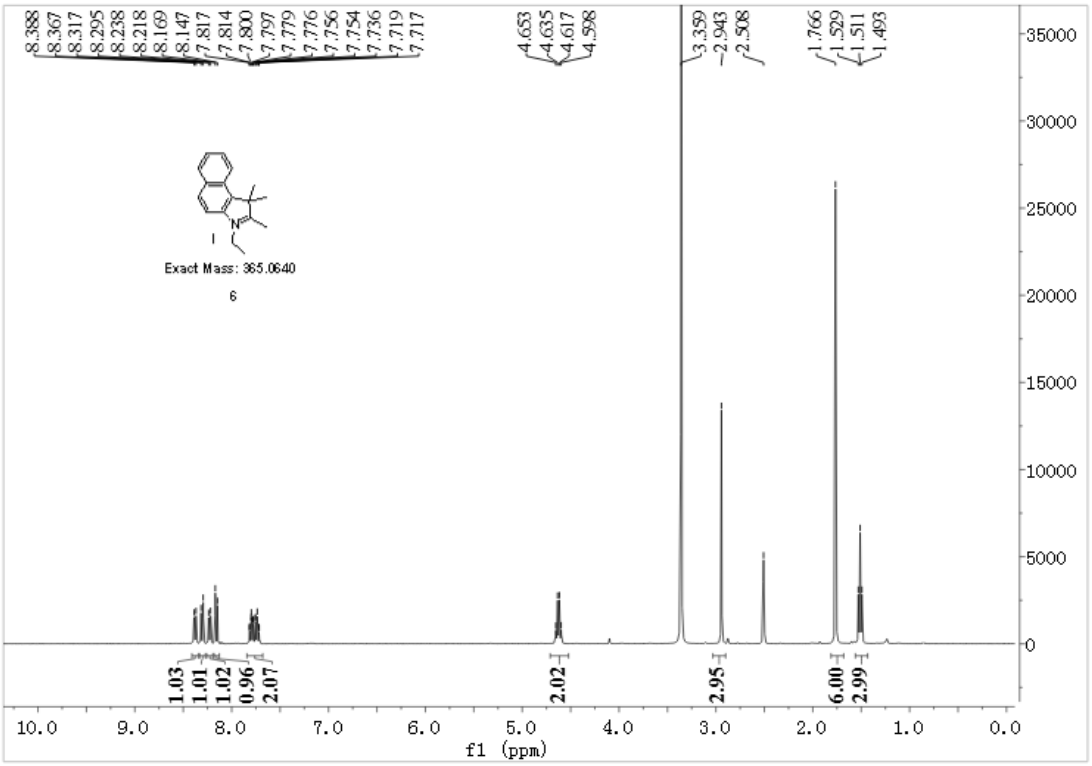
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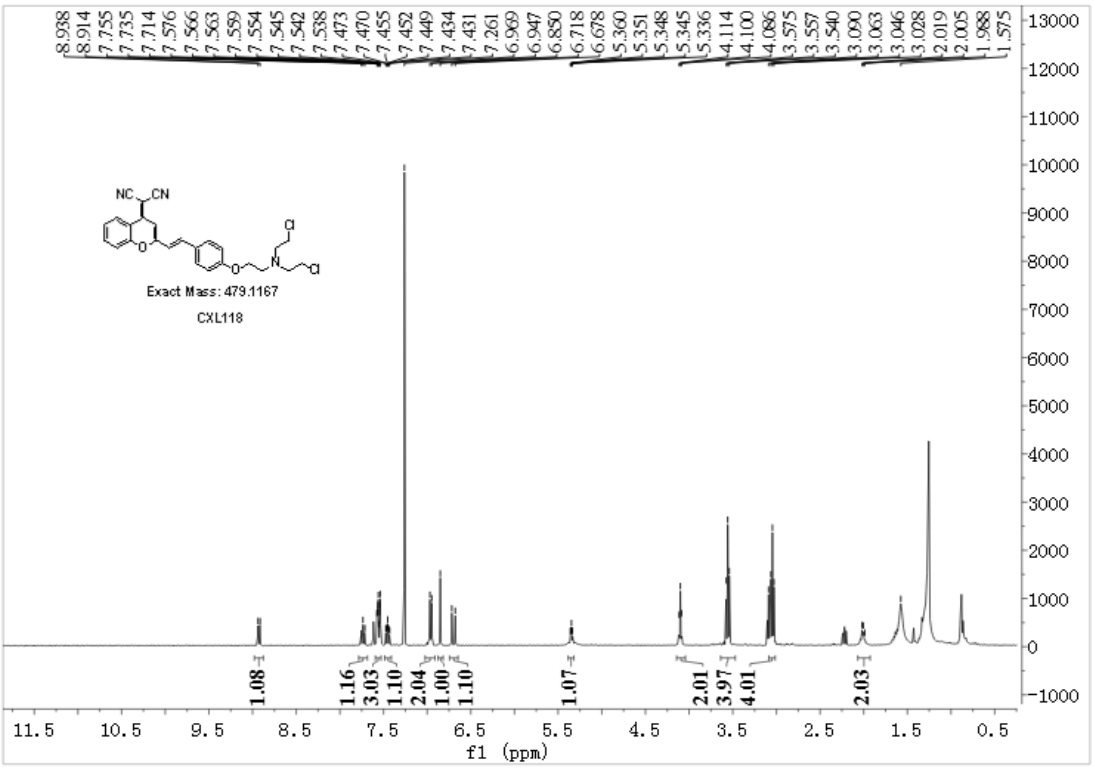
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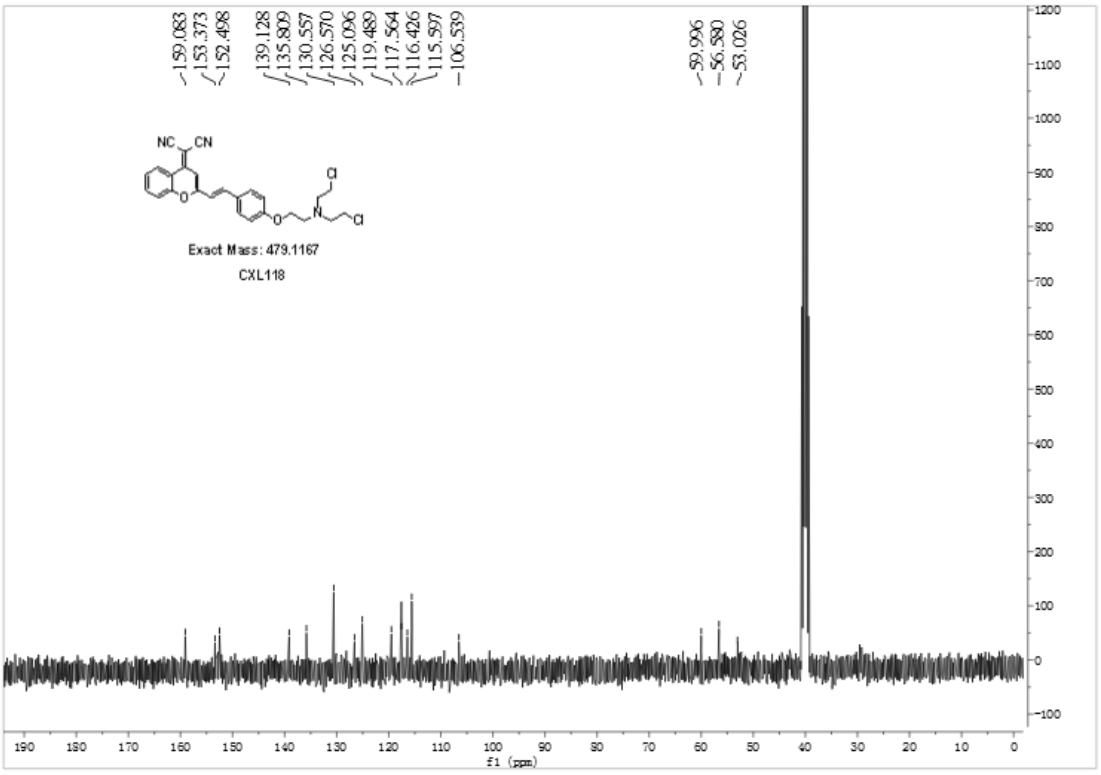
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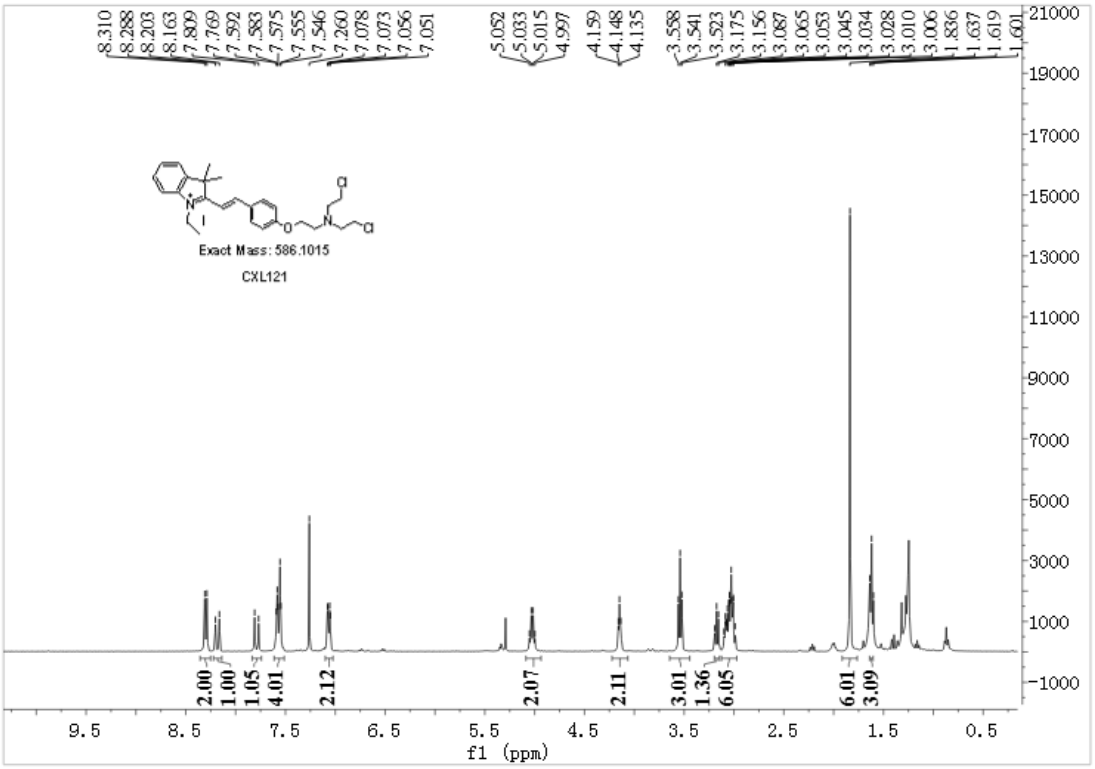
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3.087
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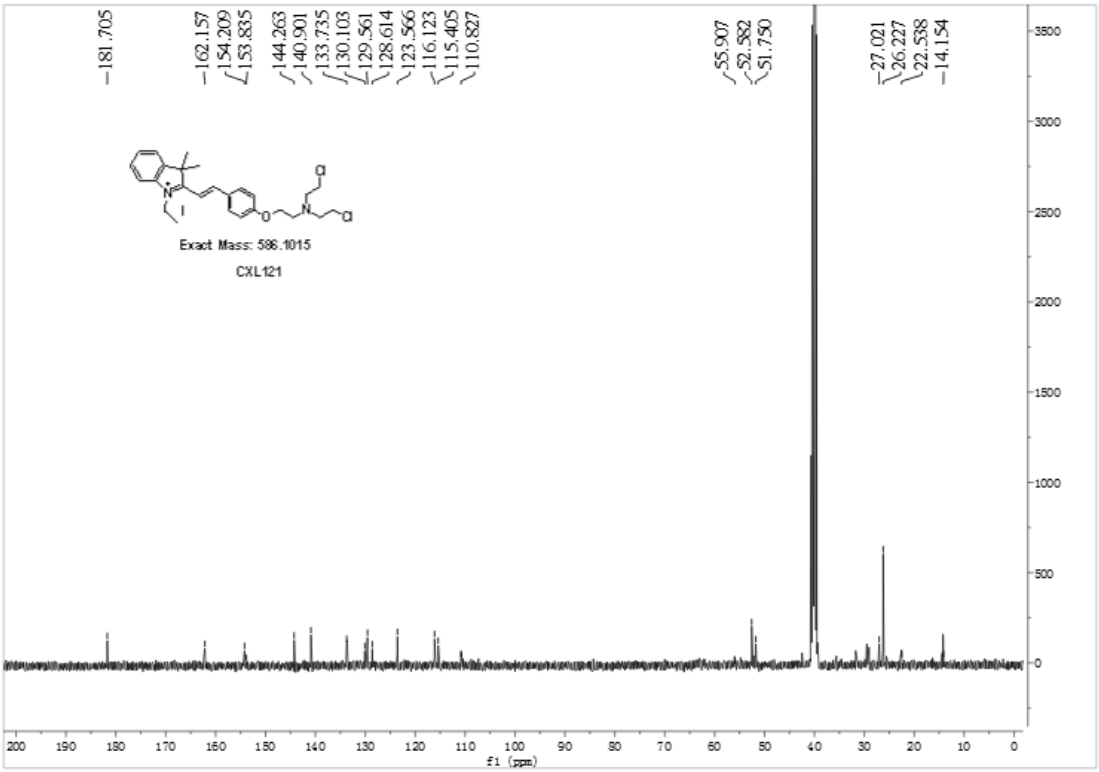
f1 (ppm)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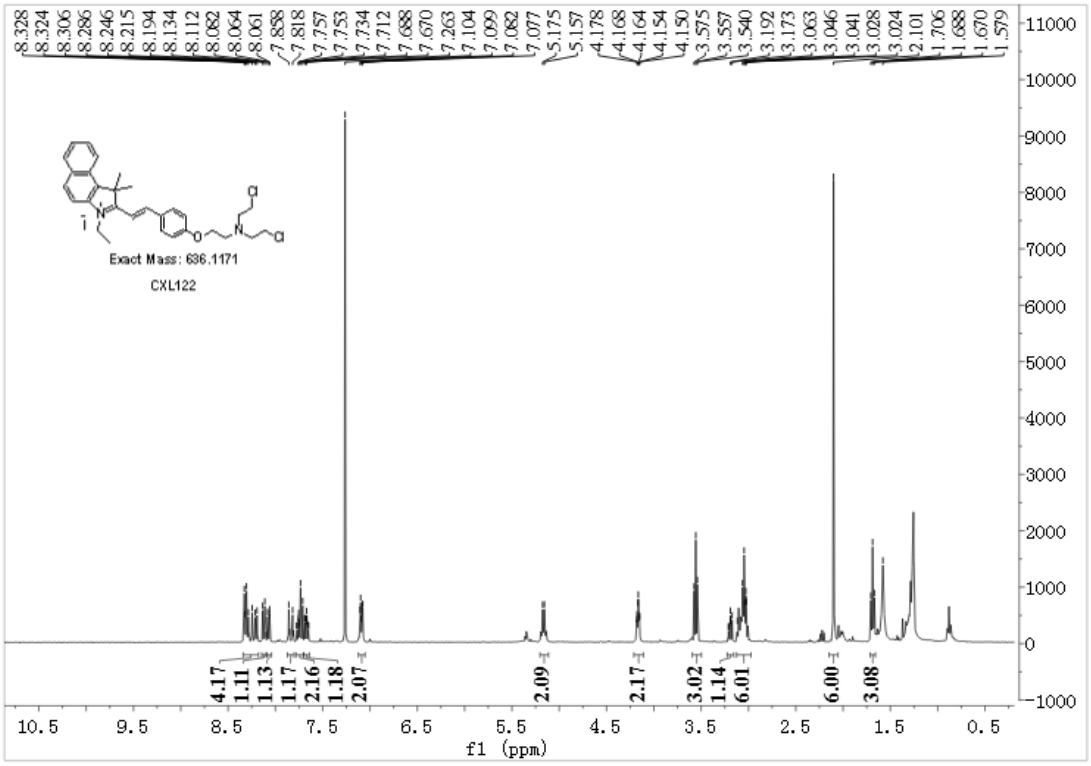
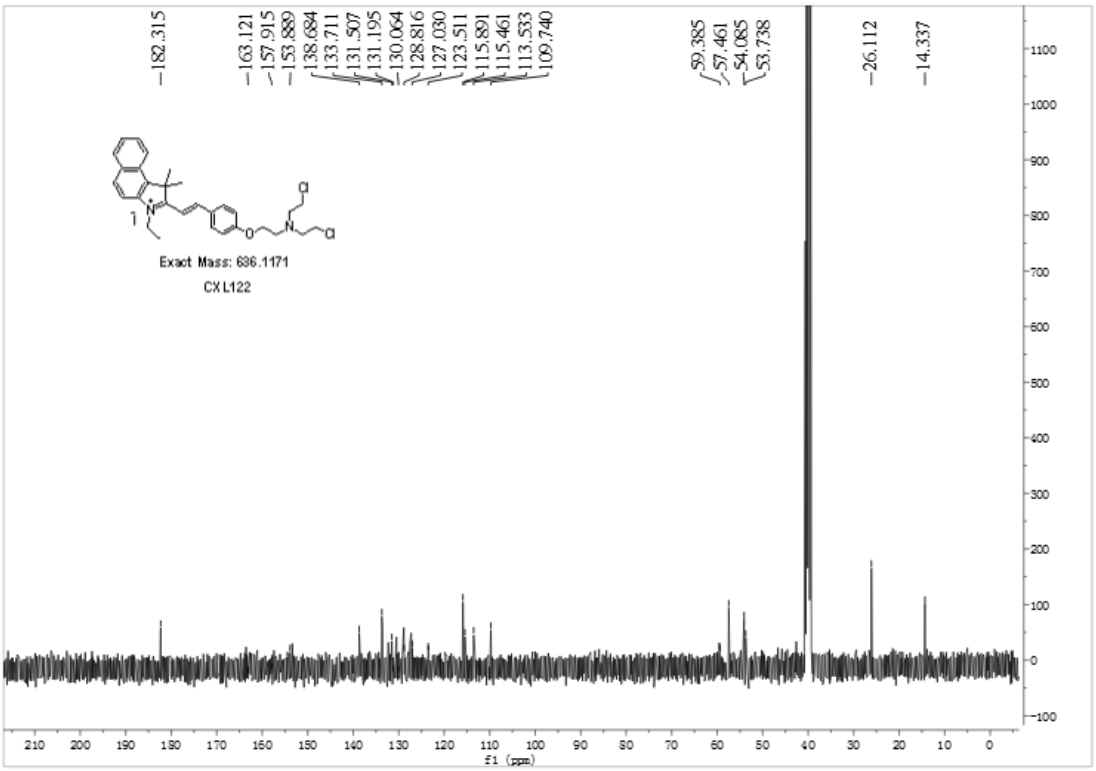
153.835
144.263
140.901
133.735
130.103
129.561
128.614
123.566
116.123
115.405
110.827
55.907
52.582
51.750
27.021
26.227
22.538
14.154
Exact Mass: 586.1015
CXL121
f1 (ppm)


Exact Mass: 636.1171
CXL122
f1 (ppm)


Exact Mass: 636.1171
CXL122
f1 (ppm)