Supporting Information

Discovery of Novel 4-Arylisochromenes as Anticancer Agents Inhibiting Tubulin Polymerization

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Contents

1. Chemistry

1.1 General

Most chemicals and solvents were purchased from commercial sources. Further purification and drying by standard methods were employed when necessary. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker-300 spectrometers in the indicated solvents (TMS as internal standard). Data are reported as follows: chemical shift in ppm (d), multiplicity (s =singlet, d =doublet, t =triplet, q =quartet, brs = broad singlet, m = multiplet), coupling constant (Hz), and integration. High Resolution Mass measurement was performed on Agilent QTOF 6520 mass spectrometer with electron spray ionization (ESI) as the ion source. Purity of all tested compounds was ≥ 95%, as estimated by HPLC (Aglilent Technologies 1260 Infinity) analysis on the Aglilent C18 column (4.6 × 150 mm, 5 μm). Flash column chromatography was carried out using commercially available silica gel (200-300 mesh) under pressure. Optical rotation was measured on a Perkin Elmer 341 Polarimeter at 589 nm. Chiral HPLC enantioseparation were performed on a shimadzu LC-20AD (UV dection at λ = 214 nm), equipped with a CHIRALPAK AD-H (0.46 cm I.D. × 15 cm L).

1.2 General procedure for synthesis of intermediate 12a-e.

To a solution of precursors with weinreb amides **11a-e** (1mmol) in dry THF (10 mL), *t*-BuLi (2.2 mmol) was added at -78 °C, and after 1 min, the reaction was quenched by the addition of sat. NH_4Cl (10 mL). The reaction mixture was then extracted with EtOAc (3 × 25 mL). The combined organic extracts were then washed with brine (25 mL), dried over Na_2SO_4 and concentrated in vacuo. Following flash column chromatography afforded **12a-e** in 72-93% yields.

1.2.1 Intermediate 12a

White solid (165 mg, yield 92.6%), ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.01 (d, J = 8.7 Hz, 1H), 6.91 (d, J = 8.6 Hz, 1H), 6.66 (s, 1H), 4.85 (s, 2H), 4.32 (s, 2H), 3.87 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.68, 164.19, 144.18, 128.94, 123.12, 114.11, 108.66, 73.32, 68.02, 55.56; HR-MS (ESI) m/z: calcd for $C_{10}H_{11}O_3$ $[M+H]^+$ 179.0703, found

179.0701; The spectra is accordance with the previous report [1].

1.2.2 Intermediate 12b

White solid (205 mg, yield 72.1%), ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.86 (d, *J* = 8.6 Hz, 1H), 7.38 (s, 5H), 6.99 (d, *J* = 8.7 Hz, 1H), 5.03 (s, 2H), 4.70 (s, 2H), 4.22 (s, 2H), 3.99 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.96, 157.17, 141.93, 136.97, 136.17, 128.54, 128.46, 128.40, 123.83, 123.41, 111.18, 74.85, 72.85, 64.01, 55.95; HR-MS (ESI) m/z: calcd for $C_{17}H_{17}O4$ $[M+H]^+$ 285.1121, found 285.1120.

1.2.3 Intermediate 12c

White solid (183 mg, yield 87.9%), ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.90 (d, *J* = 8.6 Hz, 1H), 7.01 (d, *J* = 8.7 Hz, 1H), 5.00 (s, 2H), 4.34 (s, 2H), 4.00 (s, 3H), 3.90 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.90, 157.13, 143.36, 135.57, 123.69, 123.38, 111.21, 72.87, 63.75, 60.58, 55.88; HR-MS (ESI) m/z: calcd for $C_{11}H_{13}O_4$ $[M+H]^+$ 209.0808, found 209.0810.

1.2.4 Intermediate 12d

White solid (225 mg, yield 79.1%), ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.56 (s, 1H), 7.51 - 7.28 (m, 5H), 6.65 (s, 1H), 5.18 (s, 2H), 4.83 (s, 2H), 4.31 (s, 2H), 3.95 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.64, 137.05, 136.29, 128.60, 128.38, 128.09, 127.51, 126.17, 119.30, 109.82, 106.44, 72.99, 70.92, 67.63, 56.22; HR-MS (ESI) m/z: calcd for $C_{17}H_{17}O_4$ $[M+H]^+$ 285.1121, found 285.1123.

1.2.5 Intermediate 12e

White solid (224 mg, yield 87.1%), ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.07 (d, *J* = 8.7 Hz, 1H), 6.96 (d, *J* = 8.7 Hz, 1H), 4.94 (s, 2H), 4.32 (s, 2H), 4.00 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 191.61, 160.16, 143.35, 131.85, 123.88, 111.83, 106.69, 72.98, 67.68, 56.62; HR-MS (ESI) m/z: calcd for $C_{10}H_{10}BrO_3$ $[M+H]^+$ 256.9808, found 256.9803.

1.3 General procedure for synthesis of intermediate 12f and 12g.

To a solution of **13f** or **13g (**1mmol**)** in MeCN (10 mL), K_2CO_3 (1.2mmol) and ethyl thioglycolate (1.2mmol) were added. After stirring for 2 h, the reaction mixture was then extracted with EtOAc (3 × 25 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Then the obtained crude products were dissolved into CH_3OH (5 mL), following by the addition of 10% NaOH aqueous (5 mL). After stirring at 80 °C for 1 h, the reaction mixture was acidified to pH 5 by the addition of 10% aqueous HCl, the residues were extracted with EtOAc (3 × 25 mL), dried over anhydrous Na_2SO_4, and concentrated in vacuo to afford **14f** or **14g**, which were directly used for next step without purification. To a solution of intermediates **14f** or **14g (**1mmol**)** in CH_2Cl_2 (10 mL), a drop of DMF was added as the catalyzer, then oxalyl chloride (1.2 mmol) was slowly added at 0 °C, the mixture was stirred at ambient temperature for 1h. Then the solvent was removed in vacuo, and the residue

was dissolved in chlorobenzene (10 mL), anhydrous $SnCl_4$ (1.2 mmol) was added under N_2 atmosphere. The mixture was stirred for 2h, and the solvent was removed in vacuo. The residue was then extracted with EtOAc (3 × 25 mL). The combined organic extracts were washed with brine (25 mL), dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by flash column chromatography using PE/EA (5:1,V/V) as an eluent to afford the product **12f** and **12g** in 55-60% yields.

1.3.1 Intermediate 12f

White solid (107 mg, yield 55.1% over two steps), ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.07 (d, *J* = 8.8 Hz, 1H), 6.86 (dd, *J* = 8.8, 2.2 Hz, 1H), 6.67 (s, 1H), 3.87 (s, 2H), 3.86 (d, *J* = 2.9 Hz, 3H), 3.52 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 190.09, 163.04, 144.07, 131.51, 125.25, 113.31, 112.40, 55.52, 36.95, 30.92; HR-MS (ESI) m/z: calcd for $C_{10}H_{11}O_2S$ $[M+H]^+$ 195.0474, found 195.0476.The spectra is accordance with the previous report [2].

1.3.2 Intermediate 12g

White solid (134 mg, yield 59.7% over two steps), ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.91 (d, *J* = 8.8 Hz, 1H), 6.91 (d, *J* = 8.8 Hz, 1H), 3.95 (s, 2H), 3.93 (s, 3H), 3.83 (s, 3H), 3.47 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 190.16, 156.40, 144.31, 135.90, 126.08, 125.67, 110.60, 60.48, 55.86, 36.49, 24.62; HR-MS (ESI) m/z: calcd for $C_{10}H_{13}O_3S$ $[M+H]^+$ 225.0580, found 225.0582 .

1.4 General procedure for synthesis of compound 17a-g.

To a solution of **12a-g (**1.69 mmol**)** in EtOH (10 mL), *p*-toluenesulfonhydrazide (375mg, 2.01mmol) was added. After stirring for 2 h, then the mixture was stirred at 90 °C. After completion, the reaction was cooled to room temperature, the precipitated solid was filtrated and washed by cooled EtOH to afford sulfonyl hydrazones **15a-g** as yellow solids, which was used for next step directly. To a solution of intermediate **15a-g** (0.58 mmol) in 1,4-dioxane (2 mL), 5-bromo-1,2,3-trimethoxybenzene **16** (173mg, 0.7mmol), $PdCl_2(CH_3CN)_2$ (21mg, 0.06mmol), Xphos (28mg, 0.06mmol), *t*-BuOLi (93mg, 1.16mmol) were added. The mixture was stirred in sealed tube at 90 °C for 2 h. The reaction mixture was filtrated, and the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography using PE/EA (5:1, V/V) as an eluent to afford the product **17a-g** in 45-69% yields.

1.4.1 Compound 17a.

White solid (85 mg, yield 44.6%), ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.99 (d, *J* = 8.5 Hz, 1H), 6.75 (dd, *J* = 8.5, 2.7 Hz, 1H), 6.66 (d, *J* = 10.2 Hz, 2H), 6.58 (s, 2H), 5.07 (s, 2H), 3.89 (s, 3H), 3.86 (s, 6H), 3.81 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.41,

152.79, 141.26, 136.75, 131.15, 129.66, 123.89, 123.22, 119.63, 112.33, 109.96, 105.66, 68.01, 60.42, 55.65, 54.91; HR-MS (ESI) m/z: calcd for $C_{19}H_{21}O_5$ $[M+H]^+$ 329.1384, found 329.1384.

1.4.2 Compound 17c.

White solid (105 mg, yield 50.5%), ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.75 (s, 2H), 6.63 (s, 1H), 6.58 (s, 2H), 5.21 (s, 2H), 3.89 (s, 3H), 3.86 (s, 3H), 3.86 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.76, 151.39, 143.70, 141.44, 136.74, 131.13, 124.53, 121.98, 119.21, 117.75, 110.64, 105.76, 62.79, 60.41, 55.64, 55.33; HR-MS (ESI) m/z: calcd for $C_{20}H_{23}O_6$ $[M+H]^+$ 359.1489, found 359.1484.

1.4.3 Compound 17e.

White solid (103 mg, yield 43.6%), ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.94 (d, J = 8.5 Hz, 1H), 6.75 (d, J = 8.5 Hz, 1H), 6.63 (s, 1H), 6.55 (s, 2H), 5.27 (s, 2H), 3.89 (s, 6H), 3.86 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 154.41, 152.84, 141.63, 136.88, 130.65, 129.49, 125.62, 121.80, 118.76, 110.12, 109.37, 105.81, 67.52, 60.43, 55.89, 55.66; HR-MS (ESI) m/z: calcd for $C_{19}H_{20}BrO_5$ $[M+H]^+$ 407.0489, found 407.0481.

1.4.4 Compound 17f.

White solid (96 mg, yield 48.1%), ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.00 (d, J = 8.2 Hz, 1H), 6.73 (d, J = 8.3 Hz, 2H), 6.56 (s, 2H), 6.39 (s, 1H), 3.89 (s, 3H), 3.83 (s, 9H), 3.82 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 159.09, 152.61, 138.22, 136.79, 135.82, 130.95, 127.47, 126.84, 117.33, 112.08, 111.82, 105.23, 60.45, 55.64, 54.88, 30.86; HR-MS (ESI) m/z: calcd for $C_{19}H_{21}O_4S$ $[M+H]^+$ 345.1155, found 345.1156.

1.4.5 Compound 17g.

White solid (149 mg, yield 68.6%), ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.82 - 6.72 (m, 2H), 6.57 (s, 2H), 6.42 (s, 1H), 3.98 (s, 2H), 3.90 (s, 3H), 3.88 (s, 6H), 3.84 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.58, 152.43, 143.73, 138.20, 136.80, 135.87, 127.56, 123.70, 122.13, 118.12, 109.60, 105.35, 60.45, 55.65, 55.29, 23.15; HR-MS (ESI) m/z: calcd for $C_{20}H_{23}O_5S$ $[M+H]^+$ 375.1261, found 375.1256.

1.5 General procedure for synthesis of compound 18a and 18b.

To a solution of **17b** or **17d (**0.23 mmol**)** in THF, Pd/C (10 mg) was added. The mixture was stirred at ambient temperature under H_2 atmosphere for 2h. Pd/C was filtrated and the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography using PE/EA (5:1, V/V) as an eluent to afford the product **18a** or **18b** in 54-63% yields.

1.5.1 Compound 18a.

White solid (45 mg, yield 54.3%), ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.96 (s, 1H), 6.90 - 6.74 (m, 2H), 6.61 (s, 2H), 6.54 (s, 1H), 4.97 (s, 2H), 3.78 (s, 6H), 3.76 (s, 3H), 3.69 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 152.86, 146.58, 146.07, 142.03,

136.54, 131.21, 123.80, 119.31, 119.02, 109.78, 109.48, 105.85, 67.36, 65.92, 59.97, 55.84; HR-MS (ESI) m/z: calcd for $C_{19}H_{21}O_6$ $[M+H]^+$ 345.1333, found 345.1329.

1.5.2 Compound 18b.

White solid (52 mg, yield 62.7%), ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.95 (s, 1H), 6.82 (d, J = 8.4 Hz, 1H), 6.74 (s, 1H), 6.60 (s, 2H), 6.45 (d, J = 8.4 Hz, 1H), 5.07 (s, 2H), 3.78 (s, 3H), 3.77 (s, 6H), 3.69 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 152.81, 147.06, 141.66, 141.23, 131.29, 123.99, 119.22, 115.41, 113.14, 110.35, 110.33, 105.86, 62.53, 59.97, 55.76; HR-MS (ESI) m/z: calcd for $C_{19}H_{21}O_6$ $[M+H]^+$ 345.1332, found 345.1329.

1.6 General procedure for synthesis of compound (±)-19a-d.

To a solution of **17a-d (**0.23 mmol**)** in CH_3OH, Pd/C (10 mg) was added. The mixture was stirred at ambient temperature under H_2 atmosphere for 2 h. Pd/C was filtrated and the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography using PE/EA (5:1,V/V) as an eluent to afford the product **19a-d** in 85-96% yields.

1.6.1 Compound (±)-19a.

White solid (73 mg, 96.1%), ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.85 (d, J = 8.5 Hz, 1H), 6.64 (dd, J = 8.5, 2.7 Hz, 1H), 6.50 (d, J = 2.7 Hz, 1H), 6.31 (s, 2H), 4.78 (q, J = 15.0 Hz, 2H), 4.06 (dd, J = 10.9, 4.9 Hz, 1H), 3.97 - 3.93 (m, 1H), 3.83 - 3.78 (m, 1H), 3.76 (s, 3H), 3.72 (s, 6H), 3.72 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.56, 152.65, 138.49, 135.33, 130.19, 130.13, 127.72, 112.62, 108.07, 105.40, 71.84, 68.09, 60.30, 55.60, 54.74, 43.62; HR-MS (ESI) m/z: calcd for $C_{19}H_{23}O_5$ $[M+H]^+$ 331.1540, found 331.1535.

1.6.2 Compound (±)-19b.

White solid (71 mg, 89.1%), ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.75 (s, 1H), 6.77 (d, J = 8.4 Hz, 1H), 6.47 (s, 2H), 6.34 (d, J = 8.4 Hz, 1H), 4.70 (d, J = 5.0 Hz, 2H), 3.97 - 3.91 (m, 2H), 3.78 - 3.76 (m, 1H), 3.74 (s, 3H), 3.68 (s, 6H), 3.61 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 153.09, 145.44, 141.57, 139.75, 136.56, 129.71, 122.57, 119.79, 110.73, 106.56, 71.08, 64.74, 60.38, 56.27, 43.51; HR-MS (ESI) m/z: calcd for $C_{19}H_{23}O_6$ $[M+H]^+$ 347.1489, found 347.1492.

1.6.3 Compound (±)-19c.

White solid (72 mg, 86.95), ^{1}H NMR (300 MHz, $CDCl_3$) δ6.77 – 6.69 (m, 2H), 6.38 (s, 2H), 4.92 (d, J = 2.0 Hz, 2H), 4.11– 4.06 (m, 1H), 4.05 – 3.92 (m, 2H), 3.87 (s, 3H), 3.85 (s, 3H), 3.83 (s, 3H), 3.80 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.64, 149.85, 143.50, 138.39, 138.36, 128.91, 128.28, 124.29, 110.58, 105.44, 71.22, 64.51, 60.30, 59.68, 55.62, 55.25, 43.61; HR-MS (ESI) m/z: calcd for $C_{20}H_{25}O_6$ $[M+H]^+$ 361.1646, found 361.1642.

1.6.4 Compound (±)-19d.

White solid (68 mg, 85.1%), ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.75 (s, 1H), 6.63 (s, 1H), 6.49 (s, 2H), 6.32 (s, 1H), 4.78 - 4.58 (m, 2H), 4.01 - 3.91 (m, 2H), 3.80 - 3.76 (m, 1H), 3.73 (s, 3H), 3.70 (s, 6H), 3.63 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 152.65, 146.37, 144.95, 139.19, 128.05, 125.03, 115.39, 109.49, 107.71, 106.06, 71.08, 67.38, 59.89, 55.82, 55.45, 43.02; HR-MS (ESI) m/z: calcd for $C_{19}H_{23}O_6$ $[M+H]^+$ 347.1489, found 347.1484.

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1.7 The enantioseparation of (±)-19b

Chiral HPLC enantioseparation of **(±)-19b** were performed on a shimadzu LC-20AD (UV dection at λ = 214 nm), equipped with a CHIRALPAK AD-H (0.46 cm I.D. × 15 cm L) using EtOH as the mobile phase with the flow rate of 0.5 mL/min. **(±)-19b** were prepared using HPLC-grade EtOH and filtered with a membrane filter (nylon, 0.45 μm pore size, 13 mm diameter) in a stainless steel filter holder prior to injection. The separated **(-)-19b** ($[a]_D^{20}$ **= -4.5°, c = 1.0)** and **(+)-19b** ($[a]_D^{20}$ **= +4.4°, c = 1.0)** were collected and concentrated in vacuo. The obtained of chromatograms of **(±)-19b**, **(+)-19b** and **(-)-19b** were shown in Figure S5.

1.8 ECD calculations of (*R*)-19b and (*S*)-19b

(*R*)-19b and **(*S*)-19b** were drawn with Chemdraw 13 and energy-minimized using the LigPrep module of the Schrödinger suite. All the optimized conformers were used for ECD calculations using time-dependent density functional theory (TDDFT) using B3LYP/6-311++G(2d,p) in methanol. A total of 30 excited states were calculated, and only singlet excited states were considered. ECD curves were generated with SpecDis version 1.64 software and were given linewidth using a Gaussian function using a half bandwidth of 0.16 eV, for comparison with the experimental data.

2. Pharmacology

2.1 *In vitro* anti-proliferative assay

HepG2, KB, HCT-8, MDB-MD-231, K562, H22 and LO2 cells were purchased from Nanjing KeyGen Biotech Co. Ltd. (Nanjing, China). The cytotoxicity of the test compounds was determined using the MTT assay. Briefly, the cell lines were incubated at 37 °C in a humidified 5% CO_2 incubator for 24 h in 96-microwell plates. After medium removal, 100 mL of culture medium with 0.1% DMSO containing the test compounds at different concentrations was added to each well and incubated at 37 °C for another 72 h. The MTT (5 mg/mL in PBS) was added and incubated for another 4 h, the optical density was detected with a microplate reader at 490 nm. The IC_{50} values were calculated according to the dose-dependent curves. All the experiments were repeated in at least three independent experiments.

2.2 *In vitro* tubulin polymerization inhibitory assay

An amount of 2 mg/mL tubulin (Cytoskeleton) was resuspended in PEM buffer containing 80 mM piperazine-N,N'-bis(2-ethanesulfonic acid) sequisodium salt PIPES (pH 6.9), 0.5 mM EGTA, 2 mM $MgCl_2$, and 15% glycerol. Then the mixture was preincubated with compounds or vehicle DMSO on ice. PEG containing GTP was added to the final concentration of 3 mg/mL before detecting the tubulin polymerization reaction. After 30 min, the absorbance of different concentrations was detected by a spectrophotometer at 340 nm at 37 °C. The area under the curve was used to determine the concentration that inhibited tubulin polymerization by 50% (IC_{50}), which was calculated with GraphPad Prism Software version 5.02.

2.3 Competitive Inhibition Assays.

The competitive binding activity of inhibitors was evaluated using a radiolabeled [3H]colchicine competition scintillation proximity (SPA) assay. In brief, 0.08 μM [3H]colchicine was mixed with **(±)-19b** (1 μM, 5 μM), **(*R*)-(+)-19b** (1 μM, 5 μM), or CA-4 (1 μM, 5 μM) and biotinylated porcine tubulin (0.5 μg) in a buffer of 100 μL containing 80 mM PIPES (pH 6.8), 1Mm EGTA, 10% glycerol, 1mM $MgCl_2$, and 1mM GTP for 2 h at 37 °C. Then streptavidin-labeled SPA beads (80 μg) were added to each mixture. The radioactive counts were measured directly with a scintillation counter.

2.4 Immunofluorescence staining

K562 cells were seeded into 6-well plates and then treated with vehicle control 0.1% DMSO, **(±)-19b** (5 nM, 10 nM, 20 nM) for 24 h. The cells were fixed with 4% paraformaldehyde and then penetrated with PBS for three times. After blocking for 20

min by adding 50-100 μL goat serum albumin at room temperature, cells were incubated with a monoclonal antibody (anti-α-tubulin) at 37 °C for 2 h. Then the cells were washed three times by PBS following staining by fluorescence antibody and labeling of nuclei by 4,6-diamidino-2-phenylindole (DAPI). Cells were finally visualized using a fluorescence microscope (OLYMPUS, Japan).

2.5 Cell cycle analysis

K562 cells were seeded into 6-well plates and incubated at 37 °C in a humidified 5% CO_2 incubator for 24 h, and then treated with or without **(±)-19b** at indicated concentrations for another 48 h. The collected cells were fixed by adding 70% ethanol at 4 °C for 12 h. Subsequently, the cells were resuspended in PBS containing 100 mL RNase A and 400 mL of propidium iodide for 30 min. The DNA content of the cells was measured using a FACS Calibur flow cytometer (BectoneDickinson, San Jose, CA, USA).

2.6 Cell apoptosis analysis

After treatment with or without **(±)-19b** at indicated concentrations for 48 h, the cells were washed twice in PBS, centrifuged and resuspended in 500 mL AnnexinV binding buffer. The cells were then harvested, washed and stained with 5 mL Annexin V-APC and 5 mL 7-AAD in the darkness for 15 min. Apoptosis was analyzed using a FACS Calibur flow cytometer (BectoneDickinson, San Jose, CA, USA).

2.7 Mitochondrial membrane potential analysis

After treatment with or without **(±)-19b** at indicated concentrations for 48 h, the cells were washed in PBS and resuspended in 500 mL JC-1 incubation buffer at 37 °C for 15 min. Then, **(±)-19b** was immediately assessed for a red fluorescence using a microplate reader (ELx80, Bio-Tek, USA). The fluorescent signal of monomers was measured with an excitation wavelength of 488 nm. The percentage of cells with healthy or collapsed mitochondrial membrane potentials was monitored by flow cytometry analysis (Bectone-Dickinson, San Jose, CA, USA).

2.8 Wound healing assay

K562 cells were grown in 6-well plates for 24 h. Scratches were made in confluent monolayers using 200 μL pipette tip. Then, wounds were washed twice with PBS to remove non-adherent cell debris. The media containing different concentrations (5 nM, 10 nM, 20 nM) of the compound **(±)-19b** were added to the petridishes. Cells which migrated across the wound area were photographed using phase contrast microscopy at 0 h and 24 h.

2.9 Tube formation assay

EC Matrigel matrix was thawed at 4 ℃ overnight, and HUVECs suspended in DMEM were seeded in 96-well culture plates at a cell density of 50,000 cells/well after polymerization of the Matrigel at 37 ℃ for 30 min. They were then treated with 20 μL different concentrations (5 nM, 10 nM, 20 nM) of compound **(±)-19b** or vehicle for 6h at 37 ℃. Then, the morphological changes of the cells and tubes formed were observed and photographed under inverted microscope (OLYMPUS, Japan).

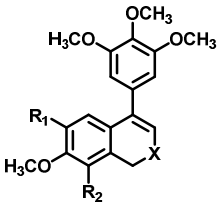
2.10 *In vivo* anti-tumor evaluation

Five-week-old male Institute of Cancer Research (ICR) mice were purchased from Shanghai SLAC Laboratory Animals Co. Ltd. A total of 1×10^6 H22 cells were subcutaneously inoculated into the right flank of ICR mice according to protocols of tumor transplant research, to initiate tumor growth. After incubation for one day, mice were weighted and at random divided into six groups of six animals. The groups treated with **(±)-19b** and CA-4 were administered 15, 30 mg/kg in a vehicle of 10% DMF/2% Tween 80/88% saline, respectively. The positive control group was treated with PTX (8 mg/kg) every 2 days by intravenous injection. The negative control group received a vehicle of 10% DMF/2% Tween 80/88% saline through intravenous injection. Treatments of **(±)-19b** and CA-4 were done at a frequency of intravenous injection one dose per day for a total 21 consecutive days while the positive group was treated with PTX one dose per two days. The mice were sacrificed after the treatments and the tumors were excised and weighed. The inhibition rate was calculated as follows: Tumor inhibitory ratio (%) = (1-average tumor weight of treated group/average tumor weight of control group) × 100%.

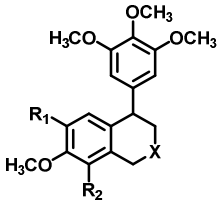
2.11 Molecular modelling

In our study, the X-ray structure of the CA-4-α,β-tubulin complex was downloaded from the Protein Data Bank (PDB code: 5lyj). The protein was prepared by removal of subunits C-F, water molecules and CA-4 using Discovery Studio modules. The docking procedure was performed by employing DOCK program in Discovery Studio 3.0 software, and the structural image was obtained using PyMOL software.

Table S1. Anti-proliferative Activity of Target Compounds Against HepG2 Cells[a]



17a-g, 18a-b



(±)-19a-d

Compd.	X	R_1	R_2	IC_{50} values (μM)	Compd.	X	R_1	R_2	IC_{50} values (μM)
17a	O	H	H	0.17±0.01	**(±)-19a**	O	H	H	0.15±0.01
17c	O	H	OCH_3	0.46±0.02	**(±)-19b**	O	H	OH	**0.015±0.001**
17e	O	H	Br	1.6±0.1	**(±)-19c**	O	H	OCH_3	0.20±0.03
17f	S	H	H	0.20±0.01	**(±)-19d**	O	OH	H	> 10
17g	S	H	OCH_3	0.21±0.01	**8**	-	-	-	23.7±0.9
18a	O	OH	H	> 10	**CA-4**	-	-	-	0.009±0.001
18b	O	H	OH	**0.026±0.001**					

[a]MTT methods; cells were incubated with indicated compounds for 72 h (means ± SD, n = 3).
[b] Inhibition of tubulin polymerization activity.

Table S2. Inhibition of Tubulin Polymerization[a] and Colchicine Binding to Tubulin[b]

Compd.	inhibition of colchicine binding (%) inhibition ± SD	
	1 μM	5 μM
(±)-19b	76.4 ± 4.4	89.6 ± 4.8
(R)-(+)-19b	79.4 ± 3.3	92.6 ± 3.2
CA-4	81.2 ± 1.9	93.7 ± 4.4

[a] The tubulin assembly assay measured the extent of assembly of 2 mg/mL tubulin after 60 min at 37 °C. Data are presented as mean from three independent experiments.
[b] Tubulin, 1 μM; [3H]-colchicine, 5 μM; and inhibitors, 1 or 5 μM.

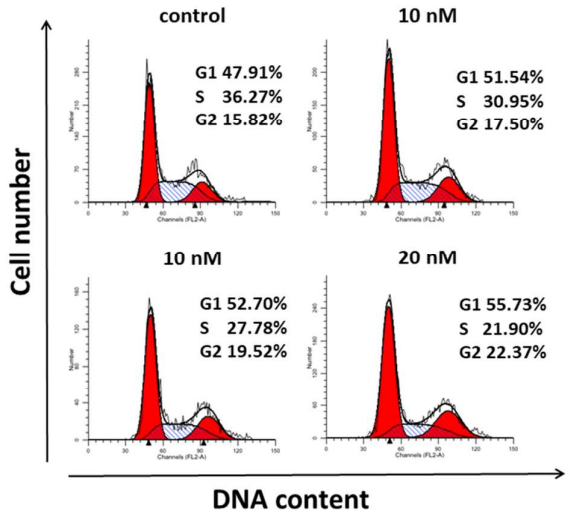


Figure S1. Effect of **(±)-19b** on cell cycle progression of K562 cells.

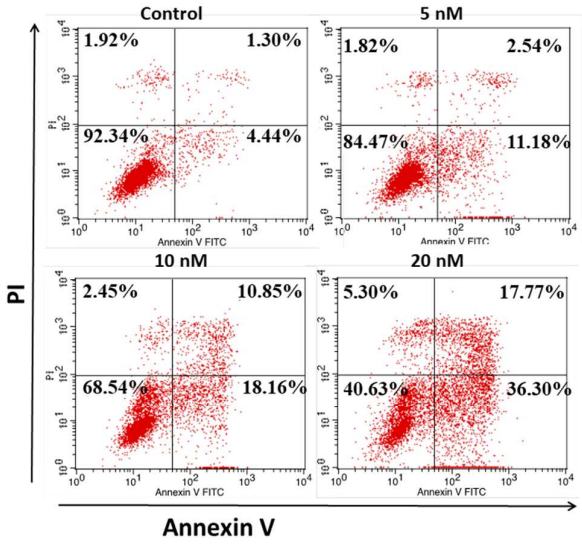


Figure S2. Effect of **(±)-19b** on apoptosis of K562 cells.

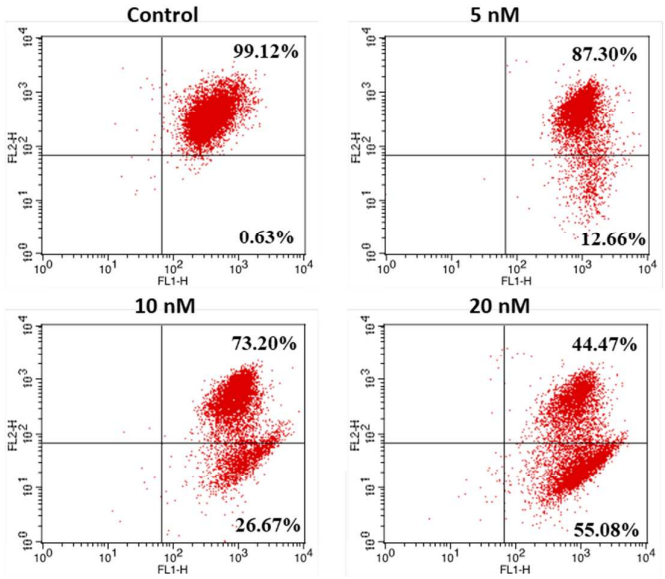


Figure S3. Effect of **(±)-19b** on the mitochondrial membrane potentials of K562 cells.

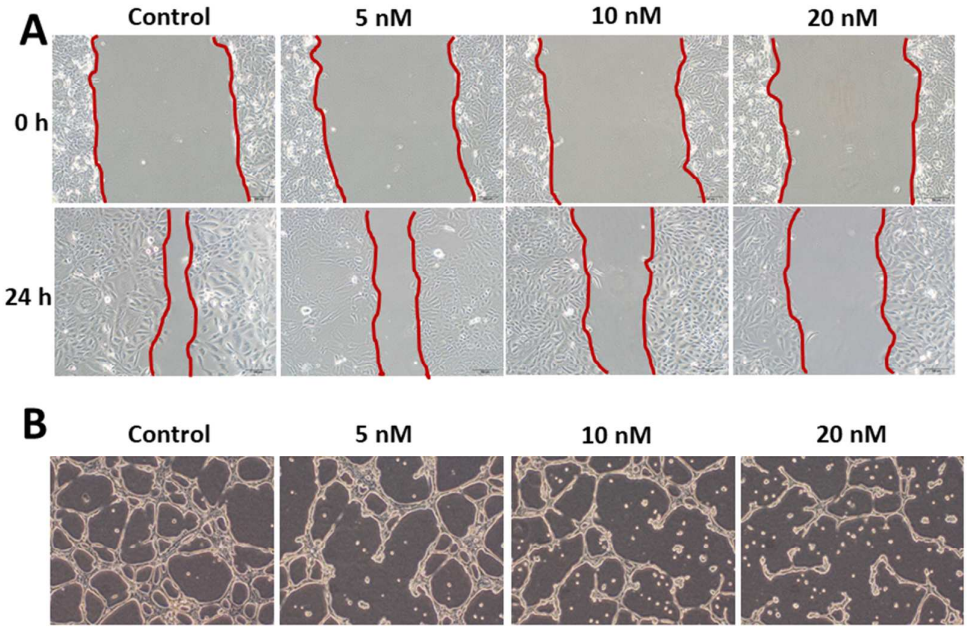
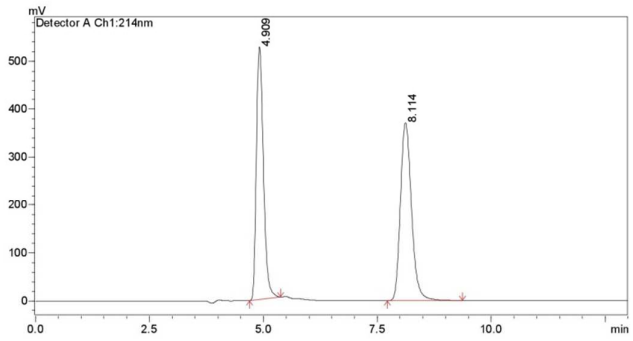


Figure S4. Effects of **(±)-19b** on the HUVECs migration (A) and tube formation (B).

A)

Column	:	CHIRALPAK AD-H(ADH0CD-UE022)
Column size	:	0.46 cm I.D. × 15 cm L
Injection	:	1.0 ul
Mobile phase	:	EtOH=100%
Flow rate	:	0.5 ml/min
Wave length	:	UV 214 nm
Temperature	:	35 °C
HPLC equipment	:	Shimadzu LC-20AD CP-HPLC-05
Sample name	:	Raw Material

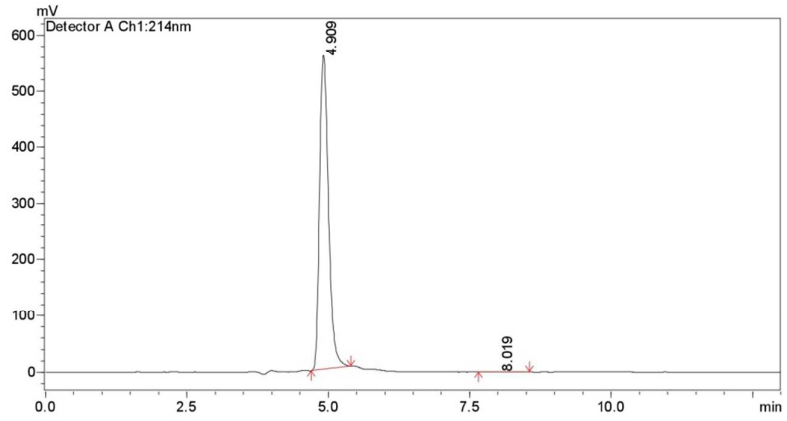
<Chromatogram>



<Column Performance Report>

Peak#	Ret. Time	Area	Area%	T.Plate#	Tailing F.	Resolution
1	4.909	5668725	47.5000	4745.471	1.369	--
2	8.114	6265425	52.5000	5479.410	1.244	8.861

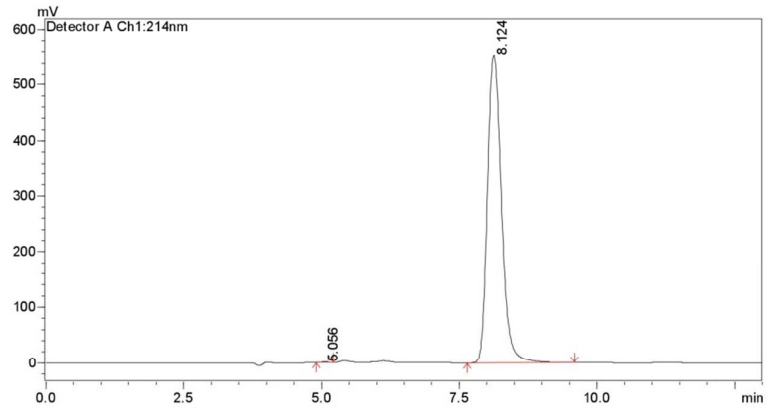
B)



<Column Performance Report>

Peak#	Ret. Time	Area	Area%	T.Plate#	Tailing F.	Resolution
1	4.909	6121587	99.7937	4621.019	1.383	--
2	8.019	12653	0.2063	6683.168	1.113	9.128

C)



<Column Performance Report>

Peak#	Ret. Time	Area	Area%	T.Plate#	Tailing F.	Resolution
1	5.056	8956	0.0909	7294.083	1.071	--
2	8.124	9843187	99.9091	5070.405	1.261	8.852

Figure S5. (A**)** Chromatograms of the separation of **(±)-19b**; (B**)** Chromatograms of the separation of **(-)-19b**; (C**)** Chromatograms of the separation of **(+)-19b**.

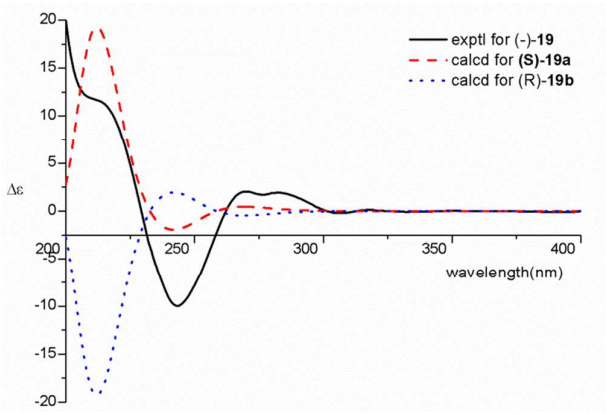


Figure S6. Comparison of ECD spectra: experimental ECD of (-)-**19b** (shown in black), calculated ECD of (*S*)-**19b** (shown in red), and calculated ECD of (*R*)-**19b** (shown in blue).

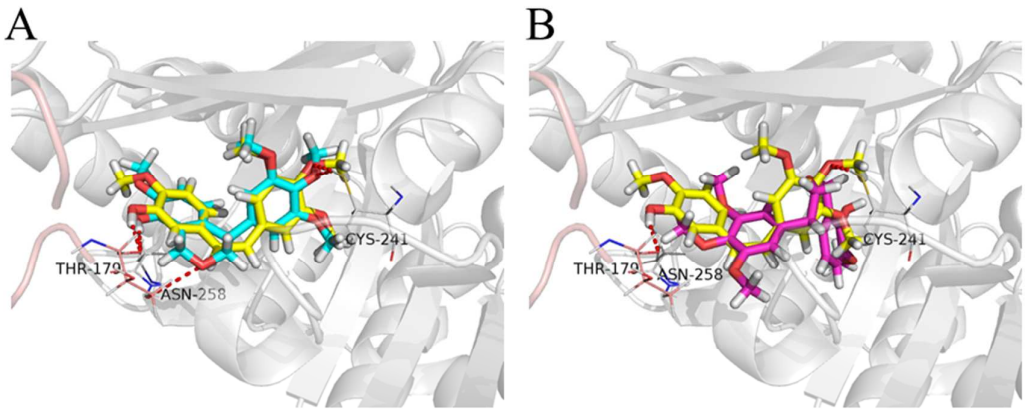
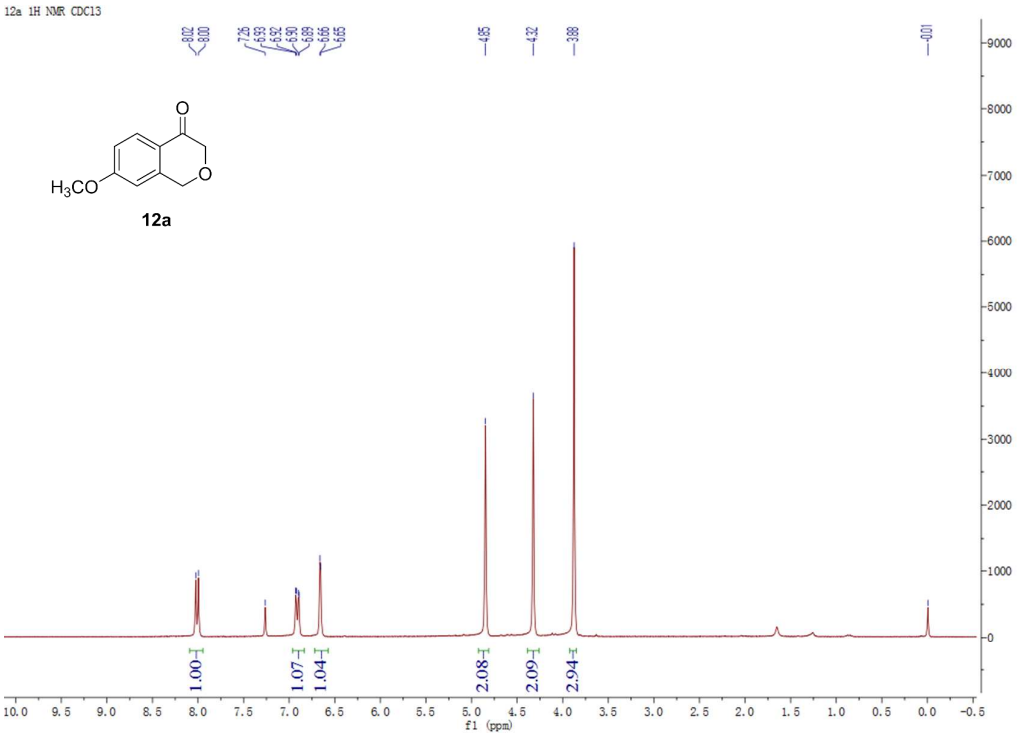
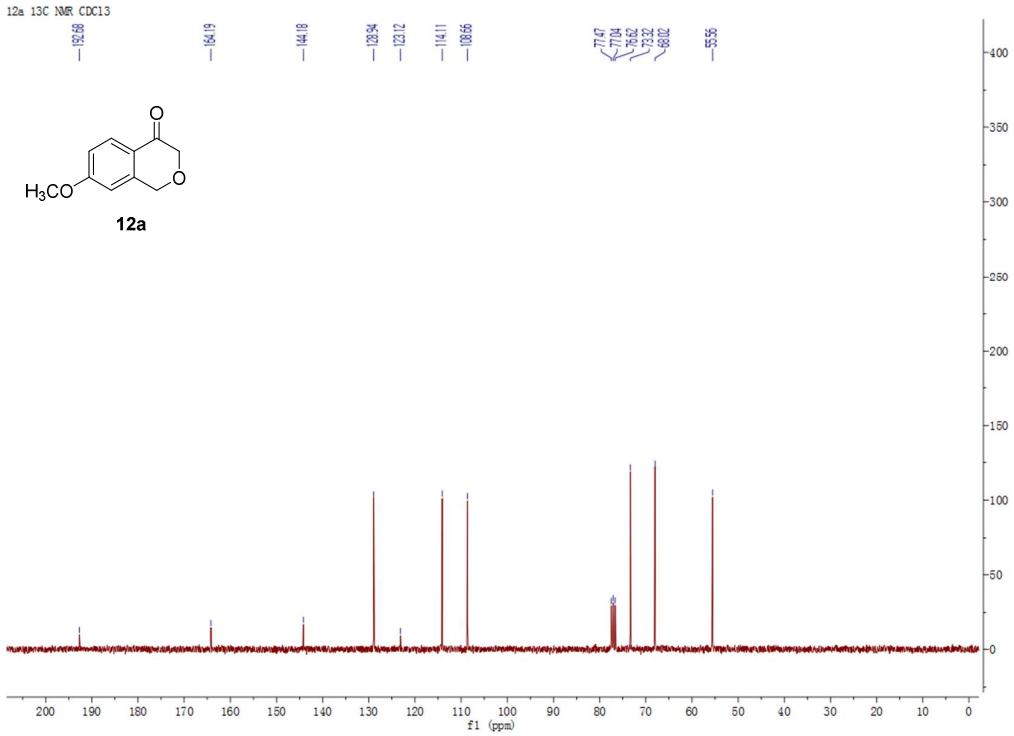


Figure S7. Proposed binding models for two enantiomers of **(±)-19b** with tubulin (PDB code: 5lyj). (A) CA-4 (shown in yellow), ***(R)*-(+)-19b** (shown in blue); (B) CA-4 (shown in yellow), ***(S)*-(-)-19b** (shown in violet).

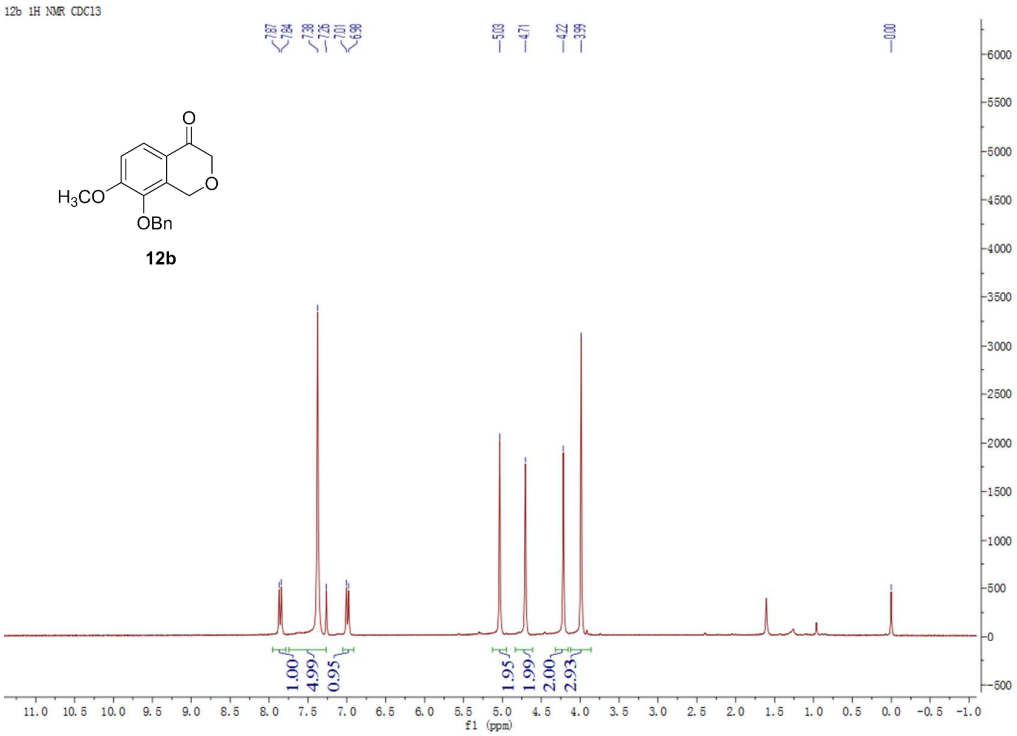
3. Copies of ^{1}H NMR spectra for intermediate 12a-g



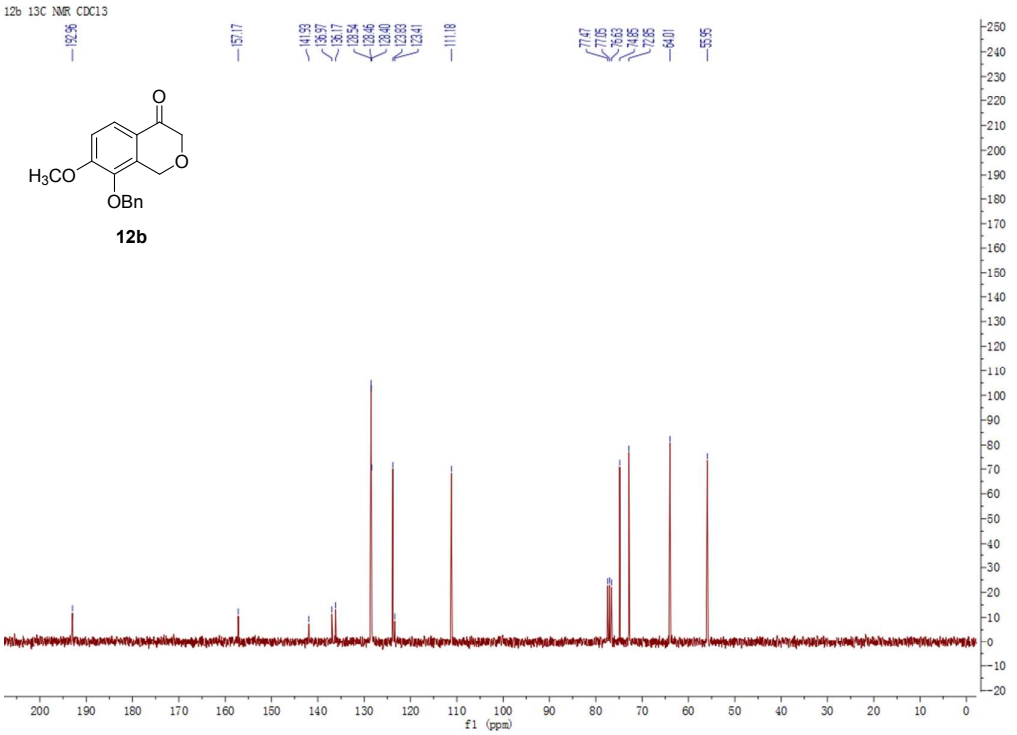
^{1}H NMR (300 MHz) of **12a** in $CDCl_3$



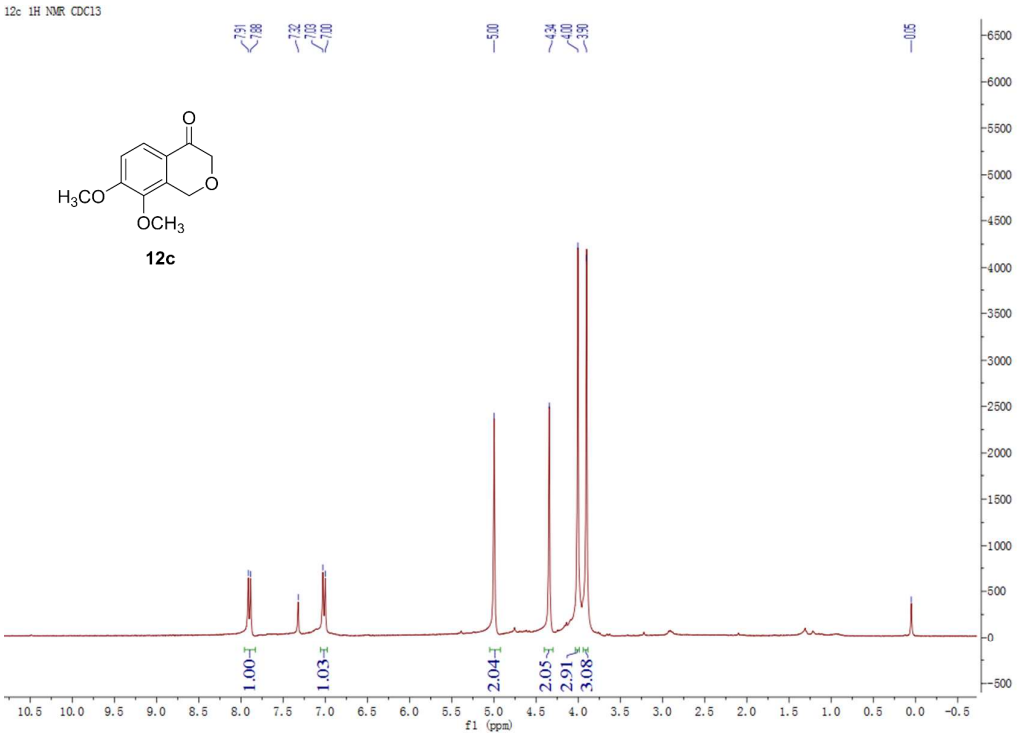
^{13}C NMR(300MHz) of 12a in $CDCl_3$



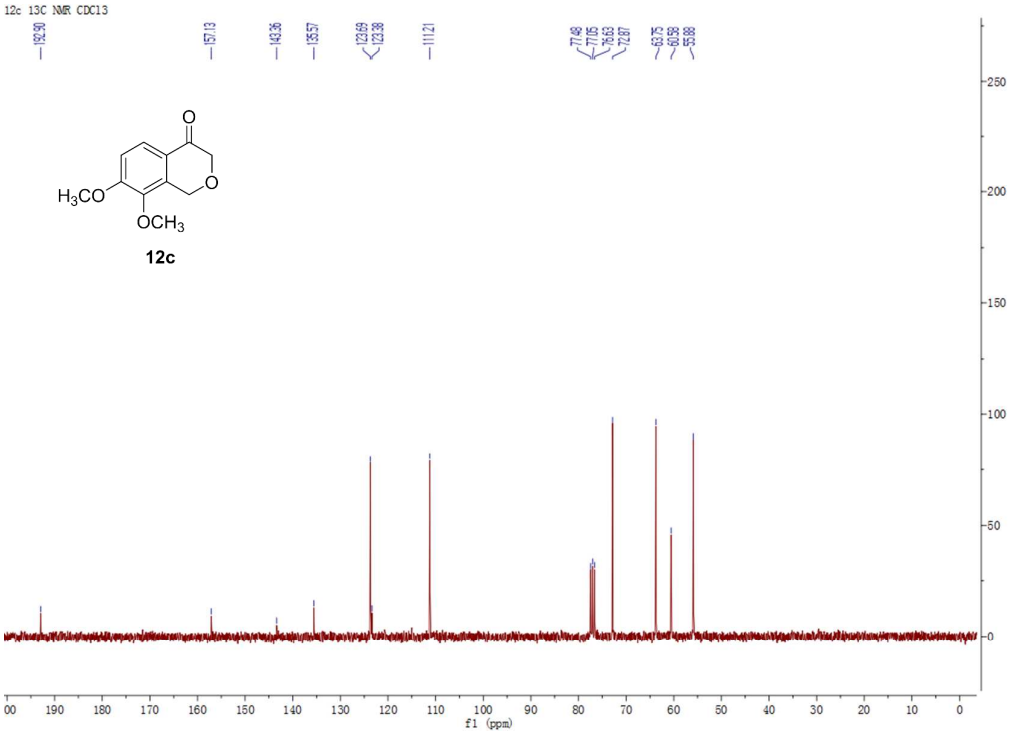
^{1}H NMR (300 MHz) of **12b** in $CDCl_3$



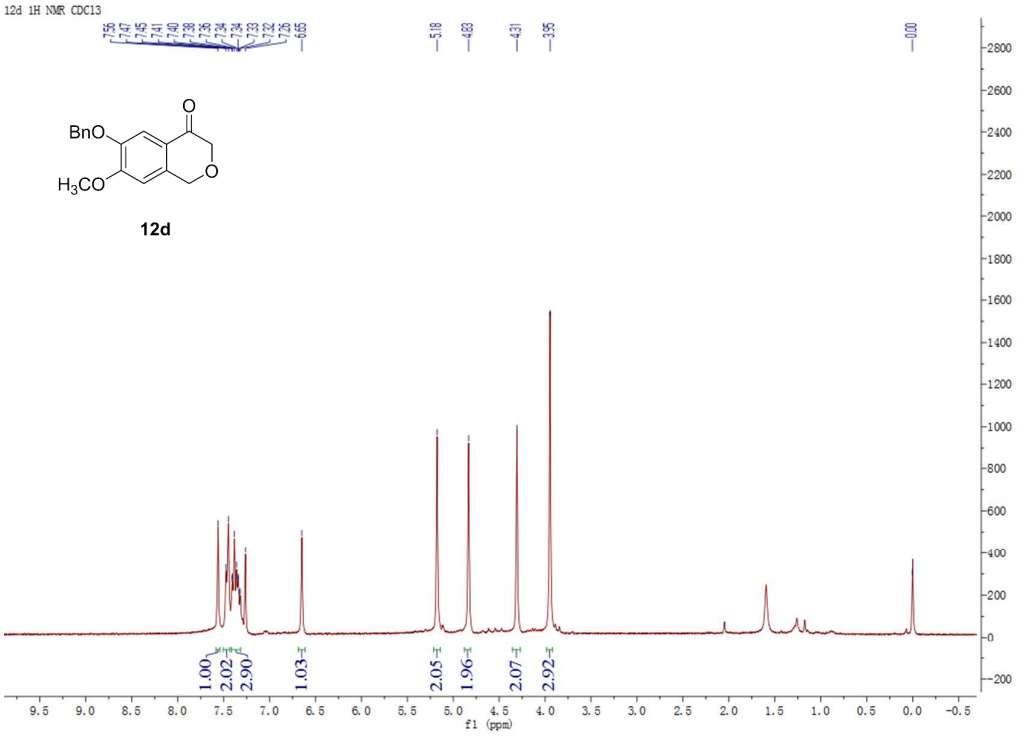
^{13}C NMR (300MHz) of **12b** in $CDCl_3$



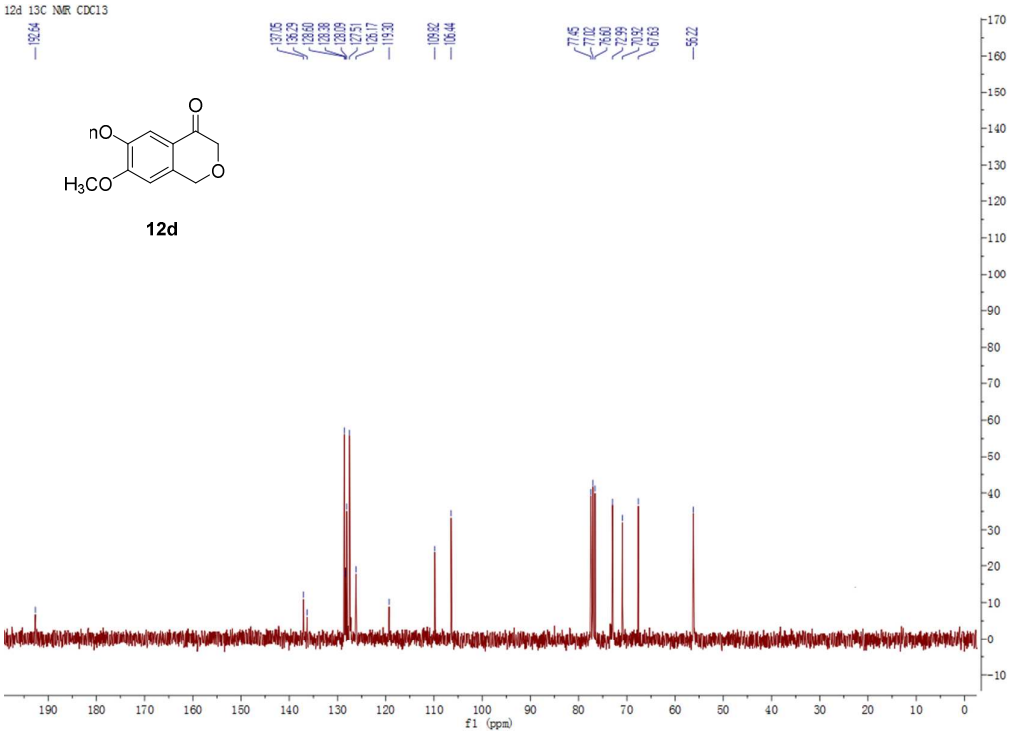
^{1}H NMR (300 MHz) of **12c** in $CDCl_3$



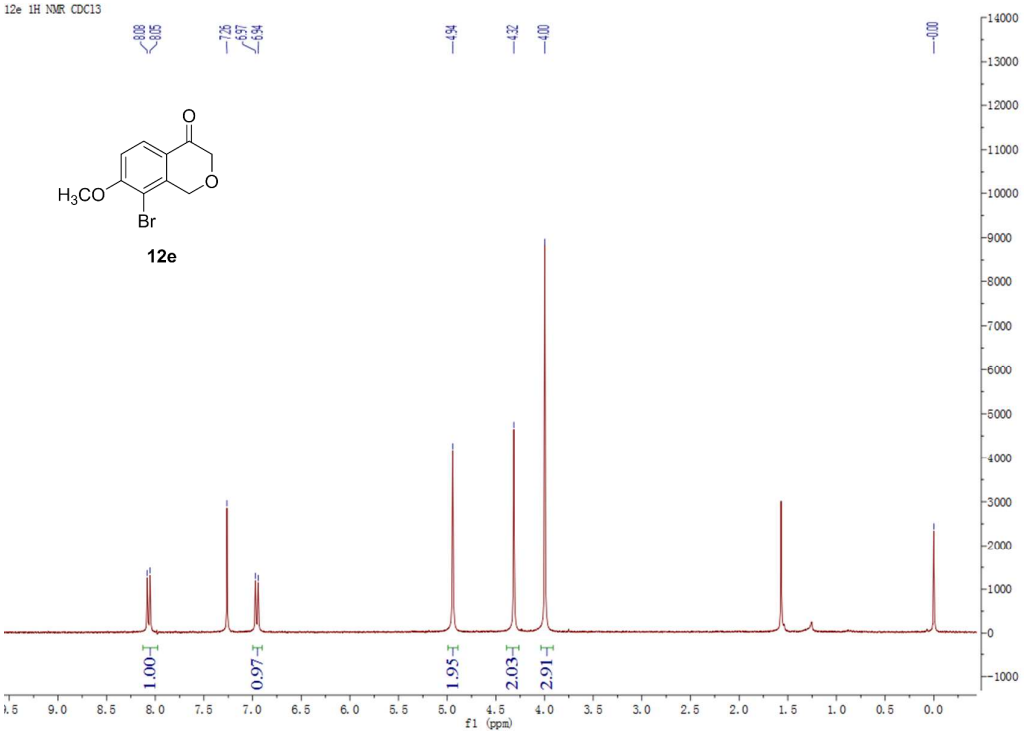
^{13}C NMR (300 MHz) of **12c** in $CDCl_3$



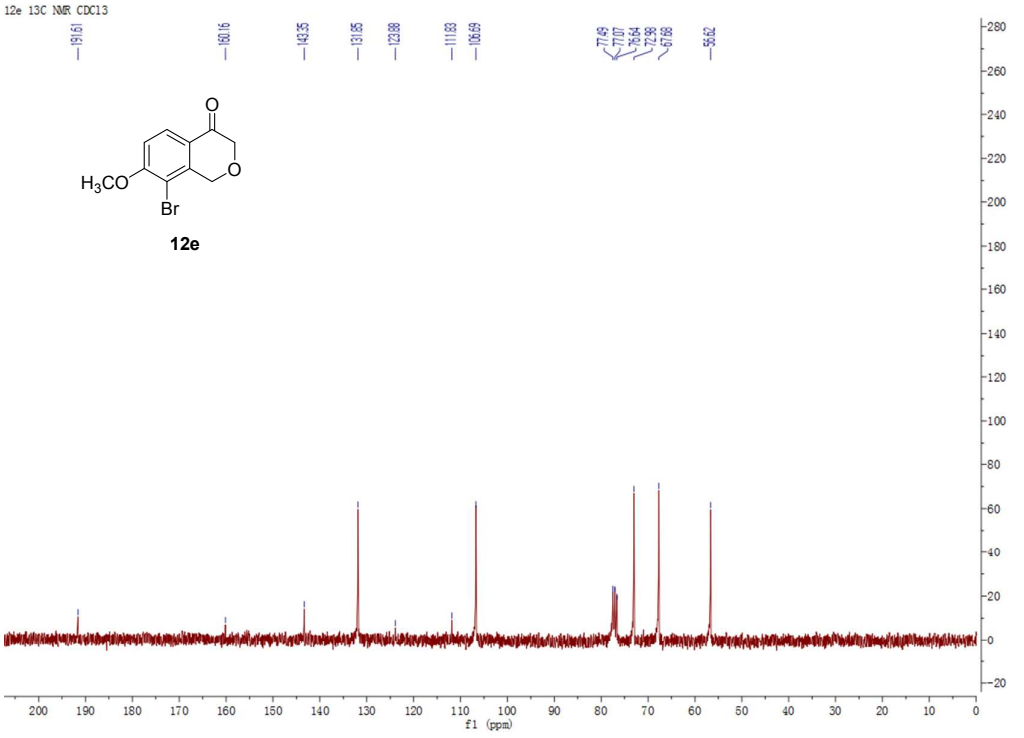
^{1}H NMR (300 MHz) of **12d** in $CDCl_3$



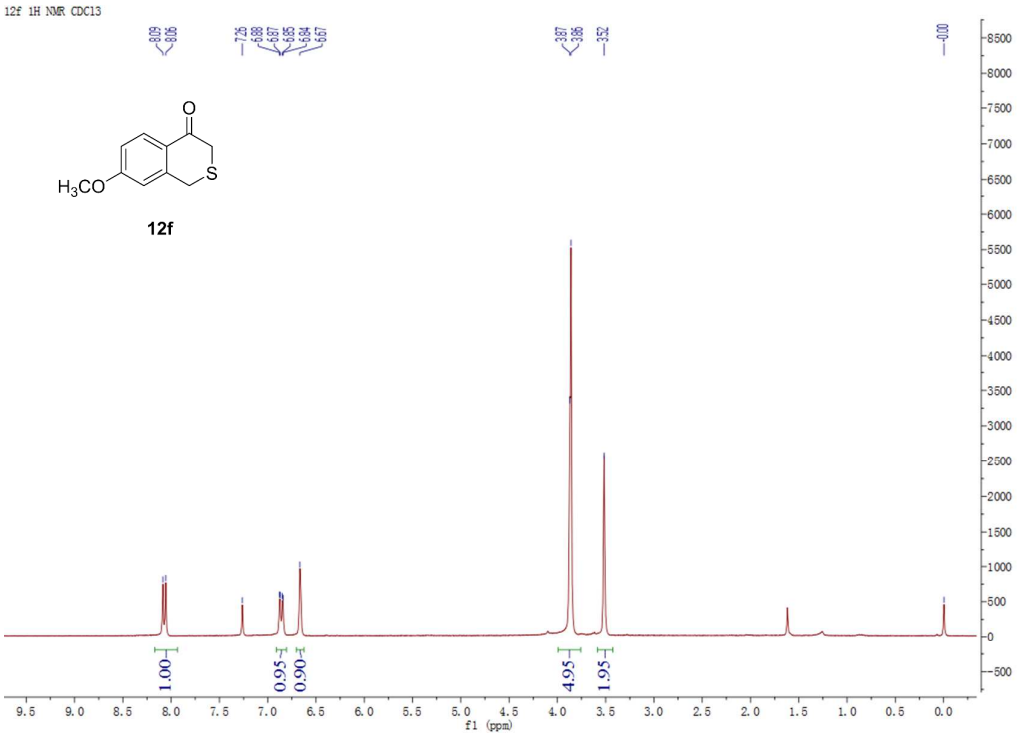
^{13}C NMR (300 MHz) of **12d** in $CDCl_3$



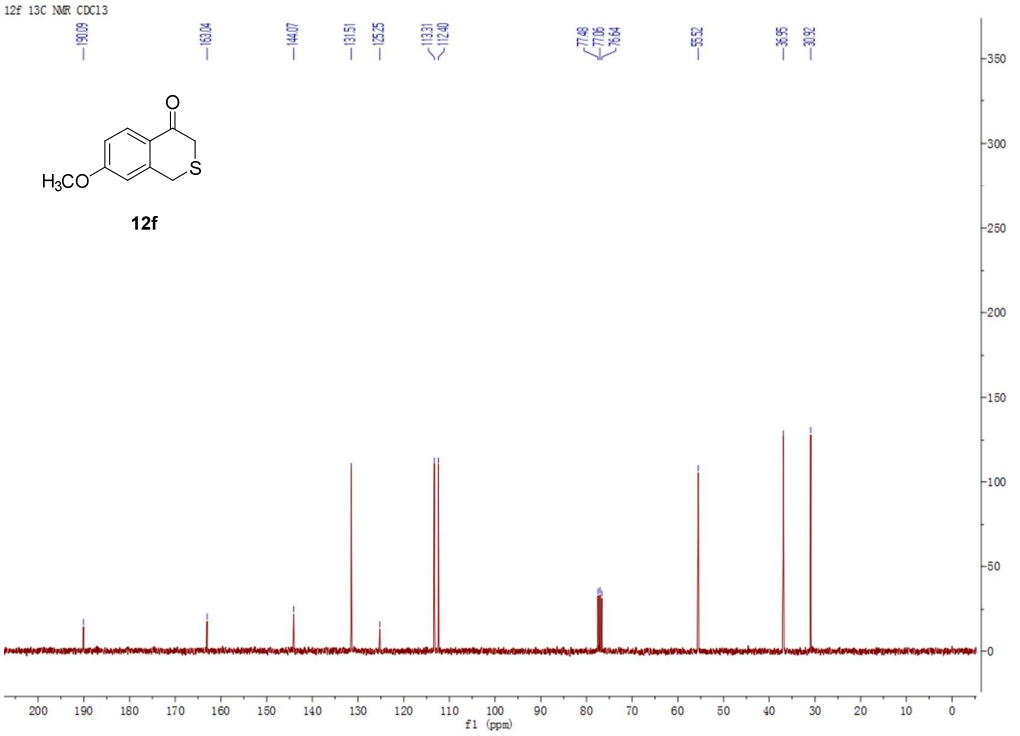
^{1}H NMR (300 MHz) of **12e** in $CDCl_3$



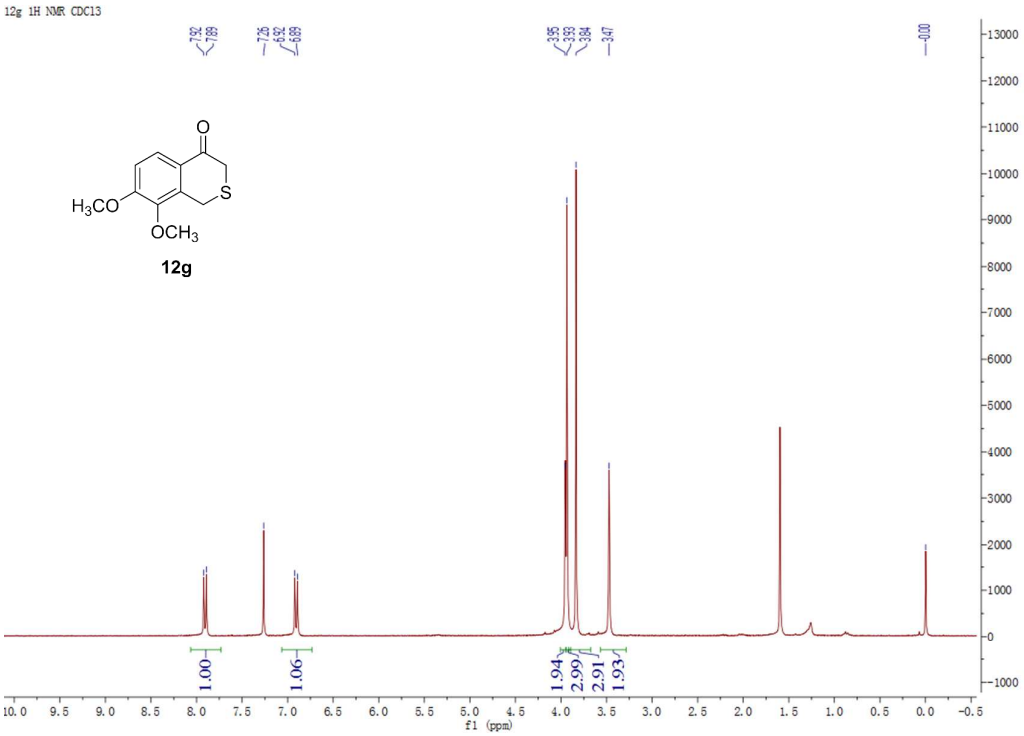
^{13}C NMR (300 MHz) of **12e** in $CDCl_3$



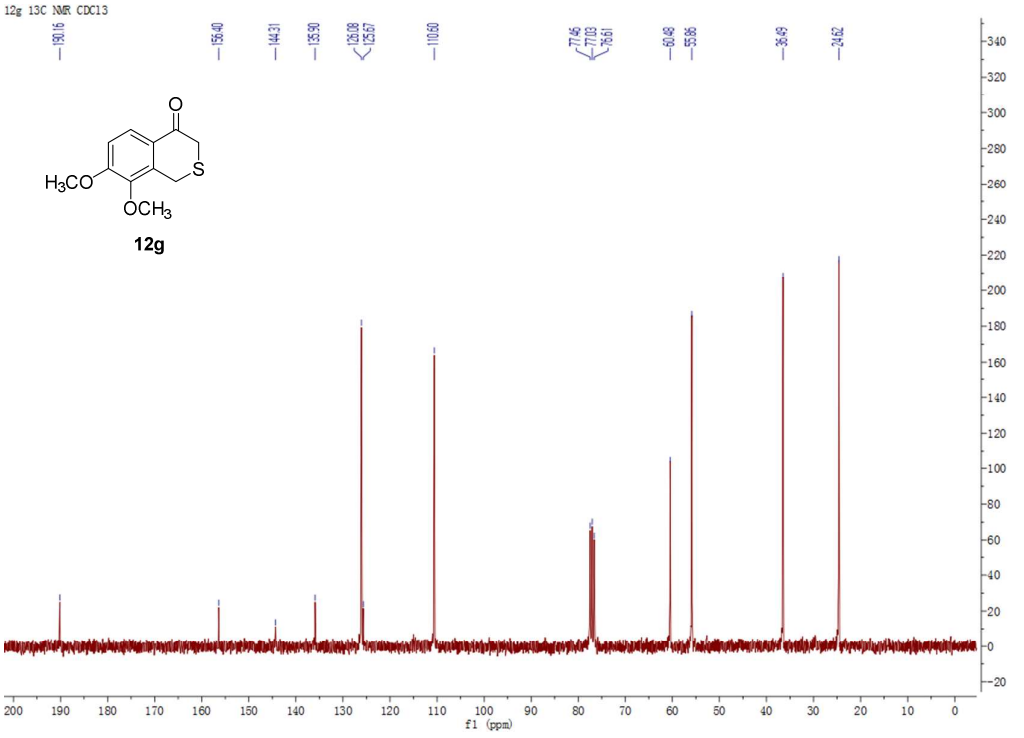
^{1}H NMR (300 MHz) of **12f** in $CDCl_3$



^{13}C NMR (300 MHz) of **12f** in $CDCl_3$

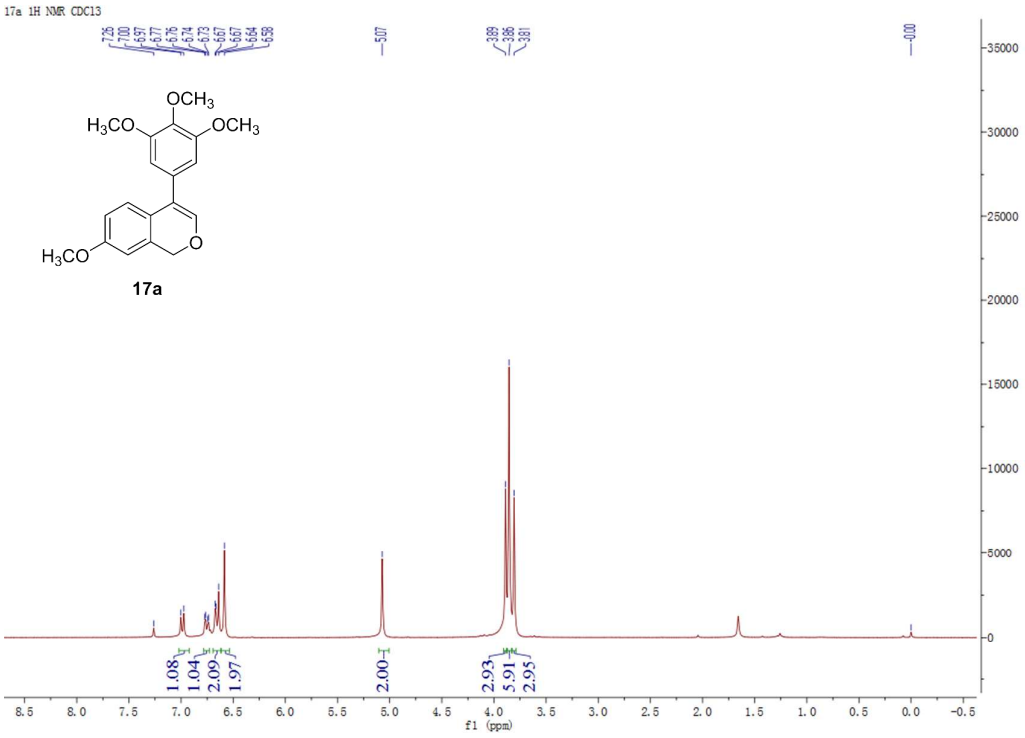


^{1}H NMR (300 MHz) of **12g** in $CDCl_3$

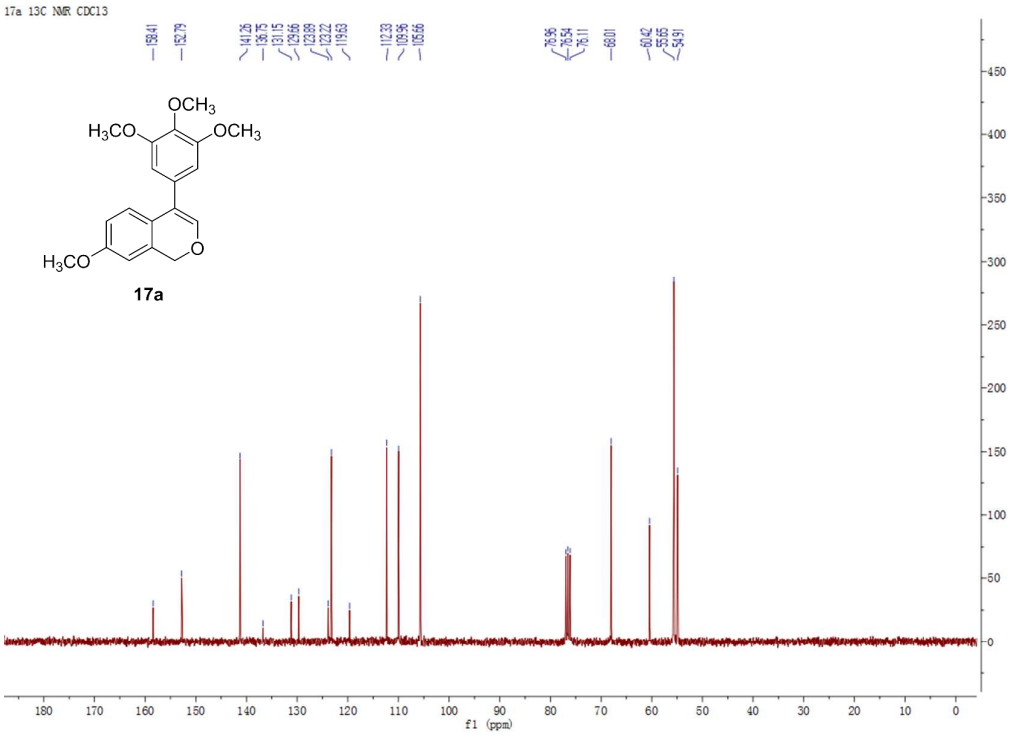


^{13}C NMR (300 MHz) of **12g** in $CDCl_3$

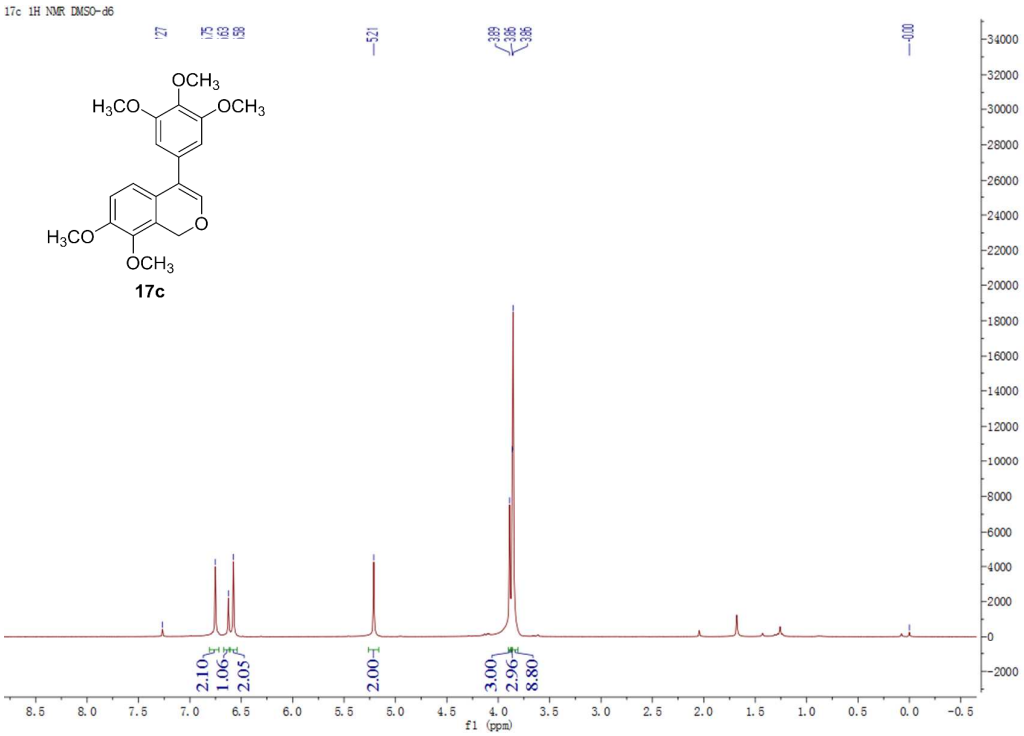
Copies of ^{1}H and ^{13}C NMR spectra for the title compounds



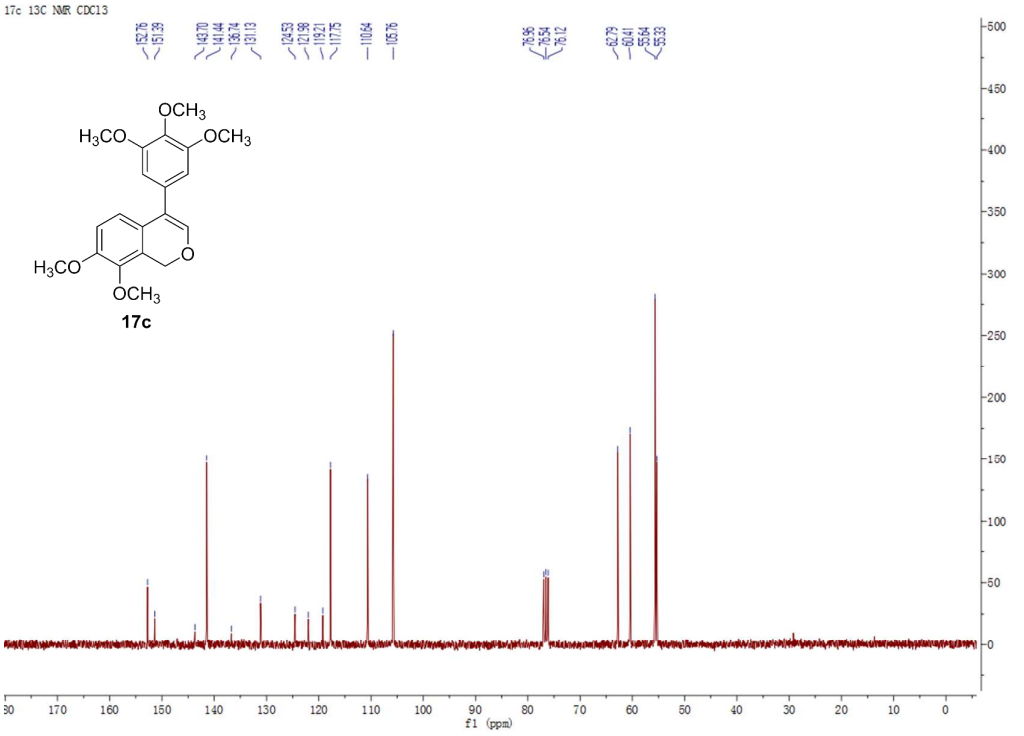
^{1}H NMR (300 MHz) of **17a** in $CDCl_3$



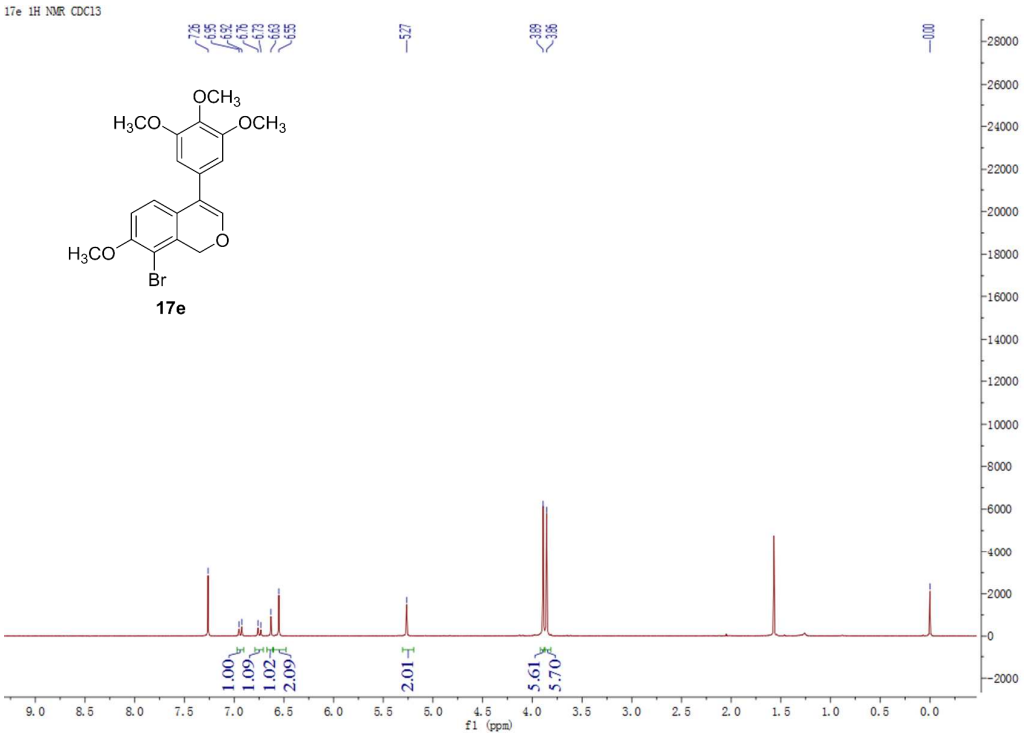
^{13}C NMR (75 MHz) of **17a** in $CDCl_3$



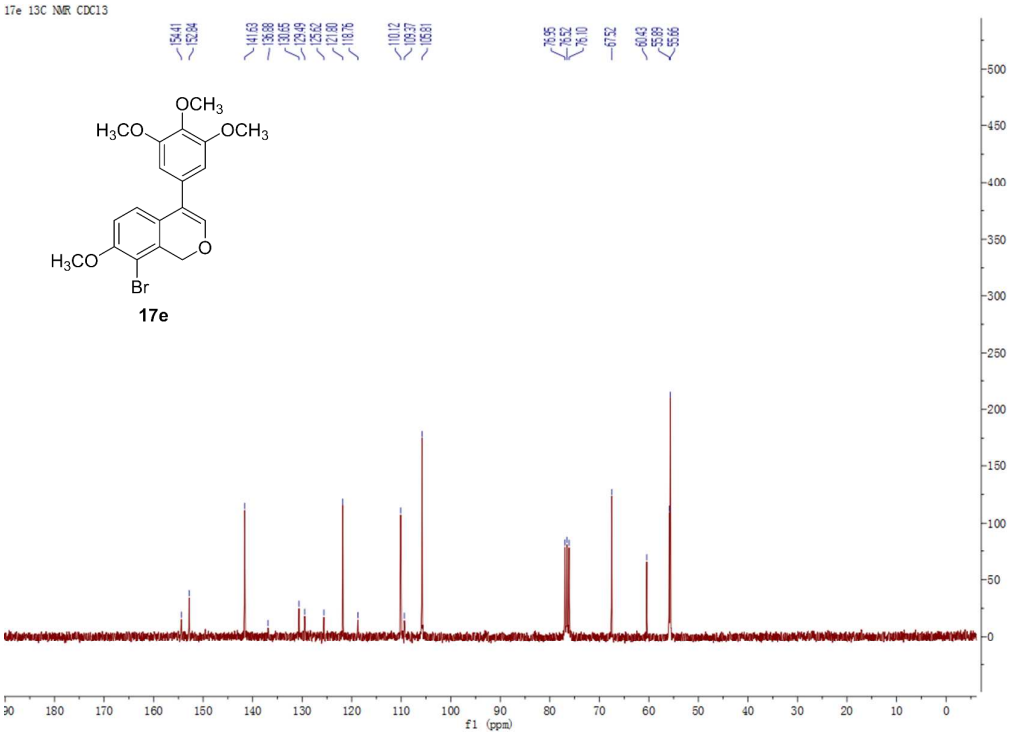
^{1}H NMR (300 MHz) of **17c** in $CDCl_3$



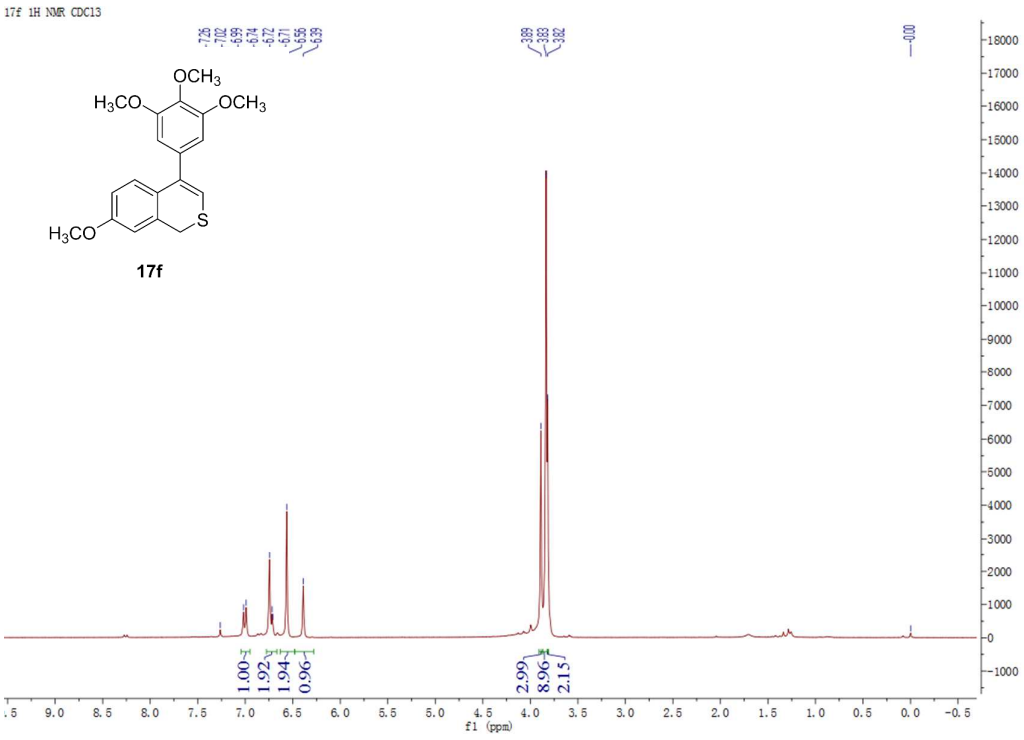
^{13}C NMR (75 MHz) of **17c** in $CDCl_3$



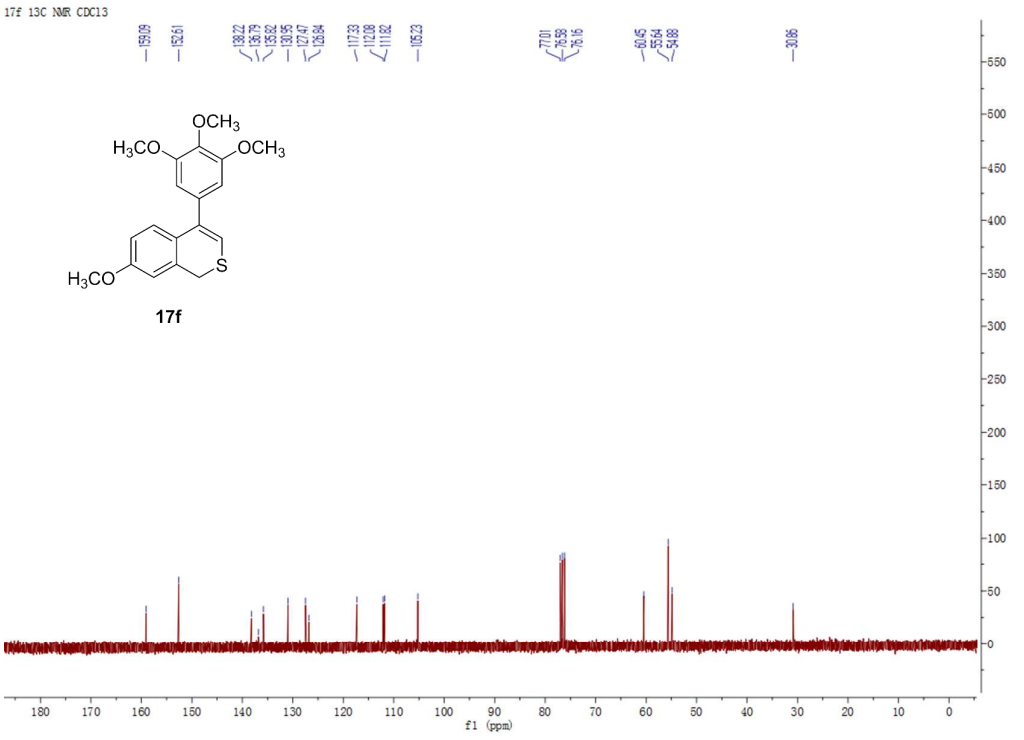
^{1}H NMR (300 MHz) of **17e** in $CDCl_3$



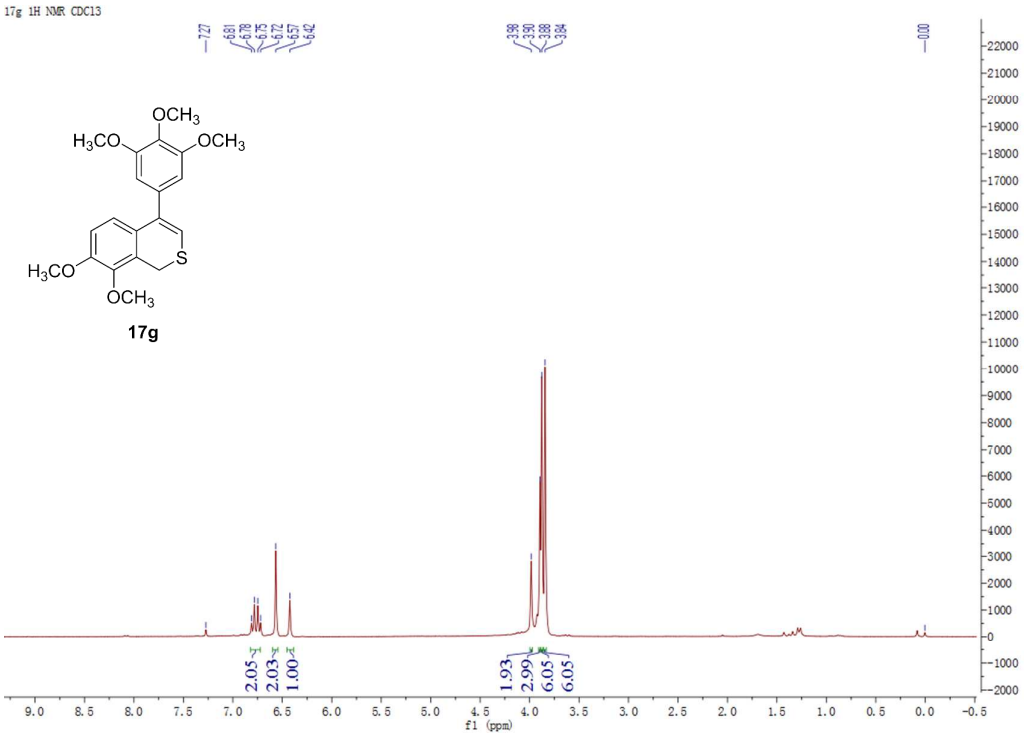
^{13}C NMR (75 MHz) of **17e** in $CDCl_3$



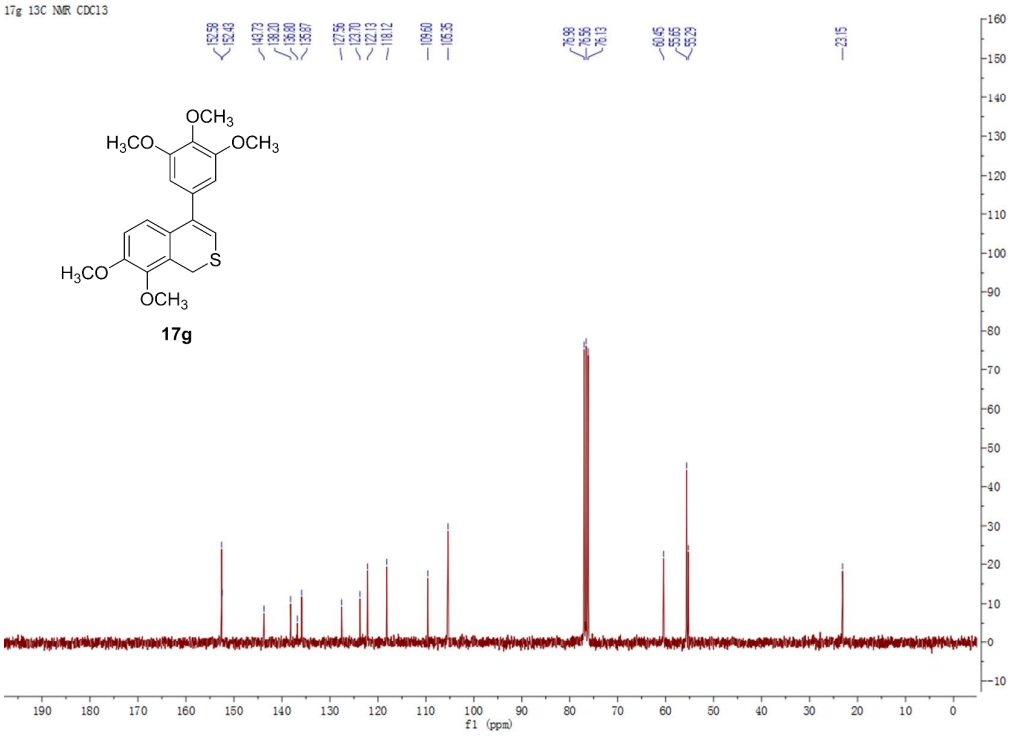
^{1}H NMR (300 MHz) of **17f** in $CDCl_3$



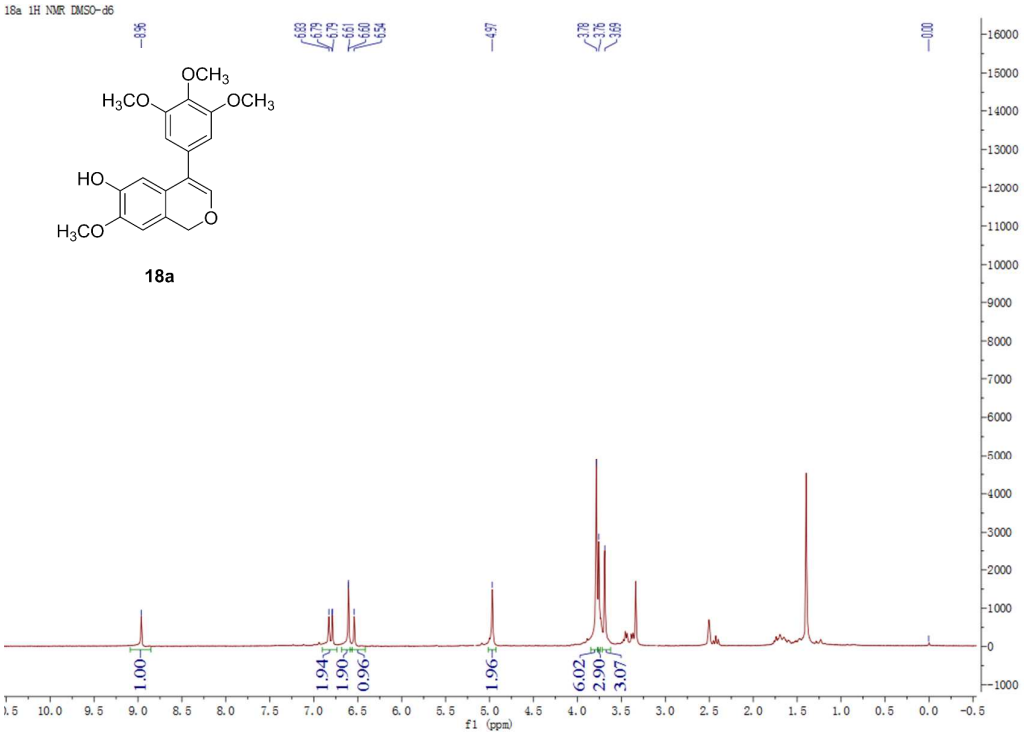
^{13}C NMR (75 MHz) of **17f** in $CDCl_3$



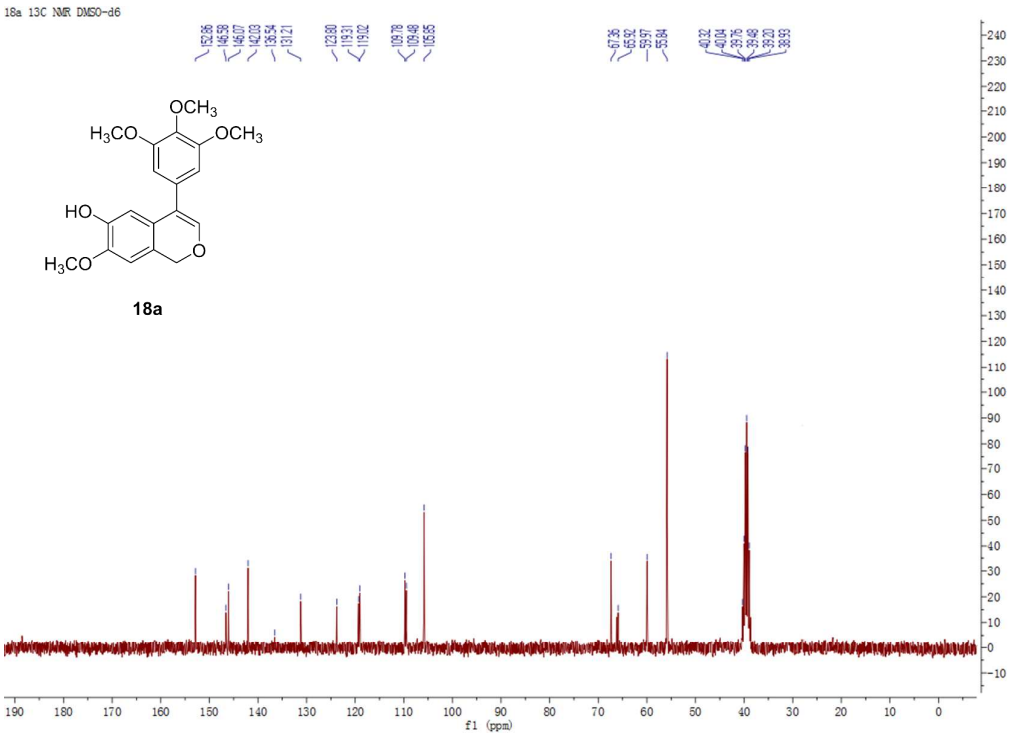
^{1}H NMR (300 MHz) of **17g** in $CDCl_3$



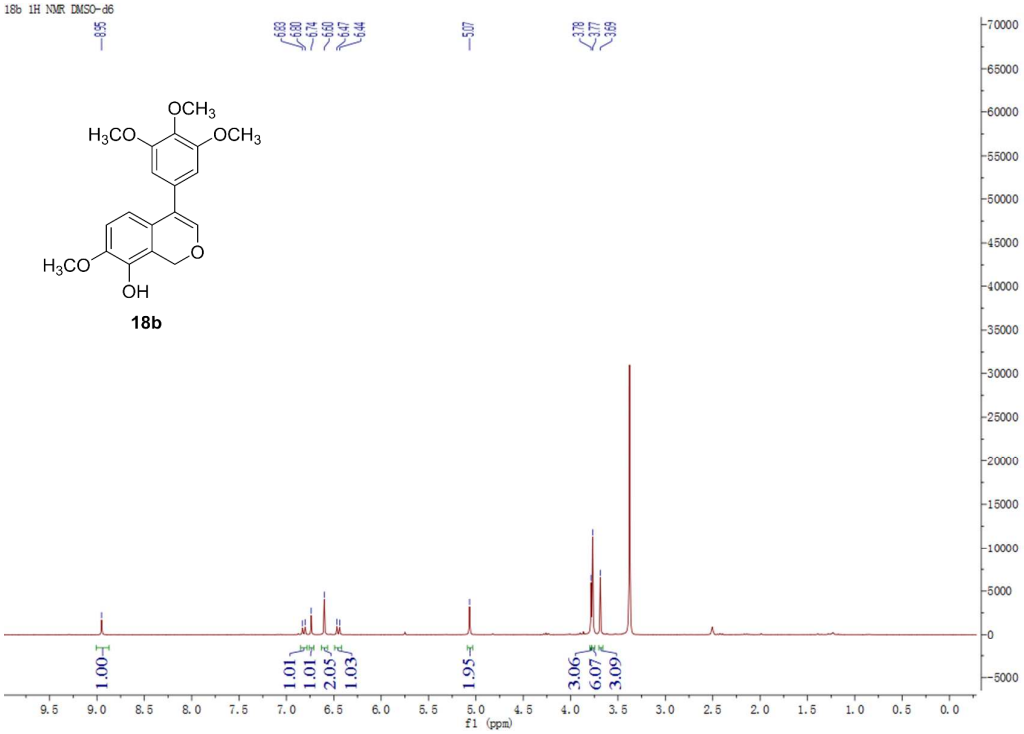
^{13}C NMR (75 MHz) of **17g** in $CDCl_3$



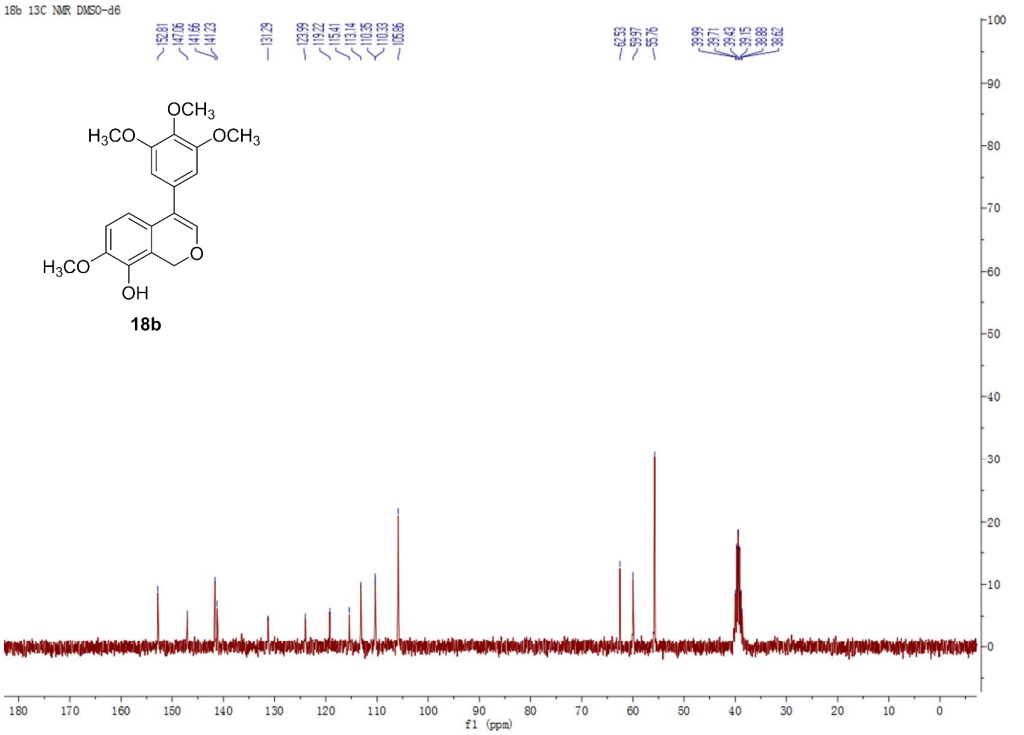
^{1}H NMR (300 MHz) of **18a** in DMSO-d_6



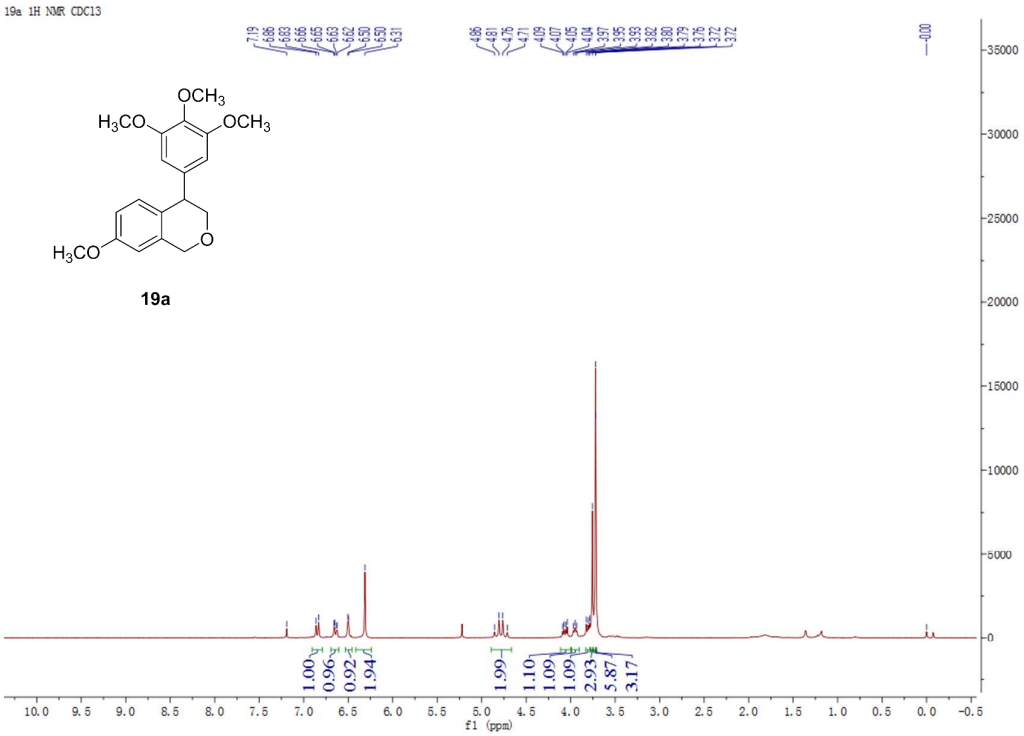
^{13}C NMR (75 MHz) of **18a** in DMSO-d_6



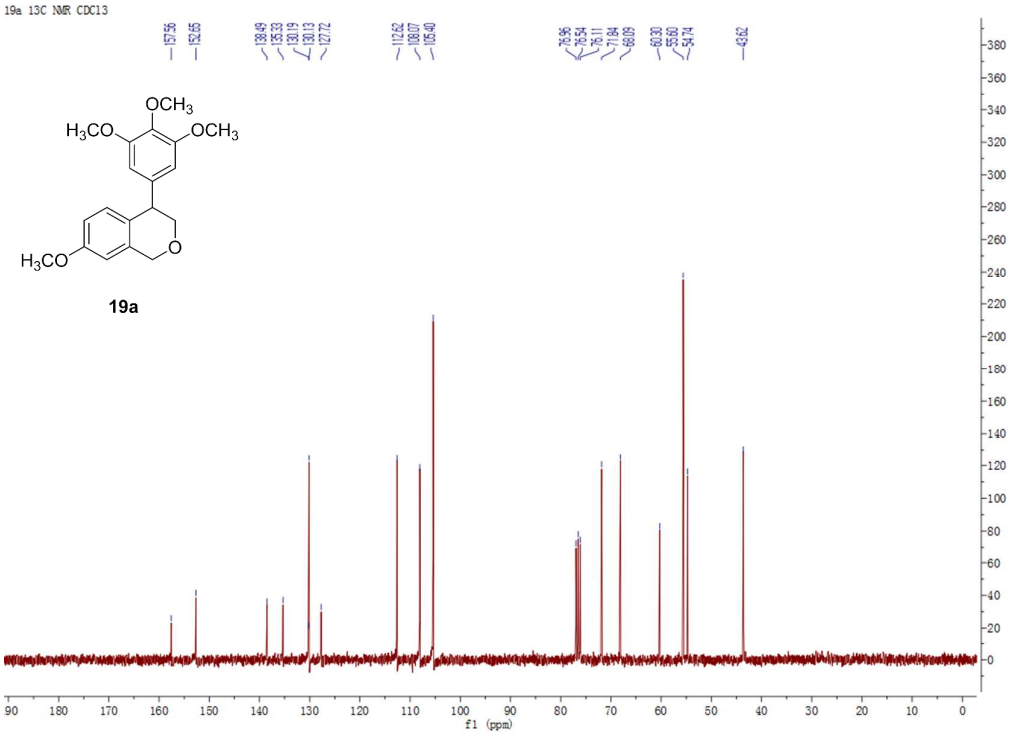
^{1}H NMR (300 MHz) of **18b** in DMSO-d_6



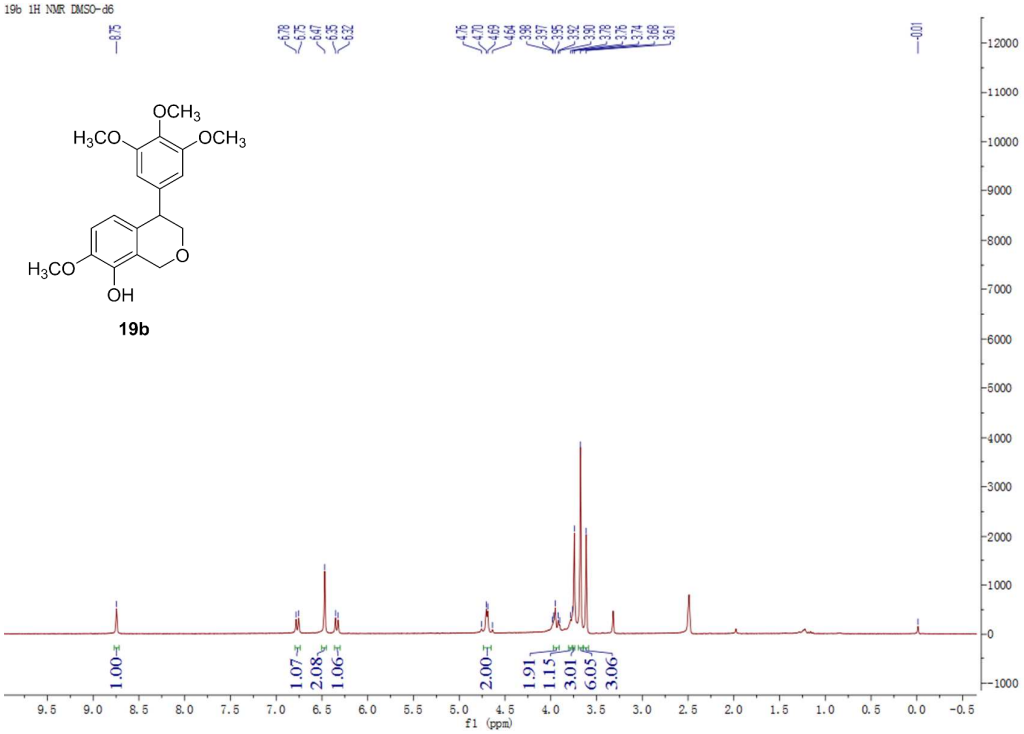
^{13}C NMR (75 MHz) of **18b** in DMSO-d_6



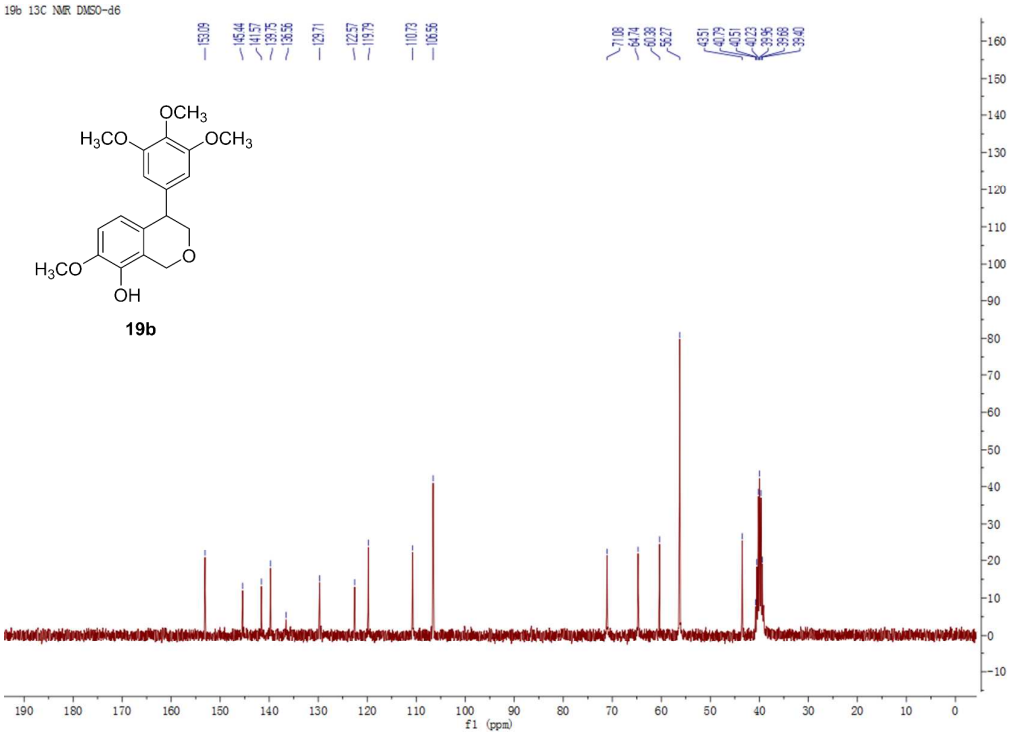
^{1}H NMR (300 MHz) of **19a** in $CDCl_3$



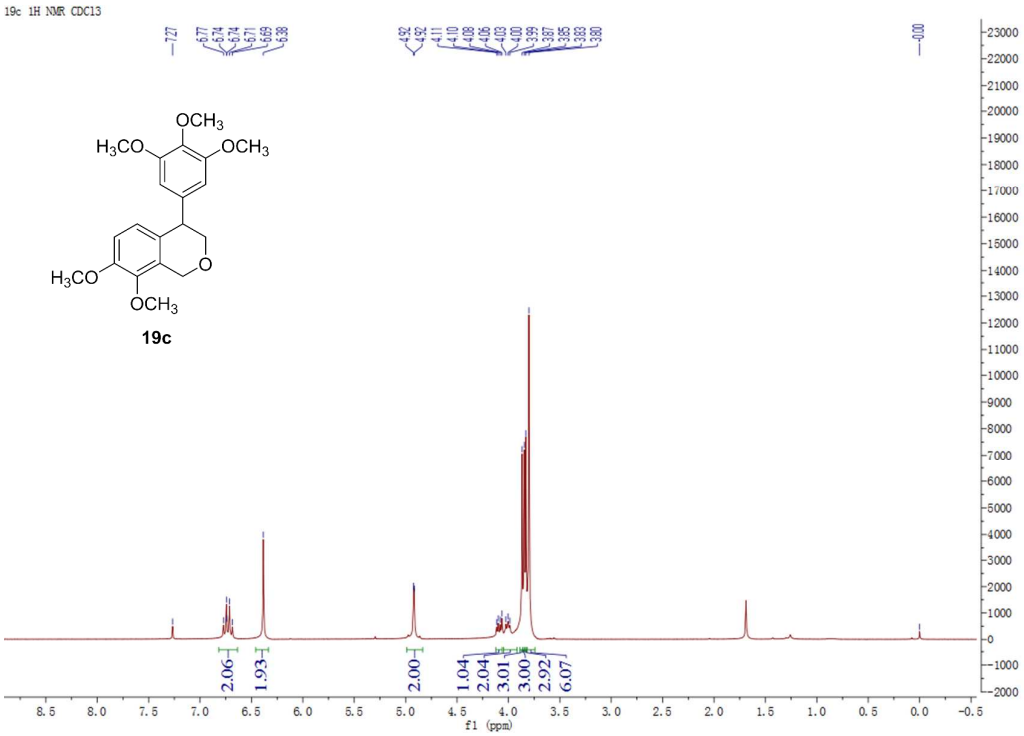
^{13}C NMR (75 MHz) of **19a** in $CDCl_3$



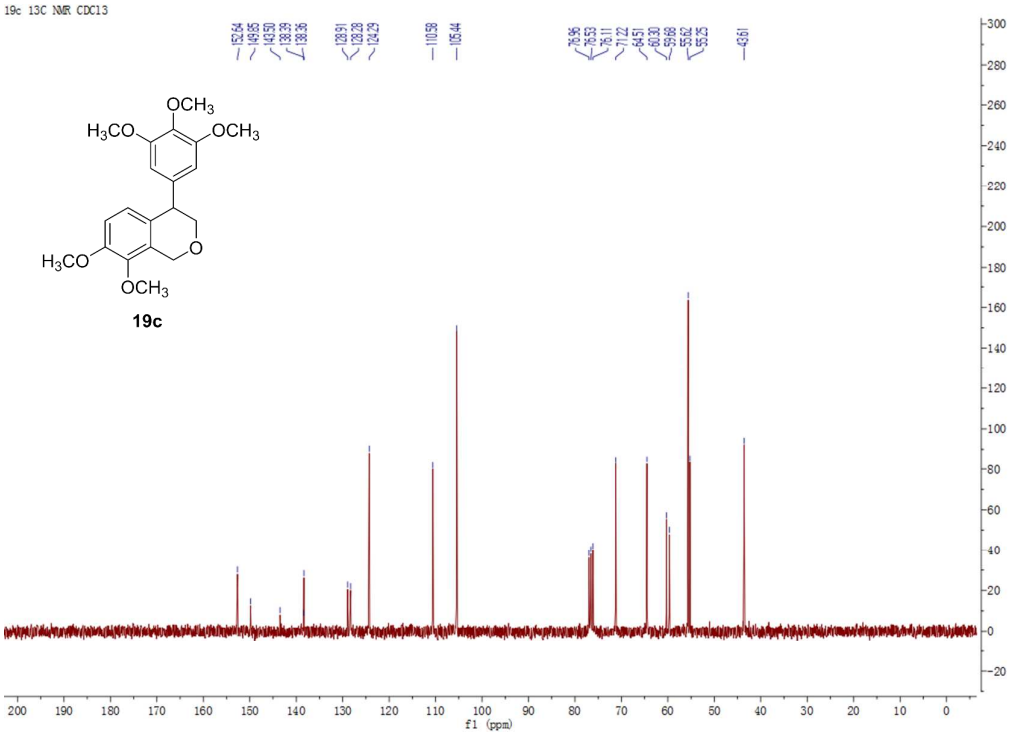
^{1}H NMR (300 MHz) of **19b** in DMSO-d_6



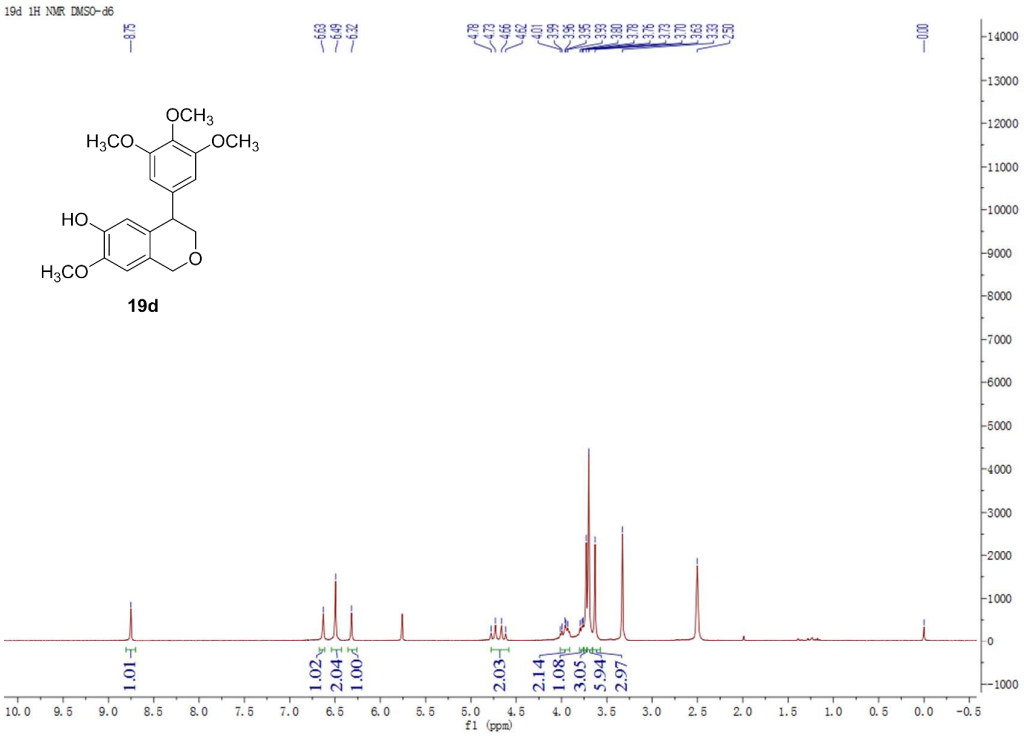
^{13}C NMR (75 MHz) of **19b** in DMSO-d_6



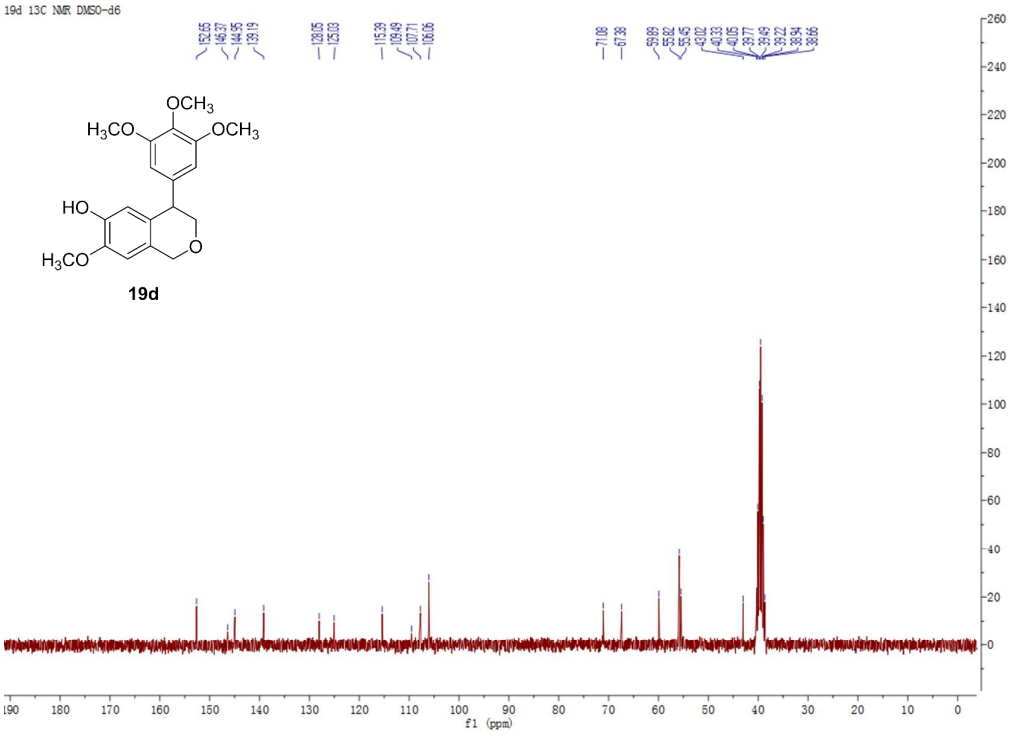
^{13}C NMR (75 MHz) of **19c** in $CDCl_3$



^{13}C NMR (75 MHz) of **19c** in $CDCl_3$



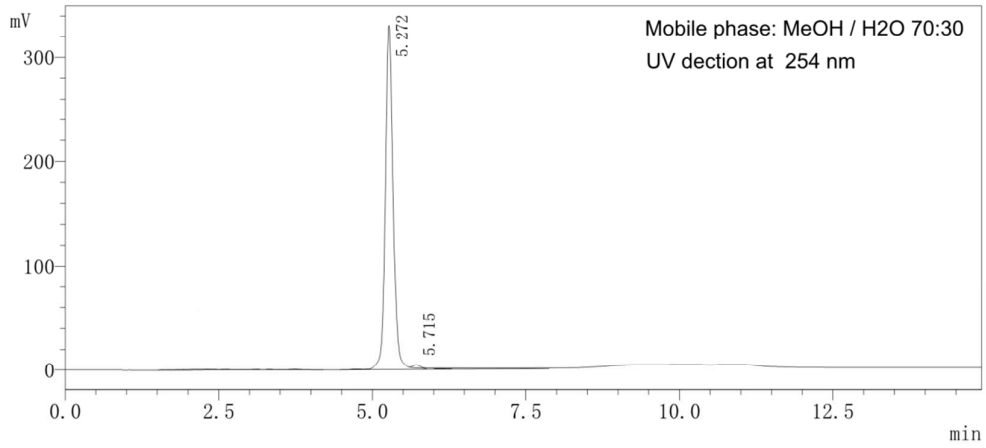
^{1}H NMR (300 MHz) of **19d** in DMSO-d_6



^{13}C NMR (75 MHz) of **19d** in DMSO-d_6

4. The HPLC traces of 18b and 19b

18b Purity = 96.7%



19b Purity = 97.3%