Supporting Information

A high-affinity subtype-selective fluorescent probe for estrogen receptor β imaging in living cells

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‡ These two authors contributed equally to this work.

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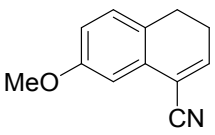
1. Materials and instruments

Starting materials were used without purification which was purchased from Aldrich, Acros, Aladin-reagent, and Alfa-Aesar. The solvents were distilled or purified before use if necessary. Reaction progress was monitored using analytical thin-layer chromatography (TLC). Visualization was achieved by UV light (254 nm and 356 nm). ^{1}H NMR, ^{13}C NMR and ^{19}F NMR were measured on a Bruker Biospin AV400 (400 MHz) instrument. Chemical shifts are reported in ppm (parts per million) and are referenced to either tetramethylsilane or the solvent. Melting points were determined on X-4 Beijing Tech melting point apparatus, and the data were uncorrected. All UV spectra were recorded with a SHIMADZU UV-2600, and fluorescence spectra were recorded with a HITACHI F-4600. Cell imaging was observed with Leica-LCS-SP8 confocal laser scanning microscope.

2. Synthesis of WAY-202196 and FPNM

Compound **WAY-202196** was synthesized according to reported procedures.[1]

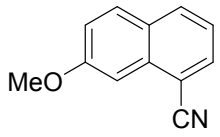
7-Methoxy-3, 4-dihydronaphthalene-1-carbonitrile (**2**)



To a mixture of commercially available 7-methoxy-1-tetralone (**1**) (19.82 g, 0.115 mol), zinc iodide (0.865 g, 2.7 mmol), and toluene (50 mL) was added trimethylsilyl cyanide (12.5 g. 0.125 mol). The mixture was stirred overnight at room temperature. Pyridine (175 mL) was added and phosphorus oxychloride (50 mL) was then added dropwise. The mixture was heated to reflux for 6 h and then pouring into brine, washing, and the mixture was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and then filtered and concentrated in vacuum. The product was purified by column chromatography (10% ethyl acetate in petroleum ether). Yield: 14.46 g (68 %), white solid (mp 51-52 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.04 (d, J = 8.3 Hz, 1H), 6.97 (d, J = 2.5 Hz, 1H), 6.87 (t, J = 4.7 Hz, 1H), 6.78 (dd, J = 8.3, 2.6 Hz, 1H), 3.79 (s, 3H), 2.75 (t, J = 8.2 Hz, 2H), 2.45 (m, J = 8.2, 4.8 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.46, 144.38, 129.24, 128.62, 125.86, 116.84, 114.15,

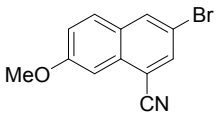
114.04, 110.04, 55.15, 24.89, 23.87.

7-Methoxy-1-naphthonitrile (**3**)



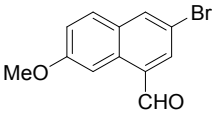
A mixture of **2** (9.95g, 53.1 mmol) and 10 % Pd/C (0.61g, 0.57 mmol) in p-cymene (50 mL) was heated to reflux overnight. The reaction was cooled to room temperature and filtered through Celite. The Celite was rinsed with ethyl acetate, and the combined organics were removed under vacuum. The product was purified by column chromatography (10 % ethyl acetate in petroleum ether). Yield: 4.3 g (44 %), white solid (mp 71-73 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (d, J = 8.2 Hz, 1H), 7.86 (dd, J = 7.2, 1.1 Hz, 1H), 7.79 (d, J = 9.0 Hz, 1H), 7.44 (d, J = 2.4 Hz, 1H), 7.36 (dd, J = 8.1, 7.4 Hz, 1H), 7.25 (dd, J = 9.0, 2.5 Hz, 1H), 3.99 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.64, 133.98, 132.78, 132.76, 130.05, 128.22, 122.42, 120.49, 118.04, 108.32, 102.76, 55.40.

3-Bromo-7-methoxy-1-naphthonitrile (**4**)



To a mixture of **3** (2.42 g, 15.4 mmol) and glacial acetic acid (10 mL) was added bromine (5.18 g, 32.3 mmol). The mixture was stirred at 90 °C for 6 h, then HCl (40 mL of 12 N solution) and $SnCl_2$ (6.9 g, 30.8 mmol) were added, and the mixture was allowed to stir at 90 °C for 1 h. The resulting solution was cooled to room temperature and poured into water. The resulting yellow precipitate was collected by filtration and purified by column chromatography (10% ethyl acetate in petroleum ether). Yield: 1.54 g (38 %), white solid (mp 213-215 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, J = 1.6 Hz, 1H), 7.80 (d, J = 1.9 Hz, 1H), 7.61 (d, J = 9.0 Hz, 1H), 7.28 (d, J = 1.5 Hz, 1H), 7.17 (dd, J = 8.9, 2.3 Hz, 1H), 3.89 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.00, 135.18, 134.87, 132.69, 129.25, 127.81, 121.75, 116.66, 115.00, 110.11, 102.89, 55.64.

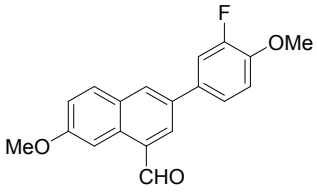
3-Bromo-7-methoxy-1-naphthaldehyde (**5**)



To a solution of **4** (3.89 g, 14.69 mmol) in toluene (150 mL) at -78 °C was added DIBAL (14.7 mL, 1M). The solution was allowed to stir for 1 h at -78 °C and then allowed to warm to room temperature slowly overnight. Methanol (5 mL) and water (5 mL) were added and the mixture was stirred for 30 min. After pouring into brine, and washing, then mixture

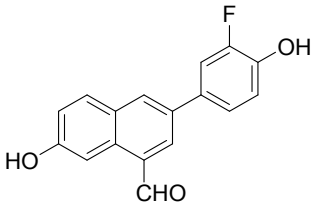
was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and then filtered and concentrated in vacuum. The product was purified by column chromatography (20% ethyl acetate in petroleum ether). Yield: 2.1 g (54 %), yellow solid (mp 95-97 °C). ^{1}H NMR (400 MHz, acetone-d_6) δ 10.33 (s, 1H), 8.62 (d, J = 2.5 Hz, 1H), 8.32 (d, J = 2.0 Hz, 1H), 8.14 (d, J = 2.1 Hz, 1H), 7.90 (d, J = 9.1 Hz, 1H), 7.29 (dd, J = 9.1, 2.6 Hz, 1H), 3.96 (s, 3H). ^{13}C NMR (101 MHz, acetone-d_6) δ 193.09, 161.20, 139.72, 136.84, 132.29, 131.28, 131.00, 129.85, 120.84, 115.70, 103.75, 55.46.

3-(3-Fluoro-4-methoxyphenyl)-7-methoxy-1-naphthaldehyde (**6**)



Under argon atmosphere, the mixture of **5** (0.263 g, 1 mmol), (3-fluoro-4-methoxyphenyl)boronic acid (0.33 g, 2.2 mmol), tetrakis-(triphenylphosphine)-palladium (0.0058 g, 0.005 mmol), and potassium carbonate (0.276g, 2 mmol) in an oxygen-free toluene solution (10 mL) was stirred at 120 °C for 24 h. After cooling to room temperature, the aqueous layer was diluted with 5% HCl solution and extracted with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and then filtered and concentrated in vacuum, and the product was purified by column chromatography (20% ethyl acetate in petroleum ether). Yield: 251 mg (81 %), yellow solid (mp 120-122 °C). ^{1}H NMR (400 MHz, acetone-d_6) δ 10.44 (s, 1H), 8.72 (d, J = 2.5 Hz, 1H), 8.40 (t, J = 2.5 Hz, 2H), 7.99 (d, J = 9.0 Hz, 1H), 7.73-7.56 (m, 2H), 7.38 -7.19 (m, 2H), 3.97 (s, 3H), 3.95 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 193.69, 160.45, 152.56 (d, J = 243.9 Hz), 147.24 (d, J = 10.7 Hz), 136.65, 133.63 (d, J = 1.8 Hz), 132.38 (d, J = 6.5 Hz), 131.97 (d, J = 10.0 Hz), 131.66, 130.93, 130.40, 129.91, 129.58, 128.47 (d, J = 12.2 Hz), 122.41 (d, J = 3.3 Hz), 120.09, 114.41 (d, J = 19.1 Hz), 113.62, 103.20, 56.18, 55.36.

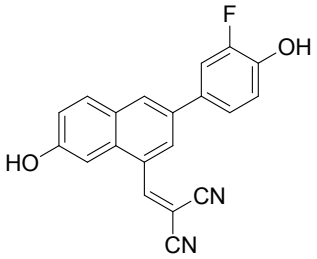
3-(3-Fluoro-4-hydroxyphenyl)-7-hydroxy-1-naphthaldehyde (**7**)



Compound **6** (0.311 g, 1 mmol) was added to pyridine hydrochloride (3 g) at 190 °C for 2 h. After cooling to room temperature, the aqueous layer was diluted with 5% HCl solution and extracted with ethyl acetate. The

combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and then filtered and concentrated in vacuum, and the residue was purified by flash chromatography (10-30% ethyl acetate in petroleum ether). Yield: 205 mg (73 %), yellow solid (mp 258-260 °C). ^{1}H NMR (400 MHz, acetone-d_6) δ 10.40 (s, 1H), 9.13 (s, 1H), 8.90 (d, J = 1.4 Hz, 1H), 8.69 (d, J = 2.4 Hz, 1H), 8.38 (t, J = 1.9 Hz, 2H), 7.99 (d, J = 8.9 Hz, 1H), 7.65 (m, 1H), 7.58-7.50 (m, 1H), 7.28 (m, 1H), 7.15 (t, J = 8.8 Hz, 1H). ^{13}C NMR (101 MHz, acetone-d_6) δ 194.68, 159.27, 152.71 (d, J = 240.3 Hz), 145.45 (d, J = 13.1 Hz), 137.77, 134.18, 132.51 (d, J = 6.2 Hz), 132.40), 131.82), 131.56, 131.44, 130.29, 123.71 (d, J = 3.1 Hz), 120.21, 119.17 (d, J = 3.1 Hz), 115.09 (d, J = 19.4 Hz), 107.62.

2-((3-(3-Fluoro-4-hydroxyphenyl)-7-hydroxynaphthalen-1-yl) methylene) malononitrile (**FPNM**)



To a round bottom flask containing a solution of **7** (0.028 g, 0.1 mmol) and malononitrile (0.014g, 0.2 mmol) in ethanol (5 mL), pyridine hydrochloride (0.023g, 0.2 mmol) was added and the mixture left stirring at 90 °C. The reaction was monitored by TLC and was completed within 24 h. After cooling to room temperature, the aqueous layer was diluted with 5% HCl solution and extracted with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and then filtered and concentrated in vacuum, and the residue was purified by flash chromatography (20% ethyl acetate in petroleum ether). Yield: 28.2 mg (86 %), red solid (mp 227-229 °C). ^{1}H NMR (400 MHz, acetone-d_6) δ 9.25 (s, 1H), 9.03 (s, 1H), 8.98 (s, 1H), 8.50 (d, J = 1.4 Hz, 1H), 8.34 (s, 1H), 7.99 (d, J = 8.9 Hz, 1H), 7.57 (m, 1H), 7.52 (d, J = 2.1 Hz, 1H), 7.48 (m, 1H), 7.32 (m, 1H), 7.19-7.07 (m, 1H). ^{13}C NMR (101 MHz, acetone-d_6) δ 159.04, 157.84, 152.13 (d, J = 238.0 Hz), 145.13 (d, J = 12.7 Hz), 133.67 (d, J = 1.9 Hz), 132.46, 131.82 (d, J = 6.3 Hz), 131.53, 131.28, 129.25, 127.60, 127.30, 123.13 (d, J = 3.1 Hz), 120.12, 118.67 (d, J = 3.2 Hz), 114.52 (d, J = 19.3 Hz), 114.18, 113.75, 105.31, 84.63. ^{19}F NMR (376 MHz, acetone-d_6) δ -137.42. HRMS (ESI) calcd for $C_{20}H_{11}FN_2O_2$ $[M + K]^+$, 369.0436; found 369.0432.

3. Estrogen receptor binding affinity

Relative binding affinities were determined by a competitive radiometric binding assay as previously described,[2, 3] using 2 nM [^{3}H]-estradiol as tracer ([2,4,6,7-^{3}H]-estra-1,3,5(10)-triene-3,17β-diol, 70-115 Ci/mmol, Perkin-Elmer, Waltham, MA), and purified full-length human ERα and ERβ were purchased from PanVera/Invitrogen (Carlsbad, CA). Incubations were for 18-24 h at 0 °C. Hydroxyapatite (BioRad, Hercules, CA) was used to absorb the receptor−ligand complexes, and free ligand was washed away. The binding affinities are expressed as relative binding affinity (RBA) values with the RBA of estradiol set to 100%. The values given are the average ± range or SD of two to three independent determinations. Estradiol binds to ERα with a K_d of 0.2 nM and to ERβ with a K_d of 0.5 nM; these values were determined by scatchard analysis using the binding assay protocol described previously.[2]

4. Live-cell imaging

MCF-7 was grown using RPMI medium, DU-145 and MDA-MB-231 were grown using DMEM medium containing 10% fetal calf serum (FCS) in an atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were plated in 35 mm glass-bottom culture dishes and allowed to adhere for 24 h, immediately before the experiments, the cells were washed with PBS buffer. The competition experiments were conducted by incubating cells with probes (10 μM) at 37 °C for 0.5 h. Then the cells were incubated with 4', 6-diamidino -2-phenylindole dihydrochloride (DAPI). the cells were observed with Leica-LCS-SP8 confocal laser scanning microscope. Next, the cells were treated with E_2 (100 μM) for 10 min and were observed for imaging.

For the co-staining experiments, cells were incubated with the probe and **EE_2-FI** (10 μM) at 37 °C for 0.5 h. Then the cells were incubated with 4', 6-diamidino -2-phenylindole dihydrochloride (DAPI). Then the cells were observed with Leica-LCS-SP8 confocal laser scanning microscope. Next, the cells were treated with E_2 (100 μM) for 10 min and were observed for imaging.

For immunofluorescence staining, the cells were incubated with the probe (10 μM) at

37 °C for 0.5 h. Then the cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 10min, washed 3 times with PBS and incubated 12 h at 37 °C with Monoclonal anti-ERβ antibody (1:200, purchased from Sigma-Aldrich). After incubation, the culture dishes were washed with PBS and incubated with DyLight 488 AffiniPure Goat Anti-Mouse IgG (1:200, purchased from Abbkine). Then after washing with PBS, the cells were incubated with DAPI. Then the cells were observed with Leica-LCS-SP8 confocal laser scanning microscope. Excitation for compound **FPNM** was from a 405 nm laser with emission of 550-750 nm. Excitation for anti-ERβ antibody and **EE_2-Fl** were from a 488 nm laser with emission of 490-550 nm.

5. Molecular modelling

Docking experiment was conducted with the crystal structure of ERβ LBD that was extracted from PDB: 1YYE.[1] Probe **FPNM** was docked into the three-dimensional structure of ERβ LBD with AutoDock software (version 4.2).[4, 5] Crystallographic coordinates of **FPNM** was created by Biochemoffice. Preparations of ligand and the protein were performed with AutoDockTools (ADT). A docking cube with edges of 60 Å, 60 Å, and 58 Å in the *X*, *Y*, and *Z* dimensions, respectively (a grid spacing of 0.375 Å), which encompassed the whole active site, was used throughout docking. On the basis of the Lamarckian genetic algorithm (LGA), 80 runs were performed for each ligand with 500 individuals in the population.[6] The figures were prepared using PyMOL.

6. Figures S1-2

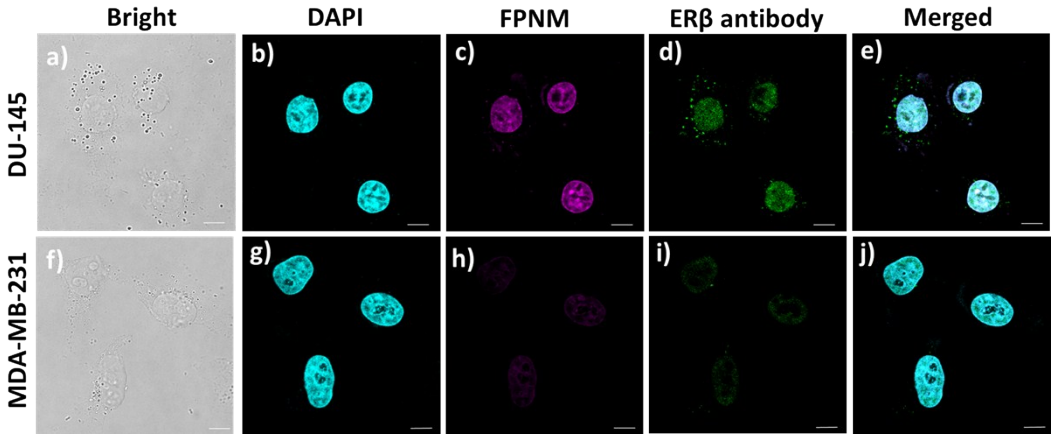


Fig. S1 Co-localization study of cells with **FPNM** (10.0 μM) and ERβ antibody. Scale bar: 10 μm.

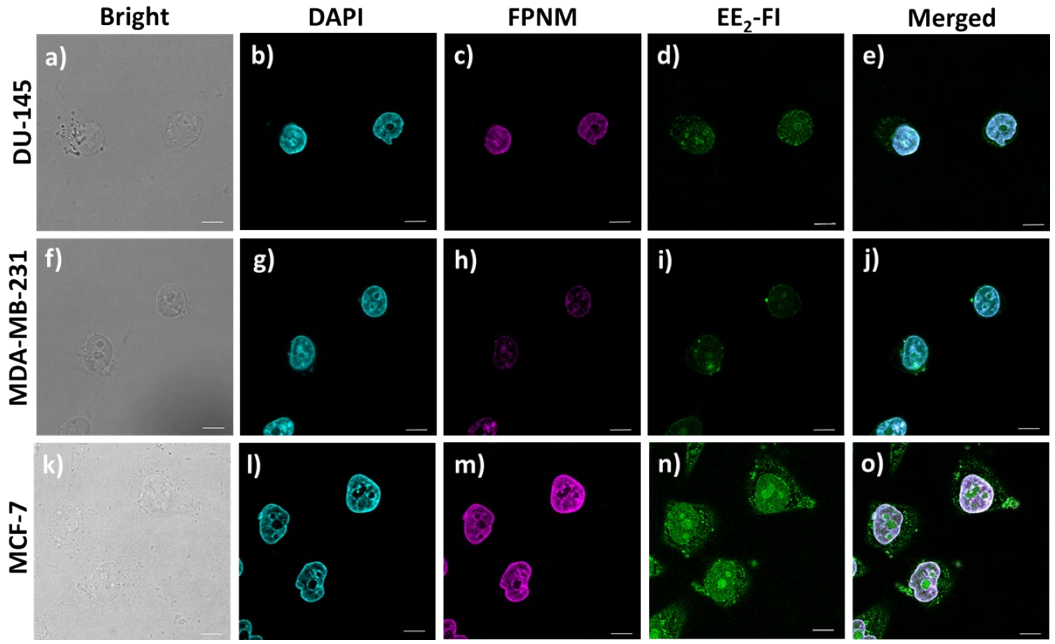
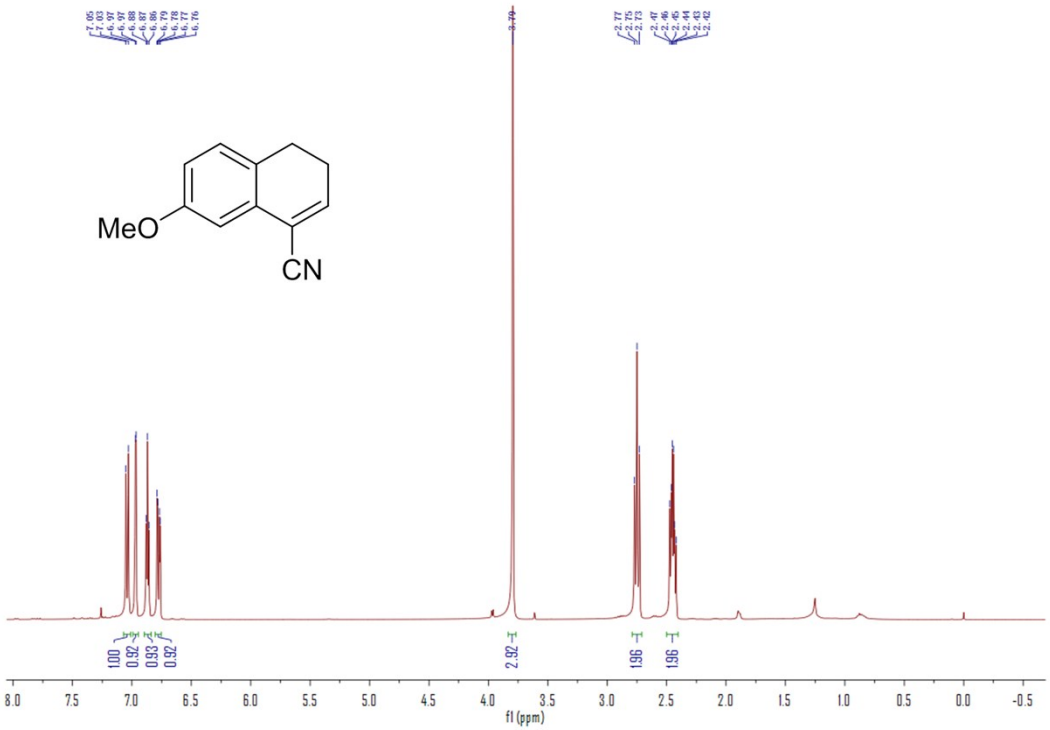


Fig. S2 Co-staining of cells with **FPNM** (10.0 μM) and **EE_2-Fl** (10.0 μM). Scale bar: 10 μm.

7. ^{1}H NMR, ^{13}C and ^{19}F Spectra

^{1}H NMR spectrum of 7-Methoxy-3, 4-dihydronaphthalene-1-carbonitrile (2)



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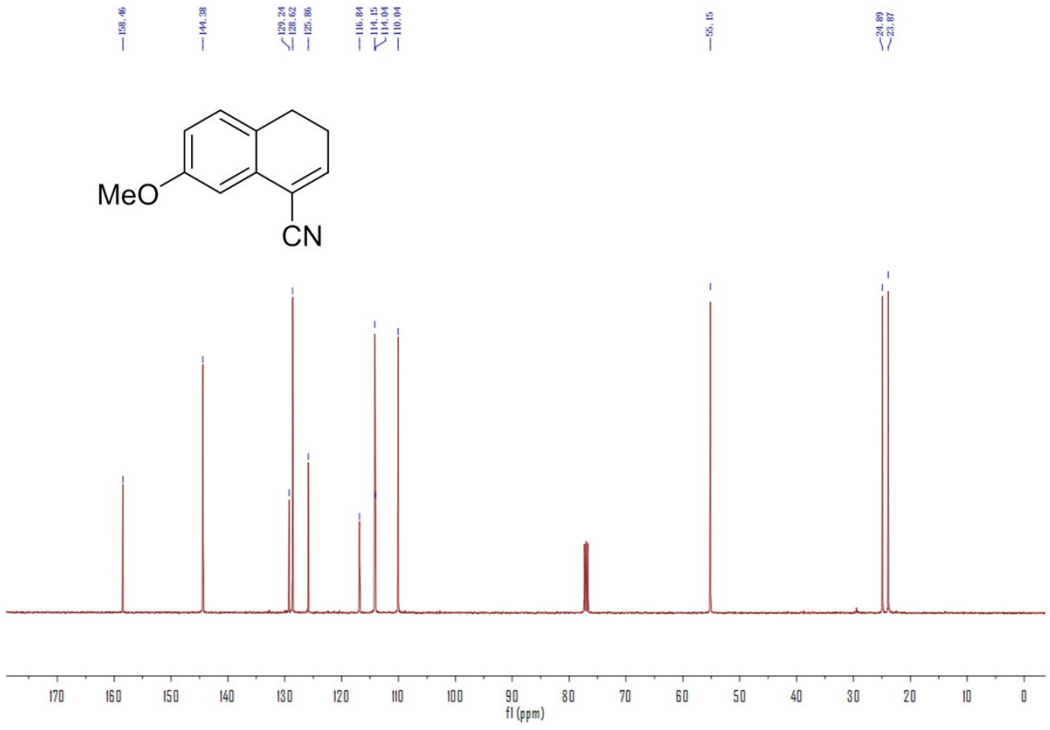
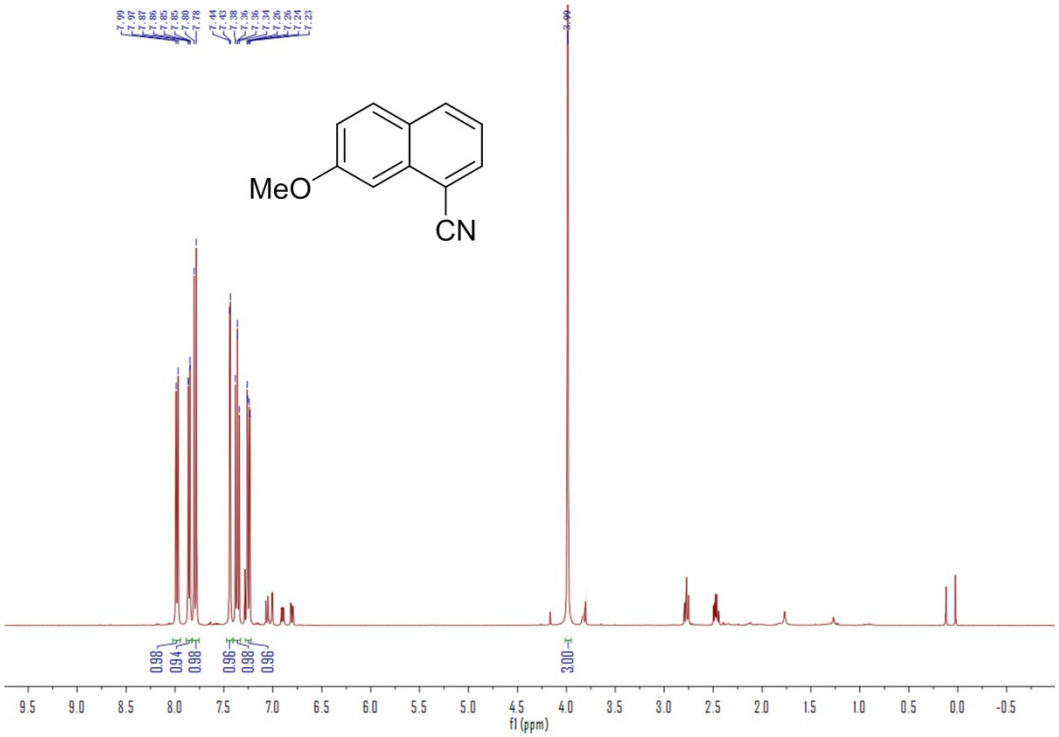


^{1}H NMR spectrum of 7-Methoxy-1-naphthonitrile (3)



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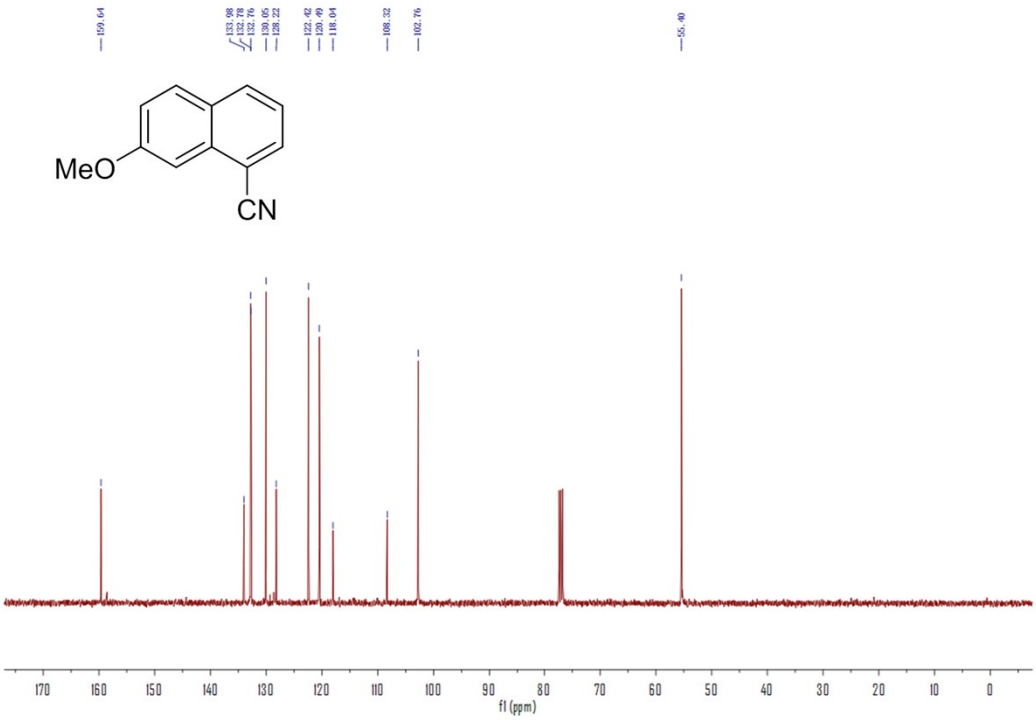

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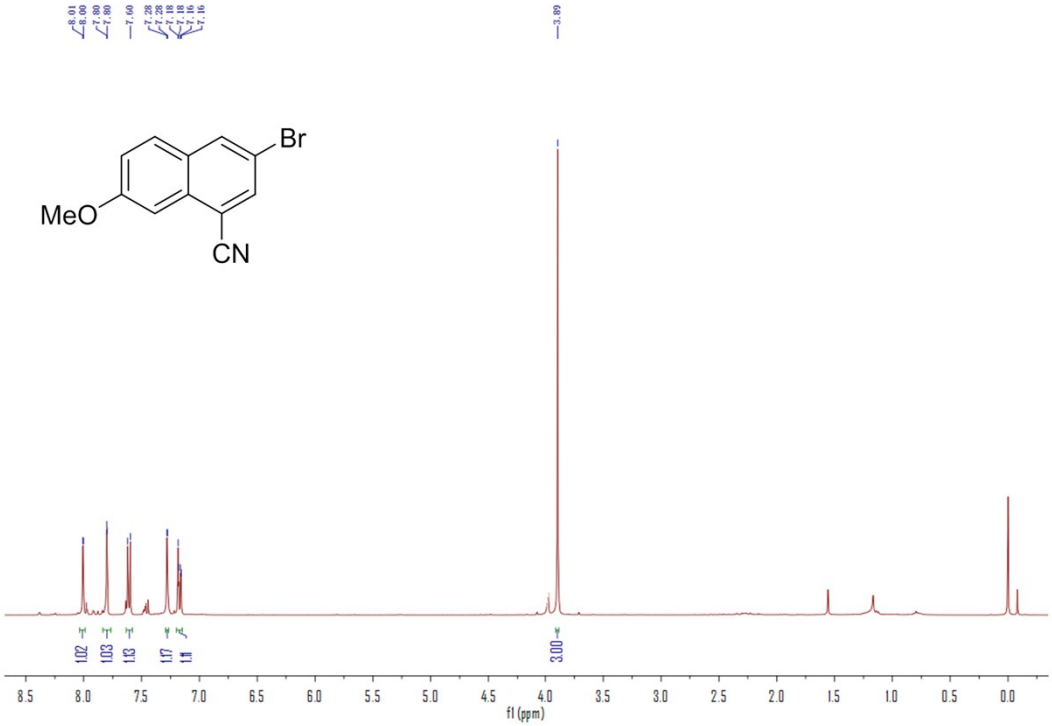

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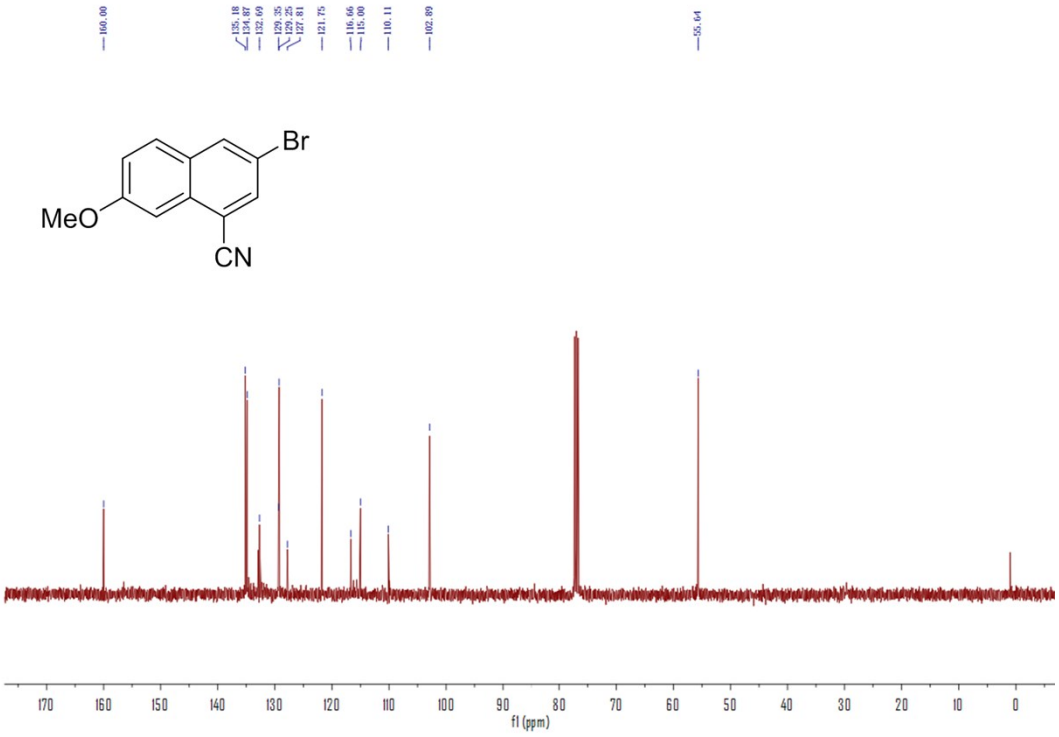

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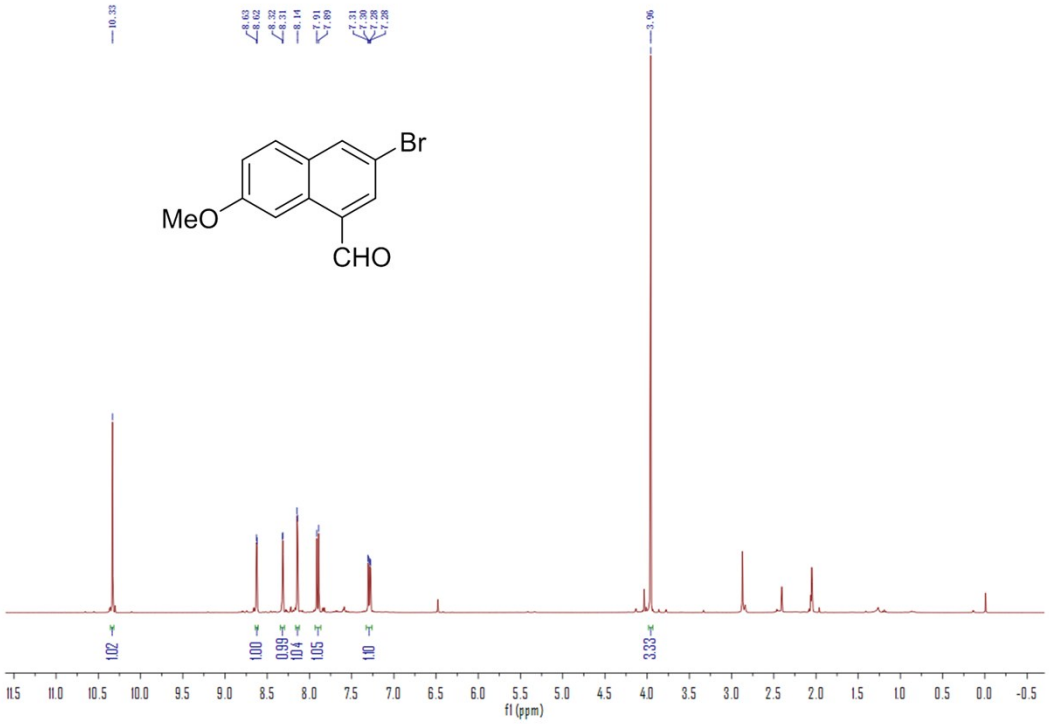

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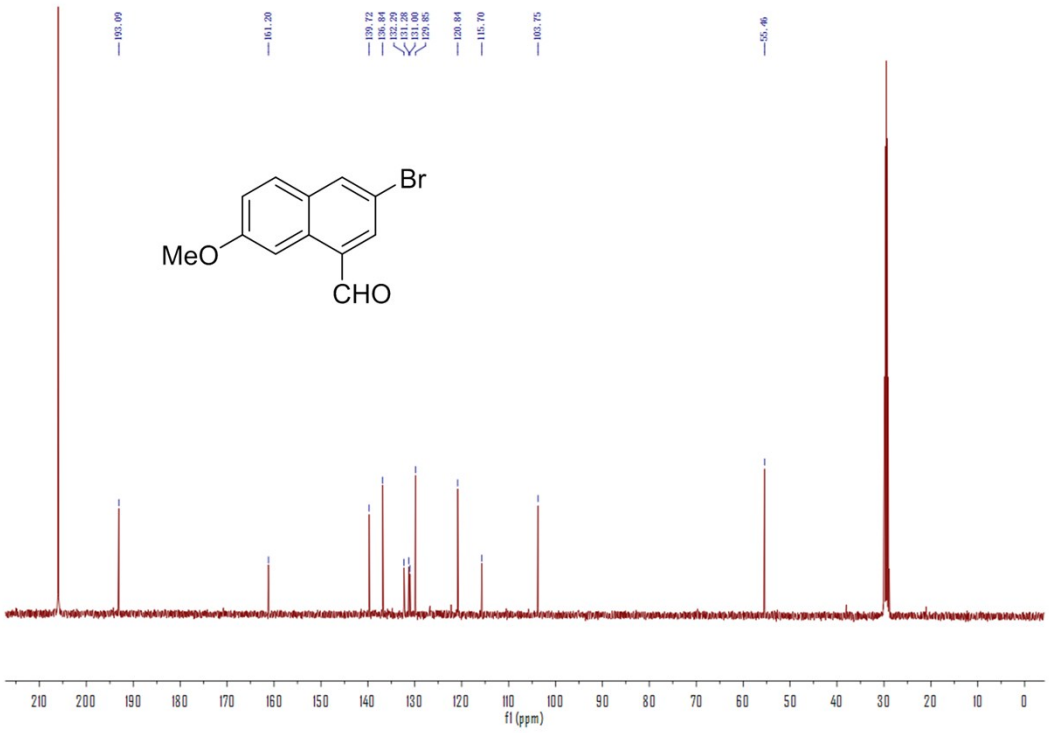

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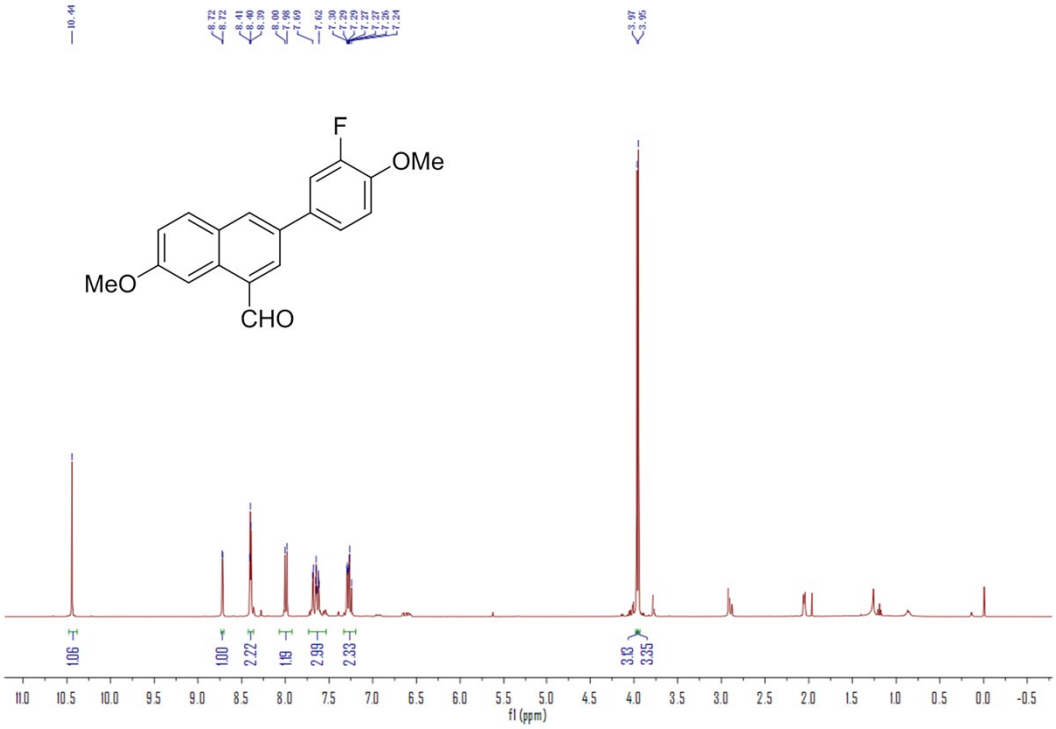

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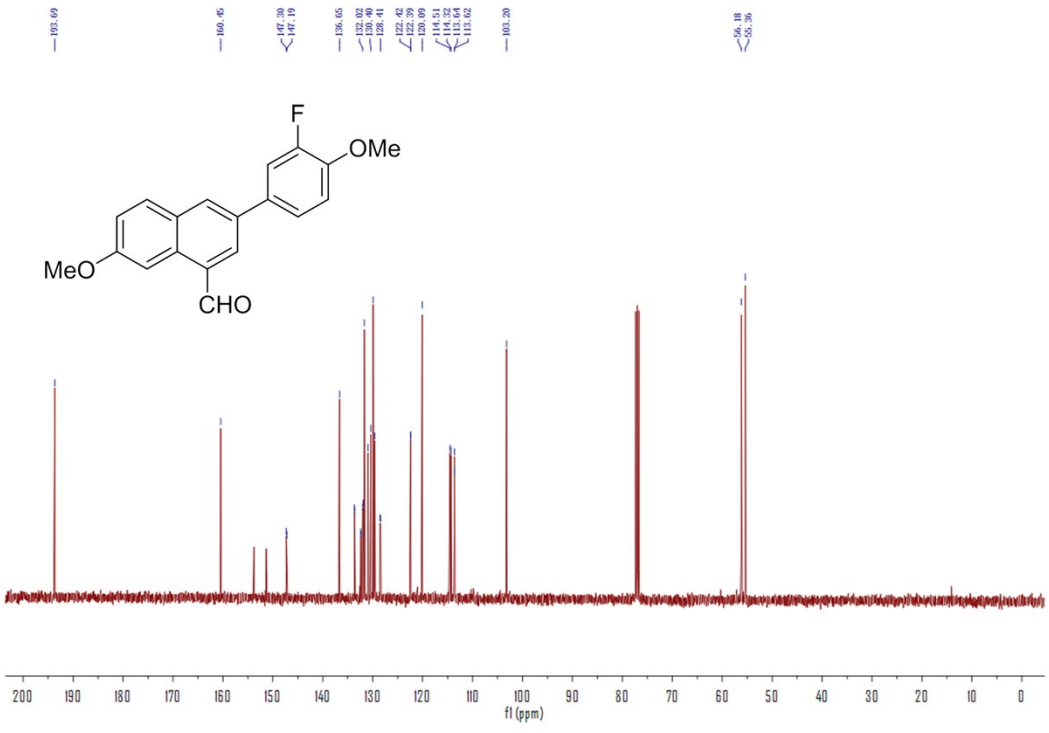

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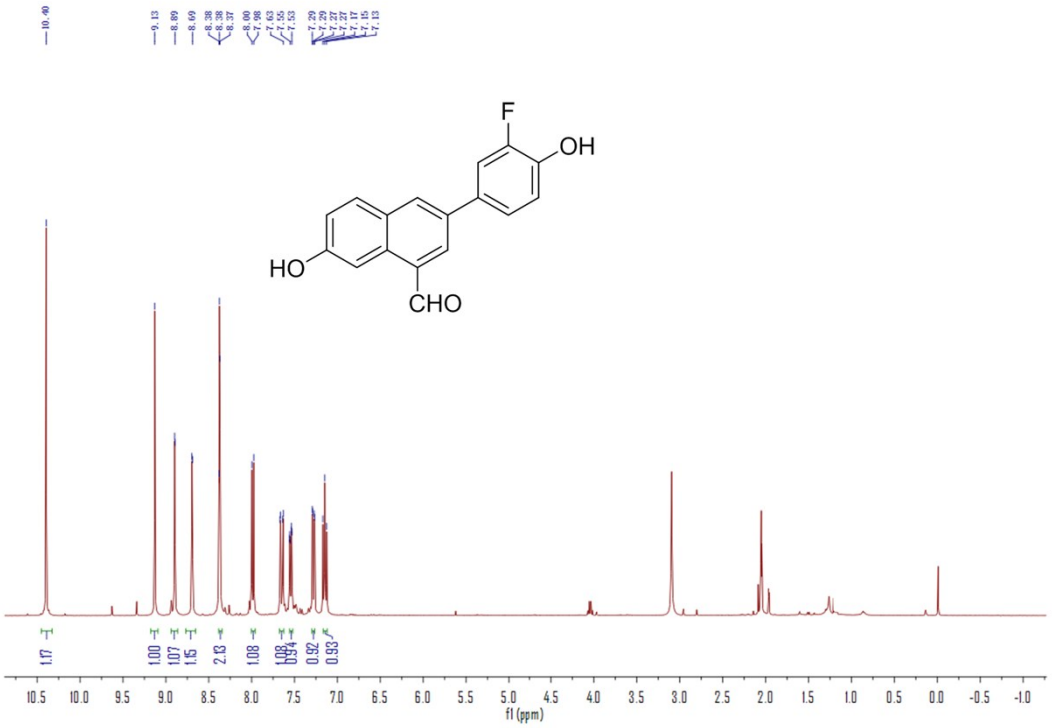

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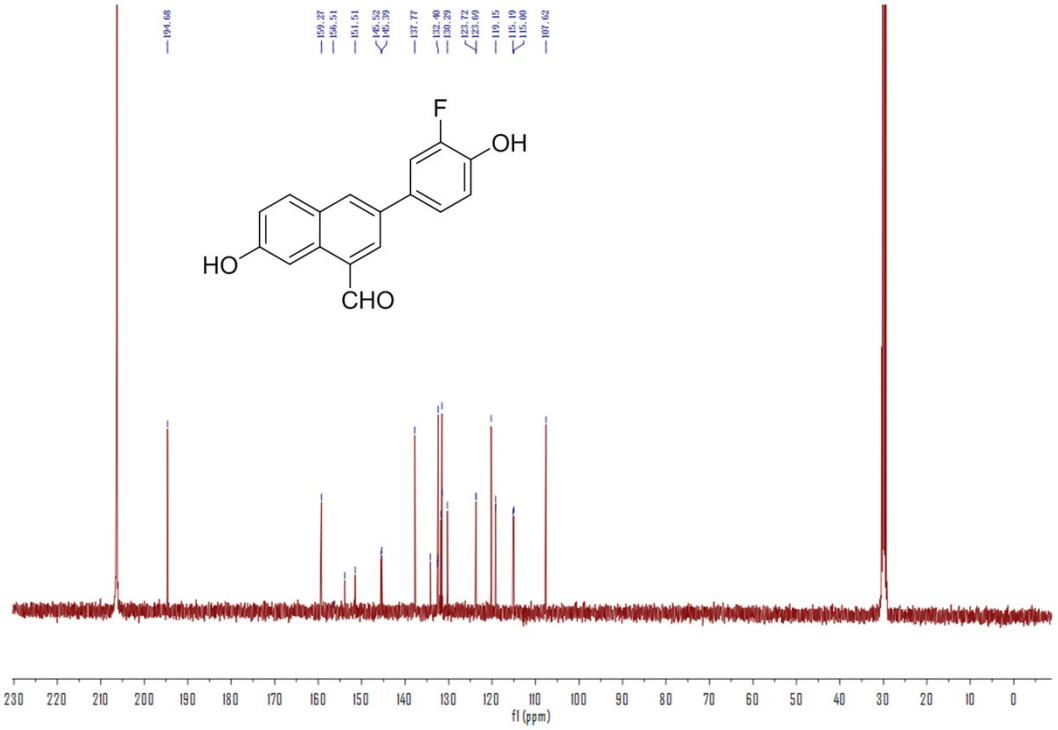

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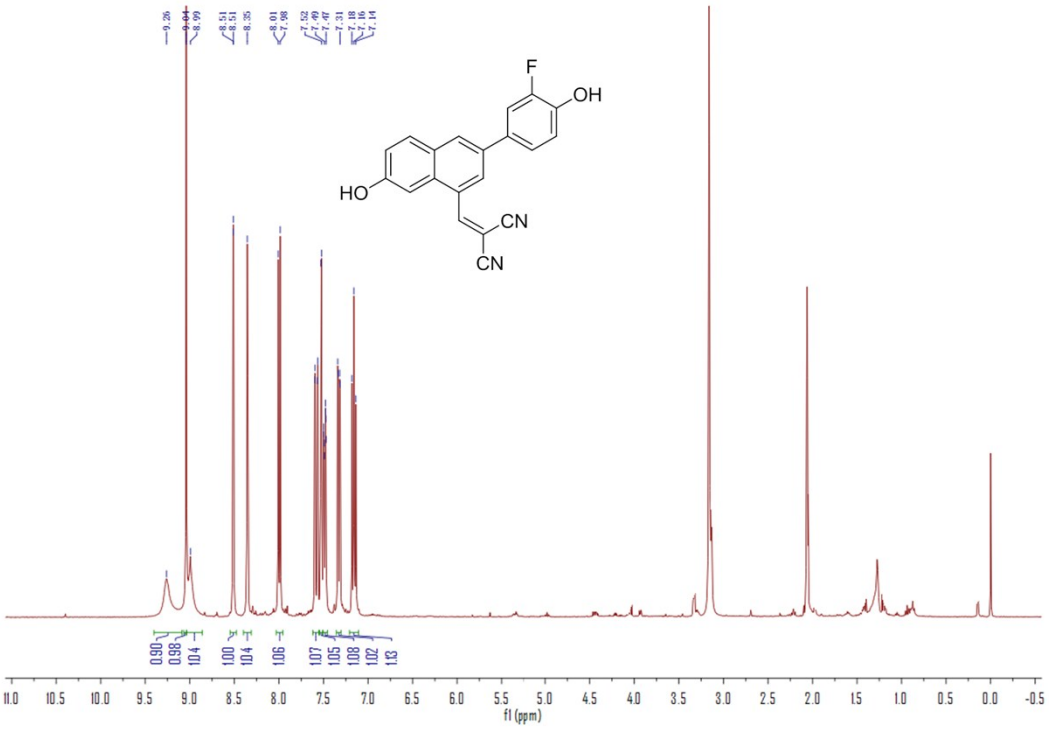

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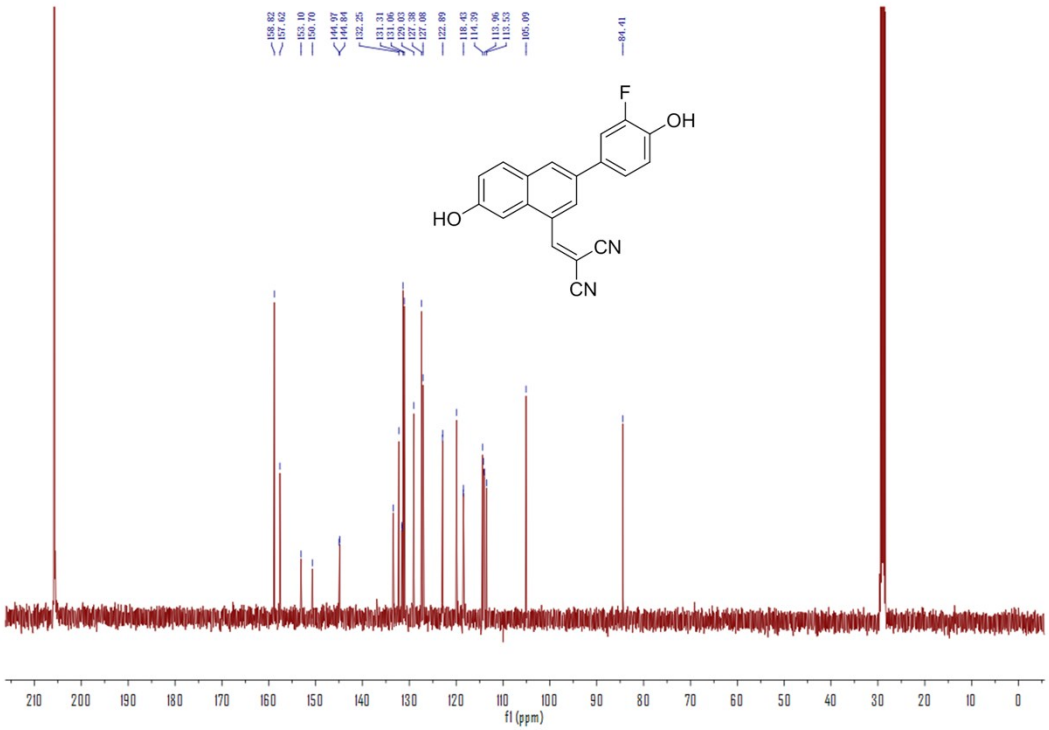

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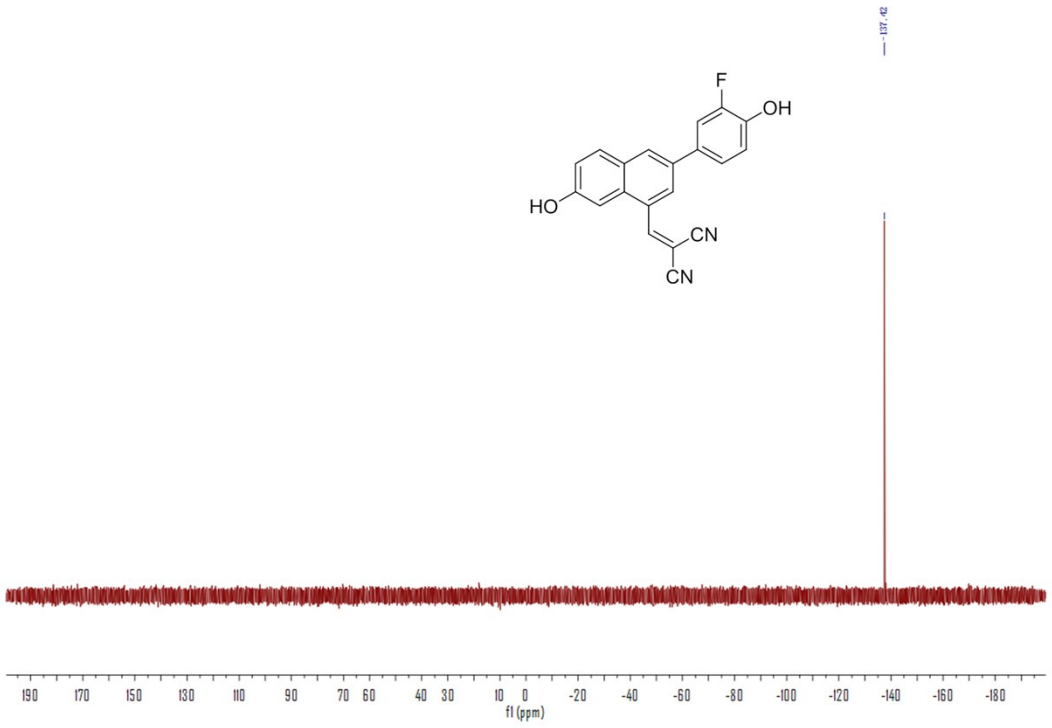

8. References

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