

< Supporting Information >

Discovery of highly selective FLT3 kinase inhibitor from phenotypic cell viability profiling

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I. General information

^1H and ^{13}C NMR spectra were recorded on a Bruker DRX-300 (Bruker Biospin, Germany) and Varian Inova-500 (Varian Assoc., Palo Alto, USA), and chemical shifts were measured in ppm downfield from internal tetramethylsilane (TMS) standard. Multiplicity was indicated as follows: s (singlet); d (doublet); t (triplet); q (quartet); m (multiplet); dd (doublet of doublet); dt (doublet of triplet); br s (broad singlet), etc. Coupling constants were reported in Hz. Routine mass analyses were performed on LC/MS system equipped with a reverse phase column (C-18, 50 x 2.1 mm, 5 μm) and photodiode array detector using electron spray ionization (ESI). Solvents and organic reagents were purchased from commercial vendors and used without further purification unless otherwise mentioned. The progress of reaction was monitored using thin-layer chromatography (TLC) (silica gel 60 F₂₅₄ 0.25 mm), and components were visualized by observation under UV light (254 and 365 nm) or by treating the TLC plates with ninhydrin followed by heating. All reactions were conducted in oven-dried glassware. Ez-cytox kit for WST assay was purchased from Daeil Co. (Korea) and was used for the cell viability test. All antibodies for immunoblotting were purchased from Cell signaling (USA) and Abcam (UK). All dose dependent graphs were figured by GraphPad Prism 5. Data are the mean measurements from at least three different independent experiments and SD. For the Ez-cytox-based cell cytotoxicity test, the absorbance of 96-well plate was measured by BioTek Synergy HT Microplate reader. Immunoblotting was developed by ChemiDocTM (BioRAD, USA). Kinase profiling assay was conducted by Reaction Biology Corporation (USA) as well as *in vitro* kinase assay using HotSpotSM kinase assay.

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Table S1. Representative result for cell viability profiling result. Cell viability was measured by WST assay for 6 different cell lines. In-house compound library, constructed by pDOS strategy, was treated for 24 h with 1 μ M as final concentration. Each compound was tested in duplicate. Results are mean value and designated by % which is normalized by DMSO as control. Data is illustrated by color coding from red for low value to green color for high value.

III. Western blot analysis

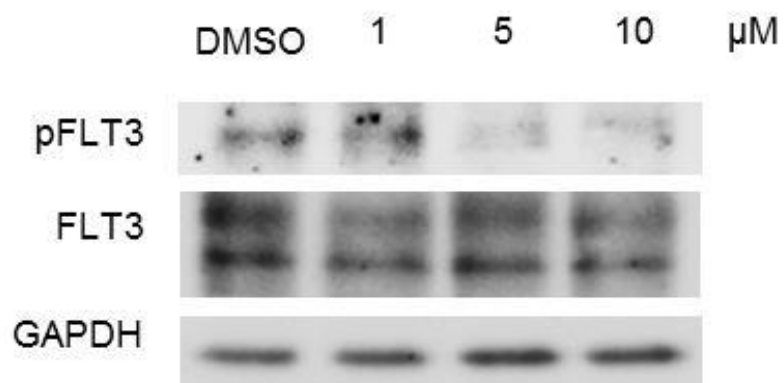


Figure S1. Western blot analysis result for inhibitory effect against FLT3 activity of **4g** in MV-4-11 cells. Compound **4g** was treated for 4 h in dose-dependent manner.

IV. Comparison of cell viability for MV-4-11 cells and HL60 cells

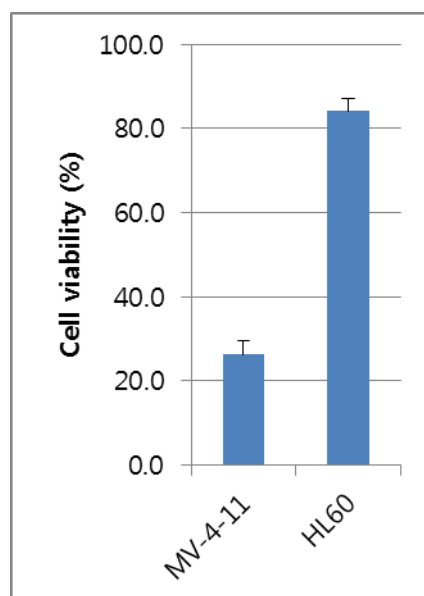


Figure S2. Cell viability assay against FLT3-positive MV-4-11 cells and FLT3-negative HL60 AML cell lines upon treatment with **4g** at the final concentration of 1 μ M in 24 h. Cell viability was normalized to DMSO control as 100 %. Each experiment was performed in triplicate; data was figured as mean and SD.

V. Experimental procedure for biological assay

Cell culture

All cell lines were obtained from American Type Culture Collection [ATCC, Manassas, VA, USA]. MCF7, MDA-MB-231, U87-MG and HL60 cell lines were cultured in RPMI 1640 [GIBCO, Invitrogen] supplemented with heat-inactivated 10 % (v/v) fetal bovine serum [GIBCO, Invitrogen] and 1 % (v/v) antibiotic-antimycotic solution [GIBCO, Invitrogen]. NIH3T3 and L6 cells were maintained in DMEM [GIBCO, Invitrogen] supplemented with heat-inactivated 10 % (v/v) fetal bovine serum [GIBCO, Invitrogen] and 1 % (v/v) antibiotic-antimycotic solution [GIBCO, Invitrogen]. MV-4-11 cells were maintained in IMDM [GIBCO, Invitrogen] supplemented with heat-inactivated 10 % (v/v) fetal bovine serum [GIBCO, Invitrogen] and 1 % (v/v) antibiotic-antimycotic solution [GIBCO, Invitrogen]. Cells were maintained in a humidified atmosphere of 5 % CO₂ incubator at 37 °C, and cultured in 100 mm cell culture dish [CORNING] or T75-flask [NUNC].

In Vitro Cytotoxicity Test

Cell viability was measured by the WST (Water-soluble tetrazolium salts) assay kit, and the experimental procedure was based on the manufacturer's manual. Cells were cultured into 96-well plates for 24 h, followed by the treatment using multiblot replicator for high throughput screening or serial dilution for a dose-dependent assay. After 24 h of incubation with compounds, 10 µL of WST-1 solution, containing 2-(4-nitrophenyl)-5-(2-sulfophenyl)-3-[4-(4-sulfophenylazo)-2-sulfophenyl]-2H-tetrazolium disodium salt, was added to each well, and plates were incubated for an additional 1–2 h at 37 °C. Absorbance in 455 nm was measured by microplate reader. The percentage of cell viability was calculated by following formula: % cell viability = (mean absorbance in test wells)/(mean absorbance in control well) × 100. Each experiment was performed in triplicate at least.

Immunoblotting experiment

For Western blot analysis to confirm the apoptotic process, MV-4-11 cells were seeded on 6 well-plate. After 12 h of incubation with increasing concentration, cells were harvest. Cell lysate was obtained by treating RIPA cell lysis buffer including PIC (Roche) and PMSF (sigma) for 2 h at 0 °C. After the centrifugation of cell lysate at 14,000 rpm for 15 min, the supernatant was analyzed by SDS-PAGE and transferred into PVDF membrane, followed by 2% BSA blocking over 1 h. The samples were subjected to immunoblot analysis to detect capsase-3 and PARP cleavage with the specific primary antibody, anti-capase-3 and anti-PARP antibodies, respectively, for overnight at 4 °C, followed by washing with TBST for 2 h at room temperature. The resulting membrane was exposed into HRP-conjugated secondary antibody for 1 h at room temperature. After 1–2 h washing with TBST, the membrane was developed by ECL and the chemiluminescent signal was scanned by ChemiDoc™. The phosphorylation of STAT5, Erk1/2 and FLT3 was detected using the identical procedure for immunoblot analysis. Briefly, after the treatment of MV-4-11 cells or HL60 cells with compounds for 4 h, cells were harvested and lysed with RIPA buffer containing PIC, PMSF, NaF (sigma) and Na₃VO₄

(sigma). Samples were analyzed by immunoblotting as outlined above using anti-STAT5, anti-phospho-STAT5, anti-p44/42 MAPK, anti-phospho-p44/42 MAPK, anti-FLT3 and anti-phospho-FLT3 antibodies.

Immunoprecipitation and western blot analysis

For the analysis of phosphorylation level on FLT3, immunoprecipitation and western blotting were performed. MV-4-11 cells were seeded on 6 well-plate. After 4 h of incubation with increasing concentration, cells were harvested and lysed by IP buffer including PIC (Roche), PMSF (sigma), NaF (sigma) and Na_3VO_4 (sigma). Cell lysate was quantified by BCA assay and incubated with anti-FLT3 antibody at 4 °C for overnight, subsequently precipitated with protein G immunoprecipitation kit (sigma) instructed by manufacturer's protocol. Samples were analyzed by identical procedure for immunoblot assay, except for using anti-FLT3 or anti-pTyr as primary antibodies.

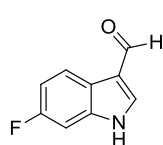
***In vitro* kinase assay and kinase profiling**

Kinase assay was conducted by Reaction Biology Corporation using HotSpotSM kinase assay. Individual reactions were carried out in the presence of 10 μM of ATP. *In vitro* FLT3 kinase assay was performed in a dose-dependent manner. To measure IC_{50} value, compound **4g** was tested in 10 different concentrations with a 3-fold serial dilution starting with 100 μM in duplicate. Kinase profiling was performed in a single dose at 10 μM of compound **4g** in duplicate. Data was represented by % enzyme activity relative to DMSO control.

VI. General synthetic procedure and compound characterization

Indole derivatives **1** and 3-aminopyrazoles **3**, used in this study as starting materials, were commercially available. Indole-3-carboxaldehyde derivatives **2** were synthesized according to the previous report.¹ The general procedure for the synthesis of heterobiaryl pyrazolopyridine analogues **4** is following: To a solution of indole-3-carboxaldehyde derivatives **2** and aminopyrazole **3** (1.0 equiv.) in MeOH (50 mM), aluminium trichloride (0.10 equiv.) was added, and the reaction mixture was heated at reflux condition as 70 °C in 20 mL-vial for 3~5 h. As the progression of this transformation, a desired product was crashed out from the reaction solution as a solid. After the completion of the reaction monitored by TLC, the reaction mixture was cooled at room temperature, and the resulting solid was slowly filtered and washed with cold MeOH and diethyl ether. Two of final products, **4a** and **4i**, were already reported in the previous paper.¹

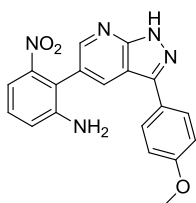
6-fluoro-1H-indole-3-carbaldehyde



¹H NMR (500 MHz, Acetone-*d*₆) δ 11.25 (br. s., 1 H), 10.04 (s, 1 H), 8.23–8.21 (m, 2 H), 7.33 (dd, 1 H), 7.10–7.06 (m, 1 H); ¹³C NMR (125 MHz, Acetone-*d*₆) δ 184.79, 161.55, 159.66, 138.17, 122.71, 121.42, 119.30, 110.78, 110.59, 98.84, 98.63. The

desired product was isolated by silica-gel flash column chromatography using EA and hexane as a white solid and purified (yield: 80%). LRMS (CI+) *m/z* calcd for C₉H₆FNO [M]⁺ 163.0, found 164.0.

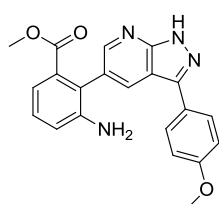
Compound 4b



¹H NMR (500 MHz, DMSO-*d*₆) δ 13.81 (br. s., 1 H), 8.35 (d, *J* = 1.96 Hz, 1 H), 8.33 (d, *J* = 1.96 Hz, 1 H), 7.97 (d, *J* = 9 Hz, 2 H), 7.31 (t, *J* = 8.19 Hz, 1 H), 7.14 (d, *J* = 8.07 Hz, 1 H), 7.07 (dd, *J* = 8.68, 2.57 Hz, 3 H), 5.37 (s, 2 H), 3.80 (s, 3 H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 159.97, 153.03, 151.74, 150.18, 149.55, 143.40, 131.61, 129.89, 128.61, 126.27, 123.68, 119.24, 115.87, 115.11, 112.79, 111.20,

55.88. The desired product was isolated by recrystallization in methanol as a yellow solid (yield: 74%). LRMS (EI+) *m/z* calcd for C₁₉H₁₅N₅O₃ [M]⁺ 361.1, found 360.0..

Compound 4c

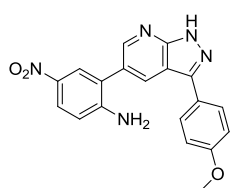


¹H NMR (500 MHz, DMSO-*d*₆) δ 13.69 (s, 1 H), 8.23 (d, *J* = 11.00 Hz, 2 H), 7.95 (d, *J* = 7.09 Hz, 2 H), 7.21 (t, *J* = 7.83 Hz, 1 H), 7.07 (d, *J* = 7.09 Hz, 3 H), 6.98 (d, *J* = 8.07 Hz, 1 H), 4.96 (br. s., 2 H), 3.81 (s, 3 H), 3.45 (s, 3 H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 168.26, 159.62, 152.59, 150.30, 148.02, 142.92, 132.41, 130.74, 128.85, 128.27, 127.05, 126.32, 122.69, 118.42, 117.50, 114.85,

112.38, 55.58. The desired product was isolated by recrystallization in methanol as a white solid (yield: 66%). LRMS (EI+) *m/z* calcd for C₂₁H₁₈N₄O₃ [M]⁺ 374.1, found 373.5.

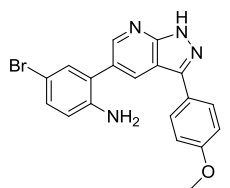
¹ S. Lee and S. B. Park, *Org. Lett.*, 2009, **11**, 5214–5217.

Compound 4d



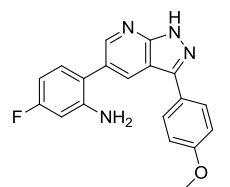
^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ 13.78 (s, 1 H), 8.54 (d, $J = 12.5$, 2 H), 8.04 (d, $J = 1.96$ Hz, 1 H), 8.01 (d, $J = 8.80$ Hz, 1 H), 7.97 (d, $J = 2.45$ Hz, 4 H), 7.08 (d, $J = 8.80$ Hz, 2 H), 6.84 (d, $J = 9.05$ Hz, 1 H), 6.58 (br. s., 2 H), 3.82 (s, 3H); ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) δ 159.97, 154.18, 153.07, 150.04, 143.56, 136.90, 136.90, 131.34, 128.69, 128.28, 126.99, 126.37, 126.20, 122.53, 115.06, 114.49, 112.67. The desired product was isolated by recrystallization in methanol as a yellow solid (yield: 72%). LRMS (EI+) m/z calcd for $\text{C}_{19}\text{H}_{15}\text{N}_5\text{O}_3$ $[\text{M}]^+$ 361.1, found 360.6.

Compound 4e



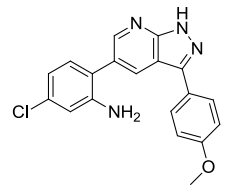
^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ 13.71 (s, 1 H), 8.50 (s, 1 H), 8.42 (s, 1 H), 7.97 (d, $J = 8.80$ Hz, 2 H), 7.22 (s, 1 H), 7.20 (d, $J = 8.31$ Hz, 1 H), 7.06 (d, $J = 8.56$ Hz, 2 H), 6.73 (d, $J = 8.31$ Hz, 1 H), 5.14 (br. s., 2 H), 3.80 (s, 3 H); ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$) δ 159.93, 152.79, 150.05, 146.27, 143.49, 130.78, 128.68, 128.67, 128.08, 126.44, 125.72, 117.73, 115.09, 115.05, 112.62, 107.70, 104.99. The desired product was isolated by recrystallization in methanol as a yellowish white solid (yield: 58%). LRMS (EI+) m/z calcd for $\text{C}_{19}\text{H}_{15}\text{BrN}_4\text{O}$ $[\text{M}]^+$ 394.0, found 393.7.

Compound 4f



^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ 12.78 (s, 1 H), 7.56 (d, $J = 1.96$ Hz, 1 H), 7.46 (d, $J = 1.71$ Hz, 1 H), 7.03 (d, $J = 8.80$ Hz, 2 H), 6.17 (m, 1 H), 6.15 (d, $J = 8.80$ Hz, 2 H), 5.64 (m, 1 H), 5.51 (m, 1 H), 4.34 (s, 2 H), 2.88 (s, 3 H); ^{13}C NMR (75 MHz, $\text{DMSO-}d_6$) δ 159.70, 152.51, 150.11, 148.66, 148.50, 143.15, 132.79, 130.43, 128.42, 128.30, 126.30, 119.83, 114.86, 112.41, 103.10, 101.20, 55.65. The desired product was isolated by recrystallization in methanol as a white solid (yield: 74%). LRMS (EI+) m/z calcd for $\text{C}_{19}\text{H}_{15}\text{FN}_4\text{O}$ $[\text{M}]^+$ 334.1, found 333.8.

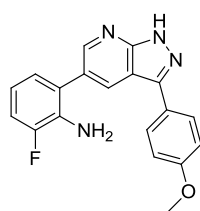
Compound 4g



^1H NMR (500 MHz, $\text{Acetone-}d_6$) δ 12.79 (br. s., 1 H), 8.55 (d, $J = 1.96$ Hz, 1 H), 8.49 (d, $J = 1.96$ Hz, 1 H), 8.05 (d, $J = 9.05$ Hz, 2 H), 7.15 (d, $J = 8.07$ Hz, 1 H), 7.10 (d, $J = 8.80$ Hz, 2 H), 6.94 (d, $J = 1.96$ Hz, 1 H), 6.75 (d, $J = 8.07$ Hz, 1 H), 4.93 (br. s., 1 H), 3.87 (s, 3 H); ^{13}C NMR (125 MHz, $\text{Acetone-}d_6$) δ 159.97, 152.81, 150.16, 148.40, 143.46, 133.62, 133.09, 130.62, 128.67, 128.31, 126.51, 122.46, 116.62, 115.11, 114.79, 112.64, 55.88. The desired product was isolated by recrystallization in methanol as a white solid (yield: 66%). LRMS (EI+) m/z calcd for $\text{C}_{19}\text{H}_{15}\text{ClN}_4\text{O}$ $[\text{M}]^+$ 350.1, found 349.8.

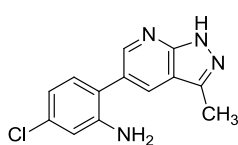
10 | SUPPORTING INFORMATION

Compound 4h



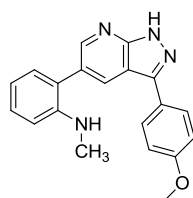
^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 13.75 (br. s., 1 H), 8.55 (d, $J = 2.45$ Hz, 1 H), 8.47 (d, $J = 2.45$ Hz, 1 H), 7.97 (m, 2 H), 7.08 (d, $J = 8.56$ Hz, 2 H), 7.08 (m, 3 H), 6.90–7.01 (m, 1 H), 6.51–6.77 (m, 1 H), 4.95 (br. s., 2 H), 3.81 (s, 3 H); ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 159.95, 152.84, 152.71, 150.83, 150.09, 143.47, 134.86, 130.68, 128.65, 128.20, 127.25, 126.39, 116.86, 115.09, 114.82, 112.60, 55.87. The desired product was isolated by recrystallization in methanol as a white solid (yield: 47%). LRMS (EI+) m/z calcd for $\text{C}_{19}\text{H}_{15}\text{FN}_4\text{O}$ $[\text{M}]^+$ 334.1, found 333.9.

Compound 4j



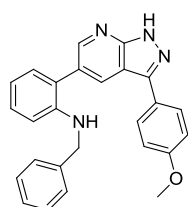
^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 13.24 (br. s., 1 H), 8.42 (s, 1 H), 8.14 (s, 1 H), 7.02 (d, $J = 7.83$ Hz, 1 H), 6.82 (s, 1 H), 6.64 (d, $J = 8.07$ Hz, 1 H), 5.25 (br. s., 2 H), 3.38 (s, 3 H); ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 152.35, 149.64, 148.02, 141.80, 133.24, 132.70, 129.66, 126.87, 122.42, 116.38, 114.51, 114.40, 12.66. The desired product was isolated by recrystallization in methanol as a white solid (yield: 30%). LRMS (EI+) m/z calcd for $\text{C}_{13}\text{H}_{11}\text{ClN}_4$ $[\text{M}]^+$ 258.1, found 257.9.

Compound 4k



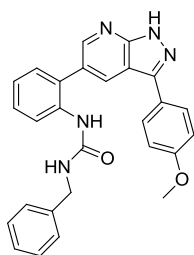
^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 13.72 (br. s., 1 H), 8.47 (s, 1 H), 8.38 (s, 1 H), 7.97 (d, $J = 8.56$ Hz, 2 H), 7.24 (t, $J = 7.83$ Hz, 1 H), 7.07 (m, 3 H), 6.70 (t, $J = 7.46$ Hz, 1 H), 6.63 (d, $J = 8.31$ Hz, 1 H), 5.09 (br. s., 1 H), 3.81 (s, 3 H), 2.63 (s., 3 H); ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$) δ 159.17, 152.05, 149.84, 147.11, 130.69, 130.21, 129.01, 128.41, 127.92, 125.76, 123.80, 115.93, 114.37, 111.95, 109.60, 55.13, 30.25. The desired product was isolated by recrystallization in methanol as a pink solid (yield: 29%). LRMS (EI+) m/z calcd for $\text{C}_{20}\text{H}_{18}\text{N}_4\text{O}$ $[\text{M}]^+$ 330.1, found 330.5.

Compound 4l



^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 13.75 (s, 1 H), 8.57 (s, 1 H), 8.48 (s, 1 H), 8.00 (d, $J=8.80$ Hz, 2 H), 7.35 (m, 2 H), 7.29 (m, 2 H), 7.20 (d, $J=7.09$ Hz, 1 H), 7.03–7.14 (m, 4 H), 6.67 (m, $J=7.09$ Hz, 1 H), 6.55 (d, $J=8.31$ Hz, 1 H), 5.71 (s, 1 H), 4.28 (s, 2 H), 3.82 (s, 3 H); ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 159.71, 152.63, 150.28, 146.10, 143.17, 140.80, 131.41, 130.80, 129.23, 128.73, 128.42, 127.33, 126.98, 126.31, 124.60, 116.69, 114.86, 112.57, 111.20, 55.66, 46.79. The desired product was isolated by recrystallization in methanol as a white solid (yield: 47%). LRMS (EI+) m/z calcd for $\text{C}_{26}\text{H}_{22}\text{N}_4\text{O}$ $[\text{M}]^+$ 406.2, found 406.3.

Compound 4m

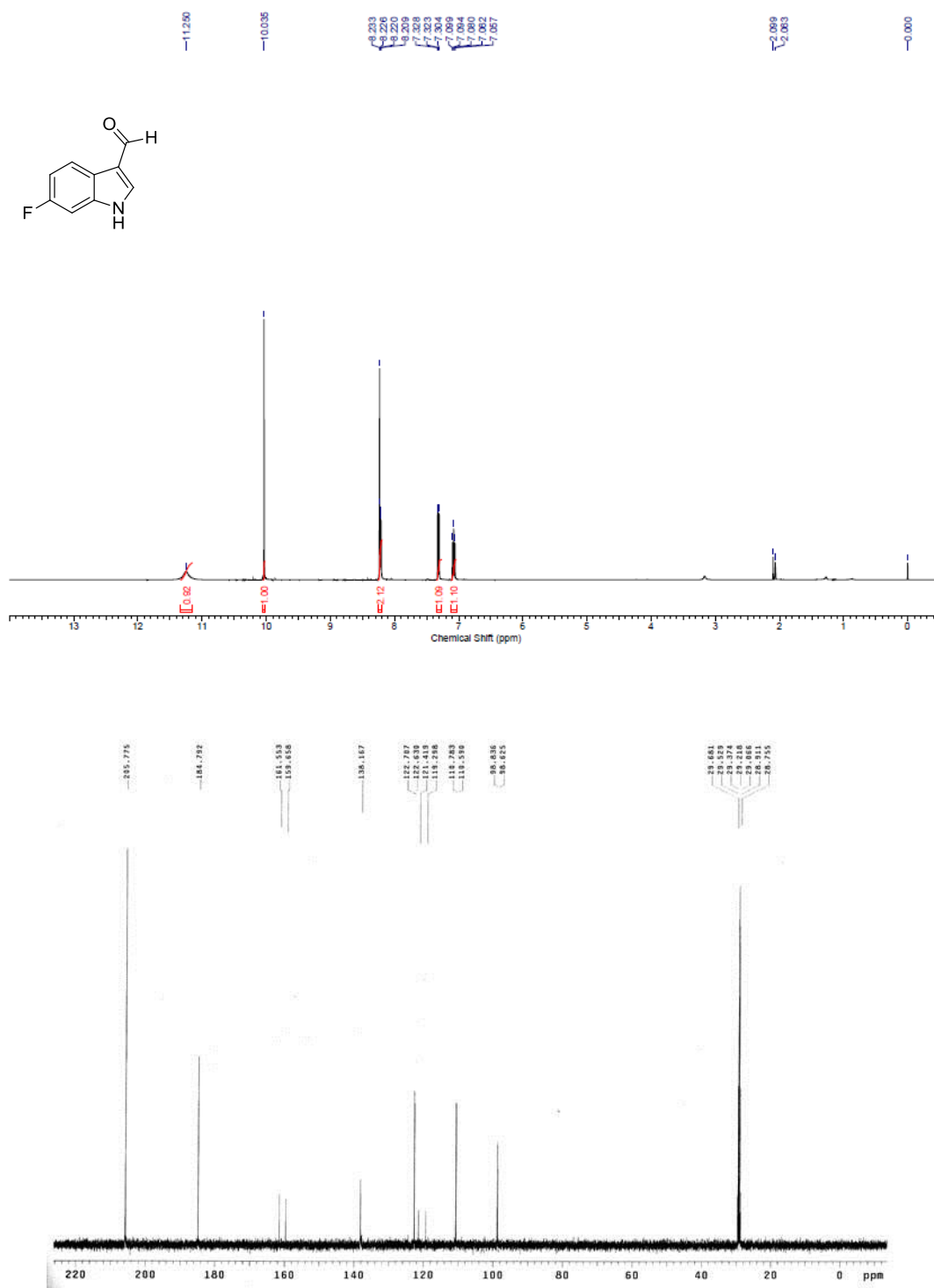


^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ 13.80 (br. s., 1 H), 8.50 (s, 1 H), 8.45 (s, 1 H), 7.99 (m, 3 H), 7.66 (s., 1 H), 7.30–7.40 (m, 2 H), 7.17–7.26 (m, 3 H), 7.11–7.16 (m, 3 H), 6.98–7.09 (m, 2 H), 6.87 (s, 1 H), 4.21 (s, 2 H), 3.81 (s, 3 H); ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$) δ ppm 159.74, 155.90, 152.68, 150.09, 143.29, 140.50, 137.89, 131.43, 130.87, 139.26, 128.68, 128.39, 128.34, 127.46, 127.11, 126.21, 123.24, 122.62, 114.89, 112.39, 55.65, 43.21. The desired product was isolated by recrystallization in methanol as a white solid (yield: 62%). LRMS (EI+) m/z calcd for $\text{C}_{27}\text{H}_{23}\text{N}_5\text{O}_2$ $[\text{M}]^+$ 449.2, found 449.9.

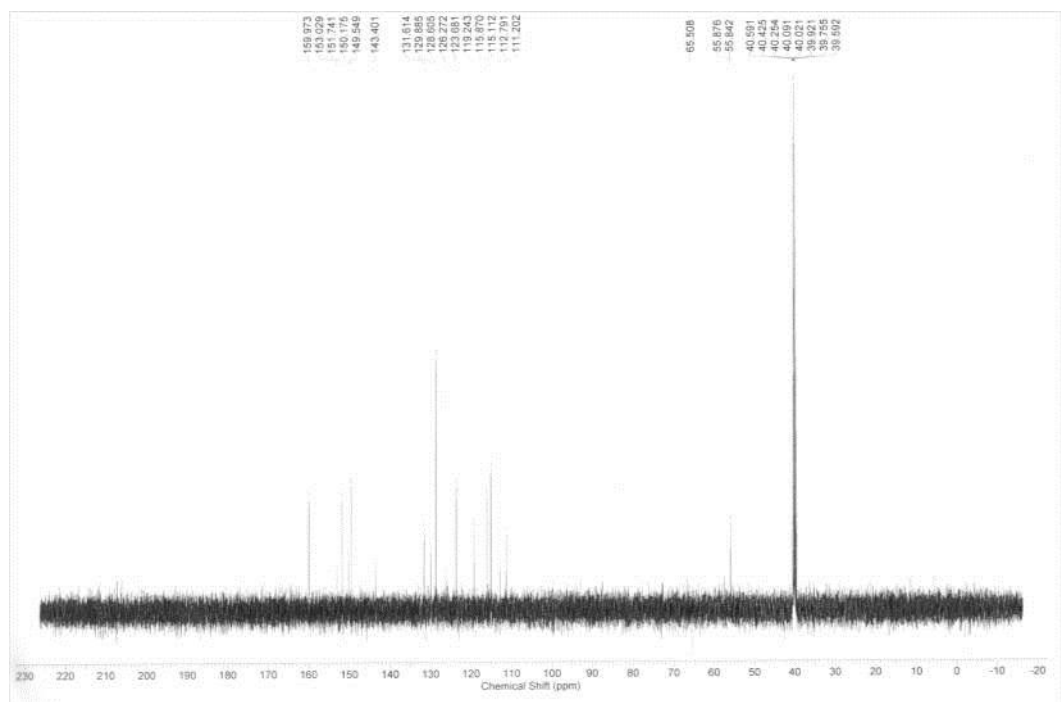
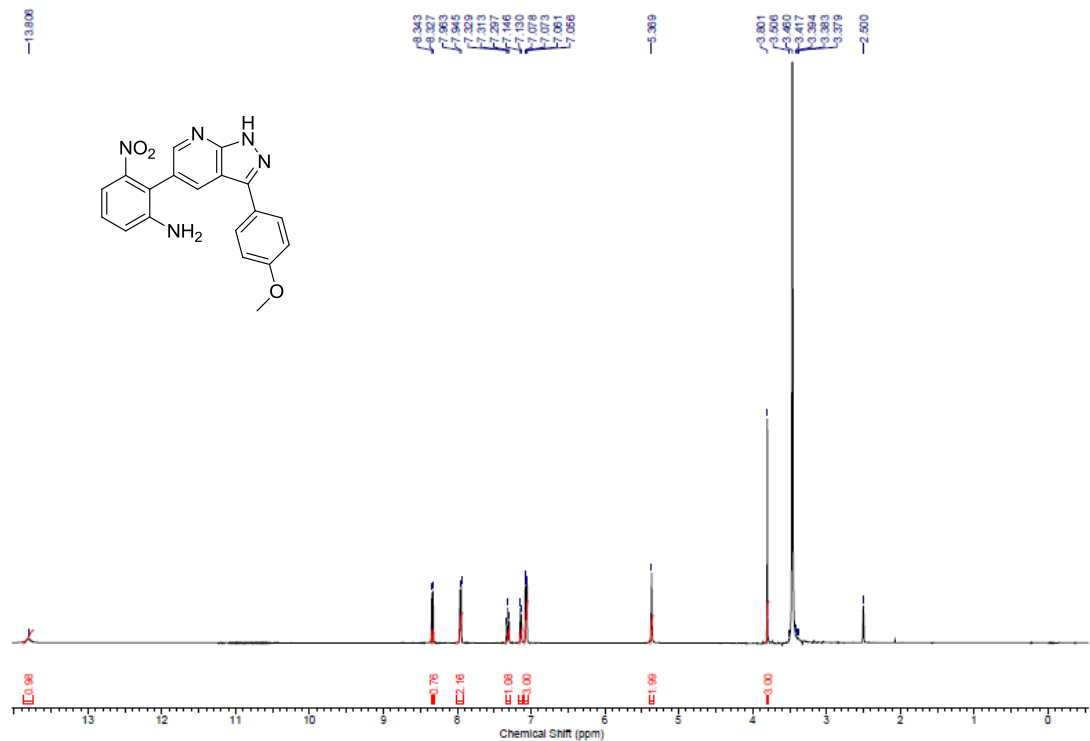
12 | SUPPORTING INFORMATION

VI. ^1H and ^{13}C NMR spectra of all new compounds.

6-fluoro-1H-indole-3-carbaldehyde

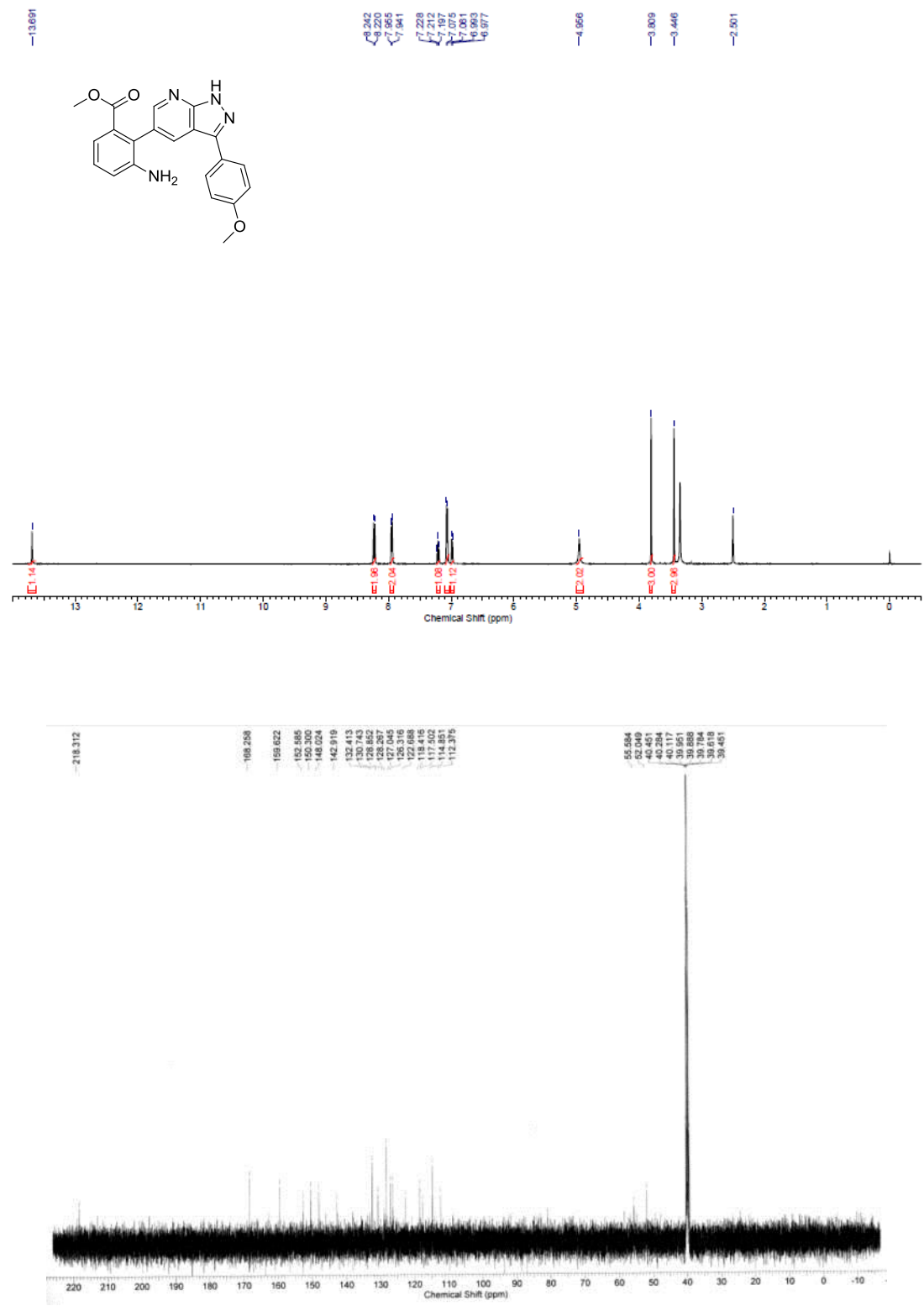


Compound 4b

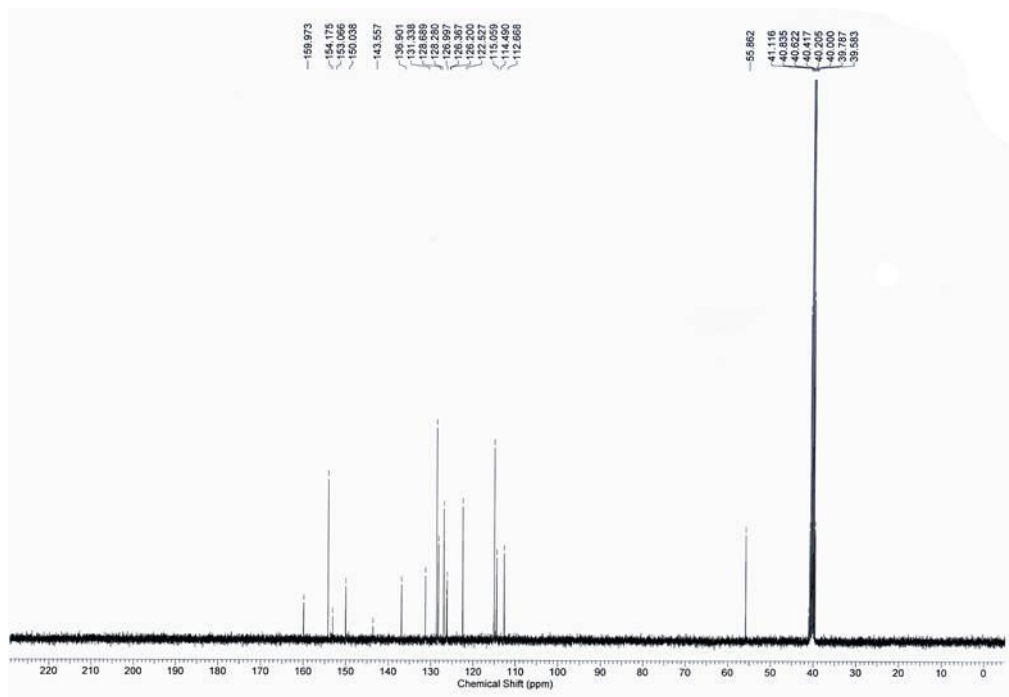
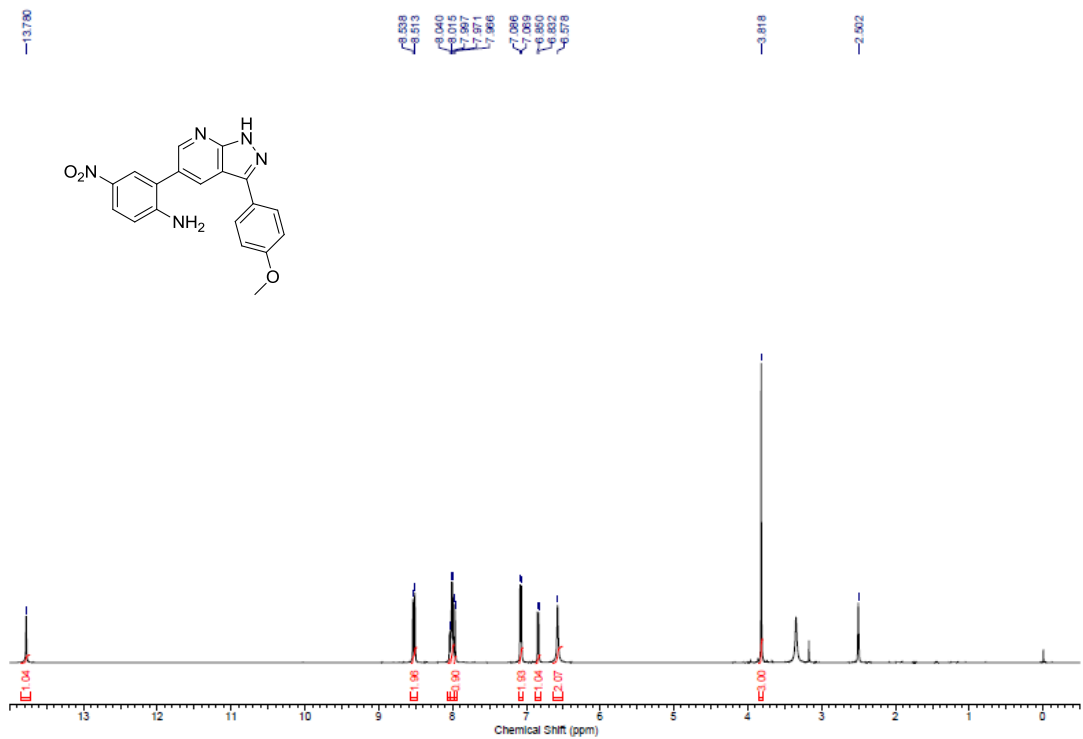


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Compound 4c

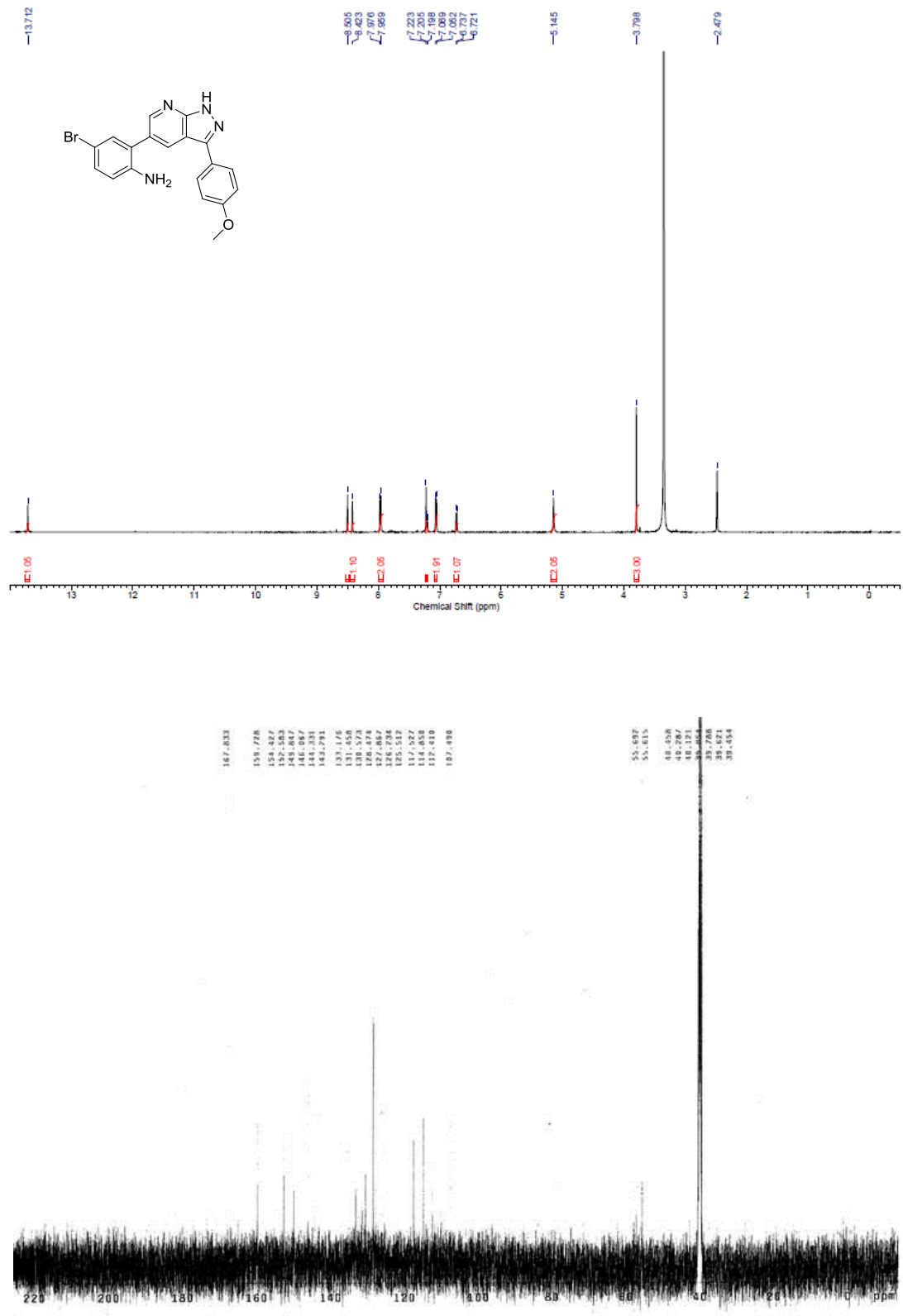


Compound 4d

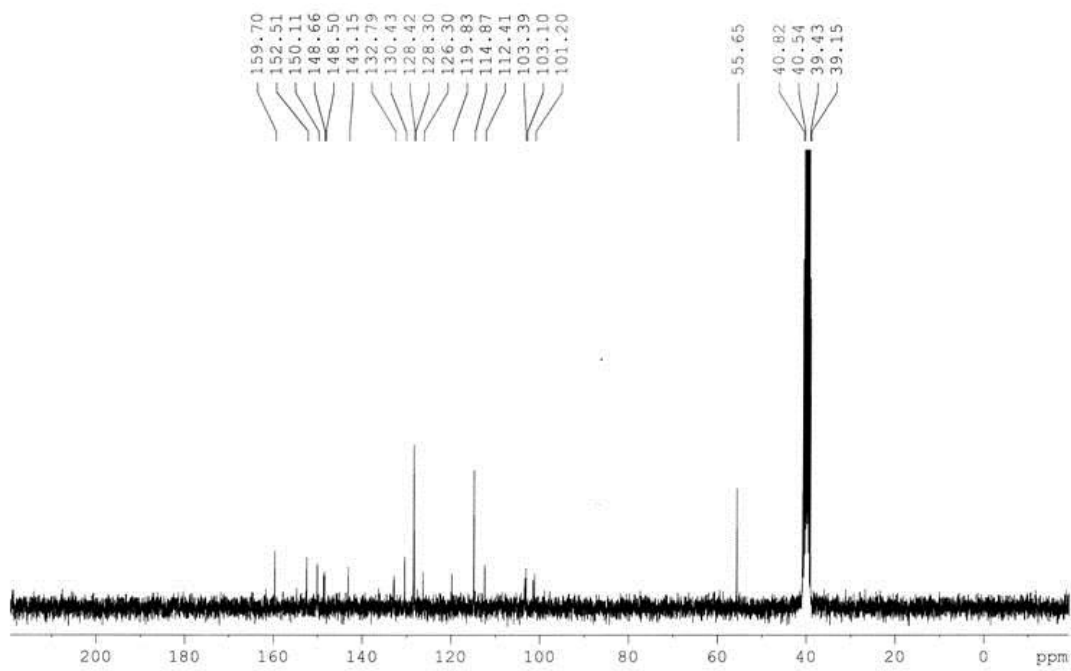
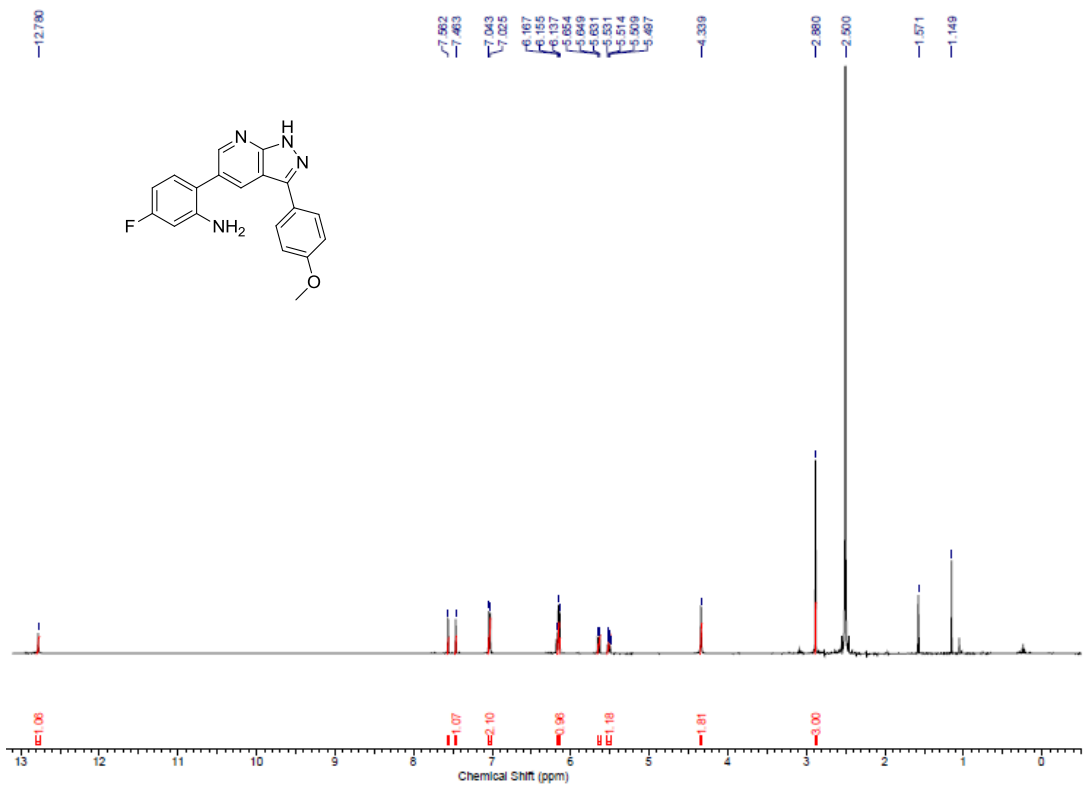


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Compound 4e

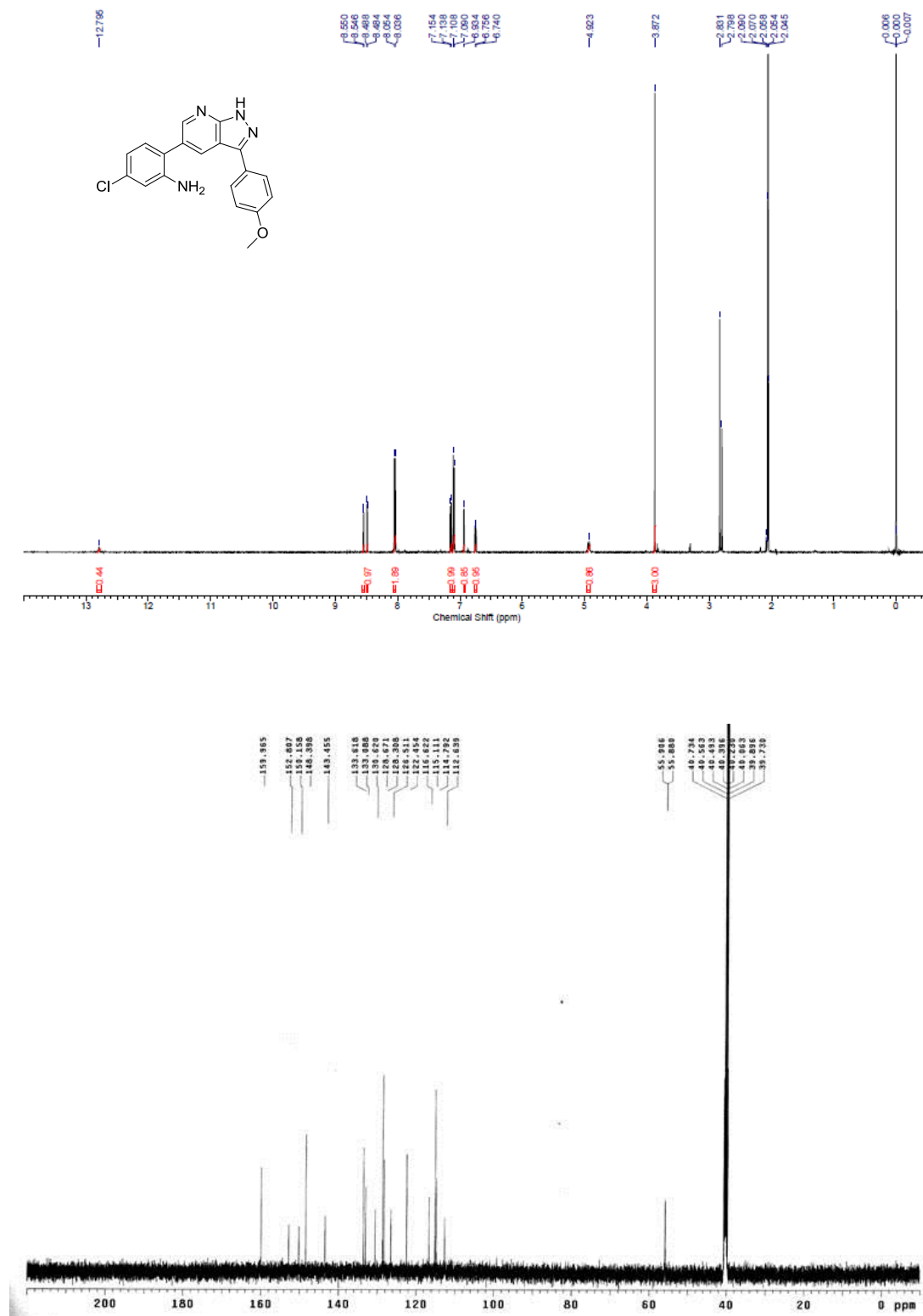


Compound 4f

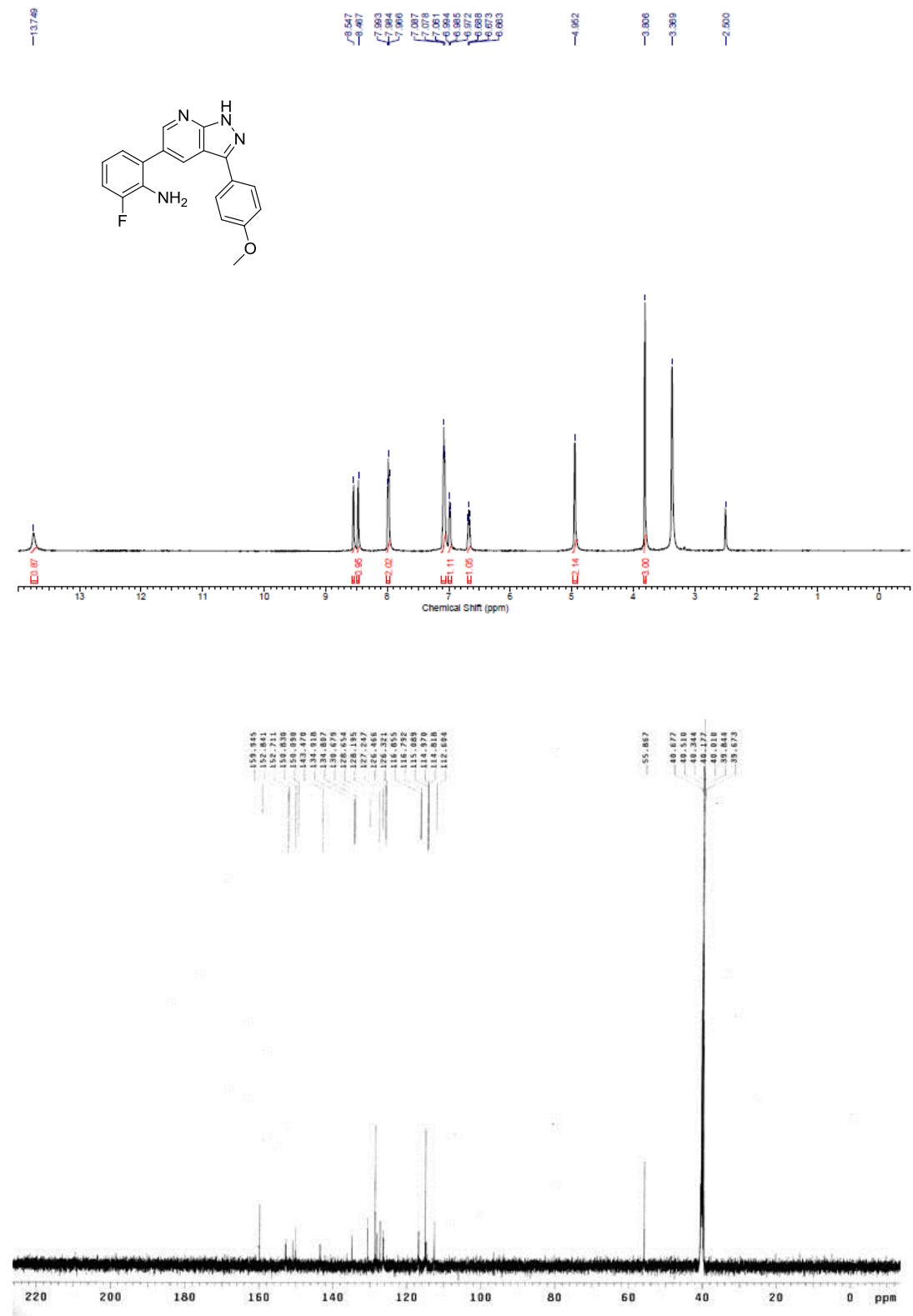


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Compound 4g

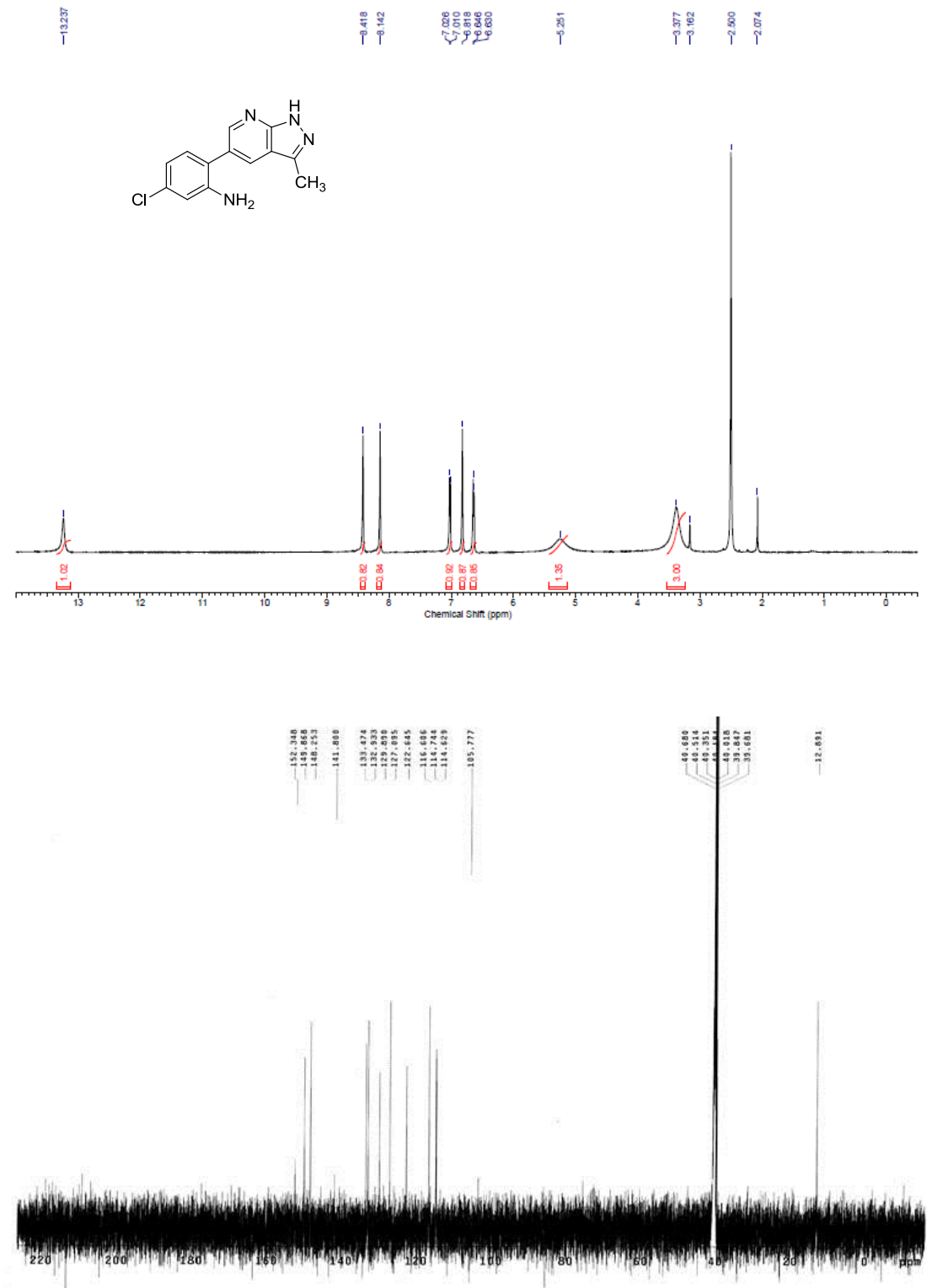


Compound 4h

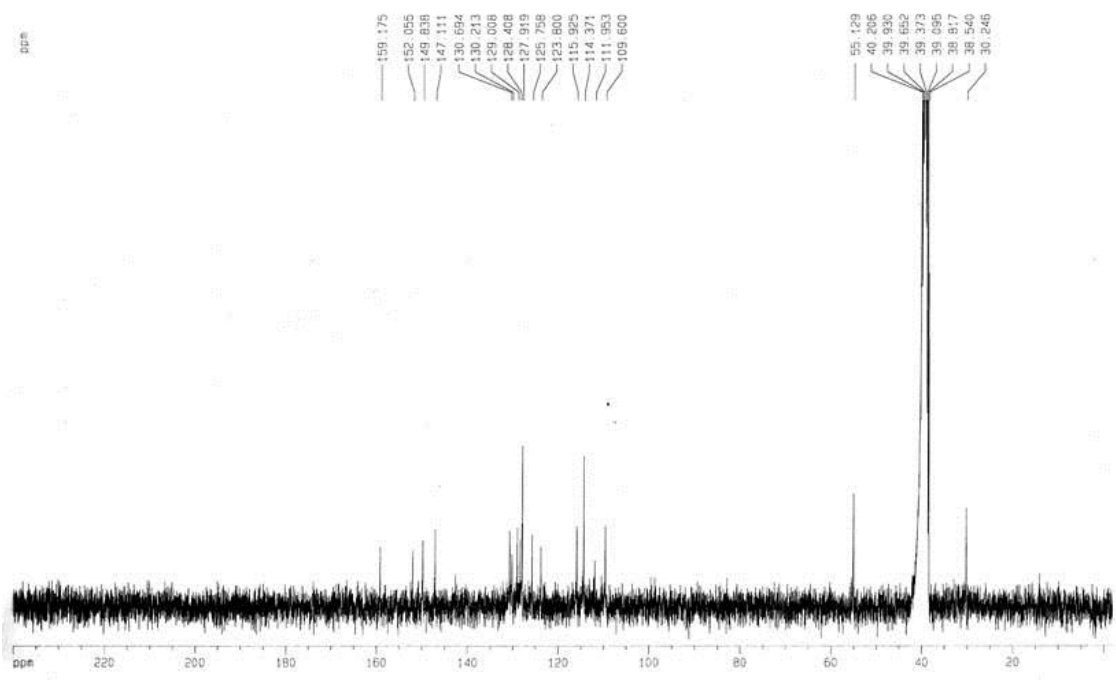
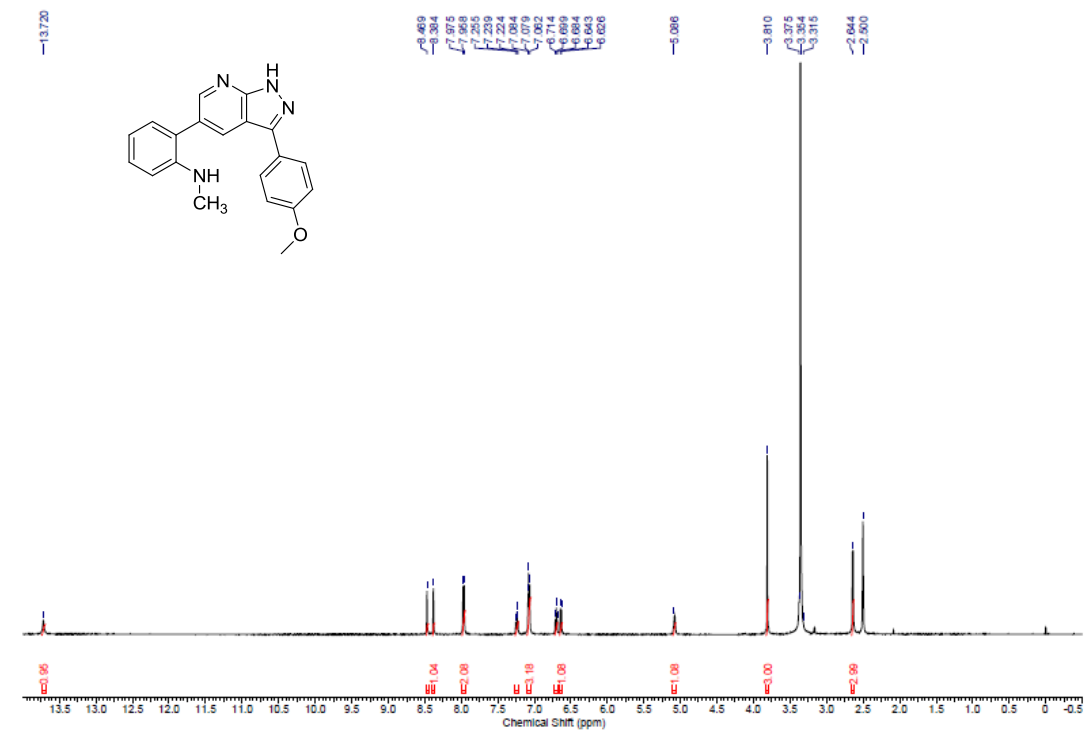


20 | SUPPORTING INFORMATION

Compound 4j

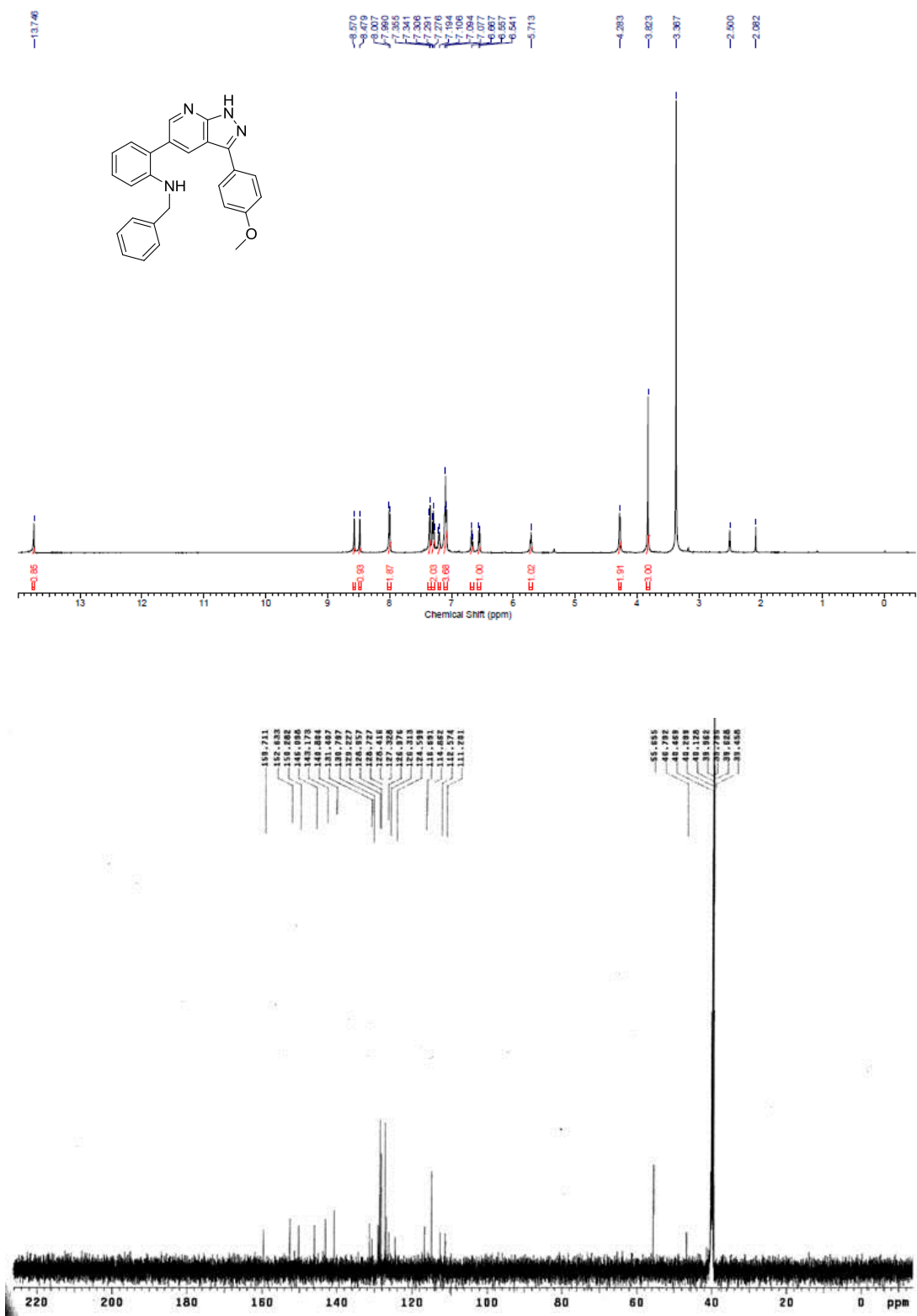


Compound 4k



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Compound 4l



Compound 4m

