SUPORTING INFORMATION

Discovery of a new pyrido[2,3-*d*]pyridazine-2,8-dione derivative as a potential anti-inflammatory agent through COX-1/COX-2 dual inhibition

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Chemistry

Materials and instrumentation. Reagent-grade solvents were applied and purified via standard methods if necessary. The reactions were monitored on thin-layer chromatography (TLC) using UV light. The melting point data of the synthesized compounds were obtained on a micro melting point apparatus and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance-300 NMR spectrometer or a Bruker Avance-500 NMR spectrometer in $CDCl_3$ with TMS as an internal reference. Chemical shifts and *J* values were expressed in δ units (ppm) and in hertz (Hz), respectively. ESI(+)-MS and tandem ESI(+)-MS/MS were acquired using a hybrid high-resolution and high-accuracy microTof (Q-TOF) mass spectrometer (Bruker). For ESI(+)-MS, the energy for the collision-induced dissociations (CDI) was optimized for each component. For data acquisition and processing, the Q-TOF-control data analysis software (Bruker Scientific) was used.

Synthesis and characterization of compounds

General method for the synthesis of 3,5-disubstituted-pyrido[2,3-*d*]pyridazin-2,8-dione derivatives 4a-g and 5a-g.

Method A. The prepared compound **2** or **3**[1] (1.0 mmol, 1.0 equiv.) and monohydrate of hydrazine (4.0 mmol, 4.0 equiv.) were dissolved in the solution of EtOH and MeCN (1:1 v/v). The mixture was stirred at 80 °C for 6 h (R= NO_2, Br, Cl, F, H, Me) or 16 h (R= OMe). The reaction mixture was cooled in an ice bath, the precipitated solid was filtered, washed with cold ethanol (10 mL), and dried under reduced pressure to obtain the pure product without the need for further purification steps.

Method B (one-pot). The β-enamino diketone **1**[2] (1.0 mmol, 1.0 equiv.) and active methylene reagents (malonitrile or ethyl cyanoacetate, 1.0 mmol, 1.0 equiv.) were dissolved in EtOH (5 mL), and the mixture stirred under reflux for 8-24 h. After being monitored by TLC, hydrazine monohydrate (4.0 mmol, 4.0 equiv.) was added to reaction, and the mixture stirred under reflux for more 6-16 h. The reaction mixture was cooled in an ice bath, the precipitated solid was then filtered, washed with cold ethanol (10 mL), and dried under reduced pressure to obtain the pure product without the need for further purification steps.

3-Cyano-5-(4-nitrophenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**4a**): Yellow solid, yield: 80%, mp > 330.0 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 7.83 (*d*, 2H, 4-NO_2-C_6H_4, *J* = 8.7 Hz), 8.95 (*s*, 1H, H4), 8.35 (*d*, 2H, 4-NO_2-C_6H_4, *J* = 8.7 Hz). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 106.4 (C3), 111.8 (CN), 118.0 (C4a), 124.2, 131.0, 141.7, 148.0 (4-NO_2-C_6H_4), 143.3 (C4), 144.5 (C5), 146.2 (C8a), 158.1 (C8), 167.3 (C2). **HRMS** (ESI+) *m/z* calcd for $C_{14}H_8N_5O_4$ $[M+H]^+$: 310.0571, found 310.0589.

3-Cyano-5-(4-bromophenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**4b**): Yellow solid, yield: 75%, mp > 311.3 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 7.49 (*d*, 2H, 4-Br-C_6H_4, *J* = 8.4 Hz), 7.73 (*d*, 2H, 4-Br-C_6H_4, *J* = 8.4 Hz), 8.03 (*s*, 1H, H4). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 107.1 (C3), 112.1 (CN), 117.6 (C4a), 123.0, 131.7, 132.0, 134.3 (4-Br-C_6H_4), 145.1 (C4), 145.1 (C5), 145.2 (C8a), 157.7 (C8), 166.4 (C2). **HRMS** (ESI+) *m/z* calcd for $C_{14}H_8BrN_4O_2^+$ $[M+H]^+$: 342.9825, found 342.9857.

3-Cyano-5-(4-chlorophenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**4c**): Yellow solid, yield: 88%, mp > 330.1 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 7.55 (*d*, 2H, 4-Cl-C_6H_4, *J* = 8.8 Hz), 7.58 (*d*, 2H, 4-Cl-C_6H_4, *J* = 8.8 Hz), 7.85 (*s*, 1H, H4); **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 105.4 (C3), 112.0 (CN), 118.4 (C4a), 129.1, 131.4, 134.1, 134.2 (4-Cl-C_6H_4), 143.1 (C4), 145.4 (C5), 147.5 (C8a), 159.0 (C8), 168.9 (C2); **HRMS** (ESI+) *m/z* calcd for $C_{14}H_8ClN_4O_2^+$ $[M+H]^+$: 299.0330, found 299.0336.

3-Cyano-5-(4-fluorophenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**4d**): Yellow solid, yield: 88%, mp > 300.0 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 7.34 (*dd*, 2H, 4-F-C_6H_4, *J* = 8.8, 8.8 Hz), 7.57 (*dd*, 2H, 4-F-C_6H_4, *J* = 8.8, 8.8 Hz), 7.89 (*s*, 1H, H4). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 106.0 (C3), 112.3 (CN), 118.2 (C4a), 115.9 (*d*, $J^2_{C\text{-}F}$= 21.7 Hz, 4-F-C_6H_4), 131.7 (*d*, $J^4_{C\text{-}F}$= 3.17 Hz, 4-F-C_6H_4), 131.8 (*d*, $J^3_{C\text{-}F}$= 8.63 Hz, 4-F-C_6H_4), 143.5 (C4), 145.5 (C5), 146.6 (C8a), 158.6 (C8), 162.9 (*d*, $J^1_{C\text{-}F}$= 246.0 Hz, 4-F-C_6H_4), 168.1 (C2). **HRMS** (ESI+) *m/z* calcd for $C_{14}H_8FN_4O_2^+$ $[M+H]^+$: 283.0626, found 283.0632.

3-Cyano-5-phenyl-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**4e**): Yellow solid, yield: 82%, mp > 306.0 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 7.52 (*s*, 5H, C_6H_5), 7.83 (*s*, 1H, H4). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 105.4 (C3), 112.2 (CN), 118.4 (C4a), 129.0, 129.3, 129.5, 135.3 (C_6H_4), 143.2 (C4), 146.5 (C5), 147.4 (C8a), 159.1 (C8), 169.0 (C2). **HRMS** (ESI+) *m/z* calcd for $C_{14}H_9N_4O_2^+$ $[M+H]^+$: 265.0720, found 265.0730.

3-Cyano-5-(4-methylphenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**4f**): Yellow solid, yield: 72%, mp > 325.7 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 2.38 (*s*, 3H, CH_3), 7.36 (*d*, 2H, 4-CH_3-C_6H_4, *J* = 8.0 Hz), 7.41 (*d*, 2H, 4-CH_3-C_6H_4, *J* = 8.0 Hz), 7.96 (*s*, 1H, H4). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 21.3 (CH_3), 107.2 (C3), 112.4 (CN), 117.6 (C4a), 129.4, 129.6, 132.2, 138.9 (4-CH_3-C_6H_4), 144.2

(C4), 144.9 (C3a), 146.1 (C5), 157.5 (C8), 166.1 (C2). **HRMS** (ESI+) *m/z* calcd for $C_{15}H_{11}N_4O_2^+$ $[M+H]^+$: 279.0877, found 279.0885.

3-Cyano-5-(4-methoxyphenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**4g**): Yellow solid, yield: 61%, mp > 308.3 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 3.83 (*s*, 3H, OCH_3), 7.36 (*d*, 2H, 4-OCH_3-C_6H_4, *J* = 8.7 Hz), 7.69 (*d*, 2H, 4-OCH_3-C_6H_4, *J* = 8.7 Hz), 7.85 (*s*, 1H, H4). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 55.7 (OCH_3), 107.5 (C3), 112.5 (CN), 117.5 (C4a), 114.5, 127.2, 130.9, 160.2 (4-OCH_3-C_6H_4), 144.3 (C4), 144.5 (C8a), 145.8 (C5), 157.1 (C8), 165.4 (C2). **HRMS** (ESI+) *m/z* calcd for $C_{15}H_{11}N_4O_3^+$ $[M+H]^+$: 295.0826, found 295.0837.

3-ethyloxycarbonyl-5-(4-nitrophenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**5a**): Yellow solid, yield: 85%, mp > 210.0 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 1.22 (*t*, 3H, $OCH_2C\underline{H}_3$, *J* = 7.1 Hz), 4.22 (*q*, 2H, $OC\underline{H}_2CH_3$, *J* = 7.1 Hz), 7.84 (*d*, 2H, 4-NO_2-C_6H_4, *J* = 8.9 Hz), 7.95 (*s*, 1H, H4), 8.39 (*d*, 2H, 4-NO_2-C_6H_4, *J* = 8.9 Hz). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 14.1 ($OCH_2\underline{C}H_3$), 61.6 ($O\underline{C}H_2CH_3$), 112.1 (C3), 129.1 (C4a), 124.1, 130.8, 140.5, 148.1 (4-NO_2-C_6H_4), 137.6 (C8a), 138.9 (C4), 143.8 (C5), 153.7 (C8), 158.1 (C2), 164.0 (C=O). **HRMS** (ESI+) *m/z* calcd for $C_{16}H_{12}N_4O_6^+$ $[M+H]^+$: 357.0830, found 357.0813.

3-ethyloxycarbonyl -5-(4-bromophenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**5b**): Yellow solid, yield: 70%, mp: 299.0-300.8 °C; **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 1.22 (*t*, 3H, $OCH_2C\underline{H}_3$, *J* = 7.1 Hz), 4.21 (*q*, 2H, $OC\underline{H}_2CH_3$, *J* = 7.1 Hz), 7.50 (*d*, 2H, 4-Br-C_6H_4, *J* = 8.4 Hz), 7.76 (*d*, 2H, 4-Br-C_6H_4, *J* = 8.4 Hz), 7.89 (*s*, 1H, H4). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 14.4 ($OCH_2\underline{C}H_3$), 61.7 ($O\underline{C}H_2CH_3$), 112.4 (C3), 129.1 (C4a), 123.4, 131.7, 132.2, 133.7 (4-Br-C_6H_4), 138.1 (C8a), 139.5 (C4), 144.8 (C5), 154.0 (C8), 158.5 (C2), 164.3 (C=O). **HRMS** (ESI+) *m/z* calcd for $C_{16}H_{12}BrN_3O_4^+$ $[M+H]^+$: 390.0084, found 390.0066.

3-ethyloxycarbonyl-5-(4-chlorophenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**5c**): Yellow solid, yield: 77%, mp > 258.0 °C. **^{1}H NMR** (300.06 MHz, DMSO-d_6) δ 1.23 (*t*, 3H, $OCH_2C\underline{H}_3$, *J* = 7.1 Hz), 4.23 (*q*, 2H, $OC\underline{H}_2CH_3$, *J* = 7.1 Hz), 7.60 (*q*, 4H, 4-Cl-C_6H_4, *J* = 8.8 Hz), 7.96 (*s*, 1H, H4). **^{13}C NMR** (75.46 MHz, DMSO-d_6) δ 14.6 ($OCH_2\underline{C}H_3$), 61.9 ($O\underline{C}H_2CH_3$), 112.7 (C3), 129.3 (C4a), 129.5, 131.7, 133.5, 134.9 (4-Cl-C_6H_4), 138.1 (C8a), 139.8 (C4), 144.9 (C5), 154.1 (C8), 158.5 (C2), 164.5 (C=O). **HRMS** (ESI+) *m/z* calcd for $C_{16}H_{12}ClN_3O_4^+$ $[M+H]^+$: 346.0589, found 346.0569.

3-ethyloxycarbonyl-5-(4-fluorophenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**5d**): Yellow solid, yield: 83%, mp: 253.6-255.2 °C. **^{1}H NMR** (300.06 MHz, DMSO-d_6) δ 1.21 (*t*, 3H, $OCH_2C\underline{H}_3$, *J* = 7.1 Hz), 4.17 (*q*, 2H, $OC\underline{H}_2CH_3$, *J* = 7.1 Hz), 7.37 (*dd*, 2H, 4-F-C_6H_4, *J* = 8.9, 8.9 Hz), 7.56 (*dd*, 2H, 4-F-C_6H_4, *J* = 8.9, 8.9 Hz), 7.77 (*s*, 1H, H4). **^{13}C NMR** (75.46 MHz, DMSO-d_6) δ 14.1 ($OCH_2\underline{C}H_3$), 61.4 ($O\underline{C}H_2CH_3$), 112.2 (C3), 115.8 (*d*, $J^2_{C\text{-}F}$ = 21.8 Hz, 4-F-C_6H_4), 128.7 (C4a), 130.6 (*d*, $J^4_{C\text{-}F}$ = 3.1 Hz, 4-F-C_6H_4), 131.6 (*d*, $J^3_{C\text{-}F}$ = 8.6 Hz, 4-F-C_6H_4), 137.5 (C8a), 139.5 (C4), 144.6 (C5), 153.5 (C8), 158.0

(C2), 162.8 (*d*, $J^{1}_{C\text{-}F}$ = 246.7 Hz, 4-F-C_6H_4), 163.9 (C=O). **HRMS** (ESI+) *m/z* calcd for $C_{16}H_{12}FN_3O_4^+$ $[M+H]^+$: 330.0885, found 330.0874.

3-ethyloxycarbonyl-5-phenyl-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**5e**): Yellow solid, yield: 78%, mp > 229.3 °C. **^{1}H NMR** (300.06 MHz, DMSO-d_6) δ 1.21 (*t*, 3H, $OCH_2C\underline{H}_3$, *J* = 7.1 Hz), 4.21 (*q*, 2H, $OC\underline{H}_2CH_3$, *J* = 7.1 Hz), 7.55 (*s*, 5H, C_6H_5), 7.97 (*s*, 1H, H4). **^{13}C NMR** (75.46 MHz, DMSO-d_6) δ 14.0 ($OCH_2\underline{C}H_3$), 61.4 ($O\underline{C}H_2CH_3$), 112.1 (C3), 129.5 (C4a), 128.5, 128.8, 129.2, 134.0 (C_6H_5), 137.6 (C8a), 139.7 (C4), 145.4 (C5), 153.5 (C8), 157.9 (C2), 163.8 (C=O). **HRMS** (ESI+) *m/z* calcd for $C_{16}H_{13}N_3O_4^+$ $[M+H]^+$: 312.0979, found 312.0967.

3-ethyloxycarbonyl-5-(4-methylphenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**5f**): Yellow solid, yield: 73%, mp > 249.0 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 1.20 (*t*, 3H, $OCH_2C\underline{H}_3$, *J* = 7.1 Hz), 2.39 (*s*, 3H, CH_3), 4.17 (*q*, 2H, $OC\underline{H}_2CH_3$, *J* = 7.1 Hz), 7.33 (*d*, 2H, 4-CH_3-C_6H_4, *J* = 8.0 Hz), 7.39 (*d*, 2H, 4-CH_3-C_6H_4, *J* = 8.0 Hz), 7.82 (*s*, 1H, H4). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 14.0 ($OCH_2\underline{C}H_3$), 20.9 (CH_3), 61.3 ($O\underline{C}H_2CH_3$), 112.1 (C3), 128.5 (C4a), 129.1, 129.3, 131.2, 138.9 (4-CH_3-C_6H_4), 137.5 (C8a), 139.7 (C4), 145.3 (C5), 153.4 (C8), 157.9 (C2), 163.9 (C=O). **HRMS** (ESI+) *m/z* calcd for $C_{17}H_{15}N_3O_4^+$ $[M+H]^+$: 326.1135, found 326.1117.

3-ethyloxycarbonyl-5-(4-methoxyphenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**5g**): Yellow solid, yield: 71%, mp: 253.4-255.3 °C. **^{1}H NMR** (300.06 MHz, DMSO-d_6) δ 1.23 (*t*, 3H, $OCH_2C\underline{H}_3$, *J* = 7.1 Hz), 3.84 (*s*, 3H, OCH_3), 4.23 (*q*, 2H, $OC\underline{H}_2CH_3$, *J* = 7.1 Hz), 7.11 (*d*, 2H, 4-OCH_3-C_6H_4, *J* = 8.8 Hz), 7.48 (*d*, 2H, 4-OCH_3-C_6H_4, *J* = 8.8 Hz), 8.02 (*s*, 1H, H4), 13.30 (*s*, 1H, NH). **^{13}C NMR** (75.46 MHz, DMSO-d_6) δ 14.0 ($OCH_2\underline{C}H_3$), 55.4 ($O\underline{C}H_2CH_3$), 61.3 (OCH_3), 112.2 (C3), 114.2, 126.3, 130.6, 160.1 (4-OCH_3-C_6H_4), 128.4 (C4a), 137.5 (C8a), 139.8 (C4), 145.1 (C5), 153.4 (C8), 157.9 (C2), 163.9 (C=O). **HRMS** (ESI+) *m/z* calcd for $C_{17}H_{15}N_3O_5^+$ $[M+H]^+$: 342.1084, found 342.1068.

***General method for the* Synthesis of 3-carboxy-5-substituted-pyrido[2,3-*d*]pyridazin-2,8-diones 6a-g**

To the synthesized compound **5** (1.0 mmol, 1.0 equiv.) a solution of sodium hydroxide (4M, 15mL) was added. The mixture was stirred at room temperature for 6 hours. Afterward, 10 mL of water was added, and the mixture was acidified to a pH of 1 using a 37% HCl solution. The preciptated solid was then filtered, washed with cold water (30 mL), and dried under reduced pressure to obtain the pure product without the need for further purification steps.

3-Carboxy-5-(4-nitrophenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**6a**): Yellow solid, yield: 85%, mp: 299.7-301.5 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 7.83 (*d*, 2H, 4-NO_2-C_6H_4, *J* = 8.8 Hz), 8.38 (*d*, 2H, 4-NO_2-C_6H_4, *J* = 8.8 Hz), 8.40 (*s*, 1H, H4), 13.23 (*ls*, 1H, OH), 13.62 (*ls*, 1H, NH). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 112.1 (C3), 129.1 (C4a), 124.1, 130.8, 140.5, 148.1 (4-NO_2-C_6H_4), 137.6 (C8a), 138.9 (C4), 143.8 (C5), 153.7 (C8), 158.1 (C2), 164.0 (C=O). **HRMS** (ESI+) *m/z* calcd for $C_{14}H_8N_4O_6^+$ $[M+H]^+$: 328.0444, found 328.0426.

3-Carboxy-5-(4-bromophenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**6b**): Yellow solid, yield: 80%, mp: 301.6-303.4 °C. **^{1}H NMR** (300.06 MHz, DMSO-d_6) δ 7.52 (*d*, 2H, 4-Br-C_6H_4, *J* = 8.4 Hz), 7.80 (*d*, 2H, 4-Br-C_6H_4, *J* = 8.4 Hz), 8.24 (*s*, 1H, H4), 13.55 (*ls*, 1H, NH). **^{13}C NMR** (75.46 MHz, DMSO-d_6) δ 113.6 (C3), 124.7 (C4a), 123.2, 131.3, 131.9, 133.0 (4-Br-C_6H_4), 137.6 (C8a), 141.3 (C4), 144.3 (C5), 153.5 (C8), 162.7 (C2), 164.0 ($\underline{C}O_2H$). **HRMS** (ESI+) *m/z* calcd for $C_{14}H_8BrN_3O_4^+$ $[M+H]^+$: 360.9698, found 360.9682.

3-Carboxy-5-(4-chlorophenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**6c**): Yellow solid, yield: 81%, mp: 296.2-298.1 °C. **^{1}H NMR** (300.06 MHz, DMSO-d_6) δ 7.59 (*d*, 2H, 4-Cl-C_6H_4, *J* = 8.5 Hz), 7.67 (*d*, 2H, 4-Cl-C_6H_4, *J* = 8.5 Hz), 8.24 (*s*, 1H, H4), 13.54 (*ls*, 1H, NH). **^{13}C NMR** (75.46 MHz, DMSO-d_6) δ 113.7 (C3), 124.7 (C4a), 129.0, 131.1, 132.7, 134.5 (4-Cl-C_6H_4), 137.6 (C8a), 141.4 (C4), 144.3 (C5), 153.6 (C8), 162.8 (C2), 164.1 ($\underline{C}O_2H$). **HRMS** (ESI-) *m/z* calcd for $C_{14}H_8ClN_3O_4$ $[M-H]^-$: 316.0125, found 316.0154.

3-Carboxy-5-(4-fluorophenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**6d**): Yellow solid, yield: 80%, mp > 350.0 °C. **^{1}H NMR** (300.06 MHz, DMSO-d_6) δ 7.43 (*dd*, 2H, 4-F-C_6H_4, *J* = 8.9, 8.9 Hz), 7.62 (*dd*, 2H, 4-F-C_6H_4, *J* = 8.9, 8.9 Hz), 8.25 (*s*, 1H, H4), 13.51 (*ls*, 1H, NH). **^{13}C NMR** (75.46 MHz, DMSO-d_6) δ 113.9 (C3), 115.9 (*d*, $J^2_{C\text{-}F}$ = 21.8 Hz, 4-F-C_6H_4), 124.5 (C4a), 130.3 (*d*, $J^4_{C\text{-}F}$ = 3.1 Hz, 4-F-C_6H_4), 131.5 (*d*, $J^3_{C\text{-}F}$ = 8.6 Hz, 4-F-C_6H_4), 137.6 (C8a), 141.6 (C4), 144.5 (C5), 153.5 (C8), 162.8 (*d*, $J^1_{C\text{-}F}$ = 247.0 Hz, 4-F-C_6H_4), 162.9 (C2), 164.0 ($\underline{C}O_2H$). **HRMS** (ESI+) *m/z* calcd for $C_{14}H_8FN_3O_4^+$ $[M+H]^+$: 302.0572, found 302.0546.

3-Carboxy-5-phenyl-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**6e**): Yellow solid, yield: 90%, mp > 345.0 °C. **^{1}H NMR** (300.06 MHz, DMSO-d_6) δ 7.58 (*m*, 5H, C_6H_5), 8.29 (*s*, 1H, H4), 13.51 (*ls*, 1H, NH). **^{13}C NMR** (75.46 MHz, DMSO-d_6) δ 113.9 (C3), 124.4 (C4a), 128.9, 129.2, 129.6, 133.8 (C_6H_5), 137.6 (C8a), 141.8 (C4), 145.4 (C5), 153.6 (C8), 163.0 (C2), 164.0 ($\underline{C}O_2H$). **HRMS** (ESI+) *m/z* calcd for $C_{14}H_9N_3O_4^+$ $[M+H]^+$: 284.0666, found 284.0645.

3-Carboxy-5-(4-methylphenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**6f**): Yellow solid, yield: 78%, mp 292.3-293.1 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 2.50 (*s*, 3H, CH_3), 7.39 (*d*, 2H, 4-CH_3-C_6H_4, *J = 7.*8 Hz), 7.44 (*d*, 2H, 4-CH_3-C_6H_4, *J* = 7.8 Hz), 8.30 (*s*, 1H, H4), 13.47 (*ls*, 1H, NH). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 20.9 (CH_3), 113.9 (C3), 124.3 (C4a), 129.1, 129.4, 130.9, 137.5

(4-CH_3-C_6H_4), 139.2 (C8a), 141.9 (C4), 145.3 (C5), 153.5 (C8), 162.9 (C2), 163.9 ($\underline{C}O_2H$). **HRMS** (ESI+) *m/z* calcd for $C_{15}H_{11}N_3O_4^+$ $[M+H]^+$: 298.0822, found 298.0807.

3-Carboxy-5-(4-methoxyphenyl)-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**6g**): Yellow solid, yield: 70%, mp: 225.6-227.5 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 3.84 (*s*, 3H, OCH_3), 7.13 (*d*, 2H, 4-OCH_3-C_6H_4, *J* = 8.7 Hz), 7.49 (*d*, 2H, 4-OCH_3-C_6H_4, *J* = 8.7 Hz), 8.32 (*s*, 1H, H4), 13.43 (*ls*, 1H, NH). **^{13}C NMR** (75.46 MHz, DMSO-d_6) δ 55.4 (OCH_3), 114.3 (C3), 124.2 (C4a), 114.2, 126.1, 130.6, 160.2 (4-OCH_3-C_6H_4), 137.8 (C8a), 141.9 (C4), 145.2 (C5), 153.6 (C8), 163.2 (C2), 164.2 ($\underline{C}O_2H$). **HRMS** (ESI+) *m/z* calcd for $C_{15}H_{11}N_3O_5^+$ $[M+H]^+$: 314.077, found 314.0743.

***General method* for the synthesis of 3-cyano-7-phenyl-5-substituted-pyrido[2,3-*d*]pyridazin-2,8-diones 7a-g**

The compound **2** (1.0 mmol, 1.0 equiv.), phenylhydrazine (4.0 mmol, 4.0 equiv.) and *p*-toluenesulfonic acid (1.0 mmol, 1.0 equiv.) were dissolved in the solution of EtOH and MeCN (1:1 v/v). The mixture was stirred at 80 °C for 24 hours. The reaction mixture was then cooled in an ice bath, the precipitated solid was filtered, washed with cold ethanol (10 mL), and dried under reduced pressure to obtain the pure product without the need for further purification steps.

3-Cyano-5-(4-nitrophenyl)-7-phenyl-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**7a**): Yellow solid, yield: 70%, mp > 331.5 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 7.47 (*m*, 1H, C_6H_5), 7.54 (*m*, 2H, C_6H_5), 7.58 (*m*, 2H, 4-NO_2-C_6H_4), 7.64 (*m*, 2H, C_6H_5), 7.69 (*m*, 2H, 4-NO_2-C_6H_4), 8.38 (*s*, 1H, H4). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 111.4 (C3), 111.7 (CN), 114.9 (C4a), 125.7, 128.3, 128.7, 128.8, 129.3, 129.6, 133.2, 141.0 (4-NO_2-C_6H_4 and C_6H_5), 138.6 (C8a), 144.8 (C5), 146.2 (C4), 152.2 (C8), 158.6 (C2). **HRMS** (ESI+) *m/z* calcd for $C_{20}H_{11}N_5O_4^+$ $[M+H]^+$: 386.0884, found 386.0858.

3-Cyano-5-(4-bromophenyl)-7-phenyl-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**7b**): Yellow solid, yield: 71%, mp > 350.0 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 7.46 (*t*, 1H, C_6H_5, *J* = 7.4 Hz), 7.54 (*t*, 2H, C_6H_5, *J* = 7.8 Hz), 7.59 (*d*, 2H, 4-Br-C_6H_4, *J* = 8.5 Hz), 7.67 (*m*, 2H, C_6H_5), 7.76 (*d*, 2H, 4-Br-C_6H_4, *J* = 8.5 Hz), 8.46 (*s*, 1H, H4), 13.34 (*ls*, 1H, NH). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 111.5 (C3), 111.6 (CN), 114.9 (C4a), 123.3, 131.5, 131.8, 132.5 (4-Br-C_6H_4), 125.7, 128.4, 128.8, 141.0, (C_6H_5), 138.6 (C8a), 143.9 (C5), 146.3 (C4), 152.2 (C8), 158.7 (C2). **HRMS** (ESI+) *m/z* calcd for $C_{20}H_{11}BrN_4O_2^+$ $[M+H]^+$: 419.0138, found 419.0187.

3-Cyano-5-(4-chlorophenyl)-7-phenyl-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**7c**): Yellow solid, yield: 66%, mp > 350.0 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 7.46 (*t*, 1H, C_6H_5, *J* = 7.4 Hz), 7.54 (*t*, 2H, C_6H_5, *J* = 7.8 Hz), 7.65 (*m*, 6H, 4-Cl-C_6H_4 and C_6H_5), 8.45 (*s*, 1H, H4), 13.32 (*ls*, 1H, NH). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 111.5 (C3), 111.7 (CN), 114.9 (C4a), 125.7, 128.4, 128.8,

128.9, 131.3, 132.1, 134.6, 141.0 (4-Cl-C_6H_4 and C_6H_5), 138.6 (C8a), 143.8 (C5), 146.3 (C4), 152.3 (C8), 158.7 (C2). **HRMS** (ESI+) *m/z* calcd for $C_{20}H_{11}ClN_4O_2^+$ $[M+H]^+$: 375.0643, found 375.0643.

3-Cyano-5-(4-fluorophenyl)-7-phenyl-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**7d**): Yellow solid, yield: 60%, mp > 330.0 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 7.40 (*dd*, 2H, 4-F-C_6H_4, *J* = 8.9, 8.9 Hz), 7.47 (*t*, 1H, C_6H_5, *J* = 7.4 Hz), 7.55 (*t*, 2H, C_6H_5, *J* = 7.8 Hz), 7.69 (*m*, 4H, 4-F-C_6H_4 and C_6H_5), 8.32 (*s*, 1H, H4), 13.35 (*ls*, 1H, NH). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 111.5 (C3), 111.8 (CN), 114.9 (C4a), 115.8 (*d*, $J^2_{C\text{-}F}$= 21.9 Hz, 4-F-C_6H_4), 125.7, 128.4, 128.8, 141.0 (C_6H_5), 129.8 (*d*, $J^4_{C\text{-}F}$= 3.2 Hz, 4-F-C_6H_4), 131.8 (*d*, $J^3_{C\text{-}F}$= 8.7 Hz, 4-F-C_6H_4), 138.6 (C8a), 144.0 (C5), 146.3 (C4), 152.3 (C8), 158.7 (C2), 162.9 (*d*, $J^1_{C\text{-}F}$= 246.9 Hz, 4-F-C_6H_4). **HRMS** (ESI+) *m/z* calcd for $C_{20}H_{11}FN_4O_2^+$ $[M+H]^+$: 359.0939, found 359.0933.

3-Cyano-5,7-diphenyl-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**7e**): Yellow solid, yield: 60%, mp > 313.2 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 7.47 (*t*, 1H, C_6H_5, *J* = 7.4 Hz), 7.56 (*m*, 5H, C_6H_5 – A and B), 7.64 (*m*, 2H, C_6H_5 - B), 7.69 (*m*, 2H, C_6H_5 - A), 8.38 (*s*, 1H, H4), 13.34 (*ls*, 1H, NH). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 111.4 (C2), 111.7 (CN), 114.9 (C4a), 125.6, 128.3, 128.7, 128.8, 129.3, 129.6, 133.2, 141.0 (C_6H_5 – A and B), 138.6 (C8a), 144.8 (C5), 146.2 (C4), 152.2 (C8), 158.6 (C2). **HRMS** (ESI+) *m/z* calcd for $C_{20}H_{12}N_4O_2^+$ $[M+H]^+$: 341.1033, found 341.1060.

3-Cyano-5-(4-methylphenyl)-7-phenyl-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**7f**): Yellow solid, yield: 51%, mp > 328.4 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 2.40 (*s*, 3H, CH_3), 7.37 (*d*, 2H, C_6H_5, *J* = 7.9 Hz), 7.46 (*m*, 1H, C_6H_5), 7.53 (*m*, 4H, 4-CH_3-C_6H_4 and C_6H_5), 7.67 (*m*, 2H, 4-CH_3-C_6H_4), 8.36 (*s*, 1H, H4), 13.27 (*ls*, 1H, NH). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 20.9 (CH_3), 111.4 (C2), 111.7 (CN), 114.9 (C4a), 125.7, 128.3, 128.8, 129.3, 129.4, 130.4, 139.3, 141.1 (4-CH_3-C_6H_4 and C_6H_5), 138.6 (C8a), 144.8 (C5), 146.3 (C4), 152.2 (C8), 158.7 (C2). **HRMS** (ESI+) *m/z* calcd for $C_{21}H_{14}N_4O_2^+$ $[M+H]^+$: 355.1190, found 355.1162.

3-Cyano-5-(4-methoxyphenyl)-7-phenyl-pyrido[2,3-*d*]pyridazin-2,8(*1H,7H*)-dione (**7g**): Yellow solid, yield: 50%, mp: 329.1-331.1 °C. **^{1}H NMR** (500.13 MHz, DMSO-d_6) δ 3.84 (*s*, 3H, OCH_3), 7.11 (*d*, 2H, C_6H_5, *J* = 8.8 Hz), 7.45 (*m*, 1H, C_6H_5), 7.55 (*m*, 4H, 4-OCH_3-C_6H_4 and C_6H_5), 7.68 (*d*, 2H, 4-OCH_3-C_6H_4, *J* = 7.4 Hz), 8.38 (*s*, 1H, H4). **^{13}C NMR** (125.77 MHz, DMSO-d_6) δ 55.8 (OCH_3), 111.8 (C2), 112.2 (CN), 114.7 (C4a), 115.4, 125.9, 126.1, 128.8, 129.2, 131.3, 141.6, 160.8 (4-OCH_3-C_6H_4 and C_6H_5), 139.0 (C8a), 145.0 (C5), 146.8 (C4), 152.6 (C8), 159.2 (C2). **HRMS** (ESI+) *m/z* calcd for $C_{21}H_{14}N_4O_3^+$ $[M+H]^+$: 371.1139, found 371.1108.

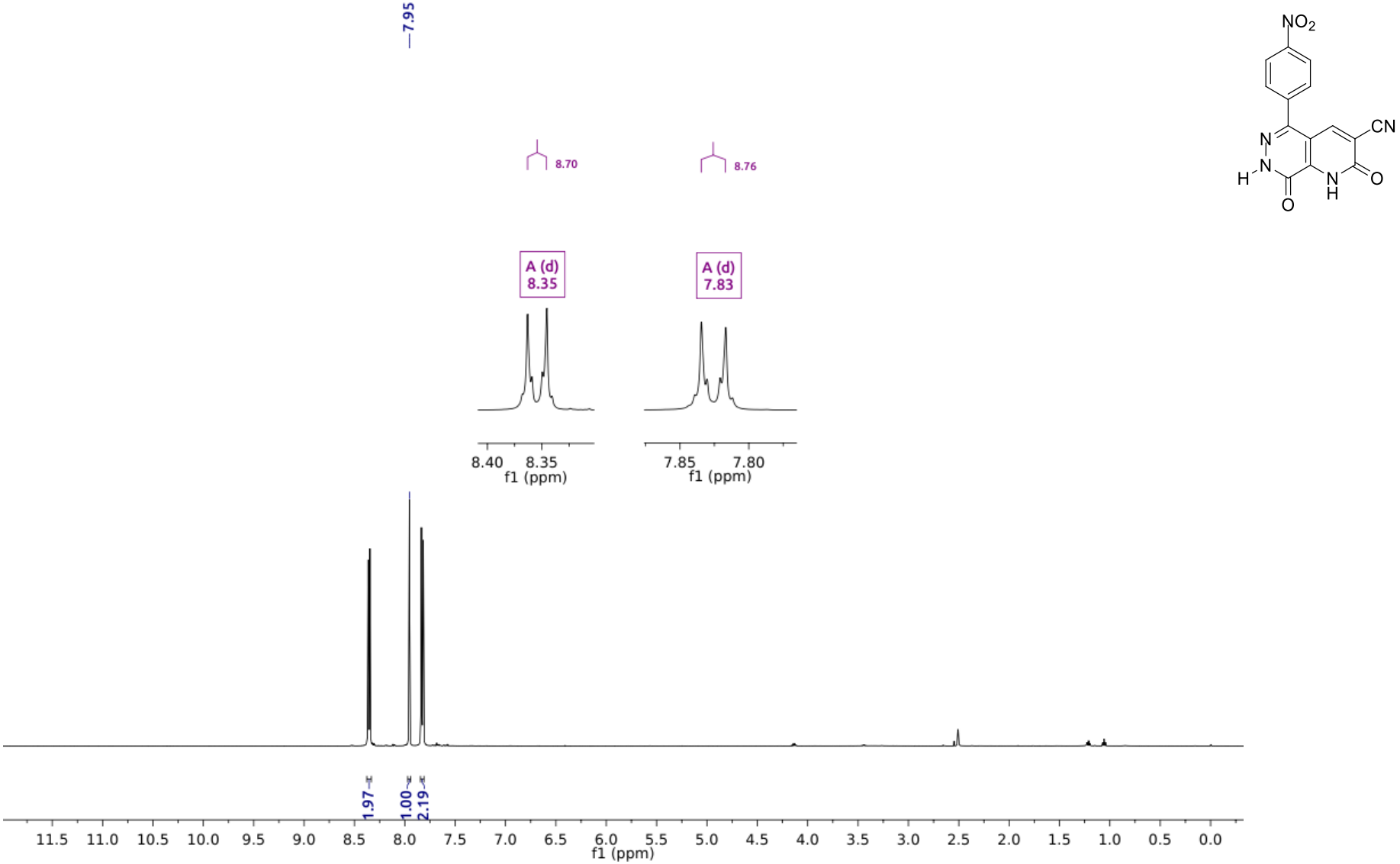

Figure S1 – ^{1}H NMR spectrum of compound **4a** in DMSO-d_6 at 500.13 MHz.

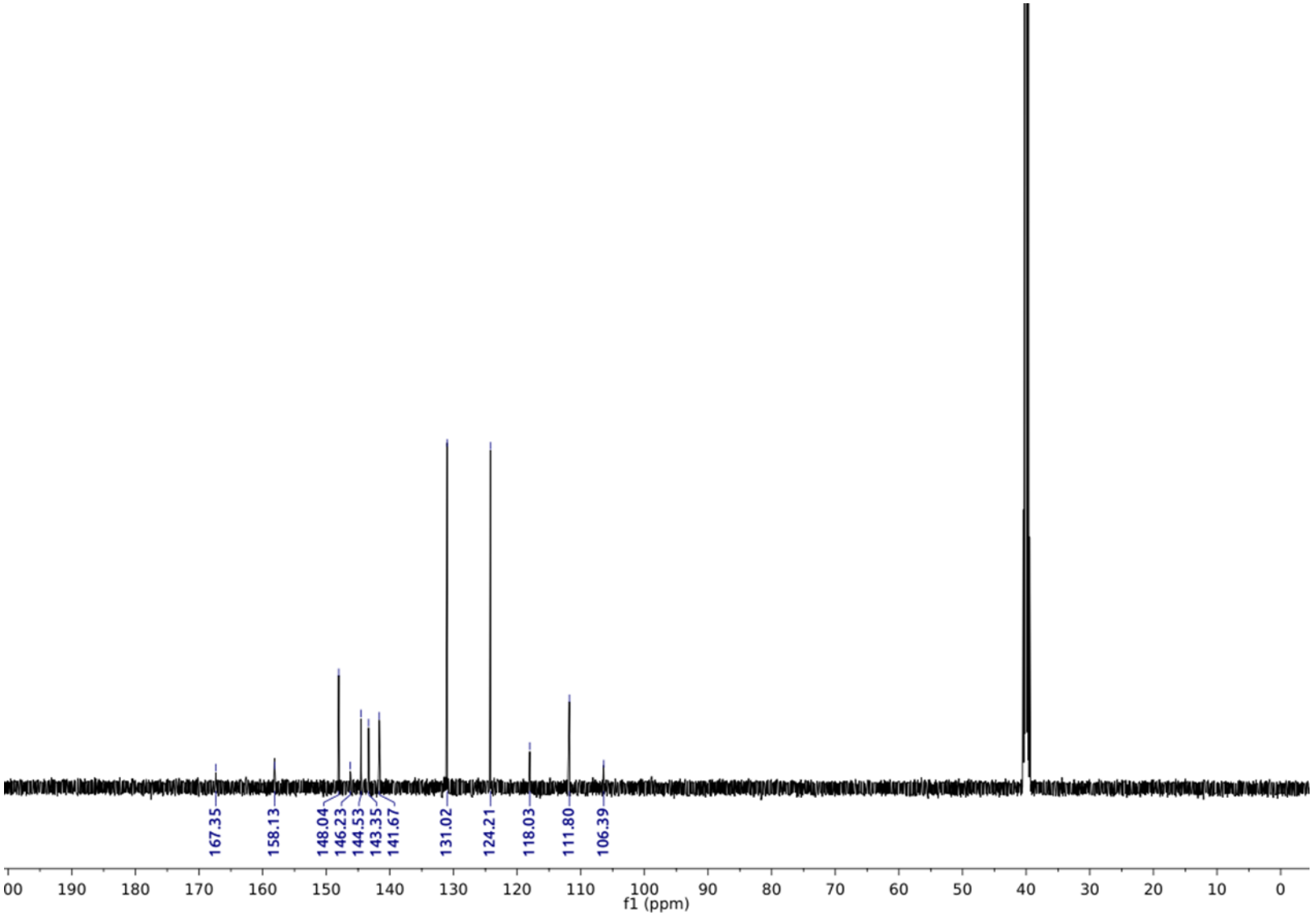

Figure S2 – ^{13}C NMR spectrum of compound **4a** in DMSO-d_6 at 125.77 MHz.

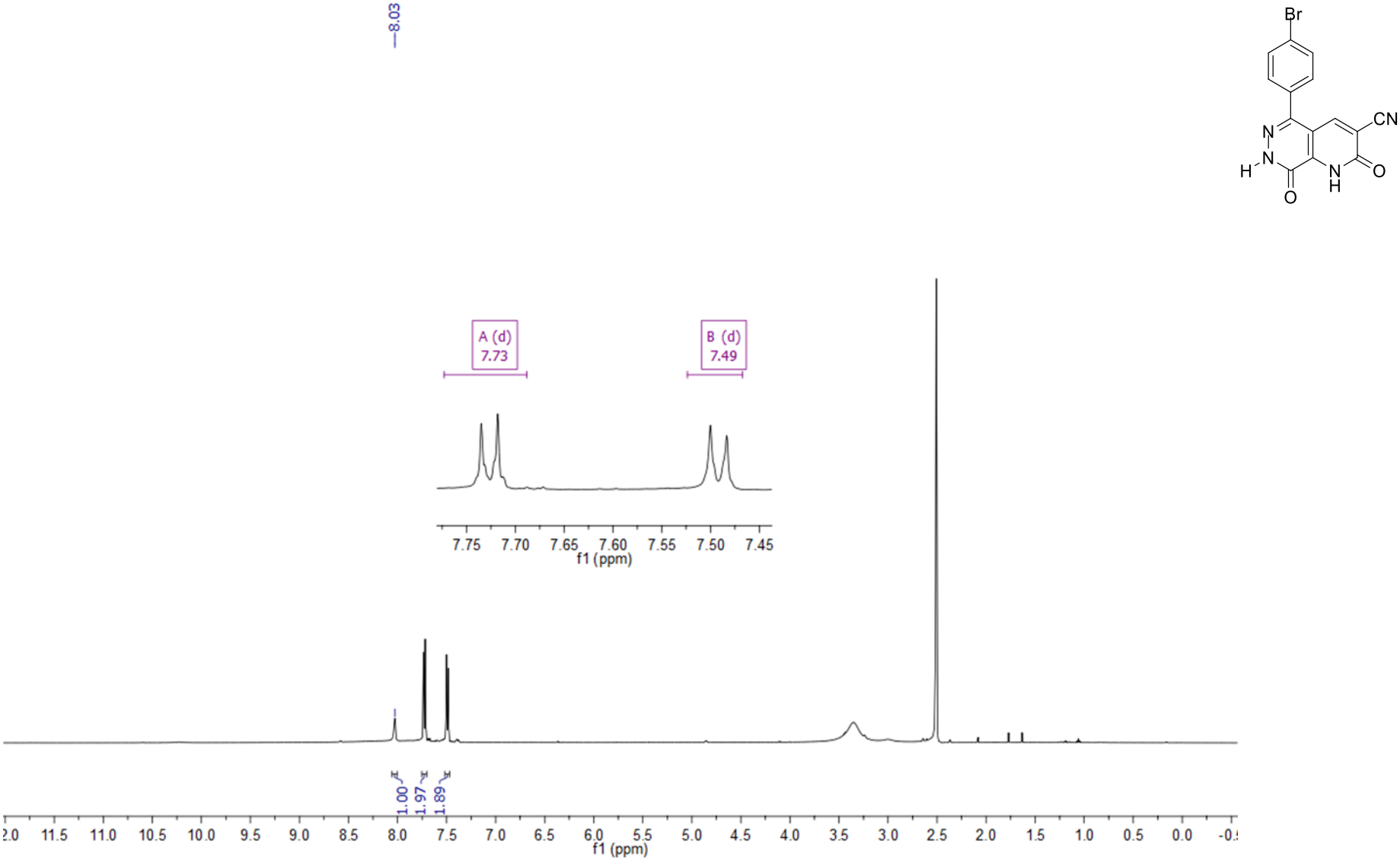

Figure S3 – ^{1}H NMR spectrum of compound **4b** in DMSO-d_6 at 500.13 MHz

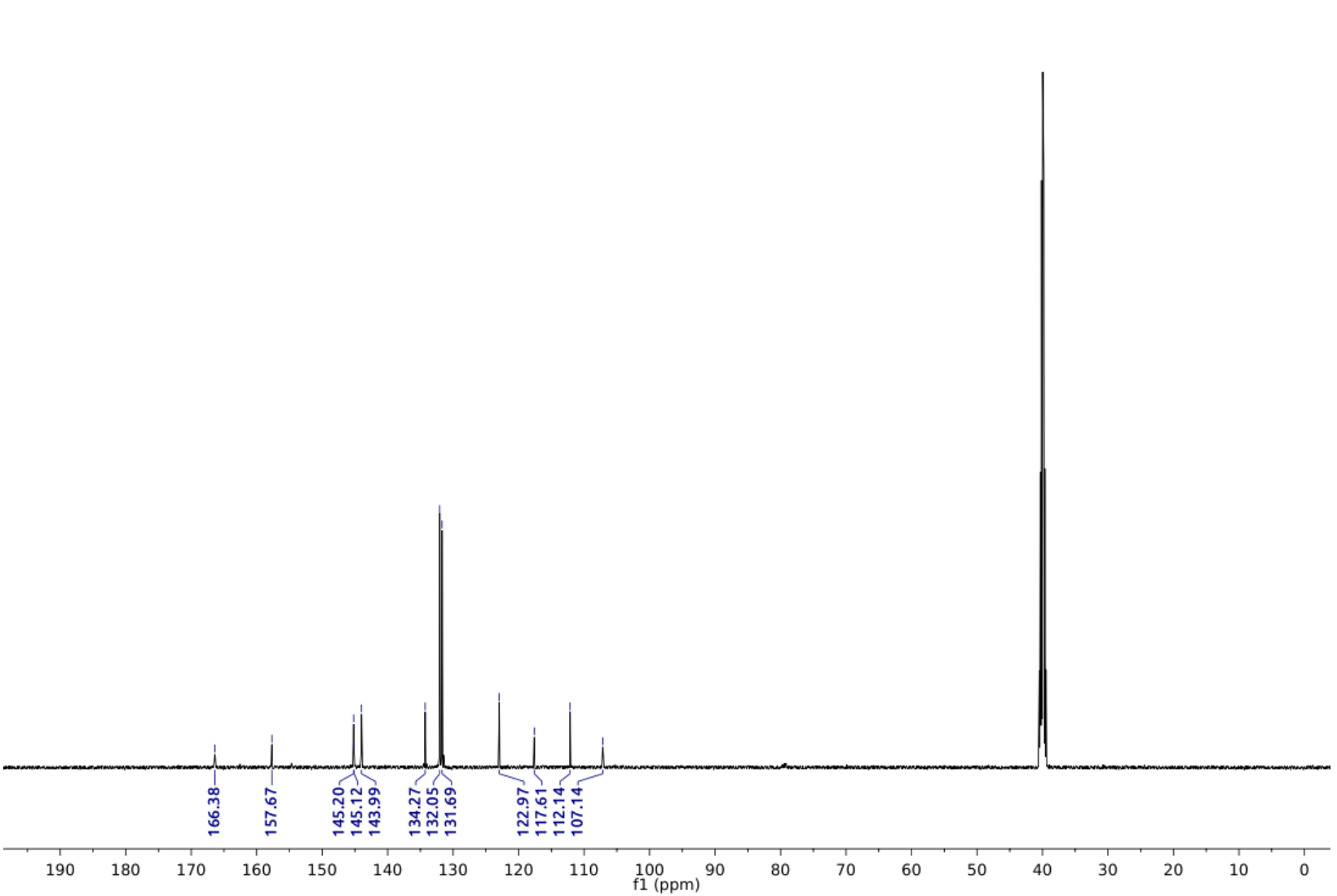

Figure S4 – ^{13}C NMR spectrum of compound **4b** in DMSO-d_6 at 125.77 MHz

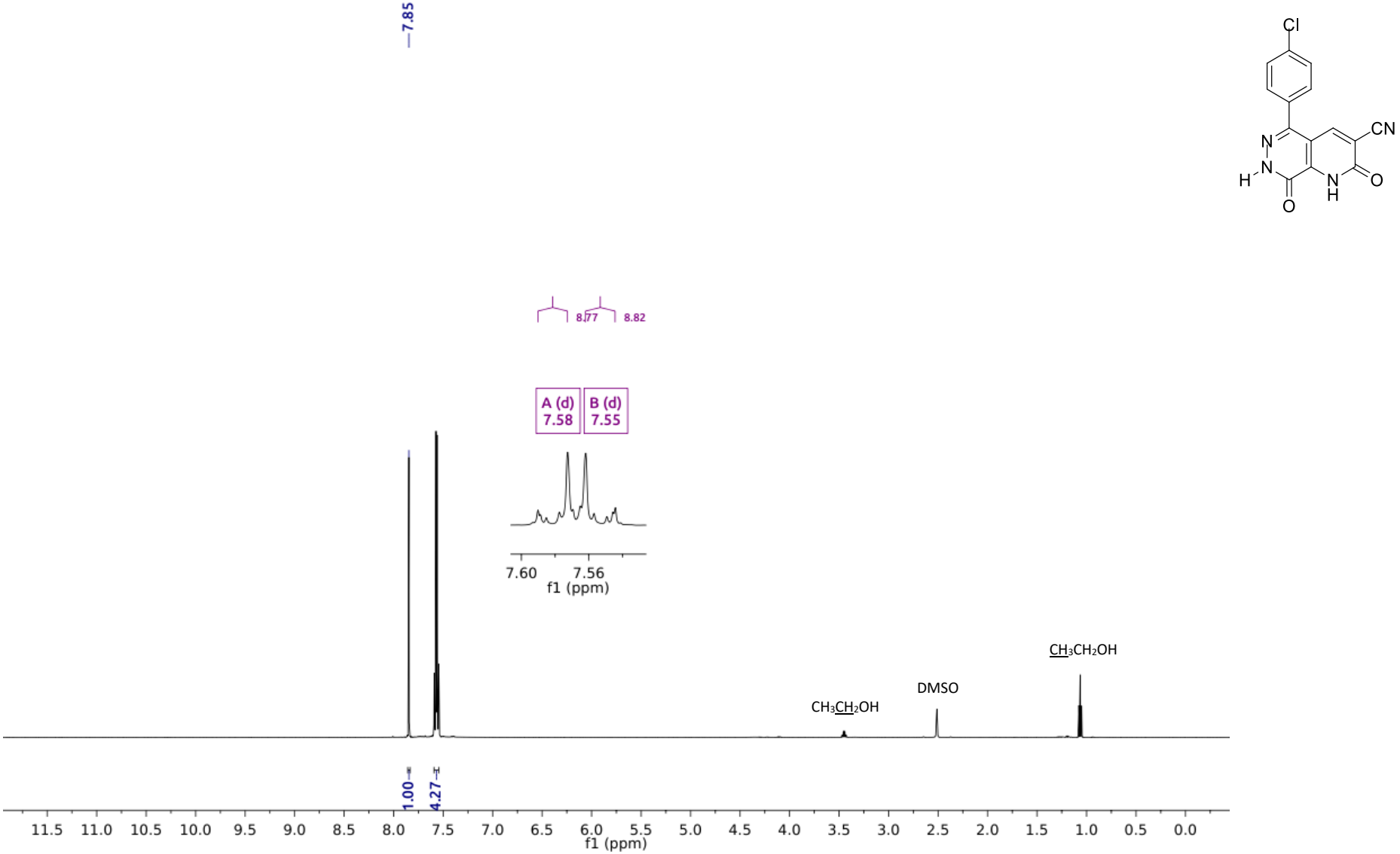

Figure S5 – ^{1}H NMR spectrum of compound **4c** in DMSO-d_6 at 500.13 MHz

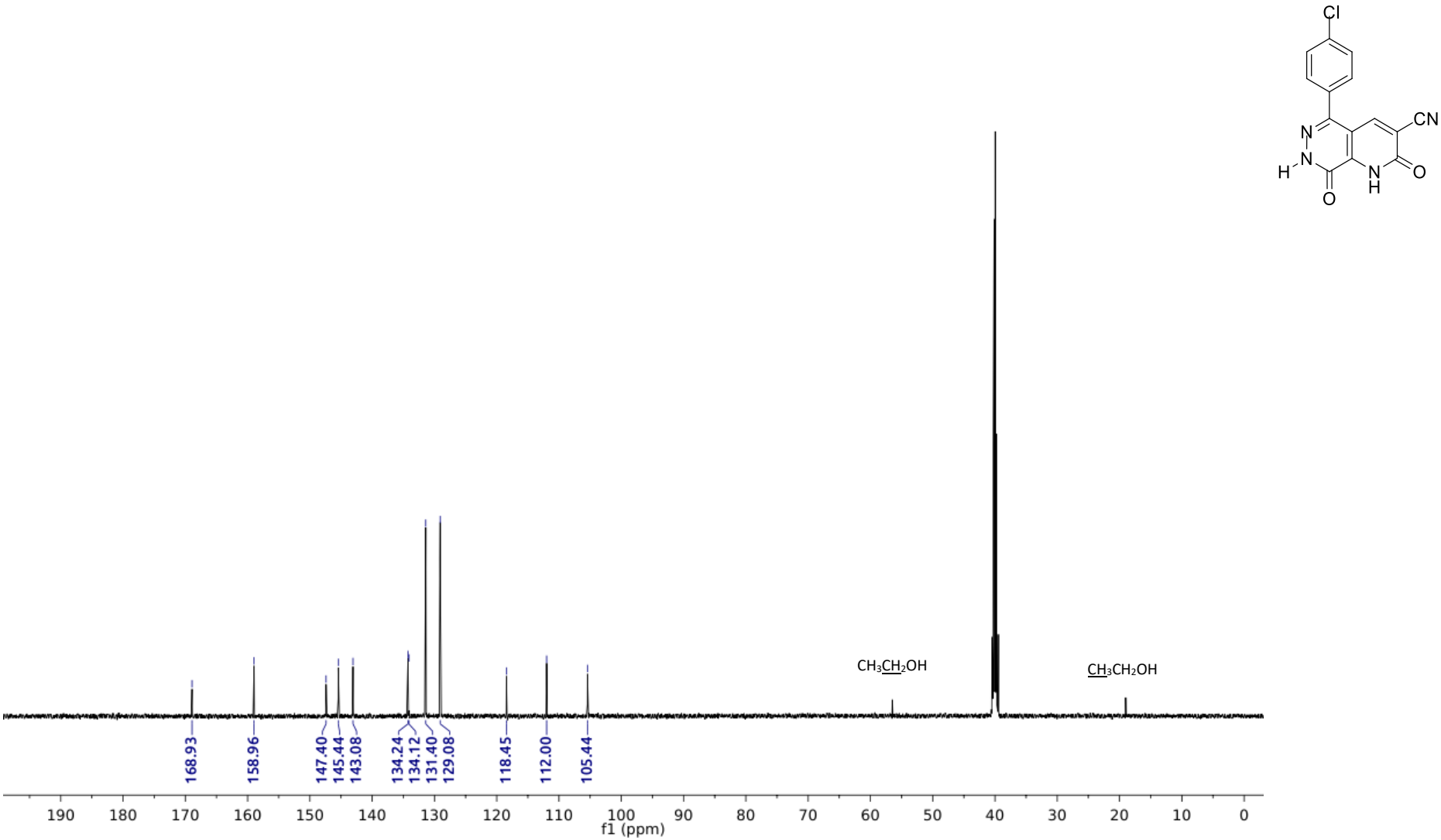

Figure S6 – ^{13}C NMR spectrum of compound **4c** in DMSO-d_6 at 125.77 MHz

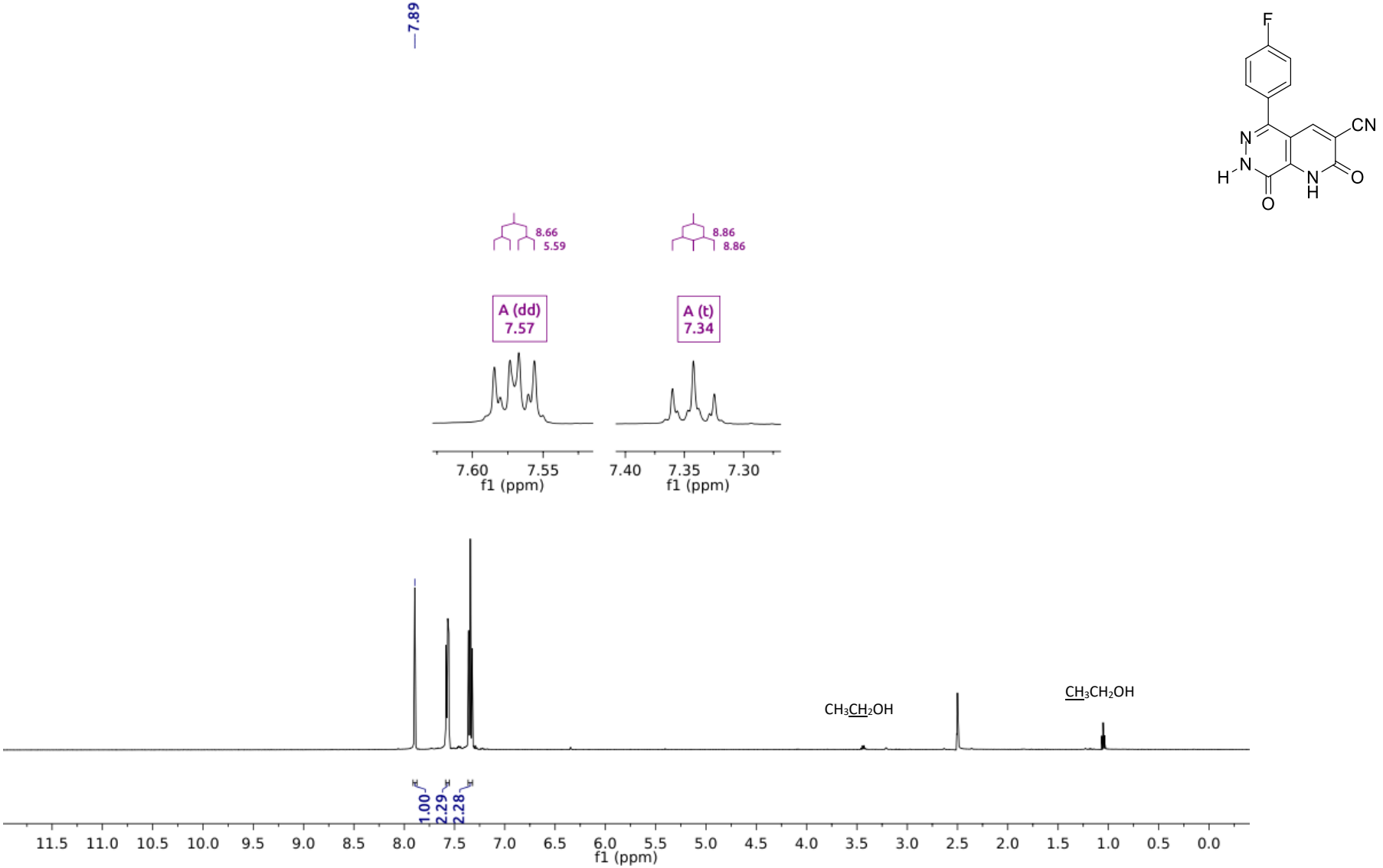

Figure S7 – ^{1}H NMR spectrum of compound **4d** in DMSO-d_6 at 500.13 MHz

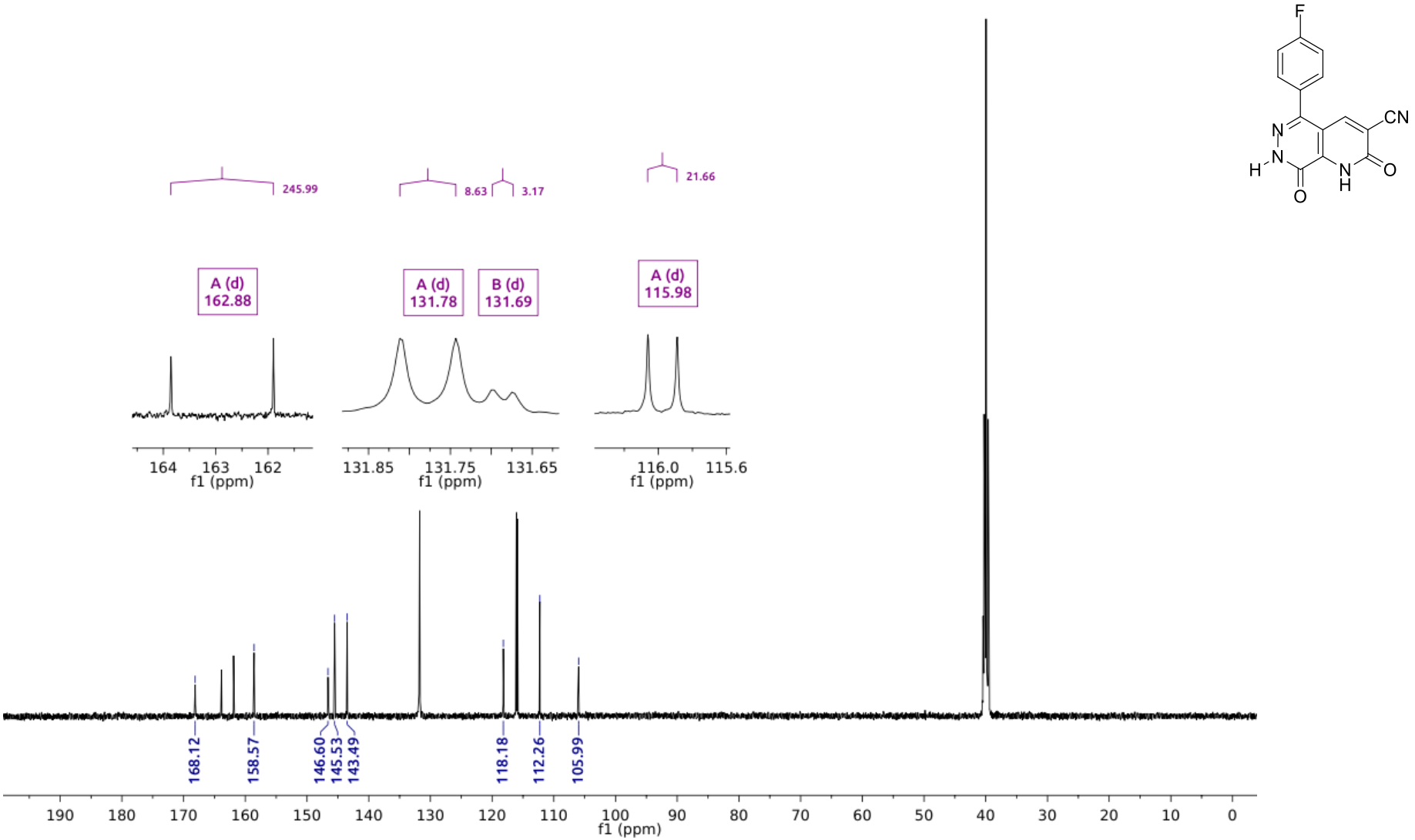

Figure S8 – ^{13}C NMR spectrum of compound **4d** in DMSO-d_6 at 125.77 MHz

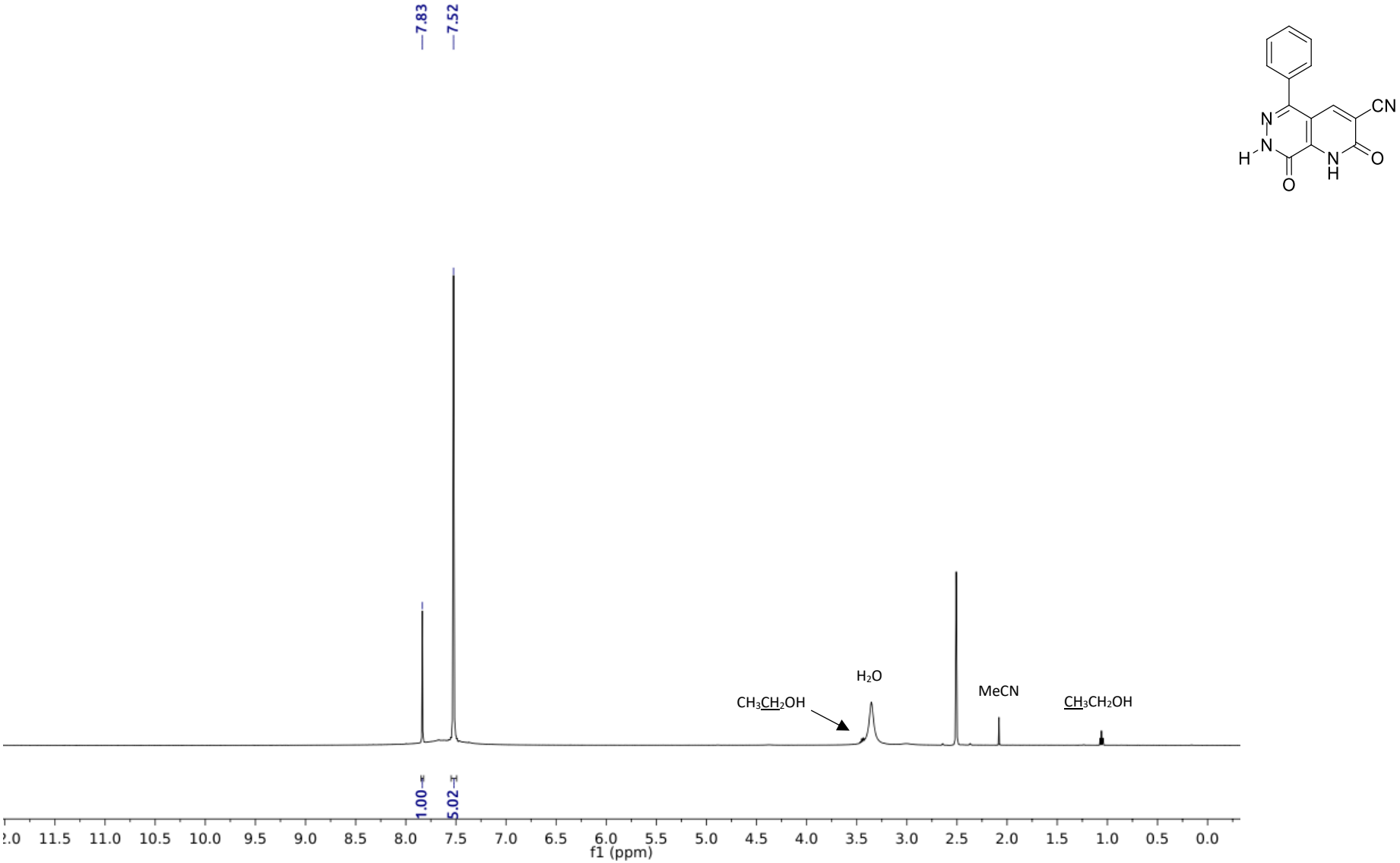

Figure S9 – ^{1}H NMR spectrum of compound **4e** in DMSO-d_6 at 500.13 MHz

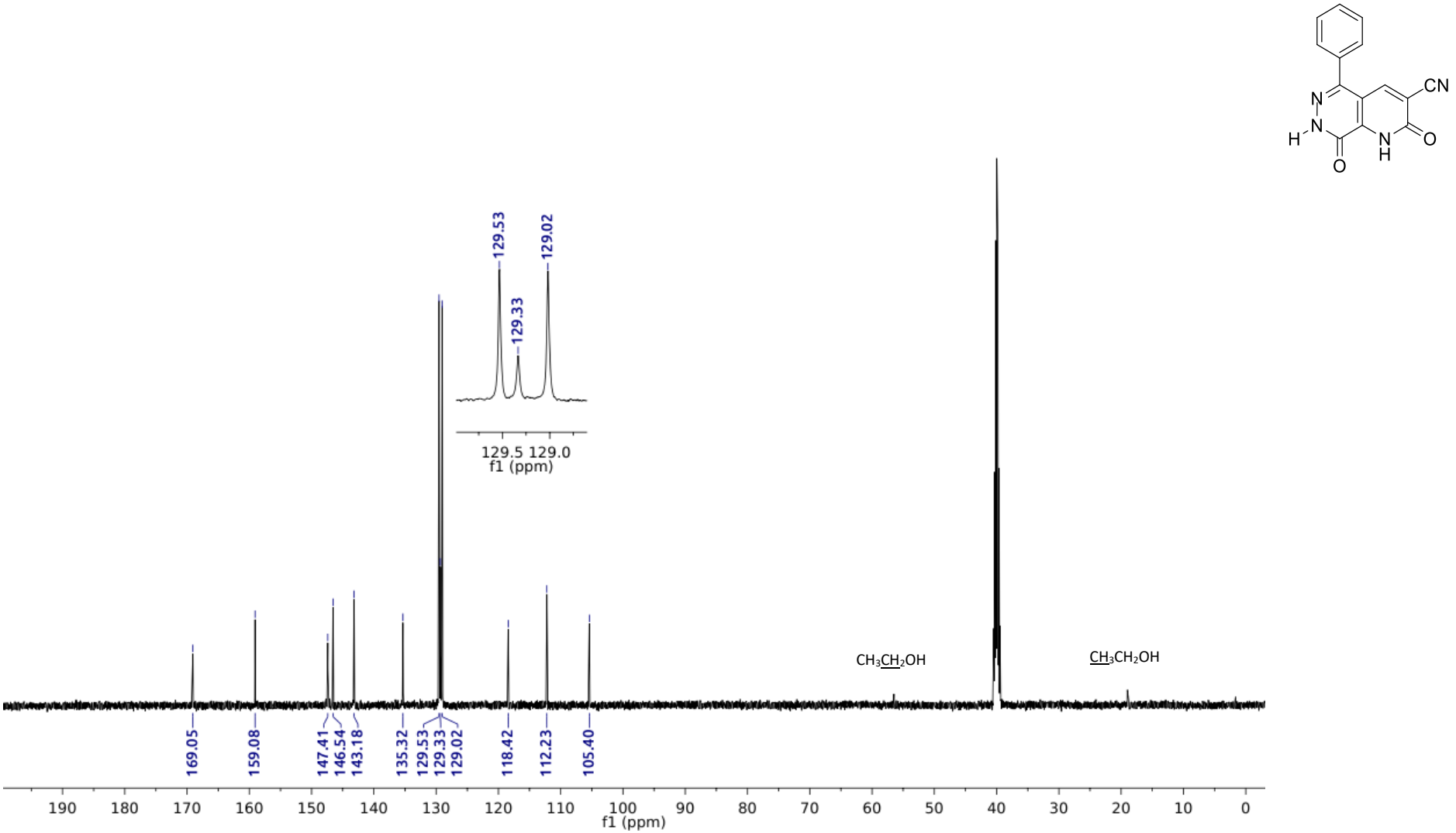

Figure S10 – ^{13}C NMR spectrum of compound **4e** in DMSO-d_6 at 125.77 MHz

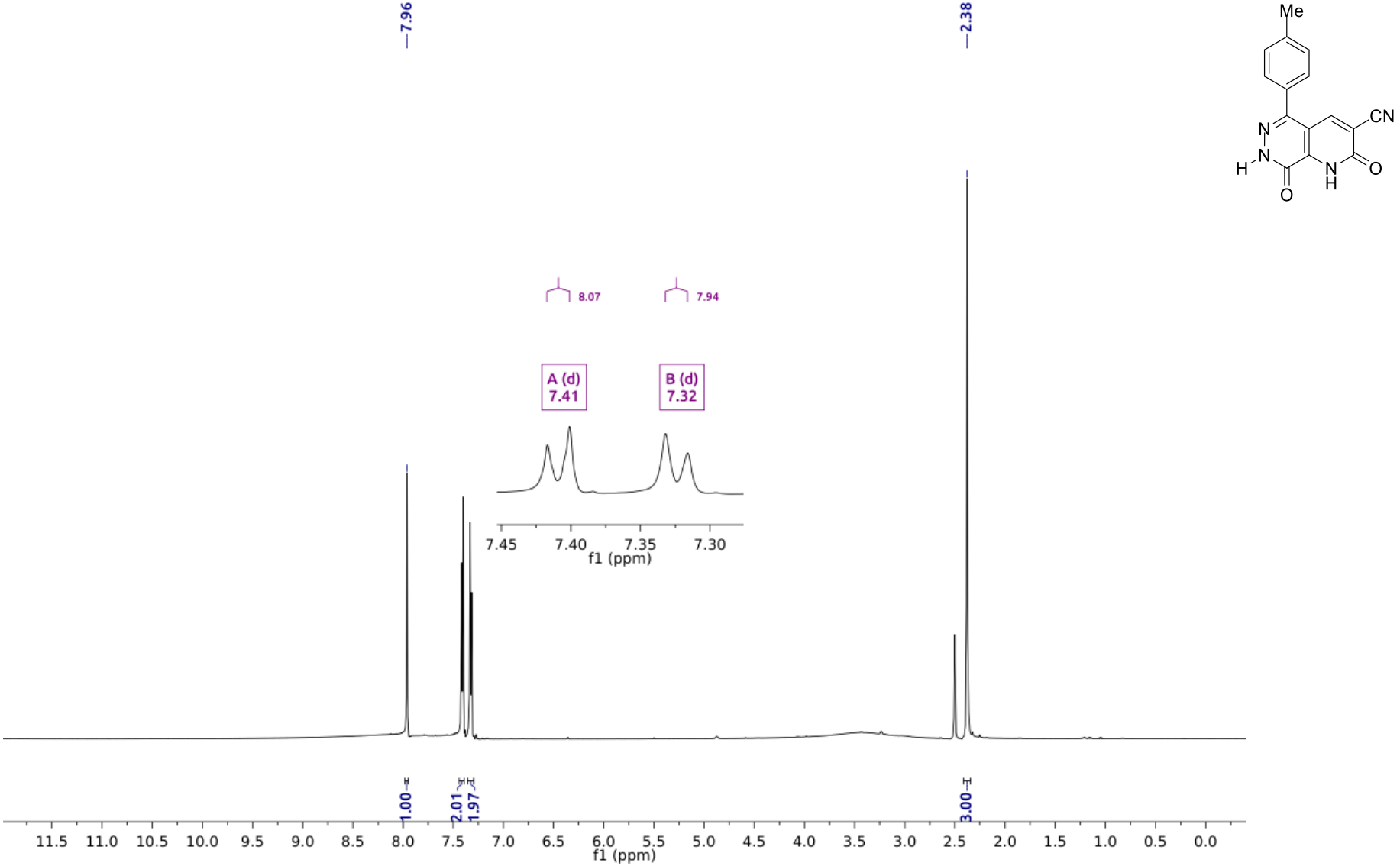

Figure S11 – ^{1}H NMR spectrum of compound **4f** in DMSO-d_6 at 500.13 MHz

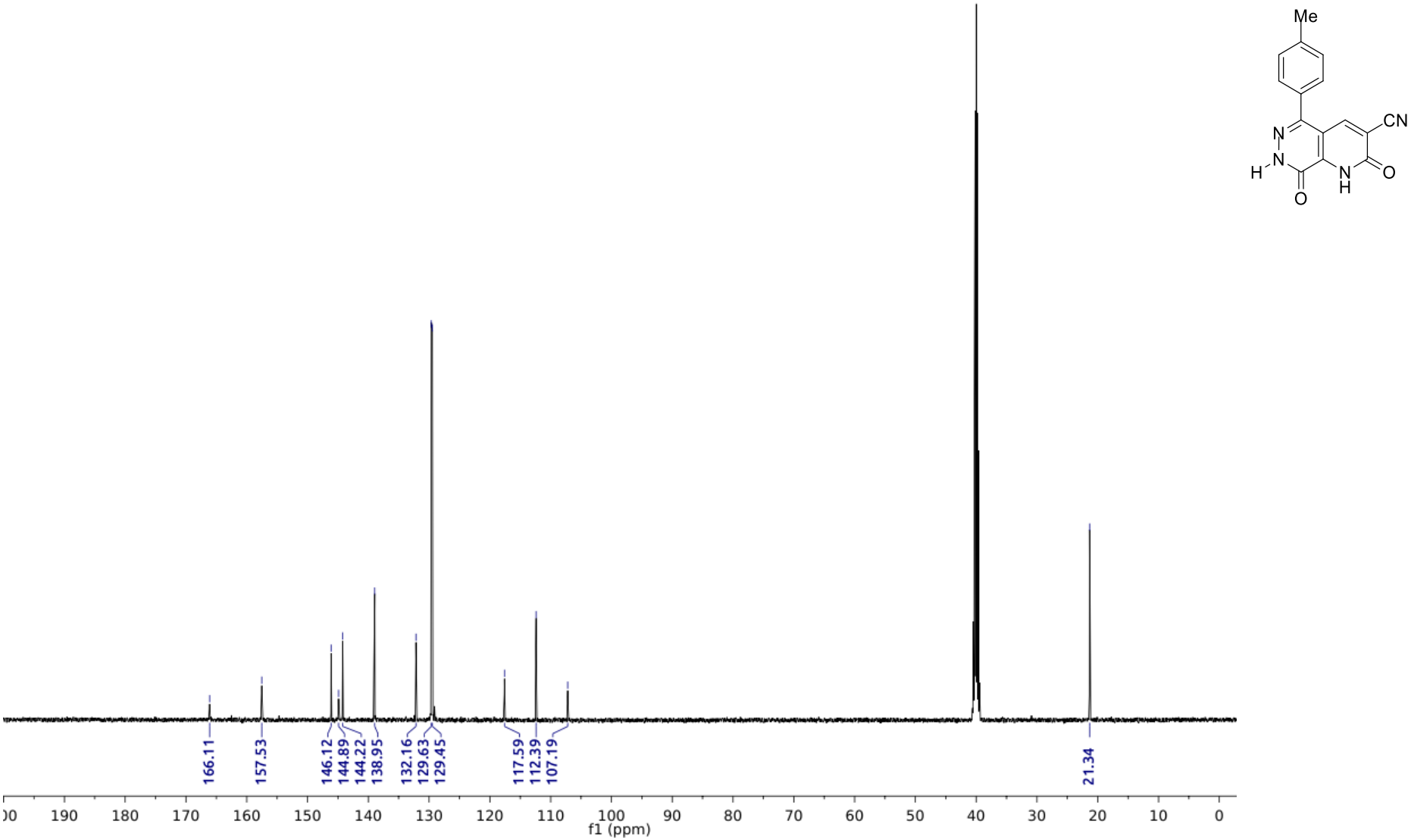

Figure S12 – ^{13}C NMR spectrum of compound **4f** in DMSO-d_6 at 125.77 MHz

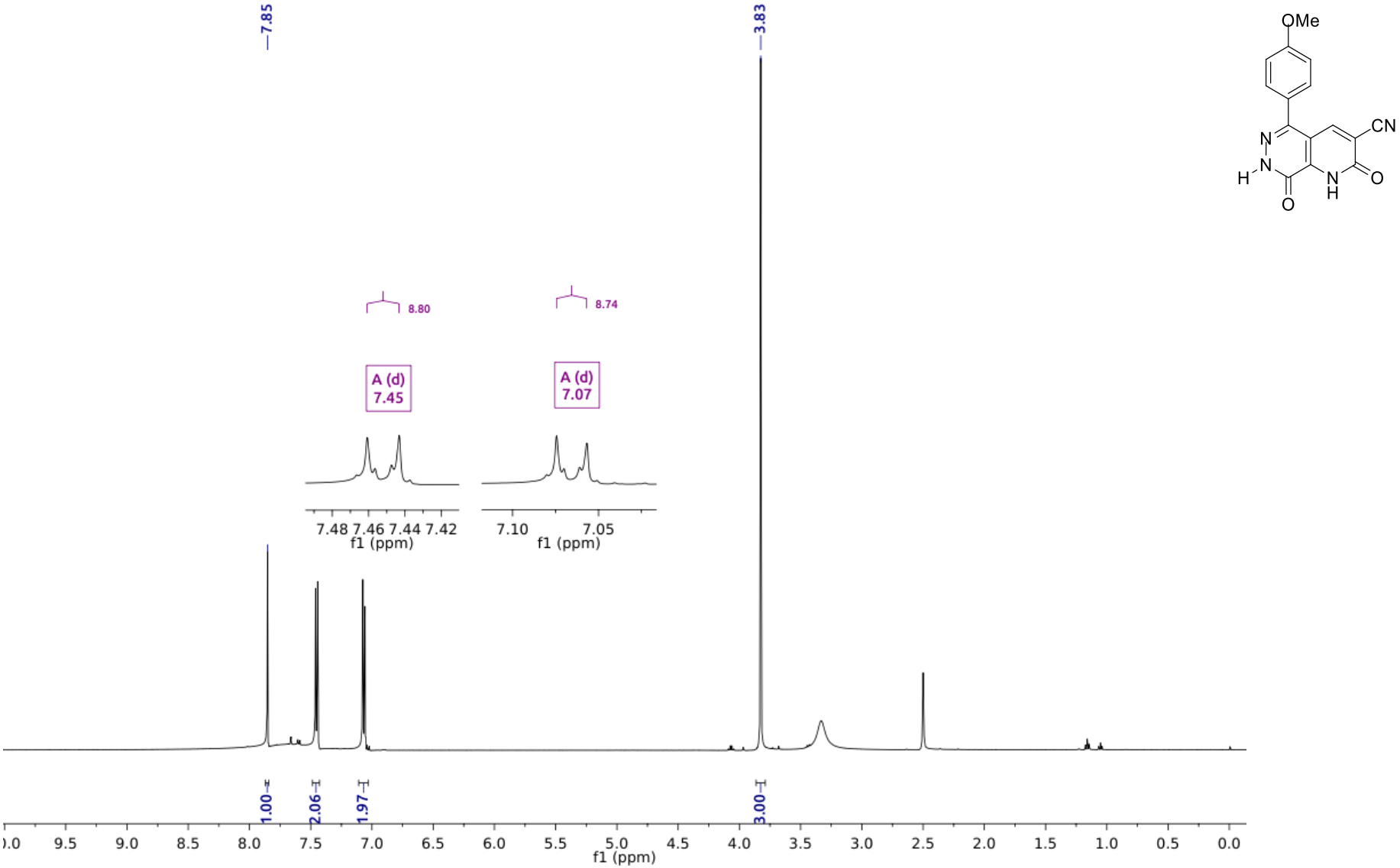

Figure S13 – ^{1}H NMR spectrum of compound **4g** in DMSO-d_6 at 500.13 MHz

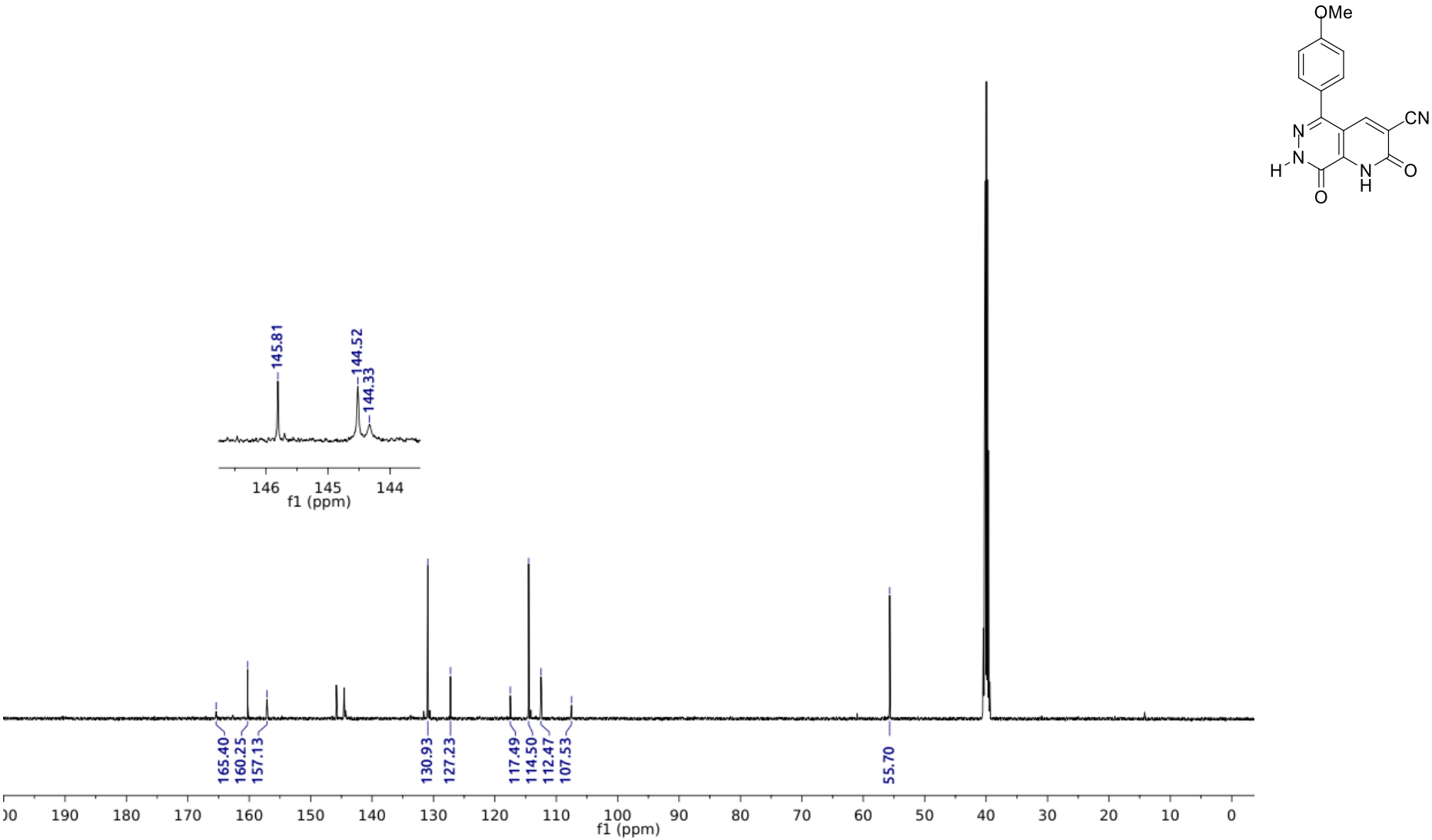

Figure S14 – ^{13}C NMR spectrum of compound **4g** in DMSO-d_6 at 125.77 MHz

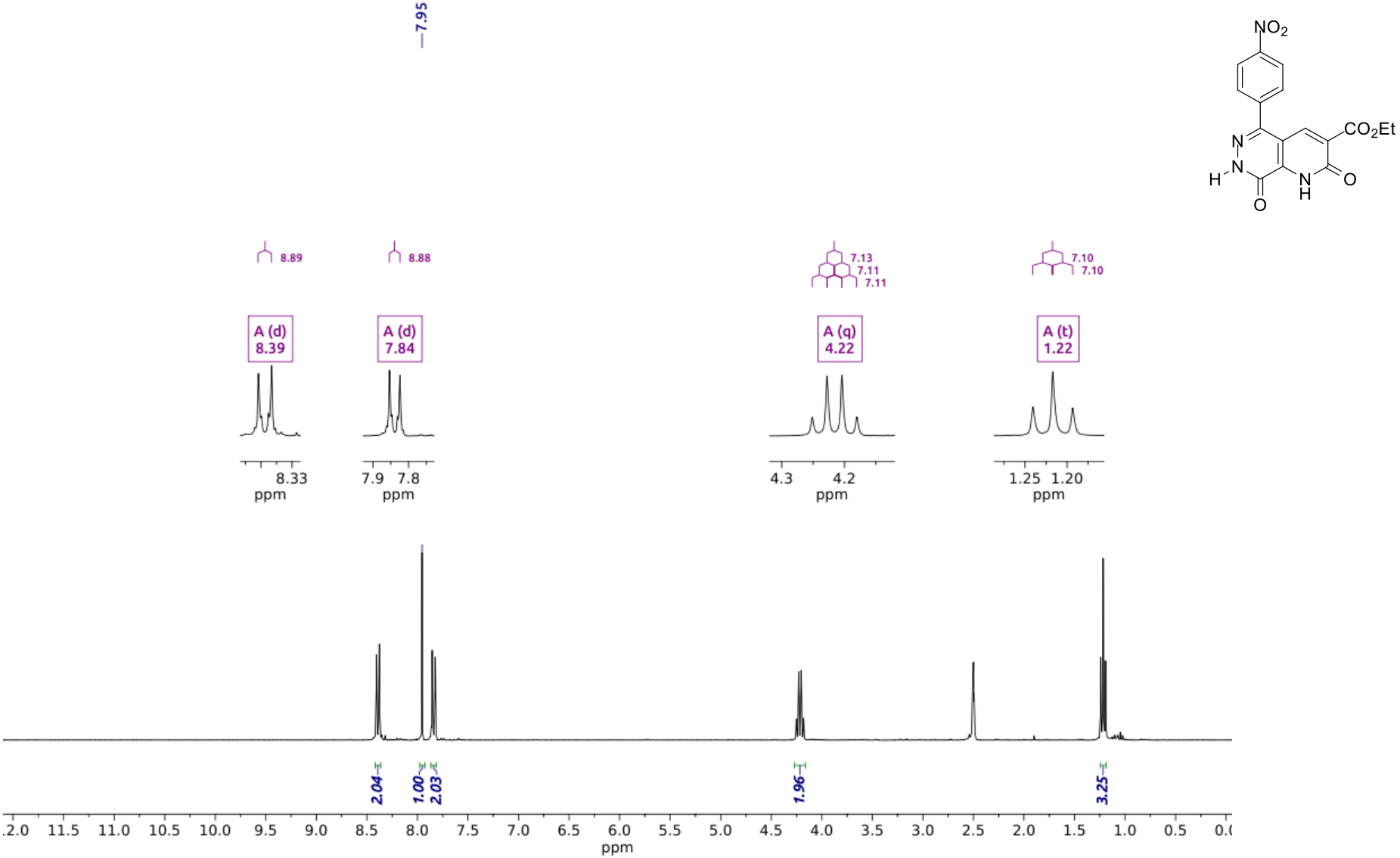

Figure S15 – ^{1}H NMR spectrum of compound **5a** in DMSO-d_6 at 300.06 MHz

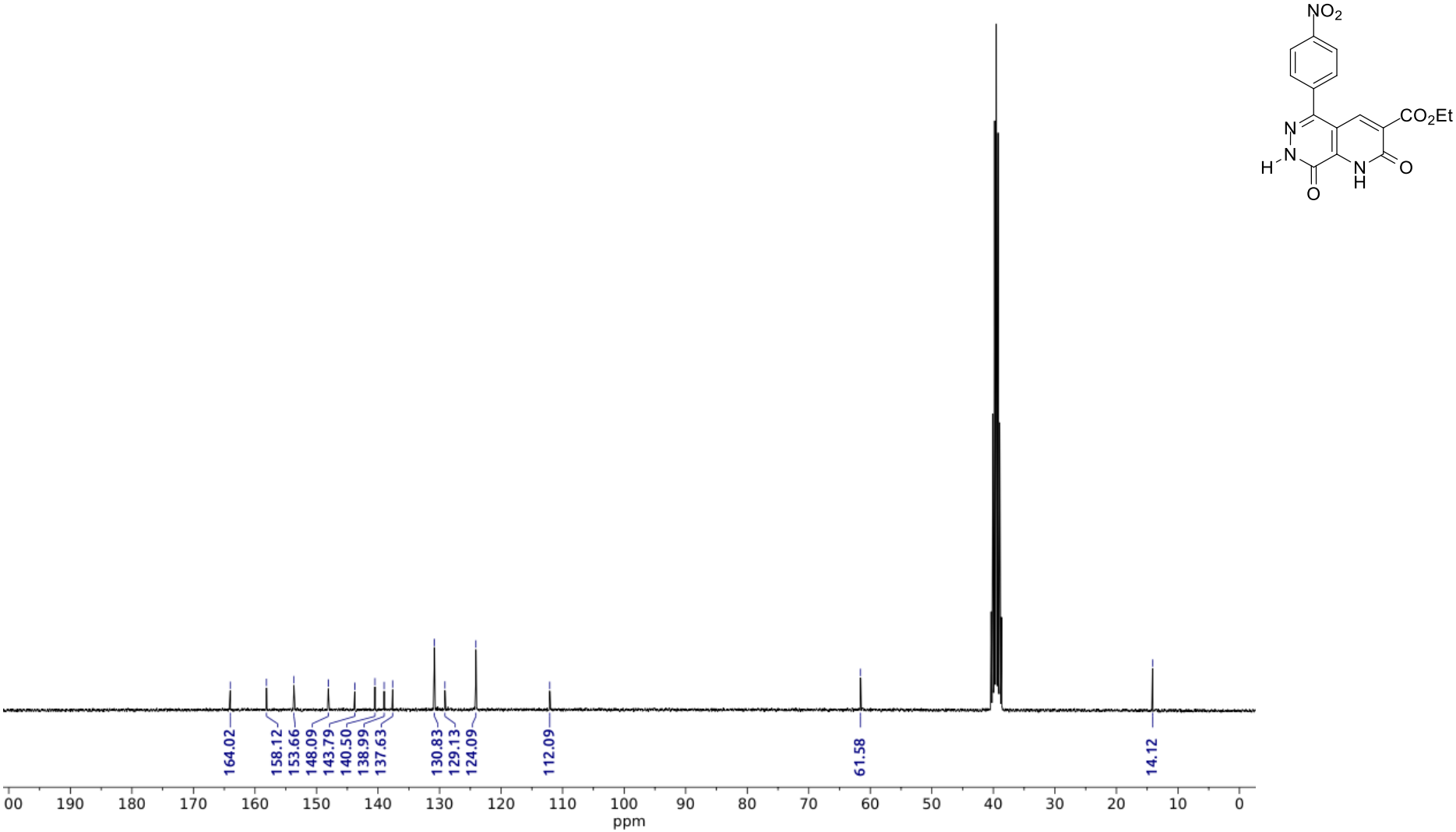

Figure S16 – ^{13}C NMR spectrum of compound **5a** in DMSO-d_6 at 75.46 MHz

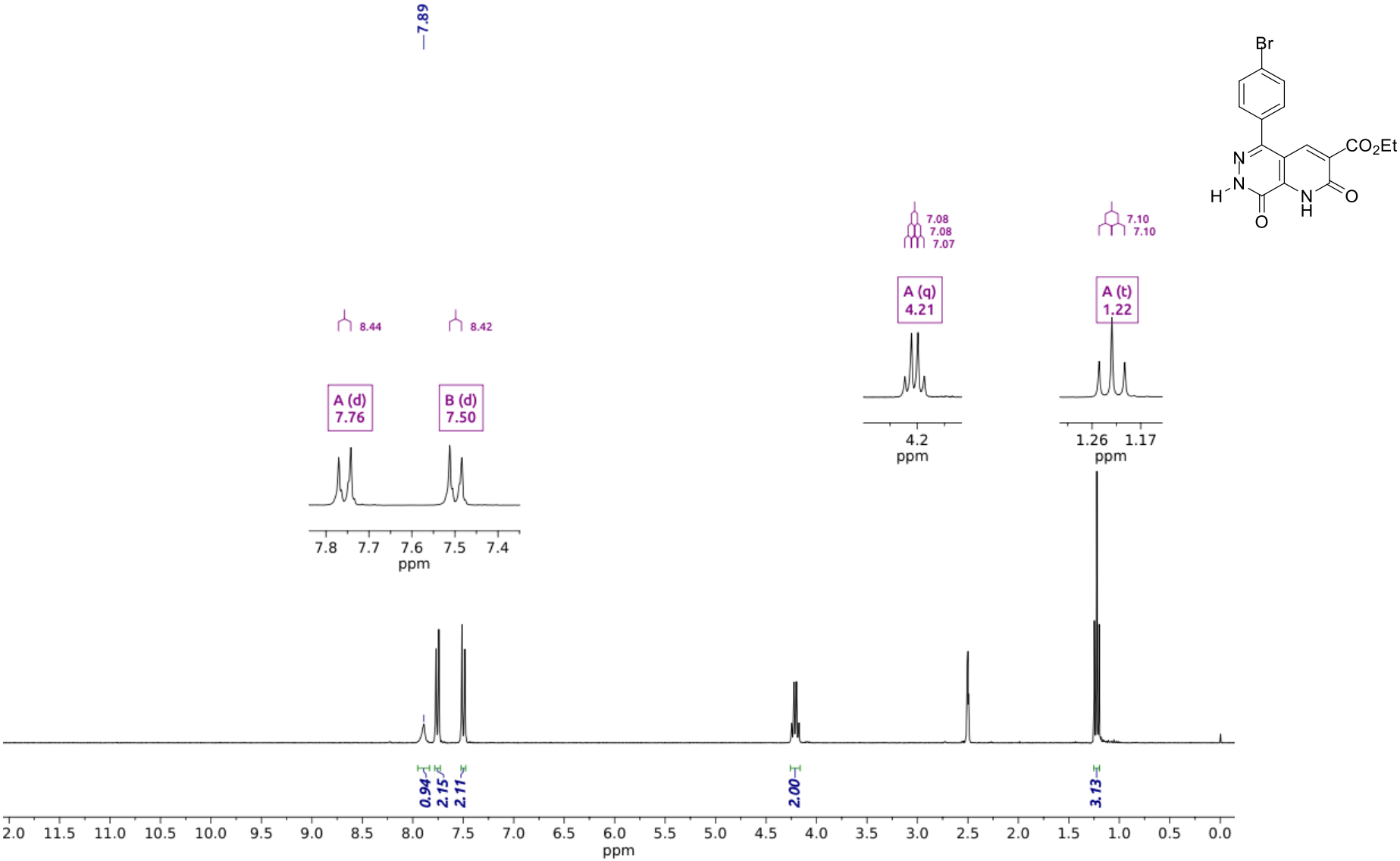

Figure S17 – ^{1}H NMR spectrum of compound **5b** in DMSO-d_6 at 300.06 MHz

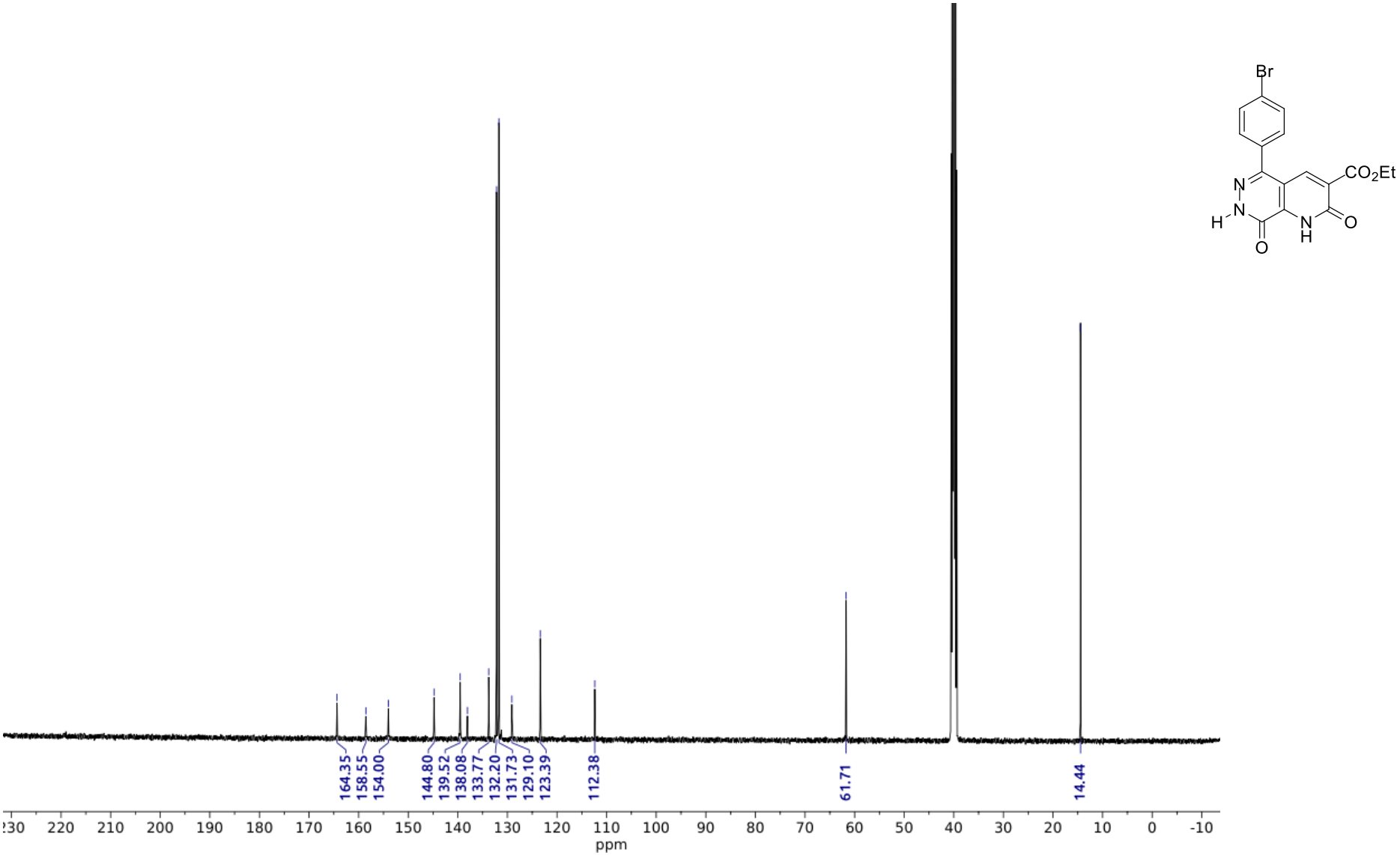

Figure S18 – ^{13}C NMR spectrum of compound **5b** in DMSO-d_6 at 75.46 MHz

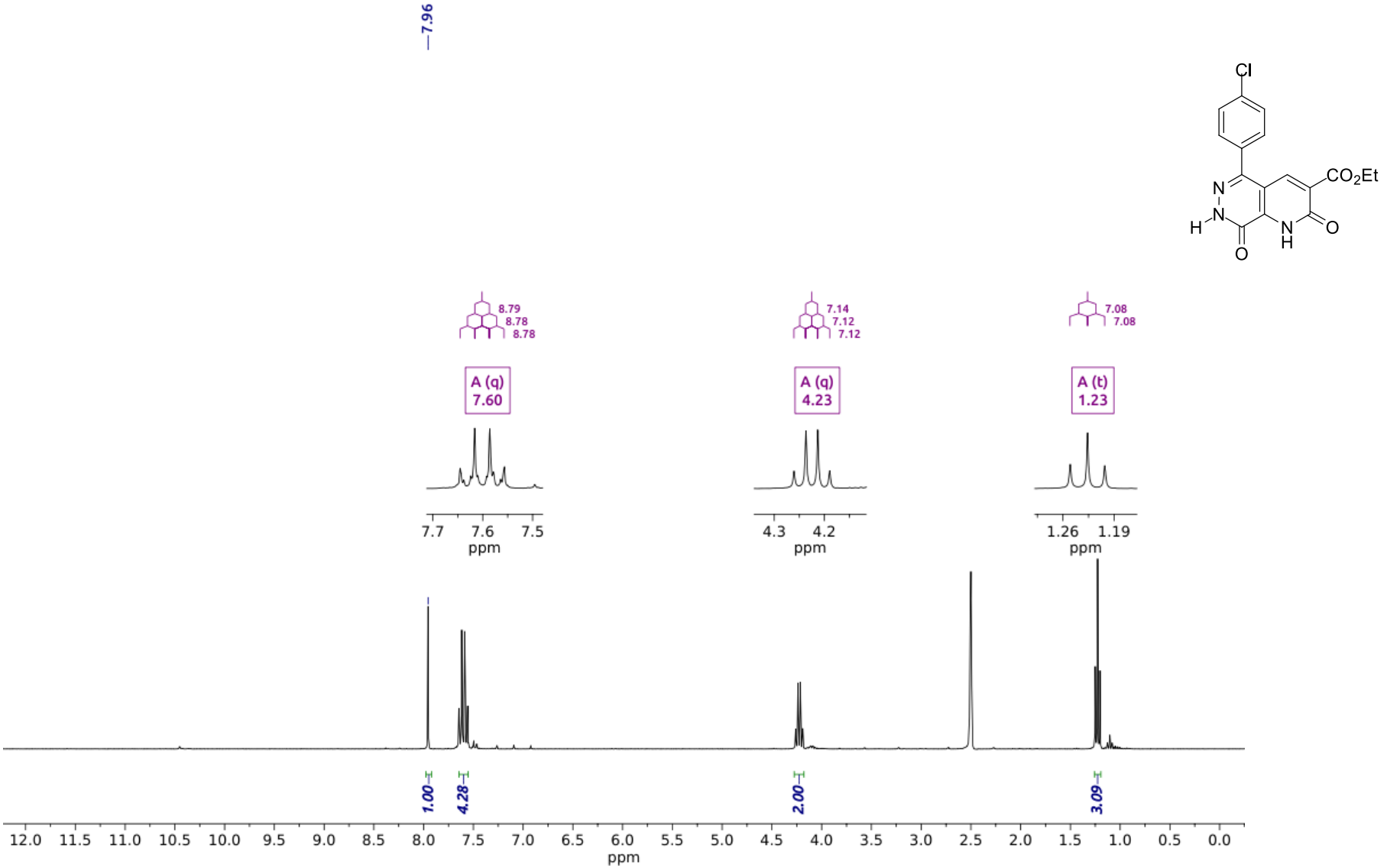

Figure S19 – ^{1}H NMR spectrum of compound **5c** in DMSO-d_6 at 300.06 MHz

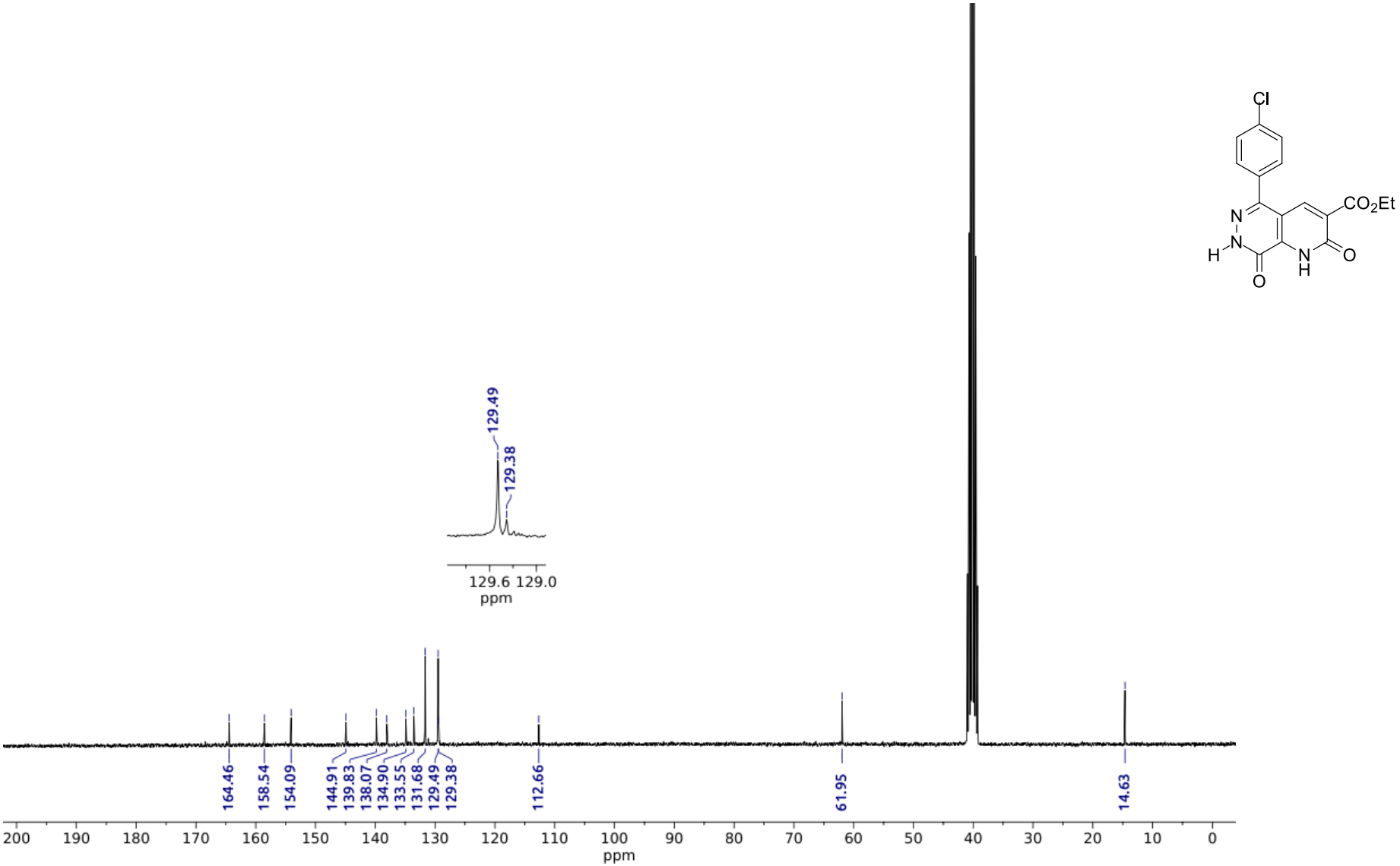

Figure S20 – ^{13}C NMR spectrum of compound **5c** in DMSO-d_6 at 75.46 MHz

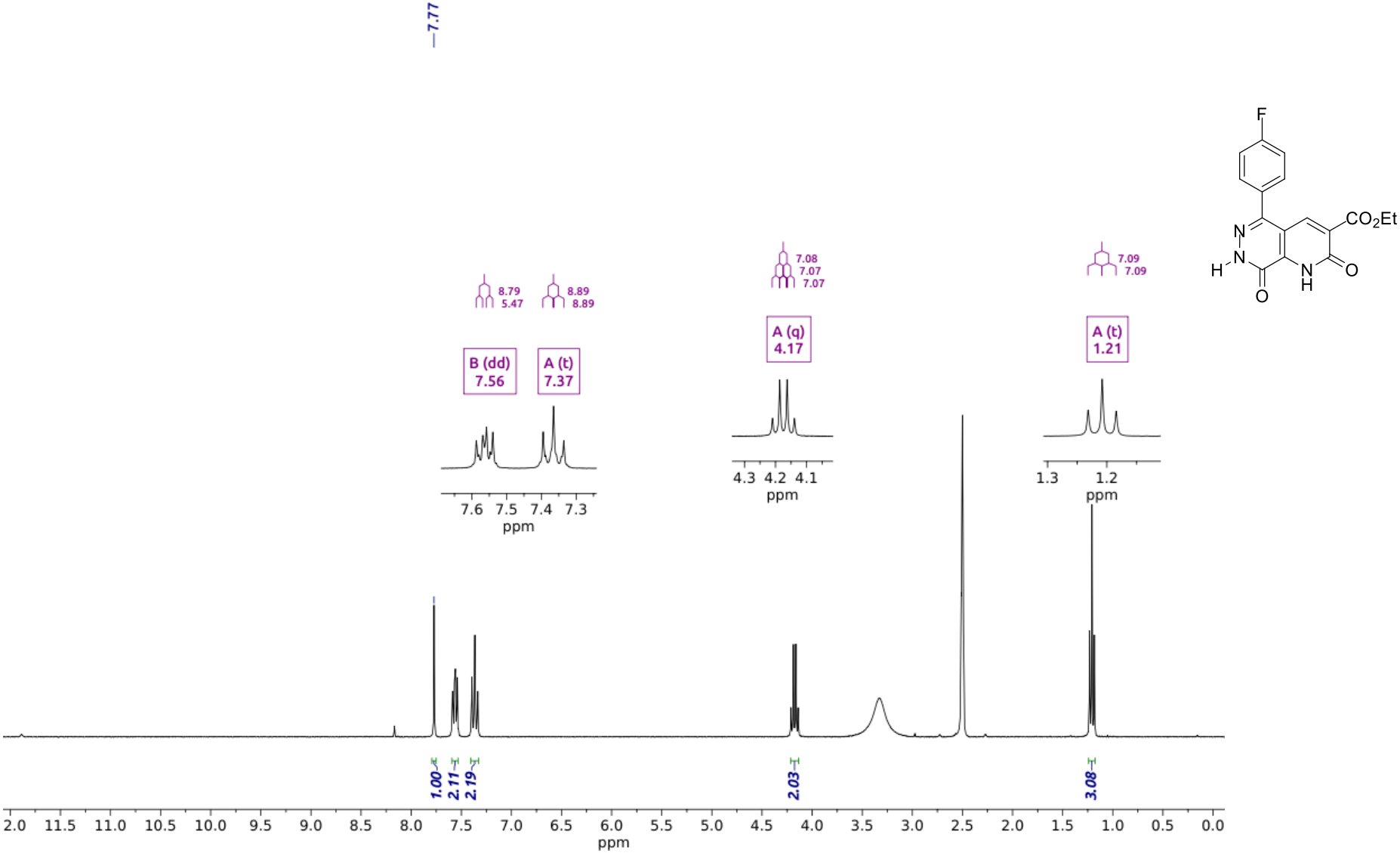

Figure S21 – ^{1}H NMR spectrum of compound **5d** in DMSO-d_6 at 300.06 MHz

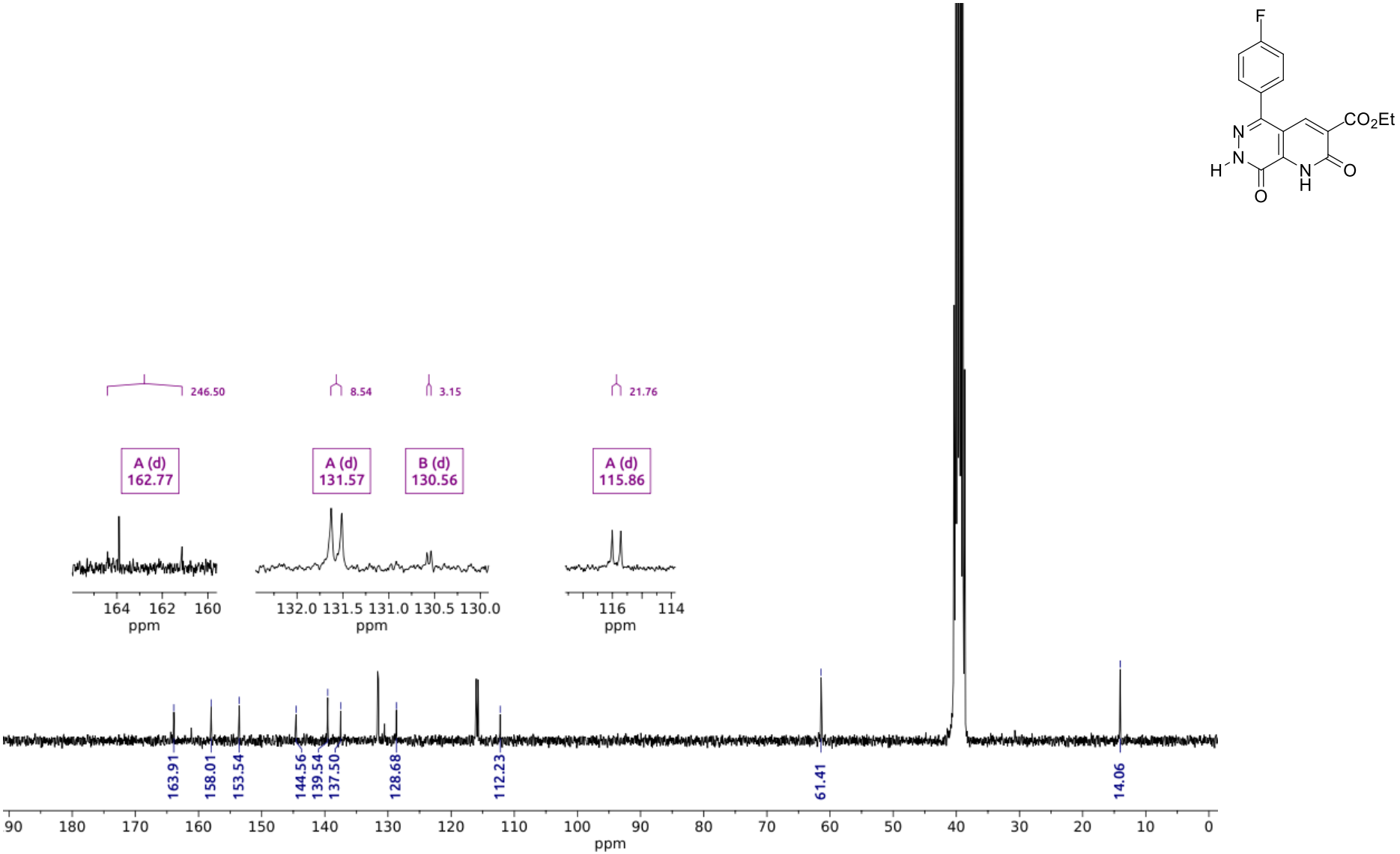

Figure S22 – ^{13}C NMR spectrum of compound **5d** in DMSO-d_6 at 75.46 MHz

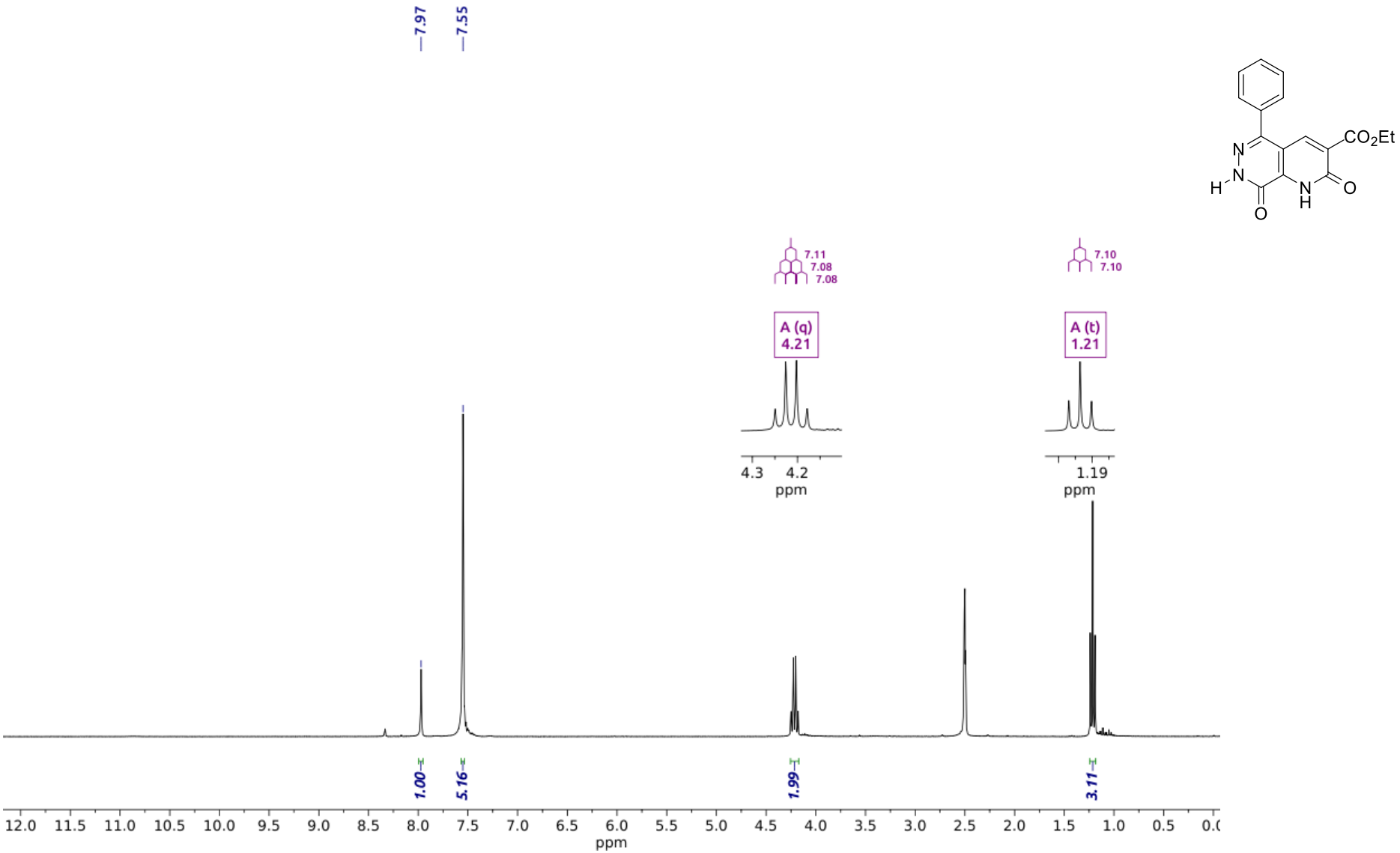

Figure S23 – ^{1}H NMR spectrum of compound **5e** in DMSO-d_6 at 300.06 MHz

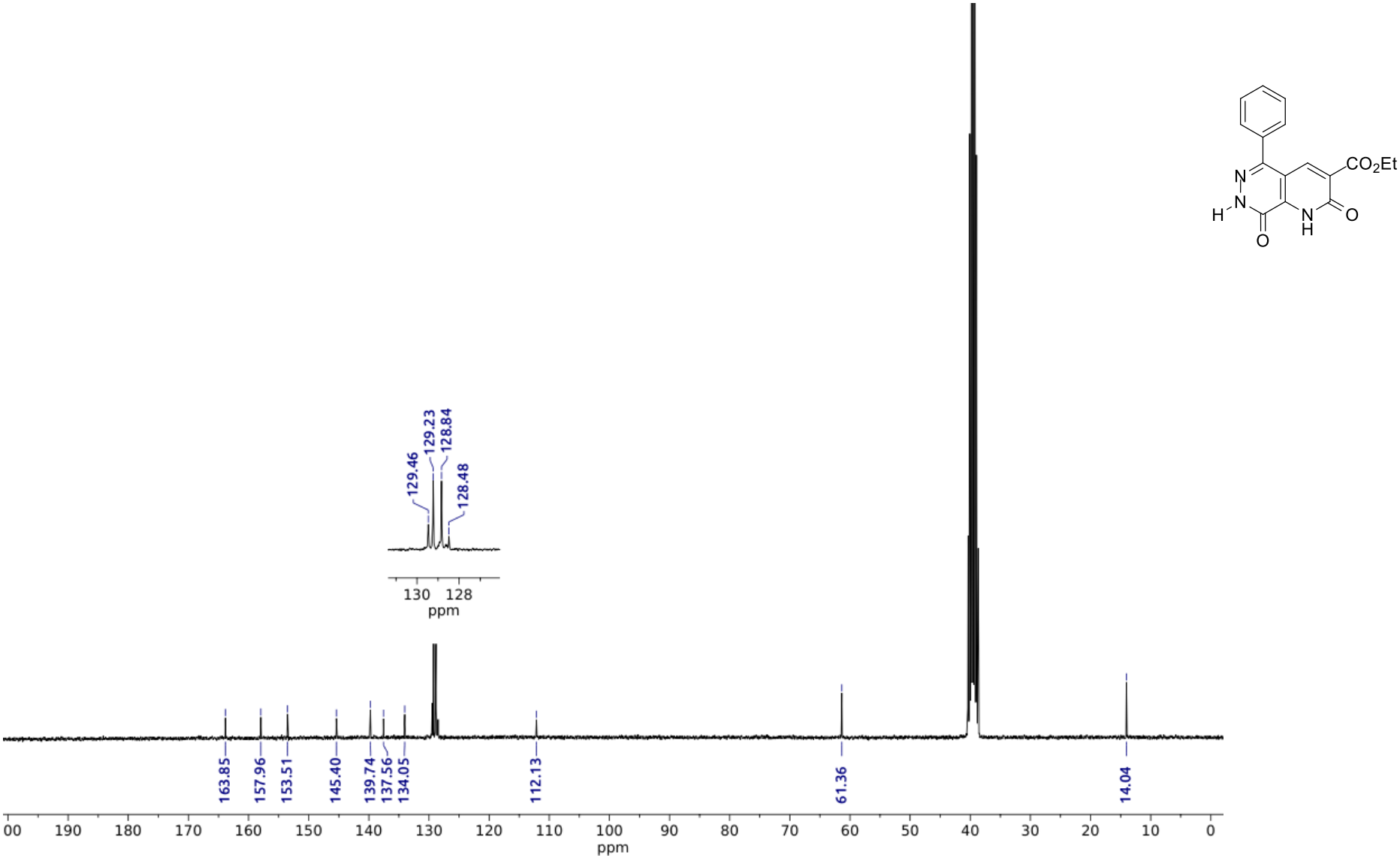

Figure S24 – ^{13}C NMR spectrum of compound **5e** in DMSO-d_6 at 75.46 MHz

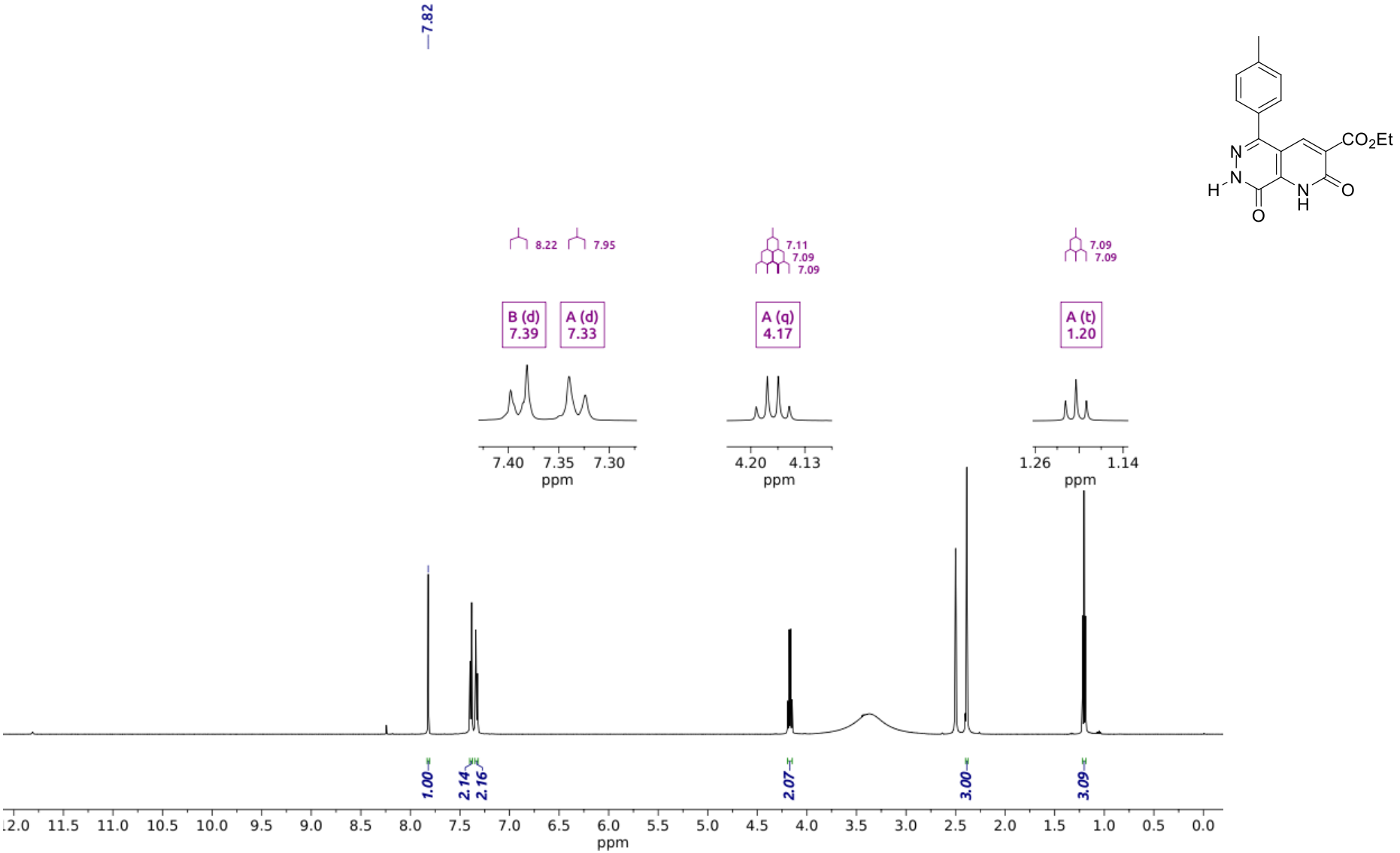

Figure S25 – ^{1}H NMR spectrum of compound **5f** in DMSO-d_6 at 500.13 MHz

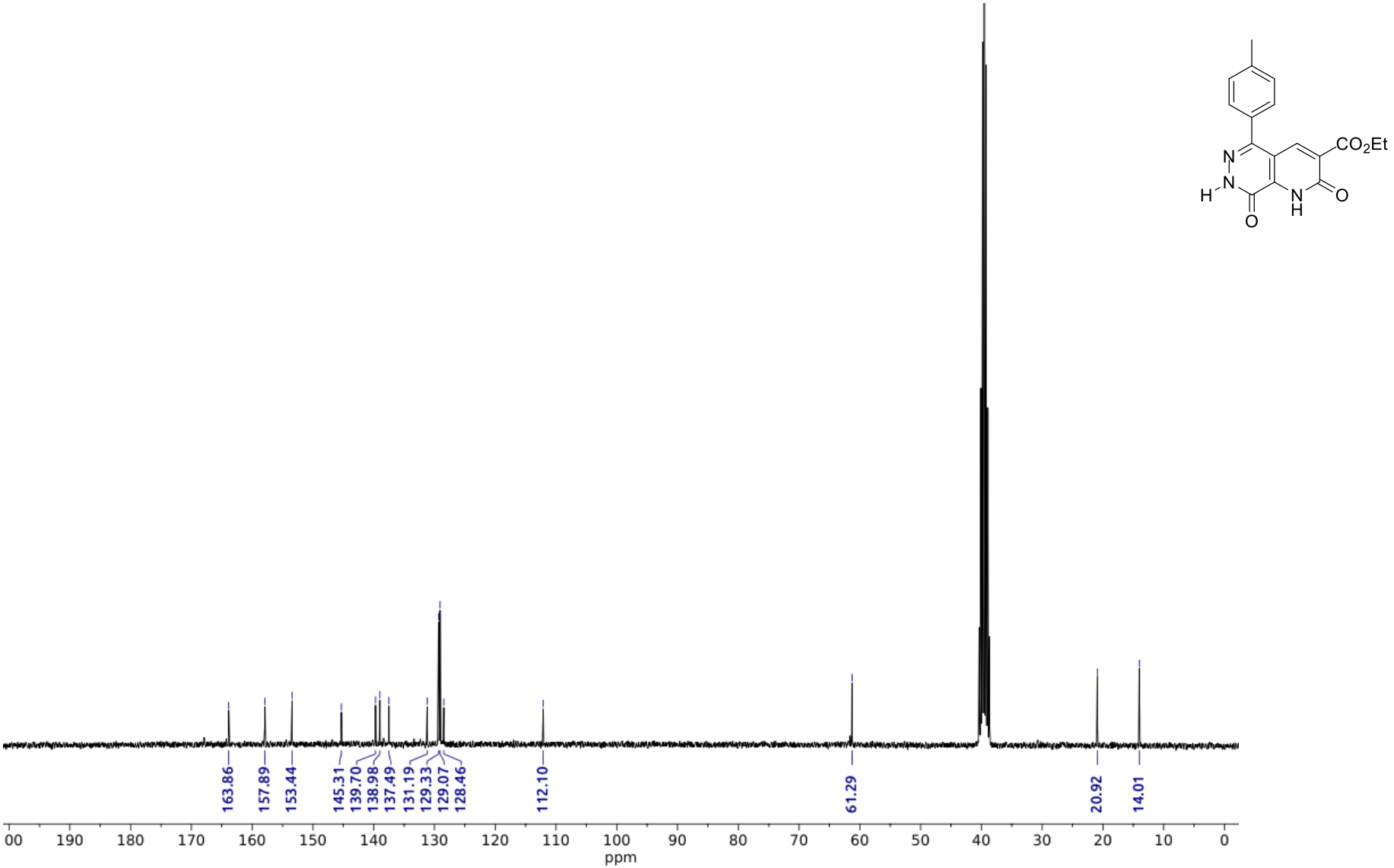

Figure S26 – ^{13}C NMR spectrum of compound **5f** in DMSO-d_6 at 125.77 MHz

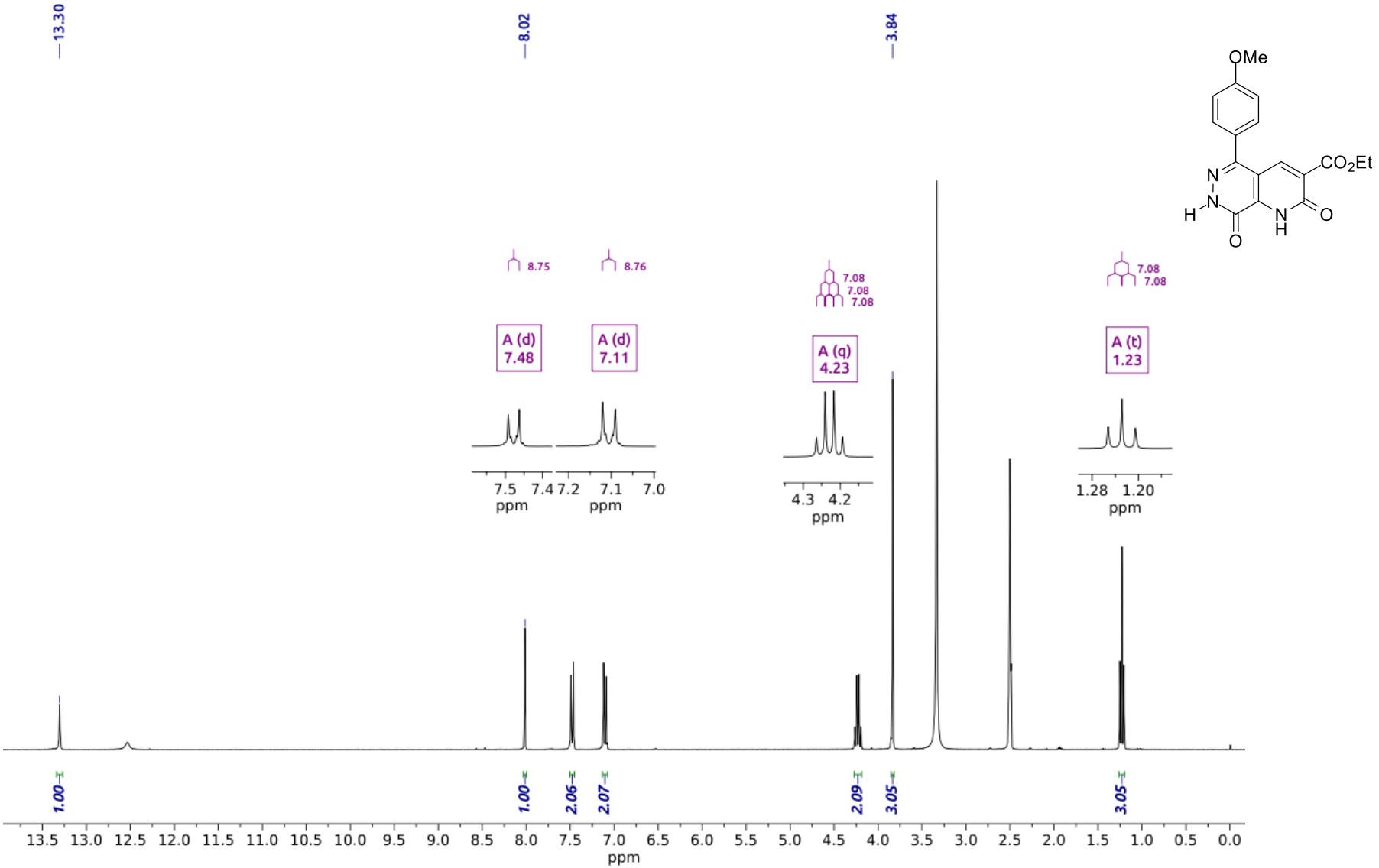

Figure S27 – ^{1}H NMR spectrum of compound **5g** in DMSO-d_6 at 300.06 MHz

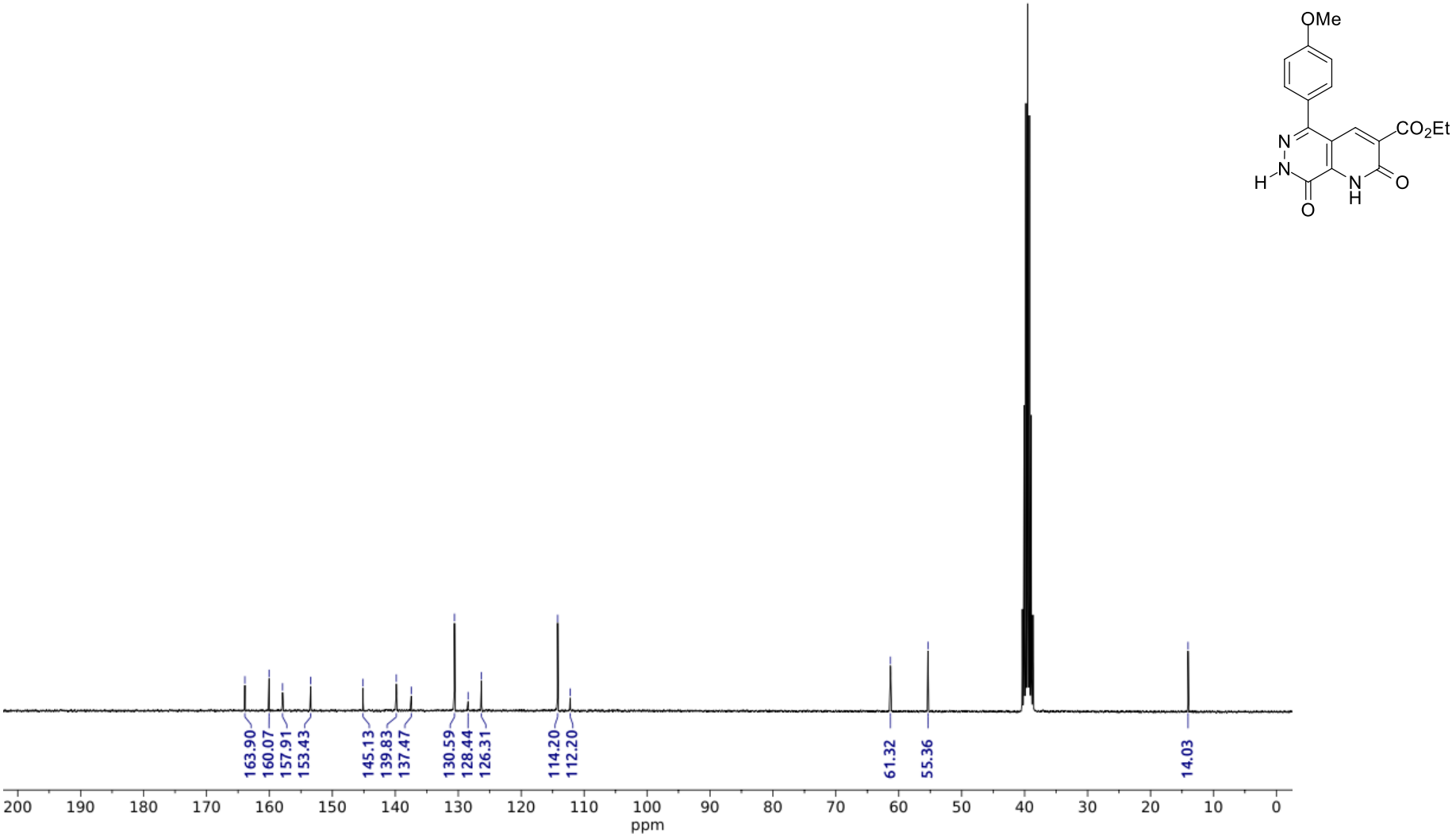

Figure S28 – ^{13}C NMR spectrum of compound **5g** in DMSO-d_6 at 75.46 MHz

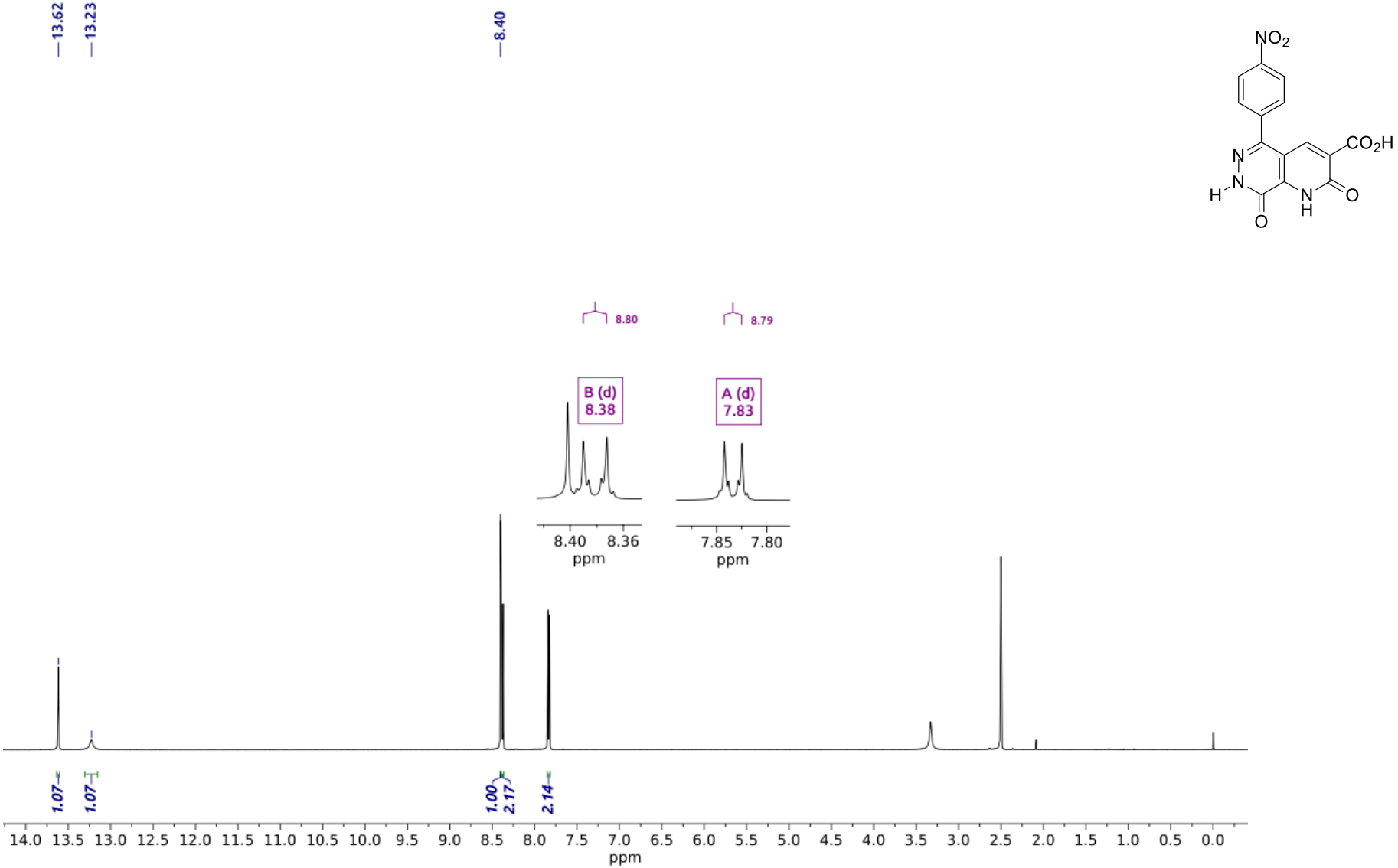

Figure S29 – ^{1}H NMR spectrum of compound **6a** in DMSO-d_6 at 500.13 MHz

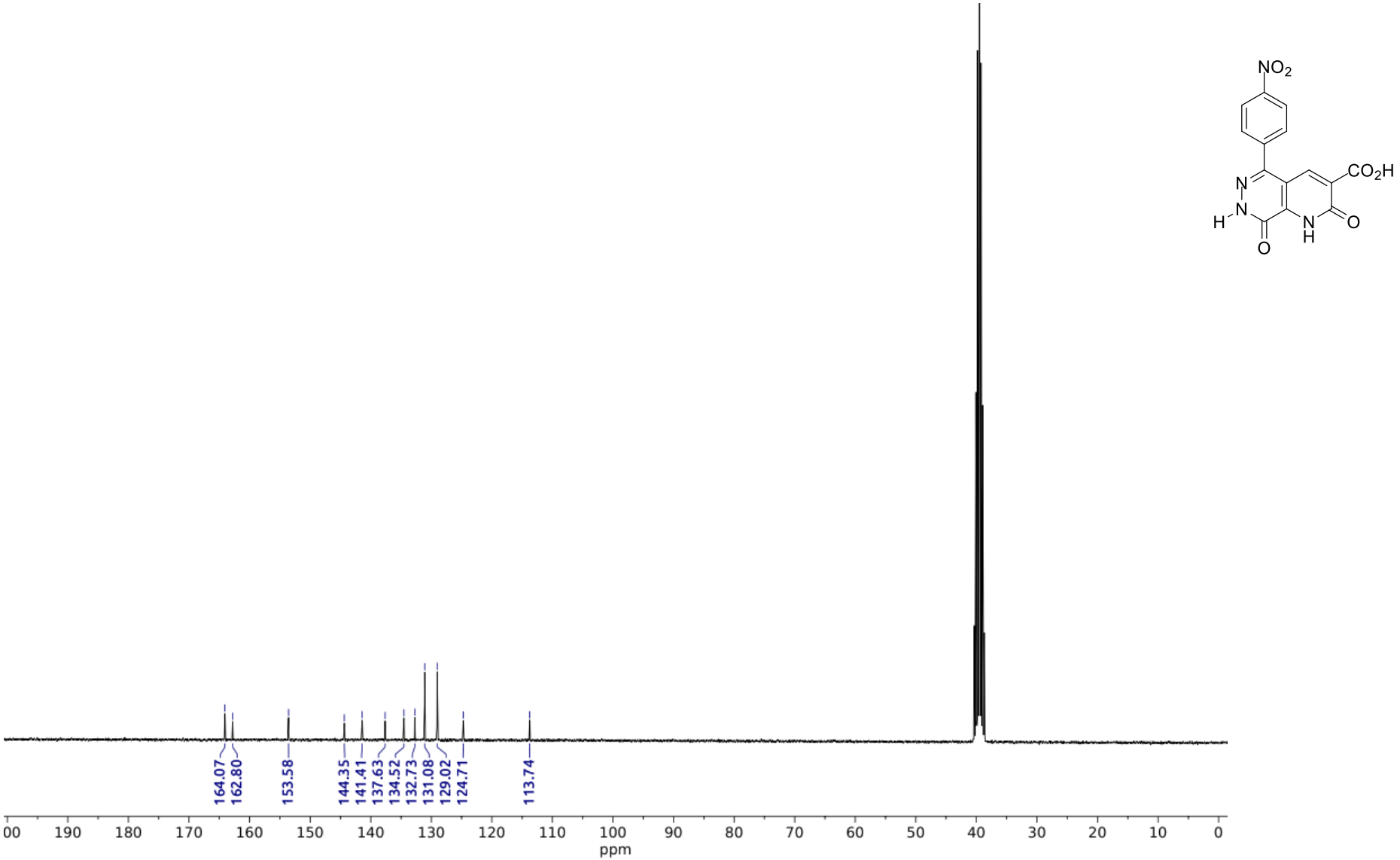

Figure S30 – ^{13}C NMR spectrum of compound **6a** in DMSO-d_6 at at 75.46 MHz

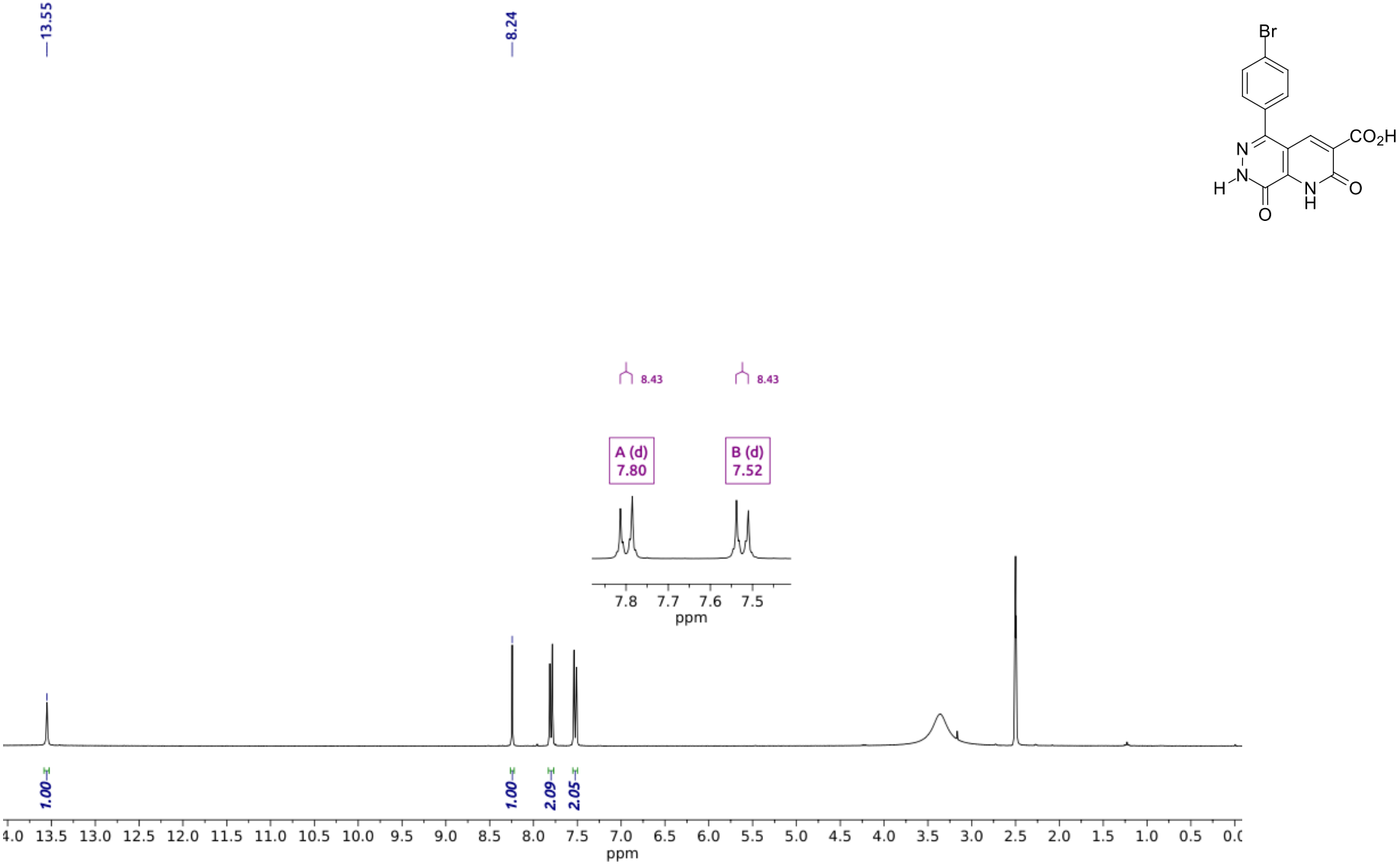

Figure S31 – ^{1}H NMR spectrum of compound **6b** in DMSO-d_6 at 300.06 MHz

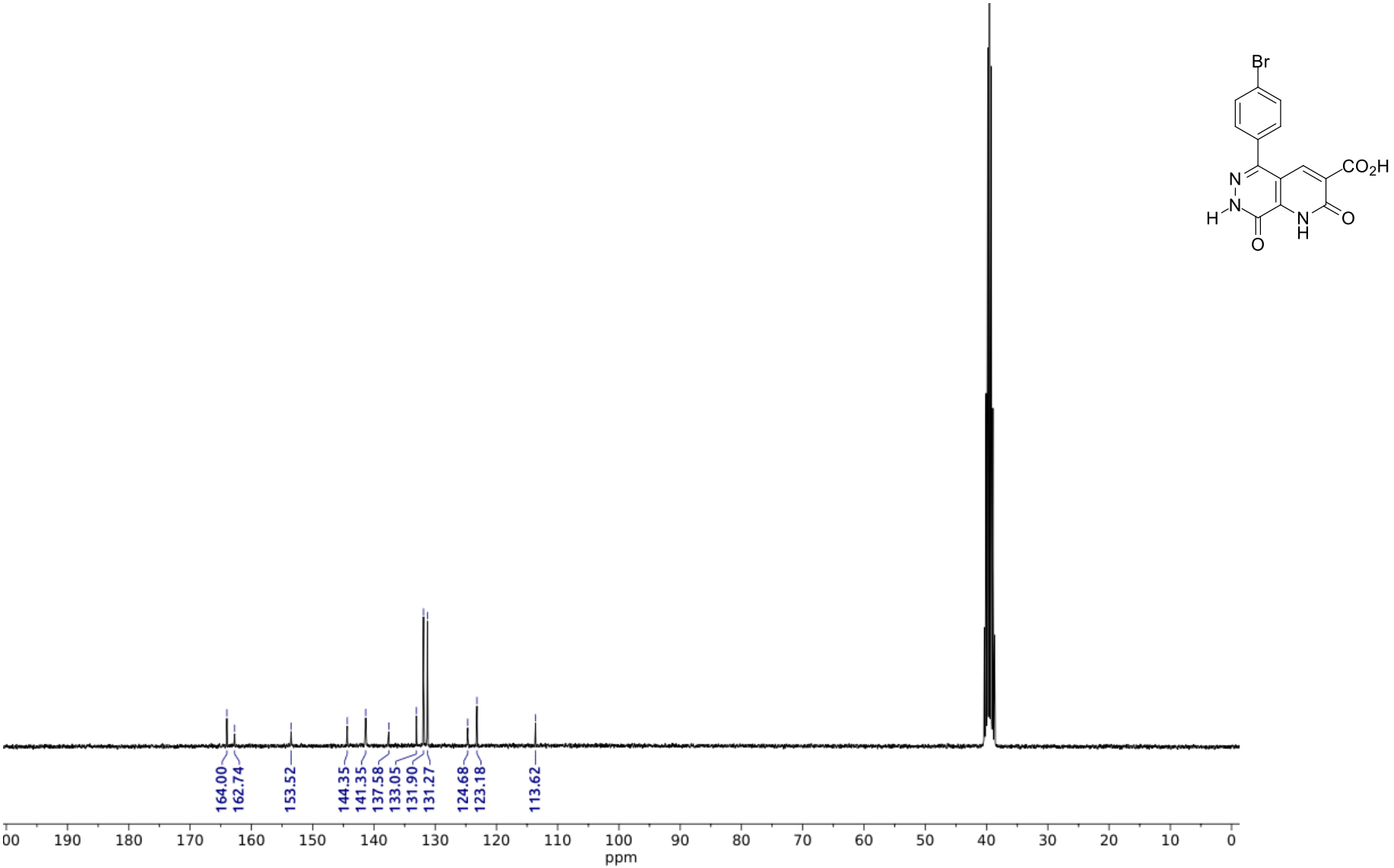

Figure S32 – ^{13}C NMR spectrum of compound **6b** in DMSO-d_6 at 75.46 MHz

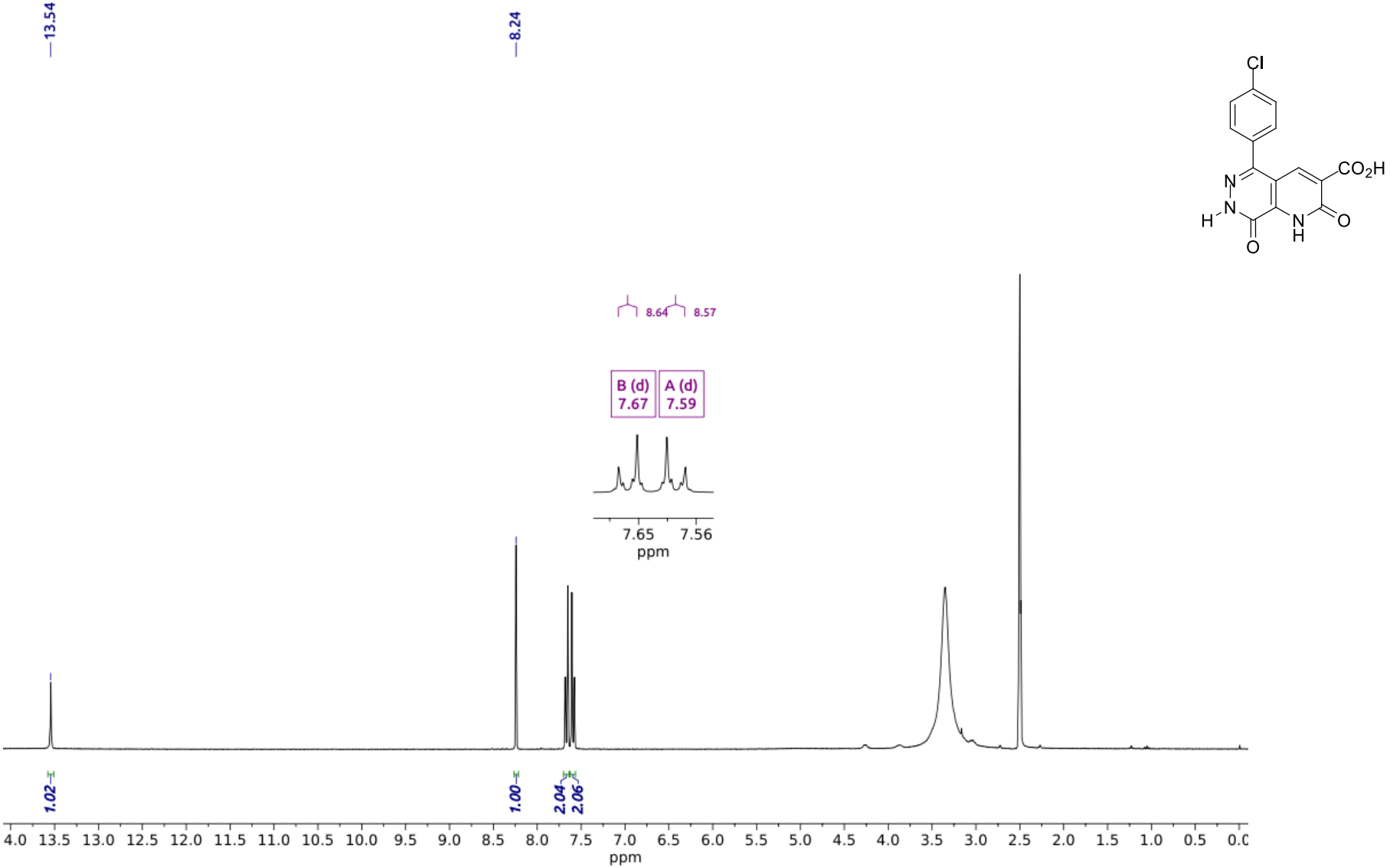

Figure S33 – ^{1}H NMR spectrum of compound **6c** in DMSO-d_6 at 300.06 MHz

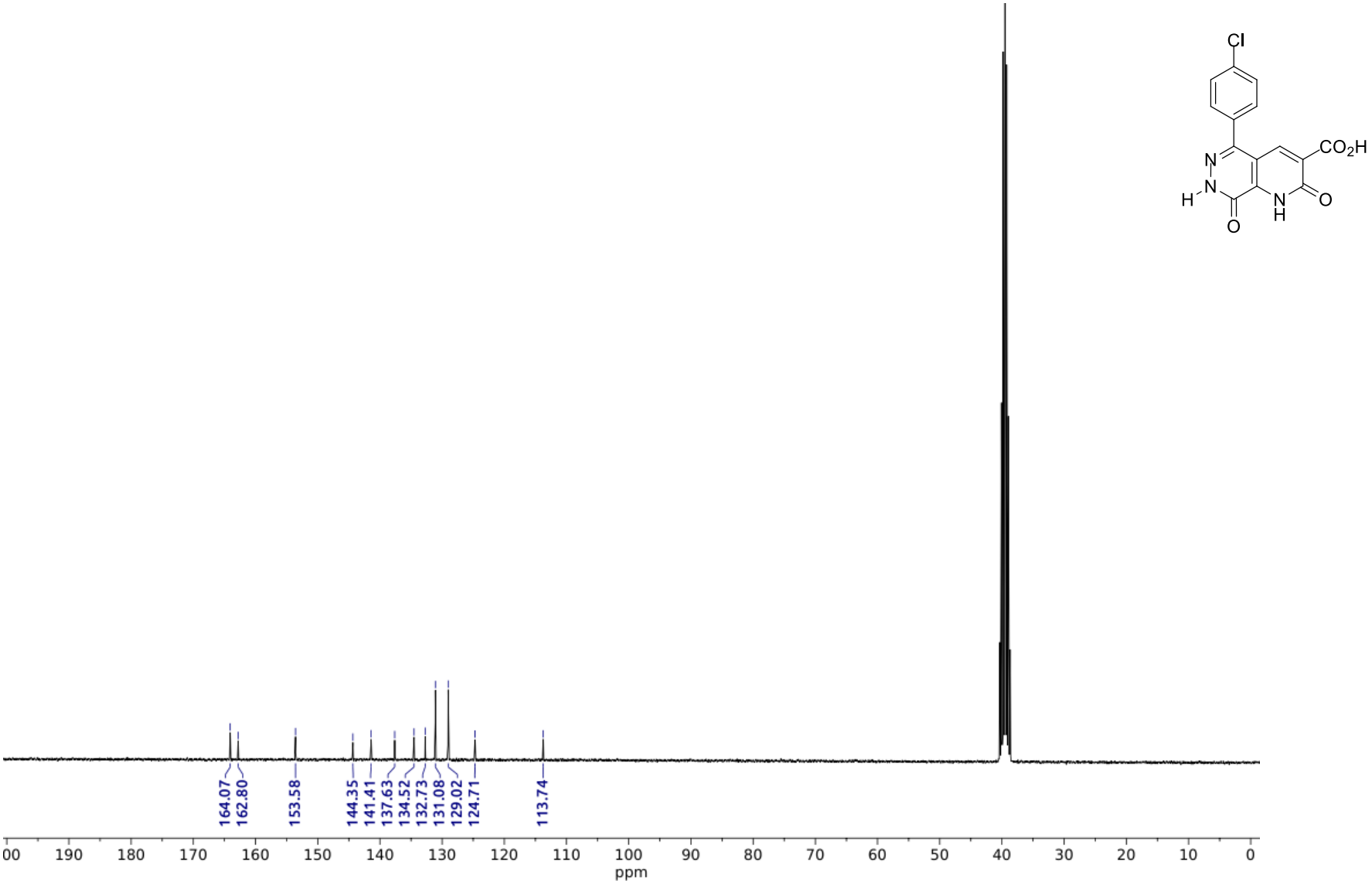

Figure S34 – ^{13}C NMR spectrum of compound **6c** in DMSO-d_6 at 75.46 MHz

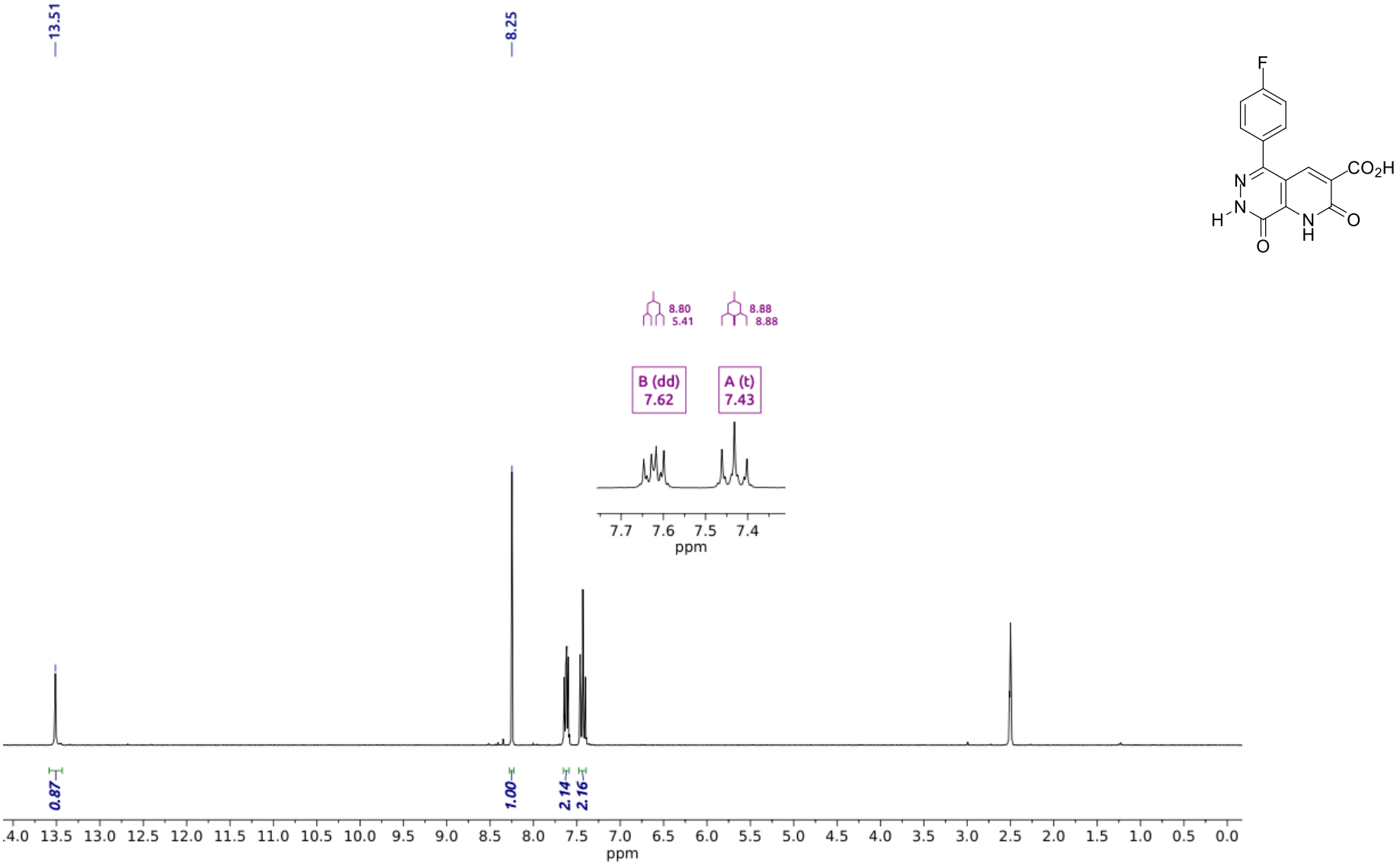

Figure S35 – ^{1}H NMR spectrum of compound **6d** in DMSO-d_6 at 300.06 MHz

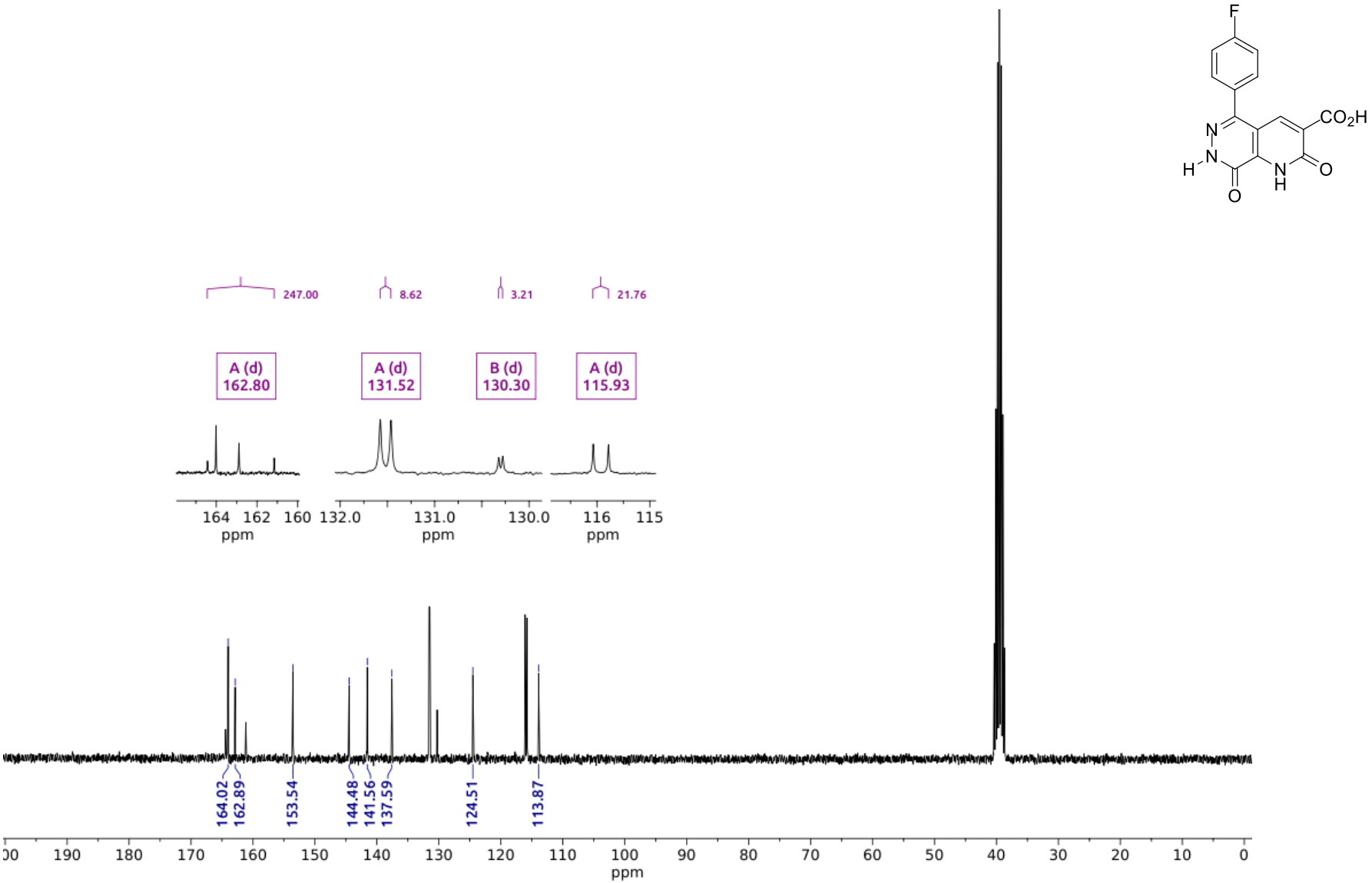

Figure S36 – ^{13}C NMR spectrum of compound **6d** in DMSO-d_6 at 75.46 MHz

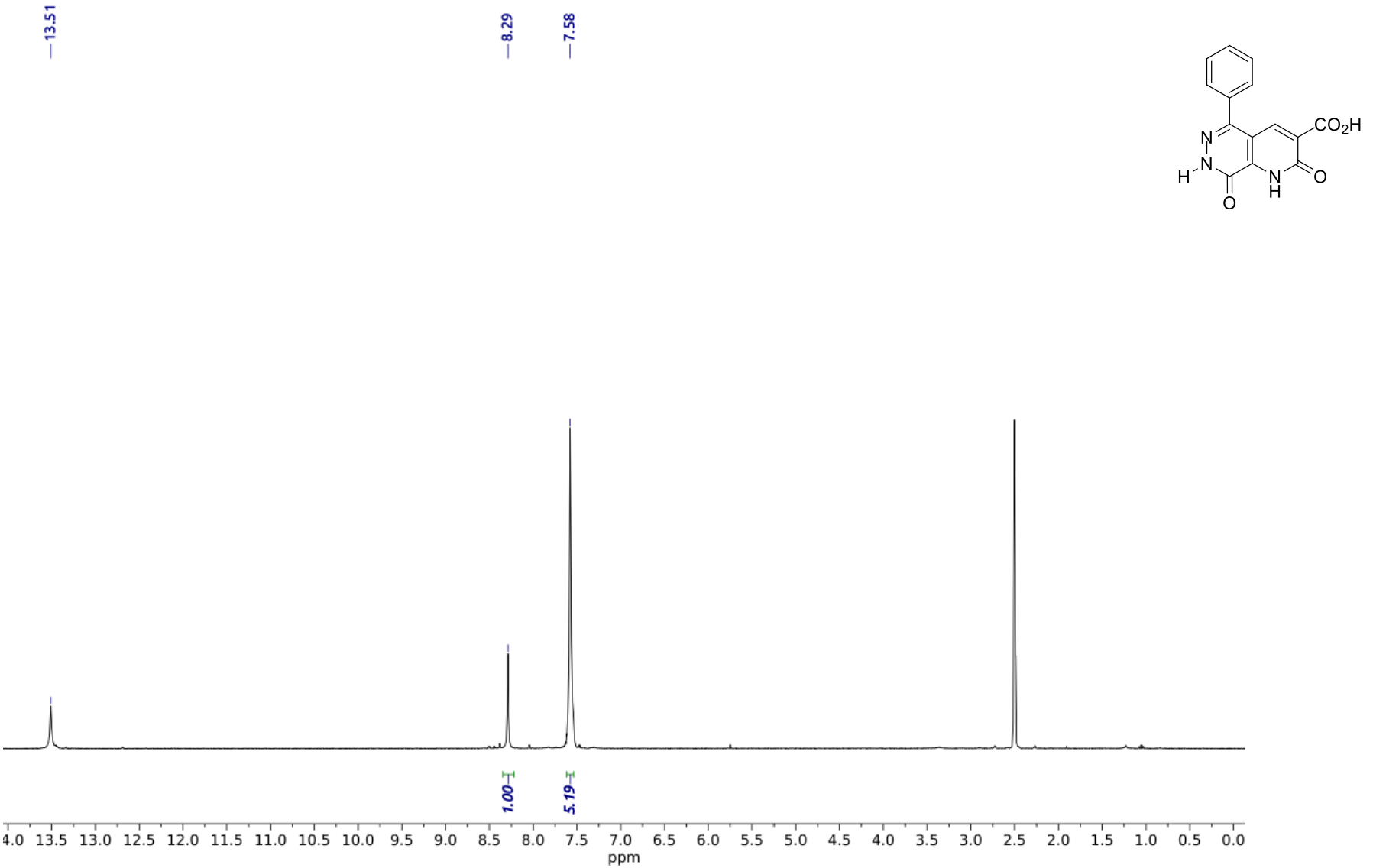

Figure S37 – ^{1}H NMR spectrum of compound **6e** in DMSO-d_6 at 300.06 MHz

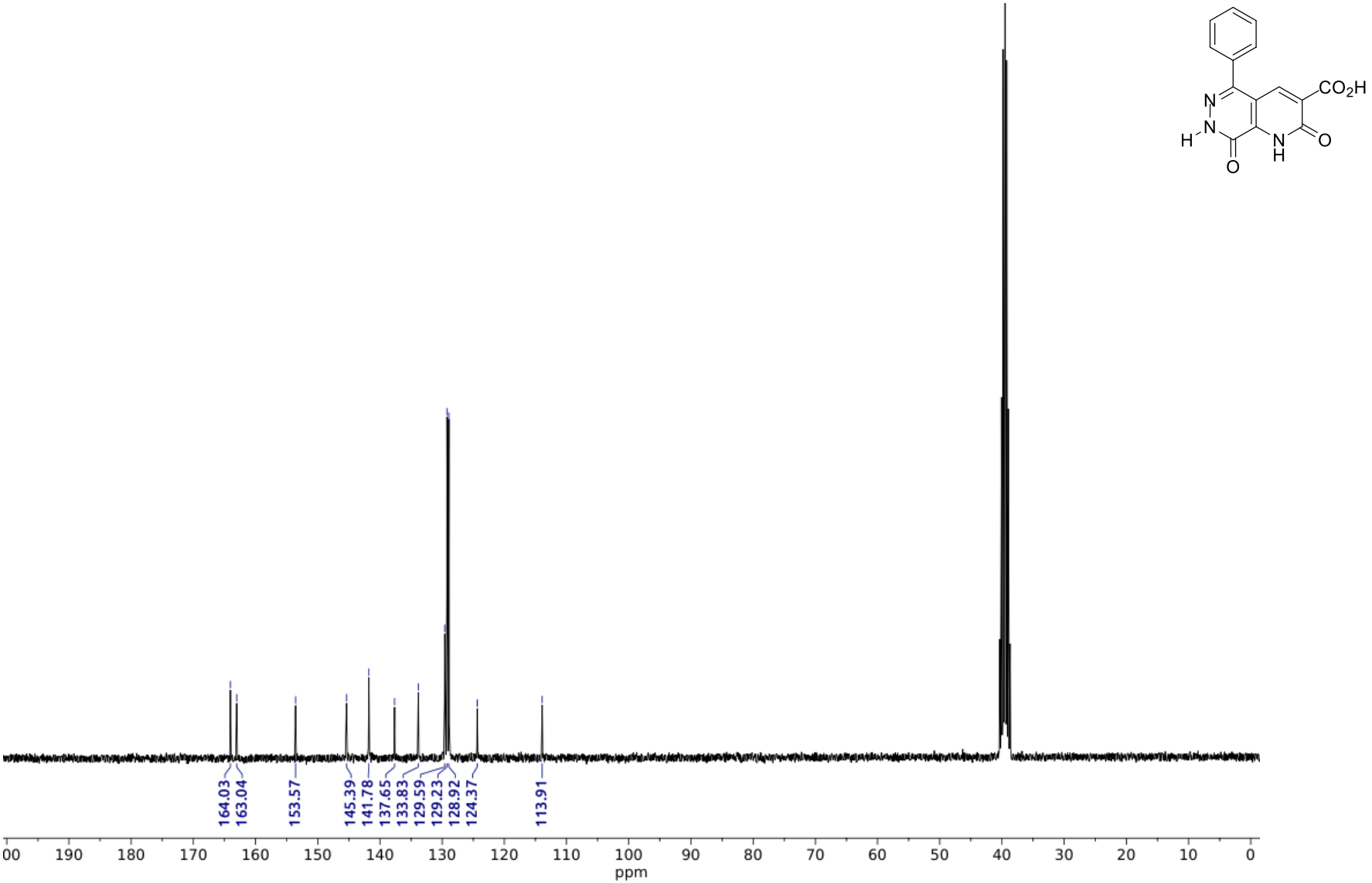

Figure S38 – ^{13}C NMR spectrum of compound **6e** in DMSO-d_6 at 75.46 MHz

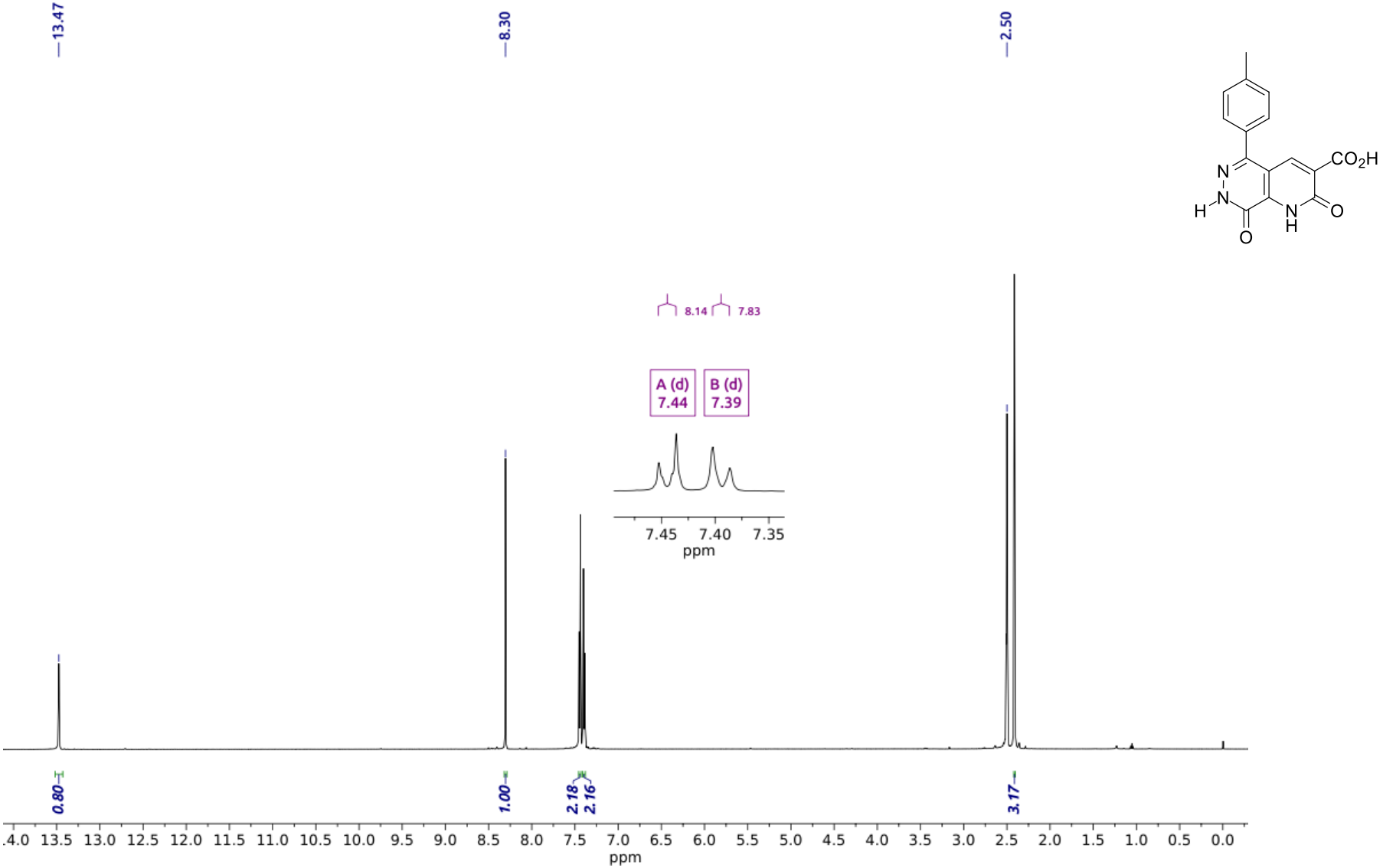

Figure S39 – ^{1}H NMR spectrum of compound **6f** in DMSO-d_6 at 500.13 MHz

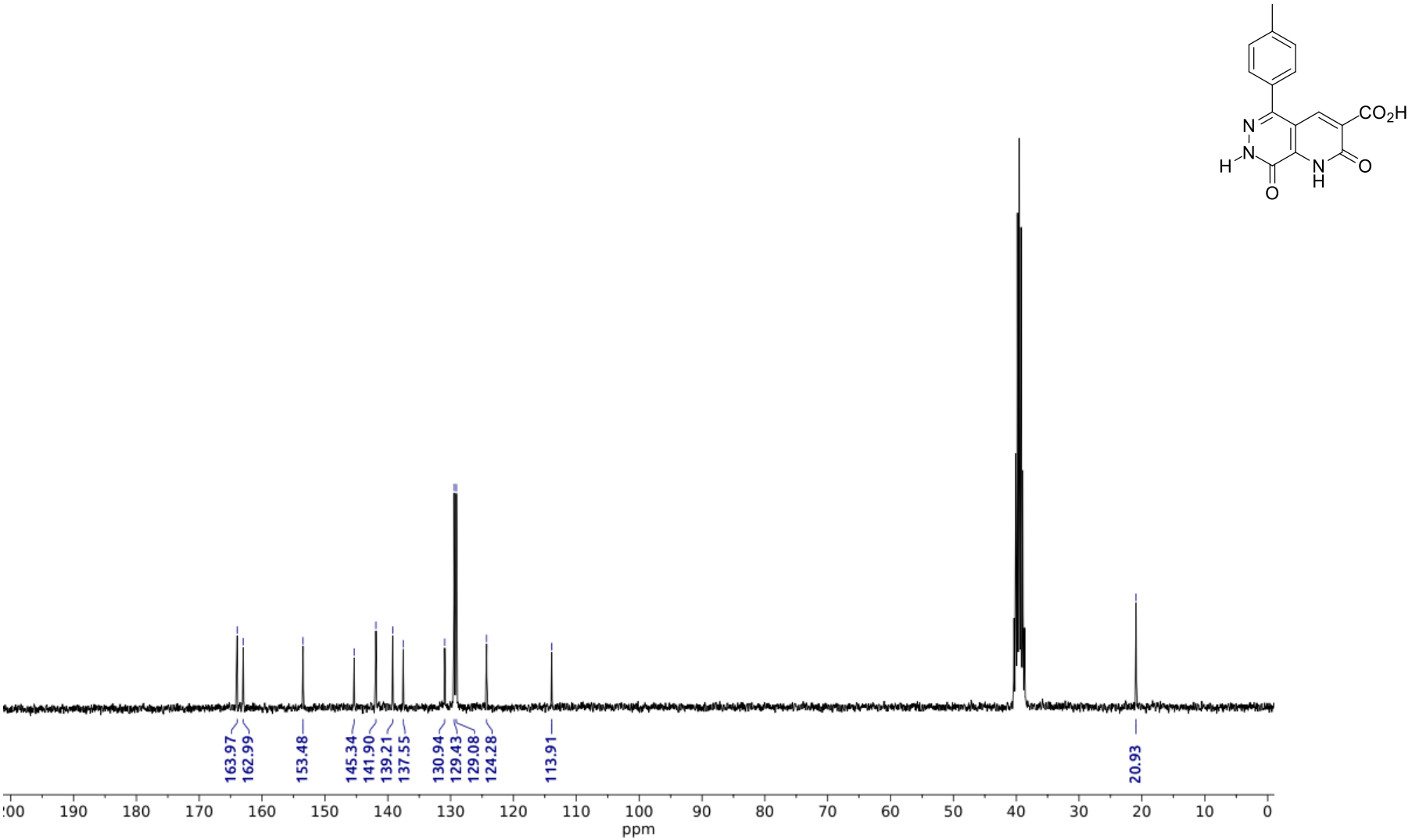

Figure S40 – ^{13}C NMR spectrum of compound **6f** in DMSO-d_6 at 125.77 MHz

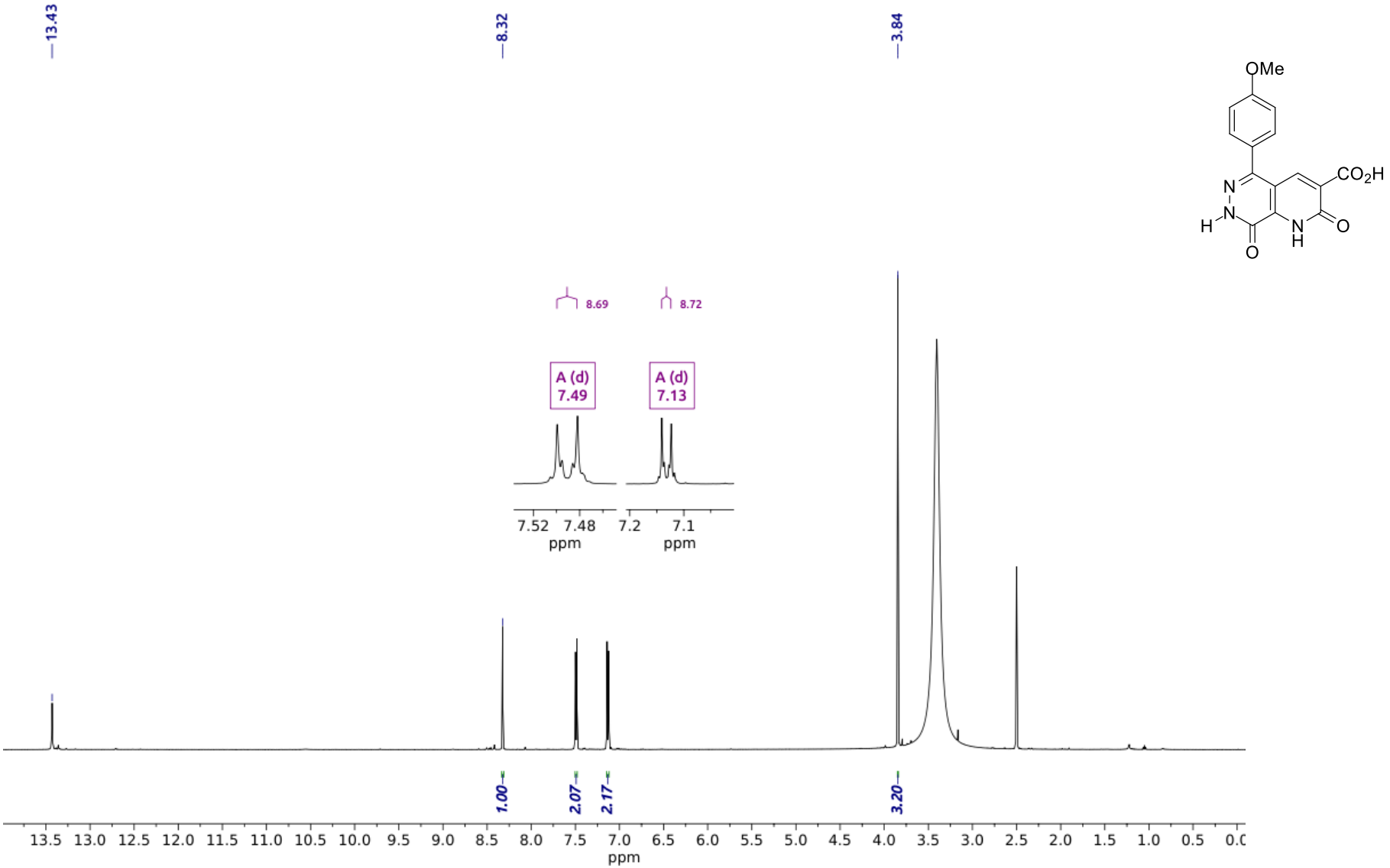

Figure S41 – ^{1}H NMR spectrum of compound **6g** in DMSO-d_6 at 500.13 MHz

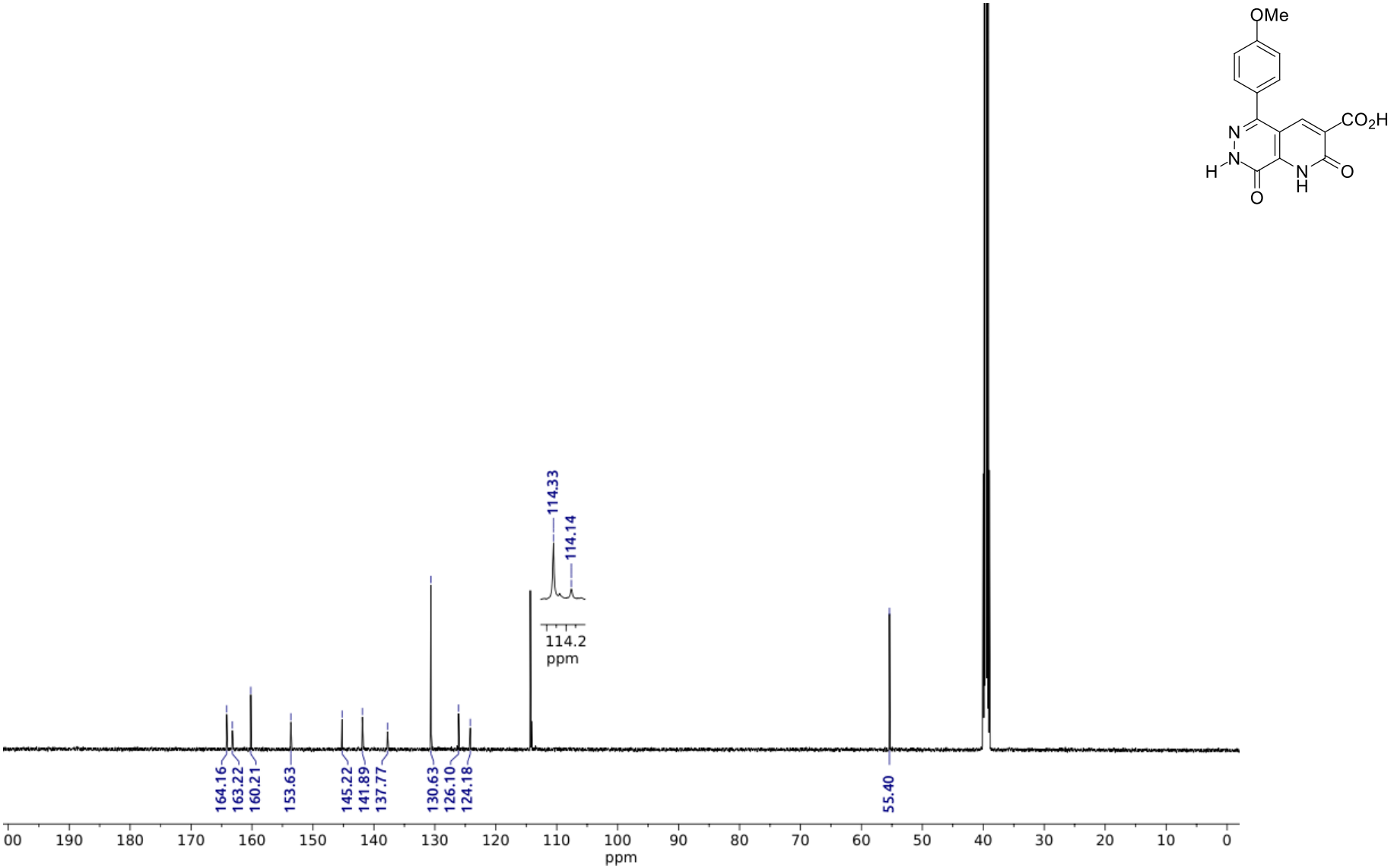

Figure S42 – ^{13}C NMR spectrum of compound **6g** in DMSO-d_6 at 125.77 MHz

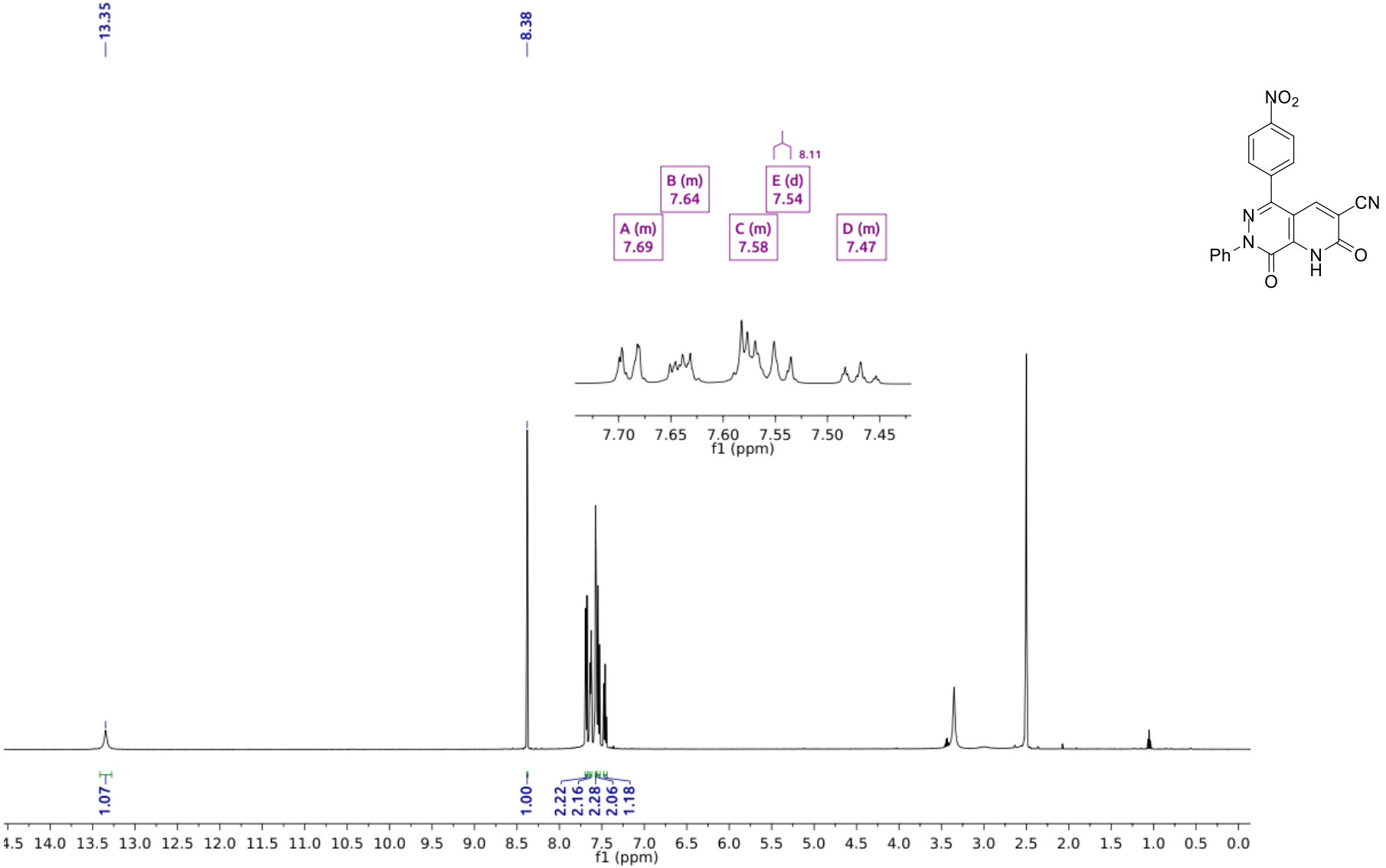

Figure S43 – ^{1}H NMR spectrum of compound **7a** in DMSO-d_6 at 500.13 MHz

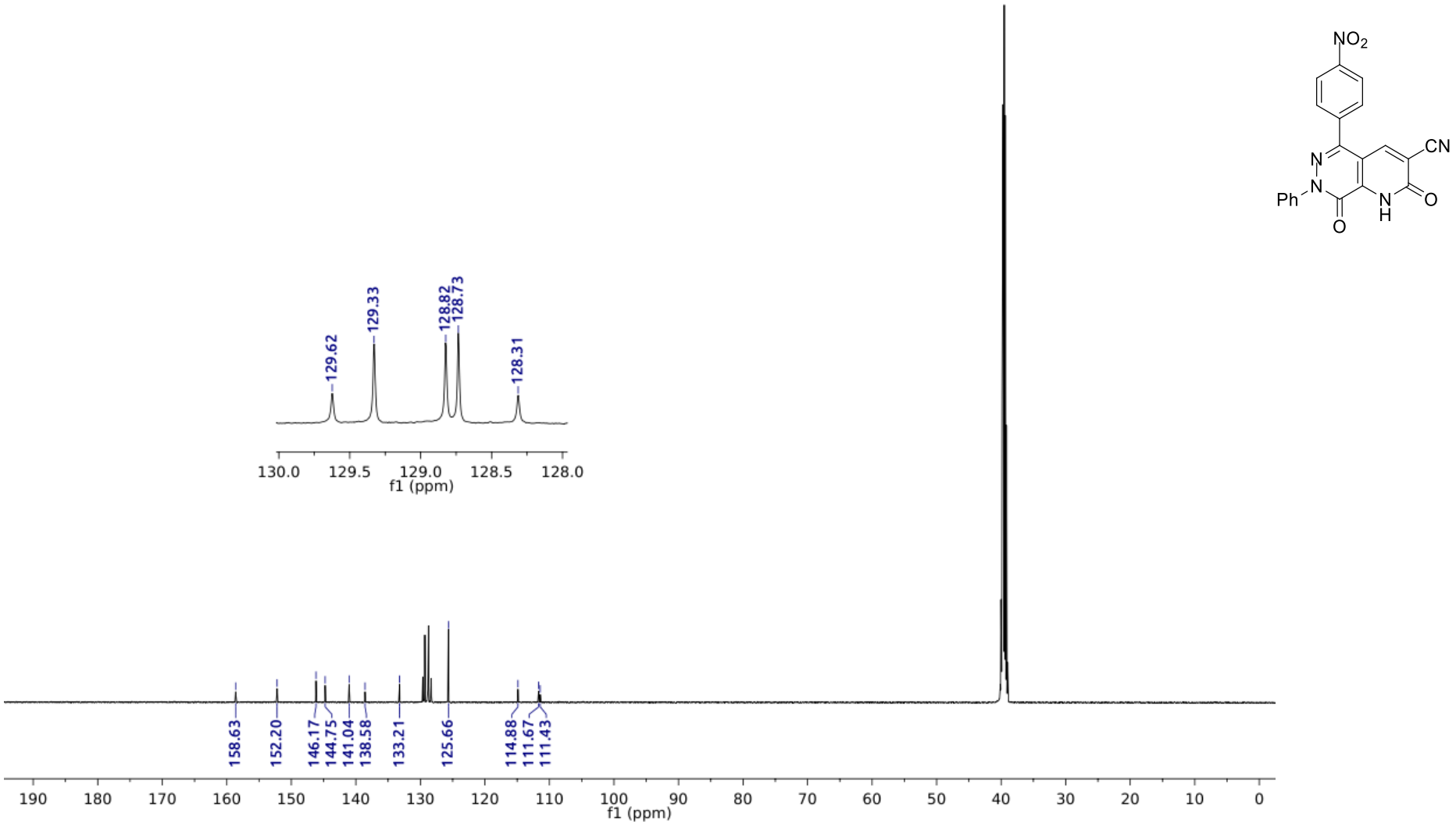

Figure S44 – ^{13}C NMR spectrum of compound **7a** in DMSO-d_6 at 125.77 MHz

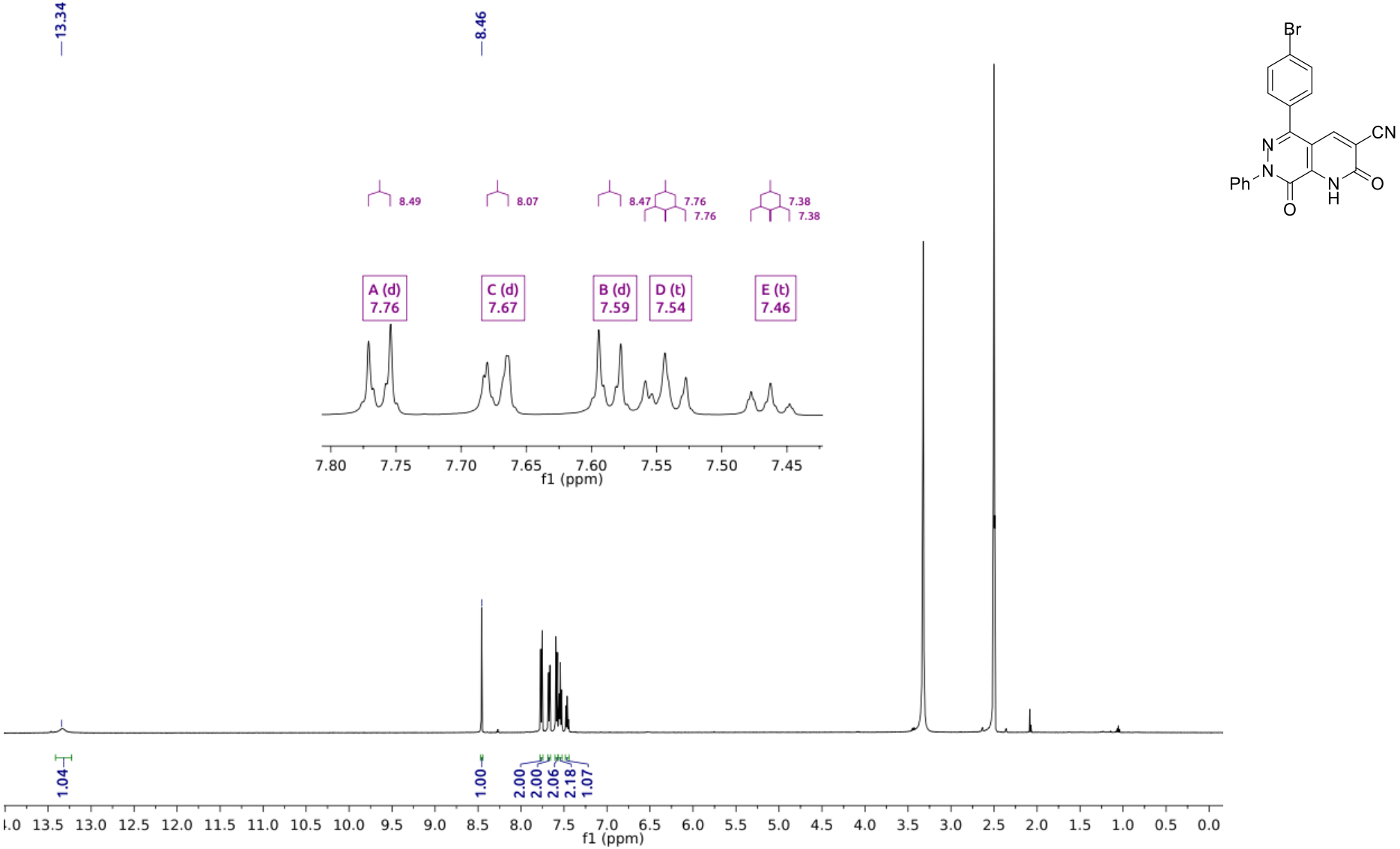

Figure S45 – ^{1}H NMR spectrum of compound **7b** in DMSO-d_6 at 500.13 MHz

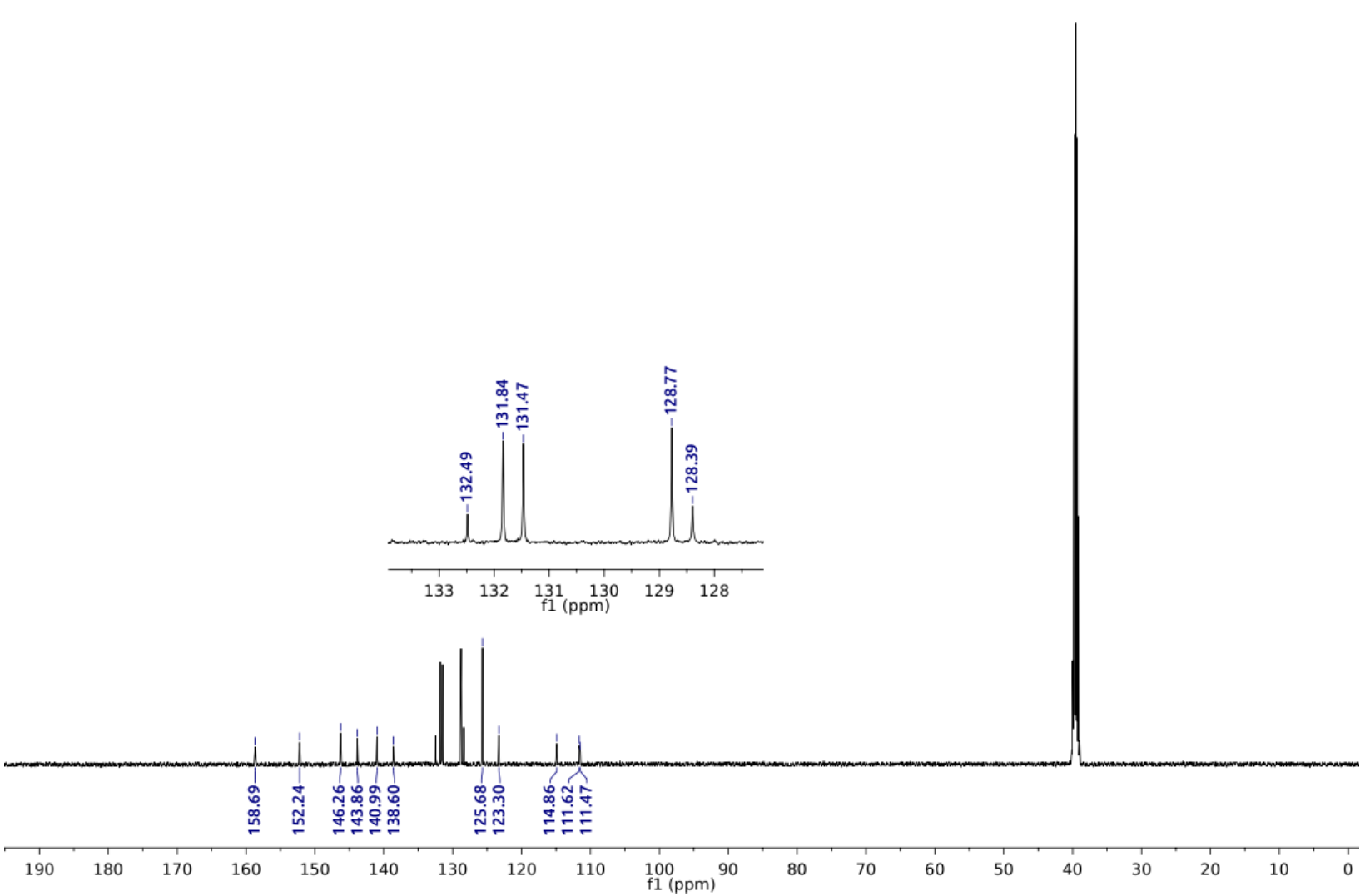

Figure S46 – ^{13}C NMR spectrum of compound **7b** in DMSO-d_6 at 125.77 MHz

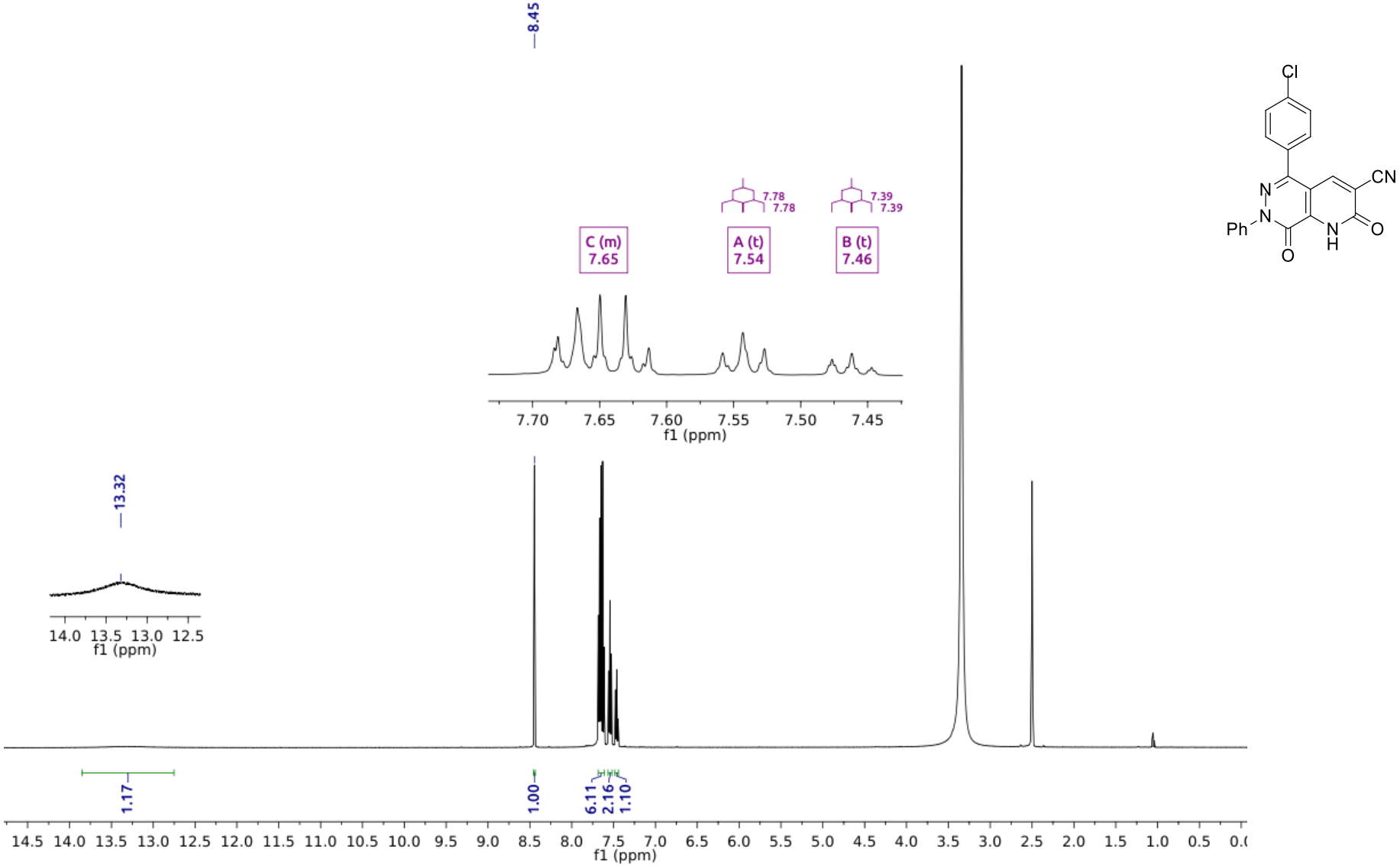

Figure S47 – ^{1}H NMR spectrum of compound **7c** in DMSO-d_6 at 500.13 MHz

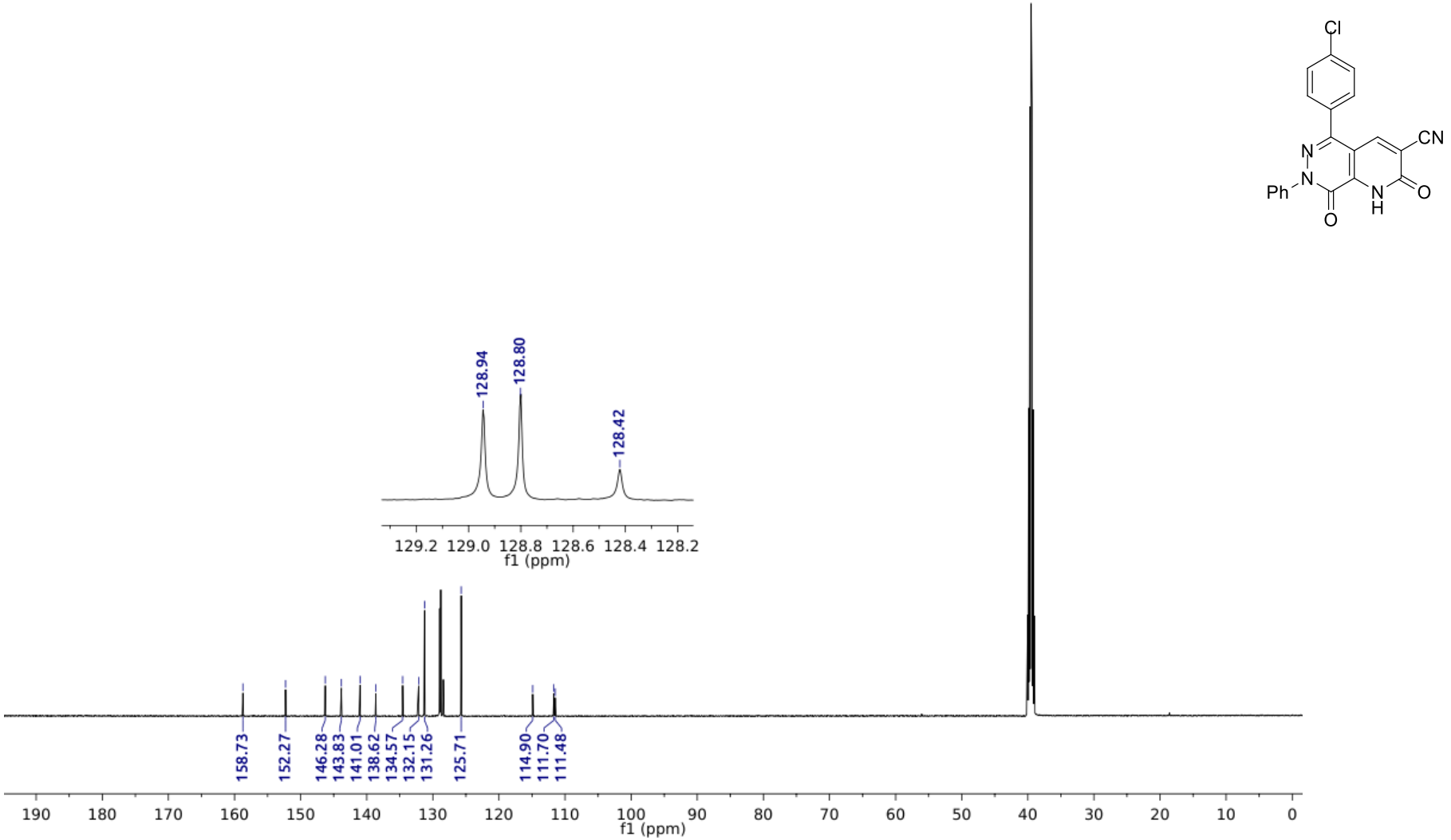

Figure S48 – ^{13}C NMR spectrum of compound **7c** in DMSO-d_6 at 125.77 MHz

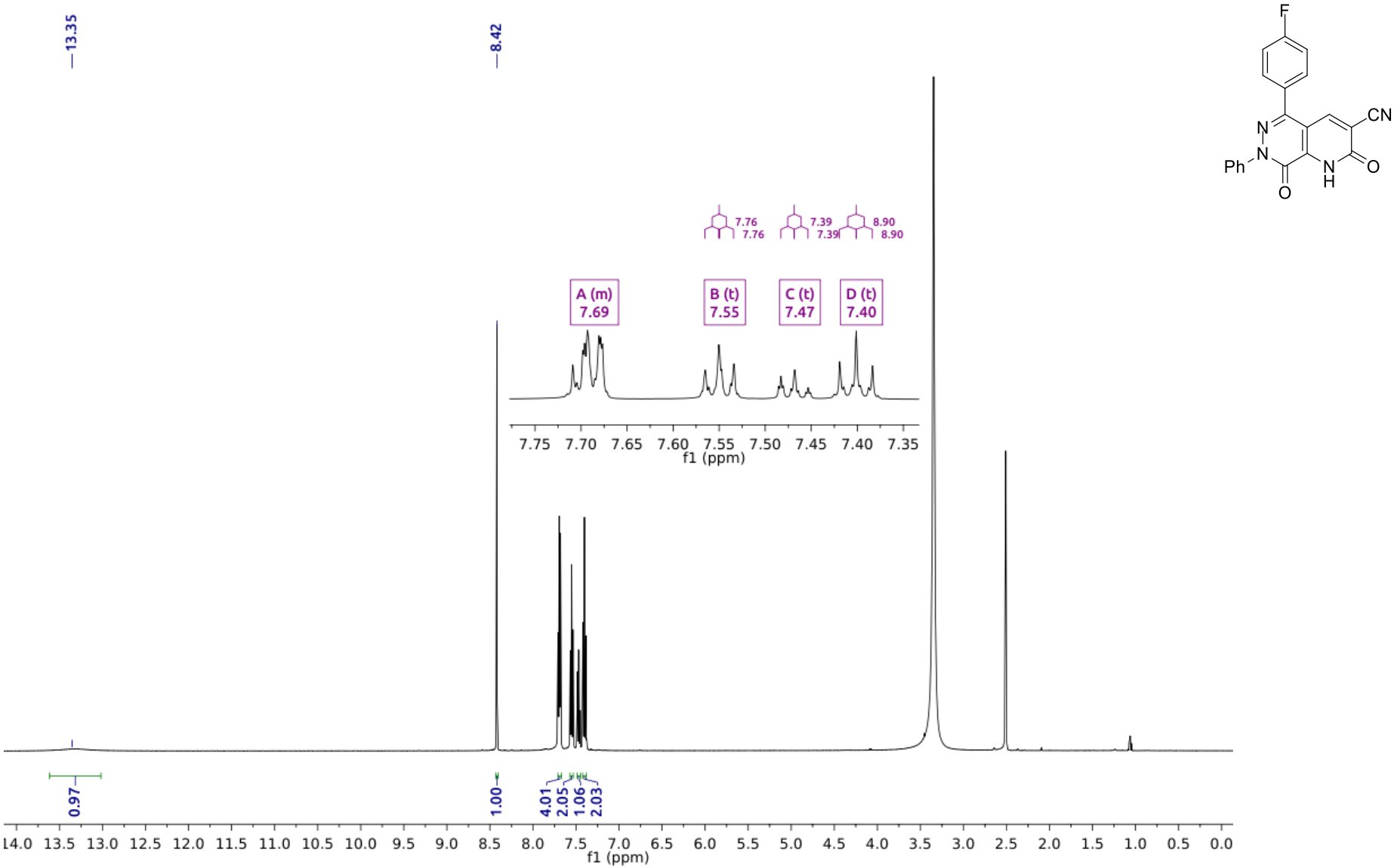

Figure S49 – ^{1}H NMR spectrum of compound **7d** in DMSO-d_6 at 500.13 MHz

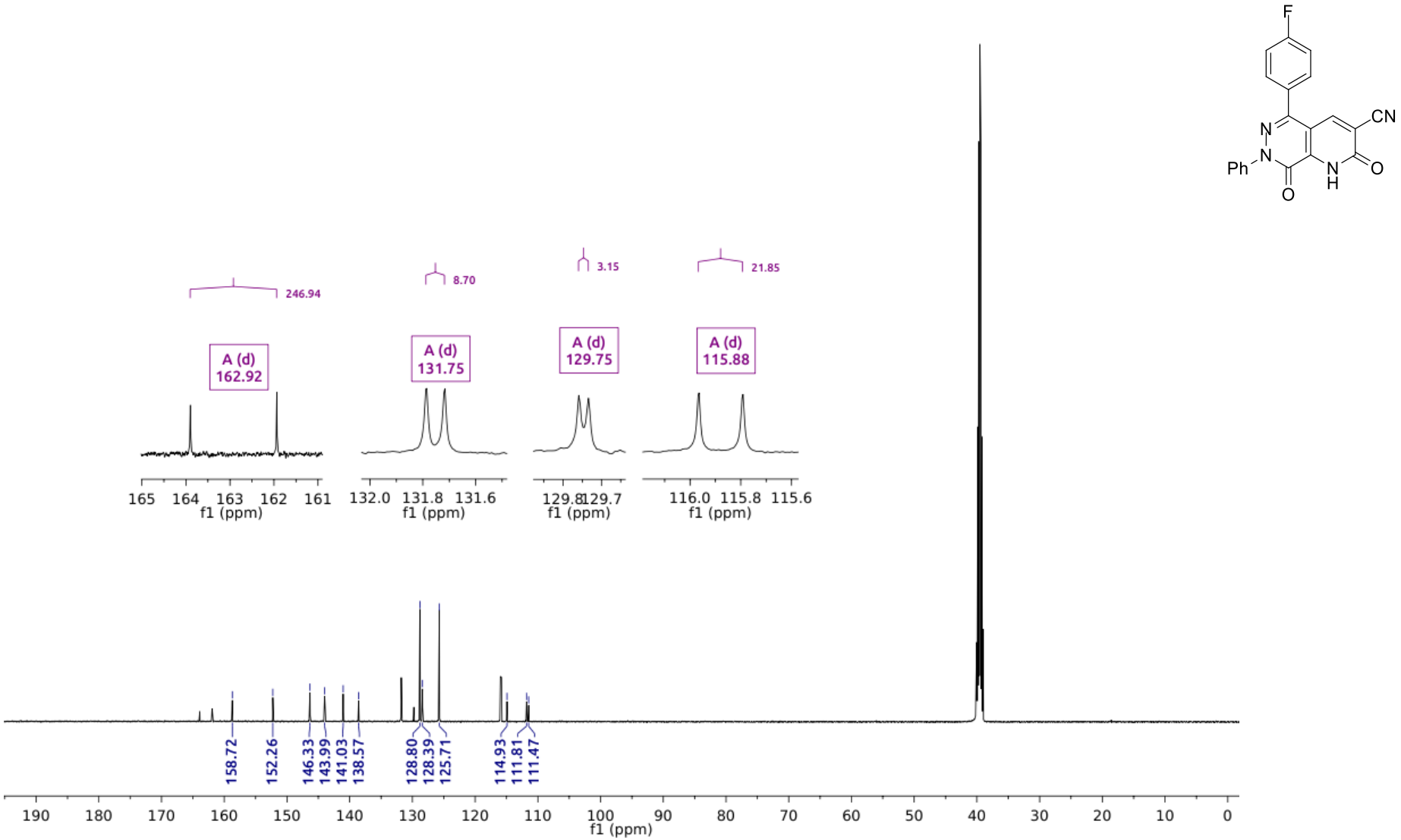

Figure S50 – ^{13}C NMR spectrum of compound **7d** in DMSO-d_6 at 125.77 MHz

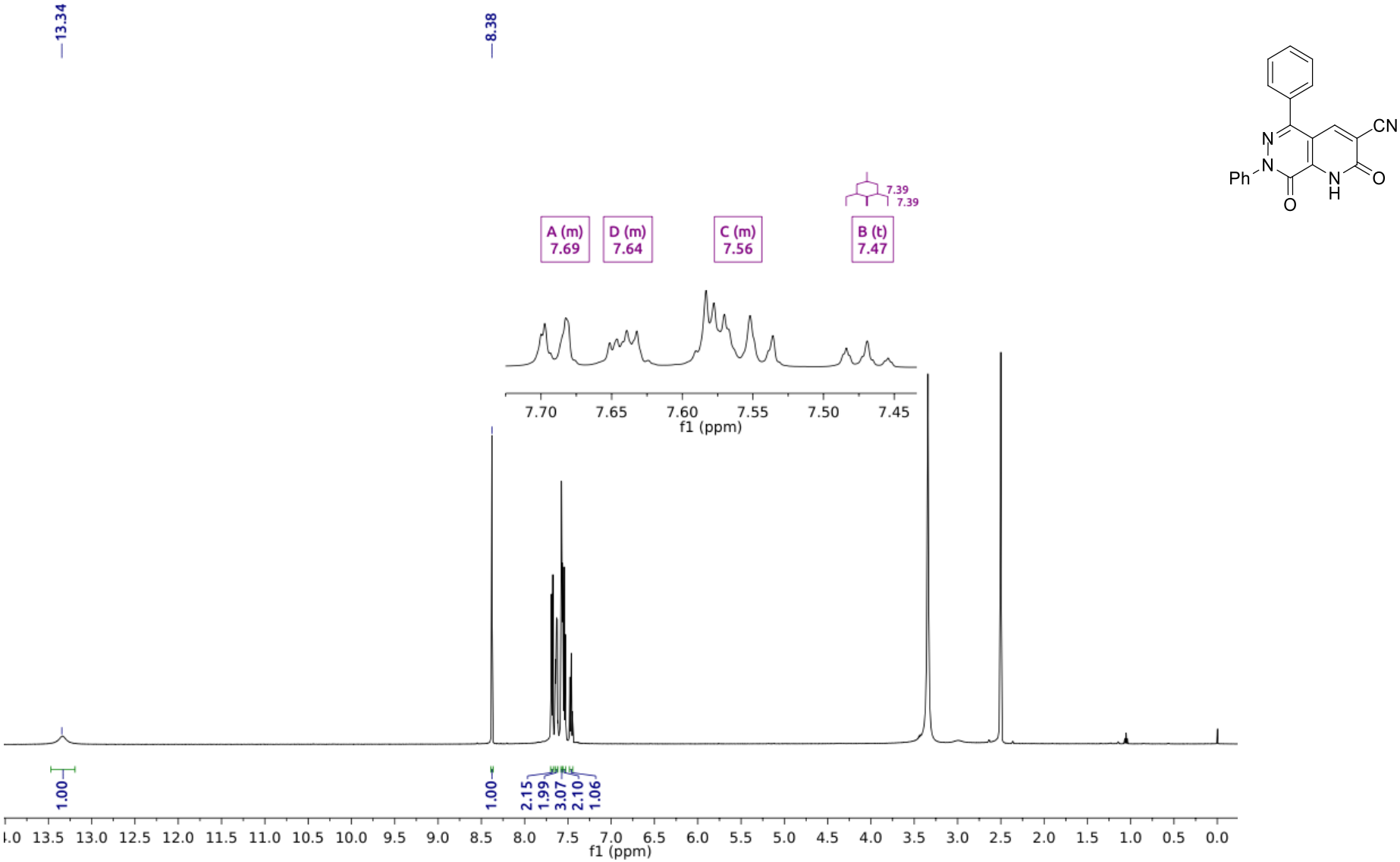

Figure S51 – ^{1}H NMR spectrum of compound **7e** in DMSO-d_6 at 500.13 MHz

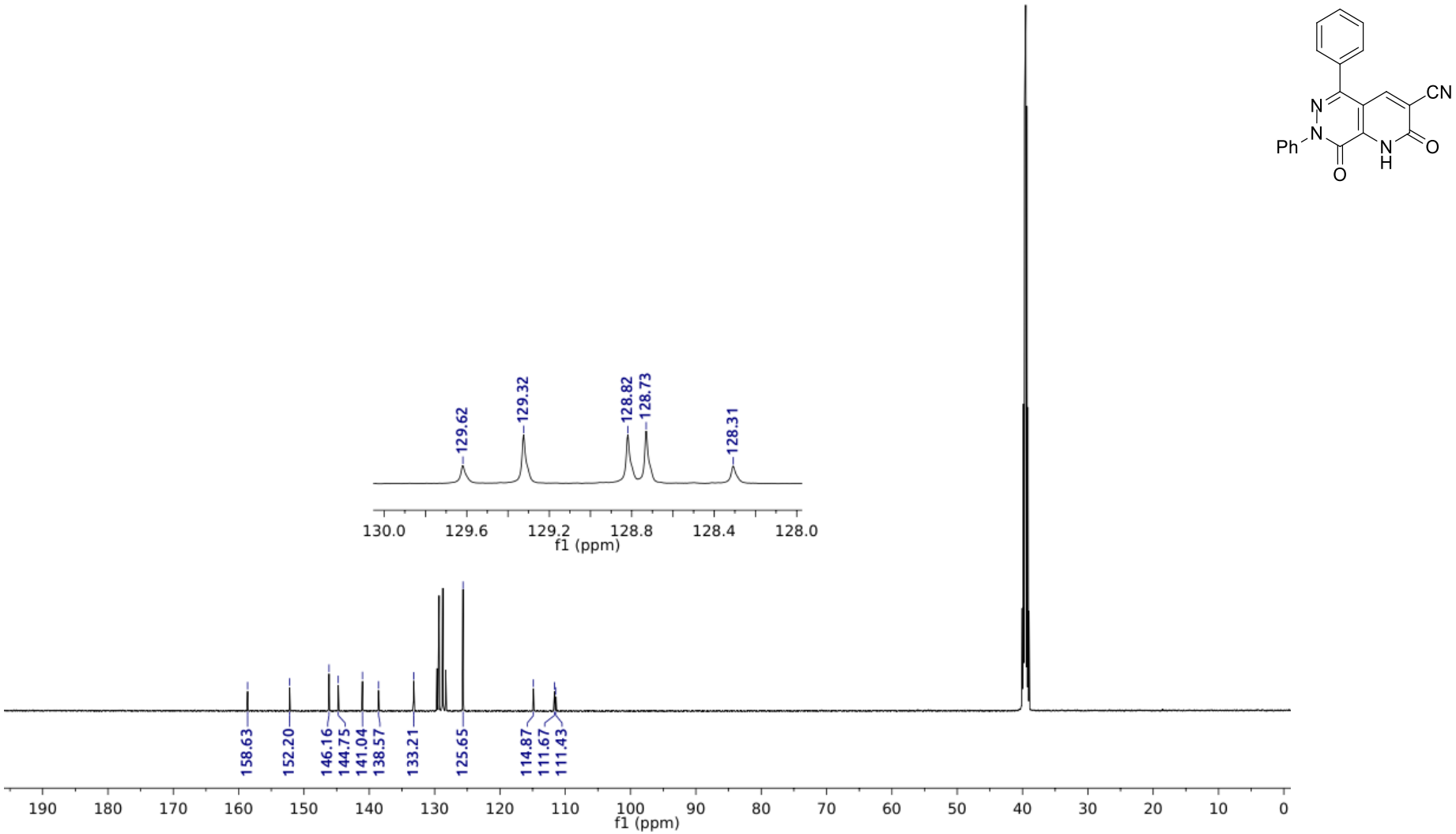

Figure S52 – ^{13}C NMR spectrum of compound **7e** in DMSO-d_6 at 125.77 MHz

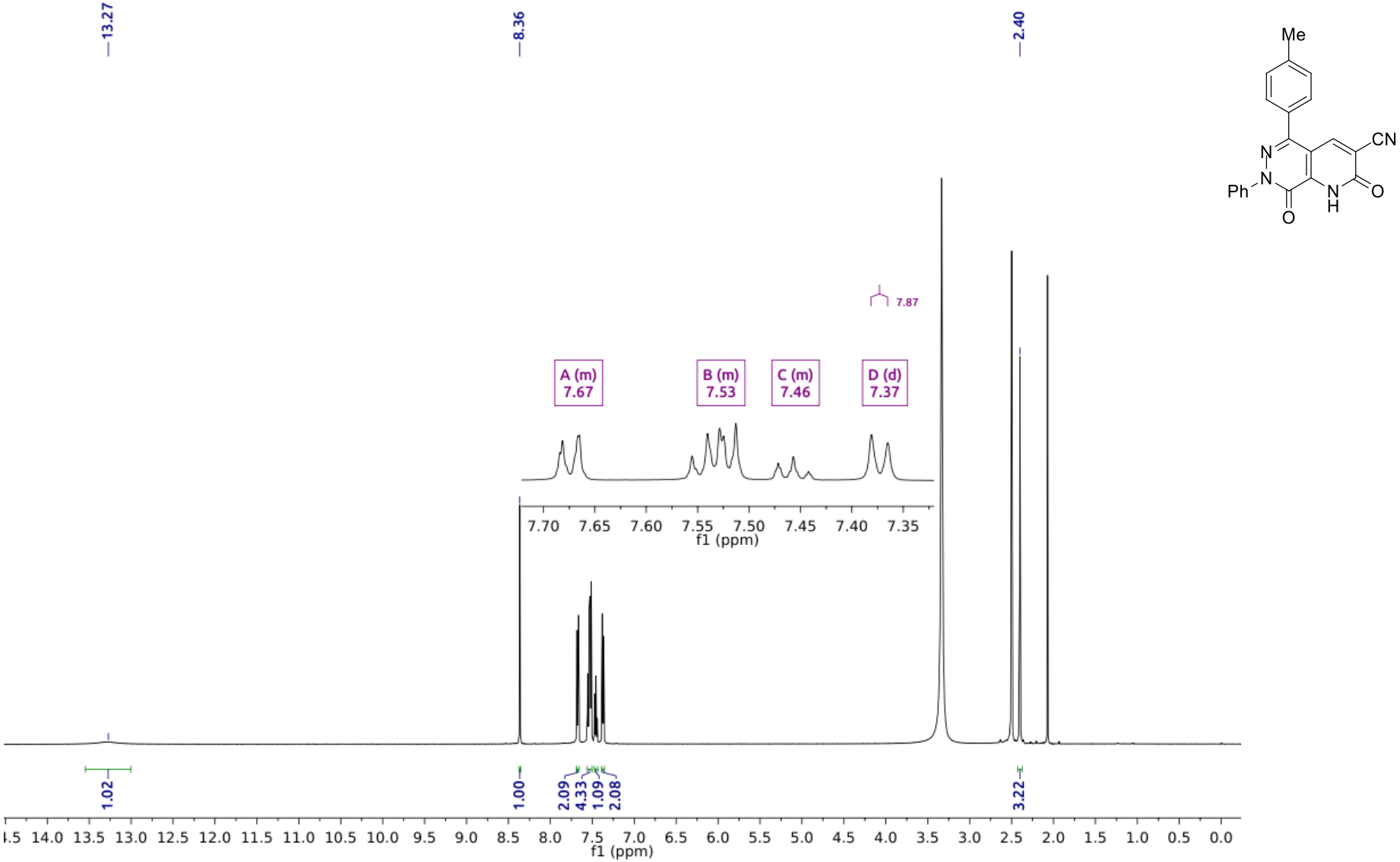

Figure S53 – ^{1}H NMR spectrum of compound **7f** in DMSO-d_6 at 500.13 MHz

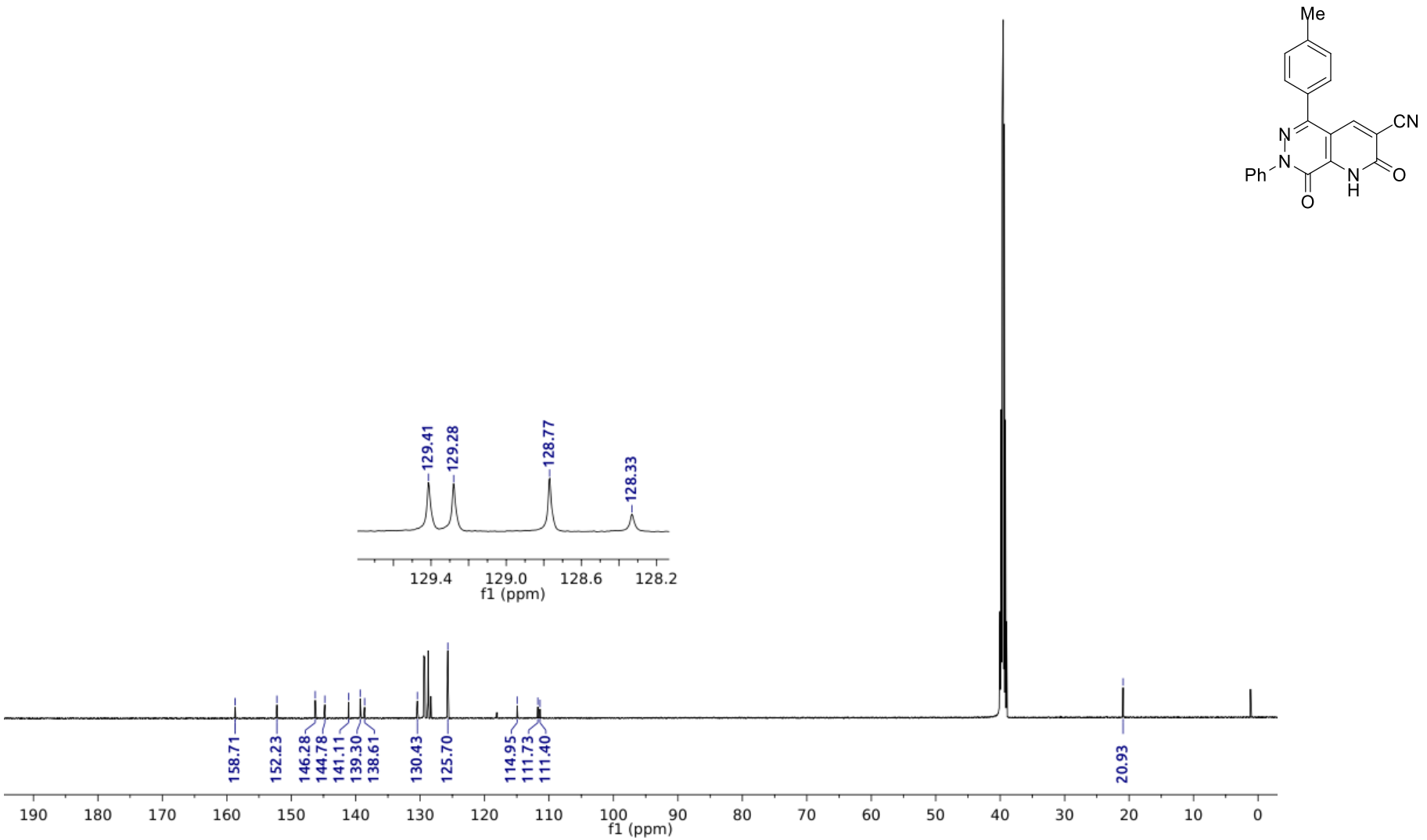

Figure S54 – ^{13}C NMR spectrum of compound **7f** in DMSO-d_6 at 125.77 MHz

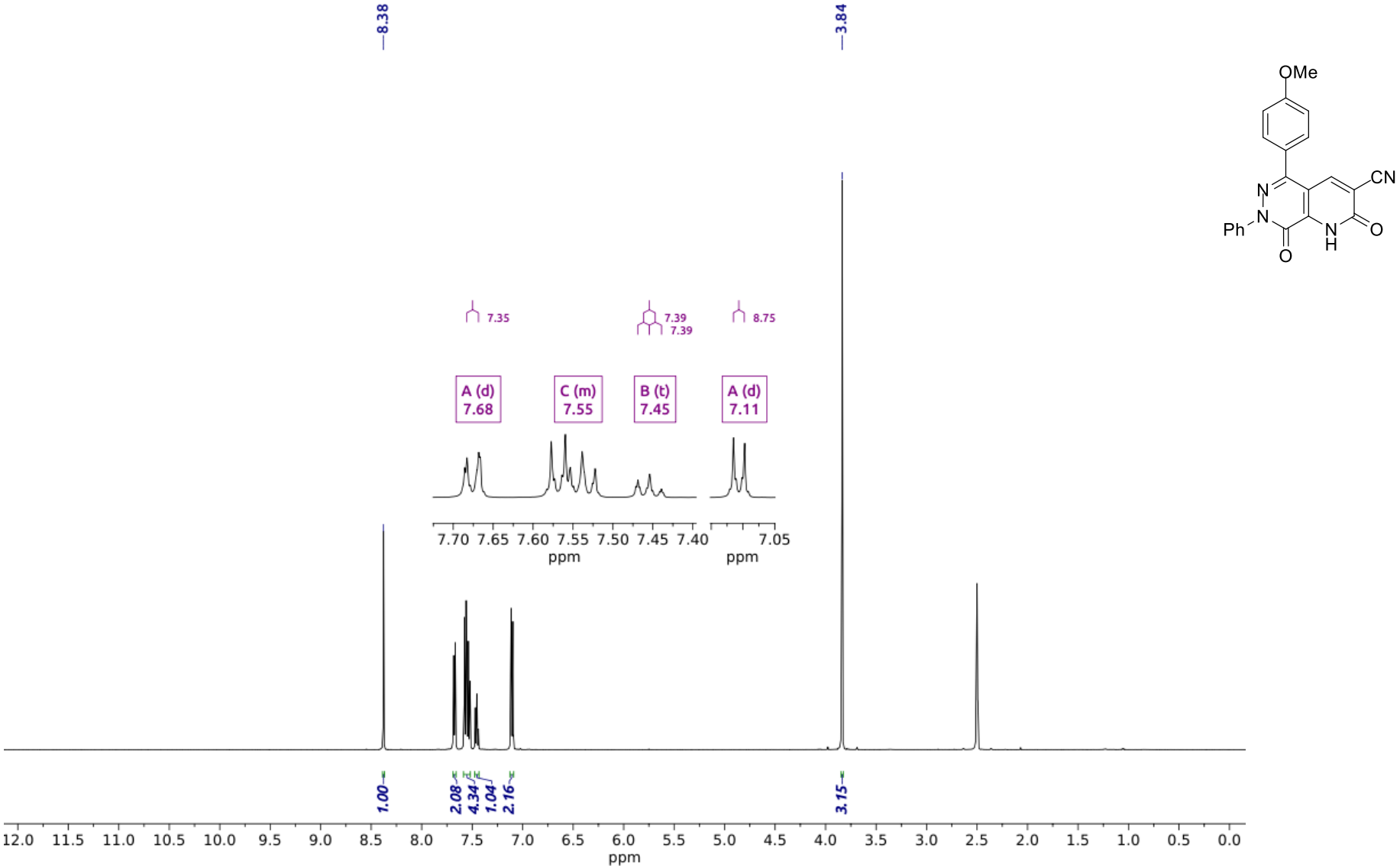

Figure S55 – ^{1}H NMR spectrum of compound **7g** in DMSO-d_6 at 500.13 MHz

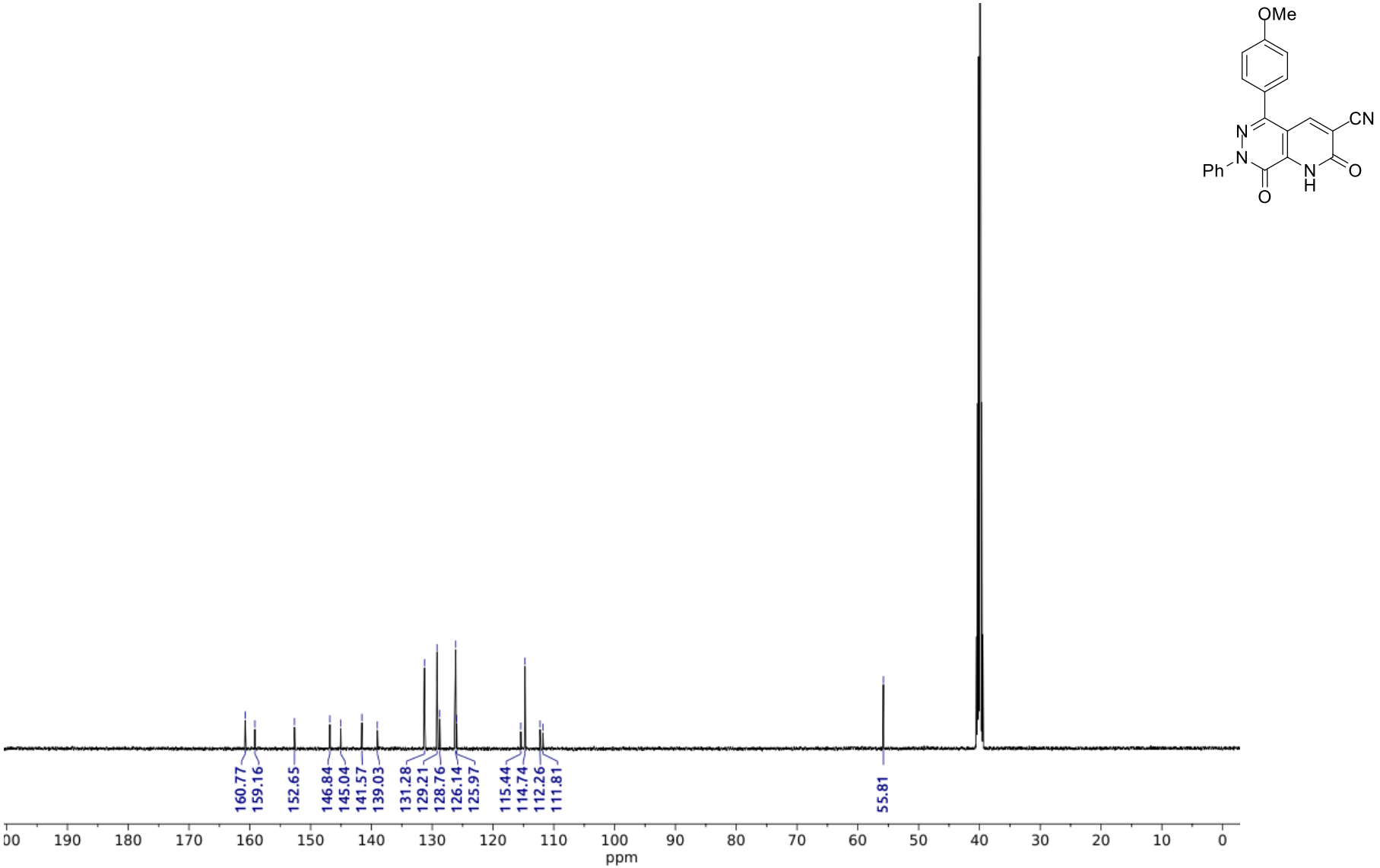

Figure S56 – ^{13}C NMR spectrum of compound **7g** in DMSO-d_6 at 125.77 MHz

Anti-inflammatory Activity

Mouse ear edema assay

Male Swiss mice weighing between 20 and 30 grams were used in this study. The animals were supplied by the Central Bioterio of State University of Maringá and were housed in the vivarium of the Laboratory of Inflammation at the same institution. They were maintained under controlled conditions, including a temperature of 22°C and had unrestricted access to water and food. The experimental protocol received approval from the Ethics Committee for Animal Experimentation at State University of Maringá (ECAE/UEM 4846281017).

Ear edema was induced by topically applying croton oil (CO, 200 µg per ear), previously diluted in a 70% acetone solution (vehicle), to the inner part of the left ear of the mice. In the right ear, only the vehicle was applied as a noninflamed control. After CO application, the animal groups (*n* = 7/group) received (*i*) 20 µl of 70% acetone (inflamed control), (*ii-iii*) 20 µL of the tested compounds at concentrations of 1.25 or 0.625 mg per ear, diluted in a 70% acetone solution (acetone/water 7:3 v/v - vehicle), or (*iv*) indomethacin (1 mg/ear, used as reference anti-inflammatory drug) diluted in 70% acetone on the left ear. Higher doses of the tested compounds were not tested due to the limited solubility. After 6 hours, the animals (*n* total = 49) were anesthetized, sacrificed, and their ears were sectioned into 6.0 mm diameter disks, which were then weighed (mg). Equation 1 was used to calculate the percentage of edema inhibition.

Equation 1.

$$inhibition\ (\%) = \frac{left\ ear\ weight_{inflamed\ control} - left\ ear\ weight_{treated}}{left\ ear\ weight_{inflamed\ control} - right\ ear\ weight_{\ non-inflamed\ control}} x\ 100$$

The data were subject to GraphPad Prism software (version 5.0) and statistically analyzed using analysis of variance (ANOVA) followed by Tukey's test. The results for ear edema values were presented as the mean ± standard error of the mean (SEM). A significance level of $p < 0.05$ was considered statistically significant.

Cytotoxicity assay

For cytotoxicity testing in fibroblasts (ATCC® CCL-1, Manassas, USA), cells were prepared at a concentration of 2.5×10^5 cells/mL in DMEM medium supplemented with 10% FBS. They were added to 96-well plates, incubated at 37 °C in a 5% CO_2 atmosphere for 24 hours to achieve confluence. After incubations, cells were then treated or not with different compound concentrations (ranging from 100 to 1000 µM) diluted in DMEM for 72 hours. For cytotoxicity

testing in macrophages (TIB-67; American Type Culture Collection, Manassas, VA, USA), cells were prepared at a concentration of 5×10^5 cells/mL in RPMI medium supplemented with 10% FBS. They were added to 96-well plates, incubated at 37 °C in a 5% CO_2 atmosphere for 24 hours to achieve confluence. After incubation, cells were treated or not with different compound concentrations (ranging from 100 to 1000 µM) diluted in RPMI for 48 hours. After treatment, the medium was removed, and cells were incubated with MTT (2 mg/mL) for 4 hours. Then, DMSO was added for solubilization of the formazan, and the absorbance was analyzed using a microplate reader (BIO-TEK Power WaveXS spectrophotometer) at 492 nm. The percentage of viable cells was calculated in relation to the control to determine the cytotoxic concentration that affects 50% of the cells (CC_{50}).

In vitro COX-1/COX-2 inhibition assay

The compound **7c** was assessed for its ability to inhibit human recombinant cyclooxygenase-2 (COX-2) and ovine cyclooxygenase-1 (COX-1) using a cyclooxygenase inhibitor screening assay kit (catalogue 560131, Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's suggested procedure. The absorbance of the 96-well plate was measured at 405 nm using a microplate reader (Asys Expert Plus, Biochrom, Berlin, Germany). The percentage of inhibition of the compound at two different concentrations (1.95 and 31.25 µM) was determined by identifying the %B/B0 (% Bound/Maximum Bound) on the standard curve ($y = -12.24\ln(x) + 114.88$; $R^2 = 0.9957$) and reading the corresponding values.

Docking Molecular

The crystal structure of human COX-2 bounded to rofecoxib encoded by PDB ID: 5KIR[3] and the human COX-1[4] bounded to Indomethacin-(R)-alpha-ethyl-ethanolamide with PDB ID: 2OYE were downloaded from Protein Data Bank (PDB), before to perform the docking studies.

Docking protocol into the COX-2 active site was validated by redocking the rofecoxib co-crystallized and the docked pose was compared with the initial pose using root mean square deviation (RMSD), which resulted in almost the same position of rofecoxib co-crystallized (RMSD = 0.9148 Å). For the COX-1, the docking protocol was also validated by redocking the indomethacin-(*R*)-alpha-ethyl-ethanolamide co-crystallized into the COX-1 active site. The docking pose was almost at the same position of crystallized ligand docked (RMSD = 0.8581 Å).

The chemical structure **7c** was drawn and submitted to geometry optimization followed by conformational analysis, by the method of systematic search with torsion angle increment set of 30° in the range 0-360° using DFT B3LYP/6-311G* basis in the gas phase. All calculations were

performed using Spartan'08 for Windows software. The lowest energy conformer for the chemical structure was saved in mol2 file before to use in docking studies.

Molecular docking studies were performed using iGemdock 2.159 and Gold software in which the individual binding pose of **7c** was assessed and submitted to dock in the active site of the COX-2 (PDB: 5KIR) and COX-1 (PDB: 2OYE). iGemdock docking calculations were performed at drug screening Docking Accuracy Setting with GA parameters set for population size, generation and number of solutions as 200, 70, and 3, respectively, and Gemdock score function of hydrophobic and electrostatic (1:1 preference). iGemdock software was used to infer the biological interactions, such as hydrogen bonding, van der Waals, and electrostatic, between biological receptor and the compound studied. GOLD molecular docking software used a genetic algorithm to perform an automatic search with the efficiency of 100% and a range of 100 to 12,500 operations. This software was applied to calculate the 100 possible conformations of the compounds which may bind to the active site of the protein. Default parameters such as population size of 100, selection pressure (1.1), number of islands of 1, niche size 2, operator weights for migrating 0, mutate, and crossover 100, were also applied. GOLD scoring functions used were the GoldScore and rescore with the ChemScore function.

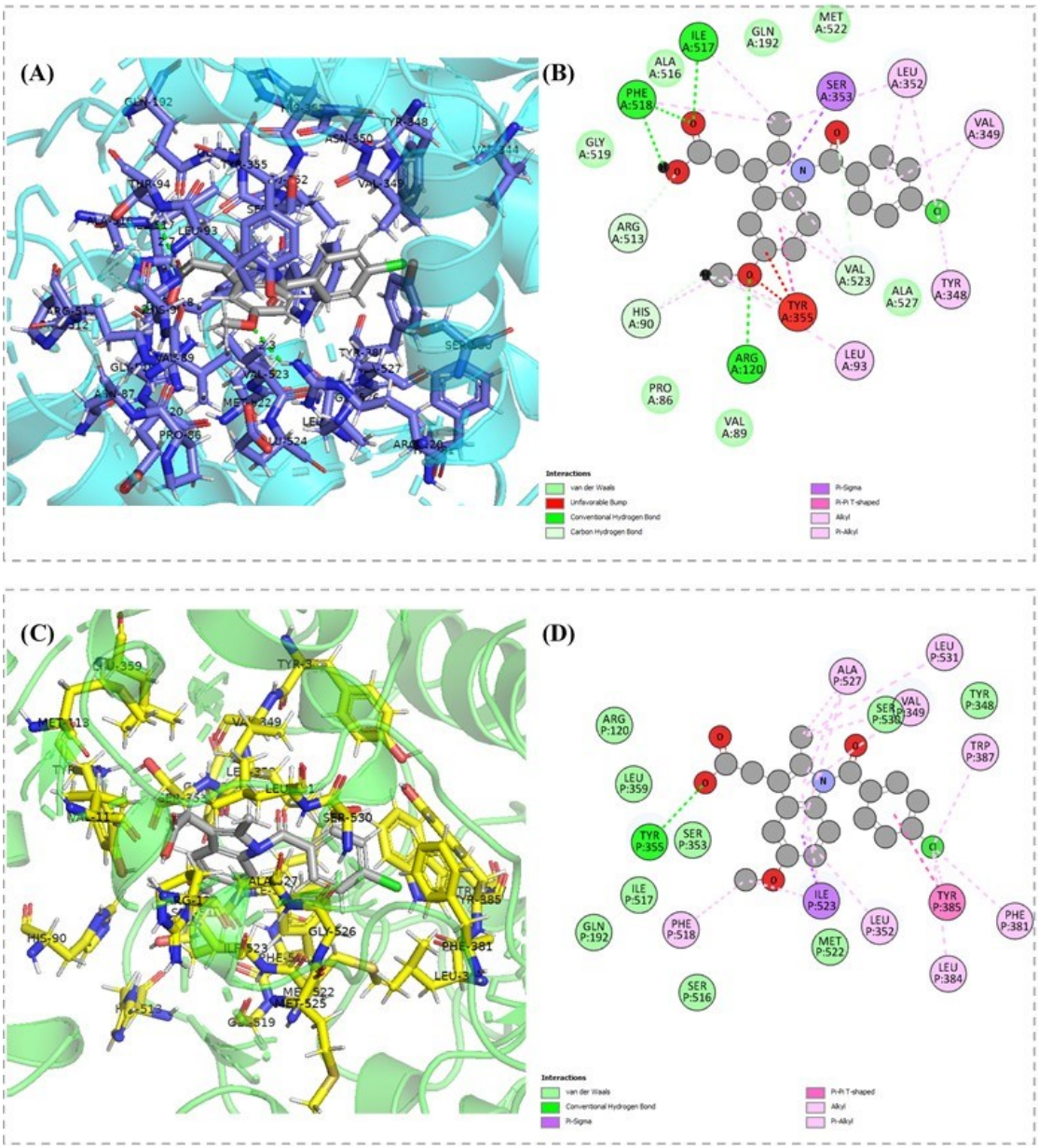

Figure S57. (A) 3D docking diagram of Indomethacin (Gray sticks models) and COX-2 residues (Blue sticks models). (B) 2D mode of interaction of the compound Indomethacin into COX-2 analysed by Discovery Studio Client v20.1.0. (C) 3D docking diagram of Indomethacin (Gray sticks models) and COX-1 residues (Yellow sticks models). (D) 2D mode of interaction of the compound Indomethacin into COX-1 analysed by Discovery Studio Client v20.1.0.

Table S1 Docking results of indomethacin into COX-1 (PDB: 2OYE)[a]

Pocket	Amino acid	Interaction	Distance (Å)
Proximal binding	Val349	Pi-alkyl with indole ring; Alkyl with methyl	
	Tyr355	H-bond with O-H of the carboxyl group	2.61
	Ile523	Pi-sigma with indole ring; Alkyl with methoxy group	
	Ala527	Pi-alkyl with indole ring; Alkyl with methyl	
Central binding	Leu352	Pi-alkyl with indole ring	
	Leu384	Alkyl with Cl atom	
	Tyr385	Pi-Pi T-shaped with 4-chlorophenyl ring	
	Trp387	Pi with Cl atom	
	Phe518	Pi-alkyl with methoxy group	
	Phe381	Pi with Cl atom	
	Leu531	Alkyl with methyl	

[a] Binding score -61.84 Kcal/mol.

Table S2 Docking results of indomethacin into COX-2 (PDB: 5KIR)[a]

Pocket	Amino acid	Interaction	Distance (Å)
Proximal binding	Arg120	H-bond with O of methoxy group	2.33
	Val349	Pi-alkyl and alkyl with 4-chlorophenyl ring	
	Ser353	Pi-sigma with indole ring	
	Tyr355	Unfavorable interaction with indole ring	
	Val523	CH-bond with C(O) of benzoyl group	
Central binding	Leu352	Pi-alkyl and alkyl with 4-chlorophenyl ring	
	Phe518	H-bond with OH and C(O) of carboxyl group	2.08
	Tyr348	Pi with Cl atom	
	Ile517	H-bond with C(O) of carboxyl group	2.73
COX-2 side	Arg513	CH-bond with OH of carboxyl group	
	His90	CH-bond with methoxy group	

[a] Binding score -60.05 Kcal/mol.

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3) B. J. Orlando, M. G. Malkowski, *Acta Crystallogr. Sect. F, Struct. Biol. Commun.,* 2016, **72**, 772–776.

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