Supporting Information

Computational Design, Synthesis, and Assessment of 3-(4-(4-(1,3,4-Oxadiazol-2-yl)-1H-Imidazol-2-yl)phenyl)-1,2,4-Oxadiazole Derivatives as Effective Epidermal Growth Factor Receptor Inhibitors: A Prospective Strategy for Anticancer Therapy

Nilesh Raghunath Khedkar,[a]* Milind Sindkhedkar[a] Alex Joseph[b†]

[a]*Novel Drug Discovery & Development, Lupin Research Park, Lupin Ltd. Pune-412115, India.*

[b]*Department of Pharmaceutical Chemistry, Manipal College of Pharmaceutical Sciences, Manipal. Academy of Higher Education, Manipal, Karnataka-576104, India.*

[†]*Deceased on 19th Dec 2023.*

Email: *milindsindkhedkar@lupin.com*

Supporting Information

Table of Contents

1. **Chemistry Experimental and analytical data for Intermediate 24-28**

Ethyl 2-(4-cyanophenyl)-1H-imidazole-4-carboxylate (24): Compound **22** (10 g, 71.4 mmol), $Pd(OAc)_2$ (1.6 g, 7.14 mmol) and 4-bromobenzonitrile (**23**) (9.7 g, 71.4 mmol) were dissolved in 1,4-dioxane (100 mL). To this solution, an aqueous solution of Cs_2CO_3 (46 g, 142.8mmol) was added under stirring. The resultant reaction mixture was heated at 110 °C for 12 hours. After completion of reaction, the reaction was allowed to room temperature and diluted with ethyl acetate (200 mL). The mixture was filtered and diluted with water (100 mL). The organic solvent was separated. The aqueous layer was again extracted with ethyl acetate (50 mL), combined organic solvents was dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by column chromatography ethyl acetate/hexane (3:7) to afford pure compound **24** (12.6 g, 73% yield) as off white solid. MP: 156-159 ^oC, ^1H NMR (400 MHz, DMSO-d_6): δ 1.42 (t, 3H, J = 7.2 Hz, CH_3), 4.44 (q, 2H, J = 7.2 Hz, CH_2), 7.85 (d, 2H, J = 8.1 Hz, Ar-H), 7.92 (d, 2H, J = 8.1 Hz, Ar-H), 8.34 (s, 1H, Ar-H), 8.62 (brs, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 18.3, 62.4, 111.6, 119.5, 126.2, 128.3, 129.6, 133.5, 140.5, 149.6, 163.5; MS (ESI): m/z 242 $[M+H]^+$.

2-(4-cyanophenyl)-1H-imidazole-4-carbohydrazide (25): A mixture of compound **(24)** (12 g, 49.8mmol) and hydrazine hydrochloride (6.7 g, 99.5 mmol) in ethanol (100 ml) was stirred at reflux for 6 hours. The reaction mixture was allowed to room temperature and was cooled down to 0 ^oC. The solid product was precipitated, filtered solid, washed with cold water and dried under vacuo to afford product **25** (8.2 g, 73% yield) as white solid. Obtained solid was carried forward in next step without further treatment. MP: 165-167 ^oC, ^1H NMR (400 MHz, DMSO-d_6): δ 4.86 (brs, 2H, NH_2), 7.86 (d, 2H, J = 8.2 Hz, Ar-H), 7.93 (d, 2H, J = 8.2 Hz, Ar-H), 8.23 (brs, 1H, NH), 8.34 (s,

1H, Ar-H), 8.62 (brs, 1H, Imidazole-NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 111.6, 118.8, 119.5, 128.3, 129.6, 133.5, 134.7, 149.6, 163.4; MS (ESI): m/z 228 $[M+H]^+$.

4-(4-(5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl)-1H-imidazol-2-yl)benzonitrile (27): Compound **25** (8 g, 35.2 mmol) was dissolved in $POCl_3$ (60 mL) and added isonicotinic acid (**26**) (4.33 g, 35.2 mmol). The resulting reaction mixture was stirred at 80 ^oC for 12 hours. After completion of reaction, solvent was evaporated and crude compound was extracted with ethyl acetate (200 mL). The organic layer was washed with aqueous $NaHCO_3$ solution (100 mL X 2) and water. The separated organic layer was dried with anhydrous Na_2SO_4, filtered, and concentrated under vacuo. The crude compound was purified by column chromatography with ethyl acetate/hexane (4:6) to afford pure compound **27** (7.9 g, 72% yield) as off white solid. MP: 186-189 ^oC, ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.75 (d, 2H, J = 6.9 Hz, Ar-H), 7.85 (d, 2H, J = 8.2 Hz, Ar-H), 7.94 (d, 2H, J = 8.2 Hz, Ar-H), 8.37 (s, 1H, Ar-H), 8.65 (brs, 1H, Imidazole-NH), 8.84 (d, 2H, J = 6.9 Hz, Ar-H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 111.6, 118.8, 119.5, 120.7, 127.4, 128.3, 129.6, 133.5, 134.7, 149.6, 150.5, 162.7, 164.5; MS (ESI): m/z 315 $[M+H]^+$.

(E)-N'-Hydroxy-4-(4-(5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl)-1H-imidazol-2-yl)benzimidamide (28): To a solution of compound **27** (7.6 g, 24.2 mmol) in methanol (70 ml) was added Et_3N (10 ml, 72.6 mmol) and $NH_2OH.HCl$ (2.4 g, 72.6 mmol). The resulting reaction mixture was refluxed for 6 hours. The reaction mixture was concentrated under vacuum, the residue was diluted with water and extracted with EtOAc. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuo. The compound was washed with diethyl ether to give pure compound **28 (**7.1 g, 85% yield) as off white solid. MP: 193-195 ^oC, ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.88 (s, 2H, NH_2), 7.76 (d, 2H, J = 7.0 Hz, Ar-H), 7.87 (d, 2H, J = 8.3 Hz, Ar-H), 7.96 (d, 2H, J = 8.3 Hz, Ar-H), 8.37 (s, 1H, Ar-H), 8.66 (brs, 1H, Imidazole-NH), 8.85 (d, 2H, J = 7.0 Hz, Ar-H), 14.36 (s, 1H, -OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 118.8, 120.7, 127.4, 128.3, 128.6, 129.6, 130.7, 134.7, 149.6, 150.5, 151.5, 162.7, 164.5; MS (ESI): m/z 348 $[M+H]^+$.

2. Chemistry Experimental and analytical data for final compounds 30a-j

3-(4-(4-(5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl)-1H-imidazol-2-yl)phenyl)-5-(3,4,5-trimethoxyphenyl)-1,2,4-oxadiazole (30a): A solution of 3,4,5-trimethoxybenzoic acid (**29a**) (305mg, 1.44 mmol) and (E)-N'-hydroxy-4-(4-(5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl)-1H-imidazol-2-yl)benzimidamide **(28)** (500 mg, 1.44 mmol) in acetonitrile (20 ml), to this CDI (466 mg, 2.88 mmol) was added and stirred for 10 minutes at RT. The resulting reaction mixture was refluxed for 12 hours. After completion of reaction, reaction mass was cooled to room temperature. To the cooled stirred solution was quenched with aqueous ammonia. After quenching, product was extracted with ethyl acetate (120 mL). The organic layer was washed with water dried over with Na_2SO_4, filtered and concentrated under vacuo. This crude product was purified by column chromatography using ethyl acetate: hexane (6:4) as eluent to afford pure compound **30a** (610.5 mg, 81% yield) as off white solid. MP: 265-267 ^oC, ^1H NMR (400 MHz, DMSO-d_6): δ 3.77 (s, 6H, -OCH_3), 3.86 (s, 3H, -OCH_3), 7.34 (s, 2H, Ar-H), 7.75 (d, 2H, J = 7.0 Hz, Ar-H), 7.89 (d, 2H, J = 8.4 Hz, Ar-H), 7.98 (d, 2H, J = 8.4 Hz, Ar-H), 8.38 (s, 1H, Ar-H), 8.68 (brs, 1H, Imidazole-NH), 8.84 (d, 2H, J = 7.0 Hz, Ar-H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 57.3, 57.8, 111.6, 119.8, 120.7, 126.3, 127.3, 127.7, 128.3, 128.7, 129.6, 134.7, 140.0, 149.6, 150.5, 154.2, 162.7, 164.5, 167.4, 175.4; MS (ESI): m/z 524 $[M+H]^+$; HRMS (ESI): m/z calculated for $C_{27}H_{22}N_7O_5$ $[M+H]^+$ 524.1677 found 524.1671.

5-(3,5-dimethoxyphenyl)-3-(4-(4-(5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl)-1H-imidazol-2-yl)phenyl)-1,2,4-oxadiazole (30b): Compound **30b** was prepared by the method described for **30a**, employing **28** (500 mg, 1.44 mmol), 3,5-dimethoxybenzoic acid (**29b**) (262 mg, 1.44mmol), & CDI (466 mg, 2.88 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (6:4) to afford the pure compound **30b** (621.3 mg, 88% yield) as off white solid. MP: 260-262 ^oC, ^1H NMR (400 MHz, DMSO-d_6): δ 3.71 (s, 6H, -OCH_3), 6.65 (s, 1H, Ar-H), 7.46 (s, 2H, Ar-H), 7.75 (d, 2H, J = 7.1 Hz, Ar-H), 7.90 (d, 2H, J = 8.5 Hz, Ar-H),

7.99 (d, 2H, *J* = 8.5 Hz, Ar-H), 8.38 (s, 1H, Ar-H), 8.68 (brs, 1H, Imidazole-NH), 8.84 (d, 2H, *J* = 7.1 Hz, Ar-H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 57.4, 102.7, 115.2, 118.8, 120.7, 126.3, 127.4, 127.8, 128.3, 128.6, 129.6, 134.7, 149.6, 150.4, 161.9, 162.7, 164.5, 167.4, 175.5; MS (ESI): m/z 494 $[M+H]^+$; . HRMS (ESI): m/z calculated for $C_{26}H_{20}N_7O_4$ $[M+H]_+$ 494.1571 found 494.1564.

5-(4-methoxyphenyl)-3-(4-(4-(5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl)-1H-imidazol-2-yl)phenyl)-1,2,4-oxadiazole (30c): Compound **30c** was prepared by the method described for **30a**, employing **28** (500 mg, 1.44 mmol), 4-methoxybenzoic acid (**29c**) (219 mg, 1.44mmol) & CDI (466 mg, 2.65 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (6:4) to afford the pure compound **30c** (505.2 mg, 76% yield) off white solid. MP: 257-259 ^{o}C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.90 (s, 3H, -OCH_3), 7.21 (d, 2H, *J* = 8.2 Hz, Ar-H), 7.74 (d, 2H, *J* = 7.2 Hz, Ar-H), 7.84 - 7.92 (m, 4H, Ar-H), 8.01 (d, 2H, *J* = 8.5 Hz, Ar-H), 8.39 (s, 1H, Ar-H), 8.67 (brs, 1H, Imidazole-NH), 8.84 (d, 2H, *J* = 7.2 Hz, Ar-H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 57.4, 115.3, 118.8, 120.7, 124.5, 126.3, 127.5, 128.3, 128.8, 128.6, 130.1, 134.7, 149.6, 150.5, 160.8, 162.7, 164.5, 167.4, 175.6; MS (ESI): m/z 464 $[M+H]^+$; HRMS (ESI): m/z calculated for $C_{25}H_{18}N_7O_3$ $[M+H]^+$ 464.1466 found 464.1474.

5-(4-chlorophenyl)-3-(4-(4-(5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl)-1H-imidazol-2-yl)phenyl)-1,2,4-oxadiazole (30d): Compound **30d** was prepared by the method described for **30a**, employing **28** (500 mg, 1.44 mmol), 4-chlorobenzoic acid (**29d**) (225 mg, 1.44mmol) & CDI (466 mg, 2.88 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (6:4) to afford the pure compound **30d** (498.6 mg, 74% yield) as a white solid. MP: 276-279 ^{o}C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.75 (d, 2H, *J* = 7.1 Hz, Ar-H), 7.85 - 7.96 (m, 4H, Ar-H), 8.10 - 8.18 (m, 4H, Ar-H), 8.40 (s, 1H, Ar-H), 8.68 (brs, 1H, Imidazole-NH), 8.85 (d, 2H, *J* = 7.1 Hz, Ar-H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 118.8, 120.7, 124.5, 126.3, 127.3, 128.4, 128.8, 129.2, 129.6, 130.7, 134.4, 134.7, 149.6, 150.6, 162.7, 164.6, 167.4, 175.6; MS (ESI): m/z 467 $[M-H]^+$; HRMS (ESI): m/z calculated for $C_{24}H_{15}ClN_7O_2$ $[M+H]^+$ 468.097 found 468.092.

5-(4-bromophenyl)-3-(4-(4-(5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl)-1H-imidazol-2-yl)phenyl)-1,2,4-oxadiazole (30e): Compound **30e** was prepared by the method described for **30a**, employing **28** (500 mg, 1.44 mmol), 4-bromobenzoic acid (**29e**) (290 mg, 1.44mmol) & CDI (466 mg, 2.88 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (6:4)to afford the pure compound **30e (**630.5 mg, 86% yield) as off white solid. MP: 282-284 ^{o}C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.74 (d, 2H, J = 7.2 Hz, Ar-H), 7.87 - 7.98 (m, 4H, Ar-H), 8.11 - 8.19 (m, 4H, Ar-H), 8.41 (s, 1H, Ar-H), 8.67 (brs, 1H, Imidazole-NH), 8.84 (d, 2H, J = 7.2 Hz, Ar-H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 118.8, 120.7, 123.3, 124.5, 126.3, 127.2, 128.3, 128.6, 129.6, 130.3, 132.7, 134.7, 149.6, 150.5, 162.7, 164.5, 167.4, 175.4; MS (ESI): m/z 513 $[M+2]^+$; HRMS (ESI): m/z calculated for $C_{24}H_{15}BrN_7O_2$ $[M+H]^+$ 512.0465 found 512.0473.

5-(4-nitrophenyl)-3-(4-(4-(5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl)-1H-imidazol-2-yl)phenyl)-1,2,4-oxadiazole (30f): Compound **30f** was prepared by the method described for **30a**, employing **28** (500 mg, 1.44 mmol), 4-nitrobenzoic acid (**29f**) (240 mg, 1.44mmol) & CDI (466 mg, 2.88 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (6:4)to afford the pure compound **30f** (601.6 mg, 87% yield) as off white solid. MP: 290-292 ^{o}C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.74 (d, 2H, J = 7.2 Hz, Ar-H), 7.99 (d, 2H, J = 8.4 Hz, Ar-H), 8.12 (d, 2H, J = 8.4 Hz, Ar-H), 8.37 (d, 2H, J = 8.6 Hz, Ar-H), 8.43 (s, 1H, Ar-H), 8.56 (d, 2H, J = 8.6 Hz, Ar-H), 8.68 (brs, 1H, Imidazole-NH), 8.84 (d, 2H, J = 7.2 Hz, Ar-H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 118.8, 120.7, 121.8, 124.5, 126.3, 127.3, 128.2, 128.5, 129.6, 130.4, 134.7, 149.6, 150.5, 162.7, 164.5, 165.3, 167.4, 175.6; MS (ESI): m/z 479 $[M+H]^+$; HRMS (ESI): m/z calculated for $C_{24}H_{15}N_8O_4$ $[M+H]^+$ 479.1211 found 479.1217.

5-(3,5-dinitrophenyl)-3-(4-(4-(5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl)-1H-imidazol-2-yl)phenyl)-1,2,4-oxadiazole (30g): Compound **30g** was prepared by the method described for **30a**, employing **28** (500 mg, 1.44 mmol), 3,5-dinitrobenzoic acid (**29g**) (305 mg, 1.44mmol) & CDI (466 mg, 2.88 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (6:4) to afford the pure compound **30g** (618.4 mg, 82% yield) as off white solid. MP: 309-311 ^{o}C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.75 (d,

2H, *J* = 7.3 Hz, Ar-H), 8.03 (d, 2H, *J* = 8.5 Hz, Ar-H), 8.15 (d, 2H, *J* = 8.5 Hz, Ar-H), 8.45 (s, 1H, Ar-H), 8.70 (brs, 1H, Imidazole-NH), 8.84 (d, 2H, *J* = 7.3Hz, Ar-H), 8.95 - 9.01 (m, 3H, Ar-H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 116.3, 118.8, 120.4, 120.7, 126.3, 127.4, 127.8, 128.3, 128.7, 129.6, 134.7, 149.6, 150.5, 162.7, 164.5, 165.3, 167.4, 175.4; MS (ESI): m/z 524 $[M+H]^+$; HRMS (ESI): m/z calculated for $C_{24}H_{14}N_9O_6$ $[M+H]^+$ 524.1062 found 524.1067.

4-(3-(4-(4-(5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl)-1H-imidazol-2-yl)phenyl)-1,2,4-oxadiazol-5-yl)benzonitrile (30h): Compound **30h** was prepared by the method described for **30a**, employing **28** (500 mg, 1.44 mmol), 4-cyanobenzoic acid (**29h**) (212 mg, 1.44mmol) & CDI (466mg, 2.88 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (6:4) to afford the pure compound **30h (**571.8 mg, 87% yield) as off white solid. MP: 289-291 ^{o}C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.74 (d, 2H, *J* = 7.3 Hz, Ar-H), 7.96 - 8.01 (m, 4H, Ar-H), 8.10 - 8.16 (m, 4H, Ar-H), 8.41 (s, 1H, Ar-H), 8.67 (brs, 1H, Imidazole-NH), 8.83 (d, 2H, *J* = 7.3 Hz, Ar-H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 111.6, 118.8, 119.5, 120.7, 124.5, 126.3, 127.4, 127.8, 128.3, 128.7, 129.6, 133.5, 134.7, 149.6, 150.4, 162.7, 164.4, 167.5, 175.4; MS (ESI): m/z 459 $[M+H]^+$; HRMS (ESI): m/z calculated for $C_{25}H_{15}N_8O_2$ $[M+H]^+$ 459.1312 found 459.1319.

3-(4-(4-(5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl)-1H-imidazol-2-yl)phenyl)-5-(p-tolyl)-1,2,4-oxadiazole (30i): Compound **30i** was prepared by the method described for **30a**, employing **28** (500 mg, 1.44 mmol), 4-methylbenzoic acid (**29i**) (196 mg, 1.44mmol) & CDI (466 mg, 2.88 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (6:4) to afford the pure compound **30i (**520.4 mg, 81% yield) as white solid. MP: 254 – 256 ^{o}C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.39 (s, 3H, -CH_3), 7.38 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.65 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.74 (d, 2H, *J* = 7.3 Hz, Ar-H), 7.88 (d, 2H, *J* = 8.4 Hz, Ar-H), 7.97 (d, 2H, *J* = 8.4 Hz, Ar-H), 8.41 (s, 1H, Ar-H), 8.67 (brs, 1H, Imidazole-NH), 8.83 (d, 2H, *J* = 7.3 Hz, Ar-H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 26.3, 118.8, 120.7, 124.5, 126.3, 127.3, 127.8, 128.4, 128.8, 129.6, 130.1, 134.7, 142.5, 149.6, 150.4, 162.7, 164.3, 167.4, 175.4; MS (ESI): m/z 448 $[M+H]^+$; HRMS (ESI): m/z calculated for $C_{25}H_{18}N_7O_2$ $[M+H]$+ 448.1516 found 448.1523.

N,N-Dimethyl-4-(3-(4-(4-(5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl)-1H-imidazol-2-yl)phenyl)-1,2,4-oxadiazol-5-yl)aniline (30j): Compound **30j** was prepared by the method described for **30a**, employing **28** (500 mg, 1.44 mmol), 4-(dimethylamino)benzoic acid (**29j**) (238 mg, 1.44mmol) & CDI (466 mg, 2.88 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (6:4) to afford the pure compound **30j (**515.6 mg, 75% yield) as white solid. MP: 262 – 264 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.86 (s, 6H, -N(CH_3)$_2$), 7.13 (d, 2H, J = 7.0 Hz, Ar-H), 7.68 (d, 2H, J = 7.0 Hz, Ar-H), 7.74 (d, 2H, J = 7.2 Hz, Ar-H), 7.87 (d, 2H, J = 8.4 Hz, Ar-H), 7.97 (d, 2H, J = 8.4 Hz, Ar-H), 8.38 (s, 1H, Ar-H), 8.67 (brs, 1H, Imidazole-NH), 8.84 (d, 2H, J = 7.2 Hz, Ar-H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 45.3, 113.2, 118.8, 120.7, 124.5, 126.3, 127.4, 128.4, 128.7, 129.6, 130.4, 134.7, 149.6, 150.5, 151.9, 162.7, 164.3, 167.4, 175.4; MS (ESI): m/z 477 $[M+H]^+$; HRMS (ESI): m/z calculated for $C_{26}H_{21}N_8O_2$ $[M+H]^+$ 477.1782 found 477.1789.

3. Biological Experimental

3.1. MTT Assay

The antiproliferative activities of analogues **30a–j** was assessed using the standard MTT assay on four human cancer cell lines (DU-145, HEPG2, A549, and PC-3) *in vitro*. DU-145, HEPG2, and A549 cells were cultured in RPMI-1640 medium, while PC-3 cells were maintained in DMEM/F12, all within a humidified atmosphere containing 5% CO2 at 37 °C. The culture media, DMEM/F12 and RPMI-1640, were supplemented with 10% fetal bovine serum (FBS). The solid investigated compounds were dissolved in DMSO (dimethyl sulfoxide) at a concentration of 60 mM, followed by dilution with the medium to obtain a range of concentrations. Cells were seeded at a density of 5×103 cells/well in a 96-well plate and incubated at 37 °C for attachment. Upon reaching 70%-80% confluence, the medium was replaced, and the cells were exposed to the new medium containing the tested analogues. Etoposide and DMSO (0.1%) were added to the wells as positive and negative controls, respectively. The cells with tested analogues were then incubated at 37°C for 48 h. Following treatment, MTT solution (5 mg/mL) was added (10 µL/well), and the plates were incubated at 37°C for 4 h. Subsequently, the

medium in the well was replaced with DMSO (150 µL), and the mixture was gently shaken for 10 minutes. The absorbance of the suspension was determined at 570 nm using a microplate reader (Infinite® 200 PRO Nano Quant – Switzerland Tecan). The formula % inhibition = $(Abs_{control} - Abs_{compound})/Abs_{control} \times 100\%$ was utilized to calculate the inhibition percentage. The IC_{50} values of the tested compounds and Etoposide were determined using the prism statistical package (GraphPad Software, San Diego, CA, U.S.A.) after treatment with drugs of various concentrations [1,2,3]

3.2. Compounds 30a-c, $EGFR^{WT}$ and $EGFR^{T790M}$ kinase inhibition assay (Biochemical assay)

Compounds (**30a-c**) underwent assessment for inhibitory activity against both the wild-type ($EGFR^{WT}$) and $EGFR^{T790M}$ strains. The test protocol involved the addition of 25 µL of the master mixture (6 µL Kinase assay buffer + 1 µL ATP (500 µM) + 1 µL PTK substrate + 17 µL water) to each well. Subsequently, 5 µL of the inhibitor solution was added to the wells labeled as "test inhibitor." Meanwhile, for the "positive control" and "blank," 5 µL of the same solution without the inhibitor (inhibitor buffer) were added. In the wells designated as "blank," 20 µL of Kinase assay buffer was added. The amount of EGFR necessary for the assay was calculated, and the enzyme was diluted to 1 ng/µL with Kinase assay buffer. The reaction was initiated by adding 20 µL of diluted EGFR enzyme to the wells designated as "positive control" and "test inhibitor control." Incubation occurred at 30 ◦C for 40 min, followed by the addition of 50 µL of Kinase-Glo Max reagent to each well. The plate was then incubated at room temperature for 15 min, and luminescence was measured using the microplate reader [4,5].

3.3. Cell Cycle Analysis

Flow-activated cell sorting analysis was employed to investigate the impact of analogue **30a** on the cell cycle of HEPG2 human colon cancer cells. The cells were initially seeded in a 6-well plate and allowed to incubate overnight at 37 °C. Upon reaching 70%-80% confluence, the cells were treated with compound **30a** at concentrations of 0.11, 0.22, and 0.44 µM in fresh medium for a duration of 24 hours. Post-treatment, cells from both the control and treatment groups underwent digestion, followed by washing with phosphate-

buffered saline and fixation in 75% pre-cooled ethanol at 4 °C overnight (for over 18 hours). Subsequently, they were rinsed with phosphate-buffered saline and stained with a solution containing 50 µg/mL of propidium iodide supplemented with 50 µg/mL of RNase at 37 °C for 30 minutes. The fluorescence intensity of the stained cells was then assessed using flow cytometry [1,4].

3.4. Cell Apoptosis Analysis

HEPG2 cells were initially plated in 6-well plates at a density of 3×10^5 cells per well. Subsequently, the cells were incubated for 24 hours in the presence or absence of compound **30a** at concentrations of 0.11, 0.22, and 0.44 µM. Following the incubation period, cells were harvested and treated with 5 µL of Annexin-V/FITC in binding buffer (composed of 10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl2 at pH 7.4) at room temperature for 15 minutes. A PI solution (10 µL) was then introduced to the medium for an additional 10-minute incubation. Flow cytometry was employed to collect nearly 10,000 events for each sample, and the obtained data were analyzed using FlowJo 7.6 analysis software. The percentage of apoptotic cells was subsequently calculated [6].

3.5. Western Blot Analysis

HEPG2 cells were cultured in 6-cm dishes and allowed to grow for 24 hours. Subsequently, the cells were treated with compound **30a** at specified concentrations (0.44 µM) for an additional 24 hours. After treatment, cell lysates were obtained using ice-cold lysis buffer. For Western blot analyses, 50 µg of total protein from each cornea homogenate was subjected to denaturation by boiling for 5 minutes in a mixture comprising 2% SDS and 5% β-mercaptoethanol. The denatured proteins were loaded onto individual lanes of a 12% SDS–PAGE gel and electro-transferred onto a nylon membrane using a T-77 ECL semi-dry transfer unit over a 2-hour period. Following transfer, the membrane was subjected to blocking in TBS buffer containing 0.05% Tween and 5% non-fat milk for one hour. Subsequent steps included incubation with specified antibodies, namely rabbit monoclonal anti-EGFR, rabbit monoclonal antibody specific to EGFR, and beta-actin (β-actin) rabbit polyclonal antibodies, all obtained from Abcam (USA). A peroxidase-conjugated rabbit secondary

antibody was then applied. Semiquantitative analysis was conducted using densitometry, where the densities of the bands were measured and graphically represented in a histogram.

4. Molecular Modeling Studies

4.1. Docking Protocol

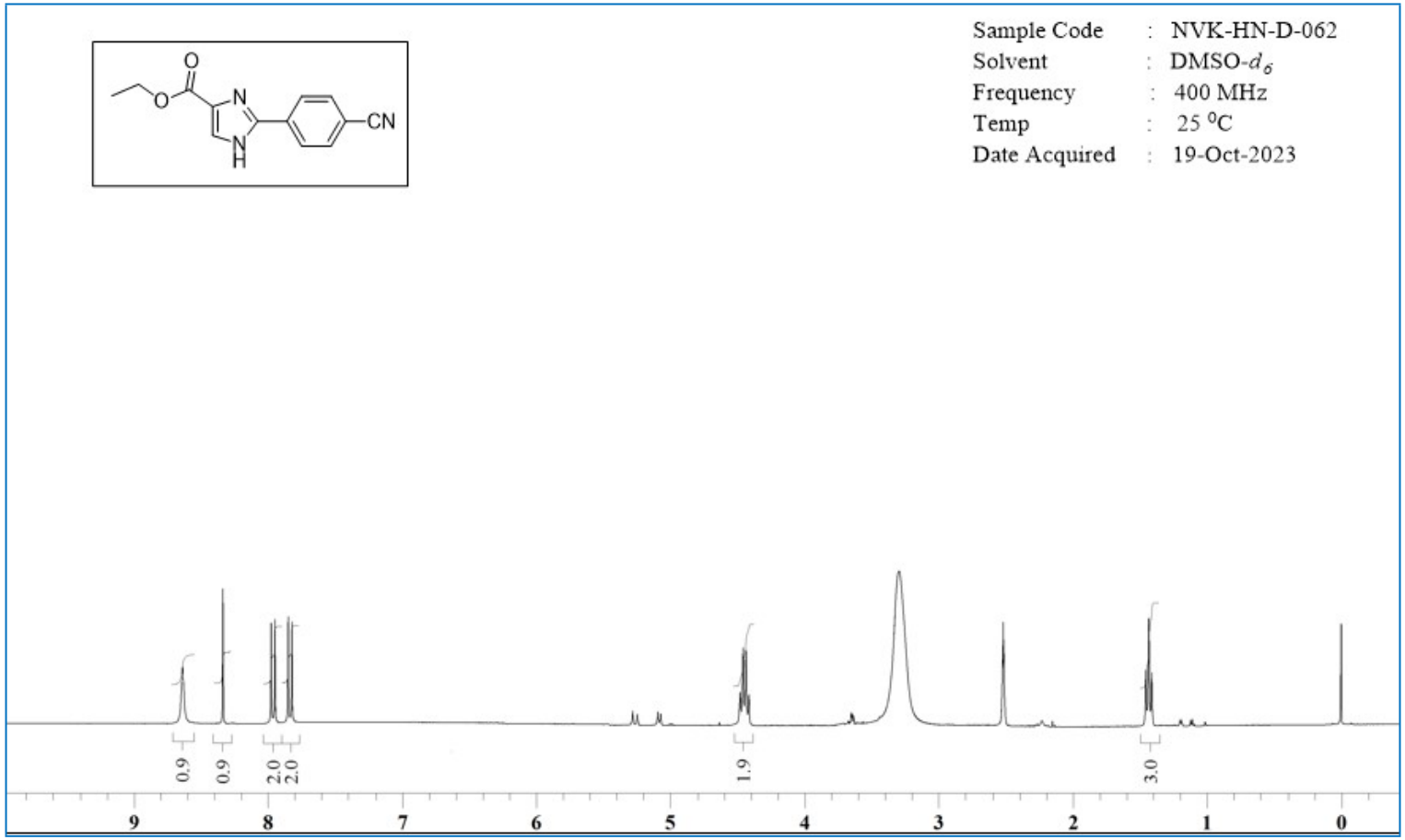
The fragment-based drug design (FBDD) adhered to standard guidelines provided by Schrödinger Maestro (2021-3 release). Subsequently, the 3-(4-(4-(1,3,4-Oxadiazol-2-yl)-1H-Imidazol-2-yl)phenyl)-1,2,4-Oxadiazole derivatives (**30a-j**) obtained were subjected to docking in the active site of mechanistic targets of EGFR ($EGFR^{WT}$, PDB ID: 1M17) proteins. This process utilized Schrödinger Maestro (2021-3 release) and Auto-Dock Tools 4.2 [7]. In silico docking encompassed the conversion of .pdb files to .pdbqt, potential calculation at the grid box, and Auto-Dock execution to identify optimal ligand positions based on binding energy. The selection of the best conformation for each docked ligand was based on the lowest binding free energy (Kcal/mol). To validate the results, the co-crystal ligand was redocked.

4.2. ADMET Studies

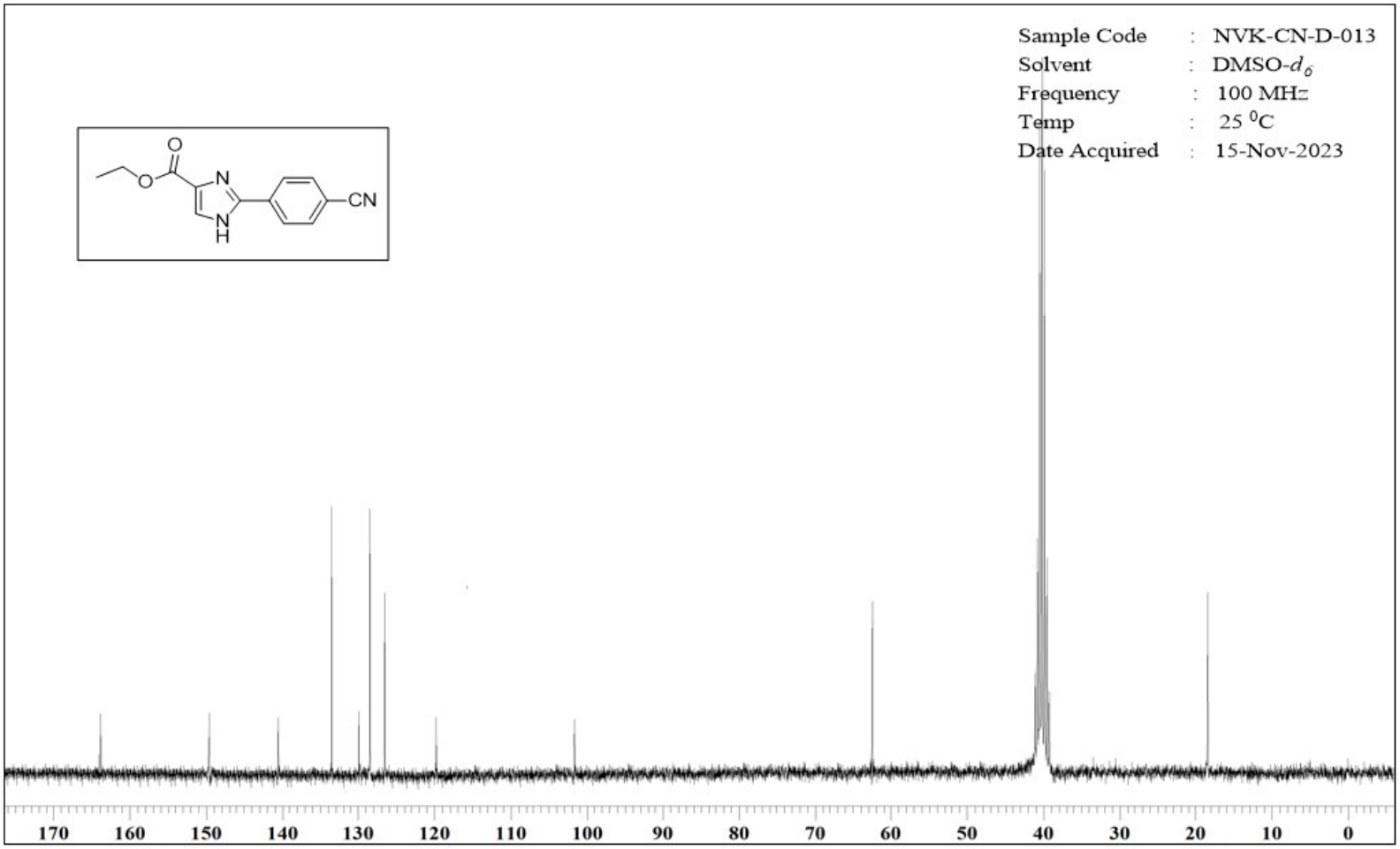
The investigation of chemical structures relies significantly on molecular descriptors, encompassing both physico-chemical and geometric aspects. In this study, the Data Warrior software [8] was employed to scrutinize the synthesized molecules, focusing on their pharmacological properties and drug-likeness. This involved the computation of Lipinski parameters and the Topological Polar Surface Area (TPSA) score. Additionally, the study encompassed an assessment of toxicity parameters, including tumorigenicity, reproductive effects, irritancy, and the presence of harmful functional groups, using the same software

5. Analytical Data of Intermediates synthesized

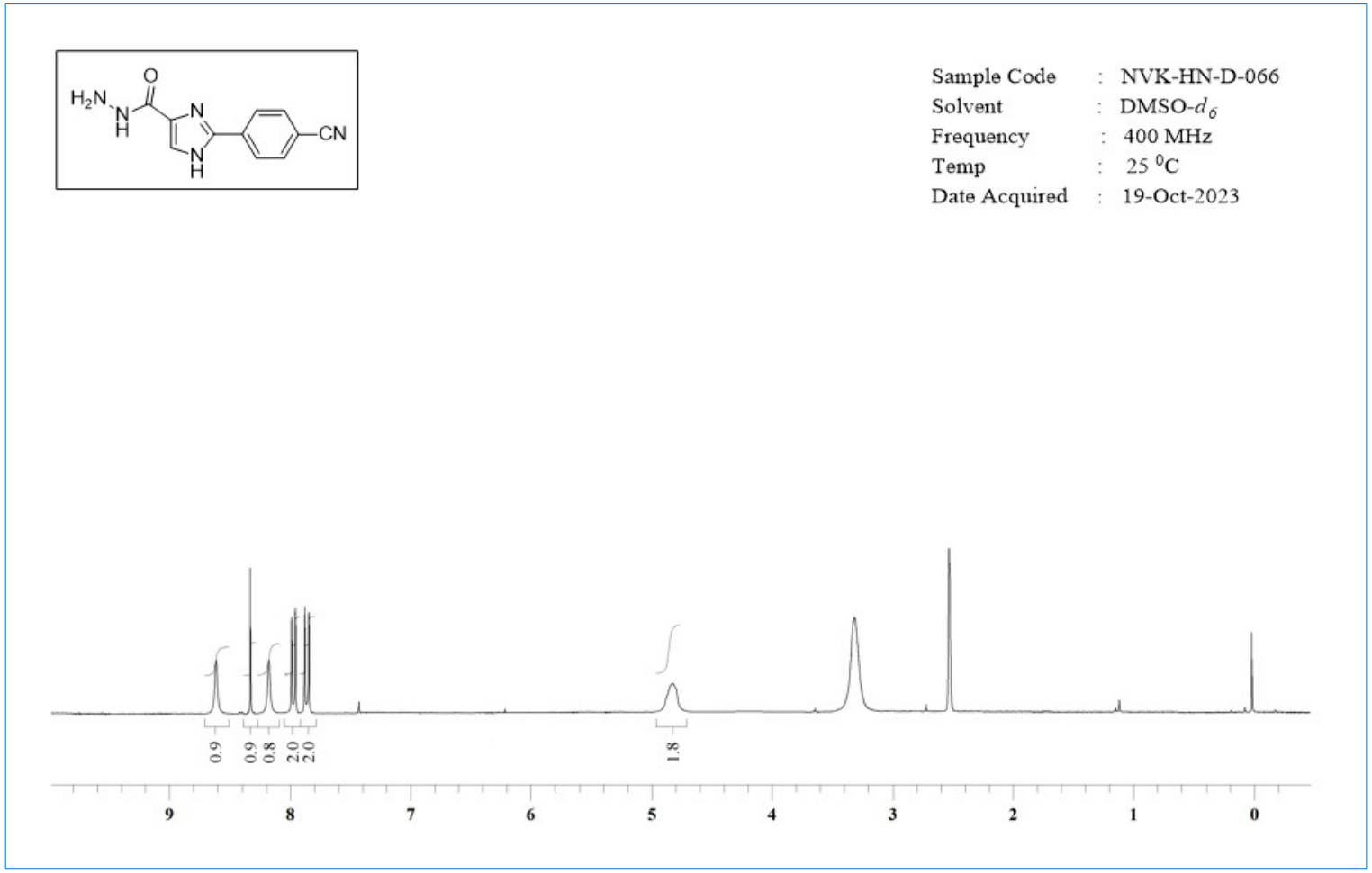
5.1. Intermediate 24, ^{1}H NMR (400 MHz, DMSO-d_6)



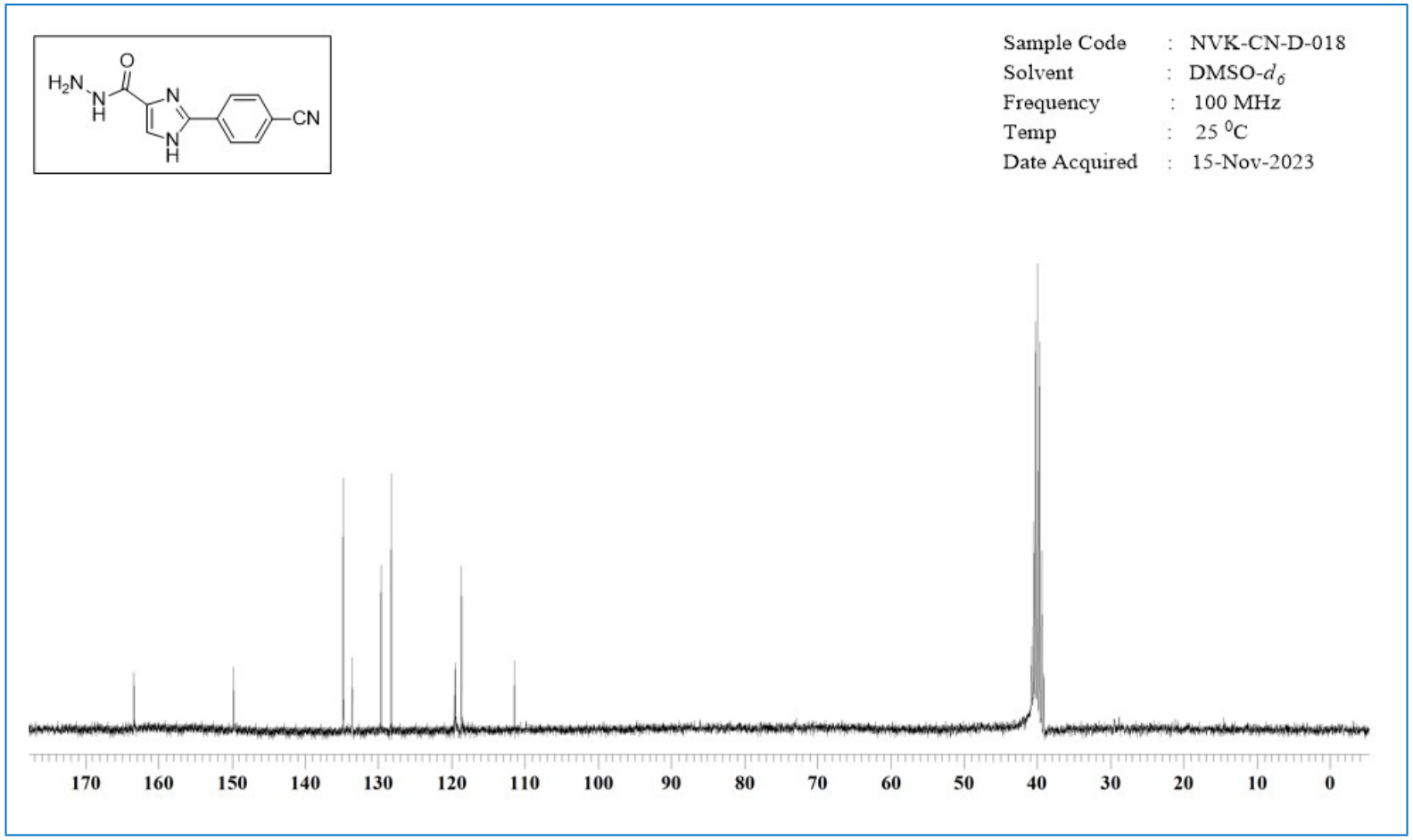
5.2. Intermediate 24, ^{13}C NMR (400 MHz, DMSO-d_6)



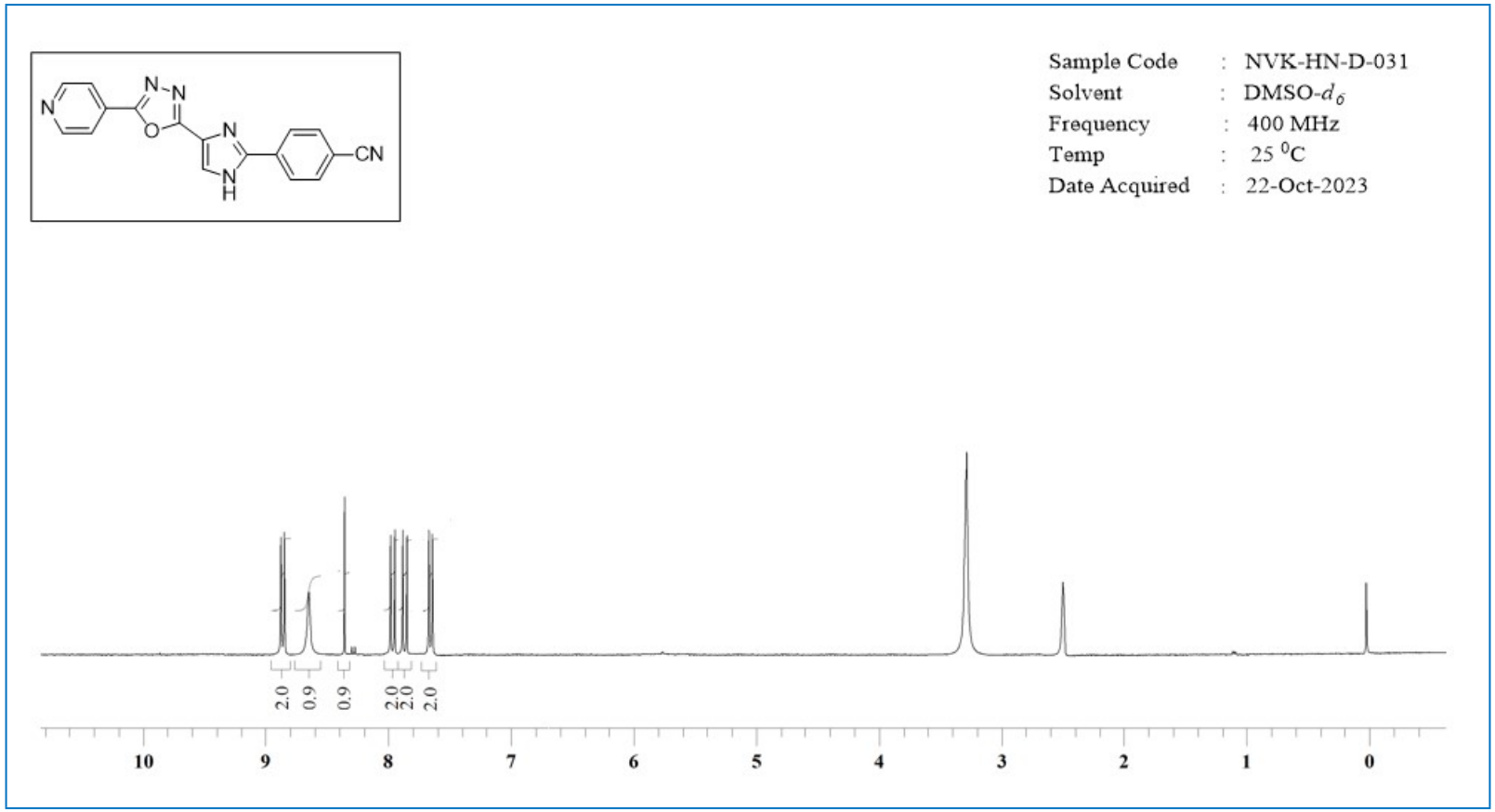
5.3. Intermediate 25, ^{1}H NMR (400 MHz, DMSO-d_6)



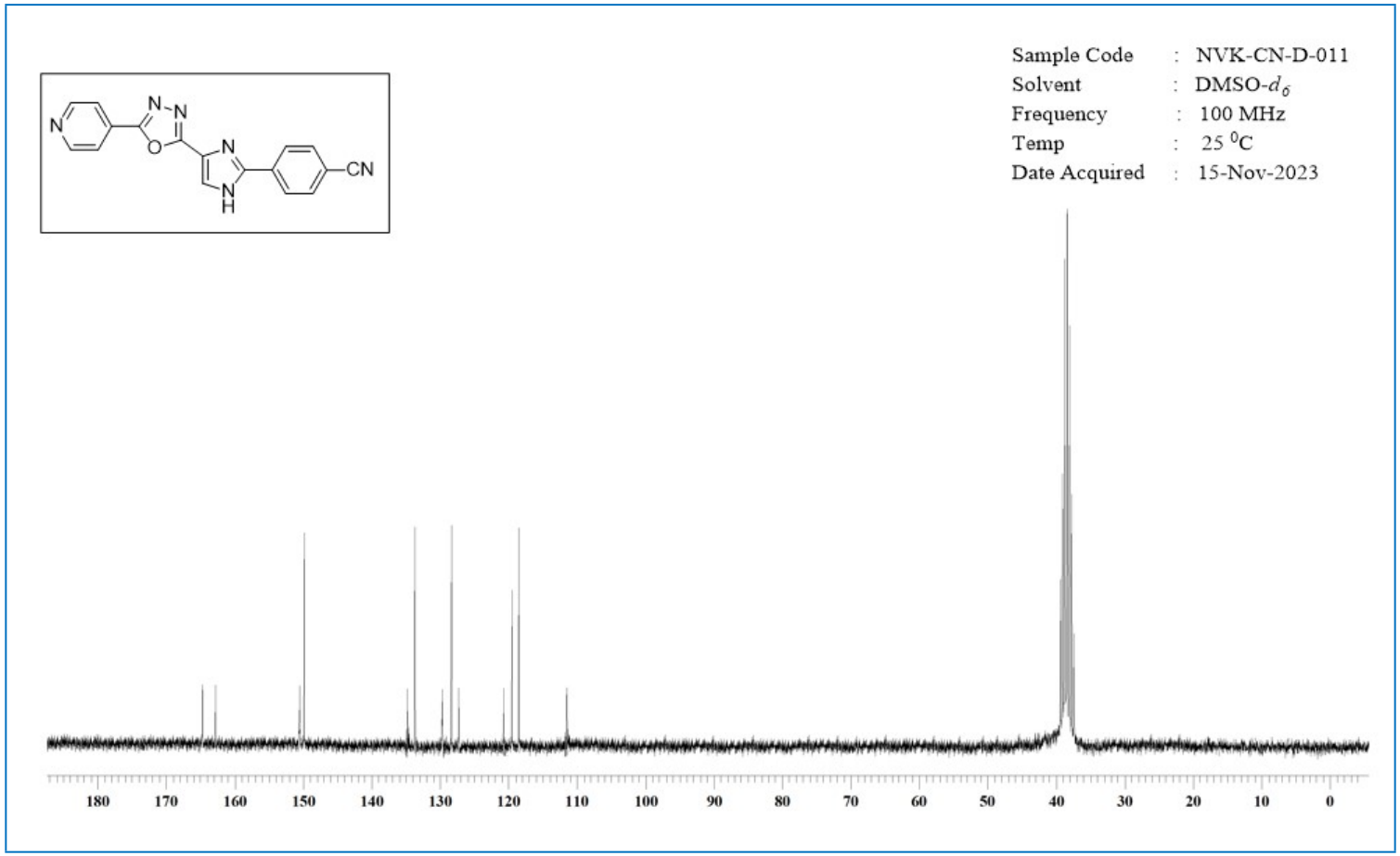
5.4. Intermediate 25, ^{13}C NMR (100 MHz, DMSO-d_6)



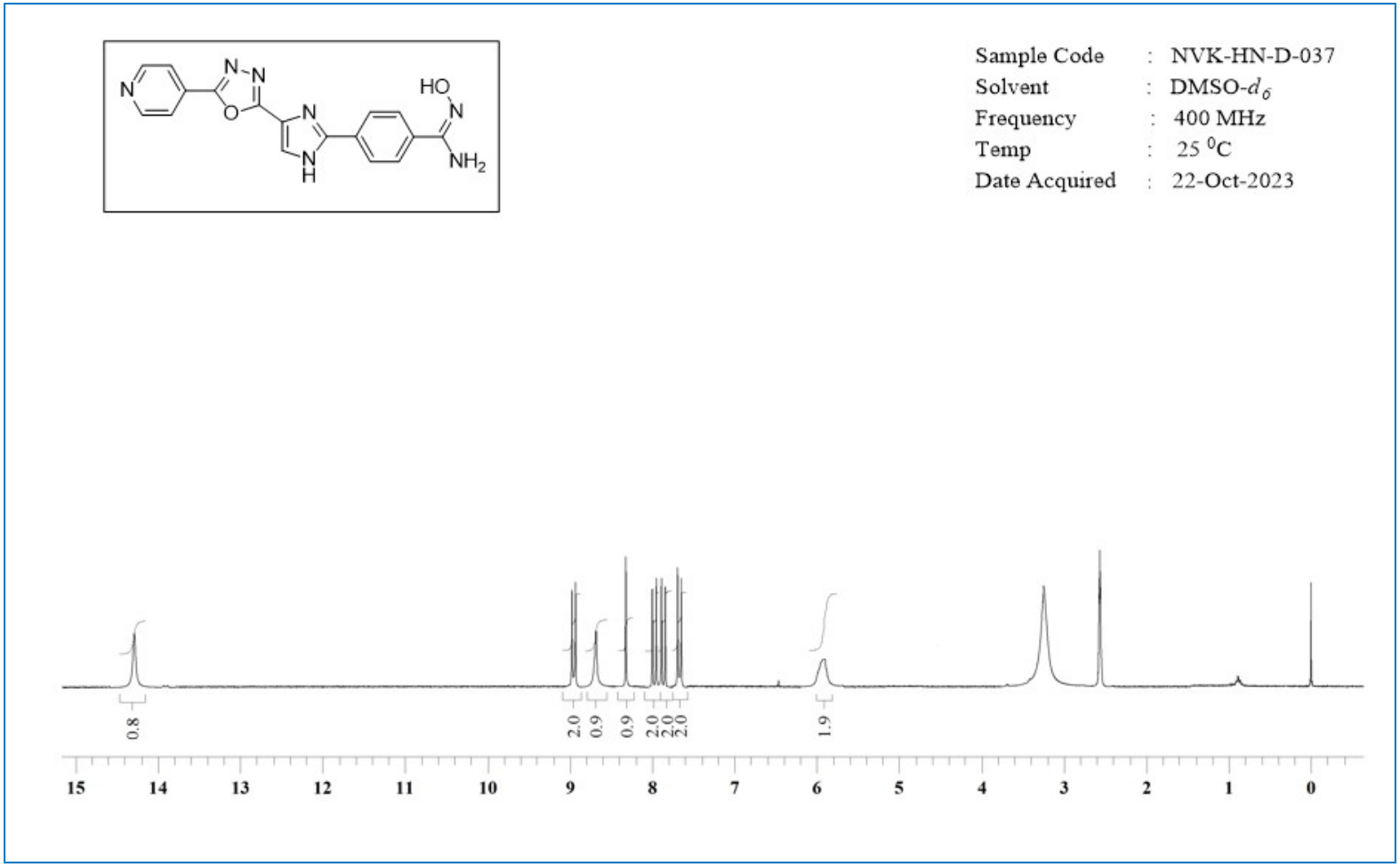
5.5. Intermediate 26, ^{1}H NMR (400 MHz, DMSO-d_6)



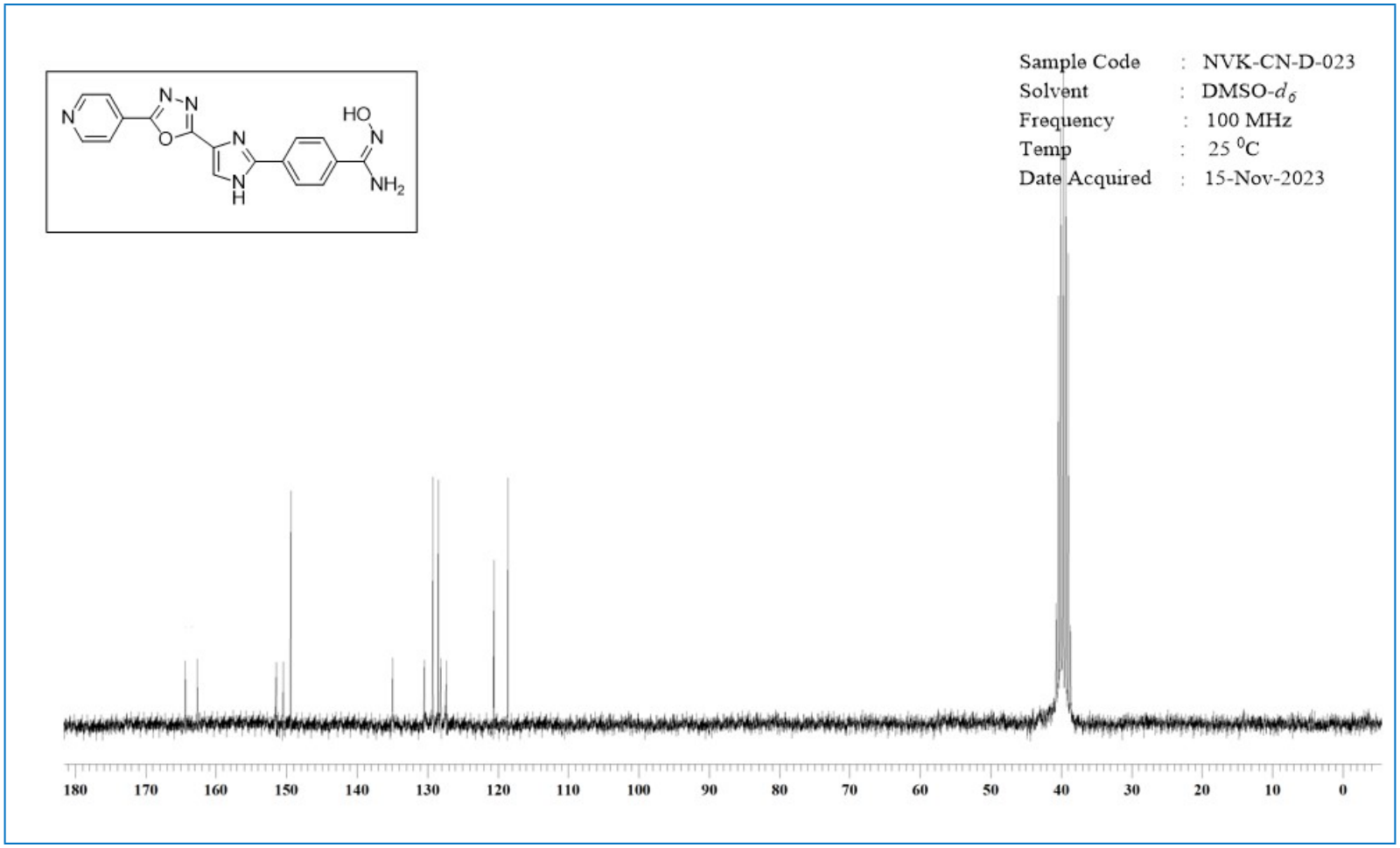
5.6. Intermediate 26, ^{13}C NMR (100 MHz, DMSO-d_6)



5.7. Intermediate 27, ^{1}H NMR (400 MHz, DMSO-d_6)

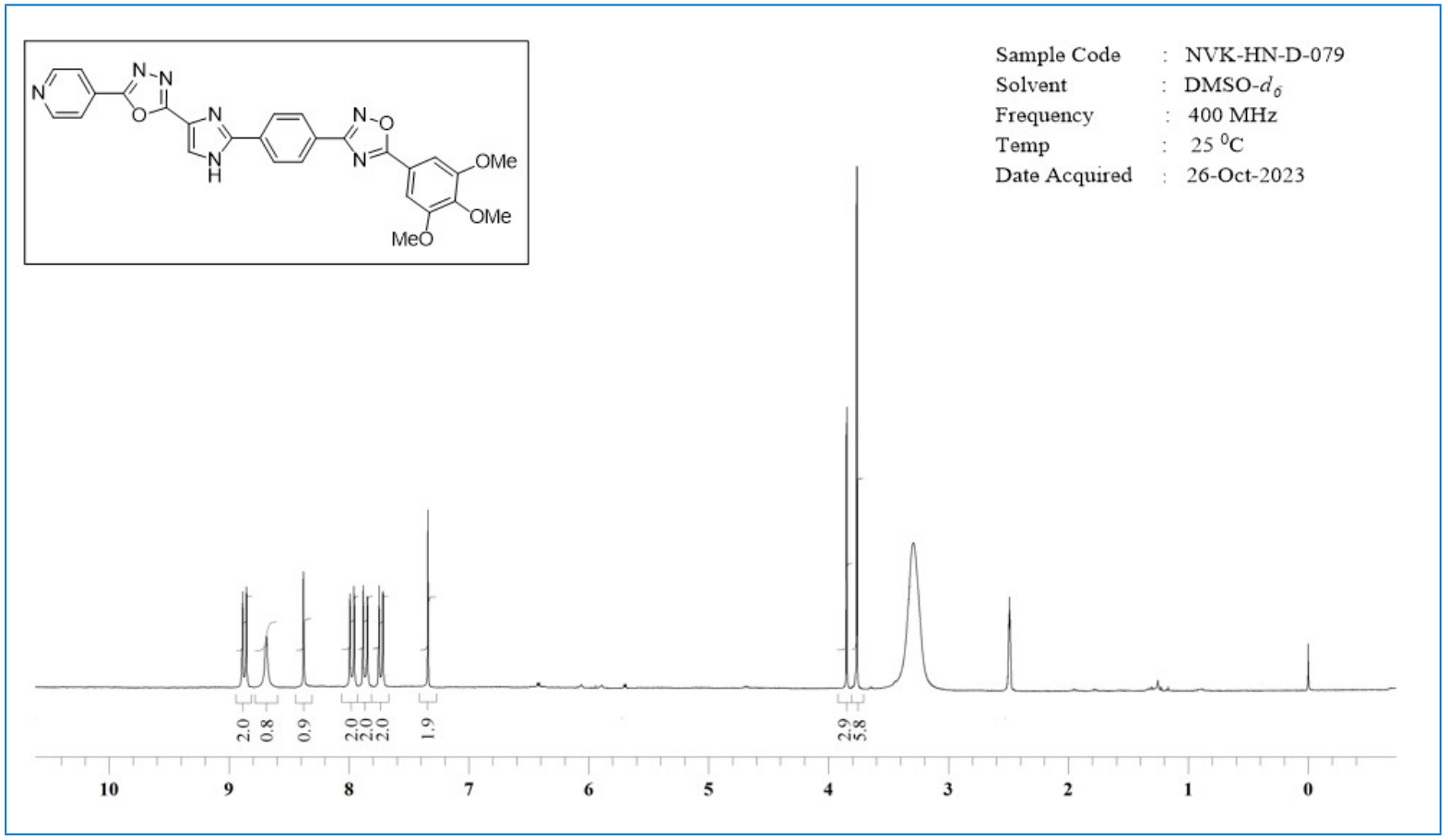


5.8. Intermediate 27, ^{13}C NMR (100 MHz, DMSO-d_6)

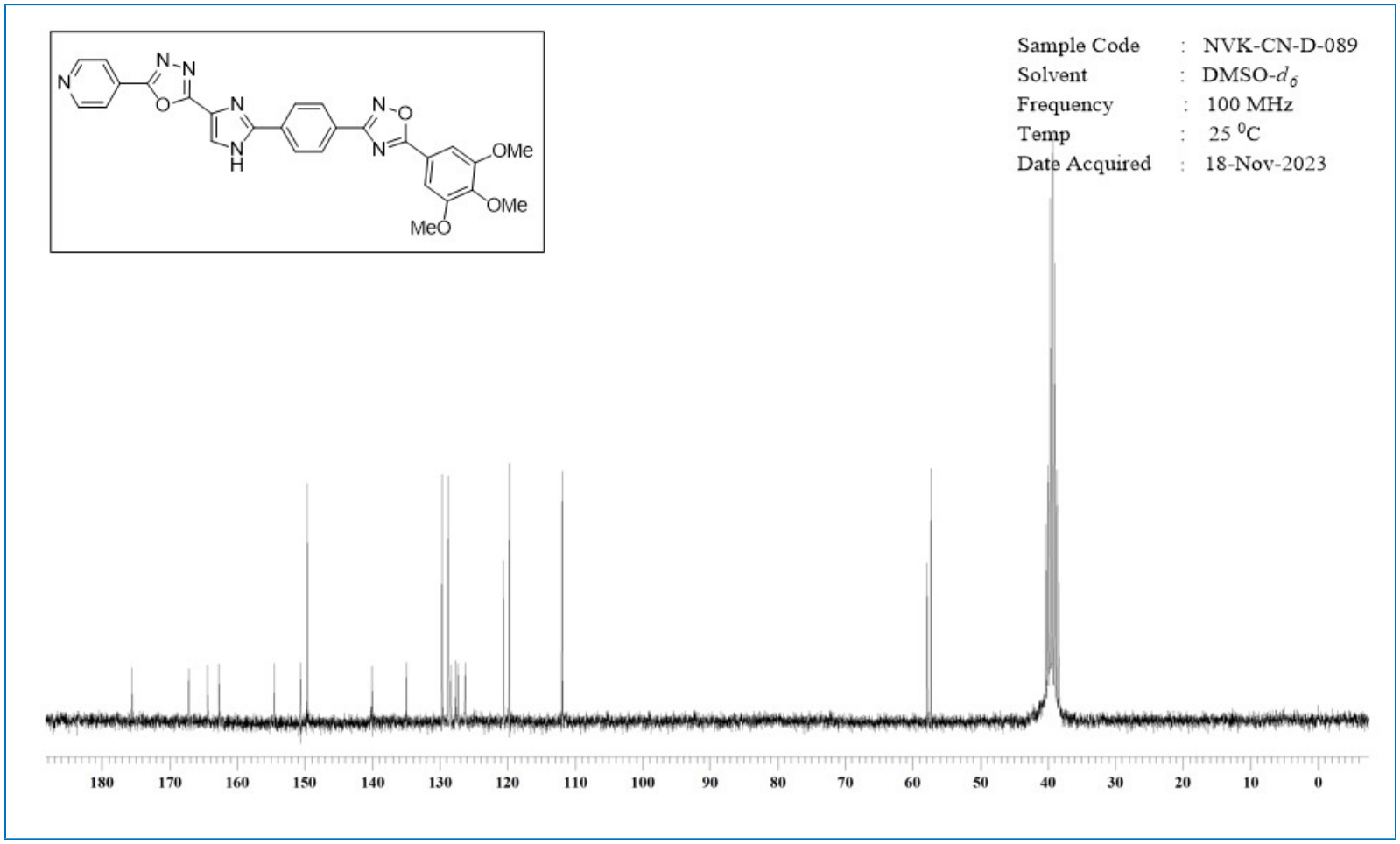


6. Analytical Data of the Final Compounds

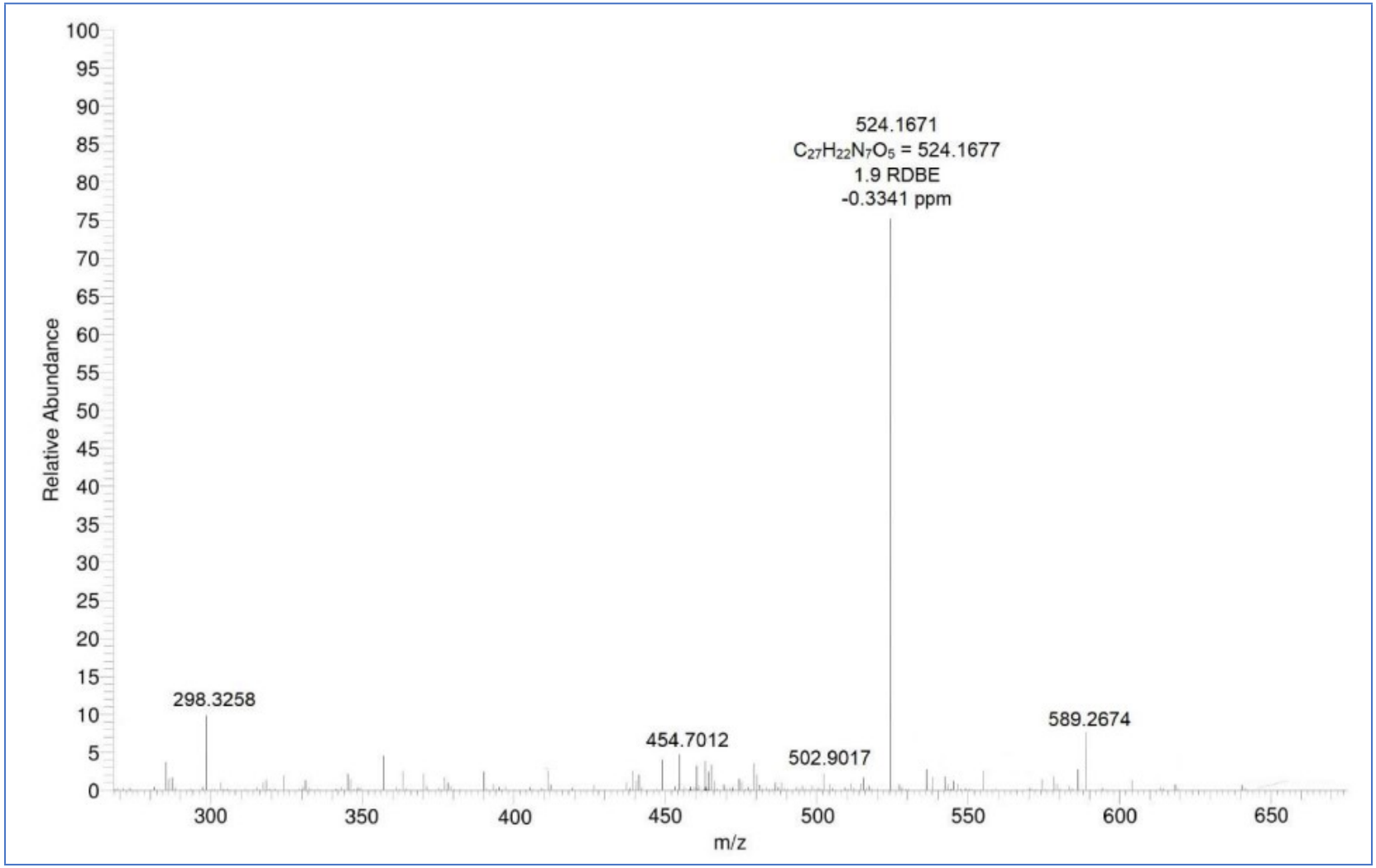
6.1. Compound 30a, ^{1}H NMR (400 MHz, DMSO-d_6)



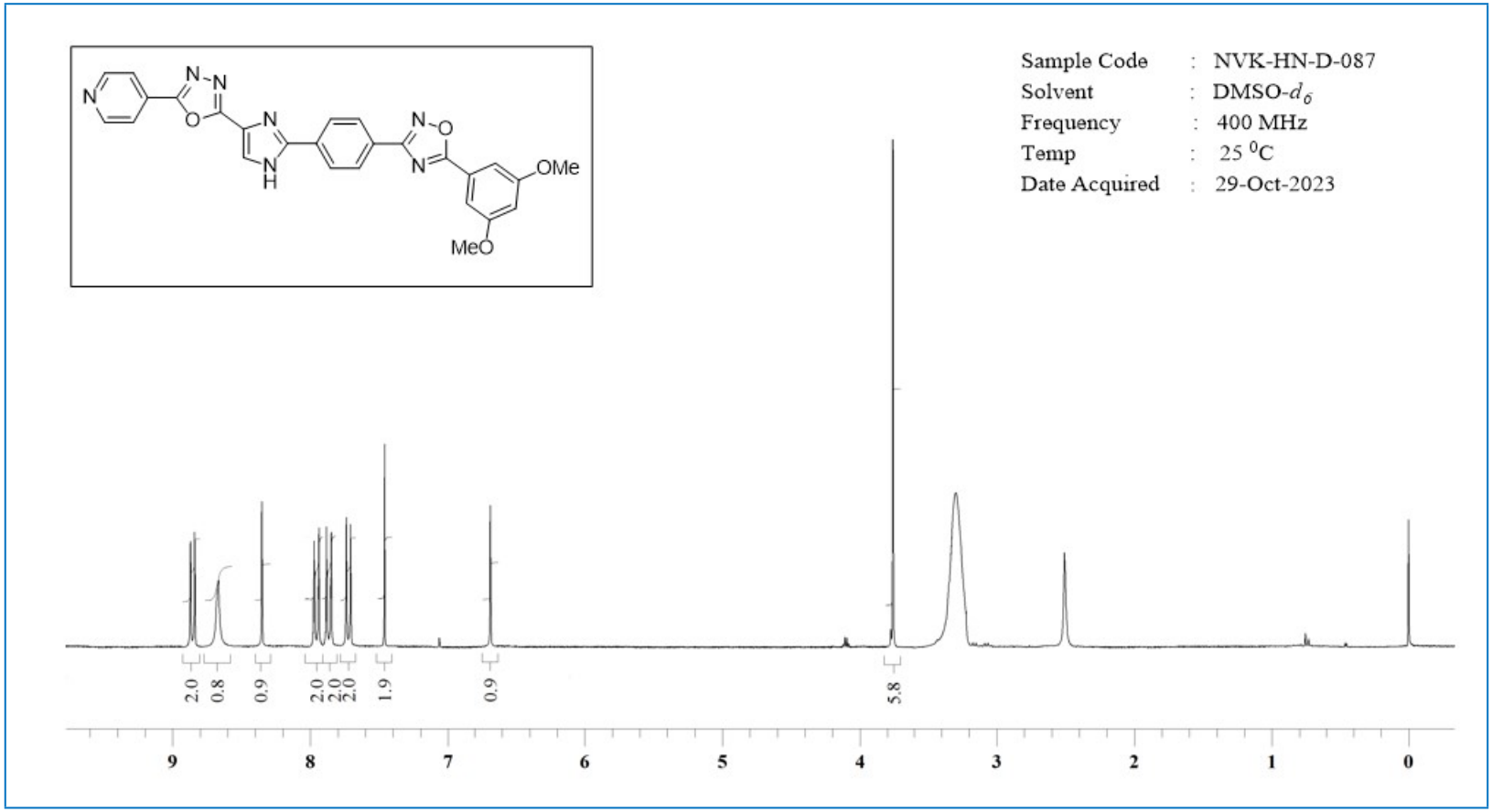
6.2. Compound 30a, ^{13}C NMR (100 MHz, DMSO-d_6)



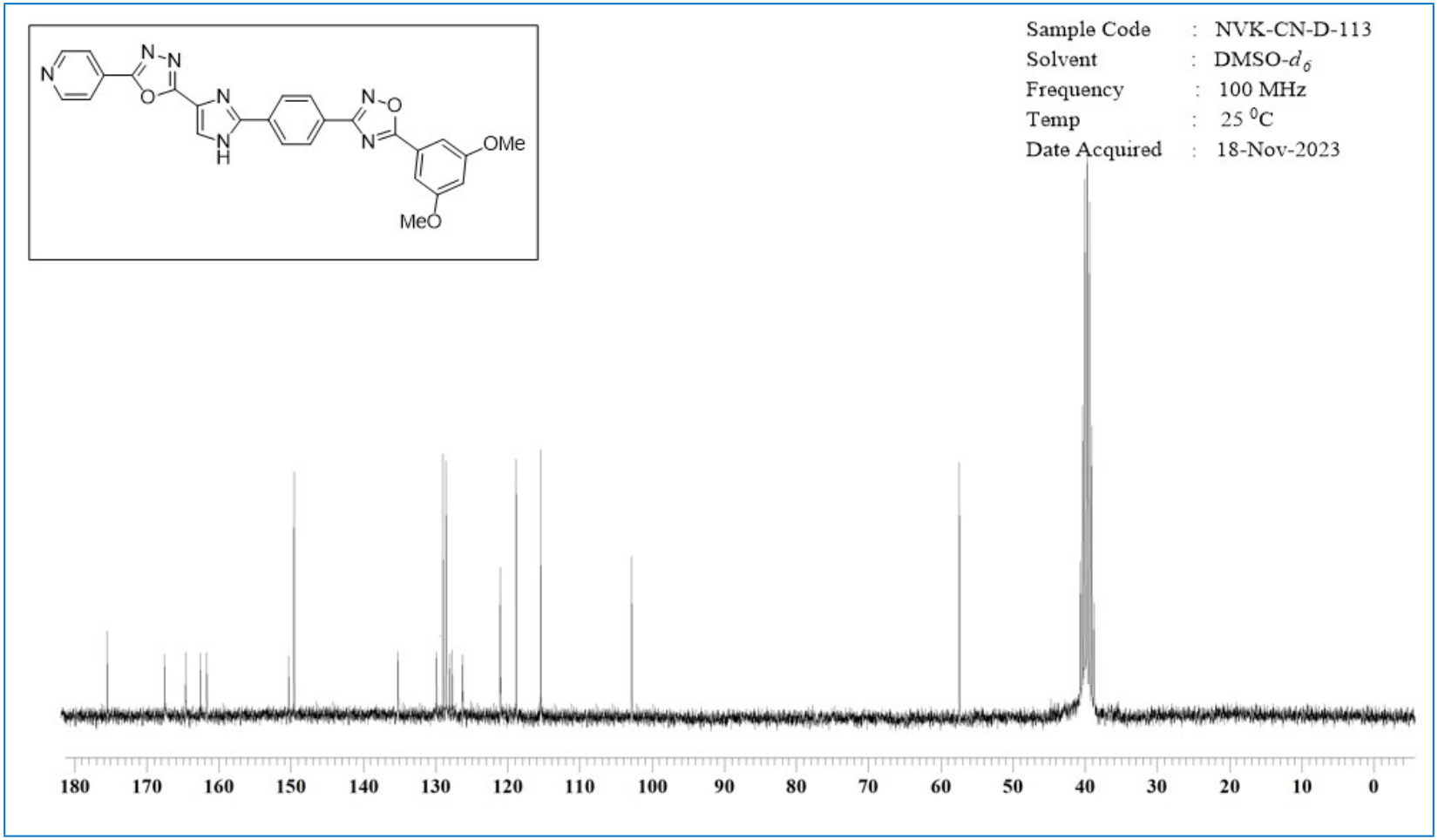
6.3. Compound 30a, HRMS Spectra



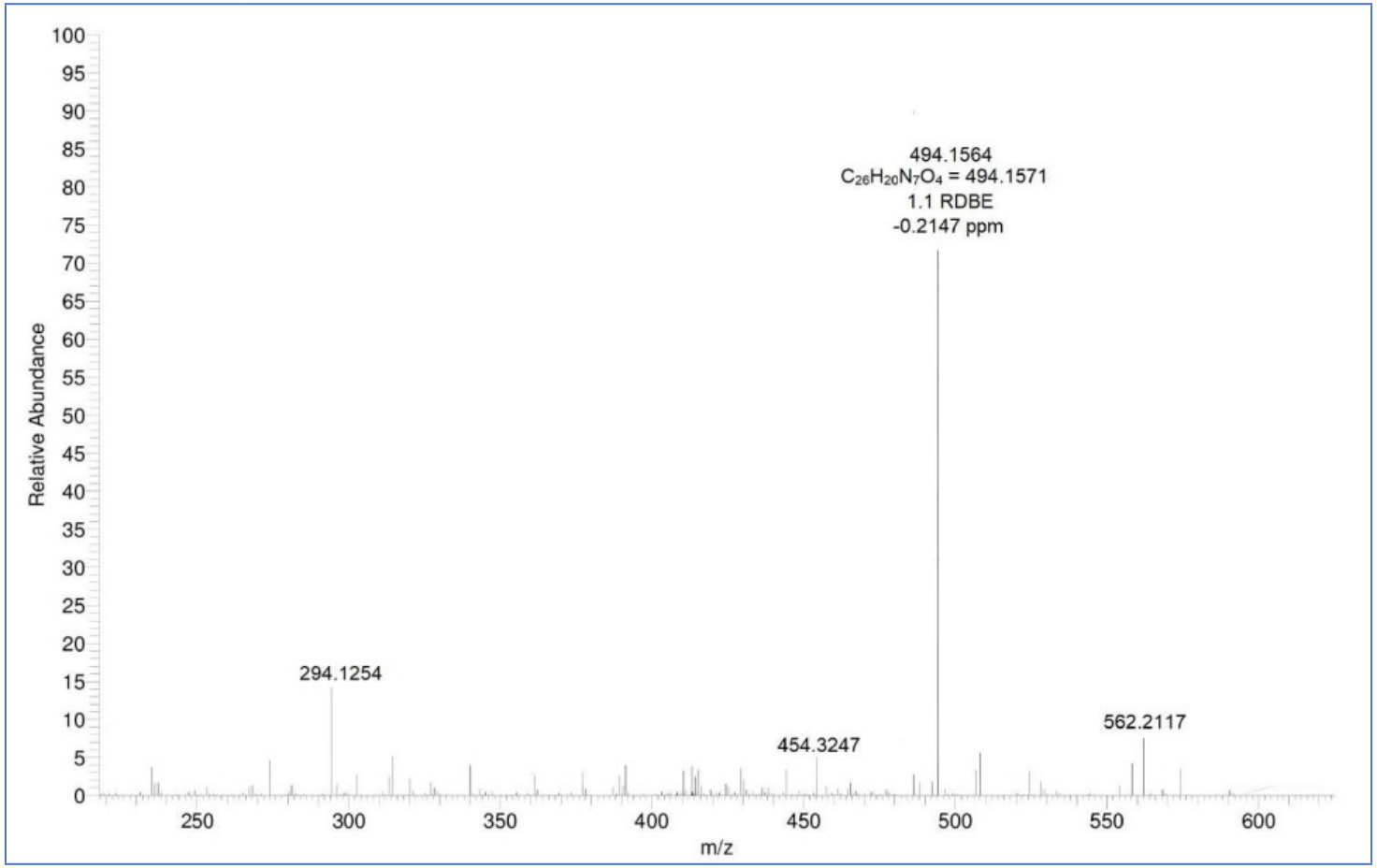
6.4. Compound 30b, 1H NMR (400 MHz, DMSO-d_6)



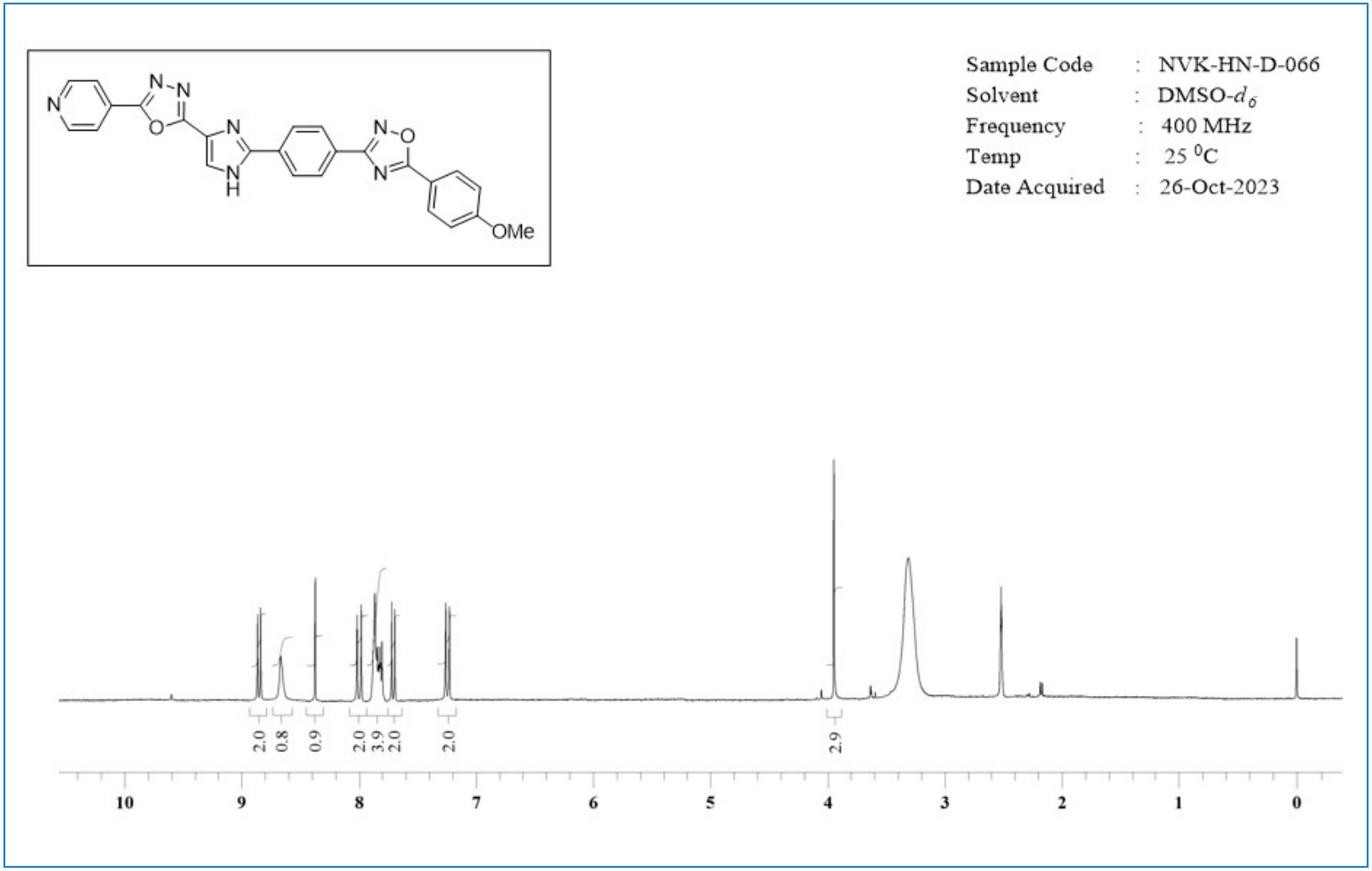
6.5. Compound 30b, ^{13}C NMR (100 MHz, DMSO-d_6)



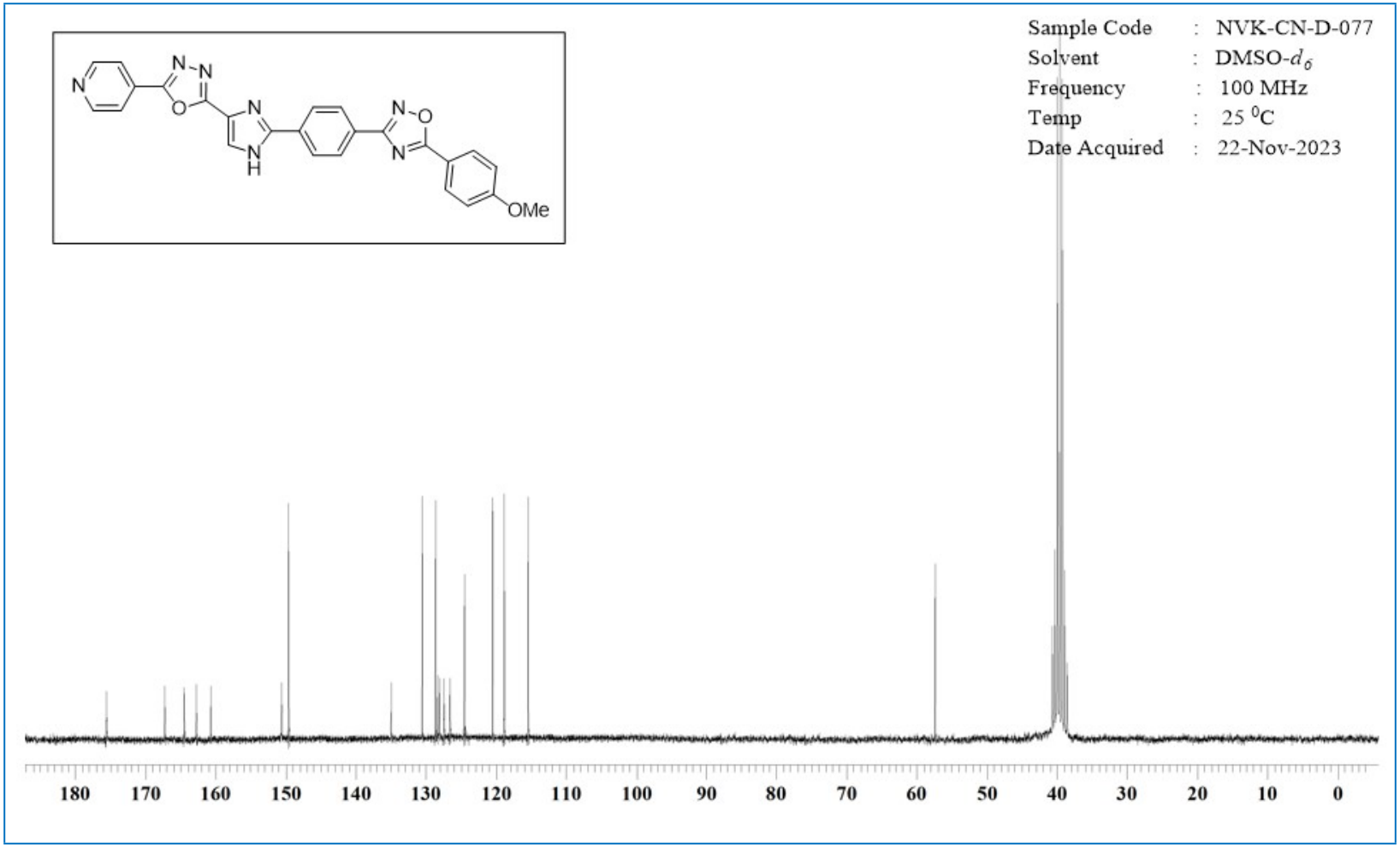
6.6. Compound 30b, HRMS Spectra



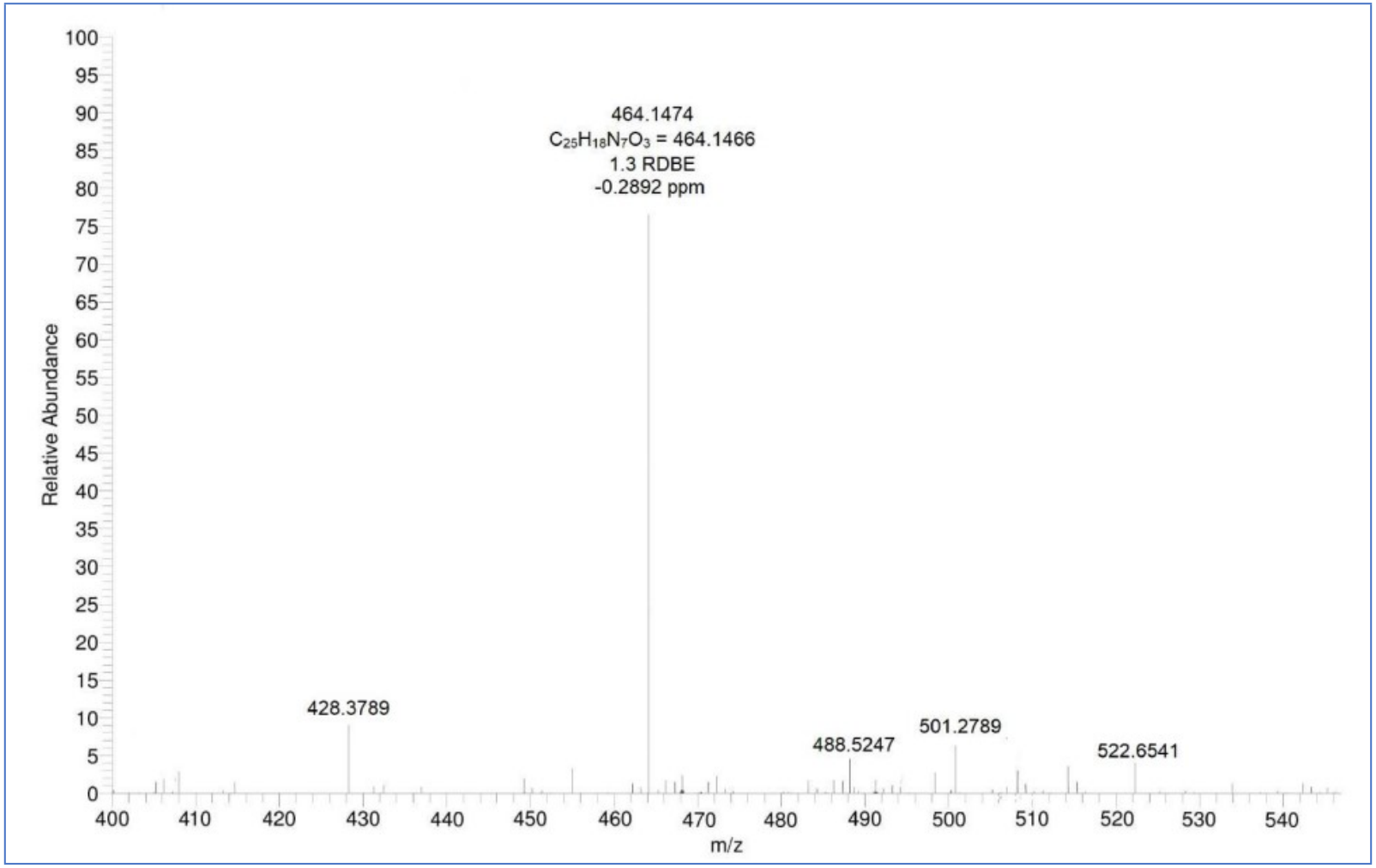
6.7. Compound 30c, ^{1}H NMR (400 MHz, DMSO-d_6)



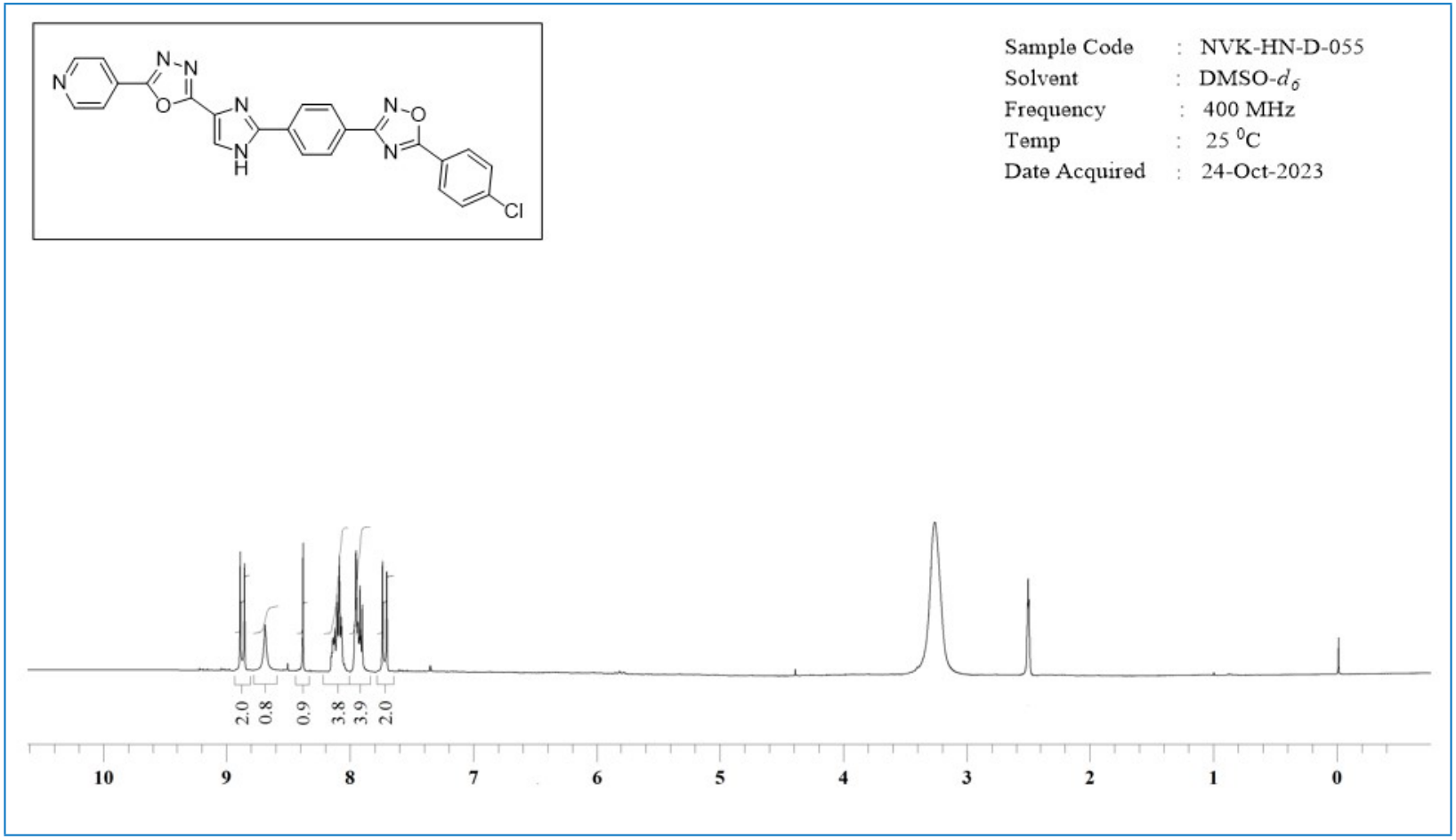
6.8. Compound 30c, ^{13}C NMR (100 MHz, DMSO-d_6)



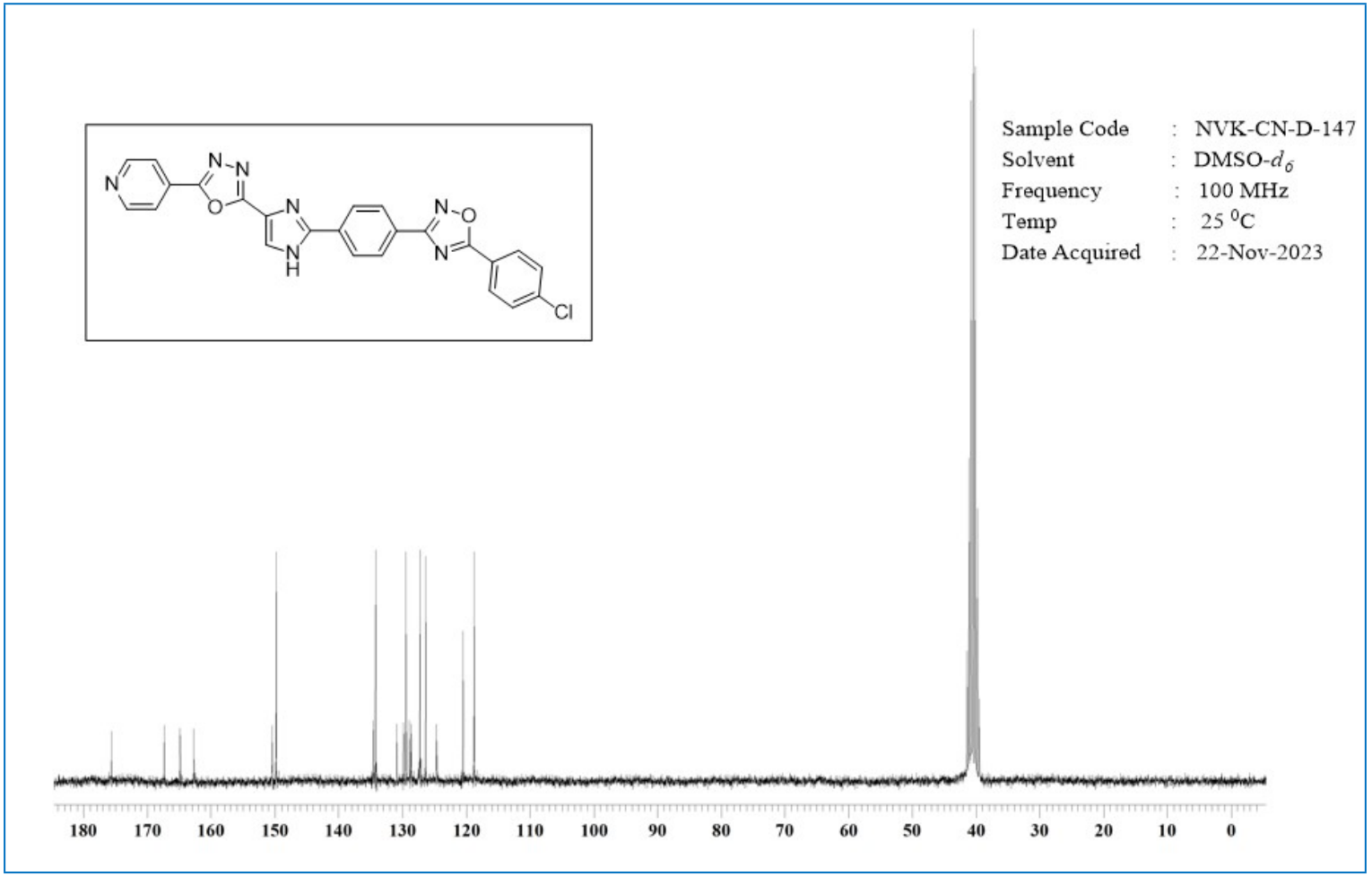
6.9. Compound 30c, HRMS Spectra



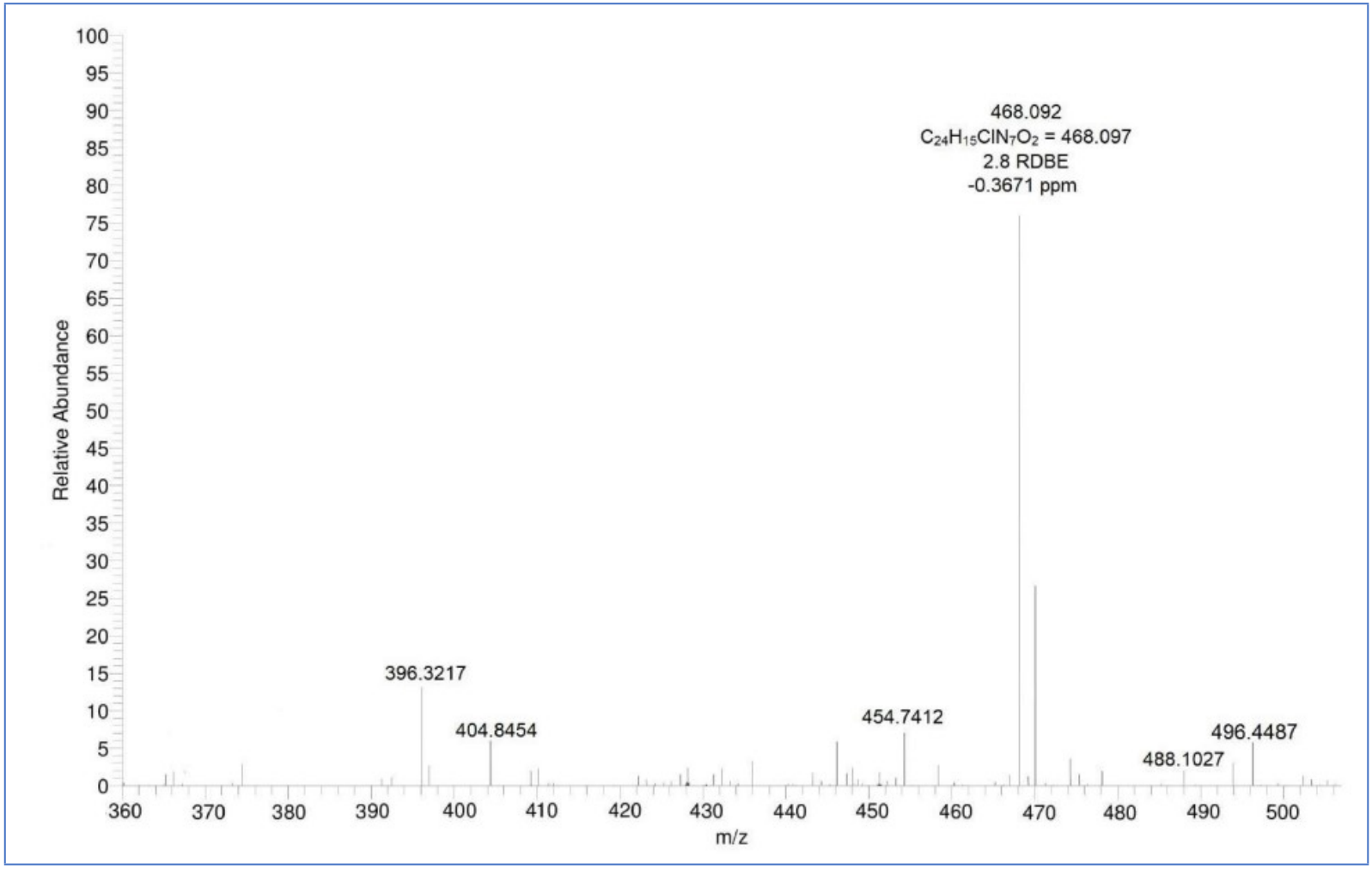
6.10. Compound 30d, ^{1}H NMR (400 MHz, DMSO-d_6)



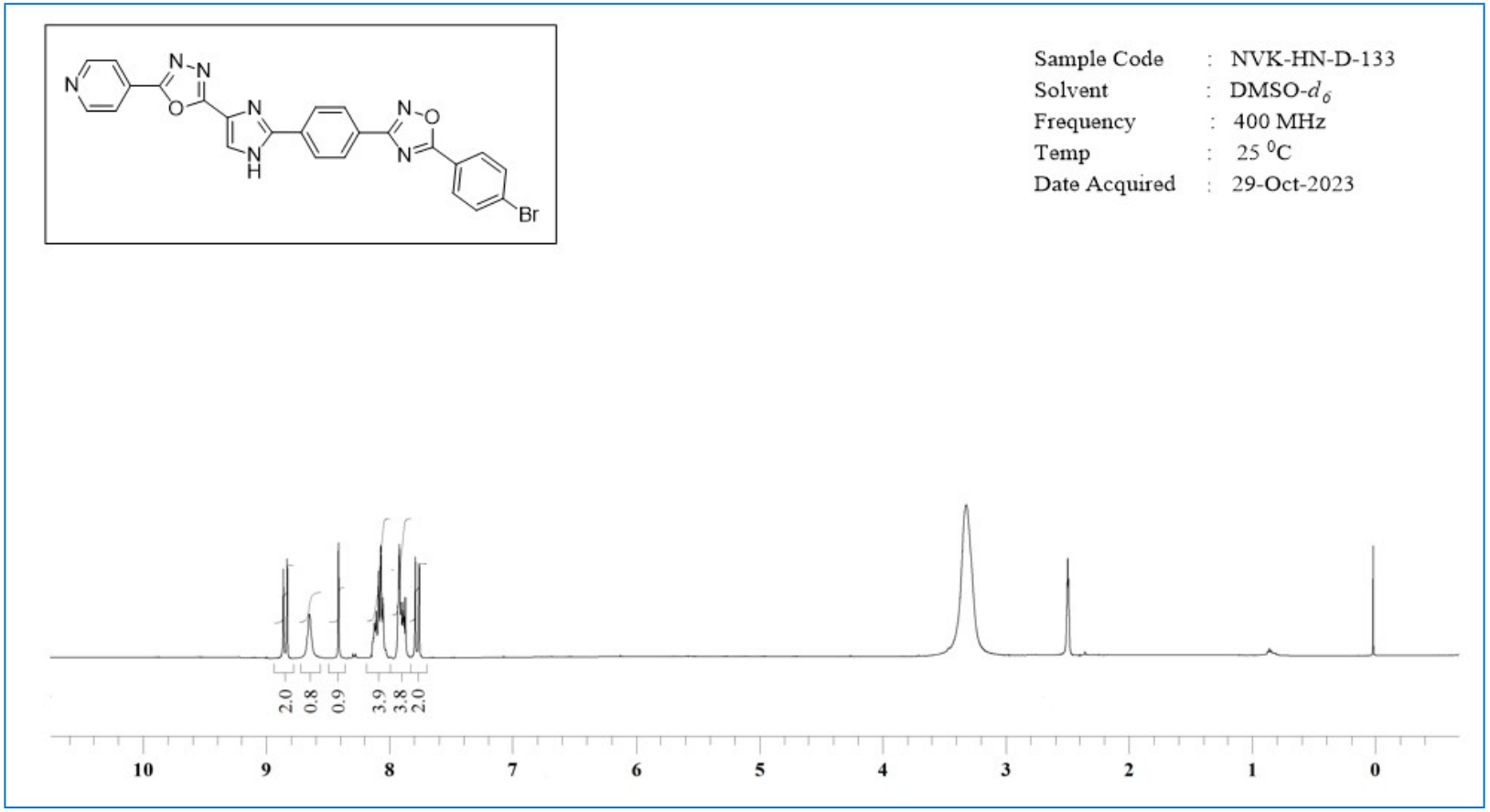
6.11. Compound 30d, ^{13}C NMR (100 MHz, DMSO-d_6)



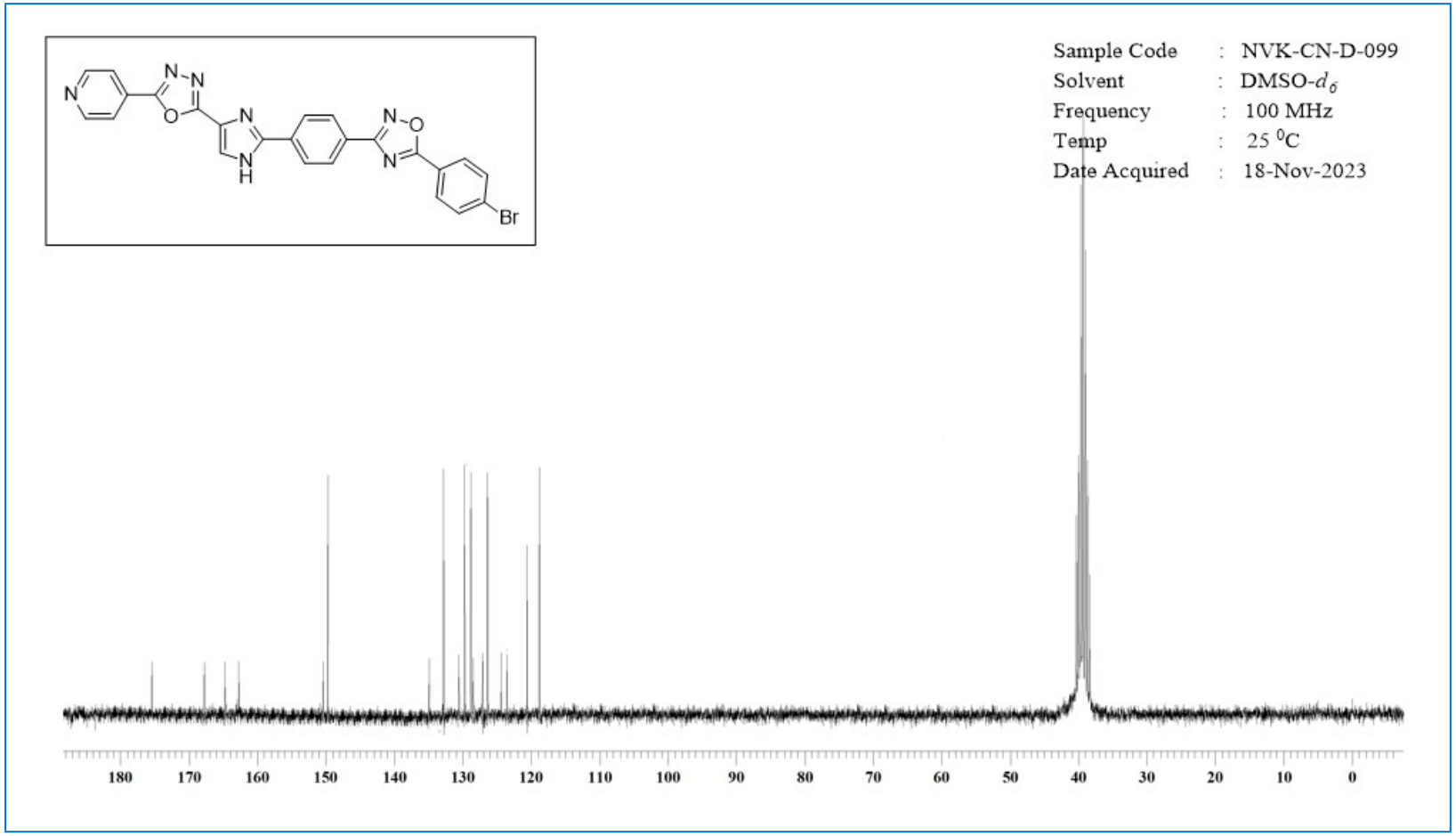
6.12. Compound 30d, HRMS Spectra



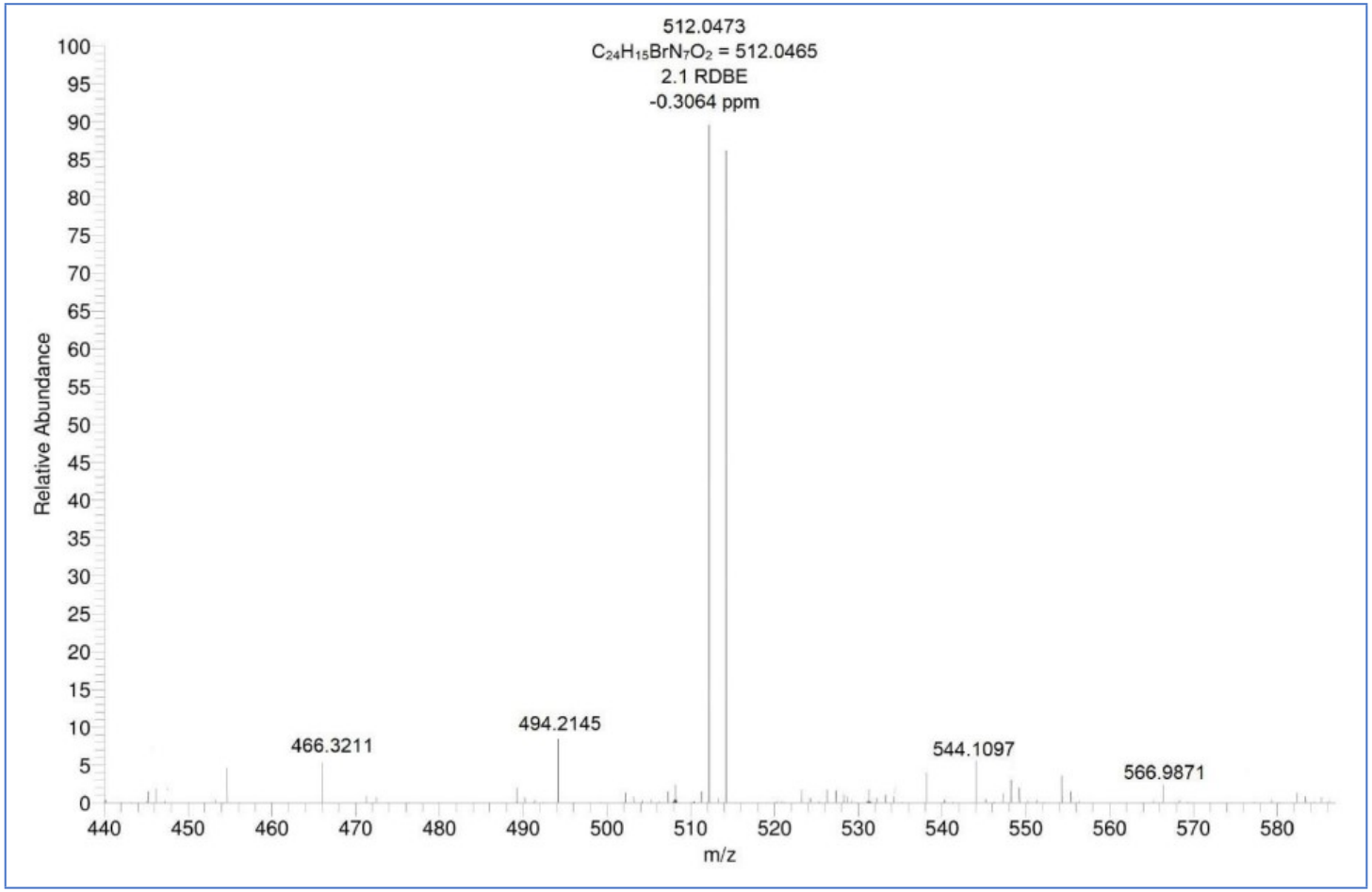
6.13. Compound 30e, ^{1}H NMR (400 MHz, DMSO-d_6)



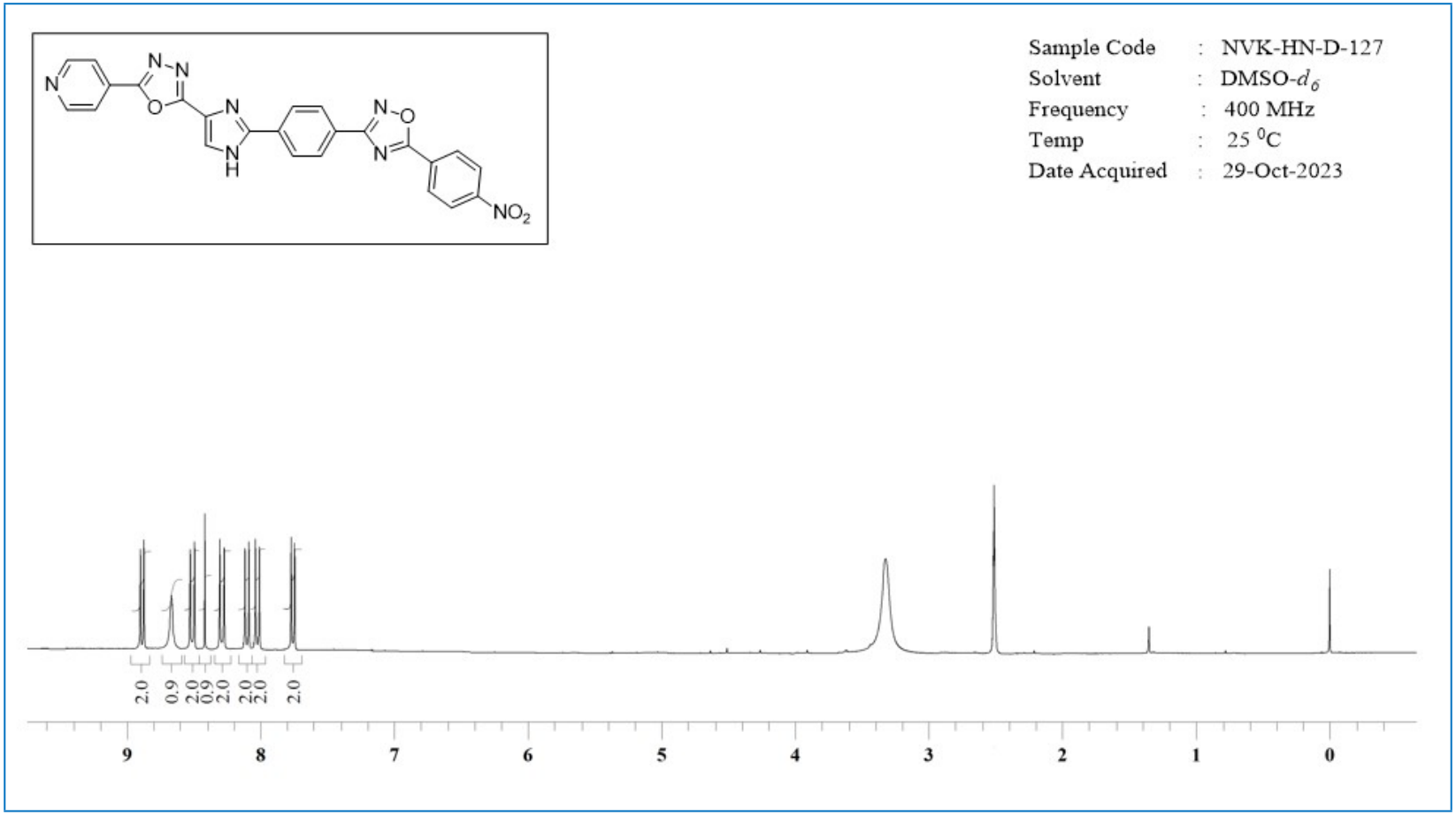
6.14. Compound 30e, ^{13}C NMR (100 MHz, DMSO-d_6)



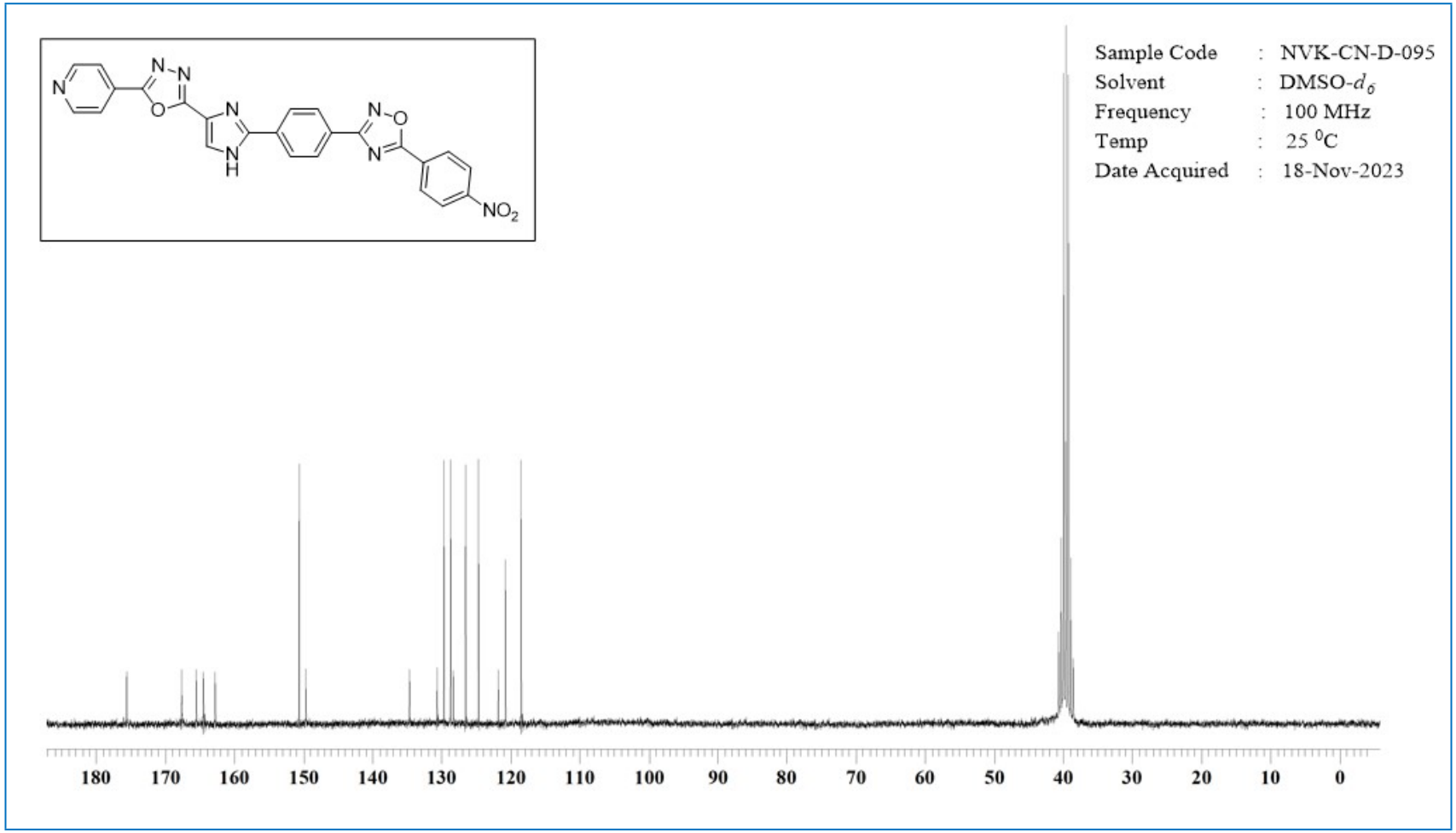
6.15. Compound 30e, HRMS Spectra



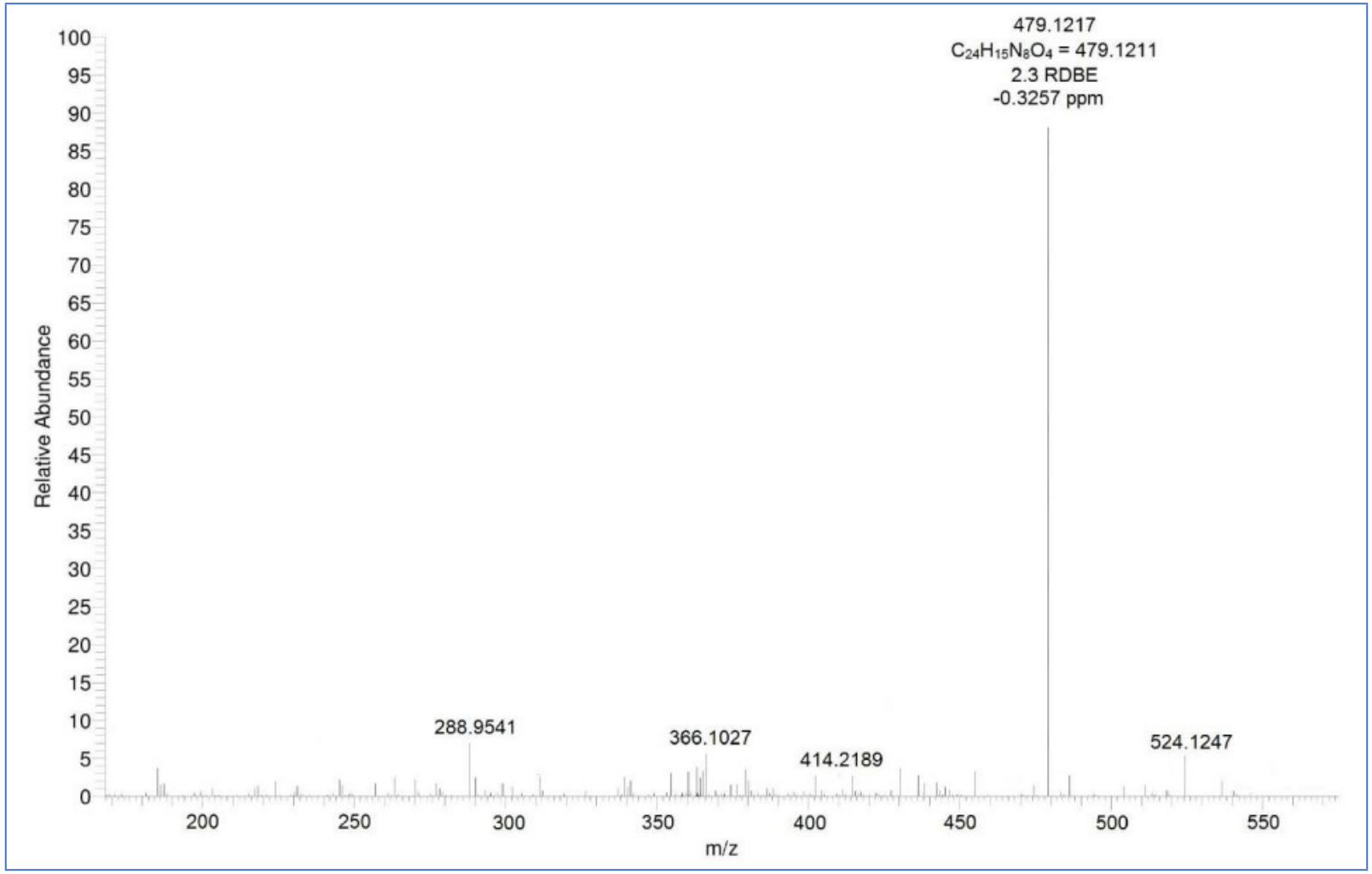
6.16. Compound 30f, ^{1}H NMR (400 MHz, DMSO-d_6)



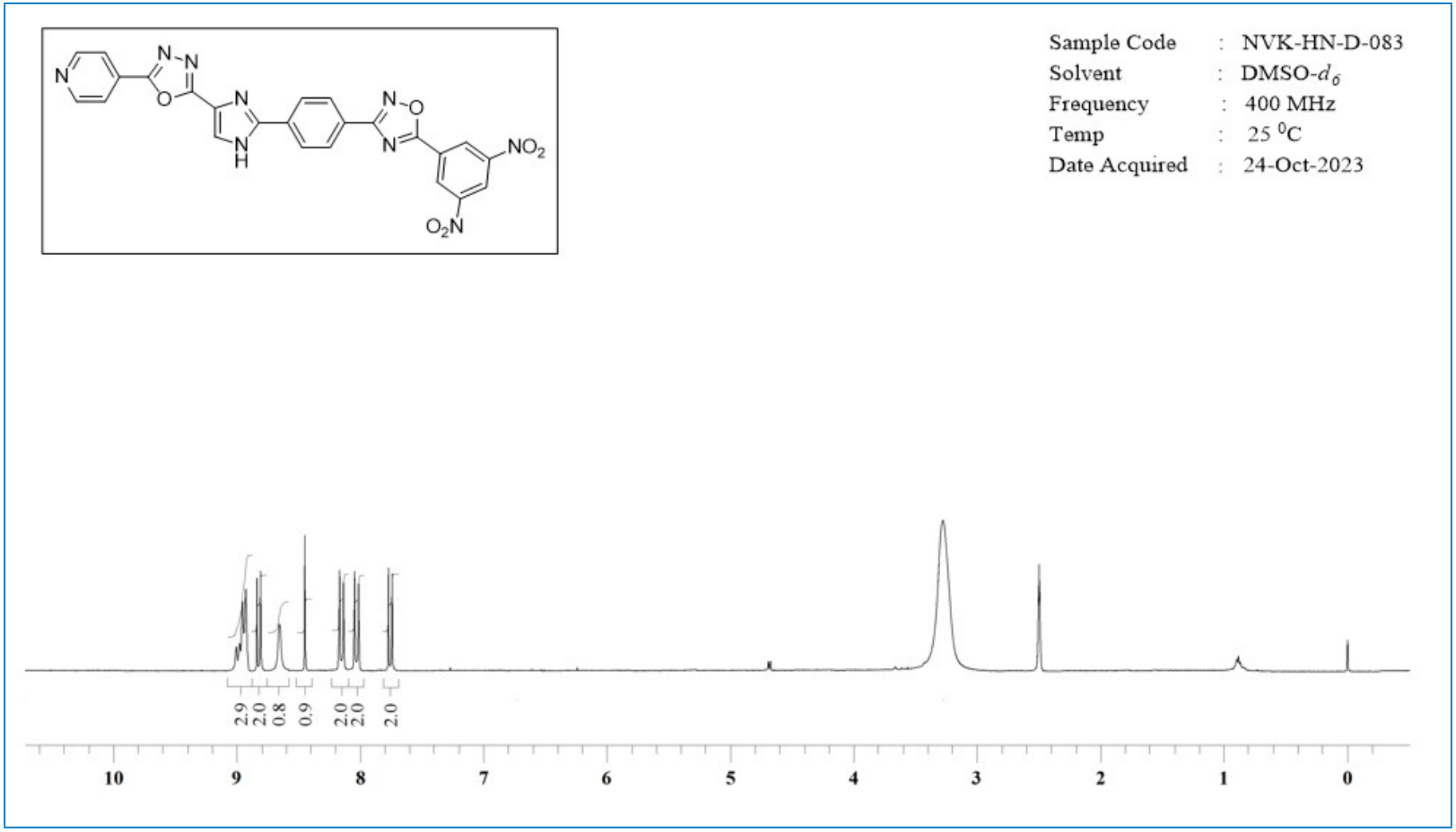
6.17. Compound 30f, ^{13}C NMR (100 MHz, DMSO-d_6)



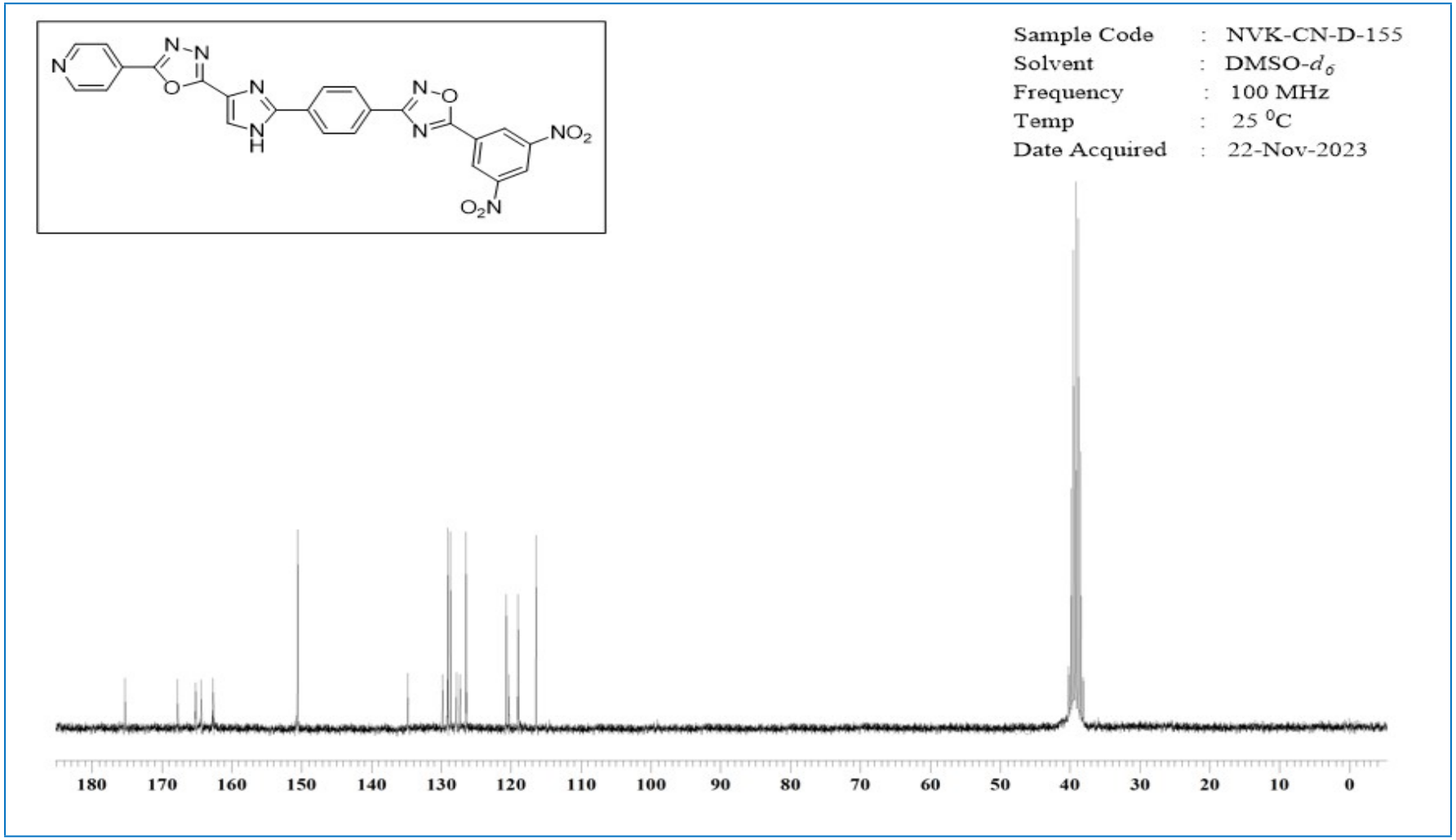
6.18. Compound 30f, HRMS Spectra



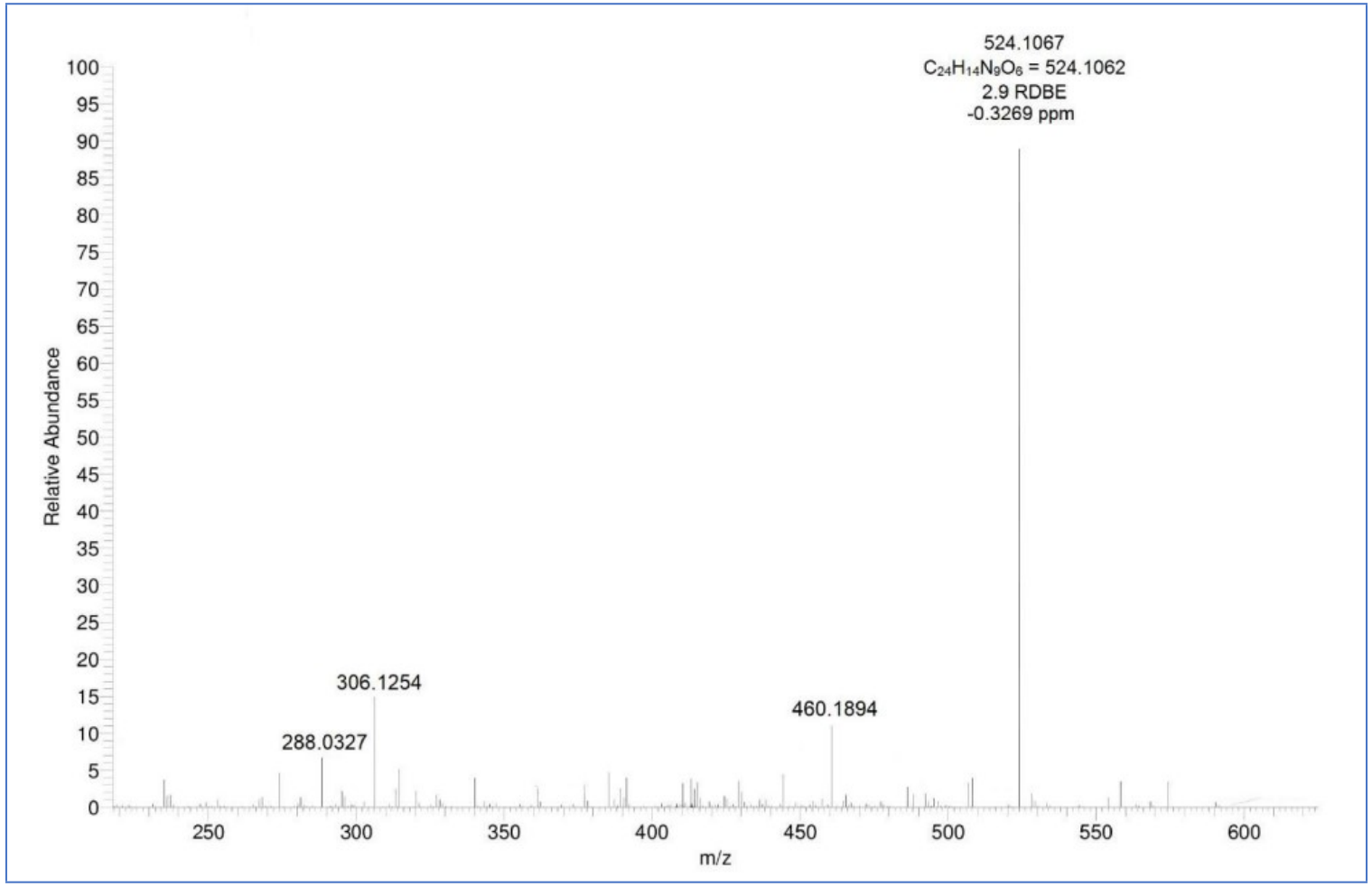
6.19. Compound 30g, ^{1}H NMR (400 MHz, DMSO-d_6)



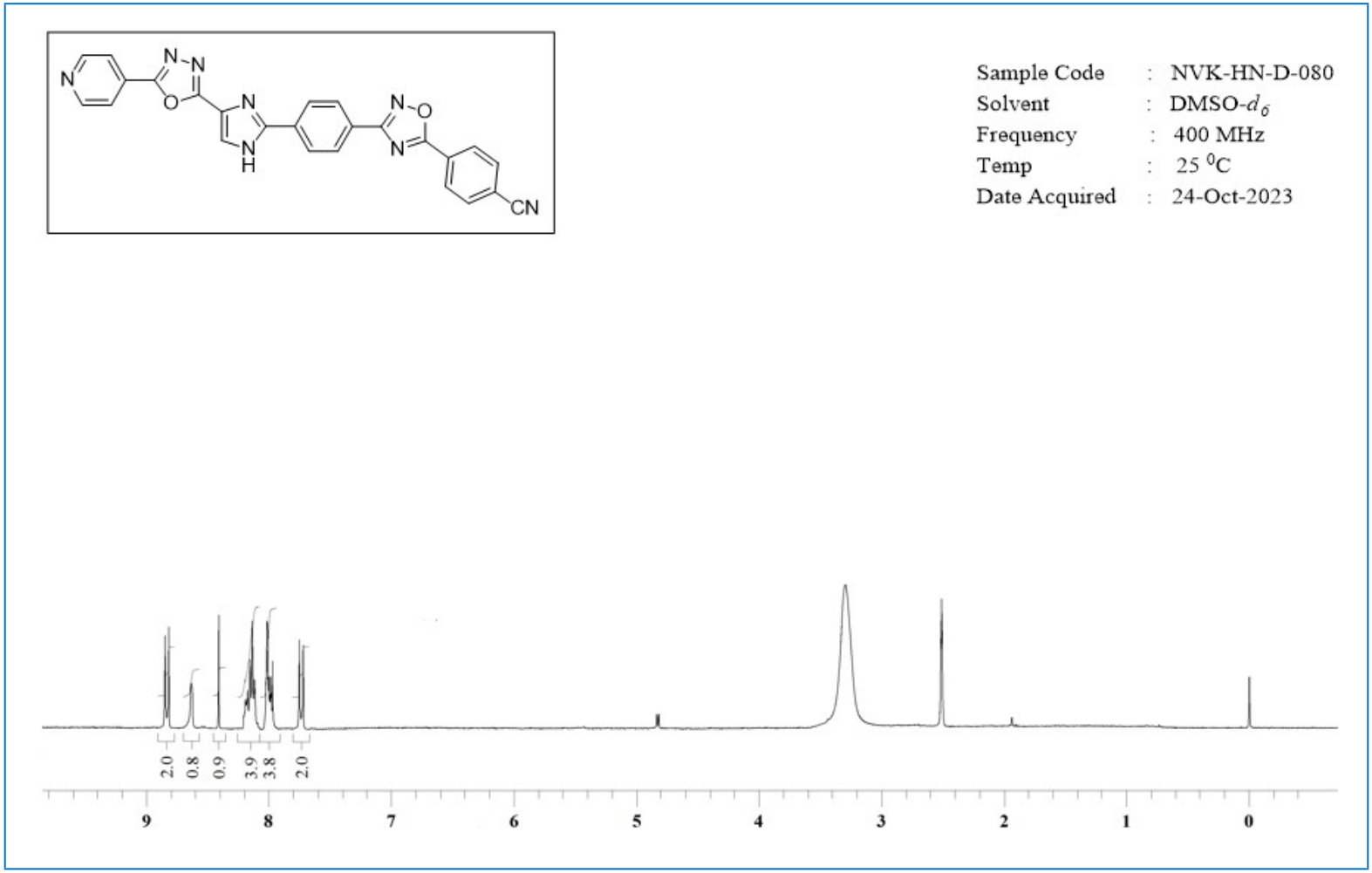
6.20. Compound 30g, ^{13}C NMR (100 MHz, DMSO-d_6)



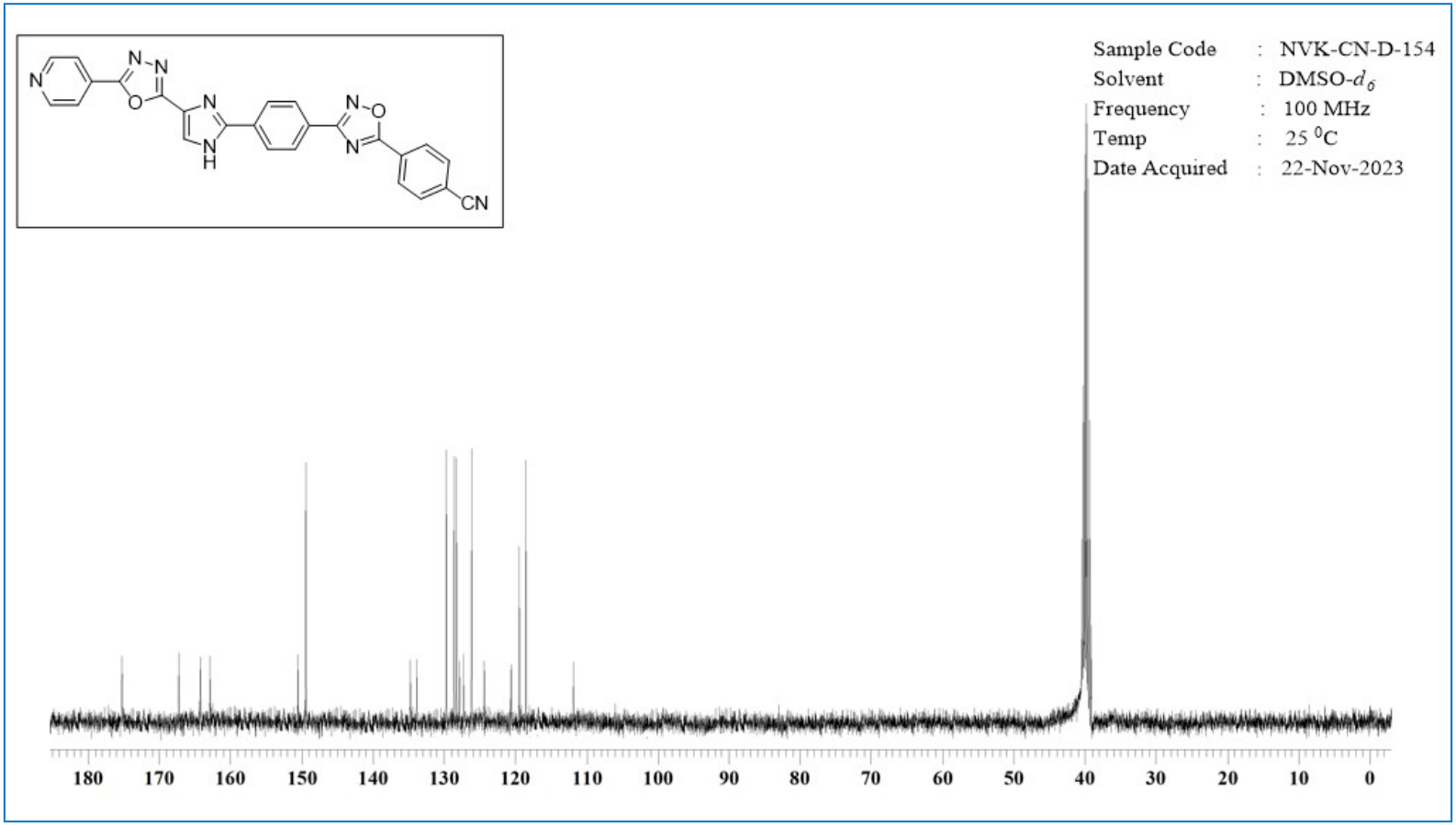
6.21. Compound 30g, HRMS Spectra



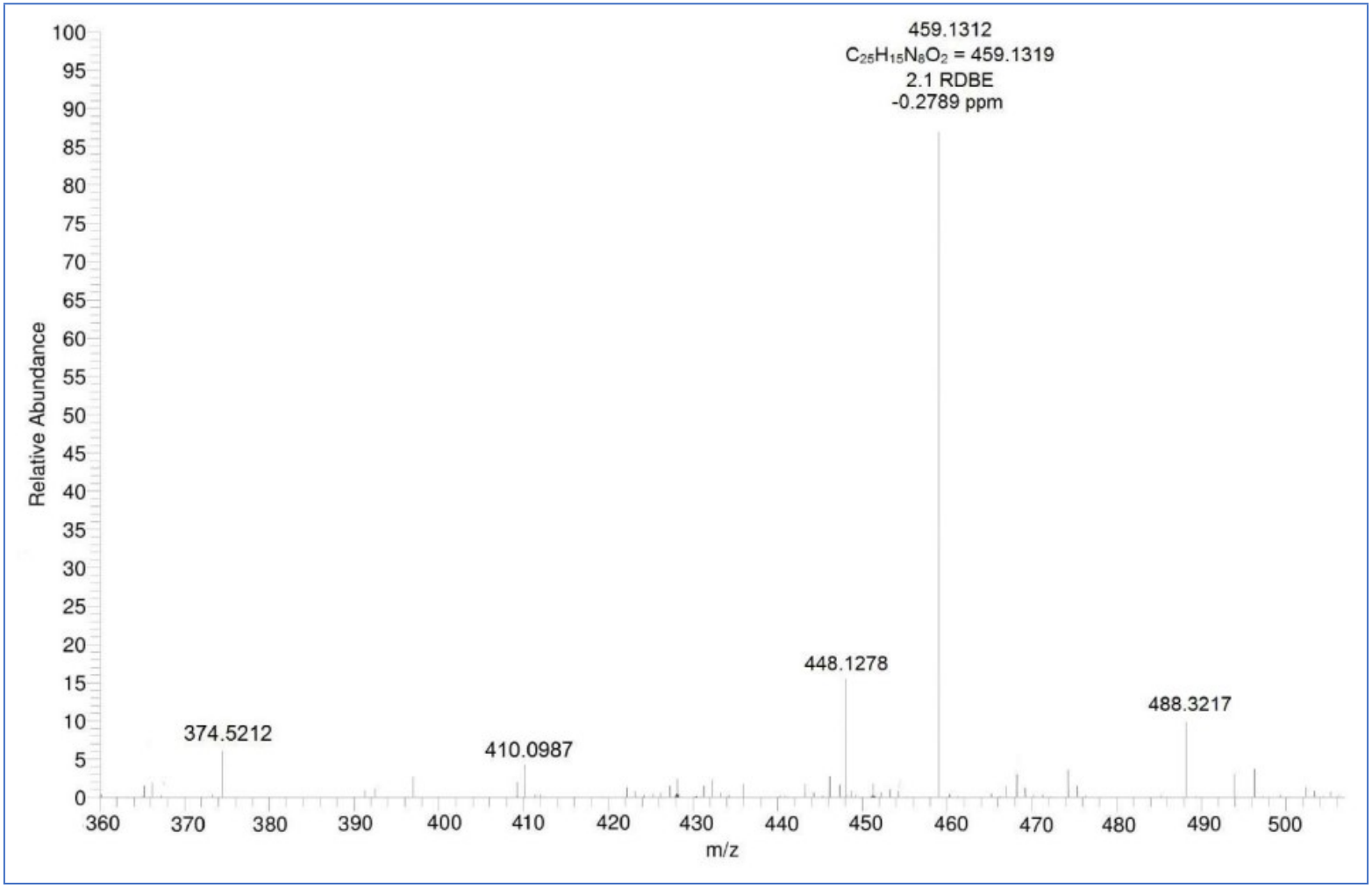
6.22. Compound 30h, ^{1}H NMR (400 MHz, DMSO-d_6)



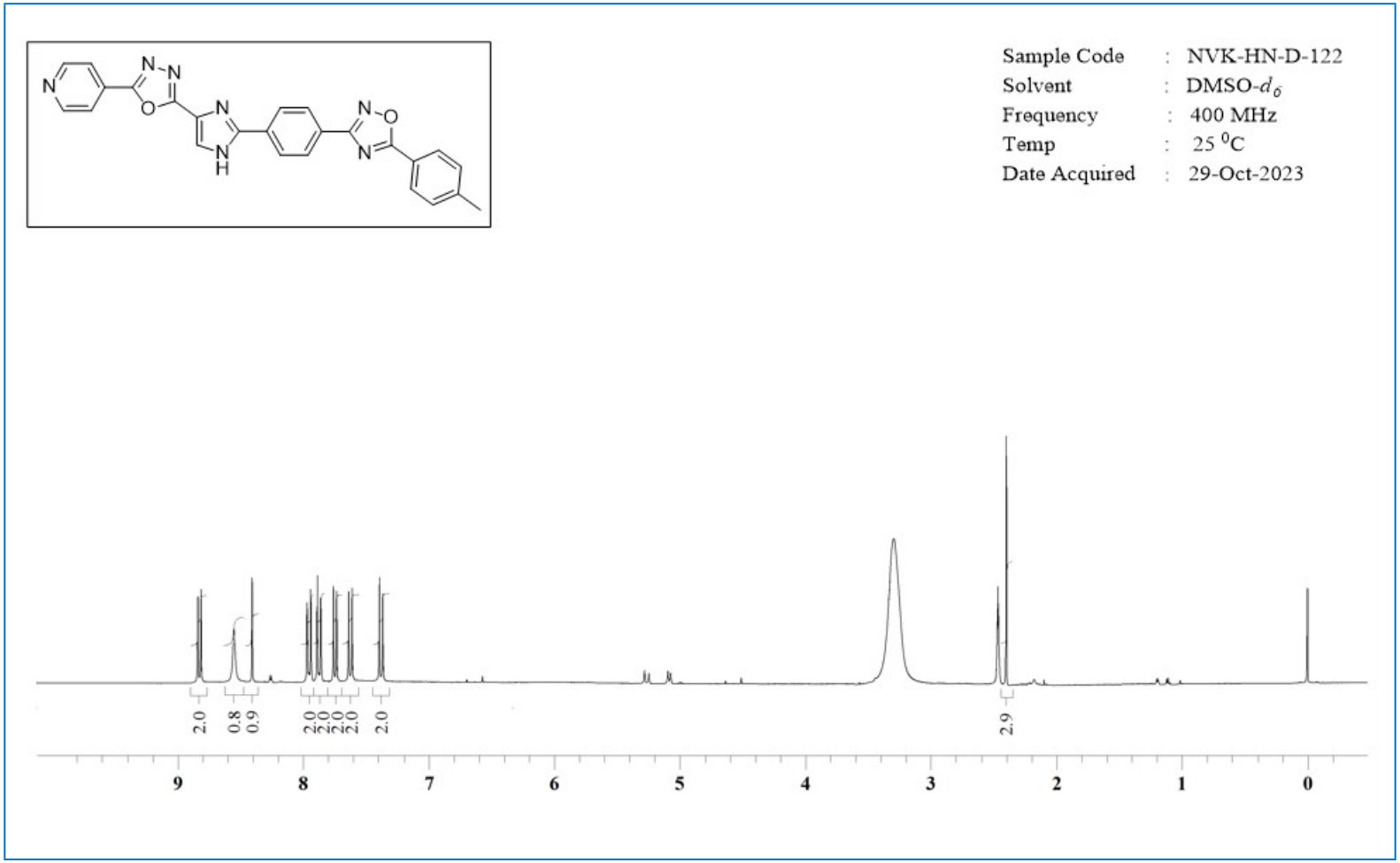
6.23. Compound 30h, ^{13}C NMR (100 MHz, DMSO-d_6)



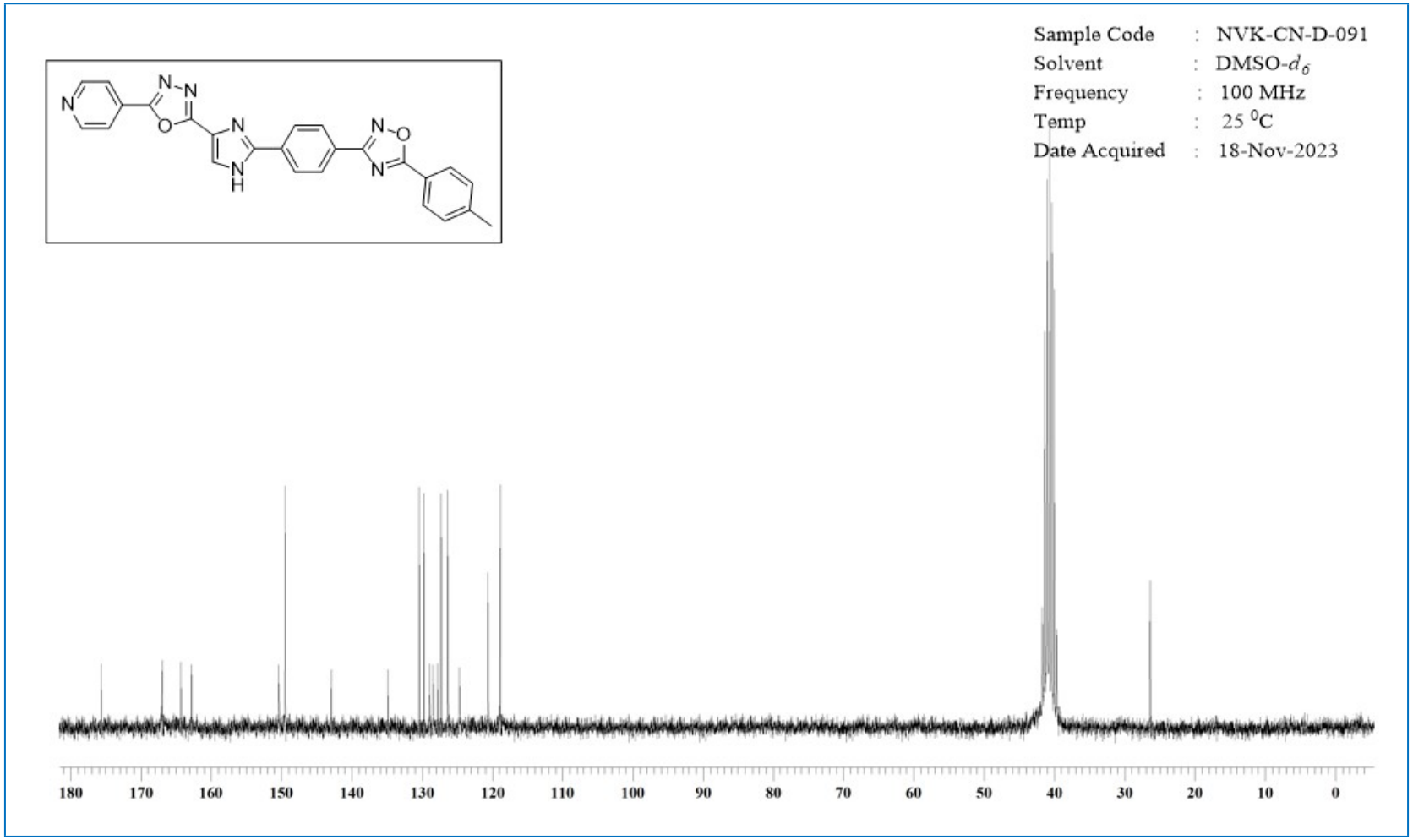
6.24. Compound 30h, HRMS Spectra



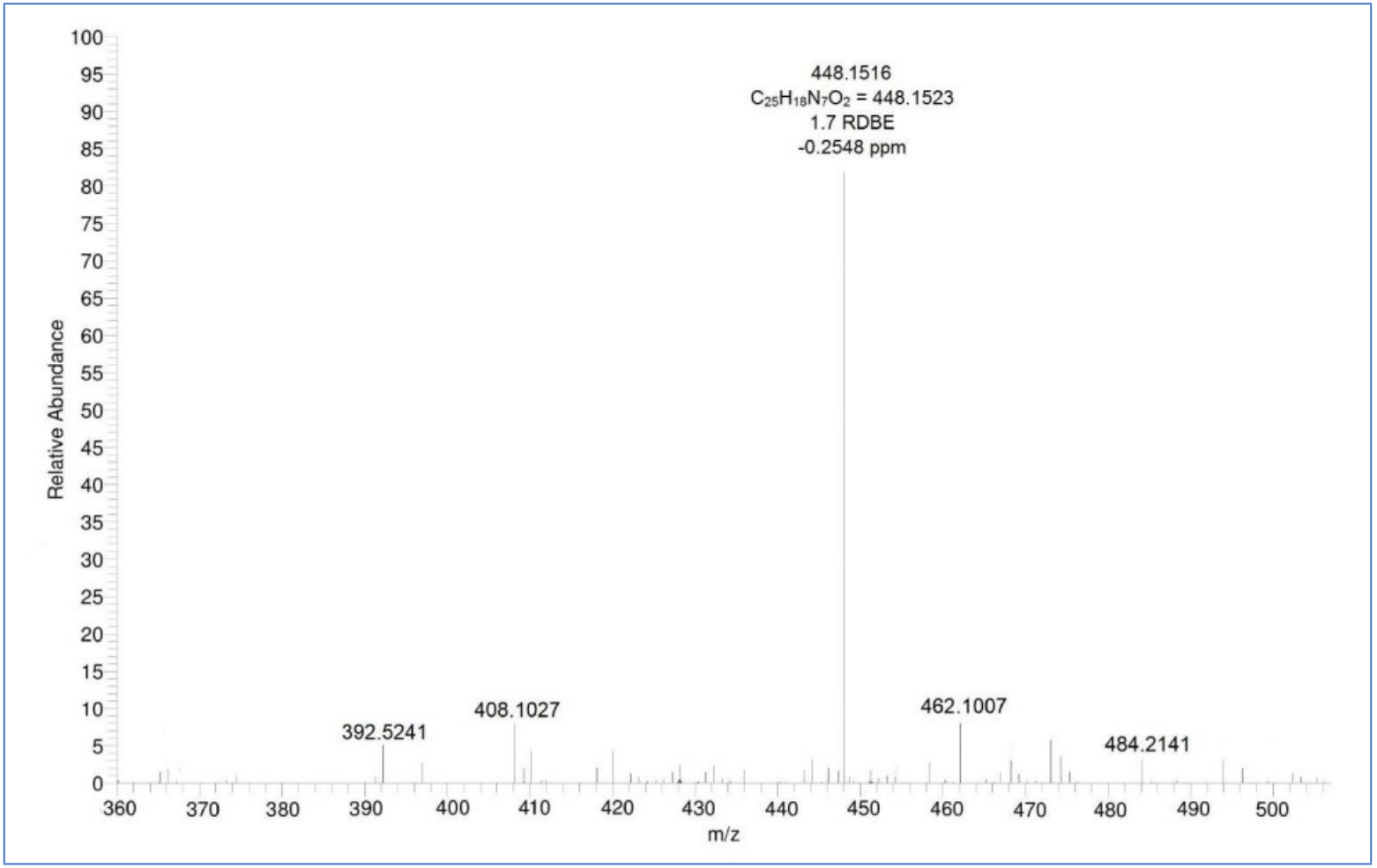
6.25. Compound 30i, ^{1}H NMR (400 MHz, DMSO-d_6)



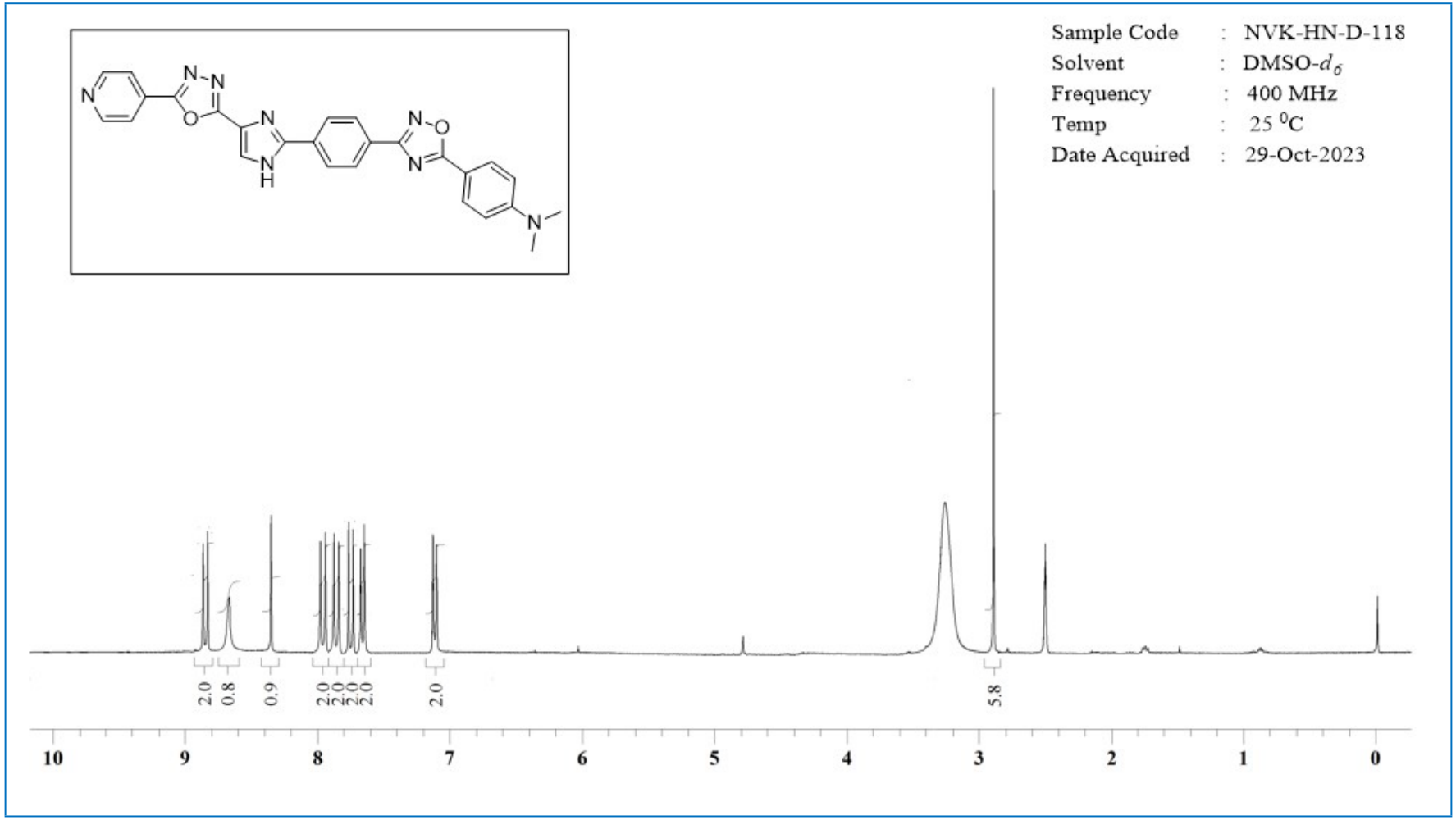
6.26. Compound 30i, ^{13}C NMR (100 MHz, DMSO-d_6)



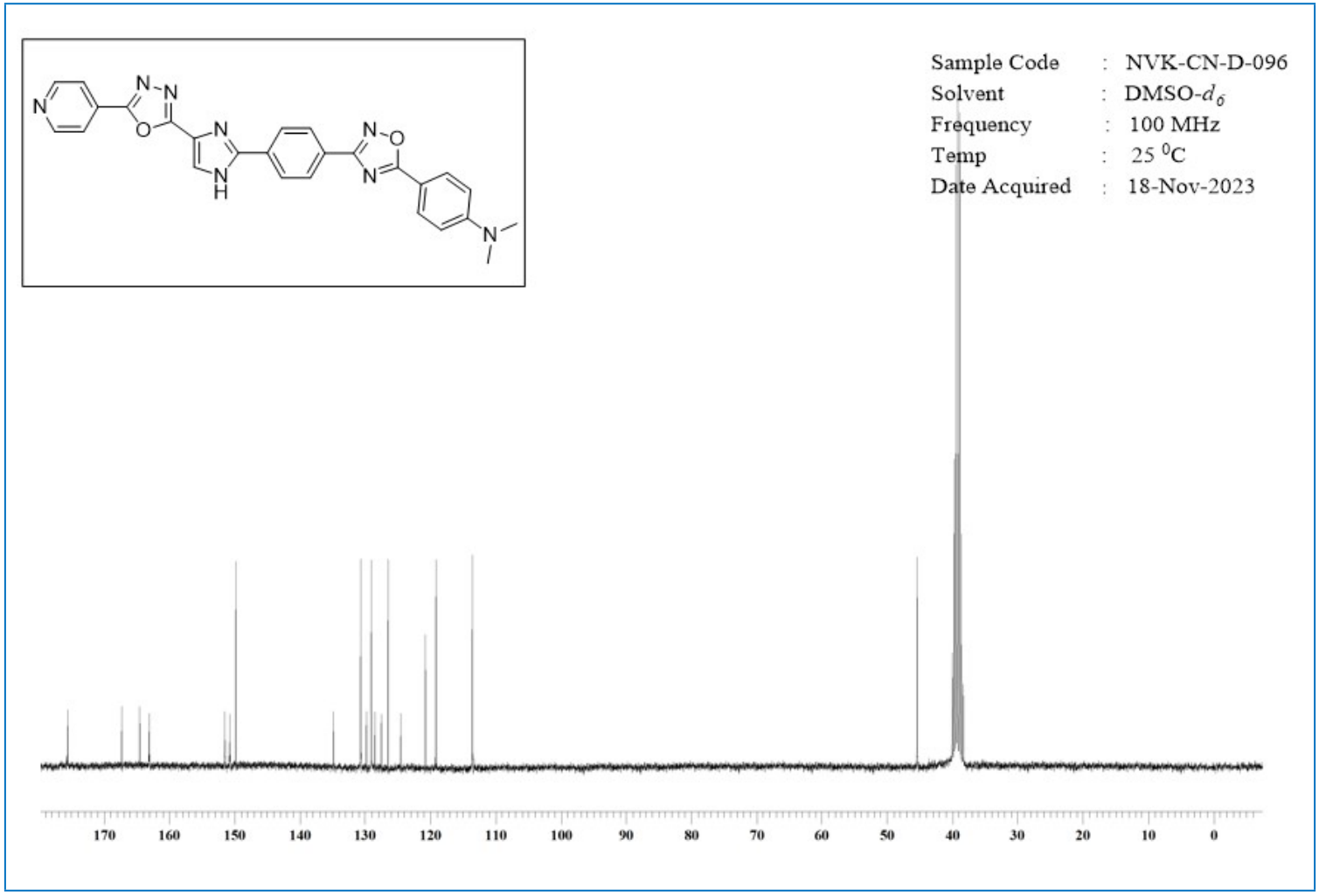
6.27. Compound 30i, HRMS Spectra



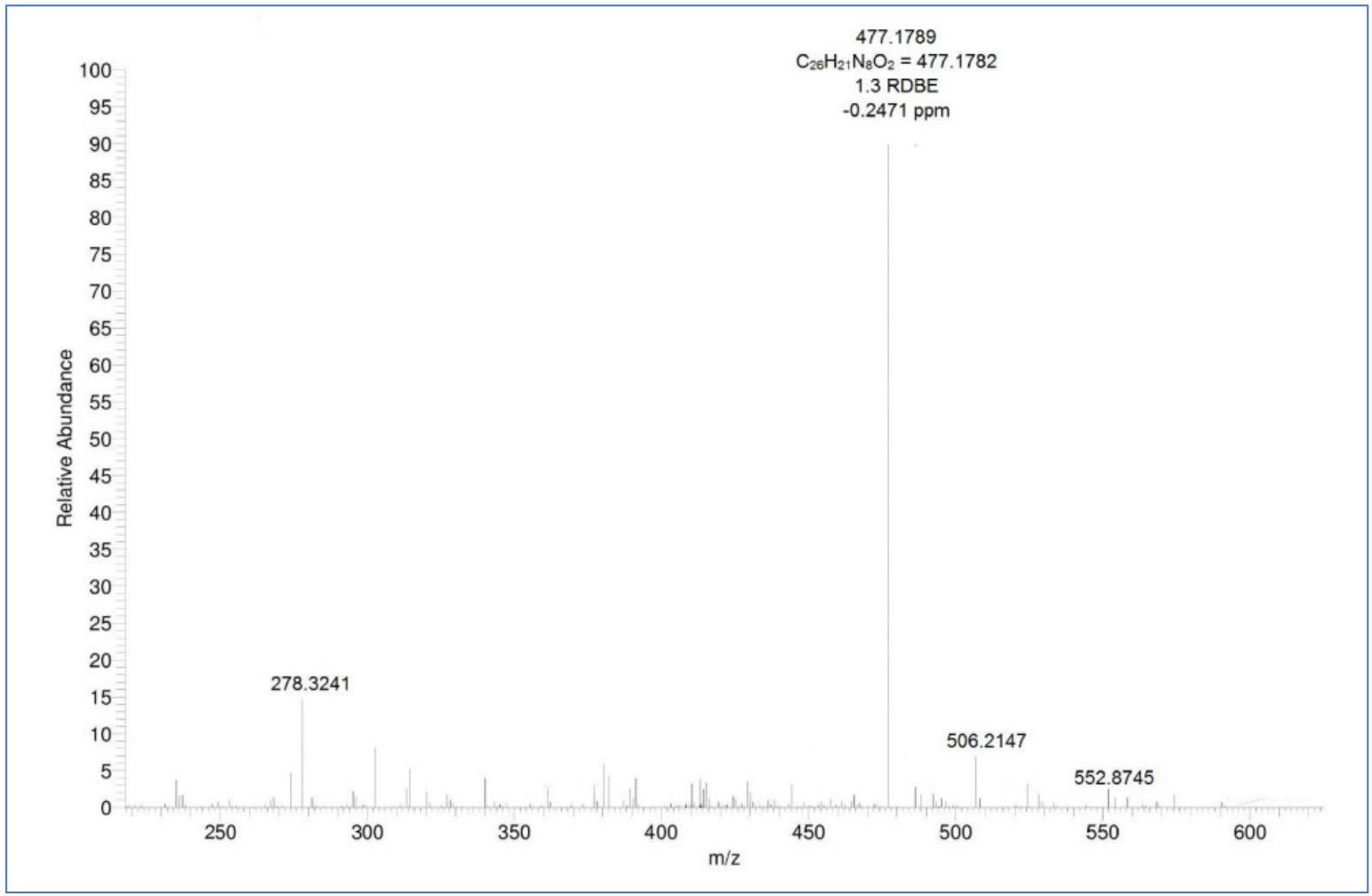
6.28. Compound 30j, ^{1}H NMR (400 MHz, DMSO-d_6)



6.29. Compound 30j, ^{13}C NMR (100 MHz, DMSO-d_6)



6.30. Compound 30j, HRMS Spectra



7. Table S1: Binding affinity values of all synthesized compounds against $EGFR^{WT}$ (PDB ID: 1M17)

Compound	Binding Affinity (Kcal/mol)
	1M17 (PDB ID: 1DI8)
30a	-9.468
30b	-9.361
30c	-9.401
30d	-9.012
30e	-8.966
30f	-8.896
30g	-8.987
30h	-8.739
30i	-9.113
30j	-8.989
Co-crystal Ligand	-9.131

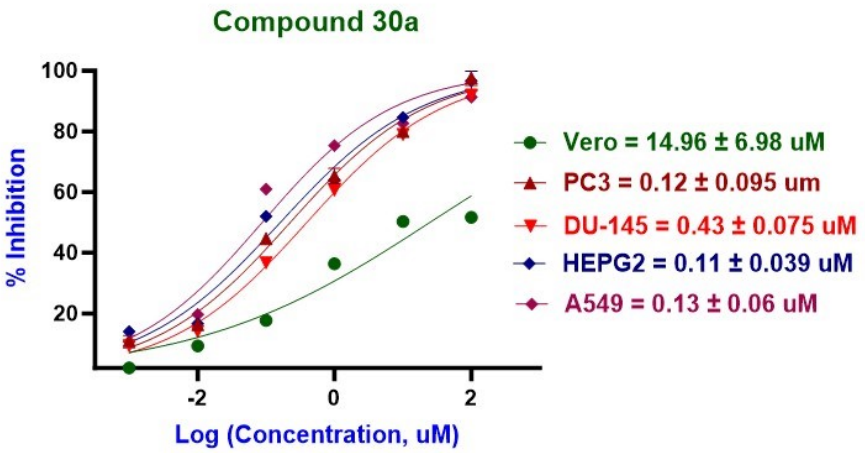
8. Table S2: In silico Bioavailability parameters of synthesized compounds

Compound	Mol Wt	cLogP	cLogS	HBA	HDA	TPSA	PSA	Druglikeness	Rof5
30a	523.51	3.38	-9.17	12	1	400.35	147.1	2.79	2
30b	493.48	3.45	-9.15	11	1	378.09	137.87	2.79	1
30c	463.46	3.52	-9.13	10	1	355.83	128.64	2.79	0
30d	467.88	4.20	-9.85	9	1	348.99	119.41	2.80	0
30e	512.33	4.32	-9.95	9	1	352.2	119.41	0.96	1
30f	478.43	2.67	-9.57	12	1	357.24	165.23	-2.35	1
30g	523.42	1.75	-10.03	15	1	380.91	211.05	-2.35	2
30h	458.44	3.43	-9.88	10	1	355.28	143.2	-1.53	0
30i	447.46	3.94	-9.46	9	1	345.83	119.41	2.69	0
30j	476.50	3.49	-9.15	10	1	368.39	122.65	3.59	0

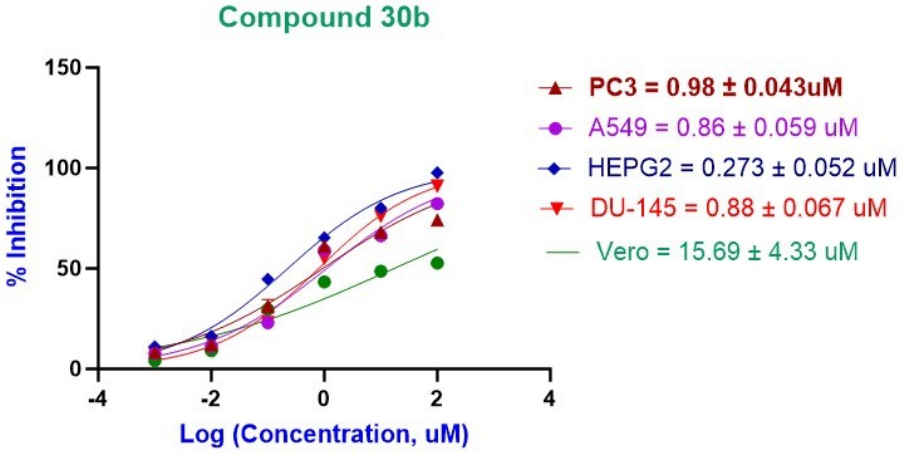
9. Table S3: Toxicity studies of synthesized compounds

Compound	Mutagenic	Tumorigenic	Reproductive Effective	Irritant
30a	none	none	none	none
30b	none	none	none	none
30c	none	none	none	none
30d	none	none	none	none
30e	none	none	none	none
30f	none	none	none	none
30g	none	none	none	none
30h	none	none	none	none
30i	none	none	none	none
30j	none	high	none	none

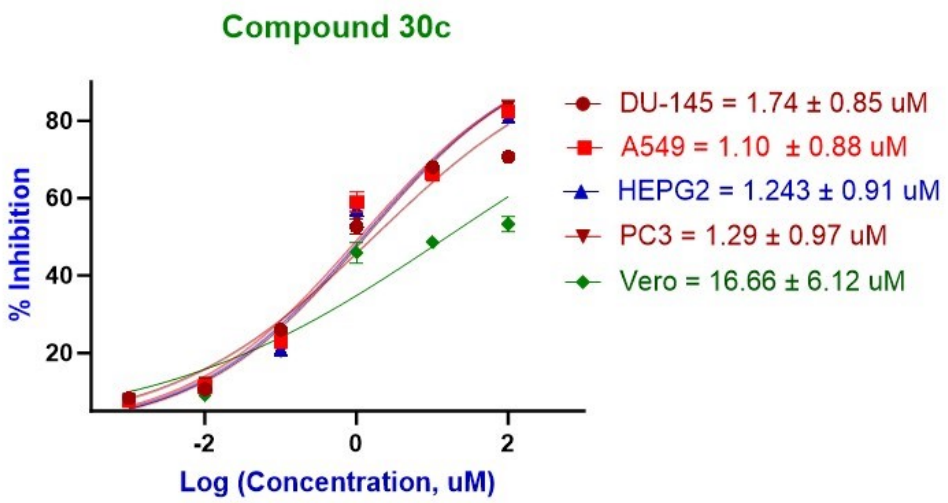
10. Inhibitory graph for compound 30a-c against all the cell lines tested and DRC curves for reference Erlotinib and compound 30a-c against WT & Mutant EGFR:



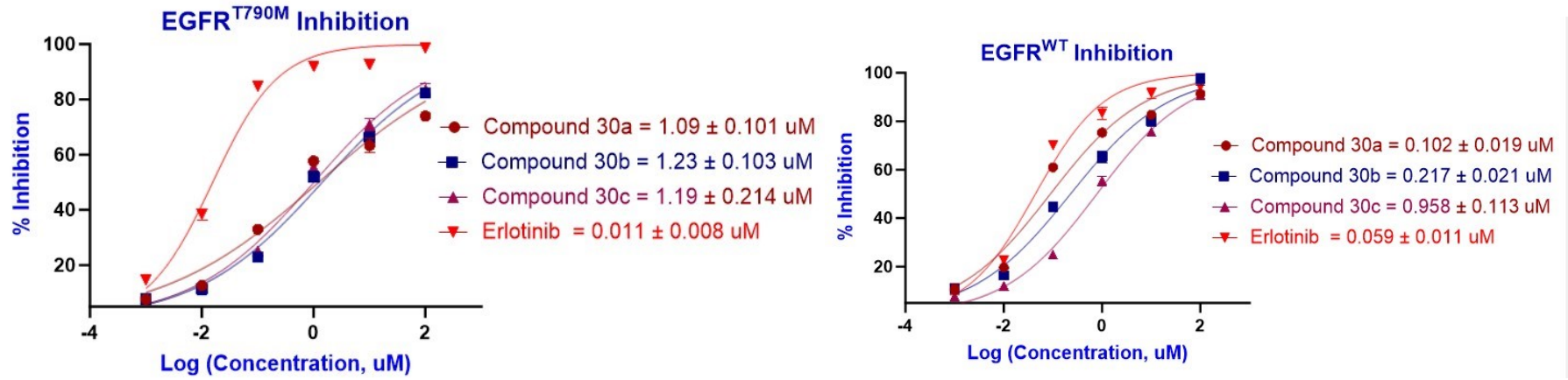
A



B


Compound 30c
% Inhibition
80
60
40
20
-2
0
2
Log (Concentration, uM)
DU-145 = 1.74 ± 0.85 uM
A549 = 1.10 ± 0.88 uM
HEPG2 = 1.243 ± 0.91 uM
PC3 = 1.29 ± 0.97 uM
Vero = 16.66 ± 6.12 uM

C



D E

Figure S1: IC_{50} graphs for reference Erlotinib and compound **30a-c.**

11. References

1. N. Rezki, M.A. Almehmadi, S. Ihmaid, A.M. Shehata, A.M. Omar, H.E.A. Ahmed, M. R. Aouad, Novel scaffold hopping of potent benzothiazole and isatin analogues linked to 1,2,3-triazole fragment that mimic quinazoline epidermal growth factor receptor inhibitors: Synthesis, antitumor and mechanistic analyses, Bioorg. Chem. 103 (2020), 104133; DOI: 10.1016/j.bioorg.2020.104133.
2. K.H.C. Demirci, H.C. Baser, Bioassay techniques for drug development by Atta-ur Rahman, M. Iqbal Choudhary (HEJRIC, University of Karachi, Pakistan), William J. Thomsen (Areana Pharmaceuticals, San Diego, CA). Harwood Academic Publishers, Amsterdam, The

Netherlands. 2001. xii + 223 pp. 15.5 × 23.5 cm. $79.00. ISBN 90-5823-051-1, J. Natural Products 2002, 65, 1086-1087; DOI: 10.1021/np020725b.

3. A. P. Demchenko, Beyond annexin V: fluorescence response of cellular membranes to apoptosis. Cytotechnology 65, 157–172 (2013). DOI: 10.1007/s10616-012-9481-y.
4. T. A. Warhi, A. Ahmed, A. L. Karmalawy, A. A. Elmaaty, M. A. Alshubramy, M. A. Motaal, T. A. Majrashit, M. Asem, A. Nabil, W. M. Eldehn, M. Sharaky, Biological evaluation, docking studies, and in silico ADME prediction of some pyrimidine and pyridine derivatives as potential EGFRWT and EGFRT790M inhibitors. J. Enzyme Inhib. and Med. Chem. 2023; 38:1, 176-191; DOI: 10.1080/14756366.2022.2135512.
5. M. Rosner, K. Schipany, M. Hengstschläger, Merging high-quality biochemical fractionation with a refined flow cytometry approach to monitor nucleocytoplasmic protein expression throughout the unperturbed mammalian cell cycle. Nat Protoc 8, 602–626 (2013). DOI: 10.1038/nprot.2013.011.
6. N. Teraiya, S.S. Karki, A. Chauhan, "Synthesis, Anticancer Evaluation and Molecular Docking of Hexahydroquinoline Derivatives as Mcl-1 Inhibitors and Apoptosis Inducers," Anti-Cancer Agents in Med. Chem. 22 (2021), DOI: 10.2174/1871520621666211021133558.
7. M. Maher, A.E. Kassab, A.F. Zaher, Z. Mahmoud, Novel pyrazolo[3,4-d]pyrimidines: design, synthesis, anticancer activity, dual EGFR/ErbB2 receptor tyrosine kinases inhibitory activity, effects on cell cycle profile and caspase-3-mediated apoptosis, J. Enzyme Inhib. and Med. Chem. 34 (2019), DOI: 10.1080/14756366.2018.1564046.
8. T. Sander, J. Freyss, M. von Korff, C. Rufener, “Data Warrior: an open-source program for chemistry aware data visualization and analysis,” J. Chem. Inf. Model. 55 2015 (2):460-73, https://doi.org/10.1021/ci500588j.