Supplementary material

Monoamine oxidase inhibitory activity of novel Pyrazoline analogues: Curcumin based design and synthesis

Vishnu Nayak Badavath[1], İpek Baysal[2], Gulberk Ucar[2,*], Barij Nayan Sinha[1], Venkatesan Jayaprakash[1,*]

[1]Department of Pharmaceutical Science and Technology, Birla Institute of Technology, Mesra, Ranchi, Jharkhand 835 215, India.

[2]Department of Biochemistry, Faculty of Pharmacy, Hacettepe University, Sıhhiye 06100, Ankara, Turkey.

***Corresponding authors:**

Venkatesan Jayaprakash: VJ; Email: drvenkatesanj@gmail.com; Phone: +91-9470137264

Gulberk Ucar: GU; Email: gulberk.ucar@gmail.com; Phone: +90-5323412866

Experimental procedures

Experimental Procedures

Materials and methods: All the chemicals and solvents for synthesis were purchased from Aldrich. Unless otherwise mentioned the solvents were used without purification. Reactions were monitored by TLC on precoated silica gel plates (Kieselgel 60 F 254,Merck) and the spots were detected under UV light (254 nm). Purification was performed by column chromatography using silica gel (particle size 100-200 mesh, CDH). Melting points were determined using Optimelt (Stanford Research Systems, Sunnyvale, CA 94089) by capillary method and are uncorrected. ^{1}H-NMR spectra were recorded on Varian 400 MHz instrument in DMSO-d_6 as a solvent. ^{13}C-NMR spectra were recorded on 100 MHz instrument in DMSO-d_6 as a solvent. Chemical shifts are reported in parts per million (d) downfield with respect to tetramethylsilane (TMS, δ=0.0) as internal standard. Spin multiplicities are given as **s** (singlet), **d** (doublet), **t** (triplet), **q** (quartet) and **m** (multiplet) as well as **b** (broad). Coupling constants (*J*) are given in hertz. Mass spectra were recorded by The ESI-MS Electro spray Ionization technique. hMAO-A and hMAO-B (both recombinant, expressed in baculovirus-infected BTI insect cells), and other chemicals were purchased from Sigma-Aldrich (Munich, Germany). The Amplex®-Red MAO assay kit was purchased from Cell Technology Inc., Mountain View, CA, USA. Molecular modeling studies were carried out on RHEL-5.0 Operating system installed on Dell Precision workstation with Intel core 2 quad processor and 8 GB RAM, using AutoDock4.2 molecular Docking software.

1. Chemistry:

Procedure for the hydroxyl protection of 4-hydroxy-3-methoxyacetophenone (1)

A solution of3,4-dihydro-α-pyran (89.68 M,2.98 equiv.) in dichloromethane was added drop wise in a solution of 4-hydroxy-3-methoxyacetophenone (30 M,1 equiv.) and pyridinium *p*-toluene sulphonate (0.72 M, 0.05 equiv.) in dichloromethane. The reaction mixture was then stirred at room temperature for 12h. The reaction mixture was washed with water, the organic layer dried with anhydrous sodium sulphate and concentrated to obtain the protected acetophenone.[1]

General procedure for the synthesis of chalcone (2)

Protected 3-methoxy-4-hydroxy acetophenone (**1**, 0.01M) and benzaldehyde (0.01M) were dissolved in ethanol (10 mL). Then, a solution of 60% sodium hydroxide was added to the resulting solution with continuous stirring at <10 ^{o}C. The reaction mixture was allowed to keep at room temperature for about 48 h with occasional shaking. After 48 h. it was poured into ice-cold water, and then neutralized to pH-2 using 6N hydrochloric acid. The yellow precipitate obtained was filtered, washed, dried and the crude was used for preparing **3**.[2]

Procedure for the deprotection of chalcone (3)

The protected chalcone intermediate (**2**) was dissolved in 20 mL ethanol. Then, *p*-toluenesulphonic acid (0.12 M) was added to the chalcone and stirred for 12 h. at room temperature. The reaction mixture was then diluted with water, neutralized with sodium carbonate and extracted with ethyl acetate. The extract was dried with anhydrous sodium sulphate and concentrated to obtain**3**.[1]

2-methoxy-4-(5-phenyl-4, 5-dihydro-1H-pyrazol-3-yl)-phenol (4) The chalcone (**3**, 2gm) was treated with hydrazine hydrate (10 mL) in ethanol (50mL) and refluxed for 3 h. Then the hot reaction mixture was poured into ice-cold water. The solid separated out was filtered, washed with water, dried and recrystallized from ethanol to afford desired compound.[3] Yield: 72 %; mp: 150 ^{o}C; ^{1}H-NMR (400MHz, DMSO-d_6): δ (ppm) 2.74-2.81 (H_A, dd, 1H, J_{AM}=16Hz, J_{AX}=10.56Hz), 3.35-3.42 (H_M, dd, J_{MA}=16Hz, J_{MX}=12Hz), 3.779 (s, 3H, -OC$\underline{H}_3$), 4.79-4.740 (H_X, 1H, merged Hx proton and appeared as triplet), 6.745-6.76 (d, 1H, *J*= 6Hz, Ar-$\underline{H}$), 6.95-6.98 (d, 1H, Ar-$\underline{H}$), 7.23-7.26 (m, 2H), 7.30-7.37 (m, 5H), 9.19 (s, 1H, Ar-O$\underline{H}$); ^{13}C-NMR (100MHz, DMSO-d_6): δ (ppm) 41.46 (pyr-C4), 55.88 (Ar-O$\underline{C}H_3$), 64.02 (pyr-C5), 108.99, 115.34, 118.40, 119.58, 125.30, 127.09, 127.63, 128.83, 131.92, 143.60, 146.78, 147.85, 149.73 (pyr-C3); ESI-MS (m/z): 269.2 $(M+1)^+$.

(3-(4-hydroxy-3-methoxyphenyl)-5-phenyl-4,5-dihydro-1H-pyrazol-1-yl)(phenyl) methanone (5) To the pyrazoline (**4**, 0.001M) in pyridine (10 mL), benzoyl chloride (0.002M) was added. The reaction mixture was refluxed for 3 h. and poured over crushed ice mixed with 6*N* hydrochloric acid. The solid separated out was filtered, washed with water, dried and recrystallized from ethanol to afford compound (**5).**[4] Yield: 55%; mp: 120-122^{o}C; ^{1}H-NMR (400MHz, DMSO-d_6): δ (ppm) 3.179-3.123 (H_A, dd, 1H, J_{AM}=17.8Hz, J_{AX}=4.8Hz), 3.764 (s, 3H, -OC$\underline{H}_3$), 3.805-3.861 (H_M, dd, J_{MA}=17Hz, J_{MX}=4.4Hz), 5.759-5.719 (H_X, dd, 1H, J_{XM}=11.4Hz, J_{XM}=4.8Hz), 6.816-6.83 (d, 1H, *J*= 5.6Hz, Ar-$\underline{H}$), 7.158-7.18 (m, 1H, Ar-$\underline{H}$), 7.22-7.28 (m, 3H, Ar-$\underline{H}$), 7.33-7.39 (m, 2H, Ar-$\underline{H}$), 7.429-7.472 (m, 4H, Ar-$\underline{H}$), 7.87-7.95 (m, 2H, Ar-$\underline{H}$), 9.59 (bs, 1H, Ar-O$\underline{H}$); ^{13}C-NMR (100MHz, DMSO-d_6): δ (ppm) 42.12 (pyr-C4), 56.07 (Ar-O$\underline{C}H_3$), 60.97 (pyr-C5), 110.89, 116.09, 121.12, 122.76, 125.94, 127.11, 128.15, 129.03, 129.21, 129.75, 130.00, 131.19, 133.31, 135.21, 143.00, 148.18, 149.72, 155.97, 165.41, 167.81 (-$\underline{C}$=O); ESI-MS (m/z): 373.3 $(M+1)^+$.

3-(4-hydroxy-3-methoxyphenyl)-N, 5-diphenyl-4,5-dihydro-1H-pyrazole-1-carbothioamide (6) To the stirred solution of pyrazoline (**4**, 1 equiv.) in absolute ethanol, was added phenyl isothiocyanate (2 equiv.) and refluxed for 2 h. The obtained crude precipitate of product was filtered, washed with petroleum ether, dried, and was recrystallized from methanol to afford the desired compound **6**.[5] Yield: 75%; mp: 178-180 ^{o}C; ^{1}H-NMR (400MHz, DMSO-d_6): δ (ppm) 3.145-3.197 (H_A, dd, 1H, J_{AM}=18Hz, J_{AX}=3.13Hz), 3.846 (s, 3H, -OC$\underline{H}_3$), 3.86-3.91(H_M, dd, J_{MA}=12Hz, J_{MX}=8Hz), 5.99-6.028 (H_X, dd, 1H, J_{XM}=11.6Hz, J_{XM}=4Hz), 6.81-6.83 (m, 1H, *J*=8Hz, Ar-$\underline{H}$), 7.13-7.24 (m, 4H, Ar-$\underline{H}$), 7.28-7.35 (m, 5H, Ar-$\underline{H}$), 7.54-7.56 (d, 2H, *J*=8Hz, Ar-$\underline{H}$), 7.61 (s, 1H, Ar-$\underline{H}$), 9.66 (s, 1H, Ar-O$\underline{H}$), 10.04 (s, 1H, -N$\underline{H}$-); ^{13}C-NMR (100MHz, DMSO-d_6): δ (ppm) 42.69 (pyr-C4), 56.37 (Ar-O$\underline{C}H_3$), 63.64 (pyr-C5), 111.19, 115.79, 122.26, 122.42, 125.40, 125.86, 126.11, 127.42, 128.52, 129.05, 104.12, 143.29, 148.33, 150.15, 156.30 (pyr-C3), 173.75 (-$\underline{C}$=S); ESI-MS (m/z): 404.3 $(M+1)^+$.

2-methoxy-4-(5-phenyl-1-tosyl-4, 5-dihydro-1H-pyrazol-3-yl)-phenol (7)*P*-Toluene sulphonyl chloride (0.0025 M) was dissolved in tetrahydrofuran (2 mL) with stirring. The stirred mixture was cooled in an ice bath to 10-15 ^{0}C; followed by gradual addition of a solution of compound (**4**, 0.0012 M**)** in tetrahydrofuran so that the temperature was maintained between 10-15 ^{o}C. Stirring was continued for 15 min. after the addition was complete. The solid separated out was filtered, dried and recrystallized from methanol to afford compound (**7**).[4] Yield: 69%; mp: 128-130 ^{o}C; ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 2.268 (s, 3H, Ar-C$\underline{H}_3$), 3.682-3.748 (H_A, dd, 1H, J_{AM}=18Hz, J_{AX}=8.8Hz), 3.827 (s, 3H, -OC$\underline{H}_3$), 3.895-3.862 (H_M, dd, J_{MA}=17.6Hz, J_{MX}=9.2Hz), 5.216-5.26 (t, 1H, merged H_X proton and appeared as triplet), 6.908-6.929 (d, 1H, *J*=8.4Hz, Ar-$\underline{H}$), 7.086-7.105 (d, 2H, *J*=7.6Hz, Ar-$\underline{H}$), 7.376-7.392 (d, 1H, *J*=6.4Hz, Ar-$\underline{H}$), 7.428-7.492 (m, 8H, Ar-$\underline{H}$), 10.01 (s, 1H, Ar-O$\underline{H}$); ^{13}C-NMR (100MHz, DMSO-d_6): δ (ppm) 21.26 (Ar-$\underline{C}H_3$), 41.43 (pyr-C4), 56.26 (Ar-O$\underline{C}H_3$), 61.21 (pyr-C5), 111.83, 116.07, 120.01,

123.90, 125.98, 128.49, 128.63, 129.52, 129.87, 134.69, 138.43, 145.83, 148.36, 151.99 (pyr-C3); ESI-MS (m/z): 423.3 $(M+1)^+$.

Phenyl-3-(4-hydroxy-3-methoxyphenyl)-5-phenyl-4, 5-dihydro-1H-pyrazole-1-carboxylate (8) To the solution of pyrazoline (**4**) in ethanol (10 mL), was added an equimolar quantity of phenylchloroformate drop-wise at <10 °C with stirring. An equimolar quantity of potassium carbonate was added and stirring continued for another 20 min. The reaction mixture was then filtered and the filtrate upon evaporation provided desired compound.[3] Yield:60 %; mp:190-192 ^oC; ^{1}H-NMR (400MHz, DMSO-d_6): δ (ppm) 3.193-3.36 (H_A, dd, 1H, J_{AM}=14.8Hz, J_{AX}=8Hz), 3.813 (s, 3H, -OC$\underline{H}_3$), 3.86-3.94 (H_M, dd, J_{MA}=20Hz, J_{MX}=12Hz), 5.56 (bs, 1H, merged H_X proton), 6.83-6.85 (d, 2H, *J*= 8Hz, Ar-$\underline{H}$), 7.17-7.20 (dd, 2H, Ar-$\underline{H}$), 7.30-7.28 (t, 2H, *J*=7.6Hz, *J*=8.4Hz, Ar-$\underline{H}$), 7.34-7.39 (m, 7H, Ar-$\underline{H}$), 9.61 (bs, 1H, Ar-O$\underline{H}$); ^{13}C-NMR (100MHz, DMSO-d_6): δ (ppm) 43.11 (pyr-C4), 56.13 (Ar-O$\underline{C}H_3$), 61.66 (pyr-C5), 110.38, 115.91, 121.45, 122.12, 122.67, 125.93, 128.01, 129.32, 129.82, 143.03, 148.27, 149.71, 150.70, 151.24, 155.73 (-$\underline{C}$=O); ESI-MS (m/z): 389.2 $(M+1)^+$.

Ethyl-3-(4-hydroxy-3-methoxyphenyl)-5-phenyl-4, 5-dihydro-1H-pyrazole-1-carboxylate (9) To the solution of pyrazoline (**4**) in ethanol (10 mL), was added an equimolar quantity of ethylchloroformate drop-wise at <10 °C with stirring. An equimolar quantity of potassium carbonate was added and stirring continued for another 15 min. The reaction mixture was then filtered and the filtrate upon evaporation provided desired compound.[3] Yield: 50%; mp: 148-150 ^oC; ^{1}H-NMR (400MHz, DMSO-d_6): δ (ppm) 1.07-1.10 (t, 3H, -CH_2-C$\underline{H}_3$), 3.071-3.128 (H_A, dd, 1H, J_{AM}=18Hz, J_{AX}=4.8Hz), 3.767-3.817 (H_M, dd, J_{MA}=12.2Hz, J_{MX}=7.6Hz), 3.827 (s, 3H, -OC$\underline{H}_3$), 4-4.06 (q, 2H, -C$\underline{H}_2$), 5.37-5.4 (H_X, dd, 1H, J_{XM}=11.4Hz, J_{XM}=4.8Hz), 6.80-6.84 (m, 2H, Ar-$\underline{H}$), 7.125-7.15 (m, 1H, Ar-$\underline{H}$), 7.17-7.19 (d, 1H, *J*=7.2Hz, Ar-$\underline{H}$), 7.26-7.30 (m, 1H, Ar-$\underline{H}$), 7.31-7.36 (m, 2H, Ar-$\underline{H}$), 7.52 (s, 1H, Ar-$\underline{H}$); ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 14.86 (-CH_2-$\underline{C}H_3$-), 42.82 (pyr-$\underline{C}$4), 56.06 (Ar-O$\underline{C}H_3$), 61.22 (pyr-$\underline{C}$5), 61.37 (-$\underline{C}H_2$-CH_3-), 110.20, 110.61, 115.54, 115.83, 121.06, 122.88, 125.83, 127.73, 129.11, 143.40, 147.91, 148.15, 149.40 (pyr-$\underline{C}$3), 154.18 (-$\underline{C}$=O);ESI-MS (m/z): 341.2 $(M+1)^+$.

4-(1,5-diphenyl-4, 5-dihydro-1H-pyrazol-3-yl)-2-methoxyphenol (10) The solution of chalcone (**3**, 0.01 M) and phenylhydrazine (0.02 M) in ethanolic sodium hydroxide (0.025 M, 20 mL) was refluxed for 4 h. After cooing the reaction mixture was poured into ice water and neutralized with dilute hydrochloric acid then the separated out was filtered and crystallized from proper solvent, if solid not obtained then extract the whole mixture with dichloromethane and then organic layer was separated and dried over Na_2SO_4 and evaporation of solvent provide the desired compound.[6] Yield: 56%; brown thick mass; ^{1}H-NMR (400MHz, DMSO-d_6): δ (ppm) 3.03-3.08 (H_A, dd, 1H, J_{AM}=16Hz, J_{AX}=4Hz), 3.57-3.72 (H_M, dd, J_{MA}=17.Hz, J_{MX}=12Hz), 3.81 (s, 3H, -OC$\underline{H}_3$), 5.36-5.4 (H_X, dd, 1H, J_{XM}=12Hz, J_{XM}=4Hz), 6.65-6.87 (m, 2H), 6.95-7.02 (t, 1H), 7.10-7.5 (m, 9H, Ar-$\underline{H}$), 7.67-7.91 (m, 1H, Ar-$\underline{H}$), 9.5 (s, 1H, Ar-O$\underline{H}$); ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 43.54 (pyr-$\underline{C}$4), 56.0 (Ar-O$\underline{C}H_3$), 63.78 (pyr-$\underline{C}$5), 110.43, 111.51, 114.48, 116.22, 118.46, 119.01, 121.79, 126.09, 128.77, 129.10, 129.23, 129.35, 132.81, 133.81, 144.99, 146.03, 146.22, 147.69, 148.30 (pyr-$\underline{C}$3); ESI-MS (m/z): 343.2 $(M-1)^-$.

1-(3-(4-hydroxy-3-methoxyphenyl)-5-phenyl-4, 5-dihydro-1H-pyrazol-1-yl) ethanone (11)A mixture of chalcone(**3**, 1 M) in 4 mL of acetic acid and hydrazine monohydrate 80% (4 M) was refluxed for 4 h. The mixture was then poured onto ice-water (25 mL) mixture toget crude pyrazoline derivative, which was then purified by recrystallization from ethanol to afford pure desired compound.[7] Yield: 76 %; mp: 100-102 ^oC; ^{1}H-NMR (400MHz, DMSO-d_6): δ (ppm) 2.293 (s, 3H, -C$\underline{H}_3$), 3.08-3.13 (H_A, dd, 1H, J_{AM}=16Hz, J_{AX}=4Hz), 3.789-3.823 (m, 4H, merged H_M proton and -OC$\underline{H}_3$ proton), 5.48-5.52 (H_X, dd, 1H, J_{XM}=12Hz, J_{XM}=4Hz), 6.81-6.83 (d, 1H,

J= 8Hz, Ar-H), 6.85-6.87 (d, 2H, *J*=8Hz, Ar-H), 7.15-7.19 (t, 1H, *J*=8Hz, J=8Hz, Ar-H), 7.238-7.204 (d, 1H, *J*=12Hz, Ar-H), 7.32-7.30 (d, 1H, *J*=8Hz, Ar-H), 7.44 (s, 1H, Ar-H), 7.515-7.490 (dd, 1H, Ar-H), 9.57 (s, 1H, Ar-OH); ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 26.81 (-CO-CH_3-), 55.66 (pyr-C4), 56.41 (Ar-OCH_3), 56.83 (pyr-C5), 111.46, 111.64, 114.83, 115.87, 122.83, 123.79, 123.93, 128.94, 129.33, 147.95, 149.55, 152.15, 154.86 (pyr-C3), 167.51 (-C=O); ESI-MS (m/z): 311.2 $(M+1)^+$.

3-(4-hydroxy-3-methoxyphenyl)-5-phenyl-4, 5-dihydro-1H-pyrazole-1-carbothioamide (12) The chalcone (**3**, 0.01M) was treated with thiosemicarbazide (0.0125M), sodium hydroxide (0.025M) and ethanol (20 mL) and refluxed for 6 h. The reaction mixture while hot was poured in ice-cold water and acidified with dilute hydrochloric acid. The precipitate obtained was filtered, dried and recrystallized from methanol.[5]Yield: 60%; mp: 158-160 ^oC; ^{1}H-NMR (400MHz, DMSO-d_6): δ (ppm) 3.06-3.11 (d, 1H, merged H_A protons), 3.78-3.87 (m, merged H_M protons and -OCH_3 protons), 5.87-5.89 (d, 1H, merged H_X protons), 6.77-6.79 (d, 1H, *J*=8Hz, Ar-H), 6.86-6.84 (d, 1H, *J*=8Hz, Ar-H), 7.01-7.22 (m, 3H), 7.39-7.35 (m, 1H), 7.43-7.50 (m, 1H, Ar-H), 7.56 (s, 1H), 7.93-7.87 (d, 1H), 9.60 (s, 1H), 9.96 (s, 1H); ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 42.93 (pyr-C4), 56.11 (Ar-OCH_3), 63.12 (pyr-C5), 110.70, 115.89, 117.86, 127.05, 127.56, 128.71, 129.17, 130.99, 131.47, 134.36, 146.04, 147.90, 155.56 (pyr-C3), 176.68 (-C=S); ESI-MS (m/z): 328.2 $(M+1)^+$.

3-(4-hydroxy-3-methoxyphenyl)-5-phenyl-4, 5-dihydro-1H-pyrazole-1-carboximidamide (13) To the solution of chalcone (**3**, 0.01 M) in ethanol was added an aqueous sodium hydroxide (0.02 M), aminoguanidine hydrochloride (0.02M.) and then, refluxed for 6 h. TLC monitoring was extensively done, the reaction mixture was cooled and poured into the crushed ice, and neutralized with dilute hydrochloric acid the solid mass which separated out was filtered, washed carefully with water, dried and recrystallized from ethanol give the pure desired compound.[5] Yield: 60%; mp:105^oC; ^{1}H-NMR (400MHz, DMSO-d_6): δ (ppm)3.17-3.23 (d, 1H, merged H_A protons), 3.708-3.764 (H_M, dd, J_{MA}=12Hz, J_{MX}=8Hz), 3.80 (s, 3H, Ar-OCH_3), 4.57 (s, 1H, Ar-OH), 5.59-5.73 (d, 1H, merged H_X protons), 6.54 (d, 1H, *J*=8Hz, Ar-H), 6.70-6.73 (m, 1H, -NH_2), 7.10-7.13 (t, 1H, *J*=8Hz, *J*=4Hz, Ar-H), 7.18-7.20 (d, 2H, *J*=8Hz, Ar-H), 7.25-7.34 (m, 2H, Ar-H), 7.36-7.41 (m, 3H, Ar-H), 7.41 (s, 1H, Ar-H), 7.82-7.86 (m, 1H, =NH); ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 43.46 (pyr-C4), 55.90 (Ar-OCH_3), 61.26 (pyr-C5), 110.50, 116.09, 118.31, 126.61, 129.03, 129.74, 132.25, 133.32, 147.38, 148.33, 149.69 (pyr-C3), 155.18 (H_2N-C=NH); ESI-MS (m/z): 311.2 $(M+1)^+$.

2. Biochemistry:

Determination of hMAO-A and -B activities:

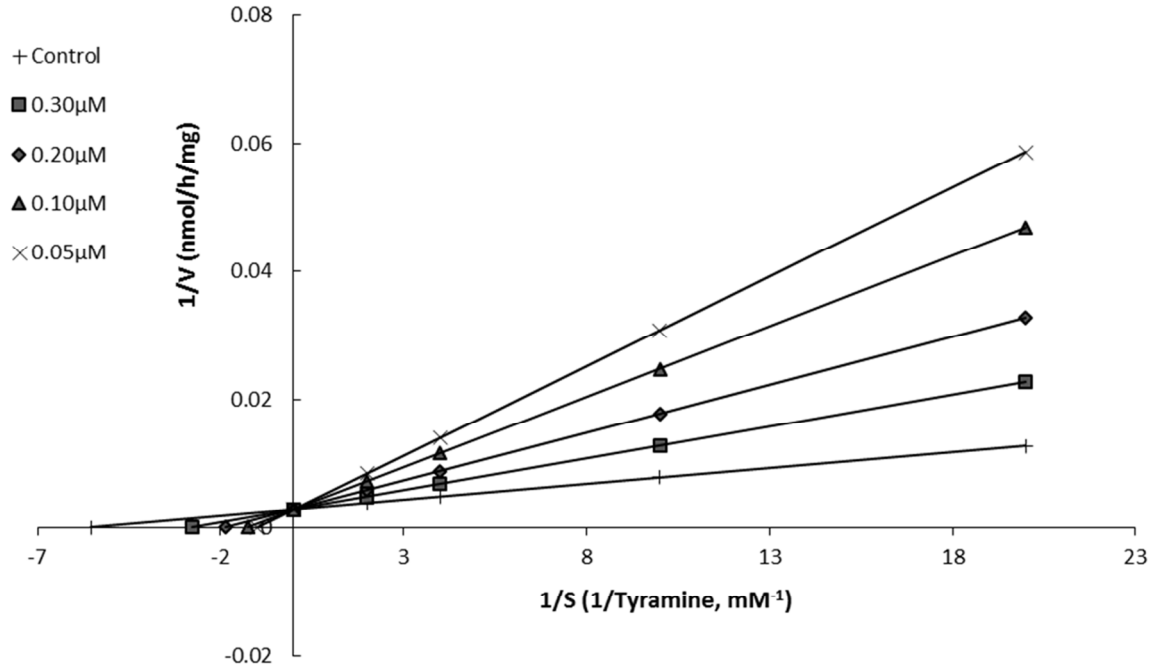
hMAO-A and hMAO-B activities were determined using p-tyramine (0.05-1.00 mM) as substrate. Specific activity was calculated as 140.05±7.90 pmol/mg/min (n=3) for hMAO-A and 122.50±7.12 pmol/mg/min (n=3) for hMAO-B. The inhibitory effects of the synthesized compounds on hMAO activities were determined by a fluorimetric method.[8,9]Study medium contained 0.1 mL of sodium phosphate buffer (0.05 M, pH 7.4), various concentrations of the newly synthesized compounds or reference compounds, and adequate amounts of recombinant hMAO-A or hMAO-B. This mixture was incubated for 15 min at 37^{o}C in the dark fluorimeter chamber. Reaction was started by adding 200 µM Amplex Red reagent, 1 U/mL horseradish peroxidase (HRP), and p-tyramine (concentration range 0.05-1.00 mM). The production of H_2O_2 catalyzed by MAO isoforms was detected using Amplex®-Red reagent, a non-fluorescent probe reacting with H_2O_2 in the presence of HRP. The fluorescent product resorufin was then quantified at 37^{o}C in a fluorescence reader with excitation at 545 nm, and emission at 590 nm for 15 min. The specific fluorescence emission was calculated after subtraction of the background activity. In our experimental conditions, this background activity was negligible.Control experiments were carried out by replacing the compound and reference inhibitors. The possible capacity of compounds to quench the fluorescence generated in the reaction mixture was determined by adding these compounds to solutions containing only the Amplex Red reagent or resorufin in a sodium phosphate buffer. The new compounds and reference inhibitors themselves did not react directly with Amplex®-Red reagent or did not effect the fluorescence generated in the reaction mixture. Newly synthesized compounds did not cause any inhibition on the activity of HRP in the test medium.

3.2.2. Kinetic experiments:

The synthesized compounds were dissolved in DMSO with a maximum concentration of 1% (w/v) and used in the concentration range of 0.10-30.00 µM. Kinetic data for the interaction of the enzymes with the compounds were analyzed with the Microsoft Excel package program. The slopes of the Line weaver-Burk plots were plotted versus the inhibitor concentration and the Ki values were determined from the x-axis intercept as -Ki. The specificity index was expressed as SI=Ki (MAO-A)/Ki (MAO-B). Protein was determined according to Bradford[10] using bovine serum albumin as the standard.Lineweaver-Burk plots corresponding the inhibition of hMAO-A by various concentrations of compound **7**has been presented in **Figure 1**.

3.2.3. Reversibility experiments:

Reversibility was assessed using a centrifugation-ultra filtration method previously reported.[11] Recombinant enzymes were incubated with the compounds or standard inhibitors in 0.05 M sodium phosphate buffer, pH 7.4, for 1 h at 37 °C. An aliquot was stored at 4 °C for MAO activity measurement. Another sample was placed in an Ultrafree-0.5 centrifuge tube (Nalgene®, NY, USA) with a 30-kDa Biomax membrane (Millipore Corp., Bedford, MA, USA) and centrifuged at 9000 x g for 20 min at 4°C. The enzyme retained in the 30-kDa membrane was resuspended in the buffer at 4°C and centrifuged, and this process was repeated once more. The enzyme retained in the membrane was resuspended in buffer and used for MAO determination. Control experiments were performed by replacing the test drugs with appropriate vehicle dilutions. The corresponding values of percent MAO inhibition were separately calculated for the samples with and without repeated washing.Reversibility data presented in the **Table 1**.



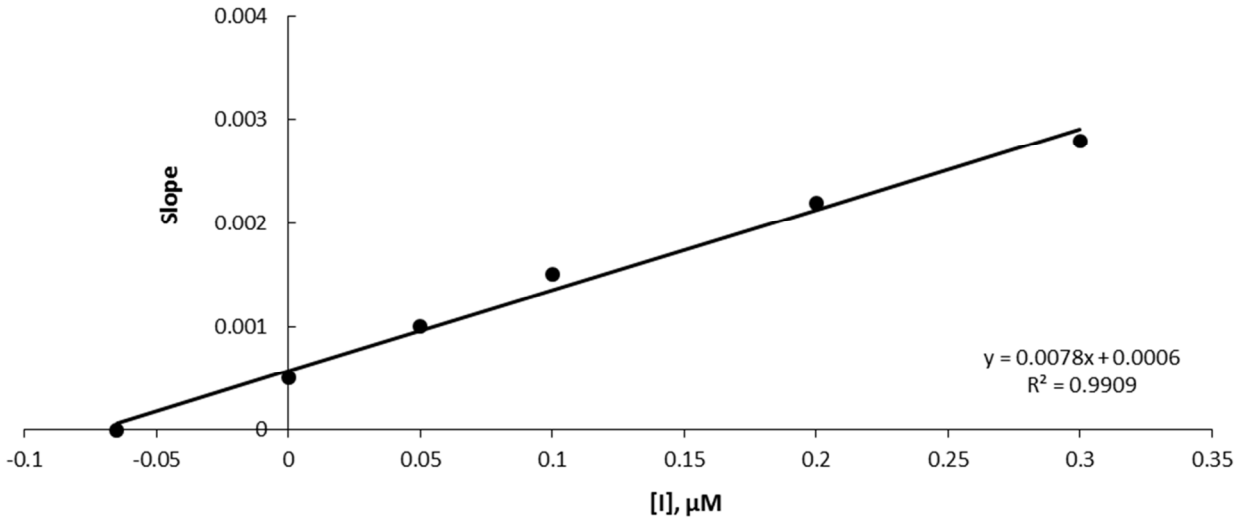


Figure 1. Lineweaver-Burk plots corresponding the inhibition of hMAO-A by various concentrations of compound **7**. Second graph presents the slopes of the Lineweaver–Burk plots versus inhibitor concentration (Ki = 0.065 µM).

Table 1. Reversibility of hMAO-A and –B inhibition by the newly synthesized 2-methoxy-4-(5-phenyl-4,5-dihydro-1H-pyrazol-3-yl) phenol derivatives

Compounds*	hMAO-A inhibition(%)		hMAO-B inhibition(%)	
	Before washing	After repeated washing	Before washing	After repeated washing
Selegiline	-	-	71.44±2.80	70.20±2.06
Moclobemide	87.21±3.12	8.88±0.32	-	-
Lazabemide	-	-	79.96±2.17	11.05±0.95
Curcumin	77.33±3.00	12.55±1.02	-	-
4	-	-	76.80±5.09	13.06±1.08
5	82.00±4.21	12.76±0.90	-	-
6	85.77±3.10	12.04±1.00	-	-
7	92.06±3.00	7.49±0.29	-	-
8	89.75±2.95	9.17±0.61	-	-
9	87.16±3.25	10.79±1.05	-	-
10	87.00±4.02	9.79±0.54	-	-
11	85.55±2.76	11.63±0.74	-	-
12	75.56±2.19	14.00±0.88	73.89±3.94	14.22±1.02
13	68.33±2.92	15.90±1.12	-	-

*Synthesized and the reference compounds were tested at 0.50 μM of concentration. Selegiline is the irreversible MAO-B inhibitor; Lazabemide is the reversible MAO-B inhibitör and moclobemide is the reversible MAO-A inhibitör.Each value represents the mean±SEM of three independent experiments.

3. Molecular docking simulation:

In order to understand the interaction at molecular level, compounds **4-13** were docked with X-ray crystal structure of hMAO-A (PDB: 2BXR) and hMAO-B (PDB:2BYB) using AutoDock 4.2. Docking protocol employed by other group and our group has reported earlier communications.[3–6,13]Ligand structures were drawn using build panel & prepared using Ligprep module implemented Maestro-8.4 (Schrodinger LLC).Energy minimization is carried out using MMFF force field. Structures were saved in .pdb format and rewritten using open bable for AutoDock compatible atom type. For docking, grid parameter file (.gpf) and docking parameter files (.dpf) were written using MGL Tools-1.5.6. Receptor grids were generated using 60×60×60 Grid points in xyz with grid spacing of 0.375Å. Grid box was centered on N5 atom of FAD. Map types were generated using autogrid 4.2. Docking was carried out with following parameters with number of runs: 50, population size: 150, number of evaluations: 2,500,000 and number of generations: 27,000, using autodock 4.2. Analysis of docking results was done using MGL Tools-1.5.6. Top scoring molecule in the largest cluster was analyzed for its interaction with the protein. The docking results are presented in **Table 2**.

Table 2. Calculated K_i values corresponding to the inhibition of hMAO isoforms by the newly synthesized 2-methoxy-4-(5-phenyl-4,5-dihydro-1H-pyrazol-3-yl) phenol derivatives

Compound	MAO-A Calc. Ki (µM)		Isomer potency (R/S)	Racemic potency MAO-A_{rac} =[(R+S)/2]	MAO-B Calc. Ki (µM)		Isomer potency(R/S)	Racemic potency MAO-B_{rac}=[(R+S)/2]	Selectivity MAO-A_{rac}/MAO-B_{rac}
	R	S			R	S			
4	4.44	6.32	0.70	5.38	0.42	0.48	0.9	0.5	11.96
5	7.97	0.29	27.48	4.13	89.35	11.71	7.6	50.5	0.08
6	0.46	0.72	0.64	0.59	18.35	13.04	1.4	15.7	0.04
7	0.02	0.08	0.25	0.05	361470	215.76	1675.3	180842.9	0.00
8	0.1	0.37	0.27	0.24	4.38	3.84	1.1	4.1	0.06
9	0.35	0.49	0.71	0.42	224.41	62.59	3.6	143.5	0.0029
10	1.79	3.36	0.53	2.58	25.65	6.21	4.1	15.9	0.16
11	3.11	3.22	0.97	3.17	57.15	8.17	7.0	32.7	0.10
12	0.63	2.88	0.22	1.755	4.66	2.42	1.9	3.5	0.50
13	7.67	30.8	0.25	19.24	237.98	30.98	7.7	134.5	0.14

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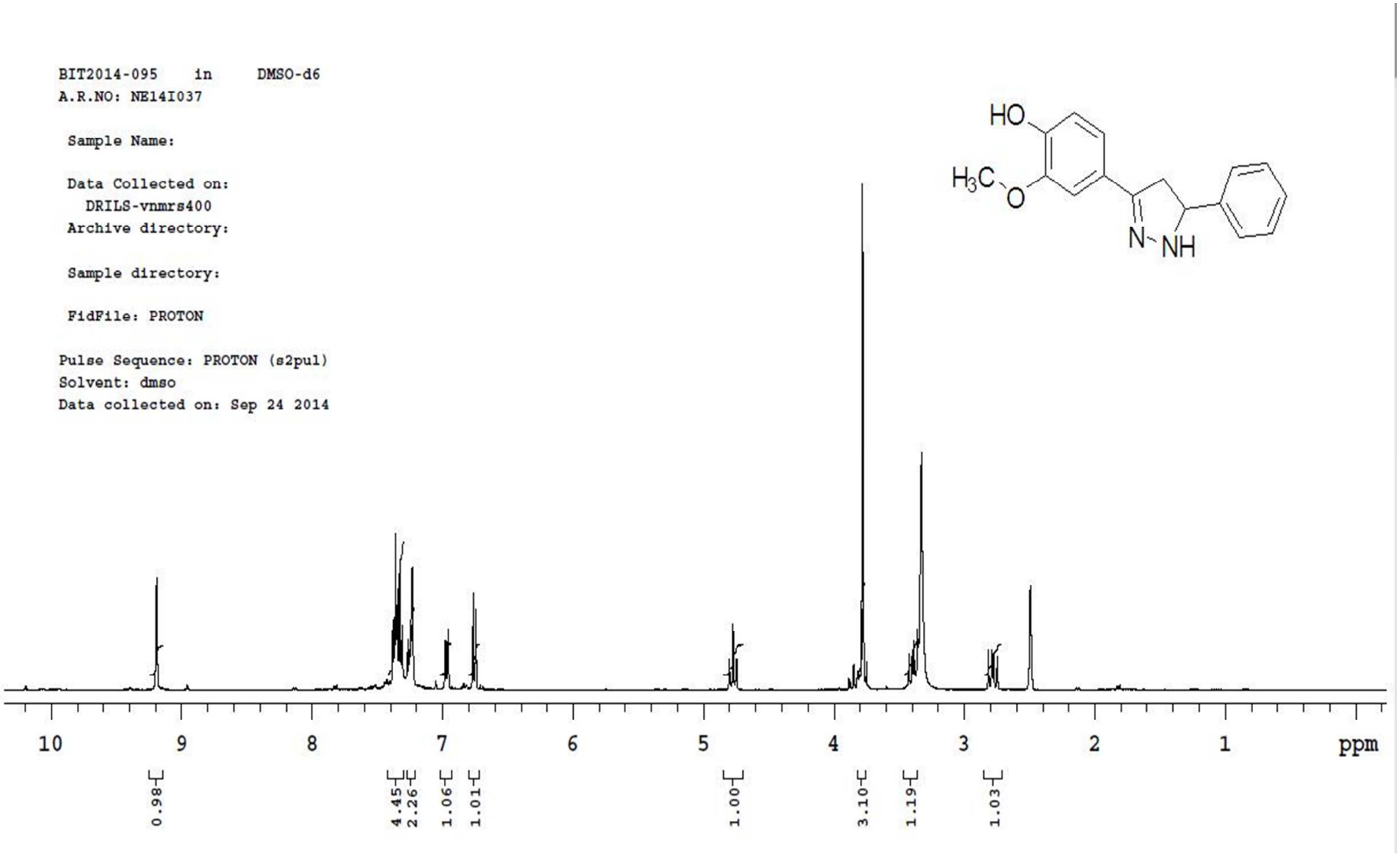

^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 4

Sample Name	BIT2014-095	**Position**	Vial 61	**Sample type**		**Instrument Name**	Instrument 1
User Name		**Inj Vol**	0.2	**Data Filename**	180914M010.d	**ACQ Method**	test.m
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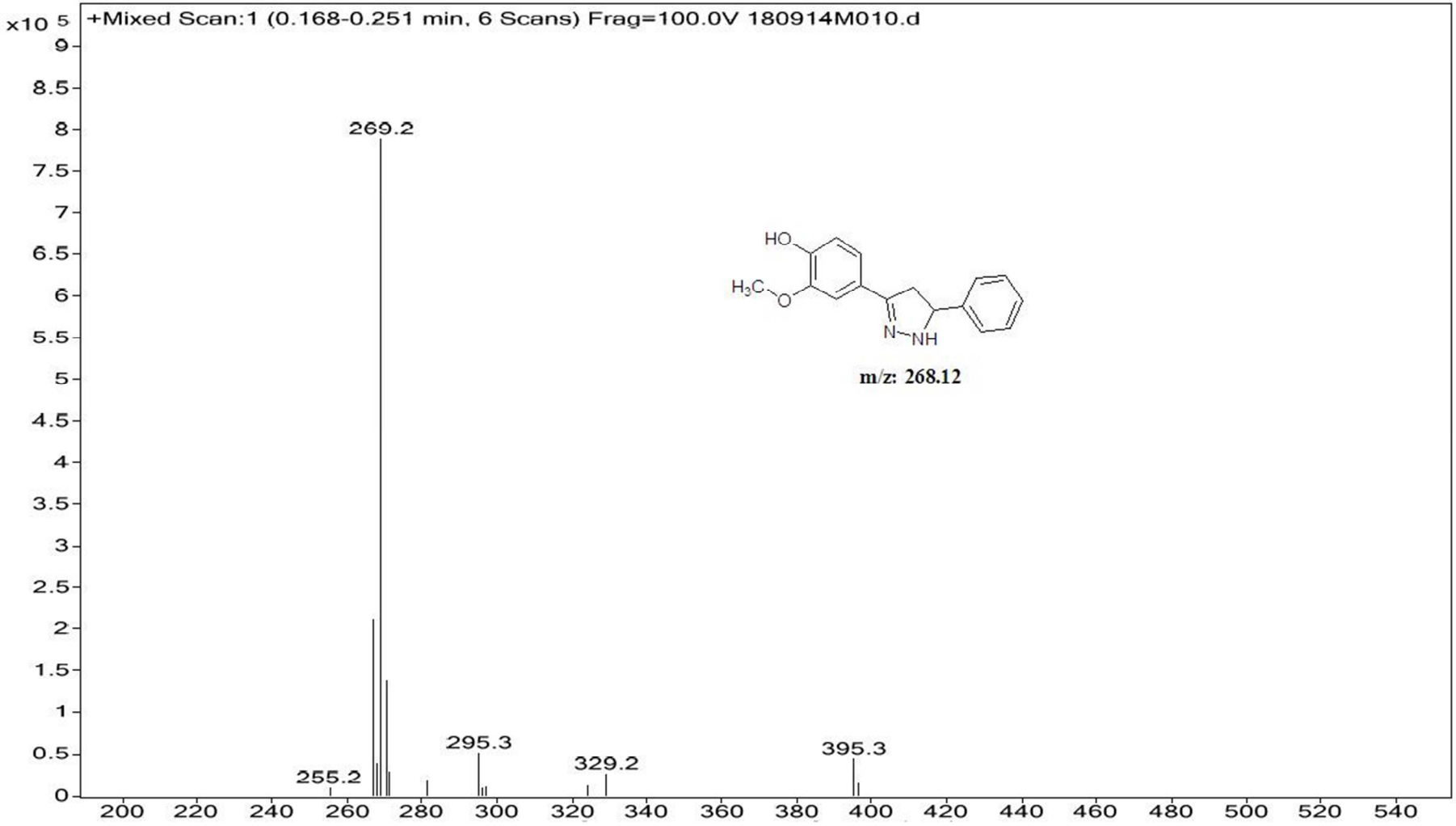
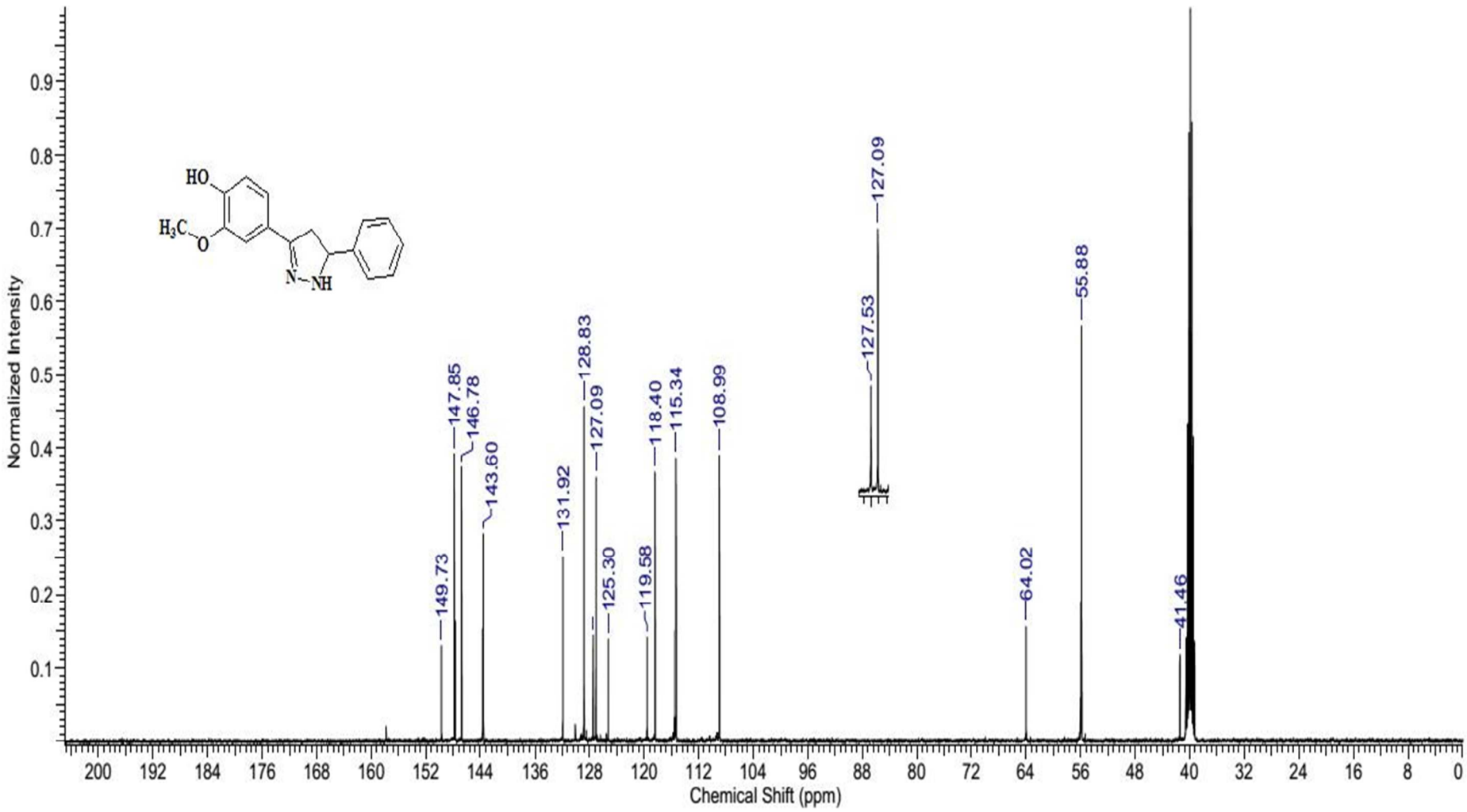
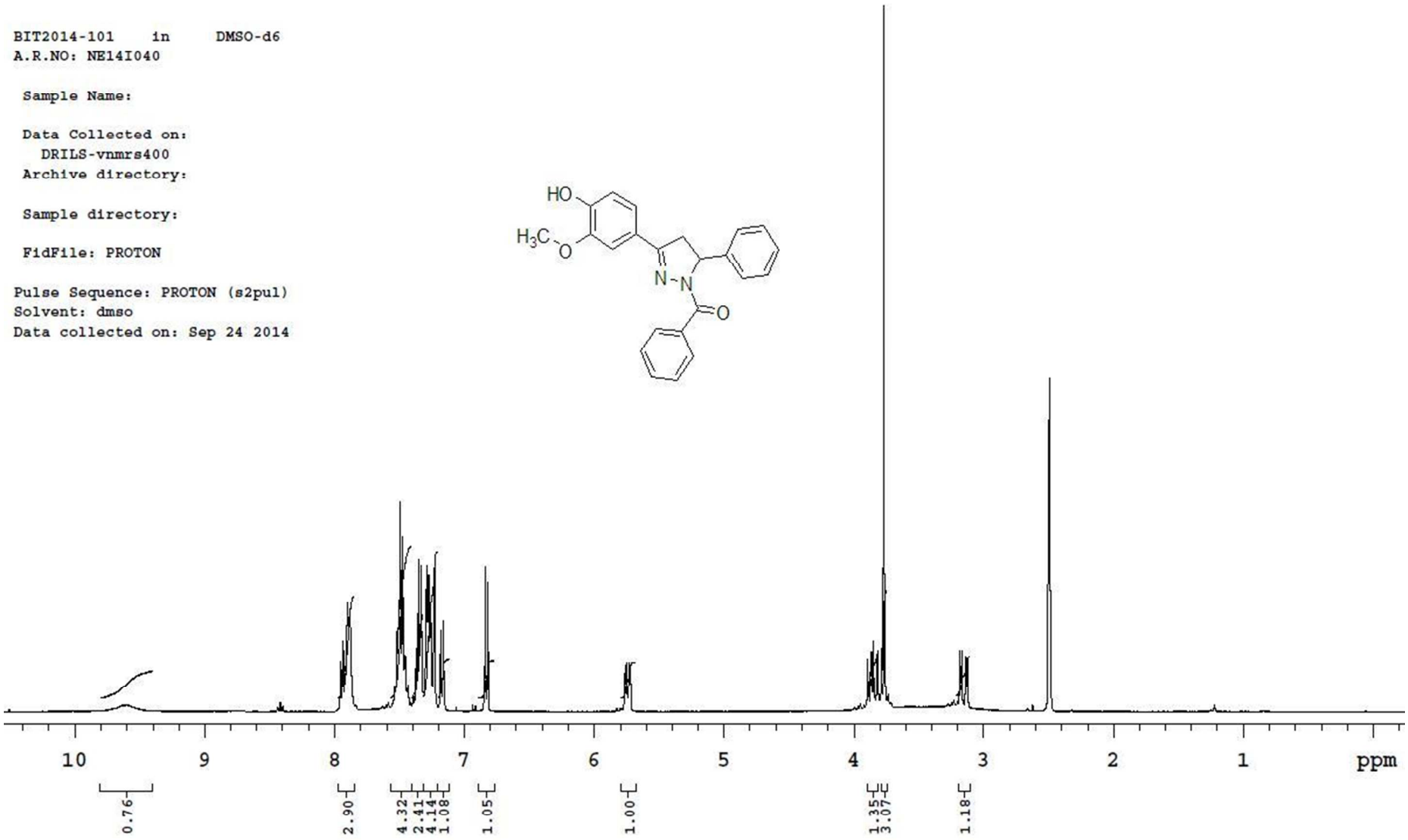


ESI-MS spectrum of compound 4

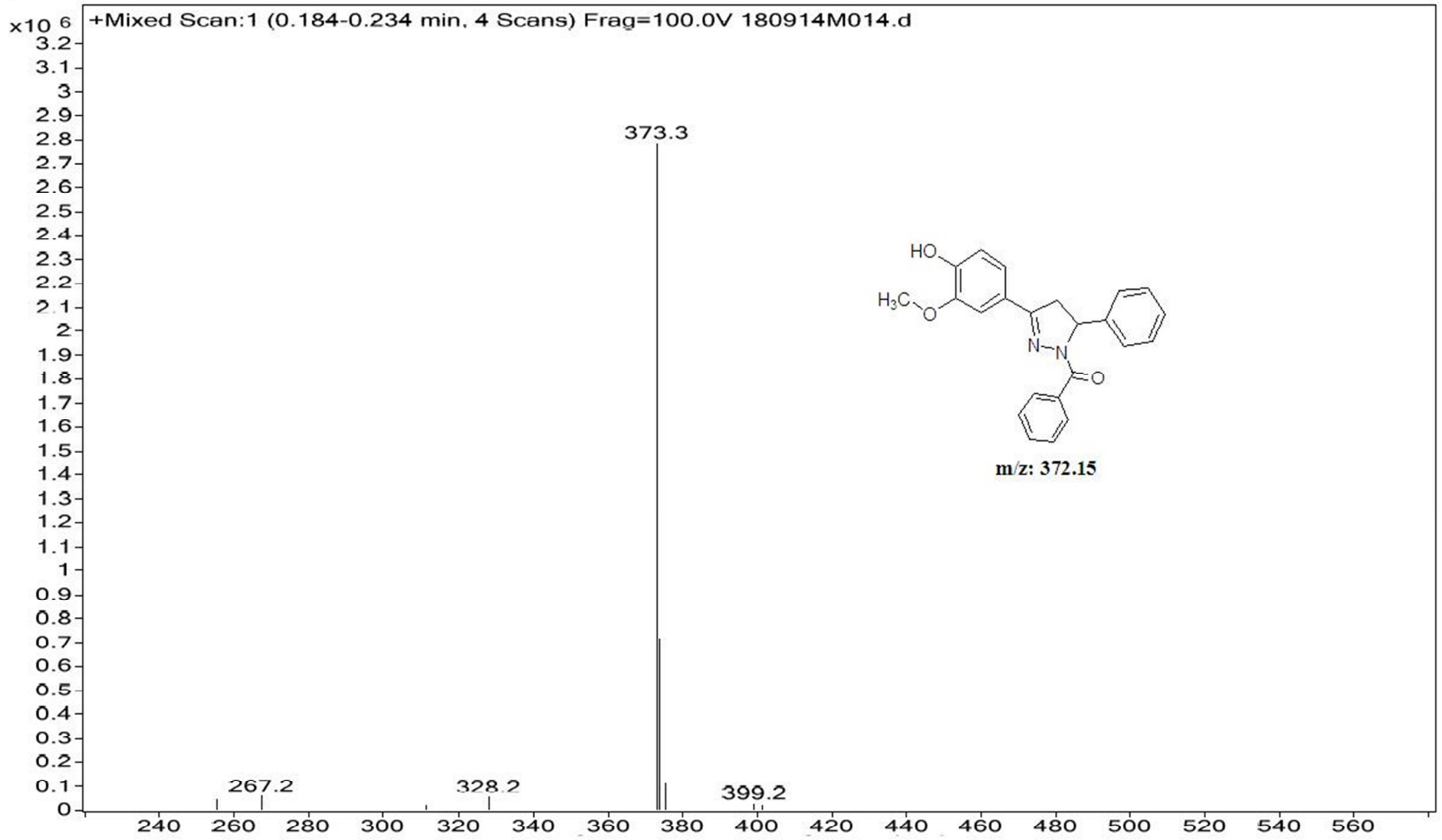


^{13}C-NMR (100 MHz, DMSO-d_6) spectrum of compound 4

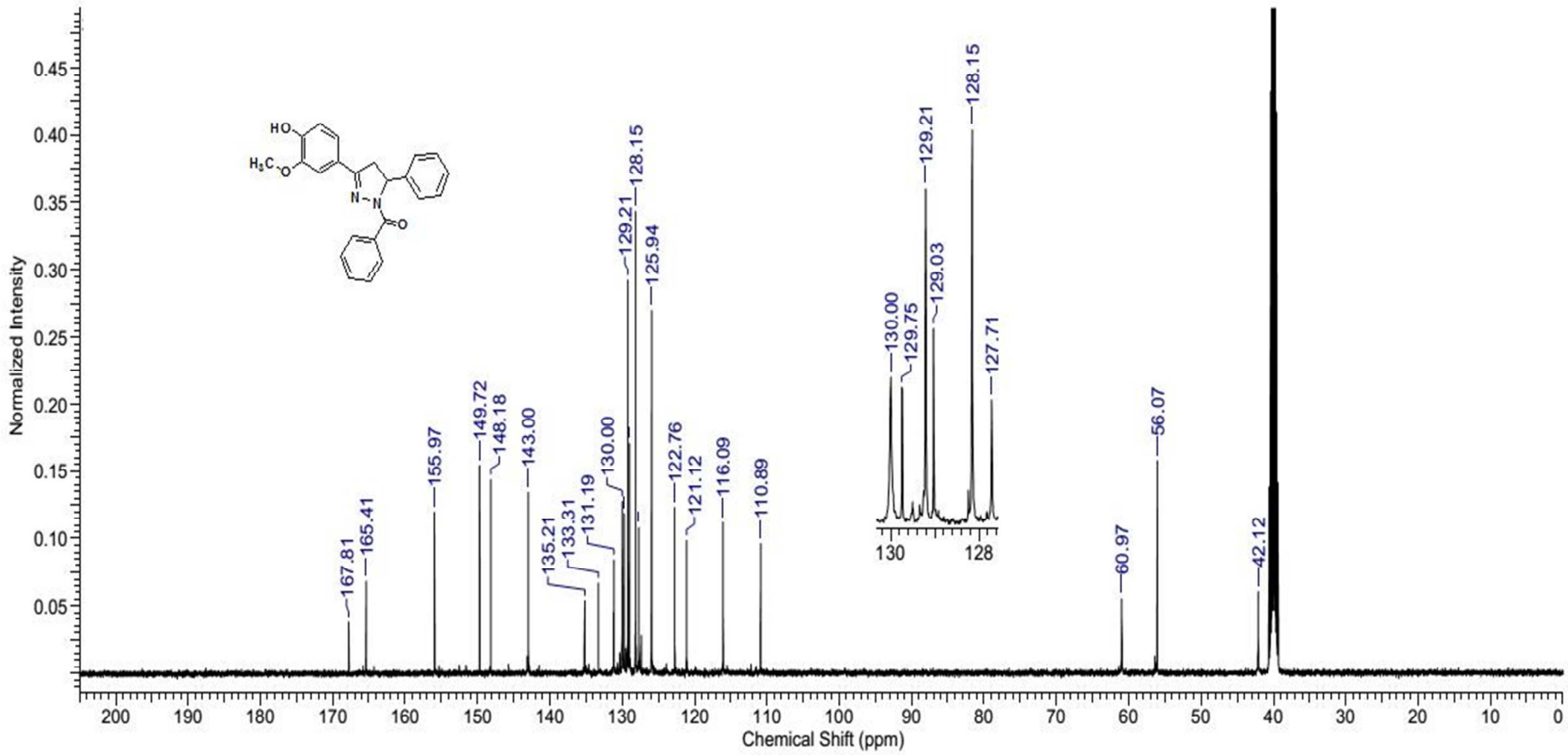


^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 5

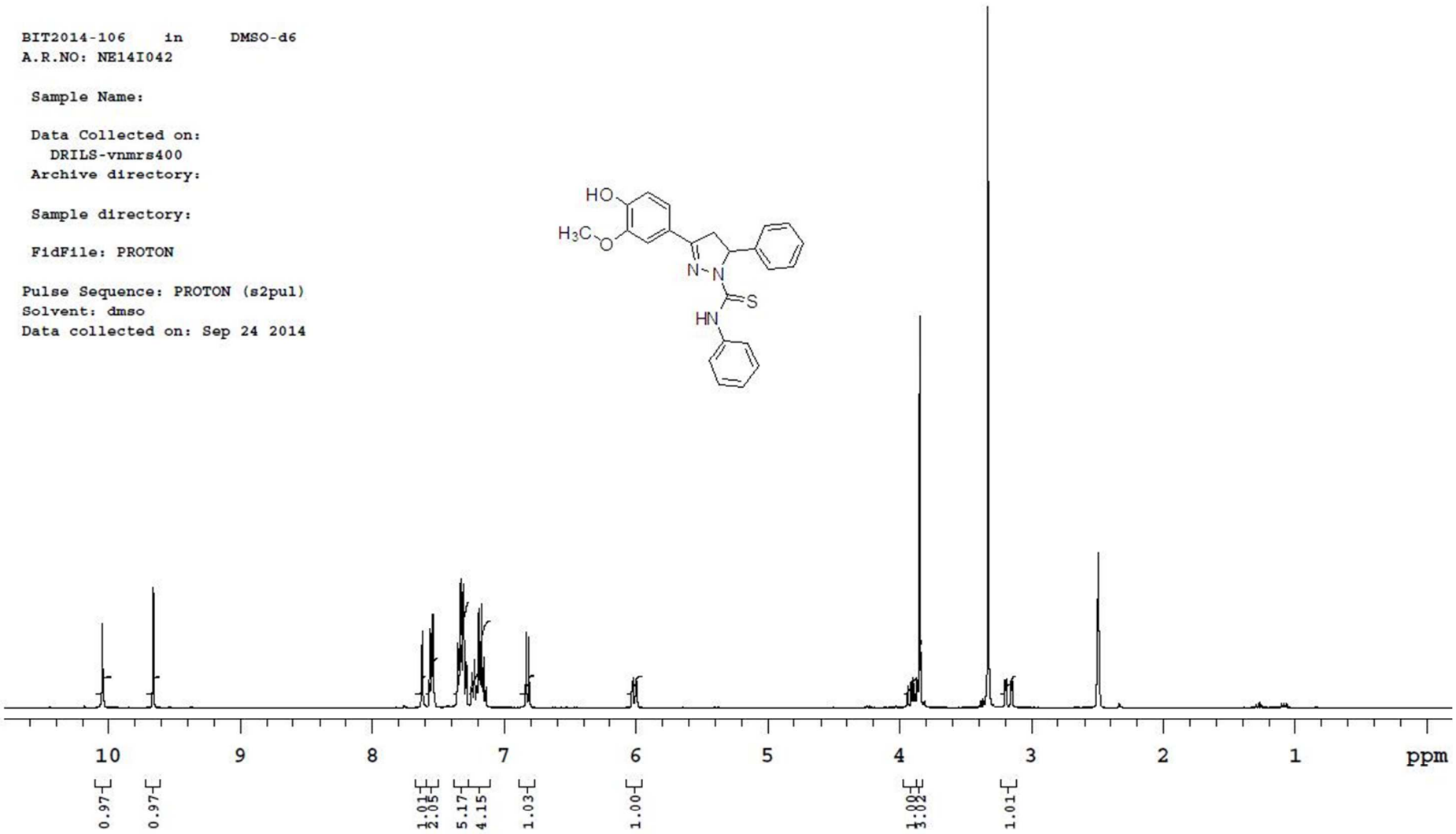
Sample Name	BIT2014-101	**Position**	Vial 65	**Sample type**		**Instrument Name**	Instrument 1
User Name		**Inj Vol**	0.2	**Data Filename**	180914M014.d	**ACQ Method**	test.m
AR NO :	ME14I025	**Acquired Time**	9/18/2014 2:37:57 PM				



ESI-MS spectrum of compound 5

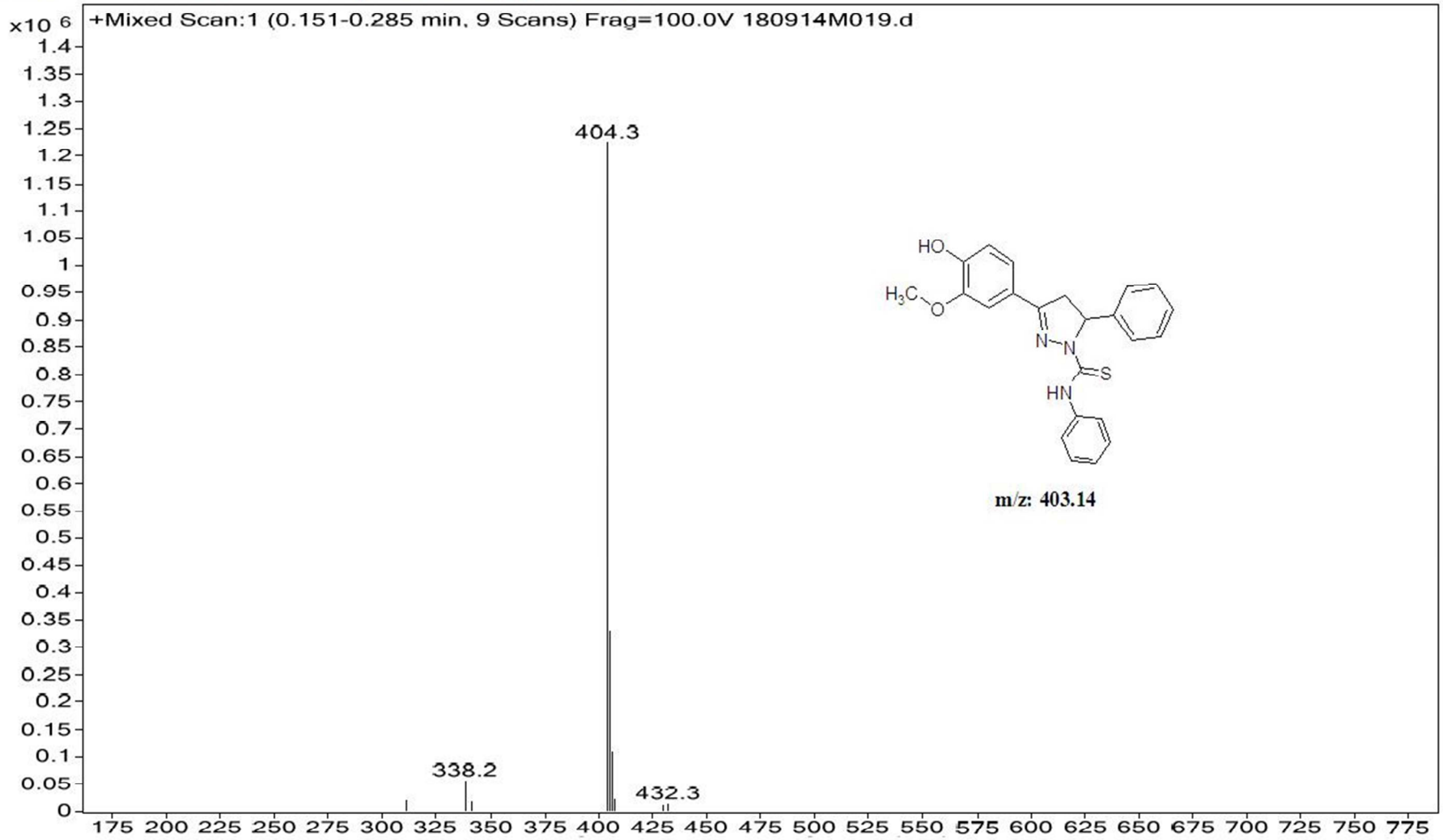


^{13}C-NMR (100 MHz, DMSO-d_6) spectrum of compound 5

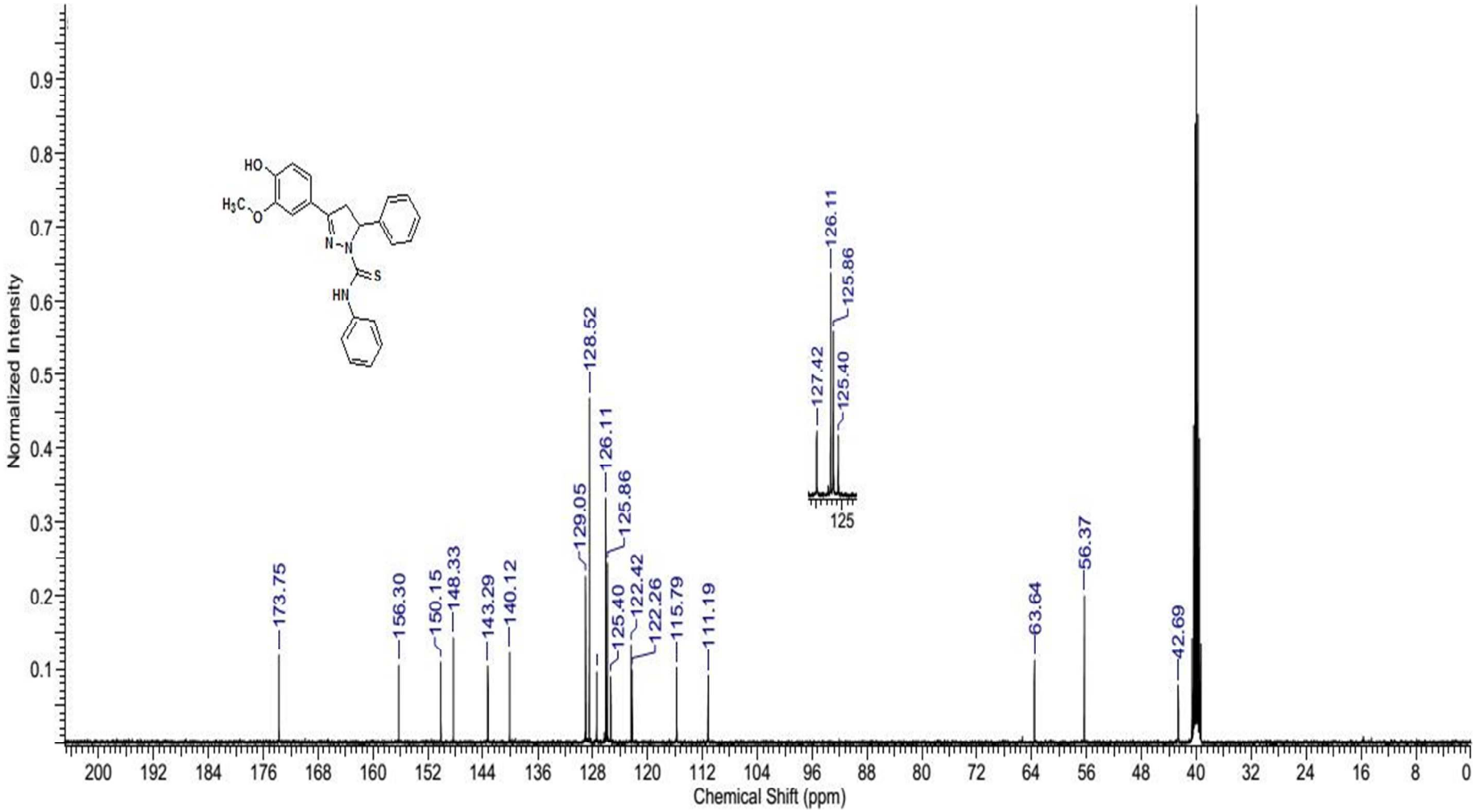


^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 6

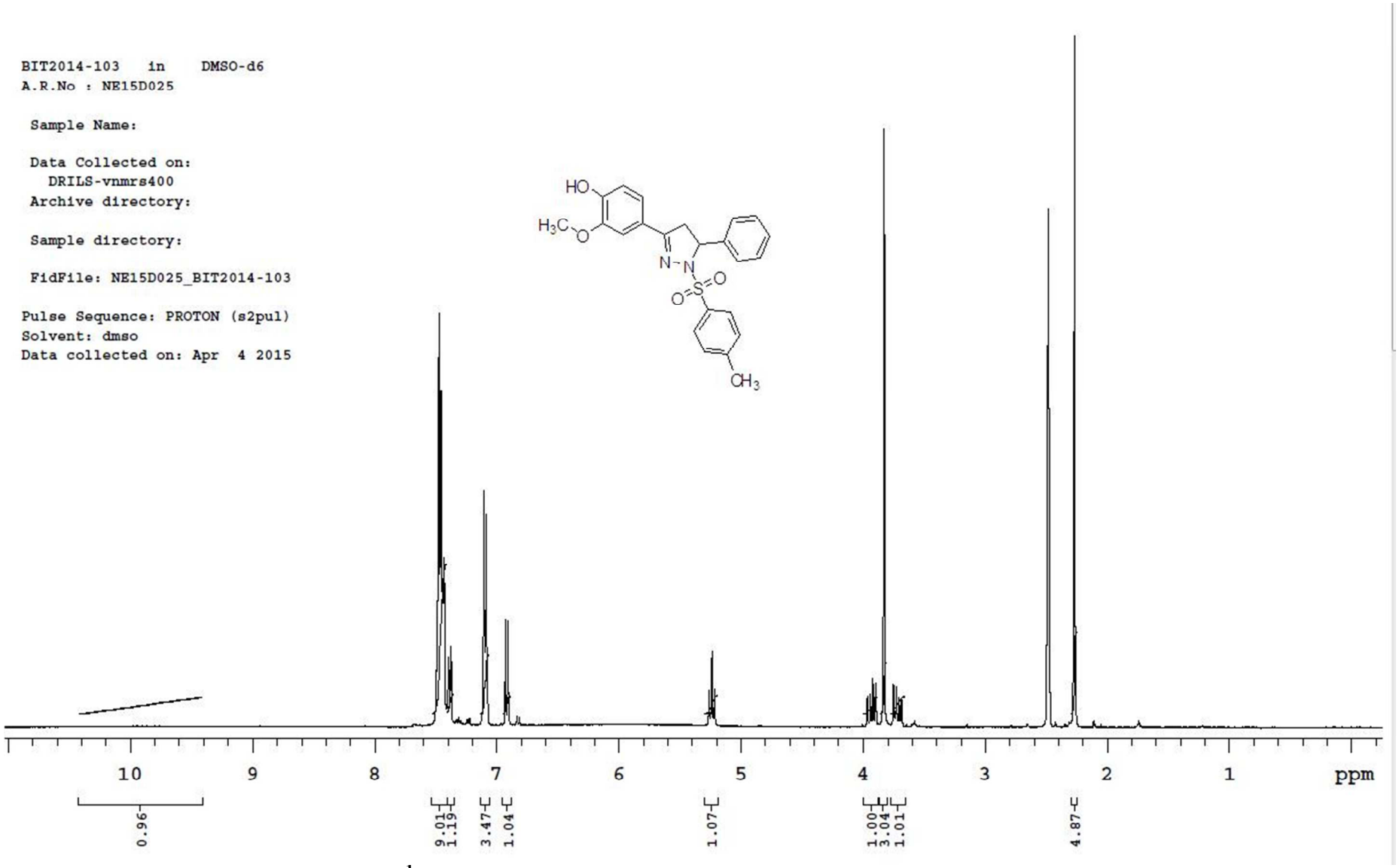
Sample Name	BIT2014-106	**Position**	Vial 70	**Sample type**		**Instrument Name**	Instrument 1
User Name		**Inj Vol**	0.2	**Data Filename**	180914M019.d	**ACQ Method**	test.m
AR NO :	ME14I030	**Acquired Time**	9/18/2014 2:46:07 PM				



ESI-MS spectrum of compound 6

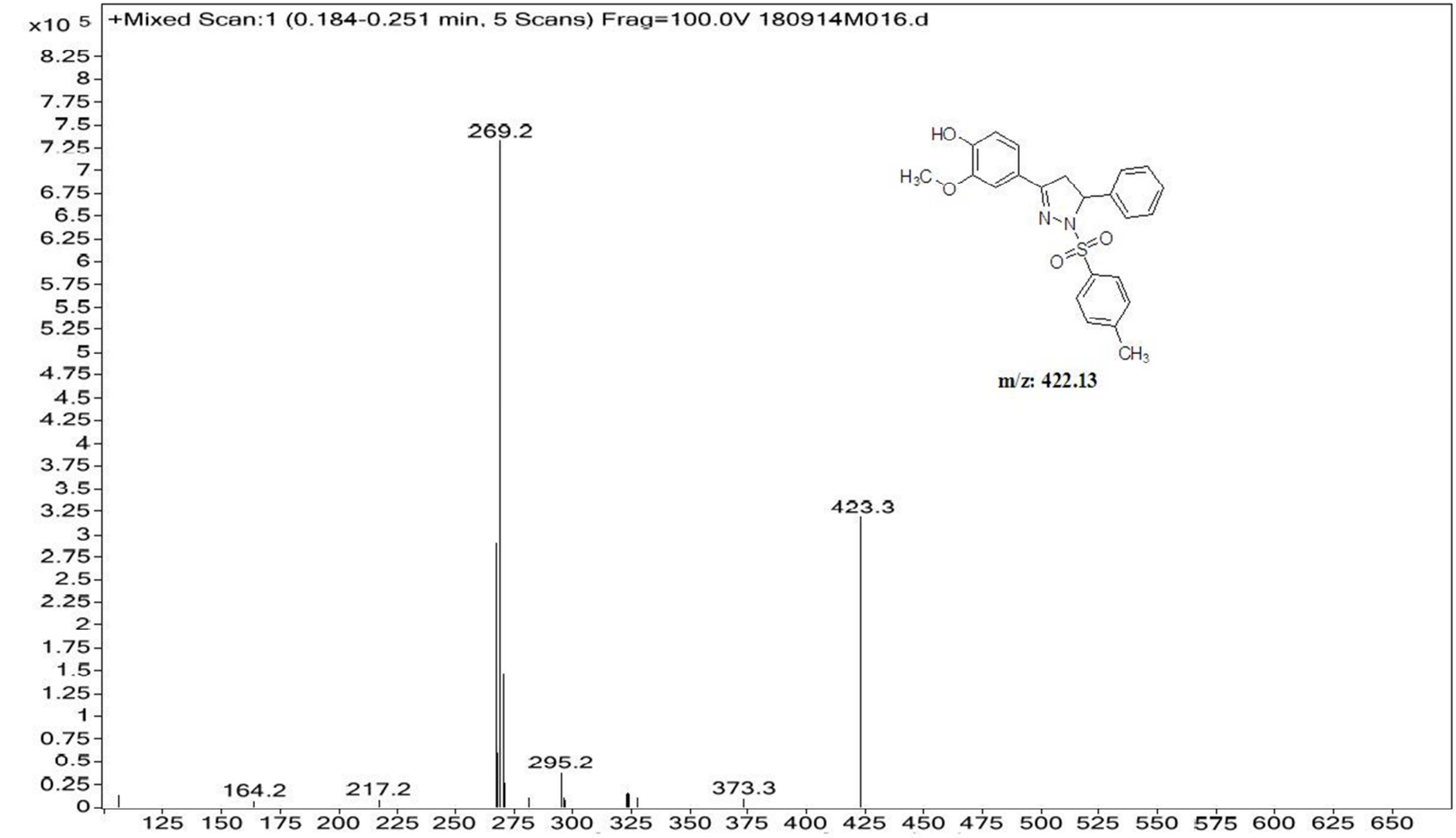


^{13}C-NMR (100 MHz, DMSO-d_6) spectrum of compound 6

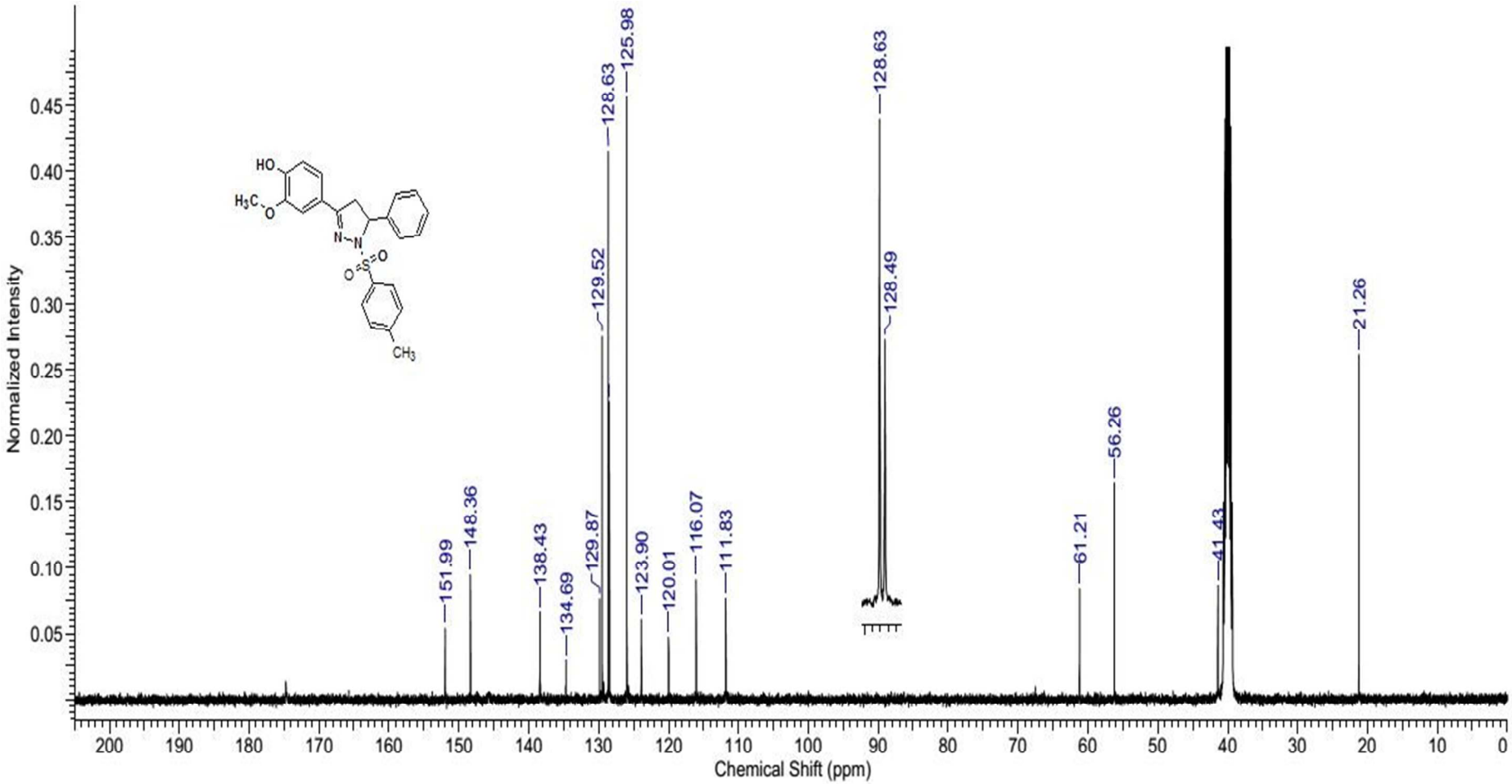


^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 7

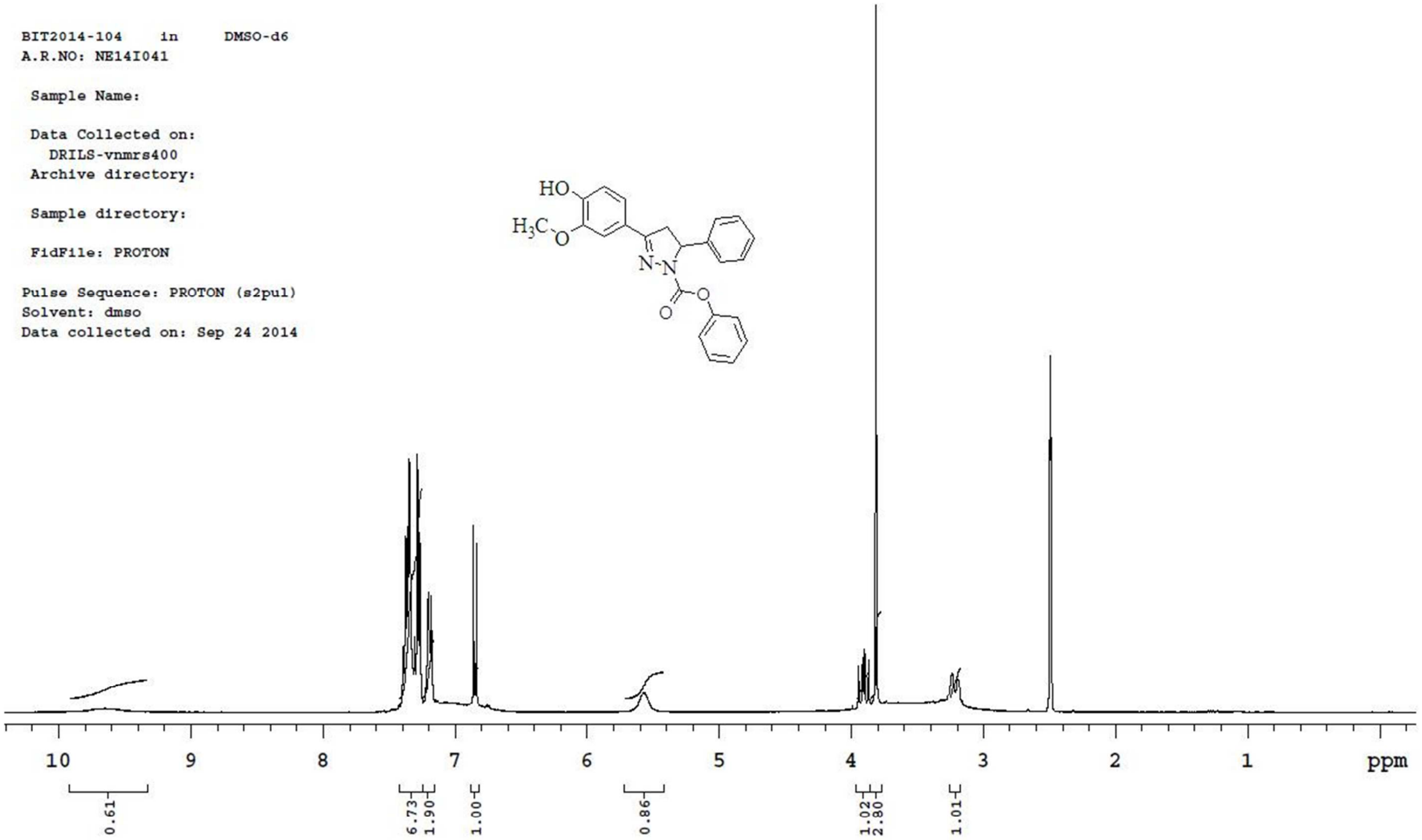
Sample Name	BIT2014-103	**Position**	Vial 67	**Sample type**		**Instrument Name**	Instrument 1
User Name		**Inj Vol**	0.2	**Data Filename**	180914M016.d	**ACQ Method**	test.m
AR NO :	ME14I027	**Acquired Time**	9/18/2014 2:41:10 PM				



ESI-MS spectrum of compound 7

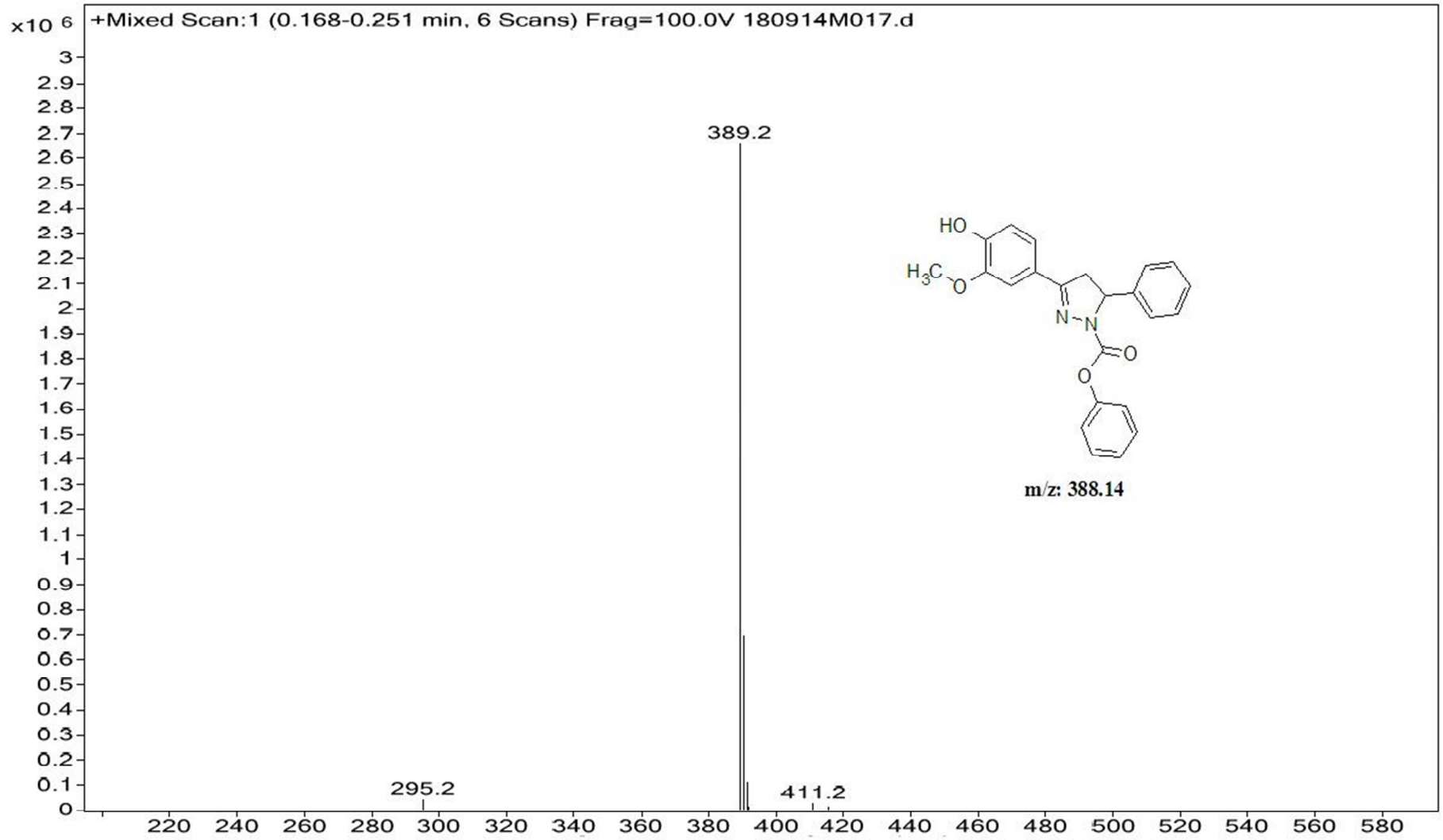


^{13}C-NMR (100 MHz, DMSO-d_6) spectrum of compound 7

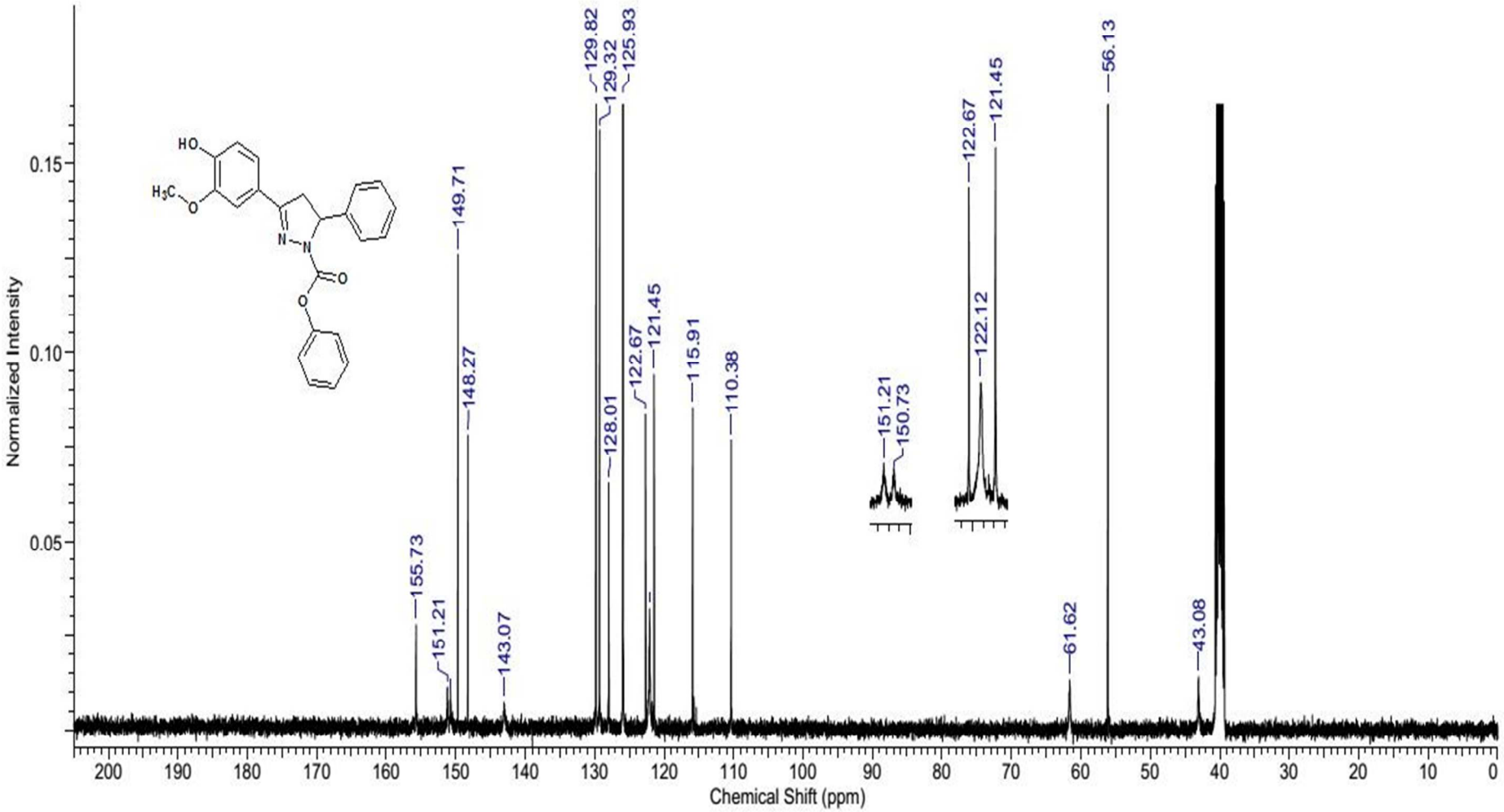


^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 8

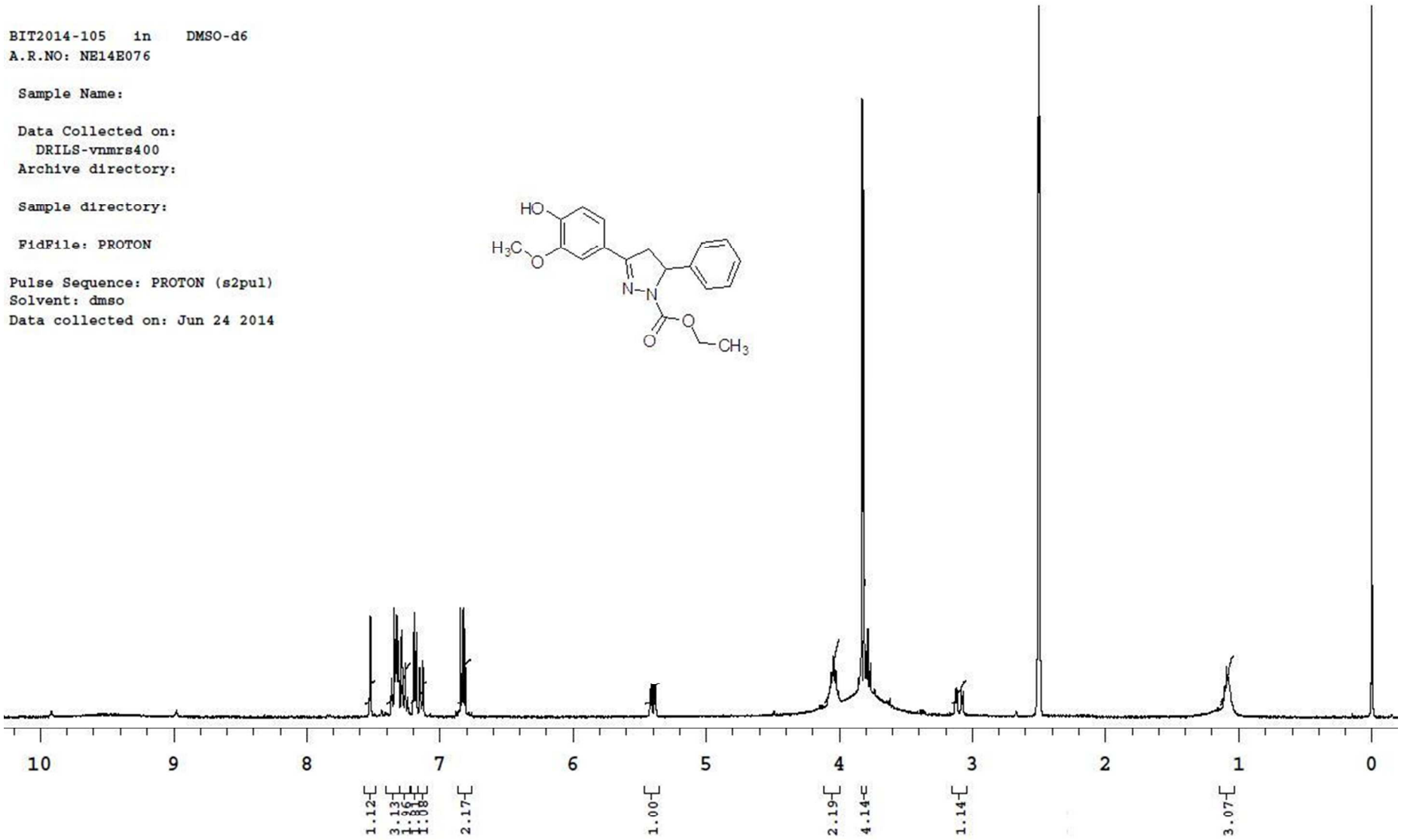
Sample Name	BIT2014-104	**Position**	Vial 68	**Sample type**		**Instrument Name**	Instrument 1
User Name		**Inj Vol**	0.2	**Data Filename**	180914M017.d	**ACQ Method**	test.m
AR NO :	ME14I028	**Acquired Time**	9/18/2014 2:42:47 PM				



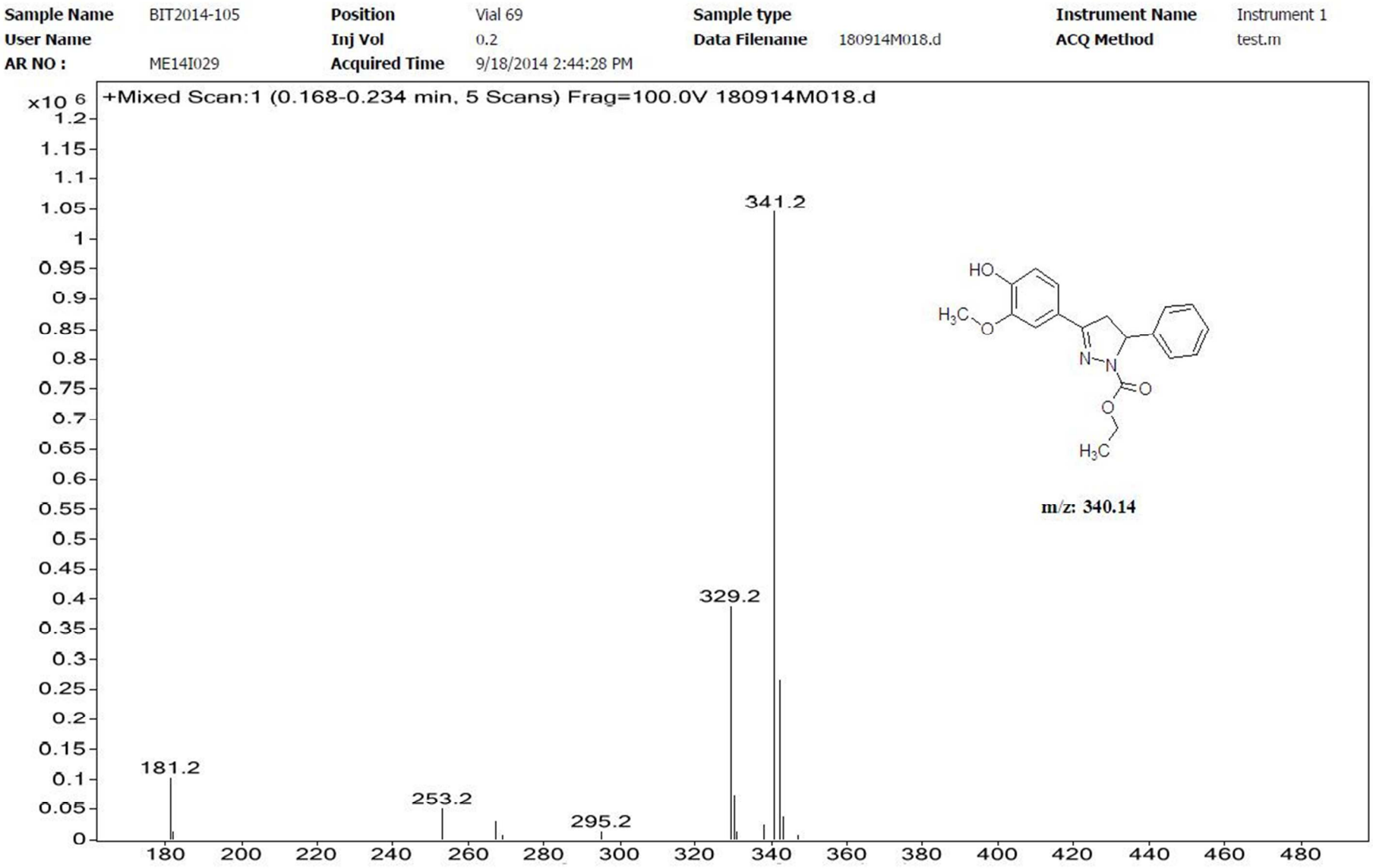
ESI-MS spectrum of compound 8



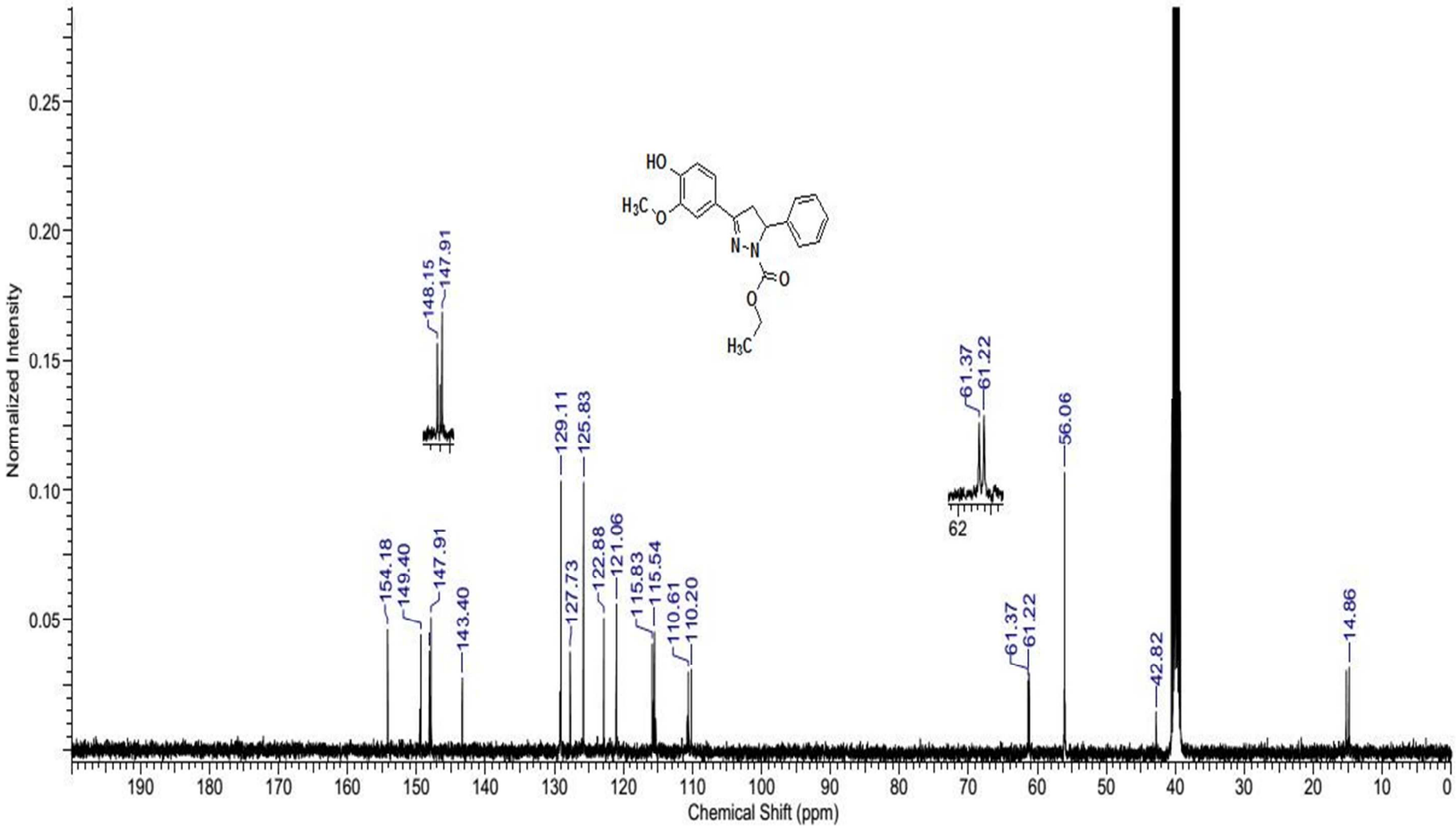
^{13}C-NMR (100 MHz, DMSO-d_6) spectrum of compound 8



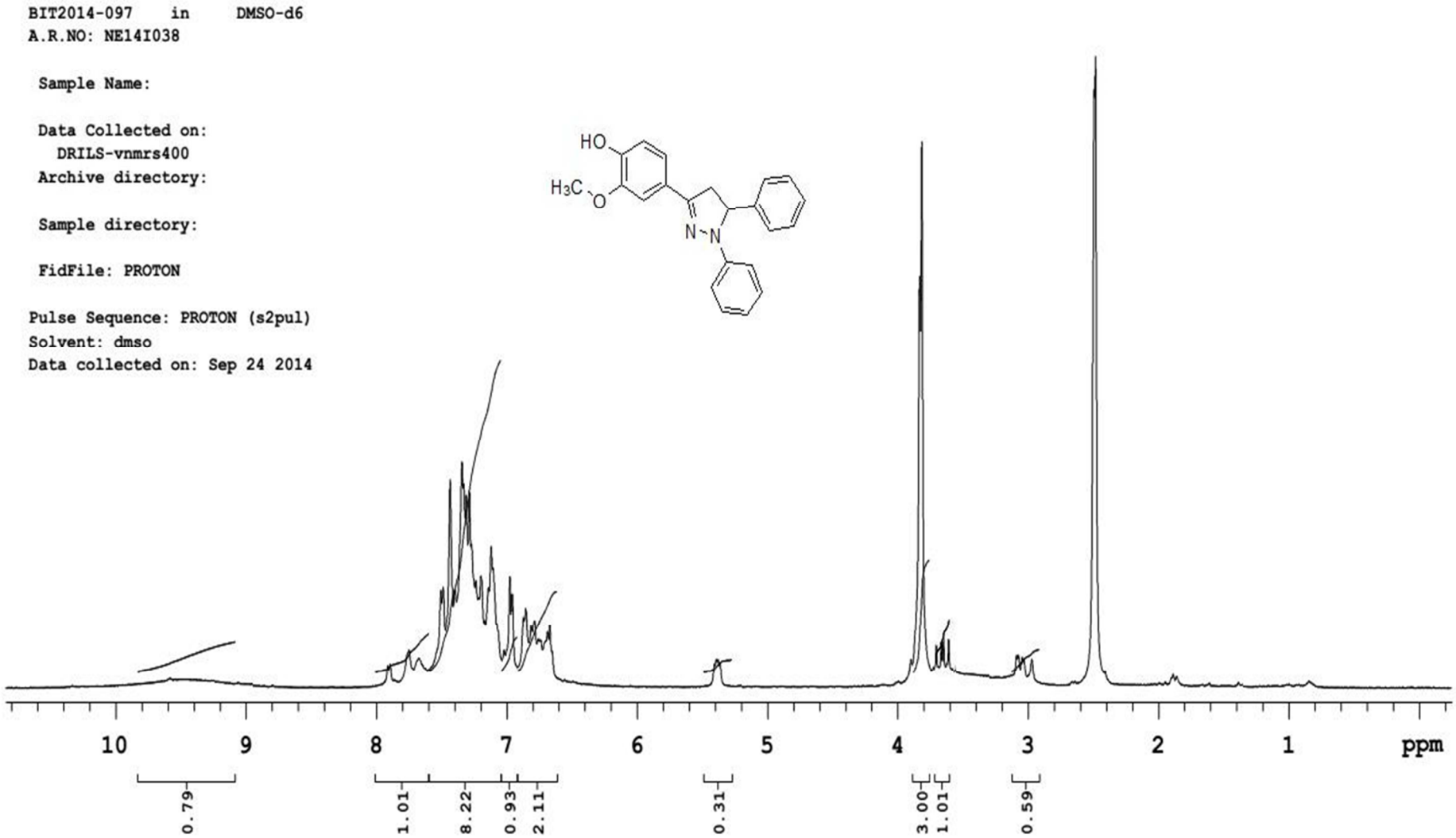
^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 9



ESI-MS spectrum of compound 9



^{13}C-NMR (100 MHz, DMSO-d_6) spectrum of compound 9



^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 10

Sample Name	BIT2014-097	**Position**	Vial 63	**Sample type**		**Instrument Name**	Instrument 1
User Name		**Inj Vol**	0.2	**Data Filename**	180914M012.d	**ACQ Method**	test.m
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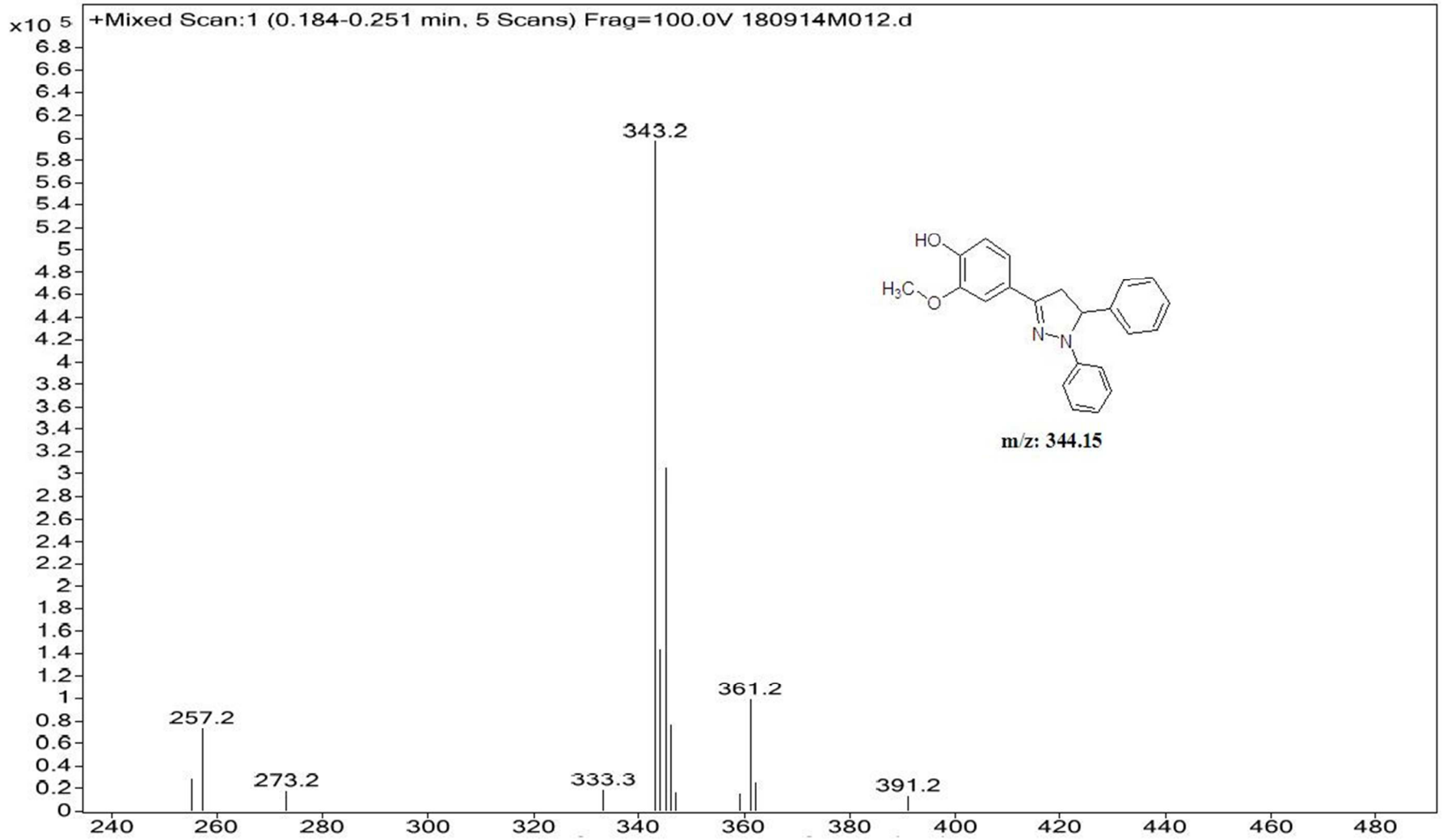
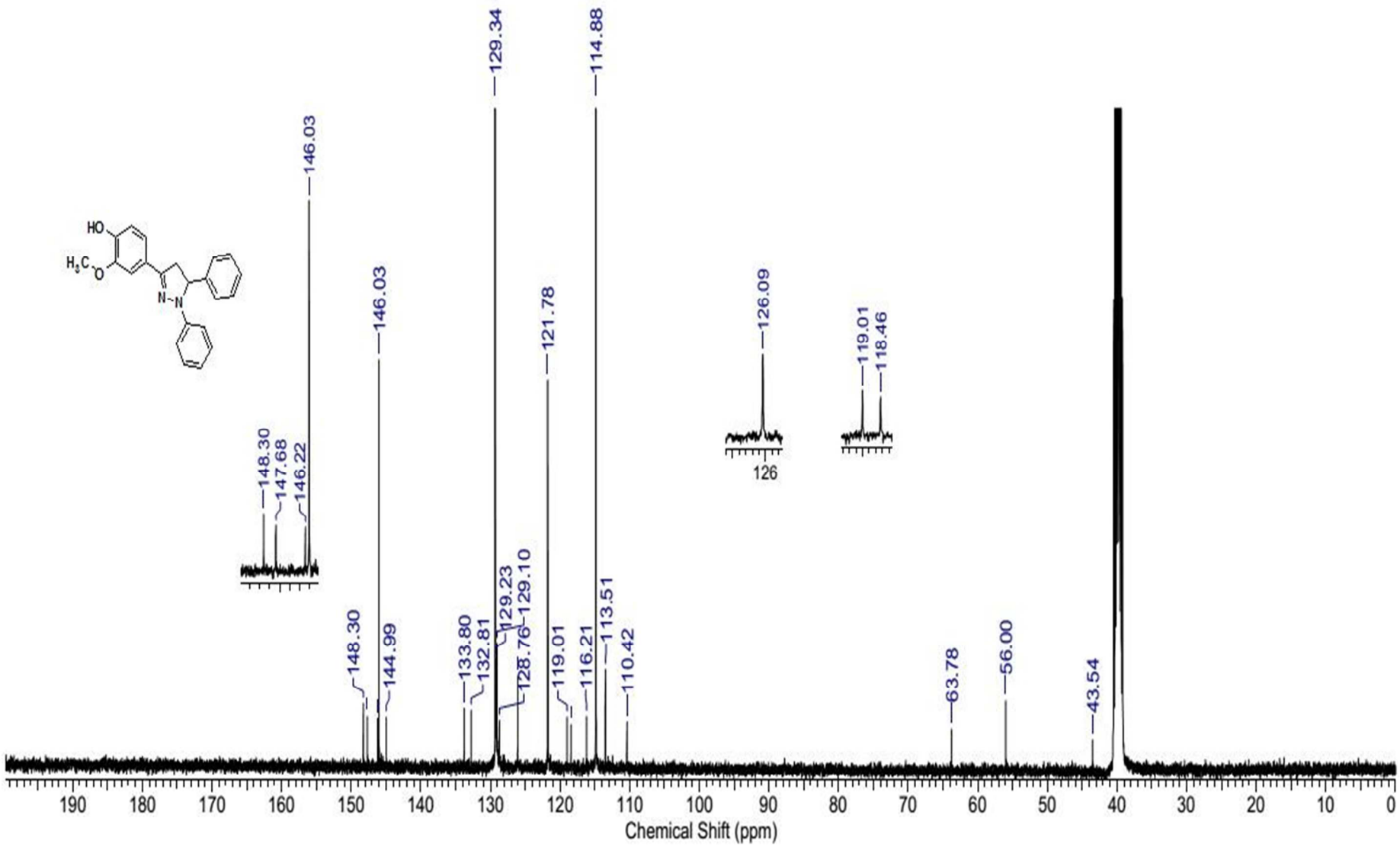
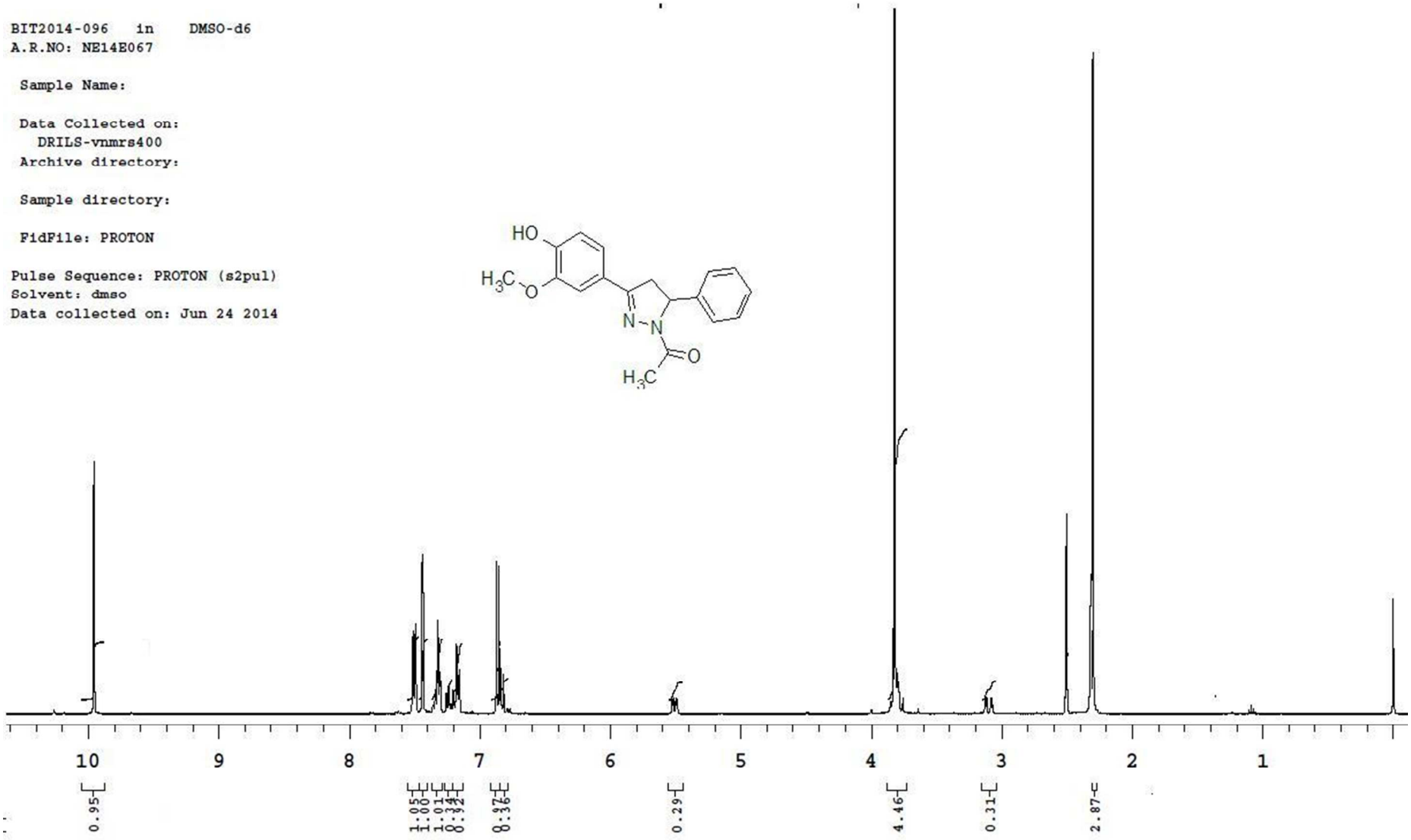


ESI-MS spectrum of compound 10



^{13}C-NMR (100 MHz, DMSO-d$_6$) spectrum of compound 10



^{1}H-NMR (400 MHz, DMSO-d_6)spectrum of compound 11

Sample Name	BIT2014-096	**Position**	Vial 62	**Sample type**		**Instrument Name**	Instrument 1
User Name		**Inj Vol**	0.2	**Data Filename**	180914M011.d	**ACQ Method**	test.m
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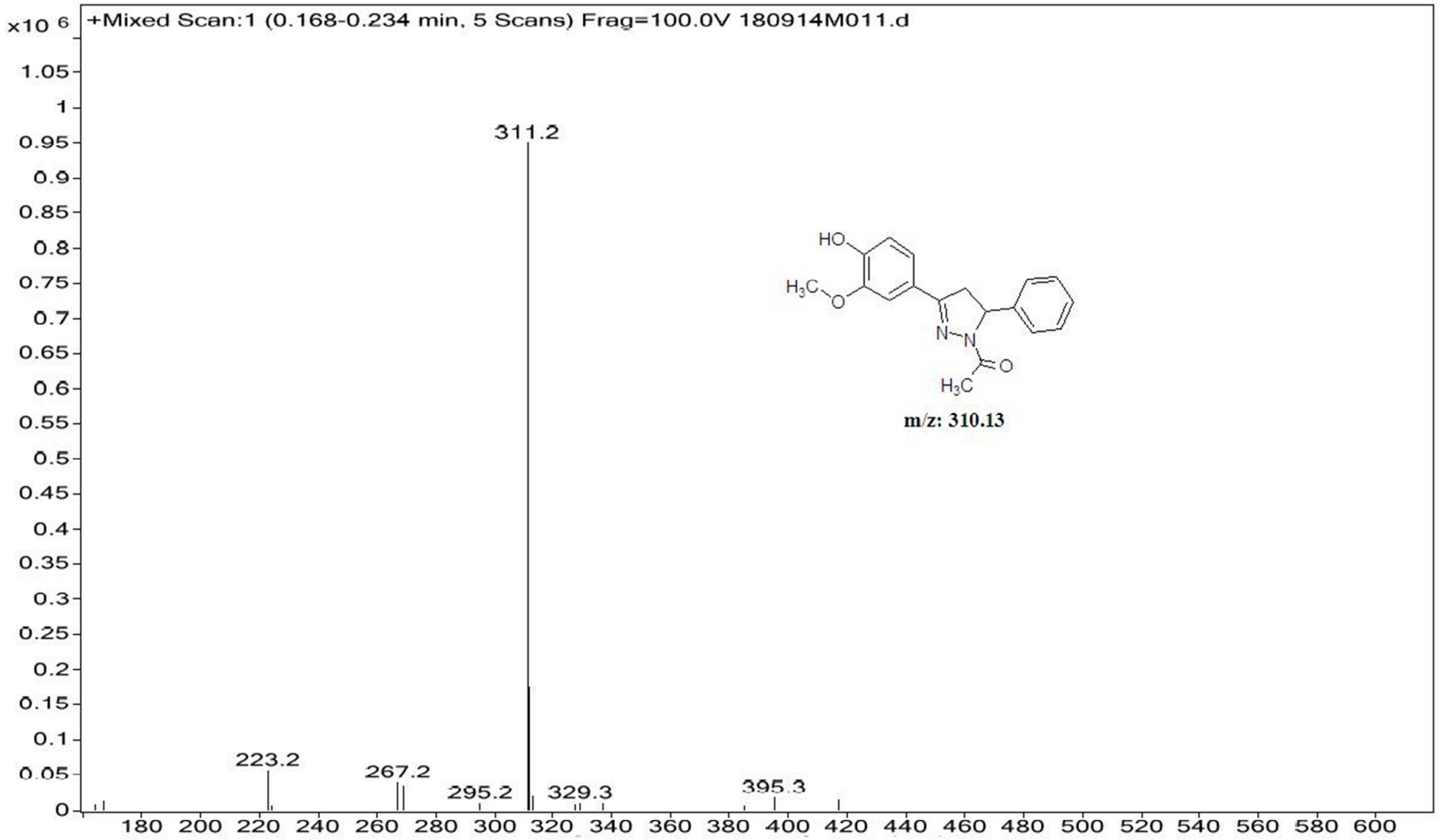
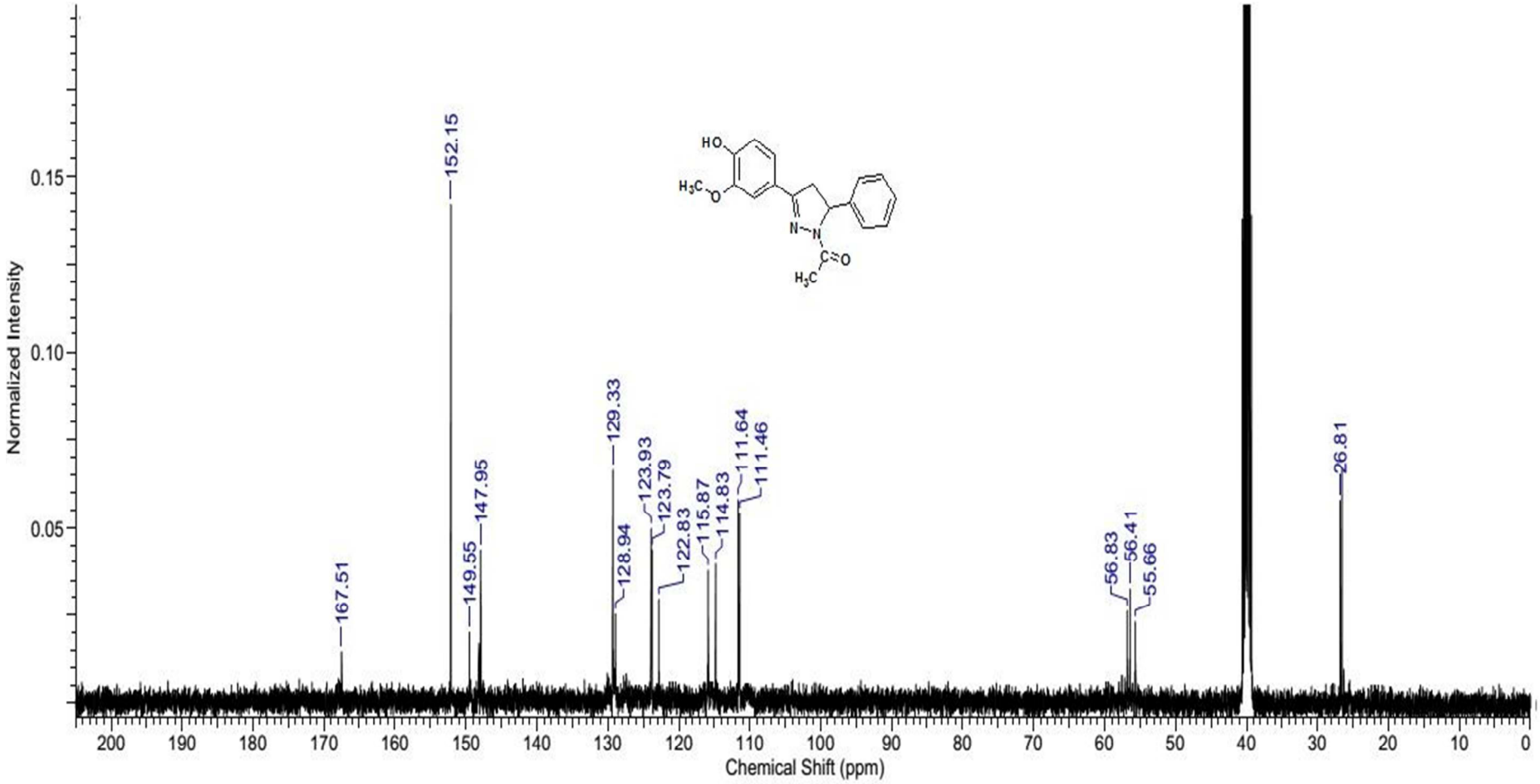
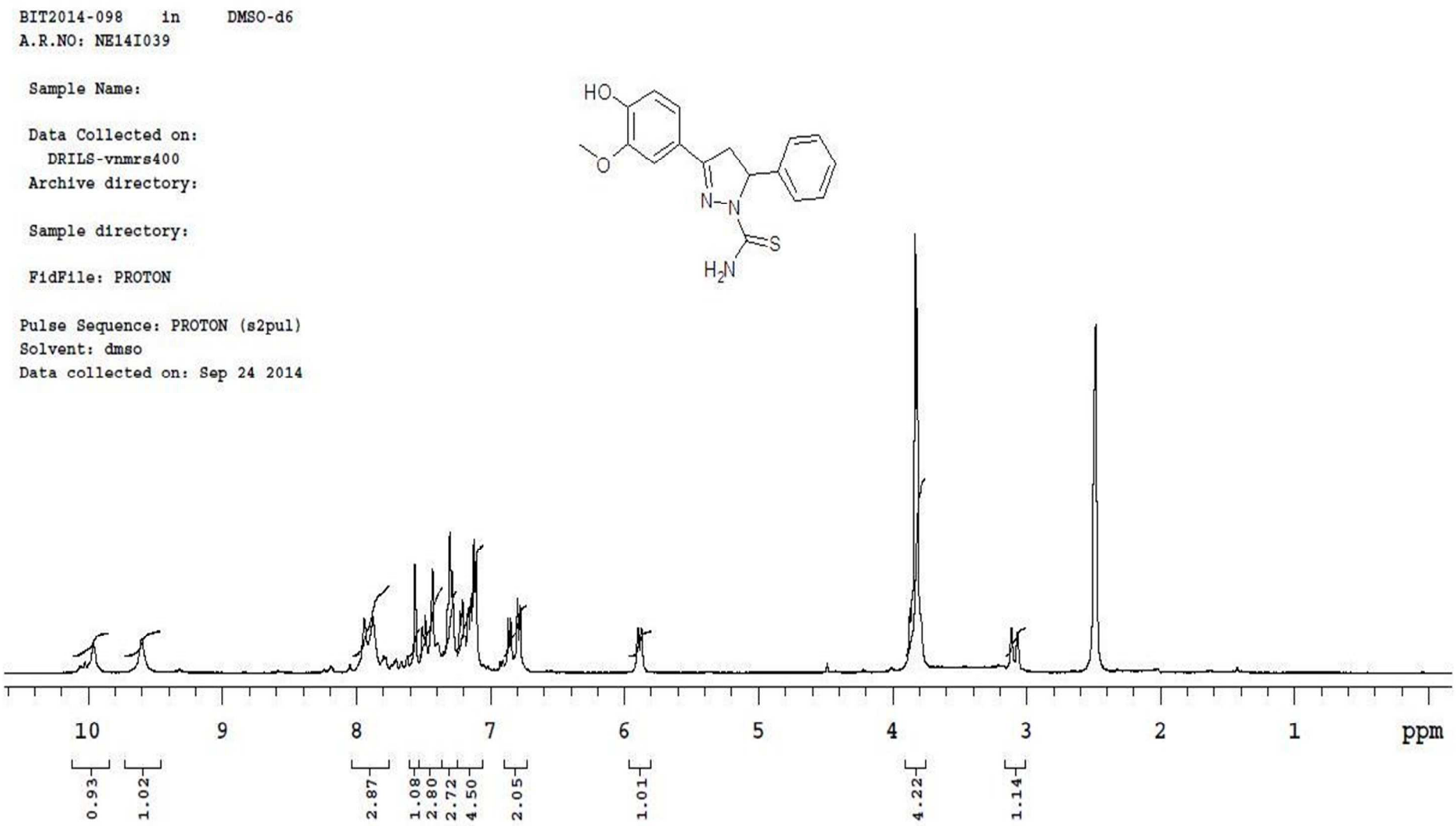


ESI-MS spectrum of compound 11



^{13}C-NMR (100 MHz, DMSO-d$_6$) spectrum of compound 11



^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 12

Sample Name	BIT2014-098	**Position**	Vial 64	**Sample type**		**Instrument Name**	Instrument 1
User Name		**Inj Vol**	0.2	**Data Filename**	180914M013.d	**ACQ Method**	test.m
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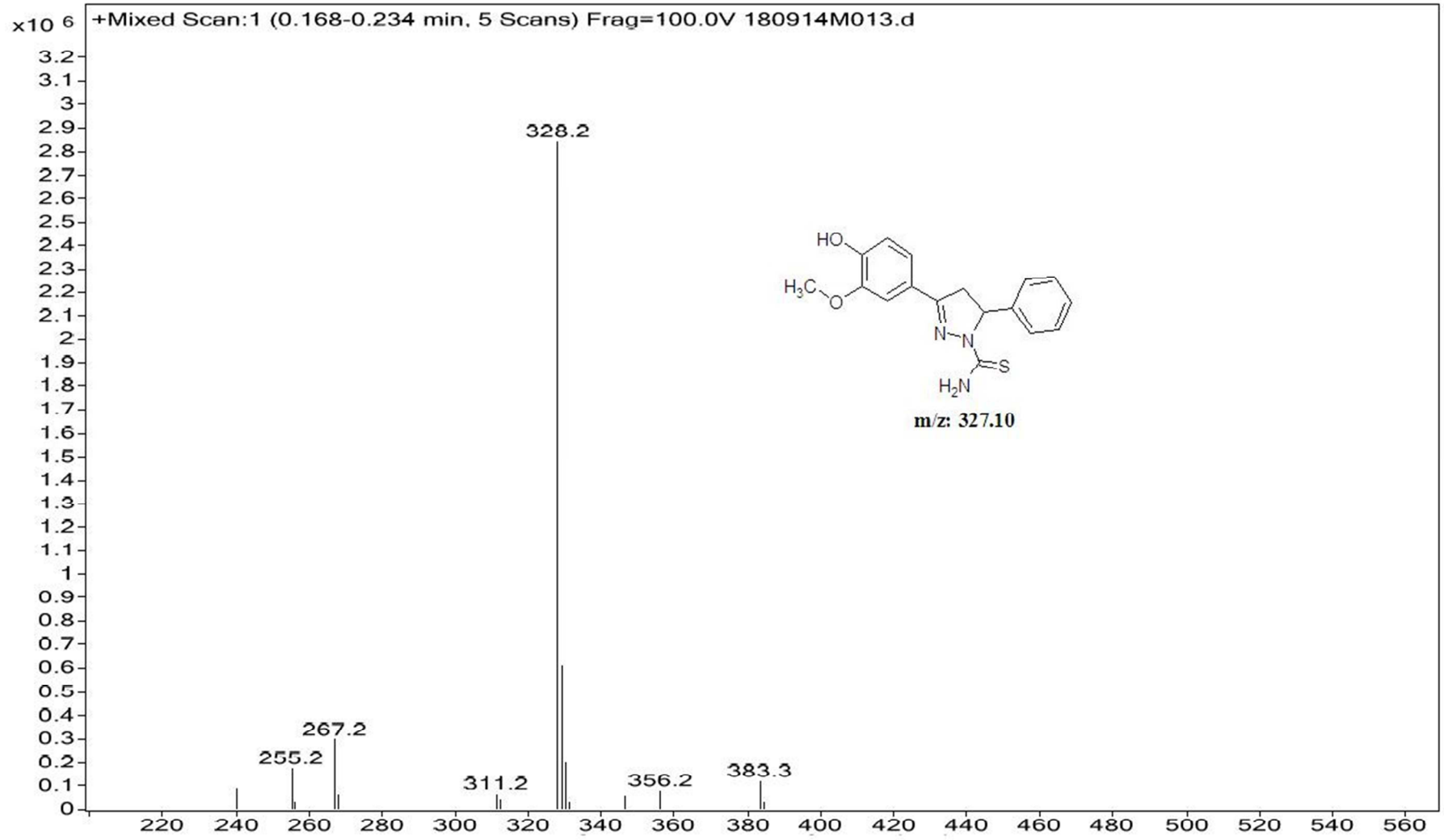
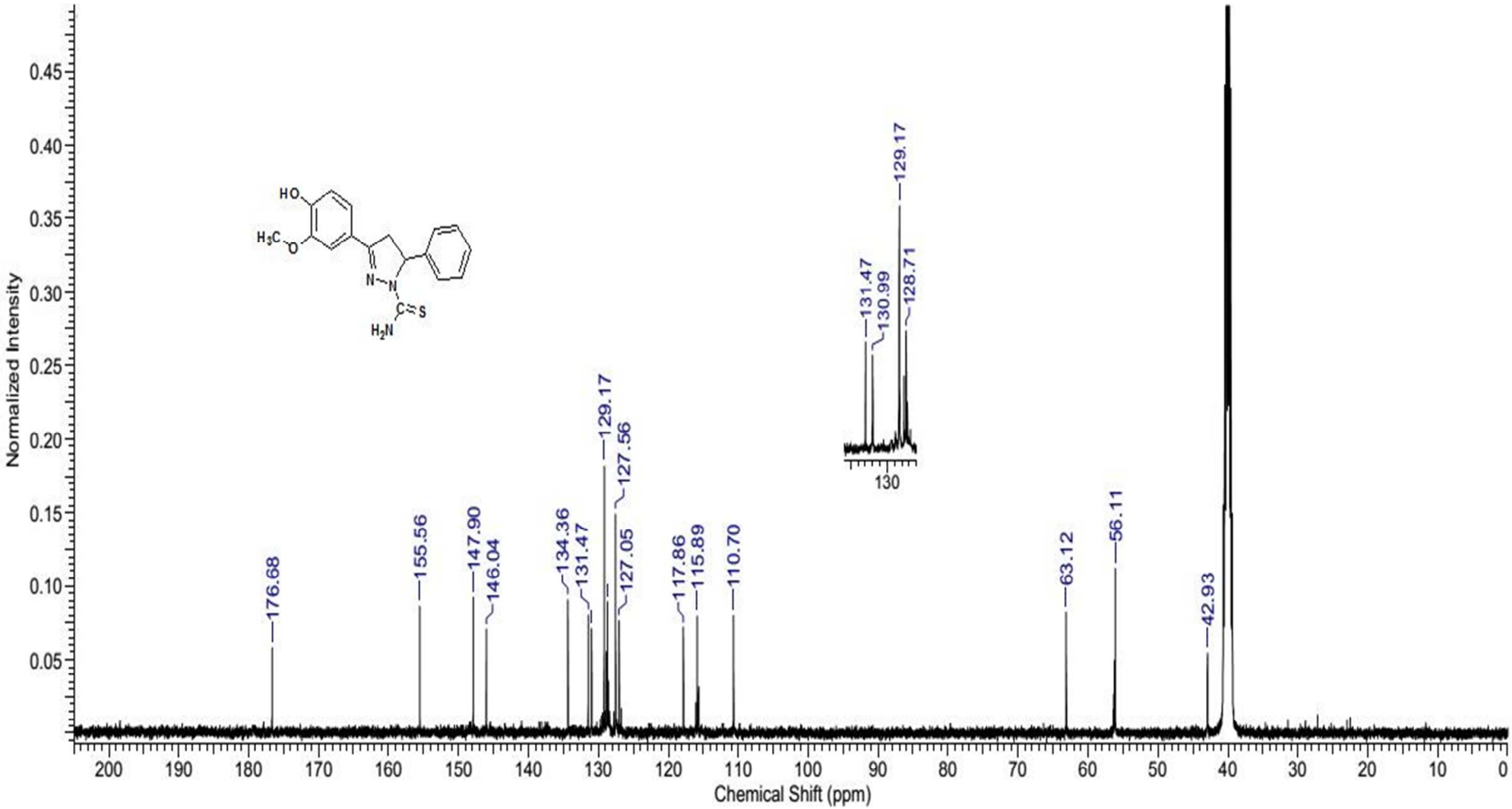
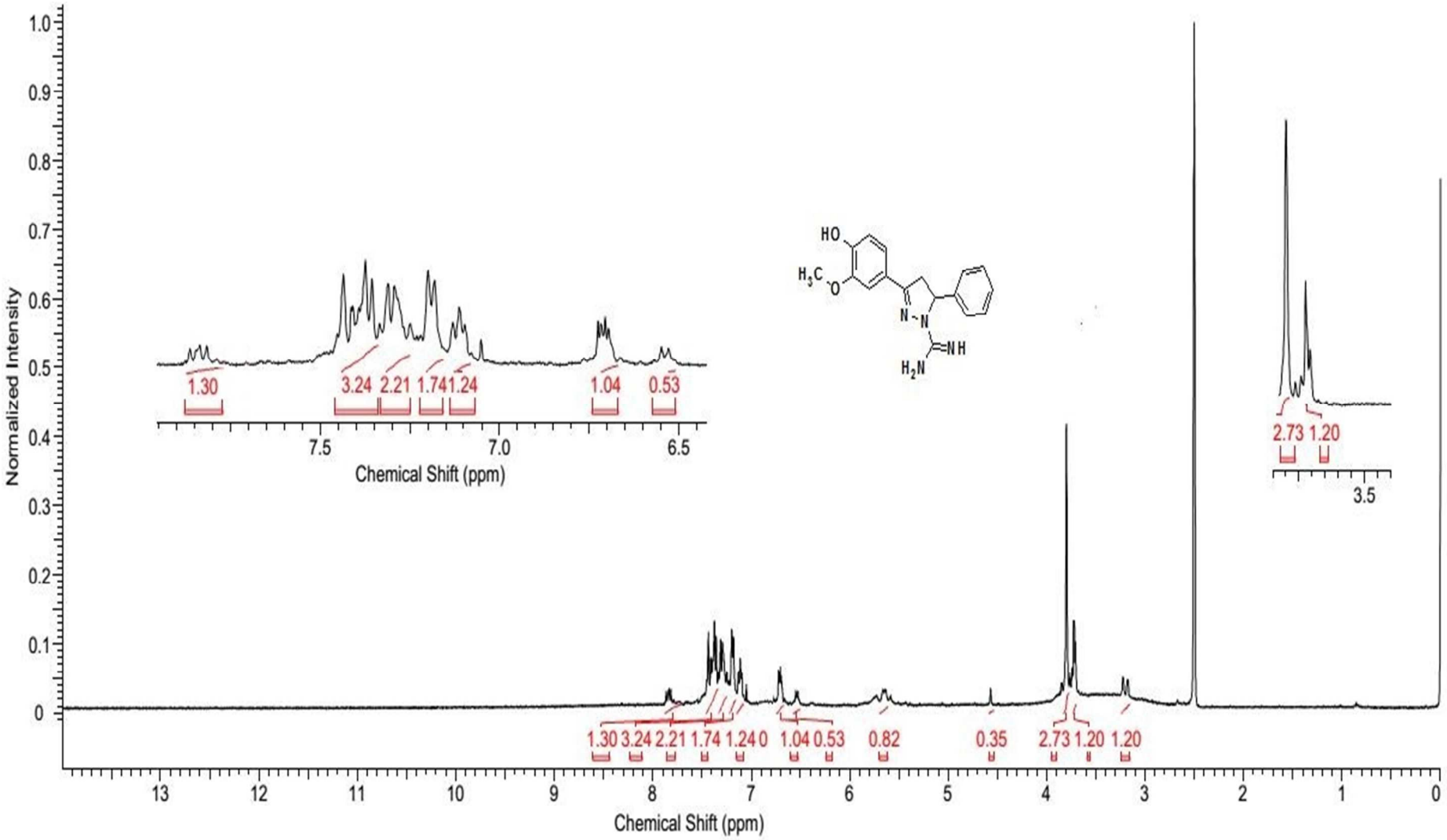


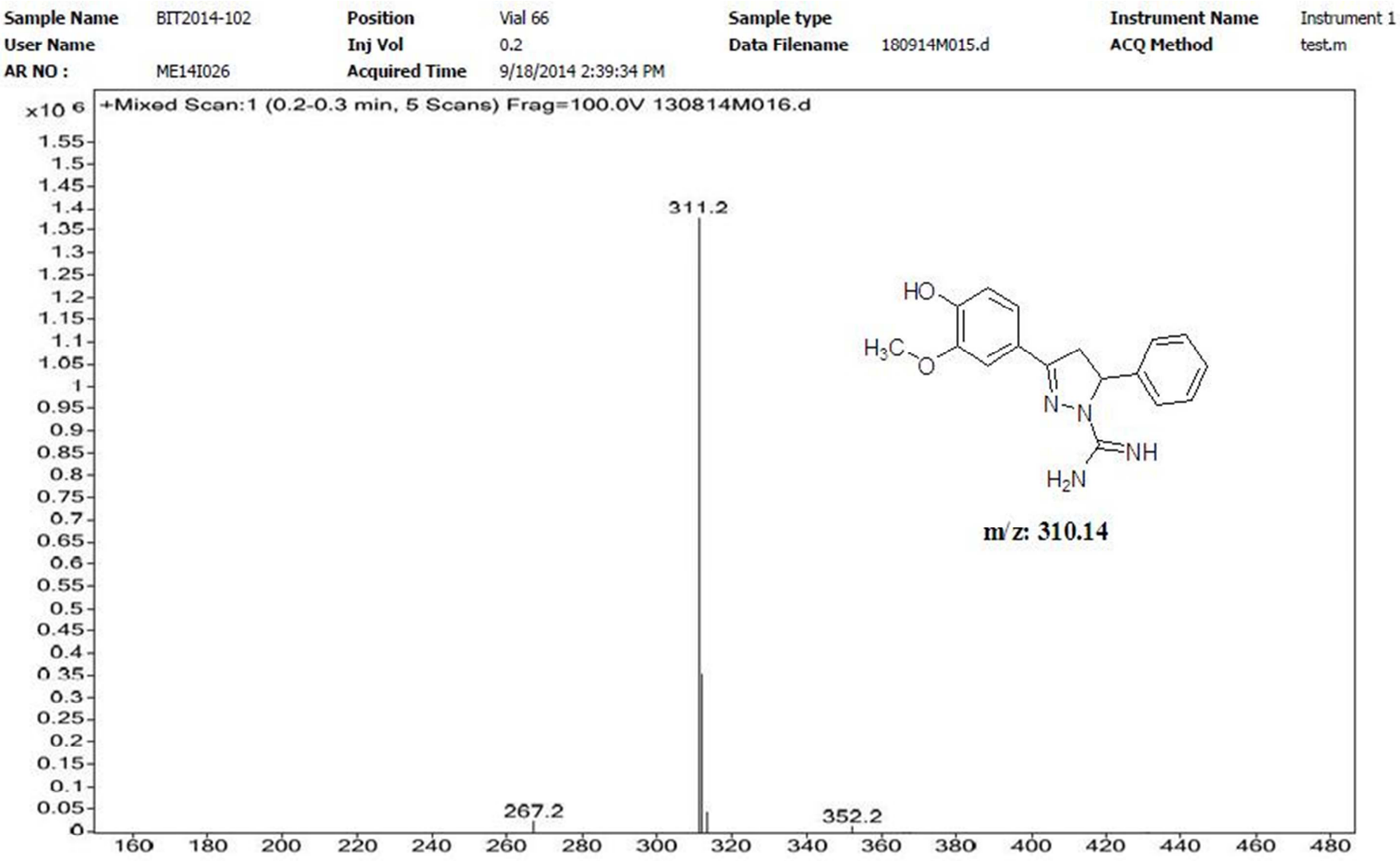
ESI-MS spectrum of compound 12



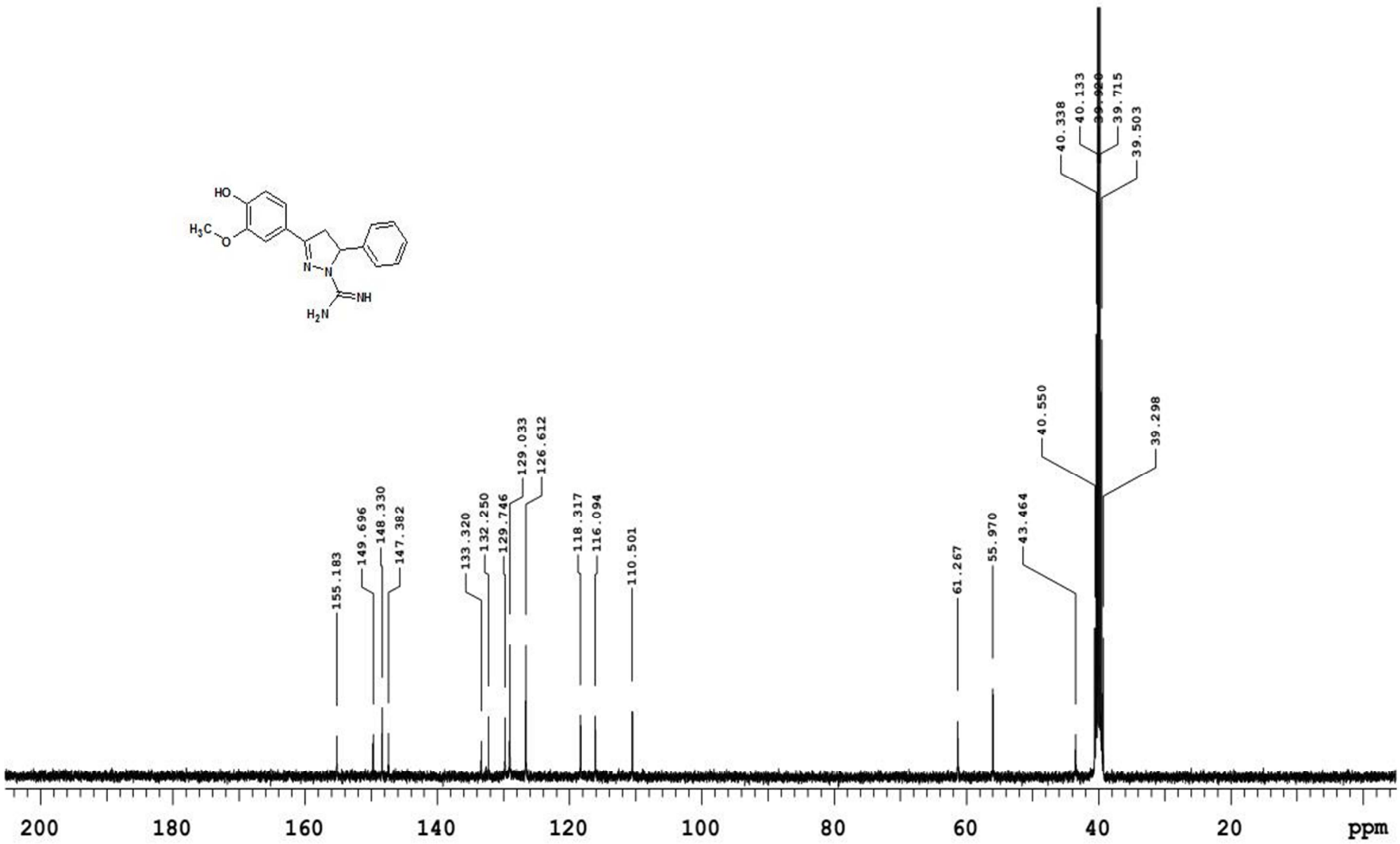
^{13}C-NMR (100 MHz, DMSO-d_6) spectrum of compound 12



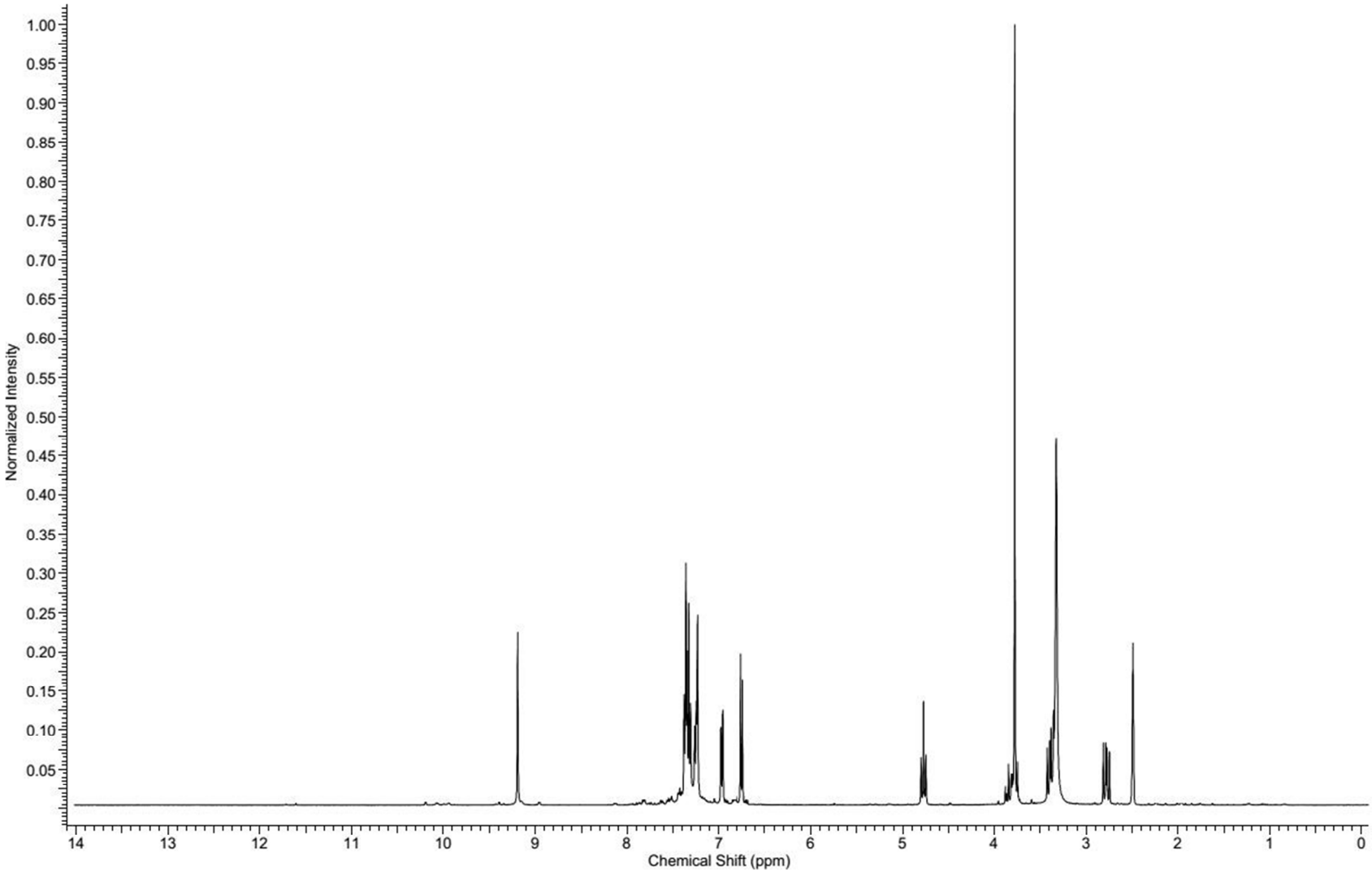
^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 13



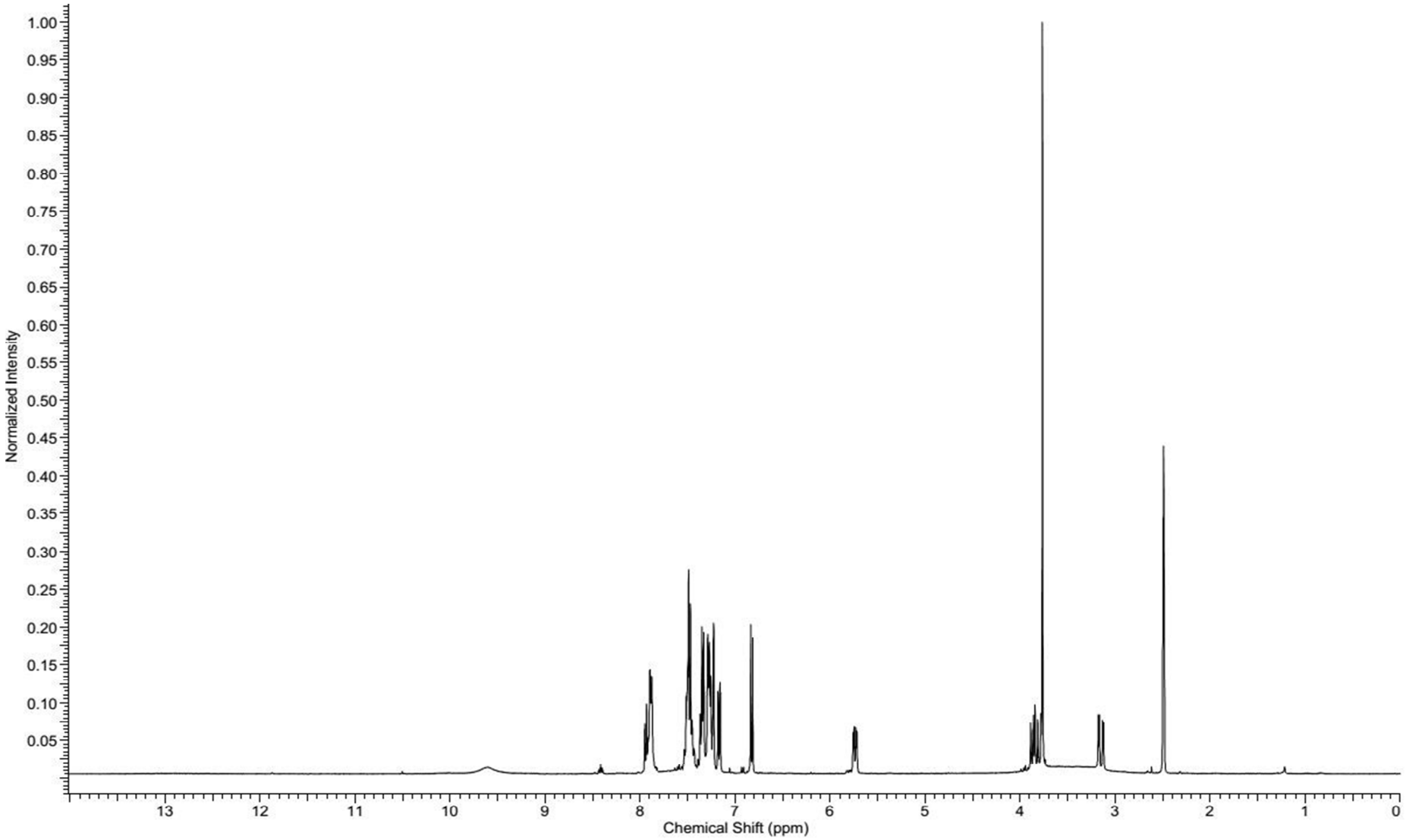
ESI-MS spectrum of compound 13



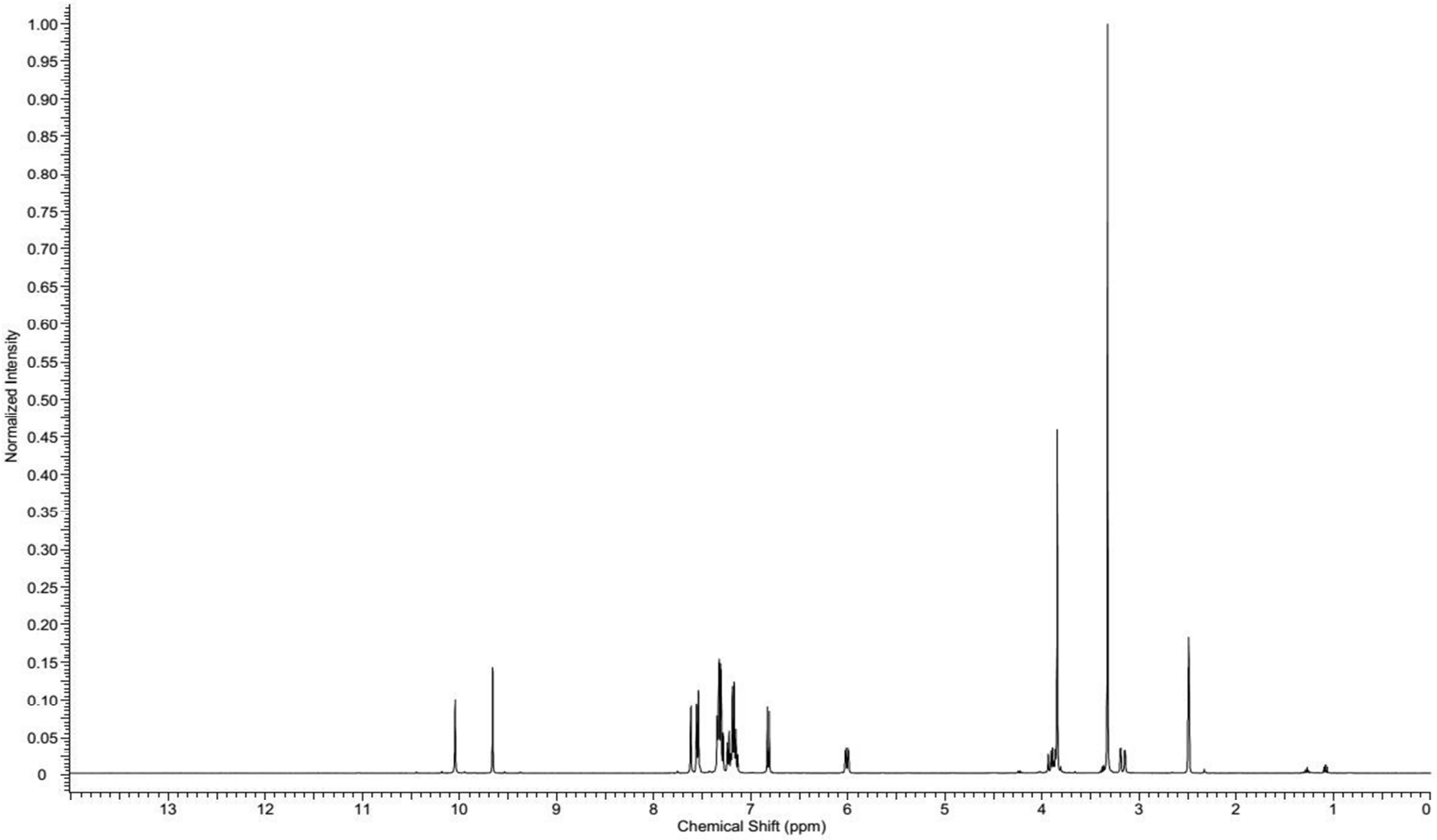
^{13}C-NMR (100 MHz, DMSO-d_6) spectrum of compound 13



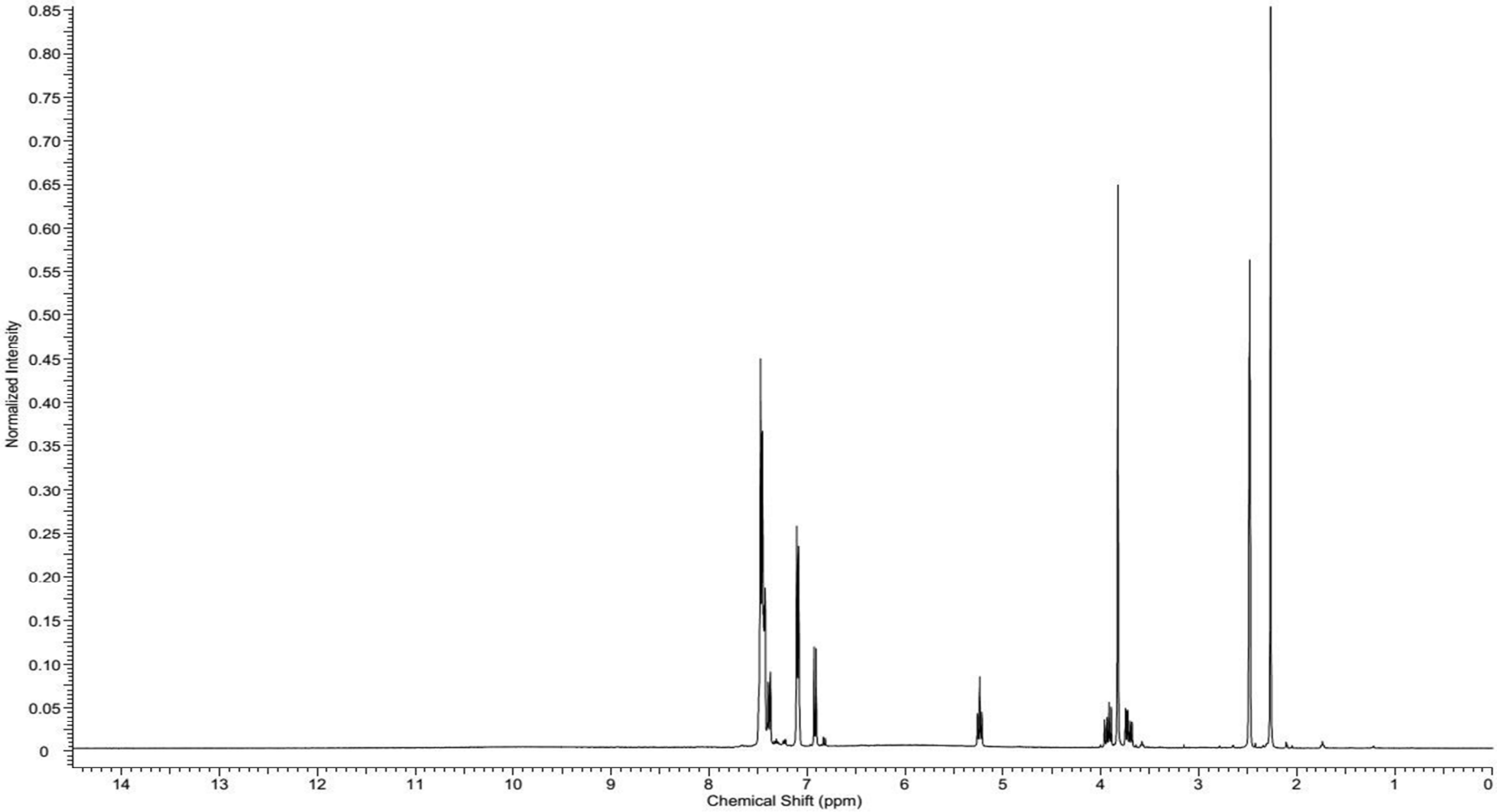
0-14 ppm ^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 4



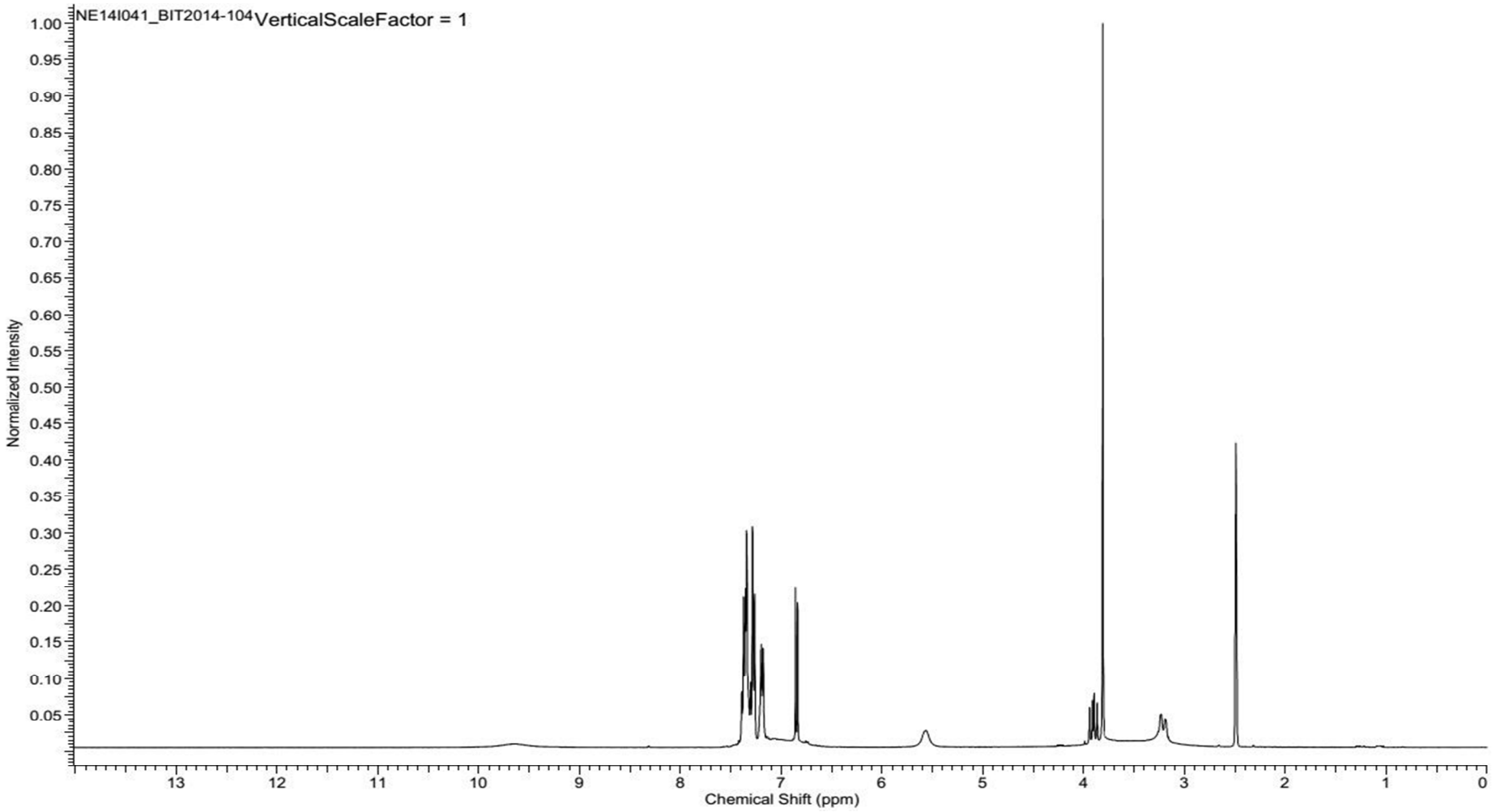
0-14 ppm ^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 5



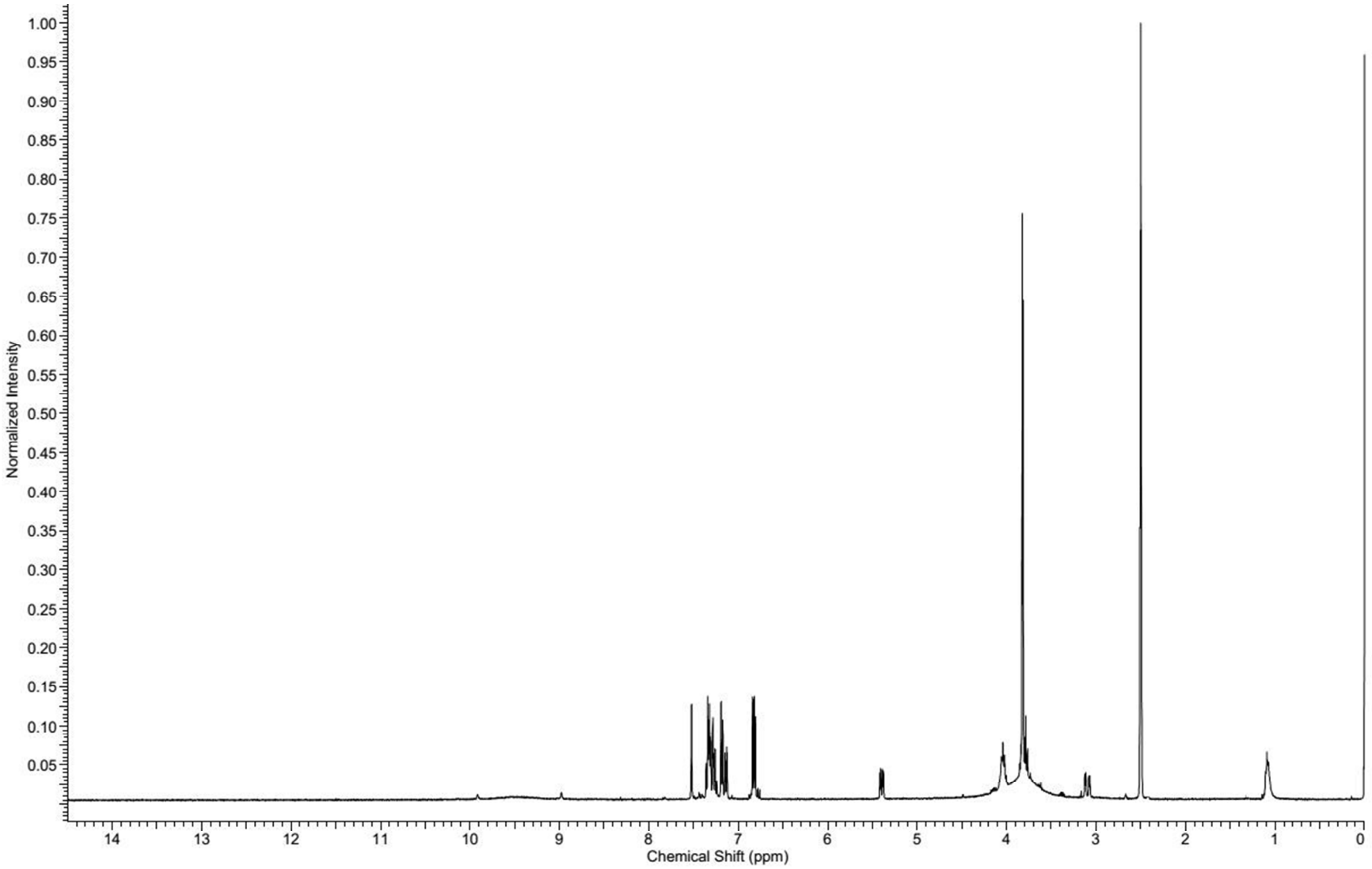
0-14 ppm ^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 6



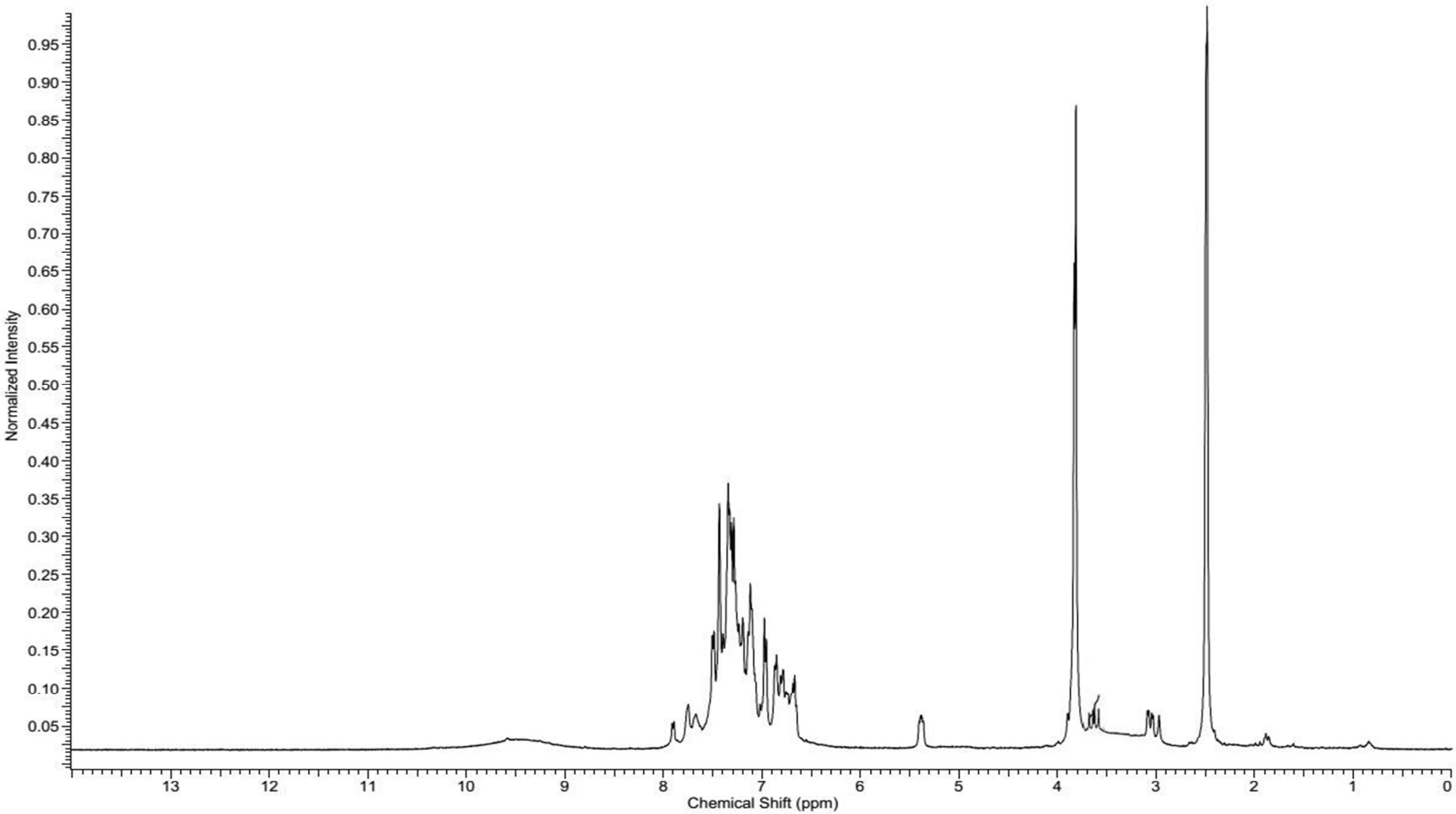
0-14 ppm ^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 7



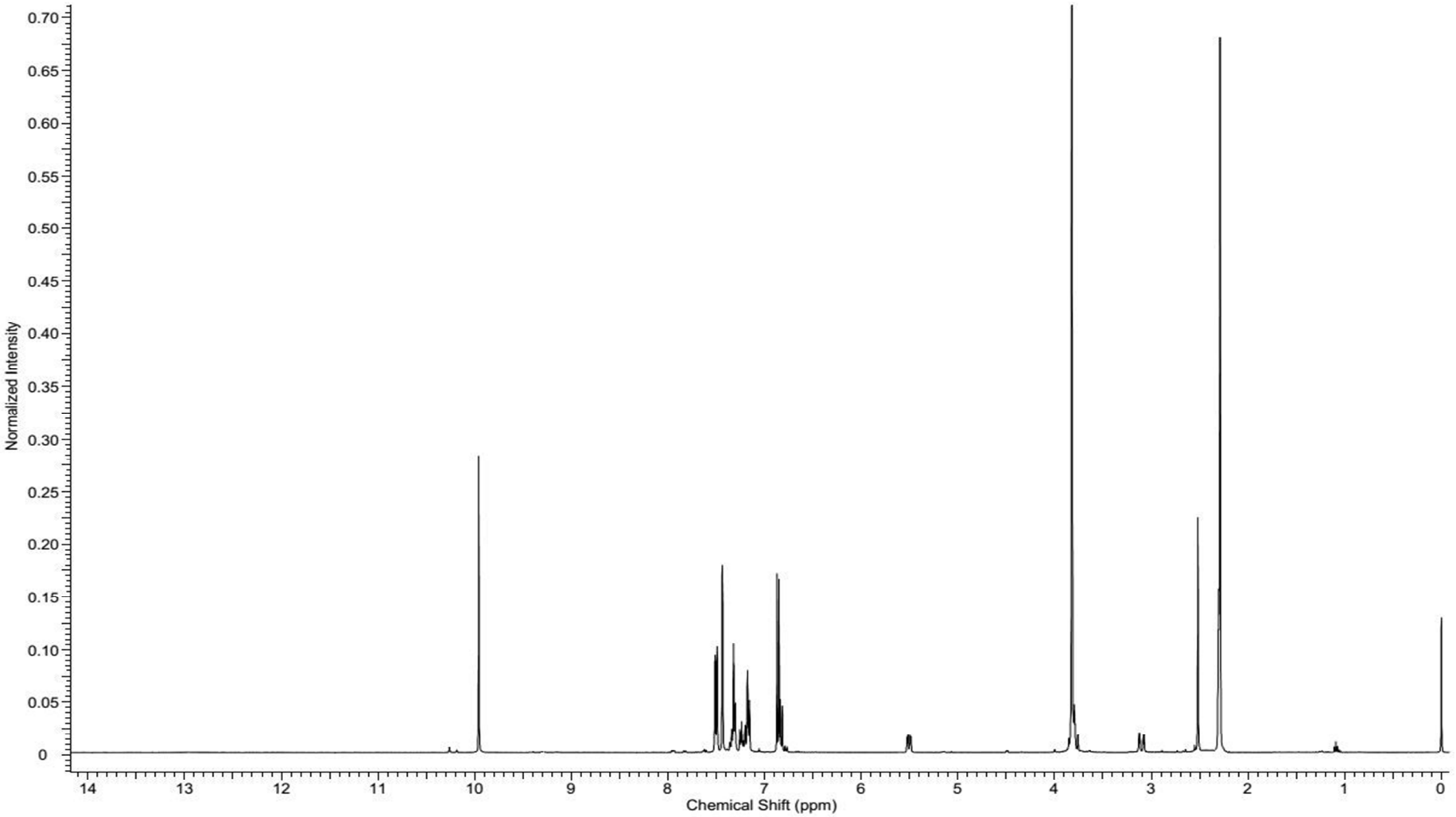
0-14 ppm ^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 8



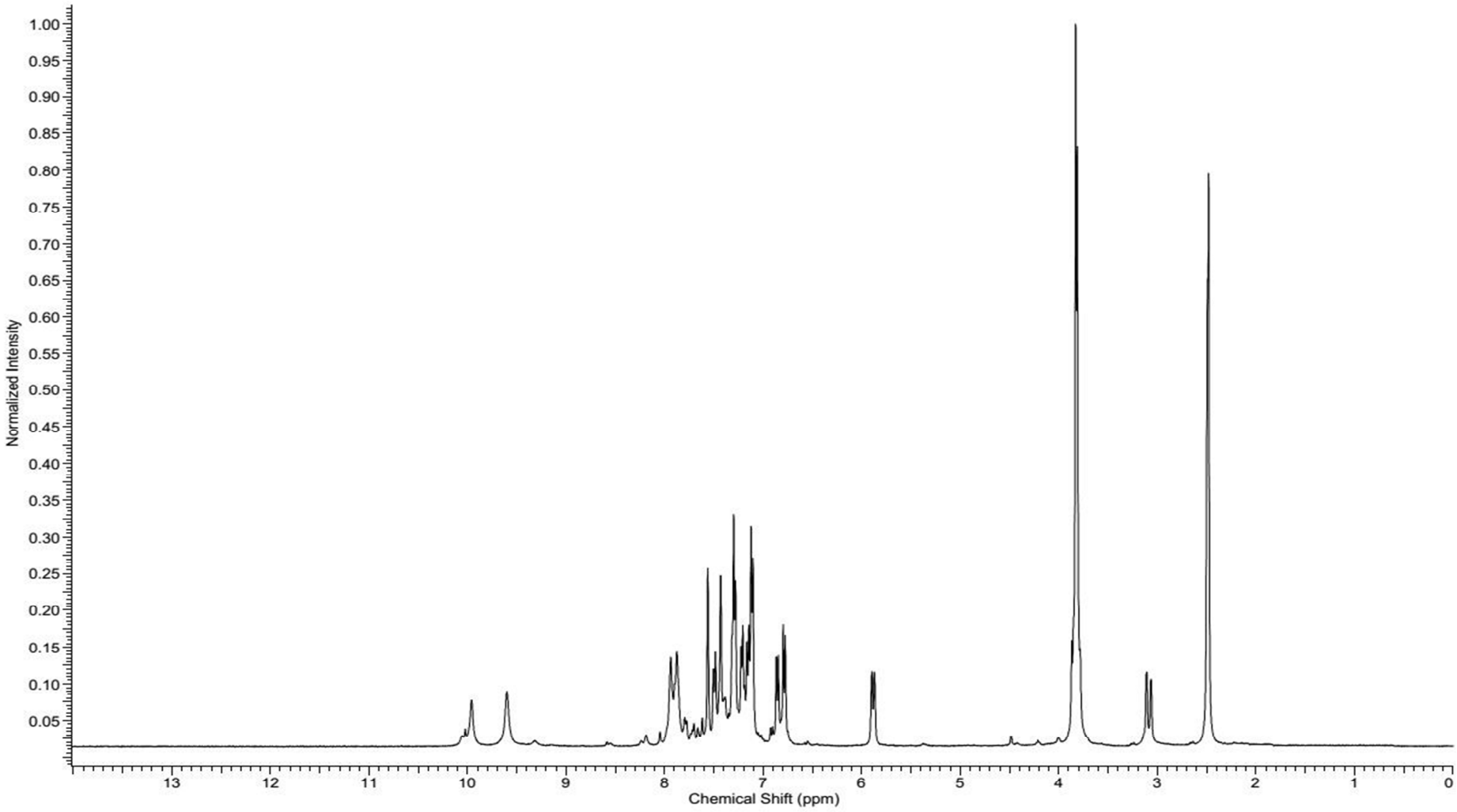
0-14 ppm ^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 9



0-14 ppm ^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 10



0-14 ppm ^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 11



0-14 ppm ^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of compound 12