

Synthesis and study of 2-Acetyl amino-3-[4-(2-amino-5-sulfo-phenylazo)-phenyl]-propionic acid: A new class of inhibitor for Hen egg white lysozyme amyloidogenesis

Sibaprasad Maity, Ravi Kumar, Suman Kumar Maity, Poulami Jana, SantuBera and DebasishHaldar*

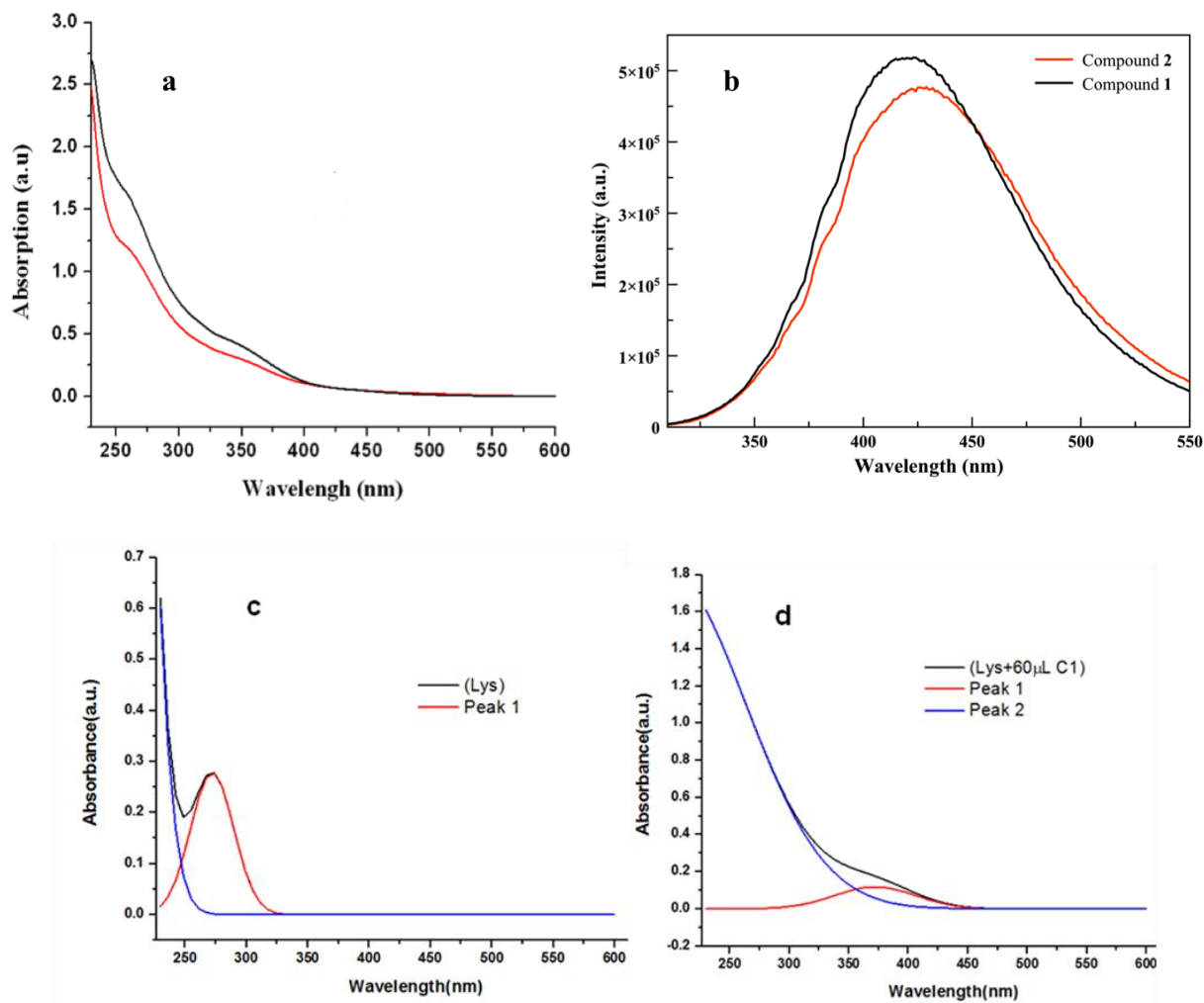
Department of Chemical Sciences, Indian Institute of Science Education and Research Kolkata, Mohanpur, West Bengal 741252, India,

Fax: +913325873020; Tel: +913325873119;

E-mail: deba_h76@yahoo.com; deba_h76@iiserkol.ac.in

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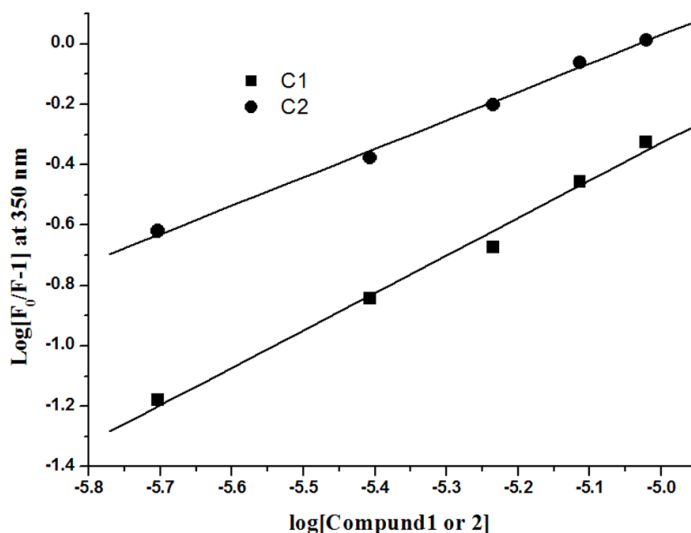
ESI Figure 1: (a) UV-Visible Spectra and (b) Emission spectra (excitation at 293 nm) of compound **1**(black)and **2**(red) (2×10^{-4} M) for both. (c) Deconvolution of UV-Visible Spectra of HEWL showing only one band. (d) Deconvolution of UV-Visible Spectra of HEWL with compound **1** showing two bands.

Binding Constant(K) and Number of binding site (n)

We have calculated binding constant for both the compounds using the equation

$$\log[F_0/F-1] = \log K + n \log[Q]$$

Where K and n are binding constant and number of binding site respectively.



ESI Figure 2: Plot $\log[F_0/F-1]$ vs $\log[\text{Compound 1 or 2}]$ for both the compound.

ESI Table 1: Binding constant parameters for both the compounds.

Compound	$K/10^4$	n	Linear Coefficient (R)
1	73.21 ± 0.0002	1.24	0.9951
2	5.52 ± 0.00015	0.94	0.9980

Materials and Methods

General

L-phenylalanine, sulphanilic acid and Ethyltrifluoroacetate were purchased from Sigma chemicals. Acetyl chloride, H_2SO_4 , HNO_3 , $NaNO_2$ were purchased from Merck Germany. Hen egg white lysozyme was purchased from SRL.

Amino acids Synthesis

The amino acids were synthesized by conventional nitration, reduction and diazo coupling methods. The acetyl or trifluoro acetyl group was used for N-terminal protection and the C-terminus was protected as a methyl ester. The intermediates and final compounds were purified and characterized by 400 MHz or 500 MHz 1H & ^{13}C NMR spectroscopy, FTIR, Mass spectroscopy and elemental analysis.

Synthesis

(a) **Compound 4:** 6.6g (40 mmol) of L-phenylalanine was dissolved in 60 mL of MeOH and cooled in ice bath. Then 8 ml of $SOCl_2$ was added dropwise and stirred for 8h. The excess solvent was evaporated under rotary vacuum and the dried solid product was dissolved in dilute $NaHCO_3$ solution and extracted with ethyl acetate. The organic phase was then dried with anhydrous Na_2SO_4 , evaporated under rotary vacuum and finally under vacuum pump to get waxy solid pure compound **4**.

Yield: 5.98g (33.4 mmol, 83.5%)

Melting point: 83-84°C

^1H NMR (400 MHz, DMSO- d_6 , δ_{ppm}): 7.275-7.188 (m, 5H, aromatic protons), 3.578-3.570 (m, 1H, C^αH), 3.563 (s, 3H, OCH_3), 2.904-2.775 (m, 2H, C^βH), 1.981 (b, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO- d_6 , δ_{ppm}): 175.47, 137.96, 129.25, 128.17, 126.32, 55.79, 51.37, 40.79. Mass spectra: m/z 180.2612 $[\text{M}+\text{H}]^+\text{M}_{\text{cal}} = 179.1025$ Anal. Calcd for $\text{C}_{10}\text{H}_{13}\text{NO}_2$ (179.09): C, 67.02; H, 7.31; N, 7.82. Found: C, 66.33; H, 7.55; N, 7.63.

(b) **Compound 5a**: 2.8g (15.6 mmol) of compound **4** was dissolved in 80 mL pyridine and cooled in ice-bath. Then 1.33 mL (18.72 mmol) of acetyl chloride was added to the solution and stirred for 5h at room temperature. Then the pyridine was evaporated under rotary vacuum and small amount of water was added and evaporated again. The product was dissolved in ethyl acetate and washed with 1N HCl solution three times and then brine solution. The organic layer was dried over anhydrous sodium sulphate and evaporated under rotary evaporator. The crude product was purified by column chromatography using silica-gel as stationary phase and ethyl acetate-hexane mixture (1:6) as eluent to get pure white solid compound **5a**.

Yield: 2.92g (13.2 mmol, 84.6%)

Melting point: 92-93°C

^1H NMR (400 MHz, CDCl_3 , δ_{ppm}): 7.278-7.268 (m, 3H, aromatic protons), 7.090-7.072 (m, 2H, aromatic protons), 6.061-6.043 (d, 1H, $J=7.2$ Hz, NH), 4.881-4.848 (m, 1H, C^αH), 3.701 (s, 3H, OCH_3), 3.122-3.090 (m, 2H, C^βH), 1.963 (s, 3H, CH_3). ^{13}C NMR (100 MHz, CDCl_3 , δ_{ppm}): 172.07, 169.62, 135.76, 129.14, 128.49, 127.04, 53.05, 52.26, 37.73, 23.02. FTIR (cm^{-1}): 3332, 3038, 2946, 1760, 1646, 1533, 1446, 1366, 1200. Mass spectra: observed m/z 244.3714 $[\text{M}+\text{Na}]^+\text{M}_{\text{cal}} 221.1052$. Anal. Calcd for $\text{C}_{12}\text{H}_{15}\text{NO}_3$ (221.10): C, 65.14; H, 6.83; N, 6.33. Found: C, 64.98; H, 6.12; N, 6.24.

(c) **Compound 5b**: 2.8g (15.6 mmol) of compound **4** was dissolved in 30 mL MeOH and 1.48 mL (18.72 mmol) of CF_3COOEt and 2.17 mL (15.6 mmol) of triethyl amine were added and stirred for 24h. Then methanol was evaporated under rotary evaporator and the product was dissolved in ethyl acetate and washed with 1N HCl three times and then with brine solution. The organic layer was dried over anhydrous Na_2SO_4 and evaporated and dried under vacuum. The crude product was purified by column chromatography using silica-gel as stationary phase and ethyl acetate-hexane mixture (1:5) as eluent to get pure white solid compound **5b**.

Yield: 3.1g (11.3 mmol, 72.4%)

Melting point: 84°C

^1H NMR (400 MHz, CDCl_3 , δ_{ppm}): 7.224-7.185 (m, 3H, aromatic protons), 7.003-6.988 (m, 2H, aromatic protons), 6.901-6.887 (d, 1H, NH), 4.806-4.772 (m, 1H, C^αH), 3.691 (s, 3H, OCH_3), 3.142-3.078 (m, 2H, C^βH). ^{13}C NMR (100 MHz, CDCl_3 , δ_{ppm}): 170.39, 156.34, 134.55, 129.08, 128.73, 127.50, 53.48, 52.76, 37.14. FTIR (cm^{-1}): 3337, 3037, 2960, 2932, 1750, 1644, 1538, 1437, 1376, 1299, 1266, 1220, 1193,

1174. Mass spectra: observed m/z 298.1348 $[M+Na]^+$ M_{cal} 275.0769. Anal. Calcd for $C_{12}H_{12}F_3NO_3$ (275.08): C, 52.37; H, 4.39; N, 5.09; Found: C, 51.84; H, 4.43; N, 5.28.

(d) **Compound 6a**: 7 mL H_2SO_4 and 7 mL of HNO_3 was mixed in a round bottom flask, kept in ice-bath. Then 2.8g (12.65 mmol) of compound **5a** was added to the mixture portion wise with stirring. Then the solution was heated at $60^\circ C$ for 30 min. Then the reaction mixture was added slowly to ice cooled water and the crude product was extracted with ethyl acetate. The organic layer was washed with fresh water repeatedly. The organic layer then dried over anhydrous Na_2SO_4 and evaporated in vacuum. The product was purified by column chromatography using silica-gel as stationary phase and ethyl acetate-hexane mixture (1:3) as eluent to get pure white semi-solid compound **6a**.

Yield: 1.4g (5.26 mmol, 41.6%)

1H NMR (400 MHz, $CDCl_3$, δ_{ppm}): 8.167-8.145 (d, 2H, $J=8.8$ Hz, aromatic protons), 7.290-7.269 (d, 2H, $J=8.2$ Hz, aromatic protons), 6.023-6.005 (b, 1H, $d=7.2$ Hz, NH), 4.936-4.918 (m, 1H, $C^\alpha H$), 3.747 (s, 3H, OCH_3), 3.290-3.191 (m, 2H, $C^\beta H$) 2.008 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ_{ppm}): 171.67, 169.86, 146.91, 143.89, 130.06, 123.51, 52.75, 52.49, 37.59, 22.84. FTIR (cm^{-1}): 3301, 3066, 2950, 2845, 1745, 1645, 1603, 1524, 1437, 1372, 1340. Mass spectra: observed m/z 267.2354 $[M+H]^+$, 289.1046 $[M+Na]^+$ M_{cal} 266.0903. Anal. Calcd for $C_{12}H_{14}N_2O_5$ (266.09): C, 54.13; H, 5.30; N, 10.52; Found: C, 54.24; H, 5.18; N, 10.58.

(e) **Compound 6b**: The preparation of compound **6b** was same as the preparation of compound **6a** described above.

1H NMR (400 MHz, $CDCl_3$, δ_{ppm}): 8.171-8.148 (d, 2H, $J=9.2$ Hz, aromatic protons), 7.256-7.234 (d, 2H, $J=8.8$ Hz, aromatic protons), 6.875-6.860 (d, 1H, $J=6.0$ Hz, NH), 4.899-4.867 (m, 1H, $C^\alpha H$), 3.795 (s, 3H, OCH_3), 3.359-3.247 (m, 2H, $C^\beta H$). ^{13}C NMR (100 MHz, $CDCl_3$, δ_{ppm}): 169.86, 156.83-156.45, 147.43, 142.30, 130.12, 123.94, 53.22, 37.06. FTIR (cm^{-1}): 3314, 3116, 2961, 2854, 1750, 1713, 1607, 1561, 1520, 1437, 1349, 1285, 1161. Mass spectra: observed m/z 321.2414 $[M+H]^+$, M_{cal} 320.0620. Anal. Calcd for $C_{12}H_{11}F_3N_2O_5$ (320.06): C, 45.01; H, 3.46; N, 8.75; Found: C, 45.12; H, 3.48; N, 8.58.

(f) **Compound 7a**: 1g of Compound **6a** was dissolved in 100 mL MeOH. This solution was passing through hydrogenation apparatus (H-Cube model no: HC 2-ss) at full hydrogen mode at room temperature. The product solution was collected from other side and dried under rotary evaporator and the product was purified by column chromatography using Silica gel (100-200 mesh) using ethyl acetate-hexane (2:1) as eluent.

Yield: 646mg (2.73 mmol, 51.9 %)

1H NMR (400 MHz, $CDCl_3$, δ_{ppm}): 6.872-6.852 (d, 2H, $J=8.0$ Hz, aromatic protons), 6.625-6.605 (d, 2H, $J=8.0$ Hz, aromatic protons), 5.971-5.951 (d, 1H, $J=8.0$ Hz, NH), 4.814-4.794 (m, 1H, $C^\alpha H$), 3.716 (s, 3H, OCH_3), 3.612 (b, 2H, NH₂) 3.009-2.985 (m, 2H, $C^\beta H$), 1.973 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ_{ppm}): 172.26, 169.67, 145.09, 130.06, 125.56, 115.40, 53.24, 52.26, 36.96, 23.11. FTIR (cm^{-1}): 3354, 3057, 2953, 2850, 1739, 1664, 1518, 1438, 1373, 1280, 1219, 1128, 1010. Mass spectra: observed m/z 236.2176 $[M+H]^+$, 259.2060 $[M+Na]^+$ M_{cal} 236.1161. Anal. Calcd for $C_{12}H_{16}N_2O_3$ (236.11): C, 61.00; H, 6.83; N, 11.86; Found: C, 60.87; H, 6.92; N, 11.68.

(g) **Compound 7b**: The preparation of compound **7b** was same as the preparation of compound **7a** described above.

Melting point: 95°C

¹H NMR (400 MHz, CDCl₃, δ_{ppm}): 6.845-6.824 (d, 2H, J=8.4 Hz, aromatic protons), 6.800-6.782 (d, 1H, J= 7.2 Hz, NH), 6.627-6.605(d, 2H, J=8.8 Hz, aromatic protons), 4.822-4.803(m, 1H, C^αH), 3.780 (s, 3H, OCH₃), 3.668 (b, 2H, NH₂), 3.095-3.083 (m, 2H, C^βH). ¹³C NMR (100 MHz, CDCl₃, δ_{ppm}): 170.49, 158.36-157.24, 145.78, 130.04, 123.98, 115.41, 53.62, 52.78, 36.41. FTIR (cm⁻¹): 3429, 3277, 3112, 3020, 2960, 1736, 1708, 1644, 1612, 1561, 1515, 1437, 1368, 1285, 1244, 1161. Mass spectra: observed m/z 291.1417 [M+H]⁺, M_{cal} 290.0878. Anal. Calcd for C₁₂H₁₃F₃N₂O₃ (290.08): C, 49.66; H, 4.51; N, 9.65; Found: C, 49.81; H, 4.62; N, 9.74.

(h) **Compound 1**: 500mg (2.11 mmol) of compound **7a** was dissolved in 10 mL dilute HCl solution kept it in ice bath. 200 mg of sodium nitrite was dissolved in 20 mL distilled water and it was also kept in ice-bath. After 15 minute 10 mL of sodium nitrate solution was added to ice cooled solution of compound **7a** and kept it in ice-bath. 365mg (2.11 mmol) sulfanilic acid was dissolved in 5 mL 5% sodium carbonate solution with heating and kept it in ice-bath. After 15 min, the diazonium salt of the compound **7a** was added to sulfanilic acid solution with stirring. Then the mixture solution was basified with addition of solid sodium carbonate and stirred at 60°C for 6h. After completion of reaction, solution was acidified with dilHCl. The water was evaporated under rotary evaporator and methanol was added and filtered. The filtrate was then evaporated and again the solid was dissolved in methanol and filtered to remove the NaCl and Na₂CO₃. Finally the product (200 mg) was purified by soxhlet extraction (200 mL, 90% ethanol) to give 145 mg pure compound **1**.

¹H NMR (400 MHz, DMSO-d₆, δ_{ppm}): 7.683-7.584 (m, 3H, aromatic protons Sulfa), 7.404-7.382 (d, J= 8.8 Hz, 2H, aromatic protons phe), 6.697-6.675 (d, J= 8.8 Hz, 2H, aromatic protons Phe), 6.604 (b, 1H, NH Phe), 5.562 (b, 2H, NH₂), 4.553 (b, 1H, C^αH), 2.962-2.944 (m, 2H, C^βH), 1.877 (s, 3H, COCH₃). ¹³C NMR (125 MHz, DMSO-d₆, δ_{ppm}): 158.48, 148.15, 129.94, 128.46, 127.70, 127.52, 125.97, 124.01, 114.80, 63.01, 29.02, 21.08. FTIR (cm⁻¹): 3462, 2928, 1637, 1527, 1384, 1267, 1128, 1039. Mass spectra: observed m/z 425.7977 [M+H+NH₄]⁺⁺, 430.5769 [M+H+Na]⁺⁺ M_{cal} 406.0947. Anal. Calcd for C₁₇H₁₈N₄O₆S (406.09): C, 50.24; H, 4.46; N, 13.79; Found: C, 50.46; H, 4.25; N, 13.91.

(i) **Compound 2**: The preparation of compound **2** was same as the preparation of compound **1** described above.

¹H NMR (400 MHz, DMSO-d₆, δ_{ppm}): 8.477 (b, 1H, NH Phe), 8.216-8.195 (d, J= 9.2 Hz, 2H, aromatic protons Phe), 7.848-7.826 (d, J=8.8 Hz, 2H, aromatic protons Phe), 7.619-7.312 (m, 3H, aromatic protons Sul), 5.504 (b, 2H, NH₂), 4.649-4.581 (m, 1H, C^αH), 3.158-3.145 (m, 2H, C^βH). ¹³C NMR (125 MHz, DMSO-d₆, δ_{ppm}): 158.66-157.11, 154.11, 127.72, 127.11, 127.01, 125.55, 123.40, 120.89, 118.51, 116.12, 114.14, 113.74, 62.82, 29.64, 21.64. FTIR (cm⁻¹): 3444, 2922, 2852, 1685, 1637, 1384, 1269, 1209, 1141, 1035. Mass spectra: observed m/z 463.1848 [M+3H]³⁺, 507.1592 [M+2Na+H]³⁺ M_{cal} 460.0664. Anal. Calcd for C₁₇H₁₅F₃N₄O₆S (460.06): C, 44.35; H, 3.28; N, 12.17; Found: C, 44.12; H, 3.22; N, 12.08.

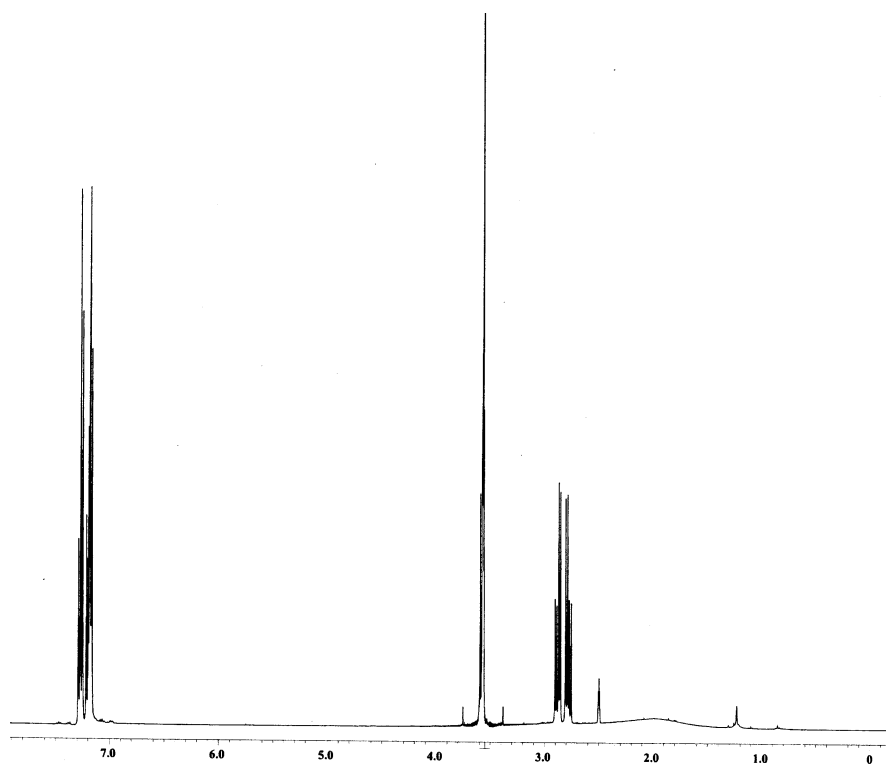
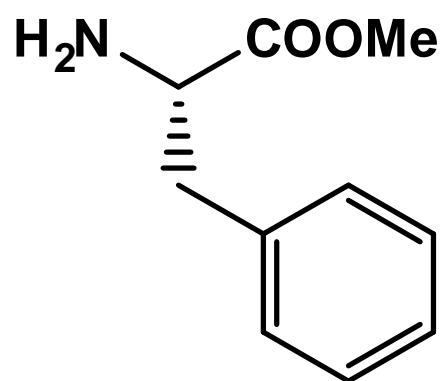


Figure 1: ^1H NMR (400 MHz, $\text{DMSO}-d_6$, δ_{ppm}) of compound **4**.

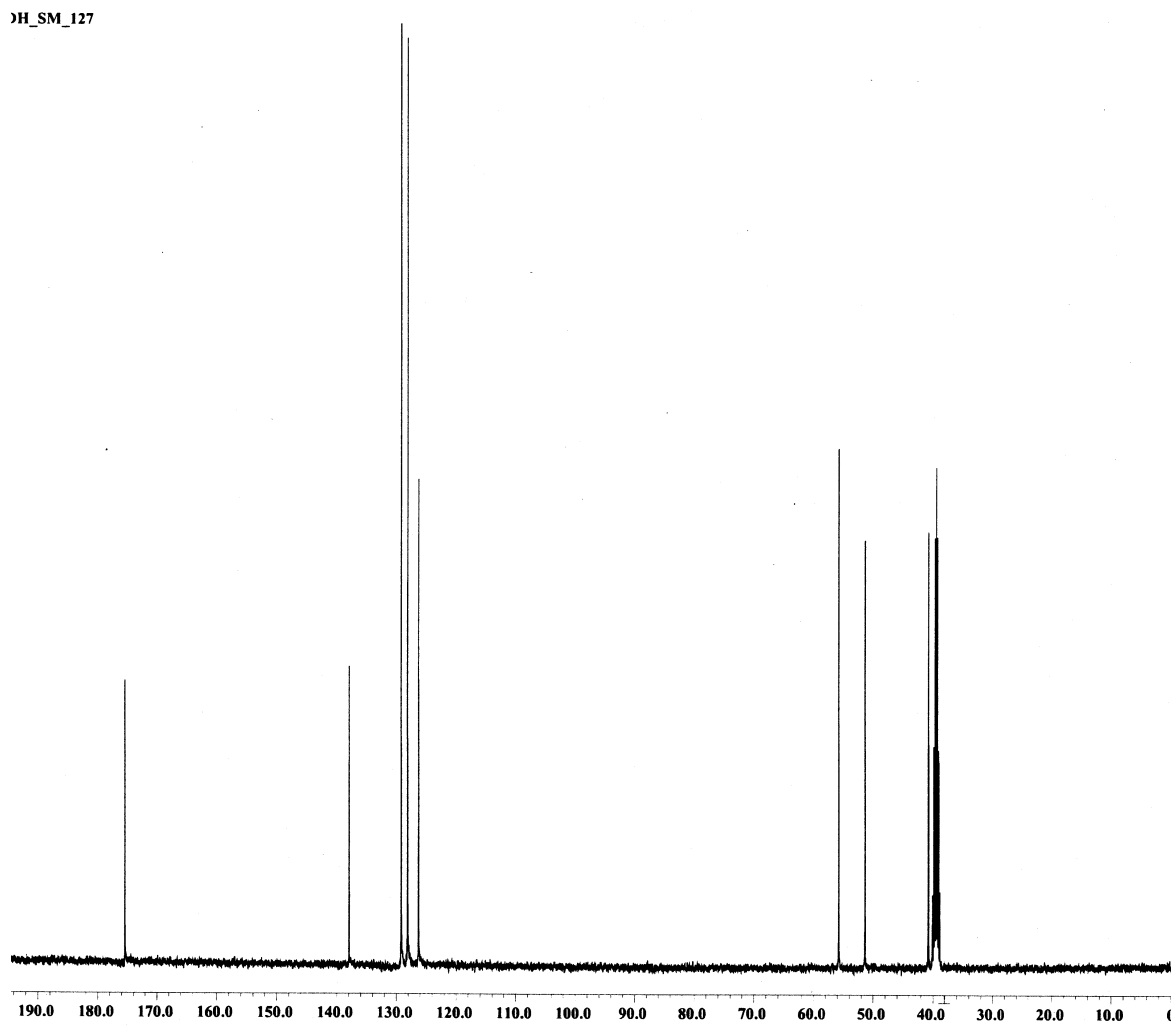


Figure 2: ¹³C NMR (100 MHz, DMSO-*d*₆, δ_{ppm}): of compound **4**.

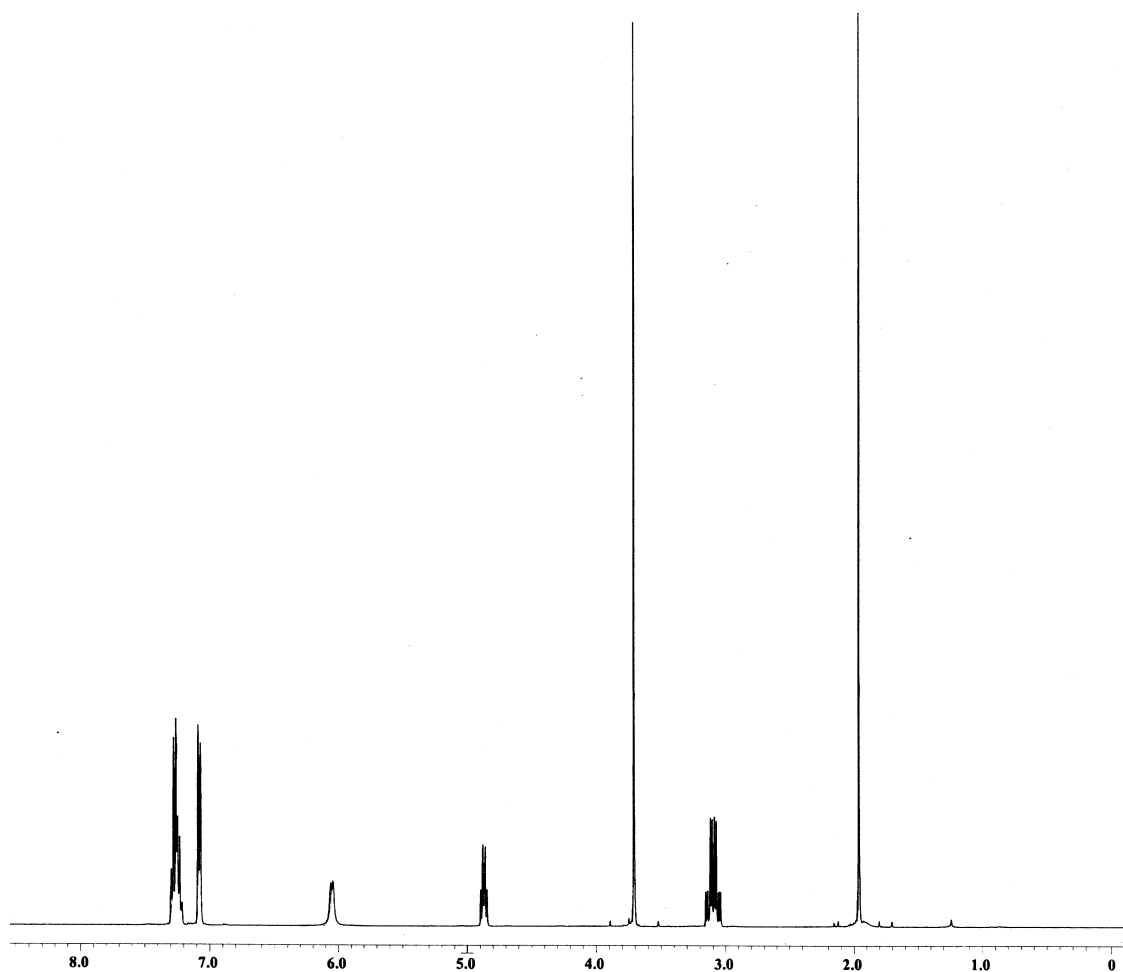
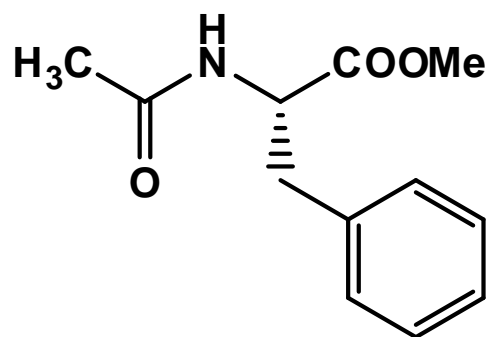


Figure 3: ¹H NMR (400 MHz, CDCl₃, δ_{ppm}) of compound **5a**.

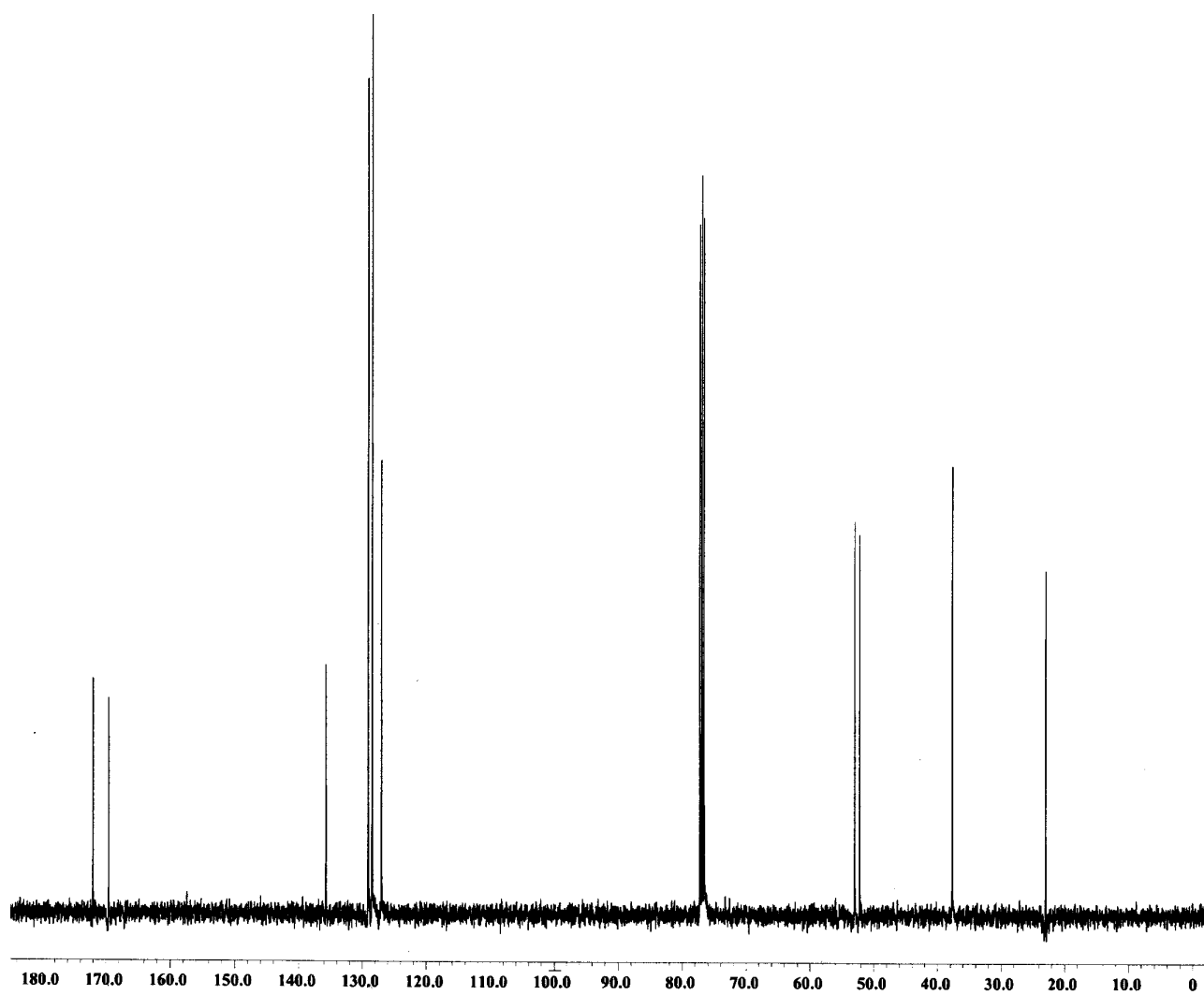


Figure 4: ^{13}C NMR (100 MHz, CDCl_3 , δ_{ppm}): of compound **5a**.

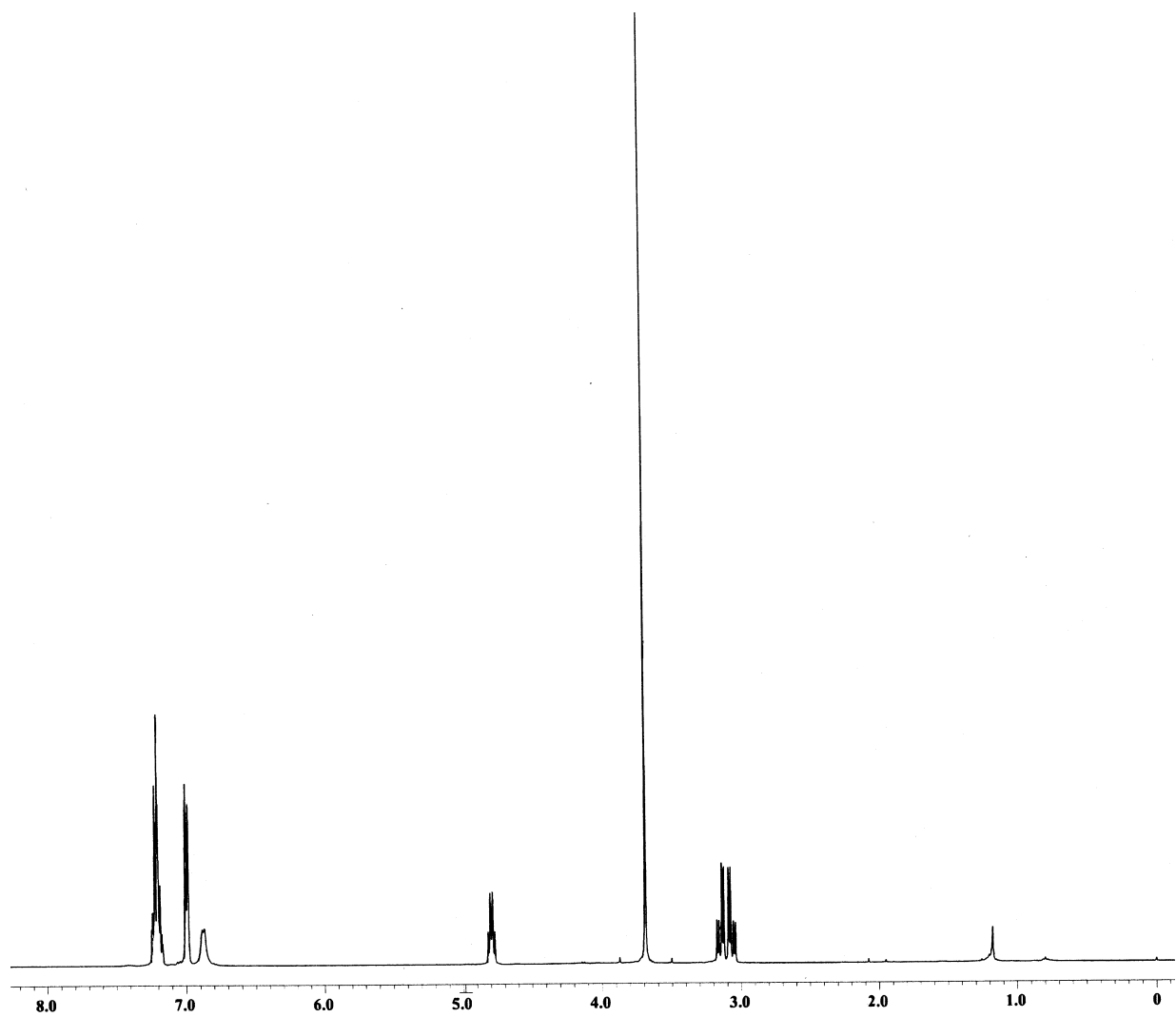
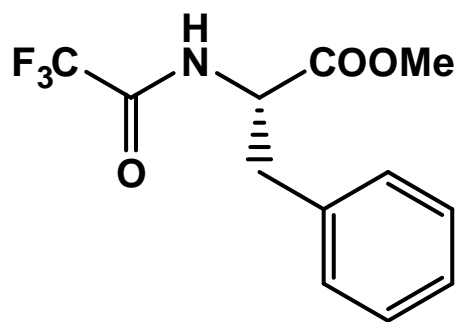


Figure 5: ^1H NMR (400 MHz, CDCl_3 , δ_{ppm}) of compound **5b**.

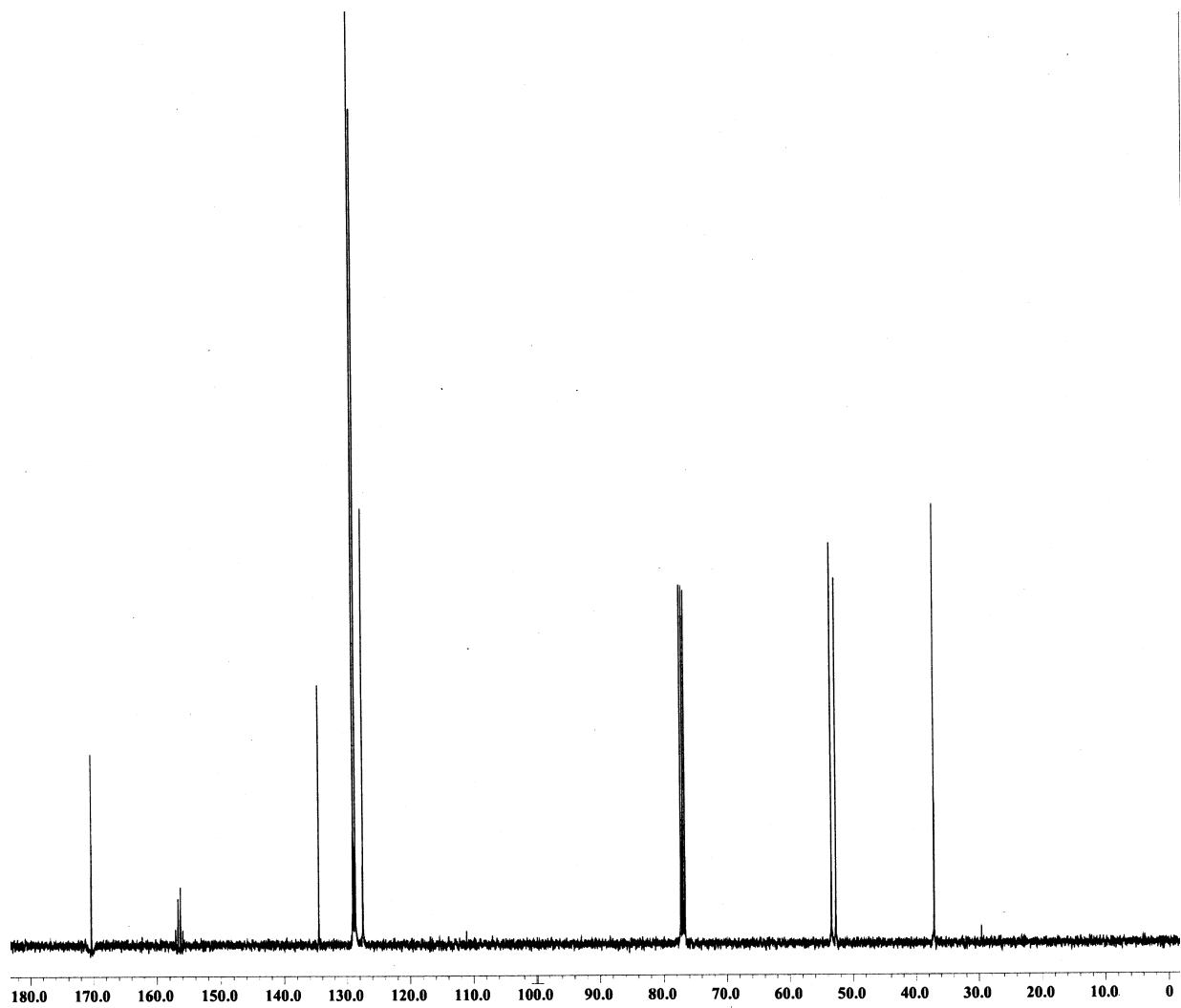


Figure 6: ^{13}C NMR (100 MHz, CDCl_3 , δ_{ppm}) of compound **5b**.

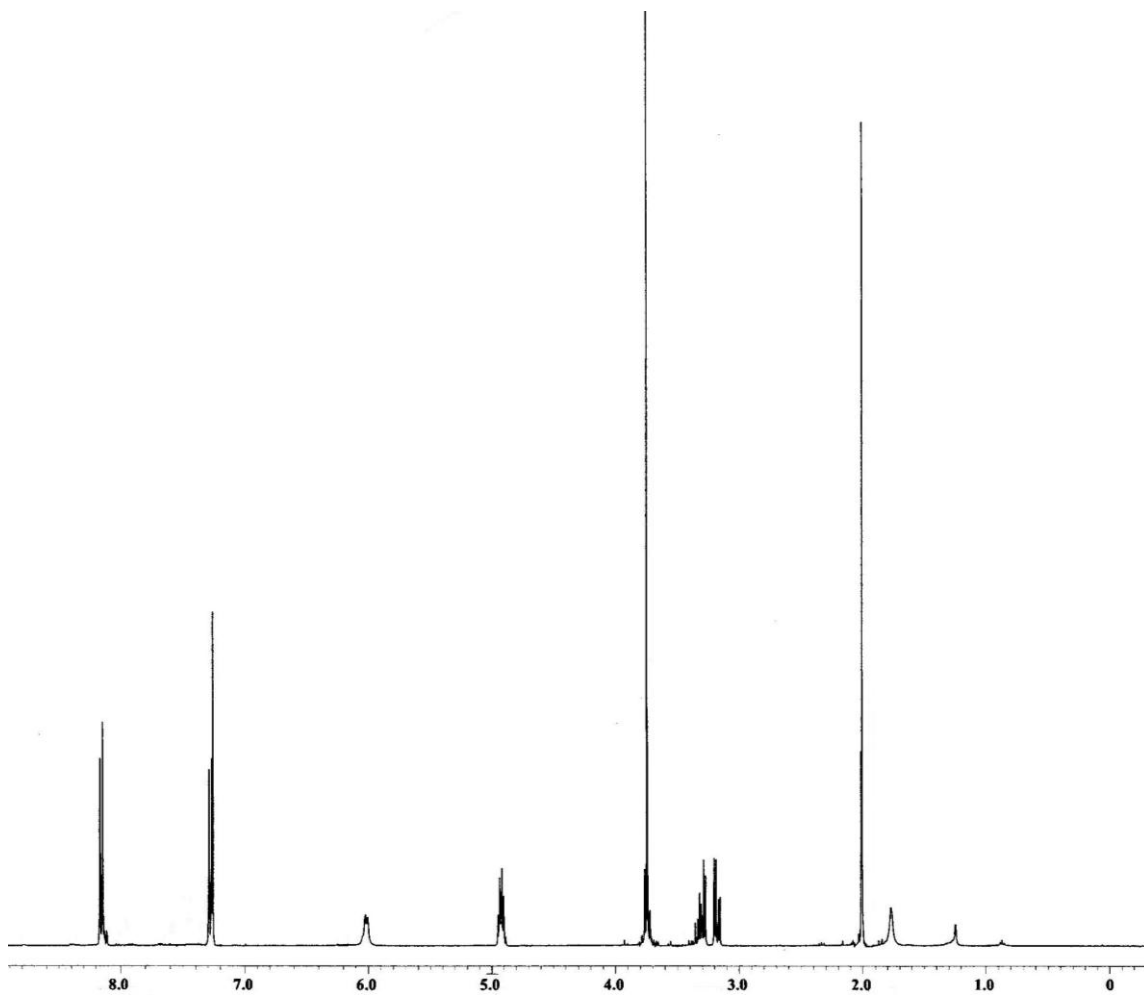
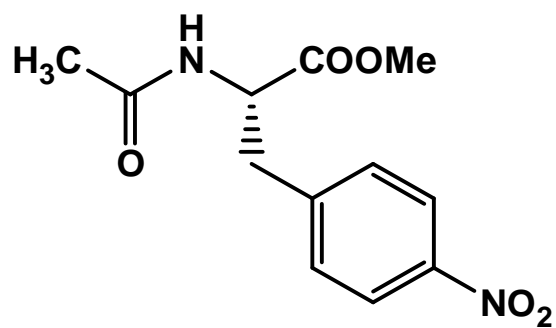


Figure 7: ¹H NMR (400 MHz, CDCl₃, δ_{ppm}) of compound **6a**.

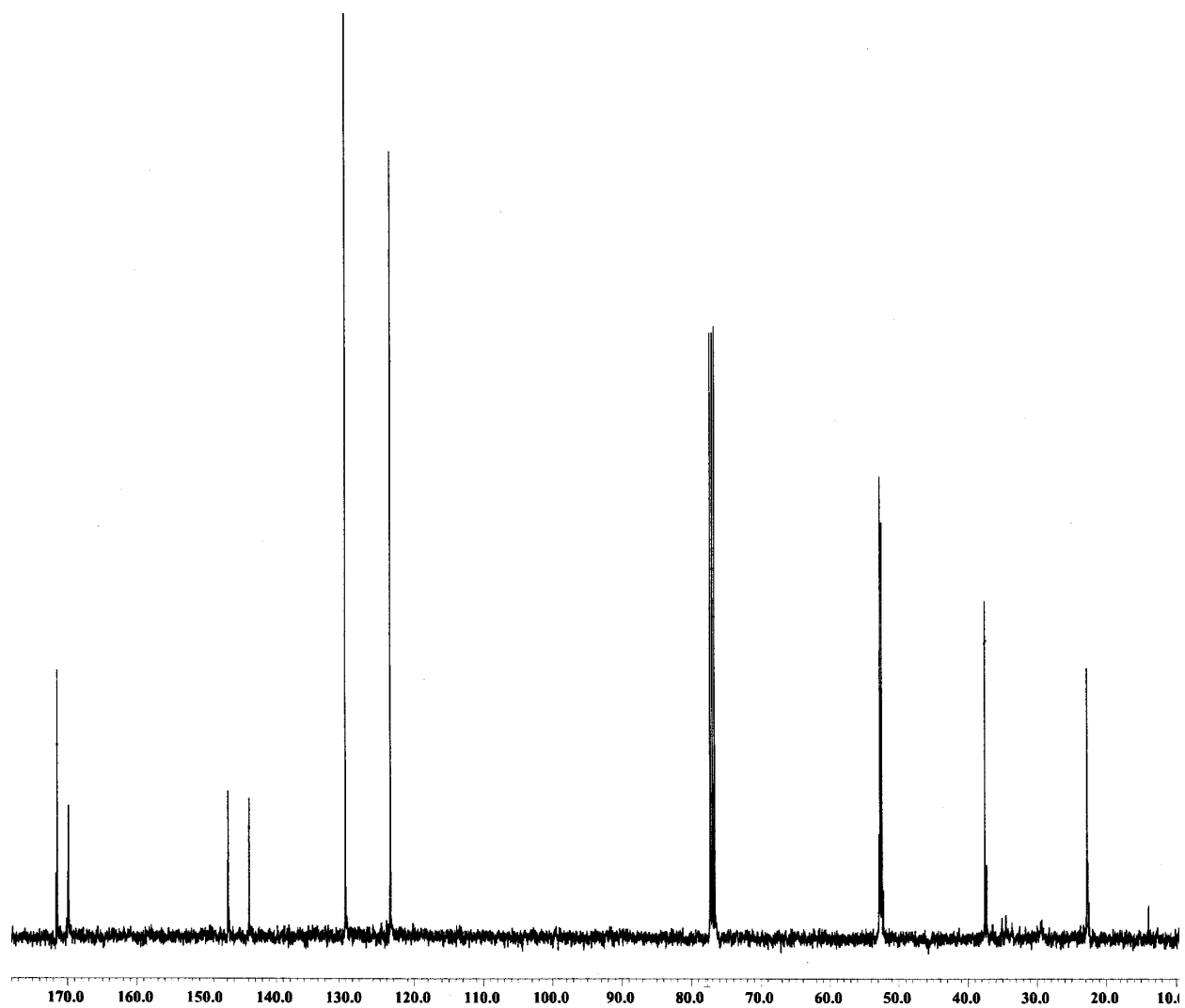


Figure 8: ¹³C NMR (100 MHz, CDCl₃, δ_{ppm}) of compound **6a**.

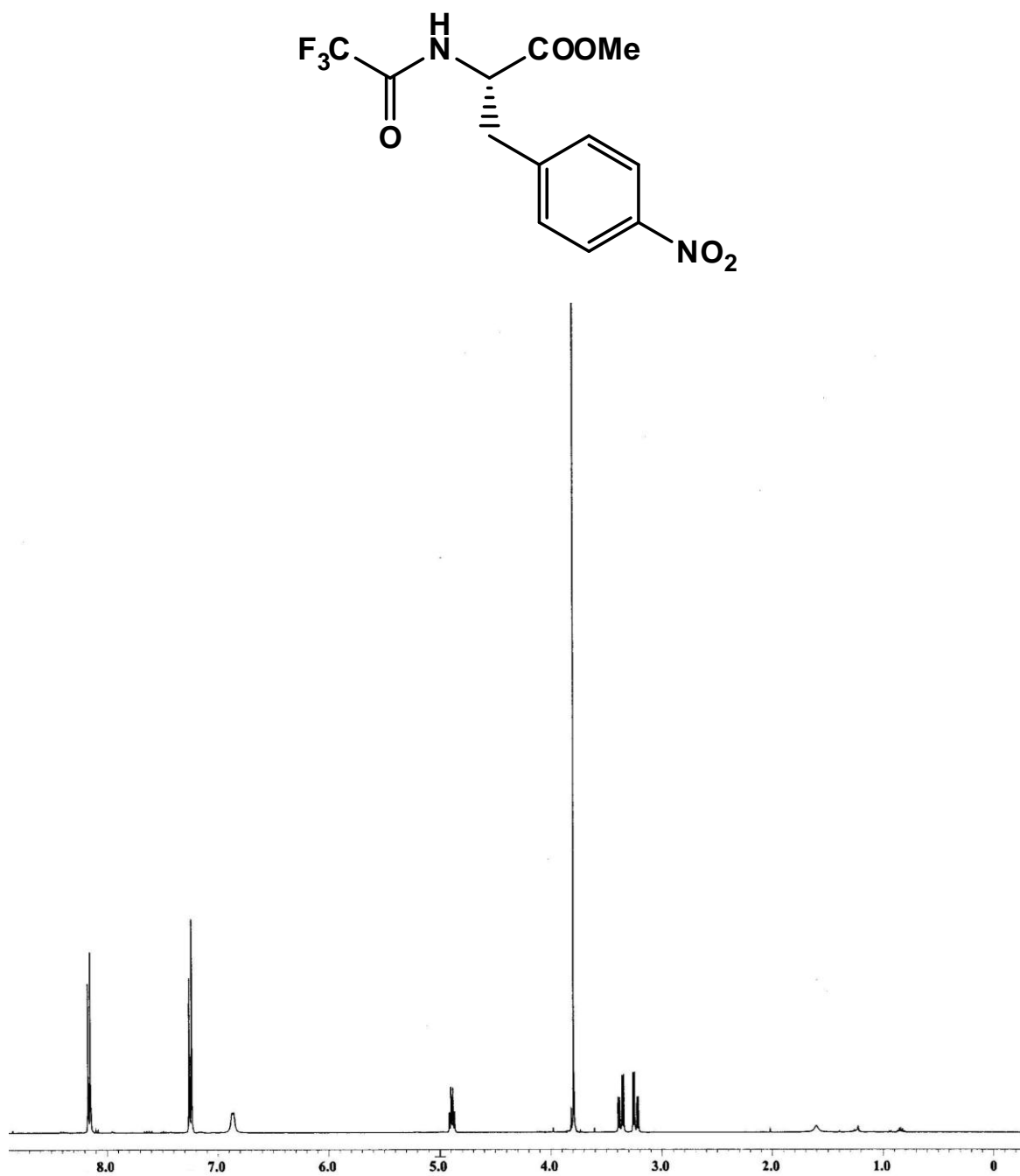


Figure 9: ^1H NMR (400 MHz, CDCl_3 , δ_{ppm}) of compound **6b**.

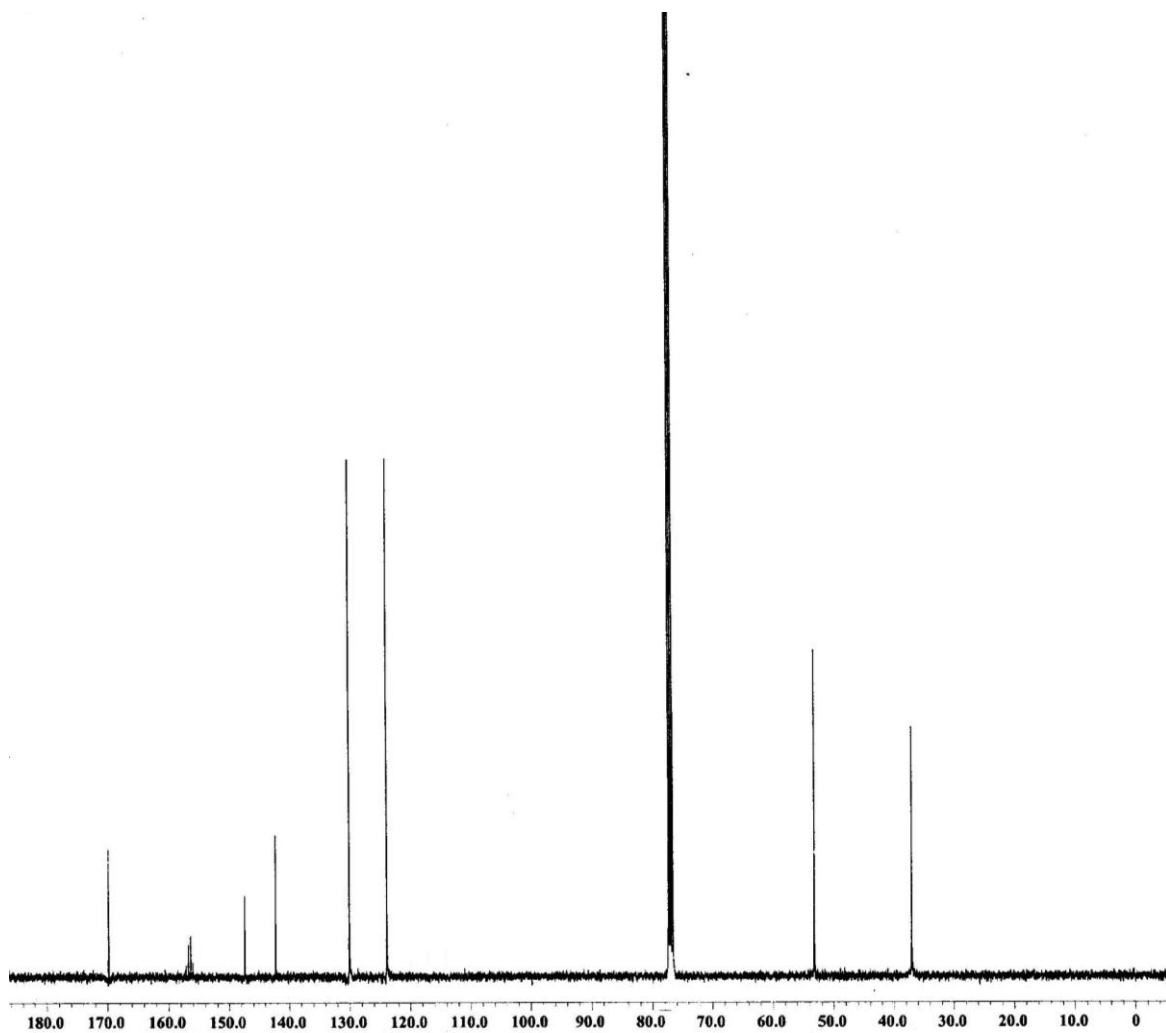


Figure 10: ^{13}C NMR (100 MHz, CDCl_3 , δ_{ppm}) of compound **6b**.



Figure 11: ¹H NMR (400 MHz, CDCl₃, δ_{ppm}) of compound **7a**.

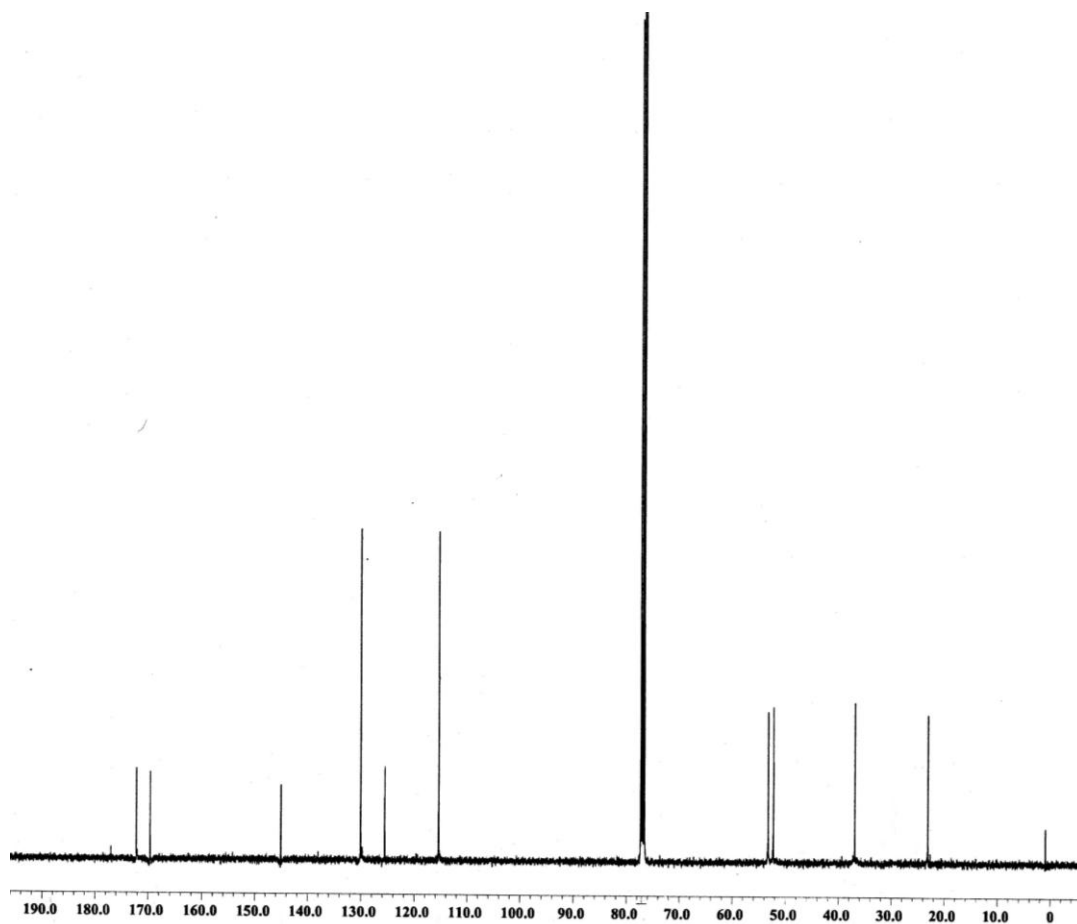


Figure 12: ^{13}C NMR (100 MHz, CDCl_3 , δ_{ppm}) of compound **7a**.

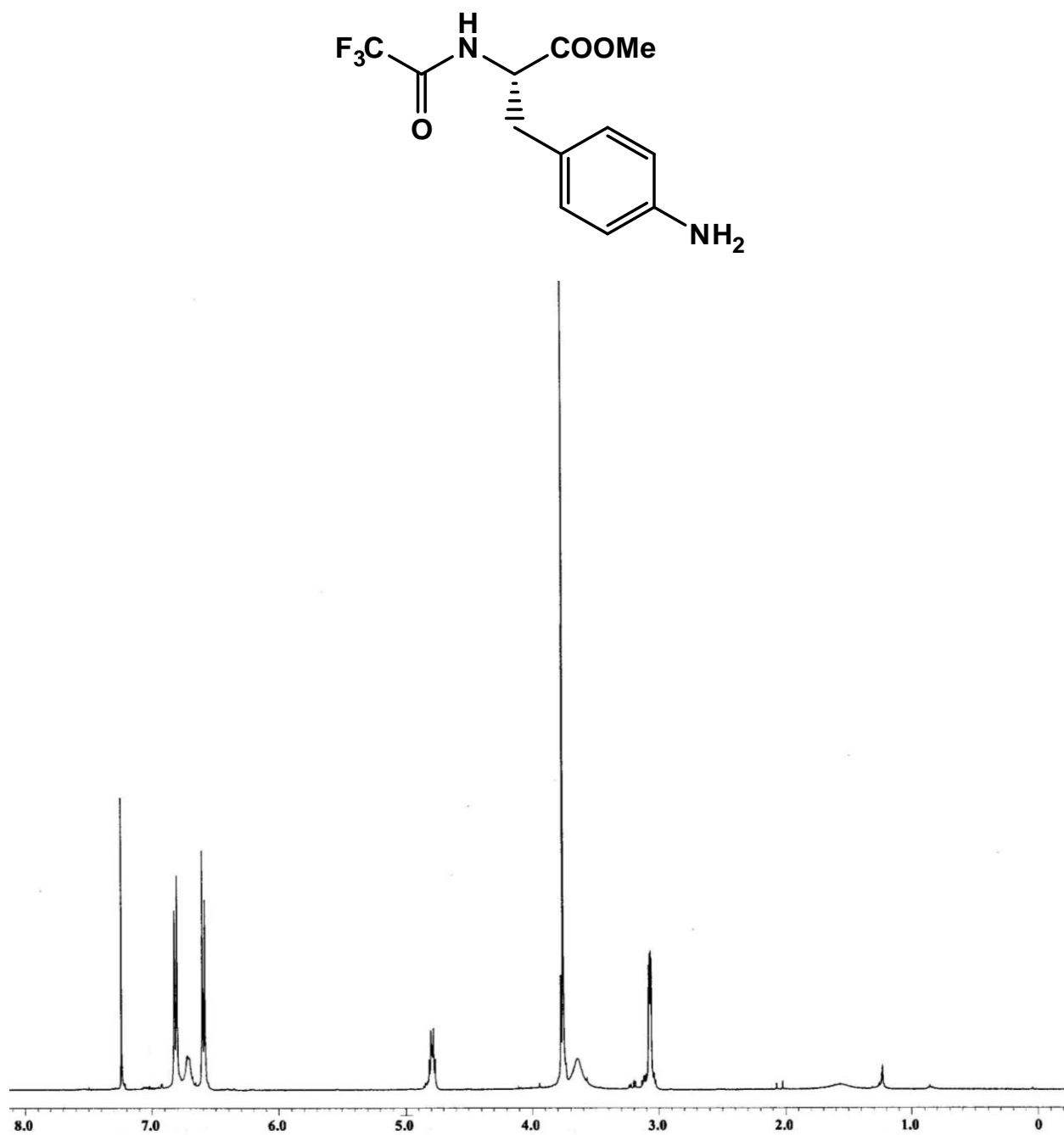


Figure 13: ¹H NMR (400 MHz, CDCl₃, δ_{ppm}) of compound **7b**.

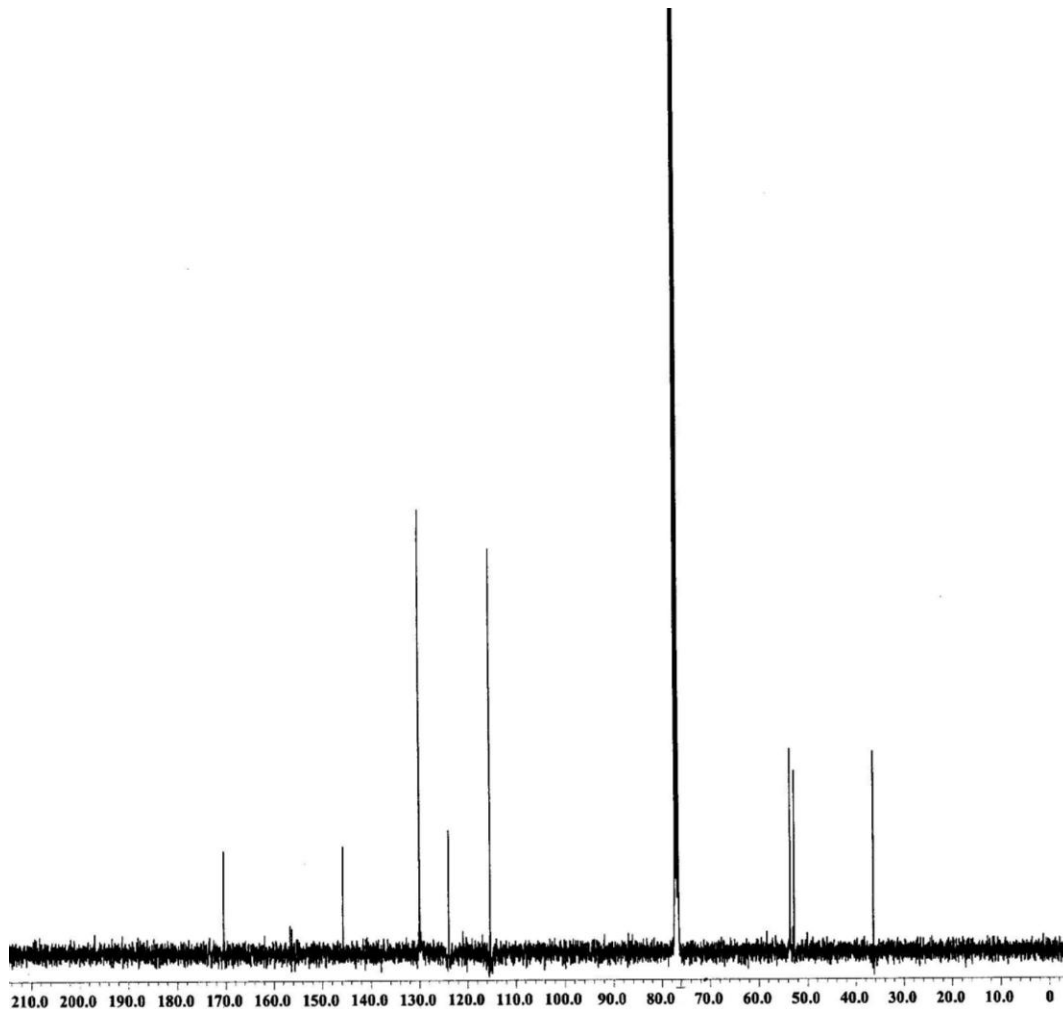


Figure 14: ^{13}C NMR (100 MHz, CDCl_3 , δ_{ppm}) of compound **7b**.

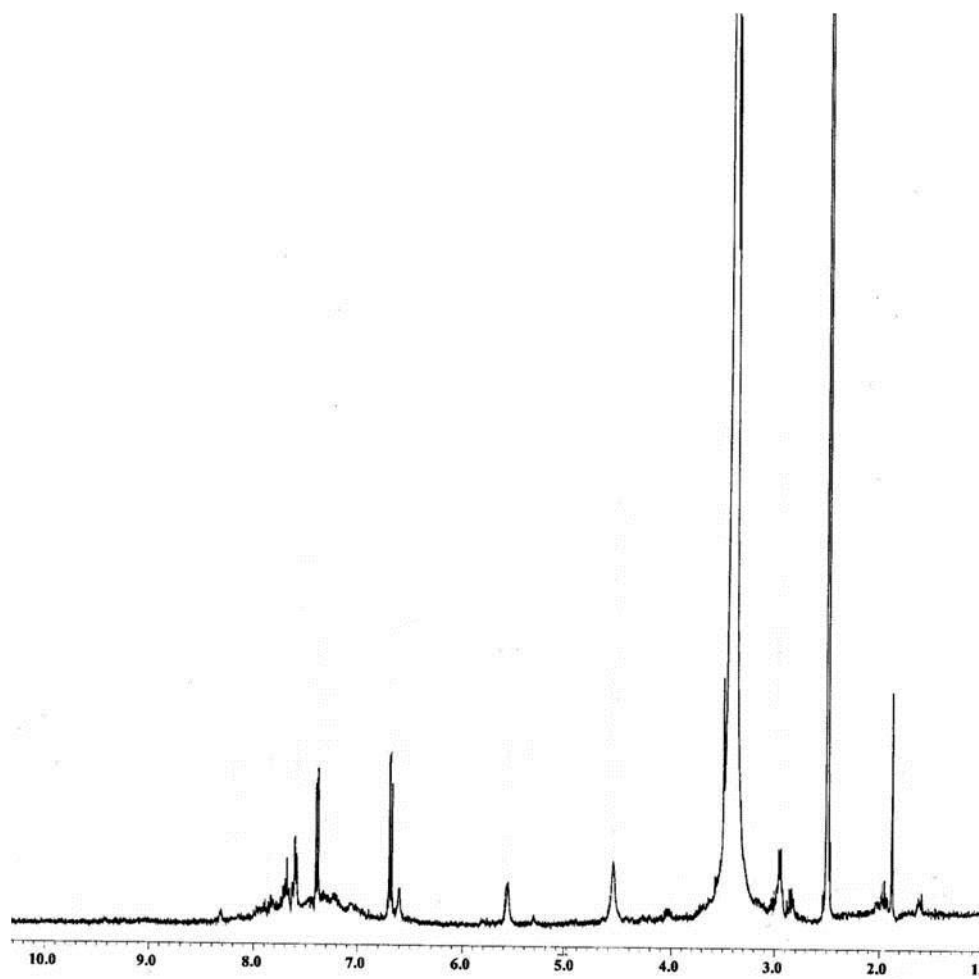
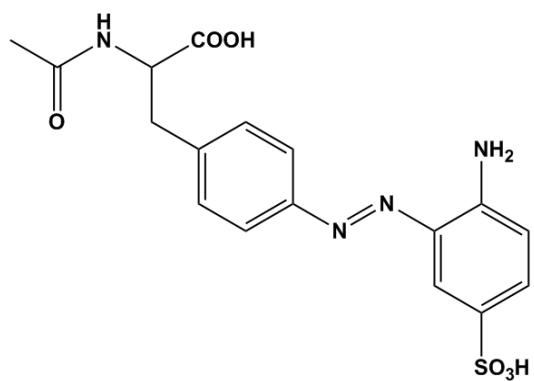


Figure 15: ¹H NMR (400 MHz, DMSO-d₆, δ_{ppm}) of compound **1**.

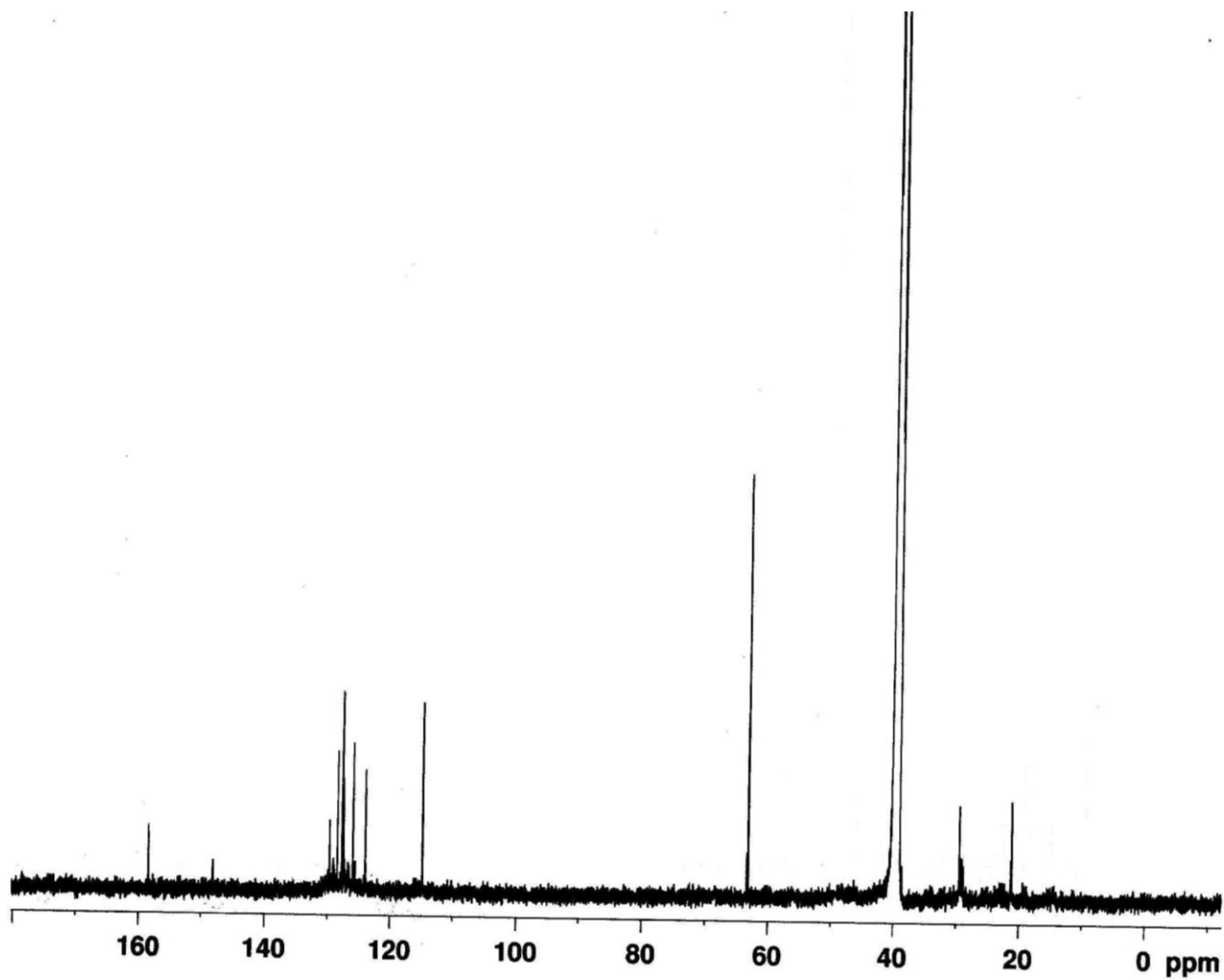


Figure 16: ^{13}C NMR (125 MHz, DMSO- d_6 , δ_{ppm}) of compound **1**.

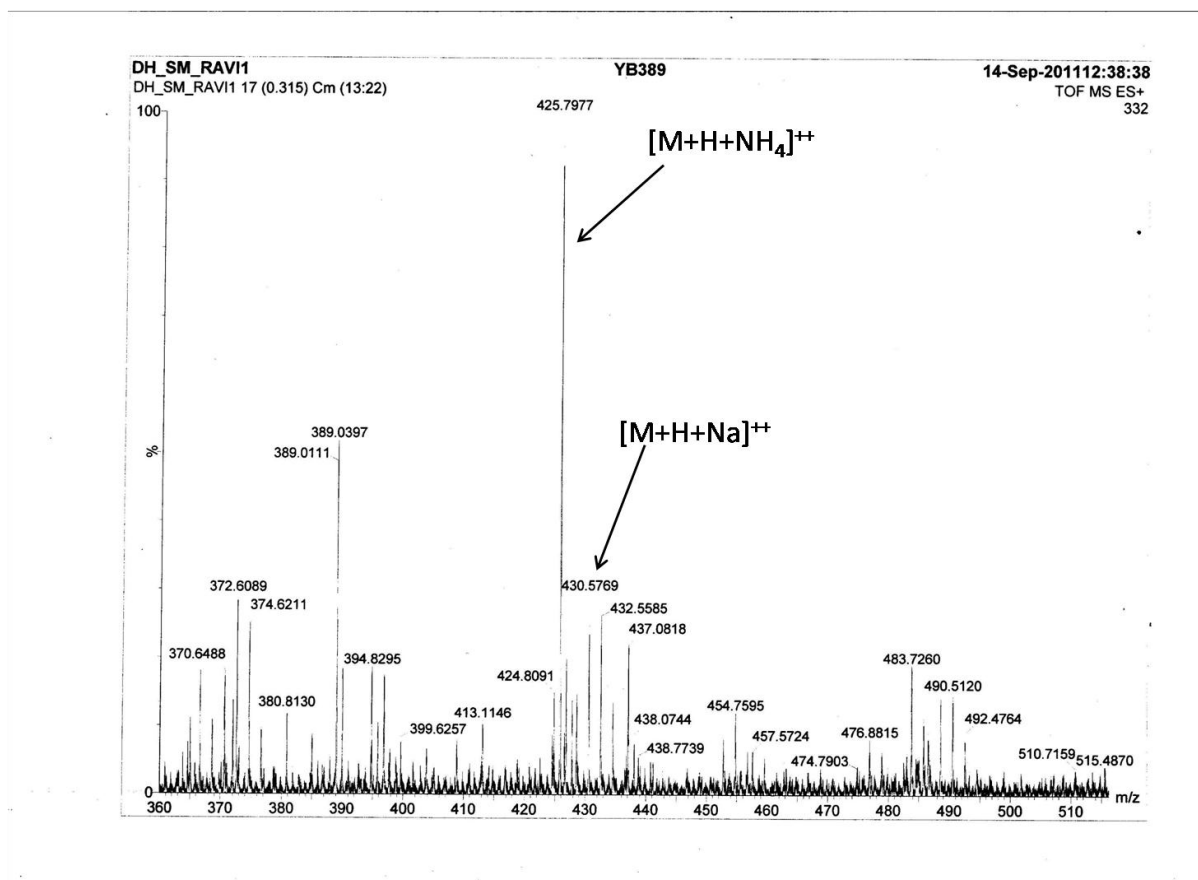
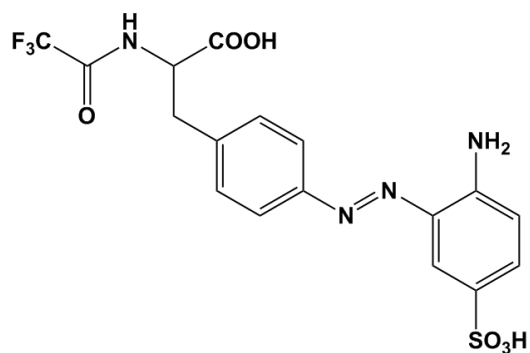


Figure 17: Partial mass spectra of Compound 1.



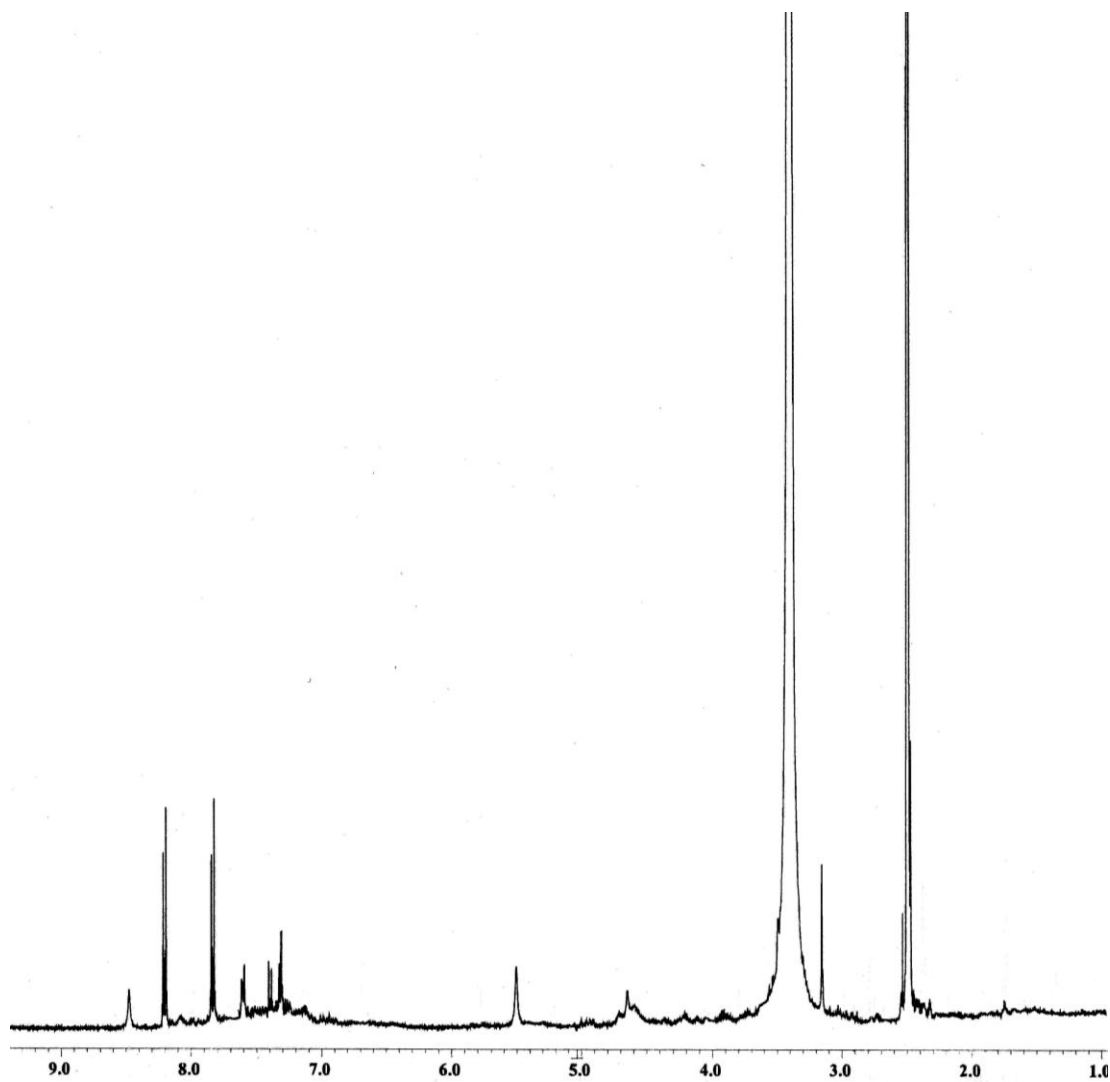


Figure 18: ¹H NMR (400 MHz, DMSO-d₆, δ_{ppm}) of compound **2**.

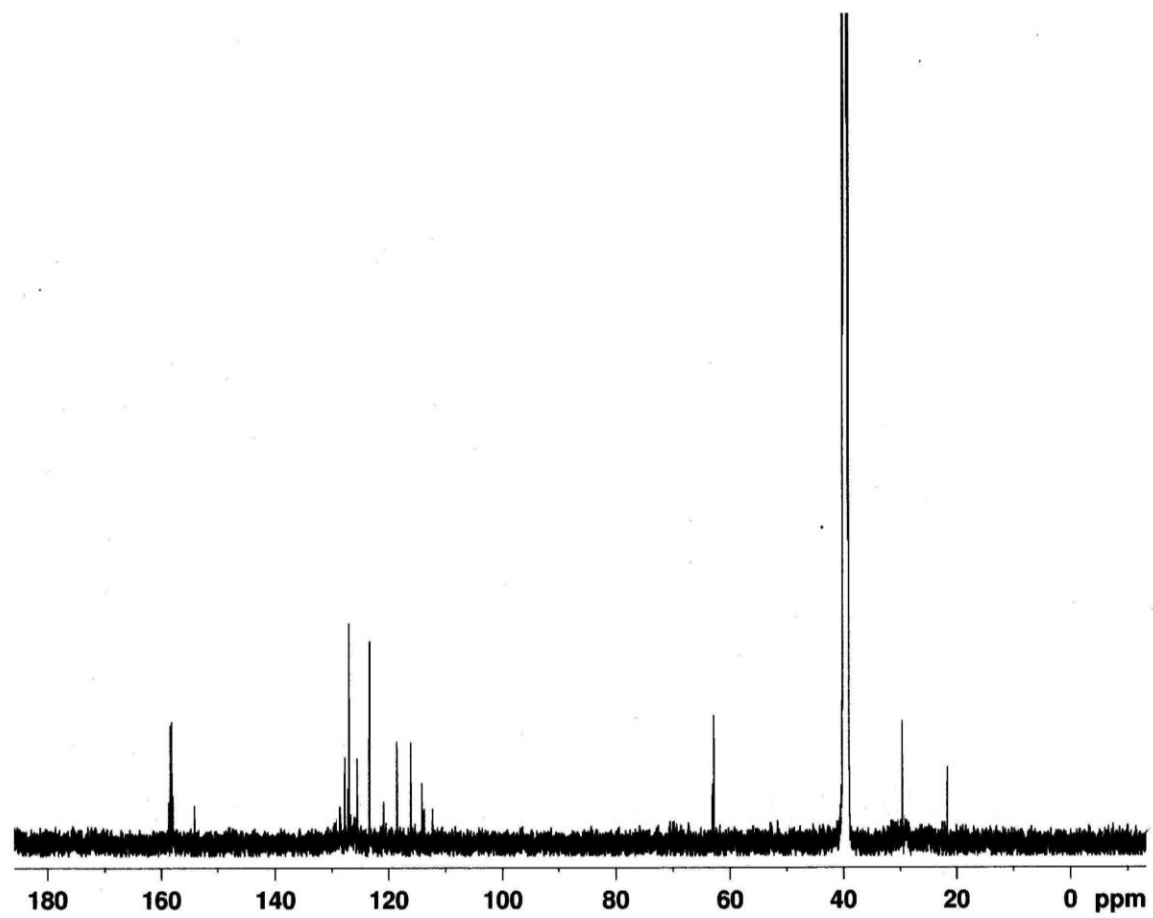


Figure 19: ¹³C NMR (125 MHz, DMSO-d₆, δ_{ppm}) of compound **2**.

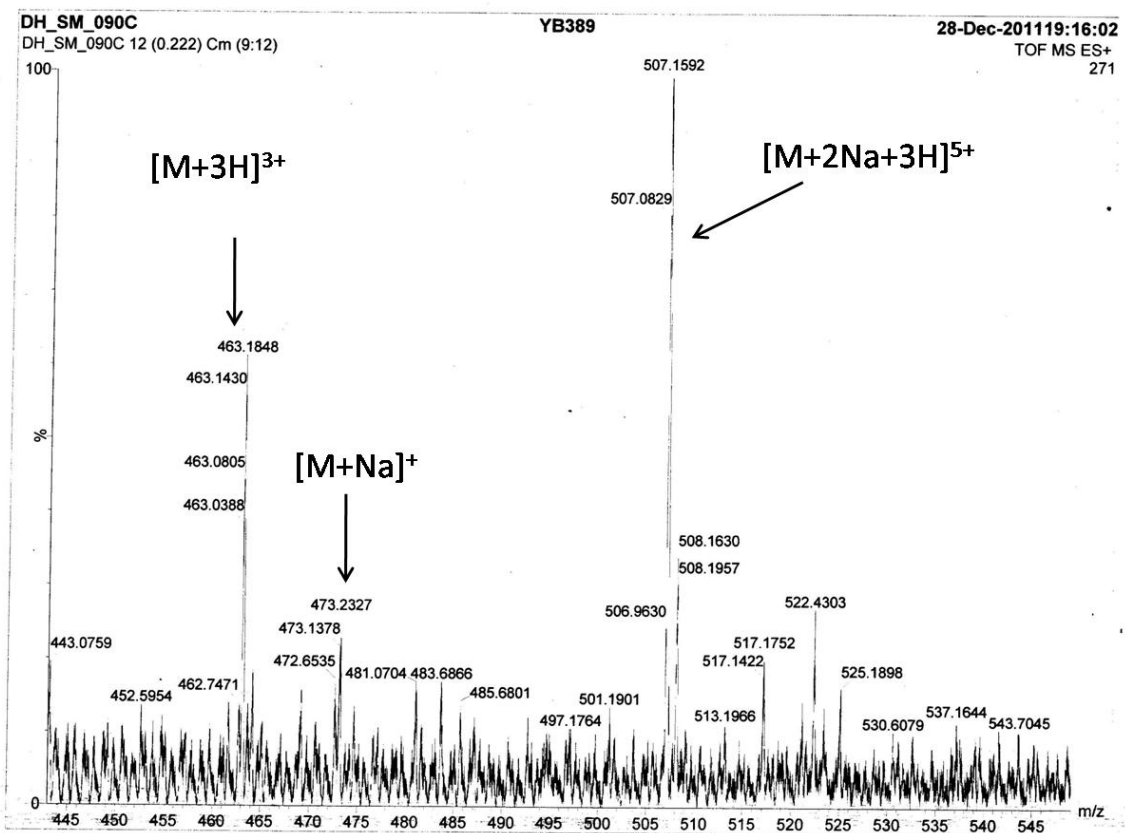


Figure 20: Partial mass spectra of compound 2.