Supporting Information

Enzymatic amide bond formation: synthesis of aminooxo-acids through a *Mycobacterium smegmatis* acyltransferase

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Contents

1. General information

NMR spectra were recorded on a Brucker Avance 600 MHz spectrometer employing the residual signal of the deuterated solvent as internal standard. Chemical shifts (δ) are expressed in ppm and coupling constants (J) in Hertz (Hz). Merck Silica gel 60 F254 (aluminum foil) plates were used for TLC analysis (Sigma Aldrich, Milan, Italy); flash column chromatography was performed on Merck Silica gel (230–400 mesh) (Sigma Aldrich, Milan, Italy). Detection of TLC analyses has been performed under UV light at 254 and 365 nm. Organic solutions were concentrated using a Buchi rotary evaporator below 40 °C at 25 torr. Spectrophotometric assays were performed using a spectrophotometer bought from Eppendorf, Milan, Italy. MS spectra were recorded using electrospray ionization (ESI) technique on a Waters Micromass Q-Tof micro mass spectrometer.

2. Chemicals

Cell growing and strain maintaining media as well as commercially available reagents and solvents were purchased from Thermo Fischer Scientific or Merck (Sigma Aldrich, Milan, Italy).

3. Cloning, Overexpression and Purification of MsAcT

Protein expression and purification were performed following previously reported protocols by Contente *et al.*[1] Figure S1 shows the crude extract, pellet and pure protein analyzed by SDS-PAGE. The monomer of MsAcT is 25.6 kDa. Typically, starting from 2 g of wet cell paste, it was possible to obtain 130 mg of pure protein (9 mg/mL).

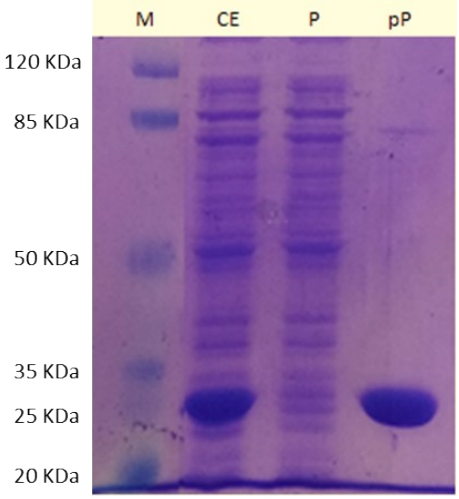


Figure S1: Lane 1: Marker (M), Lane 2: Crude Extract (CE), Lane 3: Pellet (P), Lane 4: Pure Protein (pP).

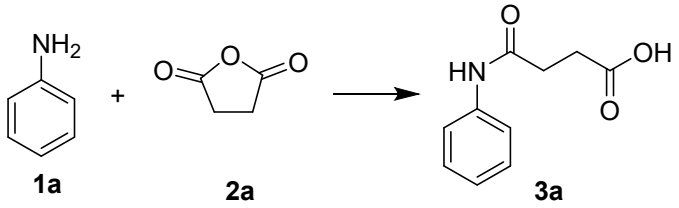
[1] M. L. Contente, A. Pinto, F. Molinari and F. Paradisi, *Adv. Synth. Catal.*, 2018, **360**, 4814–4819.

4. Activity assay of MsAcT enzyme

Enzyme activity measurements were performed following previously reported protocols by Contente *et al.*[1] employing *p*-nitrophenylacetate as substrate. The specific activity (U/mg) was expressed as µmol of product generated per minute per milligram of enzyme. MsAcT final specific activity: 120 U/mg.

5. Optimization of the reaction conditions

Batch reactions using MsAcT were performed in 10 mL screw cap tubes; 1 mL reaction mixture in 0.1 M phosphate buffer pH 8.0 containing different concentrations (0.1-1 M) of aniline (**1a**), 10% v/v DMSO, 1 mg/mL enzyme, and fixed amount of succinic anhydride **2a** (1 eq.) were left under magnetic stirring at 25 °C (Table S1).



1a **2a** **3a**

Entry	Concentration (M)	Product (% yield)[a]
1	0.1	88
2	0.25	88
3	0.5	89
4	1	90

[a] Isolated yield after 1h of reaction

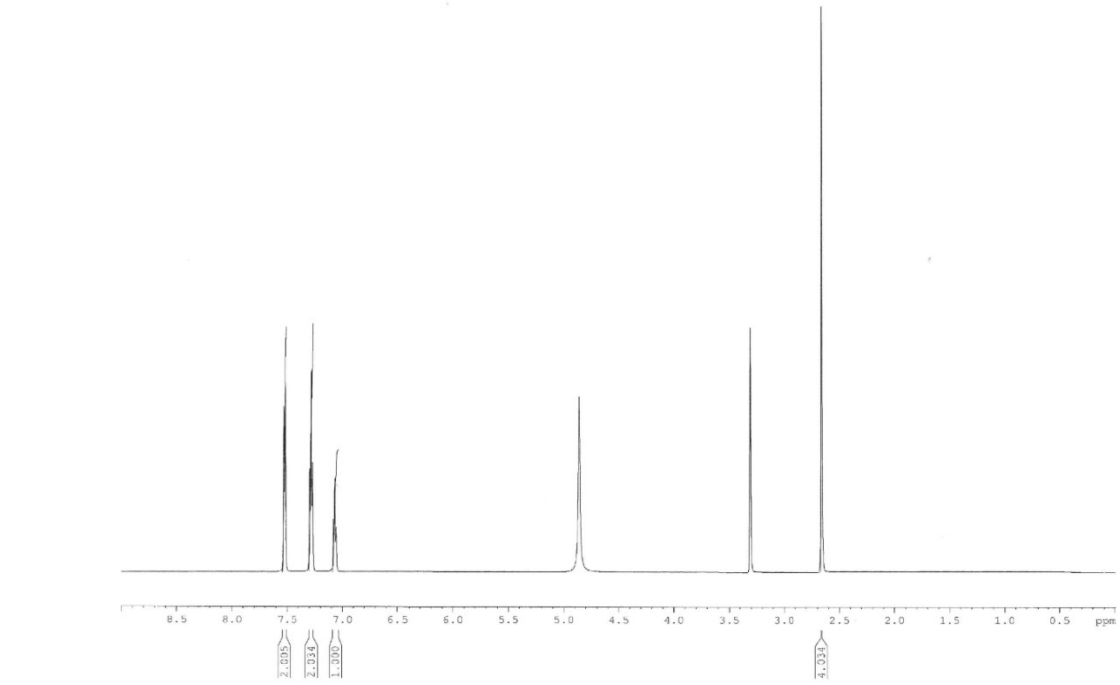
6. Batch reactions

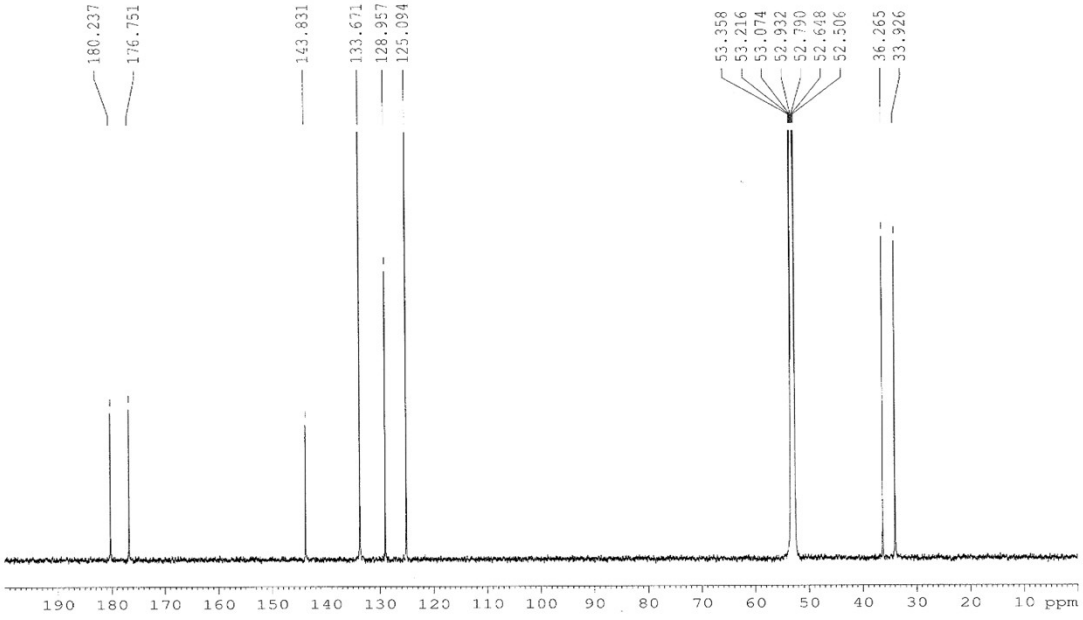
Batch reactions were performed in 10 mL screw cap tubes; 2 mL reaction mixture in 0.1 M phosphate buffer, pH 8.0 containing 1 M amine, 1 eq. anhydride, 10% v/v DMSO, 1 mg/mL enzyme was left under magnetic stirring at 25 °C. Samples withdrawn at different reaction times (30 min, 1 h, 2 h, 5 h, 24 h, 48 h) were monitored by TLC (cyclohexane/EtOAc 7:3 + 0.1% CH_3COOH or 0.1% TEA). In the reaction mixture EtOAc was added and the organic phase was washed with 1 N HCl and subsequently with brine. The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified by flash chromatography (cyclohexane/EtOAc 7:3 → 1:1) giving the desired products.

7. NMR spectra

4-Oxo-4-(phenylamino)butanoic acid (3a).

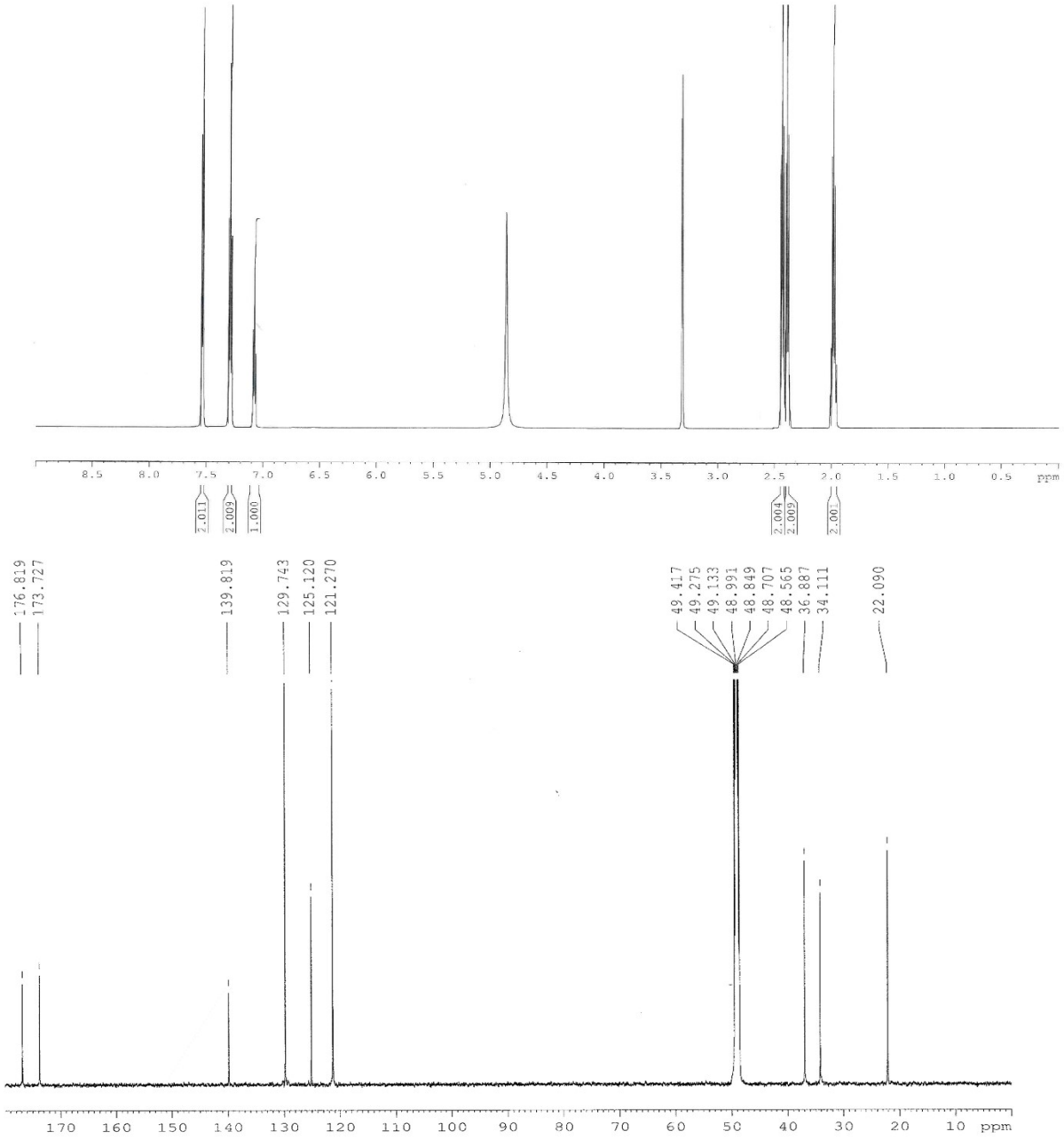
^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.52 (2H, d, J = 8.0 Hz), 7.28 (2H, t, J = 8.0 Hz), 7.06 (1H, t, J = 8.0 Hz), 2.68 – 2.65 (4H, m) ppm. ^{13}C-NMR (c): δ = 180.2, 176.8, 143.8, 133.7 (2C), 129.0, 125.1 (2C), 36.3, 33.9 ppm.





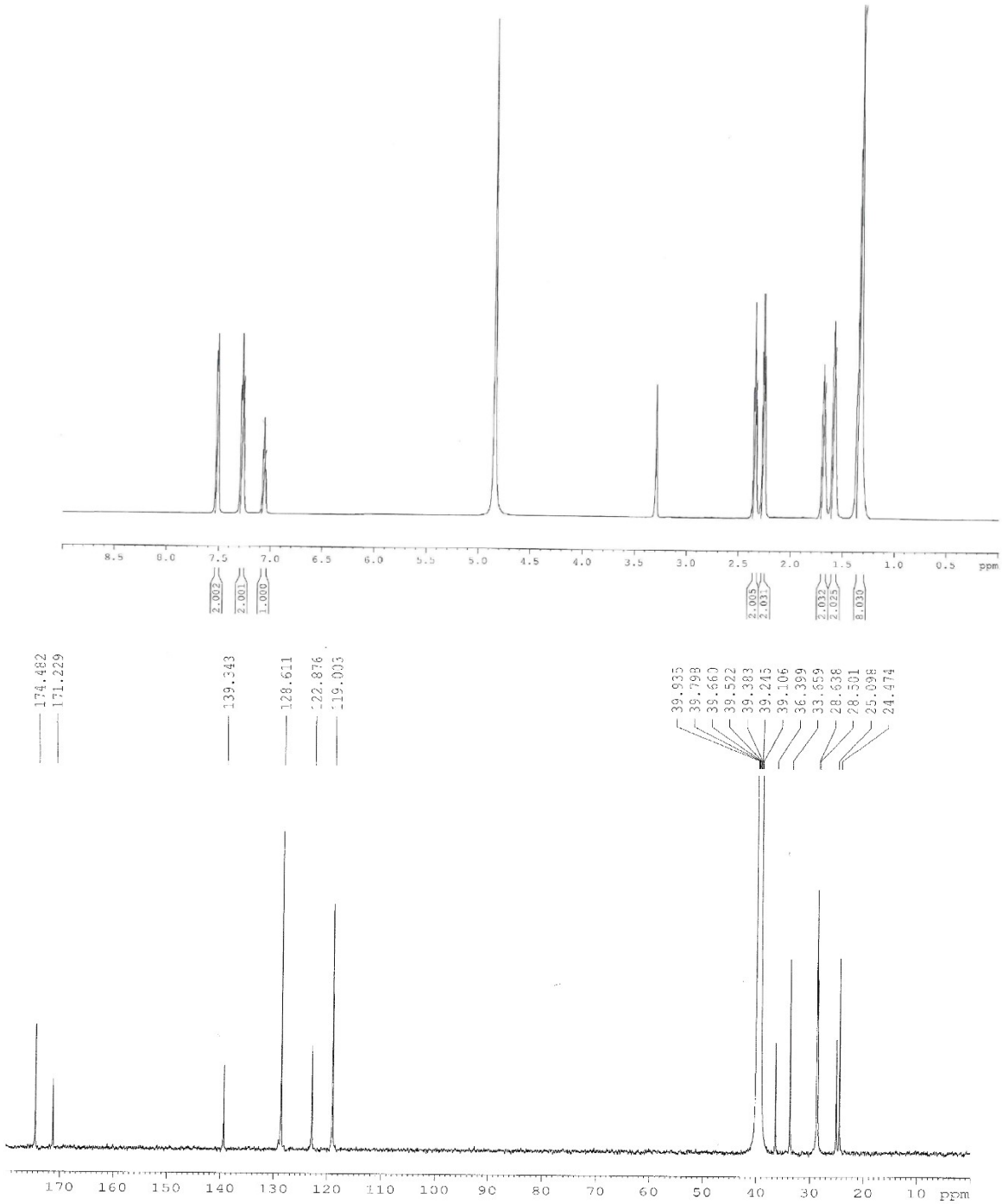
5-Oxo-5-(phenylamino)pentanoic acid (3b).

^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.53 (2H, d, *J* = 8.0 Hz), 7.29 (2H, t, *J* = 8.0 Hz), 7.07 (1H, t, *J* = 8.0 Hz), 2.44 (2H, t, *J* = 7.3 Hz), 2.39 (2H, t, *J* = 7.3 Hz), 2.01 – 1.95 (2H, m) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 176.8, 173.7, 139.8, 129.7 (2C), 125.1, 121.3 (2C), 36.9, 34.1, 22.1 ppm.



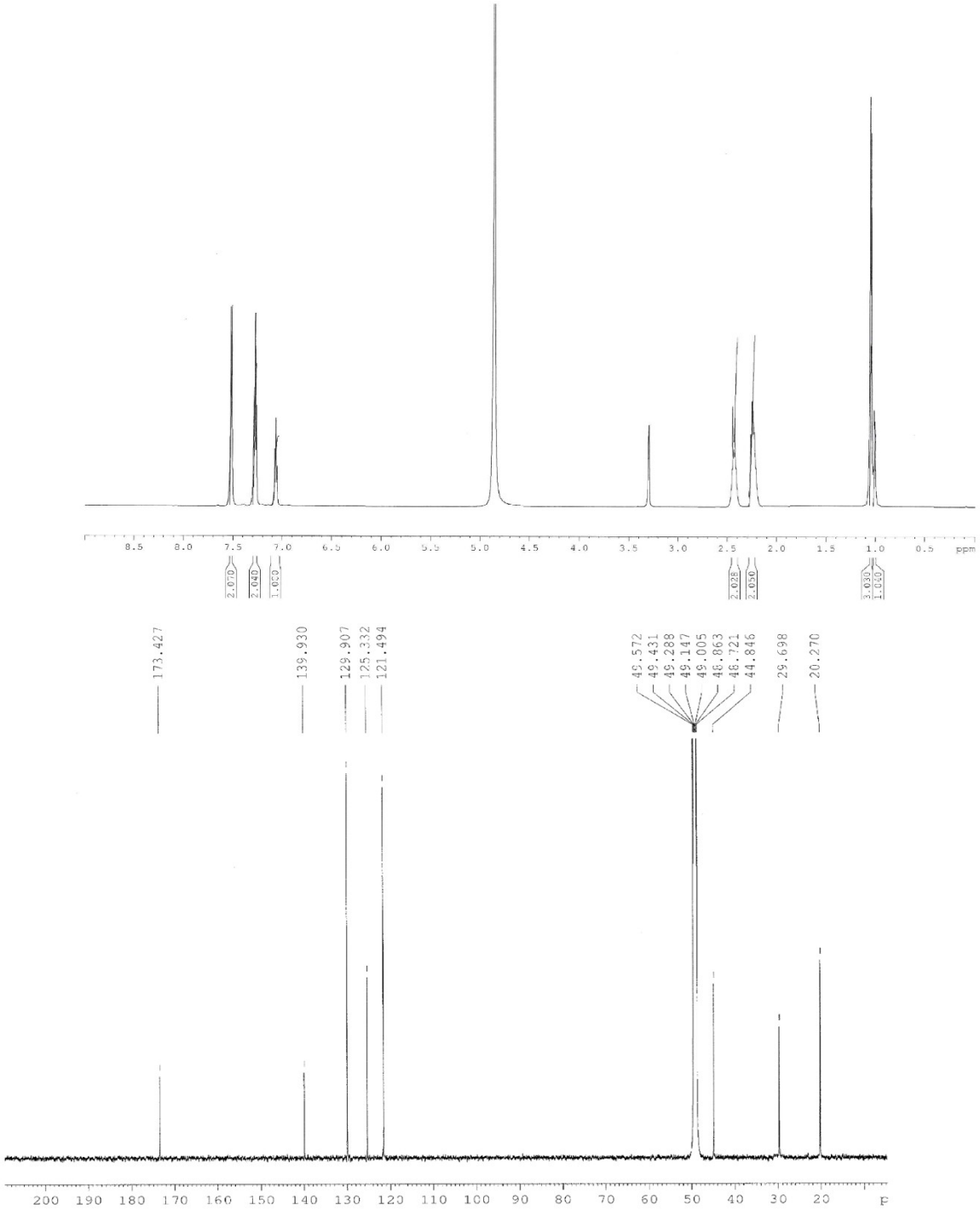
10-Oxo-10-(phenylamino)decanoic acid (3c).

^{1}H-NMR (600 MHz CD_3OD-d_4): δ = 7.51 (2H, d, *J* = 8.4 Hz), 7.27 (2H, t, *J* = 8.4 Hz), 7.06 (1H, t, *J* = 8.4 Hz), 2.34 (2H, t, *J* = 7.3 Hz), 2.26 (2H, t, *J* = 7.3 Hz), 1.70 – 1.65 (2H, m), 1.60 – 1.56 (2H, m), 1.36 – 1.29 (8H, m) ppm. ^{13}C-NMR (150 MHz, DMSO-d_6): δ = 174.5, 171.2, 139.3, 128.6 (2C), 122.9, 119.0 (2C), 36.4, 33.7, 28.6 (3C), 28.5, 25.1, 24.5 ppm.



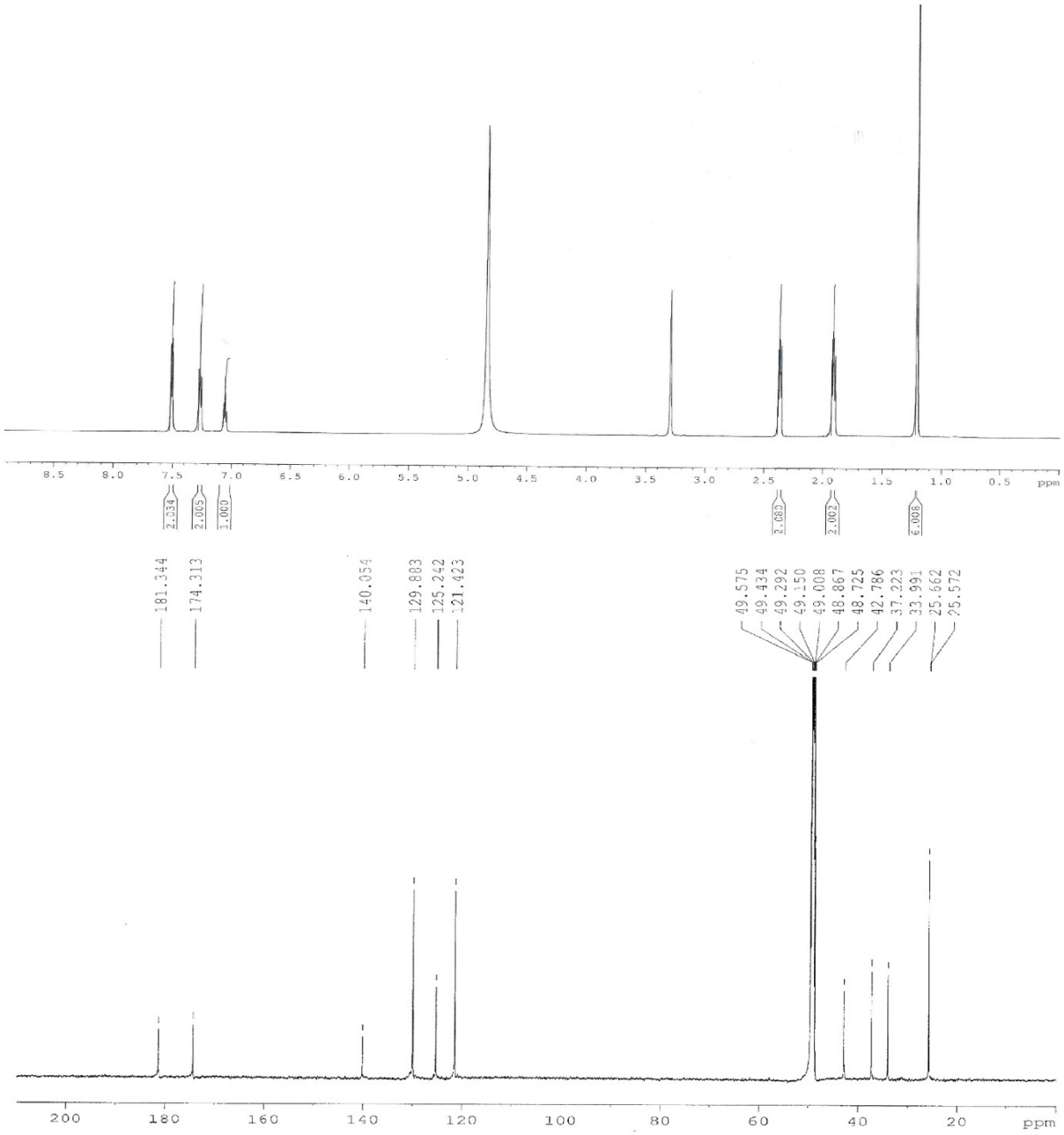
3-Methyl-5-oxo-5-(phenylamino)pentanoic acid (3d).

^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.52 (2H, d, *J* = 7.8 Hz), 7.27 (2H, t, *J* = 7.8 Hz), 7.06 (1H, t, *J* = 7.8 Hz), 2.44 – 2.41 (2H, m), 2.26 – 2.23 (2H, m), 1.04 (3H, d, *J* = 6.2 Hz), 1.01 (1H, d, *J* = 6.2 Hz) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 173.4 (2C), 139.9, 129.9 (2C), 125.3, 121.5 (2C), 44.8 (2C), 29.7, 20.3 ppm. MS (ESI): *m/z* = 244.1 $[M+Na]^+$.



2,2-Dimethyl-5-oxo-5-(phenylamino)pentanoic acid (3e).

^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.51 (2H, d, *J* = 8.0 Hz), 7.27 (2H, t, *J* = 8.0 Hz), 7.05 (1H, t, *J* = 8.0 Hz), 2.38 – 2.35 (2H, m), 1.92 – 1.90 (2H, m), 1.20 (6H, s) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 181.3, 174.3, 140.0, 129.9 (2C), 125.2, 121.4 (2C), 42.8, 37.2, 34.0, 25.7, 25.6 ppm.



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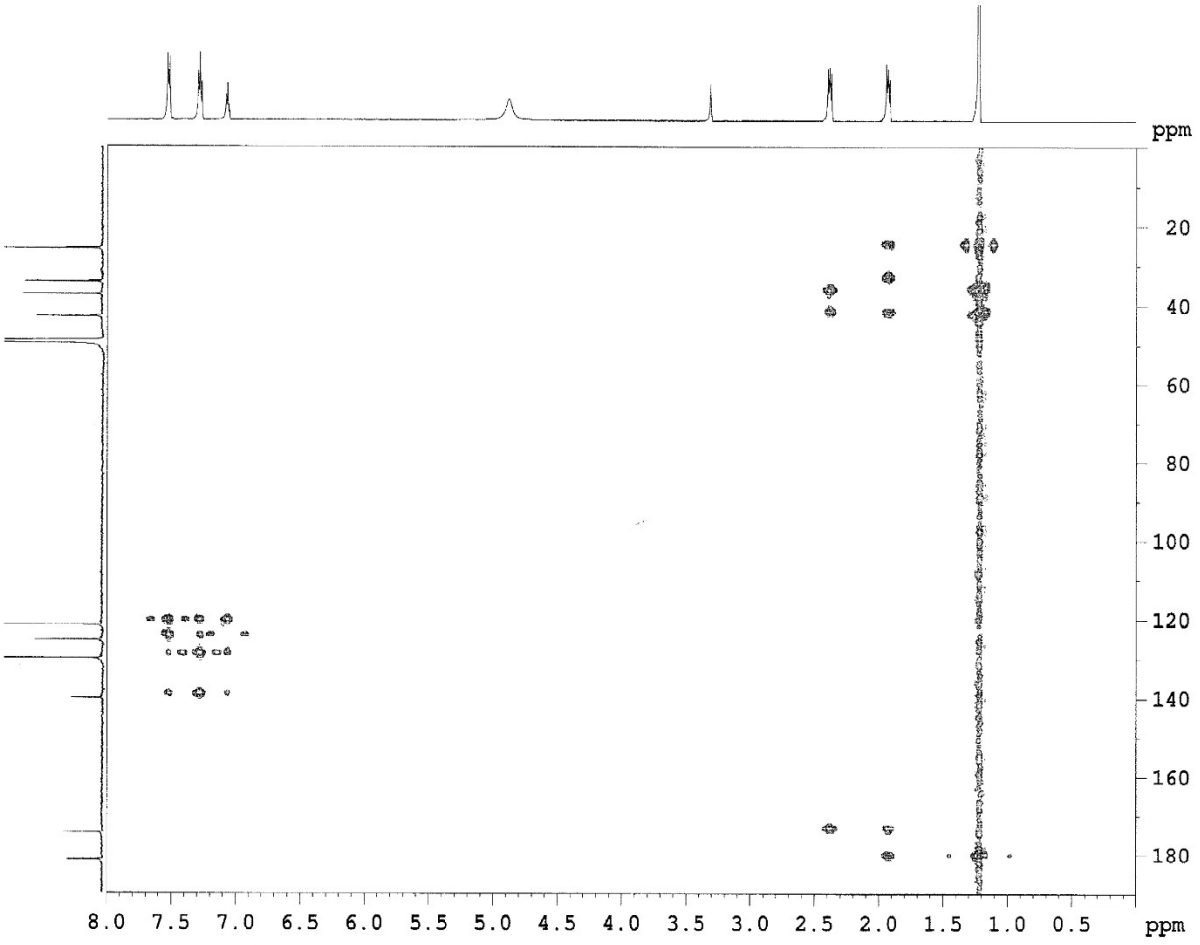
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120
140
160
180
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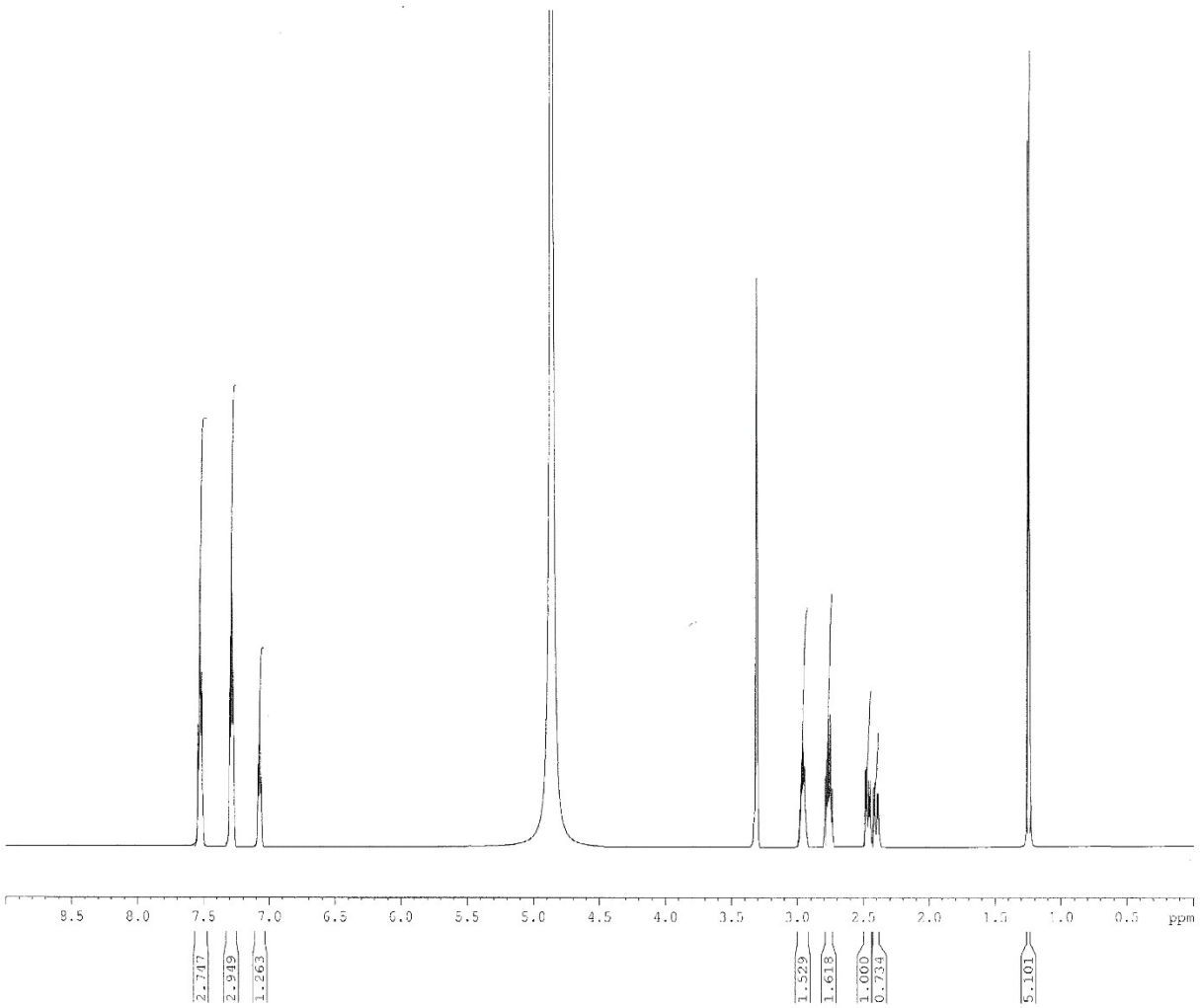
ppm

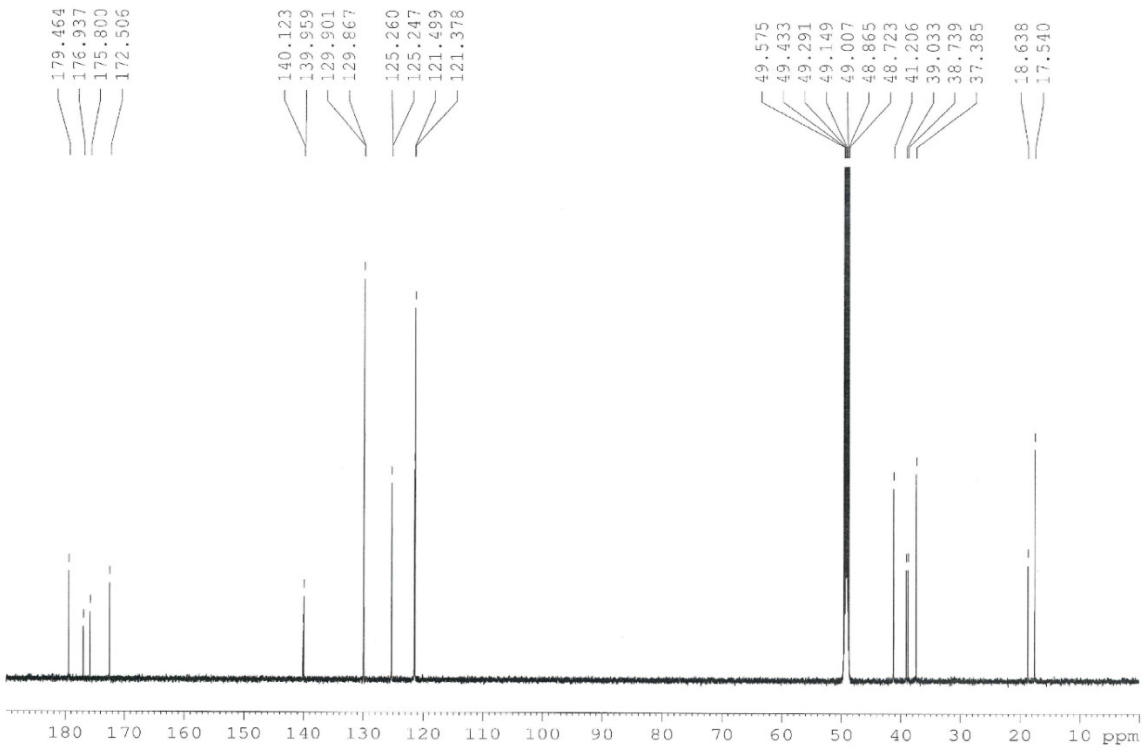
2-Methyl-4-oxo-4-(phenylamino)butanoic acid (3f).

^{1}H-NMR (600 MHz, CD_3OD-d_4): 7.52 (2H, d, *J* = 8.0 Hz), 7.28 (2H, t, *J* = 8.0 Hz), 7.07 (1H, t, *J* = 8.0 Hz), 2.99 – 2.92 (1H, m), 2.79 – 2.73 (1H, m), 2.46 (1H, dd, *J* = 15 Hz *J* = 6.6 Hz), 1.23 (3H, s) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 179.5, 172.5, 140.0, 129.9 (2C), 125.3, 121.4 (2C), 41.2, 37.4, 17.5 ppm.

3-Methyl-1-phenylpyrrolidine-2,5-dione (4f).

^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.53 (2H, d, *J* = 8.0 Hz), 7.28 (2H, t, *J* = 8.0 Hz), 7.07 (1H, t, *J* = 8.0 Hz), 2.99 – 2.92 (1H, m), 2.79 – 2.73 (1H, m), 2.40 (1H, dd, *J* = 15 Hz *J* = 6.6 Hz), 1.25 (3H, s) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 176.9, 175.8, 140.1, 129.9 (2C), 125.2, 121.5 (2C), 39.0, 38.7, 18.6 ppm.

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49.291
49.149
49.007
48.865
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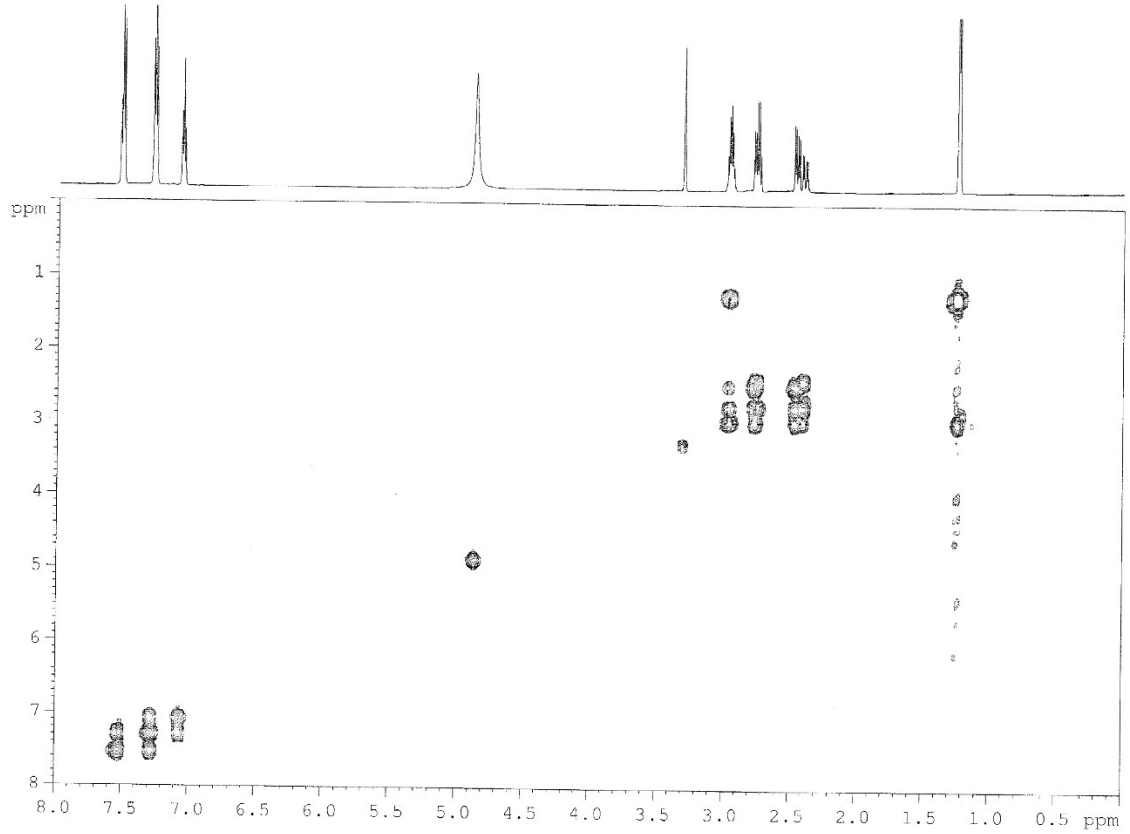
COSY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5
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7
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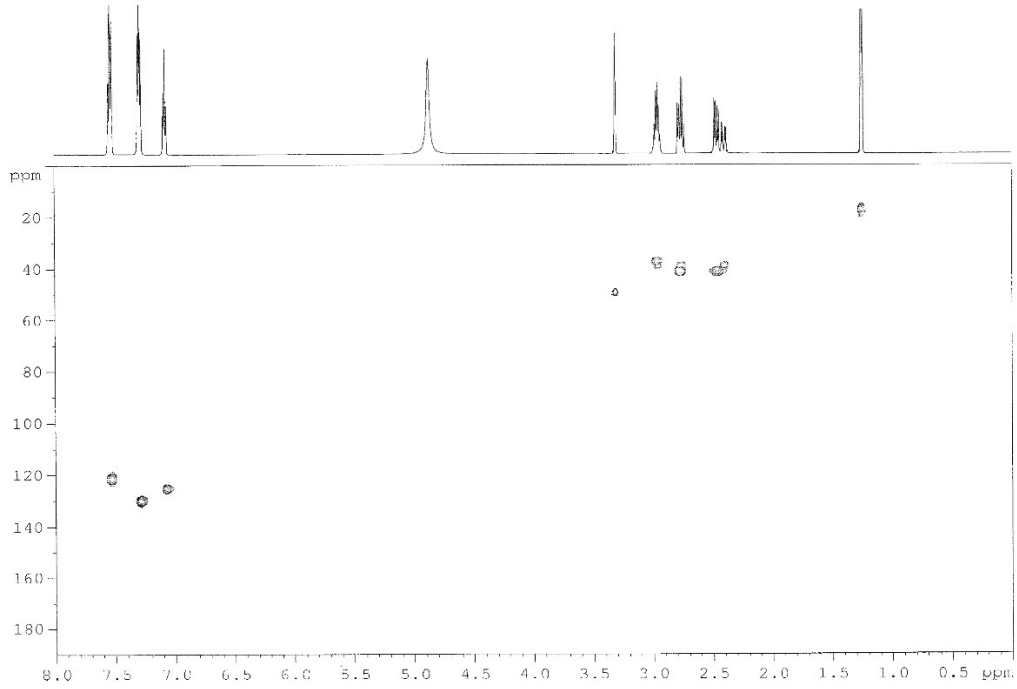
HSQC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140
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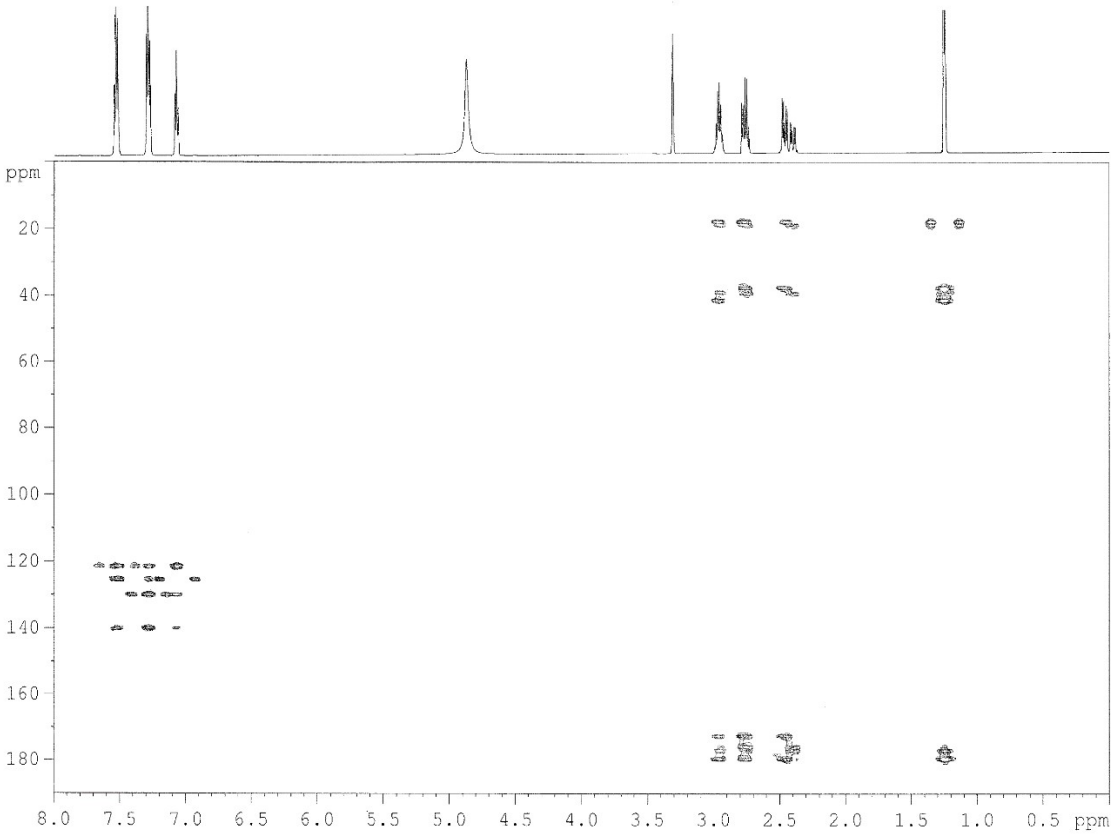
HMBC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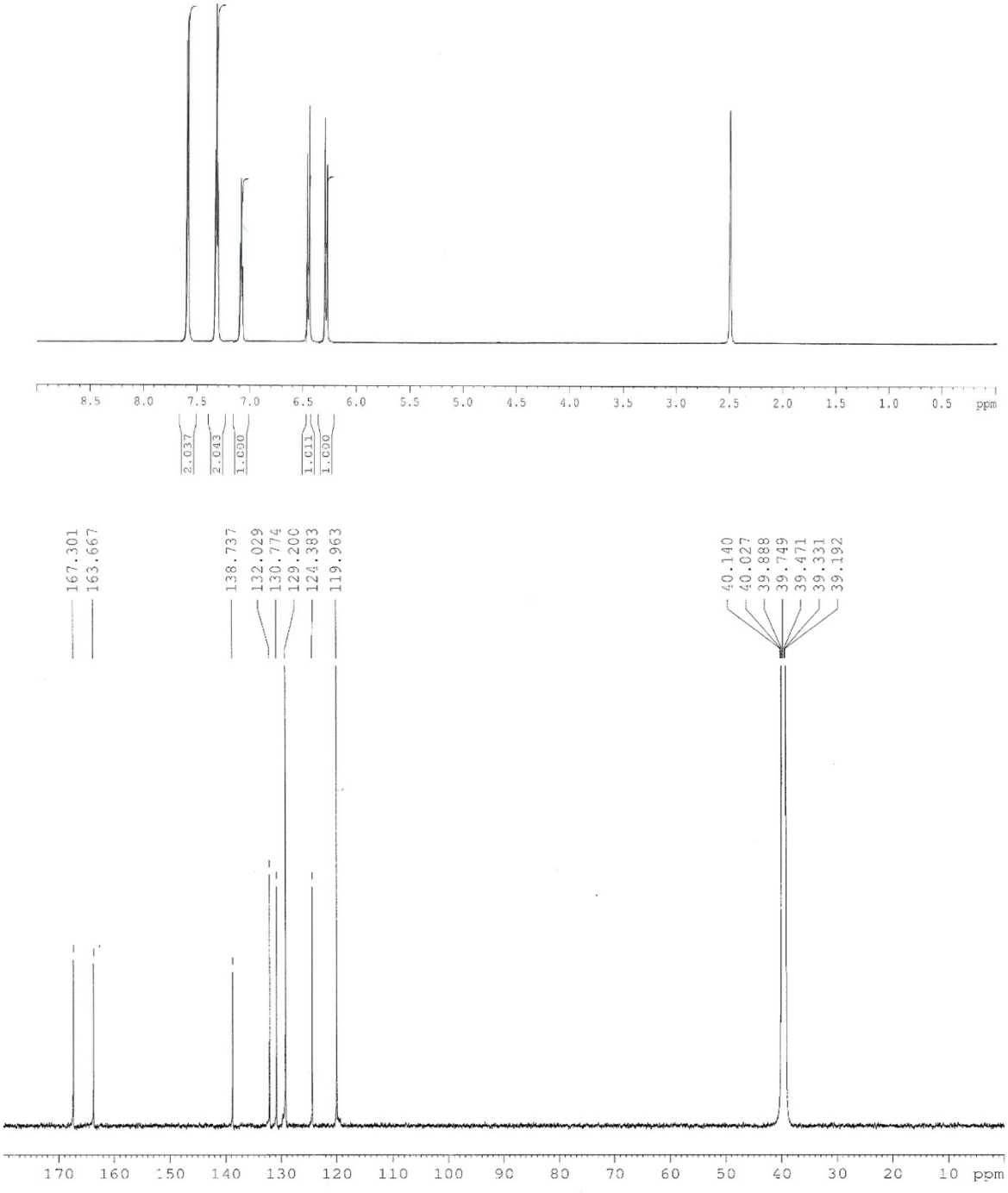
80
100
120
140
160
180
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm

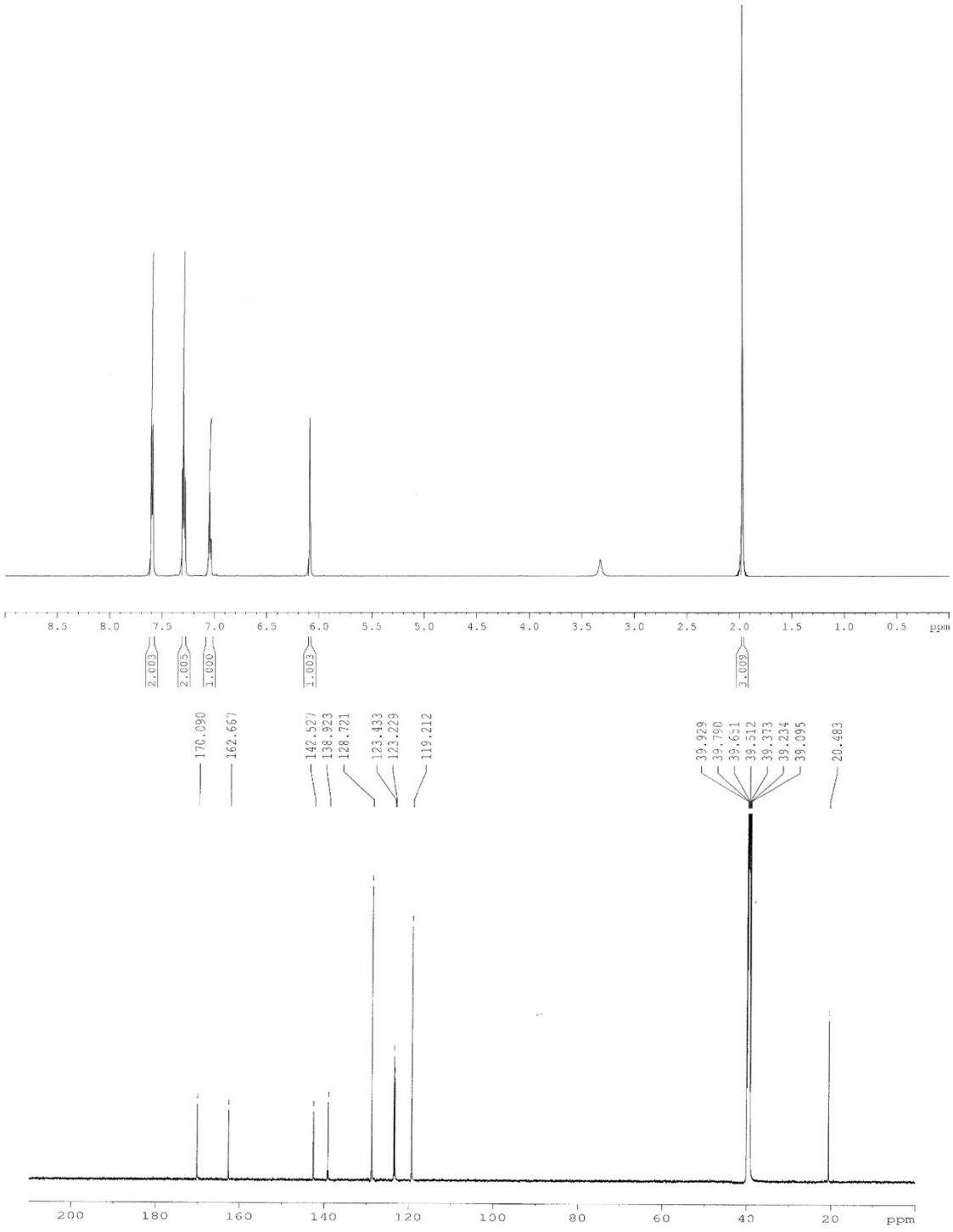
(*E*)-4-oxo-4-(phenylamino)but-2-enoic acid (3g).

^{1}H-NMR (600 MHz, DMSO-d_6): δ = 7.58 (2H, d, J = 7.8 Hz), 7.31 (2H, t, J = 7.8 Hz), 7.08 (1H, t, J = 7.8 Hz), 6.45 (1H, d, J = 12 Hz), 6.28 (1H, d, J = 12 Hz) ppm. ^{13}C-NMR (150 MHz, DMSO-d_6): δ = 167.3, 163.7, 138.7, 132.0, 130.8, 129.2 (2C), 124.4, 120.0 (2C) ppm.



2-Methyl-4-oxo-4-(phenylamino)but-2-enoic acid (3h).

^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.59 (2H, d, *J* = 7.8 Hz), 7.29 (2H, t, *J* = 7.8 Hz), 7.04 (1H, t, *J* = 7.8 Hz), 6.09 (1H, s), 1.97 (3H, s) ppm. ^{13}C-NMR (150 MHz, DMSO-d_6): δ = 170.1, 162.7, 142.5, 138.9, 128.7 (2C), 123.4, 123.2, 119.2 (2C), 20.5 ppm.



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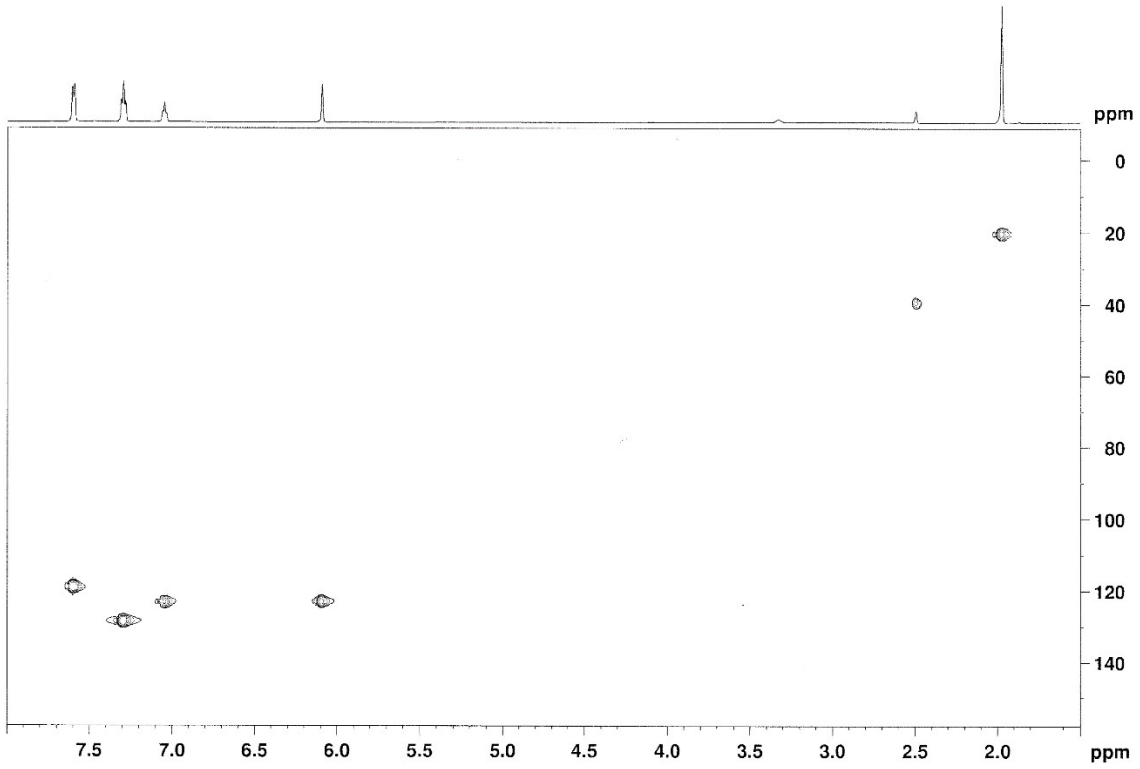
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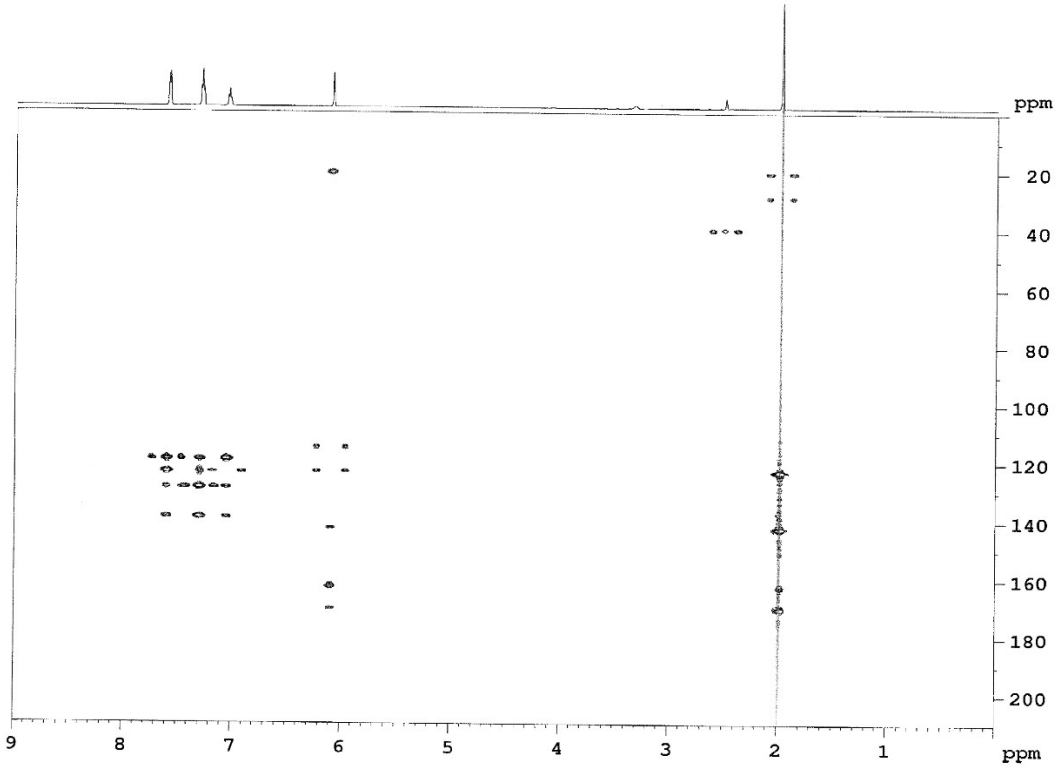
ppm

HMBC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140
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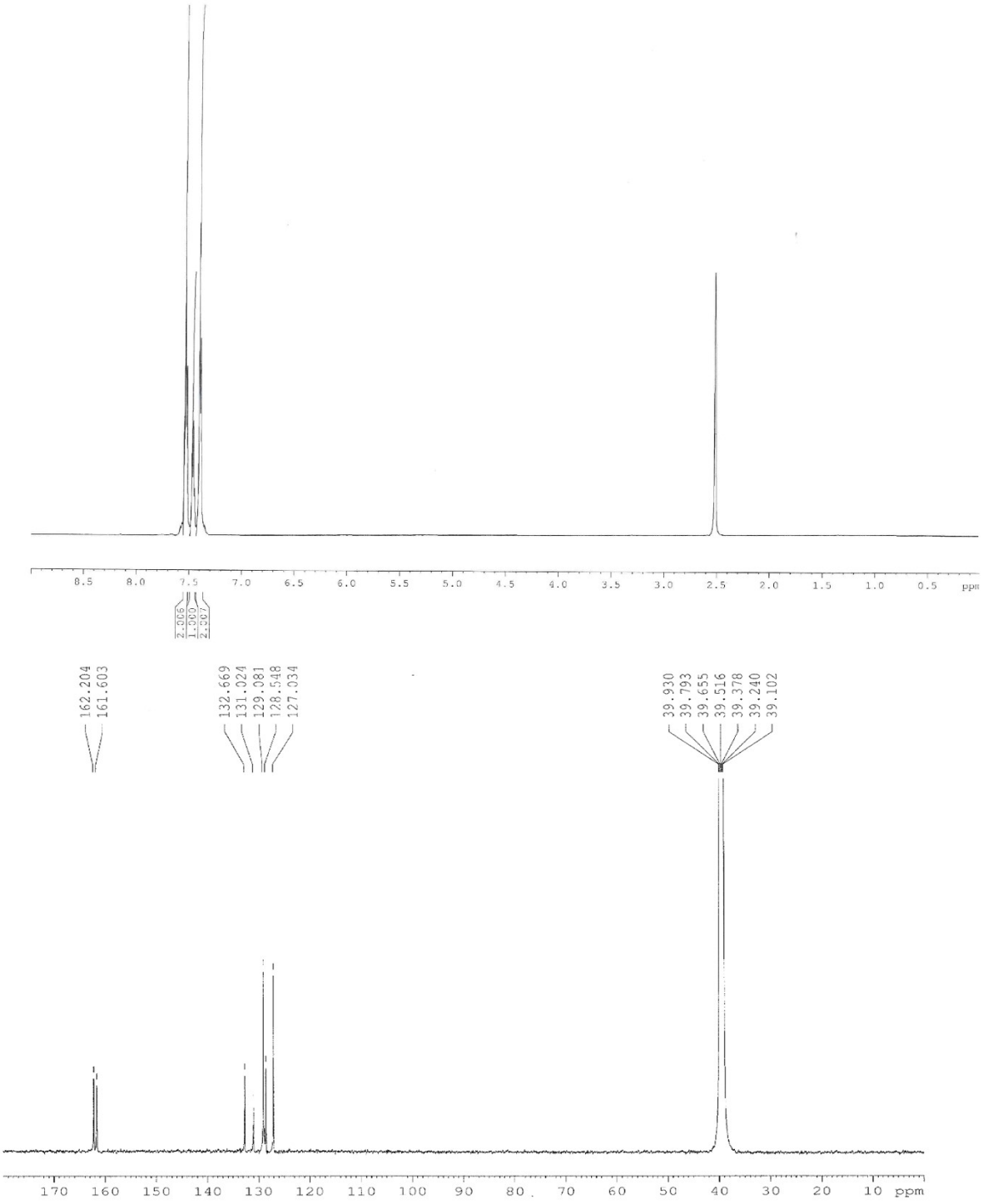
7
6
5
4
3
2
1
ppm

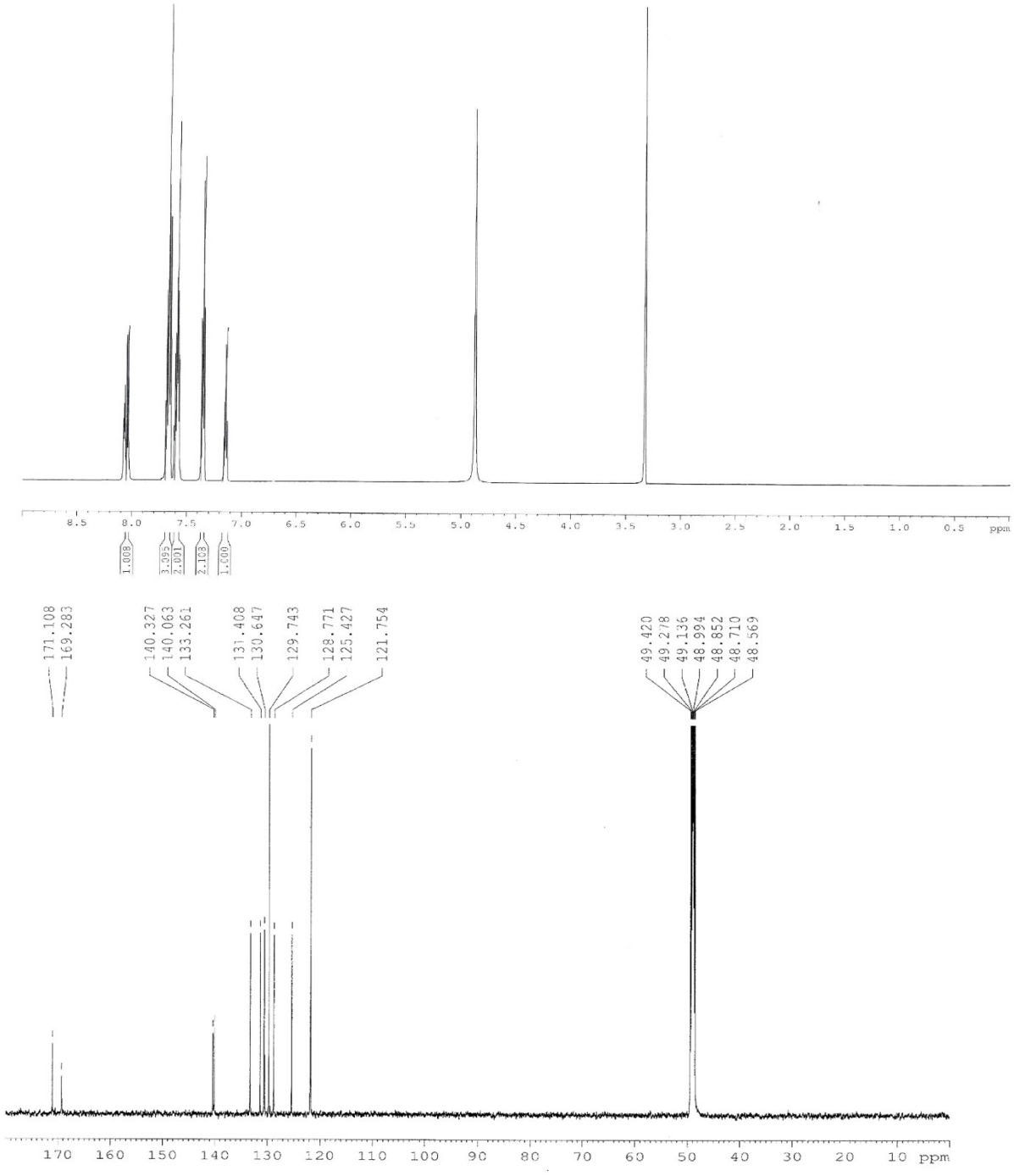
2,3-Dichloro-4-oxo-4-(phenylamino)but-2-enoic acid (3j).

^{1}H-NMR (600 MHz, DMSO-d_6): δ = 7.53 (2H, t, J = 7.7 Hz), 7.46 (1H, t, J = 7.6 Hz), 7.39 (2H, d, J = 7.6 Hz) ppm. ^{13}C-NMR (150 MHz, DMSO-d_6): δ = 162.2, 161.6, 132.7 (2C), 131.0, 129.1 (2C), 128.5, 127.0 (2C) ppm.



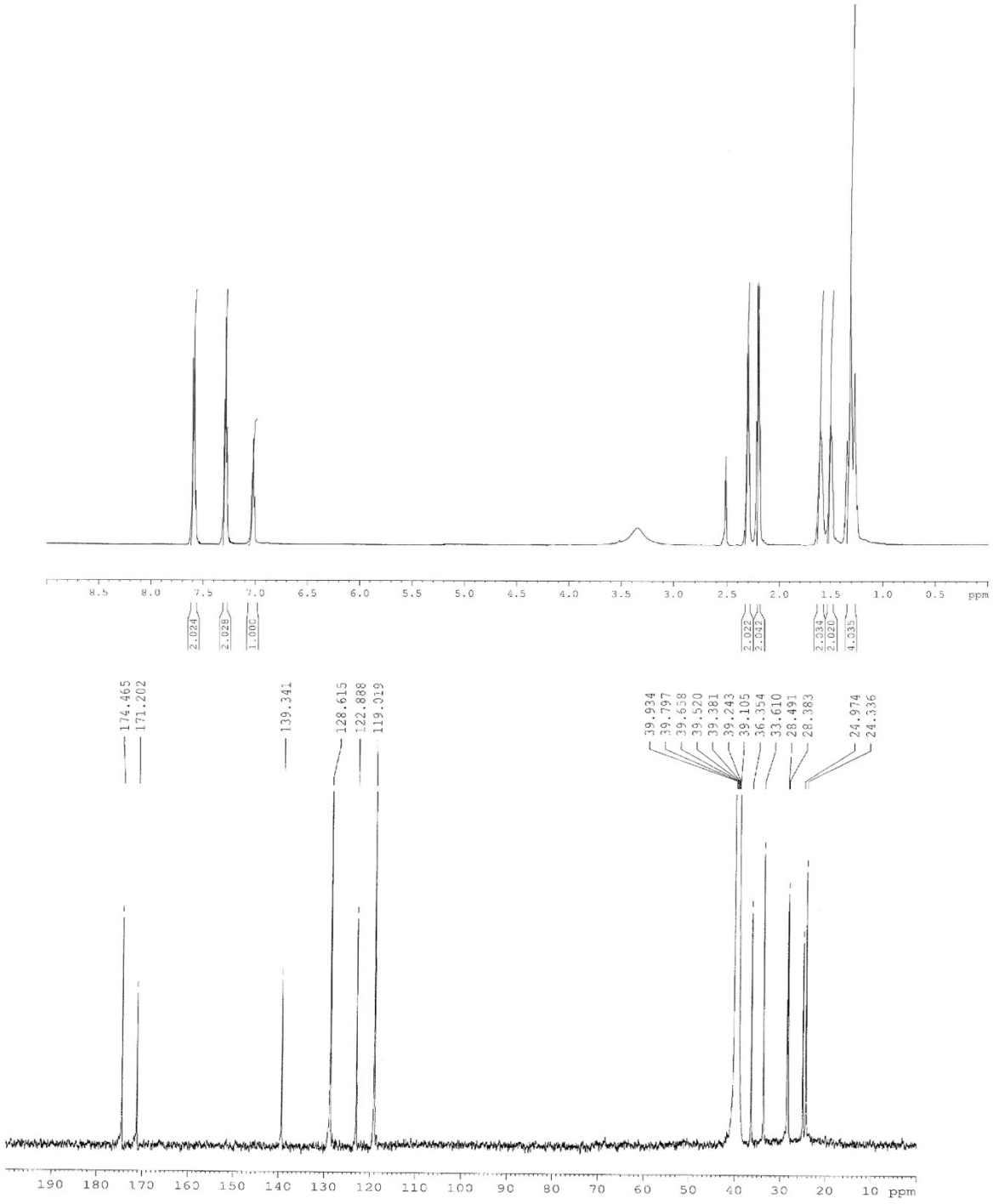
2-(Phenylcarbamoyl)benzoic acid (3k).

^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 8.04 (1H, d, J = 8.2 Hz), 7.69 – 7.65 (3H, m), 7.60 – 7.57 (2H, m), 7.35 (2H, t, J = 8.2 Hz), 7.14 (1H, t, J = 8.2 Hz) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 171.1, 169.2, 140.3, 140.1, 133.3, 131.4, 130.6, 129.7 (3C), 128.8, 125.4, 121.8 (2C) ppm.



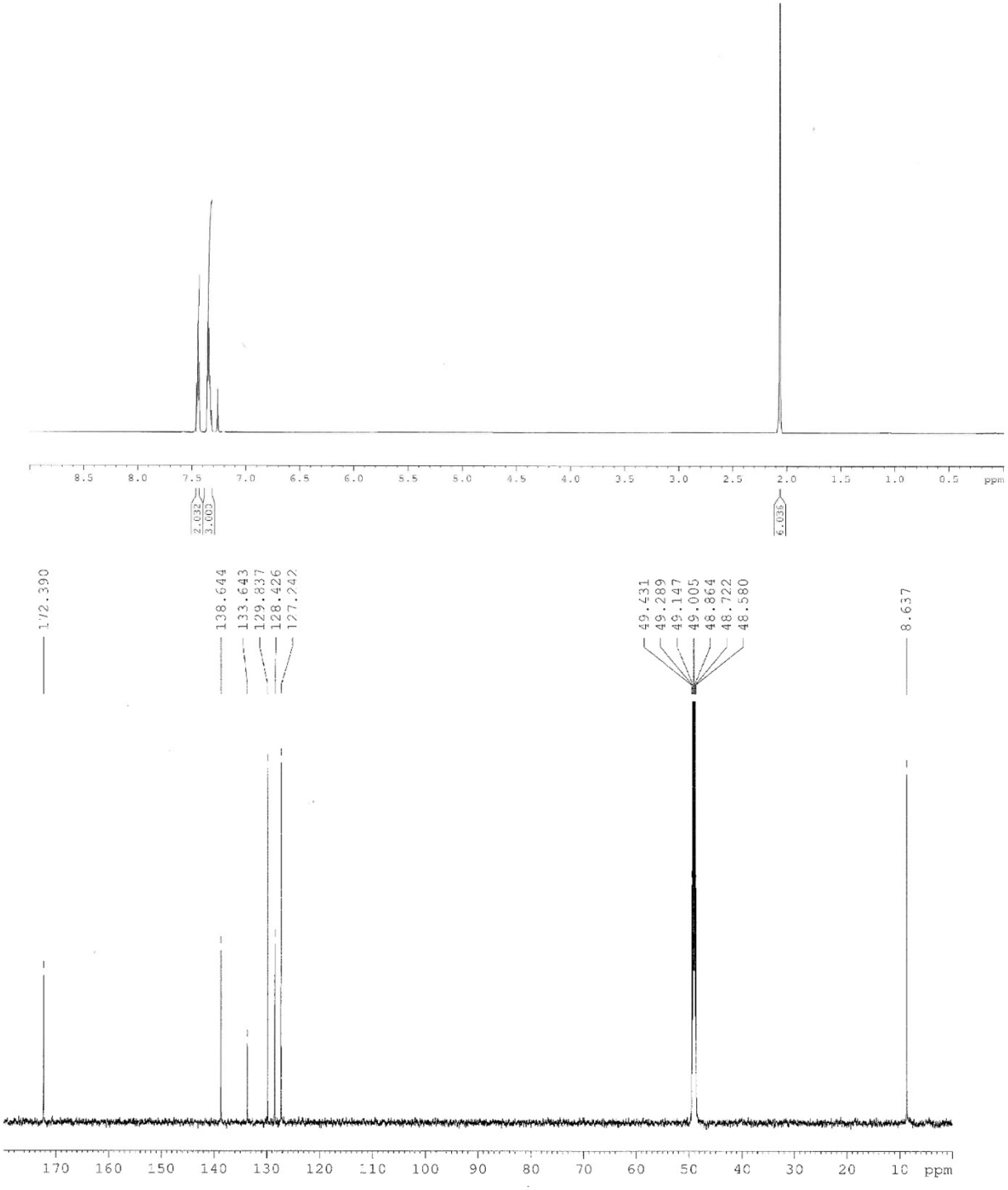
8-Oxo-8-(phenylamino)octanoic acid (3n).

^{1}H-NMR (600 MHz, DMSO-d_6): δ = 7.59 (2H, d, J = 8.0 Hz), 7.28 (2H, t, J = 8.0 Hz), 7.02 (1H, t, J = 8.0 Hz), 2.32 – 2.28 (2H, m), 2.21 – 2.18 (2H, m), 1.63 – 1.56 (2H, m), 1.53 – 1.48 (2H, m), 1.34 – 1.27 (4H, m) ppm. ^{13}C-NMR (150 MHz, DMSO-d_6): δ = 174.5, 171.2, 139.3, 128.6 (2C), 122.9, 119.0 (2C), 36.4, 33.6, 28.5, 28.4, 25.0, 24.3 ppm.



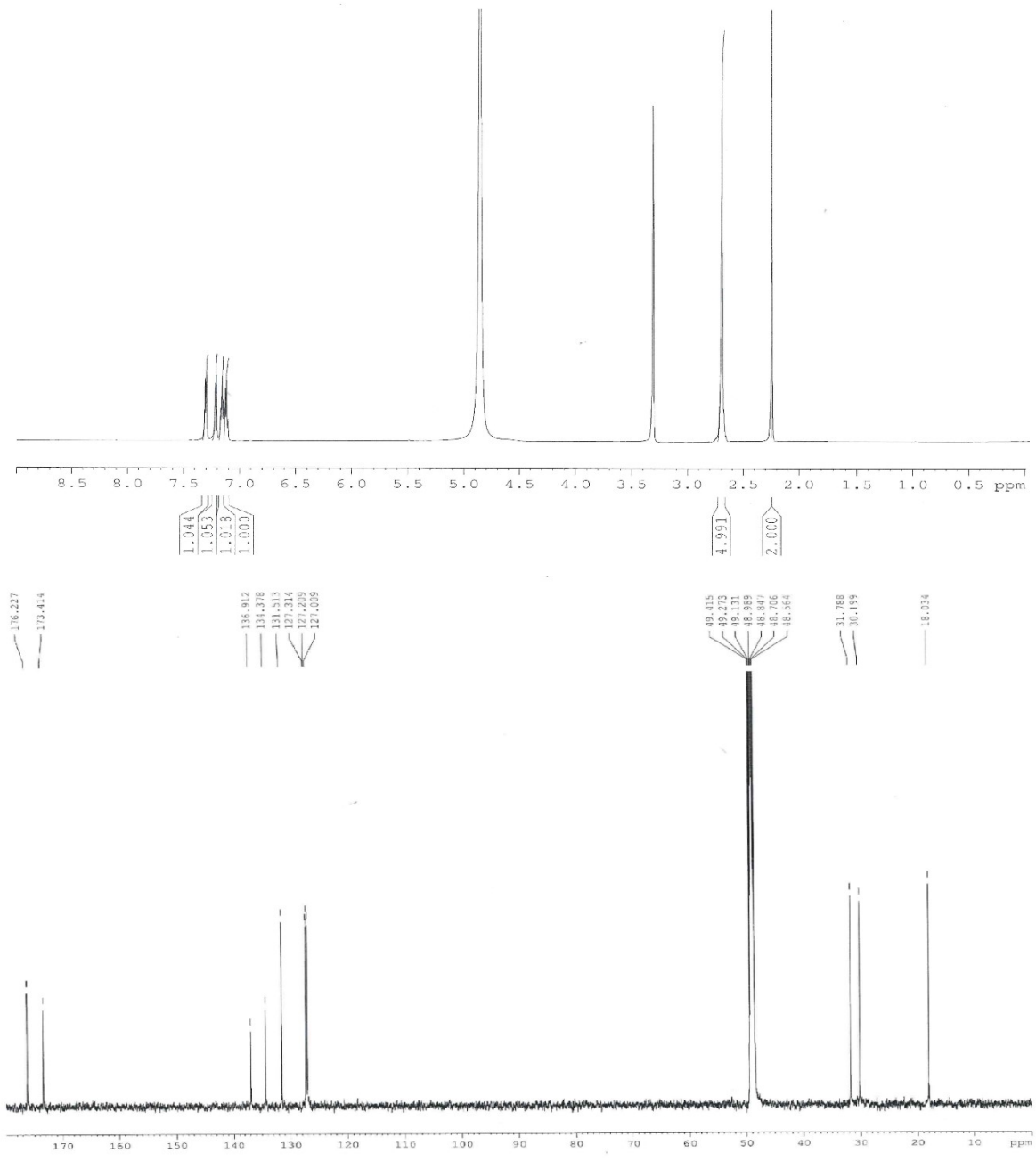
3,4-Dimethyl-1-phenyl-1*H*-pyrrole-2,5-dione (4i).

^{1}H-NMR (600 MHz, $CDCl_3$): δ = 7.44 (2H, t, J = 7.8 Hz), 7.35 – 7.32 (3H, m), 2.06 (6H, s) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 172.4 (2C), 138.6 (2C), 133.6, 129.8 (2C), 128.4, 127.2 (2C), 8.6 (2C) ppm. MS (ESI): m/z = 202.3 $[M+H]^+$.



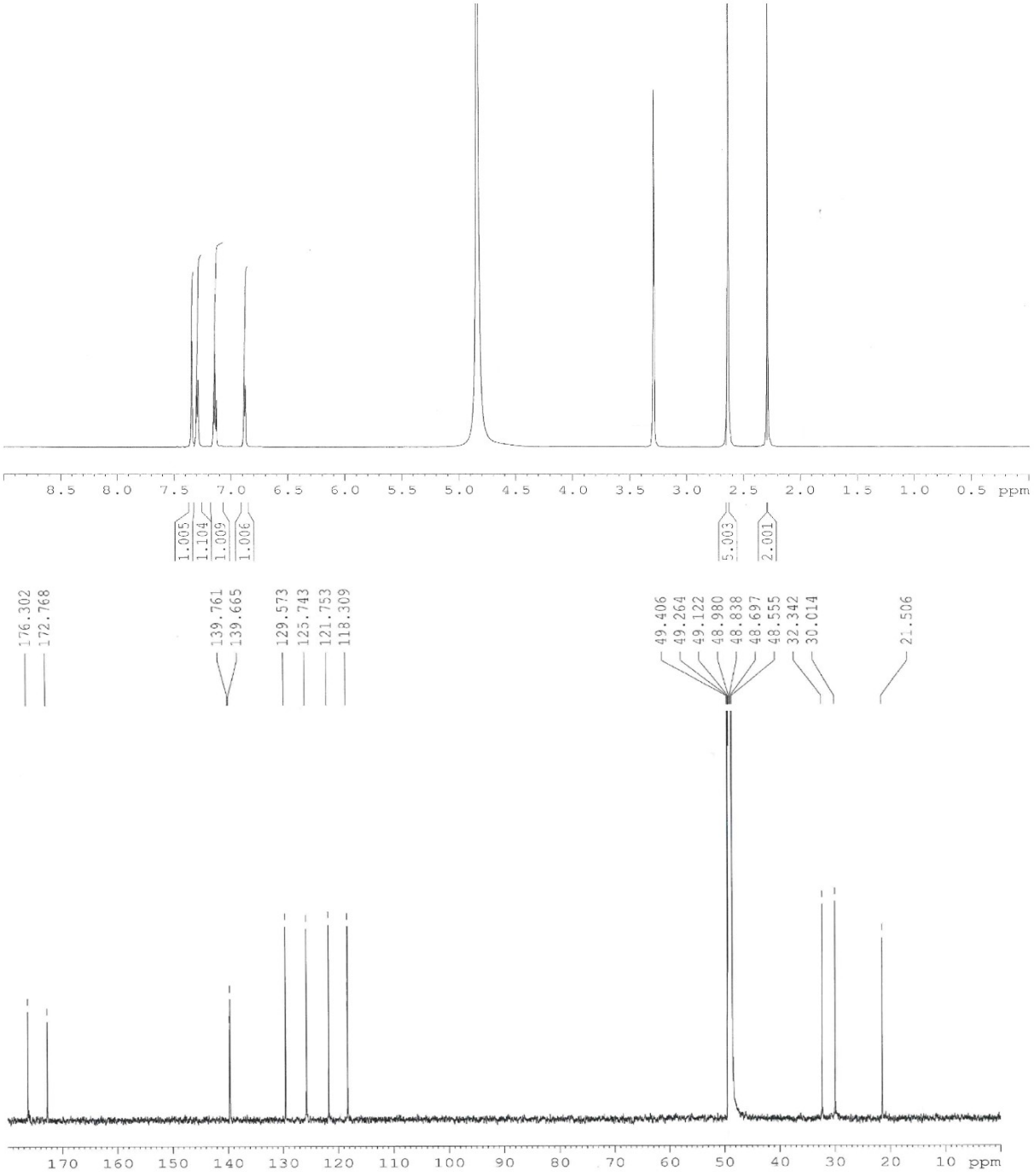
4-Oxo-4-(*o*-tolylamino)butanoic acid (5b).

^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.31 (1H, d, *J* = 7.6 Hz), 7.22 (1H, d, *J* = 7.6 Hz), 7.16 (1H, t, *J* = 7.6 Hz), 7.12 (1H, t, *J* = 7.6 Hz), 2.71 – 2.68 (5H, m), 2.25 – 2.22 (2H, m) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 176.2, 173.4, 136.9, 134.4, 131.5, 127.3, 127.2, 127.0, 31.8, 30.2, 18.0 ppm.



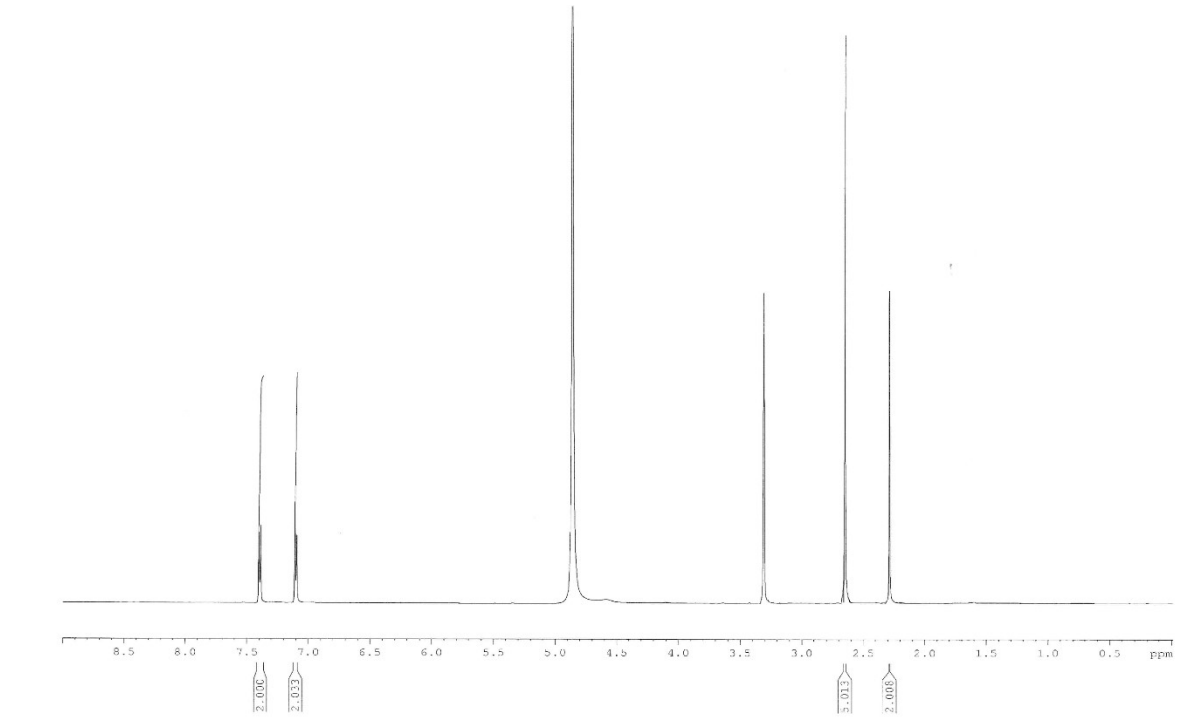
4-Oxo-4-(*m*-tolylamino)butanoic acid (5c).

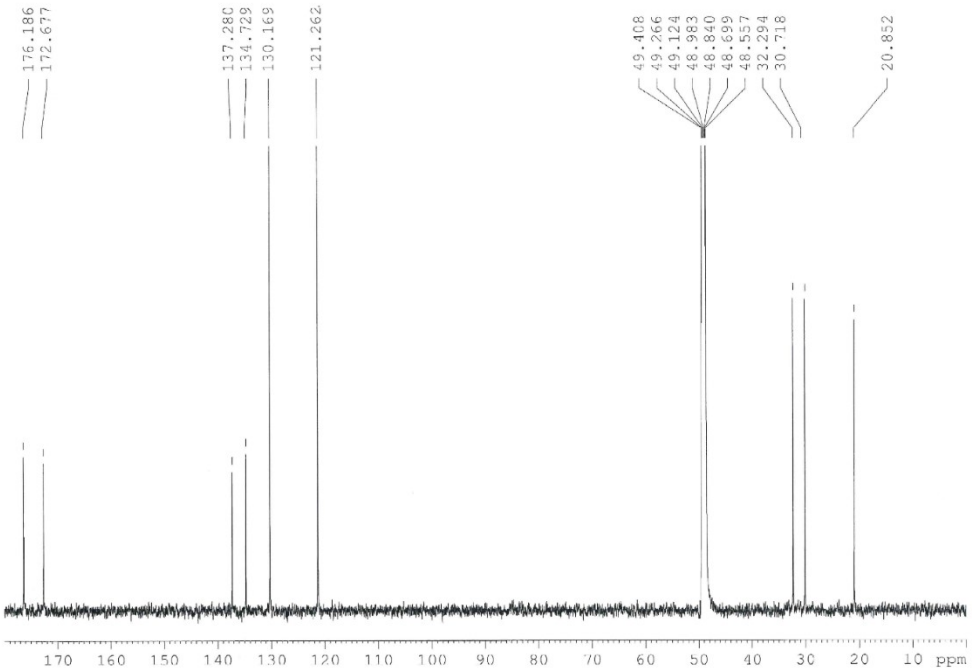
^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.34 (1H, s), 7.29 (1H, d, J = 8.0 Hz), 7.14 (1H, t, J = 8.0 Hz), 6.88 (1H, d, J = 8.0 Hz), 2.66 – 2.62 (5H, m), 2.31 – 2.28 (2H, m) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 176.3, 172.8, 139.8, 139.7, 129.6, 125.7, 121.8, 118.3, 32.3, 30.0, 21.5 ppm.



4-Oxo-4-(*p*-tolylamino)butanoic acid (5d).

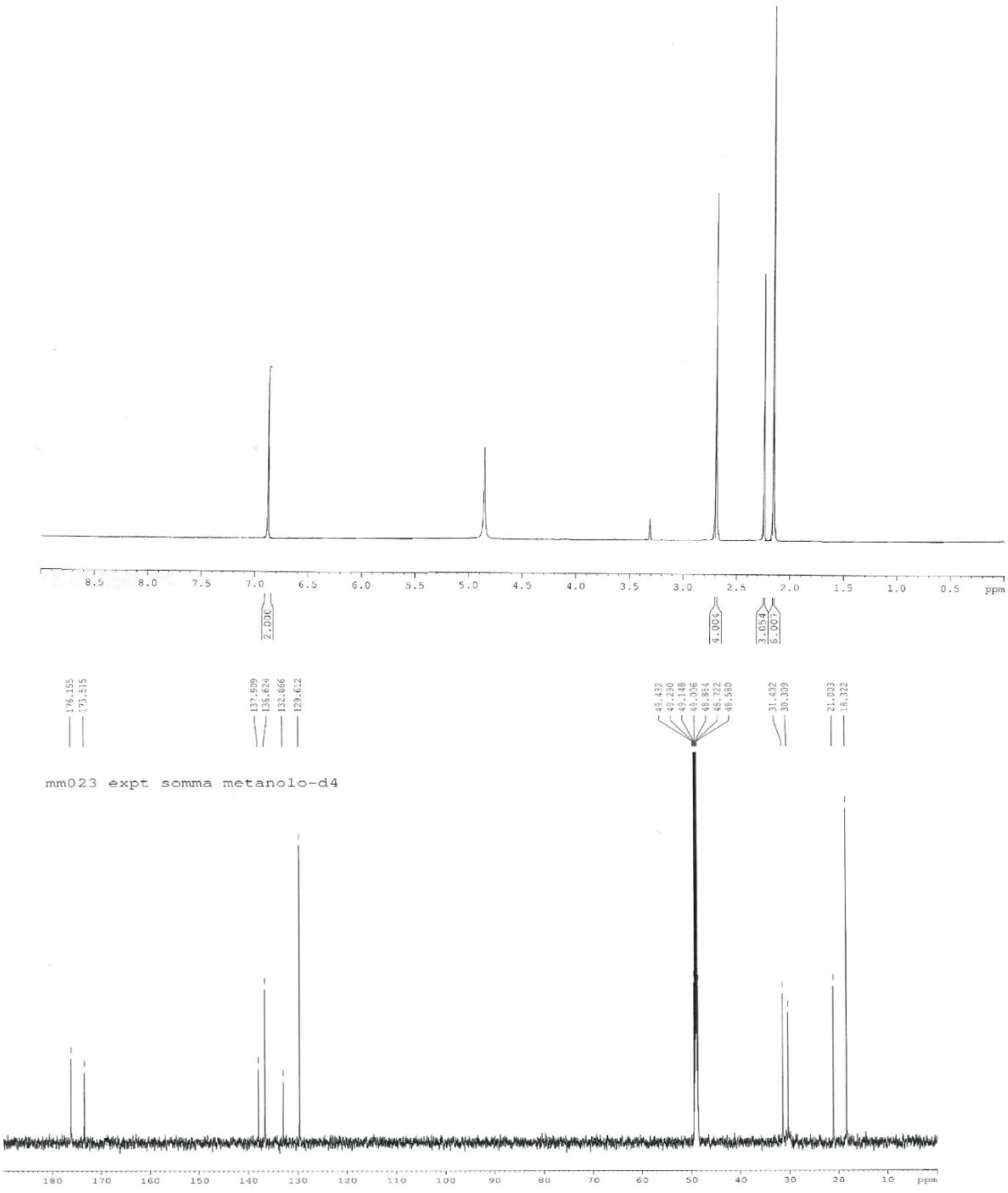
^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.39 (2H, d, J = 8.4 Hz), 7.10 (2H, d, J = 8.4 Hz), 2.66 – 2.64 (5H, m), 2.30 – 2.27 (2H, m) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 176.2, 172.7, 137.3, 134.7, 130.2 (2C), 121.3 (2C), 32.3, 30.7, 20.8 ppm.





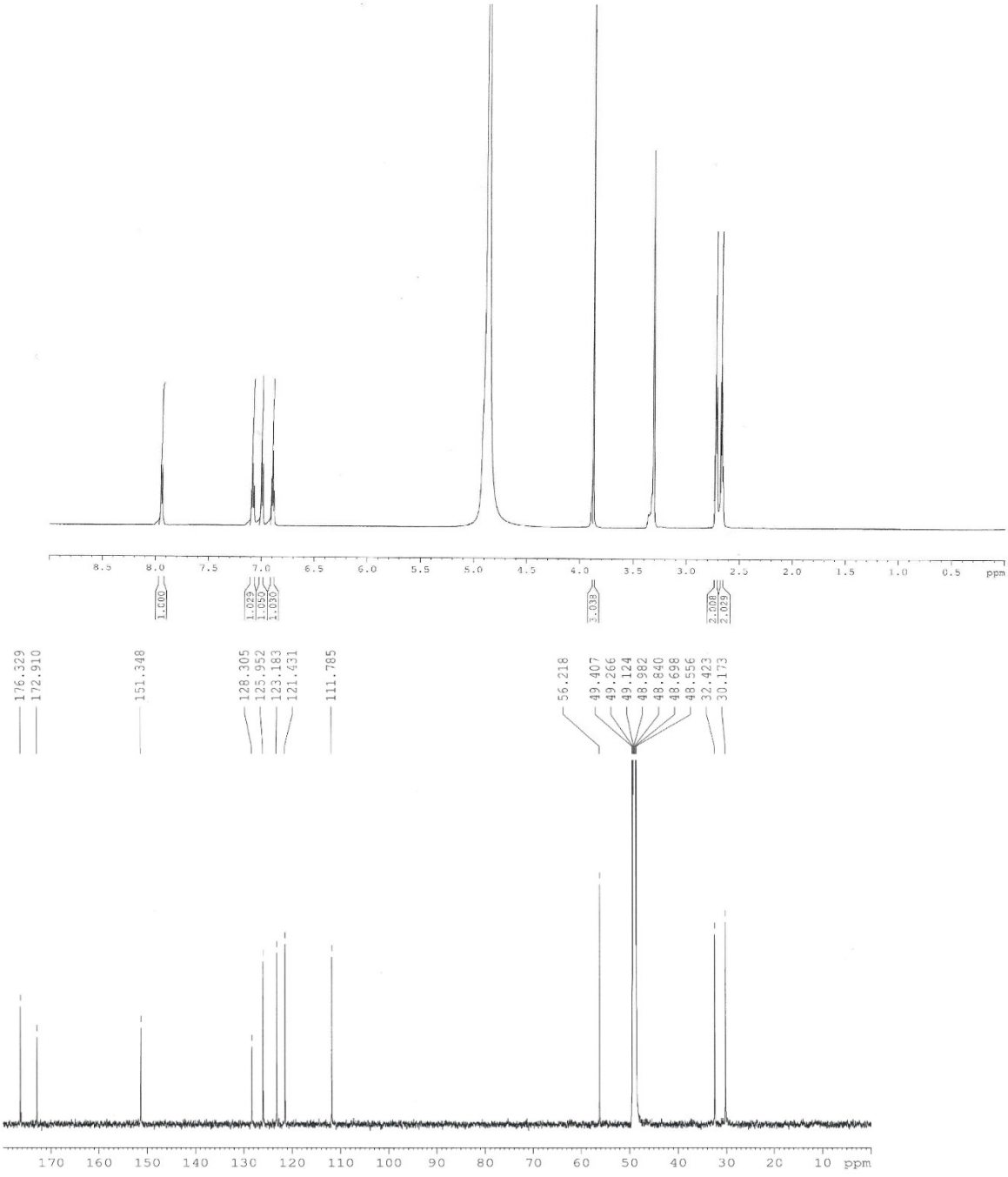
4-(Mesitylamino)-4-oxobutanoic acid (5e).

^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 6.88 (2H, s), 2.70 – 2.68 (4H, m), 2.24 (3H, s), 2.15 (6H, s) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 176.2, 173.5, 137.9, 136.6 (2C), 132.9, 129.6 (2C), 31.4, 30.3, 21.0 18.3 (2C) ppm.



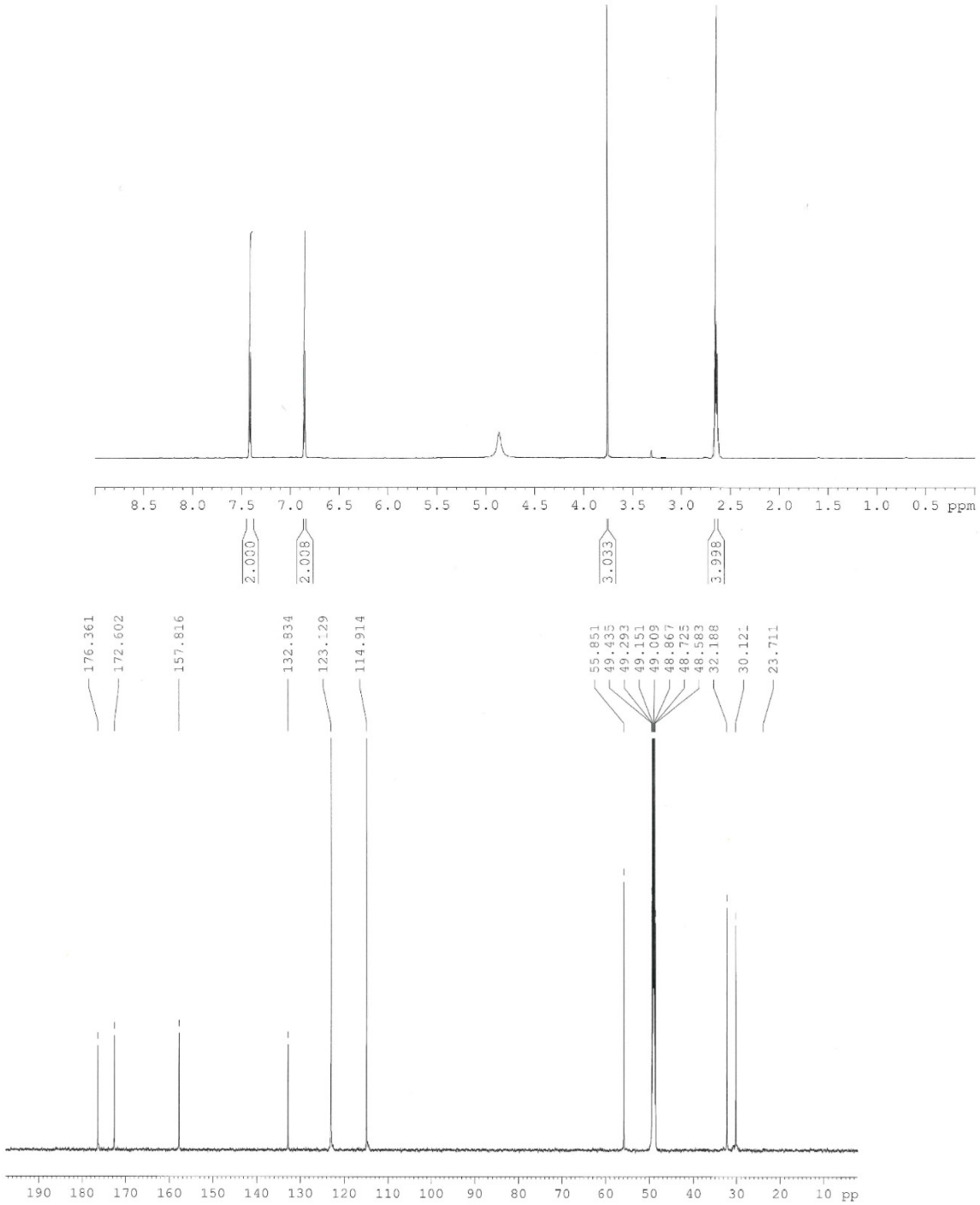
4-((2-Methoxyphenyl)amino)-4-oxobutanoic acid (5f).

^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.94 (1H, d, *J* = 7.8 Hz), 7.08 (1H, t, *J* = 7.8 Hz), 6.99 (1H, d, *J* = 7.8 Hz), 6.89 (1H, t, *J* = 7.8 Hz), 3.87 (3H, s), 2.73 – 2.71 (2H, m), 2.68 – 2.65 (2H, m) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 176.3, 172.9, 151.3, 128.3, 126.0, 123.2, 121.4, 111.8, 56.2, 32.4, 30.2 ppm.



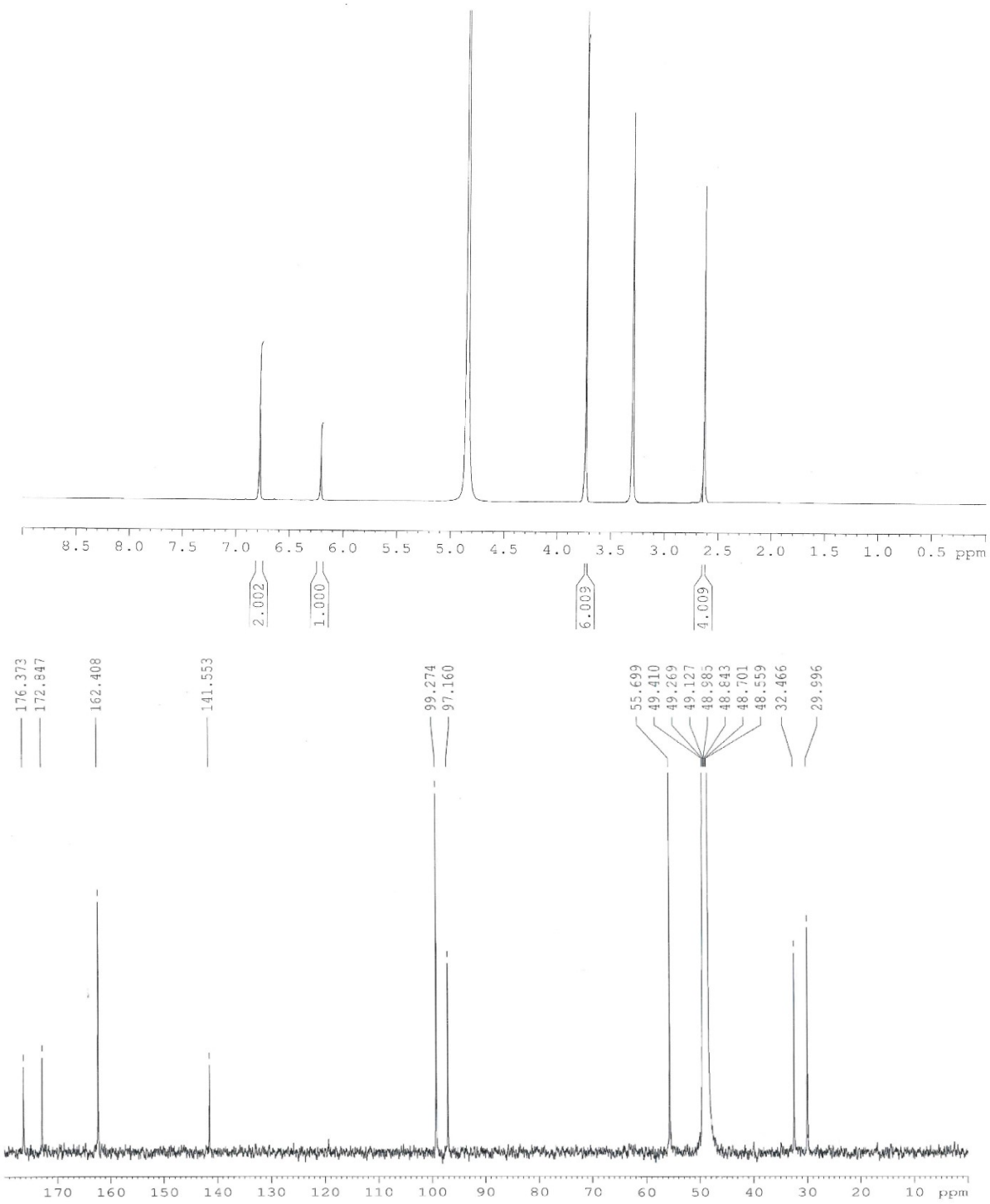
4-((4-Methoxyphenyl)amino)-4-oxobutanoic acid (5g).

^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.41 (2H, d, J = 8.6 Hz), 6.85 (2H, d, J = 8.6 Hz), 3.75 (3H, s), 2.66 – 2.64 (4H, m) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 176.4, 172.6, 157.8, 132.8, 123.1 (2C), 114.9 (2C), 55.8, 32.2, 30.1 ppm.



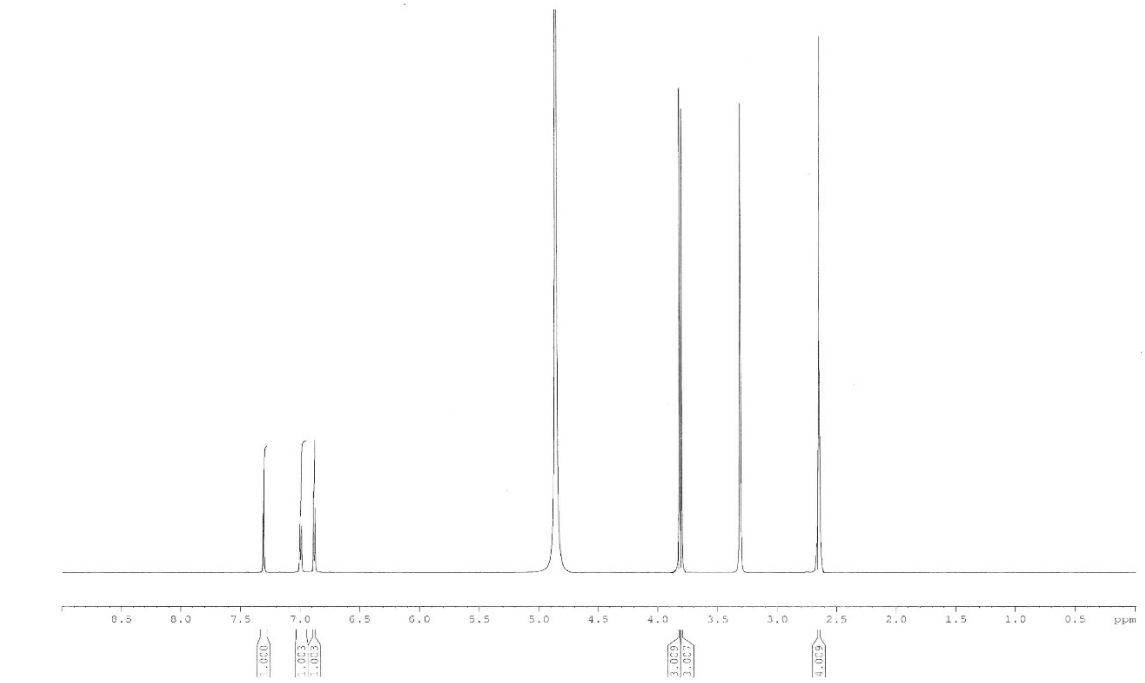
4-((3,5-Dimethoxyphenyl)amino)-4-oxobutanoic acid (5h).

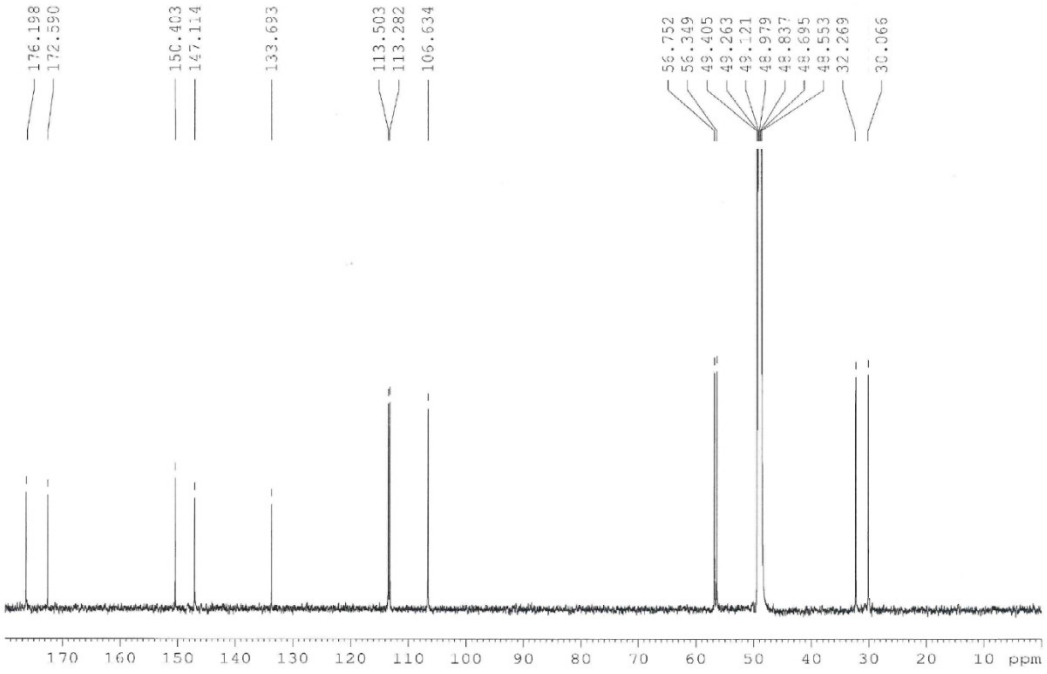
^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 6.77 (2H, s), 6.20 (1H, s), 3.73 (6H, s), 2.65 – 2.62 (4H, m) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 176.4, 172.8, 162.4 (2C), 142.6, 99.3 (2C), 97.2, 55.7 (2C), 32.2, 30.1 ppm.



4-((3,4-Dimethoxyphenyl)amino)-4-oxobutanoic acid (5i).

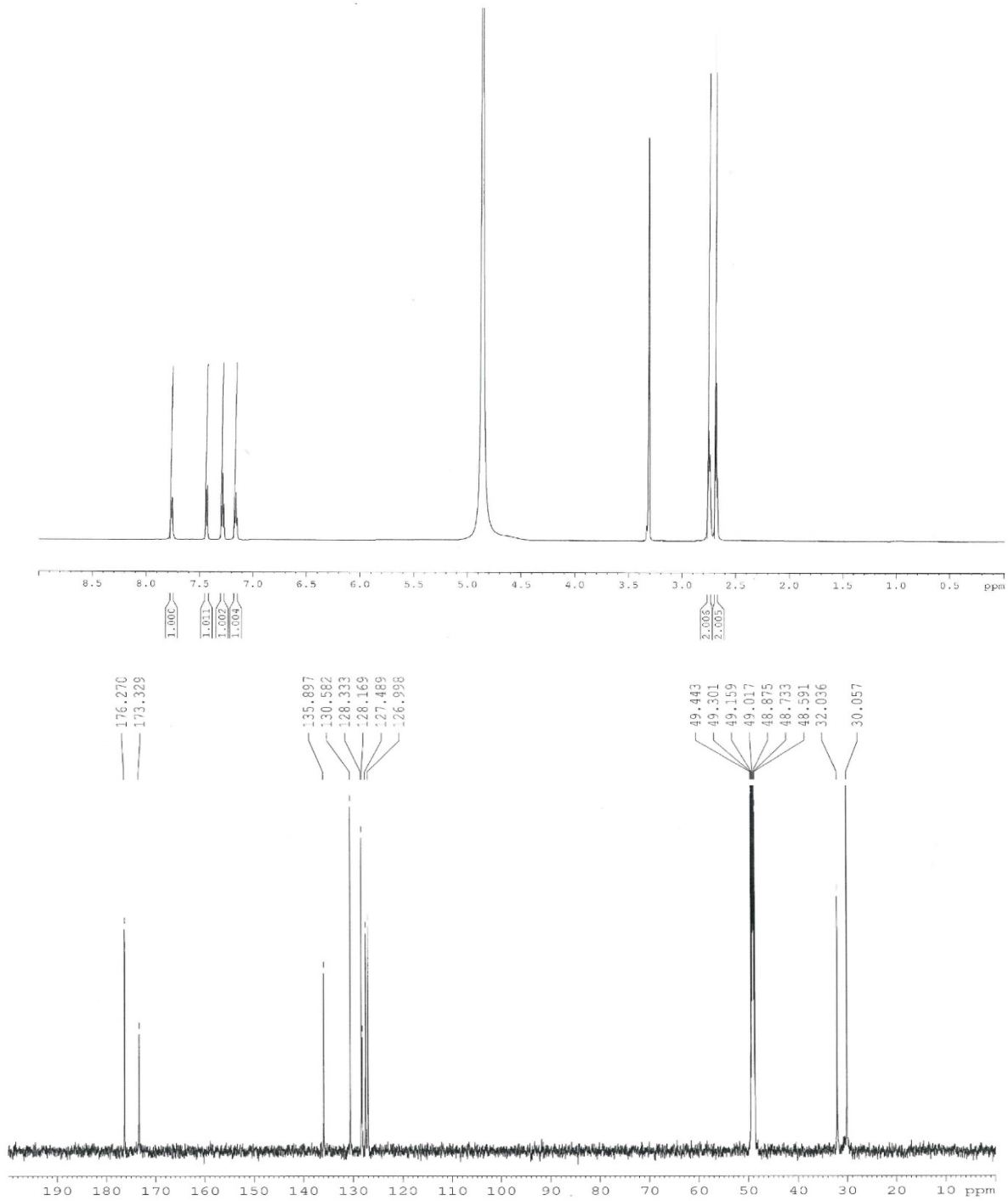
^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.30 (1H, d, *J* = 2.2 Hz), 7.00 (1H, dd, *J* = 8.4 *J* = 2.2 Hz), 6.88 (1H, d, *J* = 8.4 Hz), 3.82 (3H, s), 3.80 (3H, s), 2.66 – 2.64 (4H, m) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 176.2, 172.6, 150.4, 147.1, 133.7, 113.5, 113.3, 106.6, 56.8, 56.4, 32.3, 30.1 ppm.





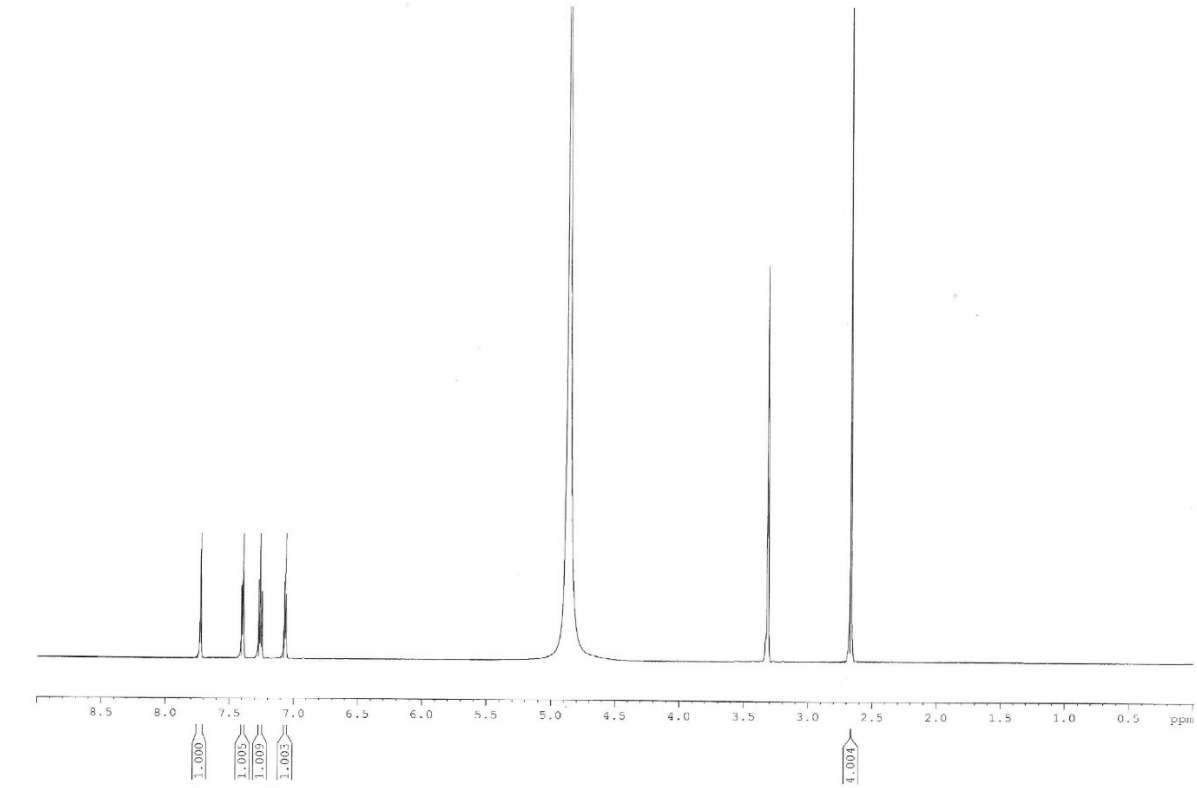
4-((2-Chlorophenyl)amino)-4-oxobutanoic acid (5j).

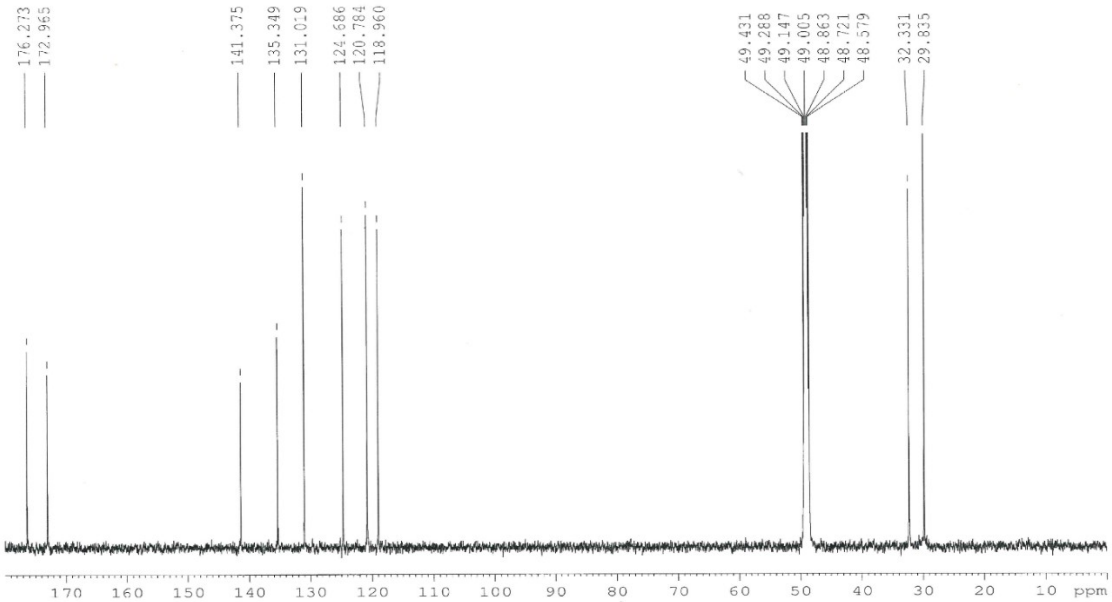
^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.76 (1H, d, *J* = 7.7 Hz), 7.43 (1H, d, *J* = 7.7 Hz), 7.28 (1H, t, *J* = 7.7 Hz), 7.16 (1H, t, *J* = 7.7 Hz), 2.76 – 2.74 (2H, m), 2.69 – 2.67 (2H, m) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 176.3, 173.3, 135.9, 130.6, 128.3, 128.2, 127.5, 127.0, 32.0, 30.1 ppm.



4-((3-Chlorophenyl)amino)-4-oxobutanoic acid (5k).

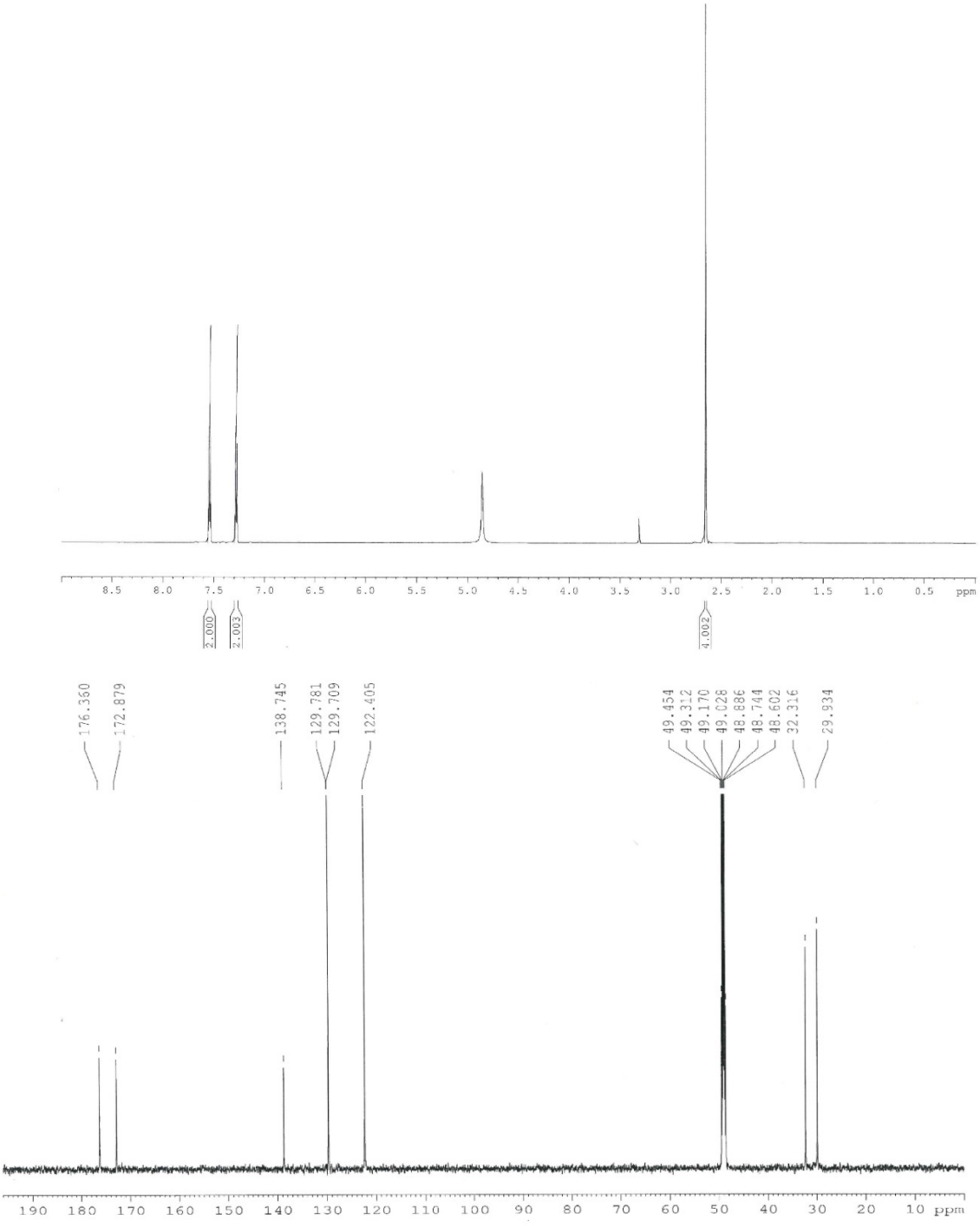
^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.72 (1H, s), 7.39 (1H, d, *J* = 8.4 Hz), 7.26 (1H, t, *J* = 8.4 Hz), 7.06 (1H, d, *J* = 8.4 Hz), 2.68 – 2.65 (4H, m) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 176.3, 173.0, 141.4, 135.3, 131.0, 124.7, 120.8, 119.0, 32.3, 29.8 ppm. MS (ESI): *m/z* = 250.3 [M+Na].





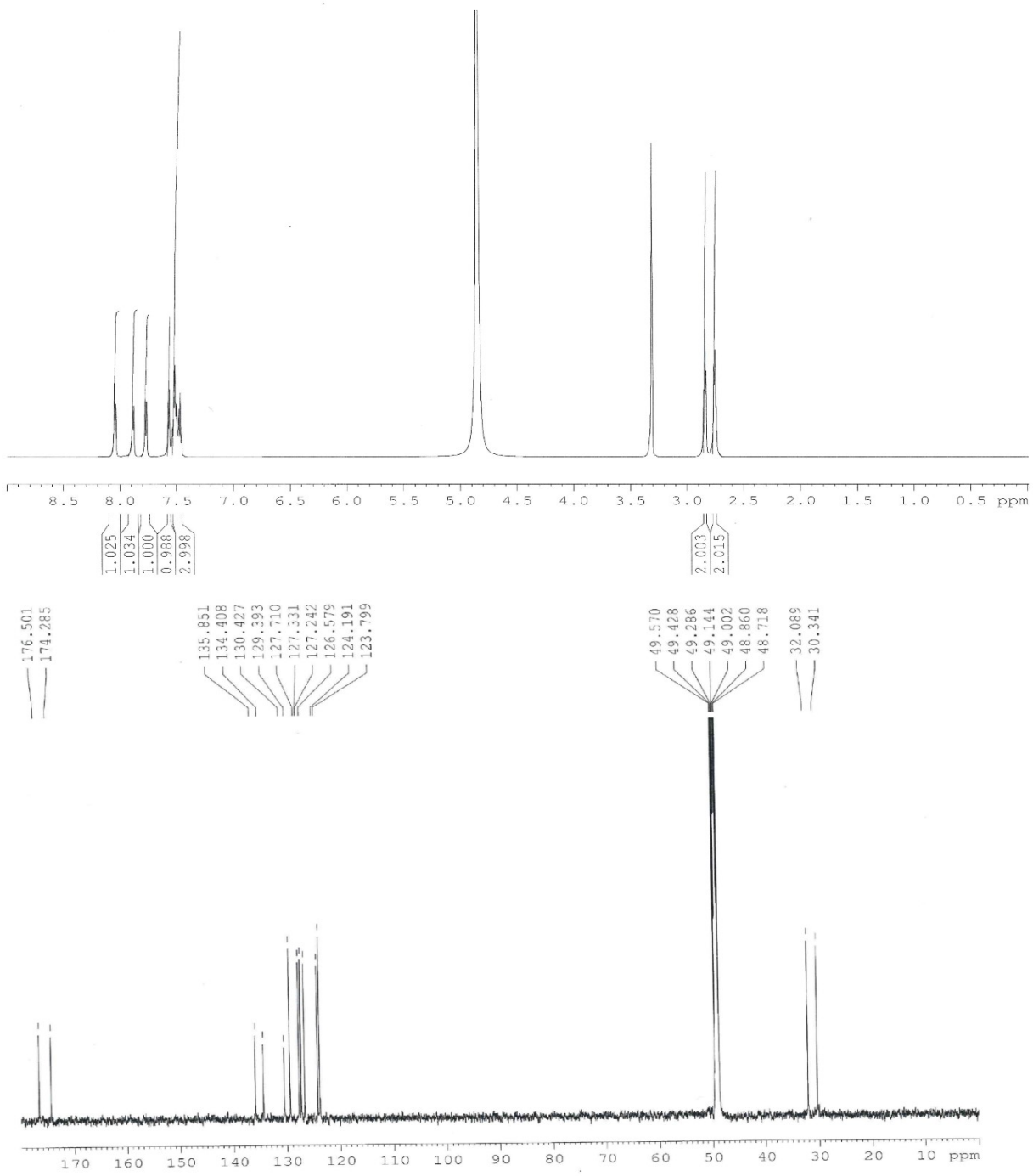
4-((4-Chlorophenyl)amino)-4-oxobutanoic acid (5l).

^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.54 (2H, d, *J* = 8.6 Hz), 7.28 (2H, d, *J* = 8.6 Hz), 2.66 – 2.64 (4H, m) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 176.4, 172.9, 138.7, 129.8, 129.7 (2C), 122.4 (2C), 32.3, 29.9 ppm.



4-(Naphthalen-1-ylamino)-4-oxobutanoic acid (5p).

^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 8.04 (1H, d, *J* = 8.4 Hz), 7.88 (1H, d, *J* = 7.4 Hz), 7.77 (1H, d, *J* = 8.4 Hz), 7.56 (1H, d, *J* = 7.4 Hz), 7.53 – 7.45 (3H, m), 2.84 (2H, t, *J* = 6.7 Hz), 2.75 (2H, t, *J* = 6.7 Hz) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 176.5, 174.3, 135.8, 134.4, 130.4, 129.4, 127.7, 127.3, 127.2, 126.6, 124.2, 123.8, 32.1, 30.3 ppm.



4-Oxo-4-((4-(1,2,2-triphenylvinyl)phenyl)amino)butanoic acid (5q).

^{1}H-NMR (600 MHz, CD_3OD-d_4): δ = 7.26 (2H, d, J = 8.4 Hz), 7.09 – 7.04 (9H, m), 7.00 – 6.95 (6H, m), 2.62 – 2.60 (4H, m) ppm. ^{13}C-NMR (150 MHz, CD_3OD-d_4): δ = 176.3, 172.7, 145.2, 145.1, 145.0, 142.2, 141.9, 140.7, 138.3, 132.8 (2C), 132.3 (4C), 129.2, 128.8 (2C), 128.7 (2C), 128.6 (2C), 127.7, 127.5 (2C), 127.4, 120.1 (2C), 32.4, 30.0 ppm.