Electronic Supplementary Material (ESI) for RSC Medicinal Chemistry.

Synthesis and antibacterial activity of 2-benzylidene-3-oxobutanamide derivatives against resistant pathogens

Ankur Sood,[a] and Venkitasamy Kesavan * [a]

[a] Department of Biotechnology, Indian Institute of Technology, Madras, Chennai-60036, India.

Supplementary Information:

General Consideration:

All the reactions were carried out under air atmosphere in round-bottom flasks. All reagents and solvents used for reactions were commercial grade and solvent were distilled prior to use. TLC was performed on pre-coated Merck silica gel aluminium plates visualised by irradiation with UV light and alkaline $KMNO_4$ were used as TLC staining solution. 1H-NMR and 13C-NMR were recorded on a Bruker AV 500 MHz using DMSO-d6 as solvent and multiplicity indicated as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublet). Coupling constants J are reported in Hz. Chemical shift is represented in δ. High resolution mass spectra were obtained by ESI using Waters/Micro mass QTOF mass spectrometer.

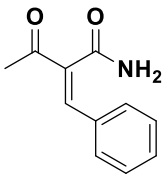
Synthesis and Characterization Data of Analogs

General Procedure for the Synthesis of Compounds 15-44.

A mixture of benzaldehyde or substituted benzaldehyde (25 mmol, 1.25 equivalents), acetoacetamide (20 mmol, 1.0 equivalent) & L-proline (4 mmol, 0.2 equivalents) in EtOH (10 ml) was stirred at room temperature for 4-5 hours (monitored by TLC in Hexane/EtOAc 50%).

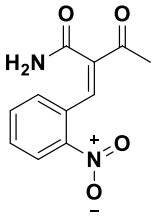
After completion of the reaction, water was added slowly to the mixture to precipitate the product. The solid was filtered out, washed with a modest quantity of petroleum ether/EtOAc (5:1) and dried under vacuum anhydrous $CaCl_2$ to afford the pure product.

***(Z)*-2-Benzylidene-3-oxobutanamide (15)**



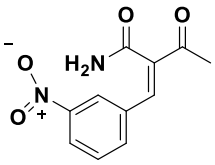
Off-white solid (92%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.89 (s, 1H), 7.76-7.74 (m, 2H), 7.60 (s ,1H), 7.55 (s,1H), 7.49-7.48 (m, 3H) 2.42 (s, 3H). ^{13}C NMR (DMSO-D6, 126 MHz): δ = 196.59, 169.62, 138.54, 138.43, 133.92, 130.73, 130.3, 129.46, 129.36, 129.20, 26.78. HRMS (T0F) m/z calcd for $C_{11}H_{11}NO_2$ $[M+H]^+$ 190.0790. found 190.0757.

***(Z)*-2-(2-Nitrobenzylidene)-3-oxobutanamide (16)**



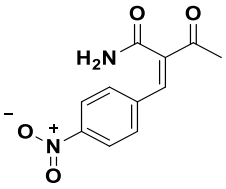
Off-white solid (90%). 1H NMR (DMSO-d6, 500 MHz): δ = 8.20 (d, J= 8.0 Hz, 1H), 7.90 (s, 1H), 7.80 (t, J=7.5 Hz, 1H), 7.72-7.64 (m, 3H), 7.42 (s,1H), 2.38 (s, 3H). ^{13}C NMR (DMSO-d6, 126 MHz): δ = 196.24, 167.83, 147.64, 140.35, 136.24, 134.53, 130.82, 130.77,130.55, 125.29, 27.20. HRMS (ESI) m/z calcd for $C_{11}H_{10}N_2O_4$ $[M+Na]^+$ 257.0533. found 257.0569.

***(Z)*-2-(3-Nitrobenzylidene)-3-oxobutanamide (17)**



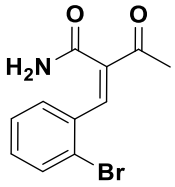
Off-white solid (90%). 1H NMR (DMSO-d6, 500 MHz): δ = 8.59 (t, J= 1.9 Hz, 1H), 8.26 (d,d, J= 7.4 Hz, J=7.5 Hz, 1H), 8.09 (d, J=7.8 Hz, 1H), 7.90 (s, 1H), 7.76-7.68 (m, 3H), 2.41 (s, 3H). ^{13}C NMR (DMSO-d6, 126 MHz): δ = 196.56, 168.82, 148.47, 140.61, 136.28, 136.02, 135.87, 130.79, 124.92, 124.32, 26.87. HRMS (ESI) m/z calcd for $C_{11}H_{10}N_2O_4$ $[M+Na]^+$ 257.0533. found 257.0571.

***(Z)*-2-(4-Nitrobenzylidene)-3-oxobutanamide (18)**



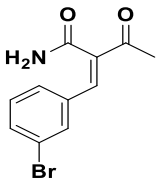
Yellow solid (90%). 1H NMR (DMSO-d6, 500 MHz): δ = 8.29 (d, J= 8.9 Hz, 2H), 7.91 (d, J=8.7 Hz, 3H), 7.66 (s, 1H), 7.64 (s, 1H), 2.09 (s, 3H). ^{13}C NMR (DMSO-d6, 126 MHz): δ = 207.01, 168.67, 148.20, 141.32, 140.75, 135.79, 131.06, 124.25, 31.17, 26.96. HRMS (ESI) m/z calcd for $C_{11}H_{10}N_2O_4$ $[M+H]^+$ 235.0719. found 235.0722.

***(Z)*-2-(2-Bromobenzylidene)-3-oxobutanamide (19)**



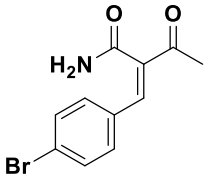
White solid (85%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.86 (s, 1H), 7.76-7.73 (m, 2H), 7.58 (s, 2H), 7.43 (t, J=7.5 Hz, 1H), 7.36 (d,d, J=7.7 Hz, J=7.8 Hz, 1H), 2.38 (s, 3H). ^{13}C NMR (DMSO-d6, 126 MHz): δ = 195.95, 168.54, 140.24, 136.18, 133.99, 133.41, 132.05, 130.06, 128.39, 125.18, 27.10. HRMS (ESI) m/z calcd for $C_{11}H_{10}BrNO_2$ $[M+H]^+$ 267.9973. found 267.9982.

***(Z)*-2-(3-Bromobenzylidene)-3-oxobutanamide (20)**



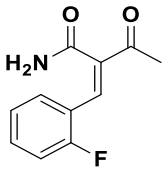
Light yellow solid (82%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.91 (t, 3.13 Hz, 1H), 7.84 (s, 1H), 7.68 (d, J=7.8 Hz, 1H) 7.63-7.61 (m, 2H), 7.48 (s, 1H), 7.40 (d,d, J=7.9 Hz, J=7.8 Hz 1H), 2.37 (s, 3H). ^{13}C NMR (DMSO-d6, 126 MHz): δ = 196.44, 169.08, 139.71, 136.59,136.49, 133.14, 132.31, 131.28, 129.24, 122.41, 26.81. HRMS (ESI) m/z calcd for $C_{11}H_{10}BrNO_2$ $[M+Na]^+$ 289.9787. found 289.9845.

***(Z)*-2-(4-Bromobenzylidene)-3-oxobutanamide (21)**



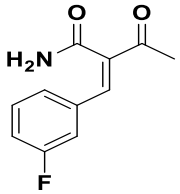
White solid (87%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.83 (s, 1H), 7.66-7.61 (m, 4H), 7.57 (s,1H), 7.48 (s, 1H), 2.35 (s, 3H). 13C NMR (DMSO-d6, 126 MHz): δ = 196.43, 169.25, 139.16, 137.02, 133.30, 132.23, 132.17, 132.16, 132.10, 124.17, 26.84. HRMS (ESI) m/z calcd for $C_{11}H_{10}BrNO2$ $[M+Na]^+$ 289.9787. found 289.9853.

***(Z)*-2-(2-Fluorobenzylidene)-3-oxobutanamide (22)**



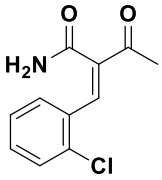
White solid (72%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.87 (s, 1H), 7.78 (d,d, J=7.8 Hz, J=7.14 Hz, 1H), 7.58 (s, 1H), 7.54 (s,1H), 7.51-7.47 (m, 1H), 7.33-7.29 (m. 1H), 7.25 (t, J=7.3, 1H), 2.37 (s, 3H). 13C NMR (DMSO-d6, 126 MHz): δ =195.97, 168.97, 160.91 (d, J=250.47 Hz), 140.10, 132.83 (d, J=8.8 Hz), 129.58 (d, 1.2 Hz), 129.10 (d, J=6.1 Hz), 125.18 (d, J=3.4 Hz), 121.93 (d, J=11.5 Hz), 116.19 (d, J=21.6 Hz), 27.00. HRMS (ESI) m/z calcd for $C_{11}H_{10}FNO_2$ $[M+Na]^+$ 230.0588. found 230.0642.

***(Z)*-2-(3-Fluorobenzylidene)-3-oxobutanamide (23)**



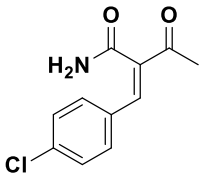
White solid (75%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.86 (s, 1H), 7.62 (s, 1H), 7.54-7.46 (m, 4H), 7.28 (t, J=9.7 Hz, 1H), 2.37 (s, 3H). 13C NMR (DMSO-d6, 126 MHz): δ = 196.39, 169.18, 162.52 (d, J=243.43 Hz), 139.61, 136.75 (d, J=2.3 Hz), 136.40 (d, J=8.0 Hz), 131.19 (d, J=8.3 Hz), 126.72 (d, J=2.46 Hz), 116.85 (d, 160.5 Hz), 116.68 (d, 161.7 Hz), 26.83. HRMS (ESI) m/z calcd for $C_{11}H_{10}FNO_2$ $[M+Na]^+$ 230.0588. found 230.0619.

***(Z)*-2-(2-Chlorobenzylidene)-3-oxobutanamide (25)**



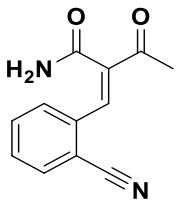
White solid (83%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.86 (s, 1H), 7.77 (d,d, J=7.7 Hz, J=1.65 Hz, 1H), 7.64 (s,1H), 7.56 (d,d, J= 9.14 Hz, J=6.68 Hz, 2H), 7.44 (t, J=7.6 Hz, 1H), 7.38 (t, 7.6 Hz, 1H), 2.38 (s, 3H). 13C NMR (DMSO-d6, 126 MHz): δ = 195.97, 168.66, 140.42, 134.70, 133.57, 132.28, 131.93, 130.19, 129.95, 127.87, 27.09. HRMS (ESI) m/z calcd for $C_{11}H_{10}ClNO2$ $[M+Na]^+$ 246.0292. found 246.0342.

***(Z)*-2-(4-Chlorobenzylidene)-3-oxobutanamide (27)**



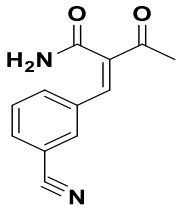
White solid (94%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.83 (s, 1H), 7.71(d, J=8.5 Hz, 2H), 7.5 (s,1H), 7.51 (d,d J=8.6 Hz, J= 6.5 Hz, 3H), 2.36 (s, 3H). 13C NMR (DMSO-d6, 126 MHz): δ = 196.38, 169.26, 139.11, 136.89, 135.28, 132.96, 132.93, 131.91, 129.40, 129.29, 26.83. HRMS (ESI) m/z calcd for $C_{11}H_{10}ClNO2$ $[M+Na]^+$ 246.0292. found 246.0345.

***(Z)*-2-(2-Cyanobenzylidene)-3-oxobutanamide (28)**



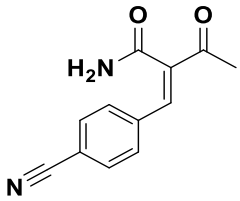
White solid (89%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.92 (d, J=8.4 Hz, 2H), 7.88 (s, 1H), 7.83 (d, J=8.3 Hz, 2H), 7.62 (s, 1H), 7.56 (s, 1H), 2.39 (s, 3H). 13C NMR (DMSO-d6, 126 MHz): δ =196.42, 168.82, 140.88, 138.79, 136.21, 133.12, 133.00, 130.62, 130.09, 118.98, 112.50, 26.93. HRMS (ESI) m/z calcd for $C_{12}H_{10}O_2N_2$ $[M+Na]^+$ 237.0634. found 237.0701.

***(Z)*-2-(3-Cyanobenzylidene)-3-oxobutanamide (29)**



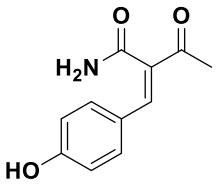
White solid (82%). 1H NMR (DMSO-d6, 500 MHz): δ = 8.08 (s, 1H), 7.98 (d, J=8.0 Hz, 1H), 7.90 (d, J=5.7 Hz, 2H), 7.67 (d, J=7.8 Hz, 2H), 7.55 (s, 1H), 2.38 (s, 3H). 13C NMR (DMSO-d6, 126 MHz): δ = 196.34, 168.94, 140.32, 135.91, 135.41, 134.48, 133.72, 133.26, 130.54, 118.83, 112.23, 26.88. HRMS (ESI) m/z calcd for $C_{12}H_{10}O_2N_2$ [M+Na] $^+$ 237.0634. found 237.0682.

***(Z)*-2-(4-Cyanobenzylidene)-3-oxobutanamide (30)**



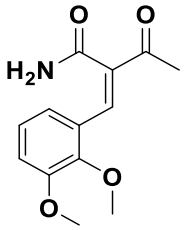
Light yellow solid (91%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.92 (d, J=8.2 Hz, 2H), 7.88 (s, 1H), 7.83 (d, J=8.5 Hz, 2H), 7.62 (s, 1H), 7.56 (s, 1H), 2.39 (s, 3H). 13C NMR (DMSO-d6, 126 MHz): δ = 196.42, 168.82, 140.88, 138.79, 136.21, 133.23, 133.00, 130.62, 130.35, 118.98, 112.50, 26.93. HRMS (ESI) m/z calcd for $C_{11}H_{10}O_2N_2$ [M+H] $^+$ 215.0742. found 215.0820.

***(Z)*-2-(4-Hydroxybenzylidene)-3-oxobutanamide (32)**



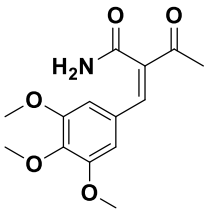
Yellow solid (78%). 1H NMR (DMSO-d6, 500 MHz): δ = 10.10 (s, 1H), 7.74 (s,1H), 7.57 (d, J=8.7 Hz, 2H), 7.46 (s, 1H), 7.37 (s, 1H), 6.80 (d, J=8.7 Hz, 2H), 2.31 (s, 3H). 13C NMR (DMSO-d6, 126 MHz): δ = 196.09, 170.17, 160.17, 138.66, 135.54, 132.70, 124.73, 124.65, 116.30, 116.12, 26.64. HRMS (ESI) m/z calcd for $C_{11}H_{11}NO_3$ [M+H] $^+$ 206.0817. found 206.0828.

***(Z)*-2-(2,3-dimethoxybenzylidene)-3-oxobutanamide (33)**



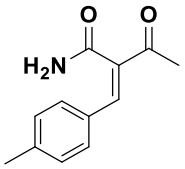
Off White solid (83%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.78 (s, 1H), 7.63 (s, 1H), 7.52 (s, 1H), 7.31 (d, J=7.6 Hz, 1H), 7.13 (d, J= 7.6 Hz, 1H), 7.08 (t, J=8.6, 1H), 3.82 (s, 3H), 3.70 (s, 3H), 2.35 (s, 3H). 13C NMR (DMSO-d6, 126 MHz): δ = 196.12, 169.48, 152.87, 148.45, 139.06, 132.24, 127.85, 124.50, 120.55, 115.27, 61.43, 56.27, 26.96. HRMS (ESI) m/z calcd for $C_{13}H_{15}NO_4$ [M+Na $^+$ 272.0893. found 272.0949.

***(Z)*-2-(3,4,5-dimethoxybenzylidene)-3-oxobutanamide (34)**



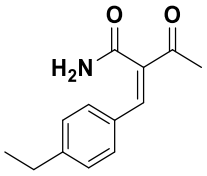
Light yellow solid (83%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.77 (s, 1H), 7.61 (s, 1H), 7.45 (s, 1H), 7.12 (s, 2H), 3,76 (s, 6H), 3.70 (s, 3H), 2.35 (s, 3H). 13C NMR (DMSO-d6, 126 MHz): δ = 196.29, 192.39, 169.88, 153.80, 153.17, 139.69, 138.57, 137.91, 129.32, 108.09, 107.17, 60.61, 56.30, 26.73. HRMS (ESI) m/z calcd for $C_{14}H_{17}NO_5$ [M+Na] $^+$ 302.0999. found 302.1058.

***(Z)*-2-(4-Methylbenzylidene)-3-oxobutanamide (35)**



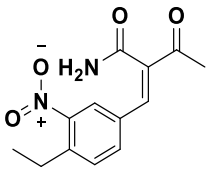
White solid (83%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.77 (s, 1H), 7.60 (d, J=8.1 Hz, 2H), 7.51 (s, 1H), 7.45 (s, 1H), 7.24 (d, J=8.0 Hz, 2H), 2.35 (s, 3H) 2.32 (s, 3H). 13C NMR (DMSO-d6, 126 MHz): δ = 196.35, 169.73, 140.77, 138.33, 137.79, 131.16, 130.98, 130.42, 130.26, 129.82, 26.76, 21.48. HRMS (ESI) m/z calcd for $C_{12}H_{13}NO_2$ [M+H] $^+$ 204.1025 found 204.1001.

***(Z)*-2-(4-Ethylbenzylidene)-3-oxobutanamide (36)**



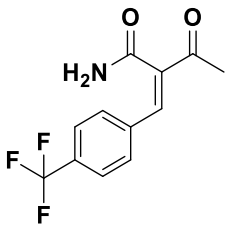
Yellow solid (92%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.77 (s, 1H), 7.62 (d, J=8.2 Hz, 2H), 7.51 (s, 1H), 7.45 (s, 1H), 7.27 (d, J=8.2 Hz, 2H), 2.62 (q, J=7.5 Hz, 2H), 2.35 (s, 3H) 1.17(t, J= 7.5 Hz, 3H). 13C NMR (DMSO-d6, 126 MHz): δ = 196.35, 169.72, 146.94, 138.31, 137.83, 131.42, 130.68, 130.52, 130.30, 128.64, 28.54, 26.77, 15.77. HRMS (ESI) m/z calcd for $C_{13}H_{15}NO_2$ $[M+Na]^+$ 240.0955 found 240.1051.

***(Z)*-2-(4-Ethyl-3-nitrobenzylidene)-3-oxobutanamide (37)**



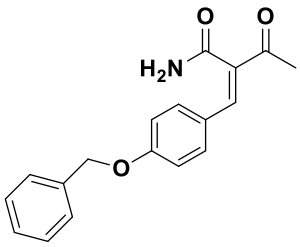
Yellow solid (78%). 1H NMR (DMSO-d6, 500 MHz): δ = 8.24 (d, J=1.07 Hz, 1H), 7.92 (d,d, J=8.1 Hz, J=1.7 Hz, 1H), 7.87 (s, 1H), 7.66 (s, 1H), 7.61 (d, J=8.13 Hz, 1H), 7.59 (s, 1H), 2.83 (m, 2H), 2.38 (s, 3H), 1.21 (t, J=7.4 Hz, 3H). 13C NMR (DMSO-d6, 126 MHz): δ = 196.41, 191.85, 168.95, 149.55, 140.05, 135.80, 134.26, 133.42, 132.30, 132.30,125.48, 26.83,25.63, 15.14. HRMS (ESI) m/z calcd for $C_{13}H_{14}N_2O_4$ $[M+Na]^+$ 285.0908 found 285.0846.

***(Z)*-2-(4-(trifluoromethyl) benzylidene)-3-oxobutanamide (38)**



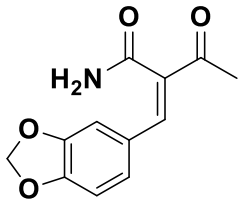
Yellow solid (83%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.87 (d, J=8.0 Hz, 3H), 7.81 (d, J=8.5 Hz, 2H), 7.60 (s, 2H), 2.39 (s, 3H). 13C NMR (DMSO-d6, 126 MHz): δ = 196.47, 168.90, 140.60, 138.25, 136.49, 130.62, 130.15 (d, J=31.92 Hz), 126.01, 125.96 (d, J=11.11 Hz), 125.53, 123.37, 26.90. HRMS (ESI) m/z calcd for $C_{12}H_{10}NO_2F_3$ $[M+Na]^+$ 280.0556 found 280.0617.

***(Z)*-2-(4-benzyloxy benzylidene)-3-oxobutanamide (39)**



Off white solid (93%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.77 (s, 1H), 7.67 (d, J=8.8 Hz, 2H), 7.50 (s, 1H), 7.44 (t, J=8.2 Hz, 3H), 7.39 (t, J=7.3, 2H), 7.34-7.32 (d,d, J=7.2 Hz, J=5.0 Hz, 1H), 7.07 (d, J=8.8 Hz, 2H), 5.16 (s, 2H), 2.33 (s, 3H) . 13C NMR (DMSO-d6, 126 MHz): δ = 196.17, 191.79, 169.96, 160.48, 138.13, 137.11, 136.53, 132.38, 132.28, 128.95, 128.45, 126.52, 115.77, 115.54, 70.13, 69.84, 26.70. HRMS (ESI) m/z calcd for $C_{18}H_{17}NO_3$ $[M+Na]^+$ 318.1101 found 318.1171.

***(Z)*-2-(benzo- [1,3-dioxol-5ylmethylene-3-oxobutanamide (40)**



Light Yellow solid (73%). 1H NMR (DMSO-d6, 500 MHz): δ = 7.80 (s, 1H), 7.56 (s, 1H), 7.41 (s, 1H), 7.29 (d, J=1.7 Hz, 1H)), 7.24 (d,d, J=9.8 Hz, J=6.6 Hz, 1H), 7.00 (d, J=8.1 Hz, 1H), 6.08 (s, 2H), 2.32 (s, 3H). 13C NMR (DMSO-d6, 126 MHz): δ = 196.18, 169.90, 149.61, 148.21, 138.22, 136.75, 127.98, 127.10, 108.23, 102.19, 26.70. HRMS (ESI) m/z calcd for $C_{11}H_{12}NO_4$ $[M+Na]^+$ 256.0636 found 256.0638.

Biological Procedure and Analysis of Analogs

Primary Antimicrobial Screening

Primary antimicrobial screening study by whole cell growth inhibition assays, using the provided samples at a single concentration, in duplicate (n=2). The inhibition of growth is measured against 5 bacteria: *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Staphylococcus aureus, and 2 fungi: Candida albicans and Cryptococcus neoformans*.

Assay Parameters

Test concentration: 32 µg/mL in ≤1% DMSO.

QC: Duplicate (n=2)

Control MIC: Pass

Plates: Non-Binding Surface, 384 well plate

Media: Bacteria- Cation-adjusted Mueller Hinton broth. Fungi-Yeast Nitrogen Base

Read Out: Bacteria- OD600

C. albicans- OD530

C. neoformans- Resazurin OD600-57

Methods

Sample preparation

Samples were prepared in DMSO and water to a final testing concentration of 32 µg/mL in 384-well, non-binding surface plate (NBS) for each bacterial/fungal strain, and in duplicate (n=2), and keeping the final DMSO concentration to a maximum of 1% DMSO.

Antimicrobial Assay

Procedure

All bacteria were cultured in Cation-adjusted Mueller Hinton broth (CAMHB) at 37 °C overnight. A sample of each culture was then diluted 40-fold in fresh broth and incubated at 37 °C for 1.5-3 h. The resultant mid-log phase cultures were diluted (CFU/mL measured by OD600), then added to each well of the compound containing plates, giving a cell density of 5×10^5 CFU/mL and a total volume of 50 µL. All the plates were covered and incubated at 37 °C for 18 h without shaking.

Antifungal Assay

Procedure

Fungi strains were cultured for 3 days on Yeast Extract-Peptone Dextrose (YPD) agar at 30 °C. A yeast suspension of 1 x 10^6 to 5 x 10^6 CFU/mL (as determined by OD530) was prepared from five colonies. The suspension was subsequently diluted and added to each well of the compound-containing plates giving a final cell density of fungi suspension of 2.5×10^3 CFU/mL and a total volume of 50 µL. All plates were covered and incubated at 35 °C for 24 h without shaking.

Analysis

Inhibition of bacterial growth was determined measuring absorbance at 600 nm (OD600), using a Tecan M1000 Pro monochromator plate reader. The percentage of Growth inhibition is calculated for each well, using the negative control (media only) and positive control (bacteria without inhibitors) on the same plate as reference.

Growth inhibition of *C. albicans* was determined measuring absorbance at 530 nm (OD530), while the growth inhibition of *C. neoformans* was determined measuring the difference in absorbance between 600 and 570 nm (OD600-570), after the addition of resazurin (0.001% final concentration) and incubation at 35 °C for additional 2 h. The absorbance was measured using a HTX plate reader. The percentage of growth inhibition was calculated for

each well, using the negative control (media only) and positive control (fungi without inhibitors) on the same plate for additional 2 h.

The significance of the inhibition values was determined by modified Z-scores, calculated using the median and MAD of the samples (no controls) on the same plate. Samples with inhibition value above 80% and Z-Score above 2.5 for either replicate (n=2 on different plates) were classed as actives. Samples with inhibition values between 50 - 80% and Z-Score above 2.5 for either replicate (n=2 on different plates) were classed as partial actives. Samples with inhibition values between 50 - 80% and Z-Score above 2.5 for either replicate (n=2 on different plates) were classed as partial actives.

Antibiotic standards preparation and Quality control

Colistin and Vancomycin were used as positive bacterial inhibitor standards for Gram negative and Gram-positive bacteria, respectively. Fluconazole was used as a positive fungal inhibitor standard for *C. albicans* and *C. neoformans*. The antibiotics were provided in 4 concentrations, with 2 above and 2 below its MIC value and plated into the first 8 wells of the 384-well NBS plates. The quality control (QC) of the assays was determined by the antimicrobial controls and the Z'-factor (using positive and negative controls). Each plate was deemed to fulfil the quality criteria (pass QC), if the Z'-factor was above 0.4, and the antimicrobial standards showed full range of activity, with full growth inhibition at their highest concentration, and no growth inhibition at their lowest concentration.

Microbial Strains

Organism	**Strain**	**Description**
Escherichia coli	ATCC 25922	FDA control strain
Klebsiella pneumoniae	ATCC 700603	MDR
Acinetobacter baumannii	ATCC 19606	Type strain
Pseudomonas aeruginosa	ATCC 27853	Quality control strain
Staphylococcus aureus	ATCC 43300	MRSA
Candida albicans	ATCC 90028	CLSI reference
Cryptococcus neoformans	ATCC 208821	H99 - Type strain

Antibiotic standards

MIC determined by BMD method, CA-MHB, Corning 3640 384 NBS plates	*E. coli*	*Klebsiella pneumoni-ae*	*Acinetobacter baumannii*	*Pseudomonas aeruginosa*	*Staphylo-coccs aureus*
Compound	**MIC (µg/mL)**				
Colistin - sulfate	**0.125**	**0.25**	**0.25**	**0.25**	
Vancomycin - HCl					**1**

Hit- Confirmation

Antimicrobial screening, Cytotoxicity and Haemolysis

Study

Hit Confirmation of active compounds by whole cell growth inhibition assays was conducted as an 8-point dose response to determine the Minimum Inhibitory Concentration (MIC), in duplicate (n=2). The inhibition of growth is measured against those microorganisms that showed susceptibility to the compounds tested in the Primary Screen.

Included in the Hit Confirmation were 5 bacteria: *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Staphylococcus aureus*, and 2 fungi *Candida albicans* and *Cryptococcus neoformans*.

In addition to determining MIC, active compounds were counter screened for cytotoxicity against a human embryonic kidney cell line, HEK293, by determining their CC50 value. The compounds were also screened for haemolysis of human red blood cells.

Assay Parameters

Assay Parameters	**Bacteria**	**Fungi**	**HEK 293**	**Haemolysis**
Test concentration	32 - 0.25 µg/mL ≤0.5% DMSO	32 - 0.25 µg/mL ≤0.5% DMSO	32 - 0.25 µg/mL ≤0.5% DMSO	32 - 0.25 µg/mL ≤0.5% DMSO
QC	Duplicate (n=2) Control MIC: Pass	Duplicate (n=2) Control MIC: Pass	Duplicate (n=2) Control CC50: Pass	Duplicate (n=2) Control HC10: Pass
Plates	Non-Binding Surface (NBS), 384-well plate	Non-Binding Surface (NBS), 384-well plate	TC, 384-well black wall/clear bottom	Polypropylene 384-well and polystyrene 384-well plates

Media	Cation-adjusted Mueller Hinton broth	Yeast Nitrogen Base	DMEM supplemented with 10% FBS	0.9% NaCl
Read out	OD600	OD630 Resazurin OD600-570	Resazurin F560/590	OD405

Sample Preparation

Samples were prepared in DMSO and water to a final testing concentration of 32 μg/mL or 20 μM (unless otherwise indicated in the data sheet) and serially diluted 1:2 fold for 8 times. Each sample concentration was prepared in 384-well plates, non-binding surface plate (NBS; Corning 3640) for each bacterial/fungal strain, tissue-culture treated (TC-treated; Corning 3712/3764) black for mammalian cell types and polypropylene 384-well (PP; Corning 3657) for haemolysis assays, all in duplicate (n=2), and keeping the final DMSO concentration to a maximum of 0.5%.

Antibacterial Assay

Procedure

All bacteria were cultured in Cation-adjusted Mueller Hinton broth (CAMHB) at 37 °C overnight. A sample of each culture was then diluted 40-fold in fresh broth and incubated at 37 °C for 1.5-3 h. The resultant mid-log phase cultures were diluted (CFU/mL measured by OD600), then added to each well of the compound containing plates, giving a cell density of $5x10^5$ CFU/mL and a total volume of 50 μL. All the plates were covered and incubated at 37 °C for 18 h without shaking.

Analysis

Inhibition of bacterial growth was determined measuring absorbance at 600 nm (OD600), using a Tecan M1000 Pro monochromator plate reader. The percentage of growth inhibition was calculated for each well, using the negative control (media only) and positive control (bacteria without inhibitors) on the same plate as references. The percentage of growth inhibition was calculated for each well, using the negative control (media only) and positive control (bacteria without inhibitors) on the same plate. The MIC was determined as the lowest concentration at which the growth was fully inhibited, defined by an inhibition ≥ 80%.

Cytotoxicity Assay

Procedure

HEK293 cells were counted manually in a Neubauer haemocytometer and then plated in the 384-well plates containing the compounds to give a density of 5000 cells/well in a final

volume of 50 μL. DMEM supplemented with 10% FBS was used as growth media and the cells were incubated together with the compounds for 20 h at 37 °C in 5% CO_2.

Analysis

Cytotoxicity (or cell viability) was measured by fluorescence, ex: 560/10 nm, em: 590/10 nm (F560/590), after addition of 5 μL of 25 μg/mL resazurin (2.3 μg/mL final concentration) and after incubation for further 3 h at 37 ° C in 5% CO2. The fluorescence intensity was measured using a Tecan M1000 Pro monochromator plate reader, using automatic gain calculation.

CC50 (concentration at 50% cytotoxicity) were calculated by curve fitting the inhibition values *vs.* log(concentration) using a sigmoidal dose-response function, with variable fitting values for bottom, top and slope. In addition, the maximal percentage of cytotoxicity is reported as DMax, indicating any compounds with partial cytotoxicity. The curve fitting was implemented using Pipeline Pilot's dose-response component, resulting in similar values to curve fitting tools such as GraphPad's Prism and IDBS's XlFit. Cytotoxic samples were classified by CC50 ≤ 32 μg/mL in replicate (n=2 on different plates).

Haemolysis Assay

Procedure

Human whole blood was washed three times with 3 volumes of 0.9% NaCl and then resuspended in same to a concentration of 0.5 x 10^8 cells/mL, as determined by manual cell count in a Neubauer haemocytometer. The washed cells were then added to the 384-well compound-containing plates for a final volume of 50 μL. After a 10 min shake on a plate shaker the plates were then incubated for 1 h at 37 °C. After incubation, the plates were centrifuged at 1000*g* for 10 min to pellet cells and debris, 25 μL of the supernatant was then transferred to a polystyrene 384-well assay plate.

Analysis

Haemolysis was determined by measuring the supernatant absorbance at 405 mm (OD405). The absorbance was measured using a Tecan M1000 Pro monochromator plate reader. HC10 and HC50 (concentration at 10% and 50% haemolysis, respectively) were calculated by curve fitting the inhibition values *vs*. log(concentration) using a sigmoidal dose-response function with variable fitting values for top, bottom, and slope. The curve fitting was implemented using Pipeline Pilot's dose-response component, resulting in similar values to curve fitting tools such as GraphPad's Prism and IDBS's XlFit. Haemolysis samples were classified by HC10 ≤ 32 μg/mL in replicate (n=2 on different plates).

Antibiotic, Cytotoxic and Haemolytic Standards Preparation and Quality Control

Colistin and Vancomycin were used as positive bacterial inhibitor standards for Gram-negative and Gram-positive bacteria, respectively. Fluconazole was used as a positive fungal inhibitor standard for *C. albicans and C. neoformans.* Tamoxifen was used as a positive

cytotoxicity standard. Melittin was used as a positive haemolytic standard. Each antibiotic standard was provided in 4 concentrations, with 2 above and 2 below its MIC or CC50 value and plated into the first 8 wells of column 23 of the 384-well NBS plates.

Tamoxifen and melittin was used in 8 concentrations in 2-fold serial dilutions with 50 μg/mL highest concentration. The quality control (QC) of the assays was determined by Z'-Factor, calculated from the Negative (media only) and Positive Controls (bacterial, fungal or cell culture without inhibitor), and the Standards.

Control

Antimicrobial susceptibility of tested strains

Values are the average of ≥ 6 independent biological replicates. All values are within the expected range as per CLSI guidelines.

Antibiotic standards

MIC determined by BMD method, CA-MHB, Corning 3640 384 NBS plates	***Escherichia coli*** FDA Control ATCC25922	***Klebsiella pneumophila*** ESBL ATCC 700603	***Acinetobacter baumannii*** Type strain ATCC 19606	***Pseudomonas aeruginosa*** QC strain ATCC 27853
Compound	MIC (μg/ml)			
Colistin- sulfate	0.125	0.25	0.25	0.25

MIC determined by BMD method, CA-MHB, Corning 3640 384 NBS plates	***Staphylococcus aureus*** MRSA ATCC 43300
Compound	MIC (μg/ml)
Vancomycin- HCl	1

Time-Kill Kinetics Test – Antimicrobial Efficacy Testing

The Time-kill kinetics assay is used to study the activity of an antimicrobial agent against a bacterial strain and can determine the bactericidal or bacteriostatic activity of an agent over time.

Procedure

Preparation of inoculum:

The bacterial suspensions for inoculation are prepared from fresh overnight cultures on Muller Hinton Broth (MHB). The isolated colonies are suspended in sterile saline and the culture turbidity is adjusted to 0.5 McFarland's standard. The suspension is diluted further to get required inoculum (i.e., 5 X 10^7 CFU/mL).

Assay specifications:

- Medium: Muller Hinton II broth cation adjusted (BD BBL 212322)
- Testing organism: Gram-positive, Gram-negative strains
- Size of inoculum: 5 X 10^8 CFU/mL
- Temperature: 37 °C
- End point: CFU reduction
- Reference standards: Vancomycin HCl for Staphylococcus aureus MRSA and Colistin Sulphate for Acinetobacter baumannii

Preparation and concentration of test and reference compounds:

The test compound is dissolved using 100% DMSO for stock preparation and subsequent dilutions are made with the recommended medium as per the guideline.

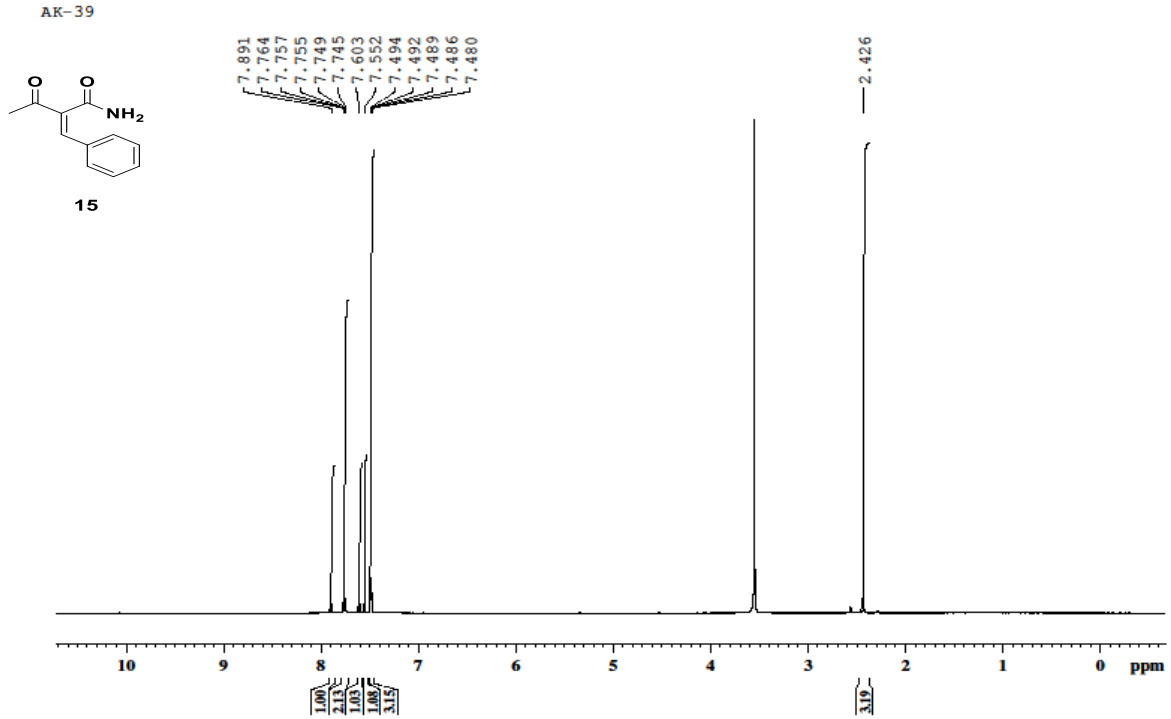
The minimum inhibitory concentration of the test compound (or) antibiotic against the organism to be tested is determined. The concentrations of the antibiotic to be tested for kill kinetics are based upon the MIC of the antibiotic.

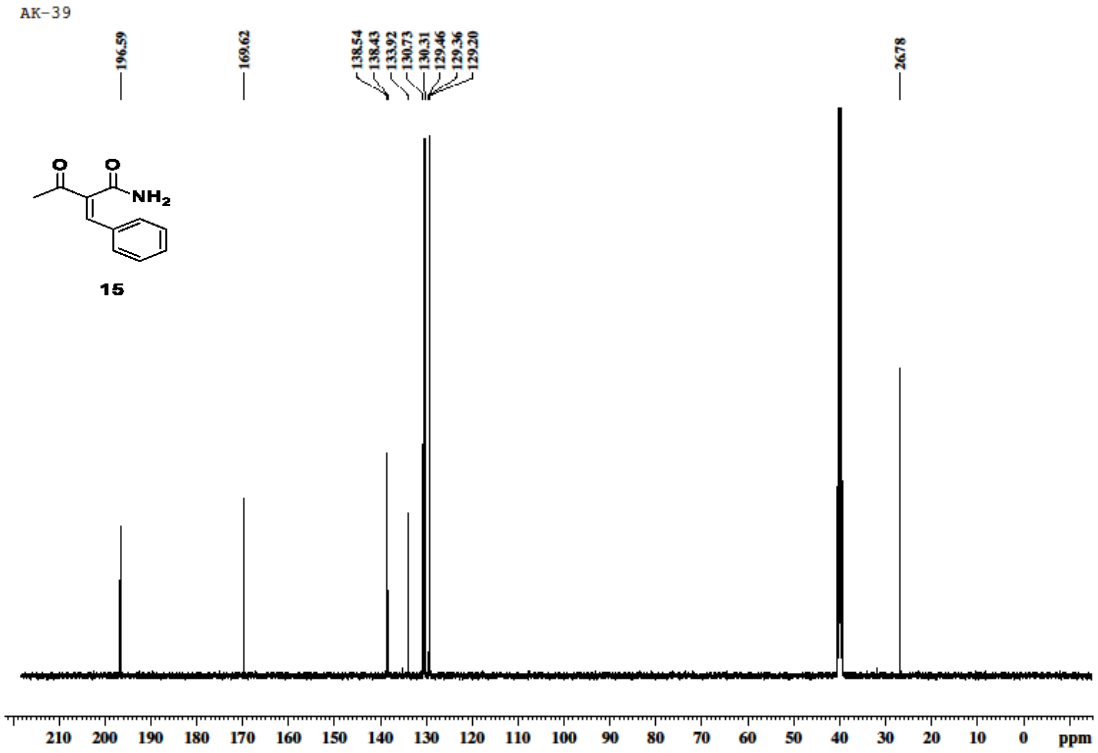
Procedure

An overnight culture of cells *Staphylococcus aureus MRSA* (ATCC 700698) and *Acinetobacter baumannii Type strain* (ATCC 19606) was diluted 1:10,000 in MHB and incubated at 37 °C with aeration at 220 r.p.m. for 2 h (early exponential).

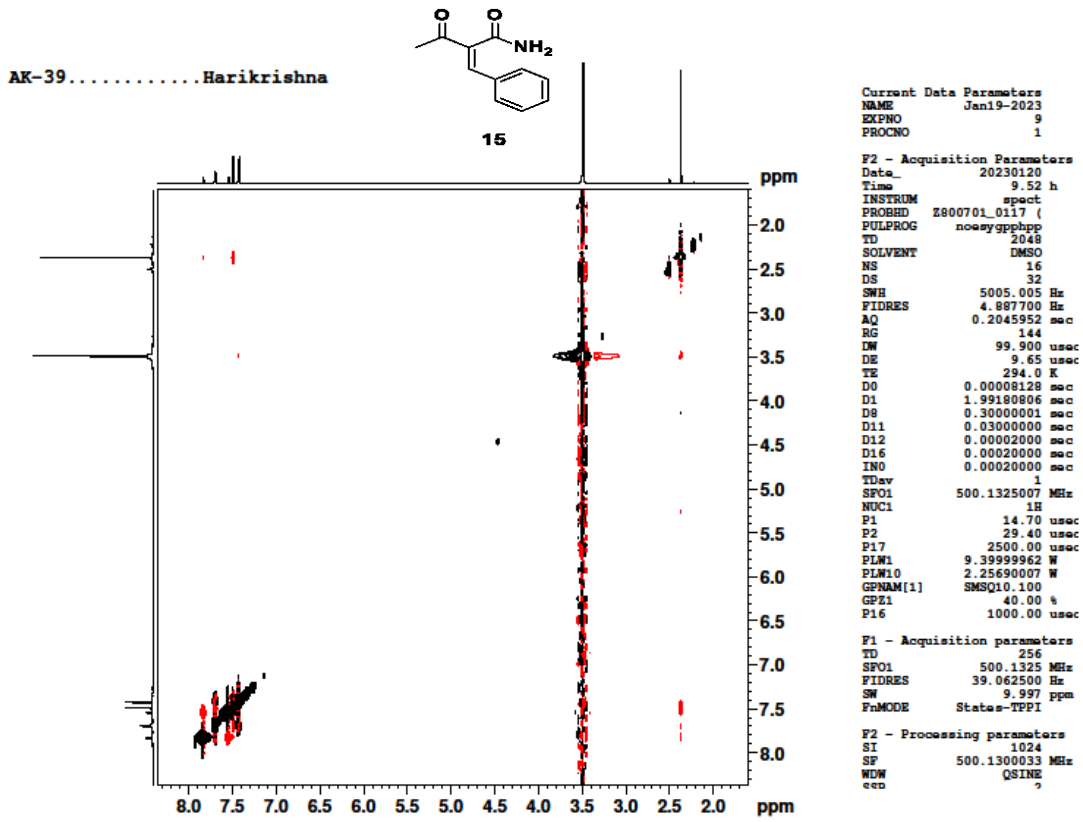
Bacteria were then challenged with active compounds at 2.5 × MIC, 5 × MIC, 10 × MIC (a desirable concentration at the site of infection) comparing with antibiotics clinically used in culture tubes at 37 °C and 220 r.p.m. At intervals, 100 µl aliquots were removed, tenfold serially diluted suspensions were plated on MHA plates and incubated at 37 °C overnight.

1H and 13C NMR Spectra of Analogs:

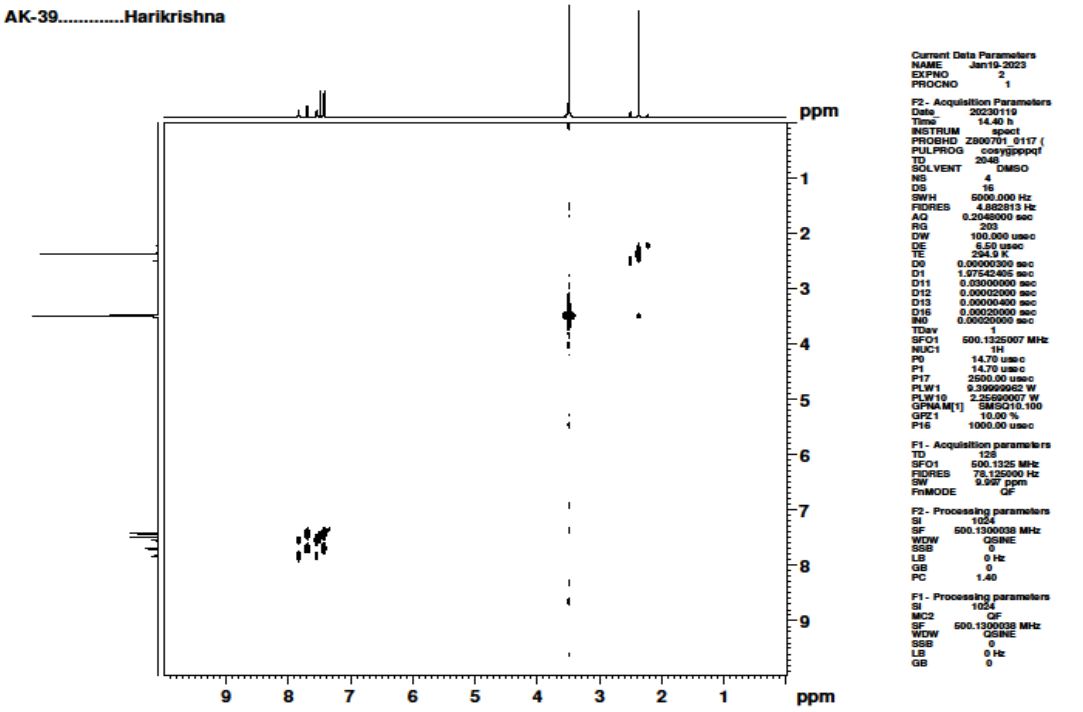




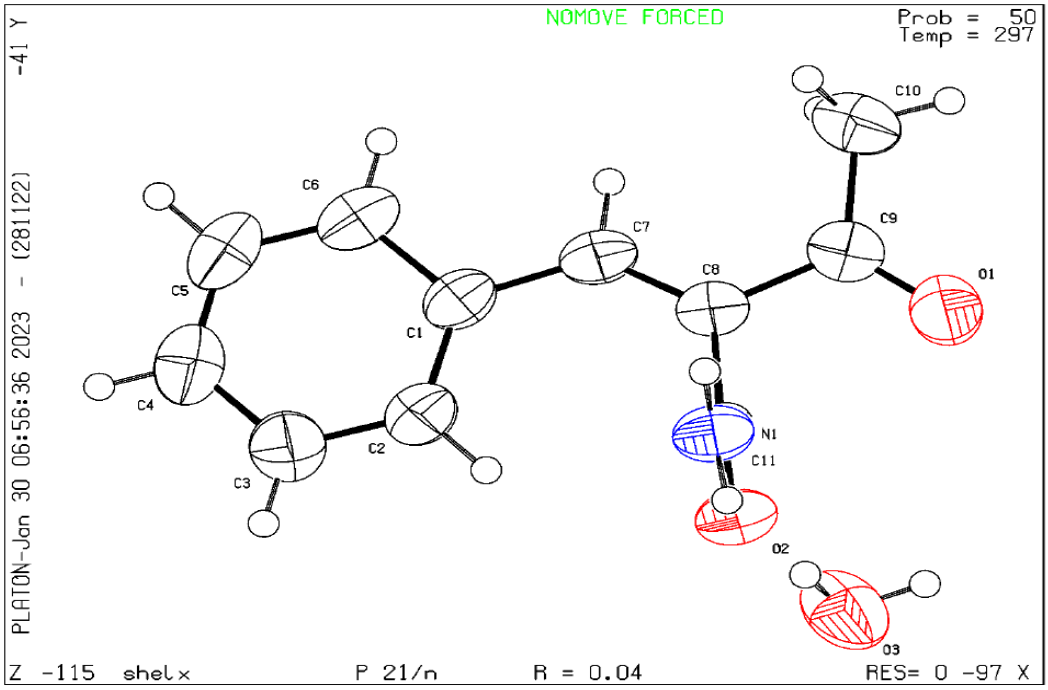
NOESY of COMPOUND 15



COSY of compound 15



ORTEP diagram for Compound 15 (CCDC-2238743)



Structure factor report

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Bond precision: C-C = 0.0020 A Wavelength=1.54178

Cell: a=11.1110(7) b=7.3567(4) c=13.9127(8)
alpha=90 beta=98.039(3) gamma=90

Temperature: 297 K

	Calculated	Reported
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Space group	P 21/n	P 21/n
Hall group	-P 2yn	-P 2yn
Moiety formula	C11 H11 N O2, H2 O	?
Sum formula	C11 H13 N O3	C11 H13 N O3
Mr	207.22	207.22
Dx,g cm-3	1.222	1.222
Z	4	4
Mu (mm-1)	0.740	0.740
F000	440.0	440.0
F000'	441.45	
h,k,lmax	13,8,16	13,8,16
Nref	2057	2039
Tmin,Tmax	0.924,0.967	0.680,0.753
Tmin'	0.864	

Correction method= # Reported T Limits: Tmin=0.680 Tmax=0.753 AbsCorr = MULTI-SCAN

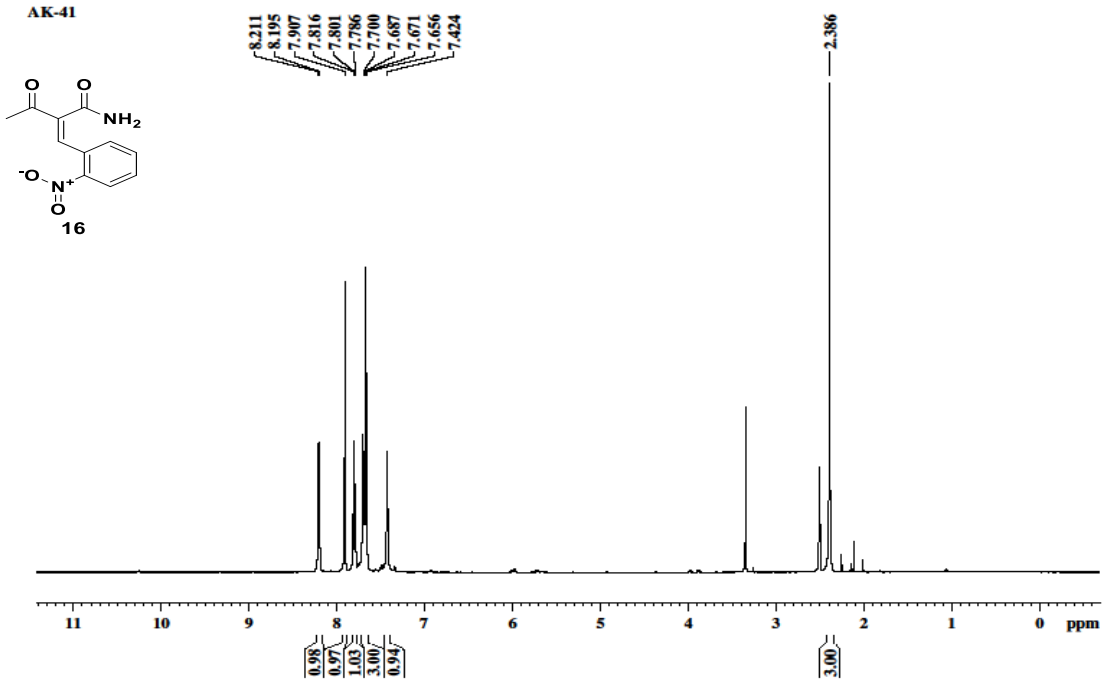
Data completeness= 0.991 Theta(max)= 68.239

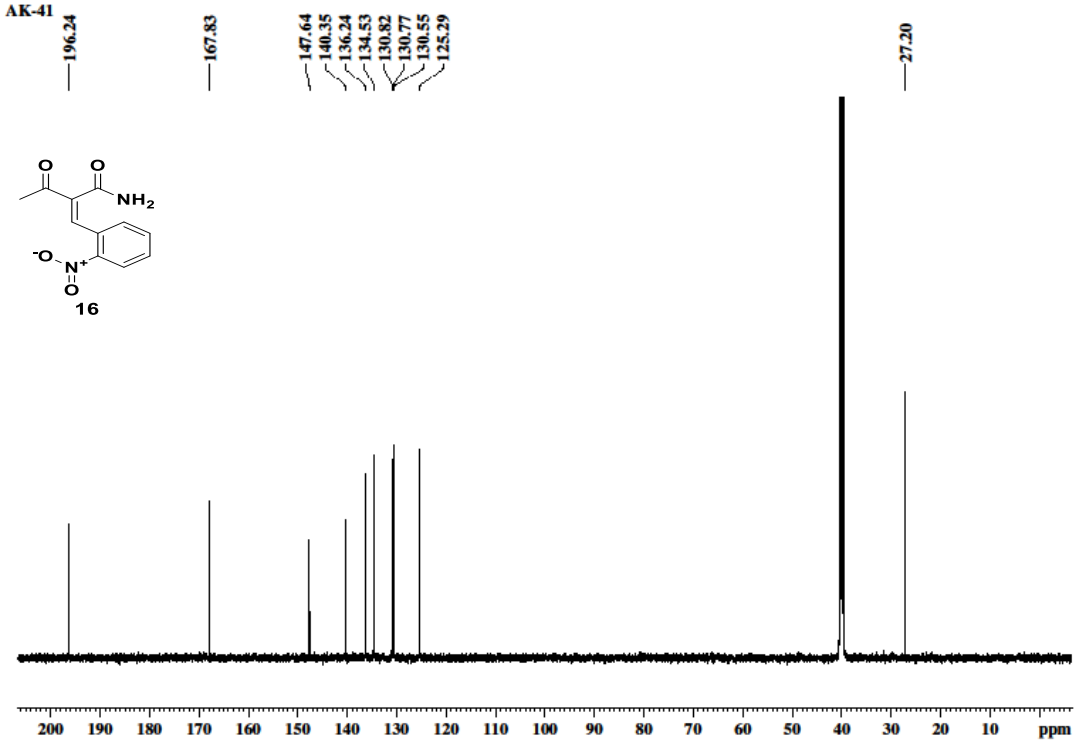
R(reflections)= 0.0388(1782) wR2(reflections)= 0.1186(2039)

S = 1.047 Npar= 149

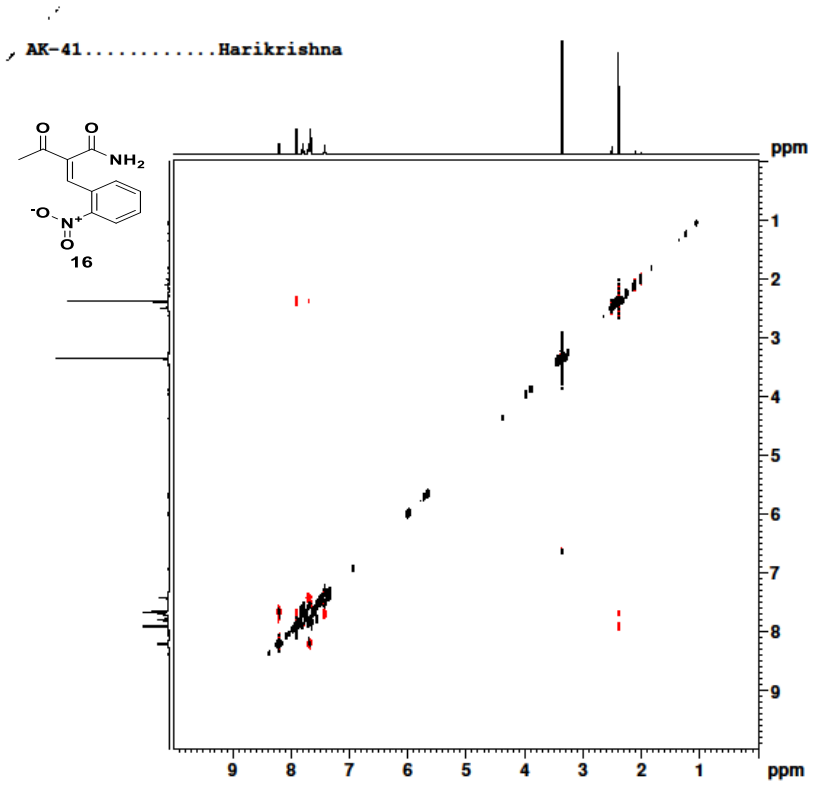
Structure factor report for compound 15

1H and 13C NMR Spectra of Compound 16





NOESY of COMPOUND 16

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NUC1                          1H
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P2                         29.40 usec
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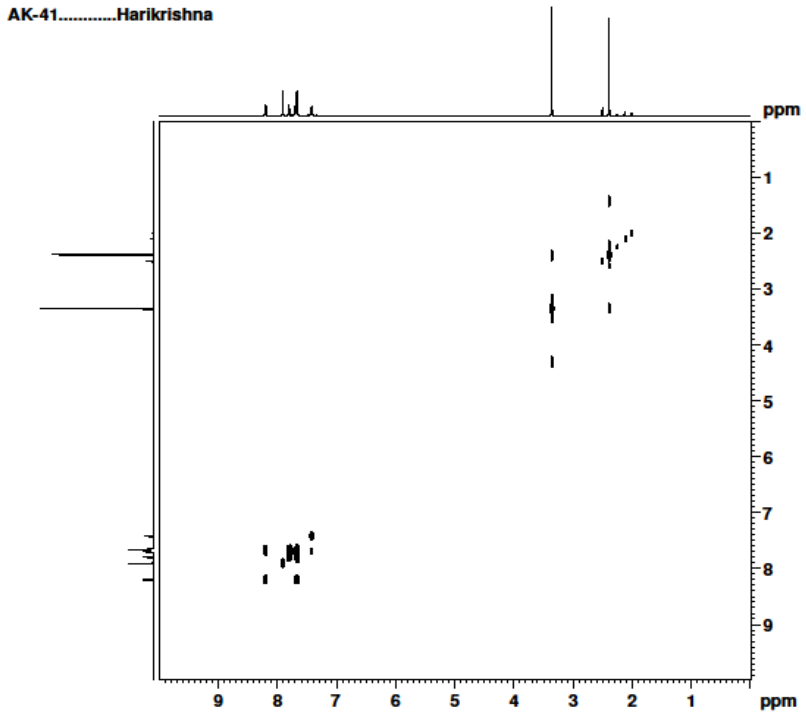
COSY of Compound 16

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FnMODE                QF

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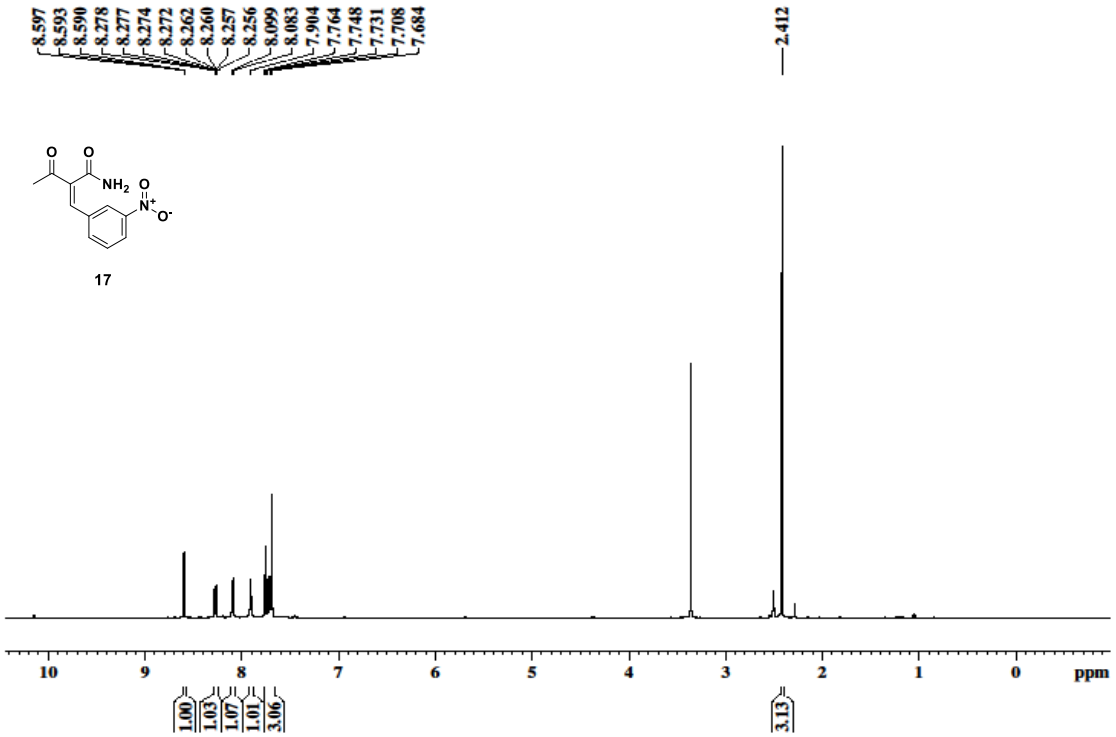
AK-43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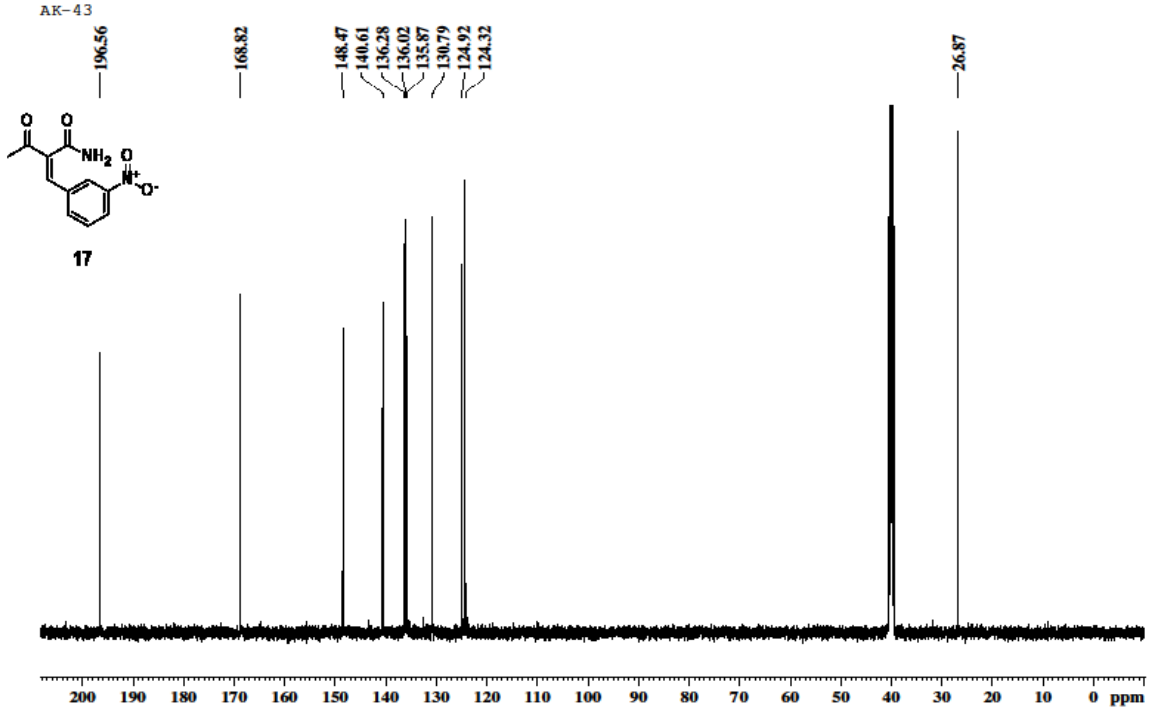
AK-43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30
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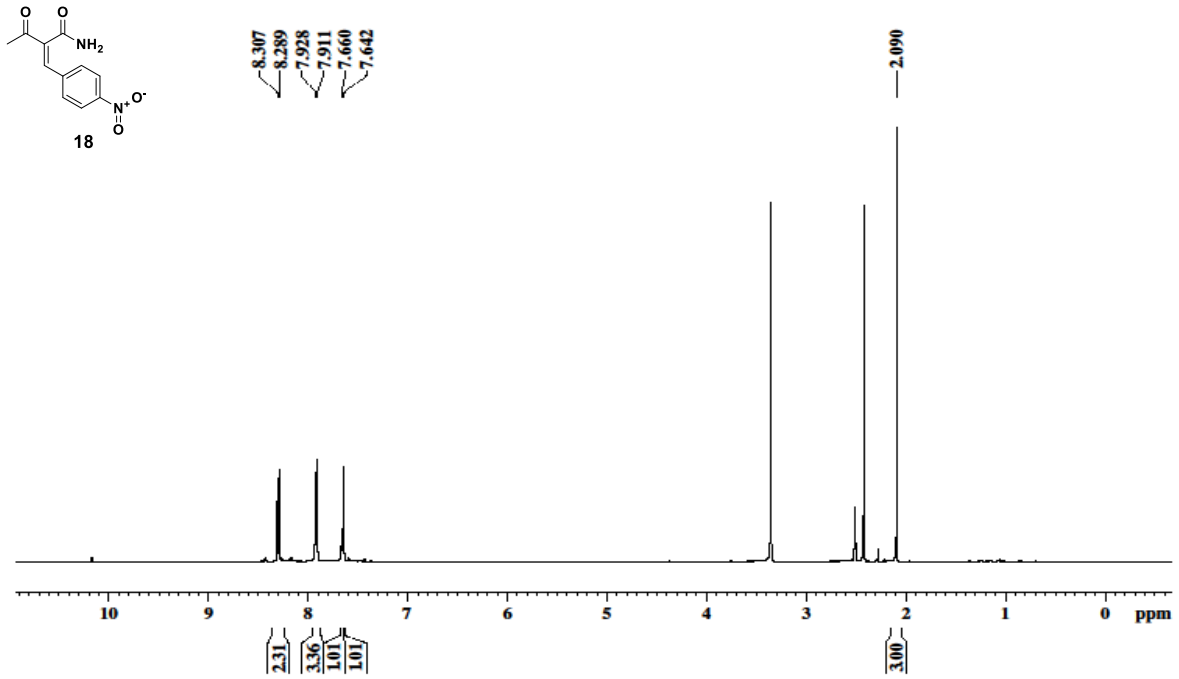
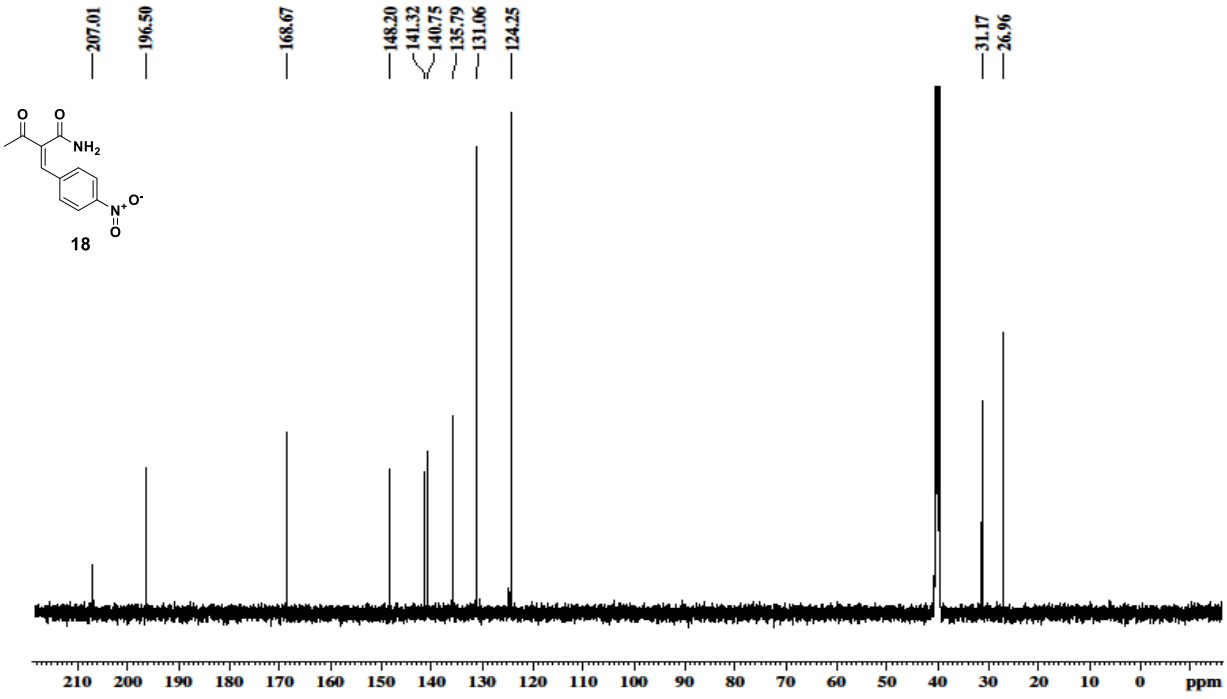
0 ppm

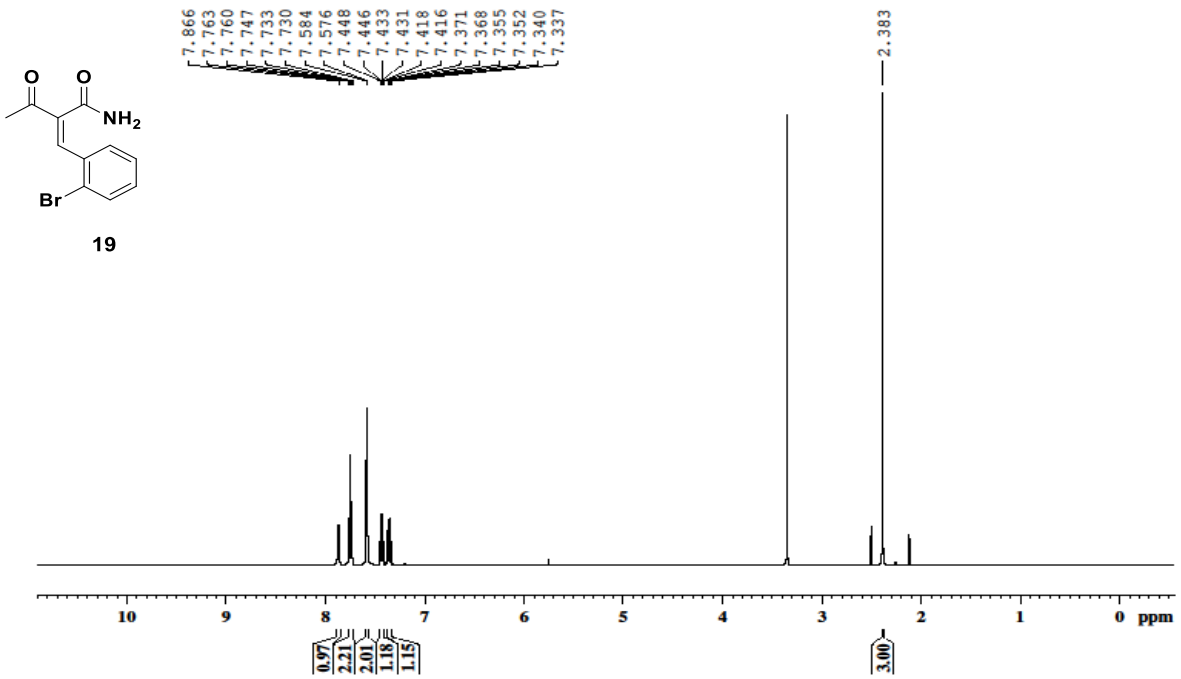
AS-47-45



AS-47-45

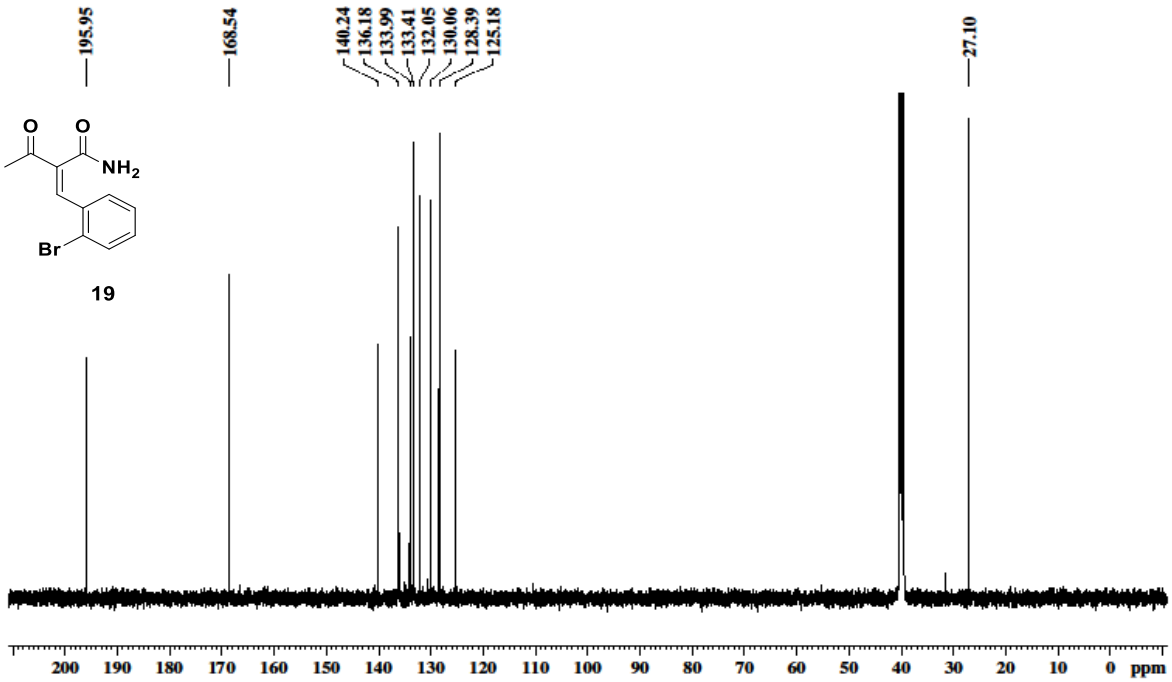


AK-47



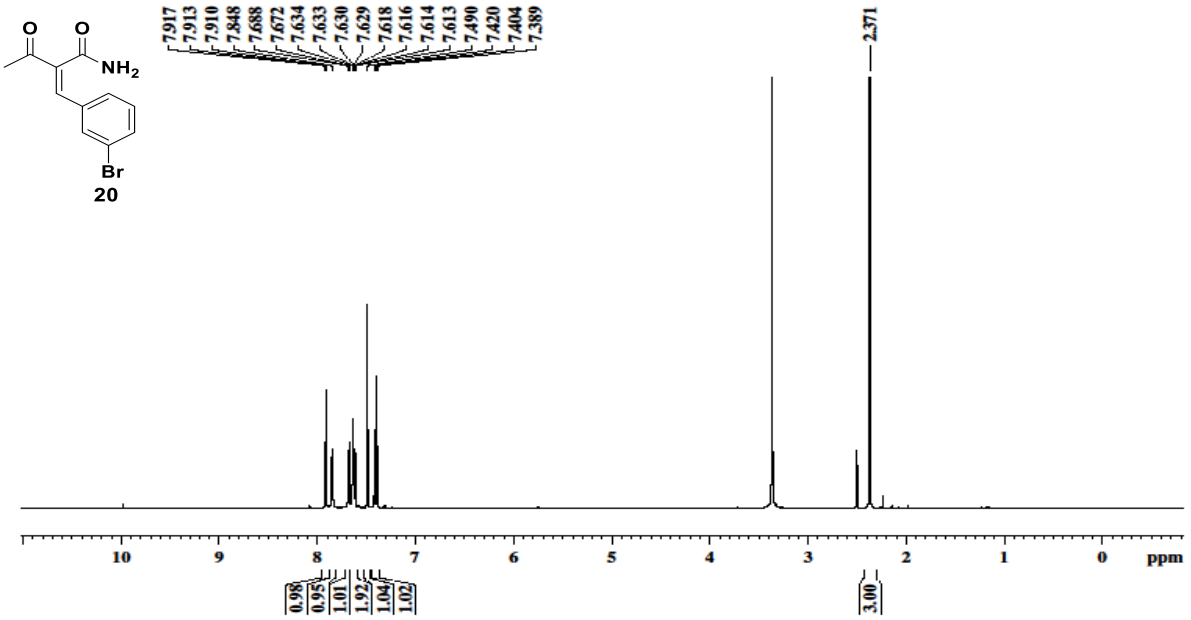
19

AK-47

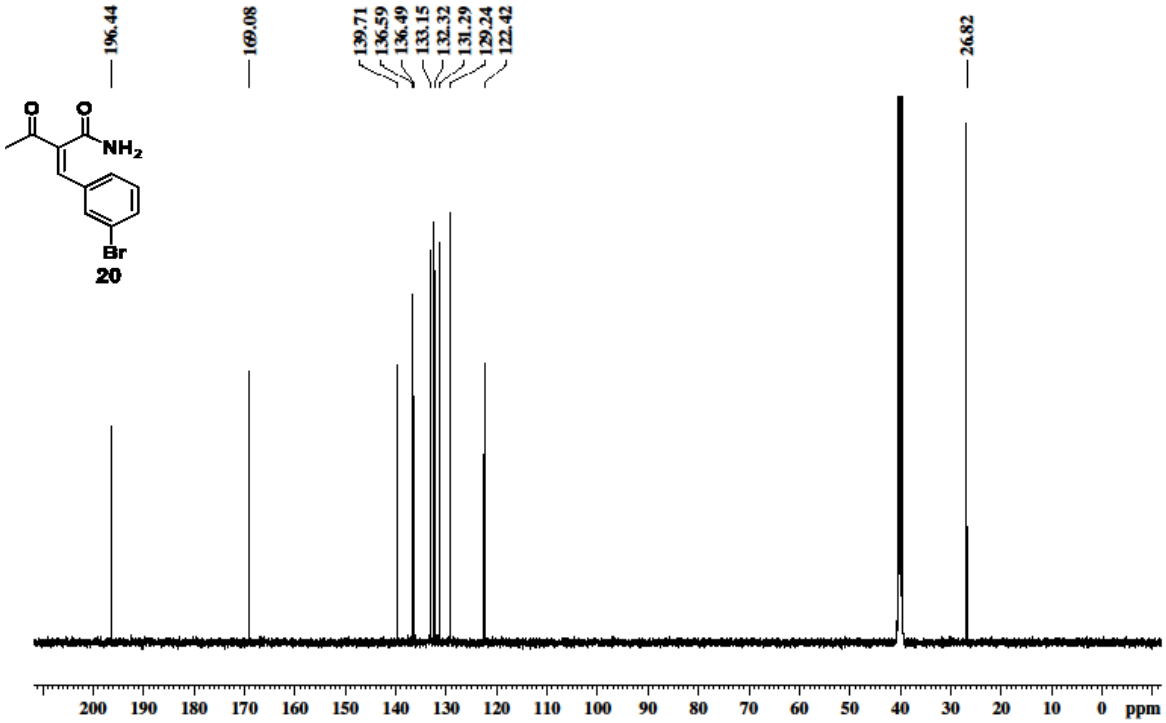


19

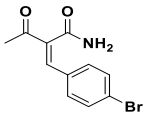
AS-1


7.917
7.913
7.910
7.848
7.688
7.672
7.634
7.633
7.630
7.629
7.618
7.616
7.614
7.613
7.490
7.420
7.404
7.389
2.371
NH2
Br
20
10
9
8
7
6
5
4
3
2
1
0
ppm
0.98
0.95
1.01
1.92
1.04
1.02
3.00

AS-1

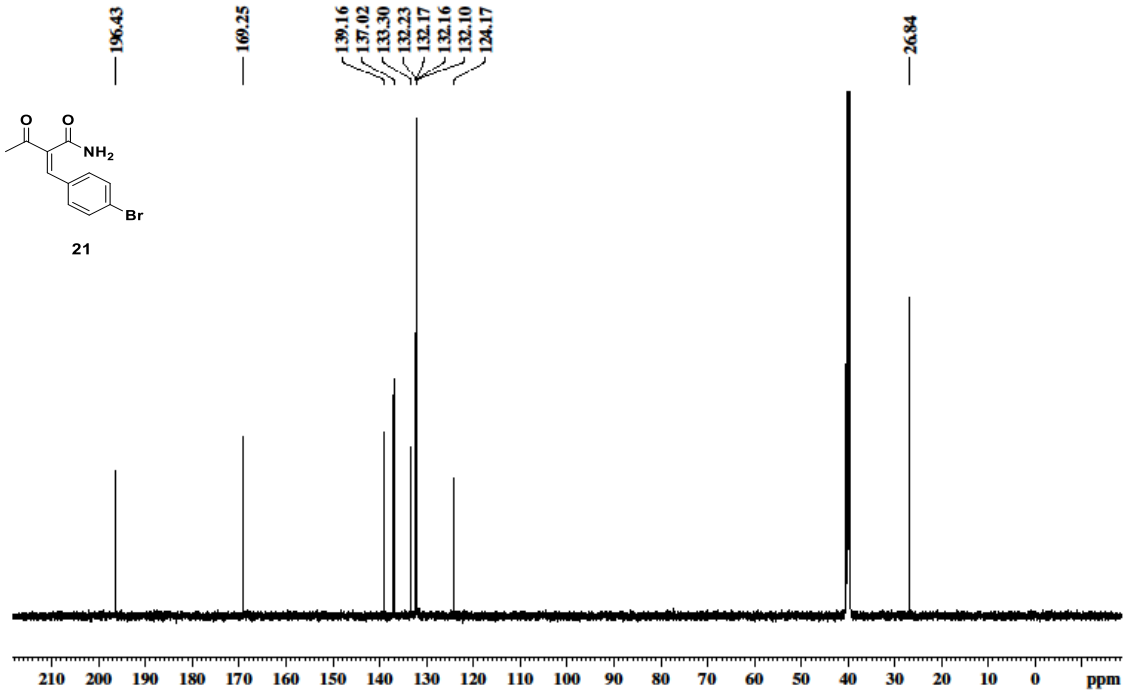

196.44
169.08
139.71
136.59
136.49
133.15
132.32
131.29
129.24
122.42
26.82
NH2
Br
20
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm

AK-49



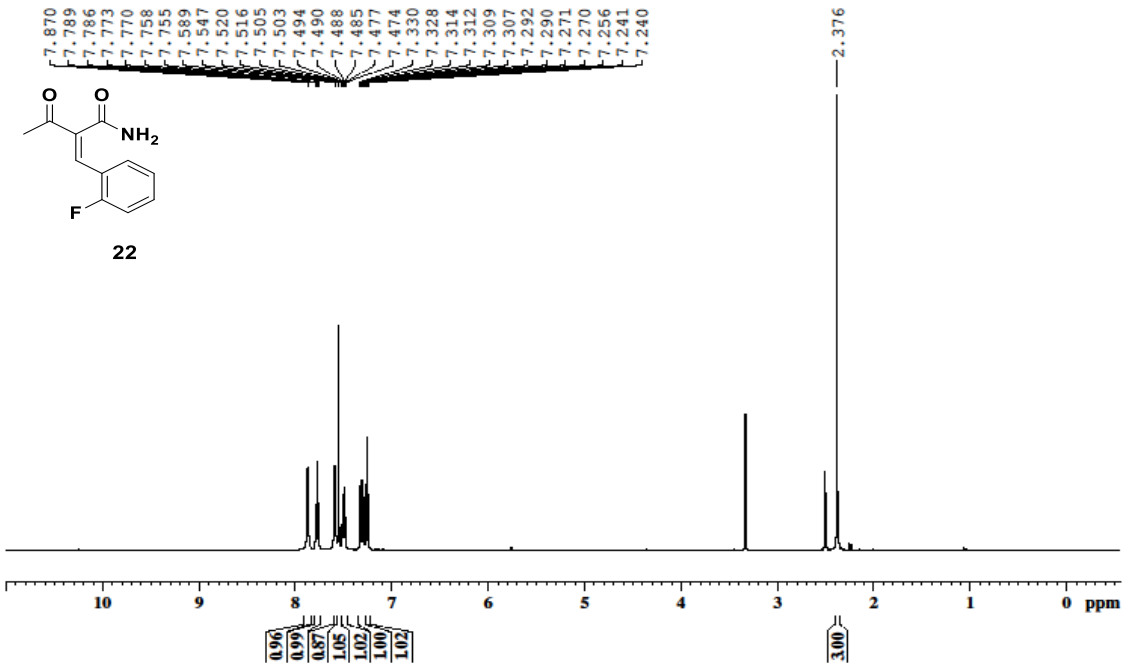
21

AK-49

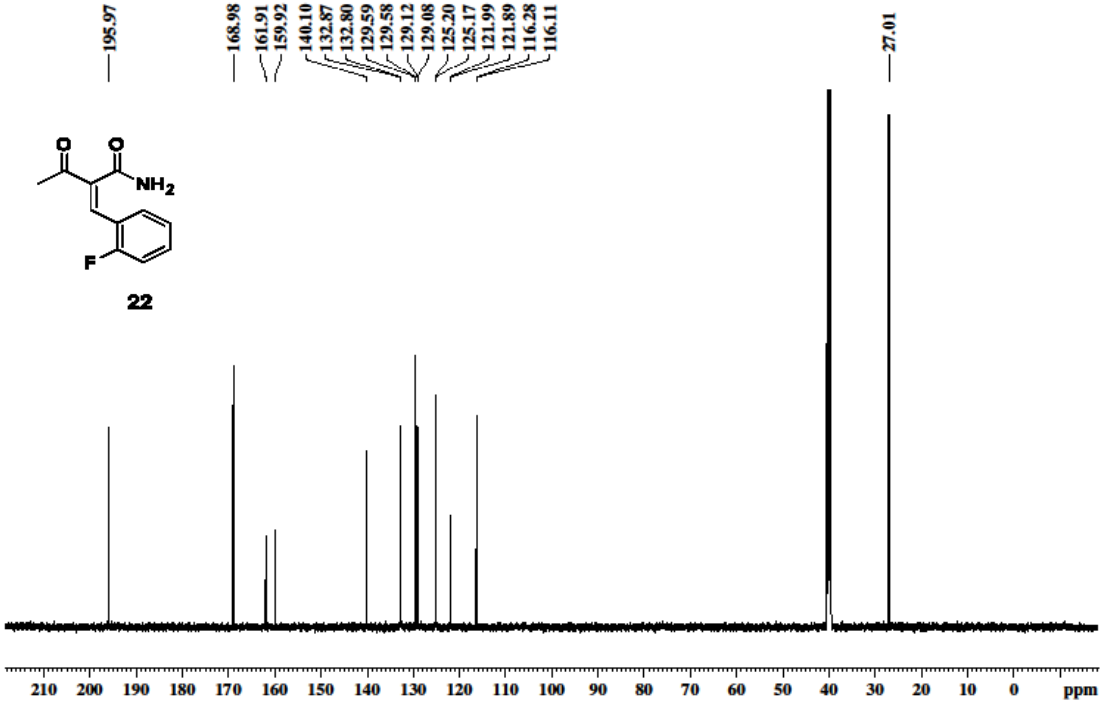


21

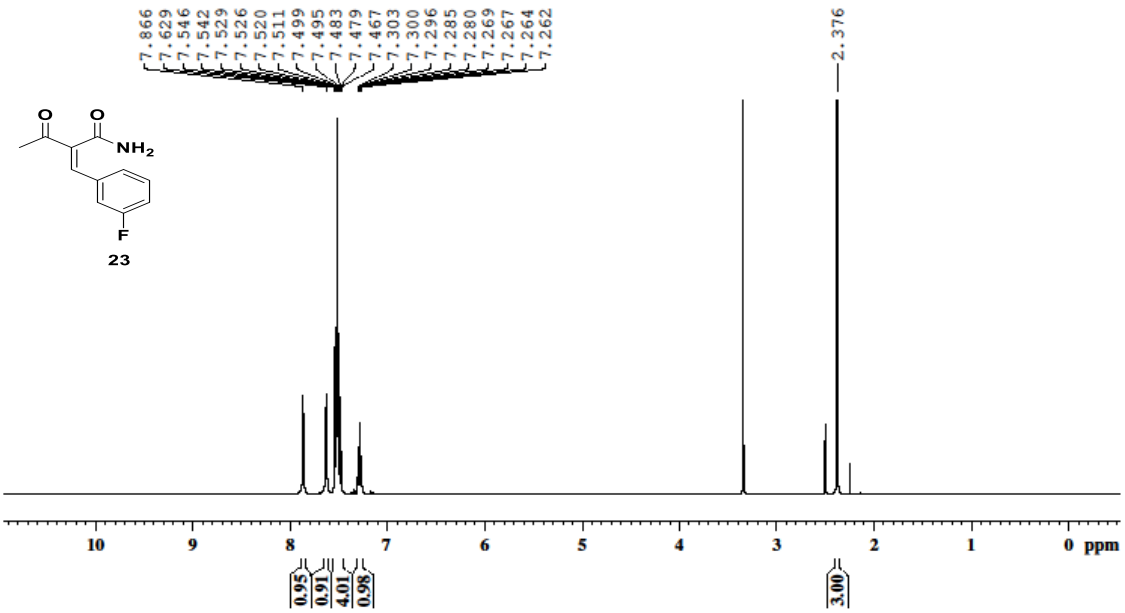
AK-51


7.870
7.789
7.786
7.773
7.770
7.758
7.755
7.589
7.547
7.520
7.516
7.505
7.503
7.494
7.490
7.488
7.485
7.477
7.474
7.330
7.328
7.314
7.312
7.309
7.307
7.292
7.290
7.271
7.270
7.256
7.241
7.240
2.376
O
O
NH2
F
22
10
9
8
7
6
5
4
3
2
1
0
ppm
0.96
0.99
0.87
1.05
1.02
1.00
1.02
3.00

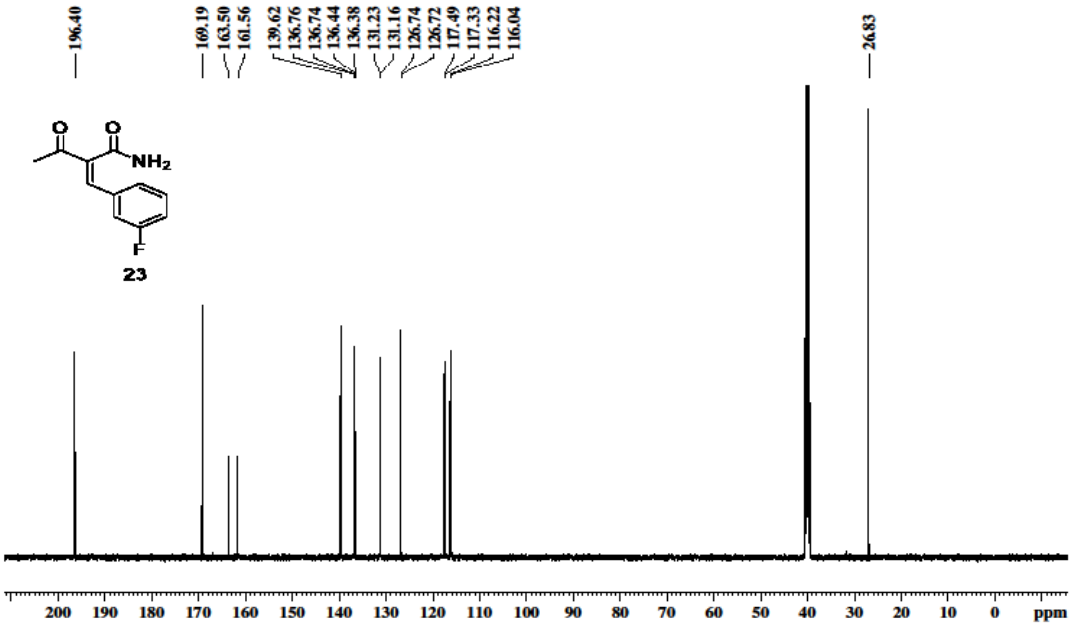
AK-51


195.97
168.98
161.91
159.92
140.10
132.87
132.80
129.59
129.58
129.12
129.08
125.20
125.17
121.99
121.89
116.28
116.11
27.01
O
O
NH2
F
22
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm

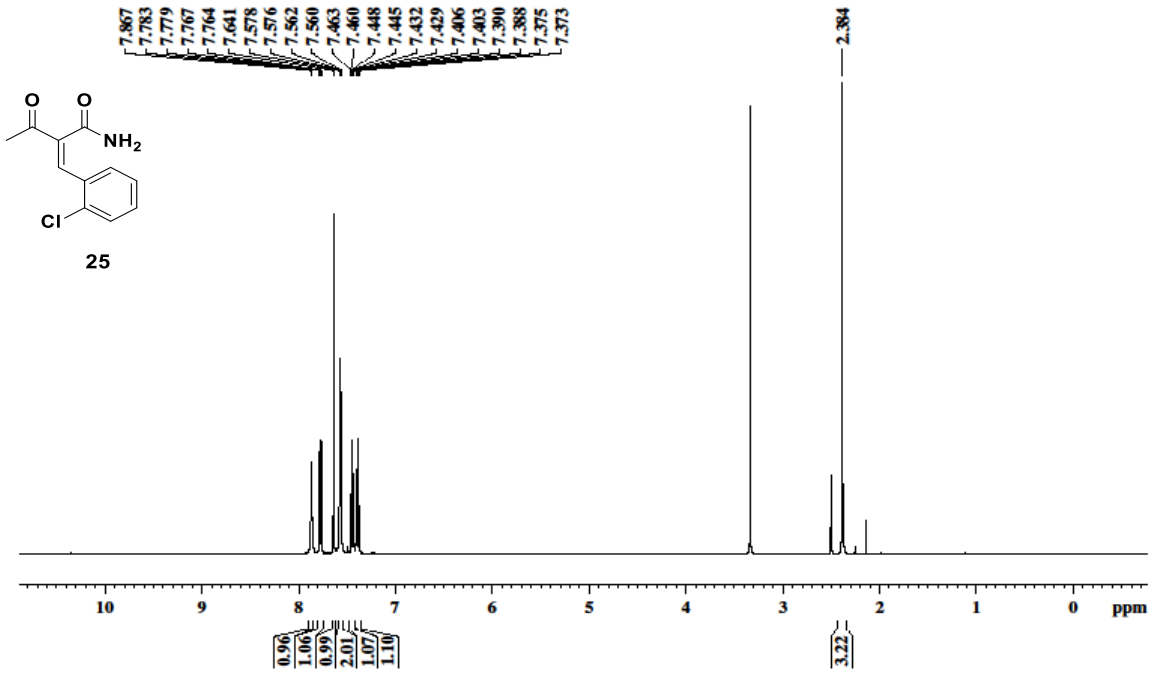
AS-2.


7.866
7.629
7.546
7.542
7.529
7.526
7.520
7.511
7.499
7.495
7.483
7.479
7.467
7.303
7.300
7.296
7.285
7.280
7.269
7.267
7.264
7.262
2.376
O
O
NH2
F
23
10
9
8
7
6
5
4
3
2
1
0 ppm
0.95
0.91
4.01
0.98
3.00

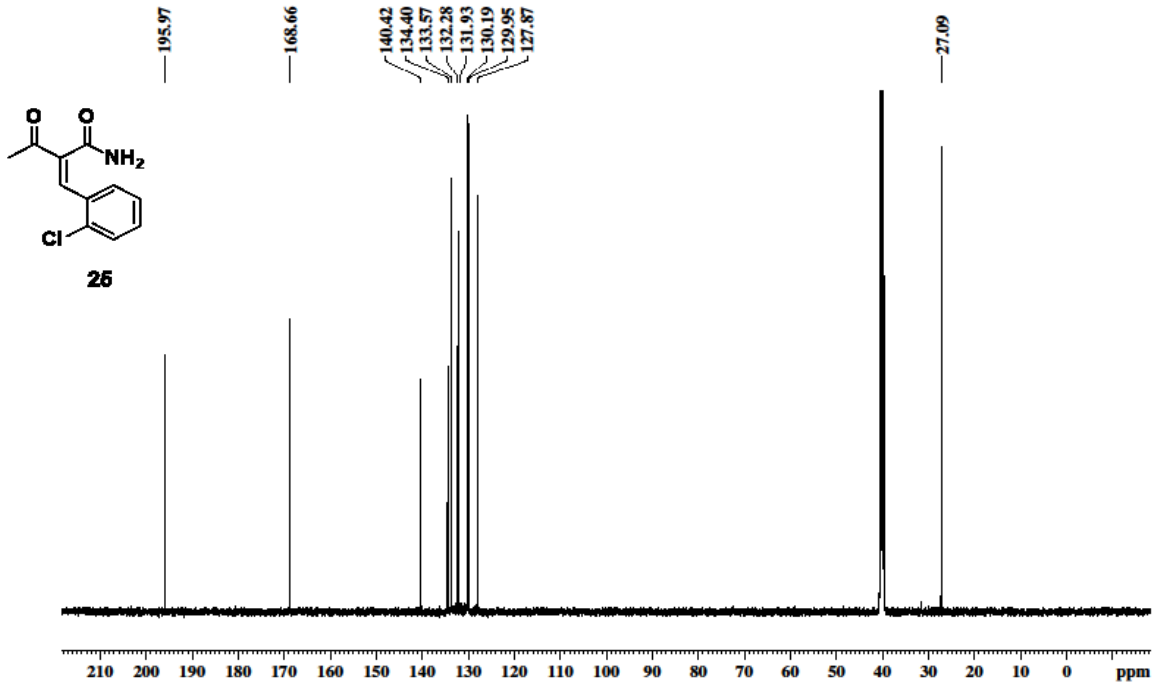
AS-2


196.40
169.19
163.50
161.56
139.62
136.76
136.74
136.44
136.38
131.23
131.16
126.74
126.72
117.49
117.33
116.22
116.04
26.83
O
O
NH2
F
23
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm

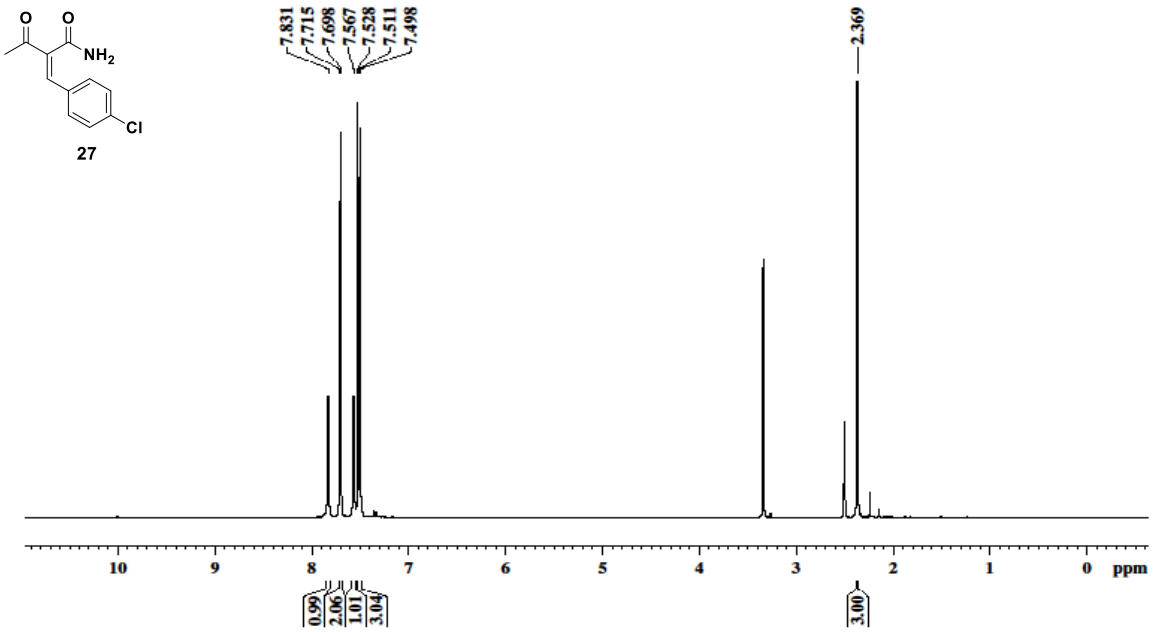
AK-55


7.867
7.783
7.779
7.767
7.764
7.641
7.578
7.576
7.562
7.560
7.463
7.460
7.448
7.445
7.432
7.429
7.406
7.403
7.390
7.388
7.375
7.373
2.384
O
O
NH2
Cl
25
10
9
8
7
6
5
4
3
2
1
0
ppm
0.96
1.06
0.99
2.01
1.07
1.10
3.22

AK-55

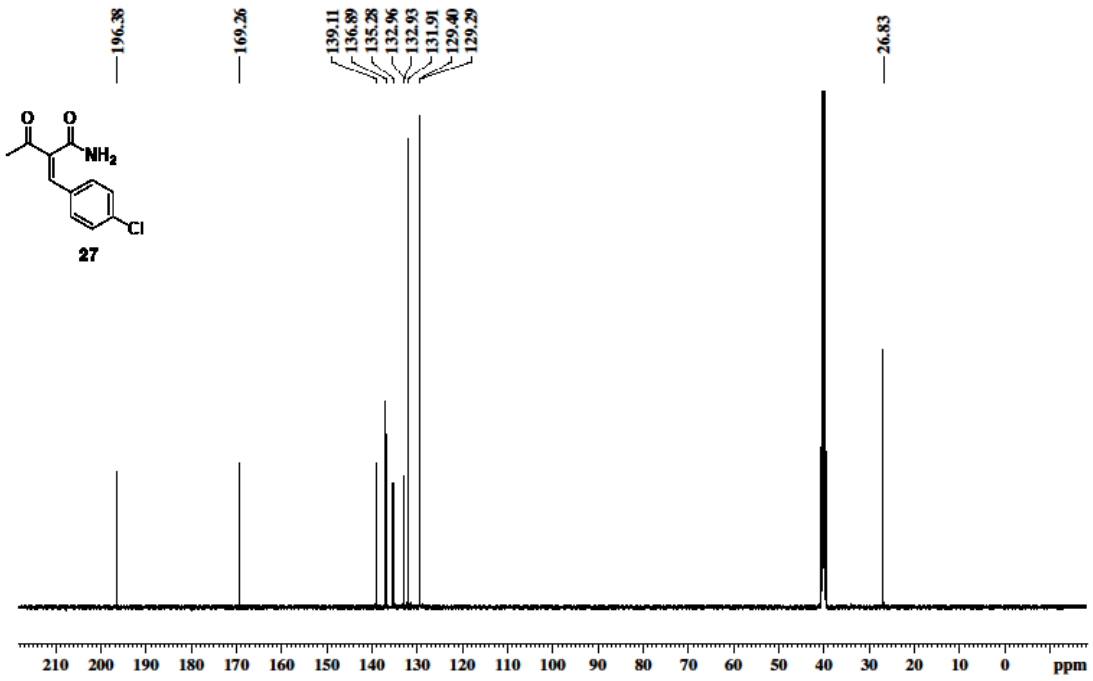

195.97
168.66
140.42
134.40
133.57
132.28
131.93
130.19
129.95
127.87
27.09
O
O
NH2
Cl
25
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm

AK-57



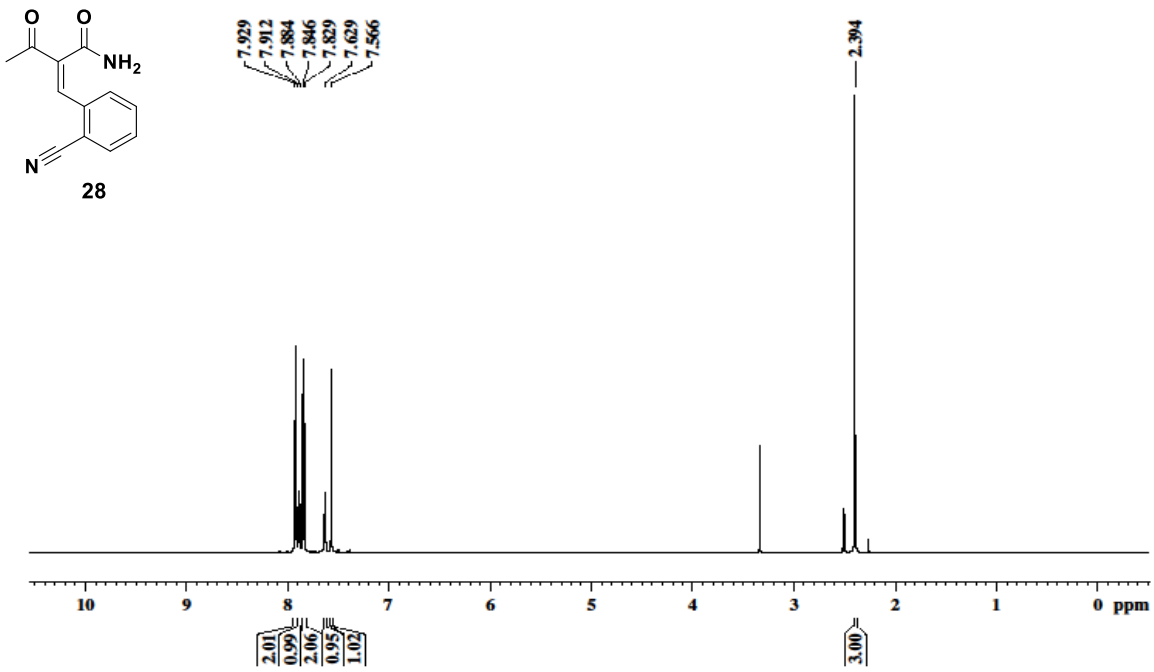
27

AK-57



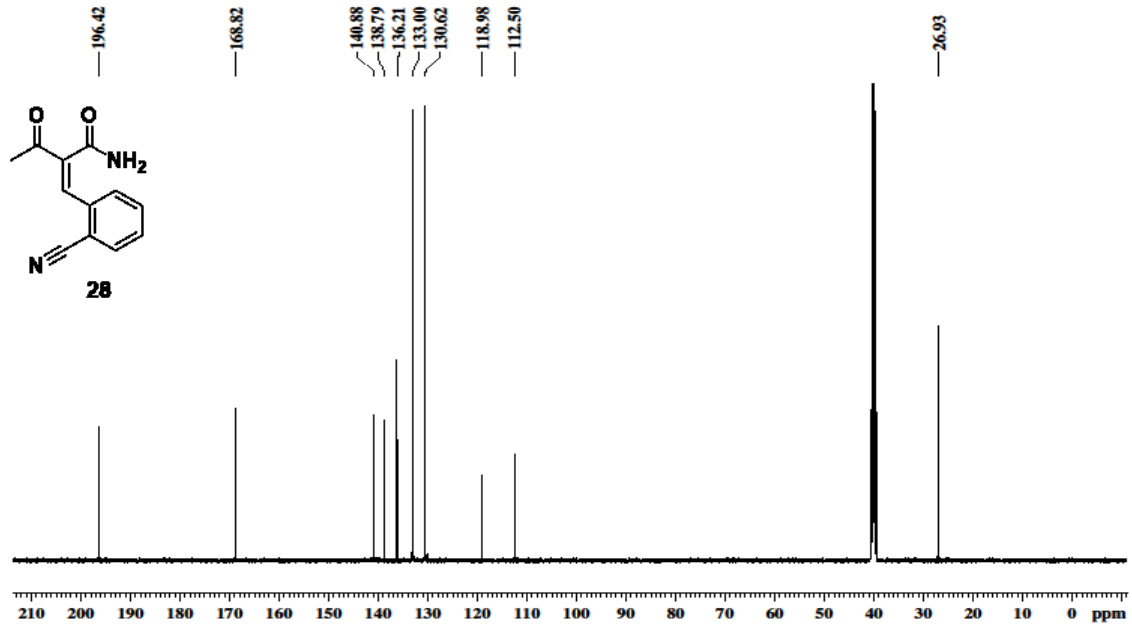
27

AK-61



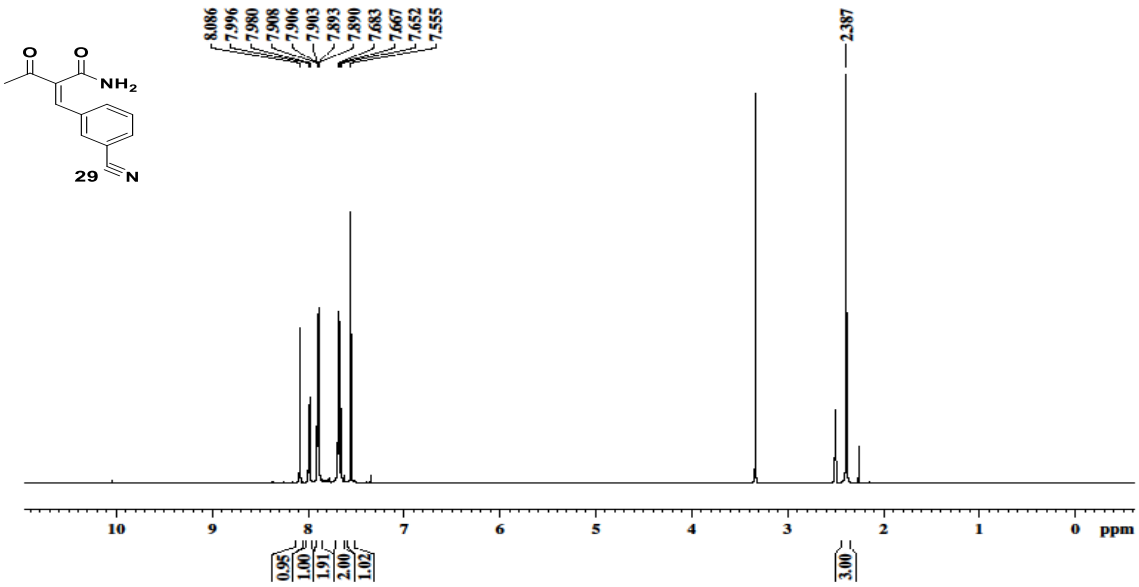
28

AK-61

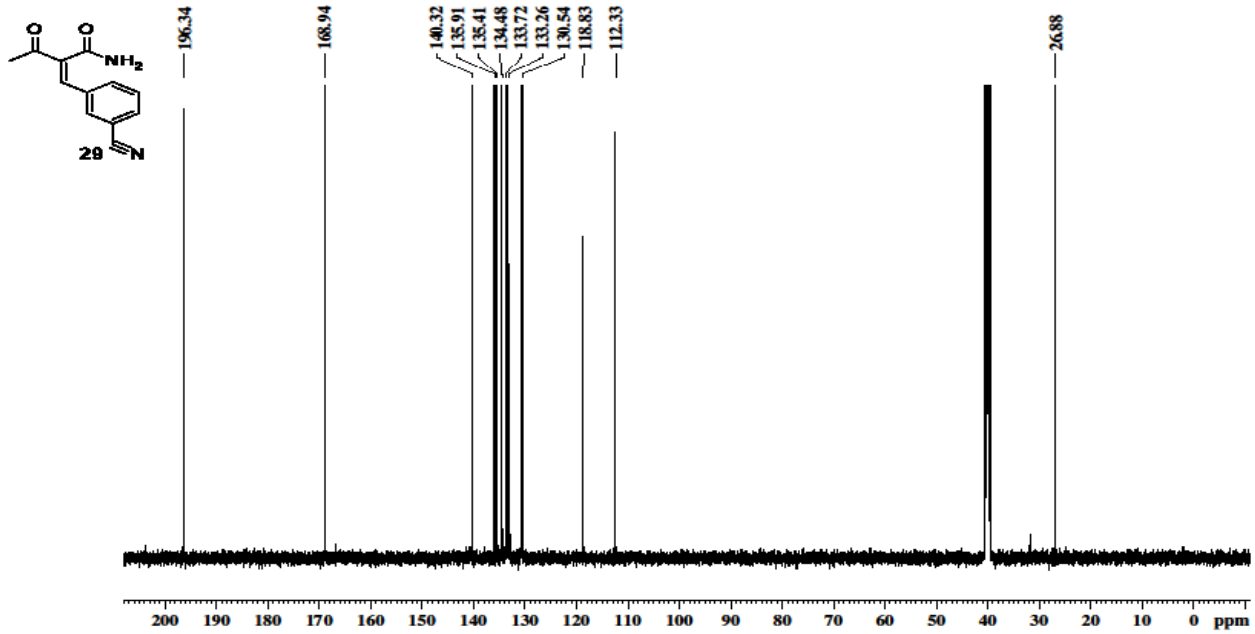


28

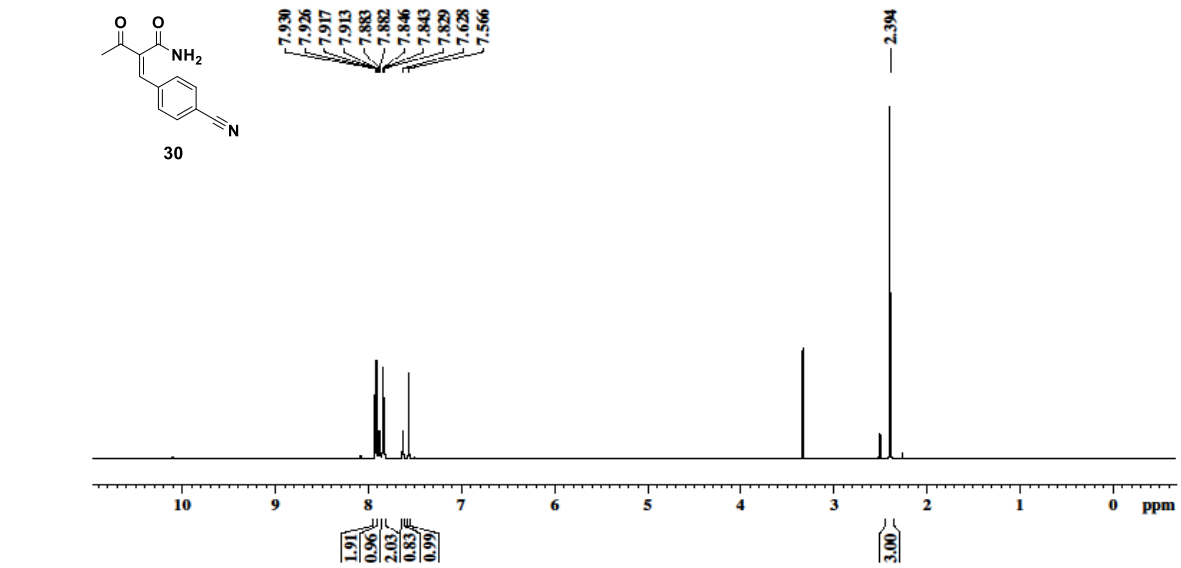
AS-3


8.086
7.996
7.980
7.908
7.906
7.903
7.893
7.890
7.683
7.667
7.652
7.555
2.387
O
O
NH2
29
N
10
9
8
7
6
5
4
3
2
1
0
ppm
0.95
1.00
1.91
2.00
1.02
3.00

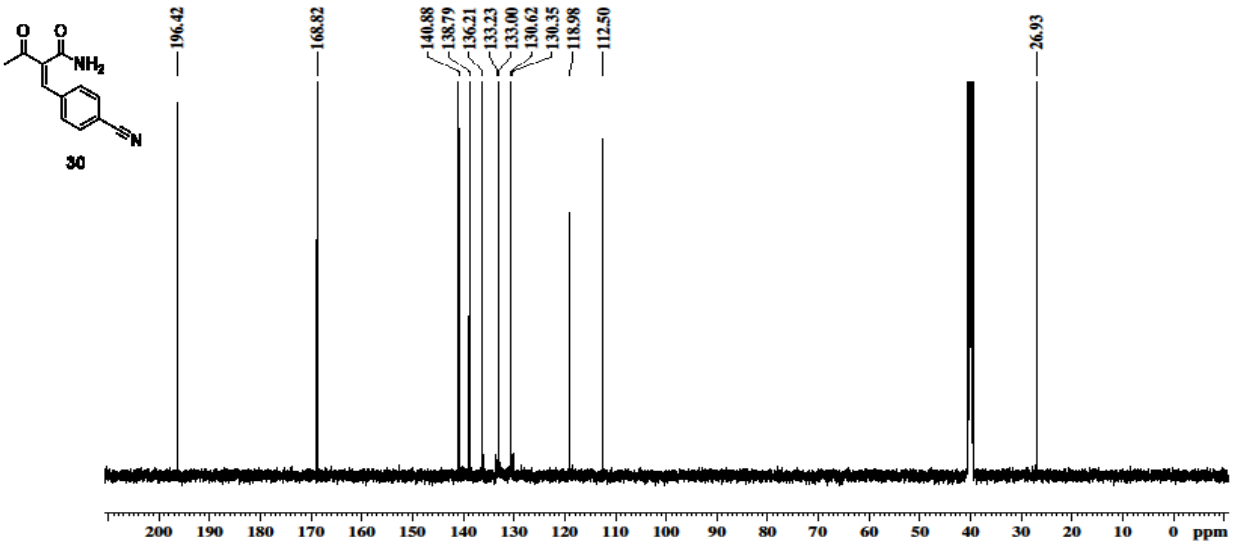
AS-3


196.34
168.94
140.32
135.91
135.41
134.48
133.72
133.26
130.54
118.83
112.33
26.88
O
O
NH2
29
N
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm

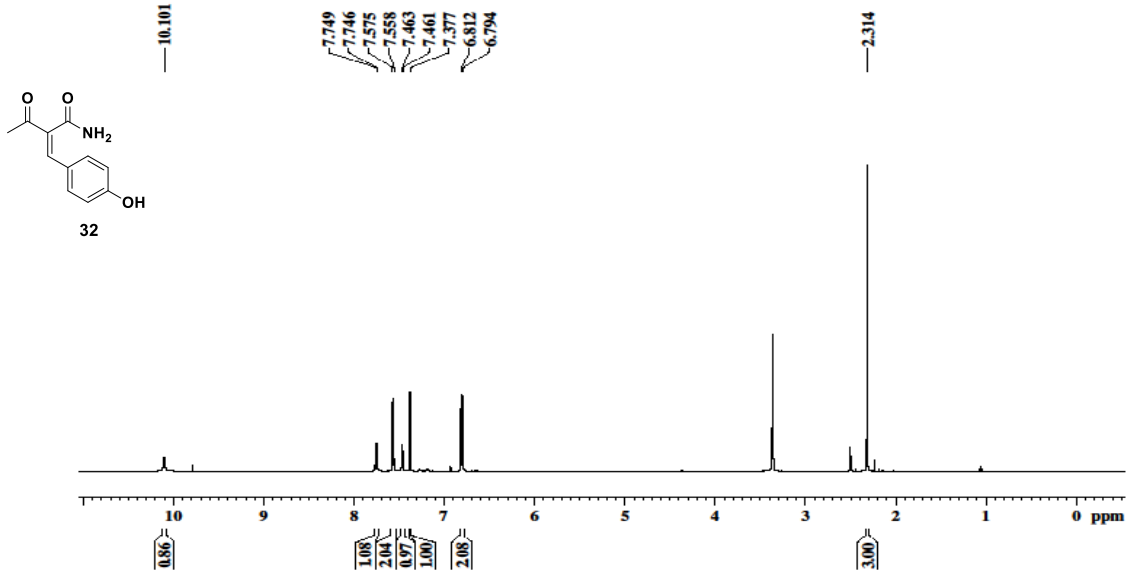
AS-4.


7.930
7.926
7.917
7.913
7.883
7.882
7.846
7.843
7.829
7.628
7.566
2.394
30
10
9
8
7
6
5
4
3
2
1
0
ppm
1.91
0.96
2.03
0.83
0.99
3.00

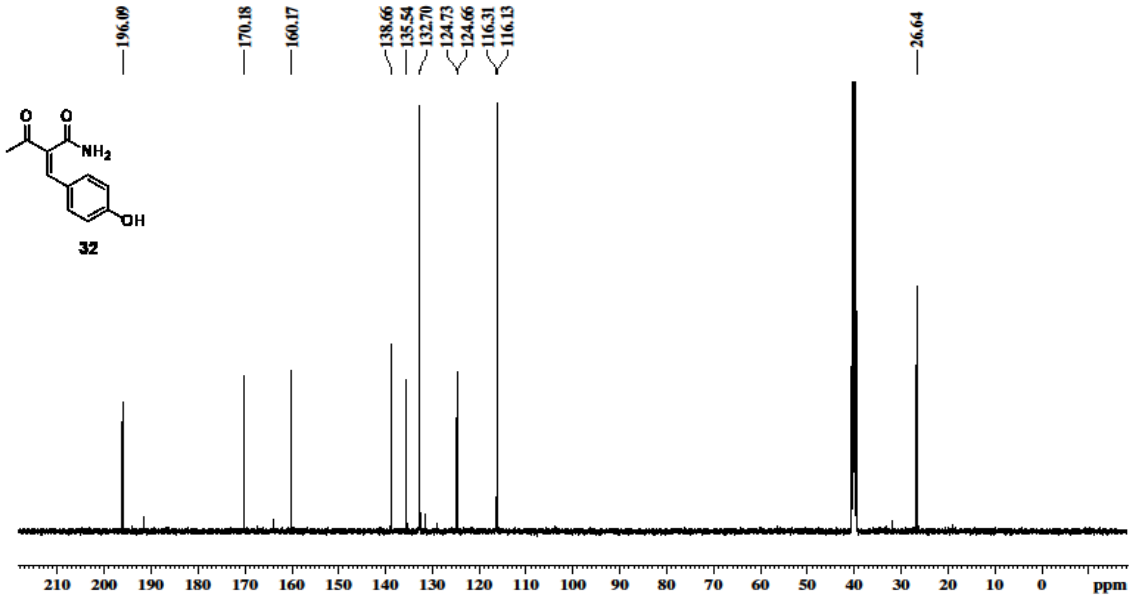
AS-4


196.42
168.82
140.88
138.79
136.21
133.23
133.00
130.62
130.35
118.98
112.50
26.93
30
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm

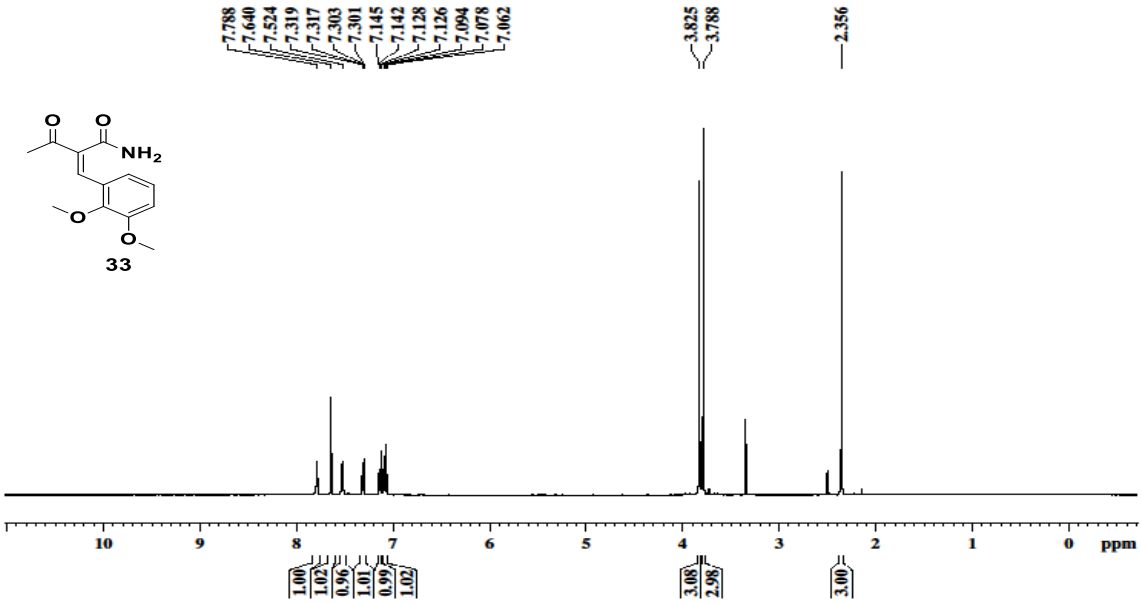
AS-10


10.101
7.749
7.746
7.575
7.558
7.463
7.461
7.377
6.812
6.794
2.314
NH2
OH
32
10
9
8
7
6
5
4
3
2
1
0 ppm
0.86
1.08
2.04
0.97
1.00
2.08
3.00

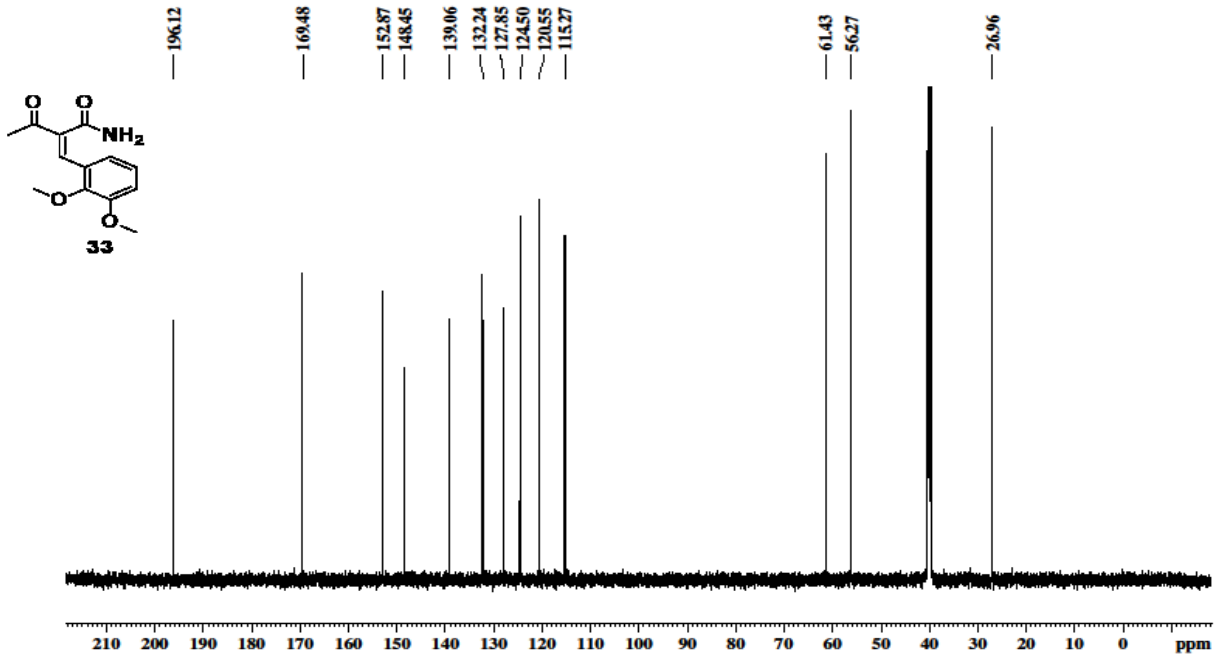
AS-10


196.09
170.18
160.17
138.66
135.54
132.70
124.73
124.66
116.31
116.13
26.64
NH2
OH
32
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm

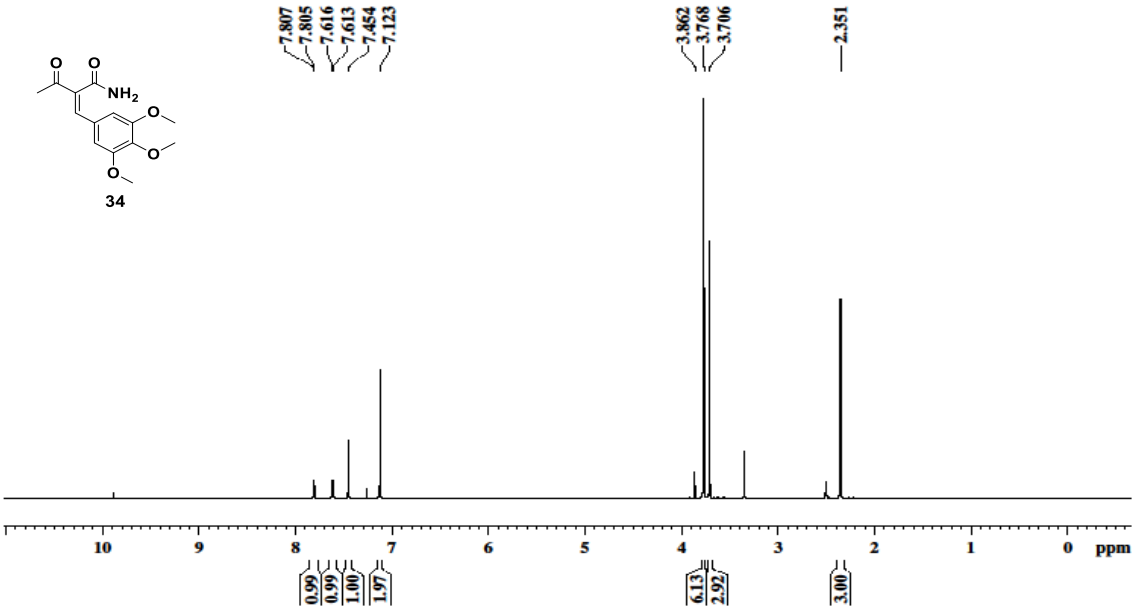
AK-47-77



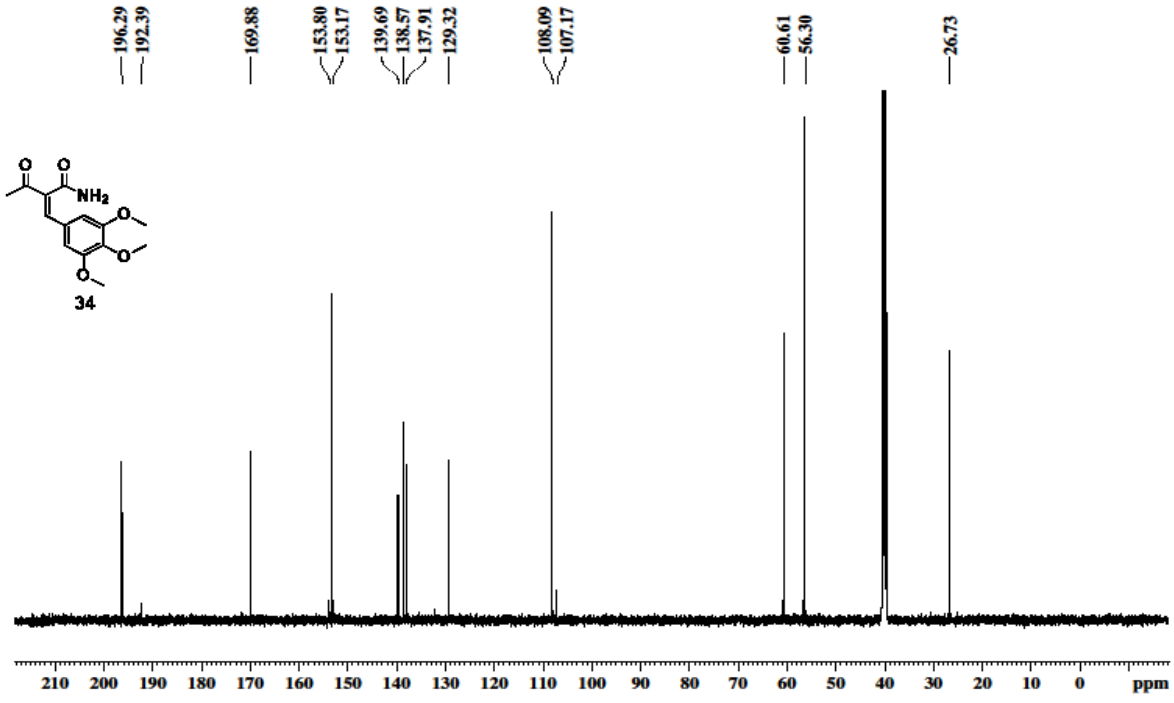
AK-47-77



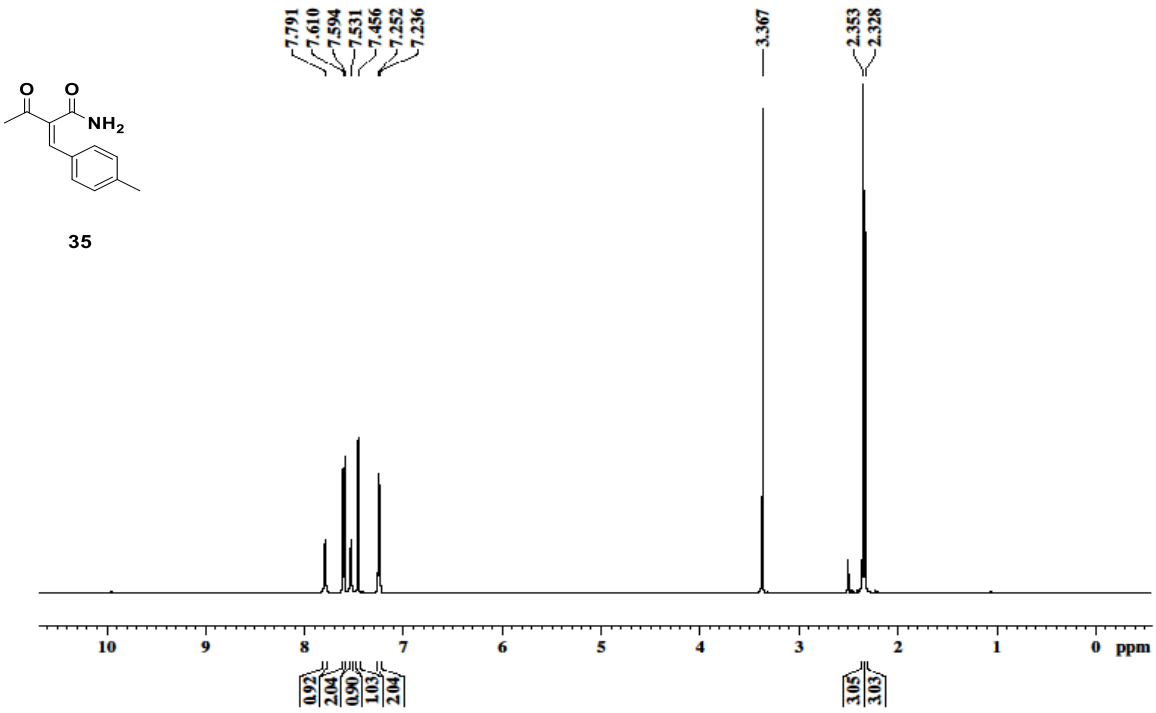
AK-47-79



AK-47-79

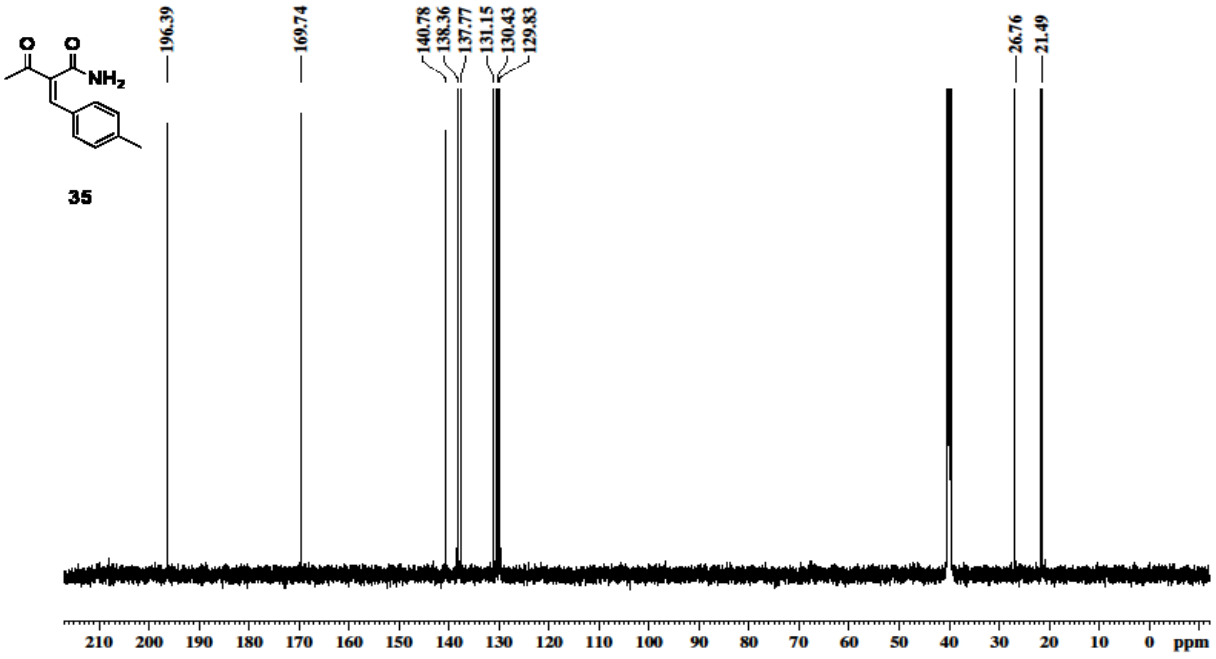


AK-47-81



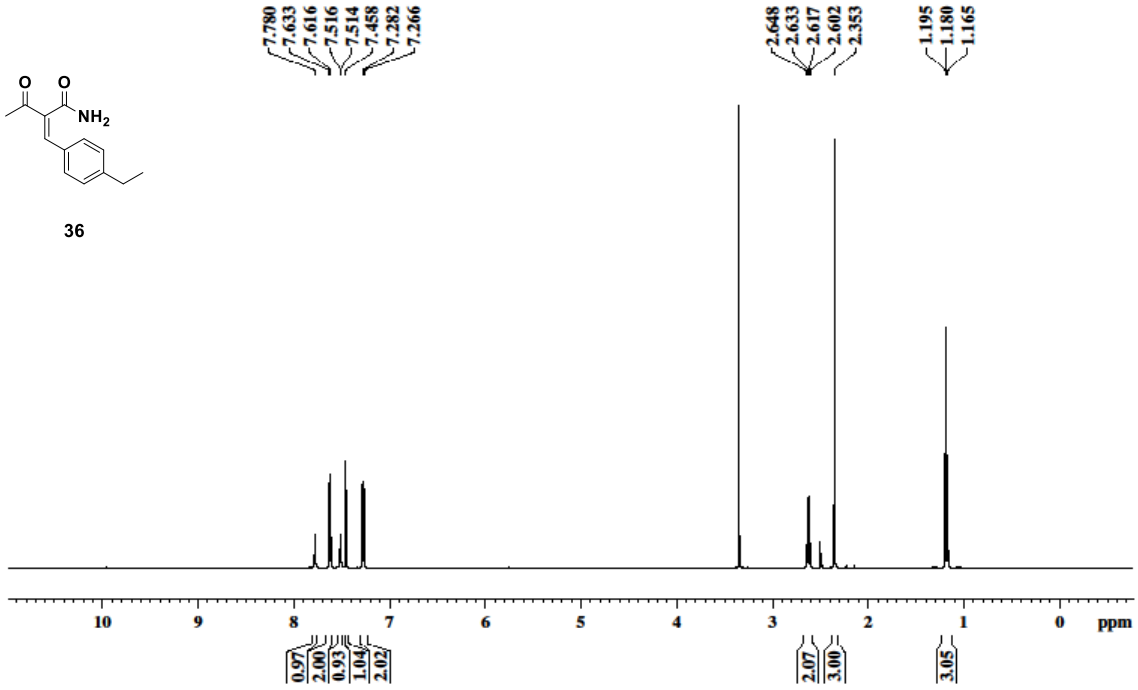
35

AK-47-81



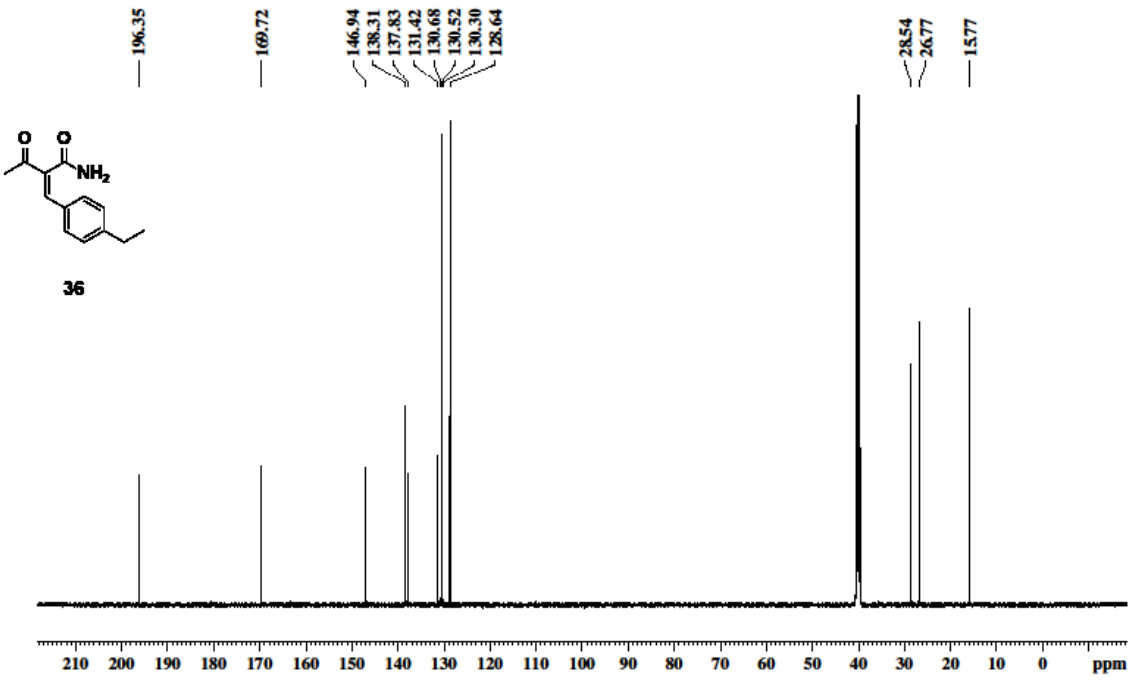
35

AS-6


7.780
7.633
7.616
7.516
7.514
7.458
7.282
7.266
2.648
2.633
2.617
2.602
2.353
1.195
1.180
1.165
O
O
NH2
10
9
8
7
6
5
4
3
2
1
0
ppm
0.97
2.00
0.93
1.04
2.02
2.07
3.00
3.05

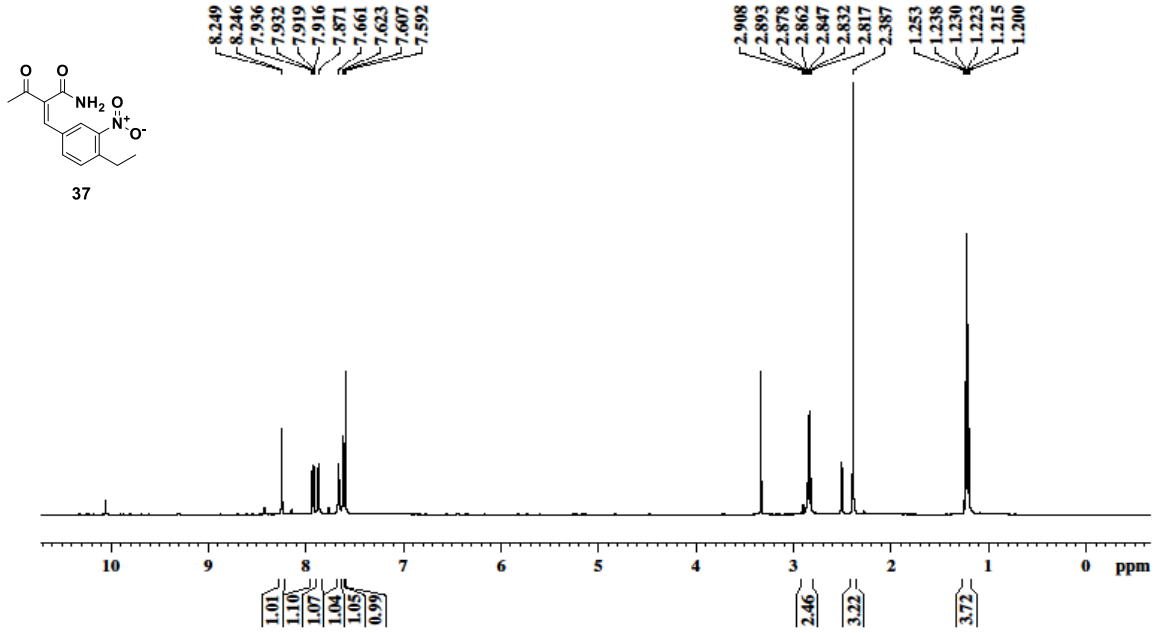
36

AS-6.


196.35
169.72
146.94
138.31
137.83
131.42
130.68
130.52
130.30
128.64
28.54
26.77
15.77
O
O
NH2
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm

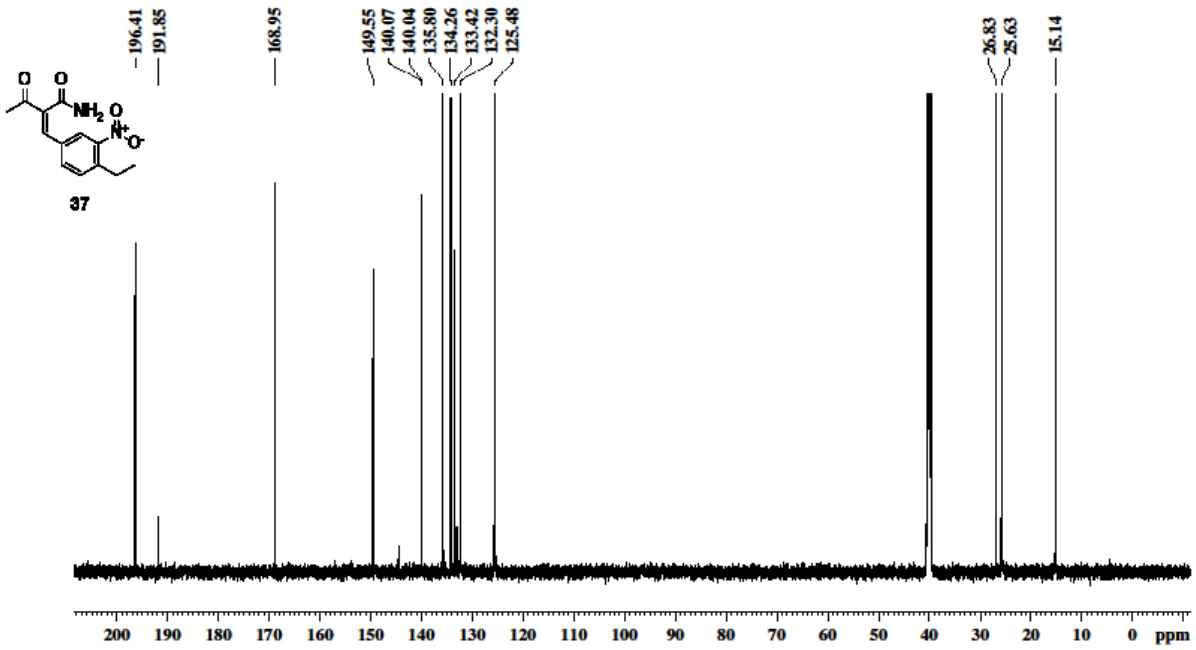
36

AK-75



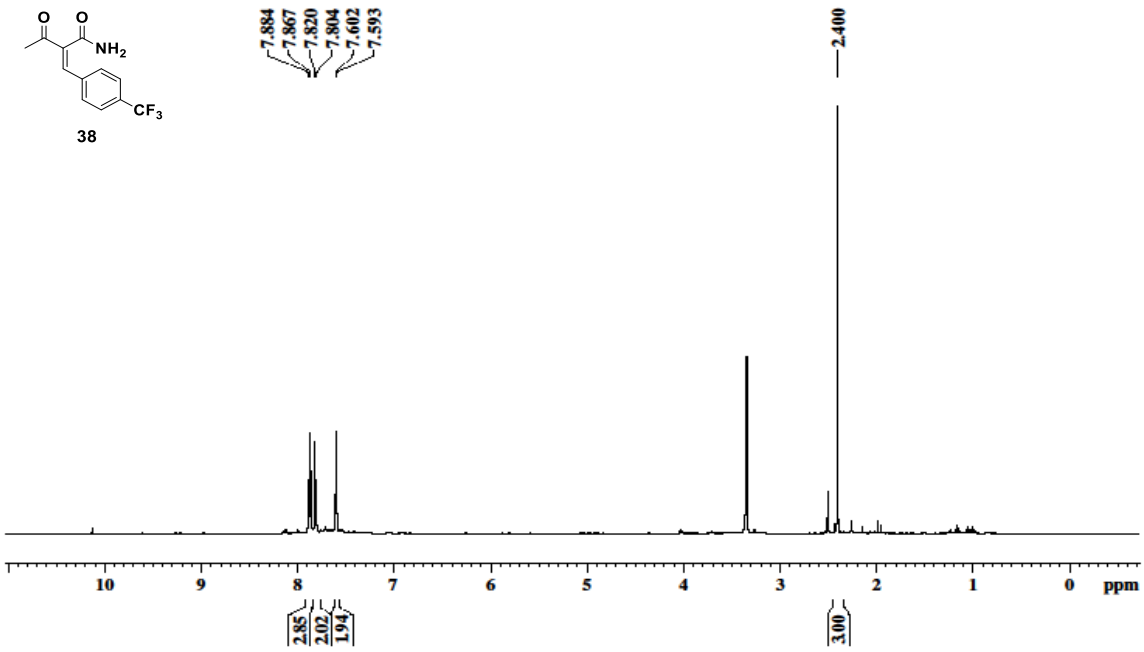
37

AK-75

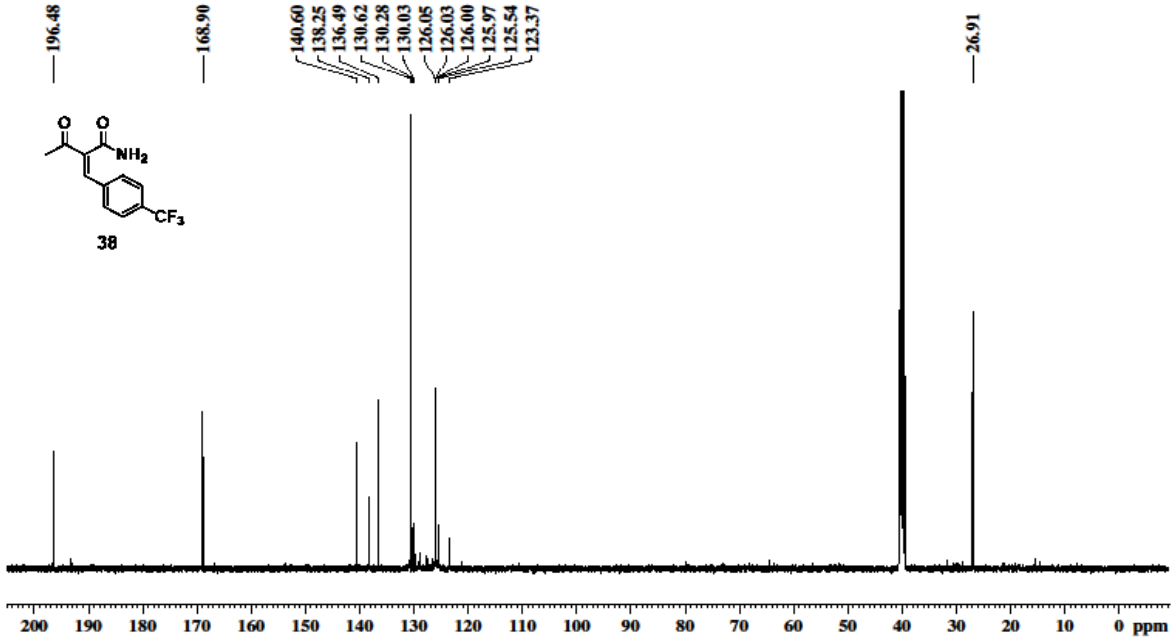


37

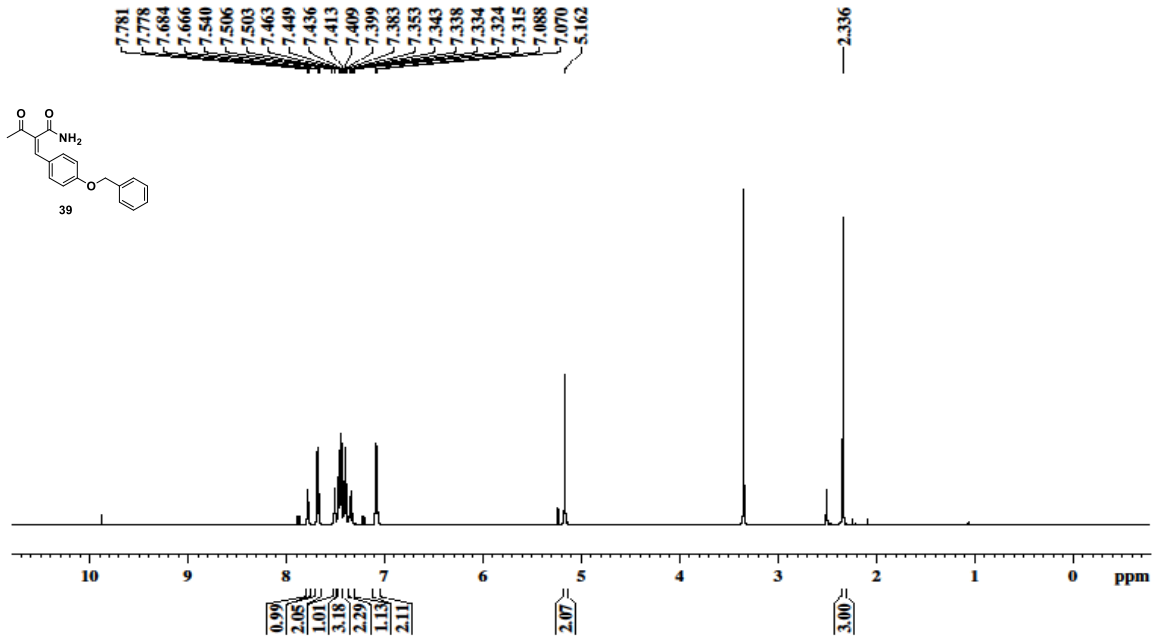
AS-7.


7.884
7.867
7.820
7.804
7.602
7.593
2.400
38
10
9
8
7
6
5
4
3
2
1
0
ppm
2.85
2.02
1.94
3.00

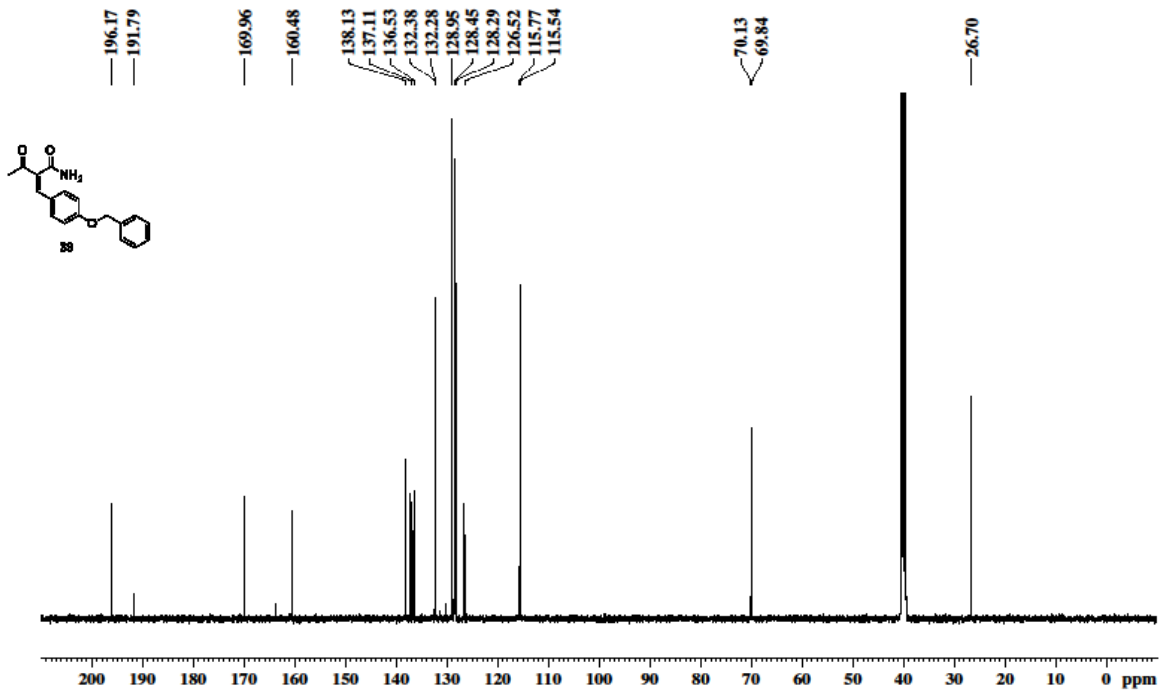
AS-7


196.48
168.90
140.60
138.25
136.49
130.62
130.28
130.03
126.05
126.03
126.00
125.97
125.54
123.37
26.91
38
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0 ppm

AS-8


7.781
7.778
7.684
7.666
7.540
7.506
7.503
7.463
7.449
7.436
7.413
7.409
7.399
7.383
7.353
7.343
7.338
7.334
7.324
7.315
7.088
7.070
5.162
2.336
O
O
NH2
O
39
10
9
8
7
6
5
4
3
2
1
0
ppm
0.99
2.05
1.01
3.18
2.29
1.13
2.11
2.07
3.00

AS-8.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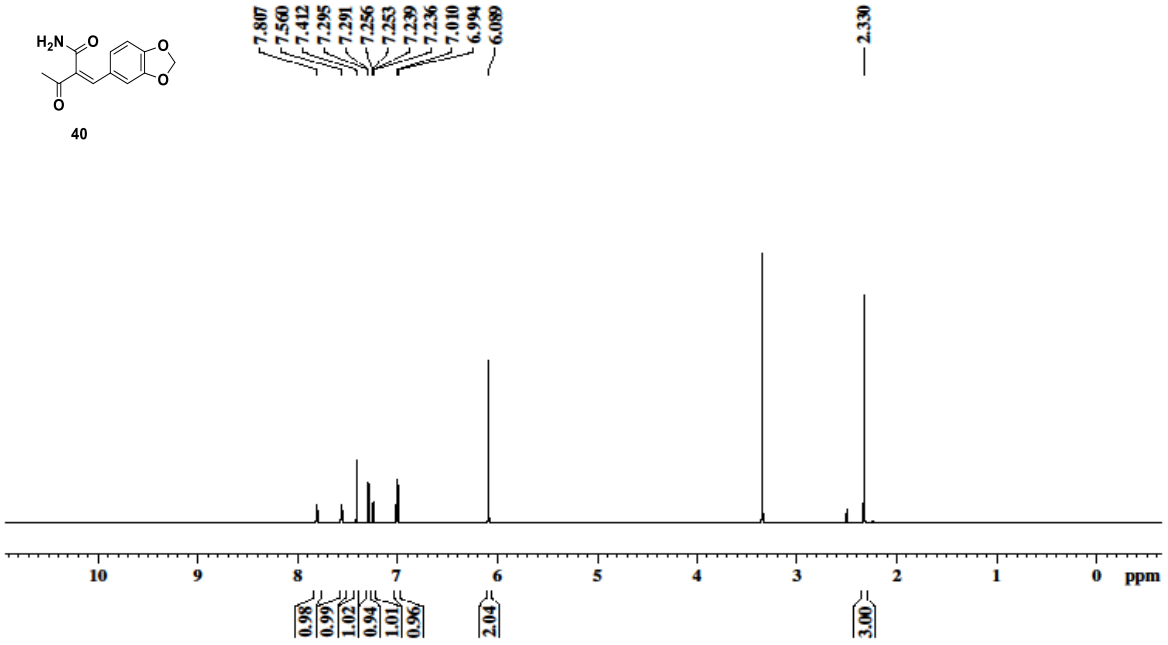
191.79
169.96
160.48
138.13
137.11
136.53
132.38
132.28
128.95
128.45
128.29
126.52
115.77
115.54
70.13
69.84
26.70
O
O
NH2
O
39
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm

AS-9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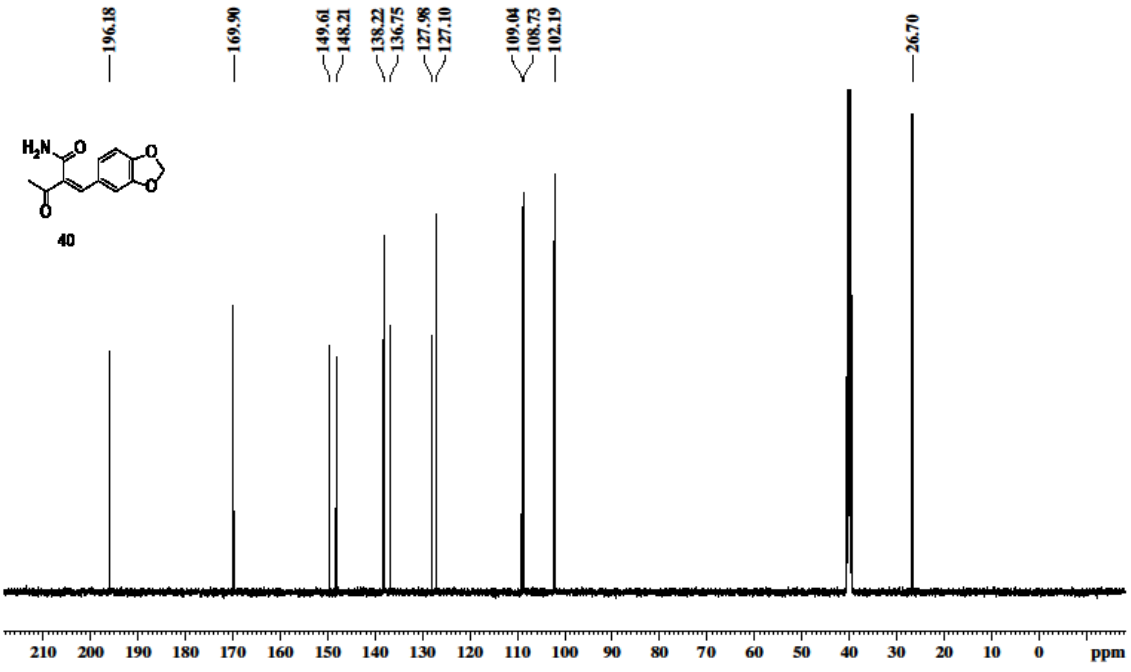
7.560
7.412
7.295
7.291
7.256
7.253
7.239
7.236
7.010
6.994
6.089
2.330
40
10
9
8
7
6
5
4
3
2
1
0
ppm
0.98
0.99
1.02
0.94
1.01
0.96
2.04
3.00

AS-9


196.18
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148.21
138.22
136.75
127.98
127.10
109.04
108.73
102.19
26.70
40
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm