Discovery of a tetrazolyl β-carboline with in vitro and in vivo osteoprotective activity in estrogen-deficientcondition

Anirudha Karvande,[‡a] Shahnawaz Khan,[‡b] Irfan Khan,[c] Deepti Singh,[c] Vikram Khedgikar,[a] Priyanka Kushwaha,[a] Naseer Ahmad,[a] Priyanka Kothari,[a] Anupam Dhasmana,[d] Ruchir Kant,[e] Ritu Trivedi*[a] and Prem M. S. Chauhan*[c]

[a]Endocrinology Division, CSIR-Central Drug Research Institute (CSIR-CDRI), Lucknow- 226031, India

[b]Chemistry Division, BHUPAL NOBLES' UNIVERSITY, Udaipur-313001, India.

[c]Medicinal and Process Chemistry Division, CSIR- Central Drug Research Institute, Lucknow-226031, U.P., India

[d]Research Himalayan School of Bio sciences, Swami Rama Himalayan University, Dehradun, India

[e]Molecular and Structural Biology Central Drug Research Institute, CSIR, Lucknow- 226031, India

[‡]A.K. and[‡]S.K. contributed equally to this work.

*Corresponding author: **RituTrivedi**, PhD,

Endocrinology Division, CSIR-Central Drug Research Institute, Sector 10, Jankipuram Extension,

Sitapur Road, Lucknow 226031, Uttar Pradesh, INDIA.

Telephone Number: +91-522 2772450; Fax Number: +91-522 2771941

Email: ritu_trivedi@cdri.res.in.

*Corresponding author: **Prem M. S. Chauhan**, PhD,

Division of Medicinal and Process Chemistry, CSIR-Central Drug Research Institute, Sector 10,

Jankipuram Extension, Sitapur Road, Lucknow 226031, Uttar Pradesh, INDIA.

Email: pms_chauhan@cdri.res.in

Table of contents

1.Experimental section

*General chemistry:*All reagents were commercial and were used without further purification. Chromatography was carried on silica gel (60-120 and 100-200 mesh). All reactions were monitored by thin-layer chromatography (TLC), silica gel plates with fluorescence F254 were used. Melting points were taken in open capillaries on Complab melting point apparatus and are presented uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded using BrukerSupercon Magnet DRX spectrometer (operating at 300 and 400 MHz for ^{1}H and 50, 75, 100 MHz for ^{13}C) using $CDCl_3$, DMSO-d_6 as solvent and tetramethylsilane (TMS) as an internal standard. Chemical shifts are reported in parts per million. Electrospray ionization mass spectra (ESIMS) were recorded on Thermo Lcq Advantage Max-IT. High-resolution mass spectra (HRMS) were recorded on 6520 Agilent Q TofLC-MS/MS (Accurate mass). Elemental analyses were performed on a Vario EL-III C, H, N, S analyzer (Germany) and Carlo-Erba-1108 C, H, N elemental analyzer (Italian), and values were within ±0.5% of the calculated values; therefore, these compounds meet the criteria of > 95% purity. A purity of ≥ 95% has been established for compounds, which showed good *in vivo* activity*.*

*Representative procedure compounds (8a- 8p):*Solution of **4a or 4b** (1 eq.) [1, 2]amine (1.2 eq.) and isocyanide (1.2 eq.) was stirred in anhydrous methanol (5mL) at RT for 10 min. Thereafter, sodium azide (3 eq.) was added and the resulting mixture was further stirred for 6h. On completion of the reaction (checked by TLC analysis), the methanol was removed *in vacuo*. The crude product was subjected to silica gel column chromatography using chloroform/methanol as the mobile phase to afford the desired compounds.

Charterization of compounds:

methyl   1-((benzylamino)(1-(tert-butyl)-1H-tetrazol-5-yl)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate (8a) - solid, Yield 78% (365mg), mp = 126-128 [0]C; ^{1}H NMR (400 MHz, CDCl$_3$): δ=10.67 (s, 1H), 8.72 (s, 1H), 8.07 (d, *J*=7.6 Hz, 1H), 7.57-7.49 (m, 2H), 7.24-7.17 (m, 6H), 6.05 (s, 1H), 3.91 (s, 3H), 3.78 (d, *J*=12.8 Hz, 1H), 3.67 (d, *J*=12.8 Hz, 1H), 1.67 (s, 9H) ppm; ^{13}C NMR (100 MHz, CDCl$_3$): δ 166.3, 154.6, 140.6, 140.3, 138.6, 136.4, 135.4, 130.5, 129.2, 128.6, 128.5, 127.5,

121.6, 121.4, 120.9, 118.1, 112.5, 62.5, 60.2, 52.4, 51.8, 30.1 ppm; HRMS (ESI) Calcd. for $C_{26}H_{28}N_7O_2^+$ [M+H]$^+$470.2299 Found 470.2296.

Methyl1-((1-tert-butyl-1H-tetrazol-5-yl)(4-methylbenzylamino)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate(8b) - solid, Yield 92% (444mg); mp = 163-165 ^{0}C ; ^{1}H NMR (400 MHz, CDCl$_3$): δ=10.66 (s, 1H), 8.72 (s,1H), 8.06 (s, 1H), 7.55 (s, 2H), 7.24-7.031 (m, 5H), 6.05 (s, 1H), 3.91 (s, 3H), 3.75 (d, *J*=11.2 Hz, 1H), 3.62 (d, *J*=12 Hz, 1H), 2.22 (s, 3H), 1.68 (s, 9H) ppm; ^{13}C NMR (100 MHz, CDCl$_3$): δ=166.3, 154.6, 140.6, 140.4, 137.3, 136.3, 135.6, 135.5, 130.5, 129.3, 129.1, 128.4, 121.6, 121.4, 120.8, 118.1, 112.5, 62.5, 60.1, 52.4, 51.6, 30.1, 21.1 ppm; HRMS (ESI) Calcd. for $C_{27}H_{20}N_7O_2^+$ [M+H]$^+$ 484.2456 Found 485. 2451.

Methyl1-((1-tert-butyl-1H-tetrazol-5-yl)(4-ethylpiperazin-1-yl)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate (8c) - solid, Yield 87% (414mg); mp = 146-148 ^{0}C; ^{1}H NMR (400 MHz, CDCl$_3$): δ=10.72 (s, 1H), 8.73 (s, 1H), 8.09 (d, *J*=7.6 Hz, 1H), 7.66 (d, *J*=8Hz, 1H), 7.57 (t, *J*=7.6Hz, 1H), 7.28-7.19 (m, 1H), 5.65 (s, 1H), 3.92 (s, 3H), 2.50-2.29 (m, 10H), 1.84 (s, 9H), 0.98 (t, *J*=6.8 Hz, 3H) ppm; ^{13}C NMR (100 MHz, CDCl$_3$): δ=166.2, 154.1, 140.6, 139.7, 136.2, 135.2, 130.0, 129.1, 121.5, 121.3, 120.7, 118.2, 112.5, 68.3, 62.5, 52.8, 52.4, 52.1, 51.7, 30.6, 11.9 ppm; HRMS (ESI) Calcd. For $C_{25}H_{33}N_8O_2^+$ [M+H]$^+$477.2721 Found 477. 2719.

methyl1-((1-tert-butyl-1H-tetrazol-5-yl)(tert-butylamino)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate (8d) **-** solid, Yield 89 % (388mg); mp = 157-160 ^{0}C; ^{1}H NMR (400 MHz, DMSO-d$_6$): δ= 11.78 (s, 1H), 8.85 (s, 1H), 8.38 (d, *J*=7.6 Hz, 1H), 7.86 (d, *J*=8 Hz, 1H), 7.62 (d, *J*=7.2 Hz, 1H), 7.32 (d, *J*=7.2 Hz, 1H), 6.12 (d, *J*=8.4 Hz, 1H), 3.87 (s, 3H), 1.82 (s, 9H), 0.97 (s, 9H) ppm; ^{13}C NMR (100 MHz, DMSO-d$_6$): δ=165.5, 155.8, 144.3, 140.7, 135.1, 134.8, 129.1, 128.7, 121.8, 120.5, 120.2, 117.3, 112.7, 62.2, 55.4, 51.9, 51.5, 29.5, 29.2 ppm; HRMS (ESI) Calcd. for $C_{23}H_{30}N_7O_2^+$ [M+H]$^+$ 436.2456 Found 436.2456.

methyl1-((1-tert-butyl-1H-tetrazol-5-yl)(3,4-dichlorobenzylamino)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate (8e) **-** solid, Yield 87 % (468mg); mp = 168-169 ^{0}C; ^{1}H NMR (400 MHz, CDCl$_3$): δ =10.75 (s, 1H), 8.83 (s, 1H), 8.17 (d, *J*=7.6 Hz, 1H), 7.69 (q, *J*=7.6 Hz, 2H), 7.41-7.28 (m, 3H), 7.13 (d, *J*=7.2 Hz, 1H), 6.12 (s, 1H), 4.05 (s, 3H), 3.79 (d, *J*=13.2 Hz, 1H), 3.70 (d, *J*=13.2 Hz, 1H), 1.78 (s, 9H) ppm; ^{13}C NMR (100 MHz, CDCl$_3$): δ=154.3, 140.7, 139.6, 138.8, 136.4, 135.2,

132.4, 131.4, 130.8, 130.4, 129.4, 127.7, 121.6, 121.4, 121.1, 118.3, 112.6, 62.4, 60.4, 52.4, 50.5, 30.1 ppm; HRMS (ESI) Calcd. for $C_{26}H_{26}Cl_2N_7O_2^+$ [M+H]$^+$538.1520 Found 538.1521.

ethyl1-((1-tert-butyl-1H-tetrazol-5-yl)(tert-butylamino)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate (8f) - solid, Yield 91 % (408mg); mp = 139-142 ^{0}C; ^{1}H NMR (400 MHz, CDCl$_3$): δ=10.56 (s, 1H), 8.65 (s, 1H), 8.06 (d, *J*=8 Hz, 1H), 7.55-7.47 (m, 2H), 7.24-7.18 (m, 1H), 6.18 (s, 1H), 4.38-4.30 (m, 2H), 2.68 (brs, 1H), 1.80 (s, 9H), 1.37 (t, *J*=7.2 Hz, 3H), 1.01 (s, 9H) ppm; ^{13}C NMR (100 MHz, CDCl$_3$): δ=165.8, 156.6, 142.4, 140.5, 136.3, 135.5, 130.2, 128.8, 121.5, 121.4, 120.6, 117.4, 112.4, 62.9, 61.1, 55.4, 52.3, 30.3, 29.5, 14.4 ppm; HRMS (ESI) Calcd. for $C_{24}H_{32}N_7O_2^+$ [M+H]$^+$450.2612 Found 450.2610.

ethyl1-((1-tert-butyl-1H-tetrazol-5-yl)(4-methylpiperazin-1-yl)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate (8g) - solid, Yield 88 % (418mg); mp = 176-178 ^{0}C; ^{1}H NMR (400 MHz, CDCl$_3$): δ=10.73 (s, 1H), 8.78 (s, 1H), 8.16 (d, *J*=7.6 Hz, 1H), 7.73 (d, *J*=8.4 Hz, 1H), 7.61 (s, 1H), 7.34 (d, *J*=7.6 Hz, 1H) 5.72 (s, 1H), 4.55-4.50 (m, 1H), 4.40-4.36 (m, 1H), 2.57 (m, 8H), 2.27 (s, 3H), 1.92 (s, 9H), 1.45 (t, *J*=7.2 Hz, 3H) ppm; ^{13}C NMR (100 MHz, CDCl$_3$): δ=165.7, 154.1, 140.6, 139.6, 136.5, 135.1, 130.0, 129.1, 121.6, 121.3, 120.6, 118.1, 112.5, 68.3, 62.6, 61.2, 55.2, 51.6, 45.8, 30.6, 14.4 ppm; HRMS (ESI) Calcd. for $C_{25}H_{33}N_8O_2^+$ [M+H]$^+$477.2721 Found 477. 2720.

ethyl1-((1-tert-butyl-1H-tetrazol-5-yl)(4-ethylpiperazin-1-yl)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate (8h) - solid, Yield 85 % (416mg); mp = 142-143 ^{0}C; ^{1}H NMR (400 MHz, CDCl$_3$): δ=10.73 (s, 1H), 8.78 (s, 1H), 8.16 (d, *J*=8 Hz, 1H), 7.72 (d, *J*=8 Hz, 1H), 7.63-7.59 (m, 1H), 7.34 (t, *J*=7.2 Hz, 1H), 5.72 (s, 1H), 4.55-4.50 (m, 1H), 4.40-4.36 (m, 1H), 2.56-2.37 (m, 10H), 1.93 (s, 9H), 1.45 (t, *J*=6.8 Hz, 3H), 1.06 (t, *J*=8 Hz, 3H) ppm; ^{13}C NMR (100 MHz, CDCl$_3$): δ=165.7, 154.1, 140.6, 139.7, 136.5, 135.1, 130.0, 129.1, 121.6, 121.3, 120.6, 118.1, 112.5, 68.3, 62.6, 61.2, 52.9, 52.1, 51.7, 30.7, 14.4, 11.9 ppm; HRMS (ESI) Calcd. for $C_{26}H_{35}N_8O_2^+$ [M+H]$^+$ 491.2878 Found 491.2879.

ethyl1-((1-tert-butyl-1H-tetrazol-5-yl)(4-methoxybenzylamino)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate (8i) - solid, Yield 92 % (471mg); mp = 161-162 ^{0}C; ^{1}H NMR (400 MHz, CDCl$_3$): δ=10.73 (s, 1H), 8.81 (s, 1H), 8.18 (d, *J*=7.6 Hz, 1H), 7.67-7.61 (m, 2H), 7.36 (t, *J*=7.2 Hz, 1H), 7.26 (d, *J*=7.6 Hz, 2H), 6.87 (d, *J*=7.2 Hz, 2H), 6.14 (s, 1H), 4.53-4.43 (m, 2H), 3.81 (s, 1H), 3.79 (s, 3H),

3.72 (s, 1H), 2.83 (s, 1H), 1.78 (s, 9H) 1.48 (t, J=7.2 Hz, 3H) ppm; ^{13}C NMR (100 MHz, CDCl$_3$): δ=165.8, 159.1, 154.7, 140.6, 140.3, 136.6, 135.4, 130.7, 130.4, 129.8, 129.1, 121.6, 121.4, 120.8, 117.9, 114.0, 112.5, 62.4, 61.2, 59.8, 55.3, 51.2, 30.1, 14.4 ppm; HRMS (ESI) Calcd. for C$_{28}$H$_{32}$N$_7$O$_3{}^+$[M+H]$^+$514.2561 Found 514.2560.

methyl1-((1-tert-butyl-1H-tetrazol-5-yl)(4-(pyrimidin-2-yl)piperazin-1-yl)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate (8j) **-** solid, Yield 89 % (468mg); mp = 180-181 ^{0}C; ^{1}H NMR (400 MHz, CDCl$_3$): δ=10.88 (s, 1H), 8.85 (s, 1H), 8.30 (d, J=4 Hz, 2H), 8.20 (d, J=7.6 Hz, 1H), 7.76-7.63 (m, 2H), 7.39-7.35 (m, 2H), 6.50 (s, 1H), 4.02 (s, 3H), 3.87-3.84 (m, 4H), 2.61 (s, 2H), 2.48 (s, 2H), 1.93 (s, 9H) ppm; ^{13}C NMR (100 MHz, CDCl$_3$): δ=166.2, 161.5, 157.7, 153.9, 140.7, 139.2, 136.3, 135.2, 130.2, 129.2, 121.6, 121.3, 120.8, 118.3, 112.6, 110.2, 68.5, 62.6, 52.4, 51.6, 43.7, 30.6 ppm; HRMS (ESI) Calcd. for C$_{27}$H$_{31}$N$_{10}$O$_2{}^+$[M+H]$^+$526.2626 Found 526.2623.

ethyl1-((1-tert-butyl-1H-tetrazol-5-yl)(morpholino)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate (8k) **-** solid, Yield 77 % (356mg); mp = 165-167 ^{0}C; ^{1}H NMR (300 MHz, CDCl$_3$): δ=10.81 (s, 1H), 8.82 (s, 1H), 8.19 (d, J=7.5 Hz, 1H), 7.76-7.62 (m, 2H), 7.38-7.29 (m, 1H), 5.76 (s, 1H), 4.55-4.43 (m, 2H), 3.74 (s, 4H), 2.57 (s, 2H), 2.42 (s, 2H), 1.94 (s, 9H), 1.49 (t, J=6.6 Hz, 3H) ppm; ^{13}C NMR (75 MHz, CDCl$_3$): δ=165.6, 153.6, 140.7, 138.8, 136.7, 135.1, 130.1, 129.1, 121.6, 121.3, 120.7, 118.1, 112.5, 68.7, 66.9, 62.66, 61.2, 52.2, 30.6, 14.4 ppm; HRMS (ESI) Calcd. for C$_{24}$H$_{20}$N$_7$O$_3{}^+$[M+H]$^+$464.2405 Found 464.2407.

ethyl1-((4-(benzo[d][1,3]dioxol-5-ylmethyl)piperazin-1-yl)(1-tert-butyl-1H-tetrazol-5-yl)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate (8l) **-** solid, Yield 79 % (470mg); mp = 186-188 ^{0}C; δ=^{1}H NMR (300 MHz, CDCl$_3$): δ=10.83 (s, 1H), 8.80 (s, 1H), 8.19 (d, J=7.8 Hz, 1H), 7.76-7.62 (m, 2H), 7.37-7.32 (m, 1H), 6.82 (s, 1H), 6.72 (s, 2H), 5.93 (s, 2H), 5.75 (s, 1H), 4.57-4.36 (m, 2H), 3.43 (s, 2H), 2.58 (s, 8H), 1.94 (s, 9H), 1.49 (t, J=6.9 Hz, 3H) ppm; ^{13}C NMR (75 MHz, CDCl$_3$): δ=165.7, 154.1, 147.6, 146.6, 140.6, 139.6, 136.5, 135.1, 131.6, 130.0, 129.0, 122.2, 121.5, 121.3, 120.6, 117.9, 112.5, 109.4, 107.8, 100.8, 68.3, 62.5, 62.4, 61.2, 52.9, 51.7, 30.7, 14.4 ppm; HRMS (ESI) Calcd. for C$_{32}$H$_{37}$N$_8$O$_4{}^+$ [M+H]$^+$ 597.2933 Found 597.2933.

ethyl1-((1-tert-butyl-1H-tetrazol-5-yl)(4-(furan-2-carbonyl)piperazin-1-yl)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate (8m) **-** solid, Yield 92 % (511mg); mp = 174-175 ^{0}C, ^{1}H NMR (300 MHz,

CDCl$_3$): δ=10.82 (s, 1H), 8.83 (s, 1H), 8.19 (d, *J*=7.5 Hz, 1H), 7.75-7.62 (m, 2H), 7.43-7.29 (m, 2H), 7.00 (s, 1H), 6.46 (s, 1H), 5.78 (s, 1H), 4.58-4.33 (s, 2H), 3.85 (s, 4H), 2.60-2.48 (m, 4H), 1.92 (s, 9H), 1.49 (t, *J*=6.9 Hz, 3H) ppm; ^{13}C NMR (75 MHz, CDCl$_3$): δ=165.5, 158.8, 153.6, 147.7, 143.6, 140.7, 138.6, 136.8, 135.1, 130.3, 129.2, 121.6, 121.3, 120.8, 118.1, 116.7, 112.6, 111.3, 68.4, 62.7, 61.2, 51.8, 30.6, 14.4 ppm; HRMS (ESI) Calcd. for C$_{29}$H$_{33}$N$_8$O$_4$$^+$ [M+H]$^+$ 557.2620 Found 557.2619.

ethyl1-((1-tert-butyl-1H-tetrazol-5-yl)(4-(4-fluorophenyl)piperazin-1-yl)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate (8n) **-** solid, Yield 89 % (483mg); mp = 185-186 ^{0}C; ^{1}H NMR (400 MHz, DMSO-d$_6$): δ=11.65 (s, 1H), 8.90 (s, 1H), 8.39 (d, *J*=7.6 Hz, 1H), 7.94 (d, *J*=8 Hz, 1H), 7.63 (t, *J*=7.6 Hz, 1H), 7.33 (t, *J*=7.2 Hz, 1H), 6.99 (d, *J*=8.4 Hz, 2H), 6.89 (d, *J*=4 Hz, 2H), 6.07 (s, 1H), 5.73 (s, 2H), 3.84 (s, 3H), 3.14 (s, 4H), 2.88 (s, 2H), 2.65 (s, 2H), 1.76 (s, 9H) ppm; ^{13}C NMR (100 MHz, DMSO-d$_6$): δ=165.4, 153.2, 147.5, 140.7, 140.5, 135.2, 130.2, 129.2, 121.6, 121.2, 120.8, 118.4, 117.9, 117.8, 116.9, 115.7, 115.4, 112.6, 68.4, 62.6, 52.5, 51.6, 50.2, 30.6 ppm; HRMS (ESI) Calcd. for C$_{30}$H$_{34}$FN$_8$O$_2$$^+$ [M+H]$^+$ 543.2627 Found 543.2630.

methyl1-((1-cyclohexyl-1H-tetrazol-5-yl)(4-methylpiperazin-1-yl)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate (8o) **-** solid, Yield 86 % (419mg); mp = 172-173 ^{0}C; ^{1}H NMR (400 MHz, DMSO-d$_6$): δ=12.18 (s, 1H), 8.90 (s, 1H), 8.39 (d, *J*=7.6 Hz, 1H), 7.85 (d, *J*=8 Hz, 1H), 7.66 (t, *J*=7.2 Hz, 1H), 7.34 (t, *J*=7.2 Hz, 1H), 5.96 (s, 1H), 5.49 (brs, 1H), 3.86 (s, 3H), 3.62 (s, 4H), 2.37-2.35 (m, 1H), 2.06 (s, 1H), 1.97-1.82 (m, 5H), 1.72-1.55 (m, 5H), 1.44-1.13 (m, 4H) ppm; ^{13}C NMR (100 MHz, DMSO-d$_6$): δ=165.5, 152.4, 141.1, 138.9, 136.3, 135.8, 129.1, 129.1, 122.1, 120.8, 120.5, 117.8, 112.8, 66.1, 63.4, 54.8, 52.0, 51.2, 33.2, 32.2, 25.1, 24.9 ppm; HRMS (ESI) Calcd. for C$_{26}$H$_{33}$N$_8$O$_2$$^+$ [M+H]$^+$489.2721 Found 489.2723.

methyl1-((1-tert-butyl-1H-tetrazol-5-yl)(2-methoxy-2-oxoethylamino)methyl)-9H-pyrido[3,4-b]indole-3-carboxylate (8p) **-** solid, Yield 84 % (378mg); mp = 156-157 ^{0}C; FT-IR (KBr) ν (cm^{-1}): 3365, 2963, 1755, 1349, 1274, 1139, 1041, 883, 767; ^{1}H NMR (400 MHz, CDCl$_3$): δ=11.19 (s, 1H), 8.81 (s, 1H), 8.17 (d, *J*=8 Hz, 1H), 7.69-7.59 (m, 2H), 7.36 (t, *J*=7.6 Hz, 1H), 6.11 (s, 1H), 3.94 (s, 3H), 3.79 (s, 3H), 3.68-3.44 (m, 2H), 3.15 (s, 1H), 1.77 (s, 9H) ppm; ^{13}C NMR (100 MHz, CDCl$_3$): δ 173.3, 166.3, 154.1, 140.7, 140.2, 136.1, 135.6, 130.1, 129.1, 121.6, 121.3, 120.7, 118.3, 112.5,

62.4, 60.1, 52.3, 48.2, 30.1 ppm; HRMS (ESI) Calcd. for $C_{22}H_{26}N_7O_4^+$ [M+H]$^+$452.2041 Found 452.2040.

Biological methods

Chemicals and reagents:Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA). All fine chemicals were purchased from Sigma Aldrich (St. Louis, MO). High-performance liquid chromatography grade acetonitrile was obtained from Merck India Ltd. (Mumbai, India).

Culture of rat calvarial osteoblasts: Rat calvarial osteoblasts(RCOs) were obtained following the previously published protocol of sequence digestion. [3] Briefly, Calvaria from 1-2 day old Sprague Dawley rats pups (both sexes) were pooled. After surgical isolation from the skull and the removal of sutures and adherent mesenchymal tissues, calvaria were subjected to five sequential (10–15 min) digestions at 37 °C in a solution containing 0.1% Dispase and 0.1% collagenase P. Cells released from the second to fifth digestions were pooled, centrifuged, resuspended, and plated in T-25 cm^2 flasks in α-MEM containing 10% FBS and 1% penicillin/streptomycin (complete growth medium).

Osteoblast differentiation: For the measurement of alkaline phosphatase (ALP) activity, RCOs at ~80% confluence were trypsinized and 2×10^3 cells per well were seeded in 96-well plates. Two additional columns containing only media (No cells) were prepared to check interference of the testing compound either on the assay substrate or having OD absorption at 405 nm. Cells were treated with different concentrations of compound for 48 h in α-MEM supplemented with 5% charcoal-treated FBS, 10 mM β- glycerophosphate, 50 µg·mL^{-1} ascorbic acid, and 1% penicillin or streptomycin (osteoblast differentiation medium). At the end of the incubation period, total ALP activity was measured using p- nitrophenyl phosphate (PNPP) as substrate and absorbance was read at 405 nm using SpectraMax Paradigm Multi well Elisa plate reader (Molecular Devices). [4]

Cytotoxicity assay: The toxicity of synthesized compounds was tested on RCOs. Cells were cultured in the absence or presence of compounds at various concentrations (1 pM to1µM) for

48h. The cell viability was determined by using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay.[5]

RNA isolation and Real-Time Polymerase Chain Reaction (RT-PCR): At the endof the treatment, RCOs were homogenized using 1mlof TRIzol reagent (Invitrogen), and total RNA was extracted according to themanufacturer's protocol. For bone-specific gene expression analysis,frozen femur bones were crushed in liquid nitrogen and homogenized using 1ml of TRIzol reagent (Invitrogen), followed by total RNA extraction using manufacturer's protocol. Primers were designed using the Universal Probe Library (Roche Applied Sciences) for the selected genes and given in **Table S2** (supporting information).[3] For real-time PCR, cDNA was synthesized with Revert Aid cDNA synthesis kit (Fermentas, Austin, TX, U.S.) using 2.0 µg of total RNA. SYBR green chemistry was used to perform quantitative determination of relative expression of transcripts for all genes. All genes were analyzed using the Light Cycler 480 (Roche Molecular Biochemicals, Indianapolis, IN, U.S.) real-time PCR machine.TheqPCR reaction was performed for quantitative comparative measurement of the expression of osteoblast specific genes Runx-2, Col-1, BMP-2, and OCN following an optimized protocol described before.[3] Transcript levels were normalized to housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. PCR analysis was carried out using comparative CT ($\Delta\Delta$CT) method.[6]

Mineralized nodule formation assay: RCOs (10,000 to 15,000 cells/cm^2 cells per well) were seeded on to 12-well plates (in duplicate plates) by using trypan blue and haemocytometerin osteoblast differentiation medium. RCOs were cultured with or without test compounds for 21 days and after every 48 h media were changed. At the end of the experiment, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 15 min. The fixed cells were stained with alizarin red-S (ARS) (40 mM, pH 4.5) for 30 min followed by washing with tap water. Stained cells were first photographed under a light microscope, and alizarin stain was then extracted by using 10% (v/v) acetic acid with shaking at room temperature for 30 min. Cells were scrapped out from wells and centrifuged (2000g for 15 min), and the supernatant was collected. To the supernatant, 10% (v/v) ammonium hydroxide was added to bring the pH of the solution to 4.5 for color formation. The

absorbanceof the solution was read at 405 nm. Differences in Alizarin Red S staining due to cell proliferation were accounted for by normalization of optical densities to cell number as previously described.[7]

BrdU assays:For BrdU incorporation assay, RCOs (2 x 10^3 cells per well) were seeded on 96-well plate(in duplicate plates) according to manufacturer's protocol (Abcam). After growth in regular media cells were treated with compound **8g** with active doses for 48h in differentiation media. BrdU was added to the media. Then the cells were incubated for 24h.One plate has been assessed for cell number and the other for BrdU incorporation. After incubation, cells were fixated and incubated with the BrdU antibody at RT for 1hr. Followed by this washing is performed and incubated with peroxidase tagged secondary antibody for 30 min at RT. After incubation washing was performed and TMB peroxidase substrate is added and incubated for 30 min in dark. The reaction was stopped by stopping solution and measured at 450 nm. Differences in BrdU incorporation due to cell proliferation were accounted for by measuring BrdU (+) osteoblasts (% of total).

Western blot analysis: RCOs were grown 60–70% confluence followed by treatment with compounds 8j,8i, and 8j with active dose for 48 h and in next experiment cells were treated with compound **8g** (100pM) and 17 β-estradiol (10nM) in presence of TNFα (10 ng·ml^{-1}) for 24 hours. The cells were washed with cold phosphate buffered saline (PBS), and whole cell lysates were prepared by the addition of lysis buffer, Sigma Aldrich (St. Louis, MO, U.S.), containing a protease and phosphatase inhibitor mixture, Sigma Aldrich (St. Louis, MO, U.S.). Nuclear and cytosolic fractions were separated using manufacture's protocol (CelLyticNuCLEAR Extraction Kit, Sigma-Aldrich). Aliquots of 20–40 µg of cell lysates were separated on SDS–PAGE under reducing conditions (Bio-Rad, Hercules, CA, U.S.) and then transferred to polyvinylidenedifluoride (PVDF) membranes (Millipore, Watford, U.K.). The membrane was blocked for nonspecific binding in 5% nonfat dry milk and followed by incubation with a primary antibody (Abcam, Cambridge, U.S.) at 4°C overnight. Membranes were washed and were probed with a horseradish peroxidase-conjugated secondary antibody (Abcam, Cambridge,

U.S.). Western blot signals were detected and visualized by an enhanced chemiluminescence system (GE Healthcare Life Sciences, India). [8]

Immunocytochemistry: Rat primary osteoblast were incubated in medium with or without compound **8g** (100 pM) and 17 β-estradiol (10 nM) in presence of TNFα (10 ng·ml^{-1}) and were grown in Lab-Tek Chamber Slides (Nunc, Denmark) for 24 h. For immunocytochemistry, cells were fixed with 4% paraformaldehyde (PFA) followed by permeabilization with 0.1% triton X-100 and incubation in primary antibody (NF-кB) for overnight. Cy3 goat anti-Rabbit Invitrogen used as secondary antibody. Fluorescence was captured using a fluorescent microscope (Eclipse80i, Nikon, Tokyo, Japan) with the aid of appropriate filter (excitation 552 nm and emission 570 nm).[8]

Measurement of intracellular ROS: Respiratory burst was assessed by measuring oxidation of DCFH-DA with a fluorescence reader (wavelength Ex 485nm/Em535nm) capable of reading microtiter plates. Osteoblast cells after treatment for 24 h were washed twice with the serum-free medium in 96 wells plate. DCFH-DA at 10 µg·mL^{-1} was added to regular culture medium with 2% serum. The plate was incubated in dark for 30 min. ROS generation was assessed using fluorescence reader (Biotek).

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay for antioxidant activity: Radical scavenging activities of the compound were determined by DPPH radical scavenging assay. [9]DPPH is a dye which produces free radicals and is used to evaluate the antioxidant property of plant extract and their derivative compounds. DPPH produces free radical that gives a violet color solution and this violet solution is reduced in the presence of an antioxidant molecule, changes are measured in spectrophotometric measurement. DPPH was dissolved in methanol, and the compoundwas serially diluted from 1000 µg·ml^{-1}to 1.95 µg·ml^{-1}. DPPH radicals and different concentrations of compound were used as 1:1 ratio. The DPPH solution with and without compound at each concentrationwas allowed to keep at room temperature for 30 minutesthen measured at 517 nm. Changes are measured due to radical scavenging. The DPPH scavenging activity/ Free radical scavenging activity (Anti-oxidant activity) of the compound was measured as follows: DPPH scavenging effect (%) = [(Abc − Abs) / Abc] × 100. (Abc is the valueof

DPPH without the sample (compound); Abs is the value of DPPH with compound concentrations from 1.95 µg·ml^{-1} to 1000 µg·ml^{-1}.

Nitric Oxide free radical scavenging activity: Nitric oxide free radical scavenging activities of compound **8g** was determined by NO detection.[10] Sodium nitroprusside(SNP) is a nitric oxide donor which produces free radicals. To check the nitric oxide free radical scavenging activity, 1 mg·ml^{-1} compound **8g** was dissolved in methanol. Compound **8g** was serially diluted from 1000 µg·ml^{-1} to 1.95 µg·ml^{-1}. 0.5ml volume was taken from each concentration and 2.0mL of sodium nitroprusside (10mM) was added to each tube. All samples with and without **8g** were incubated for 150 minutes. After the incubation of all samples, an equal volume of Griess reagent was added to each sample and the absorbance of chromophore was measured at 546 nm. The percentage free radical scavenging activity was calculated: % Scavenging = [(Abc – Abs) / Abc] × 100, where Abc is absorbance of control and Abs is for sample (**8g**).

Apoptosis assay: RCOs were grown to 50–60% confluence, followed by serum withdrawal for 3h and treatment with compound **8g** (100pM) and E2 (10nM) in presence of TNFα for 24h in α-MEM containing 0.5% FBS. Annexin-V/PI staining for FACS analysis was carried out using Annexin-V-FITC Apoptosis Detection Kit (Invitrogen) according to manufacturer's instructions.[11]

***In vivo* experiments:** All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India and approved by the Institutional Animal Ethical Committee (IAEC) at Central Drug Research Institute, (CDRI), Lucknow, India. 10-12 weeks old adult female Sprague Dawley rats (SD) were used for the study. Animals were housed at 21 °C in 12 h light per 12 h dark cycles. Normal chow diet and water were provided ad libitum. Ten rats per group were taken for the study. The animals were ovariectomized and left for 12 weeks for osteopenia to develop (Figure S1). Thereafter, the animals were divided into groups as follows: sham (ovary intact) + vehicle (1% gum acacia in distilled water)[12], Ovx + vehicle, Ovx + 1.0, Ovx + 5.0 mg·kg^{-1}·day^{-1} body weight dose of compound **8g** and standard control group Ovx + 17 β-estradiol (100µg·kg^{-1} five days a week, subcutaneously). Rats were treated with compound **8g** or vehicle

once daily for 12 weeks by oral gavage. After the period of 12 weeks, animals were sacrificed and the left and the right femurs were separated and collected for analysis of bone mineral density (BMD), trabecular microarchitecture and qRT-PCR analysis. Uteri were collected after all fat tissue was trimmed from uterine horns and weighed. μCT experiments were carried out using Sky Scan 1076 micro-CT scanner (Aartselaar, Belgium) as previously reported.[13, 14] For *ex vivo* experiments bone marrow from the femur of the vehicle and compound **8g** treated rats was flushed and cultured in osteoblast differentiation medium (10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μg·mL^{-1} ascorbic acid) for 18 days. ARS stain was used for staining mineralized nodules followed by extraction of the stain for quantitation.[15, 16]

Analysis of bone turnover markers: Serum samples separated after autopsy were used for the experiment. On the basis of our previously published protocols, serum CTX and PINP were determined by enzyme-linked immunosorbent assay kits by following the manufacturer's protocols (Immunodiagnostic Systems Inc.). [17,11]

Fluorochrome labeling and bone histomorphometry: For determination of new-bone formation in vivo, each rat received intraperitoneal administration of fluorochromes tetracycline (20 mg·kg^{-1}) and calcein (20 mg·kg^{-1}) following a previously published protocol.[18] At autopsy, femur bone was dissected out and (50 μm thickness) cross sections were cut using an Isomet slow speed bone cutter (Buehler, Lake Bluff, IL, U.S.). Photographs of the sections were taken under a fluorescent microscope aided with appropriate filters. Histomorphometric analysis for bone formation, such as for the determination of mineral appositional rate (MAR) and bone formation rate (BFR), was performed using Leica-Qwin software (Leica Microsystems Inc., Buffalo Grove, IL, U.S.) as described in our previously published protocol.[3]

Bone strength: Femora were subjected to three-point bending using a bone strength tester (model TK-252C; Muromachi Kikai, Co. Ltd, Tokyo Japan). [14,19]

In vivo toxicity and liver histology: Following treatment with compound **8g**, uterus and livers from different groups were harvested and fixed in 4% formaldehyde. A sample of each uterus and liver was dehydrated in ascending grades of isopropanol, cleared in xylene, and embedded in paraffin wax using standard procedures. Transverse sections (5 μm) were stained with

Erhlichshematoxylin and eosin, and representative images were captured using a Leica Qwincamera.[20] Total uterine area, luminal area, and luminal epithelial height was measured using Image Pro Plus software (Media Cybernetics, Silver Springs, MD).[21]

Statistical analysis: Data are expressed as the mean ± SEM. The data obtained in experiments with multiple treatments were subjected to one-way ANOVA followed by post hoc Newman–Keuls multiple comparison test of significance and student's t-test was used for experiments with only two treatments using GraphPad Prism 5.04 software.[40]

2. HPLC report of compound 8g

CDRI SAIF GLC & HPLC LAB
Lucknow

SAMPLE INFORMATION

Sample Name:	PM-UGT--02-C	Acquired By:	System
Sample Type:	Unknown	SampleName PM-UGT--02-C	
Vial:	1		
Injection #:	2	Acq. Method Set:	GENRAL HPLC
Injection Volume:	5.00 ul	Processing Method:	PMUGT02
Run Time:	30.0 Minutes	Channel Name:	265.0nm
Date Acquired:	3/9/2017 4:38:08 PM IST		
Date Processed:	3/9/2017 5:05:03 PM IST		

Auto-Scaled Chromatogram

Peak Results

	Name	RT	Area	Height	% Area
1		4.137	282063	6945	0.48
2		5.240	58317151	2644232	99.41
3		17.180	66704	4485	0.11

Reported by User: System
Report Method: CDRI_Report
Report Method ID 47962
Page: 1 of 1

Project Name: RKP
Date Printed:
3/9/2017
5:05:39 PM Asia/Calcutta

3. X-ray crystallographic structure of compound 8b
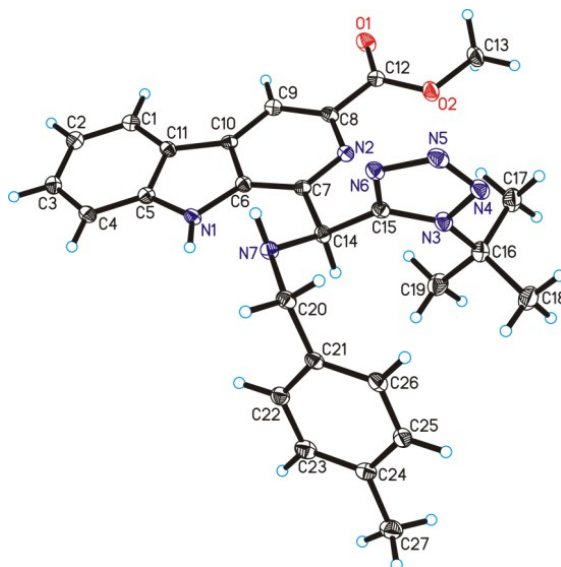
The crystal data of **8b**: $C_{27}H_{29}N_7O_2$, M = 483.57, Monoclinic, P2(1)/n , a = 9.814(2) Å, b = 9.916(2) (2)Å, c = 25.156(5) Å, β = 90.477(4) °, V = 2448.0(9) Å^3, Z = 4, D_c = 1.312 g cm^{-3}, μ (Mo-Kα) = 0.087 mm^{-1}, $F(000)$ = 1024, rectangular block, 15541 reflections measured (R_{int} = 0.0873), 6035 unique, wR$_2$ = 0.1895 for all data, conventional $R1$= 0.0633 for 3730 Fo > 4σ(Fo) and 0.1147 for all 6035 data, S = 1.073 for all data and 334 parameters.Unit cell determination and intensity data collection was performed on a Bruker SMART APEX CCD area-detector at 100(2) K. Structure solutions by direct methods and refinements by full-matrix least-squares methods on F^2. Programs: SMART 32(Bruker), SAINT (Bruker, 2001) ,SHELXTL-NT [Bruker AXS Inc.: Madison, Wisconsin, USA 1997]. CCDC (deposit No: 1542868) contains the supplementary crystallographic data. These data can be obtained free of charge from www.ccdc.cam.uk/conts/retrieving. html or from the Cambridge Crystallographic Data Center,12 Union Road, Cambridge,CB21EZ, U. K; Fax: (internat.) + 44-1223/336-033; E-mail: deposit@ccdc.cam.ac.uk.

4. Copies of ^{1}H and ^{13}C spectra of compounds
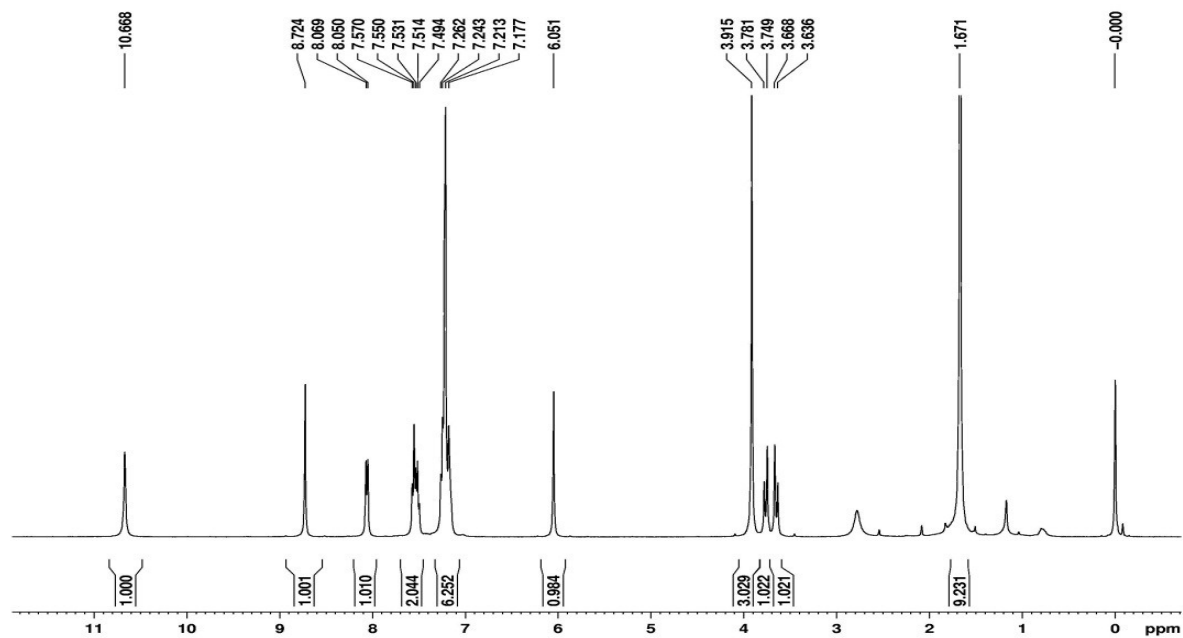
Fig.S1.^{1}H spectra of Compound 8a
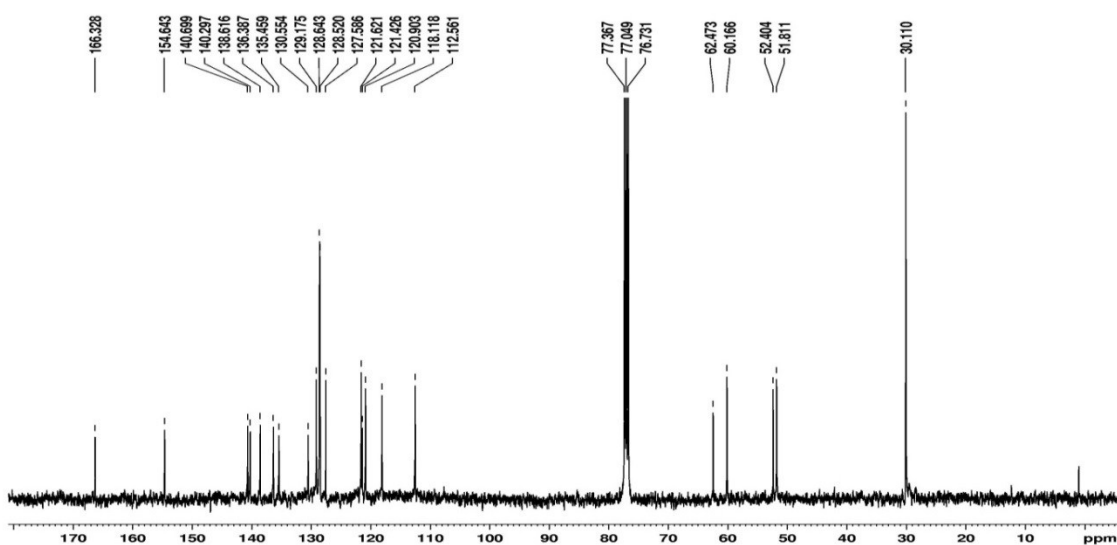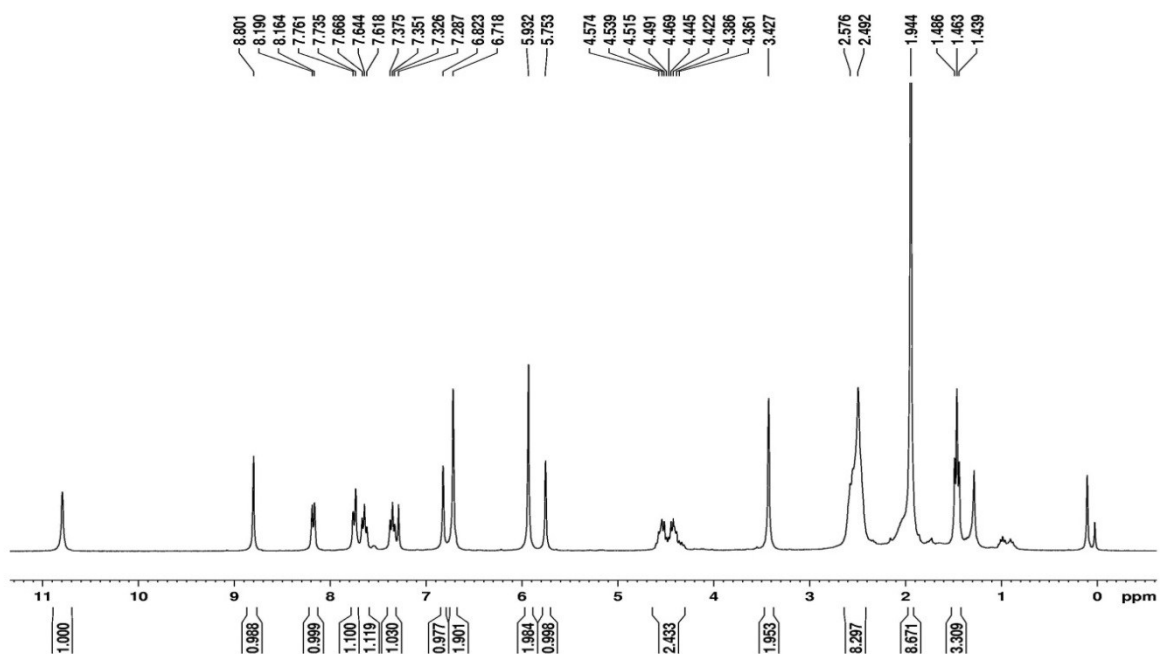

Fig.S2.^{13}C spectra of Compound 8a
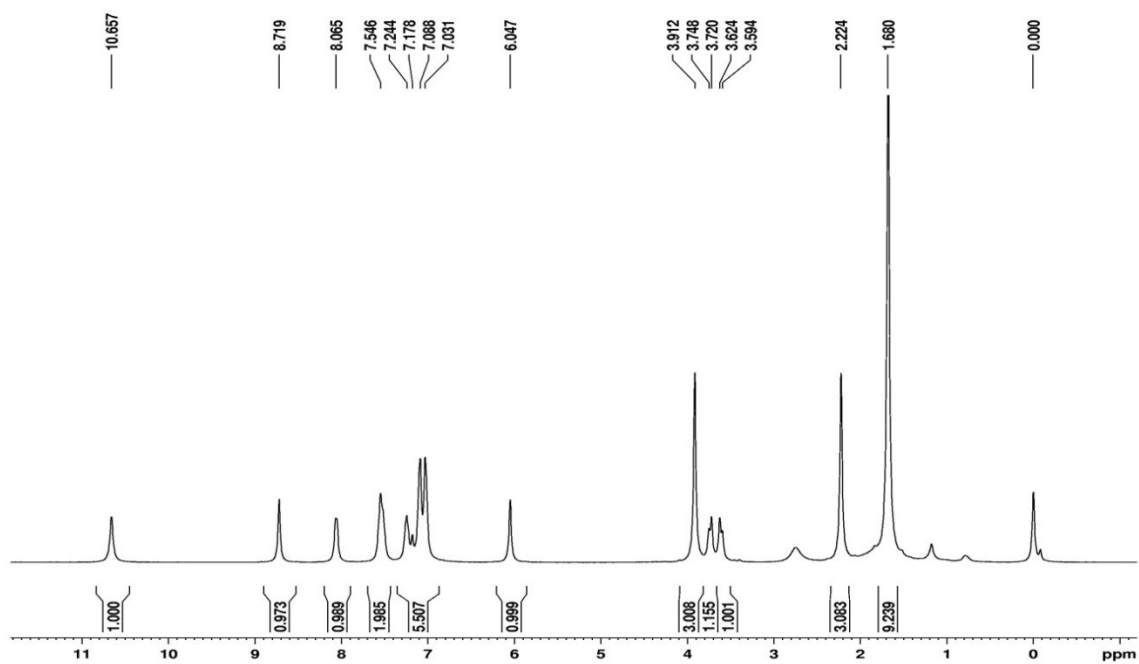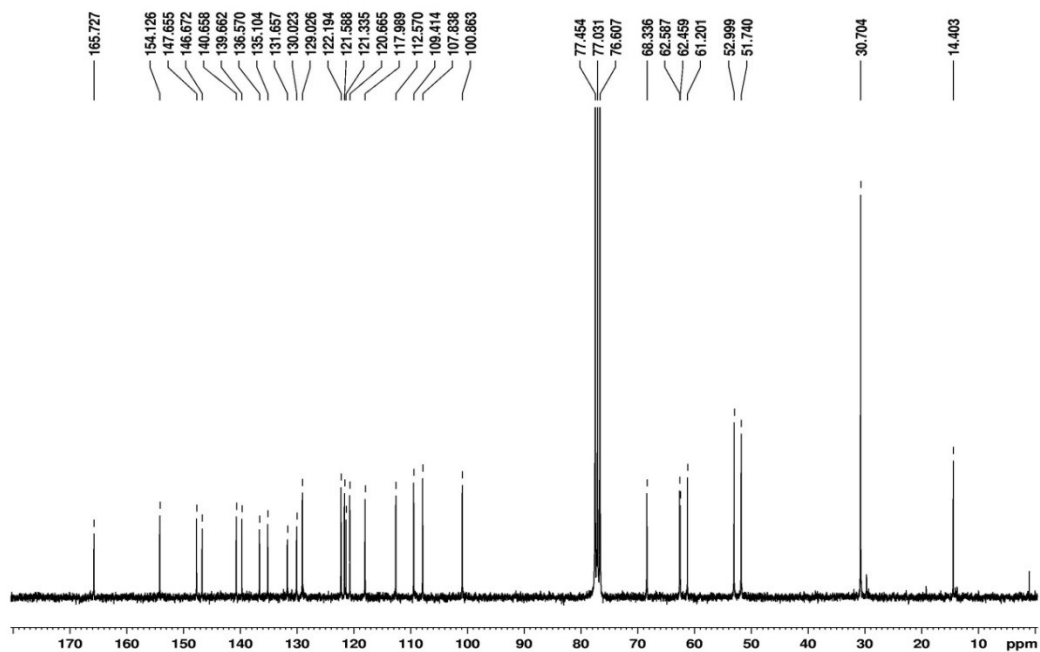
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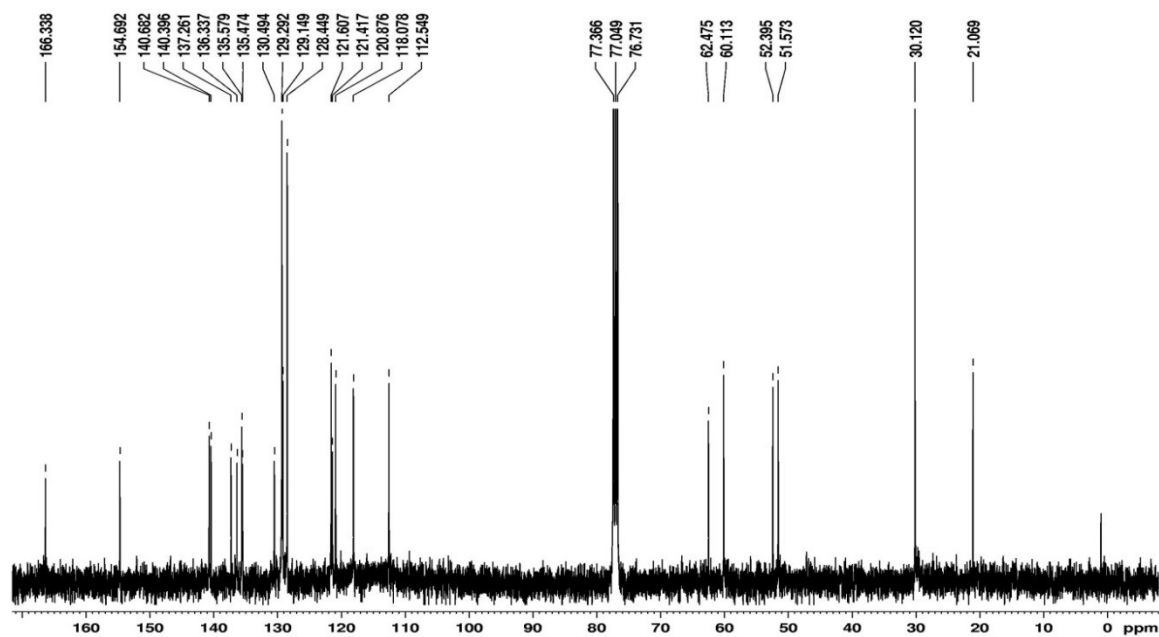
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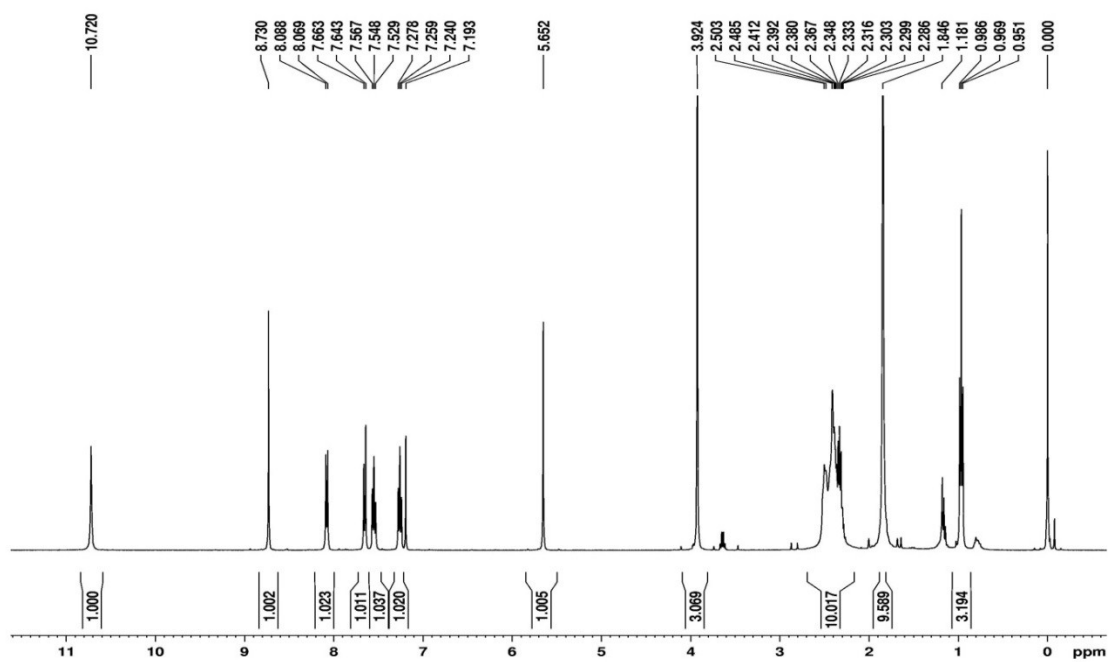
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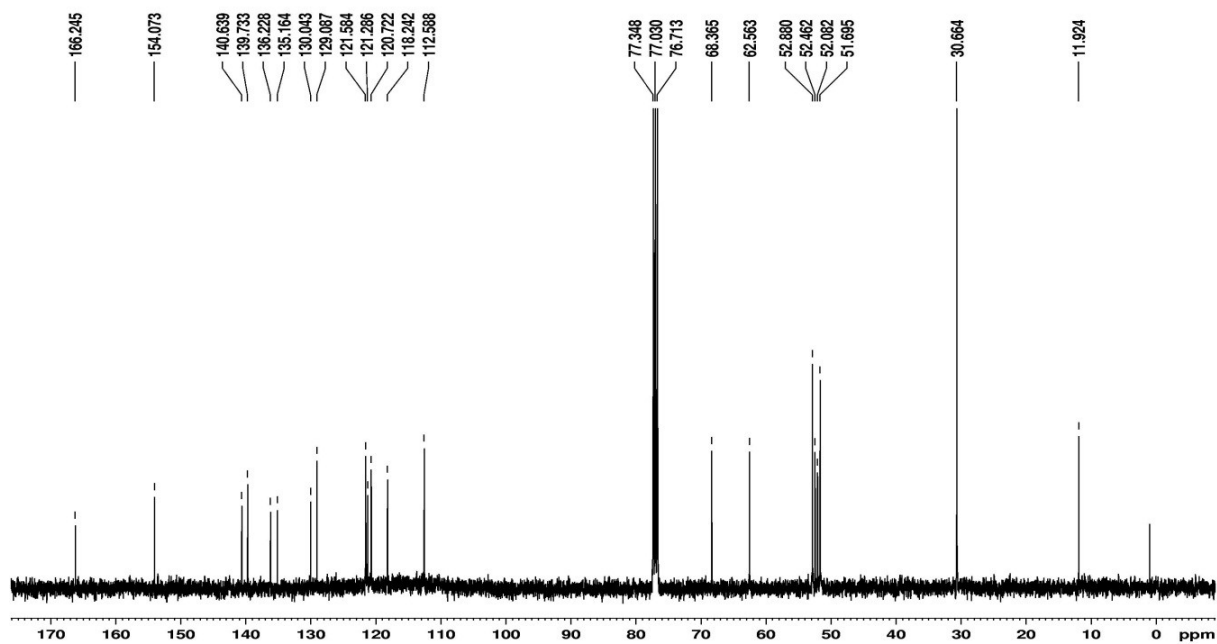
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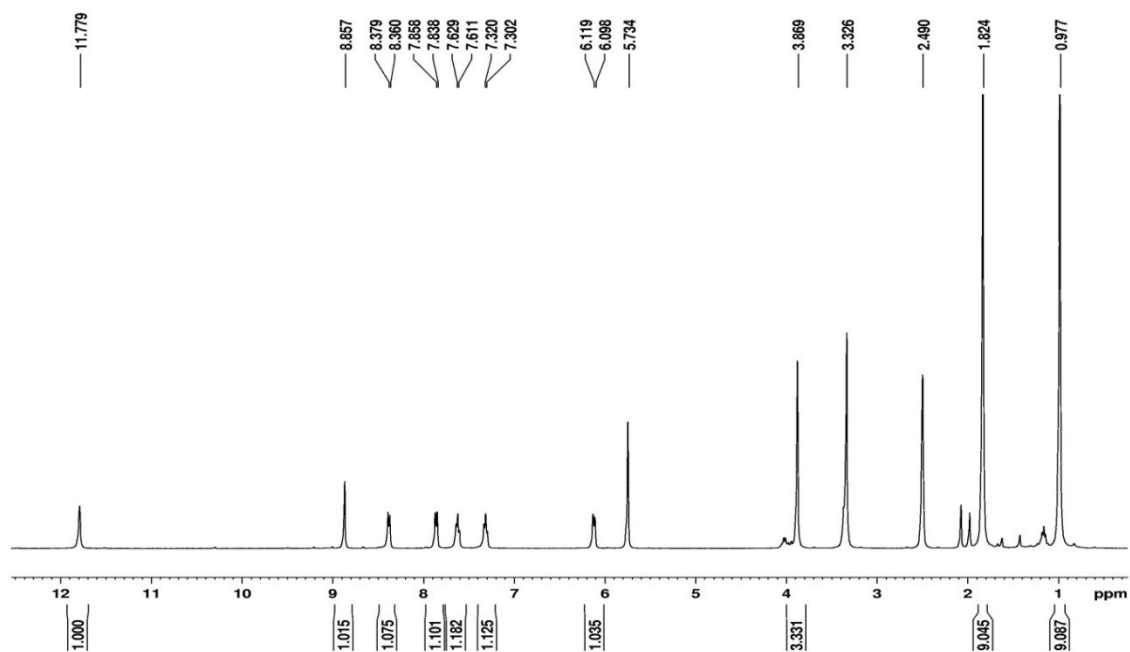
Fig. S7.^{1}H spectra of Compound 8d
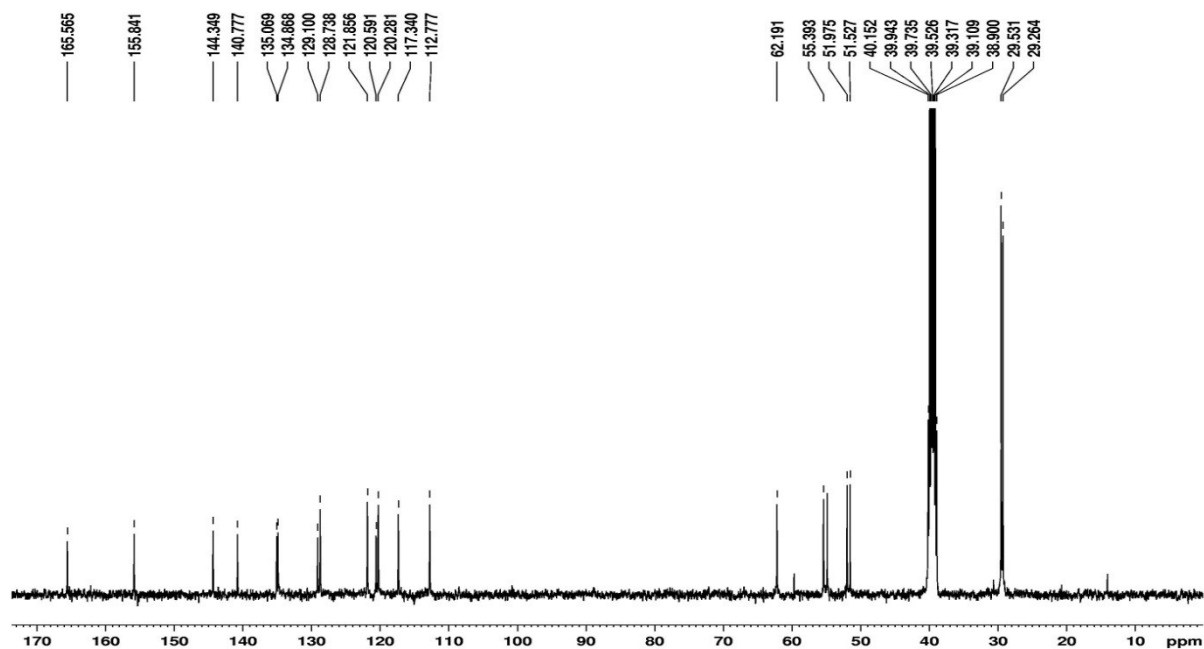
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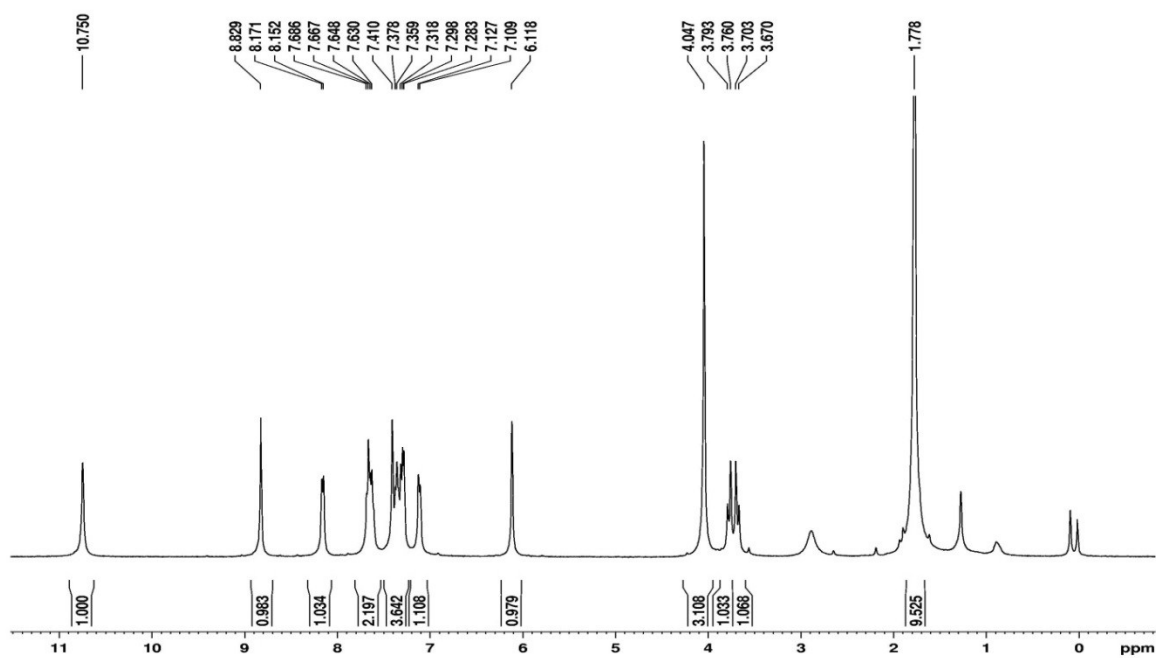
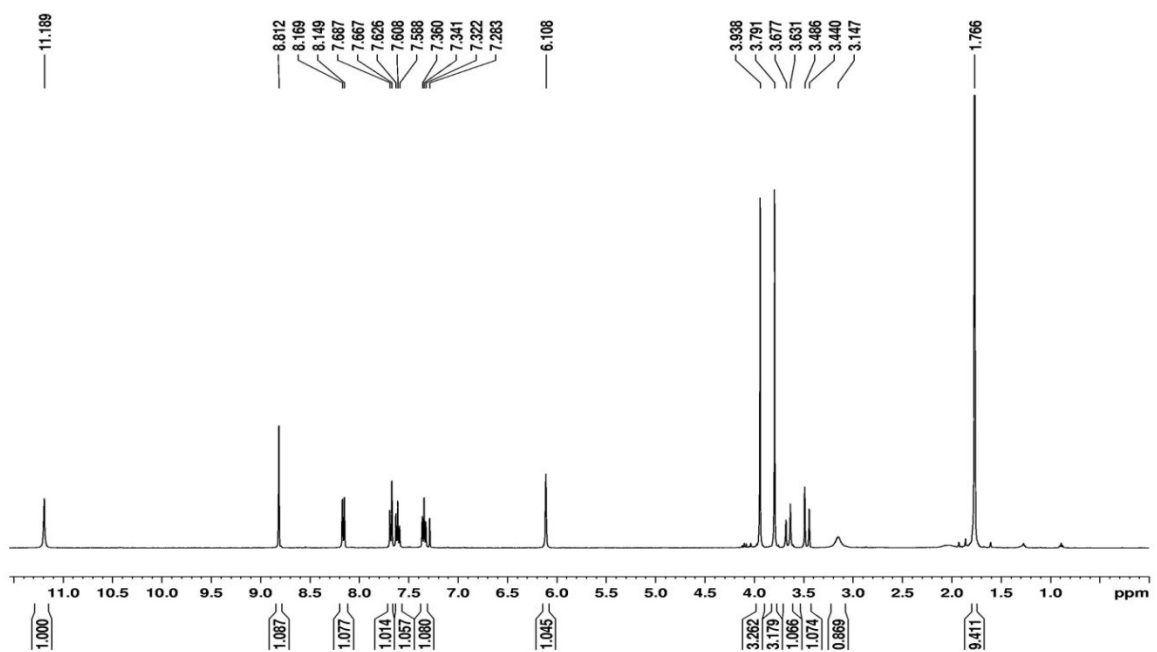**Fig.S9.^{1}H spectra of Compound 8e**
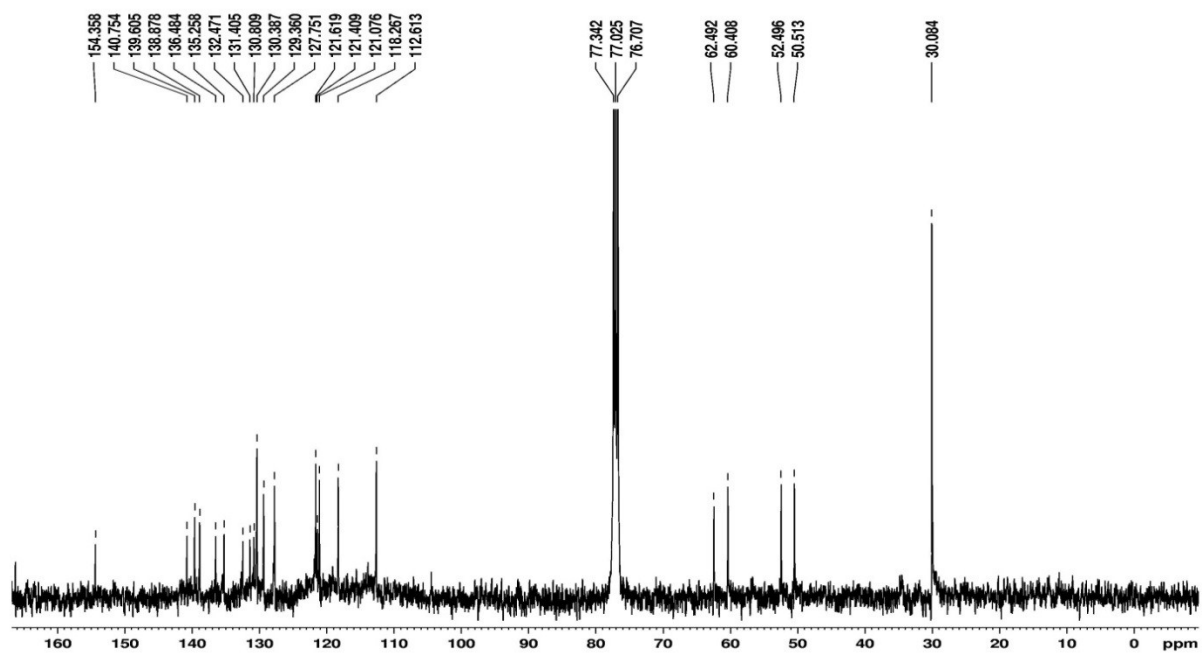
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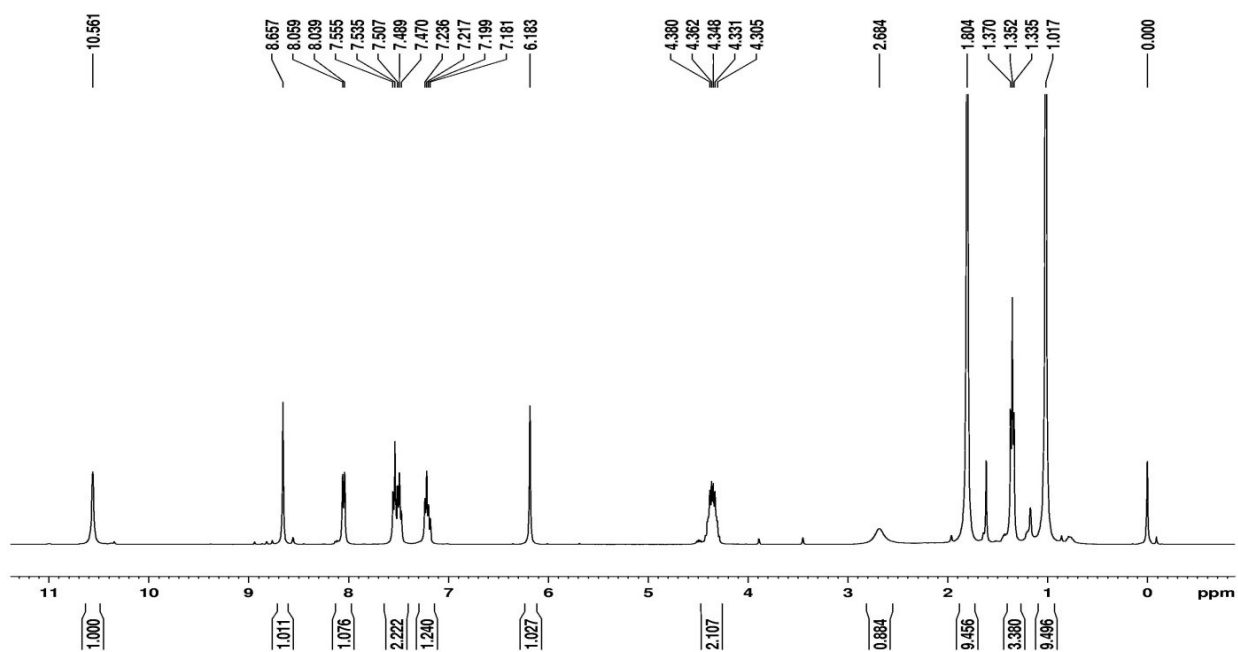
Fig.S11.^{1}H spectra of Compound 8f
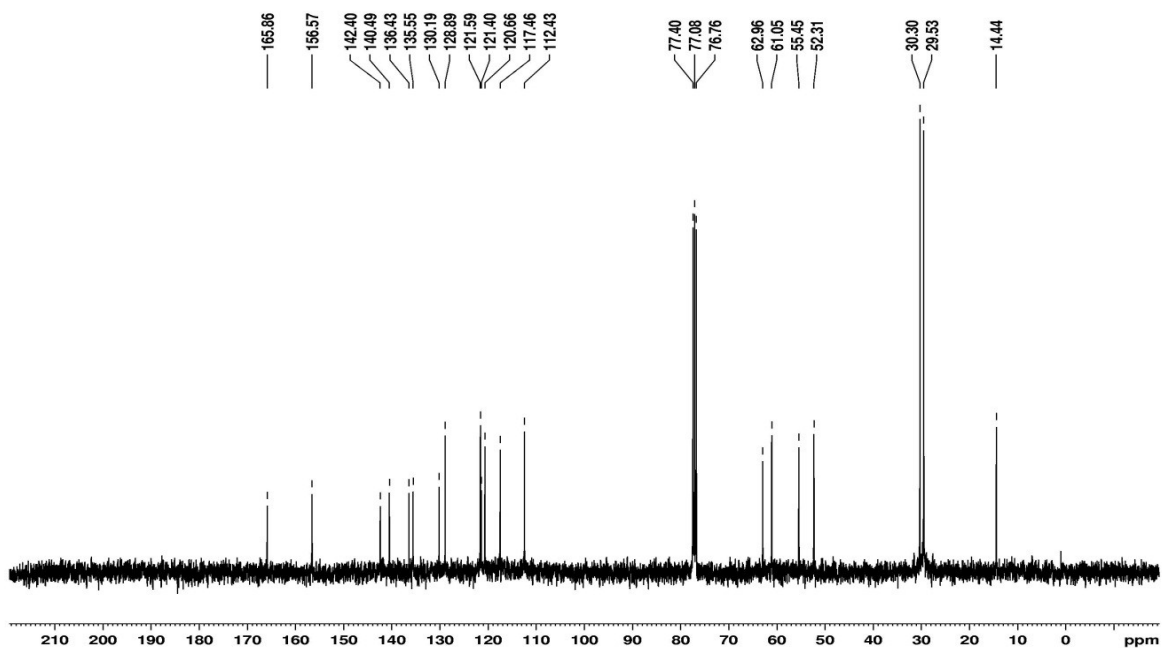
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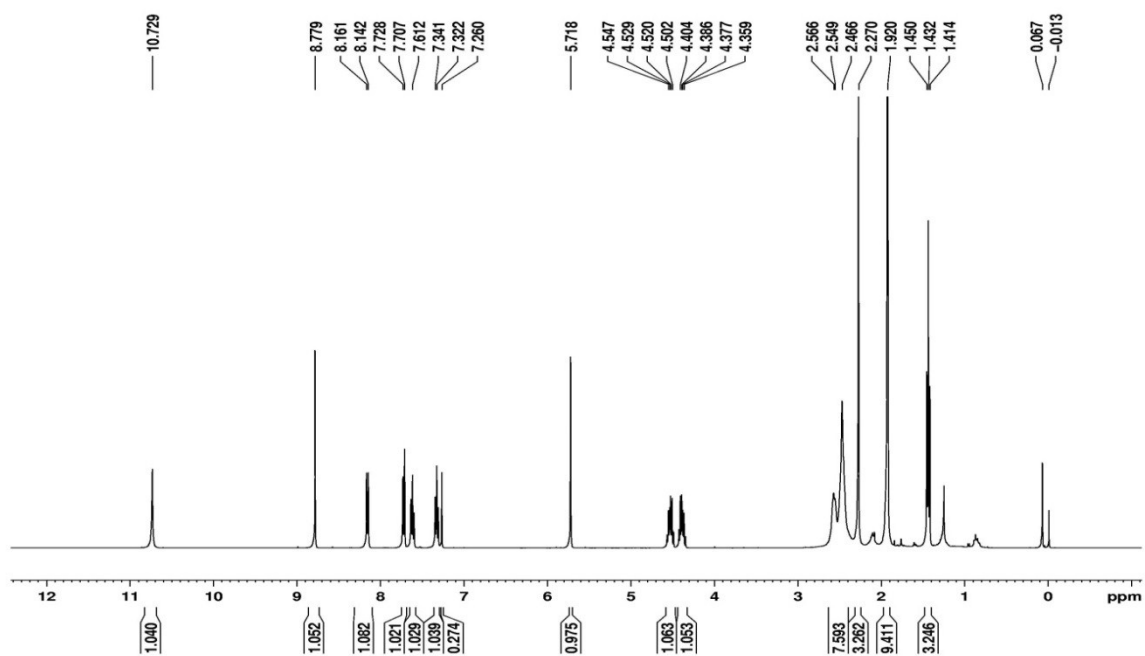
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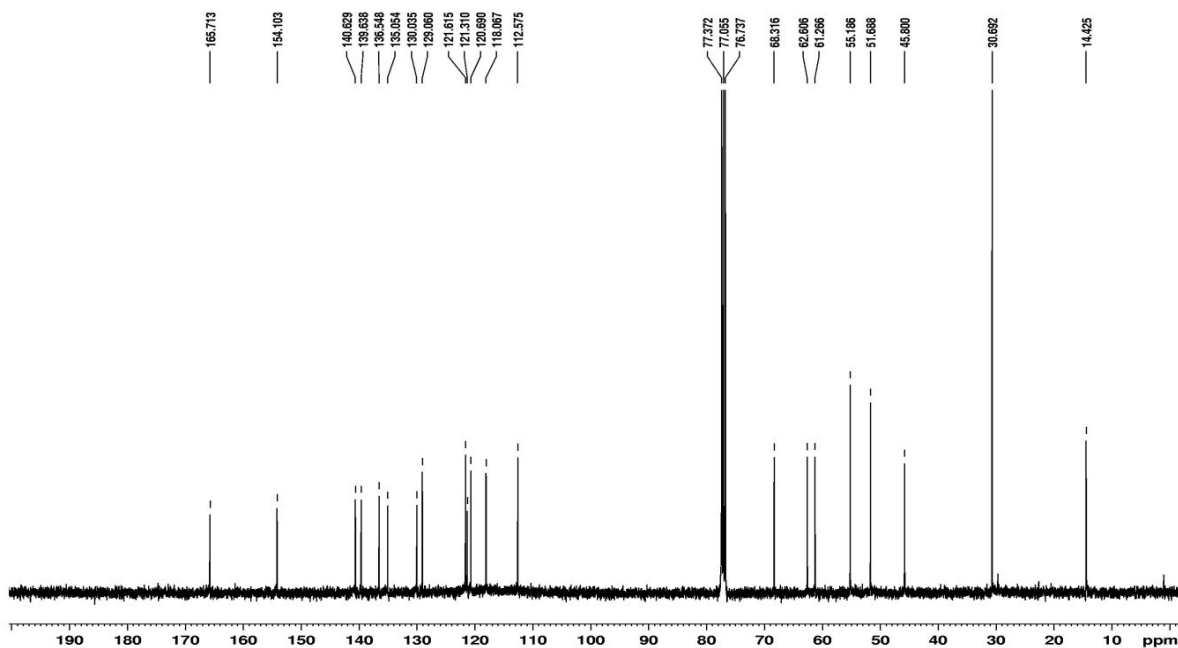
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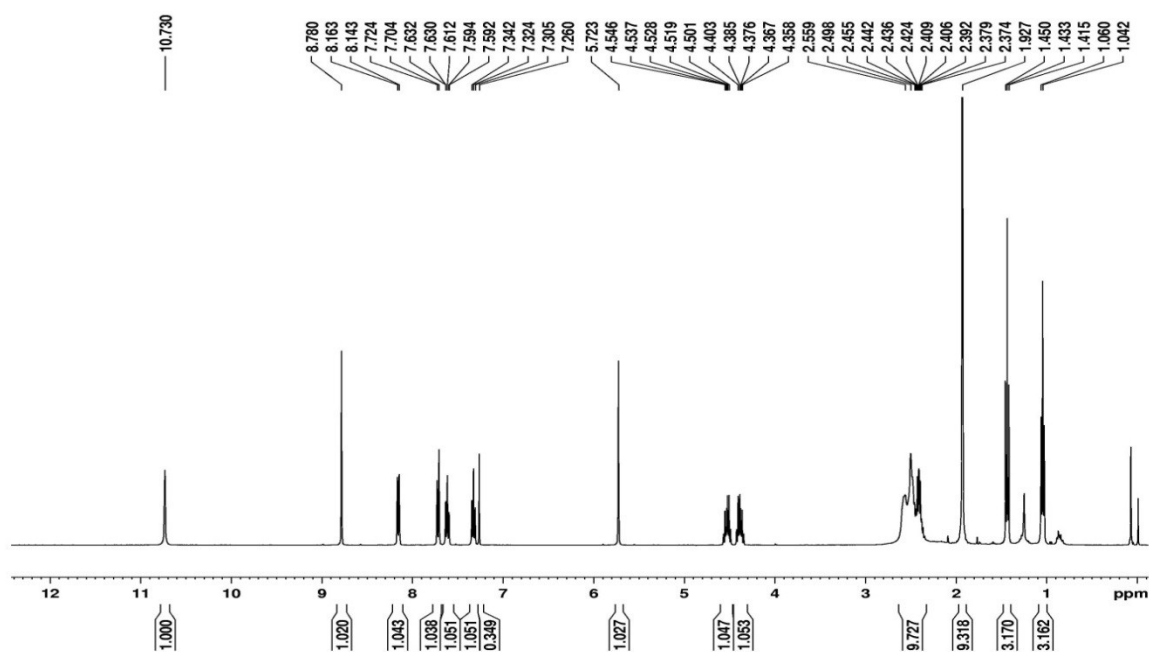
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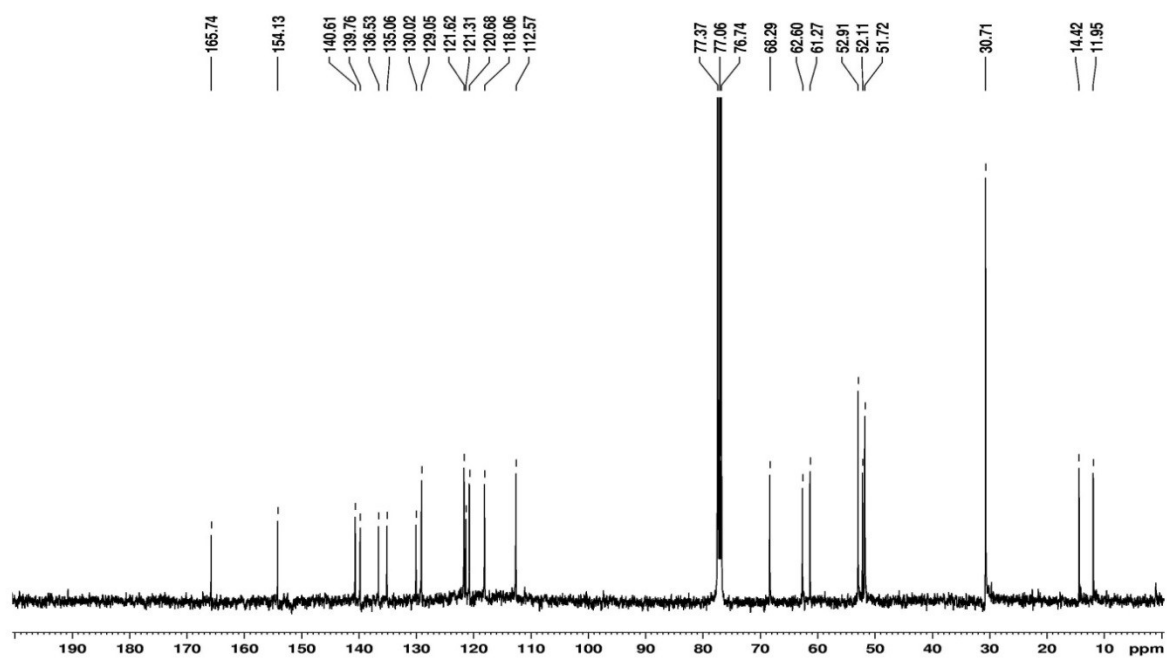
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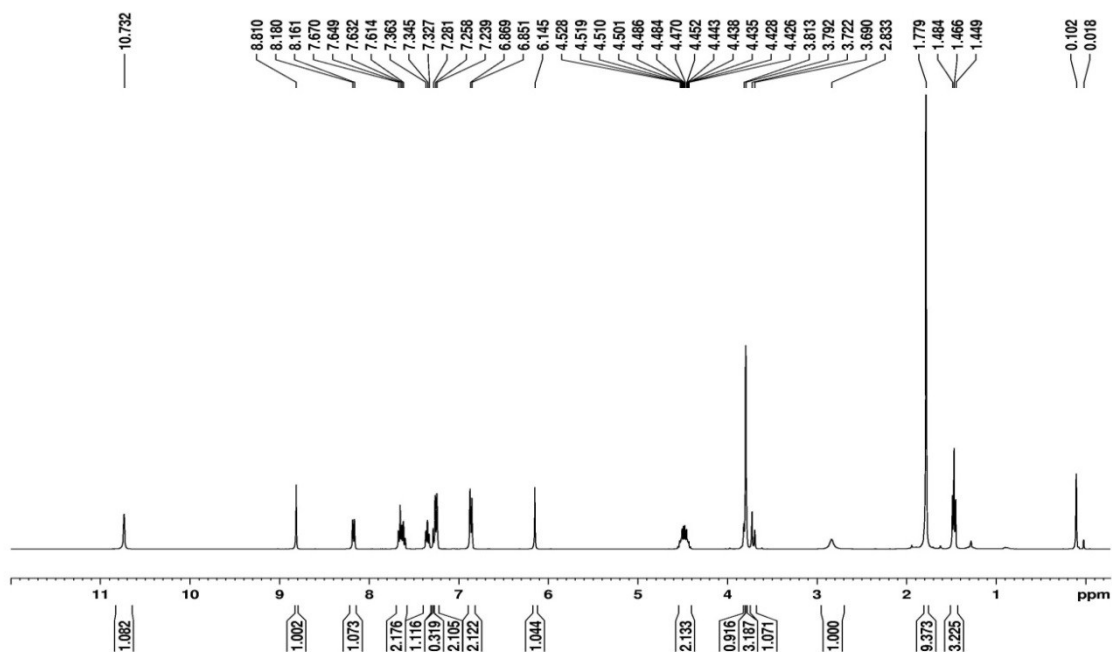
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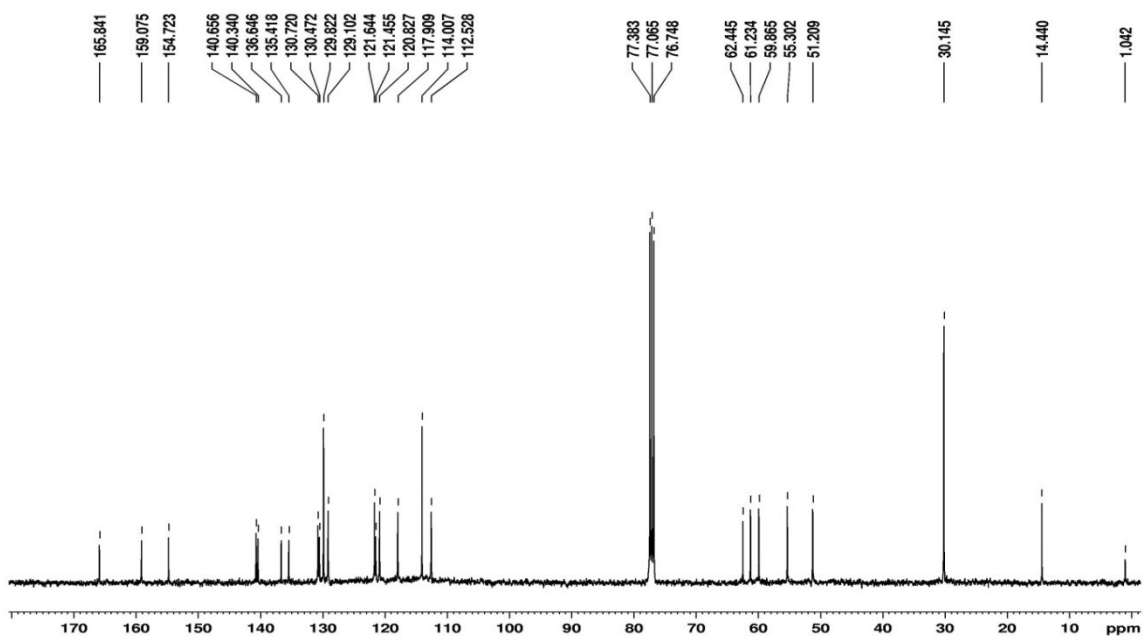
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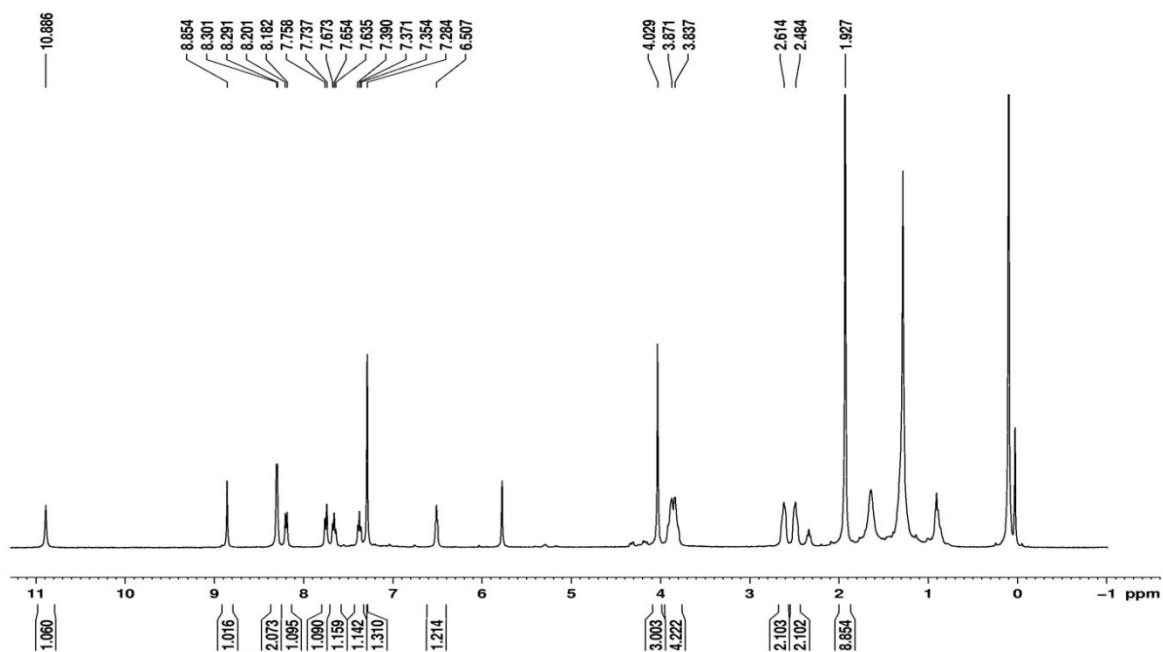
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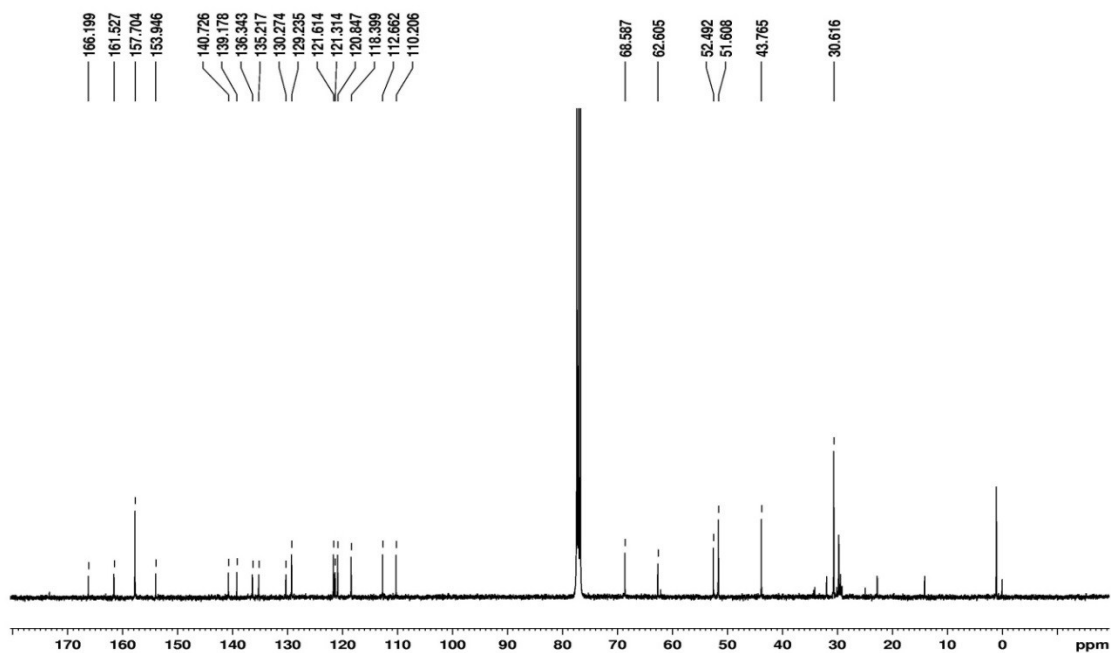
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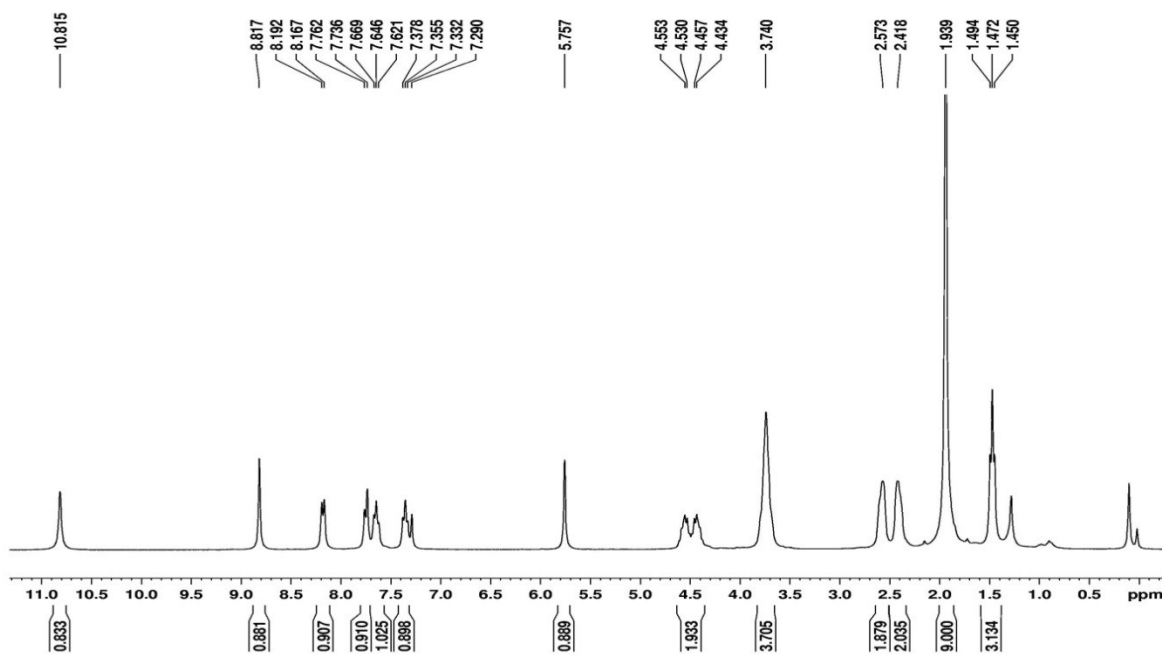
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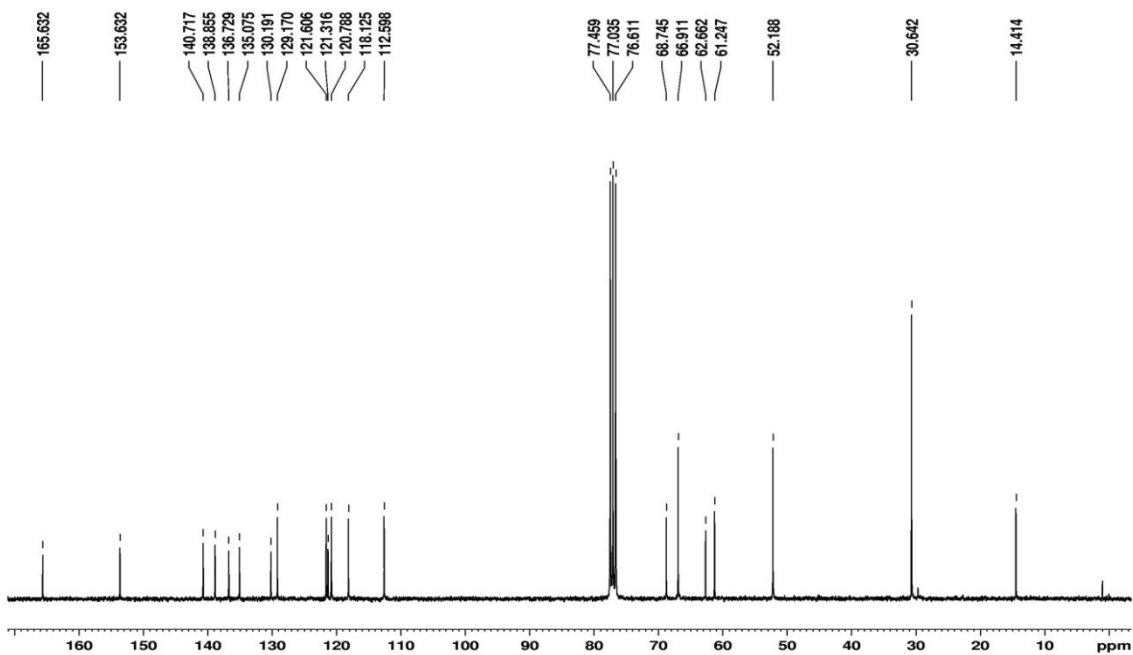
Fig.S22.^{13}C spectra of Compound 8k

Fig.S23.[1]H spectra of Compound 8l

Fig.S24.[13]C spectra of Compound 8l
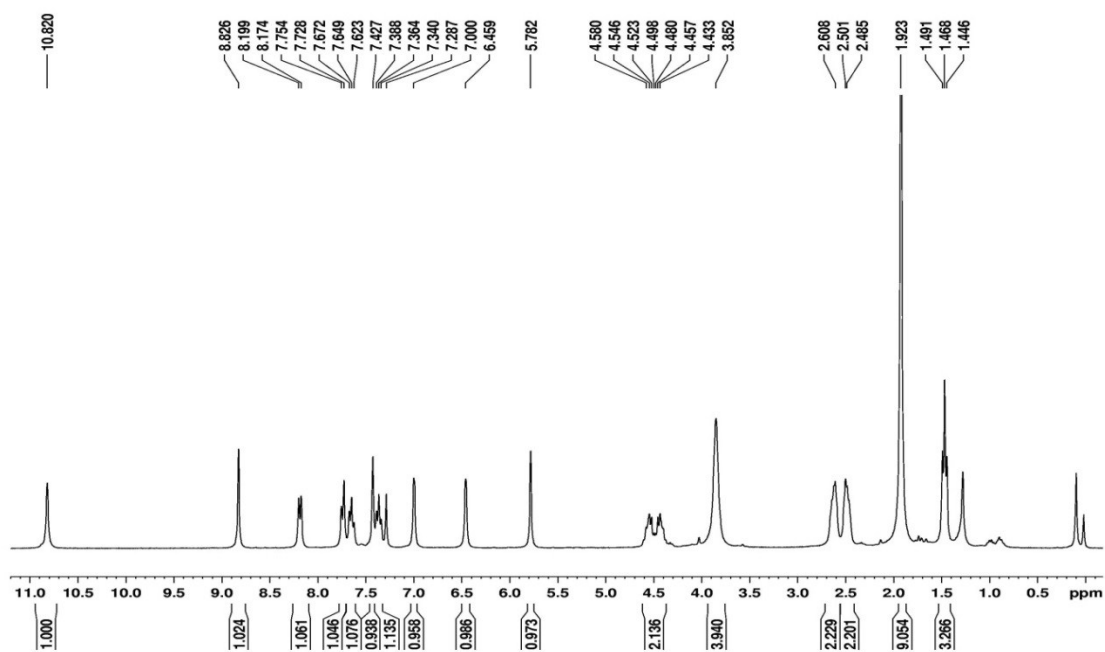
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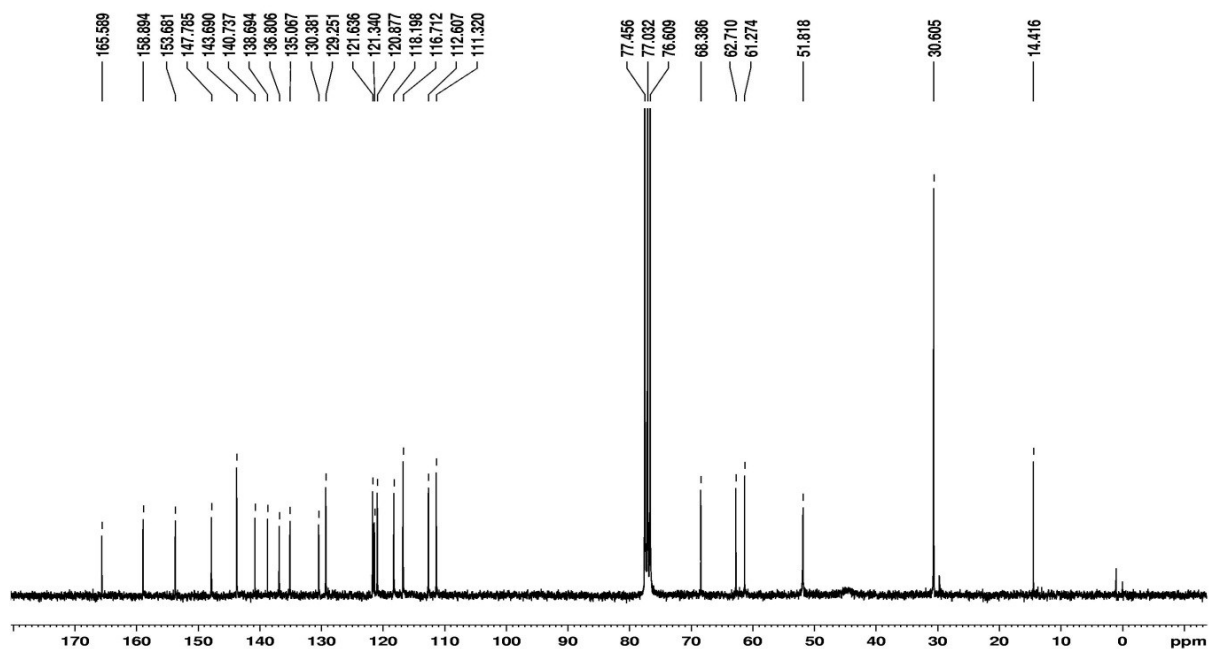
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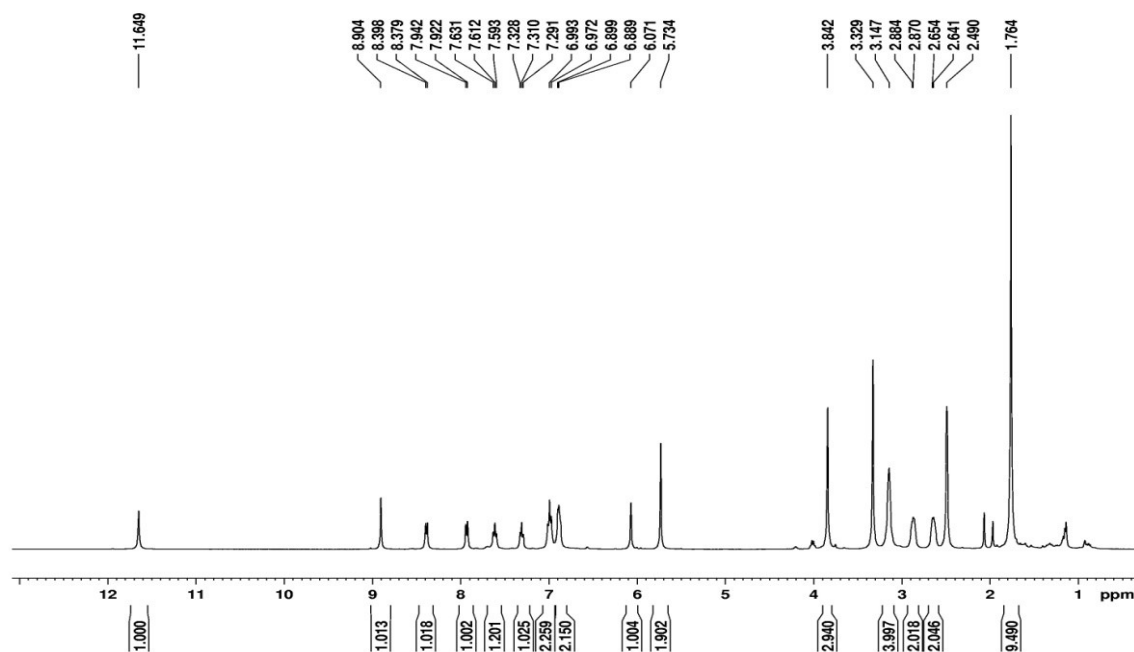
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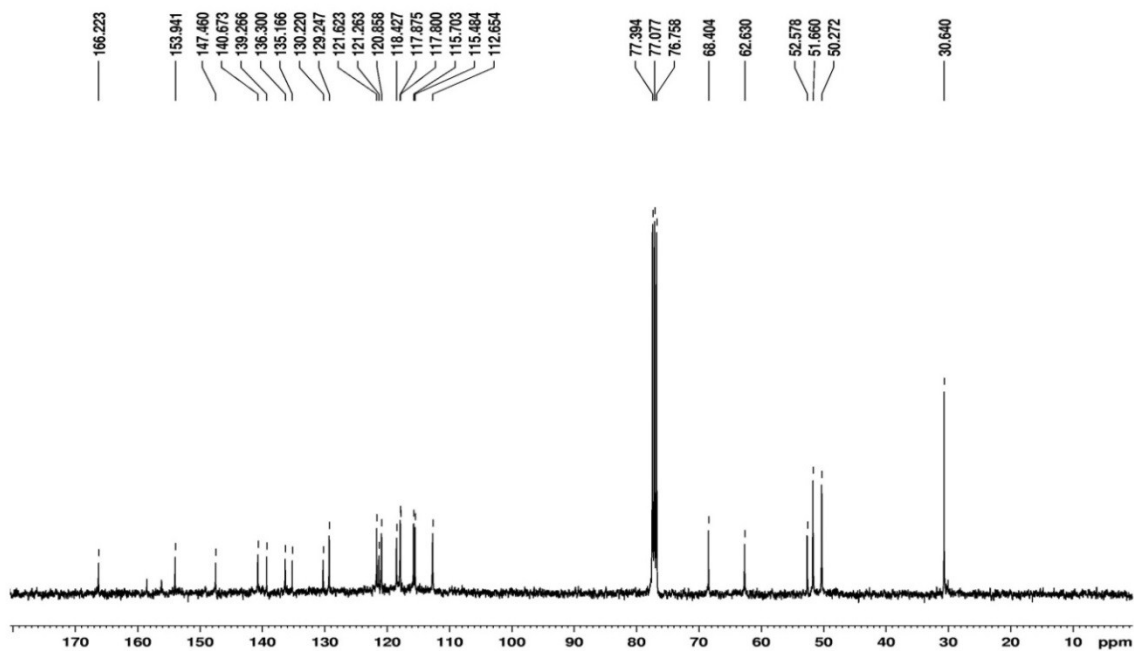
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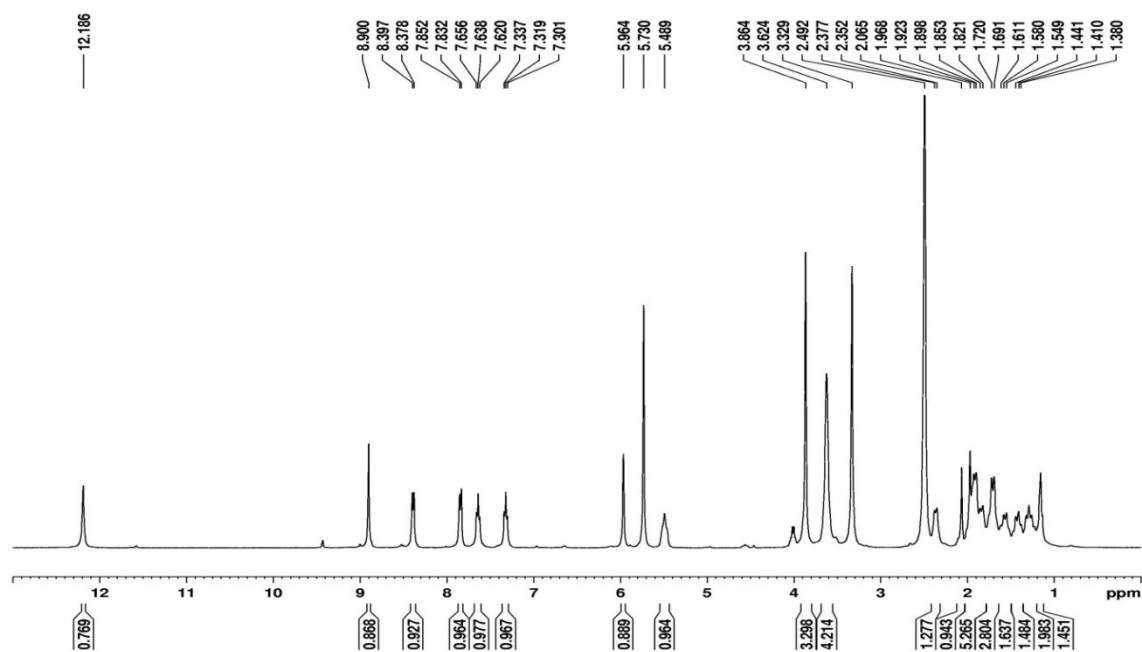
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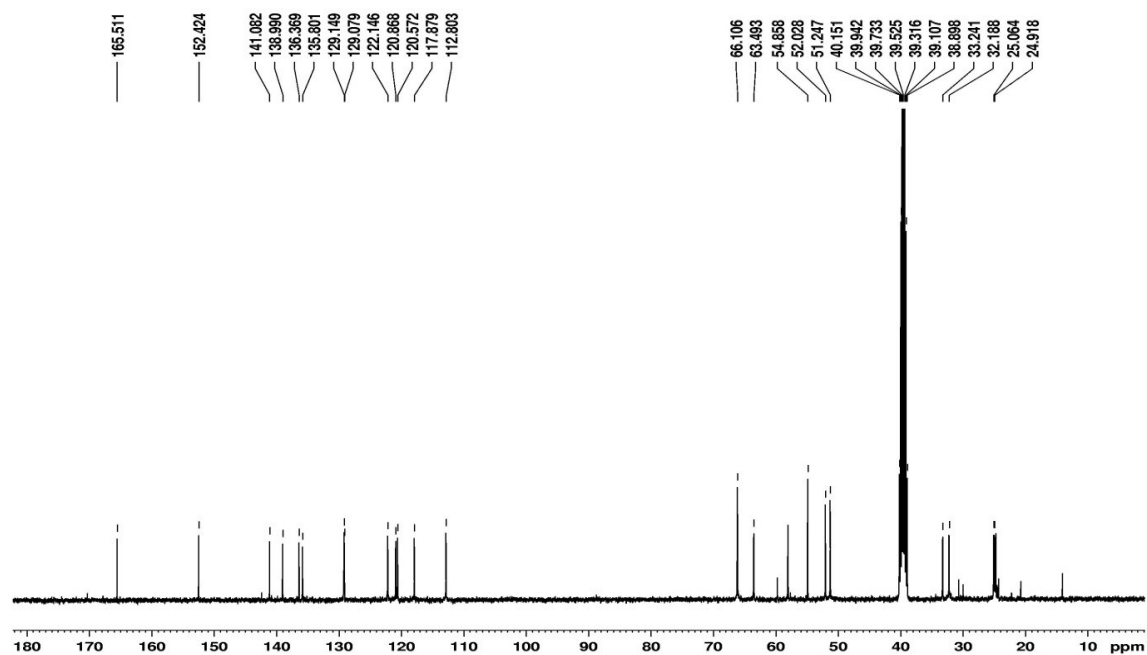
Fig.S30.¹³C spectra of Compound 8o

Fig.S31.¹H spectra of Compound 8p
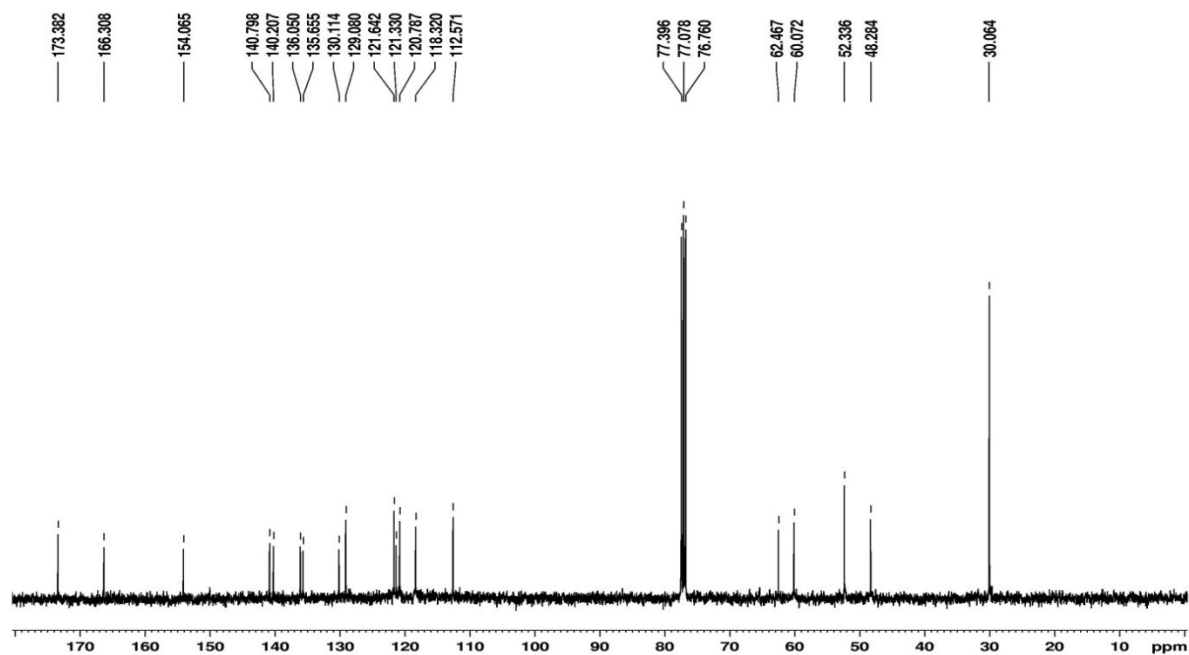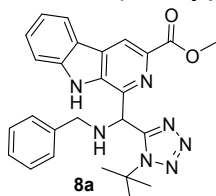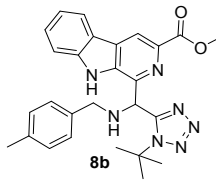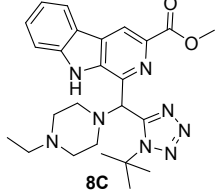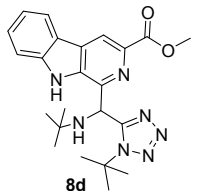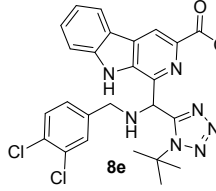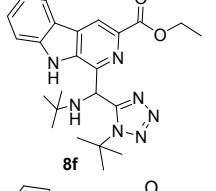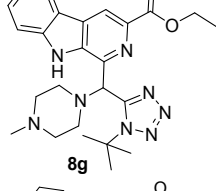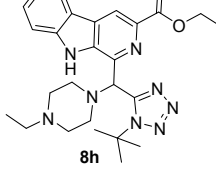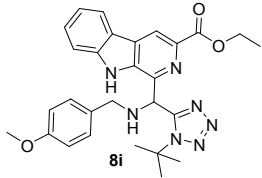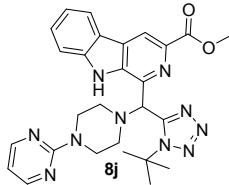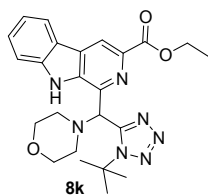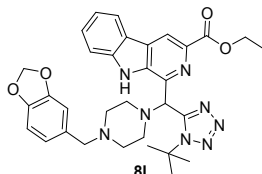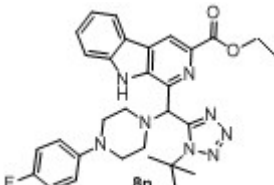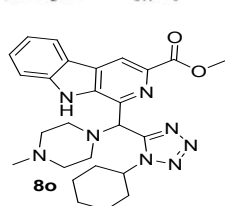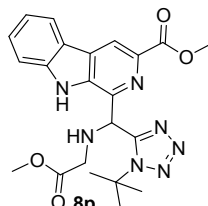
Fig.S32.¹³C spectra of Compound 8p

5. Table S1. Screening of compounds for PAINS

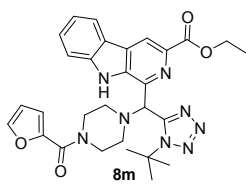| Entry	Product (8a-8p)	SMILE ID	PIANS Filter result
1	8a	CC(C)(C)n1nnnc1C(NCc2ccccc2)c4nc(cc3c5ccccc5nc34)C(=O)OC	Passed
2	8b	CC(C)(C)n1nnnc1C(NCc2ccc(C)cc2)c4nc(cc3c5ccccc5nc34)C(=O)OC	Passed
3	8C	COC(=O)c2cc1c5ccccc5nc1c(n2)C(NCN3CCN(CC3)CC)c4nnnn4C(C)(C)C	Passed
4	8d	CC(C)(C)n1nnnc1C(NC(C)(C)C)c3nc(cc2c4ccccc4nc23)C(=O)OC	Passed
5	8e	CC(C)(C)n1nnnc1C(NCc2ccc(Cl)c(Cl)c2)c4nc(cc3c5ccccc5nc34)C(=O)OC	Passed
6	8f	CC(C)(C)n1nnnc1C(NC(C)(C)C)c3nc(cc2c4ccccc4nc23)C(=O)OCC	Passed
7	8g	CCOC(=O)c2cc1c5ccccc5nc1c(n2)C(NN3CCN(C)CC3)c4nnnn4C(C)(C)C	Passed
8	8h	CCOC(=O)c2cc1c5ccccc5nc1c(n2)C(NN3CCN(CC3)CC)c4nnnn4C(C)(C)C	Passed

9	8i	CC(C)(C)n1nnnc1C(NCc2ccc(OC)cc2)c4nc(cc3c5ccccc5nc34)C(=O)OCC	Passed
10	8j	COC(=O)c2cc1c6ccccc6nc1c(n2)C(c3nnnn3C(C)(C)C)N4CCN(CC4)c5ncccn5	Passed
11	8k	CCOC(=O)c2cc1c5ccccc5nc1c(n2)C(c3nnnn3C(C)(C)C)N4CCOCC4	Passed
12	8l	CCOC(=O)c2cc1c7ccccc7nc1c(n2)C(c3nnnn3C(C)(C)C)N4CCN(CC4)Cc5ccc6OCOc6c5	Passed
13	8m	O=C(N1CCN(CC1)C(c3nc(cc2c4ccccc4nc23)C(=O)OCC)c5nnnn5C(C)(C)C)c6ccco6	Passed
14	8n	CCOC(=O)c6cc2c1ccccc1[nH]c2c(C(c3nnnn3C(C)(C)C)N5CCN(c4ccc(F)cc4)CC5)n6	Passed
15	8o	CN1CCN(CC1)C(c2nnnn2C3CCCCC3)c5nc(cc4c6ccccc6nc45)C(=O)OC	Passed
16	8p	OC(O)(O)n1nnnc1C(NCC(=O)OC)c3nc(cc2c4ccccc4nc23)C(=O)OC	Passed

6. Table S2. Primer sequence of various genes used for qRT-PCR

Gene Symbol	Gene Name	Primer Sequence	Accession no.
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	F- CAGCAAGGATACTGAGAGCAAGAG R- GGATGGAATTGTGAGGGAGATG	NM_017008.4
BMP-2	Bone morphogenetic protein2	F- CGGCTGCGGTCTCCTAA R- GGGAAGCAGCAACACTAGA	NM_017178.1
Runx-2	Runt-related transcription factor2	F- CCACAGAGCTATTAAAGTGA R- AACAAACTAGGTTTAGAGTCATCAAGC	NM_001278483.1
Col-1	Type 1 Collagen	F- CATGTTCAGCTTTGTGGACCT R- GCAGCTGACTTCAGGGATGT	NM_053304.1
OCN	Osteocalcin	F- ATAGACTCCGGCGCTACCTC R- CCAGGGGATCTGGGTAGG	NM_013414.1

qRT-PCR, quantitative real-time polymerase chain reaction; F, forward; R, reverse.
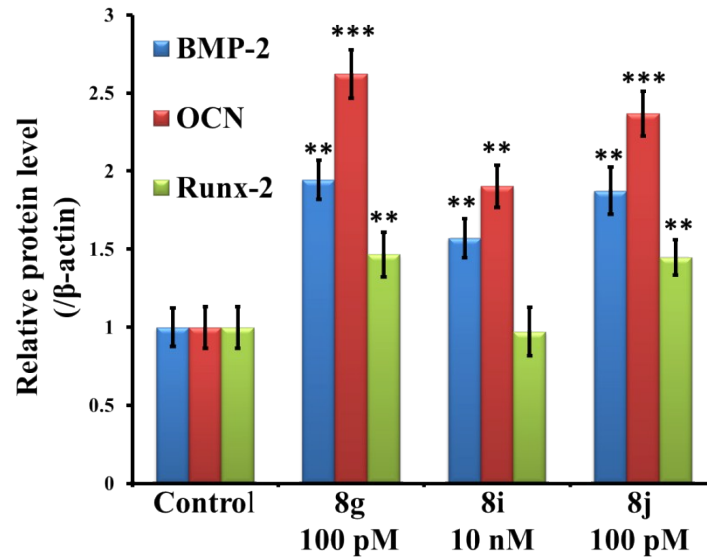
Fig.S33. Potent compoundspromote osteoblast differentiation.ROCs were treated with or without various compounds for 48h. Proteins extracted from cell lysates were transblotted on to a membrane and probed with primary antibodies followed by the corresponding secondary antibodies normalized with β-actin.The graph shows the densitometric analysis (fold change) of the observed change in expression of the osteoblast differentiation BMP-2, OCN and Runx-2 proteins after the treatment of respective compounds at their effective concentrations.Values represent mean ± SEM of three independent experiments: ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ compared with untreated cells taken as control.
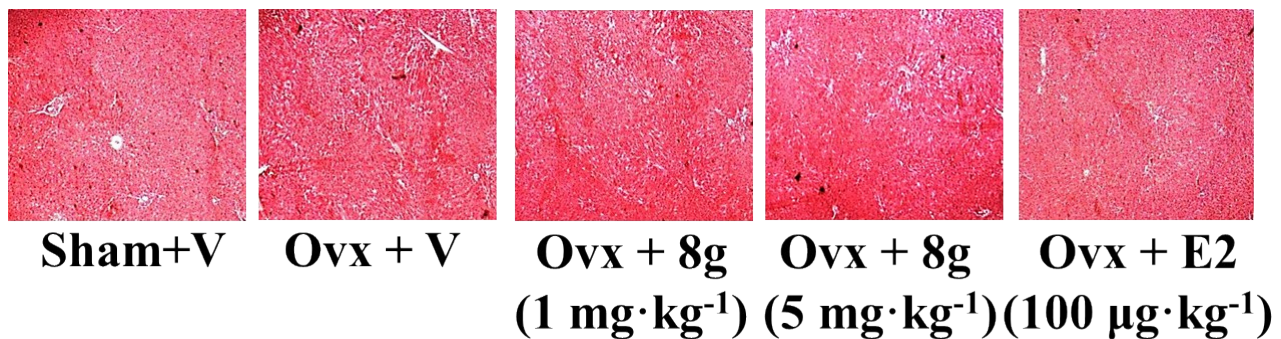
Fig. S34 Compound 8g shows no toxicity. Representative images of transverse sections (5.0µm) of livers followed by hematoxylin and eosin staining after 12 weeks of treatment to the rats.Vehicle represents 1% gum acacia dissolved in distilled water.

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