

Solvent-free peptide synthesis assisted by microwave irradiation: Environmentally benign synthesis of bioactive peptides

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SUPPORTING INFORMATION

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1. General information

All starting materials were purchased from Sigma-Aldrich/Chem-Impex and used further without any additional purification. Analytical thin-layer chromatography (TLC) was performed using aluminium plates precoated with silica gel (0.25 mm, 60 Å pore-size) and impregnated with a fluorescent indicator (254 nm). Visualization on TLC was achieved by the use of UV light (254 nm), treatment with 10% ninhydrin in ethanol or staining with iodine. Flash column chromatography was undertaken using preloaded silica gel (400-630 mesh) cartridges on Biotage® automated purification system. Proton nuclear magnetic resonance spectra (^1H NMR) were recorded on Avance III 400 Bruker (400 MHz). Proton chemical shifts are expressed in parts per million (ppm, δ scale) and are referenced to residual protium in the NMR solvent (CDCl_3 , δ 7.26 and CD_3OD , δ 3.31). The following abbreviations were used to describe peak patterns when appropriate: br = broad, s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet. Coupling constants, J , were reported in Hertz unit (Hz). Carbon 13 nuclear magnetic resonance spectroscopy (^{13}C NMR) was recorded on Avance III 400 Bruker (100 MHz) and was fully decoupled by broad band decoupling. Chemical shifts were reported in ppm referenced to the centre line at a 77.0 and 49.0 ppm of CDCl_3 and CD_3OD . High resolution mass spectra were taken with Maxis-Bruker using ESI-TOF method. HPLC analysis was performed on the Shimadzu Prominence using SupelcosilTM LC-18 column (25 cm $\times$ 4.6 mm, 5 μm) run for 60 min with a flow of 1 mL/min, using a gradient of 95-5%; where buffer A was 0.1% TFA in H_2O and buffer B was 0.1% TFA in CH_3CN and detection at 220 nm. All MW-irradiation reactions were carried out on a CEM Discover® microwave reactor.

2. Experimental procedure

2.1. General experimental procedure for dipeptides (2a-m)

In a 10 mL MW vial equipped with a magnetic stir bar, amino acid (AA_2) methyl ester-xHCl (1 mmol) was taken followed by DIEA (3 mmol). Boc- AA_1 -OH (1.2 mmol) was added followed by DIC (1.2 mmol) and HONB (1.2 mmol). After allowing mixing with magnetic stir for 2-3 min, reaction mixture was subjected to MW irradiation (CEM Discover® microwave reactor) with gas cooling (pressure of 40 psi was maintained during irradiation) for 15 min at 40W with magnetic stirring, and a temperature limit of 60 °C (reaction time refers to the hold time at the desired set temperature). After the completion of reaction, the reaction mixture was purified on automated flash column chromatography system (Biotage®) to give Boc- AA_1 - AA_2 -OMe.

2.2. General experimental procedure for N- α -Boc-protected peptides (3a-f, 4a-c, 5a)

Boc- AA_1 - AA_2 -OMe (1 mmol) was taken into a round bottom flask and subjected to deprotection with 6N HCl (5 mL) at 25 °C for 15 min and the resulting AA_1 - AA_2 -OMe (1 mmol) was dried completely. In a 10 mL MW vial equipped with a magnetic stir bar, hydrochloride salt of dipeptide was taken and neutralized with DIEA (3 mmol). Boc- AA_3 -OH (1.2 mmol) was added followed by DIC (1.2 mmol) and HONB (1.2 mmol). After allowing mixing with magnetic stir for 2-3 min, reaction mixture was subjected to MW-irradiation (CEM Discover® microwave

reactor) with gas cooling (pressure of 40 psi was maintained during irradiation) for 15 min at 40W with magnetic stirring, and a temperature limit of 60 °C (reaction time refers to the hold time at the desired set temperature). After the completion of reaction, the reaction mixture was purified on automated flash column chromatography system (Biotage®) to afford tripeptides (**3a-f**). The deprotection and coupling cycle described above was repeated to obtain tetrapeptides (**4a-c**) and pentapeptide (**5a**).

2.3. General method for the removal of methyl ester group

To a suspension of the desired peptide (1 equiv) in ethyl alcohol (5 mL) was added potassium carbonate (1.2 equiv), and the mixture was stirred for 60 min at 25 °C. The solvent of reaction mixture was removed under reduced pressure.

2.4. General method for the removal of benzyl ester/benzyl ether/Z groups

To a suspension of the desired peptide (1 equiv), 10% Pd-C (10 equiv) in ethyl alcohol (5 mL) was added ammonium formate (5 equiv), and the mixture was heated with stirring at 60 °C for 10 h. The reaction mixture was filtered through a celite pad, and solvent was removed under reduced pressure. The resulting residue was purified on a preparative HPLC using a Shimadazu system. Method: 40 min gradient, 5-90% CH₃CN-H₂O-0.1% CF₃CO₂H at 220 nm.

2.5. General method for the workup of peptides

On completion of chemical synthesis of the peptide chain, the final step is the extraction or precipitation. Depending on the solubility of the peptide, extraction or precipitation method was used. The solvents used for extraction or precipitation includes diethyl ether, ethyl acetate, and glacial acetic acid. Many different approaches to this problem have been established, but the procedure most widely used for peptides has been treatment with cold ether. After completion of synthesis, ether was added directly to the reaction vessel, and peptide mixture was stirred for 30-60 sec. The mixture was filtered and residue was washed three times with cold ether. The purity of crude peptide was analyzed using analytical HPLC and if peptide was more than 90% pure, then lyophilization was performed directly for final storage. Otherwise isolation or purification (using Flash or HPLC chromatography) of the crude peptide followed by lyophilization is the final step in this process.

3. Product Characterization Data

Boc-Phe-Ile-OMe (**2a**)

¹H NMR (400 MHz, CD₃OD): δ = 7.30-7.21 (m, 5H), 4.41-4.38 (m, 1H), 4.37-4.34 (m, 1H), 3.69 (s, 3H), 3.09 (dd, *J* = 5.8, 13.8 Hz, 1H), 2.82 (dd, *J* = 9.2, 13.7 Hz, 1H), 1.92-1.84 (m, 1H), 1.38 (s, 9H), 1.31-1.24 (m, 1H), 1.24-1.17 (m, 1H), 0.95-0.90 (m, 6H); ¹³C NMR (100 MHz, CD₃OD): δ = 173.0, 171.9, 156.2, 137.1, 129.0, 128.0, 126.2, 79.3, 56.7, 55.8, 51.1, 37.6, 37.1, 27.2, 24.8, 14.5, 10.2; IR (neat): ν 3308, 2968, 2929, 2879, 1752, 1683, 1646, 1535, 1437, 1391, 1366, 1173, 1017, 989, 860, 716, 701; HRMS (ESI-TOF): calculated for [M+H⁺] 393.2389; found 393.2384; HPLC: *t*_R = 47.80 min, 97%.

Boc-Phe-His-OMe (2b)

^1H NMR (400 MHz, CD_3OD): δ = 7.72 (s, 1H), 7.27-7.19 (m, 5H), 6.92 (s, 1H), 4.70 (t, J = 6.1 Hz, 1H), 4.32 (dd, J = 5.4, 8.9 Hz, 1H), 3.67 (s, 3H), 3.19-3.11 (m, 1H), 3.10-3.07 (m, 1H), 3.07-3.01 (m, 1H), 2.82-2.74 (m, 1H), 1.35 (s, 9H); ^{13}C NMR (100 MHz, CD_3OD): δ = 172.9, 171.3, 156.2, 137.2, 134.1, 128.9, 128.0, 126.3, 117.8, 79.3, 55.9, 52.3, 51.6, 37.7, 28.1, 27.3; IR (neat): ν 3406, 2956, 1757, 1661, 1601, 1448, 1391, 1365, 1275, 1206, 1176, 1094, 763; HRMS (ESI-TOF): calculated for $[\text{M}+\text{H}^+]$ 417.2138; found 417.2138; HPLC: t_{R} = 30.87 min, 92.4%.

Boc-Ser(Bzl)-Ile-OMe (2c)

^1H NMR (400 MHz, CD_3OD): δ = 7.37-7.22 (m, 5H), 4.53 (d, J = 5.0 Hz, 2H), 4.42 (d, J = 5.8 Hz, 1H), 4.32 (s, 1H), 3.75-3.69 (m, 2H), 3.68 (s, 3H), 1.90-1.83 (m, 1H), 1.44 (s, 9H), 1.31-1.27 (m, 1H), 1.24-1.13 (m, 1H), 0.92-0.85 (m, 6H); ^{13}C NMR (100 MHz, CD_3OD): δ = 171.9, 171.5, 156.3, 137.8, 128.0, 127.5, 127.3, 79.5, 72.9, 69.7, 56.8, 54.5, 51.1, 37.3, 27.3, 24.8, 14.4, 10.3; IR (neat): ν 3398, 3263, 2929, 2856, 1705, 1651, 1534, 1435, 1379, 1368, 1169, 1073, 967, 740, 698; HRMS (ESI-TOF): calculated for $[\text{M}+\text{H}^+]$ 423.2495; found 423.2495; HPLC: t_{R} = 50.65 min, 96.1%.

Boc-Trp-His-OMe (2d)

^1H NMR (400 MHz, CD_3OD): δ = 7.61-7.52 (m, 2H), 7.32 (d, J = 8.0 Hz, 1H), 7.11-7.05 (m, 2H), 7.04-6.97 (m, 1H), 6.79 (s, 1H), 4.68-4.62 (m, 1H), 4.40-4.32 (m, 1H), 3.63 (s, 3H), 3.21 (dd, J = 5.8, 14.6 Hz, 1H), 3.10-3.04 (m, 1H), 3.02 (d, J = 9.0 Hz, 1H), 3.00-2.94 (m, 1H), 1.36 (s, 9H); ^{13}C NMR (100 MHz, CD_3OD): δ = 173.3, 171.5, 156.2, 136.6, 134.9, 127.5, 123.3, 120.9, 118.4, 117.9, 110.9, 109.5, 79.3, 55.5, 52.6, 51.4, 29.3, 28.6, 27.7; IR (neat): ν 3331, 3127, 2782, 1729, 1692, 1653, 1532, 1438, 1367, 1292, 1173, 972, 798, 781, 752; HRMS (ESI-TOF): calculated for $[\text{M}+\text{H}^+]$ 456.2247, found 456.2247; HPLC: t_{R} = 30.95 min, 91.7%.

Boc-Val-Ile-OMe (2e)

^1H NMR (400 MHz, CD_3OD): δ = 4.29 (d, J = 6.0 Hz, 1H), 3.80 (d, J = 7.3 Hz, 1H), 3.60 (s, 3H), 1.94-1.87 (m, 1H), 1.83-1.79 (m, 1H), 1.34 (s, 9H), 1.21-1.17 (m, 1H), 1.17-1.11 (m, 1H), 0.87-0.80 (m, 12H); ^{13}C NMR (100 MHz, CD_3OD): δ = 174.7, 173.4, 157.9, 80.9, 61.4, 58.2, 52.4, 38.4, 33.1, 28.4, 26.3, 19.8, 18.7, 15.9, 11.7; IR (neat): ν 3736, 3336, 2973, 2483, 1685, 1527, 1366, 1251, 1176, 1090, 1043, 1013, 880, 784; HRMS (ESI-TOF): calculated for $[\text{M}+\text{H}^+]$ 345.2389; found 345.2382; HPLC: t_{R} = 46.23 min, 92.3%.

Boc-His(Bzl)-His-OMe (2f)

^1H NMR (400 MHz, CD_3OD): δ = 7.64 (s, 1H), 7.57 (s, 1H), 7.37-7.28 (m, 3H), 7.22 (d, J = 6.8 Hz, 2H), 6.86 (d, J = 6.3 Hz, 2H), 5.13 (s, 2H), 4.64 (t, J = 6.3 Hz, 1H), 4.29 (dd, J = 5.3, 8.5 Hz, 1H), 3.65 (s, 3H), 3.11-3.06 (m, 1H), 3.03 (d, J = 7.5 Hz, 1H), 2.99-2.89 (m, 1H), 2.77 (dd, J = 8.9, 14.7 Hz, 1H), 1.37 (s, 9H); ^{13}C NMR (100 MHz, CD_3OD): δ = 172.9, 171.6, 156.2, 137.3, 136.9, 136.8, 134.9, 128.5, 127.7, 127.2, 117.4, 79.3, 52.5, 51.4, 50.3, 30.1, 28.6, 27.3; IR (neat):

ν 3292, 2924, 1674, 1501, 1391, 1366, 1259, 1168, 1048, 750; HRMS (ESI-TOF): calculated for $[M+H]^+$ 497.2512; found 497.2514; HPLC: t_R = 26.43 min, 98.7%.

Boc-Trp-Ile-OMe (2g)

1H NMR (400 MHz, CD_3OD): δ = 7.48 (d, J = 7.5 Hz, 1H), 7.21 (d, J = 8.0 Hz, 1H), 7.01-6.93 (m, 2H), 6.93-6.86 (m, 1H), 4.31 (t, J = 7.0 Hz, 1H), 4.25 (d, J = 6.0 Hz, 1H), 3.51 (s, 3H), 3.11 (dd, J = 6.1, 14.4 Hz, 1H), 2.95 (dd, J = 7.4, 14.4 Hz, 1H), 1.73-1.64 (m, 1H), 1.32-1.22 (s, 9H), 1.11-1.05 (m, 1H), 1.05-0.97 (m, 1H), 0.79-0.72 (m, 6H); ^{13}C NMR (100 MHz, CD_3OD): δ = 173.3, 171.8, 156.2, 136.6, 127.5, 123.2, 120.9, 118.4, 118.0, 110.9, 109.5, 79.3, 56.7, 55.4, 51.1, 37.1, 27.6, 24.8, 14.4, 10.3; IR (neat): ν 3331, 2964, 2926, 2855, 1746, 1668, 1602, 1504, 1456, 1392, 1366, 1259, 1170, 1095, 763, 749; HRMS (ESI-TOF): calculated for $[M+H]^+$ 432.2498, found 432.2494; HPLC: t_R = 46.93 min, 95.1%.

Boc-Asp(Bzl)-Lys(Z)-OMe (2h)

1H NMR (400 MHz, CD_3OD): δ = 7.37-7.33 (m, 5H), 7.33-7.26 (m, 5H), 5.13 (s, 2H), 5.05 (s, 2H), 4.51 (t, J = 6.5 Hz, 1H), 4.39 (dd, J = 4.8, 8.8 Hz, 1H), 3.68 (s, 3H), 3.09 (t, J = 6.9 Hz, 2H), 2.86 (dd, J = 5.3, 16.3 Hz, 1H), 2.72 (dd, J = 8.0, 16.3 Hz, 1H), 1.89-1.78 (m, 1H), 1.75-1.64 (m, 1H), 1.53-1.46 (m, 2H), 1.43 (s, 9H), 1.39-1.33 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD): δ = 172.4, 170.4, 136.9, 135.9, 128.1, 128.0, 127.7, 127.5, 127.3, 79.5, 66.2, 65.9, 52.2, 51.3, 51.0, 40.0, 30.7, 29.3, 28.8, 27.2, 22.3; IR (neat): ν 3310, 2933, 1716, 1674, 1524, 1456, 1390, 1366, 1274, 1259, 1170, 1027, 818, 749, 698; HRMS (ESI-TOF): calculated for $[M+H]^+$ 600.2921; found; 600.2919; HPLC: t_R = 30.29 min, 90.5%.

Boc-Met-Ile-OMe (2i)

1H NMR (400 MHz, CD_3OD): δ = 4.41 (d, J = 5.8 Hz, 1H), 4.23 (d, J = 5.8 Hz, 1H), 3.72 (s, 3H), 2.61-2.51 (m, 2H), 2.10 (s, 3H), 2.06-1.98 (m, 1H), 1.93-1.84 (m, 2H), 1.45 (s, 9H), 1.33-1.22 (m, 2H), 0.96-0.90 (m, 6H); ^{13}C NMR (100 MHz, CD_3OD): δ = 173.3, 171.9, 156.4, 79.3, 56.8, 53.4, 51.2, 37.0, 29.7, 27.3, 24.8, 15.9, 14.6, 13.9; IR (neat): ν 3336, 2967, 1744, 1658, 1523, 1456, 1366, 1275, 1260, 1170, 764; HRMS (ESI-TOF): calculated for $[M+H]^+$ 377.2110; found 377.2107; HPLC: t_R = 42.73 min, 97.9%.

Boc-Abu-Ile-OMe (2j)

1H NMR (400 MHz, $CDCl_3$): δ = 6.52 (br. s., 1H), 4.58 (dd, J = 5.0, 8.8 Hz, 1H), 4.02 (d, J = 6.5 Hz, 1H), 3.74 (s, 3H), 1.95-1.89 (m, 1H), 1.89-1.83 (m, 1H), 1.64 (td, J = 7.3, 14.2 Hz, 1H), 1.45 (s, 9H), 1.43-1.39 (m, 1H), 1.21-1.15 (m, 1H), 0.96 (t, J = 7.5 Hz, 3H), 0.90 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.1, 171.7, 156.6, 80.0, 56.3, 55.8, 52.0, 37.8, 28.2, 25.3, 25.0, 15.4, 11.5, 9.9; IR (neat): ν 3297, 2977, 2937, 2878, 1747, 1682, 1656, 1542, 1457, 1391, 1366, 1293, 1251, 1171, 1085, 1047, 1031, 1003, 978, 866, 763, 645; HRMS (ESI-TOF) calculated for $[M+H]^+$ 331.2233; found 331.2237; HPLC: t_R = 32.11 min, 90.6%.

Boc-Lys(Z)-Pro-OMe (2k)

^1H NMR (400 MHz, CD_3OD): δ = 7.39-7.27 (m, 5H), 5.07 (s, 2H), 4.51-4.44 (m, 1H), 4.32 (br. s., 1H), 3.85-3.78 (m, 2H), 3.64 (s, 3H), 3.15 (t, J = 5.9 Hz, 2H), 2.23 (br. s., 1H), 2.01 (d, J = 7.8 Hz, 2H), 1.98-1.89 (m, 2H), 1.79-1.68 (m, 2H), 1.66-1.57 (m, 1H), 1.57-1.51 (m, 2H), 1.48 (s, 9H); ^{13}C NMR (100 MHz, CD_3OD): δ = 172.5, 171.0, 156.5, 136.7, 128.4, 128.1, 128.0, 79.6, 66.5, 58.6, 51.4, 46.9, 40.5, 32.2, 29.1, 28.9, 24.9, 23.4, 21.6; IR (neat): ν 3394, 2966, 2844, 1702, 1638, 1532, 1454, 1366, 1259, 1168, 1054, 1033, 1016, 749, 699; HRMS (ESI-TOF) calculated for $[\text{M}+\text{H}^+]$ 492.2710; found 492.2709; HPLC: t_{R} = 30.18 min, 93.1%.

Boc-Phg-Ile-OMe (2l)

^1H NMR (400 MHz, CDCl_3): δ = 7.38-7.32 (m, 5H), 5.18 (br. s., 1H), 4.55 (dd, J = 4.9, 8.4 Hz, 1H), 3.66 (s, 3H), 1.90 (td, J = 7.2, 14.5 Hz, 1H), 1.43 (s, 9H), 1.28 (dd, J = 7.8, 18.6 Hz, 1H), 1.20 (dd, J = 7.0, 14.1 Hz, 1H), 0.94-0.89 (m, 6H); ^{13}C NMR (100 MHz, CDCl_3): δ = 171.6, 169.9, 136.9, 129.0, 129.0, 128.4, 127.2, 127.3, 80.1, 56.7, 52.0, 37.9, 28.2, 25.1, 15.4, 11.5; IR (neat): ν 3310, 2976, 1662, 1520, 1275, 1260, 1170, 1048, 764, 750; HRMS (ESI-TOF) calculated for $[\text{M}+\text{H}^+]$ 379.2233; found 379.2223; HPLC: t_{R} = 42.12 min, 94.4%.

Boc-Val-Tyr(Bzl)-OMe (2m)

^1H NMR (400 MHz, CDCl_3): δ = 7.46-7.37 (m, 4H), 7.36-7.32 (m, 1H), 7.02 (d, J = 8.5 Hz, 2H), 6.89 (d, J = 8.5 Hz, 2H), 5.05 (s, 2H), 4.87-4.81 (m, 1H), 3.94-3.88 (m, 1H), 3.72 (s, 3H), 3.08 (d, J = 5.3 Hz, 2H), 2.13-2.05 (m, 1H), 1.46 (s, 9H), 0.94 (d, J = 6.8 Hz, 3H), 0.89 (d, J = 6.5 Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ = 171.7, 171.2, 157.9, 136.9, 130.2, 128.5, 127.9, 127.8, 127.5, 114.9, 69.9, 59.8, 53.2, 52.3, 37.1, 30.8, 28.3, 19.1, 17.6; IR (neat): ν 3340, 3027, 2969, 2936, 2871, 2476, 1742, 1681, 1665, 1525, 1451, 1369, 1311, 1277, 1250, 1175, 1112, 1081, 1052, 1018, 934, 876, 828, 803, 729, 693, 621, 565, 538; HRMS (ESI-TOF) calculated for $[\text{M}+\text{H}^+]$ 485.2651; found 485.2647; HPLC: t_{R} = 31.57 min, 98.6%.

Boc-Ile-Phe-Ile-OMe (3a)

^1H NMR (400 MHz, CD_3OD): δ = 7.30-7.18 (m, 5H), 4.63 (s, 1H), 4.38-4.33 (m, 1H), 4.14-4.07 (m, 1H), 3.66 (s, 3H), 3.07 (s, 1H), 2.95-2.87 (m, 1H), 1.88-1.80 (m, 1H), 1.74-1.64 (m, 1H), 1.44 (s, 9H), 1.31-1.22 (m, 2H), 1.22-1.14 (m, 1H), 1.09 (d, J = 6.0 Hz, 1H), 0.91-0.80 (m, 12H); ^{13}C NMR (100 MHz, CD_3OD): δ = 172.8, 171.8, 171.7, 156.4, 136.7, 129.1, 128.0, 126.3, 79.2, 60.2, 59.2, 56.7, 54.0, 51.1, 37.7, 37.0, 27.4, 24.8, 24.3, 14.5, 10.3, 10.1; IR (neat): ν 3328, 2964, 2925, 1750, 1674, 1601, 1455, 1260, 1174, 1092, 748; HRMS (ESI-TOF): calculated for $[\text{M}+\text{H}^+]$ 506.323; found 506.325; HPLC: t_{R} = 48.93 min, 94.1%.

Boc-Thr(Bzl)-Phe-Ile-OMe (3b)

^1H NMR (400 MHz, CD_3OD): δ = 7.31-7.25 (m, 5H), 7.23-7.15 (m, 5H), 4.73 (t, J = 6.8 Hz, 1H), 4.51 (d, J = 11.5 Hz, 1H), 4.40 (d, J = 11.5 Hz, 1H), 4.30 (d, J = 6.3 Hz, 1H), 4.14-4.10 (m, 1H), 3.97 (dd, J = 3.6, 5.9 Hz, 1H), 3.65 (s, 3H), 3.05 (dd, J = 6.5, 13.6 Hz, 1H), 2.92 (dd, J =

7.5, 13.8 Hz, 1H), 1.77 (dd, $J = 6.5, 13.3$ Hz, 1H), 1.43 (s, 9H), 1.40-1.35 (m, 2H), 1.15 (d, $J = 6.3$ Hz, 3H), 0.87-0.82 (m, 6H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 171.6, 171.2, 138.3, 136.6, 129.1, 128.1, 127.9, 127.6, 127.3, 126.4, 79.6, 74.9, 71.0, 58.7, 56.8, 54.2, 51.0, 37.7, 37.0, 27.2, 24.9, 15.2, 14.4, 10.2$; IR (neat): ν 3339, 3266, 3089, 3031, 2972, 2931, 2877, 2411, 1743, 1691, 1645, 1529, 1454, 1391, 1366, 1300, 1248 1174, 1112, 1056, 1016, 928, 861, 773, 738, 696, 608, 500; HRMS (ESI-TOF) calculated for $[\text{M}+\text{H}^+]$ 584.3336; found 584.3330; HPLC: $t_{\text{R}} = 46.97$ min, 91.9%.

Boc Asp(Bzl)-Val-Tyr(Bzl)-OMe (3c)

^1H NMR (400 MHz, CDCl_3): $\delta = 7.44\text{-}7.40$ (m, 3H), 7.39-7.38 (m, 1H), 7.37 (t, $J = 1.8$ Hz, 1H), 7.36-7.34 (m, 4H), 7.34-7.31 (m, 1H), 7.04 (d, $J = 8.8$ Hz, 2H), 6.93-6.89 (m, 2H), 5.11 (d, $J = 1.8$ Hz, 2H), 5.03 (s, 2H), 4.82 (td, $J = 6.1, 7.9$ Hz, 1H), 4.55-4.48 (m, 1H), 4.23 (dd, $J = 5.8, 8.5$ Hz, 1H), 3.71 (s, 3H), 3.12-3.07 (m, 1H), 3.06-2.97 (m, 2H), 2.79 (dd, $J = 6.8, 17.3$ Hz, 1H), 2.24-2.14 (m, 1H), 1.46 (s, 9H), 0.92 (d, $J = 6.8$ Hz, 3H), 0.84 (d, $J = 6.8$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): $\delta = 171.8, 171.7, 170.2, 157.9, 136.9, 135.3, 130.8, 130.2, 128.6, 128.5, 128.4, 128.2, 127.9, 127.5, 115.0, 69.9, 66.9, 58.5, 53.2, 52.3, 50.7, 36.9, 35.7, 30.4, 29.7, 28.2, 19.1, 17.4$; IR (neat): ν 3313, 3033, 3034, 2959, 2871, 1758, 1725, 1686, 1664, 1644, 1545, 1524, 1453, 1434, 1390, 1367, 1301, 1275, 1253, 1209, 1171, 1122, 1048, 1029, 934, 811, 731, 694; HRMS (ESI-TOF) calculated for $[\text{M}+\text{H}^+]$ 690.3390; found 690.3401; HPLC: $t_{\text{R}} = 38.40$ min, 93.5%.

Boc-His(Bom)-Phe-Ile-OMe (3d)

^1H NMR (400 MHz, CDCl_3): $\delta = 7.49$ (s, 1H), 7.39-7.31 (m, 6H), 7.25 (d, $J = 7.5$ Hz, 2H), 7.15 (d, $J = 7.3$ Hz, 2H), 6.88 (s, 1H), 4.64 (q, $J = 6.8$ Hz, 1H), 4.55 (s, 2H), 4.49 (s, 2H), 4.46 (dd, $J = 5.1, 8.4$ Hz, 1H), 4.38 (d, $J = 5.8$ Hz, 1H), 3.68 (s, 3H), 3.15-3.08 (m, 1H), 3.04-2.94 (m, 3H), 1.86-1.75 (m, 1H), 1.38 (s, 9H), 1.33 (dd, $J = 4.9, 7.7$ Hz, 1H), 1.16-1.04 (m, 1H), 0.87 (t, $J = 7.3$ Hz, 3H), 0.82 (d, $J = 7.0$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): $\delta = 171.6, 170.8, 170.1, 155.4, 138.4, 136.3, 135.8, 129.7, 129.3, 128.9, 128.7, 128.6, 128.4, 128.1, 127.0, 80.4, 72.8, 69.8, 60.0, 56.6, 54.4, 54.0, 52.2, 37.9, 37.6, 29.6, 25.1, 15.3, 11.4$; IR (neat): ν 3301, 2967, 1744, 1648, 1498, 1455, 1391, 1366, 1249, 1170, 1089, 1027, 927, 820, 743, 698, 660, 496; HRMS (ESI-TOF) calculated for $[\text{M}+\text{H}^+]$ 650.3553; found 650.3494; HPLC: $t_{\text{R}} = 39.8$ min, 95.4%.

Boc-Ala(2-naphthyl)-Phe-Ile-OMe (3e)

^1H NMR (400 MHz, CDCl_3): $\delta = 7.84\text{-}7.78$ (m, 3H), 7.64 (s, 1H), 7.50-7.46 (m, 2H), 7.33 (d, $J = 8.5$ Hz, 1H), 7.21-7.17 (m, 3H), 7.06 (d, $J = 7.3$ Hz, 2H), 4.87 (br. s., 1H), 4.65-4.59 (m, 1H), 4.43 (dd, $J = 5.0, 8.3$ Hz, 1H), 3.70 (s, 3H), 3.22-3.20 (m, 2H), 3.09 (dd, $J = 5.9, 13.9$ Hz, 1H), 2.92 (dd, $J = 7.5, 13.8$ Hz, 1H), 1.76 (ddd, $J = 2.1, 4.7, 9.2$ Hz, 1H), 1.35 (s, 9H), 1.32-1.29 (m, 1H), 1.08-1.02 (m, 1H), 0.86 (t, $J = 7.4$ Hz, 3H), 0.79 (d, $J = 6.8$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): $\delta = 171.5, 171.1, 170.0, 136.1, 133.8, 133.4, 132.4, 129.2, 128.6, 128.5, 128.0, 127.6, 127.5, 127.2, 127.0, 126.2, 125.8, 79.1, 56.6, 54.4, 52.0, 38.0, 37.6, 28.1, 25.0, 15.2, 11.4$; IR

(neat): ν 3297, 3063, 2968, 2936, 2878, 1747, 1682, 1657, 1538, 1457, 1391, 1366, 1293, 1250, 1171, 1085, 1047, 1030, 1015, 978, 929, 913, 880, 784, 762, 645, 503; HRMS (ESI-TOF) calculated for $[M+H]^+$ 590.3230; found 590.3228; HPLC: t_R = 54.72 min, 93.9%.

Boc-Pro-Met-Ile-OMe (3f)

1H NMR (400 MHz, $CDCl_3$): δ = 4.50-4.46 (m, 1H), 4.30 (d, J = 5.8 Hz, 1H), 4.18-4.15 (m, 1H), 3.66 (s, 3H), 3.49-3.43 (m, 1H), 3.38-3.32 (m, 1H), 2.56-2.44 (m, 2H), 2.18 (d, J = 8.5 Hz, 1H), 2.05 (s, 3H), 2.00 (d, J = 6.3 Hz, 1H), 1.85-1.73 (m, 5H), 1.47-1.42 (m, 2H), 1.38 (s, 9H), 0.90-0.84 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.9, 154.7, 80.5, 56.6, 52.0, 42.1, 37.4, 29.9, 29.6, 28.3, 25.0, 23.4, 15.5, 14.9, 11.5; IR (neat): ν 3310, 2968, 1745, 1648, 1539, 1402, 1275, 1260, 1206, 1163, 1124, 1030, 764, 750; HRMS (ESI-TOF) calculated for $[M+H]^+$ 474.2638; found 474.2633; HPLC: t_R = 34.0 min, 97.8%.

Boc-Ala-Thr(Bzl)-Phe-Ile-OMe (4a)

1H NMR (400 MHz, CD_3OD): δ = 7.35-7.26 (m, 5H), 7.25-7.16 (m, 5H), 4.76 (dd, J = 6.5, 7.8 Hz, 1H), 4.55-4.51 (m, 1H), 4.43 (d, J = 12.8 Hz, 2H), 4.31 (d, J = 6.3 Hz, 1H), 4.14-4.02 (m, 2H), 3.66 (s, 3H), 3.12 (dd, J = 6.0, 13.8 Hz, 1H), 2.93 (dd, J = 7.9, 13.9 Hz, 1H), 1.81 (br. s., 1H), 1.48-1.44 (m, 1H), 1.42 (s, 9H), 1.30 (t, J = 3.4 Hz, 6H), 1.21 (br. s., 1H), 0.89-0.83 (m, 6H); ^{13}C NMR (100 MHz, CD_3OD): δ = 174.6, 171.6, 158.5, 138.1, 136.1, 128.9, 128.0, 127.9, 127.6, 127.3, 126.3, 75.2, 71.0, 56.8, 54.2, 51.0, 41.2, 36.9, 22.1, 15.2, 14.4, 10.2; IR (neat): ν 3383, 3287, 3036, 2976, 2931, 2871, 2453, 1736, 1709, 1685, 1633, 1544, 1518, 1484, 1456, 1406, 1385, 1363, 1308, 1255, 1213, 1170, 1130, 1069, 1042, 1026, 926, 860, 766, 745, 701, 647, 587, 508; HRMS (ESI-TOF) calculated for $[M+H]^+$ 655.3707; found 655.3714; HPLC: t_R = 44.27 min, 96.6%.

Boc-Lys(Z)-Asp(Bzl)-Val-Tyr(Bzl)-OMe (4b)

1H NMR (400 MHz, $CDCl_3$): δ = 7.43-7.38 (m, 5H), 7.37-7.32 (m, 10H), 7.05 (d, J = 8.5 Hz, 2H), 6.89 (d, J = 8.5 Hz, 2H), 5.12 (s, 2H), 5.10 (s, 2H), 5.01 (s, 2H), 4.83-4.76 (m, 2H), 4.26 (dd, J = 5.9, 8.4 Hz, 1H), 4.09-4.01 (m, 1H), 3.69 (s, 3H), 3.24-3.17 (m, 2H), 3.13-3.04 (m, 2H), 3.02-2.96 (m, 1H), 2.76 (dd, J = 6.7, 17.2 Hz, 1H), 2.21-2.13 (m, 1H), 1.91-1.82 (m, 1H), 1.69-1.62 (m, 1H), 1.54-1.49 (m, 2H), 1.46 (s, 9H), 1.42-1.36 (m, 2H), 0.87 (d, J = 6.5 Hz, 3H), 0.83 (d, J = 7.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.7, 170.3, 170.1, 157.8, 157.7, 136.9, 135.2, 130.2, 128.6, 128.2, 128.1, 127.9, 127.4, 114.9, 69.9, 67.0, 66.6, 58.8, 54.9, 53.3, 52.6, 49.7, 40.0, 36.9, 31.9, 29.7, 29.5, 28.3, 22.4, 19.0, 17.4; IR (neat): ν 3277, 2933, 1734, 1685, 1641, 1563, 1541, 1456, 1367, 1274, 1260, 1188, 1033, 818, 763, 849; HRMS (ESI-TOF) calculated for $[M+H]^+$ 952.4708; found 952.4696; HPLC: t_R = 50.75 min, 94.7%.

Boc-Met-Ala(2-naphthyl)-Phe-Ile-OMe (4c)

1H NMR (400 MHz, CD_3OD): δ = 7.80-7.74 (m, 3H), 7.67 (s, 1H), 7.43 (td, J = 3.1, 6.3 Hz, 2H), 7.35 (dd, J = 1.6, 8.4 Hz, 1H), 7.26-7.21 (m, 5H), 5.02-4.99 (m, 1H), 4.75-4.71 (m, 1H),

4.69 (dd, $J = 5.6, 7.9$ Hz, 1H), 4.31 (d, $J = 6.3$ Hz, 1H), 3.68 (s, 3H), 3.23 (dd, $J = 5.5, 14.1$ Hz, 1H), 3.12 (dd, $J = 6.1, 13.9$ Hz, 1H), 3.00-2.95 (m, 1H), 2.94-2.89 (m, 1H), 2.10-2.01 (m, 2H), 1.84 (s, 3H), 1.81-1.78 (m, 1H), 1.64-1.56 (m, 2H), 1.41 (d, $J = 9.0$ Hz, 1H), 1.29 (s, 9H), 1.20-1.16 (m, 1H), 0.91-0.85 (m, 6H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 171.8, 157.8, 136.6, 134.5, 133.5, 132.4, 129.0, 128.0, 127.6, 127.4, 127.2, 127.1, 126.9, 126.3, 125.6, 125.2, 80.0, 56.8, 54.4, 54.2, 51.1, 37.5, 36.9, 31.6, 29.3, 24.8, 14.4, 13.0, 10.2$; IR (neat): ν 3298, 2926, 2931, 1742, 1645, 1549, 1453, 1275, 1260, 1170, 1130, 1021, 764, 749, 701; HRMS (ESI-TOF) calculated for $[\text{M}+\text{H}^+]$ 721.3635; found 721.3626; HPLC: $t_R = 37.98$ min, 92.7%.

Boc-Arg-Lys(Z)-Asp(Bzl)-Val-Tyr(Bzl)-OMe (5a)

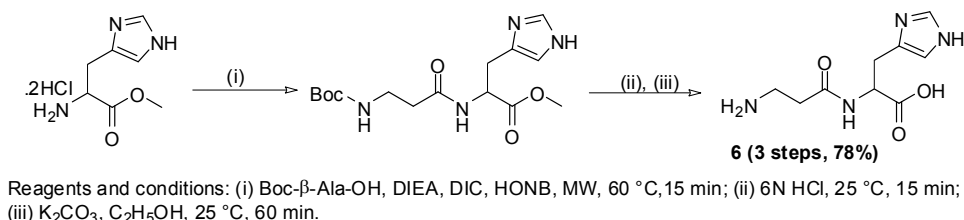
^1H NMR (400 MHz, CD_3OD): $\delta = 7.44\text{--}7.29$ (m, 15H), 7.04 (d, $J = 8.3$ Hz, 2H), 6.88 (d, $J = 8.5$ Hz, 2H), 5.23 (s, 2H), 5.09 (s, 2H), 5.07 (s, 2H), 4.82-4.74 (m, 2H), 4.25-4.13 (m, 3H), 3.99 (dd, $J = 5.3, 14.3$ Hz, 2H), 3.89 (dd, $J = 5.8, 13.8$ Hz, 2H), 3.67 (s, 3H), 3.17-3.08 (m, 3H), 3.08-3.02 (m, 1H), 3.02-2.92 (m, 2H), 2.89-2.79 (m, 1H), 2.20-2.11 (m, 1H), 1.79-1.71 (m, 2H), 1.70-1.58 (m, 2H), 1.55-1.47 (m, 1H), 1.41 (s, 9H), 0.93-0.88 (m, 2H), 0.86 (d, $J = 6.8$ Hz, 3H), 0.83 (d, $J = 6.8$ Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 171.8, 171.6, 170.4, 170.1, 163.5, 160.7, 157.8, 156.6, 155.7, 136.9, 136.6, 135.3, 134.5, 130.2, 128.9, 128.8, 128.5, 128.3, 128.0, 127.9, 127.8, 127.5, 114.9, 114.0, 80.3, 69.9, 69.0, 67.0, 66.9, 66.5, 58.8, 53.3, 52.2, 44.0, 40.3, 36.9, 35.5, 33.8, 31.9, 30.3, 29.1, 28.9, 28.2, 22.7, 22.5, 19.1$; IR (neat): ν 3902, 3748, 3283, 2952, 1658, 1636, 1562, 1541, 1456, 1368, 1275, 1260, 1189, 816, 764, 749, 699; HRMS (ESI-TOF) calculated for $[\text{M}+\text{H}^+]$ 1376.6454; found 1376.6415; HPLC: $t_R = 47.17$ min, 94.7%.

4. Synthesis and Characterization of bioactive peptides

4.1. Synthetic schemes for bioactive peptides

4.1.1. Carnosine (6)

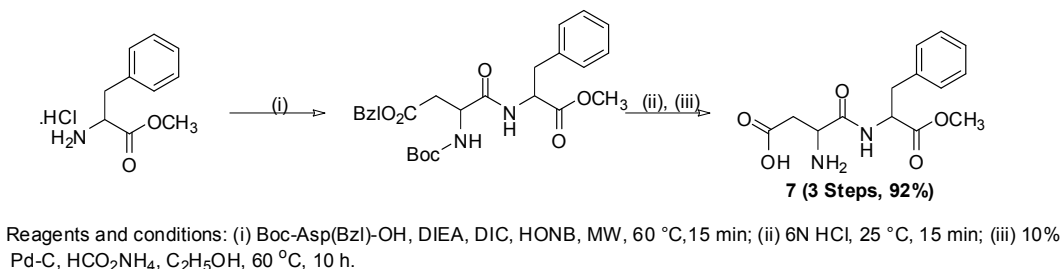
Carnosine (6) was synthesized starting from L-histidine methyl ester·2HCl in 78% overall yield, using synthetic scheme 1. The key step of the synthesis was the successful coupling of side-chain reaction group containing L-histidine methyl ester·2HCl, confirming the reactive group tolerance of the procedure.



Scheme 1. Synthesis of carnosine

4.1.2. Aspartame (7)

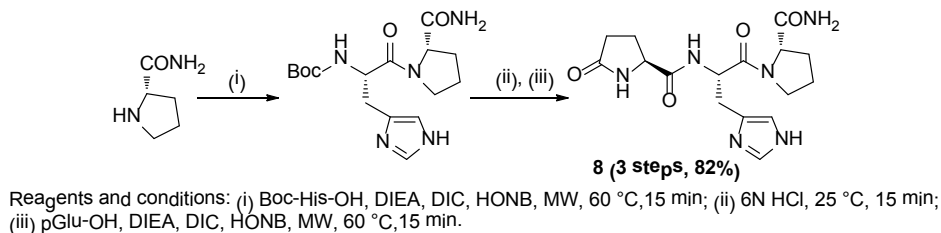
The aspartame (7) was synthesized starting from Phe-OMe·HCl in 92% overall yield, using synthetic scheme 2.



Scheme 2. Synthesis of aspartame

4.1.3. Thyrotropin-releasing hormone (TRH, 8)

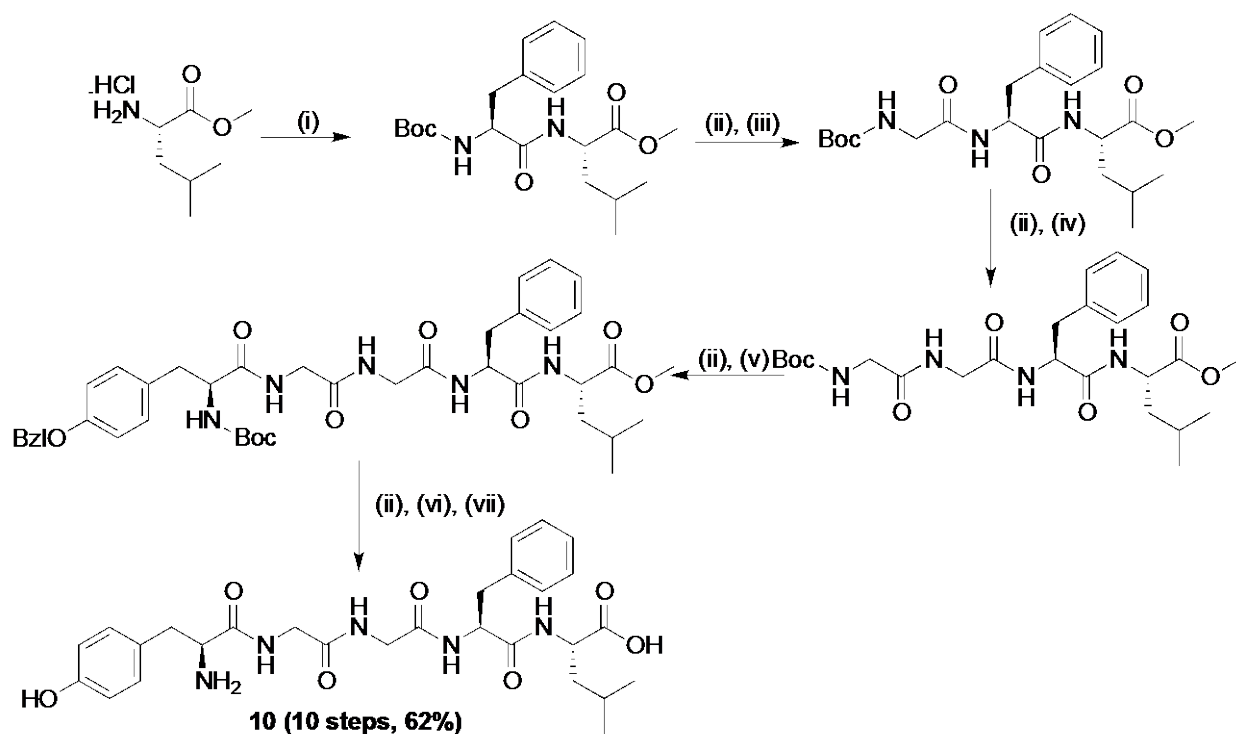
The TRH (8) was synthesized starting from L-ProNH₂ in 82% overall yield, using synthetic scheme 3. The peptide was synthesized in high overall yield, while using the reactive group containing His residue.



Scheme 3. Synthesis of TRH

4.1.4. Leu-enkephalin (10)

The Leu-enkephalin (10) was synthesized in ten steps starting from Leu-OMe·HCl in 62% overall yield, using synthetic scheme 4.



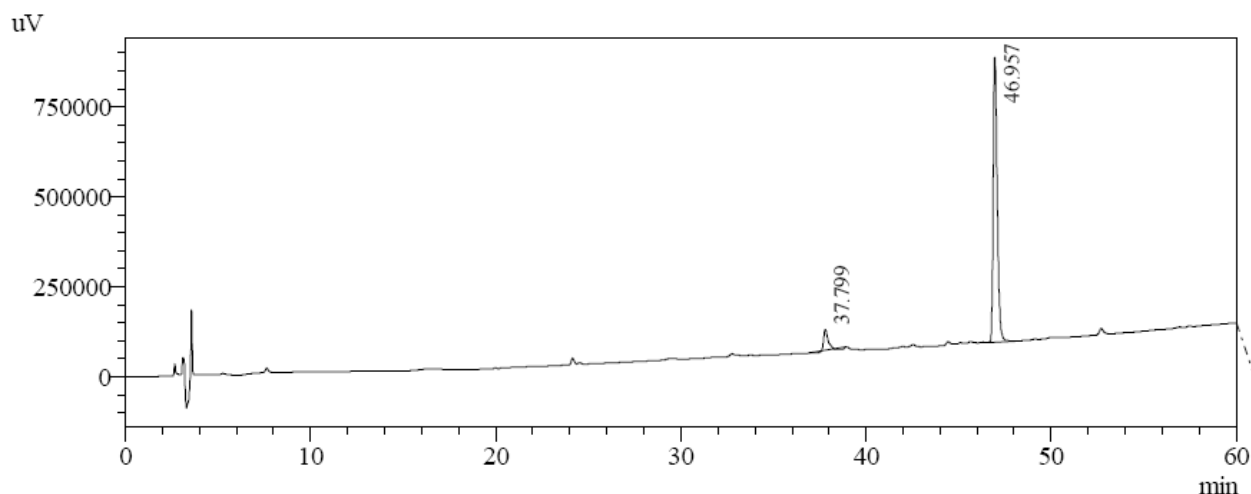
Reagents and conditions: (i) Boc-Phe-OH, DIEA, DIC, HONB, MW, 60 °C, 15 min; (ii) 6N HCl, 25 °C, 15 min; (iii) Boc-Gly-OH, DIEA, DIC, HONB, MW, 60 °C, 15 min; (iv) Boc-Gly-OH, DIEA, DIC, HONB, MW, 60 °C, 15 min; (v) Boc-Tyr(Obzl)-OH, DIEA, DIC, HONB, MW, 60 °C, 15 min; (vi) K₂CO₃, C₂H₅OH, 25 °C, 60 min; (vii) 10% Pd-C, HCOONH₄, C₂H₅OH, 60 °C, 10 h.

Scheme 4. Synthesis of Leu-enkephalin

4.2. Characterization data and HPLC chromatograms of 6, 7, 8, 9 and 10

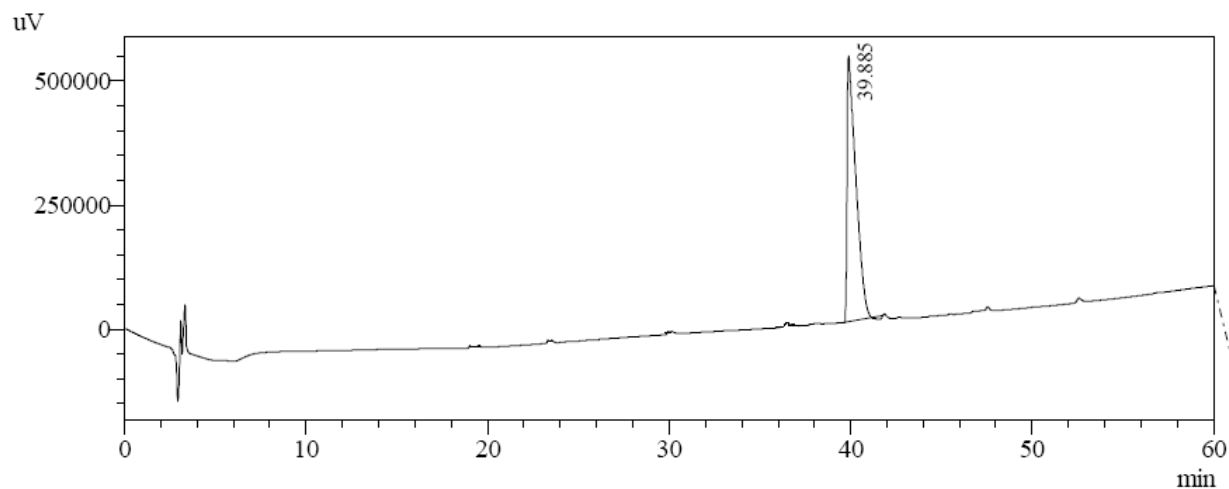
Carnosine (6)

¹H NMR (400 MHz, CD₃OD): δ = 7.76 (s, 1H), 6.98 (s, 1H), 4.75 (t, *J* = 7.6 Hz, 1H), 3.20-3.11 (m, 1H), 3.10-3.07 (m, 1H), 3.06-3.01 (m, 2H), 2.82-2.79 (m, 2H); ¹³C NMR (100 MHz, CD₃OD): δ = 172.9, 171.6, 156.3, 137.1, 134.6, 55.9, 52.6, 37.7, 28.6; IR (neat): ν 3406, 2956, 1757, 1391, 1365, 1275, 1206, 1176, 1094, 763; HRMS (ESI-TOF): calculated for [M+H]⁺ 227.1144; found 227.1149; HPLC: *t*_R = 46.96 min, 95.3%.



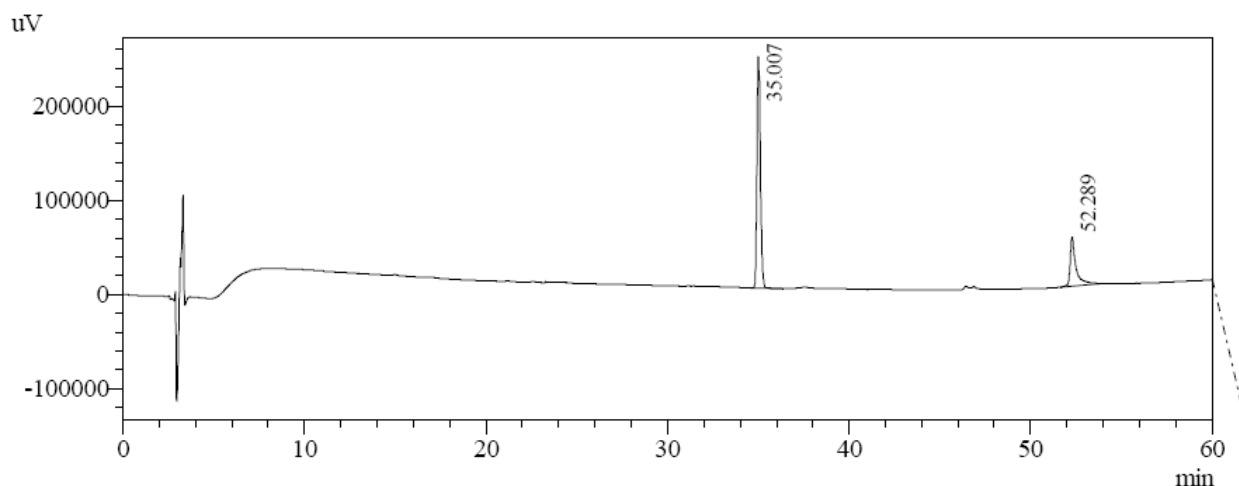
Aspartame (7)

^1H NMR (400MHz, CD_3OD): δ = 7.39 (d, J = 7.3 Hz, 2H), 7.32 (t, J = 7.4 Hz, 2H), 7.27-7.23 (m, 1H), 4.83 (td, J = 6.1, 7.7 Hz, 1H), 4.55 (d, J = 3.3 Hz, 1H), 3.71 (s, 3H), 3.18-3.12 (m, 1H), 3.12-3.06 (m, 1H), 2.91 (dd, J = 4.1, 16.9 Hz, 1H), 2.61 (dd, J = 6.7, 16.9 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ = 171.4, 171.2, 135.9, 129.2, 128.6, 127.7, 127.1, 53.5, 52.3, 50.8, 42.0, 37.3; IR (neat): ν 3290, 2952, 1734, 1674, 1541, 1456, 1367, 1275, 1260, 861, 764; HRMS (ESI-TOF) calculated for $[\text{M}+\text{H}^+]$ 295.1294; found 295.1294; HPLC: t_{R} = 39.89 min, 97.6%.



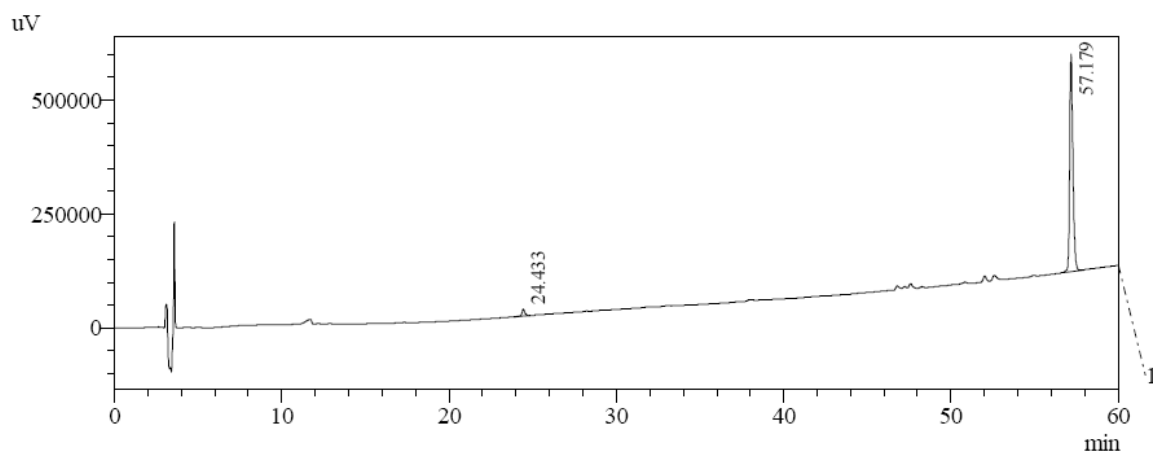
Thyrotropin-releasing hormone (TRH, 8)

^1H NMR (400MHz, CD_3OD): δ = 7.76 (s, 1H), 7.11 (s, 1H), 4.96-4.93 (m, 1H), 4.57-4.50 (m, 1H), 4.19-4.14 (m, 1H), 3.22-3.16 (m, 2H), 2.33-1.97 (m, 10H); ^{13}C NMR (100 MHz, CD_3OD): δ = 177.2, 175.3, 170.2, 139.3, 135.6, 118.6, 66.3, 57.1, 55.3, 52.3, 30.2, 28.7, 21.1; IR (neat): ν 3190, 2898, 1714, 1650, 1523, 1412, 1167, 1002, 869, 784; HRMS (ESI-TOF) calculated for $[\text{M}+\text{H}^+]$ 363.1781; found 363.1781; HPLC: t_{R} = 35.01 min, 93.7%.



Thymopentin (9)

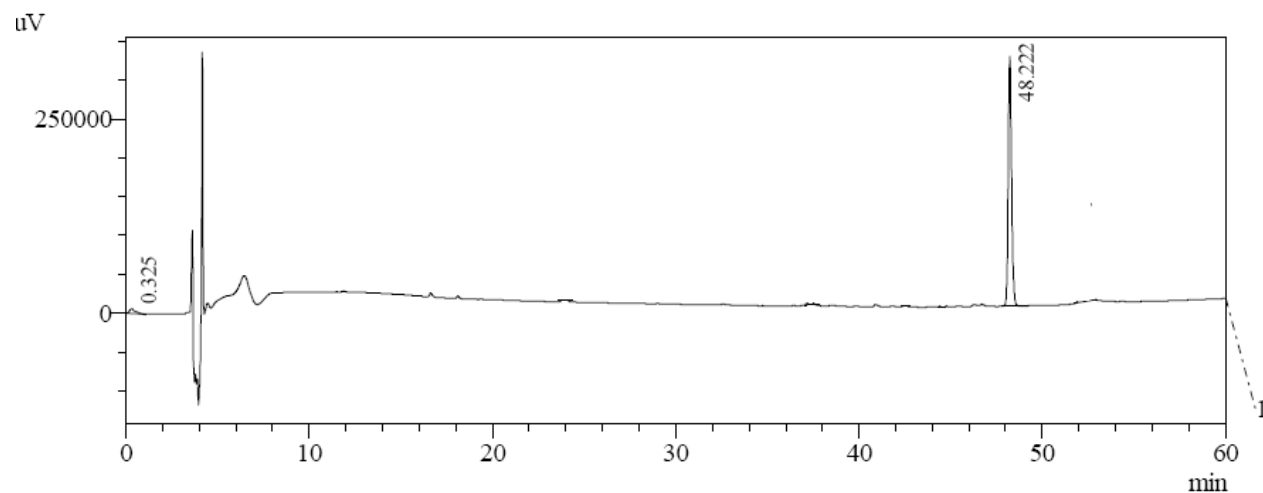
^1H NMR (400MHz, CD_3OD): δ = 7.13 (d, J = 7.4 Hz, 2H), 6.98 (d, J = 7.3 Hz, 2H), 4.80-4.71 (m, 3H), 4.25-4.18 (m, 2H), 3.97 (dd, J = 5.3, 14.3 Hz, 2H), 3.86 (dd, J = 5.8, 13.8 Hz, 2H), 3.17-3.08 (m, 3H), 3.07-3.02 (m, 1H), 3.01-2.92 (m, 2H), 2.87-2.79 (m, 1H), 2.20-2.11 (m, 1H), 1.78-1.71 (m, 2H), 1.65-1.54 (m, 2H), 1.50-1.47 (m, 1H), 0.93-0.88 (m, 2H), 0.86 (d, J = 6.8 Hz, 3H), 0.83 (d, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD): δ = 171.8, 171.6, 170.4, 170.1, 163.5, 160.7, 136.9, 130.2, 128.9, 69.9, 67.0, 66.9, 66.5, 58.8, 52.2, 44.0, 40.3, 36.9, 35.5, 33.8, 31.9, 30.3, 29.1, 19.1; IR (neat): ν 3902, 2952, 1658, 1636, 1562, 1541, 1456, 1368, 1275, 1260, 1189, 816, 699; HRMS (ESI-TOF) calculated for $[\text{M}+\text{H}^+]$ 680.3731; found 680.3738; HPLC: t_{R} = 57.17 min, 97.1%.



Leu-enkephalin (10)

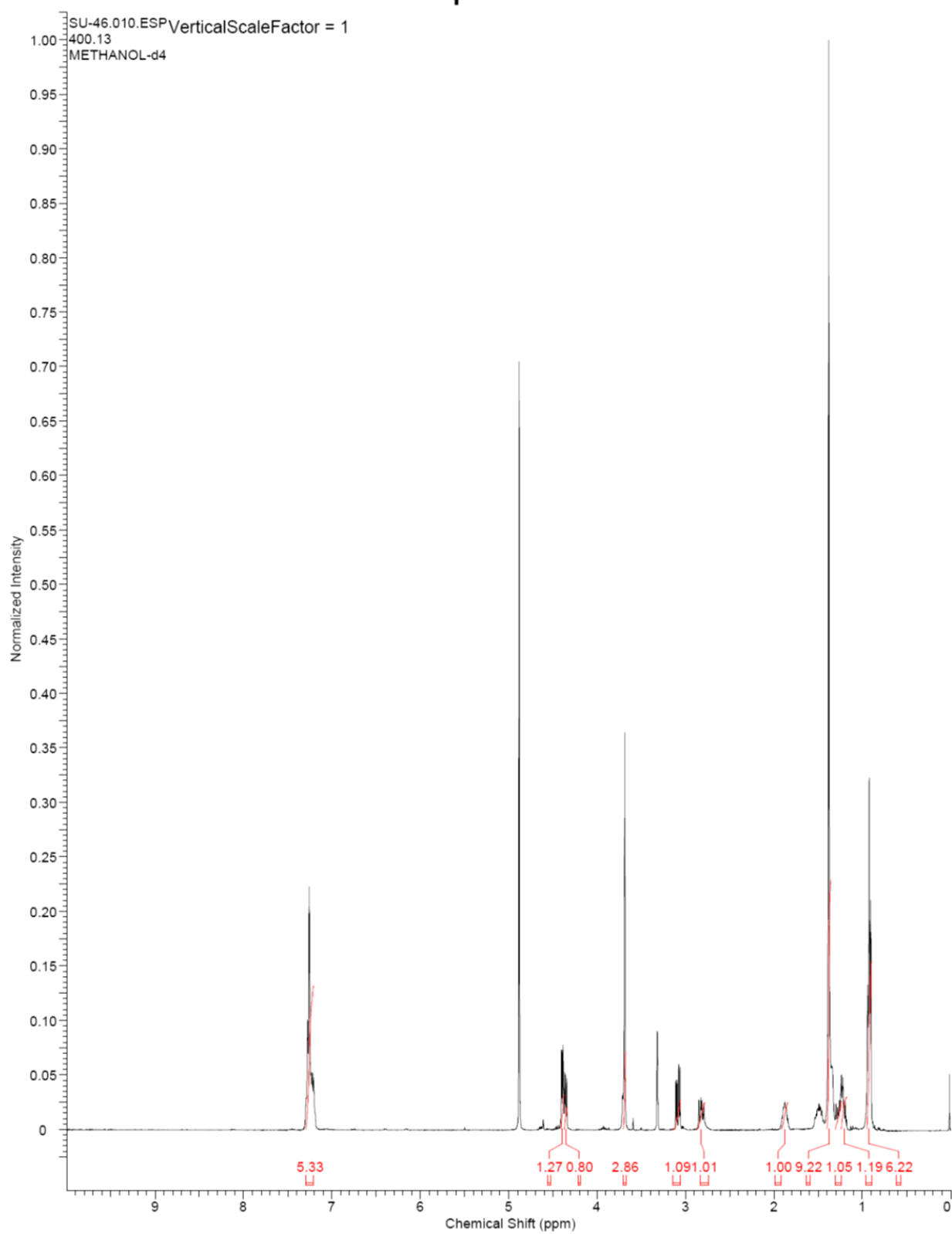
^1H NMR (400MHz, CD_3OD): δ = 7.42 (d, J = 6.4 Hz, 2H), 7.33 (d, J = 6.3 Hz, 2H), 7.27-7.19 (m, 5H), 4.74 (t, J = 6.1 Hz, 1H), 4.56-4.50 (m, 1H), 4.32 (dd, J = 5.4, 8.9 Hz, 1H), 4.10 (s, 4H), 3.19-3.11 (m, 1H), 3.10-3.07 (m, 1H), 3.07-3.01 (m, 2H), 2.82-2.74 (m, 1H), 1.75-1.70 (m, 2H), 0.91-0.90 (m, 6H); ^{13}C NMR (100 MHz, CD_3OD): δ = 175.0, 174.7, 171.7, 171.5, 155.7, 136.1,

130.6, 130.2, 125.6, 122.3, 58.1, 56.7, 54.3, 42.0, 38.1, 37.6, 24.8, 22.9; IR (neat): ν 3317, 3064, 2964, 2876, 2475, 2419, 1742, 1690, 1653, 1510, 1450, 1385, 1364, 1250, 1206, 1147, 1089, 1030, 781, 758, 740, 696; HRMS (ESI-TOF) calculated for $[M+H]^+$ 556.2771; found 556.2765; HPLC: t_R = 48.22 min, 98.7%.

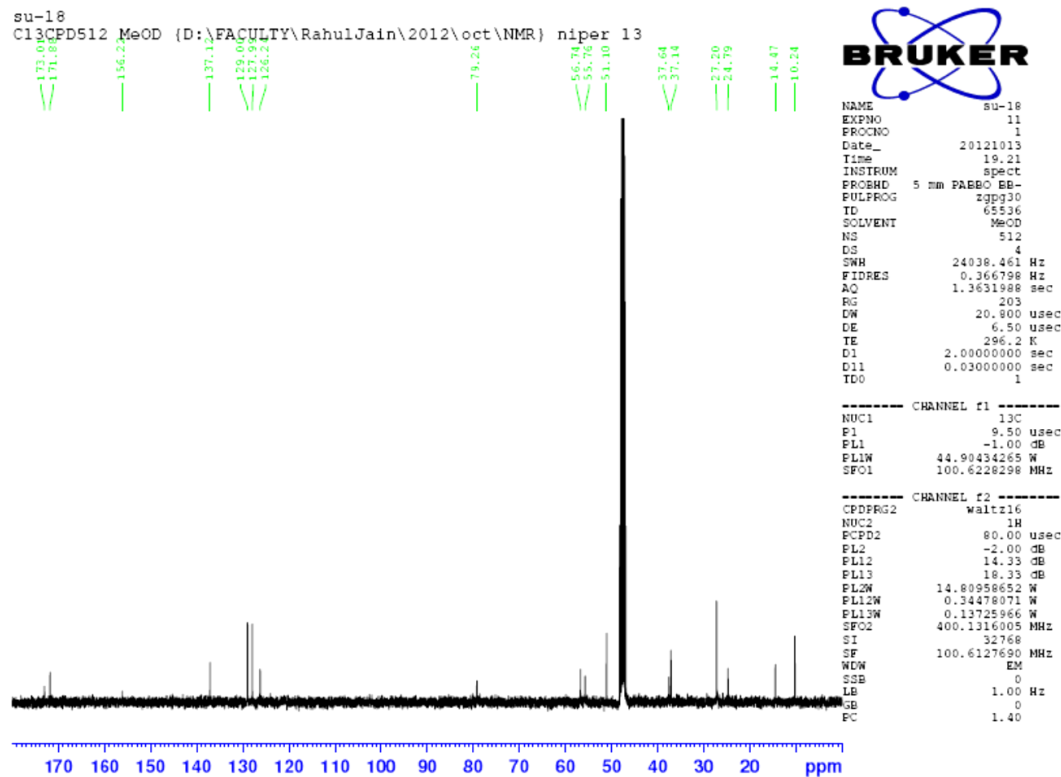


5. Representative NMR Spectrum of Peptides

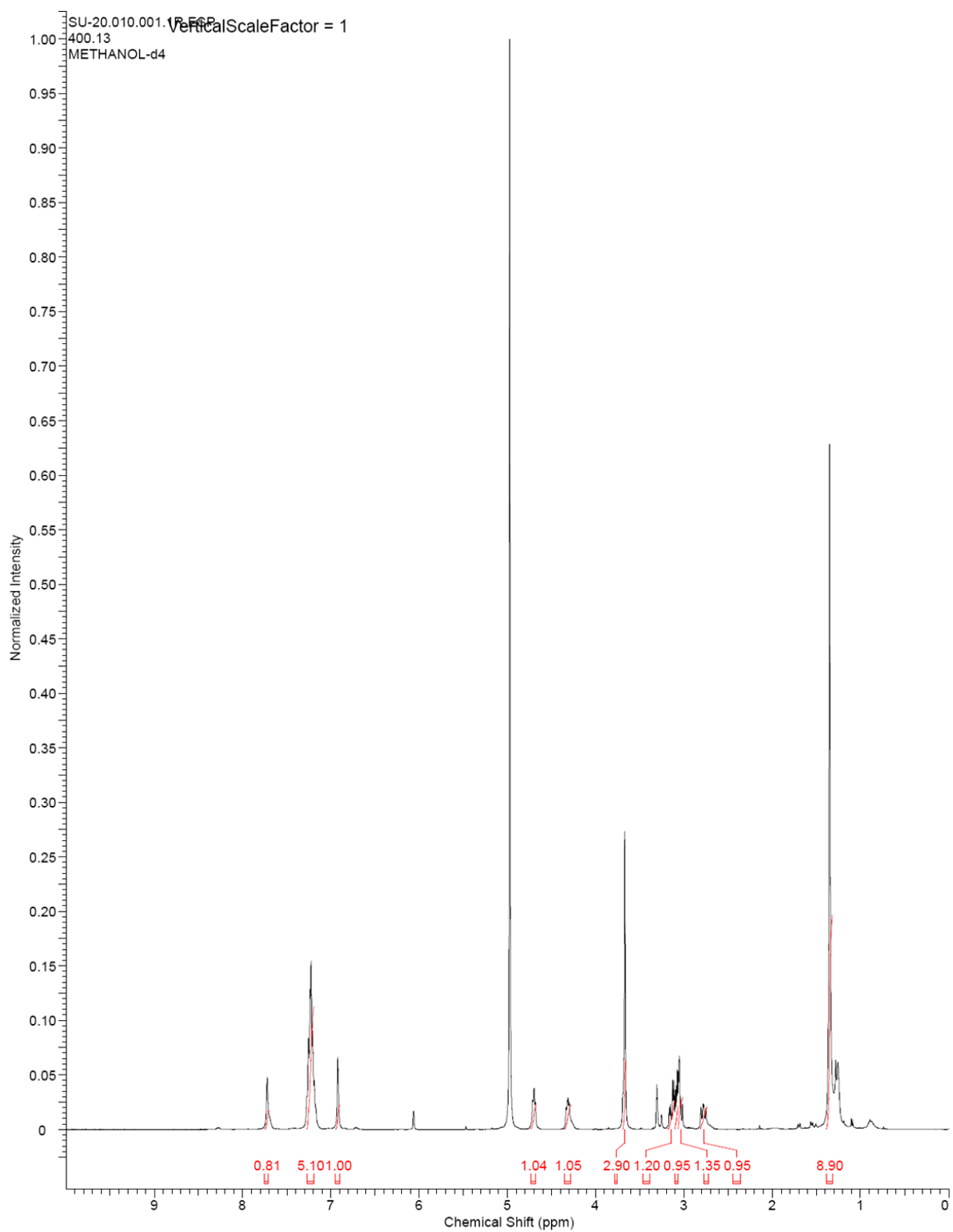
¹H spectrum of 2a



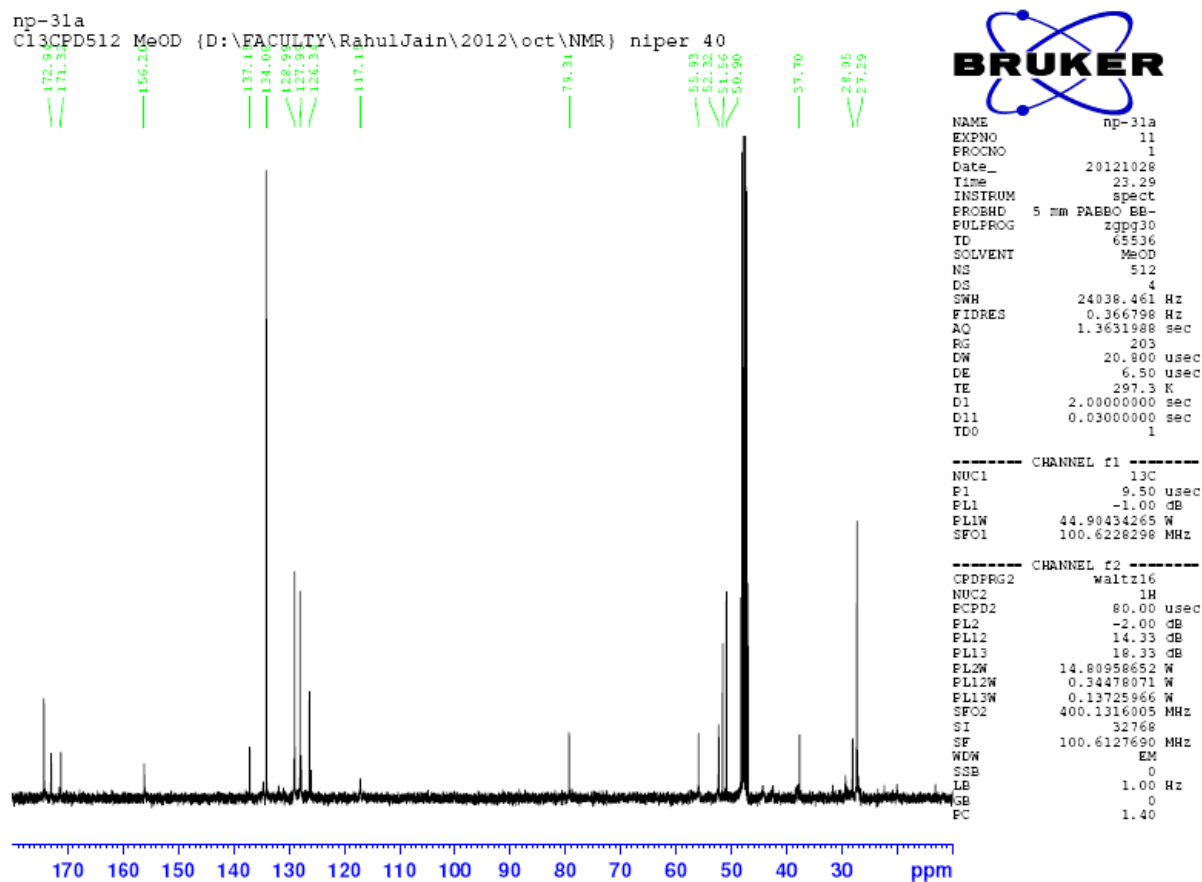
¹³C spectrum of 2a



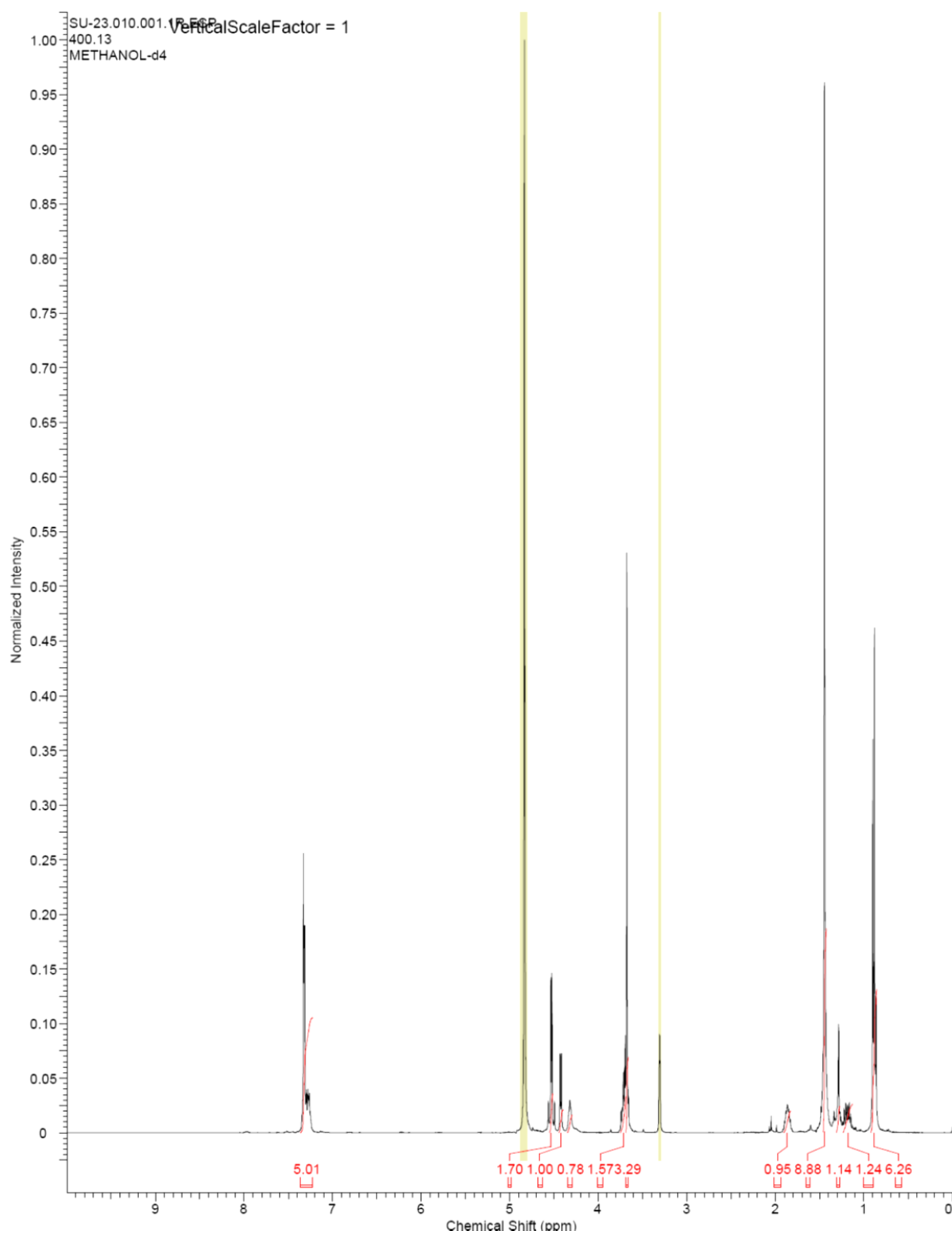
¹H spectrum of 2b



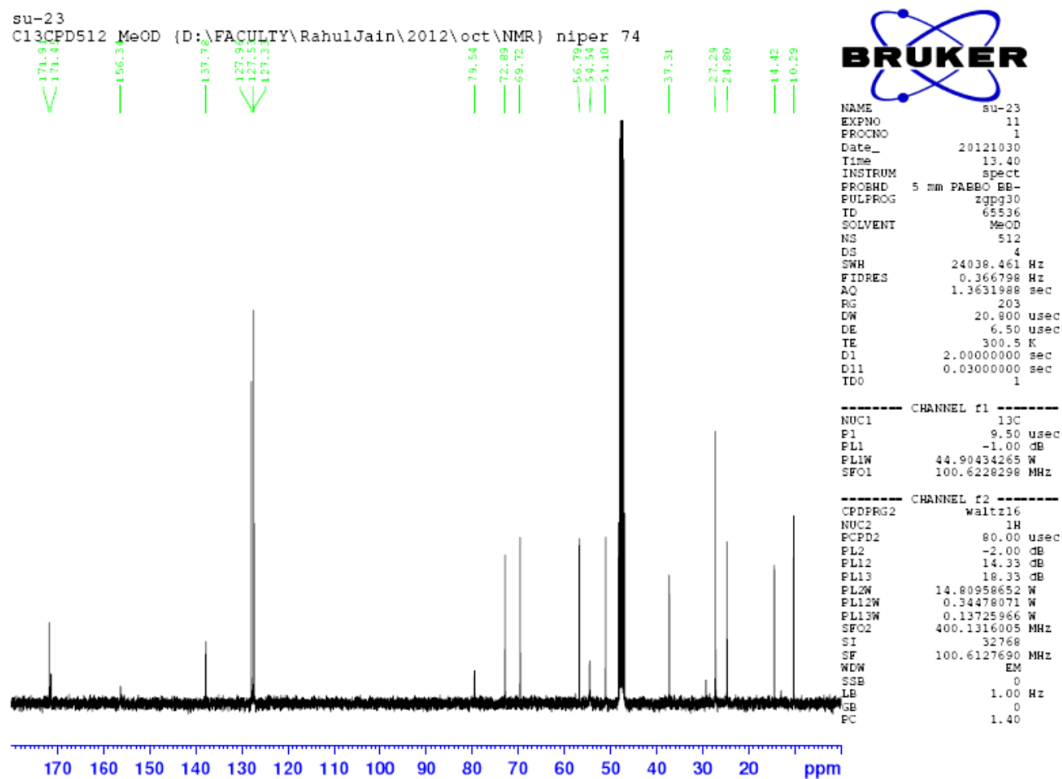
¹³C spectrum of 2b



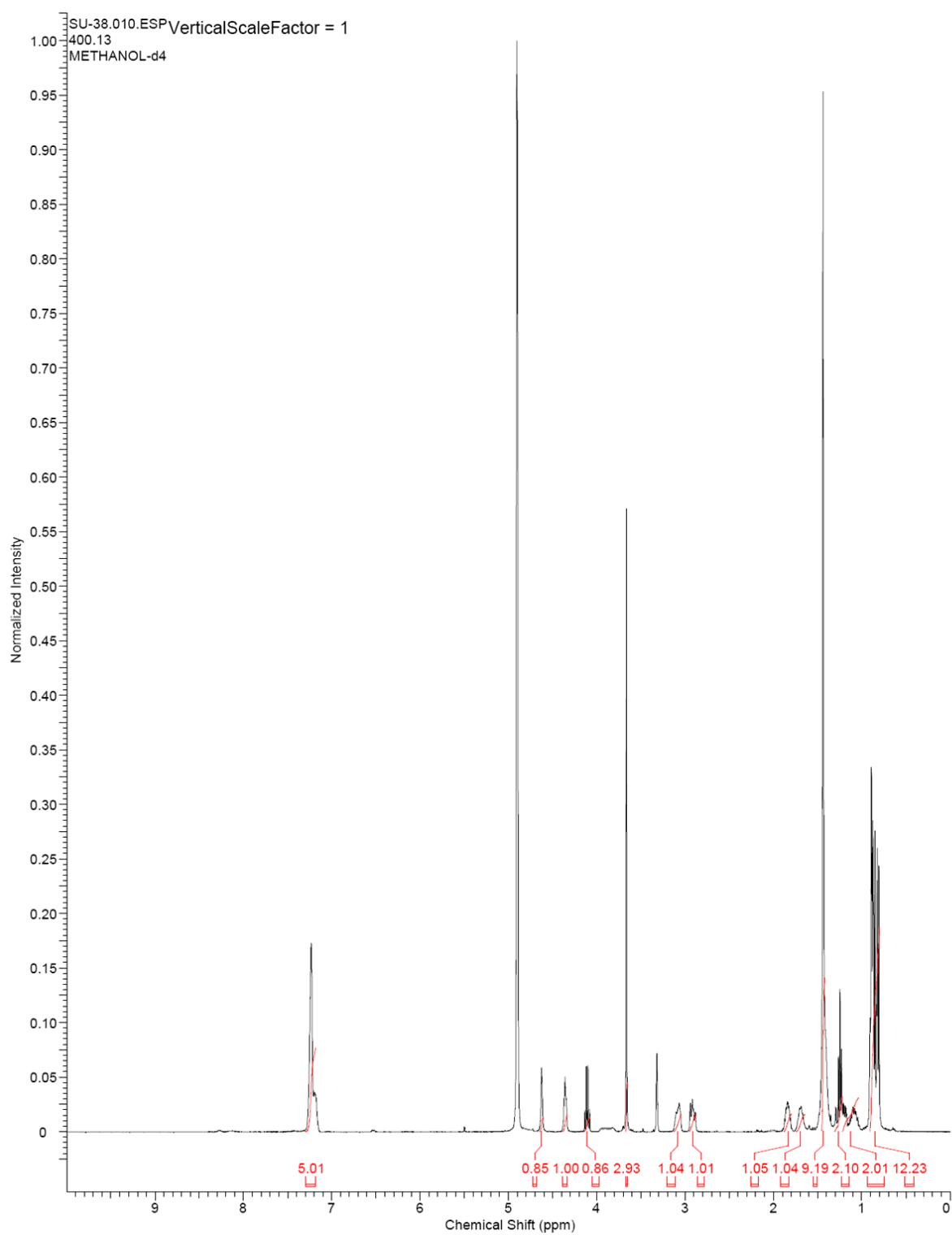
¹H spectrum of 2c



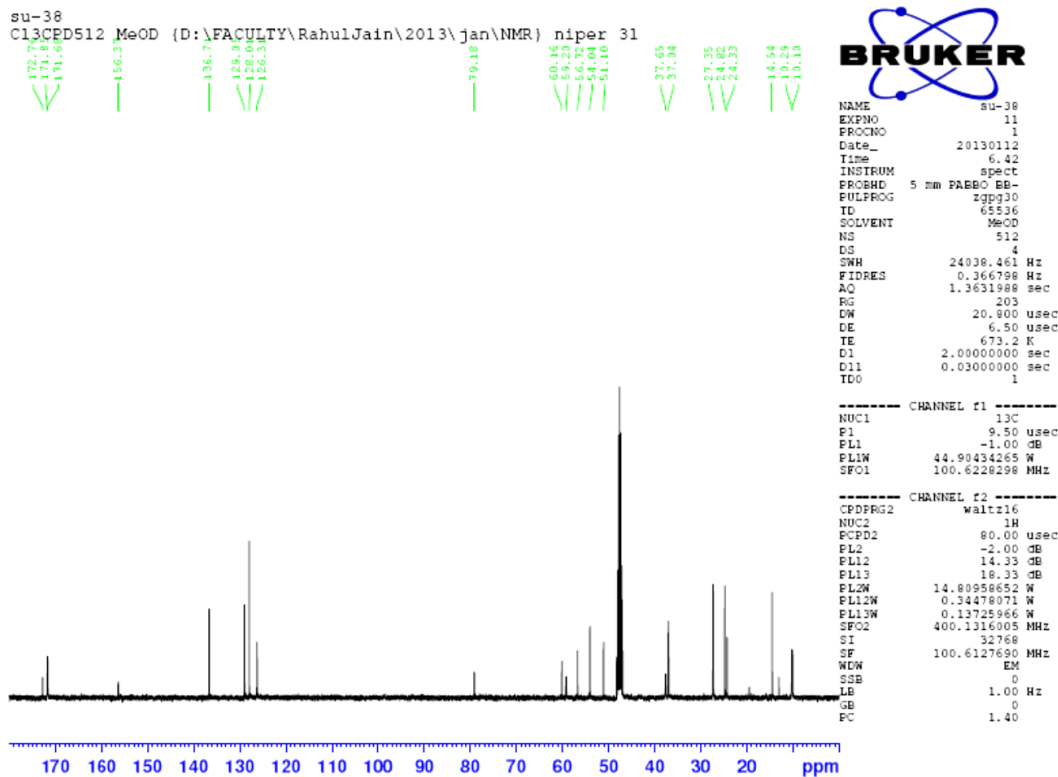
¹³C spectrum of 2c



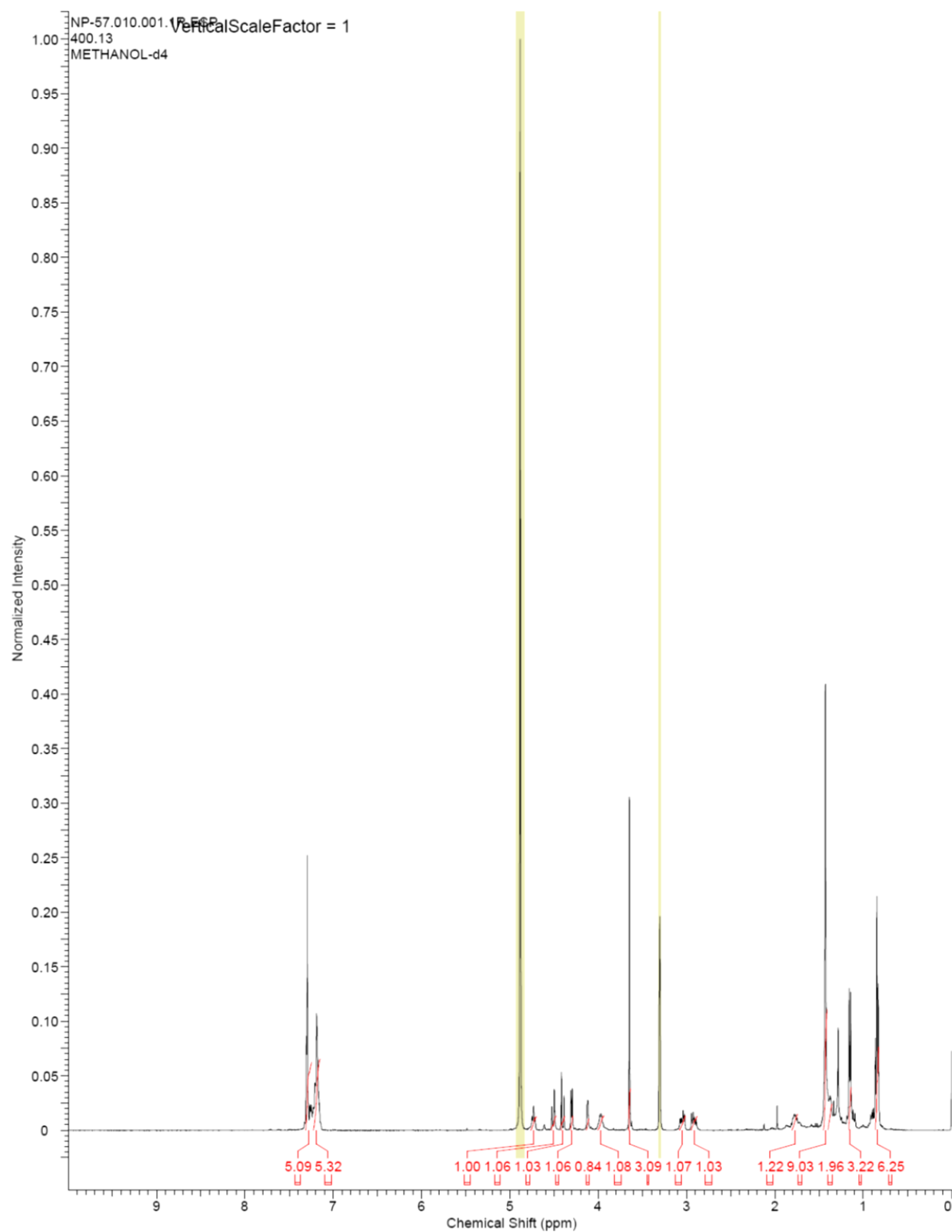
¹H spectrum of 3a



¹³C spectrum of 3a



¹H spectrum of 3b



¹³C spectrum of 3b

