

Supporting Information

for

Peptide synthesis: ball-milling, in solution, or on solid support, what is the best strategy?

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[§]<http://www.greenchem.univ-montp2.fr>

Experimental procedures and characterization data of peptides

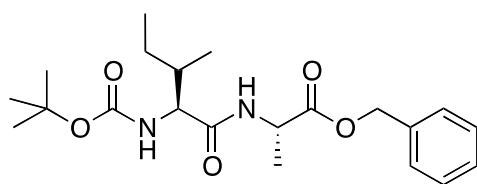
General information

Reagents were purchased from Sigma Aldrich, Fluka, Propeptide, Novabiochem or Senn Chemicals and used without further purification. All the amino acid derivatives employed in this study displayed L absolute configuration. The milling treatments were carried out in a Retsch Mixer Mill 200. SPPS was performed at the "SynBio3 Platform" (IBMM, Université de Montpellier) using a Liberty Blue peptide synthesizer (CEM). Analyses were performed at the "Plateforme Technologique Laboratoire de Mesures Physiques" (IBMM, Université de Montpellier). ¹H NMR spectra were recorded either on a Bruker Avance I 300 MHz or on a Bruker Avance III HD 400 MHz spectrometer and are reported in ppm using residual solvent as an internal standard (CDCl₃ at 7.26 ppm, DMSO-*d*₆ at 2.50 ppm, CD₃OD at 3.31 ppm and D₂O at 4.79 ppm). Data are reported as s = singlet, d = doublet, t = triplet, q = quadruplet, qt = quintuplet, m = multiplet; coupling constant in Hz; integration. ¹³C NMR spectra were

recorded either on a Bruker Avance I 75 MHz or on a Bruker Avance III HD 101 MHz and are reported in ppm using residual solvent as an internal standard (CDCl₃ at 77.16 ppm, DMSO-*d*₆ at 39.52 ppm and CD₃OD at 49.00 ppm). Mass spectra were obtained by LC–ESIMS using a Waters Alliance 2695 as LC, coupled to a Waters ZQ spectrometer with electrospray source, a simple quadrupole analyzer and a UV Waters 2489 detector. Purity of products was determined by HPLC (Agilent technologies 1220 infinity LC) using Ony Monolithic HD–C18 (50 × 4.6 mm) column and a UV detector (λ = 214 nm); samples were injected with a volume of 5 μ L. The solvent system used water (0.1% TFA) and acetonitrile (0.1% TFA) as eluent with a flow of 3mL/min (0–100% in 3 min).

Operating procedures and analytical data

Boc-Ile-Ala-OBn [70691-54-2]¹



Synthesis in solution

p-TsOH·H-Ala-OBn (239.9 mg, 0.683 mmol, 1.0 equiv), Boc-Ile-OH (189.5 mg, 0.819 mmol, 1.2 equiv), Oxyma (116.2 mg, 0.818 mmol, 1.2 equiv) and *N,N*-diisopropylethylamine (475 μ L, 2.727 mmol, 4.0 equiv) were introduced in a round-bottomed flask and solubilized in a minimum amount of DMF (1 mL) under magnetic stirring. After a few minutes of stirring, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (145 μ L, 0.819 mmol, 1.2 equiv) was added. Aliquots of the reaction mixture were withdrawn periodically and immediately quenched with a 1:1 MeCN/1 N aqueous HCl solution. Conversion was determined by HPLC analysis.

Table of conversion

Time (min)	0.0	10.0	20.0	30.0	60.0	120.0	180.0
Conversion in solution (%)	0.0	30.6	46.5	57.2	74.6	90.6	97.6

After completion of the reaction, the mixture was concentrated under vacuum and EtOAc (20 mL) and deionized water (15 mL) were added. The aqueous phase was extracted with EtOAc (2 × 10 mL) and the combined organic phase was washed with 1 N aqueous HCl solution (2 × 10 mL), 1 N aqueous NaOH solution (2 × 10 mL) and saturated aqueous NaCl solution (2 × 10 mL), dried over MgSO₄, filtered and concentrated under vacuum to furnish the desired product as a pale yellow solid (236.5 mg, 88% yield, 96% HPLC purity).

Synthesis in ball-mill

p-TsOH·H-Ala-OBn (239.8 mg, 0.682 mmol, 1.0 equiv), Boc-Ile-OH (189.6 mg, 0.820 mmol, 1.2 equiv), Oxyma (116.5 mg, 0.820 mmol, 1.2 equiv), NaH₂PO₄ (327.9 mg, 2.733 mmol, 4.0 equiv) and EtOAc (450 μ L, η = 0.45 μ L/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was subjected to grinding for 2 min in the mixer mill operated at 25 Hz. *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide (145 μ L,

0.819 mmol, 1.2 equiv) was added and the jar was subjected to grinding for 8 min in the mixer mill operated at 25 Hz. A first aliquot of the reaction mixture was withdrawn, immediately quenched with 1:1 MeCN/1 N aqueous HCl solution and analyzed by HPLC to determine the conversion. The jar was subjected to further grinding in the mixer mill operated at 25 Hz until next aliquot was withdrawn. This operation was repeated until completion of the reaction.

Table of conversion

Time (min)	0.0	10.8	20.0	29.6	39.5	48.8	59.8
Effective milling time (min)	0.0	8.0	16.0	24.0	32.0	40.0	48.0
Conversion in ball-mill (%)	0.0	96.2	98.1	98.8	98.9	99.4	100.0

The reaction mixture was treated with EtOAc (20 mL) and deionized water (20 mL). The aqueous phase was extracted with EtOAc (2×10 mL) and the combined organic phase was washed with 1 N aqueous HCl solution (2×10 mL), 1 N aqueous NaOH solution (2×10 mL) and saturated aqueous NaCl solution (2×10 mL), dried over MgSO₄, filtered, concentrated under vacuum to furnish the desired product as a white solid (238.8 mg, 89% yield, 93% HPLC purity).

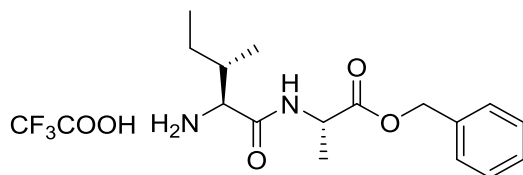
Characterization

¹H NMR (300 MHz, CDCl₃): δ 7.41 – 7.28 (5H), 6.42 (br d, J = 6.3 Hz, 1H), 5.20 (d, J = 12.3 Hz, 1H), 5.14 (d, J = 12.0 Hz, 1H), 5.05 (br d, J = 7.8 Hz, 1H), 4.63 (qt, J = 7.2 Hz, 1H), 3.95 (br t, J = 7.5 Hz, 1H), 1.86 (m, 1H), 1.56 – 1.33 (13H), 1.12 (m, 1H), 0.97 – 0.82 (6H).

¹³C NMR (101 MHz, CDCl₃): δ 172.6, 171.3, 155.9, 135.4, 128.7, 128.6, 128.3, 80.0, 67.3, 59.2, 48.2, 37.5, 28.4, 24.9, 18.4, 15.6, 11.5.

MS (ESI): m/z 393.2 [M+H]⁺

TFA·H-Ile-Ala-OBn



Synthesis in solution

Boc-Ile-Ala-OBn (213.5 mg, 0.544 mmol, 1.0 equiv) was solubilized in a 1:1 TFA/CH₂Cl₂ mixture (1 mL) in a round-bottomed flask with magnetic stirring. After 60 min agitation, the reaction mixture was concentrated under vacuum to furnish the desired product as a yellow solid (234.3 mg, >99% yield, 99% HPLC purity).

Synthesis in ball-mill

Boc-Ile-Ala-OBn (672.0 mg, 1.712 mmol, 1.0 equiv) and trifluoroacetic acid (TFA, 655 μ L, 5.0 equiv) were introduced in a 15 mL PTFE grinding jar equipped with one PTFE ball (10 mm diameter). The jar was subjected to grinding for 4 h in the mixer mill operated at 25 Hz. The reaction mixture was recovered with CH_2Cl_2 and concentrated under vacuum to furnish the desired product as a pale yellow solid (703.2 mg, >99% yield, 100% HPLC purity).

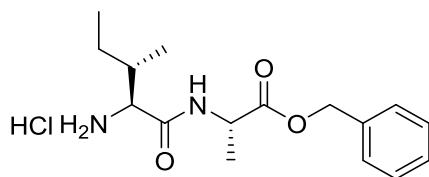
Characterization

^1H NMR (300 MHz, CD_3OD): δ 7.40 – 7.27 (5H), 5.19 (d, J = 12.3 Hz, 1H), 5.13 (d, J = 12.3 Hz, 1H), 4.54 (q, J = 7.3 Hz, 1H), 3.71 (d, J = 5.6 Hz, 1H), 1.90 (m, 1H), 1.57 (m, 1H), 1.43 (d, J = 7.3 Hz, 3H), 1.31 – 1.10 (1H), 0.99 (d, J = 6.9 Hz, 3H), 0.91 (t, J = 7.4 Hz, 3H).

^{13}C NMR (75 MHz, CD_3OD): δ 173.4, 169.3, 137.1, 129.6, 129.4, 68.1, 58.9, 38.0, 25.3, 17.2, 14.8, 11.6.

MS (ESI): m/z 292.9 $[\text{M}+\text{H}]^+$

HCl·H-Ile-Ala-OBn [178313-32-1]²



Synthesis in ball-mill

Boc-Ile-Ala-OBn (210.8 mg, 0.537 mmol, 1.0 equiv) was submitted to gaseous HCl for 3.5 h. HCl·H-Ile-Ala-OBn was recovered as a white solid (181.1 mg, >99% yield, 97% HPLC purity).

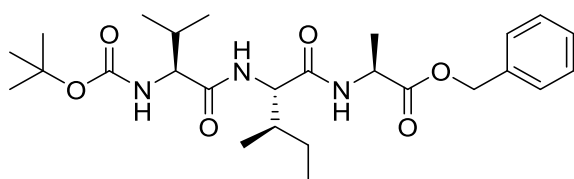
Characterization

^1H NMR (300 MHz, D_2O): δ 7.49 – 7.38 (5H), 5.20 (s, 2H), 4.53 (q, J = 7.3 Hz, 1H), 3.86 (d, J = 5.6 Hz, 1H), 2.01 – 1.83 (1H), 1.91 (m, 1H), 1.57 – 1.48 (1H), 1.45 (d, J = 7.1 Hz, 3H), 1.17 (m, 1H), 0.92 (d, J = 6.9 Hz, 3H), 0.85 (t, J = 7.4 Hz, 3H).

^{13}C NMR (101 MHz, D_2O): δ 173.7, 168.9, 135.0, 128.82, 128.77, 128.5, 67.8, 57.5, 49.0, 36.3, 24.0, 15.8, 13.6, 10.5.

MS (ESI): m/z 293.3 $[\text{M}+\text{H}]^+$

Boc-Val-Ile-Ala-OBn



Synthesis in solution

TFA-H-Ile-Ala-OBn (178.1 mg, 0.438 mmol, 1.0 equiv), Boc-Val-OH (114.7 mg, 0.528 mmol, 1.2 equiv), Oxyma (74.9 mg, 0.527 mmol, 1.2 equiv) and *N,N*-diisopropylethylamine (305 μ L, 1.751 mmol, 4.0 equiv) were introduced in a round-bottomed flask and solubilized in a minimum of DMF (700 μ L) with magnetic stirring. After a few minutes, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (93.1 μ L, 0.526 mmol, 1.2 equiv) was added. Aliquots of the reaction mixture were withdrawn periodically and immediately quenched with 1:1 MeCN/1 N aqueous HCl solution. The conversion was determined by HPLC analysis.

Table of conversion

Time (min)	0.0	5.0	10.0	15.0	20.0	30.0	40.0	60.0
Conversion in solution (%)	0.0	17.4	27.1	37.3	45.6	59.9	69.1	82.8

After stirring for 15 h, the reaction mixture was concentrated under vacuum and EtOAc (20 mL) and deionized water (20 mL) added. The aqueous phase was extracted with EtOAc (2 $\times$ 10 mL) and the combined organic phase was washed with 1 N aqueous HCl solution (2 $\times$ 10 mL), 1 N aqueous NaOH solution (2 $\times$ 10 mL) and saturated aqueous NaCl solution (2 $\times$ 10 mL), dried over MgSO₄, filtered and concentrated under vacuum to furnish the desired product as a pale white solid (165.8 mg, 77% yield, 90% HPLC purity).

Synthesis in ball-mill

HCl-H-Ile-Ala-OBn (111.3 mg, 0.338 mmol, 1.0 equiv), Boc-Val-OH (88.8 mg, 0.409 mmol, 1.2 equiv), Oxyma (57.9 mg, 0.407 mmol, 1.2 equiv), NaH₂PO₄ (162.4 mg, 1.354 mmol, 4.0 equiv) and EtOAc (450 μ L, η = 0.93 μ L/mg) were introduced in a 15 mL PTFE grinding jar equipped with one stainless steel ball (10 mm diameter). The jar was subjected to grinding for 2 min in the mixer mill operated at 25 Hz. Then, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (71.9 μ L, 0.406 mmol, 1.2 equiv) was added and the jar was subjected to grinding for 3 min in the mixer mill operated at 25 Hz. A first aliquot of the reaction mixture was withdrawn, immediately quenched with 1:1 MeCN/1 N aqueous HCl solution and analyzed by HPLC to determine the conversion. The jar was subjected to further grinding in the mixer mill operated at 25 Hz until next aliquot was withdrawn. This operation was repeated until completion of the reaction.

Table of conversion

Time (min)	0.0	5.0	10.0	20.0	30.0	40.0	60.0
Effective milling time (min)	0.0	3.0	6.0	14.0	22.0	30.0	50.0
Conversion in ball-mill	0.0	95.3	98.8	100.0	100.0	100.0	100.0

To the reaction mixture was added EtOAc (25 mL) and deionized water (20 mL). The aqueous phase was extracted with EtOAc (2 $\times$ 10 mL) and the combined organic phase was washed with 1 N aqueous HCl solution (2 $\times$ 10 mL), 1 N aqueous NaOH solution (2 $\times$ 10 mL) and saturated aqueous NaCl solution (2 $\times$ 10 mL), dried over MgSO₄, filtered, concentrated

under vacuum to furnish the desired product as a white solid (148.4 mg, 89% yield, 99% HPLC purity).

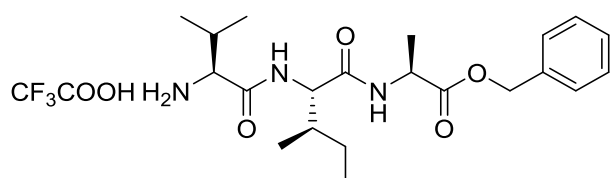
Characterization

¹H NMR (400 MHz, CDCl₃): δ 7.37 – 7.28 (5H), 6.90 (br d, *J* = 6.8 Hz, 1H), 6.80 (br d, *J* = 8.4 Hz, 1H), 5.25 (br d, *J* = 8.3 Hz, 1H), 5.18 (d, *J* = 12.0 Hz, 1H), 5.12 (d, *J* = 12.4 Hz, 1H), 4.61 (qt, *J* = 7.2 Hz, 1H), 4.33 (t, *J* = 8.0 Hz, 1H), 3.94 (br t, *J* = 7.2 Hz, 1H), 2.09 (m, 1H), 1.94 – 1.82 (1H), 1.56 – 1.46 (1H), 1.42 (s, 9H), 1.38 (d, *J* = 7.2 Hz, 3H) 1.11 (m, 1H), 0.95 – 0.83 (12H).

¹³C NMR (101 MHz, CDCl₃): δ 172.5, 172.0, 170.7, 156.1, 135.5, 128.7, 128.5, 128.3, 80.0, 67.2, 60.3, 57.8, 48.2, 37.0, 30.8, 28.4, 24.9, 19.4, 18.2, 18.1, 15.4, 11.3.

MS (ESI): *m/z* 492.1 [M+H]⁺

TFA·H-Val-Ile-Ala-OBn



Synthesis in solution

Boc-Val-Ile-Ala-OBn (141.5 mg, 0.288 mmol, 1.0 equiv) was solubilized in a 1:1 TFA/CH₂Cl₂ mixture (1 mL) in a round-bottomed flask with magnetic stirring. After 90 min agitation, the reaction mixture was concentrated under vacuum to furnish the desired product as a yellow solid (146.8 mg, >99% yield, 92% HPLC purity).

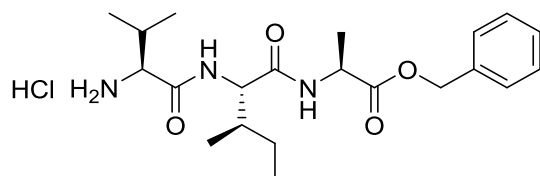
Characterization

¹H NMR (400 MHz, CD₃OD): δ 7.45 – 7.28 (5H), 5.17 (d, *J* = 12.4 Hz, 1H), 5.13 (d, *J* = 12.0 Hz, 1H), 4.44 (q, *J* = 7.3 Hz, 1H), 4.27 (d, *J* = 8.0 Hz, 1H), 3.74 (d, *J* = 5.7 Hz, 1H), 2.17 (m, 1H), 1.81 (m, 1H), 1.59 (m, 1H), 1.39 (d, *J* = 7.3 Hz, 3H), 1.18 (m, 1H), 1.07 – 0.97 (m, 6H), 0.94 (d, *J* = 6.8 Hz, 3H), 0.88 (t, *J* = 7.4 Hz, 3H).

¹³C NMR (101 MHz, CD₃OD): δ 173.6, 172.9, 169.5, 137.2, 129.6, 129.3, 67.9, 59.4, 59.2, 38.1, 31.7, 25.9, 18.9, 17.8, 17.3, 15.7, 11.3.

MS (ESI): *m/z* 392.3 [M+H]⁺

HCl·H-Val-Ile-Ala-OBn



Synthesis in ball-mill

Boc-Val-Ile-Ala-OBn (121.2 mg, 0.247 mmol, 1.0 equiv) was submitted to gaseous HCl for 16 h. HCl·H-Val-Ile-Ala-OBn was recovered as a white solid (101.1 mg, 96% yield, 97% HPLC purity).

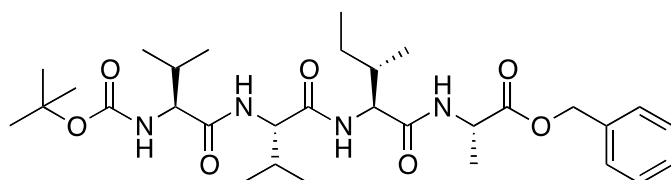
Characterization

¹H NMR (400 MHz, D₂O): δ 7.49 – 7.39 (5H), 5.21 (s, 2H), 4.43 (q, *J* = 7.3 Hz, 1H), 4.19 (d, *J* = 8.3 Hz, 1H), 3.85 (d, *J* = 6.1 Hz, 1H), 2.19 (m, 1H), 1.87 – 1.74 (1H), 1.55 – 1.46 (1H), 1.43 (d, *J* = 7.3 Hz, 3H), 1.17 (m, 1H), 1.02 – 0.98 (6H), 0.92 – 0.80 (6H).

¹³C NMR (101 MHz, D₂O): δ 174.0, 172.6, 169.2, 135.2, 128.8, 128.7, 128.4, 67.7, 58.2, 48.9, 36.0, 30.1, 24.5, 17.6, 16.8, 16.5, 15.9, 14.5, 14.3, 10.0.

MS (ESI): *m/z* 392.3 [M+H]⁺

Boc-Val-Val-Ile-Ala-OBn



Synthesis in solution

TFA·H-Val-Ile-Ala-OBn (121.6 mg, 0.241 mmol, 1.0 equiv), Boc-Val-OH (62.8 mg, 0.289 mmol, 1.2 equiv), Oxyma (41.2 mg, 0.290 mmol, 1.2 equiv) and *N,N*-diisopropylethylamine (168 μL, 0.965 mmol, 4.0 equiv) were introduced in a round-bottomed flask and solubilized in a minimum amount of DMF (1.4 mL) with magnetic stirring. After a few minutes, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (51.1 μL, 0.289 mmol, 1.2 equiv) was added. Aliquots of the reaction mixture were withdrawn periodically and immediately quenched with 1:1 MeCN/1 N aqueous HCl solution. The conversion was determined by HPLC analysis.

Table of conversion

Time (min)	0.0	5.0	10.0	20.0	40.0	60.0	120.0	180.0
Conversion in solution (%)	0.0	14.7	20.8	32.0	55.3	68.1	87.1	98.2

After stirring for 16 h, the reaction mixture was concentrated under vacuum and EtOAc (30 mL) and deionized water (25 mL) added. The aqueous phase was extracted with EtOAc (2 × 10 mL) and the combined organic phase was washed with 1 N aqueous HCl solution (2 × 10 mL), 1 N aqueous NaOH solution (2 × 10 mL) and saturated aqueous NaCl solution (2 × 10 mL), dried over MgSO₄, filtered and concentrated under vacuum to furnish the desired product as a white solid (91.1 mg, 64% yield, 85% HPLC purity).

Synthesis in ball-mill

HCl·H-Val-Ile-Ala-OBn (53.2 mg, 0.124 mmol, 1.0 equiv), Boc-Val-OH (32.5 mg, 0.150 mmol, 1.2 equiv), Oxyma (22 mg, 0.155 mmol, 1.2 equiv), NaH₂PO₄ (60.0 mg, 0.500 mmol,

4.0 equiv) and EtOAc (450 μL , $\eta = 2.37 \mu\text{L}/\text{mg}$) were introduced in a 15 mL PTFE grinding jar equipped with one stainless steel ball (10 mm diameter). The jar was subjected to grinding for 2 min in the mixer mill operated at 25 Hz. Then, *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide (26.4 μL , 0.149 mmol, 1.2 equiv) was added and the jar was subjected to grinding for 3 min in the mixer mill operated at 25 Hz. A first aliquot of the reaction mixture was withdrawn, immediately quenched with 1:1 MeCN/1 N aqueous HCl solution and analyzed by HPLC to determine the conversion. The jar was subjected to further grinding in the mixer mill operated at 25 Hz until next aliquot was withdrawn. This operation was repeated until completion of the reaction.

Table of conversion

Time (min)	0.0	5.0	10.0	20.0	30.0	40.0	60.0
Effective milling time (min)	0.0	3.0	6.0	14.0	22.0	30.0	50.0
Conversion in ball-mill (%)	0.0	72.4	87.2	100.0	100.0	100.0	100.0

To the reaction mixture was added EtOAc (30 mL) and deionized water (25 mL). The aqueous phase was extracted with EtOAc ($2 \times 10 \text{ mL}$) and the combined organic phase was washed with 1 N aqueous HCl solution ($2 \times 10 \text{ mL}$), 1 N aqueous NaOH solution ($2 \times 10 \text{ mL}$) and saturated aqueous NaCl solution ($2 \times 10 \text{ mL}$), dried over MgSO_4 , filtered, concentrated under vacuum to furnish the desired product as a white solid (57.4 mg, 78% yield, 88% HPLC purity).

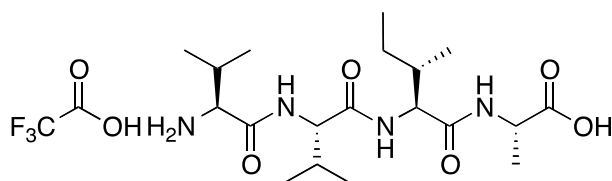
Characterization

^1H NMR (400 MHz, DMSO- d_6): δ 8.38 (d, $J = 6.4 \text{ Hz}$, 1H), 7.80 (d, $J = 8.8 \text{ Hz}$, 1H), 7.68 (d, $J = 8.7 \text{ Hz}$, 1H), 7.41 – 7.28 (5H), 6.82 (d, $J = 8.9 \text{ Hz}$, 1H), 5.09 (br s, 2H), 4.36 – 4.15 (3H), 3.78 (br t, $J = 8.0 \text{ Hz}$, 1H), 2.00 – 1.84 (2H), 1.74 – 1.61 (1H), 1.47 – 1.32 (10H), 1.28 (d, $J = 7.3 \text{ Hz}$, 3H), 1.05 (m, 1H), 0.90 – 0.69 (18H).

^{13}C NMR (101 MHz, DMSO- d_6): δ 172.2, 171.3, 170.7, 170.6, 155.5, 136.0, 128.4, 128.1, 128.0, 127.8, 78.0, 65.9, 60.1, 57.4, 56.3, 47.7, 36.7, 30.7, 30.1, 28.2, 24.2, 19.3, 19.2, 18.3, 18.1, 16.8, 15.0, 10.9.

MS (ESI): m/z 591.3 $[\text{M}+\text{H}]^+$

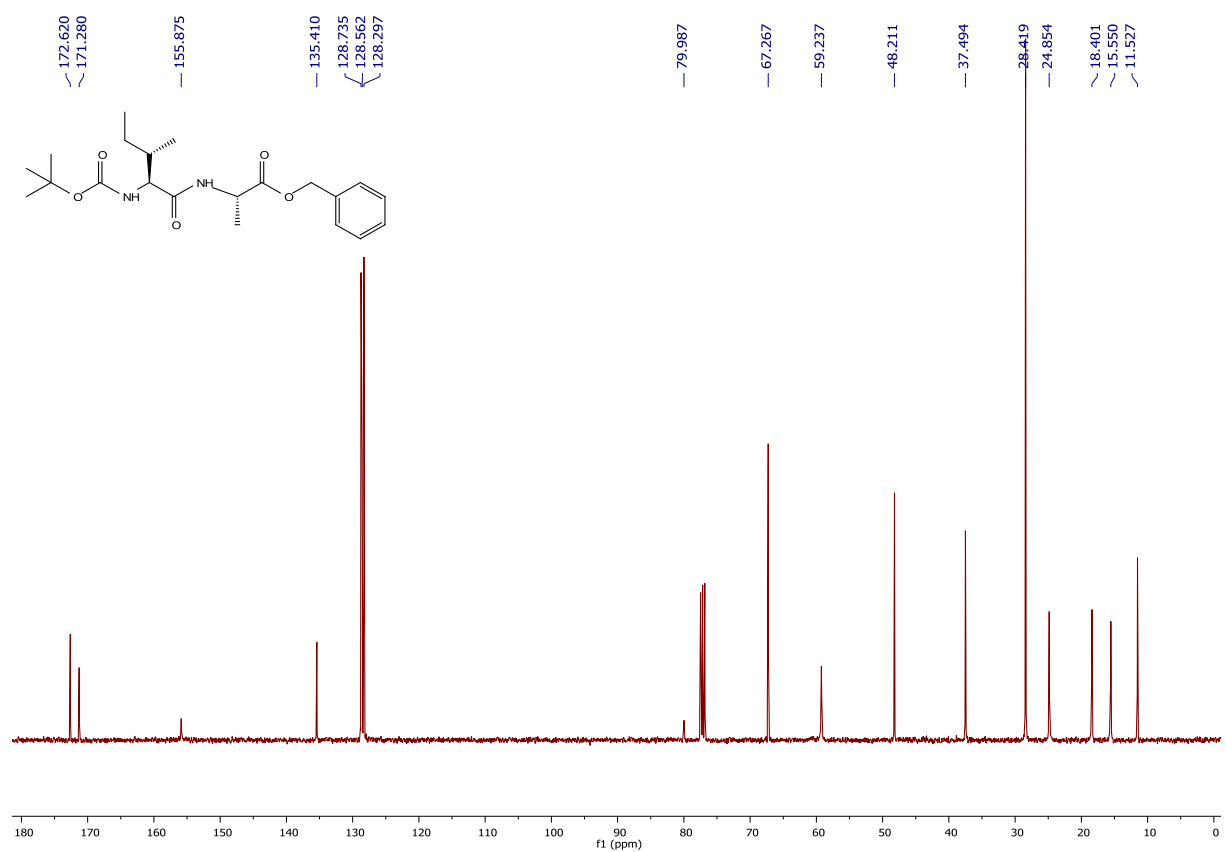
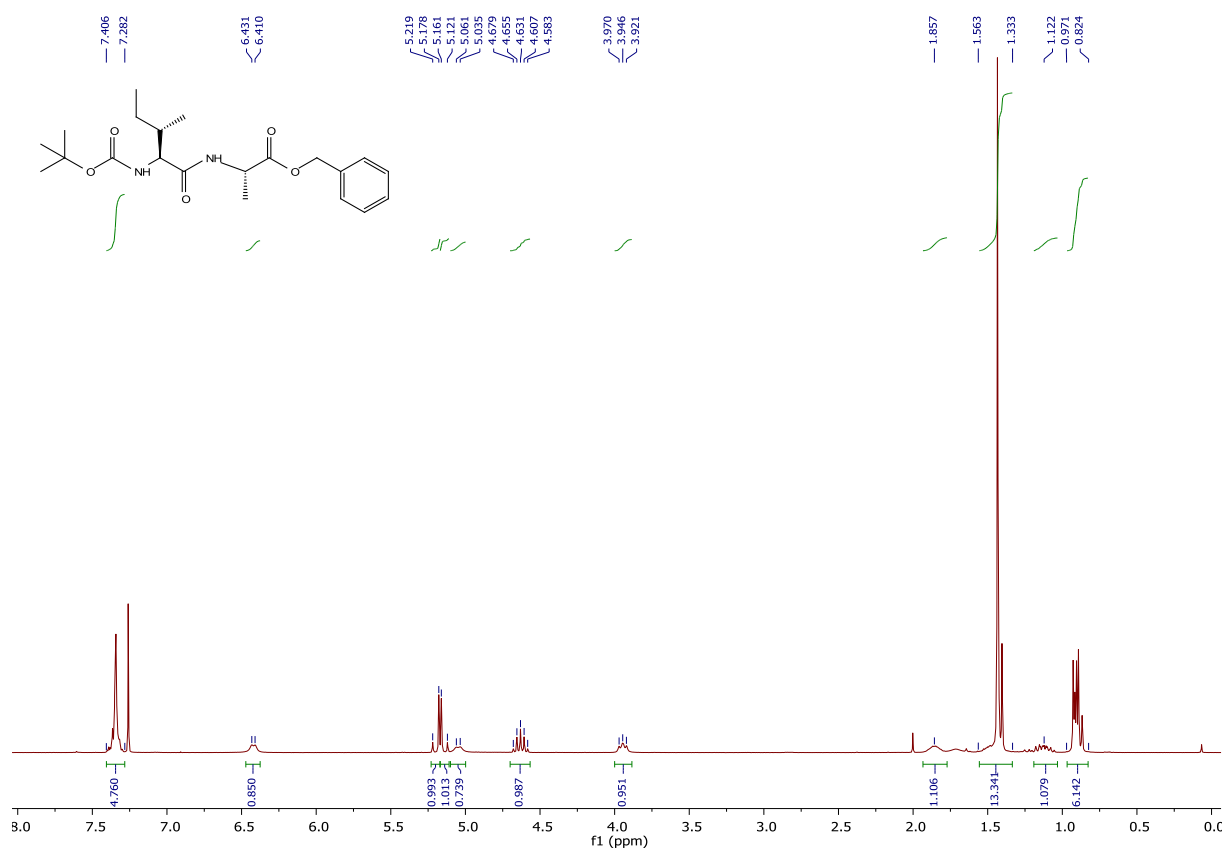
TFA·H-Val-Val-Ile-Ala-OH

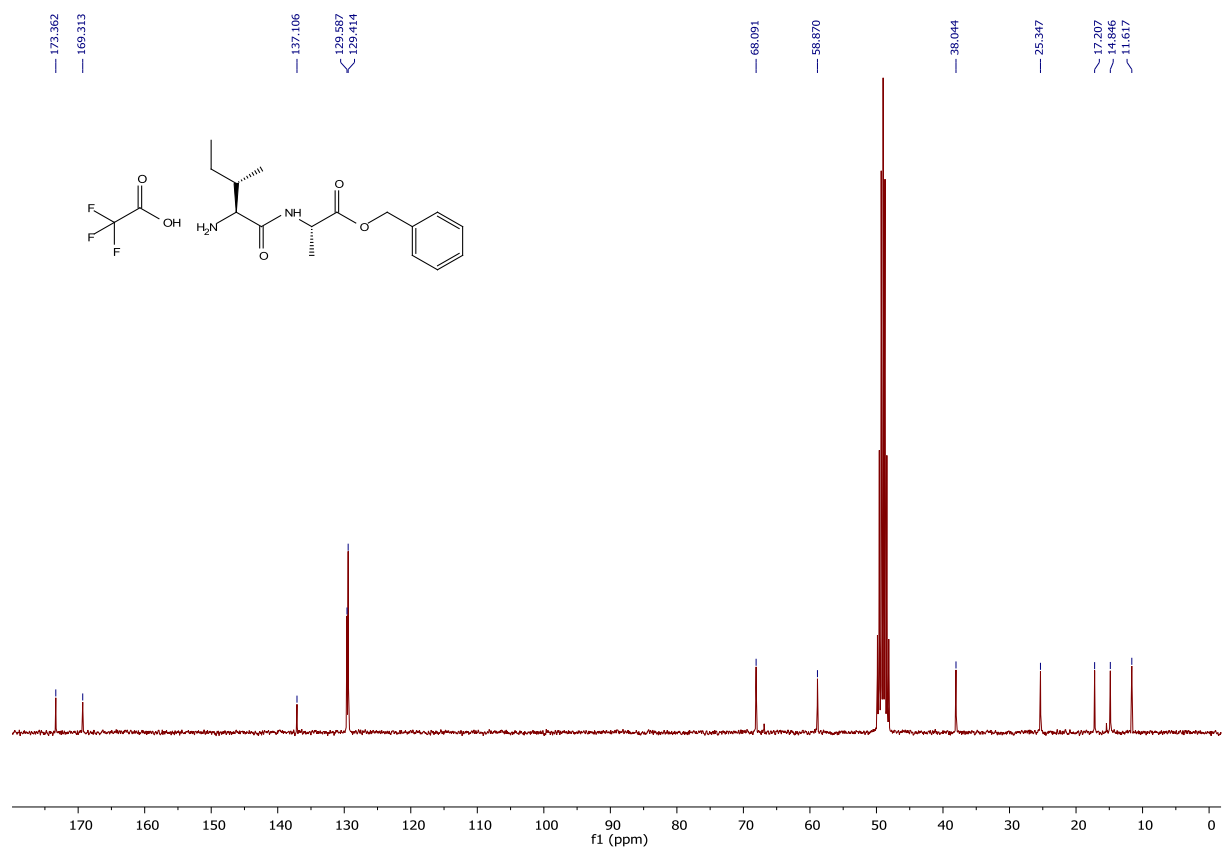
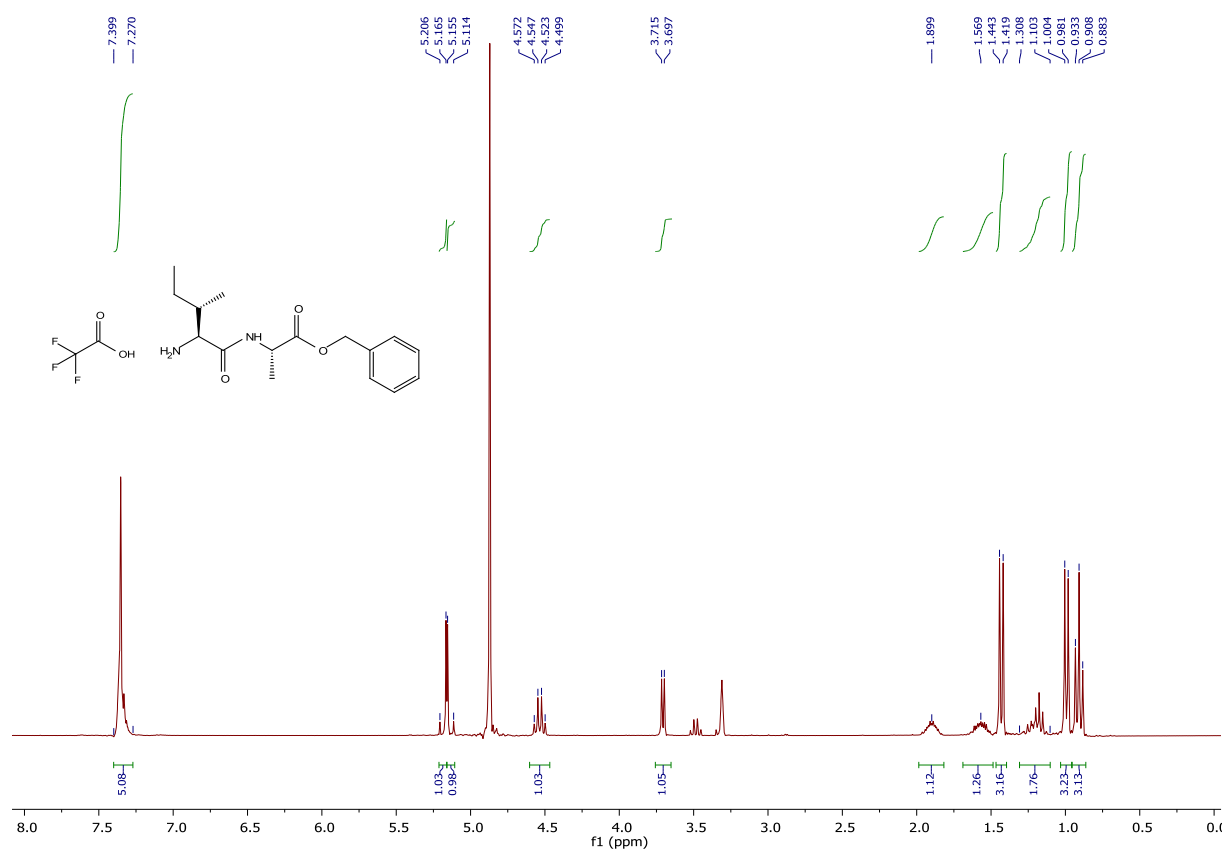


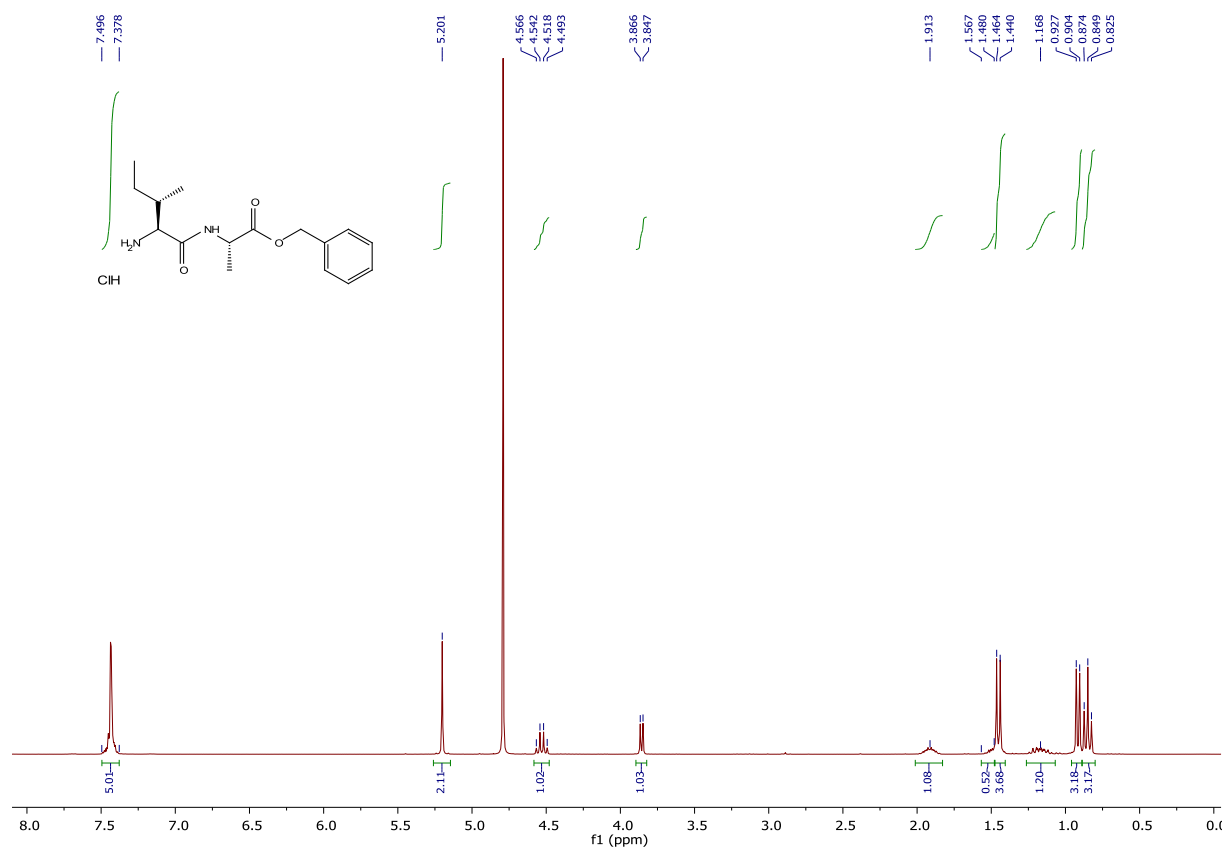
Solid-phase peptide synthesis

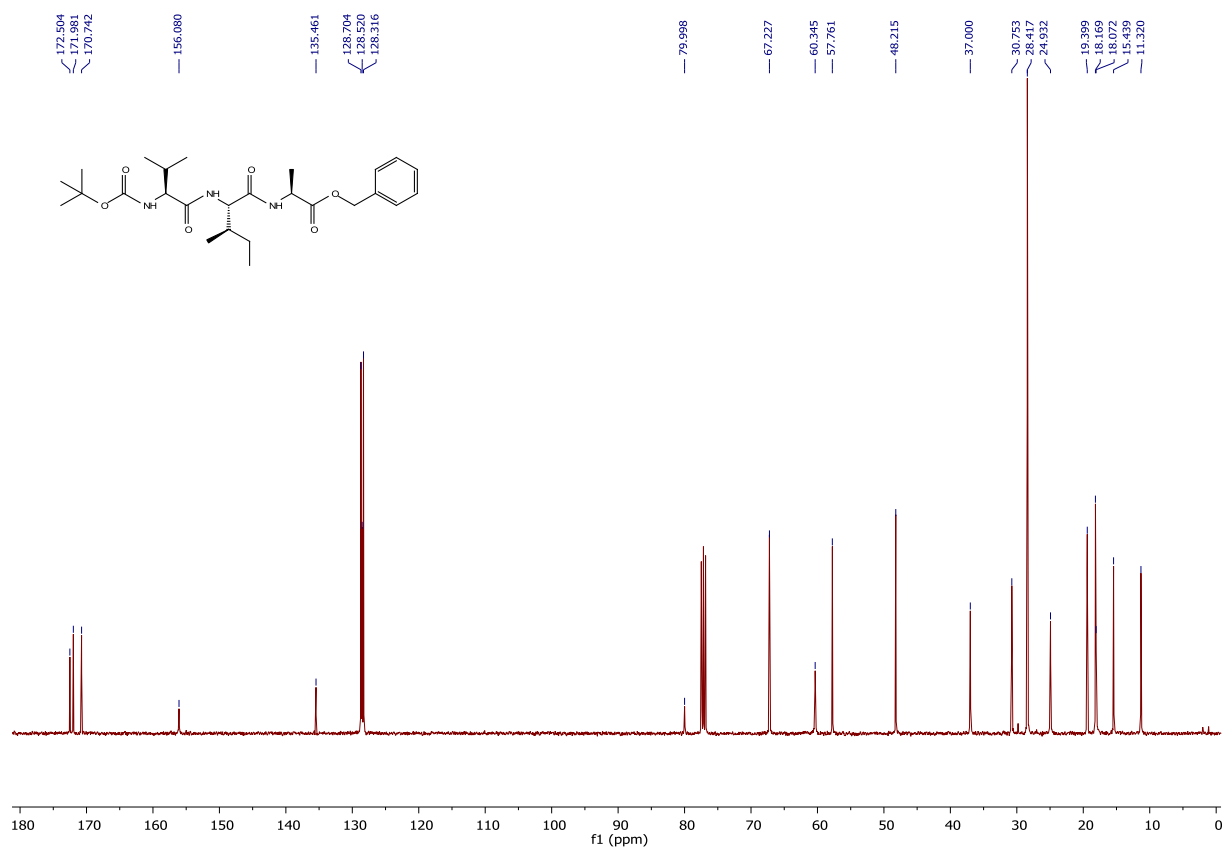
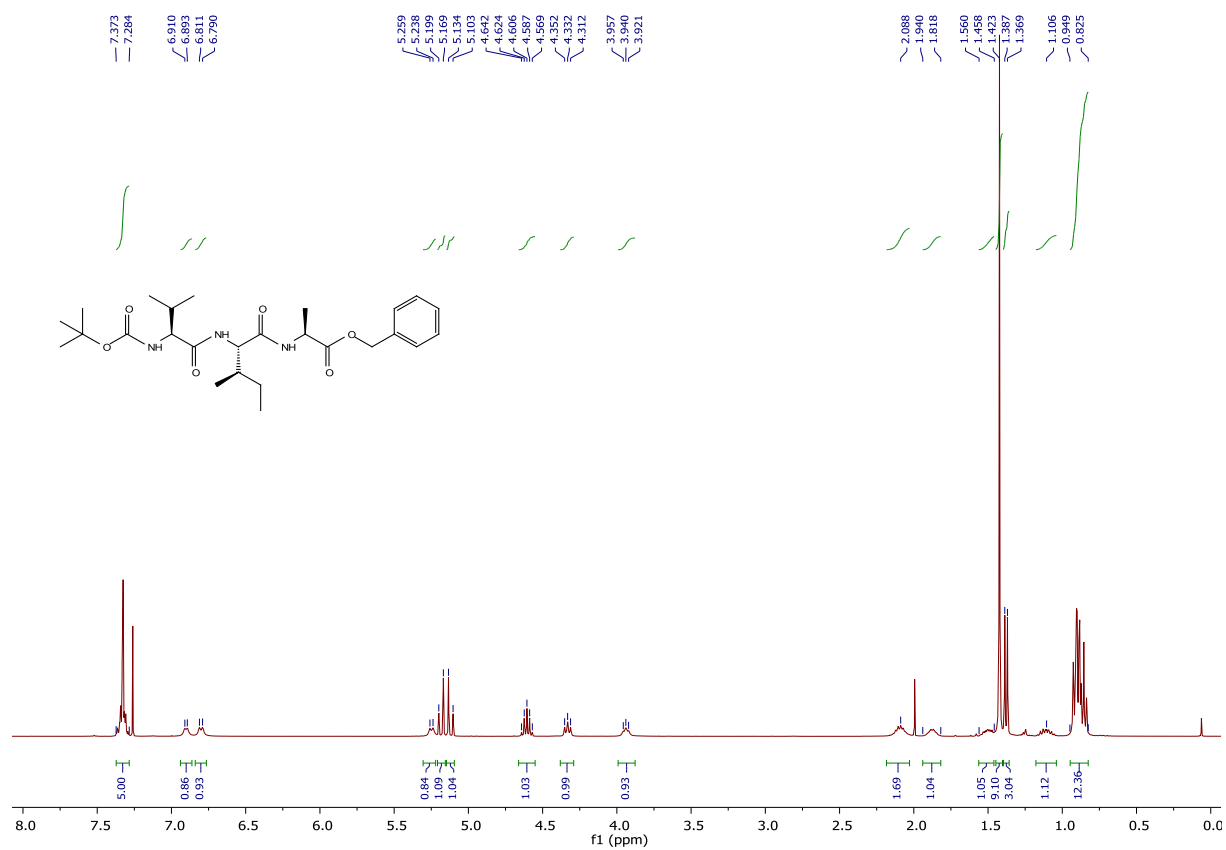
The peptide chains were elongated by means of a Liberty Blue peptide synthesizer (CEM) employing standard Fmoc chemistry. The syntheses were conducted on a 0.1 mmol scale starting with Fmoc-Ala-Wang resin (0.8 mmol/g) with a 5-fold excess of Fmoc-protected L-amino acids (0.2 M in DMF, 2.5 mL, 0.5 mmol, 5.0 equiv), 0.5 M DIC in DMF (1 mL, 0.5 mmol, 5.0 equiv) as coupling reagent, 1 M Oxyma in DMF (0.5 mL, 0.5 mmol, 5.0 equiv) for 7 min at 70 °C. The deprotection steps were carried out with piperidine/DMF 1:4 (v/v) (14 mL, 28.3 mmol, 283.5 equiv) over 3 min at 70 °C. The second coupling and deprotection of Fmoc-Ile-Ala-OHMP was performed during 90 min at room temperature. After the assembly was complete, the peptide-resin was washed with CH₂Cl₂ (70 mL) and cleavage was performed with TFA/TIS/H₂O 94:3:3 (10 mL) for 2 h at room temperature. The peptide was precipitated by adding cold Et₂O, the solution was decanted, and the solid was triturated with cold Et₂O, which was again decanted and filtrated. After lyophilization, the peptide was obtained as a fluffy white solid (28.0 mg, 54% yield, 96% purity, MS (ESI): m/z 401.3 [M+H]⁺).

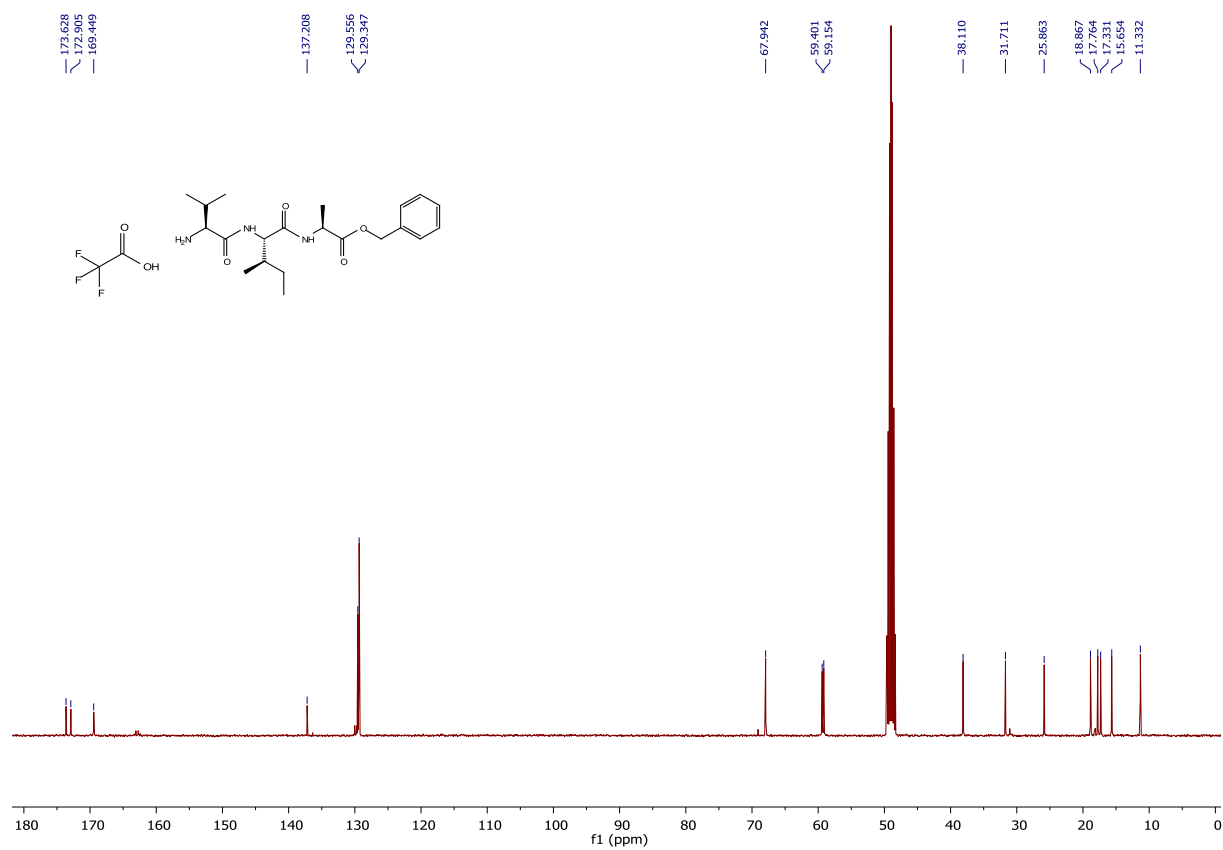
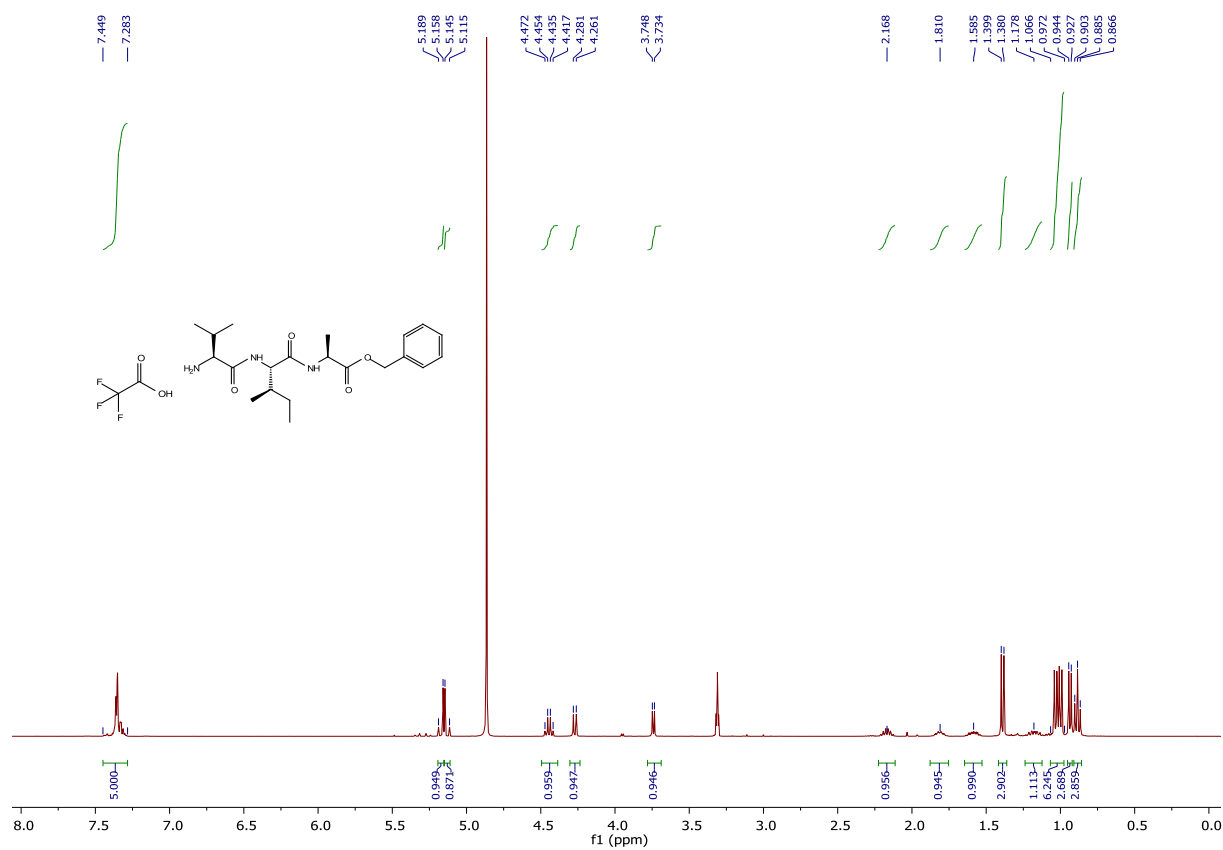
¹H and ¹³C spectra

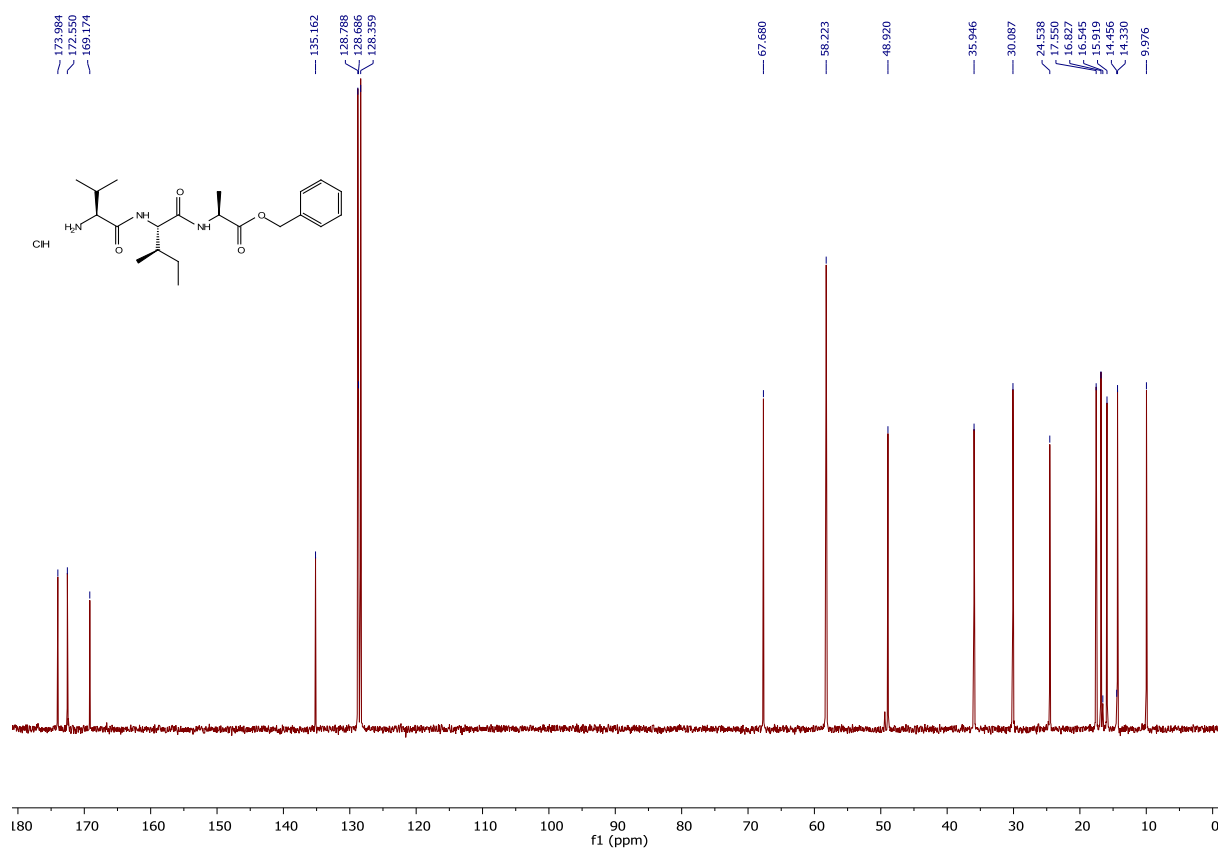
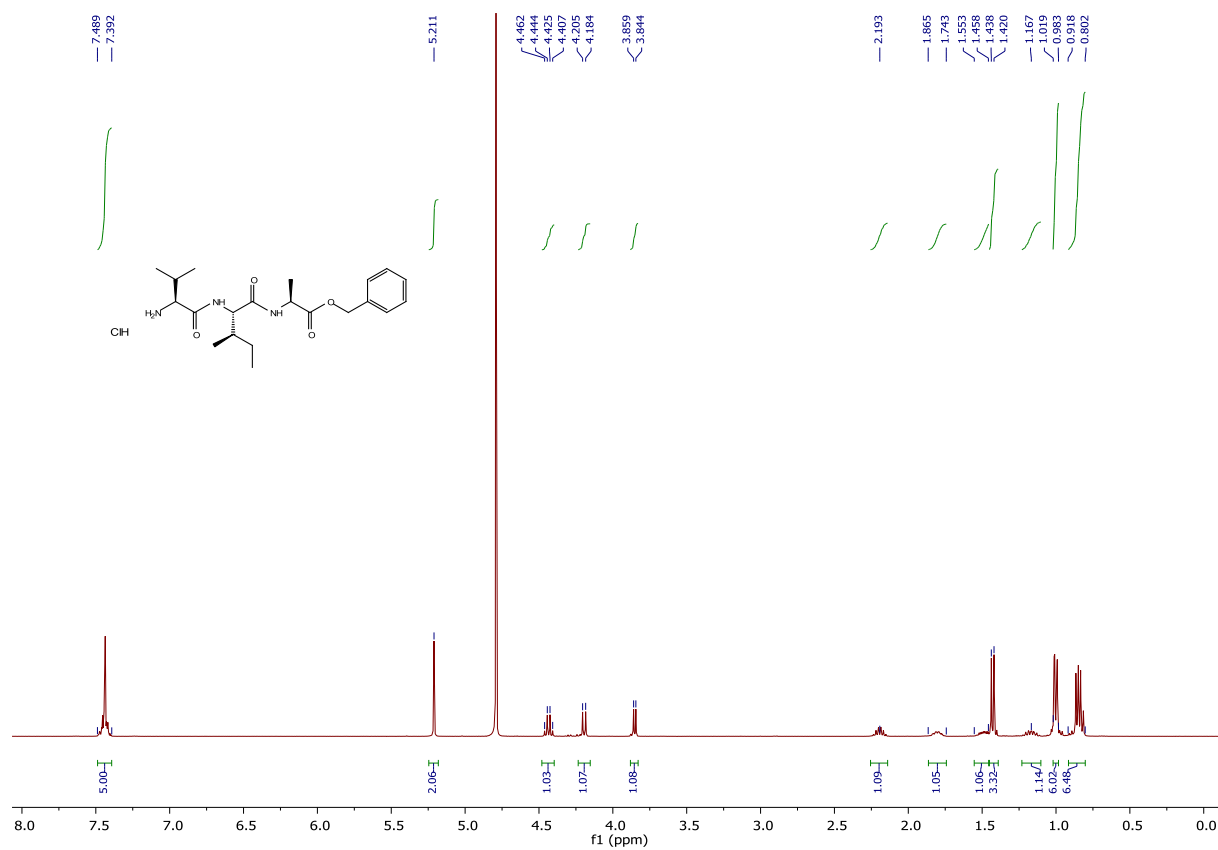


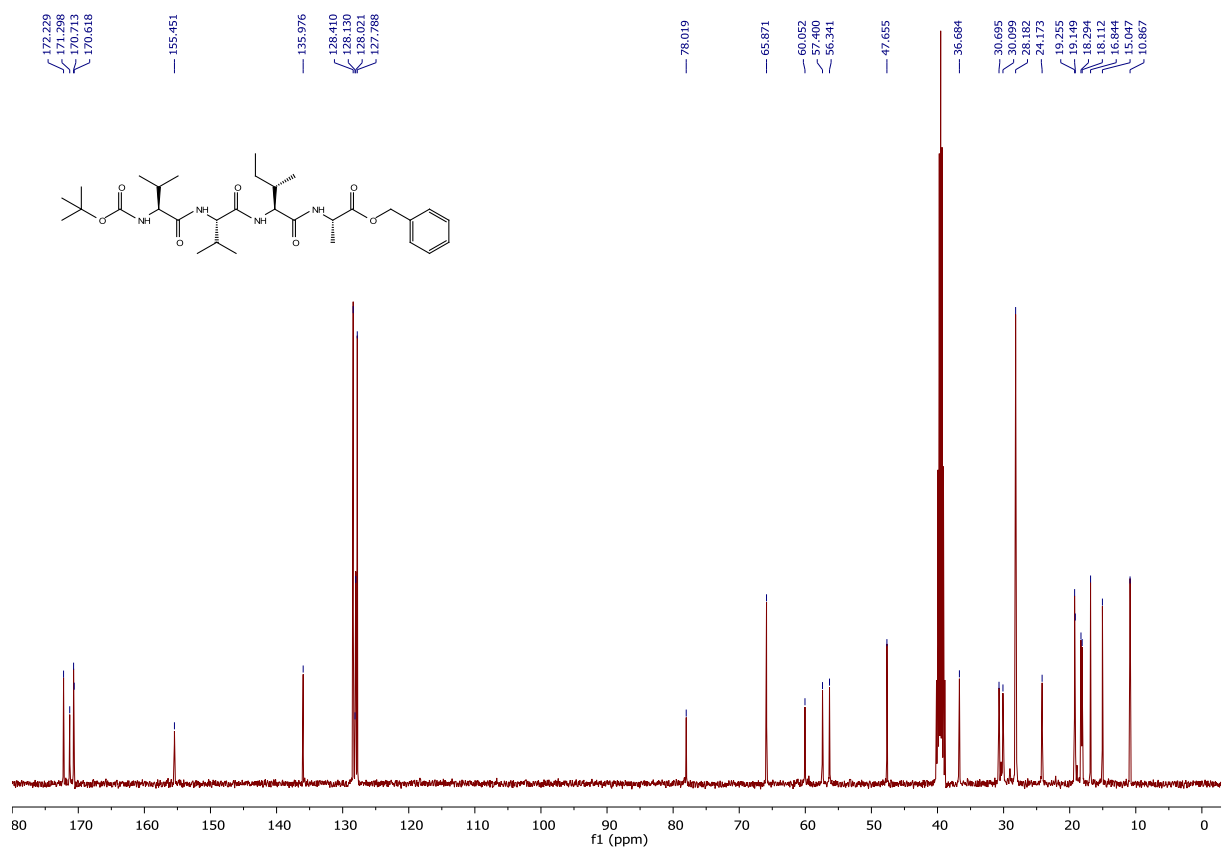
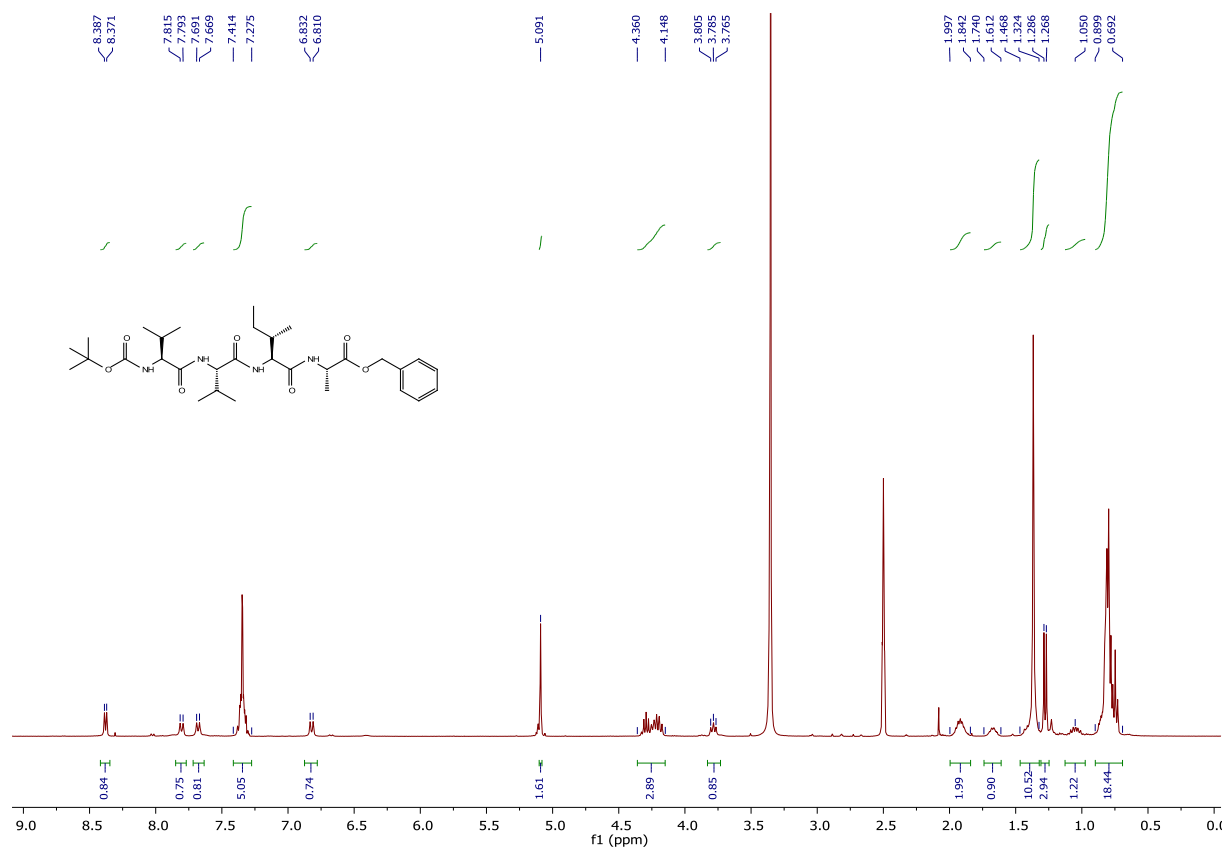












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