Electronic Supplementary Material (ESI) for Chemical Communications

Universal strategy for preparing protected *C*-terminal peptides on solid phase through an intramolecular *click* chemistry-based handle

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SUPPLEMENTARY INFORMATION

GENERAL

General methods: Protected amino acid derivatives, Fmoc-Rink-OH, HOBt and 2-CTC resin were obtained from Iris Biotech (Marktredwitz, Germany). HMPS resin, HMBA and HMPA linkers were obtained from Novabiochem (Läufelfingen, Switzerland). DIEA and DIPCDI were obtained from Aldrich (Milwaukee, WI), TFA was from Scharlau (Barcelona, Spain), and HCTU and Oxyma were from Luxembourg Industries (Tel Aviv, Israel). DMF, DCM, Et_2O, piperidine and acetonitrile (HPLC grade) were obtained from SDS (Peypin, France). All commercial reagents and solvents were used as received. THF was obtained from Scharlau and purified using a Pure-Solv MD-2 solvent system (Innovative Technology, Inc.)

Solid-phase syntheses were performed in polypropylene syringes fitted with a polyethylene porous disc. Solvents and soluble reagents were removed by suction. Removal of the Fmoc group was carried out with piperidine–DMF (1:4, v/v) (1 × 1 min, 2 × 10 min). Washings between deprotection, coupling, and final deprotection steps were carried out with DMF (5 × 1 min) and DCM (5 × 1 min). Peptide synthesis transformations and washes were performed at 25 °C.

SunFire® C18 reversed-phase HPLC analytical column (4.6 mm × 100 mm, 3.5 µm), and XBridge BEH130 C18 column, (4.6 mm x 100 mm, 3.5 µm) were obtained from Waters (Ireland). Analytical RP-HPLC was carried out on a Waters instrument comprising a separation module (Waters 2695), automatic injector, photodiode array detector (Waters 2998), and system controller (Empower login). UV detection was at 220 and 254 nm, and linear gradients of ACN (+0.036% TFA) into H_2O (+0.045% TFA) were run at 1.0 mL·min^{-1} flow rate over 8 min. RP-HPLC-ESMS was performed on a Waters Micromass ZQ spectrometer. Linear gradients of ACN (+0.07% formic acid) into H_2O (0.1% formic acid) were run at 0.3 mL·min^{-1} flow rate over 8 min.

SPPS of T-20 fragment H-(27-36)-DKP$_{handle}$ using as attachment to the resin a DKP *click* handle (0.1 mmol scale): Trt-Lys(Fmoc)-D-Pro-OH as a dipeptidyl linker

The synthesis was performed manually.

Attachment of the diketopiperazine group that forms the dipeptidyl linker to the resin

HMPS resin (106.2 mg, 0.98 mmol/g) was swelled with DCM (5 × 1 min; 3 mL each) and DMF (5 × 1 min; 3 mL each) at 25 °C and then filtered. Fmoc-D-Pro-OH (135 mg, 4 eq) and DIPCDI (31 µL, 2 eq) in DCM-DMF (15:1 (v/v), 2.5 mL) were then added to the resin. DMAP (4.9 mg, 0.4 eq) in DCM (0.5 mL) was then added, and the mixture was left to stand at 25 °C for 2 h. A recoupling of Fmoc-D-Pro-OH was carried out for 16 h. The resin was washed with DCM (5 × 1 min; 3 mL each) and DMF (5 × 1 min; 3 mL each). The resin was then capped using acetic anhydride (47 µL, 5 eq) and DIEA (85 µL, 5 eq) in DMF (2.5 mL) for 30 min at 25 °C. After capping, the resin was washed with DMF (5 × 1 min; 3 mL each) and DCM (5 × 1 min; 3 mL each). The Fmoc group was then removed and a 0.95 mmol/g resin loading was determined by UV quantification. To introduce the second amino acid (L-Lys) of the dipeptidyl linker, a mixture of Trt-L-Lys(Fmoc)-OH (198 mg, 3 eq), HOBt (50 mg, 3 eq) and DIPCDI (50 µL, 3 eq) in DMF (2 mL) was shaken for 5 min at 25 °C, and then added to the resin. The mixture was left to stand at 25 °C for 16 h. No recoupling was required according to the chloranil test. The resin was washed with DMF (5 × 1 min; 3 mL each) and DCM (5 × 1 min; 3 mL each), and then the Fmoc group was removed.

Incorporation of a Rink-amide handle group

Fmoc-Rink-OH (175 mg, 3 eq), HOBt (50 mg, 3 eq) and DIPCDI (50 µL, 3 eq) in DMF (2 mL) were shaken for 5 min at 25 °C, and then added to the previously described resin. The mixture was left to stand at 25 °C for 1 h. No recoupling was required according to the ninhydrin test. The resin was washed with DMF (5 × 1 min; 3 mL each) and DCM (5 × 1 min; 3 mL each), and then the Fmoc group was removed.

T-20 fragment H-(27-36)-DKP$_{handle}$ elongation by SPPS

The first amino acid, Fmoc-^{36}Phe-OH, was coupled to the resin comprising the dipeptidyl linker and a Rink-amide handle group described above, and then the following amino acids were coupled. A mixture of Fmoc-Xaa-OH (3 eq), HCTU (140 mg, 3 eq), DIEA (110 µL, 6 eq) in DMF (2 mL) was shaken for 30 s at 25 °C, and then added to the resin. The mixture was left to stand at 25 °C for 1 h, and then a ninhydrin test was performed. When the ninhydrin test was positive, a recoupling was carried out (using the same coupling conditions). The resin was then capped using acetic anhydride (47 µL, 5 eq) and DIEA (85 µL, 5 eq) in DMF (2.5 mL) for 15 min at 25 °C. The Fmoc group was then removed. Washes between coupling,

deprotection and capping steps were performed using DMF (5 × 1 min; 3 mL each) and DCM (5 × 1 min; 3 mL each).

Analysis - Cleavage of T-20 fragment H-(27-36)-NH_2 from the Rink-amide handle group

A small portion of peptidyl-resin (5 mg) was treated with 1 mL of a mixture TFA–TIS–H_2O (95:2.5:2.5) for 1 h at 25 °C in order to cleave the peptide from the Rink-amide handle group and to fully remove the amino acid side-chains. The unprotected peptide was obtained in 83% purity, as determined by analytical RP-HPLC (linear gradient from 25% to 50% ACN over 8 min; t_R = 5.25 min). RP-HPLC-ESMS showed the target peptide (linear gradient from 5% to 100% of ACN over 8 min; t_R = 6.47 min; *m/z* calculated for $C_{68}H_{86}N_{16}O_{14}$, 1351.5; found, 1352.2 $[M+H]^+$, 676.9 $[(M+2H)/2]^{2+}$, where M is the MW of the fully unprotected T-20 fragment H-(27-36)-NH_2).

Trt protecting group removal of the L-Lys residue of the dipeptidyl linker, formation of the diketopiperazine moiety and cleavage from the resin.

In a first step, the Trt protecting group of the L-Lys residue of the dipeptidyl linker was removed by treating the peptidyl-resin (15 mg) with 0.2% (v/v) TFA in DCM (2 × 5 min; 2 mL each) at 25 °C. In a second step, the peptidyl-resin was then neutralized by washing with 5% (v/v) DIEA in DCM (2 × 5 min; 2 mL each) at 25 °C. RP-HPLC analysis after the first step did not show peptide cleavage from the resin or from the Rink-amide handle group, and after the second step, almost negligible peptide comprising the DKP moiety was observed (linear gradient from 5% to 100% ACN over 8 min). Finally, the fully protected peptide comprising the DKP *C*-terminal protecting moiety was obtained by treating the peptidyl-resin from the second step with 5% (v/v) piperidine in THF at 25 °C (5 × 5 min, 2 mL each). This step can also be performed with 5% (v/v) piperidine in DMF, 5% (v/v) pyrrolidine in THF or 5% (v/v) pyrrolidine in DMF. THF was then removed by evaporation under reduced pressure and the resulting fully protected peptide comprising the DKP *C*-terminal protecting group was obtained as a diastereomeric mixture (1:1) due to the Rink-amide handle group. The mixture was analyzed by RP-HPLC (linear gradient from 80% to 100%; t_R = 4.20 min and 4.63 min) and RP-HPLC-ESMS (linear gradient from 80% to 100% ACN over 8 min; t_R = 2.64 min; *m/z* calculated for $C_{143}H_{181}N_{19}O_{28}$, 2614.1; found, 2616.2 $[M+H]^+$, 1308.0 $[(M+2H)/2]^{2+}$, where M is the MW of the fully protected T-20 fragment H-(27-36)-DKP_{handle} comprising the DKP moiety).

In order to quantify the amount of the remained target peptide attached to the resin via the DKP *click* handle, the resin was treated with 2 mL of TFA–TIS–H_2O (95:2.5:2.5) for 1 h at 25 °C. RP-HLPC analysis showed that there was less than 1% left of target fragment on the resin (linear gradient from 5% to 100% ACN over 8 min).

SPPS of T-20 fragment H-(27-36)-DKP$_{handle}$ using as attachment to the resin a DKP *click* handle (5 mmol scale): Trt-Lys(Fmoc)-D-Pro-OH as a dipeptidyl linker

The SPPS was performed manually using HMPS resin (5.0977 g, 0.98 mmol/g). Introduction of the dipeptidyl linker, Rink-amide handle group, first amino acid, and coupling cycles were performed as described in the previous synthesis except for Fmoc-^{28}Lys(Boc)-OH, which was coupled as follows: a mixture of Fmoc-^{28}Lys(Boc)-OH (3 eq), DIPCDI (2.3 mL, 3 eq), HOBt (2.3 g, 3 eq) in DMF (100 mL) was shaken for 5 min at 25 °C, and then added to the resin prepared according to the preceding step in the elongation sequence. The mixture was left to stand at 25 °C for 16 h. A recoupling was carried out using HCTU (6.2 g, 3 eq), DIEA (5.2 mL, 6 eq) in DMF (100 mL). This mixture was shaken for 30 s at 25 °C, and then added to the resin. The mixture was left to stand at 25 °C for 2 h. The resin was washed with DMF (5 × 1 min; 100 mL each) and DCM (5 × 1 min; 100 mL each), and then was capped using acetic anhydride (2.4 mL, 5 eq), DIEA (4.4 mL, 5 eq) in DMF (100 mL) for 1 h at 25 °C. The resin was washed with DMF (5 × 1 min; 100 mL each) and DCM (5 × 1 min; 100 mL each), and then the Fmoc group was removed.

At this point, an aliquot of peptidyl-resin (5 mg) was cleaved from the Rink-amide handle group by treating the resin with 1 mL of a mixture of TFA–TIS–H_2O (95:2.5:2.5) for 1 h at 25 °C. The unprotected peptide was obtained in 72% purity, as determined by analytical RP-HPLC (linear gradient from 25% to 50% of ACN over 8 min, t_R = 5.25 min).

In this case, the Trt protecting group of the L-Lys of the dipeptidyl linker was removed by treating the peptidyl-resin (1.57 g) with 0.5% (v/v) TFA in DCM (2 × 5 min; 20 mL each) at 25 °C. Neutralization was performed as described earlier by washing the peptidyl-resin with 5% (v/v) DIEA in DCM (2 x 5 min; 20 mL each) at 25 °C. Diketopiperazine formation was carried out by treating the peptidyl-resin with 5% (v/v) piperidine in THF (5 × 5 min; 20 mL each). THF was removed under reduced pressure and the resulting crude was washed with

pre-cooled (4 °C) Et_2O (3 × 50 mL). 642.5 mg of the fully protected T-20 fragment H-(27-36)-DKP_{handle} comprising the DKP *C*-terminal protecting moiety were obtained.

SPPS of T-20 fragment Boc-(17-26)-OH

SPPS of T-20 fragment Boc-(17-26)-OH was performed manually by linear Fmoc SPPS. Only the last amino acid was Boc-protected (Boc-Glu(*t*Bu)-OH).

2-CTC resin (5.0054 g) was swelled with DCM (5 × 1 min; 50 mL each) and DMF (5 × 1 min; 50 mL each) at 25 °C. The first amino acid, Fmoc-^{26}Leu-OH (1.8 g, 1 eq) and DIEA (8.7 mL, 10 eq) in DCM (50 mL) were added to the resin, and then the mixture was left to stand at 25 °C for 1h. The resin was then capped by adding MeOH (0.8 µL/mg resin; 4 mL) to the resin for 15 min at 25 °C. After capping, the resin was washed with DCM (5 × 1 min; 50 mL each) and DMF (5 × 1 min; 50 mL each). The Fmoc group was then removed and a 0.89 mmol/g resin loading was determined by UV quantification.

To elongate the peptide, a mixture of Fmoc-Xaa-OH (3 eq), Oxyma (1.9 g, 3 eq), DIPCDI (2.3 mL, 3 eq) in DMF was shaken for 5 min at 25 °C, and then added to the resin. The mixture was left to stand at 25 °C for 16 h. No recouplings were required according to the ninhydrin test. Washes between couplings and deprotection steps were performed using DMF (5 × 1 min; 100 mL each) and DCM (5 × 1 min; 100 mL each).

Analysis - Cleavage of T-20 fragment H-(17-26)-OH from the 2-CTC resin

An aliquot of peptidyl-resin (5 mg) was treated with 1 mL of TFA–TIS–H_2O (95:2.5:2.5) for 1 h at 25 °C in order to cleave the peptide from the 2-CTC resin and to fully remove the amino acid side-chains. The T-20 fragment H-(17-26)-OH was obtained in 85.7% purity, as determined by analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R = 3.90 min). The peptide was analyzed by RP-HPLC-ESMS (linear gradient from 5% to 100% of ACN over 8 min; t_R = 6.71 min; *m/z* calculated for $C_{53}H_{89}N_{13}O_{21}$, 1244.5; found, 1244.7 $[M+H]^+$, where M corresponds to the fully unprotected T-20 fragment H-(17-26)-OH).

Cleavage of the fully protected T-20 fragment Boc-(17-26)-OH

The peptidyl resin (1.094 g) was treated with 1% (v/v) TFA in DCM (5 × 1 min; 50 mL each) at 25 °C, all 5 mixtures were poured into H_2O (20 mL). This aqueous mixture was then evaporated under reduced pressure and the crude was lyophilized. The fully protected T-20 fragment Boc-(17-26)-OH was obtained (510 mg) and analyzed by RP-HPLC-ESMS (linear

gradient from 95% to 100% of ACN over 8 min; t_R = 7.84 min; *m/z* calculated for $C_{117}H_{165}N_{13}O_{25}$, 2153.6; found, 2153.8 $[M+H]^+$, where M corresponds to the fully protected T-20 fragment Boc-(17-26)-OH). Analytical RP-HPLC showed one peak with 85.7% purity (linear gradient for 95% to 100% of ACN over 8 min; t_R = 6.06 min).

No partial deprotection of the fully protected T-20 fragment Boc-(17-26)-OH was observed by RP-HPLC-ESMS (linear gradient from 50% to 100% ACN over 8 min).

Assembly of T-20 fragment H-(17-36)-NH_2 by fragment coupling in solution

Fully protected T-20 fragment Boc-(17-26)-OH (10 mg, 4.6 µmol), HOBt (2.2 mg, 3 eq) were dissolved in DCM (350 µL) and DIPCDI (2.2 µL, 3 eq) was then added to the mixture. The mixture was shaken for 5 min at 25 °C, and then added to a solution of fully protected T-20 fragment H-(27-36)-DKP_{handle} comprising the DKP *C*-terminal protecting moiety (12 mg, 4.6 µmol) in DCM (350 µL). The resulting mixture was stirred at 25 °C for 16 h. The fragment coupling was monitored by analytical RP-HPLC (linear gradient from 95% to 100% ACN over 8 min); no starting material was observed after 16 h.

The solvent was evaporated under reduced pressure yielding the fully protected T-20 fragment Boc-(17-36)-DKP_{handle} whose *C*-terminal function was protected with the DKP moiety. An aliquot of the crude peptide (1 mg) was treated with 1 mL of TFA–DMB–TIS (92.5:5:2.5) for 1 h at 25 °C. The mixture was evaporated and a 0.5% (v/v) aqueous NH_3 (1 mL) solution was added and left to stand for 16 h at 25 °C to remove the remained *N*-carboxy groups from the side chains of the Trp residues. The fully unprotected T-20 fragment H-(17-36)-NH_2 was obtained with 60.2% purity, as determined by analytical RP-HPLC (linear gradient from 30% to 40% ACN over 8 min; t_R = 5.94 min). RP-HPLC-ESMS showed the target peptide (linear gradient from 30% to 40% of ACN over 8 min; t_R = 6.84 min; *m/z* calculated for $C_{121}H_{173}N_{29}O_{34}$, 2577.8; found, 1290.0 $[(M+2H)/2]^{2+}$, where M is the MW of the T-20 fragment H-(17-36)-NH_2).

SPPS of the tripeptide H-Phe-Ala-Leu-OH using as attachment to the resin a DKP *click* handle: Trt-Dap(Fmoc)-D-Pro-OH as a dipeptidyl linker

The synthesis was performed manually.

Attachment of the diketopiperazine group that forms the dipeptidyl linker to the resin

HMPS resin (103.6 mg) was swelled with DCM (5 × 1 min; 3 mL each) and DMF (5 × 1 min; 3 mL each) at 25 °C and then filtered. Fmoc-D-Pro-OH (132 mg, 4 eq) and DIPCDI (30 µL, 2 eq) in DCM-DMF (15:1 (v/v), 2.5 mL) were then added to a resin. DMAP (4.8 mg, 0.4 eq) in DCM (0.5 mL) was then added to the resin, and the mixture was left to stand at 25 °C for 2 h. A recoupling of Fmoc-D-Pro-OH was carried out for 16 h at 25 °C. The resin was washed with DCM (5 x 1 min) and DMF (5 x 1 min). The resin was then capped using acetic anhydride (46 µL, 5 eq) and DIEA (86 µL, 5 eq) in DMF (2.5 mL) for 30 min at 25 °C. After capping, the resin was washed with DMF (5 × 1 min; 3 mL each) and DCM (5 × 1 min; 3 mL each). The Fmoc group was then removed and a 0.98 mmol/g resin loading was determined by UV quantification. To introduce the second amino acid (L-Dap) of the dipeptidyl linker, a mixture of Trt-L-Dap(Fmoc)-OH (173 mg, 3 eq), HOBt (47 mg, 3 eq) and DIPCDI (47 mL, 3 eq) in DMF (2 mL) was shaken for 5 min at 25 °C, and then added to the resin. The mixture was left to stand at 25 °C for 1 h. No recoupling was required according to the chloranil test. The resin was washed with DMF (5 x 1 min) and DCM (5 x 1 min), and then the Fmoc group was removed.

Incorporation of HMPA handle group

A mixture of HMPA (55 mg, 3 eq), HOBt (47 mg, 3 eq) and DIPCDI (47 µL, 3 eq) in DMF (2 mL) was added to the previously described resin, and then left to stand at 25 °C for 1 h. The resin was then washed with DMF (5 x 1 min; 3 mL each) and DCM (5 x 1 min; 3 mL each). No recoupling was required, according to the ninhydrin test.

Tripeptide elongation by SPPS

Fmoc-Leu-OH (144 mg, 4 eq) and DIPCDI (30 µL, 2 eq) in DCM-DMF (15:1 (v/v), 2.5 mL) were added to the resin comprising the dipeptidyl linker and the HMPA handle group described above. DMAP (4.8 mg, 0.4 eq) in DCM (0.5 mL) was then added and the mixture was left to stand at 25 °C for 2 h. The amino acid was recoupled for 16 h at 25 °C. After recoupling, the resin was washed with DMF (5 x 1 min; 3 mL each) and DCM (5 x 1 min; 3 mL each). The resin was then capped using acetic anhydride (46 µL, 5 eq) and DIEA (86 µL, 5 eq) in DMF (2.5 mL) for 30 min at 25 °C. After capping, the resin was washed with DCM (5 × 1 min; 3 mL each) and DMF (5 × 1 min; 3 mL each). The Fmoc group was then removed and a 0.94 mmol/g resin loading was determined by UV quantification. Next, a mixture of the respective Fmoc-

Xaa-OH (3 eq), HOBt (47 mg, 3 eq) and DIPCDI (47 µL, 3 eq) in DMF (2 mL) was added to the resin, and then left to stand at 25 °C for 1 h. No recoupling was required, according to the ninhydrin test. The resin was washed with DMF (5 × 1 min; 3 mL each) and DCM (5 × 1 min; 3 mL each), and then the Fmoc group was removed.

Analysis - Cleavage of the tripeptide from HMPA handle group

A small portion of resin (5 mg) was cleaved from the HMPA handle group by treating the resin with 1 mL of TFA–TIS–H_2O (95:2.5:2.5) for 1 h at 25 °C. RP-HLPC-ESMS analysis confirmed the identity of Phe-Ala-Leu (linear gradient from 5% to 100% of ACN over 8 min; t_R = 5.53 min; *m/z* calculated for $C_{18}H_{27}N_3O_4$, 349.4; found, 350.3 $[M+H]^+$, where M is the MW of the tripeptide).

Trt protecting group removal of the L-Dap residue of the dipeptidyl linker, formation of the diketopiperazine moiety and cleavage from the resin.

In a first step, the Trt protecting group of the L-Dap of the dipeptidyl linker was removed by treating the peptidyl-resin (15 mg) with 0.2% (v/v) TFA in DCM (2 × 5 min; 2 mL each) at 25 °C. In a second step, the peptidyl-resin was then neutralized by washing with 5% (v/v) DIEA in DCM (2 × 5 min; 2 mL each) at 25 °C. RP-HPLC analysis after the first step did not show any peptide cleavege from the resin or from the HMPA handle group, and after the second step, no peptide comprising a DKP moiety was observed (linear gradient from 5% to 100% ACN over 8 min). Finally, the peptide comprising the diketopiperazine *C*-terminal protecting moiety was obtained by treating the peptidyl-resin from the second step with 5% (v/v) piperidine in THF at 25 °C (5 × 5 min; 2 mL each). THF was then removed by evaporation under reduced pressure and the resulting peptide comprising the DKP *C*-terminal protecting group was analyzed by RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; $t_{R[M]}$ = 4.62 min, $t_{R[M+HMPA]}$ = 5.31 min). Over-incorporation of the HMPA handle group was determined by analytical RP-HPLC-ESMS (linear gradient from 5% to 100% ACN over 8 min; $t_{R[M+H]}$ = 5.90 min, $t_{R[M+HMPA+H]}$ = 6.41 min; *m/z* calculated for $C_{35}H_{46}N_6O_8$, 678.8; found, 679.4 $[M+H]^+$, 843.4 $[M+HMPA+H]^+$; where M is the MW of tripeptide comprising the DKP moiety).

SPPS of BUBU Enkephalin using as attachment to the resin a DKP *click* handle: Alloc-Lys(Fmoc)-D-Pro-OH as a dipeptidyl linker

The synthesis was performed manually.

Attachment of the diketopiperazine group that forms the dipeptidyl linker to the resin

HMPS resin (312.1 mg) was swelled with DCM (5 × 1 min; 5 mL each) and DMF (5 × 1 min; 5 mL each) at 25 °C and then filtered. Fmoc-D-Pro-OH (396 mg, 4 eq) and DIPCDI (93 µL, 2 eq) in DCM-DMF (15:1 (v/v); 3 mL) were added to the resin. DMAP (15 mg, 0.4 eq) in DCM (0.5 mL) was then added to the resin, and the mixture was left to stand at 25 °C for 2 h. The first amino acid was recoupled for 16 h at 25 °C. After recoupling, the resin was washed with DCM (5 x 1 min; 5 mL each) and DMF (5 x 1 min; 5 mL each), and then capped using acetic anhydride (150 µL, 5 eq) and DIEA (270 µL, 5 eq) in DMF (3 mL) for 30 min at 25 °C. After capping, the resin was washed with DCM (5 × 1 min; 5 mL each) and DMF (5 × 1 min; 5 mL each). The Fmoc group was then removed and a 0.98 mmol/g resin loading was determined by UV quantification. To introduce the second amino acid (L-Lys) of the dipeptidyl linker, a mixture of Alloc-L-Lys(Fmoc)-OH (390 mg, 3 eq), COMU (384 mg, 3 eq) and DIEA (305 µL, 6 eq) in DMF (3 mL) was shaken for 30 s at 25 °C, and then added to the resin. The mixture was left to stand at 25 °C for 1 h. No recoupling was required, according to the chloranil test. The resin was washed with DMF (5 × 1 min; 5 mL each) and DCM (5 × 1 min; 5 mL each), and then the Fmoc group was removed.

Introduction of HMBA handle group

A mixture of HMBA (137 mg, 3 eq), COMU (384 mg, 3 eq) and DIEA (305 µL, 3 eq) in DMF (3 mL) was added to the resin, and the mixture was left to stand at 25 °C for 1 h. No recoupling was required, according to the ninhydrin test. The resin was washed with DMF (5 × 1 min; 5 mL each) and DCM (5 × 1 min; 5 mL each).

BUBU Enkephalin elongation by SPPS

Fmoc-Thr(*t*Bu)-OH (487 mg, 4 eq) and DIPCDI (93 µL, 2 eq) in DCM-DMF (15:1 (v/v); 3 mL) was added to the resin comprising the dipeptidyl linker and a HMBA handle group describe above. DMAP (15 mg, 0.4 eq) in DCM (0.5 mL) was then added, and the mixture was left to stand at 25 °C for 2 h. The amino acid was recoupled for 16 h at 25 °C. After recoupling, the resin was washed with DCM (5 x 1 min; 5 mL each) and DMF (5 x 1 min; 5 mL each). The resin was then capped using acetic anhydride (150 µL, 5 eq) and DIEA (270 µL, 5 eq) in DMF (3 mL) for 30 min at 25 °C. After capping, the resin was washed with DMF (5 × 1 min; 5 mL each) and DCM (5 × 1 min; 5 mL each). The Fmoc group was then removed and a 0.94 mmol/g resin loading was determined by UV quantification. Next, a mixture of the respective Fmoc-

Xaa-OH (3 eq), COMU (384 mg, 3 eq) and DIEA (305 µL, 3 eq) in DMF (4 mL) was shaken for 30 s at 25 °C, and then added to the resin. The mixture was left to stand at 25 °C for 1 h. When the ninhydrin test was positive, a recoupling was carried out (using the same conditions). The resin was washed with DMF (5 × 1 min; 5 mL each) and DCM (5 × 1 min; 5 mL each), and then the Fmoc group was removed.

Alloc protecting group removal of the L-Lys residue of the dipeptidyl linker, formation of the diketopiperazine moiety and cleavage from the resin.

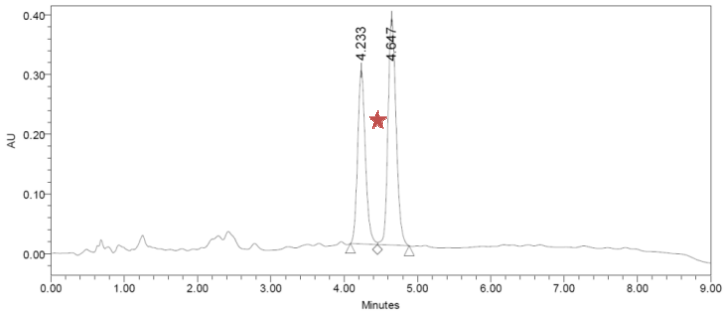
To remove the Alloc group, the peptidyl-resin (15 mg) was treated with $Pd(PPh_3)_4$ (0.1 eq) and $PhSiH_3$ (10 eq) in DCM (3 × 15 min; 2 mL each) at 25 °C. In a second step, the peptidyl-resin was then washed with 5% (v/v) DIEA in DCM (2 x 5 min; 2 mL each) at 25 °C. RP-HPLC analysis after the first step did not show cleavage from the resin or from the HMBA handle group, and after the second step, almost negligible peptide comprising a DKP moiety was observed (linear gradient from 5% to 100% ACN over 8 min). The BUBU Enkephalin peptide comprising the DKP *C*-terminal protecting moiety was obtained by treatment with 5% (v/v) piperidine in THF (2 × 5 min; 2 mL each). THF was removed by evaporation under reduced pressure and the resulting crude was analyzed by RP-HPLC (linear gradient from 30% to 60% of ACN over 8 min; $t_{R[M]}$ = 5.43 min, $t_{R[M+HMBA]}$ = 5.78 min). Over-incorporation of the HMBA handle group was determined by analytical RP-HPLC-ESMS (linear gradient from 5% to 100% of ACN over 8 min; $t_{R[M+H]+}$ = 6.61 min, $t_{R[M+HMBA+H]+}$ = 6.70 min; *m/z* calculated for $C_{60}H_{85}N_9O_{13}$, 1140.3; found, 1140.7 $[M+H]^+$, 1274.6 $[M+HMBA+H]^+$; where M is the MW of the BUBU Enkephalin peptide comprising the DKP moiety).

Diketopiperazine *C*-terminal protecting group removal by catalytic hydrogenation

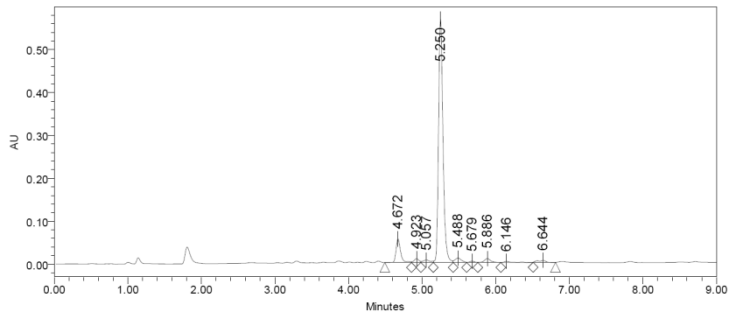
A stirred solution of *C*-terminal protected BUBU Enkephalin and Pd/C (10% w/w) in MeOH was hydrogenated under H_2 atmosphere for 2 h. The mixture was filtered over a Celite pad, washed with MeOH, evaporated under reduced pressure and analyzed by RP-HPLC-ESMS (linear gradient from 5% to 100% of ACN over 8 min; t_R = 6.45 min; *m/z* calculated for $C_{41}H_{62}N_6O_{10}$, 798.9; found, 799.56 $[M+H]^+$, where M is the MW of BUBU Enkephalin peptide).

RP-HPLC of T-20 fragments

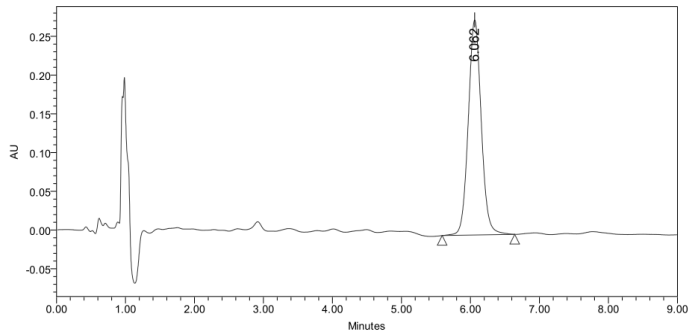
a) fully protected T-20 fragment H-(27-36)-DKP_{handle}



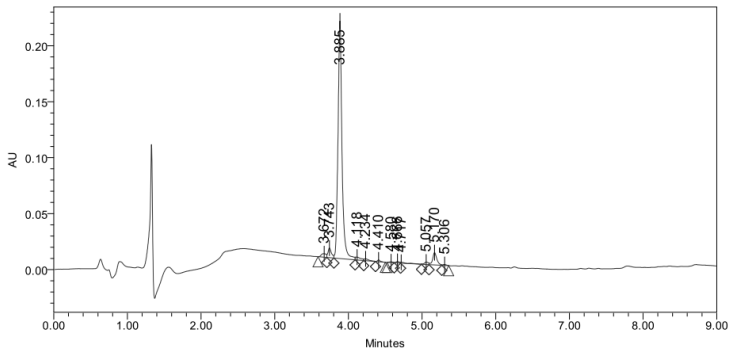
b) unprotected T-20 fragment H-(27-36)-NH_2



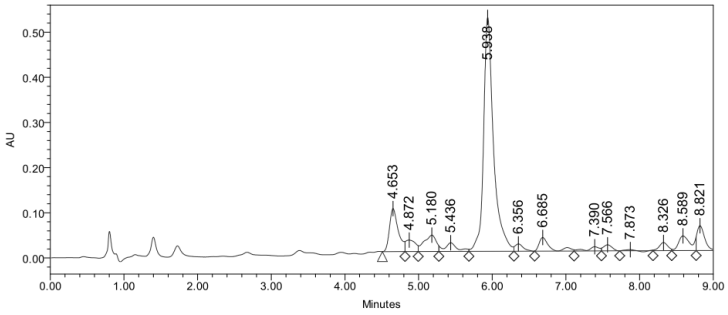
c) fully protected T-20 fragment Boc-(17-26)-OH



d) unprotected T-20 fragment H-(17-26)-OH

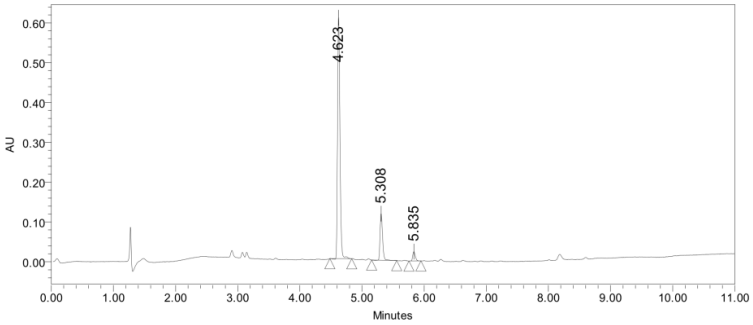


e) unprotected T-20 fragment H-(17-36)-NH_2



RP-HPLC of Phe-Ala-Leu

Phe-Ala-Leu comprising the diketopiperazine *C*-terminal protecting moiety



RP-HPLC of BUBU Enkephalin

BUBU Enkephalin comprising the diketopiperazine *C*-terminal protecting moiety