Structure activity relationship study on the peptide hormone preptin, a novel bone-anabolic agent for the treatment of osteoporosis

Zaid Amso,[a,b] Renata Kowalczyk,[a] Maureen Watson,[c] Young-Eun Park,[c] Karen E. Callon,[c] David S. Musson,[c] Jillian Cornish,[c] and Margaret A. Brimble[*a,b]

[a.] *School of Chemical Sciences, The University of Auckland, 23 Symonds St, Auckland 1142, New Zealand. E-mail: m.brimble@auckland.ac.nz; Phone: (+64) 9 373 7599 ext. 88259*

[b.] *Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand.*

[c.] *School of Medicine, The University of Auckland, Private Bag 92019, Auckland 1010, New Zealand.*

Supporting information

General methods

Materials

All reagents were acquired as reagent grade from commercial sources and used without further purification. Solvents for RP-HPLC were purchased as RP-HPLC grade and used without further purification. 6-Chloro-1-hydroxybenzotriazole (6-Cl-HOBt) was purchased from Aapptec (Louisville, Kentucky). *O*-(6-chlorobenzotriazol-1-yl)-*N*,*N*,*N'*,*N"*-tetramethyluronium hexafluorophosphate (HCTU), *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU), (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), di-*tert*-butyl dicarbonate (Boc_2O), and 2-chlorotrityl chloride resin were purchased from GL Biochem (Shanghai, China). *N,N*-dimethylformamide (DMF) (AR grade), and acetonitrile (CH_3CN) [high-performance liquid chromatography (HPLC) grade], and hydrochloric acid (HCl) were purchased from Scharlau (Barcelona, Spain). *N,N'*-diisopropylethylamine (iPr_2EtN), piperazine, triisopropylsilane (iPr_3SiH), *N,N'*-diisopropylcarbodiimide (DIC), phenylsilane ($PhSiH_3$), Hoveyda-Grubbs' II catalyst, Grubbs' II catalyst, 2,2,2-trifluoroethanol (TFE), formic acid, and caffeine were purchased from Sigma–Aldrich (Sydney, Australia). Aminomethyl polystyrene resin was synthesised "in house" as previously described in the literature.[1] Dichloromethane (CH_2Cl_2) was purchased from ECP Limited (Auckland, New Zealand). Fmoc-homoleucine was purchased from Chem-Impex International (Illinois, USA). Fmoc-1-amino-1-cyclobutane carboxylic acid and Fmoc-AA-O-CH_2-Phi-OCH_2-CH_2-COOH (Fmoc-AA-HMPP) were purchased from PolyPeptide Laboratories Group (Strasbourg, France). Diethyl ether (Et_2O) was purchased from Avantor Performance Materials. Trifluoroacetic acid (TFA) was purchased from Halocarbon (River Edge, USA). Dimethyl sulfoxide (DMSO) was purchased from Romil Ltd (Cambridge, UK). 4-(4-Hydroxymethyl-3-methoxyphenoxy)butyric acid (HMPB linker) was purchased from NovaBioChem (Läufelfingen, Switzerland). Tetrakis(triphenylphosphine)palladium ($Pd(PPh_3)_3$) was synthesised "*in house*" as previously described.[2]

The following Fmoc-amino acids were purchased from GL Biochem: Fmoc-norleucine, Fmoc-norvaline, Fmoc-allylglycine-OH (Fmoc-Agl), Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-Ala-OH, and Fmoc protected amino acids with the following side chain protection: Fmoc-Asp(*t*Bu)-OH (*t*Bu = *tert*-butyl) , Fmoc-Asp-O*t*Bu, Fmoc-Ser(*t*Bu)-OH, Fmoc-

Thr(*t*Bu)-OH, Fmoc-Gln(Trt)-OH (Trt = triphenylmethyl), Fmoc-Arg(Pbf)-OH (Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl), Fmoc-Tyr(*t*Bu)-OH, Fmoc-Asp(OAllyl)-OH and Fmoc-Asp-OAllyl.

RP-HPLC and LC-MS

Analytical RP-HPLC spectra were acquired on a Dionex P680 system using an analytical column (Phenomenex Gemini C_{18}, 250 x 4.6 mm; 5 µm) at a flow rate of 1 mL min^{-1}, using gradient systems as indicated in the figures. A linear gradient of 5% solvent B to 65% was used (where solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in CH_3CN) with detection at 210 nm and 254 nm. The ratio of products was determined by integration of spectra recorded at 210 nm or 214 nm. LCMS spectra were acquired on an Agilent Technologies 1120 Compact LC equipped with a Hewlett Packard 1100 MSD mass spectrometer using an analytical column (Agilent Zorbax 300SB-C_3, 3.0 mm x 150 mm, 3.5 µm) at a flow rate of 0.3 mL min^{-1} using gradients specified in the text. High-resolution mass spectra were obtained on a Bruker micrOTOFQ mass spectrometer. Semi-preparative RP-HPLC was performed on a Waters 600E system using a semi-preparative column (Waters XTerrra® C_{18}, 300 mm x 19 mm, 10 µm) at a flow rate of 10 mL min^{-1} and eluted using a one-step slow gradient protocol with a detection at 210 nm.[3] Fractions were collected, analysed by RP-HPLC and LC-MS, pooled and lyophilised three times with 10 mM aq HCl.

Peptide synthesis

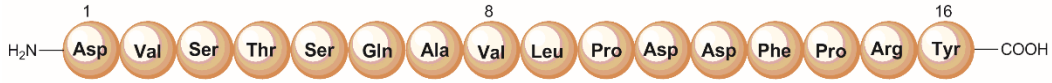
1. **Synthesis of peptides 1-17 and 20-23**

For peptides **1**-**16** and **20**-**23**, a solution of Fmoc-Tyr(*t*Bu)-O-CH_2-phi-OCH_2-CH_2-COOH (127.5 mg, 0.2 mmol) and DIC (31 µL, 0.2 mmol) in CH_2Cl_2/DMF (*v/v*; 2:1, 3 mL) was added to pre-swollen (CH_2Cl_2, 3 mL, 20 min) aminomethyl polystyrene resin (110.0 mg, 0.1 mmol, loading 0.91 mmol g^{-1}) and the mixture gently agitated for 4 h, at room temperature, filtered and washed with DMF (4 x 3 mL). A negative Kaiser test[4] confirmed the coupling. For peptide **17** however, Fmoc-Ala-O-CH_2-phi-OCH_2-CH_2-COOH (97.9 mg, 0.2 mmol) was used instead for the loading step.

Extension of the *C*-terminal amino acid was performed *via* Fmoc-SPPS on a Liberty Microwave Peptide Synthesiser (CEM Corporation, Mathews, NC). All amino acid couplings were performed as single coupling cycles, with the exception of Fmoc-Arg(Pbf)-OH where a double coupling cycle was performed as part of a synthetic protocol recommended by CEM Microwave Technology. Couplings were performed using a mixture of Fmoc-amino acid (0.4 mmol, 0.2 M), HCTU (0.36 mmol, 0.45 M) and *i*Pr_2EtN (0.8 mmol, 2M) in DMF for 5 min, at 25 W and a maximum temperature of 75 °C, except Fmoc-Arg(Pbf)-OH that was initially coupled for 25 min at room temperature which was followed by the second coupling for 5 min, at 25 W and a maximum temperature of 72 °C. The Fmoc group was removed using 5% (*w/v*) solution of piperazine with 0.1 M 6-Cl-HOBt in DMF (1 x 30 sec then 1 x 3 min). A 30 sec deprotection cycle was followed by a second deprotection for 3 min using microwave conditions of 62 W and a maximum temperature of 75 °C.

The resulting peptides were released from the resin with concomitant removal of the side chain protecting groups by treatment with TFA/*i*Pr_3SiH/H_2O (*v/v/v*; 95:2.5:2.5, 5 mL) at room temperature for 2 h. The resin was removed by filtration, washed with TFA (2 x 3 mL) and the combined filtrates were concentrated. The crude peptides were recovered by precipitation with cold diethyl ether, isolated by centrifugation (2 x 10 min; 3000 rpm), re-suspended with H_2O/CH_3CN (*v/v*; 1:1), and lyophilised.

Synthesis of peptide 1



Peptide **1** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (174 mg). RP-HPLC purification afforded peptide **1** as an amorphous solid (103 mg, 59% yield, 99% purity); R_t 12.69 min; *m/z* (ESI-MS) 1809.8 ($[M+H]^+$ requires 1809.9), Fig. S1.

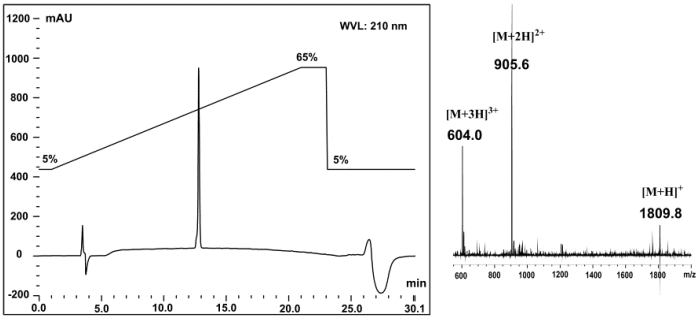


Figure S1. RP-HPLC and ESI-MS traces of peptide **1**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 2

Peptide **2** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (168 mg). RP-HPLC purification afforded peptide **2** as an amorphous solid (108 mg, 64% yield, 99% purity); R_t 12.66 min; *m/z* (ESI-MS) 1765.6 ($[M+H]^+$ requires 1765.9), Fig. S2.

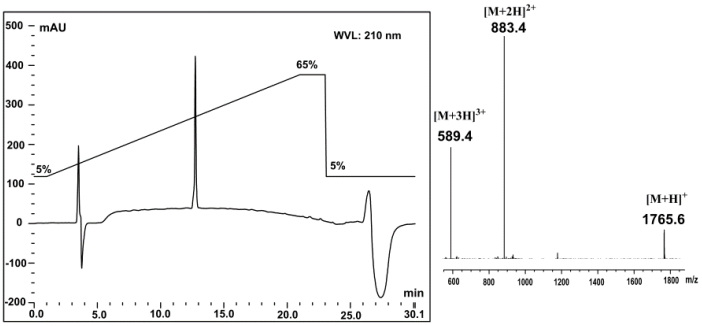


Figure S2. RP-HPLC and ESI-MS traces of peptide **2**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 3

Peptide **3** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (171 mg). RP-HPLC purification afforded peptide **3** as an amorphous solid (104 mg, 61% yield, 99% purity); R_t 12.48 min; *m/z* (ESI-MS) 1781.6 ($[M+H]^+$ requires 1781.9), Fig. S3.

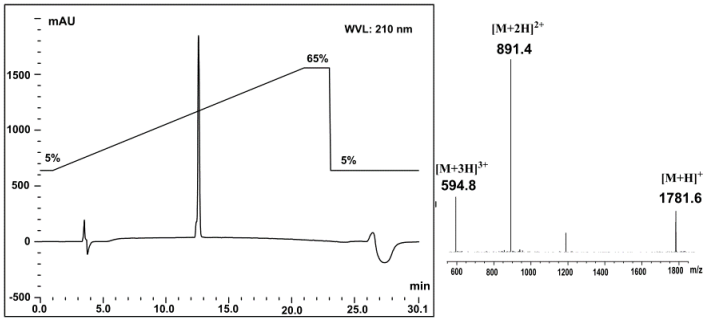


Figure S3. RP-HPLC and ESI-MS traces of peptide **3**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 4

Peptide **4** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (169 mg). RP-HPLC purification afforded peptide **4** as an amorphous solid (101 mg, 60% yield, 99% purity); R_t 12.80 min; *m/z* (ESI-MS) 1793.6 ($[M+H]^+$ requires 1793.9), Fig. S4.

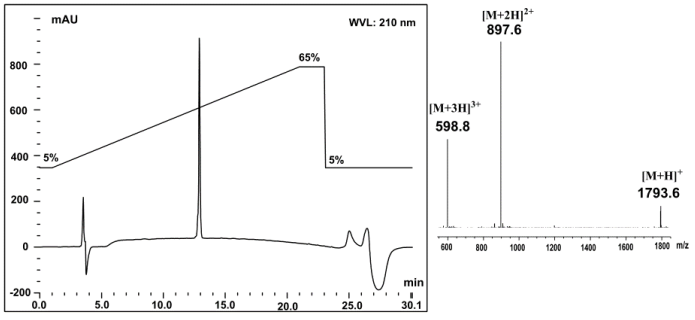


Figure S4. RP-HPLC and ESI-MS traces of peptide **4**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 5

Peptide **5** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (172 mg). RP-HPLC purification afforded peptide **5** as an amorphous solid (88 mg, 51% yield, 99% purity); R_t 12.80 min; *m/z* (ESI-MS) 1779.6 ($[M+H]^+$ requires 1779.9), Fig. S5.

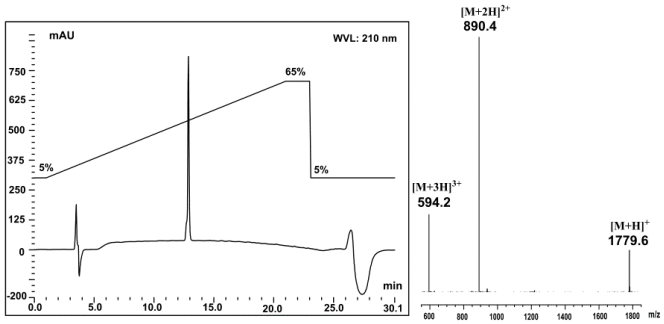


Figure S5. RP-HPLC and ESI-MS traces of peptide **5**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 6

Peptide **6** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (166 mg). RP-HPLC purification afforded peptide **6** as an amorphous solid (95 mg, 57% yield, 98% purity); R_t 12.90 min; *m/z* (ESI-MS) 1794.4 ($[M+H]^+$ requires 1793.9), Fig. S6.

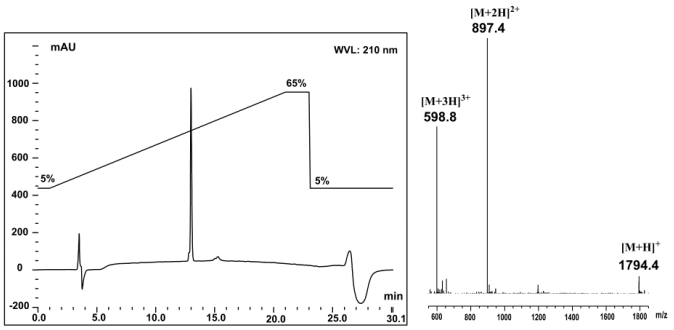
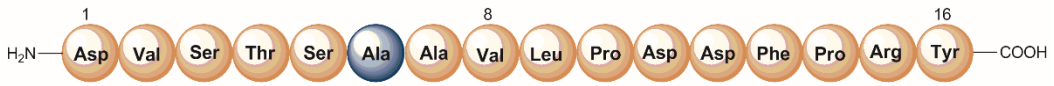


Figure S6. RP-HPLC and ESI-MS traces of peptide **6**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 7



Peptide **7** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (151 mg). RP-HPLC purification afforded peptide **7** as an amorphous solid (85 mg, 56% yield, 99% purity); R_t 12.60 min; *m/z* (ESI-MS) 1752.8 ($[M+H]^+$ requires 1752.8), Fig. S7.

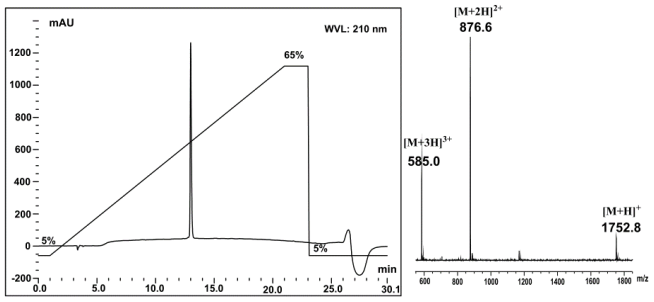


Figure S7. RP-HPLC and ESI-MS traces of peptide **7**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 8

Peptide **8** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (157 mg). RP-HPLC purification afforded peptide **8** as an amorphous solid (79 mg, 50% yield, 99% purity); R_t 12.30 min; *m/z* (ESI-MS) 1796.0 ($[M+H]^+$ requires 1795.9), Fig. S8.

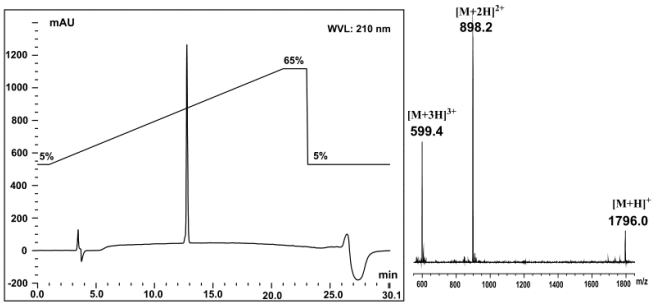
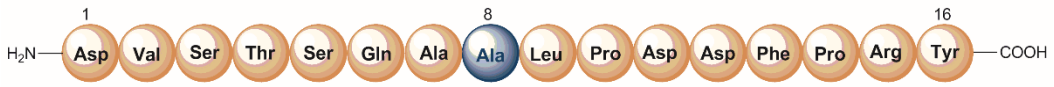


Figure S8. RP-HPLC and ESI-MS traces of peptide **8**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 9



Peptide **9** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (153 mg). RP-HPLC purification afforded peptide **9** as an amorphous solid (50 mg, 33% yield, 99% purity); R_t 11.80 min; *m/z* (ESI-MS) 1781.8 ($[M+H]^+$ requires 1781.8), Fig. S9.

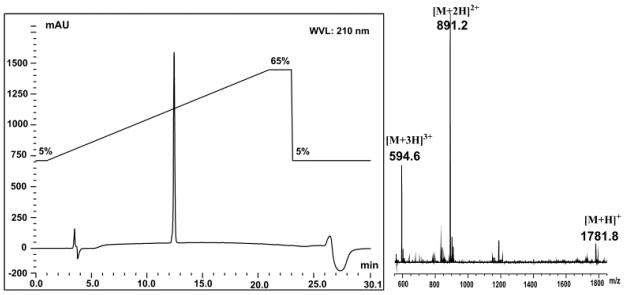
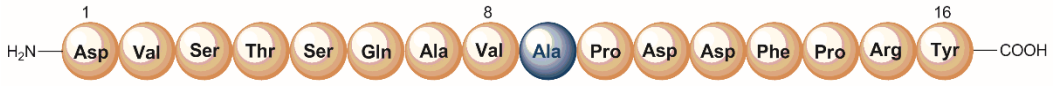


Figure S9. RP-HPLC and ESI-MS traces of peptide **9**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 10



Peptide **10** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (170 mg). RP-HPLC purification afforded peptide **10** as an amorphous solid (80 mg, 47% yield, 99% purity); R_t 12.80 min; *m/z* (ESI-MS) 1767.8 ($[M+H]^+$ requires 1767.8), Fig. S10.

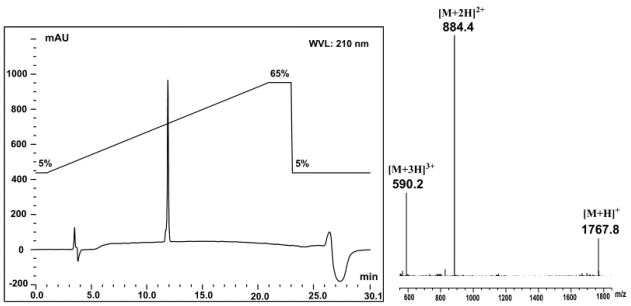
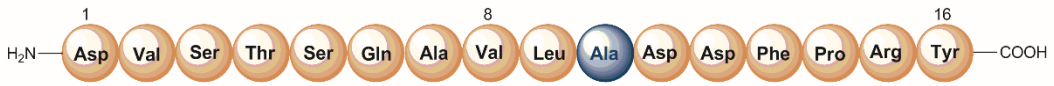


Figure S10. RP-HPLC and ESI-MS traces of peptide **10**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 11



Peptide **11** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (165 mg). RP-HPLC purification afforded peptide **11** as an amorphous solid (18 mg, 11% yield, 99% purity); R_t 12.70 min; *m/z* (ESI-MS) 1783.8 ($[M+H]^+$ requires 1783.8), Fig. S11.

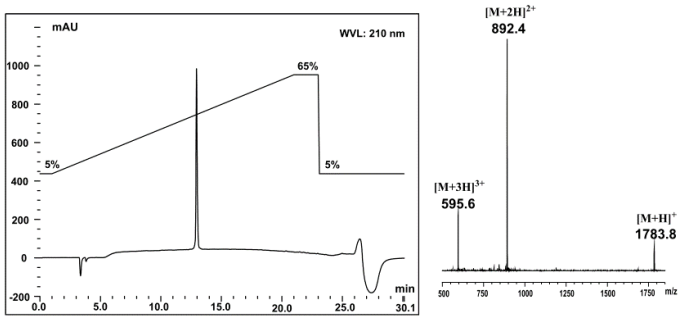
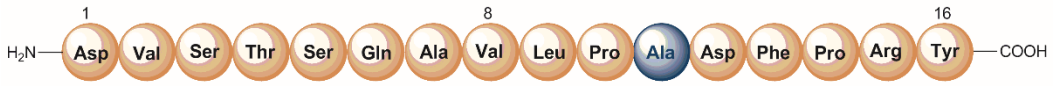


Figure S11. RP-HPLC and ESI-MS traces of peptide **11**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 12



Peptide **12** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (161 mg). RP-HPLC purification afforded peptide **12** as an amorphous solid (79 mg, 49% yield, 99% purity); R_t 12.80 min; *m/z* (ESI-MS) 1765.8 ($[M+H]^+$ requires 1765.9), Fig. S12.

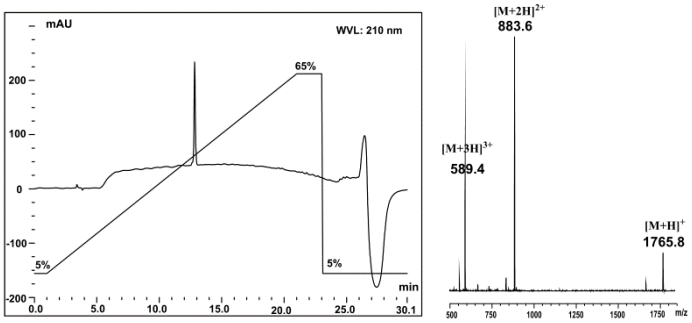
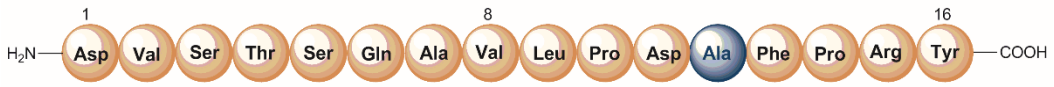


Figure S12. RP-HPLC and ESI-MS traces of peptide **12**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 13



Peptide **13** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (147 mg). RP-HPLC purification afforded peptide **13** as an amorphous solid (91 mg, 62% yield, 99% purity); R_t 12.90 min; *m/z* (ESI-MS) 1765.6 ($[M+H]^+$ requires 1765.9), Fig. S13.

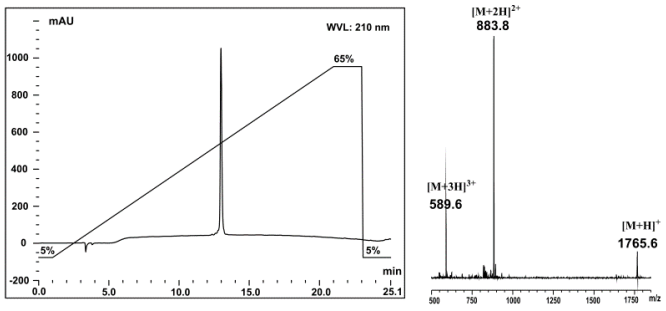
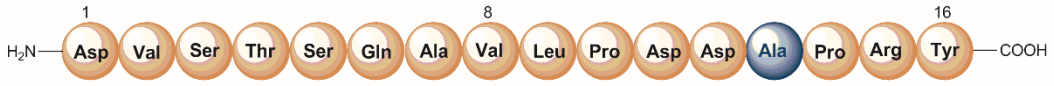


Figure S13. RP-HPLC and ESI-MS traces of peptide **13**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 14



Peptide **14** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (158 mg). RP-HPLC purification afforded peptide **14** as an amorphous solid (49 mg, 31% yield, 99% purity); R_t 11.10 min; *m/z* (ESI-MS) 1734.0 ($[M+H]^+$ requires 1733.8), Fig. S14.

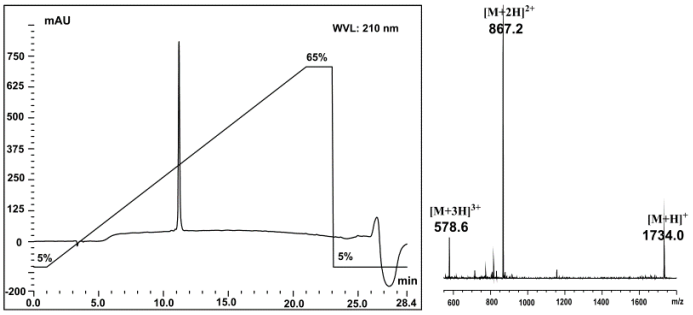
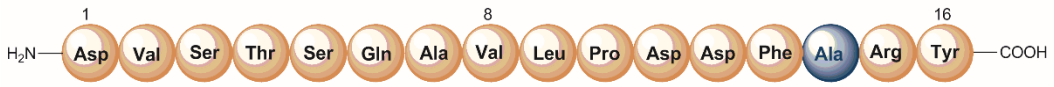


Figure S14. RP-HPLC and ESI-MS traces of peptide **14**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 15



Peptide **15** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (162 mg). RP-HPLC purification afforded peptide **15** as an amorphous solid (63 mg, 39% yield, 99% purity); R_t 12.70 min; *m/z* (ESI-MS) 1783.2 ($[M+H]^+$ requires 1783.8), Fig. S15.

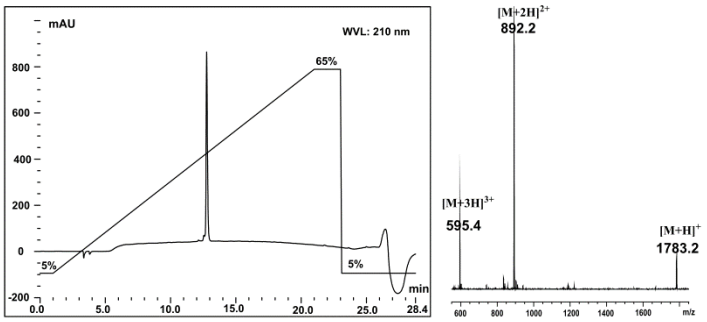
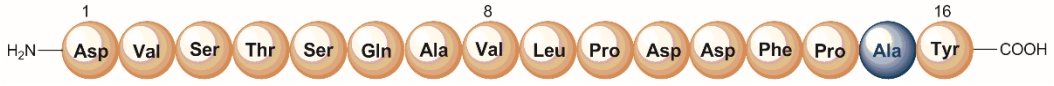


Figure S15. RP-HPLC and ESI-MS traces of peptide **15**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 16



Peptide **16** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (155 mg). RP-HPLC purification afforded peptide **16** as an amorphous solid (56 mg, 36% yield, 99% purity); R_t 13.60 min; *m/z* (ESI-MS) 1724.8 ($[M+H]^+$ requires 1724.8), Fig. S.16.

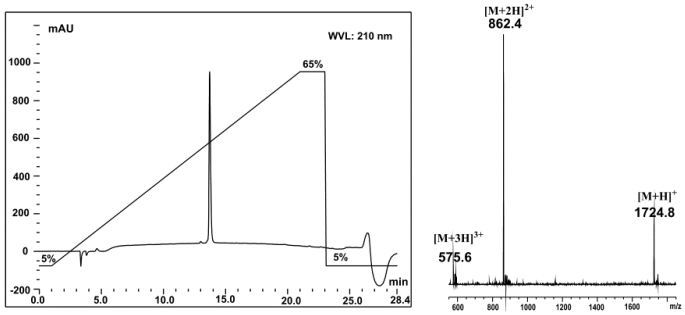
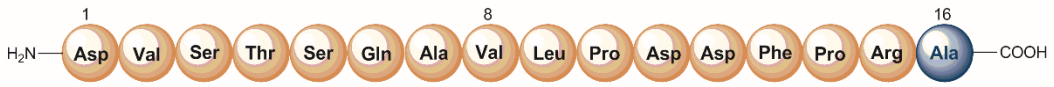


Figure S16. RP-HPLC and ESI-MS traces of peptide **16**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 17



Peptide **17** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (169 mg). RP-HPLC purification afforded peptide **17** as an amorphous solid (88 mg, 52% yield, 99% purity); R_t 12.10 min; *m/z* (ESI-MS) 1718.0 ($[M+H]^+$ requires 1717.8), Fig. S17.

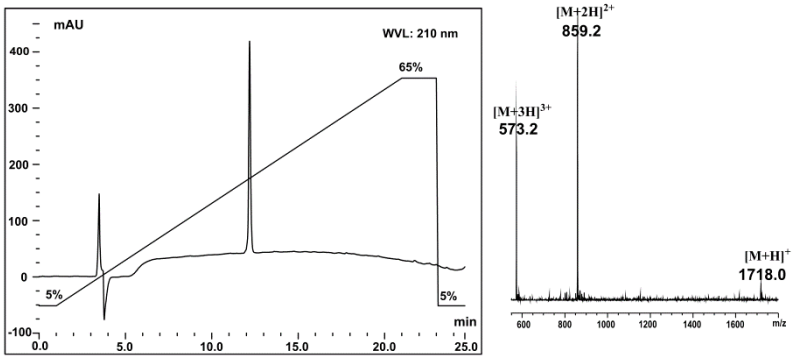


Figure S17. RP-HPLC and ESI-MS traces of peptide **17**, linear gradient of 5%B to 65%B over 20 min, *ca* 3%B per minute.

Synthesis of peptide 20

Peptide **20** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (142 mg). RP-HPLC purification afforded peptide **20** as an amorphous solid (75 mg, 53% yield, 99% purity); R_t 17.20 min; *m/z* (ESI-MS) 1835.6 ($[M+H]^+$ requires 1835.9), Fig. S18.

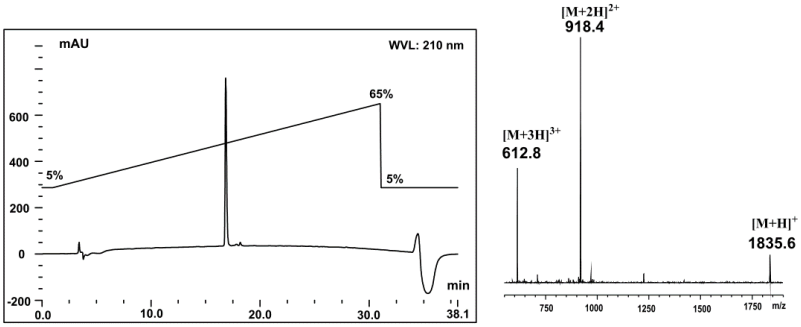


Figure S18. RP-HPLC and ESI-MS traces of peptide **20**, linear gradient of 5%B to 65%B over 30 min, *ca* 2%B per minute.

Synthesis of peptide 21

Peptide **21** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (156 mg). RP-HPLC purification afforded peptide **21** as an amorphous solid (59 mg, 38% yield, 98% purity); R_t 17.80 min; *m/z* (ESI-MS) 1849.8 ($[M+H]^+$ requires 1849.9), Fig. S19.

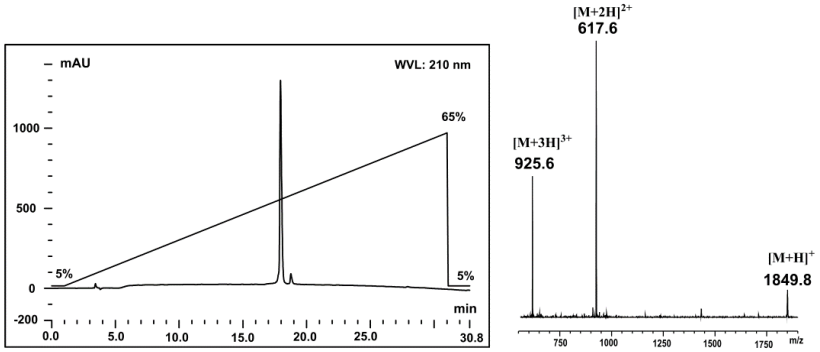


Figure S19. RP-HPLC and ESI-MS traces of peptide **21**, linear gradient of 5%B to 65%B over 30 min, *ca* 2%B per minute.

Synthesis of peptide 22

Peptide **22** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (172 mg). RP-HPLC purification afforded peptide **22** as an amorphous solid (69 mg, 40% yield, 99% purity); R_t 16.60 min; *m/z* (ESI-MS) 1821.8 ($[M+H]^+$ requires 1821.9), Fig. S20.

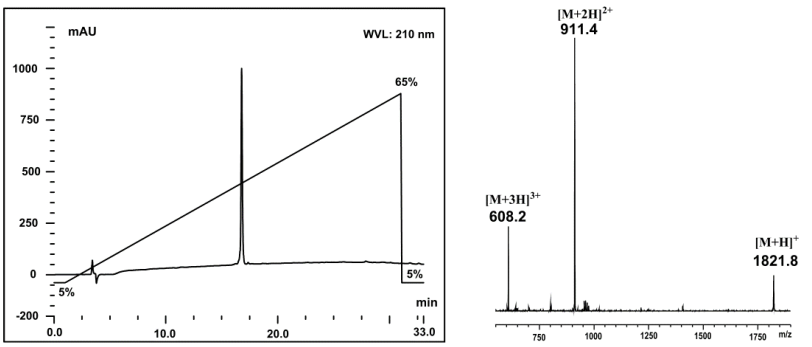


Figure S20. RP-HPLC and ESI-MS traces of peptide **22**, linear gradient of 5%B to 65%B over 30 min, *ca* 2%B per minute.

Synthesis of peptide 23

Peptide **23** was synthesised using microwave-enhanced Fmoc-SPPS on an aminomethyl polystyrene resin (0.1 mmol), which following the cleavage conditions in the general procedure afforded the crude peptide (155 mg). RP-HPLC purification afforded peptide **23** as an amorphous solid (47 mg, 30% yield, 99% purity); R_t 16.80 min; *m/z* (ESI-MS) 1819.6 ($[M+H]^+$ requires 1819.9), Fig. S21.

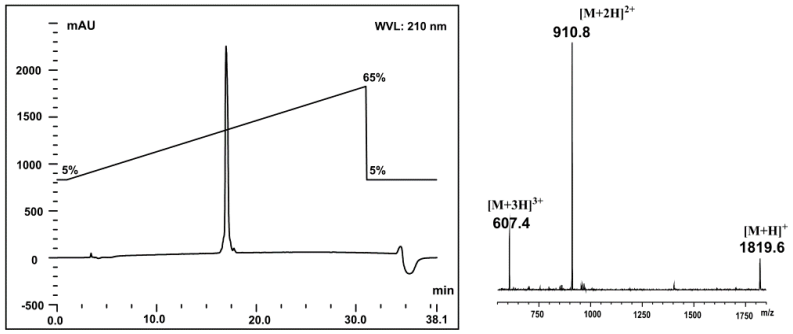


Figure S21. RP-HPLC and ESI-MS traces of peptide **23**, linear gradient of 5%B to 65%B over 30 min, *ca* 2%B per minute.

2. Data for head-to-tail macrocycle 24

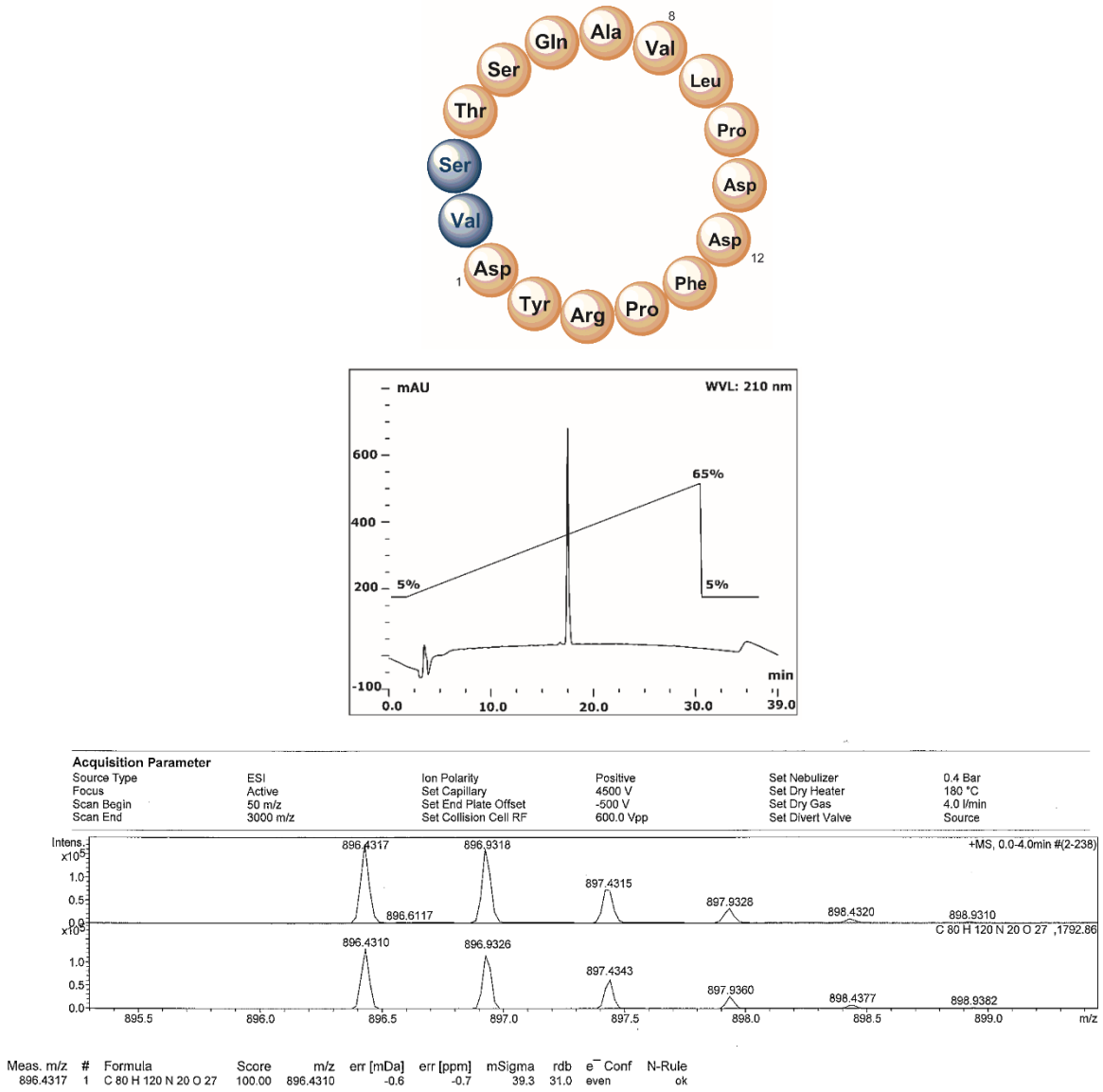
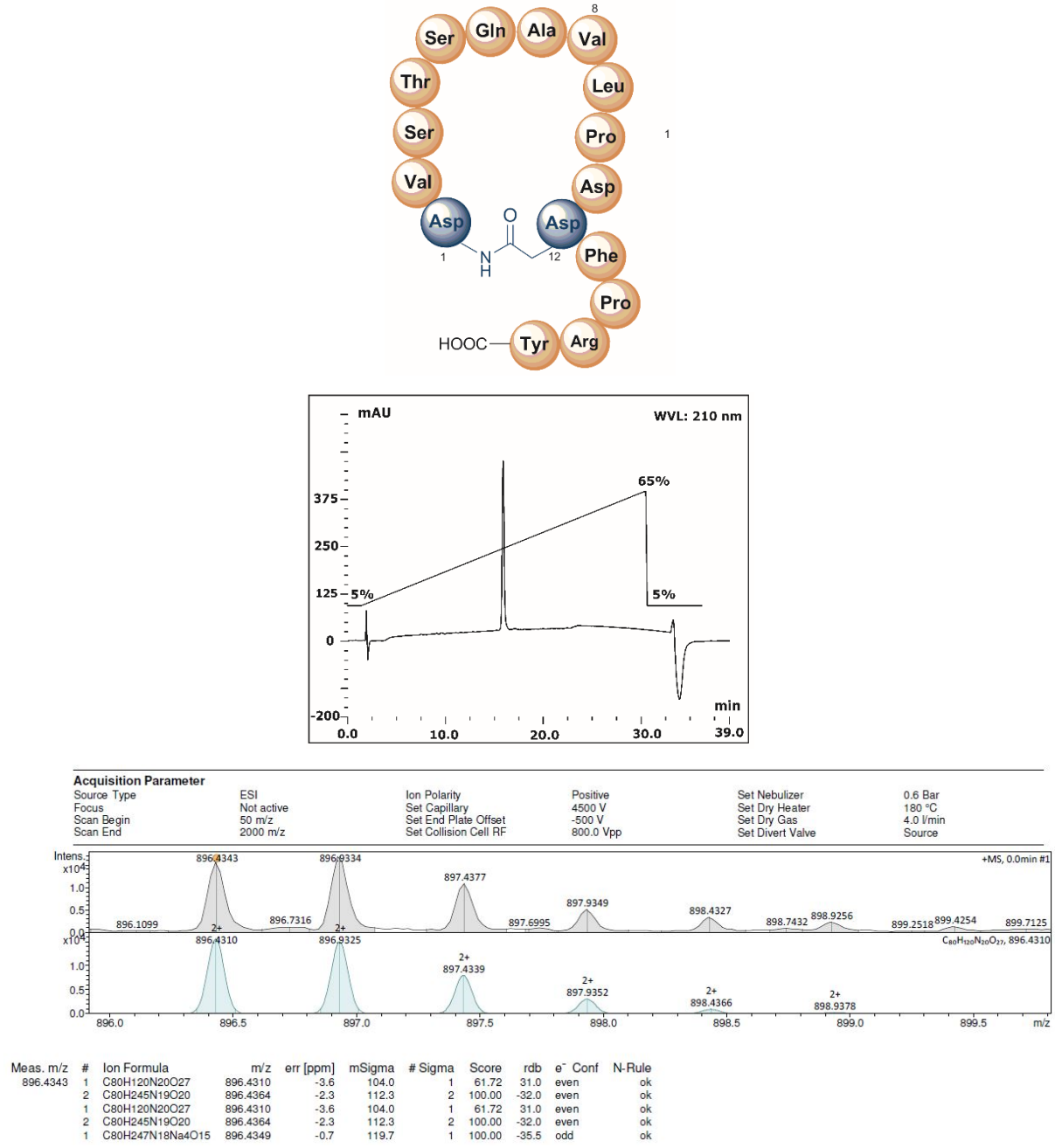


Figure S22. RP-HPLC and HRESI-MS traces of peptide **24**, linear gradient of 5%B to 65%B over 30 min, *ca* 2%B per minute.

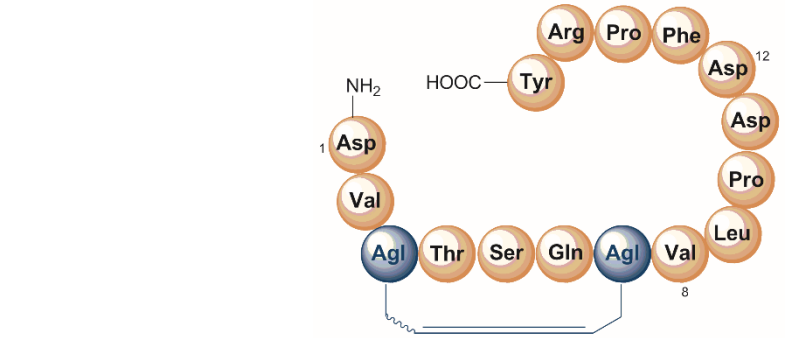
3. Data for head-to-side chain macrocycle 25

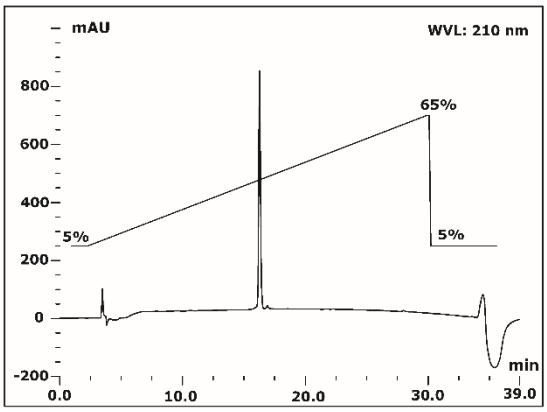


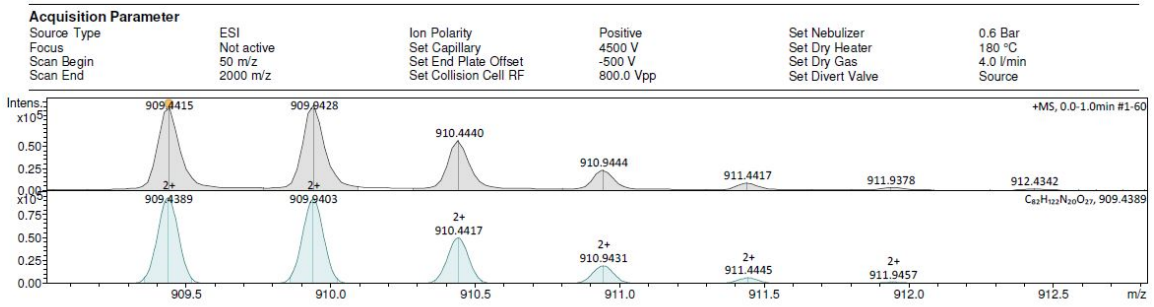
Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e⁻ Conf	N-Rule
896.4343	1	C80H120N20O27	896.4310	-3.6	104.0	1	61.72	31.0	even	ok
	2	C80H245N19O20	896.4364	-2.3	112.3	2	100.00	-32.0	even	ok
	1	C80H120N20O27	896.4310	-3.6	104.0	1	61.72	31.0	even	ok
	2	C80H245N19O20	896.4364	-2.3	112.3	2	100.00	-32.0	even	ok
	1	C80H247N18Na4O15	896.4349	-0.7	119.7	1	100.00	-35.5	odd	ok

Figure S23. RP-HPLC and HRESI-MS traces of peptide **25**, linear gradient of 5%B to 65%B over 30 min, *ca* 2%B per minute.

4. Data for cyclic dicarba analogue 26







Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e⁻ Conf	N-Rule
909.4415	1	C82H122N20O27	909.4389	-2.9	30.5	1	100.00	32.0	even	ok
	2	C82H247N19O20	909.4442	-3.0	36.6	2	64.93	-31.0	even	ok
	1	C82H122N20O27	909.4389	-2.9	30.5	1	100.00	32.0	even	ok
	2	C82H247N19O20	909.4442	-3.0	36.6	2	64.93	-31.0	even	ok
	1	C82HN21Na4O28	909.4440	2.8	33.9	1	57.91	91.0	even	ok
	2	C82H249N18Na4O15	909.4427	1.4	44.7	2	93.86	-34.5	odd	ok
	3	C82H368N22Na4O4	909.4424	-1.1	48.5	3	100.00	-92.0	even	ok

Figure S24. RP-HPLC and HRESI-MS traces of peptide **26**, linear gradient of 5%B to 65%B over 30 min, *ca* 2%B per minute.

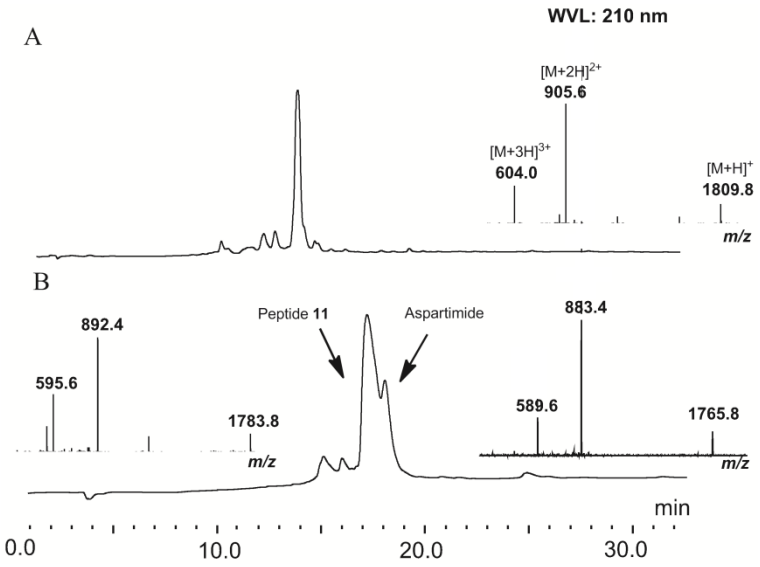


Figure S25. Comparison of the analytical RP-HPLC chromatogram of: (A) Crude preptin (1-16) **1**. (B) Crude peptide **11** (Pro-10 to Ala-10). Agilent Zorbax 300SB-C_3 column (C_3, 3.0 x 150 mm; 3.5 µm), gradient 5-65% B over 30 min, 0.3 mL min^{-1} flow rate.

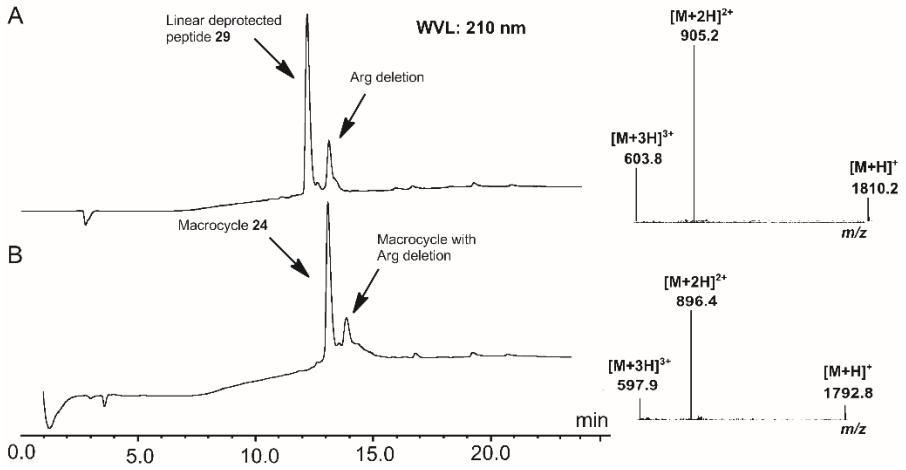


Figure S26. Analytical LC-MS chromatograms for (A) crude linear peptide **29** (B) crude head-to-tail cyclic preptin (1-16) **24**. Agilent Zorbax 300SB-C_3 column (3.0 x 150 mm; 3.5 µm), gradient 5-95% B over 22 min, 0.3 mL min^{-1} flow rate.

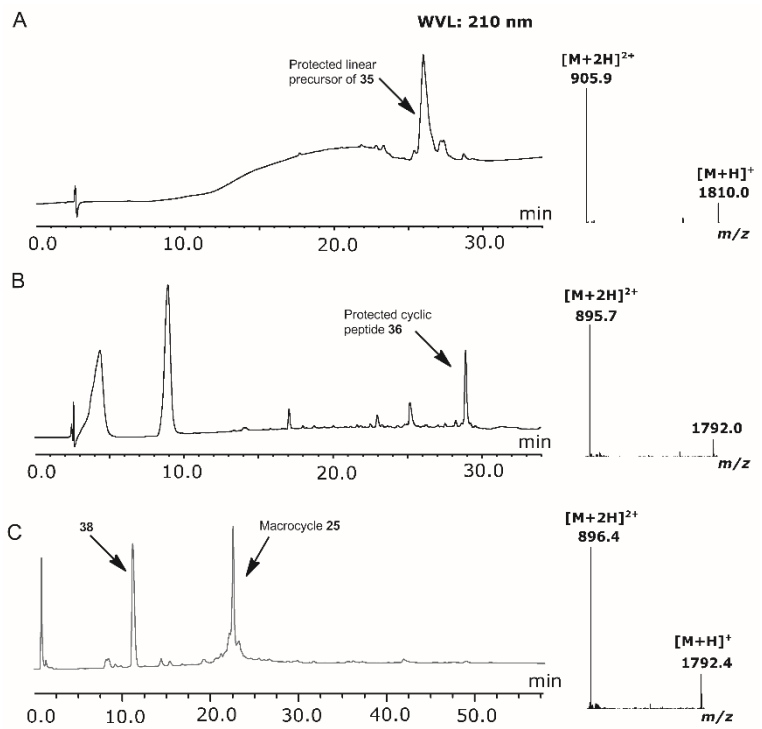


Figure S27. Analytical LC-MS chromatograms for the synthesis of macrocycle **25**. (A) The linear peptide **35**; (B) crude cyclic peptide **36**, analysed by Agilent Zorbax 300SB-C_3 column (3.0 x 150 mm; 3.5 µm), gradient 5-95% B over 30 min, 0.3 mL min^{-1} flow rate; (C) The condensation reaction to yield **25**, analysed by Agilent Zorbax 300SB-C_3 column (3.0 x 150 mm; 3.5 µm), gradient 5-65% B over 60 min, 0.3 mL min^{-1} flow rate.

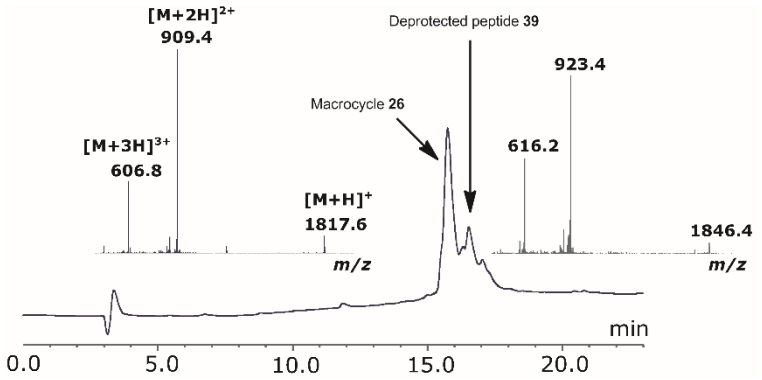


Figure S28. Analytical LC-MS chromatograms for the synthesis of macrocycle **26** showing the mass and retention time difference between **26** and its linear precursor. Analysed by Agilent Zorbax 300SB-C_3 column (3.0 x 150 mm; 3.5 µm), gradient 1-61% B over 20 min, 0.3 mL min^{-1} flow rate.

Circular dichroism (CD) spectroscopy. All CD spectra were recorded using a Pi Star-180 (Applied Photophysics, UK) spectrometer at 20 ^{o}C with a cell of 0.1 cm path length in the range from 180 nm to 300 nm at 0.5 nm intervals with a 5 s response time. Each CD spectrum measurement represents the average of four scans obtained with a 2 nm optical bandwidth. Baseline spectrum was collected with the buffer alone and then subtracted from the raw peptide spectra. The measurements were performed at peptide concentrations of 50 µM in 20 mM sodium phosphate buffer, pH 7.21 in 1 mm quartz cuvettes (Hellma Analytics, Germany). Data are expressed as mean residue ellipticities [Θ] in (deg cm^2 dmol^{-1}), calculated as follows:

$$\Theta = S / (10 \times c \times L \times n)$$

Where S is the raw CD signal (millidegrees), c is the peptide concentration (M), L is the cuvette path length (cm), and n is the number of peptide bonds.

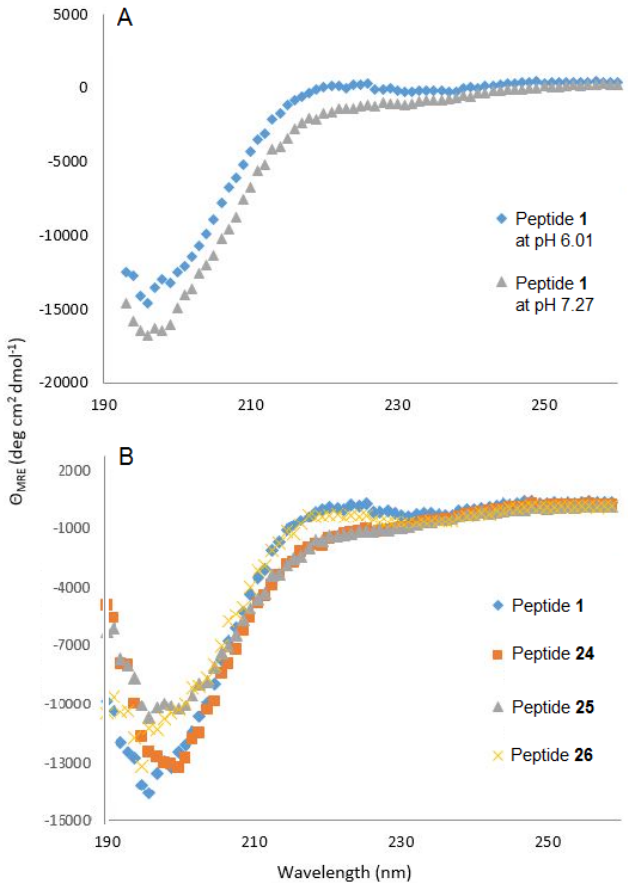


Figure S29. Circular dichroism spectroscopy data acquired at 50 µM in 20 mM phosphate buffer of (A) preptin (1-16) **1** at pH 7.27 and pH 6.01; and (B) peptides **1**, and **24**-**26** at pH 7.27.

References

1. P. W. R. Harris, S. H. Yang and M. A. Brimble, *Tetrahedron Lett.,* 2011, **52**, 6024-6026.

2. D. R. Coulson, L. C. Satek and S. O. Grim, Tetrakis (Triphenylphosphine) Palladium (0). In *Inorganic Syntheses*, A., C. F., Ed. John Wiley & Sons, Inc.: 2007; Vol. 13, pp 121-124.

3. (a) P. W. R. Harris, D. J. Lee and M. A. Brimble, *J. Pept. Sci.,* 2012, **18**, 549-555; (b) Y. Chen, C. T. Mant and R. S. Hodges, *J. Chromatogr. A.,* 2007, **1140**, 112-120.

4. E. Kaiser, R. L. Colescott, C. D. Bossinger and P. I. Cook, *Anal. Biochem.,* 1970, **34**, 595-598.

5. J. L. Stymiest, B. F. Mitchell, S. Wong and J. C. Vederas, *Org. Lett.,* 2003, **5**, 47-49.