Supporting Information

Helical structure in cyclic peptides: effect of N-methyl amides versus esters

Chongyang Wu,[a] Huy N. Hoang,[a] Timothy A. Hill,[a] Junxian Lim,[a] W. Mei Kok,[a] Kalyani Akondi,[a] Ligong Liu,[a] and David P. Fairlie*

Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science, Institute for Molecular Bioscience, University of Queensland, Brisbane, Qld 4072, Australia

Email : d.fairlie@imb.uq.edu.au

1. List of Abbreviations **2**

2. Peptide synthesis **4**
2.1 Peptide assembly *4*
2.2 Cyclization *13*
2.3 Cleavage *13*
2.4 HPLC methods *13*

3. CD spectroscopy **14**

4. NMR spectroscopy **14**

5. Structure calculations **15**

6. Computational modelling **16**

7. Flow cytometry **16**

8. Characterization of peptides by proton NMR spectroscopy **17**

9. Peptide analysis by mass spectrometry **23**

10. NMR solution structure data **24**

11. Modelling of idealised alpha helices, Raw CD spectra and NMR-derived structures **30**

1. List of Abbreviations

Ac_2O	Acetic Anhydride
DEAD	Diethyl azodicarboxylate
DIAD	Diisopropyl azodicarboxylate
DIC	N,N′-Diisopropylcarbodiimide
DIPEA	Diisopropylethylamine
DMF	Dimethylformamide
DMAP	4-Dimethylaminopyridine
DMPA	2,2-Dimethoxy-2-phenylacetophenone
DMSO	Dimethyl Sulfoxide
DSS	Trimethylsilylpropane sulfonate
equiv	Equivalent
ESI-MS	Electrospray ionization mass spectrometry
EtOAc	Ethyl Acetate
TOF-MS	Time-of-flight mass spectrometry
Fmoc	9-fluorenylmethyloxycarbonyl
HATU	*N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-Methylmethanaminium hexafluorophosphate *N*-oxide
HBTU	2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl uranium Hexafluorophosphate
HOAt	1-Hydroxy-7-azabenzotriazole
HPLC	High-performance liquid chromatography
HSQC	Heteronuclear single quantum correlation
HR-MS	High-resolution mass spectroscopy
Hz	Hertz
$^3J_{XY}$	X and Y coupling constant across 3 bonds
K	Kelvin
LC	Liquid Chromatography
Me	Methyl
MeOH	Methanol
MS	Mass Spectrometry
NBS	N-bromo succinimide
NMP	N-methyl Pyrolidone
PE	Petroleum Ether

ppb	Parts per billion
PPh_3	Triphenylphosphine
p-TsOH	p-Toluenesulfonic acid
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
RP	Reverse Phase
ROE	Rotating frame nuclear overhauser enhancement
ROESY	Rotating frame nuclear overhauser enhancement specyroscopy
rp-HPLC	Reserved-phase high performance liquid chromatography
rt	Room temperature
RMSD	Root-mean-square deviation
TBAF	Tetra-n-butylammonium fluoride
THF	Tetrahydrofuran
TFA	Trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
TLC	Thin Layer Chromatography
TMS	Trimethylsilyl
TIS	Triisopropylsilane
TOCSY	Total correlation spectroscopy
UV	Ultraviolet

2. Peptide synthesis

2.1 Peptide assembly

Protected amino acids and resins were obtained from ChemImpex and Novabiochem. TFA, piperidine, DIPEA, DCM and DMF (peptide synthesis grade was reagent grade unless otherwise stated). Peptides were synthesized using Fmoc solid support chemistry on Rink amide resin (low loading 0.38 mmol/g; Novabiochem) at a 100 μM scale on a Symphony Multiplex Synthesizer. Amino acids (4 eq.) were activated using HCTU (4 eq.) and DIPEA (8 eq.) in DMF (2 × 20 min) prior to remove N-terminal Fmoc protecting group using 20% piperidine in DMF (2 × 5 min).

Synthesis of N-methylated peptides (2a-e)

Peptides **2b-c** were synthesized on solid phase by using commercially available Fmoc-*N*-methyl-amino acids. Peptide **2a** was synthesized on 0.1 mmol scale with Rink-amide resin (0.35 mmol/g). Standard peptide coupling conditions (HBTU, DIPEA) were used for the incorporation of normal amino acids. After the deprotection of Fmoc to give the *N*-terminal amine (NH_2-KLLLD-resin), which is ready for N-methylation. A solution of *O*-NBS-Cl (0.4 mmol, 89 mg), collidine (1.0 mmol, 132 μL) in NMP 3mL was added to the resin-bound free amine peptide, which was shaken gently for 15 min. The reaction solution was removed, followed by washing the resin DCM and DMF for 4 times. Then a solution of PPh_3 (0.5 mmol, 132 mg), MeOH (1 mmol, 40 μL) in anhydrous THF 3 mL was added to cover the peptide-bound resin. DIAD (0.5 mmol, 97 μL) in 1 mL THF was added dropwise, which was repeated twice (2 × 2h). The N-methylation reaction finished, which was confirmed by mini-cleavage, followed by LC-MS.

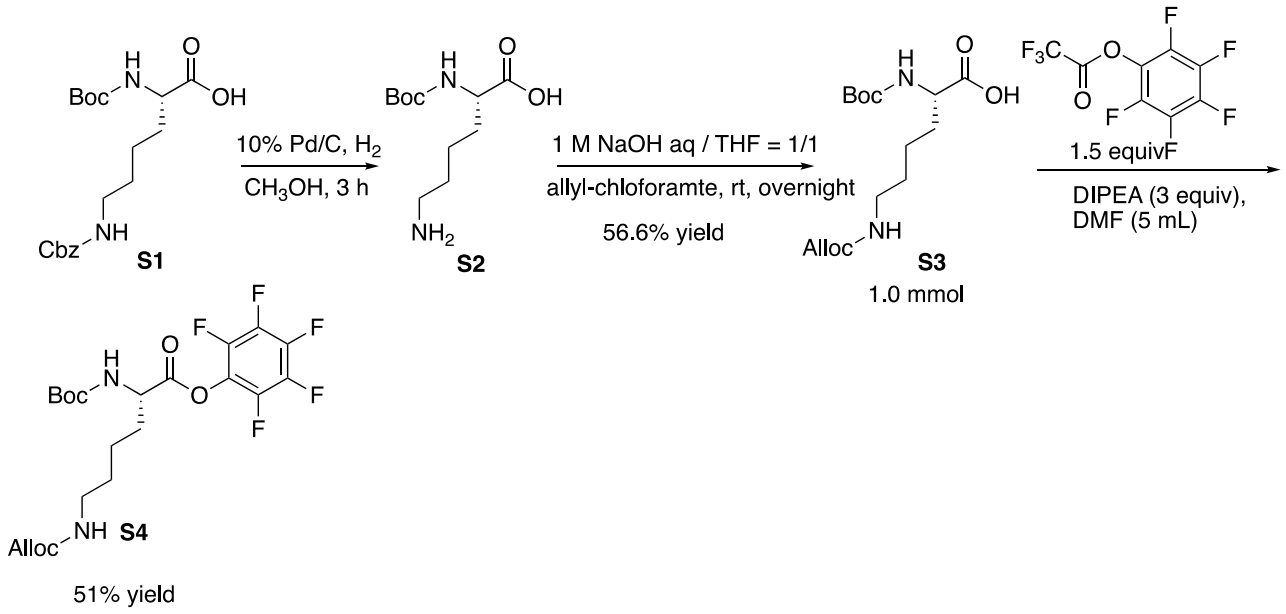
O-NBS deprotection was achieved by using 2-mercaptoethanol (1 mmol, 71 μL) and DBU (0.5 mmol, 75 μL) in NMP for 3 h. After deprotection, the resin was capped with a solution of Ac_2O (200 μL) and DIPEA (100 μL), followed by removing the side chain protecting groups of Lys and Asp, and cyclization on solid phase to give the product **2a**.

Synthesis of compound **2d** and **2e**. Fmoc-Lys(Alloc)-OH and Fmoc-Asp(OPip)-OH were employed for incorporation of non-standard amino acids at positions 1 and 5 respectively. The peptide Ac-Lys(Alloc)-Leu-Leu-Leu-Asp(OPip) was assembled on the solid support as described in the general procedure. The Alloc protecting group was removed by treating the resin with a solution of phenylsilane (24 equiv) and $Pd(PPh_3)_4$ (0.1 equiv). N_2 was bubbled through the reaction mixture for 10 min and the resin was washed with DCM. A solution of 2-nitrobenzenesulfonyl chloride (4 equiv)

and DIPEA (4 equiv) in DCM was added to the resin-bound free-amine peptide and shaken for 30 min. The resin was filtered and washed with DCM and DMF. The N-methylation procedure was conducted by treatment with a solution of methyl iodide (4 equiv) and MTBD (6 equiv), and the reaction was agitated overnight. For subsequent *o*-NBS deprotection, the peptide was treated with a solution of mercaptoethanol (10 equiv) and DBU (3 equiv) in DMF for 5 min. The deprotection procedure was repeated and the resin washed with DMF (5x). The resin was then washed with DCM and treated repeatedly with 2% TFA in DCM (5 x 2 min). Subsequently, the peptide was cleaved by TFA acidolysis to afford the crude linear peptide. The linear peptide was then treated with a solution of PyBOP (4 equiv) and DIPEA (4 equiv) in DMF, with overnight stirring. The reaction mixture was reduced *in vacuo* and the crude cyclized peptide was redissolved in 50% acetonitrile in H_2O and purified by RP-HPLC.

Synthesis of building blocks for ester-containing helical peptides 3a-e

Synthesis of Boc-Lys(Alloc)-OPfp (S4)

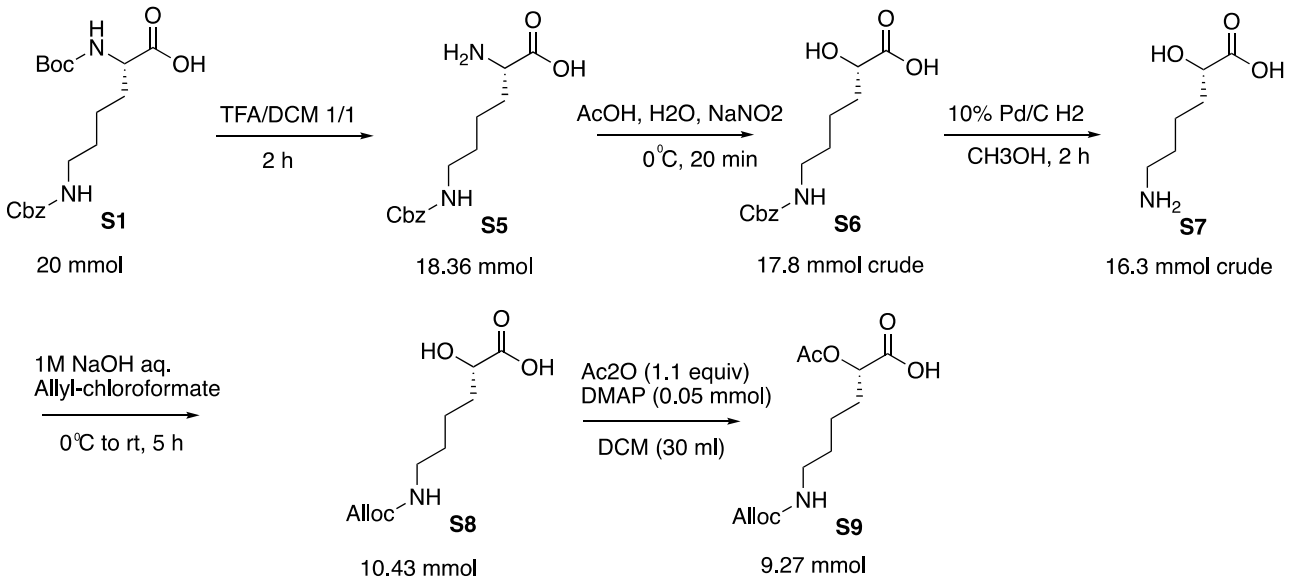


N^{α}-Boc-N^{ε}-Lys(Z)-OH (**S1**) (3.8 g, 10 mmol) was dissolved in MeOH (60 mL) and 10% Pd/C (380 mg) was added under a H_2 atmosphere at 1 atm. The reaction mixture was stirred for 2 h and then was filtrated through celite. The product was concentrated *in vacuo* to dryness. The crude intermediate N^{α}-Boc-N^{ε}-Lys-OH (**S2**) was dissolved in 1M aq NaOH:THF (2:1, 15 mL). Allyl chloroformate (935 μL, 8.8 mmol) was added dropwise. The reaction mixture was stirred at r.t. overnight. THF was removed under vacuum, the residue was dissolved in EtOAc (30 mL) in an ice bath. The solution was adjusted to pH 3 with 1M aq HCl, extracted by EtOAc (30 mL × 2). The

combined organic phases were washed with saturated NaCl solution and dried over anhydrous $MgSO_4$. The concentrated residue was purified by flash chromatography (Petroleum:EtAcOHA = 1:1) to give N^{α}-Boc-N^{ε}-Lys(Alloc)-OH (**S3)**, 1.87 g, 56% yield.

N^{α}-Boc-N^{ε}-Lys(Aloc)-OH (**S3**) (1 mmol, 330 mg) was dissolved in anhydrous DMF (5 mL). DIPEA (3 mmol, 524 μL) was added. Pfp-TFA (2.0 mmol, 340 μL) was added dropwise at r.t. and the mixture was left stirring overnight. Upon completion, the reaction was quenched by slow addition of 10% critic acid (10 mL) and extracted with EA (20 mL ×3). The combined organic layers were washed by saturated $NaHCO_3$ solution (20 mL) and brine solution (20 mL). The organic solvent was removed *in vacuo*. The residue was purified by flash chromatography with a gradient of PE:EtOAc (10:1 to 4:1). After concentration of the combined product fractions, the title compound (**S4**) was obtained as white flakes (252 mg, 51% yield). ^{1}H-NMR (600 MHz, $CDCl_3$): δ = 5.92 (m, 1H), 5.30 (dd, *J* = 17.3 Hz, 1.5 Hz, 1H), 5.21 (dd, *J* = 10.4 Hz, 1.2 Hz, 1H), 5.18 (d, *J* = 7.1 Hz, 1H), 4.78 (t, 1H), 4.60 (m, 1H), 4.57 (d, J = 5.2 Hz, 2H), 3.22 (m, 2H), 1.99 (m, 1H), 1.87 (m, 1H), 1.64-1.55 (m, 2H), 1.54-1.48 (m, 2H). ^{13}C{1H}-NMR (150 MHz, $CDCl_3$): δ = 169.1, 156.5, 155.3, 141.9, 140.5, 140.3, 138.8, 138.7, 137.1, 132.9, 117.7, 80.6, 65.6, 53.3, 40.2, 31.6, 29.5, 22.2. ESI-M/Z: *m/z* 519.1 [M+Na^{+}].

Synthesis of S9 (Building block for **3a**).



N^{α}-Boc-N^{ε}-Lys(Z)-OH (**S1**) (7.6 g, 20 mmol) was dissolved in DCM–TFA (50 mL, 1:1) and stirried at r.t. for 2 h before removing the solvent under a N_2 gas flow. AcCN–H_2O (30 mL, 1:1) was added, followed by freeze-drying to give the crude N^{α} -N^{ε}-Lys(Z)-OH (**S5**). The crude product was dissolved in 50% AcOH (150 mL). A solution of $NaNO_2$ (7 equiv, 9.8 g) in H_2O (40 mL) was added dropwise to the mixture in an ice bath. The reaction was stirred at 0 °C for 20 min then r.t. for 30 min, while the reaction progress was monitored by LC-MS. After completion (1 h), the mixture was concentrated

in vacuo to give the crude α-hydroxy acid **S6**. A suspension of the α-hydroxy acid and 10% Pd/C (500 mg) in 100 mL CH_3OH was stirred vigorously under H_2 atmosphere (1 atm) for 2 h. The reaction mixture was filtered through a celite pad and washed with MeOH. The crude product was concentrated *in vacuo*, treated with H_2O (50 mL), and washed by DCM to remove any impurity. The aqueous phase was lyophilized to give the crude product **S7** (*S*)-6-amino-2-hydroxyhexanoic acid[30] (2.4 g, 17.8 mmol)).

To a stirred solution of (*S*)-6-amino-2-hydroxyhexanoic acid in 1 N NaOH (40 mL) at 0 °C, allyl chloroformate (1.1 equiv, 1.9 mL) was added, then the ice bath was removed, and the reaction was stirred at room temperature for 5 h. The mixture was washed with diethyl ether (20 mL) to remove any organic impurities. The aqueous phase was concentrated to half of the original volume, adjusted to pH 3 with 1 N HCl, extracted with DCM (20 mL ×3). The combined DCM phases were washed with saturated NaCl solution. Compound **S8** was obtained after concentration of the organic phase. ^{1}H-NMR (600 MHz, $CDCl_3$): δ = 5.91 (m, 1H), 5.30 (d, *J* = 17.0 Hz, 1H), 5.21 (d, *J* = 10.4 Hz, 1H), 4.93 (m, 1H), 4.55 (d, *J* = 4.9 Hz, 1H), 4.25 (m, 1H), 3.20 (m, 2H), 1.86 (m, 1H), 1.74 (m, 1H), 1.58-1.43 (m, 4H). ^{13}C{^{1}H}-NMR (150 MHz, $CDCl_3$): δ = 177.8, 156.7, 132.8, 117.8, 70.0, 66.3, 40.6, 33.4, 29.5, 21.7.

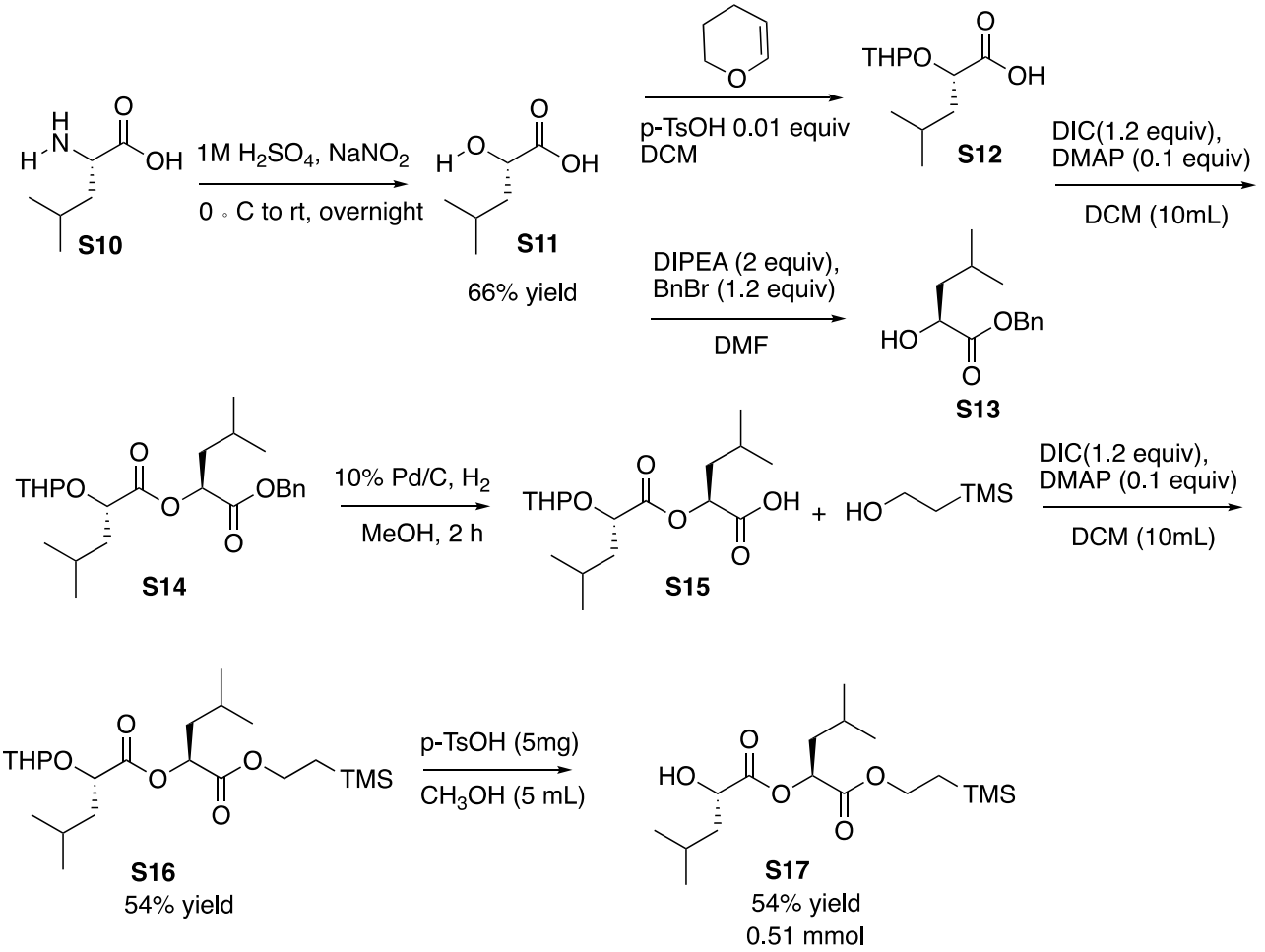
The Alloc protected α-hydroxy acid **S8** was dissolved in DCM (30 mL), Ac_2O (1.1 equiv, 1.17 mL) and DMAP (0.05 equiv, 64 mg) were added. After 7 h, another portion of Ac_2O (1.17 mL) was added to progress the reaction to completion. DCM was removed and the crude was stirred with THF–H_2O (1:1, 20 mL) overnight. THF was removed and the pH was adjusted to basicity with $NaHCO_3$ solution. The aqueous phase was washed with diethyl ether to remove any organic impurities. The aqueous phase was acidified to pH 3 with 1 N HCl, extracted with ethyl acetate, and the organic phase was dried over $MgSO_4$, filtered and concentrated to give **S9** (2.53 g, 46 % yield). ^{1}H-NMR (600 MHz, $CDCl_3$): δ = 5.92 (m, 1H), 5.31 (d, *J* = 17.4 Hz, 1H), 5.21 (d, *J* = 10.3 Hz, 1H), 5.02 (m, 1H), 4.84 (m, 1H), 4.56 (d, *J* = 4.6 Hz, 2H), 3.20 (m, 2H), 2.15 (s, 3H), 1.89 (m, 2H), 1.55 (m, 2H), 1.47 (m, 2H). ^{13}C{^{1}H}-NMR (150 MHz, $CDCl_3$): δ = 174.5, 170.7, 156.4, 132.9, 117.8, 71.7, 65.6, 40.7, 30.5, 29.5, 22.3, 20.6.

Synthesis of S17

L-Leucine (**S10**) (34.65 mmol, 4.54 g) was dissolved in 1 M H_2SO_4 (69 mL) in an ice bath, then a solution of $NaNO_2$ (6 equiv, 14.35 g) in water (42 mL) was added dropwise. The mixture was stirred at 0 °C for 2 h, then r.t. for another 14 h. After extraction with ethyl acetate (50 mL × 3), the combined organic phases were dried with $MgSO_4$. After filtration and concentration, the crude solid product

was recrystallized with petroleum ether–diethyl ether to afford (*S*)-2-hydroxy-4-methylpentanoic acid (**S11**) as a white solid (877 mg, 66 % yield). ^{1}H-NMR (600 MHz, $CDCl_3$): δ = 4.29 (dd, *J* = 9.1, 4.4 Hz, 1H), 1.96-1.88 (m, 1H), 1.68-1.59 (m, 2H), 0.97 (d, *J* = 6.6 Hz, 6H). ^{13}C{^{1}H}-NMR (150 MHz, $CDCl_3$): δ = 180.0, 68.9, 43.2, 24.5, 23.2, 21.4. (NMR is consistent with literature data (H. Guyon, A. Boussonnière, A.-S. Castanet, *J. Org. Chem.* **2017**, *82*, 4949-4957).

(*S*)-2-Hydroxy-4-methylpentanoic acid (9.09 mmol, 1.2 g) was dissolved in DMF (20 mL), followed by addition of DIPEA (2 equiv, 3.18 mL) and benzyl bromide (1.2 equiv, 1.30 mL) at 0 °C. The mixture was stirred overnight. After removing solvent, the crude residue was diluted with ethyl acetate (50 mL), washed with 10% citric acid solution and saturated brine solution. The organic phase was dried over $MgSO_4$ before removing the solvent under vacuum. The benzyl protected product **S13** was obtained as a colourless oil (1.85 g, 8.33 mmol, 91 % yield). ^{1}H-NMR (600 MHz, $CDCl_3$): δ = 7.40-7.34 (m, 5H), 5.21 (d, *J* = 12.1 Hz, 2H), 4.26-4.2 (ddd, *J* = 8.8, 5.8, 4.7 Hz, 1H), 1.93-1.84 (m, 1H), 1.62-1.54 (m, 2H), 0.94 (d, J = 6.6 Hz, 3H), 0.93 (d, J = 6.6 Hz, 3H). ^{13}C{^{1}H}-NMR (150 MHz, $CDCl_3$): δ = 175.8, 135.2, 128.7, 128.6, 128.3, 69.2, 67.3, 43.4, 24.4, 23.3, 21.6.



S12 was prepared based on a modified method (O. Kuisle, E. Quinoa, R. Riguera, *J. Org. Chem.* **1999**, *64*, 8063-8075). To a stirred suspension of (*S*)-2-hydroxy-4-methylpentanoic acid **S11** (10

mmol, 1.32 g) and *p*-TsOH (0.02 equiv, 38 mg) in chloroform (20 mL) at 0 ºC was added dropwise dihydropyran (1.75 equiv, 1.28 mL). After 5 min, the ice bath was removed. The reaction mixture was stirred at room temperature for another 2.5 h, then extracted with 0.2 N KOH (20 mL × 2). The combined aqueous phases were acidified with 1 M HCl to pH 3-4 and extracted with DCM (40 mL × 2). The combined DCM phases were washed with saturated NaCl solution, filtered, and concentrated *in vacuo*. Flash column chromatography gave the protected compound (**S12**) as a colourless solid (1.62g, 75 % yield). ^{1}H-NMR (600 MHz, $CDCl_3$): δ = 4.56 (dd, *J* = 6.6 Hz, 2.6 Hz, 1H), 4.10 (dd, *J* = 9.2 Hz, 4.3 Hz, 1H), 4.01 (m, 1H), 3.51 (m, 1H), 1.86 (m, 1H), 1.83-1.75 (m, 2H), 1.72-1.66 (m, 2H), 1.62-1.57 (m, 1H), 1.57-1.52 (m, 2H), 0.94 (d, *J* = 6.6 Hz, 3H), 0.92 (d, *J* = 6.6 Hz, 3H). ^{13}C{^{1}H}-NMR (150 MHz, $CDCl_3$): δ = 175.6, 102.4, 77.5, 64.8, 41.3, 30.8, 24.9, 24.4, 23.2, 21.7, 20.5.

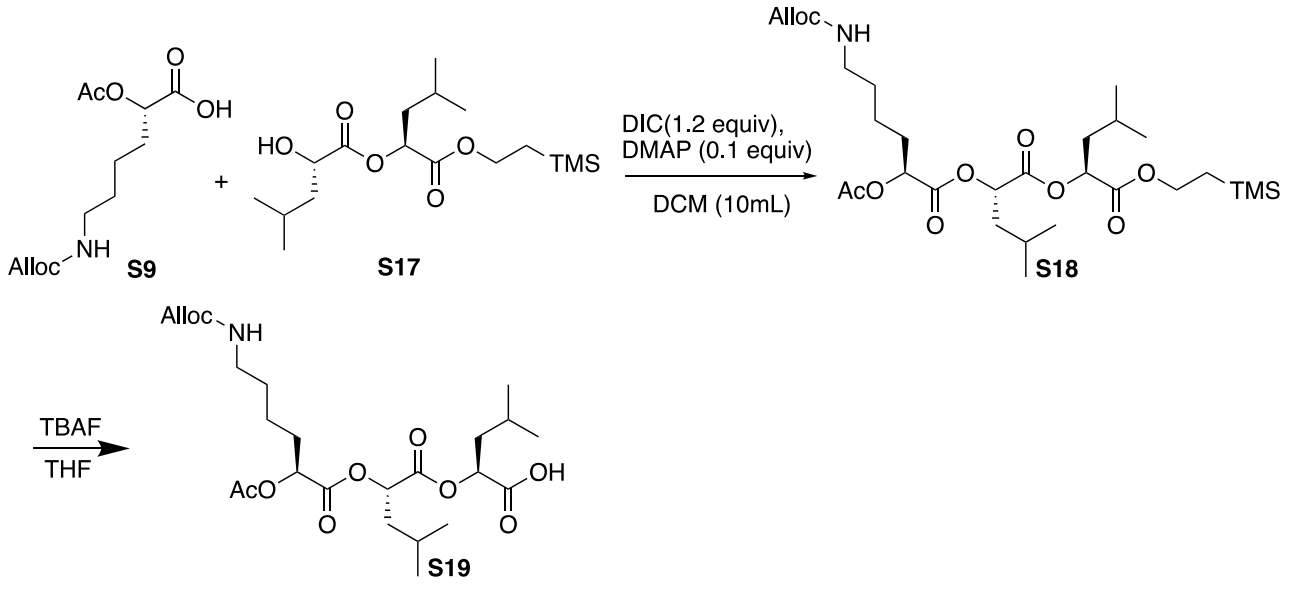
The solution of the **S12** (7.49 mmol, 1.62 g) in anhydrous DCM (30 mL) and DIC (1.5 equiv, 1.76 mL) was stirred and 46 mg of DMAP (0.05 equiv) was added. After 10 min, the hydroxyl partner **S13** (1.1 equiv, 8.33 mmol) was added in small portions. The mixture was stirred at r.t. for 24 h, then concentrated *in vacuo*. The crude product was purified by flash column chromatography (petroleum ether–ethyl acetate 9:1) to afford **S14** (1.35g, 43 % yield), with recovery of unreacted hydroxyl partner **S13** (0.9 g). ^{1}H-NMR (600 MHz, $CDCl_3$): δ = 7.38-7.31 (m, 5H), 5.17-5.13 (m, 3H), 4.67 (t, *J* = 3.4 Hz, 1H), 4.43 (dd, *J* = 9.8 Hz, 4.0 Hz, 1H), 3.83 (m, 1H), 3.50 (m, 1H), 1.92 (m, 1H), 1.85-1.79 (m, 2H), 1.75-1.65 (m, 4H), 1.58-1.50 (m, 4H), 0.94-0.91 (m, 12H). (^{1}H for major isomer, dr 5:1). ^{13}C{^{1}H}-NMR (150 MHz, $CDCl_3$): δ = 173.2, 170.2, 128.6, 128.4, 128.3, 100.4, 97.2, 72.5, 71.1, 67.1, 62.3, 41.6, 39.6, 30.3, 25.4, 24.6, 24.5, 23.3, 23.0, 21.5, 21.4, 19.1.

S14 (1 mmol, S6 mg) was dissolved in CH_3OH (10 mL), the benzyl group was removed under 1 atm H_2 in the presence of 42 mg of 10 % Pd/C for 2 h. After filtration through a celite pad, the solvent was removed. The crude product **S15** was used directly without further purification. To a stirred solution of **S15** in 8 mL of DCM, DIC (1.2 equiv, 188 μL) and DMAP (0.1 equiv, 12.2 mg) was added. After adding TMS-ethanol (1.5 equiv, 215 μL), the mixture was stirred at room temperature overnight. After removal of DCM, the reaction mixture was purified by flash column chromatography to give **S16** (228 mg, 53 % yield). Finally, the THP protecting group was deprotected in the presence of 5 mg of p-TsOH in 5 mL CH_3OH for 4 h. After removal of solvent, the concentrated reaction mixture was purified by flash column chromatography over a gradient of 0-10 % EA in PE to provide product **S17** (175 mg, 94 % yield). ^{1}H-NMR (600 MHz, CDCl3): δ = 5.10 (dd, *J* = 9.66 Hz, 3.76 Hz, 1H), 4.26 (m, 1H), 4.22 (m, 2H), 4.57 (d, *J* = 6.50 Hz, 1H), 1.96 (m, 1H), 1.81 (m, 1H), 1.69 (m, 1H), 1.68 (m, 1H), 1.61 (m, 1H), 1.01 (m, 1H), 0.98 (d, *J* = 6.59 Hz, 6H), 0.96 (d, *J* = 6.59 Hz, 3H), 0.93

(d, *J* = 6.59 Hz, 3H). $^{13}C\{1H\}$-NMR (151 MHz, $CDCl_3$): δ = 177.1, 171.8, 73.5, 70.6, 65.5, 45.1, 41.2, 26.2, 26.0, 24.9, 24.6, 23.0, 22.9(6), 18.9.

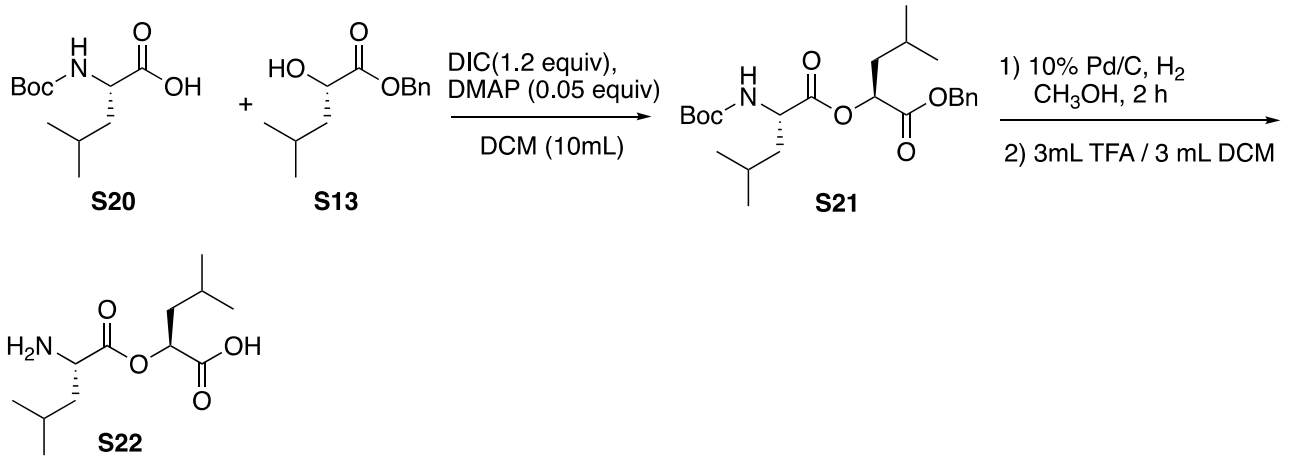
Synthesis of S19 (Building block for **3d**)

The carboxylic acid **S9** (0.64 mmol, 175 mg) and the alcohol **S17** (0.51 mmol, 1 equiv, 175 mg) were coupled in the presence of DIC (1.2 equiv, 120 µL) and DMAP (0.1 equiv, 7.8 mg) in 8 mL of DCM at r.t. overnight. The concentrated crude mixture was purified by flash column chromatography (PE:EA = 4:1) to provide 216 mg of product **S18** (71 % yield). ^{1}H-NMR (600 MHz, $CDCl_3$): δ = 5.89 (m, 1H), 5.27 (dd, *J* = 17.2 Hz, 1.2 Hz, 1H), 5.17 (d, *J* = 10.5 Hz, 1H), 5.10 (dd, *J* = 9.6 Hz, 4.1 Hz, 1H), 5.06 (dd, *J* = 9.4 Hz, 3.8 Hz, 1H), 4.99 (t, 6.1 Hz, 1H), 4.52 (d, *J* = 5.4 Hz, 2H), 4.18 (m, 2H), 3.17 (m, 2H), 2.10 (s, 3H), 1.93-1.88 (m, 2H), 1.87-1.74 (m, 6H), 1.64 (m, 1H), 1.54 (m, 1H), 1.50-1.48 (m, 3H), 0.98 (m, 2H), 0.96 (d, *J* = 6.41 Hz, 3H), 0.94 (d, *J* = 6.41 Hz, 3H), 0.93 (d, *J* = 6.41 Hz, 3H), 0.90 (d, *J* = 6.41 Hz, 3H), 0.01 (s, 9H). $^{13}C\{^{1}H\}$-NMR (151 MHz, $CDCl_3$): δ = 170.5, 170.2, 170.0, 169.8, 156.3, 133.1, 117.5, 71.8, 71.7, 71.3, 65.4, 63.9, 40.5, 39.6, 39.4, 30.4, 29.3, 24.6, 23.0, 21.8, 21.5, 21.4, 20.6, 17.3, -1.6.



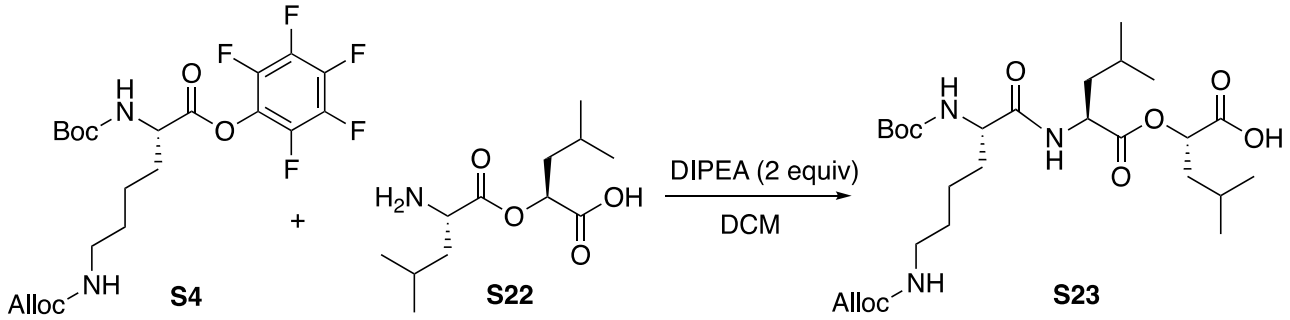
S18 was dissolved in 5 mL of THF, TBAF (1.0 M in THF, 0.36 mL) was added slowly. The reaction progress was monitored by TLC. After 4 h, THF was removed in vacuo. The residue was dissolved in 30 mL of ethyl acetate, acidified by 10 % citric acid solution to pH 3, followed by extraction with EA twice. The combined organic phases were washed with saturated NaCl solution, dried over $MgSO_4$, concentrated *in vacuo* to give the deprotected product **S19**, which was used directly on solid phase to access peptide **3d**.

Synthesis of S23 (Building block for **3c**)



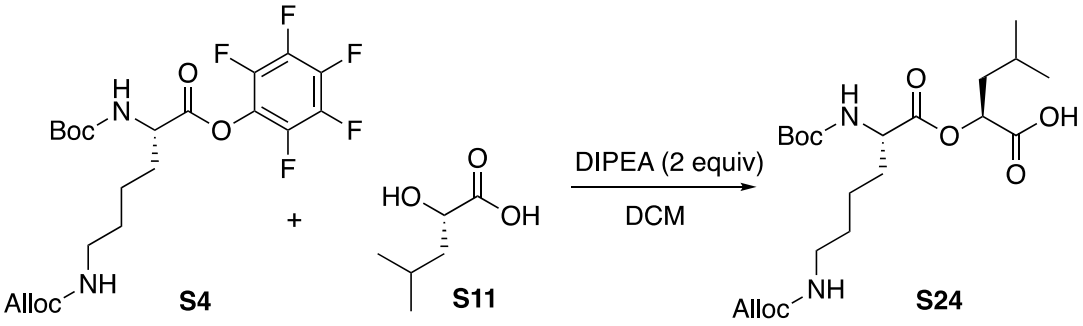
N^{α}-Boc-L-Leu-OH (**S20**) (2mmol, 499 mg) was dissolved in 10 mL of DCM, DIC (2 equiv, 313 µL) and DMAP (0.05 equiv, 12 mg) was added sequentially. After five minutes, the hydroxyl partner **S13** (1.73 mmol, 384 mg) was added, the reaction was stirred at room temperature for 4 h. The reaction mixture was filtered. The filtrate was concentrated and the residue purified by flash column chromatography (PE:EA = 10:1) to give the isolated product **S21** (644 mg, 74 % yield). ^{1}H-NMR (600 MHz, CDCl3): δ = 7.37-7.31 (m, 5H), 5.17 (d, *J* = 12.2 Hz, 1H), 5.12 (d, *J* = 12.4 Hz, 1H), 4.85 (d, *J* = 8.7 Hz, 1H), 4.35 (dt, *J* = 9.3, 4.8 Hz, 1H), 1.84-1.78 (m, 1H), 1.78-1.71 (m, 2H), 1.70-1.62 (m, 2H), 1.43 (s, 9H), 1.42-1.37 (m, 1H). ^{13}C{^{1}H}-NMR (150 MHz, $CDCl_3$): δ = 173.3, 170.2, 155.4, 135.2, 128.6, 128.5, 128.3, 79.8, 71.6, 67.1, 51.8, 41.5, 39.6, 28.3, 24.7, 24.5, 22.98, 22.93, 21.6(2), 21.6(0).

S21 was dissolved in 10 mL of CH_3OH, 10 % Pd/C (64 mg) was added. The reaction mixture was stirred under 1 atm H_2 atmosphere. After completion (4 h), the reaction mixture was filtered and the filtrate was concentrated under vacuum. The residue was dissolved in TFA (8 mL) to deprotect the Boc group over 10 mins. After removal of TFA by bubbling N_2 gas through the mixture, the product was dissolved in 50% AcCN in H_2O (10 mL), followed by freeze-drying to give **S22**, which was used without further purification.



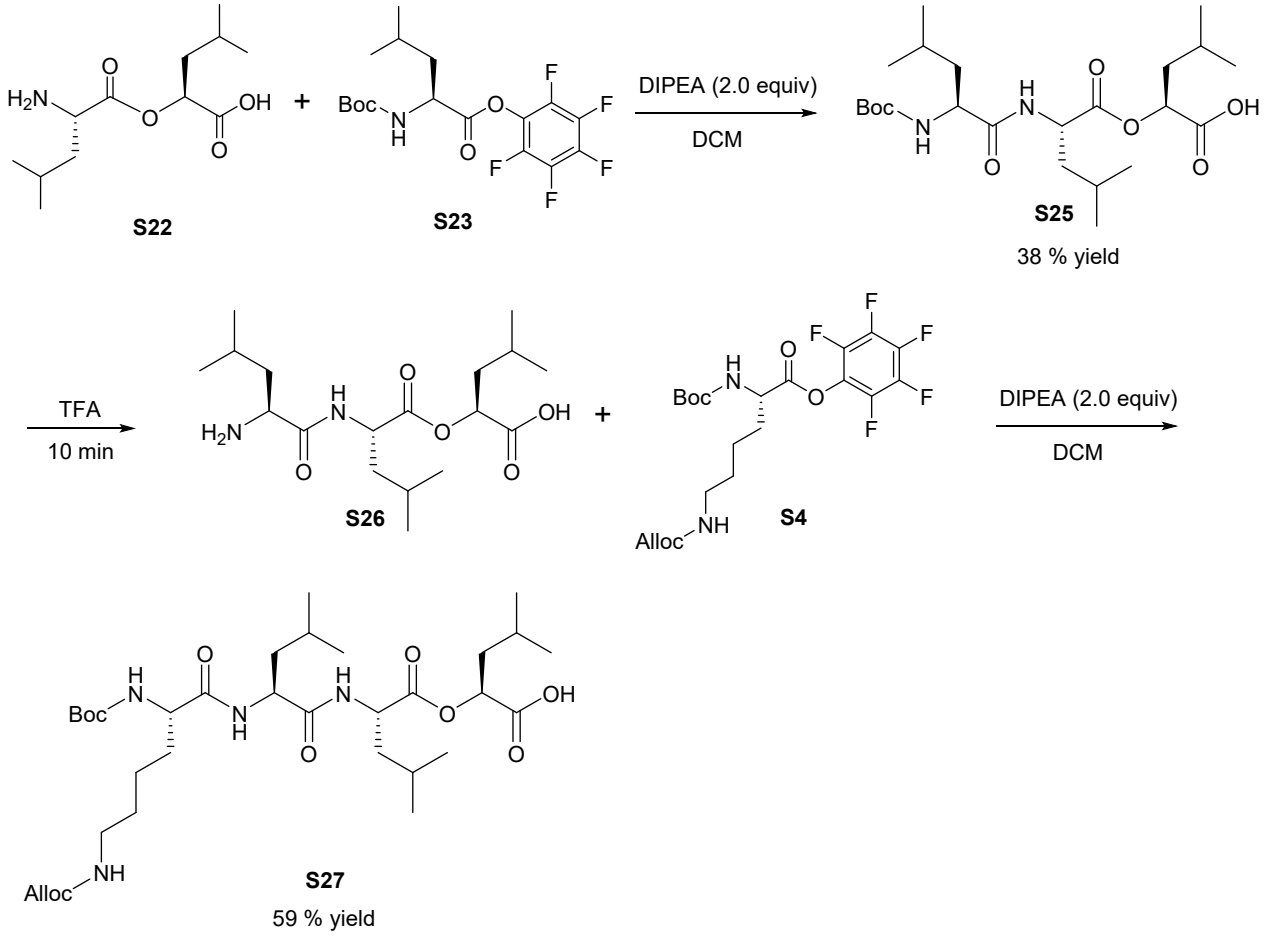
To a stirred suspension of **S4** (0.5 mmol, 252 mg) in 5 mL of DCM, **S22** (0.42 mmol, 102 mg) was added. After 3 hs, the reaction mixture was concentrated and purified by flash column chromatography (PE:EA:AcOH = 100:100:1) to give **S23**. ESI-MS: *m/z* 558.8 [M+H$^+$].

Synthesis of S24 (Building block for **3b**)



To a stirred suspension of **S4** (0.47 mmol, 236 mg) in 5 mL of DCM, **S11** (0.47 mmol, 62 mg) and DIPEA (1 equiv, 87 μL) were added. The mixture was stirred overnight. **S11** (20 mg) was added and the mixture was stirred for another 3 h. Concentration followed by purification using flash column chromatography (PE:EA:AcOH = 100:100:1) to give **S24** (60 mg, 28.7% yield), with some recovered **S4**. ESI-MS: *m/z* 445.3 [M+H$^+$]. (Due to the low yield, **S24** was directly used for SPPS to assemble the compound **3b**, no epimerized peptide compound was found).

Synthesis of S27 (Building block for **3e**)



To a stirred suspension of **S23** (1.0 mmol, 397 mg) in 5 mL of DCM, **S22** (1.1 mmol, 270 mg) and DIPEA (1.1 equiv, 192 μL) were added. The mixture was stirred at room temperature for 2 h. The solvent was removed under vacuum and the resulting product was purified by flash column chromatography (PE:EA = 1:1 to DCM:MeOH:AcOH = 90:10:0.1) to give **S25**. The compound was treated with TFA for 10 min. TFA, water and CH_3CN were removed under a nitrogen flow followed by lyophilisation. Rp-HPLC was then used to purify **S26** to a white solid (32 % yield).

To a stirred suspension of **S26** (0.12 mmol, 45 mg) in 5 mL of DCM, **S4** (1.2 equiv, 75 mg) and DIPEA (2.0 equiv, 45 μL) were added. The mixture was stirred at room temperature for 4 h. The solvent was removed under vacuum and the resulting residue was purified by Rp-HPLC to afford **S27** as a white solid after lyophilization (59 % yield). The compound was subjected solid phase synthesis to assemble **3e**, which was fully characterized by HRMS and NMR (see ahead).

2.2 Cyclization

Formation of lactam bridge constraints through cyclization was conducted on resin for peptides **2a-e** and **3a-e**. Side chain protecting groups of aspartic acid and lysine were first removed. Phenylisopropyl (OPip) esters and methyl trityl (Mtt) protecting groups were removed by allowing 5 × 10 mL portions of 3% TFA in DCM to drip through the resin. The resin was then washed with 2 × 10 mL portions of DCM, 2 × 10 mL portions of DMF and 2 × 10 mL portions of 5% DIPEA in DMF. Allyl esters were removed by treating the peptide resin with $Pd(PPh_3)_4$ (5 mol%) N,N-dimethylbarbituric acid (5eq) in DCM under argon for 2h, repeating the procedure a second time. Cyclization was achieved on peptide-resin with BOP or PyBOP (2 eq), DIPEA (2 eq), HOAt (2eq) in DMF overnight at room temperature with periodic nitrogen bubbling through the mix.

2.3 Cleavage

Peptides were cleaved from the resin using TFA/TIPS/H_2O (95/2.5/2.5) for 2 h. Solutions were filtered from the resin and the cleavage mixture removed *in vacuo*. The resulting residues were treated with ice-cold diethyl ether to precipitate the peptides. Ether was removed by decantation. The crude peptides were dried under nitrogen, dissolved in acetonitrile-water (1:1) and lyophilised. Crude peptides were purified by reversed-phase HPLC on a Waters 486 system equipped with a Rheodyne semi-preparative injector with a 5 mL loop volume on a Phenomenex Luna C18 15 μm column, 250 mm x 22 mm, at 20 mL/min using linear gradient elution (solvent A is water and 0.1% TFA; solvent B is 90% MeCN, 10% water, and 0.1% TFA) and UV detection at 214 nm.

2.4 HPLC methods

Analytical HPLC was performed using an Agilent 1200 Series instrument with a diode-array detector on a Phenomenex Luna 5 μm, C18 250 mm× 4.60 mm column. Methods include the following gradients: A (0-40% B in 20 min), B (20-80% B in 20 min), C (30-70% B in 20 min), D (0-60% B in 20 min), E (20-100% B in 20 min), F (0-100% B in 30 min). Some samples were also examined using a Shimadzu UFLC system, using an Eclipse Plus C18 1.8 um (2.1 x 100 mm) column with an eluting flow rate of 0.5 mL/min and gradient 0 to 100% % buffer B (90% CH3CN/10% H2O/0.1% TFA in buffer A, 0.1% TFA in water) over 20 minutes (method G). The solvent gradient was A (0.1% TFA in MilliQ water) and B (CH_3CN/MilliQ H_2O = 90:10, 0.1% TFA).

3. CD spectroscopy

CD measurements were performed using a Jasco model J-710 spectropolarimeter, which was routinely calibrated with (1*S*)-(+)-10-camphorsulfonic acid. A stock solution of 1–5 mg of peptide was dissolved in 600 μL of 18 MΩ deionised water, 60 μL D_2O and 10 μL of 50 mM DSS were added as an internal standard. Accurate concentrations of these solutions were then determined using the PULCON method (Dreier, L. and G. Wider, *Concentration measurements by PULCON using X-filtered or 2D NMR spectra.* Magn Reson Chem, **2006**. 44 Spec No: p. S206-12). 90° pulses were accurately determined and then 1D Spectra were acquired using the standard watergate sequence with a ns= 16, d1= 30s to ensure complete relaxation of proton signals. Integration of well resolved signals compared to the internal DSS standard were used to determine concentration of peptide solutions using the following equation:

$$[Peptide] = [DSS] \times \left(\frac{Integral_{Peptide} \times \#H_{DSS}}{Integral_{DSS} \times \#H_{peptide}} \right) \qquad (Eq.\ 1)$$

where [*Peptide*] is the peptide concentration, [*DSS*] is the concentration of DSS in the NMR tube (746 μM). *#H* is the number of protons corresponding to the *Integral* (in absolute units) for the peptide signal or DSS signal.

An appropriate amount of the NMR stock solutions was then used to prepare the CD solution making up the difference with 10 mM Phosphate Buffer (pH 7.4) or TFE. Spectra were recorded at room temperature (298K), with a 0.1 cm Jasco quartz cell over the wavelength range 260-185 nm at 50 nm/min, with a bandwidth of 1.0 nm, response time of 1 s, resolution step width of 1 nm and sensitivity of 20-50 Mdeg. Each spectrum represents the average of 5 scans. Spectra were analysed using the spectral analysis software and smoothed using the 'adaptive smoothing' function.

4. NMR spectroscopy

1D and 2D ^{1}H-NMR spectra were recorded on a Bruker Avance III DRX-600 spectrometer with cryo-probe. 2D ^{1}H-spectra were recorded in phase-sensitive mode using time-proportional phase incrementation for quadrature detection in the *t*1 dimension. The 2D experiments included TOCSY (standard Bruker mlevgpph pulse program), ROESY (standard Bruker roesygpph pulse program), NOESY and dqfCOSY (standard Bruker dqfcosygpph pulse program). TOCSY spectra were acquired over 6887 Hz with 2048 complex data points in *F2*, 256 increments in *F1* and 8 scans per increment. ROESY and NOESY spectra were acquired over 6887 Hz with 4096 complex data points in *F2*, 512 increments in *F1* and 32 scans per increment. TOCSY, ROESY and NOESY spectra were acquired with several isotropic mixing times of 80 ms for TOCSY, 300 ms for ROESY. For all NMR experiments, water suppression was achieved using modified WATERGATE. For 1D ^{1}H NMR spectra acquired in H_2O/D_2O (9:1), the water resonance was suppressed by low power irradiation during the relaxation delay (1.5 to 3.0 s). The variable temperature NMR experiments were performed in 10^{o}C increments over the range of 278-318K. Spectra were processed using Topspin (Bruker, Germany). The *t*1 dimensions of all 2D spectra were zero-filled to 1024 real data points with 90˚ phase-shifted QSINE bell window functions applied in both dimensions followed by Fourier transformation and fifth order polynomial baseline correction. ^{1}H chemical shifts were referenced to DSS (δ 0.00 ppm) in water. $^3J_{NHCH\alpha}$ coupling constants were measured from 1D ^{1}H NMR and dqf-COSY spectra.

5. Structure calculations

Distance restraints used in calculating the structures for **3a-c** in H_2O/D_2O (9:1) and **3d** in CD_3CN/H_2O (7:3) were derived from ROESY spectra (recorded 293-298K) using mixing time of 300ms. ROE cross-peak volumes obtained manually by counting number of contours which were classified manually as strong (upper distance constraint ≤ 2.7Å), medium (≤ 3.5Å), weak (≤ 5.0Å) and very weak (≤ 6.0 Å). Standard pseudoatom distance corrections were applied for non-stereospecifically assigned protons (K. Wüthrich, M. Billeter, W. Braun, *J. of Mol. Biol.* **1983**, *169*, 949-961). To address the possibility of conformational averaging, intensities were classified conservatively and only upper distance limits were included in the calculations to allow the largest possible number of conformers to fit the experimental data. Backbone dihedral angle restraints were inferred from $^3J_{NHCH\alpha}$ coupling constants in 1D spectra, ϕ was restrained to –65 ± 30° for $^3J_{NHCH\alpha}$ ≤ 6Hz and to -120 ± 30° for $^3J_{NHCH\alpha}$ ≥ 8Hz. There was clearly no evidence at all for *cis*-amides about peptide bonds (i.e. no CHα-CHα (*i*, *i+1*) ROEs) in the ROESY spectra, so all ψ-angles were set to *trans* (ψ = 180˚). Starting structures with randomised ϕ and ψ angles and extended side chains were generated using

an *ab initio* simulated annealing protocol. The calculations were performed using the standard forcefield parameter set (PARALLHDG5.2.PRO) and topology file (TOPALLHDG5.2.PRO) in XPLOR-NIH with in-house modifications to generated amide bond between Lys and Asp side chains, N-CH_3 and ester bonds. Refinement of structures was achieved using the conjugate gradient Powell algorithm with 4000 cycles of energy minimisation and a refined forcefield based on the program CHARMm. Structures were visualised with Pymol and analysed for distance (> 0.2 Å) and dihedral angle (>5°) violations using noe.inp files. Final structures contained no distance violations (> 0.2 Å) or angle violations (> 5°).

6. Computational modelling

The putative three-dimensional α-helical structures for peptides **2a-c** were modelled using 3D Builder module from Maestro/Schrodinger package (version 2020-3) under backbone Phi and Psi dihedral angles constraints of -58 and -48 degrees, respectively. Energy minimisation was applied under OPLS-3e force field to produce the final structures for **2a**-**c**, van der Waals radii were generated and displayed by Pymol software (version 2.5.4).

The solvation energy calculation was performed on the Current Energy module of MacroModel program in the Schrodinger 2020-3 suite using default settings. All calculations were computed under OPLS4 forcefield with water as the solvent at a temperature of 300K.

7. Flow cytometry

HeLa cervical adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC). HeLa cells were seeded into 12-well plates at a density of 3×10^5 cells/mL and incubated overnight at 37°C. The following day, cells were washed once with phosphate buffered saline (PBS), then 10 μM of peptide diluted in serum-free media was added and incubated at 37 °C for 1 h. After incubation, the cells were washed with PBS twice and dissociated with 0.25 % trypsin EDTA for 10 min on ice. Dissociated cells were collected in cold PBS supplemented with 1% serum and centrifuged at 500 g for 5 min at 4 °C. Cells were resuspended in cold PBS with 5 μL trypan blue (5 μg/mL), and 5 μL 7-AAD were added prior to flow cytometry. Fluorescence was measured using a Gallios Flow Cytometer (Beckman Coulter). Data were analysed using FlowJo software and presented as mean fluorescence intensity (± SEM) of at least three independent repeats.

8. Characterization of peptides by proton NMR spectroscopy

^{1}H NMR resonance assignments and chemical shifts (δ ppm) for **2a** in H_2O/D_2O (9:1) at 298K.

Residue	NH or NMe	H α	H β	Other
Ac-(NMe-K1)	2.96	4.62	1.90, 1.63	Ac 2.07; γa 1.36, γb 1.30; δ1a 1.59, δ1b 1.40; ε1a 3.30, ε1b 2.91;
L2	8.09	4.12		1.58-1.46 (2βH + 1γH)
L3	7.74	4.26		1.60-1.49 (2βH + 1γH)
L4	7.88	4.09		1.58-1.46 (2βH + 1γH)
D5-NH_2	8.13	4.61	2.73, 2.59	T1 7.08, T2 7.26

Amide NH coupling constants (Hz) and Δδ/T (ppb/deg) for **2a** in H_2O:D_2O (9:1) at 298K.

Residue	K1-NMe	L2	L3	L4	D5	NT1	NT2	NH-side
$^3J_{NH - CH\alpha}$ (Hz)	N/A	6.1	7.7	6.2	7.9	N/A	N/A	N/A

^{1}H NMR resonance assignments and chemical shifts (δ ppm) for **2a** in DMSO-d_6 at 298K.

Residue	NH or NMe	H α	H β	Other
Ac-(NMe-K1)	2.94	4.89	1 βH 2.03	Ac 2.01; γa 1.35, γb 1.20; 2δH + 1 βH 1.60-1.40; ε1a 3.25, ε1b 2.94;
L2	8.21	4.14		1.60-1.42 (2βH + 1γH)
L3	7.23	4.31		1.57-1.37 (2βH + 1γH)
L4	8.12	3.93		1.69-1.40 (2βH + 1γH)
D5-NH_2	7.85	4.34	2.44, 2.37	T1 7.19, T2 7.02

Amide NH coupling constants (Hz) and Δδ/T (ppb/deg) for **2a** in DMSO-d_6 at 298K.

Residue	K1-NMe	L2	L3	L4	D5	NT1	NT2	NH-side
$^3J_{NH - CH\alpha}$ (Hz)	N/A	8.6	8.3	4.7	8.1	N/A	N/A	N/A

^{1}H NMR resonance assignments and chemical shifts (δ ppm) for **2b** (*trans*) in D_2O/H_2O (9:1) at 298K.

Residue	NH or NMe	H α	H β	Other
Ac-K1	8.21	4.58	1.78, 1.60	Ac 1.91; γ1a 1.42, γ1b 1.31; δ1a 1.56, δ1b 1.43; ε1a 3.23, ε1b 2.99;
L2-NMe	2.97	5.04	1.75, 1.58	γ2 1.38; δ2a 0.86, δ2b 0.75;
L3	7.33	4.22		δ 3a 0.83, δ 3b 0.81; 1.60-1.48 (2βH and 1γH)
L4	7.99	4.23		δ 4a 0.79, δ 4b 0.84; 1.55-1.46 (2βH and 1γH)
D5-NH_2	8.28	4.60	2.68, 2.54	T1 7.05, T2 7.40

Amide NH coupling constants and Δδ/T for **2b** (trans) in H_2O/D_2O (9:1) at 298K.

Residue	K1	NMe-L2	L3	L4	D5	NT1	NT2	NH-side
$^{3}J_{NH\text{-}CH\alpha}$ (Hz)	6.2	N/A	6.5	7.0	8.1	N/A	N/A	N/A

^{1}H NMR resonance assignments and chemical shifts (δ ppm) for **2b** (*trans*) in DMSO-d_6 at 298K.

Residue	NH or NMe	H α	H β	Other
Ac-K1	8.16	4.66	1.81, 1.47	Ac 1.82; γ1a 1.37, γ1b 1.27; δ1a 1.46, δ1b 1.31; ε1 3.03 (2H);
L2-NMe	2.90	5.09	1.64-157 (2H)	γ2 1.40; δ2a 0.89, δ2b 0.81;
L3	6.91	4.23		δ 3a 0.88, δ 3b 0.86; 1.52-1.45(2βH and 1γH)
L4	8.05	4.22		δ 4a 0.90, δ 4b 0.86; 1.60 β4a, γ4 and β4b (1.50-1.44)
D5-NH_2	7.92	4.43	2.42-2.35 (2H)	T1 7.02, T2 7.23

Amide NH coupling constants and Δδ/T for **2b** (*trans*) in DMSO-d_6 at 298K.

Residue	K1	NMe-L2	L3	L4	D5	NT1	NT2	NH-side
$^{3}J_{NH\text{ - }CH\alpha}$ (Hz)	7.6	N/A	7.6	7.4	8.4	N/A	N/A	N/A

^{1}H NMR resonance assignments and chemical shifts (δ ppm) for **2c** (*cis*) in D_2O/H_2O (9:1) at 298K. (*trans*/*cis* = 1/2)

Residue	NH or NMe	H α	H β	Other
Ac-K1	8.32	4.29	1.70, 1.58	Ac 1.96; γ 1.26 (2H); δ1a 1.43, δ1b 1.40; ε1a 3.37, ε1b 2.86;
L2	7.95	4.87	1.68, 1.36	γ2 1.62; δ2 0.85 (6H);
L3-NMe	2.63	4.92	1.87, 1.51	δ 0.89(6H); γH 1.44;
L4	8.73	4.17	1.64, 1.49	δ 0.79 (6H); γH 1.43;
D5-NH_2	8.35	4.79	2.77, 2.60	T1 7.08, T2 7.23

Amide NH coupling constants and Δδ/T for **2c** (*cis*) in H_2O/D_2O (9:1) at 298K.

Residue	K1	L2	NMe-L3	L4	D5	NT1	NT2	NH-side
$^{3}J_{NH - CH\alpha}$ (Hz)	7.7	6.2	N/A	6.6	8.6	N/A	N/A	N/A

^{1}H NMR resonance assignments and chemical shifts (δ ppm) for **2c** (*cis*) in DMSO-d_6 at 298K. (*trans*/*cis* = 1/5)

Residue	NH or NMe	H α	H β	Other
Ac-K1	8.04	4.07	1.49 (2H)	Ac 1.84; γ 1.21 (2H) δ1a 1.32, δ1b 1.29; ε1a 3.23, ε1b 2.86;
L2	8.17	4.69	1.55, 1.44	γ2 1.54; δ2 0.86 (6H);
L3-NMe	2.59	4.56	1.40 (2βH + 1γH)	δ3a 0.96, δ3b 0.92
L4	7.81	4.22	1.57-1.47 (2βH + 1γH)	δ4a 0.87, δ4b 0.85
D5-NH_2	7.87	4.55	2.44 (2H)	T1, T2 7.10 (overlap)

Amide NH coupling constants and Δδ/T for **2c** (*cis*) in DMSO-d_6 at 298K.

Residue	K1	L2	NMe-L3	L4	D5	NT1	NT2	NH-side
$^{3}J_{NH - CH\alpha}$ (Hz)	7.5	8.4	N/A	7.3	8.5	N/A	N/A	N/A

Amide NH coupling constants for (**2c**) in in H_2O/D_2O (9:1) at 298K.

Residue	K1	L2	NMe-L3	L4	D5	NT1	NT2	NH-side
$^{3}J_{NH-CH\alpha}$ (Hz)/*cis*	7.72	6.20	N/A	6.56	8.58	N/A	N/A	N/A
$^{3}J_{NH-CH\alpha}$ (Hz)/*trans*	6.13	7.34	N/A	7.30	7.94	N/A	N/A	N/A

[a] N/A indicates not applicable.

^{1}H NMR resonance assignments and chemical shifts (δ ppm) for **3a** in H_2O/D_2O (9:1) at 298K.

Residue	**NH**	**H α**	**H β**	**Other**
Ac-oK1	N/A	4.75	1.87, 1.80	Ac 2.11; Y1a 1.45, Y1b 1.22; δ1a 1.50, δ1b 1.38; ε1a 3.46, ε1b 2.66; side NH 7.93;
Leu2	8.47	4.03	1.61, 1.50	δ2a 0.86, δ2b 0.81; γ 1.59;
Leu 3	7.58	4.12	1.49-1.59 (2βH, 1γH)	δ3a 0.84, δ3b 0.80;
Leu 4	7.47	4.08	1.57, 1.48	δ4a 0.81, δ4b 0.79; γ1.59;
D5-NH_2	7.99	4.58	2.81, 2.58	T1 7.15, T2 7.11

Amide NH coupling constants and Δδ/T for **3a**.

Residue	oK1	L2	L3	L4	D5	NT1	NT2	NH-side
$^{3}J_{NH\text{ - }CH\alpha}$ (Hz)	N/A	4.4	6.0	6.2	7.1	N/A	N/A	N/A
Δδ/K (-ppb)	N/A	5.59	7.3	0.98	5.13	0	7.12	9.23

^{1}H NMR resonance assignments and chemical shifts (δ ppm) for **3b** in H_2O/D_2O (9:1) at 298K.

Residue	**NH**	**H α**	**H β**	**Other**
Ac-K1	8.46	4.17	1.80 (2H)	Ac 1.98; Y1a 1.48, Y1b 1.16; δ1a 1.49, δ1b 1.38; ε1a 3.50, ε1b 2.63; side NH 8.01;
o-Leu2	N/A	4.76	1.78, 1.54	δ2a 0.86, δ2b 0.81; γ 1.67;
Leu 3	7.68	4.18	1.53-1.62 (2βH, 1γH)	δ3a 0.85, δ3b 0.80;
Leu 4	7.64	4.04	1.47-1.57 (2βH, 1γH)	0.78-0.83 (6 δH)
D5-NH_2	7.56	4.58	2.85, 2.54	T1 7.18, T2 7.03

Amide NH coupling constants and Δδ/T for **3b**.

Residue	K1	oL2	L3	L4	D5	NT1	NT2	NH-side
$^{3}J_{NH\text{-}CH\alpha}$ (Hz)	3.4	N/A	6.5	5.1	7.0	N/A	N/A	N/A
Δδ/K (-ppb)	6.43	N/A	7.40	2.80	1.21	0.45	7.71	9.07

^{1}H NMR resonance assignments and chemical shifts (δ ppm) for **3c** in H_2O/D_2O (9:1) at 298K.

Residue	**NH**	**H α**	**H β**	**Other**
Ac-K1	8.06	4.05	1.74, 1.67	Ac 1.94; γ 1a 1.43, γ 1b 1.22; δ1a 1.50, δ1b 1.35; ε1a 3.42, ε1b 2.70; side NH 7.92;
Leu2	8.33	4.20	1.61, 1.56	δ2a 0.86, δ2b 0.81; γ 1.58;
o-Leu 3	N/A	4.81	1.77, 1.51	0.80-0.83 (2 δH), γH 1.64;
Leu 4	7.98	4.09	1.61, 1.54	δ4a 0.81, δ4b 0.79; γ1.64;
D5-NH_2	8.15	4.54	2.79, 2.56	T1 7.16, T2 7.00

Amide NH coupling constants and Δδ/T for **3c**.

Residue	K1	L2	oL3	L4	D5	NT1	NT2	NH-side
$^{3}J_{NH - CH\alpha}$ (Hz)	4.8	5.2	N/A	5.8	6.8	N/A	N/A	N/A
Δδ/K (-ppb)	6.71	5.79	N/A	5.00	5.36	0	7.36	9.2

^{1}H NMR resonance assignments and chemical shifts (δ ppm) for **3d** in CD_3CN/H_2O (7:3) at 293K.

Residue	**NH**	**H α**	**H β**	**Other**
Ac-oK1	N/A	4.84	1.93, 1.84	Ac 2.10; γ 1a 1.50, γ 1b 1.25; δ1a 1.50, δ1b 1.41; ε1a 3.49, ε1b 2.68; side NH 7.44;
oL2	N/A	4.96	1.79, 1.67	δ2a 0.94, δ2b 0.91; γ 0.94;
oL3	N/A	4.88	1.80, 1.56	δ3a 0.92, δ3b 0.86;
Leu 4	7.31	4.10	1.52-1.56 (2H)	δ4a 0.87, δ4b 0.85; γ 1.64;
D5-NH_2	7.28	4.56	2.73, 2.50	T1 6.83, T2 6.76

Amide NH coupling constants and Δδ/T for **3d** in CD_3CN/H_2O (7:3) at 293K.

Residue	L4	D5	NT 1	NT 2	NH-side
$^{3}J_{NH - CH\alpha}$ (Hz)	5.8	7.4	N/A	N/A	N/A
Δδ/K (-ppb)	3.2	0	0.3	7.1	6.5

^{1}H NMR resonance assignments and chemical shifts (δ ppm) for **3e** in H_2O/D_2O (9:1) at 298K.

Residue	**NH**	**H α**	**H β**	**Other**
Ac-K1	8.12	4.13	1.74, 1.60	Ac 1.92; γ 1 1.34 (2H); δ1a 1.55, δ1b 1.42; ε1a 3.22, ε1b 2.95; side NH 7.80;
L2	8.23	4.19	1.58, 0.79	δ2a 0.85, δ2b 0.79; γ 1.58; β2a 1.58, β2b 0.79;
L3	7.87	4.44		δ3a 0.85, δ3b 0.81; 1.64-1.55 (2βH, 1γH);
oL4	N/A	4.90	1.69, 1.49	δ4a 0.84, δ4b 0.81; γ 1.66; β4a 1.69, β4b 1.49;
D5-NH_2	8.44	4.67	2.70, 2.56	T1 7.35, T2 7.08;

Amide NH coupling constants and Δδ/T for **3e** in in H_2O/D_2O (9:1) at 298K.

Residue	K1	L2	L3	oL4	D5	NT1	NT2	NH-side
$^{3}J_{NH - CH\alpha}$ (Hz)	6.3	6.5	8.0	N/A	7.9	N/A	N/A	N/A
Δδ/K (- ppb)	8.1	6.5	6	N/A	8.7	5.3	6	7.3

9. Peptide analysis by mass spectrometry

#	Peptide	Formula	[M + H^+/Na^+] calculated	[M + H^+/Na^+] found (HRMS)	R_t (min)/(Method[a])
2a	Ac-cyclo-(1,5)-(N-Me-K)LLLD-NH_2	$C_{31}H_{55}N_7O_7Na^+$	660.4060	660.4055	14.3/C
2b	Ac-cyclo-(1,5)-K(N-Me-L)LLD-NH_2	$C_{31}H_{56}N_7O_7^+$	638.4241	638.4240	15.1/B
2c	Ac-cyclo-(1,5)-KL(N-Me-L)LD-NH_2	$C_{31}H_{56}N_7O_7^+$	638.4241	638.4240	14.1/B
2d	Ac-cyclo(1,5)-[KLLLD]-NHMe	$C_{31}H_{56}N_7O_7^+$	660.406	660.406	3.6/G
2e	Ac-(1,5)-[K(ε-NMe)LLLD]-NH_2	$C_{31}H_{56}N_7O_7^+$	660.406	660.406	18.6/F
3a	Ac-cyclo-(1,5)-oKLLLD-NH_2	$C_{30}H_{53}N_6O_8$	625.3919	625.3915	12.8/E
3b	Ac-cyclo-(1,5)-KoLLLD-NH_2	$C_{30}H_{53}N_6O_8$	625.3919	625.3923	13.0/E
3c	Ac-cyclo-(1,5)-KLoLLD-NH_2	$C_{30}H_{53}N_6O_8$	625.3919	625.3924	12.7/E
3d	Ac-cyclo-(1,5)-oKoLoLLD-NH_2	$C_{30}H_{51}N_4O_{10}$	627.3600	627.3598	16.5/E
3e	Ac-cyclo-(1,5)-KLLoLD-NH_2	$C_{30}H_{52}N_6O_8Na^+$	647.3739	647.3742	12.1/E

[a]Methods B, C, E, F and G are described in section 2.4

10. NMR solution structure data

ROE-derived distances, $^{3}J_{NH\text{-}CH\alpha}$ derived ϕ-angle restraints and hydrogen bond restraints used for calculating the solution structure of **3a** in H_2O/D_2O (9:1) at 298K.

1	Acetyl 1 Hα*	Leu 3 HN	6.5 Å; Weak + 1.5Å correction
2	Acetyl 1 Hα*	Leu 4 HN	7.5 Å; Very Weak + 1.5Å correction
3	Lys 2 Hβ2	Leu 3 HN	5.0 Å; Weak
4	Lys 2 Hβ2	Leu 4 HN	6.0 Å; Very Weak
5	Lys 2 Hγ2	Ala 3 HN	6.0 Å; Vey Weak
6	Leu 3 Hα	Lys 2 HZ	6.0 Å; Very Weak
7	Leu 3 Hα	Leu 4 HN	3.5 Å; Medium
8	Leu 3 Hα	Asp 6 HN	5.0 Å; Weak
9	Leu 3 HN	Leu 4 HN	3.5 Å; Medium
10	Leu 3 Hα	Asp 6 Hβ2	5.0 Å; Weak
11	Leu 3 Hα	Leu 2 Hγ2	6.0 Å; Very Weak
12	Leu 4 Hα	Asp 6 H2	6.0 Å; Vey Weak
13	Leu 4 Hα	Leu 5 HN	3.5 Å; Medium
14	Leu 5 Hα	Asp 6 H1	6.0 Å; Very weak
15	Leu 5 Hβ2	Asp 6 HN	5.0 Å; Weak
16	Leu 5 Hβ1	Asp 6 HN	5.0 Å; Weak
17	Leu 5 HN	Leu 6 HN	5.0 Å; Weak
18	Leu 5 Hα	Asp 6 HN	3.5 Å; Medium
19	Asp 6 Hα	Asp 6 H1	5.0 Å; Weak
20	Asp 6 Hα	Asp 6 H2	5.0 Å; Weak
21	Asp 6 Hβ2	Leu 2 HZ	3.4 Å; Medium
22	Asp 6 Hβ1	Leu 2 HZ	5.0 Å; Weak
23	Asp 6 HN	Asp 6 H1	6.0 Å; Very Weak
24	Asp 6 Hβ1	Asp 6 H1	6.0 Å; Very Weak
25	Asp 6 Hβ2	Asp 6 H1	6.0 Å; Very Weak
26	Asp 6 HN	Asp 6 H1	7.0 Å; Weak

ϕ-angle restraints

	Residue	$^{3}J_{NH\text{-}CH\alpha}$	ϕ-dihedral angle restraint
1	Leu 2	4.38	-57 ± 30°
2	Leu 3	6.00	-60 ± 30°

Hydrogen-bond restraints

	Donor	Acceptor	H-O Distance	N-O Distance
1	Asp 6 H1	Leu 3 O	1.88[-.3 Å,+.42 Å]	2.88[-.3 Å,+.42 Å]
2	Asp 6 NH	Leu 2 O	1.88[-.3 Å,+.72 Å]	2.88[-.3 Å,+.62 Å]
3	Leu 5 NH	Acetly1 O	1.88[-.3 Å,+.42 Å]	2.88[-.3 Å,+.32 Å]

ROE-derived distances, $^{3}J_{NH\text{-}CH\alpha}$ derived ϕ-angle restraints and hydrogen bond restraints used for calculating the solution structure of **3b** in H_2O/D_2O (9:1) at 298K.

1	Acetyl 1 Hα*	Lys 2 HN	4.2 Å; Strong + 1.5Å correction
2	Acetyl 1 Hα*	Leu 4 HN	6.5 Å; Weak + 1.5Å correction
3	Acetyl 1 Hα*	Leu 5 HN	7.5 Å; Very Weak + 1.5Å correction
4	Lys 2 Hα	Lys 2 Hγ2	6.0 Å; Very Weak
5	Lys 2 Hα	Lys 2 Hδ2	6.0 Å; Vey Weak
6	Leu 3 Hα	Leu 4 HN	3.5 Å; Medium
7	Leu 3 Hα	Leu 5 HN	6.0 Å; Very Weak
8	Leu 3 Hα	Leu 6 HN	5.0 Å; Weak
9	Leu 3 Hα	Lys 2 HZ	6.0 Å; Very Weak
10	Leu 3 Hα	Lys 2 Hγ2	5.0 Å; Weak
11	Leu 3 Hα	Asp 6 H1	6.0 Å; Very Weak
12	Leu 3 Hβ1	Leu 4 HN	5.0 Å; Weak
13	Leu 3 Hγ	Lys 2 HN	6.0 Å; Vey Weak
14	Leu 4 Hα	Leu 5 HN	3.5 Å; Medium
15	Leu 4 Hα	Asp 6 HN	6.0 Å; Very weak
16	Leu 5 Hα	Asp 6 HN	3.5 Å; Medium
17	Leu 5 Hα	Asp 6 H1	6.0 Å; Very weak
18	Asp 6 HN	Asp 6 H2	6.0 Å; Vey Weak
19	Asp 6 Hα	Asp 6 H1	5.0 Å; weak
20	Asp 6 Hα	Asp 6 H2	3.5 Å; Medium
19	Asp 6 HN	Asp 6 H1	6.0 Å; Very weak

ϕ-angle restraints

	Residue	$^{3}J_{NH\text{-}CH\alpha}$	ϕ-dihedral angle restraint
1	Lys 1	3.36	-57 ± 30°
3	Leu 4	5.11	-60 ± 30°

Hydrogen-bond restraints

	Donor	Acceptor	H-O Distance	N-O Distance
1	Asp 6 H1	Leu 3 O	1.88[-.3 Å,+.42 Å]	2.88[-.3 Å,+.32 Å]
2	Asp 6 NH	Leu 2 O	1.88[-.3 Å,+.42 Å]	2.88[-.3 Å,+.32 Å]
3	Leu 5 NH	Acetly1 O	1.88[-.3 Å,+.42 Å]	2.88[-.3 Å,+.32 Å]

ROE-derived distances, $^{3}J_{NH\text{-}CH\alpha}$ derived ϕ-angle restraints and hydrogen bond restraints used for calculating the solution structure of **3c** in H_2O/D_2O (9:1) at 298K.

1	Acetyl 1 Hα*	Lys 2 HN	4.2 Å; Strong + 1.5Å correction
2	Acetyl 1 Hα*	Leu 3 HN	6.5 Å; Weak + 1.5Å correction
3	Acetyl 1 Hα*	Leu 5 HN	7.5 Å; Very Weak
4	Acetyl 1 Hα*	Lys 2 Hα	6.0 Å; Very Weak
5	Lys 2 Hγ2	Lys 2 Hα	6.0 Å; Vey Weak
6	Lys 2 Hγ1	Lys 2 Hα	5.0 Å; Weak
7	Lys 2 Hα	Leu 3 HN	2.7 Å; Strong
8	Lys 2 Hα	Leu 5 HN	5.0 Å; Weak
9	Lys 2 HN	Asp 3 HN	5.0 Å; Medium
10	Lys 2 Hε	Asp 6 Hβ1	5.0 Å; Weak
11	Leu 3 Hα	Asp 6 HN	6.0 Å; Very Weak
12	Leu 3 Hα	Asp 6 Hβ2	6.0 Å; Vey Weak
13	Leu 4 Hα	Leu 5 HN	3.5 Å; Medium
14	Leu 4 Hα	Asp 6 HN	6.0 Å; Very weak
15	Leu 4 Hα	Asp 6 H1	6.0 Å; Very weak
16	Leu 4 Hβ1	Leu 5 HN	5.0 Å; Weak
17	Leu 5 HB1	Asp 6 HN	6.0 Å; Very weak
18	Leu 5 Hα	Asp 6 HN	3.5 Å; Medium
19	Leu 5 Hα	Asp 6 H1	6.0 Å; Very weak
20	Leu 5 HN	Asp 6 HN	5.0 Å; Weak
21	Leu 5 Hβ2	Asp 6 HN	5.0 Å; Weak
22	Asp 6 HN	Asp 6 H1	5.0 Å; Weak
23	Asp 6 Hα	Asp 6 H2	5.0 Å; Weak
24	Asp 6 Hα	Asp 6 H1	5.0 Å; Weak
25	Asp 6 Hα	Lys 2 HZ	6.0 Å; Very Weak
26	Asp 6 Hβ2	Lys 2 HZ	3.5 Å; Medium
27	Asp 6 Hβ1	Lys 2 HZ	5.0 Å; Weak

ϕ-angle restraints

	Residue	$^{3}J_{NH\text{-}CH\alpha}$	φ-dihedral angle restraint
1	Lys 1	4.80	-60 ± 30°
2	Leu 2	5.22	-65 ± 30°
3	Leu 4	5.76	-65 ± 30°

Hydrogen-bond restraints

	Donor	Acceptor	H-O Distance	N-O Distance
1	Asp 6 H1	Leu 3 O	1.88[-.3 Å,+.42 Å]	2.88[-.3 Å,+.32 Å]
2	Asp 6 NH	Leu 2 O	1.88[-.3 Å,+.72 Å]	2.88[-.3 Å,+.62 Å]
3	Leu 5 NH	Acetly1 O	1.88[-.3 Å,+.72 Å]	2.88[-.3 Å,+.62 Å]

ROE-derived distances, $^{3}J_{NH\text{-}CH\alpha}$ derived ϕ-angle restraints and hydrogen bond restraints used for calculating the solution structure of **3d** in 70% CD_3CN : 30% D_2O at 298K.

1	Acetyl 1 Hα*	Leu 2 Hα	6.5 Å; Weak + 1.5Å correction
2	Acetyl 1 Hα*	Leu 5 HN	7.5 Å; Very Weak + 1.5Å correction
3	Lys 2 Hα	Leu 5 HN	5.0 Å; Weak
4	Lys 2 Hα	Asp 6 HN	6.0 Å; Very Weak
5	Lys 2 Hδ1	Lys 2 Hγ2	5.0 Å; Weak
6	Lys 2 Hδ2	Lys 2 Hγ1	5.0 Å; Weak
7	Leu 3 Hα	Lys 2 HZ	6.0 Å; Very Weak
8	Leu 3 Hα	Leu 5 HN	6.0 Å; Very Weak
9	Leu 3 Hα	Leu 6 HN	6.0 Å; Very Weak
10	Leu 3 Hα	Leu 2 Hγ2	5.0 Å; Weak
11	Leu 3 Hα	Asp 6 Hβ2	5.0 Å; Weak
12	Leu 4 Hβ1	Leu 5 HN	5.0 Å; Weak
13	Leu 4 Hα	Asp 6 H1	6.0 Å; Very Weak
14	Leu 4 Hα	Leu 5 HN	3.5 Å; Medium
15	Leu 4 Hα	Asp 6 HN	6.0 Å; Very Weak
16	Leu 5 Hα	Asp 6 HN	3.5 Å; Medium
17	Asp 6 HN	Asp 6 HN	5.0 Å; Weak
18	Asp 6 Hα	Asp 6 H2	5.0 Å; Weak
19	Asp 6 Hα	Leu 2 HZ	6.0 Å; Very Weak
20	Asp 6 Hα	Asp 6 H1	5.0 Å; Weak
21	Asp 6 Hβ2	Leu 2 HZ	3.5 Å; Medium
22	Asp 6 Hβ2	Asp 6 H1	6.0 Å; Very Weak
23	Asp 6 Hβ1	Leu 2 HZ	5.0 Å; Weak
24	Asp 6 HN	Asp 6 H2	6.0 Å; Very Weak

ϕ-angle restraints

	Residue	^{3}JNH-CHα	φ-dihedral angle restraint
1	Leu 4	5.77	-60 ± 30°

Hydrogen-bond restraints

	Donor	Acceptor	H-O Distance	N-O Distance
1	Asp 6 H1	Leu 3 O	1.88[-.3 Å,+.42 Å]	2.88[-.3 Å,+.42 Å]
2	Asp 6 NH	Leu 2 O	1.88[-.3 Å,+.42 Å]	2.88[-.3 Å,+.42 Å]
3	Leu 5 NH	Acetly1 O	1.88[-.3 Å,+.72 Å]	2.88[-.3 Å,+.62 Å]

Table S2. RMSD for the NMR structures compared to putative α-helix or 3_{10}-helix.

Peptide	**3a**	**3b**	**3c**	**3d**
Ensembles (Å)[a]	0.360	0.187	0.225	0.320
α-helix (Å)[b]	0.209	0.237	0.280	0.239
3_{10}-helix (Å)[c]	0.811	0.837	0.848	0.841
Cα(*i*)-Cα(*i*+4) distance (Å)	5.788	5.736	5.763	5.711

[a] Twenty lowest energy structures superimposed on each other.[b,c] Twenty lowest energy structures superimposed on idealised α-helix or 3_{10}-helix.

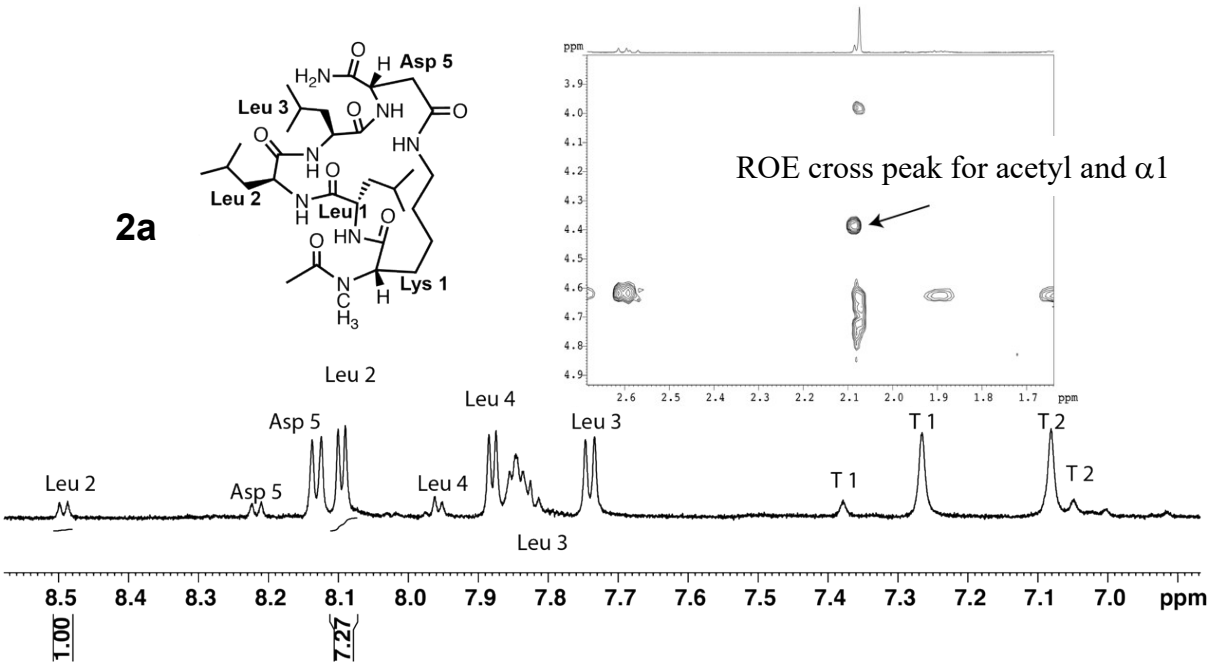


Figure S1. ^{1}H NMR amide chemical shift region for **2a** and ROE cross peak between acetyl cap and α1 (Lys Hα) in H_2O:D_2O (9:1).

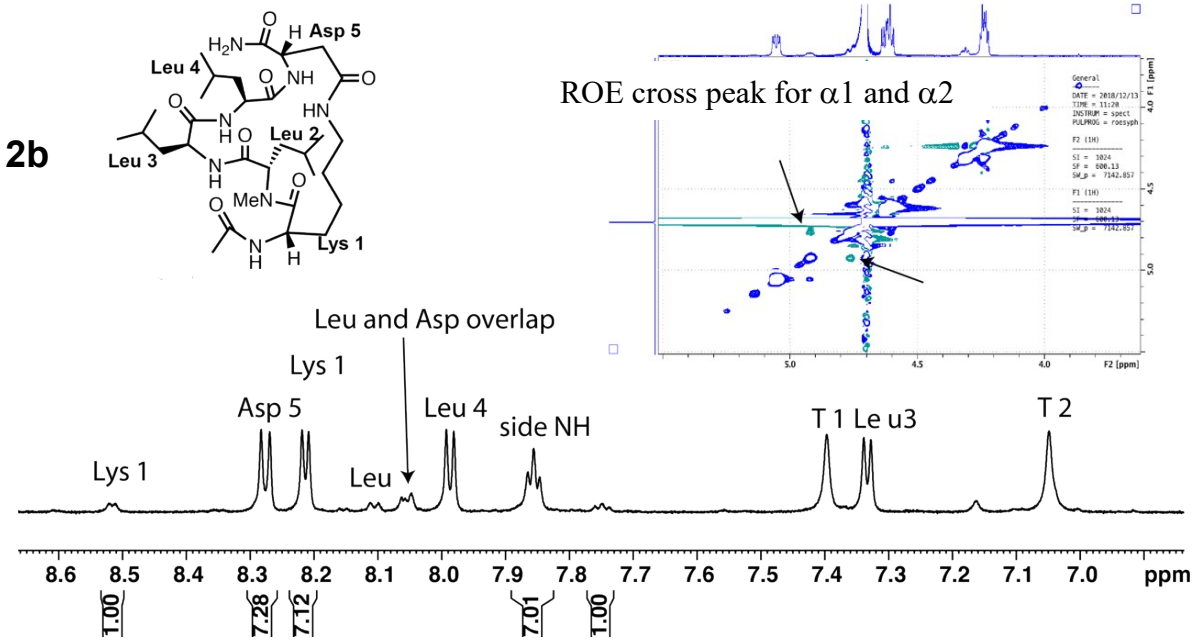


Figure S2. ^{1}H NMR amide chemical shift region for **2b** and ROE cross peak between Hα1 (Lys 1 Hα) and Hα2 (Leu 2 Hα) in H_2O:D_2O (9:1).

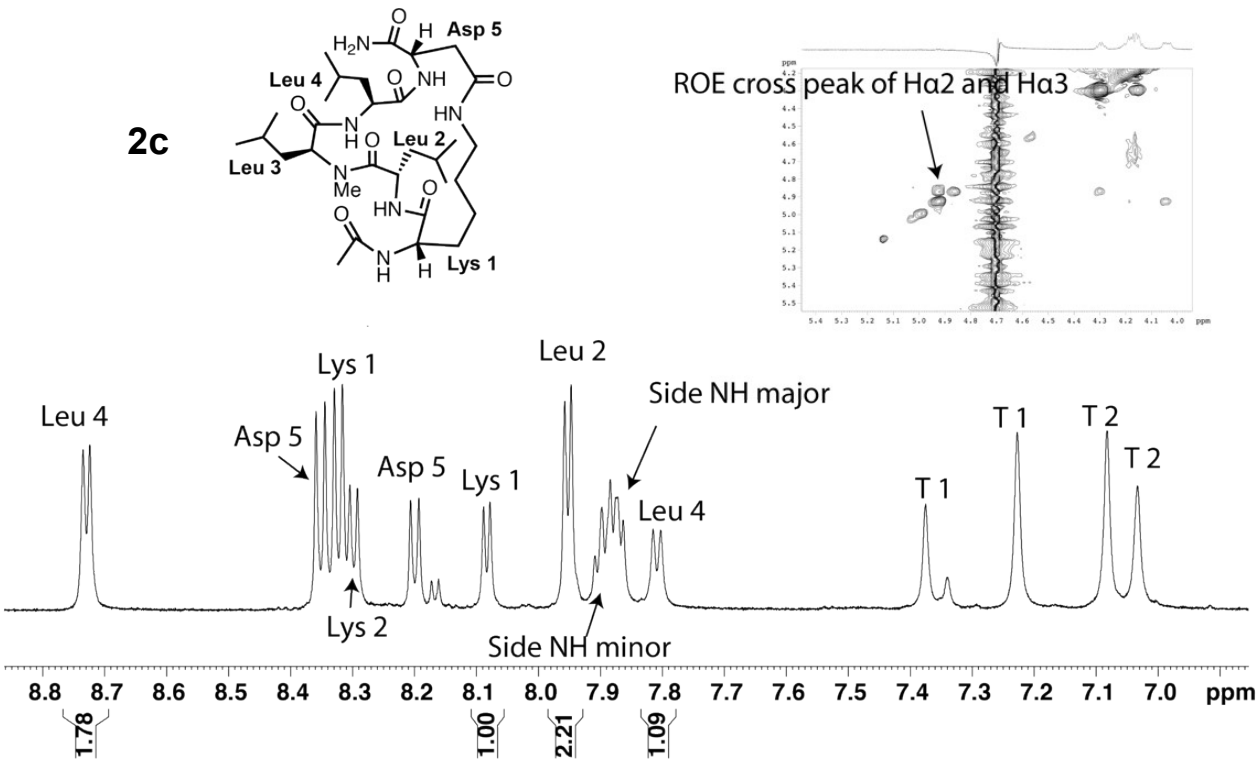


Figure S3. ^{1}H NMR amide region for **2c** and ROE cross peak between Hα2 (Leu 2 Hα)and Hα3 (Leu 3 Hα) in H_2O:D_2O 9:1.

11. Modelling of idealised alpha helices, Raw CD spectra and NMR-derived structures

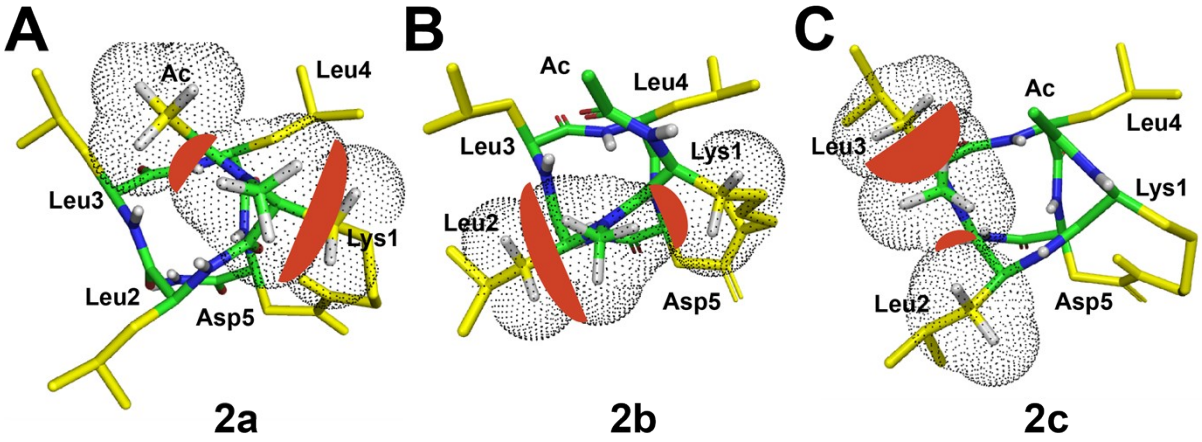


Figure S4. Graphical models for idealised alpha helices for peptides **2a (A)**, **2b (B)** and **2c** (**C**) viewed down the helix axis, showing van der Waals radii (black dotted spheres) for amide N-methyl and neighbouring Cβ methylene groups. Red regions show steric clash of N-methyl with neighbouring Cβ–methylene groups in **2a-c**.

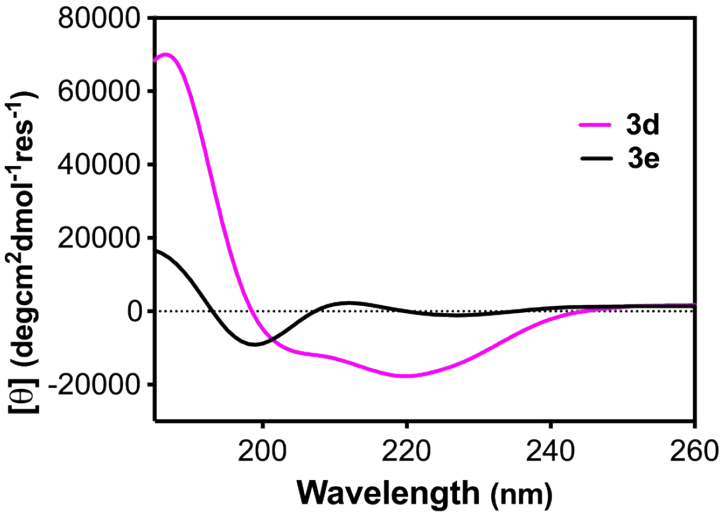


Figure S5. CD spectra for esters **3d** (250 μM 70% CD_3CN for solubility) and **3e** (100 μM) in phosphate buffer (10mM, pH 7.4, 298K). Indicates no helical structure in **3e**.

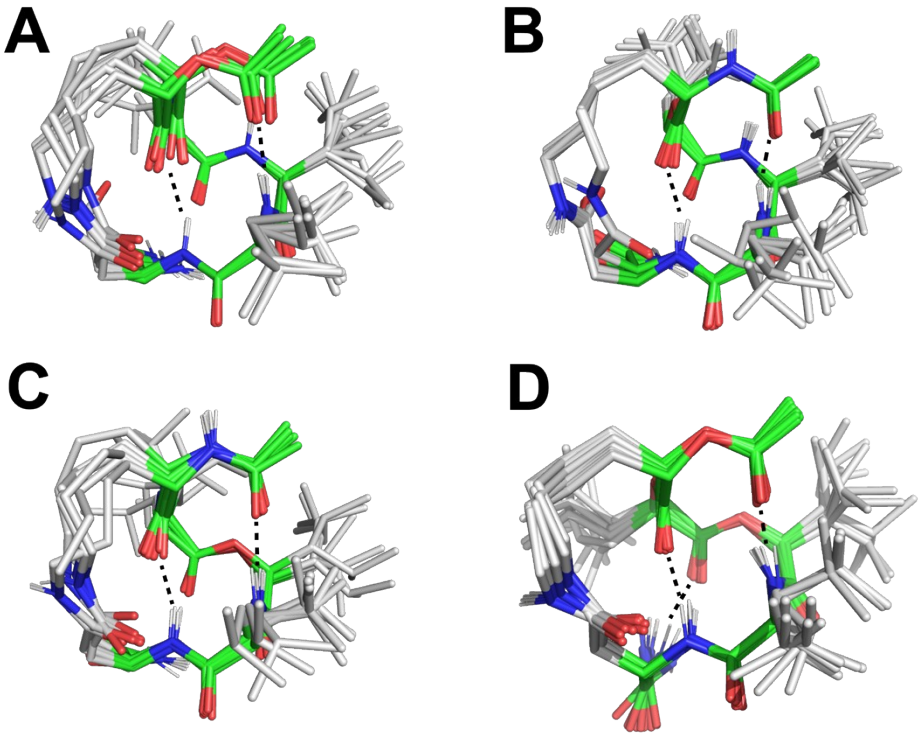


Figure S6. NMR solution structures for the cyclic depsipeptides **3a** (A), **3b** (B), **3c** (C) in 90% H_2O: 10% D_2O and **3d** (D) in 70% CD_3CN : 30% H_2O. Peptide backbone carbon, oxygen and nitrogen atoms are in green, red and blue, respectively, whereas side chain carbon atoms are grey for clarity. Hydrogen bonds are represented as dashed lines. The N-terminal acetyl group or corresponding ester is shown at the top of structures **3a-d**.

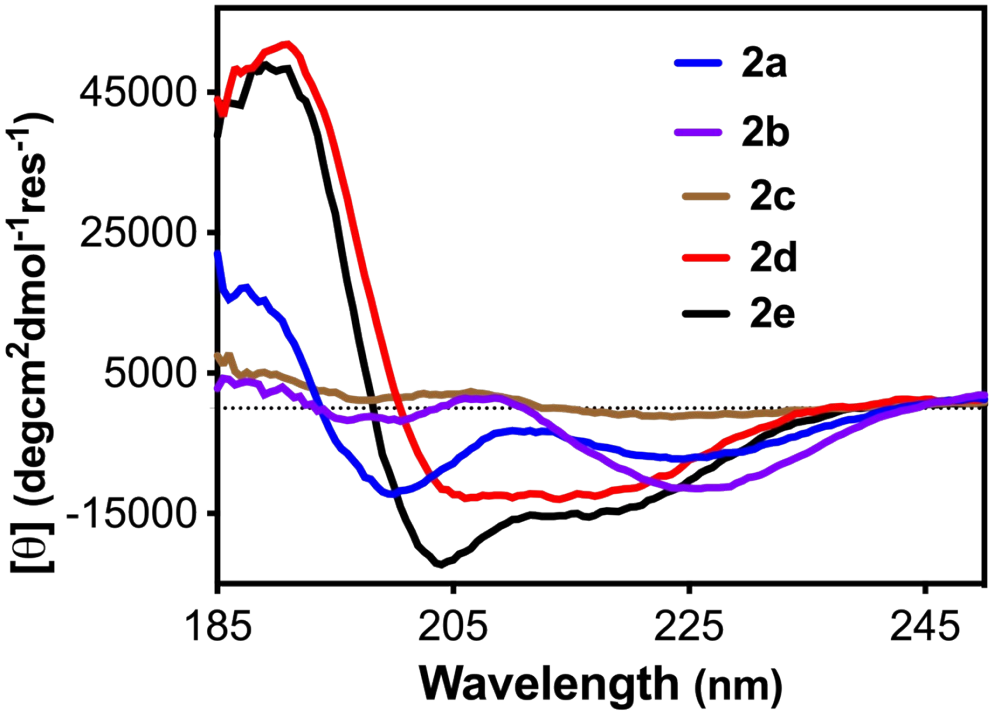


Figure S7. Raw CD spectra for N-methylated peptides **2a-e** (100 μM) in phosphate buffer (10mM, pH 7.4, 298K).

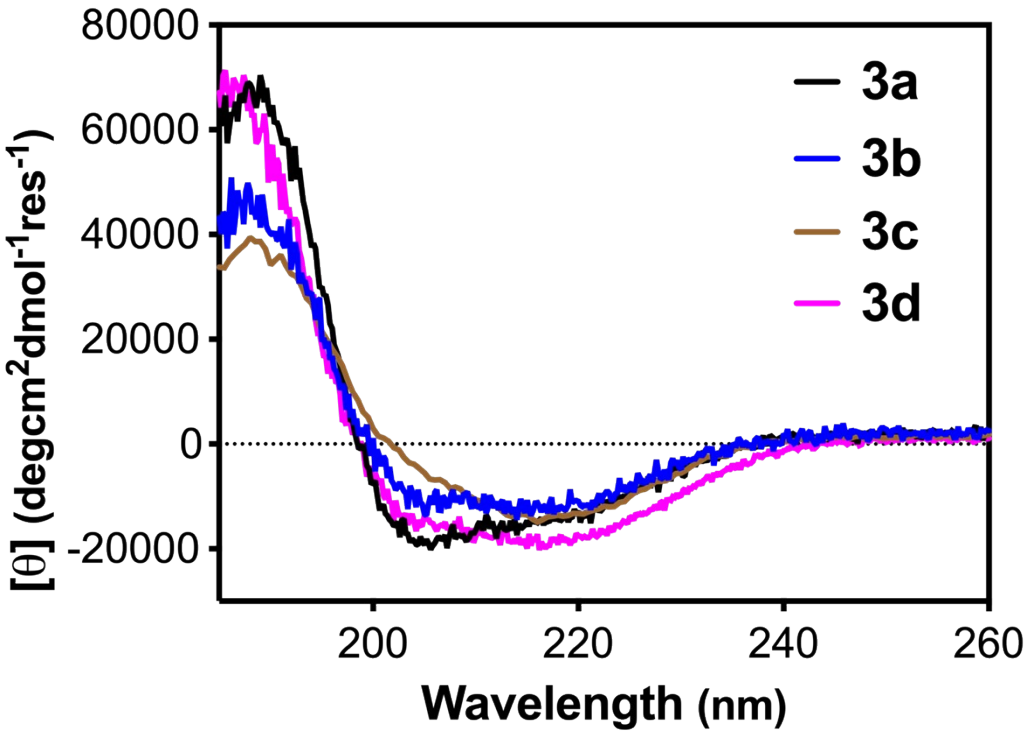


Figure S8. Raw CD spectra for monoesters **3a-c** (100 μM) in phosphate buffer (10 mM, pH 7.4, 298K) and triester **3d** (250 μM) in 70% CD_3CN in water for solubility.

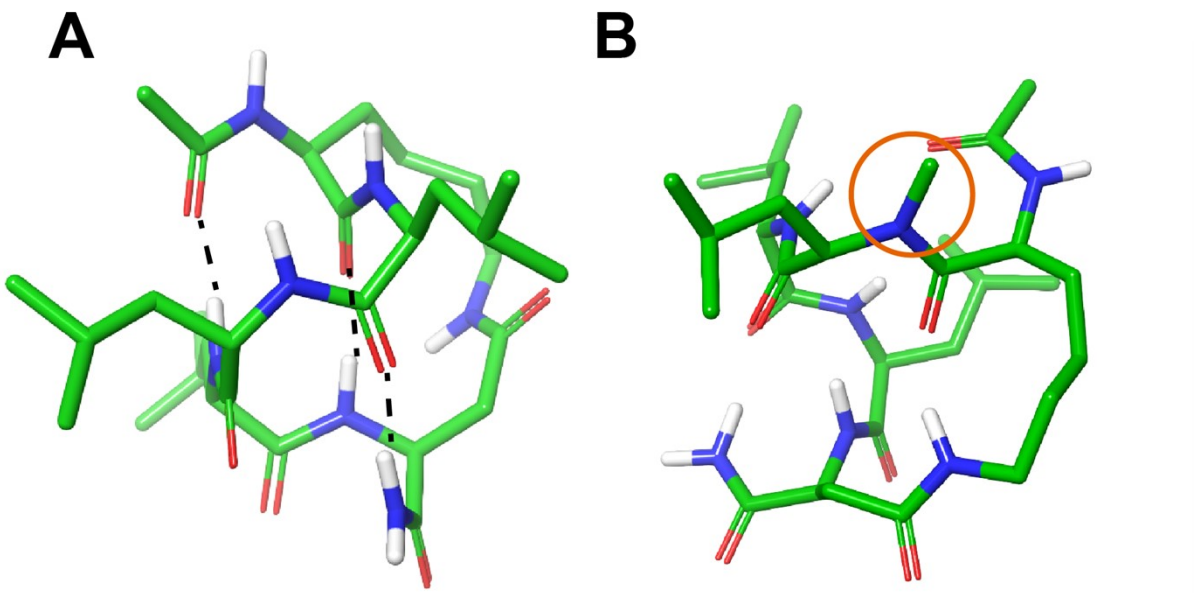


Figure S9. Energy minimised structures in a simulated water environment (generated by MacroModel module in Schrodinger suite 2022) for **1** (A) versus **2b** (B). These results indicate alpha-helical structure for **1** (A), with three i to i+4 hydrogen bonds (dashed lines), but no helical structure and no intramolecular hydrogen bonds for **2b** (B). Red circle highlights N-methyl group on residue 2.