# SUPPORTING INFORMATON

# Flexibility is Important for Inhibition of the MDM2/p53 Protein-Protein Interaction by Cyclic β-Hairpins

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## 1. Nomenclature

The following amino acid abbreviations have been used in this supporting information: P = Proline, $^{D}$P = $^{D}$Proline, G = Glycine, F = Phenylalanine, S = Serine, L = Leucine, D = Aspartic acid, W = Tryptophan, E = Glutamic acid, F(Br) = 4-Bromo-phenylalanine, F(Cl) = 4-Chloro-phenylalanine, W(Cl) = 6-Chloro-tryptophan. For side chain positions the following notations have been used: alfa (α), beta (β), gamma (γ), delta (δ) and epsilon (ε).

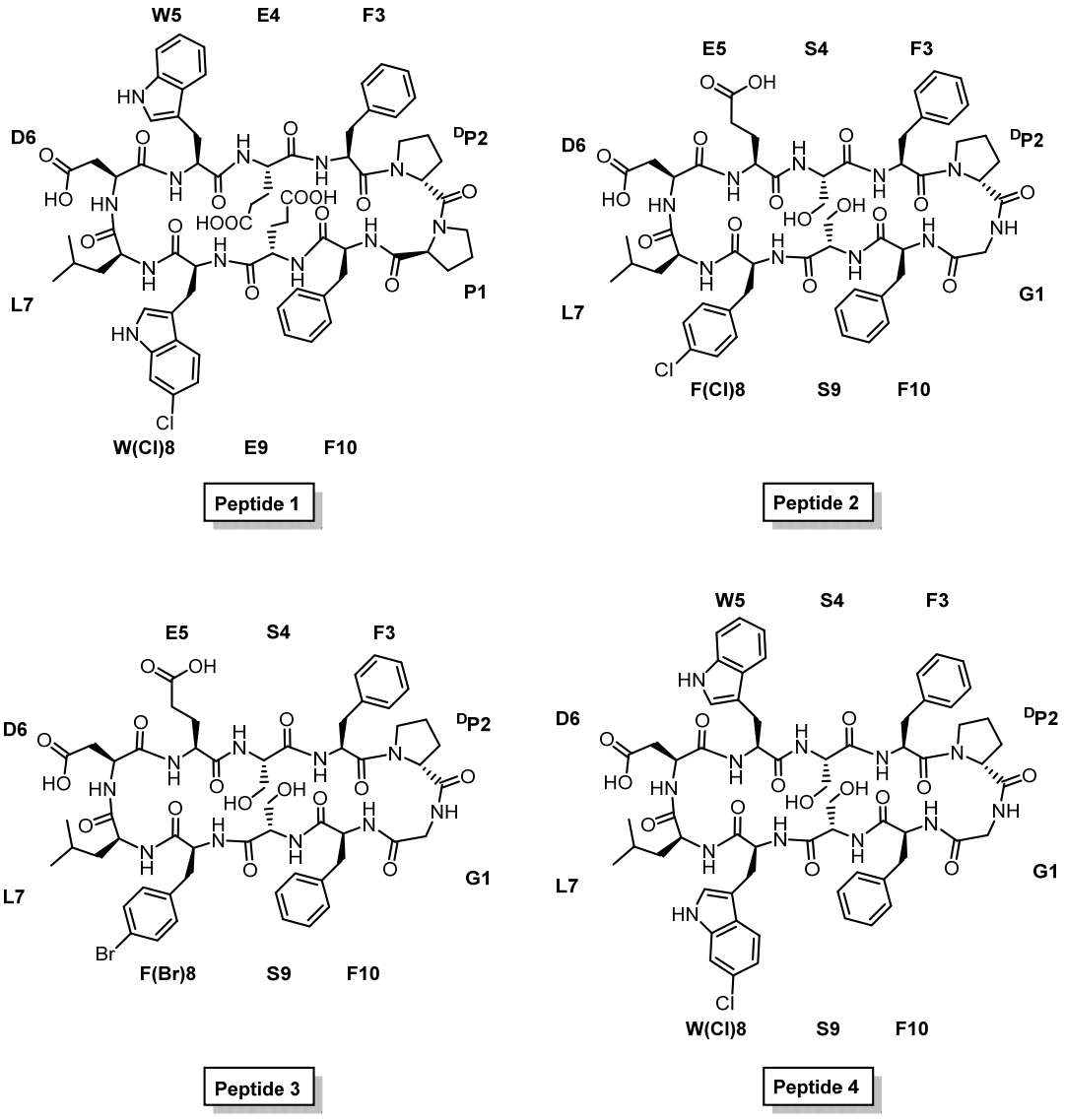



**Figure S1**. Structures of the investigated cyclic peptides, with the numbering used and the amino acids given as one letter acronyms.

## 2. Synthesis

*General*

The synthetic schemes for peptides **1**-**4** are outlined in scheme S1–3. Commercially available reagents and solvent were used without further purification. Fmoc-protected amino acids, glycine substituted 2-chlorotrityl resin (loading 0.46 mmol/g) and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) were purchased from AAPPtec. Dimethyl formamide (DMF, ACS reagent grade), piperidine (99%), diisopropylamine (DIPEA), *O*-*tert*-butyl-serine substituted 2-chlorotrityl resin (loading 1.1 mmol/g), glutamic acid 5-*tert*-butyl ester substituted 2-chlorotrityl resin (1 mmol/g) and 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) were obtained from Sigma Aldrich. 3-(Trimethylammonium)propyl carbonate-functionalized silica gel (loading 0.46 mmol/g) was obtained from Sigma Aldrich/Silicycle inc. $C_{18}$ – cartridges were obtained from Isolute. LCMS analysis was performed on a API SCIEX 150 EX Perkin Elmer ESI-MS (30 eV) connected to a Perkin Elmer gradient pump system and a C8 column (Gemini) using acetonitrile and MilliQ-water with 0.1% formic acid as mobile phase with a gradient of 5 to 95% acetonitrile over 4 min. The purity of the peptides were determined by HPLC on a C8 column (Gemini) using acetonitrile and MilliQ-water with 0.1% formic acid as mobile phase with a gradient of 5 to 95% acetonitrile over 8 min, followed by 4 min of 95% acetonitrile. All peptides were of >95% purity.

*Linear peptide*

Solid phase peptide synthesis using a PS3 Peptide Synthesizer (Protein Technologies Inc) was carried out following the standard Fmoc-protecting strategy on 2-chlorotrityl resin on 0.3 mmol scale. *Tert*-butyl (S, E, D) and Boc (W) were used as side chain protecting groups. Coupling reactions were performed using TBTU (0.6 mmol) as coupling reagent, DIPEA (0.4 M, 3 ml/coupling) and DMF (3 ml/coupling) as solvent. Double couplings (2 × 60 min, except for the first coupling in the synthesis and coupling to $^{D}$P for which 2 × 90 min used) were used for the peptide coupling steps. Two eq. were used for all amino acids except for where W(Cl)8  1.1 eq. were used. Capping with 20% acetic anhydride in DMF (5 ml) for 20 min was done after every coupling. Fmoc-deprotection was accomplished with 20% piperidine in DMF (5 ml). After completion of the synthesis of the linear peptide, the resin was transferred to a disposable syringe fitted with a porous polystyrene frit. Removal of DMF was accomplished by washing with DCM. The resin was dried under nitrogen gas and vacuum and was then stored at -18 °C.

*Cleavage from the resin*

The resin was first swelled in DCM for at least 30 min and the linear peptides were then cleaved by treatment with 1% TFA in DCM for at least 3 × 30 min using a tube rotator. The cleavage was monitored with LCMS analysis. In between every cleavage the resin was washed with DCM until the color of the resin went from red to beige/light brown. The combined filtrates were concentrated under a stream of nitrogen gas and the peptide was precipitated from ice cooled DEE, 20–30 ml DEE/1 ml DCM. The vial was then put in the fridge overnight. The crude peptides were isolated as white solids by filtration.

*Cyclization of Peptide **2***

Peptide **2** was cyclized following a previously published procedure[1] with some modifications. The crude linear peptide (~0.15 mmol) was suspended in dry DMF (24 ml) and transferred to a

syringe. HATU (0.45 mmol) was dissolved in dry DMF (24 ml) and transferred to a second syringe. The peptide and the HATU solutions were then added under nitrogen atmosphere at room temperature using a syringe pump (rate = 0.03 ml/min) to a round bottomed flask containing HATU (0.015 mmol), DIPEA (0.9 mmol) and dry DMF (16 ml). *Note, the amount of solvent used is dependent on the solubility of the peptide. The concentration used in the procedure of Malesevic et al. was used as a starting point.*[1] After the addition, the undissolved peptide was suspended in dry DMF (10 ml) and added to the reaction mixture in one portion. LCMS analysis showed full conversion of the starting material. The reaction mixture was filtered through a plug of 3-(trimethylammonium)propyl carbonate-functionalized silica gel (2.64 mmol, loading 0.46 mmol/g). The plug was rinsed with DMF until no product could be observed by LCMS in the filtrate. The DMF (~100 ml) solution was then cooled on ice and slowly, while cooling, diluted with distilled water to a final volume of ~200 ml. The solution was then passed through a $C_{18}$ cartridge (5 g) which had been conditioned with DMF and distilled water (1:1). A gradient of water in acetonitrile (50–0%) was used as mobile phase. The solvent was removed under reduced pressure and the crude cyclic peptide was obtained as a white solid.

*Cyclization of Peptide **3***
Peptide **3** was cyclized using the same procedure as Peptide **2** starting from 0.17 mmol crude peptide.

*Cyclization of Peptide **1** and Peptide **4***
Peptide **1** and peptide **4** were synthesized using the same procedure as peptide **2** starting from 0.11 and 0.23 mmol crude peptide, respectively, except that the reaction mixture was not filtered through 3-(trimethylammonium)propyl carbonate-functionalized silica gel. Instead, directly after the cyclization, the reaction mixture was cooled on ice and diluted to approximately (1:1) with distilled water and then passed through the $C_{18}$ cartridge (5 g) as described for peptide **2**.

*Side chain deprotection*
To the crude cyclic peptide a mixture of TFA:$H_2O$:TIPS (95:2.5:2.5) was added. The mixture was stirred for at least 40 min at room temperature until LCMS analysis showed full deprotection of all side chain protection groups for peptide **2**-**4**. The solvents were removed under a stream of nitrogen gas followed by reduced pressure.
For Peptide **1**, a mixture of TFA:$H_2O$:TIPS (95:2.5:2.5) was added and after 2 h starting material was still observed by LCMS analysis. The solvents were removed under a stream of nitrogen gas and a fresh mixture of TFA:$H_2O$:TIPS (95:2.5:2.5) was added. The reaction mixture was stirred for additional 1 h whereupon LCMS analysis showed full conversion of starting material. The solvents were removed under a stream of nitrogen gas followed by reduced pressure.

*Purification by RP-HPLC*

*Peptide **1***
The crude cyclic peptide was dissolved in acetonitrile/0.1% aqueous formic acid (40:60) and a few drops of DMSO, filtered through a PTFE membrane and purified by RP-HPLC on a VWR LaPrep preparative HPLC using a Gemini $C_{18}$ column (10 μm, 250 × 21.2 mm, Phenomenex). Acetonitrile and 0.1% aqueous formic acid were used as mobile phases with a gradient of 20–95% acetonitrile over 40 min at a flow rate of 20 ml/min and UV detection at 220 nm. After

evaporation **1** was isolated as a white solid (73 mg). MS *m/z* $[M+H]^+$ calculated for $C_{70}H_{81}ClN_{12}O_{16}$: 1381.6. Found: 1381.7. See figure S15 for HPLC chromatogram.

*Peptide **2***

The crude cyclic peptide was dissolved in acetonitrile/0.1% aqueous formic acid (40:60) and a few drops of DMSO, filtered through a PTFE membrane and purified by RP-HPLC on a VWR LaPrep preparative HPLC using a Gemini $C_{18}$ column (10 µm, 250 × 21.2 mm, Phenomenex). Acetonitrile and 0.1% aqueous formic acid were used as mobile phases with a gradient of 10–95% acetonitrile over 35 min at a flow rate of 20 ml/min and UV detection at 220 nm. After evaporation **2** was isolated as a white solid (27 mg). MS *m/z* $[M+H]^+$ calculated for $C_{55}H_{69}ClN_{10}O_{16}$: 1161.5. Found: 1162.0. See figure S16 for HPLC chromatogram.

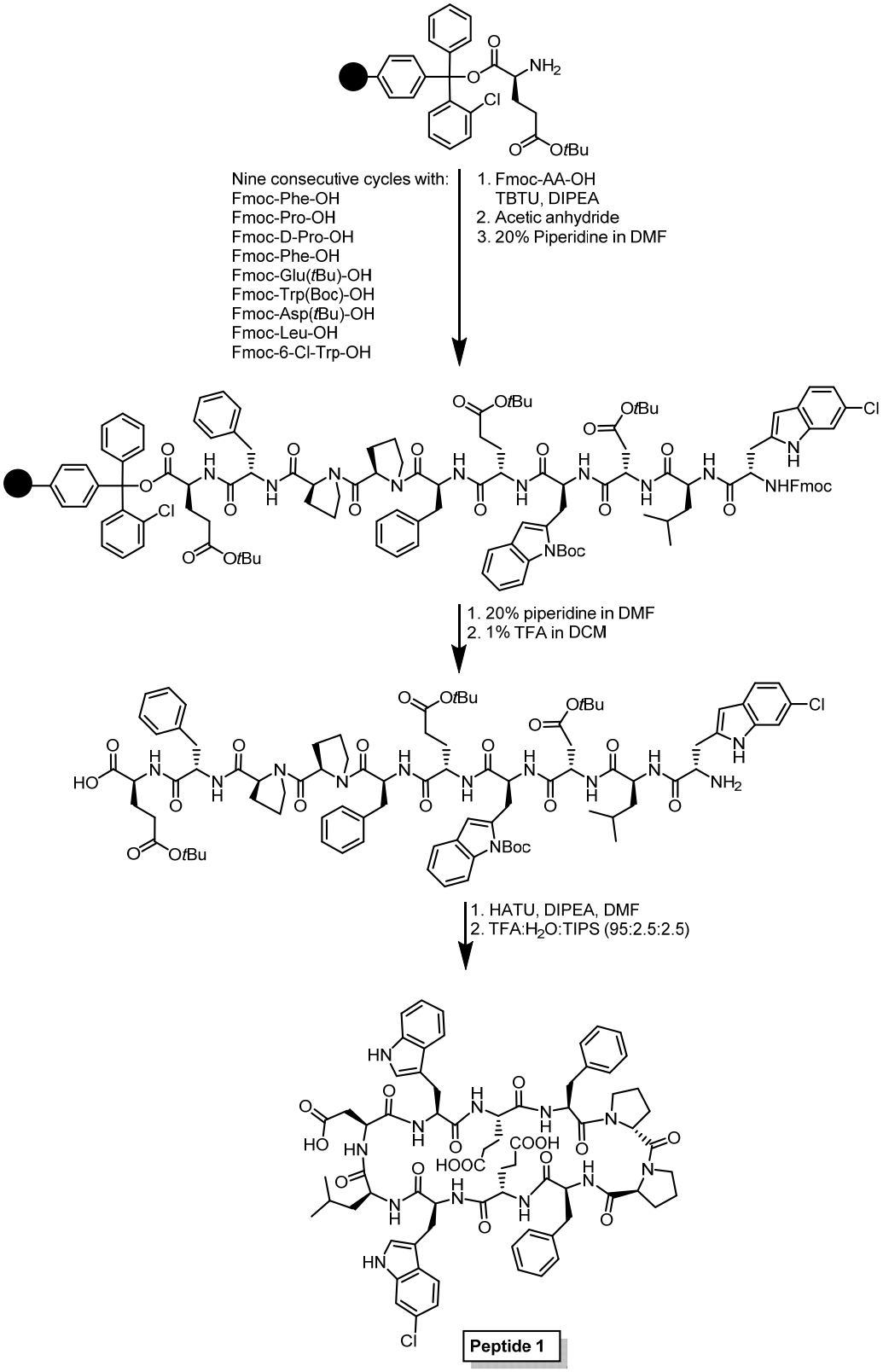
*Peptide **3***

The crude cyclic peptide was dissolved in acetonitrile/0.1% aqueous formic acid (40:60) and a few drops of DMSO, filtered through a PTFE membrane and purified by RP-HPLC on a VWR LaPrep preparative HPLC using a Gemini $C_{18}$ column (10 µm, 250 × 21.2 mm, Phenomenex). Acetonitrile and 0.1% aqueous formic acid were used as mobile phases with a gradient of 20–95% acetonitrile over 30 min at a flow rate of 20 ml/min and UV detection at 220 nm. After evaporation **3** was isolated as a white solid (58 mg). MS *m/z* $[M+H]^+$ calculated for $C_{55}H_{69}BrN_{10}O_{16}$: 1205.4. Found: 1205.7. See figure S17 for HPLC chromatogram.

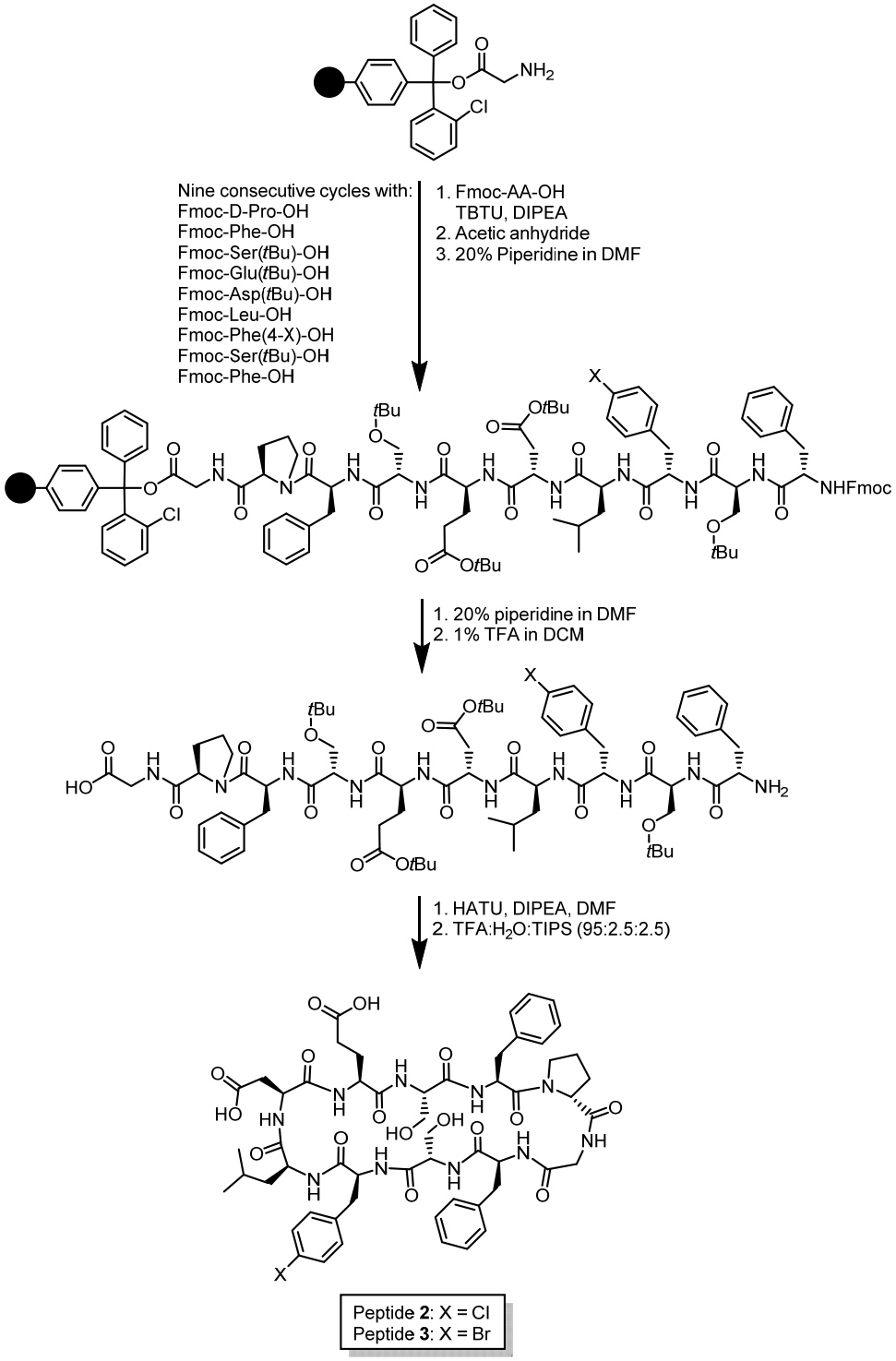
*Peptide **4***

The crude cyclic peptide was dissolved in acetonitrile/0.1% aqueous formic acid (50:50) and few drops of DMSO, filtered through a PTFE membrane and purified by RP-HPLC on a VWR LaPrep preparative HPLC using a Gemini $C_{18}$ column (10 µm, 250 × 21.2 mm, Phenomenex). Acetonitrile and 0.1% aqueous formic acid were used as mobile phases with a gradient of 20–95% acetonitrile over 30 min at a flow rate of 20 ml/min and UV detection at 220 nm. After evaporation **4** was isolated as a white solid (40 mg). MS *m/z* $[M+H]^+$ calculated for $C_{63}H_{73}ClN_{12}O_{14}$: 1257.5. Found: 1257.6. See figure S18 for HPLC chromatogram.

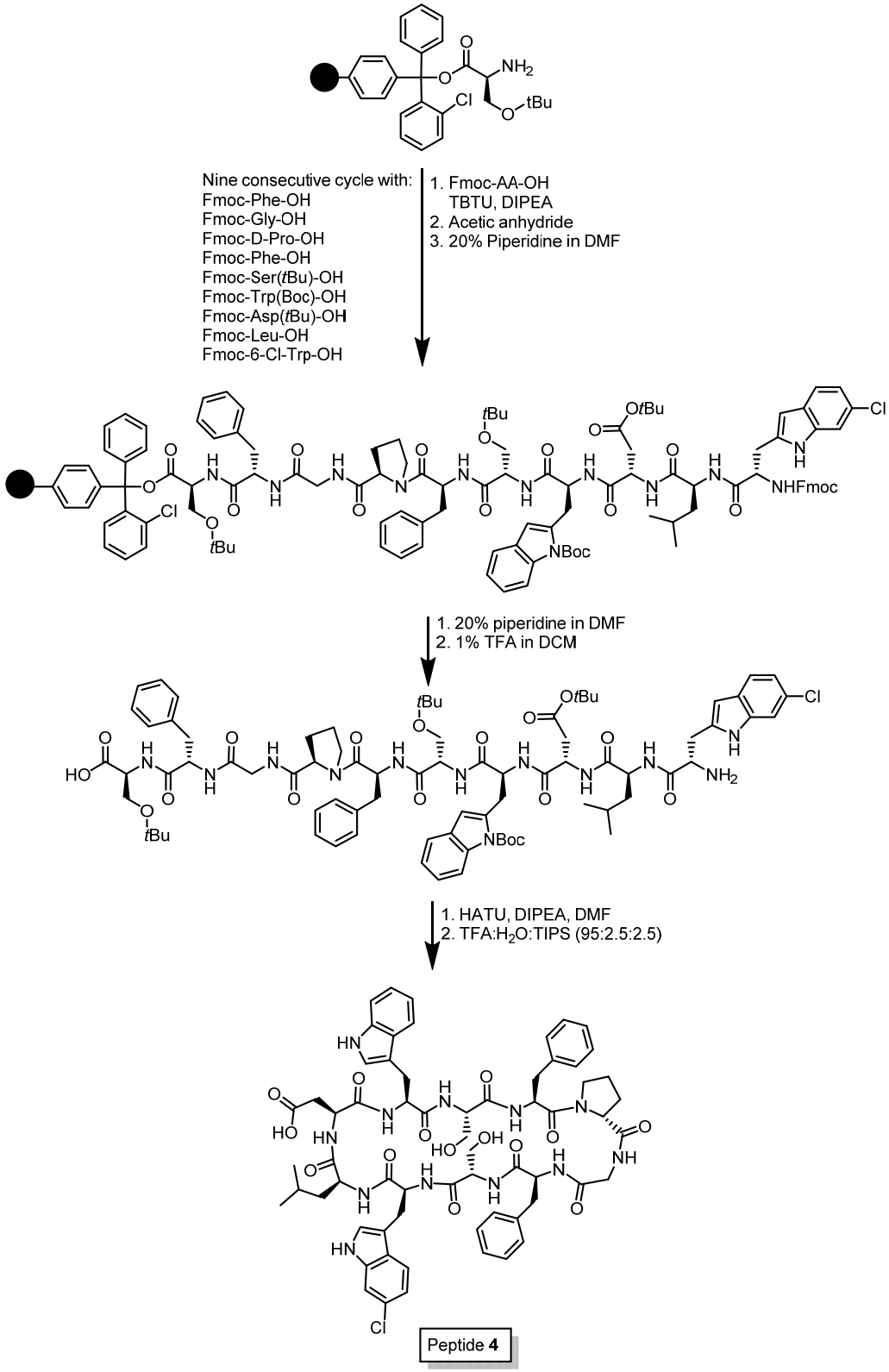
**Scheme S1**. Synthesis of the cyclic Peptide **1**.



**Scheme S2**. Synthesis of the cyclic Peptides **2** and **3**.



**Scheme S3**. Synthesis of the cyclic Peptide **4**.



## 3. NMR Spectroscopy

### *3.1 Chemical shift assignment and J couplings*

Protons were assigned from TOCSY and NOESY NMR spectra recorded at 25 °C on a 900 MHz BRUKER Avance III HD NMR spectrometer equipped with a TCI cryoprobe in DMSO-$d_6$. The chemical shifts are shown in Tables S1–S4. $^3J_{CH\alpha\text{-}NH}$ coupling constants were determined from $^1$H NMR spectra measured on a 400 MHz Varian spectrometer using DMSO-$d_6$ as solvent. The coupling constants are given in Table S5.

**Table S1**. $^1$H NMR chemical shift assignment (δ, ppm) for Peptide **1** at 25 °C in DMSO-$d_6$.

| Residue | Hα | Hα1 | Hα2 | Hβ | Hβ1 | Hβ2 | Hγ | Hγ1 | Hγ2 | Hδ | Hδ1 | Hδ2 | HN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **P1** | 4.22 | - | - | 1.74 | - | - | 1.53 | - | - | - | 3.69 | 3.43 | - |
| **$^D$P2** | 4.46 | - | - | 2.03 | - | - | 1.75 | - | - | 3.39 | - | - | - |
| **F3** | 4.90 | - | - | - | 3.08 | 2.80 | - | - | - | - | - | - | 8.59 |
| **E4** | 4.83 | - | - | 1.83 | - | - | 2.23 | - | - | - | - | - | 8.60 |
| **W5** | 4.76 | - | - | - | 3.20 | 2.91 | - | - | - | - | - | - | 7.83 |
| **D6** | 4.27 | - | - | - | 2.80 | 2.70 | - | - | - | - | - | - | 8.23 |
| **L7** | 3.48 | - | - | - | 1.62 | 1.41 | 1.06 | - | - | 0.70 | - | - | 8.73 |
| **W(Cl)8** | 4.67 | - | - | - | 3.23 | 2.97 | - | - | - | - | - | - | 8.60 |
| **E9** | 5.02 | - | - | - | 1.89 | 1.74 | 2.24 | - | - | - | - | - | 8.84 |
| **F10** | 4.82 | - | - | - | 3.09 | 2.93 | - | - | - | - | - | - | 7.50 |

**Table S2.** $^1$H NMR chemical shift assignment (δ, ppm) for Peptide **2** at 25 °C in DMSO-$d_6$.

| Residue | Hα | Hα1 | Hα2 | Hβ | Hβ1 | Hβ2 | Hγ | Hγ1 | Hγ2 | Hδ | Hδ1 | Hδ2 | HN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **G1** | 3.51 | - | - | - | - | - | - | - | - | - | - | - | 8.40 |
| **$^D$P2** | 4.22 | - | - | - | 1.97 | 1.73 | - | 1.84 | 1.60 | - | 3.50 | 3.00 | - |
| **F3** | 4.78 | - | - | - | 3.01 | 2.90 | - | - | - | - | - | - | 8.71 |
| **S4** | 4.85 | - | - | - | 3.65 | 3.53 | - | - | - | - | - | - | 8.55 |
| **E5** | 4.56 | - | - | 1.92 | - | - | 2.24 | - | - | - | - | - | 7.51 |
| **D6** | 4.37 | - | - | - | 2.84 | 2.70 | - | - | - | - | - | - | 8.20 |
| **L7** | 3.65 | - | - | - | 1.62 | 1.43 | 1.25 | - | - | - | 0.83 | 0.77 | 8.56 |
| **F(Cl)8** | 4.54 | - | - | - | 3.08 | 2.88 | - | - | - | - | - | - | 8.30 |
| **S9** | 4.70 | - | - | - | 3.61 | 3.47 | - | - | - | - | - | - | 8.52 |
| **F10** | 4.78 | - | - | - | 2.99 | 2.88 | - | - | - | - | - | - | 7.70 |

**Table S3**. $^1$H NMR chemical shift assignment (δ, ppm) for Peptide **3** at 25 °C in DMSO-$d_6$.

| Residue | Hα | Hα1 | Hα2 | Hβ | Hβ1 | Hβ2 | Hγ | Hγ1 | Hγ2 | Hδ | Hδ1 | Hδ2 | HN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **G1** | 3.52 | - | - | - | - | - | - | - | - | - | - | - | 8.41 |
| **$^D$P2** | 4.22 | - | - | - | 1.96 | 1.73 | - | 1.83 | 1.60 | - | 3.49 | 2.98 | - |
| **F3** | 4.79 | - | - | - | 3.00 | 2.89 | - | - | - | - | - | - | 8.71 |
| **S4** | 4.86 | - | - | - | 3.65 | 3.52 | - | - | - | - | - | - | 8.57 |
| **E5** | 4.56 | - | - | 1.92 | - | - | 2.24 | - | - | - | - | - | 7.50 |
| **D6** | 4.37 | - | - | - | 2.84 | 2.70 | - | - | - | - | - | - | 8.21 |
| **L7** | 3.64 | - | - | - | 1.63 | 1.42 | 1.23 | - | - | 0.83 | 0.77 | - | 8.56 |
| **F(Br)8** | 4.54 | - | - | - | 3.05 | 2.86 | - | - | - | - | - | - | 8.29 |
| **S9** | 4.71 | - | - | - | 3.60 | 3.46 | - | - | - | - | - | - | 8.53 |
| **F10** | 4.78 | - | - | - | 2.99 | 2.88 | - | - | - | - | - | - | 7.70 |

**Table S4.** [1]H NMR chemical shift assignment (δ, ppm) for Peptide **4** at 25 °C in DMSO-$d_6$.

| Residue | Hα | Hα1 | Hα2 | Hβ | Hβ1 | Hβ2 | Hγ | Hγ1 | Hγ2 | Hδ | Hδ1 | Hδ2 | HN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **G1** | - | 3.60 | 3.52 | - | - | - | - | - | - | - | - | - | 8.47 |
| **$^{D}$P2** | 4.20 | - | - | - | 1.94 | 1.73 | - | 1.82 | 1.59 | - | 3.49 | 2.97 | - |
| **F3** | 4.68 | - | - | - | 2.98 | 2.86 | - | - | - | - | - | - | 8.61 |
| **S4** | 4.70 | - | - | - | 3.68 | 3.53 | - | - | - | - | - | - | 8.36 |
| **W5** | 4.67 | - | - | 3.11 | - | - | - | - | - | - | - | - | 7.86 |
| **D6** | 4.33 | - | - | - | 2.77 | 2.62 | - | - | - | - | - | - | 8.10 |
| **L7** | 3.64 | - | - | - | 1.59 | 1.40 | 1.13 | - | - | - | 0.73 | 0.71 | 8.42 |
| **W(Cl)8** | 4.57 | - | - | - | 3.20 | 3.00 | - | - | - | - | - | - | 8.50 |
| **S9** | 4.80 | - | - | - | 3.69 | 3.52 | - | - | - | - | - | - | 8.28 |
| **F10** | 4.77 | - | - | 2.98 | - | - | - | - | - | - | - | - | 7.70 |

**Table S5**. $^{3}J_{CH\alpha,NH}$ for Peptides **1-4**[a].

| | $^{3}J_{CH\alpha,NH}$ | | | |
|---|---|---|---|---|
| **Residue[b]** | **Peptide 1** | **Peptide 2** | **Peptide 3** | **Peptide 4** |
| **1** | - | - | - | - |
| **2** | - | - | - | - |
| **3** | - | 7.8 | 7.5 | - |
| **4** | - | - | 7.3 | 7.4 |
| **5** | 8.4 | 7.4 | 8.2 | - |
| **6** | 7.7 | 6.2 | 8.0 | 6.8 |
| **7** | - | - | - | - |
| **8** | 7.3 | 7.4 | 8.6 | 7.3 |
| **9** | 7.9 | - | 7.3 | 6.6 |
| **10** | 8.9 | 7.5 | 9.2 | 8.5 |

[a] [1]H NMR was recorded on 400 MHz agilent spectrometer. [b]For residue identity see Figure S1.

### *3.2 NOE build-up analysis*

NOESY spectra were recorded on a 900 MHz BRUKER Avance III HD NMR spectrometer equipped with a TCI cryoprobe. NOE build-ups were recorded without solvent suppression with mixing times of 200, 300, 400, 500, 600 and 700 ms. The relaxation delay was set to 2.5 s, and 16 scans were recorded with 16384 points in the direct dimension and 512 points in the indirect dimension. Distances were calculated using geminal methylene protons (1.78 Å) as reference. The NOE peak intensities were calculated using normalisation of both cross peaks and diagonal peaks according to $([\text{cross peak}_1 \times \text{cross peak}_2]/[\text{diagonal peak}_1 \times \text{diagonal peak}_2])^{0.5}$ (table S7, S9, S11 and S13). At least 5 mixing times giving a linear ($R^2$>0.98) initial NOE rate for every distance were used to determine $\sigma_{ij}$ build-up rates (Figures S2–S5) according to the equation $r_{ij}=r_{ref}(\sigma_{ref}/\sigma_{ij})^{(1/6)}$, where $r_{ij}$ is the distance between protons i and j in Ångström and $\sigma_{ij}$ is the normalized intensity obtained from NOESY experiments. The calculated distances are given in Tables S6, S8, S10 and S12.

**Table S6.** Interproton distances (Å) for Peptide **1** derived from NOE build-up measurements.

| | Chemical shift | | | | |
|---|---|---|---|---|---|
| **Residue** | **f1 (ppm)** | **f2 (ppm)** | **σ** | **$R^2$** | **$r_{ij}$ (Å)** |
| E9NH-F10NH | 8.84 | 7.50 | 0.0000452 | 0.98 | 2.93 |
| L7NH-W5NH | 8.73 | 7.83 | 0.0000455 | 0.99 | 2.93 |
| L7NH-D6NH | 8.73 | 8.23 | 0.0004500 | 0.99 | 2.00 |
| D6NH-W(Cl)8α | 8.23 | 4.67 | 0.0001248 | 0.99 | 2.48 |
| W5NH-W(Cl)8α | 7.83 | 4.67 | 0.0000344 | 0.99 | 3.07 |
| W5NH-L7α | 7.83 | 3.48 | 0.0000474 | 0.99 | 2.91 |
| W5NH-E9α | 7.83 | 5.02 | 0.0000598 | 0.99 | 2.80 |
| D6NH-L7α | 8.23 | 3.48 | 0.0006154 | 0.99 | 1.90 |
| W5NH-D6α | 7.83 | 4.27 | 0.0002381 | 0.99 | 2.22 |
| F10NH-P1α | 7.50 | 4.22 | 0.0000486 | 0.99 | 2.90 |
| F10NH-$^{D}$P2α | 7.50 | 4.46 | 0.0000571 | 0.99 | 2.82 |
| F3α-$^{D}$P2α | 4.90 | 4.46 | 0.0000166 | 0.99 | 3.46 |
| W5α-D6α | 4.76 | 4.27 | 0.0000099 | 0.99 | 3.78 |
| W(Cl)8α-L7α | 4.67 | 3.48 | 0.0000938 | 0.98 | 2.60 |
| $^{D}$P2α-P1α | 4.46 | 4.22 | 0.0000164 | 0.98 | 3.47 |
| D6α-L7α | 4.27 | 3.48 | 0.0000337 | 0.98 | 3.08 |
| D6NH-W5α | 8.23 | 4.76 | 0.0000407 | 0.99 | 2.98 |
| E9α-W(Cl)8α | 5.02 | 4.67 | 0.0000364 | 0.99 | 3.04 |
| L7β1-L7β2 (ref) | 1.62 | 1.41 | 0.0009032 | 0.99 | 1.78 |

**Table S7.** Normalized peak areas for Peptide **1** derived from NOE build-up measurements.

| | Normalized peak area/mixing time | | | | | |
|---|---|---|---|---|---|---|
| **Residue** | **200 ms** | **300 ms** | **400 ms** | **500 ms** | **600 ms** | **700 ms** |
| E9NH-F10NH | 0.003676 | 0.006614 | 0.010331 | 0.014581 | 0.019880 | 0.026507 |
| L7NH-W5NH | 0.008670 | 0.011161 | 0.016680 | 0.021104 | 0.026460 | - |
| L7NH-D6NH | 0.304476 | 0.351683 | 0.398150 | 0.449640 | 0.480479 | - |
| D6NH-W(Cl)8α | 0.037983 | 0.048780 | 0.060440 | 0.074964 | 0.087290 | - |
| W5NH-W(Cl)8α | 0.006351 | 0.008505 | 0.012823 | 0.015759 | 0.019949 | - |
| W5NH-L7α | 0.008513 | 0.012733 | 0.017819 | 0.021701 | 0.027724 | - |
| W5NH-E9α | 0.010590 | 0.015452 | 0.021750 | 0.027487 | 0.034457 | - |
| D6NH-L7α | 0.570301 | 0.644136 | 0.713095 | 0.774281 | 0.812929 | - |
| W5NH-D6α | 0.082148 | 0.108857 | 0.133530 | 0.154283 | 0.178480 | - |
| F10NH-P1α | 0.008787 | 0.014200 | 0.018469 | 0.023392 | 0.028705 | 0.033151 |
| F10NH-$^{D}$P2α | 0.006794 | 0.010958 | 0.016328 | 0.021977 | 0.028532 | 0.035073 |
| F3α-$^{D}$P2α | 0.001741 | 0.003212 | 0.004736 | 0.006248 | 0.008544 | - |
| W5α-D6α | 0.001812 | 0.002673 | 0.003446 | 0.004574 | 0.005814 | 0.009825 |
| W(Cl)8α-L7α | 0.006065 | 0.011901 | 0.019830 | 0.029498 | 0.040132 | 0.052882 |
| $^{D}$P2α-P1α | 0.002519 | 0.003352 | 0.005215 | 0.006710 | 0.009022 | - |
| D6α-L7α | 0.003107 | 0.004832 | 0.007825 | 0.011412 | 0.015407 | 0.019652 |
| D6NH-W5α | 0.009749 | 0.012945 | 0.017230 | 0.021418 | 0.025856 | - |
| E9α-W(Cl)8α | 0.004633 | 0.007770 | 0.010307 | 0.014250 | 0.018426 | 0.022959 |
| L7β1-L7β2 | 0.283505 | 0.401429 | 0.509532 | 0.598927 | 0.672235 | 0.735364 |

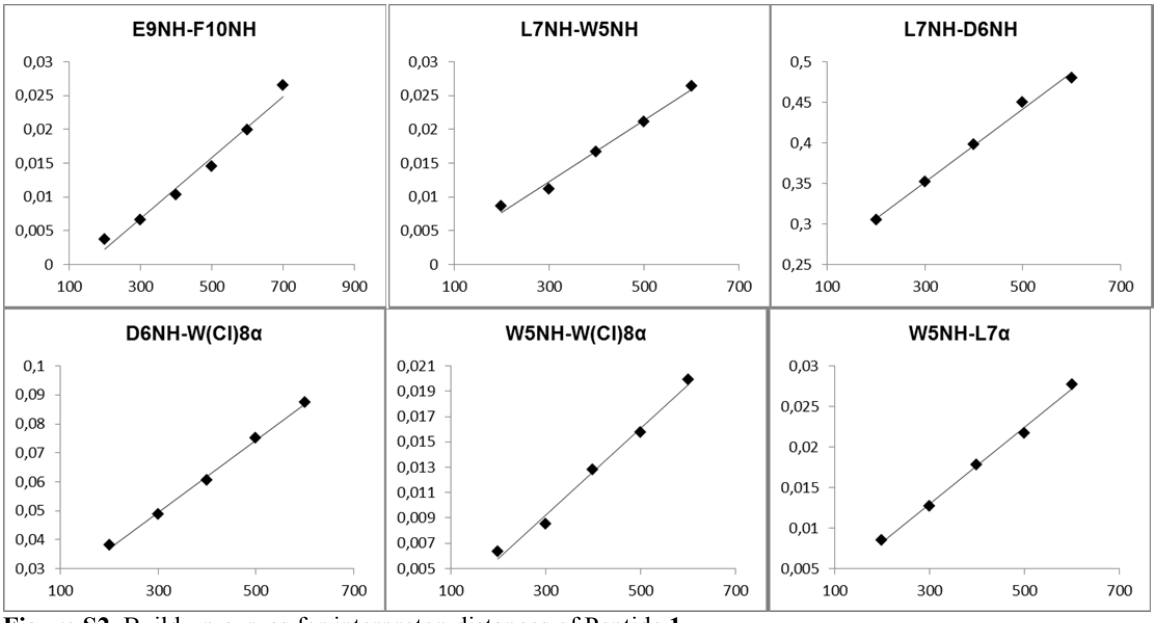



**Figure S2**. Build-up curves for interproton distances of Peptide **1**.

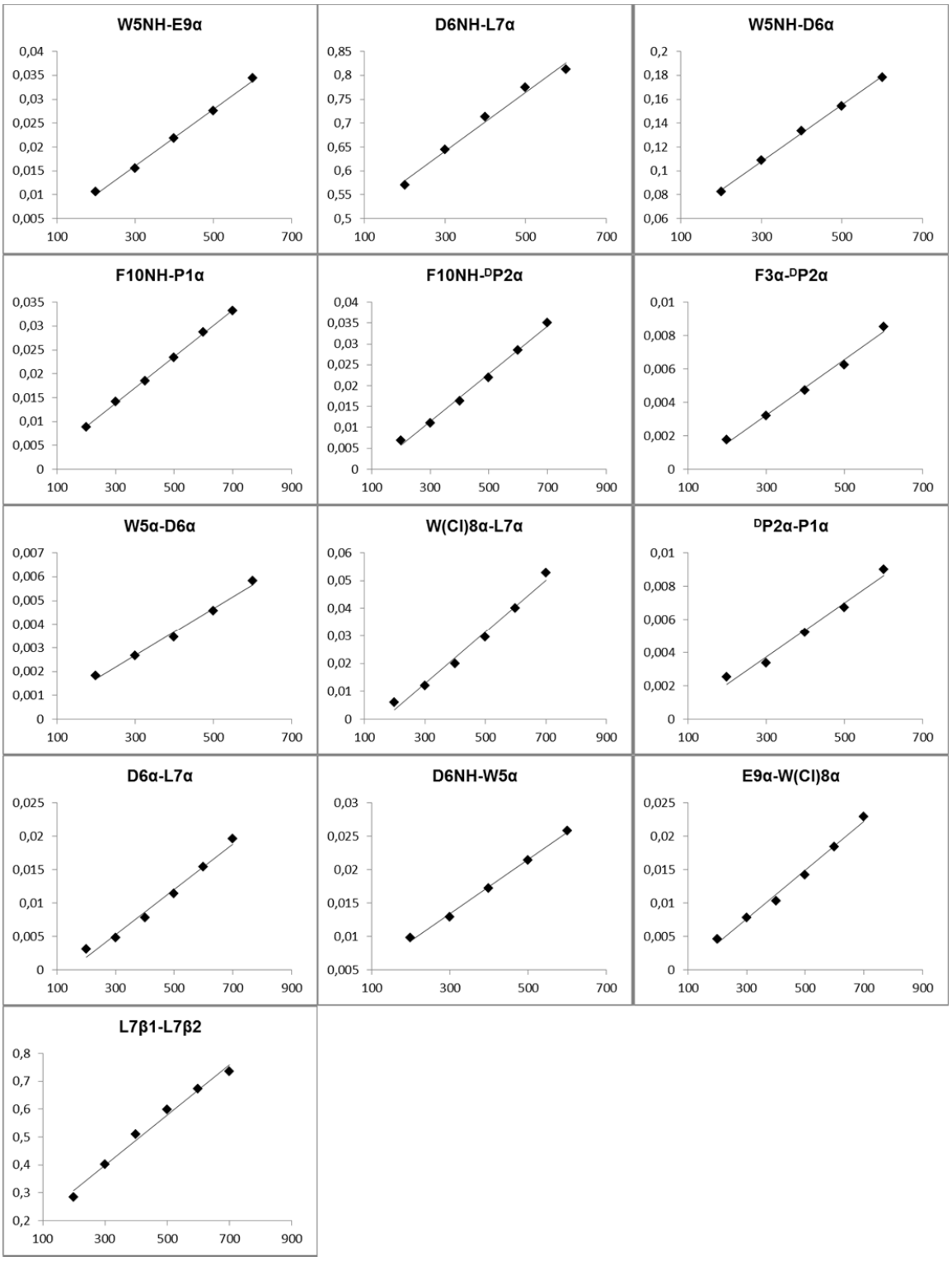



**Figure S2 continued**. Build-up curves for interproton distances of Peptide **1**.

**Table S8**. Interproton distances (Å) for Peptide **2** derived from NOE build-up measurements.

| | **Chemical shift** | | | | |
|---|---|---|---|---|---|
| **Residue** | **f1 (ppm)** | **f2(ppm)** | **σ** | **$R^2$** | **$r_{ij}$ (Å)** |
| F3NH-$^{D}$P2α | 8.71 | 4.22 | 0.0000089 | 0.99 | 3.71 |
| F3NH-S9α | 8.71 | 4.70 | 0.0001751 | 0.99 | 2.26 |
| F3NH-G1NH | 8.71 | 8.40 | 0.0000202 | 0.99 | 3.24 |
| F3NH-F10NH | 8.71 | 7.70 | 0.0000599 | 0.99 | 2.70 |
| G1NH-F10NH | 8.40 | 7.70 | 0.0001982 | 0.99 | 2.21 |
| G1NH-$^{D}$P2α | 8.40 | 4.22 | 0.0003608 | 0.99 | 2.00 |
| F(Cl)8NH-S4α | 8.30 | 4.85 | 0.0001186 | 0.99 | 2.41 |
| F(Cl)8NH-E5NH | 8.30 | 7.51 | 0.0000680 | 0.99 | 2.64 |
| D6NH-E5NH | 8.20 | 7.51 | 0.0002337 | 0.99 | 2.15 |
| F10NH-$^{D}$P2α | 7.70 | 4.22 | 0.0000433 | 0.99 | 2.85 |
| E5NH-D6α | 7.51 | 4.37 | 0.0001395 | 0.99 | 2.35 |
| S4α-S9α | 4.85 | 4.70 | 0.0002180 | 0.99 | 2.18 |
| S9β1-S9β2 (ref) | 3.61 | 3.47 | 0.0007310 | 0.99 | 1.78 |

**Table S9.** Normalized peak areas for Peptide **2** derived from NOE build-up measurements.

| | Normalized peak area/mixing time | | | | | |
|---|---|---|---|---|---|---|
| **Residue** | **200 ms** | **300 ms** | **400 ms** | **500 ms** | **600 ms** | **700 ms** |
| F3NH-$^{D}$P2α | 0.001091 | 0.001923 | 0.002592 | 0.003591 | 0.004800 | 0.005413 |
| F3NH-S9α | 0.022648 | 0.040224 | 0.051892 | 0.072932 | 0.091253 | 0.110373 |
| F3NH-G1NH | 0.005226 | 0.006560 | 0.008722 | 0.010733 | 0.012802 | 0.015252 |
| F3NH-F10NH | 0.011379 | 0.016289 | 0.022806 | 0.029153 | 0.034186 | 0.041271 |
| G1NH-F10NH | 0.042789 | 0.062916 | 0.081244 | 0.102129 | 0.124431 | 0.140432 |
| G1NH-$^{D}$P2α | 0.068008 | 0.106573 | 0.137844 | 0.177498 | 0.213902 | 0.248244 |
| F(Cl)8NH-S4α | 0.014332 | 0.023674 | 0.033757 | 0.047318 | 0.059102 | 0.073378 |
| F(Cl)8NH-E5NH | 0.013998 | 0.020998 | 0.027428 | 0.035165 | 0.042526 | 0.047127 |
| D6NH-E5NH | 0.049698 | 0.073096 | 0.096578 | 0.121282 | 0.145224 | 0.165041 |
| F10NH-$^{D}$P2α | 0.005249 | 0.008838 | 0.012672 | 0.017017 | 0.021953 | 0.026835 |
| E5NH-D6α | 0.023246 | 0.036828 | 0.049747 | 0.063895 | 0.078816 | 0.092845 |
| S4α-S9α | 0.094584 | 0.118538 | 0.134766 | 0.161849 | 0.184167 | 0.202367 |
| S9β1-S9β2 | 0.238014 | 0.307828 | 0.403515 | 0.474851 | 0.531269 | 0.601371 |

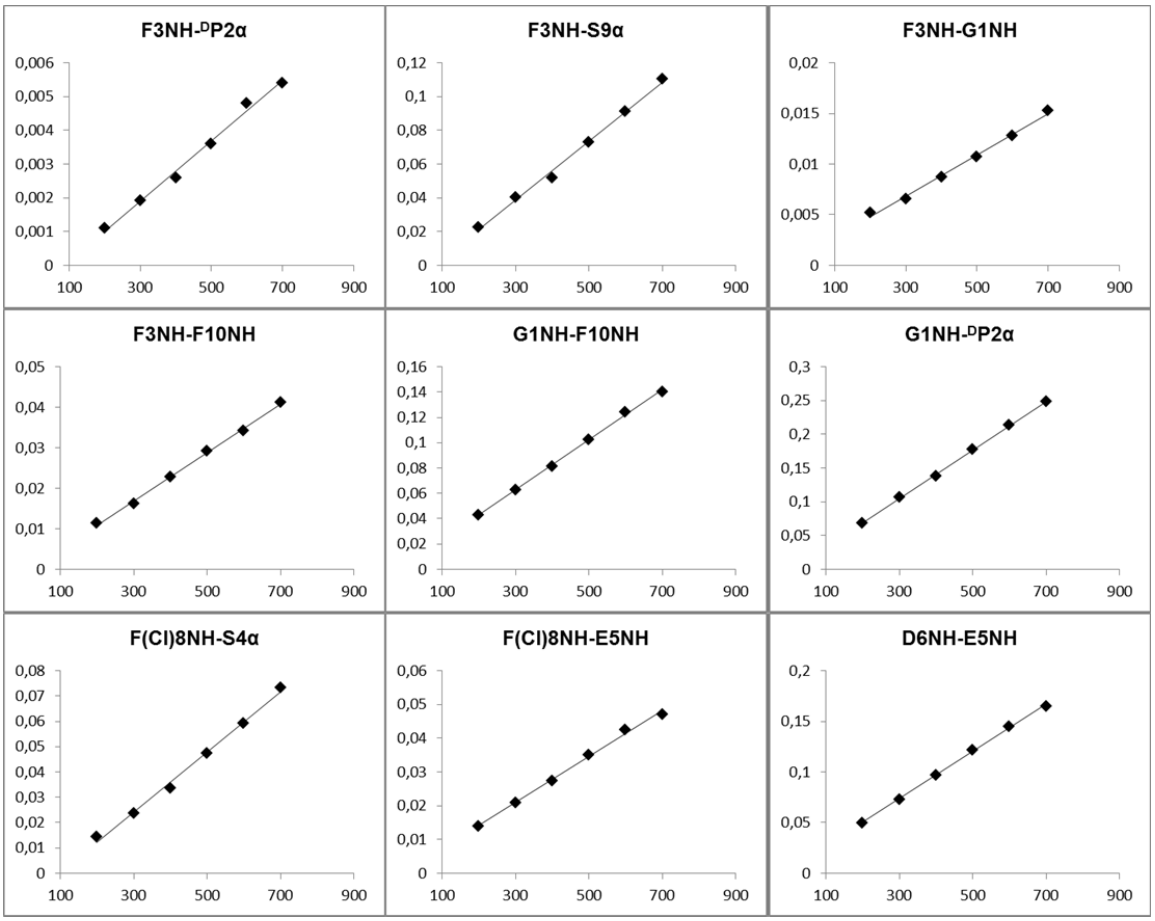



**Figure S3**. Build-up curves for interproton distances of Peptide **2**.

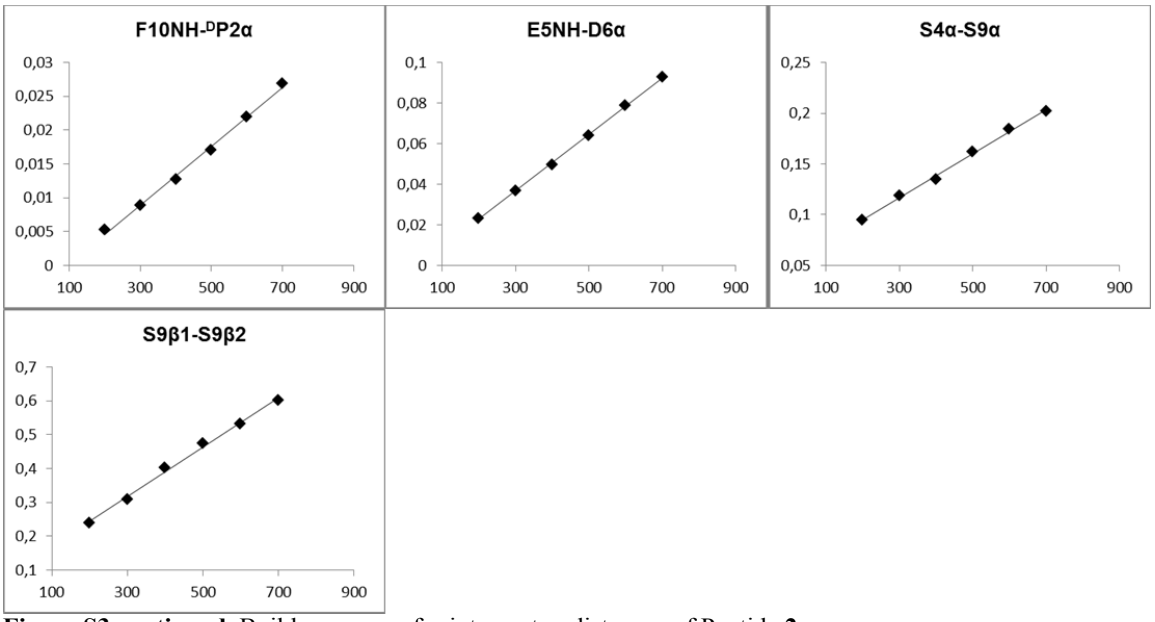



**Figure S3 continued**. Build-up curves for interproton distances of Peptide **2**.

**Table S10.** Interproton distances (Å) for Peptide **3** derived from NOE build-up measurements.

| | Chemical shift | | | | |
|---|---|---|---|---|---|
| **Residues** | **f1 (ppm)** | **f2 (ppm)** | **σ** | **$R^2$** | **$r_{ij}$ (Å)** |
| F3NH-S9α | 8.71 | 4.71 | 0.0001876 | 0.99 | 2.28 |
| F3NH-F(Br)8NH | 8.71 | 8.29 | 0.0000420 | 0.99 | 2.92 |
| F3NH-$^{D}$P2α | 8.71 | 4.22 | 0.0000070 | 0.99 | 3.94 |
| F3NH-S9NH | 8.71 | 8.53 | 0.0000198 | 0.98 | 3.31 |
| F3NH-G1NH | 8.71 | 8.41 | 0.0000232 | 0.99 | 3.22 |
| F3NH-F10NH | 8.71 | 7.70 | 0.0000694 | 0.99 | 2.69 |
| S9NH-S4α | 8.53 | 4.86 | 0.0000222 | 0.99 | 3.25 |
| G1NH-$^{D}$P2α | 8.41 | 4.22 | 0.0002927 | 0.99 | 2.11 |
| G1NH-F10NH | 8.41 | 7.70 | 0.0001996 | 0.99 | 2.25 |
| F(Br)8NH-D6NH | 8.29 | 8.21 | 0.0000181 | 0.99 | 3.36 |
| F(Br)8NH-E5NH | 8.29 | 7.50 | 0.0000741 | 0.99 | 2.66 |
| F(Br)8NH-S4α | 8.29 | 4.86 | 0.0001027 | 0.99 | 2.52 |
| F(Br)8NH-D6α | 8.29 | 4.37 | 0.0000125 | 0.99 | 3.57 |
| D6NH-E5NH | 8.21 | 7.50 | 0.0002374 | 0.99 | 2.19 |
| F10NH-$^{D}$P2α | 7.70 | 4.22 | 0.0000484 | 0.99 | 2.85 |
| F10NH-S4α | 7.70 | 4.86 | 0.0000207 | 0.98 | 3.29 |
| E5NH-S9α | 7.50 | 4.71 | 0.0000600 | 0.99 | 2.75 |
| D6β2-D6β1 (ref) | 2.84 | 2.70 | 0.0008186 | 0.99 | 1.78 |

**Table S11.** Normalized peak areas for Peptide **3** derived from NOE build up measurements.

| | Normalized peak area/mixing time | | | | | |
|---|---|---|---|---|---|---|
| **Residues** | **200 ms** | **300 ms** | **400 ms** | **500 ms** | **600 ms** | **700 ms** |
| F3NH-S9α | 0.055364 | 0.073648 | 0.092856 | 0.111903 | 0.130058 | - |
| F3NH-F(Br)8NH | 0.003926 | 0.006688 | 0.010421 | 0.014390 | 0.019198 | 0.025008 |
| F3NH-$^{D}$P2α | 0.000770 | 0.001323 | 0.001872 | 0.002513 | 0.003403 | 0.004264 |
| F3NH-S9NH | 0.003851 | 0.005218 | 0.007163 | 0.008774 | 0.010940 | 0.013940 |
| F3NH-G1NH | 0.004296 | 0.006476 | 0.008372 | 0.011040 | 0.013543 | 0.015777 |
| F3NH-F10NH | 0.012996 | 0.019566 | 0.026366 | 0.033528 | 0.040296 | 0.047702 |
| S9NH-S4α | 0.002537 | 0.004309 | 0.006013 | 0.008289 | 0.010665 | 0.013831 |
| G1NH-$^{D}$P2α | 0.058054 | 0.088537 | 0.116189 | 0.147681 | 0.176909 | 0.203604 |
| G1NH-F10NH | 0.037661 | 0.057416 | 0.076123 | 0.097398 | 0.117327 | 0.137153 |
| F(Br)8NH-D6NH | 0.010021 | 0.011450 | 0.012984 | 0.015776 | 0.016656 | 0.019014 |
| F(Br)8NH-E5NH | 0.013577 | 0.020646 | 0.027732 | 0.035211 | 0.042558 | 0.050776 |
| F(Br)8NH-S4α | 0.012247 | 0.020222 | 0.029163 | 0.039390 | 0.050607 | 0.063888 |
| F(Br)8NH-D6α | 0.001704 | 0.002678 | 0.003835 | 0.004983 | 0.006523 | 0.007909 |
| D6NH-E5NH | 0.047289 | 0.067751 | 0.095958 | 0.114534 | 0.138024 | 0.167597 |
| F10NH-$^{D}$P2α | 0.005987 | 0.009805 | 0.014212 | 0.018606 | 0.024324 | 0.030286 |
| F10NH-S4α | 0.001615 | 0.002967 | 0.004425 | 0.006924 | 0.008933 | 0.012057 |
| E5NH-S9α | 0.012678 | 0.018046 | 0.023922 | 0.029945 | 0.036717 | - |
| D6β2-D6β1 | 0.206016 | 0.277741 | 0.372328 | 0.423559 | 0.543374 | 0.609443 |

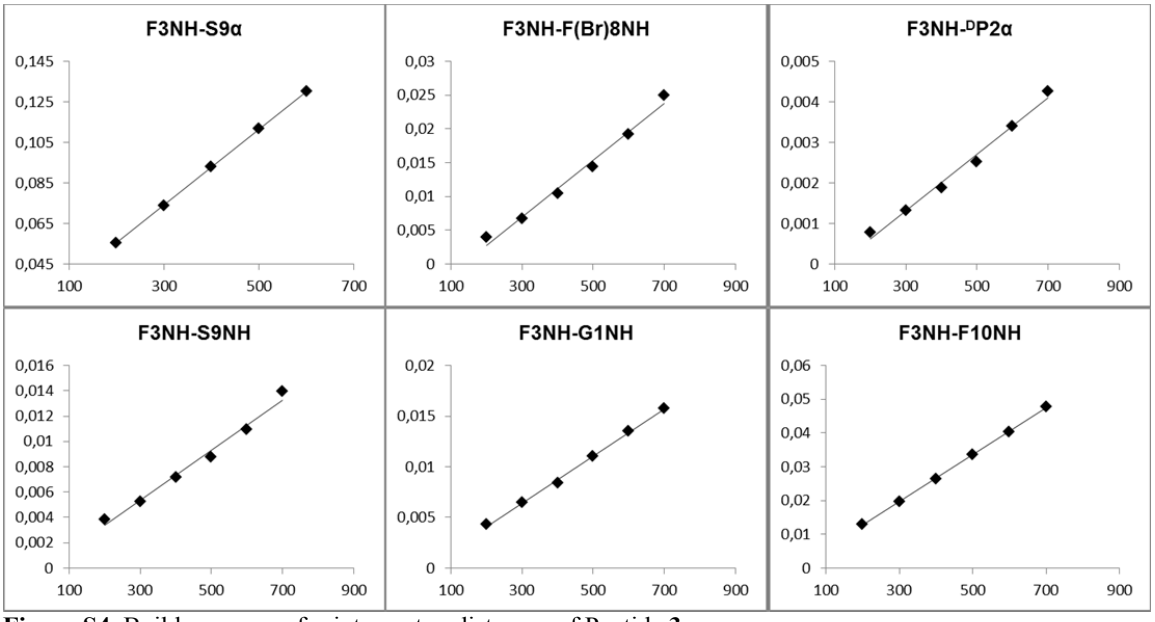



**Figure S4.** Build-up curves for interproton distances of Peptide **3**.

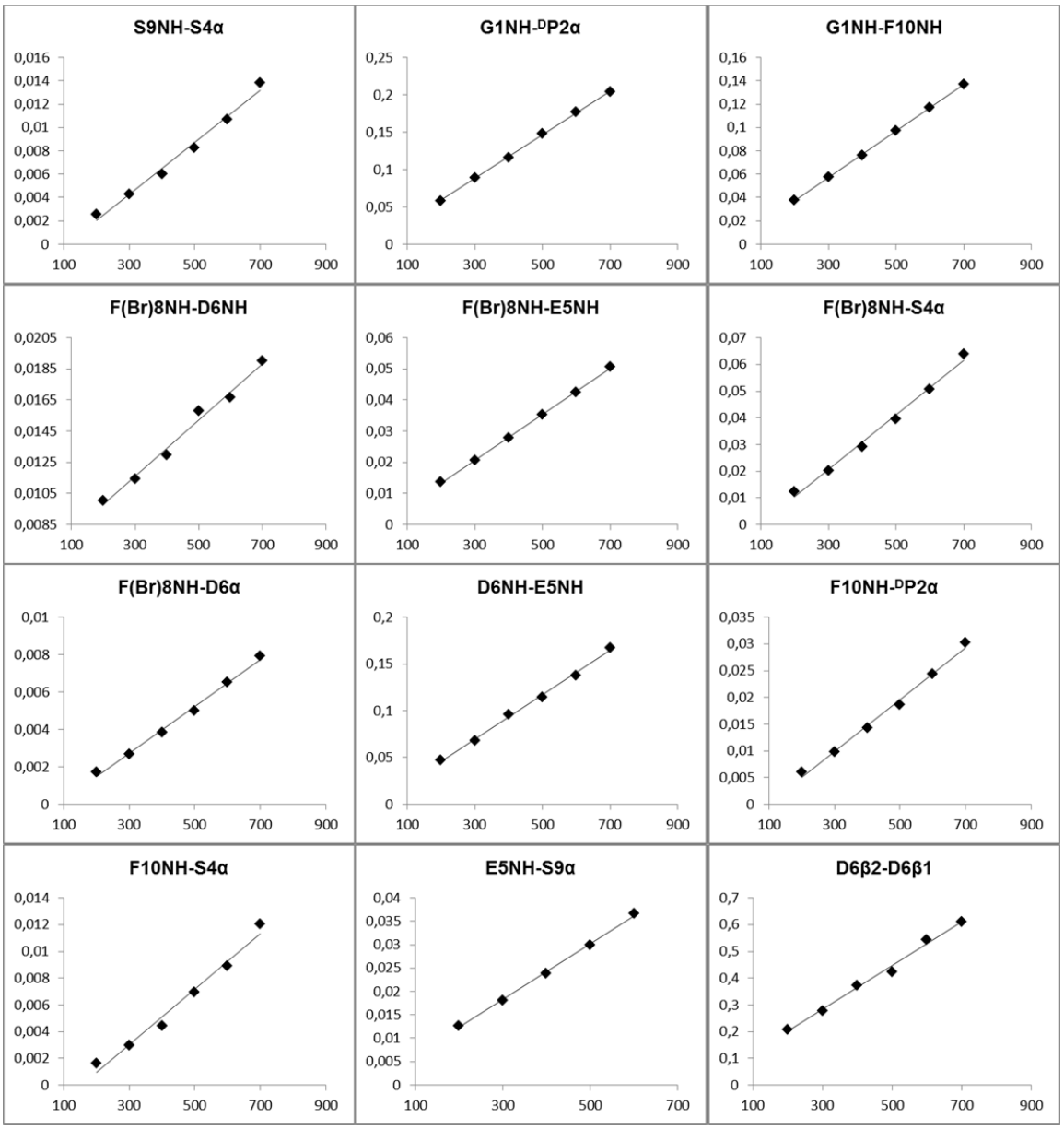



**Figure S4 continued**. Build-up curves for interproton distances of Peptide **3**.

**Table S12**. Interproton distances (Å) for Peptide **4** derived from NOE build-up measurements.

| | Chemical shift | | | | |
|---|---|---|---|---|---|
| **Residues** | **f1 (ppm)** | **f2(ppm)** | **σ** | **$R^2$** | **$r_{ij}$ (Å)** |
| F3NH-S4NH | 8.61 | 8.36 | 0.0000687 | 0.99 | 2.67 |
| F3NH-F10NH | 8.61 | 7.70 | 0.0000394 | 0.99 | 2.93 |
| F3NH-F10α | 8.61 | 4.77 | 0.0000984 | 0.99 | 2.52 |
| W(Cl)8NH-W5NH | 8.50 | 7.86 | 0.0000515 | 0.99 | 2.81 |
| W(Cl)8NH-S9NH | 8.50 | 8.28 | 0.0000535 | 0.99 | 2.79 |
| G1NH-F10NH | 8.47 | 7.70 | 0.0001724 | 0.99 | 2.29 |
| G1NH-$^{D}$P2α | 8.47 | 4.20 | 0.0002927 | 0.99 | 2.10 |
| L7NH-D6NH | 8.42 | 8.10 | 0.0001391 | 0.99 | 2.38 |
| L7NH-W(Cl)8α | 8.42 | 4.57 | 0.0003536 | 0.99 | 2.04 |
| S4NH-W5NH | 8.36 | 7.86 | 0.0000868 | 0.99 | 2.57 |
| S9NH-F10NH | 8.28 | 7.70 | 0.0000981 | 0.99 | 2.52 |
| S9NH-F10α | 8.28 | 4.77 | 0.0004976 | 0.99 | 1.92 |
| D6NH-W5NH | 8.10 | 7.86 | 0.0002145 | 0.99 | 2.21 |
| D6NH-L7α | 8.10 | 3.64 | 0.0001356 | 0.99 | 2.39 |
| W5NH-D6α | 7.86 | 4.33 | 0.0001919 | 0.99 | 2.25 |
| F10NH-$^{D}$P2α | 7.70 | 4.20 | 0.0000469 | 0.99 | 2.85 |
| $^{D}$P2β1-$^{D}$P2β2 (ref) | 1.94 | 1.73 | 0.0007898 | 0.99 | 1.78 |

**Table S13.** Normalized peak areas for Peptide **4** derived from NOE build-up measurements.

| | Normalized peak area/mixing time | | | | | |
|---|---|---|---|---|---|---|
| **Residues** | **200 ms** | **300 ms** | **400 ms** | **500 ms** | **600 ms** | **700 ms** |
| F3NH-S4NH | 0.019706 | 0.024698 | 0.031001 | 0.037043 | 0.045550 | 0.054056 |
| F3NH-F10NH | 0.007161 | 0.010912 | 0.014694 | 0.018533 | 0.022434 | 0.027063 |
| F3NH-F10α | 0.015090 | 0.024592 | 0.033345 | 0.043848 | 0.053484 | 0.064530 |
| W(Cl)8NH-W5NH | 0.011093 | 0.016231 | 0.021185 | 0.026366 | 0.031459 | 0.036943 |
| W(Cl8)NH-S9NH | 0.019089 | 0.022862 | 0.027040 | 0.032977 | 0.039052 | 0.045655 |
| G1NH-F10NH | 0.039778 | 0.055954 | 0.072931 | 0.090975 | 0.107487 | 0.125941 |
| G1NH-$^{D}$P2α | 0.057783 | 0.087100 | 0.116133 | 0.145164 | 0.174797 | 0.204230 |
| L7NH-D6NH | 0.028346 | 0.039540 | 0.053460 | 0.066714 | 0.082394 | 0.097371 |
| L7NH-W(Cl)8α | 0.070457 | 0.106652 | 0.141737 | 0.175731 | 0.211421 | 0.248307 |
| S4NH-W5NH | 0.013141 | 0.020565 | 0.028475 | 0.036718 | 0.046230 | 0.056875 |
| S9NH-F10NH | 0.017869 | 0.026703 | 0.035946 | 0.045852 | 0.055845 | 0.067058 |
| S9NH-F10α | 0.095738 | 0.145862 | 0.195816 | 0.244170 | 0.295953 | 0.344305 |
| D6NH-W5NH | 0.052826 | 0.072088 | 0.093431 | 0.115099 | 0.136960 | 0.159720 |
| D6NH-L7α | 0.041721 | 0.058146 | 0.078889 | 0.102721 | 0.124632 | 0.151871 |
| W5NH-D6α | 0.031728 | 0.050475 | 0.069243 | 0.087635 | 0.107771 | 0.127992 |
| F10NH-$^{D}$P2α | 0.005359 | 0.009234 | 0.013258 | 0.017890 | 0.023099 | 0.028959 |
| $^{D}$P2β1-$^{D}$P2β2 | 0.205122 | 0.296898 | 0.384428 | 0.463484 | 0.535165 | 0.599223 |

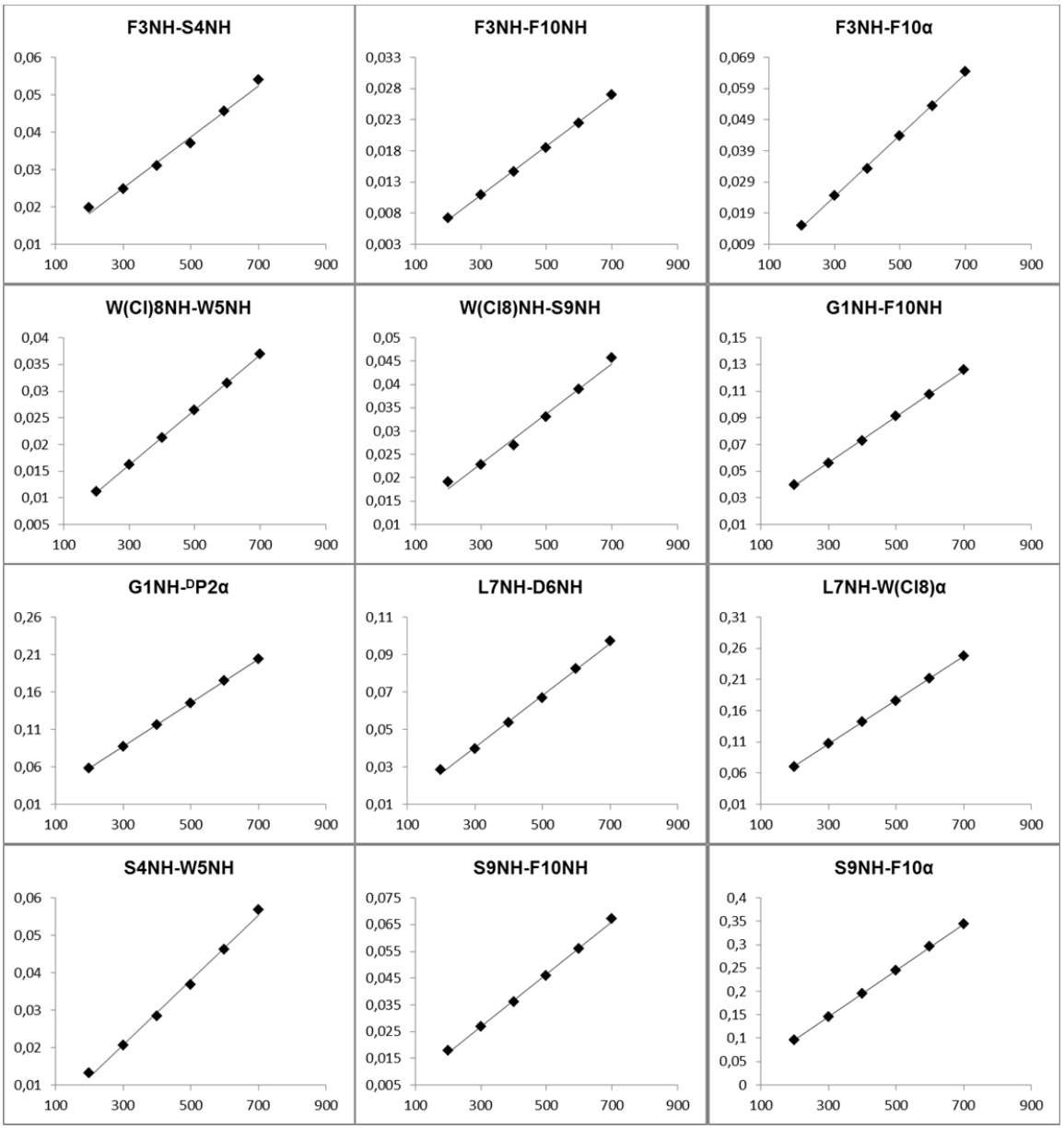



**Figure S5**. Build-up curves for interproton distances of Peptide **4**.

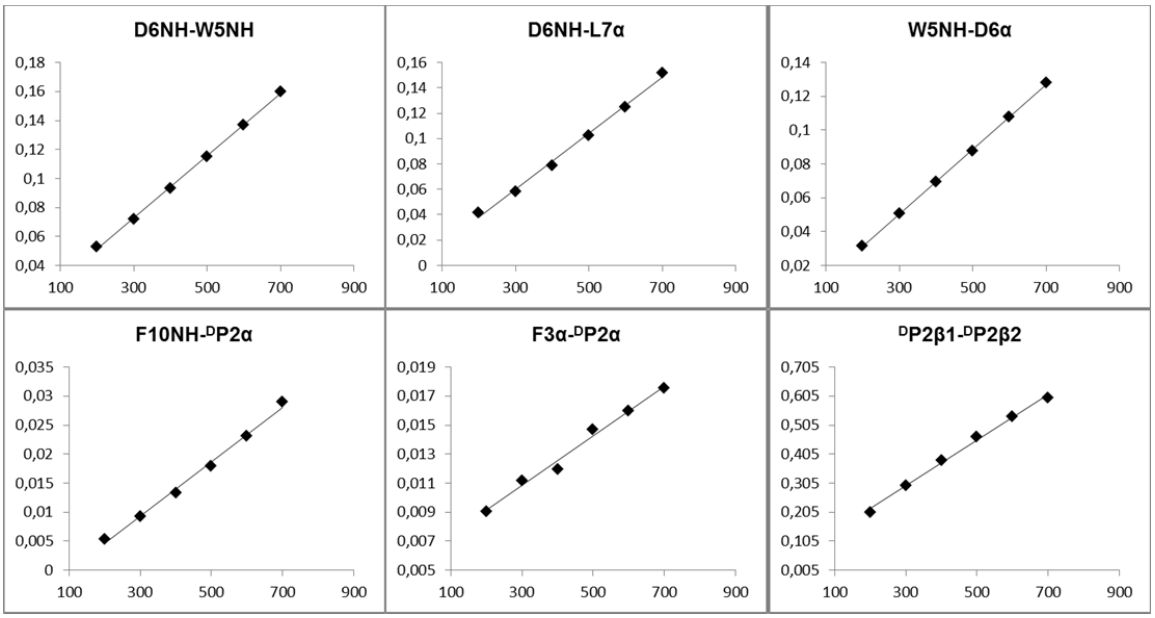



**Figure S5 continued**. Build-up curves for interproton distances of Peptide **4**.

### *3.3 Amide temperature coefficients*

Amide temperature coefficients, $\Delta\delta_{NH}/\Delta T$ (ppb $K^{-1}$), were obtained by running $^1H$ and TOCSY spectra at 25, 30, 35, 40 and 45 °C on a 500 MHz or 600 MHz Varian spectrometer in DMSO-$d_6$. The amide temperature coefficient ($\Delta\delta_{NH}/\Delta T$) were obtained from $(\delta_{T,high}-\delta_{T,low})/(T_{high}-T_{low})$, which provides negative values but are reported as positive values. $\Delta\delta_{NH}/\Delta T < 3$ indicates a strong intramolecular hydrogen bond, $\Delta\delta_{NH}/\Delta T = 3–5$ indicates that the amide proton is in equilibrium between a solvent exposed and an intramolecular hydrogen bond and $\Delta\delta_{NH}/\Delta T >5$ that the amide proton is solvent exposed.[2] Plotting δNH against temperature gave $R^2 > 0.99$ for all amide protons. The DMSO-$d_6$ residual solvent signal was used as chemical shift reference, with its temperature dependence taken in consideration following published calibration data.[3] The results are summarized in Tables S14–S17.

**Table S14**. Amide proton temperature coefficients $\Delta\delta_{NH}/\Delta T$ (ppb $K^{-1}$) in DMSO-$d_6$ for Peptide **1**.

| | Chemical shift (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Temp. (°C)** | **F3** | **E4** | **W5** | **D6** | **L7** | **W(Cl)8** | **E9** | **F10** |
| 25 | 8.527 | 8.510 | 7.754 | 8.161 | 8.664 | 8.510 | 8.773 | 7.422 |
| 30 | 8.500 | 8.484 | 7.731 | 8.110 | 8.628 | 8.484 | 8.729 | 7.404 |
| 35 | 8.470 | 8.433 | 7.706 | 8.059 | 8.584 | 8.452 | 8.683 | 7.385 |
| 40 | 8.442 | 8.384 | 7.682 | 8.010 | 8.542 | 8.415 | 8.638 | 7.367 |
| 45 | 8.414 | 8.335 | 7.659 | 7.962 | 8.500 | 8.377 | 8.592 | 7.349 |
| | **F3** | **E4** | **W5** | **D6** | **L7** | **W(Cl)8** | **E9** | **F10** |
| **ΔδNH** | 0.113 | 0.175 | 0.095 | 0.199 | 0.164 | 0.133 | 0.181 | 0.073 |
| **ΔδNH/ΔT** | 5.7 | 8.8 | 4.8 | 10.0 | 8.2 | 6.7 | 9.1 | 3.7 |

**Table S15.** Amide proton temperature coefficients $\Delta\delta_{NH}/\Delta T$ (ppb $K^{-1}$) in DMSO-$d_6$ for Peptide **2**.

| | Chemical shift (ppm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Temp. (°C)** | **G1** | **F3** | **S4** | **E5** | **D6** | **L7** | **F(Cl)8** | **S9** | **F10** |
| 25 | 8.333 | 8.651 | 8.473 | 7.445 | 8.127 | 8.486 | 8.231 | 8.433 | 7.640 |
| 30 | 8.283 | 8.615 | 8.426 | 7.429 | 8.089 | 8.441 | 8.200 | 8.379 | 7.623 |
| 35 | 8.237 | 8.578 | 8.379 | 7.413 | 8.050 | 8.395 | 8.169 | 8.326 | 7.606 |
| 40 | 8.179 | 8.540 | 8.332 | 7.397 | 8.019 | 8.348 | 8.137 | 8.274 | 7.588 |
| 45 | 8.146 | 8.502 | 8.284 | 7.381 | 7.985 | 8.300 | 8.106 | 8.223 | 7.570 |
| | | | | | | | | | |
| $\Delta\delta_{NH}$ | 0.187 | 0.149 | 0.189 | 0.064 | 0.142 | 0.186 | 0.125 | 0.210 | 0.070 |
| $\Delta\delta_{NH}/\Delta T$ | 9.3 | 7.4 | 9.4 | 3.2 | 7.1 | 9.3 | 6.2 | 10.5 | 3.5 |

**Table S16.** Amide proton temperature coefficients $\Delta\delta_{NH}/\Delta T$ (ppb $K^{-1}$) in DMSO-$d_6$ for Peptide **3**.

| | Chemical shift (ppm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Temp. (°C)** | **G1** | **F3** | **S4** | **E5** | **D6** | **L7** | **F(Br)8** | **S9** | **F10** |
| 25 | 8.349 | 8.657 | 8.493 | 7.422 | 8.141 | 8.503 | 8.223 | 8.459 | 7.624 |
| 30 | 8.309 | 8.620 | 8.449 | 7.407 | 8.105 | 8.460 | 8.193 | 8.411 | 7.606 |
| 35 | 8.268 | 8.582 | 8.399 | 7.391 | 8.069 | 8.413 | 8.163 | 8.362 | 7.587 |
| 40 | 8.227 | 8.544 | 8.351 | 7.376 | 8.033 | 8.375 | 8.132 | 8.313 | 7.569 |
| 45 | 8.186 | 8.505 | 8.302 | 7.360 | 7.997 | 8.332 | 8.101 | 8.263 | 7.550 |
| | | | | | | | | | |
| | **G1** | **F3** | **S4** | **E5** | **D6** | **L7** | **F(Br)8** | **S9** | **F10** |
| $\Delta\delta_{NH}$ | 0.163 | 0.152 | 0.191 | 0.062 | 0.144 | 0.171 | 0.122 | 0.196 | 0.074 |
| $\Delta\delta_{NH}/\Delta T$ | 8.1 | 7.6 | 9.6 | 3.1 | 7.2 | 8.6 | 6.1 | 9.8 | 3.7 |

**Table S17**. Amide proton temperature coefficients $\Delta\delta_{NH}/\Delta T$ (ppb $K^{-1}$) in DMSO-$d_6$ for Peptide **4**.

| | Chemical shift (ppm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Temp. (°C)** | **G1** | **F3** | **S4** | **W5** | **D6** | **L7** | **W(Cl)8** | **S9** | **F10** |
| 25 | 8.410 | 8.555 | 8.304 | 7.796 | 8.043 | 8.363 | 8.431 | 8.209 | 7.629 |
| 30 | 8.362 | 8.511 | 8.245 | 7.770 | 8.001 | 8.310 | 8.390 | 8.167 | 7.611 |
| 35 | 8.311 | 8.472 | 8.186 | 7.742 | 7.960 | 8.266 | 8.345 | 8.125 | 7.593 |
| 40 | 8.260 | 8.433 | 8.126 | 7.715 | 7.919 | 8.211 | 8.303 | 8.082 | 7.574 |
| 45 | 8.209 | 8.393 | 8.067 | 7.687 | 7.879 | 8.160 | 8.261 | 8.039 | 7.556 |
| | **G1** | **F3** | **S4** | **W5** | **D6** | **L7** | **W(Cl)8** | **S9** | **F10** |
| $\Delta\delta_{NH}$ | 0.201 | 0.162 | 0.237 | 0.109 | 0.164 | 0.203 | 0.170 | 0.170 | 0.073 |
| $\Delta\delta_{NH}/\Delta T$ | 10.0 | 8.1 | 11.8 | 5.5 | 8.2 | 10.1 | 8.5 | 8.5 | 3.7 |

## 4. Computational conformation analysis

Conformational searches were performed using the Monte Carlo algorithm with intermediate torsion sampling, 50000 Monte Carlo steps, and RMSD cut-off set to 2.0 Å, followed by Molecular Mechanics energy minimization, with the software Macromodel (v.9.1) as implemented in the Schrödinger package. Two independent searches were performed using OPLS-2005 or Amber* as force field, and with water solvation model. Energy minimization was performed using the Polak-Ribiere type conjugate gradient (PRCG) with maximum iteration steps set to 5000. All conformations within 42 kJ/mol from the global minimum were saved. The ensembles from the conformational searches using OPLS-2005 and Amber* were combined and elimination of redundant conformtions by comparsions of the heavy atom coordinates applying the RMSD cutoff 1.5-2.5 Å was preformed. The results are summarized in Table S18.

**Table S18.** Results of the conformational analysis.

| | Number of conformations | | | | |
|---|---|---|---|---|---|
| | Total[a] | Within 12.6 kJ/mol[b] | Within 42.0 kJ/mol[c] | RMSD 1.5 Å[d] | RMSD 2.5 Å[d] |
| Peptide **1** OPLS | 403 | 22 | 402 | 394 | - |
| Amber* | 159 | 14 | 159 | | |
| Peptide **2** OPLS | 208 | 6 | 207 | - | 101 |
| Amber* | 520 | 41 | 518 | | |
| Peptide **3** OPLS | 281 | 20 | 281 | - | 101 |
| Amber* | 292 | 21 | 292 | | |
| Peptide **4** OPLS | 423 | 32 | 423 | 347 | - |
| Amber* | 239 | 39 | 239 | | |

[a]Total number of unique conformations found. The seven conformations with lowest energy were found on average at least seven times for all peptides. [b]Conformations found within 12.6 kJ/mol (3.0 kcal/mol) of the global minimum. [c]Conformations found within 42.0 kJ/mol (10.0 kcal/mol) of the global minimum. [d]Conformations obtained after redundant conformation elimination with the root-mean-square deviation cutoff 1.5 or 2.5 Å for heavy atoms. This conformation ensemble is used as input in the NAMFIS analysis.

## 5. Identification of solution ensembles using the NAMFIS algorithm

Solution ensembles were determined by fitting the experimentally measured distances and coupling constants to those back-calculated for computationally predicted conformations following previously described protocols.[4] Dihedral angles were calculated from experimental

coupling constants using a Karplus equation specifically developed for peptides.[5,6] NOE-derived distances are presented in Tables S6, S8, S10 and S12 whereas coupling constants are presented in Table S5. The results of the NAMFIS-analysis are given in Table S19.

**Table S19.** Results of the NAMFIS-analyses for Peptides **1**-**4**.

| Peptide **1** | | Peptide **2** | | Peptide **3** | | Peptide **4** | |
|---|---|---|---|---|---|---|---|
| Conf. no.[a] | %Population[b] | Conf. no.[a] | %Population[b] | Conf. no.[a] | %Population[b] | Conf. no.[a] | %Population[b] |
| 1 | 6 | 1 | 9 | 1 | 13 | ***1*** | 7 |
| 2 | 38 | ***2*** | 24 | 2 | 13 | 2 | 3 |
| 3 | 3 | 3 | 16 | ***3*** | 27 | ***3*** | 8 |
| 4 | 3 | ***4*** | 32 | 4 | 7 | ***4*** | 10 |
| ***5*** | 18 | 5 | 6 | ***5*** | 13 | 5 | 22 |
| ***6*** | 6 | ***6*** | 5 | ***6*** | 21 | 6 | 14 |
| 7 | 3 | 7 | 7 | 7 | 6 | 7 | 16 |
| 8 | 10 | | | | | ***8*** | 14 |
| 9 | 12 | | | | | 9 | 4 |

[a]The structures of the most populated conformations are shown in Figures S6–S9. Hairpin conformations are indicated by numbers in *italic*. [b] The hairpin population in solution was calculated to 24% for Peptide **1**, 61% for Peptide **2**, 61% for Peptide **3** and 39% for Peptide **4**.

**Table S20.** Experimentally determined and back-calculated (NAMFIS) interproton distances (Å) and coupling constants (Hz) for the solution ensemble of Peptide **1**.

| Assign. | Calculated | Experimental |
|---|---|---|
| Interproton distances (Å) | | |
| E9NH-F10NH | 3.59 | 2.93 |
| L7NH-W5NH | 4.03 | 2.93 |
| L7NH-D6NH | 2.67 | 2.00 |
| D6NH-W(Cl)8α | 3.55 | 2.48 |
| W5NH-W(Cl)8α | 4.67 | 3.07 |
| W5NH-L7α | 4.19 | 2.91 |
| W5NH-E9α | 4.39 | 2.80 |
| D6NH-L7α | 2.42 | 1.90 |
| W5NH-D6α | 2.84 | 2.22 |
| F10NH-P1α | 3.22 | 2.90 |
| F10NH-$^{D}$P2α | 3.66 | 2.82 |
| F3α-$^{D}$P2α | 4.51 | 3.46 |
| W5α-D6α | 4.52 | 3.78 |
| W(Cl)8α-L7α | 4.47 | 2.60 |
| $^{D}$P2α-P1α | 4.62 | 3.47 |
| D6α-L7α | 4.53 | 3.08 |
| D6NH-W5α | 4.66 | 2.98 |
| E9α-W(Cl)8α | 4.49 | 3.04 |
| *RMSD error* | | *1.18* |
| Coupling constants (Hz) | | |
| W5 | 7.3 | 8.4 |
| D6 | 6.6 | 7.7 |
| W(Cl)8 | 6.3 | 7.3 |
| E9 | 8.9 | 7.9 |
| F10 | 8.7 | 8.9 |
| *RMSD error* | | *0.94* |

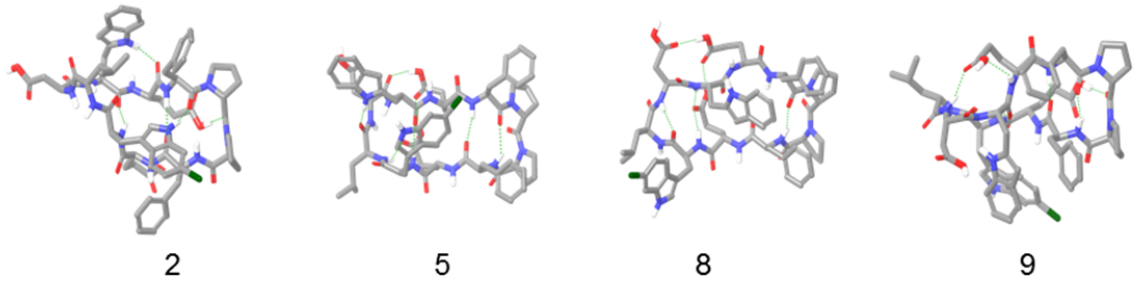



**Figure S6.** The most populated solution conformations of Peptide **1**, as selected by the NAMFIS-analysis. Populations in % are given in Table S19. Hydrogen bonds are indicated by green dotted lines. Non-polar hydrogens are omitted for clarity.

**Table S21**. Experimentally determined and back-calculated (NAMFIS) interproton distances (Å) and coupling constants (Hz) for the solution ensemble of Peptide **2**.

| Assign. | Calculated | Experimental |
|---|---|---|
| Interproton distances (Å) | | |
| F3NH-$^{D}$P2α | 5.05 | 3.71 |
| F3NH-S9α | 2.94 | 2.26 |
| F3NH-G1NH | 3.34 | 3.24 |
| F3NH-F10NH | 3.37 | 2.70 |
| G1NH-F10NH | 2.33 | 2.21 |
| G1NH-$^{D}$P2α | 2.35 | 2.00 |
| F(Cl)8NH-S4α | 2.88 | 2.41 |
| F(Cl)8NH-E5NH | 3.26 | 2.64 |
| D6NH-E5NH | 2.37 | 2.15 |
| F10NH-$^{D}$P2α | 3.70 | 2.85 |
| E5NH-D6α | 2.68 | 2.35 |
| S4α-S9α | 2.35 | 2.18 |
| *RMSD error* | | *0.60* |
| Coupling constants (Hz) | | |
| F3 | 7.1 | 7.8 |
| E5 | 7.5 | 7.4 |
| D6 | 6.7 | 6.2 |
| F(Cl)8 | 7.6 | 7.4 |
| F10 | 8.1 | 7.5 |
| *RMSD error* | | *0.47* |

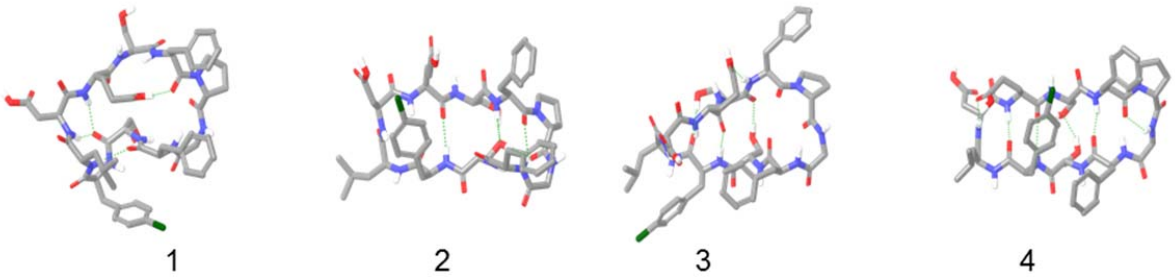



**Figure S7.** The most populated solution conformations of Peptide **2**, as selected by the NAMFIS-analysis. Populations in % are given in Table S19. Hydrogen bonds are indicated by green dotted lines. Non-polar hydrogens are omitted for clarity.

**Table S22.** Experimentally determined and back-calculated (NAMFIS) interproton distances (Å) and coupling constants (Hz) for the solution ensemble of Peptide **3**.

| Assign. | Calculated | Experimental |
|---|---|---|
| Interproton distances (Å) | | |
| F3NH-S9α | 2.89 | 2.28 |
| F3NH-F(Br)8NH | 3.90 | 2.92 |
| F3NH-$^{D}$P2α | 5.03 | 3.94 |
| F3NH-S9NH | 4.16 | 3.31 |
| F3NH-G1NH | 3.67 | 3.22 |
| F3NH-F10NH | 3.41 | 2.69 |
| S9NH-S4α | 4.20 | 3.25 |
| G1NH-$^{D}$P2α | 2.41 | 2.11 |
| G1NH-F10NH | 2.57 | 2.25 |
| F(Br)8NH-D6NH | 3.44 | 3.36 |
| F(Br)8NH-E5NH | 3.40 | 2.66 |
| F(Br)8NH-S4α | 3.40 | 2.52 |
| F(Br)8NH-D6α | 5.11 | 3.57 |
| D6NH-E5NH | 2.29 | 2.19 |
| F10NH-$^{D}$P2α | 3.61 | 2.85 |
| F10NH-S4α | 4.57 | 3.29 |
| E5NH-S9α | 3.75 | 2.75 |
| *RMSD error* | | *0.84* |
| Coupling constants (Hz) | | |
| F3 | 6.9 | 7.5 |
| S4 | 7.4 | 7.3 |
| E5 | 7.9 | 8.2 |
| D6 | 7.3 | 8.0 |
| F(Br)8 | 6.9 | 8.6 |
| S9 | 6.8 | 7.3 |
| F10 | 8.4 | 9.2 |
| *RMSD error* | | *0.82* |

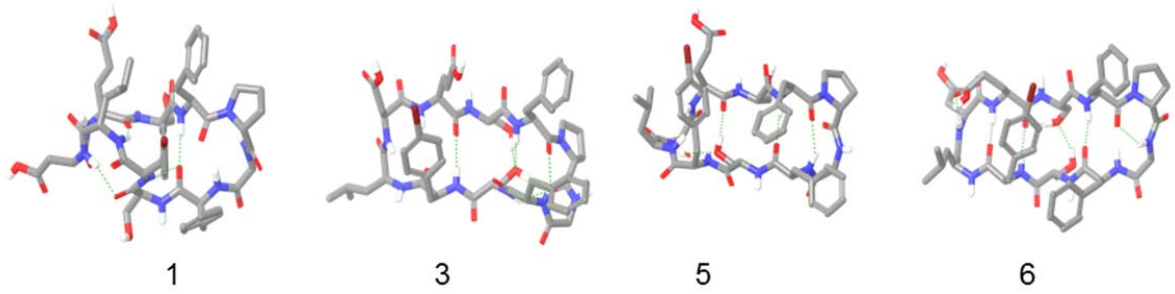



**Figure S8.** The most populated solution conformations of Peptide **3**, as selected by the NAMFIS-analysis. Populations in % are given in Table S19. Hydrogen bonds are indicated by green dotted lines. Non-polar hydrogens are omitted for clarity.

**Table S23**. Experimentally determined and back-calculated (NAMFIS) interproton distances (Å) and coupling constants (Hz) for the solution ensemble of Peptide **4**.

| Assign. | Calculated | Experimental |
|---|---|---|
| Interproton distances (Å) | | |
| F3NH-S4NH | 2.87 | 2.67 |
| F3NH-F10NH | 3.62 | 2.93 |
| F3NH-F10α | 2.92 | 2.52 |
| W(Cl)8NH-W5NH | 3.37 | 2.81 |
| W(Cl8)NH-S9NH | 2.55 | 2.79 |
| G1NH-F10NH | 2.51 | 2.29 |
| G1NH-$^{D}$P2α | 2.25 | 2.10 |
| L7NH-D6NH | 2.65 | 2.38 |
| L7NH-W(Cl)8α | 2.36 | 2.04 |
| S4NH-W5NH | 3.05 | 2.57 |
| S9NH-F10NH | 3.08 | 2.52 |
| S9NH-F10α | 2.34 | 1.92 |
| D6NH-W5NH | 2.36 | 2.21 |
| D6NH-L7α | 2.60 | 2.39 |
| W5NH-D6α | 2.96 | 2.25 |
| F10NH-$^{D}$P2α | 3.43 | 2.85 |
| *RMSD error* | | *0.43* |
| Coupling constants (Hz) | | |
| S4 | 7.4 | 7.4 |
| D6 | 8.1 | 6.8 |
| W(Cl)8 | 7.0 | 7.3 |
| S9 | 7.0 | 6.6 |
| F10 | 8.4 | 8.5 |
| *RMSD error* | | *0.62* |

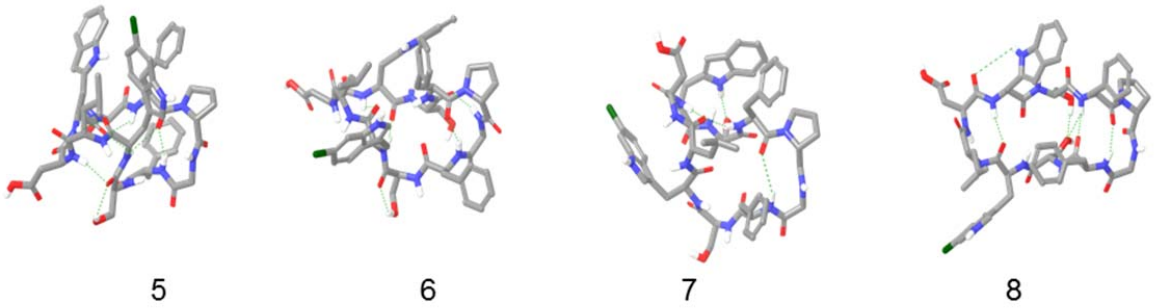



**Figure S9.** The most populated solution conformations of Peptide **4**, as selected by the NAMFIS-analysis. Populations in % are given in Table S19. Hydrogen bonds are indicated by green dotted lines. Non-polar hydrogens are omitted for clarity.

## 6. Biological evaluation

### *6.1 Fluorescence polarization assay*

The fluorescence polarization (FP) assay was performed in black 384-well microtiter plates at a final volume of 20 µL per well. Assay plates were prepared by addition of 20 µL of assay buffer (10 mM Tris (pH 8.0), 42.5 mM NaCl, and 0.0125% Tween-20) containing 1 µM GST-MDM2 (1–188) and Texas Red labeled wild-type p53 peptide (15 nM, amino acids 15–29: GSGSSQETFSDLWKLLPEN) to each well using a WellMate instrument (Matrix). For testing, a dilution series of small molecules (spanning 10 mM to 0.5 µM in DMSO in 1:3-dilution) was added by direct addition to the assay plate by Biomek FX lab automation workstation (Beckman Coulter. Inc., Fullerton, CA) using pin transfer (100ss pins, V&P scientific) giving a test dilution series spanning 130 µM to 6.6 nM with a final concentration of 1.3% DMSO. The assay mixture was incubated for 1 hour at room temperature and the fluorescence polarization signal was measured on an EnVision multilabel plate reader fitted with a 555-nm excitation filter, 632-nm static and polarized filters and a Texas Red FP dichroic mirror. Unlabeled WT-p53 peptide was used as a positive control and DMSO was used as a negative control. Technical triplicate data was normalized to the positive (100% inhibition) and negative (0% inhibition) controls on the corresponding row of the 384-well plate (the percentage inhibition = 100 × (sample result – negative control)/(positive control mean – negative control)). Two to seven independent experiments of normalized data were combined into a data set and then fit using a non-linear regression in GraphPad Prism with the formula log(inhibitor) vs. response – variable slope (four parameters). Additionally, the residuals of the curve fit were plotted to determine the fit of the theoretical curve. $IC_{50}$ and 95% confidence intervals (CI) were determined from these graphs.

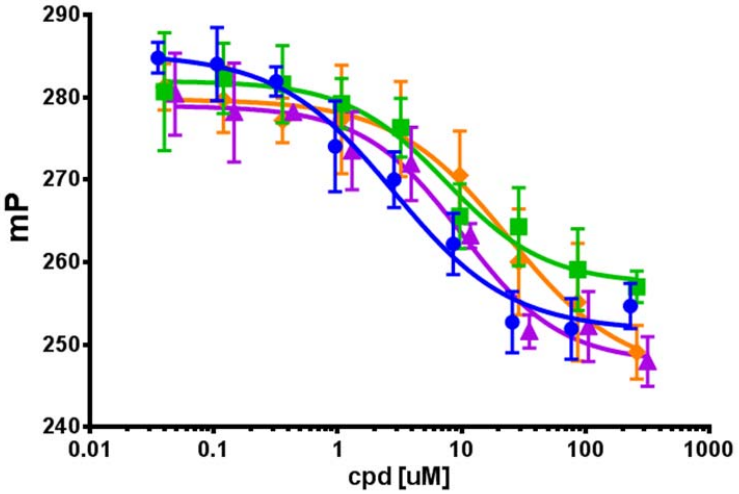



**Figure S10.** Fluorescence polarization-based MDM2 binding assay. Inhibition curves for Peptide **4** (green). Peptide **3** (violet), Peptide **2** (orange) and Peptide **1** (blue). Wild-type p53 peptide (amino acids 15 – 29: GSGSSQETFSDLWKLLPEN) was used as positive control. $IC_{50}$ = 7.56 [95% CI, 3.42–16.69]µM (Peptide **4**), 10.10 [95% CI, 5.67–17.99] µM (Peptide **3**) 23.94 [95% CI, 7.72–74.30]µM (Peptide **2**) and 2.86 [95% CI, 1.61–5.10] µM (Peptide **1**). CI = confidence interval.

### *6.2 Surface plasmon resonance measurements*

*Biotinylation of MDM2*

MDM2 was minimally biotinylated by reaction with EZ-Link Sulfo-NHS-LC-LC-Biotin (Thermo Scientific). The biotin reagent was added to the protein at a 0.5:1 molar ratio and the reaction was incubated on ice for 3–4 hours. Unconjugated biotin was removed by processing the samples through two Zeba Spin Desalting Columns (Thermo Scientific) that had been equilibrated with storage buffer (20 mM Bis-Tris pH 6.5, 200 mM NaCl, 20 mM dithiothreitol, 20% glycerol). Bovine serum albumin (BSA) was added to the reaction at a final concentration of 0.1 mg/mL immediately prior to processing through the spin columns to improve recovery.[7] The biotinylated protein was aliquotted, flash-frozen and stored at -80 °C for use in subsequent binding experiments.

*Equilibrium affinity analysis of compound binding by surface plasmon resonance*

SPR experiments were conducted at 20 °C using a SensiQ Pioneer FE optical biosensor (SensiQ Technologies). Neutravidin (Thermo Scientific) was covalently immobilized on a polysaccharide hydrogel-coated gold surface (COOH5 chip; SensiQ Technologies) using routine amine coupling chemistry in immobilization buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.005% Tween20). Carboxyl groups on the hydrogel were activated with N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), and neutravidin was injected in 10 mM sodium acetate pH 5.0 until immobilization levels of ~3700-4100 RU were achieved. Remaining active sites were blocked by reaction with ethanolamine. The instrument was primed with binding buffer (20 mM Tris pH 8.0, 100 mM NaCl, 5 mM dithiothreitol, 0.005% Tween20, 5% glycerol, 5% DMSO), and biotinylated MDM2 was injected until ~335 RU of protein was captured.

Peptide analytes were prepared in running buffer as a 3-fold dilution series starting at 5 µM for **1**, 200 µM for **2** and **3**, and 50 µM for **4** and were injected in duplicate at a flow rate of 100 µL/min.

A series of buffer-only (blank) injections was included throughout the experiment to account for instrumental noise. The data were processed, double-referenced, solvent corrected and analyzed[8,9] using the software package Qdat (version 2.5.0.106, BioLogic Software). The kinetic rate constants and/or equilibrium dissociation constants were determined by fitting the data to a 1:1 interaction model.

**Table S24**. Equilibrium affinity analyses of the investigated peptides.

| Analyte | $K_D$ (μM) | Rmax (RU) |
|---|---|---|
| Peptide **1** | 0.127 ± 0.001 | 25.60 ± 0.07 |
| Peptide **2** | 7.0 ± 0.10 | 19.87 ± 0.07 |
| Peptide **3** | 5.73 ± 0.09 | 18.61 ± 0.07 |
| Peptide **4** | 2.50 ± 0.02 | 33.81 ± 0.08 |

**Table S25**. Kinetic analysis of Peptide **1**.

| Analyte | $k_a$ ($M^{-1}$ $s^{-1}$) | $k_d$ ($s^{-1}$) | $K_D$ (nM) | Rmax (RU) |
|---|---|---|---|---|
| Peptide **1** | 8.30 (±0.02) x $10^5$ | 1.074 (± 0.001) x $10^{-1}$ | 129.3 ± 0.3 | 26.03 ± 0.01 |

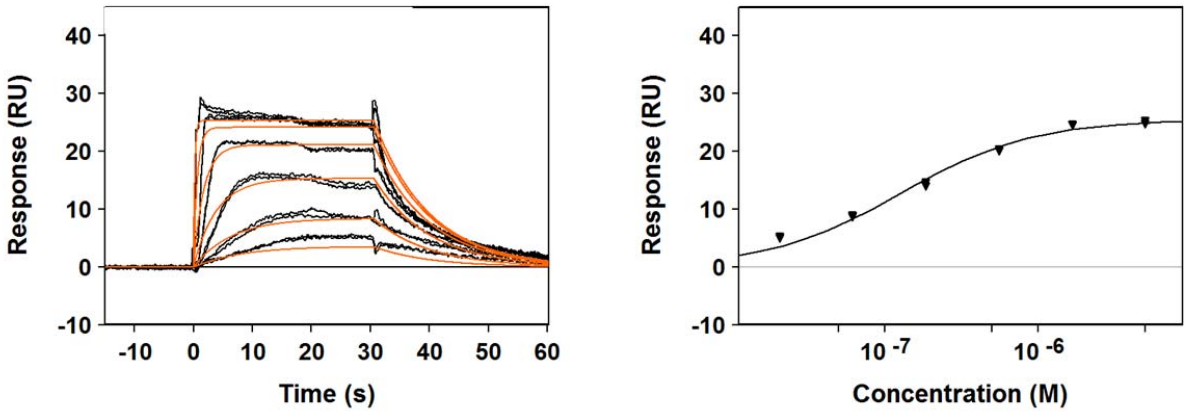



**Figure S11.** Left: Sensorgram (black lines) and kinetic analysis fit (orange lines) for Peptide **1**. Right: binding isotherm for equilibrium affinity analysis for Peptide **1**.

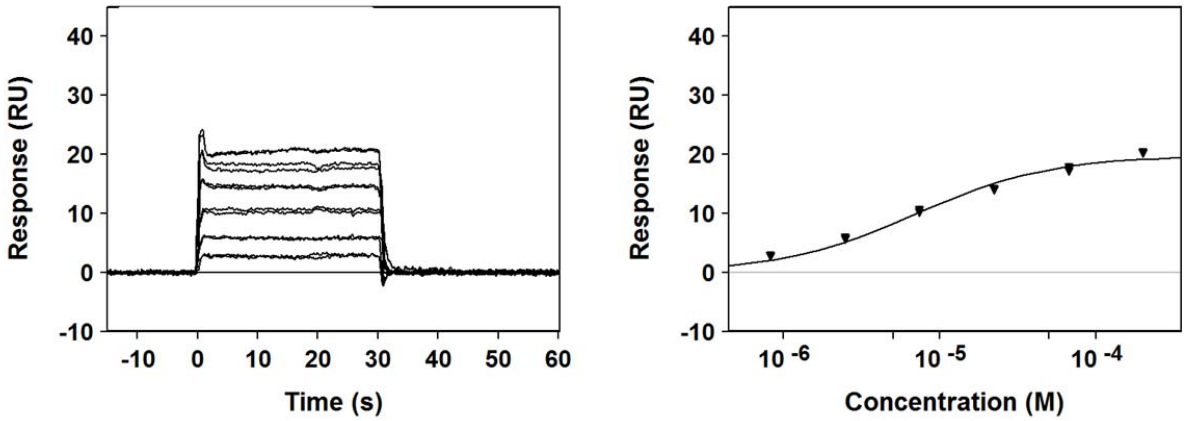



**Figure S12**. Left: Sensorgram (black lines) for Peptide **2**. Right: binding isotherm for equilibrium affinity analysis for Peptide **2**.

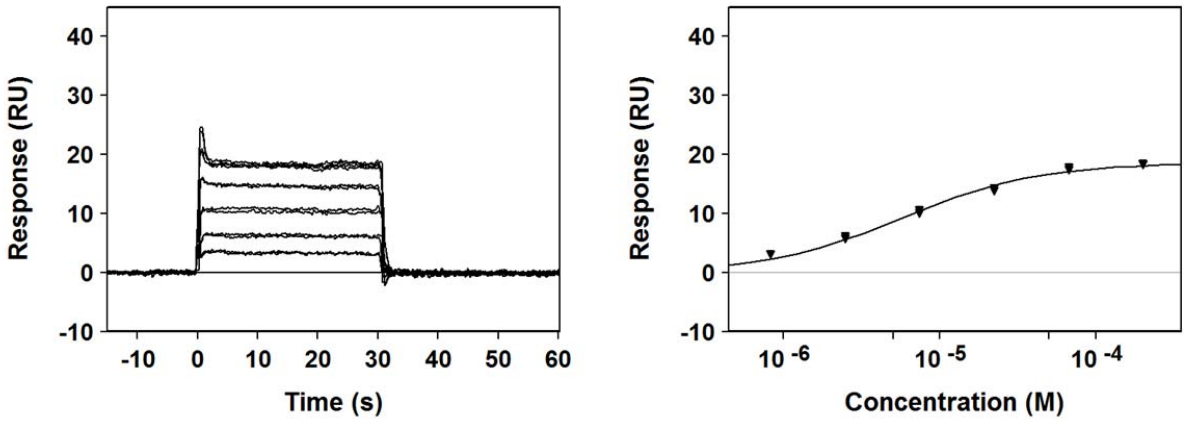



**Figure S13**. Left: Sensorgram (black lines) for Peptide **3**. Right: binding isotherm for equilibrium affinity analysis for Peptide **3**.

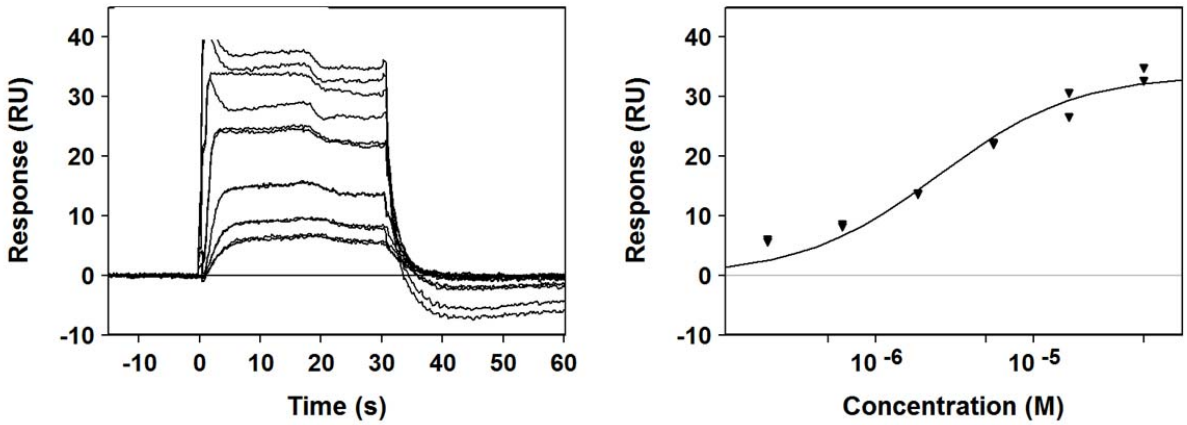



**Figure S14**. Left: Sensorgram (black lines) for Peptide **4**. Right: binding isotherm for equilibrium affinity analysis for Peptide **4**.

## 7. HPLC chromatogram of peptides 1-4

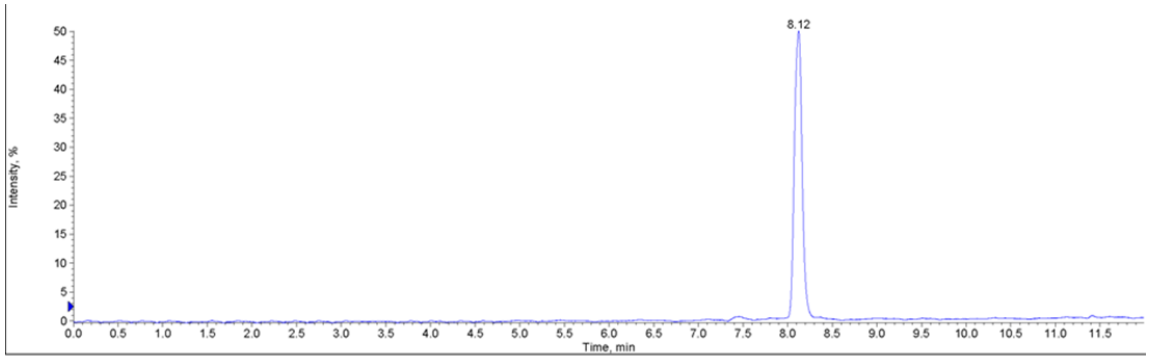



**Figure S15**. HPLC chromatogram of peptide **1** at 254 nm.

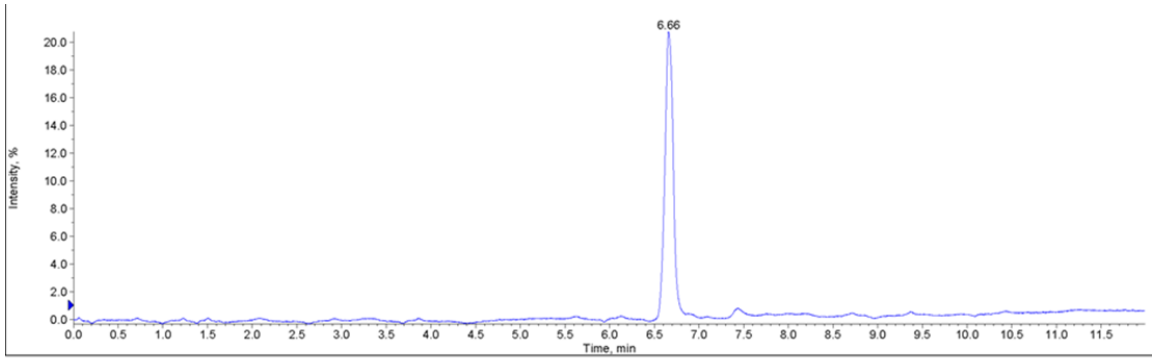



**Figure S16**. HPLC chromatogram of peptide **2** at 254 nm.

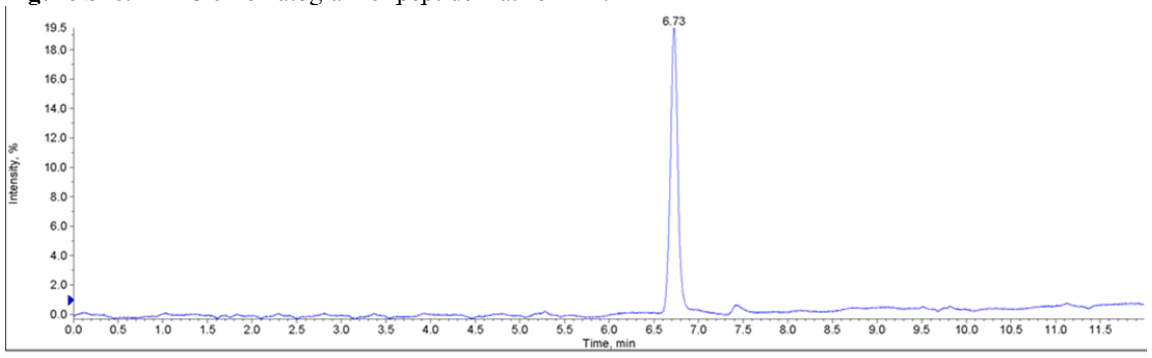



**Figure S17**. HPLC chromatogram of peptide **3** at 254 nm.

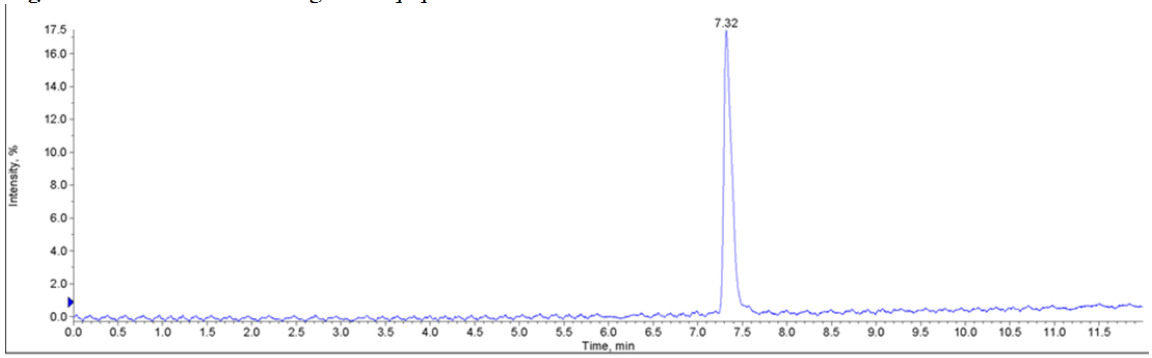



**Figure S18**. HPLC chromatogram of peptide **4** at 254 nm.

## 8. References

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