Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

for

Angew. Chem. Int. Ed. Z52659

69451 Weinheim, Germany

Consecutive Cyclic Pentapeptide Modules Form Short Alpha-Helices that are Very Stable to Water and Denaturants**

Nicholas E. Shepherd, Giovanni Abbenante, David P. Fairlie*

Centre for Drug Design and Development, Institute for Molecular Bioscience, University of Queensland, Brisbane, Qld 4072, Australia.

d.fairlie@imb.uq.edu.au

General. Fmoc-Asp(OAllyl)-OH and tetrakis(triphenylphosphino)palladium were obtained from Sigma-Aldrich (Sydney, Australia). Boc-Lys(Fmoc)-OH, Rink Amide MBHA resin and other L-amino acids were obtained from Novabiochem (Melbourne, Australia). Benzotriazol-1-yl-1,1,3,3-tetramethyluronium (HBTU) and benzotriazol-1-yloxy-tris(dimethylamino)-phosphonium (BOP) were obtained from Richelieu Biotechnologies (Quebec Canada). All other reagents were of peptide synthesis grade and obtained from Auspep (Melbourne, Australia).

Peptide Synthesis.
Ac-(KARAD)$_n$-NH$_2$, n=2 (**4**), *n=3* (**5**). Peptides **4** and **5** were prepared by manual stepwise solid phase peptide synthesis using HBTU/DIPEA activation for Fmoc chemistry [1] on Rink Amide MBHA resin (substitution 0.78mmol.g^{-1}, 0.5mmol, 648mg). Four equivalents of amino acid and eight equivalents of diisopropylethylamine (DIPEA) were employed in each coupling step (45mins). Fmoc deprotections were achieved with 3×5min treatments with excess 1:1 piperidine:DMF. Coupling yields were monitored by quantitative ninhydrin assay [2] and double couplings were employed for yields below 99.6%. After assembly of the first 10 residues, the peptide resin was washed, dried and split into two portions, one portion was acetylated, whilst to the other was added the final 5 residues. N-terminal acetylation was achieved by treating the fully protected peptide with 4 equivalents of glacial acetic acid, 4 equivalents of HBTU, and 8 equivalents of DIPEA. The peptides were simultaneously deprotected and cleaved from the resin by 2hr treatment of the washed and dried resin in 95% TFA, 2.5% TIPS, 2.5% H_2O (15µl per 10mg resin). The solution was then filtered, the filtrate concentrated *in vacuo* and the peptide precipitated with cold diethyl ether. The peptide precipitate was filtered washed with copious amounts of diethyl ether, redissolved in 1:1 acetonitrile/water and lyophilised. The crude peptides were purified by rp-HPLC (Vydac C18 column, 300Å. 22 × 250mm, 214nm, Solvent A = 0.1% TFA in H_2O, Solvent B = 0.1% TFA, 10% H_2O in Acetonitrile. Gradient: 0%B to 100%B over 30 mins. (**4**) Yield 20% (isolated). [R_t=12.65min]. MS: [M+H$^+$] (calc.) = 1142.63 (expt.) = 1142.75. ;[M+2H$^+$]/2 (calc.) 571.85 (expt.) =571.86. (**5**) Yield 30% (isolated) [R_t=13.16min]. MS: [M+2H$^+$]/2 (calc.) = 842.46 (expt.) = 842.64; [M+3H$^+$]/3 (calc.) 561.98 (expt.) = 562.08.

*NH$_2$-(cyclo1-5)-KARAD-NH$_2$ (***6**). Peptide **6** was prepared by manual stepwise solid phase peptide synthesis using HBTU/DIPEA activation for Fmoc chemistry [1] on Rink

Amide MBHA resin (substitution 0.78mmol.g^{-1}, 1.56mmol, 2000mg). Four equivalents of amino acid and eight equivalents of diisopropylethylamine (DIPEA) were employed in each coupling step (45mins), except for Fmoc-Asp(OAllyl)-OH and Boc-Lys(Fmoc)-OH where only 2 eqivalents were used. Fmoc deprotections were achieved with 3×5min treatments with excess 1:1 piperidine:DMF. Coupling yields were monitored by quantitative ninhydrin assay [2] and double couplings were employed for yields below 99.6%. After the assembly was complete, the allyl ester of aspartic acid was removed by treating the peptide resin with $Pd(PPh_3)_4$ (0.05eq) and diethylamine (5eq) in DCM, under argon and in the dark for 2hrs. After which the peptide was washed with DCM, DMF and 0.5% diethyldithiocarbamate in DMF. 2mg of resin was subjected to cleavage and the progress of the reaction monitored by MS. This process was repeated if necessary. Following Allyl ester deprotection the N(ζ)-Fmoc group was removed by treatment with piperidine (1:1 in DMF). Cyclisation was effected on-resin using 1.5 eq BOP, 2eq DIPEA in DMF/Benzene (2:1). The reaction was monitored by cleaving ~2mg resin and subjecting the residue to MS, total reaction time was approximately 48-72 hours. The peptides were simultaneously deprotected and cleaved from the resin by 2hr treatment of the washed and dried resin in 95% TFA, 2.5% TIPS, 2.5% H_2O (15µl per 10mg resin). The solution was then filtered, the filtrate concentrated *in vacuo* and the peptide precipitated with cold diethyl ether. The peptide precipitate was filtered washed with copious amounts of diethyl ether, redissolved in 1:1 acetonitrile/water and lyophilised. The crude peptides were purified by rp-HPLC (Vydac C18 column, 300Å. 22 × 250mm, 214nm, Solvent A = 0.1% TFA in H_2O, Solvent B = 0.1% TFA, 10% H_2O in Acetonitrile. Gradient: 0%B to 100%B over 30 mins. (**6**) Yield 30% (isolated). [R_t=12.82min]. MS: [M+H$^+$] (calc.) = calc. 541.31 (expt.)=541.39 .

Boc-(cyclo1-5)-KAR(Pbf)AD-OH (**7**). Peptide **7** was synthesised in an analogous manner to peptide **6**, however using trityl chloride resin (0.95mmol.g^{-1}, 1.28g, 1.16mmol). Cleavage was achieved using 50ml 10% acetic acid, 20% 2,2,2-trifluoroethanol, 70% DCM for 2hrs. After lyophilisation the crude peptide was deemed pure enough by analytical HPLC and used without further purification. Yield 50%. MS: [M+H$^+$] (calc) = 893.43 (expt.) = 893.67]

Ac-(cyclo1-5,6-10)-KARADKARAD-NH$_2$ (**2**). DIPEA (135µL, 0.38 mmol) was added to a solution Peptide **7** (154mg, 0.17mmol), peptide **6** (102mg, 0.19mmol, and BOP (80mg, 0.18mmol) in DMF (5mL). After stirring (2h, RT), solvent was evaporated in vacuo, the residue dissolved in H_2O/MeCN (1:1), lyophilised and purified (rpHPLC). The product was treated with TFA/TIPS 19:1 (1h, 20°C), evaporated, and reacted (2h, 20°C) with AcOH (15ul, 0.26mmol), 0.5M HBTU (500 µL 0.25mmol) and DIPEA (90uL, 0.52mmol). Solvent was removed in vacuo, H_2O/MeCN (1:1) added, lyophilised and purified (rpHPLC) to yield **2** (19.1mg, 10% isolated). MS [M+H$^+$] (calc.) 1106.6 (expt.) 1106.97, [M+2H]/2 (calc.) = 554.3 (expt.) = 554.04. Anal. rpHPLC: 14.8 min. (Gradient 0%-100% acetonitrile over 30 min).

Ac-(cyclo1-5,6-10,11-15)-KARADKARADKARAD-NH$_2$ (**3**). DIPEA (135µL, 0.38 mmol) was added to a solution Peptide **7** (66mg, 0.077mmol), peptide **6** (42mg, 0.074mmol, and BOP (52mg, 0.154mmol) in DMF (5mL). After stirring (2h, RT), solvent was evaporated in vacuo, the residue dissolved in H_2O/MeCN (1:1), lyophilised and purified (rpHPLC). The product (34mg, 0.024mmol) was treated with TFA/TIPS 19:1 (1h, 20°C), evaporated, and reacted (2h, RT) with peptide **7** (20mg,

0.024mmol), BOP (15mg, 0.034mmol), and lastly DIPEA (50ul, 0.24mmol). The solvent was evaporated in vacuo, the residue dissolved in H_2O/MeCN (1:1), lyophilised and purified (rpHPLC). The product was once again treated with TFA/TIPS 19:1 (1h, 20°C), evaporated, and reacted with AcOH (2ul, 0.0132mmol), BOP 7mg, 0.016mmol) and DIPEA (19uL, 0.138mmol) for 2hrs at RT. The solvent was removed in vacuo, H_2O/MeCN (1:1) added, lyophilised and purified (rpHPLC) to yield **3** (7.8mg, 5.5%(isolated). MS $[M+2H^+]/2$ (calc.) = 815.44 (expt.) = 815.55. [M+3H]/3 (calc.) = 543.97 (expt.) = 544.03. Anal. rpHPLC: 15.09 min.

NMR Spectroscopy.

Samples for NMR analysis of peptide **2** were prepared by dissolving the peptide 3mg in 450ul H_2O and 50ul D_2O (5mmol) and adjusting the pH of the solution to 4.5 by adding HCl or NaOH and stirring for 30 mins. 1D and 2D ^{1}H NMR spectra were recorded on both Bruker ARX-500 and Bruker Avance DMX-750 spectrometers at 278K. All spectra were recorded in the phase sensitive mode using time proportional phasing incrementation[3]. 2D experiments included TOCSY using MLEV-17 spin lock sequence with a mixing time of 100ms, NOESY with a mixing time of 300ms. Water suppression was achieved using watergate W5 pulse sequences with gradients using double echo[4]. 2D TOCSY and NOESY experiments were recorded over 7936.5 Hz with 4096 complex data points in F2 and 512 increments in F1 with 16 and 48 scans per increment respectively. Spectra were processed using XWINNMR (Bruker, Germany). The *t*1 dimensions of all 2D spectra were zero filled with 2048 real data points, and 90° phase-shifted sine bell window functions applied in both dimensions followed by fourier transformation and fifth order polynomial baseline correction. Chemical shifts were referenced to TSP an internal standard at 0.00ppm. Processed spectra were analyzed using the program SparkyNMR[5] and assigned using the sequential assignment technique[6].

Structure Calculations.

Cross peaks in NOESY spectra were integrated and calibrated in SparkyNMR[5], and distance constraints derived using the standard CALIBA function in DYANA[7]. Corrections for pseudo atoms were added to distance constraints where needed. Backbone dihedral angle restraints were inferred from $^3J_{NHC\alpha}$ coupling constants in 1D spectra at 278K and 288K, ϕ was restrained to $-65 \pm 30°$ for $^3J_{NHC\alpha} \leq 6$Hz. Peptide bond ω angles were all set to trans, and structures were calculation without explicit hydrogen bond restraints. Stereospecific assignments of β-methylene protons and χ_1 dihedral angles were derived from 1D ^{1}H spectra $^3J_{\alpha\beta}$ and set to $-60 \pm 30°$ for both aspartic acid residues. Initial structures were generated using a torsion angle simulated annealing protocol in DYANA until no violations were obtained. Final structures were calculated using XPLOR 3.851. Starting structures with randomised ϕ and ψ angles and extended side chains were generated using an ab initio simulated annealing protocol [8]. The calculations were performed using the standard forcefield parameter set (PARALLHDG.PRO) and topology file (TOPALLHDG.PRO) in XPLOR with in house modifications to generated lactam bridges between lysing and aspartic acid residues. Refinement of structures was achieved using the conjugate gradient Powell alogrithm with 1000 cycles of energy minimsation and a refined forcefield based on the program CHARMm[9]. Structures were visualised with MOLMOL[10] and InsightII[11].

Table S1. ^{1}H NMR resonance assignments and chemical shifts* (δ ppm) for **2** in 90 % H_2O:10% D_2O (278 K, pH 4.5).

Residue	NH	Hα	Hβ	Other
Ac-Lys	8.45	4.18	1.78, 1.94	Hγ 1.16, Hδ 1.42, 1.60, NHζ 8.42, Ac-CH_3 2.11
Ala	8.64	4.15	1.16	
Arg	8.16	4.09	1.77, 1.85	Hγ 1.65, Hδ 3.24, NHε 7.32
Ala	8.18	4.18	1.47	
Asp	9.21	4.61	2.76, 2.86	
Lys	7.93	4.39	1.95, 1.99	Hγ 1.25, Hδ 1.40, 1.62, NHζ 8.15
Ala	8.02	4.13	1.52	
Arg	7.71	4.07	1.78, 1.96	Hγ 1.65, Hδ 3.24, NHε 7.32
Ala	7.89	4.14	1.49	
Asp-NH_2	8.36	4.66	2.68, 3.02	NH_2 7.51, 7.03

*Referenced to TSP 0.00ppm., Ac= acetyl

Table S2. $^{3}J_{NHCH\alpha}$ Coupling Constants for **2** (278K-308K*)

	Lys 1	**Ala 2**	**Arg 3**	**Ala 4**	**Asp 5**	**Lys 6**	**Ala 7**	**Arg 8**	**Ala 9**	**Asp 10**
$^{3}J_{NHCH\alpha}$* (H□)	2.21	2.39	5.15	5.29	4.79	4.67	3.31	4.67	4.36	7.05
ϕ restraint (°)	-65 ±30	-65 ±30	-65 ±30	-65 ±30	-65 ±30	-65 ±30	-65 ±30	-65 ±30	-65 ±30	NA

* observed coupling constants did not change over this temperature range

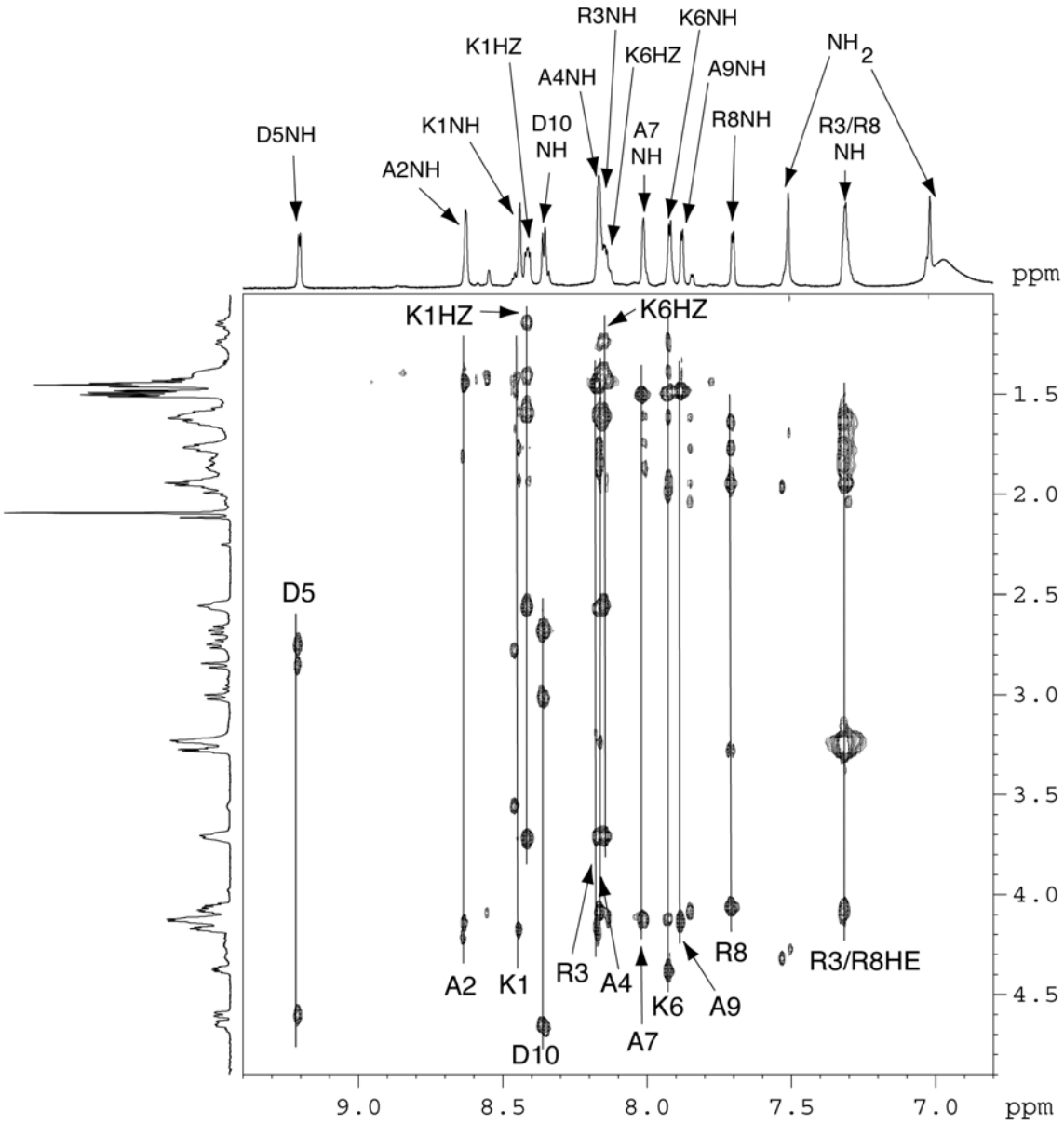

Figure S1. Section from the 750MHz TOCSY spectrum for **2** in 90 % H_2O:10% D_2O (278 K, pH 4.5). Individual spin systems are indicated by solid lines.

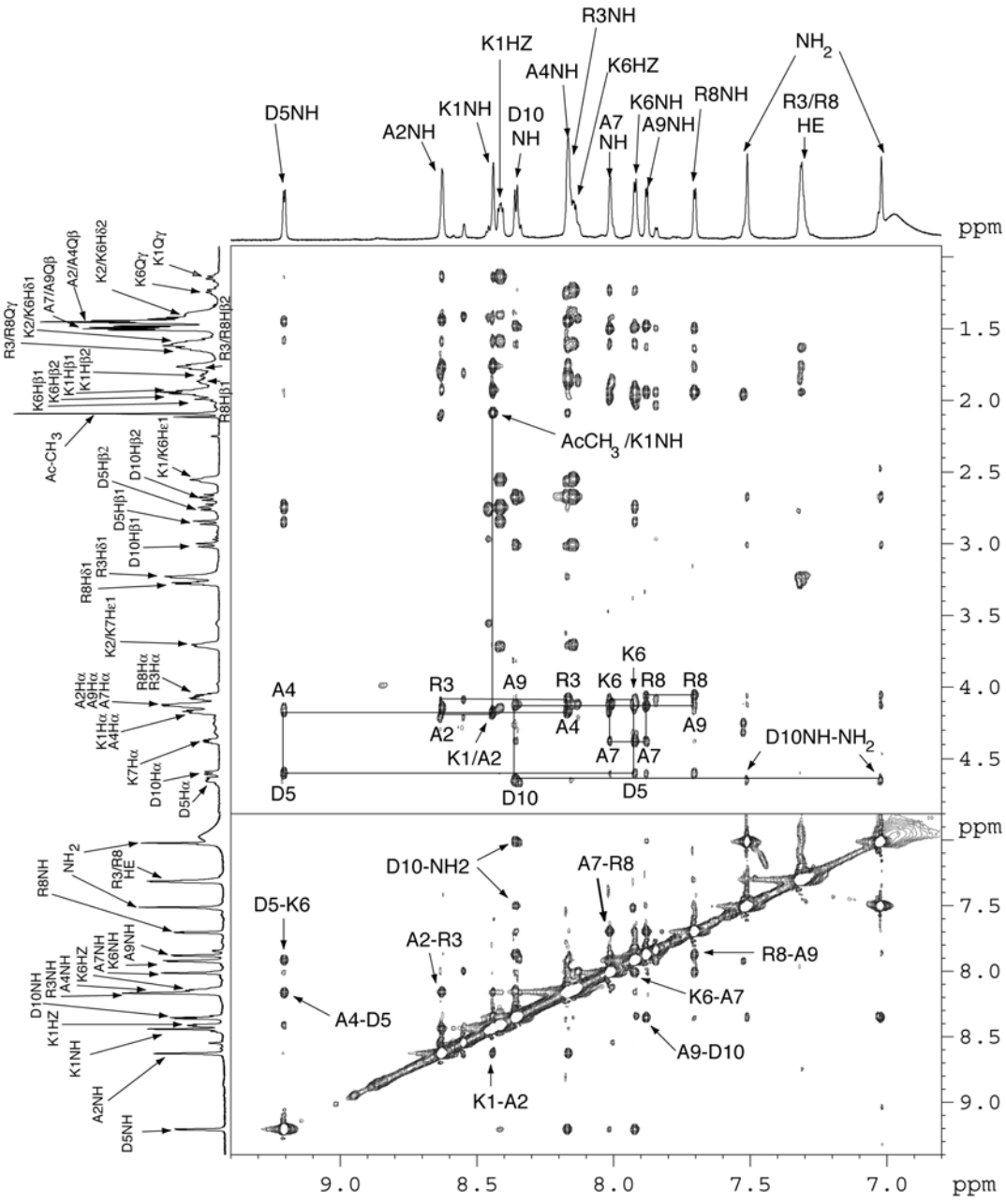

Figure S2. Sections from the 750MHz NOESY spectrum for **2** in 90 % H_2O:10% D_2O (278 K, pH 4.5). Sequential connectivities are represented by solid lines.

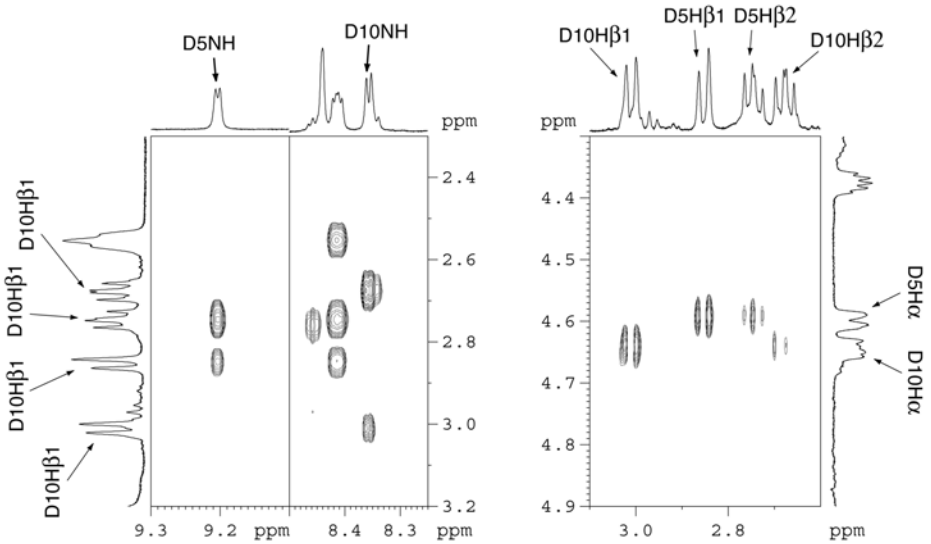

Figure S3. Sections from the 750MHz NOESY spectrum used for stereospecific assignment of aspartic acid 5 and 10 β-methylene protons.

Table S3. Coupling constant and NOE information used for stereospecific assignment of aspartic acid 5 and 10 β-methylene protons.

	Asp-5	**Asp-10**
$^3J_{H\alpha\text{-}H\beta1}$	0 Hz (<5Hz)	0 Hz (<5Hz)
$^3J_{H\alpha\text{-}H\beta2}$	16.37 Hz (>10Hz)	16.37 Hz (>10Hz)
NOE volume H_N-$H_{\beta1}$*	4.34 e+06 (weak)	5.68 e+06 (weak)
NOE volume H_N-$H_{\beta2}$*	1.21 e+07 (strong)	1.79 e+07 (strong)
NOE volume H_α-$H_{\beta1}$*	1.30 e+07 (strong)	1.65 e+07 (strong)
NOE volume H_α-$H_{\beta2}$*	7.76 e+06 (weak)	7.51 e+06 (weak)
χ_1 assignment	-60^o ($\pm 30^o$)	-60^o ($\pm 30^o$)

*volumes calculated using integrate function in SparkyNMR[5].

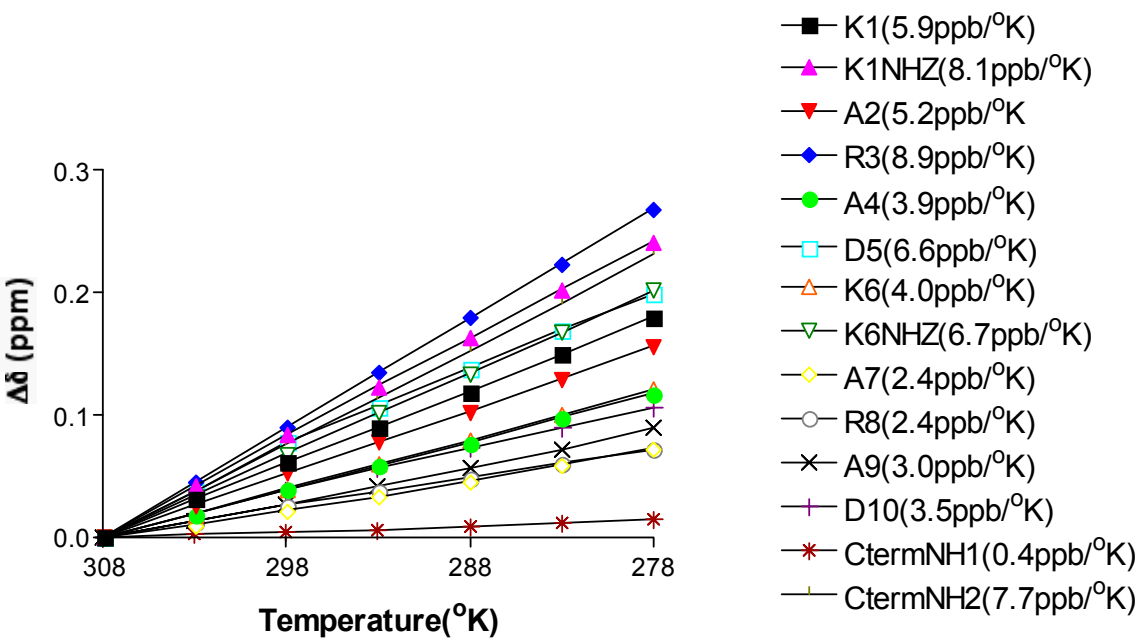

Figure S4. Temperature dependence of the amide NH chemical shifts for **2**. Line slopes indicating temperature co-efficients ($\Delta\delta/T$) for each residue are shown (in brackets) in the legend.

Table S4. NOE derived distance restraints used for calculating the solution structure of **2** in 90% H_20:10% D_20.

Atom A	Atom B	Upper distance restraint (Å) and comment.
Ac-CH_3	Lys1-NH	3.5 Å, strong
Ac-CH_3	Lys1 –Hα	5.0 Å medium ± 1.6 Å correction
Ac-CH_3	Ala2-NH	5.0 Å medium ± 1.6 Å correction
Ac-CH_3	Arg3-NH	5.0 Å medium ± 1.6 Å correction
Ac-CH_3	Arg3-Hδ#	6.20 Å very weak ± 1.6 Å correction
Lys1-NH	Lys1-Hβ2	2.83 ± 1.03 Å strong
Lys1-NH	Lys1-Hβ1	2.83 ± 1.03 Å strong
Lys1-NH	Lys1-Hβ#	2.74 ± 0.94 Å strong
Lys1-NH	Lys1 Hγ#	4.09 ± 2.29 Å weak
Lys1-NH	Lys1-Hδ#	4.09 ± 2.29 Å weak
Lys1-NH	Ala2-NH	3.045 ± 1.245 Å medium
Lys1-NH	Arg3-NH	3.65 ± 1.85 Å weak
Lys1-Hβ2	Ala2-NH	3.215 ± 1.415 Å medium
Lys1-Hβ1	Ala2-NH	3.215 ± 1.415 Å medium
Lys1-Hβ#	Lys1-HZ	4.09 ± 2.29 Å weak
Lys1-Hβ#	Ala2-NH	2.97 ± 1.17 Å medium
Lys1-Hγ#	Lys1-HZ	4.09 ± 2.29 Å weak
Lys1-Hγ#	Ala2-NH	4.09 ± 2.29 Å weak
Lys1-Hγ#	Ala2-Hα	4.09 ± 2.29 Å weak

Lys1- Hγ#	Asp5-NH	4.09 ± 2.29 Å weak
Lys1- Hγ#	Asp5-Hβ1	4.59 ± 2.29 Å very weak
Lys1- Hγ#	Asp5-Hβ2	5.09 ± 2.29 Å very weak
Lys1-Hδ1	Asp5-NH	4.15 ± 1.85 Å weak
Lys1-Hδ2	Asp5-NH	4.15 ± 1.85 Å weak
Lys1-Hδ#	Lys1-HZ	2.84 ± 1.04 strong
Lys1-Hδ#	Ala3-NH	4.09 ± 2.29 Å weak
Lys1-Hδ#	Asp5-Hβ2	4.09 ± 2.29 Å weak
Lys1-Hδ#	Asp5-Hβ1	4.09 ± 2.29 Å weak
Lys1-Hε2	Asp5-NH	3.65 ± 1.85Å medium
Lys1- Hε1	Asp5-NH	3.65 ± 1.85Å medium
Lys1- Hε#	Asp5-NH	3.575 ± 1.775Å medium
Lys1-HZ	Ala2-Hα	3.65 ± 1.85 Å medium
Lys1-HZ	Asp5-NH	3.65 ± 1.85 Å medium
Lys1-HZ	Asp5-Hα	3.65 ± 1.85 Å medium
Lys1-HZ	Asp5-Hβ2	3.075 ± 1.275Å strong
Lys1-HZ1	Asp5-Hβ1	3.465 ± 1.665 Å medium
Lys1-HZ1	Asp5-NH	3.495 ± 1.695 Å medium
Ala2-NH	Arg3-NH	3.0 ± 1.2Å strong
Ala2-NH	Asp5-NH	3.65 ± 1.65Å medium
Ala2-Hα	Asp5-NH	3.65 ± 1.85 Å medium
Ala2-Hα	Asp5-Hβ2	3.125 ± 1.325 Å strong
Ala2-Hα	Asp5-Hβ1	3.395 ± 1.495 Å medium
Ala2-Hβ#	Asp5-NH	4.165 ± 2.365 Å weak
Arg3-NH	Arg3-Hβ#	2.75 ± 0.95 Å strong
Arg3-NH	Arg3-Hδ#	4.09 ± 2.29 Å weak
Arg3-Hα	Asp5-NH	3.54 ± 1.74 Å medium
Arg3-Hα	Lys6-NH	3.65 ± 1.85 Å medium
Arg3-Hα	Lys6-Hβ#	3.5 ± 1.7 Å medium
Arg3-Hβ#	Arg3-HE	4.09 ± 2.29 weak
Ala4-NH	Asp5-NH	2.905 ± 1.105 Å strong
Ala4-NH	Lys6-NH	3.2 ± 1.4 Å medium
Ala4-Hα	Ala7-NH	3.65 ± 1.85 Å medium
Ala4-Hα	Arg8-NH	3.65 ± 1.85 Å medium
Asp5-NH	Asp5-Hβ2	2.86 ± 1.06 Å strong
Asp5-NH	Lys6-NH	2.97 ± 1.17 Å strong
Asp5-NH	Lys6-Hβ2	3.62 ± 1.82 Å medium
Asp5-NH	Lys6-Hβ1	3.62 ± 1.82 Å medium
Asp5-NH	Lys6-Hβ#	3.62 ± 1.82 Å medium
Asp5-NH	Ala7-NH	3.65 ± 1.85 Å medium
Asp5-NH	Arg8-NH	3.65 ± 1.85 Å medium
Asp5-Hα	Ala7-NH	3.23 ± 1.43 strong
Asp5-Hα	Arg8-NH	3.34 ± 1.54 Å strong
Asp5-Hα	Arg8-Hβ2	3.375 ± 1.625 Å medium
Asp5-Hα	Arg8-Hβ1	3.375 ± 1.275 Å strong
Asp5-Hα	Arg8-Hβ#	3.375 ± 1.275 Å strong
Asp5-Hα	Arg8-Hγ#	4.09 ± 2.29 Å weak

Asp5-Hα	Arg8-Hδ#	4.09 ± 2.29 Å weak
Asp5-NH	Ala9-NH	3.525 ± 1.725 Å medium
Asp5-Hβ2	Lys6-NH	3.11 ± 1.31 Å strong
Asp5-Hβ1	Lys6-NH	3.42± 1.62 Å medium
Lys6-NH	Lys6-Hβ2	2.845± 1.045 Å strong
Lys6-NH	Lys6-Hβ1	2.845± 1.045 Å strong
Lys6-NH	Lys6-Hβ#	2.69± 0.895 Å strong
Lys6-NH	Lys6-Hγ#	4.09 ± 2.29 Å weak
Lys6-NH	Lys6-Hδ2	4.15 ± 1.85 Å weak
Lys6-NH	Lys6-Hδ1	4.15 ± 1.85 Å weak
Lys6-NH	Ala7-NH	2.815 ± 1.015 Å strong
Lys6-NH	Arg8-NH	3.51 ± 1.71 Å medium
Lys6-Hα	Arg8-NH	3.245 ± 1.445 Å medium
Lys6-Hα	Ala9-NH	3.34 ± 1.54 Å medium
Lys6-Hα	Ala9-Hβ#	4.165 ± 2.365 Å medium
Lys6-Hα	Asp10-NH	3.48 ± 1.68 Å medium
Lys6-Hβ2	Ala7-NH	3.28 ± 1.48 Å strong
Lys6-Hβ1	Ala7-NH	3.28 ± 1.48 Å strong
Lys6-Hβ#	Ala7-NH	3.025 ± 1.225 Å strong
Lys6-Hγ#	Lys6-HZ	3.95 ± 2.15 Å weak
Lys6-Hγ#	Ala7-NH	4.09 ± 2.29 Å weak
Lys6-Hγ#	Ala7-Hα	4.09 ± 2.29 Å weak
Lys6-Hγ#	Asp10-NH	4.09 ± 2.29 Å weak
Lys6-Hγ#	Asp10-Hβ2	4.09 ± 2.29 Å weak
Lys6-Hγ#	Asp10-Hβ1	4.09 ± 2.29 Å weak
Lys6-Hδ2	Asp10-NH	3.9 ± 1.85 Å weak
Lys6-Hδ1	Asp10-NH	3.65 ± 1.85 weak
Lys6-Hδ#	Asp10-Hβ2	4.09 ± 2.29 Å weak
Lys6-Hδ#	Asp10-Hβ1	4.09 ± 2.29 Å weak
Lys6-HZ	Ala7-NH	3.65 ± 1.85 Å weak
Lys6-HZ	Asp10-NH	3.65 ± 1.85 Å weak
Lys6-HZ	Asp10-Hα	3.65 ± 1.85 Å weak
Lys6-HZ	Asp10-Hβ2	2.955 ± 1.155 Å strong
Lys6-HZ	Asp10-Hβ1	3.34 ± 1.54 Å medium
Ala7-NH	Arg8-NH	2.985 ± 1.185 Å strong
Ala7-NH	Ala9-NH	3.57 ± 1.77 Å medium
Ala7-Hα	Asp10-NH	3.045 ± 1.245 Å strong
Ala7-Hα	Asp10-Hβ2	3.075 ± 1.275 Å strong
Ala7-Hα	Asp10-Hβ1	3.605 ± 1.805 Å medium
Ala7-Hα	Asp10-HT1	3.65 ± 1.85 Å medium
Ala7-Hα	Asp10-HT2	2.9 ± 1.1 Å strong
Arg8-NH	Arg8-Hβ#	2.79 ± 0.99Å strong
Arg8-NH	Arg8-Hγ#	4.09 ± 2.29 Å weak
Arg8-NH	Arg8-Hδ#	4.09 ± 2.29 Å weak
Arg8-NH	Ala9-NH	2.875 ± 1.075 Å strong
Arg8-NH	Asp10-NH	3.03 ± 1.23 Å strong
Arg8-Hα	Ala9-NH	2.625 ± 0.825 Å strong

Arg8-Hα	Asp10-HT1	3.65 ± 1.85 Å medium
Arg8-Hα	Asp10-HT2	3.65 ± 1.85 Å medium
Arg8-Hβ2	Arg8-HE	3.65 ± 1.85 Å medium
Arg8-Hβ1	Arg8-HE	3.65 ± 1.85 Å medium
Ala9-NH	Asp10-NH	2.845 ± 1.045 Å strong
Ala9-NH	Asp10-HT2	3.65 ± 1.85 Å medium
Asp10-NH	Asp10-Hβ2	2.735 ± 0.935 Å strong
Asp10-Hβ2	Asp10-HT1	3.15 ± 1.35 Å strong
Asp10-Hβ1	Asp10-HT1	3.15 ± 1.35 Å strong

CD Spectroscopy.

CD experiments were performed on a Jasco Model J-710 spectropolarimeter which was routinely calibrated with (1S)-(+)-10-camphorsulfonic acid. Temperature control was achieved using a Neslab RTE-111 circulating water bath. Spectra were recorded in a 0.1cm Jasco cell between 310-185nm at 50 nm/min wit a band width of 1.0nm, response time of 2 sec, resolution step width of 0.1nm and sensitivity of 20, 50 or 100 mdeg. Each spectrum represents the average of 5 scans with smoothing to reduce noise. Peptide samples for CD spectroscopy were dissolved in distilled water (~1mg/ml). Each stock solution was diluted to a final concentration of 50uM in 10mM sodium phosphate buffer (pH7.4), with or without additives (2,2,2-trifluoroethanol (TFE) or guanidine.HCl). Guanidine.HCl denaturation experiments were performed according to ref 12.

Accurate concentration determination of stock solutions were obtained by 1D ^{1}H NMR using the method of Larive *et. al.*[13] (425µl of peptide stock solution, 50µl of D_2O, and 25µl of 10.077mM DSS as an internal standard).

Peptide/ Concentration	$[\vartheta]_{222}$	$[\vartheta]_{208}$	$[\vartheta]_{192}$
2 (32µM)	-32340	-24957	104187
2 + TFE (32µM)	-27542	-25210	99734
3 (37µM)	-31987	-23842	100811
3 + TFE (37µM)	-30302	-26664	101480
4 (44µM)	-1836	-8552	-12237
4 + TFE (44µM)	-7528	-10889	2754
5 (43µM)	-3852	-9788	-8024
5 + TFE (43µM)	-16882	-20002	32244
2 (16µM)	-30560	-25722	99836
2 (64µM)	-31140	-24547	102263
2 (128µM)	-31824	-25925	91078*
3 (18.5µM)	-31826	-24193	97838
3 (74µM)	-33172	-25267	106650
3 (148µM)	-33096	-25615	89947*

* Due to the high peptide concentrations, signal dampening effects were observed at this wavelength (ie. **2** (128µM) HT =774, **3** (148µM) HT = 865)

Trypsin Digestion.

Solutions of helical cycle **2** (25uM) and linear peptide **4** (26uM) were incubated with trypsin (1ug/ml) in 25mM ammonium carbonate buffer (pH=8) at room temperature. Aliquots were taken at 30seconds, 1 minute, 5 minutes, 15 minutes, 30 minutes, 1 hour and 2 hours, and diluted with an equivalent volume of 3% trifluoroacetic acid. The resultant solutions were analysed using a 2 x 75mm, 3 μm, Aqua C-18 column (Phenomenex) equilibrated in aqueous formic acid (0.1%). Peptide cleavage products were eluted using a linear gradient of acetonitrile from 0 to 80% in aqueous 0.01% formic acid over 20 minutes at a rate of 300 μL/min. Rate of degradation of either **2** or **4** was quantified by determining extracted ion counts of chromatograms relative to control solutions (containing no enzyme) using a QSTAR PULSAR Electrospray QqTOF Mass Spectrometer and analyzed using BioMultiview (SCIEX Software). Retention time of cycle **2** = 11.64 minutes. Retention time of linear peptide **4** = 7.43 minutes.

References

[1] Engelbretsen, D. R.; Garnham, B. G.; Bergman, D. A.; Alewood, P. F. *Tetrahedron Lett.* **36**: 8871 (1995).

[2] Sarin, V.; Kent, S. B. H.; Tan, J. P.; Merrifield, R. B. *Anal. Biochem.* **117:** 147 (1981).

[3] Marion, D.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **113**: 967 (1983).

[4] Liu, M.; Mao, X.; He, C.; Huang, H.; Nicholson, J. K.; Lindon, J.C. *J. Magn. Reson.* **132**: 125 (1998).

[5] T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco.

[6] Wüthrich, K. *NMR of Proteins and Nucleic Acids.* Wiley-Interscience. New York. (1986).

[7] Güntert, P., Mumenthaler, C. & Wüthrich, K. *J. Mol. Biol.* **273**: 283-298 (1997).

[8] Nilges, M.; Gronenborn, A. M.; Brünger, A. T.; Clore, G. M. *Prot. Eng.* **2**: 27 (1988).

[9] Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D.; States, D. J.; Swaminathan, S.; Karplus, M. *J. Comput. Chem.* **4**: 187 (1983).

[10] Koradi, R., Billeter, M., and Wüthrich, K. *J Mol Graphics* **14**, 51-55 (1996) .

[11] Version 2000, Molecular Simulations Inc. San Diego CA.

[12] Creighton, T. E. *Protein Structure: A Practical Approach*. 2nd Ed. Oxford University Press, New York. pp299-321. (1997).

[13] Larive, C. K.; Jayawickrama, D.; Orfi, L. *Appl. Spec.* **51**: 1531 (1997).