Effects of Chirality and Side Chain Length in $C_{\alpha,\alpha}$-Dialkylated Residues on β-Hairpin Peptide Folded Structure and Stability

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SUPPORTING INFORMATION

Contents

General Information. HCTU, PyAOP, Fmoc-protected α-amino acids, and Fmoc-protected $C_{\alpha,\alpha}$-dialkylated α-amino acids were purchased from NovaBioChem, Chem-Impex, or ChemSpace. NovaPEG Rink Amide Resin was purchased from NovaBioChem. Solvents and all other reagents were purchased from Fisher Scientific or Millipore Sigma used as received without further purification.

Peptide Synthesis. Peptides were synthesized using Fmoc solid-phase synthesis methods with a CEM Mars manual peptide synthesizer on NovaPEG Rink Amide resin. Couplings were completed with a 90 second ramp to 90 °C and a total run time of 3.5 minutes using Fmoc-protected amino acid (6 equiv relative to resin), HCTU (6 equiv), and DIEA (10 equiv) in *N*-methyl-2-pyrrolidone. Deprotections were completed with a 90 second ramp to 90 °C and a total run time of 3 minutes using an excess of 20% 4-methylpiperidine in DMF. After each coupling or deprotection cycle, the resin was washed three times with DMF. Residue Asn6 was double coupled in each peptide.

For peptides **3a-3b**, residue X13 (6 equiv) was coupled with PyAOP (6 equiv). For peptides **4a-4b,** residue X13 (4 equiv) was coupled with PyAOP (4 equiv). Directly following the $C_{\alpha,\alpha}$-dialkylated α-amino acid at position 13, residue Phe12 was double coupled with PyAOP (6 equiv). After this double coupling, the peptide was capped by acetylation with a solution of 8:2:1 DMF:DIEA:Ac_2O. In syntheses where capping was not employed, multiple deletion products were seen resulting from incomplete coupling of the residue following the bulky $C_{\alpha,\alpha}$-dialkylated residue.

Prior to cleavage, the resin was washed three times each with DMF, dichloromethane, and methanol then dried under vacuum. Peptides were cleaved from resin using a solution of TFA/H_2O/TIS (95%/2.5%/2.5%) for 3 hours. After precipitating in cold diethyl ether, the solutions were centrifuged and the pelleted solids were dissolved in 10% acetonitrile in water containing 0.1% TFA and sonicated for 30 minutes. Peptides were purified by RP-HPLC using a Phenomenex Luna C18 column with gradients between 0.1% TFA in H_2O and 0.1% TFA in acetonitrile then lyophilized. Peptide identity and purity were determined by mass spectrometry (ESI-MS, Table S1) and analytical RP-HPLC, respectively (Figure S1).

NMR Sample Preparation and Data Collection. NMR samples were prepared by dissolving 2-3 mg peptide in 650 µL de-gassed buffer solution (50 mM phosphate, 9:1 H_2O/D_2O, uncorrected pH 6.3) to make ~2 mM solutions. 3-(Trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS, 50 mM in water) was added to a final concentration of ~0.2 mM DSS in the sample. The NMR tube headspace was purged with a stream of nitrogen prior to capping.

NMR spectra of peptides were recorded on a Bruker Avance-700 spectrometer. Chemical shifts are reported relative to DSS (0 ppm). TOCSY, NOESY, and COSY pulse programs used excitation-sculpted gradient-pulse solvent suppression. All experiments were obtained using 2048 data points in the direction dimension and 512 data points in the indirect dimension. TOCSY were acquired with a mixing time of 80 ms and NOESY were acquired with a mixing time of 200 ms.

NMR Data Analysis. Backbone ^1H resonance assignments for peptides **1** and **2** were previously reported, and the data are included here for comparison to new compounds (Table S2, S3).[1] Backbone ^1H resonance assignments for peptides **3a**, **3b**, **4a**, and **4b** (Table S4-S7) were assembled manually from the corresponding 2D NMR experiments described above using the NMRFAM-SPARKY[2] and POKY[3] software packages.

Folded populations for each peptide were estimated using the separation of diastereotopic H_α signals for residue G10. TOCSY cross-peaks were selected on both sides of the diagonal corresponding the geminal coupling between these protons, and the chemical shift for each resonance tabulated from the value observed in the direct dimension (Figure S2). The resulting difference in resonance frequency (Δδ) was

used to calculate folded population and ΔG_{fold} as previously described.[4] Reference $\Delta\delta$ values of 0 ppm and 0.310 ppm were used for the fully unfolded and fully folded states, respectively.[1] For peptides **4a** and **4b**, the above analysis was repeated for a series of TOCSY spectra acquired on the same sample at a range of temperatures from 278 to 318 K (Figure S3, Table S8, S9) and fit to obtain thermodynamic parameters for the folding equilibrium following methods described.[4]

NMR Structure Determination For peptides **4a** and **4b**, full ^{1}H resonance assignment was completed, and a folded structure of each determined using simulated annealing with NMR-derived restraints. A structure of peptide **1** was calculated using the same methods and data from previously reported experiments.[1] NMR structure calculations were performed using ARIA (Ambiguous Restraints for Iterative Assignment, version 2.3)[5] and CNS (Crystallography & NMR System, version 1.2).[6] Parameter and topology definitions for the artificial α(Et)Nva monomer were assembled by hand based on analogous atom types already present in the force field. ARIA settings were modified from program defaults based on reported optimizations to improve model quality and convergence.[7] The structure calculation proceeded in two rounds. The first run used as input the resonance assignments for the peptide and a list of unassigned integrated NOESY peaks. NOE distance restraints were generated automatically by ARIA from this information in iterative fashion over the calculation. The ensemble resulting from run 1 was used to estimate the rotational correlation time of the peptide using the program HYDROPRO[8] as well as assess backbone hydrogen bonds present in the structure. The second simulated annealing run was initiated from the run 1 ensemble and proceeded identically to the first run, except for the addition of hydrogen bond restraints and the use of the spin diffusion correction option in ARIA.[9] The set of 10 lowest energy models resulting from the second run were taken as the final NMR ensemble of each peptide (Table S10). As discussed in the text, the NMR data set for **4a** was used to calculate the structure of its enantiomer (***ent*-4a**). Coordinates and associated experimental data have been deposited in the PDB (**1**: 8T0G, **ent-4a**: 8T0H, **4b**: 8T0I) and BMRB (**1**: 31089, ***ent*-4a**: 31090, **4b**: 31091).

Figure S1. Pure analytical HPLC traces for peptides **2-4b.** Each peptide was analyzed using an increasing gradient (20% to 30%) of acetonitrile in water with 0.1% trifluoroacetic acid.

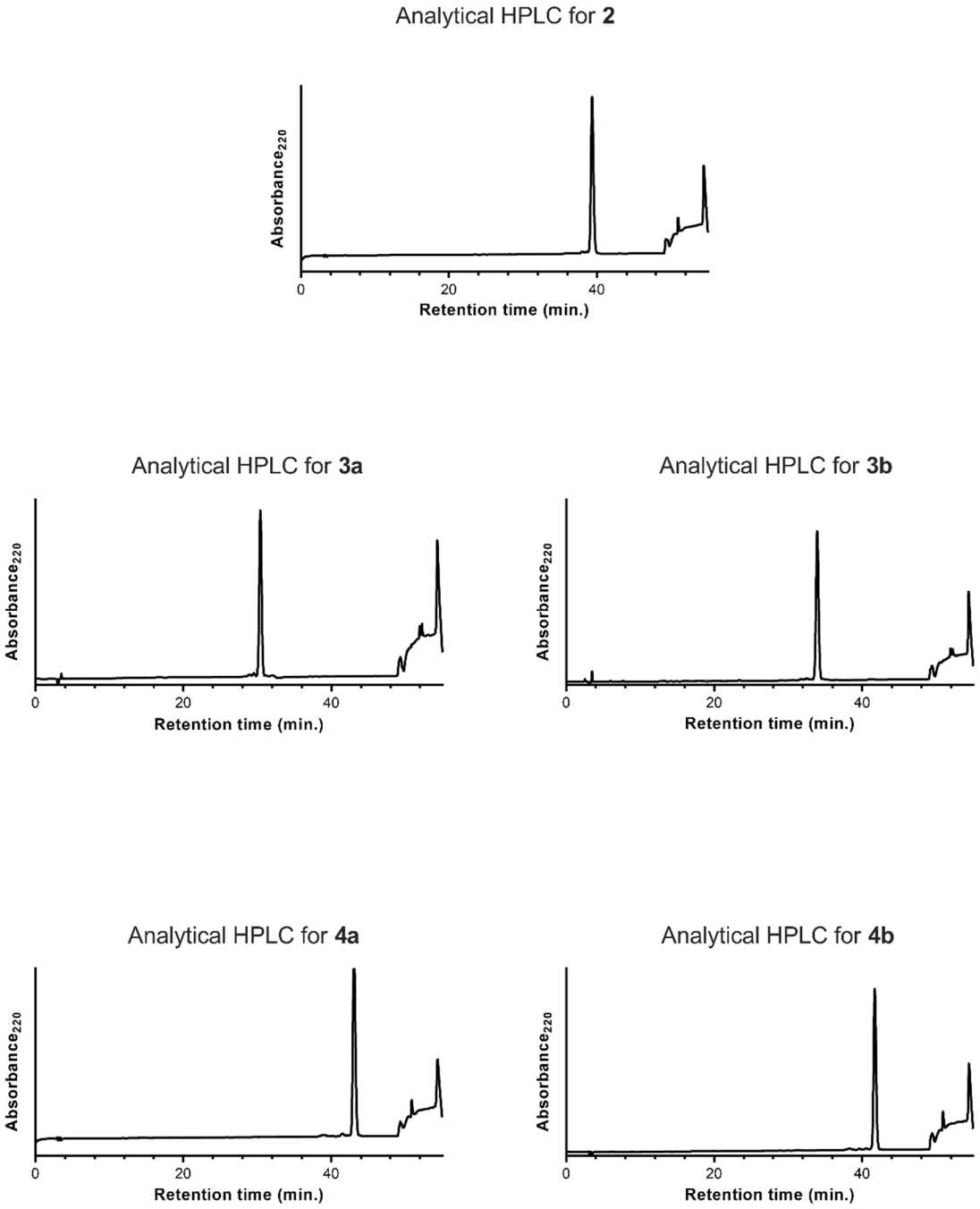

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Figure S2. Selected regions of the TOCSY spectra of peptides **1**-**4b** at 298 K. Dashed lines indicate chemical shifts of the two diastereotopic protons of Gly_{10}.

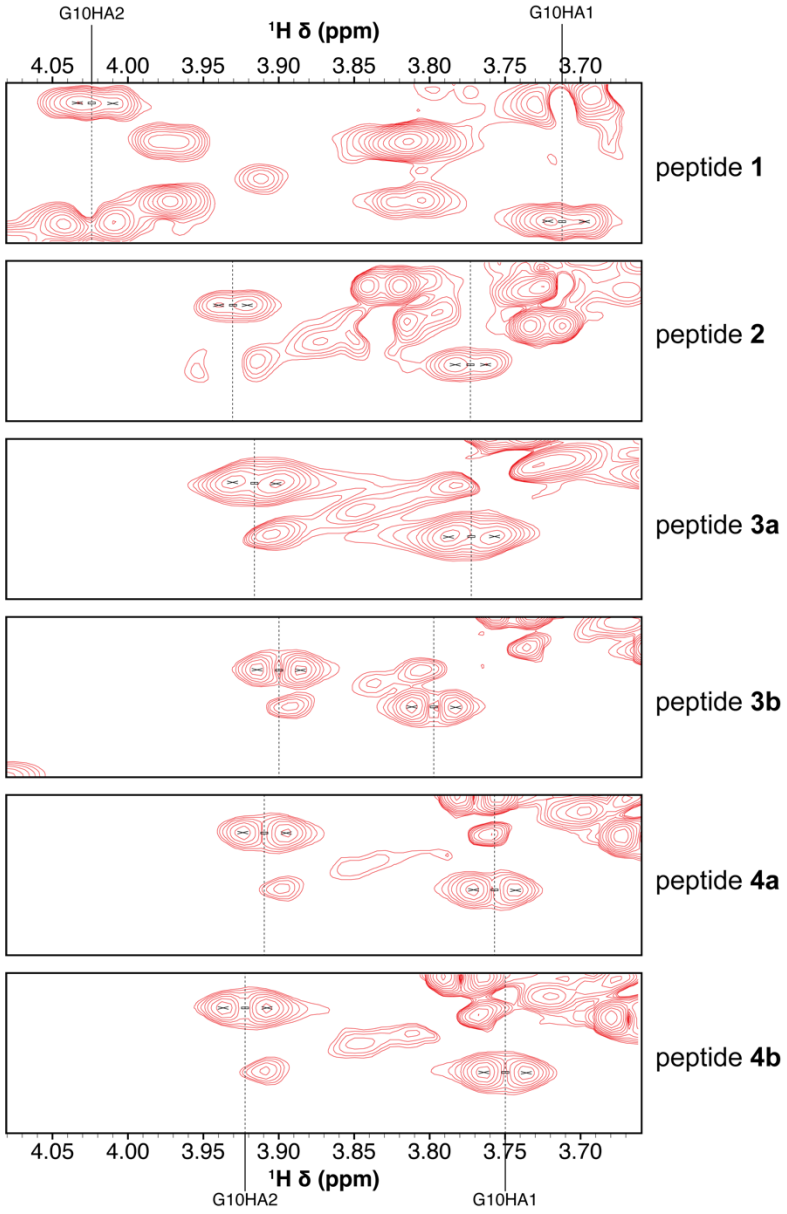

Figure S3. Thermal denaturation melt NMR spectra for peptides **4a** and **4b**. Dashed lines indicate chemical shifts of the two diastereotopic protons of Gly_{10} at 278 K.

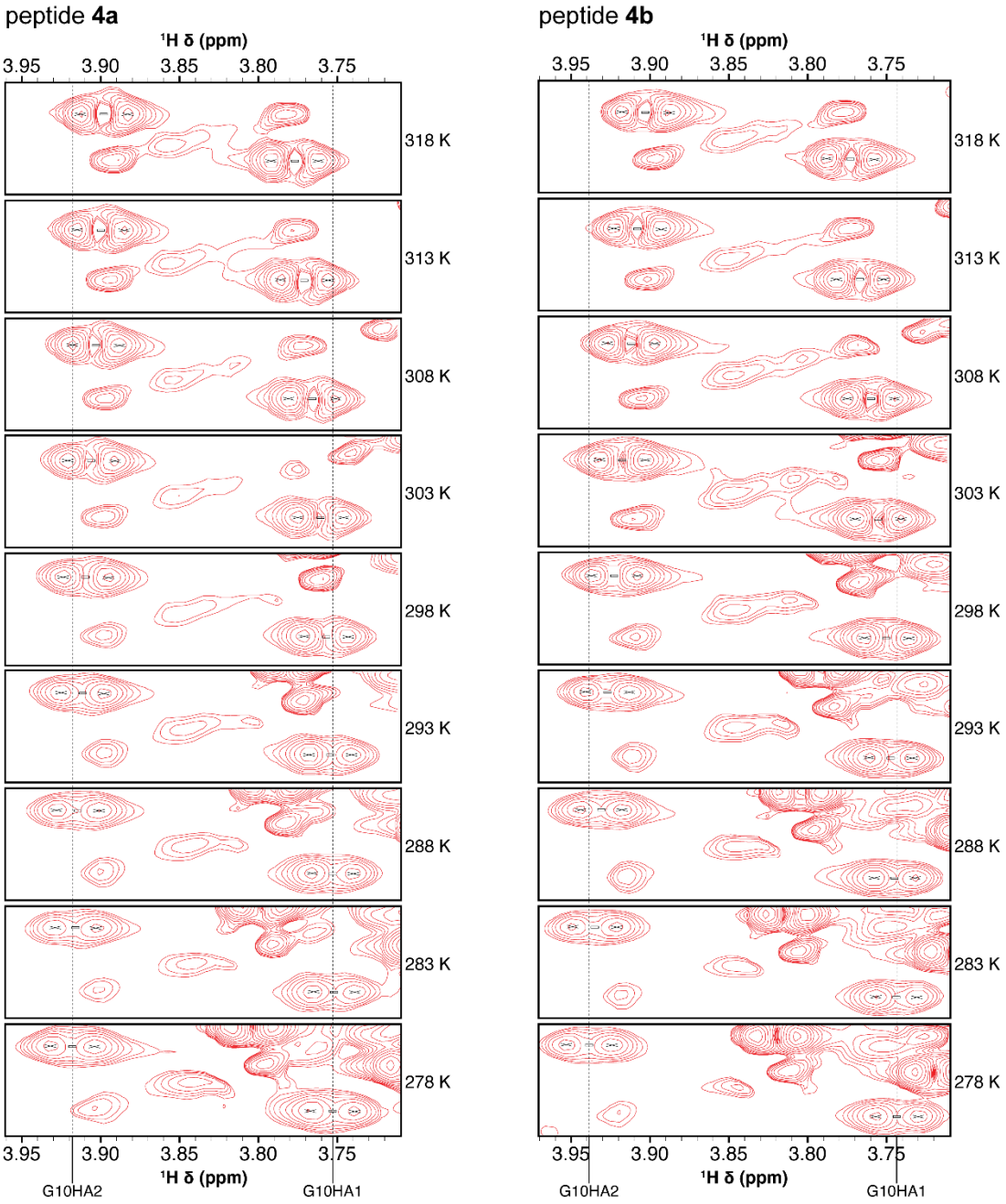

Table S1. ESI-MS Data for Peptides **2**-**4b**.

Peptide	Calculated *m/z* $(M+H)^+$	Observed *m/z* $(M+H)^+$
2	1869.88	1869.89
3a	1855.86	1855.87
3b	1855.86	1855.87
4a	1883.90	1883.91
4b	1883.90	1883.91

Table S2. Backbone Chemical Shift Assignments for Peptide **1** (298 K).

Residue	Atom	Chemical Shift (ppm)
G1	$H_{\alpha1}$	*
G1	$H_{\alpha2}$	*
E2	H	7.817
E2	H_{α}	4.453
W3	H	8.747
W3	H_{α}	4.859
A4	H	8.868
A4	H_{α}	4.760
Y5	H	8.774
Y5	H_{α}	3.648
N6	H	7.716
N6	H_{α}	4.957
P7	H_{α}	3.972
A8	H	7.835
A8	H_{α}	4.207
T9	H	7.062
T9	H_{α}	4.398
G10	H	8.318
G10	$H_{\alpha1}$	3.720
G10	$H_{\alpha2}$	4.032
K11	H	7.344
K11	H_{α}	4.704
F12	H	8.642
F12	H_{α}	4.816
A13	H	8.734
A13	H_{α}	4.684
W14	H	8.659
W14	H_{α}	4.622
T15	H	8.357
T15	H_{α}	4.398
E16	H	8.338
E16	H_{α}	4.789

*Not included due to ambiguous assignment.

Table S3. Backbone Chemical Shift Assignments for Peptide **2** (298 K).

Residue	Atom	Chemical Shift (ppm)
G1	$H_{\alpha1}$	*
G1	$H_{\alpha2}$	*
E2	H	*
E2	H_{α}	4.419
W3	H	8.384
W3	H_{α}	4.646
A4	H	8.292
A4	H_{α}	4.371
Y5	H	8.021
Y5	H_{α}	4.203
N6	H	8.031
N6	H_{α}	4.861
P7	H_{α}	4.132
A8	H	7.982
A8	H_{α}	4.239
T9	H	7.424
T9	H_{α}	4.313
G10	H	8.240
G10	$H_{\alpha1}$	3.758
G10	$H_{\alpha2}$	3.925
K11	H	7.568
K11	H_{α}	4.353
F12	H	8.195
F12	H_{α}	4.717
X13	H	8.073
W14	H	7.591
W14	H_{α}	4.922
T15	H	8.200
T15	H_{α}	4.324
E16	H	8.172
E16	H_{α}	4.229

*Not included due to ambiguous assignment.

Table S4. Backbone Chemical Shift Assignments for Peptide **3a** (298 K).

Residue	Atom	Chemical Shift (ppm)
G1	$H_{\alpha1}$	*
G1	$H_{\alpha2}$	*
E2	H	*
E2	H_{α}	4.213
W3	H	8.237
W3	H_{α}	4.578
A4	H	8.087
A4	H_{α}	4.219
Y5	H	7.913
Y5	H_{α}	4.178
N6	H	8.010
N6	H_{α}	4.765
P7	H_{α}	4.094
A8	H	7.953
A8	H_{α}	4.161
T9	H	7.421
T9	H_{α}	4.213
G10	H	8.169
G10	$H_{\alpha1}$	3.689
G10	$H_{\alpha2}$	3.827
K11	H	7.571
K11	H_{α}	4.183
F12	H	8.138
F12	H_{α}	4.455
X13	H	8.112
W14	H	7.343
W14	H_{α}	4.610
T15	H	7.785
T15	H_{α}	4.155
E16	H	7.878
E16	H_{α}	4.104

*Not included due to ambiguous assignment.

Table S5. Backbone Chemical Shift Assignments for Peptide **3b** (298 K).

Residue	Atom	Chemical Shift (ppm)
G1	$H_{\alpha1}$	*
G1	$H_{\alpha2}$	*
E2	H	*
E2	H_{α}	*
W3	H	8.286
W3	H_{α}	4.667
A4	H	8.120
A4	H_{α}	4.284
Y5	H	8.277
Y5	H_{α}	4.565
N6	H	8.089
N6	H_{α}	4.839
P7	H_{α}	4.187
A8	H	8.050
A8	H_{α}	4.240
T9	H	7.539
T9	H_{α}	4.251
G10	H	8.207
G10	$H_{\alpha1}$	3.802
G10	$H_{\alpha2}$	3.898
K11	H	7.705
K11	H_{α}	4.284
F12	H	7.967
F12	H_{α}	4.336
X13	H	4.330
W14	H	7.547
W14	H_{α}	4.641
T15	H	7.705
T15	H_{α}	4.162
E16	H	7.891
E16	H_{α}	4.110

*Not included due to ambiguous assignment.

Table S6. Backbone Chemical Shift Assignments for Peptide **4a** (298 K).

Residue	Atom	Chemical Shift (ppm)
G1	$H_{\alpha1}$	*
G1	$H_{\alpha2}$	*
E2	H	*
E2	H_{α}	4.367
W3	H	8.346
W3	H_{α}	4.645
A4	H	8.226
A4	H_{α}	4.325
Y5	H	7.965
Y5	H_{α}	4.275
N6	H	8.069
N6	H_{α}	4.485
P7	H_{α}	4.147
A8	H	7.995
A8	H_{α}	4.247
T9	H	7.451
T9	H_{α}	4.302
G10	H	8.242
G10	$H_{\alpha1}$	3.767
G10	$H_{\alpha2}$	3.909
K11	H	7.568
K11	H_{α}	4.318
F12	H	8.230
F12	H_{α}	4.688
X13	H	8.154
W14	H	7.484
W14	H_{α}	4.831
T15	H	8.032
T15	H_{α}	4.301
E16	H	8.045
E16	H_{α}	4.202

*Not included due to ambiguous assignment.

Table S7. Backbone Chemical Shift Assignments for Peptide **4b** (298 K).

Residue	Atom	Chemical Shift (ppm)
G1	$H_{\alpha1}$	*
G1	$H_{\alpha2}$	*
E2	H	*
E2	H_{α}	4.419
W3	H	8.384
W3	H_{α}	4.646
A4	H	8.292
A4	H_{α}	4.371
Y5	H	8.021
Y5	H_{α}	4.203
N6	H	8.031
N6	H_{α}	4.861
P7	H_{α}	4.132
A8	H	7.982
A8	H_{α}	4.239
T9	H	7.424
T9	H_{α}	4.313
G10	H	8.241
G10	$H_{\alpha1}$	3.758
G10	$H_{\alpha2}$	3.925
K11	H	7.568
K11	H_{α}	4.353
F12	H	8.195
F12	H_{α}	4.717
X13	H	8.072
W14	H	7.591
W14	H_{α}	4.922
T15	H	8.200
T15	H_{α}	4.324
E16	H	8.172
E16	H_{α}	4.229

*Not included due to ambiguous assignment.

Table S8. Gly10 $H_{\alpha 1}$ and $H_{\alpha 2}$ Chemical Shift Assignments for Peptide **4a** as a Function of Temperature.

temp (K)	G10 $H_{\alpha 1}$	G10 $H_{\alpha 2}$	Δδ (ppm)	fraction folded	ΔG (kcal/mol)
278	3.753	3.918	0.165	0.532	-0.071
283	3.752	3.916	0.164	0.529	-0.065
288	3.753	3.914	0.161	0.519	-0.044
293	3.754	3.912	0.158	0.510	-0.023
298	3.757	3.909	0.152	0.490	0.023
303	3.760	3.906	0.146	0.471	0.070
308	3.766	3.903	0.137	0.442	0.143
313	3.771	3.900	0.129	0.416	0.211
318	3.777	3.898	0.121	0.390	0.282

Table S9. Gly10 $H_{\alpha 1}$ and $H_{\alpha 2}$ Chemical Shift Assignments for Peptide **4b** as a Function of Temperature.

temp (K)	G10 $H_{\alpha 1}$	G10 $H_{\alpha 2}$	Δδ (ppm)	fraction folded	ΔG (kcal/mol)
278	3.743	3.939	0.196	0.632	-0.299
283	3.744	3.935	0.191	0.616	-0.266
288	3.745	3.931	0.186	0.600	-0.232
293	3.747	3.927	0.18	0.581	-0.189
298	3.750	3.922	0.172	0.555	-0.130
303	3.755	3.918	0.163	0.526	-0.062
308	3.760	3.912	0.152	0.490	0.024
313	3.767	3.908	0.141	0.455	0.113
318	3.773	3.903	0.130	0.419	0.206

Table S10. Statistics for NMR Structure Calculations of Peptides **1**, ***ent*-4a**, and **4b**

	1	***ent*-4a**	**4b**
Experimental restraints			
Total NOEs	247	232	260
Unambiguous NOEs	231	208	220
Intra-residue	114	124	121
Sequential ($\|i - j\| = 1$)	50	47	54
Medium-range ($1 < \|i - j\| < 5$)	23	16	11
Long-range ($\|i - j\| \geq 5$)	44	21	34
Ambiguous NOEs	16	24	40
H-bonds	12	6	8
Violations			
NOE >0.5 Å	0.4 ± 0.5	1.4 ± 1.0	2.1 ± 1.1
NOE rmsd (Å)	0.037 ± 0.02	0.13 ± 0.06	0.10 ± 0.05
H-bond >0.5 Å	0	0	0
Ensemble rmsd			
Backbone heavy atoms	0.61 ± 0.23	1.33 ± 0.33	1.52 ± 0.34
All heavy atoms	0.86 ± 0.19	1.50 ± 0.34	1.79 ± 0.30
Geometry analysis			
rmsd bonds (Å)	0.0031 ± 0.0002	0.0030 ± 0.0001	0.0028 ± 0.0002
rmsd angles (°)	0.38 ± 0.01	0.43 ± 0.01	0.44 ± 0.02
rmsd impropers (°)	1.03 ± 0.14	0.83 ± 0.13	0.92 ± 0.16
Ramachandran analysis			
Favored (%)	94.0	82.1	83.6
Allowed (%)	5.3	15	14.3
Disallowed (%)	0.7	2.9	2.1

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