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A General Method for Constraining Short Peptides to an α-Helical Conformation

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Abstract: A method for constraining short peptides (<20 residues) of arbitrary sequence to an α-helical conformation (~100% helical in H_2O at 25 °C) is presented. Glutamine residues at positions *i* and *i* + 7 of the peptides were tethered with an alkanediyl chain between the side chain nitrogen atoms. Peptides containing this tether were readily synthesized on the solid phase by amide formation between an α,ω-diaminoalkane and the side chain carboxylates of glutamate residues. The resulting cyclic peptides were studied by NMR and CD and were found to adopt an α-helical conformation in aqueous solution. The α-helix was thermally stable to ≥40 °C. Corresponding untethered control peptides with *N*-methylglutamine at the *i* and *i* + 7 positions lacked helicity under the same conditions. Analogous peptides were also prepared for comparison using the thiolysine cross-linking method described previously [Jackson, D. Y.; King, D. S.; Chmielewski, J.; Singh, S.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 9391−9392].

Introduction

The α-helix is the most prevalent secondary structure element observed in proteins. However, investigations of the chemical and physical properties of isolated α-helices have been hampered by the inability of most linear peptides to sustain an α-helical conformation in aqueous solution.[1,2] A general method allowing the study of isolated α-helices would constitute a useful tool for experimental investigation of theories on the formation and stability of α-helices, accurate measurements of the energetics of binding interactions involving α-helices, and "epitope reduction" of α-helical protein domains involved in biologically important interactions.

A variety of methods for stabilizing α-helical peptides have been described previously. Such peptides have been stabilized by modification of the solvent (e.g., with trifluoroethanol) and by dimerization at hydrophobic interfaces.[3,4] Short α-helical peptides have been stabilized from the end(s) by stabilization of the intrinsic helix dipole[5] and by incorporation of naturally-

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occurring capping motifs[6−8] as well as organic templates.[9−12] Noncovalent side chain constraints that have been used for α-helix stabilization include hydrophobic interactions,[13] salt bridges,[14,15] and metal ion chelation by both natural[16−18] and unnatural[19] amino acids. Finally, α-helices have been stabilized by covalent side chain tethers: side chain to side chain lactamization between residues *i* and *i* + 3, *i* and *i* + 4, or *i* and *i* + 7,[20−25] and disulfide bonds between residues *i* and *i* + 4[26] or *i* and *i* + 7.[27]

Earlier work on the bee venom peptide apamin[28] as a scaffold for presentation of α-helical peptide sequences[29,30] served as a starting point for our design of an amide-based tether. Apamin presents a C-terminal helix that is stabilized by two disulfide bonds to a structured N-terminal loop. The utility of apamin as a general scaffold for helix display is limited by the fact that the N-terminus of the helix is "capped" and cannot be extended. Synthetic apamin peptides in which either of the cysteines is replaced by a pair of alanines show a marked decrease in helicity as evaluated by circular dichroism[31] and proton NMR;[32] comparison of the peptide lacking the Cys3−Cys15 pair with that lacking the Cys1−Cys11 pair suggests that an *i* to *i* + 7 tether is more effective than an *i* to *i* + 3 tether for inducing helicity.

We evaluated the *i* to *i* + 7 tethering scheme recently described by Jackson *et al.*[27] Although peptides with this disulfide tether appear helical by CD, NMR studies suggest a loss of helicity surrounding the D-amino acid in the *i* position (see the Supporting Information), consistent with the observation that D-amino acids tend to destabilize helical peptides by approximately 1 kcal mol^{-1}.[33] The method also requires a multistep synthesis of D- and L-thiolysine. Hence, we sought to develop an alternative *i* to *i* + 7 tethering scheme that could be implemented using commercially available L-amino acids.

We established the following criteria for a method that would be generally useful for our applications: (1) The method must allow the presentation of arbitrary amino acid sequences. (2) It must maintain helicity approaching 100% in H_2O at room temperature in short peptides (less than 20 residues). (3) It must be synthetically straightforward and compatible with solid-phase peptide synthesis chemistry. (4) It must allow variation of solvent (e.g., osmolarity and pH) and temperature. We report here a new method for constraining peptides to an α-helical conformation with an alkanediyl tether between amide side chains at residues *i* and *i* + 7. The initial design of the tether is described, along with the synthesis and structural characterization by NMR and CD of several short tethered α-helical peptides. Structural studies of analogous nontethered peptides and peptides constrained using the thiolysine "lock" are also presented for comparison.

Results and Discussion

Design Considerations. As a tether we chose an alkanediyl chain between the side chain nitrogen atoms of glutamine residues at positions *i* and *i* + 7. This structure was compatible with our chemical criteria and was expected to provide a minimum of strain and steric hindrance. Straightforward peptide-compatible chemistries with which to construct the desired tether include amide and disulfide bonds. Disulfides, however, introduce an unwanted 90° twist into the linkage. A representative set of protein crystal structures from the Brookhaven Database[34] was searched for all occurrences of glutamine in an α-helical context (with $\phi = -60° \pm 30°$ and $\psi = -45° \pm 30°$). The resulting data set was used to determine the side chain rotamer distributions of naturally occurring helical glutamine residues. In general, amino acid residues in an α-helical context have $\chi_1 \approx -60°$, a conformation suitable for the *i* + 7 position of a side chain linker. Glutamine has a relatively high population (14.6%) of the $\chi_1 = 180°$ rotamer, representing a suitable conformation for the *i* position that points the side chain toward the C-terminal end of the helix. Rotamer combinations were identified that minimized the Nε2−Nε2 distance between the *i* and *i* + 7 side chains in a model helical peptide; the optimized distances ranged from 5.3 to 7 Å.

Model building suggested that a 4-methylene "bridge" could optimally link these two glutamine side chains without incurring unfavorable torsional interactions. Models of 3-, 4-, and 5-methylene-bridged helical peptides were constructed using distance geometry methods[35] followed by energy minimization. All residues except the linked glutamines were alanine. The conformational stabilities of helical peptides were assessed using 1 ns of unconstrained molecular dynamics at 298 K following an initial 100 ps equilibration period during which harmonic restraints (25 kcal mol^{-1} Å^{-1}) were applied to maintain helicity. As a control, a polyalanine helix was calculated for 1 ns in the presence of identical restraints.

Peptides containing a 3-methylene bridge maintained a consistent helical conformation but showed significant "bending"

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Table 1. Structures of Peptides **1**−**4**

1	Ac **T N X D L A A R R Z Q Q** NH_2	**a:** protected, on resin, X=Glu(OAll), Z=Glu(OFm)
		b: X=Z=Gln(NMe)
2	Ac **A E X A A A K F L Z A H A** NH_2	**c:** X–Z=Gln(N(CH_2)$_3$N)Gln
		d: X–Z=Gln(N(CH_2)$_4$N)Gln
		e: X–Z=Gln(N(CH_2)$_5$N)Gln
		f: protected, on resin, X=Z=Glu(OFm)
3	Ac **T N X D L A A R R Z Q Q** NH_2	**a:** X=D-Thiolys(Acm), Z=L-Thiolys(Acm)
		b: X–Z=D-Thiolys-S–S-L-Thiolys
4	Ac **A E X A A A K F L Z A H A** NH_2	

of the helix axis. Peptides containing a 4-methylene bridge maintained helicity with little distortion, having backbone dihedral angles comparable to those of the control peptide; χ_1 and χ_2 angles of the tethered glutamines did not change during the simulation. Peptides based on a 5-methylene bridge transiently escaped out of a helical conformation into nested turns centered around the $i + 5$ residue. Multiple side chain rotamers were also observed in the $i + 7$ residue. On the basis of these observations, it seemed that the 4-methylene bridge would be of optimal length, although the behavior of the 5-methylene-bridged peptide may have been an artifact of the simple *in vacuo* simulation.

Synthesis. Amino acid sequences for trial peptides were based on the C-terminal helix of apamin[36−38] (peptides **1** and **2**, "apamin") and on S-peptide derived from the C-peptide from RNAse A[6,39] (peptides **3** and **4**, "C-tide"). The complete sequences of these peptides are shown in Table 1.

Linear protected peptides **1a**, **1f**, and **2a** were synthesized by standard Merrifield methods using BOC chemistry. Control peptides **1b** and **2b** were elaborated from **1f** and **2a** by simultaneous deprotection of both glutamate residues followed by coupling with methylamine (Figure 1). Attempted synthesis of **1d** from **1f** by double deprotection and coupling with 1,4-butanediamine gave an impure product in poor yield. Constrained peptides **1c**−**e** and **2c**−**e** were elaborated from **1a** and **2a** by removal of the 9-fluorenylmethyl ester from Glu3, coupling with the appropriate alkanediamine, removal of the allyl ester from Glu10, and cyclization (Figure 1). Yields were improved by the use of mono-BOC-protected alkanediamine in the first coupling step (R = BOC) and by the use of a polystyrene resin with 2% DVB cross-linker. The completed peptides were cleaved from the resin with HF and purified by preparative HPLC.

Thiolysine-based peptides **3a** and **4a** were synthesized in the linear acetamidomethyl-protected form using standard Merrifield methods and FMOC chemistry, followed by cleavage from the resin with trifluoroacetic acid/triethylsilane (9:1 v/v) and purification by preparative HPLC. These were converted into the disulfide forms **3b** and **4b** in solution by simultaneous Cys deprotection and oxidation with acetic acid and molecular iodine in 2,2,2-trifluoroethanol.

Peptides **1**−**4** were characterized by mass spectrometry and by quantitative amino acid analysis. All peptides gave results consistent with the intended structures. Experimental and calculated molecular weights and AAA values are reported in the Supporting Information.

Protected D- (**7**) and L-thiolysine (**10**) were prepared as shown

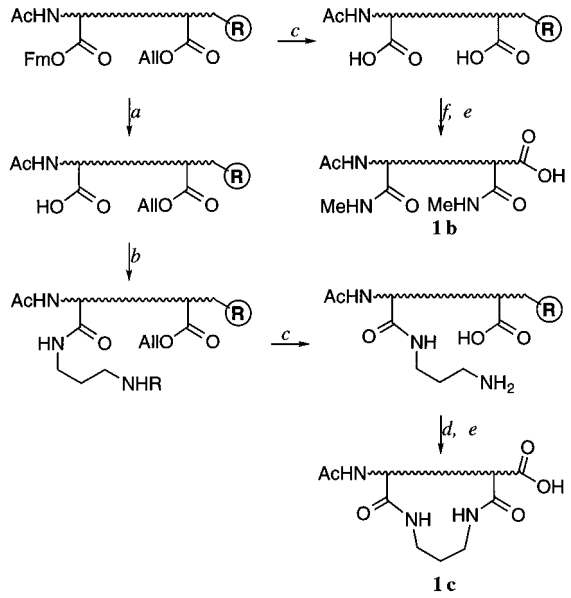

Figure 1. Synthesis of **1b** and **1c**. *a*, 20% piperidine/DMA; *b*, H_2-$NCH_2CH_2CH_2NHR$ (R = H or BOC), BOP, DIPEA, CH_2Cl_2; *c*, Pd-(PPh_3)$_4$, 20% piperidine/DMA; (R = BOC) TFA/CH_2Cl_2/anisole/$HSCH_2CH_2SH$ 9:9:1:1 v/v; *d*, BOP, DIPEA, CH_2Cl_2; *e*, HF/anisole/EtSMe 20:2:1 v/v, 0 °C; *f*, CH_3NH_2, BOP, CH_2Cl_2.

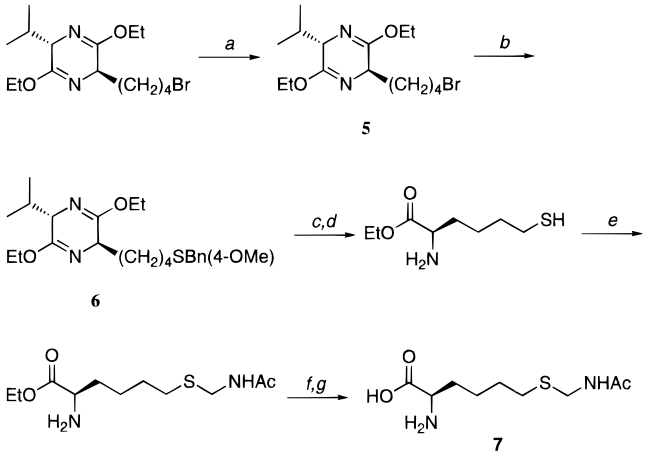

Figure 2. Synthesis of N-Fmoc-S-Acm-D-thiolysine (**7**): *a*, ^{n}BuLi, THF, −78 °C; $Br(CH_2)_4Br$; *b*, 4-MeOBnSH, KO^{t}Bu, THF; *c*, 0.25 M HCl, THF/H_2O; *d*, $Hg(OAc)_2$, TFA; H_2S; *e*, acetamidomethanol, TFA; *f*, LiOH, THF/H_2O; *g*, Fmoc-OSu, dioxane, $NaHCO_3$.

in Figure 2. The Schöllkopf reagent[40] was treated with *n*−butyllithium followed by 1,4-dibromobutane to give the known (bromobutyl)pyrazine **5**. The bromide was displaced with the potassium salt of 4-methoxytoluenethiol to give **6**. The pyrazine was hydrolyzed with aqueous HCl, and the thiol was deprotected with $Hg(OAc)_2$ in TFA followed by H_2S. The crude thiolysine ethyl ester was then reprotected with acetamidomethanol in TFA. The ester was hydrolyzed with LiOH, and the free S-protected amino acid was N-protected with Fmoc-*N*-hydroxysuccinimide in dioxane to give **7**. The same procedures were used for the synthesis of **10**.

Structural Characterization. Methods. The peptides were primarily characterized using 2D ^{1}H NMR; details are provided in the Supporting Information. Resonance positions were obtained by standard sequential assignment methods.[41] The degree of helicity of each peptide was judged from TOCSY

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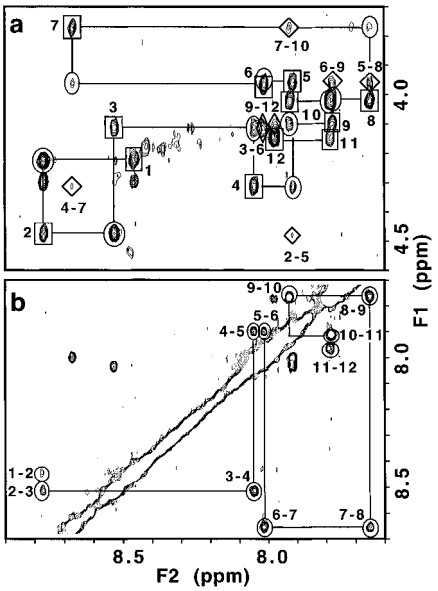

Figure 3. H^N-H^α (a) and H^N-H^N (b) sections of the ROESY spectrum of **1c**. The spectrum was collected at 280 K, pH 5.0, 500 MHz and a peptide concentration of 1.5 mM with a 4.5 kHz spin-lock mixing pulse of 200 ms duration. Lines connect the ROEs by which sequential assignments were made. Rectangular, oval, and diamond-shaped boxes denote intraresidue, sequential, and (i, $i + 3$) correlations, respectively.

and ROESY spectra by the presence of intense sequential H^N-H^N ROE cross-peaks, the presence of i and $i + 3$ $H^\alpha-H^N$ or $H^\alpha-H^\beta$ ROE cross-peaks, and ${}^3J_{H^N-H^\alpha} < 6.0$ Hz. A representative ROESY spectrum of diamide-constrained peptide **1c** is shown in Figure 3; a section of the TOCSY spectrum of this peptide is show in the Supporting Information. Additional spectra (NOESY and higher-sensitivity ROESY) were acquired and used as a basis for structure determination using distance geometry (DG) and restrained molecular dynamics (rMD). Circular dichroism spectra were acquired on aqueous solutions of **1**−**4** between 20 and 120 μM at 280 K and pH 5 to confirm the results of NMR studies; variable-temperature CD studies were used to evaluate the helicity of peptides at higher temperatures.

Helicity of 1c. NMR. Summaries of the H^N-H^N and $H^\alpha-H^N$ ROEs between neighboring residues are depicted in Figure 4 for **1c** and for the corresponding control peptide **1b**. These data indicate that peptide **1c** is helical between residues Asn2 and Gln10. Beyond Gln10, ${}^3J_{H^N-H^\alpha}$ rises above 6.0 Hz, but some medium range ROEs are still present, suggesting partial or transient helical character. Such fraying at helix termini is commonly observed in NMR studies of peptides and proteins. The ^{1}H chemical shifts of **1c** change by <0.02 ppm over the concentration range 8.0−0.06 mM; this suggests that the helical conformation is not stabilized by a self-association event. $H^\alpha-H^N$ (i, $i + 4$) interactions were also observed in higher-sensitivity ROESY spectra of **1c**, indicating that the helical conformation adopted is not of the 3_{10} type, but rather is of the regular α-helical variety.[42]

Interproton distance restraints were generated from the ROESY and NOESY data, and were used as a basis for calculating a structure for **1c**. Nearly half (66) of the 141 restraints were between amino acids two to four residues apart in the primary sequence, as expected for an α-helical conformation. Dihedral angle restraints, based on observed ${}^3J_{H^N-H^\alpha}$ and ${}^3J_{H^\alpha-H^\beta}$, were also used in these calculations, but explicit hydrogen bond restraints were not utilized. The final ensemble

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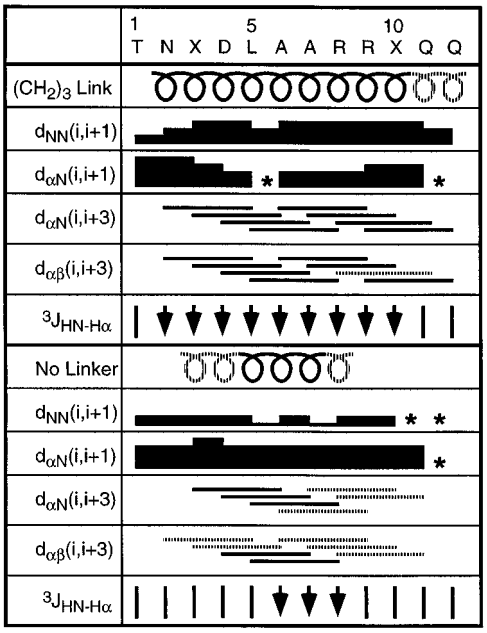

Figure 4. ROE and ${}^3J_{H^N-H^\alpha}$ data for **1c** and **1b**. For the d_{NN} and $d_{\alpha N}$ rows, observation of the sequential ROE is indicated by a bar connecting two residues, the thickness of the bar indicating the relative intensity of the ROE. The downward pointing arrows indicate ${}^3J_{H^N-H^\alpha}$ less than 6.0 Hz. Observed medium-range ROEs ($H^\alpha-H^N$ (i, $i + 3$) and $H^\alpha-H^\beta$ (i, $i + 3$)) are indicated by the lines in the lower part of the figure; dotted lines and stars indicate ROEs that could not be unambiguously observed because of chemical shift degeneracy. The coil motif above the primary sequence indicates the region deduced to have helical structure from the NMR data; the dashed coil indicates sections of peptide where only some of the NMR data indicate helical character.

Table 2. Amide Hydrogen Exchange Rate Constants[a] and Protection Factors[b] for Peptides **1b** and **1c**

residue	log k (**1b**)	log k (**1c**)	protection factor
Thr1	−2.44	−2.48	∼1
Asn2	nd	nd	
Gln3	nd	nd	
Asp4	−2.72	−2.84	1.3
Leu5	−2.69	−3.51	6.7
Ala6	−2.73	−3.77	10.9
Ala7	−2.51	−3.55	11.1
Arg8	nd	nd	> 26
Arg9	nd	−3.21	> 20
Gln10	nd	−3.29	>25
Gln11	nd	nd	
Gln12	nd	nd	

[a] The rate constants are expressed in units of s^{-1}. nd indicates that the exchange was sufficiently fast that no peak was observed in the NMR spectrum acquired 300 s after addition of D_2O. In these cases, assuming that >90% of the hydrogen atoms have exchanged in 300 s allows a lower limit of 0.013 s^{-1} (log $k = -1.89$) to be calculated for the rate constant. [b] Protection factors are calculated as the rate constant for peptide **1c** divided by that of peptide **1b**.

of 20 structures is depicted in Figure 5. The structures agree with the input data very well, with no distance restraint violations above 0.1 Å, no dihedral angle violations above 1.0°, and a mean restraint violation energy term of 0.10 ± 0.09 kcal mol^{-1}. The backbone atoms of residues Thr1 to Gln10 are well-defined (average RMS deviation from the mean structure 0.38 ± 0.08 Å), but the two C-terminal glutamine residues are not. The side chains of Thr1, Gln3, Asp4, Leu6, and Gln10 have well-defined χ_1 values, but only Gln10 has a consistent value of χ_2 in all structures.

Hydrogen-bonding interactions observed both in the calculated structure of **1c** and by solvent exchange experiments also support an α-helical structure for **1c**. $H^N(i)-O(i-4)$ hydrogen

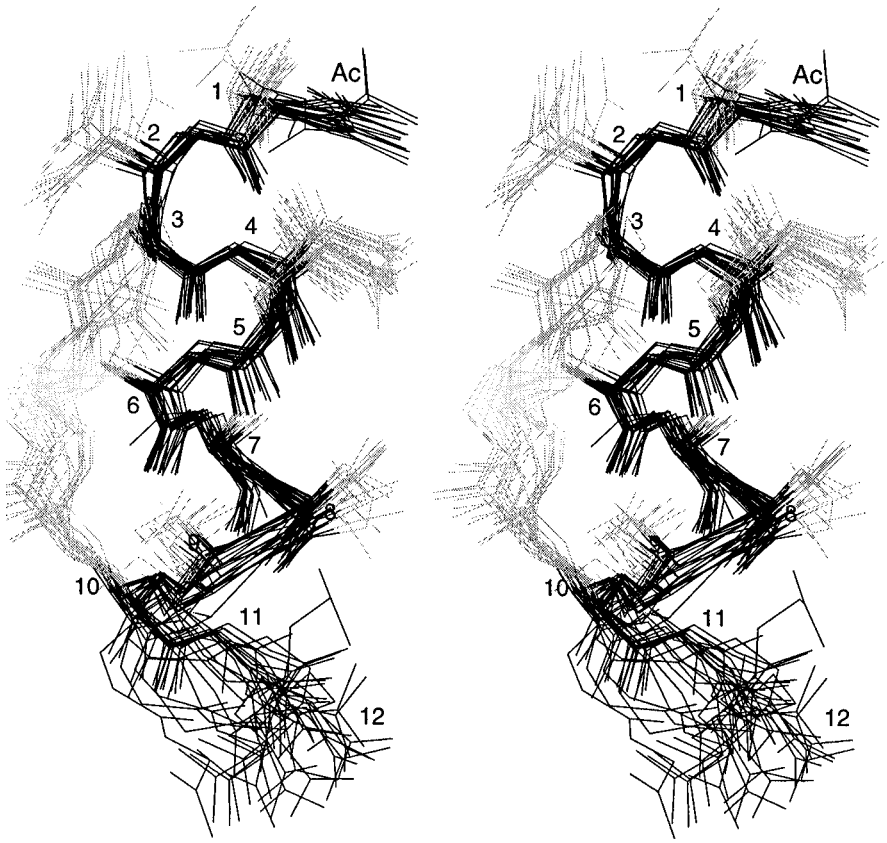

Figure 5. Ensemble of 20 rMD structures calculated using NMR data for peptide **1c**. The structures were overlaid using the N, C^{α}, and C atoms of residue Thr1 to Gln10. Backbone and side chain heavy atoms are connected by solid and dotted lines, respectively. The side chains of Arg8 and Arg9 have been truncated at C^{γ}, and all side chain atoms of Gln11 and Gln12 have been omitted for clarity.

bonds are observed to the amide protons of Leu5, Ala6, and Gln10 in >90% of the structures and to the amide protons of Ala7, Arg8, and Arg9 in ∼50% of the structures. $H^N(i)-O(i-3)$ hydrogen bonds are present for the latter protons in 25−35% of the structures, suggesting that there is a slight distortion of the helix in this region. Interestingly, hydrogen bonds from Asp4 H^N to Thr1 $O^{\gamma 2}$ are present in 80% of the structures, indicating that an N-cap hydrogen-bonding interaction[42] is present even in this short peptide. Solvent exchange rates for the amide hydrogen atoms of **1c** are up to 25-fold slower than those of **1b** (see Table 2), providing experimental evidence for the hydrogen bonding observed in the calculated structures. However, the amide proton of Asp4 is not noticeably protected from exchange; hence, the N-cap hydrogen bond may be more transient.

The backbone dihedral angles throughout the tethered region are close to those expected for an ideal α-helix (mean $\phi = -63° \pm 8°$, mean $\psi = -42° \pm 8°$). Exceptions are ψ of Ala6, which is approximately 15° lower than expected (closer to that expected for a 3_{10} helix), and ψ of Gln10, which is higher than ideal (reflecting fraying beyond the tethered region). The deviation at Ala6 may be the result of the short tether present in this peptide. Although the diamide linkage is not well defined, the side chains of Gln3 and Gln10 do adopt conformations close to those predicted by the initial modeling experiments described above (Gln3 $\chi_1 = -173° \pm 17°$, $\chi_2 = 34° \pm 47°$; Gln10 $\chi_1 = -71° \pm 7°$, $\chi_2 = 174° \pm 22°$). Thus, the ^{1}H NMR data support an α-helical solution structure for **1c** from Asp2 to Gln10 with an N-terminal capping box and a very slight distortion in the central turn of the helix.

Stability by CD. The helicity of **1c** was confirmed by CD spectrometry. At 7 °C, **1c** showed 84% helicity (evaluated by per-residue molar ellipticity at 222 nm), as compared to 20% helicity for control peptide **1b** (Figure 6). The integrity of the **1c** helix was retained under thermal denaturation conditions. Even at 97 °C, the ellipticity of **1c** was substantially more negative than that of **1b** at 7 °C, and after recooling most of the helical character of **1c** was restored (data shown in the Supporting Information).

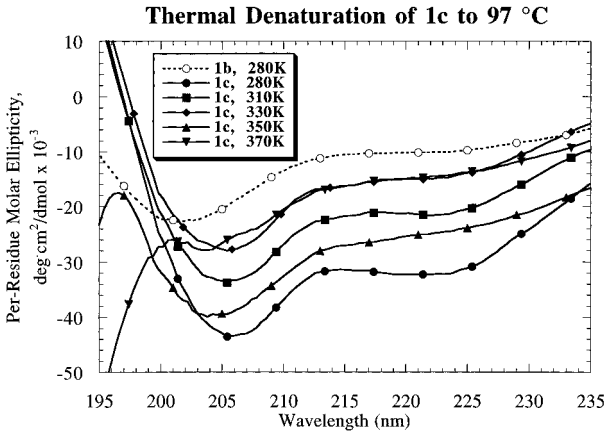

Figure 6. CD spectra of **1c** at 280, 310, 330, 350, and 370 K.

Comparison of Peptides. A summary of both ^{1}H NMR and CD data for peptides **1**−**4** is given in Table 3. Detailed summaries of ^{1}H NMR and graphs of CD data for these peptides are given in the Supporting Information. Comparison of peptides **1c**−**1e** and **2c**−**2e** with corresponding control peptides **1b** and **2b** shows that incorporation of diamide tethers of three, four, or five carbon atoms in peptides **1** and **2** clearly reduces the mean value of $^3J_{H^N-H^{\alpha}}$ in the restrained region, increases the number of observable (i, $i + 3$) ROEs, and increases the percent helicity observed by CD. The results for peptide **4**

Table 3. Evaluation of Peptide Helicity[a]

peptide	description	mean $^3J_{H^N-H^\alpha}$ in constrained region	fraction of medium-rang ROEs obsd	percent helicity by CD
1b	apamin, *N*-methyl-Gln control	6.0	0.14	20
1c	apamin, 1,3-propanediyl linker	5.0	0.69	84
1d	apamin, 1,4-butanediyl linker	5.2	0.56	63
1e	apamin, 1,5-pentanediyl linker	5.0	0.69	100
2b	C-tide, *N*-methyl-Gln control	5.9	0.08	32
2c	C-tide, 1,3-propanediyl linker	4.8	0.75	60
2d	C-tide, 1,4-butanediyl linker	4.8	0.43	82
2e	C-tide, 1,5-pentanediyl linker	4.9	0.80	63
3a	apamin, *S*-Acm thiolys control	6.0	0.03	10
3b	apamin, thiolys disulfide	6.0	0.08	35
4a	C-tide, *S*-Acm thiolys control	6.0	0.05	19
4b	C-tide, thiolys disulfide	6.0	0.48	27

[a] Values below 6 for the mean three-bond H^N-H^α coupling constant indicate helicity. Medium-range $i - i + 3$ ROEs are expressed as the observed fraction of the total number of such ROEs possible, with very weak ROEs counted as half. The percent helicity as determined by CD is derived as by Jackson.[42]

indicate that the formation of the disulfide bond does constrain the peptide to be helical (Table 3). However, a number of medium-range ROEs could not be observed and the $^3J_{H^N-H^\alpha}$ values were >6.0 Hz for the two thiolysine residues and Leu9 in **4b**, indicating a distortion from an ideal helical structure in the region of the D-thiolysine residue. With the apamin sequence, incorporation of the thiolysine disulfide appears to impart an increase in helicity as judged by CD (**3b** *vs* **3a**); however, the ^{1}H NMR data for **3b** show none of the characteristics of a true helix.

Conclusion

We have devised a simple method for constraining small peptides to an α helical conformation. This *i* to *i* + 7 amide-based tether is successful as a general method for inducing α-helicity in small peptides. It allows complete sequence variability in all residues except the two directly involved in the tether and is successful with short (12 and 13 residue) peptides; comparison of helical peptides **1c**−**1e** with nonhelical peptide **1b** shows that the helicity is achieved by introduction of the linker rather than being a property of the primary sequence. The method induces helicity approaching 100% in aqueous solution at room temperature and substantial helicity at higher temperatures. The tethered peptides are synthesized by standard solid-phase (Merrifield) chemistry requiring only commercially available reagents. This method should be generally useful for studies of biologically active helical regions of proteins; for the experimental study of helix formation, propagation, and stability; and for physical organic experiments on the interactions of helical peptides with their environments.

Experimental Section

Details for the syntheses of peptides **1**−**4** and for D- and L-Boc-thiolys(*S*-Acm)OH (**7** and **10**) and for experimental methods for ^{1}H NMR and CD spectroscopy are provided in the Supporting Information.

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Supporting Information Available: Details of synthetic and analytical methods, additional references for the Experimental Section, tables of ^{1}H NMR data for peptides **1**−**4** , summaries of ROE and coupling data for peptides **1**−**4**, a section of the TOCSY spectrum of peptide **1c**, a graph of thermal denaturation of **1c**, and CD spectra of peptides **1**−**4** (29 pages). See any current masthead page for ordering and Internet access instructions.

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