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Design of a Peptide Hairpin Containing a Central Three-Residue Loop

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Abstract: The construction of **a** designed β-hairpin structure, *containing a central* three-residue loop has been successfully achieved in the synthetic nonapeptide Boc-Leu-Phe-Val-^{D}Pro-^{L}Pro-^{D}Ala-Leu-Phe-Val-OMe (**2**). The design is based on expanding the two-residue loop established in the peptide β-hairpin Boc-Leu-Phe-Val-^{D}Pro-^{L}Pro-Leu-Phe-Val-OMe (**1**). Characterization of the registered β-hairpins in peptides **1** and **2** is based on the observation of key nuclear Overhauser effects (NOEs) in $CDCl_3$ and CD_3OH. Solvent titration and temperature dependence of NH chemical shifts establish the identity of NH groups involved in interstrand hydrogen bonding. In peptide **2**, the antiparallel registry is maintained, with the formation of a ^{D}Pro-^{L}Pro-^{D}Ala loop, stabilized by a 5→1 hydrogen bond between Val3 CO and Leu7 NH groups (C_{13}, α-turn) and a 3→1 hydrogen bond between ^{D}Pro4 CO and ^{D}Ala6 NH groups (C_7, γ-turn). NMR derived structures suggest that in peptide **2**, ^{D}Ala(6) adopts an α_L conformation. In peptide **1**, the ^{D}Pro-^{L}Pro segment adopts a type II′ β-turn. Replacement of ^{D}Ala (6) in peptide **2** by ^{L}Ala in peptide **3** yields a β-hairpin conformation, with a central ^{D}Pro-^{L}Pro two-residue loop. Strand slippage at the C-terminus results in altered registry of the antiparallel strands.

Introduction

β-Hairpins are widely distributed in proteins**.** Hairpin formation in polypeptide chains is nucleated by chain reversals facilitated by connecting loops, that contain one or more residues. Two-residue loops of defined stereochemistry have been most frequently observed as hairpin nucleating segments, with type I′ and II′ β-turns being the most common.[1] The tendency of the 'prime turns' to nucleate β-hairpins has been used successfully in generating synthetic models incorporating ^{D}Pro-Xxx (where Xxx = Gly, ^{L}Ala, ^{D}Ala, Aib, Ac_6c, and Ac_8c) or Asn-Gly segments as the folding nucleus.[2] Tight, two-residue turns have been employed in the construction of multistranded β-sheet structures in de novo designed peptides.[3] Three-residue turns are the second most frequently observed class of hairpin **forming** loops. An analysis of hairpins in protein structures revealed that the most widely observed loop conformation was $\alpha_R\alpha_R\alpha_L$, which represents a type I β-turn followed by a residue in the left-handed α-helical region.[1d,4] Three-residue loops, notably the G1 bulge, have been characterized in a monomeric β-hairpin derived from the N-terminus of ubiquitin, although this peptide has non-native registry of the antiparallel strands.[5] As part of a program to engineer peptide hairpins with three-residue loops, we have been examining the conformational properties of designed nonapeptides containing a centrally positioned three-residue loop segment. Our approach is based on the principle that antiparallel structures may be generated by preferentially limiting the range of conformations of the central peptide segment, which facilitates the chain reversal. The use of a heterochiral diproline segment permits sharp chain reversal. The weight of available evidence suggests that polypeptide hairpin folding is dictated by turn characteristics, with cross-strand interactions adding stability after nucleation of hairpin.[5] This report describes the successful construction of a peptide hairpin containing a central three-residue loop. The present approach is based on an attempt to expand well-defined two-residue loop hairpins by insertion of an additional residue, without disruption of strand registry.

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(4) α_R, α_L refers to residue conformations lying in the right- and left-handed regions of ϕ,ψ space [α_R ($\phi = -140°$ to $-30°$ and $\psi = -90°$ to $45°$), α_L ($\phi = 20°$ to $125°$ and $\psi = -45°$ to $90°$)] respectively (Table 2, ref 1d).
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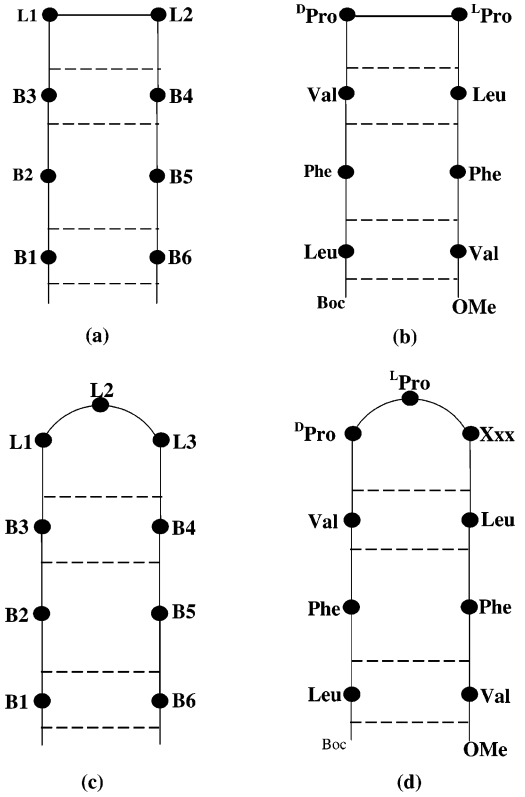

Figure 1. Schematic diagram of β-hairpins with two residue loops (a, b) and three residue loops (c, d).

Figure 1 schematically illustrates the parent two-residue hairpin and target three-residue hairpin structures. The following peptide sequences have been investigated:

Boc-Leu-Phe-Val-^{D}**Pro**-^{L}**Pro**-Leu-Phe-Val-OMe (**1**)

Boc-Leu-Phe-Val-^{D}**Pro**-^{L}**Pro**-^{D}**Ala**-Leu-Phe-Val-OMe (**2**)

Boc-Leu-Phe-Val-^{D}**Pro**-^{L}**Pro**-^{L}**Ala**-Leu-Phe-Val-OMe (**3**)

The parent hairpin (peptide **1**) has the strong type II′ β-turn forming ^{D}Pro-^{L}Pro sequence located centrally.[6] Previous studies on cyclic peptides have established the ability of this dipeptide sequence to facilitate appropriate antiparallel strand registry.[6] The tripeptide segment Leu-Phe-Val was chosen for the potential β-strands, since earlier work has established β-hairpin conformations in model peptides incorporating this segment.[2e,7] Peptides **2** and **3** were designed to examine the impact of insertion of a ^{D}Ala/^{L}Ala residue immediately following the β-turn segment. The results presented in this paper demonstrate that insertion of ^{D}Ala preserves a β-hairpin conformation, with expansion of the connecting loop to a three-residue turn, while in the case of ^{L}Ala the two-residue turn nucleated hairpin is maintained, with the ^{L}Ala residue being accommodated into the C-terminus strand, resulting in slippage of the antiparallel registry.

Experimental Section

Materials and Methods. The peptides were synthesized by classical solution phase procedures using a racemization-free fragment condensation strategy. The *tert*-butoxycarbonyl (Boc) group was used for *N*-terminal protection and the *C*-terminal was protected as a methyl ester. Deprotections were performed using 98% formic acid and saponification for the N- and C-terminus, respectively. Couplings were mediated by *N*,*N*-dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBT). The final step in the synthesis of peptide **1** was achieved by the fragment condensation of Boc-Leu-Phe-Val-OH and **HN**-^{D}Pro-^{L}Pro-Leu-Phe-Val-OMe. Peptides **2** and **3** were synthesized by the fragment condensation of Boc-Leu-Phe-Val-OH and HN-^{D}Pro-^{L}Pro-Xxx-Leu-Phe-Val-OMe (Xxx = ^{D}Ala **2**/^{L}Ala **3**), using DCC/HOBt as coupling agents. All the intermediates were characterized by ^{1}H NMR spectroscopy (80 MHz) and thin-layer chromatography (TLC) on silica gel and used without further purification. The final peptides were purified by reverse phase, medium-pressure liquid chromatography (C18, 40−60 μ) and high performance liquid chromatography (HPLC) on a reverse phase C18 column (5−10 μ, 7.8 mm × 250 mm) using methanol/water gradients. The purified peptides were characterized by electrospray ionization mass spectrometry (ESI-MS). MNa^{+}_{obs} **Peptide 1** = 1067 (M_{cal} = 1044), MNa^{+}_{obs} **Peptide 2** = 1138 (M_{cal} = 1115) and MNa^{+}_{obs} **Peptide 3** = 1138 (M_{cal} = 1115) and by complete assignment of 500 MHz ^{1}H NMR spectra.

NMR experiments were carried out on a Bruker DRX500 spectrometer. One-dimensional (1D) and 2D spectra were recorded at a peptide concentration of ~3 mM in $CDCl_3$ and CD_3OH at 300 K. Delineation of exposed NH groups was achieved by titrating $CDCl_3$ solution with low concentrations of DMSO-d_6. In the case of CD_3OH, intramolecular hydrogen bonding was probed by recording 1D spectra at five different temperatures between 278−323 K at 10 K intervals and determining the temperature coefficients of amide proton chemical shifts.

Resonance assignments were carried out with the help of 1D and 2D spectra. Residue specific assignments were obtained from TOCSY[8] experiments, while ROESY[9] spectra permitted sequence specific assignments. All 2D experiments were recorded in phase sensitive mode using the TPPI (time proportional phase incrementation) method. A data set of 1024 × 450 was used for acquiring the data. The same data set was zero filled to yield a data matrix of size 2048 × 1024 before Fourier transformation. A spectral width of 6000 Hz was used in both dimensions. Mixing times of 100 and 200 ms were used for TOCSY and ROESY, respectively. Shifted square sine bell windows were used while processing. All processing was done using BRUKER XWINNMR software.

Structure Calculations. Structure calculations were performed using DYANA v 1.5 software.[10] NOEs were classified as strong (2.5 Å), medium (3.5 Å), and weak (5.0 Å) by visual inspection, and used as upper distance limits, whereas the lower limit was set at 1.8 Å in all cases. Hydrogen bond constraints were obtained from temperature coefficients of amide proton chemical shifts in the case of CD_3OH and from DMSO titration data in the case of $CDCl_3$. A total of 54 distance constraints for peptide **1** and 68 distance constraints for peptide **2** were used for structure calculations. In addition, six dihedral restraints for peptide **1** and seven dihedral restraints for peptide **2** which were obtained from $^{3}J_{NHC}\alpha_{H}$ coupling constants were also added later for structure refinement. The best 10 structures out of the calculated 25 structures were selected and superposed using MOLMOL.[11]

Results and Discussion

Peptides **1**−**3** yielded well-resolved 500 MHz ^{1}H NMR spectra in both $CDCl_3$ and CD_3OH. Sequential resonance assignments were readily achieved using a combination of TOCSY and ROESY spectra. The NMR parameters for all three peptides are listed in Table 1. The solvent sensitivity of NH

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Table 1. NMR Parameters for Peptides **1**−**3** in $CDCl_3$ and CD_3OH Solutions[a]

	peptide **1**					peptide **2**					peptide **3**				
	chemical shift (ppm)					chemical shift (ppm)					chemical shift (ppm)				
residue	NH	$C^{\alpha}H$	$^3J_{NH\,C^{\alpha}H}$	$\Delta\delta^b$ (ppm)	dδ/dT (ppb/k)	NH	$C^{\alpha}H$	$^3J_{NH\,C^{\alpha}H}$	$\Delta\delta^b$ (ppm)	dδ/dT (ppb/k)	NH	$C^{\alpha}H$	$^3J_{NH\,C^{\alpha}H}$	$\Delta\delta^b$ (ppm)	dδ/dT (ppb/k)
Leu(1)	5.10 (6.16)	4.26 (4.24)	8.4 (8.7)	0.95	1.08	5.10 (6.28)	4.10 (4.14)	8.1 (8.8)	0.90	2.6	5.54 (6.81)	4.15 (4.18)	7.0 (8.9)	0.84	8.15
Phe(2)	7.11 (8.19)	5.44 (5.42)	C (8.9)	C	10.09	6.48 (8.05)	5.53 (5.49)	9.1 (8.0)	1.49	6.9	6.75 (8.19)	5.21 (5.25)	8.5 (8.7)	0.68	10.0.5
Val(3)	8.77 (9.03)	4.60 (4.54)	9.6 (9.8)	0.01	4.25	8.73 (9.00)	4.97 (4.95)	9.0 (9.3)	0.02	6.9	8.35 (8.67)	4.54 (4.55)	8.8 (9.5)	−0.58	3.4
^{D}Pro (4)	–	4.59 (4.82)	–			–	4.45 (4.60)	–			–	4.56 (4.68)	–	–	–
^{L}Pro(5)	–	4.68 (4.78)	–			–	4.70 (4.65)	–			–	4.56 (4.51)	–	–	–
^{D}Ala/^{L}Ala (6)	–	–	–			7.70 (7.98)	4.36 (4.29)	7.0 (6.9)	0.01	3.5	7.72 (7.88)	4.40 (4.40)	8.2 (8.0)	−0.17	2.35
Leu (6)/Leu (7)	7.76 (7.98)	4.39 (4.60)	8.3 (8.6)	0.12	2.03	7.58 (8.07)	4.43 (4.55)	8.0 (9.3)	0.17	4.2	7.11 (7.99)	4.50 (4.35)	c (7.9)	0.84	7.84
Phe (7)/Phe(8)	6.60 (8.61)	4.60 (4.58)	8.2 (7.7)	1.91	9.23	6.59 (8.63)	4.98 (4.86)	7.4 (7.6)	1.83	9.3	8.32 (8.55)	4.73 (4.63)	7.1 (8.3)	−0.51	8.72
Val (8)/Val(9)	7.70 (8.17)	4.25 (4.10)	9.1 (9.8)	0.23	2.90	7.61 (8.13)	4.27 (4.10)	8.8 (8.7)	0.15	4.3	7.01 (7.99)	4.44 (4.27)	6.0 (7.9)	0.58	9.65

[a] The values in parentheses correspond to those in CD_3OH solution. [b] $\Delta\delta = \delta$ ($CDCl_3$ + DMSO-d_6) − δ ($CDCl_3$); c = overlapped.

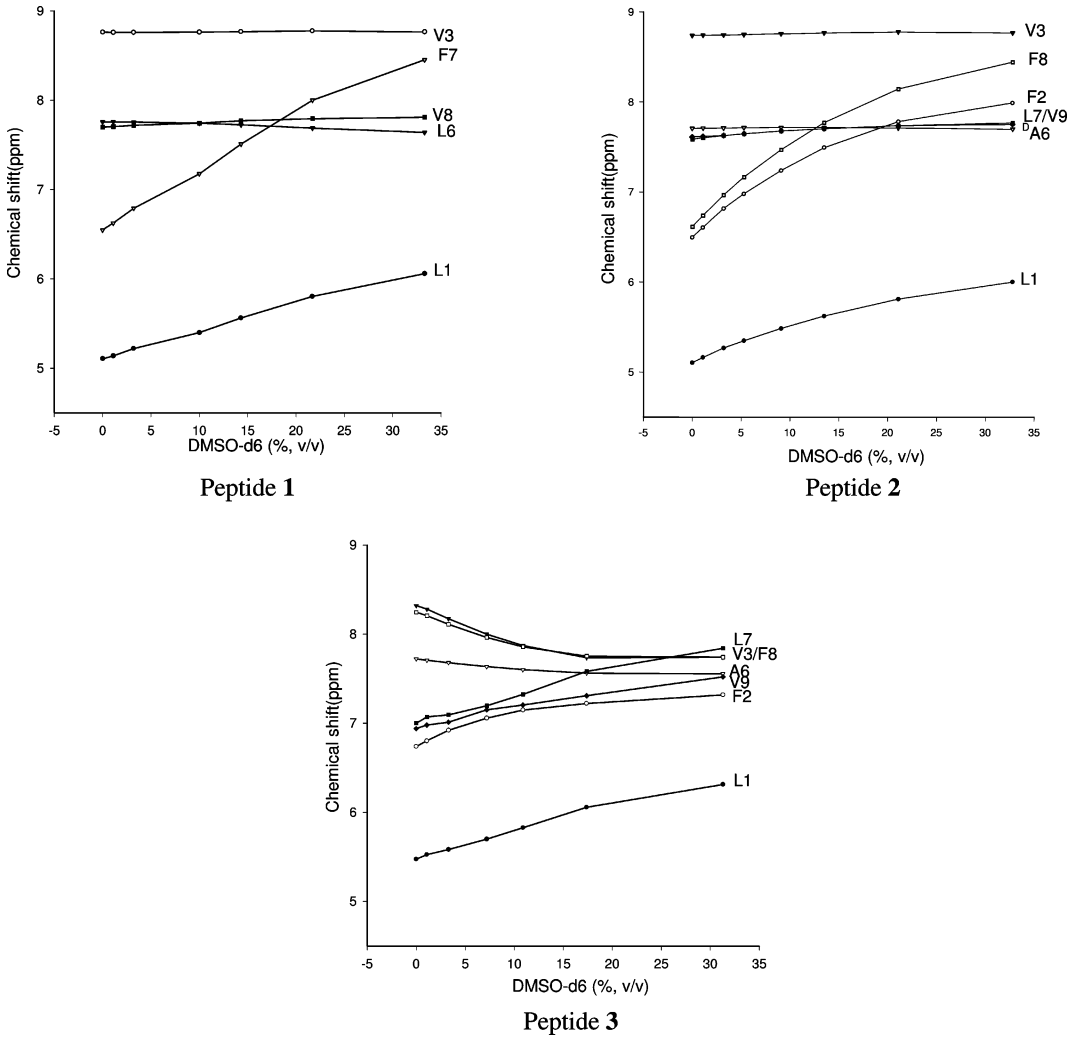

Figure 2. Solvent dependence of NH chemical shifts at varying concentrations of $(CD_3)_2SO$ in $CDCl_3$ to probe exposed vs hydrogen-bonded amides.

chemical shifts in the peptides was probed by addition of varying concentrations of the strongly hydrogen-bonding solvent DMSO to peptides in the poorly interacting solvent, $CDCl_3$. The solvent titration curves are shown in Figure 2. Temperature coefficients of NH chemical shifts in methanol were determined and are listed in Table 1. Nuclear Overhauser effects (NOEs) were determined using ROESY spectra. A comparison of the NH−NH and $C^{\alpha}H-C^{\alpha}H$ NOEs are shown in Figures 3 and 4.

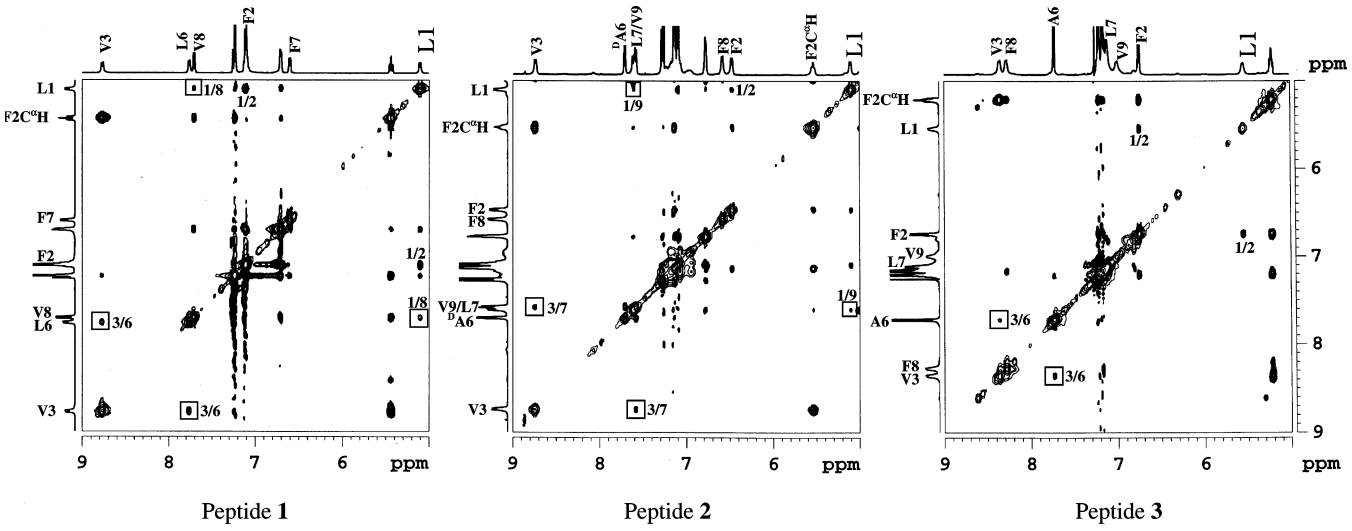

Figure 3. Partial 500 MHz ROESY spectra of peptides **1**−**3** in $CDCl_3$ highlighting NH↔NH NOEs.

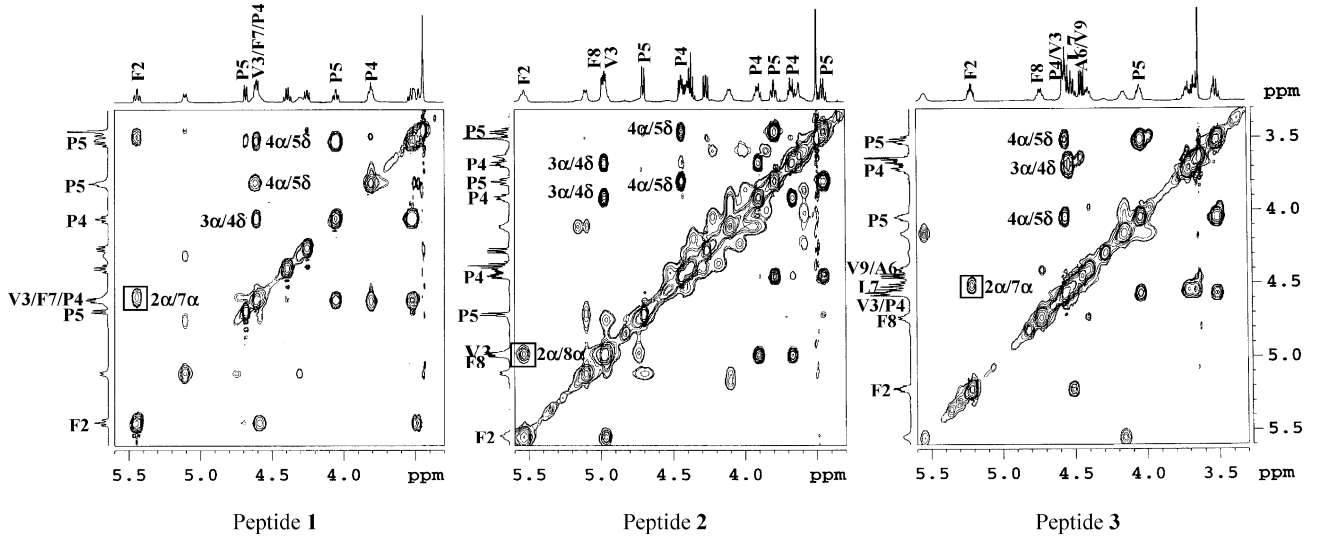

Figure 4. Partial 500 MHz ROESY spectra of peptides **1**−**3** illustrating $C^{\alpha}H$↔$C^{\alpha}H$ and $C^{\alpha}H$↔$C^{\delta}H$ NOEs observed for the peptides in $CDCl_3$.

Conformations of Peptide 1. In peptide **1** three NH groups, V3, L6, and V8, show limited solvent dependence of chemical shifts in $CDCl_3$−DMSO mixtures and low $d\delta/\delta T$ values in CD_3-OH, suggesting their solvent-shielded nature in both solvents. Indeed, in an ideal β-hairpin structure (Figure 5) these three NH groups are anticipated to form intramolecular hydrogen bonds. In addition, Leu1 NH also displays a low-temperature coefficient in CD_3OH suggesting that the terminal hydrogen bond may be favored in this solvent. Relatively large $^3J_{NHC^{\alpha}H}$ values (>8.0 Hz) for residues **1**−**3** and **6**−**8** are also supportive of extended strand conformations for these segments.

Critical NOEs characteristic of β-hairpin conformations are also observed (Figures 3 and 4). Most notably, Figure 3 illustrates the d_{NN} NOEs between Leu1 and Val8 and Val3 and Leu6 NH groups, which are diagnostic of strand registry. The $d_{\alpha\alpha}$ NOE between Phe2 $C^{\alpha}H$ and Phe7 $C^{\alpha}H$ is shown in Figure 4. An NOE between Val3 $C^{\alpha}H$ and ^{D}Pro4 $C^{\delta}H$ confirms the trans conformation about the Val3-^{D}Pro4 bond, while the ^{D}Pro4 $C^{\alpha}H$↔^{L}Pro5 $C^{\delta}H$ NOE is characteristic of a ψ value of ∼−120° for ^{D}Pro4, which is necessary for accommodating a type II′ β-turn about the 4−5 segment. Structure calculations using NMR-derived distance constraints result in the β-hairpin structures illustrated in Figure 6. The backbone dihedral angles for one of the NMR structures listed in Table 2 compares well with those determined crystallographically for the sequence -Leu-Phe-Val-^{D}Pro-Gly-Leu-Phe-Val-OMe, which constitutes the C-terminus of a synthetic 17-residue peptide, which contains a β-hairpin segment.[12]

Conformations of Peptide 2. Figure S1 (in Supporting Information) shows a chemical shift correlation diagram for peptides **1**−**3**. The data for the established hairpin Boc-Leu-Phe-Val-^{D}Pro-Gly-Leu-Phe-Val-OMe is also shown for comparison. A clear feature of peptide **1** and the parent sequence Boc-Leu-Phe-Val-^{D}Pro-Gly-^{D}Leu-Phe-Val-OMe is the distinct low-field position of the intramolecularly hydrogen-bonded NH groups of Val3, Leu6, and Val8, as compared to the high-field NH groups of Phe2 and Phe7. In peptide **2**, despite the insertion of a central ^{D}Ala residue at position 6, the clear separation of the chemical shifts into two sets of NH groups is maintained. This strongly suggests that the cross-strand hydrogen-bonding pattern is maintained in peptide **2**. Inspection of solvent titration data in Figure 2 also reveals that Phe2 and Phe8 NH groups

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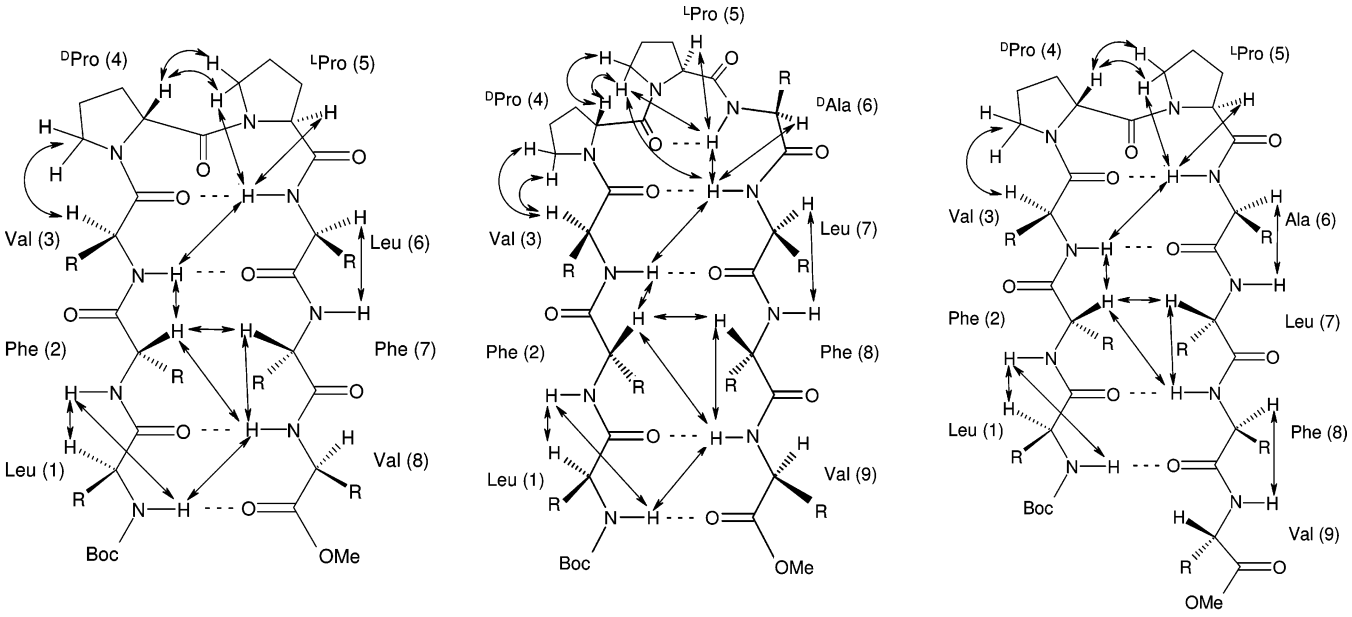

Figure 5. Schematic representation of the proposed β-hairpin structures of peptides **1**−**3**. The expected hydrogen bonds are shown by dashed lines. Observed NOEs are highlighted by double edged arrows.

show a large solvent dependence, clearly indicating their exposed nature. In contrast, Val3, Leu7, ^{D}Ala6, and Val9 NH groups show almost no solvent dependence, strongly suggesting their involvement in intramolecular hydrogen bonding. The moderate solvent dependence of Leu1 is anticipated, because fraying of secondary structures at the termini is widely observed. Interestingly, the ^{D}Ala6, Leu7, and Val9 NH groups show low-temperature coefficients in CD_3OH solution. Notably, Leu1 NH also has a low-temperature coefficient (Table 1). Large $^3J_{NHC^\alpha H}$ values are also observed for all the anticipated strand residues, with the exception of Phe8. The hairpin conformation is confirmed by the presence of the diagnostic Phe2 $C^\alpha H \leftrightarrow$Phe8 $C^\alpha H$ $d_{\alpha\alpha}$ cross-strand NOE and further supported by long-range interstrand Leu1 NH$\leftrightarrow$Val9 NH, Val3 NH$\leftrightarrow$Leu7 NH (d_{NN}) NOEs. Figure 5 summarizes the critical observed NOEs. A most important feature of the NMR data for peptide **2** is the strongly solvent-shielded nature of the ^{D}Ala6 and Leu7 NH groups, suggesting that the central loop must adopt a conformation in which successive NH groups are internally hydrogen bonded. The two potential hydrogen bonds stabilizing a three-residue loop ^{D}Pro-^{L}Pro-^{D}Ala, maintaining the registry of antiparallel strands, are shown in Figure 5. The loop structure requires a 5$\rightarrow$1 hydrogen bond between Val3 CO and Leu7 NH groups (C_{13}, α-turn)[12] and a 3$\rightarrow$1 between ^{D}Pro4 CO and ^{D}Ala6 NH groups (C_7, γ-turn). An alternative hydrogen bond pattern in which the Val3 CO participates in a bifurcated interaction with both ^{D}Ala6 and Leu7 NH groups may also explain the low-temperature coefficient of the ^{D}Ala6 NH proton. A structure calculation using NMR-derived restraints results in a family of conformations in which the two-loop hydrogen bonds and antiparallel strand registry are maintained. The hydrogen bond parameters for the Val3 CO$\cdots$HN Leu7 interaction (5$\rightarrow$1) are N$\cdots$O 3.32Å, $\angle$NHO 131.9° and $\angle$HNO 35.4°, while for ^{D}Pro4 CO$\cdots$HN ^{D}Ala6 interaction (3$\rightarrow$1) the parameters are N$\cdots$O 2.86Å, $\angle$NHO 119.9° and $\angle$HNO 42.3°. Figure 6 shows representative NMR-derived structures, and averaged backbone torsion angles for a family of 10 structures are listed in Table 2. Clearly, insertion of a ^{D}Ala residue into the sequence of peptide **1** results in the expansion of the central two-residue loop into a three-residue loop, with maintenance of the antiparallel β-strand registry.

Conformations of Peptide 3. Inspection of the distribution of NH chemical shifts (Figure S1) for peptide **3** in $CDCl_3$ suggests distinct conformational differences as compared to those for peptides **1** and **2**. Most dramatically, Phe8 NH appears at an extremely low field position (8.32 ppm) as compared to that in peptide **2** (6.59 ppm). A significant difference is also evident for Leu7 NH, which appears at 7.11 ppm in peptide **3** and 7.58 ppm in peptide **2**. Differences in structures of peptides **1** and **2** are also evident from the solvent titration curves illustrated in Figure 2. Phe2, Leu7, and Val9 NH groups show the largest chemical shifts upon increasing DMSO concentration, suggesting their solvent-exposed nature. As in the other cases, Leu1 NH shows a high degree of solvent sensitivity. Ala6 NH shows very little solvent dependence, indicative of involvement in intramolecular hydrogen bonding. The Val3 and Phe8 NH groups show an upfield shift, albeit small, at low DMSO concentrations (10−35% v/v). This behavior is indicative of their solvent-shielded nature. The critical NOEs observed for peptide **3** are Val3 NH$\leftrightarrow$Ala6 NH, Phe2 $C^\alpha H \leftrightarrow$Leu7 $C^\alpha H$. Figure 5 shows the observed NOEs on a schematic model. A β-hairpin structure, stabilized by a central ^{D}Pro-^{L}Pro type-II′ β-turn is consistent with the observed NMR data. The NH groups of Val3, L6, and V8 are involved in intramolecular hydrogen bonds, with slippage of the C-terminus strand as compared to data for peptide **1**. In this conformation, Val9 is outside the hairpin structure.

Discussion

An analysis of three-residue loops in a data set of 250 nonhomologous protein structures revealed that as many as 52 examples had the conformational motif $\alpha_R\alpha_R\alpha_L$, which corresponds to a type-I β-turn followed by a residue in the left-handed α-helical region of conformational space.[1d] Several attempts in

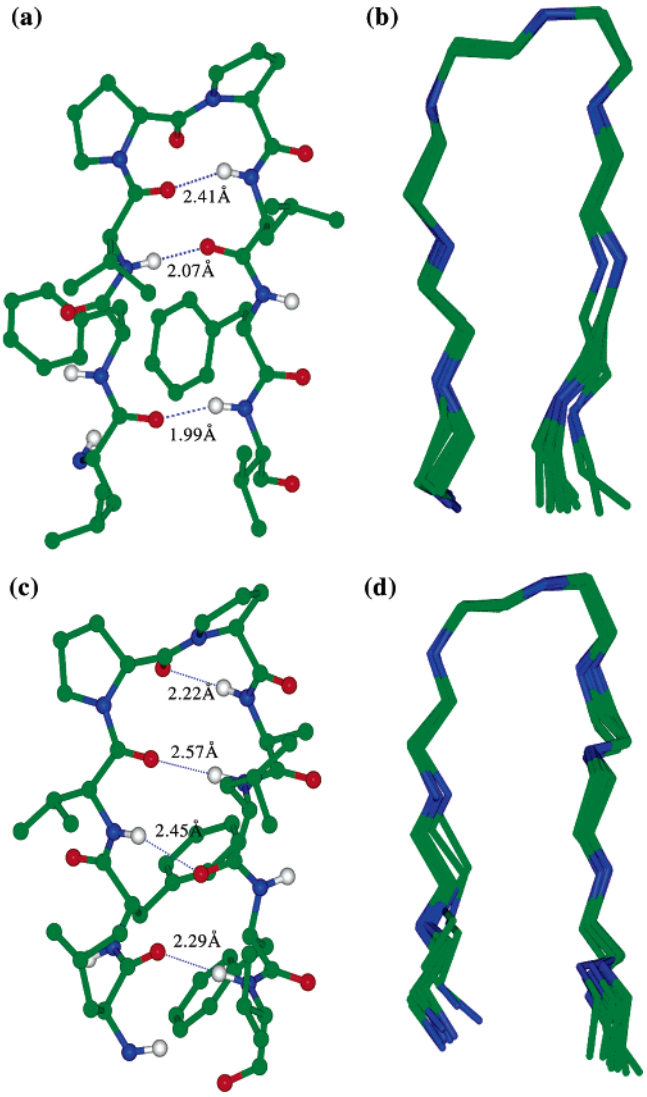

Figure 6. Solution structures of peptides **1** and **2** using NOE information from the ROESY spectra of the individual peptides in $CDCl_3$. (a and c) Ball and-stick representation of one of the best structures of peptides **1** and **2**, respectively. (b) Superposition of 10 best structures calculated for peptide **1**. Mean backbone rmsd: 0.24 ± 0.06 Å. Mean global heavy atom rmsd: 0.96 ± 0.20 Å. (d) Superposition of 10 best structures calculated for peptide **2**. Mean global backbone rmsd: 0.23 ± 0.05 Å. Mean global heavy atom rmsd: 0.88 ± 0.19Å.

Table 2. Average Backbone Torsion Angles Derived from NMR Data for Peptide Hairpins[a]

	peptide **1**		peptide **2**	
	torsional angles (deg)		torsional angles (deg)	
residue	ϕ	ψ	ϕ	ψ
Leu(1)	–	86.6 ± 10.0	–	138.8 ± 24.8
Phe(2)	−139.9 ± 12.9	169.7 ± 2.7	−122.5 ± 11.6	116.0 ± 21.1
Val(3)	−151.4 ± 4.9	90.2 ± 1.9	−147.9 ± 6.9	156.4 ± 2.0
^{D}Pro(4)	75.0 ± 5.6	−137.1 ± 3.9	75.0 ± 4.1	−148.0 ± 3.6
^{L}Pro(5)	−75.0 ± 2.9	4.3 ± 0.7	−75.0 ± 2.7	81.0 ± 4.2
^{D}Ala (6)	–	–	40.1 ± 0.1	61.2 ± 6.7
Leu(6)/Leu(7)	−119.6 ± 2.1	154.3 ± 16.9	−106.7 ± 7.3	73.2 ± 6.5
Phe(7)/Phe (8)	−119.3 ± 6.0	150.3 ± 13.8	−96.8 ± 7.4	112.2 ± 13.3
Val(8)/Val(9)	−119.4 ± 14.7	–	−88.9 ± 3.5	–

[a] Averaged over 10 structures with rmsd of ~0.24 Å for backbone atoms. ω values were restrained at 180°.

this laboratory to design stable β-hairpins based on this motif using ^{L}Pro-Gly-^{D}Ala as the turning segment, did not result in sequences, which displayed antiparallel strand registry. We therefore chose to systematically examine the effects of expanding a well-defined two-residue loop by insertion of a residue at the C-terminal side. Our choice of ^{L}Ala and ^{D}Ala was intended to provide a means of populating α_R/α_L conformations at the inserted residue. The studies reported here establish that the expansion of a ^{D}Pro-^{L}Pro template into a three-residue ^{D}Pro-^{L}Pro-^{D}Ala loop is indeed possible, with maintenance of antiparallel strand registry.

The observed loop conformation can be described as an α-turn stabilized by 5→1 (C_{13}) hydrogen bond, incorporating an internal γ-turn stabilized by a 3→1 (C_7) hydrogen bond. This connecting loop has been obtained by inserting a residue with an α_L conformation at the C-terminus of a type-II′ β-turn. α-Turns in protein structures have been analyzed in some detail.[13a] Several examples have indeed been observed in the connecting loops of β-hairpins in proteins. The loop conformation determined in the engineered peptide **2**, thus indeed corresponds closely to that observed in a specific protein-derived loop family (family g4; $\phi_1 = +60°$, $\psi_1 = -140°$; $\phi_2 = -95°$, $\psi_2 = +75°$; $\phi_3 = +60°$, $\psi_3 = +40°$) and also to a minimum energy conformation observed on the basis of a grid search of conformational space of a system of four linked peptide units (three sets of ϕ, ψ torsional values).[13a]

The present study establishes that expansion of the loop size in designed β-hairpins may be achieved by a careful choice of residues, which bias the local conformational choice in a centrally positioned segment. For small loops, like two- and three-residue loops, the formation of a local chain reversal undoubtedly facilitates hairpin formation, with cross-strand interactions, interstrand hydrogen bonds, and side-chain contacts providing stability to the folded structures. Extensive studies on hairpins formed using a central two-residue loop suggest that turn formation is indeed a nucleating event.[2,3] For larger loops one must consider the interplay between antiparallel strand registry and turn formation. The observation of the slipped hairpin structure in peptide **3**, which contains the ^{D}Pro-^{L}Pro-^{L}Ala loop segment, is notable. In principle, the ^{L}Ala residue could have adopted an α_L conformation, with the energy penalty being compensated by the formation of an additional turn hydrogen bond as observed in peptide **2**. The tendency of amino acid residues to adopt the unfavorable sense of twist of local helical conformations is documented in the case of helical structures when D-residues are accommodated in an α_R conformation.[15] It is clear that energetics appear to favor the two-residue loop slipped hairpin conformation. In our preliminary stage of rational design, we have limited the range of peptides studied to completely apolar sequences, which can be examined in apolar solvents, which do not strongly compete for backbone hydrogen bonding sites. In aqueous solvents cross-strand interactions may promote clustering of hydrophobic chains, resulting in a greater role for sequence effects in hairpin design. In our approach sequence effects are minimized by the choice of apolar residues

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and organic solvents as models for investigation. This permits a greater understanding of the role of short-range forces in nucleating polypeptide chain folding. Conformationally constrained residues provide an opportunity to limit the range of accessible conformations at specific points along a polypeptide chain. Insertion of D-amino acids allows biased sampling of regions of ϕ, ψ space, which are generally inaccessible to L-amino acids. ^{D}Pro is a particularly useful residue because of the additional constraint of the pyrrolidine ring, which limits the ϕ value $\sim+60^0 \pm 20°$. The present study establishes that ^{D}Pro-^{L}Pro-^{D}Xxx segments may be usefully employed in nucleating β-hairpin structures with a central three-residue loop. Notably, the observed loop conformation stabilized by two intramolecular hydrogen bonds (5→1, 3→1), is not maintained in the isolated model peptide Piv-^{D}Pro-^{L}Pro-^{D}Ala-NHMe (unpublished). Cooperative hydrogen-bond formation in a registered antiparallel hairpin must contribute to selection of a favorable loop conformation. The engineering of larger loops merits further attention. The design of β-sheets with the features observed in natural proteins poses a more formidable challenge.[16]

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Supporting Information Available: NMR structural calculations: statistical data, list of restraints of peptides **1** and **2**, stick plots of observed NH chemical shifts for peptides **1**−**3**, and partial 500 MHz ROESY spectra of peptides **1**−**3** in $CDCl_3$ and CD_3OH at 300 K illustrating NH↔C^{α}H and NH↔NH NOEs. This material is available free of charge via the Internet at http://pubs.acs.org.

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