Peptide Design. Structural Evaluation of Potential Nonhelical Segments Attached to Helical Modules

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Abstract: The conformations of three decapeptides containing a helical heptapeptide module attached to a potentially helix destabilizing tripeptide segment have been investigated in single crystals. X-ray diffraction studies of the sequence Boc-Gly-Dpg-Xxx-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe (Xxx = Leu (**1**), Pro (**2**), and Ala (**3**); Dpg = α,α-di-*n*-propylglycine; Aib = α-aminoisobutyric acid) reveal helical conformations for the segment 2−9 in all three peptides. In **1** and **2** Gly(1) is not accommodated in the right-handed helix and adopts a left-handed helical conformation with positive ϕ, ψ values. The terminal blocking group extends away from the helix in **1** and **2**. In **3** the helix is continuous, encompassing residues **1**−**9**. The Dpg residues in all three cases adopt helical conformations, even when flanked by two helix destabilizing residues as in **2**. These findings suggest that the higher α,α-dialkyl residues are good helix promoters although theoretical calculations suggest the existence of a pronounced energy minimum in fully extended regions of conformational space. None of the peptides pack efficiently. The register between helices in the head-to-tail region is not good, with disordered water molecules serving as hydrogen bond bridges and as space fillers. The crystallographic parameters follow. **1**: Xxx = Leu, $C_{54}H_{98}N_{10}O_{13}\cdot 2H_2O\cdot C_3H_7OH$, $P2_12_12_1$, a = 16.399(3) Å, b = 18.634(3) Å, c = 23.241(4) Å. **2**: Xxx = Pro, $C_{53}H_{94}N_{10}O_{13}\cdot xH_2O$, $P2_12_12_1$, a = 16.468(4) Å, b = 18.071(4) Å, c = 23.397(5) Å. **3**: Xxx = Ala, $C_{51}H_{92}N_{10}O_{13}\cdot xH_2O$, $P2_12_12$, a = 19.289(7) Å, b = 35.950(12) Å, c = 9.570(3) Å.

Introduction

Nonstandard amino acids with strong conformational preferences may be used to direct the course of polypeptide chain folding, by imposing local stereochemical constraints, in *de novo* approaches to peptide design.[1] The strong helix inducing properties of α-aminoisobutyric acid (Aib),[2] the first member of the series of α,α-di-*n*-alkyl glycines, have been well documented by numerous crystal structure analyses of short peptides[3] that form 3_{10}-helices and longer peptides[4] that form mixed 3_{10}-/α-helices and α-helices. Incorporation of even a single Aib residue at the center of a 7 or 9 residue sequence is sufficient to stabilize a two-to-three turn helical structure.[4a] This ability to construct stereochemically well defined helical peptide modules may be used to advantage in a "Meccano (or Lego) Set" approach to the design of super-secondary structure motifs.[1]

Construction of a helix−linker−helix motif with a well-defined orientation of the two cylindrical peptide elements could be achieved, in principle, by controlling the stereochemistry of the linking segment. While construction of continuous helical modules encompassing three-to-four helical turns has been achieved, with structures of 13 to 16 residue sequences being characterized in crystals,[5] control over linking sequence conformations has proved more difficult. Initial attempts to generate peptides with two distinct helical segments have used ϵ-aminocaproic acid (Acp),[1c] D-residues,[6] and Pro[7] as interrupting elements. While Acp incorporation results in clearly demarcated helical segments, D-residues and Pro have been accommodated in the framework of right-handed peptide helices in 16-residue sequences.

The higher α,α-dialkyl amino acids like α,α-di-*n*-propylglycine (Dpg) have been shown to adopt fully extended (C_5, ϕ = ψ = 180°) conformations in crystal structures of short homopeptides.[8] In longer heteromeric sequences they are accommodated in helical structures.[9] Coexistence of both extended and helical conformations of Dpg in crystals of a tripeptide, Boc-Leu-Dpg-Val-OMe, suggests that the two conformations are approximately isoenergetic,[10] with environmental factors being critical in tilting the balance. It was therefore of interest

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(2) (a) Abbreviations used: Aib, α-aminoisobutyric; Dpg, α,α-di-*n*-propylglycine; Boc, *tert*-butyloxycarbonyl. All chiral amino acids are of the L configuration. (b) Conventions used for torsion angles in peptides follow the IUPAC-IUB Commission on Biochemical Nomenclature. *Biochemistry* **1970**, *9*, 3471−3479.

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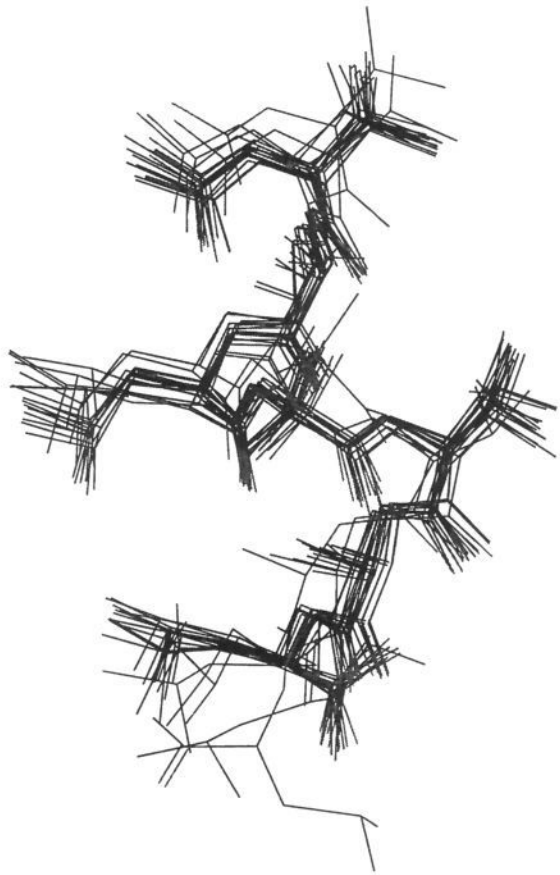

Figure 1. Superposition of conformations of 18 independent −Val-Ala-Leu-Aib-Val-Ala-Leu− modules determined crystallographically in oligopeptides.[12] The rms deviation for all non-hydrogen atoms in these structures is 1.25 Å.

to explore the possibility of using Dpg residues to stabilize fully extended conformations in potential linking segments between helices.

We describe in this report the structures, in crystals, of three decapeptides **Boc-Gly-Dpg-Xxx-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe** (Xxx = Leu (**1**), Pro (**2**), Ala (**3**)). The −Val-Ala-Leu-Aib-Val-Ala-Leu− segment (residues 4−10) has been chosen as the helical module. This sequence adopts a helical (3_{10}, $3_{10}/\alpha$, or α) conformation in a large number of peptides[1,4a,5,11] Figure 1 shows a superposition of crystallographically characterized conformations for this peptide module.[12] The Gly-Dpg-Xxx sequence was chosen as a potential nonhelical linking segment. Gly residues have the highest propensity for occurring in connecting elements (irregular loops) in protein structures[13] and have a strong tendency to act as secondary structure breakers.[14] The Dpg residue was positioned next to Gly with the expectation that destabilization of a helical fold might force the Dpg residue into an extended structure. The Xxx group was varied, with Pro being introduced to break the possibility of a 4→1) hydrogen bond stabilized Gly-Dpg 3_{10}-turn (Type IIIβ). Further, Pro residues can function as C-terminal helix terminators by interrupting hydrogen bond formation and can also serve to nucleate helix formation when placed at the amino terminal end of helices.[15] In peptides **1** and **2**, residues 2−9 form a helical stretch, with Gly(1) adopting positive ϕ, ψ values, while in peptide **3** a continuous helix is observed for residues 1−9. In all cases the Dpg at position 2 adopts a helical conformation.

Table 1. Crystal and Diffraction Parameters of Boc-Gly-Dpg-Xxx-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe[a]

peptide	**1** (Xxx = Leu)	**2** (Xxx = Pro)	**3** (Xxx = Ala)
empirical formula	$C_{54}H_{98}N_{10}O_{13}$	$C_{53}H_{94}N_{10}O_{13}$	$C_{51}H_{92}N_{10}O_{13}$
cocrystallized solvent	$2H_2O{\cdot}C_3H_7OH$	$\sim 2H_2O$	$\sim 3.8H_2O$
crystal habit	colorless hexagonal plate	striated opaque plate	corroded edges/ surfaces thin elongated plate
crystal size (mm)	0.90 × 0.75 × 0.20	0.41 × 0.75 × 0.12	0.53 × 1.1 × 0.05
crystallizing solvent	IprOH/H_2O	MeOH/H_2O	MeOH/H_2O
space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12$
a (Å)	16.399(3)	16.468(4)	19.289(7)
b (Å)	18.634(3)	18.071(4)	35.950(12)
c (Å)	23.241(4)	23.397(5)	9.570(3)
α (deg)	90	90	90
β (deg)	90	90	90
γ (deg)	90	90	90
vol (Å^3)	7101(2)	6960(3)	6636(4)
Z	4	4	4
mol wt	1095.4 + 96.1	1079.4 + 36.0	1053.4 + 68.5
density (g/cm^3) (calcd)	1.114	1.064	1.123
F(000)	2600	2424	2438
temp (°C)	−40	−40	−40
no. of unique reflcns	4769	5030	4848
no. of obsd reflcns $\|\|F\| > 3\sigma(F)\|$	3939	3704	3060
weights		0.00025	
final R (obs data)	10.4[b]	9.0	12.8
resolution (Å)	0.93	0.93	0.93
data/parameter ratio	5.4:1	5.3:1	4.4:1

[a] For all the crystals, Cu Kα radiation (λ = 1.54178 Å) was used with a $\theta/2\theta$ scan, and a scan width of $2.0 + 2\theta(\alpha_1 - \alpha_2)$ and a scan speed of 14 deg/min. Standards reflections were read every 97 measurements. Standards remained constant (within 3%) for **1** and **2**. Data for **3** were measured from slightly split crystal (the best one available) and 80 reflections were dropped due to bad background readings. All crystals were coated with microscope immersion oil. [b] Reflections 002, 020, 012, and 122 were omitted from the least-squares refinement.

Experimental Procedures

The peptides Boc-Gly-Dpg-Xxx-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe (**1**−**3**) were synthesized by conventional solution phase procedures as described elsewhere for Aib[16] and Dpg[17] peptides and purified by HPLC

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Table 2. Comparison of Backbone Torsion Angles in Decapeptides

	peptide	M[a]	1	2	3
residue	Xxx		Leu	Pro	Ala
Gly	ϕ_1		+78	+73	−60
	ψ_1		+22	+26	−25
	ω_1		−176	−176	−173
Dpg	ϕ_2	−56 (Aib)	−51	−52	−46
	ψ_2	−34	−47	−51	−35
	ω_2	−178	−175	−177	−180
Xxx	ϕ_3		−78	−68	−62
	ψ_3		−20	−26	−30
	ω_3		171	177	−180
Val	ϕ_4	−58	−65	−63	−69
	ψ_4	−37	−47	−45	−43
	ω_4	180	178	180	176
Ala	ϕ_5	−65	−58	−59	−62
	ψ_5	−37	−43	−40	−44
	ω_5	178	−178	179	176
Leu	ϕ_6	−72	−66	−67	−59
	ψ_6	−40	−41	−43	−45
	ω_6	178	177	−179	−174
Aib	ϕ_7	−50	−57	−59	−57
	ψ_7	−45	−41	−37	−34
	ω_7	−179	176	180	−176
Val	ϕ_8	−72	−73	−77	−98
	ψ_8	−42	−41	−41	−9
	ω_8	180	176	175	172
Ala	ϕ_9	−59	−65	−70	−91
	ψ_9	−33	−46	−43	−36
	ω_9	177	−168	168	−176
Leu	ϕ_{10}	−89	−177	−113	−107
	ψ_{10}	−8	−13	−16	−9
	ω_{10}	171	−179	−178	−165

[a] A model Aib containing peptide Boc-Aib-Val-Ala-Leu-Aib-Val-Ala-Leu-Aib-OMe[24] containing the heptapeptide module sequence for comparison with the Gly-Dpg-Xxx containing decapeptides.

on reverse phase C_{18} (10 μm) columns using methanol−water gradients. Crystals were grown by slow evaporation from organic solvent−water mixtures as listed in Table 1. Crystals of each of the peptides presented some difficulties with respect to stability for data collection. The best data sets for peptides **1**, **2**, and **3** were obtained after quickly immersing the crystal in microscope immersion oil after removing it from its mother liquor and then cooling rapidly to −40 °C in a stream of cold N_2 gas. Actually, the initial data for **1** (Xxx = Leu) were collected at room temperature, from which the structure was solved. The later low-temperature data yielded a better *R* factor and solvent molecules whose positions were defined much more precisely.

The structure for **1** was obtained by the location of a helical fragment of known geometry (the backbone and C^β atoms from another helical peptide) by a Patterson search function, packing, and direct phasing methods in the PATSEE program.[18] The procedure was almost successful in that rotation of the model fragment was correct, but the translation was in error along one axial direction. A repetition of the translation part of the procedure, after knowing the correct placement, did not give any choices that correspond to the correct placement. Partial structure development of the fragment showed a ghost molecule superimposed on the atoms from the model. The correct placement of the fragment was half way between the two images. The structure of **3** (Xxx = Ala) was readily obtained by the PATSEE procedure using a model composed of the backbone atoms from $C^\alpha(3)$ to $C^\alpha(9)$ and the C^β atoms in peptide **1**. Crystal **2** (Xxx = Pro) is nearly isomorphous with **1**, hence the PATSEE procedure was not needed for deriving the structure. Full-matrix anisotropic least-squares refinement was performed on the C, N, and O atoms. In the final stages of refinement, H atoms were added in idealized positions and allowed to ride with the C or N atom to which each was bonded. The *R* factors, Table 1, are best for **1** and **2**, 10.4 and 9.0%, respectively, at a resolution of 0.93 Å. They are higher for **3**, 12.8% due to crystal imperfections. Fractional coordinates for peptides **1**−**3** are provided as supporting information. Conformational angles are listed in Table 2 and hydrogen bond parameters in Table 3.

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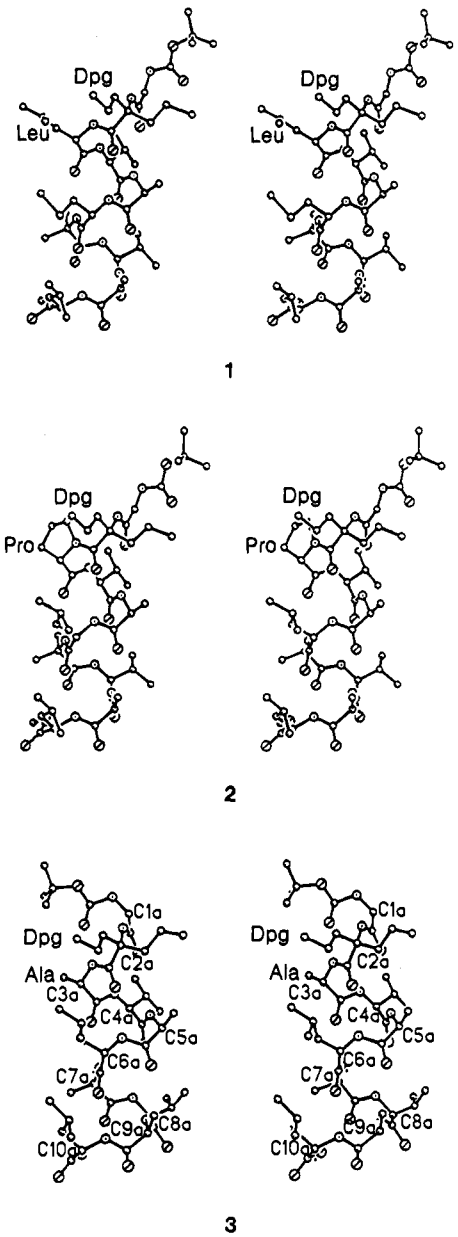

Figure 2. Comparison of helices formed by Boc-Gly-Dpg-Xxx-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe where Xxx = Leu (**1**), Pro (**2**), and Ala (**3**). **1** and **2** are essentially isostructural with the backbone extended at Gly^1. The major change associated with placing Ala in position 3 is the folding of the backbone at Gly^1 in **3**.

Results and Discussion

Molecular Conformation. The conformations of peptides **1**, **2**, and **3** are shown in Figure 2. The three helices are oriented in the same manner to permit easy visual comparisons of the folding patterns. Peptides **1** (Xxx = Leu) and **2** (Xxx = Pro) are isostructural and crystallize into almost identical cells. The only difference is cocrystallized solvent (Table 1), which is discussed later. In peptides **1** and **2** there is a change in the sign of the ϕ, ψ torsion angles at Gly(1), placing this residue in a left-handed (α_L) conformation (ϕ_{Gly} is +78° and +73° and ψ_{Gly} is +22° and +26° in peptides **1** and **2**, respectively). A consequence of this feature is that the Boc group extends away from the helix. Replacement of residue 3 by Ala in peptide **3** results in ϕ, ψ values of −60°, −55° at Gly(1) placing this residue also in a right-handed helical conformation. At first

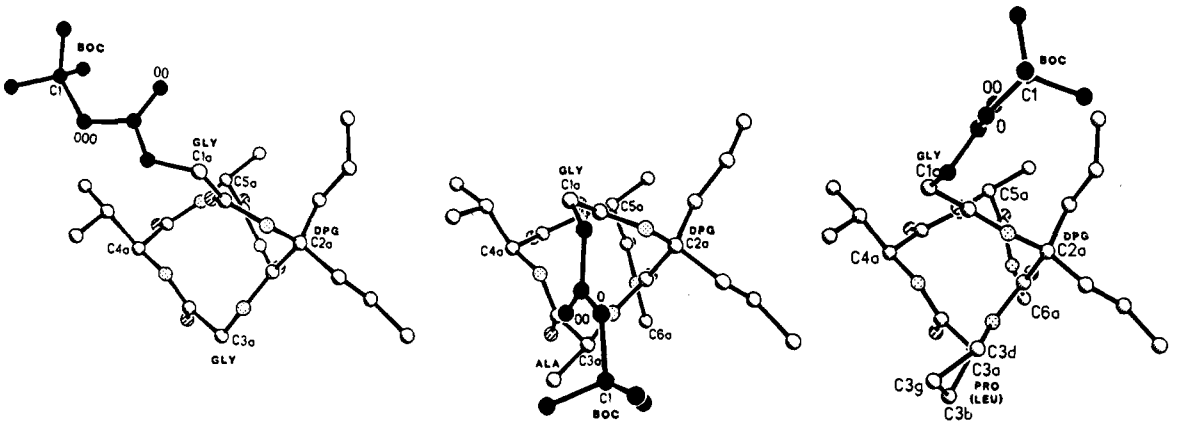

Figure 3. Projections down the helix axis of the Gly(3) (unpublished), Ala(3) and (Pro(3)/Leu(3)) (this study) analogs of Boc-Gly-Dpg-Xxx-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe. The last four residues are omitted for clarity. The Boc end group is blackened in order to emphasize the three different conformations that it assumes with respect to the helix. The torsion angles at Gly(1), $\phi_1(N_1-C^\alpha)$, and $\psi_1(C^\alpha-C')$ are −94°, −162°; −60°, −25°; and +73°, +26°.

Table 3. Hydrogen Bond Values for N−O and O−O (Å) in Peptides **1**−**3**

type	donor	acceptor	**1** (Xxx = Leu)	**2** (Xxx = Pro)	donor	acceptor	**3** (Xxx = Ala)
head-to-tail	N1	O8	2.93	2.93			
	N2	O9	2.98	2.93			
water bridge	N3	W1	2.93		N1	W2, W3	2.86
		O9	2.95		W2, W3	O9	2.70
					N2	W6	3.06
					W6	O10	2.80
4→1					N3	O0	2.97
	N4	O1	2.94	2.87	N4	O1	2.93
transition					N5	O2	3.16
5→1	N5	O1	3.09	3.13			
	N6	O2	3.18	3.20	N6	O2	2.97
	N7	03	2.95	2.98	N7	O3	2.94
	N8	O4	3.00	3.04	N8	O4	3.42
	N9	O5	2.95	2.91	N9	O5	3.20
	N10	O6	2.94	2.93	N10	O6	2.85
solvent	W1[a]	W2	2.76		W7	O7	2.97
					W5	O8	3.00
	W2	O0	2.99	2.92	W5	W6	2.72
	IprOH[b]	O8	2.97	2.87	W1	O10	2.98
					W1	W3	2.70
	W3[c]	O10		2.95	W3	W6	2.83

[a] Missing in **2**. [b] Replaced by water in **2**. [c] Missing in **1**.

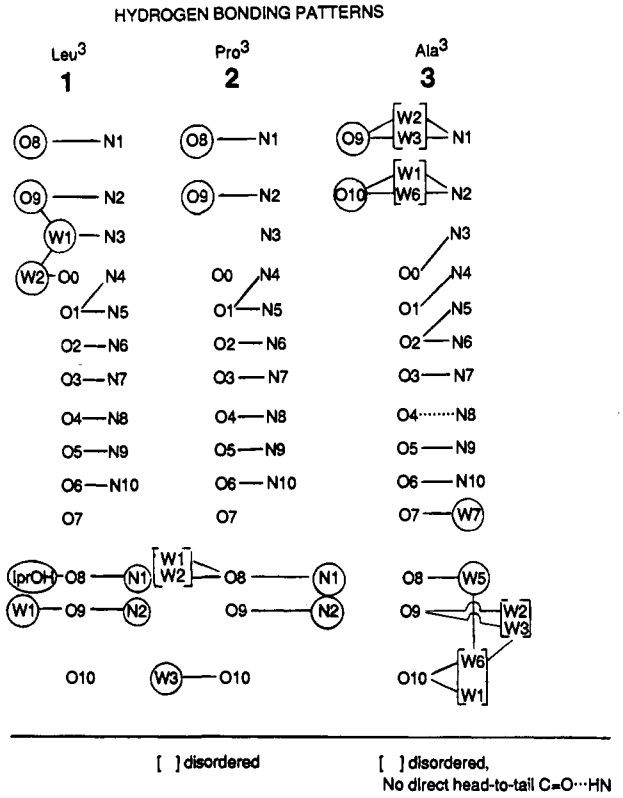

Figure 4. A schematic diagram depicting the hydrogen bonding patterns in peptides **1** to **3**. Intramolecular 5→1 1-hydrogen bonds (α-helix) are indicated by horizontal lines, 4→1 hydrogen bonds (3_{10}-helix) are indicated by slanted lines, and transition areas between 3_{10}- and α-helices are shown by ∠. Circled atoms are involved in head-to-tail hydrogen bonds with water or other solvent molecules. The dotted N8···O4 distance in **3** is 3.42 Å, very long for a hydrogen bond.

glance, it may appear that decreasing the size of the side chain at $C^\alpha(3)$ has a direct effect on the folding of the backbone at the N-terminus to continue the helix. However, this generalization does not hold since the crystal structure of an analog where Ala(3) is replaced by Gly(3) produces an entirely different conformation at Gly(1), which adopts extended ϕ, ψ values ($\phi_{Gly(1)} = -94°$ and −96°, $\psi_{Gly(1)} = -162°$ and −153° for the two crystallographically independent molecules of Boc-Gly-Dpg-Gly-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe, unpublished). Figure 3 shows a comparison of the three types of orientation of N-terminus blocking residue observed in the decapeptides examined so far.

In peptides **1** and **2**, residues 2−9 are accommodated into a continuous helical fold. In peptide **3** (Xxx = Ala) the helix spans the entire length of the sequence encompassing residues 1−9. The type of helix formed is illustrated schematically in Figure 4, where the hydrogen bonding patterns are shown. In **1** and **2** the α-helix is well formed except for the first N−H···O hydrogen bond where a transition between a 3_{10}-helix and an α-helix occurs at O(1). **3** begins with a longer 3_{10}-helical segment with three 4→1 hydrogen bonds. The α-helical portion in **3** is distorted by a N(8)···O(4) separation of 3.42 Å and an H(8)···O(4) separation of 2.70 Å. Peptides **1**−**3** provide another example of facile transitions between 3_{10}- and α-helical hydrogen bonding patterns.[4c]

Crystal Packing

Head-to-Tail Hydrogen Bonds. In crystals, helical peptides align themselves into columns by heat-to-tail hydrogen bonding, almost without exception. The individual helices are repeated by a simple translation or by a 2-fold screw translation. In the present cases, direct head-to-tail N(1)H···O(8) and N(2)H···O(9) hydrogen bonds are present in peptides **1** and **2** (Figure 5). In the nearly isomorphous structures of **1** and **2**, N(3) (Leu(3))

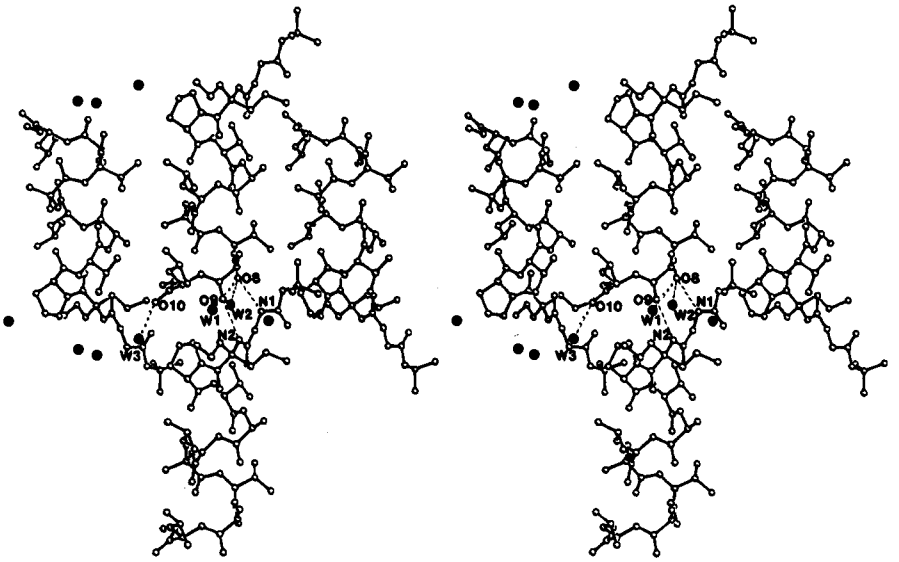

Figure 5. Stereo diagram of packing for crystal **2** showing direct head-to-tail hydrogen bonding, N1···O8 and N2···O9, as well as water molecules (darkened) hydrogen bonded to O8 and O10. Crystal **1** is nearly isomorphous and differences from **2** in intermolecular hydrogen bonding are shown schematically in Figure 4. The axial directions are ↓ for x and → for y.

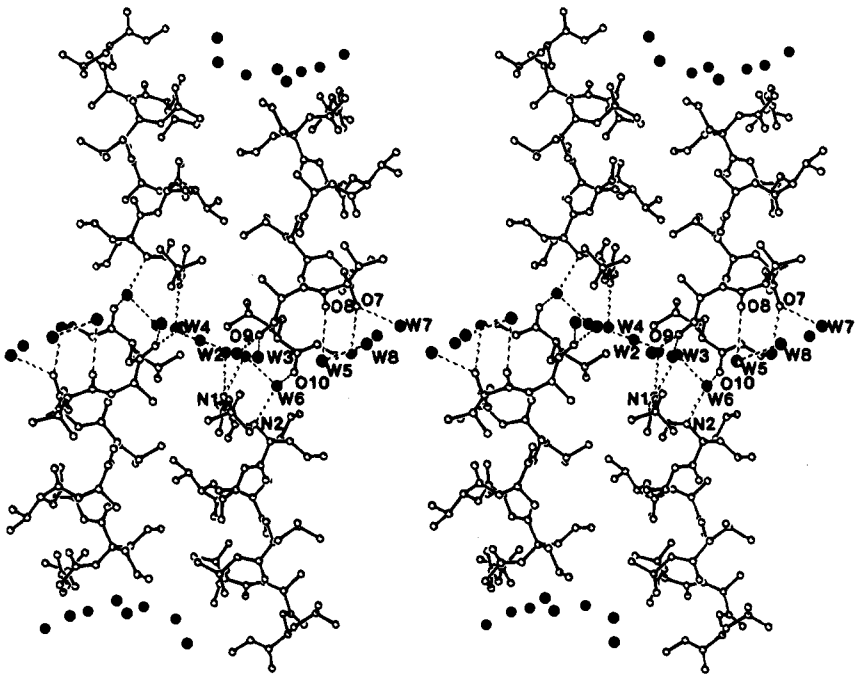

Figure 6. Stereo diagram of packing for crystal **3** in which a layer of water molecules (darkened) separates the head and tail region between the helices. This is a rare example of the absence of direct NH···O=C bonds between helices. The axial directions are → for x and ↑ for y.

in **1** forms a hydrogen bond with water W(1), which in turn bonds to O(9) and W(2), while N(3) (Pro(3)) in peptide **2** is not capable of hydrogen bonding and W(1) is missing, the space being occupied by the pyrrolidine ring. Further, the 2-propanol molecule in **1** is replaced by two partially occupied sites (but not at the same time by W(1A) and W(2A)) in **2**.

Peptides **3** represents an unusual occurrence in which there are no direct head-to-tail NH···O hydrogen bonds. Instead, a layer of water molecules, perpendicular to the helix axis separates peptide molecules. The water molecules serve as bridges in forming hydrogen bonds between the head of one peptide, N(1) and N(2), and the tail of another, O(7) to O(10) (Figure 6). The register between successive helices in a column does not permit direct NH···O head-to-tail bonds.

Aggregation of Peptide Columns. The presence of two propyl chains in the Dpg residues does not appear to have any effect on the crystal packing of the helical molecules, as compared to related molecules without these residues. All the lateral contacts are between nonpolar hydrocarbon chains separated by normal van der Waals distances. In all three peptides the helical columns pack in an antiparallel motif. In the large number of crystal structures of hydrophobic helices examined thus far, water molecules have been found in the lateral regions between columns only when insertion results in hydrogen bonds. There are as yet no examples of water molecules in purely apolar cavities in peptide crystals.[4a,19] Recent studies of proteins in solution and crystals have focussed on the possibility of finding non-hydrogen bonded water molecules in apolar cavities.[20] The crystallinity of hydrophobic helical peptides must in large measure arise from the regular

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association into helical columns followed by lateral close packing of the cylindrical columns. In principle, association of these large molecules in organic solvents used for crystallization must be driven by solvophobic forces.[21] The ease of crystallization of this class of peptide helices may be contrasted with the difficulties in growing single crystals of medium-sized oligopeptides which are conformationally flexible or form β-sheet structures.

Implications of Design

In two of the three decapeptides, Boc-Gly-Dpg-Xxx-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe (Xxx = Leu (**1**) and Pro (**2**)), the N-terminus Gly(1) residue of the putative Gly-Dpg-Xxx linking segment adopts a conformation that results in helix termination. An extended conformation has also been established in the decapeptide, where residue 3 is also Gly (unpublished). While the peptides were synthesized with the hope that Dpg(2) would be coaxed into a fully helical conformation, this expectation was not realized. In all the decapeptides, Dpg adopts a right-handed helical conformation. These results suggest that while helix interruption may be facilitated by introducing conformationally flexible residues like Gly, the higher α,α-dialkyl residues may not be good candidates for stabilizing extended local conformations in linking segments. Indeed, these residues appear to be comfortably accommodated in helical structures, in a wide range of sequence contexts,[9,22] despite the fact that theoretical calculations show the occurrence of two energy minima corresponding to fully extended and helical regions of ϕ, ψ space, with the former being energetically marginally more favorable.[23]

The present study reinforces the view that the heptapeptide helical segment containing a single, centrally located Aib residue can indeed be used as a stable, pre-fabricated element in a modular approach to the construction of larger, supersecondary structures. The α_L conformation at Gly(1) in peptides **1**, **2**, and **3** results in an extension of the terminal Boc group away from the body of the helix. Attempts are underway to structurally characterize a 17 residue peptide in which the Gly-Dpg-Xxx sequence separates two helical modules. Studies in progress also attempt to establish conformational control at linking segments by use of D-Pro residues.

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Supporting Information Available: Tables of atomic coordinates, bond lengths, bond angles, anisotropic temperature factors, and H atom coordinates for **1**, **2**, and **3** (28 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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