Solid- and Solvated-State Conformation of the Free Tetrapeptide Glycyl-L-prolyl-D-leucylglycine by X-ray and Proton Nuclear Magnetic Resonance Spectroscopy

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Abstract: The conformation of the free linear peptide Gly-L-Pro-D-Leu-Gly has been determined by both single-crystal X-ray diffraction analysis and ^{1}H NMR spectroscopy. In the solid state the molecules of peptide are stabilized by head-to-tail interactions within dimers packed according to a double right-handed helix, whereas in Me_2SO solution the conformation fits rather well a type II β-turn with a Gly(4) → Gly(1) intramolecular hydrogen bond. In the crystal this latter is replaced by a 4 → 1′ intermolecular hydrogen bond stabilizing the two strands of the double helix. Minor energetic changes are involved between these two different conformations since mainly one bond, at the hinge L-Pro-D-Leu, has to be twisted to account for both the β-turn and the double helix.

Isolation in the past few years of short linear peptides exhibiting interesting biological properties and easily obtainable by synthesis has remarkably increased the conformational studies on peptides. However, despite their large number and variety, only a few of them led to crystals that obviously has limited the X-ray diffraction studies.[1-3] In contrast, NMR investigations on conformation and stability of linear peptides are numerous[4] but only in limited cases have comparisons with crystal structures been possible.

In solution, the results usually agree with folded conformations, mainly β-turns (type I or II), where the common feature is a 4 → 1 hydrogen bond.[4-5] In crystals, such foldings may also be observed: they occur, however, when the N-terminus end or the two ends are blocked with bulky groups that strongly decrease or eliminate the terminal charge effects.[2]

Concerning the free peptides, the few available examples illustrate the charge effects with, as a consequence, the predominance of the head-to-tail intermolecular interactions leading to (i) dimers stabilized by two intrastrand NH_3^+/COO^- bridges, as found in Tyr-Gly-Gly-Phe,[6] or (ii) β-sheet structures, with linear conformations stacked in infinite layers via intermolecular NH_3^+/COO^- bonds as observed in Gly-Gly-Phe-Leu[6] and Trp-Met-Asp-Phe-NH_2.[7]

In the course of NMR analyses on stabilization of β-turns in free tetrapeptides in solution,[8] the obtention of a single crystal of Gly-L-Pro-D-Leu-Gly (to our knowledge the first free peptide with the L–D sequence being crystallized to date) has allowed the present comparative study. We report the synthesis of the peptide in natural abundance and also labeled in Pro(2) with both ^{13}C and ^{2}H, and we correlate its structure and conformation in the crystal and solution.

Experimental Section

Labeled Amino Acids. The doubly enriched (85% ^{13}C and 98% ^{2}H) proline was prepared in the Service de Biochimie, Commissariat à l'Energie Atomique, Saclay, by R. Mermet-Bouvier from hydrolysates of green algae *Spirulina maxima*[9] grown in 99% D_2O/90% ^{13}C ammonium carbonate media.

Gly-L-Pro-D-Leu-Gly and Gly-L-[^{13}C,^{2}H]-Pro-D-Leu-Gly were synthesized by the solid-phase method. (Chloromethyl)polystyrene/1% divinyl benzene (0.32 mM Cl/g) was substituted with *N*-(*tert*-butyloxycarbonyl)glycine by the cesium salt method.[10,11] All the amino acids were coupled as *N*-*tert*-butyloxycarbonyl derivatives with the aid of dicyclohexylcarboxydiimide as the coupling reagent in $CHCl_2$. *N*-(*tert*-Butyloxycarbonyl)-L-[^{13}C,^{2}H]-proline was prepared by the Schnabel method[12] in a similar way to the natural proline derivative. Completions in the coupling reaction were monitored by using the ninhydrin test.[13] The cleavage of the *N*-*tert*-butyloxycarbonyl group was performed with 40% trifluoroacetic acid solution in dichloromethane followed by neutralization with 10% triethylamine in the same solvent. The protected peptides were removed from the resin by the action of doubly distilled hydrogen fluoride in the presence of anisole over a 1-h period at 0 °C. The crude peptides were purified by partition chromatography on Sephadex *G*-25 using 1-butanol/pyridine/0.1% acetic acid in water (5/3/11 v/v). The white product (89% yield) was found homogeneous by thin-layer chromatography in both basic and acid eluent systems: R_f 0.4 in 1-butanol/pyridine/0.1% acetic acid in water (5/3/11 v/v) and R_f 0.15 in 1-butanol/acetic acid/water (4/1/5 v/v).

Crystallization. Elongated plates were obtained from methanol/water mixtures by a vapor equilibrium. They were stabilized in a 65% methylpentanediol solution and sealed in glass capillaries in the presence of the stabilizing solution.

X-ray Structure Determination. Crystal data are as follows: system monoclinic, space group $C2$ ($Z = 4$) with cell parameters $a = 22.05$, $b = 7.93$, and $c = 13.39$ Å and $\beta = 111\ (6)°$ ($v = 2184.6$ Å^3). With the aid of an automatic four-circle diffractometer (Cu Kα radiation monochromatized by graphite), 1802 reflections were scanned up to $\theta = 55°$. After corrections from Lorentz polarization but not from absorption, 1553 of them were considered as observed with $I \geq 2\sigma(I)$. The multisolution method[14] failed to solve the structure. The final result has been deduced from a Patterson search method[15] used in the less symmetric space group $P2_1$ ($Z = 4$), deduced by rotation around b and translation (the new parameters are $a = 13.306$, $b = 7.93$, and $c = 21.13$ Å and $\beta = 105.1°$). The starting model was a proline residue with different conformations, selected from the literature.[16] Among four possible rotation/translation parameters, only one led, after Fourier synthesis, to a correct development of the structure. All the atoms were located on

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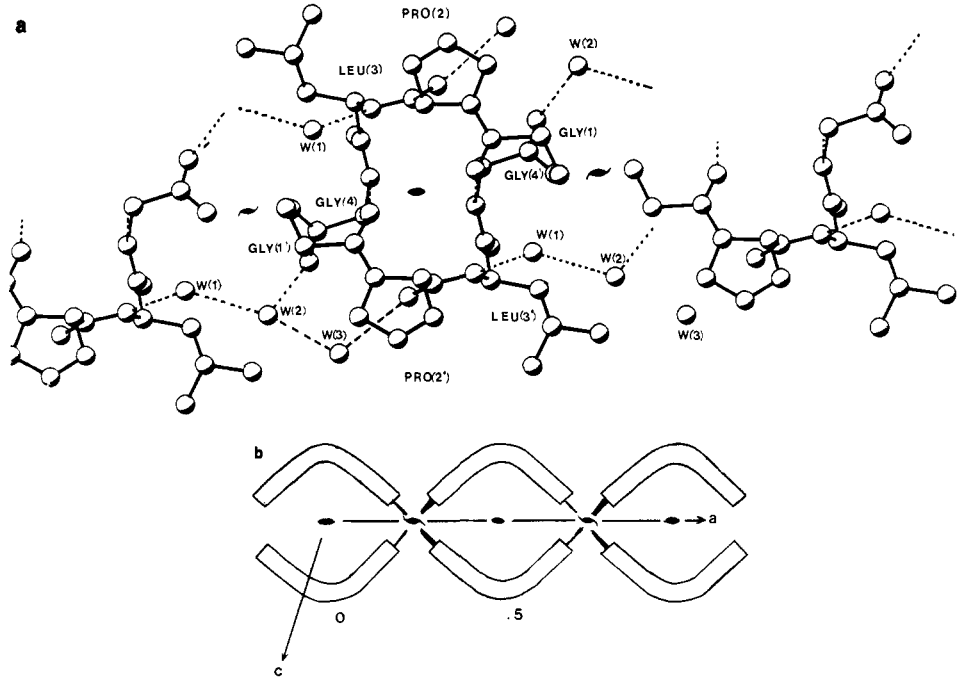

Figure 1. Projection of the helicoidal association along the *b* axis (upper) and schematic drawing of the infinite double helix running along *a* and *b*.

Table I. Intermolecular (Hydrogen Bonds) Distances within the Double Helix in the Crystal

	molecule 1[a]		molecule 2[a]			
atom	sym. no.	transl.	sym. no.	transl.	distance, Å	remarks
Gly(1)C*O*···Gly(4)N*H*	1	0, 0, 0	2	0, 0, 2	2.86	intrahelix, parallel to the *C*2 axis
Gly(4)COO$^-$···Gly(1)NH$_3^+$	1	0, 0, 0	2	0, 0, 2	2.81	ionic interaction intrahelix
Gly(4)COO$^-$···Gly(1')NH$_3^+$	1	0, 0, 0	3	−1, 0, 0	2.75	ionic interaction between helices
Pro(2)C*O*···W(3)	1	0, 0, 0	1	0, 0, 0	2.84	in the helix groove
W(3)····W(2)	1	0, 0, 0	1	0, 0, 0	2.66	in the helix groove
W(2)····W(1)	1	0, 0, 0	3	−1, 0, 0	2.76	in the helix groove
W(1)···Leu(3)C*O*	1	0, 0, 0	1	−1, −1, 0	2.63	between helices (laterally)
W(1)···Leu(3)N*H*	1	0, 0, 0	1	−1, 0, 0	2.94	between helices (laterally)
W(1)···Gly(1)NH$_3^+$	1	0, 0, 0	2	0, 0, 2	2.83	
W(3)···W(3)	1	0, 0, 0	2	0, 0, 1	3.26	through the *C*2 axis

[a] Symmetries: 1, x, y, z; 2, $\bar{x}, y, \bar{z}$; 3, $^1/_2 + x, ^1/_2 + y, z$; 4, $^1/_2 - x, ^1/_2 + y, \bar{z}$. Translations: along *a*, *b*, and *c*, respectively.

subsequent F_o maps, and three molecules of water were found in the cell. The refinements were performed in the original *C*2 space group by block-diagonal matrix least squares with individual isotropic thermal factors to an *R* value of 0.14 ($R = \sum||F_o| - |F_c||/\sum|F_o|$). At this step, difference Fourier syntheses showed 17 of the hydrogens from a total of 32. Their electronic contributions were added in the following steps of refinement, performed with anisotropic thermal factors. After three cycles of refinements plus difference Fourier, 10 more hydrogens were located. At the end of the process, the *R* value converged to 0.08.

A rather high anisotropy was observed in a single direction. The analysis in terms of the rigid body model is relevant of a ±0.4-Å vibrating motion along a vector aligned on the *C*2 axis of the space group. After subtraction of this effect from individual thermal factors, a large residual motion can still be noticed for the C^γ atom of Pro(2) ($\langle B \rangle \sim 10$ Å^2). Such a high value is a general feature and many authors have pointed out the C^γ as the hinge point of the proline ring flexibility. This can be analyzed both as a dynamic vibration[17] or as a statistical disorder[18-19] (C^γ endo/C^γ exo) depending on the intensity of the motion. It is interesting to note that the more the residue is preceding proline in planar conformation ($\psi_1 = -170°$), the more the C^γ atom is found agitated.

^{1}H NMR. The samples used for ^{1}H NMR measurements were taken at concentrations ranging from 1×10^{-4} to 5×10^{-1} M. Before dissolution in Me_2SO-d_6, the selected ionization state was achieved by adjusting the pH of a solution of peptide in H_2O with 1 M HCl or NaOH. After lyophilization, the peptide was dried under vacuum at 60 °C. For the proton–proton NOE experiments (performed at 500 MHz), the peptide was dissolved in water previously treated with Chelex. The sample tube was sealed after degasing of the solution. Spectra were obtained at 250 MHz (Cameca TSN 250 spectrometer) and at 500 MHz (Brucker WH-500 spectrometer) in the Fourier transform mode. The heteronuclear vicinal coupling constants $^3J(^{13}C'_{Pro}-H^\alpha_{Leu})$ was measured from the ^{1}H NMR spectrum of Gly-L-[^{13}C,^{2}H]Pro-D-Leu-Gly by simultaneous decoupling of both the amide and the C^β-methylene protons of the D-leucine, as previously reported.[8]

In all cases, chemical shifts are given downfield from internal tetramethylsilane.

Results

Single-Crystal Conformation. The positional and thermal parameters as well as the distances (Å) and angles have been deposited.[20] They are consistent with equivalent data on other peptide structures and will be not discussed further.

The most striking feature in this crystal structure is the head-to-tail association of two molecules in a dimeric fashion (Figures 1 and 2) around the crystallographic binary axis and strongly stabilized by hydrogen network (Table I): four bonds within the dimer and four others between different dimers of the packing. Three molecules of water are also included in the crystal and participate in the network by numerous bondings cross-linking the dimers.

The four ligands of the water molecule denoted W(1) are (i) the N–H of D-Leu(3) ($d_{N\cdots W}$ = 2.94 Å) of the peptide molecule

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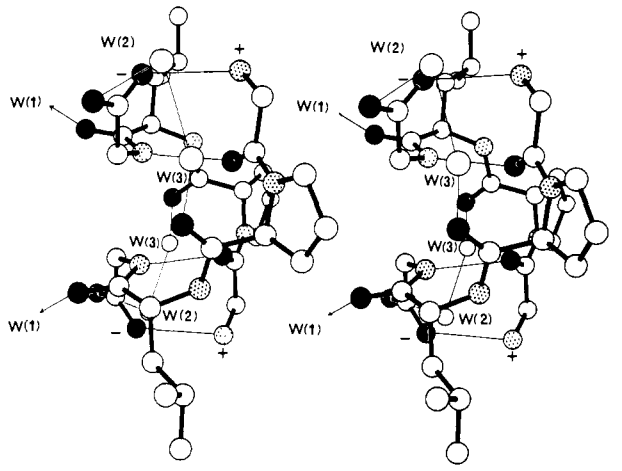

Figure 2. Stereoscopic view of the dimer. The hydrogen bonds are indicated in light lines, within the double helix and the molecules of water inside the groove of the helix. The *C*2 axis is horizontal, across the middle of the figure.

situated one cell above (*b* axis), (ii) the carbonyl oxygen (CO) of D-Leu(3) ($d_{O\cdots W}$ = 2.63 Å), (iii) the N^+-terminal atom of Gly(1) ($d_{N\cdots W}$ = 2.83 Å), and (iv) the second molecule of water W(2). This latter participates in three interactions with (i) the preceding W(1) molecule, (ii) the COO^--terminal group of Gly(4) ($d_{O\cdots W}$ = 2.79 Å), and (iii) the third molecule of water, W(3). This last one is further bonded with the Pro(2) carbonyl group ($d_{W\cdots O}$ = 2.84 Å).

In the single conformer, the IUPAC dihedral angles show only minor changes from the type II β-turn, mainly one ψ value (Table II). The stereo view of the dimer (2 × Gly-L-Pro-D-Leu-Gly, Figure 2) along the *a* axis clearly depicts a half-turn of a double right-handed helix, an unusual conformation, in which the 4 → 1 intramolecular hydrogen bond characterizing the β-turn is replaced by two interstrand hydrogen bonds (4 → 1′) involving however, the same donor [Gly(4) N–H] and acceptor [Gly(1) CO] ($d_{N\cdots O}$ = 2.86 Å). All together the two strands are maintained on a half-turn by these two hydrogen bonds and also by two more NH_3^+/COO^- ionic interactions (d = 2.81 Å). The dimer is further prolongated by two other NH_3^+/COO^- bonds toward the next dimer, thus leading to an infinite double helix along the *a* axis by stacking half-turn segments (Figure 1). In these conditions, the role of the three molecules of water becomes more evident in the stabilizing process as two of them—W(2) and W(3)—are located in the helix groove and tightly maintained by four hydrogen bonds while the molecule W(1) is dedicated to interhelix cross-linkages via the interactions occurring on D-Leu(3) residues of two different double helices.

To minimize the conformational energy of the double-stranded helix, the C-terminal residue Gly(4) is turned outward from the helix. Apparently, this induces strain on the peptide bond connecting D-Leu(3) and Gly(4), which is found to deviate about 17° from the ideal ω_3 = 180°. Such a distortion has been observed in cyclic strained peptides with all-trans peptide bonds[21] and recently in a linear alternating DL hexapeptide backbone.[22] In contrast, Gly(1) is found in a perfect planar arrangement in respect to L-Pro(2): $\psi_1 = -179°$; $\omega_1 = 157°$.[17]

^{1}H NMR Conformation. Data for the peptide dissolved in Me_2SO-d_6 in its cationic and neutral forms are reported in Table III. They are from experiments performed at 5 × 10^{-2} M. At any ionic state, cis ⇄ trans equilibrium of proline leads to two populations of peptide conformation where the trans conformer is the predominant one (60 ± 5%). The following will be more concerned with the neutral, zwitterionic trans conformer, as it

Table II. Comparison of Crystal Structure and Solution Conformations (Rounded Angle Values)[a] in Gly-L-Pro-D-Leu-Gly

	Gly(i = 1)		L-Pro(i = 2)		D-Leu(i = 3)		Gly(i = 4)	
	cryst.	soln	cryst.	soln	cryst.	soln	cryst.	soln
ϕ_i			−71°	−60°	+89°	+90°	−81°	−70°; −170°
ψ_i	180°		163°	120°	+14°	+0°	−4°/177°	
ω_i	176°		176°		+163°			
χ_i			8°		+59°			
H bond	C=O, intermol	C=O, intramol or intermol?	C=O, solvated	disorder	N–H, solvated	N–H, exposed to solv	N–H, intermol	N–H, intramol or intermol?
ionic NH_3^+/COO^-	intermol	intramol or intermol?					intermol	intramol or intermol?

[a] Using the Bystrov relationships: see ref 25.

Table III. NMR Parameters of Trans Conformer of Gly-L-Pro-D-Leu-Gly in Me_2SO at 5 × 10^{-2} M[a]

	Gly(1),	Pro(2)			Leu(3)						Gly(4)			
	$\delta(H^\alpha)$	$\delta(H^\alpha)$	$^3J(\alpha\beta)$	$^3J(\alpha\beta')$	$\delta(H^\alpha)$	$^3J(\alpha\beta)$	$^3J(\alpha\beta')$	δ(NH)	T coeff, 10^{-3} ppm/°C	^3J-(HC$^\alpha$NH)	^3J-(^{13}C′$_{Pro}$-H$^\alpha_{Leu}$)	$\delta(H^\alpha)$	δ(NH)	T coeff, 10^{-3} ppm/°C	^3J-(HC$^\alpha$NH)
cation	3.79	4.39	4.1	7.9	4.29	5.8	9.0	8.33	−4.0	8.6		3.74	8.11	−3.7	5.9
zwitterion	3.72	4.40	3.2	6.8	4.21	4.4	10.0	8.32	−5.3	8.6	2.0	3.55(B); 3.42(A)	7.51	+0.8	5.3 4.4

[a] Chemical shifts (δ) are given in ppm downfield from internal Me_4Si; coupling constants are given in Hz. Assignments A and B of the Gly(4) methylene protons are described in the text.

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corresponds to the one observed in the crystal.

The two vicinal coupling constants $^3J(H^AC^\alpha-NH)$ and $^3J(H^BC^\alpha-NH)$ characterizing Gly(4) display distinct values in the neutral form. Assignment of the high-field methylene proton (H^A) to H^α-proton (*pro-R*) of amino acids[23,24] and comparisons of the θ^A and θ^B angles in the $NH-C^\alpha H^A H^B$ moiety yields two possible φ_4 values: −170° and −70°, from the Bystrov et al. relationship.[25] Considered together with the chemical shift nonequivalence of H^A and H^B (0.11 ppm) and the NH-proton temperature coefficient ($\sim 0.8 \times 10^{-3}$ ppm/deg), these data suggest the coexistence of a limited number of conformations in the C-terminal part of the molecule. The 8.6-Hz value of the vicinal constant $^3J(HC^\alpha-NH)$ found in D-Leu(3) leads to four possible φ_3 angles: +150°, +90°, −45°, and −65°. Their comparison with those derived from the vicinal coupling constant $^3J(^{13}C'_{Pro}-H^\alpha_{Leu}) = 2$ Hz measured in the ^1H NMR spectrum of the labeled proline by using the corresponding relationship[25] suggests the two positive values to be allowable.[8] On the other hand, the vicinal coupling constants $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ are indicative of a Leu side chain mostly with $\chi_1 = +60°$ and a little with $\chi_1 = -180°$ (the rotamer with $\chi_1 = -60°$ is completely excluded). The NH-proton of D-Leu(3) shows a temperature coefficient clearly in favor of its exposure to solvent.

In proline, the coupling constants $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ are better interpreted in terms of flexible pyrrolidine ring than of single discrete conformations. The same conclusion applies to Gly(1), whose two methylene protons display equivalence of their chemical shifts.

Discussion

The most relevant NMR and crystal X-ray structure data are compared in Table III.

The NMR parameters $\varphi_3 = +90°$ and $\varphi_4 = -170°$ and the temperature coefficient of the Gly(4) NH-proton are consistent with a type II β-turn ($\varphi_2/\psi_2 = -60°/+120°$, $\varphi_3/\psi_3 = +80°/0°$, and 4 → 1 hydrogen bond).[5] This in fact seems reasonable if we consider the propensity to folding of Pro-D-X sequences in peptides.

On the other hand the structure of Gly-Pro-D-Leu-Gly in the solid state could be considered as a distorted β-turn. This gives a good illustration of the determinant role played by a single ψ angle on the whole conformation of a peptide: a deviation in ψ_2 of ~40° from the standard value destroys the ideal type II β-turn, though the angles at the hinge, are preserved. The change is sufficient for precluding the formation of the 4 → 1 intramolecular bond, which is now replaced by a 4 → 1′ hydrogen bond stabilizing a dimer. Therefore, even if most of the NMR data fit (vide infra) a type II β-turn in solution, this structure to be confirmed requires accurate informations on both the ψ_2 angle and the hydrogen bonds, especially 4 → 1. In fact, the existence at 500 MHz of a negative NOE between the L-Pro(2) H^α- and the D-Leu(3) NH-protons, not observed in the corresponding L–L sequence, is consistent with a more or less CIS arrangement ($\psi_2 \sim 120°$) of the two above-mentioned protons characterizing the type II β-turns[26-28] Yet, since a NOE effect can occur even with $\psi_2 = 163°$ (crystal value), the existence of distortion in the β-turn is still possible in solution.

To obtain information about the nature of the hydrogen bonds in solution, we examined the effects of peptide concentration on the NH-proton chemical shifts, the NH temperature dependencies, and the $^3J(HC^\alpha NH)$ coupling constants. There were no visible effects in the range of concentration 5×10^{-1} to below 1×10^{-4} M, suggesting that intermolecular hydrogen bonds are either weakly represented or remain unaffected in the limits of concentration explored. Alternatively, intermolecular and intramolecular hydrogen bonds could compete in a concentration-dependent equilibrium involving the type II β-turn (monomer) and the double-helix structure (dimer): no change on the above experimental parameters can be then observed since the two conformations share the same bond donor [Gly(4) NH] and acceptor [Gly(1) CO] and differ one from the other by only their ψ_2 and φ_4 angles. [The two $^3J(HC^\alpha-NH)$ values in Gly(4) fit equally well the −70° and −170° angular values. The first fits the crystal structure and the second the β-turn conformation.]

The role played by the ionic forces also deserves mention. We have seen that they are involved both in the intrahelical 4 → 1′ hydrogen bond and in the crystal structure head-to-tail interactions. The orientation of Gly(4) outward from the molecule and the distortion of ω_3 are possible consequences of these strong interactions. Their effects can also be propagated to ψ_2 and might, therefore, be considered as the origin of the distorted β-turn. In Me_2SO solution, their influence cannot be negligible, as pointed out by the significant variation induced in the spectrum by the deprotonation of the carboxyl group.[8] Among the peptide residues, Gly(4), located at the C end of the molecule, is by far the most affected. The modifications taking place on Gly(4) clearly indicate its stabilization with ionization.

However, it is once again difficult to appreciate whether the ionic interactions (NH_3^+/COO^-) take place inside a single molecule (monomer) or between elements in dimer. In the highly probable hypothesis of an equilibrium between monomers and dimers, these interactions could serve as driving forces to the 4 → 1 or 1′ hydrogen bonds. This is supported by the fact that the existence of the hydrogen bonds is subordinated to the existence of the ionic interactions: when the latter are abolished, the former are no longer observed (cationic species of the peptide).

Conclusion

The present work indicates that in solution an alternative conformation for the β-turn is an unexpected right-handed double helix that conserves most of the β-turn features. The major reason for not having a true β-turn in the crystal appears to be the strong head-to-tail interactions where ionic forces (NH_3^+/COO^-) are dominant. This is consistent with the fact that blocked peptides containing L–D sequences, as, for instance, isobutyryl-L-Pro-D-Ala-isopropylamide[29] and *N*-Ac-L-Pro-D-Lac-methylamide,[30] assume typical type II β-turns.

In contrast, free peptides lead at best to distorted β-turns,[6] the most favorable conformation being a flat extended pleated sheet structure.[7] In solution (Me_2SO), intramolecular and intermolecular interactions may compete for shaping the predominant folded conformation.

Registry No. Gly-L-Pro-D-Leu-Gly, 86863-01-6; Gly-L-[^{13}C,^2H]-Pro-D-Leu-Gly, 86940-54-7.

Supplementary Material Available: Tables containing positional parameters and the mean *U* recalculated factor, anisotropic thermal factors, and distances and angles between atoms (3 pages). Ordering information is given on any current masthead page.

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