Peptide Conformation. 12.[1] Conformation of Cyclo-(L-Pro$_3$) in Solution

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The 270- and 500-MHz ^{1}H NMR spectra of cyclo-(L-Pro$_3$) have been completely analyzed. The molecule shows effective C_3 symmetry. A number of previous spectral assignments had to be revised on the basis of ^{1}H chemical shifts and changes in these shifts caused by the addition of benzene, Me_2SO, or the shift reagent Eu(fod)$_3$. The ^{1}H coupling constants are best interpreted in terms of an α^+ (envelope) conformation. The high mobility of the γ-CH_2 group easily transforms this structure to twist conformations.

The conformation of the pyrrolidine ring in proline derivatives is a much-discussed problem[2] that has been investigated by theoretical calculation methods,[3,4] NMR spectroscopy,[5-7] and X-ray structure techniques.[8-11] The synthetic peptide cyclo-(L-Pro$_3$) (1)[12] has played an important role in these earlier studies since a ^{1}H NMR analysis existed[5] which appeared to be confirmed by a later crystallographic analysis.[8,9] A Karplus relationship was derived from the coupling constants of 1 and was used to determine the conformations of various linear and cyclic proline-containing peptides.[13] Originally, we planned to use 1 as a model substance for an investigation of the considerably more complicated spin system of cyclo-(L-Pro$_2$-D-Pro).[14,15] A thorough analysis of the 270- and later 500-MHz ^{1}H NMR spectra of 1 led to a new interpretation which requires changes in the previously published signal assignments.

Prior to studying the 220-MHz ^{1}H NMR spectrum of 1, Deber et al.[5] examined cyclo-(L-Pro$_2$-L-Hyp) (2). The spin system of Hyp is simplified by the replacement of one γ-proton in the *pro-R* position by a hydroxyl group, which also leads to an increased dispersion of chemical shifts. Directly measurable coupling constants and those obtained by spectrum simulation were used in an appropriate Karplus equation[13] to compute the dihedral angles for the hydroxyproline residue in the cyclic tripeptide 2. The coupling constants of 2 were then used as a starting basis for the simulation of the proline spin system in 1. This approach assumes that both tripeptides have similar conformations. However, current opinion suggests that Hyp prefers a relatively immobile five-membered-ring conformation while Pro possesses a greater flexibility.[2] The most important results of this NMR study were the apparent C_3 symmetry in the spectrum and the description of the pyrrolidine ring conformation as nearly planar with the nitrogen atom rotated out of the plane (N^- form). Both observations require a planar cis peptide bond, in contradiction to the model studies of Venkatachalam,[16] who found that the peptide bond must be nonplanar (Ω = 20-30°) when the pyrrolidine ring is planar. Rothe et al.[17,18] also suggested a twisted peptide bond based on the

Table I. ^{1}H Coupling Constants for Cyclo-(L-Pro$_3$) and the Derived Dihedral Angles

coupling[f]	coupling constant: L-Hyp[a]	L-Pro[b]	L-Pro[c]	L-Pro[d]	θ[e]
$^3J_{1,2}$, $\alpha\beta^t$	1.4	1.4	0.8	0.69	87
$^3J_{1,3}$, $\alpha\beta^c$	7.0	7.0	7.2	7.31	28
$^4J_{1,4}$, $\alpha\gamma^t$			0.3	−0.54	
$^4J_{1,5}$, $\alpha\gamma^c$			0.2	−0.18	
$^4J_{1,6}$, $\alpha\delta^c$				<0.1	
$^4J_{1,7}$, $\alpha\delta^t$				−0.26	
$^2J_{2,3}$, $\beta^t\beta^c$	−13.5	−12.0	−12.3	−12.38	
$^3J_{2,4}$, $\beta^t\gamma^t$	7.5	2.5	8.5	8.55	18
$^3J_{2,5}$, $\beta^t\gamma^c$		10.0	2.4	2.44	104
$^4J_{2,6}$, $\beta^t\delta^c$			0.4	−0.66	
$^4J_{2,7}$, $\beta^t\delta^t$			0.1	0.12	
$^3J_{3,4}$, $\beta^c\gamma^t$	5.0	9.5	9.7	10.03	150
$^3J_{3,5}$, $\beta^c\gamma^c$		6.5	10.0	9.66	0
$^4J_{3,6}$, $\beta^c\delta^c$			0.2	−0.22	
$^4J_{3,7}$, $\beta^c\delta^t$			0.2	0.33	
$^2J_{4,5}$, $\gamma^t\gamma^c$		−15.0	−12.5	−12.33	
$^3J_{4,6}$, $\beta^t\delta^c$		6.5	6.8	6.92	133
$^3J_{4,7}$, $\gamma^t\delta^t$		9.0	9.8	10.22	0
$^3J_{5,6}$, $\gamma^c\delta^c$	7.0	9.5	8.9	9.16	14
$^3J_{5,7}$, $\gamma^c\delta^t$	3.0	4.0	4.3	4.66	121
$^2J_{6,7}$, $\delta^c\delta^t$	−14.0	−12.5	−12.3	−12.31	

[a] Reference 5; data for 2 iterated, error limits ±0.5 Hz, simulation line width 2.5 Hz. [b] Reference 5; derived from L-Hyp and iterated, error limits ±0.5 Hz for H_α and H_δ and ±1.0 Hz for H_β and H_γ. [c] This work, 270 MHz; values were iterated with an improved LAOCOON program. [d] This work, 500 MHz; iterated with the PANIC program, error limits ±0.05 Hz. [e] Dihedral angle (±5°) for vicinal protons calculated with eq 2. [f] Superscripts c and t stand for cis and trans, respectively.

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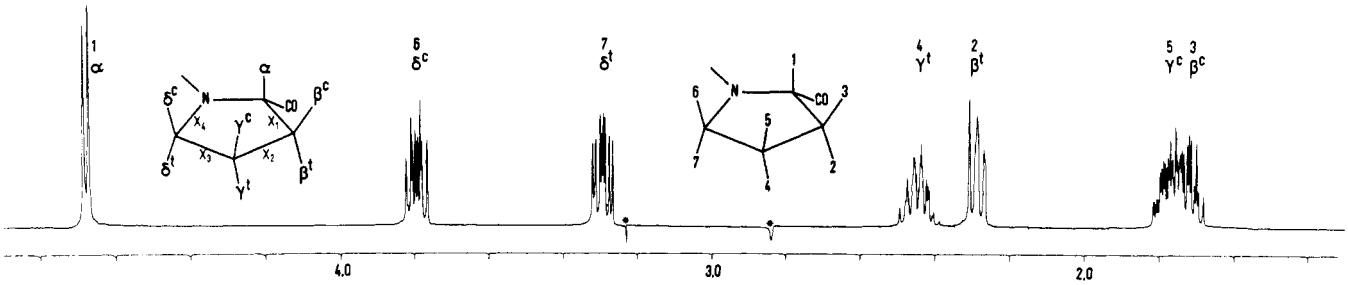

Figure 1. 500-MHz ^{1}H NMR spectrum of cyclo-(L-Pro$_3$) in 60:40 $CDCl_3/C_6D_6$. No line-shape manipulation was made. The signals marked with an asterisk are folded solvent signals.

Table II. ^{1}H NMR (270 MHz) Chemical Shifts for Cyclo-(L-Pro$_3$) in Various Solvents at 20 °C

		chemical shift δ				induced shift (Δ), ppm[b]		
			$CDCl_3/C_6D_6$					
no.	signal[a]	$CDCl_3$	3:2	1:9	$CDCl_3$/ Me_2SO, 3:2	$CDCl_3$/ C_6D_6	$CDCl_3$/ Me_2SO	LIS
1	α	5.07	4.68	4.21	5.45	−0.86	+0.38	0.063
2	β^t	2.37	2.29	2.17	2.12	−0.20	−0.25	0.041
3	β^c	1.91	1.72	1.38	1.82	−0.54	−0.09	0.036
4	γ^t	2.48	2.44	2.42	2.26	−0.06	−0.22	0.031
5	γ^c	1.92	1.76	1.53	1.85	−0.39	−0.07	0.019
6	δ^c	3.82	3.79	3.77	3.66	−0.05	−0.16	0.064
7	δ^c	3.32	3.29	3.27	3.03	−0.05	−0.29	0.068

[a] The indices c (cis) and t (trans) indicate orientation relative to H_α. [b] The shift in the solvent mixture minus the shift in pure $CDCl_3$. [c] After addition of 1 molar equiv of Eu(fod)$_3$ to a solution containing 1 in 1:1 $CDCl_3/C_6D_6$. Qualitatively similar results are obtained in pure $CDCl_3$.[14]

nonequivalence of the δ protons in the ^{1}H NMR spectrum as well as the appearance of a low-frequency, weak absorption maximum in the CD spectrum. In contradiction, the position of the amide I band in the IR spectrum at 1630 cm^{-1} is evidence for a planar peptide bond.[5] Within the limits of error, the torsion angles determined by NMR are in agreement with X-ray data[8,9] and confirm a planar peptide bond.

A later publication from Anteunis[7] exchanged the chemical shift assignments for the δ protons of Pro. The assignment uncertainties as well as the rather larger error limits for coupling constants made it apparent that a thorough analysis of the ^{1}H NMR spectrum of 1 with modern high-field spectrometers is desirable. At the same time this work would serve as a test of the methods to be used for the analysis of the ^{1}H NMR spectrum of cyclo-(L-Pro$_2$-D-Pro).

Results

The 270-MHz ^{1}H NMR spectrum of 1 in $CDCl_3$ solution exhibits five groups of signals for the seven protons of the Pro spin system. The addition of benzene to the solution improves the chemical shift dispersion so that all seven proton multiplets are separated in a solvent mixture of 90% deuteriobenzene with 10% deuteriochloroform.[14] A mixture of 60% $CDCl_3$ and 40% C_6D_6 was used to obtain the 500-MHz ^{1}H NMR spectra used for a complete spin simulation (Figure 1). Table I presents a summary of the ^{1}H NMR data for Pro in 1 as obtained from the literature and from iterative spin simulation of the 270-MHz spectrum and the 500-MHz spectrum. The most consistent data were obtained with the 500-MHz spectrum which was analyzed with the iterative spin-simulation program PANIC.[19] An important part of the analysis is the proper assignment of the chemical shifts for the various Pro ring protons (Figure 1). ^{1}H homodecoupling experiments allow an unambiguous assignment of protons to their respective carbon atoms (i.e., α, β, γ, δ); however, as already mentioned, the literature contains conflicting assignments concerning configuration. The assignments presented here are based on several independent experiments (Table II): decoupling experiments and the analysis of the ^{1}H–^{1}H scalar coupling constants, changes in chemical shifts induced by benzene (ASIS), changes in chemical shifts induced by Me_2SO (SIS), changes in chemical shifts induced by Eu(fod)$_3$ (LIS), and consideration of the anisotropic influence of the carbonyl group on the chemical shifts.

Without doubt the C_α proton can be assigned to the signal at 4.68 ppm. Decoupling at this position produces a drastic change at 1.72 ppm but only a small effect on the pattern at 2.29 ppm. Decoupling at 1.72 ppm produces the expected singlet at 4.68 ppm and a strong effect at 2.29 ppm. Decoupling of δ-protons causes no large effect at 2.29 ppm so that the signals at 1.72 ppm and 2.29 ppm can be assigned immediately to β-protons. Further decouplings lead to the complete assignments shown in Table II.

It is unusual that H_α appears as a broad doublet ($J_{1,3}$ = 7.3 Hz) rather than a doublet of doublets or triplet pattern as is common for proline-containing oligopeptides[20] or for polyproline.[21] This is the result of a nearly vanishing vicinal coupling to the low-field β-proton at 2.29 ppm. Similarly, the small coupling between H_β (low field) and H_γ (high field) of $^3J_{2,5}$ = 2.5 Hz and the coupling between H_γ (high field) and H_δ (high field) of $^3J_{5,7}$ = 4.7 Hz are important for the analysis. Small vicinal couplings can occur only when the corresponding dihedral angle approaches 90°, i.e., only between trans-standing protons in a five-membered ring. Thus, the protons 1, 2, 5, and 7 have a vicinal trans configuration.

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Quantitative analysis of the ASIS effect[22-24] (Table II) shows, in general, a shift to high field for all protons. The shift is particularly strong for protons lying "above" the nine-membered macrocycle of the peptide backbone (α, β^c, γ^c). This is consistent with our expectation based on models, whereby benzene complexes preferentially to that side of the peptide bond which is cis relative to H_α. The δ-protons lie in the area of the carbonyl group and should, therefore, show a weaker ASIS effect.

To our knowledge there is no satisfactory discussion in the literature of the effect of Me_2SO upon the ^1H chemical shift of tertiary amides. The observed SIS value in Table II can be understood if one makes the assumption that the polar solvent Me_2SO results in an increase in the polarization of the peptide bond. Thus, the α-proton should shift to low field because of reduced electron density at the amide nitrogen. All other protons will shift to high field, with the largest shifts being observed for protons that are closest to the carbonyl oxygen which has increased electron density. Consideration of molecular models suggests the largest high-field SIS for β^t and γ^t.

The lanthanide shift reagent $Eu(fod)_3$ binds to the peptide carbonyl group[25] and causes chemical shift changes that are qualitatively similar to those caused by Me_2SO. In this case, however, all LIS shifts are to low field with the relative effects in the following order: $\beta^t > \beta^c$; $\gamma^t > \gamma^c$; $\delta^t \simeq \delta^c$ (Table II). Molecular models indicate that the protons β^t and γ^t are nearer than their geminal neighbors to the probable location of the lanthanide ion.

The diamagnetic anisotropy of the carbonyl group[26] causes low-field shifts for β^t and γ^t. Furthermore, given an appropriate conformation, the carbonyl will also exert a strong downfield shift on the δ^c proton of the neighboring Pro residue. The δ^t proton stands perpendicular to the peptide bond plane and experiences only a small influence from the carbonyl group.

All the chemical shift and coupling constant data presented here represent a self-consistent data set that is in complete agreement with the signal assignments given in Table I. Comparison of the coupling constants of this work with those of ref 5 indicates that three of the published values differ from our values by an amount which lies outside the published error limits. At first it was thought that an accidental interchange of $^3J_{2,4}$ and $^3J_{2,5}$ had occurred in ref 5. However, we were unable to obtain a calculated spectrum which even approximated the experimental ones when we used either the published constants or a set in which the two couplings mentioned above were interchanged. The iteratively refined couplings of this work give an excellent match of calculated and experimental spectra at both 270 and 500 MHz.

Discussion

In solution and at room temperature the peptide framework of cyclo-(L-Pro_3) possesses C_3 symmetry (at least on the average on the time scale of the NMR measurement). Only one seven-spin system is to be seen in the ^1H NMR spectrum (Figure 2). ^{13}C NMR has also shown that only a single type of pyrrolidine ring is present.[27] The conformation of the pyrrolidine ring can be

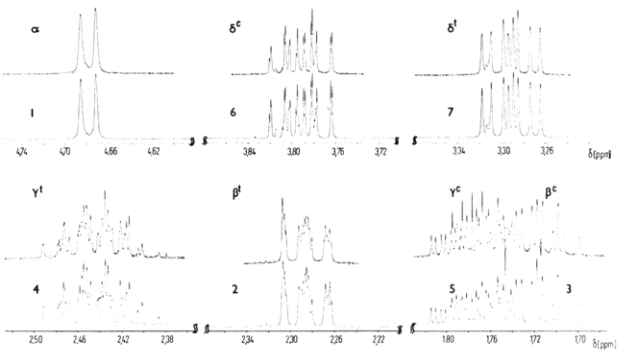

Figure 2. 500-MHz ^1H NMR spectrum of cyclo-(L-Pro_3) in 60:40 $CDCl_3/C_6D_6$. The individual spin multiplets have been expanded. In each case the upper trace is the resolution-enhanced spectrum, and the bottom trace is the final iterated theoretical spectrum obtained by using the parameters of Table I and a Lorentzian line width of 0.75 Hz (α), 0.5 Hz (δ^t, γ^t, β^t), 0.4 Hz (γ^c, β^c), and 0.35 Hz (δ^c).

Figure 3. Dependence of the vicinal coupling constants upon torsion angle: left and middle, two extreme cases for C_β–C_γ; right, representation of the torsion angles for a hypothetically linearized C_β–C_γ–C_δ.

analyzed by considering the dependence of the vicinal couplings upon dihedral angles. This can be accomplished by using the appropriately modified Karplus equation[13] in which substance class and constitution have been taken into account. The equation used by Deber et al.[5] for the α,β vicinal coupling can be written as in eq 1. Using X-ray

$$^3J = 9.5 \cos^2 \theta - 1.0 \cos \theta + 1.4 \quad (1)$$

data for cyclo-(L-Pro_3), Kopple et al.[28] have modified eq 1 to give eq 2. Equation 2 was used to calculate the

$$^3J = 9.4 \cos^2 \theta - 1.4 \cos \theta + 1.6 \quad (2)$$

dihedral angles tabulated in Table I. Unfortunately, the error by such a procedure is relatively great since electronic effects have been neglected. Thus, the Karplus equation cited above gives only a band of possible coupling constants. In the references mentioned, the error limits are $\pm$0.5 Hz (α,β coupling) and $\pm$1 Hz (β,γ coupling). The results presented in this work reduce these limits to $\pm$0.05 Hz for all vicinal couplings. Moreover, the value of $^3J_{1,2}$, which serves as a calibration point for eq 1 and 2 with $\theta = 90°$,[13,28] has been reduced to 0.7 Hz. Thus, a significant correction to the Karplus equations mentioned above is required.

The Karplus relationship for vicinal coupling constants predicts that cis-standing pairs of protons in a Pro ring (Figure 3) should have equal couplings (i.e., $J_{2,4} = J_{3,5}$ and $J_{4,7} = J_{5,6}$), even when several conformations are in equilibrium. The experimental data indicate conclusively that this equality does not occur. The cause of this effect could be bond angle deformation or directional dependence of vicinal couplings in the fragment X–CH_2–CH_2–Y.

The sum of the vicinal (cis) coupling constants discussed above gives an indication of the degree of torsion about

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Table III. Comparison of Experimental x Angles[a] for Cyclo-(L-Pro$_3$)

	NMR results[b]		X-ray data, deg
	ref 5	this work	
χ_1	30	30 (1, 2), 28 (1, 3)	29.0 to 34.6
χ_2	−10	−18 (2, 4), −15 (2, 5), −30 (3, 4), 0 (3, 5)	−17.6 to −29.5
χ_3	−5	13 (4, 6), 0 (4, 7), 14 (5, 6), 1 (5, 7)	−0.9 to 13.0
χ_4	25[c]	20[c]	8.2 to 20.9

[a] χ angle defined according to IUPAC.[29] [b] In degrees ±5°; the numbers of the protons observed are given in parentheses. [c] Determined from a Dreiding model after setting the other angles.

the respective C–C single bonds in the Pro ring. From eq 2 and Figure 3 it is clear that this sum (e.g., $J_{2,4} + J_{3,5}$) will be a maximum for $\theta = 0°$ or when the cis-standing protons are eclipsed. From Table I we find that $(J_{2,4} + J_{3,5}) < (J_{4,7} + J_{5,6})$, which means that torsion is greater for χ_2 than for χ_3. Under the crude assumption that the Karplus equation (eq 2) can be used for all vicinal couplings in the Pro ring, one obtains the θ values presented in Table I. These can be converted to χ angles which are found in Table III. The χ values derived from NMR data are in excellent agreement with the X-ray data. Inspite of the interchanged assignments for the δ-protons, Deber et al.[5] obtained χ angles which differ significantly from our results only for χ_3. The values presented here indicate that the proline ring adopts essentially an α^+ conformation as proposed by Anteunis et al.[7] Thus, the atoms C_β, C_γ, C_δ, and N lie nearly coplanar, as was also found for proline rings A II and A III in the X-ray structure. Dreiding models also show a similar conformation.

Using the more specific Karplus equation from Altona et al.,[30] one derives a phase angle P in the range 108–134° (the S conformational range, χ_2 = negative) from the coupling constants of the α,β and β,γ bond. The couplings for γ and δ-protons result in a wider range of P values (103–160°), depending on which coupling constant is used for the evaluation. A phase angle $P = 126°$ corresponds to the proposed $\alpha^+ = \alpha^E = \alpha$-endo conformation.

These conclusions assume that no significant twist exists in the peptide bond and that only one proline conformation dominates the equilibrium or that this equilibrium can be described by a single averaged conformation. In the crystal lattice, however, two molecules are found with different conformations, indicating that the energy barrier for conformational change is low. The X-ray data show that the bond lengths to C_γ are shorter than for other C–C bonds and that the thermal parameters for C_γ are higher. This indicates that C_γ has a greater mobility.[8] Spin-lattice relaxation times for ^{13}C (NT_1 values) demonstrate that the β-, γ-, and δ-carbons have significantly higher mobility than C_α and that C_γ may have slightly more mobility (ca. 10%) than its neighboring carbons.[27] We and other workers have found a correlation between ^{13}C NT_1 values in solution and thermal parameters in the crystal.[2,14,31] Molecular models show that movement of C_γ can easily convert the envelope conformation (α^+, A II, A II), which is favored by our results, into the twist conformation (A I, B I, B II, B III) observed in the crystal.[9]

A thorough discussion of the long-range couplings ^4J in the proline ring is not possible at this time since a well-developed theory of these couplings is not available. Examination of Table I, however, does suggest that systematic stereochemical relationships may exist for these couplings. There are four pairs of ^4J couplings. Each pair involves one cis relationship and one trans relationship, e.g., $^4J_{1,5}$ (cis) and $^4J_{1,4}$ (trans) or $^4J_{2,7}$ (cis) and $^4J_{2,6}$ (trans). In each case the coupling ^4J between cis-standing protons is smaller in magnitude than the ^4J for the corresponding trans protons. A complete analysis of the ^1H NMR spectrum of cyclo-(L-Pro$_2$-D-Pro) is in progress and indicates that the above systematic behavior of ^4J also holds, at least for the D-Pro spin system.

Experimental Section

The 500-MHz ^1H NMR spectra were obtained with a Bruker WM-500 spectrometer using a 5-mm sample containing 12 mg/mL of cyclo-(L-Pro$_3$) in 60:40 $CDCl_3/C_6D_6$ at 300 K. The data-acquisition parameters were as follows: spectral width, 3000 Hz; data table, 64K; pulse width, 4 μs; 128 transients; digital resolution, 0.09 Hz; resolution enhancement with parameters chosen to give the best compromise between resolution and signal-to-noise.

Iterative spin simulation was performed with the PANIC program.[19] Considerable manipulation of coupling constants was first necessary to create a calculated spectrum which displayed all the features of the experimental spectrum to a good approximation. Particularly important was the determination of the best set of signs for the long-range couplings. These were determined by trial and error chiefly by considering relative signal intensities and fine-splitting patterns. The signs presented in Table I gave the best fit to the experimental spectrum but do not represent experimentally determined values. For iteration purposes all resolved transitions in the experimental spectrum were assigned to corresponding transitions in the calculated spectrum. All shifts and couplings were freely iterated simultaneously to give a fit with a root-mean-square deviation of 0.073 Hz. Error limits for iterated parameters are ±0.05 Hz.

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