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SYNTHESIS AND NMR ANALYSIS OF CYCLO-[85% ^{13}C-ASP]-PRO: ^{13}C–^{13}C VICINAL COUPLING CONSTANTS AND CONFORMATION

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Abstract—The dipeptide cyclo(Asp-Pro) where the aspartic acid residue was 85% ^{13}C enriched, was synthesized with the aim of analyzing its conformation in solution by using ^{1}H–^{1}H, ^{13}C–^{1}H and ^{13}C–^{13}C coupling constant parameters. The values of these couplings agree well with each other and show that the side chain of the aspartic acid residue adopts privileged conformations the proportions of which vary somewhat with pH, and more weakly, with a change in solvent. The ^{13}C–^{13}C interresidue coupling constants $^{3}J_{C_\beta\text{-}C_\gamma}$ and $^{3}J_{C_\beta\text{-}C_{\gamma'}}$ obtained after long accumulation of the signals of unenriched carbons, have different values; they show puckering in the pyrrolidine ring similar to that found in cyclo(Leu-Pro) in the solid state. It was concluded that ^{13}C–^{13}C coupling constants represent an excellent means of determining the side chain conformation (whenever the incorporation of an enriched amino acid into the peptide is possible) that will find applications particularly in the case of peptides with complicated proton spectra.

Cyclic dipeptides which are increasingly considered as metabolic intermediates[1,2] and derivatives of which exhibit antiviral properties,[3] have been extensively studied either in the solid state by X-ray crystallography or in solution by several spectroscopic methods.[4] As diketopiperazines have restricted rotational freedom, the number of the conformations is limited; and thus they constitute excellent substrates for collecting parameters useful for analysing more complicated peptides. The following rules which are true for both solutions and solids have thus been established.

(1) The diketopiperazine ring is planar or almost planar when either the two residues are glycine or one of the two α-branched residues is of the D-form.

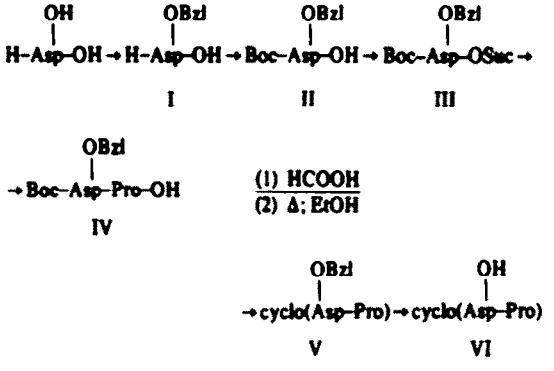
(2) The diketopiperazine ring is buckled (boat conformation) in all other cases. Proline and derivatives accentuate the buckling and limit the motions. In all L-L or Gly-L-diketopiperazines examined, proline itself adopts exclusively the envelope C_δ–C_β *endo* conformation.

(3) Aromatic residues lead to the flagpole-boat conformation. There are no particular features to note concerning the side chains of other residues except that conformations with $\chi_1 = +60°$ are often preferred in solution. Only few data concerning polar side-chains in diketopiperazines exist.

The present NMR study of cyclo(L-Asp-L-Pro) (Fig. 1) was undertaken in order to investigate the side-chain arrangements of the Asp and Pro residues and their effects on the diketopiperazine ring buckling. The technique of inserting ^{13}C-enriched amino acids for peptide conformation analysis was developed several years ago;[5–10] many ^{13}C–^{13}C vicinal coupling constants in addition to those of ^{1}H–^{1}H and ^{13}C–^{1}H have been measured and interpreted in conformational terms. Results show that ^{13}C–^{13}C coupling constants are valuable parameters for probing side-chain and backbone conformations of peptides in solution; they give information on molecular aspects unexplorable by ^{1}H NMR and ^{13}C NMR dealing with ^{13}C natural abundance compounds.

EXPERIMENTAL

Material and instrumentation. Uniformly 85% ^{13}C enriched amino acids were prepared on a large scale from Spirulina maxima as previously reported.[6] The synthesis of ^{13}C enriched Asp-Pro diketopiperazine was carried out according to the following scheme:



The preparation of the β-benzylester of ^{13}C enriched aspartic acid[11] yielded 42% β-benzylaspartate(I), 39% tosylate of the dibenzylester of aspartic acid and 12% of copper complexed aspartic acid. Products II and III were prepared by classical methods, III was then condensed with free proline in dimethylformamide according to the method by Savrda.[12] The removal of the Boc group by formic acid[13] and subsequent heating in ethanol gave the diketopiperazine V in good yield. The final product VI was obtained quantitatively by catalytic hydrogenation of V in ethanol/water solution, m.p.: 273–276°. (Found: C, 55.6; H, 6.0; N, 14.1. Calc. for $^{12}C_5{}^{13}C_4H_{12}O_3N_2$: C, 55.8; H, 6.1; N, 14.0%).

The ^{13}C NMR samples in $^{2}H_2O$ at 2.10^{-1} M to 3.10^{-1} M concentrations contained dioxane as internal reference (taken at 67.4 ppm from TMS). The spectra were recorded at 25.2 MHz and 20 MHz on a Varian XL 100-12 WG and a CFT 20 spectrometer respectively at 30°C probe temp. 8K data points were generally used for a spectral width of 500 Hz permitting an intrinsic resolution of 0.125 Hz. Each sample was run two to three times and the final values of δ and J are arithmetic averages. Proton broad band decoupling was applied to measure δ from the spectra of the ^{13}C natural abundance and $J_{C,C}$ from

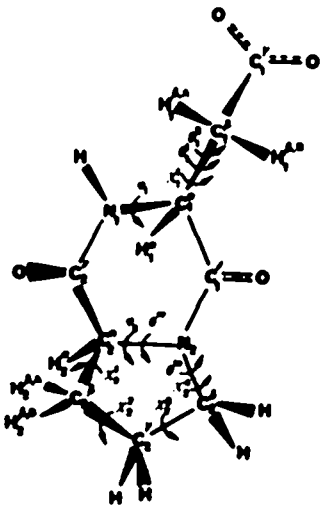


Fig. 1. Dihedral angles, bonds, atoms and main conformational features in cyclo(L-Asp-L-Pro). The numbering and lettering given in this figure is used throughout the present paper in the text and in the Tables. The dihedral angles ϕ_1, χ_1^1, θ_1^1, θ_1^2 of Asp (residue 1) and ϕ_2, χ_2^1, θ^{III} (defined by $C_1^{\alpha}N_2C_2^{\alpha}C_2^{\beta}$) of Pro (residue 2) can be estimated directly from NMR data (see Results and Discussion).

those of the ^{13}C enriched compounds. The spectra with proton couplings were recorded with gated proton decoupling in which the decoupler was off during data acquisition. The ^1H NMR samples at about 0.1 M concentration either in ^2H_2O (internal reference TSP-d_4) or in DMSO-d_6 (int. reference TMS) were run at 250 MHz on the Cameca TSN-250 spectrometer in the C.W. mode. The ^1H–^1H coupling constants were obtained with ±0.1 Hz precision. For titrations, the pH was adjusted by concentrated NaO^2H and ^2HCl and measured directly inside either the 10 mm ^{13}C NMR tube or the 5 mm ^1H NMR tube. None of the pH readings was corrected for deuterium isotope effects.

Spectral analysis and procedures. The ^1H–^1H and ^{13}C–^1H coupling constants were measured in the ^1H and ^{13}C NMR spectra of the natural abundance compound. The directly bonded ^{13}C–^{13}C couplings were determined from spectra of the ^{13}C labelled samples (Fig. 2). The ^{13}C–^{13}C couplings over two and three bonds needed high resolution spectra and were measured either directly at the ^{13}C enriched atom signals for the intra Asp couplings or at ^{13}C natural abundance signals of proline for the inter Asp-Pro couplings.

Analysis of 85% ^{13}C enriched amino acid spectra has been reported elsewhere:[6] it was shown that the residual 15% of ^{12}C in the C atoms complicate the spectra to such an extent that incorporation of more than one labelled residue in the same peptide[8] was not permitted. However, ^{13}C–^{13}C long range couplings, albeit weak compared to the one bond couplings are often easily readable especially on the carboxyl multiplets.[8,9] Couplings involving both ^{13}C enriched and ^{13}C natural abundance atom positions form simpler multiplets at the ^{13}C natural abundance resonance signal; their detection, however, demands long accumulation times.

Proton coupled ^{13}C spectra of the carboxyl group were analysed in ^{13}C resonance mode as the X part of an ABCX system according to Hansen *et al.*[14] and Espersen and Martin.[15] Such couplings were measured in ^{13}C natural abundance cyclo-(Asp-Pro) at the C_1^{γ} signal which appears as a pseudo quadruplet.

Several ^1H–^1H coupling constants were determined in cyclo(Asp-Pro) at ^{13}C natural abundance either dissolved in ^2H_2O as a function of pH or in DMSO-d_6 only for the un-ionised (COOH) state (solubility in the organic solvent is weak for the ionised species). They concern mainly the vicinal couplings H_1^{α}–$H_1^{\beta A}$, $H_1^{\beta A}$–$H_1^{\beta B}$ and H_1^{α}–N_1H (in DMSO-d_6).[16] For proline only the H_2^{α} signal was analysed because of the complexity of the $H_2^{\beta A}$, $H_2^{\beta B}$ multiplet due to additional couplings with H_2^{γ} protons. In addition, one $^5J_{H_1^{\alpha}-H_2^{\gamma}}$ coupling was found at both signals involved.

Fractions of rotamer populations were calculated from the $^3J_{H_1^{\alpha}-H_1^{\beta A}}/^3J_{H_1^{\alpha}-H_1^{\beta B}}$, the $^3J_{H_1^{\alpha}-C_1^{\gamma}}$ and the $^3J_{C_1^{\gamma}-C_1^{\beta}}$ coupling constants data using the following parameters:

$$J_g^{H,H} = 2.6\ \text{Hz}, \quad J_t^{H,H} = 13.6\ \text{Hz},^{17}$$

$$J_g^{H,C} = 0.4\ \text{Hz}, \quad J_t^{H,C} = 11.9\ \text{Hz},^{14}$$

and

$$J_g^{C,C} = 0.5\ \text{Hz}, \quad J_t^{C,C} = 5.9,$$

for proton-proton, carbon-13-proton and carbon-13-carbon-13 couplings respectively. The latter values were arrived at by comparing ^{13}C couplings with the ^1H couplings in several amino acids, when they were applied to free amino acids and C-terminal or acid residues in peptides (work in progress). They agree well with their counterparts in the relationships proposed by Barfield *et al.*[18] for butanoic acid and the Marshall and Miller data[19] for aliphatic carboxylic acids. The Kopple *et al.*[20] relationship for side chain arrangements was used for proline.

RESULTS AND DISCUSSION

^{13}C-NMR chemical shifts. ^{13}C chemical shifts of all C atoms measured for the un-ionised and the ionised form together with the pH effects are assembled in Table 1. The resonances are assigned on the basis of those of the free amino acids, by analysing the pH dependence effects on the chemical shifts and by comparing the ^{13}C-labelled and ^{13}C natural abundance compound spectra (Fig. 2). For proline they are about the same as the ones found for any proline containing diketopiperazine.[8,9] During deprotonation of the side chain γ-carboxylic group, all the aspartic acid carbons are shifted downfield. The effect decreases regularly from the C_1^{γ} end to the C_1^{α} end, simply describing a phenomenon dependent on the carbon-carboxyl distance, such as it had already been shown for C-terminal amino acids in peptides.[21] The averaged pK value of the carboxyl group, estimated from the chemical shift curves of all Asp carbons is about 4.25.

^1H NMR chemical shifts. ^1H chemical shifts for two pH values are given in Table 2. Again they were assigned from the comparison with ^1H resonances of related compounds and with the assistance of the pH effects. In this work we designate by A the β-proton of the Asp residue characterized by the signal having the smaller chemical shift and giving rise to the larger coupling with the α-proton at neutral pH. If we take into account the generally accepted steric factors, then rotamer I is found to be more populated than rotamer II. This assignment is further corroborated by the $^3J_{C_1^{\alpha}-C_1^{\gamma}}$ data which emerge as a good means for assigning the A and B β-protons (see rotamers in Table 4). In agreement with the ^{13}C results, the pH dependence of the H_1^{α}, $H_1^{\beta A}$ and $H_1^{\beta B}$ proton chemical shifts yields of pK of 4.25.

Coupling constants: $^1J_{C,C}$, $^2J_{C,C}$ and $^2J_{H,H}$ coupling constants (Table 3). The geminal $^2J_{H_1^{\beta A}-H_1^{\beta B}}$ coupling constant in the aspartic acid residue varies from −17.8 Hz to −16.9 Hz in the course of ionisation of the carboxylic group and thus depicts this process inadequately. Three one-bond ^{13}C–^{13}C couplings are available in the ^{13}C enriched aspartic residue: $^1J_{C_1^{\alpha}-C_1^{\alpha}}$, $^1J_{C_1^{\gamma}-C_1^{\beta}}$ (sp^2–sp^3 type) and $^1J_{C_1^{\alpha}-C_1^{\beta}}$ (sp^3–sp^3 type). $^1J_{C_1^{\gamma}-C_1^{\beta}}$ (the greatest of the three values at acid pH) which involves the carbon of the ionisable group diminishes from 56 Hz to 51 Hz, whereas $^1J_{C_1^{\alpha}-C_1^{\alpha}}$ and $^1J_{C_1^{\alpha}-C_1^{\beta}}$

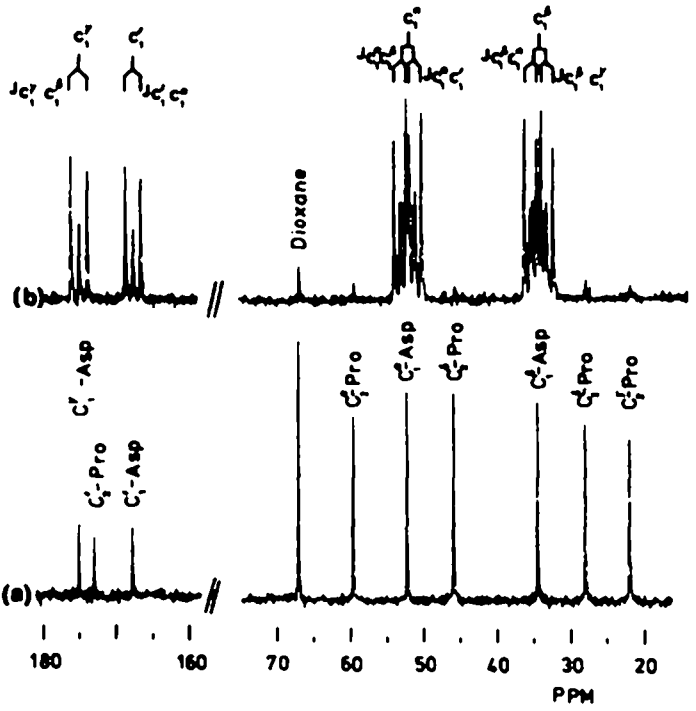


Fig. 2. ^{13}C NMR spectra of: (a) natural ^{13}C abundance cyclo (Asp-Pro), (b) cyclo ([85% ^{13}C-Asp]-Pro) in $^{2}H_2O$ at pH 1.55.

Table 1. ^{13}C chemical shifts in cyclo(Asp-Pro)

pH	Asp				Pro				
	C'_1	C^{α}_1	C^{β}_1	C^{γ}_1	C'_2	C^{α}_2	C^{β}_2	C^{γ}_2	C^{δ}_2
1.55	167.39	52.68	35.09	174.70	172.67	60.00	28.68	22.71	46.37
6.40	168.16	53.76	38.20	178.40	172.68	59.91	28.62	22.73	46.33
$\Delta\delta$	0.77	1.08	3.01	3.70	- 0.01	-0.09	- 0.06	0.02	- 0.04

Table 2. ^{1}H chemical shifts in cyclo(Asp-Pro)

pH	Asp			Pro				
	H^{α}_1	$H^{\beta,B}_1$	$H^{\beta,A}_1$	H^{α}_2	$H^{\beta,A-B}_2$		H^{γ}_2	H^{δ}_2
1.44	4.55	3.01	2.96	4.36	2.32	2.04	2.0	3.55
6.02	4.45	2.80	2.69	4.36	2.33	2.05	2.0	3.55

conserve their initial value of 52 Hz and ~40 Hz respectively, during the deprotonation process of the carboxylic group. At the same time an almost linear relationship between the C_1^{γ} chemical shifts and the $^{1}J_{C_1^{\gamma}-C_1^{\beta}}$ variations is observed, the slope being 1.3 Hz/ppm (for C-termanal glycine in di and tripeptides this slope was about 1.5 Hz/ppm[6]).

The two ^{13}C geminal couplings $^{2}J_{C_1^{\gamma}-C_1^{\alpha}}$ and $^{2}J_{C_1'-C_1^{\beta}}$ are about equal for a given pH: ~1.5 Hz in the neutral form and slightly smaller in the anion. Apparently, ion-

Table 3. One bond and two bond coupling constants in cyclo(Asp-Pro)

Ionization state	$^1J_{C_1^i-C_1^\alpha}$	$^1J_{C_1^\alpha-C_1^\beta}$	$^1J_{C_1^\beta-C_1^\gamma}$	$^2J_{C_1^\alpha-C_1^\gamma}$	$^2J_{C_1^i-C_1^\beta}$	$^2J_{H_1^{\beta,A}-H_1^{\beta,B}}$
$C_1^\gamma OOH$	52.0	40.3	56.0	2.0	1.8	- 17.5
$C_1^\gamma OO^-$	52.0	40.0	51.0	1.2	1.7	- 16.9

isation of the carboxylic group does not affect the geminal couplings to a great extent in this compound. Several authors have attempted to correlate geminal coupling with torsional angles, but further investigations are needed to assess the validity of this approach for an Asp residue.

Vicinal ^{1}H–^{1}H, ^{13}C–^{1}H *and* ^{13}C–^{13}C *intraresidue coupling constants and the aspartic acid side chain arrangement.* The vicinal coupling constants measured in both natural abundance and ^{13}C enriched cyclo (Asp-Pro) are listed in Table 4, together with the fractions of the rotamer populations. They have been estimated by using the three sets of ^3J_g and ^3J_t coupling constants given in the Experimental. The good agreement that exists between the values of rotamer population obtained by each method shows the validity of the use of ^{13}C couplings in this approach. Moreover, the ^{13}C–^{13}C couplings yield unequivocally rotamer I, and therefore eliminate the ambiguity arising from the impossibility to differentiate rigorously between the $H^{\beta,A}$ and $H^{\beta,B}$ protons, i.e. between rotamer I and II. Given the errors associated with estimates of rotamer populations, this task is most easily accomplished when the fractions of rotamer I and II are very different as is the case at neutral pH.

In the uncharged form of cyclo(Asp-Pro) rotamer III is found in greater amount than the two others in both solvents; this situation is often encountered in cyclo-dipeptides,[4] less often in linear peptides, and can be explained mainly by steric factors which are specific to diketopiperazines. Clearly, dimethylsulfoxide and pH effects introduce only small changes in the rotamer distribution; undoubtedly side chain charges, solvation forces and H-bonds are not the major factors influencing the side chain organization in this compound.

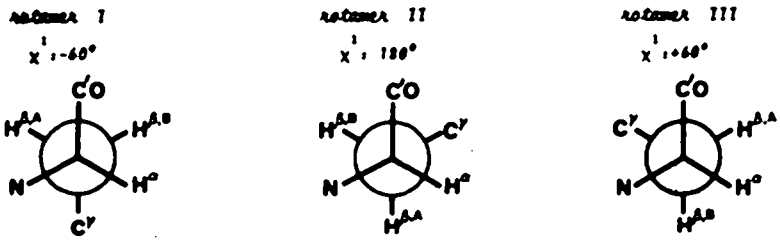
Vicinal ^{1}H–^{1}H, ^{13}C–^{13}C *and* ^{1}H–^{1}H *five bond coupling constants: the pyrrolidine and the diketopiperazine ring conformations.* In dimethylsulfoxide the NH signal of the aspartic acid residue appears as a singlet, i.e. $^3J_{H_1^\alpha-N_1H} \sim 0$–1 Hz. According to this value the dihedral angle ϕ_1 (aspartic acid residue) must be $-30°$ to $-40°$, which already suggests that the diketopiperazine ring assumes a boat conformation (bowsprit–boat) with the α-protons located in quasi-axial position and the two side chain C^α–C^β bonds in quasi-equatorial position. In aqueous solution the Asp and Pro α-proton signals show well resolved extra splittings (2.5 Hz) assigned to the long range coupling ^{5}J with each other. Five-bond $^1H_1^\alpha$–$^1H_2^\alpha$ couplings are often observed in cyclic peptides[24] and have been extensively studied by Davies and Khaled[25] in a large series of compounds in correlation with their conformation. According to the relationship

$$^5J = nA^\circ \sin^4 \theta$$

where A° is a constant (1.4 Hz in ^2H_2O) for syncoplanar

Table 4. Vicinal coupling constants and side chain arrangement of aspartic acid in cyclo(Asp-Pro). (For spectral analysis and rotamer calculation, see Experimental section)

	^2H_2O (COOH state)				^2H_2O (COO^- state)				DMSO (COOH state)			
Coupled Nuclei	^3J (Hz)	rotamers I	II	III	^3J (Hz)	rotamers I	II	III	^3J (Hz)	rotamers I	II	III
H_1^α - $H_1^{\beta,A}$, $H_1^{\beta,B}$	5.2, 5.1	0.24	0.29	0.53	6.6, 4.7	0.36	0.19	0.45	5.3, 6.5	0.25	0.35	0.40
C_1^i - C_1^γ	2.0	0.27			2.5	0.37			1.75	0.23		
H_1^α - C_1^γ	6.0			0.49	5.7			0.46	5.8			0.47



C$^{\alpha}$H groups, n equals the number of equivaleat coupling paths (i.e. n = 2 for diketopiperazines) and θ equals $|\phi - 60°|$, one obtains a value for the dihedral angle ϕ_1 of about $-35°$, $-40°$. Considering the remarkable agreement between the ϕ values obtained by the two different sets of coupling constants, it can be reasonably concluded that the diketopiperazine ring assumes about the same buckled "bowsprit" conformation in the two solvents.

The H_2^{α}–$H_2^{\beta,A}$ and H_2^{α}–$H_2^{\beta,B}$ proton coupling constants in proline measured at the α-proton signal are 10.2 Hz and 6.2 Hz for both the neutral and the anionic form in ^2H_2O solution. According to the Kopple *et al.* curve, these values correspond to torsional angles of 155° or 0° and 35° or 135° (rounded to the nearest five degrees). The values of 155° and 35° converge to a quite fixed value for the dihedral angle χ_2^1 of $-35°$ (Fig. 1). Two interresidue ^{13}C–^{13}C vicinal coupling constants are also related to the geometry of the pyrrolidine ring: on the one hand $^3J_{C_1'-C_\gamma^2}$ characterized by a value of 1.95 Hz describes the torsional angle θ^{IV}; on the other hand, $^3J_{C_1'-C_2^\gamma}$ of 2.9 Hz is related to θ^{III}.[26]

In the absence of a suitable relationship directly applicable to peptide side chains, the significance of the interresidue ^{13}C–^{13}C coupling data in terms of angular dependence is checked against the crystallographic parameters of the analogous compound cyclo(Leu-Pro) reported by Karle.[27] We note (Table 5) that proline containing L–L cyclic dipeptides show about the same conformational features throughout, a fact which encourages the present comparison: the diketopiperazine ring assumes a significant boat–bowsprit buckling; the pyrrolidine ring conformer is of C_s–C_β *endo* type (C_s = envelope; C_β *endo*: the C_β atom is above the plane formed by the other four atoms of the ring and on the same side as the CO group).[24,28] The validity of the comparison, however, requires the conservation of the main conformational features in both the solid and solution states. The following data indicate that possible distortions occurring in cyclo(Asp-Pro) have relatively small effects: the values of $^3J_{H_2^\alpha-H_2^{\beta,A}}$ and $^3J_{H_2^\alpha-H_2^{\beta,B}}$ in proline lead to a unique value of χ_2^1, in perfect agreement with the X-ray data; the ϕ_1 value (Asp residue) determined by both $^3J_{H_1^\alpha-N_1H}$ and $^3J_{H_1^\alpha-H_2^\alpha}$ fits well with the crystallographic data.

It is concluded that the cyclopeptide assumes about equal conformations in solution and in the solid state so that $^3J_{C_1'-C_\gamma^2} = 1.95$ Hz and $^3J_{C_1'-C_2^\gamma} = 2.9$ Hz describe approximately the value of $\theta^{IV} \sim 150°$ and $\theta^{III} \sim 170°$ respectively which are derived from the X-ray data.[27] This accuracy is largely sufficient for the present purpose as the trends rather than a rigorous correspondence are sought. From these results it becomes clear that the conformation of the pyrrolidine ring of cyclo (Asp-Pro) in solution is mainly of C_s–C_β *endo* type ($\chi_2^1 \sim -30°$ and $\chi^4 \sim -10°$) as it is encountered in cyclo(Leu-Pro). From a general point of view it appears that the couplings

Table 5. Comparison of cyclo(Leu-Pro) X-ray data and cyclo(Asp-Pro) NMR data

cyclo(Asp$_1$-Pro$_2$)			cyclo(Leu$_1$-Pro$_2$)
Coupling constants (Hz)		Angles derived from NMR data a)	Angles derived from X-ray data b)
^1H - ^1H	^{13}C - ^{13}C		
$^3J_{H_2^\alpha-H_2^{\beta,A}} = 10.2$		χ_2^1 : - 35°	χ_2^1: - 32°
$^3J_{H_2^\alpha-H_2^{\beta,B}} = 6.7$			
			χ_2^2: 36°
			χ_2^3: - 25°
			χ_2^4: 4°
$^3J_{H_1^\alpha-N_1H} = 0-0.2$		ϕ_1: -20° to -40°	ϕ_1, ϕ_2: -42°
$^5J_{H_1^\alpha-H_2^\alpha} = 2.4$		ϕ_1, ϕ_2: -40° to -45°	
			ϕ_1, ϕ_2 : 34°
			ω_1, ω_2: 6°
	$^3J_{C_1'-C_2^\gamma} = 2.9$		θ^{III} : 170°
	$^3J_{C_1'-C_\gamma^2} = 1.95$		θ^{IV} : 150°

a) ref. [14,16,17] b) ref. [27]

$^3J_{C_1'-C_2'}$ and $^3J_{C_1'-C_2''}$ provide useful information about the proline ring puckering. It can be stated that only when $^3J_{C_1'-C_2'} = {}^3J_{C_1'-C_2''}$ is the ring planar.

CONCLUSION

The present work demonstrates that $^{13}C-^{13}C$ coupling constants are well adapted for an approach to peptide conformation, especially when side chains are involved. This applies clearly to aspartic acid and also to proline in the simple compound cyclo (Asp-Pro) but will be of special interest in peptides having more complicated ^1H NMR spectra, notably when suitable relationships for the angular dependence of $^{13}C-^{13}C$ couplings will be established.

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