The Solution Structure of [Ala⁴]-Desdimethylchlamydocin: A ^{1}H N.M.R. Relaxation Study

Paolo Mascagni and William A. Gibbons *
Department of Pharmaceutical Chemistry, School of Pharmacy, University of London, London WC1N 1AX
Daniel H. Rich
School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706, U.S.A.
Neri Niccolai
Dipartmento di Chimica, Universita di Siena, 53100 Siena, Italy

Many fungal peptides exhibit plant toxicity in a host-specific manner. Here we report a proton relaxation and two dimensional (2D) n.m.r. study of the [Ala⁴]-desdimethyl analogue of the fungal tetrapeptide chlamydocin. Interproton distances calculated from n.m.r. parameters agreed substantially with the corresponding distances in the crystalline form of dihydrochlamydocin. A solution conformational analysis was performed based on n.m.r. distance measurements and φ, ψ, and ω angles of the four residues plus χ_i rotamer populations. These data support the use of proton relaxation parameters as a basis for accurate solution conformational analysis. As a corollary, the data can also indicate within experimental error that the crystal and solution conformations of chlamydocin and the [Ala⁴]-desdimethyl analogue, respectively, are identical.

Cyclic tetrapeptides have served as models for peptide conformational studies by theoretical,[1–4] crystallographic,[5,6] and spectroscopic[7–10] techniques. They exhibit unusual conformations and conformational interconversions and the biological activities[11] of several representatives including tentoxins, chlamydocins, AM-toxins, and HC-toxins have been elucidated; in some cases, the functions have been rationalized in conformational terms.[11] The majority of the n.m.r. studies of tetrapeptides have involved principally one dimensional methods and conformations were derived from chemical shifts, dihedral scalar coupling constants, and/or hydrogen bonding patterns. Here we report a continuation of the studies of the chlamydocins[7] in which we apply two dimensional (2D) methods and proton relaxation parameters to determine the configuration of [Ala⁴]-desdimethylchlamydocin, a cyclic tetrapeptide of structure (Gly¹-Phe²-Pro³-Ala⁴).[12]

Results and Discussion

(i) ^{1}H *N.M.R. Assignments and* ^{2}J *and* ^{3}J *Coupling Constants.*—A more complete set of assignments and scalar coupling constants of the ^{1}H n.m.r. spectrum (Figure 1) of the title compound[7] was obtained by 1D difference double resonance[13] at 270 and 600 MHz and proton 2D *J*-resolved spectroscopy.[14] ^{1}H Chemical shifts and coupling constants are shown in Table 1. $^3J_\varphi$ Coupling constants were used to derive r_φ distances from the appropriate Karplus relationship.[15] The large values of 10.0 and 10.6 Hz for the Phe² and Ala⁴ amide protons are consistent with r_φ interproton distances of 3.0 ± 0.2 Å.[16]

(ii) *Interproton Distances from N.O.E.s and Cross-relaxation Rates.*—The observed n.O.e.s are related to the relaxation parameters σ and *R* by equation (1):

$$\mathrm{NOE}_i(j) = \frac{\sigma_{ij}}{R_i} - \sum_{(k \neq i)} \frac{\sigma_{ik}}{R_i} \cdot \frac{(I_{zk} - I_{ok})}{I_{oi}} \qquad (1)$$

where ($R_i = 1/T_i^{SE}$) is the monoselective spin lattice relaxation rate and T_i^{SE} the corresponding relaxation time. The second term on the right of equation (1), the cross-polarization term, accounts for the indirect contributions to an observed n.O.e. that result from the partial saturation of protons other than *i* and *j*, or by cross relaxation *via* another nucleus. In our case the effect of other protons was within experimental error and not taken into account. Assuming that the n.O.e. relaxation

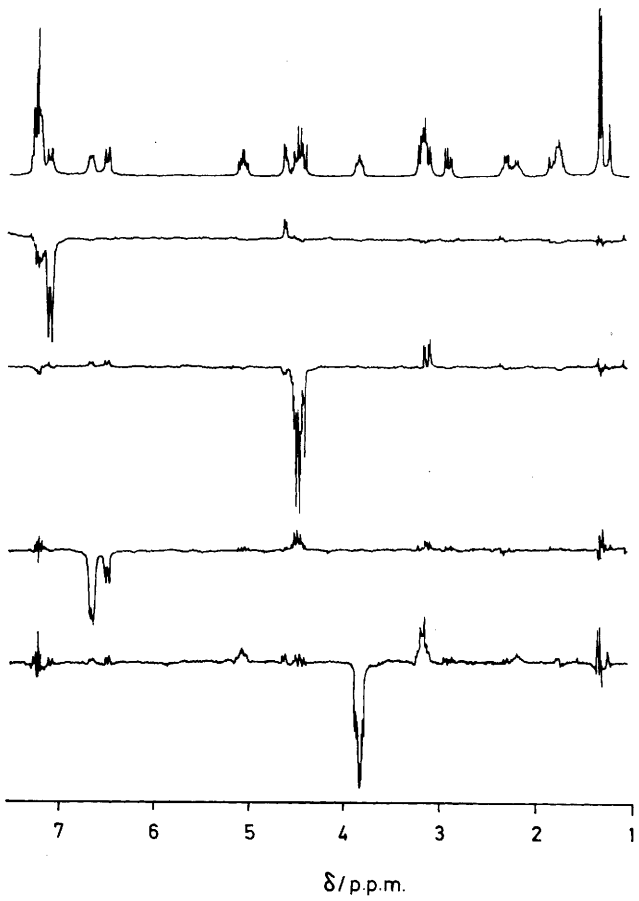

Figure 1. In the top trace the off-resonance 270 MHz ^{1}H n.m.r. spectrum of [Ala⁴]-desdimethylchlamydocin in $CDCl_3$ is shown; the lower traces refer to difference spectra. Negative peaks result from the irradiated proton; positive peaks are due to the homonuclear Overhauser enhancements (n.O.e.) discussed in the text.

Table 1. Scalar coupling constants (J/Hz) for [Ala^4]-desdimethylchlamydocin[a]

Gly^1		Phe^2		Pro^3		Ala^4	
α^u-α^d	−13.8	NH-α	10.9[b]	α-β^d	2.2	NH-α	11.5[b]
NH-α^d	3.3	α-β^d	9.4	α-β^u	7.9	α-β	6.9
NH-α^u	9.9	α-β^u	6.0	β^d-β^u	12.9		
		β^u-β^u	−13.2	β^d-γ^d	1.5		
				β^d-γ^u	8.2		
				β^u-γ^d	8.2		
				β^u-γ^u	9.0		
				γ^d-γ^u	−12.0		
				γ^d-δ^d	10.0		
				γ^d-δ^u	9.0		
				γ^u-δ^d	4.5		
				γ^u-δ^u	9.0		
				δ^d-δ^u	−8.5		

[a] Superscript u = Upfield signal; d = downfield signal. [b] Corrected $J_{\alpha N}$ coupling constants.[15]

mechanisms are dipolar (IDD),[17] in the extreme narrowing conditions, $(\omega_o\tau_c)^2 \ll 1$, then:

$$\sigma_{ij} = (\text{const.})(1/r_{ij}^6)(\tau_c^{ij}) \quad (2)$$

Under these conditions, the theory[18] also yields the following:

$$F = R_i(\text{NS})/R_i(\text{SE}) = 1.5 \quad (3)$$

where R_i(NS) is the non-selective spin lattice relaxation rate. The experimental non-selective and mono-selective relaxation rates for the investigated compound are listed in Table 2. Measured F^i ratios of CH and CH_2 protons range from 1.4 to 1.4_5, indicating that paramagnetic impurities and/or relaxation mechanisms other than the IDD do not contribute significantly to the proton relaxation pathway of this peptide. It can also be stated that the efficient dipole–dipole interactions are modulated by correlation times which satisfy the extreme narrowing conditions $\omega_o^2\tau_c^2 \ll 1$. The Phe^2 and Gly^2 amide protons exhibit lower F^i ratios, suggesting that the ^{14}N–^{1}H dipolar interaction yields a significant relaxation contribution, as previously described.[19]

(*a*) *Distances from σ values.*[20] In order to calculate interproton distances from equation (2), the cross relaxation parameters (σ_{ij}) and correlation times τ_c must be known. The use of the selective excitation method[18,20,21] allowed the determination of the mono-selective relaxation rates (R_i^{SE}) which in turn could be used to derive σ values from equation (1). The σ values thus obtained are listed in Table 3. The constant geminal H_α–H_α interproton distance of 1.8 Å in the Gly residue and the appropriate σ value were then used to evaluate τ_c from the simplified equation (2). The value $\tau_{Gly\alpha-\alpha} = 4.3 \times 10^{-11}$ s was found. When the correlation time for the Phe^2-H_β and Pro^3-H_δ geminal protons was calculated, τ_c values of 3.7×10^{-11} and 4.5×10^{-11} s, respectively, were obtained. This observation strengthens the conclusion that an average τ_c of 4.4×10^{-11} s modulates all backbone proton-proton relaxation. The slightly faster τ_c calculated for the Phe^2-H_β geminal interaction may arise from χ_i internal motion. This value of τ_c and the corresponding σ values then permitted the calculation of the interproton distances shown in Table 4.

(*b*) *Distances from the N.O.E. Ratio Method.*[16,20] Since σ_{ij} and R_i are modulated by the same correlation time, in the ratio of any two σ values all the terms except for the $1/r^6$ terms in equation (2) cancel; in cases where irradiation of two different protons induce an n.O.e. at the same proton, the following applies:

$$\frac{\text{NOE}_i(j)}{\text{NOE}_i(l)} = \frac{r_{il}^6}{r_{ij}^6} \quad (4)$$

If one distance is known the other can then be calculated. Using this approach several interproton distances were derived and compared in Table 4 with those obtained from σ values and from crystallographic data of dihydrochlamydocin; the conformation of the latter was found to be identical with that of the title compound.[5] All the distances calculated from the n.O.e. ratio method and cross-relaxation rates agree with each other (±0.1 Å) and with the crystal structure distances, proving that proton relaxation parameters can be used to determine the conformation of the peptide. The interproton distances involving both Gly^1-H_α protons could not be compared with crystallographic data because in dihydrochlamydocin the Gly residue is replaced by the Aib residue. An approximate estimate of the two r_φ distances was made from the non-equivalent first-order coupled glycyl geminal protons. Thus the larger $^3J_\varphi$ coupling constant (9.9 Hz) between the Gly^1-NH and the upfield H_α protons and the smaller value for the other $^3J_\varphi$ coupling constant (3.3 Hz) establish the vicinal angle φ as being *ca.* 75°,[15] consistent with the measured values r_α^u (Gly) ≃ 3.0 Å and r_α^d (Gly) ≃ 2.3 Å. (Superscripts u and d refer to the upfield and downfield protons.) Furthermore, as observed in a previous paper[7] on the hydrogen bonding of the title compound, the Gly^1 downfield alpha proton is eclipsed by the carbonyl moiety of the Ala^4 residue, whereas the Phe^2-NH proton is hydrogen bonded to the same carbonyl. From this observation it follows that Gly^1-H^d and the Phe^2-NH are eclipsed whereas Gly^1-H^u is *trans* to Phe^2-NH, consistent with the fact that r(Phe^{2}NH-Gly^{1}H^d) is smaller than r(Phe^{2}NH-Gly^{1}H^u).

(iii) *Dihedral Angles* φ, ψ, *and* ω.—φ *Angles.* The φ angles of Gly^1, Phe^2, and Ala^4 residues were derived from suitable Karplus relationships.[15] Values of −109° (−120° when using $^3J_{corr}$) and −102° (−120° when using $^3J_{corr}$) were found for φ of Ala^4 and Phe^2 respectively. The two $^3J_\varphi$ coupling constants (9.9 and 3.3 Hz) for the Gly^1 residue yielded a torsion angle of 75°.

ψ *Angles.* The knowledge of r_ψ(Ala^4) allowed the determination of the corresponding ψ torsion angle;[22] the r_ψ(Ala^4) value of 2.2 Å corresponds to a ψ(Ala^4) angle of 100 ± 20° which is consistent with the crystallographic value ψ(AEO) = 104°. The angle ψ(Pro^3) was assumed to be −60° by virtue of the unusual $^{13}C_\beta$ high-field resonance.[7,23]. Finally the correlation between $^2J_{\alpha\alpha}$ and the ψ angles for Gly residues[15] yielded two possible ψ(Gly^1) values of −55 and −130°.

ω *Angles.* Space filling models were built using the distances

Table 2.

H_i	Entry	δ[a]	R_i(NS)[b]	R_i(SE)[c]	F^i[d]	$(I_z - I_0)/I_0$[f]														
						1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Phe Arom	1	7.2[b]	0.4_6	(0.3_2)	(0.4_2)	−100				7.1						6.7		6.7				
Ala NH	2	7.11	0.9_9	(0.8_3)	(1.1_9)		−100				13.2											
Gly NH	3	6.74	1.2_0	1.0_0	1.2_0			−100				18					6.7					
Phe NH	4	6.54	1.0_9	0.8_7	1.1_8				−100				7.0									
Phe H_α	5	5.09	1.1_3	0.8_0	1.4_0	8.0				−100				5.3		3.3		4.0				
Pro H_α	6	4.64	0.8_8	0.6_2	1.4_2		11.4				−100											7.3
Ala H_α	7	4.51	1.0_1	0.7_1	1.4_2			9.1				−100										
Gly H_α^d	8	4.46	2.0_7	1.4_6	1.4_2				4.0				−100				32.2					
Pro H_δ^d	9	3.86	2.1_2	1.4_7	1.4_4					11.4				−100	34.0					8.2	2.2	
Pro H_δ^u	10	3.21	*e*	*e*						7.0				28.6	−100					3.5	11.0	
Phe H_β^d	11	3.20	1.9_5	1.3_4	1.4_5					4.0						−100		26.3				
Gly H_α^u	12	3.14	1.7_5	1.2_3	1.4_2			8.2					26.8				−100					
Phe H_β^u	13	2.93	1.8_9	1.3_0	1.4_5				3.0							23.3		−100				
Pro H_β^d	14	2.33	1.7_1	(1.2_0)	(1.4_2)														−100			22.6
Pro H_γ^d	15	2.23	1.5_5	(1.0_9)	(1.4_2)															−100	17.6	
Pro H_γ^u	16	1.79	1.4_8	(1.0_4)	(1.4_2)										6.8					19.1	−100	
Pro H_β^u	17	1.77	1.9_1	(1.3_4)	(1.4_2)						5.0								24.0			−100
Ala H_β	18	1.34	1.7_4		(1.4_2)																	

[a] In p.p.m. from internal Me_4Si. [b] Non-selective spin-lattice relaxation rates (s^{-1}). [c] Selective spin-lattice relaxation rates (s^{-1}). Values in parentheses were estimated from R(NS) and an assumed F^i. [d] $F^i = R(NS)/R(SE)$. Values in parentheses were assumed from an average of F^i values calculated for CH and NH protons. [e] Relaxation rates not determined owing to strong spectral overlapping. [f] Proton–proton Overhauser enhancements (%).

Table 3. Cross-relaxation rates ms^{-1} [a]

σ_{1-5}	Phe (Ar)/Phe H_α	57[b]	σ_{8-12}	Gly H_α^d/Gly H_α^u	396
σ_{1-11}	Phe (Ar)/Phe H_β^d	90[b]	σ_{9-5}	Pro H_δ^d/Phe H_α	91
σ_{1-13}	Phe (Ar)/Phe H_β^u	87[b]	σ_{10-5}	Pro H_δ^u/Phe H_α	56
σ_{2-6}	Ala NH/Pro H_α	82	σ_{10-9}	Pro H_δ^u/Pro H_δ^d	420
σ_{3-7}	Gly NH/Ala H_α	128	σ_{11-5}	Phe H_β^d/Phe H_α	32
σ_{3-12}	Gly NH/Gly H_α^u	82	σ_{11-13}	Phe H_β^d/Phe H_β^u	341
σ_{4-8}	Phe NH/Gly H_α^d	102	σ_{12-8}	Gly H_α^u/Gly H_α^d	391
σ_{5-9}	Phe H_α/Pro H_δ^d	78	σ_{13-11}	Phe H_β^u/Phe H_β^d	312
σ_{5-11}	Phe H_α/Phe H_β^d	45	σ_{17-6}	Pro H_β^u/Pro H_α	44[b]
σ_{5-13}	Phe H_α/Phe H_β^u	52			

[a] Average cross-relaxation rates calculated from the experimentally determined n.O.e.s and R^i(SE) values. [b] Average cross-relaxation rates calculated from n.O.e.s and experimental and estimated R^i(SE) values.

and the φ and ψ angles measured above. A single conformation possessing four transoid ω angles was thus obtained. This agrees with theoretical predictions.[24,25] These results were then confirmed by computer modelling. A linear peptide with the same sequence as the title compound was used. The set of φ, ψ, and ω angles without the Gly^1 ω angle was incorporated in the model; a distance between the carbonyl carbon of Gly^1 and the Phe^2 nitrogen that is consistent with a single C–H covalent bond was found indicating that ring closure is achievable using our experimentally derived parameters.

(iv) *Side Chain Structure and Rotamer Populations.*—The classical analysis for the C_α–C_β bond rotation of Phe^2 yielded p_{+60}, p_{-60}, and p_{+180} rotamer populations of 0.0_7, 0.6_2, and 0.3_1 respectively; these are consistent with the averaging of $^3J_{\alpha\beta}$ due to internal motion. The p_{-60} and p_{+180} rotamers predominate. It is worth noting that interchange between these two preferred conformations can reduce the reorientational lifetime of the H_{β_1}–H_{β_2} vector and, as observed in section (ii), a correlation time faster than the molecular one must be expected. The rotamer populations obtained for the Phe^2 residue were then used to determine the averaged Phe (α–β) distances from the relaxation data as follows.[20] Based on the reasonable assumption that the times for the $Phe^2(\alpha\beta_1)$ and $Phe^2(\alpha\beta_2)$ vectors are the same, the known rotamer populations and the interproton *gauche–gauche* (2.49 Å) and *trans–gauche* (3.07 Å)[26] distances in the p_{60}, p_{-60}, and p_{180} rotamers lead to equations (5) and (6):

$$r_{\alpha\beta_2}^{-6} = p_{60}\left(\frac{1}{2.4_9}\right)^6 + p_{180}\left(\frac{1}{3.0_7}\right)^6 + p_{-60}\left(\frac{1}{2.4_9}\right)^6 \quad (5)$$

$$r_{\alpha\beta_1}^{-6} = p_{60}\left(\frac{1}{3.0_7}\right)^6 + p_{180}\left(\frac{1}{1.24_9}\right)^6 + p_{-60}\left(\frac{1}{2.4_9}\right)^6 \quad (6)$$

The two Phe α–β interproton distances of 2.7_4 and 2.5_1 Å thus obtained are in satisfactory agreement with those derived from cross-relaxation rates (Table 4). The $^3J_\chi$ coupling constants were used to determine the non-classical χ_i rotation in the prolyl residue. χ Values were derived graphically using the appropriate Karplus relationships[27] and discrimination among the several values thus obtained was based on energetically allowed conformations of proline.[28,29] The analysis gave values of $\chi_1 = -20°$, $\chi_2 = 27°$, and $\chi_3 = -20°$ in good agreement with the crystallographic data of dihydrochlamydocin (−30, 28, and −11.5°).

(v) *Backbone Conformation.*—The r_φ and r_ψ distances and the torsional angles listed in Table 4 and Table 5 were used for molecular modelling. The conformation thus obtained is consistent with that proposed from preliminary studies of the title compound and with the crystal structure of dihydrochlamydocin:[11] a γ turn between the Ala^4-NH and the Phe^2 carbonyl group and a second γ turn between the Phe^2-NH and the Ala^4 carbonyl moiety stabilize the all-transoid conformation of [Ala^4]-desdimethylchlamydocin (Figure 2).

Conclusions

Crystallographic internuclear distance measurement is routinely used to determine the conformation of natural and biological macromolecules, and although in recent years crystallographic data has been re-examined to determine the role played by internal motions, these have not basically influenced the crystallographic conformational analysis. The measurement of internuclear distances by n.m.r. spectroscopy in solution is more recent; however, it can be difficult to interpret the data,

Table 4. Comparison of the interproton distances of [Ala^4]-desdimethylchlamydocin calculated by different methods

	N.O.e. ratio method	σ parameters	Crystallographic determination
H(8)–H(12) (Gly H_α^d/Gly H_α^u)	1.8[a]		
H(8)–H(3) (Gly H_α^d/Gly NH)	*ca.* 3[b]		
H(12)–H(3) (Gly H_α^u/Gly NH)	2.3_0	2.3_0	
H(8)–H(4) (Gly H_α^u/Phe NH)	2.2_1	2.2_1	
H(12)–H(4) (Gly H_α^u/Phe NH)	*ca.* 3[c]		
H(3)–H(7) (Gly NH/Ala H_α)	2.2_6	2.1_5	2.3_0
H(4)–H(13) (Phe NH/Phe H_β^u)	2.3_2		2.6_6
H(4)–H(5) (Phe NH/Phe H_α)	*ca.* 3		2.8_0
H(5)–H(13) (Phe H_α/Phe H_β^d)	2.4_2	(2.5_1)[c] 2.4_8	2.4_9[d]
H(5)–H(11) (Phe H_α/Phe H_β^d)	*ca.* 3	(2.7_4)[c] 2.7_0	2.9_0[d]
H(5)–H(9) (Phe H_α/Pro H_δ^d)	2.3_4	2.3_2	2.3_4
H(5)–H(10) (Phe H_α/Pro H_δ^u)		2.4_5	2.5_0
H(11)–H(13) (Phe H_β^d/Phe H_β^u)	1.8[a]		
H(9)–H(10) (Pro H_δ^d/Pro H_δ^u)	1.8[a]		
H(6)–H(2) (Pro H_α/Ala NH)	2.2_5	2.3_0	2.2_8
H(6)–H(17) (Pro H_α/Pro H_β^u)		2.5_5[e]	2.5_3
H(2)–H(7) (Ala NH/Ala H_α)	*ca.* 3[b]		2.8_0

[a] Constant geminal interproton distance from standard bond angles and standard bond lengths.[26] [b] N.O.e.s of *ca.* 2% were observed between the protons in question, consistent with interproton distances of ≥3 Å.[22] [c] Averaging around the Phe^2(α-β) bond was considered in calculating these distances (see text). [d] From the crystal structure, $Phe^2(\chi_1) = -60°$, and standard bond angles and bond lengths.[26] [e] Interproton distance calculated on the basis also of an assumed R_i(SE).

Table 5. Torsional angles and rotamer populations for [Ala⁴]-desdimethylchlamydocin[a-c]

	Gly¹	Phe²	Pro³	Ala⁴
φ	75	−102	(83.0)	−109
	(71.8)	(−105.5)		(−105.5)
ψ	−55		−60	100
	(−63.7)	(94.4)	(−72.8)	(104.7)
ω	transoid	transoid	transoid	transoid
	(162)	(−165.7)	(−156.5)	(−163.7)
		$P_{180}^{\alpha\beta}$ 0.3_1	−20	
			(−30)	
χ_i		$P_{-60}^{\alpha\beta}$ 0.6_2	27	
			(26.2)	
		$P_{60}^{\alpha\beta}$ 0.0_7	−20	
			(−11.4)	

[a] Values in parentheses are from the crystal structure of dihydrochlamydocin. [b] All values are ±20°. [c] The nomenclature used here for φ, ψ, and ω is that for the IUPAC.[30] r_φ and r_ψ are simply the φ-dependent and ψ-dependent interproton distances NH_i–$H_{\alpha i}$ and $H_{\alpha i}$–NH_{i+1} (see ref. 16a).

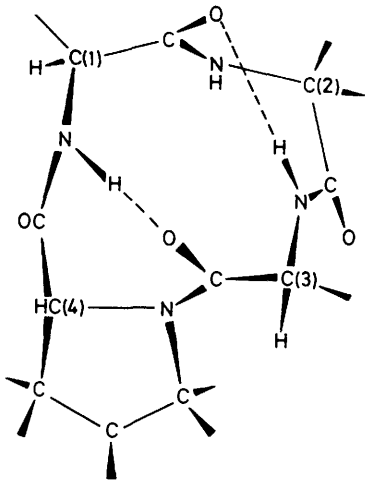

Figure 2. The spatial structure proposed for [Ala⁴]-desdimethylchlamydocin in chloroform solution

since the varieties, modes, and amplitude of motions that affect the measurements are greater. Nevertheless progress has been made and good agreement between n.m.r. and crystallographic distance measurements has been achieved in some cases.

We have described here a conformational analysis of a derivative of the host specific fungal toxin, chlamydocin, using n.O.e.s, relaxation rates, and scalar coupling constants. The interproton distances and angles calculated from each of these measurements are self-consistent and agree with the corresponding parameters calculated from the crystal structure of a closely related compound. This gives some confidence in the application of n.m.r. internuclear distance measurements to peptide conformational analysis and to the basic assumptions governing relaxation mechanisms and motions. Eventually our knowledge of the latter will have to be highly refined and improved but this should not influence the basic n.m.r. methodology.

Experimental

Samples were prepared by dissolving [Ala⁴]-desdimethylchlamydocin (3 mg) in 99.98% deuteriochloroform (0.3 ml). 1D n.m.r. techniques were performed on a Bruker WH-270 spectrometer equipped with a Nicolet 1180 computer; 2D *J*-resolved spectra were taken on a Nicolet NT-200 spectrometer equipped with a Nicolet 1280 computer. The spin–spin analysis of the D-prolyl ring was completed with the aid of 600 MHz spectra (Carnegie Mellon Institute, Pittsburgh, Pennsylvania). Reported *J* coupling constants have been refined by computer simulation of 200, 270, and 600 MHz spectra. Proton spin-lattice relaxation rates were measured with the inversion recovery $(180°\text{-}\tau\text{-}90°\text{-}T)_n$ pulse sequence. Selective spin-lattice relaxation rates were determined by generating a 180° selective pulse with the decoupler channel (typical duration of the pulse 20 ms). Semilog plots of $(I_o - I_\tau)/2I_o$ *vs.* τ were used to calculate initial relaxation rates, experimental error of which was estimated to be ±2%. Homonuclear n.O.e.s were generated by presaturating selected proton signals with a low-power 5-s decoupler pulse. Quantification of the observed n.O.e.s was achieved by measuring the integral of the peaks obtained by difference spectra (see Figure 1). All n.m.r. measurements were carried out at 26 ± 1 °C.

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