80949-68-4; **30**, 70913-12-1; **31**, 50397-92-7; **32**, 62937-86-4; **33**, 65086-21-7; **34**, 85282-96-8; **36**, 85282-97-9; **43**, 80949-70-8; **44**, 85282-98-0; **45**, 85282-99-1; **46**, 70913-17-6; **47**, 70913-19-8; **48**, 70913-18-7; **49**, 70913-20-1; **50**, 85283-00-7; *o*-bromovinylbenzene, 2039-88-5; diphenylmethylcyclopropenylium perchlorate, 72612-89-6; benzyne, 462-80-6; **18**, 51310-25-9; **12**, 67177-31-5; 2-methyl-3-phenylindanone, 52957-74-1; *o*-bromoisopropenylbenzene, 7073-70-3; α-carboxy-*o*-toluic acid, 89-51-0; *o*-(2-hydroxyethyl)benzyl alcohol, 6346-00-5; *o*-(2-chloroethyl)benzyl chloride, 78317-75-6; *o*-vinylbenzyl chloride, 22570-84-9.

Conformational Analysis by NMR Spectrometry of the Highly Substituted Cyclic Tetrapeptides, Chlamydocin and Ala⁴-Chlamydocin. Evidence for a Unique Amide Bond Sequence in Dimethyl-d_6 Sulfoxide

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Contribution from the School of Pharmacy, University of Wisconsin—Madison, Madison, Wisconsin 53706. *Received August 26, 1982*

Abstract: The solution conformations of chlamydocin, *cyclo*(Aib-Phe-D-Pro-L-Aoe), and Ala⁴-chlamydocin, *cyclo*[Aib-Phe-D-Pro-L-Ala), have been investigated by ^1H and ^{13}C nuclear magnetic resonance spectroscopy in dimethyl-d_6 sulfoxide. Two conformations of these molecules are present in ratios of approximately 6:4. The conformers interconvert slowly on the NMR time scale, and this slow interconversion is due to a cis–trans isomerization of one amide bond. The 12-membered ring system of conformer I in dimethyl-d_6 sulfoxide is characterized by four transoid amide bonds, with two bis γ-turn intramolecular hydrogen bonds. Conformer II, which is found only in mixed solvents that contain high concentrations of Me_2SO, has a cis,trans,trans,trans amide bond sequence. Low-temperature NOE was utilized to determine amide bond geometry in conformer II. Circular dichroism data in different solvents are recorded. Approximate torsional angles of the minor conformer (conformer II) derived from the NMR data and Dreiding models are Aib ϕ +70°, ψ +75°, ω −160°; Phe ϕ +150°, ψ −105°, ω +20°; D-Pro ϕ +85°, ψ −140°, ω +165°; Ala (Aoe) ϕ −105°, ψ +80°, ω −160°, respectively. The cis,trans,trans,trans ring conformation of a cyclic tetrapeptide has not been described previously.

Chlamydocin, *cyclo*[Aib¹-L-Phe²-D-Pro³-L-(2-amino-8-oxo-9,10-epoxydecanoic acid)] (**1**),[1-3] is a cytostatic agent that appears to have an unusual mechanism of action. At concentrations near 2 nM, chlamydocin inhibits tritiated thymidine incorporation into calf thymus lymphocytes stimulated with phytohemaglutinin,[4] an assay that is sensitive to any agent interfering with rapid normal uptake of DNA precursors by the rapidly growing cells.[5] This strong inhibition parallels chlamydocins's behavior in an in vivo mouse mastocytoma assay (EC_{50} = 0.3 ng/mL).[2] The site of action is not known, but the low effective concentrations in these assay systems necessitate a very specific interaction between this site and chlamydocin, an interaction that requires both the cyclic tetrapeptide ring system and the intact epoxy ketone group.[2,3]

Conformations for the chlamydocin ring system have been determined in the solid state[6] and in nonpolar solvents.[7] The all-transoid[1c] bis γ-turn conformation (Figure 1F) was first identified in dihydrochlamydocin (**2**), the reduced carbonyl analogue of **1**, by Flippen and Karle[6] and subsequently was found for (Gly¹,Ala⁴)-chlamydocin (**3**), *cyclo*(D-Phe-Pro)$_2$ (**4**),[8] Ala⁴-chlamydocin (**5**), and chlamydocin in chloroform solution.[7] However, the all-transoid conformation is readily disrupted by hydrogen-bonding solvents. In view of the importance of the cyclic tetrapeptide ring system to the biological activity of chlamydocin, we have studied its conformation in dimethyl sulfoxide.

We report here the results of NMR studies to determine the conformations of **1** and **5** in dimethyl sulfoxide. Low-temperature nuclear Overhauser effect (NOE) difference spectra[9,10] were used to assign amide bond geometries in one conformation that rapidly interconverts at room temperature. Conformations are proposed for **1**, **5**, and (Gly¹,Ala⁴)-chlamydocin (**3**) in Me_2SO. Evidence is presented for a previously unobserved cyclic tetrapeptide conformation with three trans and one cis amide bond in the 12-membered ring system.

Experimental Section

Detailed descriptions of experimental methods used for solvent titrations, for concentration and temperature dependency measurements of amide proton chemical shifts, and for Tempo titration measurements were reported in earlier papers.[7,8] Dimethyl-d_6 sulfoxide and 2,2,6,6-tetramethyl-1-piperdinyloxy free radical (Tempo) were obtained from Aldrich Co., Inc. For NOE experiments in Me_2SO-d_6 the spectra were obtained at 50 °C in order to reduce the correlation times and to increase NOE enhancements. In some cases, spectra were obtained in mixed solvent pairs of Me_2SO-d_6/chloroform-*d* at lower temperatures to suppress conformational interconversions and saturation transfer. Samples of **1** and **5**, stored in Me_2SO for months, were recovered by evaporation of solvent in vacuo at room temperature and reanalyzed by TLC and NMR.

cyclo[Aib-L-Phe-D-Pro-L-[3,3,3-$^2H_3$]Ala] (**6**) was synthesized from 3,3,3-trideuterioalanine by using the methods reported for the synthesis of the protio compound.[11]

(1) (a) Abstracted in part from: Jasensky, R. D., Ph.D. Thesis, University of Wisconsin, Madison, WI, 1979. (b) Abbreviations used follow IUPAC-IUB tentative rules as described in: *J. Biol. Chem.* **1972**, *247*, 977. Additional abbreviations used: Aib, α-aminoisobutyric acid; Aoe, 2-amino-8-oxo-9,10-epoxydecanoic acid. Superscripts to amino acids in a peptide chain designate the point of substitution relative to the parent compound. (c) Transoid amide bonds are defined as amide bonds with the torsion angle ω deviating from 0° or 180°.

(2) Stahelin, H.; Trippmacher, A. *Eur. J. Cancer*, **1974**, *10*, 801-808.

(3) Closs, A.; Huguenin, R. *Helv. Chim. Acta* **1974**, *57*, 533-545.

(4) Rich, D. H.; Jasensky, R. D.; Mueller, G. C.; Anderson, K. E. *J. Med. Chem.* **1981**, *24*, 567-572.

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(6) Flippen, J.; Karle, I. *Biopolymers* **1976**, *15*, 1081-1092.

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(9) Noggle, J. H.; Schirmer, R. E. "The Nuclear Overhauser Effect"; Academic Press: New York, 1971.

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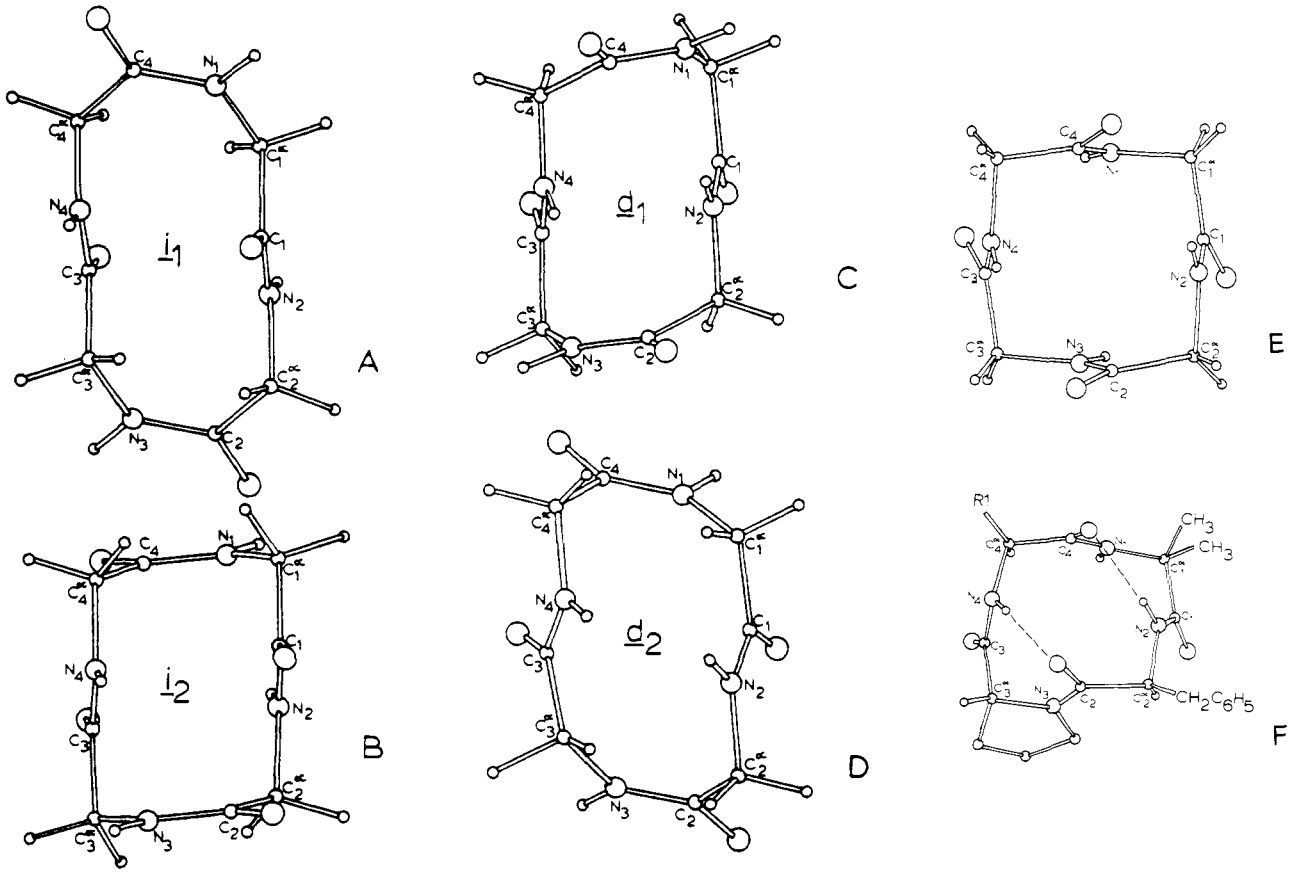

Figure 1. Conformations of cyclic tetrapepties: (A) i_1 symmetry; (C) d_1 symmetry; (E) S_4, all-transoid amide bond symmetry; (F) bis γ-burn, all-transoid amide bond conformation. Chlamydocin (**1**) is R = $-(CH_2)_5COCHCH_2O$; dihydrochlamydocin (**2**) is R = $-(CH_2)_5CH(OH)CHCH_2O$.

Table I. 270-MHz Proton NMR Data for Conformers I and II of Chlamydocin in Dimethyl-d_6 Sulfoxide[a]

residue	δ	^3J, Hz	$\Delta\delta/\Delta T$[b]	Δ solv[c]
		Conformer I		
Phe NH	7.72 (d)	10.0	−2.33	+0.18
α-CH	4.97 (ddd)	10.0, 7.7, 6.0		−0.19
Aib NH	8.05 (s)		−6.33	+1.98
Aoe NH	6.95 (d)	10.3	0	−0.15
α-CH	4.18–4.32 (m)			+0.33
Pro α-CH	4.73 (d)	7.1		+0.06
		Conformer II		
Phe NH	6.88 (d)	6.0	−3.00	
α-CH	4.18–4.32 (m)			
Aib NH	8.01 (s)		−9.33	
Aoe NH	8.56 (d)	7.8	−4.00	
α-CH	4.18–4.32 (m)			
Pro α-CH	4.85 (br dd)	8.0, 1.8		

[a] Concentration 26.7 mg/0.35 mL, 25 °C (δ from Me_4Si) in Me_2SO-d_6. [b] $\times 10^{-3}$ ppm/deg. Temperature range studied: 26.4 to 56.5 °C for *cyclo*(Aib-Phe-D-Pro-Ala) (17.2 mg/0.32 mL); 25 to 55 °C for chlamydocin (21.5 mg/0.35 mL). [c] $\delta_{CHCl_3\text{-}d} - \delta_{Me_2SO\text{-}d_6}$.

Results

^{1}H NMR of Chlamydocin in Me_2SO-d_6. The 270-MHz proton NMR spectrum is shown in Figure 2. The spectrum shows two sets of resonances of unequal intensity for each set of protons. The major conformer is designated conformer I and the minor conformer, conformer II. When conventional decoupling methods were carried out to assign proton resonances, saturation transfer was observed due to interconversion between conformers. For example, upon irradiation of the peak at 4.53 ppm (assigned to one Phe C$^\alpha$H), partial saturation of the PheC$^\alpha$H was observed in the other conformer. The peak intensity at 4.93 ppm (which is assigned to Phe C$^\alpha$H in conformer II) decreases but is not completely canceled. Although the proton coupled to the Phe[1]

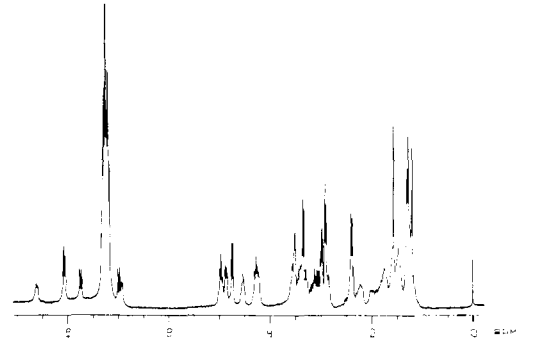

Figure 2. 270-MHz proton NMR spectrum of chlamydocin in Me_2SO-d_6 at 25 °C. Peptide concentration 7.3 mg/0.35 mL.

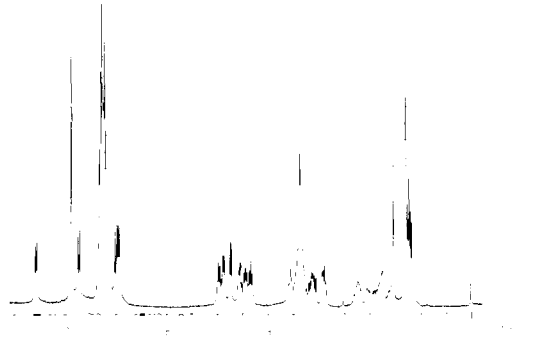

Figure 3. 270-MHz proton NMR spectrum of *cyclo*(Aib-Phe-D-Pro-Ala) in Me_2SO-d_6 at 25 °C. Peptide concentration 7.4 mg/0.31 mL.

(11) Pastuszak, J.; Gardner, J. H.; Singh, J.; Rich, D. H. *J. Org. Chem.* **1982**, *47*, 2982–2987.

Table II. 270-MHz Proton NMR Data for Conformers I and II of *cyclo*(Aib-Phe-D-Pro-Ala)[a] in Dimethyl-d_6 Sulfoxide

residue	δ	^3J, Hz	$\Delta\delta/\Delta T$[b]	Δ solv[c]
		Conformer I		
Phe NH	7.69 (d)	10.3	−1.99	−0.15
α-CH	4.94 (ddd)	10.3, 7.5, 6.5		−0.34
Aib NH	7.85 (s)		−5.96	+1.74
Ala NH	6.97 (d)	10.3	0	−0.28
α-CH	4.42 (m)		−0.99	−0.05
Pro α-CH	4.72 (d)	7.2		−0.02
		Conformer II		
Phe NH	6.92 (d)	6.8	−2.65	
α-CH	4.53 (m)			
Aib NH	7.85 (s)		−5.30	
Ala NH	8.54 (d)	7.8	−4.30	
α-CH	4.32 (m)			
Pro α-CH	4.85 (br dd)	7.8, 1.8		

[a] 10.4 mg/0.35 mL, 25 °C (δ from Me_4Si) in Me_2SO-d_6. [b,c] Defined in Table I.

$C^\alpha H$ at 4.53 ppm is cleanly decoupled, the proton coupled to the Phe[11] $C^\alpha H$ at 4.93 ppm is not decoupled, indicating incomplete saturation of the nonirradiated conformer. Thus double resonance experiments were carried out, permitting the assignment of all peaks (Table I).

^1H NMR of Ala[4]-Chlamydocin in Me_2SO-d_6. Figure 3 shows the 270-MHz proton NMR spectrum of Ala[4]-chlamydocin in Me_2SO-d_6. The complexity of this spectrum is very similar to that of chlamydocin in Me_2SO-d_6 except for the absence of the Aoe resonances (Figure 2). The two sets of protons again reveal two conformers. Careful comparison of both spectra suggests two small differences between the compounds: (1) Both Aib NHs for Ala[4]-chlamydocin appear at 7.85 ppm. In chlamydocin these are separated (8.01 ppm and 8.05 ppm). (2) The Ala $C^\alpha H$ resonances in Ala[4]-chlamydocin for both conformers are separated (4.32 and 4.42 ppm) but overlap (at 4.18–4.32 ppm) in chlamydocin. These differences could indicate minor conformational differences between the two molecules.

The temperature dependence of each amide resonance was measured (Tables I and II). The data establish that all amide protons in conformer II are solvent exposed. Amide protons in conformer II not only shift upfield but also coalesce much faster than the amide protons in conformer I.

Free Radical Titrations with Tempo. Free radical titration of amide protons is a very useful tool for detecting hydrogen bonding of amide protons especially in cases where temperature changes could alter conformational equilibria.[12] Figure 4 shows the line broadening of the peptide amide protons produced by adding small amounts of 2,2,6,6-tetramethyl-1-piperidinyloxy free radical to the solution (highest concentration at top of figure). The data clearly suggest that the Phe NH (7.72 ppm) and Aoe NH (6.95 ppm) in conformer I are shielded from the solvent or from intermolecular interactions because these resonances are less broadened than the corresponding resonances in the other conformer. In contrast, the peaks at 8.56 and 6.88 ppm, which are assigned to Aoe NH and Phe NH in conformer II, respectively, are exposed to the solvent. The effect of line broadening on either Aib NH is not clear, and selective broadening is not obvious. It is possible that the bulky geminal methyl groups of Aib may prevent close approach of the spin label. Tempo titration of Ala[4]-chlamydocin (not shown) gave identical results.

The data from the temperature dependence studies are consistent with the results of the Tempo titrations. The Phe NH and the Ala NH in conformer I appear to be shielded from solvent, while the Aib NH is exposed to solvent. On the other hand, it is clear that all three amide protons in conformer II are exposed to solvent. These results suggest that in conformer I the Phe NH and the Ala NH form intramolecular hydrogen bonds, but the Aib NH does not. In contrast, in conformer II there are no intramolecular hydrogen bonds.

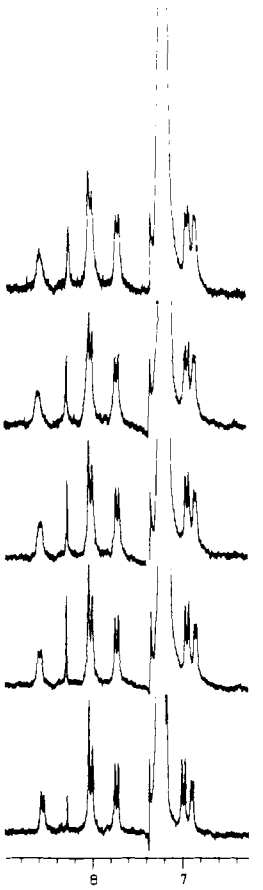

Figure 4. 2,2,6,6-Tetramethyl-1-piperdinyloxy free radical titration of chlamydocin in Me_2SO-d_6. Initial peptide concentration 25.5 mg/0.32 mL of Me_2SO-d_6. From bottom to top: 0, 5, 8, 15, and 25 μL of 5% Tempo in Me_2SO-d_6 solution were added. The peak at 8.3 ppm is from traces of chloroform.

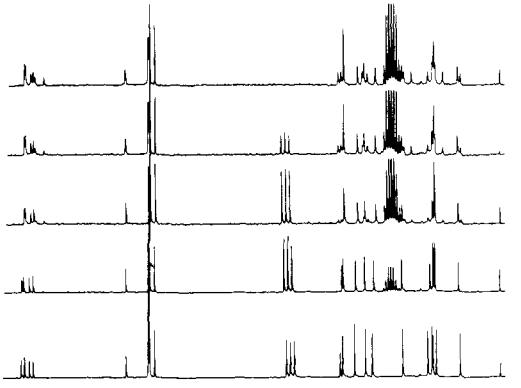

Figure 5. Carbon-13 NMR spectra of *cyclo*(Aib-Phe-D-Pro-Ala) in Me_2SO–chloroform mixtures. Concentration range 42.2–70.0 mg/mL. From top to bottom [given as % Me_2SO-d_6 in $CDCl_3$ (v/v)]: 100%, 80%, 60%, 20% and 0%.

Carbon-13 NMR Spectra of Ala[4]-Chlamydocin. ^{13}C NMR spectra of Ala[4]-chlamydocin in variable mixtures of Me_2SO in chloroform are shown in Figure 5. As described previously,[7] Ala[4]-chlamydocin has one set of resonances in pure chloroform, indicating a single conformation with four transoid amide bonds and two γ-turns. The β- and γ-carbons of proline resonate at 25.08 and 24.76 ppm in chloroform-*d*, indicating that the Phe-Pro bond must be trans and that D-proline is part of an inverse γ-turn with ψ (D-Pro) = 60°.[13,14]

(12) Kopple, K. D.; Go, A. *J. Am. Chem. Soc.* **1977**, *99*, 7968 and references therein.

(13) Siemion, I.; Wieland, T.; Pook, K. *Angew. Chem., Int. Ed. Engl.* **1975**, *14*, 702–703.

Table III. Carbon-13 NMR Chemical Shifts of Chlamydocin and Ala[4]-Chlamydocin in Dimethyl-d_6 Sulfoxide

	chlamydocin		Ala[4]-chlamydocin	
carbon	conformer I	conformer II	conformer I	conformer II
Phe α	52.15		52.17	
β	35.92	35.00	35.92	35.16
Pro α	57.26	58.35	57.21	58.29
β	24.11	32.56	24.11	32.40
γ	23.84	21.02	23.73	20.91
δ	45.67	48.81	45.61	48.65
Aib α	57.26	(59.34)	57.21	(59.32)
β	24.92		24.76	
β'	24.11		24.38	
Ala α			49.84	50.44
β			15.49	14.46
Aoe α	55.04	54.50		
S[a]	36.19	24.92		
	29.25	22.27		
	27.95			
E[b]	52.77 (α)			
	45.67 (β)			
Epoxy C=O	207.27			
C=O	174.93	171.57	174.44	171.35
	174.39	170.16	174.01	170.65
	174.23	167.18	172.17	167.35
	172.22			

[a] Side chain of Aoe group. [b] Epoxy moiety of Aoe side chain.

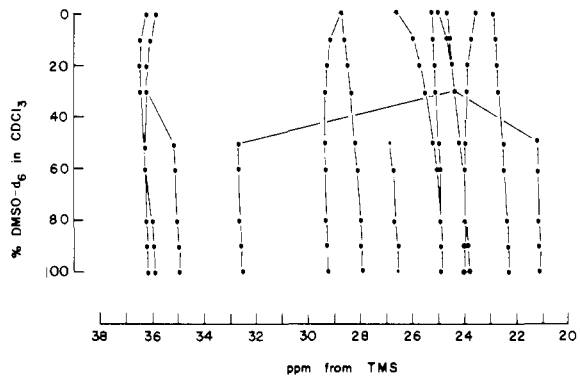

Figure 6. Effect of solvent composition on partial ^{13}C NMR spectrum of chlamydocin. Peptide concentration 26.72 mg/0.32 mL; 35900 scans.

However, as Me_2SO-d_6 is added to the chloroform solution, additional resonances appear in the ^{13}C NMR and ^1H NMR spectra, and the emerging resonances at 32.40 and 20.91 ppm in 60% Me_2SO-d_6 establish the presence of a cis Phe-Pro amide bond in conformer II. The intensity of these *cis*-Pro signals increases in neat Me_2SO. The doubling of the proton resonances in both the ^1H NMR spectrum (Figure 2) and the ^{13}C NMR spectrum (Figure 5 and Table III) establishes the presence of two decoalesced conformations. Recovery of Ala[4]-chlamydocin from Me_2SO established these are conformational interconversions and not epimerizations.

Carbon-13 NMR Spectra of Chlamydocin in Mixed Solvents. The carbon NMR spectrum of chlamydocin taken in Me_2SO-d_6 (Figure 6) is consistent with multiple ring conformations revealing that the conformation of chlamydocin also is extremely dependent on the nature of the solvent. Careful solvent titration was utilized to assign the two sets of carbons. As was found in the case of Ala[4]-chlamydocin, the emerging peaks at 32.56 and 21.02 ppm correspond to the β- and γ-carbons of the Pro residue, establishing that the Phe-Pro amide bond is cis in one conformer. The additional peaks at 24.11 and 23.84 ppm establish the existence of a trans Phe-Pro amide bond in conformer I. The ratio of conformers, based on the peak intensity, is approximately 6:4, as was

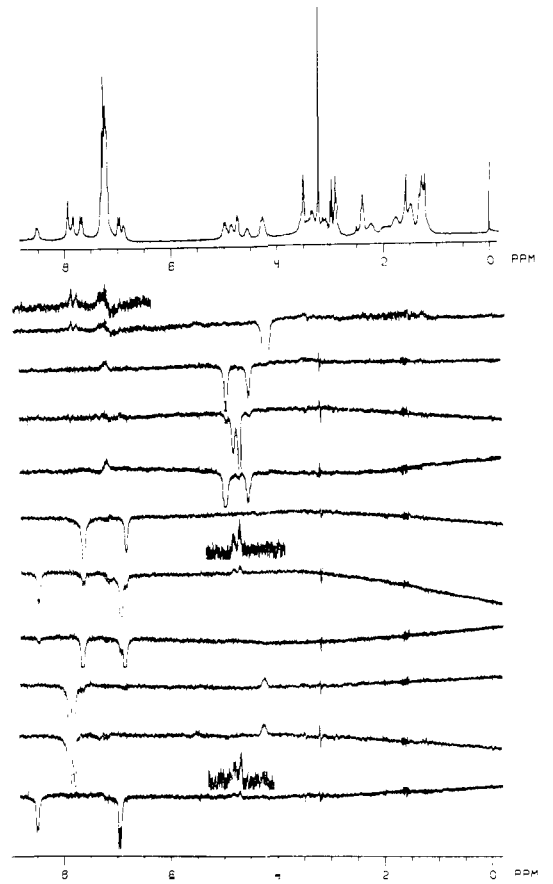

Figure 7. NOE difference spectra of Ala[4]-chlamydocin in Me_2SO-d_6 at 50 °C.

also found in the ^1H NMR spectra.

NOE Effects in Ala[4]-Chlamydocin in Me_2SO-d_6. Nuclear Overhauser effect (NOE) difference spectra[9,10] (270-MHz) were taken at 50 °C (Figure 7). Unfortunately, some of the peaks overlap with each other (Aib NHs and Phe NH) and some lines are broadened. All of the observed intensity changes are positive, indicating the compound is "rapid tumbling".[15]

At 50 °C saturation transfer from one conformer to another occurs. For example, irradiation of the peak at 8.54 ppm (Ala NH in conformer II) produces two sets of negative peaks in the difference spectrum (bottom, Figure 7); i.e., both the Ala NH proton at 8.54 ppm in conformer II and the Ala NH at 6.97 ppm in conformer I are saturated by irradiation at 8.54 ppm. Saturation transfer is observed for all irradiated peaks at the temperature of these experiments. These results are predictable from the rapid interconversion between conformer I and conformer II in solution; i.e., the half-life for conformational interconversion ($T_{1/2}$) is equal to or faster than T_1, the longitudinal relaxation time. See ref 16 for a prior example of this effect.

In order to suppress saturation transfer effects on the NOE, experiments were carried out on the specifically deuterated [3,3,3-$^2H_3$]Ala-chlamydocin derivative (**6**) at low temperature in a mixed solvent system of 20% chloroform in dimethyl sulfoxide (Figure 8). ^{13}C and ^1H NMR data obtained in this solvent system established that both conformers I and II were present. Because of the increased viscosity of the mixed solvent system, the NOE enhancements were less than obtained in pure chloroform at 25 °C or in dimethyl sulfoxide at 50 °C. Nevertheless, the enhancements were reproducible and larger than the incomplete cancellations of the aromatic and aliphatic protons. The critical comparisons were the enhancements observed at 10 and 50 °C

(14) Pease, L.; Watson, C. *J. Am. Chem. Soc.* **1978**, *100*, 1279–1289. Pease, L.; Niu, C. H.; Zimmermann, G. *J. Am. Chem. Soc.* **1979**, *101*, 184–191.

(15) Leach, S. J.; Némethy, G.; Scheraga, H. *Biochem. Biophys. Res. Commun.* **1977**, *75*, 207–215.

(16) Roques, B. P.; Combrisson, S.; Wasylishen, R. *Tetrahedron* **1976**, *32*, 1517–1521.

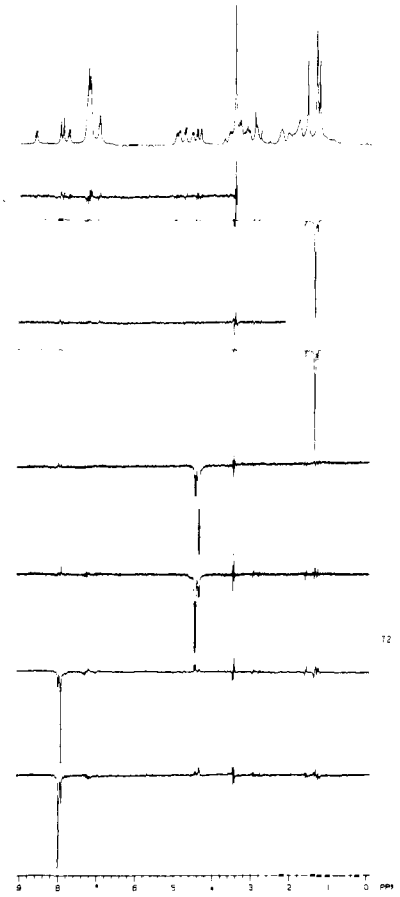

Figure 8. NOE difference spectra of *cyclo*[Aib-Phe-D-Pro[3,3,3-$^2H_3$]-Ala] in 80% Me_2SO-d_6/$CHCl_3$-d (v/v) at 10°C. Negative peaks show position of saturating f_2 irradiation.

when protons at 4.75 and 4.4 ppm and 7.97 and 8.0 ppm were irradiated. These correspond to the $C^\alpha H$ and NH of Ala^I and Ala^{II}, respectively, and provide data to assign the amide bond configuration between Ala (Aoe) and Aib. At 50 °C irradiation at ~4 ppm clearly enhances both Aib NH resonances at 7.5 ppm (Figure 7). At 10 °C irradiation at 4.4 ppm enhances the Aib^I NH at 7.97 ppm while irradiation at 4.25 ppm enhances the 8.0 ppm resonance (Figure 8). Selective irradiation of the NH resonances clearly enhances the corresponding Ala $C^\alpha H$. These data indicate that the Ala $C^\alpha H$ and Aib NH are close both in conformer I and in conformer II. Thus the Ala-Aib amide bond is trans in conformer II. No enhancements of Ala $C^\alpha H$ were observed when either Aib C^β-methyl group was irradiated.

Circular Dichroism Studies. CD spectra of chlamydocin, Ala^4-chlamydocin (**5**), dihydrochlamydocin (**2**), and Gly^1,Ala^4-chlamydocin (**3**) in methanol and water are shown in Figure 9. Comparisons among the Asu^4-chlamydocin[17] (Asu = α-aminosuberic acid), Ala^4-chlamydocin, chlamydocin were used to assign unusual features of the chlamydocin spectrum. The negative ellipticity at 270 nm is due to the chiral ketone in **1** as this is absent in dihydrochlamydocin (**2**). The epoxide contributes the positive ellipticity at 225 nm. This peak is more positive in **2** (partial CD shown) and not positive in the Ala^4 analogue **5**. The CD spectra of Ala^4- and Asu^4-chlamydocin are essentially identical, and thus represent the CD of the cyclic tetrapeptide ring system lacking the epoxy ketone side chain. The CD spectra of the Gly^1 analogue **3** in water or methanol differ substantially from that of the Ala^4-analogue **5** in these solvents.

Major Conformation (Conformer I) of Chlamydocin and Ala^4-Chlamydocin. The conformation of chlamydocin in chloroform has been described previously.[7] In the present study,

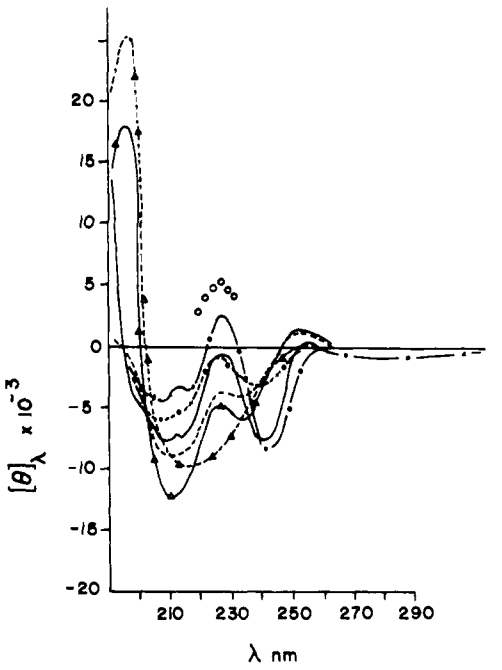

Figure 9. Circular dichroism spectra of chlamydocin, *cyclo*(Aib-Phe-D-Pro-Ala), and *cyclo*(Gly-Phe-D-Pro-Ala) in polar solvents. (—) Ala^4-chlamydocin in methanol; (---) Ala^4-chlamydocin in water; (-·-) chlamydocin in methanol; (·---·) chlamydocin in water; (Δ) Gly^1,Ala^4-chlamydocin in methanol; (--Δ--) Gly^1,Ala^4-chlamydocin in water; (O) dihydrochlamydocin in methanol—partial spectrum.

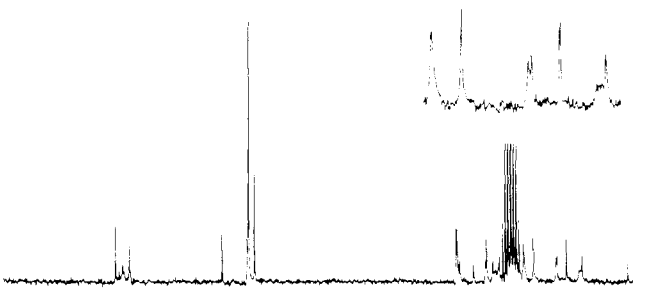

Figure 10. Carbon-13 NMR spectrum of (Gly^1,Ala^4)-chlamydocin in Me_2SO-d_6. Peptide concn 14.2 mg/0.35 mL; 44000 scans. Insert: Region between 14 and 36 ppm.

essentially identical results were obtained for conformer I of chlamydocin in Me_2SO-d_6 solution. The coupling constants, $^3J_{NH-C^\alpha H}$, observed for the Phe^I NH and Aoe^I NH are as large as those observed in neat chloroform-d (Table I). These large $^3J_{NH-C^\alpha H}$, ≥10.0 Hz, indicate that Θ^α_{NH} is ~180°.[18] In addition, the small temperature coefficients ($\alpha\delta/\Delta T$) for Phe^I NH (~0 ppm/°C) reveal that these protons are shielded from the solvent when compared to the solvent exposed Aib NHs. Also, the chemical shifts of the two NHs (Phe, Aoe) are nearly independent of the solvent polarity (Δ solv in Table I). The C^β and C^α of the Pro residue in conformer *I* resonate at 24.11 and 23.84 ppm indicating a trans Phe-Pro amide bond.[18,19]

From these data it is concluded that conformation I for chlamydocin in dimethyl sulfoxide is the same as that found for chlamydocin in neat chloroform and is shown in Figure 1F.

The ^1H NMR results, i.e., temperature dependence coefficients ($\alpha\delta/\Delta T$), solvent dependency, and the large NH–$C^\alpha H$ coupling constants for Ala^4-chlamydocin (shown in Table II), are in good agreement with those found for chlamydocin. Similarly, the ^{13}C NMR results (Table III) establish a trans Phe-Pro amide bond (the C^β and C^α of Pro resonate at 24.11 and 23.73 ppm, respectively). On the basis of these spectroscopic similarities it is

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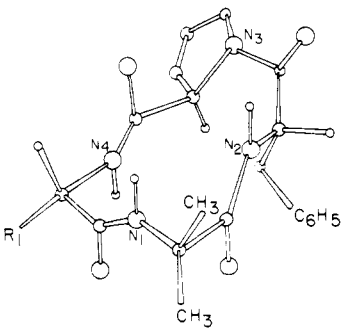

Figure 11. Schematic drawing of minor conformer II of Ala⁴-chlamydocin in Me_2SO-d_6 with the cis,trans,trans,trans amide bond sequence.

concluded that conformer I of Ala⁴-chlamydocin is the same as that found for chlamydocin (Figure 1F).

Minor Conformation (Conformer II) of Chlamydocin and Ala⁴-Chlamydocin. The ^{13}C NMR data show that 40% of the chlamydocin molecules are in conformation II, which must have a cis Phe-Pro amide bond on the basis of the Pro C^{β} and C^{α} resonances (Table III).[20] On the basis of energy calculations[21-24] and established conformations of cyclic tetrapeptides[25,26] an amide bond sequence with two cis amide bonds was possible. In addition, we considered the possibility that conformer II might have a new cyclic tetrapeptide conformation—one with a single cis amide bond.

NOE difference spectra were obtained at 50 °C in an attempt to establish the amide bond geometries. Unfortunately, at this temperature interconversion between conformers I and II was sufficiently rapid relative to T_1 that saturation transfer occurred. As a result, NOE enhancements were observed for protons in both conformations regardless of the conformation of the proton irradiated. Related transfers of NOE enhancements have been reported for NOE studies of interconverting amide bond geometries in *N*-acetylproline,[16,27] where cis to trans acetylproline interconversions are faster than T_1.[28,29]

To suppress the rate of conformational interconversions NOE experiments were carried out at low temperatures in mixed solvent systems. This experiment (Figure 8) was successful in that NOE enhancements could be observed without saturation transfer from one conformer to another (note the absence of the second negative peak). This result is possible when the half-life for conformational interconversion is longer than the longitudinal relaxation time (T_1).[16] The low-temperature NOE data are consistent with a trans amide bond for Ala-Aib in conformer II. Thus the amide bond geometry for conformer II appears to be cis,trans,trans,trans. The existence of the trans Ala-Aib amide bond rests on the observable NOE between Ala^{II} $C^{\alpha}H$ and Aib^{II} NH at low temperatures where saturation transfer from conformer I to II is minimal. Corroborating NOE enhancement data were obtained by irradiating the Aib^{II} C^{β} protons while Ala^{II} $C^{\alpha}H$ on the [3,3,3-$^2H_3$]-L-Ala derivative (**6**) was being observed. No enhancement of Ala $C^{\alpha}H$ was observed, which is consistent with a trans amide bond. The only alternative conformation possible for II would have two trans and two cis amide bonds, e.q., compound **7**, but this conformation would be expected to give the missing NOE enhancements because of the proximity of the Ala C^{α} and Aib C^{β} protons.

Table IV. Torsional Angles[a] for Proposed Solution Conformations of Chlamydocin and *cyclo*(Aib-Phe-D-Pro-Ala) in Dimethyl-d_6 Sulfoxide

	Aib	Phe	D-Pro	Ala (Aoe)
		Major Conformer		
ϕ	+60°	−120°	+60°	−110°
ψ	−50°	+120°	−55°	+110°
ω	+160°	−160°	+160°	−160°
		Minor Conformer		
ϕ	+70°	+150°	+85°	−105°
ψ	+75°	−105°	−140°	+80°
ω	−160°[b]	+20°	+165°	−160°

[a] For definition of torsion angles see ref 31. All values are ±20°. [b] Values are approximate torsion angles for conformations allowing ω twist angles.

On the basis of Tempo titration and temperature dependency data, all amide protons in conformer II are solvent exposed. The coupling constants for the two NH–$C^{\alpha}H$ interactions are relatively small when compared with those in conformer I. These establish either fast ψ,ϕ' rotations or vicinal bond angles restricted to about 30° or 140°. On the basis of $^3J_{NH-C^{\alpha}H}$ values,[35] the most probable average conformation is shown in Figure 11. Model building studies indicate that all amide bonds would be nonplanar (transoid) with ω twist angles near ±15–25°. Comparable nonplanar amide bonds have been observed in the all-transoid conformation of chlamydocin (15–25°)[6] and larger ones in CC-1065 (53° twist)[28] and are not energetically prohibitive.[29,30] The congruent data for Ala⁴-chlamydocin (**2**) lead to a closely related conformation. The torsion angles for both compounds are listed in Table IV.

Discussion

While studying the solution conformations of the cytostatic cyclic tetrapeptide chlamydocin, *cyclo*[Aib^1-L-Phe^2-D-Pro^3-L-(2-amino-8-oxo-9,10-epoxydecanoic acid)^4] (**1**),[3] and the related cyclic tetrapeptides Ala⁴-chlamydocin (**5**) and (Gly^1,Ala^4)-chlamydocin (**3**),[7,8] we found the ring system conformation of these molecules to be remarkably sensitive to solvent. In chloroform the ring system of chlamydocin has approximately 2-fold rotational symmetry, as shown in Figure 1F, that is characterized by two 1←3 intramolecular hydrogen bonds (γ-turns)[31] and four transoid amide bonds.[1c] This same conformation is found in the closely related cyclic tetrapeptides Ala⁴-chlamydocin (**5**) and (Gly^1,Ala^4)-chlamydocin (**3**),[8] and all three are very similar to the crystal structure of dihydrochlamydocin (**2**)[6], a biologically inactive analogue of **1**.

However, small amounts of hydrogen bonding solvents such as dimethyl sulfoxide perturb the intramolecular 1←3 hydrogen bonds of compounds **1**–**5**, causing partitioning into new conformations with cis X-Pro amide bonds.[7,8] Because the polar solvent conformations may be relevant to the biologically active conformation of chlamydocin, as well as to the theoretical conformations of cyclic tetrapeptides, we carried out NMR studies to determine the conformation of chlamydocin (**1**) and the Ala⁴-analogue **5** in dimethyl sulfoxide.

The results described here establish that in dimethyl sulfoxide–chloroform solvent mixtures containing more than 50% dimethyl sulfoxide, the cyclic tetrapeptide ring system equilibrates between two conformations, present in ratios of about 6:4. Conformer I is clearly the all-transoid conformation (Figure 1F). Conformer II, however, has a cis,trans,trans,trans amide bond sequence (Figure 11). The existence of the cis amide bond between L-Phe-D-Pro is unambiguously established by the ^{13}C NMR data, and the presence of a trans amide bond between Ala-Aib is proven by the NOE experiment at 10 °C. Recovery of cyclic tetrapeptide with correct chirality from Me_2SO solutions left standing several months establishes that conformer II is not an epimer of **1** (or **5**). Racemization of Phe in the cyclic tetrapeptide, *cyclo*(L-

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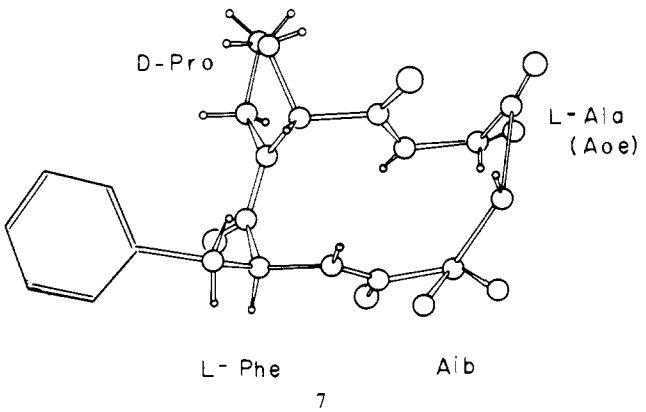
Pro-L-Phe-L-Pro-L-Ala), apparently occurs upon standing in solution.[32,33]

The conformation proposed for the chlamydocin ring system in Me_2SO has not been reported previously and apparently has not been examined in the reported molecular calculations. Theoretical calculations have shown that the 12-membered ring of cyclic tetrapeptides can adopt at least three distinct conformations (Figure 1, conformations A–E) depending on the sequence of chiral amino acids and tertiary amide bonds.[21-26,34] The *d*-symmetry conformation is formed by "ring-flipping" one end of the *i*-symmetry conformation while the S_4 conformation requires two cis to trans amide bond isomerizations. When chiral amino acids are present, four orientations of the carbonyl groups are possible. Only the symmetrical conformations shown in Figure 1 have been calculated. Close approximations of several of these conformations have been observed in model cyclic tetrapeptide systems. The i_1-symmetry conformer was found first[25] and is the most commonly observed.[26,34] Approximate d_1 symmetry was found in the crystal structure of *cyclo*(Phe-Pro-Ala-Pro)[32,33] while the epimer *cyclo*(D-Phe-Pro-Ala-Pro) is an asymmetric version of d_2. The all-trans amide bond conformer with S_4 symmetry (Figure 1) has not been observed unambiguously, but a related bis γ-turn all-transoid cyclic tetrapeptide conformation has been established in solution[7,8] for chlamydocin peptides **1** and **3–5** and in crystalline sample for **2**.[6] The effect of solvent on these conformations has been ignored in the theoretical calculations and in most solution conformation studies, and potentially strained or highly substituted systems have not been considered.

The existence of conformer II for a cyclic tetrapeptide has not been anticipated by molecular calculations. Examination of molecular models suggests that the chlamydocin ring system adopts the cis,trans,trans,trans amide bond sequence in Me_2SO over a cis,trans,cis,trans conformer **7** because severe transannular steric



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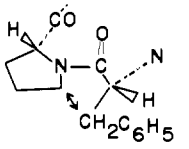
interactions destabilize conformer **7**. The configurational sequence of amino acids in **1** (and **5**) places both the Phe C^β and Aib C^β over the peptide ring at distances approximately 2.9 Å from the Pro C^α and Ala C^α, respectively, in **7**. The first interaction has been found in *cyclo*(D-Phe-L-Pro-L-Ala-L-Pro) (**8**) where the D-Phe C^β lies over the cyclic peptide ring near the Pro C^α.[33] The fact that **1** and **5** do not adopt conformation **7** indicates the second interaction (or sum of both) destabilizes conformer **7** relative to the proposed conformation. It should be noted that energy calculations[22,23] indicate that steric interactions between Ala C^β and Aib C^β are absent in the all-transoid conformer I; thus they are not likely to destabilize a cis,trans,trans,trans conformation in which the Ala and Aib side chains are separated by a trans amide bond. Theoretical calculations allowing for distortion of all ring bond angles and lengths are needed to accurately determine all possible conformations for these highly substituted cyclic tetrapeptides.

The proposed conformation II for compounds **1** and **5** also clarifies NMR data, suggesting multiple conformations of (Gly[1],Ala[4])-chlamydocin (**3**) in Me_2SO. If the conformation proposed for chlamydocin and **5** is correct, then replacement of Aib by Gly should increase the energetically allowed conformations by removing the steric interaction between Ala C^β and Aib C^β in either of the two possible cis,trans,cis,trans conformations related to **7**. In fact, ^{13}C NMR data for (Gly[1],Ala[4])-chlamydocin establish several additional cis Phe-Pro conformers in Me_2SO (see Figure 10). In addition to the resonances for the cis (20.7 and 31.9 ppm) and trans (23.99 and 24.32 ppm) Phe-Pro amide bonds, at least four Ala C^β signals (15.55, 15.98, 16.25, and 16.58 ppm) are evident. The ^{13}C NMR data therefore suggest that in solution (Gly[1],Ala[4])-chlamydocin (**3**) is a mixture of all four known cyclic tetrapeptide ring conformations (e.g., bis γ-turn, Figure 1F; cis,trans,cis,trans, i_2 and d_1; and cis,trans,trans,trans, Figure 11).

The chlamydocin system represents the first time a conformationally labile cyclic tetrapeptide has been studied in detail in both polar and nonpolar solvents. Most other cyclic tetrapeptides are soluble only in highly polar organic solvents (e.g., TFA, Me_2SO) due to the lack of long aliphatic side chains and the presence of one to three amide NH groups. Cyclic tetrapeptides with three or four *N*-methylamide groups cannot adopt all-transoid conformations,[23] while *cyclo*(L-Phe-L-Pro-L-Ala-L-Pro), which is soluble in chloroform,[32] cannot adopt the all-transoid conformation due to the unfavorable L-Phe-L-Pro steric interaction shown in **9**. It is clear that the conformations of chlamydocin peptides **1**



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and **3–5** depend on solvent. In chloroform, and presumably in other relatively nonpolar solvents, these peptides adopt the all-transoid conformation, which is stabilized by the hydrogen bonds in the bis γ-turns, whereas in Me_2SO and other polar solvents the trans,trans,trans,cis amide bond conformations accumulate for **1** and **5** while **3** adopts additional centrosymmetric conformations. The apparent preference of cyclic tetrapeptides for cis,trans,cis,trans conformations, even when all-transoid bis-γ-turn conformations are possible, probably is a result of the poor solubility of these compounds in nonpolar solvents. Under these circumstances NMR studies would not be feasible in nonpolar solvents where γ-turns would stabilize conformation. In addition crystallization from polar organic solvents for X-ray crystallographic studies would favor the centrosymmetric forms that are usually isolated. The relative energies of the respective cyclic tetrapeptide ring systems with and without attached molecules of solvation remain to be determined.

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