Reaction 2 is controlled by an activation process so that a change of dielectric properties affects reorganization of the solvent around both the reactant and the activation complex. The reaction is enhanced in polar media. Results in Figure 2b show that the slopes, $\partial \ln k_2/\partial(1/\epsilon)$, depend on the nature of the nonaqueous component. The slopes of the straight lines are -60, -120, and -170 for water–ethanol, water–glycerol, and water–sucrose solutions, respectively. This indicates existence of specific solvation and interaction of reacting species with the solvent shell at the initial and transient states.

References and Notes

(1) (a) Boris Kidrič Institute of Nuclear Sciences; (b) California Institute of Technology.
(2) (a) Part 1, O. I. Mićić and B. Čerček, *J. Phys. Chem.*, **78**, 285 (1974); (b) M. J. Blandamer and J. Burgess, *Chem. Soc. Rev.*, **4**, 55 (1975).
(3) (a) J. Khaladji, *Ann. Chim. (Paris)*, **13**, 555 (1958); (b) L. Eldjarn, *Acta Chem. Scand.*, **5**, 677 (1951).
(4) M. Z. Hoffman and E. Hayon, *J. Am. Chem. Soc.*, **94**, 7950 (1972).
(5) G. E. Adams, G. S. McNaughton, and B. D. Michael in "The Chemistry of Ionization and Excitation", G. R. A. Johnson and G. Scholes, Ed., Taylor and Francis, London, 1976, p 281.
(6) M. Bonifačić, K. Schäfer, H. Möckel, and K.-D. Asmus, *J. Phys. Chem.*, **79**, 1496 (1975).
(7) (a) V. M. Marković, D. Nikolić, and O. I. Mićić, *Int. J. Radiat. Phys. Chem.*, **6**, 224 (1974); (b) O. I. Mićić and M. T. Nenadović, *J. Phys. Chem.*, **80**, 940 (1976).
(8) J. R. McPhee, *Biochem. J.*, **64**, 22 (1956).
(9) J. Timmermans, "Physico-Chemical Constants of Binary Systems", Vol. 4, Interscience, New York, N.Y., 1960.
(10) J. S. Moore and A. F. Norris, *Int. J. Radiat. Biol.*, **29**, 489 (1976).
(11) J. V. Davies, W. Griffiths, and G. O. Phillips in "Pulse Radiolysis", M. Ebert, J. P. Keen, A. J. Swallow, and J. H. Baxendale, Ed., Academic Press, New York, N.Y., 1965, p 181.
(12) M. Simić, P. Neta, and E. Hayon, *J. Phys. Chem.*, **73**, 3794 (1969).
(13) P. Warrick, Jr., J. J. Auborn, and E. M. Eyring, *J. Phys. Chem.*, **76**, 1184 (1972).
(14) Y. Nishijima and G. Oster, *Bull. Chem. Soc. Jpn.*, **33**, 1649 (1960).
(15) "International Critical Tables", Vol. 5, McGraw-Hill, New York, N.Y., 1929, p 157.
(16) P. A. Carapellucci, *J. Am. Chem. Soc.*, **98**, 3016 (1976).
(17) (a) C. Walling, "Free Radicals in Solution", Wiley, New York, N.Y., 1957, p 76; (b) R. M. Noyes, *Prog. React. Kinet.*, 1, 152. (1961).

Conformational Studies of Tentoxin by Nuclear Magnetic Resonance Spectroscopy. Evidence for a New Conformation for a Cyclic Tetrapeptide

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Abstract: The conformations of the phytotoxic cyclic tetrapeptide tentoxin, *cyclo*-(L-MeAla[1]-L-Leu[2]-MePhe[(*Z*)Δ][3]-Gly[4]) and the analogues, L-Pro[1]-tentoxin and *N*-L-[*methyl*-^{13}C]Ala[1]-tentoxin, have been studied by ^{1}H and ^{13}C NMR in chloroform, and by ultraviolet and circular dichroism spectroscopy in methanol. The data from these studies indicate that the three compounds have essentially the same conformation and that the conformation previously proposed for tentoxin is incorrect. A new conformation is proposed for tentoxin with the following torsion angles: ϕ_1, $-80°$, ψ_1, $-10°$; ϕ_2, $-120°$, ψ_2, $+70°$; ϕ_3, $-90°$, ψ_3, $-20°$; ϕ_4, $-120°$, ψ_4, $+70°$. The new ring conformation differs from the centrosymmetric conformation previously proposed for tentoxin by the sign reversal of the torsion angles ψ_4,ϕ_1 while ϕ_2,ψ_2, ϕ_3,ψ_3, and all ω torsional angles are unchanged. The conformation proposed for these compounds is a new conformation for the 12-membered cyclic tetrapeptide ring system.

Tentoxin (**1**) is a phytotoxic cyclic tetrapeptide produced by the plant pathogen *Alternaria tenuis*.[2] When applied to germinating seedlings, tentoxin causes chlorosis in some plant species but has little apparent effect on others.[3] This selective toxicity has been linked to the presence in susceptible species of a high affinity ($k_{assoc} = 2 \times 10^8$ M) tentoxin binding site[4] on chloroplast coupling factor 1 (CF_1), a key protein involved in chloroplast synthesis of ATP.[5] Resistant species usually contain a form of CF_1 which does not bind tentoxin tightly.

The configuration and sequence[6] of the amino acids in tentoxin and the configuration of the double bond in the *N*-methyldehydrophenylalanyl residue (MePhe[(*Z*)Δ])[7] are well established and have been confirmed by total synthesis.[8,9] In addition, a conformation was proposed[6] for tentoxin (Figure 1a), by analogy to that observed by Dale and Titlestad for several model cyclic tetrapeptides (Figure 1b),[10] based on the close similarity in the ^{1}H NMR chemical-shift and coupling constant data for the two systems.[6c] However, whereas the Dale–Titlestad conformation (Figure 1b) has been confirmed by x-ray crystallography,[11] no substantiating evidence for the proposed tentoxin conformation has been reported.

We first came to question the proposed tentoxin conformation[6] as a result of synthesizing the analogue D-MeAla[1]-tentoxin (**2**) which differs from **1** only in the stereochemistry of the methyl group at C-11 (see Figures 1a and 1b for the numbering used here). Examination of molecular models of tentoxin in conformation **1a** revealed that the alanine β-carbon was about 2.3 Å from the glycine α-carbon at C-8. This carbon–carbon distance is very close and should be very hindered.[12] It was expected that the alanine β-carbon would be in an unhindered, "pseudoequatorial" position in the D-MeAla analogue **2** and therefore would be no less stable than tentoxin **1**. In fact, when synthesized, D-MeAla[1]-tentoxin was found to exist in several conformations, two of which could be isolated,[9] and this occurrence of multiple conformations was inconsistent with conformation **1a**.

As a result we decided to test the proposed tentoxin conformation by synthesizing the analogue, L-Pro[1]-tentoxin (**3**), in which L-proline replaces L-*N*-methylalanine. The five-membered ring of proline limits the C^δ–N–C^α–C^β vicinal bond angle to values less than ±0–20° whereas, in the proposed conformation for tentoxin (Figure 1a), the C–N–C^α–C^β vicinal bond angle is about 130°. Thus, L-Pro[1]-tentoxin (**3**) cannot exist in a conformation directly analogous to that proposed for tentoxin. If **3** were synthesized and found to have similar conformational and biological properties to tentoxin **1**, then the proposed conformation shown in Figure 1a could not be correct.

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Table I. NMR Parameters of Tentoxin and L-Pro-Tentoxin[a]

	1-position	Leucine	Methyldehydrophenylalanine	Glycine
	Alanine			
Tentoxin	$C^{\alpha}H$ 4.3	$C^{\alpha}H$ 4.16 (8.9)	C_6H_5 7.27	CH_i 5.2 (10, 15)
	$C^{\beta}H_3$ 1.52	$C^{\delta}H_3$ 0.52	$C^{\beta}H$ 7.75	CH_o 3.51 (2, 15)
	N-Me 2.77	$C^{\delta}H_3$ 0.61	*N*-Me 3.19	NH 7.97 (10, 2)
		NH 7.2 (8.9)		
	Proline			
L-Pro-Tentoxin	$C^{\alpha}H$ 4.49	$C^{\alpha}H$ 4.4 (7)	C_6H_5 7.41	CH_i 5.13 (14.8, 10.5)
	$C^{\beta}H$ 2.41	$C^{\delta}H_3$ 0.51	$C^{\beta}H$ 7.76	CH_o 3.54 (14.8, 1)
	$C^{\beta}H$ 1.59-2	$C^{\delta}H_3$ 0.63	*N*-Me 3.19	NH 7.93 (10.5, 1)
	$C^{\gamma}H_2$			
	$C^{\delta}H_2$ 3.54	NH 7.3 (7)		

[a] Taken in chloroform-*d* solutions with tetramethylsilane added as internal reference. Data are reported in parts per million from Me_4Si with coupling constants in hertz.

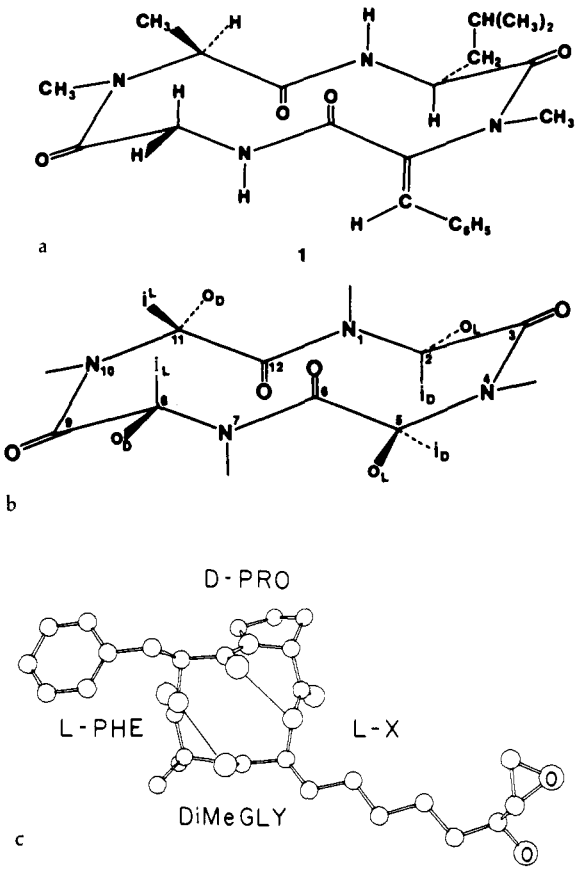


Figure 1. Conformation of cyclic tetrapeptides: (a) conformation proposed for tentoxin;[6] (b) cyclotetrasarcosyl;[10] (c) dihydrochlamydocin.[18]

L-Pro[1]-tentoxin (**3**) was synthesized by cyclization of H-Pro-Leu-MePhe[(*Z*)Δ]-Gly-OTcp in pyridine at 90 °C as described[9] and isolated in 30% yield. The ^1H NMR spectra of **1** and **3** in chloroform-*d* are shown in Figures 2 and 3 and the chemical-shift and coupling constant data are given in Table I. The spectrophotometric properties of tentoxin **1** and L-Pro[1]-tentoxin (**3**) are remarkedly similar, particularly the chemical shifts and coupling constant data for the glycyl protons and the dehydrophenylalanyl vinyl proton. As described later in this text, these latter resonances are very sensitive to conformational changes. The circular dichroism spectra for tentoxin and L-Pro[1]-tentoxin are shown in Figure 4. Furthermore, the biological activity of L-Pro[1]-tentoxin as an inhibitor of the CF_1-ATPase was indistinguishable from that of tentoxin (Figure 5).

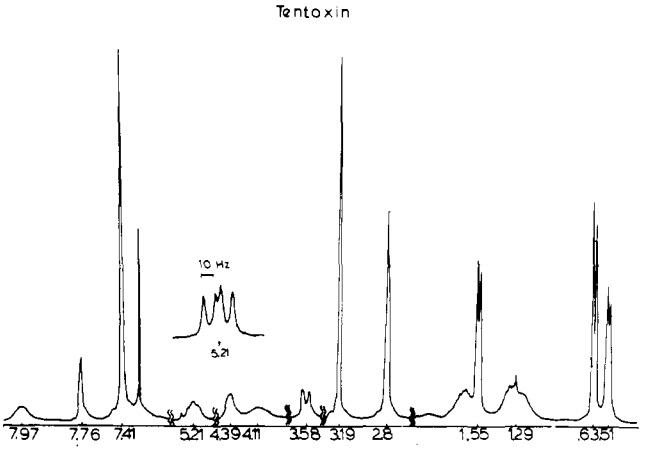


Figure 2. ^1H NMR spectrum (270 MHz) of tentoxin in chloroform-*d*. Values reported are in parts per million from internal tetramethylsilane. Singlet at 7.23 ppm is from chloroform. Insert: 90-MHz spectrum of glycyl inner porton.

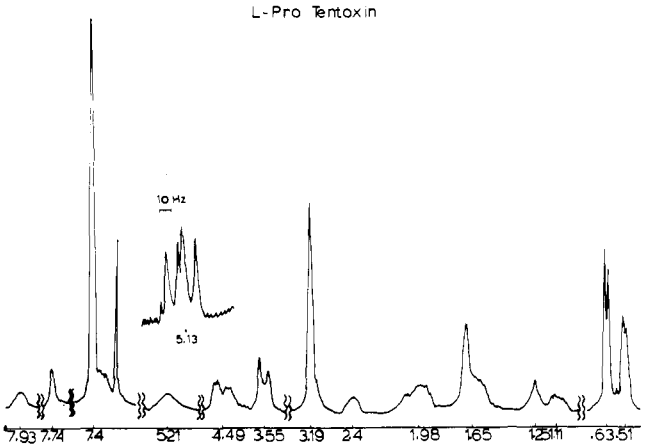


Figure 3. ^1H NMR spectrum (270 MHz) of L-Pro[1]-tentoxin taken in chloroform-*d*. Values reported are in parts per million from internal Me_4Si. Singlet at 7.2 ppm is due to chloroform. Insert: 90-MHz spectrum of glycyl inner proton.

The close similarity between the spectrophotometric and biological data of tentoxin and L-Pro[1]-tentoxin indicates that their conformations must be closely related. Since L-Pro[1]-tentoxin cannot adopt a conformation analogous to the one shown in Figure 1a, the conformation proposed[6] for tentoxin cannot be correct. We report here conformational studies of tentoxin (**1**), L-Pro[1]-tentoxin (**3**), and *N*-L-[*methyl*-^{13}C]-

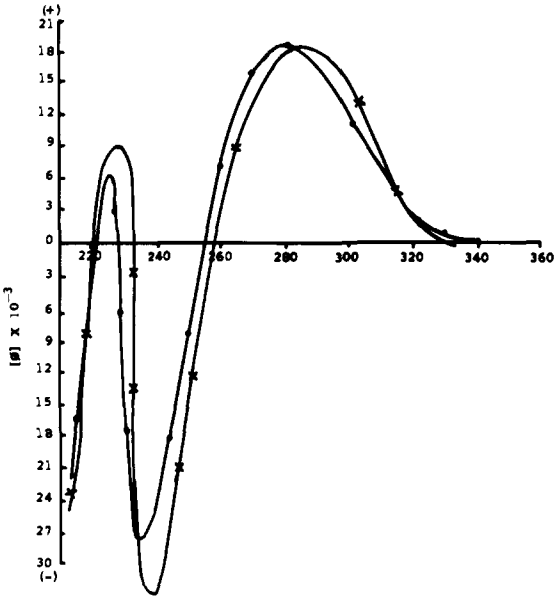


Figure 4. Circular dichroism spectrum of tentoxin (×—×) and L-Pro¹-tentoxin (•—•) in methanol.

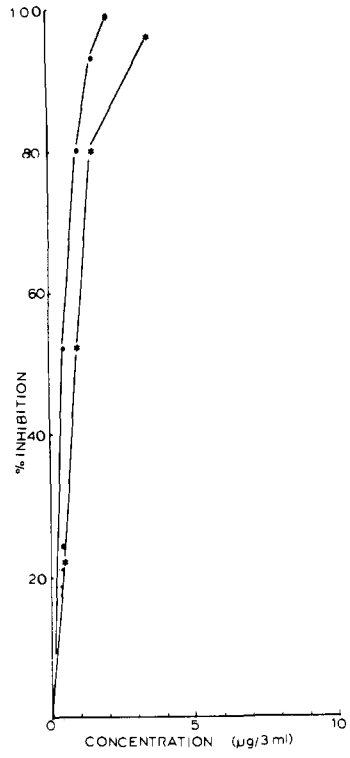


Figure 5. Inhibition of coupled electron transport in lettuce chloroplasts by the tentoxin analogues tentoxin (•—•); L-Pro¹-tentoxin (*—*).

Ala¹-tentoxin (**4**) and propose conformations for these molecules that show the two cyclic peptides are conformationally congruent and are different from the one originally proposed for tentoxin.

Experimental Section

The syntheses of tentoxin, L-Pro¹-tentoxin, and the ^{13}C-labeled analogue **7** by cyclization of the corresponding trichlorophenyl esters in pyridine have been reported.[9] Cyclic tetrapeptides **1**, **3**, and **7** used in this study were analytically pure. The ^1H NMR spectra at 270 MHz were obtained on a Bruker WH270 spectrometer in the Department of Biochemistry, University of Wisconsin—Madison, and at 90 MHz using a Bruker HX-90E pulse Fourier transform NMR spectrometer interfaced with a Nicolet 1080 computer and disk unit.

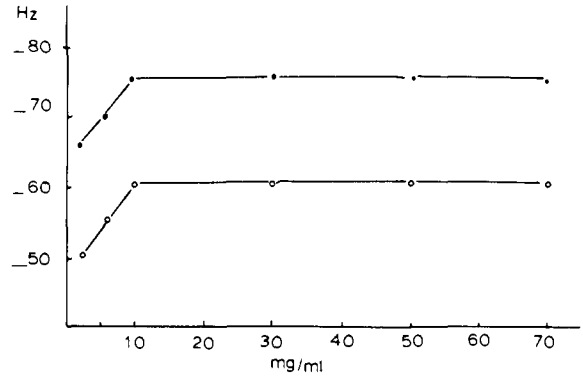


Figure 6. Concentration dependence of L-Pro¹-tentoxin amide protons in chloroform-*d*. Scale: glycine NH (●) 750–780 Hz; leucine NH (○) 650–680 Hz.

Spectra of peptides were obtained at concentrations between 1 and 40 mg/mL in chloroform-*d* solutions containing tetramethylsilane as internal standard. The ^{13}C NMR spectra were recorded on the Bruker HX-90E. The solution concentration was varied between 1 and 40 mg/mL. Above 10 mg/mL concentrations, a loss in resolution was observed.

Ultraviolet spectra were determined in methanol using a Cary 14 spectrometer. The circular dichroism spectra were measured on a Cary 16 equipped with circular dichroism attachment.

The biological activity of L-Pro¹-tentoxin was determined following the reported procedure.[4]

Results

Analysis of the Conformation of L-Pro¹-Tentoxin (3). Solvent Exposure of Peptide Protons. Concentration-dependent chemical shifts for amide protons have been interpreted to indicate intermolecular hydrogen bonding while concentration-independent chemical shifts can indicate either the proton is intramolecularly hydrogen bonded or is shielded from solvent.[13] The concentration dependence of the chemical shift of the glycine amide proton of L-Pro¹-tentoxin (**3**)[14] in chloroform is shown in Figure 6.

At concentrations greater than 11 mg/mL the chemical shift does not vary appreciably. This indicates that the molecules are becoming intermolecularly hydrogen bonded through aggregation. The ^{13}C NMR spectra (not shown) taken at these higher concentrations show a marked broadening of the carbon resonances. Aggregation of peptides at higher concentrations has been shown previously to contribute to line broadening.[15]

At concentrations between 2.5 and 10 mg/mL the glycyl NH chemical shift is concentration dependent with a slope equal to 0.0063 ppm/mM. This concentration dependency is less than that observed for nonintramolecularly hydrogen-bonded protons in protected tetrapeptides in chloroform (0.012 ppm/mM) but greater than that attributed to intramolecularly hydrogen-bonded protons (slope = 0.000 ppm/mM)[13] and indicates that the glycyl NH in L-Pro¹-tentoxin is probably not intramolecularly hydrogen bonded, but may be shielded somewhat from solvent. Some intermolecular aggregation may persist even at the higher dilutions.

Configuration Sequence of Amide Bonds. In addition to preventing the occurrence of certain conformations, the substitution of L-proline for L-*N*-methylalanine enables the geometry about the Gly-Pro amide bond to be determined by ^{13}C NMR.[16] The ^{13}C NMR of L-Pro¹-tentoxin showed only five resonances (21.7, 21.92, 23.93, 22.18, and 30.77 ppm) between 0 and 32 ppm and these are assignable to the prolyl β- and γ- and leucyl γ- and two δ-carbons. Since the leucyl γ-carbon reported resonates between 24 and 26 ppm, the prolyl γ-carbon must be the 22.18-ppm (or lower) signal, and therefore the Gly-Pro amide bond is cis ($\Delta^{\beta,\gamma}$ = 8.6 ppm).

The ^{13}C NMR carbonyl chemical shift also has been reported to provide information about amide bond geometry. Grathwohl et al.[17] have shown that the ^{13}C NMR chemical shift of a glycine carbonyl carbon is about 166 ppm in a cis amide bond and about 169 ppm in a trans amide bond. Carbonyl carbons of alanine resonate at 169 ppm in cis amide bonds and at 172 ppm in trans amide bonds (Table II).

The chemical shift at 165.47 ppm in L-Pro[1]-tentoxin (Table II) is consistent with the already assigned *cis*-Gly-Pro amide bond, and the resonances at 173.2 and 172.56 ppm are consistent with trans amide bonds. The resonance at 170.7 ppm cannot be assigned to either a cis or a trans amide bond on the basis of the ^{13}C NMR data but is assigned a cis configuration on the basis of ultraviolet absorption data which are presented in detail in the following section titled The MePhe[(*Z*)Δ] Unit. Thus, L-Pro[1]-tentoxin contains no more than two cis amide bonds and one *cis*-Gly-Pro amide bond.

Because of their relatively low-field chemical shifts the two trans amide bonds in L-Pro[1]-tentoxin may not be planar, and ω may deviate from 180° by 10–20°. Grathwohl et al.[17] have proposed that cyclotetraglycyl has four transoid amide bonds in Me_2SO because the chemical shifts of the carbonyl carbons are about 1 ppm downfield from a planar trans amide bond. In chlamydocin, **5a,** which has four transoid amide bonds (ω = 162°, −166°, 156°, −164°) in the crystalline state,[18] the carbonyl chemical shifts (Table II) are about 4 ppm downfield from model trans amide chemical shifts.[19] Although suggestive, the two examples do not provide sufficient information correlating ω with ^{13}C NMR carbonyl resonances to decide if the secondary amide bonds are trans or transoid in L-Pro[1]-tentoxin. The chemical shifts of the carbonyl carbons in tentoxin **1** are consistent with planar trans amide bonds, and therefore we have assumed that all the amide bonds are essentially planar (ω = 0 or 180 ± 5°), and have used the corresponding τ angle of 111° found in *cyclo*-$(Sar)_4$.[11]

Examination of Dreiding molecular models suggested two possible ring conformations with alternating cis,trans,cis,trans amide bond sequences. The major difference between these two is found in the orientation of the CH=C—C=O chromophore; in one it is S-cis and in the other it is S-trans. The other possible amide bond sequences, cis-cis-trans-trans, cis-trans-trans-trans, etc., which can be constructed from cyclic tetrapeptides containing one or two cis amides, were examined but these were rejected either because of severe steric interactions or ring strain apparent in these conformations or because they were inconsistent with the NMR data.

The following paragraphs contain our analysis of the NMR and UV data which is used to provide partial conformational units. These units are combined to specify the conformation of the 12-membered ring system.

The Glycyl Unit. It is clear from the data in Table I that the glycyl α protons in cyclic tetrapeptides can be nonequivalent in the ^1H NMR and subject to anisotropic effects not seen in larger or more flexible peptides. This nonequivalency has been thoroughly studied in cyclotetrasarcosyl (Figure 1b)[10] in which two distinct sets of glycyl α protons are observed. Those in the 2 and 8 inner positions are eclipsed with the carbonyl groups in the 12 and 6 positions, respectively, and these protons are strongly deshielded (5.2 ppm). The outer protons in the 2 and 8 positions are in more equatorial-like positions and are observed near 3.2 ppm.[10] Geminal coupling in the 2 and 8 positions is about 15 Hz.

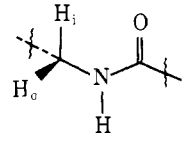
In contrast the protons in the 5 and 11 positions are subjected to different chemical and magnetic environments and the inner and outer protons are observed at about 4.3 and 3.6 ppm, respectively, in the ^1H NMR with a geminal coupling constant of 18 Hz.[10] Neither proton in the 5 or 11 position is eclipsed by a carbonyl group and consequently neither is as deshielded as the 2 (and 8) inner protons. By means of multistep rotational processes (described in detail in ref 6c and 20) it is possible for the 2 and 8 protons to exchange with the 5 and 11 protons. This does not occur at ambient temperature in cyclotetrasarcosyl (coalescent temperature ca. 180 °C), but does occur when both *N*-methyl groups in positions 1 and 7 are removed.[10] *cyclo*-(Gly-Sar-Gly-Sar) coalesces in the NMR at about 20 °C.[10]

Table II. ^{13}C NMR Carbonyl Resonances as a Function of Amide Bond Geometry[a]

cyclo-(Gly-Gly)[b,c]	166.0 (Gly, cis)
cyclo-(Gly-Ala)[b,c]	165.9 (Gly, cis); 168.6 (Ala, cis)
cyclo-(Ala-Ala)[b,c]	–; 169.0 (Ala, cis)
TFA·Gly-Gly-Gly-Ala	168.6 (Gly, trans) 168.8 (Gly, trans)
TFA·Gly-Gly-Ala-Ala	168.9 (Gly, trans); 171.8 (Ala, trans)
cyclo-(L-MeAla-L-Leu-MePhe[(*Z*)Δ]-Gly)[d] (**1**)	164.74, 170.024, 171.53, 171.74
cyclo-(L-Pro-L-Leu-MePhe[(*Z*)Δ]-Gly)[d] (**3**)	165.47, 170.7, 172.56, 173.2
Chlamydocin (**5**)[e]	171.7, 172.7, 174.4, 175.5
cyclo-(Gly-Gly-Gly-Gly)[b,c]	169.8

[a] Chemical shifts are in parts per million from internal Me_4Si. [b] In Me_2SO. [c] Data taken from ref 17. [d] $CDCl_3$. [e] R. D. Jasensky, unpublished data.

In L-Pro[1]-tentoxin (Table I) the chemical shift of the glycyl outer (3.55 ppm) and inner (5.2 ppm) protons is similar to those in the 8 position of cyclotetrasarcosyl (3.25 and 5.3 ppm). The $^3J_{CH-C^{\alpha}H}$ coupling constant in L-Pro[1]-tentoxin is 10.5 Hz which is consistent with a vicinal bond angle of 170 ± 20°.[21] Also, the geminal coupling (14.8 Hz) is similar to the 8 position of cyclotetrasarcosyl. Together these data indicate the partial structure **6** in which the inner proton and the *N*-methyldehydrophenylalanylcarbonyl group are approximately coplanar (±20°).



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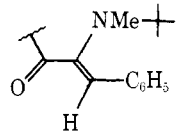
The effect of temperature on the ^1H NMR spectrum of L-Pro[1]-tentoxin in chloroform was studied over the temperature range −30 to +55 °C. The coupling constants and line widths of the α protons were not changed at the higher temperatures and no evidence for the beginning of some coalescence process was observed. No broadening of line width was observed at −30 °C which indicates that the glycine proton resonances do not arise from the time averaging of multiple ring conformations. This is in contrast to the ^1H NMR spectrum of *cyclo*-(Sar-Gly-Sar-Gly), in which coalescence, caused by rapid ring flipping processes and ϕ,ψ' bond rotations,[10,20] was observed at 20 °C. The absence of coalescence at 55 °C indicates the conformation about the glycyl residue is relatively rigid. The ^1H NMR spectrum of tentoxin did not show any line broadening for glycine at 120 °C, the highest temperature tested.

The MePhe[(*Z*)Δ] Unit. The total synthesis of tentoxin[8,9] established a *Z* configuration for the olefin group. In linear peptides this unit absorbs in the ultraviolet spectrum at 278 nm ($\epsilon \simeq 18\,000$).[9] In tentoxin the chromophore is essentially unchanged [λ_{max} 284 nm (ϵ 20 670)] which indicates that the CH=C—C=O unit is conjugated and nearly planar in the cyclic structure. Further evidence for coplanarity comes from the analysis of *cyclo*-(Gly-Phe[(*Z*)Δ]-D-Pro-L-Ala) (**7**).[22] Analysis of Dreiding molecular models shows that in **7** the

carbonyl group and the double bond cannot be coplanar whether **7** is in a cis,trans,cis,trans conformation or an all transoid conformation. The ultraviolet spectrum of **7** shows that the Phe[(*Z*)Δ] residue has a low extinction coefficient [λ_{max} 282 nm (ϵ 13 000)] which is consistent with reduced overlap of the C=C and C=O orbitals.

The high extinction coefficient for tentoxin (20 670) also supports the assumption that the amide bond between the leucyl- and *N*-methyldehydrophenylalanyl residues is cis. Dreiding models of tentoxin in a conformation with an all-trans or with a cis,trans,trans,trans amide bond sequence show that the carbonyl group and the double bond could not be coplanar in these conformations, but would be nearly perpendicular to each other and therefore would be expected to have ultraviolet extinction coefficients closer to that observed with the model peptide **7** (ϵ 13 000).

The chemical shift of the vinyl proton of the dehydrophenylalanyl residue is sensitive to its orientation to the carbonyl group. In L-Pro^1-tentoxin and in tentoxin the vinyl proton is found at 7.74 ppm. In other tentoxin analogues[9] chemical shifts of 7.75 and 7.25 ppm are observed. The low-field resonance (7.74 ppm) is consistent with an S-cis[23] CH=C—C=O configuration, i.e., **8**. Supporting this is the observation that the vinyl proton of **9** resonates at 7.55 ppm while the isomer **10** resonates at 6.5 ppm.[24] Protons eclipsed by the carbonyl



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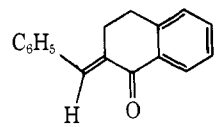

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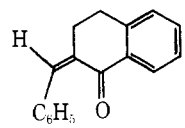

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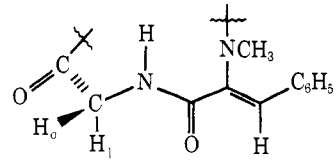
group are more strongly deshielded. Evidence that the vinyl proton in L-Pro^1-tentoxin is deshielded by an S-cis carbonyl group is also provided by cyclic tetrapeptide **7** where the vinyl proton, which cannot be eclipsed by the carbonyl group, absorbs at 6.55 ppm.[22]

The MePhe[(*Z*)Δ]-Gly Unit. When the partial data for the glycyl- and methyldehydrophenylalanyl residues are combined and the two units joined, a conformation of the MePhe[(*Z*)-Δ]-Gly face of L-Pro^1-tentoxin emerges in which the vinyl proton, the carbonyl group, and the glycyl inner proton are approximately coplanar (±30°). This is illustrated in partial structure **11**.



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The Conformation of L-Pro^1-Tentoxin. When the partial conformation **11** is incorporated into the cyclic tetrapeptide, *cyclo*-(L-Pro-L-Leu-MePhe[(*Z*)Δ]-Gly) (**3**) which contains the conformationally restrictive proline residue, the major features of the conformation are almost completely defined. The question remains whether the Leu-H$^\alpha$-NH vicinal coupling constant (7 Hz) is due to a distinct conformation with a Leu-NH-C$^\alpha$H vicinal bond angle of ±135° or whether the 7 Hz is an averaged *J* value caused by rapid rotation about the ψ_{Pro},ϕ_{Leu} angles. It is probable that the leucyl $^3J_{NH-CH}$ arises from rapid rotation because in most cyclic peptides, and in proteins, ψ_{Pro},ϕ_{Leu} rotation is too fast to be observed by NMR. Only in (L-Pro-Gly-L-Pro-Gly) is the rotation sufficiently slow to be observed.[25] In this case in Me_2SO, line broadening is evident at 0 °C and, at −30 °C, two sets of resonances are observed which coalesce at about −15 °C. At −30 °C the chemical shifts and coupling constants of the nonexchangeable protons of L-Pro^1-tentoxin (and tentoxin) are close to the values obtained at 30 °C (±0.02 ppm) except for the leucyl protons. The leucyl α proton shifts from 4.11 ppm at 30 °C to 4.34 ppm at −30 °C and the δ resonance at 0.51 ppm is shifted farther upfield to 0.44 ppm at −30 °C. Comparable upfield shifts (0.1 ppm) of the leucyl β and γ protons are evident. These results indicate that the conformation about the leucine residue is being affected to the greatest extent by the lowering of temperature and this is consistent with a slowing down of the rotations about ψ_{Pro},ϕ_{Leu}, and possibly also the rotations about the leucyl side chain (χ_1).

The data described indicate that in chloroform L-Pro^1-tentoxin has the conformation shown in Figure 7a. The torsion angles for this conformation are given in Table III.

Conformational Analysis of Tentoxin (1) and *N*-[*methyl*-^{13}C]Alanyl^1-Tentoxin (4). Because the ^{1}H NMR, CD, and biological properties of tentoxin and L-Pro^1-tentoxin are so similar (Figures 2-4) their conformations also should be similar. In order to be certain that the alanyl *N*-Me-C$^\alpha$H vicinal bond angle in tentoxin is similar to the prolyl C$^\delta$N-C$^\alpha$-H vicinal bond angle in L-Pro^1-tentoxin, analogue **4** was synthesized[9] in which the *N*-methyl group on the L-alanine was enriched in ^{13}C. The undecoupled ^{13}C NMR spectrum for **4** is shown in Figure 8. The ^{13}C-N-C$^\alpha$-H coupling constant measured is 5.0 Hz. Using the $^3J_{^{13}CN-CH}$ dihedral angle relationship derived by Ovchinnikov and Ivanov[26] a value of 5.0 Hz indicates a C$^\delta$-N-C$^\alpha$-H vicinal bond angle of about 127°. The corresponding vicinal bond angle in proline is 135°. Thus, ϕ_1 and ψ_2 for the *N*-methylalanyl^1 residue in tentoxin are very nearly the same as the L-Pro residue in L-Pro^1-tentoxin, and the conformation of tentoxin is as shown in Figure 7b. The torsion angles for tentoxin are given in Table III.

Discussion

The conformation observed for tentoxin and L-Pro^1-tentoxin is a new conformation for the 12-membered cyclic tetrapeptide ring system. Previously, two types of cyclic tetrapeptide conformations had been observed in *cyclo*-(Gly)$_4$[17,26] and *cyclo*-(Sar).[10,11] Grathwohl et al. proposed that *cyclo*-(Gly)$_4$ in solution occurs predominantly in an S_4 symmetrical conformation with the principal symmetry axis perpendicular to the cyclo peptide ring.[17] Theoretical studies of model peptides have indicated that closure of the tetrapeptide could be achieved by reducing the angle τ[28] at the α-carbon atom from 109.5 to 95° or by making the peptide amide groups nonplanar with ω deviating from 180° by ±15-25° or by a combination of these two modifications,[29] and the conformation of *cyclo*-(Gly)$_4$ appears to be consistent with these modifications.

Recently several biologically active cyclic tetrapeptides have been found in nature.[30] The study of one of these, chlamydocin (**5a**) which is a highly cytostatic cyclic tetrapeptide,[32] has provided another example of an all-transoid conformation. An x-ray study of an analogue, dihydrochlamydocin (**5b**), revealed that, in the solid state, **5b** contained four transoid amide bonds with ω twist angles of 18, 14, 24 and 16°[18] (Figure 1c). In addition an average angle of 105° for τ was found for the four corners of the ring system. Thus, to close the chlamydocin tetrapeptide ring, both ω and τ deviate from the normal Pauling-Corey geometry.[33]

In contrast to these systems, *cyclo*-(Sar)$_4$ has been shown by NMR and x-ray studies to have a centrosymmetric con-

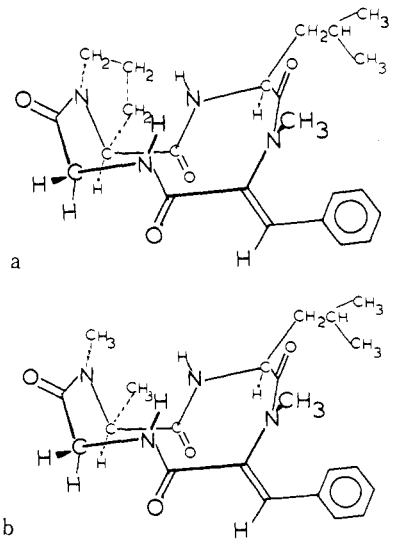

Figure 7. Schematic representation of conformation of (a) L-Pro[1]-tentoxin and (b) tentoxin in chloroform.

Table III. Torsion Angles[a] for L-Pro[1]-Tentoxin and Tentoxin

Residue	L-Pro[1]-Tentoxin	Tentoxin
L-Pro	$\phi = -80°$ [a]	-85 to $-90°$ [a]
(L-MeAla)	$\psi = -10°$	0 to 10°
L-Leu	$\phi = -120°$	-120 to $-130°$
	$\psi = +70°$	$+55$ to $+65°$
MePhe[(*Z*)Δ]	$\phi = -90°$	$-90°$
	$\psi = -20°$	$-20°$
Gly	$\phi = -120$ to $-130°$	-120 to $-130°$
	$\psi = +70°$	$+55$ to $+65°$

[a] Values of ϕ,ψ ±20°.

formation with the amide bond sequence cis,trans,cis,trans (Figure 1b).[10,11] The amide bonds in *cyclo*-(Sar)$_4$ are nearly planar with an average ω twist angle of 5–6° and the angle τ averages 111°. No explanation has been given why *cyclo*-(Sar)$_4$ or *cyclo*-(Sar-Gly)$_2$ adopts alternating cis,trans,cis,trans amide conformations rather than all-transoid conformations. However, since *cyclo*-(Gly)$_4$, which has no α or N substitution, adopts the S_4 conformation it must be the most stable cyclic tetrapeptide conformation as was predicted by the theoretical studies.[29] Methylation of two or more amide nitrogens probably introduces transannular steric interactions which destabilize the preferred S_4 conformation.

The conformation of tentoxin in chloroform is related to the *cyclo*-(Sar)$_4$ conformation (Figure 1b) except that the Gly-MeAla (or Gly-Pro) end of the ring system has undergone a "ring-flip". This "ring-flip" process is analogous to a cyclohexane chair to boat interconversion and corresponds to the reversal of the signs, but not the magnitude, of the ψ_4,ϕ_1 torsion angles. This "ring-flip" process apparently removes a steric interaction between the β-carbon of L-MeAla and the Gly α-carbon. These atoms would be only 2.3 Å apart in the *cyclo*-(Sar)$_4$ centrosymmetric conformation (Figure 1a). Because *cyclo*-(Sar)$_4$ does not adopt the unsymmetrical ring-flip conformation of tentoxin either in solution or in the solid state, the unsymmetrical ring-flip conformation must be less stable than the centrosymmetric conformation. The energy difference between the two ring conformations must be less than the steric interaction that would develop between the Gly and MeAla β-carbon atoms. A recent study of Ramakrishnan and Mangula[36] has shown that the centrosymmetric conformation of *cyclo*-(Sar)$_4$ is about 1 kcal more stable than the conformation in which one end of the ring has been "flipped".

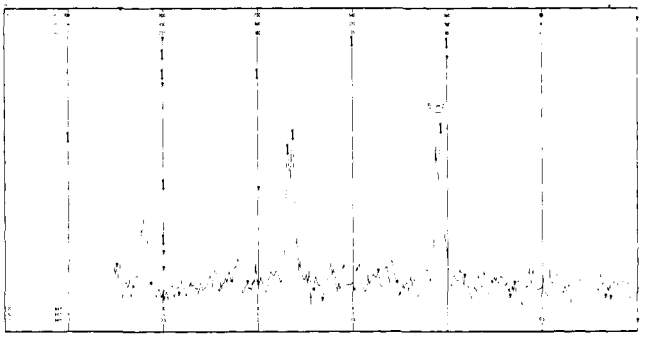

Figure 8. Partial undecoupled ^{13}C NMR spectrum for L-[*methyl*-^{13}C]-alanyl[1]-tentoxin in chloroform. Tetramethylsilane was added as internal reference.

Here also the "ring-flipping" process corresponds to the sign reversal of the torsion angles ψ_4,ϕ_1 while ϕ_2,ψ_2, ϕ_3,ψ_3, and all ω torsion angles are unchanged.

With the exception of the unsaturation in the 5 position, the tentoxin conformation is very similar to the unsymmetrical conformation of a proposed intermediate in the multistep process required for interconversion of the 2 and 8 positions with the 5 and 11 positions of *cyclo*-(Sar)$_4$,[20] and to that calculated by Ramakrishnan.[36] The tentoxin conformation would also be related to the conformation proposed on symmetry arguments for *cyclo*-(L-Pro-Gly)$_2$.[25] The preferred configurational sequence of chiral amino acids in the tentoxin type conformation would be L,L,L,L. *cyclo*-(L-Ala)$_4$[34,35] could adopt this conformation without developing severe transannular steric interactions or it could adopt the all-transoid conformation.

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References and Notes

(1) Taken in part from the Ph.D. Thesis of Pradip K. Bhatnagar, University of Wisconsin—Madison, 1977. Financial support from the National Institutes of Health (GM 19311) is gratefully acknowledged.
(2) G. E. Templeton, *Microb. Toxins*, **8**, 160–192 (1972).
(3) N. D. Fulton, K. Bollenbacher, and G. E. Templeton, *Phytopathology*, **55**, 49–51 (1965).
(4) J. A. Steele, T. F. Uchytil, R. D. Durbin, P. Bhatnagar, and D. H. Rich, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 2245–2248 (1976).
(5) H. S. Penefsky, *Enzymes, 3rd Ed.*, **10**, 375 (1974).
(6) (a) W. L. Meyer, L. F. Kuyper, R. B. Lewis, G. E. Templeton, and S. H. Woodhead, *Biochem. Biophys. Res. Commun.*, **56**, 234–240 (1974); (b) W. L. Meyer, L. F. Kuyper, D. W. Phelps, and A. W. Cordes, *J. Chem. Soc., Chem. Commun.*, 339 (1974); (c) W. L. Meyer, L. F. Kuyper, D. W. Phelps, and A. W. Cordes, *J. Am. Chem. Soc.*, **97**, 3802 (1975).
(7) All amino acids except glycine are of the L configuration unless noted. Standard abbreviations for amino acids, protecting groups, and peptides are recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.*, **247**, 977, (1972)) are used. Additional abbreviations are: MePhe[(*Z*)Δ], *N*-methyl-(*Z*)-dehydrophenylalanine; MeAla, *N*-methylalanine; Boc, *tert*-butoxycarbonyl; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; TEA, triethylamine; Tcp, trichlorophenol; DCU, dicyclohexylurea. The symbol *Z* designates the configuration of the olefin (zusammen) (IUPAC Nomenclature Commission, *J. Org. Chem.*, **35**, 28 (1970)).
(8) D. H. Rich and P. Mathiaparanam, *Tetrahedron Lett.*, 4037–4040 (1974).
(9) D. H. Rich, P. Bhatnagar, P. Mathiaparanam, J. A. Grant, and J. P. Tam, *J. Org. Chem.*, **43**, 296 (1978).
(10) J. Dale and K. Titlestad, *J. Chem. Soc., Chem. Commun.*, 1403 (1970); 656 (1969).
(11) P. Groth, *Acta Chem. Scand.*, **24**, 780 (1970).
(12) For comparison, the carbon–carbon distance between methyl groups in a 1,3-diaxially substituted cyclohexane is 2.6 Å. This interaction is destabilized by about 5.5 kcal; N. L. Allinger and M. A. Miller, *J. Am. Chem. Soc.*, **83**, 2145 (1961).
(13) K. D. Kopple, A. Go, and D. R. P. Lipanskas, *J. Am. Chem. Soc.*, **97**, 6830 (1975).
(14) The leucine NH proton lies under the aromatic protons. Downfield shifts upon dilution were observed for this proton comparable to that observed for the Gly NH but the shifts were not quantitated.

(15) J. P. Meraldi and V. J. Hruby, *J. Am. Chem. Soc.*, **98**, 6408 (1976), and references cited therein.
(16) D. E. Dorman and F. A. Bovey, *J. Org. Chem.*, **38**, 2379 (1973); D. A. Torchia, J. R. Lyerla, Jr., and C. M. Deber, *J. Am. Chem. Soc.*, **96**, 5009 (1974); V. F. Bystrov, *Prog. Nucle. Magn. Resonance Spectrosc.*, **10**, 41 (1976).
(17) C. Grathwohl, A. Tun-kyi, A. Bundi, R. Schwyzer, and K. Wüthrich, *Helv. Chim. Acta*, **58**, 415 (1975).
(18) J. L. Flippen and I. L. Karle, *Biopolymers*, **15**, 1081 (1976).
(19) ^{13}C NMR data obtained by R. D. Jasensky. We thank Dr. J. Pless of Sandoz Ltd. for sending a sample of chlamydocin.
(20) J. Dale and K. Titlestad, *Acta Chem. Scand., Ser. B*, **29**, 353 (1975).
(21) V. F. Bystrov, V. T. Ivanov, S. L. Portnova, T. A. Balashova, and Yu. A. Ovchinnikov, *Tetrahedron*, **29**, 873 (1973).
(22) D. H. Rich and R. D. Jasensky, unpublished results.
(23) Nomenclature from R. S. Mulliken, *Rev. Modern Phys.*, **14**, 265 (1942).
(24) G. L. Martin and M. L. Martin, *Prog. Nucl. Magn. Resonance Spectrosc.*, **8**, 163–259 (1972).
(25) C. M. Deber, E. T. Fossell, and E. R. Blout, *J. Am. Chem. Soc.*, **96**, 4015 (1974).
(26) Yu. A. Ovchinnikov and V. T. Ivanov, *Tetrahedron*, **31**, 2177 (1975).
(27) R. Schwyzer, B. Iselin, W. Rittel, and P. Sieber, *Helv. Chim. Acta*, **39**, 872 (1956).
(28) G. N. Ramachandran and V. Sasisekharan, *Adv. Protein Chem.*, **23**, 283 (1968).
(29) C. Ramakrishnan and K. P. Sarathy, *Biochim. Biophys. Acta*, **168**, 402 (1968).
(30) Cf.: (a) fungisporin, see R. O. Studer, *Experientia*, **25**, 898 (1969). (b) Roccanin, see G. Bohman, *Tetrahedron Lett.*, 3065 (1970); G. Bohman-Lindgren, *ibid.*, 4625 (1972). (c) Chlamydocin, see A. Closse and R. Huguenin, *Helv. Chim. Acta*, **57**, 533 (1974). (d) AM-I toxin, see T. Ueno, T. Nakashima, Y. Hayashi, and H. Fukami, *Agric. Biol. Chem.*, **39**, 1115–1122 (1975). This toxin has also been called alternariolide; cf. T. Okuni, Y. Ishita, A. Sugawara, Y. Mori, K. Sawai, and T. Matsumo, *Tetrahedron Lett.*, 335–336 (1975). For the synthesis of AM-toxin I, see S. Lee, H. Aoyagi, Y. Shimohigashi, and N. Izumiya, *Tetrahedron Lett.*, 843–846 (1976). (e) Cyl-2, see A. Hirota, A. Suzuki, K. Aizawa, and S. Tamura, *Agric. Biol. Chem.*, **37**, 955–956 (1973).
(31) A. Closse and R. Huguenin, *Helv. Chim. Acta*, **57**, 533 (1974).
(32) H. Stahelin and A. Trippmacher, *Eur. J. Cancer*, **10**, 801 (1974).
(33) L. Pauling, "The Nature of the Chemical Bond", 3rd ed, Cornell University Press, Ithaca, N.Y., 1960, p 282.
(34) J. Dale and K. Titlestad, *J. Chem. Soc., Chem. Commun.*, 255 (1972).
(35) M. Fridkin, A. Patchornik, and E. Katchalski, *J. Am. Chem. Soc.*, **87**, 4646 (1965).
(36) G. Manjula and C. Ramakrishnan, "Proceedings of the Fifth American Peptide Symposium", M. Goodman and J. Meienhofer, Eds., Wiley, New York, N.Y., 1977, pp 296–299.

Isolation and Conformational Analysis of Two Conformers of D-Methylalanine[1]-tentoxin

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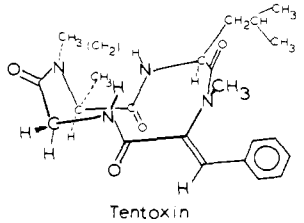
Abstract: D-MeAla[1]-tentoxin, an analogue of the phytotoxic cyclic tetrapeptide, *cyclo*-(L-MeAla[1]-L-Leu[2]-MePhe[(*Z*)Δ]-Gly), has been found to exist in multiple conformations which can be separated by thin-layer chromatography (TLC) and isolated at 4 °C. The two conformers are designated **2U** (upper) and **2L** (lower) based on their faster and slower mobilities, respectively, on TLC. The activation energy (E_a) for the equilibrium **2U** ⇌ **2L** is 23 ± 1 kcal/mol. The conformations of each of the purified conformers, **2U** and **2L**, as well as the analogue, D-Pro[1]-tentoxin, have been studied by ^1H and ^{13}C NMR, and by ultraviolet and circular dichroism spectroscopy. The data show that at room temperature conformer **2L** is a mixture of rapidly equilibrating conformers $\mathbf{2L_1}$ and $\mathbf{2L_2}$ and that the E_a for interconversion is 13 ± 1 kcal. The conformations of $\mathbf{2L_1}$ and $\mathbf{2L_2}$, determined at −30 °C, have the following torsion angles: $\mathbf{2L_1}$ ϕ_1 +60°, ψ_1 −150°; ϕ_2 −120°, ψ_2 +60°; ϕ_3 −90°, ψ_3 +160°; ϕ_4 130°, ψ_4 −85°; $\mathbf{2L_2}$, ϕ_1 60°, ψ_1 135°; ϕ_2 −140°, ψ_2 70°; ϕ_3 −80°, ψ_3 −30°; ϕ_4 0°, ψ_4 −90°. A conformation is proposed for **2U**, based on ^1H NMR and CD data, with the following torsion angles: ϕ_1 60°, ψ_1 −160°; ϕ_2 −60°, ψ_2 −60°; ϕ_3 90°, ψ_3 −20°; ϕ_4 140°, ψ_4 −80°. A comparison between the conformations of D-MeAla[1]-tentoxin and the dihydro analogue, *cyclo*-(D-MeAla-L-Leu-L-MePhe-Gly), indicates that dehydro amino acid residues can affect the conformational space available to a peptide.

The cyclic tetrapeptide tentoxin, *cyclo*-(L-MeAla-L-Leu-MePhe[(*Z*)Δ]-Gly) (**1**), is a phytotoxin that causes chlorosis in some plant species but not in others.[1,2] This selectivity has been linked to the presence in sensitive species of a tentoxin binding site on chloroplast coupling factor 1 (CF_1), an isolable protein involved in the photosynthesis of ATP.[3] The structure of tentoxin **1** is known[4] and the conformation of tentoxin shown for **1** has been proposed on the basis of ^1H NMR studies.[5]



Tentoxin

1

In order to study the relationships between the structure, including conformation, and biological activity of tentoxin **1**, as well as to explore the effect of the configurational sequence of constituent amino acids on the conformation of the 12-membered ring system, we have synthesized several tentoxin analogues.[6-8] One of these, *cyclo*-(D-MeAla-L-Leu-MePhe[(*Z*)Δ]-Gly) (**2**), which corresponds to the replacement of L-MeAla with D-MeAla in the 1 position, was found to exhibit unusual conformational properties. We report here the isolation and conformational analysis by NMR of two conformations of D-MeAla[1]-tentoxin (**2**).

Experimental Section

D-MeAla[1]-tentoxin (**2**) was synthesized in 48% yield by cyclization of the linear peptide, D-MeAla-L-Leu-MePhe[(*Z*)Δ]-Gly-O-Tcp, following the reported procedure.[8] The product was purified by preparative silica gel thin-layer chromatography (TLC) eluting with 5% ethanol in ethyl acetate. Two major bands (R_f 0.45, 0.30) were detected and isolated by extraction of the silica gel with ethyl acetate. When the extraction was carried out at 25 °C, each fraction was found to contain approximately equal amounts of both components (R_f 0.45, 0.30), but when the extraction was done at 4 °C each fraction contained only a single component. The smaller R_f component is designated **2L** and the higher R_f component is designated **2U**. The samples of **2U** used in these studies were prepared by equilibrating pure **2L** at room temperature and then rechromatographing the mixture at 4 °C. This procedure was used to eliminate the possible contamination

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