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

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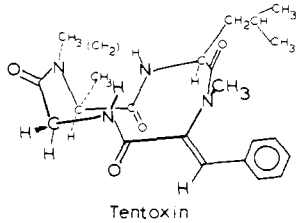
Abstract: D-MeAla[1]-tentoxin, an analogue of the phytotoxic cyclic tetrapeptide, *cyclo*-(L-MeAla[1]-L-Leu[2]-MePhe[(*Z*)Δ]-Gly), has been found to exist in multiple conformations which can be separated by thin-layer chromatography (TLC) and isolated at 4 °C. The two conformers are designated **2U** (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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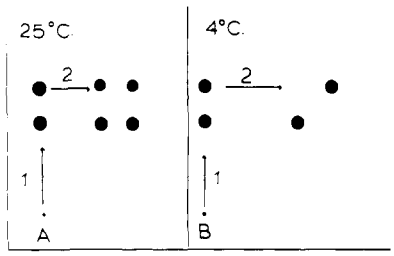


Figure 1. Schematic drawing of TLC properties of D-MeAla[1]-tentoxin. (A) TLC plate maintained at 25 °C after first elution, then developed at right angles in the same solvent system. (B) TLC plate maintained at 4 °C during and after the first elution, then redeveloped at right angles to the direction of the first elution.

of **2U** with tentoxin which has a similar R_f in the TLC solvent system.

The ^{1}H 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-90-E pulse Fourier transform NMR spectrometer interfaced with a Nicolet 1080 computer and disk unit. 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. Solution concentration was varied between 1 and 40 mg/mL. Above 10 mg/mL concentrations, a loss in resolution was observed.

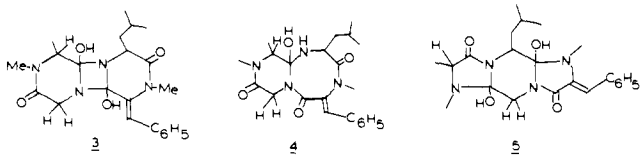
Ultraviolet spectra were determined in methanol using a Cary 14 spectrometer. The circular dichroism spectra were measured on a Cary 16 equipped with a circular dichroism attachment.

Results

Isolation and Characterization of Conformers 2U and 2L. D-MeAla[1]-tentoxin (**2**) was prepared in 48% yield following the reported procedure.[8] Preparative and analytical TLC data established that cyclotetrapeptide **2** was a mixture of two components, designated **2U** and **2L**, which were separable by preparative TLC and present in approximately equal ratios. These components were shown to be in equilibrium by the experiment shown in Figure 1A. D-MeAla[1]-tentoxin **2** was chromatographed on a TLC plate. The plate was let stand at room temperature for 12 h and then redeveloped in the same solvent at right angles to the initial direction of elution. After the second development, four spots were found corresponding to two spots (R_f 0.45, 0.30) developing from each of the components separated by the first elution. Thus each component is in equilibrium with the other.

A study of the temperature dependence established that interconversion is very slow at 4 °C (Figure 1B). Even after 12 h at 4 °C, no equilibration between **2U** and **2L** is observed. These results established that it was possible to isolate each component (**2U** and **2L**) at 4 °C.

The ^{1}H NMR spectrum of the mixture of conformers of D-MeAla[1]-tentoxin (**2U, 2L**) is shown in Figure 2A. The doubling of signals for the phenyl, the two *N*-methyl, and the glycyl protons clearly indicates that two forms of **2** are present and the TLC data previously described establish that both forms are in equilibrium. The question arises whether these are conformers of **2** or one of the possible tautomeric forms **3–5** which could form by one or two transannular insertion reactions. Structures **3–5** were eliminated on the basis of NMR



3 **4** **5**

evidence. Eight amide carbonyl carbons were found in the ^{13}C NMR spectrum of **2**, and the glycine proton coupling constants

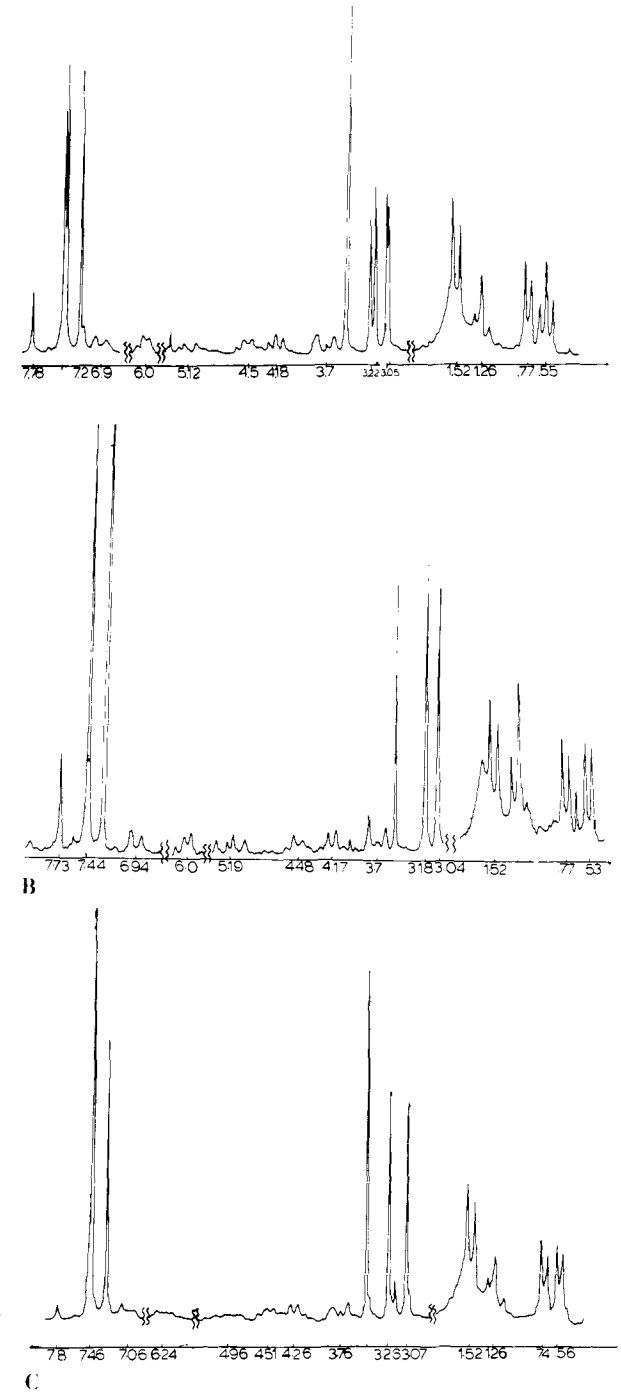


Figure 2. ^{1}H NMR spectra of D-MeAla[1]-tentoxin conformers at 270 MHz in chloroform-*d* at 25 °C. Values reported are in parts per million downfield from internal tetramethylsilane. (A) Mixture of **2U** and **2L** before separation. (B) Spectrum of more mobile on TLC form of D-MeAla[1]-tentoxin (**2U**). (C) Spectrum of less TLC mobile form of D-MeAla[1]-tentoxin (**2L**). Solvent impurities at 3.5 and 7.23 ppm are from methanol and chloroform used to extract the silica gel.

indicated that the glycyl α protons must be adjacent to an NH group in both conformers **2U** and **2L**. Linear structures for **2** were rejected because neither TLC band was ninhydrin positive indicating that no free amino group was present. Thus, it was concluded that **2U** and **2L** were conformers of **2.**

Calculation of Free Energy of Activation for Interconversion of 2U ⇌ 2L. Using dynamic ^{1}H NMR spectroscopy it was possible to calculate the free energy of activation for the interconversion of **2U** ⇌ **2L** by two methods. The coalescence temperature for D-MeAla[1]-tentoxin in bromoform is 130 °C, the difference between resonances is 4.23 Hz, and the differ-

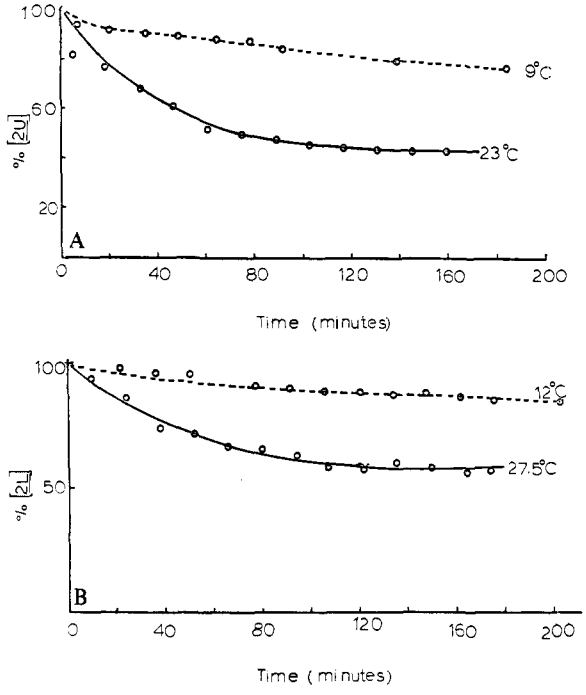


Figure 3. Kinetics of equilibration between conformers of D-MeAla^1-tentoxin. (A) Disappearance of **2U** with respect to time at 9 and 23 °C. (B) Disappearance of **2L** with respect to time at 12 and 27.5 °C. Kinetics were followed by measuring the decrease in the corresponding N-CH_3 resonances in the ^{1}H NMR in chloroform-*d*.

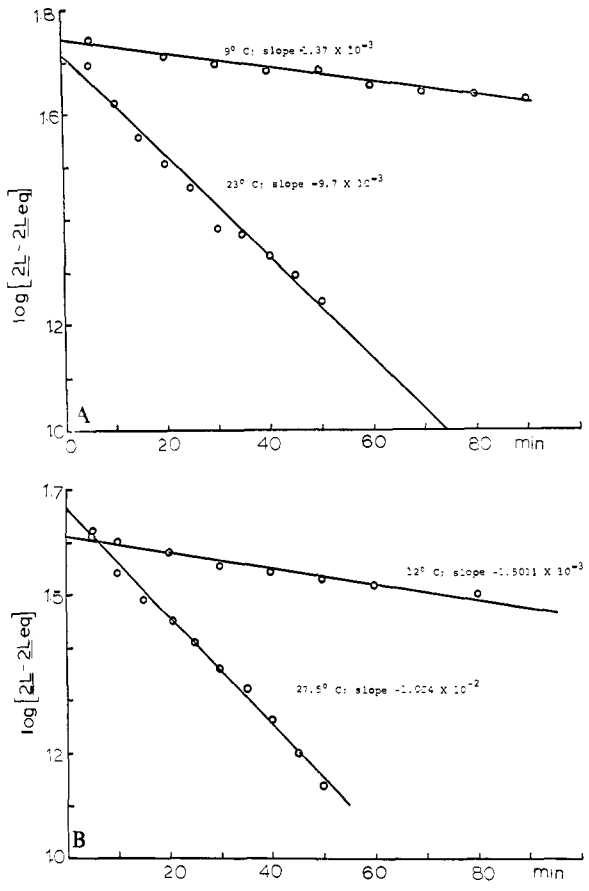


Figure 4. Data in Figure 3 replotted according to the equation for a first-order approach to equilibrium: $\log([\mathbf{2L}] - [\mathbf{2L}_{eq}]) = -[(k_f + k_r)/2.303] \cdot \tau + \log([\mathbf{2L}] - [\mathbf{2L}_{eq}])$. (A) [**2L**] is concentration of species **2L** at time τ; [$\mathbf{2L}_{eq}$] is concentration of **2L** at equilibrium. (B) [**2U**] is concentration of species **2U** at time τ; [$\mathbf{2U}_{eq}$] is concentration of **2U** at equilibrium.

ence between the relative concentration of each conformer is 0.1 mole fraction. Using these values and the method of Shanan-Atidi and Bar-Eli,[9] the free energy of activation is 22 ± 1 kcal/mol and the free-energy difference between the two conformers is 250 cal/mol.

Because it was possible to isolate each conformer it was possible to estimate the Arrhenius activation energy (E_a) for the reversible process using the following equation for a first-order approach to equilibrium:

$$\log \frac{k_1}{k_2} = \frac{E_a}{2.303R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right)$$

where E_a = Arrhenius activation energy: k_1 and k_2 = rate constants at temperatures T_1 and T_2: and R = 1.98.

The rate for the forward and reverse reactions can be followed by monitoring the time-dependent changes in the ^{1}H NMR spectrum of each pure conformer. Figure 3A shows the rate of disappearance of **2U** at 9 and 23 °C and Figure 3B shows the rate of disappearance of **2L** at 12 and 27.5 °C. The plots of log ($A - A_{eq}$) vs. time are shown in Figures 4A and 4B. The values 23.1 and 24.19 kcal/mol were calculated for the activation energies.

From these results it is concluded that the free energy of activation for interconversion of D-MeAla^1-tentoxin conformers is about 23 ± 1 kcal and the two conformers are separated by less than 1 kcal/mol. This value for the activation energy is reflected in the short half-life for interconversion (about 20 min) at room temperature. The observed activation energy of 23 kcal/mol is near the minimum barrier permitting separation of two rotamers.[10]

Conformational Analysis of 2U and 2L by NMR. The ^{1}H NMR spectra of the upper (**2U**) and lower spots (**2L**) (at room temperature) are shown in Figures 2B and 2C. The chemical shifts and coupling constants are given in Table I.

A preliminary analysis of the data in Table I indicated that at least one of the conformers **2U** or **2L** was unlike any cyclic tetrapeptide conformation known to us,[11-13] and therefore it was decided to synthesize and study D-Pro^1-tentoxin (**6**). The replacement of D-MeAla with D-Pro in the 1 position would allow us to determine the geometry of the Gly-Pro amide bond and to confirm that the 12-membered ring system retained the basic cis,trans,cis,trans amide bond sequence found in tentoxin.[14]

D-Pro^1-tentoxin (**6**) was synthesized as previously described.[8] TLC and ^{1}H NMR data (Figure 5, Table II) showed that the D-Pro analogue **6**, like the D-MeAla^1-tentoxin **2**, was an equilibrating mixture of conformers. However, while these could be separated by TLC, the rate of interconversion is somewhat faster and hampered our attempts to purify each conformer. Nevertheless, the ^{1}H NMR and CD data for the D-MeAla^1 **2** and D-Pro^1 **6** mixtures (Figure 6) established that the conformations of the two molecules were similar, and would have the same amide bond sequences.

The ^{13}C NMR spectrum of D-Pro^1-tentoxin **6** showed five sets of resonances between 20 and 35 ppm (20.57, 21.01, 21.23, 24.3, 31.45, and 31.79 ppm). Those at 24.3 (2 peaks) and 31.45 and 31.79 ppm were well resolved and assignable to the leucyl γ-carbons and prolyl β-carbons, respectively, as paired sets of conformers. Only three lines were resolvable from 21 to 22 ppm but the signal intensities suggested overlap of several resonances.

If the two resonances at 24.3 are due to the Leu γ-carbons only, then the prolyl γ-carbon lies under the 20–22 resonance and the Gly-Pro bond is cis. This assignment appears reasonable as the difference between the 24.3- and 31.45-ppm resonances is 7.15 ppm which lies outside the <6.0-ppm difference for trans amide bonds.[15] A carbonyl carbon resonance at 165 ppm is also consistent with the *cis*-Gly-Pro bond.[12] Also, the

Table I. ^{1}H NMR Data for D-MeAla[1]-tentoxin Conformers[a]

	D-MeAla[1]		L-Leu		MePhe[(Z)Δ]		Gly	
2L	H^α	4.37	H^α	4.21	*N*-Me	3.25	H_o	3.48
	H^β	1.50	H^δ	0.58 (d)	C_6H_5	7.41	H_i	4.9 (br s)
				0.74 (d)				
	N-Me	3.06	NH	6.34	H^β	7.22	NH	6.93
2U	H^α	4.48	H^α	4.17 (6)	*N*-Me	3.18	H_o	3.7 (<1, 16)
	H^β	1.52	H^δ	0.53	C_6H_5	7.44	H_i	5.19 (10, 16)
				0.77				
	N-Me	3.04	NH	6.0 (6)	H^β	7.73	NH	6.94 (10, <1)

[a] Chemical shifts reported in parts per million from internal Me_4Si in chloroform-*d* at 27 °C. Coupling constants reported in hertz. Only the vicinal NH-C$^\alpha$H and ring geminal coupling constants are reported.

Table II. ^{1}H NMR Spectrum of Equilibrium Mixture of D-Pro[1] Conformers[a]

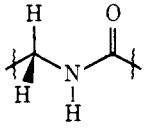
D-Pro[1]		L-Leu		MePhe-[(Z)Δ]			Gly	
H^α	4.34 (9)	H^α	4.48 (6)	*N*-Me	3.2	H_o	3.72 (16, <2)	
H^β	2.2-1.9	H^δ	0.54	C_6H_5	7.45	H_i	5.11 (16, 10)	
			0.77		7.38		4.60 (br s)	
H^γ	1.93	NH	6.42 (6)	H^β	7.74	NH	7.33 (10, <2)	
H^δ	3.6				7.17			

[a] Chemical shifts reported in parts per million from internal Me_4Si in chloroform at 27 °C. Coupling constants reported in hertz.

^{1}H NMR $^3J_{NH-C^\alpha H}$ for proline appears as a doublet which is consistent with the *cis*-Gly-Pro bond.[16] The Leu-MePhe[ZΔ]-amide bond is assigned a cis configuration on the basis of the high extinction coefficient (ε 19 800) for **2** and **6** as was found for tentoxin.[5] The ^{13}C NMR data and the CD/UV data together indicate that the conformations of D-MeAla[1]- and D-Pro[1]-tentoxin are similar and that each contains a cis,trans,cis,trans amide bond sequence.

Analysis of ^{1}H NMR Data for D-MeAla[1]-Tentoxin (Lower) (2L). The ^{1}H NMR spectrum (Figure 2C) of D-MeAla[1]-tentoxin (**2L**) at room temperature does not resemble the typical *cyclo*-(Sar)$_4$ peptide spectra. The signals are broad, the glycyl protons are not resolved nor separated by the 2 ppm usually seen in these systems, and the vinyl proton is broadened and upfield. The line width and chemical shift of the vinyl proton and glycyl protons suggested that the lower conformer was not a single species but two (or more) rapidly equilibrating conformers. This hypothesis was not unreasonable since Dale and Titlestad[11] had shown that (Sar-Gly-Sar-Gly) coalesced at 20 °C.

Thus, it was decided to measure the ^{1}H NMR spectrum of **2L** at −30 °C (Figure 7 and Table III). At −30 °C two conformations are clearly visible. A glycine unit typical of the *cyclo*-(Sar)$_4$ system (5.1 ppm, J = 15.5, 10 Hz; 3.68 ppm, J = 15.5 Hz) is evident and indicates the partial structure **7** for



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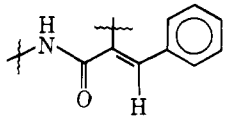
the reasons described previously.[5] A deshielded vinyl proton (7.77 ppm) appears and this is consistent with the partial S-cis structure of **8** for the reasons described previously in the de-



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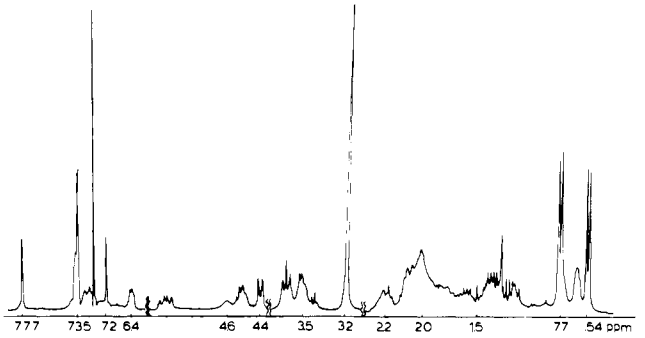
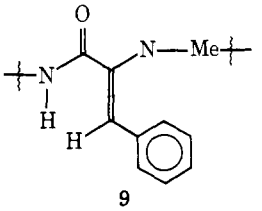


Figure 5. ^{1}H NMR spectrum (270 MHz) of D-Pro[1]-tentoxin at 25 °C in chloroform-*d*. Spectrum is of mixture of conformers. Signal at 7.23 ppm is from chloroform.

termination of the tentoxin conformation.[5] A second vinyl proton resonance appears at 7.26 ppm and can be assigned to the *S-trans*-dehydrophenylalanine **9**.[5,17] A second set of gly-



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cine resonances is found at 4.22 ppm (J = 18, 7.5 Hz), 3.5 ppm (18 Hz), and NH 7.86 ppm (7.5 Hz). The 7.5-Hz $^3J_{NH-CH_i}$ is consistent with vicinal bond angles ±30 or ±140°, and the $^3J_{NH-CH_o}$ < 1-2 Hz is consistent with a vicinal bond angle of 90 ± 20°. Because these new resonances are seen only at −30 °C, it is unlikely that the 7.5-Hz coupling constant is an average ^3J from equilibrating rotomers.

The data in Table III are compatible with the conformations shown in Figure 8. It should be stressed that the deshielded vinyl proton (7.77 ppm) belongs to conformer $\mathbf{2L_2}$ where it is adjacent to the carbonyl and that the vinyl proton in $\mathbf{2L_1}$ is the 7.26-ppm signal. Notice that it is not possible for the glycyl inner proton, the carbonyl group, and the vinyl proton to become coplanar simultaneously. Rotation of the ψ_1,ϕ_2 angle of [MeAla[1]-Leu[2]] is probably fast and the Leu-NH-H$^\alpha$ coupling constant (~6 Hz) is consistent with an average J caused by rapid equilibration among rotomers. The other glycyl unit (4.22 ppm, J = 18, 7.5 Hz) is consistent with $\mathbf{2L_2}$ with coupling between the NH and both glycyl protons. The 18-Hz geminal coupling is consistent with the inner-outer geometry and not the pseudo-axial-equatorial geometry but this is not the same arrangement of atoms found in the 5 and 11 positions of *cyclo*-(Sar)$_4$ and therefore the numerical agreement (18 Hz) may be coincidental. The vicinal bond angles Gly-NH-CH$_i$ = −30° and Gly-NH-CH$_o$ = +90° are in good agreement with angles estimated from the coupling constant data.

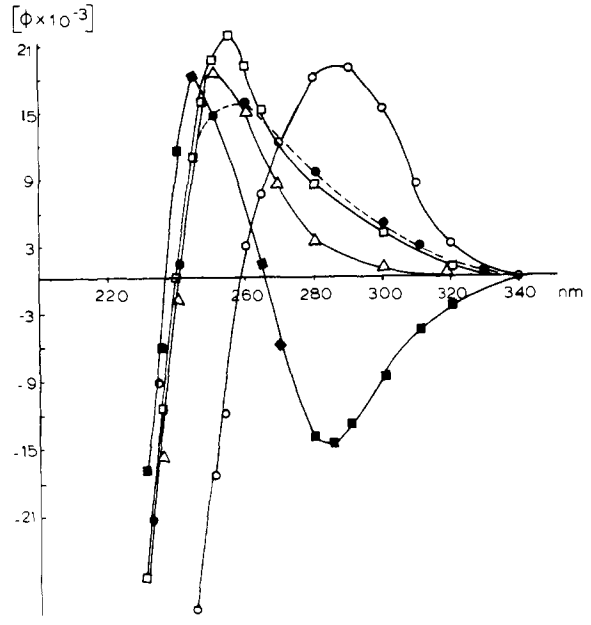


Figure 6. Circular dichroism spectra of tentoxin analogues at 25 °C in methanol: tentoxin (**1**) (○—○); D-MeAla[1]-tentoxin, mixture of both conformers **2L** and **2U** (□—□); D-Pro[1]-tentoxin, mixture of both conformers (△—△); lower R_f conformer of D-MeAla[1]-tentoxin (**2L**) (●- -●); higher R_f conformer of D-MeAla[1]-tentoxin (**2U**) (■—■).

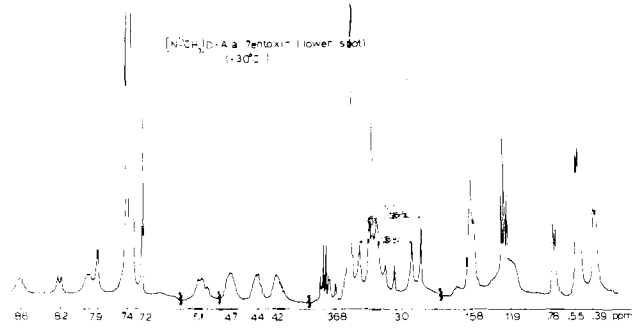
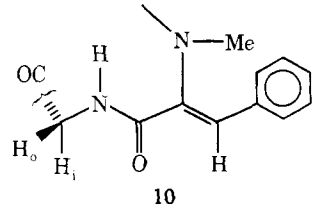


Figure 7. ^{1}H NMR spectrum (270 MHz) of lower R_f conformer of D-MeAla[1]-tentoxin (**2L**) taken at −30 °C in deuteriochloroform. Compound used was labeled with ^{13}C on the alanine *N*-methyl group (75% ^{13}C). Signal at 3.52 ppm from residual methanol.

Analysis of the ^{1}H NMR Data for D-MeAla[1]-Tentoxin (Upper) (2U). The data for this conformer (Table I) are similar in many respects to that of tentoxin. For example, the downfield glycyl inner proton (5.19 ppm, J = 16, 10 Hz) and vinyl proton (7.73 ppm) are almost identical with those of tentoxin[5] and indicate that a similar MePhe[(*Z*)Δ]-Gly geometry predominates and the vinyl proton, the glycyl inner proton, and the dehydrophenylalanyl carbonyl must be approximately coplanar (±30°) (**10**).



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However, there are major differences between the spectra of the compounds. The Leu-NH appears at 6.0 ppm. This is an exceptionally upfield amide proton that strongly suggests it must be in a shielding environment. The Leu-NH is not shielded in tentoxin[5] nor in any of the model *cyclo*-(Sar-Gly)$_2$ peptides.[11] The other major difference between **2U** and **1** is

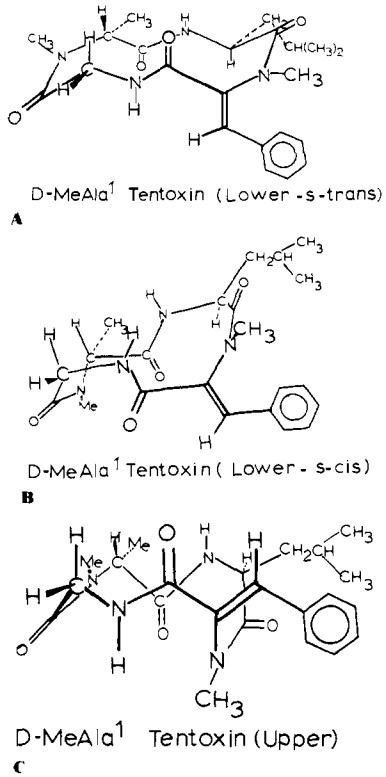


Figure 8. Schematic representations of conformations found for D-MeAla[1]-tentoxin: (A) conformer **2L$_1$**; (B) conformer **2L$_2$**; (C) conformer **2U**.

seen in the circular dichroism spectra of tentoxin and the D-MeAla[1]- and D-Pro[1]-tentoxins (Figure 6). The ellipticity in all tentoxin analogues is positive near 280 nm except in the case of **2U**. This indicates that there are major conformational differences between **2U** and **2L** particularly in the region of the dehydrophenylalanyl residue.

Amide Bond Sequence of 2U. The previously described ^{13}C NMR spectrum of the D-Pro[1]-tentoxin mixture establishes both conformations as having the *cis*-Gly-Pro bond. The geometry between Leu-MePhe[(*Z*)Δ] was assigned a cis amide bond based on the high extinction coefficient (ε 19 700) for the mixture of D-MeAla[1]-tentoxin (**2**) conformers. It was not possible to weigh each conformer separately but the extinction coefficients for each conformer could not differ by much since the UV absorbance did not change with time when allowing either conformer to equilibrate.

Thus, the amide bond sequence for both D-MeAla[1]-tentoxin (**2**) conformers was assumed to be cis-trans-cis-trans. This, plus the MePhe[(*Z*)Δ]-Gly geometry (**7**) previously described on the basis of the ^{1}H NMR data, limited the upper conformer (**2U**) to the conformation proposed in Figure 8C in which there is a rapid rotation of the $\psi_{Ala},\phi_{Leu'}$ angle.

Conformation 8C is a minor variation of the conformation found for a cyclotetradepsipeptide[18] and is consistent with the anomalous spectral data. It explains the upfield absorption of the Leu-NH because this is directly over the double bond where it would be shielded. Also, this conformation is consistent with the circular dichroism data in that the orientations of the double bond and the Leu α proton across the ring from the double bond are almost exactly reversed from the orientation of these units in tentoxin. This is consistent with the opposite signs but similar ellipticities in the CD spectra due to the dehydrophenylalanine absorption.

Discussion

D-MeAla[1]-tentoxin **2** was synthesized to study the effects of configurational sequence of the constituent amino acids on

Table III. ^{1}H NMR Data for D-MeAla[1]-tentoxin Conformers[a]

	D-MeAla[1]		L-Leu		MePhe[(Z)Δ]		Gly	
2L$_1$[b]	H^α	4.67	H^α	4.41 (7)	*N*-Me	3.33[c]	H_o	3.68 (<2. 15.5)
	H^β	1.58	H^δ	0.39[c]	C_6H_5	7.49[c]	H_i	5.1 (10. 15.5)
				0.55				
	N-Me	3.18[c]		0.77	H^β	7.26	NH	8.22 (10)
			NH	8.6 (7)				
2L$_2$[b]	H^α	4.67	H^α	4.41	*N*-Me	3.27[c]	H_o	3.5 (<1. 18)
	H^β	1.58	H^δ	0.39[c]	C_6H_5	7.39[c]	H_i	4.22 (7.5. 18)
				0.55				
	N-Me	3.03[c]		0.77	H^β	7.77	NH	7.8 (7.5)
			NH	8.57				

[a] Chemical shifts reported in parts per million from internal Me_4Si. Coupling constants in hertz. Only vicinal NH-C$^\alpha$H and ring geminal coupling constants reported. [b] Data obtained at −30 °C in deuteriochloroform. [c] Assignment of δ protons to individual conformer is ambiguous.

the biological activity and conformational properties of tentoxin. At the outset it seemed probable that of the known cyclic tetrapeptide conformations, D-MeAla[1]-tentoxin would adopt a conformation closest to *cyclo*-(Sar)$_4$. The *cyclo*-(Sar)$_4$ conformation prefers a L,L,D,D (or D,D,L.L) sequence of chiral amino acids[11] and would readily accept the configurational sequence (Gly,D,L,Δ) found in **2.**

In fact three conformations have been detected for D-MeAla[1]-tentoxin and these can be separated by thin-layer chromatography into two components designated the lower and upper conformers. At 25 °C the lower R_f material **2L** is actually a rapidly equilibrating mixture of two *cyclo*-(Sar)$_4$ conformers, **2L$_1$** and **2L$_2$** (Figure 8A,B), which differ by a rotation about the $\psi_3.\phi_4$ bonds, and which can be observed at −30 °C. The activation energy (E_a) for this rotation can be calculated by coalescence ^{1}H NMR spectrometry.[9] The coalescence temperature was estimated to be 4 °C and the E_a calculation to be ca. 13 ± 1 kcal/mol. This value is comparable to the activation energy (15 kcal/mol) in Me_2SO for the ψ,ϕ' rotation in *cyclo*-(L-Pro-Gly-L-Pro-Gly).[19]

Surprisingly, D-MeAla[1]-tentoxin (and D-Pro[1]-tentoxin) also adopts an additional conformation (designated **2U** because it is more mobile on TLC) which is separated from **2L** by an energy barrier of 23 ± 1 kcal/mol. The circular dichroism spectrum shows that the orientation of the Leu α proton to the double bond of the methyldehydrophenylalanine system in **2U** is very different from the arrangement found in tentoxin. The opposite sign for the ellipticity suggests that the orientation of these two units in conformer **2U** is approximately a mirror image to the orientation found in tentoxin, and this is the arrangement of these two units in the conformation proposed for **2U** (Figure 8C). Conformation **2U** also provides a rationalization for the unusually high-field leucine NH resonance in the ^{1}H NMR. This proton lies in the shielding cone of the double bond in **2U** and would be expected to be found upfield.

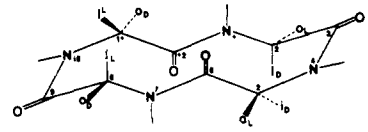
Molecular models show that conformer **2U** is derived from conformer **2L$_1$** by "ring-flipping" the Leu-MePhe (Δ) end of the 12-membered ring system. This "ring-flipping" process is analogous to a chair-to-boat conformational interconversion in a cyclohexane ring system. Formally the process corresponds to rotations about the ϕ_2,ψ_2 and ϕ_3,ψ_3 bond angles leading to a conformation in which the signs of $\psi_{Leu},\phi_{MePhe(\Delta)}$ are reversed but the magnitudes are unchanged. The ϕ,ψ values for Gly and MeAla plus all ω values do not change. Torsion angles for conformers **2L$_1$**, **2L$_2$**, and **2U** are given in Table IV. The major difference between **2L$_1$** and **2U** is in the sign of the ψ_{Leu} and $\phi_{MePhe(\Delta)}$ torsion angles.

Recent calculations by Ramakrishnan and Mangula[20] have shown that several cis,trans,cis,trans conformations are possible for cyclotetrasarcosyl and that these are about 1 kcal/mol

Table IV. Torsion Angles (Degrees) for D-MeAla[1]-tentoxin Conformers[a]

Residue		**2L$_1$**	**2L$_2$**	**2U**
D-MeAla	ϕ	+60	+60	+60
	ψ	−150	−135	−160
L-Leu	ϕ	−120	−140	−60
	ψ	+60	+70	−60
MePhe[(Z)Δ]	ϕ	−90	−80	+90
	ψ	+160	−30	−20
Gly	ϕ	+130	0 ± 10	+140
	ψ	−85	−90	−80

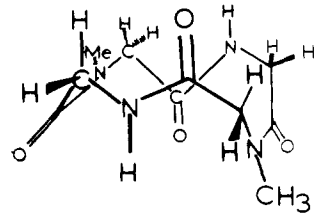
[a] All values of ϕ,ψ are ±10-20°: ω is 180 or 0 ± 10°.



11

higher energy state than the centrosymmetric form **11** found in solution[11] and in the crystal state.[21] With the exception of the double bond in the MePhe[(Z)Δ] residue. these calculated conformations closely resemble those found for D-MeAla[1]-tentoxin. Conformer **2L$_1$** is analogous to the centrosymmetric *cyclo*-(Sar)$_4$ conformation **11** with respect to the 12-membered ring system. and **2L$_2$** is derived from **2L$_1$** by a 180° rotation about $\psi_{MePhe(\Delta)},\phi_{Gly}$.

The Ramakrishnan-Mangula calculations[20] also show that. in the absence of steric limitations imposed by α substitution on the constituent amino acids, a "ring-flipped" conformation **12** corresponding to a sign reversal of ψ_2,ϕ_3 is possible for a



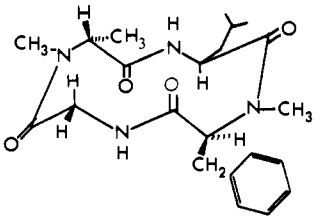
12

cyclic tetrasarcosine and differs from the centrosymmetric conformation by only about 1 kcal/mol. Thus, with the exceptions of the α-substitution patterns and the MePhe[(Z)Δ] double bond, the "ring-flipped" *cyclo*-(Sar)$_4$ conformation calculated by Ramakrishnan and Mangula is closely analogous

to conformer **2U** we propose on the basis of the NMR and CD data for D-MeAla[1]-tentoxin.

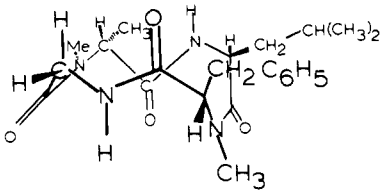
Many dehydro amino acid containing peptides have been identified in nature.[22] It is important to determine if the dehydro residue has any function in addition to the biosynthetic[23] and alkylating[24] roles proposed for these amino acid derivatives.

A comparison between the conformations of D-MeAla[1]-tentoxin **2** and the dihydro analogue, D-MeAla[1]-dihydrotentoxin (**13**), illustrates that dehydro amino acid residues can



N-Me-D-Ala-
N-Me-L-Phe DHT
13

affect the conformational space available to a peptide. ^{1}H NMR evidence indicates that D-MeAla[1]-dihydrotentoxin (**13**) has the conformation shown.[7] With the exception of the α substituents, the ring conformation is centrosymmetric. ^{1}H NMR and TLC data show that the conformation of **13** is very stable and that the conformer **14**, which would be formed by



14

"flipping" the Leu-L-MePhe end of the ring system and which is analogous to **2U** except for the double bond, is not observed within the limits of detection by NMR.

Molecular models of **14** reveal that the MePhe β-carbon is only 2.5 Å from the Leu α-carbon and, depending on small changes in the ring torsion angles, between 1.5 and 2.3 Å from the Leu β-carbon. Either of these interactions is very hindered and would be destabilized by more than 5 kcal/mol.[26] In contrast, molecular models of **2U** show that the dehydrophenylalanyl β-carbon is about 3.4 Å from the leucyl α-carbon so that the severe steric hindrance does not develop and, consequently, conformer **2U** is present. Thus it is clear that the presence of a dehydro residue in a peptide permits certain conformations not possible when that unit is saturated.

These results can be important to the interpretation of structure-activity studies of dehydro amino acid containing peptides which have been modified by reduction or by addition of a nucleophile. Loss of biological activity as a result of either of these synthetic procedures indicates that the dehydro residue is important to activity of the molecule but this could be due to either loss of an alkylating functional group or to a change in the conformational space available to the peptide. The effect of reduction of the dehydrophenylalanine residue in tentoxin on the biological activity has been described.[7,25]

A comparison between the biological activities of **2L** and **2U** has been carried out. The activity of **2L** approaches that of tentoxin while **2U** is much less active. These results will be reported in detail separately.[27]

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