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Use of ^{1}H Nuclear Magnetic Resonance Spectroscopy for Sequence and Configuration Analysis of Cyclic Tetrapeptides. The Structure of Tentoxin[1,2a–d]

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Received July 23, 1974

Abstract: Tentoxin, a metabolite of *Alternaria tenuis* Nees. which induces chlorosis in germinating seedlings of many dicotyledonous plant species, was previously shown to be a cyclic tetrapeptide containing one unit each of glycine, L-leucine, *N*-methylalanine, and *N*-methyldehydrophenylalanine. Detailed analysis of ^{1}H NMR data from tentoxin and its dihydro, *N,N*-dimethyl, and *N,N*-dimethyldihydro derivatives permits deduction of the full structure, sequence, and configuration of the natural product as cyclo(L-leucyl-*N*-methyl-(*Z*)-dehydrophenylalanyl-glycyl-*N*-methyl-L-alanyl), with the conformation depicted in structure **1**.

Tentoxin is a phytotoxic metabolite of the pathogenic fungus *Alternaria tenuis* Nees. [*A. alternata* (Fries) Keissler] (ATCC 24127) which causes the cotyledons of germinating cotton and many other dicotyledonous plants to develop severe variegated chlorosis.[3] Additional interest in tentoxin developed with its identification as a cyclic tetrapeptide containing one unit each of glycine, L-leucine, *N*-methylalanine, and *N*-methyldehydrophenylalanine,[1a,3e,f,4,5] for previously no cyclic tetrapeptide had been recognized in nature,[6] and the presence of an α,β-unsaturated α-amino acid unit in its structure placed tentoxin among a small but growing group of antibiotic and phyto-

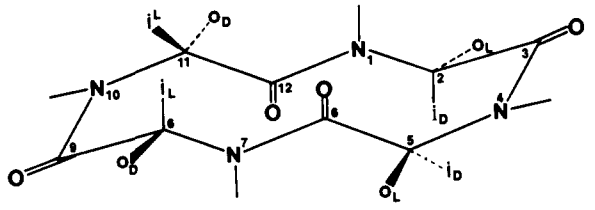
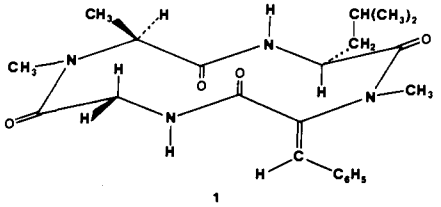


Figure 1. Conformation of the cyclotetrapeptide ring in cyclotetrasarcosyl.[12] Notations D and L denote the configuration of an amino acid unit which will locate its α-alkyl group in the indicated outer (o) or inner (i) conformation.

toxic peptides and related substances which contain this unusual type of functional system.[7] In this paper, we describe in detail the interpretation of ^{1}H NMR data which alone allowed the initial deduction that tentoxin corresponds to the complete amino acid sequence, configuration, and conformation shown in structure **1**.[1b] Although this sequence



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and configuration have been amply confirmed by X-ray structure determination of the dihydro derivative[8] and subsequent synthesis,[9] we believe that these ^{1}H NMR interpretations remain of significant value in their own right. To the best of our knowledge, ^{1}H NMR spectroscopy has not been previously utilized in this way for sequence and configuration analysis in the cyclopeptide field, where conventional sequencing methods often suffer from the absence of end groups, and techniques similar to those illustrated here may well be useful for exploitation in future cases.

The foundations for this analysis were derived from Dale and Titlestad's ^{1}H NMR studies of cyclic tetrapeptides containing glycyl and alanyl units, many of them N-methylated, which led to the proposal that in solution such systems quite commonly adopt the conformation shown in Figure 1.[10,11] This was also found by X-ray crystallography to correspond to the structure of c-Sar$_4$ in the solid state.[12] In view of many resemblances between ^{1}H NMR properties of tentoxin and its derivatives and data reported by Dale and Titlestad for the simpler systems, it seemed probable that this conformation was also involved throughout the tentoxin series, and thus this basic ring geometry was adopted as a working hypothesis during formulation of the sequence and configuration analysis. The absolute configuration of the leucyl unit in tentoxin, previously determined by enzymatic techniques to be L,[4] was taken as a configurational reference point.

Before proceeding with the ^{1}H NMR interpretations, it is necessary to consider certain of the properties of this fundamental cyclotetrapeptide conformation.[10] In the absence of different substituents on the four α carbons and nitrogens, the structure is centrosymmetric, and thus the eight sites for α substituents fall into two enantiomeric sets of four conformationally different locations (2 and 8 inner, 2 and 8 outer, 5 and 11 inner, and 5 and 11 outer[11]). Primarily because of nonbonded interaction between the two syn inner substituents on the same end of the ring, inner substituents are more crowded than are outer substituents,[13] so in the absence of other effects a conformation in which bulky substituents are located in outer positions should be energetically preferred. An L-amino acid residue will have its large α substituent in the outer conformation if it is located at position 2 or 5 (shown as o_L in Figure 1) but in the inner conformation if it is located at 8 or 11 (i_L); the converse is true for a D-amino acid residue (o_D and i_D).

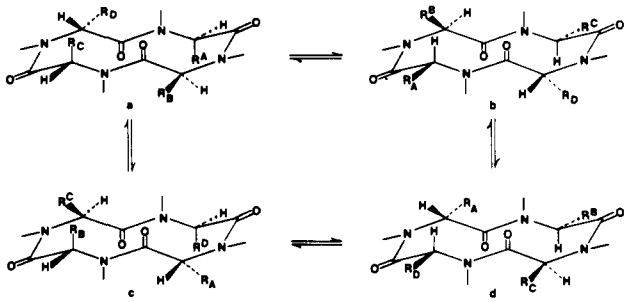


Figure 2. Conformational interconversions available to a D-(R_A)-L-(R_B)-L-(R_C)-D-(R_D) cyclotetrapeptide.

Finally it should be observed that by means of two rotational processes it is possible for the ring to be converted from one conformation to an analogous one which has the same basic skeletal arrangement but in which certain substituent conformations have changed from 2,8 to 5,11 types and from inner to outer.[10] One such process is a complete inversion of the puckered 12-membered ring, similar to the familiar cyclohexane chair–chair inversion. If the four α substituents of conformer a are labeled A, B, C, and D as in Figure 2, it will be seen that this converts conformer a into conformer b, in which residue A is shifted from a type 2 conformational location to type 8, etc. The net result is that positions 2 and 8 become interchanged as do 5 and 11, with every substituent which was originally outer becoming inner and vice versa. The other type of interconversion involves rotation of the two cis amides to trans and vice versa, which converts conformer a to c (or b to d). This results in shifting the amino acid which was at position 2 to position 5 and that which was at position 8 to position 11 *without* changing the inner and outer nature of their substituents, while the amino acid which was at 5 moves to 8 and that from 11 moves to 2 *with* a change of outer substituents to inner and vice versa. As shown in Figure 2, combinations of these two rotations, which appear to usually have accessible activation energies,[10,16] should allow a given tetrapeptide to settle in any of these four possible conformations (a, b, c, or d) which best accommodates its particular steric and other interactions.

Numerous features in the ^{1}H NMR spectrum of tentoxin (Table I) require location of its glycyl unit at position 2 or 8 of the basic framework rather than at 5 or 11. The combination of a large and a very small coupling constant between the glycyl NH and its α-CH's is in good accord with the pair of values expected from the experimental dihedral angle–vicinal coupling relationship of Bystrov et al.[17] for a methylene group at the former but not the latter location.[18] The geminal CH_2 coupling is also in good agreement with the 14.5–15 Hz reported by Dale and Titlestad for 2- and 8-methylenes in model systems[10] but is unacceptably small in comparison with the 18–19 Hz found for 5- or 11-methylenes,[10] where the CH_2 orientation allows better hyperconjugative spin interaction with the adjacent carbonyl π system.[19] Finally, the CH_2 protons have chemical shifts similar to those reported for 2- or 8-methylenes in the model peptides;[10] particularly the inner proton shift and the separation of the two resonances differ drastically from those characteristic of a 5- or 11-methylene.[20]

The leucyl unit of tentoxin also shows a large NH–C_αH spin-coupling. This requires that an inner proton be involved,[18] and its location is thereby restricted to position 2 or position 5, where an L-amino acid can have an inner α hydrogen.

Table I. ^{1}H NMR Properties of Tentoxin and Derivatives in Deuteriochloroform Solution[a]

	Compd 1[b]	2	3a	3b
Glycine unit				
τ NR	~2.03	6.87	3.65	6.91
τ CH (i)	4.93	5.73	5.80	5.70
τ CH (o)	6.49	6.42	6.41	6.45
J CH_2	15.0	17.5	17.2	17.6
J NH–C_αH (i)	10.1		9.5	
J NH–C_αH (o)	[c]		6.0	
Alanine unit				
τ NCH_3	7.23	7.50	7.33	7.46
τ C_αH	5.70	4.54	4.79	4.62
τ CCH_3	8.48	8.72	8.80	8.76
Leucine unit				
τ NR	~2.80	7.15	4.50	7.12
τ C_αH	~5.84	~5.53	~6.00	~5.72
τ CCH_3	9.48	9.00	9.17	9.12
τ CCH_3	9.39	9.00	9.17	9.12
J NH–C_αH	8.9		8.1	
Phenylalanine unit				
τ NCH_3	6.86	6.81	7.18	7.22
τ C_αH			4.34	4.38
τ C_βH	2.43	3.10	6.86	6.78
τ C_βH			6.97	7.06
τ C_6H_5	2.73	2.74	2.80	2.79

[a] Chemical shifts in τ units ±0.03 (±0.05 where indicated ~), coupling constants in Hz, ±0.3. Where resonance assignments were not completely apparent from chemical shifts and splitting patterns alone, they were made with assistance of appropriate proton–proton spin-decoupling and INDOR spectra and other data described in the discussion. Chemical shifts and coupling constants of glycyl $NHCH_2$ and *N*-methylphenylalanyl $CHCH_2$ systems were derived by fitting spectra to theoretical ABC patterns using the program LAOCOON II kindly provided by Professor A. A. Bothner-By of Carnegie-Mellon University. [b] Benzene free. [c] Unresolved; less than 2 Hz.

Placement of the glycyl unit at 2 or 8 and the L-leucyl unit at 2 or 5 leaves six possibilities for the amino acid locations in tentoxin, those with the 2,5,8,11 sequence (a) -Leu-MeΔPhe-Gly-MeAla-, (b) -Leu-MeAla-Gly-MeΔPhe-, (c) -Gly-Leu-MeΔPhe-MeAla-, (d) -Gly-Leu-MeAla-MeΔPhe-, (e) -MeΔPhe-Leu-Gly-MeAla-, and (f) -MeAla-Leu-Gly-MeΔPhe-. Each of these could involve either a D- or an L-*N*-methylalanyl unit and a *Z* or an *E* double bond configuration so 24 potential structures for the toxin molecule require further examination. However, the number of reasonable possibilities becomes remarkably reduced upon consideration of several additional features in the ^{1}H NMR spectrum.[21]

The chemical shift of the leucyl α proton is τ 5.84, whereas those expected are about τ 5.1 for a 2i proton and about τ 6.0 for a 5i proton.[20] Thus if the leucyl unit is located at position 5, its environment must be normal, but if it is at position 2, its α proton must be subject to a considerable shielding influence. That the latter situation is not implausible is evident from the very unusual shielding of the isobutyl group's anisochronous methyls. These resonances are 0.3–0.5 ppm upfield from a normal position and from their position in spectra of all other tentoxin derivatives we have examined. Only the styryl unit could reasonably produce this shielding, and the methyls must therefore be oriented over the plane of its π system. No matter what rotational arrangement is taken within the isobutyl group, such a juxtaposition of its methyls and the styryl unit cannot be attained by the relative leucyl–dehydrophenylalanyl locations -2-L-Leu-11-(*Z*)-MeΔPhe-, -5-L-Leu-8-(*E*)-MeΔPhe-, or -5-L-Leu-11-(*Z*)-MeΔPhe-, and thus structures containing these combinations are unacceptable.[22]

To return to the chemical shift of the leucyl α proton, the shielding which it must experience if it is at C-2 is readily interpretable if the benzylidene group is at C-5 (*E* or *Z*), for the proton then lies directly over the double bond. However, such shielding cannot be accommodated by location of the dehydro system at position 11, and this datum therefore allows exclusion of all structures with -2-Leu-11-MeΔPhe-. If the leucyl unit is at position 5, its α proton chemical shift is normal, not shielded; this would certainly be in accord with simultaneous location of either a (*Z*)- or an (*E*)-benzylidene group at 8 or 11, for in neither instance is the 5i proton in the environment of the π system. Prediction of the shielding effect of a 2-exo double bond on a 5i proton is difficult to make with assurance from models;[23] accordingly we did not exclude such structures from consideration on the basis of this datum alone.

The chemical shift of the glycine 2i or 8i proton shows no evidence of shielding by the π system. This would certainly not be the case for the -8-Gly-11-MeΔPhe- arrangement,[23] so all such structure are excluded. Alanine's *C*-methyl resonance is likewise not at unusually high field (if anything it is somewhat less shielded than normal), so its protons cannot be over the unsaturation as would be required if an L-*N*-methylalanyl unit and the dehydro system were paired in either sense between positions 8 and 11. And finally, *N*-methylalanine's α proton chemical shift is at far too high a field to be of the 2i or 8i type unless it is shielded by the benzylidene group. A 2-L-*N*-methylalanyl unit is thus excluded, for this would require a shielding benzylidene group at 5, and such a combination is inconsistent with the requirements for 2- or 8-Gly and 2- or 5-Leu.

The only structures for tentoxin which are not rendered highly improbable by at least one and usually more of the foregoing ^{1}H NMR criteria are (a) cyclo(-2-L-Leu-5-MeΔPhe-8-Gly-11-MeAla-) (four diastereomeric forms), (b) cyclo(-2-MeΔPhe-5-L-Leu-8-Gly-11-MeAla-) (four diastereomers), (c) cyclo(-2-Gly-5-L-Leu-8-D-MeAla-11-(*E*)-MeΔPhe-), and (d) cyclo(-2-Gly-5-L-Leu-8-(*Z*)-MeΔPhe-11-D-MeAla-). Although analysis of the tentoxin spectrum alone can be pressed further, it is more expedient at this point to turn to the spectrum of *N,N*-dimethyltentoxin for further limitation of the possibilities.

Exposure of tentoxin to dimsyl sodium and methyl iodide in dimethyl sulfoxide[24] or to sodium hydride and methyl iodide in dimethylformamide[25] converts it cleanly to an *N,N*-dimethyl derivative. Numerous ^{1}H NMR spectral features (Table I) as well as a 10-nm hypsochromic shift in its ultraviolet absorption[26] make it apparent that a conformational change (Figure 2) has occurred upon formation of this derivative from tentoxin. The sarcosine CH_2 coupling and CH shifts are now in accord with a 5 or 11 location of that residue.[27] *N*-Methylalanine's α proton chemical shift is too far downfield to correspond to anything but an unshielded 2i or 8i proton, and this unit must thus be either L and at 2 or D and at 8. It also must not be directly followed in the sequence by the dehydro system, which would shield its α proton from 5 or 11, respectively. These conclusions alone, independent of the tentoxin analysis, are consistent with but 12 structures and, of these, all but those with the 2,5,8,11 arrangements -L-MeAla-L-MeLeu-MeΔPhe-Sar- (two diastereomers), -MeΔPhe-Sar-D-MeAla-L-MeLeu- (two diastereomers), and -L-MeLeu-(*Z*)-MeΔPhe-D-MeAla-Sar- correspond to sequences and/or configurations which had been discarded as incompatible with the tentoxin data. Of the ten structures for tentoxin which were retained after analysis of its spectrum, the four cyclo(-2-MeΔPhe-5-L-Leu-8-Gly-11-MeAla-) diastereomers contain the now prohibited -MeAla-MeΔPhe- sequence, as does cyclo(-2-Gly-5-L-Leu-8-D-MeAla-11-(*E*)-MeΔPhe-). The latter is

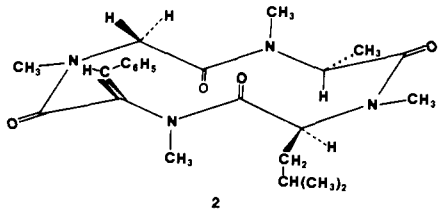
also discordant with the conclusion that glycine and *N*-methylalanine are adjacent (one at a 2,8 location and the other at a 5,11 location in dimethyltentoxin).

If one considers the -2-MeΔPhe-5-Sar-8-D-MeAla-11-L-MeLeu- isomers of dimethyltentoxin from a conformational point of view, they, too, may be dismissed. By the rotational processes discussed above (Figure 2), they could become -2-D-MeAla-5-L-MeLeu-8-MeΔPhe-11-Sar- conformers with a 2i-methyl and 5o-isobutyl rather than the 8o-methyl-11i-isobutyl forms. Inasmuch as all other interactions in the competitive conformations would be identical, it seems almost certain that those with the bulkier isobutyl outside would be preferred. D-*N*-Methylalanine would thus be at 2, not 8, and this is not in accord with the presence of a 2i alanyl α proton which is indicated by ^{1}H NMR.[28]

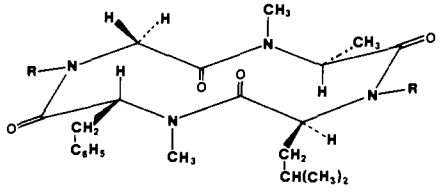
Although again it is possible to carry analysis of this spectrum further, a more direct process is to turn to the spectrum of dihydrotentoxin, with the three remaining possibilities for tentoxin (-L-Leu-MeΔPhe-Gly-L-MeAla- and -Gly-L-Leu-(*Z*)-MeΔPhe-D-MeAla-) foremost in mind. Hydrogenation of tentoxin in methanol over 5% palladium on carbon cleanly produces a single diastereomeric dihydro derivative in which the carbon–carbon double bond has been saturated. Analysis of its spectrum (Table I) is particularly direct, because the anisotropic effect of the double bond has been removed, and all four amino acids have α protons. The α proton resonances of *N*-methylphenylalanine and *N*-methylalanine are too far downfield to be anything but 2,8i types. Thus these two amino acids must be of opposite absolute configurations, they must be nonadjacent in the peptide molecule, and the conformation of this derivative contains either -2-L-MeAla-8-D-MePhe- or -2-L-MePhe-8-D-MeAla-. This datum alone excludes numerous possibilities, including the sequence -Gly-Leu-MePhe-MeAla- which was involved in one of the structures remaining after the foregoing analyses of tentoxin and dimethyltentoxin. These conclusions are supported by the observations that leucine's α proton chemical shift and its spin-coupling to the NH are those expected for a 5i or 11i hydrogen, and that glycine's α protons have the proper chemical shifts, mutual coupling, and NH–C_αH couplings for a 5 or 11 location. Since leucine is L, it must be at the 5 rather than the 11 position, and thus only two structures for dihydrotentoxin satisfy its ^{1}H NMR spectrum, cyclo(-2-L-MeAla-5-L-Leu-8-D-MePhe-11-Gly-) and cyclo(-2-L-MePhe-5-L-Leu-8-D-MeAla-11-Gly-). The latter sequence was excluded on several grounds from analysis of the spectrum of tentoxin alone.

Dihydrotentoxin may also be converted to an *N,N*-dimethyl derivative, and the ^{1}H NMR properties of this compound are very similar to those of its precursor (Table I). Analysis of the α proton chemical shifts and sarcosine's geminal coupling constant in this spectrum also leads to the conclusions that the methylated phenylalanyl and alanyl residues are distributed between positions 2 and 8 with their α protons in the inner orientations, and that sarcosine and *N*-methylleucine are in the 5,11 types of location.

Thus only one amino acid sequence for tentoxin, -Leu-MeΔPhe-Gly-MeAla-, is mutually compatible with these analyses of the ^{1}H NMR and conformational behavior of the natural product and its three derivatives, with the *N*-methylalanyl unit of the L configuration and the *N*-methylphenylalanyl unit in dihydrotentoxin of the D configuration. Accordingly we may assign the structure and conformation cyclo(-2-L-Leu-5-MeΔPhe-8-Gly-11-L-MeAla-) (**1**) to tentoxin, cyclo(-2-L-MeAla-5-L-MeLeu-8-MeΔPhe-11-Sar-) (**2**) to dimethyltentoxin, cyclo(-2-L-MeAla-5-L-Leu-8-D-MePhe-11-Gly-) (**3a**) to dihydrotentoxin, and **3b** to its dimethyl derivative, respectively, and turn to the sole remaining question, that concerning the geometric isomerism at the double bonds of tentoxin and its dimethyl derivative. This configuration should not be altered by the N-methylation conditions and is therefore the same in the two substances.



2



3a: R = H
3b: R = CH_3

The fact that tentoxin adopts conformation **1** in itself serves as evidence regarding the double-bond configuration. The 5-exocyclic double bond and the 6,7-amide group are coplanar, or nearly so, and in such an arrangement there would be a very serious nonbonded interaction between the glycine NH and a *cis*- but not a *trans*-β-phenyl group. The (*E*)-benzylidene system (cis) could adopt the alternative conformation -2-L-MeAla-5-L-Leu-8-(*E*)-MeΔPhe-11-Gly- (cf. **2** with N–H rather than N–CH_3 at 4 and 10 and an (*E*)-MeΔPhe unit), at the energetic cost of converting the two CONH groups from trans to cis and losing coplanarity and any consequent conjugative stabilization of the benzylidene and carbonyl groups,[26] but with the energetic gain of moving the alanine *C*-methyl from inside to outside and relieving the NH–β-phenyl interaction. The NH–β-phenyl interaction appears so severe that it would dominate this balance, and accordingly the fact that tentoxin is -5-MeΔPhe-8-Gly- rather than -8-MeΔPhe-11-Gly- indicates that the *N*-methyldehydrophenylalanyl unit is *Z*.[29] This implication is supported by the observation that the phenyl of a (*Z*)-benzylidene system is appreciably better disposed than is that of its *E* isomer to produce the observed shielding of leucine's *gem*-dimethyl protons.[30]

Additional evidence that the dehydro system is *Z* is found in the remarkable chemical-shift difference between the vinyl protons of tentoxin and its dimethyl derivative. In structure **1** this proton is very close to and in the nodal plane of the 6,7-amide π system, and on shifting to the 8-location after dimethylation (**2**) it exchanges this very deshielding environment for one where, if anything, it is slightly shielded by the 9,10-amide's π system. The very considerable upfield shift accompanying methylation is thus in excellent accord with the (*Z*)-dehydro structures. It would not be anticipated for an (*E*)-dehydro unit, for the pair of structures involved in that instance would not alter the environment of the vinyl proton in the required manner.

Thus these data lead to an assignment of the full structure of tentoxin as cyclo(L-leucyl-*N*-methyl-(*Z*)-dehydrophenylalanyl-glycyl-*N*-methyl-L-alanyl), with the conformation shown in structure **1**. Correspondingly dimethyltentoxin is formulated as the (*Z*)-dehydrophenylalanyl structure **2**. It may be noted that in this analysis every other possibility has been discarded on the basis of several independent lines of reasoning. It should be emphasized that with the exception of the absolute configuration of the leucyl residue, *this analysis has allowed deduction of a unique sequence, configuration, and conformation* of tentoxin and each of its

derivatives *solely from* ^1H *NMR data.* In principle, analogous analysis should be possible for other cyclic tetrapeptides, provided the ^1H NMR data provide reasonable assurance that the basic ring geometry of Figure 1 is present.

These structures also accommodate other data which have been mentioned without interpretation in the preceding discussion. Foremost, perhaps, is the stereospecificity with which tentoxin undergoes hydrogenation. The 5-*N*-methyldehydrophenylalanyl unit of tentoxin (**1**) is quite open to catalyst approach at its outer face, but approach to the inner face would be extremely hindered. Thus hydrogenation would be expected to introduce a 5o proton, producing the D-configuration which has been deduced on independent grounds.

The change in conformation which occurs upon methylation of tentoxin (**1** → **2**) is clearly a consequence of the proximity of the N-7 substituent and the olefinic hydrogen in structure **1**. Whereas interaction of the olefinic hydrogen with a 7-NH is energetically tolerable, the corresponding interaction with a bulkier 7-*N*-methyl is too costly in energy to remain. The change in conformation to remove this interaction is in this case also facilitated by the fact that all four peptide bonds are of the $CONCH_3$ type, so there is little energetic preference as to which two are cis and which two are trans.

The *N*-methyl chemical shifts of these peptides also correlate well with those reported for simpler systems[10] if one takes into account appropriate perturbations due to the anisotropy of the phenyl and benzylidene groups. The glycyl and leucyl *N*-methyl resonances of **2** and **3b** are identified by their absence from spectra of the corresponding dimethyl-d_6 derivatives. With this information, particular resonances can be readily assigned to the α-saturated amino acid units (Table I) on the basis of analogy with the reported data,[10] which showed sarcosyl cis amide and trans amide *N*-methyl resonances near τ 6.95 and 7.15, respectively, with the latter shifted to about τ 7.35 by the 2o-methyl in an *N*-methylalanyl unit,[10] provided it is assumed that a 5o-alkyl group would produce an analogous upfield shift of a cis amide *N*-methyl resonance, and that 2o and 5o isobutyl and benzyl groups would have effects similar to those of methyl. It may be noted that the dehydrophenylalanyl *N*-methyl resonances of **1** and **2**, for which no earlier analogy is available, fall somewhat to low field from the others; assessment of the relative significance of several factors in producing this deshielding will require further data.

The credibility of this ^1H NMR analysis would obviously be strengthened and the validity of the interpretative technique tested by independent and direct confirmation of the configuration of the *N*-methylalanyl units in these compounds and of the *N*-methylphenylalanyl units in the dihydro substances. Accordingly amino acid mixtures from hydrolysis of tentoxin and dihydrotentoxin were exposed to L-leucine *N*-carboxy anhydride to produce corresponding mixtures of L-leucyl dipeptides.[32] Chromatographic comparison of the latter with appropriate authentic dipeptides demonstrated the presence of L-leucine and L-*N*-methylalanine, but not their D enantiomers in both hydrolysates, and of D-*N*-methylphenylalanine, but not its L enantiomer in that from dihydrotentoxin.[1b,33] Very recently, we have also demonstrated by X-ray crystallography that dihydrotentoxin indeed corresponds to the full structure and conformation **3a**.[8] The amino acid sequence, configurations and, in one case, the conformation assigned from the ^1H NMR analysis are thus completely confirmed, and the validity of that analysis is correspondingly supported. It is pertinent to note that had the configurational results been available prior to completion of the ^1H NMR analysis (as in fact they were not), they would have facilitated that analysis by allowing immediate discard of all the contending structures with D-*N*-methylalanyl and L-*N*-methylphenylalanyl units, rather than depending upon ^1H NMR interpretations to eliminate these structures.[1b] However, we would emphasize that the ^1H NMR technique as described can (and did) allow deduction of these configurations *without* relying on these data, which we view as one of the substantial powers of the ^1H NMR method. We would suggest, therefore, that the general nature of the ^1H NMR analysis is perhaps an even more important result of this work than is per se the assignment of a full and correct structure to tentoxin, for similar analytical techniques would appear to have potentially wide utility in deducing sequences, configurations, and conformations in the cyclic peptide field.

Experimental Section[5]

Spectroscopic data were obtained with Perkin-Elmer 337 (ir), Cary 14 (uv) and 60 (ord), and Varian A-60 and Bruker HFX-90 (NMR) spectrometers, the HFX-90 (90 MHz) being used for INDOR and spin-decoupled ^1H NMR spectra. The ^1H NMR reference was Me_4Si as an internal standard. Medium resolution mass spectra were obtained with a Hitachi RMU-6E double-focusing spectrometer with direct sample introduction at an ionization potential of 80 eV; high resolution spectra were obtained from the Florida State University Mass Spectrometry Center. Observed masses of the molecular ion and the most abundant fragments are reported, with parenthetical indication of compositions (if high resolution data were obtained) and relative intensities as percent of base peak intensity. Automated ion-exchange analysis of amino acids and peptides was carried out with a Beckman 120C amino acid analyzer. Melting points were determined on a microscope hot stage (indicated m) or under N_2 in sealed capillaries (c) and are corrected for stem exposure. Microanalyses are by Alfred Bernhardt, Mulheim, Germany (indicated B), Spang Microanalytical Laboratory, Ann Arbor, Mich. (S), or Geller Laboratories, Charleston, W. Va. (G).

Isolation of Tentoxin (1).[34] An 80% ethanol solution of crude tentoxin, obtained by continuous ether extraction of culture filtrates[3b] from *Alternaria tenuis*,[3c] was passed successively through cation and anion exchange resins Dowex 50W-X8 (hydrogen form) and Dowex 2-X8 (hydroxide form), evaporated, and the residue was chromatographed with methanol over basic alumina. Fractions showing chlorosis-inducing activity in the cucumber bioassay[3c] were combined, crystallized, and recrystallized from benzene to afford a benzene solvate of pure tentoxin as colorless prisms: mp 172–175° (m); ir ($CHCl_3$) 3345, 1670, 1630 cm^{-1}; ORD in CH_3OH ($[\alpha] \times 10^{-3}$ in degrees (λ, nm))[35] −0.18 (380), −0.12 (370), −0.07 (360), +0.09 (350), +0.39 (340), +0.98 (330), +1.86 (320, peak), +1.30 (310), −0.15 (300), −2.70 (290), −7.44 (280), −11.50 (270), −13.60 (258, trough), −3.83 (240), +1.58 (233, peak), −9.0 (225); ^1H NMR ($CDCl_3$) τ 2.69 (intensity variable, from C_6H_6 of crystallization), otherwise similar to that described in Table I with slight differences in some chemical shifts;[1a] mass spectrum 414.2270 ($C_{22}H_{30}N_4O_4$, 30), 188.0950 ($C_{11}H_{12}N_2O$, 15), 132.0807 ($C_9H_{10}N$, 15), 131.0733 (C_9H_9N, 21), 116.0498 (C_8H_6N, 20), 114.0559 ($C_5H_8NO_2$, 18), 58.0657 (C_3H_8N, 100). Material of this purity, containing variable amounts of C_6H_6 of crystallization, was used in all degradation and derivatization experiments. This procedure afforded 5 mg of crystalline **1** per l. of culture filtrate.

Anal. Calcd for $C_{22}H_{30}N_4O_4 \cdot \frac{1}{4}C_6H_6$ (molecular ion 414.2266): C, 65.03; H, 7.32; N, 12.91. Found (G) (molecular ion 414.2270): C, 65.01; H, 7.24; N, 12.66.

Benzene was removed by storage at 100° (3 mm) for 24 hr or by recrystallization from ether–pentane to afford pure **1**: mp 168–172° (m); uv max (H_2O) 285 nm (ϵ 17,500[36]); uv max (95% C_2H_5OH) 282 nm (ϵ 20,700); ^1H NMR see Table I.

Anal. Calcd for $C_{22}H_{30}N_4O_4$: C, 63.75; H, 7.30; N, 13.52. Found (B): C, 63.80; H, 6.98; N, 13.39.

***N,N*-Dimethyltentoxin (2).** A mixture of 125 mg (5.21 mmol) of NaH (prepared by repeatedly washing 250 mg of a 50% dispersion in mineral oil with hexane) and 25 mg (60 μmol) of **1** in 2 ml of dimethylformamide (dried over molecular seive 4A) was stirred under N_2 at 20° for 15 min, treated with 0.23 ml (3.7 mmol) of CH_3I, stirred for 20 min at 20°, diluted with 5 ml of brine, and ex-

tracted with CH_2Cl_2 which was washed with brine, dried ($MgSO_4$), and evaporated in vacuo.[25] The residual 27 mg (100%) of yellow oil was chromatographed on 8 g of 100–200 mesh silicic acid (Bio-Sil A, Bio-Rad Laboratories, Inc.). Elution with chloroform removed mineral oil, and 95:5 chloroform–methanol afforded the dimethyl derivative **2** as a pale-yellow oil which solidified after dissolution in hot pentane and evaporation. Crystallization could not be induced, but repeated dissolution in excess pentane, partial evaporation to a cloudy emulsion, and chilling to effect formation of a solid precipitate afforded an analytical sample of **2** as a colorless amorphous powder: mp 88–107° (m); ir ($CHCl_3$) 1680, 1645 cm^{-1}; uv max (H_2O) 275 nm (ϵ 15,100); ^1H NMR see Table I; mass spectrum 442.2578 ($C_{24}H_{34}N_4O_4$, 40), 260.1407 ($C_{14}H_{18}N_3O_2$, 30), 202.1105 ($C_{12}H_{14}N_2O$, 100), 201.1016 ($C_{12}H_{13}N_2O$, 42), 131.0735 (C_9H_9N, 58), 116.0504 (C_8H_6N, 43), 114.0557 ($C_5H_8NO_2$, 86).

Anal. Calcd for $C_{24}H_{34}N_4O_4$: C, 65.14; H, 7.74; N, 12.66. Found (S): C, 64.69; H, 7.79; N, 11.90.

***N,N*-Bis(trideuteriomethyl)tentoxin.** A mixture of 109 mg of a 50% dispersion of NaH in mineral oil (2.27 mmol of NaH) and 1 ml of Me_2SO was heated at 85° for 30 min under N_2, frozen in ice, treated with 27.8 mg (60 μmoles) of **1** and 1 ml of Me_2SO,[24] brought to room temperature, and 0.4 ml of CD_3I (Stohler Isotope Chemicals, 99.5 atom % D) was added in two portions with cooling to prevent boiling. The solution was stirred for 1.25 hr, diluted with 5 ml of water, and extracted with $CHCl_3$ which was dried ($MgSO_4$) and evaporated. The residue was chromatographed with 80:20 chloroform–acetone on a silica gel GF thick-layer plate. Extraction of the band at R_f 0.30 with chloroform–methanol and evaporation left 13.4 mg of the dimethyl-d_6 derivative as an oil: ^1H NMR ($CDCl_3$) like **2** without τ 6.87 and 7.15 singlets; mass spectrum 448.2922 ($C_{24}H_{28}D_6N_4O_4$, 34), 263.1578 ($C_{14}H_{15}D_3N_3O_2$, 24), 205.1285 ($C_{12}H_{11}D_3N_2O$, 100), 204.1202 ($C_{12}H_{10}D_3N_2O$, 44), 131.0732 (C_9H_9N, 64), 116.0499 (C_8H_6N, 47), 114.0551 ($C_5H_8NO_2$, 90).

Dihydrotentoxin (3a). A mixture of 50 mg (0.12 mmol) of **1** and 111 mg of 5% palladium on carbon[37] in 5 ml of CH_3OH was stirred for 50 hr at room temperature under 1 atm of H_2 and filtered. A CH_3OH suspension (25 ml) of the catalyst was refluxed for 2 hr, filtered, and the combined CH_3OH solutions were evaporated to afford 50 mg (100%) of **3a** as a colorless solid which recrystallized from chloroform–dimethyl sulfoxide or from methanol as colorless prisms: mp 277–279° (m);[36] ir ($CHCl_3$) 1670 cm^{-1}; ^1H NMR see Table I; mass spectrum 416.2392 ($C_{22}H_{32}N_4O_4$, 1), 274.1314 ($C_{15}H_{18}N_2O_3$, 18), 269.1200 ($C_{15}H_{15}N_3O_2$, 15), 140.1442 ($C_9H_{18}N$, 14), 134.0966 ($C_9H_{12}N$, 100), 127.0615 ($C_6H_9NO_2$, 20).

Anal. Calcd for $C_{22}H_{32}N_4O_4$: C, 63.44; H, 7.74; N, 13.45. Found (B): C, 63.23; H, 7.56; N, 13.31.

***N,N*-Dimethyldihydrotentoxin (3b).** Methylation of 25 mg (60 μmol) of **3a** as described for **1** afforded 24 mg (90%) of crude **3b** as a colorless oil. Chromatography on silica gel (CH_3OH elution) and repeated recrystallization from ether–pentane afforded the pure dimethyl derivative **3b** as a colorless powder, mp 132–133.5° (m), or as colorless needles, mp 164–166° (m): ir ($CHCl_3$) 1665 cm^{-1}; ^1H NMR see Table I; mass spectrum 444.2744 ($C_{24}H_{36}N_4O_4$, 27), 288.1475 ($C_{16}H_{20}N_2O_3$, 100), 226.1190 ($C_{10}H_{16}N_3O_3$, 29), 216.1021 ($C_{13}H_{14}NO_2$, 70), 156.0890 ($C_7H_{12}N_2O_2$, 45), 134.0966 ($C_9H_{12}N$, 27), 127.0634 ($C_6H_9NO_2$, 31), 100.1126 ($C_6H_{14}N$, 43).

Anal. Calcd for $C_{24}H_{36}N_4O_4$: C, 64.84; H, 8.16; N, 12.60. Found (B): C, 64.68; H, 8.11; N, 12.60.

The corresponding ***N,N*-bis(methyl-d_3) derivative** was prepared identically and chromatographically purified but not crystallized: ^1H NMR ($CDCl_3$) like **3b** without τ 6.91 and 7.12 singlets; mass spectrum 450.3135 ($C_{24}H_{30}D_6N_4O_4$, 30), 291.1674 ($C_{16}H_{17}D_3N_2O_3$, 79), 229.1382 ($C_{10}H_{13}D_3N_3O_3$, 23), 216.1023 ($C_{13}H_{14}NO_2$, 54), 159.1987 ($C_7H_9D_3N_2O_2$, 27), 134.0997 ($C_9H_{12}N$, 17), 127.0683 ($C_6H_9NO_2$, 16), 103.1307 ($C_6H_{11}D_2N$, 26).

Amino Acid Composition and Configuration Analysis of Peptides.[1] A solution of ca. 10 mg of peptide and 2 ml of 6 *N* HCl in a sealed ampoule was heated at 100° for 21–24 hr, cooled, and evaporated in vacuo. The residue was separated by two-dimensional TLC on silica gel using 69:31 1-propanol–water (R_f^a), followed by 85:15 phenol–water (R_f^b),[38a] products being detected by ninhydrin–copper nitrate spray[38b] and identified by comparison of color and mobility with independently chromatographed authentic samples (DL-MeAla prepared by reaction of α-bromopropionic acid with methylamine[39] or by N-methylation of Z-L-Ala,[40] saponification, and treatment with HBr–acetic acid;[41] other synthetic references described below). Spots (R_f^a, R_f^b, color) from the hydrolysate of **1** corresponded to Gly (0.30, 0.20, yellow), Leu (0.51, 0.45, purple), Sar (0.20, 0.34, yellow), MeAla (0.25, 0.40, pink), and CH_3NH_2·HCl (0.00, 0.26, pink). Spots from the hydrolysate of the platinum-catalyzed hydrogenation product of **1** (below) corresponded to Gly, Leu, MeAla, MePhe (0.44, 0.63, pink), *N*-methyl-β-cyclohexylalanine (0.54, 0.77, pink), and CH_3NH_2·HCl.

A chilled (2°) solution of the evaporated hydrolysate corresponding to 20 μmol of peptide in 2 ml of pH 10.2 borate buffer[32] was stirred at high speed in a Vortex stirrer, treated with 3.5 mg (22 μmol) of L-leucine *N*-carboxy anhydride (prepared from Z-L-Leu by the PCl_5–ether procedure[42] and recrystallized from *n*-hexane), stirred for an additional 2.00 min with momentary interruption and shaking each 0.5 min, and quickly treated with 0.80 ml of of 1.0 *N* HCl, all operations being conducted as described by Manning and Moore[32] in a cold room (5°). Such solutions were refrigerated pending ion-exchange chromatography, which was conducted on a 0.9 × 55 cm column of Beckman PA-28 resin with buffers 0.2 *N* in sodium citrate, using one or more of the following systems of pH, temperature, and flow rate conditions as necessary: (A) 5.42, 55°, 68.2 ml/hr; (B) 4.14, 52°, 69.8 ml/hr; (C) 4.10 for the first 200 min and then 5.40, 40°, 54.5 ml/hr. L-Leucyl dipeptide peaks were identified by comparing elution volumes with those of identically L-leucylated and chromatographed authentic samples as follows (system, elution volume in milliliters): Gly (B, 227); Sar (B, 160 and 185); L-Leu (A, 86; B, 113; appears in all chromatograms from coupling of the *N*-carboxy anhydride); D-Leu (A, 99; B, 113); L-MeAla (B, 300); DL-MeAla (B, 261 and 300);[43] L-MePhe (C, 658); D-MePhe (C, 639). The hydrolysate from **1** showed peaks corresponding to dipeptides from Gly, Sar, L-Leu, and L-MeAla but none of the other aforementioned amino acids; that from **3a** showed peaks corresponding to dipeptides from Gly, L-Leu, L-MeAla, and D-MePhe, but not the others.

Platinum-Catalyzed Hydrogenation of Tentoxin.[1a] A solution of 20 mg of **1** in 200 ml of 95% ethanol containing 10 mg of PtO_2 was hydrogenated at 1 atm for 1.5 hr, 282 nm absorption decreasing to less than 1% of its initial value. Filtration and evaporation left 18 mg of a mixture of dihydro-, hexahydro-, and octahydrotentoxin as a colorless solid: ^1H NMR ($CDCl_3$) contains aromatic and vinyl resonance; mass spectrum shows molecular ions at *m/e* 416, 420, and 422. This sample was hydrolyzed without further purification.

L-*N*-Methylalanine and L- and D-*N*-Methylphenylalanine.[1] These amino acids were prepared from L-Ala, L-Phe, and D-Phe respectively by N-benzylation (benzaldehyde–$NaBH_4$ "Method A"), N-methylation, and debenzylation using the procedures of Quitt, Hellerbach, and Vogler.[44] L-MeAla had mp 300–301° (c), $[\alpha]^{25}D$ 10.1° (*c* 1, 6 *N* HCl) (lit.[44] mp 270° dec, $[\alpha]^{21}D$ 11.5° (*c* 1, 6 *N* HCl)). L-MePhe had mp 253–254° with sublimation (m), $[\alpha]^{25}D$ 26.5° (*c* 1, 6 *N* HCl) (lit.[44] mp 260° dec, $[\alpha]^{21}D$ 26.6° (*c* 1, 6 *N* HCl)). D-MePhe had mp 250–253° with sublimation (m), $[\alpha]^{25}D$ −24.7° (*c* 1, 6 *N* HCl).

DL-*N*-Methyl-β-cyclohexylalanine.[1a] An 85-mg sample of crude DL-MePhe (mp 238–250°; prepared from DL-Phe by the sequence described above[44]) in 5 ml of glacial acetic acid containing 15 mg of PtO_2 was hydrogenated at room temperature (1 atm) for 4 hr, the theoretical amount of H_2 being absorbed. Filtration and evaporation in vacuo left 87 mg (100%) of the saturated amino acid as a colorless solid: mp 195–197°; ^1H NMR ($CDCl_3$) no aromatic or vinyl resonance. This sample was used without further purification in chromatographic identification of hydrolysis products from hydrogenated tentoxin.

Acknowledgments. We are grateful to Mr. W. Thaanum of the Applied Physics Corp. for temporary use of a Model 60 spectropolarimeter.

References and Notes

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(2) (a) This research was partially supported by research Grant CSRS-916-15-24 to the Arkansas Agricultural Experiment Station, by National Science Foundation Undergraduate Research Participation Grant GY-35 and Research Instrument Grants GP-6978 (Hitachi mass spectrometer) and GP-18291 (Bruker NMR spectrometer) to the Department of Chemistry, and by the University of Arkansas Research Reserve Fund. (b) Presented in part at the 1970 Southeast-Southwest Regional Meeting and the 1972 Midwest Regional Meeting of the American Chemical Society, New Orleans, La., Dec 3, 1970, and Columbia, Mo., Nov 10, 1972; (c) abstracted in part from M.S. theses of C.I.G. (1967) and S.H.W. (1973), University of Arkansas; (d) published with approval of the Director, Arkansas Agricultural Experiment Station; (e) Department of Chemistry; (f) National Science Foundation Undergraduate Research Participant, 1966; (g) National Defense Education Act Fellow, 1971-1974; (h) National Institutes of Health Predoctoral Fellow, 1965-1967.

(3) (a) N. D. Fulton, K. Bollenbacher, and B. J. Moore, *Phytopathology*, **50**, 575 (1960); (b) N. D. Fulton, K. Bollenbacher, and G. E. Templeton, *ibid.*, **55**, 49 (1965); (c) G. E. Templeton, C. I. Grable, N. D. Fulton, and K. Bollenbacher, *ibid.*, **55**, 1079 (1965); **57**, 516 (1967); (d) C. I. Grable, G. E. Templeton, and W. L. Meyer, *ibid.*, **56**, 897 (1966); (e) G. E. Templeton, C. I. Grable, N. D. Fulton, and W. L. Meyer, Proceedings of the 1967 Mycotoxin Research Seminar, U.S. Department of Agriculture, Washington, D.C., June 8-9, 1967, p 27; (f) G. E. Templeton, W. L. Meyer, C. I. Grable, and C. W. Sigel, *Phytopathology*, **57**, 833 (1967); (g) for a recent review, see G. E. Templeton in "Microbial Toxins", Vol. 8, S. J. Ajl, S. Kadis, and A. Ciegler, Ed., Academic Press, New York, N.Y., 1972, pp 178-187.

(4) J. T. Daniel and G. E. Templeton, *Proc. Arkansas Acad. Sci.*, **24**, 47 (1970).

(5) Previously unrecorded experimental details of that research, the results of which were announced in ref 3f and discussed in ref 1a, are included in the Experimental Section of the present paper. This work needs further comment on but two points to be substantially complete. First, the unrecognized tenacity with which tentoxin retains benzene of crystallization rendered the early microanalytical results inadequate for determination of a unique molecular formula and necessitated the initial deduction of $C_{22}H_{30}N_4O_4$ from a low-resolution MS determination of molecular weight, ir and ^1H NMR data, and valence considerations;[1a] this composition has now been confirmed both by high-resolution MS and by microanalysis of a benzene-free sample. Secondly, the fifth ninhydrin-positive product detected by TLC in the hydrolysate of tentoxin[1a] has chromatographic properties identical with those of sarcosine; apparently hydrolysis is to some extent preceded by a retro-aldol reaction at the conjugated system.

(6) For subsequent examples, cf. (a) *fungisporin*, R. O. Studer, *Experientia*, **25**, 898 (1969); (b) *roccanin*, G. Bohman, *Tetrahedron Lett.*, 3065 (1970); G. Bohman-Lindgren, *ibid.*, 4625 (1972); (c) *chlamydocin*, A. Closse and R. Huguenin, *Helv. Chim. Acta*, **57**, 533 (1974). Bitter peptide PB-II, for which a cyclic tetrapeptide structure was proposed in 1972, has been shown to be a dipeptide by T. Shiba and K. Nunami, *Tetrahedron Lett.*, 509 (1974).

(7) B. W. Bycroft, *Nature (London)*, **224**, 595 (1969), and references therein; R. B. Pringle, *Plant Physiol.*, **48**, 756 (1971); W. B. Turner, "Fungal Metabolites", Academic Press, London, 1971, pp 320-327, and references therein; E. Gross in "Chemistry and Biology of Peptides, Proceedings of the Third American Peptide Symposium", J. Meienhofer, Ed., Ann Arbor Science Publishers, Inc., Ann Arbor, Mich., 1972, pp 671-678, and references therein; B. W. Bycroft, *ibid.*, pp 665-670; E. Gross, H. Kiltz, and E. Nebelin, *Hoppe-Seyler's Z. Physiol. Chem.*, **354**, 810 (1973); T. Okuno, Y. Ishita, K. Sawai, and T. Matsumoto, *Chem. Lett.*, 635 (1974); A. Dossena, R. Marchelli, and A. Pochini, *J. Chem. Soc., Chem. Commun.*, 771 (1974).

(8) W. L. Meyer, L. F. Kuyper, D. W. Phelps, and A. W. Cordes, *J. Chem. Soc., Chem. Commun.*, 339 (1974).

(9) D. H. Rich and P. Mathiaparanam, *Tetrahedron Lett.*, 4037 (1974).

(10) J. Dale and K. Titlestad, *Chem. Commun.*, 656 (1969); 1403 (1970); 255 (1972); cf. J. Dale, *Pure Appl. Chem.*, **25**, 469 (1971).

(11) The numbering convention of ref 10, shown in Figure 1, is adopted here, as are the terms "inner" and "outer" (i and o, *cf.* Figure 1) to describe the two types of substituent conformation at each tetrahedral α carbon.

(12) P. Groth, *Acta Chem. Scand.*, **24**, 780 (1970).

(13) The separation of the 2i and 5i protons is about 2.22 Å in c-Sar_4[14] compared with 2.5 Å between syn-axial hydrogens of cyclohexane.[15]

(14) Distances and dihedral angles were computed from the published crystallographic data of Groth.[12] We are grateful to Professor A. W. Cordes for the computer programs used in this calculation, and for education in their use.

(15) E. L. Eliel, N. L. Allinger, S. J. Angyal, and G. A. Morrison, "Conformational Analysis", Interscience, New York, N.Y., 1965, p 43.

(16) C. M. Deber, E. T. Fossel, and E. R. Blout, *J. Am. Chem. Soc.*, **96**, 4015 (1974), and references therein.

(17) V. F. Bystrov, V. T. Ivanov, S. L. Portnova, T. A. Balashova, and Y. A. Ovchinnikov, *Tetrahedron*, **29**, 873 (1973). For other theoretical and experimental relationships between peptide NH-C_αH dihedral angles and vicinal couplings, all of which are consistent with the deductions described here, see references cited in that paper and also K. D. Kopple, A. Go, R. H. Logan, Jr., and J. Savrda, *J. Am. Chem. Soc.*, **94**, 973 (1972); F. A. Bovey, A. I. Brewster, D. J. Patel, A. E. Tonelli, and D. A. Torchia, *Acc. Chem. Res.*, **5**, 193 (1972); M. Barfield and H. L. Gearhart, *J. Am. Chem. Soc.*, **95**, 641 (1973); V. N. Solkan and V. F. Bystrov, *Tetrahedron Lett.*, 2261 (1973); N. S. Ostlund and M. J. Pruniski, *J. Magn. Reson.*, **15**, 549 (1974); M. T. Cung, M. Marraud, and J. Neel, *Macromolecules*, **7**, 606 (1974).

(18) The N(CH_3)-C_αH dihedral angles in c-Sar_4 are 175 (H_{2i}), 63 (H_{2o}), 151 (H_{5i}), and 32° (H_{5o}).[14] These would correspond[17] to NH-CH_2 couplings of approximately 10.4 (i) and 1.8 Hz (o) for a 2- or 8-CH_2 and 8.2 (i) and 6.0 Hz (o) for a 5- or 11-CH_2, and to NH-CHR couplings of 9.9, 1.7, 7.8, and 5.7 Hz for 2,8i, 2,8o, 5,11i, and 5,11o α-methine protons, respectively. One observed value of 10 Hz has been reported for the NH-H_2; coupling in an alanyl unit.[10]

(19) Cf. N. S. Bhacca and D. H. Williams, "Applications of NMR Spectroscopy in Organic Chemistry", Holden-Day, San Francisco, Calif., 1964, pp 57-61.

(20) Approximate values used in this analysis for the chemical shifts characteristic of the various α-proton types were developed from consideration of the data reported in ref 10 and are as follows (τ scale, chloroform): 2i-CH_2, 5.1 (Gly) or 4.7 (Sar); 2o-CH_2, 6.6 (Gly) or 6.8 (Sar); 5i-CH_2, 5.7 (Gly or Sar); 5o-CH_2, 6.4 (Gly or Sar); 2i-CHR, 5.1 (NHCHR) or 4.7 (NMeCHR); 5i-CHR, 6.0. Derivation of values in this group for proton types not included among the compounds of ref 10 and types which in that work were not examined in chloroform involved the following assumptions, justified as indicated: (a) 5,11-CH_2 shifts of Gly units should be nearly the same as those of Sar units (cf. the similarity between dihydrotentoxin and dimethyldihydrotentoxin, Table I); (b) N-1 methylation will probably cause the same 0.4-0.5 ppm downfield shift on a 2i-CHR as it does on a 2i-CH_2 (the effect is probably largely a consequence of an alteration of the anisotropy of the C-12-N-1 amide π system, in the nodal plane of which the 2i proton lies); (c) the inner proton of a Leu or Phe unit will approximately resemble that of an Ala unit; and (d) a change from chloroform to trifluoroacetic acid as solvent will have approximately the same influence on an Ala 5i proton as it has on a Sar 5i proton (data in ref 10 suggest that the corresponding solvent effect does appear to be constant for 2i protons in Gly, Sar, and Ala units). These values will surely be subject to some refinement as data from additional substances become available.

(21) Most of the features about to be discussed are also incompatible with some structures which have been already discarded by the deduction that glycine is at 2 or 8 and leucine is at 2 or 5 and thus provide further important evidence against those alternatives. For brevity, throughout the remainder of this paper spectral features will usually be explicitly discussed only in terms of structures which have not been eliminated earlier in the discussion, even though they may also militate against previous dismissed possibilities. Nonetheless, it is an interesting exercise to consider each piece of data with respect to all possible combinations of sequence and configuration, to realize the full weight of data against every structure except that which is ultimately assigned.

(22) That such strong shielding would be observed for a number of other combinations is improbable, for they involve either rather unlikely rotational arrangements of the isobutyl group or distances between the methyls and the π system which seem rather great. However, in this development of the analysis, we have chosen to exclude at this point only those structures for which shielding appears virtually impossible rather than only improbable.

(23) An exocyclic double bond at C-5 projects almost directly down from the ring, as does a 2i proton, so the latter is almost directly over the double bond's center and would surely be shielded. However, a 2-exo double bond is oriented well out from the ring, and the 5i proton is much closer to the borderline between the shielding and deshielding regions of the olefinic bond and phenyl group. That in fact such an orientation of the benzylidene group is almost without effect on the 5i proton will be seen below from the spectrum of dimethyltentoxin, in which the glycine 11i proton has a normal chemical shift.

(24) S. I. Hakomori, *J. Biochem.*, **55**, 205 (1964); D. W. Thomas, *Biochem. Biophys. Res. Commun.*, **33**, 483 (1968); E. Vilkas and E. Lederer, *Tetrahedron Lett.*, 3089 (1968).

(25) G. Marino, L. Valente, R. A. W. Johnstone, and F. Mohammedi-Tabrizi, *J. Chem. Soc., Chem. Commun.*, 357 (1972).

(26) Whereas a 5- or 11-exo double bond (**1**) is coplanar with the attached carbonyl and orthogonal to the π system of the attached nitrogen, a 2- or 8-exo olefin (**2**) is coplanar with neither its attached carbonyl nor the amide group of its attached nitrogen, cf. ref 23.

(27) The argument that the chemical shifts are of the 2,8 type with the inner proton shielded by the benzylidene group not only ignores the geminal coupling criterion but leads to the sequence -Sar-MeΔPhe- at either 2,5 or 8,11, and every structure containing a -Gly-MeΔPhe- sequence was inconsistent with the foregoing analysis of the tentoxin spectrum.

(28) Analogous conformational analysis of those dimethyltentoxin structures which have 5- or 11-sarcosine units together with 2-L- or 8-D-*N*-methylalanyl units not followed by the dehydrophenylalanine indicates that conformations -2-MeAla-5-Sar-8-MeΔPhe-11-MeLeu-, -2-MeAla-5-Sar-8-MeLeu-11-MeΔPhe-, and -2-MeLeu-5-(*E*)-MeΔPhe-8-MeAla-11-Sar- would also be energetically unfavorable with respect to alternatives which are available, and they could have been ruled out on that basis. These sequences were also incompatible with the tentoxin ^1H NMR data, however.

(29) The -5-MeΔPhe-8-Gly- arrangement also provides an explanation for the fact that addition of dimethyl sulfoxide to the $CDCl_3$ ^1H NMR solution produces a strong downfield shift in leucine's NH resonance but little or no shift of the glycine NH absorption; the dehydro unit can sterically hinder Me_2SO's approach and solvation at the latter group.

(30) This is a stronger piece of evidence than it might at first seem to be. If the benzylidene group were *E* in spite of the apparently unfavorable energetic situation, the nonbonded NH-phenyl interaction would certainly cause the phenyl group to rotate out of the plane of the C=C—CONH system. In such an arrangement, it could shield the *gem*-methyls little if at all. Furthermore, it should shield both the 8-outer proton and the NH of glycine, but such effects are not observed, the former being normal in chemical shift and the latter considerably deshielded compared with the leucine NH, as would be expected from its location in the nodal plane of the π system.[31]

(31) Had this deshielding of the glycine NH been introduced as evidence for the partial structure -5-MeΔPhe-8-Gly- (or -11-MeΔPhe-2-Gly-) in deducing the limited set of structures which were compatible with the tentoxin ^{1}H NMR data, it would have eliminated five of the ten structures (two of the four sequences) with which the above presentation turned from the tentoxin spectrum to that of dimethyltentoxin. On analogous grounds, the observation that neither the alanyl *N*-methyl of tentoxin nor the glycyl, leucyl, nor alanyl *N*-methyls of dimethyltentoxin are unusually deshielded might have been used as an additional basis to exclude for those peptides partial structures which contain a 5- or 11-dehydrophenylalanyl unit followed by a 7- or 1-methyl, respectively.
(32) J. M. Manning and S. Moore, *J. Biol. Chem.*, **243**, 5591 (1968).
(33) This result regarding the configurations of the leucyl and *N*-methylalanyl units of tentoxin has also recently been reported in connection with an erroneous formulation of the amino acid sequence of tentoxin by M. Koncewicz, P. Mathiaparanam, T. F. Uchytil, L. Sparapano, J. Tam, D. H. Rich, and R. D. Durbin, *Biochem. Biophys. Res. Commun.*, **53**, 653 (1973).
(34) For an improved procedure, see S. H. Woodhead, G. E. Templeton, W. L. Meyer, and R. B. Lewis, *Phytopathology*, **65**, 495 (1975).
(35) The precision of conversion of specific to molecular rotations will suffer from uncertainty in the benzene content of this sample.
(36) The value reported earlier[1] is in error.
(37) Reduction was incomplete with lower catalyst:tentoxin ratios. It appears that **3a** is strongly adsorbed on the catalyst and acts as a catalyst poison.
(38) E. Stahl, Ed., "Thin-Layer Chromatography, A Laboratory Handbook", Springer-Verlag, Berlin, 1965: (a) p 399; (b) p 496.
(39) Cf. E. Fischer and L. v. Mechel, *Ber.*, **49**, 1355 (1916).
(40) J. R. Coggins and N. L. Benoiton, *Can. J. Chem.*, **49**, 1968 (1971).
(41) Whether racemization occurred during saponification or HBr treatment or both in this instance was not determined, cf. J. R. McDermott and N. L. Benoiton, *Can. J. Chem.*, **51**, 2555 (1973).
(42) R. R. Becker and M. A. Stahmann, *J. Biol. Chem.*, **204**, 737 (1953).
(43) The DL reference was used rather than the D isomer.[1b]
(44) P. Quitt, J. Hellerbach, and K. Vogler, *Helv. Chim. Acta*, **46**, 327 (1963).

Synthesis of Unsymmetrical Phosphodiesters by Means of Cyclic Enediol Pyrophosphates

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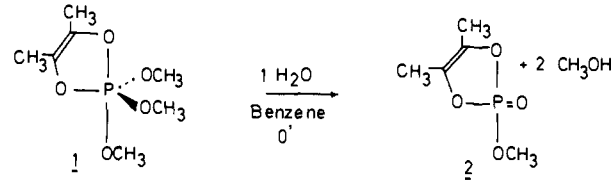
Abstract: Unsymmetrical phosphodiesters [(RO)(R'O)P(O)(OH)] are readily synthesized from the alcohols, ROH and R'OH, by means of the new reagent acetoin enediol cyclopyrophosphate. When one of the two alcohols is a diol with a primary and a secondary hydroxyl, the primary function is selectively phosphorylated in the presence of the unprotected secondary function. The method holds promise in the synthesis of oligonucleotides.

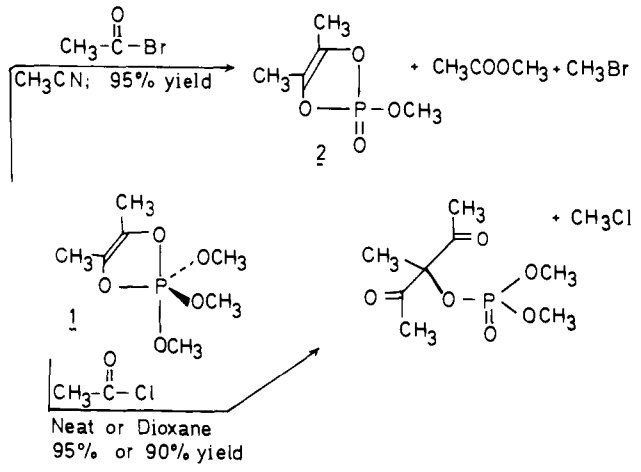
Methyl acetoin enediol cyclophosphate (2-methoxy-4,5-dimethyl-1,3,2-dioxaphosphole 2-oxide, **2**) was first obtained from the hydrolysis of the oxyphosphorane[2,3] **1**, (Scheme I).

Scheme I



A more practical synthesis of CEP-OCH$_3$[4] (**2**), based on the ability of the oxyphosphorane **1** to undergo C-acylation[5] or exocyclic O-acylation under different conditions, was announced recently[6] (Scheme II).[7]

Scheme II

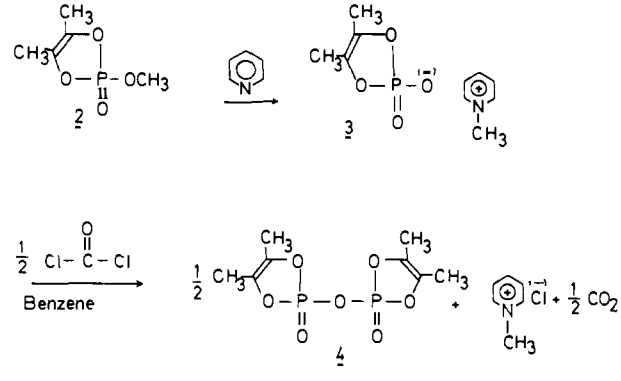


This paper describes the conversion of CEP-OCH$_3$ (**2**) into a cyclic pyrophosphate which can be used as reagent for the synthesis of unsymmetrical phosphodiesters [(RO)(R'O)P(O)(OH)].[8-19] The reagent needs no additional activation to carry out the double phosphorylation and does not generate, in most cases, symmetrical phosphodiesters as by-products. The primary alcohol of a diol can be phosphorylated selectively in the presence of an unprotected secondary alcohol. These properties suggest possible applications of the new reagent to the synthesis of oligonucleotides.[20-23]

Results

Preparation of (*N*-Methylpyridinium)$^+$ $^-$OCEP (3). This salt (**3**) (Scheme III) is obtained in high yield from the

Scheme III



reaction of the ester CEP-OCH$_3$ (**2**) with pyridine. The data summarized in Table I support the cyclic structure of the salt (**3**); note the similarity of its ^{31}P NMR shift and that of CEP-OCH$_3$ (**2**). The shifts of the acyclic analogs