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"Conformationally Constrained Biologically Active Peptides: Tentative Identification of the Antimitogenic Bioactive Conformer of the Naturally Occurring Cyclic Tetrapeptides."

Richard E. Shute,[a] Megumi Kawai,[b] and Daniel H. Rich*
School of Pharmacy
University of Wisconsin-Madison
425 North Charter Street
Madison, WI 53706

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Introduction.

In the quest for a greater understanding of the relationship between "drug" structure and biological activity the study of conformationally constrained peptides has received considerable attention.[1] Mammalian hormone systems have been a particularly fruitful field for investigation of the effects of such modifications;[2] synthetic analogs of the enkephalins,[3] α-melanotropin[4] and, preeminently, somatostatin[5] have all provided valuable examples of how flexible, biologically active compounds may be constrained by the incorporation of cyclic structures to increase their activity and receptor specificity, and often to prolong their biological response. Less frequently studied have been naturally occurring examples of cyclically constrained peptides with potent antibiotic or antitumor activity.[6,7] This is not surprising when the size and complexity of the majority of these natural products is considered. There are, however, a few cyclic peptide natural products that are of a sufficiently small size, and with sufficiently interesting biological properties, to have warranted detailed investigation into the structural and conformational requirements needed for their activity, the most noteworthy examples being the β-lactam antibiotics[8] and, more recently, the cyclosporines.[9] We describe herein the structure-activity and conformational aspects of another class of cyclic peptide natural products that possess potent biological activity, namely, the fungally derived cyclic tetrapeptides.

This family of compounds (Table 1), whose members, including HC-toxin,[10] chlamydocin,[11] WF 3161[12] and Cyl-2,[13] all possess a peptidyl 12-membered cyclic ring system, demonstrates significant bioactivity both as plant toxins[14] and, in vitro, as cytostatic and antimitogenic agents with IC_{50} values in the nanomolar range against P-815 mastocytoma cells and murine lymphocytes.[15] Such potency is usually indicative of interaction with a very specific receptor or enzyme which is present only at very low concentrations in the particular testing system. Unfortunately, although highly active in vitro, chlamydocin (and presumably the others) is rapidly inactivated in vivo,[15] which prevented development of these compounds as chemotherapeutic, antitumor agents. However, the convenience with which these cyclic peptides can be tested coupled with their extraordinary potency and the comparative rigidity of their medium-sized cyclic ring system makes the cyclic tetrapeptide natural products particularly attractive for structure-activity and conformational study.

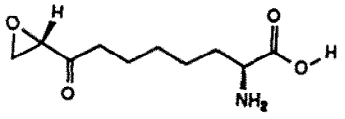
In this paper, we report some of our previously unpublished results in this area, collate all the structure-activity data that has to date been published, and attempt to identify the structural and the conformational requirements for bioactivity in these compounds. The conformational conclusions will be supported by data from force field calculations.

Table 1: The Cyclic Tetrapeptide Family of Natural Products.

COMPOUND (No.)	SEQUENCE
HC-Toxin (1)	cyclo[-L-Aoe^1-D-Pro^2-L-Ala^3-D-Ala^4-]
Cyl-1 (2)	cyclo[-L-Aoe^1-D-Tyr(OMe)^2-L-Ile^3-L-Pro^4-]
Cyl-2 (3)	cyclo[-L-Aoe^1-D-Tyr(OMe)^2-L-Ile^3-L-Pip^4-]
Chlamydocin (4)	cyclo[-L-Aoe^1-Aib^2-L-Phe^3-D-Pro^4-]
WF 3161 (5)	cyclo[-L-Aoe^1-D-Phe^2-L-Leu^3-L-Pip^4-]
[Gly]^4 HC-Toxin (6)	cyclo[-L-Aoe^1-D-Pro^2-L-Ala^3-Gly^4-]

Primary Structure-Activity Data.

HC-toxin, cyclo[-Aoe-D-Pro-Ala-D-Ala-] (1),[16] was the first cyclic tetrapeptide natural product isolated;[10] its biological activity was initially shown to be as a plant toxin on susceptible varieties of maize.[14] Later, the agent Cyl-2, cyclo[-Aoe-D-Tyr(OMe)-Ile-Pip-] (3) was similarly identified as a toxin but against a number of plant species.[13] Contrarily, the compounds chlamydocin, cyclo[-Aoe-Aib-Phe-D-Pro-] (4),[11,15] and WF-3161, cyclo[-Aoe-D-Phe-Leu-Pip-] (5)[12] were shown to be potent in vitro cytostatic agents. The presence in all of these natural products of the unusual amino acid L-2-amino-8-oxo-9(S),10-epoxydecanoic acid (L-Aoe; 7)[17] indicated not only a structural relationship, but also suggested that they should be related in their biological activity. This hypothesis was later confirmed when chlamydocin was shown to be a plant toxin with similar toxicity to HC-toxin,[18] and HC-toxin itself was shown to possess potent in vitro cytostatic and antimitogenic activity, albeit to a 10-15 fold lesser extent than chlamydocin.[19,20]



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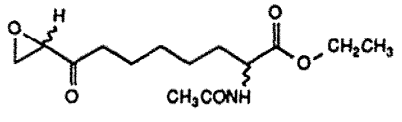
Throughout the rest of this report, it is this cytostatic and antimitogenic activity that provides the primary basis for our structure-activity conclusions. It seems likely that both the botanical and zoological receptor should be somewhat alike, but the greater amount of data, coupled with our greater interest and experience in the antimitogenic properties of these agents, prompts us to concentrate on this testing system and not on their herbicidal properties.

Early structure-activity work demonstrated that alteration of the side-chain epoxyketone functionality in 7 by reduction or ring-opening led to material with 1000-fold less cytostatic activity against mouse P-815 mastocytoma cells.[11] More recent Aoe side-chain modified compounds provided further proof that a suitably functionalized side-chain is needed for maximal bioactivity (Table 2). The notable activity of the chloromethylketone-functionalized compounds (16,19,20)[24] strongly suggests that these molecules alkylate their target receptor and that position 9 of the side-chain is intimately involved in this process. The lack of activity in analogs with other potential alkylating side-chains (e.g., 15 and 18),[21,24] coupled with the extraordinarily low IC_{50} values for the active compounds point to a highly specific active site nucleophile being involved.

The cyclic tetrapeptide ring system is required for maximal activity since derivatives of the essential amino acid Aoe, e.g., N-acetyl D,L-Aoe, ethyl ester (22) and N-isovaleryl D,L-Aoe, benzylamide (23), synthesized using methods already described,[25] were found to have IC_{50} values of >10,000 ng/ml and 800 ng/ml respectively, compared to chlamydocin which has an IC_{50} of 1-3 ng/ml.[26,27] Even allowing for bioactivity residing only in the L-enantiomers, this demonstrates the necessity for the intact peptide ring system for high potency. Subtle differences in activity,



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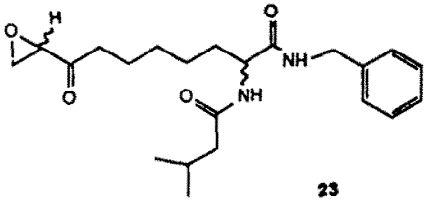

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for example, between chlamydocin (IC_{50} 1-3 ng/ml) and HC-toxin (IC_{50} 10-15 ng/ml), establish a relationship between the peptide ring system conformation or the side chain substitution and biological activity. This latter could be manifested in the presence of an aromatic residue in chlamydocin, Cyl-2 and WF-3161, which is lacking in HC-toxin. The recent isolation of another cyclic tetrapeptide natural product, [Gly]^4 HC-toxin (cyclo[-Aoe-D-Pro-Ala-Gly-]) (6),[28] which was found to be 30-40 fold less active than HC-toxin, albeit in an assay measuring inhibition of root

Table 2: Antimitogenesis Activity of Aoe-Side-chain Modified Cyclic Tetrapeptides.

CYCLIC TETRAPEPTIDE RING SYSTEM	SIDE-CHAIN[a] FUNCTIONALITY (No.)	IC_{50} (ng/ml)[b]	REFERENCE[b]
Chlamydocin	8-oxo-9(S),10-epoxy Natural Product (4)	(0.36),1-3	(15),21
Chlamydocin	8-hydroxy-9,10-epoxy (Dihydrochlamydocin) (8)	ca. 5000	(11),21
Chlamydocin	8-oxo-10-hydroxy (9)	(>10^6)	(11)
Chlamydocin	8-oxo-9,10-ene (10)	200	(11),20
Chlamydocin	8-oxo- (11)	(>10^6)	(11)
Chlamydocin	8-hydroxy-9,10-ene (12)	1500	20,22
Chlamydocin	9,10-ene (13)	400	19,22
Chlamydocin	9,10-epoxy (14)	2400	20
[Gly]^2 Chlamydocin	4-aza-5-oxo-7-maleimido (15)	>10^5	21
Chlamydocin	8-oxo-9-chloro (16)	3-10	24
HC-toxin	8-oxo-9(S),10-epoxy (Natural Product) (1)	10-15	19,24
HC-toxin	8-hydroxy-9,10-yne	10^4	20,23
HC-toxin	8-oxo-9,10-yne (17)	400	19,23
HC-toxin	8-oxo-9-diazo (18)	>2000	24
HC-toxin	8-oxo-9-chloro (19)	30-40	24
[L-Phe]^3 HC-toxin	8-oxo-9-chloro (20)	40-100	24

[a] All side chain derivatives are α-amino decanoic acid derivatives in the 1-position of the cyclic tetrapeptide (Table 1) except for the chloromethyl and diazomethyl ketone derivatives 16, 18-20 which are derived from α-amino nonanoic acids, and 15.

[b] IC_{50} values and references refer to antimitogenesis assay data; figures in brackets refer to P-815 mouse mastocytoma, cytostatic data.

growth of susceptible maize seedlings not antimitogenesis, further indicated the possible importance of this lipophilic interaction at the receptor. However, an [L-Phe]^3 HC-toxin analog, bearing a chloromethylketone functionality (20), which was indeed more lipophilic, was found to be less active, by a factor of about 2, than the corresponding HC-toxin chloromethylketone (19).[24] This rather surprising result indicated that the proposed binding site interaction was not an absolute prerequisite for increased potency, and pointed to other structural differences between the agents, e.g., ring system conformation, being responsible for activity differences.

Conformational Analysis of Cyclic Tetrapeptides.

It is known that the presence of imino acids or the incorporation of α-methylated amino acids affects considerably the conformation of linear peptides;[2,29] it seems likely, therefore, that in a more rigid, cyclic system such additional constraints might play an even more crucial role in the controlling of conformational flexibility, perhaps resulting in only one thermodynamically acceptable structure that is able to bind optimally to the receptor and elicit the maximal biological response. Analysis of the conformational differences between the cyclic tetrapeptides might therefore reveal a "bioactive conformer" which could be used as a basis in the design of possibly more effective agents.

The three-dimensional, solid state conformation of the cyclic tetrapeptide dihydrochlamydocin (8), shown in Figure 1, was established by X-ray crystallographic methods.[30] The structure is characterized by two 3→1 hydrogen bonds (the bis γ-turn conformation) and the presence of four "transoid" amide bonds, so-called because they are twisted 15° to 25° from planarity (ω = 180° ± 15-25°). Later, the solution conformation of chlamydocin was determined by NMR techniques and found to be very close to the X-ray structure (Figure 2A).[31] More importantly, however, it was

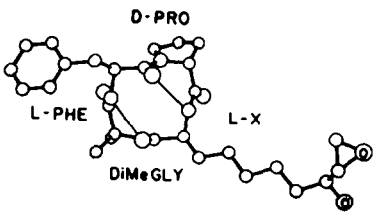
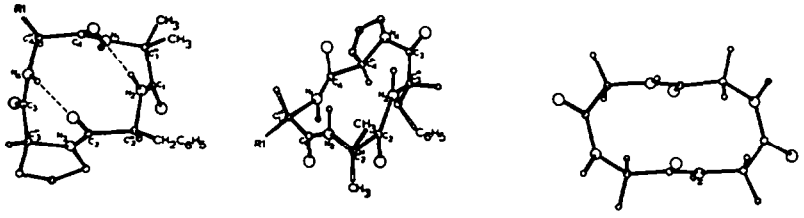


Figure 1: X-ray Crystal Structure of Dihydrochlamydocin (8).[30]



A B C

Figure 2: a: Conformation of Chlamydocin (4) in Chloroform; the trans-4 (T_4) Conformation. R = Aoe side chain; b: Conformation of Chlamydocin (4) in DMSO, the cis,trans,trans,trans (CT_3) Conformation. R = Aoe side chain; c: one of several possible cis,trans,cis,trans cyclic tetrapeptide ring conformations (CTCT). Model is of cyclic tetraglycine.

observed that addition of a hydrogen-bonding solvent, such as dimethylsulfoxide (DMSO) or methanol, to deuterochloroform ($CDCl_3$) solutions of chlamydocin promoted a new conformation.[32] Extensive NMR analysis established that this alternative conformation differed from that in the solid state in that it contained one cis amide bond (Figure 2B). In this discussion we will term this a cis,trans-3, or CT_3, conformation and the all transoid conformation will be abbreviated T_4. Thus, in DMSO, chlamydocin adopts the CT_3 conformation shown in Figure 2B in which there is a cis amide bond between Phe-D-Pro.

HC-toxin in $CDCl_3$ has been shown to exist in the intramolecularly hydrogen bond stabilized bis γ-turn (T_4) conformation, very similar to chlamydocin.[33] We have observed that HC-toxin is somewhat more stable in this conformation,[34] but addition of hydrogen bond breaking solvents (e.g., DMSO-d6) does cause conformational interconversion. Some 30% of the cyclic tetrapeptide adopts a new conformation shown from conformational analysis of the model peptide, cyclo(L-Ala-D-Ala-L-Ser(Bzl)-D-Pro),[34] to be the CT_3 conformation.

Other model cyclic tetrapeptide conformations have been determined by NMR spectroscopy as part of systematic studies of the conformation of cyclic tetrapeptides in general,[35,36] or from simplified analogs of the naturally occurring compounds.[31,32,36,37] Both the bis γ-turn (T_4) conformation, and the CT_3 structure[32,35,37,38] are observed. The latter, as noted previously, appears in dipolar aprotic solvents as a result of breakage of the intramolecular hydrogen bonds, and trans to cis isomerism of one of the amide bonds.

A third conformation found for many cyclic tetrapeptides contains an additional cis amide bond, yielding a cis,trans,cis,trans (CTCT) array (Figure 2C). Although this conformation had not been reported for any of the highly biologically active compounds related to chlamydocin, its repeated observation in model systems,[39-41] both by solution NMR methods, again in polar solvents, and by X-ray crystallography,[42] coupled with the suggestion that in certain symmetrical structures this array has unusual energetic stability,[43] prompted us to synthesize analogs which would, firstly, show this conformation in solution, and secondly, possess the side-chain epoxyketone functionality to instil the possibility for bioactivity.

Evaluation of Analogs Constrained to the Cis,Trans,Cis,Trans Conformation.

Examination of models suggested that replacement of the Aib residue in chlamydocin with L-Pro would yield a compound, cyclo[-Aoe-Pro-Phe-D-Pro-], [L-Pro]^2-chlamydocin (26), which would be unable to exist in the CT_3 conformation and would be either T_4 or CTCT.

Analog 26 was synthesized using the standard techniques employed in our laboratory.[22] The use of racemic D,L-Ade-OEt as the Aoe-precursor[25] entailed a separation of the two diastereomers at the protected linear tetrapeptide stage; this was, however, readily achieved chromatographically. Similarly, a mixture of diastereomers at the 9-position of the epoxide was produced during the last, nonstereoselective oxidative reaction in the assimilation of the side-chain functionality. This mixture was not resolved because we have shown that the configuration at this center is not crucial for bioactivity; the unnatural 9R-isomer has biological activity approximately 3-4 fold less than the natural 9S-compound.[22] The 9R,S-mixture thus has only slightly lower activity than the pure 9S-diastereomer.

The insolubility of the product in $CDCl_3$ prevented NMR data being collected in nonpolar solvents, however, the ^{1}H- and ^{13}C-NMR spectra of this material in DMSO-d6 when compared to cyclo[-Ala-Pro-Phe-D-Pro-] (27), synthesized analogously using L-Ala-OMe instead of D,L-Ade-OEt, and also insoluble in $CDCl_3$, clearly indicated that both X-Pro amide bonds had the cis configuration.[44] More detailed proton NMR-NOE experiments confirmed the CTCT conformation.[34]

A further examination of molecular models suggested that another analog cyclo[-Aoe-D-Pro-Phe-D-Pro-], [D-Pro]^2-chlamydocin (28), might also exist preferentially in the CTCT conformation. Comparison with the enantiomerically related, known cyclic tetrapeptide cyclo[-D-Phe-Pro-D-Phe-Pro-] (29)[36] suggested that 28 might adopt a bis γ-turn conformation in $CDCl_3$ but should indeed exist in the CTCT conformation in polar solvents. Synthesis of 28 was carried out analogously for 26; the product again being a mixture of diastereomeric epoxides. NMR conformational analysis indicated that, like the enantiomeric model compound cyclo[-D-Phe-Pro-D-Phe-Pro-] (29), this material existed in the all transoid, T_4 conformation in $CDCl_3$, but that in DMSO-d6, the cis,trans,cis,trans, CTCT conformation predominated.

When these compounds were tested in the antimitogenesis assay, [L-Pro]^2-chlamydocin (26) showed an IC_{50} of 7000 ng/ml. The [D-Pro]^2-chlamydocin analog (28) showed an IC_{50} of 200 ng/ml. The comparatively poor activity of 28 strongly mitigates against the cis,trans,cis,trans being the bioactive conformation, but the extremely poor activity of 26, which is "locked" in the CTCT conformation, and which demonstrates an IC_{50} value less even than N-isovaleryl Aoe, benzylamide (23), conclusively excludes consideration of the cis,trans,cis,trans conformation as the bioactive conformation.

WF 3161 as a Conformationally Constrained Analog.

WF 3161 is the most active Aoe-containing cyclic tetrapeptide tested in our antimitogenesis assay.[20] Like Cyl-2 and unlike chlamydocin, however, WF 3161 contains an L-pipecolate residue instead of D-proline, i.e., an L-6-membered imino acid as opposed to a D-5-membered residue. The configurations of the amino acids were established by means of high field proton NMR-NOE measurements, circular dichroism work and by amino acid analysis before and after digestion with amino acid oxidases. This established the sequence as cyclo[-L-Aoe-D-Phe-L-Leu-L-Pip-].[35]

NMR conformational analysis of WF 3161 in both $CDCl_3$ and DMSO-d6 revealed that this cyclic tetrapeptide ring system adopts only the CT_3 cyclic tetrapeptide ring system conformation (Figure 3); we have not been able to detect the all transoid conformation under any conditions.[35] Interestingly, conformational analysis of the agent Cyl-2 and simplified models without the Aoe-residue indicate that Cyl-2 also exists preferentially in the CT_3 conformation.[37,38]

When one compares the different conformations which have been observed for all the cyclic tetrapeptides, it is clear that all the very potent compounds are ones which contain or can adopt the CT_3 amide bond configuration in the ring system conformation (Table 3). [L-Pro]^2-chlamydocin, cyclo[-Aoe-Pro-Phe-D-Pro-] (26), which cannot adopt the CT_3 conformation, is considerably less active. However, cyclo[-Aoe-D-Pro-Phe-D-Pro-] (28), which has been shown to exist in two conformations, the all transoid in nonpolar solvents and the CTCT in polar solvents, possesses intermedi-

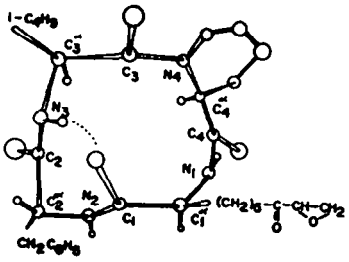


Figure 3: The Solution Conformation of WF-3161.[35] The amide bond between L-Leu-L-Pip is cis.

ate biological activity. The CT_3 conformation has not been observed for this compound but it can be envisaged that in a stepwise transition between the T_4 and the CTCT conformations where each X-Pro amide bond rotates in turn, a CT_3 conformation can be proposed as a possible metastable intermediate. It would be this energetically less favorable conformation which would bind to the receptor and would explain the intermediate activity of this compound between WF 3161 and cyclo[-Aoe-Pro-Phe-D-Pro-] (26).

These observations strongly suggest that the cis,trans,trans,trans (CT_3) amide bond conformation of the cyclic tetrapeptides is the one most closely associated with the bioactive conformation. The primary justification for this assumption is the inability to observe any conformation for WF 3161 and Cyl-2 other than the CT_3, yet they are the two most potent agents in our bioassay. The alternate conformations observed for the other cyclic tetrapeptides result in lower biological activities.

Table 3: Ring System Modified Cyclic Tetrapeptides.

COMPOUND (No.)	CONFORMATION IN NONPOLAR SOLVENTS[a]	CONFORMATION IN POLAR SOLVENTS[a]	IC_{50} (ng/ml)[b]
WF 3161 (5)	CT_3	CT_3	1-2
Cyl-2 (3)[c]	CT_3	CT_3	1-2
Chlamydocin (4)	T_4	CT_3	1-3
Chlamydocin chloromethylketone (16)	T_4	CT_3	3-10
HC-toxin (1)	T_4	(CT_3)[d]	10-15
HC-toxin cloromethylketone (19)	T_4	(CT_3)	30-40
$[L\text{-Phe}]^3$ HC-toxin chloromethylketone (20)	(T_4)	(CT_3)	40-100
$[D\text{-Pro}]^2$ Chlamydocin (28)	T_4	CTCT	200
Iva-D,L-Aoe-NH-Benzyl (23)	-	-	800
$[L\text{-Pro}]^2$ Chlamydocin (26)	CTCT	CTCT	7000
Ac-D,L-Aoe-OEt (22)	-	-	10000

[a]Conformation in brackets indicates a degree of uncertainty about the assignment because the compound has not been thoroughly analyzed in this solvent.
[b]Antimitogenesis assay.
[c]Conformational data taken from References 33 and 40.
[d]Approximately 30% CT_3 in DMSO.[34]

The one anomaly to this pattern is HC-toxin. Whereas HC-toxin demonstrates notable bioactivity and can adopt a CT_3 amide conformation, the CT_3 conformation of HC-toxin, in which the cis amide bond is between Aoe-D-Pro, differs from the CT_3 conformer for chlamydocin, in which the cis amide bond lies between Phe-D-Pro. It is quite evident that the cis amide bonds are on opposite sides of the cyclic tetrapeptide ring system (cf. Figure 4B vs Figure 2B). For HC-toxin's CT_3

conformation to be isosteric with chlamydocin's CT_3 conformation the Aoe-D-Pro tertiary amide bond must be trans and the secondary amide bond Ala-D-Ala must be cis (Figure 4C). We have not detected this conformation in solution, but the presence of cis secondary amide bonds has been observed in cyclic tetrapeptide ring systems, particularly in CTCT conformational arrays.[37] A more important precedent, however, is provided by dihydrotentoxin (30), formed by hydrogenation of another plant toxin tentoxin,[45] the crystal structure of which is shown in Figure 5.[46] Dihydrotentoxin contains two cis amide bonds, both secondary, and two trans amide bonds, both tertiary. The existence of this molecule clearly indicates that the unusual conformation proposed for HC-toxin might be thermodynamically possible.

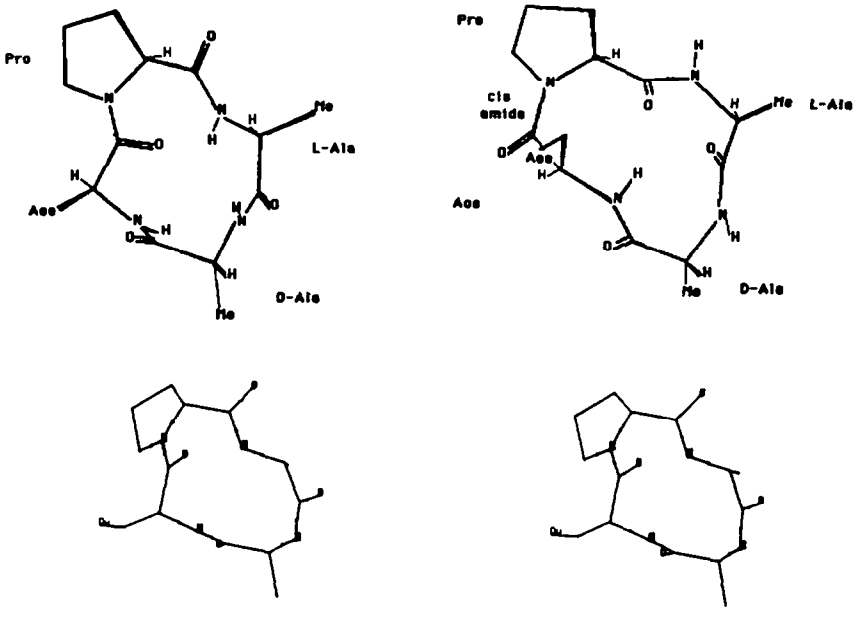


Figure 4: Ring Conformations of HC Toxin. a: The trans-4 Conformation in Chloroform.[33] See Figures 1 and 2a for related conformations. b: A CT_3 Ring System Conformation With the cis Aoe-Pro Amide Bond. Du = Aoe side chain. Hydrogen atoms are deleted. c: Proposed Bioactive CT_3 Conformation With a cis Ala-D-Ala Amide Bond shown in a crossed stereo representation. Du = Aoe side chain. Hydrogen atoms have been deleted.

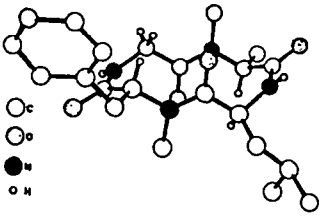


Figure 5: X-ray Crystal Structure of Dihydrotentoxin (30).[46] Note both tertiary amide bonds are trans and both secondary amide bonds are cis.

In order to test the energetic stability of the CT_3 conformation, we used computerized molecular modeling techniques and energy minimization methods to see if the ring system conformations of the three parent structures, chlamydocin, HC-toxin and WF 3161, could be constrained reasonably to one CT_3 ring system conformation. Our analysis also provided the relative energies of the various CT_3 conformations generated for all the compounds, and the otherwise unobserved T_4 conformation for WF 3161. If the force field calculations that we undertook were to be relied upon, then a clear differentiation between these various conformations should be apparent. Furthermore, they should

predict the CT_3 conformations to be lower in energy than the T_4.

The calculations were carried out on a VAX 11/785 using the SYBYL molecular graphics software supplied by Tripos Associates of St. Louis. We used the MAXIMIN energy minimizers and parameters contained in that program. The amide parameters are derived from those reported by White, who first calculated cyclic tetrapeptide conformations.[47] They should therefore be highly reliable for calculations of this type and were not modified.

Another factor which must be considered in these calculations is the possibility of unusual side-chain conformations. All non-proline side-chain containing residues were treated as alanines except for the pipecolate ring system in WF 3161. In order to evaluate multiple pipecolate ring conformations we used the SEARCH algorithm contained within SYBYL. This program enables one to estimate all such possible conformations, within reasonable limits (10° torsion angle increments) that the molecule might adopt. We found, not surprisingly, that the 6-membered pipecolate ring could exist in 2 or 3 distinctly different ring-flip conformations. One of these, however, was found to be significantly lower in energy than any of the others; we therefore focused on this lowest energy ring conformation for the pipecolate residue in our WF 3161 calculations.

The relative energies of the cyclic tetrapeptide conformations are shown in Table 4. In the absence of any attempt to fit these different structures one to another, two observations immediately emerged. Firstly, in all three cases the CT_3 cyclic tetrapeptide conformation was lower in energy than the corresponding T_4 structure. In the case of WF 3161, the CT_3 tetrapeptide ring conformation was about 5 kcal more stable than the T_4 conformation, which is consistent with our failure to detect the T_4 conformation in either polar or nonpolar solvents by NMR methods. Secondly, the differences between the CT_3 and T_4 conformations are about the same for all three cyclic tetrapeptide systems. This suggests an additional factor, not yet identified, which must destabilize the T_4 conformation of WF 3161 in solution since it has not been observed. More sophisticated calculations on these conformations particularly to include solvent contribution will be needed to identify and quantify these other effects.

We then extended the energy calculations to "force-fit" the different ring conformations to each other. This was carried out using the multi-fit subroutine contained within the MAXIMIN module of SYBYL. Multi-fit allows one to force atoms into common coordinates by using known amounts of energy, then to observe how the system responds to this stress. When chlamydocin, HC-toxin and WF 3161 were fitted in this way, we found that the molecules could adopt very similar conformations with respect to the 12 atoms in the cyclic tetrapeptide ring system.

The resultant structures for chlamydocin, HC-toxin and WF 3161 are shown superimposed in Figure 6. These structures were fitted with respect to the 4 α-carbons in the 12-membered ring system and to the Aoe side-chains. The results are consistent with the proposal that all three structures can adopt the CT_3 conformation as the bioactive conformation. It should be emphasized that the different side-chains on each ring system will alter the topology of each analog leading to the observed slight differences in biological activity.

The fact that active analogs can accommodate either L-pipecolate or D-proline suggests that this corner of the cyclic tetrapeptide system does not interact tightly with the receptor. Conformation 4C for HC-toxin is calculated to be quite stable. Its overall conformation is closely analogous to that of WF 3161. The *cis* amide bond between Leu-Pip in WF 3161 is mimicked by a *cis* amide bond between Ala-D-Ala in HC-toxin, and on the corners of the tetrapeptide ring system, the side-chains project into essentially identical space. The presence of a *cis* amide bond between Ala-D-Ala in HC-toxin may provide an explanation for the approximately 2-fold lesser activity of the $[L\text{-Phe}]^3$ HC-toxin analog (*20*). Any improved binding capability that might be provided by the aromatic side-chain may be outweighed by a greater energy barrier to the Phe-D-Ala amide bond having to rotate from *trans* to *cis* as compared to the Ala-D-Ala bond in HC-toxin itself. This has not, to date, been calculated but may result in the demonstration of greater stability for the T_4 conformation of this compound. The resultant lower stability of the CT_3 conformation would be manifested by decreased bioactivity. Similar arguments could be used to explain the lower activity of $[Gly]^4$ HC-toxin (*6*), albeit in this case with respect to a different receptor. Force field calculations taking into account these side-chain contributions should predict such results, but these have not

Table 4: Relative Energies of Multi-fitted Cyclic Tetrapeptide Conformations.

COMPOUND	T_4 CONFORMATION (kcal)	CT_3 CONFORMATION (kcal)	DIFFERENCE
WF 3161 (5)	15.58	10.7	5 kcal
Chlamydocin (4)	18.75	12.5	6 kcal
HC-toxin (1)	17.52	11.5	6 kcal

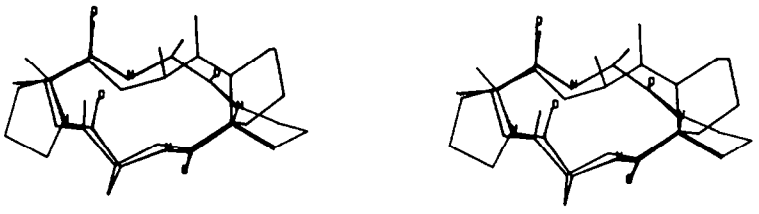


Figure 6: Crossed stereo representation of the superimposed WF 3161, Chlamydocin and HC-toxin Multi-fit CT_3 Conformations. Hydrogen atoms have been deleted for clarity. Darker lines are the WF 3161 ring system. Proline on left is in HC-toxin; proline on right is in chlamydocin.

as yet been instigated.

Finally, it once again bears reemphasizing that throughout this work we have been considering one biological testing system, the antimitogenesis assay, which yields only IC_{50} data at a fixed time point, 24 hours. As such, this gives only non-equilibrium information (for a presumed alkylation reaction) and no indication of the rate constants for the inhibitory process. It is possible that the higher activity of, for example, WF 3161 over HC-toxin, is a manifestation of a faster over a slower alkylation during some critical time period in the cell's cycle. Similarly, it may be that the second order rate constants for the binding of the peptide ring system are very different for compounds such as WF 3161 and chlamydocin, although their IC_{50} values are very similar. Without the isolated enzyme or receptor to yield more reliable kinetic data for these compounds, such possibilities cannot, at this stage, be resolved.

Conclusion.

The results we have described herein lead us to propose the bioactive conformation for all members of the cyclic tetrapeptide family of cytostatic and antimitogenic natural products to be one containing a cis,trans,trans,trans array for the four amide bonds in these compounds. This backbone conformation is shown for the representatives chlamydocin, HC-toxin, and WF 3161 in Figure 6. We suggest this bioactive conformation only with respect to the fixed time point antimitogenesis assay, firstly, because it is entirely possible, in view of the recent discoveries of multiple conformations shown, for example, by peptide hormones, that the bioactive conformation for the specific plant toxin activities of HC-toxin, chlamydocin, and Cyl-2 might be slightly, or possibly even radically different from the CT_3 conformation proposed above for antimitogenic activity; and secondly, in the absence of quantitative data on the rates of inhibition and dissociation constants for the binding of these compounds during the thermodynamically sensitive prealkylation step, the antimitogenesis bioassay may be yielding IC_{50} values that suppress differences between analogs (see Ref. 24 for a detailed discussion of this point). Isolation of the target enzyme, or enzyme systems, for these agents would result in better kinetic data becoming available which would categorically discriminate between the receptor-binding affinities of different cyclic tetrapeptide sequences and conformations. This would lead, ultimately, to conclusive proof of the bioactive conformation for these compounds.

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[a]Present address: Department of Pharmacy,
University of Nottinghma,
Nottingham NG7 2RD, UK

[b]Present address: Abbott laboratories
Abbott Park, IL 60064