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THE STRUCTURE AND CONFORMATION OF HC-TOXIN

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Difference nuclear magnetic resonance studies and amino acid oxidase experiments establish the sequence and configuration of amino acids in HC-toxin as cyclo(L-Aoe-D-Pro-L-Ala-D-Ala). HC-toxin adopts the bis-γ-turn conformation in solution previously found for the cytostatic cyclic tetrapeptide chlamydocin.

The host-specific toxin, HC-toxin, isolated from culture filtrates of Helminthosporium carbonum (cochliobolus) race 1 by Pringle (1) has attracted considerable attention both for its effects on maize (2,3) and its unusual structural properties. Recently two structures have been proposed for HC-toxin: (I) cyclo (Aoe-Ala-Ala-Pro), by Liesch et al. (4) and (II) cyclo (L-Aoe-D-Pro-L-Ala-L-Ala) by Walton et al. (5). [Aoe stands for 2-amino-8-oxo-9,10-epoxy decanoic acid, a novel amino acid previously found in CyL-2 (6) and chlamydocin (7).] The composition of amino acids in both structures are the same but the sequences differ and chirality was proposed only for II. Both cyclic tetrapeptide structures are closely related to the system found in chlamydocin, a cytostatic cyclic tetrapeptide.

While investigating the solution conformation of HC-toxin by nuclear magnetic resonance (NMR) spectroscopy, we obtained evidence confirming that the amino acid sequence is cyclo (Aoe-D-Pro-Ala-Ala) (II), but consistent

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with the second alanine residue having the D - not the L - configuration. We report here the results of the NMR and amino acid oxidase studies that confirm the sequence and chirality of amino acids and establish the overall conformation of HC-toxin in chloroform.

Materials and Methods

The isolation and the purification of HC-toxin has been reported in detail (5). Chloroform-d (99.6% atom %D) was purchased from Aldrich Chemical Co. Proton NMR spectra were recorded on a Bruker WH-270 (270 MHz) spectrometer operated in the FT mode at 30°C. Detailed descriptions of experimental methods for the NMR studies are reported in preceding papers (8,9,10). The carbon-13 NMR spectrum was recorded on a Bruker WM-300 (75.5 M Hz) at 25°C. The amino acid oxidase experiments were carried out essentially by the procedure of Closse and Huguenin (5) as reported for chlamydocin. HC-toxin (1 μmole) was hydrolyzed (6 N HCl, 110°, 12 hr), and the mixture treated separately with L-amino acid oxidase and D-amino acid oxidase as described. The reaction was stopped by boiling, the solution was filtered and the filtrate loaded directly onto the Beckman column. Amino acid analysis after digestion with L-amino acid oxidase showed 22.7 nmole Ala:2.3 nmole Pro and after reaction with D-amino acid oxidase, 27.7 nmole Ala:27.3 nmole Pro, respectively.

Results

The 270 MHz ^{1}H NMR data are summarized in Table 1. These results agree reasonably well with reported ^{1}H NMR data (4,5) although the chemical shifts and the coupling constant values are slightly different. The two alanine

Table 1. 270 MHz ^{1}H NMR data for HC-toxin[1]

[L-Aoe^1 - D-Pro^2-L-Ala^3-D-Ala^4]

		$\Delta\delta/\Delta T$[2]
7.12	(d, 1 H, J = 10.3 Hz, Ala^3 NH)	-1.73
6.32	(d, 1 H, J = 10.5 Hz, Aoe NH)	-2.97
6.25	(d, 1 H, J = 9.5 Hz, Ala^4 NH)	-5.00
4.77	(m, 1 H, Aoe α-CH)	
4.70	(d.d, 1 H, J = 1.6, 6.0 Hz, Pro α-CH)	
4.56	(m, 1 H, Ala^4 α-CH)	
4.46	(m, 1 H, Ala^3 α-CH)	
3.97	(m, 1 H, Pro δ-CH)	
3.52	(m, 1 H, Pro δ'-CH)	
3.42	(d.d, 1 H, J = 2.4, 4.6 Hz, Aoe epoxy$^{\alpha}$)	
2.99	(d.d, 1 H, J = 4.7, 5.8 Hz, Aoe epoxy$^{\beta}$)	
2.86	(d.d, 1 H, J = 2.4, 5.8 Hz, Aoe epoxy$^{\beta}$)	
2.40-2.48	(m, 6 H, Pro β, γ, Aoe ω)	
1.55-1.97	(m, 8 H, Aoe β, γ, δ, ε)	
1.32	(d, 3 H, J = 6.8 Hz, Ala^3 β-CH$_3$)	
1.27	(d, 3 H, J = 7.0 Hz, Ala^4 β-CH$_3$)	

1: Peptide concentration: 6.8 mg/0.35 mL $CDCl_3$ at 30°C.
2: Temperature range 30-56.6°C: x 10^{-3} ppm/°C

Table 2. Results of NOE experiments of HC-toxin in chloroform-d

irradiate signal (ppm)	observed NOE (ppm)	%
Ala^3 NH (7.12)	Pro_2 α-CH (4.70)	13.3
Aoe_6 NH (6.32)	Ala^4 α-CH (4.56)	13.6
Ala^4 NH (6.25)	Ala^3 α-CH (4.46)	9.0
Aoe α-CH (4.77)	Pro_2 δ-CH (3.97)	< 1.0
Pro_2 α-CH (4.70)	Ala^3 NH (7.12)	10.1
Ala^4 α-CH (4.56)	Aoe_6 NH (6.32)	7.5
Ala^3 α-CH (4.46)	Ala^4 NH (6.25)	3.5

residues were assigned by standard double resonance experiments and by nuclear Overhauser experiments (Table 2). Large coupling constants ($^3J\alpha$, NH) for Ala^3 NH and Aoe NH protons have also been observed in other polypeptide systems, (8,9,11, 12) suggesting that $^3J\alpha$, NH $\geq$ 10 Hz represents $\theta\alpha$, NH values $\simeq$ 180° for transoid peptide bonds (13). The temperature coefficients of the amide

Table 3. Carbon-13 chemical shifts of HC-toxin and chlamydocin in chloroform-d

carbon	HC-toxin	chlamydocin[a]
phe α		53.52
β		35.92
pro α	57.98	57.86
β	25.06	25.03
γ	24.96	24.76
δ	47.19	47.02
Aoe α	51.98	54.39
side chain	29.01, 28.68	28.77
	25.45	25.30
	22.75	22.86
	36.26	36.30
Epoxy α	53.36	53.36
β	46.03	45.99
Aib α		58.89
β		26.55
β'		23.57
Ala α	48.21, 47.58	
β	14.58, 14.00	
C = O	207.51 (Aoe epoxy)	207.27
	173.81	175.63
	173.75	174.33
	173.36	172.87
	171.44 (Aoe)	171.84

[a] cited in Ref. 9

protons (Table 1) establish that the L-Ala NH and the Aoe NH are shielded from solvent, consistent with two intramolecular hydrogen bonds (9).

The C^{13} NMR data for HC-toxin, listed in Table 3, are compared with those of chlamydocin (9). By analogy we were able to assign all chemical shifts of carbons except the carbonyl carbons. The Pro C^{β} and C^{α} carbons resonate at 25.06 ppm and 24.96 ppm respectively, establishing that the x-Pro bond is predominantly trans (14) and part of an inverse γ-turn for the reasons discussed in preceding papers (8,9). One major difference between the two compounds is found at the Aoe α-carbons. In HC-toxin this carbon resonates at 51.98 ppm, but in chlamydocin resonates at 54.39 ppm.

Discussion

To distinguish between the two sequences (I and II) proposed for HC-toxin we carried out difference NOE experiments. Based on our previous results with chlamydocin (8,9) a strong positive enhancement of the α-proton of D-proline is expected when the amide NH of the next residue is irradiated. Our results showed that when Ala^1-NH (7.12 ppm) was irradiated a 13% enhancement of Pro-αH was obtained (4.70 ppm). No enhancement was observed at Pro-αH when the Aoe-NH was irradiated. These data establish the partial sequence D-Pro-L-Ala- as is found in II and also establish a trans amide bond configuration. Consistent with this assignment is the finding that the Aoe α-carbon in HC-toxin resonates about 2.4 ppm upfield from the corresponding position in chlamydocin in the ^{13}C NMR. This upfield shift is probably caused by steric compression (γ-effect)[15] between the Aoe-C^{α} and Pro-C^{δ}.

Irradiation of Aoe NH at 6.32 ppm produced an enhancement of Ala^4 α-H. This indicates the Aoe NH proton is located near the Ala^4 α-H, the amide bond is trans, and the sequence must be Ala-L-Aoe not a D-Pro-Aoe. Thus the sequence for HC-toxin must be (L-Aoe-D-Pro-L-Ala-Ala) as in II (5).

The difference NOE data also suggested the chirality of Ala^4 should be reassigned from L to D. Since all observed $^3J\alpha$, NH values are large, rapid rotations about amide bonds or about the ψ, ϕ torsion angles are not expected.

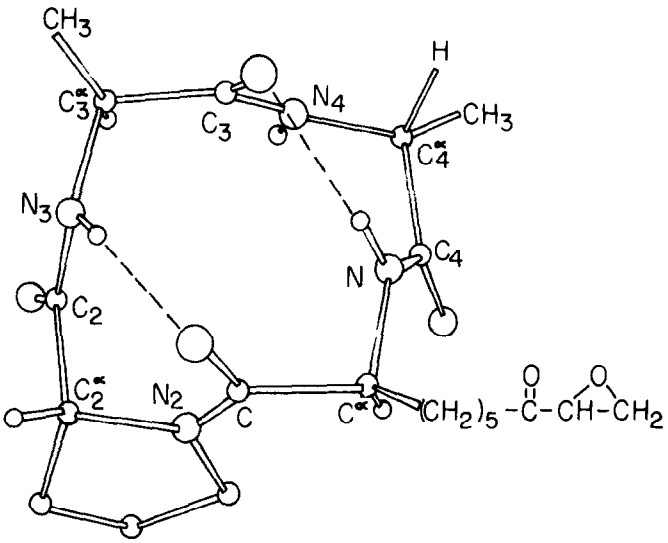

Figure 1. Schematic drawing of sequence and chloroform solution conformation of HC-toxin. Dashed lines indicate probable hydrogen bonds. Torsion angles (± 20°) are: L-Aoe, ϕ,-120; ψ, 120; ω, -160. D-Pro, ϕ, 60; ψ, -55; ω, +160. L-Ala, ϕ, -110; ψ, 110; ω, -160. D-Ala, ϕ, 60; ψ, -50; ω, 160.

The observed coupling constant (^3J NH = 9.5 Hz) for Ala^4 NH and the positive NOE enhancement between Ala^3 α-H and Ala^4 NH is reasonable if Ala^4 is a D amino acid. For an L-configuration at Ala^4 $^3J\alpha$, NH would be ~ 6 Hz and no NOE enhancement between Aoe NH and Ala^4 α-CH protons would be expected. The NOE enhancement also establishes a trans amide bond between Ala^3 and Ala^4.

HC-toxin was hydrolyzed and reacted separately with L-amino acid oxidase and D-amino acid oxidase. In both cases one equivalent of alanine was oxidized by the enzyme which established the presence of 1 L-Ala and 1 D-Ala in HC-toxin as predicted from the NMR data. Thus, the sequence and chirality of HC-toxin is cyclo (L-Aoe-D-Pro-L-Ala-D-Ala). The chirality of the epoxide group has not been assigned.

Our data also establish that the conformation of HC-toxin in chloroform is very similar to that of chlamydocin (9) and its related cyclic tetrapeptides (8). The NOE data establish that HC-toxin has four trans amide bonds. The ^{13}C NMR data establish the D-Pro-L-Ala sequence part of an inverse γ-turn. The temperature dependencies of the amide protons establish both the L-Ala and Aoe amide protons are likely to be hydrogen bonded. Together these data are

consistent with the bis-γ-turn conformation shown schematically in Figure 1. The fully characterized NMR results will be reported separately. It should be noted that the structures of the cytostatic compound chlamydocin and HC-toxin are remarkably similar. Both contain the Aoe residue and both cyclic tetrapeptide ring systems adopt the bis-γ-turn conformation in chloroform solution. Attempts to uncover the respective sites of action for these molecules are in progress.

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