Structure of the Host-Specific Toxin Produced by *Helminthosporium carbonum*[†]

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ABSTRACT: The primary structure of HC-toxin, a host-selective plant toxin produced by the fungus *Helminthosporium carbonum*, has been determined as a cyclic tetrapeptide. In addition to an uncommon α-amino acid, 2-amino-8-oxo-9,10-epoxydecanoic acid, the toxin contains one proline and two alanine residues. The sequence has been established as *cyclo*-[propylalanylalanyl-2-amino-8-oxo-9,10-epoxydecanoyl].

Certain plant pathogenic fungi, particularly species of *Alternaria* and *Helminthosporium*, produce metabolites in culture that specifically affect those host plants susceptible to the pathogen. Early work by Pringle & Scheffer (1967) and Pringle (1970, 1971) led them to postulate that the host-specific toxin from *Helminthosporium carbonum* race 1, attacking maize (HC-toxin), was a cyclic polypeptide ($C_{32}H_{50}N_6O_{11}$) containing proline, alanine, and unknown amino acids. Continuation of this research has resulted in the demonstration by Liesch et al. (1982) that HC-toxin is a tetrapeptide of alanine (two residues), proline, and the unusual 2-amino-8-oxo-9,10-epoxydecanoic acid (Aoe). The presence of the latter was deduced primarily by comparison of MS[1] and NMR spectra of the previously described fungal tetrapeptides Cyl-2 (Hirota et al., 1973) from *Cylindocladium scoparium* and chlamydocin (Closse & Hugenin, 1974) from *Diheterospora chlamydospora*. We have confirmed the amino acid composition reported by Liesch et al. (1982) independently and by different methods.

Configuration, dictated by amino acid sequence, obviously is of paramount importance in a molecule with host selectivity. There are three possible sequences for the amino acids reported for HC-toxin (Figure 1). On the basis of fragmentation during electron-impact mass spectrometry (EI-MS), Liesch et al. proposed the amino acid sequence labeled I. However, sequence assignment by EI-MS is equivocal because of the possibility of rearrangements (Biemann, 1971; Anderegg et al., 1979). This paper and another paper concerning the determination of sequence by mass spectrometry (Gross et al., 1982) provide unequivocal evidence that the sequence shown as III (Figure 1) is correct. Since this work was completed, Walton et al. (1982) also reported the sequence of amino acids to be the same as that reported here.

Materials and Methods

HC-Toxin, purified by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), inhibited root growth of susceptible maize genotypes by 50% compared to water controls at a concentration of 180–230 ng/mL (EC_{50}). Conditions for culturing *Helminthosporium carbonum* and procedures for isolating, purifying, and measuring biological activity of HC-toxin are reported by Ciuffetti et al. (1983).

Silica Gel TLC. For the monitoring of toxin purity during storage and isolation of toxin derivatives, thin-layer chromatography was performed with commercially prepared glass plates, EM 60 silica gel G (Merck-EM Reagents). The solvent system used was methyl ethyl ketone/pyridine/water/acetic acid (70:5:5:2 v/v/v/v). A 0.05% solution of bromcresol green in acetone or I_2 vapors were used to visualize toxin.

Polyamide TLC. Micropolyamide chromatography sheets, 5 × 5 cm (Schleicher & Schuell), were used for two-dimensional chromatography of dansyl [5-(dimethylamino)-naphthalene-1-sulfonyl] derivatives of amino acids and peptides. The sheets were developed with water/formic acid (200:3 v/v) in the first dimension and benzene/acetic acid (9:1 v/v) in the second as described by Gray (1967).

Amino Acid Analysis. Samples of toxin (ca. 250 μg) were hydrolyzed in 0.2 mL of 6 N HCl at 110 °C for 24 h. Hydrolyzed samples were analyzed with a Hitachi Perkin-Elmer KLH-3B amino acid analyzer by standard procedures.

Microscale Acid Hydrolysis and Derivatization. To conserve toxin, a microscale procedure was developed when dansyl derivatives were prepared from hydrolysates. Usually, 1-μL samples of an aqueous toxin solution (4 μg/mL) were added to 10 μL of 6 N HCl in melting point capillaries. The free space of the capillary was purged with N_2, and the tube was sealed in a flame. For complete hydrolysis sealed tubes were heated at 110 °C for 24 h. Partial hydrolysis, for dipeptide analysis, was performed in the same manner except that the tube was held at 50 °C for 10 h in the silicone oil bath. After hydrolysis, all samples were taken to dryness in vacuo over NaOH pellets.

Gray's (1967) method was adapted for the microscale hydrolytic methods described above. From a stock solution of 0.5 N $NaHCO_3$ and an acetone solution of dansyl chloride (8 mg/mL), a dansylating reagent was prepared immediately

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[1] Abbreviations: MS, mass spectroscopy; NMR, nuclear magnetic resonance.

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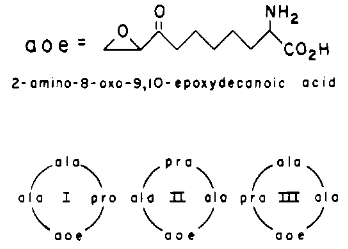


FIGURE 1: Possible amino acid sequences for HC-toxin. The diagrams are read in a clockwise direction.

prior to use by mixing equal volumes of the two solutions and centrifuging to remove small quantities of precipitated $NaHCO_3$. Dansyl derivatives of standard amino acids and dipeptides were prepared by adding 0.4 mL of the dansylating reagent to 1 mg of the standard. Standard amino acids and dipeptides (L-alanyl-L-alanine, L-alanyl-L-proline, and L-prolyl-L-alanine) were obtained from Sigma Chemical Co., St. Louis, MO. The reaction tubes were stoppered and placed in a water bath at 37 °C for 1 h. To the dried, microscale hydrolysates contained in melting point capillaries 10-μL aliquots of the dansylating reagent were added. The capillary tubes were flame sealed and incubated at 37 °C for 1 h. After the dansylation reaction, 0.005–0.08-μL samples of the resulting solution were applied to chromatography sheets for polyamide TLC analysis.

Sodium Borohydride Reduction of HC-Toxin. Approximately 2 mg of HC-toxin was placed in a 3-mL conical centrifuge tube and was taken to dryness under vacuum. The toxin was dissolved in 1 mL of dry methanol, prepared by refluxing MeOH over Mg turnings and I_2 and distilling. A 1-mL solution of 3 mg of $NaBH_4$/mL of dry MeOH was added. The reaction mixture was stoppered, mixed thoroughly, but gently, and allowed to stand 20 h at room temperature. After reduction, the solution was added to 4 mL of H_2O and extracted 6 times with 2-mL aliquots of $CHCl_3$. The combined extract was concentrated under N_2 and subjected to TLC on silica gel.

Opening of Oxirane Ring of HC-Toxin. The product of $NaBH_4$ reduction was taken to dryness in a 12-mL conical centrifuge tube. Acetic acid (2 mL) was added, and the mixture was refluxed for 2.5 h. The solution was lyophilized and acetylated as described below.

Acetylation of Derivatives. Samples were taken to complete dryness in vacuo; 0.5 mL of dry pyridine and 0.5 mL of acetic anhydride were added, and the mixtures were stirred at room temperature overnight.

Instrumentation. Ultraviolet absorption spectra of a 40-mg sample of toxin in 1 mL of D_2O were obtained with a Hewlett-Packard 8450A UV/vis spectrophotometer. Infrared absorption spectra were obtained with a Perkin-Elmer 283 infrared spectrophotometer. HC-Toxin was incorporated into a KBr wafer.

Proton NMR spectra and decoupling experiments were carried out with a 360-MHz, Model 1080 Nicollet instrument with the toxin dissolved in $CDCl_3$. Proton noise-decoupled ^{13}C NMR spectra were recorded with a Varian XL-100 100-MHz spectrometer.

Mass spectral experiments were performed in the Midwest Center for Mass Spectrometry, University of Nebraska—Lincoln. Chemical ionization (CI) with peak matching and full-scan high-resolution electron impact (EI) spectra were obtained with a Kratos MS-50 double-analyzer mass spectrometer. Collision-induced decomposition (CID) spectra from fast-atom bombardment mass spectrometry (FAB-MS) were obtained on a Kratos MS-50 triple analyzer.

Results

HC-Toxin, freshly purified by HPLC, gave a single band (R_f 0.74) by silica gel TLC when developed plates were exposed to iodine vapors or sprayed with bromcresol green. Because storage at 4 °C in water over a period of several months caused the appearance of an additional compound (R_f 0.55), called conversion product (Ciuffetti et al., 1983), the purity of our preparations was confirmed before each of the structural studies to be described. Neither the HC-toxin nor the conversion product reacted with ninhydrin.

Amino acid analysis of 6 N HCl hydrolysates of the toxin indicated the presence of alanine and proline in a molar ratio of 2:1, respectively. Some fluctuations in the base line of the amino acid analyzer recorder tracing were noted along with a strong ammonia peak. Only 50–60% of the weight of HC-toxin hydrolyzed could be accounted for by the quantities of alanine and proline detected by the amino acid analyzer. Complete acid hydrolysates of HC-toxin were reacted with dansyl chloride (by the microscale method), and the resulting products were separated by polyamide TLC. Intense fluorescent spots corresponding to the dansyl derivatives of alanine, proline, and NH_3 were observed along with several very weak fluorescent spots that did not match derivatives of the common amino acids. The fact that the toxin contained two common amino acids, but did not react with ninhydrin, indicated that all amino groups were protected.

Chemical ionization mass spectrometry (CI-MS) gave a protonated molecular ion at a nominal mass of 437. Three peak matching trials yielded high-resolution masses that were within 1, 0.5, and 0.5 ppm of the calculated mass of m/z 437.24001 for the ion ($C_{21}H_{32}N_4O_6 + H^+$). By high-resolution electron-impact mass spectrometry, a molecular ion was observed at m/z 436.2333, which agrees with a calculated mass of m/z 436.2323 for a molecule with an empirical formula of $C_{21}H_{32}N_4O_6$. With such an empirical formula and the presence of alanine and proline in a 2:1 ratio, only two alanine and one proline residues could be present in the molecule. Thus, the remainder of the empirical formula $C_{10}H_{15}NO_3$ represented unknown structure. Besides the possibility of an unusual amino acid residue, an ester was considered.

Ultraviolet absorption spectra of a solution of 40 mg of HC-toxin/mL of D_2O were recorded. The molecular absorptivity coefficients were calculated for the three observed maxima, on the basis of the molecular weight determined by MS. The results were as follows: λ_{max} 234 nm, $\epsilon = 21.6$; λ_{max} 246 nm, $\epsilon = 21.0$; λ_{max} 284 nm, $\epsilon = 20.7$. Although the absorption maxima were similar to those previously published (Pringle, 1970), the absorptivity coefficients were much lower and did not indicate the presence of unsaturated carbon bonds. The absorption at 284 nm was characteristic of carbonyl functions, and the remaining maxima, although not typical, were attributed to amide linkages.

The infrared absorption spectrum was similar to that previously described (Pringle, 1970). The spectrum was dominated by a broad asymmetrical absorption band at 1684–1630 cm^{-1}. Of particular importance were the three bands attributable to epoxides at 1233, 930, and 865 cm^{-1}.

Proton noise-decoupled ^{13}C NMR spectra of HC-toxin were obtained with D_2O as a solvent. The 21 resonance signals observed were consistent with the empirical formula determined by mass spectrometry. The observed shifts and eventual assignments are shown in Table I. The signal at 198.2 ppm was assigned to a ketone. Four carboxylic acid derivatives, consistent with peptide linkages, were observed at 168.4, 169.5,

Table I: ^{13}C NMR Spectral Assignments of HC-Toxin, in D_2O, Relative to TSP

ppm	assignment
11.5, 14.5	Ala$_1$-β, Ala$_2$-β
15.6, 18.5, 21.3, 23.7	Pro-γ, Aoe-γ, Aoe-δ, Aoe-ε
26.0, 27.7	Pro-β, Aoe-β
34.6	Aoe-ζ
43.8, 45.2, 47.1, 49.0, 49.3, 52.8	Aoe-α, Aoe-θ, Aoe-ι, Ala$_1$-α, Ala$_2$-α, Pro-δ
55.7	Pro-α
168.4, 169.5, 170.3, 171.9	carbonyl of Ala$_1$, Ala$_2$, Pro, and Aoe
198.2	Aoe-η (ketone)

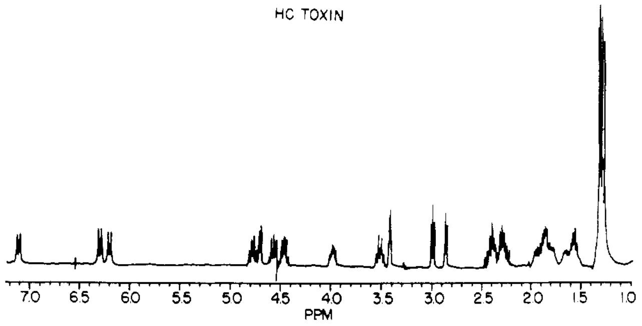


FIGURE 2: Proton NMR spectrum of HC-toxin in $CDCl_3$. Chemical shifts are relative to tetramethylsilane.

170.3, and 171.9 ppm. This suggested that the unknown structure was a single carboxylic acid derivative joined to the three common amino acids by a peptide linkage. The empirical formula of the unknown moiety, the lack of unsaturation, and lack of more than one ketone or aldehyde suggested a ring in the unknown acid.

Proton NMR spectra of HC-toxin in $CDCl_3$ (Figure 2) indicated three amide protons at 6.22, 6.32, and 7.13 ppm that were exchangeable in D_2O. The postulated ring, suggested by IR to be an oxirane, was supported by resonances at 2.88, 2.99, and 3.43 ppm. The three proton signals suggested a terminal epoxide. Thus, an amino acid, presumably an α-amino acid containing a ketone and a terminal epoxide, was postulated as the fourth amino acid in a cyclic tetrapeptide.

For cyclic peptides, mass spectrometric fragmentation mechanisms predict the appearance of relative intense imine fragment ions representative of each constituent α-amino acid residue minus CO plus one proton ($^+NH_2{=}CH{-}R$). An alanine residue should yield $^+NH_2{=}CH{-}CH_3$, *m/z* 44, and a proline residue should yield c-$(CH_2)_3CHN^+$, *m/z* 70. An α-amino acid with a $C_8H_{13}O_2$ R group should yield an *m/z* 170 peak. The high-resolution EI-MS of HC-toxin gave a peak at *m/z* 170.1182 with an intensity of 46.3% relative to that of the proline ring fragment at *m/z* 70.0656, which was the base peak. The mass of an ion with an empirical formula of $C_9H_{16}N_1O_2$ is only 0.4 ppm greater than the observed mass for the peak at *m/z* 170. This supported the conclusion that the unknown component was due to a single α-amino acid containing two oxygen atoms and a ring in the R group. Because NMR suggested a terminal epoxide and a ketone function, a series of HC-toxin derivatives were prepared to establish the presence of those groups and gain information of the location of the ketone. Silica gel TLC was performed at each step to isolate and purify the derivatives. First HC-toxin was reduced with $NaBH_4$ to selectively reduce the ketone function to hydroxyl group. The product, called reduced toxin, had an R_f of 0.69. Acetylation of the reduced toxin yielded the monoacetyl derivative, R_f 0.80. Then the epoxide ring was opened by refluxing the reduced toxin in acetic acid, followed by acetylation. The resulting triacetyl derivative had an R_f of 0.82. High-resolution EI-MS spectra of the three derivatives were compared to each other and to native HC-toxin. Table II shows the expected R group of the amino acid, the masses observed for the molecular ions, and ion fragments arising from the derivatized R groups. In all spectra, the proline residue ion at *m/z* 70 was the base peak, and observed masses were within 2 ppm of the appropriate empirical formulas. Collison-induced decomposition (CID) spectra of each of the residue ions (*m/z* 170, 172, 214, and 316) were obtained with the third sector of a Krotoes MS-50 triple analyzer. These spectra confirmed that derivatization of the residue had occurred as predicted and indicated that the ketone function of the unusual amino acid was adjacent to the epoxide group. Also, the appearance of an *m/z* 98 ion, $C_6H_{12}N$, in the high-resolution spectra confirmed that the epoxy ketone was a terminal group in the α-amino acid. The unusual amino acid appeared to be 2-amino-8-oxo-9,10-epoxydecanoic acid (Aoe). Proton NMR decoupling experiments confirmed the structure for Aoe. Table III shows the chemical shifts, assignments, decoupling frequencies applied, and resulting decoupling.

Assignment of the protons of the two alanyl and one prolyl residues made it possible to correlate the structure for the remainder of the molecule. Consistent with the mass spectral evidence, Aoe appeared to be an α-amino acid in amide linkage

Table II: Electron-Impact, High-Resolution Masses Observed for Selected Ions from HC-Toxin and Three of Its Derivatives

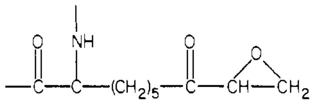
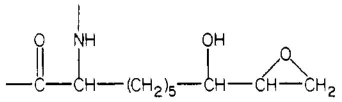
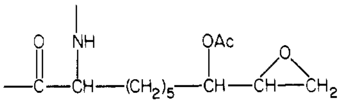
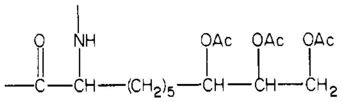
derivative	derivatized amino acid residue	molecular ion (*m/z*)	residue ion (*m/z*)	rel intensity of residue ion (%)
HC-toxin		436.2333	170.1182	46.3
reduced toxin		438.2458	172.1340	31.9
monoacetyl derivative		480.2575	214.1444	27.2
triacetyl derivative		582.2912	316.1770	47.7

Table III: Proton NMR Spectral Assignments of HC-Toxin in $CDCl_3$, Relative to Me_4Si

chemical shift (ppm)	assignments, integration, and coupling constants (J in Hz)	decoupling irradiation (ppm)	effect of decoupling
1.28	Ala_1-β, 3 H, $^3J_{\alpha\beta} = 7.04$	4.59	d → s
1.32	Ala_2-β, 3 H, $^3J_{\alpha\beta} = 7.04$	4.49	d → s
1.24–1.36	Aoe-γ, 2 H; Aoe-δ, 2 H	1.57	m → sim
1.57	Aoe-ϵ, 2 H	1.298, 2.287, 2.340	m → sim
1.65	Aoe-βU, 1 H, $^3J_{\alpha\beta U} = 7.68$	1.298	m → d of d
1.80	Aoe-βD, 1 H, $^3J_{\alpha\beta D} = 7.68$	1.298	m → d of d
1.86	Pro-βU, 1 H, $^3J_{\alpha\beta U} = 7.80$	2.287, 2.34, 2.40, 4.75	m → sim
1.93	Pro-γU, 1 H, $^3J_{\gamma U\delta U} = 6.64$, $^3J_{\gamma U\delta D} = 4.93$	2.287, 2.34, 2.40, 3.52, 4.00	m → sim
2.30	Pro-γD, 1 H, $^3J_{\gamma D\delta U} = 6.62$, $^3J_{\gamma D\delta D} = 8.55$	1.87, 1.94, 2.40, 3.52, 4.00	m → sim
2.38	Pro-βD, 1 H, $^3J_{\alpha\beta D} = 2.19$	1.87, 1.94, 4.75	m → sim
2.26–2.46	Aoe-ζ, 2 H	1.57	m → d of d
2.88	Aoe-ιU, 1 H, $^3J_{\iota U\iota D} = 5.64$, $^3J_{\iota U\theta} = 3.92$	2.85, 3.43	d of d → sim
2.99	Aoe-ιD, 1 H, $^3J_{\iota U\iota D} = 5.64$, $^3J_{\iota D\theta} = 3.66$	2.85, 3.43	d of d → sim
3.43	Aoe-θ, 1 H, $^3J_{\iota U\theta} = 3.92$, $^3J_{\iota D\theta} = 3.66$	2.85	d of d → d
		3.00	d of d → s
3.53	Pro-δU, 1 H, $^3J_{\gamma D\delta D} = 6.62$, $^3J_{\gamma U\delta U} = 6.64$, $^2J_{\gamma U\delta D} = -9.95$	1.94, 2.287, 4.00	d of t → sim
3.99	Pro-δD, 1 H, $^3J_{\gamma D\delta D} = 4.93$, $^3J_{\gamma U\delta D} = 8.85$, $^2J_{\delta U\delta D} = -9.95$	1.94, 2.287, 3.52	m → sim
4.49	Ala_2-α, 1 H, $^3J_{N\alpha} = 10.25$, $^3J_{\alpha\beta} = 7.04$	7.10	d of q → d
		1.298	d of q → q
4.59	Ala_1-α, 1 H, $^3J_{N\alpha} = 9.73$, $^3J_{\alpha\beta} = 7.04$	6.10	d of q → d
		1.298	d of q → q
4.71	Pro-α, 1 H, $^3J_{\alpha\beta U} = 7.80$, $^3J_{\alpha\beta D} = 2.19$	1.87, 2.40	d of d → d
4.79	Aoe-α, 1 H, $^3J_{N\alpha} = 10.39$, $^3J_{\alpha\beta U} = 7.68$, $^3J_{\alpha\beta D} = 7.68$	6.30, 1.65, 1.80	d of t → d of d
			d of t → t
6.22	Ala_1-NH, 1 H, $^3J_{N\alpha} = 9.73$	4.59	d → s
6.32	Aoe-NH, 1 H, $^3J_{N\alpha} = 10.39$	4.75	d → s
7.13	Ala_2-NH, 1 H, $^3J_{N\alpha} = 10.25$	4.49	d → s

to the remaining residues. The presence of a fourth amide carbon atom in the ^{13}C NMR spectrum, a proton in the ^1H NMR spectrum that resonated in the region of the α-proton of amino acids, and the coupling of that proton (4.79 ppm) to a single amide proton (6.32 ppm) support that conclusion. The three protons of the terminal epoxide function were all intercoupled but showed no additional coupling, confirming that the oxirane ring was adjacent to the ketone function. There remained four methylene groups, which the decoupling experiments showed to be an aliphatic chain between the β-methylene and the terminal epoxy ketone.

The remaining major structural feature of HC-toxin to be determined was the sequence of amino acids in the toxin molecule. The amino acid composition allows the three possible sequences shown in Figure 1.

Although Aoe is not stable under acid hydrolysis conditions, each of these sequences should be uniquely determined by partial acid hydrolysis and identification of resulting dipeptides of the common amino acids. The presence of two dipeptides and absence of a third should distinguish all three sequences. Sequence I should yield Ala-Ala and Ala-Pro but not Pro-Ala; II should yield Ala-Pro and Pro-Ala but not Ala-Ala; III should yield Pro-Ala and Ala-Ala but not Ala-Pro. Dansyl derivatives of the three possible dipeptides, Ala-Ala, Ala-Pro, and Pro-Ala, were separable by polyamide TLC (Figure 3).

By adjustment of time and temperature, optimum hydrolytic conditions for the production of dipeptides from HC-toxin were found. Comparisons of the mobilities of the dansylated products to those of standards showed that the sequences Ala-Ala and Pro-Ala were present while Ala-Pro was absent. The presence or absence of the three dipeptides in question was confirmed by rechromatographing the products mixed with one of each of the standards. These results showed that the sequence must be sequence III, in which, as shown in Figure 4, the carboxyl group of Aoe forms an amide linkage with the secondary amino group of proline and the two alanine residues are adjacent. The sequence is substantiated by the observation of an ion (m/z 126.0559) corresponding to the sequence Ala-Ala in our EI-MS spectra and by the FAB spectrometry (ms/ms/ms) reported by Gross et al. (1982).

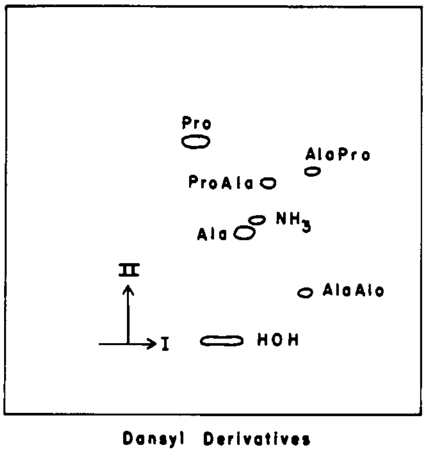


FIGURE 3: Tracing of a two-dimensional thin-layer polyamide sheet showing locations of the dansyl derivatives of alanine and proline and dansyl derivatives of dipeptides of alanine and proline. (Solvent I) Water/formic acid (200:3 v/v); (solvent II) benzene/acetic acid (9:1 v/v).

Discussion

The usefulness of the computer-based natural product recognition system used by Liesch et al. (1982) was demonstrated by our confirmation of the structure of the unusual

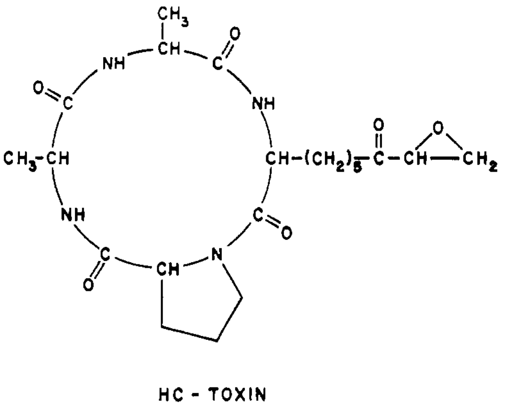


FIGURE 4: Proposed structure of HC-toxin.

α-amino acid 2-amino-8-oxo-9,10-epoxydecanoic acid (Aoe), which was determined by conventional methods. Proton NMR decoupling experiments were critical in our structural studies. Although there is good agreement between their and our spectra, decoupling led to several distinctly different and more complete assignments. The α-proton resonances assigned to alanine residues differ, and we were able to assign each of the three amide protons to specific amino acids. Some assignments of proline and Aoe protons were different, and because of overlapping signals, their assignments could be ascertained only by decoupling. Proton NMR assignments were not reported by Walton et al. (1982), but their spectrum was similar to ours except for some explainable differences (Ciuffetti et al., 1983).

The sequence of amino acids in the toxin is the major difference between our results and those of Liesch et al. (1982). Results from degradation studies reported here and the mass spectrometric data reported elsewhere (Gross et al., 1982) for HC-toxin strongly indicate that the carboxyl group of proline does not form an amide linkage with the α-amino group of Aoe as it does in chlamydocin (Closse & Hugenin, 1974). In HC-toxin, the linkage is just the reverse.

To understand the biological activity of a host-specific toxin, stereochemical conformation of a molecule must be considered in addition to the primary structure. Space-filling models of HC-toxin indicate that the peptide ring is constrained. Nonpolar R groups appear outside the ring with the nitrogen and several oxygen atoms on the interior. The peptide linkages may have cis to trans conformations that do not seem readily interchangeable due to the ring constraints. Five asymmetric carbon atoms are present in the molecule. The preferred conformation for biological activity may not be apparent until the sterochemistry is determined. The necessity of an intact oxirane ring for toxicity is reported in a following paper (Ciuffetti et al., 1983), and the effect of other functional groups and molecular conformation on biological activity is being investigated.

Registry No. HC-Toxin, 83209-65-8.

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