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STRUCTURE OF AN AMINO ACID ANALOG OF THE HOST-SPECIFIC TOXIN FROM *HELMINTHOSPORIUM CARBONUM*

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Abstract: The structure of an analog to the host-specific plant toxin from *Helminthosporium carbonum* has been determined to be cyclo [prolylalanylglycyl-2-amino-8-oxo-9,10-epoxydecanyl], which has a glycine residue in place of the D-alanine residue of the toxin.

Four cyclic tetrapeptides, containing the unusual amino acid, 2-amino-8-oxo-9,10-epoxydecanoic acid (Aoe), have been isolated from fungi that differ phylogenetically (1-7). All are biologically active, but in different biological systems. Two of these are plant toxins. The host-specific toxin from *Helminthosporium carbonum* (HC-toxin) affects only varieties of maize that are susceptible to the fungus. In contrast, Cyl-2, produced by another plant pathogen, *Cylindrocladium scoparium*, is toxic to several diverse plant species (8). Antitumor activity has been detected for two chemically related tetrapeptides, WF-3161, isolated from *Petriella guttulata* (7) and chlamydocin, from *Diheterospora chlamydosporia* (5). In addition to the Aoe residue, all four of these cyclic tetrapeptides contain an imino acid moiety, either proline or pipecolic acid. Thus, chemical differences among these tetrapeptides reside largely in the nature and sequence of the two remaining amino acid residues, which may have a significant effect on their biological activities and specificities. We report here the characterization of another cyclic tetrapeptide from *H. carbonum* containing proline, alanine, glycine and 2-amino-8-oxo-9,10-epoxydecanoic acid. This toxin, called HC-toxin analog, differs from HC-toxin by the replacement of a D-alanine residue with a glycine residue.

The glycine-containing HC-toxin analog was isolated by the procedures developed for HC-toxin (1). It was detected initially by spraying TLC plates with 4-(p-nitrobenzyl)-pyridine reagent (9) to which epoxide functions react. The analog had not been detected previously in cultural filtrates of *H. carbonum* because of its low concentration (<5% of HC-toxin) and the use of non-specific TLC spray reagents. Purification by TLC and HPLC indicated the analog was slightly more polar than HC-toxin.

The EC_{50} (concentration of toxin that inhibits root growth of susceptible maize seedlings by 50%) of the analog was about 7.0 µg/ml, whereas HC-toxin has an EC_{50} near 0.2 µg/ml (10). Although lower in toxicity, the analog was selectively active against susceptible genotypes and did not inhibit root growth of resistant varieties of maize at 10.0 µg/ml.

Amino acid analysis by HPLC of phenylthiocarbamyl derivatives (11) yielded a molar ratio of 1.28:0.96:1.00 for alanine, proline and glycine, respectively.

Proton NMR (Table I) with a 360 MHz, model 108 Nicolet instrument revealed the analog was structurally similar to HC-toxin. Readily apparent differences were observed in only three regions: (a) near 1.3 ppm, the analog exhibited the presence of one instead of two methyl groups; (b) one proton signal was present at 3.12 ppm in the analog spectrum but absent in the HC-toxin spectrum; (c) in the analog spectrum, one amide proton exhibited additional splitting. Hence the spectrum was consistent with the replacement of one of the alanine residues of HC-toxin with a glycine residue. Decoupling experiments permitted complete, consistent assignments as shown in Table I.

Table I. Proton NMR Spectral Assignments of Glycine - HC-Toxin in $CDCl_3$, Relative to Me_4Si

Chemical Shift (ppm)	Assignments, integration, and Coupling Constants (J in H_Z)	Decoupling irradiation (ppm)	Effect of Decoupling
7.0	Ala-NH,1H	4.52	d → s
6.5	gly-NH,1H, $J_{N\alpha 2}$ = 3.65, $J_{N\alpha'}$ = 10.43	4.47 3.12	d of d → s d of d → s
6.25	Aoe-NH,1H, $J_{N\alpha}$ = 10.78	4.7	d → s
4.75	Aoe-α,1H, $J_{N\alpha}$ = 10.78, $J_{\alpha\beta}$ = 7.63	6.25, 1.88 1.55	m → Sim
4.70	pro-α,1H, $J_{\alpha\beta'}$ = 7.54, $J_{\alpha\beta 2}$ = 2.03	2.36, 1.88	d of d → d
4.52	Ala-α,1H, $J_{\alpha\beta}$ = 6.85	1.30 7.0	d of q → d d of q → d
4.42	gly-α,1H, $J_{\alpha,\alpha 2}$ = 13.76, $J_{N\alpha'}$ = 10.43	6.5 3.12	d of d → d d of d → d
3.90	pro-δ_2,1H, $J_{\delta,\delta 2}$ = 10.07	3.5, 2.36 1.88	m → Sim
3.50	pro-δ_1,1H, $J_{\delta,\delta 2}$ = 10.07	3.92, 2.36 1.88	m → Sim
3.39	Aoe-θ,1H, $J_{\theta\iota 2}$ = 2.44, $J_{\theta\iota 1}$ = 4.70	2.98 2.85	d of d → d d of d → d
3.12	gly-α_2,1H, $J_{N\alpha 2}$ = 3.65, $J_{\alpha,\alpha 2}$ = 13.76	4.47 6.50	d of d → d d of d → d
2.98	Aoe-ι,1H, $J_{\theta\iota}$ = 4.70, $J_{\iota,\iota 2}$ = 5.77	3.39 2.85	d of d → d d of d → d
2.85	Aoe-ι_2,1H, $J_{\theta\iota 2}$ = 2.44, $J_{\iota,\iota 2}$ = 5.77	3.39 2.98	d of d → d d of d → d
2.36	pro-β_2,1H, $J_{\alpha\beta 2}$ = 2.03	4.70, 1.88	m → Sim
2.27	pro-γ_2,1H	3.92, 3.5	m → Sim
2.4-2.2	Aoe-ζ,2H	1.55	m → Sim
2.0-1.7	pro-γ,1H; pro-β_1,1H; Aoe-β_1,1H	4.7, 3.92 3.5, 2.36 1.55, 1.30	m → Sim
1.65-1.28	Aoe-γ,2H; Aoe-δ,2H; Aoe-ε,2H; Aoe-β_2,1H	4.75, 2.3	m → Sim
1.29	Ala-β,3H, $J_{\alpha\beta}$ = 6.85	4.52	d → s

Fast Atom Bombardment (FAB) mass spectrometry with a Kratos MS-50 triple analyzer, yielded an $(M+1)^+$ ion at m/z 423, which corresponds to an empirical formula of $C_{20}H_{30}N_4O_6$ for the neutral molecule. Using two different matrices, glycerol and a dithiothreitol/dithioerythritol mixture, the intensity of $(M+H)^+$ was 5.4% and 45.4%, respectively, of the base peak which was due to the matrix in both cases.

The elemental composition of HC-toxin analog was established by peak matching the $(M+H)^+$ ion in the FAB mass spectrum and by high resolution electron impact (EI) mass spectrometry using CsI in glycerol as a source of reference masses. The mass of $(M+H)^+$ was determined to be 423.22669 amu, within 5.6 ppm of the mass of an ion with the formula $C_{20}H_{30}N_4O_6$. The EI mass spectrum yielded a molecular ion at m/z 422.2163, a 0.3 ppm deviation from the mass calculated for $C_{20}H_{30}N_4O_6$.

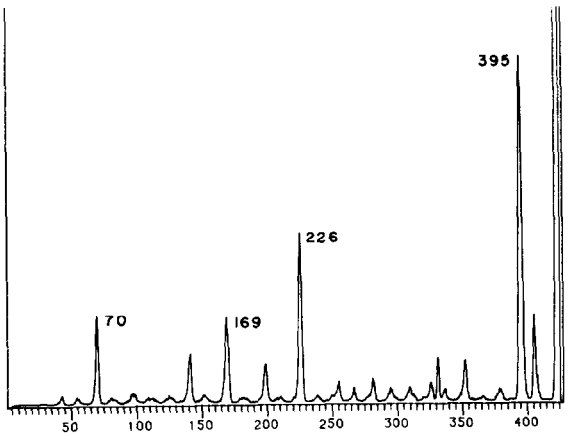
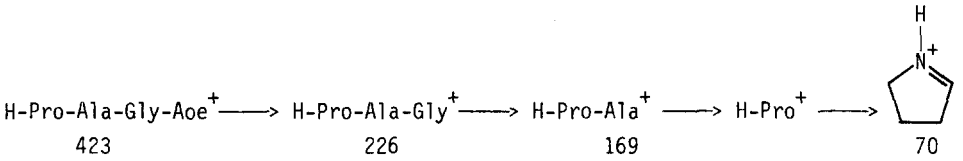


Figure 1. CAD spectrum of the $(M+H)^+$ ion of HC-toxin analog

Other ions in the EI mass spectrum implied a strong similarity to HC-toxin, particularly m/z 170, m/z 169, and m/z 70, which indicate the presence of Aoe, alanine-proline and proline respectively. These residues account for all but C_2H_3NO which is consistent with a glycine residue. In addition, ion signals at m/z 254 and m/z 154 are consistent with formulas for $(Gly\text{-}Aoe)^+$ and $(Gly\text{-}Pro)^+$ respectively.



Scheme 1

To determine the amino acid sequence Collision Activated Decomposition (CAD) following FAB ionization was used. The CAD spectrum of $(M+H)^+$ at m/z 423 showed principal decomposition products at 395, 226, 169, 141 and 70 m/z (Figure 1). The loss of CO from $(M+H)^+$ yields m/z 395 while the other ions may be accounted for by decomposition of the ring-opened $(M+H)^+$ ion as indicated in scheme 1.

Since m/z 226 contained proline, alanine and glycine residues (empirical formula confirmed by peak matching) its CAD spectrum further defines the sequence of residues and corroborate the above proposal. As shown in Figure 2, ions at m/z 169 and m/z 70 were observed. Thus, we propose the structure of the HC-toxin analog to be that shown in figure 3.

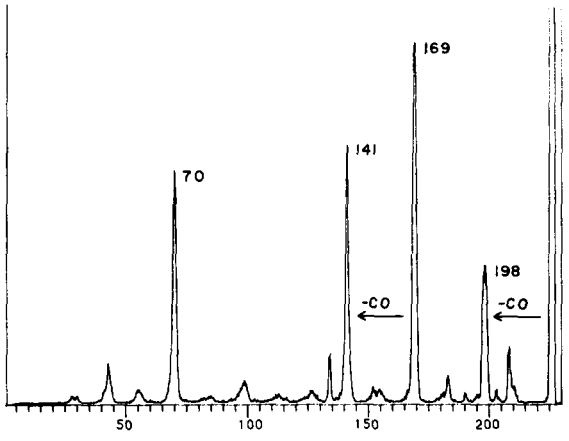


Figure 2. CAD spectrum of the m/z 226.

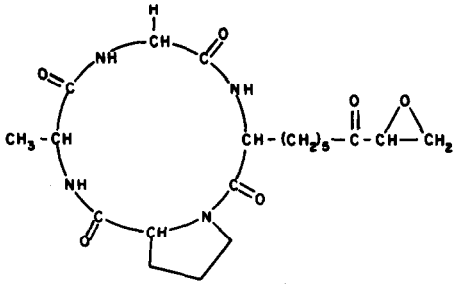


Figure 3. Structure of HC-toxin analog.

The relatively small structural difference between HC-toxin and the analog (a methyl group) causes a large difference in toxicity on susceptible maize genotypes. Although the epoxide group of Aoe is critical for toxicity (10), other amino acid residues apparently play an important role in determining the molecule's biological activity.

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