Isolation and Structure of an Inactive Product Derived from the Host-Specific Toxin Produced by *Helminthosporium carbonum*†

Lynda M. Ciuffetti, Mark R. Pope,‡ Larry D. Dunkle, J. M. Daly, and Herman W. Knoche*

ABSTRACT: Storage in aqueous solutions and acidic conditions resulted in the formation of an inactive product from the host-specific toxin (HC-toxin) produced by the fungal pathogen of maize *Helminthosporium carbonum* race 1. This conversion provided an opportunity to examine structural features required for specificity and toxicity with this toxin. The conversion product was obtained in good yields by acid treatment of HC-toxin and further purified by chromatographic methods. Proton nuclear magnetic resonance, mass spectrometry, and degradation and derivation reactions established that the conversion product was *cyclo*-[prolylalanylalanyl-2-amino-8-oxo-9,10-dihydroxydecanoyl], which is easily formed by hydrolysis of the oxirane ring of the 2-amino-8-oxo-9,10-epoxydecanoic acid moiety of HC-toxin. Thus, at least one structural requirement for toxin activity in maize genotypes susceptible to the pathogen is an intact oxirane ring. The conversion product is inactive at concentrations at least 100 times greater than native toxin and shows no competitive effects with it. Its presence in toxin preparations may explain conflicting data about the specific biological activity of HC-toxin.

In a previous paper (Pope et al., 1983), we presented evidence for the structure of HC-toxin, the host-specific toxin produced by the foliar pathogen of maize, *Helminthosporium carbonum* Ullstrup race 1. The toxin is a cyclic tetrapeptide with a molecular weight of 436, an empirical formula of $C_{21}H_{32}N_4O_6$, and a sequence *cyclo*-[Pro-Ala-Ala-Aoe], where Aoe is 2-amino-8-oxo-9,10-epoxydecanoic acid. By monitoring the purity and activity of HC-toxin preparations during isolation, storage, and structural analyses, we observed a contaminating peptide in preparations that had reduced biological activity. Evidence of a single structural modification of the toxin and the consequence on biological activity are presented in this paper. The results document the essentiality of the oxirane ring of Aoe for toxic activity in maize.

Materials and Methods

Toxin Isolation and Purification. Single-spore isolates of *Helminthosporium carbonum* race 1 were obtained from diseased leaf tissue of susceptible maize genotypes and maintained on potato dextrose agar. For toxin production, the fungus was grown for 19 days on liquid modified-Fries' medium (Pringle & Scheffer, 1967). Initial isolation procedures were as described by Pringle & Scheffer (1967). However, in all stages involving evaporation of solvents or concentration of solutions in vacuo, the vacuum apparatus was purged with nitrogen.

Culture filtrates were filtered through cheesecloth, concentrated in vacuo at 30 °C, and deproteinized with methanol. Any precipitate remaining after evaporation of the methanol was removed by filtration, and the filtrate was extracted 4 times with an equal volume of chloroform. The combined chloroform extracts were concentrated to dryness at 30 °C, and the residue was solubilized in ethanol/ether (1:20 v/v) for 24 h at 5 °C.

The ethanol/ether was decanted and evaporated in vacuo, and the residue was dissolved in a small volume of ethanol. After addition of 5 volumes of water and then 4–5 volumes of ether, the mixture was centrifuged at 8000*g* for 30 min, and the lower (ethanol/water) phase was removed and concentrated to dryness. The residue containing the toxin was dissolved in a small volume of water and stored at 5 °C.

The enriched toxin preparation was further purified by preparative thin-layer chromatography (TLC) on silica gel G (SG-G) or silica gel 60 (SG-60) with HPLC-grade acetonitrile as a solvent. A small portion of the toxin band (R_f 0.45 or 0.56 on SG-G or SG-60, respectively) was detected with bromocresol green, and the remainder of the band was removed from the plate. Toxin was eluted from silica gel 3 times with methanol. The methanol was evaporated in vacuo, and the residue was dissolved in a small volume of water.

High-performance liquid chromatography (HPLC), the final purification step, was done on a Beckman Model 110 A with an Altex C8 column (250 × 4.5 mm, 5-μm particles, Ultrasphere octyl) and an isocratic solvent system (18.5% acetonitrile in water) at a flow rate of 0.6 mL/min. The effluent was monitored by absorbance at 206 nm. The toxin, which eluted at 46 min, was collected, dried in vacuo at 30 °C, dissolved in water, and stored at 5 °C.

Bioassays. Activity of toxin preparations was determined by a modified root growth inhibition bioassay (Dunkle, 1979). Seeds of susceptible (K61 × Pr; Pr × K61; or Pr) and near-isogenic resistant (K61 × Pr 1; Pr 1 × K61; or Pr 1) genotypes were germinated at 24 °C until the radicle had just emerged from the seed (1–2 mm). Seedlings were incubated for 96 h in 9-cm petri dishes containing 15 mL of water or dilutions of the toxin preparations. Root lengths were measured and used to determine the concentration that inhibited root growth by 50% (EC_{50}) compared with water controls of the same genotype. The effect of preparations on the rate and extent of electrolyte leakage from susceptible and resistant seedlings was determined as previously described (Dunkle & Wolpert, 1981).

† From the U.S. Department of Agriculture, Agricultural Research Service, Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana 47907 (L.M.C. and L.D.D.), and the Department of Agricultural Biochemistry, University of Nebraska, Lincoln, Nebraska 68583 (M.R.P., J.M.D., and H.W.K.). *Received December 22, 1982.* This is Journal Paper No. 9286 from the Purdue Agricultural Experiment Station and Paper No. 7048, Journal Series, from the Nebraska Agricultural Experiment Station. The research was partially supported by National Science Foundation Grant PCM-7920685. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or imply approval to the exclusion of other products that also may be suitable.

‡ Present address: Department of Biochemistry, University of Wisconsin, Madison, WI 53706.

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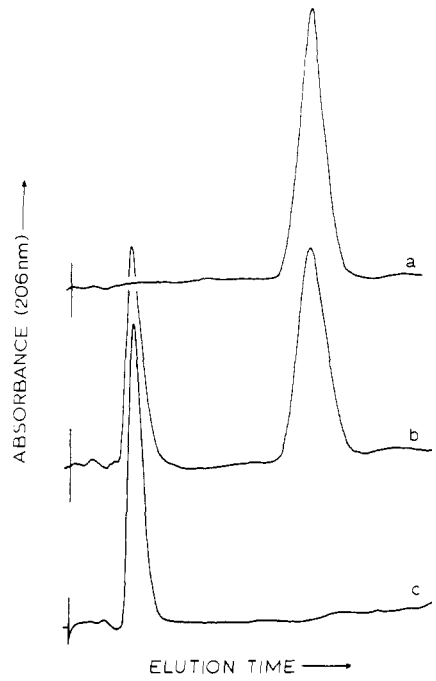

FIGURE 1: High-performance liquid chromatography recordings of (a) pure HC-toxin, (b) a mixture of HC-toxin and its degradation product, called conversion product, and (c) pure conversion product. HPLC conditions are given under Materials and Methods.

Analytical Procedures. Amino acid compositions were determined by hydrolysis in 6 N HCl at 110 °C for 24 h and by standard procedures with a Durrum D 500 analyzer. Dansyl chloride [5-(dimethylamino)naphthalene-1-sulfonyl chloride] was used to prepare amino acid derivatives for separation on polyamide sheets according to the method of Gray (1967). Standard dansyl amino acids were purchased from Sigma Chemical Co.

Solvents for TLC with silica gel were as follows: (solvent A) propanol/acetic acid/water (100:3:200 v/v); (solvent B) acetonitrile (HPLC grade); (solvent C) methyl ethyl ketone/pyridine/water/acetic acid (70:10:10:2 v/v); (solvent D) methyl ethyl ketone/pyridine/water/acetic acid (70:15:15:2 v/v); (solvent E) 0.1% trifluoroacetic acid in water/acetonitrile (1:99 v/v). All solvents except solvent A separated HC-toxin from the major contaminating peptide. Procedures for proton NMR, mass spectrometry, and acetylation are described in the previous paper (Pope et al., 1983).

Results

Purified HC-toxin migrated on silica gel as a single spot in several TLC solvent systems and eluted as a single UV-absorbing peak with isocratic (Figure 1a) or gradient solvent systems in HPLC. The preparation inhibited root growth of susceptible seedlings by 50% at 180–230 ng/mL (Figure 2) but did not affect root growth of the resistant seedlings at concentrations up to 5 μg/mL, the highest concentration tested. Loss of electrolytes from susceptible seedlings treated with 5 μg of toxin/mL began only after a relatively long period of time (12–15 h). The effect of toxin, however, was selective; resistant seedlings were not affected (Figure 3).

Our initial procedures of toxin purification involved the use of an acidic HPLC solvent system consisting of 12% acetonitrile and 0.1% trifluoroacetic acid (TFA) in water and evaporation of solvents or concentration of solutions in vacuo without precautions to minimize exposure of samples to air (see Materials and Methods). When such toxin preparations were analyzed by HPLC with various solvent systems, a second peak, referred to as conversion product, was detected. Figure

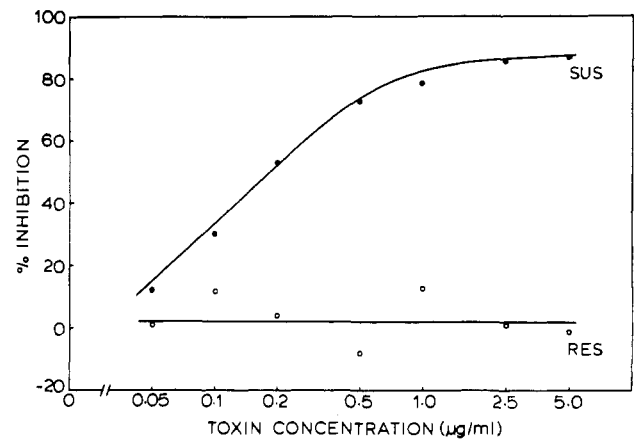

FIGURE 2: Root growth inhibition bioassay of HC-toxin with near-isogenic lines of maize susceptible (●) or resistant (○) to *H. carbonum* race 1. Each data point is the mean value from four separate bioassays of different toxin preparations.

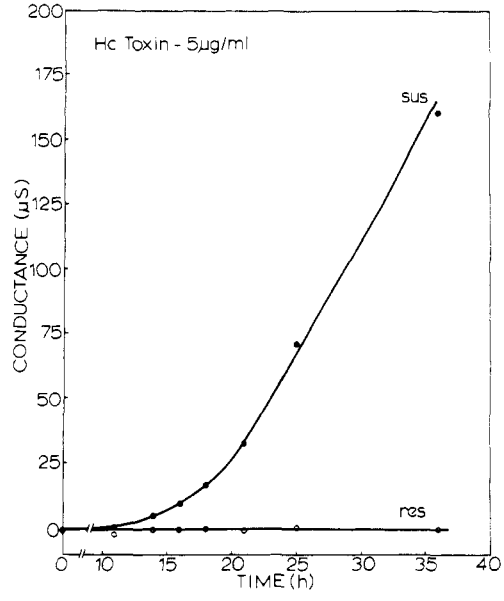

FIGURE 3: Loss of electrolytes from susceptible (●) or resistant (○) maize seedlings incubated in 5 μg of HC-toxin/mL of water.

1b shows the elution of conversion product at 18 min and toxin at 46 min with 18.5% acetonitrile/water. Conversion product was detected in purified toxin preparations if the aqueous solutions were stored at 5 °C for long periods of time (3–4 weeks). Likewise, conversion product was detectable in stored culture filtrates and was present in higher quantities if culture filtrates had an unusually low pH (pH <3). Thus, the quantity of conversion product formed was dependent upon procedures and conditions of toxin isolation.

Experiments in which toxin, purified by HPLC, was processed in the presence or absence of TFA were performed, and in vacuo concentration steps were done with toxin exposed to nitrogen or air. Relatively large quantities of conversion product (22.8% of the total weight of sample determined by peak integration) were generated when the toxin was dried in the presence of TFA and exposed dry to air. On the other hand, when TFA was eliminated from the solvent and solutions were concentrated under N_2, conversion product, if detected at all, was present in trace quantities (<1%).

For biological testing and structural analyses, conversion product was produced by drying HPLC-purified HC-toxin preparations in vacuo in the presence of TFA and air. Yields approached 75% by this method. Final purification of conversion product was performed by HPLC (Figure 1c), but in

Table I: Effect of Conversion Product on Activity of HC-Toxin in Root Growth Inhibition Bioassays

concn		
toxin (μg/mL)	conversion product (μg/mL)	rel act.[a] (%)
0.2[b]	0	100.0
0.2	0.2	109.3
0.2	0.4	105.2
0.2	0.6	95.2
0.2	0.8	94.2
0.2	1.0	99.0
0.2	2.0	105.4
0.3	0.7	90.4
0.4	0.6	104.9
0.5	0.5	106.1
0.7	0.3	100.0

[a] The activity of the mixture indicated by the first two columns divided by the activity of HC-toxin alone at the concentration given in the first column, expressed as percentage. [b] In these experiments, root growth of K61 × Pr seedlings was inhibited 57.8% at this concentration.

some cases, preparative TLC in solvent B was used as a preliminary step.

Conversion product did not inhibit root growth of susceptible seedlings at concentrations up to 50 μg/mL and did not induce electrolyte leakage from susceptible seedlings at 25 μg/mL. Resistant seedlings were not affected by conversion product in either bioassay.

HC-Toxin preparations that contained conversion product had a higher EC_{50} than preparations devoid of conversion product. To determine whether the reduced activity was due to competitive inhibition or simply the result of a proportional decrease in the amount of active toxin in the sample, varying quantities of toxin were bioassayed in the presence or absence of conversion product. Results of these experiments indicated that conversion product did not inhibit or stimulate the activity of HC-toxin, since the same concentrations of toxin exhibited comparable activities regardless of the presence or absence of conversion product (Table I). Increasing the concentration of conversion product to 10-fold greater than toxin near the EC_{50} did not affect biological activity. Thus, the EC_{50} values for HC-toxin/conversion product mixtures reflected the relative contribution of HC-toxin to the weight of the sample.

The formation of an inactive compound from HC-toxin under relatively mild conditions suggested that few, if more than one, structural changes had occurred. Elucidation of the change(s) offered an opportunity to gain an insight into structure–activity relationships for the toxin molecule.

The common amino acid content of conversion product was found to be identical with that of HC-toxin. Dansylation of acid hydrolysates of conversion product established the presence of alanine and proline, the amino acid analyses indicated a ratio of 2 alanine:1 proline. As with HC-toxin, very small amounts of other residues that did not correspond to any known amino acids were detected by both methods.

Chemical ionization (CI) and electron impact (EI) mass spectrometry of conversion product yielded spectra virtually indistinguishable from those of HC-toxin, including apparent molecular ions corresponding to $C_{21}H_{32}N_4O_6$. Other evidence had suggested that the product was identical with HC-toxin except that the epoxy group of the 2-amino-8-oxo-9,10-epoxydecanoic acid moiety had been hydrolyzed to form the 9,10-dihydroxy derivative. The same apparent molecular ion and the high degree of similarity between the spectra of HC-toxin and conversion product could be explained easily by dehydration of the vicinal dihydroxy groups of conversion

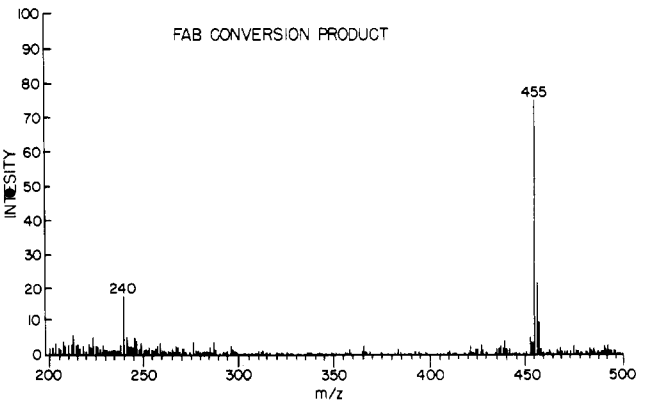

FIGURE 4: Fast atom bombardment mass spectrum of a conversion product derived from HC-toxin.

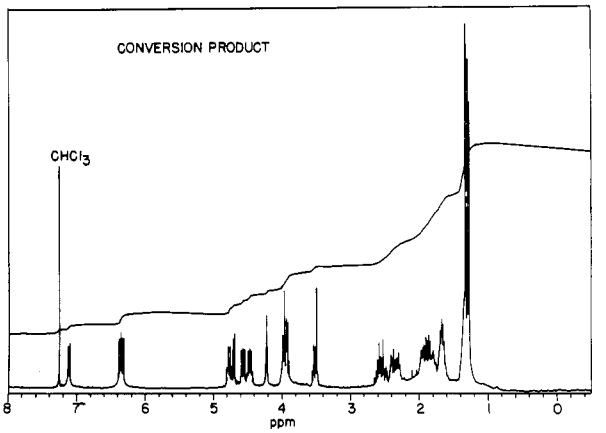

FIGURE 5: Proton NMR spectrum of a conversion product derived from HC-toxin in $CDCl_3$. Chemical shifts are relative to tetramethylsilane.

product when heated (90–150 °C) by the direct-inlet probe of the mass spectrometers. However, fast atom bombardment mass spectrometry did yield an intense peak at m/z 455 (Figure 4). Peak matching established the mass to be within 1 ppm of 455.250576, which corresponds to an empirical formula of $C_{21}H_{34}N_4O_7 + H^+$ or HC-toxin plus H_2O.

Proton NMR revealed that the signals assigned to the protons of the epoxide groups, 2.88, 2.99, and 3.43 ppm, in HC-toxin were not present in the spectrum of conversion product (Figure 5). However, different signals corresponding to hydroxyl proton signals at 3.49 ppm, Aoe-θ (1 H) at 4.23 ppm, Aoe-iU (1 H) at 3.92, and Aoe-iD (1 H) at 3.98 ppm were present. Decoupling experiments were used to aid in the assignments of signals that are presented in Table II. To eliminate the signals of the hydroxyl protons, deuterium exchange with D_2O was performed, and the signal at 3.49 ppm decreased in intensity as expected. Finally, conversion product was acetylated. Proton NMR revealed two additional signals (six protons) near 1.30 ppm and the absence of the hydroxyl proton signals at 3.49 ppm when the spectra of the acetylated derivative and conversion product were compared. Decoupling experiments with the acetylated derivative confirmed assignments for conversion product and supported the conclusion that two acetate groups were present at the 9,10 positions of the Aoe moiety. EI mass spectrometry of the diacetyl derivative of conversion product yielded an apparent molecular ion at a nominal mass of m/z 538, consistent with the expected empirical formula $C_{25}H_{38}N_4O_9$. IR spectra of the diacetate derivative also supported the proposed structure. An intense ester band at 1746 cm^{-1} and an acetate band at 1221 cm^{-1} were observed in spectra of the diacetate derivative. Although the

Table II: Proton NMR Assignments for Conversion Product Derived from HC-Toxin

chemical shift[a] (ppm)	assignments	coupling constants (Hz)
1.28	Ala_1-β (3 H) (d)	$^3J_{\alpha\beta} = 6.92$
1.32	Ala_2-β (3 H) (d)	$^3J_{\alpha\beta} = 6.83$
1.26–1.40	Aoe-γ (2 H)	
	Aoe-δ (2 H) (m)	
1.67	Aoe-ϵ (2 H) (m)	
1.70	Aoe-βU (1 H) (m)	$^3J_{\alpha\beta} = 7.78$
1.79	Aoe-βD (1 H) (m)	$^3J_{\alpha\beta} = 7.78$
1.87	Pro-βU (1 H) (m)	$^3J_{\alpha\beta U} = 7.81$
1.95	Pro-γU (1 H) (m)	$^3J_{\gamma U\delta U} = 7.5$
		$^3J_{\gamma U\delta D} = 4.5$
2.31	Pro-γD (1 H) (m)	$^3J_{\gamma D\delta U} = 7.5$
		$^3J_{\gamma D\delta D} = 8.0$
2.39	Pro-βD (1 H) (m)	
2.51	Aoe-ζU (1 H) (d, t)	$^3J_{\delta\zeta} = 7.27$
		$^2J_{\zeta\zeta} = -17.28$
2.60	Aoe-ζU (1 H) (d, t)	$^3J_{\delta\zeta} = 7.27$
		$^2J_{\zeta\zeta} = -17.28$
3.49	Aoe-θ,ι-OH (2 H) (s)	
3.52	Pro-δU (1 H) (d, t)	$^3J_{\delta\delta} = -10.0$
3.92	Aoe-ιU (1 H) (d, d)	$^3J_{\theta\iota} = 3.30$
		$^2J_{\iota\iota} = -11.85$
3.98	Aoe-ιD (1 H) (d, d)	$^3J_{\theta\iota} = 3.30$
		$^2J_{\iota\iota} = -11.85$
3.99	Pro-δD (H) (m)	
4.23	Aoe-θ (1 H) (t)	$J_{\theta\iota} = 3.30$
4.48	Ala_2-α (1 H) (d, q)	$^3J_{\alpha\beta} = 6.83$
		$^3J_{\alpha NH} = 10.12$
4.58	Ala_1-α (1 H) (d, q)	$^3J_{\alpha\beta} = 6.92$
		$^3J_{\alpha NH} = 9.68$
4.71	Pro-α (1 H) (d, d)	$^3J_{\alpha\beta U} = 7.81$
		$^3J_{\alpha\beta D} = 1.86$
4.79	Aoe-α (1 H) (d, t)	$^3J_{\alpha\beta} = 7.78$
		$^3J_{\alpha NH} = 10.30$
6.28	Ala_1-NH (d)	$^3J_{\alpha NH} = 9.68$
6.36	Aoe-NH (d)	$^3J_{\alpha NH} = 10.30$
7.13	Ala_2-NH (d)	$^3J_{\alpha NH} = 10.12$

[a] Relative to tetramethylsilane in $CDCl_3$.

1233-cm^{-1} band, attributed to the epoxide group of HC-toxin, would have been obscured, the other two epoxide bands at 930 and 865 cm^{-1} were not present in the spectra of the diacetate derivative of conversion product. Consequently, conversion product is proposed to be *cyclo*-[prolylalanylalanyl-2-amino-8-oxo-9,10-dihydroxydecanoyl] (Figure 6).

Discussion

The facile hydrolysis of the oxirane ring of HC-toxin to form conversion product, particularly under acidic conditions, was not surprising. However, the lack of biological activity or competitive effects by the resulting product was striking. Although the amounts of conversion product available limited tests at high concentrations, no biological activity could be detected for conversion product at concentrations more than 100 times greater than the EC_{50} for pure HC-toxin. Furthermore, the presence of conversion product in bioassay solutions did not have an effect, either inhibitory or stimulatory, on the activity of HC-toxin. Apparently, the hydrolysis of the epoxy function destroys the interaction between the molecule and the hypothetical toxin receptor site(s).

The lack of activity for conversion product may explain some of the conflicting reports concerning the EC_{50} of HC-toxin. Leisch et al. (1982) indicated 1 μg/mL for the EC_{50} value of their preparations used for structural studies, and Pringle & Scheffer (1967) reported a value of 500 ng/mL for the EC_{50}. Walton et al. (1982), who used TFA in their HPLC solvent system, reported an EC_{50} of 500 ng/mL also, whereas our preparations exhibited an EC_{50} of about 200 ng/mL.

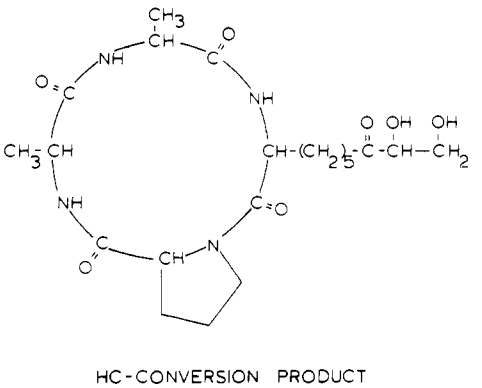

FIGURE 6: Proposed structure of conversion product.

Because the CI and EI mass spectra of conversion product and HC-toxin were nearly indistinguishable, the presence of conversion product in preparation could go undetected by those methods. FAB mass spectrometry should reveal both components if they were present in sufficient proportions. The same should apply to proton NMR since the protons of the epoxy group, 2.88, 2.99, and 3.43 ppm, and one of the protons of the dihydroxy function, 4.23 ppm, are unique and readily detectable in the spectra of HC-toxin and conversion product, respectively. The spectrum shown by Walton et al. (1982) clearly shows a signal near 4.23 ppm, although it is ignored in their discussion. Estimations from their integrator tracings for the signals near 4.23, 2.88, and 2.99 ppm indicate that conversion product may have constituted 40 ± 10% of their toxin preparation. Roughly, this corresponds to the differences in the EC_{50} values reported by Walton et al. (1982) and ourselves (Pope et al., 1983). Further, Walton et al. (1982) detected an additional compound eluting ahead of the toxin in HPLC and suggested that this compound may be a stable conformer of the toxin. However, in our preparations, we have not detected any compounds other than conversion product and have no evidence for other toxic metabolites.

The apparent requirement for an epoxide group may provide clues about the nature of key biochemical reactions involved in HC-toxin's action. In addition to an epoxide group, there may be strict requirements for other structural features such as the carbonyl adjacent to the epoxy group or the tertiary structure of the cyclic peptide. Studies are under way to determine the biological activities of several derivatives prepared by modification of the epoxy–keto function of Aoe.

Registry No. HC-conversion product, 85800-05-1.

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