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PURIFICATION AND STRUCTURE OF THE HOST-SPECIFIC TOXIN FROM HELMINTHOSPORIUM CARBONUM RACE 1

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The host-specific toxin from Helminthosporium carbonum race 1 was purified from culture filtrates by solvent extraction, gel filtration, and high pressure liquid chromatography. High resolution mass spectrometry of the purified toxin gave a MW of 436.2318 and an elemental composition $C_{21}H_{32}N_4O_6$. Amino acid analysis and proton and ^{13}C-NMR indicated a peptide containing four amino acids. Their sequence was determined by gas chromatography mass spectrometry. Finally, digestion of the amino acids with D- and L-amino acid oxidases gave the complete structure cyclo[(L-2-amino-9, 10-epoxy-8-oxodecanoyl)-D-prolyl-L-alanyl-L-alanyl].

Introduction

The fungal genus Helminthosporium causes a number of economically important diseases of crop plants. Several Helminthosporium species produce toxins that have the same host range as the fungus and which during infection play a primary role in disease development (1,2). Helminthosporium carbonum Ullstrup [Cochliobolus carbonum Nelson] race 1 is pathogenic on maize varieties which are homozygous recessive at the nuclear Hm locus. A low molecular weight compound present in culture filtrates of H. carbonum race 1, HC-toxin, has the same host-specificity as the fungus (3,4). Several lines of evidence indicate that production of this toxin by the fungus is essential for pathogenicity (1).

We report here a complete chemical structure for HC-toxin. While the presently described work was in progress, a structure for HC-toxin, cyclo[(2-amino-9,10-epoxy-8-oxodecanoyl)-alanyl-alanyl-prolyl], was published (5).

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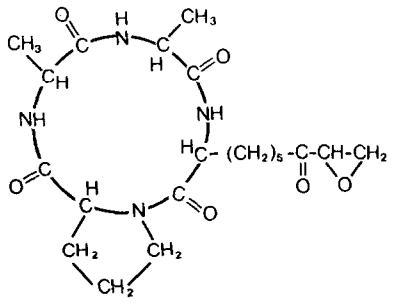


Figure 1. Proposed structure of the host-specific toxin from Helminthosporium carbonum race 1.

Although our results support the same amino acid composition, we propose a different amino acid sequence, namely cyclo[(2-amino-9,10-epoxy-8-oxodecanoyl)-prolyl-alanyl-alanyl] (Fig. 1). Furthermore, we present additional mass spectral evidence for the structure of the epoxide amino acid and describe the absolute configurations of the α-carbons of the four amino acids.

Materials and Methods

H. carbonum race 1 was isolated from lesions on artificially-inoculated field-grown maize (Zea mays L.) in September, 1981. Culture filtrates (total volume 1000 ml) were produced and taken through chloroform extraction as described (3). HC-toxin activity was followed during purification using a modified root growth bioassay and the maize hybrids Pr x K61 (genotype hm hm, susceptible) and Pr1 x K61 (genotype Hm hm, resistant) (3). The chloroform extracts of culture filtrates were evaporated, dissolved in absolute ethanol, evaporated again, and redissolved in 5 ml distilled H_2O, with filtering as necessary to remove insoluble material. This solution was applied to a 2.6 cm x 40 cm G-15 Sephadex column (V_o = 50 ml, V_t = 150 ml) and washed with H_2O. A peak of host-specific toxin activity corresponded with a peak of absorption at 230 nm. Column fractions were pooled, lyophilized, and dissolved in 5-10 ml H_2O.

HC-toxin was purified on a Waters high pressure liquid chromatograph (HPLC) with a Model 660 gradient programmer running at 23°C, on a Waters μBondapak C-18 reverse phase column, 30 cm x 3.9 mm. The eluant was monitored by absorbance at 254 nm. Aliquots (0.1 - 0.5 ml) from G-15 Sephadex column fractions showing toxin activity were injected and eluted with a linear gradient of 0.1% trifluoroacetic acid in H_2O (solvent A) to 0.1% trifluoroacetic acid in acetonitrile (solvent B), 0-60% solvent B in 30 or 60 min, at a flow rate of 1.5 ml/min. HC-toxin thus purified was used for structural studies.

For amino acid analysis a sample of HC-toxin was hydrolyzed in 6N HCl at 110°C for 24 hr, and the amino acids separated and identified on a Beckman 119 CL amino acid analyzer.

NMR spectra were measured on a Bruker Model WM 300 spectrometer at 300 MHz (^1H) or 75.5 MHz (^{13}C) at 25°C, in $CDCl_3$. A total of 91,000 ^{13}C scans were collected at 100 scans/min.

The fast atom bombardment (FAB) mass spectrum was obtained on a Varian MAT 731 mass spectrometer fitted with an Iontech FAB power supply and gun. Xenon gas was used as the atom beam with an operating current of 20 μA. A sample of HC-toxin was dissolved in glycerol and 50% acetic acid (1:1) and approximately

one µl was placed on a stainless steel probe. The high resolution peak matching measurement was made using one of the glycerol cluster ions as reference mass at a mass resolution of approximately 7000.

Sequencing of HC-toxin by gas chromatography mass spectrometry (GCMS) required approximately 50 µg of HC-toxin. It was hydrolyzed in a sealed evacuated tube in 100 µl of 6N HCl at 105°C for 8 min, and the partial acid hydrolyzate was methylated, trifluoroacetylated, reduced to the polyamino alcohols, and silylated as described (7). A small portion (1-2 µl) was injected directly onto the gas chromatograph equipped with an on-column injector and a fused silica column. The GCMS system was a Varian 3700 gas chromatograph coupled via an open slit interface to a Finnegan MAT 212 mass spectrometer. A Finnegan SS 200 data system controlled the scanning functions and data acquisition. The scan rate was 1.7 sec/decade and mass range was 100-700 with a mass resolution of 1000. The fused silica column was a 30 m x 0.32 mm ID SE30 column (J & W Scientific, Rancho Cordova, CA) programmed from 45°C at 9°C/min to 110°C, then 3°C/min to 320°C.

Amino acid configurations were determined by digestion of a sample of completely hydrolyzed HC-toxin with either D-amino acid oxidase (from hog kidney, 85 units/ml, Sigma) or L-amino acid oxidase (from snake venom Type 1 crude, 0.5 units/mg, Sigma) (6). An aliquot of each reaction mixture was spotted on Whatman #1 filter paper and developed by ascending chromatography for 3.5 hr in phenol:H_2O (4:1). Amino acids were detected with ninhydrin.

Results

HC-toxin activity eluted from the HPLC after 15 min as a single, symmetrical, broad peak. No interfering peaks were seen at 230 nm or 280nm. Another smaller peak eluting just before HC-toxin had a similar 230/254 nm absorption ratio (approximately 10) and may be a minor toxin species or a stable conformer.

Figure 2 shows the host-specific nature of purified HC-toxin. Pr x K61 (susceptible) and Pr1 x K61 (resistant) differ only at the nuclear Hm locus. Our toxin preparation caused 50% inhibition of susceptible root growth at 0.5 µg/ml, comparable to the purified preparations of others (3,5).

Purified HC-toxin in absolute ethanol showed a strong shoulder of absorption at 230 nm ($\varepsilon_{1\ cm}$ = 3800)(8). There was no significant absorption above 265 nm.

Amino acid analysis of hydrolyzed HC-toxin yielded proline and alanine in the ratio 1:2, plus three or four other components, none of which corresponded to any common amino acid or were greater than 19% of the alanine content, as found earlier (8). On the basis of the yield of alanine, purity of HPLC-purified HC-toxin was 89%.

Proton NMR indicated three amide, four α-carbon, six methyl, and three epoxide neighboring protons (Fig. 3). The pattern of coupling was consistent

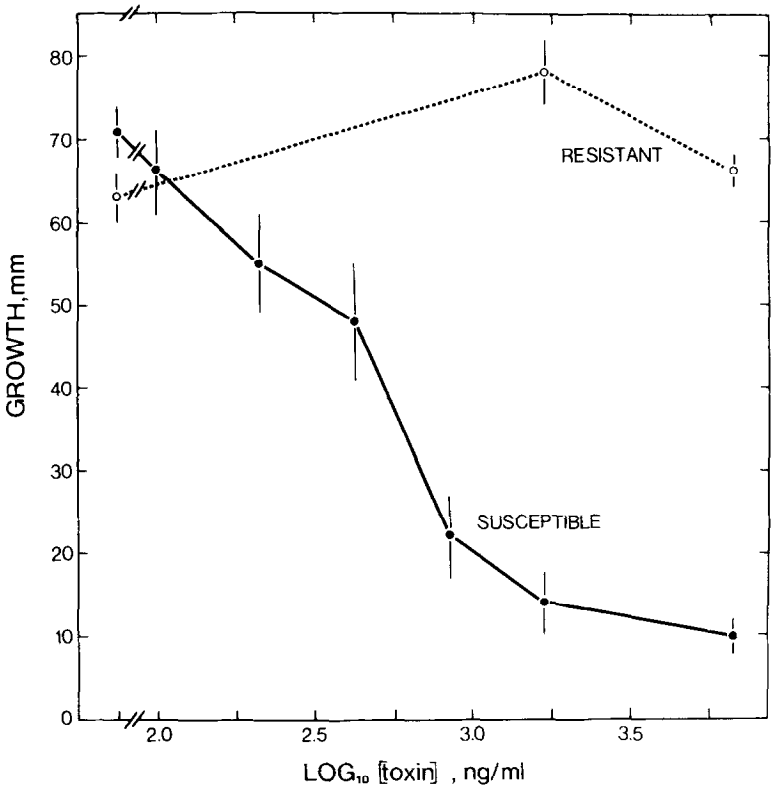


Figure 2. Bioassay of purified HC-toxin. Seeds were soaked in H_2O for 3 h, wrapped in damp paper towels, and germinated in the dark for 60 h. Seeds with primary roots between 15 and 30 mm in length were placed in 100 mm glass petri plates containing 5 ml H_2O and a piece of Whatman #1 filter paper, plus toxin in H_2O at different concentrations, and incubated in the dark for another 48 h. Growth is a measure of net elongation during the 48 h. Each concentration was done in duplicate, four seeds per plate. Error bars indicate $\pm$ 1 SE.

with four amide linkages and a terminal epoxide (Fig. 3). Our ^{13}C NMR spectrum was almost identical to that of Liesch et al. (5), including a carbonyl carbon at 207.5 ppm and a total of twenty-one carbon atoms.

A low resolution FAB spectrum of HC-toxin gave an intense protonated molecular ion (MH^+) of M/Z = 437, as well as a much less intense protonated dimer (M_2H^+) of M/Z = 873. By high resolution peak matching, an exact mass of 436.2318 was obtained for the molecular ion of HC-toxin, which, assuming one proline and two alanine residues, gave a unique element formula of $C_{21}H_{32}N_4O_6$ (calculated = 436.2321). A FAB mass spectrum of a sample of HC-toxin treated for 30 min with 1N HCl/methanol at 25°C caused disappearance of the peak at 437 and new peaks at MH^++32, MH^++35, and MH^++37, which would correspond to addition

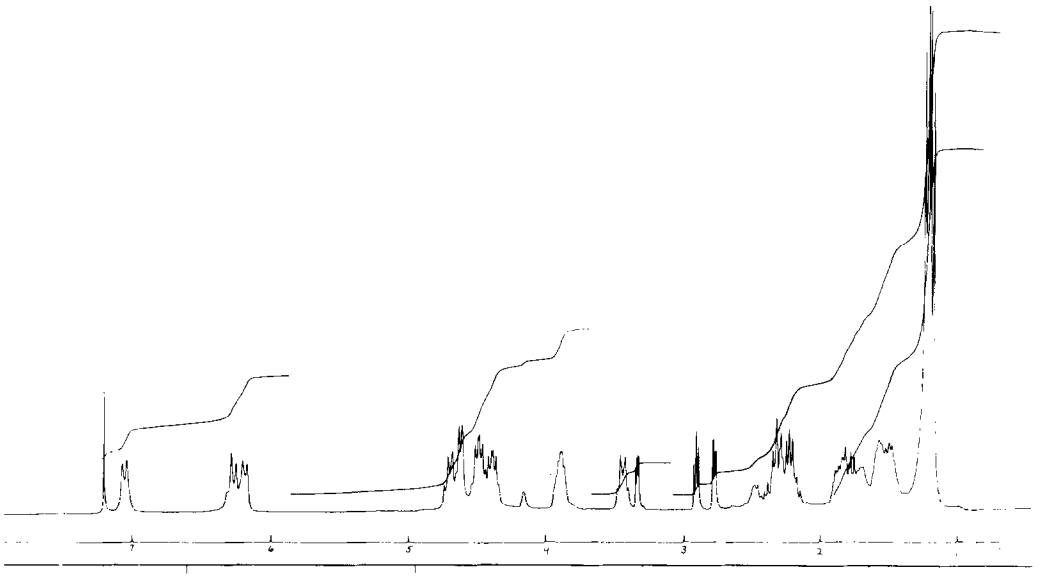


Figure 3. Proton NMR spectrum of HPLC-purified HC-toxin in $CDCl_3$. Decoupling experiments indicated that the following signals were due to protons on adjacent atoms: 1.17 and 4.37; 1.25 and 4.50; 2.75, 2.90, and 3.35; 6.20 and 4.70; 6.32 and 4.37; and 7.07 and 4.50. The signal at 7.70 ppm is assigned to $CDCl_3$.

of methanol and HCl to the epoxide. The presence of a strong fragmentation ion MH^+-71 (M/Z = 366) in the FAB spectrum probably represents the carbonyl-induced cleavage of the epoxyketone moiety, suggesting that the carbonyl is vicinal to the terminal epoxide.

The identifications of the peptide spectra in the GCMS experiment of the partial acid hydrolyzate of HC-toxin along with their calculated retention indices are listed in Table 1. The peptides containing only proline and alanine were easily identified and the tripeptide Pro-Ala-Ala (Fig. 4) was alone sufficient to sequence HC-toxin as Pro-Ala-Ala-Aoe, where Aoe represents the epoxyketone-containing residue.

Table 1. Peptides identified by GCMS in partial acid hydrolyzate of HC-toxin.

Retention Index	
1300	Ala-Ala
1470	Pro-Ala
1795	Pro-Ala-Ala
2440	Ala-Aoe1
2650	Aoe1-Pro
3000	Aoe1-Pro-Ala
3360	Ala-Aoe1-Pro-Ala (weak)

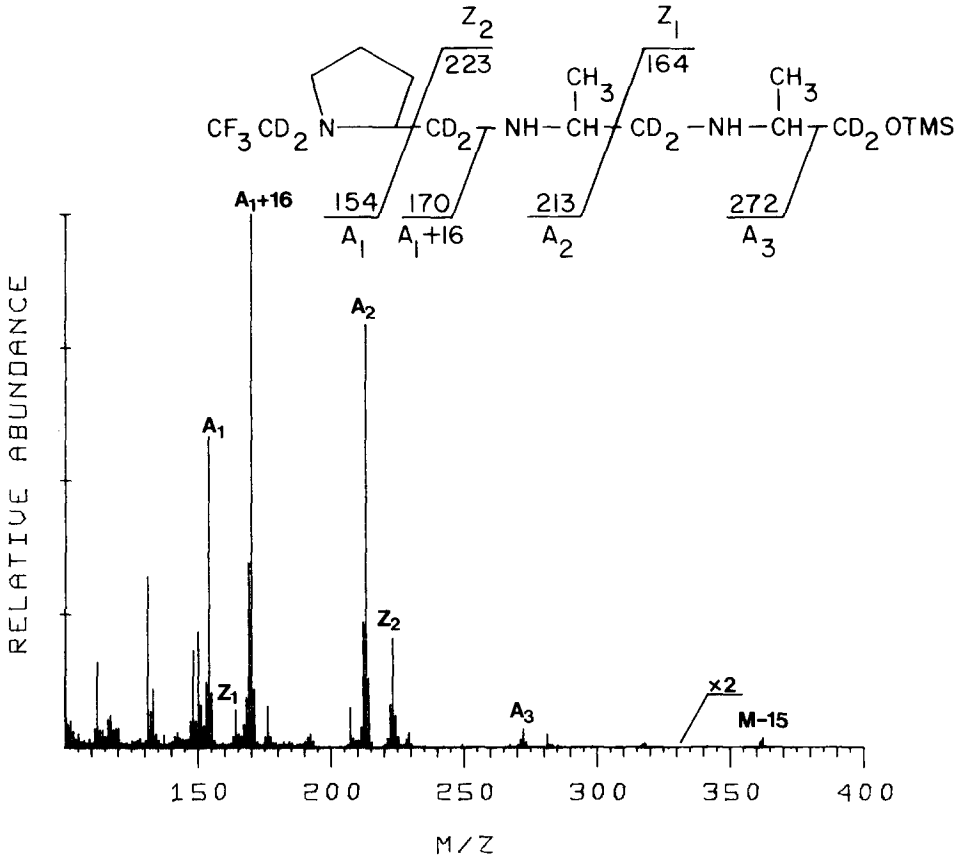
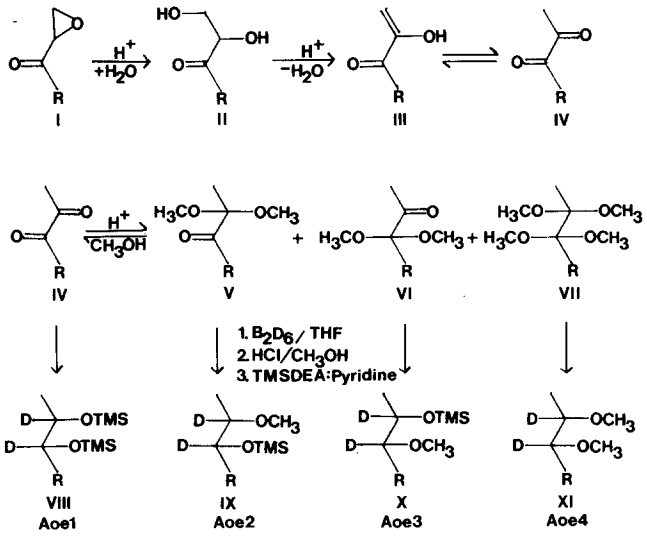


Figure 4. Mass spectrum of the polyaminoalcohol derivative of the tripeptide Pro-Ala-Ala.

Although the conversion of peptides to their corresponding polyaminoalcohol derivatives produces a single unique species for each common amino acid, we found four derivatives of the epoxyketone-containing residue, referred to as Aoe1-4 (scheme 1). It is proposed (scheme 1) that those derivatives are formed first by protonation of the epoxide in 6N HCl (I) followed by hydrolysis to the ketonediol (II). Protonation followed by elimination of the β-hydroxy group would then produce the ketone enol (III), which is likely in tautomeric equilibrium with the diketone (IV). This reaction has been previously demonstrated for a variety of α,β-epoxyketones (9).

In the subsequent derivatization steps, we would first expect the diketone (IV) to form various methyl ketals (V-VII) during methylation in methanolic HCl. Ketals are known (10) to undergo reductive cleavage to ethers by diborane



Scheme 1

in tetrahydrofuran; we expect the analagous reaction using deuterated diborane (B_2D_6). The ketone(s) would be expected to form the alcohol(s), which would add the trimethylsilyl group (TMS) during silylation in trimethylsilyl diethylamine (TMSDEA). In conjunction, these steps would produce four derivatives (VIII-XI), Aoe1-4.

Although the diketone derivative, Aoe1, was the most abundant species, representing approximately 70%, all four derivatives were found alone or in proline and/or alanine-containing peptides. In Figure 5 we show both the mass spectra of Aoe1 and of Ala-Aoe1, where the A_i and Z_i ions refer to the usual C-C bond cleavage α to the amine (11), and some of the other fragmentations are represented with dotted lines. One can see from these two spectra how the additional OTMS groups dominate the fragmentation pattern. The loss of 118 ($CH_3\overset{+}{C}D{=}\overset{+}{O}TMS$) and 90 ($H\overset{+}{O}{=}TMS$), the latter involving a proton transfer, occur in a number of primary and/or secondary fragmentation processes, conferring additional complexity to the usual dominant A_i and Z_i ions. It is clear, however, from the additional Aoe-containing peptides (Table 1) that the sequence of HC-toxin is cyclo(Pro-Ala-Ala-Aoe).

It is interesting to note that the diastereomers of Aoe1-containing peptides were baseline resolved under the conditions used for their gas chromatographic

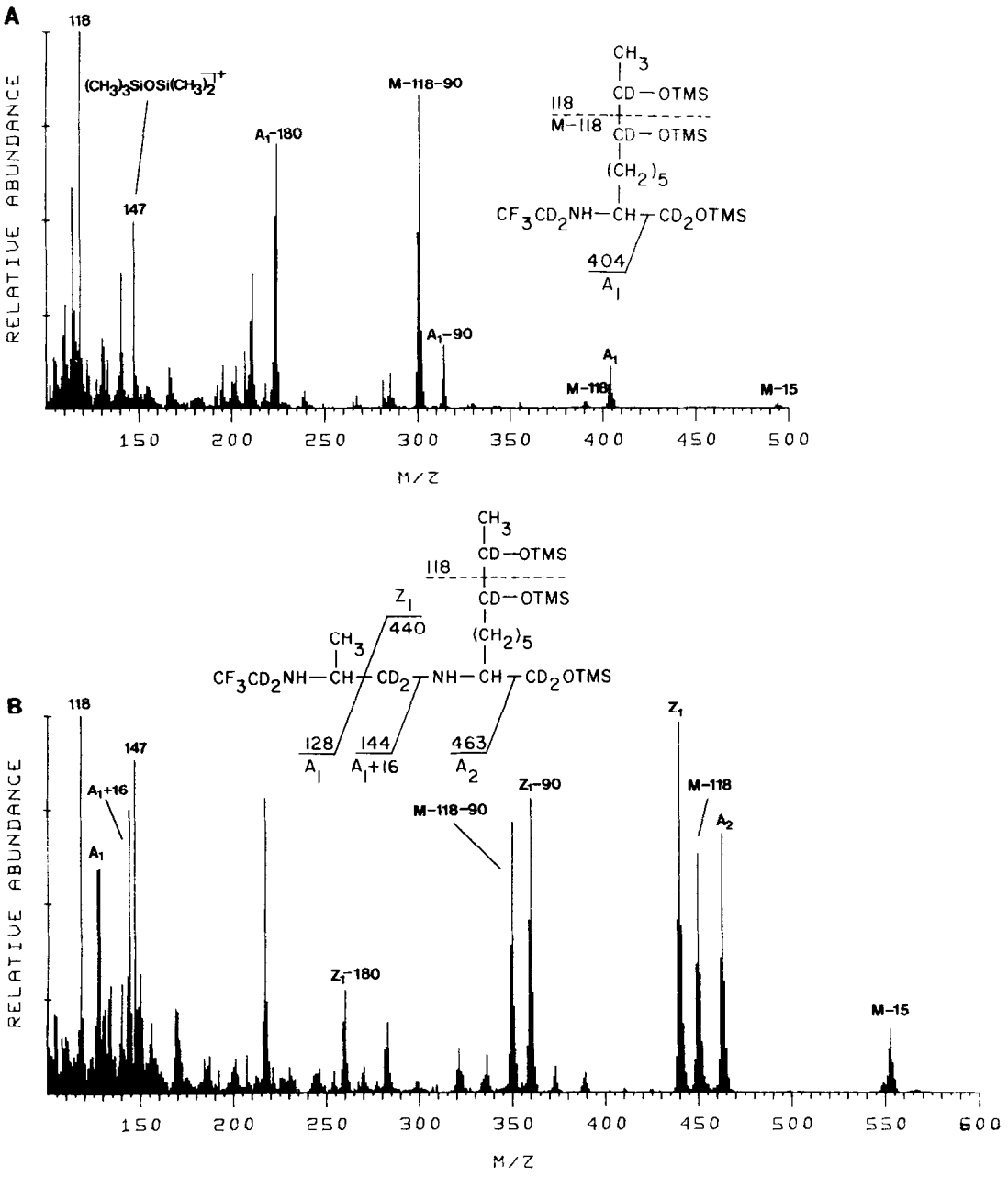


Figure 5. A: Mass spectrum of the major polyaminoalcohol derivative of 2-amino-9,10-epoxy-8-oxodecanoic acid (Aoe1). B: Mass spectrum of the polyaminoalcohol derivative of Alanyl-2-amino-9,10-epoxy-8-oxodecanoic acid (Ala-Aoe1).

separation; this was initially a source of confusion until it was realized that the mass spectra were in fact identical.

Paper chromatography of an HC-toxin acid hydrolyzate revealed ninhydrin-positive spots at R_f 0.58 (purple with ninhydrin) corresponding to alanine,

R_f 0.89 (yellow with ninhydrin) corresponding to proline, and a poorly resolved spot between R_f's 0.50 and 0.83 (purple with ninhydrin), probably corresponding to the breakdown products of the epoxide amino acid (6). After digestion with D-amino acid oxidase, the proline spot disappeared, and after digestion with L-amino acid oxidase the alanine and putative epoxide amino acid spots disappeared. Therefore, we conclude that the proline in HC-toxin has the D-configuration, and that both alanine residues and the epoxide amino acid have the L-configuration at their α-carbons. The alanine and proline assignments are consistent with the observation that when polyaminoalcohol derivatives of standard L-Ala-L-Ala and L-Pro-L-Ala were coinjected with the derivatized HC-toxin hydrolyzate, the sample Ala-Ala coeluted with L-Ala-L-Ala whereas the sample Pro-Ala was baseline resolved from L-Pro-L-Ala.

Discussion

Using a different purification scheme we have isolated and characterized a cyclic peptide which agrees in molecular weight and amino acid composition with that of Liesch et al. (5). Thus, we can be reasonably certain that this molecule (Fig. 1) is identical to the one they characterized and is responsible for the host-specific toxicity of H. carbonum race 1 culture filtrates. Our structure (Fig. 1) differs, however, in the sequence of amino acids. As has been pointed out previously (12), electron ionization-mass spectrometry of underivatized peptides, especially cyclic peptides, does not give unequivocal sequence information, whereas GCMS produces unambiguous sequence data and is therefore particularly well-suited for cyclic peptides.

The unusual epoxide amino acid in HC-toxin, deduced by Liesch et al. (5) and confirmed by us, has been found in two other fungal metabolites, Cyl-2 and chlamydocin (6,13). In both chlamydocin and HC-toxin, the epoxide amino acid has the L-configuration and is adjacent to D-proline, but HC-toxin differs in having the D-proline on the carboxyl side of the epoxide amino acid.

Acknowledgements

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