^{13}C Labeling Indicates That the Epoxide-Containing Amino Acid of HC-toxin Is Biosynthesized by Head-to-Tail Condensation of Acetate

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HC-toxin, cyclo(D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-9,10-epoxi-8-oxodecanoic acid, is a potent inhibitor of histone deacetylase. Previous molecular genetic studies indicated that HC-toxin biosynthesis requires a dedicated fatty acid synthase. The incorporation of [^{13}C]acetate into HC-toxin was studied using NMR. The pattern of incorporation of ^{13}C was consistent with the carbons of Aeo being derived from head-to-tail condensation of acetate.

HC-toxin, a cyclic tetrapeptide with the structure cyclo-(D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-9,10-epoxi-8-oxodecanoic acid (Figure 1), is a virulence determinant for the producing fungus, *Cochliobolus carbonum*, on its host, maize (*Zea mays*).[1] HC-toxin and other Aeo-containing cyclic tetrapeptides cause detransformation of oncogene-transformed mammalian cells and have cytostatic activity against a variety of cell types, including several important protozoal parasites.[2] The site of action of Aeo-containing peptides is histone deacetylase, an enzyme that is emerging as a critical regulator of chromatin structure and gene regulation.[3−7]

There are five known Aeo-containing cyclic tetrapeptides, as well as a number of synthetic derivatives.[8,9] The 8-carbonyl and the intact 9,10-epoxide of Aeo are critical for biological activity. Reduction of the 8-carbonyl is particularly important because this reaction is the basis of resistance of maize to HC-toxin and hence *C. carbonum*.[10] The absolute necessity of the terminal epoxide, however, has been recently challenged by the discovery of two biologically active compounds related to the Aeo-containing peptides but lacking the terminal epoxide. One, apicidin, has 2-amino-8-oxodecanoic acid in place of Aeo yet is comparable to HC-toxin in its ability to inhibit histone deacetylase.[2] The other compound is identical to chlamydocin (cyclo[α-aminoisobutyrate-L-Phe-D-Pro-L-Aeo]) except that the epoxide is replaced by a 9-hydroxy group.[11]

HC-toxin production in *C. carbonum* is controlled by a single Mendelian locus, *TOX2*.[9] At the molecular level, *TOX2* is complex, containing multiple copies of multiple genes. Genes of *TOX2* that have been identified to date include *HTS1*, encoding a 570-kDa tetrapartite peptide synthetase; *TOXA*, encoding an HC-toxin efflux carrier of the major facilitator superfamily; and *TOXC*, encoding a fatty acid synthase beta subunit.[12−16] The product of *HTS1* is the central enzyme in HC-toxin biosynthesis, but nothing in its sequence suggests that it could have any role in Aeo biosynthesis.

The backbone of Aeo is aminodecanoic acid, *TOXC* encodes a fatty acid synthase beta subunit, and *TOXC* is required only for HC-toxin biosynthesis.[16] *TOXC* is not involved in primary metabolism because *TOXC* is present only in Tox2$^+$ isolates of *C. carbonum*, and *TOXC* mutants have no phenotype other than loss of HC-toxin production.[16] Therefore, a plausible role for the product of *TOXC* is in the biosynthesis of Aeo. If the product of *TOXC* contributes to the synthesis of the backbone of Aeo, then Aeo is predicted to be synthesized by head-to-tail condensation of acetate. Wessel et al.[17] showed that [^{14}C]acetate is incorporated into HC-toxin in short-term feeding studies, but the pattern of labeling was not analyzed. Here we use an alternative method, feeding [^{13}C]acetate to fungal cultures and analyzing the labeled HC-toxin by NMR, to study the biogenic origin of Aeo.

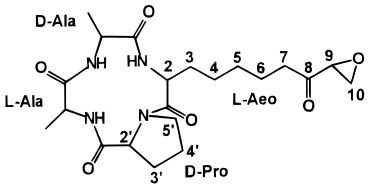

Figure 1. Structure of HC-toxin. The chirality of carbon 9 of Aeo is *S*.[29]

Table 1. Proton NMR Assignments for HC-toxin[a]

	Aeo	Pro	D-Ala	L-Ala
N	6.25		6.14	7.07
2	4.73	4.67	4.55	4.42
3	1.77, 1.61	2.37, 1.82	1.22	1.28
4	1.22, 1.28	2.25, 1.93		
5	1.25, 1.25	3.97, 3.48		
6	1.51, 1.52			
7	2.39, 2.20			
8				
9	2.97, 2.83			
10	3.38			

[a] Chemical shifts are indicated in ppm.

The complete assignments of the ^{1}H and ^{13}C NMR signals of HC-toxin have not previously been made.[1,18−21] HMQC combined with 2-D TOCSY provided nearly complete proton and carbon resonance assignments for HC-toxin (Tables 1 and 2). The only ambiguities that persist are the assignments of signals from protons on the same methylene carbons of Aeo and proline. However, for this study these assignments are not critical insofar as the identities of the carbons are the most important biogenically.

Based on the assignments of the carbon atoms from these studies, HC-toxin labeled by feeding fungal cultures with

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Table 2. Carbon NMR Assignments for HC-toxin[a]

	Aeo	Pro	D-Ala	L-Ala
1	171.6	173.5	173.8	173.8
2	51.7	57.6	45.5	47.8
3	28.4	24.5	12.9	13.4
4	25.2	24.4		
5	27.8	46.8		
6	22.4			
7	35.9			
8	207.5			
9	53.4			
10	45.9			

[a] Chemical shifts are indicated in ppm.

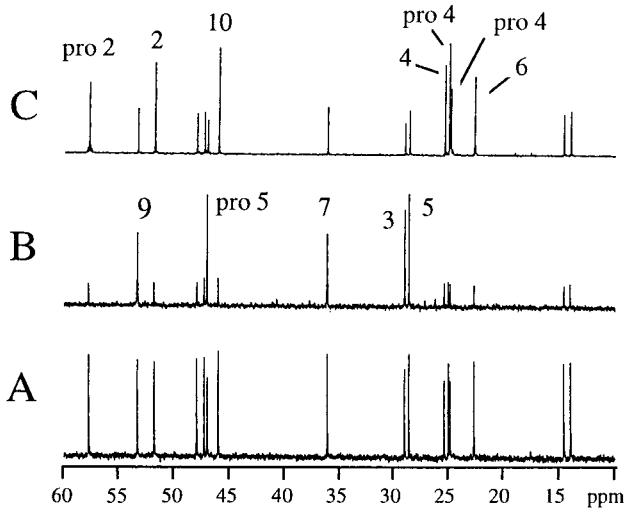

Figure 2. ^{13}C NMR spectra of (A) native HC-toxin and HC-toxin from culture filtrates of *C. carbonum* grown in the presence of either (B) 1-[^{13}C]acetate or (C) 2-[^{13}C]acetate.

1-[^{13}C]acetate or 2-[^{13}C]acetate was purified and analyzed by solution-state high resolution 1D ^{13}C NMR (Figure 2). There was a clear difference in the enrichment pattern when the fungus was incubated with 1-[^{13}C]acetate as compared to 2-[^{13}C]acetate (Figures 2B, C). 1-[^{13}C]Acetate enriched the ^{13}C signals of carbons 3, 5, 7, and 9 of Aeo (Figure 2B) whereas 2-[^{13}C]acetate enriched the signals of carbons 2, 4, 6, 8, and 10 (Figure 2C). The relative ^{13}C signal intensities of the native, 1-[^{13}C], and 2- [^{13}C] labeled HC-toxins were compared and analyzed in order to determine the degree of isotopic enrichment. These values were derived by first normalizing all of the ^{13}C resonance intensities to the intensity of the ^{13}C signal from carbon 2 of L-alanine within each spectrum. The degree of enrichment was then determined by calculating the ratio of each normalized resonance intensity in the labeled samples with its counterpart in the intensities from the native HC-toxin. As seen in Table 3, there is an alternating pattern of isotopic enrichment in the Aeo decanoic acid backbone, as expected if the Aeo side chain is formed by head-to-tail condensation of acetate. Determining the relative enrichment of the carbonyl resonances was more difficult due to their longer relaxation times. To acquire the data in a reasonable amount of time, the pulse delay had to be shorter than what would permit complete relaxation of the resonances with long T_1 values such as the carbonyl resonances. The intensities of these resonances are reduced more than those with shorter T_1 values, and the resulting errors are higher. Therefore, the carbonyl resonance values must be considered only semiquantitative.

Acetate condensation to produce unsaturated linear alkanoic acids can be catalyzed by either polyketide synthases or fatty acid synthases. Although these two classes of enzymes catalyze many of the same reactions, their domain organizations are distinct.[22] Earlier, we had described a gene, *TOXC*, that is dedicated to HC-toxin biosynthesis and whose predicted product is a fatty acid synthase β subunit.[16] Considering that (a) the other amino acids present in HC-toxin, alanine and proline, are primary metabolites, (b) the product of *HTS1* has no domains other than those typical of other cyclic peptide synthetases, and hence probably activates, but does not synthesize, Aeo,[13] and (c) the studies described here showing that the Aeo backbone is derived from head-to-tail condensation of acetate, the most reasonable function of the product of *TOXC* is to participate in the biosynthesis of the Aeo backbone.

Table 3. Quantitative Analysis of Enrichment of Relevant Carbon Atoms in HC-toxin after Labelling with 1-[^{13}C]acetate or 2-[^{13}C]acetate[a]

residue	2-[^{13}C]	1-[^{13}C]
Aeo CO	1.40	***2.52***
Aeo 2	***2.30***	0.80
Aeo 3	1.03	***4.32***
Aeo 4	***2.57***	0.80
Aeo 5	1.05	***4.25***
Aeo 6	***2.67***	0.87
Aeo 7	1.04	***3.84***
Aeo 8	***2.19***	1.13
Aeo 9	1.13	***6.10***
Aeo 10	***2.49***	0.68
L and D-Ala CO	0.90	1.46
L-Ala 2	1.03	0.88
L-Ala 3	1.00	1.00
D-Ala 2	0.99	0.84
D-Ala 3	1.09	1.08
Pro CO	1.16	1.88
Pro 2′	***2.87***	0.61
Pro 3′	***3.85***	0.84
Pro 4′	***2.45***	1.15
Pro 5′	1.03	***4.00***

[a] Sites of isotopic enrichment are shown in bold italic type.

Our studies indicate that all 10 carbons of Aeo are derived from acetate. The product of *TOXC* is a likely candidate enzyme for catalysis of its condensation. However, we cannot conclude that the *TOXC* protein catalyzes the complete synthesis of the Aeo backbone, because it might make a smaller acetate polymer that is then extended by another enzyme such as a polyketide synthase. Sterigmatocystin biosynthesis in *Aspergillus nidulans* involves genes encoding α and β fatty acid synthase subunits that are distinct from the genes involved in primary fatty acid biosynthesis. The product of the sterigmatocystin-dedicated fatty acid synthase (FAS) is hexanoic acid, which is then elongated by condensation with acetyl-CoA catalyzed by a dedicated polyketide synthase.[23]

The importance in HC-toxin biosynthesis of *TOXC*, encoding a fatty acid synthase β subunit, and the studies reported here indicating that Aeo is biogenically a fatty acid together indicate that Aeo biosynthesis must also require the participation of a fungal fatty acid synthase α subunit. This α subunit could be the one involved in primary fatty acid biosynthesis or could be dedicated to HC-toxin biosynthesis.

In the [^{13}C]acetate labeling studies, the ^{13}C label was also incorporated into the proline residue of HC-toxin, but not into the alanine residues (Figure 2). Whereas alanine is directly synthesized from pyruvate, proline is synthesized from α-ketoglutarate, an intermediate of the citric acid cycle. When exogenous acetate is metabolized, it is incor-

porated into the citric acid cycle after derivatization with CoA, and, therefore, it is not unexpected that amino acids biosynthesized from citric acid cycle intermediates would also become labeled.

Experimental Section

Culture and Isolation of HC-Toxin. *Cochliobolus carbonum* Nelson (anamorph *Bipolaris zeicola* [Nisak. and Miyake] or *Helminthosporium carbonum* Ullstrup) race 1 strain SB111 (ATCC 90305) was grown for 10 days in still culture on modified Fries's medium at ambient temperature.[1] The culture filtrate was filtered through a layer of Miracloth (Calbiochem, CA). Proteins and other high molecular weight compounds were precipitated overnight with an equal volume of MeOH at 4 °C and removed by filtration. After evaporation of the MeOH under reduced pressure, the remaining H_2O phase was extracted three times with equal volumes of $CHCl_3$. The $CHCl_3$ was evaporated under reduced pressure, and the final reddish oil was redissolved in H_2O. Further purification was done by reversed-phase (C18) HPLC eluted with a linear gradient of 100% H_2O to 60% MeCN in 30 min, at a flow rate of 1 mL/min.[1] Fractions containing HC-toxin were pooled and rechromatographed under the same conditions. Finally, the toxin samples were lyophilized and redissolved in $CDCl_3$.

Biosynthetic Conditions. The optimal conditions for [^{13}C]-acetate acid incorporation into HC-toxin were determined by preliminary [^{14}C]acetate acid labeling experiments (data not shown). Two days after initial inoculation of flasks of medium with fungal spores, [^{13}C]acetic acid (sodium salt, 1-[^{13}C] or 2-[^{13}C], Sigma) was added to the fungal cultures at a final concentration of 12 mM. Cultures were allowed to grow for a further 8 days, and HC-toxin was purified from the culture filtrates by $CHCl_3$ extraction and HPLC as described above.

NMR Methods. HC-toxin (ca. 2 mg) was dissolved in 600 μL $CDCl_3$ in a 5-mm Wilmad 528pp NMR tube. The NMR data were acquired on a 500-MHz Varian VXR500 NMR spectrometer at 25 °C. The spectra were processed using Varian's VNMR software and NMRPipe software[24] on a Silicon Graphics Indigo 2XZ computer. A combination of homonuclear 1D and 2D (^{1}H, ^{13}C) and heteronuclear 2D (^{1}H−^{13}C) experiments were used in the analysis.[25−28] A sweep width of 4189 Hz in each dimension was used, and 2048 acquired points in t_2, 512 complex t_1 increments, and a total of 32 scans per t_1 with a pulse delay of 2.0 s were collected. The processing parameters of the TOCSY spectrum were as follows: t_2, first point multiplication by 0.5, apodization with a cosine squared shifted by a factor of 0.35, zero-order baseline correction; t_1, apodization with a cosine squared shifted by a factor of 0.35, zero filling to 2048 points, and zero-order baseline correction.

The heteronuclear multiple-quantum coherence (HMQC, also called ^{1}H-detected ^{1}H−^{13}C correlated spectroscopy) data were acquired with a sweep width of 4189 Hz in t_2 (proton dimension) and 31 422 Hz in t_1 (carbon dimension). A total of 32 scans per t_1 increment were acquired. All spectra were referenced to the $CHCl_3$ peak at 7.24 ppm for ^{1}H and 77 ppm for ^{13}C. 1D H and C NMR spectra were acquired with the same parameters.

Supporting Information Available: Copies of ^{1}H and ^{13}C NMR spectra of HC-toxin, upfield portion of the 2D-TOCSY spectrum of HC-toxin, and upfield portion of the 2D-HMQC spectrum of HC-toxin (3 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from ACS; see any current masthead page for ordering information.

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