

A NEW HOST SPECIFIC TOXIN FROM HELMINTHOSPORIUM CARBONUM

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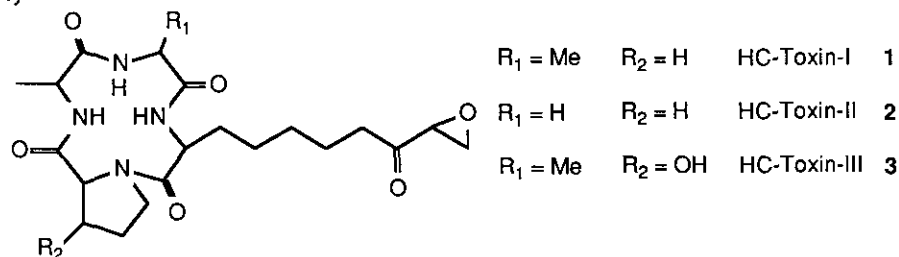
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Abstract-The structure and sequence of a new HC-toxin analog **3** produced by H. carbonum is reported.

The cyclic tetrapeptide HC-toxin-1 **1**, elaborated by the fungus H. carbonum shows host-specific toxicity toward susceptible strains of maize.³ In addition to two units of alanine and one proline moiety, this toxin contains the unusual amino acid, 2-amino-8-oxo-9,10-epoxydecanic acid (Aoe) which has also been found in other bioactive fungal metabolites.³⁻⁶ We have examined culture filtrates of H. carbonum⁷ and have isolated two additional toxic cyclic tetrapeptides; the previously isolated HC-toxin-II **2**⁶ with a glycine in place of one alanine unit; and HC-toxin-III **3**⁷ in which the proline of **1** is replaced by trans-3-hydroxyproline.

The isolation and purification of the HC-toxins was accomplished as previously described.⁶ From a 10-20L harvest of culture filtrate, we were typically able to obtain 0.480 grams of **1**, 4.0 mg of **2**, and 2.4 mg of HC-toxin-III **3** in a chromatographically pure state. The purified toxins exhibited similar levels of toxicity.⁷



Fast atom bombardment (FAB) mass spectroscopy^{8,9} of **2** gave an (M+1)⁺ ion at m/z 423. High resolution FAB-MS (Csl, glycerol) established the empirical formula of HC-toxin-II **2** as C₂₀H₃₀N₄O₆ (calcd. C₂₀H₃₁N₄O₆ 423.22436 amu, found 423.22558 amu; +2.88 ppm). Similarly HC-toxin-III **3** displayed an (M+1)⁺ ion at 453; and an empirical formula of C₂₁H₃₂N₄O₇ (calcd. C₂₁H₃₃N₄O₇ 453.23493, found 453.23221; -6.0 ppm). Comparison with the known HC-toxin-I **1** (C₂₁H₃₂N₄O₆) suggested that **2** and **3** differed by a CH₂ and an oxygen atom respectively.

A low resolution FAB-MS of **1** displayed fragment ions at m/z 240 (M- 196), 170 and 70 (base) which have been previously been assigned¹⁰ as the tripeptide unit Ala-Ala-Pro, and the protonated iminium ions derived from Aoe and Pro, respectively. HC-toxin-II **2** also exhibited fragment ions at m/z 170 and 70 (base); the presence of glycine in **2** is suggested by the observation of a fragment ion (FAB-MS) at m/z 226 (M- 196) which might arise from the fragment Ala, Gly, Pro. However HC-toxin-III **3**, while apparently possessing Aoe (m/z 170), had a base peak at m/z 86 (C₄H₈NO; peak matching) which we tentatively attributed to a hydroxy proline residue. Also in agreement with the presence of a hydroxy proline residue was the ion observed at m/z 256 (M- 196), which corresponds to a tripeptide fragment ion composed of two Ala units and a hydroxyproline.

Amino acid analysis¹¹ of **2** yielded a 1.00: 1.07: 0.76 ratio of Gly, Ala, and Pro; while analysis of HC-toxin-III **3** indicated Ala and an unidentified amino acid an a 2.44: 1 ratio. Comparison of this unknown residue with Pro, cis- and trans-3-¹² and -4-hydroxy proline demonstrated that the unknown amino acid was trans-3-hydroxy proline.

¹³C-NMR data (68.9 MHz, CDCl₃) for **2** and **3** is presented in Table 1. As expected from the MS-derived empirical formulae, HC-toxins-II **2** and -III **3** possess 20 and 21 carbons respectively. Four amide carbonyls are observed which is consistent with cyclic tetrapeptide structures and in agreement with their chromatographic

TABLE 1

¹³C-NMR of HC-Toxins-II 2 and -III 3 (68.9 MHz, CDCl₃, CHCl₃ ref.)

Amino Acid	Carbon	HC-Toxin-II 2	HC-Toxin-III 3
Ala	α	47.9	47.3, 47.6
	β	14.5	14.1, 14.7
Gly	α	44.3	
Aoe	α	52.1	51.6
	β γ δ	22.7, 25.4, 28.7	22.6, 25.4, 28.4
	ε ζ	29.0, 36.2	28.9, 35.9
	ι θ	53.4, 46.1	53.4, 46.7
Pro	α	57.8	
	β	25.0	
	γ	24.9	
	δ	47.0	
3-Hydro	α		66.0
	β		71.0
	γ		34.0
	δ		45.0
Amide C=O		171.3	170.4
		172.1	173.5
		174.3	173.7
		174.3	173.9
Aoe	η	200.3	201.9

TABLE 2

¹H-NMR HC-Toxin-II 2 (250 MHz, CDCl₃, CHCl₃ ref.)

HC-TOXIN-II-2

7.04(d)	J _{Nα} = 9.95 Hz 1H Ala NH	
6.60(dd)	J _{Nα2} = 3.63 Hz	J _{Nα1} = 10.4 Hz 1H Gly NH
6.28(d)	J _{Nα} = 10.85 Hz 1H Aoe NH	
4.72(m)	J _{Nα} = 10.85 Hz 1H Aoe _α	
4.68(dd)	J _{αβ1} = 7.23 Hz	J _{αβ2} = 2.70 Hz 1H Pro _α
4.46(m)	J _{Nα} = 9.95 Hz	J _{αβ} = 6.80 Hz 1H Ala _α
4.42(dd)	J _{Nα1} = 10.40 Hz	J _{α1α2} = 13.56 Hz 1H Gly _{α1}
3.91(m)	1H Pro _{δd}	
3.50(m)	1H Pro _{δv}	
3.39(dd)	J _{θ1} = 4.75 Hz	J _{θ2} = 2.73 Hz 1H Aoe _θ
3.12(dd)	J _{Nα2} = 3.63 Hz	J _{α1α2} = 13.56 Hz 1H Gly _{α2}
2.96(dd)	J _{ι1ι2} = 5.88 Hz	J _{ι1θ} = 4.75 Hz 1H Aoe _{L1}
2.83(dd)	J _{ι1ι2} = 5.88 Hz	J _{ι2θ} = 2.73 Hz 1H Aoe _{L2}
2.37(m)	1H Pro _{βd}	
2.27(m)	1H Pro _{γd}	
2.45-2.20(m)	2H Aoe _ε	
1.92(m)	1H Pro _{γv}	
1.84(m)	1H Pro _{βv}	
1.85(m)	1H Aoe _{βd}	
1.75-1.37	7H Aoe _{vδε}	
1.30(d)	J _{αβ} = 6.80 Hz 1H Ala _β	

similarity to **1**. Noteworthy differences are 1-CH₃ in **2** (Ala) vs. 2-CH₃ (2-Ala) and two downfield resonances in **3** (66.0 and 71.0 ppm) due to trans-3-hydroxyproline.

The ¹H-NMR (250 MHz, CDCl₃) data for HC-toxins-II **2** and -III **3** with assignments¹³ from extensive decoupling and COSY¹⁴ experiments appears in Tables 2 and 3 respectively. The components of **2** Ala, Pro, Gly, and Aoe are characterized by three NH, five α-H, three epoxide-H, and one -CH₃ resonance in accord with the data previously reported.⁶

The ¹H-NMR spectrum of **3** was very similar to **1** with the exception of those resonances associated with the hydroxyproline residue. However the region between 4.40 and 4.80 ppm contained five resonances, compared to the four signals found in the same region of HC-toxin-I **1**. Examination of the COSY spectrum of that region of **3** established that three of the resonances were the α- hydrogens of the two Ala and Aoe residues. The two remaining resonances at δ 4.68 and 4.60 ppm were identified as the trans-3-hydroxyproline β- and α- hydrogens, respectively. Irradiation of the Ala-NH at 7.11 ppm resulted in a 11% nOe of the resonance at δ 4.60 ppm. Based upon the previously noted¹⁵ observation of significant nOe at the α-H of the proline of **1** upon irradiation of an Ala-NH preceding it and an inspection of models, the signal at δ 4.60 ppm is assigned as the hydroxyproline α-H. Also this establishes the proximity of the Ala to the hydroxyproline moiety. Further analysis of the COSY spectrum allowed the resonance at δ 4.68 ppm to be assigned as follows. The two hydroxyproline δ- hydrogens (4.20, 3.67 ppm) are strongly coupled to the two γ- hydrogens (2.60, 2.00 ppm). The γ- H at δ 2.60 ppm is further coupled to the resonance at 4.68 ppm; this resonance is the β-H, and consistent with its downfield position the carbon bearing this H must also be the attachment point of the hydroxyl group, thus confirming the presence of trans-3-hydroxyproline.

With the constitutions of HC-toxins-II **2** and -III **3** established as (Ala, Gly, Aoe, Pro) and (Ala, Ala, Aoe, trans-3-Hypro) respectively, the correct sequence must now be determined. The previously characterized HC-toxin-I **1** has been sequenced by FAB-

TABLE 3
¹H-NMR HC-Toxin-III 3 (250 MHz, CDCl₃, CHCl₃ ref.)
HC-Toxin-III 3

7.11(d)	J _{Nα} = 10.53 Hz	1H Ala ₁ NH	
6.26(d)	J _{Nα} = 10.40 Hz	1H Aoe NH	
6.03(d)	J _{Nα} = 9.80 Hz	1H Ala ₂ NH	
4.77(m)	J _{Nα} = 10.40 Hz	1H Aoe _α	
4.68(d)	J _{βγ} = 3.85 Hz	1H 3-HyPro _β	
4.60(s)		1H 3-HyPro _α	
4.54(dq)	J _{Nα} = 9.80 Hz		J _{αβ} = 7.03 Hz 1H Ala _{2α}
4.41(dq)	J _{Nα} = 10.53 Hz		J _{αβ} = 6.83 Hz 1H Ala _{1α}
4.20(m)	J _{δuδd} = 9.75 Hz	1H 3-HyPro _{δd}	
3.67(m)	J _{δuδd} = 9.75 Hz	1H 3-HyPro _{δu}	
3.40(dd)	J _{θL2} = 2.43 Hz		J _{θL1} = 4.27 Hz 1H Aoe _θ
2.97(dd)	J _{L1L2} = 5.79 Hz		J _{L1θ} = 4.27 Hz 1H Aoe _{L1}
2.83(dd)	J _{L1L2} = 5.79 Hz		J _{L2θ} = 2.43 Hz 1H Aoe _{L2}
2.60(m)		1H 3-HyPro _{γd}	
2.41(dt)	J _{ζdζv} = 17.43 Hz		J _{ζde} = 7.20 Hz 1H Aoe _{ζd}
2.26(dt)	J _{ζdζv} = 17.43 Hz		J _{ζve} = 7.20 Hz 1H Aoe _{ζv}
2.00(m)		1H 3-HyPro _{γv}	
1.80(m)		1H Aoe _{βd}	
1.57(m)		1H Aoe _{βv}	
1.53(m)		2H Aoe _ε	
1.60(s)		1H 3-HyPro OH	
1.35-1.20(m)		4H Aoe _{γδ}	
1.29(d)	J _{αβ} = 6.83 Hz	3H Ala _{1β}	
1.24(d)	J _{αβ} = 7.03 Hz	3H Ala _{2β}	

TABLE 4
nOe DIFFERENCE EXPERIMENTS : HC-TOXINS- II 2 and - III 3

	<u>H-IRRADIATED</u>	<u>H-ENHANCED</u>	<u>%-nOe</u>	<u>PARTIAL SEQUENCE</u>
<u>HC-TOXIN-II 2</u>	GLY-NH	Ala-α	15%	Ala-Gly
	Aoe-NH	Gly-α	9%	Gly-Aoe
	Ala-NH	Pro-α	13%	Pro-Ala
	Pro-δ _{upfield}	Aoe-α	3%	Aoe-Pro
	Pro-δ _{downfield}	Aoe-α	5%	Aoe-Pro
		Pro-δ _{upfield}	15%	
<u>HC-TOXIN-III 3</u>	Ala ₁ -NH	3-HyPro-α	11%	3-HyPro-Ala ₁
	Aoe-NH	Ala ₂ -α	9%	Ala ₂ -Aoe
	Ala ₂ -NH	Ala ₁ -α	11	Ala ₁ -Ala ₂
	3-HyPro-δ _{upfield}	Aoe-α	4%	Aoe-3-HyPro
		3-HyPro-δ _{downfield}	21%	
	3-HyPro-δ _{downfield}	Aoe-α	13%	Aoe-3-HyPro
	3-HyPro-δ _{upfield}	18%		

CAD-MS¹⁵, GC-MS of derivitized partial hydrolysates^{15c}, 2D-TLC of derivitized partial hydrolysates^{15d}, and ¹H-NMR nOe difference spectroscopy.^{15a,b}

The sequences of **2** and **3** were determined by the nOe difference technique. As applied to **2** and **3** this method yielded the data in Table 4 from which the overlapping partial structures were obtained. From this data structures **2** and **3** for HC-toxins-II and -III respectively, are unambiguously established.

The data presented above establishes the structures of **2** and **3** as Ala, Gly, Pro, Aoe and Ala, Ala, trans-3-hydroxyproline, and Aoe respectively. The isolation and structure elucidation of other active components of the culture filtrate of H. carbonum is currently underway. These results will be reported in due course.

EXPERIMENTAL

Reagent grade solvents were employed for extractions; column- and thin layer chromatography and were used as received. HPLC separations were performed with HPLC-grade solvents from American Burdick and Jackson, Muskegon, Mi. . Flash chromatography was performed according to the method of Still¹⁶; conditions are reported as follows: (column diameter in mm; solvent system; grams of silica gel employed; fraction size collected) TLC was performed on 0.25mm silica gel plastic backed plates (Macherery- Nagel, D-5160) which were developed with 4:3 acetone-methylene chloride. HC-Toxins were visualized on TLC plates as blue spots when sprayed with 0.05% bromocresol green in acetone followed by heating. HPLC was performed on a Varian-5000 instrument, equipped with a variable wavelength UV-detector, set at 215 nm. Normal phase HPLC separations utilized a 0.4 x 25 cm Whatman Partisil 10 column eluted isocratically with 96:4 hexane-ethanol at 4.0 ml per minute. Reverse phase separations were performed with a 0.78 x 30 cm Waters m- Bondapac C₁₈ column eluted with the following gradient: 7% aqueous ethanol to 20% aqueous ethanol over 30 min; 20% aqueous ethanol for 15 min; followed by a recycle to 7% aqueous ethanol over 20 min.

¹H- and ¹³C-NMR spectra were recorded in CDCl₃ at 250 MHz and 68.9 MHz, respectively, on a Bruker WM-250 spectrometer. All chemical shifts are reported in ppm relative to internal CHCl₃. ¹³C-NMR spectra were acquired with broadband noise decoupling; COSY spectra were obtained as 1K x 512W data matrices using a $\pi/2$ -T- $\pi/4$ -AQ pulse sequence. Data were multiplied by a gaussian function prior to

transformation. DEPT¹⁷ subspectra were obtained using the following pulse sequence $^1\text{H}(\pi/2-\Delta-\pi-\theta-\Delta-\text{BB})$, $^{13}\text{C}(-\pi/2-\pi-\text{AQ})$; editing was performed using the procedure described by Bendall and Pegg¹⁸. nOe experiments employed a 2-second decoupled irradiation, followed by a $\pi/2$ observation pulse and acquisition of the FID. FID's were multiplied by an exponential function before transformation such that a 2Hz- line broadening was introduced. Subtraction of a transformed off- resonance control spectrum from the transformed on resonance spectrum produced the nOe difference spectrum from which the nOe's were determined¹⁹. The ratio of the area of the enhanced peak to the area of the irradiated peak (normalized to equal unity) determined the % nOe. FAB-MS were obtained⁸ on a Varian Mat CH5-DF double focussing mass spectrometer. EI/MS were obtained²⁰ on a Finnigan 4000 mass spectrometer equipped with an INCOS 4201 data system.

The initial stages of the isolation and purification ⁷ of the HC-toxins from a culture of Helminthosporium carbonum (race 1) was accomplished by Sephadex LH-20 chromatography of the crude CHCl_3 extract of the culture filtrate. Variable quantities of the crude HC-toxins were obtained as a function of the culture size, age, viability, etc.

In a typical isolation , 2-5 ml of the Sephadex eluate was dissolved in CH_2Cl_2 (50 ml), leaving behind a small amount of insoluble dark-colored material. The CH_2Cl_2 solution was washed with distilled water (3x30ml) , brine (50ml) and dried (Na_2SO_4). Concentration in vacuo yielded an orange-brown gum.

This material (2.24g from one run) was purified by flash chromatography (60mm , 2-step gradient: 3 | 1:11:1 hexane-acetone-methylene chloride, 4 | 4:3 acetone-methylene chloride; 300g 230-400 mesh; 1x3l, 1x300ml, 1x700ml, 1x3l).

After concentration fraction 1 yielded 1.45g of a pale yellow foam. Comparison of this sample to HC-Toxin-1 **1** isolated previously demonstrated that it consisted primarily of **1**. The $^1\text{H-NMR}$ spectrum of this material compared favorably with that of authentic **1**. Fraction 4 yielded 0.350g of an orange brown oil which illicited host specific toxicity (TLC: streak R_f 0.55- 0.20). This material was purified further by flash chromatography (40mm, 8:12:3 acetone-methylene chloride-ethanol, 100g 230-400 mesh; 50ml initial fraction followed by 20 ml fractions). Fractions 8 and 9 were combined to yield 0.181g of a yellow oil which was active in the bioassay (TLC: two poorly resolved spots, R_f 0.30).

This oil was dissolved in H₂O and passed through a Waters C₁₈-Sep-Pak; flushed with 40% aqueous ethanol. Reverse phase HPLC of 35 mg of this material separated it into three major components, retention times 25, 35 and 47 min respectively. Normal phase HPLC of the 25 min. retention time peak (11.0 mg) separated it into two components (retention times 25 and 33 min) which were designated HC-Toxin-II **2** (4.0 mg) and HC-Toxin-III **3** (2.4 mg) in order of the elution.

HC-Toxin-II **2** cyclo[Ala-Gly-(2-amino-8-oxo-9,10-epoxy-decanoyl)-Pro]

EI-MS (70 eV): 422(M⁺, 5.0), 170 (76.6), 70 (base). FAB-MS (glycerol): 423 M⁺+1, 48), 226(M-196, 10), 170(13), 70(base). High resolution FAB-MS (Csl, glycerol) for C₂₀H₃₁N₄O₆, calculated 423.22436, found 423.22558; +2.88ppm. ¹H- and ¹³C-NMR: see tables.

HC-Toxin-III **3** cyclo[Ala-Ala-(2-amino-8-oxo-9,10-epoxy-decanoyl)-trans-3-Hypro]

EI-MS (70 ev): 452(M⁺, 3.9), 170(base), 86(55.4). FAB-MS (glycerol): 453(M⁺+1, 19), 170(30), 156(M-196, 12), 86(base). High-Resolution FAB-MS (Csl, glycerol) for C₂₁H₃₃N₄O₇, calculated 453.23493, found 453.23221; -6.0ppm. ¹H- and ¹³C-NMR see tables.

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