Tetrahedron Vol. 38, No. 1, pp. 45 to 48, 1982
Printed in Great Britain.

0040-4020/82/010045-04$03.00/0
© 1982 Pergamon Press Ltd.

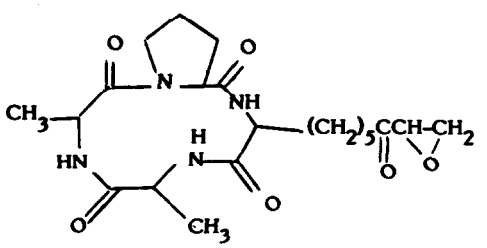
STRUCTURE OF HC-TOXIN, A CYCLIC TETRAPEPTIDE FROM *HELMINTHOSPORIUM CARBONUM*

JERROLD M. LIESCH*, CHARLES C. SWEELEY+, GLENN D. STAFFELD+, MATT S. ANDERSON+, DARRELL J. WEBER●, AND ROBERT P. SCHEFFER▲

*Merck Institute for Therapeutic Research, P.O. Box 2000, Rahway, NJ 07065; +Department of Biochemistry, Michigan State University, East Lansing, MI 48824; ●Department of Botany, Brigham Young University, Provo, UT 84601; ▲Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI 48824 U.S.A..

(*Received in U.S.A. 31 August 1981*)

Abstract - *Helminthosporium carbonum*, which is pathogenic to maize, produces a metabolite, HC-toxin (1), with selective toxicity to susceptible host genotypes. Resistant genotypes and non host plants are tolerant. The structure of 1 ($C_{21}H_{32}N_4O_6$) was determined as cyclo[(2-amino-9,10-epoxy-8-oxodecanoyl)-alanyl-alanyl-prolyl] based upon spectral evidence.



1

Helminthosporium carbonum Ullstrup race 1 selectively parasitizes certain genotypes of maize causing necrotic lesions on the leaves. Scheffer and Ullstrup[1] found that the fungus produces a toxic substance named HC-toxin which has the same specificity for maize genotypes as does the producing fungus. Sensitive tissue is affected by the toxin at very low concentrations (0.1 μg/ml), whereas resistant plants will tolerate much higher concentrations with no obvious damage.[2] The toxin was shown to be required for successful colonization of susceptible plants.[3] HC-toxin causes various physiological changes in sensitive cells, including an immediate but temporary increase in negative electropotential across the cell membrane,[4] an increase in dark fixation of CO_2, and an increase in the uptake of nitrate and other solutes. Eventually, exposure to HC-toxin leads to an increase in tissue respiration and loss of materials from the cell. HC-toxin appears to have a primary effect on the plasma membrane.[5]

HC-toxin previously was isolated from culture filtrates and partially characterized.[6,7] Pringle[6] reported that the toxin has a molecular formula $C_{32}H_{50}N_6O_{10}$ based upon elemental analysis, and did not react with ninhydrin. Alanine and proline in a 2 to 1 ratio were found among the hydrolytic products and a third ninhydrin-positive component was detected.[7] A structure was not proposed.

Several peptidic fungal products affecting higher plants have been reported. The host selective toxins[8-10] from *Alternaria mali* affecting apple are closely related cyclic tetra-depsipeptides. Cyclic tetrapeptides include tentoxin[11] from *Alternaria alternata* f. *tenuis*, and Cyl-2[12] from *Cylindrocladium scoparium* Morgan and chlamydocin[13] from *Diheterospora chlamydospora* which both contain the novel amino acid 2-amino-9,10-epoxy-8-oxodecanoic acid.[12] In this paper, we report the structure of HC-toxin (1), which is a cyclic tetrapeptide of the same structural family as Cyl-2 and chlamydocin.

The metabolite was isolated from cultures of *H. carbonum* by chloroform extraction followed by chromatography to afford fine colorless needles. HC-toxin (1) is a neutral substance with empirical formula $C_{21}H_{32}N_4O_6$. Amino acid analysis of an acidic hydrolyzate of 1 indicated the presence of proline and alanine (1:2). Proline plus two alanine residues joined via amide bonds

account for the elements $C_{11}H_{17}N_3O_3$, thus leaving $C_{10}H_{15}NO_3$ unassigned in the formula $C_{21}H_{32}N_4O_6$ for 1.

Examination of the ^{1}H-NMR spectrum of 1 (Table 1) indicated that the unknown subunit exhibits resonances at 1.51-1.87 (8H), 2.28 (2H), 2.80, 2.93, 3.35, and 4.71 ppm. Similarly, the ^{13}C-NMR spectrum of 1 (Table 2) contains resonances at approximately 24 (3C), 29, 36, 50 (3C), 170, and 200 ppm attributable to the $C_{10}H_{15}NO_3$ subunit. The ^{1}H-NMR resonance at 4.71 ppm (1H, d of t, J = 7.5, 9.5) suggests that the subunit is an α-amino acid wherein the C3 carbon is a methylene group (-CO-CHNH-CH_2-). Resonances at 2.80 (1H, d of d, J = 2.5, 6.0), 2.93 (1H, d of d, J = 4.5, 6.0), and 3.35 ppm (1H, d of d, J = 2.5, 4.5) imply a terminal epoxy group,

Table 1. HC-toxin (1) ^{1}H-NMR ($CDCl_3$, 25°C, 300 MHz).

	δ(ppm)	Assignment
1.19	(3H,d,J=7.0)	ALA_1-β
1.23	(3H,d,J=7.0)	ALA_2-β
1.51 1.58 1.75 1.87	(2H,m) (2H,m) (2H,m) (2H,m)	epoxyAA-β γ δ ε
2.22	(2H,m)	PRO-β/γ
2.28	(2H,m)	epoxyAA-ζ
2.33	(2H,m)	PRO-β/γ
2.80	(1H,d of d, J=2.5,6.0)	epoxyAA-ι
2.93	(1H,d of d, J=4.5,6.0)	epoxyAA-ι
3.35	(1H,d of d, J=2.5,4.5)	epoxyAA-θ
3.44	(1H,d of t, J=7.0,10.0)	PRO-δ
3.90	(1H,m)	PRO-δ
4.40	(1H,d of q, J=7.0,9.5)	ALA_1-α
4.49	(1H, d of q, J=7.0,9.5)	ALA_2-α
4.63	(1H,d of d, J=2.0,8.0	PRO-α
4.71	(1H,d of t, J=7.5,9.5)	epoxyAA-α
6.23 6.27 7.06	(1H,d,J=9.5) (1H,d,J=9.5 (1H,d,J=9.5)	NH-ALA_1,ALA_2,epoxyAA

Table 2. ^{13}C-NMR Spectra of HC-toxin (1), (a, $CDCl_3$, 20 MHz; b, $CDCl_3$ plus chromium acetylacetone, 45.3 MHz).

δ(ppm) a	δ(ppm) b	Assignment
13.6 14.5	14.1 14.7	ALA_1-C3,ALA_2-C3
22.6 24.3 24.3 25.0	22.8 24.9 25.1 25.5	PRO-C4,epoxyAA-C4,C5,C6
28.6 29.0	28.7 29.1	PRO-C3,epoxyAA-C3
36.2	36.3	epoxyAA-C7
45.7 46.4 47.1 47.9 51.2 52.9	46.1 47.1 47.4 48.0 51.9 53.4	ALA_1-C2,ALA_2-C2,epoxyAA-C2,C9,C10,PRO-C5
57.4	57.8	PRO-C2
- - - -	171.4 173.2 173.7 173.7	ALA_1-C1,ALA_2-C1,PRO-C1,epoxyAA-C1
-	203.5	epoxyAA-C8

whose assignment is corroborated by typical epoxide bands[14] at 1230, 925, and 865 cm^{-1} in the IR spectrum of 1. Lastly, the resonance at 203.5 ppm in the ^{13}C-NMR (Table 2) of 1 indicates a ketone group.

An amino acid moiety (-CO-CHNH-CH_2-), terminal epoxide (-$\overbrace{CH\text{-}CH_2}$O), and ketone group (C=O) account for the elements $C_6H_7NO_3$, leaving four methylene groups (C_4H_8) unassigned in the $C_{10}H_{15}NO_3$ subunit. Comparison of the structural features of 1 deduced from the spectroscopic data, with the structures of known natural products using SQUINT,[15] a new computer-based natural products structure recognition system, disclosed a net structural similarity with the previously reported metabolite chlamydocin.[13] Comparison of the spectroscopic data for chlamydocin with that of 1 allowed final assignment of the $C_{10}H_{15}NO_3$ α-amino-epoxyketo acid subunit in 1 as 2-amino-9,10-epoxy-8-oxodecanoic acid (epoxyAA), which is also present in chlamydocin and Cyl-2. Thus, 1 is identified as a cyclic tetrapeptide containing proline, two alanine, and epoxyAA residues.

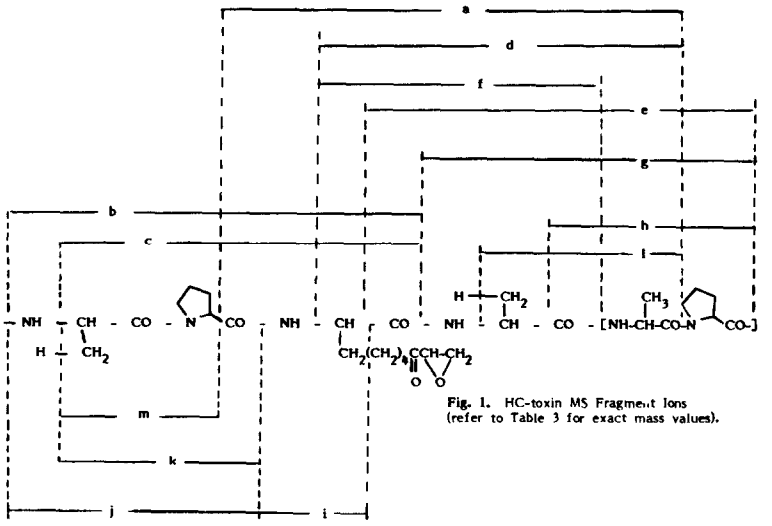


Fig. 1. HC-toxin MS Fragment Ions (refer to Table 3 for exact mass values).

The sequence of the four amino acids remained to be determined, and this was accomplished by examination of the high resolution mass spectrum of 1 (Table 3 and Fig. 1). The observed dipeptide fragments (Fig. 1) ALA-ALA (l), epoxyAA-ALA (f), and ALA-PRO (j) uniquely define the relative sequence ALA-ALA-PRO-epoxyAA with unspecified head-to-tail orientation. Critical fragment ions k and m from the ALA-PRO dipeptide unit, j, permit the absolute assignment ALA→PRO since these fragments contain only one nitrogen. Of the two possible dipeptide sequences ALA→PRO and PRO→ALA, the PRO→ALA sequence cannot readily yield fragment ions which have lost NH (or NH_2) such as in k and m. Furthermore, ions of compositions k and m are not reasonably expected to arise from elsewhere in the molecule. The dipeptide sequence ALA→PRO is therefore indicated, thus allowing assignment of 1 as the structure of HC-toxin.

Some of the results obtained in this laboratory differ from those of Pringle.[6,7] The most significant difference is the molecular weight value obtained and consequently, the number of residues present. However, the molecular formula $C_{32}H_{50}N_6O_{10}$ previously reported from elemental analysis data is simply a multiple (≈1.5) of that which we obtained by high resolution mass spectrometry ($C_{21}H_{32}N_4O_6$) and therefore does not present a contradiction. The unusual hydroxyamino acid detected in the acidic hydrolyzate of 1 by Pringle is probably a degradation product of the epoxyAA residue.

Table 3. Significant Ions in HR-EI-MS of HC-toxin (mass values averaged from four spectra; refer to Fig. 1 for assignments).

m/e	Formula	Δm/e,(mmu)	Identity
436.2320	$C_{21}H_{32}N_4O_6$	(-0.2)	M^+
408.2374	$C_{20}H_{32}N_4O_5$	(0.1)	$(M-CO)^+$
393.2136	$C_{19}H_{29}N_4O_5$	(-0.2)	$(M-CH-CH_2)^+$ (O)
367.1755	$C_{17}H_{25}N_3O_6$	(1.2)	a
365.1949	$C_{18}H_{27}N_3O_5$	(-0.2)	b
350.1867	$C_{18}H_{26}N_2O_5$	(2.5)	c
324.1685	$C_{16}H_{24}N_2O_5$	(.0)	d
267.1216	$C_{12}H_{17}N_3O_4$	(-0.3)	e
253.1302	$C_{13}H_{19}NO_4$	(-1.2)	f
239.1285	$C_{11}H_{17}N_3O_3$	(1.5)	g
196.0848	$C_9H_{12}N_2O_3$	(.0)	h
170.1175	$C_9H_{16}NO_2$	(-0.6)	i + H
169.1102	$C_9H_{15}NO_2$	(-0.1)	i
168.0888	$C_8H_{12}N_2O_2$	(-1.1)	j
152.0717	$C_8H_{10}NO_2$	(0.5)	k
126.0541	$C_6H_8NO_2$	(-1.4)	l
124.0766	$C_7H_{10}NO$	(0.4)	m
70.0646	C_4H_8N	(-1.3)	CH_2CH_2 / CH_2 CH / NH

HC-toxin (1), chlamydocin, and Cyl-2 comprise a family of neutral phytotoxic fungal pathogens which contain 2-amino-9,10-epoxy-8-oxodecanoic acid. Structural similarity is further extended by the presence of a secondary α-amino acid (proline, or pipecolic acid in Cyl-2) at the N-terminus of the epoxyAA residue, thus defining a common dipeptide unit PRO(or PIP)→epoxyAA. Major structural variability is exhibited in the two remaining amino acid subunits, and these structural variations apparently must correlate with the observed host specific toxicity of these metabolites. The assignment of the structure of HC-toxin will allow further physiological studies on this interesting fungal phytotoxin.

EXPERIMENTAL

The m.p. is uncorrected. NMR spectra were obtained on Varian SC-300 (^{1}H), Varian CFT20 (^{13}C), and Bruker WH180 (^{13}C) instruments using $CDCl_3$ as the solvent. The chemical shift is presented in δ(ppm) from the internal standard tetramethylsilane. Mass spectra were recorded on LKB-9000, Varian MAT CH5, and Varian MAT 731 instruments. The infrared spectrum was obtained on a Perkin-Elmer 421 instrument as a KBr pellet.

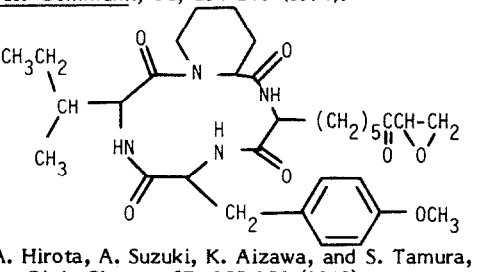
Isolation of HC-toxin (1). Concentrated filtrates were obtained from cultures of H. carbonum race 1 grown in Fries medium plus 0.1% yeast extract.[16] An equal volume of methanol was added, the mixture was allowed to stand for 24 hr, and then filtered to remove the precipitate, which was washed with cold 50% aqueous methanol. The filtrates were combined and the methanol was removed. The remaining aqueous solution was extracted five times with successive 100-ml volumes of chloroform to remove toxin. The combined chloroform extracts were dried with anhydrous sodium sulfate, filtered to remove the desicant, and the chloroform was removed by evaporation under reduced pressure. The residue was purified further by counter current distribution using n-butanol, glacial acetic acid, and water (4:1:5, v/v). The active fraction was evaporated to dryness and dissolved in 100 ml of absolute ethyl ether. The solution was stored at 5°C and the toxin slowly crystallized over 3 days as fine colorless needles. HC-toxin is soluble in water, has limited solubility in lower alcohols, acetone, ethyl ether, and chloroform, and is insoluble in petroleum ether and carbon tetrachloride. Crystalline HC-toxin at 1.0 μg/ml gave 50% inhibition of seedling root growth when assayed by the standard procedure.[5]

Physical Properties of HC-toxin (1), m.p. 150°C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3350 (broad), 2930, 1650-1700 (broad), 1525, 1440, 1230, 925, and 865. ^{1}H-NMR: Table 1. ^{13}C-NMR: Table 2. MS m/e (abundance): 436 (M^+, 4.5%), 408 (1.0), 393 (1.5), 367 (3.5), 365 (1.0), 350 (3.0), 324 (14.5), 267 (3.5), 253 (5.5), 239 (1.5), 196 (3.0), 170 (50.0), 169 (3.2), 168 (3.0), 152 (5.5), 126 (3.5), 124 (4.0), and 70 (100); and Table 3.

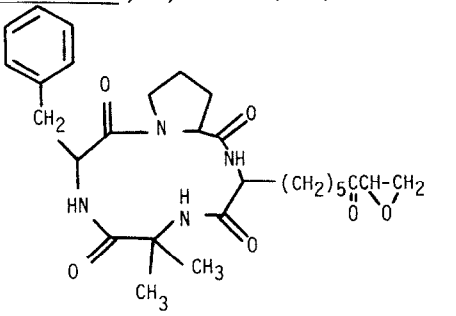
Acknowledgements - The ^{13}C NMR spectra of 1 were obtained through the courtesy of George Slump, the Upjohn Company, Kalamazoo, Michigan, and Otto Ganso, Department of Chemistry, Michigan State University. This work was supported in part by research grants from the National Institutes of Health, RR00480 (CCS), and the National Science Foundation, PCM-7611916 (RPS).

REFERENCES

1 R.P. Scheffer and A.J. Ullstrup, Phytopathology, 55, 1037-1038 (1965).
2 R.B. Pringle and R.B. Scheffer, Phytopathology, 57, 1169-1172 (1967).
3 J.C. Comstock and R.P. Scheffer, Phytopathology, 63, 24-29 (1973).
4 J.M. Gardner, R.P. Scheffer, and N. Higinbotham, Plant Physiol., 54, 246-249 (1974).
5 R.P. Scheffer, Physiological Plant Pathology, Encyclopedia of Plant Physiology, New Series, IV (R. Heitefuss, and P.H. Williams, eds.), Springer-Verlag, Berlin, 247-269 (1976).
6 R.B. Pringle, Plant Physiol., 46, 45-49 (1970).
7 R.B. Pringle, Plant Physiol., 48, 756-759 (1971).
8 S. Lee, H. Aoyagi, Y. Shimohigashi, N. Izumiya, T. Ueno, and H. Fukami, Tetrahedron Lett., 843-846 (1976).
9 T. Okuno, Y. Sawai, and T. Matsumoto, Chem. Lett. (Chem. Soc. Japan), 1974, 635-638 (1974).
10 T. Ueno, T. Nakashima, Y. Hayashi, and H. Fukami, Agr. Biol. Chem., 39, 1115-1122 (1975).
11 W.L. Meyer, L.F. Kuyper, R.B. Lewis, G.E. Templeton, and S.H. Woodhead, Biochem. Biophys. Res. Commun., 56, 234-240 (1974).
12



A. Hirota, A. Suzuki, K. Aizawa, and S. Tamura, Agr. Biol. Chem., 37, 955-956 (1973).
13



A. Closse and R. Huguenin, Helv. Chim. Acta, 57, 533-545 (1974).
14 K. Nakanishi, Infrared Absorption Spectroscopy, Holden-Day, Inc., San Francisco (1962).
15 J.M. Liesch and G. Albers-Schonberg, manuscript in preparation.
16 R.B. Pringle, and R.P. Scheffer, Phytopathology, 53, 785-787 (1963).