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Full Papers

Characterization of victorin C, the major host-selective toxin from *Cochliobolus victoriae*: structure of degradation products

V. Macko[1], T.J. Wolpert, W. Acklin, B. Jaun, J. Seibl, J. Meili and D. Arigoni[1]

Boyce Thompson Institute, Cornell University, Ithaca (New York 14853, USA), and Laboratorium für Organische Chemie, Eidgenössische Technische Hochschule, CH-8092 Zürich (Switzerland), 18 June 1985

Summary. Several host-selective toxins have been isolated in pure form from culture filtrates of *Cochliobolus victoriae*. Acid hydrolysis of the major toxin, victorin C (apparent mol.wt 796), produced five fragments to which structures **1**–**5** have been assigned. Spectroscopic techniques revealed that the toxin contains an additional subunit corresponding to **6**; thus all the components of victorin C are accounted for.
Key words. Host-selective toxins; *Cochliobolus victoriae; Helminthosporium victoriae;* victorin C; oats; blight of oats; unusual amino acids.

Many disease-inducing plant pathogenic fungi produce toxic metabolites which are injurious to plants at low concentrations. Those pathogen-produced substances that affect selectively only certain varieties or genotypes of a given plant species have been termed host-specific toxins or host-selective toxins. These toxins are thought to play an important role in disease, because they can induce most or all of the disease symptoms and because toxin production by the pathogen often parallels its virulence[2,3].

Among the most elusive of the selective toxins is victorin, also known as HV-toxin. Victorin was discovered as a result of an outbreak of a new disease of oats in North America in the late 1940's. The fungus *Helminthosporium victoriae* was first described by Meehan and Murphy as the causal agent of the disease called Victoria blight of oats[4]. These authors also reported that culture filtrates from the fungus contained a toxin which caused symptoms typical of Victoria blight disease on susceptible but not on resistant plants[5].

Early work by Pringle and Braun[6,7] led them to postulate that victorin was composed of a small peptide and a nitrogen containing terpenoid, victoxinine. The structure of victoxinine was later elucidated by Dorn and Arigoni[8], but despite extensive research over the years the structure of victorin itself has remained unknown. Recently two groups reported on the purification of victorin but no structural information was given[9,10]. We have independently purified several toxic components from culture filtrates of the fungus and now report on the characterization and structure of the degradation products of the major toxin, henceforth designated as victorin C.

Materials and methods

Cochliobolus victoriae Nelson (*Helminthosporium victoriae* Meehan and Murphy) isolate 033 and isolate 1 were obtained from R.P. Scheffer and H.E. Wheeler, respectively. For toxin production the fungus was grown for 14–21 days at 25 °C under constant illumination in still culture in 500-ml Erlenmeyer flasks containing 100 ml of modified liquid Fries' medium[11] supplemented with either 0.1 % yeast extract or with oat flakes[12].

Culture filtrates and fractions from each purification step were tested for toxin activity by placing excised oat leaves in 4-ml vials containing 0.5 ml of test solutions. The root growth assay was performed essentially as described by Luke and Wheeler[11] using susceptible and resistant oat varieties, Park and Rodney, respectively.

Culture fluid was separated from mycelium by filtration through cheese cloth. Extraction, concentration and initial fractionation of the toxin were accomplished in a

single step by passing approximately 50–60 l of culture filtrate through a 2.5 × 60 cm column of reverse-phase C_{18} packing (Waters Ass. preparative C_{18} 55–105 µm). A number of metabolites, including all compounds displaying toxic activity, were retained on the column while many components of the culture medium, such as inorganic salts, amino acids, carbohydrates, etc. were completely eluted with water. The retained materials were then eluted with a 1600 ml linear gradient of 0–100% H_2O-acetonitrile at 10 ml/min. Fractions showing host-selective activity were analyzed by injecting a small portion of the fraction into an analytical reverse phase HPLC column (Waters Resolve) eluting with 25% acetonitrile/0.1% TFA at 1 ml/min and monitoring at 254 nm. In addition, fractions from the analytical column were collected and assayed for toxicity. Fractions from the preparative column which displayed an identical behavior in terms of biological activity and of retention time when injected into the analytical column were pooled and concentrated in vacuo. In the next step pooled fractions were chromatographed by gel filtration on a 2.5 × 210 cm column of P-2 (Biorad) with 10 mM NaCl in H_2O as the mobile phase. Final purification and desalting were accomplished by pumping the active P-2 fractions directly onto a C_{18} reversed phase HPLC column (Z module with Novapak cartridge, Waters Assoc.) at 3 ml/min. After loading, the column was eluted with a 2-h gradient of 0–15% H_2O-acetonitrile at 3 ml/min. Active fractions were then pooled, concentrated in vacuo and used for spectral analyses.

Hydrolysis experiments. a) Mild hydrolysis: 40 mg of victorin C were incubated in 10 ml of 6 N HCl at r.t. for 22 h. The sample was dried in vacuo, redissolved in water and chromatographed by HPLC as described above for the toxin. b) Drastic hydrolysis: 50 mg of victorin C were dissolved in 10 ml of 6 N HCl and heated in a sealed tube at 110 °C for 18 h. The crude reaction mixture was taken to dryness and separated by TLC (Merck 'soft plus' silica gel plates developed in isopropanol:58% ammonium hydroxide 7:3) followed by gel filtration chromatography (Biogel P-2, Biorad).

FAB measurements were carried out with a Kratos MS 50 instrument, equipped with a M-Scan gun at 8 kV accelerating voltage. CI spectra were obtained on a Finnigan 3300 Quadrupole instrument under 0.5 torr of methane.

NMR spectra of solutions (D_2O with 0.2% deut. TFA) were recorded either on a Bruker WM-300 (300 MHz ^1H; 75.35 MHz ^{13}C) or on a Bruker WM-500 spectrometer. Homonuclear ^1H-shift correlated 2 D spectra were obtained by the conventional COSY-45 pulse sequence[13] using a 16-phase cycle and 32 scans/FID. Typical data sizes were 2K(t2) × 512(t1), leading to 1K × 1K absolute value presentations made symmetric by triangular multiplication.

Results and discussion

Five fractions displaying host-selective toxic activity were isolated from the culture fluids of *C. victoriae* and designated as victorins A, B, C, D and E, respectively, according to their order of elution from the reversed phase HPLC column. The main fraction, victorin C, accounted for 85–90% of the total activity, displaying half-maximal inhibition of susceptible root growth at 80–120 pg/ml. The preparation behaved as homogeneous when chromatographed on various columns as well as in TLC; its chemical purity was eventually confirmed by NMR techniques. This paper is concerned with the chemical characterization of victorin C, which we found was the major toxin produced by the two isolates on two liquid media. Although a direct comparison was not made, this compound is probably identical with the main toxin fraction reported by Walton and Earle[10] and Keen et al.[9].

Victorin C was obtained as a white amorphous solid, soluble in H_2O and DMSO and less soluble in methanol and ethanol, $[\alpha]_D = -76°$ (c = 1, H_2O), $[\alpha]_D = -60.5°$ (c = 1, 0.1% TFA). The UV spectrum of the compound in H_2O displayed $\lambda_{max} = 256$ nm with a shoulder at 225 nm. IR spectroscopy (KBr) revealed bands at 2960, 2930, 2880 sh, 2860 sh, 1680, 1619, 1528, 1470, 1435, 1385, 1335, 1320, 1112, 883, and 750 cm^{-1}. Preliminary attempts to obtain information on the mol.wt by EI- and CI-MS were unsuccessful. FAB-MS using glycerol as a matrix gave, both in the positive and the negative mode, a set of ions corresponding to fragments ranging from 467 to 945, all of which were accompanied by m + 2 and m + 4 isotope peaks. Differentiation of matrix-related products from compound-specific ions was achieved by cross checking with spectra determined in a different matrix, 3-nitro-benzylalcohol[14]. In this way it was possible to detect an apparent molecular mass of 796 which must include 3 Cl atoms, as judged by the characteristic isotope pattern of ions in the molecular ion region.

Initial interpretation of the ^1H- and ^{13}C-NMR spectra of victorin C (cf. tables) was hampered by the fact that about ⅓ of all the atoms display a dichotomous behavior leading to a confusing redundance of signals. By following the changes in the relative intensities of cognate dichotomous signals (from 1:1 to 2:1, the sum being unchanged) caused by the addition of a small amount of TFA it was determined that the redundance is not due to sample heterogeneity, but rather reflects the co-occurrence of two forms of the same molecule which are not in a rapid equilibrium. With this understanding of the spectra it was eventually possible to detect the presence of 33 nonexchangeable protons and, with less confidence, of 31 carbon atoms. Specifically, the appearance of a cluster of signals around 172 ppm in the ^{13}C-NMR spectrum suggested, in connection with the IR evidence, the presence of several amide bonds and it was felt that an analysis of the degradation products generated by hydrolysis might provide an insight into the composition of the compound.

Mild hydrolysis of victorin C with HCl at r.t. gave several degradation products, the most polar of which was eluted from the column with water and identified as glyoxylic acid, **1**, by TLC-comparison with authentic material and by formation of its 2,4-dinitrophenylhydrazone. A complementary fragment with mol.wt 740, desglyovictorin C, could be detected among the less polar products of the hydrolysis. Analysis of its NMR spectrum revealed that the removal of glyoxylic acid is linked with the disappearance of the signals of a dichotomous proton at 5.36 and 5.37 ppm and of two carbon signals, one at 89.4 ppm and the other belonging to the cluster of singlets in the region

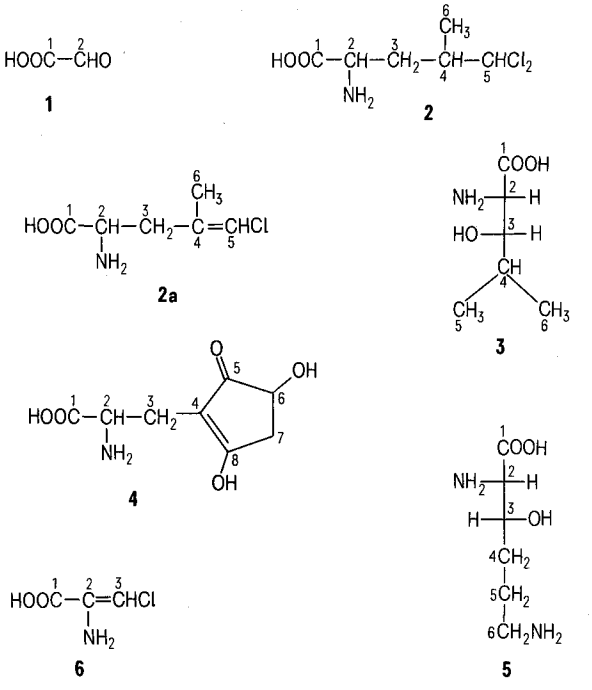
165–176 ppm. These results certify that in victorin C the aldehydo group of the glyoxylic acid moiety must be present in a masked form with an sp^3 carbon atom.

Under more forceful conditions of hydrolysis the toxin was degraded to a set of ninhydrin positive, low mol.wt compounds, which after separation and purification were analyzed by MS and by NMR. The NMR data obtained for the single compounds are recorded in tables 1 and 2 along with the corresponding signals in the spectra of victorin C. The connectivities indicated in table 1 were derived for the latter by single irradiation experiments and/or by analysis of the corresponding homocorrelated 2 D ^{1}H-NMR COSY spectrum.

As determined by FAB-MS (glycerol matrix), the component with the highest Rf (0.61) had a mol.wt of 199 (2 Cl atoms), corresponding to the composition $C_6H_{11}O_2NCl_2$. From this and from the ^{1}H-NMR data (cf. table 1) it was concluded that the compound must represent one of the stereoisomers of 5,5-dichloroleucine (Cl_2-leu), **2**. In agreement with this assignment the compound was converted into leucine upon treatment with a Zn/Cu couple in H_2O:THF (1:5) at 70 °C for 3 h[15]. The same dichloroamino acid, or a stereoisomer thereof, has been detected previously as a component of the marine organism *Dysidea herbacea*[16].

The next component on the TLC plate, Rf 0.53, was present only in minor amounts and was identified from its mass spectrum (mol.wt 174, containing one Cl atom) and from the NMR data* as 5-chloro-4,5-bisdehydroleucine, **2a**. Signals corresponding to **2a** are not present in the NMR spectrum of victorin C and therefore the compound must have been formed during the hydrolysis through dehydrochlorination of **2**.

The third compound from the hydrolysis, Rf 0.43, was an amino acid $C_6H_{13}O_3N$, mol.wt 147. The NMR data (cf.



Carbon numbers as indicated are used for identification of NMR signals in the tables.

Table 1. ^{1}H-NMR data[a] of victorin C and of its hydrolysis compounds

	Carbon No.[b]	Free compound	Victorin C[c] Major	Minor
Glyox. acid (**1**)	2	5.30 (s)[d]	5.36 (s)	5.37
Cl_2-leu (**2**)	2	4.17 (dd, 10, 5)	4.57 (dd, 10, 4)	
	3	2.25 (*d*dd, *15*, 10, 5)	2.15 (m)	
		2.1 (*d*dd, *15*, 10, 5)	1.9 (m)	
	4	2.47 (m)	2.32 (m)	
	5	6.15 (d, 3)	6.10 (d, 3)	
	6	1.22 (3H, d, 7)	1.17 (3H, d, 6.6)	
OH-leu (**3**)	2	4.31 (d, 2.7)	5.1 (br)	5.0 (d, 10)
	3	3.56 (dd, 2.7, 9.7)	4.7 (dd, 9, 10)	?
	4	2.01 (d, hp, 9.7, 6.6)	2.2 (m)	2.0
	5	0.99 (3H, d, *6.6*)	1.09 (3H, d, 6.8)	0.93
	6	0.98 (3H, d, 6.6)	1.04 (3H, d, 6.8)	0.89
Victala (**4**)	2	4.14/4.17 (dd, 6.7, 6.0)	4.4 (d, 6)	4.1 (br)
	3	2.78 (*d*d, *15*, 6.7)	2.8 (2H, m)	?
		2.71 (*d*d, *15*, 5.8)		
	6	4.60 (dd, 6.5, 2.0)	4.33 (br)	4.37 (d, 7)
	7	2.90 (*d*d, *18*, 6.5)	3.38 (dd, 18, 7)	3.26
		2.34/2.35 (*d*d, *18*, 2.0)	2.75 (d, 18)	2.55
OH-lys (**5**)	2	4.08 (d, 4.1)	4.41 (d, 6)	
	3	4.24 (t, d, 9.5, 4.1)	4.0 (dd, 12, 6)	
	4	1.5–2 (4H, m)	1.60 (2H, m)	
	5		1.75 (m)	
			1.9 (m)	
	6	3.06 (2H, t, 7.3)	3.03 (2H, t, 7.6)	
aClaa (**6**)	3		7.52 (br)	7.66 (s)

[a] δ values relative to external DSS = 0 ppm; in parentheses: number of hydrogens (if more than one), multiplicities, J values in Hz (br = broad signal, m = multiplet). Number in *italics* refer to geminal J values between identified partners. [b] Connectivities as obtained from the ^{1}H-NMR of victorin C (cf. text) are indicated by square brackets linking the connected C-atoms. [c] As discussed in the text some of the atoms display dichotomous signals with different intensities; dichotomous signals for the major component are listed together with the non dichotomous ones under 'major'; additional signals due to the minor component are listed under 'minor'. [d] Present as the hydrate. [e] Ca 1:1 mixture of stereoisomers.

tables) suggested the presence of a β-hydroxyleucine (OH-leu) and comparison with authentic samples of racemic *erytho*-[17] and *threo*-β-hydroxy leucine[18] verified that the degradation product is the *erythro* isomer **3**.

The fourth compound from the hydrolysis, Rf 0.16, was obtained as a mixture of nonseparable stereoisomers, as judged by the doubling of some signals in the NMR spectra. Extensive FAB-MS experiments indicated a mol.wt of 201 and revealed the presence of five protons which were easily exchanged in D_2O. According to the ^{1}H-NMR spectrum, 6 additional protons are present in two independent $-CH_2-CHX-$ moieties (cf. table 1). The compound showed a marked UV absorption λ_{max} = 254 nm, ε = 9370 in 4 mN TFA, shifted to λ_{max} = 269 nm, ε = 14,800 in 10 mN NH_4OH) characteristic of an enolized β-dicarbonyl group and thus the degradation product retains a chromophore similar to that of the original toxin; this chromophore is also responsible for the strong absorption band at 1633 cm^{-1} in the IR spectrum (KBr) of the material. In addition, the ^{13}C-NMR spectrum of the compound (cf. table 2) proved the presence of 8 carbon atoms, 4 of which belong to the sp^2-type. From all this evidence the composition $C_8H_{11}O_5N$ and the unusual constitution **4** can be derived for the new compound, henceforth referred to as victalanin (victala).

Table 2. ^{13}C-NMR data[a] of victorin C and of its hydrolysis compounds

	Carbon No.		Victorin C[b] Major	Minor
Glyox. acid (**1**)	1	173.8 [c]	[d]	
	2	87.1 [c]	89.4 d	
Cl_2-leu (**2**)	1	173.7	[d]	
	2	52.6	53.8 d	
	3	34.3	36.0 t	
	4	41.4	42.7 d	
	5	79.4	81.3 d	
	6	15.9	16.8 q	
OH-leu (**3**)	1	172.0 [c]	175.8 s[f]	173.6[f]
	2	57.7 [c]	60.8 d	55.7
	3	78.9 [c]	86.0 d	81.2
	4	33.1 [c]	31.4 d	35.0
	5	21.3 [c]	19.4 q	21.1
	6	20.8 [c]	17.0 q	19.0
Victala (**4**)	1	174.3 [g]	d	
	2	54.8 [g]	51.6 d	55.7
	3	24.7 [g]	26.4 t	32.7
	4	113.3/113.4 [g]	116.5 s	117.5
	5	201.8/202.0 [g]	208.5 s	209.5
	6	70.9/ 71.0 [g]	72.7 d	72.6
	7	43.1 [g]	38.0 t	37.0
	8	197.9/198.3 [g]	189.1 s	
OH-lys (**5**)	1	172.5	d	
	2	59.9	61.0 d	
	3	70.8	72.5 d	
	4	25.8	25.8 t	
	5	32.4	32.6 t	
	6	41.5	42.0 t	
aClaa (**6**)	1		164.0 s	162.0
	2		134.2 s	133.5
	3		135.7 d	

[a] δ values relative to external TSP = 0 ppm; multiplicities are given for the 'major' victorin C signals as observed in the off-resonance or in the DEPT spectrum. [b] See footnote c in table 1. [c] Present as the hydrate. [d] Any of the following four singlets: 165.8, 172.7, 175.1 and 176.3. [e] Measured on the synthetic specimen. [f] Tentatively assigned on the basis of the observed dichotomy. [g] Ca 1:1 mixture of steroisomers.

This last ninhydrin positive compound had an even mol.wt of 162 (determined by CI-MS), later shown to correspond to $C_6H_{14}O_3N_2$. From the ^{1}H-NMR data (cf. table 1) it could be concluded that this compound is a caproic acid substituted with heteroatoms at C-2, C-3 and C-6. Heteronuclear decoupling experiments carried out on the intact toxin disclosed a correlation between the signal of the β-proton at 4.0 ppm and the ^{13}C signal at 72.5 ppm, thus locating an oxygen substituent at C-3. The structural issue was eventually settled in favor of *threo*-β-hydroxy-lysine (OH-lys), **5**, through direct comparison with synthetic specimens of the *threo*- and *erythro*-racemates[19].

Taking into account that each hydrolytic step in the degradation of the toxin requires the addition of one H_2O molecule it is possible to deduce the mol.wt of the residue which had escaped detection during hydrolysis from the apparent mol.wt of the toxin, 796, and from the structure of the degradation compounds; a value of 139 is obtained if all the fragments are arranged in a noncyclic fashion, whereas a value of 121 would result from a cyclic arrangement of the same fragments. The structure of the elusive fragment must include the hitherto unlocalized third Cl atom, an odd number of nitrogen atoms, as well as structural elements responsible for the NMR signals of victorin C not matched by corresponding signals in the isolated degradation fragments, namely a dichotomous proton signal at 7.52/7.66 ppm and three ^{13}C signals at 134.2/133.5 ppm (dichotomous, s), 135.7 ppm (d), and 164/162 ppm (dichotomous, s). These stringent requirements are met uniquely by the composition $C_3H_4O_2NCl$ (mol.wt 121) and its expansion into the structure of α-amino-β-chloro-acrylic acid (aClaa), **6**. The shoulder at 225 nm in the UV spectrum of the toxin is in keeping with the presence of a chromophoric group corresponding to **6**. In addition, it is clear that a compound with structure **6** would escape detection as a ninhydrin positive material because of further hydrolysis under the drastic conditions that led to its formation.

From the available evidence it can now be concluded that the apparent mol.wt of victorin C corresponds to the composition $C_{31}H_{43}O_{12}N_6Cl_3$ and that the toxin is assembled from the fragments **1**–**6** by a process which involves elimination of six molecules of H_2O and results in a cyclic array of the subunits**. An earlier claim[7] implying the presence of victoxinine among the constituents of the host-specific toxic factor from *C. victoriae* is not supported by our work and is most probably rooted in the impure nature of the original preparations.

The biosynthetic origin of the unusual amino acids **2**–**6** requires some comment. Compounds **3** and **5** are likely to stem from the oxidation of the corresponding proteinogenic amino acids. Formation of α-amino β-chloroacrylic acid, **6**, can be explained easily by invoking as as precursor dichloropyruvate, itself available through a mechanistically plausible biological chlorination of pyruvate. It is tempting to postulate that dichloropyruvate may also serve as a precursor of dichloroleucine, **2**, when channeled into the known pathway of leucine biosynthesis[20]. Finally, formation of victalanine, **4**, can be visualized starting from tyrosine through a process which involves oxydation, ring contraction and subsequent loss of one carbon atom via decarboxylation.

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Abbreviations used: FAB, fast atom bombardment; CI, chemical ionization; TFA, trifluoracetic acid; THF, tetrahydrofuran; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

* Signals at δ 6.15, 1H, s (C–6–H); δ 3.85, 1H, dd, $J_1 = 9$, $J_2 = 4.5$ (C–2–H; δ 2.75, 1H, dd, $J_1 = 15$, $J_2 = 4.5$ and δ 2.6, 1H, dd, $J_1 = 15$, $J_2 = 9$ (C–3–H_2); δ 1.89, 3H, s (C–5–H_3).

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1 To whom inquiries concerning this paper should be addressed.
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Studies on the fungal phytotoxin victorin: structures of three novel amino acids from the acid hydrolyzate

J.B. Gloer[1], J. Meinwald, J.D. Walton[2] and E.D. Earle

Departments of Chemistry and Plant Breeding and Biometry, Cornell University, Ithaca (New York 14853, USA), 2 September 1985

Summary. The host-selective phytotoxin victorin, produced by the fungus *Cochliobolus victoriae*, was found to be at least partially peptidic in nature, and did not contain victoxinine. The exact mass of the M-H ion was measured by FABMS as 795.1877. Derivatives of three major acid hydrolysis products were isolated. The structures of the corresponding amino acids were assigned as 2S,3R-3-hydroxyleucine, 5,5-dichloroleucine, and 3-hydroxylysine. A fourth hydrolysis product was also isolated and partially characterized. Tritiated leucine, but not ^{35}S, was incorporated into victorin by the fungus in vivo.
Key words. Cochliobolus victoriae; Helminthosporium; Bipolaris; Avena.

Fungi which produce toxins are responsible for many important crop diseases. Some of these toxins exhibit host-selectivity, that is, they affect only particular varieties or genotypes of a plant species. Such specificity suggests that structural knowledge of these toxins could lead to better understanding of the fundamental mechanisms of plant pathogenesis.

Victorin, also known as HV-toxin, was the first host-selective toxin discovered and is the most toxic and most selective of the twenty or so currently known[3,4]. Victorin is produced by the plant pathogenic fungus *Cochliobolus victoriae* Nelson (imperfect stage *Helminthosporium victoriae* Meehan and Murphy or *Bipolaris victoriae* Shoem.) which parasitizes certain cultivars of oat, *Avena*