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Molecular Features Affecting the Biological Activity of the Host-Selective Toxins from *Cochliobolus victoriae*[1]

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ABSTRACT

The structures of the toxins produced by *Cochliobolus victoriae*, victorin B, C, D, E, and victoricine, have recently been established. These toxins and modified forms of victorin C were tested for their effect on dark CO_2 fixation in susceptible oat (*Avena sativa*) leaf slices. Half-maximal inhibition of dark CO_2 fixation occurred with the native toxins in the range of 0.004 to 0.546 micromolar. An essential component for the inhibitory activity of victorin is the glyoxylic acid residue, particularly its hydrated aldehyde group. Removal of glyoxylic acid completely abolished the inhibitory activity of victorin, and the reduction of the aldehydo group transformed the toxin into a protectant. Conversion of victorin to its methyl ester resulted in diminution of inhibitory activity to 10% of the original activity of the toxin, whereas derivatization of the ϵ-amino group of the β-hydroxylysine moiety resulted in a decrease of inhibitory activity to 1% of that of victorin C. However, the derivatized toxin retained its host selectivity. In addition, the opening of the macrocyclic ring of the toxin drastically reduced the inhibitory activity.

The fungus *Cochliobolus victoriae* Nelson (*Helminthosporium victoriae* Meehan and Murphy) is the causal agent of victoria blight of oats. Victorin, the host-selective toxin produced by the fungus, is considered the primary determinant of pathogenicity because it selectively affects only those genotypes of oats that are susceptible to the fungus. Furthermore, the toxin appears to elicit the same biochemical response of the host as does infection with the pathogen (2, 7, 10, 15). An understanding of the structure-activity relationship of the toxin could, therefore, provide an experimental basis for studies of its mode of action and eventually should lead to an understanding of the molecular mechanisms of disease development.

Recently (5, 13, 14), we have isolated and determined the structure of five naturally occurring forms of the toxin designated victorin B, C, D, E, and victoricine, which are closely related, highly active peptides containing an unusual 12-membered ring. The predominant form in culture filtrates is victorin C, which accounts for 85 to 90% of the total activity and inhibits root growth of susceptible seedlings by 50% at 80 to 120 pg/ml (5). An additional toxin, victorin M, was isolated and its structure determined by Kono *et al. (4)*. The structural differences among naturally occurring multiple species of the toxins could provide, in a quantitative bioassay, an initial insight into the innate structural requirements for toxicity and selectivity. This naturally occurring diversity of structure combined with the potential to produce a number of chemical derivatives, alterations, and deletions of various functional groups on the molecule prompted us to investigate those features of the molecule that are essential for biological activity. Further, we wanted to determine the structural features of the compound that can be altered with little or no effect on the host selectivity. The information can be then utilized for the construction of labeled probes and the characterization of putative receptors.

MATERIALS AND METHODS

The production and purification of victorin C (**1**), victorins B, D, and E (**2–4**) and victoricine (**5**) (Table I) have been described previously (5). All toxin derivatives were purified by C_{18} reversed-phase column chromatography (Z module μBondapak, Waters Assoc.) The samples were chromatographed with a 2-h linear gradient of 0 to 20% acetonitrile in 10 mM aqueous KH_2PO_4 at a flow rate of 3 ml/min. Compounds were detected by their absorbance at 254 nm, and 6-ml fractions were collected. After chromatography, the samples were concentrated *in vacuo* and desalted with a disposable C_{18} cartridge (Sep-Pak; Waters Assoc.) The cartridge, prewashed with acetonitrile and equilibrated with water, was loaded with the sample and purged with water to remove the salts, and finally, the sample was eluted from the cartridge with 50% acetonitrile:water. After desalting, the samples were concentrated *in vacuo*. All derviatives displaying toxic activity were chromatographed at least three times to ensure complete removal of residual underivatized toxin.

The preparation of DC (**6**),[2] DSC (**7**), RC (**8**), and MEC (**9**) was described previously (14). Additional compounds were prepared as described below. In each case the structure of the toxin derivative was confirmed by NMR and/or fast atom bombardment-mass spectrometry.

Preparation of the Bolton-Hunter Analog of Victorin C. Four milligrams of sulfosuccinimidyl-3-(4 hydroxyphenyl)-propionate (so-called Bolton-Hunter reagent) (Pierce Chemical Co.) were added to a solution of 2 mg victorin C in 1 mL 0.1 M Mes buffer, pH 6.5. After incubation at room temperature for 4 h the toxin derivative, BHC (**10**), was purified by HPLC (MS: $M+H^+ = 945$, 3 Cl).

Preparation of the Biotinylated Analog of Victorin C. Four milligrams of *N*-hydroxysuccinimidobiotin (Pierce Chemical Co.) were dissolved in 0.1 mL of dimethylsulfoxide and subsequently added to 1.4 mL of 0.1 M Mes, pH 6.5, containing 4 mg

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[2] Abbreviations: DC, desglyovictorin C (**6**); DSC, desglyosecovictorin C (**7**); RC, reduced form of victorin C (**8**); MEC, methylated victorin C (**9**); BHC, Bolton-Hunter analog of victorin C (**10**); BIC, biotinylated analog of victorin C (**11**); SC, secovictorin C (**12**).

victorin C. After 2 h at room temperature, BIC (**11**) was purified by HPLC (MS: $M+H^+ = 1023$, 3 Cl).

Preparation of Secovictorin C. One-hundred-fifty milligrams of *N*-hydroxysuccinimide were added to a solution of 88 mg of glyoxylic acid in 4 mL of dioxane. Next, *ca.* 400 mg of dicyclohexylcarbodiimide were added, the solution was left at room temperature for 1 h and was then filtered, and the filtrate containing the succinimidyl ester (GSE) was used for further reactions (11). Approximately 1 mg of DSC, 7, was dissolved in 1.5 mL of 0.1 M potassium phosphate, pH 6.0. To this solution were added four successive 50-μL aliquots of the GSE solution at intervals of 10 min. The solution was then filtered through a glass fiber filter, and the product, SC (**12**), was purified by HPLC (MS: $M+H^+ = 815$, 3 Cl).

Plant material. Oat (*Avena sativa*) cultivars 'Park' and 'Rodney,' susceptible and resistant to *C. victoriae*, respectively, were planted at a density of *ca.* 100 seedlings in a 15-cm diameter pot and placed in a growth chamber. Plants were grown under a 16-h photoperiod at 23°C light and 20°C dark with 60% relative humidity.

Dark CO_2 Fixation Assay. Assays were performed essentially as described by Daly and Barna (1) for corn and Duvick *et al.* (3) for sugarcane. The oldest leaves from 8-d-old seedlings were used for all assays. The apical section (approximately 0.5 cm) and basal section (approximately 3 cm) were removed from the blades, and the remaining leaf segment, with a fairly uniform width of *ca.* 0.5 cm was used for sectioning. The leaf segments were sliced into 2 × 5 mm slices with an apparatus similar to that described by Rathnam (6), allowing slices to fall directly into distilled water. The leaf slices were blotted dry and 20 slices were placed in each 7 ml scintillation vial containing 0.475 ml of 20 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.0, + 2 % sucrose. Chl content, as measured by the method of Wintermans and DeMots (12), averaged 2 to 3 μg/slice.

The effect of test compounds on dark CO_2 fixation was determined by adding 25 μL of test solution to the vials. The vials were covered with an aluminum foil jacket and placed in racks inside a covered, circulating, 25°C water bath. After 4 h, 25 μL aliquots of 60 mM $NaHCO_3$ containing 10 μCi/ml ^{14}C $NaHCO_3$ were added to each vial. The vials were sealed with a serum stopper and incubated in the water bath for an additional 15-min period. After the labeling period, the stoppers were removed and the reaction was terminated by adding 0.25 mL of 1:2 (w/v) trichloroacetic acid:water. The vials were then left under a fume hood overnight to remove unreacted $^{14}CO_2$. The next morning 4.5 mL of scintillation fluid (Scinti Verse E, Fischer Scientific Co.) was added to each vial, and the samples were counted on a Beckman LS-100.

A standard assay involved seven concentrations of the test compound over a range of four orders of magnitude with three replicate vials per concentration. The concentrations of test materials were typically selected to give a response from 0 to 90% inhibition of dark CO_2 fixation. The concentration of compound affecting 50% inhibition of dark CO_2 fixation (EC_{50}) was interpolated from graphs of the percent inhibition plotted against the log of the concentration. The EC_{50} was selected for direct comparison of the activities of the various compounds because this was the midpoint of the most linear portion of the curve.

Leaf Bioassay. A simple and convenient method for directly assaying column eluants was developed. An aliquot, typically 50 μL, of each fraction diluted 1:10 with water was added to a 1.5 × 4.5 cm glass vial, and the cut end of an 8-cm apical segment of the primary leaf of a 7- to 10-d-old oat seedling was placed into this solution. After 24 h, the oat leaves were visually rated for symptom development. Oat leaves tolerated a 1:10 dilution of 25% acetonitrile + 0.1% trifluoroacetic acid without any obvious adverse effects; thus, the fractions from the analytical HPLC column could be assayed, after dilution, directly.

RESULTS

Derivatization of victorin C, **1**, under the conditions described resulted, in all cases, in one major product which was readily separated from underivatized toxin by HPLC. In all cases where a toxin derivative displayed biological activity, the compound was chromatographed a minimum of three times to ensure that there was no activity due to trace contamination by the unreacted, native toxin. Further confirmation of the biological activity of toxin derivatives was achieved by isocratic elution of the derivative on an analytical C_{18} column under conditions in which the derivative was clearly separated from native toxin as determined by absorption at 254 nm. Fractions were collected from the isocratic elution and assayed directly by the leaf assay method. In all cases, biological activity was associated only with the UV peak corresponding to the derivative (data not shown).

The results of a typical assay are illustrated in Figure 1. The logarithmic plot displays a nearly linear response from 0 to 90% inhibition of dark CO_2 fixation over a range of four orders of magnitude. Four-hour exposures to toxin were selected as a matter of convenience. It should be noted, however, that at concentrations of the toxin giving less than complete inhibition, an increase in the length of the exposure increased the sensitivity of the assay, *i.e.* the EC_{50} decreased. Consequently, all assays were performed identically with careful attention to the duration of toxin treatment.

The results of the assays are summarized in Table I. The predominant component of the naturally occurring toxin complex, victorin C, **1**, displayed an EC_{50} of 36.9 nM and was considered the standard with which all its derivatives and the minor toxins were compared. Toxin B, **2**, which differs from toxin C only by the absence of one chlorine atom in the *N*-terminal amino acid residue, displayed essentially identical biological activity.

Toxin D, **3**, lacks the free OH group on the 2-alanyl-3,5-dihydroxy-Δ^2-cyclopentenone-1 (victala) residue of toxin C, **1**. This structural difference appeared to enhance the biological activity approximately threefold, decreasing the EC_{50} from 36.9 nM of victorin C, **1**, to 10.8 nM for victorin D, **3**. In contrast,

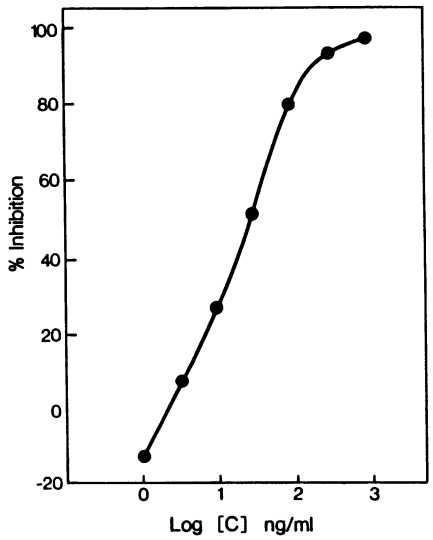
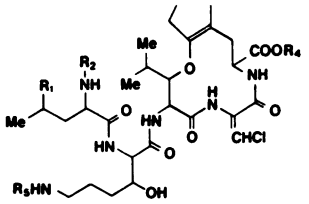
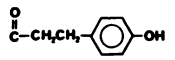
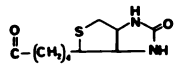


FIG. 1. Inhibition of dark CO_2 fixation in Park oat leaf slices as a result of exposure to varying concentrations of victorin C. Dark CO_2 fixation was measured for 15 min after a 4-h pretreatment with toxin at 25°C. Each point represents the average of three replicates.

Table I. *Biological Activity of Structural Variants of Host-Selective Toxins from Cochliobolus victoriae*

The general structure of the toxins and toxin derivatives is presented at the top of the table. Reference to the substituents presented to the right of the name of the compound, under columns R_1 to R_5 will complete the fine structure of the compound. The EC_{50} was derived from the log of the dry weight of the compound (ng/mL) which inhibited dark CO_2 fixation in oat leaf slices by 50% of controls after 4 h incubation at 25°C. The log EC_{50} was interpolated from a plot of log concentration versus % inhibition of dark CO_2 fixation in an experiment typically involving seven different concentrations with three replicates per concentration. Each experiment was repeated at least three times except where noted. The Ec_{50} value presented represents the average nanomolar concentration derived from three to five experiments (except where noted) followed by the standard error of the mean. In those cases where a compound displayed no toxic activity the EC_{50} was given a value of 0.



	R_1	R_2	R_3	R_4	R_5	EC_{50} nM ± SE
victorin C, 1	$CHCl_2$	CO-CH<(OH, OH)	OH	H	H	36.9 ± 8.34
desglyovictorin C, 6 (DC)	$CHCl_2$	H	OH	H	H	0
desglyosecovictorin C, 7 (DSC) (see Fig. 3)						0
reduced victorin C, 8 (RC)	$CHCl_2$	$CO-CH_2-OH$	OH	H	H	0 protective* (2940 ± 1140)
methylated victorin C, 9 (MEC)	$CHCl_2$	CO-CH<(OH, OH)	OH	CH_3	H	283 ± 45.2
Bolton-Hunter victorin C, 10 (BHC)	$CHCl_2$	CO-CH<(OH, OH)	OH	H		3890 ± 329
biotinylated victorin C, 11 (BIC)	$CHCl_2$	CO-CH<(OH, OH)	OH	H		2820 ± 452
secovictorin C, 12 (SC) (see Fig. 3)						16,000**
victorin B, 2	CH_2Cl	CO-CH<(OH, OH)	OH	H	H	43.9 ± 14.1
victorin D, 3	$CHCl_2$	CO-CH<(OH, OH)	H	H	H	10.8 ± 2.36
victorin E, 4	CCl_3	CO-CH<(OH, OH)	OH	H	H	4.2**
victoricine, 5 (see Fig. 2)						546 8.8

* This compound was found to prevent or reduce the effect of victorin C when applied 0.5 h previous to the introduction of victorin C. The numbers in parenthesis represent the dose of RC necessary for 50% protection against 100 ng/ml of victorin C. ** Because of limited quantities of this compound the EC_{50} presented is interpolated from the values derived from one experiment.

victoricine, **5**, which has a six-membered dihydroxylated aromatic ring in the fifth amino acid residue and lacks the two chlorine atoms in the first amino acid residue (Fig. 2), shows a marked reduction in biological activity. This toxin displayed an EC_{50} of 546 nM. MEC, **9**, showed a *ca.* 10-fold decrease in activity compared to the activity of the native toxin. The EC_{50} of MEC, **9**, was 283 nM compared to 36.9 nM for victorin C, **1**. Although the activity of MEC, **9**, was reduced to 10% of that of the native toxin, the derivative retained its host-selective activity; that is, it was toxic only to the toxin-sensitive genotype of oats and had no effect on the resistant variety.

Formation of either the biotin derivative, **11**, or the Bolton-Hunter (4-hydroxyphenyl propionate) derivative, **10**, involving the ϵ-amino group of the β-hydroxylysine residue of victorin C, **1**, resulted in approximately a 100-fold decrease in the activity compared to the native toxin. These compounds displayed EC_{50} values of 2.82 μM and 3.89 μM, respectively. However, both of these derivatives still retained host-selective activity.

In contrast to the relatively minor effects of alteration of the free carboxylic acid function or the ϵ-amino group of the victorin C, **1**, alterations of the hydrated aldehydic group of the glyoxylic acid residue had a dramatic effect on the activity of the molecule. Removal of the glyoxylate residue, as in the DC, **6**, completely abolished toxic activity. In accord with expectations, the acylation of DC, **6**, with glyoxylic acid, which resulted in the reformation of victorin C, **1**, completely restored the toxic activity (data not shown). Furthermore, reduction of the glyoxylic acid moiety of victorin C, **1**, to a glycolic acid residue (Table I) also

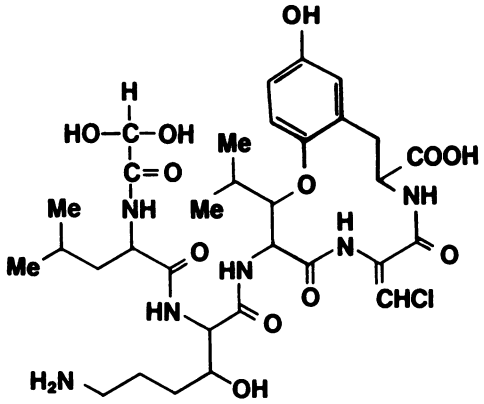
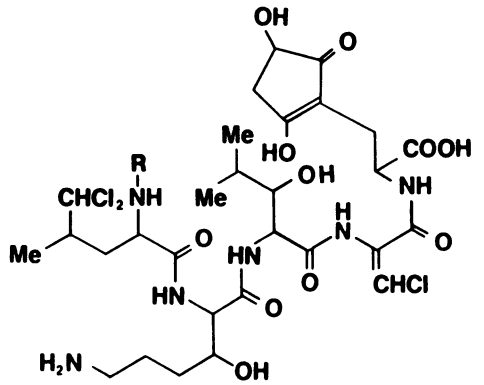


FIG. 2. The structure of victoricine. This compound is identical to victorin C with the exception of a six-membered aromatic ring in the C-terminal amino acid residue and the absence of Cl_2 on the *N*-terminal amino acid residue.



12 secovictorin C (R=CO-CH(OH)(OH))

7 desglyosecovictorin C (R=H)

FIG. 3. Structure of secovictorin C (**12**), the linearized form of victorin C. In this compound the macrocyclic ring formed by a linkage between residues three and five in victorin C has been hydrolyzed, resulting in a compound identical to victorin C except that it lacks the macrocyclic ring. Structure of desglyosecovictorin C (**7**). This compound is identical to secovictorin C except that it lacks the glyoxyl moiety.

abolished the toxic activity. However, RC, **8**, retained biological activity. Pretreatment of leaf slices with RC, 8, prevented or reduced the effects of subsequent additions of native, biologically active victorin C, **1**. Pretreatment of leaf slices with approximately a 24:1 molar excess of RC:C resulted in a 50% reduction of the activity of the toxin. DC, **6**, was far less effective as a protectant than RC, **8**. The protection afforded by varying concentrations of DC, **6**, against 100 ng/mL of victorin C, **1**, did not display a linear dose-response, and the extrapolated concentration required for 50% protection was far beyond the solubility of the compound.

The contribution of the 12-membered ring to the biological activity of the toxin was assessed by assays of SC, **12**. The corresponding DSC, **7**, displayed no biological activity either as a toxin or a protectant as expected for a compound lacking the biologically essential glyoxylic acid residue. Reintroduction of this moiety produced SC, **12**, (Fig. 3), a compound differing from the native toxin only in the linear rather than the cyclic arrangement of the last three subunits. Assays of SC, **12**, showed that the compound had the lowest specific activity of all the active forms or derivatives of the toxin with an EC_{50} of 16.0 μM.

DISCUSSION

The naturally occurring diversity in the structures of victorins B, C, D, E, and victoricine, coupled with the fact that they all display host-selective activity, indicates that certain structural features of the toxins are not essential for biological activity.

Comparison of **1**, **2**, and **3** shows that loss of a chlorine atom from the *N*-terminal amino acid has no effect on activity and removal of the free hydroxyl group from the C-terminal amino acid (victorin D, **3**) apparently enhances the activity of the molecule. Victorin E, **4**, was not obtained in sufficient quantity for repetitive quantitative assays. However, a single experiment showed that it was at least as active as victorin C, **1**, from which it differs by an additional chlorine atom in the N-terminal amino acid. Introduction of a six-membered aromatic ring in the C-terminal amino acid residue and removal of two Cl atoms from the *N*-terminal amino acid residue as in victoricine, **5**, reduces the activity of the molecule without affecting its selectivity. Because the biological activity seems to be insensitive to the number of Cl atoms in the *N*-terminal leucine unit, as judged by comparison of **1** with **2** and **4**, the observed reduction of biological activity in victoricine, **5**, is likely to be caused largely, if not exclusively, by the change in the C-terminal residue. In fact, comparison of the activities of **1**, **3**, and **5** suggest that the nature and the substitution pattern on the 12-membered macrocyclic ring is important in the association of the toxin with the host's active-site. This suggestion is further supported by the observation that MEC, **9**, in which the carboxyl group of the C terminus has been modified also displays a reduced activity while retaining the selective toxicity.

A further indication of the importance of the macrocyclic ring for toxic activity come from the experiments with SC, **12**. This compound is the linear form of victorin C, **1**, formed by opening the ether linkage between the third and fifth amino acid residues, and it displays the lowest specific activity of all the toxic compounds tested. The low residual activity detected for SC, **12**, suggests that this compound can, to some extent, approach the conformation of the closed ring form which seems necessary for association with the active site in the host.

The latent aldehyde group of the hydrated glyoxylic acid residue proved to be essential for activity. Removal of the glyoxylic acid residue as in DC, **6**, resulted in a complete loss of all toxic activity. The conversion of the glyoxylic to a glycolic acid residue (conversion of **1** to **8**) resulted in the loss of toxic activity; however, RC, **8**, retained considerable biological activity as a protectant that is able to prevent the effect of subsequent additions of toxin and, therefore, retains its ability to associate with the active site in the host. Thus, the glyoxylic acid residue appears important in two respects: the masked aldehydic function of the native toxin C, **1**, (and quite probably of the other toxins) is essential for toxic activity and, moreover, the glyoxylate residue is important for the association of the toxin with its active site involving an interaction that can be mimicked by the glycolate residue as in **8**. It is tempting to speculate that the aldehyde group may mediate a covalent binding of the toxin to its site of action in the host as many aldehydes spontaneously bind to protein (*cf.* Ref. 8). In this connection, the very low relative toxicity reported by Kono *et al.* (4) for toxin M, a victorin C cognate containing a glycine rather than a glyoxylic acid residue is best viewed as the result of a slow transamination which converts toxin M into victorin C.

Although acylation of the ϵ-amino group of the β-hydroxylysine residue (BIC, **11**; BHC, **10**) did cause marked reductions in the activity of the toxin, the fact that these derivatives still retained selective toxic activity implies that these modifications do not prevent the association of the toxin with its active site but merely reduce the efficiency of the association, *e.g.* by uptake, transport, or binding. This demonstrates that the free ϵ-amino

group of the toxin is not essential to activity and, further, allows for the simple production of derivatives of the toxin potentially useful in probing for the site-of-action in the host. For example, the BHC, **10**, derivative can be radioactively labeled to a high specific activity with ^{125}I which, in turn, may allow for the toxin-mediated radiolabeling of the host receptor. BIC, **11**, may facilitate the introduction of a biotin moiety to the toxin receptor through the association of the toxin with its site of action. This, in turn, may facilitate the purification of the receptor by the use of an avidin affinity column (9). This potential for the production of active derivatives of the toxins, combined with an increased understanding of the structural features that are essential for biological activity, should contribute substantially to future efforts toward an understanding of the site and mode-of-action of the toxins. Ultimately, efforts in this area may lead to a description of the molecular events of the host-parasite interaction involved in victoria blight of oats.

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