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DESMETHOXYVIRIDIOL, A NEW TOXIN FROM *NODULISPORIUM HINNULEUM*

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Abstract—A new mycotoxin obtained from culture extracts of *Nodulisporium hinnuleum* was determined to be desmethoxyviridiol ($C_{19}H_{16}O_5$). The toxin had an oral median lethal dose of 4·2 mg/kg in 1-day-old cockerels. Desmethoxyviridiol also produced plant-growth regulating and phytotoxic effects in plant systems. The physical and chemical characteristics of desmethoxyviridiol are described.

INTRODUCTION

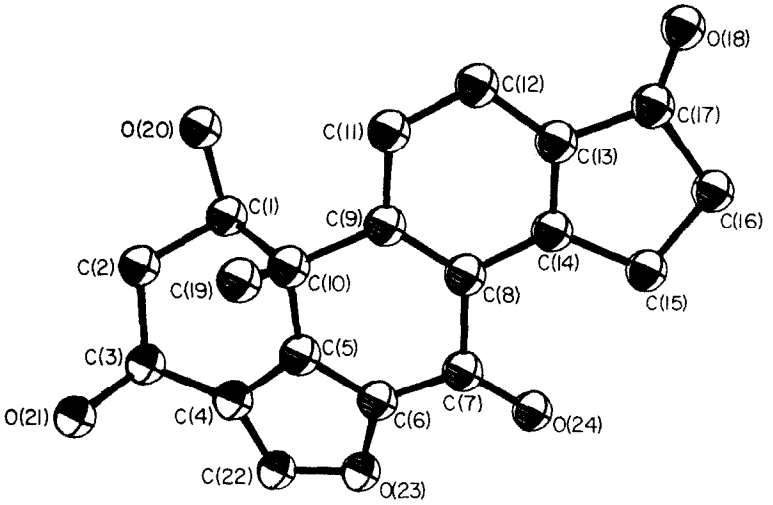
Since the discovery of the aflatoxins in 1960 an awareness has developed concerning potential contamination of feed and food supplies by toxic fungal metabolites. In order to accurately assess the extent of this threat to animal health, considerable effort is still needed to determine which toxigenic fungi are commonly found contaminating agricultural commodities and to identify their toxic metabolites.

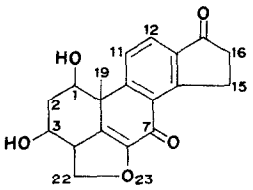
A toxigenic mold isolated from peanut seed produced in Virginia during the 1972 growing season was identified as *Nodulisporium hinnuleum* Smith (ATCC 24911). We wish to report the biological effects of the major toxic metabolite produced by this mold in both animal and plant systems, the physical and chemical characteristics of the toxin, and the chemical structure of this metabolite as elaborated by its physical and chemical characteristics and by single crystal X-ray diffraction studies.

RESULTS AND DISCUSSION

Crystalline toxin (mp 155–157°C) appeared on TLC at R_f 0·35 as a blue spot after sulfuric acid spray treatment [1]. It was soluble in Et_2O, $ChCl_3$. EtOAc, Me_2O; moderately soluble in toluene, MeOH and EtOH; insoluble in hexane and H_2O.

Results of a single crystal X-ray diffraction experiment showed the crystals were monoclinic with a = 7·295(9), b = 15·19(3), c = 7·90(1) Å and β = 121·12(3). The systematic extinction on OkO (absent if $k = 2n + 1$) combined with the known optical activity uniquely indicated space group $P2_1$. A calculated and observed density of $\sim 1{\cdot}44$ g/cm^3 combined with high resolution mass spectral data indicated that the asymmetric unit





Desmethoxyviridiol (1)

Fig. 1. Computer generated perspective drawing of the non-hydrogen atoms of desmethoxyviridiol.

was $C_{19}H_{16}O_5$. A perspective drawing of the X-ray model is given in Fig. 1. This X-ray determination defines only the relative stereochemistry. The absolute stereochemistry is inferred from the close relationship to viridiol and viridin, and hence the steroids [2]. In view of this, the trivial name desmethoxy-viridiol is proposed.

UV analysis showed λ_{max}^{EtOH} 251 (ϵ_{max} $3{\cdot}0 \times 10^4$) and 322 nm (ϵ_{max} $1{\cdot}6 \times 10^4$). The UV spectrum was similar to the spectrum of viridiol (λ_{max} 250 and 317 nm). The IR spectrum showed γ_{max} 3390 (OH), 1670 (C = 0), 825 (2 adjacent aromatic H's) and 760 cm^{-1}. The HRP mass spectrum showed a molecular ion peak at *m/e* 324·0994 (M*) with a computer-calculated molecular formula of $C_{19}H_{16}O_5$. The mass spectrum showed major fragment ions at *m/e* 306, *m/e* 280 and *m/e* 265. The LRP chemical-ionization mass spectrum showed a protonated molecular ion peak at nominal mass *m/e* 325 with fragment ions only at *m/e* 307 and *m/e* 281.

The NMR spectrum of toxin showed the following features which were consistent with the structure (Fig. 1) determined by X-ray crystallography. A chemical shift for a one-proton singlet at δ 7·77 was observed for the olefinic proton located in the furan ring at C(22) (I). Chemical shifts for a strongly coupled AB system located at C(11) and C(12) of the aromatic system resonated as two one-proton doublets at δ 7·81 and δ 8·61 (J 17 Hz). A coupling constant of 17 Hz was consistent with that expected for *ortho* substituted protons. The indanone resonances were an A_2B_2 system [protons on C(16) and C(15)] consisting of a pair of complex two-proton chemical shifts at δ 3·65 (multiplet) and δ 2·65 (multiplet), respectively. A signal for the tertiary methyl group resonating at δ 1·59 as a 3-proton singlet was assigned to the methyl group at C(10). The methylene protons at C(2) appeared as an unstructured multiplet at $\simeq\delta$ 2·55, superimposed on the methylene protons located on C(15). The methine protons on C(1) and C(3) resonated at δ 4·07 and δ 4·82 as an ill-defined triplet and a

multiplet, respectively. Chemical shifts for the two OH groups positioned at C(1) and C(3) were not clearly evident presumably due to exchange and/or coupling. The NMR spectrum of the diacetate derivative of desmethoxyviridiol was very similar and showed chemical shifts for two additional 3-proton singlets at δ 2·04 and δ 2·19 (Me–C=O).

The oral LD_{50} of toxin to day-old cockerels was 4·2 mg/Kg or 171 μg/cockerel. In the majority of instances, the first signs of toxicity occurred within the 1st hr after dosing. During this time cockerels became dull, listless and weak with beginning signs of ataxia. Following was progressive incoordination until complete prostration occurred. In all cases coma followed by convulsions preceded death.

The onset of signs and lethal effects depended on dosage level. At a dosage level of 300 μg/cockerel clinical signs and death occurred within two hours after administration of toxin. At a dosage level of 600 μg/cockerel onset of signs and death occurred within one hour after dosing.

Wheat coleoptiles were significantly ($P < 0{\cdot}01$) inhibited at 10^{-3}; 10^{-4}; 10^{-5}; and 10^{-6} M and, respectively, showed 100; 100; 96; 58% inhibition relative to controls. Oat 1st internodes were plasmolyzed at 10^{-3} M and were significantly ($P < 0{\cdot}01$) inhibited at 10^{-4}; 10^{-5}; 10^{-6}; and 10^{-7} M, that is, 100; 100; 50 and 40%, respectively, relative to controls.

Within 48 hr after treatment corn plants had necrotic lesions and many leaves had the appearance of soft rot. This was followed, in several cases, by stem collapse. Subsequently new growth appeared, but after 2 weeks it was evident that the growth of the new shoots was inhibited at 10^{-2} and 10^{-3} M; there was also some stunting at 10^{-4} M. These effects lasted several weeks. Effects in bean plants were almost negligible. Slight malformations were produced only in the first trifoliates at 10^{-2} and 10^{-3} M and there was slight overall stunting in bean plants at 10^{-2} M relative to controls. In tobacco plants there was no stunting at any time during the experiment and only one leaf exhibited slight malformation.

From the data obtained in experiments with intact plants, it appeared that the toxin selectively inhibited the growth of monocotyledons, yet had relatively little or no effect on dicotyledons. These observations may warrant more research on this compound with a view to developing a selective herbicide for grasses.

It is becoming increasingly more evident that fungal metabolites isolated as animal toxins (mycotoxins) are also capable of potent plant-growth regulating effects [3–6]. These compounds or their derivatives may provide a potentially rich source of agrichemicals. In view of the fact that they are of biological origin and presumably biodegradable, harmful residues may not be the problem they have been in the past with synthetic pesticides.

EXPERIMENTAL

Production and purification of toxin. The toxin was produced by fungal cultures grown statically in liquid medium for 14 days at 27° and subsequently extracted with hot $CHCl_3$. The $CHCl_3$ extracts from 3·6 l. of culture medium were evaporated to dryness and the residue applied to a silica gel column (5·0 × 45 cm) packed in a slurry of hexane. The column was eluted sequentially with 1·5 l. each of hexane, Et_2O, $CHCl_3$, EtOAc, Me_2CO and MeOH. Gross fractions of each solvent were collected and the biological activity, as measured by acute toxicity to orally dosed 1-day-old cockerels, was found exclusively in the EtOAc fraction. This fraction was further purified on a second Si gel column (3·5 × 45 cm) eluted with a linear gradient from $CHCl_2$ to EtOAc (20 column vol.). Toxicity was associated with fractions 130–170 and 250–330 suggesting the presence of two separate toxins. Both series of fractions were separately combined and evaporated to dryness. Non-polar residue (tubes 130–170) was not sufficiently pure to crystallize. The polar residue (tubes 250–330) was crystallized at room temp from EtOAc. This publication will be concerned only with the chemical and biological characterization of the latter fractions.

Physical and chemical analyses. The toxin was analyzed by TLC using 20 × 20 cm glass plates coated with silica gel GH-R (0·50 μm thickness), using toluene-EtOAc-HCO_2H (5:4:1). The toxin was visualized by spraying TLC plates with 50% ethanolic H_2SO_4 and heating for 5 min at 100°.

Mp's were determined on a Kofler apparatus and were uncorrected. UV spectra were taken in MeOH. The UV spectrophotometer was calibrated with a holmium oxide standard. IR spectra were taken as thin films coated onto KBr windows.

HRP mass spectral analyses were made with an A.E.I. MS-9 mass spectrometer. Samples were introduced into the ion-source by the direct-probe method and ionization was effected by electron-impact at 70 eV. The ion-source temperature was maintained at 200° and high-resolution measurements were made by peak matching using perfluorokerosene as the internal standard.

NMR spectra were obtained at a frequency of 60 MHz. Spectra of the toxin were taken in $CDCl_3$ and $(CD_3)_2CO$ using TMS as internal standard.

X-ray diffraction analyses. Crystals of the toxin were submitted to a single crystal X-ray diffraction experiment. A complete set of 1166 independent diffraction maxima with $\theta \leqslant 60°$ for Ni-filtered CuKα radiation was collected on a computer controlled 4-circle diffractometer. After correction for Lorentz, background and decay effects 1041 were judged observed ($I_0 - \leqslant 3\sigma[I_0]$). These intensities were converted to normalized

structure factors and the structure routinely solved by a weighted, iterative tangent formula approach [7]. Anisotropic full matrix least-squares refinements have presently converged to a normal crystallographic discrepancy index of 0·107.

The following library of crystallographic programs was used: C. R. Hubbard, C. O. Quicksall and R. A. Jacobson, "The Fast Fourier Algorithm and the Programs ALFF, ALFFDP, ALFFPROJ. ALFFT and FRIEDL," USAEC Report IS-2625, Iowa State University-Institute for Atomic Research, Ames, Iowa, 1971; W. R. Busing, K. O. Martin and H. A. Levy, "ORFLS, A Fortran Crystallographic Least Squares Program," USAEC Report ORNL-TM-305, Oak Ridge National Laboratory, Oak Ridge, Tenn., 1965; C. K. Johnson "ORTEP, A Fortran Thermal-Ellipsoid Plot Program," USAEC Report ORNL-3794, Oak Ridge National Laboratory, Oak Ridge, Tenn., 1965.

Animal and plant bioassay. Preparations for animal bioassay were administered in corn oil suspension [8]. Samples were dosed orally via crop intubation into 1-day-old Dekalb cockerels at a vol. of 1 cm^3/cockerel. Dosage levels used for LD_{50} determination were 100 μg (2·5 mg/Kg), 200 μg (5·0 mg/Kg) and 300 μg/cockerel (7·5 mg/Kg) with a total of 40 replications of each dosage level.

Solutions of the toxin for plant bioassay were formulated [9] at concentrations ranging from 10^{-3} to 10^{-7} M. These were bioassayed using coleoptiles from 4-day-old etiolated [10] wheat seedlings (*Triticum aestivum* L., "Wakeland") and 1st internodes from 5-day-old etiolated [11] oat plants (*Avena sativa* L., Mo-0205). Ten 4 mm sections of either wheat coleoptiles, or oat 1st internodes, were placed in test-tubes containing 2 ml test solns. These were incubated for 24 hr at 21° in a roller tube apparatus. Greenhouse tests were made with toxin solns on corn (*Zea mays* L., "Norfolk Market White"), bean (*Phaseolus vulgaris* L., "Black Valentine") and tobacco seedlings (*Nicotiana tobacum* L., "Hicks"), in triplicate expts. Solutions were formulated as previously described [9] at concentrations from 10^{-2} to 10^{-4} M. Week-old corn seedlings received 0·1 ml per plant in the leaf Whorl (actual amounts per plant; $10^{-2} = 320\,\mu g$; $10^{-3} = 32\,\mu g$; $10^{-4} = 3{\cdot}2\,\mu g$). Ten-day-old bean seedlings received 1 ml of spray soln per pot (that is, $10^{-2} = 3200\,\mu g$, $10^{-3} = 320\,\mu g$, $10^{-4} = 32\,\mu g$) and there were 3 plants per pot. One ml of each soln was sprayed onto individual 6-week-old tobacco plants.

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