SPECIALIA

Characterization of the host-specific toxins produced by *Helminthosporium sacchari*, the causal organism of eyespot disease of sugarcane[1]

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Summary. Investigation of the host-specific toxin complex from *H. sacchari* has led to the isolation of 3 isomeric glycosidic components $C_{39}H_{64}O_{22}$, each active at 2×10^{-11} moles. The 3 isomers consist of 4 galactose units linked to an aglycone residue $C_{15}H_{24}O_2$.

Several species of plant pathogenic fungi release toxins which selectively damage susceptible plants and produce all or most of the symptoms of the disease. Selective pathotoxins or host specific toxins (HST) can be used in the place of living pathogens as disease inducing agents to study the sequence of events in pathogenesis and the molecular basis of plant disease resistance and susceptibility[4].

For almost three decades many studies of HST have been conducted with impure preparations of unknown composition. Investigations into the mode of action of these toxins have been hampered and interpretation of existing data has been difficult[5]. Previous reports on the HST from *H. sacchari*, the causal organisms of eyespot disease of sugarcane, have described conflicting results. An early claim[6] that the toxin is identical with 2-hydroxycyclopropyl-α-galactopyranoside has not been substantiated in further work. Livingston and Scheffer have suggested that there is only one active form of the toxin (tentatively assigned a mol.wt of 1028), composed of 5 galactose units and a triply unsaturated $C_{15}H_{21}$ moiety[7,8]. Beier, in turn, has proposed as a working hypothesis that the toxin contains 2 galactose units and an aglycone $C_{15}H_{22}O_2$[9]. Our own investigation of this problem has now led to results which differ in many details from all previous proposals.

Helminthosporium sacchari was obtained from the American Type Culture Collection, as ATCC No. 2468, and was maintained on sugarcane leaf agar. For comparative studies, a strain of *H. sacchari* was provided by Dr G.A. Strobel. For toxin production, the fungus was grown for 21–23 days in still culture in 500-ml Erlenmeyer flasks containing 100 ml of modified liquid Fries' medium[10] supplemented with 0.1% yeast extract. The culture filtrate and fractions from subsequent purification steps were tested under standard assay conditions[11]. For this test a susceptible clone of sugarcane, Co 453, and a resistant one, CP76-1343, were obtained from Dr J.L. Dean, USDA, ARS, Canal Point, Florida and maintained in the greenhouse.

The culture filtrate was passed through a column of Ambersorb XE 348 (Rohm and Haas Co.). All the toxin activity was retained on this column and then eluted with a linear gradient of water to acetone. Fractions with toxin activity were pooled and chromatographed on a column of silica gel 60 (E. Merck, particle size 40–63 μm) with 80% acetonitrile in water or with chloroform-methanol-water 60:39:1. Efficient enrichment of the toxin activity was obtained by droplet countercurrent chromatography (DCC)[12] in the descending mode with the 2 phases of a n-butanol-acetone-water 33:10:50 mixture at a flow rate of 0.1 ml/min. Final purification was achieved by high performance liquid chromatography on a column of μ-Bondapak C_{18} (Waters Assoc.) 7.8 mm × 30 cm with 22% acetonitrile in water at a flow rate of 2 ml/min. Toxin activity in the effluent was associated with 3 symmetrical peaks (I, II, and III) at 10, 14, and 18 min, respectively, detected by a differential refractometer. As determined from peak areas and bioassays, toxins I, II and III comprised more than 99% of the total activity. Consequently, further work was restricted to these 3 fractions. When tested in a leaf puncture bioassay, the 3 isomers showed the same specific activity, producing symptoms on sugarcane at minimum levels of 2×10^{-11} moles for the susceptible clone Co 453 and 2×10^{-7} moles for the resistant CP 76-1343.

After DCC, our toxin preparation was indistinguishable from a reference preparation provided by Dr G.A. Strobel when chromatographed on silica gel 60 TLC plates with a number of solvent systems and when bioassayed.

Field desorption mass spectrometry (FDMS) of the 3 separated toxins gave in each case a cationized pseudomolecular ion of 907 $(M+Na)^+$, suggesting that each isomer had a mol.wt of 884 daltons. Another diagnostic ion in FDMS was 745, corresponding to a loss of a monosaccharide unit (162) from the pseudomolecular ion[13].

Mass spectral analysis of the *H. sacchari* toxin isomer II, by negative chemical ionization with CF_2Cl_2 (Freon 12) as reagent gas[a,b]

Peaks (m/e)	Relative intensity (%)[c]	Proposed origin of fragment
739	1.6	$M(884)-C_6H_{12}O_6+Cl^-$
722	8.3	$M(884)-C_6H_{10}O_5$
595	75	$M(884)-2\times C_6H_{10}O_5+Cl^-$
559	91	$M(884)-2\times C_6H_{12}O_6+Cl^-$
433	16	$595-C_6H_{10}O_5$
359	100	$595-C_{15}H_{24}O_2$
197	83	$433-C_{15}H_{24}O_2$

[a] The ions corresponding to ^{37}Cl have been omitted for the sake of clarity. [b] Mass spectra of isomers I and III showed the same peaks as isomer II but with different relative intensities. [c] Relative intensities varied considerably during the run and with individual isomers, but typically, as shown above, they greatly diminished with increasing m/e. Thermal degradation of the sample occurred even under the conditions used, resulting in a background in the low mass region. Consequently, intensities are recorded relative to the most intense peak (m/e 359) above mass 150.

Negative chemical ionization (NCI) mass spectral analysis with CF_2Cl_2 as a reagent gas[14-16] provided additional details about the structure of the toxin. The NCl mass spectra of toxins I, II and III (table) were almost identical except for relative intensities of the peaks. Fragment-Cl-adduct ions in the spectrum corresponded to a sequential loss of 4 monosaccharide units from the molecular ion. The loss of 236 mass units from the ion at m/e 595 was attributed to the elimination of an aglycone moiety. Except for the shift in mass due to the aglycone, the fragmentation pattern of the toxin was similar to that obtained for di- and trisaccharides analyzed under similar conditions; in every case Cl-adducts of fragments corresponded to losses of monosaccharides or monosaccharides minus water.

Analysis of the hydrolysis products of the toxin (0.05 M trifluoroacetic acid at 90 °C for 1 h) indicated the presence of only 1 monosaccharide that cochromatographed in GLC (after silylation) and TLC with galactose. The identity of the aglycone(s) is not known, but the electron impact (EI) mass spectrometry provided some clues. In the EI mode, the mass spectra of the 3 isomeric toxins showed an ion at m/e 218 with further ions in the lower mass region. The ion at m/e 218 apparently originated from the aglycone moiety, 236, with a loss of 18 mass units (H_2O). From high resolution mass measurement of the ion at m/e 218 of toxin isomer II an empirical formula $C_{15}H_{24}O_2$ for the aglycone moiety was calculated.

From these data it can be concluded that the 3 toxins have the composition $C_{39}H_{64}O_{22}$, corresponding to a 4-fold galactosidation of a $C_{15}H_{24}O_2$ aglycone. Extended ^1H- and ^{13}C-NMR investigations, to be reported in detail elsewhere, confirm this conclusion and reveal in addition that the 3 isomers differ only in the position of 1 double bond in the aglycone component.

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Polyacetylenes from the sponge *Petrosia ficiformis* found in dark caves[1]

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Summary. Several high molecular weight polyacetylenes have been isolated from the sponge *Petrosia ficiformis* found in dark caves. These compounds are related to, but different from, the polyacetylenes isolated from the same sponge living in its usual habitat.

Usually the mediterranean sponge *Petrosia ficiformis* displays a red-brown colour due to the presence of the symbiotic alga *Aphanocapsa feldmanni*[3]; however *P.ficiformis* found in dark caves lacks this symbiotic alga and therefore appears white. In the course of a study[4] on the secondary metabolites of the sponge *P.ficiformis* (red-brown) and of its predator, the nudibranch *Peltodoris atromaculata*, we have recently isolated[5] the high molecular weight polyacetylene mixtures **1** and **2** from both invertebrates.

We wish to report now the isolation of related compounds from *P.ficiformis* (white) which also provokes a positive food response by the nudibranch[4].

The ether-soluble fraction from the acetone extracts of the sponge was separated into 2 main fractions containing mixtures of acetylenic compounds, by chromatography on silica gel. The less polar mixture was further divided into 2 fractions (**a** and **b**) by preparative HPLC (μBondapak C_{18}; $CH_3OH \cdot H_2O$, 9:1).

Fraction a (0.033% dry weight of the sponge). Inspection of

$$HC{\equiv}C{-}\underset{\displaystyle OH}{\underset{|}{CH}}{-}CH{=}CH{-}CH_2{-}R^1{-}CH_2{-}CH{=}CH{-}C{\equiv}C{-}\underset{\displaystyle OH}{\underset{|}{CH}}{-}C{\equiv}C{-}CH_2{-}R^2{-}CH_2{-}CH{=}CH{-}\underset{\displaystyle OH}{\underset{|}{CH}}{-}C{\equiv}CH$$

1, $R^1+R^2=C_nH_{2n-6}$; n = 25, 28 **2**, $R^1+R^2=C_nH_{2n-4}$; n = 28, 31, 34