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Communication

Characterization of the Host-Specific Pathotoxin Produced by *Helminthosporium maydis*, Race T, Affecting Corn with Texas Male Sterile Cytoplasm[1]

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The fungus *Helminthosporium maydis*, race T, causal agent of a damaging epidemic of corn blight in 1970–1971, produces a toxin that affects corn carrying Texas male sterile (TMS) cytoplasm but not corn with normal (N) cytoplasm. Crude toxin preparations affect a variety of biochemical and physical processes in susceptible (TMS) corn, including mitochondrial oxidation (*1*), ion leakage (*2*), photosynthesis (*3*), and dark CO_2 fixation (*3*). Because crude preparations limit studies of the primary site of action, we developed procedures for isolation of pure toxin.

Culture filtrates (10 liters) and 70% acetone homogenates of mycelium (approximately 1000 g) were treated with Norit A (3% w/v), and toxin material was desorbed with chloroform. After flash evaporation of solvent at 45°C, the residual red oil was redissolved in a minimum volume of chloroform. A slightly pigmented precipitate, formed after refrigeration overnight, was reprecipitated from warm methanol or acetone. Yields ranged from 50 to 300 mg. The colorless toxin (C: 63.84, H: 9.18, O: 26.68) had a sharp melting point (125–126°C) and exhibited the requisite biological specificity for TMS corn. Only 4 to 8 ng were required to cause complete killing of an entire first true leaf of TMS corn, while 20 μg was taken up by N corn leaves without visible effect.

Positive tests for carbonyl (2:4-dinitrohydrazine), hydroxyl (triphenyltetrazolium chloride at 100°C), and active methylene (sodium nitroprusside) were obtained; but tests for α-ketol (triphenyltetrazolium chloride at 37°C), aldehyde (Fehling's test), ester (alkaline hydroxylamine–ferric chloride), carboxyl (hydroxylamine–ferric chloride), epoxide (sodium thiosulfate), and carbon–carbon double bond (tetranitromethane) were negative. The Lieben reaction indicated a methyl ketone function (iodoform). Infrared spectra showed broad bands at 3420 and 1095 cm^{-1} attributable to hydroxyl groups,

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carbonyl at 1713 cm^{-1}, active methylene at 1400 cm^{-1}, and methyl and methylene at 1455 and 2880 cm^{-1}, respectively.

In over 50 solvent and support systems used, toxin preparations chromatographed as a single, diffuse component. With Anasil GF silica gel and chloroform–methanol (94:6) as solvent, a major band at R_f 0.30 (70–80% by spot intensity and yield) and two minor bands of equal intensity at R_f 0.45 and 0.15 were obtained. Because the ir and nmr spectra, as well as biological activity, appeared to be identical for all three components after separation and isolation by tlc, it was not possible to distinguish between production of artifacts on this support and, for example, the existence of natural isomeric or homologous species. Mr. Ron Betts and Professor Carl Tipton of Iowa State independently have obtained by different methods a toxin which appears identical to ours by several criteria. They kindly furnished us with the information that commercially prepared plates of Merck EK 60 silica gel will separate up to nine bands. We have confirmed their findings with commercial plates and, as in their experience, find that plates of Merck EM 60 prepared in the laboratory are not effective.

With chloroform–methanol (85:15), preparative tlc (3 mg per plate) gives seven bands with the following R_f values. The relative order of staining intensity with iodine vapor is indicated in parentheses and is used to code the bands: 0.25 (7); 0.30 (6); 0.35 (5); 0.40 (2); 0.43 (1); 0.46 (3); 0.49 (4). Band 1 (R_f 0.43) consistently is the most abundant in terms of stain intensity and recovery, but band 2 frequently is only equal to, or even less than, band 3. Overall recoveries from plates are low (30–60%), in part because some of the isolated components are not always stable. For example, isolated band 3 (R_f 0.46) upon rechromatography was observed to produce significant amounts of materials with R_f values of 0.4, 0.3, and 0.2 which can not be readily explained as initial impurities. Band 1 rarely evidenced instability.

After a second chromatographic purification, bands 1, 2, and 3 were tested individually for biological activity, but it was necessary to combine bands 5, 6, and 7. There was not enough of band 4 for an accurate weighing. At 10 ng/ml, each fraction caused 50–70% increases of state 4 NADH oxidation (*4*) by susceptible mitochondria when compared to control mitochondria, while 10 μg/ml had no effect on resistant mitochondria. The unchromatographed toxin from which they were prepared had the same quantitative, specific effects. Whether natural products or artifacts of chromatography, it thus appears that all components are equally host specific and toxic. Similarly, ir spectra of the original toxin and bands 1, 2, and 3 appeared identical, except for a shift in a minor absorption at 830 cm^{-1} in the original toxin to 790 cm^{-1} for each band. Electron impact high-resolution mass spectra were essentially the same, with base peaks corresponding to $C_6H_9O_2$ and highest observable peaks corresponding to $C_{33}H_{48}O_5$ for band 2 and $C_{33}H_{46}O_4$ for bands 1 and 3.

The pmr and cmr of purified bands and of the original toxin provided no evidence for tertiary or quaternary ring structure and indicated only two methyl groups. Band 1 and 2 toxins were reduced to hydrocarbons by the method of Cope *et al.* (*5*), except that platinum oxide was used as a catalyst to reduce carbonyl functions (*6*). The hexane-soluble products were separated by a gas chromatograph (OV-1 column) interfaced with a low-resolution mass spectrometer. The major component of each toxin fraction had the retention time, the molecular ion, and the paraffin fragmentation pattern of a C_{41} hydrocarbon. Table 1 compares the distribution of hydrocarbons from C_{35} to C_{45}

TABLE 1

PERCENTAGE DISTRIBUTION[a] OF HYDROCARBONS PREPARED FROM RACE T TOXIN AND ANALYZED BY gc–ms

Sample	Number of carbons					
	35	37	39	41	43	45
1/2/78 toxin	2	10	11	55	16	6
5/17/78	3	10	29	46	12	3
Band 1 toxin	1	tr	3	95	tr	tr
Band 2 toxin	1	2	tr	93	4	tr

[a] Estimated from gc peaks; approximately 50% total yield for unchromatographed toxin and 85% for individual bands.

obtained from two different unchromatographed toxin preparations and bands 1 and 2. In the light of the small differences in R_f values, contamination of bands 1 and 2 is not unexpected. The fact that the two major components from tlc separation are C_{41}

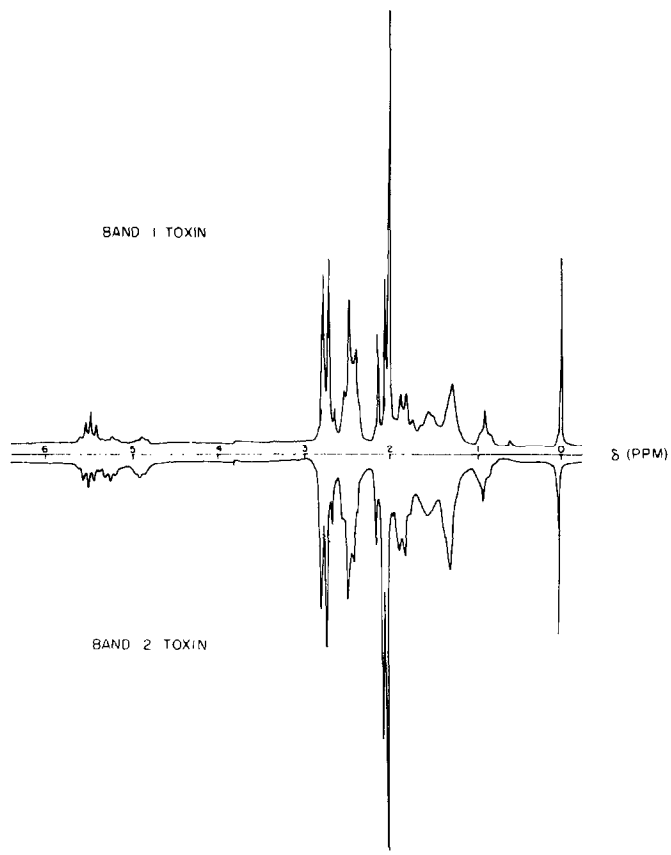

FIG. 1. The pmr spectra of band 1 (upper) and band 2 (lower) toxin. The absorptions at δ5.48, δ5.22, and δ4.90 integrate for 3:1:1 protons and 2:2:2 protons for band 1 and band 2 toxins, respectively. The differences are obvious at high amplitude.

compounds is consistent with results obtained with unchromatographed toxin. Bands 1, 2, and 3 were acetylated and the acetate derivatives purified by tlc. The pmr of acetylated bands 1 and 2 are drawn in Fig. 1 so as to emphasize their similarity.

A major absorption at δ4.1 ppm in the pmr spectra of band 1 toxin, assigned to hydroxyl functions, was absent in spectra of band 1 toxin acetate, while three signals due to acetyl protons were present at δ2.02, 2.04, and 2.08, integrating for a total of 15 protons. Poorly resolved absorptions at 4.5 to 4.8 ppm in the spectrum of the underivatized toxin were shifted to sharper absorptions at δ5.48 (3 H), δ5.22 (1 H), and

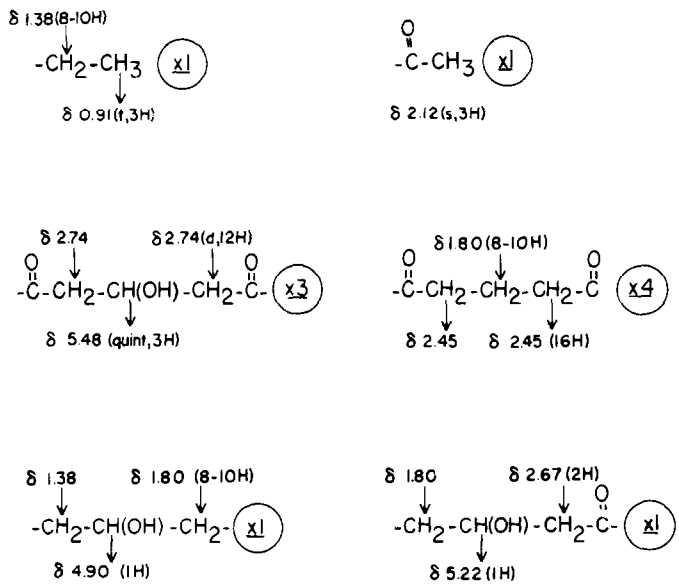

FIG. 2. Type and number of functional groups in band 1 race T toxin suggested by decoupling pmr spectra of toxin acetate. Arrows above protons indicate irradiating frequency (ppm); arrows below protons indicates response observed at indicated chemical shifts. Tetramethylsilane was the internal reference. Data in parentheses give the nature of the signal observed at that frequency, along with the number of protons obtained from integration. Numbers in circles indicate the total number of each function based on proton integration. Although 4 is the number assigned to the 1,5-diketone function, this may include one 1,7-diketone function.

δ4.90 (1 H) ppm (Fig. 1), corresponding to five methine protons of acetylated secondary alcohols, three of which are in the same immediate chemical environment. At high gain, the absorption at δ5.48 was a quintet in both 60 and 100 MHz spectra with a spacing of 6.0 Hz. Figure 2 summarizes the proton chemical shifts and coupling for band 1 toxin based on decoupling data for the toxin acetate. Not included are 8 to 10 protons with shifts from δ1.40 to 1.70 attributable to methylene functions.

The cmr of band 1 toxin in pyridine-5*d* showed complex signals in the carbonyl absorption region at δ209 ppm, but no signals from 80 to 200 ppm. Signals at 70.7 and 68.0, and 64.4 ppm (relative intensities 3, 3, 8) in proton noise decoupling spectra gave three doublets in gated decoupling spectra and can be ascribed to oxygen substituted methine carbons. The upper sets of numbers in Fig. 3 give some observed ^{13}C chemical

shifts for toxin in pyridine-5*d*. The lower sets of numbers are calculated ^{13}C shifts based on data from Stothers (*7*) or Johnson and Jankowski (*8*). Not listed are chemical shifts of δ23.6, 25.7, 28.9, and 32.3 ppm, which can be assigned to methylene carbons bridging these functions. Weak signals at 31.5, 51.6, and 42.4 ppm were interpreted as carbons shown as equivalent in the functions of Fig. 2 but in slightly different environments due to neighboring functions (e.g., 42.4 versus 42.8 or 31.5 versus 32.3). The pmr and cmr spectra of band 1 toxin appear identical to those of the original toxin mixture or to band 3 toxin. Band 1 toxin can be distinguished from band 2 toxin, which integrates for two protsons each at δ5.48, 5.22, and 4.90 ppm, instead of 3 : 1 : 1 (fig. 1). Band 2 toxin appears to have six hydroxyls comprising three equivalent pairs.

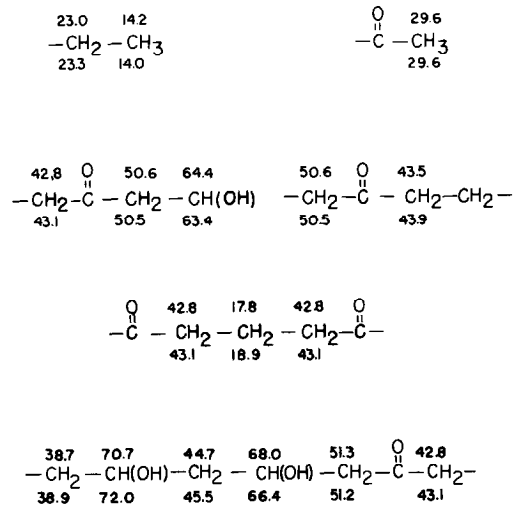

FIG. 3. Observed (upper numbers) and calculated (lower numbers) ^{13}C chemical shifts for postulated functional groups of band 1 toxin. Underivatized toxin in pyridine-5*d* with tetramethylsilane as reference. The observed and calculated shifts for toxin acetate in $CDCl_3$ show similar agreement.

For band 1 toxin, the spectal data lead to the expectation of an empirical formula of $C_{41}H_{68}O_{13}$. Conversion of toxin to the trimethyl silyl ether by a modification (*9*) of the procedure developed for polyols (*10*) yielded upon low-resolution mass spectroscopy a series of fragments (Table 2) corresponding to sequential loss of 5 $(CH_3)_3SiOH$ groups. The largest mass was 1110 (expected 1128). Coating the capillary with silyl-8 resulted in detection of the postulated parent ion, as determined by peak matching with the nearest suitable reference peak of tris-(pentadecafluoroheptyl)-*S*-triazine (Table 2).

The expected parent ion (mass 1202) was not found for band 2 (Table 2), but good agreement was obtained with masses corresponding to M^+-18 and M^+-18–90 for the 6-TMS derivative of a structure of empirical formula $C_{41}H_{70}O_{13}$.

The loss of water can be rationalized as the cyclization, during heating, of a 1,5-diketone function (Figs. 1 and 2) similar to the base-catalyzed formation of cyclohexenones reported by Stork and Borch (*11*). Consideration of the structure of Fig. 4 suggests cyclization of the terminal 1,5-diketone function, followed by fragmentation adjacent to the carbon carrying a silyl ether function, yielding a fragment

TABLE 2

LOW- AND HIGH-RESOLUTION MASS SPECTROSCOPY OF TMS[a] ETHERS FROM TOXIN BANDS

Band	Mass and relative abundance						
1	1110	1020	930	840	750	660	570[c]
	(15)	(61)	(65)	(100)[b]	(81)	(62)	
2	1184	1094	1004	914	824	734	644
	(15)	(47)	(85)	(100)[b]	(92)	(74)	(54)
3	1068	978	888	798	708	618	528[c]
	(13)	(39)	(52)	(95)	(100)[b]	(95)	

		M^+	$(M-18)^+$	$(M-18-90)^+$
1	Calc	1128.6637	1110.6530	1020.6030
($C_{56}H_{108}O_{13}Si_5$)	Obs	1128.6624	1110.6560	1020.6037
2	Calc	1202.7188	1184.7083	1094.6582
($C_{59}H_{118}O_{13}Si_6$)	Obs	ND	1184.7055	1094.6652
3	Calc	1086.6531	1068.6425	978.5924
($C_{54}H_{106}O_{12}Si_5$)	Obs	1086.6530	1068.6425	978.5871

[a] $(CH_3)_3SiOH$ (MW = 90).
[b] For masses above 600.
[c] Observed, but expected isotopic masses not obvious.

with a *m/e* of 211.1154 (found 211.1165). This mass is prominent in spectra of bands 1 and 2.

Band 3 yielded a sequence of peaks in low-resolution spectra which are consistent with a structure of empirical formula $C_{39}H_{66}O_{12}$ possessing five hydroxyl groups (Table 2). A fourth component subsequently has been shown to be $C_{39}H_{68}O_{12}$. These four components account for 50–70% of the materials separated by tlc and are in good agreement with the results of Table 1.

We tentatively propose the structure in Fig. 4 for band 1 race T toxin. Although we recognize the precise location of functions is not established, high-resolution mass spectroscopy of band 1 resulted in assignment of 700 ion fragments, none of which are inconsistent with the proposal. Preliminary studies of high-resolution spectra of the TMS derivative show the prominent presence (and without consideration or rearrangements) of the masses expected for the 10 carbons on the left in Fig. 3. In addition, the masses expected for the internal 1,5- and 1,7-diketone functions were

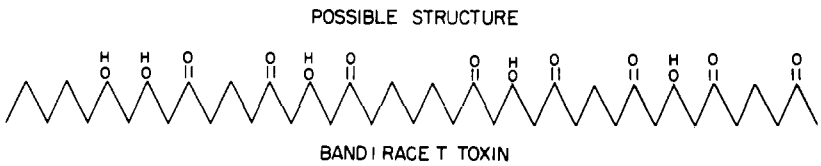

FIG. 4. Proposed structure of band 1 toxin obtained from cultures of *Helminthosporium maydis*, race T. Band 2 toxin differs only by the reduction of a carbonyl group.

obtained. The structure for band 2 should differ from that of Fig. 3 only by the presence of one hydroxyl instead of carbonyl.

It should be remarked that the lengths of the molecules so far characterized approximate the length of a membrane bilayer. This characteristic may be important in explaining the high biological activity in susceptible corn.

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