Studies on the Host-Specific Pathotoxins Produced by *Helminthosporium maydis*, race T

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Two host-specific pathotoxins, Band 1- and Band 2-toxins, were isolated from the toxin complex obtained from the culture broth and mycelial mat of *Helminthosporium maydis*, race T, the fungus causing Southern corn blight disease. Chemical and spectrometric studies showed them to be polyketo-polyhydroxy compounds with C_{41} carbon chains. Band 2-toxin is identical to Band 1-toxin except that Band 2 toxin has an additional hydroxyl group in place of one of the keto-group of Band 1-toxin.

As has been reported^{1,2)} the phytopathogenic *Bipolaris* (*Helminthosporium*) *maydis*, race T, causal agent of southern corn blight, produces a host-specific pathotoxin complex that has a specific effect on leaves of sensitive corn. Toxin complex was obtained as follows: culture filtrates and extracts of the mycelium were treated with charcoal (Norit A), the complex was precipitated from its chloroform extract by refrigeration and purified by reprecipitation from warm methanol or acetone. The complex (mp 125~126°C, C: 63.84; H: 9.18, O: 26.68) exhibited biological specificity for corn with Texas male (Tms) cytoplasm, but did not effect normal (N) cytoplasm corn. Only 4 to 8 ng of the toxin complex were required to cause complete killing effect on

TMS corn, while 20,000 ng was taken up by N corn leaves without visible effect. The toxin complex was separated into seven to nine spots on silica gel TLC (9:1, chloroform-methanol). We have isolated four toxins from the complex, examined their physicochemical and biological properties and have proposed structures for Band 1- and Band 2-toxins, which are the major components of the complex.^{1,2)} In the present paper we wish to present the complete details of the process for the assignment of the structures **I** and **II** to the toxins.

Band 1-toxin melts at 132~133°C and, Band 2-toxin at 127~128°C, and their molecular formulae were determined as $C_{41}H_{68}O_{13}$ and $C_{41}H_{70}O_{13}$, respectively, by high

Band 1 toxin

Band 2 toxin

resolution- and field-desorption mass spectrometry and elemental analysis.^{1,2)} Infrared spectra of both toxins showed broad bands at 3420 and 1095 to 1040 cm^{-1} attributable to hydroxyl groups, and bands at 1713 cm^{-1} (carbonyl) and at 1400 cm^{-1} (active methylene).

The ^1H -NMR spectra of these two toxins suggested that the toxins have many common partial structures. The cluster of peaks situated between 4.5 and 4.8 ppm in the spectra of underivatized toxin was shifted to lower fields by acetylation and was resolved into three quintets at 5.48 (3H for Band 1-, 2H for Band 2-toxins), 5.22 (1H; 2H) and 4.90 (1H; 2H) ppm, corresponding to five (for Band 1-toxin) or six (for Band 2-toxin) methine protons of acetylated secondary alcohols. This result indicated that three protons (5.48 ppm) for Band 1-toxin, and the three pairs of protons in Band 2-toxin (5.48, 5.22 and 4.90 ppm), are in essentially identical chemical environments. These peaks appeared as quintets in both 60- or 100 MHz ^1H -NMR, with a coupling constant of 6.0 Hz. The results of decoupling studies suggested that molecules of Band 1- and Band 2-toxins contain the following partial structures:

Partial structures	Number	
	Band 1-toxin	Band 2-toxin
CH_3CH_2-	1	1
$\text{CH}_3\text{CO}-$	1	1
$-\text{COCH}_2\text{CH}(\text{OH})\text{CH}_2\text{CO}-$	3	2
$-\text{COCH}_2\text{CH}_2\text{CH}_2\text{CO}-$	3	3
$-\text{CH}_2\text{CH}(\text{OH})\text{CH}_2\text{CH}(\text{OH})\text{CH}_2\text{CO}-$	1	2

The ^{13}C -NMR spectrum of Band 1-toxin acetate is shown in Fig. 1. The ^{13}C -NMR of Band 2-toxin acetate is different from this spectrum only by the presence of additional signals, designated by the arrows in Fig. 2.

Although both toxin acetates absorb at 43.5 and 23.6 ppm, these signals for Band 1-toxin acetate correspond to 2 carbons each, but only for one carbon each in Band 2-toxin acetate, suggesting that the latter molecule does not contain a ζ -dioxo ($-\text{CO}(\text{CH}_2)_5\text{CO}-$) group. While the Band 1-toxin shows absorptions also at 34.1, 71.0, 38.3, 67.7 and 46.5 ppm (one carbon each) attributable to

$-\text{CH}_2\text{CH}(\text{OH})\text{CH}_2\text{CH}(\text{OH})\text{CH}_2\text{CO}-$ group, Band 2-toxin absorbs at 38.3, 67.7 and 46.5 ppm (two carbons each) and, additionally, at 25.1, 34.0 and 70.9 ppm (one carbon each). The examination of the above data makes it fairly clear that two partial structures, *i.e.* a ζ -dioxo-group and a β -oxy- δ -oxo- ($-\text{COCH}_2\text{CH}(\text{OH})\text{CH}_2\text{CO}-$) group are replaced by a structure:

FIG. 1. ^{13}C -NMR of Band 1-Toxin Acetate in CDCl_3 .

FIG. 2. ^{13}C -NMR of Band 2-Toxin Acetate in CDCl_3 .

Thus it may be concluded that the reduction of either one of the carbonyl groups in the partial structure $-\text{CO}(\text{CH}_2)_5\text{CO}-$ of Band 1-toxin converts it to Band 2-toxin.

The assignment of ^{13}C -NMR signals of Band 1-toxin (and its acetate) and Band 2-toxin (and its acetate) are summarized in Table I. The calculated values were obtained using data available for aliphatic alcohols and ketones.^{3,4)}

PRFT (Partially Relaxed Fourier Transform) spectra of Band 2-toxin acetate without degassing gave relatively long relaxation times for carbon nuclei at 14.0 (3.3 sec), 18.1 (1.9), 22.5 (3.1), 29.6 (5.6), 31.5 (2.0) and 43.0 ppm (3.0). Other carbon nuclei gave shorter relaxation times between 0.5 to 0.9 sec. The carbons with longer relaxation times can be ascribed to the carbon nuclei at the both sides of the chain.

The presence of β -oxyoxo groups and the absence of β -dioxo group in these toxins has been suggested by spectroscopic and chemical evidence.^{1,2)} Accordingly, each toxin was oxidized with Jones' CrO_3 reagent in acetone, to convert hydroxyl groups into ketones to form β -dioxo groups. Analysis of the mass and ^1H -NMR spectra showed that the products from both toxins were identical. The oxidized toxins were refluxed in alkaline solution to cause β -dioxo cleavage,⁵⁾ and from each hydrolysate

were isolated glutaric, pimelic and β -hydroxyoctanoic acids as main acidic products, as shown by GC-MS comparison with authentic specimens. No detectable amount of similar acidic components were present in hydrolysates of the underivatized toxins. The isolation of two dibasic acids from the CrO_3 oxidation substantiated the supposition of the presence of ζ -dioxo or ζ -oxyoxo and δ -dioxo groups. Examination of the results revealed that the number of the possible structures that are in accord with these data are restricted to three for Band 1-toxin (Fig. 3) and five for Band 2-toxin (Fig. 4). The precise positions of ζ -dioxxygenous and δ -dioxo groups in the carbon chain required further study in order to establish the complete structures of the toxins.

Conversion of the Band 1- and 2-toxins to a hydrocarbon by removal of all oxygen groups was carried out according to Cope *et al.*,⁶⁾ with slight modifications. Each toxin was hydrogenated to a polyalcohol by catalytic hydrogenation with Adams platinum oxide in methanol-acetic acid (9:1) until keto groups disappeared. The resultant polyalcohols were converted to iodinated hydrocarbons in the following two-step treatment to avoid side reactions: in the milder first treatment the polyalcohols were refluxed for 24 hr in a boiling mixture of red phosphorus, hydriodic acid and *n*-heptane. The iodides were dehalo-

TABLE I. ASSIGNMENT OF ^{13}C -NMR OF BANDS 1 AND 2 TOXINS (FREE AND ACETATE)

Functions	R = H ^a			R = Ac ^b		
	Calc.	Band 1 obs.	Band 2 obs.	Calc.	Band 1 obs.	Band 2 obs.
CH ₃	14.0	14.0 (1)	14.0 (1)	14.0	14.0 (1)	14.0 (1)
CH ₂	23.3	23.0 (1)	23.0 (1)	23.0	22.5 (1)	22.5 (1)
CH ₂	32.6	32.3 (1)	32.3 (1)	32.3	31.5 (1)	31.5 (1)
CH ₂	26.7	25.7 (1)	25.7 (1)	27.5	24.7 (1)	24.7 (1)
CH ₂	38.7	38.9 (1)	38.9 (1)	35.2	34.1 (1)	34.1 (1)
CH-(OR)	72.0	70.7 (1)	70.7 (1)	74.2	71.0 (1)	71.0 (1)
CH ₂	45.5	44.7 (1)	44.7 (1)	38.3	38.3 (1)	38.3 (1)
CH-(OR)	66.4	68.0 (1)	68.0 (1)	69.9	67.7 (1)	67.7 (1)
CH ₂	51.2	51.3 (1)	51.3 (1)	48.1	46.5 (1)	46.5 (1)
CO						
CO						
CH ₂	43.1	42.8 (3) ^c	42.8 (3) ^c	43.1	42.8 (3) ^c	42.8 (3) ^c
CH ₂	18.9	17.8 (3) ^d	17.8 (3) ^d	18.9	17.8 (3) ^d	17.8 (3) ^d
CH ₂	43.1	42.8 (3)	42.8 (3)	43.1	42.8 (3)	42.8 (3)
CO						
CO						
CH ₂	50.5	50.6 (3)	50.6 (2)	46.9	46.1 (3)	46.1 (2)
CH-(OR)	63.4	64.4 (3)	64.4 (2)	65.0	64.4 (3)	64.4 (2)
CH ₂	50.5	50.6 (3)	50.6 (2)	46.9	46.1 (3)	46.1 (2)
CO						
CO						
CH ₂	43.9	43.5 (1)	—	43.9	43.5 (1)	—
CH ₂	23.6	23.6 (1)	—	23.6	23.6 (1)	—
CH ₂	28.5	28.9 (1)	—	28.5	28.9 (1)	—
CH ₂	23.6	23.6 (1)	—	23.6	23.6 (1)	—
CH ₂	43.9	43.5 (1)	—	43.9	43.5 (1)	—
CO						
CO						
CH ₂	43.9	—	43.5 (1)	43.9	—	43.5 (1)
CH ₂	23.6	—	23.9 (1)	23.6	—	23.6 (1)
CH ₂	29.6	—	29.6 (1)	29.6	—	29.0 (1)
CH ₂	27.5	—	25.8 (1)	27.5	—	25.1 (1)
CH ₂	38.7	—	38.7 (1)	35.2	—	34.0 (1)
CH-(OR)	72.0	—	70.7 (1)	74.2	—	70.9 (1)
CH ₂	45.5	—	44.7 (1)	38.3	—	38.3 (1)
CH-(OR)	66.4	—	68.0 (1)	69.9	—	67.7 (1)
CH ₂	51.2	—	51.3 (1)	48.1	—	46.5 (1)
CO						
CO						
CH ₃	29.5	29.6 (1)	29.6 (1)	29.5	29.6 (1)	29.6 (1)

^a In C₅D₅N^b In CDCl₃.^c One of these carbons was observed at 43.0 ppm.^d One of these carbons was observed at 18.1 ppm.

FIG. 3. Possible Structures for Band 1-Toxin.

FIG. 4. Possible Structures for Band 2-Toxin.

genated with LiAlH_4 in tetrahydrofuran. The products were again halogenated as above but without *n*-heptane, and then were similarly dehalogenated. Each of the products from two toxins was hydrogenated with Adams platinum oxide as catalyst and was purified on silica gel columns with *n*-hexane to yield two fractions each. The first fraction of each gave an identical product that showed a molecular ion peak of m/e 576.6594 ($n\text{-C}_{41}\text{H}_{84}$, calc. 576.6573). The two second eluates both from Band 1- and Band 2-toxins were mixtures of products. According to the MS fragmentation patterns, each of the mixtures seemed to consist of products carrying a single 4H-pyran ring on the carbon chain. The 4H-pyran ring must have come from cyclization of δ -dioxo or δ -oxyoxo groups during the above reductive treatments.^{7,8)} GC-MS (as shown in Fig. 5) of the mixtures gave identical parent ion peaks of $\text{C}_{41}\text{H}_{82}\text{O}$ (m/e 590.6399, calc. 590.6362) and

fragmentation peaks. In high resolution GC-MS, three pairs of prominent peaks which can be interpreted as the result of cleavage of the molecule on each side of 4H-pyran ring were observed. The MS data gave evidence for the ultimate structures of the toxins are summarized in Fig. 6. This enabled us to choose structure 2 of Fig. 3 as the structure for Band 1-toxin, and also to limit the number of possible structures to two (Fig. 4-3 and 4-4) for Band 2-toxin. The fragments obtained by high-resolution EI-MS of the underivatized toxins are in accord with the above presumed structures and, further, the presence of two dehydrated fragments from the Band 2-toxin ($\text{C}_{33}\text{H}_{48}\text{O}_5$ and $\text{C}_{25}\text{H}_{36}\text{O}_5$), indicates that the position of the hydroxyl group in question is at C24, thus establishing the structure of the Band 2-toxin as number 3 in Fig. 4.

Other fragments ($\text{C}_{15}\text{H}_{24}\text{O}_2$, $\text{C}_{15}\text{H}_{22}\text{O}_4$) are also in accord with the presence of the ζ -

FIG. 5. Mass Spectrum of 4H-Pyran Compounds from C_{41} Toxin.

$C_{10}H_{19}O$	Obs.	155.1436	Calc.	155.1436	$C_{36}H_{71}O$	Obs.	519.5593	Calc.	519.5504
$C_{14}H_{27}O$	Obs.	211.2100	Calc.	211.2062	$C_{32}H_{63}O$	Obs.	463.4902	Calc.	463.4776
$C_{18}H_{35}O$	Obs.	267.2694	Calc.	267.2668	$C_{28}H_{55}O$	Obs.	407.4253	Calc.	407.4252

FIG. 6. HR-GC-MS of 4H-Pyran Compounds.

FIG. 7. HR-MS Fragments from Band 2-Toxin.

oxygenous groups covering C18 to C24 as shown in Fig. 7. The high resolution MS of TMS derivatives of Band 1- and Band 2-toxins gave prominent peaks at m/e 173.1361 for

and m/e 211.1165 for

The loss of water can be rationalized by the cyclization during heating of a δ -dioxo group similar to the base-catalyzed formation of cyclohexanones.⁹⁾ These peaks also support the structure of both terminal groups in the

toxins.

Based on the procedures described above, the respective structures of the toxins are presented as shown I and II.

Both toxins show nearly identical biological activities.^{10,11)}

EXPERIMENTAL

Melting points were measured on a Yanaco microscope hot plate and uncorrected. IR spectra were obtained with a Beckman Acculab 6, a Shimadzu IR-27 and JASCO IRA-1 with films on KBr pellets or disks. UV spectra in

methanol were obtained with a Shimadzu double beam spectrometer UV 200 or with a Beckman DB instrument. Low resolution mass spectrometer were obtained with a Hitachi RMU-6M(G) mass spectrometer with a direct inlet system or a gas chromatographic inlet system using OV-1 column (4 mm $\times$ 50 cm). High resolution mass spectra were obtained with an AEI-M5902, an AEI-MS5076 or with a JEOL JMS-D300. ^1H -NMR and ^{13}C -NMR spectra were obtained with a Varian EM360 instrument (60 MHz), a JEOL JNM-PMX60 (60 MHz), a Varian XL-100, a JEOL FX-100 (100 MHz) or a JEOL FX-90 Q (90 MHz). Thin layer chromatography was carried out on Merck silica gel 60 F_{254} (0.25 mm for detection, 0.5 mm for preparative). Gas chromatographic analysis was done with a Shimadzu GC-4B (OV-1, 4 mm $\times$ 50 cm).

Isolation of Band 1- and Band 2-toxins. *Helminthosporium maydis*, race T, freshly isolated from the leaf lesions, was inoculated into the bottles containing 800 ml of modified Fries medium and grown under 12,000 lux of continuous fluorescent light.¹²⁾ Mycelium obtained by filtration of cultures through Miracloth was homogenized for 15 minutes in one liter of 70% aqueous acetone. The culture filtrates were treated with 3% (w/v) Norit A for 2 hrs with stirring. After recovery of the Norit A and washing with water, it was added to the acetone homogenate and stirred for another 2 hrs. The recovered Norit A was washed several times with acetone. Toxin was desorbed by three 12-hour extraction with CHCl_3 -MeOH (95:5), 1/4 to 1/5 volume of the filtrate. The use of pure chloroform in desorption resulted in less pigmented preparation but was less efficient. Purer preparations can also be obtained by treatment of the original culture filtrate with 3% (w/v) animal charcoal, desorption of toxin with 80% acetone homogenate of the mycelium, followed by treatment of the acetone with Norit A. The yield of toxin is lower by this procedure because the adsorption on animal charcoal is not complete.

The slightly yellow CHCl_3 -MeOH eluates were combined and evaporated to dryness under vacuum. Temperature during drying did not exceed 45°C. The red oil was dissolved in a minimum volume of CHCl_3 and kept at 2~4°C overnight. The resulting colorless precipitate was collected on a sintered glass filter and washed with cold CHCl_3 . The precipitate was dissolved in hot methanol and allowed to stand at 2~4°C overnight followed by collection of the precipitate and drying under vacuum. Yields of precipitate was 50 to 300 mg from 10 liters culture. The precipitate of the toxin complex (295 mg) was fractionated using silica gel preparative TLC plate with chloroform-methanol (9:1) as a solvent. The developed TLC was visualized by spraying with water or exposing to iodine vapor. Band 1-toxin (R_f 0.43) and Band 2-toxin (R_f 0.40) bands were scraped off and eluted with hot methanol. This procedure was repeated two to three times. The yields of purified toxins were 19.5 mg for Band

1-toxin (mp 132~133°C) and 22.3 mg for Band 2-toxin (mp 127~128°C). Direct extraction of the crude culture filtrates followed by chromatography on silicic acid, Sephadex LH-20 and Sephadex G-15 columns was possible, but 70 to 80% of the biological activity was lost.

Assay for host-specific activity. Routine semi-quantitative estimation of the biological activity was based on the maximum dilution factor required to cause wilting and necrosis of second and third leaves of susceptible W64A-Tms corn 16~18 hr after placing the basal end of the leaf in toxin solution. In each routine assay, resistant corn (W64A-N) was tested at the lowest dilution. Quantitative data were obtained by measurement of the inhibition of dark CO_2 fixation by leaf disks of corn.^{13,14)} As the solubility of the toxins was low (0.5 mg/ml), stock solutions of toxin were handled as follows. Amounts varying from 1 to 5 mg were weighed with Cahn electrobalance and were transferred to 2 to 5 ml Reactivials (Pierce Chemical Co.). After adding the desired volume of MeOH, the vials were heated to 75°C for complete dissolution. After cooling to 35~45°C, aliquots for dilution were removed by insertion of a microliter syringe through the stopper.

Isolation of the toxin acetates. In an attempt to improve chromatographic separation, and because of the limited solubility in available NMR solvents, the toxin complex was acetylated as follows. Fifty milligrams of toxin complex was dissolved in 5 ml of pyridine plus 3 ml of acetic anhydride and kept at 4°C overnight. The solution was concentrated *in vacuo*. The toxin acetates (60 mg) were chromatographically separated three times using silica gel 60 (200~400 mesh ASTM) column with benzene-ethyl acetate-methanol-acetic acid (50:50:3:1). Fifteen milligrams of the main component was obtained. This was separated by HPLC using Zorbax-SIL column (2 mm $\times$ 25 cm, solvent; dichloromethane-isopropanol 98:2, column pressure; 200 kg/cm²). From this experiment, four components were isolated as single peaks. Peak A had a retention time of 3.1 min, peak B 3.9 min, peak C 4.3 min and peak D 6.4 min, respectively. The amount of each peak was 0.5 mg for peak A, 1.2 mg for peak B, 1.6 mg for peak C and 1.1 mg for peak D. Peak C gave the same spectra as Band 1-toxin acetate in ^1H -NMR and ^{13}C -NMR.

Band 1-toxin and Band 2-toxin acetates were separated by preparative TLC using Merck silica gel F_{254} TLC (0.25 mm). The solvent system was benzene-ethyl acetate-methanol-acetic acid (50:50:2:1, R_f of Band 1-toxin acetate was 0.22 and Band 2-toxin acetate was 0.31) or benzene-isopropanol-acetic acid (95:5:1, R_f of Band 1-toxin acetate was 0.13 and Band 2-toxin acetate was 0.17). Ten mg of Band 1-toxin acetate gave 4.5 mg of the pure acetate, and 8 mg of Band 2-toxin acetate gave 4.5 mg of the pure acetate.

Physicochemical properties of Band 1-toxin, Band 2-toxin and their acetates.^{1,2)}

Band 1-toxin: UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (ϵ); 280 (154). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} ; 3420 (ν O-H), 2940, 2860 (ν C-H), 1712 (ν C=O), 1400 (active methylene), 1090, 1065 1040 (ν C-OH). MS of penta-TMS derivative m/e , 1128.6624 (M^+ , 1128.6637 Calcd. for $\text{C}_{56}\text{H}_{108}\text{O}_{13}\text{Si}_5$). HR-FD-MS of sodium complex m/e , 791.4436 (M^+ , 791.4556 Calcd. for $\text{C}_{41}\text{H}_{68}\text{O}_{13}\text{Na}$). Elemental analysis of pentaacetate, Found: C, 62.15; H, 7.97; O, 29.07. Calcd. for $\text{C}_{51}\text{H}_{78}\text{O}_{18}$: C, 62.57; H, 7.97; O, 29.44. Molecular weight determination of pentaacetate by V.P.O. method, 1040 (Calcd. for $\text{C}_{51}\text{H}_{78}\text{O}_{18}$ 978). $[\alpha]_{\text{D}}^{25} = +12.0^\circ$ ($c=0.12$, MeOH).

Band 2-toxin: UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (ϵ); 280 (154). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} ; 3420 (ν O-H), 2940, 2860 (ν C-H), 1712 (ν C=O), 1400 (active methylene), 1090, 1065, 1040 (ν C-OH). MS of hexa-TMS derivative m/e , 1184.7055 ($(\text{M}-18)^+$, 1184.7083 Calcd. for $\text{C}_{56}\text{H}_{116}\text{O}_{12}\text{Si}_6$). FD-MS of sodium complex m/e 793 (M^+ , 793 Calcd. for $\text{C}_{41}\text{H}_{70}\text{O}_{13}\text{Na}$). Elemental analysis of hexaacetate, Found: C, 61.66; H, 8.01. Calcd. for $\text{C}_{53}\text{H}_{82}\text{O}_{19}$: C, 62.23; H, 8.02. $[\alpha]_{\text{D}}^{25} = +17.5^\circ$ ($c=0.16$, MeOH).

*MS of TMS derivatives.*¹⁾

A toxin (100 to 200 μg) was dissolved in dry pyridine which was kept over KOH pellets (0.5 ml), and 0.1 ml of hexamethyldisilazane and 0.05 ml of trimethylchlorosilane were added.¹⁵⁾ After 1 hr the reaction mixture was concentrated *in vacuo*.¹⁶⁾ The product was dissolved in *n*-hexane (100 μl) or carbon tetrachloride (100 μl) and analyzed by EI mode with silyl-8 coated capillary tubes.

Low-resolution MS of TMS derivatives of Band 1- and Band 2-toxins; m/e , 1110 (15%), 1020 (61), 930 (65), 840 (100), 750 (81) and 660 (62) for Band 1-toxin, 1184 (15), 1094 (47), 1004 (85), 914 (100), 824 (92), 734 (74) and 644 (54) for Band 2-toxin.

High-resolution MS of TMS derivatives of Band 1- and Band 2-toxins; M^+ 1128.6624 (calcd. 1128.6637), $(\text{M}-18)^+$ 1110.6560 (1110.6530), $(\text{M}-18-90)^+$ 1020.6037 (1020.6030) for Band 1-toxin, $(\text{M}-18)^+$ 1184.7055 (calcd. 1184.7083), $(\text{M}-18-90)^+$ 1094.6652 (1094.6582) for Band 2-toxin. Other useful peaks were observed at m/e 173.1361 (calcd. 173.1362 for $\text{CH}_3(3\text{H}_2)_4\text{CH}(\text{O}-\text{TMS})$) and m/e 211.1165 (calcd. 211.1165 for $\text{CH}_3\text{CO}(\text{CH}_2)_3\text{COCH}_2\text{CH}(\text{O}-\text{TMS})-\text{H}_2\text{O}$). For the reference of HR-MS, tris-(pentadecafluoroheptyl)-S-triazone was used.

Proton NMR of Band 1- and Band 2-toxins acetates.

δ 5.48 (quintet, $J=6$ Hz, 3H for Band 1-toxin and 2H for Band 2-toxin), 5.22 (quintet, $J=6$ Hz, 1H, 2H), 4.90 (quintet, $J=6$ Hz, 1H, 2H, 2.74 (doublet, $J=6$ Hz, 12H, 8H), 2.67 (doublet, $J=6$ Hz, 2H, 4H), 2.45 (triplet like, $J=6$ Hz, 16H, 14H), 2.12 (singlet, 3H), 2.08 (singlet, 3H, 6H), 2.04 (singlet, 3H, 6H), 2.02 (singlet, 9H, 6H), 1.80 (quartet like, 8H, 10H), 1.5 (multiplet, 6H, 6-8H), 1.3 (multiplet, 8H, 10H) and 0.91 ppm (triplet, 3H, 3H).

The results of decoupling studies.

[5.48 ppm](coupling) [2.74 ppm]
[2.67 ppm]— — — — [5.22 ppm] — — — — [1.80 ppm]
[1.80 ppm]— — — — [4.90 ppm] — — — — [1.30 ppm]
[2.45 ppm]— — — — [1.80 ppm]
[2.38 ppm]— — — — [1.50 ppm]
[1.30 ppm]— — — — [0.91 ppm]

Carbon-13 NMR of Band 1- and Band 2-toxins acetates.

Off-resonance spectra; quartets at 14.0, 21.0 and 29.6 ppm, triplets at 17.8, 18.1, 22.5, 24.7, 25.1 (only in Band 2-toxin), 29.0 (Band 2-toxin), 31.5, 34.0 (Band 2-toxin), 34.1, 38.3, 42.8, 43.0, 43.5, 46.1 and 46.5 ppm, doublets at 64.4, 67.7, 70.9 (Band 2-toxin) and 71.0, and singlets around 170 and 207 ppm.

The results of selective decoupling using single-frequency $^{13}\text{C}-\{^1\text{H}\}$ technique are shown below.

irr. (^1H)	obs. (^{13}C)
δ 5.46 ppm	64.4, 67.7 (doublet to singlet)
4.88	67.7, 70.9, 71.0 (doublet to singlet)
2.71	46.1, 46.5 (triplet to singlet)
2.46	42.8, 43.0, 43.5 (triplet to singlet)
2.12	21.0, 29.6 (quartet to singlet)
1.79	17.8, 18.1, 38.3 (triplet to singlet)
1.56	23.6, 24.7, 34.1 (triplet to singlet)
1.25	22.5, 29.0, 31.5 (triplet to singlet)
0.88	14.0 (quartet to singlet)

*Alkaline degradation of oxidized toxin.*²⁾ Toxin complex (2.5 mg) was dissolved in 2 ml of warm acetone and 0.2 ml of Jones reagent (1.34 g of CrO_3 was dissolved in 1.2 ml of concentrated H_2SO_4 and diluted with distilled water to 10 ml) was added. The solution was stirred for 3 to 5 min at 40°C . The upper layer of acetone solution was collected by decantation and concentrated *in vacuo*. The residue was dissolved in 80% ethanol–20% water containing 3% KOH (5 ml) and refluxed for 18 hrs. The reacted solution was concentrated and extracted with two 10 ml portions of diethyl ether after acidification with dilute hydrochloric acid. The extract was dried with MgSO_4 , concentrated *in vacuo* and treated with diazomethane in diethyl ether to form methyl esters. The products were analyzed by GC and GC-MS (OV-1). Glutaric (about 40 μg , base peak at m/e 101), pimelic (40 μg , base peak at m/e 129) and β -hydroxyoctanoic acids (60 μg , base peak at m/e 103) were identified through comparison with authentic specimens. β -hydroxyoctanoic acid was synthesized using Reformatsky reaction^{17,18)} from *n*-hexanal and bromoacetic acid methyl ester. The yields of above acids were about 5%, 10%, and 12%, respectively. Band 1-toxin gave the same products as the toxin complex, *i.e.* glutaric (5% yield), pimelic (15%) and β -hydroxyoctanoic acids (12%). Band 2-toxin also gave the same products as above, glutaric (7% yield), pimelic (15%) and β -hydroxyoctanoic acids (20%). Oxidized Band 1- and Band 2-toxins gave the same products identified by ^1H -NMR and mass fragmentation

patterns using the in-beam method. No detectable amount of similar acidic components were present in the hydrolysates of the toxin themselves.

*Conversion of the toxin to hydrocarbon.*²⁾ About 3 to 5 mg toxin was reduced using Adams platinum oxide (40 mg) in methanol-acetic acid (9:1, 10 ml) for 72 to 96 hrs until keto groups disappeared. The catalyst was separated and the solvent evaporated. The oily residue (3 to 5 mg) and red-phosphorus (20 mg) were heated under reflux for 24 hrs in excess constant boiling hydriodic acid (3 ml) and *n*-heptane (2 ml). Water (3 ml) was added to the mixture, followed by extraction with 5 ml portions of chloroform. The combined extracts were washed with 2% sodium thiosulfate solution (1 ml), then with water (3 ml, three times), and dried over magnesium sulfate. Removal of solvent gave an iodine-containing oil which was dissolved in tetrahydrofuran (5 ml) and refluxed overnight with lithium aluminum hydride (15 mg). Work-up was accomplished by successive addition of water (15 μ l), 15% sodium hydroxide solution (15 μ l) and water (45 μ l). The precipitate was washed with chloroform (10 ml). Then the tetrahydrofuran and the chloroform solution were combined and, after evaporation of the solvents, the residue was re-halogenated using the above method (red-phosphorus and hydriodic acid) without *n*-heptane. Then the product was dehalogenated with lithium aluminum hydride as above. The product was hydrogenated using Adams platinum oxide in *n*-hexane-ethyl acetate (2:1, 10 ml) for one hour. The catalyst was separated and the solvent evaporated. Then the product was separated using silica gel column chromatography. The first eluate of *n*-hexane contained hydrocarbon which was identified with authentic *n*-C₄₁H₈₄ (synthesized from stearyl bromide and behenic acid¹⁸⁻²⁰⁾). The yield of hydrocarbon was 20 to 30%. The second eluate of *n*-hexane was a complex of 4H-pyran compounds (yield, about 15%) which gave M⁺ ion at *m/e* 590.6399 (C₄₁H₈₂O, calcd. 590.6362) in HR-GC-MS and gave (M+1)⁺ at *m/e* 591 in CI-GC-MS using isobutane. Toxin complex, Band 1-toxin and Band 2-toxin gave the same products and gave the same characteristic peaks at *m/e* 155, 211, 267, 407, 463 and 519 in low-resolution mass spectra. These characteristic peaks were analyzed by HR-GC-MS and following fragments were obtained.

fragments	complex	Band 1	Band 2	calcd.
C ₁₀ H ₁₉ O	155.1436	155.1478	155.1433	155.1436
C ₃₆ H ₇₁ O	519.5533	519.5517	519.5481	519.5504
C ₁₄ H ₂₇ O	211.2100	211.2082	211.2071	211.2062
C ₃₂ H ₆₃ O	463.4902	463.4903	463.4909	463.4876
C ₁₈ H ₃₅ O	267.2694	267.2690	267.2660	267.2688
C ₂₈ H ₅₅ O	407.4253	407.4271	407.4203	407.4252

These prominent peaks can be interpreted as the result of cleavage of the molecule on each side of 4H-pyran ring of three components.

Fragments in HR-MS (EI mode) of Band 1- and Band 2-toxins.²⁾

Fragments	Band 1-toxin	Band 2-toxin	Calcd.
C ₈ H ₁₃ O ₃	157.0879	157.0840	157.0864
C ₁₀ H ₁₉ O ₃	187.1330	187.1286	187.1333
C ₁₅ H ₂₄ O ₂	236.1775	236.1784	236.1775
C ₁₆ H ₂₄ O ₃	264.1716	264.1747	264.1724
C ₁₅ H ₂₂ O ₄	266.1520	266.1540	266.1518
C ₂₅ H ₃₆ O ₅	—	416.2557	416.2562
C ₃₃ H ₄₈ O ₅	—	524.3504	524.3502

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