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6-EPIOPHIOBOLIN A AND 3-ANHYDRO-6-EPIOPHIOBOLIN A—HOST SPECIFIC PHYTOTOXINS OF *DRECHSLERA MAYDIS* (RACE T)

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Key Word Index—*Zea mays*; Gramineae; Texas-male-sterile; *Drechslera maydis*; phytotoxins; 6-epiophiobolin; 6-epiophiobolin A; 3-anhydro-6-epiophiobolin A.

Abstract—6-Epiophiobolin A and 3-anhydro-6-epiophiobolin A were isolated from *Drechslera maydis* race T, the causal agent of Southern corn leaf blight in Texas-male-sterile (Tms) corn. The isolation of these compounds was followed throughout the fractionation protocol by a mitochondrial electron transport assay designed to identify those compounds that were specific for their inhibition of malate oxidation in Tms corn. These phytotoxins were not present in race O of the fungus, which, although pathogenic to corn, is not specific for Tms corn.

INTRODUCTION

Severe crop losses in Texas-male-sterile (Tms) corn occurred between 1969 and 1972 as a result of infection by *Drechslera maydis* (*Helminthosporium maydis*), the causal agent of Southern corn leaf blight [1, 2]. Although *D. maydis* was known to infect corn [3, 4], it soon became clear that there were two races of *D. maydis* that could be distinguished by their virulence on Tms corn [5, 6]. Race O or 'normal' fungus was found to infect a number of corn lines and cause lesion development while infection of Tms corn with race T fungus resulted in massive coalescing lesions resulting in death of the entire plant [6]. The rapidity with which the lesions spread from a single infection site suggested to Lim and Hooker [7] that the fungus employed a toxin as a disease determinant, and these workers were able to isolate a toxic fraction from infected corn leaves which would induce typical symptoms in Tms corn but not in normal corn. The host-specific nature of the *D. maydis* race T toxin has since been confirmed by subjecting various corn lines to culture filtrate and culture extracts of the fungus [8, 9].

There have been several attempts to isolate and chemically characterize the *D. maydis* race T toxin, but as yet there is no consensus as to its identity. It has been reported to be a low-M_r polypeptide [7, 10], a mixture of terpenoid alcohols [11, 12], a derivative of mannitol [13], and a C_{41}-polyketide [14]. In view of these conflicting reports, we began a programme to isolate and chemically characterize the *D. maydis* race T phytotoxin(s), and these studies led to the characterization of two terpenoids, namely 6-epiophiobolin A and 3-anhydro-6-epiophiobolin A [15]. The evidence that these compounds are present in *D. maydis* race T and are important in its pathogenicity to Tms corn is summarized below.

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RESULTS

Phytotoxin isolation

Extraction. The culture fluids from *D. maydis* race T, isolate A-T, and *D. maydis* race O, isolate A-O, were tested for host-specific activity by leaf lesion and malate-DCPIP mitochondrial oxidation assays (Table 1). The culture filtrate from isolate A-T was found to contain host-specific bioactivity as measured by both assays (Table 1), but considerable non-host-specific bioactivity was present in the culture filtrates of both isolates. The host-specific bioactivity from isolate A-T as well as the non-host-specific bioactivity from both isolates (A-T and A-O) partitioned into the organic phase upon extraction of the culture filtrates with dichloromethane, chloroform or ethyl acetate (Table 1). In later preparations, removal of bioactivity from the isolate A-T culture filtrate was accomplished by adsorption to Amberlite XAD-2 resin and elution with ethanol (Table 1).

Host-specific bioactivity was recovered from the mycelial mat of isolate A-T by extraction with dichloromethane-methanol (2:1) as tested by both assays (Table 1). Non-host-specific bioactivity was also extracted from A-T and A-O mycelial mats under these conditions, and qualitatively it appeared that a greater proportion of non-host-specific activity was present in these mycelial extracts.

Sephadex LH-20 chromatography. Shown in Fig. 1 are typical elution profiles for the extracts from the isolate A-T culture filtrate (Fig. 1A), isolate A-T mycelial mat (Fig. 1B), and isolate A-O culture filtrate (Fig. 1C). The profile for the isolate A-O mycelial mat extract was similar to that of the A-O culture filtrate extract (Fig. 1C) and is not shown.

The results of leaf lesion assays of isolates A-T and A-O as lesion length on Tms corn versus Sephadex LH-20 fraction number are shown in Fig. 2. While lesions varied from four to 15 cm in length between assays, the host-

Table 1. Leaf lesion and mitochondrial oxidation assays of *D. maydis* isolate A-T and A-O extracts on Tms and N corn

	Leaf lesion*				Mitochondrial oxidation†			
	A-T		A-O		A-T		A-O	
Extract	Tms	N	Tms	N	Tms	N	Tms	N
					Percent inhibition			
Culture filtrate	+4	+2	+2	+2	100	30	40	45
Organic extract	+4	+2	+2	+2	100	35	45	40
Aqueous residue	+1	+1	0	0	10	15	15	10
XAD-2 effluent	0	0	—‡	—	<10	<10	—	—
XAD-2 EtOH eluant	+4	+2	—	—	100	35	—	—
Mycelial extract	+4	+3	+3	+3	100	65	70	70

*1:10 dilution; legend: 0, <0.5 cm; +1, 0.5–1.0 cm; +2, 1.0–1.5 cm; +3, 1.5–2.0 cm; +4, >2.0 cm.

†1:100 dilution; values are relative to controls run with the same mitochondrial preparation; standard error ± 5%.

‡Not tested.

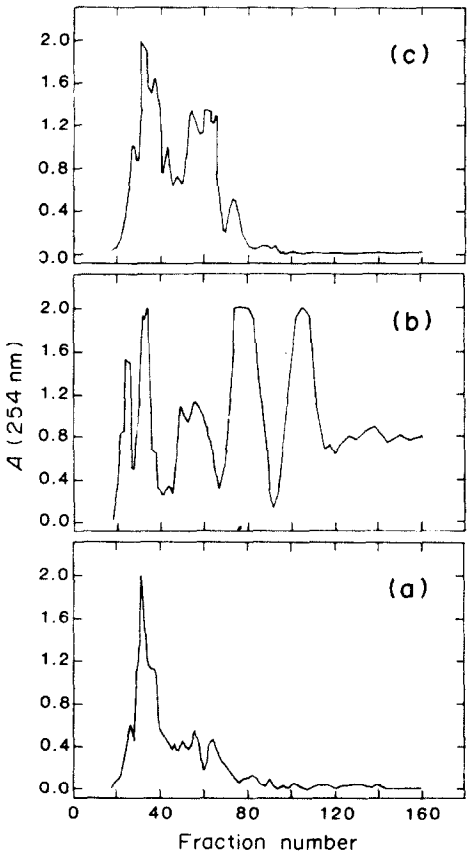


Fig. 1. Fractionation of the dichloromethane extracts from the *D. maydis* isolate A-T culture filtrate (a), isolate A-T mycelial mat (b), and isolate A-O culture filtrate (c) by chromatography on Sephadex LH-20.

specific toxicity of the first A-T fraction was reproducible over a dilution range of 1:10–1:100 of the original culture concentration. Lesions on N corn varied from 0.0–1.0 cm in length in a random pattern for both isolate A-T and isolate A-O.

Inhibition of DCPIP reduction in Tms mitochondria with malate as substrate was measured across the Sephadex LH-20 elution profiles of the isolate A-T and A-O culture extracts at 1:600 dilution as shown in Fig. 3. As with the leaf lesion assay, host-specific bioactivity was observed in the early eluting fractions of the isolate A-T culture extract (Fig. 1A). At 1:3600 dilution, isolate A-O fractions no longer inhibited Tms mitochondria while the response due to isolate A-T fractions was undiminished. Assays with N corn mitochondria showed that the isolate A-O fractions inhibited reduction in N and Tms corn mitochondria equally, while isolate A-T inhibition was specific for Tms corn.

In a comparison of Sephadex LH-20 fractions derived by chromatography of the isolate A-T culture filtrate (see Fig. 1A) and A-T mycelial mat (see Fig. 1B) at 1:600 dilution, both extracts contained host-specific bioactivity in the first two early eluting fractions (Fig. 4). The isolate A-T mat extract also contained material which inhibited both Tms and N corn mitochondria, and a similar activity profile was observed for the isolate A-O mat extract.

Silica gel chromatography. Further purification of fraction A-T-1 (Fig. 3) was accomplished using a three-part step gradient on silica gel. The sample was loaded in dichloromethane and a coloured band was eluted, followed by a five bed volume column wash. A second coloured band was removed by elution with five bed volumes of acetonitrile, and finally, the remainder of the coloured material at the top of the column was removed by elution with methanol. Malate-DCPIP mitochondrial assays established that no inhibitory activity was present in fractions collected after the five bed volume wash in any elution step. The three fractions were dried by evaporation under a stream of nitrogen, redissolved in acetonitrile or methanol, and bioassayed by malate-DCPIP mitochondrial oxidation in both Tms and N corn. At *ca* 1 ppm dilution, the dichloromethane fraction was not bioactive and the acetonitrile and methanol fractions were, but the acetonitrile fraction was consistently more bioactive than the methanol fraction in repeat experiments.

Reverse-phase HPLC. The acetonitrile and methanol fractions from column chromatography were further

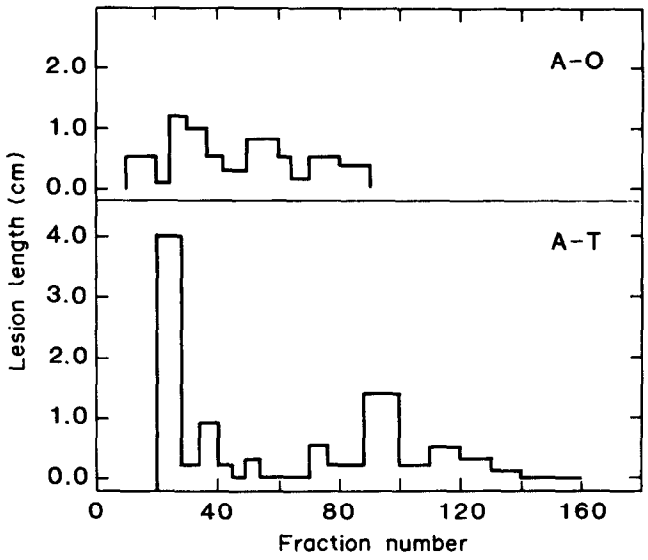


Fig. 2. Leaf lesion assays of the fractions obtained by Sephadex LH-20 column chromatography of the dichloromethane extracts of isolate A-T culture filtrate (Fig. 1A) and isolate A-O culture filtrate (Fig. 1C). Fractions were diluted 1:10 in 50% ethanol and 50 μl aliquots were applied. Fraction number refers to the original tube number (Fig. 1).

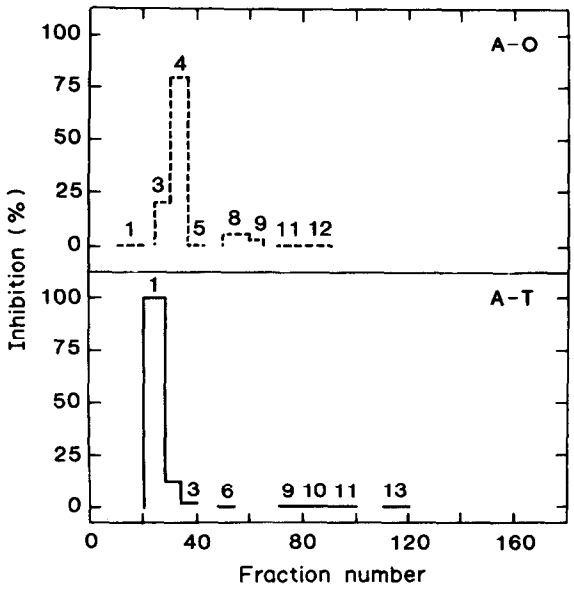


Fig. 3. Malate-DCPIP oxidation assays of selected Sephadex LH-20 fractions derived from isolate A-T (see Fig. 1A) and isolate A-O (see Fig. 1C). Numerals refer to pooled fraction number. Fraction number refers to original tube number (Fig. 1). Dashed line: percent inhibition equal in N and Tms corn mitochondria. Solid line: inhibition specific for Tms corn mitochondria.

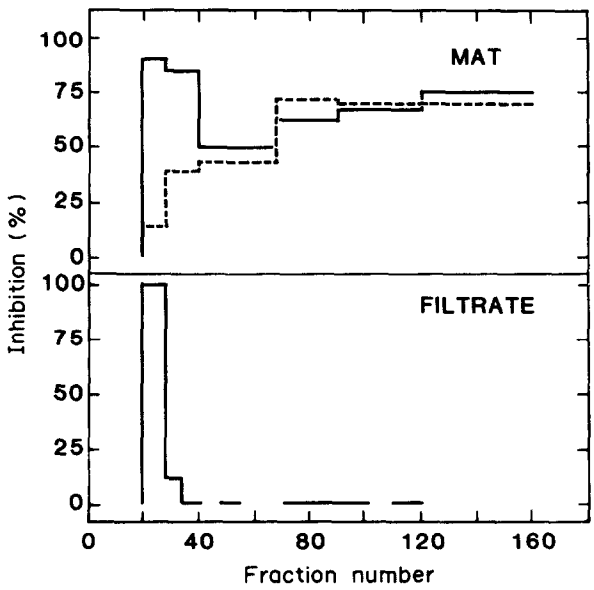


Fig. 4. Malate-DCPIP oxidation assays of Sephadex LH-20 fractions of isolate A-T mycelial mat (from Fig. 1B) and seleted Sephadex LH-20 fractions of isolate A-T culture filtrate (from Fig. 1A). Fraction number refers to the original tube number (Fig. 1). Both Tms mitochondria (solid line) and N mitochondria (dashed line) were assayed at 1:600 dilution of sample.

analysed by reverse-phase HPLC. Samples were dissolved in 50% acetonitrile, injected on to the column, and eluted with a 45 min linear gradient of 9:1 water–acetonitrile to 100% acetonitrile at a flow rate of 4 ml/min. The column effluent was monitored by absorbance at 254 nm and 195 nm. The methanol fraction (chromatogram not shown) contained a broad tailing band of material and a number of smaller peaks. Malate-DCPIP mitochondrial assays indicated that the region of maximal bioactivity was under the broad band. The

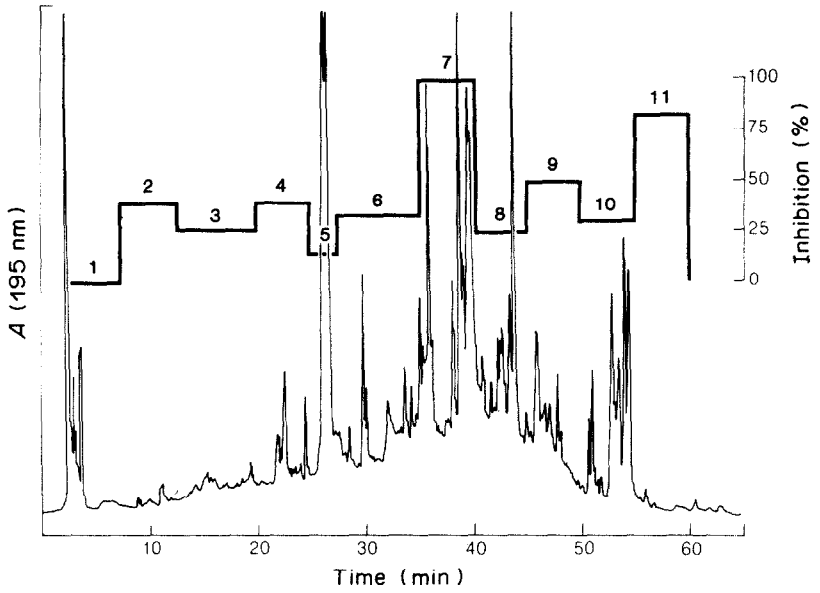


Fig. 5. Fractionation of the acetonitrile fraction from silica gel chromatography by reverse phase HPLC. Fractions were assayed by malate-DCPIP mitochondrial oxidation as described in the text. Numerals refer to fraction number.

acetonitrile fraction, shown at 195 nm in Fig. 5, was much better resolved and was therefore chosen over the methanol fraction for further work. The effluent in Fig. 5 was analysed by the malate-DCPIP mitochondrial assay, and the percent inhibition of oxidation in Tms mitochondria is shown at the top of Fig. 5. Two major and three minor regions of inhibition were observed, and all but the last were consistent with absorbance at 195 nm. Due to the mechanics of sample collection, the inhibition observed in Fraction 11 is most likely an overlap of the late-eluting peaks in Fraction 10.

Fraction 7 (Fig. 5), designated ACN-7, was reapplied to the column and eluted with a 30 min linear gadient of 13:7 water–acetonitrile to 1:1 water–acetonitrile at a flow rate of 4 ml/min. The elution profile of ACN-7 and the percent inhibition of mitochondrial oxidation in Tms corn is shown in Fig. 6. Fraction 1 (Fig. 6) was identified as residual material from the earlier portion of the previous chromatographic separation (Fig. 5). At this level of inhibition of mitochondrial oxidation, the order of inhibition by Fractions 1 and 7 (Fig. 6) is not significant. Fraction 7 (Fig. 6), designated ACN-7.7, was further fractionated on the same reverse-phase column by isocratic elution with water–acetonitrile (21:29) at 4 ml/min. The elution profile together with the resultant malate-DCPIP mitochondrial oxidation assay profile, are shown in Fig. 7. Two regions of bioactivity are observed in Fig. 7. The first region is comprised of Fractions 6 and 7, both of which are host-specific in their inhibition. The second region, comprised of Fractions 8 and 9, exhibited a mixture of effects. Fraction 8 inhibited oxidation in Tms and N mitochondria equally, while Fraction 9 showed moderate selectivity for inhibition of oxidation in Tms mitochondria. Fractions were not assayed at other concentrations in order to conserve sample.

Fraction 6 (Fig. 7), designated ACN-7.7.6, was purified to homogeneity by reapplication to the reverse phase

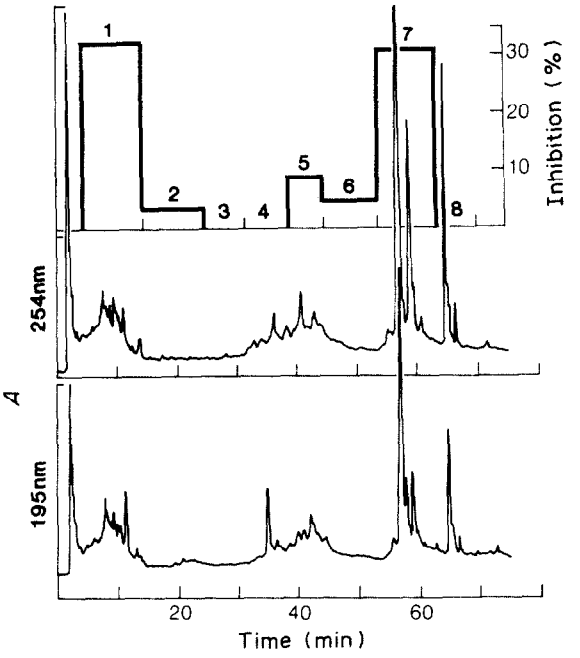


Fig. 6. Fractionation of fraction ACN-7 (Fig. 5) by reverse phase HPLC. Fractions were assayed by malate-DCPIP mitochondrial oxidation as described in the text. Numerals refer to fraction number.

column. Fraction 7 (Fig. 7), designated ACN-7.7.7, was reapplied to the reverse-phase column and was found to contain both ACN-7.7.6 and ACN-7.7.7. The peak corresponding to ACN-7.7.7 was collected and again applied to the reverse phase column, and again the peak corre-

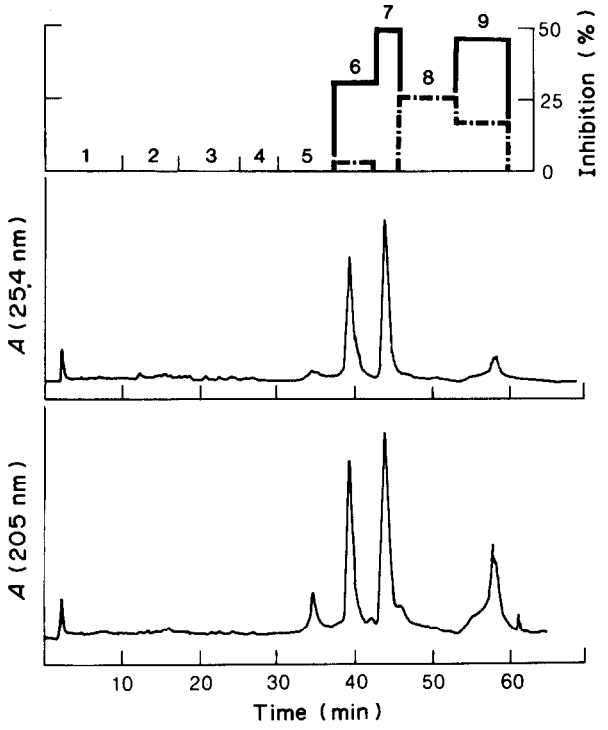


Fig. 7. Fractionation of fraction ACN-7.7 (Fig. 6) by reverse phase HPLC. Fractions were assayed by malate-DCPIP mitochondrial oxidation with Tms mitochondria (solid line) and N mitochondria (dashed line) as described in the text. Numerals refer to fraction number.

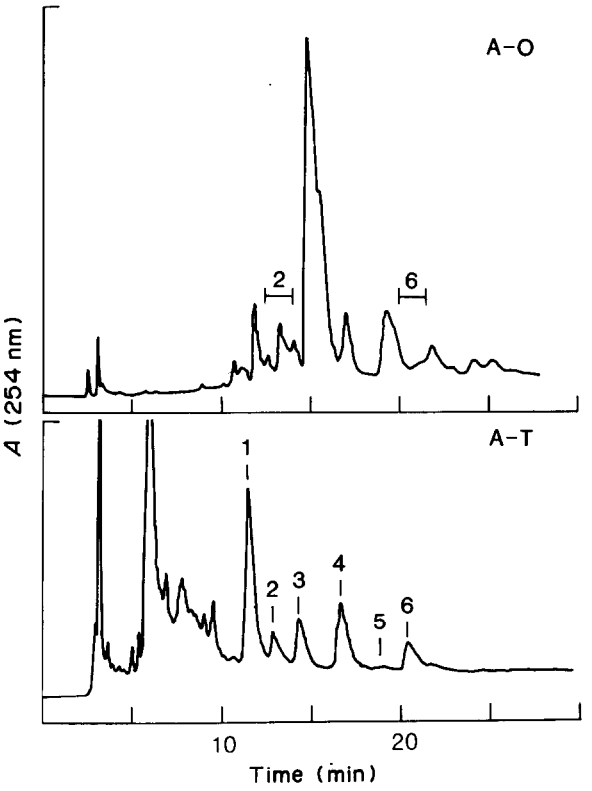


Fig. 8. Fractionation of ACN-7 from isolate A-T (Fig. 5) and the corresponding fraction from isolate A-O by normal-phase HPLC. Numerals refer to isolate A-T fraction numbers and the corresponding regions in the isolate A-O chromatogram.

sponding to ACN-7.7.6 was present. The recovered weight of ACN-7.7.7 decreased with each separation and the absorbance ratio of ACN-7.7.6 to ACN-7.7.7 increased, indicating that ACN-7.7.7 was being converted to ACN-7.7.6 at some point in the separation.

Normal-phase HPLC. In an effort to minimize the conversion of ACN-7.7.7 to ACN-7.7.6, normal-phase HPLC on silica was investigated. Aliquots from fraction ACN-7 (Fig. 5) were dissolved in 7:3 hexane–ethyl acetate and were chromatographed on a silica column in the same solvent at a flow rate of 4.5 ml/min. The elution times of ACN-7.7.6 and ACN-7.7.7 were established by comparison with samples previously purified by reverse-phase HPLC (Fig. 7). Fraction ACN-7 from isolate A-T is compared to a similar fraction from isolate A-O in Fig. 8. In the isolate A-T profile, Peak 2 corresponds to ACN-7.7.6 and Peak 6 corresponds to ACN-7.7.7. The comparable peaks appeared to be missing from isolate A-O, and chemical ionization mass spectrometry confirmed this conclusion (results discussed below). Malate-DCPIP mitochondrial oxidation assays demonstrated that Peaks 2 and 6 (Fig. 8) were host-specific for Tms corn; no inhibition of mitochondrial oxidation in N corn was observed. Peak 6 (Fig. 8), corresponding to ACN-7.7.7, remained stable in this solvent system and both Peak 2 and Peak 6 were purified to homogeneity by reapplication to the silica column.

Preparative isolation protocol. Based upon experiences with the isolation protocol just described, a more efficient protocol was developed for the preparative isolation of the two bioactive components designated ACN-7.7.6 and ACN-7.7.7. Harvested cultures were separated into filtrate and mycelial mat fractions. The filtrate was treated with an adsorbent, Amberlite XAD-2, and after washing with water to remove all non-adsorbed components, bioactive components were removed by elution with 95% ethanol. The mycelial mat was homogenized and extracted with dichloromethane–methanol (2:1), and the extract was combined with the above 95% ethanol eluant and evaporated to dryness. The combined extracts were chromatographed on Sephadex LH-20 as described in Fig. 1, and bioactive fractions were pooled, evaporated to dryness, then chromatographed on a short silica gel column as described previously. The acetonitrile fraction was separated on a reverse-phase C-18 Sep Pak, with sequential elution with acetonitrile and dichloromethane. The acetonitrile eluant was evaporated and the residue was dissolved in ethyl acetate and passed through a Silica Sep-Pak. The eluant was evaporated to dryness and subjected to normal-phase HPLC as described in Fig. 8. Fractions corresponding to ACN-7.7.6 (Peak 2) and ACN-7.7.7 (Peak 6) were collected, and the individual components were purified to homogeneity by reapplication to the column as needed.

Structural characterization

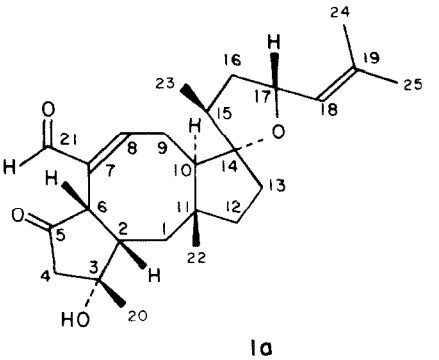
Positive-ion ammonia CIMS. Normal-phase HPLC of comparable isolate A-T and A-O fractions (Fig. 8) sug-

gested that Peaks 2 and 6 in the A-T profile, corresponding to ACN-7.7.6 and ACN-7.7.7, respectively, did not occur in the isolate A-O profile. To confirm this, similar regions in the two chromatographic profiles were compared by CIMS with ammonia as the reagent gas. Peak 2 (ACN-7.7.6) in the isolate A-T profile was found to give a mass ion at m/z 399.7 which represented 35% of the total ion current, whereas the identical region in the isolate A-O profile did not give this ion. The largest observed mass ion in the Peak 2 region of the isolate A-O profile was 383.1, representing 5% of the total ion current. Peak 6 (ACN-7.7.7) in the isolate A-T profile gave a mass ion at m/z 417.8, representing 30% of the total ion current, and again, this ion was not observed in the A-O profile. The largest mass ion observed in the Peak 6 region of the A-O profile was m/z 391.1, which represented 3% of the total ion current. These results confirmed the observation that components ACN-7.7.6 (Peak 2, Fig. 8) and ACN-7.7.7 (Peak 6, Fig. 8) were present only in extracts of isolate A-T.

High Resolution EIMS. Given in Table 2 are fragmentation ions of components ACN-7.7.6 and ACN-7.7.7 together with computer-generated elemental assignments. Isotopic ratios did not indicate the presence of nitrogen, sulphur, halogens, or higher elements in the parent ion. The parent ion of ACN-7.7.7 (m/z 400.2648) indeed did contain one molecule of water more than the parent ion of ACN-7.7.6 (m/z 382.3526), consistent with the results of CIMS. In addition, the M_rs observed for these parent ions in high resolution EIMS demonstrate that the ions observed in positive-ion ammonia CIMS represented ammonium cluster ions.

A search of the literature for known *Drechslera* metabolites revealed that Nozoe *et al.* [16] and Canonica *et al.* [17] had independently characterized a C_{25}-terpenoid (M_r 400) and its dehydration product (M_r 382) as ophiobolin A (**1a**) and 3-anhydroophiobolin A(**2**), respectively. Nozoe *et al.* [18] later reported a set of mass fragmentation ions characteristic of the ophiobolin A ring nucleus, namely m/z 273, 176 and 165. As these fragments were also observed in the mass spectra of components ACN-7.7.6 and ACN-7.7.7, an authentic sample of ophiobolin A was obtained for comparison. The mass spectral fragmentation pattern of ophiobolin A (Table 2) was found to be virtually identical to that of component ACN-7.7.7, with the exception that an ion of m/z 364 was not present in the spectrum of ophiobolin A. Incidentally, an ion of m/z 364 was detected in a low resolution EIMS of ophiobolin A.

^{1}H NMR Spectroscopy. The ^{1}H NMR spectra of both ACN-7.7.6 and ACN-7.7.7 were very similar to the spectrum of ophiobolin A, but careful analysis of the chemical



1a

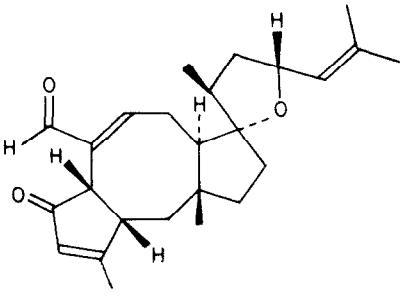


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Table 2. M_rs and elemental compositions of fragments observed in the HR-EIMS of components ACN-7.7.6 and ACN-7.7.7, and ophiobolin A

			ACN-7.7.6		ACN-7.7.7		Ophiobolin A	
C	H	O	m/z	%	m/z	%	m/z	%
25	36	4	—	—	400.2648	9.7	400.2631	12.3
25	34	3	382.2526	24.1	382.2491	6.9	382.2507	11.3
25	32	2	364.2441	3.5	364.2357	2.0		—
19	26	4		—	318.1815	11.8	318.1694	10.5
19	24	3	300.1741	19.2	300.1716	13.4	300.1716	16.8
19	23	2	283.1683	7.2	283.1710	4.5	283.1705	10.8
17	21	3	273.1492	21.7	273.1516	18.5	273.1506	23.8
17	19	2	255.1374	7.0	255.1476	5.6	255.1401	9.6
16	21	2	245.1543	9.8	245.1568	9.5	245.1543	7.7
15	19	2	231.1381	3.2	231.1347	5.0	231.1328	8.4
11	12	2	176.0812	53.7	176.0812	9.8	176.0779	8.8
11	17	1	165.1268	35.3	165.1245	69.8	165.1269	70.0
9	13	1	137.1004	7.5	137.0987	6.1	137.0980	13.9
8	13	0	109.1073	100	109.1046	100	109.1068	100

shifts and multiplicities of the resonances indicated important differences. Proton-proton decoupling experiments led to the assignment of most of the resonances in the three spectra, and these are tabulated in Table 3. The assigned resonances were numbered according to the convention proposed by Tsuda *et al.* [19], as indicated in structure **1a**.

The ^{1}H NMR spectra of ophiobolin A (**1**) and ACN-7.7.7 both gave integral values for 36 protons, as expected. Significant differences were observed in the chemical shift and multiplicity of several of the resonances, however, in particular those ascribable to H-2, -4, -6, -8, and -9. Otherwise, the spectra were remarkably similar.

Because of the fact that the major differences in these spectra were attributable to protons attached to the fused cyclopentanone and cyclooctene rings, it was suspected that ophiobolin A and ACN-7.7.7 might differ in their geometry of ring fusion at C-2 and C-6. A series of N.O.E. difference spectra were therefore obtained for both ophiobolin A and ACN-7.7.7. For ophiobolin A (**1b**, shown in stereochemical perspective), irradiation of the H-6 resonance (δ 3.26) gave a pronounced N.O.E. of the H-2 resonance (δ 2.36). Furthermore, irradiation of the C-22 methyl resonance (δ 0.83) gave a pronounced N.O.E. for both the H-2 (δ 2.36) and the H-6 (δ 3.26) resonances. A N.O.E. was also detected for H-6 (δ 3.26) upon irradiation of the H-9α resonance at δ 2.25. These results

Table 3. Assignments of resonances in the ^{1}H NMR spectra of ophiobolin A(**1**), and components ACN-7.7.7 and ACN-7.7.6 (Fig. 7) (300 MHz, $CDCl_3$, TMS as int. standard)

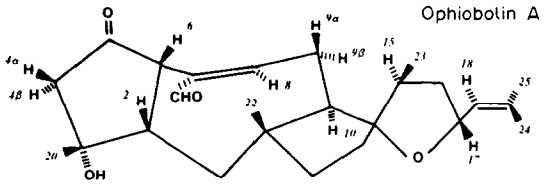
H*	Ophn A (**1**)†	ACN-7.7.7 (**3**)‡	ACN-7.7.6 (**4**)
2	2.36 *ddd*, 3.0, 10.6, 12.6 §	2.12 *ddd*, 4.5, 10.6, 12.2	2.64 *complex*
4α	2.50 *d*, 19.3	2.41 *dd*, 1.6, 16.8	
4β	2.81 *d*, 19.3	3.06 *d*, 16.8	6.04 *quint*, 1.5
6	3.26 *d*, 10.6	3.35 *br d*, 10.9	3.42 *d*, 4.2
8	7.21 *dd*, 8.2, 8.6	6.87 *dd*, 2.3, 6.9	6.82 *dd*, 2.6, 6.7
9α	2.25 *dt*, 8.7 11.4	2.33 *ddd*, 7.1 13.7, 19.1	2.34 *ddd*, 6.7, 13.8, 19.3
9β	2.42 *dd*, 8.2, 11.4	2.78 *ddd*, 2.3, 4.4, 19.1	2.84 *ddd*, 2.6, 3.9, 19.3
10		2.62 *dd*, 4.3, 13.8	2.67 *dd*, 3.9, 13.9
17	4.42 *dt*, 5.8, 8.6	4.61 *dt*, 7.0, 8.6	4.60 *dt*, 7.1, 8.7
18	5.15 *dsept*, 1.4, 8.6	5.14 *dsept*, 1.4, 8.6	5.13 *dsept*, 1.4 8.7
20	1.36 *s*	1.42 *s*	2.05 *m*
21	9.23 *s*	9.21 *s*	9.32 *s*
22	0.83 *s*	0.85 *s*	0.88 *s*
23	1.09 *d*, 7.1	1.04 *d*, 7.0	1.05 *d*, 6.9
24	1.71 *d*, 1.4	1.68 *d*, 1.4	1.67 *d*, 1.4
25	1.74 *d*, 1.4	1.71 *d*, 1.4	1.71 *d*, 1.4
OH	3.22 *br s*	3.34 *br s*	—

*Resonances not assigned were in a complex region of the spectrum and were obscured by other resonances.

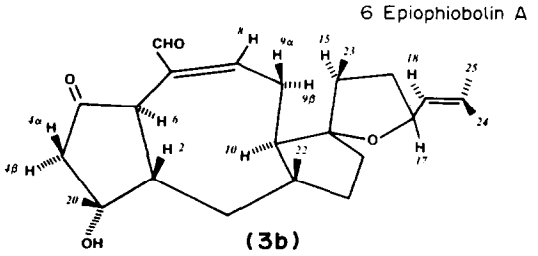
†Values are in agreement with those previously reported in refs [16, 17].

‡Values are in agreement with those previously reported in ref. [20].

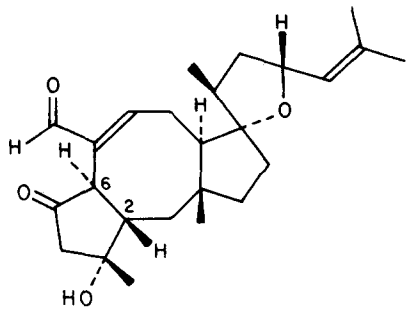
§Listed sequentially are the chemical shift (δ), multiplicity, and coupling constant(s) (Hz).



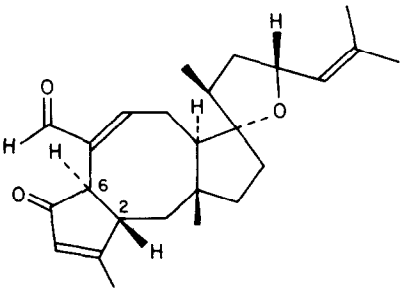
(1b)



(3b)



(3a)



(4)

indicate that the C-22 methyl group is in close proximity to both H-2 and H-6 whereas H-9α is in close proximity only to H-6. Indeed, these are exactly the results expected based upon inspection of a molecular model of ophiobolin A.

A different pattern of N.O.E.'s was observed for ACN-7.7.7, however. Irradiation of H-6 (δ 3.35) gave rise to an N.O.E. at δ 2.62 (H-10) and not at δ 2.12 (H-2), and conversely, irradiation of H-10 (δ 2.62) gave rise to an N.O.E. at H-6 (δ 3.35). These results therefore establish that the cyclopentanone and cyclooctene rings of ACN-7.7.7 are *trans*-fused, as suspected. Component ACN-7.7.7 is therefore 6-epiophiobolin A (**3a**). Inspection of a

molecular model of **3a** (as represented by **3b**) indeed reveals that H-6 and H-10 are in close proximity.

The ^{1}H NMR spectrum of ACN-7.7.6 was very similar to that of ACN-7.7.7 except for the absence of the H-4 methylene and hydroxyl proton resonances and the presence of a new vinylic proton resonance (δ6.04). The C-20 methyl resonance (δ2.05) of ACN-7.7.6 was also shifted considerably downfield relative to its position in ACN-7.7.7 (δ1.42). Component ACN-7.7.6 is therefore a 3-anhydro-ophiobolin derivative. The stereochemistry of ring fusion at C-2 and C-6 was determined by N.O.E. experiments as described above. Irradiation of the H-6 resonance (δ3.42) resulted in an N.O.E. at H-10 (δ2.67) and not at H-2, and conversely, irradiation at H-10 gave an N.O.E. at H-6. Therefore, component ACN-7.7.6 is 3-anhydro-6-epiophiobolin A (**4**).

Comparative bioassays

3-Anhydro-6-epiophiobolin A(**4**) and ophiobolin A (**1**) were compared in a series of DCPIP mitochondrial oxidation assays using both Tms and N corn mitochondria with malate or succinate as substrates. The results for malate and succinate oxidation in Tms mitochondria are shown in Fig. 9. The concentration at which 50% inhibition of oxidation occurred in Tms mitochondria (I_{50}) was found to be 15 μM for 3-anhydro-6-epiophiobolin A (**4**) when malate was used as the substrate. Over the same range of concentrations, **4** did not inhibit oxidation in Tms mitochondria when succinate was used as the substrate (Fig. 9). Ophiobolin A (**1**) was found to inhibit oxidation in Tms mitochondria, but at much higher concentrations; i.e. the I_{50} for **1** was found to be 290 μM when malate was used as the substrate and 650 μM when succinate was used as the substrate. Ophiobolin A (**1**) and 3-anhydro-6-epiophiobolin. A (**4**) were also tested for their ability to inhibit oxidation in N mitochondria at the concentration (290 and 15 μM, respectively) found to give 50% inhibition of malate oxidation in Tms mitochondria; ophiobolin A was found to inhibit malate oxidation by 58% and succinate oxidation by 18% in N mitochondria (at 290 μM), whereas **4** did not inhibit oxidation of either malate or succinate in N mitochondria at a concentration of 15 μM.

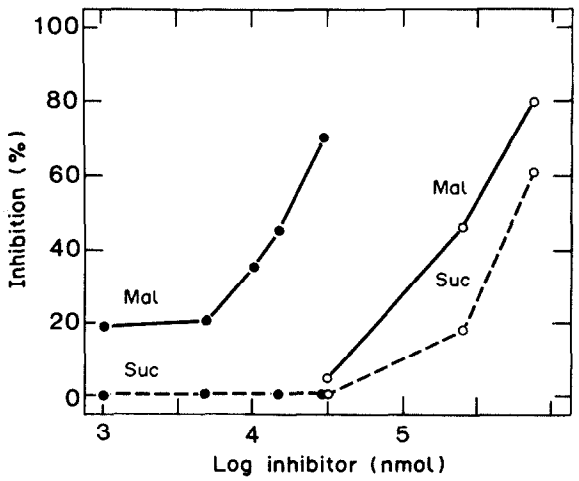


Fig. 9. Inhibition of malate- and succinate-DCPIP Tms mitochondrial oxidation by 3-anhydro-6-epiophiobolin A (**4**, ●——● and ●----●, respectively) and ophiobilin A (**1**, ○——○ and ○----○, respectively). Assays were performed as described in the text.

Due to a limited sample size, 6-epiophiobolin A (**3**) was compared to ophiobolin A (**1**) and 3-anhydro-6-epiophioblin A (**4**) at only two concentrations. At concentrations of 0.5 and 2.5 μM, **3** inhibited malate oxidation in Tms mitochondria by 40 and 53%, respectively, giving an I_{50} value of *ca* 2.4 μM. Neither succinate oxidation in Tms mitochondria nor malate or succinate oxidation in N mitochondria were inhibited by these concentrations (0.5 and 2.5 μM) of 6-epiophiobolin A (**3**).

DISCUSSION

Because of conflicting reports as to the identity of the *D. maydis* race T phytotoxin, a different method of isolation was deliberately chosen in order to provide a fully independent approach coupled with this isolation protocol was a routine assay for phytotoxin activity that guided the selection of fractions for further evaluation.

Of the approximately 50 assays that have been reported for host-specific effects of *D. maydis* race T on Tms corn (see [21] for a review), a mitochondrial oxidation assay was chosen for routine use because of extensive evidence that mitochondria are the site of action of the toxin [21–30]. The actual assay chosen for these studies was the reduction of 2,6-dichlorophenol-indophenol (DCPIP) in the presence of malate [31], as previously recommended by others for use as part of an isolation protocol [32, 33]. The original assay was modified in several important respects, however. First, cyanide ion was included in the assay as recommended by Gengenbach and Green [25]. In the presence of KCN, DCPIP replaces cytochrome *a* as an electron acceptor from cytochrome *c* oxidase and, in its absence, competes with cytochrome *a* for reducing equivalents [34]. Hence, KCN is required to ensure maximal rates of DCPIP reduction. Second, bovine serum albumin was removed from the assay media after it was observed that it reduced the level of inhibition by non-specific adsorption of the toxin. Third, the replacement of Tris buffer with 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) buffer was found to increase Tms mitochondrial viability. Finally, mannitol was used as an osmoticant in place of sucrose with a resultant increase in mitochondrial stability, as suggested by Quail [35]. It has also been shown that mitochondria in mannitol-containing media are more sensitive to race T toxin than in sucrose media [36, 37].

In order to identify those fractions in the isolation protocol that were host-specific for Tms-corn, malate-DCPIP mitochondrial assays were always performed in parallel with mitochondria derived from both a Tms corn line (W64A Tms × A297) and its non-Tms parent (N corn, W64A × A297). Furthermore, the isolation protocol was carried out in parallel with extracts from *D. maydis* race O fungus and mitochondrial assays and leaf lesion assays were performed at critical stages of purification of the phytotoxin. These studies were included in order to establish whether the phytotoxin ultimately isolated from race T fungus was also present in race O fungus.

These studies ultimately led to the isolation of two host-specific phytoxins from race T fungus, namely 6-

epiophiobolin A (**3**) and 3-anhydro-6-epiophiobolin A (**4**). These two epiophiobolin derivatives were indeed not present in race O fungus, as judged by HPLC of extracts of the race T and race O fungi under identical conditions and analysis of the eluted components by chemical ionization mass spectrometry (Fig. 8). Compounds **3** and **4** were not the only host-specific toxins present in the race T fungus, however. Once it was clear that multiple toxins existed, as previously postulated [11, 22, 38–40], the decision was made to focus initially on the characterization of one toxin in the belief that its structure would be the key to a possible family of toxins. The acetonitrile fraction from silica gel column chromatography was therefore chosen over the methanol fraction both because it contained a higher specific activity and was better resolved by reverse-phase HPLC (Fig. 5). Fraction 7 (Fig. 5) was similarly chosen for further examination because it contained a small number of peaks that were readily resolved by reverse phase HPLC (Figs 6 and 7). It should be mentioned, however, that fractions ACN-4 (Fig. 5) and ACN-7.1 (Fig. 6), which are both bioactive, were examined by ^{1}H NMR spectroscopy and both spectra (data not given) contained characteristic 6-epiophiobolin resonances for H-8, H-17, H-18 and H-21. These spectra were different from those of 3 and 4 but were not further analysed. The data clearly suggest that a family of epiophiobolin toxins is present in the race T fungus. It is also clear from the HPLC-bioassay profiles (Figs 5–7) that other toxins are present, however, and it is not known whether all of these are members of the epiophiobolin class or are those proposed earlier by other workers [14, 15].

The purified pathotoxins (**3, 4**) were finally compared with ophiobolin A (**1**) as inhibitors of mitochondrial respiration in corn. Ophiobolin A (**1**) inhibited both malate and succinate oxidation in Tms as well as N corn mitochondria (Fig. 9). 6-Epiophiobolin A (**3**) and 3-anhydro-6-epiophiobolin A (**4**), however, specifically inhibited malate oxidation in Tms corn mitochondria. The oxidation of malate in Tms mitochondria was *ca* 100-fold more sensitive to 6-epiophiobolin A (**3**) than to ophiobolin A (**1**) and *ca* 20-fold more sensitive to 3-anhydro-6-epiophiobolin A (**4**) than to ophiobolin A (**1**). These levels of inhibition are best considered a rough comparison rather than an absolute measure of toxicity, however, since the variance observed in the inhibition of Tms corn mitochondria by various toxin preparations may be more a function of the age of the mitochondria, their method of preparation, and assay conditions [30, 32]. Never-the-less, both of the epiophiobolins (**3, 4**) exhibited remarkable selectivity as inhibitors of malate oxidation in Tms corn mitochondria; i.e. at the concentrations where **3** (2.4 μM) and **4** (15 μM) gave 50% inhibition of malate oxidation in Tms mitochondria, neither inhibited mitochondrial malate oxidation in N corn and neither inhibited mitochondrial succinate oxidation in either Tms or N corn. These results are consistent with the previously reported effects of the toxin; i.e. malate–pyruvate oxidation and the associated α-ketoglutarate oxidation have been reported to be completely inhibited by phytotoxin extracts [30, 32, 33, 36, 37, 41–46], whereas succinate oxidation in Tms corn has been reported to be either weakly inhibited or weakly simulated by addition of toxin [21, 30, 32, 36]. The inhibition of malate, pyruvate and α-ketoglutarate oxidation together with the mixed effects on succinate oxidation has led several workers to conclude that the phytotoxin inhibits mitochondrial electron transport prior to ubiquinone [30, 33, 45]. The mechanism by which the toxin exerts this effect, however, is not known.

EXPERIMENTAL

Materials. Samples of *Drechslera maydis* races T and O were obtained from Professor O. C. Yoder of Cornell University. Isolate A- O was from line 141-2-3-race O-sex type A. Isolate A-T, line 141-2-1- race T-sex type A, was defined by Professor Yoder (personal communication), as nearly isogenic to isolate A-O. Corn lines W64A Tms × A297 and W64A × A297 were obtained from the Minnesota Crop Improvement Association, St. Paul, Minnesota.

Culture of D. maydis. D. maydis isolates were cultured by a modification of the method of ref. [25]. Leaves infected with *D. maydis* were surface sterilized with 50% EtOH, placed on Difco potato-dextrose agar plates and wet with several drops of sterile H_2O. Isolates were incubated at room temp. for 7 days then transferred to ground-corn-leaf agar plates made from 40–45 *g* fr. corn leaves/l Difco Bacto-Agar [47].

Prior to inoculation of fresh cultures, stock isolates were transferred to fresh-ground corn leaf agar plates. After 72 hr these plates were divided into 1 cm squares under sterile conditions. Fresh culture media was then inoculated with one section per litre. The culture medium consisted of 2% dextrose, 0.01% NH_4NO_3, 0.01% KH_2PO_4, 0.005% $MgSO_4$, 0.0013% $CaCl_2$, 0.001% NaCl and 0.05% of a micronutrient stock consisting of 0.12% $FeCl_2$, 0.17% $ZnSO_4$, 0.07% $MnSO_4$, and 0.05% $CuSO_4$ per litre of stock soln. Cultures were incubated either on a gyrorotatory shaker for 10 days at 23° or in still culture at room temp. until grown to confluence (*ca* 21 days). Cultures were harvested by filtration through 4-ply cheesecloth. The mycelial mat was stored at 0° in Al foil for later extraction.

Extraction of phytotoxin. In early preps, the culture filtrate (1 l) was concd 10-fold by evapn under vacuum at 40°. The concentrate was diluted with 200 ml MeOH and refrigerated overnight. The soln was vacuum-filtered through Whatman analytical grade paper and the filtrate was evapd under vacuum to remove MeOH. The resultant aq soln (*ca* 100 ml) was extracted (× 2) with two vols of EtOAc and the pooled EtOAc extracts were evapd to dryness under vacuum. The residue was dissolved in 100 ml EtOAc, filtered, and evapd to dryness. The residue (*ca* 250 mg/l of culture) was stored at 0°.

In later preparations, the culture filtrate was concentrated on a column (5 × 100 cm) of Amberlite XAD-2. The resin was preconditioned before each use by extraction sequentially with MeOH, MeCN, $CHCl_3$, MeCN and H_2O for a minimum of 12 hr each in a Soxhlet extractor. The resin was then slurry packed into the column and kept wet until use to avoid bead fracture. The culture filtrate was passed through the column at 750 ml/hr, and after 10 l of culture filtrate had been processed, the column was washed sequentially with distilled H_2O (1 l), 95% EtOH (2 l) and distilled H_2O (1 l). The EtOH and final H_2O washes were pooled and set aside. Another 10 l of filtrate was then processed and the cycle was repeated until all culture filtrate (up to 40 l) was processed. In a final wash, *i*PrOH (2 l) was passed through the column and was pooled with the combined EtOH and second H_2O washes. Solvents were removed by evapn under vacuum at 35° and the residue (*ca* 60 mg/l of culture filtrate) was stored at 4°.

Extraction of the mycelial mat (*ca* 500 g) was accomplished with CH_2Cl_2–MeOH (1 l, 2:1) in a 2 l Erlenmeyer flask. The flask was stoppered and the soln was stirred magnetically for

12 hr then filtered through cheesecloth. The extracted mat was extracted twice more as above and more, if necessary, until it disintegrated. The resulting extracts were evapd to dryness under vacuum at 35°. Residual H_2O was present in the first extract, so its residue was re-extracted with CH_2Cl_2–MeOH (0.5 l, 2:1) to remove organic-insoluble material. Solvents were removed as before and the oily residue (*ca* 9 mg/g of mat) was stored at 0°.

Liquid chromatography of extracts. Residues (*ca* 1 g) from the previous extracts were dissolved in 3–5 ml of CH_2Cl_2–MeOH (2:1) and loaded on to a column (2 × 100 cm) of Sephadex LH-20, equilibrated in the same solvent. The column was eluted with the same solvent at a flow rate of 21 ml/hr and fractions (3.5 ml) were either collected and assayed for their absorbance at 254 nm or assayed automatically with an in-line UV column monitor. Bioactive fractions were dried, dissolved in CH_2Cl_2, and loaded on a column of silica gel contained in a 5 ml syringe. The column was eluted sequentially with 25 ml portions of CH_2Cl_2, MeCN and MeOH, and each eluant was collected and evapd to dryness under vacuum. The three fractions were bioassayed in 50% aq MeCN. The MeCN fraction was chosen for further chromatography. It was loaded on a Waters C-18 Sep-Pak, which was eluted sequentially with MeCN and CH_2Cl_2. The MeCN eluant was dried by evapn under vacuum then rechromatographed on silica gel as described previously.

HPLC was conducted on a Waters Associates model ALC/GPC-244 liquid chromatograph equipped with a Waters model 660 solvent programmer and a Waters model 440 UV detector measuring absorbance at 254 nm and a Beckman model 155 variable wavelength detector connected in series.

Reverse phase HPLC was carried out with a 6-μm particle size DuPont Zorbax-ODS column (0.94 × 25 cm) with a 5 μm particle size Brownlee Spheri-5 RP-18 guard column (0.46 × 3 cm). Normal phase HPLC was performed on a DuPont 6 μm particle size Zorbax-Sil column (0.94 × 25 cm) with a 5 μm particle size Brownlee Spheri-5 silica guard column (0.46 × 3 cm). All samples were filtered through a 0.2 μm Rainin Nylon 66 membrane. Further filtration was provided by a Rheodyne 5 μm low-dead-volume filter between the injector and the guard column. All solvents, including water, were HPLC grade. Typically, *ca* 2 mg of sample was injected in each HPLC run.

Leaf lesion assay. The assay used was a slight modification of the method reported by Gengenbach *et al.* [23]. Tms and N corn plants were grown a minimum of 1 m in height. Mature leaves were punctured with a 22 gauge needle and a 5 cm length of pipe cleaner was inserted. A 50 μl aliquot of sample in 50% ethanol was applied to the base of the pipe cleaner. Samples and controls were tested in duplicate, and lesions were measured at 72 hr.

Mitochondrial electron transport assay. This assay was developed from a coupled dye reduction first described by Peterson *et al.* [46] as modified by Gengenbach and Green [25]. Corn seeds (300 ml, dry vol.) were washed with Orthocide, soaked in distilled H_2O for 0.5 hr, rinsed with H_2O, placed on plastic trays lined with damp paper towels, and covered with Al foil. After 3–5 days incubation, the etiolated coleoptiles were exised with a scalpel at 4° and placed in a chilled mortar with 50 ml of grinding buffer (0.5 M mannitol, 10 mM HEPES, 10 mM K-Pi buffer, 1 mM EDTA, 0.2% bovine serum albumin, pH 7.2). The material was homogenized with a pestle for 1 min, filtered through 4 ply cheesecloth, diluted with grinding buffer to 150 ml, and poured into chilled centrifuge tubes. The above steps were performed at 4°. Cellular debris was sedimented at 1500 g for 5 min and the supernatant was decanted into clean centrifuge tubes.

Mitochondria were sedimented by centrifugation for 5 min at 30000 *g* then resuspended in 15 ml of 0.4 M mannitol, 25 mM HEPES buffer (pH 7.2). Further debris was sedimented by centrifugation for 5 min at 1500 *g* and the supernatant was decanted into clean tubes. An equal vol. of 0.6 M mannitol, 25 mM HEPES buffer (pH 7.2) was layered under the mitochondrial suspension, and the mitochondria were centrifuged through the more concd buffer for 20 min at 17000 *g*. The supernatant was decanted and the pellet was resuspended in 1 ml of the 0.4 M buffer with a camel hair brush. The suspension was transferred with a pipet to a clean test tube and stored on ice until use. All buffers were made fresh and kept on ice, and centrifugations were run at 2°.

Electron transport was monitored by the reduction of DCPIP ($E = 21\ mM^{-1}\ cm^{-1}$ at 600 nm [48]) from the blue iminoquinone to the colourless indophenol. Both malate dehydrogenase and succinate dehydrogenase were assayed. The assay buffer consisted of 0.4 M mannitol, 25 mM HEPES, 1 mM KCN and 30 mM substrate (either malate or succinate), pH 7.2. DCPIP was dissolved in distilled H_2O in quantities such that a 15 μl aliquot gave a final concentration of 50 μM and an absorbance of 1.05 at 600 nm when diluted to 1.0 ml with assay buffer and measured in a 1.0 cm cuvette. The mitochondrial concentration was adjusted to cause a decrease in DCPIP absorbance of 0.2 absorbance units/min. Samples to be assayed were dissolved in either 50% MEOH or 50% MeCN. The reference and controls consisted of mitochondria, buffer and sample solvent. The order of addition to the cuvette was mitochondrial suspension, buffer, sample or control, and DCPIP to initiate the reaction. The assay mixture (1.00 ml) was rapidly mixed after capping with a piece of Parafilm and placed in the spectrophotometer. Data were acquired continuously for 3–5 min. Succinate and malate assays were alternated and duplicated. Controls were run before and after sets of sample assays. Percent inhibition was calculated as the decrease in DCPIP reduction relative to controls.

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Note added in proof: After submission of this manuscript, a paper [Sugawara, F., Strobel, G., Strange, R. N., Siedow, J. N., Van Duyne, G. D. and Clardy, J. (1987) *Proc. Natl Acad. Sci. U.S.A.* **84**, 3081] appeared which described the isolation of **3** and **4** and structurally related 6-epiophiobolins from *D. maydis* and evaluation of their phytotoxic properties. Compound **3** was reported to be selectively toxic to corn bearing Tms cytoplasm when assayed in a dark CO_2 fixation assay.

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