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TOXINS FROM WEED PATHOGENS, I. PHYTOTOXINS FROM A *BIPOLARIS* PATHOGEN OF JOHNSON GRASS[1]

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ABSTRACT.—Two strongly phytotoxic metabolites, prehelminthosporol [**1**] and dihydroprehelminthosporol [**4**], have been isolated from culture filtrates of a *Bipolaris* species pathogenic to Johnson grass. Prehelminthosporol [**1**] occurs naturally as a mixture of epimers. The isomers have been separated as the corresponding acetyl derivatives, and the stereochemistry at the hemiacetal carbon has been determined by nOe experiments. Four other structurally related sesquiterpenes, helminthosporal acid [**6**], helminthosporol [**8**], helminthosporic acid [**9**], and isosativenediol [**10**], were also isolated but showed no significant activity.

Plant pathogens inflict disease on weeds as well as crop plants. Knowledge of the phytotoxins produced by plant pathogens can lead to a better understanding of the chemical basis for plant disease resistance and susceptibility (1). Identification of the toxins produced by weed pathogens could provide leads for the development of new herbicides (2–4) or even result in the use of these toxins as natural herbicides. A fungus of the genus *Bipolaris* was isolated as a pathogen of Johnson grass [*Sorghum halepense* (L.) Pers.], one of the worst weeds in tropical and subtropical areas of the world. We have found that prehelminthosporol [**1**] and dihydroprehelminthosporol [**4**], both metabolites of this fungus, are toxic towards Johnson grass in leaf spot assays.

RESULTS AND DISCUSSION

Electrolyte leakage, leaf immersion, and leaf spot assays detected toxicity of a *Bipolaris* culture filtrate toward Johnson grass and sorghum [*Sorghum bicolor* (L.) Moench]. We selected the leaf spot assay, using sorghum as the test plant, to guide the fractionation on the basis of its simplicity and reproducibility and because the response observed was rapid, clear, and very similar to that caused by the fungus in the field. Sorghum (Pioneer 8300) was used for the bioassay because of its genetic homogeneity and uniform germination. The procedure for testing aqueous samples involved placing a droplet of the test solution over a slightly injured area of the leaf.

The fungus was grown on CZ8 medium (5). The culture filtrate was separated from the mycelial mat and extracted with EtOAc. Removal of the solvent produced the organic crude extract that, when resuspended in 2% EtOH and tested, caused effects identical to the original culture filtrate. The extracted culture filtrate showed no activity. Due to the low H_2O solubility of the organic crude extract and of the resulting chromatographic fractions, we adopted a modification of the leaf spot assay to determine their activity. This modification has been previously used in the isolation of various phytotoxins (6,7). An organic solution of the components to be tested is adsorbed on Si gel and the impregnated adsorbent placed on the leaf. A small amount of H_2O is then added to the Si gel.

Purification of the active crude extract was initiated by redissolving it in a mixture of H_2O/MeOH and extracting the resulting suspension with hexane (low polarity fraction), EtOAc (medium polarity fraction), and *n*-BuOH (high polarity fraction). Bioassay of the three fractions showed that the low polarity fraction possessed the strongest

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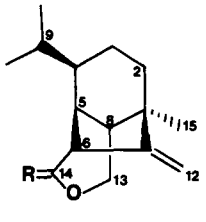
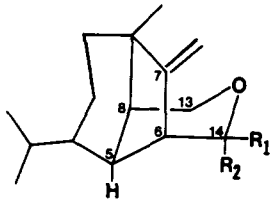
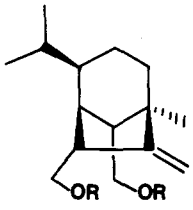


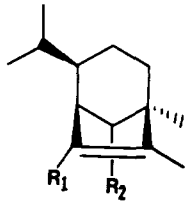
1 R=H, OH
2 R=H, OCOMe
3 R=O



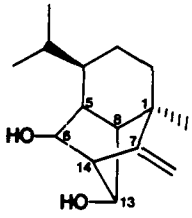
2a R_1=H, R_2=OCOMe
2b R_1=OCOMe, R_2=H



4 R=H
5 R=COMe



	R_1	R_2
6	CHO	COOH
7	CHO	CHO
8	CHO	CH_2OH
9	COOH	CH_2OH



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activity. Separation of the low polarity fraction by flash chromatography (8) using stepwise elution yielded 19 major fractions.

Testing of all fractions allowed the recognition of two "toxic zones" with strongest activity located in fractions B and M. The single compound present in toxic fraction B was identified as prehelminthosporol [**1**] by comparison of our spectroscopic data with that reported for **1** and its derivatives (9,10). Prehelminthosporol is a sesquiterpene previously isolated from culture filtrates of *Helminthosporium sativum* (9,11), a fungus now reclassified as *Bipolaris sorokiniana* and known to cause seedling blight, foot and root rot, head blight, and leaf spot of cereals and grasses (12–14). Prehelminthosporol has also been reported from culture filtrates of *Cochliobolus sativus* (10) and *Cochliobolus setariae* (11,15). *C. sativus* and *C. setariae* are the perfect stages of *B. sorokiniana* and *Bipolaris setariae*, respectively (16). Prehelminthosporol [**1**], a colorless oil, has a molecular formula $C_{15}H_{24}O_2$ (mol wt 236). In the ^{1}H- and ^{13}C-nmr spectra of **1** two sets of signals are observed. This is in agreement with the report (10) that **1** occurs as a mixture of epimers at the hemiacetal carbon. Previous workers have reported that the acetylated derivative of prehelminthosporol **2** occurs as a single isomer (10,15). We have found that acetylation (Ac_2O/pyridine, room temperature) of the hemiacetal, in fact, yields two products in an approximate ratio of 4:1. Both acetates have been obtained in pure form by flash chromatographic purification of the mixture. The ^{1}H-nmr spectrum of each epimeric acetate presents a singlet at ca. 2 ppm corresponding to the

acetyl methyl group, and the expected downfield shifts of the hemiacetal protons of both isomers (4.94 to 5.75 and 4.67 to 5.61 ppm) are also observed. Oxidation of **1** with pyridinium chlorochromate (17) afforded the corresponding lactone **3** as a single compound, confirming the hemiacetal carbon as the epimeric center. The stereochemistry at the anomeric carbon (C-14) of each epimeric acetate was determined by nOe experiments. Irradiation of the hemiacetal proton signal of the minor epimer (5.61, br s) resulted in an 11% enhancement of the signal corresponding to the bridgehead proton (H-5). A similar experiment on the major epimer resulted in only a 4% enhancement of H-5. These results indicate that H-5 and H-14 have the same orientation in the minor epimer. The stereochemistry of the major and minor epimeric acetates can then be assigned as shown in **2a** and **2b**, respectively.

The second toxic metabolite, obtained by purification of fraction M, has the formula $C_{15}H_{26}O_2$ (mol wt 238) as determined by hrms. The ir spectrum of this metabolite shows a strong hydroxyl group absorption at 3335 cm^{-1}, as well as a band at 3076 cm^{-1} indicating the presence of an exocyclic double bond (18). In the ^{1}H-nmr spectrum three methyl group signals appear as two doublets ($J = 7$ Hz) at 0.96 and 0.84 ppm and a singlet at 0.99 ppm. Decoupling experiments show that the one-proton broad triplet ($J = 8$ Hz) at 2.52 ppm is part of a $ROCH_2\dot{C}H\text{-}\dot{C} = CH_2$ structural unit, coupled to two vinylic protons at 4.90 and 4.79 ppm (d, 3 Hz), as well as to the protons of an oxygen-bearing methylene at 3.68 (dd, 11, 6 Hz) and 3.46 ppm (dd, 11, 9 Hz). Similarly, the proton at 1.57 ppm (br t, 8 Hz) was found to be coupled to the protons of a second oxygen-bearing methylene at 3.66 (dd, 11, 6.5 Hz) and 3.25 ppm (dd, 11, 9 Hz). Treatment of the metabolite with a mixture of Ac_2O/pyridine at room temperature resulted in the formation of a diacetyl derivative. In the ^{1}H-nmr spectrum of the acetylated compound, the signals corresponding to the two methylene groups bearing oxygens have experienced downfield shifts of 0.3–0.5 ppm; these acylation shift values are characteristic of primary alcohols (19). On the basis of the preceding spectroscopic data, the phytotoxic metabolite from fraction M has been assigned structure **4**. Compound **4** has been previously reported as a metabolite of *Helminthosporium victoriae* (20,21), the causal agent of blight disease in oats (22), and as an intermediate in the synthesis of victoxinine (20,21). Our ir and ms data are in agreement with those published (21). This, however, is the first report of ^{1}H- and ^{13}C-nmr data for compound **4**. Confirmation of the proposed structure **4** was obtained by treatment of **1** with $NaBH_4$. The reduction product is identical to **4** by tlc, nmr, ms, and ir. We propose the name dihydroprehelminthosporol for compound **4**.

Four other compounds, all structurally related to prehelminthosporol [**1**] and dihydroprehelminthosporol [**4**], were also isolated and characterized from the low polarity fraction. None of these metabolites showed biological activity when tested by the leaf spot assay. Helminthosporal acid [**6**] was obtained by preparative tlc purification of a fraction containing **4**. Helminthosporal acid has been reported previously as the product resulting from Ag_2O oxidation of helminthosporal [**7**] (23,24) as well as Jones oxidation of helminthosporol [**8**] (9,25). This is the first report of **6** as a natural product. Helminthosporal acid [**6**] has the formula $C_{15}H_{22}O_3$ (mol wt 250) as determined by hrms. The ir spectrum of **6** shows two carbonyl bands at 1702 and 1670 cm^{-1}. The aldehyde was shown to be part of the α,β-unsaturated system by reduction of **6** with $NaBH_4$. The resulting product does not show the uv absorption at λ max 267 observed in the uv spectrum of the starting material. Helminthosporol [**8**] and its air oxidation product helminthosporic acid [**9**] were obtained in pure form by preparative tlc purification. Both metabolites were first isolated from culture filtrates of *H. sativum* in screening projects to find new plant growth regulators among metabolites of phytopathogenic fungi (26–28).

Isosativenediol [**10**], obtained as colorless needles by dry flash chromatography (7), has been previously isolated from mycelia and culture filtrates of *C. setariae* and *H. sativum* (11,29).

The results listed in Table 1 were determined by considering the time required for the test metabolite to cause a visual lesion, as well as the extent of that lesion. Prehelminthosporol [**1**], its derivatives **2a** and **3**, and dihydroprehelminthosporol [**4**] caused lesions very similar in appearance to those induced by the fungus in the field. The lesions appear on the plant as a reddish brown area (0.3–0.5 cm in diameter) limited by a black circle. The circle is surrounded by a narrow chlorotic zone. Even though **1** and **4** have comparable toxicity at the highest level tested (25 μg/5μl), **1** maintins its toxicity at a lower concentration (2.5 μg/5μl). It is interesting to note that only the major epimer **2a** of *O*-acetylprehelminthosporol possesses biological activity. Prehelminthosporol lactone [**3**] also shows toxicity. In both cases the biological activity is lower than that of prehelminthosporol. These observations suggest that the hemiacetal as well as the configuration at the anomeric carbon of prehelminthosporol is essential for full activity. Acetylation of **4** afforded the diacetyl derivative **5**, which lacked activity. In this case, the diol appears to be essential for biological activity. Prehelminthosporol affected uninjured leaves at concentrations of 25 and 12.5 μg/5μl; however, **4** was not toxic under these conditions.

TABLE 1. Phytotoxicity[a] of *Bipolaris* Metabolites and Their Derivatives Towards Sorghum [*Sorghum bicolor* (L.) Moench] and Johnson grass [*Sorghum halepense* (L.) Pers.].

Metabolite	Concentration (μg/5μl droplet)				
	25.0	12.5	5.0	2.5	1.25
Prehelminthosporol [**1**]	4	4	2	1	0
Prehelminthosporol acetate **2a**	2	0	0	0	0
Prehelminthosporol acetate **2b**	0	NT	NT	NT	NT
Prehelminthosporol lactone [**3**]	3	1	0	0	0
Dihydroprehelminthosporol [**4**]	4	3	1	0	0
O,O-Diacetyldihydroprehelminthosporol [**5**]	0	NT	NT	NT	NT
6, 8, 9, 10	0	NT	NT	NT	NT
Control	0	0	0	0	0

[a]1 = slightly toxic, 2 = moderately toxic, 3 = very toxic, 4 = extremely toxic, 0 = not toxic, NT = not tested.

This is the first report of biological activity for prehelminthosporol and dihydroprehelminthosporol.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—For flash cc E. Merck Si gel 60 (230–400 mesh) was employed. Analytical tlc was carried out on E. Merck DC-Alufolien, kieselgel 60 F-254 (0.2 mm thickness). The chromatograms were examined under uv light (254 nm) and visualized by dipping in phosphomolybdic acid reagent (5% phosphomolybdic acid in 5% H_2SO_4 with a trace of ceric sulfate), followed by blotting and careful charring on top of a hot plate. Preparative tlc was carried out on E. Merck precoated tlc plates of Si gel 60 F-254 (20 × 20 cm, 0.25 mm thickness) or E. Merck DC-Alufolien, kieselgel 60 F-254 (0.2 mm thickness). Materials were detected by examining the plate under a uv lamp (254 nm) or by cutting a thin strip from each side, followed by visualization of the strips with the phosphomolybdic acid reagent. Microscope slides coated (approximately 0.3 mm) with E. Merck Si gel GF-254 (type 60) for tlc were prepared in the laboratory and used to adsorb the samples to be assayed.

Uv spectra were obtained on a Perkin-Elmer Lambda 4B UV/VIS spectrophotometer. Ir spectra were recorded on a Mattson Instruments Sirius 100 FT-IR controlled by a Mattson Instruments Starlab computer. Hrms were determined in a A.E.I. MS-902 mass spectrometer. Cims and lrms were obtained using

a Hewlett Packard 5985-B mass spectrometer. ^{1}H-nmr spectra were determined on a Bruker WM-250 spectrometer. ^{13}C-nmr spectra were obtained using either a Bruker WM-250 or an IBM-NR100AS spectrometer.

CZ8 medium consists of a 1:1 mixture of V-8 juice and GCLDA medium. To prepare V-8 juice medium: One 6-oz can of V-8 juice (Campbell Soup Co., California, USA) is diluted to 1 liter with distilled H_2O, and 1 g of $CaCO_3$ is added. GCLDA medium is prepared as follows: 40 g of young corn leaves are boiled in 0.6 liter of distilled H_2O for 20 min. The leaves are removed by filtration through cheesecloth, and 0.75 g of KH_2PO_4, 1.5 g of $MgSO_4$, and H_2O to 1 liter are added to the filtrate. The two parts are combined, and the medium is filtered through four layers of cheesecloth to clarify and then autoclaved for 20 min.

CULTURING AND WORK-UP OF *BIPOLARIS* SP. (ATCC 64838).—The fungus was isolated from infected Johnson grass leaves and maintained in slant tubes on potato dextrose agar (PDA) at 4°. The fungus was transferred from slants to petri dishes containing PDA. The plates were placed in a temperature (25°) and relative humidity (75%) controlled growth chamber with alternate 12-h periods of light and darkness. Cultures were allowed to grow for 2 weeks or until the fungus covered most of the surface of the plate. Spores from one plate were removed with a scalpel and used to inoculate 0.5 liter of liquid CZ8 medium. Liquid cultures were grown in a shaker at 100 rpm, room temperature, and under natural light for 3 days. For large scale production of the fungus, 0.5 liter of a three-day culture was used to inoculate 10 liters of CZ8 medium containing 20 ml of antifoaming agent (Thomas Scientific Co., Swedesboro, New Jersey). The fermentation was stirred at 350 rpm for 2 days at 30°, and aerated at 10,000 cc/min. Gravity filtration through four layers of cheesecloth separated the mycelial mat from the culture filtrate. The filtrate was either extracted directly with EtOAc in a separatory funnel or concentrated under reduced pressure (bath temperature 35–40°) to approximately 1/10 of its original volume and then extracted with EtOAc in a continuous liquid-liquid extractor. Both procedures gave similar yields of organic crude extract (ca. 90–100 mg/liter).

LEAF-SPOT ASSAY.—*Aqueous samples.*—The test solution (20 μl) was placed over a slightly injured area of the leaf (gently scratched with a cut-off artist's brush). The leaf was maintained in a petri dish in a moist atmosphere at room temperature. An initial effect was observed within 48 h.

Water-insoluble samples.—A 0.5% solution of the test component in an organic solvent (5 μl) was adsorbed on a 0.5 × 0.5 cm square of Si gel, and the solvent was allowed to evaporate. The impregnated Si gel was then placed on the leaf and the adsorbent wet with 20 μl of H_2O. An active extract or fraction induced an initial effect within 36 h.

FRACTIONATION AND PURIFICATION OF THE ORGANIC CRUDE EXTRACT.—The crude organic extract was suspended in a 9:1 mixture of H_2O-MeOH (ca. 100 ml of solution/200 mg of crude), and the resulting aqueous solution extracted three times with hexane (ratio of solvent to suspension, 2:1, 1:1, 1:1), three times with EtOAc (same ratios as above), and once with *n*-BuOH (2:1). Drying (anhydrous Na_2SO_4) and evaporation of the corresponding solvents yielded the low, medium, and high polarity fractions, respectively. Leaf-spot assay of the three extracts indicated that the strongest activity was located in the low polarity fraction.

ISOLATION OF PREHELMINTHOSPOROL [**1**].—The toxic, low-polarity fraction (271 mg) was purified by flash cc (3 × 20 cm column). Stepwise elution with C_6H_6-Me_2CO mixtures (95:5 to 70:30) yielded 19 major fractions (A to S). Bioassay results showed that the strongest biological activity was located in fractions B (21 mg) and M (35 mg). Fraction B, a colorless oil, showed a single component on tlc. The pure metabolite was identified as prehelminthosporol [**1**]: tlc R_f 0.70 (C_6H_6-Me_2CO, 80:20), 0.58 (C_6H_6-Et_2O, 60:40), 0.52 (petroleum ether-Me_2CO, 85:15); ir (thin film on KBr) 3404, 3067, 1655 cm^{-1}; ^{1}H nmr (250 MHz, $CDCl_3$), major epimer 4.94 ppm (1H, d, 2.7 Hz, H-14), 4.90 (1H, s, H-12), 4.81 (1H, s, H-12), 3.99 (1H, dd, 11.5, 2 Hz, H-13), 3.57 (1H, dd, 11.5, 2 Hz, H-13), 3.18 (1H, br s, OH), 2.69 (1H, br s, H-6), 2.52 (1H, br s, H-5), 1.16 (3H, s, H-15), 0.94 (3H, d, 6 Hz, H-10 or H-11), 0.88 (3H, d, 6 Hz, H-10 or H-11), minor isomer 4.84 (1H, s, H-12), 4.73 (1H, s, H-12), 4.67 (1H, br s, H-14), 3.93 (1H, dd, 11.5, 1.5 Hz, H-13), 3.77 (1H, dd, 11.5, 2 Hz, H-13), 2.73 (1H, br s, OH), 2.61 (1H, br s, H-6), 2.09 (1H, br s, H-5), 1.19 (3H, s, H-15), 0.97 (3H, d, 6 Hz, H-10 or H-11), 0.87 (3H, d, 6 Hz, H-10 or H-11); ^{13}C nmr (62.8 MHz, $CDCl_3$) of epimeric mixture 157.83 ppm (s, C-7), 157.25 (s, C-7′), 101.92 (t, C-12), 101.07 (t, C-12′), 97.09 (d, C-14), 96.00 (d, C-14′), 67.64 (t, C-13), 64.71 (t, C-13′), 51.64 (d, C-5, C-6 or C-8), 50.48 (d, C-5, C-6 or C-8, and C-5′, C-6′ or C-8′), 48.87 (d, C-5′, C-6′ or C-8′), 46.54 (d, C-5, C-6 or C-8), 44.78 (s, C-1), 44.53 (d, C-5′, C-6′ or C-8′), 43.60 (s, C-1′), 41.95 (t, C-2 and C-2′), 36.96 (d, C-4 and C-4′), 30.73 (d, C-9 and C-9′), 25.79 (t, C-3 and C-3′), 20.91 (q, C-10, C-11 or C-15, and C-10′, C-11′ or C-15′), 20.81 (q, C-10, C-11 or C-15), 20.16 (q, C-10′, C-11′ or C-15′), 18.88 (q, C-10, C-11 or C-15, and C-10′, C-11′ or C-15′); hrms *m/z* [M]$^+$ calcd for $C_{15}H_{24}O_2$, 236.1776, found 236.1796; cims (isobutane) 237 (37%), 219 (100%), 191 (77%).

O-ACETYLPREHELMINTHOSPOROL [**2**].—Prehelminthosporol [**1**] (4.4 mg) was treated with Ac_2O (1 ml) and pyridine (0.5 ml). After stirring overnight at room temperature, the reaction mixture was poured into ice H_2O (20 ml) and extracted with EtOAc (3 × 20 ml). The organic layer was successively washed (2 × 25 ml) with H_2O, HCl 5%, H_2O, NaOH 5%, H_2O and brine (1 × 25 ml). Drying (anhydrous Na_2SO_4) and evaporation of the solvent afforded the crude acetylated product (5.7 mg), which showed the presence of two products on tlc. Flash chromatography purification (C_6H_6, 0.4 × 10 cm column) yielded each epimer in pure form. Major epimer **2a** (2.6 mg): tlc R_f 0.47 (petroleum ether-Me_2CO, 95:5), 0.73 (C_6H_6-Me_2CO, 95:5); ir (thin film on KBr) 3070, 1749, 1657 cm^{-1}; ^1H nmr (250 MHz, $CDCl_3$) 5.75 ppm (1H, d, 3 Hz, H-14), 4.92 (1H, s, H-12), 4.77 (1H, s, H-12), 3.89 (1H, dd, 10, 2 Hz, H-13), 3.83 (1H, dd, 10, 2 Hz, H-13), 2.67 (1H, br s, H-6), 2.57 (1H, br s, H-5), 2.07 (3H, s, OAc), 1.18 (3H, s, H-15), 0.92 (3H, d, 6 Hz, H-10 or H-11), 0.85 (3H, d, 6 Hz, H-10 or H-11); ^{13}C nmr (62.8 MHz, $CDCl_3$) 156.09 ppm (s, C-7), 102.62 (t, C-12), 97.04 (d, C-14), 66.54 (t, C-13), 51.00 (d, C-5, C-6 or C-8), 47.28 (d, C-5, C-6 or C-8), 46.46 (d, C-5, C-6 or C-8), 43.87 (s, C-1), 41.76 (t, C-2), 37.69 (d, C-4), 30.76 (d, C-9), 25.74 (t, C-3), 21.11 (q, C-10, C-11, C-15 or OAc), 20.90 (q, C-10, C-11, C-15 or OAc), 20.65 (q, C-10, C-11, C-15 or OAc), 20.10 (q, C-10, C-11, C-15 or OAc); hrms *m/z* $[M]^+$ calcd for $C_{17}H_{26}O_3$, 278.1882, found 278.1888. Minor epimer [**2b**] (0.4 mg); tlc R_f 0.25 (petroleum ether-Me_2CO, 95:5), 0.64 (C_6H_6-Me_2CO, 95:5); ^1H nmr (250 MHz, $CDCl_3$) 5.61 ppm (1H, br s, H-14), 4.95 (1H, s, H-12), 4.81 (1H, s, H-12), 4.06 (1H, dd, 11, 2 Hz, H-13), 3.64 (1H, br d, 11 Hz, H-13), 2.60 (1H, br s, H-6), 2.11 (1H, br s, H-5), 2.09 (3H, s, OAc), 1.18 (3H, s, H-15), 0.92 (3H, d, 6 Hz, H-10 or H-11), 0.86 (3H, d, 6 Hz, H-10 or H-11); lrms 278 (13%), 219 (21%).

PREHELMINTHOSPOROL LACTONE [**3**].—Prehelminthosporol (8.8 mg, 0.038 mM) in 1 ml of CH_2Cl_2 was added to a suspension of pyridinium chlorochromate (32.8 mg, 0.152 mM, ratio of oxidant to alcohol 4:1) in 2 ml of the same solvent. The mixture was stirred at room temperature, and the progress of the reaction was monitored by tlc. After 4 h of reaction, no starting material could be detected by tlc, and 10 ml of Et_2O was added. The residue was washed twice with 5-ml portions of Et_2O, and the resulting solution was passed through a small bed of Si gel (230–400 mesh). Evaporation of the solvent afforded 7.1 mg of crude oxidized product. Pure prehelminthosporol lactone [**3**] (5.6 mg) was obtained by flash chromatography purification (C_6H_6, 0.4 × 10 cm column) of the crude. Tlc R_f 0.23 (hexane-Me_2CO, 95:5), 0.50 (C_6H_6-Me_2CO, 95:5); ir (thin film on KBr) 3083, 1741, 1660 cm^{-1}; ^1H nmr (250 MHz, $CDCl_3$) 5.17 ppm (1H, s, H-12), 4.83 (1H, s, H-12), 4.42 (1H, d, 10 Hz, H-13), 4.22 (1H, dd, 10, 4 Hz, H-13), 3.35 (1H, br s, H-6), 2.42 (1H, br s, H-5), 1.21 (3H, s, H-15), 0.91 (3H, d, 6 Hz, H-10 or H-11), 0.87 (3H, d, 6 Hz, H-10 or H-11); hrms *m/z* $[M]^+$ calcd for $C_{15}H_{22}O_2$, 234.1620, found 234.1621; cims (isobutane) 235 (100%).

ISOLATION OF DIHYDROPREHELMINTHOSPOROL [**4**].—Preparative tlc purification (Et_2O-C_6H_6, 70:30, 3×) of the toxic fraction M (35 mg) resulted in the isolation of pure dihydroprehelminthosporol [**4**] (6.1 mg): tlc R_f 0.43 (Et_2O-C_6H_6, 70:30, 3×), 0.34 (Et_2O-hexane, 70:30, 4×), 0.34 (C_6H_6-Me_2CO, 80:20); ir (thin film on KBr) 3335, 3076, 1647 cm^{-1}; ^1H nmr (250 MHz, $CDCl_3$) 4.90 ppm (1H, d, 3 Hz, H-12), 4.79 (1H, d, 3 Hz, H-12), 3.68 (1H, dd, 11, 6 Hz, H-14), 3.66 (1H, dd, 11, 6.5 Hz, H-13), 3.46 (1H, dd, 11, 9 Hz, H-14), 3.25 (1H, dd, 11, 9 Hz, H-13), 2.52 (1H, br t, 8 Hz, H-6), 2.37 (1H, s, H-5), 1.57 (1H, br t, 8 Hz, H-8), 0.99 (3H, s, H-15), 0.96 (3H, d, 7 Hz, H-10 or H-11), 0.84 (3H, d, 7 Hz, H-10 or H-11); ^{13}C nmr (25 MHz, $CDCl_3$) 158.69 ppm (s, C-7), 104.50 (t, C-12), 66.44 (t, C-13 or C-14), 63.11 (t, C-13 or C-14), 57.39 (d, C-5, C-6 or C-8), 49.27 (d, C-5, C-6 or C-8), 47.28 (s, C-1), 45.67 (d, C-5, C-6 or C-8), 42.72 (t, C-2), 37.71 (d, C-4), 30.61 (d, C-9), 24.97 (t, C-3), 21.34 (q, C-10, C-11 or C-15), 20.66 (q, C-10, C-11 or C-15), 20.00 (q, C-10, C-11 or C-15); hrms *m/z* $[M]^+$ calcd for $C_{15}H_{26}O_2$, 238.1933, found 238.1928; cims (CH_4): 239 (16%), 221 (100%), 208 (16%), 203 (78%), 191 (86%).

O,O-DIACETYLDIHYDROPREHELMINTHOSPOROL [**5**].—Dihydroprehelminthosporol [**4**] (5.1 mg) was stirred overnight with Ac_2O (1 ml) and pyridine (0.5 ml). The mixture was poured over 30 ml of ice H_2O and extracted with EtOAc (3 × 15). Work-up of the organic phase followed by flash chromatography purification (C_6H_6-Et_2O, 95:5, 0.4 × 10 cm column) of the crude product yielded 5 mg of pure *O,O*-diacetyldihydroprehelminthosporol [**5**]: tlc R_f 0.50 (C_6H_6-Et_2O, 90:10); ir (thin film on KBr) 3074, 1744, 1655 cm^{-1}; ^1H nmr (250 MHz, $CDCl_3$) 4.99 ppm (1H, d, 2.5 Hz, H-12), 4.86 (1H, d, 2.5 Hz, H-12), 4.18 (1H, dd, 11, 6 Hz, H-14), 4.04 (1H, dd, 11, 8 Hz, H-13), 3.75 (1H, t, 11 Hz, H-13), 3.70 (1H, dd, 11, 8 Hz, H-13), 2.65 (1H, m, H-6), 2.23 (1H, s, H-5), 2.04 (3H, s, OAc), 2.03 (3H, s, OAc), 1.69 (1H, br t, 8 Hz, H-8), 1.01 (3H, s, H-15), 0.93 (3H, d, 6.5 Hz, H-10 or H-11), 0.84 (3H, d, 6.5 Hz, H-10 or H-11); hrms *m/z* $[M - C_2H_4O_2]^+$ (parent ion not observed) calcd for $C_{17}H_{28}O_2$, 262.1991, found 262.1992; cims (CH_4) 263 (13%), 203 (100%).

REDUCTION OF PREHELMINTHOSPOROL [**1**].—Prehelminthosporol [**1**] (7 mg) in 4 ml of MeOH was treated with an excess of $NaBH_4$. The mixture was stirred at room temperature. After 60 min of reac-

tion, no starting material was observed by tlc. The mixture was poured over ice H_2O (30 ml), and extracted with EtOAc (3 × 30 ml). The organic layer was washed with H_2O and brine (1 × 30 ml), dried (anhydrous Na_2SO_4), the solvent removed, and the resulting crude purified by flash chromatography (C_6H_6-Me_2CO, 80:20, 0.4 × 10 cm) to yield 6.3 mg of pure synthetic dihydroprehelminthosporol [**4**], identical by tlc, ^1H nmr, ms, ir, and biological activity to the natural product.

ISOLATION OF HELMINTHOSPORAL ACID [**6**].—In addition to dihydroprehelminthosporol [**4**], fraction M (35 mg) showed the presence of a uv-quenching compound. Following preparative tlc of the mixture (Et_2O-C_6H_6, 70:30, 4×), the compound was obtained pure and identified as helminthosporal acid [**6**] (1.1 mg): tlc R_f 0.46 (Et_2O-hexane, 70:30, 5×), 0.62 (Et_2O-C_6H_6, 70:30, 3×); ir (thin film on KBr) 1702, 1670, 1631 cm^{-1}; uv λ max (MeOH) 267 (4392); ^1H nmr (250 MHz, $CDCl_3$) 9.99 ppm (1H, s, H-14), 3.39 (1H, s, H-5), 2.35 (1H, s, H-8), 2.02 (3H, s, H-12), 1.17 (3H, s, H-15); hrms *m/z* $[M]^+$ calcd for $C_{15}H_{22}O_3$, 250.1542, found 250.1563; cims (isobutane) 251 (100%), 233 (1%).

ISOLATION OF HELMINTHOSPOROL [**8**] AND HELMINTHOSPORIC ACID [**9**].—The tlc of fraction J (16.5 mg), from the purification of the low polarity fraction, displayed two major components. Both compounds were visible when the plate was observed under short-wave uv light and obtained pure by preparative tlc (CH_2Cl_2-Me_2CO, 80:20, 2×). The more polar compound was identified as helminthosporic acid [**9**] (3.6 mg); tlc R_f 0.11 (CH_2Cl_2-Me_2CO, 90:10), 0.24 (C_6H_6-Me_2CO, 80:20); ir (thin film on KBr) 3343, 1670, 1623 cm^{-1}; ^1H nmr (250 MHz, $CDCl_3$) 3.66 ppm (1H, dd, 11, 4 Hz, H-13), 3.39 (1H, dd, 11, 9 Hz, H-13), 3.15 (1H, s, H-5), 2.00 (3H, s, H-12), 1.63 (1H, dd, 9, 4 Hz, H-8), 1.02 (3H, d, 6 Hz, H-10 or H-11), 0.96 (3H, s, H-15), 0.77 (3H, br s, H-10 or H-11); hrms *m/z* $[M]^+$ calcd for $C_{15}H_{24}O_3$, 252.1725, found 252.1725; cims (isobutane) 253 (5%), 235 (100%). The less polar compound was identified as helminthosporol [**8**] (1.2 mg): tlc Rf 0.50 (CH_2Cl_2-Me_2CO, 90:10), 0.47 (C_6H_6-Me_2CO, 80:20); ^1H nmr (250 MHz, $CDCl_3$) 10.03 ppm (1H, s, H-14), 3.63 (1H, dd, 11.5, 5.5 Hz, H-13), 3.32 (1H, dd, 11, 8 Hz, H-13), 2.00 (3H, s, H-12), 1.66 (1H, dd, 8, 5 Hz, H-8), 1.05 (3H, d, 6 Hz, H-10 or H-11), 1.02 (3H, s, H-15), 0.75 (3H, d, 5 Hz, H-10 or H-11).

O-ACETYLHELMINTHOSPORIC ACID.—Helminthosporic acid [**9**] (2.3 mg), Ac_2O (1 ml), and pyridine (0.5 ml) were stirred overnight at room temperature. The mixture was poured over ice H_2O (25 ml) and extracted with EtOAc (3 × 15 ml). After the usual work-up, 2 mg of crude acetylated product was obtained. Preparative tlc purification (C_6H_6-Me_2CO, 80:20) produced 0.5 mg of pure *O*-acetylhelminthosporic acid: tlc R_f 0.44 (petroleum ether-Me_2CO, 85:15), 0.68 (C_6H_6-Me_2CO, 80:20); ir (thin film on KBr) 1744, 1673, 1621 cm^{-1}; ^1H nmr (250 MHz, $CDCl_3$) 4.12 ppm (1H, dd, 11, 5 Hz, H-13), 3.80 (1H, dd, 11, 9 Hz, H-13), 3.06 (1H, bs, H-5), 2.03 (3H, s, H-12 or OAc), 2.00 (3H, s, H-12 or OAc), 1.01 (3H, d, 6 Hz, H-10 or H-11), 0.98 (3H, s, H-15), 0.78 (3H, d, 6 Hz, H-10 or H-11).

ISOLATION OF ISOSATIVENEDIOL [**10**].—Dry flash chromatography purification (C_6H_6-Me_2CO, 95:5, 0.4 × 10 cm column) of fraction F (6.1 mg), obtained from the separation of the low polarity crude fraction, yielded 1.1 mg of pure, crystalline isosativenediol [**10**]: tlc R_f 0.36 (C_6H_6-Me_2CO, 90:10), 0.47 (petroleum ether-Me_2CO, 85:15); ir (thin film on KBr) 3443, 3388, 3074, 1658 cm^{-1}; ^1H nmr (250 MHz, $CDCl_3$) 4.97 ppm (1H, s, H-12), 4.67 (1H, s, H-12), 4.39 (1H, br s, H-6 or H-13), 3.64 (1H, br s, H-6 or H-13), 2.70 (1H, s, H-14), 2.62 (1H, br s, H-5 or H-8), 2.55 (1H, br s, H-5 or H-8), 1.03 (3H, d, 6 Hz, H-10 or H-11), 0.97 (3H, s, H-15), 0.92 (3H, d, 6 Hz, H-10 or H-11); hrms *m/z* $[M]^+$ calcd for $C_{15}H_{24}O_2$, 236.1776, found 236.1777; cims (isobutane) 219 (100%).

O,O-DIACETYLISOSATIVENEDIOL.—Isosativenediol [**10**] (0.8 mg) was treated with Ac_2O (1 ml) and pyridine (0.5 ml). The mixture was stirred overnight at room temperature, poured over ice H_2O (25 ml), and extracted with EtOAc (3 × 15 ml). Usual work-up of the organic extract yielded 1.4 mg of crude product. Purification by flash chromatography (C_6H_6-Me_2CO, 99:1, 0.4 × 15 cm column) yielded of 0.6 mg of pure *O,O*-diacetylisosativenediol: tlc R_f 0.32 (C_6H_6-Me_2CO, 98:2), 0.48 (hexane-Me_2CO, 85:15); ir (thin film on KBr) 3077, 1741, 1662 cm^{-1}; ^1H nmr (250 MHz, $CDCl_3$) 5.14 ppm (1H, s, H-12), 4.88 (1H, br s, H-6 or H-13), 4.81 (1H, s, H-12), 4.65 (1H, br s, H-6 or H-13), 3.09 (1H, br s, H-14), 2.74 (1H, br s, H-5 or H-8), 2.05 (3H, s, OAc), 1.96 (3H, s, OAc), 1.04 (3H, s, H-15), 0.88 (3H, d, 6 Hz, H-10 or H-11), 0.87 (3H, d, 6 Hz, H-10 or H-11); lrms 320 (0.5%), 260 (1.2%), 218 (25%), 200 (17%); cims 261 (100%), 201 (1%).

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