Prehelminthosporol and Prehelminthosporol Acetate: Plant Growth Regulating Properties

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Prehelminthosporol, extracted from *Dreschlera sorokiana*, and its synthetic derivative, prehelminthosporol acetate, were tested for biological activity. Both inhibited wheat coleoptile growth at 10^{-3} and 10^{-4} M ($P < 0.01$), and both significantly promoted growth at 10^{-5} M. Neither induced response in tobacco plants. Prehelminthosporol, at 10^{-2} M, caused the first true leaves of bean plants (33%) to bend over within 48 h. The acetate induced only necrosis in beans. Stunting and chlorosis were induced in corn plants by both compounds. Prehelminthosporol was not toxic to chicks at 450 mg/kg.

Helminthosporol, isolated from *Helminthosporium sativum* [*Dreschlera sorokiana*], is a plant growth regulator that promotes shoot growth in rice seedlings but inhibits the growth of wheat seedlings (Tamura et al., 1963, 1965). Synthetic helminthosporol analogues induce different responses in rice and lettuce seedlings (Tamura and Sakurai, 1964). Helminthosporol has been implicated as the toxin responsible for the natural destruction of cereal crops in North America. Unfortunately, helminthosporol and the associated metabolite helminthosporal are most probably artifacts arising from the precursors prehelminthosporol (octahydro-5-isopropyl-8-methyl-9-methylene-4,8-methano-1*H*-2-benzopyran-3-ol) and prehelminthosporal, respectively (DeMayo et al., 1965). However, the biological activities of either prehelminthosporol or prehelminthosporal, which are structurally different from their products, have not been reported.

During the course of examining fungal extracts for biological activity we isolated pure prehelminthosporol from *Dreschlera sorokiana*. We now report the biological activity of prehelminthosporol (Figure 1, I) and synthetically prepared prehelminthosporol acetate (Figure 1, II).

MATERIALS AND METHODS

Production, Isolation, and Purification of Prehelminthosporol. *Dreschlera sorokiana* (ATCC accession No. 42957) was isolated from infected maize roots collected in southern Georgia and was cultured on potato–dextrose–agar slants at 26 °C for 14 days. Cultures were transferred to 2.8-L Fernbach flasks, each containing 100 g of shredded wheat, 200 mL of Difco mycological broth (pH 4.8), 2% yeast extract, and 20% sucrose (Kirksey and Cole, 1974). They were incubated for 19 days at about 26 °C in the laboratory; then each flask was extracted with 300 mL of acetone and the mycelia and the substrate were ground with a Super Dispax homogenizer. The pulp was filtered on a Büchner funnel, using two sheets of Whatman No. 1 filter paper, under vacuum. The filtrate was reduced in volume to an aqueous phase on a rotary evaporator at 50 °C under reduced pressure and partitioned against 2 volumes of ethyl acetate. The ethyl acetate fractions were combined, passed through anhydrous sodium sulfate, and reduced in volume by vacuum distillation at 50 °C to yield a crude extract (approximately 90 mL). This was placed on the top of the silica in a chromatography column containing silica gel (70–230 mesh) (9.0-cm diameter × 12-cm length) that had been packed as a slurry in benzene (Figure 2). Stepwise elution with 1 L each of benzene, absolute diethyl ether, ethyl acetate, acetone, and methanol followed. Each solvent drained to the top of the silica gel before addition of the next sequential solvent. The bulk of each fraction was reduced in volume, and approximately 10 μL of each was bioassayed (vide infra) in the wheat coleoptile bioassay. Both the benzene and ether fractions were inhibitory to coleoptiles. These were bulked together and then added to a silica gel (70–230 mesh) chromatography column (4.0 × 46 cm) that had been packed in benzene, and 625 mL of benzene was allowed to percolate through the column. Following this, the column was eluted with a linear gradient of benzene–ethyl acetate (1.0 L of benzene and 1.0 L of ethyl acetate) and 25-mL fractions were collected. From each fraction, 20 μL was taken, evaporated to dryness under nitrogen, and bioassayed. Contents of the tubes showing activity (tubes 47–65; 1.175–1.625 L from the start of the linear gradient) were bulked, reduced in volume, and added to a freshly prepared silica gradient column under conditions identical with those just described.

Again, the biological activity of each fraction was noted, and these were bulked and placed on a Sephadex LH-20 column (5.0 × 65 cm) that had been packed in methanol and eluted by using methanol as the mobile phase at a flow rate of 0.19 mL/min. Column eluate was collected in bulk until the daylight visible brown-yellow broad band was three-quarters down the column. Then individual 2.3-mL fractions were collected, and 5 μL of each was bioassayed. Tubes containing the active substance (tubes 33–175; 75.9–402.5 mL from the time that the brown-yellow band was three-quarters down the column) were added together, reduced in volume, and further chromatographed on Bio-Beads S-X2 (200–400 mesh, Bio-Rad Laboratories). The 3.5 × 40 cm column was packed and run in benzene. Approximately 12-mL fractions were collected after the initial dark brown-yellow band was three-quarters through the column, and 10 μL of each was bioassayed. Again, the active fractions were pooled, reduced in volume, and chromatographed, this time on RP-2 (silanized silica gel 60, 70–230 mesh, E. M. Reagents). Columns were 3 × 20 cm, and the solid phase was packed in acetonitrile–water (1:1 v/v). The same solvent was used for the mobile phase. As the first colored band exited the column 15-mL fractions were collected, 5 μL of which was bioassayed. Tubes

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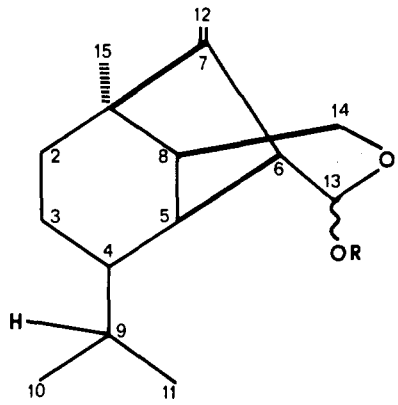

I. R = H
II. R = $CH_3C(=O)-$

Figure 1. Structure of prehelminthosporol and prehelminthosporol acetate.

containing active material (tubes 2–8; 15–120 mL) were bulked together, and final cleanup of the active metabolite was accomplished by reverse-phase, C_{18} (contents of a Prep PAK-500; Waters Associates) open column chromatography (column size 1.2 × 35 cm) using acetonitrile–water (1:1 v/v). All fractions were bioassayed, and the active fractions (tubes 2–8; 24–96 mL) were mixed and evaporated, under vacuum, to an oily residue.

Preparation of Prehelminthosporol Acetate (II). Ninety milligrams of I (0.38 mmol) was mixed with 0.5 mL of pyridine (6.2 mmol) and 1.0 mL of acetic anhydride (0.01 mol) and left at 27 °C overnight. Ice was added to the reaction mixture which was then partitioned against ethyl acetate. The ethyl acetate was reduced under vacuum, and the product was cleaned on a silica gel 60 (70–230 mesh) chromatography column (1 × 15 cm) by using ethyl acetate–benzene (1:4 v/v) elution. The elution of II was monitored by thin-layer chromatography and was isolated as a colorless oil.

Physical and Chemical Analysis. Infrared (IR) spectra of I and II were obtained with a Beckman IR 4210 equipped with a 4× beam condenser. Samples were prepared as thin films on KBr windows.

Proton decoupled natural abundance ^{13}C NMR spectra were obtained on a Varian Associates FT-80A spectrometer. Samples were prepared (~50 mg/0.5 mL) in 5-mm tubes by using deuteriochloroform containing a trace of tetramethylsilane (Me_4Si) as a reference. Conditions to obtain the spectra were pulse angle 45°, delay between pulses 1.5 s, sweep width 5 kHz, data points 8K, sensitivity enhancement −0.5, and noise bandwidth of the decoupler −2000. Single-frequency off-resonance proton decoupled (sford) spectra were obtained by offsetting the decoupler 400 Hz upfield from Me_4Si in order to aid in distinguishing the types of carbons. Proton spectra were obtained on identical solutions at 250 MHz by using a Bruker WM 250 spectrometer.

Low-resolution (LRP) mass spectra were obtained with a Hewlett-Packard 5985 spectrometer, by the direct probe method.

Purification steps for I and II were monitored by thin-layer chromatography on silica gel 60, F-254 (E. M. Laboratories, Inc.), with an ethyl acetate–benzene (1:4 v/v) developing solvent. Developed plates were sprayed with anisaldehyde (Stahl, 1965) and heated to 100 °C.

Bioassays. Four sets of bioassays were used to determine the biological activity of I and II in plants. In the primary assay, wheat coleoptiles were used. Wheat seeds (*Triticum aestivum* L., cv. Wakeland) were grown on moist sand, in trays, in the dark at 22 ± 1 °C for 4 days (Hancock et al., 1964). The etiolated shoots were harvested, and the apical 2 mm were removed in a Van der Weij guillotine and discarded. The next 4 mm of the coleoptile was saved for bioassay. Fractions to be assayed, which were obtained from column chromatography, were either 5, 10, or 20 μL in volume. They were evaporated to dryness under nitrogen at 60 °C, and 2 mL of phosphate–citrate buffer containing 2% sucrose at pH 5.6 (Nitsch and Nitsch, 1956) was added to each test tube. Ten coleoptiles were placed in each test tube, and the tubes were rotated for 24 h at 22 °C at 0.25 rpm in the dark. All manipulations were carried out under a green safelight (Nitsch and Nitsch, 1956). Following treatment, the images (×3) of the coleoptile segments were measured using a photographic enlarger (Cutler and Vlitos, 1962). Both I and II were assayed by dissolving the appropriate weight of each in 50 μL of acetone (Cutler, 1968) and adding phosphate–citrate buffer to a volume of 10 mL to give a 10^{-3} M solution. A dilution series was made from the 10^{-3} M stock to give 10^{-4}, 10^{-5}, and 10^{-6} M solutions. Data were analyzed statistically (Kurtz et al., 1965).

Metabolites I and II were also tested for activity on greenhouse grown plants. Appropriate weights of each metabolite were dissolved in acetone, and then water containing 0.1% Tween 20 was added (final solution contained 10% acetone) to give concentrations of 10^{-2}, 10^{-3}, and 10^{-4} M. Six-week-old tobacco plants (*Nicotiana tabacum* L., cv. Hick's) were sprayed with 1 mL of each solution per plant (for I, 2360, 236, or 23.6 μg; for II, 2780, 278 or 27.8 μg, respectively). Eleven-day-old bean plants (*Phaseolus vulgaris* L., cv. Black Valentine) were treated with 1 mL of each concentration of either I or II per pot. Each pot contained four bean plants. Twelve-day-old corn plants (*Zea mays* L., cv. Norfolk Market White) were treated with 100 μL of each solution which was pipetted into individual leaf whorls (10^{-2}, 10^{-3}, and 10^{-4} M concentrations were 236, 23.6, or 2.36 μg for I and 278, 27.8, or 2.78 μg for II, respectively). Each pot contained four corn plants. All experiments were triplicated, and observations were made at 7 and 14 days after treatment. Control plants were treated with solutions that contained the appropriate amounts of Tween 20, acetone, and water, in all cases.

The vertebrate bioassay used 1-day-old chicks. They were dosed via crop intubation with corn oil as the carrier (1 mL of corn oil/chick), and only prehelminthosporol was tested. The metabolite was dissolved in acetone, corn oil was added, and then the acetone was removed under vacuum at 70 °C on a rotary evaporator. After the apparent removal of all the acetone, the evaporator reservoir was emptied and dried. The sample was again subjected to distillation to ensure complete removal of the acetone (Kirksey and Cole, 1974). Controls were prepared identically.

RESULTS AND DISCUSSION

Physical and Chemical Characteristics. *Dreschlera sorokiana* produced approximately 248 mg of prehelminthosporol from 72 flasks of shredded wheat media in 19 days. Initial chromatography of the crude extract from silica gel (9 × 12 cm column) gave benzene and diethyl ether fractions that were biologically active. When both active fractions were combined and subjected to silica gel column chromatography using a benzene–ethyl acetate linear gradient, biological activity was noted in tubes 47–65 (1.175–1.625 L, from the first collected fraction). When

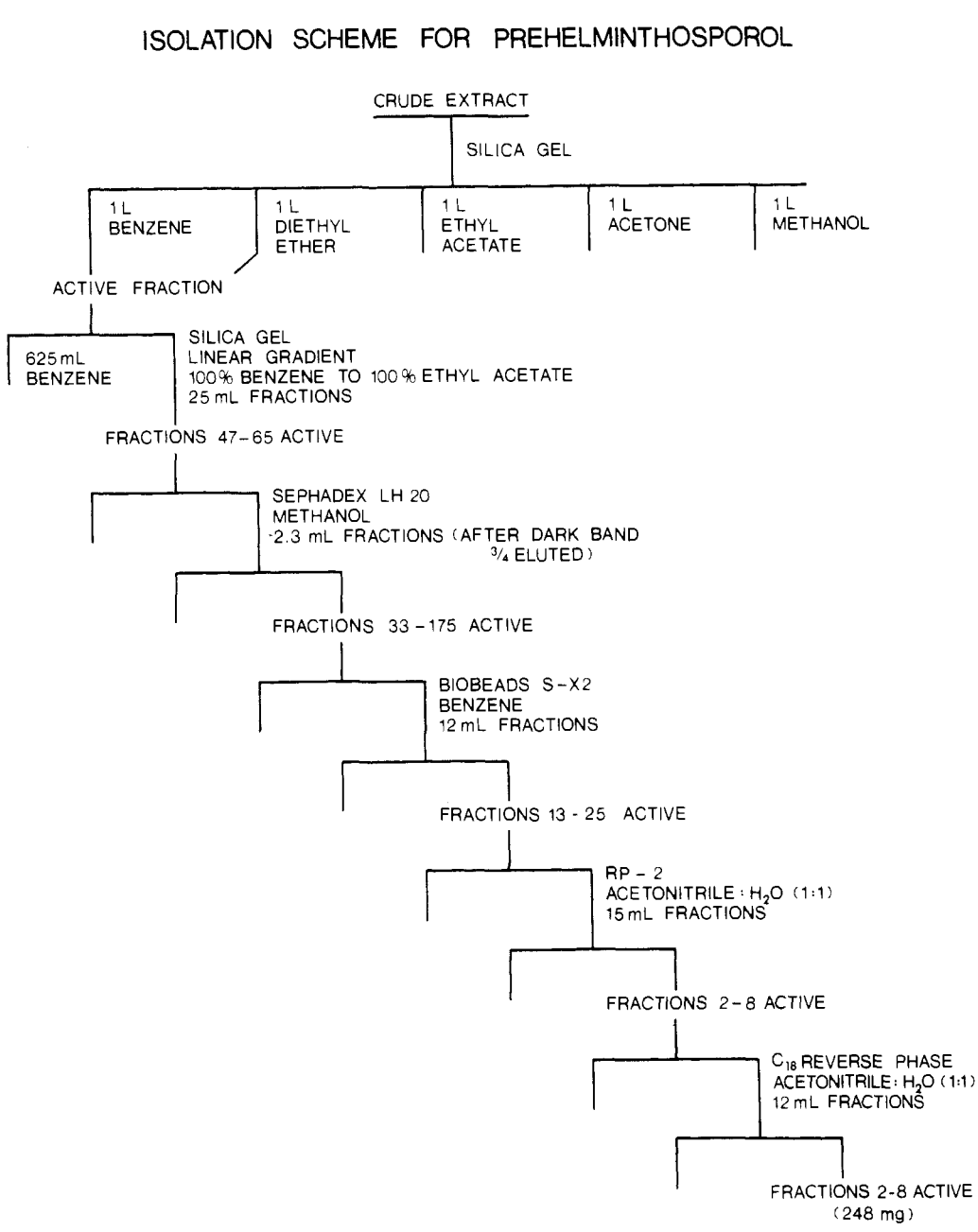

Figure 2. Isolation flow scheme.

the active material was placed on a Sephadex LH-20 column and eluted with methanol, biological activity was observed in tubes 33–175 (75.9–402.5 mL measured from the time that the brown-yellow broad band was visible three-fourths down the column). Further purification on Bio-Beads S-X2 with benzene yielded active material in tubes 13–25 (156–300 mL). Using an RP-2 column with acetonitrile–water (1:1 v/v) as the mobile phase and collecting fractions from the time that the first colored band exited the column, activity was located in tubes 2–8 (15–120 mL). In the final cleanup on C_{18} reverse-phase chromatography using acetonitrile–water (1:1 v/v), biological activity was observed in tubes 2–8 (24–97 mL).

Both I and II were liquids, metabolite I was a pale yellow color and II was clear.

The major IR bands and probable assignments for I were as follows: 3400 (fairly sharp, OH), 2950 (CH_3), 2920 (CH_2), 2862 (CH_3), 2840 (CH_2), 1735 (shoulder, weak), 1720 (medium), 1650 (strong, —CH=CH_2), 1446 (strong, —CH=CH_2), 1380 (CH_3), 1362, 1300 (weak), 1246, 1218, 1170, 1111 and 1088 and 1052 and 1040 (C–O–C stretching), 1010, 956, 915, 872 (very strong), 809 (strong), 725 cm^{-1}. Wavenumbers and probable assignments for II were as follows: 3070 (very small, very sharp), 2950 (CH_3), 2920 (CH_2), 2862 (CH_3), 2840 (shoulder, CH_2), 1740 (very strong ester), 1652 and 1450 (—CH=CH_2), 1367 (—O—CO—C-H_3), 1235 and 1215 (both very strong, acetate bands), 1178, 1160, 1145 and 1125 and 1046 (C—O<<C stretching), 1007, 945, 932, 880, 845, 822, 786, 732 cm^{-1}. No UV absorptions were observed for I or II (no conjugations).

The LRP mass spectral analysis of I indicated a molecular ion peak (M^+) at m/z 236.2, and the computer program (ELCOMP, Hewlett-Packard) allowed for the molecular formula $C_{15}H_{24}O_2$. There were fragment ions at m/z 219 ($M^+ - OH$), 193 ($M^+ - C_3H_7$), and 190 ($M^+ - H_2O - C_2H_4$ (McLafferty, 1967). The M^+ for II was located at m/z 278 and fragment ions were noted at 235 ($M^+ - CH_3CO$) and 232 ($M^+ - H_2O - C_2H_4$). The latter fragment is sometimes noted even with esters (McLafferty, 1967).

The 20-MH_z ^{13}C NMR spectrum of I exhibits a total of 24 peaks (Table I). Intensity considerations suggest that six of the peaks are due to two carbons each. These data relative to the mass spectral data suggest that I exists as a mixture of two C_{15} isomers. The 250-MHz ^1H spectrum

Table I. ^{13}C NMR Chemical Shifts of Prehelminthosporol (Ia and Ib) and Its Acetate (II)[a,b]

carbon/compd	Ia[c]	Ib	II
1	44.8 s	43.7 s	46.6 s
2	42.0 t	42.0 t	41.9 t
3	25.9 t	25.9 t	25.8 t
4	37.0 d	37.0 d	37.8 d
5	50.6 d	48.9 d	47.4 d
6	51.6 d	50.6 d	51.1 d
7	157.9 s	157.2 s	156.2 s
8	46.6 d	46.6 d	46.6 d
9	30.8 d	30.8 d	30.9 d
10	18.9 q[d]	20.3 q[d]	20.2 q[d]
11	21.1 q[d]	20.9 q[d]	20.8 q[d]
12	102.1 t	101.1 t	102.8 t
13	97.2 d	96.2 d	97.1 d
14	67.7 t	64.8 t	66.7 t
15	21.2 q[d]	21.1 q[d]	21.1 q[d]
$CH_3C=O$			169.9 s
$CH_3C=O$			21.3 q[d]

[a] In ppm downfield from Me_4Si. [b] Multiplicity data from sford spectra, s, singlet; d, doublet; t, triplet; q, quartet. [c] Major isomer. [d] Assignments uncertain.

Table II. ^1H Chemical Shifts of Prehelminthosporol (Ia and Ib) and Its Acetate (II)[a]

proton/ compd	Ia[b]	Ib	II
2	1.2	1.2	1.16
3	1.35	1.35	1.40
4	1.66	1.66	1.7
5	2.51	2.67	2.59
6	3.30 (J = 12.5 Hz)	3.40	2.68
8	2.07	2.58	2.10
9	1.35	1.35	1.3
10	0.85 (J = 6.5 Hz)	0.87 (J = 6.6 Hz)	0.87 (J = 6.6 Hz)
11	0.96 (J = 6.5 Hz)	0.93 (J = 6.6 Hz)	0.92 (J = 6.2 Hz)
12a	4.79	4.71	4.78
12b	4.89	4.81	4.93
13	4.65 (J = 12.5 Hz)	4.92 (J = 2.9 Hz)	5.76 (J = 3.3 Hz)
14a	3.92 (J = 11.0 Hz)	3.97 (J = 1.9, 11.4 Hz)	3.84 (J = 1.9, 11.5 Hz)
14b	3.65 (J = 11.0 Hz)	3.75 (J = 2.2, 11.4 Hz)	3.90 (J = 2.2, 11.5 Hz)
15	1.16	1.18	1.19
$CH_3C=O$			2.09

[a] In ppm downfield from Me_4Si. [b] Major isomer.

of I also shows a doubling of certain peaks, indicating a mixture of isomers. Acetylation of I to produce the monoacetate II results in a ^{13}C NMR spectrum for II consisting of 17 peaks. Comparison of the ^1H NMR data for I (Table II) with the data in the literature (DeMayo et al., 1965) suggests that I is prehelminthosporol. The ^1H NMR data for II is consistent within that reported for prehelminthosporol acetate (Aldridge and Turner, 1970). In the same report it was stated that prehelminthosporol exists as a mixture of epimers (C_{13}). Integration of the ^1H and ^{13}C NMR spectra in our experiments shows that the two epimers are present in the ratio of ~55:45. Acetylation results in a single compound. Inspection of models of the two epimers of I (Ia and Ib) and of the proton–proton coupling constants in the ^1H NMR spectra suggests that the acetate (II) is formed from the epimer that is better able to minimize steric interactions between the acetate group and the remainder of the molecule. Interestingly, the configuration of the acetate at C_{13} corresponds to the less abundant epimer (Ib) of prehelminthosporol.

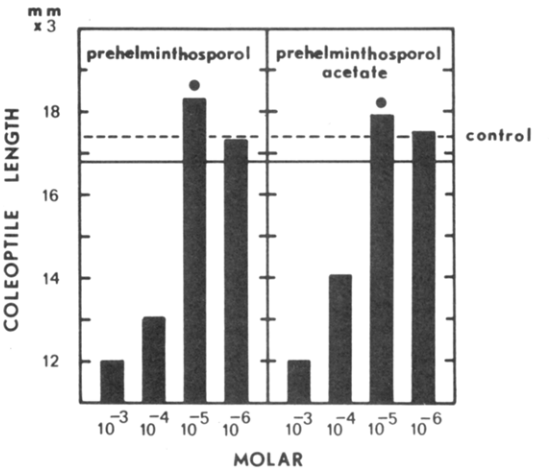

Figure 3. Effects on the growth of etiolated wheat coleoptile (*T. aestivum* L., cv. Wakeland) by prehelminthosporol and prehelminthosporol acetate. Significant inhibition ($P < 0.01$): below solid line. (●) is significant promotion ($P < 0.01$). Control: dashed line.

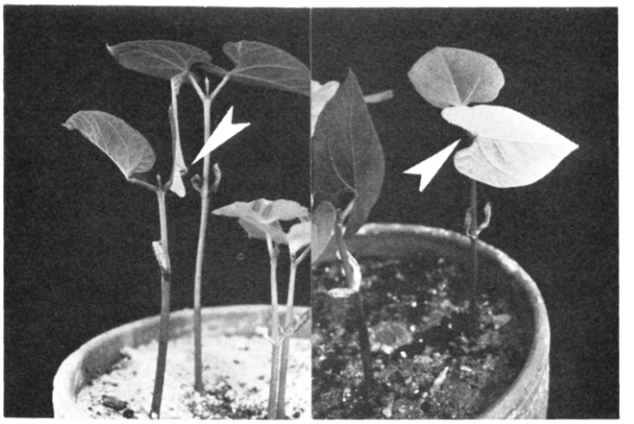

Figure 4. Bending response produced by prehelminthosporol on the pulvinus of bean plants (*P. vulgaris* L., cv. Black Valentine) 24 h after treatment with 10^{-2} M solutions.

Attempts to separate the two epimers of prehelminthosporol by HPLC were unsuccessful. Although the epimers seemed to be separated by HPLC and TLC, solutions of the two isolated compounds resulted in NMR spectra identical with that of the original epimeric mixture. These observations and the formation of one acetate indicate that rapid equilibrium of epimers through the intermediate aldose occurs under very mild conditions.

The R_f values for the metabolite and the synthetic derivative, when developed in the ethyl acetate–benzene solvent on silica gel 60 plates, were for prehelminthosporol (I) 0.50–0.59 and for prehelminthosporol acetate (II) 0.80–0.82 (very strong) and 0.85–0.92 (very light). Both I and II appeared as bright brick-orange spots after developed plates were sprayed with anisaldehdye and heated.

Bioassay Results. Both I and II significantly inhibited ($P < 0.01$) the growth of wheat coleoptiles at 10^{-3} and 10^{-4} M while at 10^{-5} M growth was significantly promoted (Figure 3). Superficially, these responses appear to resemble those induced by indole-3-acetic acid in the wheat coleoptile bioassay where 10^{-3} M solutions inhibit growth and 10^{-4}, 10^{-5}, and 10^{-6} M solutions significantly promote growth (H. G. Cutler, unpublished data; Phillips, 1971). Furthermore, I induced bending of the pulvinus in beans (vide infra), but II did not. Thus, the possible auxin-like properties of I, and possibly II, need further study.

It is interesting to note that I and the acetate II were active in the wheat bioassay. Even though II was slightly

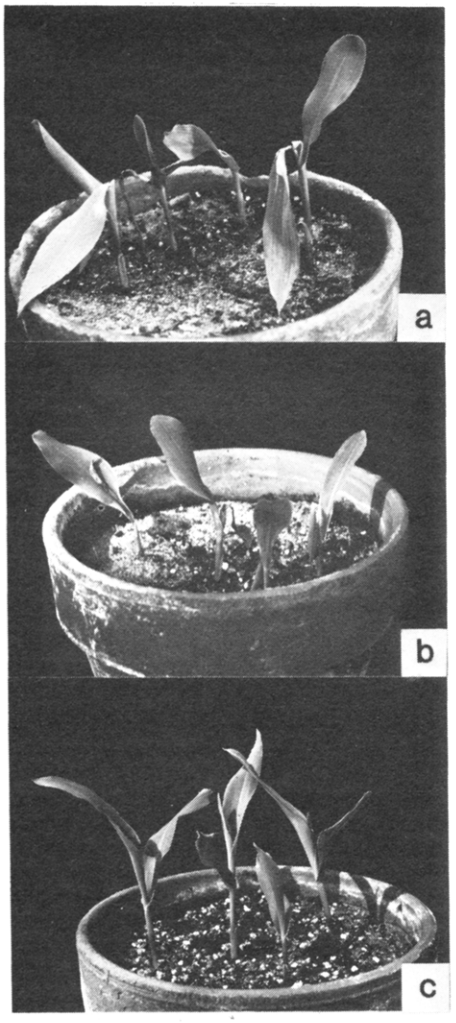

Figure 5. (a) Stem collapse and general necrosis induced by prehelminthosporol application on corn plants (*Z. mays* L., cv. Norfolk Market White) 24 h after treatment with 10^{-2} M solutions. (b) Stem collapse and marginal leaf necrosis induced by prehelminthosporol acetate application to corn plants 48 h after treatment with 10^{-2} M solutions. (c) Control corn plants treated with 0.1% Tween 20 plus 10% acetone in water after 48 h.

less active than I, acetylation did not radically change the properties of the parent molecule as happens with other growth regulators (Cutler et al., 1978, 1979).

Greenhouse-grown tobacco plants were not affected by either I or II. When beans were treated with I at 10^{-2} M, approximately 21% of the first true leaves bent over at the pulvinus so that the underside of the leaves were uppermost (Figure 4) and showed some necrotic lesions within 24 h. At 48 h the number of plants showing the bending effect had risen to 33% at 10^{-2} M. Unlike prehelminthosporol, the acetate (II) did not induce bending at the pulvinus but caused marginal leaf burn at 10^{-2} M, 24 and 48 h after treatment. The 10^{-3} and 10^{-4} M treatments produced no apparent effects. All plants looked normal 2 weeks after treatment.

Within 24 h of treatment with I at 10^{-2} M, all stems of corn collapsed within the leaf whorls (Figure 5a), and there was general necrosis. Initially there were no visible effects at 10^{-3} and 10^{-4} M. However, at 48 h all plants showed some chlorosis or necrosis at 10^{-3} M and slight necrosis at 10^{-4} M. At 10^{-2} M the acetate (II) produced necrosis of leaves in the whorls within 24 h and stem collapse within 48 h (Figure 5b). Slight necrosis occurred at 10^{-3} M. All plants started to recover 1 week after treatment.

Day-old chicks were dosed with concentrations of prehelminthosporol up to 450 mg/kg. No effects were observed.

It would be premature to state that the effects observed in *H. sativum* infected corn are due solely to the production of prehelminthosporol. But perhaps prehelminthosporol in concert with other toxins, including prehelminthosporal, induce the total pathology. We did not find helminthosporol in our separation and concur that it is an artifact (DeMayo et al., 1965).

Because both I and II exhibit approximately equal activity in various plant bioassays, it is apparent that biological activity is not associated with the free OH group. Apparently some other structural features in the molecule are responsible. Because of the nontoxic properties of I to chicks and the selective action of I and II to different plant species, the basic structure is an interesting one with which to study the effects of synthetic structural variations relative to biological activity.

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