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PHYTOTOXINS OF *ASCOCHYTA HYALOSPORA*, CAUSAL AGENT OF LAMBSQUARTERS LEAF SPOT

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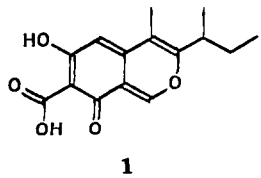
ABSTRACT.—Three phytotoxins, ascochytine [**1**], pyrenolide A [**2**], and a new metabolite hyalopyrone [**3**], were isolated from culture filtrates of *Ascochyta hyalospora*, the causal agent of lambsquarters leaf spot. All three toxins were phytotoxic on nine weed species. Ascochytine and pyrenolide A caused dose-dependent increase in electrolyte leakage from lambsquarters leaf, inhibited most bacterial and fungal growth, and also inhibited sorghum root growth.

Ascochyta hyalospora (Cooke and Ell.) Boerema, Mathur and Neergaard causes leaf spot and stem necrosis on lambsquarters (*Chenopodium album* L.). The causal agent was first reported to be *Diplodia hyalospora* (1), subsequently described as *Pleospora chemopodii* (2) and only recently renamed *Ascochyta hyalospora* (3). This pathogen attacks seeds, seedlings, leaves, and young stems of lambsquarters (3). Many species of the genus *Ascochyta* produce large amounts of the easily isolated, bright yellow azophilone ascochytine [**1**]. The role of ascochytine in plant disease has been studied (4–8). Epoxydon (9), brefeldin A (10), pachybasin (11), and triprenylchlorophenols (12) have been previously reported from the genus. In the course of our study of phytotoxins produced by plant pathogenic fungi (13–15) we have investigated the toxins produced by *Asc. hyalospora* and found them, with the exception of ascochytine, to be different from the metabolites previously reported from this genus.

RESULTS AND DISCUSSION

Ascochytine was readily isolated from the culture filtrate of *Asc. hyalospora* by acid-base extraction followed by crystallization from hexane/MeOH in a yield of 13 mg/liter. Neutral metabolites were fractionated by preparative Si gel tlc. A non-polar toxin isolated in a yield of 5 mg/liter proved to be identical to pyrenolide A [**2**] by ir, ^{1}H nmr, ^{13}C nmr, cims, and hrms (16). The pyrenolides have been previously reported only from *Pyrenophora teres* as fungal morphogenic factors.

The most polar phytotoxin, hyalopyrone [**3**], was located on preparative tlc by fluorescence quenching. Hyalopyrone does not reduce phosphomolybdic acid. In consequence, it is also locatable on tlc as a white spot surrounded by the pale blue background produced during heating of the tlc plate dipped in this reagent. Hyalopyrone was isolated as a pale yellow syrup (12 mg), migrating as a single substance on tlc developed in five solvent systems. Its ^{1}H-nmr spectrum (Table 1) establishes the presence of a C-methyl triplet (0.88 ppm), two C-methyl doublets (1.23, 1.30), a C-methyl singlet (1.94), a methylene multiplet (1.63), a methylene doublet (2.63), one heteroaromatic singlet (6.15 ppm), and two further methine multiplets (2.91, 4.19). These proton assignments, together with one exchangeable, alcoholic proton, agree with the hrms



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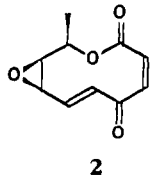

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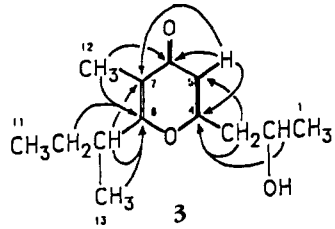

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TABLE 1. ^{1}H- and ^{13}C-nmr Chemical Shift Assignments for Hyalopyrone [3] in $CDCl_3$ at 500 MHz.

Position	^{1}H nmr	^{13}C nmr
1	1.30, d, J=7	23.23
2	4.19, sex, J=7	65.75
3	2.63, d, J=7	43.19
4	—	164.94
5	6.15, s	113.20
6	—	180.13
7	—	120.04
8	—	167.47
9	2.91, sex, J=7	36.87
10	1.63, m	27.64
11	0.88, t, J=7	11.89
12	1.94, s	9.24
13	1.23, d, J=7	17.81

elemental composition $C_{13}H_{20}O_3$ (calcd 224.1413, found 224.1427). The coupling pattern and homonuclear COSY establish the nature of the four substituents on the oxygenated chromophore as a hydrogen (6.15 ppm), a methyl (1.94 ppm), an isobutyl, and a 2′-hydroxypropyl group. The remaining atoms, C_5O_2, form the core of the oxygenated chromophore. The chemical shifts of the five chromophoric carbon resonances occur at 113.20, 120.04, 164.94, 167.47, and 180.13. Correlation of aliphatic ^{13}C-nmr signals with ^{1}H-nmr signals shown in Table 1 was made by heteronuclear COSY. In addition, the aromatic proton singlet correlates with δ_C113.20 ppm. The carbonyl nature of the 180.13 ppm carbon is confirmed by strong ir absorption at 1650 cm^{-1}.

Four chromophores having composition C_5O_2 come into consideration for hyalopyrone: α- or γ-pyrone, or α- or β-acylfuran. Hyalopyrone has maxima at 215 nm (8700) and 255 (22,500), which do not fit the wavelengths of α-acylfurans (17,18) or α-pyrones (19). The relative intensities of the two absorption maxima of hyalopyrone are not those of β-acylfurans (20,21). The uv spectrum is consistent with that of other similarly substituted γ-pyrones (19,22). Strong carbonyl absorption in the ir of hyalopyrone in thin film occurs at 1650 cm^{-1}, consistent with the 1543–1675 cm^{-1} range of γ-pyrones (19,22) but not the 1705–1739 cm^{-1} range of α-pyrones (19). The C-4 carbonyl resonance of γ-prones bearing only alkyl and hydrogen substitutents occurs between 180 and 181 ppm, the C-2 and C-6 resonances between 152 and 166 ppm, and the C-3 and C-5 resonances between 99 and 127 ppm (22–24). These ranges are consistent with resonances assigned to the hyalopyrone in Table 1.

The aromatic proton signal at 6.15 ppm correlates with the ^{13}C signal at 113.20 ppm. Both the ^{1}H and ^{13}C resonances are consistent with chemical shift data for the β position, but not the α position, of a γ-pyrone (25–29). The three alkyl substituents were located by heteronuclear multiple bond correlations shown on structure **3**. An alternative bis-norsesquiterpene structure having the hydroxypropyl group at α-C of the γ-pyrone, the methyl at the β-C and the isobutyl at the ε-C was rejected on the basis of unlikely long range heteronuclear correlations and the absence of some expected shorter range correlations. The existence of chirality implied by structure **3** was confirmed by the observation of a negative cd extremum at 234 nm. The configuration of the two chiral centers is unknown.

The *Ascochyta* metabolites were phytotoxic in three assays: detached leaf spot necrosis, leaf electrolyte leakage, and root growth inhibition. In the leaf necrosis assay

TABLE 2. Phytotoxicity of *Ascochyta hyalospora* Cell-free Culture Filtrate (Cf) and Toxins **1–3** on Weed Leaves.[a]

Weed	Compound								
	Cf	**1**			**2**			**3**	
	(20 µl)	100[b]	50	25	50	25	10	100	50
Lambsquarters	18	19	11	5	13	5	2	5	4
Prickly sida	19	35	20	6	4	0	0	13	7
Sicklepod	15	16	13	9	2	0	0	4	3
Morningglory	7	9	9	8	3	0	0	8	5
Johnsongrass	31	24	10	10	18	7	3	15	11
Sorghum	30	28	18	12	10	8	2	23	18
Bentgrass	3	12	8	7	0	0	0	9	6
Ragweed	2	8	5	5	6	3	0	18	10
Watercress	16	20	5	2	1	0	0	7	5
Jimsonweed	18	22	15	11	2	0	0	3	0

[a]Table entries are necrotic area (mm^2).
[b]Concentration in µg.

both johnsongrass and sorghum were more sensitive to unfractionated culture filtrate toxicity than was the host, lambsquarters (Table 2). This was reflected in the greater toxicity of the major metabolite ascochytine to johnsongrass and sorghum. Pyrenolide A and ascochytine possessed comparable activity towards the host plant lambsquarters, but hyalopyrone was considerably less active. Ascochytine and pyrenolide A also possessed very comparable activity against lambsquarters in causing electrolyte leakage (Figure 1) and in inhibiting sorghum root growth (Figure 2). Pyrenolide A is as phytotoxic as ascochytine and should be considered as a possible factor in *Asc. hyalospora* pathogenicity.

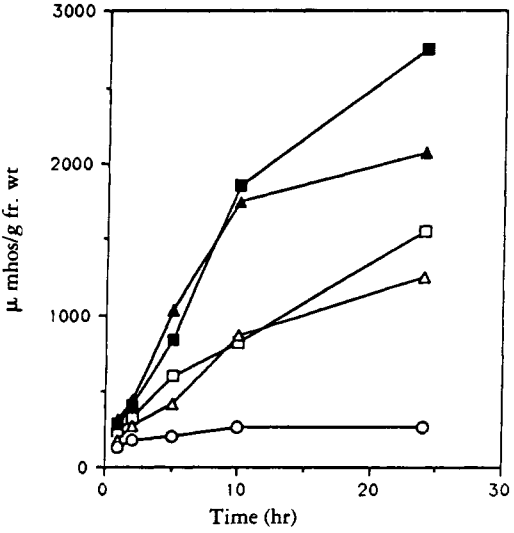

FIGURE 1. Electrolyte leakage caused by ascochytine [**1**] and pyrenolide A [**2**] on lambsquarters leaves: ⊟ ascochytine 500 µg/ml; ■ ascochytine 1000 µg/ml; △ pyrenolide A 500 µg/ml; ▲ pyrenolide A 1000 µg/ml; ⊖ distilled H_2O control.

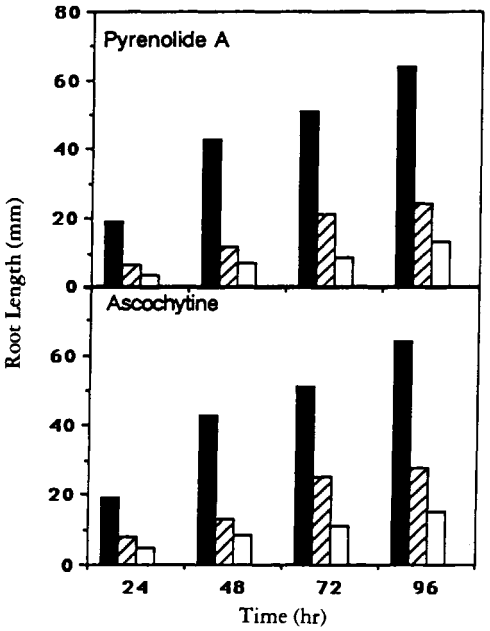

FIGURE 2. Effect of ascochytine [**1**] and pyrenolide A [**2**] on sorghum root growth. Uniformly pregerminated seeds were placed on agar and overlaid with Whatman 3MM paper impregnated with 250 (▨) or 500 (□) μg of pyrenolide A or ascochytine. Paper wet with distilled H_2O was used as a control (■). The Petri plates were oriented vertically to permit geotropic root growth. Diffusion of the yellow toxin ascochytine was complete within 6 h. Root length was measured at 24 h. There was no root growth when the paper was impregnated with 1000 μg of either toxin.

The *Ascochyta* metabolites had selective activity at 500 μg against bacteria and fungi in the antibiotic disk assay (Table 3). Ascochytine was strongly inhibitory to *Agrobacterium tumefaciens, Corynebacterium flaccumfaciens,* and *Colletotrichum acutatum,* but did not inhibit growth of *Erwinia carotovora, Aspergillus flavus,* or *Penicillium notatum.* Pyrenolide A inhibited *Cor. flaccumfaciens, Pen. notatum* and *Col. acutatum.* Hyalopyrone had moderate inhibitory activity against all of the fungi and against *Cor. flaccumfaciens.* None of the metabolites inhibited growth of *E. carotovora.*

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Low resolution cims and eims were obtained using a Hewlett Packard 5985-B mass spectrometer, and hrms was determined on the A.E.I. MS-902. The nmr spectra were measured on a GE 500 Omega spectrometer in $CDCl_3$. The cd measurements were made in a 1 cm cell on JASCO J-600 spectropolarimeter. Analytical tlc was carried out on E. Merck DC-Alufolien, Kieselgel 60 F-254 (0.2-mm thickness). Preparative tlc was carried out on E. Merck Si gel 60 F-254 plates (0.5-mm thickness). A pure culture of *Asc. hyalospora* strain 805 was isolated from lambsquarters leaf and supplied by Dr. George Templeton, University of Arkansas. This strain has been deposited in the American Type Culture Collection as #76079.

FERMENTATION AND PREPARATION OF EXTRACT.—An agar block from a 20-day-old culture of *Asc. hyalospora* strain 805 on potato dextrose agar was inoculated into 1-liter flasks containing 250 ml potato dextrose broth. Stationary cultures were incubated at 26° for 20 days. The bright yellow, fluorescent culture filtrate (10 liters) was adjusted to pH 3 and extracted 3× with 10 liters of recycled $CHCl_3$. The biologically inactive H_2O layer was discarded.

TABLE 3. Effect of *Ascochyta hyalospora* Toxins Against Assorted Microorganisms.[a]

Microorganism	Compound				
	1		2		3
	500[b]	1000	500	1000	500
Escherichia coli	8	10	0	0	0
Agrobacterium tumefaciens	26	35	4	22	0
Erwinia carotovora	0	0	0	0	0
Corynebacterium flaccumfaciens	30	42	22	34	12
Aspergillus flavus	0	0	0	0	10
Penicillium notatum	0	10	16	22	12
Colletotrichum acutatum	18	20	21	32	16

[a]Table entries are inhibitory zone (diameter in mm).
[b]Concentration in μg.

ASCOCHYTINE [**1**].—The toxic residue (400 mg) after evaporation of $CHCl_3$ was partitioned between $CHCl_3$ and 0.4% Na_2CO_3. All of the yellow color was extracted into aqueous Na_2CO_3. Acidification of the aqueous layer, back-extraction into $CHCl_3$, and evaporation of $CHCl_3$ gave yellow needles. Recrystallization from hexane/MeOH gave yellow crystals of ascochytine with physical and spectral properties identical to those reported in the literature (30).

PYRENOLIDE A [**2**].—The initial $CHCl_3$ extract containing alkali-insoluble metabolites was fractionated on Si gel preparative tlc developed with C_6H_6-Me_2CO (3:2). A non-polar, fluorescence-quenching band was eluted to give 50 mg of a brown syrup identified spectroscopically as pyrenolide A (16).

HYALOPYRONE [**3**].—A polar zone from the preparative tlc of the crude $CHCl_3$ extract gave 12 mg of pale yellow syrup which quenched fluorescence on tlc plates but did not react with phosphomolybdic acid (white spot on blue background). This metabolite was identified spectroscopically as hyalopyrone: tlc R_f 0.31 [C_6H_6-Me_2CO (60:40)], 0.50 [$CHCl_3$-MeOH (90:10)], 0.28 [CH_2Cl_2-Me_2CO (70:30)]; hrms 224.1427 (calcd for $C_{13}H_{20}O_3$, 224.1413); eims *m/z* 224 (13%), 209 (100%), 196 (37%), 182 (41%), 165 (52%), 151 (44%); uv λ max (MeOH) 215 nm (e 8700), 255 (22,000), no NaOH shift; ir (thin film) 1650 cm^{-1}; cd in MeOH $[\theta]_{234}$ −11,400 deg·cm^2·$decimol^{-1}$.

TEST PLANTS.—Lambsquarters and nine other weeds, prickly sida (*Sida spinosa* L.), morningglory (*Ipomoea* sp.), jimsonweed (*Datura stramonium* L.), sorghum [*Sorghum bicolor* (L.) Moench], johnsongrass [*S. halepense* (L.) Person], watercress (*Nasturtium officinale* R. Br.); sicklepod (*Cassia obtusifolia* L.), ragweed (*Ambrosia artemisifolia* L.), and bentgrass (*Agrostis alba* L.) were used for the leaf bioassay. The plants were grown and maintained in a greenhouse.

BIOLOGICAL TESTING.—Phytotoxicity was determined on lambsquarters and nine different weed species by a detached leaf assay (13). Electrolyte leakage from lambsquarters leaf tissues was determined by the method described previously (31). Antimicrobial disk assays were performed by standard methods (32).

ROOT GROWTH INHIBITION ASSAY.—A known weight of the toxins (500 and 1000 μg of ascochytine and pyrenolide A) in EtOAc was absorbed on to a piece of autoclaved Whatman 3 mm paper cut to the size of the experimental agar zone (Figure 3) (33). Solvent was evaporated from the paper. A solvent blank paper was also prepared and dried. Grain sorghum seeds were soaked in sterile H_2O for 5–6 h, then placed between moistened papers in a plastic box at 25°. Seeds in excess of the actual number to be used in the bioassay were started in order to allow selection of uniformly germinated seedlings. The box was arranged vertically to facilitate straight and geotropic growth of roots between papers. After 24 h, five seedlings of very uniform root length (0.5 cm) were selected.

An agar Petri plate (90 mm) was prepared as indicated in Figure 3. Agar was scooped out to provide aerial growth space. Holes (3 mm) were cut just at the air-agar interface, and the germinated seeds were inserted. The holes kept the seeds from falling when the plates were raised to the vertical position to allow geotropic growth. A marking pen was used to draw a line across the plate to mark the location of the root tips at the beginning of the assay. A channel was cut into the agar 3 cm below the holes to prevent test compounds from being diluted by diffusion throughout the plate. The paper strip containing absorbed test material was laid on top of the agar strip containing the seeds. The paper quickly became wet from the H_2O agar, after which the plate was raised to the vertical position. A control consisted of a similar plate on which

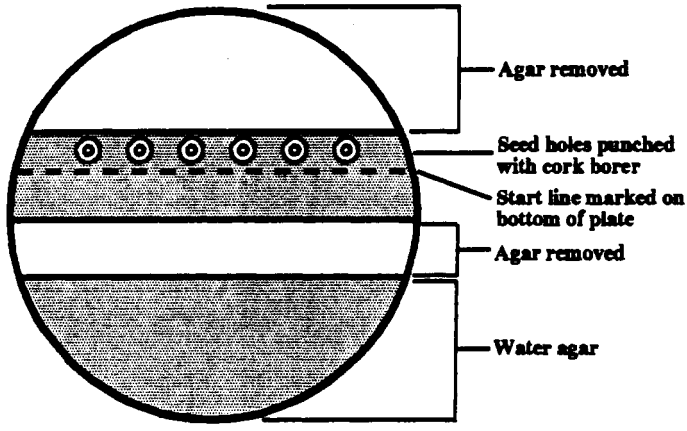

FIGURE 3. Agar plate root growth inhibition assay (see Experimental).

was laid the blank paper. Root growth below the marking pencil line was recorded at time intervals. Inhibition was detected in some cases as early as 4 h after set-up and was quite evident by 24 h.

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