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Interference Competition among Coprophilous Fungi: Production of (+)-Isoepoxydon by *Poronia punctata*

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(+)-Isoepoxydon has been established as the major causative agent of interference competition between *Poronia punctata* (NRRL 6457), a late fungal colonist of cattle dung, and two early-occurring dung colonists, *Ascobolus furfuraceus* (NRRL 6460) and *Sordaria fimicola* (NRRL 6459). This compound was isolated from ethyl acetate extracts of liquid cultures of *P. punctata* by silica gel chromatography and identified by mass spectrometry and proton and carbon nuclear magnetic resonance spectroscopy. The isolation process was guided by in vitro bioassays for antifungal activity against *A. furfuraceus* and *S. fimicola*. (+)-Isoepoxydon has been implicated as an intermediate in the biosynthesis of patulin, a mycotoxin produced by *Penicillium* spp., but no patulin could be detected in cultures of *P. punctata*.

Antagonism between species of fungi has been observed frequently in studies of natural fungal communities (2–6, 10, 12, 14–16). It has been proposed that such interactions are important factors in fungal community organization and in the pattern of fungal succession within individual substrate. In many cases, the mechanism of antagonism appears to involve the production of a chemical agent by one species which inhibits the growth of another. Wicklow and Hirschfield (16) have described this phenomenon in the context of a competitive hierarchy among coprophilous fungal species. These workers have reported that *Poronia punctata* (Linnaeus ex Fries), a late colonist of cattle dung from the grasslands of the western United States (appearing on the dung after 6 to 10 months of exposure in the field), exhibits antagonism towards several of its natural competitors in vitro. Identification of the metabolite(s) responsible for such effects would provide a tool with which to investigate the ecology of competition within fungal communities and could lead to the discovery of new antifungal agents. We have isolated a metabolite from liquid cultures of *P. punctata* which is responsible for these antagonistic effects, and we report here details of the isolation, identification, and biological activity of this compound.

MATERIALS AND METHODS

General. A strain of *P. punctata* (NRRL 6457), originally isolated from an aged dung pat (>2 years of field exposure) collected in the Pawnee National Grasslands in western Colorado (1), and cultures of two widely distributed early colonists of herbivore dung, *Ascobolus furfuraceus* (NRRL 6460) and *Sordaria fimicola* (NRRL 6459), were obtained from the Agricultural Research Service Culture Collection at the Northern Regional Research Laboratory in Peoria, Ill. Stock cultures were maintained on Difco potato dextrose agar (PDA) slants at 4°C. Cattle dung broth and cattle dung agar were prepared from dried dung pats by methods described previously (11). Glucose-cattle dung broth was prepared by supplementing cattle dung broth with glucose to a final concentration of 3%. All culture media were sterilized by autoclaving at 121°C and 15 lb/in^2 for 15 min. Column chromatography was done with silica gel (80-230 mesh; Baker). Thin-layer chromatography (TLC) was performed on glass plates coated with silica gel F-254 (0.25 mm thickness; E. Merck), and TLC spots were visualized by examination under a UV lamp or by exposure to iodine vapor. Reversed-phase high-pressure liquid chromatography (HPLC) was carried out on a Beckman model 332 gradient system with an Altex semipreparative ODS column (5 μm, 10 by 250 mm, 2.0 ml/min, monitored at 215 nm). Quantitative HPLC analysis of the antifungal metabolite was performed by correlation of peak integrals with standard curves generated by injection of known quantities of the metabolite. Proton and carbon-13 nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WM-360 spectrometer at 360 and 90.7 MHz, respectively. Electron impact mass spectra were obtained at 70 eV with a Hewlett-Packard 5985B mass spectrometer with direct inlet probe. The high-resolution chemical ionization mass spectrum was obtained with a VG 7070E spectrometer with methane as the reagent gas.

Bioassays. *A. furfuraceus* and *S. fimicola*, two species of early-occurring dung colonists, were used as test organisms. Assays for direct competition between *P. punctata* and each test organism were performed by inoculating the fungi 25 mm apart on a petri dish containing PDA. *P. punctata* was inoculated 48 h prior to introduction of the test organism because of its relatively slow growth rate. In all trials, a zone extending approximately 10 mm from the edge of the *P. punctata* colony was uninvaded by the early colonists. Similar results were obtained with a cattle dung agar substrate, although the uninvaded zones were approximately 50% smaller.

Assays for activity of culture extracts, column fractions, and purified metabolites against the test organisms were done with two different paper disk-agar diffusion assay techniques. In one assay, filter paper disks (12.5 mm diameter) were impregnated with measured amounts of solutions containing known concentrations of sample to be tested. After evaporation of solvent, the disks were placed in the center of a petri dish (100 by 15 mm) containing sterile PDA (7 ml). Controls were prepared in the same manner, but only pure solvent was added to the disks. After 4 h, the disk was removed and the center of the plate was inoculated with the test organism. Growth on the treated plates was compared

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with growth on untreated controls by comparing colony diameters at 12-h intervals. Each colony diameter was obtained by averaging values measured for three different cross-sections of the colony. All trials were run concurrently in triplicate. In the second assay, the filter paper disk was allowed to remain on the agar surface and the test organism was inoculated 25 mm from the center of the disk. The culture was then incubated at 25 to 28°C for 48 to 72 h. No metabolites were present in controls, and the growth of the test organism was unimpeded. If the disk contained a metabolite antagonistic to the test organism, colony deformation occurred in the area about the disk due to interference with the normal growth of the test organism. The physical characteristics of the peripheral hyphae of affected colonies were compared with those of the controls by examination under a microscope (45× magnification).

Cultivation of *P. punctata*. Two-liter Erlenmeyer flasks, each containing 450 ml of potato dextrose broth (Difco), were inoculated with several 1-cm^2 plugs of *P. punctata* taken from 4-day-old petri dish cultures. Flask cultures were incubated at 25 to 28°C and aerated by agitation on an orbital shaker at 200 rpm. Production of the antagonistic metabolite was monitored by daily bioassay of the culture filtrate and in later experiments by TLC. Antifungal activity reached a maximum after approximately 5 days. Similar results were obtained when glucose-dung broth was used, but potato dextrose broth was preferred for chemical studies because it contains substantially fewer contaminating extractables.

Isolation of the antagonistic component. The filtered culture broth (900 ml) was extracted three times with dichloromethane (300 ml) and three times with ethyl acetate (300 ml). The ethyl acetate fractions were combined, dried ($MgSO_4$), and evaporated to afford 454 mg of an orange oil which contained most of the antifungal activity. The oil was chromatographed on a silica gel column (2 by 45 cm) with 95:5 (vol/vol) chloroform-methanol, collecting 4-ml fractions. Fractions 38 to 58 were combined to give an antifungal compound (163 mg) as a colorless oil which showed a single spot on TLC at R_f 0.25 (6:1, chloroform-methanol) and a single peak by reversed-phase HPLC with 25:75 methanol-water (retention time, 7.23 min). The antifungal metabolite was identified as (+)-isoepoxydon (Fig. 1) by analysis of the spectral data given in the Appendix.

Extraction of the air-dried mycelium (2.38 g) with ethyl acetate afforded 15.4 mg of a colorless oil which contained virtually none of the antagonistic component (<0.1 mg as determined by quantitative HPLC). Thus, practically all of the antagonistic component was excreted into the medium.

Quantitation of isoepoxydon. Production of isoepoxydon by *P. punctata* began near the end of the growth phase. After a 36- to 48-h lag phase and a 24-h period of rapid mycelial growth, a sharp increase in antifungal activity of the culture filtrate was observed. After 5 days, fungal growth and production of isoepoxydon had effectively ceased. Correlation of isoepoxydon production with air-dried cell mass at 24-h intervals beginning 48 h after inoculation afforded the following results (given as approximate cell mass/isoepoxydon concentration, each in milligrams per milliliter): 48 h, 0.5/<0.002; 72 h, 1.7/0.04; 96 h, 2.6/0.21; 120 h, 2.8/0.29; 144 h, 2.9/0.30.

RESULTS AND DISCUSSION

Analysis of the high-resolution chemical ionization mass spectrum of the antifungal metabolite suggested the molecular formula $C_7H_8O_4$, and the carbon-13 NMR spectrum confirmed the presence of seven carbons. The UV, infrared, and carbon-13 NMR spectra suggested the presence of an α,β-unsaturated ketone moiety. The proton NMR spectrum showed signals suggesting the presence of 1 olefinic hydrogen, 1 hydroxymethylene unit, 1 CH-OH methine proton, 2 hydroxyl protons, and 2 vicinal protons of an epoxide ring. Homonuclear proton NMR decoupling experiments established the connection of these units (Fig. 1). Comparison of all the spectral data and the specific rotation of the isolated compound with literature values for the possible stereoisomers which possess this connectivity allowed assignment of the stereochemistry as shown. This compound, (+)-isoepoxydon, has been isolated previously from a patulin-negative mutant strain of *Penicillium urticae* (9) and has been implicated as an intermediate in the biosynthesis of patulin (Fig. 2), an important mycotoxin. Chromatographic comparison (HPLC and TLC) of the organic-soluble extracts with an authentic sample of patulin (Sigma Chemical Co.) indicated that patulin was not produced by *P. punctata* in either of the media described above, even in older cultures. (+)-Isoepoxydon has also been reported as a lettuce seedling germination inhibitor produced by an unidentified fungus isolated from a leaf surface (7).

Assays of isoepoxydon for activity against the test organisms showed symptoms of hyphal damage identical to those observed in assays for direct competition between *P. punctata* and the test organisms. To determine whether both effects were indeed caused by isoepoxydon and to determine whether isoepoxydon was produced by *P. punctata* on a solid substrate, plugs (1 by 2 cm) of the agar medium were cut from zones uninvaded by the test organisms after assays for direct competition. Analysis of extracts of the plugs by TLC and HPLC confirmed the presence of isoepoxydon as a major component. Again, no patulin could be detected.

The antagonistic activity of isoepoxydon toward the early dung colonists *A. furfuraceus* and *S. fimicola* is summarized in Table 1. At low concentrations, growth of the test organisms was delayed relative to controls, but no other difference could be observed. When larger amounts were introduced, however, severe stunting of growth was observed, and hyphae were poorly developed. Application of a 2-mg sample delayed the growth of *S. fimicola* by 4 days relative to controls and completely prevented spore germination and growth of *A. furfuraceus* even after 1 week. When test organisms were inoculated onto a plate near a filter paper disk impregnated with the metabolite, the colonies formed were abnormally shaped as a result of growth

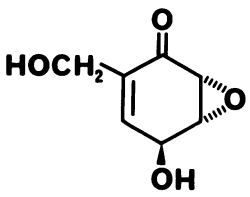

FIG. 1. (+)-Isoepoxydon.

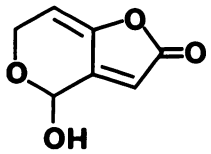

FIG. 2. Patulin.

TABLE 1. Inhibition of radial growth of competitor fungi by (+)-isoepoxydon as determined by the paper disk assay

Quantity applied (mg/disk)	Avg colony diam (mm)[a] at incubation time (h):										
	S. fimicola						*A. furfuraceus*				
	12	24	36	48	60	72	24	48	72	96	120
0 (controls)	21	38	56	81	85	85	10	25	59	85	85
0.25	11	25	41	60	80	85	—	9	22	38	54
0.50	8	19	32	48	65	85	—	—	10	21	38
1.00	—	10	14	20	28	39	—	—	—	8	17
2.00	—	—	—	—	—	9	—	—	—	—	—

[a] Values are averages derived from three concurrent trials. —, Complete inhibition of growth. A value of 85 indicates that the petri dish was completely covered with mycelial growth.

inhibition in the region near the disk. The magnitude of the observed effect was directly related to the quantity of material applied to the disk. The hyphal strands at the edge of this zone were brown and abnormally slender and exhibited an unusually high degree of branching relative to the edge of control colonies. After a delay of several days, however, the test organisms grew sluggishly through the region, eventually encompassing the disk with fragile, vacuolate hyphae.

These results are somewhat different from those observed in assays for direct competition, in which inoculated test organisms never encroached closer than 10 mm to the edge of *P. punctata* colonies. This difference cannot be ascribed to volatility, but is probably due to the high degree of reactivity of isoepoxydon, which may decompose slowly under the assay conditions or be slowly metabolized by the test organisms. Assays for direct competition would yield different results because the living *P. punctata* colonies could continue to produce isoepoxydon as it is lost. Such a mechanism would also prevent a very large buildup of isoepoxydon under natural conditions, which might be toxic or autoinhibitory to *P. punctata*. Although many factors are involved in competition among coprophilous fungi, the large quantities of isoepoxydon produced by *P. punctata* in culture, the essentially complete excretion of this metabolite into the surrounding medium, its production on cattle dung media, and its activity against potential fungal competitors suggest that the compound may provide a competitive advantage for *P. punctata*.

Despite the activity of isoepoxydon against competitor fungi, it was inactive in paper disk-agar diffusion assays against the yeasts *Saccharomyces cerevisiae* (ATCC 2601) and *Candida albicans* (ATCC 14053). In addition, isoepoxydon had no effect on the growth or germination of *P. punctata* relative to controls. However, the production of isoepoxydon by the control colonies prevents exclusion of the possibility that it may exert some effect on *P. punctata*.

Recently, a series of sesquiterpenoids with antibacterial activity, known as punctatins, have been isolated from a horse dung isolate of *P. punctata* (8). We encountered several sesquiterpenoids in our culture filtrates, including punctatin B (Fig. 3), but these did not exhibit any activity against *S. fimicola* or *A. furfuraceus* or any synergistic effects when tested as mixtures with isoepoxydon.

Isoepoxydon is now known to be produced by both *P. punctata* and *Penicillium urticae*, two fungi with quite different life cycles, ecological niches, and taxonomic classifications. Strongman et al. (13) suggest that certain secondary metabolic pathways leading to compounds with specific activities may be conserved across wide taxonomic lines. Alternatively, the selection process may have guided the separate evolution of these pathways. Mycotoxins are abundant and widespread in nature, but little is known of their roles in the life cycles of the producing organisms. One reason proposed for the evolution of the biosynthetic abilities associated with mycotoxin production is that such compounds may be useful to the producing organisms as chemical defenses under certain circumstances. The production of isoepoxydon by *P. punctata* and its activity against competitor fungi lend support to this argument, since production of isoepoxydon could help *P. punctata* colonies to exclude other fungi from consuming available, vital nutrient resources. We have recently found that other coprophilous fungal species also produce agents effective against potential competitors, and studies of these agents and their effects are under way in our laboratory.

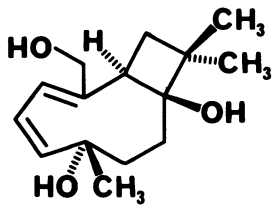

FIG. 3. Punctatin B.

APPENDIX

(+)-Isoepoxydon gave the following spectral data: $[\alpha]_D$ (methanol; c = 7.5): +192°; UV (methanol): 239 (ε5400), 283 nm (ε200); infrared ($CHCl_3$): 3,345 (OH), 1,671 cm^{-1} (C = 0); EIMS: m/z 138 (M − H_2O; 1.0% of base peak), 121 (3), 112 (15), 110 (19), 97 (12), 96 (16), 82 (18), 81 (26), 71 (33), 69 (28), 55 (52), 53 (100), 43 (48), 41 (47), 39 (94), 31 (50); CIMS: m/z 157 (M + H; base peak); 1H NMR (acetone-d_6): signals at 6.70 (*m*, 1H), 4.84 (broad *s*, 1H, D_2O exchangeable), 4.65 (*m*, 1H), 4.25 (broad *d*, J = 16 Hz, 1H), 4.15 (broad *d*, J = 16 Hz, 1H), 4.09 (broad *s*, 1H, D_2O exchangeable), 3.77 (*ddd*, J = 3.7, 2.6, 1.4 Hz, 1H), and 3.40 ppm (*dd*, J = 3.7, 1.2 Hz, 1H); ^{13}C NMR (acetone-d_6): signals at 194.3 (*s*), 139.2 (*d*), 137.0 (*s*), 63.3 (*d*), 59.0 (*t*), 58.8 (*d*), and 54.1 ppm (*d*); HRCIMS: observed; m/z 157.0492 (M + H). Calculated for $C_7H_8O_4$ + H: 157.0500.

Treatment of (+)-isoepoxydon with an excess of acetic anhydride (10% solution in pyridine) gave a single, aromatized acetylation product which was assigned as tetra-*O*-acetyl-2,3,5-trihydroxybenzyl alcohol by analysis of the following data: EIMS ions at m/z 282 (M − CH_3CO, 0.6% of base peak), 240 (2), 222 (1), 198 (0.6), 180 (11), 138 (39), 110 (4), 69 (11), 43 (100); 1H NMR ($CDCl_3$): 7.09 (*d*, 1H, J = 3.5 Hz), 7.03 (*d*, 1H, J = 3.5 Hz), 5.03 (*s*, 2H), 2.30 (*s*, 3H), 2.27 (*s*, 3H), 2.25 (*s*, 3H), 2.06 (*s*, 3H).

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