Journal of Natural Products
Vol. 51, No. 5, pp. 879-883, Sep-Oct 1988

INTERFERENCE COMPETITION AMONG NATURAL FUNGAL COMPETITORS: AN ANTIFUNGAL METABOLITE FROM THE COPROPHILOUS FUNGUS *PREUSSIA FLEISCHHAKII*

HOLLY A. WEBER and JAMES B. GLOER*

Department of Chemistry, University of Iowa, Iowa City, Iowa 52242

ABSTRACT.—Compound **1** (2,3′-dihydroxy-4-methoxy-5′,6-dimethyldiphenyl ether) has been established as the major causative agent of interference competition between *Preussia fleischhakii*, a fungal colonist of rabbit dung, and two early successional dung colonists, *Ascobolus furfuraceus* and *Sordaria fimicola*. Compound **1** was isolated from EtOAc extracts of liquid cultures of *P. fleischhakii* by Si gel chromatography and reversed-phase hplc and was identified by analysis of ^{1}H-nmr, ^{13}C-nmr, and ms data. The isolation process was guided by in vitro bioassays for antifungal activity against *A. furfuraceus* and *S. fimicola*. These results suggest that studies of interference competition observed in relatively unexplored natural fungal communities may be a valuable, ecologically rational approach to the discovery of antifungal agents.

Antagonism between species of fungi has been observed frequently in studies of natural fungal communities (1–6). It has been proposed that such interactions are important factors in determining the organization, composition, and pattern of succession within these fungal ecosystems (1–3). In many cases, the mechanism of this antagonism appears to involve the production by one species of a chemical agent that inhibits the growth of competitors (1–4,6).

Reports of interspecies competition among coprophilous (dung-colonizing) fungi (3–5) have led us to investigate the chemical basis for these observations (1). Studies of metabolites responsible for such effects would provide further insight into the ecology of competition within fungal communities and could lead to the discovery of new antifungal agents. Chemical studies of coprophilous fungi are relatively rare, since they are not commonly encountered in soil samples. We have isolated from cultures of the coprophilous fungus *Preussia fleischhakii* (Auerswald) Cain (NRRL A-24068) a metabolite that inhibits the growth of potential fungal competitors, and we report here details of the isolation, identification, and biological activity of this compound.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—A culture of *P. fleischhakii* was isolated from rabbit dung collected from the Curtis Prairie Arboretum at the University of Wisconsin, Madison by Dr. D.T. Wicklow of the USDA Northern Regional Research Center. *Ascobolus furfuraceus* Persoon per Hooker (NRRL 6460) and *Sordaria fimicola* (Roberge) Cesati et de Notaris (NRRL 6459), two widely distributed early colonists of herbivore dung, were employed as test organisms. All cultures were obtained from the ARS culture collection, USDA Northern Regional Research Center, Peoria, Illinois. Stock cultures were maintained on Difco potato dextrose agar (PDA) slants at 4°. Culture media were sterilized by autoclaving at 121° and 15 psi for 15 min. Column chromatography employed Si gel (80–230 mesh; Baker). Tlc was performed using glass plates precoated with Si gel F-254 (0.25 mm thickness, E. Merck), and spots were visualized by examination under a uv lamp (254 nm) or by exposure to iodine vapor. Reversed-phase hplc was carried out using a Beckman model 332 gradient system with an Altex semipreparative ODS column [5-μm; 10 × 250 mm; MeOH-H_2O (70:30) at 2.0 ml/min; monitored by uv detection at 215 nm]. ^{1}H- and ^{13}C-nmr spectra were recorded in $CDCl_3$ on a Bruker WM-360 spectrometer at 360 and 90.7 MHz, respectively, and nmr assignments were based on the results of ^{13}C-^{1}H decoupling experiments. All mass spectra were obtained by electron impact ionization (direct inlet probe) with a VG ZAB-HF spectrometer.

BIOASSAYS.—*A. furfuraceus* and *S. fimicola*, two species of early successional dung colonists, were employed as test fungi. Assays for direct competition between *P. fleischhakii* and each test organism were performed in vitro by inoculating the fungi at a distance of 25 mm apart on a Petri dish containing PDA. *P. fleischhakii* was inoculated 24 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. fleischhakii* colony

was not invaded by the test organism. These assays were repeated using glucose dung agar prepared by supplementing dung agar (7) with glucose to a concentration of 3%. Analogous results were obtained, although the uninvaded zones in each case were approximately 30% smaller.

Antifungal activities of extracts and column fractions were determined semiquantitatively using paper disk/agar diffusion assay methods described previously (1). Activity of the antagonistic agent was quantitated using standard agar dilution techniques and is reported in terms of IC_{50} values (concentration of metabolite required to reduce fungal growth by 50%) and MIC values (minimum concentration required to completely prevent fungal growth). The brine shrimp toxicity assay was performed according to procedures of Meyer *et al.* (8), and brine shrimp eggs were obtained from Carolina Biological Supply Company.

CULTIVATION OF *P. FLEISCHHAKII*.—Two-liter Erlenmeyer flasks, each containing 400 ml of potato dextrose broth (Difco), were inoculated with several 1-cm^2 plugs of *P. fleischhakii* taken from 3-day-old Petri dish cultures (PDA). Flask cultures were incubated at 25-28° and aerated by agitation on an orbital shaker at 200 rpm. Production of the antagonistic metabolite was monitored by bioassay of the culture filtrate, and in later experiments by tlc. Activity of the filtrate against both *A. furfuraceus* and *S. fimicola* reached a maximum after 14 days.

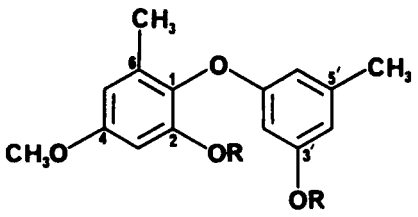
ISOLATION AND ANALYSIS OF THE ANTAGONISTIC COMPONENTS [**1**].—The filtered culture broth (1200 ml) was extracted with EtOAc (4 × 500 ml), and the EtOAc fractions were combined, dried ($MgSO_4$), and evaporated to afford 166.5 mg of an orange oil which contained most of the antifungal activity. The oil was chromatographed on a Si gel column (2 × 45 cm) with 200 ml of $CHCl_3$, followed by 200 ml each of 1, 3, 5, and 8% $CHCl_3$/MeOH, and 5-ml fractions were collected. Fractions 78–95 were combined and purified further by reversed-phase hplc to afford 18 mg of the antifungal compound **1** as a colorless oil that crystallized from $CHCl_3$/petroleum ether. Compound **1** has the following properties: mp 120.5–121.5°; R_f 0.48 [$CHCl_3$-MeOH (9:1)]; hplc retention time 11.25 min; uv λ max (MeOH) 225 (ε 21000), 277 (ε 4500), 281 nm (ε 5100); ir (neat) 3570, 3420, 2970, 1585, 1485, 1315, 1215, 1155, 980, 970 cm^{-1}; eims (70 eV) *m/z* $[M]^+$ 260 (100%; base peak), 245 (7.2), 230 (9.8), 227 (12), 199 (9.0), 153 (74), 152 (42), 135 (12), 125 (54), 108 (14), 107 (31), 93 (9.7), 79 (23), 77 (39), 69 (14), 65 (8.2); ^1H and ^{13}C nmr see Table 1; hreims observed *m/z* 260.1049 $[M]^+$, calculated for $C_{15}H_{16}O_4$, 260.1048.

ACETYLATION OF COMPOUND **1**.—A sample of compound **1** (9.2 mg) was dissolved in a solution of Ac_2O (0.2 ml) in pyridine (1 ml). After stirring at room temperature for 18 h, the solvent was evaporated and the residue partitioned between H_2O and CH_2Cl_2 (2 ml each). The organic phase was washed twice more with H_2O (2 × 2 ml), and subsequently dried ($MgSO_4$) and evaporated to give 10.9 mg (89.6% yield) of diacetylation product **2** as an oil that exhibits the following properties: R_f 0.74 [$CHCl_3$-MeOH (9:1)]; hplc retention time 27.9 min; uv λ max (MeOH) 224 (ε 18400), 270 (ε 3200), 278 nm (ε 3400); ir (neat) 2940, 2860, 1760, 1600, 1585, 1375, 1215, 1165, 1135, 1050, 990, 905 cm^{-1}; eims (30 eV) *m/z* $[M]^+$ 344 (36% of base peak), 302 (98), 260 (100), 245 (5.4), 230 (5.2), 227 (6.1), 199 (4.5), 153 (21), 152 (10), 125 (17), 107 (5.4), 77 (4.6), 43 (15); ^1H and ^{13}C nmr see Table 1.

RESULTS AND DISCUSSION

STRUCTURE DETERMINATION.—Analysis of the high resolution mass spectrum of the antifungal metabolite suggested the molecular formula $C_{15}H_{16}O_4$, and the ^{13}C-nmr spectrum verified the presence of 15 carbons. The ^1H- and ^{13}C-nmr spectra revealed the presence of 1,3,5-trisubstituted and 1,2,3,5-tetrasubstituted aromatic rings, two aromatic methyl substituents, one methoxy group, and two phenolic hydroxy groups. The remaining oxygen atom was assigned to an ether linkage. Thus, the metabolite was proposed to be a pentasubstituted diphenyl ether derivative. Comparison of preliminary data for the isolated compound with literature values (uv, mp) implied that this metabolite might be identical to compound LL-VI25α [**1**], which has been previously reported only as a metabolite of an unidentified fungus (9,10). Although the structure of compound LL-VI25α was established through a multistep synthesis (10), sufficiently detailed spectral data for direct comparison (^{13}C nmr, ir, ms, complete ^1H nmr) were not reported, and structural isomers could not be ruled out. Thus, ^1H-^1H and ^{13}C-^1H nmr decoupling experiments were employed in our laboratory to definitively establish the connectivity of the isolated compound.

Irradiation of the aromatic methyl proton signal at 2.22 ppm simplified each of the three mutually coupled aromatic proton multiplets at 6.29, 6.26, and 6.10 ppm to



1 R=H
2 R=Ac

varying degrees, thereby placing this methyl group on the 1,3,5-trisubstituted ring. Irradiation of the second aromatic methyl resonance collapsed the doublet of quartets at 6.30 ppm to a sharp doublet and sharpened the doublet at 6.43 slightly, thereby indicating its location on the tetrasubstituted ring, and suggesting an orientation ortho to the proton at 6.30 ppm and para to the proton at 6.43. This arrangement was verified by long-range ^{13}C-^{1}H decoupling experiments wherein only the signal at 6.30 showed coupling to the methyl carbon. Irradiation of the methoxy proton resonance sharpened the signals for both of these aromatic protons, positioning the methoxy group on the tetrasubstituted ring, and placing it ortho to both protons. This assignment was supported by observation of nOe enhancements of 8% and 7% for the signals at 6.43 and 6.30 ppm upon irradiation of the methoxy proton resonance.

These data narrowed the possible structures to **1** and an alternative structure in which the hydroxy and aryloxy substituents on the tetrasubstituted ring are interchanged. Differentiation between these possibilities was achieved by examination of the ^{13}C-nmr data for the diacetyl derivative of **1**. Acetylation of the hydroxyl groups in either structure would result in characteristic downfield shifts of the carbon signals ortho and para to the newly formed acetoxy group. Signals for protons meta to the

TABLE 1. Nmr Assignments for Compounds **1** and **2**.

Carbon	Compound			
	1		**2**	
	^{13}C	^{1}H	^{13}C	^{1}H
C-1	133.4 (s)	—	138.0 (s)	—
C-2	149.2 (s)	—	143.8 (s)	—
C-3	99.3 (d)	6.43 (br d; 2.9)	106.9 (d)	6.55 (br d; 3.0)
C-4	157.0 (s)	—	156.5 (s)	—
C-5	108.1 (d)	6.30 (dq; 2.9, 0.5)	113.1 (d)	6.66 (dq; 3.0, 0.6)
C-6	132.5 (s)	—	133.6 (s)	—
C-1′	158.7 (s)[a]	—	158.6 (s)	—
C-2′	99.4 (d)	6.10 (br dd; 2.2, 2.1)	105.9 (d)	6.32 (br dd; 2.5, 2.2)
C-3′	156.6 (s)[a]	—	151.4 (s)	—
C-4′	110.2 (d)[b]	6.29 (m)[c]	115.8 (d)[d]	6.53 (m)[e]
C-5′	141.1 (s)	—	140.4 (s)	—
C-6′	108.0 (d)[b]	6.26 (m)[c]	114.0 (d)[d]	6.47 (m)[e]
4-OMe	55.5 (q)	3.77 (br s)	55.6 (q)	3.77 (br s)
6-Me	16.3 (q)	2.04 (br s)	16.5 (q)	2.14 (br s)
5′-Me	21.5 (q)	2.22 (br s)	21.5 (q)	2.26 (br s)
2,3′-Ac	—	—	21.1 (q)	2.22 (s)
			20.5 (q)	2.04 (s)
2,3′-OH	—	var	—	—

[a-e]Proton and carbon assignments with identical superscript letters are interchangeable.

acetoxy group would be relatively unaffected. Treatment of the natural product with Ac_2O/pyridine afforded a diacetate **2** in which the carbon signals at 99.3, 108.1, and 133.4 ppm were shifted downfield to 106.9, 113.1, and 138.0 ppm, respectively. The signals at 132.5 and 157.0 ppm were relatively unaffected by acetylation (133.6 and 156.5), and the signal at 149.2 was shifted upfield (to 143.8 ppm) as expected for the carbon attached to the acetoxy group. Thus, both methine protons are ortho or para to the hydroxyl group in the natural product. Because only the methine proton attached to the carbon at 99.3 ppm is coupled to the hydroxyl-group-bearing carbon, the natural product must have structure **1**. Compound **1** appears to be derived from the polyketide pathway as a tetraketide with a loss of one carbon.

BIOLOGICAL ACTIVITY.—In assays of compound **1** for antifungal activity, the test organisms showed symptoms of hyphal damage identical to those observed in assays for direct competition between *P. fleischhakii* and the test organisms. These symptoms included brownish coloration, abnormally slender hyphae, and an unusually high degree of branching. In order to establish that this competitive effect is indeed caused by compound **1** and to determine whether this compound is produced by *P. fleischhakii* on a solid substrate, 1- × 2-cm plugs of the agar medium were cut from zones uninvaded by the test organisms after assays for direct competition. Analysis of EtOAc extracts of the plugs by hplc confirmed the presence of compound **1** as a major component. Similar results were obtained using glucose-dung agar.

When either test organism was inoculated onto a plate near a filter paper disk impregnated with the metabolite, the colonies formed were abnormally shaped as a result of growth inhibition in the region near the disk. The hyphal strands at the edge of this zone were abnormally slender, discolored, and exhibited an unusually high degree of branching relative to the edges of control colonies. Compound **1** exhibited approximate IC_{50} values of 50 μg/ml and 100 μg/ml against *S. fimicola* and *A. furfuraceus*, respectively. The corresponding MIC values were 75 μg/ml and 250 μg/ml. Compound **1** is also mildly toxic to brine shrimp, exhibiting an LC_{50} value of 125 μg/ml.

Although there are many factors involved in competition among coprophilous fungi, the production of **1** by *P. fleischhakii* in culture, its production on dung media, and its activity against potential fungal competitors in vitro suggest that the compound may provide a natural competitive advantage for *P. fleischhakii*. However, studies of the production and effects of **1** within the natural substrate are necessary in order to permit conclusions about the importance of this compound in interspecies competition. Efforts to detect levels of this metabolite produced in solid-substrate fermentations on dung are underway in our laboratory, and studies of other antagonistic agents produced by coprophilous fungi are also in progress. To our knowledge, this account represents the first report of a secondary metabolite from a member of the genus *Preussia*.

ACKNOWLEDGMENTS

The authors thank Dr. D.T. Wicklow of the USDA Northern Regional Research Center for providing the cultures and for helpful discussions.

LITERATURE CITED

1. J.B. Gloer and S.M. Truckenbrod, *Appl. Environ. Microbiol.*, **54**, 861 (1988).
2. D.B. Strongman, J.D. Miller, L. Calhoun, J.A. Findlay, and N.J. Whitney, *Bot. Mar.*, **30**, 21 (1987).
3. D.T. Wicklow and B.J. Hirschfield, *Can. J. Microbiol.*, **25**, 855 (1979).
4. N. Singh and J. Webster, *Trans. Br. Mycol. Soc.*, **61**, 487 (1973).
5. F.E.O. Ikediugwu and J. Webster, *Trans. Br. Mycol. Soc.*, **54**, 181 (1970).
6. M.A. Stillwell, F.A. Wood, and G.M. Strunz, *Can. J. Microbiol.*, **15**, 501 (1969).
7. R.B. Stevens, "Mycology Guidebook," University of Washington Press, Seattle, 1981, p. 659.

8. B.N. Meyer, N.R. Ferrigni, J.E. Putnam, L.B. Jacobsen, D.E. Nichols, and J.L. McLaughlin, *Planta Med.*, 31 (1983).
9. W.J. McGahren, W.W. Andres, and M.P. Kunstmann, *J. Org. Chem.*, **35**, 2433 (1970).
10. J.R. Cannon, T.M. Cresp, B.W. Metcalf, M.V. Sargent, and J.A. Elix, *J. Chem. Soc., Perkin Trans. 1*, 1200 (1972).

Received 25 February 1988