Thallophytic Allelopathy: Isolation and Identification of Laetisaric Acid

WILLIAM S. BOWERS, HARVEY C. HOCH, PHILIP H. EVANS, MASATO KATAYAMA

***Laetisaria arvalis*, a soil-dwelling basidiomycete fungus, secretes an allelopathic agent that induces rapid hyphal lysis in several phytopathogenic fungi. The active compound was isolated from chloroform:methanol extracts of *L. arvalis* mycelia and characterized as a previously unknown hydroxy fatty acid, *(Z,Z)*-9,12-8-hydroxyoctadecadienoic acid.**

PYTHIUM ULTIMUM, AN OOMYCETE fungus, is a major root pathogen of many crops in temperate regions of the world. *Laetisaria arvalis* Burds., a basidiomycete fungus (*1*), is effective as a biological control agent in reducing soil-borne diseases of several crops incited by *Pythium ultimum* and other phytopathogenic fungi including *Rhizoctonia solani* and *Phoma betae* (*2*).

During investigations of the underlying control mechanism, we found that combination of growing cultures of *P. ultimum* and *L. arvalis* on an agar-coated microscope slide (*3*) resulted in rapid lysis of the *P. ultimum* hyphae (Fig. 1). This suggested that the observed suppression of *P. ultimum* growth might be due to a diffusable toxin rather than mycoparasitism. Although both aqueous and organic extracts of *L. arvalis* were initially found to induce cytoplasmic lysis of *P. ultimum*, the organic extracts were far more potent. From *L. arvalis* we have isolated and identified a previously undescribed compound that effectively inhibits the growth of *P. ultimum* and certain other phytopathogenic fungi.

Mycelia of *L. arvalis* cultures were extracted with chloroform:methanol (9:1 by volume), and the solvent was evaporated to yield an active crude extract (*4*). Column chromatography of the extract on Florisil and sequential preparative silica thin-layer chromatography (TLC) (*5*) yielded a pure compound (*6*), which we have characterized and named laetisaric acid.

The mass spectrum of the methyl ester of laetisaric acid (prepared by treatment with diazomethane) showed a base peak at the mass-to-charge *(m/z)* ratio 93 and a strong peak at *m/z* 292 due to a loss of water from the molecular ion, suggesting a molecular formula of $C_{19}H_{34}O_3$. Proton nuclear magnetic resonance (^{1}H NMR) spectra of laetisaric acid methyl ester exhibited 34 protons, suggesting a methyl ester of a fatty acid with a nonconjugated dienol system (*7*). In further support of the structural assignments, the methyl ester yielded methyl stearate after catalytic hydrogenation (*8*). The physical constants [gas chromatographic (GC) retention time and mass spectra] were identical to those of authentic methyl stearate. The geometry of the two double bonds was *cis (Z)* (coupling constant, J values of 10.7 and 11.2 Hz in the ^{1}H NMR spectrum of the methyl ester). The evidence for the position of the double bonds was obtained from ozonolysis products, whose mass spectrum and GC retention times were identical to those of authentic *n*-hexanal. These results, combined with the ^{1}H NMR analyses, indicated double bonds located at C-9 and C-12 and a hydroxyl group at C-8. These assumptions were further supported by mass spectra of the trimethylsilyl (TMS) ethers and the oxidized derivatives which showed characteristic ion peaks at *m/z* 239 ($C_5H_{11}CH$=$CHCH_2$–CH=CHCH=OTMS) and *m/z* 165 ($C_5H_{11}CH$=$CHCH_2CH$=CHC=O), respectively, due to alpha-cleavage.

Confirmation of the structure of laetisaric acid was obtained by a total synthesis of 8-hydroxylinoleic acid (*9*). Physical and biological activity data from the natural and synthetic laetisaric acid were coincidental.

Crude *L. arvalis* mycelial extracts, chromatographic fractions, and pure isolated and synthetic laetisaric acid were evaluated for activity against growth of *P. ultimum* by a radial growth assay. Material to be tested was incorporated into agar-coated petri plates, and disks of *P. ultimum* mycelia were placed onto the solidified agar. The dose-response curve of pure laetisaric acid was nearly linear and highly reproducible (Fig. 2).

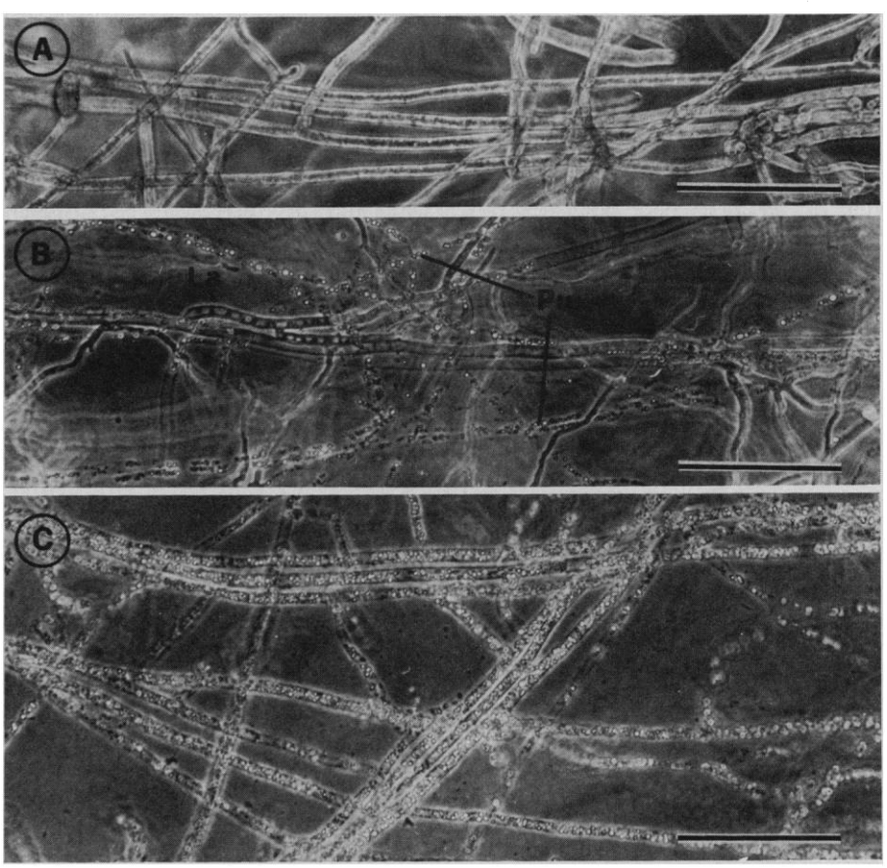

Fig. 1. (A to C) Phase-contrast light micrographs of *P. ultimum* hyphae. All scale bars are 100 μm. (A) The cytoplasm of normal hyphae appears homogeneous, with various organelles clearly visible. (B) Lipid bodies are characteristically prominent in lysed hyphae grown in culture with *L. arvalis*. (C) Hyphae exposed to $1.3 \times 10^{-4}M$ laetisaric acid undergo the same kind of cytoplasmic disorganization found during culture with *L. arvalis*.

W. S. Bowers and P. H. Evans, Department of Entomology, University of Arizona, Tucson, AZ 85721.
H. C. Hoch, Department of Plant Pathology, New York State Agricultural Experiment Station, Geneva, NY 14456.
M. Katayama, Faculty of Agriculture, Nagoya University, Nagoya, Japan.

We believe that this is the first report of an unsaturated fatty acid produced by a fungus inducing lysis in another fungal species. Fatty acids, particularly linoleic acid, occur in fungi in large quantities (*10*), and the lipid content of *P. ultimum* is as great as 48 percent of the dry weight (*11*). The allelopathic agent, laetisaric acid, is the 8-hydroxy derivative of the abundant fatty acid, linoleic acid. Although slight fungicidal activity of some fatty acids is known (*12*), we find by extrapolation from linear regression analysis that laetisaric acid inhibits growth of *P. ultimum* by 50 percent at a concentration of 22 μg/ml in our bioassay (Fig. 2). Since linoleic acid has no effect on growth at this concentration, the 8-hydroxy substitution is clearly necessary for activity. Although a closely related compound, 8-hydroxy palmitic acid, is reported to be an endogenous spore germination inhibitor of the fern *Lygodium japonicum (13)* and 8-hydroxy methyl linoleate was found as a minor product of autoxidation of methyl linoleate (*14*), laetisaric acid has not been described previously to our knowledge. Preliminary investigations indicate fungicidal activity of laetisaric acid against *Rhizoctonia solani, Mucor globosus, Mucor racemosus, Verticillium albo-atrum, Phoma betae, Phytophthora megasperma, Fusarium solani*, and *Fusarium oxysporium*. The phycomycetous fungi exhibit greater sensitivity to laetisaric acid than the ascomycetous and basidiomycetous fungi.

Mounting anxiety over the environmental insults of certain synthetic pesticides, coupled with the rapid appearance of resistance to them by pathogens and insects, has prompted renewed interest in the development and use of biological methods of pest control. Many organisms with a potential for biological control applications are recognized, although few have been successfully commercialized. Ignorance of their specific mode of action and pathological consequences for humans, domestic animals, and wildlife has inhibited commercial interest. Our investigations establish laetisaric acid as the lytic agent responsible for the observed inhibitory action of *L. arvalis* on fungal growth. Since hydroxy acids undergo facile metabolism in animals, toxicity to them by laetisaric acid is unlikely. The observed lytic action in fungi may be due to their failure to metabolize hydroxy acids. Alternatively, the insertion of a hydroxy acid into a polarized bimolecular lipid membrane might be expected to disrupt its structural integrity, initiating the observed hyphal disintegration.

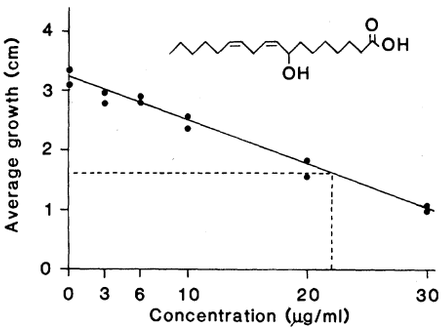

Fig. 2. Linear regression plot of radial growth of *P. ultimum* versus concentration of laetisaric acid (structure shown) in the growth medium. The assay was performed by placing a plug of growing *P. ultimum* mycelia onto the center of a petri plate containing laetisaric acid in PDA. The radial growth of mycelia in all plates was measured when the growth on the untreated plates neared the edge (24 to 30 hours after inoculation). Each data point is the mean of four measurements of a growing *P. ultimum* colony [slope ± SE, −0.071 ± 0.010 cm/(μg/ml)]. The dashed line shows the concentration (22 μg/ml) corresponding to 50 percent growth inhibition.

The discovery of laetisaric acid has several implications for biological control applications. Analytical chemical techniques applied to the selection of high laetisaric acid–yielding strains of the natural soil-inhabiting organism *L. arvalis* may be useful in reducing the rate of fungicide applications to crop plants. Historically, natural products have often provided the basis from which commercial plant protection products, useful in agriculture, have been developed [for example, pyrethrins leading to pyrethroids (*15*), physostigmine to the carbamate insecticides (*16, 17*), and insect juvenile hormone III to insect growth regulants (*18, 19*)]. Similarly, the novel structure of laetisaric acid may be susceptible to synthetic chemical optimization, leading to the development of biorational fungicides.

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3. Sterilized microscope slides were dipped in molten potato dextrose agar (PDA) and placed on V-shaped glass rods in petri plates containing water-saturated filter paper. Portions of the growing margin of *P. ultimum* colonies (maintained on PDA) were transferred to the slides and incubated at 21°C for 40 to 44 hours. The slide cultures were conditioned by flooding for 1 hour with 4 percent methanol in potato dextrose broth and draining before addition of the test solutions. Cytoplasmic responses to the solutions were observed with a phase-contrast microscope at ×160 and ×400. The hyphae were apparently lysed when no cytoplasmic movement could be observed, prominent lipid bodies in all hyphal regions were obvious, and no further growth occurred after 24 hours.
4. *Laetisaria arvalis* was grown at 23°C for 3 to 4 weeks on 20 ml of potato dextrose broth in petri plates. For extraction the mycelial mats were pooled from 200 petri plates, drained of excess culture broth, mixed in a blender for 30 seconds, and allowed to sit for 30 minutes at 23°C. Two volumes of chloroform:methanol (9:1 by volume) were added to the mixture and again blended. The organic phase was separated by centrifugation and filtered through silicone-treated phase separation paper (Whatman) before being concentrated under vacuum. The crude extract was stored under nitrogen at −20°C.
5. Crude extract (3 g) was applied to a chromatographic column containing 90 g of Florisil (deactivated with 7 percent water by weight) and eluted with 300 ml of 50 percent chloroform in methanol followed by 400 ml of methanol. The active component eluted in the methanol fraction. Preparative TLC was performed on 0.5-mm-thick silica plates developed in benzene:ethyl acetate:formic acid (80:20:1 by volume), then further separated twice in benzene:ethyl acetate:formic acid (80:40:1). A portion of each plate was visualized by spraying with 10 percent phosphomolybdic acid in ethanol and heating at 100°C for 5 minutes. Bands were scraped and eluted with ethyl acetate. The eluent was filtered and evaporated under nitrogen to yield laetisaric acid.
6. Analytical TLC was performed on silica plates in chloroform:diethyl ether:formic acid (80:20:0.5 by volume) and visualized by spraying with 10 percent phosphomolybdic acid in ethanol and heating to 100°C for 5 minutes. Capillary gas chromatography was performed on a Hewlett-Packard 5880A with a 12-m dimethyl silicone column, nitrogen carrier, flame ionization detection, and temperature programing from 100° to 250°C (10°C per minute).
7. Proton NMR spectra were measured on a Bruker WH-400 FT instrument with samples in deuterochloroform with tetramethylsilane as internal standard. The presence of a nonconjugated dienol moiety was apparent from the following signals: 4.45 (1H, dt, J = 8.4, 6.3 Hz), 5.31 (1H, dtt, J = 10.7, 7.1, 1.4 Hz), 5.38 (1H, ddt, J = 11.2, 8.4, 1.4 Hz), 5.41 (1H, dtt, J = 10.7, 7.1, 1.5 Hz), 5.47 (1H, dt, J = 11.2, 7.2 Hz), 2.87 (1H, dt, J = 15.5, 7.1 Hz), and 2.80 (1H, dt, J = 15.5, 7.2 Hz). Other signals were: 0.89 (3H, t, J = 6.7 Hz), 1.2 to 1.7 (17H), 2.05 (2H, q, J = 7.1 Hz), 2.30 (2H, t, J = 7.5 Hz), and 3.67 (3H, s). NMR signal peaks are abbreviated as s = singlet, t = triplet, q = quartet, dt = doublet triplet, dtt = doublet triplet triplet, and ddt = doublet doublet triplet.
8. In catalytic hydrogenation, the active methyl ester gave methyl stearate as a result of hydrogenolysis and subsequent hydrogenation of a dienol system.
9. Total synthesis of 8-hydroxylinoleic acid (laetisaric acid) was achieved starting with 1-heptyne and 1,8-octanediol.
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