Synthetic Optimization of Laetisaric Acid for Fungicidal Activity

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Analogues of laetisaric acid (8-hydroxy-9(Z),12(Z)-octadecadienoic acid) were synthesized and examined for growth inhibition of the fungus *Pythium ultimum*. The most active analogue is a 12-carbon α-hydroxy fatty acid.

Bioactive natural products often provide the resources for the development of new pest control products following biologically guided synthetic optimization programs, e.g. the pyrethroids from natural pyrethrins (Janes, 1984). Allelopathic interactions between higher plants and microbes may also provide the elements needed for the genetic engineering of pest resistance into higher plants (Kosuge and Nester, 1984). The recently described allelopathic basidiomycete fungus *Laetisaria arvalis* (Odvody et al., 1980; Burdsall et al., 1980) inhibits growth of the plant pathogenic fungi *Rhizoctonia solani*, *Pythium ultimum*, and *Phoma betae* in both laboratory and field trials. Accordingly, *L. arvalis* is being investigated as a biological control agent for diseases caused by these organisms (Odvody et al., 1980; Lewis and Papavizas, 1980; Hoch and Abawi, 1979; Martin et al., 1982, 1984). We have isolated the active antibiotic produced by *L. arvalis* that incites cytoplasmic lysis and growth inhibition of numerous fungi and characterized it as 8-hydroxyoctadeca-9(Z),12-(Z)-dienoic acid (laetisaric acid) (Bowers et al., 1986).

We established a synthesis program to investigate the elements of chemistry required for biological activity and to seek more active analogues. Biological activity was quantified by growth inhibition of the phytopathogenic fungus *P. ultimum*.

MATERIALS AND METHODS

Synthetic laetisaric acid analogues were tested for growth inhibition of the oomycete fungus *P. ultimum*. An innoculum of growing *P. ultimum* mycelia placed in the center of an 8-cm-diameter potato dextrose agar (PDA) Petri plate gives uniform radial growth and reaches the edge of the plate with 48 h at 23 °C. Radial growth of *P. ultimum* is linearly related to time, and inhibition of growth by laetisaric acid is linear with dose (Bowers et al., 1986). These traits assure accurate and reproducible growth inhibition bioassays when candidate synthetic compounds are incorporated into the PDA growth medium. Synthetic compounds were examined for growth inhibition by incorporation into molten PDA at the desired concentration and poured into Petri plates (Evans et al., 1984). A 4-mm-diameter plug of growing *P. ultimum* mycelia is placed in the center of the cooled PDA Petri plate. The resulting radial growth is measured on the experimental plates when the growth on untreated plates reaches the edge.

The total synthesis of laetisaric acid incorporates methods reported for the synthesis of *Z* unsaturated fatty acids along with a Grignard coupling of the appropriate aldehyde and acetylenic intermediate to produce the desired chain length, position and degree of unsaturation, and location of the hydroxylation. Unless otherwise noted, all reagents are from Aldrich Chemical Co. Capillary gas chromatography was performed on a Hewlett-Packard 5880 with a 12-m methylsilicone column using nitrogen carrier gas and flame ionization detection. NMR spectra (90, 250, 400 MHz) were taken in deuteriochloroform with tetramethylsilane. Infrared spectra were from NaCl plates on a Perkin-Elmer 1420.

1,8-Octanediol (14.6 g, 100 mmol) in 200 mL of chloroform plus 50 mL of ethylene glycol dimethyl ether was stirred at 25 °C with 190 mg (1 mmol) of *p*-toluenesulfonic acid. While the mixture was stirred at 15 °C, 8.41 g (100 mmol) of 3,4-dihydropyran was added dropwise and the reaction maintained at 15–20 °C for 2 h (Greene, 1981). Aqueous saturated sodium carbonate (40 mL) was added and the reaction vacuum evaporated to a volume of approximately 50 mL. The reaction was extracted two times with 100 mL of diethyl ether and the ethereal extract washed with water and brine, dried over anhydrous magnesium sulfate, and vacuum evaporated to give 20.68 g of crude product. The product, containing starting diol and mono- and bis(tetrahydropyranyl) ether, was separated on 300 g of Florisil (60–100 mesh, Floridin Co.) deactivated by addition of 7% water and eluted with 25% ethyl acetate in hexane. The desired 1-(tetrahydropyranyloxy)octanol (THP-octanol) eluted between 700 and 1200 mL to give 23.7 mmol (24% yield).

1-THP-octanol (6.6 g, 23.7 mmol) in 25 mL of dichloromethane was added to a solution of 750 mg (3.48 mmol) of pyridinium chlorochromate in 125 mL of dichloromethane and the resultant mixture stirred at 23 °C for 2 h (Piancatelli et al., 1982). The reaction was extracted with 100 mL of diethyl ether and the reaction residue washed three times with 25-mL portions of diethyl ether. The combined ethereal extracts were filtered through 30 g of 7% water–Florisil and vacuum evaporated to give 6.4 g (23.1 mmol) (98% yield) of a pale yellow liquid, which by silica thin-layer chromatography appeared pure and was less polar than the starting alcohol. Infrared spectroscopy revealed a lack of a broad hydroxyl absorption between 3100 and 3600 cm^{-1} and the appearance of a peak at 1720 cm^{-1}, indicating that the alcohol was oxidized to 1-(tetrahydropyranyloxy)octanal.

The synthesis of 1,4-decadiyne follows the procedure of Ege et al. (1961) and Rachlin et al. (1961). To 15.4 g (0.16 mol) of 1-heptyne in 200 mL of anhydrous tetrahydrofuran (THF) under 1 atm nitrogen was added dropwise 0.17 mol of 3 M ethylmagnesium bromide ethereal solution, and the reaction was stirred at 60 °C for 1 h. At 35 °C, 0.5 g (5 mmol) of copper(I) chloride and 23.8 g (0.2 mol) of propargyl bromide were added and stirred at 35–40 °C for 1 h. The reaction was poured into 100 mL of saturated aqueous ammonium chloride and extracted two times with 100 mL of diethyl ether. The combined organics were washed with water and brine and dried over anhydrous magnesium sulfate. Vacuum evaporation of the ether and THF left 16.9 g of yellow oil. Vacuum distillation at 5 mmHg between 56 and 65 °C gave 6.65 g (50 mmol) (31% yield) of 1,4-decadiyne. Infrared spectra agreed with reported data (Ege et al., 1961) and the NMR spectrum integrated to 14 protons, with the signals for the two

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protons from the C-3 position appearing at 3.15 ppm.

To 2.95 g (22 mmol) of 1,4-decadiyne in 30 mL of anhydrous THF was added dropwise 23 mmol of a 3 M diethyl ether solution of ethylmagnesium bromide under 1 atm of nitrogen. The reaction was refluxed at 55 °C for 30 min and then cooled to 20 °C before the dropwise addition of 6.1 g (22 mmol) of the THP-octanol in 10 mL of anhydrous THF while stirring and maintaining the temperature below 20 °C. After 1 h, 50 mL of saturated aqueous ammonium chloride was added and the reaction mixture extracted two times with diethyl ether. The combined organics were washed with water and brine and dried over anhydrous magnesium sulfate. Vacuum evaporation gave 6.2 g of yellow oil that was purified on a 200-g column of 7% water-deactivated Florisil and eluted with ethyl acetate to give 4.74 g (13.2 mmol) (57% yield) of a colorless oil. Silica thin-layer chromatography developed with 20% ethyl acetate in hexane revealed a pure product different from the starting decadiyne or aldehyde. Infrared spectroscopy gave broad absorption between 3100 and 3600 cm^{-1}, indicating the expected product of the Grignard coupling, a secondary alcohol, 1-(tetrahydropyranyloxy)-9,12-octadecadiyn-8-ol.

1-(Tetrahydropyranyloxy)-9,12-octadecadiyn-8-ol (4.0 g, 11.2 mmol) in 60 mL of methanol, 400 mg of palladium on calcium carbonate (Lindlar catalyst), and 0.8 mg of quinoline were stirred at 23 °C in 1.1 atm hydrogen for 15 h (Fieser and Fieser, 1967). The reaction was filtered and the solvent evaporated to give 4.2 g of a yellow oil. This crude product was loaded on 120 g of 7% water-deactivated Florisil and eluted with ethyl acetate to give 3.8 g (10.5 mmol) (93% yield) of a colorless oil, which by proton NMR gave signals (Bowers et al., 1986) characteristic of the nonconjugated dienol structure 1-(tetrahydropyranyloxy)octadeca-9,12-dien-8-ol.

1-(Tetrahydropyranyloxy)octadeca-9,12-dien-8-ol (3.8 g, 10.5 mmol) in 8 mL of anhydrous pyridine stirring at 0 °C was added to 6 mL of acetic anhydride. After 30 min of stirring at 0 °C and 3.5 h at 23 °C the reaction mixture was poured into 50 mL of ice water and extracted two times with 100 mL of diethyl ether. The ethereal extract was washed sequentially with 1 N HCl, water, and brine and dried over anhydrous sodium sulfate to give 4.2 g of an oil that was purified by elution from 120 g of 7% water-deactivated Florisil with 50% ethyl acetate in hexane to give 3.7 g (9.1 mmol) (87% yield) of a colorless oil. This product gave a single less polar spot on silica thin-layer chromatography developed in 50% ethyl acetate–hexane. Infrared spectroscopy showed loss of the broad hydroxyl absorption between 3100 and 3600 cm^{-1} and the presence of a peak at 1230 cm^{-1}, indicating that the product is 8-acetoxy-1-(tetrahydropyranyloxy)octadeca-9,12-diene.

To 0.82 g (2 mmol) of 8-acetoxy-1-(tetrahydropyranyloxy)octadeca-9,12-diene in 7 mL of ethanol was added a solution of pyridinium *p*-toluenesulfonate (50.4 mg, 0.2 mmol) in 9.5 mL of ethanol. After the mixture was stirred at 50 °C for 3.5 h, the solvent was removed under vacuum and the residue purified by silica preparative thin-layer chromatography developed in ethyl acetate–hexane (3:7) to give 0.6 g (1.83 mmol) (92% yield) of a colorless oil. The behavior of the product on thin-layer and capillary gas chromatography and the appearance of a large infrared absorption between 3100 and 3700 cm^{-1} indicated that the product was 8-acetoxyoctadeca-9,12-dien-1-ol.

To 0.52 g (1.59 mmol) of 8-acetoxyoctadeca-9,12-dien-1-ol in 10 mL of acetone was added 9.6 mL of chromic acid in sulfuric acid (Jones reagent) (Fieser and Fieser, 1967) at 0 °C and the mixture stirred for 20 min and at 23 °C for 10 min. The excess Jones reagent was destroyed with methanol and the mixture concentrated under vacuum, diluted with 50 mL of water, and extracted with ethyl acetate. The ethyl acetate was washed with water and saturated brine, dried over anhydrous sodium sulfate, and evaporated to give a yellow oil that was purified on preparative silica thin-layer chromatography, eluting with chloroform–ethyl acetate–formic acid (80:20:1) to give 0.48 g (1.42 mmol) (89% yield) of 8-acetoxylinoleic acid as a colorless oil. Infrared spectroscopy revealed the lack of a broad hydroxy absorption and the appearance of a strong 1710-cm^{-1} absorption.

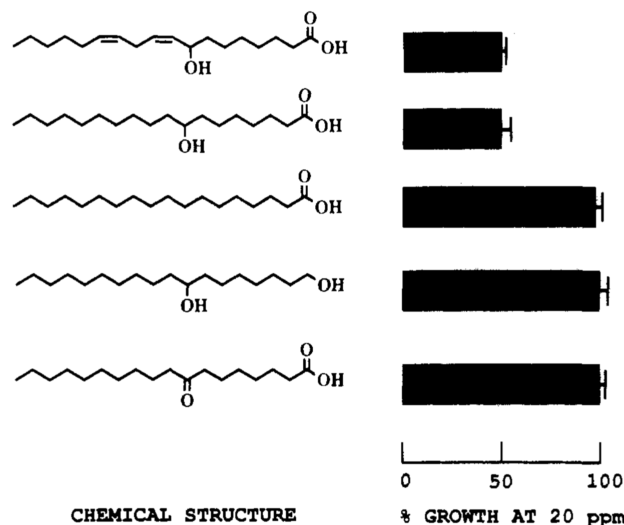

Figure 1. Inhibition of growth of *P. ultimum* on PDA Petri plates containing 20 μg/mL each of the illustrated compounds. 100% is growth of *P. ultimum* on plates without added compound. Each solid bar is the mean of eight measurements with 1 standard deviation indicated.

To 0.45 g (1.32 mmol) of 8-acetoxylinoleic acid in 30 mL of methanol was added a solution of potassium carbonate (0.91 g, 6.62 mmol) in 2.5 mL of water at 23 °C. After being stirred for 18 h, the solution was evaporated under vacuum, diluted with water, acidified with 1 N HCl, and extracted with ethyl acetate. The extract was washed with water and saturated brine, dried over anhydrous sodium sulfate, and evaporated to yield a colorless oil. The product was purified by preparative thin-layer chromatography in benzene–ethyl acetate–formic acid (80:40:1) to give 0.32 g (1.08 mmol) (82% yield) of laetisaric acid as a colorless oil. The capillary gas chromatographic retention times, infrared spectra, and NMR spectra were identical with those of natural laetisaric acid (Bowers et al., 1986). Biological activity as measured by inhibition of growth of *P. ultimum* was similar for the natural and synthetic product.

RESULTS AND DISCUSSION

Several active analogues were discovered that provide insight into the putative mode of action of laetisaric acid. Figure 1 illustrates some of the functional group requirements for fungicidal activity. The occurrence of 9(*Z*) and 12(*Z*) unsaturation in laetisaric acid apparently is not required for activity since the saturated analogue, 8-hydroxystearic acid, has activity equivalent to that of laetisaric acid. Modification of the terminal carboxyl or substitution of the hydroxy group results in the loss of activity. Similarly, transposition of the hydroxy group to positions other than carbon 8 of 18-carbon acids results in loss of activity (Figure 2). The activity of 8-hydroxy acids of various chain lengths indicates that the 18-carbon saturated analogue of laetisaric acid is the most active analogue (Figure 3). Significantly, we find that fungal

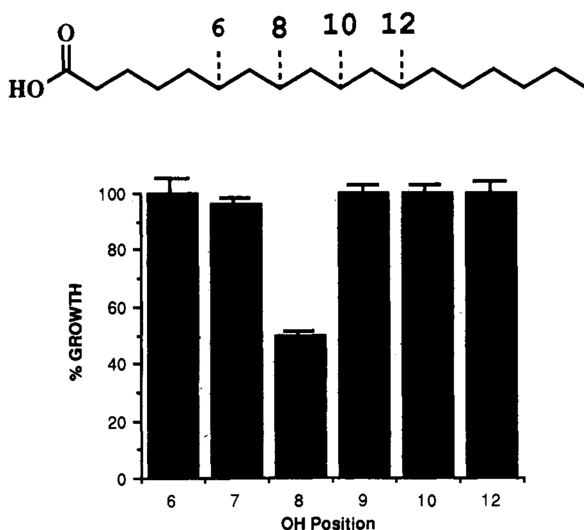

Figure 2. Fungistatic activity of synthetic octadecanoic (C-18) acids hydroxylated at the indicated carbons. An inoculum of *P. ultimum* is placed on a PDA Petri plate containing 20 μg/mL synthetic hydroxylated octadecanoic acid. Percent growth is reported compared to that of *P. ultimum* on plates without added compound. Each solid bar is the mean of eight measurements with 1 standard deviation indicated.

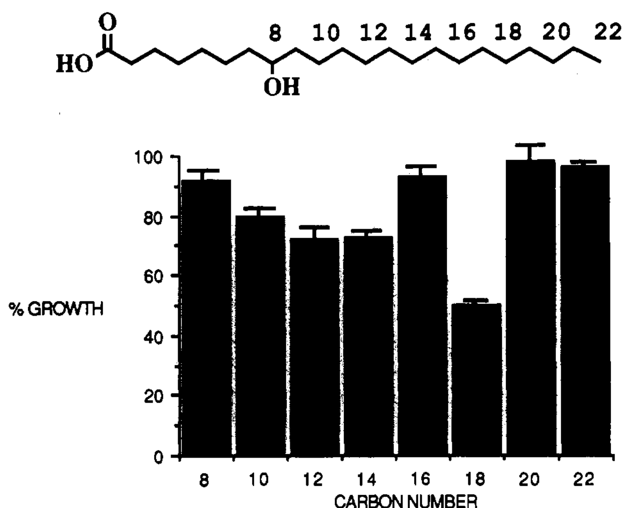

Figure 3. Fungistatic activity of the indicated chain length saturated 8-hydroxy fatty acid. An inoculum of *P. ultimum* is placed on a PDA Petri plate containing 20 μg/mL synthetic hydroxylated fatty acid. Percent growth is reported compared to that of *P. ultimum* on plates without added compound. Each solid bar is the mean of eight measurements with 1 standard deviation indicated.

growth inhibition by synthetic 8-hydroxystearic acid and 2-hydroxydodecanoic acid is linear and similar on a mole basis (8-hydroxystearic acid, ID_{50} = 70 mM; 2-hydroxydodecanoic acid, ID_{50} = 60.5 mM) (Figure 4). These observations suggest that *P. ultimum* is unable to metabolize laetisaric acid (via lipid β-oxidation) beyond a 12-carbon α-hydroxy acid. Thus, the putative allelopathic agent may be 2-hydroxydodecadienoic acid. If true, this biotransformation–activation sequence of hydroxylation of a ubiquitous natural fatty acid (linoleic acid) appears uniquely targeted to disrupt discrete aspects of fungal metabolism since animals and higher plants freely metabolize α-hydroxy acids (Fulco and Mead, 1961; Newcome and Stumpf, 1952).

When hyphae of *P. ultimum* come into contact with *L. arvalis*, laetisaric acid, or active analogues, cytoplasmic streaming is rapidly disrupted, followed by cellular disorganization and the appearance of lipid bodies (Bowers

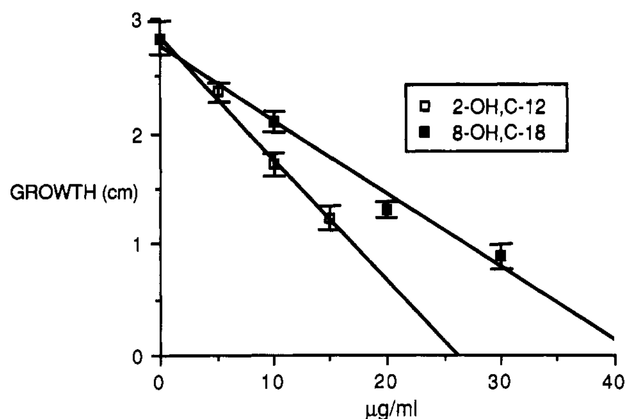

Figure 4. Radial growth of *P. ultimum* vs. concentration of 8-hydroxystearic acid and 2-hydroxydodecanoic acid on PDA Petri plates. Each point is the mean of eight measurements with ±1 standard deviation indicated. Each curve has a correlation coefficient (r) of −0.99.

et al., 1986). This lytic activity of laetisaric acid and analogues, which can be metabolized to a 2-hydroxy, 12-carbon chain acid, may signal a mode of action involving the disruption of specific lipid membrane structures in the fungal cells.

The relative structural simplicity of 2-hydroxydodecanoic acid suggests numerous obvious chemical modifications for possible optimization of biological activity including substitution of the 2-hydroxy terminus with other polar, poorly metabolized groups, while retaining in the molecule the necessary 12-carbon hydrophobic hydrocarbon chain. Synthesis and activity of several of these detergent-like compounds will be reported.

Fatty acids and their salts are generally regarded as having very low mammalian toxicity and are commonly used as general disinfectants. Some fatty acids are effective fungicides (Wyss et al., 1945) and are used in agriculture for control of apple powdery mildew and apple scab (Clifford and Hislop, 1975; Burchill and Swait, 1977). Fatty acids are used as adjuvants for pesticides and herbicides and for the control of *Olpidium* and *Pythium* diseases in hydroponic plant culture (Tomlinson and Thomas, 1986; Stanghellini and Tomlinson, 1987). The likely origin of laetisaric acid (8-hydroxylinoleic acid) from the ubiquitous linoleic acid seems obvious. Knowledge of the biosynthesis of laetisaric acid by *L. arvalis* may provide a basis for the genetic engineering of laetisaric acid into crop plants or associated commensal microorganisms, conferring endogenous resistance to plant diseases. Structure–biological activity relationships derived from this and similar studies will provide leads for the synthesis of analogues optimized for fungicidal activity. An understanding of the mode of action of laetisaric acid may, by inference from activity of the synthetic analogues, supply direction for the genetic introduction of this or similar allelochemics into crop plants. The elucidation of the molecular mode of action of fatty acids as fungicides may also lead to the design of safe and selective biorational plant disease control agents.

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