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A NOVEL ANTIFUNGAL FURANONE FROM *Pseudomonas aureofaciens*, A BIOCONTROL AGENT OF FUNGAL PLANT PATHOGENS

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Abstract—*Pseudomonas aureofaciens* (= *P. chlororaphis*) strain 63-28 is a biocontrol agent active against many soil-borne fungal plant pathogens and shows antifungal activity in culture assays. 3-(1-Hexenyl)-5-methyl-2-(5H)furanone was isolated from culture filtrates of this bacterium. The purified furanone showed antifungal activity against *Pythium ultimum*, *Fusarium solani*, *Fusarium oxysporum*, and *Thielaviopsis basicola*. The ED_{50}S for spore germination of these fungi were 45, 54, 56, and 25 μg/ml, respectively. The compound also inhibited the germ tube growth of *Rhizoctonia solani* growing from microsclerotia, with an ED_{50} of 61 μg/ml. The compound is the reduced form of furanones previously described from this bacterium: 3-(1-hexenyl)-5-hydroxy-5-methyl-2-(5H)-furanone and 3-(1-hexenyl)-5-hydroxymethyl-2-(5H)-furanone. This volatile antifungal furanone has structural similarity to other antifungal furanones produced by actinomycetes (*Streptomyces* spp.), fungi (*Trichoderma harzianum*), and higher plants (*Pulsatilla* and *Ranuculus* spp.). This is the first report of 3-(1-hexenyl)-5-methyl-2-(5H)-furanone produced by a bacterium.

Key Words—2(5H)-Furanone, butenolide, lactone, butyrolactone, antibiotic, biological control, *Pseudomonas chlororaphis*, *Pythium ultimum*, *Rhizoctonia solani*, *Fusarium solani*, *Fusarium oxysporum*, *Thielaviopsis basicola*.

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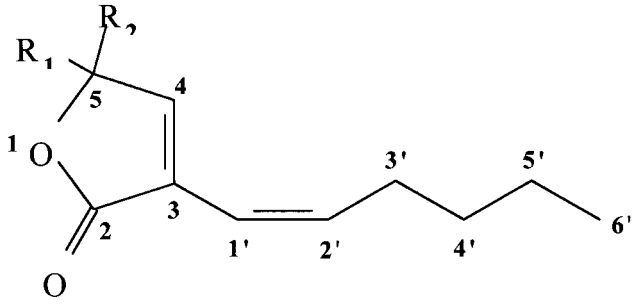
INTRODUCTION

Fluorescent members of the bacterial genus *Pseudomonas* have been widely investigated as biological control agents against soil-borne plant pathogenic fungi such as *P. ultimum*, the causal agent of pre- and postemergence seedling rots, especially in greenhouses and nurseries. The production of anti-fungal antibiotics, such as 2,4-diacetylphloroglucinol (Maurhofer et al., 1992), phenazine-1-carboxylic acid (Thomashow and Weller, 1988), pyoluteorin (Howell and Stipanovic, 1980), and pyrrolnitrin (Pfender et al., 1993) contributes, in part, to the biological control activity exhibited by some *Pseudomonas* species (Thomashow and Weller, 1996). *Pseudomonas aureofaciens* (=*P. chlororaphis*) strain 63-28 has been tested as a potential biocontrol agent to protect greenhouse crops, including tomatoes (Gagné et al., 1993), cucumbers (McCullagh et al., 1996), poinsettia (Seresinhe et al., 1997), and chrysanthemum (Hill and Peng, 1999). Earlier studies on the ultrastructural effects of *P. aureofaciens* 63-28 on *Pythium ultimum* in pea roots revealed distortion of the hyphae, strong plasmalemma retraction, cytoplasmic disorganization, and organelle breakdown, that are characteristic of hyphal responses to antifungal metabolites (Benhamou et al., 1996). We have since detected four compounds in culture supernatants of this bacterium, especially in a medium amended with 2% glucose (Gamard et al., 1996). Two of the antifungal compounds have been recently identified as the butyrolactones (*Z*)-4-hydroxy-4-methyl-2-(1-hexenyl)-2-butenolide (**2**) and (*Z*)-4-hydroxymethyl-2-(1-hexenyl)-2-butenolide (**3**) (Gamard et al., 1997) (Figure 1). Both compounds inhibited the in vitro growth of the plant pathogens *Pythium ultimum* Trow and *Phytophthora cryptogea* Pethybr. & Lafferty.

A third, chromatographically distinct antifungal compound, provisionally termed FI, is also produced by *P. aureofaciens* strain 63-28 and is the focus of this communication. To understand further the ability of *P. aureofaciens* strain 63-28 to reduce disease incidence caused by plant pathogens such as *P. ultimum*, we describe herein the structural characterization of FI, its antifungal activity against five plant pathogenic fungi, and speculate on its role in the biocontrol properties of *P. aureofaciens* strain 63-28. In addition, we compare the structure to other antifungal 2(5H)-furanones.

METHODS AND MATERIALS

Biological Material. Pseudomonas aureofaciens (=*P. chlororaphis*) strain 63-28 was obtained from Agrium Inc, Saskatoon, Saskatoon, Canada. The strain was isolated from the roots of a canola plant near Winnipeg, Manitoba, Canada. Cultures were maintained at −80°C in nutrient broth + 10% glycerol. Fungal cultures were from the collection of Dr. R. Linderman, USDA-ARS Corvallis,



1 $R_1 = H$ $R_2 = CH_3$

2 $R_1 = OH$ $R_2 = CH_3$

3 $R_1 = H$ $R_2 = CH_2OH$

FIG. 1. Structure of three antifungal furanones (**1**, **2**, and **3**) produced by *Pseudomonas aureofaciens*.

Oregon, and were stored on 2% agar slants. Cultures tested included *Pythium ultimum* strain N1 (from the collection of the first author), *Fusarium oxysporum* Schlechtend.:Fr., *Fusarium solani* (Mart.) Sacc., *Thielaviopsis basicola* (Berk. & Broome) Ferraris, and *Rhizoctonia solani* Kühn. Sporangia of *P. ultimum* were produced in 0.5% agar plates and microsclerotia of *R. solani* were produced on frozen cut bean pods and stored at −20°C (van Bruggen and Arneson, 1986). Microconidia of the other fungi were produced on potato dextrose agar plates.

Bioassays. The crude extract of a 100-ml culture was dissolved in 1 ml of methanol and fractionated on aluminum TLC plates (20 × 20 cm, 0.2 mm thick, 60 F_{254}, Merck, Darmstadt, Germany) developed in chloroform–acetone (9 : 1) and the fraction containing compound **1** (R_F = 0.60–0.67) was scraped from the plates and resuspended in 5 ml methanol. For a control treatment, silica was scraped from plates developed without the antibiotic. The methanol was evaporated at room temperature under vacuum (Speed Vac), and the sample was resuspended in 5 ml sterile distilled water. The absorbance of the samples at 255 nm was measured to calculate the concentration from the extinction coefficient. In the first set of bioassays, compound **1** was diluted with sterile distilled water to give concentrations of 100%, 50%, 25%, 12.5%, and 0% for testing in the bioassay. In the second and third set of experiments, 50%, 33%, 25%, and 0% were tested. Seventy-five microliters of the sample was mixed with 100 μl of nutrient broth + 2% glucose containing sporangia of *P. ultimum*, microscle-

rotia of *R. solani*, or microconidia of *F. oxysporum*, *F. solani*, or *T. basicola*. Three replicate samples of each fungus–antibiotic mixture were placed into the wells of a 96-well polystyrene ELISA plate. At various times from 4 to 24 hr, the germination of the spores and germ tube growth were observed through an inverted microscope. Percent germination was calculated by counting the number of spores where the length of the germ tube exceeded the diameter of the spore, divided by the total number of spores counted. Germ tube growth was measured with an ocular micrometer as the total length of the germ tube from the spore wall to the hyphal tip. Germination or hyphal growth was expressed as a percentage of the control without the antifungal compound and transformed to probits. The probit values were regressed against the log of the concentration of compound **1** using a linear regression. The ED_{50} and ED_{95} values were calculated from the regression.

Production, Extraction, and Purification of Antifungal Compound. The bacterial strain *Pseudomonas aureofaciens* 63-28 was grown in four 500-ml Erlenmeyer flasks, each containing 250 ml of nutrient broth + 2% glucose. Flasks were seeded with a loop of bacterial cells from a 24-hr-old bacterial culture growth on Kings medium B (King et al., 1954). Flasks were incubated at 25°C on an orbital shaker at 150 rpm. After 48 hr, bacterial cells were removed from the culture by centrifugation at 2000*g*. The supernatant was extracted three times with 1/10 volume ethyl acetate in a separatory funnel. The ethyl acetate fraction was evaporated to dryness, in vacuo, at 40°C with a rotary evaporator and resuspended in 10 ml of chloroform–methanol (20 : 1). The most nonpolar antifungal fraction with the longest retention time on HPLC (termed FI in Gamard et al., 1997) was purified using silica gel flash chromatography. A 1-cm-diameter × 80-cm flash chromatography column was filled to 15 cm with a slurry of silica gel (40-μm particle size) mixed with chloroform. A 0.5-cm layer of washed quartz sand was sprinkled on the top of the silica layer. The 10-ml sample from the rotary evaporator was dried onto silica gel, which was loaded on the top of the silica layer in the column. The column was eluted with 100% chloroform. A flow controller was fitted to the top of the column, and the column was pressurized with nitrogen to produce a flow of 2 ml/min. Fractions of 2-ml were collected and tested with thin-layer chromatography developed in chloroform–methanol (20 : 1) to determine which fractions contained the UV-absorbing compound. Compound **1** was present in fractions 14–38. The fractions were combined and evaporated to dryness with nitrogen. N_2 was passed over the sample overnight to remove any residual chloroform, prior to analysis.

NMR spectra were measured on Bruker DRX-600, AM-400 and AC-300 spectrophotometers. Mass spectra were obtained on a Kratos MS 50 TC spectrometer, and IR spectra were recorded on a Nicolet 5DXB FT-IR. The molar extinction coefficient (ϵ) of compound **1** at 255 nm was determined to be 3226 A/M × cm (J. Rahematpura, University of Dusseldorf, Dusseldorf, Germany).

RESULTS

Biological Activity. Compound **1** showed activity against all tested fungi which included an oomycete (*Pythium ultimum*), three deuteromycetes (*Fusarium oxysporum*, *F. solani*, and *Thielaviopsis basicola*) and one basidiomycete (*Rhizoctonia solani*). This compound inhibited the germination of propagules, with ED_{50} values ranging from 25 to 61 μg/ml (Table 1). *T. basicola* was the most sensitive and *R. solani* was the least sensitive. At the higher concentrations, germ tube growth was also reduced. The morphology of the germ tubes of *Pythium ultimum* was visibly altered. In the presence of **1**, germ tubes had a convoluted and swollen appearance and were shorter than the straight, slender germ tubes observed in the absence of **1**.

Structure Determination. The structure of compound **1** is shown in Figure 1. Resonances detected in ^{13}C NMR and DEPT spectra indicated the presence of one ester carbonyl (δ 173.1), four sp^2 carbons (δ 148.6, 139.8, 128.6, and 116.6), an oxygen-substituted methine (δ 77.4), three sp^3 methylenes (δ 31.2, 29.4, and 22.4), and two methyls (δ 19.2 and 13.8). A molecular formula of $C_{11}H_{16}O_2$, for **1**, was established by high-resolution mass spectrometry and was fully consistent with the number of carbon centers detected in the ^{13}C NMR spectrum. Three of the four degrees of unsaturation present were accounted for by the ester carbonyl and the two double bonds formed by the sp^2 carbons. The remaining degree of unsaturation required a ring.

All one-bond and two- and three-bond heteronuclear couplings were established with the modified HMBC NMR experiment (Seto et al., 1996) Long-range ^1H–^{13}C couplings were observed between the proton (δ 7.16, broad singlet) attached to C-4 (δ 148.6) and the C-2 ester carbonyl (δ 173.1), the C-3 and C-1′ olefinic carbons (δ 128.6 and 116.6, respectively), the C-5 oxymethine (δ 77.4), and the C-6 methyl (δ 19.2), whose attached proton resonance was a doublet with a coupling constant of 7 Hz. Additional long-range couplings were

TABLE 1. ANTIFUNGAL ACTIVITY OF COMPOUND **1** AGAINST FIVE PLANT PATHOGENIC FUNGI[a]

Fungus	ED_{50}	ED_{95}
Pythium ultimum	45	108
Fusarium oxysporum	56	169
Fusarium solani	54	153
Thielaviopsis basicola	25	79
Rhizoctonia solani	61	423

[a] ED_{50} and ED_{95} values are derived from a regression of germination as a percent of control (probit) vs concentration of **1** ($\log_{10}$). All regressions were statistically significant, $P < 0.05$.

observed between the H-5 oxymethine proton (δ 5.09, bq) and the C-3 and C-4 olefinic carbons (δ 128.6 and 148.6, respectively) and the C-6 methyl carbon (δ 19.2). These two sets of long-range correlations, the number of oxygen atoms in the molecular formula, and a required ring structure collectively establish the presence of the 3,5-substituted furanone ring within this compound. The couplings observed between the H_3-6 doublet (coupling constant 7 Hz) and both C-4 (δ 148.6) and C-5 (δ 77.4) established that the C-6 methyl is attached to C-5 of the furanone ring. HMBC correlations between the H-1′ olefinic proton (δ 6.07, dm, J = 11.5 Hz) and the C-2 ester carbonyl (δ 173.1), the C-4 carbon (δ 148.6), the olefinic C-2′ carbon (δ 139.8), and the C-3′ methylene signal (δ 29.4), in addition to a long-range coupling between H-2′ and C-3 are consistent with attachment of the side chain at the three-position of the ring.

Heteronuclear couplings observed between H-2′ (δ 5.94, dt, J = 11.5, 7.0 Hz) and C-3′, C-1′, and the C-4′ methylene carbon (δ 31.2) as well as couplings between the H-6′ methyl protons (δ 0.92, t, J = 7.0 Hz) and both C-4′ and C-5′ (δ 22.4) are evidence for the 1-hexenyl side chain. The base-peak in the EI mass spectrum at m/z = 137 formed by allylic fragmentation between C-3′ and C-4′ further supports the assigned structure of the side chain. The *cis* configuration of the alkene was assigned based on the observed 11.5 Hz couplings between H-1′ and H-2′; this was further substantiated by the absence of an 900–700 cm^{-1} IR absorption that is characteristic of conjugated *trans* double bonds (Christie, 1982).

3-(1-Hexenyl)-5-methyl-2-(5H)-furanone. $[\alpha]_D^{22}$ = 9.6° (*c* 0.50, $CHCl_3$; IR (film) 2957, 2930, 2871, 1754, 1318, 1199, 1119, 1090, 1071, 1027 cm^{-1}; ^3H NMR ($CDCl_3$, 300 MHz) δ 7.16 (bs, 1H, H-4), 6.07 (dm, 1H, J = 11.5, <1 Hz, H-1′), 5.94 (dt, 1H, J = 11.5, 7.0 Hz, H-2′), 5.09 (bq, 1H, J = 7.0 Hz, H-5), 2.22 (qd, 2H, J = 7.0, 1.5 Hz, H_2-3′), 1.50–1.25 (m, 4H, H_2-4′, H_2-5′) 1.47 (d, 3H, J = 7.0 Hz, H-6), 0.92 (t, 3H, J = 7.0 Hz, H-6′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.1 (s, C-2), 148.6 (d, C-4), 139.8 (d, C-2′), 128.6 (s, C-3), 116.6 (d, C-1′), 77.4 (d, C-5), 31.2 (t, C-4′), 29.4 (t, C-3′), 22.4 (t, C-5′), 19.2 (q, C-6), 13.8 (q, C-6′); EI-MS m/z (rel intensity) 180 [M^+, 62], 165 [M^+-CH_3, 6], 151 [M^+-CH_2CH_3, 22], 137 [M^+-$CH_2CH_2CH_3$, 100], 123 [M^+-$CH_2CH_2CH_2CH_3$, 22], 93 [98]; HR-EI-MS m/s 180.1150 ($C_{11}H_{16}O_2$, Δ 0.1 mmu).

DISCUSSION

Compound **1** is the most abundant form of the three furanones thus far isolated from *P. aureofaciens* strain 63-28 cultures. Unlike other antibiotics that are produced in the stationary phase, these furanones (**1**, **2**, and **3**) are detectable in liquid culture during the mid–late exponential phase 8–12 hr after bacterial inoculation. However, the levels of **2** and **3** decline after 20 hr, while compound **1**

continues to increase from 20 to 60 hr. *P. ultimum* is able to germinate quickly in response to plant exudates, colonize the seed coat within 10 hr, and infect the embryo within 24–48 hr (Lifshitz et al., 1986), thus **1**, **2**, and **3** may be produced early enough to inhibit this pathogen. Furthermore, the production of several structurally related metabolites active against a variety of fungal pathogens may increase the ability of *P. aureofaciens* 63-28 to reduce agricultural diseases under a variety of growth conditions. In addition, these low-molecular-weight furanones are volatile. This may also give the bacterium an advantage, since inhibitory concentrations could build up around the microcolonies of the bacterium, enabling it to antagonize fungi over a greater distance than would be afforded by a nonvolatile antibiotic. Most of the antibiotics characterized from *Pseudomonas* spp. are not volatile, except for HCN.

The furanones produced by *P. aureofaciens* resemble the butyrolactone autoinducers that regulate aerial mycelia formation (Kelemen and Buttner, 1998) and antibiotic production (Horinouchi and Beppu, 1994; Yamada et al., 1987) within the genus *Streptomyces*. The gram-negative plant pathogen *Xanthomonas campestris* pv *campestris* also produces a related lactone that functions as a regulator of extracellular polysaccharide and pigment biosynthesis (Chun et al., 1997). However, within other gram-negative bacteria such as the *Pseudomonas*, *Agrobacterium*, *Rhizobium*, and *Erwinia* species, *N*-acyl homoserine lactone derivatives function as autoinducers or quorum sensing signals (Pierson et al., 1998). Generally these compounds are involved in the regulation of virulence factors, antibiotics, rhamnolipids, bioluminescence, and other cellular functions. The potential functions of **1**, **2**, and **3** in quorum-sensing or as pheromones in strain 63-28 have not been investigated, so it is not certain whether the furanones produced by *P. aureofaciens* function in a regulatory pathway or simply act as fungal inhibitors.

We have observed that compound **1** alters the hyphal morphology of *Pythium ultimum*, resulting in a convoluted, abnormal appearance. Similar effects on fungal morphology are caused by protoanemonin (**4**) (Figure 2), a fungal toxin produced by plants such as *Pulsatilla* and *Ranunculus* spp. (Martin et

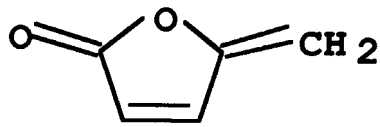


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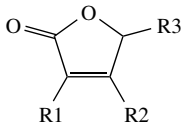
FIG. 2. Structure of protoanemonin (**4**).

TABLE 2. DERIVATIVES OF 2(5H)-FURANONE KNOWN TO EXHIBIT ANTIFUNGAL OR ANTIBACTERIAL ACTIVITY



R1	R2	R3	Chemical name	Reference
H	H	H	crotonic acid	Hisao (1966)
H	H	CH_3	β-angelica lactone	Dal Pozzo et al. (1972) Sakurai et al. (1968)
CH_2O-Glucosyl	H	H	β-miroside	Lorimer et al. (1995)
$(CH_2)_5CH_3$	H	CH_3		Rezanka et al. (1994)
$CH(OH)CH(CH_3)$-CH_2CH_3	CH_3	OH		Braun et al. (1995)
$CHCH_2$	H	OH		Els et al. (1958) Lorezen et al. (1995)
$CH_2CH(OH)CH_3$	$CH_2(CH)_4CH_3$	H	harzianolide	Ordentlich et al. (1992) Almassi et al. (1991)

al., 1990; Mares, 1987; Misra and Dixit, 1980). Protoanemonin is also known to disrupt organelle formation in both *Microsporum cookei* and *Trichophyton mentagrophytes* (Mares, 1989; Mares and Fasulo, 1990). The saturated 2(3H)-furanone version of this compound, dihydro-5-methylene-2(3H)-furanone, has been tested as a fungicide against *Fusarium graminearum* on wheat and showed an ED_{50} of 183 μg/ml against conidia (Wu et al., 1995). Many other examples of 2(5H)-furanone derivatives are known to have antifungal as well as antibacterial activity (Table 2). Generally, these compounds are 3,5-diakyl derivatives of the 2(5H)-furanone structure. Sakurai et al. (1968) demonstrated that maximum activity was observed when chain lengths at the 5 position were four to six carbons long and contained a double bond. It has also been postulated that the conjugated α,β-unsaturated carbonyl may alter protein structure or activity by reacting with cysteine residues. The inactivation of protoanemonin by the addition of cysteine indirectly supports this proposal (Mares, 1987). Regardless of the mode of action, it is evident that 2(5H)-furanone-based compounds are effective antifungal metabolites. For *P. aureofaciens*, these furanones represent a new class of antifungal metabolites that may play a role in the ability of this bacterium to control plant pathogenic fungi.

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