Novel Butyrolactones with Antifungal Activity Produced by *Pseudomonas aureofaciens* Strain 63-28

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The bacterium *Pseudomonas aureofaciens* 63-28 is antagonistic to several plant pathogenic fungi, including *Pythium* spp. The bacterium produced at least four antifungal metabolites active against *Pythium ultimum* and *Phytophthora cryptogea* when tested in culture for antifungal activity. Two of these compounds were identified as the novel butyrolactones (*Z*)-4-hydroxy-4-methyl-2-(1-hexenyl)-2-butenolide and (*Z*)-4-hydroxymethyl-2-(1-hexenyl)-2-butenolide, by using NMR and GC-MS. All compounds were different from other antibiotics produced by *Pseudomonas* spp., including pyoluteorin, pyrrolnitrin, and 2,4-diacetylphloroglucinol, as determined by HPLC. This is the first report of butyrolactones with antifungal activity produced by a saprophytic *Pseudomonas* spp.

Antibiotic production by soil bacteria of the genus *Pseudomonas* has been established as one mechanism involved in the biological control of diverse soilborne fungal phytopathogens[1,2]. A single strain of *Pseudomonas* can produce several different antibiotics, and a similar spectrum of antibiotic production has been described in different strains. For instance, *Pseudomonas fluorescens* strains CHA0 and Pf-5 both synthesize 2,4-diacetylphloroglucinol, pyoluteorin, and hydrogen cyanide[3,4]. KEEL *et al.*[5] showed that biosynthesis of 2,4-diacetylphloroglucinol was a widely distributed phenotype among fluorescent *Pseudomonas* spp. worldwide. Other antibiotics produced by *Pseudomonas* spp. include pyrrolnitrin[6,7], phenazines[8,9] and oomycin A[10].

Pseudomonas aureofaciens strain 63-28 has been investigated as a potential biological control agent to protect and improve the yield of diverse greenhouse crops, including tomatoes[11,12]. This strain was classified originally as *Pseudomonas fluorescens*, but was recently re-identified as *Pseudomonas aureofaciens*, based on MIS fatty acid profiles performed by J. W. KLOEPPER at Auburn University, AL, and J. J. GERMIDA at University of Saskatchawan, Canada. This strain was selected from a collection of over 4000 isolates, based on its plant growth-promoting activity on canola in greenhouse and field trials[13]. When grown in culture, *P. aureofaciens* 63-28 inhibits the growth of *Pythium ultimum* Trow, *Rhizoctonia solani* Kühn, *Phytophthora cryptogea* Pethybr. & Lafferty, and *Cladosporium herbarum* (Pers.: Fr.) Link , and produces a minimum of four different antifungal compounds[14,15]. None of the metabolites produced by 63-28 exhibit chromatographic properties of known antifungal compounds commonly produced by *Pseudomonas* spp., including 2,4-diacetylphloroglucinol, pyoluteorin, and pyrrolnitrin[16]. We report here the identification of the structures of two compounds produced by *P. aureofaciens* 63-28, by use of NMR techniques and mass spectrometry.

Materials and Methods

Bacterium and Growth Conditions

P. aureofaciens strain 63-28 was characterized by KLOEPPER *et al.*[13]. The strain was provided by Agrium, Inc. (Saskatoon, Canada) and Premier Tech Ltd. (Rivière-du-Loup, Canada). The bacterium was maintained at −20°C in tryptic soy broth (BBL, Becton Dickinson) supplemented with 25% glycerol. Agar-diffusible antifungal activity was detected on Bacto nutrient agar (Difco Laboratories) supplemented with 2% D-glucose (NA-Glc). For extraction and purification of antibiotics, cultures were grown in Bacto nutrient broth supplemented with 2% D-glucose (NB-Glc), pH = 6.0. All bacterial cultures were grown at 25°C.

Detection of Agar-diffusible Antifungal Activity

The antifungal activity of *P. aureofaciens* strain 63-28 was tested against the fungi *P. ultimum*, *R. solani*, and *P. cryptogea* #26756. These fungi were from the collection of Dr. T. PAULITZ and were stored at 10°C on water agar (1.5% granulated agar, BBL, Becton Dickinson) and routinely grown on potato dextrose agar (Bacto PDA, Difco) at 25°C. Plugs of mycelium (5-mm diameter) were cut from the edges of expanding colonies on PDA and placed at the center of 100 × 15 mm petri dishes containing NA-Glc medium. *P. aureofaciens* strain 63-28 was transferred to three different spots approximately 2 cm from the mycelium plug. The plates were incubated at 25°C for 2, 4, and 5 days, for *P. ultimum*, *R. solani* and *P. cryptogea*, respectively. Tests were also performed by inoculating the bacteria 48 hours before the fungi. Controls consisted of plates inoculated with the fungi only. The relative amount of agar-diffusible antifungal compound(s) was estimated by measuring the size of the inhibition zone between the edges of the bacterial colony and the fungal mycelium. Each treatment had three replicate plates and the experiments were conducted three times. The results were analysed by analysis of variance, with a Fischers protected LSD test to determine mean separations.

Extraction and Purification of Antibiotics

P. aureofaciens strain 63-28 was grown in 6 liters of NB-Glc medium (in six 2-liter Erlenmeyer flasks) for 5 days at 25°C on a rotary shaker at 150 rpm. Cultures were inoculated with a loop of bacteria grown on a plate of NA-Glc medium. The whole cell culture was lyophilized and rehydrated in sterile distilled water (concentrated 10-fold). The concentrate was extracted twice with 600 ml of chloroform, and after separation from the aqueous phase, the combined chloroform phases were evaporated to dryness *in vacuo* at 40°C. The dry residue was redissolved in acetone and fractionated by thin layer chromatography (TLC), using TLC glass plates (0.5 mm silica-gel $60F_{254}$. Merk, Darmstadt, Germany). After development in chloroform-acetone (9 : 1), the plates were divided into six sections from top to bottom (fractions 0 to V), which were scraped off the plates. These sections corresponded to Rf values of 1.00~0.81, 0.81~0.63, 0.63~0.44, 0.44~0.29, 0.29~0.15, and 0.15~0.00, respectively. Compounds were eluted from the silica with acetone and the eluates were recovered by filtration on Whatman paper no. 1. After evaporation *in vacuo* at 40°C, the residues were dissolved in 1.5 ml of acetone and tested for antifungal activity using a disk bioassay. Sterile filter paper disks (3 mm diameter) were soaked with 20 or 50 μl of each fraction. Control disks were soaked with acetone. Disks were placed on PDA plates, at 2 cm around a plug of fresh growing mycelium. Plates were incubated at 25°C and inhibition zones around disks were recorded after 2, 4 and 5 days, for *P. ultimum*, *R. solani* and *P. cryptogea*, respectively. Tests were done twice.

Antifungal activity was also directly observed on TLC plates using a modification of the *Cladosporium* bioassay of DAHIYA *et al.*[17] The isolate of *C. herbarum* was from the collection of Dr. T. PAULITZ, and was stored on PDA at 10°C. Crude extracts of 63-28 cultures were separated on TLC aluminum sheets (0.2 mm silica gel 60-F_{254} Merck, Darmstadt, Germany) developed in chloroform-acetone (9 : 1). *C. herbarum* cultures were grown on PDA for 5 days at 25°C. Spores were collected by adding 10 ml of warm, distilled water (45°C) to the petri dish and scraping the surface of the mycelium with a glass rod. The spore suspension was mixed with 10 ml of molten PDA (20 g/liter; 45°C) and spread with a glass rod on the dried TLC sheets as a thin agar layer. TLC sheets were subsequently incubated in a moist chamber at room temperature for 2 days. Spots with antifungal activity appeared as white zones on a green background formed by the mycelium and spores of *C. herbarum*. The fractions exhibiting the highest antifungal activities in both the disk bioassay and the *Cladosporium* bioassay were selected for further isolation of active compounds using successive preparative TLC and preparative high-performance liquid chromatography (HPLC).

High Performance Liquid Chromatography (HPLC)

The HPLC analyses were performed on a multisolvent delivery system (Waters 600E) controlled by a Waters 600 controller, equipped with a photodiode array detector, and a Waters 717 autosampler. The system was equipped with a Waters C_{18} reverse phase column model RCM 8 × 100 mm for analytical chromatography, or with a Waters C_{18} reverse phase column model RCM 25 × 200 mm for preparative chromatography. Samples were run at 0.8 ml/minute for analytical purposes and at 6 ml/minute for preparative purposes. The mobile phase solvents were of HPLC grade, filtered on a Millipore filtration system holding a 0.45 μm pore size membrane, and degassed under vacuum. The following ternary linear gradient was used for comparative analysis and for the purification of compounds FIV and FI-: 0 to 12 minutes-45% water, 30% acetonitrile, 25% methanol; 20 to 23 minutes-0% water, 90% acetonitrile, 10%

methanol; 26 to 35 minutes - 45% water, 30% acetonitrile, 25% methanol. For purification of compound FIII, the following gradient was used: 0 minute - 35% water, 40% acetonitrile, 25% methanol; 8 minutes - 10% water, 80% acetonitrile, 10% methanol; 10 minutes - 0 % water, 90% acetonitrile, 10% methanol; 12 to 22 minutes - 35% water, 40% acetonitrile, 25% methanol. Chromatograms were monitored by UV absorbance (range 220 ~ 400 nm), and spectra were analyzed with the Millenium 2.1 software. Active unknown compounds were compared to the reference antibiotics 2,4 diacetylphloroglucinol, pyrrolnitrin and pyoluteorin, provided by B. NOWAK-THOMPSON, USDA-ARS, Corvallis, OR. They were analyzed individually and by co-injection with a mixture of the reference antibiotics. It was possible to determine the structural identification of two of the compounds after their final purification by preparative HPLC.

Antibiotic Structure Determination

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity-500 MHz spectrometer at room temperature in deuterated chloroform ($CDCl_3$). The chemical shifts are presented in δ (ppm) values relative to TMS using residual protonated solvent ($CHCl_3$) as an internal reference. Various 2D experiments were recorded to assign the structure; these are COSY (COrrelated SpectroscopY) (absolute value), and three phase sensitive experiments: HMQC (Heteronuclear Multiple Quantum Experiment), HMBC (Heteronuclear Multiple Bond Correlation), and NOESY (NOE2D spectroscopy). The phases were detected using the hypercomplex mode.

Mass spectra were obtained on a Kratos MS25RFA, using an ionization source of EI, CI.

Results

Antifungal Activity Detected on Agar Medium and in TLC-fractions of *P. aureofaciens* 63-28

P. aureofaciens 63-28 produced inhibition zones of 4.5 ~ 9.2 mm against all three fungi on NA-Glc medium when the fungi and bacterium were inoculated simultaneously, and of 11.6 ~ 19 mm when the bacterium was inoculated 48 hours before the fungi. Of the six TLC fractions that were bioassayed, fractions I, III, IV and V showed significant activity against *P. ultimum* and *P. cryptogea* (Table 1). No clear zone of inhibition was observed against *R. solani*. However, density of mycelia was decreased around the disks. When *R. solani* was tested against larger aliquots of fractions I, III, IV, and V (50 μl), only fraction I showed an inhibition zone (1.5 mm), which disappeared by day 6. The *Cladosporium* bioassay done on a TLC chromatogram of a total extract of 63-28 showed the presence of four inhibitory spots with Rfs of 0.67, 0.41, 0.24, and 0.07, corresponding to fractions I, III, IV, and V, respectively.

Table 1. Fractionated bioactivity of a crude culture extract of *Pseudomonas aureofaciens* 63-28 using a disk bioassay.[a]

TLC fractions		Inhibition zone (mm) ± SEM		
		P. ultimum	*R. solani*	*P. cryptogea*
Control	(50 μl)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0	(50 μl)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
I	(20 μl)	4.0 ± 0.0	0.0[b] ± 0.0	3.5 ± 0.5
II	(50 μl)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
III	(20 μl)	7.0 ± 2.5	0.0 ± 0.0	9.0 ± 1.0
IV	(20 μl)	8.0 ± 0.0	0.0 ± 0.0	7.0 ± 0.0
V	(20 μl)	5.5 ± 0.5	0.0 ± 0.0	4.0 ± 0.0

[a] Bacterium grown for 5 days in NB-Glc medium and medium extract fractionated on TLC with chloroform-acetone. Paper filter disks were soaked with an aliquot (volume in parentheses) of each fraction extract dissolved in acetone. Control consisted of disks soaked with acetone only.

[b] 0.0: No clear inhibition zone around the disk, but fungal mycelial density decreased compared to control disk.

Table 2. Retention times and wavelengths of maximum UV absorbance of purified active compounds and reference antibiotics.

Compounds	Separate runs		Combined run[a]	
	Rt (minute)	A_{max} (nm)	Rt (minute)	A_{max} (nm)
FI	26.3	254	26.3	254
FIII	20.1	263	20.5	263
FIV	14.6	254	14.4	254
Pyrrolnitrin	26.8	<220	26.8	<220
Pyoluteorin	10.6	307	10.7	307
2,4 Diacetylphloroglucinol	21.2	271	22.9	271

[a] For the combined run, acidified water (acetic acid 2%) was used for better resolution.

HPLC Analysis of Purified Active Compounds and Reference Antibiotics

Table 2 shows the retention times and wavelength of maximum UV absorbance for the purified active compounds isolated from fractions I, III and IV and reference antibiotics. Compound FIV eluted off the column well after pyoluteorin, and had a similar UV spectrum to compound FI, with maximum UV absorbance at 254 nm. Compound FIII eluted off just before the

Table 3. NMR spectroscopic data for the compound FIV.

Position	^{1}H NMR				^{13}C NMR		
	Integration	δ (ppm)-mult	J (Hz)	COSY	δ (^{13}C)[a]	HMBC (τ=0.1)	NOESY[b]
6′	3H	0.917 (t)	7.1	5′	13.78	C5′, C4′	
5′	2H	1.363 (sixtet)	7.3	6′, 4′	22.23	C6′, C4′, C3′	
4′	2H	1.465 (quint.)	7.5	5′, 3′	30.82	C6′, C5′, C3′, C2′	
3′	2H	2.260 (q)	7.1	4′, 2′ weak 4	29.43	C5′, C4′, C2′, C1′	**4**, *4′*, *2′*
6	1Ha	3.771 (dd)	12.2, 5.4	6b, 5	62.94	C4	**6b**, *5*, *4*
6	1Hb	3.990 (dd)	12.2, 3.7	6a, 5		C4, C5	**6a**, *5*, *4*
5	1H	5.116 (br. t)	—	6a, 6b, 4	81.72	—	**4**, **6a**, **6b**, **3′**
2	1H	5.978 (dt)	11.7, 7.3	3′, 1′	140.59	C3, C4′, C3′	**1′**, *4′*, *3′*
1	1H	6.087 (br. d)	11.7	3′, 2′	116.16	C2, C4, C2′, C3′	**2′**, *3′*
4	1H	7.156 (br. s)		5	144.03	C2, C3, C5	**3′**, **5**, *6a*, *6b*
3	—	—			130.27		
2					173.01		

[a] The chemical shift of the quaternary carbons comes from the HMBC experiment.
[b] The typeface used for the proton labels indicates qualitatively the strength of the NOE effect: Bold face + underline indicates strong NOE, bold face only indicates medium NOE and italic indicates small NOE.

diacetylphloroglucinol standard, with a retention time of 20 minutes. Compound FIII had a maximum UV absorbance of 263 nm, unlike phloroglucinol with a maximum UV absorbance at 271 nm. Compound FI had a retention time of 26 minutes and eluted from the column just before the pyrrolnitrin standard. However, the UV spectrum of FI was different from pyrrolnitrin, with a maximum UV absorbance at 254 nm, whereas pyrrolnitrin had a maximum absorbance <220 nm.

Identification of Compound FIV

The ^{1}H NMR spectrum of the FIV sample displayed signals for a short mono unsaturated aliphatic chain (6 carbons) as can be seen from the COSY spectrum data (Table 3). The double bond was assigned as the *cis*-configuration due to the 11.7 Hz coupling constant for the 2 olefinic protons. A second spin system was also identified containing three deshielded protons that were each adjacent to an oxygen atom based on the mass spectrometry data and chemical shifts of observed proton resonances. Two of these protons were assigned as diastereotopic (H_A and H_B) due to an observed COSY correlation.

Protonated ^{13}C NMR resonances were assigned from ^{1}H-^{13}C correlations observed in the HMQC experiment. Chemical shifts of quaternary carbons (carbonyl and olefinic signals) were assigned based on $^2J_{CH}$ and $^3J_{CH}$ couplings identified in the HMBC spectrum. The H2′ olefinic proton (5.98 ppm) showed correlation to C3′ (29.6 ppm) and C4′ (31.2 ppm), and a previously unassigned quaternary olefinic carbon resonance at 130.1 ppm (C3). The H-1′ proton (6.09 ppm) also showed correlation to the C3′ olefinic carbon as well as C4 (144.0 ppm) and a carbonyl resonance (C2) at 173.0 ppm that was suggestive of an ester moiety. The most deshielded olefinic proton H4 (7.16 ppm) was also correlated with the carbonyl ester, to the quaternary olefinic C3 (130.1 ppm) and to the oxygenated carbon assigned to C5 at 81.8 ppm.

Using this information, two possibilities can describe the compound: a six-membered ring lactone bearing an hydroxy group at C5 with the methylene C6 deshielded by the oxygen of the lactone group, or a five-membered ring lactone substituted at C5 with an hydroxy-methyl group. These two possible structures were distinguished by an observed coupling in the ^{1}H NMR spectrum between the protons at C6 and an exchangeable proton located at 1.91 ppm (broad triplet).

From the NMR data, we postulated a compound with 11 carbons, 15 hydrogens attached to carbons, and one carbonyl oxygen. There was at least another heteroatom that deshielded the proton and methine α to the CH_2OH and a heteroatom next to the carbonyl to make it an ester or amide. A tentative formula was proposed based on the NMR data-$C_{11}H_{16}O_2+X$.

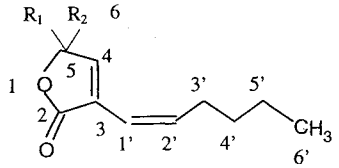
A molecular weight of 196 for both compounds FIII and FIVwas determined by mass spectrometry. Only two molecular formulas with a MW 196 are consistent for $C_{11}H_{16}O_2+X$: $C_{11}H_{16}O_3$ and $C_{11}H_{20}N_2O$. With the formula $C_{11}H_{20}N_2O$, two nitrogens and four extra protons would have to be positioned, and the oxygen eliminated. Therefore, the only logical formula that satisfies both the NMR and MS data is $C_{11}H_{16}O_3$. Upon assembling the molecule, we obtained the structure

shown in Fig. 1, which is (*Z*)-4-hydroxymethyl-2-(1-hexenyl)-2-butenolide.

Identification of Compound FIII

NMR and MS data of compound FIII were very similar to compound FIV, suggesting that FIII was an isomer (Table 4). There was an additional methyl at 1.74 ppm and a loss of the H5 resonance observed for compound FIV. From the MS data, the base peak (*m/z* 135) was attributed to H_2O elimination and a loss of C_3H_7 β to the olefin. A second fragmentation resulting in a very intense (99.7%) peak (*m/z* 139) was explained by the loss of a methyl group and the C_3H_7 cleavage β to the olefin. Using the same rationalization as for compound FIV, the molecular ion peak $m/z=196$, the molecular formula for FIII is $C_{11}H_{16}O_3$. A correlation in the HMBC experiment between the methyl protons located at C6 position and the protonated olefinic carbon (145 ppm) ($^3J_{CH}$) indicates that the structure shown in Fig. 1 is the only acceptable isomer. The molecule is (*Z*)-4-hydroxy-4-methyl-2-(1-hexenyl)-2-butenolide.

Fig. 1. Structure of compounds FIII and FIV.



	R_1	R_2
FIII	OH	CH_3
FIV	CH_2OH	H

Discussion

Several new antifungal compounds produced by *P. aureofaciens* strain 63-28 were detected *via* HPLC and bioassays. They showed strong activity against *P. ultimum* and *P. cryptogea*, but not *R. solani*. Production of these compounds appeared to be induced by glucose in the medium (GAMARD, unpublished data). This is similar to the observations with oomycin A, which is produced in the presence of glucose by *P. fluorescens* strain HV37A[18]. High levels of glucose may have suppressed the production of some antibiotics. For example, pyoluteorin synthesis is usually repressed in the presence of glucose[4]. Based on HPLC analysis and comparison with three of the most commonly studied antibiotics of *Pseudomonas* spp., 2,4-diacetylphloroglucinol, pyrrolnitrin and pyoluteorin, 63-28 did not produce detectable amounts of these antibiotics. However, over 50 different antibiotics have been characterized from the species of *P. fluorescens*[19], with a dozen active in biological control[16].

Two of the antifungal compounds produced by *P. aureofaciens* strain 63-28 were purified and identified as isomeric butyrolactone derivatives, (*Z*)-4-hydroxy-4-methyl-2-(1-hexenyl)-2-butenolide (FIII) and (*Z*)-4-hydroxymethyl-2-(1-hexenyl)-2-butenolide (FIV). Their isolation was directed by bioassay activities against *C. herbarum*. Both compounds exhibited different chromatographic properties and differed only in the substitution group on the C5 of the lactone ring, with an hydroxymethyl group on the FIV compound and a methyl and a hydroxy group in the case of FIII.

To the best of our knowledge, (*Z*)-4-hydroxy-4-methyl-2-(1-hexenyl)-2-butenolide and (*Z*)-4-hydroxymethyl-2-(1-hexenyl)-2-butenolide have not been de-

Table 4. NMR spectroscopic data for the compound FIII.

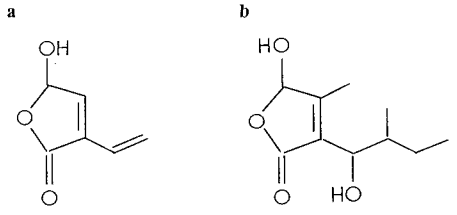
Position	^{1}H NMR			^{13}C NMR		
	Integration	δ (ppm)-mult	*J* (Hz)	δ (^{13}C)	HMBC ($\tau=0.1$)	HMBC ($\tau=0.07$)
6′	3H	0.925 (t)	7.3	13.73	H5′, H4′	
5′	2H	1.372 (sixtet)		22.24	H6′, H4′, H3′	
4′	2H	1.473 (quint.)	8.1	30.96	H6′, H5′, H3′, H2′	
6	3H	1.742 (s)		34.80	145.38, 104.02	
3′	2H	2.250 (q)		29.55	H5′, H4′, H2′, H6	
2′	1H	6.03 (o. dt)	≈12.7 (d)	141.89	129.62, H4′, H3′	
1′	1H	6.05 (d)	≈12.5	115.58	145.71, 129.62	
4	1H	6.964 (s)		145.44	129.62	3-C, 5-C, 115.77, 170.5
3				129.62		
5				104.02		
2				145.36 (br)		

scribed previously, and this is the first report of butyrolactones with antifungal activity produced by a Gram-negative bacterium. The structure of FIII and FIV closely resembles the antibiotic PA-147, a compound with weak antibacterial activity isolated from an unidentified *Streptomyces* strain (Fig. 2a)[20]. FIII and FIV are also structurally similar to butenolide 3 (Fig. 2b), which is one of four analogs isolated from *Streptomyces antibioticus* strain Tü 99 that exhibited antibacterial activity but not antifungal activity[21].

FIII and FIV are also comparable to another class of actinomycete metabolites known as autoregulators. These butyrolactone autoregulators act as signal molecules, initiating and regulating endogenous cytodifferentiation and production of secondary metabolites by the Actinomycetes. The compound shown in Fig. 3a was isolated from an unidentified marine actinomycete[22]. The virginiae butenolide C (Fig. 3b) was isolated from *Streptomyces virginiae*[23]. The A-factor (Fig. 3c) was isolated from *Streptomyces griseus*, and it is the most well-characterized autoregulator factor in the order Actinomycetale[24].

Fig. 2. Antibiotic compounds structurally related to FIII and FIV.

a) PA-147, isolated from *Streptomyces* sp.; b) Butenolide 3, isolated from *S. antibioticus*.

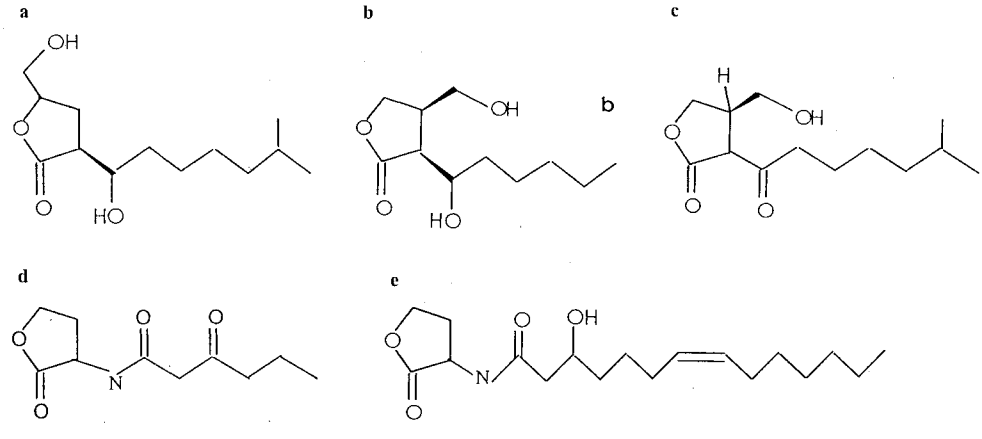


Gram-negative bacteria like *Pseudomonas* spp. produce homoserine lactone compounds that function as autoinducers[25]. Homoserine lactones (HSL) are small diffusible molecules, which act as communication signals between bacterial cells, in a population density-dependent manner. VAI (Fig. 3d) was the first of its kind to be isolated from the fish symbiont *Vibrio fisheri*[26]. In the past few years, HSL were found to be widespread among Gram-negative bacteria[27], and the coexistence of several different homoserine lactone autoinducing systems in a single strain has been demonstrated[28,29]. HSL autoinducers regulate physiological processes, which are usually related to or involved in a bacteria-host relationship, such as bioluminescence in *V. fischeri*[30], Ti plasmid transfer in the plant pathogen *Agrobacterium tumefaciens*[31], root nodulation by the symbiont *Rhizobium leguminosarum*[25], synthesis of virulence factors by the plant pathogen *Erwinia carotovora*[25] or the human pathogen *Pseudomonas aeruginosa*[29]. HSL were also shown to regulate the production of an antibiotic in *E. carotovora*[32]. Although the structure of the soluble inducer is not known, an HSL is involved in the production of phenazine in the soil biological control agent *P. aureofaciens* 30-84[33].

Until recently, antibiotic activity in neither the butyrolactone autoregulators from actinomycetes, nor the homoserine lactones from Gram-negative bacteria was known. However, it has been demonstrated that a homoserine lactone compound produced by *R. leguminosarum* (Fig. 3e) acts both as a bacteriocin and an autoinducer activity as well[34,35]. Among *Streptomyces* species, there are a few metabolites possessing both

Fig. 3. Autoregulatory factors and autoinducers structurally related to FIII and FIV.

a) Autoregulator from a marine actinomycete; b) Virginiae butanolide C from *S. virginiae*; c) A-factor from *S. griseus*; d) Autoinducer from *V. fischeri* (VAI); e) Bacteriocin *small* of *R. leguminosarum*.



properties too, but their structures are quite different from butyrolactones. Lincomycin is produced by *Streptomyces lincolnensis*, and it showed antibiotic activity against *S. alboniger* when used at high levels ($2 \sim 10\,\mu g$/assay disk) and stimulated aerial mycelial formation when used at low levels ($0.002 \sim 1\,\mu g$/disk)[36]. Looking for an endogenous signal in *S. alboniger*, pamamycin was isolated, which also showed a dual antibiotic and stimulator activity[36]. *P. aureofaciens* strain 63-28 and the purified compounds FIII and FIV were tested in a bioassay constructed to detect homoserine lactone activity. The indicator strain, *A. tumefaciens* NT1 (pJM749, pSVB33)[37], contains a *lacZ* fusion in a gene that is activated in the presence of any one of several exogenous acyl-homoserine lactones but does not produce any homoserine lactone[31]. No activity was detected after a week of incubation of a mixed culture of *P. aureofaciens* 63-28 and the indicator strain of *Agrobacterium* (GAMARD, unpublished results). The *Agrobacterium* autoinducing system has a certain level of specificity, and the structures of FIII and FIV are probably too different from the homoserine lactone compounds to be analogs of the *Agrobacterium* system. Because of their close relatedness to the actinomycete butyrolactone autoregulators, and because some molecules showed a dual feature of antibiotic and regulatory activity, it is possible that FIII and FIV could play a double role in *P. aureofaciens*.

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