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Structure, chemistry, and biological activity of pseudophomins A and B, new cyclic lipodepsipeptides isolated from the biocontrol bacterium *Pseudomonas fluorescens*

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Abstract

Pseudophomins A and B are cyclic lipodepsipeptides isolated from *Pseudomonas fluorescens* strain BRG100, a bacterium with potential application for biocontrol of plant pathogens and weeds. Their chemical structures were established by a combination of spectroscopic data, X-ray crystallography, and selective chemical degradation. This unique chemical degradation allowed the unambiguous determination of the absolute configuration of the amino acid residue Leu-1, due to γ-lactam formation followed by selective cleavage of the adjacent N(8)–C(7) bond. To the best of our knowledge this is the first application of γ-lactam formation to the determination of absolute configuration of an adjacent amino acid. Pseudophomin B showed higher antifungal activity against the phytopathogens *Phoma lingam*/*Leptosphaeria maculans* and *Sclerotinia sclerotiorum* than pseudophomin A, and is likely to be the main component responsible for the antifungal activity of EtOAc extracts of strain BRG100. By contrast, pseudophomin A showed stronger inhibition of green foxtail (*Setaria viridis*) root germination than pseudophomin B.

Keywords: Antifungal; Biocontrol; Depsipeptide; Lipodepsipeptide; *Leptosphaeria maculans*; *Phoma lingam*; *Pseudomonas fluorescens*; *Sclerotinia sclerotiorum*

1. Introduction

Microorganisms such as soil bacteria and plant pathogens can be used for biological control and development of biorational products to reduce populations of weeds (bioherbicides) or plant pathogens (biofungicides), and thus prevent the continuous use of chemical herbicides and fungicides. A mechanism often responsible for root disease suppression and potential control of soilborne plant pathogens appears to involve the production of antibiotic compounds or bioactive metabolites (Faull and Powell, 1995). Direct correlations have been established between antibiotic production and disease suppression. In fact, the genes for the biosynthesis of some antibiotics involved in biocontrol have been isolated and their regulation has been studied (Faull and Powell, 1995; Dowling and O'Gara, 1994). Bacteria of the genus *Pseudomonas* comprise a large group of potential biocontrol species as a result of their ability to produce a diverse array of antifungal metabolites. For example, *Pseudomonas fluorescens* can produce a very wide-range of secondary metabolites such as phenazines (Faull and Powell, 1995; Dowling and O'Gara, 1994), siderophores (Faull and Powell, 1995), pyrrolnitrin (Dowling and O'Gara, 1994), 2,4-diacetylphloroglucinol (Dowling and O'Gara, 1994), lipodepsipeptides (Laycock et al., 1991), or a combination of several bioactive metabolites. Such metabolites can have antifungal and herbicidal activity, depending on the particular strain. In addition, the diversity of bioactive metabolites produced by biocontrol agents is highly dependent on environmental factors. In particular, the carbon sources may have a dramatic influence on the type and levels of antibiotics produced (Rosales et al., 1995).

As part of a program toward understanding the mechanism of action and potential application of new biological control agents, we investigated the production of antifungal and herbicidal metabolites by the

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bacterium *P. fluorescens* strain BRG100. We have established that the major metabolites, named pseudophomins A (**1**) and B (**2**), are new cyclic depsipeptides active against plant pathogens. The chemical structures of these compounds were established by a combination of spectroscopic techniques, chemical derivatization and degradation, and X-ray crystallography. The absolute configuration was determined using a remarkable chemical degradation methodology applicable to this type of cyclic lipodepsipeptide. Here we report the structure determination of pseudophomins A (**1**) and B (**2**), and their antifungal and herbicidal activities.

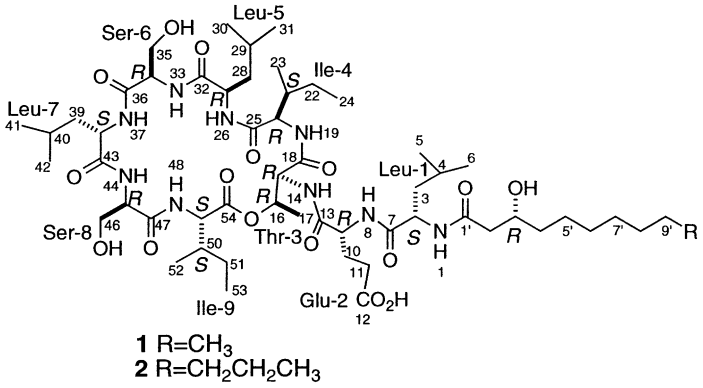
2. Results and discussion

EtOAc extracts from culture supernatants of *P. fluorescens* strain BRG100 were bioassayed against four fungal phytopathogens [*Phoma lingam* (Tode ex Fr.) Desm., sexual stage = *Leptosphaeria maculans* (Desm.) Ces. et de Not.; *Alternaria brassicae* (Berk.) Sacc., *Sclerotinia sclerotiorum* (Lib.) de Bary, and *Rhizoctonia solani* Kuhn] and the weed green foxtail (*Setaria viridis*). Most of the herbicidal activity remained in the aqueous phase, whereas the antifungal activity was found in the extract residue. The EtOAc extracts were combined, concentrated, and fractionated by column chromatography; resulting fractions were combined and bioassayed for antifungal and herbicidal activity, employing fungal radial growth and root growth assays, as described in the Experimental. No substantial herbicidal activity was detected in any of the fractions. The fractions that showed antifungal activity were combined and were subjected to further fractionation on C_{18} reversed-phase silica gel. HPLC analysis of the combined antifungal fractions showed two major peaks, with retention times

Table 1
^{1}H and ^{13}C NMR spectroscopic assignments of pseudophomin B (**2**) in CD_3OD

Residue	C #	^{13}C[a] δ (ppm) (mult)	^{1}H δ (ppm), multiplicity (J in Hz)	HMBC	Residue	C #	^{13}C[a] δ (ppm) (mult)	^{1}H δ (ppm), multiplicity (J in Hz)	HMBC
C_{12} HO-acid	1′	174.2 (s)				22	25.7 (*t*)	1.45, *m* 1.29	
	2′	43.6 (*t*)	2.45 *d* (7)	3′, 1′		23	16.3 (*q*)	1.01, *d* (6.5)	
	3′	68.8 (*d*)	4.08, *m*	1′, 4′		24	9.9 (*q*)	0.97	
	4′	39.7 (*t*)	1.74, *m*			25	174.9 (s)		
	5′	25.7 (*t*)	1.29		Leu-2	NH		7.28, *d*, (7)	
	6′	29.7 (*t*)	1.29			27	62.5 (*d*)	3.76, *dt* (8, 3.5)	29, 32
	7′	29.7 (*t*)	1.29			28	37.5 (*t*)	1.45, *m*	
	8′	32.1 (*t*)	1.29			29	35.2 (*d*)	2.12, *br m*	
	9′	29.7 (*t*)	1.29			30	21.7 (*q*)	0.91	
	10′	29.5 (*t*)	1.29			31	22.7 (*q*)	0.92, *d* (6)	
	11′	22.7 (*t*)	1.29			32	172.3 (s)		
	12′	13.5 (*q*)	0.92		Ser-1	NH		7.60, *d* (4)	
Leu-1	NH		7.75, *d*			34	56.4 (*d*)	4.39, *m*	32, 35
	2	54.8 (*d*)	4.08, *m*	1′		35	62.8 (*t*)	3.93, *d*	
								4.13, *d*	
	3	39.4 (*t*)	1.52, *m*			36	174.2 (s)		
	4	24.8 (*d*)	1.66		Leu-3	NH		7.21, *d* (7)	
	5	22.2 (*q*)	0.91			38	53.6 (*d*)	4.39, *m*	39, 40, 43
	6	22.6 (*q*)	1.29			39	41.0 (*t*)	1.94	
	7	175.5 (s)				40	24.8 (*d*)	1.63, *m*	
Glu	NH		9.19, *d* (4)			41	20.0 (*q*)	0.91, *d*	
	9	57.3 (*d*)	4.05, *m*	7, 10, 11, 13		42	20.3 (*q*)	0.93, *d*	
	10	27.0 (*t*)	2.12, *br m*			43	174.1 (s)		
	11	33.6 (*t*)	2.39, *m*	12, 9, 10	Ser-2	NH		8.28, *d* (8)	
	12	180.6 (s)				45	56.6 (*d*)	4.47, *m*	43, 46, 47
	13	176.1 (s)				46	61.9 (*t*)	3.78, *d*	
								4.05	
Thr	NH		8.49, *d* (6.5)			47	171.9 (s)		
	15	60.9 (*d*)	4.14, *m*	13, 16, 17	Ile-2	NH		7.18, *d* (9.5)	
	16	69.5 (*d*)	5.49, *dq*, (11, 6)	15, 17, 54		49	56.6 (*d*)	4.64, *dd* (10, 3)	47, 50, 51, 52, 54
	17	17.6 (*q*)	1.39, *d* (6)	15, 16		50	36.5 (*d*)	1.99, *m*	
	18	172.3 (s)				51	24.6 (*t*)	0.97, *m*	
Ile-1	NH		8.76, *d* (7)			52	15.2 (*q*)	0.95, *d* (6)	
	20	53.4 (*d*)	4.05, *m*			53	11.3 (*q*)	0.91	
	21	24.7 (*d*)	1.97, *m*			54	168.9 (s)		

[a] Multiplicities were established using the DEPT pulse sequence.



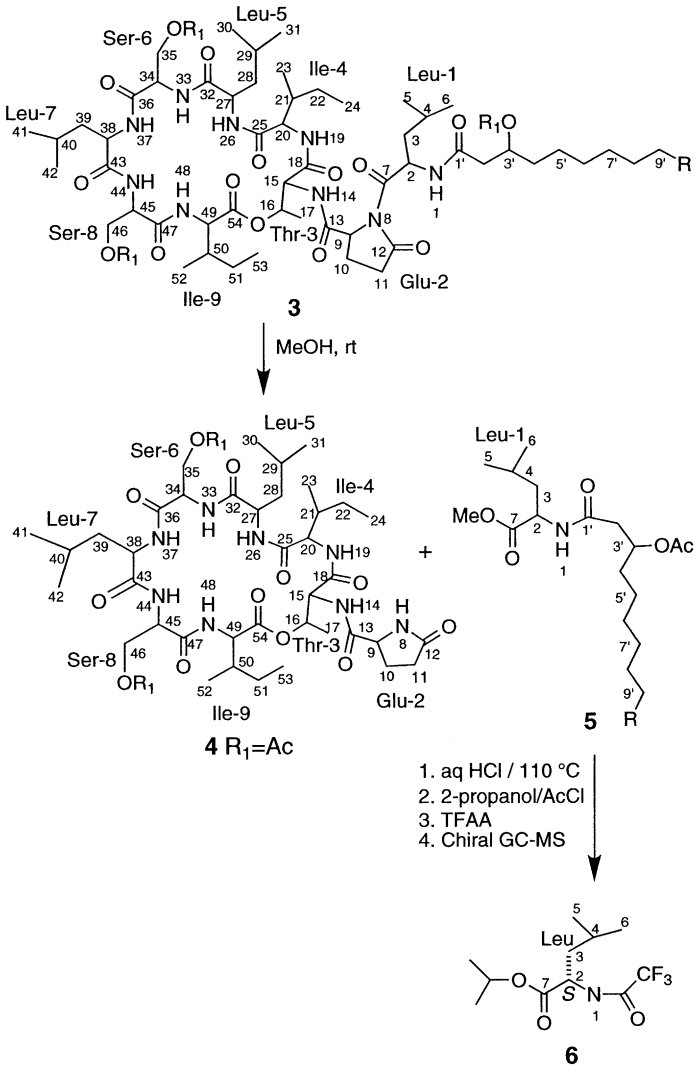
of 16.7 and 21.7 min (system A), along with some minor peaks. This fraction was further separated on C_{18} reversed-phase silica gel to yield pseudophomin B (**2**, 40 mg, $r_t = 21.7$ min, white amorphous powder), which was crystallized from aqueous acetonitrile to yield needle-like crystals. The remaining portion (54 mg) was further purified by reversed-phase HPLC to yield pseudophomin A (**1**, 35 mg, $r_t = 16.7$ min, white amorphous powder), which was recrystallized from CH_2Cl_2–MeOH.

Pseudophomin B was obtained as optically active ($[\alpha]_D$ −15, EtOH) colourless needles; both the ^1H and ^{13}C NMR spectroscopic data of pseudophomin B were

Table 2
^1H and ^{13}C NMR spectroscopic assignments of pseudophomin A (**1**) in DMSO-d_6

Residue	C #	^{13}C[a] δ (ppm) (mult)	^1H δ (ppm), multiplicity (J in Hz)	HMBC	Residue	C #	^{13}C[a] δ (ppm) (mult)	^1H δ (ppm), multiplicity (J in Hz)	HMBC
C_{10} HO-acid	1′	172.9 (*s*)				23	15.8 (*q*)	0.83, *d* (6.5)	
	2′	44.2 (*t*)	2.26, *d* (6.5)	1′, 3′, 4′		24	11.7 (*q*)	0.75, *t* (7)	21
	3′	68.4 (*d*)	3.85, *m*			25	172.4 (*s*)		
	4′	37.9 (*t*)	1.37, *m*		Leu-2	NH		7.20, *br s*	
	5′	29.9 (*t*)	1.25			27	58.1 (*d*)	4.30, *m*	25, 29
	6′	25.6 (*t*)	1.25			28	30.9 (*t*)	2.26, *m*	
	7′	25.8 (*t*)	1.25			29	24.9 (*d*)	1.71, *br m*	
	8′	25.8 (*t*)	1.25			30	24.8 (*q*)	1.63	
	9′	22.9 (*t*)	1.25			31	23.9 (*q*)	0.88, *d* (8.5)	
	10′	14.7 (*q*)	0.91			32	171.0 (*s*)		
Leu-1	NH		7.66, *d* (8)		Ser-1	NH		7.77, *br s*	
	2	54.4 (*d*)	4.08, *m*	1′		34	59.1 (*d*)	4.70, *m*	35
	3	32.0 (*t*)	1.25			35	62.6 (*t*)	3.63, *m* 3.70, *m*	
	4	23.9 (*d*)	0.83			36	171.8 (*s*)		
	5	21.5 (*q*)	0.88, *d* (8.5)		Leu-3	NH		7.35, *d* (6.5)	36
	6	21.8 (*q*)	0.77			38	58.1 (*d*)	4.08, *m*	
	7	174.3 (*s*)				39	29.5 (*t*)	1.25	41
Glu	NH		8.53, *br s*			40	25.0 (*d*)	1.51, *m*	
	9	52.7 (*d*)	4.16, *m*	7, 13		41	22.8 (*q*)	0.91, *d*	
	10	27.3 (*t*)	1.86, *m*			42	23.5 (*q*)	0.91, *d*	
	11	30.9 (*t*)	2.31, *m*	13		43	174.2 (*s*)		
	12	174.4 (*s*)			Ser-2	NH		8.29, *d* (7)	
	13	173.8 (*s*)				45	57.9 (*d*)	4.30, *m*	46
Thr	NH		8.29, *d* (7)	13, 15, 16		46	62.2 (*t*)	3.74, *m* 3.80, *m*	45
	15	59.1 (*d*)	4.30, *m*			47	171.8 (*s*)		
	16	69.5 (*d*)	5.19, *dq* (10, 6)	15, 17, 54	Ile-2	NH		7.66, *d* (8)	
	17	18.1 (*q*)	1.19, *d* (6)	15, 16		49	51.9 (*d*)	4.30, *m*	
	18	171.1 (*s*)				50	37.2 (*d*)	1.90, *m*	
Ile-1	NH		8.06, *br s*			51	26.3 (*t*)	1.04, *m*	
								1.31, *m*	
	20	57.7 (*d*)	4.08, *m*	18		52	15.8 (*q*)	0.91, *d* (7)	
	21	36.6 (*d*)	1.63, *m*	20, 25		53	12.1 (*q*)	0.72, *d* (6.5)	
	22	25.6 (*t*)	1.37, *m*			54	169.8 (*s*)		

[a] Multiplicities were established using the DEPT pulse sequence.



Scheme 1. Determination of absolute configuration of pseudophomins A (**1**, R = CH_3, R_1 = H) and B (**2**, R = $CH_2CH_2CH_3$, R_1 = H); **5a**, R = CH_3, R_1 = $COCH_3$; **5b**, R = $CH_2CH_2CH_3$, R_1 = $COCH_3$.

indicative of a lipopeptidic structure. In the positive-ion FAB–MS spectrum, its molecular ion peak appeared at *m/z* 1191 and 1169 indicating ions resulting from addition of sodium $[M+Na]^+$ and proton $[M+H]^+$, respectively. The positive ion HRMS–FAB showed a peak at *m/z* 1168.7456 suggesting a molecular formula of $C_{57}H_{102}N_9O_{16}$ (calcd. *m/z* 1168.7444, $[M+H]^+$), consistent with both the ^1H and ^{13}C NMR spectra (Table 1). In addition, the ^1H NMR spectrum showed nine signals at δ_H 7.10–9.44 likely due to NH protons. Detailed analysis of the HMQC, HMBC, and COSY spectra showed the presence of nine amino acids: three leucine (Leu), two isoleucine (Ile), two serine (Ser), one threonine (Thr) and one glutamic acid (Glu) residues. After subtraction of nine amino acid residues ($C_{45}H_{78}N_9O_{14}$) from the molecular formula of pseudophomin B, the remaining fragment of the molecule contained 12 carbons, 23 hydrogens and two oxygen atoms. Furthermore, the NMR data showed that this fragment contained one aliphatic methyl (CH_3), one carbinol methine (CHOH), nine methylenes (CH_2) and a carbonyl (CO) carbon, typical of a 12-carbon fatty acid chain. Complete analysis of the data suggested that pseudophomin B had structure **2**, which was confirmed by X-ray crystallographic analysis (Quail et al., 2002).

Pseudophomin A (**1**, 35 mg) was obtained as an optically active ($[\alpha]_D$ −23, EtOH) white powder. The positive-ion MS–FAB spectrum exhibited ions at *m/z* 1163 and 1141 indicating addition of sodium $[M+Na]^+$ and proton $[M+H]^+$ to the molecular ion, respectively. The positive ion HRMS–FAB showed a peak at *m/z* 1140.7139 indicating a molecular formula of $C_{55}H_{98}N_9O_{16}$ (calc. *m/z* 1140.7132 = $[M+1]^+$), and suggesting that the compound only differed from pseudophomin B ($C_{57}H_{102}N_9O_{16} = [M+1]^+$) by two methylene units. Comparison of the NMR spectra (Tables 1 and 2) of pseudophomin A (**1**) with those of B (**2**) showed the same number of amino acid residues and MS-FAB data

indicated that both compounds only differed in the β-hydroxy fatty acid residue.

Although the relative configurations of the hydroxy acid and amino acid residues of pseudophomins A and B could be established by X-ray crystallography (Quail et al., 2002), the absolute stereochemistry required chemical degradation and derivatization as follows (Scheme 1). Pseudophomin A (**1**) or B (**2**) was acetylated to triacetates **3a** (**3**, $R=CH_3$, $R_1=Ac$) or **3b** (**3**, $R=CH_2CH_2CH_3$, $R_1=Ac$), respectively, dissolved in MeOH, and separated by reversed-phase HPLC to yield **4** ($R=Ac$; HPLC $r_t=2.3$ min) and **5a** (**5**, $R=CH_3$, HPLC $r_t=3.8$ min) in the case of pseudophomin A, or **4** and **5b** (**5**, $R=CH_2CH_2CH_3$, HPLC $r_t=5.3$ min) in the case of pseudophomin B (Scheme 1). The chemical structure of depsipeptide **4** was established by analysis of the NMR data (Table 3) and MS spectroscopic data. The γ-lactam moiety present in **4** is likely to result from formation of the mixed anhydride of Glu-2 during acetylation, followed by nucleophilic displacement of acetate to form the C(12)–N(8) imide bond, which upon methanolysis yields compounds **4** and **5**. Product **5** (**5a** or **5b)** was hydrolyzed in aqueous HCl and derivatized to yield leucine derivative **6**. This unique selective degradation allowed the unambiguous determination of the absolute configuration of the amino acid residue Leu-1, due to selective cleavage of the N(8)–C(7) bond. Although there are many examples of γ-lactam formation in proteins and peptides (Bateman et al., 1990; Baldwin et al., 1990; Abraham and Podell, 1981), to the best of our knowledge this is the first time that selective imide bond cleavage is used to determine the absolute configuration of a cyclic depsipeptide.

A literature search showed that pseudophomin A (**1**) is a diastereomer of massetolide A and pseudophomin B (**2**) is a diastereomer of massetolide C (Gerard et al., 1997). The few crystal structures of cyclic lipodepsipeptides determined to date include WLIP (white-line inducing principle), tensin, and amphisin (Sorensen et al., 2001). Interestingly, the chemical structure of pseudophomin A, including the absolute configuration of each stereogenic center, is similar to that of WLIP, except for the D-Ile-4 residue which in WLIP is replaced with D-Val residue (Han et al., 1992; Mortishire-Smith et al., 1991). WLIP is a lipodepsipeptide produced by *Pseudomonas reactans* which inhibits browning of mushrooms caused by *P. tolaasii*. Mushroom caps treated with various concentrations of a crude WLIP sample,

Table 3
^1H and ^{13}C NMR spectroscopic assignments of compound **4** ($R_1=Ac$) in $(CD_3)_2CO$

Residue	C #	^{13}C[a] δ (ppm) (mult)	^1H δ (ppm), multiplicity (*J* in Hz)	HMBC	Residue	C #	^{13}C[a] δ (ppm) (mult)	^1H δ (ppm), multiplicity (*J* in Hz)	HMBC
Glu	NH		8.70, *br s*			27	52.6 (*d*)	4.88, *m*	28, 31
	2	57.7 (*d*)	4.30, *m*	6		28	63.9 (*t*)	4.30, *m*	
	3	25.0 (*t*)	1.36, *m*			29	20.3 (*q*)	2.03, *s*	30
	4	—	2.32, *dd* (9.5, 6)	5		30	169.8 (*s*)		
	5	178.6 (*s*)				31	169.6 (*s*)		
	6	170.5 (*s*)			Leu-2	NH		7.12, *br s*	
Thr	NH		7.59, *d* (8.5)			33	57.2 (*d*)	4.46, *m*	31
	8	61.2 (*d*)	4.30, *m*	6,8,11		34	40.5 (*t*)	1.85, *m*	38
	9	70.0 (*d*)	5.38, *dq*, (12, 6)	8, 51		35	24.7 (*d*)	1.91	
	10	18.2 (*q*)	1.41, *d* (6)	8		36	22.5 (*q*)	0.99, *d* (6.5)	
	11	174.9 (*s*)				37	23.0 (*q*)	0.90, *d*	
Ile-1	NH		8.03, *br s*			38	172.7 (*s*)		
	13	61.2 (*d*)	3.98, *t* (7.5)	14, 18	Ser-2	NH		7.59, *d* (8.5)	
	14	35.9 (*d*)	2.00, *m*	16		40	53.2 (*d*)	4.66, *m*	41, 44
	15	25.9 (*t*)	1.51, *m*			41	63.6 (*t*)	4.40, *m*	40, 43
	16	15.3 (*q*)	0.96, *d* (6.5)			42	20.3 (*q*)	2.05, *s*	
	17	11.4 (*q*)	0.90, *t* (6.5)			43	170.3 (*s*)		
	18	172.7 (*s*)				44	169.6 (*s*)		
Leu-1	NH		8.10, *br s*		Ile-2	NH		7.74, *br s*	
	20	53.9 (*d*)	4.30, *m*	21, 25		46	53.4 (*d*)	4.30, *m*	44, 48, 51
	21	40.8 (t)	1.67	22, 24, 25		47	36.6 (*d*)	1.98	49
	22	24.8 (*d*)	1.91, *m*			48	25.8 (t)	1.20, *br s*	50
	23	21.0 (*q*)	0.98, *d* (6.5)			49	15.5 (*q*)	0.96, *d* (6.5)	
	24	21.9 (*q*)	0.95, *d*			50	10.8 (*q*)	0.90, *t* (6.5)	
	25	173.3 (*s*)				51	169.6 (*s*)		
Ser-1	NH		7.59, *d* (8.5)						

[a] Multiplicities were established using the DEPT pulse sequence.

Table 4
Antifungal activity of EtOAc extract from cultures of *Pseudomonas fluorescens* and pseudophomins A (**1**) and B (**2**)

Compound (concentration)	*Phoma lingam*[a] % inhibition[c]	*Alternaria brassicae*[b] % inhibition[c]	*Sclerotinia sclerotiorum*[b] % inhibition[c]	*Rhizoctonia solani*[b] % inhibition[c]
Ethyl acetate extract (2.0 mg/ml)	62%±2	47%±5	76%±7	46%±15
pseudophomin A (**1**) (0.5 mg/ml)	31%±2	23%±2	13%±4	19%±2
pseudophomin B (**2**) (0.5 mg/ml)	65%±3	51%±18	60%±2	18%±3

[a] Mycelia incubated on V8 agar medium, at 25 °C under constant fluorescent light.
[b] Mycelia incubated on potato dextrose agar (PDA), at 25 °C under constant fluorescent light.
[c] The percentage of inhibition was calculated using the formula:% inhibition = 100−[(growth in treated/growth in control)×100]±standard error; results are the means of three independent experiments conducted in triplicate.

and later inoculated with bacterial concentrations higher than the threshold value, did not develop the symptoms of the disease (Soler-Rivas et al., 1999). To date several lipodepsipeptides displaying broad bioactivity spanning from phytotoxic to antiviral, antibacterial, and antifungal, have been isolated from diverse *Pseudomonas* species (Balkovec, 1994). Recently, it was demonstrated that the cytotoxic mode of action of some lipodepsipeptides is due to formation of ion channels that are freely permeable to divalent cations thus promoting passive transmembrane ion fluxes and causing cell necrosis (Hutchison and Gross, 1997). It is worthy to note that pseudophomin B is substantially more active against the phytopathogens *P. lingam* and *S. sclerotiorum* than pseudophomin A (Table 4), and is likely to be the main component responsible for the antifungal activity of EtOAc extracts of strain BRG100. These results suggest that potential changes on transmembrane ion fluxes are not likely to be the sole molecular mechanism involved in the somewhat selective fungal growth inhibition caused by pseudophomins. Furthermore, both pseudophomins A and B showed slight inhibition (<15%) of root germination and root growth of the weed green foxtail (*S. viridis*) utilizing methods A and B; however, as shown in Table 5, stronger herbicidal activity was detected using method C. Interestingly, pseudophomin A showed stronger inhibition of green foxtail root germination than pseudophomin B. Overall our results indicate that the antifungal activity shown by cultures of *P. fluorescens* strain BRG100 is likely due to the secretion of pseudophomins A (**1**) and B (**2**). Furthermore, considering that strong herbicidal activity remained in the aqueous phase of extracted cultures, compounds **1** and **2** do not appear to be the main components responsible for the herbicidal activity shown by BRG100 liquid cultures.

Table 5
Herbicidal activity[a] of EtOAc extract from cultures of *Pseudomonas fluorescens* and pseudophomins A (**1**) and B (**2**) against seeds of green foxtail (*Setaria viridis*)

Compound (concentration)	Root elongation (mm)	% Inhibition[b]
EtOAc extract (0.6 mg/ ml)	10±1	63±6
Pseudophomin A (**1**) 5×10^{-4} M	9±2	67±6
pseudophomin B (**2**) 5×10^{-4} M	20±2	30±4

[a] Incubated at 20 °C in darkness using method C described in the Experimental.
[b] The percentage of inhibition was calculated using the formula:% inhibition = 100−[(growth in treated/growth in control)×100]±standard error; results are the means of five independent experiments.

3. Experimental

3.1. General

All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON. All solvents were HPLC grade and used as such, except for CH_2Cl_2 and $CHCl_3$ which were redistilled. Organic extracts were dried over anhydrous Na_2SO_4 and solvents removed under reduced pressure in a rotary evaporator.

HPLC analysis was carried out with a HPLC equipped with quaternary pump, automatic injector, and diode array detector (wavelength range 190–600 nm), degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 i.d.×200 mm), equipped with an in-line filter. Mobile phase, system A: 0.1% TFA in H_2O–0.1% TFA in CH_3CN (40:60) to 100% CH_3CN containing 0.1% of TFA, for 30 min, linear gradient, 1.0 ml/min; system B: 0.1% TFA in H_2O–0.1% TFA in CH_3CN (20:80) to 100% CH_3CN containing 0.1% of TFA, for 25 min, linear gradient, 1.0 ml/min. For the semi-preparative separation, an EconoSphere® ODS column (10 μm particle size silica, 10.0 i.d.×250 mm) equipped with an in-line filter was used with an isocratic elution (0.1% TFA in H_2O–CH_3CN, 35:65) for 25 min, flow rate 5.0 ml/min.

GC analysis was carried out with a Hewlett-Packard 5890 gas chromatograph, equipped with a chiral capillary column (Chirasil-Val® column, Alltech, 25 m×0.32 mm id, film thickness 0.2 μm), with He as carrier gas

(flow 30 cm/s, measured at 70 °C); detector at 240 °C; injector at 220 °C; oven temperature 70–200, at 4 °C min^{-1}; samples were injected in split mode (ca. 1:50) with CH_2Cl_2 as solvent.

NMR spectra were recorded on a Brüker AMX 300, Brüker AMX 500 or Brüker Avance 500 spectrometers; for ^1H (300 or 500 MHz), δ values were referenced to CD_3OD (CD_2HOD 3.31 ppm), $CD_3S(O)CD_3$ ($CD_2HS(O)CD_3$, 2.50 ppm), or $CD_3C(O)CD_3$ ($CD_2HC(O)CD_3$, 2.05 ppm) and for ^{13}C (75.5 or 125.8 MHz) referenced to CD_3OD (49.15 ppm), $CD_3S(O)CD_3$ (39.51 ppm), or $CD_3C(O)CD_3$ (206.68, 29.92 ppm).

Fourier transform infrared (FTIR) spectra were obtained on a Bio-Rad FTS-40 spectrometer using a diffuse reflectance cell.

Mass spectra (MS) [high resolution (HR), electron impact (EI), chemical ionization (CI) or fast-atom bombardment (FAB)] were obtained on a VG 70-VSE magnetic sector mass spectrometer using double focusing EB geometry operating at an accelerated voltage of 8 kV; EI/CI source operating with an electron energy of 70 eV and a source temperature of 200 °C; in FAB the primary Cs energy was 28 kV and the sample was introduced in a matrix containing either nitrobenzyl alcohol or glycerol, on the tip of a FAB insertion probe.

Specific rotations, $[\alpha]_D$ were determined at ambient temperature on a Perkin-Elmer 141 polarimeter using a 1 ml, 10 cm path length cell; the units are 10^{-1} deg cm^2 g^{-1} and the concentrations (c) are reported in g/100 ml.

3.2. Bacterial fermentation

A culture of *Pseudomonas fluorescens* strain BRG100 was deposited at the International Depository Authority of Canada (IDAC, Winnipeg, Manitoba) with the number IDAC 141200-1. Cultures of *P. fluorescens* BRG100 were streaked onto Pseudomonas Agar F (PAF) plates and incubated for three to four days at room temperature (20–25 °C). Bacterial inoculum was prepared by inoculating 20 ml of nutrient broth with bacterial cells grown in PAF plates, and incubating inoculum on a shaker at 150 rpm, 15 °C for 48 h. Large scale bacterial cultures (1-l flasks containing 500 ml nutrient medium: sodium phosphate, 6.0 g/l; ammonium chloride, 1.0 g/l; potassium dihydrogen orthophosphate, 3.0 g/l; sodium chloride, 2.0 g/l; magnesium sulfate, 1.23 g/l; thiamine hydrochloride, 5 mg/l; calcium chloride, 15 mg/l; glucose, 2 g/l) were obtained by inoculating each flask with 200 μl of bacterial inoculum followed by incubation at 15 °C, in a shaker at 150 rpm for 48 h.

Bacterial cultures (batches of 5 l) were combined in a 10 l container and pumped into a flow through CEPA industrial centrifuge (40,000 rpm) at 250–300 ml/min. The pellet and supernatant were separated, the pellet was collected in MeOH and the supernatant frozen immediately. The pelleted cells were extracted with MeOH, filtered through Whatman paper, then the filtrate was dried over sodium sulfate, and concentrated to dryness in a rotary evaporator.

3.3. Antifungal assays

Bioassays to determine antifungal activity were carried out with the following plant pathogens: *Phoma lingam* (Tode ex Fr.) Desm., sexual stage = *Leptosphaeria maculans* (Desm.) Ces. et de Not.; *Alternaria brassicae*, (Berk.) Sacc., isolate AB 11-1; *Sclerotinia sclerotiorum* (Lib.) de Bary, clone #33; *Rhizoctonia solani* Kuhn, isolate AG 2-1. *Phoma lingam* virulent isolate BJ 125 was grown on V8 agar under continuous light; after 15 days the fungal spores were collected and stored at −20 °C as previously reported (Pedras and Khan, 1996). *Alternaria brassicae*, isolate AB 11-1 was grown on potato dextrose agar (PDA) at 25 °C under continuous fluorescent light for 13 days (Pedras et al., 1998). *Sclerotinia sclerotiorum* and *Rhizoctonia solani*, isolate AG 2-1 were grown on potato dextrose agar (PDA), incubated under continuous light at 25 °C for 3 and 4 days respectively (Pedras and Khan, 2000).

Samples were dissolved in CH_2Cl_2–MeOH (9:1, v/v); the extracts were tested at a concentration of 2 mg/ml, while fractions, sub-fractions or pseudophomins A and B were tested at a concentration of 0.5 mg/ml. Solutions were applied onto the agar plate to make a uniform film on the agar surface, and the solvent was allowed to evaporate under a laminar flow hood. After 4 h, the plates were inoculated with mycelial plugs of actively growing cultures of the pathogen. Control plates containing solvent only were prepared and incubated in similar way. The percentage of inhibition was calculated relative to control plates by measuring the diameter of mycelial growth.

3.4. Herbicidal assays

For bioassay-guided isolation only two methods were used, referred to as method A and B since they require smaller amounts of sample (see below); a third method referred to as method C was used for determination of herbicidal activity of extracts and purified compounds.

Method A: Seeds of green foxtail (*S. viridis*) were sterilized (Javex-Tween 20-H_2O, 33:0.5:66.5, for 10 min) soaked in a solution of extract (2 mg/ml) or compound (0.5 mg/ml) prepared in sterile H_2O or H_2O–CH_3CH_2OH (90:10) for 30 min, and the coated seeds were placed in Petri plates lined with sterile moistened Whatman filter paper. The plates were sealed and placed in an incubator at 20 °C under darkness. Germination and/or root length was measured after 7 days. Controls (seeds treated with solvent) were used to determine the percentage of inhibition. Seeds that did not germinate were not considered in seedling length

measurements. Three replicate plates containing five seeds per plate were employed in each bioassay.

Method B: Seeds of green (*S. viridis*) were sterilized as earlier, germinated for three days on moist filter paper, and the germinated seeds were soaked in a solution of extract (2 mg/ml) or compound (0.5 mg/mL) prepared in sterile H_2O or H_2O–CH_3CH_2OH (90:10) for 15 min, and the coated seeds were placed in Petri plates lined with sterile moistened Whatman filter paper. The plates were sealed and placed in an incubator at 20 °C under darkness. Growth and/or root length was measured after 7 days. Controls (seeds treated with solvent) were used to determine the percentage of inhibition. Three replicate plates containing five seeds per plate were employed in each bioassay.

Method C: Seeds of green foxtail (*S. viridis*) were sterilized as above; 24-well multidish plates prepared containing agar medium (0.9%, 1.5 ml per well) amended with EtOAc extract or pseudophomins A or B dissolved in DMSO. The assay was conducted in five replicates, one seed per well was placed on agar plates, and plates were sealed and placed in an incubator at 20 °C in darkness. Germination and/or root length was measured after 7 days. Controls (non-amended medium) were used to determine the percentage of inhibition.

3.5. Isolation of pseudophomins A (1) and B (2)

EtOAc extracts (1.4 g) were obtained from culture supernatants (71 l of culture in glucose containing medium). These extracts were combined and divided into five portions, each portion was fractionated through CC using gradient elution with CH_2Cl_2–MeOH (CH_2Cl_2 to CH_2Cl_2–MeOH, 60:40). Fifty five fractions (five columns, 11 fractions per column) were collected, concentrated to dryness under vacuum, and analyzed by TLC and HPLC. Those fractions determined to be similar by TLC and HPLC were combined and were bioassayed for antifungal activity, employing the mycelial radial growth assay. The fractions that showed antifungal activity were combined (124 mg) and were subjected to further fractionation on C_{18} reversed-phase silica gel using a gradient elution with H_2O–MeOH (15:85). Fifteen subfractions were collected, similar fractions were combined and were bioassayed for antifungal activity.

HPLC analysis of the combined antifungal fractions showed two major peaks, with retention times of 16.7 min and 21.7 min, along with some minor peaks. This fraction was separated again on C_{18} reversed-phase silica gel using a gradient elution of H_2O–MeOH (15:85) to yield pseudophomin B (40 mg) (HPLC $r_t = 21.7$ min, white amorphous powder), which was crystallized from aqueous acetonitrile to yield needle-like crystals. The remaining portion (54 mg) was further separated by reversed-phase semi-preparative HPLC (dissolved in H_2O–MeOH, 10:90 at a concentration 10 mg/mL) using mobile phase 0.1% TFA in acetonitrile CH_3CN-H_2O, (65:35) for 35 min to yield pseudophomin A (35 mg, HPLC $r_t = 16.7$ min). Pseudophomin A was crystallized twice from CH_2Cl_2–MeOH (10:90). The purity of A and B was established by HPLC (0.1% TFA in H_2O–CH_3CN, 40:60 to acetonitrile) for 30 min, linear gradient, and a flow rate = 1.0 ml/min).

3.6. Acetylation of pseudophomins A (1) and B (2) and preparation of N-trifluoroacetyl isopropyl esters of leucine

Pseudophomin A or B (1 mg) was dissolved in acetic anhydride and pyridine (1:2, 300 μl) and kept overnight at room temperature. The reaction mixture was diluted with toluene and concentrated under reduced pressure. Degassed 6 M HCl (0.5 ml) was added and the mixture was heated in a sealed tube at 110 °C for 24 h. The reaction mixture was concentrated to dryness, the residue was dissolved in 2-propanol (200 μl) containing 10% AcCl (v/v) in a ReactiVial, and heated at 110 °C for 60 min. The reaction mixture was cooled, concentrated in vacuum, dissolved in CH_2Cl_2 (200 μl) and TFAA (100 μl) and kept at room temperature for 15 h. The N,O-trifluoroacetyl isopropyl ester derivative **6** was concentrated under a slow stream of argon and analyzed by GC–MS as described earlier. Larger scale separations were carried out to obtain sufficient amounts of material for complete characterization using an acetylated fraction (35 mg) containing both **1** and **2**, which was acetylated as described earlier, the reaction mixture concentrated to dryness, allowed to stand in MeOH for 15 min, and separated by CC (CH_2Cl_2–MeOH) to yield the following products: acetylated compounds **3a** (4 mg) and **3b** (3.5 mg), cyclic depsipeptide **4** (1 mg), and compounds **5a** (1.5 mg) and **5b** (1 mg).

Pseudophomin A (**1**): HPLC $r_t = 16.7$ min (system A); $[\alpha]_D^{26}$ −23 (*c* 0.25, EtOH); UV (MeOH) end absorption; IR (KBr) 3326, 2958, 1662, 1535 cm^{-1}; ^1H and ^{13}C NMR (see Table 2); HRMS–FAB 1140.7139 (calc. for $C_{55}H_{98}N_9O_{16}$, 1140.7132, $[M+H]^+$), 857.4997 (calc. for $C_{39}H_{69}N_8O_{13}$, 857.4984), 728.4561 (calc. for $C_{34}H_{62}N_7O_{10}$, 728.4558), 609.3857 (calc. for $C_{31}H_{53}N_4O_8$, 609.3863), 496.3038 (calc. for $C_{25}H_{42}N_3O_7$, 496.3022) 413.2647 (calc. for $C_{21}H_{37}N_2O_6$, 413.2651), 284.2230 (calc. for $C_{16}H_{30}NO_3$, 284.2225).

Pseudophomin B (**2**): HPLC $r_t = 21.7$ min (system A); $[\alpha]_D^{16}$ −15 (*c* 0.40, EtOH); UV (MeOH) end absorption; IR (KBr) 3325, 2958, 1661, 1533 cm^{-1}; ^1H and ^{13}C NMR (see Table 1); HRMS–FAB *m*/*z*: 1168.7456 (calc. for $C_{57}H_{102}N_9O_{16}$, 1168.7444, $[M+H]^+$), 857.4980 (calc. for $C_{39}H_{69}N_8O_{13}$, 857.4984), 728.4562 (calc. for $C_{34}H_{62}N_7O_{10}$, 728.4558), 637.4183 (calc. for $C_{33}H_{57}N_4O_8$, 637.4176), 524.3330 (calc. for $C_{27}H_{46}N_3O_7$, 524.3335), 441.2970 (calc. for $C_{23}H_{41}N_2O_6$, 441.2964), 312.2531 (calc. for $C_{18}H_{34}NO_3$, 312.2538).

Triacetate **3a**: HPLC $r_t = 13.8$ min (system B); ^{1}H NMR ($CDCl_3$) δ: 0.88, 1.01 (*d*, 6H, $J = 6$ Hz), 1.68 (*m*), 1.97 (*s*, 3 H, $CO\underline{CH_3}$)), 2.11 (*s*, 6H, ($CO\underline{CH_3}$)), 2.30 (*m*), 2.52 (*m*), 2.71 (*m*), 3.58 (*dd*, 1H $J = 11$, 5 Hz), 3.68 (*d*, 1H, $J = 5$ Hz), 3.81 (*m*), 4.06 (*dd*, 1H $J = 11$, 5 Hz), 4.17 (*m*), 4.44 (*d*, 2H $J = 6$ Hz), 4.65 (*dd*, 1H $J = 10$, 3 Hz), 6.42 (*d*, 1H, 10 Hz), 6.79 (*d*, 1H, $J = 5$ Hz), 6.99 (*d*, 1H, $J = 5$ Hz), 7.22 (1H), 7.36 (*d*, 2H, $J = 5$ Hz), 7.66 (d, 1H, $J = 9.5$ Hz), 7.70 (*d*, 1H, $J = 4.5$ Hz); MS–FAB *m/z:* 1249 $[M + H]^+$; HRMS–FAB *m/z:* 1248.7320 (calc. for $C_{61}H_{102}N_9O_{18}$ $[M + H]^+$ 1248.7342).

Tri-acetate **3b**: HPLC $r_t = 18.4$ min (system B); ^{1}H NMR ($CDCl_3$) δ: 0.92, 0.96 (*d*, 6H, $J = 6$ Hz), 1.04 (*d*, 6H, $J = 6$ Hz), 1.28 (*br s*, 19 H), 1.74 (*m*), 2.01 (s, 3 H, $CO\underline{CH_3}$), 2.14 (*s*, 6H, ($CO\underline{CH_3}$)), 2.32 (*m*), 2.52 (*m*), 3.58 (*dd*, 1H, $J = 10$, 4 Hz), 4.67 (*dd*, 1H, $J = 10$, 3 Hz), 5.00 (*m*, 1H), 5.06 (*br s*, 1H), 5.16 (*m*, 1H), 5.35 (*m*, 1H), 6.46 (*d*, 1H, $J = 9.5$ Hz), 6.67 (*br s*, 1H), 6.99 (*d*, 1H, $J = 4$ Hz), 7.27 (*s*, 1H), 7.37 (d, 2H, $J = 5.5$ Hz), 7.66 (*d*, 1H, $J = 9.5$ Hz), 7.71 (*d*, 1H, $J = 4$ Hz); ^{13}C NMR ($CDCl_3$) δ: 10.7 (*q*), 12.3 (*q*), 14.5 (*q*), 16.1 (*q*), 16.6 (*q*), 18.8 (*t*), 19.0 (*q*), 21.2 (*q*), 21.3 (*q*), 21.6 (*q*), 21.7 (*q*), 22.2 (*q*), 23.0 (*t*), 23.3 (*q*), 23.5 (*q*), 23.5 (*q*), 23.7 (*t*), 24.7 (*t*), 24.9 (*d*), 25.0 (*d*), 25.2 (*d*), 25.7 (*t*), 25.7 (*t*), 29.6 (*t*), 29.8 (*t*), 30.0 (*t*), 32.5 (*t*), 33.8 (*t*), 35.6 (*t*), 35.7 (*d*), 36.3 (*d*), 38.8 (*t*), 39.9 (*t*), 41.3 (*t*), 44.0 (*t*), 52.6 (*d*), 52.7 (*d*), 54.3 (*d*), 54.5 (*d*), 54.9 (*d*), 57.1 (*d*), 62.3 (*d*), 62.6 (*d*), 63.2 (*d*), 64.2 (*t*), 64.5 (*t*), 68.9 (*d*), 73.2 (*d*), 168.9 (*s*), 169.8 (*s*), 170.3 (*s*), 171.0 (*s*), 171.4 (*s*), 171.6 (*s*), 171.6 (*s*), 172.9 (*s*), 173.2 (*s*), 173.4 (*s*), 174.1 (*s*), 174.6 (*s*), 174.8 (*s*); MS–FAB *m/z*: 1277 $[M + H]^+$; HRMS–FAB *m/z*: 1276.7704 (calc. for $C_{63}H_{106}N_9O_{18}$ $[M + H]^+$ 1276.7655).

Cyclic peptide **4**: HPLC $r_t = 2.3$ min (system B); ^{1}H and ^{13}C NMR (see Table 3); HRMS–FAB *m/z*: 923.5111 (calc. for $C_{43}H_{71}N_8O_{13}$ $[M + H]^+$, 923.5089), 881.4996 (calc. for $C_{41}H_{69}N_8O_{13}$, 881.4984), 839.4883 (calc. for $C_{39}H_{67}N_8O_{12}$, 839.4878).

FA-Leu (**5a**): HPLC $r_t = 3.8$ min (system B); ^{1}H NMR ($CDCl_3$) δ: 0.88, 0.94 (*d*, 6H, $J = 6$ Hz), 1.24 (*br s*), 1.54 (*m*), 1.63 (*m*, 2H), 1.69 (*m*), 2.07 (*s*, 3H), 2.52 (*d*, 2H, $J = 6$ Hz), 3.74?(*s*, 3H), 4.62 (*m*, 1H), 5.14 (*dq*, 1H, $J = 12$, 6 Hz); ^{13}C NMR ($CDCl_3$) δ: 14.4, 21.5, 22.3, 23.0, 23.1, 25.2, 25.6, 29.6, 30.1, 32.1, 34.4, 41.8, 42.0, 51.1, 52.6, 71.8, 170.0, 171.1, 173.8; MS-FAB *m/z:* 358 $[M + H]^+$; HRMS-FAB *m/z:* 358.2587 (calc. for $C_{19}H_{36}NO_5$ 358.2593).

FA-Leu (**5b**): HPLC $r_t = 5.3$ min (system B); ^{1}H–NMR ($CDCl_3$) δ: 0.87, 0.94 (*d*, 6H, $J = 6$ Hz), 1.24 (*br s*), 1.58 (*br s*), 2.03 (*s*, 3H), 2.50 (*d*, 2H, $J = 6$ Hz), 3.72 (*s*, 3H), 4.62 (*m*, 1H), 5.14 (*m*, 1H), 6.07 (*d*, 1H, $J = 8$ Hz); MS–FAB *m/z:* 386 $[M + H]^+$; HRMS–FAB *m/z:* 326.2679 (calc. for $C_{19}H_{36}NO_3$ $[M–CO_2CH_3]^+$ 326.2695).

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