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Antifungal Lipopeptides from *Bacillus amyloliquefaciens* Strain BO7

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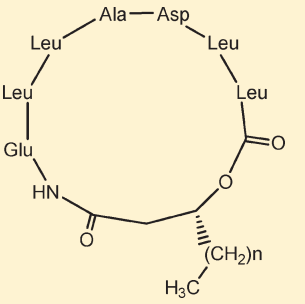
ABSTRACT: Three new lipopeptides (**1**−**3**) were isolated from the organic extract of *Bacillus amyloliquefaciens* strain (BO7). These compounds represented the major constituents (>60%) of the total cell lipids extractable with $CHCl_3$/MeOH (2:1). Elucidation of their chemical structure was carried out by spectroscopic analyses, including 1D and 2D NMR spectroscopy, mass spectrometry (MS), and secondary ion mass spectrometry (MS/MS), along with chemical degradation. The compounds are members of the surfactins family and are based on the heptapeptide Glu-Leu-Leu-Ala-Asp-Leu-Leu, N-acylated to the N-terminal by an (*R*)-3-hydroxy fatty acid with linear alkyl chains from 16:0 to 18:0 (**1**−**3**, respectively). An ester bond between the 3-hydroxyl group of the fatty acid and the carboxylic group of the C-terminal amino acid closes a 13-membered lactone ring. The bacterial lipopeptides, particularly compound **3**, displayed strong and dose-dependent antifungal activity against the plant pathogenic fungus *Fusarium oxysporum*.

1 n=12
2 n=13
3 n=14



Lipopeptides are natural compounds of bacterial origin consisting of a hydrophobic long alkyl chain linked to a hydrophilic polypeptide to form a cyclic or linear structure.[1] The majority are cyclic, generally composed of seven amino acid units and a 3-hydroxy or 3-amino fatty acid.[2] Cyclic lipopeptides have received considerable attention as key compounds in biocontrol[3,4] due to their strong antimicrobial activity, low toxicity, and high biodegradability compared to chemical pesticides.

Microbial biocontrol agents (BCAs) represent a promising alternative to chemical control, with a number of BCA formulations already available for practical use.[5] The genus *Bacillus*, a Gram-positive spore-forming bacteria, includes species considered particularly promising as BCAs against plant pathogens. Several *Bacillus* strains produce biologically active compounds including lipopeptides, with an important role in plant disease control.[6−8] Known lipopeptides from *Bacillus* spp. include iturins, surfactins, and fengycins, differing from each other in amino acid composition and sequence as well as in the structure of the long alkyl chain.

In this work we report the isolation and structural characterization of three new cyclic lipopeptides (**1**−**3**) from the *Bacillus amyloliquefaciens* strain BO7. The stereostructures of the three compounds were elucidated by NMR and MS techniques and chemical methods. The newly characterized lipopeptides exhibited a strong inhibitory activity against *Fusarium oxysporum*, an economically important fungal pathogen affecting a large number of different crop plants, highlighting their potential role in biocontrol activity of *B. amyloliquefaciens*.

RESULTS AND DISCUSSION

The cell-free culture filtrate of *B. amyloliquefaciens* strain BO7 was found to contain approximately 0.0015% w/v of $CHCl_3$/MeOH (2:1) extractable lipopeptides. Purification of these compounds was carried out by reversed-phase HPLC in isocratic mode, affording the new compounds **1** (0.0005% w/v), **2** (0.0003% w/v), and **3** (0.0002% w/v).

Compound **1**, isolated as an amorphous solid in relatively high yield, showed a molecular formula of $C_{52}H_{91}N_7O_{13}$ deduced by high-resolution FAB MS measurements and confirmed by ^{13}C NMR data (Figure 1 and Table 2). Low-resolution FABMS spectra in addition to the protonated pseudo molecular ion peak at *m/z* 1023 $[M + H]^+$ showed intense peaks, due to Na and K adducts, respectively, at *m/z* 1045 $[M + Na]^+$ and *m/z* 1061 $[M + K]^+$. Preliminary ^1H NMR analysis of compound **1** indicated its lipopeptide nature (Figure 1 and Table 1). In agreement with this result, the ^1H NMR spectrum showed N-binding protons (δ 8.60−7.50), α-protons (δ 5.30−4.00), and a long alkyl chain (δ 0.80−1.40). In addition, the ^{13}C NMR spectrum showed diagnostic signals of carboxyl groups (δ 175.5−172.1), which, together with seven nitrogen-bearing carbon signals (δ 50.5−59.8), indicated the presence of seven amide bonds (Figure 1 and Table 2).

To obtain further information on compound **1**, chemical degradation was performed by acid hydrolysis under strong

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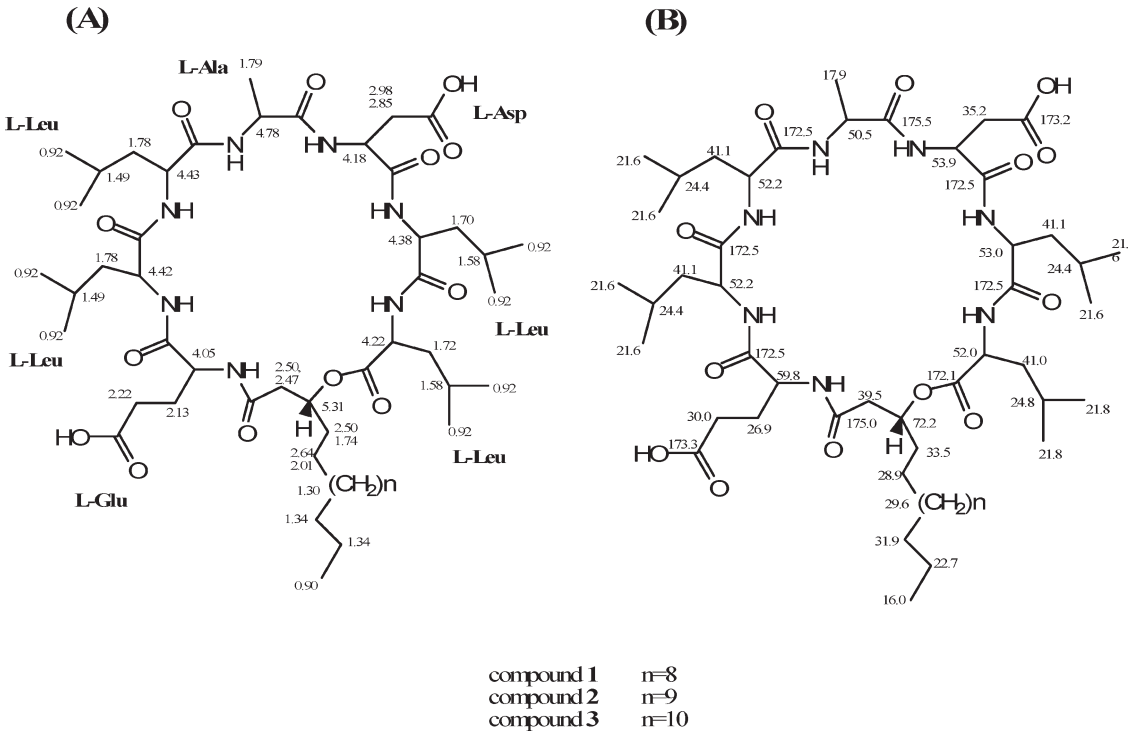

Figure 1. Chemical structure of compounds **1**−**3** purified from *Bacillus amyloliquefaciens* strain BO7. ^{1}H (A) and ^{13}C (B) NMR signals (CD_3OD) reported are those of compound **1**.

conditions. Thus, an aliquot of **1** (1.5 mg) was treated with 0.30 mL of 6 M HCl for 40 h at 110 °C. The reaction mixture was diluted with water, and the lipophilic products were extracted with $CHCl_3$. After permethylation with ethereal diazomethane and further purification, the lipophilic fraction was shown to contain 3-methoxy hexadecanoate, as identified by GC-MS.

The aqueous phase was analyzed by TLC (CH_3CN/EtOAc/CH_3CO_2H/H_2O, 20:6:4:3) and GC-MS (as *N*-trifluoroacetyl derivatives of the corresponding butyl esters) in comparison with the standards, allowing the identification of L-alanine, L-leucine, L-glutamic acid, and L-aspartic acid in the ratio 1:4:1:1. To confirm the stereochemistry of the single amino acids, an aliquot of the water phase from the hydrolysate of **1** was subjected to enzymatic oxidation catalyzed by L- and D-amino acid oxidases,[9] showing reaction with L-enzyme and remaining unchanged with D-enzyme. This procedure allowed us to unequivocally determine the L-series for all amino acids residues.

Further information of the chemical structure of **1** was obtained by tandem MS analysis of the sodium-ionized molecular ion. Taking the ion peak at *m*/*z* 1045 as precursor, obtained by simple cleavage (Figure 2A), the fragments at *m*/*z* 932 and 819 obtained by tandem MS (Figure 2B) showed a first loss of amino acid residues, which indicated the existence of a cyclic structure.[10] In particular, ion peaks at *m*/*z* 932 and 819 were indicative of the consecutive losses of two Leu residues from the C-terminal end, while a further peak at *m*/*z* 704 indicated the loss of Asp (Figure 2B). Figure 3A shows a double hydrogen transfer mechanism in the ESIMS/MS of cyclic lipopeptides.[11] Thus, starting from the molecular ion peak at *m*/*z* 1045, the difference with the ion peak at *m*/*z* 801 indicates the loss of 244 amu (Figure 3B), which is attributed to the dipeptide residue Leu-Leu-H_2O. This result confirmed the presence of two Leu residues at the C-terminal end and indicated that a Leu residue is involved in the ester bond, giving rise to the lactone ring of the cyclopeptide skeleton. Also in this fragmentation pattern, the fragment ion at *m*/*z* 686 was indicative of the consecutive loss of an Asp residue (Figure 2B). Figure 3B shows the set of fragment ions produced from compound **1** after the break of the cyclic structure by a double hydrogen transfer mechanism. Besides peaks at *m*/*z* 267 and 382 indicating the loss of two Leu and an Asp, respectively, diagnostic fragments at *m*/*z* 453 (+71, Ala), 566 (+113, Leu), and 679 (+113, Leu) allowed us to elongate the peptide chain with three further residues, Ala, Leu, and Leu, respectively, from which we deduced that glutamic acid is the N-terminal amino acid.

With these data in hand, we performed a detailed NMR analysis. Inspection of 2D NMR COSY and HOHAHA spectra allowed us to sequence all the proton multiplets in eight spin systems belonging to the seven amino acid residues and to the 3-hydroxy fatty acid residue. Association of all proton signals with those of the directly linked carbons was obtained by analysis of the 2D HSQC spectrum. Finally, study of the $^{2,3}J_{H-C}$ correlation peaks from the 2D HMBC spectrum allowed connecting the spin systems, pointing to a lipopeptide structure composed by seven amino acids with the following sequence starting from the N-terminus: Glu-Leu-Leu-Ala-Asp-Leu-Leu. The terminal N- and C-ends are linked respectively to the acid and alcohol residues of a 3-hydroxy acid with a 13-carbon side chain (3-hydroxyhexadecanoic acid) (**1**, Figure 1). Complete ^{1}H and ^{13}C NMR assignments of compound **1** are reported in Figure 1 and Tables 1 and 2. Collectively, the data obtained allowed unequivocal elucidation of the chemical structure of compound **1** (Figure 1).

Compound **2**, isolated as an amorphous solid, showed a molecular formula of $C_{53}H_{93}N_7O_{13}$ deduced by high-resolution FABMS measurements and confirmed by ^{13}C NMR data

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Table 1. ^{1}H NMR Data of 1−3 at 500 MHz in CD_3OD

	1	2	3
position	δ_H (int., mult., *J* in Hz)	δ_H (int., mult., *J* in Hz)	δ_H (int., mult., *J* in Hz)
Glu	7.58 (1H, d, 2.5, N−H)	7.56 (1H, d, 2.5, N−H)	7.58 (1H, d, 2.5, N−H)
	4.05 (1H, bt, 5.5, isα)	4.05 (1H, bt, 5.5, α)	4.04 (1H, bt, 5.5, α)
	2.13 (2H, m, *β*)	2.11 (2H, m, *β*)	2.13 (2H, m, *β*)
	2.22 (2H, t, *γ*)	2.22 (2H, t, *γ*)	2.21 (2H, t, *γ*)
Leu-2	8.22 (1H, d, 2.5, N−H)	8.20 (1H, d, 2.5, N−H)	8.22 (1H, d, 2.5, N−H)
	4.42 (1H, bt, 6.5, α)	4.42 (1H, bt, 6.5, α)	4.45 (1H, bt, 6.5, α)
	1.78 (2H, dd, 6.5, 7.5, *β*)	1.78 (2H, dd, 6.5, 7.5, *β*)	1.77 (2H, dd, 6.5, 7.5, *β*)
	1.49 (1H, dq, 5.5, 6.0, *γ*)	1.49 (1H, dq, 5.5, 6.0, *γ*)	1.48 (1H, dq, 5.5, 6.0, *γ*)
	0.92 (6H, d, 6.0, *δ*)	0.92 (6H, d, 6.0, *δ*)	0.92 (6H, d, 6.0, *δ*)
Leu-3	8.42 (1H, d, 2.5, N−H)	8.45 (1H, d, 2.5, N−H)	8.42 (1H, d, 2.5, N−H)
	4.43 (1H, bt, 6.5, α)	4.42 (1H, bt, 6.5, α)	4.45 (1H, bt, 6.5, α)
	1.78 (2H, dd, 6.5, 7.5, *β*)	1.78 (2H, dd, 6.5, 7.5, *β*)	1.79 (2H, dd, 6.5, 7.5, *β*)
	1.49 (1H, dq, 5.5, 6.0, *γ*)	1.49 (1H, dq, 5.5, 6.0, *γ*)	1.49 (1H, dq, 5.5, 6.0, *γ*)
	0.92 (6H, d, 6.0, *δ*)	0.92 (6H, d, 6.0, *δ*)	0.92 (6H, d, 6.0, *δ*)
Ala	7.80 (1H, d, 2.5, N−H)	7.80 (1H, d, 2.5, N−H)	7.80 (1H, d, 2.5, N−H)
	4.78 (1H, q, 4.0, α)	4.79 (1H, q, 4.0, α)	4.78 (1H, q, 4.0, α)
	1.79 (3H, d, 4.0, *β*)	1.77 (3H, d, 4.0, *β*)	1.79 (3H, d, 4.0, *β*)
Asp	7.93 (1H, d, 2.5, N−H)	7.96 (1H, d, 2.5, N−H)	7.94 (1H, d, 2.5, N−H)
	4.18 (1H, t, 4.5, α)	4.18 (1H, t, 4.5, α)	4.18 (1H, t, 4.5, α)
	2.98 (1H, dd, 15.0, 4.5, *β*)	2.98 (1H, dd, 15.0, 4.5, *β*)	2.98 (1H, dd, 15.0, 4.5, *β*)
	2.85 (1H, dd, 15.0, 4.5, *β*)	2.85 (1H, dd, 15.0, 4.5, *β*)	2.85 (1H, dd, 15.0, 4.5, *β*)
Leu-6	8.42 (1H, d, 2.5, N−H)	8.42 (1H, d, 2.5, N−H)	8.43 (1H, d, 2.5, N−H)
	4.38 (1H, bt, 6.5, α)	4.40 (1H, bt, 6.5, α)	4.41 (1H, bt, 6.5, α)
	1.70 (2H, dd, 6.5, 7.5, *β*)	1.71 (2H, dd, 6.5, 7.5, *β*)	1.70 (2H, dd, 6.5, 7.5, *β*)
	1.58 (1H, dq, 5.5, 6.0, *γ*)	1.59 (1H, dq, 5.5, 6.0, *γ*)	1.59 (1H, dq, 5.5, 6.0, *γ*)
	0.92 (6H, d, 6.0, *δ*)	0.92 (6H, d, 6.0, *δ*)	0.90 (6H, d, 6.0, *δ*)
Leu-7	8. 50 (1H, d, 2.5, N−H)	8. 50 (1H, d, 2.5, N−H)	8. 50 (1H, d, 2.5, N−H)
	4.22 (1H, bt, 6.5, α)	4.25 (1H, bt, 6.5, α)	4.25 (1H, bt, 6.5, α)
	1.72 (2H, dd, 6.5, 7.5, *β*)	1.74 (2H, dd, 6.5, 7.5, *β*)	1.72 (2H, dd, 6.5, 7.5, *β*)
	1.58 (1H, dq, 5.5, 6.0, *γ*)	1.59 (1H, dq, 5.5, 6.0, *γ*)	1.59 (1H, dq, 5.5, 6.0, *γ*)
	0.92 (6H, d, 6.0, *δ*)	0.92 (6H, d, 6.0, *δ*)	0.92 (6H, d, 6.0, *δ*)
β-OH acid	2.50 (1H, dd, 15.0, 5.0, α)	2.50 (1H, dd, 15.0, 5.0, α)	2.50 (1H, dd, 15.0, 5.0, α)
	2.47 (1H, dd, 15.0, 5.0, α)	2.47 (1H, dd, 15.0, 5.0, α)	2.47 (1H, dd, 15.0, 5.0, α)
	5.31 (1H, m, *β*)	5.28 (1H, m, *β*)	5.30 (1H, m, *β*)
	2.50 (1H, m, *γ*)1.74 (1H, m, *γ*)	2.50 (1H, m, *γ*)1.74 (1H, m, *γ*)	2.50 (1H, m, *γ*)1.74 (1H, m, *γ*)
	2.64 (1H, m, *δ*)2.01 (1H, m, *δ*)	2.65 (1H, m, *δ*)2.01 (1H, m, *δ*)	2.64 (1H, m, *δ*)2.00 (1H, m, *δ*)
	1.30 (20H, m, $\varepsilon-\omega_4$)	1.31 (22H, m, $\varepsilon-\omega_4$)	1.30 (24H, m, $\varepsilon-\omega_4$)
	1.34 (4H, m, ω_2, ω_3)	1.34 (4H, m, ω_2, ω_3)	1.33 (4H, m, ω_2, ω_3)
	0.90 (3H, t, *ω*)	0.90 (3H, t, *ω*)	0.90 (3H, t, *ω*)

(Tables 1 and 2). In addition to the protonated pseudomolecular ion peak at *m/z* 1037 $[M + H]^+$, low-resolution FABMS spectra also showed an intense peak due to the Na and K adduct, at *m/z* 1059 $[M + Na]^+$ and *m/z* 1075 $[M + K]^+$, respectively. Preliminary NMR analysis showed a close similarity between the spectra of compounds **1** and **2**, indicating a lipopeptide nature. Compared to compound **1**, the molecular formula of compound **2** showed the presence of an additional methylene group that could be located either on the alkyl chain or on the peptide chain. Therefore a detailed investigation on the structure of compound **2** was undertaken by running a series on 1D and 2D NMR spectra and MS and MS/MS experiments. Both NMR and MS data pointed to the same amino acid sequence between the two compounds.

Further hydrolysis of compound **2**, followed by GC-MS analysis of the water phase of the hydrolysate, confirmed the same amino acid composition as in compound **1**, suggesting that the additional methylene group must be located on the alkyl chain. Analysis of the $CHCl_3$ phase of the hydrolysate by permethylation and GC-MS analysis identified the lipophilic part of the compound as 3-hydroxyheptadecanoic acid.

Thus, the chemical structure of compound **2** is based on the same heptapeptide chain, Glu-Leu-Leu-Ala-Asp-Leu-Leu, N-acylated to the N-terminal amino acid, Glu, by a 3-hydroxy C_{17} fatty acid with saturated linear chain (17:0) (Figure 1).

Compound **3**, $C_{54}H_{95}N_7O_{13}$ by high-resolution ESIMS, was isolated in lower yield and found to be the analogue of compound

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Table 2. ^{13}C NMR Data of 1−3 at 125 MHz in CD_3OD

position	1 δ_C (mult.)	2 δ_C (mult.)	3 δ_C (mult.)
Glu	172.5 (s, C=O)	172.5 (s, C=O)	172.5 (s, C=O)
	59.8 (d, α)	59.7 (d, α)	59.8 (d, α)
	26.9 (t, β)	26.9 (t, β)	26.8 (t, β)
	30.0 (t, γ)	30.1 (t, γ)	30.0 (t, γ)
	173.3 (s, δ)	173.3 (s, δ)	173.3 (s, δ)
Leu-2	172.5 (s, C=O)	172.5 (s, C=O)	172.5 (s, C=O)
	52.2 (d, α)	52.2 (d, α)	52.2 (d, α)
	41.4 (t, β)	41.4 (t, β)	41.4 (t, β)
	24.4 (t, γ)	24.4 (t, γ)	24.4 (t, γ)
	21.6 (q, δ)	21.6 (q, δ)	21.6 (q, δ)
Leu-2	172.5 (s, C=O)	172.5 (s, C=O)	172.5 (s, C=O)
	52.2 (d, α)	52.2 (d, α)	52.2 (d, α)
	41.4 (t, β)	41.4 (t, β)	41.4 (t, β)
	24.4 (t, γ)	24.5 (t, γ)	24.6 (t, γ)
	21.6 (q, δ)	21.6 (q, δ)	21.6 (q, δ)
Ala	175.5 (s, C=O)	175.5 (s, C=O)	175.5 (s, C=O)
	50.5 (d, α)	50.5 (d, α)	50.5 (d, α)
	17.9 (q, β)	17.8 (q, β)	17.9 (q, β)
Asp	172.5 (s, C=O)	172.5 (s, C=O)	172.5 (s, C=O)
	53.9 (d, α)	53.9 (d, α)	53.9 (d, α)
	35.2 (t, β)	35.5 (t, β)	35.3 (t, β)
	173.2 (s, γ)	173.3 (s, γ)	173.3 (s, γ)
Leu-6	172.5 (s, C=O)	172.5 (s, C=O)	172.5 (s, C=O)
	53.0 (d, α)	53.0 (d, α)	53.0 (d, α)
	41.1 (t, β)	41.4 (t, β)	41.4 (t, β)
	24.4 (t, γ)	24.3 (t, γ)	24.4 (t, γ)
	21.6 (q, δ)	21.6 (q, δ)	21.5 (q, δ)
Leu-7	172.1 (s, C=O)	172.1 (s, C=O)	172.1 (s, C=O)
	52.0 (d, α)	52.0 (d, α)	52.0 (d, α)
	41.0 (t, β)	41.0 (t, β)	41.0 (t, β)
	24.8 (t, γ)	24.8 (t, γ)	24.8 (t, γ)
	21.8 (q, δ)	21.8 (q, δ)	21.8 (q, δ)
β−OH acid	175.0 (s, C=O)	175.0 (s, C=O)	175.0 (s, C=O)
	39.5 (t, α)	39.5 (t, α)	39.5 (t, α)
	72.2 (d, β)	72.2 (d, β)	72.2 (d, β)
	33.5 (t, γ)	33.4 (t, γ)	33.5 (t, γ)
	28.9 (t, δ)	28.9 (t, δ)	28.8 (t, δ)
	29.6 (t, $\varepsilon-\omega_4$)	29.6 (t, $\varepsilon-\omega_4$)	29.6 (t, $\varepsilon-\omega_4$)
	31.9 (t, ω_3)	31.9 (t, ω_3)	31.9 (t, ω_3)
	22.7 (t, ω_2)	22.7 (t, ω_2)	22.7 (t, ω_2)
	16.0 (q, ω)	16.0 (q, ω)	16.0 (q, ω)

2 with an additional methylene group. This was first suggested by MS data, since the pseudomolecular ion peak of compound **3** was 28 and 14 amu higher when compared to compounds **1** and **2**, respectively. Moreover, 1D and 2D NMR spectra of the three isolated compounds revealed a close similarity with almost superimposable spectra. Further MS/MS analysis of compound **3** indicated the same fragmentation pattern as in **1** and **2**, pointing to the same heptapeptide chain and indicating that the difference must be located on the alkyl chain. Chemical degradation of compound **3** confirmed that its chemical structure is based on the same cyclic peptide skeleton, with the only difference in the 3-hydroxy C_{18} fatty acid with saturated linear chain (18:0). Complete NMR assignments of compound **3** are reported in Tables 1 and 2.

The configuration at C-3 of fatty acids of **1**−**3** was determined on the 3-hydroxy fatty acids **4**−**6** (acid hydrolysis of **1**−**3**) after derivatization with chiral (α-methoxy-α-trifluoromethyl) phenylacetic acid (MTPA, Mosher's reagent)[12,13] with *S*- and *R*-configuration (Scheme 1), affording the (*S*)- and (*R*)-MTPA esters (**4a**/**4b**−**6a**/**6b**), respectively. Analysis of the $\Delta\delta$ ($S - R$) values according to the Mosher model (Scheme 1) for each couple of diastereomeric MTPA esters (**4a**/**4b**−**6a**/**6b**) pointed to an *R*-configuration at C-3 of compounds **4**−**6**.

Previous studies revealed a broad antimicrobial activity spectrum of LPs purified from *Bacillus* species such as *B. subtilis*[7] and *B. thuringiensis*.[14] Antifungal strains of *B. amyloliquefaciens* were shown to produce iturins,[15,16] although no surfactins have been reported from this species. We therefore tested the inhibitory activity of the purified LPs from *B. amyloliquefaciens* BO7 against the fungal pathogen *F. oxysporum* f.sp. *lycopersici* (*Fol*). LPs **1**, **2**, and **3** showed significant inhibitory activity at 100 ppm, with mycelial growth reductions of 29%, 11%, and 56%, respectively, while only compound **3** maintained a detectable activity (25%) at 50 ppm. Interestingly, double or triple combinations of LPs resulted in an increase in antifungal activity, with mixtures **1**+**3**, **2**+**3**, and **1**+**2**+**3** at a total concentration of 50 ppm producing inhibition values of 31%, 54%, and 60%, respectively. By contrast, a commercial bacterial surfactin used as a control had no inhibitory effect and even stimulated the fungal growth.

Bacterial surfactins are mainly known for their antiviral and antibacterial activities as well as their surfactant properties.[4] The antifungal activity displayed by the isolated lipopeptides represents the structural difference from previously known LPs of the surfactin family, related to the substitution of a valine residue with an alanine.[4] Thus, the three isolated LPs showed a common core based on the heptapeptide Glu-Leu-Leu-Ala-Asp-Leu-Leu, but differed from each other in the length of the side alkyl chain. Compound **3** was the most active, with an EC_{50} of 88.46 μg/mL, which is comparable to that of a surfactin purified from *B. subtilis* (EC_{50} 99.98 μg/mL) displaying strong inhibitory activity against *Penicillium digitatum*.[17] Interestingly, **3** has the longest alkyl chain, making it less polar than **1** and **2**. Although the mechanism of the antifungal action is unknown, we speculate that this structural characteristic could result in a higher affinity for the fungal cell membrane, thus allowing a better penetration into the cell and increasing its inhibitory activity.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and 10 cm microcell. FABMS (recorded in a glycerol matrix) were measured on a Prospec Fisons mass spectrometer. ESIMS experiments were performed on an Applied Biosystem API 2000 triple-quadrupole mass spectrometer. The spectra were recorded by infusion into the ESI source using MeOH as solvent. ESIMS/MS spectra were recorded on a API 2000 instrument. GC-MS analysis was performed on a Carlo Erba instrument using a HP-5 capillary column packed with 5% polyphenyl siloxane, 30 m × 0.25 mm i.d. (Agilent, USA). ^1H and ^{13}C NMR spectra were recorded on a Varian Unity Inova spectrometer at 500.13 and 125.77 MHz, respectively. Chemical shifts were referred to the residual solvent signal (CD_3OD: δ_H 3.31, δ_C 49.0).[18] The multiplicities of ^{13}C NMR resonances were determined by DEPT experiments. ^1H connectivities were determined by using COSY and HOHAHA

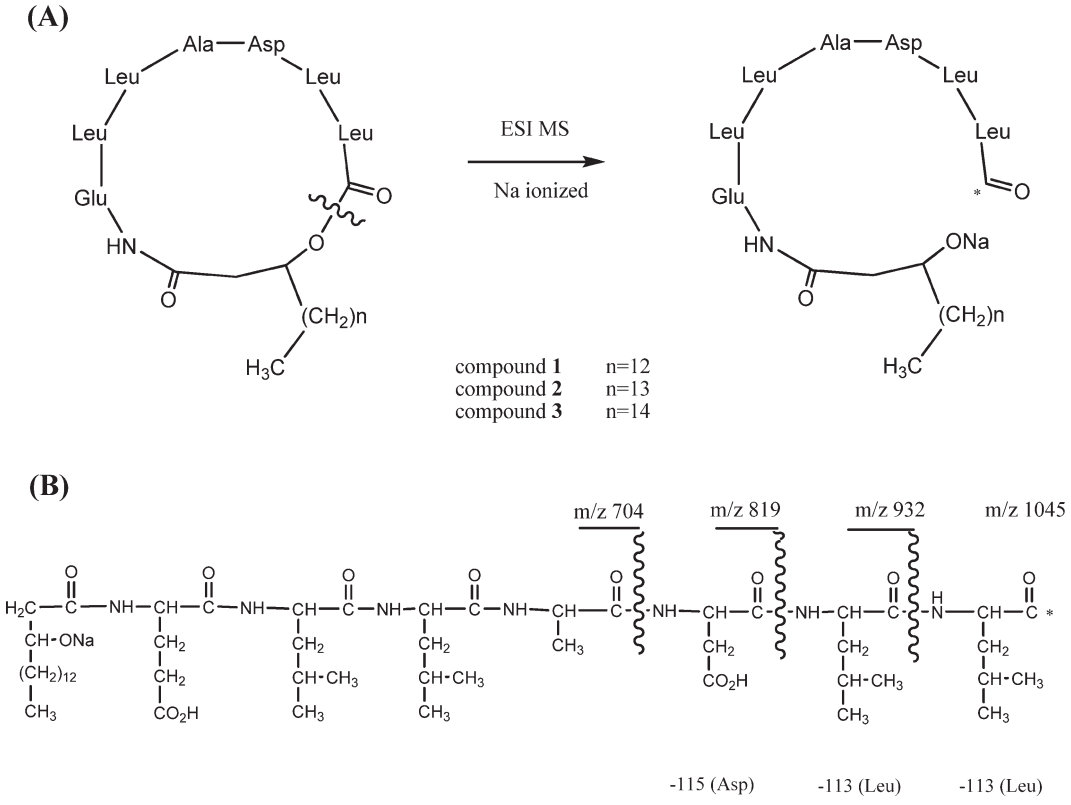

Figure 2. (A) Simple cleavage of lipopeptides in ESIMS. (B) Set of fragmentation ions obtained in the ESIMS of compound **1**.

experiments; the 2D HOHAHA experiments were performed in the phase-sensitive mode (TPPI) using the MLEV-17 (mixing time 125 ms) sequence for mixing. One-bond heteronuclear $^{1}H-^{13}C$ connectivities were determined with 2D HSQC pulse sequence with an interpulse delay set for $^{1}J_{CH}$ of 130 Hz. Two- and three-bond heteronuclear $^{1}H-^{13}C$ connectivities were determined with 2D HMBC experiments, optimized for $^{2-3}J_{CH}$ of 8 Hz. HPLC in isocratic mode was performed on a Varian 940-LC apparatus equipped with a refractive index detector by using a a μ-Bondapack C_{18} column, 7.8 mm × 300 mm, i.d. (Merck, USA). TLC on SiO_2 with $BuOH/H_2O/CH_3CO_2H$, 60:25:15 (BAW), for development was used. Spots were visualized with cerium sulfate in 2 N H_2SO_4.

Microorganisms and Growth Conditions. *Bacillus amyloliquefaciens* strain BO7 was isolated from olive orchard soil using the methodology described by Boulter et al.[19] and characterized by routine bacteriological tests (Biolog and API test) and by sequencing of the small 16S ribosomal subunit (CBS: Centraalbureau voor Schimmelcultures, Netherland). Strain BO7 was routinely cultivated in minimum salt liquid medium (MSLM: K_2HPO_4 2.5 g/L; KH_2PO4 2.5 g/L; $(NH_4)_2HPO_4$ 2.5 g/L; $MgSO_4 \times 7H_2O$ 0.2 g/L; $FeSO_4 \times 7H_2O$ 0.01 g/L; $MnSO_4 \times 7H_2O$ 0.007 g/L; sucrose 10 g/L; pH 7.5) at 28 °C and 120 rpm. Crude culture filtrate was obtained by centrifugation at 14 000 rpm for 5 min and sterilized by filtration (0.22 μm pore size). Bacterial cells were resuspended in sterile PBS pH 7.4,[20] and cell concentration was adjusted spectrophotometrically (610 nm). Bacterial strains were stored at −80 °C with 30% glycerol. *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) wild-type strain 4287 (FGSC 9935), isolated from infected tomato plants,[21] was used in the antifungal activity assays. *Fol* was cultured on solid potato dextrose agar (PDA, Liofilchem S.p.a., Italy) or in submerged culture at 150 rpm in potato dextrose broth (PDB, prepared from fresh potatoes) for 4 days at 28 °C on a rotary shaker (200 rpm). Conidial suspensions for antifungal assays were prepared as reported.[22]

Extraction and Purification of Lipopeptides 1−3. The lipopeptide fraction was separated from an aliquot (1 L) of cell-free supernatant by adjusting the pH to 2.0 using 6 N HCl and incubating overnight at 4 °C. The pellet was recovered by centrifugation for 20 min at 10 000 rpm and then extracted using a $CHCl_3$/MeOH mixture (2:1, v/v) and passed through a polytetrafluoroethylene (PTFE) filter. The extract was concentrated under vacuum by obtaining a crude lipid mixture. The final step in the purification employed high-performance liquid chromatography (HPLC) in isocratic mode on a Varian 940-LC apparatus equipped with refractive index detector. The lipid mixture was chromatographed on a C_{18} reversed-phase column (Waters μBondapak C18 3.9 × 300 mm) eluted with 0.1% of trifluoroacetic acid (TFA) in CH_3CN/H_2O (8:2, v/v), obtaining pure compound **1** (4.7 mg, t_R = 30.0 min), **2** (3.2 mg, t_R = 30.0 min), and **3** (2.0 mg, t_R = 30.0 min).

Compound ***1***. Yield: 4.7 mg; colorless, amorphous solid; $[\alpha]^{25}_{D}$ −30.7 (*c* 0.1 MeOH); IR (KBr) ν_{max} 3410, 2930, 1150, 1045 cm^{-1}; ^{1}H NMR data, see Figure 1 and Table 1; ^{13}C NMR data, see Figure 1 and Table 2; FABMS *m/z* 1023 $[M + H]^+$, *m/z* 1045 $[M + Na]^+$, 1061 $[M + K]^+$; ESIMS/MS see Figures 2 and 3.

Compound ***2***. Yield: 3.2 mg; colorless, amorphous solid; $[\alpha]^{25}_{D}$ −31.8 (*c* 0.1 MeOH); IR (KBr) ν_{max} 3413, 2928, 1151, 1045 cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; FABMS *m/z* 1037 $[M + H]^+$, *m/z* 1059 $[M + Na]^+$, 1075 $[M + K]^+$; ESIMS/MS *m/z* 1059, 946, 933, 815, 718, 700, 693, 580, 467, 396, 281.

Compound ***3***. Yield: 2.0 mg; colorless, amorphous solid; $[\alpha]^{25}_{D}$ −32.5 (*c* 0.1 MeOH); IR (KBr) ν_{max} 3411, 2931, 1150, 1044 cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; FABMS *m/z* 1051 $[M + H]^+$, *m/z* 1073 $[M + Na]^+$, 1089 $[M + K]^+$; ESIMS/MS *m/z* 1073, 960, 947, 829, 732, 714, 707, 594, 481, 410, 295.

Hydrolysis of Compounds 1−3. A 1.5 mg amount of compounds was dissolved in 6 M HCl (0.30 mL) and stirred at 110 °C for 40 h. After cooling, the solution was diluted with water, and the lipophilic products were extracted with chloroform. Both phases were concentrated

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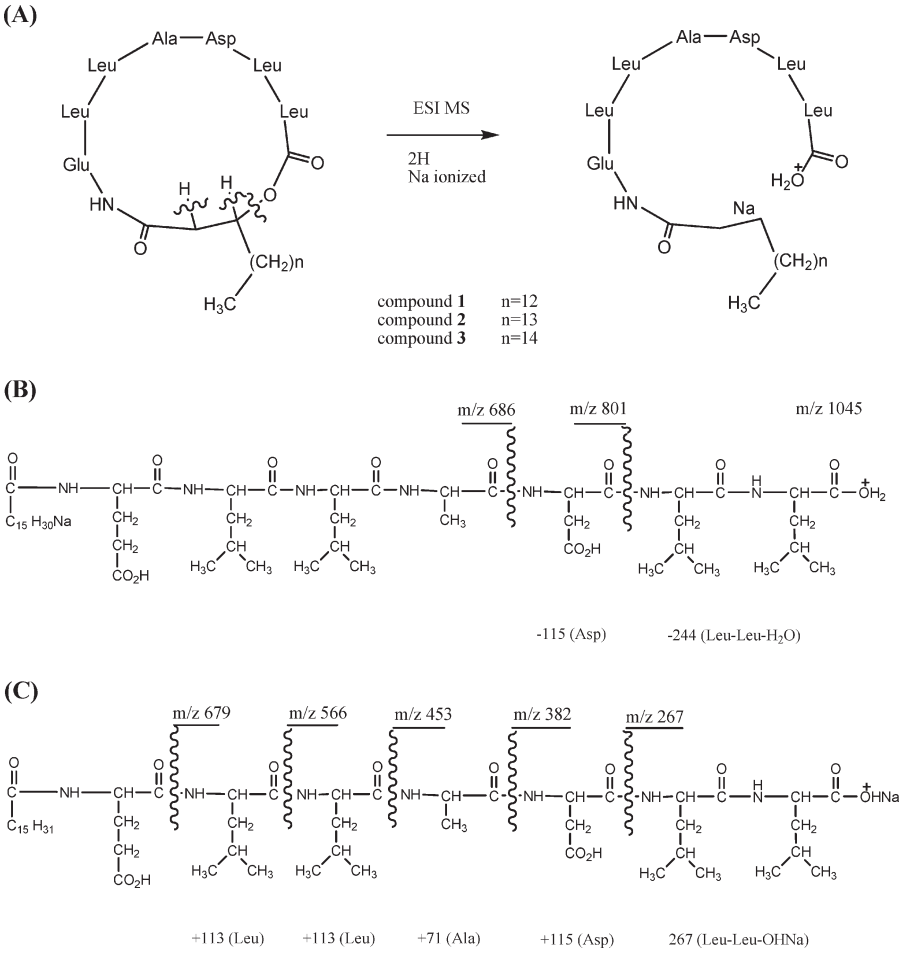
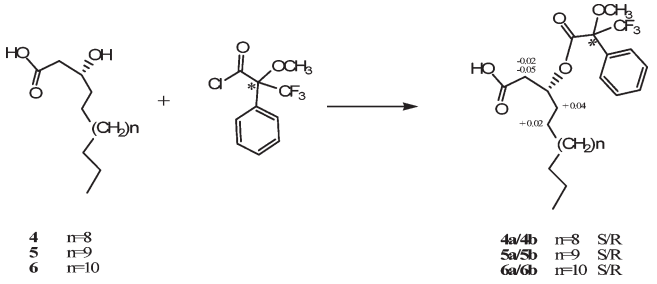


Figure 3. (A) Cleavage of lipopeptides in ESIMS according to the double hydrogen transfer mechanism of aliphatic esters. (B) Molecular and fragmentation ion peaks obtained with this cleavage are 18 units higher compared to the corresponding peaks after simple cleavage. (C) Set of fragmentation ions obtained in the ESIMS of compound **1**.

Scheme 1. (*S*)- and (*R*)-MTPA esters (4a/4b−6a/6b) of 3-Hydroxy Fatty Acids 4−6 Obtained by Acid Hydrolysis of 1−3[a]



[a] $\Delta\delta$ ($S - R$) values (in ppm) reported are those of **4a**/**4b**. Analogous values have been found for **5a**/**5b** and **6a**/**6b**.

under a stream of N_2 and analyzed separately. The lipophilic fraction, containing 3-hydroxy fatty acids **4**−**6** (Scheme 1), respectively, was methylated with ethereal diazomethane and chromatographed with a silica gel column, eluting with a gradient solvent system from hexane with increasing amount of ethyl acetate (from 20% to 70%). The fraction eluted with 50% was shown to contain a single compound identified by GC-MS and NMR (for **1**: methyl 3-methoxy hexadecanoate; for **2**: methyl 3-methoxy eptadecanoate; for **3**: methyl 3-methoxy octadecanoate). The water-phase residue was analyzed by TLC (solvent system: $CH_3CN/EtOAc/CH_3CO_2H/H_2O$, 20:6:4:3) and GC-MS as *N*-trifluoroacetyl derivatives of the corresponding butyl esters following the procedure developed by Gelpi et al.[23] A mixture of L-alanine, L-leucine, L-glutamic acid, and L-aspartic acid taken in an equimolecular ratio served as a standard. By this procedure we identified L-alanine, L-leucine, L-glutamic acid, and L-aspartic acid in the ratio 1:4:1:1. To confirm the stereochemistry of single amino acids, an aliquot of the water phase from the hydrolysate of **1** was subjected to enzymatic oxidation catalized by L- and D-amino acid oxidases (Sigma Aldrich, USA) following the procedure described by Debono et al.[9] showing reaction with the former enzyme and remaining unchanged with the latter enzyme. By this procedure, the amino acids were identified to belong to the L-series.

Synthesis of the (*S*)- and (*R*)-MTPA Esters of 3-Hydroxy Acids 4−6. Two aliquots (1 mg) of each 3-hydroxy fatty acid (**4**−**6**, Scheme 1), obtained by acid hydrolysis (6 M HCl) of compounds **1**−**3**, were dissolved in dry pyridine and allowed to react overnight with (*S*)- and (*R*)-MTPA chloride, affording the (*S*)- and (*R*)-MTPA esters (**4a**/**4b**−**6a**/**6b**, Scheme 1), respectively. Analysis of the $\Delta\delta$ ($S - R$) values obtained by ^{1}H NMR spectrum for each couple of diastereomeric MTPA esters (**4a**/**4b**−**6a**/**6b**), according to the Mosher model (Scheme 1) pointed to an *R*-configuration at C-3.

Bioassays with Purified Lipopeptides. The three purified LPs (1, 2, 3) were dissolved in ethanol, alone or in mixture, and added to the

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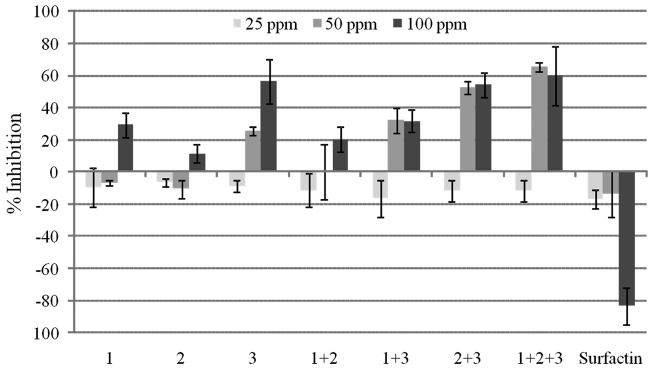

Figure 4. Antifungal activity of the purified lipopeptide compounds from *Bacillus amyloliquefaciens* strain BO7 against *Fusarium oxysporum*. Each lipopeptide, either alone or in combination, was tested at three final concentrations (100, 50, and 25 ppm), using a bioassay in microtiter plates. Fungal growth was measured spectrophotometrically at 595 nm after 72 h incubation, and inhibitory activity (IA) was calculated as the percentage of fungal biomass reduction compared to the control (EtOH). A commercial surfactin (S3523, Sigma) was included in the analysis as control. Bars indicate the standard deviation from 4 replicates.

medium at a final concentration of 25, 50, and 100 ppm. In mixed applications, each compound was used at equimolecular ratio to produce a final additive concentration of 25, 50, or 100 ppm. The solvent ethanol was used as a negative control, while a commercial bacterial surfactin (S3523, Sigma) was used as a reference compound of the surfactin family. Bioassays were conducted in a 96-well plate against microconidia of *Fol*. Samples contained a mixture of purified LPs (or ethanol as a control) mixed with PDB and 10^5 *Fol* microconidia mL^{-1}. Plates were incubated at 28 °C on a rotary shaker (200 rpm) for different times, and fungal growth was determined spectrophotometrically by measuring absorption at 595 nm, using a microplate reader (model 550, Bio-Rad). Data from bioassays were submitted to variance analysis (ANOVA) using the SPSS software (version 16.0 for Windows, SPSS Inc. Chicago, IL), and means were compared by Tukey's test. The effective lipopeptide concentration required to obtain 50% growth inhibition of *Fol* (EC_{50}) was calculated by regressing inhibition of fungal growth against the logarithm of LP concentration.

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