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Antifungal Cyclic Lipopeptides from *Bacillus amyloliquefaciens* Strain BO5A

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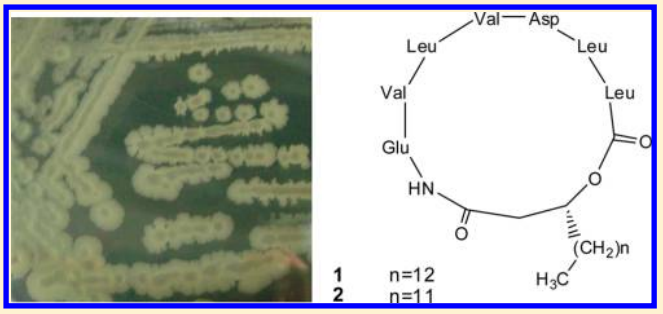
Supporting Information

ABSTRACT: A bioassay-guided fractionation of *Bacillus amyloliquefaciens* strain BO5A afforded the isolation of two new cyclic lipopeptides (**1** and **2**) as the major lipid constituents (>60%) of the $CHCl_3$–MeOH (2:1) extract. The chemical structures of the isolated metabolites were elucidated by spectroscopic methods, including 1D and 2D NMR spectroscopy, mass spectrometry (MS), secondary ion mass spectrometry (MS1, MS2), and chemical degradation. The compounds are members of the surfactins family and are based on a heptapeptide chain composed by Glu-Val-Leu-Val-Asp-Leu-Leu. Its N-terminal end is N-acylated by an (*R*)-3-hydroxy fatty acid with linear alkyl chains of 16:0 and 15:0 (**1** and **2**, respectively). The 3-hydroxyl group closes a 25-membered lactone ring with the carboxylic group of the C-terminal amino acid. The isolated compounds were tested for their inhibitory activity against the four pathogenic fungi *Fusarium oxysporum*, *Aspergillus niger*, *Botrytis cinerea*, and *Penicillium italicum* and the biocontrol fungus *Trichoderma harzianum*. Compound **2** displayed activity against all tested pathogens.



Cyclic lipopeptides are a class of bacterial secondary metabolites based on a polypeptide chain, generally composed of seven amino acids and a 3-hydroxy or 3-amino fatty acid,[1] connected in two places, giving rise to a macrocyclic structure. The N-terminal end of the polypeptide is involved in one amide bond with the carboxylic group of the fatty acid, while the 3-hydroxy or 3-amino group of the fatty acid gives rise to an ester or amide bond, respectively, with the C-terminal amino acid.[2]

Cyclic lipopeptides have received increasing attention as key compounds in biocontrol of plant disease[3,4] due to their strong antimicrobial activity, low toxicity, and low stability, i.e., easily degradable under natural conditions, compared to other chemical pesticides. In this context, many members of the *Bacillus* genus have been studied chemically because they produce lipopeptides that inhibit plant pathogens as well as having a key role in stimulating the host defense mechanism.[5−9] Known lipopeptides from *Bacillus* spp. include basic structures—named iturins, surfactins, and fengycins—differing in the amino acid composition and sequence and in the structure of the alkyl chain.

In the course of characterizing bioactive compounds from *Bacillus* spp., we recently isolated three cyclic lipopeptides from *Bacillus amyloliquefaciens* strain BO7 that showed strong and dose-dependent activity against *Fusarium oxysporum*.[10,11] We have now extended our analysis to strain BO5A of *B. amyloliquefaciens,* isolating and characterizing two new lipopeptides (**1** and **2**), whose stereostructures have been elucidated by NMR, MS techniques, and chemical methods. The isolated compounds were subjected to antifungal tests against the four pathogenic fungi *F. oxysporum*, *Aspergillus niger*, *Botrytis cinerea*, and *Penicillium italicum* and the biocontrol fungus *Trichoderma harzianum*.

RESULTS AND DISCUSSION

The cell-free culture filtrate of *B. amyloliquefaciens* strain BO5A 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 and isocratic elution, affording the new compounds **1** (8.4 mg/L) and **2** (5.8 mg/L). The purity of the isolated compounds, eluted as single peaks, was determined by ^{1}H NMR spectra (Figures S1 and S7).

Compound **1**, isolated as an amorphous solid as the major component, showed a molecular formula of $C_{53}H_{93}N_7O_{13}$, deduced by high-resolution FAB-MS measurements and consistent with ^{13}C NMR data (Figure 1 and Table 1). Low-resolution positive ion ESI-MS spectra (Figure S2) gave the pseudomolecular ion peak at *m*/*z* 1037 $[M + H]^+$ and, in addition, showed correlated peaks of Na and K adducts at *m*/*z* 1059 $[M + Na]^+$ and 1075 $[M + K]^+$, respectively. Preliminary ^{1}H NMR analysis of compound **1** indicated its lipopeptide nature (Figure 1 and Table 1), thus showing aminic protons (δ 8.60−7.50), α-amino acid protons (δ 5.30−4.00), and a long alkyl chain (δ 0.80−1.40). In addition, the ^{13}C NMR spectrum contained diagnostic signals of carboxyl groups (δ 175.5−172.1)

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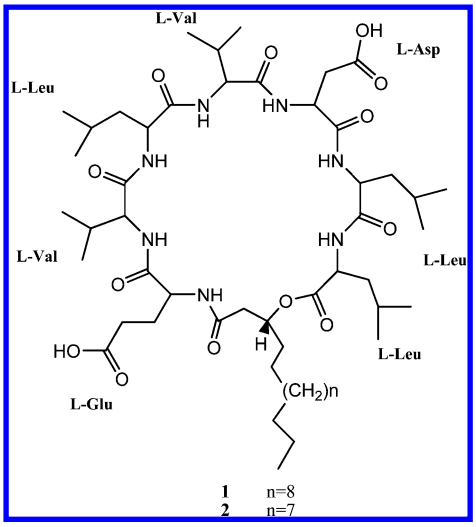

Figure 1. Structure of compounds **1** and **2** purified from *Bacillus subtilis* strain BO5A.

that, together with seven nitrogen-bearing carbon signals (δ 50.5−59.8), indicated the presence of seven amide bonds (Figure 1 and Table 1).

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 (see Experimental Section), 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), allowing the identification of valine, leucine, and glutamic and aspartic acids and the estimation of their ratios (2:3:1:1, respectively), according to peak areas. To confirm the stereochemistry of single amino acids, an aliquot of the aqueous phase from the hydrolysate of **1** was subjected to enzymatic oxidation by L- and D-amino acid oxidases,[12] showing reaction with the L-enzyme and remaining unchanged with the D-enzyme. This procedure allowed us to unequivocally determine the L-series for all amino acid residues.

Further information of the chemical structure of **1** was obtained by tandem ESI-MS analysis of the Na-ionized molecular ion (Figure S3). Taking the MS1 peak at *m/z* 1059 $[M + Na]^+$ as precursor, obtained by simple cleavage (Figure 2A), the MS2 fragments at *m/z* 946 (−113) and 833 (−113) obtained by ESI-MS1 and -MS2 (Figure 2B) showed the loss of amino acid residues after the cleavage of the ester bond (Figure 2A), thus indicating the existence of a cyclic structure.[13] The fragment at 113 amu corresponds to either leucine or iso-leucine, but according to the NMR data and the chemical degradation study must be leucine. In particular, these peaks were indicative of the consecutive losses of two Leu

Table 1. ^{1}H and ^{13}C NMR Data of 1 and 2 at 500 and 100 MHz, Respectively, in CD_3OD

	1		2	
position	δ_C mult.	δ_H mult. (*J* in Hz)	δ_C mult.	δ_H mult. (*J* in Hz)
Glu-1				
NH		7.57 d (2.5)		7.57 d (2.5)
C=O	172.4 s		172.4 s	
α	59.7 d	4.04 bt (5.5)	59.7 d	4.03 bt (5.5)
β	26.9 t	2.11 m	26.9 t	2.11 m
γ	30.1 t	2.22 t	30.1 t	2.21 t
δ	173.3 s		173.3 s	
Val-2				
NH		8.20 d (2.5)		8.20 d (2.5)
C=O	172.7 s		172.7 s	
α	52.2 d	4.42 bd (6.0)	52.2 d	4.43 bd (6.0)
β	24.5 t	1.47 dq (6.0)	24.4 t	1.46 dq (6.0)
γ	21.5 q	0.93 d (6.0)	21.6 q	0.93 d (6.0)
Leu-3				
NH		8. 40 d (2.5)		8. 40 d (2.5)
C=O	172.6 s		172.6 s	
α	52.1 d	4.43 bt (6.5)	52.2 d	4.44 bt (6.5)
β	41.4 t	1.78 dd (6.5, 7.5)	41.4 t	1.78 dd (6.5, 7.5)
γ	24.4 t	1.49 dq (5.5, 6.0)	24.5 t	1.49 dq (5.5, 6.0)
δ	21.3 q	0.92 d (6.0)	21.6 q	0.92 d (6.0)
Val-4				
NH		7.91 d (2.5)		7.91 d (2.5)
C=O	175.5 s		175.5 s	
α	50.5 d	4.46 bd (6.0)	50.5 d	4.47 bd (6.0)
β	24.4 t	1.49 dq (6.0)	24.4 t	1.49 dq (6.0)
γ	17.9 q	0.90 (d (4.0)	17.8 q	0.89 d (4.0)
Asp-5				
N−H		7.93 d (2.5)		7.93 d (2.5)
C=O	172.3 s		172.3 s	
Asp-5				
α	53.9 d	4.18 t (4.5)	53.9 d	4.18 t (4.5)
β	35.2 t	2.98, 2.85 dd (15.0, 4.5)	35.5 t	2.98, 2.85 dd (15.0, 4.5)
γ	173.2 s		173.3 s	
Leu-6				
N−H		8. 43 d (2.5)		8.43 d (2.5)
C=O	172.0 s		172.0 s	
α	53.0 d	4.38 bt (6.5)	53.0 d	4.39 bt (6.5)
β	41.1 t	1.70 dd (6.5, 7.5)	41.4 t	1.70 dd (6.5, 7.5)
γ	24.4 t	1.59 dq (5.5, 6.0)	24.3 t	1.59 dq (5.5, 6.0)
δ	21.6 q	0.91 d (6.0)	21.5 q	0.90 d (6.0)
Leu-7				
N−H		8. 51 d (2.5)		8. 50 d (2.5)
C=O	172.1 s		172.1 s	
α	52.0 d	4.24 bt (6.5)	52.0 d	4.25 bt (6.5)
β	41.0 t	1.71 dd (6.5, 7.5)	41.1 t	1.72 dd (6.5, 7.5)
γ	24.8 t	1.59 dq (5.5, 6.0)	24.9 t	1.59 dq (5.5, 6.0)
δ	21.8 q	0.92 d (6.0)	21.8 q	0.92 d (6.0)
β-OH acid				
CO_2H	175.0 s		175.0 s	
α	39.5 t	2.52, 2.46 dd (15.0, 5.0)	39.5 t	2.50, 2.47 dd (15.0, 5.0)
β	72.2 d	5.30 m	72.2 d	5.30 m
γ	33.5 t	2.50, 1.75 m	33.4 t	2.50, 1.74 m
δ	28.9 t	2.63, 2.01 m	28.9 t	2.64, 2.00 m
ε−ω_4	29.6 t	1.30 m	29.6 t	1.30 m
ω_2	31.9 t	1.34 m[a]	31.9 t	1.33 m[a]
ω_2	22.7 t	1.34 m[a]	22.7 t	1.33 m[a]
ω	16.0 q	0.92 t	16.0 q	0.92 t

[a]Overlapped with other signals.

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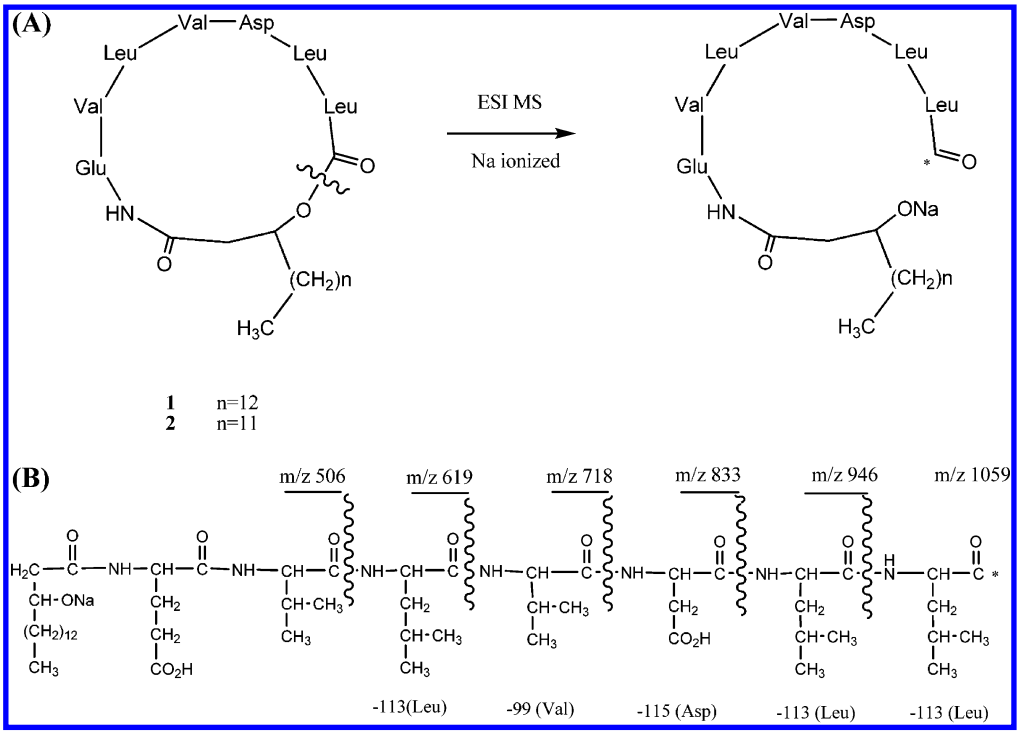

Figure 2. (A) Simple cleavage of **1** and **2** in ESI-MS. (B) Set of fragmentation ions obtained in tandem ESI-MS of compound **1**.

residues from the C-terminal end, while further peaks at *m/z* 718 (−115), 619 (−99), and 506 (−113) indicated the consecutive losses of Asp, Val, and Leu, thus showing the peptide chain sequence (Figure 2B). Figure 3A shows a double hydrogen transfer mechanism in the ESI-MS1, -MS2 of cyclic lipopeptides.[14] Starting from the molecular ion peak at *m/z* 1059 (Figure 3B), the difference from the ion peak at *m/z* 815 (−244) was attributed to the loss of the dipeptide residue Leu-Leu-H_2O. This confirmed that a Leu residue at the C-terminal end is involved in the ester bond with the 3-hydroxyl group, affording the typical 25-membered lactone ring of the cyclopeptide skeleton. Figure 3B shows the set of fragment ions produced from compound **1** after cleaving the cyclic structure by a double hydrogen transfer mechanism that confirmed the amino acid sequence found after a simple cleavage mechanism (Figure 2A). Thus, the remaining glutamic acid residue had to be located at the N-terminal end.

With these data in hand, we performed a detailed NMR analysis, running 1D and 2D COSY (Figure S4) and HOHAHA spectra, that 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 (Figure S5). Finally, study of the $^{2,3}J_{H-C}$ correlation peaks from the 2D HMBC spectrum (Figure S6) allowed the spin systems to be connected, pointing to a lipopeptide structure composed of seven amino acids with the following sequence starting from the N-terminal end: Glu-Val-Leu-Val-Asp-Leu-Leu. The N- and C-ends are attached to the acidic and alcoholic functional groups, respectively, of a 16-carbon 3-hydroxy acid (3-hydroxyhexadecanoic acid) (**1**, Figure 1). ^{1}H and ^{13}C NMR assignments of compound **1** are reported in Table 1. These data support the chemical structure of compound **1** as depicted in Figure 1.

Compound **2**, isolated as an amorphous solid, showed a molecular formula of $C_{52}H_{91}N_7O_{13}$, deduced by high-resolution FAB-MS measurements and consistent with the ^{13}C NMR data (Table 1). Low-resolution ESI-MS spectra (Figure S8) showed the pseudomolecular ion peak at *m/z* 1023 $[M + H]^+$, together with the peaks for Na and K adducts, at *m/z* 1045 $[M + Na]^+$ and 1061 $[M + K]^+$, respectively. Preliminary NMR analysis showed a close similarity between compounds **1** and **2**, indicating the lipopeptide nature of compound **2**. The latter compound had a molecular weight 14 amu lower, thus indicating the absence of a methylene group in compound **2** that could be located either on the alkyl chain or on the peptide chain. A detailed investigation of compound **2** was undertaken by obtaining 1D and 2D NMR spectra and by ESI-MS1, -MS2 experiments. Both NMR and tandem MS data indicated that **1** and **2** had the same amino acid sequence.

Hydrolysis of compound **2**, followed by GC-MS analysis of the water phase of the hydrolysate, showed the same amino acid composition for both compounds **1** and **2**, suggesting the presence of an alkyl chain one carbon shorter in **2**. This was confirmed by GC-MS analysis of the $CHCl_3$ phase of the hydrolysate after permethylation, which identified the lipophilic part of the compound as 3-hydroxypentadecanoic acid.

Thus, the chemical structure of compound **2** is based on the same heptapeptide chain, Glu-Val-Leu-Val-Asp-Leu-Leu, N-acylated to the N-terminal amino acid, Glu, by a 3-hydroxy C_{15} fatty acid with a saturated linear chain (15:0) (Figure 1).

The configuration at C-3 of the fatty acids in **1** and **2** was determined using the derived 3-hydroxy fatty acids **3** and **4** obtained by acid hydrolysis, after derivatization with chiral (*α*-methoxy-*α*-trifluoromethyl)phenylacetic acid (MTPA, Mosher's reagent)[15,16] with *S*- and *R*-configuration (Scheme 1), affording the (*S*)- and (*R*)-MTPA esters (**3a**/**3b**, **4a**/**4b**), respectively. Analysis of the $\Delta\delta(S-R)$ values according to the Mosher model (Scheme 1) for each pair of diastereomeric MTPA esters (**3a**/**3b**, **4a**/**4b**) was undertaken. The *S*-configuration at C-3 of the compounds should give positive $\Delta\delta(S-R)$ values

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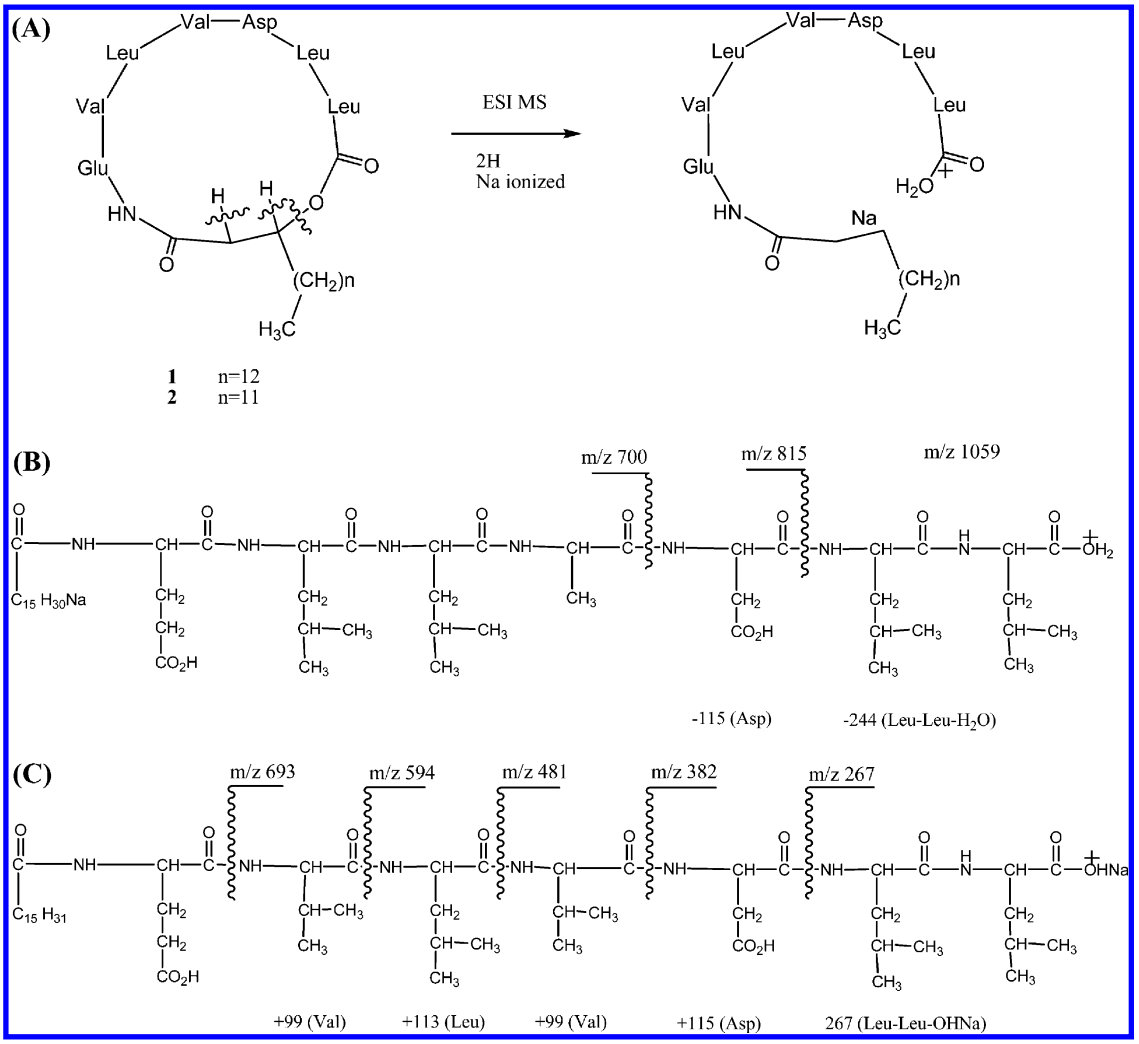
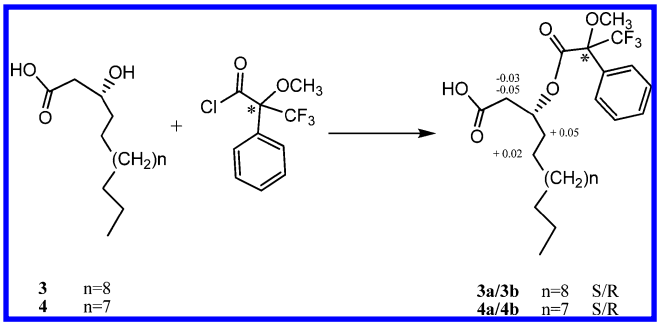


Figure 3. (A) Double hydrogen transfer cleavage of **1** and **2** in tandem ESI-MS. (B) Molecular and fragmentation ions obtained in tandem ESI-MS of compound **1**. These ion peaks are 18 amu higher than corresponding peaks after simple cleavage. (C) Set of fragmentation ions obtained in tandem ESI-MS of compound **1**.

Scheme 1. (*S*)- and (*R*)-MTPA Esters (3a/3b, 4a/4b) of 3-Hydroxy Fatty Acids 3 and 4 Obtained by Acid Hydrolysis of 1 and 2[a]



[a]$\Delta\delta(S-R)$ values (in ppm) reported are those of **3a**/**3b**. Analogous values have been found for **4a**/**4b**.

for H-2 and negative $\Delta\delta(S-R)$ for H-4/H-5, but the measured $\Delta\delta(S-R)$ values were −0.03 and −0.05 (H-2), +0.05 (H-4), and +0.02 (H-5), which corresponds to the *R*-configuration. Therefore, according to the Mosher model, the *R*-configuration at C-3 is indicated for compounds **3** and **4**, and consequently also for **1** and **2**.

Previous studies revealed a broad antimicrobial activity spectrum of lipopeptides from *Bacillus* species such as *B. subtilis*[7] and *B. thuringiensis*.[17] Antifungal strains of *B. amyloliquefaciens* were shown to produce iturins.[18,19] Only in a one case have surfactins been isolated from strain BO7 of this species, as reported from our previous work,[10] in which we isolated three surfactin derivatives that showed significant antifungal activity against *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*). Thus, this is the second report of surfactin compounds from *B. amyloliquefaciens*. Following the same methods,[10] we tested the inhibitory activity of the surfactins **1** and **2** against the same fungus and against three of the most important fungal plant pathogens, namely, the fruit rot agent *Penicillium italicum*, the air-borne pathogens *Aspergillus niger* and *Botrytis cinerea*, as well as the biocontrol agent *Trichoderma harzianum*.

The data obtained, reported in Figure 4, showed that compound **2** exhibits significant inhibitory activity at three concentrations tested (10, 50, and 100 ppm), with an average mycelial growth reduction of 57%, 43%, 38%, and 54% for *Fol*, *P. italicum*, *A. niger*, and *T. harzianum*, respectively. Compound **1** was active against *Fol* and *A. niger* with an average inhibition of 59% and 36%, respectively. Neither compound showed activity against *B. cinerea*. The mixture of both compounds

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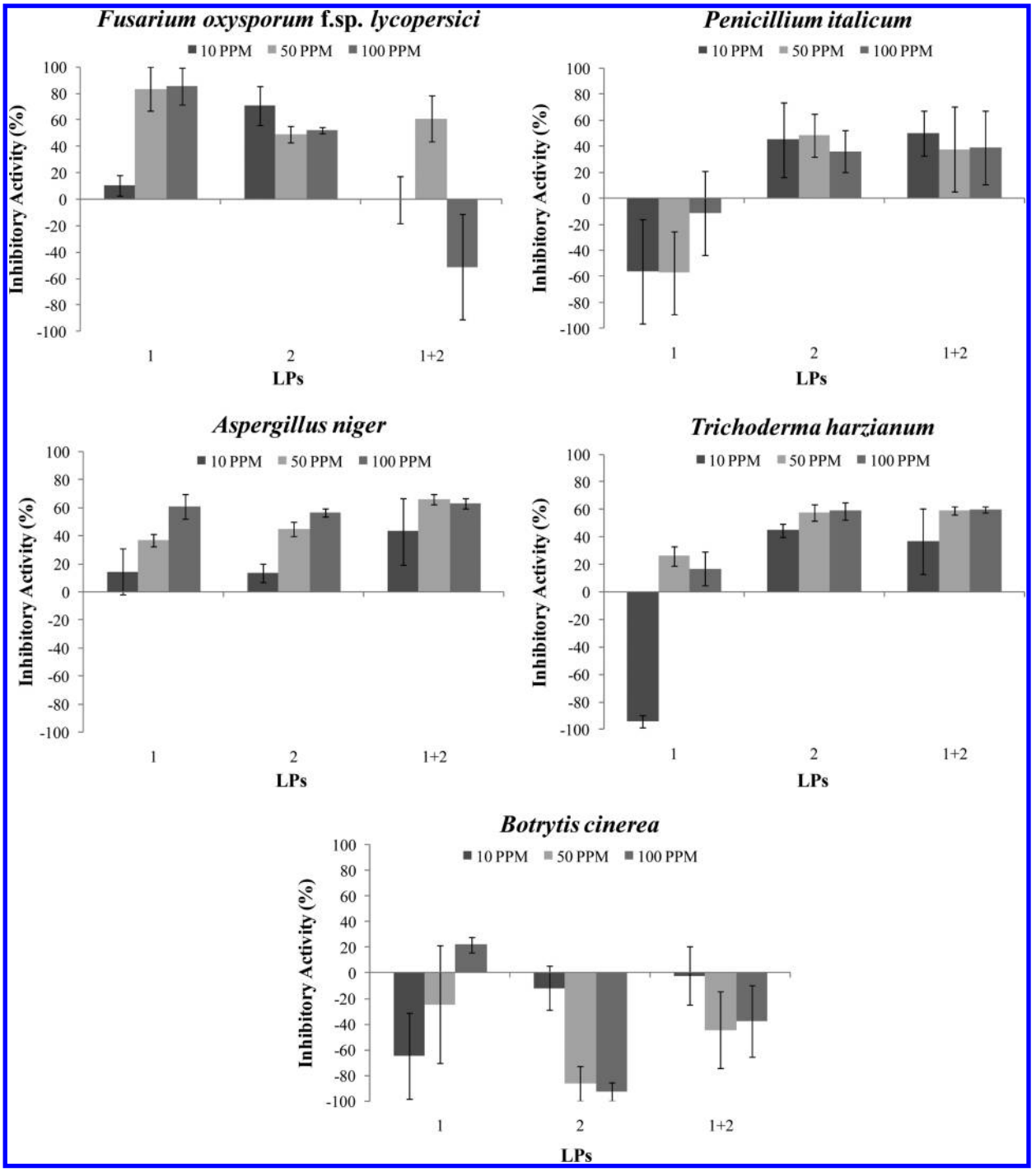

Figure 4. Antifungal activity of compounds **1** and **2** against five common fungal species. Each compound, either alone or in combination, was tested at the final concentrations of 10, 50, and 100 ppm, using a bioassay in microtiter plates. Fungal growth was measured spectrophotometrically at 595 nm after 72 h incubation, and inhibitory activity (IA) was calculated as the percentage of fungal biomass reduction compared to the control (EtOH = 0%). Bars indicate the standard deviation from four replicates.

did not supply higher activity than compounds **1** and **2** applied alone on all tested fungi (Figure 4).

There have been reports on antifungal activity of bacterial surfactins,[10,11] but these compounds are better known for their antiviral and antibacterial activities as well as their surfactant properties.[4] Although the cellular mechanism of the antifungal mode of action of such compounds is still unknown, their efficacy and availability at low cost make the isolated surfactins attractive potential natural fungicides.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell. FAB-MS (recorded in a glycerol matrix) were measured on a Prospec Fisons mass spectrometer. ESI-MS 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. ESI-MS1, -MS2 spectra were recorded on an API 2000 instrument. GC-MS analysis was performed on a Carlo Erba instrument by an Agilent Technologies

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6890N Network gas 236 using a HP-5 capillary column (30 m × 0.25 mm i.d.) packed with 5% polyphenyl siloxane, helium carrier flow 10 mL min^{-1}, and an FID detector operated at 2608. The column temperature was maintained at 70 °C for 3 min and then gradually increased (10 °C/min) to 300 °C. ^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).[20] The multiplicities of ^{13}C NMR resonances were determined by DEPT experiments. ^1H connectivities were determined by using COSY and HOHAHA 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 $^1H-^{13}C$ connectivities were determined with a 2D HSQC pulse sequence with an interpulse delay set for a $^1J_{CH}$ of 130 Hz. Two- and three-bond heteronuclear $^1H-^{13}C$ connectivities were determined with 2D HMBC experiments, optimized for a $^{2-3}J_{CH}$ of 8 Hz. HPLC in isocratic mode was performed on a Varian 940-LC apparatus equipped with a refractive index detector by using a μ-Bondapack C_{18} analytical column, 3.9 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 first by UV lamp and then with cerium sulfate in 2 N H_2SO_4 after heating as brown spots (R_f = 0.6 for compound **1** and 0.5 for compound **2**).

Production of 1 and 2 in *Bacillus* Cell Suspension: Extraction and Purification Procedures. Samples of *Bacillus amyloliquefaciens* strain BO5A were isolated from an orchard soil, collected in Larino (Campobasso, Italy), and in particular from the rhizosphere of the olive plant, using the methodology described by Boulter et al.[21] and characterized by routine bacteriological tests (Biolog and API test) and by sequencing of the small 16S ribosomal subunit (CBS: Centraalbureau voor Schimmelcultures, The Netherlands). Strain BO5A was routinely cultivated in minimum salt liquid medium (MSLM: K_2HPO_4 2.5 g/L; KH_2PO_4 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 shaken at 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,[22] and cell concentration was adjusted spectrophotometrically (610 nm). Bacterial strains were stored at −80 °C with 30% glycerol.

Bacteria cells were removed from the culture by centrifugation at 5000 rpm for 30 min. The lipopeptide fraction was obtained from an aliquot (1 L) of cell-free supernatant that has been subjected to acid precipitation, by the addition of 6 N HCl to a final pH of 2.0, and incubated overnight at 4 °C. The acid precipitate was recovered by centrifugation at 10.000 rpm for 20 min and lyophilized overnight. The lipopeptides were extracted from the powder by using a $CHCl_3$–MeOH mixture (2:1, v/v) and passed through a polytetrafluoroethylene (PTFE) filter. The extract was concentrated under vacuum to obtain a crude lipopeptide mixture. The final step in the purification employed high-performance liquid chromatography (HPLC) in isocratic mode on a Varian 940-LC apparatus equipped with a refractive index detector. The lipid mixture was chromatographed on an analytical C_{18} reversed-phase column (Waters μBondapak C_{18} 3.9 × 300 mm) eluted with 0.1% of trifluoroacetic acid (TFA) in CH_3CN-H_2O (8:2, v/v), obtaining pure compounds **1** (8.4 mg, t_R = 5.4 min) and **2** (5.8 mg, t_R = 4.2 min).

Antifungal Assays: Growth Conditions and Bioassays with Purified Lipopeptides. *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) strain 4287 (FGSC 9935) was isolated from infected tomato plants.[23] *Penicillium italicum* (PI), *Aspergillus niger* (AN), *Botrytis cinerea* (BC), and *Trichoderma harzianum* (TH) strain T-22 were obtained from the Dipartimento di Agraria, Naples, Italy. These fungal strains were 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 previously reported.[24]

The two purified LPs (**1** and **2**) were dissolved in ethanol, alone or in mixture, and added to the medium at a final concentration of 10, 50, and 100 ppm. In mixed applications, each compound was used at equimolecular ratio to produce a final additive concentration of 10, 50, or 100 ppm. The solvent ethanol was used as a negative control. Bioassays were conducted in a 96-well plate against conidia of the five chosen fungal strains (*F. oxysporum* f.sp. *lycopersici*, *P. italicum*, *A. niger*, *T. harzianum*, and *B. cinerea*). Samples contained a mixture of purified LPs (or ethanol as a control) mixed with PDB and 10^5 fungal conidia 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, USA), and means were compared by Tukey's test. The effective lipopeptide concentration required to obtain 50% growth inhibition of tested fungi (EC_{50}) was calculated by regressing inhibition of fungal growth against the logarithm of LP concentration. Commercial surfactin (C15, Sigma Aldrich) was used as a control (data not show).

Compound 1. Yield: 8.4 mg; colorless, amorphous solid; $[\alpha]^{25}_D$ −26.5 (*c* 0.1 MeOH); IR (KBr) ν_{max} 3413, 2928, 1151, 1045 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 1; HRFAB-MS (positive ion) found *m/z* 1036.6928 $[M + H]^+$, calcd for $C_{53}H_{94}N_7O_{13}$ *m/z* 1036.6914; FAB-MS (positive ion) *m/z* 1037 $[M + H]^+$, *m/z* 1059 $[M + Na]^+$, *m/z* 1075 $[M + K]^+$; ESI-MS1/MS2 *m/z* 1059, 946, 933, 815, 718, 700, 693, 580, 467, 396, 281.

Compound 2. Yield: 5.8 mg; colorless, amorphous solid; $[\alpha]^{25}_D$ −25.7 (*c* 0.1 MeOH); IR (KBr) ν_{max} 3410, 2930, 1150, 1045 cm^{-1}; ^1H NMR data, see Figure 1 and Table 1; ^{13}C NMR data, see Figure 1 and Table 1; HRFAB-MS (positive ion) found *m/z* 1022.6769 $[M + H]^+$, calcd for $C_{52}H_{91}N_7O_{13}$ *m/z* 1022.6757; FAB-MS (positive ion) *m/z* 1023 $[M + H]^+$, *m/z* 1045 $[M + Na]^+$, *m/z* 1061 $[M + K]^+$; ESI-MS1/MS2 *m/z* 1045, 932, 919, 801, 704, 686, 679, 566, 453, 382, 267.

Hydrolysis of Compounds 1 and 2. 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 under a stream of N_2 and analyzed separately. The lipophilic fraction, containing 3-hydroxy fatty acids **3** and **4** (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 amounts of ethyl acetate (from 20% to 70%). The fraction eluted with 50% hexane–ethyl acetate was shown to contain a single compound identified by GC-MS and NMR (for **1**, methyl 3-methoxyhexadecanoate; for **2**, methyl 3-methoxypentadecanoate). 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.[25] A mixture of L-valine, L-leucine, L-glutamic acid, and L-aspartic acid taken in an equimolecular ratio served as a standard. Using this procedure L-valine, L-leucine, L-glutamic acid, and L-aspartic acid were identified and their 2:3:1:1 ratios was estimated according to the peak areas. To confirm the stereochemistry of the amino acids, an aliquot of the aqueous phase from the hydrolysate of **1** was subjected to enzymatic oxidation catalyzed by L- and D-amino acid oxidases (Sigma Aldrich, USA) following the procedure described by Debono et al.,[12] 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 3 and 4. Two aliquots (1 mg) of each 3-hydroxy fatty acid (**3**, **4**; Scheme 1), obtained by acid hydrolysis (6 M HCl) of compounds **1** and **2**, were dissolved in dry pyridine and allowed to react overnight with (*S*)- and (*R*)-MTPA chloride, affording the (*S*)- and (*R*)-MTPA esters (**3a**/**3b**, **4a**/**4b**; Scheme 1), respectively. Analysis of the $\Delta\delta(S-R)$ values obtained by the ^1H NMR spectrum for each couple of diastereomeric MTPA esters (**3a**/**3b**, **4a**/**4b**), according to the Mosher model (Scheme 1), pointed to an *R*-configuration at C-3.

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■ ASSOCIATED CONTENT

Supporting Information

NMR and MS data of compounds **1** and **2** (Figures S1−S8) are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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