Bacterial Lipodepsipeptides and Some of Their Derivatives and Cyclic Dipeptides as Potential Agents for Biocontrol of Pathogenic Bacteria and Fungi of Agrarian Plants

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ABSTRACT: Biotic stresses (fungi, bacteria, insects, weeds, etc.) are some of the most important causes of the decrease in the quality and quantity of crops that could become an emergency due to a noteworthy increase in the world population. Thus, to overcome these problems, massive use of chemical pesticides has been carried out with heavy consequences for environmental pollution and food safety. An eco-friendly alternative can be using natural compound-based biopesticides with high efficacy and selectivity. Some bacterial lipodepsipeptides (tolaasins I, II, A, D, and E and WLIP methyl ester) and cyclic dipeptides (cyclo(L-Pro-L-Tyr), cyclo(D-Pro-L-Tyr), cyclo(L-Pro-L-Val), and cyclo(L-Pro-L-Leu)) were assayed against several pathogenic bacteria and fungi of important agrarian plants. Lipodepsipeptides showed strong growth inhibition of all microorganisms tested in the range of 0.1−0.8 μg/mL, while cyclodipeptides, despite preserving this ability, showed a noteworthy reduced antimicrobial activity being active only in the range of 15−900 μg/mL. Among the lipodepsipeptides and cyclic dipeptides assayed, tolaasin D and cyclo(L-Pro-L-Tyr) (also named maculosin-1) appeared to be the most toxic compounds. Some structure−activity relationships of lipodepsipeptides were also discussed along with their practical application as biopesticides in agriculture.

KEYWORDS: agrarian plant diseases, bacterial and fungal pathogens, biopesticides, lipodepsipeptides, cyclic dipeptides, antimicrobial activity

INTRODUCTION

People, since ancient times, had worldwide developed agriculture as the first human activity to produce food in high quantity and quality. This necessity increased over time for a constant increase in the world population, which will be almost 10 billion by 2050.1−3 These aspects, despite the noteworthy technological progress done in agriculture, are becoming an emergency due to the strong reduction of natural resources, the environmental pollution, and climate changes.4−6 Biotic stresses, including microbial pathogens, weeds, insects, etc., represent the main causes of severe losses in agrarian production and food safety. Up to this day, control of these damaging agents has been done with the massive use of synthetic pesticides. The latter can cause environmental pollution, induce resistance in the host plants, and are responsible for the presence of toxic residues in agricultural products.5,6 These problems prompted efforts to develop integrated pest management7 to reduce or eliminate synthetic pesticides significantly. A valid and efficacy alternative is represented by biopesticides, which are easy degradable and represent no risk for human and animal health as strongly required by consumers and by public administrators.5,7

Natural products are the most important source for finding substances with different biological activities, new carbon skeletons to overcome resistance phenomena, and potential applications as new eco-friendly solutions in various fields.6,8 Among these classes of natural bioactive metabolites, there are lipodepsipeptides and cyclic dipeptides. Lipodepsipeptides are biologically active metabolites produced by different bacteria and are constituted by three moieties: (i) a macrocyclic peptide lactone; (ii) a linear peptide; and (iii) fatty acid. These lipodepsipeptides, containing unusual amino acids also with an opposite stereochemistry, are classified according to their primary structures into two groups. Siringotoxins, siringomycins, pseudomycins, and siringostatin belong to the first group. Those containing from 18 to 25 amino acid residues, most of which have a D-stereochemistry, such as siringopeptins, fuscopeptins, tolaasins, and corpeptins, are reported in the second group.9 In the latter one, the C-terminal group forms a lactone ring constituting from 5 (corpeptins, tolaasins, and fuscopeptins) to 8 (siringopeptins) amino acids. The first reported nonapeptides were siringomyccins, a subgroup synthesized by the plant pathogenic bacterium Pseudomonas syringae pv. syringae showing antifungal activities. They targeted the fungal plasma membrane, and some studies on their mode of action were also performed.10 Successively, the other nonapeptides siringostatins, siringotoxins, and pseudomycines were produced by P. syringae pv. syringae but isolated from different infected host plants.11 Other lipodepsipeptides such as...
syringopeptines, fuscopetines, and corpeptines were produced by *P. syringae pv. syringae*, *Pseudomonas fuscovaginae*, *Pseudomonas corrugata*, and *Pseudomonas cichorii*. Lipodepsipeptides in addition to phytotoxic and antifungal activities also showed potential antibiotic activity and thus potential against the bacterial species that have developed resistance to common antibiotics.

Also, pathogenic bacteria of cultivated mushroom produce lipodepsipeptides with different biological activities such as *Burkholderia gladioli pv. agaricicola*, *Pseudomonas tolaasii*, and *Pseudomonas reactans*. The main bioactive lipodepsipeptides produced by both *Pseudomonas* strains are tolaasins I and II (1 and 2, Figure 1), which themselves differed in the substitution of the homoserine residue (Hse16) of macrocyclic lactone with a glycine residue, and the so-called white line inducing principle (WLIP, 3, Figure 2). The role that these metabolites play in the diseases and their biological activities were extensively studied. *P. tolaasii*, pathogen of *Agaricus bisporus* and *Pleurotus ostreatus*, also showed to produce, despite being in lesser amounts, other tolaasins named tolaasins A, B, C, D, and E (4–8, Figure 1). They differed from tolaasins I and II in the peptide chain, as observed in other lipodepsipeptides of bacterial origin, and preserved the \( \beta \)-hydroxyoctanoyl \( \phi \) group at the N-terminus, except for tolaasin A, in which the acyl moiety was a \( \gamma \)-carboxybutanoyl \( \phi \) chain. When tested on fungi, yeast, and bacteria, they showed antimicrobial activity against Gram-positive bacteria, which appeared to be the most sensitive, and this activity seemed to be related to the structural differences of the analogues.

The close naturally occurring cyclopeptides exhibit potent biological activities, including insecticidal, antimicrobial, antifungal, and antiproliferative. They are produced by marine organisms and plants. A subgroup of this class of natural compounds is the cyclodipeptides, also known as 2,5-diketopiperazines, which showed various biological activities and displayed strong resistance against enzymatic hydrolysis, thus attracting great interest in a variety of fields spanning from functional materials to drug discovery.

Among 2,5-diketopiperazines, the most known is maculosin-1 (cyclo(\( \text{L-Pro-L-Tyr} \)) \( 9 \), Figure 3). Compound 9 is a host-specific phytotoxin produced by *Alternaria alternata*, a pathogen of knapweed. The same fungus also synthesizes cyclo(\( \text{L-Pro-L-Phe} \)) (maculosin-2) and cyclo(\( \text{L-Pro-Ala} \)), cyclo(\( \text{L-Pro-Val} \)), cyclo(\( \text{L-Pro-Hle} \)), cyclo(\( \text{L-Pro-Leu} \)), and cyclo(\( \text{L-Pro-D-Phe} \)), as potential biocontrol agents of knapweed.

Compound 9 was also recently isolated from *Lysobacter capsici* AZ78 and showed antifungal activity against *Phytophthora infestans* and *Plasmopara viticola*, two pathogens of important crops. Some derivatives of maculosin-1 were also prepared and their antifungal activity, compared to those of the parent compound (9) and maculosin-2 cyclo(\( \text{L-Pro-L-Phe} \)) \( 10 \), Figure 3), was tested against *P. infestans*. Among them, the azido derivative of 9 showed strong antifungal activity, suggesting its potential use as a biofungicide. To corroborate these results, 9 was applied on tomato leaves to prevent the occurrence of late blight lesions. These results prompted an in-depth investigation of 2,5-diketopiperazine production by *L. capsici*. In fact, cyclo(\( \text{-Pro-L-Val} \)), cyclo(\( \text{-Pro-D-Phe} \)), cyclo(\( \text{-Pro-L-Leu} \)),...
and cyclo(D-Pro-L-Tyr) (11−14, Figure 3) were successively isolated from the same bacterial cultures and were tested together with maculosins-1 and 2 (9 and 10) against the phytopathogenic Gram-positive bacterium Rhodococcus fascians LMG. Among all the 2,5-diketopiperazines assayed, compound 11 showed toxicity similar to that of chloramphenicol, a positive control, when used at the same concentration. These results and reported data suggest that 2,5-diketopiperazines could be proposed as potential biopesticides due to their broad activity spectrum against phytopathogenic microorganisms.

Thus, this article reports the antimicrobial activity of five lipodepsipeptides (1, 2, 7, and 8), WLIP (3), two tolaasin I derivatives (15 and 16), one WLIP derivative (17), and four diketopiperazines (9, 11, 13, and 14) against several pathogenic bacteria and fungi of agrarian plants. Results of structure–activity relationships were also discussed.
**MATERIALS AND METHODS**

**General Experimental Procedures.** Optical rotation, 1H NMR spectra, electrospray ionization mass spectrometry (ESI MS) analysis, analytical and preparative thin-layer chromatography (TLC), and column chromatography were performed as previously reported.

Reverse-phase high-performance liquid chromatography (HPLC) of the tolaasin crude mixture was performed as previously reported. P. tolaasii Tolasains I, II, D, and E were produced growing P. tolaasi (strain type NCPPB21912) in liquid King’s B medium stirred culture at 25 °C as previously reported. The culture was centrifuged and lyophilized, and tolasaIns were purified from the culture filtrates (1.35 L) according to a previously reported method. Briefly, after acidification of the culture filtrates, the precipitate was discarded, and tolasaIns were precipitated by adding CaCl2. After more steps of washing with small volumes of MeOH followed by washing with small volume of Milli-Q water, the crude residue was desalted by G-10 column chromatography, and the tolaasin-containing fractions were combined and lyophilized to give a white solid residue. The tolaasin mixture (67.6 mg) was purified by HPLC using a reverse-phase semi-preparative column eluted with a gradient MeCN-0.1% TFA and afforded tolasaIns I, II, D, and E (1, 2, 7, and 8). This procedure was repeated more times to accumulate the tolaasin in discrete amounts. Tolaasins I, II, D, and E were identified by 1H NMR and ESIMS spectra in comparison with those of standard samples. Their purity was >98% as ascertained by HPLC analysis.

**Acetylation of Tolaasin I.** Tolaasin I (1, 2.0 mg) was dissolved in dry pyridine (100 μL) and acetylated with Ac2O (100 μL). The reaction was carried out at room temperature overnight and stopped by adding MeOH. Pyridine was eliminated under a N2 stream of the azo trope formed by addition of CH2Cl2. The organic residue was purified with TLC using i-PrOH/H2O (8/2, v/v) as an eluent, affording the hexacytethyl derivative of tolaasin I (15 mg, 19 mg, 88%) as an amorphous solid. Compound 15 had a 1H NMR spectrum (Figure S2, SI) that essentially differed from that of tolaasin I (Figure S1, SI), recorded under the same conditions for the presence of the singlets of five acetyl groups in the range of δ 2.2–1.99; ESIMS (+) m/z: 2260 [M + Na]+ (Figure S3, SI).

**Hydrogenation of Tolaasin I.** Tolaasin I (1, 2.7 mg) was dissolved in MeOH (1 mL) and added to a suspension of 95% PtO2/C in MeOH (1 mL) pressurized with H2 gas for 30 min under stirring. The reaction was performed with H2 at atmospheric pressure at room temperature under stirring in the dark. The reaction was completed after 24 h and stopped by filtration of the catalyst. The solution was evaporated under reduced pressure to give the tetrahydro derivative of tolaasin I (16, 2.6 mg, 96%) as an amorphous solid. The 1H NMR spectrum recorded in CD3OD (Figure S4, SI) essentially differed from that of tolaasin recorded under the same conditions (Figure S1, SI) for the absence of olefinic protons; ESIMS (+) m/z: 1900 [M + H]+ (Figure S5, SI).

**Production and Purification of WLIP.** P. reactans NCPPB13111 was grown on liquid KB medium at 25 °C under shaking, as previously reported. Briefly, the lyophilized culture filtrate (1.4 L) was dissolved in Milli-Q water (1.3 L) and centrifuged at 10 000 rpm at 15 °C for 30 min. The supernatant was filtered on a Whatman n. 42 paper disk, acidified up to pH 5 with 1 N HCl, and left at room temperature overnight. The precipitate dissolved in Milli-Q water was alkalized up to pH 7.5 with 1 N NaOH, and the solution was filtered on Whatman S/40 paper disks. It was acidified up to pH 5 with 1 N HCl. The precipitate was collected by centrifugation at 10 000 rpm at 15 °C for 30 min, oven-dried at 50 °C, and then dissolved in MeOH (100 mL). The suspension was then filtered on Whatman n. 42 paper disks, and the filtrate was evaporated under vacuum. The solid residue was washed with MeOH (10 mL), centrifuged at 8000 rpm at 15 °C for 30 min, then dissolved in MeOH (100 mL), and dried under vacuum to give crude WLIP (250 mg). The latter crystallized as white needles (216 mg) with blowing in water vapor, according to the procedure reported by Mortishire-Smith (1991). WLIP was identified by 1H NMR and ESIMS spectra in comparison with those of standard samples. Its purity was >98% as ascertained by HPLC analysis.

**Production and Purification of 2,5-Diketopiperazines.** L. capsici AZ78 cultures were obtained as previously described. The lyophilized culture filtrates (20 L) were dissolved in Milli-Q water (2 L) and extracted with EtOAc (3 × 2 L). The corresponding extract was fractionated according to the procedure previously reported. In particular, the organic extracts were combined and dried under vacuum to give a solid residue (1.56 g). The latter was chromatographed on a silica gel eluted with CHCl3/i-PrOH (9/1) and then with CHCl3/i-PrOH (7/3), yielding 10 groups of homogeneous fractions (F1–F10). The F2 residue (302 mg) was subjected to another fractionation by column chromatography, using CHCl3/i-PrOH (9/1) as an eluent. A total of 10 groups of homogeneous fractions were collected (F2.1–F2.10). The F2 residue (13 mg) appeared to be a pure metabolite, identified as cyclo(i-Pr-o-Tyr) (11). The F2.4 residue (51.3 mg) was further purified by TLC, eluted with CHCl3/i-PrOH (9/1), yielding four groups of homogeneous fractions (F2.4.1–F2.4.4). The F2.4.2 residue (36.2 mg) was further purified by more steps of TLC, giving further amounts of F11 (4 mg) and cyclo(i-Pro-o-Tyr) (13, 3.7 mg). The residue (62.5 mg) of F3 was further purified by several steps of TLC, yielding further amounts of F11 (5.5 mg), maculocin-1 (cyclo(i-Pro-o-Tyr) (9, 11.9 mg), and cyclo(i-Pro-o-Tyr) (14, 18.4 mg). Their identity was ascertained by 1H NMR and ESIMS spectra in comparison with those of standards. Their purity was >98% as ascertained by HPLC analysis.

**Minimum Inhibitory Concentrations (MIC).** Antimicrobial assay. The antimicrobial assay was carried out as described in Bassarello et al. (2004) with some modifications. Bacteria were grown in LB broth at 25 or 37 °C overnight at 150 rpm. A total of 500 μL of a suspension containing about 109 cfu mL−1 were added to 3 mL of LB soft agar (0.7%) and poured onto plates containing 7 mL of LB broth with agar 1.8%. After agar gelification, 10 μL drops of serial dilutions of different lipopeptides and their derivatives (from 0.1 to 1 μg/mL) and cyclic dipeptides (from 10 to 1000 μg/mL) were tested. After 24 ± 48 h of incubation at 25 or 37 °C, the end serial dilution inhibiting the growth of the bacteria in the area of application of 10 μL solutions was recorded. The plates containing the bacteria alone were used as a control. The experiment was performed in triplicate.

**Antifungal Assay.** The antifungal activity was performed in 24-well culture plates according to the method previously described with some modification. Serial dilutions of different lipopeptides (from 0.1 to 1 μg/mL) and cyclic dipeptides (from 10 to 1000 μg/mL) were dissolved in a volume of 500 μL of ultrapure Milli-Q and finally inoculated with 500 μL of 2× potato dextrose broth (Difco) containing the Colletotrichum truncatum plug of 4 mm × 4 mm diameter. As a control, C. truncatum plugs (4 mm × 4 mm) were grown in 2× PD broth diluted with 500 μL of ultrapure Milli-Q water, and the plates were incubated at 28 °C for 7 days. The MIC was measured as the lowest concentration of antifungal agent at which there was no visible growth of the fungus after incubation. The experiment was performed in triplicate.

**RESULTS AND DISCUSSION**

Bacteria belonging to the Pseudomonas genus were used in this study; all are causal agents of severe diseases of important agrarian plants. Among them, there is Burkholderia caryophylli (syn. Pseudomonas caryophylli) responsible for bacterial wilt of carnation resulting in serious losses in carnation production.

From its culture filtrates were isolated three polysaturated C17 fatty acids and other three metabolites; the latter were...
obtained as an interconvertible mixture and named caroyonencins A-C. The latter showed strong antibacterial activity against Gram-positive and Gram-negative bacteria such as Staphylococcus aureus, Bacillus subtilis, Escherichia coli, and Klatsiella pneumoniae but had no phytotoxicity.32,33 Although the culture filtrates exhibited phytotoxicity toward the host and nonhost plants, up to a day, phytotoxins were not isolated from B. caryophylli, but from some preliminary experiments carried out by some of the authors, they should be lipodepsipeptidides (private communication). Extensive work was done by some of the authors on the lipopolysaccharides (LPS) present in the outer membrane of this bacterium as in general, LPS plays an important role in the first process of pathogenesis and in particular in the interaction of the plant and pathogen.35 The LPSs of B. caryophylli appeared to be constituted by two homopolysaccharide chains, with the major one built up of (1 → 7)-linked α-cyrophyllose, (3,6,10-trideoxy-4-C-(d-glycero-1-hydroxyethyl)-d-erythro-d-gulo-decose) residues and the minor one made up of (1 → 7)-linked β-cyrophose, (4,8-cyclo-3,9-dideoxy-l-erythro-l-idono-nonose) residues. A third polysaccharide fraction mainly constituted by heptose and glucose was also isolated.35 The main polysaccharide, named caryophyllan, was constituted by a repeating unit of a novel 4-branched monosaccharide, named cyrophyllose, characterized as tri- deoxy-C-(R)-1-hydroxyethyl)-d-erythro-d-gulo-decose.36,37 The minor polysaccharide, named carian, was constituted by the repeating unit of two cyclic monosaccharide, named caryose, characterized as carbocyclic (4,8-cyclo-3,9-dideoxy-l-erythro-l-idono-nonose).38 Another bacterium used is P. syringae pv. panici, a worldwide diffused pathogen, which induces diseases in different plants including crops such as rice, lilac, millet, and pearl millet.39 In rice, P. syringae pv. panici induces brown stripe disease.40 Pseudomonas syringae pv. tabaci was also included among the bacteria used in this study as it induces brown spots on tobacco, a disease named wildfire, with severe economic consequences.41 The same is for P. syringae pv. siringae (Pss), which is the most polyphagous bacterium in the P. syringae complex due to its wide host range, first affecting woody and herbaceous host plants. In early 1990s, Pss caused apical necrosis of mango trees, a severe disease in Southern Spain. A lot of studies had been carried out on this pathogen, whose results are reported in some reviews as that published by Gutiérrez-Barranquero et al.42 Pseudomonas syringae pv. japonica, also included in the bacteria tested, induced the black node disease of barley (Hordeum vulgare L.) and wheat (Triticum aestivum L.) and was initially classified as Pseudomonas striaficiens var. japonica.43 The other three bacteria tested were B. subtilis, Bacillus megaterium, and E. coli, which are laboratory strains. Colletotrichum truncatum was selected, among the phytopathogenic fungi available, as the only strain to test because very low amounts of both lipodepsipeptides and cyclodipeptides were available for the antimicrobial assay. The strain of C. truncatum was isolated in Argentina as one of the most dangerous pathogens of soybean inducing anthracnose symptoms with severe epidemics and expressive yield losses.44 All the lipodepsipeptides (tolaasins and WLIP) were produced, purified, and identified as reported in detail in the Materials and Methods section. The two derivatives of tolaasin I and the methyl ester of WLIP were prepared and characterized as reported in detail in the same section and in the Supporting Information. In particular, the 1H NMR spectrum (Figure S2, SI) of the hexacetyl derivative of tolaasin I (15) essentially differed from that of tolaasin I (Figure S1, SI), recorded under the same conditions for the singlets of five acetyl groups in the range of δ 2.20–1.99. Its ESIMS (+), spectrum showed the sodiated adduct ion [M + Na]+ at m/z 2260. The 1H NMR spectrum (Figure S4, SI) of the tetrahydro derivative of tolaasin I (16) essentially differed from that of tolaasin I, recorded under the same conditions, for the absence of olefinic protons. Its ESIMS (+) spectrum showed the protonated adduct ion [M + H]+ at m/z 1990. Finally, the 1H NMR spectrum of WLIP methyl ester (17) (Figure S6, SI) essentially differed from that of WLIP (Figure S7, SI), recorded under the same conditions, for the presence as a singlet at δ 3.70 due to the ester methyl group. Its ESIMS (+) spectrum exhibited the sodiated [M + Na]+ and the protonated [M + H]+ adduct ions at m/z 1275 and 1289, respectively.

In the first experiment, the lipodepsipeptide tolaasins I, II, D, and E (1, 2, 7, and 8, Figure 1) and WLIP (3, Figure 2) and their derivatives hexacetyl- and tetrahydro-tolaasin I and WLIP methyl ester (15, 16, and 17, Figure 2) were assayed against all the plant pathogenic and nonpathogenic bacteria and the fungus C. truncatum reported above using antimicrobial and antifungal tests (Figures 4 and 5). The results obtained, summarized in Table 1, showed that among the tolaasins and their two derivatives, the compounds 1, 2, and 7 and the tetrahydro tolaasin I (16) inhibited all the bacteria and the fungus tested with a MIC in the range of 0.1–0.9 μg/mL. Just for the bacteria E. coli, the growth was not inhibited. Tolaasin E and the hexacetyl tolaasin I (8 and 15) did not show activity against the three laboratory bacterial strains of B. subtilis, B. megaterium, and E. coli. However, compounds 8 and 15 showed a MIC in the range of 3–6 and 0.7–1 μg/mL, respectively, against the pathogenic bacteria and C. truncatum. Furthermore, the sensitivity among the bacteria seems similar, while the fungus C. truncatum showed essentially different MICs.

![Figure 4](https://doi.org/10.1021/acs.jafc.1c08139)

**Figure 4.** Representative photographs of minimum inhibitory concentration of tolaasin II against the *Pseudomonas syringae* pv. syringae strain B475.

![Figure 5](https://doi.org/10.1021/acs.jafc.1c08139)

**Figure 5.** Representative photographs of minimum inhibitory concentration of tolaasin I against the *C. truncatum* strain 17-5-5.

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the amino acid residue at the 16 position of the macrolactone ring is not important for the activity as it is L-homoserine (L-Hse) in 1 and L-serine (L-Ser) in 2. L-Hse is also present at the same position in tolaasin D (7); thus, the increased activity showed by the latter, with respect to 1, could be due to the presence of a different amino acid residue at the 15 position, which is L-leucine (L-Leu) in 7 and L-iso-leucine (L-Ile) in the other two. However, the presence in the same lipodepsipeptide of L-Leu and L-Ser at 15 and 16 positions, respectively, probably induces a noteworthy decrease in antimicrobial activity as observed in tolaasin E (8). The acetylation of the hydroxyl group of the fatty acid, L-Ser, L-Hse, and the primary amino groups of D-2,4-diamino butyric acid (D-Dab) and L-lysine (L-Lys) at positions 17 and 18 of macrocyclic lactone and the hydrogenation of two residue 2-butenylbutyric acid (ΔBut) located at 1 and 13 positions of the linear peptide chain, compared to 1, significantly induced a decrease in activity.

The nonapeptide WLIP (3) that differed from tolaasins for all the three moieties such as the fatty acid, the linear side peptide chain, and the macrocyclic lactone practically did not inhibit the growth of all pathogenic bacteria, while it exhibited activity against the two laboratory Gram-positive strains B. subtilis and B. megaterium. Despite the lesser activity of tolaasins I, II, and D (1, 2, and 7), WLIP showed antifungal activity against C. truncatum with a MIC of 3 μg/mL. Its methyl ester gave very similar activity, suggesting that a lethal metabolism could work by hydrolyzing the methyl ester group under physiological conditions.

In a second experiment, using the same bioassay method and the same microorganisms, the antimicrobial activity of the 2,5-diketopiperazines, namely cyclo-(L-Pro-L-Tyr), cyclo-(L-Pro-L-Val), cyclo(L-Pro-L-Leu), and cyclo(D-Pro-L-Tyr) (9, 11, 13, and 14), was tested. The results of the bioassay, listed in Table 2, showed that all the diketopiperazines showed activity against all the bacteria except compound 9 on E. coli. The 2,5-diketopiperazine 11 was not toxic. Among the active compounds 9, 13, and 14, the highest antimicrobial activity was shown by maculosin-1 (cyclo-(L-Pro-L-Tyr), 9) with a MIC range of 15–20 μg/mL. The other two compounds (13 and 14) were less active, showing for the pathogenic bacteria and the fungus a MIC range of 500–800 μg/mL, but were more active against the laboratory bacterial strains. The antimicrobial activity of compound 9 is in agreement with its antifungal activity previously reported. The lack of activity of compound 14 also demonstrated that the configuration D or L of the amino acids that constitute the diketopiperazine is a very important feature to impart activity. In fact, diketopiperazines 9 and 14 differed only for the opposite stereochemistry of proline residue in the second one, and its activity is reduced with respect to that of 9 by 50–60 times. The amino acids which constitute the diketopiperazines also affect the activity as compound 13, which differs from compound 9 for the substitution of L-Tyr with L-Leu, showing a noteworthy reduction of activity by 40–50 times. Very surprising is the inactivity of diketopiperazine 11 as recently it showed, among the 2,5-diketopiperazines reported above, the highest activity against R. fuscans.

In conclusion, in testing the antibacterial and antifungal activity, lipodepsipeptides showed growth inhibitory activity 56–60 times higher than that of diketopiperazines. Among the lipodepsipeptides, the nonapeptides such as WLIP, tested on phytopathogenic bacteria and fungus, showed only weaker fungicide activity against C. truncatum. In lipodepsipeptides

### Table 1. Minimal Inhibitory Concentration of the Lipodepsipeptides Tested

| ID          | strain                  | WLIP methyl ester | hexacetyl tolaasin I (15) | tolaasin D (17) | tolaasin E (8) | tetrahydro tolaasin I (16) | tolaasin I (1) | tolaasin II (2) |
|-------------|-------------------------|-------------------|---------------------------|----------------|----------------|---------------------------|----------------|----------------|
| NCPPB 349  | Pseudomonas caryophylli | -                 | -                         | 0.9            | 0.1            | 0.9                       | 0.2            | 0.4            |
| ICMP3955   | Pseudomonas syringae pv. panici | -                 | -                         | 0.7            | 0.1            | 0.9                       | 0.2            | 0.4            |
| ICMP2706   | Pseudomonas syringae pv. tabaci | -                 | -                         | 0.8            | 0.1            | 0.8                       | 0.3            | 0.4            |
| B475       | Pseudomonas syringae pv. syringae | -                 | -                         | 0.8            | 0.1            | 0.8                       | 0.3            | 0.4            |
| ICMP6305   | Pseudomonas syringae pv. japonica | -                 | -                         | 0.7            | 0.1            | 0.9                       | 0.3            | 0.4            |
| PY79       | Bacillus subtilis        | 0.3               | 0.5                       | -              | 0.2            | -                         | 0.2            | 0.3            |
| QMB        | Bacillus megaterium      | 0.3               | 0.5                       | -              | 0.2            | 0.2                       | 0.3            | 0.4            |
| DHSr       | Escherichia coli         | -                 | -                         | -              | -              | -                         | -              | -              |
| 17-5-5     | Colletotrichum truncatum | 3                 | 5                         | 1              | 0.2            | 6                         | 3              | 0.6            |

*No activity.

### Table 2. Minimal Inhibitory Concentration of the Cyclo peptides Tested

| ID          | strain                  | MIC (μg/mL) |
|-------------|-------------------------|-------------|
|             |                         | L-Pro-L-Tyr | D-Pro-L-Tyr | L-Pro-L-Leu | L-Pro-L-Val |
| NCPPB 349  | Pseudomonas caryophylli | 15          | 800         | 500         | -           |
| ICMP3955   | Pseudomonas syringae pv. panici | 15          | 900         | 700         | -           |
| ICMP2706   | Pseudomonas syringae pv. tabaci | 15          | 800         | 500         | -           |
| B475       | Pseudomonas syringae pv. syringae | 15          | 800         | 600         | -           |
| ICMP6305   | Pseudomonas syringae pv. japonica | 15          | 800         | 600         | -           |
| PY79       | Bacillus subtilis        | 20          | 35          | 15          | -           |
| QMB        | Bacillus megaterium      | 20          | 30          | 30          | -           |
| DHSr       | Escherichia coli         | -           | 20          | 300         | -           |
| 17-5-5     | Colletotrichum truncatum | 20          | 800         | 500         | -           |

*No activity.
having a longer peptide side chain, the presence of some amino acid residues of the lactone ring is important to increase the activity as was the effect on the activity of tolaasin D for the presence of L-Ile instead of L-Leu residue. The derivatization of their amino acid residues of both the macrocyclic lactone ring and linear peptide side chain weakly affects inhibitory activity. Finally, for tolaasin D, considering the possibility of its large-scale production using a fermenter, a suitable bioformulation could have a potential for practical application as a bactericide and fungicide in agriculture and in particular against the pathogens of important agrarian plants that have developed resistance to the common chemical pesticides.

■ ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c08139.

1H NMR spectrum of tolaasin I in CD3OD at 500 MHz (Figure S1); 1H NMR spectrum of hexactyltolaasin I in CD3OD at 500 MHz (Figure S2); ESIMS spectrum of hexactyltolaasin I (Figure S3); 1H NMR spectrum of tetrahidrotolaasin I in CD3OD at 500 MHz (Figure S4); ESIMS spectrum of tetrahidrotolaasin I (Figure S5); 1H NMR spectrum of WLIP in CD3OD at 500 MHz (Figure S6); 1H NMR spectrum of WLIP methyl ester in CD3OD at 500 MHz (Figure S7); and ESIMS spectrum of WLIP methyl ester (Figure S8) (PDF)

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Notes
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■ REFERENCES

(1) Tilman, D.; Balzer, C.; Hill, J.; Befort, B. L. Global food demand and the sustainable intensification of agriculture. Proc. Natl. Acad. Sci. U.S.A. 2011, 108, 20260–20264.
(2) Food and Agriculture Organization of the United Nations. The Future of Food and Agriculture-Trends and Challenges: FAO: Rome, Italy, 2017.
(3) Rosegrant, M. W.; Cline, S. A. Global food security: challenges and policies. Science 2003, 302, 1917–1919.
(4) Foley, J. A.; Ramankutty, N.; Brauman, K. A.; Cassidy, E. S.; Gerber, J. S.; Johnston, M.; Mueller, N. D.; O’Connell, C.; Ray, D. K.; West, P. C.; Balzer, C.; Bennett, E. M.; Carpenter, S. R.; Hill, J.; Monfreda, C.; Polasky, S.; Rockstrom, J.; Sheehan, J.; Siebert, S.; Tilman, D.; Zaks, D. P. M. Solutions for a cultivated planet. Nature 2011, 478, 337–342.
(5) Oerke, E. C. Crop losses to pests. J. Agric. Sci. 2006, 144, 31–43.
(6) Cimmino, A.; Masi, M.; Evidente, M.; Superchi, S.; Evidente, A. Fungal phytotoxins with potential herbicidal activity: chemical and biological characterization. Nat. Prod. Rep. 2015, 32, 1629–1653.
(7) Godfray, H. C. J.; Beddington, J. R.; Crute, I. R.; Lawrence, H.; Lawrence, D.; Muir, J. F.; Sherman, P.; Roinson, S.; Thomas, S. M.; Toulmin, C. Food security: the challenge of feeding 9 billion people. Science 2010, 327, 812–818.
(8) Newman, D. J.; Cragg, G. M. Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. J. Nat. Prod. 2020, 83, 770–803.
(9) Bender, C. L.; Alarcon Chaidiz, F.; Gross, D. C. Pseudomonas syringae phytotoxins: Mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol. Mol. Biol. Rev. 1999, 63, 266–292.
(10) Takemoto, J. Y.; Brand, J. G.; Kaunin, Y. A.; Maley, V. V.; Schagina, L. V.; Blasko, K. The syringomycins: lipodepsipeptide pore formers from plant bacterium Pseudomonas syringae. In Pore-forming Peptides and Proteins; Menestrina, G.; Della Serra, M.; Lazaroivici, P., Eds.; Francis and Taylor: London, U.K., 2003; Vol. 5, pp 260–271.
(11) Ballio, A.; Grugurina, I. Bioactive Lipopeptides of Pseudomonas syringae. In Bacterial, Plant & Animal Toxins; Ascenzi, P.; Pollicelli, F.; Visca, P., Eds.; Research Signpost: Trivandrum, India, 2003; Vol. 76, pp 45–58.
(12) Scaloni, A.; Camoni, L.; Di Giorgio, D.; Scortichini, M.; Cozzolino, R.; Ballio, A. A new syringopeptin produced by a Pseudomonas syringae pv. syringae strain isolated from diseased twigs of laurel. Physiol. Mol. Plant Pathol. 1997, 51, 259–264.
(13) Ballio, A.; Bossa, F.; Camoni, L.; Di Giorgio, D.; Flamand, M. C.; Maraitte, H.; Nitti, G.; Pucci, P.; Scaloni, A. Structure of fuscopeptins, phytotoxic metabolites of Pseudomonas fuscoaginata. FEBS Lett. 1996, 381, 213–216.
(14) Emanuele, M. C.; Scaloni, A.; Lavermicocca, P.; Jacobellis, N. S.; Camoni, L.; Di Giorgio, D.; Pucci, P.; Paci, M.; Segre, A.; Ballio, A. Corceptin, a new bioactive lipodepsipeptid from cultures of Pseudomonas corrugata. FEBS Lett. 1998, 433, 317–320.
(15) Huang, C. J.; Pauwelyn, E.; Ongena, M.; Debois, D.; Leclère, V.; Jacques, P.; Bleyaert, P.; Höfte, M. Characterization of cichopeptins, new phytotoxic cyclic lipodepsipeptides produced by Pseudomonas cichorii SF1-S4 and their role in bacterial midrib rot disease of lettuce. Mol. Plant-Microbe Interact. 2015, 28, 1009–1022.
(16) Bionda, N.; Pitteloud, J. P.; Cudic, P. Cyclic lipodepsipeptides: a new class of antibacterial agents in the battle against resistant bacteria. Future Med. Chem. 2013, 5, 1311–1330.
(17) Nutkins, J. C.; Mortishire-Smith, R. J.; Packman, L. C.; Brodey, C. L.; Rainey, P. B.; Johnstone, K.; Williams, D. H. Structure determination of tolaasin, an extracellular lipodepsipeptide produced
by the mushroom pathogen, Pseudomonas tolaasii Paine. J. Am. Chem. Soc. 1991, 113, 2621−2627.

(18) Mortishire-Smith, R. J.; Nutkins, J. C.; Packman, L. C.; Brodey, C. L.; Rainey, P. B.; Johnstone, K.; Williams, D. H. Determination of the structure of an extracellular peptide produced by the mushroom saprotroph Pseudomonas reactans. Tetrahedron 1991, 47, 3645−3654.

(19) Andolfi, A.; Cimmino, A.; Canatore, P. L.; Iacobellis, N. S.; Evidente, A. Bioactive and structural metabolites of Pseudomonas and Burkholderia species causal agents of cultivated mushrooms diseases. Perspect. Med. Chem. 2008, 2, 81−112.

(20) Bassarello, C.; Lazzaroni, S.; Bifulco, G.; Lo Cantore, P.; Iacobellis, N. S.; Riccio, R.; Gomez-Paloma, L.; Evidente, A. Tolaasins A−E, five new lipodepsipeptides produced by Pseudomonas tolaasii. J. Nat. Prod. 2004, 67, 811−816.

(21) Albericio, F.; Alvarez, M.; Bruno, P.; Canedo, L.; Cuevas, C.; Francesch, A.; Garcia-Ramos, Y.; Just, X.; Martin, J.; Munt, S. et al. State of the Art in the Synthesis of Complex Natural Marine Peptides. Proceedings of the 4th Asia-Pacific International Peptide Symposium/50th Japanese Peptide Symposium; Osaka, Japan, Nov 7, 2013; pp 13−16.

(22) Daihya, R.; Daihya, S. Natural bioeffective cyclooligopeptides from plant seeds of Annona genus. Eur. J. Med. Chem. 2021, 214, No. 113221.

(23) Scalet, M.; Marchesan, S. Diketopiperazine gels: New horizons from the self-assembly of cyclic dipetides. Molecules 2021, 26, No. 3376.

(24) Sterle, A. C.; Cardellina, J. H.; Strobel, G. A. Maculosin, a host-specific phytotoxin for spotted knapweed from Pseudomonas tolaasii. J. Appl. Microbiol. 1998, 85, 8008−8011.

(25) Puopolo, G.; Cimmino, A.; Palmieri, M. C.; Giovannini, O.; Evidente, A.; Pertot, I. Lysobacter capsici AZ78 produces cyclo(L-Pro-L-Tyr), a 2,5-diketopiperazine with toxic activity against sporangia of Alternaria alternata. J. Appl. Microbiol. 2014, 117, 1168−1180.

(26) Cimmino, A.; Puopolo, G.; Perazzoli, M.; Andolfi, A.; Melch, D.; Pertot, I.; Evidente, A. Cyclo(L-Pro-L-Tyr), the fungicide isolated from Lysobacter capsici AZ78: a structure-reactivity relationship study. Chem. Heterocycl. Compd. 2014, 50, 290−295.

(27) Cimmino, A.; Bejarano, A.; Masi, M.; Puopolo, G.; Evidente, A. Isolation of 2,5-diketopiperazines from Lysobacter capsici AZ78 with activity against Rhodococcus fascians. Nat. Prod. Res. 2021, 35, 4969−4977.

(28) Peng, J. T. Resistance to Disease in Agaricus bisporus (Lange) Imbach. Ph.D. Thesis, University of Leeds, 1986.

(29) Lo Cantore, P.; Lazzaroni, S.; Coraiola, M.; Serra, M. D.; Cañadas, C.; Evidente, A.; Iacobellis, N. S. Biological characterization of white line-inducing principle (WLIP) produced by Pseudomonas reactans NCPPB1311. Mol. Plant-Microbe Interact. 2006, 19, 1113−1120.

(30) Segarra, G.; Puopolo, G.; Giovannini, O.; Pertot, I. Stepwise flow diagram for the development of formulations of non spore-forming bacteria against foliar pathogens: the case of Lysobacter capsici AZ78. J. Biotechnol. 2015, 216, 56−64.

(31) Aigrillo, B.; Mirino, S.; Tatè, R.; Gratino, O.; Gogliettino, M.; Cocca, E.; Tablì, N.; Nabhí, E.; Palmieri, G. An alternative biocontrol agent of soil-borne phytopathogens: A new antifungal compound produced by a plant growth promoting bacterium isolated from North Algeria. Microbiol. Res. 2019, 221, 60−69.

(32) Yamaguchi, T. Horticulture in Japan; Asakura Publishing Co. Ltd (in Japanese): Tokyo, Japan, 1994.

(33) Kusumi, T.; Ohtani, I.; Nishiyanuma, K.; Kakishawa, H. Caryocycines, potent antibiotics from a plant pathogen Pseudomonas caryophylli. Tetrahedron Lett. 1987, 28, 3981−3984.

(34) Molinaro, A.; Newman, M. A.; Lanzetta, R.; Parrilli, M. The structures of lipopolysaccharides from plant-associated Gram-negative bacteria. Eur. J. Org. Chem. 2009, 2009, 5887−5896.

(35) Adinolfi, M.; Corsaro, M. M.; De Castro, C.; Lanzetta, R.; Parrilli, M.; Evidente, A.; Lavermicocca, P. A novel 4-C branched sugar from the lipopolysaccharide of the bacterium Pseudomonas caryophylli. Carbohydr. Res. 1995, 267, 307−311.

(36) Adinolfi, M.; Corsaro, M. M.; De Castro, C.; Lanzetta, R.; Parrilli, M. The relative and absolute configurations of stereocenters in caryophyllose. Carbohydr. Res. 1995, 274, 223−232.

(37) Adinolfi, M.; Corsaro, M. M.; De Castro, C.; Evidente, A.; Lanzetta, R.; Magioni, L.; Parrilli, M. Biological characterization of the Art in the Synthesis of Complex Natural Marine Peptides. Proceedings of the 4th Asia-Pacific International Peptide Symposium/50th Japanese Peptide Symposium; Osaka, Japan, Nov 7, 2013; pp 13−16.

(38) Daihya, R.; Daihya, S. Natural bioeffective cyclooligopeptides from plant seeds of Annona genus. Eur. J. Med. Chem. 2021, 214, No. 113221.

(39) Scalet, M.; Marchesan, S. Diketopiperazine gels: New horizons from the self-assembly of cyclic dipetides. Molecules 2021, 26, No. 3376.

(40) Sterle, A. C.; Cardellina, J. H.; Strobel, G. A. Maculosin, a host-specific phytotoxin for spotted knapweed from Pseudomonas tolaasii. J. Nat. Prod. 1998, 85, 8008−8011.

(41) Puopolo, G.; Cimmino, A.; Palmieri, M. C.; Giovannini, O.; Evidente, A.; Pertot, I. Lysobacter capsici AZ78 produces cyclo(L-Pro-L-Tyr), a 2,5-diketopiperazine with toxic activity against sporangia of Phytophthora infestans and Plasmopara viticola. J. Appl. Microbiol. 2014, 117, 1168−1180.

(42) Cimmino, A.; Puopolo, G.; Perazzoli, M.; Andolfi, A.; Melch, D.; Pertot, I.; Evidente, A. Cyclo(L-Pro-L-Tyr), the fungicide isolated from Lysobacter capsici AZ78: a structure-reactivity relationship study. Chem. Heterocycl. Compd. 2014, 50, 290−295.

(43) Cimmino, A.; Bejarano, A.; Masi, M.; Puopolo, G.; Evidente, A. Isolation of 2,5-diketopiperazines from Lysobacter capsici AZ78 with activity against Rhodococcus fascians. Nat. Prod. Res. 2021, 35, 4969−4977.

(44) Peng, J. T. Resistance to Disease in Agaricus bisporus (Lange) Imbach. Ph.D. Thesis, University of Leeds, 1986.

(45) Lo Cantore, P.; Lazzaroni, S.; Coraiola, M.; Serra, M. D.; Cañadas, C.; Evidente, A.; Iacobellis, N. S. Biological characterization of white line-inducing principle (WLIP) produced by Pseudomonas reactans NCPPB1311. Mol. Plant-Microbe Interact. 2006, 19, 1113−1120.

(46) Segarra, G.; Puopolo, G.; Giovannini, O.; Pertot, I. Stepwise flow diagram for the development of formulations of non spore-forming bacteria against foliar pathogens: the case of Lysobacter capsici AZ78. J. Biotechnol. 2015, 216, 56−64.

(47) Aigrillo, B.; Mirino, S.; Tatè, R.; Gratino, O.; Gogliettino, M.; Cocca, E.; Tablì, N.; Nabhí, E.; Palmieri, G. An alternative biocontrol agent of soil-borne phytopathogens: A new antifungal compound produced by a plant growth promoting bacterium isolated from North Algeria. Microbiol. Res. 2019, 221, 60−69.

(48) Yamaguchi, T. Horticulture in Japan; Asakura Publishing Co. Ltd (in Japanese): Tokyo, Japan, 1994.