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Antimicrobial Activities of Novel Mannosyl Lipids Isolated from the Biocontrol Fungus *Simplicillium lamellicola* BCP against Phytopathogenic Bacteria

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Supporting Information

ABSTRACT: The antagonistic fungus *Simplicillium lamellicola* BCP has been developed as a microbial biopesticide that effectively controls the development of various plant diseases caused by both pathogenic bacteria and pathogenic fungi. Antibacterial bioassay-directed fractionation was used to isolate mannosyl lipids from *S. lamellicola* BCP, and the structures of these compounds were elucidated using spectral analysis and chemical degradation. Three novel mannosyl lipids were characterized and identified as halymecins F and G and (3*R*,5*R*)-3-*O*-*β*-D-mannosyl-3,5-dihydrodecanoic acid. Massoia lactone and (3*R*, 5*R*)-3-hydroxydecan-5-olide were also isolated from *S. lamellicola* BCP. The three novel compounds inhibited the growth of the majority of phytopathogenic bacteria that were tested, and halymecin F displayed the strongest antibacterial activity. *Agrobacterium tumefaciens* was the most sensitive to the three novel compounds, with IC_{50} values ranging from 1.58 to 24.8 μg/mL. The ethyl acetate extract of the fermentation broth from the antagonistic fungus effectively reduced the bacterial wilt caused by *Ralstonia solanacearum* on tomato seedlings. These results indicate that *S. lamellicola* BCP suppresses the development of plant bacterial diseases through the production of antibacterial metabolites.

KEYWORDS: *biocontrol, halymecin, mannosyl lipid, antimicrobial activity, Simplicillium lamellicola*

INTRODUCTION

Phytopathogenic fungi and bacteria cause massive damage and reduce the yield of crops. The resistance of plant pathogens to commercial synthetic agrochemicals, the desire for organic food, and environmental concerns have led to intensive research efforts for integrated control using antagonistic microorganisms and microorganism-derived natural products.[1,2] As biodegradable compounds, microbial natural products are attractive candidates for development as agrochemicals. A number of natural fungicides such as kasugamycin, polyoxins, validamycin, and blasticidin-S, and bactericides such as oxytetracyline and streptomycin have been isolated from microbial resources. These compounds are well-known commercial agrochemicals derived from *Streptomyces* species and have been effectively used to control phytopathogenic diseases caused by the fungi *Botrytis cinerea*, *Colletotrichum coccodes*, *Magnaporthe oryzae*, *Phytophthora infestans*, *Puccinia recondita*, and *Rhizoctonia solani* and the bacteria *Xanthomonas oryzae*, *Xanthomonas citri*, and *Pseudomonas* species.[1,3]

The fungal strain used to produce the mannosyl lipids in the present study was *Simplicillium lamellicola* BCP, which was isolated from the mycelia of *Botrytis cinerea*. This strain was previously known as *Acremonium strictum* BCP on the basis of morphological identification.[4] However, re-examination of the strain using sequence analyses of the 28S rRNA gene and ITS regions identified it as *S. lamellicola*. The antagonistic fungus effectively suppressed various plant diseases caused by pathogenic fungi, including *B. cinerea*, and *S. lamellicola* was commercialized as the microbial fungicide Acre.[5] Its antifungal mechanism is both mycoparasitism and antibiosis.[4,6] The antagonistic fungus overgrew the colonies of *B. cinerea* and caused severe lysis of the host hyphae. Hyphal growth of BCP strain inside the mycelia of *B. cinerea* was observed.[6] In addition, it produces verlamelin as an antifungal metabolite, which is active in vitro to phytopathogenic fungi, but not to phytopathogenic bacteria.[4] During evaluation of the disease control efficacy of this microbial fungicide in fields, we found that it also highly suppressed the development of tomato bacterial wilt caused by *Ralstonia solanacearum*. Therefore, the current study was performed to isolate and determine the chemical structures of the antibacterial metabolites from the

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fermentation broth of the biocontrol fungal strain *S. lamellicola* BCP. In addition, the in vitro and in vivo activities of the ethyl acetate layer and pure compounds were evaluated against phytopathogenic bacteria.

MATERIALS AND METHODS

Organisms and Culture Conditions. The following seven phytopathogenic bacterial strains were used for the antibacterial bioassay: *Acidovorax konjaci*, the causal agent of bacterial blight of konjac; *Agrobacterium tumefaciens*, the causal agent of crown gall disease; *Burkholderia glumae*, the causal agent of bacterial grain rot of rice; *Pectobacterium carotovorum* subsp. *carotovorum*, the causal agent of bacterial soft rot of potato; *Pseudomonas syringae* pv *lachrymans*, the causal agent of cucumber angular leaf spot; *R. solanacearum*, the causal agent of tomato bacterial wilt; and *Xanthomonas euvesicatoria*, the causal agent of pepper bacterial spot. All strains were grown on nutrient agar (NA) (Becton, Dickinson and Co., Sparks, MD, USA) and nutrient broth (NB), except *R. solanacearum*, which was grown in tryptone soy agar (TSA) (Becton, Dickinson and Co.) and tryptone soy broth (TSB) (Becton, Dickinson and Co.). *X. euvesicatoria* was cultured aerobically at 25 °C for 18−36 h, and the remaining strains were cultured aerobically at 30 °C for 18−36 h.

Isolation of Antibacterial Substances. Strain BCP was cultured on potato dextrose broth (PDB) medium (Becton, Dickinson and Co.) on a rotary shaker at 150 rpm and at 25 °C for 10 days as described previously by Kim et al.[4] The broth culture (10 L) was centrifuged at 10000*g* for 10 min. The obtained supernatant was mixed with an equal volume of acetone and incubated in a fume hood for 24 h. The precipitates were filtered out using filter paper, and the filtrate was concentrated under a vacuum to remove the acetone. The aqueous layer was then partitioned twice with equal volumes of ethyl acetate (EtOAc). The EtOAc layers were pooled and concentrated in a rotary evaporator at 40 °C to remove the organic solvent. The EtOAc concentrate (2.5 g) showed strong activity against *R. solanacearum* as determined by a disk diffusion assay.[4] The EtOAc concentrate was subjected to chromatography on a silica gel column [100 g of Kiesel gel 60 (230−400 mesh; E. Merck, Darmstadt, Germany), 3.4 cm × 40 cm, packed with EtOAc/methanol (MeOH) (9:1, v/v)] with successive elution with mixtures of EtOAc/MeOH/water (W) (80:10:2, v/v/v, 200 mL), EtOAc/MeOH/W (80:10:5, v/v/v, 200 mL), EtOAc/MeOH/W/acetic acid (80/10/8/1, v/v/v/v, 500 mL), and dichloromethane (DCM/MeOH/W (60:40:10, v/v/v, 500 mL), yielding 15 fractions, ABE 1−15. The fractions were monitored using thin-layer chromatography (TLC) (Kiesel gel 60GF 254, 0.25-mm layer thickness; E. Merck) with the developing solvent EtOAc/MeOH/W/acetic acid (80:10:8:1, v/v/v/v). The fractions were also assayed in vitro for growth inhibition of *R. solanacearum*. The active fractions ABE 5−8 displayed a similar pattern of primary components based on the TLC analysis and were pooled and further purified repeatedly using preparative HPLC (SCL-10A VP; Shimadzu Co., Kyoto, Japan) with a Capcell Pak C18 UG120 Å (5 μm, 20 mm × 250 mm; Shiseido Co., Tokyo, Japan). The combined fraction (460 mg) was eluted with a linear gradient from 45% MeOH to 100% MeOH for 30 min and then maintained at 100% MeOH for 10 min. The eluent (6.5 mL/min) was monitored at 204, 215, and 254 nm. Two compounds, **1** (85 mg) and **2** (23 mg), were isolated to purity by repeated preparative HPLC. Compound **3** (12 mg) was purified from fraction ABE 13 by preparative TLC (Kiesel gel 60GF 254, 0.5 mm layer thickness; E. Merck) with the developing solvent EtOAc/MeOH/W/acetic acid (80:10:8:1, v/v/v/v).

Degradative products of **1** were prepared by directed lactonization of fatty acids in the structure of **1** (12 mg), with the catalysis of 0.1 N HCl in CH_3CN (2 mL of a mixture of 1 mL of 0.1 HCl aq + 1 mL of CH_3CN). The reaction was performed at 80 °C for 2 h. The product was monitored by TLC with hexane/EtOAc (1:3, v/v) as the developing solvent. After working up the reaction, the nonpolar product was extracted twice with 1 mL of a mixture of EtOAc and hexane (3:1, v/v). The organic layers were pooled and concentrated under an N_2 stream at 40 °C until dryness. The residue was separated by preparative TLC using a mixture of hexane/EtOAc (1:3, v/v) to yield 4.2 mg of compound **4** and 5 mg of compound **5**.

The aqueous layer was concentrated to dryness and redissolved in 0.2 mL of 2 N HCl in a 7 mL screw-top vial. The vial was heated to 100 °C for 4 h to hydrolyze the sugar moiety. After hydrolysis, the mixture of products was divided into two parts. One portion was partitioned with DCM to remove the nonpolar impurities, and the aqueous layer was evaporated off under an N_2 stream. The residue was dissolved in MeOH and analyzed by TLC using DCM/MeOH/W (6:4:1, v/v/v) and was compared with authentic monosaccharides. The second portion was neutralized with $NaHCO_3$ and dried under an N_2 stream. The residue was acetylated with 0.2 mL of acetic anhydride/pyridine (1:1, v/v) at 100 °C for 45 min. After the acetylation reaction, the mixture of acetic anhydride/pyridine was removed under an N_2 stream. The residue containing the acetylated monosaccharide was dissolved into 0.2 mL of 2% $CuSO_4$ in water and partitioned twice with 0.5 mL of EtOAc. The EtOAc layers were pooled and dried under a nitrogen stream. The residue obtained after drying was dissolved in 50 μL of chloroform for GC-MS analysis. Authentic samples of glucose, rhamnose, mannose, galactose, and sorbitol (0.5 mg each) were also acetylated. The acetylated monosaccharides were analyzed by GC-MS on an SP2330 column (30 m × 0.32 mm i.d.; 0.25 μm film thickness; Supelco, Inc., Bellefonte, PA, USA) using He as the carrier gas with a temperature program of 120 °C (2 min) to 230 °C (5 °C min^{-1}) maintained for 12 min.

Structure Determination of Antibacterial Substances. The optical rotations of the purified compounds in MeOH were recorded on a JASCO J-20 automatic recording spectropolarimeter using a 1 cm path-length cell. The chemical structures were determined by mass and nuclear magnetic resonance (NMR) spectroscopy. Fast atom bombardment (FAB) analysis was performed with a glycerol matrix and Ar as the bombarding gas using a high-resolution mass spectrometer (JMS-AX505; JEOL Ltd., Tokyo, Japan). The electrospray ionization mass spectra (ESI-MS) of the purified compounds were recorded on an MSD1100 single-quadrupole mass spectrometer equipped with an ESI (Hewlett-Packard Co., Palo Alto, CA, USA). ^1H and ^{13}C NMR, COSY, HMQC, and HMBC spectra were recorded in CD_3OD (E. Merck) with a Bruker AMX-500 spectrometer (Bruker Analytische Messtechnik Gmbh, Rheinstetten, Germany).[7] The spectra were referenced to either tetramethylsilane (TMS) (^1H) or solvent signals (^{13}C). The coupling constants are reported in hertz. The chemical structures of the purified substances were analyzed on the basis of the MS and NMR spectral data and comparisons to reported values in the literature.

In Vitro Antibacterial Assay against Phytopathogenic Bacteria. An in vitro antibacterial assay was performed in 96-well microtiter plates based on a modification of the broth microdilution method.[8,9] The *R. solanacearum* strain was incubated in TSB medium, and the other bacteria were incubated in NB medium. The stock solutions of pure compounds dissolved in MeOH were diluted 100-fold with the proper growth medium for each strain and tested at final concentrations ranging from 0.39 to 100 μg/mL. Bacterial suspensions (50 μL) of each strain with an inoculum of 2×10^6 cells/mL were added to the wells containing 50 μL of 2-fold serially diluted pure compounds in the proper growth medium for a final volume of 100 μL in each well. The negative controls were treated with 1% MeOH, which corresponds to the highest concentration. The blank wells were prepared with culture medium containing pure compounds at the same test concentrations. The dilutions were prepared with three replicates, and the experiments were performed a minimum of three times. The inoculated plates were incubated at 30 °C, except for *X. euvesicatoria* at 25 °C, for 18−48 h after shaking at 300 rpm for 20 min on a microplate shaker. The optical density (OD) of each well was measured using a microplate reader (Bio-Rad, Benchmark Plus, USA) at 600 nm. The growth inhibition of each dilution was determined using the formula[9]

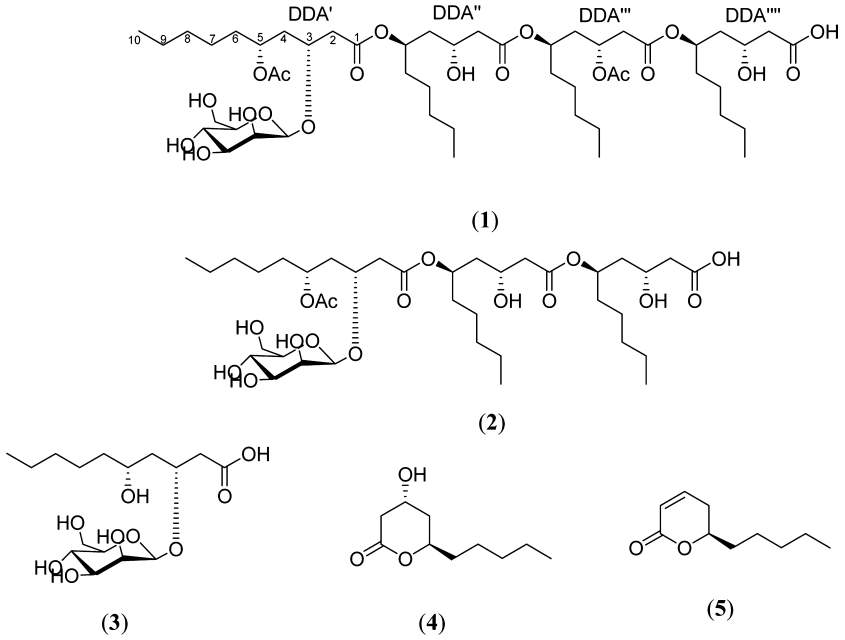

Figure 1. Chemical structures of the compounds isolated from the ethyl acetate layer of the *Simplicillium lamellicola* BCP broth. **1**, halymecin F; **2**, halymecin G; **3**, (3*R*,5*R*)-3-*O*-*β*-D-mannosyl-3,5-dihydroxydecanoic acid; **4**, (+)-(3*R*,5*R*)-3-hydroxydecan-5-olide; **5**, (−)-massoia lactone; DDA, 3, 5-dihydroxydecanoic acid.

$$\%\ \text{inhibition} = 100 \times [1 - \text{OD of treated well} / \text{OD of negative control well}]$$

where OD values of the negative control well and the OD of the treated well were corrected with the OD of the blank wells corresponding to each concentration. The IC_{50} values were derived from Probit analysis of the concentration−response data, with serially diluted concentrations of the pure compounds.

Disease Control Efficacy of the Ethyl Acetate Layer of *S. lamellicola* BCP against Tomato Bacterial Wilt. To evaluate the disease control efficacy of the EtOAc extract of *S. lamellicola* BCP culture broth against tomato bacterial wilt caused by *R. solanacearum* SL1944 (race 1, biovar 4),[10,11] 3-week-old tomato plants at the 4−5-true-leaf stage of 'Seokwang' were used. The plants were grown in vinyl pots with a volume of 170 mL in a greenhouse and were then transplanted into vinyl pots with a diameter of 7 cm (one plant per pot). Three different amounts of the EtOAc layer, 50, 100, and 200 mg, were dissolved in 2 mL of MeOH and then poured into 98 mL of distilled water containing 250 μg/mL Tween-20 to obtain test concentrations of 500, 1000, and 2000 μg/mL. *R. solanacearum* was cultured on TSA Petri dishes at 30 °C for 48 h and were subsequently washed twice from the dishes with sterile distilled water. The inoculum suspension was adjusted to an optical density OD_{600} of 0.1 (approximately 1.5×10^8 cells/mL) using sterile distilled water. The 20 mL aliquots of three EtOAc extract solutions were evenly applied to the soil of each pot. After 3 h, *R. solanacearum* was inoculated by applying 20 mL of the inoculum suspensions into the soil. The second treatment with the EtOAc layer was performed 5 days after the first treatment. Streptomycin sulfate (SM), a bactericidal antibiotic with a broad activity against both Gram-negative and Gram-positive bacteria, was used as a positive control at a concentration of 200 μg/mL. Pots treated with water containing Tween-20 (250 μg/mL) and MeOH (2%) served as the negative controls (CK). The plants were maintained in a controlled climate at 30 °C with a relative humidity of 70−80%. The disease severity was recorded on a scale of 0−5 as described by Winstead and Kelman.[12] The following scales were used: 0 = no symptoms, 1 = one leaf partially wilted, 2 = one to two leaves wilted, 3 = two to three leaves wilted, 4 = four or more leaves wilted, and 5 = death of the entire plant. The pots were arranged as a randomized complete block with five replicates per treatment. The experiment was repeated three times. The control value was calculated using the following formula: control value (%) = 100 × (disease severity of control − disease severity of treatment)/disease severity of control.

Statistical Analysis. The data obtained in this study were evaluated by one-way ANOVA, and the significance of the treatments was determined by Tukey's HSD for multiple comparisons ($P \leq 0.05$). The IC_{50} values were calculated on the basis of Probit analysis using WINPEPI software version 11.4.[13]

RESULTS AND DISCUSSION

This study focused on the identification of antibacterial metabolites produced by the biocontrol agent *S. lamellicola* BCP and the in vitro and in vivo activities of these compounds against phytopathogenic bacteria to identify either alternatives to currently used crop protection agents or lead compounds to synthesize derivatives with enhanced activities or environmental friendliness. The *S. lamellicola* BCP strain, previously identified as *A. strictum* BCP on the basis of morphological characteristics, was developed as a microbial fungicide for the control of various plant diseases, such as tomato gray mold and rice blast.[5,6] However, in the present study, we identified the strain as *S. lamellicola* BCP on the basis of sequence analysis of the 28S rRNA gene and ITS regions (Figures S1 and S2 in the Supporting Information). In addition, we found that the microbial fungicide also effectively suppressed the development of tomato bacterial wilt in fields. We previously reported that the antagonistic fungus produces verlamelin as an antifungal metabolite.[4] However, the metabolite did not show any antibacterial activity. Therefore, in this study, we characterized the antibacterial metabolites produced by *S. lamellicola* BCP.

Characterization of Substances from the Culture Broth of *S. lamellicola* BCP. When investigating the bioactivities of the EtOAc extract from the *S. lamellicola* BCP culture broth, we found that the EtOAc layer significantly inhibited the growth of *R. solanacearum* and also contained trimers and tetramers of (3*R*,5*R*)-3,5-dihydroxydecanoic acids (DDA) as the primary metabolites. Using bioassay-guided

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fractionation, the antibacterial metabolites were isolated from the EtOAc layer by various chromatographic methods.

Compound **1**, a main component (Figure 1) ($[\alpha]^{20}_{D}$ −11.9°; *c* 0.27, MeOH), was obtained as a colorless oil, and its molecular formula is $C_{50}H_{88}O_{20}$ as determined by FAB-MS ($[M + Na]^+$ 1032) and ESI-MS ($[M + Na]^+$ 1031.6) data. The FAB-MS and positive ESI-MS data (Figure 2) of **1** exhibited

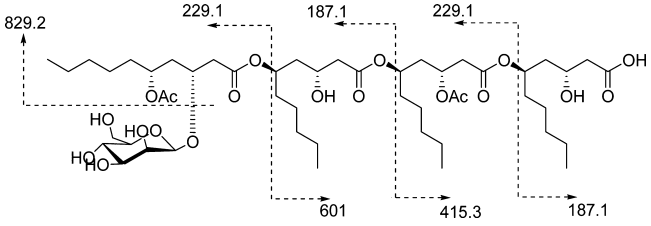

Figure 2. Fragmentation of halymecin F (**1**) ($C_{50}H_{88}O_{20}$, quasi-molecular ion peak *m/z* 1031.6 $[M + Na]^+$) generated by ESI-MS.

significant fragment peaks at *m/z* 829.2 $[M + H - 180]^+$, *m/z* 601 $[M + H - 180 - C_{12}H_{20}O_4]^+$, *m/z* 583 $[601 - H_2O]^+$, *m/z* 523 $[583 - 60]^+$, *m/z* 415.3 $[C_{22}H_{39}O_7]^+$, *m/z* 355.3 $[C_{22}H_{39}O_7 - 60]^+$, *m/z* 229.1 $[C_{12}H_{21}O_4]^+$, and *m/z* 187.1 $[C_{10}H_{19}O_3]^+$. Hydrolysis of **1** after lactonization yielded a hydrolysate containing a monosaccharide moiety. TLC analysis revealed that the sugar moiety is D-mannose on the basis of comparison to the authentic compound. GC-MS analysis of the acetylated product of the hydrolysate also confirmed the presence of pentaacetyl-β-D-mannose. These data suggest that **1** contains β-D-mannose, two acetyl groups (two losses of 60 in the MS spectrum), and $C_{10}H_{19}O_3$ units. The ^{1}H NMR spectrum of **1** (Table 1) displayed signals corresponding to four terminal methyl groups at δ 0.89 (12H, t, *J* = 7.01), two acetyl groups at δ 2.01 (3H, s) and 2.02 (3H, s), an anomeric proton at δ 4.61 (1H, brs), and a region of protons of oxygenated carbons. The ^{13}C NMR spectrum of **1** revealed the presence of 1 anomeric carbon at δ 101.18, 13 oxygenated carbons from δ 62.97 to δ 78.28, 6 carbonyl groups in the range of δ 172.47−173.63, and the methyl carbons of 2 acetyl groups at δ 21.24 (Table 1). On the basis of analysis of the COSY and HMQC spectra, assignment of the β-D-mannose moiety was verified. The proton spin system of the H1−H6 of each fatty acid was determined by COSY, and the position of the carbonyl group was determined by a cross peak between the methylene protons of the C-2 and the carbon of the carbonyl group. The long-range correlations of H-3 (δ 4.21, DDA′) with an anomeric carbon (101.18) on the HMBC spectrum demonstrated that the β-D-mannose moiety was situated at the C-3 of DDA′. The linkage of the C-5 with an acetyl group was deduced from cross peaks between the protons of the acetyl group and the C-4 (δ 39.84), C-5 (δ 72.78), and C-6 (δ 35.41) in the HMBC spectrum and the fragment peak at *m/z* 602 on the FAB-MS spectrum. The ester linkages between the carbonyl carbons with the C-5 in the remaining fatty acid chains were also deduced from the HMBC data. Furthermore, the HMBC data led to the assignment of the other acetyl group at the C-3 of DDA‴. The lactonization of **1** produced a known δ-lactone (**4**), which was identified as 3-hydroxydecan-5-olide. Its carbon chiral centers were determined as (3*R*, 5*R*) on the basis of the ^{1}H and ^{13}C NMR spectra and optical rotary power. A review of the literature and a comparison of the spectral data of **1** with mannosyl lipids suggested that the structure of **1** was a tetramer of 3,5-dihydrodecanoyl and that its structure was similar to that of halymecin B,[14] except for an additional acetyl group attached to the C-5 of DDA′. Compound **1** was named halymecin F, and its chemical structure is presented in Figure 1.

Compound **2** ($[\alpha]^{20}_{D}$ −8.99°; *c* 0.2, MeOH) was named halymecin G. Its molecular formula is $C_{38}H_{68}O_{16}$ as determined from the quasi-molecular ion peaks at *m/z* $[M + Na]^+$ 803.2 and *m/z* $[M - H]^-$ 779.7 in positive and negative ESI-MS spectra, respectively. These data suggest that **2** lacks one acetyl group and a DDA compared to **1**. The positive-FAB-MS spectrum of **2** showed significant fragment peaks at *m/z* 601 $[M + H - 180]^+$, *m/z* 373 $[C_{20}H_{37}O_6]^+$, and *m/z* 187.1 $[C_{10}H_{19}O_3]^+$. The fragmentation of 373 indicated a dimer of two DDA units without an acetyl group, and these data suggest that compound **2** is a partial structure of **1** and has an acetyl group located at DDA′. The ^{1}H and ^{13}C NMR spectra of **2** showed the presence of one acetyl group, one mannosyl moiety, four carbonyl carbons, and three terminal methyl groups. The location of the mannose moiety is at the C-3 of DDA′ via glycosidic linkage as determined by the HMBC correlation peaks at H-1 (4.61, mannose) with a carbon signal at 74.94. The stereochemical analysis for the DDAs of **2** was also performed as described for **1**, and the chemically degraded product was determined to be (3*R*,5*R*)-3-hydroxydecan-5-olide. Analysis of the 1D-NMR spectroscopic data and interpretation of the HMBC and HSQC allowed us to assign all signals of **2** and identified the compound as a monoacetate trimer of DDA units (Table 1 and Figure 1). Compound **2** was named halymecin G.

Compound **3** ($[\alpha]^{20}_{D}$ −2.9°; *c* 0.176, MeOH) has the molecular formula $C_{16}H_{30}O_9$ as determined by ESI-MS ($[M + Na]^+$ 389.2 and $[M - H]^-$ 365.4). The negative-ESI-MS spectrum of **3** showed a fragment peak at *m/z* 179.2 $[C_{10}H_{19}O_3 - H_2O]^+$, indicating a dehydrated DDA. The ^{1}H NMR spectrum of **3** exhibited a singlet of the anomeric proton H-1 (mannose) at δ 4.66 and a terminal methyl group at δ 0.91. The ^{13}C NMR spectrum had 16 carbon signals, including 6 carbons belonging to a β-D-mannose moiety and 10 carbons for one DDA. The position of the glycosidic linkage of the mannosyl and DDA was also determined by the HMBC analysis. Finally, compound **3** was identified as (3*R*,5*R*)-3-*O*-β-D-mannosyl-3,5-dihydroxydecanoic acid, and its NMR data and structure are presented in Table 1 and Figure 1.

δ-Lactones **4** and **5** were identified as (+)-(3*R*,5*R*)-3-hydroxy-decan-5-olide (EI-MS *m/z* 168 $[M - H_2O]^+$, $C_{10}H_{18}O_3$; $[\alpha]^{20}_{D}$ +39.9°; *c* 0.05, CH_2Cl_2), and (−)-massoia lactone (EI-MS *m/z* 168 $[M]^+$, $C_{10}H_{16}O_2$; $[\alpha]^{20}_{D}$ −65.9°; *c* 0.2, CH_2Cl_2),[15,16] respectively, on the basis of the analysis of the EI-MS, GC-MS, and 1D-NMR data (Figure 1; Table S1 and Figure S3 in the Supporting Information). In a previous study, compounds **4** and **5** were found as biologically active δ-lactones and were chemically synthesized from streptenol A. Optical rotary powers (**4**, $[\alpha]^{20}_{D}$ +36.9°; *c* 0.92, CH_2Cl_2; **5**, $[\alpha]^{20}_{D}$ −112.5°; *c* 1 $CHCl_3$) and MS and 1D-NMR data of these synthesized compounds were reported by Romeyke et al.[15] Although compounds **4** and **5** are chemically degraded products of compounds **1**−**3**, their presence in the EtOAc layer of the *S. lamellicola* BCP culture broth was also confirmed with TLC and GC-MS.

Halymecins were previously reported to be tetramers and trimers of (3*R*,5*R*)-3,5-dihydroxydecanoic acid isolated from *Fusarium* spp. and *Exophiala pisciphila*. All of the microorganism resources were collected from marine algae and sponges,[14,17] whereas *S. lamellicola* BCP was isolated as a

Table 1. ^{1}H and ^{13}C NMR (500 MHz) Data of Compounds 1−3 in Methanol-d_4[a]

	1		2		3	
position	^{13}C[b]	^{1}H	^{13}C[b]	^{1}H	^{13}C	^{1}H
mannose						
1′	101.18	4.61 brs	101.23	4.61 brs	100.7	4.66 brs
2′	72.76	3.83 m	72.70	3.83 m	72.71	3.87 m
3′	75.27	3.44 m	75.25	3.43 m	75.21	3.44 m
4′	68.56	3.53 m	68.60	3.52 m	68.55	3.53 m
5′	78.27	3.22 m	78.29	3.2 m	78.44	3.22 m
6′	62.97	3.71 m; 3.82 m	62.99	3.69 m; 3.83 m	62.93	3.69 m; 3.84 m
DDA′[c]						
1	173.06		173.14		173.06	
2	41.83	2.57 m; 2.85 m	42.10	2.36 m; 2.48 m	37.76	2.76 m; 2.84 m
3	74.93	4.21 m	74.94	4.17 m	78.01	4.67 m
4	39.84	1.74−1.92 m	39.82	1.82−1.91 m	36.63	1.64−1.73 m
5	72.84	5.03 m	72.80	5.03 m	70.83	4.40 m
6	35.41b	1.60 brs	35.39b	1.59 brs	33.75	1.62 brs
7	25.97c	1.31 brs	25.94c	1.29 brs	25.65	1.34 brs
8	32.83d	1.31 brs	32.79d	1.29 brs	32.77	1.34 brs
9	23.71e	1.31 brs	23.62	1.29 brs	23.61	1.34 brs
10	14.44f	0.89 t (7.01)	14.42	0.89 t (7.06)	14.41	0.91 t (6.9)
*CH_3*CO[d]	21.24	2.02 s	21.29	2.02 s		
CH_3*CO*	172.61		172.65			
DDA″						
1	172.47		173.06			
2	40.76	2.43−2.49 m	43.19	2.38−2.48 m		
3	66.77a	4.09 m	66.76a	3.54 m		
4	42.11	1.72−1.78 m	42.22	1.72−1.79 m		
5	73.71	5.05 m	73.13	5.07 m		
6	35.10b	1.61 brs	35.11b	1.59 brs		
7	25.91c	1.31 brs	25.94c	1.29 brs		
8	32.78d	1.31 brs	32.79d	1.29 brs		
9	23.60e	1.31 brs	23.62	1.29 brs		
10	14.37	0.89 t (7.01)	14.42	0.89 t (7.06)		
DDA‴						
1	172.63		175.69			
2	43.57	2.48−2.51 m	43.55	2.38−2.48 m		
3	70.02	5.28 m	66.85a	3.54 m		
4	39.30	1.81 m	42.36	1.72−1.79 m		
5	72.61	4.93 m	72.86	5.04 m		
6	35.14b	1.61 brs	35.11b	1.59 brs		
7	25.91c	1.31 brs	25.94c	1.29 brs		
8	32.82d	1.31 brs	32.79d	1.29 brs		
9	23.66e	1.31 brs	23.62	1.29 brs		
10	14.40f	0.89 t (7.01)	14.42	0.89 t (7.06)		
*CH_3*CO	21.24	2.01 s				
CH_3*CO*	172.47					
DDA⁗						
1	173.16					
2	43.55	2.43−2.48 m				
3	66.82a	4.09 m				
4	42.37	1.72−1.78 m				
5	72.84	5.03 m				
6	35.06b	1.61 brs				
7	25.91c	1.31 brs				
8	32.72d	1.31 brs				
9	23.59e	1.31 brs				
10	14.36f	0.89 t (7.01)				

[a]The chemical shifts (δ) are in ppm from TSM. The coupling constant J values (in hertzz) are given in parentheses. All assignments are based on the COSY, HMQC, and HMBC experiments. [b]Assignments followed by the same letters (a−f) in the same column may be interchangeable. [c]DDA, 3,5-dihydroxydecanoic acid. [d]CH_3CO, acetyl group

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Table 2. Antibacterial Activity of the Isolated Compounds against the Growth of Seven Phytopathogenic Bacteria

	IC_{50} (95% CI)[a]		
name	1	2	3
A. konjaci	22.5(8.5−59.9)	>100	>100
A. tumefaciens	1.58 (0.91−2.74)	1.49 (0.18−2.95)	24.8 (16.9−36.4)
B. glumae	33.2 (18.9−58.4)	>100	NI[b]
P. carotovora subsp. *carotovora*	>100	>100	NI
P. syringae pv *lachrymans*	>100	>100	>100
R. solanacearum	73.8 (41.4−106.1)	75.8 (44.3−107.4)	>100
X. euvesicatoria	72.1 (40.6−103.6)	>100	>100

[a]The antibacterial activity is expressed as the IC_{50} (concentration causing 50% growth inhibition) as determined by the broth microdilution method. Growth inhibition was observed 2 days after treatment. 95% CI, 95% confidence interval. [b]No inhibition.

mycoparasite of *B. cinerea*. The present study identifies compounds **1**−**3** for the first time.

Antibacterial Activity. The in vitro antibacterial activity of compounds **1**−**3** was tested in 96-well microtiter plates, and the results are presented in Table 2. Compound **1** displayed a broad spectrum and strong antibacterial activity compared to compounds **2** and **3**. Compound **1** caused growth inhibition of all seven phytopathogenic bacteria and was highly active against *A. konjaci*, *A. tumefaciens*, and *B. glumae*, with IC_{50} values of 25.5, 1.58, and 33.2 μg/mL, respectively. *R. solanacearum* (IC_{50} 73.8 μg/mL) and *X. euvesicatoria* (IC_{50} 72.1 μg/mL) were moderately sensitive to compound **1**. However, *P. carotovora* subsp. *carotovora* and *P. syringae* pv *lachrymans* were highly tolerant to this compound. Compound **2** also affected the cell growth of all bacteria tested. It effectively inhibited the growth of *A. tumefaciens* with an IC_{50} value of 1.49 μg/mL. *R. solanacearum* growth was inhibited by compound **2**, with an IC_{50} value of 75.8 μg/mL. Compound **3** caused good growth inhibition of *A. tumefaciens*, but had no effect against *B. glumae* and *P. carotovora* subsp. *carotovora*. Of the three compounds, compound **1** showed the strongest antibacterial activity against the phytopathogenic bacteria tested.

The EtOAc extract from the *S. lamellicola* BCP broth was evaluated for in vivo antibacterial activity against *R. solanacearum* on 3-week-old tomato seedlings (Figure 3). The extract suppressed the development of bacterial wilt in a dose-dependent manner. *R. solanacearum* caused severe wilting damage on seedlings treated with distilled water and 500 μg/mL EtOAc extract 9 days after inoculation (Figure 3B). However, the EtOAc extract at concentrations of 1000 and 2000 μg/mL displayed strong control efficacies of 85 and 100%, respectively, against *R. solanacearum* 9 days after inoculation. These disease control efficacies were higher than that of streptomycin sulfate (200 μg/mL). These results suggest that the EtOAc extract and the fermentation broth of *S. lamellicola* BCP may be used as a natural bactericide to control tomato bacterial wilt.

Biocontrol provides an alternative to the use of synthetic agrochemicals and is used as part of an overall integrated plant disease management program. Several filamentous fungal species are known mycoparasites against various phytopathogenic fungi.[18−20] The biocontrol mechanism is most likely due to the action of cell wall degradative enzymes and secondary metabolites.[21−23] In addition, the microorganisms that produce microbial polyesters (polyhydroxyalkanoates) have long been considered important in various sectors, such as the food, pharmaceutical, cosmetic, fragrance, flavor, and biofuel industries.[17]

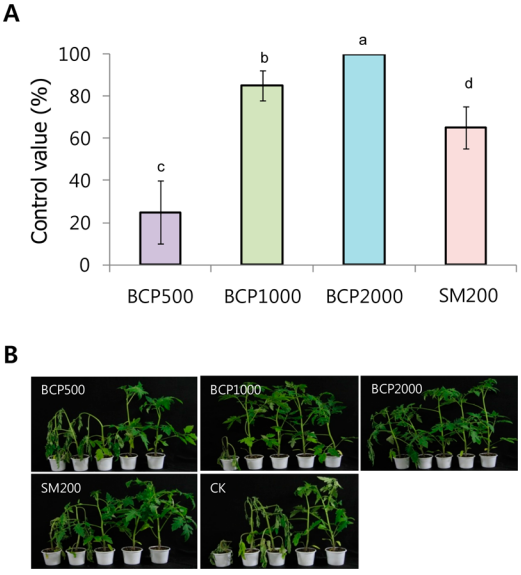

Figure 3. Control efficacy of the ethyl acetate layer of the *Simplicillium lamellicola* BCP culture broth in reducing wilting disease severity caused by *Ralstonia solanacearum* on tomato seedlings (A) and the treated plants 9 days after inoculation (B). BCP500, 500 μg/mL; BCP1000, 1000 μg/mL; and BCP2000, 2000 μg/mL, ethyl acetate extracts from the *S. lamellicola* BCP culture broth; ST200, 200 μg/mL of streptomycin sulfate; CK, untreated control. Each value represents the mean ± standard deviation of three experiments with five replicates each. Means (±SD) followed by the same letters above the bars are not significantly different (P = 0.05) in a Tukey's HSD test.

S. lamellicola BCP was reported to be a biocontrol fungus with good efficacy against *B. cinerea* and *M. grisea*.[5,6] *S. lamellicola* was previously known as *Cephalosporium lamellicola* and *Verticillium lamellicola*, which exhibited mycoparasitic interactions with phytopathogenic fungi causing rust diseases.[20,24] The strain BCP was also reported to produce verlamelin that significantly suppressed tomato late blight caused by *P. infestans*, wheat leaf rust caused by *P. recondita*, and barley powdery mildew caused by *B. graminis* f. sp. *hordei*.[4,25] In the present study, we demonstrated that *S. lamellicola* BCP produces different antimicrobial metabolites and may be considered a producer of polyesters of (3*R*,5*R*)-3,5-dihydroxydecanoic acid.

For the antimicrobial activities of substances containing (3*R*,5*R*)-3,5-dihydroxydecanoic acid, verbalactone isolated from the plant roots of *Verbascum undulatum* exhibited moderate antibacterial activity against *Salmonella enteritidis*, *Staphylococcus aureus*, and *S. epidermidis*.[26] Only a few tetramers and trimers of (3*R*,5*R*)-3,5-dihydroxydecanoic acid from three fungal strains

have previously been reported. Halymecins A, B, and C were isolated from *Fusarium* spp. FE-71-1, and halymecins D and E were isolated from *Acremonium* spp. FK-N30. Of these compounds, halymecin A is a tetramer of DDA and causes growth inhibition of bacteria, such as *Enterococcus faecium*, *Klebsiella pneumoniae*, and *Proteus vulgaris*. Halymecin A also showed strong antimicroalgal and cytotoxic activities.[14] Exophilin A was reported to be a trimer of (3*R*,5*R*)-3,5-dihydroxydecanoic acid isolated from *Exophiala pisciphila* NI10102. The trimer displayed antimicrobial activity against some Gram-positive bacterial strains, such as *Enterococcus faecium* and *Staphylococcus aureus*.[17] In our study, the mannosyl lipids halymecin F (**1**), halymecin G (**2**), and (3*R*,5*R*)-3-*O*-β-D-mannosyl-3,5-dihydroxydecanoic acid (**3**) were novel mannosyl lipids isolated from *S. lamellicola* BCP with antibacterial activities against phytopathogenic bacteria.

In addition, we successfully isolated and confirmed the presence of massoia lactone (**5**), an active metabolite member of the *Trichoderma* family,[23] in the culture broth of *S. lamellicola* BCP. Massoia lactone represents a minor component of the crude extracts of various *Trichoderma* spp., including *Trichoderma viride*. This compound was patented because it controls *B. cinerea* and *Phytopthora* species.[22] The role of *Trichoderma* antimicrobial metabolites in biocontrol remains unclear. Some of these compounds may be the major contributor to biocontrol activity for a certain strain but not for other strains.[21] The presence of massoia lactone (**5**) in *S. lamellicola* BCP suggests a relationship between the biocontrol mechanism of the strain and *Trichoderma* species. Our findings confirmed the production of mannosyl lipids **1**−**3** and massoia lactone from *S. lamellicola* BCP and the antibacterial activities of the novel mannosyl lipids against phytopathogenic bacteria. This strain may be considered a producer of polyesters of (3*R*,5*R*)-3,5-dihydroxydecanoic acid. The presence of the three novel mannosyl lipids in the fermentation broth cultures of *S. lamellicola* BCP was demonstrated in this study. Furthermore, the in vivo activities of the EtOAc extract against tomato bacterial wilt in a climate-controlled room and disease control of the microbial biopesticide Acre against tomato bacterial wilt in fields were also elucidated. These results suggest that *S. lamellicola* BCP and its mannosyl lipids could be used to control plant diseases caused by phytopathogenic bacteria.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR spectral data and EI-MS spectrum of compound **4**, in vivo antifungal activity of ABE, and neighbor-joining trees based on sequence analysis of the 28S rRNA gene and ITS regions. This material is 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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