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Identification, Fermentation, and Bioactivity Against *Xanthomonas oryzae* of Antimicrobial Metabolites Isolated from *Phomopsis longicolla* S1B4

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Bacterial blight, an important and potentially destructive bacterial disease in rice, is caused by *Xanthomonas oryzae*. Recently, this organism has developed resistance to available antibiotics, prompting scientists to find a suitable alternative. This study focused on secondary metabolites of *Phomopsis longicolla* to target *X. oryzae*. Five bioactive compounds were isolated by activity-guided fractionation from ethyl acetate extracts of mycelia and were identified by LC/MS and NMR spectroscopy as dicerandrol A, dicerandrol B, dicerandrol C, deacetylphomoxanthone B, and fusaristatin A. This is the first time fusaristatin A has been isolated from *Phomopsis* sp. Deacetylphomoxanthone B showed a higher antibacterial effect against *X. oryzae* KACC 10331 than the positive control (2,4-diacetyphloroglucinol). Dicerandrol A also showed high antimicrobial activity against Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis*) and yeast (*Candida albicans*). In addition, high production yields of these compounds were obtained at the stationary and death phases.

Keywords: *Phomopsis longicolla, Xanthomonas oryzae,* antibacterial activity, fusaristatin A

Plant diseases are caused primarily by fungal and bacterial pathogens, resulting in severe losses to agriculture and horticulture crop production every year. These losses can bring about reduction in food supplies, poor quality of agricultural products, economic hardship for growers and producers, and ultimately higher prices [8]. Among several diseases caused by bacterial, fungal, and viral pathogens that devastate rice yields globally, bacterial blight (*Xanthomonas oryzae* pv. *oryzae*), blast (*Magnaporthe grisea*), sheath blight (*Rhizoctonia solani*), sheath rot (*Sarocladium oryzae*), and tungro virus are the most critical [15].

X. oryzae pv. *oryzae* causes bacterial leaf blight of rice and has been reported in all rice-growing areas of the world. The disease was first observed by farmers in Japan in 1884. Crop losses of 10–20% under moderate conditions have been recorded in several Asian and Southeast Asian countries [15]. The bacterium enters its host through hydathodes or wounds around leaf edges and then multiplies and spreads in xylem vessels, causing disease [13].

The endophytic fungus *Phomopsis* sp. is known to be a rich source of bioactive secondary metabolites with diverse structures [11]. The antibiotic potential against some bacteria and human tumor cells of secondary metabolites from *P. longicolla* has been previously reported [16]. However, the antibacterial activity of these metabolites against *X. oryzae* strains has not yet been studied.

In this study, we investigated the antimicrobial activity of five secondary metabolites from *P. longicolla* S1B4 against *X. oryzae* and other pathogenic microorganisms, and we report the isolation, structural elucidation, and fermentation of these metabolites.

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Materials and Methods

Chemicals and Culture Media

HPLC-grade water, methanol, and acetonitrile were purchased from Burdick & Jackson (Muskegon, MI, U.S.A.). First-grade acetone,

ethyl acetate, and methanol were purchased from Duksan Pure Chemicals Co. (Seoul, Korea). Potato dextrose broth (PDB), potato dextrose agar (PDA), Mueller–Hinton broth, and yeast extract were purchased from Difco Laboratories (Detroit, MI, U.S.A.). Sucrose and *N*-Z-Amine A were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Sephadex LH-20 gel for column chromatography was purchased from GE Healthcare (Sweden). 2,4-Diacetyphloroglucinol (DAPG) was purchased from Toronto Research Chemicals (North York, ON, Canada). Antibiotics were procured from either Sigma-Aldrich or Duchefa Biochemie BV (Haarlem, The Netherlands).

Bacterial and Fungal Strains

The endophytic fungus *P. longicolla* S1B4 was isolated from a plant sample collected in Hadong-gun, Kyungnam Province, South Korea. *X. oryzae* pv. *oryzae* and other *Xanthomonas* species, *Salmonella enterica* subsp. *enterica* KACC 20110, *Erwinia amylovora* KACC 10060, *Clavibacter michiganensis* subsp. *michiganensis* KACC 20122, and *Ralstonia solanacearum* KACC 10149 were kindly provided by the Korean Agricultural Culture Collection (KACC, Suwon, Korea). *Escherichia coli* KCTC 1924, *Staphylococcus aureus* subsp. *aureus* KCTC 1916, *Bacillus subtilis* subsp. *subtilis* KCTC 1021, *Pseudomonas syringae* subsp. *syringae* KCTC 12500, *Candida albicans* KCTC 1940, and *Aspergillus oryzae* var. *oryzae* KCTC 6377 were purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea).

Preliminary Screening and Identification

Endophytic fungal isolates were cultured on PDA plates for 21 days. A 1×1 cm^2 plug of confluent growth was mixed with 1 ml of methanol overnight at room temperature and the extract was concentrated using a centrifugal vacuum evaporator. The residue was redissolved in 0.2 ml of methanol, and 35 µl was used for bioassay against *X. oryzae* by the agar diffusion method. Strains were identified based on macroscopic, microscopic, and molecular techniques including sequencing of the internal transcribed spacer (ITS) and 28S rDNA.

Fermentation Conditions

P. longicolla S1B4 was grown on a PDA plate for 5 days at 25°C. A seed culture was prepared by inoculating a loop of fungal mass into a 500-ml Erlenmeyer flask containing 200 ml of PDB and then incubating at 25°C with shaking at 150 rpm for 3 days. For production of mycelium, 25 ml of seed culture was inoculated into twenty 2-l Erlenmeyer flasks containing 500 ml of PDB [14]. Fermentation was carried out with shaking at 150 rpm at 25°C for 18 days. To study the growth pattern and production of secondary metabolites, seed culture broth [5% (v/v)] was inoculated into 250-ml flasks containing 100 ml of PDB and then incubated at 25°C and 150 rpm for 20 days [11]. Samples were harvested at 2-day intervals. Filtered mycelium was freeze-dried and dry cell weight was determined [1, 2]. The production of secondary metabolites was identified by LC/MS [7].

Extraction, Isolation, and Purification of Metabolites

The isolation procedure is summarized in Fig. 1. A 10-l volume of *P. longicolla* S1B4 fermentation broth was filtered by suction using Whatman no. 2 filter paper. Mycelium was extracted with 1 l of aqueous acetone (80%) and then filtered [3]. The filtrate was concentrated *in vacuo* to yield about 200 ml, which was then subjected twice to solvent partitioning with an equal volume of ethyl acetate. The organic layer was concentrated *in vacuo*. The crude ethyl acetate extract was passed through a Sephadex LH-20 column and eluted with aqueous methanol (80%). A 10-ml volume of eluate per tube was collected using an automatic sample collector. Fractions showing antibacterial activity against *X. oryzae* KACC 10331 were selected for further study and were further purified by reverse-phase high-performance liquid chromatography (RP–HPLC) on a YMC-Pack Pro C18 column (250×4.6 mm).

LC–ESI/MS Analysis

Samples were analyzed using an LC–MS/MS system (Varian Inc., Palo Alto, CA, U.S.A.) fitted with a Chromsep C18 column (150×2.0 mm) at a flow rate of 0.2 ml/min. Mobile phases A and B consisted of

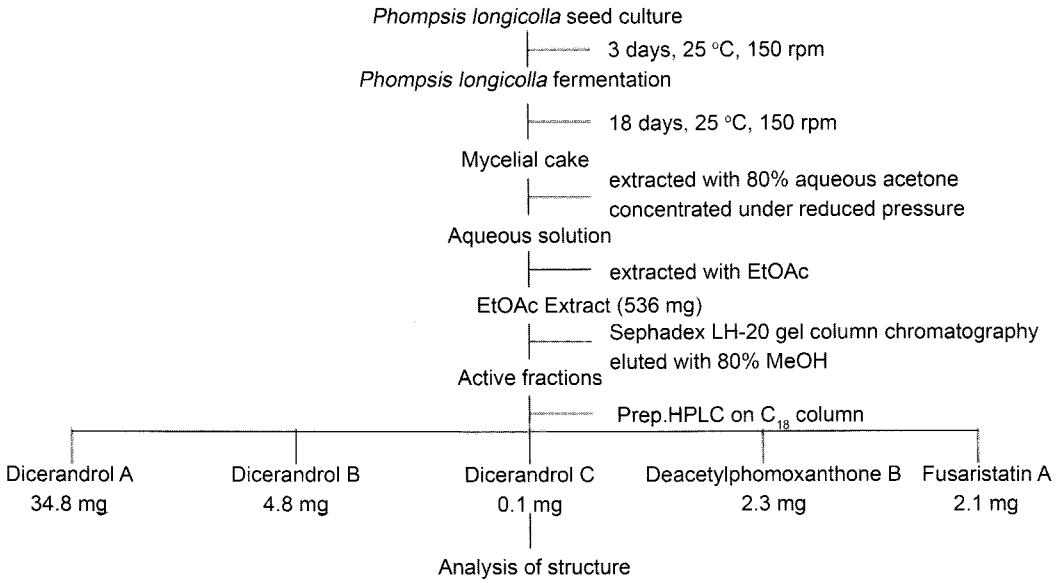

Fig. 1. Schematic representation of the procedure for isolation of compounds from *Phomopsis longicolla*.

0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. Samples were run in a linear gradient from 0 to 10% B in 2 min up to 40% B in 10 min, and up to 70% B in 20 min. The system was operated with MS Workstation software (version 6.9; Varian, Inc.). Mass spectra were acquired using an electrospray ionization (ESI) source in negative ionization mode.

Full-scan mass spectra were recorded in the range of *m/z* 50–2000. The operating parameters for samples in the negative-ion mode were as follows: spray needle voltage, 5 kV; capillary voltage, 80 V; drying temperature, 220°C; drying gas (nitrogen) pressure, 10 psi; nebulizer gas (air) pressure, 35 psi; and neutral collision gas (helium) flow rate, 1.0 ml/min. Tandem mass spectrometry was carried out using scan-type turbo data-dependent scanning (DDS). The collision-induced dissociation (CID) voltage was automatically set by DDS. The mass width was set at 3 *m/z*.

NMR Measurements

All NMR experiments were recorded at 298K on a Varian VNMRS 600 MHz spectrometer (Varian Inc.) equipped with a triple-resonance 5-mm HCN salt-tolerant cold probe. The NMR spectra of ^{1}H NMR and ^{13}C NMR were collected in acetonitrile-d_3.

Antibacterial Assays

The purified compounds were tested for antimicrobial activity using the agar diffusion method on solid medium. An M210 top agar plate was used for *X. oryzae* KACC 10331. Six-mm-diameter discs were soaked with the test sample and placed on the agar surface [4, 9]. The plates were then incubated at 28°C for 24 h. Minimal inhibitory concentrations (MICs) were determined by a modified version of the broth microdilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [5, 6]. Two-fold dilutions were prepared in broth medium to obtain a concentration range of 0.125–128 ppm (μg/ml). The MIC was defined as the lowest concentration resulting in a 90% reduction in growth [17].

Results

Screening and Identification of Endophytic Fungus *Phomopsis longicolla*

In an ongoing effort, 283 endophytic fungal isolates were screened in order to identify compounds that are bioactive against *X. oryzae* pv. *oryzae*. Based on microscopic morphology and analysis of ITS and 28S rDNA sequences, the isolate exhibiting the highest antimicrobial activity was identified as *P. longicolla* S1B4. This strain was therefore used for further study of secondary metabolite isolation.

Identification and Structure of Bioactive Compounds

Active fractions were separated by Sephadex LH-20 column chromatography and purified by RP-HPLC to obtain five pure active compounds. The structure of these compounds was identified based on ^{1}H-NMR, ^{13}C-NMR, heteronuclear multiple bond correlation (HMBC), and MS spectroscopic data and was confirmed using previously published literature (Table 1).

Three compounds obtained in the form of a pure yellow powder were identified as dicerandrols A, B, and C by NMR and MS full-scan fragmentation (*m/z*); their molecular formulas are $C_{34}H_{34}O_{14}$, $C_{36}H_{36}O_{15}$, and $C_{38}H_{38}O_{16}$, respectively.

Dicerandrol A: yellow powder; UV λ_{max} (MeOH): 224, 343 nm. ^{1}H-NMR: (600 MHz, CD_3CN) δ: 7.39 (2H, d, *J*=8.4 Hz, H-3, H-3'), 6.44 (2H, d, *J*=8.4 Hz, H-4, H-4'), 5.61 (2H, d, *J*=1.2 Hz, H-5, H-5'), 2.47 (2H, m, H-6, H-6'), 2.33 (2H, m, H-7α, H-7'α), 2.50 (2H, m, H-7β, H-7'β), 1.00 (6H, d, *J*=3.0 Hz, H-11, H-11'), 3.90 (2H, dd, *J*=4.4, 9.0 Hz, H-12α, H-12'α), 3.56 (2H, dd, *J*=6.4, 9.0, H-12β, H-12'β), 2.03 (6H, s, H-14, H-14'); ^{13}C-NMR; (150 MHz, CD_3CN) δ: 160.3 (C-1, C-1'), 118.5 (C-2, C-2'), 141.1 (C-3, C-3'), 109.0 (C-4, C-4'), 158.9 (C-4a, C-4a'), 71.8 (C-5, C-5'), 28.5 (C-6, C-6'), 34.1 (C-7, C-7'), 179.9 (C-8, C-8'), 102.3 (C-8a, C-8a'), 189.1 (C-9, C-9'), 107.3 (C-9a, C-9a'), 83.8 (C-10a, C-10a'), 17.8 (C-11, C-11'), 65.8 (C-12, C-12'), 171.4 (C-13, C-13'), 21.0 (C-14, C-14'); ESI/MS: *m/z*: 667 $[M+H]^+$. The ^{13}C-NMR spectral data of dicerandrol A showed only 17 carbon resonances, and a major absorption peak was observed for $[M+H]^+$ at *m/z*=667 (Table 1). Therefore, this compound may be a symmetric dimer, as shown in Fig. 2 [16].

Dicerandrol B: yellow powder; UV λ_{max} (MeOH): 233, 342 nm. ^{1}H-NMR: (600 MHz, CD_3CN) δ: 7.39 (2H, d, *J*=8.4 Hz, H-3, H-3'), 6.43 (1H, d, *J*=8.4 Hz, H-4), 6.36 (1H, d, *J*=8.4 Hz, H-4'), 5.61 (1H, d, *J*=1.3 Hz, H-5), 5.54 (1H, d, *J*=1.3 Hz, H-5'), 2.35 (2H, m, H-6, H-6'), 2.46 (4H, m, H-7α, H-7'α, H-7β, H-7'β), 1.01 (3H, d, *J*=3.0 Hz, H-11), 1.00 (3H, d, *J*=3.0 Hz, H-11'), 3.91 (1H, d, *J*=13.2 Hz, H-12α), 4.50 (1H, d, *J*=13.2 Hz, H-12' α), 3.56 (1H, d, *J*=13.2 Hz, H-12 β), 4.19 (1H, d, *J*=13.2, H-12' β), 2.00

Table 1. Retention time, quasi-molecular ion in positive mode, and key fragments by LC–ESI/MS of five secondary metabolites of *Phomopsis longicolla*.

No.	Metabolite	Rt (min)	MS $[M+H]^+$	MS full-scan fragmentation (*m/z*)
1	Dicerandrol A	21.8	667 $[M+H]^+$	607, 589, 529, 511
2	Dicerandrol B	24.1	709 $[M+H]^+$	649, 631, 589, 529, 511
3	Dicerandrol C	27.1	751 $[M+H]^+$	709, 691, 631, 589, 529, 511
4	Deacetylphomoxanthone B	23.8	667 $[M+H]^+$	649, 589, 529, 511
5	Fusaristatin A	27.3	659 $[M+H]^+$	546, 232, 147

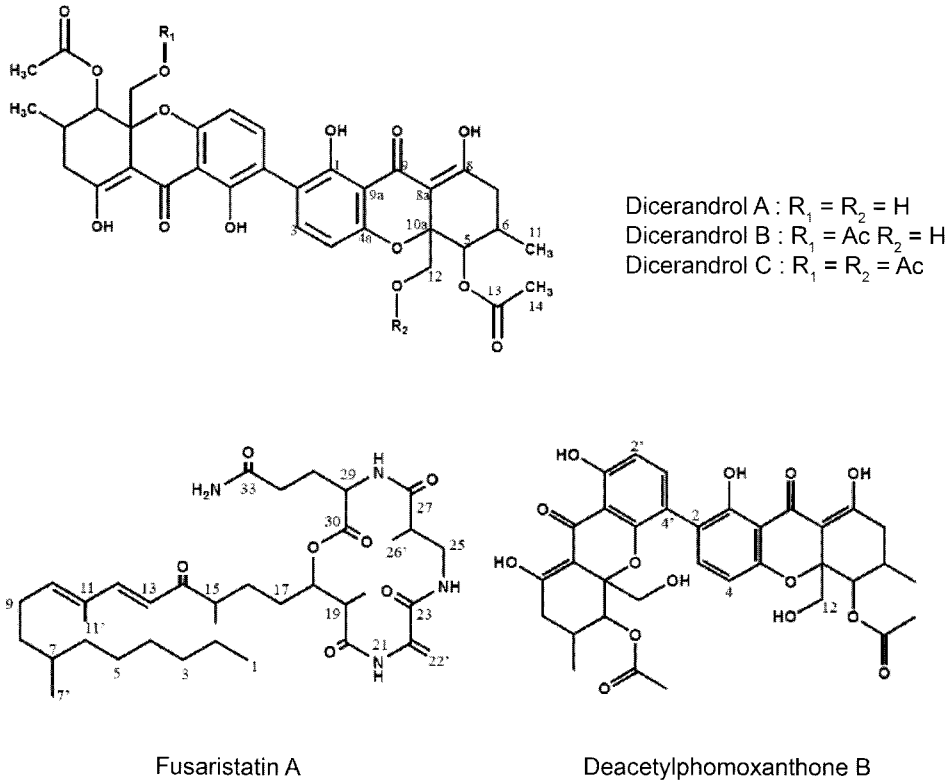

Fig. 2. Chemical structure of dicerandrols A, B, and C, deacetylphomoxanthone B, and fusaristatin A.

(3H, s, H-14), 2.04 (3H, s, H-14'), 2.03 (3H, s, H-16); ESI/MS: *m/z*: 709 $[M+H]^+$. The chemical ionization of dicerandrol B showed a mass difference of 42 units compared with dicerandrol A, which was attributed to the addition of a single acetate group to the latter. The mass difference, the loss of symmetry in the ^{1}H-NMR spectra, and the composite appearance of the NMR spectra indicate that dicerandrol B is an asymmetric dimer consisting of one monomer unit from dicerandrol A (Fig. 2) [16].

Dicerandrol C: yellow powder; UV λ_{max} (MeOH): 226, 336 nm. ESI/MS: *m/z*: 751 $[M+H]^+$. This compound was identified by MS full-scan fragmentation (*m/z*) (Table 1). The dicerandrol series exhibited a gap of 42 mass units between dicerandrol A (MW 666), dicerandrol B (MW 708), and dicerandrol C (MW 750), corresponding to CH_2CO (acetate group).

Deacetylphomoxanthone B: yellow powder; UV λ_{max} (MeOH): 227, 339 nm. ^{1}H-NMR: (600 MHz, CD_3CN) δ: 7.23 (1H, d, *J*=8.4 Hz, H-3), 6.38 (1H, d, *J*=8.4 Hz, H-4), 5.36 (1H, d, *J*=1.2 Hz, H-5), 2.40 (1H, m, H-6), 2.48 (2H, m, H-7α, H-7β), 0.93 (3H, d, *J*=6.6 Hz, H-11), 3.93 (1H, dd, *J*=4.8, 5.4 Hz, H-12α), 3.56 (1H, dd, *J*=7.2, 7.2 Hz, H-12β), 1.92 (3H, s, H-14), 6.50 (1H, d, *J*=8.4 Hz, H-2'), 7.34 (1H, d, *J*=8.4 Hz, H-3'), 5.62 (1H, d, *J*=1.8 Hz, H-5'), 2.40 (1H, m, H-6'), 2.48 (2H, m, H-7'α, H-7'β), 1.00 (3H, d, *J*=6.6Hz, H-11'), 3.79 (1H, dd, *J*=4.2, 4.2 Hz, H-12'α), 3.47 (1H, dd, *J*=7.2, 7.2 Hz, H-12'β), 2.05 (3H, s, H-14'); ^{13}C-NMR: (150 MHz, CD_3CN) δ: 160.3 (C-1), 118.9 (C-2), 140.6 (C-3), 108.9 (C-4), 158.8 (C-4a), 71.3 (C-5), 28.5 (C-6), 33.9 (C-7), 179.6 (C-8), 102.0 (C-8a), 189.2 (C-9), 107.2 (C-9a), 83.4 (C-10a), 17.5 (C-11), 65.5 (C-12), 170.9 (C-13), 21.0 (C-14), 162.5 (C-1'), 109.8 (C-2'), 140.7 (C-3'), 117.9 (C-4'), 156.5 (C-4a'), 71.8 (C-5'), 28.5 (C-6'), 34.1 (C-7'), 179.9 (C-8'), 102.4 (C-8a'), 189.3 (C-9'), 107.4 (C-9a'), 83.8 (C-10a'), 17.8 (C-11'), 65.6 (C-12'), 171.4 (C-13'), 21.1 (C-14'); Major long-range ^{1}H-^{13}C correlations (HMBC) were observed as H-3/C-4a, C-4'; H-4/C-2, C-4a, C-9a; H-5/C-6, C-7, C-8a, C-10a, C-11, C-13; H-6/C-8, C-8a, C-11; H-7/C-5, C-6, C-8, C-8a; H-11/C-5, C-6, C-7; H-12/C-5, C-10a; H-14/C-13; H-2'/C-4', C-9a'; H-3'/C-2, C-1', C-4a'; H-5'/C-6', C-7', C-8a', C-10a', C-11', C-13'; H-6'/C-8', C-8a', C-11'; H-7'/C-5', C-6', C-8', C-8a'; H-12'/C-5', C-10a'; H-14'/C-13'; ESI/MS: *m/z*: 667 $[M+H]^+$. Deacetylphomoxanthone B, with the molecular formula of $C_{34}H_{34}O_{14}$, was isolated as a yellow powder. The UV and MS full-scan fragmentation (*m/z*) spectra were similar to those of dicerandrol A (Table 1). However, the ^{13}C-NMR spectrum contained 32 carbon resonances for 34 carbons, thus indicating that deacetylphomoxanthone B is an asymmetric dimer. In addition, HMBC correlations of H-3/

C-4' and H-3'/C-2 established a C-2-C-4' linkage between two tetrahydroxanthones. Therefore, this compound was confirmed to be deacetylphomoxanthone B [11].

Fusaristatin A: colorless oil; UV λ_{max} (MeOH): 286 nm. ^{1}H-NMR: (600 MHz, CD_3CN) δ: 0.88 (3H, m, H-1), 1.27 (13H, m, H-2, H-5, H-6, H-7, H-16, H-17, H-32), 2.25 (8H, m, H-3, H-4, H-9, H-31), 0.88 (6H, m, H-7', H-26'), 1.41 (2H, m, H-8) 6.02 (1H, t, *J*=7.8 Hz, H-10), 1.08 (3H, s, H-11'), 7.23 (1H, d, *J*=15.6 Hz, H-12), 6.22 (1H, d, *J*=15.6 Hz, H-13), 2.69 (1H, m, H-15), 1.05 (3H, d, *J*=7.2 Hz, H-15'), 4.94 (1H, m, H-18), 2.83 (1H, m, H-19), 1.11 (3H, m, H-19'), 5.81 (1H, s, H-22'α), 5.39 (1H, s, H-22'β), 3.49 (1H, m, H-25α), 3.38 (1H, m, H-25β), 2.49 (1H, m, H-26), 4.36 (1H, m, H-29); ^{13}C-NMR: (150 MHz, CD_3CN) δ: 14.5 (C-1), 23.5 (C-2), 32.2 (C-3), 27.4 (C-4), 30.4 (C-5), 37.6 (C-6), 33.3 (C-7), 19.9 (C-7'), 36.9 (C-8), 27.3 (C-9), 145.0 (C-10), 134.2 (C-11), 12.5 (C-11'), 148.5 (C-12), 124.0 (C-13), 204.8 (C-14), 44.6 (C-15), 17.3 (C-15'), 28.8 (C-16), 30.5 (C-17), 77.6 (C-18), 44.5 (C-19), 15.1 (C-19'), 173.3 (C-20), 138.1 (C-22), 115.1 (C-22'), 164.7 (C-23), 42.8 (C-25), 42.5 (C-26), 14.6 (C-26'), 174.7 (C-27), 53.6 (C-29), 172.3 (C-30), 27.7 (C-31), 32.7 (C-32), 175.6 (C-33); ESI/MS: *m/z*: 659 $[M+H]^+$. Fusaristatin A was isolated as a colorless oil with a molecular formula of $C_{36}H_{58}N_4O_7$. The UV spectrum revealed an absorption maximum at 286 nm, which suggested a chromophore of conjugated double bonds or a benzene ring in the molecule. This compound, having a different skeleton from the dicerandrols, was identified by comparison of its ^{1}H-NMR, ^{13}C-NMR, and HMBC spectroscopic data with those reported in the literature (Fig. 2) [12].

Antibacterial Activity Against *Xanthomona oryzae*

A disk diffusion assay was performed to test the antibacterial activity of dicerandrols A and B against *X. oryzae* KACC 10331 (Table 2). Dicerandrol A showed higher activity than dicerandrol B and its response was dose-dependent whereas that of dicerandrol B was not. It should be noted that the degree of inhibition is not a reliable indicator of activity because the size of the zone of inhibition is based on the mobility in agar of the test compound, which can be influenced by its polarity [10].

Table 2 also shows the MIC values of dicerandrols A, B, and C, deacetylphomoxanthone B, and fusaristatin A against *X. oryzae* KACC 10331. All of these compounds exhibited high inhibitory activity against the test strain, with deacetylphomoxanthone B showing the highest activity (MIC, 4 μg/ml). Dicerandrol A, the compound obtained in the highest yield, was assayed for antimicrobial activity against seven other *X. oryzae* strains. As outlined in Table 3, this compound showed a broad spectrum of activity against these strains, with MIC values ranging from 2 to 64 μg/ml. In particular, *X. oryzae* KACC 10331 and KACC 10876 were inhibited at very low concentrations of dicerandrol A (8 and 2 μg/ml, respectively), compared with the positive control (DAPG). Dicerandrols B and C, deacetylphomoxanthone B, and fusaristatin A could be tested against only *X. oryzae* KACC 10331 because of their low yield.

Dicerandrol A was evaluated for antibacterial activity against Gram-positive and Gram-negative bacteria and

Table 2. Antimicrobial activity of *Phomopsis longicolla* secondary metabolites against *Xanthomonas oryzae* KACC 10331.

Compound	Inhibition zone diameter (mm)			MIC[a] (μg/ml)
	5 μg	10 μg	20 μg	
Dicerandrol A	7	10	13	8
Dicerandrol B	8	8	8	16
Dicerandrol C	-	-	-	>16
Deacetylphomoxanthone B	-	-	-	4
Fusaristatin A	-	-	-	128
DAPG[b]	13	15	18	8

[A]Minimal inhibitory concentration.
[b]2,4-Diacetyphloroglucinol.
- Not tested.

Table 3. Antimicrobial activity of dicerandrol A isolated from *Phomopsis longicolla.*

Microorganism	MIC value (μg/ml)	
	Dicerandrol A	Antibiotic
Gram-positive		
Staphylococcus aureus KCTC 1916	0.25	0.25[a]
Bacillus subtilis KCTC 1021	0.125	0.5[b]
Clavibacter michiganesis KACC 20122	1	0.25[c]
Gram-negative		
Xanthomona oryzae KACC 10859	64	2[d]
Xanthomona oryzae KACC 10874	8	4[d]
Xanthomona oryzae KACC 10875	8	4[d]
Xanthomona oryzae KACC 10876	2	2[d]
Xanthomona oryzae KACC 10882	64	2[d]
Xanthomona oryzae KACC 10884	16	8[d]
Xanthomona oryzae KACC 10885	16	4[d]
Escherichia coli KCTC 1924	>128	16[c]
Pseudomonas syringae KACC 10134	>128	1[b]
Salmonella enterica KACC 10763	>128	0.5[b]
Erwinia amylovora KACC 10060	32	16[c]
Ralstonia solanacearum KACC 10149	>128	0.5[b]
Yeast		
Candida albicans KCTC 7965	2	128[e]
Fungus		
Aspergillus oryzae KCTC 6377	>128	4[d]

[a]Neomycin.
[b]Kanamycin.
[c]Ampicillin.
[d]DAPG.
[e]Amphotericin B.

fungi, and the resulting MIC values are given in Table 3. This compound showed higher activity against Gram-positive bacteria than Gram-negative bacteria and fungi but exhibited a broad spectrum of activity against these groups of microorganisms.

Growth and Time-dependent Production of Secondary Metabolites

Fig. 3A shows the growth curve of *P. longicolla* S1B4, as well as the concentrations of the five antibacterial compounds over time. The dicerandrols and fusaristatin A were mainly produced in the stationary phase, with maximal production occurring on day 10. The production of deacetylphomoxanthone B was higher in the death phase than the other stages, and maximal production was observed on the 18th day of incubation. The pH of the culture medium changed from acidic to neutral (5.10 to 7.74) during the fermentation.

The antibacterial activity of ethyl acetate extracts of mycelium against *X. oryzae* KACC 10331 is presented in Fig. 3B. The results indicate that antibacterial activity was minimal during the first four days of fermentation. Moderate activity was observed from days 6 through 12 of fermentation. Maximal antibacterial activity was observed from the 14th day of incubation onward.

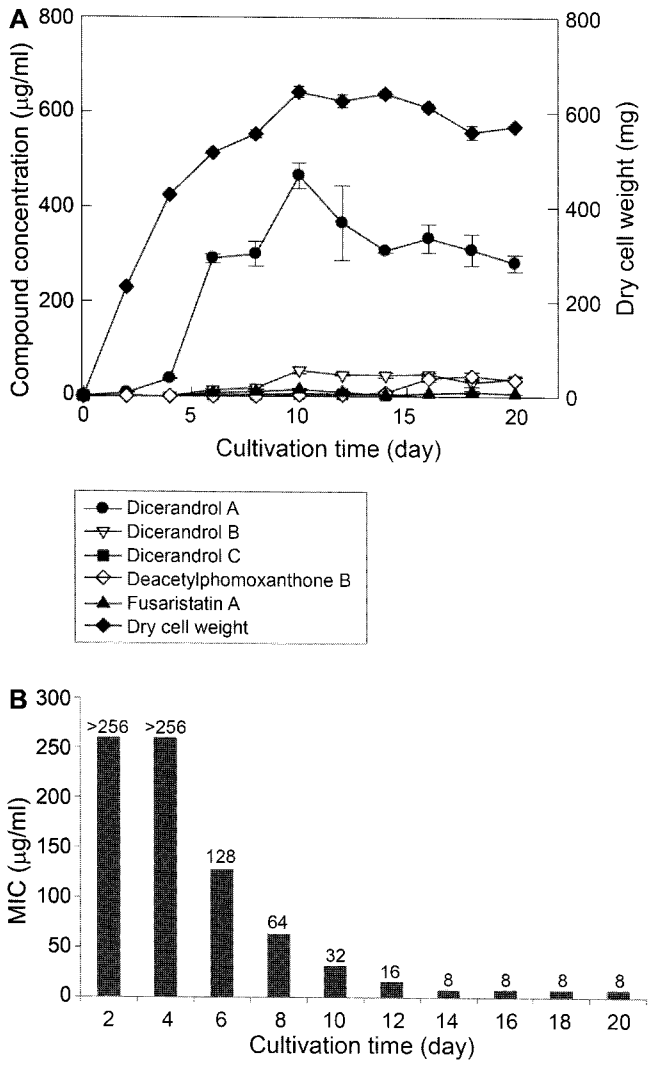

Fig. 3. Growth pattern, production of secondary metabolites, and antibacterial activity of *Phomopsis longicolla* in potato dextrose broth.

A. Dry cell weight vs. secondary metabolite production. **B.** Antibacterial activity against *Xanthomonas oryzae* KACC 10331.

DISCUSSION

In the present study, we evaluated the antimicrobial activity of dicerandrols A, B, and C, deacetylphomoxanthone B, and fusaristatin A against *X. oryzae* strains, which cause bacterial blight of rice. These compounds were previously reported to exhibit antibacterial activity and were shown to possess modest activity in human cancer cell lines. However, to our knowledge, this is the first report demonstrating the antibacterial activity of these compounds against *X. oryzae* strains.

First, we isolated dicerandrols A, B, and C, deacetylphomoxanthone B, and fusaristatin A from *P. longicolla* S1B4. Fusaristatin A has not been previously isolated from this organism. We then assayed the antimicrobial activity of all five metabolites against *Xanthomonas oryzae* KACC 10331. The results of the activity assay showed that deacetylphomoxanthone B provides higher antimicrobial activity than the other compounds. Moreover, dicerandrol A exhibited significant activity against seven other *X. oryzae* strains compared with the positive control (DAPG) [15]. The results indicate that dicerandrol A may be used as a natural preventive for bacterial blight of rice. Furthermore, a crude ethyl acetate extract of *P. longicolla* S1B4 could potentially serve as a biological agent for the prevention of this disease.

Dicerandrol A was also assayed for antimicrobial activity against pathogenic Gram-positive and Gram-negative bacteria, yeast, and fungi. This compound inhibited the growth of the three Gram-positive bacteria tested, one of five Gram-negative bacteria, as well yeast, thereby demonstrating a broad spectrum of antimicrobial activity. The results presented in this study suggest that dicerandrol A offers potential antimicrobial activity against pathogenic microorganisms and is an excellent candidate for antimicrobial applications.

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References

1. Akihiro, K., K. Fumihisa, M. Godliving, and N. Yoshitoshi. 2006. Development of optimal culture method of *Sparassis crispa* mycelia and a new extraction method of antineoplastic constituent. *Biochem. Eng. J.* **30:** 109–113.
2. Casas López, J. L., J. A. Sánchez Pérez, J. M. Fernández Sevilla, F. G. Acién Fernández, E. Molina Grima, and Y. Chisti. 2003. Production of lovastatin by *Aspergillus terreus*: Effects of the C:N ratio and the principal nutrients on growth and metabolite production. *Enzyme Microb. Tech.* **33:** 270–277.
3. Cui, C. B., M. Ubukata, H. Kakeya, R. Onose, G. Okada, I. Takahashi, K. Isono, and H. Osada. 1996. Acetophthalidin, a novel inhibitor of mammalian cell cycle, produced by a fungus isolated from a sea sediment. *J. Antibiot. (Tokyo)* **49:** 216–219.
4. Dung, N. T., J. M. Kim, and S. C. Kang. 2008. Chemical composition, antimicrobial and antioxidant activities of the essential oil and the ethanol extract of *Cleistocalyx operculatus* (Roxb.) Merr and Perry buds. *Food Chem. Toxicol.* **46:** 3632–3639.
5. Espinel-Ingroff, A., B. Arthington-Skaggs, N. Iqbal, D. Ellis, M. A. Pfaller, S. Messer, *et al.* 2007. Multicenter evaluation of a new disk agar diffusion method for susceptibility testing of filamentous fungi with voriconazole, posaconazole, itraconazole, amphotericin B, and caspofungin. *J. Clin. Microbiol.* **45:** 1811–1820.
6. Goker, H., S. Ozden, S. Yildiz, and D. W. Boykin. 2005. Synthesis and potent antibacterial activity against MRSA of some novel 1,2-disubstituted-1H-benzimidazole-*N*-alkylated-5-carboxamidines. *Eur. J. Med. Chem.* **40:** 1062–1069.
7. György, S., M. György, and P. T. Robert. 1998. Production of lovastatin by a wild strain of *Aspergillus terreus*. *Biotechnol. Lett.* **20:** 411–415.
8. Lee, C. H., H. J. Kempt, Y. Lim, and Y. H. Cho. 2000. Biocontrol activity of *Pseudomonas cepacia* AF2001 and anthelmintic activity of its novel metabolite, Cepacidine A. *J. Microbiol. Biotechnol.* **10:** 568–571.
9. Lee, S. B., K. H. Cha, S. N. Kim, S. Altantsetseg, S. Shatar, O. Sarangerel, and C. W. Nho. 2007. The antimicrobial activity of essential oil from *Dracocephalum foetidum* against pathogenic microorganisms. *J. Microbiol.* **45:** 53–57.
10. Mikani, A., H. R. Etebarian, P. L. Sholberg, D. T. O'Gorman, S. Stokes, and A. Alizadeh. 2008. Biological control of apple gray mold caused by *Botrytis mali* with *Pseudomonas fluorescens* strains. *Postharvest Biol. Tech.* **48:** 107–112.
11. Rukachaisirikul, V., U. Sommart, S. Phongpaichit, J. Sakayaroj, and K. Kirtikara. 2008. Metabolites from the endophytic fungus *Phomopsis* sp. PSU-D15. *Phytochemistry* **69:** 783–787.
12. Shiono, Y., M. Tsuchinari, K. Shimanuki, T. Miyajima, T. Murayama, T. Koseki, *et al.* 2007. Fusaristatins A and B, two new cyclic lipopeptides from an endophytic *Fusarium* sp. *J. Antibiot. (Tokyo)* **60:** 309–316.
13. Tsuge, S., H. Ochiai, Y. Inoue, T. Oku, K. Tsuno, H. Kaku, and Y. Kubo. 2004. Involvement of phosphoglucose isomerase in pathogenicity of *Xanthomonas oryzae* pv. *oryzae*. *Phytopathology* **94:** 478–483.
14. Tsumagari, N., R. Nakai, H. Onodera, A. Hasegawa, E. S. Rahayu, K. Ando, and Y. Yamashita. 2004. MPC1001, a new antitumor antibiotic produced by *Cladorrhinum* sp. *J. Antibiot. (Tokyo)* **57:** 532–534.
15. Velusamy, P., J. E. Immanuel, S. S. Gnanamanickam, and L. Thomashow. 2006. Biological control of rice bacterial blight by plant-associated bacteria producing 2,4-diacetylphloroglucinol. *Can. J. Microbiol.* **52:** 56–65.
16. Wagenaar, M. M. and J. Clardy. 2001. Dicerandrols, new antibiotic and cytotoxic dimers produced by the fungus *Phomopsis longicolla* isolated from an endangered mint. *J. Nat. Prod.* **64:** 1006–1009.
17. Zhu, Z. J., O. Krasnykh, D. Pan, V. Petukhova, G. Yu, Y. Liu, *et al.* 2008. Structure–activity relationships of macrolides against *Mycobacterium tuberculosis*. *Tuberculosis (Edinb.)* **88:** 49–63.