JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Disease Control Effect of Strevertenes Produced by *Streptomyces psammoticus* against Tomato Fusarium Wilt

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ABSTRACT: During screening of microorganisms producing antifungal metabolites, *Streptomyces psammoticus* strain KP1404 was isolated. The culture extract of this strain showed potent disease control efficacy against Fusarium wilt on tomato plants. The antifungal metabolites ST-1 and ST-2 were isolated from the culture extract using a variety of chromatographic procedures. On the basis of MS and NMR spectrometric analysis, the structures of the antifungal active compounds ST-1 and ST-2 were determined to be the polyene antibiotics strevertene A and strevertene B, respectively. In vitro, strevertenes A and B showed inhibitory effects against the mycelial growth of *Alternaria mali*, *Aspergillus oryzae*, *Cylindrocarpon destructans*, *Colletotrichum orbiculare*, *Fusarium oxysporum* f.sp. *lycopersici*, and *Sclerotinia sclerotiorum*, even at concentrations of 4−16 μg/mL. Fusarium wilt development on tomato plants was strongly retarded by treatment with 1 μg/mL of these strevertenes. The disease control efficacies of strevertenes on Fusarium wilt were as remarkable as that of benomyl.

KEYWORDS: *Streptomyces psammoticus*, *Fusarium oxysporum* f.sp. *lycopersici*, polyenes, strevertenes, plant disease control agent, Fusarium wilt

INTRODUCTION

Microbial secondary metabolites are an attractive source of antifungal drugs for researchers in the field of plant disease control, due to their chemical and biological properties. Microbial metabolites have versatile chemical structures, with diverse biological activities that exceed the scope of synthetic organic chemical compounds.[1,2] Thus, an unexpected and newly discovered antifungal compound is likely to have a new mode of action and lack cross-resistance to commercial fungicides.[3] The importance of biodegradability as a property of microbial metabolites cannot be overstated. Microbial metabolites usually degrade within a month or even a few days when exposed to agricultural environments, thereby leading to low residual and therefore less harmful levels in the agro-ecosystem.[4]

Since the first microbial metabolite, blasticidin, was introduced for the control of blast disease in rice fields, extensive screening of microbial fungicides has yielded kasugamycin, polyoxin, validamycin, and mildiomycin, which have been used per se as active ingredients of commercial agricultural fungicides. Fungicides recently introduced into the market, such as strobilurins, fludioxonil, and fenpiclonil, were developed from microbial metabolites through chemical modifications that endowed them with additional features suitable for agricultural use.[5,6] This approach using microbial metabolites as the lead compound for fungicide development has proven to be a promising and effective strategy for the development of new fungicides. More recently, whole culture broth or crude culture extracts of numerous microorganisms producing antifungal secondary metabolites have been introduced as biofungicides, and these have shown potent control efficacies, protecting against a variety of plant diseases, including chronic diseases that are difficult to control with conventional synthetic fungicides.[7−9] As a result, microbial metabolites are currently facing a revival as a source of plant disease control agents.

Vascular wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* is a devastating soilborne disease and in warmer climate regions causes severe loss of tomato plant yield.[10] The management of Fusarium wilt is a difficult task because the pathogen population can rapidly build up in soil and persist for many years, even when host plants are not present. Fusarium wilt has been controlled by integrating strategies from several different control methods such as cultivation of disease-resistant varieties and application of chemicals such as preplant soil fumigation with methyl bromide. Because new pathogenic fungal species are continuously evolving and the production and use of chemicals such as methyl bromide are to be phased out in most countries, novel control measures that reinforce current control strategies or formulate alternative strategies need to be developed.[11]

A *Streptomyces* strain was isolated during the screening for microorganisms producing antifungal metabolites. Culture extract from this strain demonstrated potent disease control efficacy against Fusarium wilt on tomato plants. We report here the isolation of the antifungal metabolites from the culture broth using a variety of chromatographic procedures and the structural elucidation of the metabolites using photochemical analyses.

Received: October 4, 2010
Accepted: January 19, 2011
Revised: January 19, 2011
Published: February 11, 2011

dx.doi.org/10.1021/jf1038585 | *J. Agric. Food Chem.* 2011, 59, 1893–1899

MATERIALS AND METHODS

Isolation of Antagonistic Actinomycetes. Soil samples used for the isolation of antagonistic actinomycetes were collected from the Guri province in Korea. Soil samples (10 g) were taken from a depth of 5−20 cm below the soil surface and suspended in 100 mL of sterile water in 1 L Erlenmeyer flasks. The flasks were incubated at 28 °C and agitated at 170 rpm for 3 h, and the soil suspension was then filtered using Whatman no. 1 filter paper.

The filtered soil suspension was then 10-fold serial diluted to 10^{-5}. Each diluted soil sample (100 μL) was plated onto humic acid vitamin agar medium (1.0 g of humic acid, 0.5 g of Na_2HPO_4, 1.71 g of KCl, 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.02 g of $CaCO_3$, 0.5 mg of thiamin−HCl, 0.5 mg of riboflavin, 0.5 mg of niacin, 0.5 mg of pyridoxine−HCl, 0.5 mg of inositol, 0.5 mg of calcium pantothenate, 0.5 mg of *p*-aminobenzoic acid, 0.25 mg of biotin, 50 mg of cycloheximide, and 15 g of agar, in 1 L of H_2O, adjusted to pH 7.2; vitamins and cycloheximide were filter-sterilized). The plates were incubated at 28 °C for 4−14 days. On the basis of colony morphological characteristics, actinomycetes colonies on the plates were selected and transferred to 3% tryptic soy agar (Difco Laboratories, Detroit, MI). Isolated actinomycetes were stored in 40% glycerol suspensions at −80 °C.

The activities of the selected actinomycetes against plant pathogenic fungi were evaluated on tryptic soy agar plates by inoculating the actinomycetes in the center of the plate. Following incubation at 28 °C for 2 days, mycelia disks (5 mm in diameter) of *Colletotrichum orbiculare*, *Diaporthe citri*, *Fusarium oxysporum* f.sp. *lycopersici*, *Magnaporthe oryzae*, *Phytophthora capsici*, and *Rhizoctonia solani* were inoculated onto both sides of the plates. Following incubation at 28 °C for 7 days, the inhibition zones were measured. The actinomycete strain KP1404 showed potent antifungal activity and was selected for further study.

Identification of Actinomycete Strain KP1404. Actinomycete strain KP1404 was grown on tryptic soy agar at 28 °C for 5 days, and a cell lysate was prepared by resuspending actinomycete cells in sterile water in a microcentrifuge tube and boiling at 100 °C for 20 min. The supernatant of the boiled sample containing genomic DNA was then used as the template for PCR.

PCR hot start premix (Bioneer Co., Daejeon, Korea) was used for amplification of the 16S rDNA from actinomycete strain KP1404 using forward primer fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer rP2 (5′-ACGGCTACCTTGTTACGACTT-3′).[12]

The PCR reaction mixture contained 100 ng of template DNA, primers fD1 and rP2 (each at a concentration of 10 pmol), 1 unit of *Taq* polymerase, 1× *Taq* polymerase buffer, 1.5 mM $MgCl_2$, and a mixture of deoxynucleoside triphosphates (dNTPs) (250 μM of each dNTP). The final volume of premix was adjusted to 20 μL by the addition of distilled water.

The PCR mixture was subjected to an initial denaturation step of 95 °C for 15 min followed by 30 cycles consisting of three steps: a 30 s denaturation step at 94 °C, a 30 s annealing step at 55 °C, and a 1 min extension step at 72 °C. A final 10 min extension step at 72 °C was then performed, and the reaction sequence was completed by cooling the reaction to 4 °C. The amplified DNA was analyzed by agarose gel (0.8%) electrophoresis and visualized by UV fluorescence following staining with ethidium bromide. The amplified 16S rDNA was purified using a PCR product purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The sequence of the purified 16S rDNA was determined using an ABI Prism 3700 automatic DNA sequencer (Applied Biosystems, Foster City, CA), and the software program DNA Star was used for sequence assembly (DNAStar Inc., Madison, WI). Analysis of the 16S rDNA sequence of the actinomycete strain KP1404 was performed using the BLAST network service at the National Center for Biotechnology Information. The 16S rDNA sequence of 1380 nucleotides was aligned with various representative nucleotide sequences from related *Streptomyces* species.

Morphological and physiological characterization studies of strain KP1404 were performed according to methods described by Waksman,[13] Shirling and Gottlieb,[14] and *Bergey's Manual of Systemic Bacteriology*.[15] Morphological characteristics, such as spore size, spore chain morphology, and spore surface ornamentation, of strain KP1404 when cultured on ISP3 medium for 10 days at 28 °C were observed by scanning electron microscopy. The chemotaxonomic characteristics of strain KP1404 were examined by performing diaminopimelic acid analysis as described previously.[8,14]

Production and Purification of Antibiotics ST-1 and ST-2. The actinomycete strain KP1404 was grown on tryptic soy agar for 5 days at 28 °C. Single colonies were transferred into 500 mL Erlenmeyer flasks containing 50 mL of tryptic soy broth. The flasks were agitated on a rotary shaker at 28 °C and 170 rpm for 2 days. A 1 mL aliquot of the culture broth was transferred into 100 mL of glucose starch molasses medium (10 g of soluble starch, 20 g of glucose, 5 mL of molasses, 5 g of yeast extract, 5 g of peptone, and 2 g of calcium carbonate, in 1 L of distilled water, pH 7.0) in a 1 L Erlenmeyer flask. The inoculated flasks were incubated on a rotary shaker at 28 °C and 200 rpm for 5 days. The culture broth (5 L) was subjected to centrifugation at 10000*g* for 35 min, and then the harvested cells were extracted with 1 L of methanol. The crude cell extracts were evaporated in vacuo to dryness, and the resulting residue was resuspended in 1 L of 2% aqueous methanol.

The crude cell extracts were purified by flash column chromatographic procedures. The columns used were a 300 mm × 100 mm i.d. glass column packed with Diaion HP-20 resin (Mitsubishi Chemical Corp., Tokyo, Japan) and a 200 mm × 120 mm i.d. glass column packed with 20−63 μm Lichropep RP-18 resin (Merck, Darmstadt, Germany). The crude cell extracts were adsorbed to Diaion HP-20 resin and then eluted with a stepwise gradient of water and methanol (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100, v/v), and the column was washed with acetone (1 L). Each fraction (500 mL) was concentrated in vacuo, and its antifungal activity was tested using the paper disk method. The active fractions were pooled and resuspended in 500 mL of 2% aqueous methanol and further purified by C18 flash chromatography. The column was eluted with a stepwise gradient of water and methanol (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100, v/v). Each fraction was concentrated in vacuo and then bioassayed using the paper disk method. The active fractions were pooled and freeze-dried. The dried sample was dissolved in dimethyl sulfoxide (10 mL) and further purified using a Gilson HPLC system (Gilson, Middleton, WI). The column used was a 250 mm × 10 mm i.d., 5 μm, Discovery C18 (Supelco, Bellefonte, PA). The chromatography was conducted at a flow rate of 2 mL/min using a linear gradient solvent system (from 57 to 74% methanol). The eluate of each peak was collected while the absorbance at 254 nm was monitored and bioassayed for antifungal activity.

Structure Elucidation of the Antibiotics ST-1 and ST-2. UV absorption spectra and high-resolution mass spectra of the purified antibiotics ST-1 and ST-2 were recorded on a quadruple time-of-flight tandem mass spectrometer (Waters, Manchester, U.K.) using the ESI-MS method. The following HR-ESI-MS spectrometry data were observed for the compounds ST-1 and ST-2: ST-1, $[M + H]^+$ calcd 581.3326, found 581.3298; ST-2, $[M + H]^+$ calcd 595.3482, found 595.3474. Additionally, nuclear magnetic resonance (NMR) spectra were recorded at room temperature on a Varian 500 MHz spectrometer (Varian, Palo Alto, CA). ^{1}H NMR spectra were measured in DMSO-d_6 (99.9% D) (Cambridge Isotope Laboratories, Andover, MA). Chemical shifts are given in δ values (ppm) referenced to the proton of the solvent as the internal standard, and coupling constants (J) are given in hertz. ^{13}C NMR spectra (125 MHz) were recorded in DMSO-d_6 (99.9% D) using broad-band proton decoupling. Distortionless enhancement by

polarization transfer spectra were obtained using Varian's automated DEPT pulse program spectra. A variety of 2D-NMR spectroscopies such as DQF-COSY (double quantum filtered correlation spectra), HMBC (heteronuclear shift correlations via multiple bond connectivites), and HSQC (heteronuclear single quantum coherence) were recorded on a Varian 500 MHz NMR spectrometer. On the basis of these results, the structures of the antibiotics were determined. $[\alpha]_D^{20}$ values of ST-1 and ST-2 (*c* 0.6%, CH_3OH) were -26 ± 2 and -23 ± 2, respectively.

In Vitro and in Vivo Antifungal Bioassays. During the purification procedures, active fractions were confirmed by the paper disk bioassay method. Sterile paper disks (8 mm in diameter) were loaded with individual fractions collected from the chromatographic procedures. A conidial suspension of *F. oxysporum* f.sp. *lycopersici* (10^7 conidia/mL) was added to molten potato dextrose agar (0.7% agar) at 40 °C. The seeded agar medium was poured into 90 mm Petri dishes and the paper disk treated with each fraction placed on the agar surface. After incubation for 1 day at 28 °C, the mycelial growth inhibition zones around the paper disks were measured.

The R_f values of antifungal compounds on TLC plates were confirmed using a bioautographic method. The concentrated active fractions were loaded onto 0.2 mm thickness, 60 F_{254} silica gel TLC plates in duplicate (Merck) and developed with butanol/acetic acid/water (4:1:1, v/v). *F. oxysporum* f.sp. *lycopersici* (10^7 conidia/mL) seeded agar medium was uniformly poured onto one of the developed TLC plates, which was placed on a water agar plate (1.5% agar in a 9 cm diameter Petri dish) after air-drying to remove the solvent. The bands on the second TLC plate were visualized by the addition of *p*-anisaldehyde−sulfuric acid. After incubation at 28 °C for 1 day, the inhibition zone on the TLC plate was compared to the bands on the second TLC plate.

The minimum inhibitory concentrations (MICs) of the antibiotics ST-1 and ST-2 against several plant pathogenic fungi were evaluated in 96-well plates (Cell Wells) (Corning Glass Corp., Corning, NY) as recommended in the CLSI M38-A protocol.[16] For the tests, 100 μL of conidial suspension of plant pathogenic fungi (*Alternaria brassicola, Alternaria mali, Aspergillus oryzae, Cercospora canescens, Colletotrichum cocodes, Colletotrichum gloeosporioides, Colletotrichum orbiculare, Cylindrocarpon destructans, F. oxysporum* f.sp. *lycopersici,* and *F. oxysporum* f.sp. *cucumerinum*) or mycelial suspension (*Diaporthe citiri, Rhizoctonia solani,* and *Sclerotinia sclerotiorum*) was dispensed into each well so that the final concentration was $(0.5-5) \times 10^4$ spores or mycelial fragments/mL. The antibiotics ST-1 and ST-2 (100 μL) were added into each well so that the final concentrations in the wells ranged from 0.125 to 64 μg/mL. After incubation at 28 °C for 1−3 days, MICs were determined by visual examination and corresponded to the lowest concentration that caused complete growth inhibition.

In vivo disease control efficacies of antibiotics ST-1 and ST-2 and the commercial fungicide benomyl against Fusarium wilt on tomato plants were evaluated under greenhouse conditions. Tomato seeds, pregerminated at 28 °C, were sown in 36 plastic pots (25 × 25 × 6 cm) containing a sterilized soil mix (peat moss, perlite, and vermiculite, 5:3:2, v/v/v). Tomato plants were raised in a greenhouse at 25 ± 3 °C for 14 days. The antibiotics ST-1 and ST-2 were dissolved in DMSO, and benomyl was dissolved in water; all were serially diluted to 1, 10, 100, and 500 μg/mL. *F. oxysporum* f.sp. *lycopersici* JCM 12575 was cultured on Czapek Dox broth (30 g of sucrose, 3 g of $NaNO_3$, 1 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, and 0.01 g of $FeSO_4 \cdot 7H_2O$, in 1 L of distilled water, pH 7.3 ± 0.2, before autoclaving) at 28 °C for 14 days. A conidial suspension (2×10^6 conidia/mL) was used as the inoculum. Wounded roots of tomato seedlings were dipped into the conidial suspension for 10 min; then the tomato seedlings were transferred into the plastic pots, and 5 mL of ST-1, ST-2, or benomyl at each concentration was applied as a soil drench. Control plants were drenched with 5 mL of sterilized water. The inoculated seedlings were placed in a dew chamber at 28 °C for 24 h and then transferred to a greenhouse. Disease severity on the tomato seedlings, based on a scale of 0−5, was estimated 10 days after inoculation (0, no visible disease symptoms; 1, leaves slightly wilted with yellowish lesions beginning to appear on leaves; 2, 30−50% of entire plant wilted; 3, 50−70% of entire plant wilted; 4, 70−90% of entire plant wilted; and 5, plant dead).

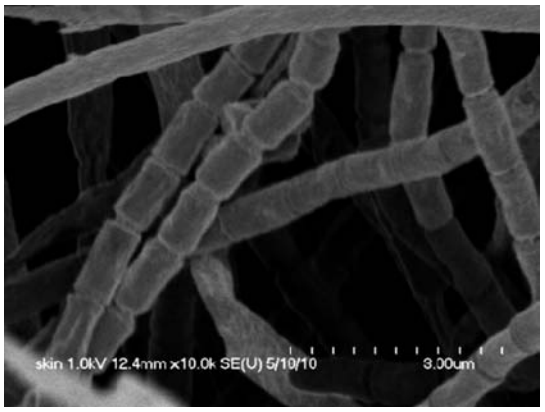

Figure 1. Scanning electron micrograph of *Streptomyces psammoticus* strain KP1404 cultured on ISP3 for 10 days at 28 °C.

RESULTS

Isolation of Actinomycete Strain KP1404. Among the 600 actinomycete strains isolated from soil samples of the Guri province in Korea, 91 strains showed antifungal activity against at least one of the plant pathogenic fungi tested. One of the antagonistic actinomycete strains, KP1404, showed potent inhibition of mycelial growth of *F. oxysporum* f.sp. *lycopersici*. Therefore, strain KP1404 was selected for further studies to evaluate its disease control efficacy and to identify its active ingredients.

Identification of Strain KP1404 Based on Morphological and Molecular Analysis. A comparison of a partial 16S rDNA sequence of 1380 nucleotides for strain KP 1404 (GenBank accession no. GU166432) with 16S rDNA sequences in the NCBI database revealed a 99.93% identity to the 16S rDNA sequence of *Streptomyces psammoticus* strain NBRC13971 (accession no. AB184554).

The morphological and physiological characteristics of strain KP1404 indicated that the strain was a *Streptomyces*. Spore chain morphology and spore surface ornamentation were observed by scanning electron microscope (Figure 1). The spore chains of strain KP1404 were of the rectiflexible type and consisted of cylindrically shaped, smooth-surfaced spores (0.8−1 μm diameter). Specific structures, such as synnemata, sclerotia, or sporangia, were not observed. The cell wall hydrolysates of KP1404 contained L,L-diaminopimelic acid. On the basis of these results, strain KP1404 was identified as *S. psammoticus*. Strain KP1404 has been deposited in the Korean Agricultural Culture Collection as *S. psammoticus* KACC 14610.

Antifungal Compounds Produced by Strain KP1404. To purify the active ingredients from the culture extract, sequential chromatographic procedures were performed. The crude cell extract (800 mg) was subjected to Diaion LH-20 column chromatography, and two fractions eluted with 60% and 80% methanol showed antifungal activity. With a bioautographic experiment on TLC plate developed with butanol/acetic acid/water (4:1:1, v/v), both active fractions revealed an oval-shaped inhibition zone at R_f 0.65. The fractions of 60 and 80% methanol

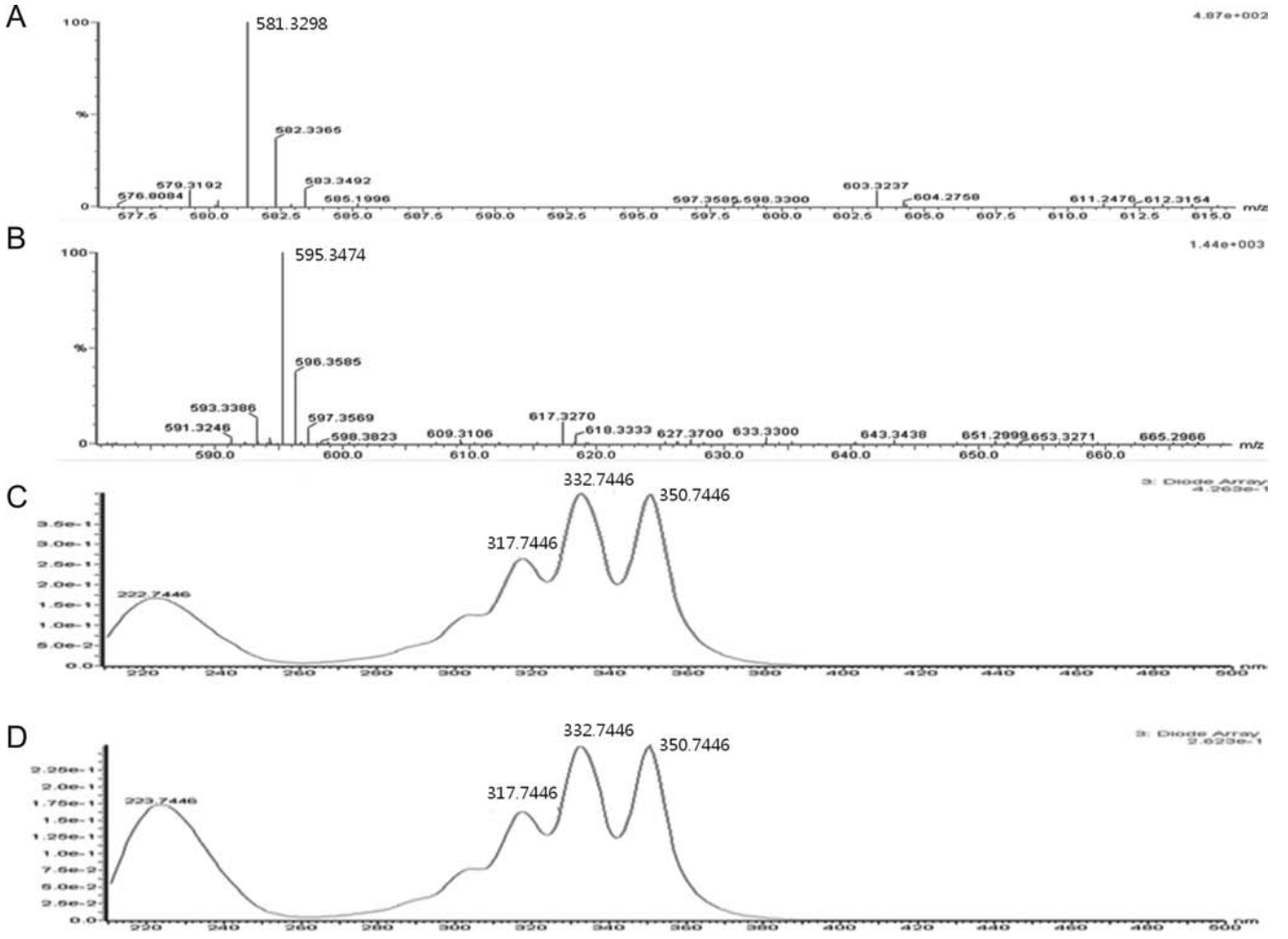

Figure 2. Mass spectra of antibiotics ST-1 (A) and ST-2 (B) and UV spectroscopic data of ST-1 (C) and ST-2 (D).

eluates were pooled and further purified using C18 column chromatography. After a stepwise elution with methanol/water (v/v), the fractions collected from the 40−100% methanol eluates showed antifungal activity. Following evaporation of the methanol under reduced pressure, the concentrate (200 mg) was dissolved in DMSO (5 mL) and then subjected to a semipreparative HPLC system equipped with a C18 reverse-phase column. Active fractions were eluted at retention times of 21.1 min (fraction 1) and 23.8 min (fraction 2). After drying under reduced pressure, fractions 1 and 2 yielded 30 mg (compound ST-1) and 20 mg (compound ST-2) of a white powdered substance, respectively.

Structures of ST-1 and ST-2. UV analyses of ST-1 and ST-2 revealed the presence of UV absorption maxima at 317, 332, and 350 nm, a characteristic pattern for conjugated pentaene structures. The molecular formulas of ST-1 ($C_{31}H_{48}O_{10}$) and ST-2 ($C_{32}H_{50}O_{10}$) were calculated from HR-ESI MS spectrometry data (Figure 2). The ^{13}C NMR spectrum of ST-1 had 31 signals, which were assigned to 2 carboxyl, 5 methylene, 10 methine, and 3 methyl groups in combination with the HSQC-DEPT results (Table 1). The pentaene moiety observed in the UV spectrum was confirmed by the presence of a spin system of 10 olefinic protons. Carbon connectivities of the compounds were determined from a combination of COSY and HMBC spectra. On the basis of spectrometric MS and NMR analyses, the structures of the antifungal compounds ST-1 and ST-2 were found to correspond to those of the polyene antibiotics strevertene A and strevertene B, respectively (Figure 3). The spectroscopic data of ST-1 and ST-2 were in complete agreement with those described previously for strevertenes A and B by Schlingmann et al.[17]

In Vitro and in Vivo Antifungal Activities of Strevertenes A and B. In vitro antimicrobial spectra of strevertenes A and B were determined by CLSI method for filamentous fungi (M38-A) in 96-well plates. With the exception of *A. brassicola* and *R. solani*, the mycelial growth of all the plant pathogenic fungi examined were inhibited by strevertenes A and B. Strevertenes A and B inhibited the growth of *A. mali*, *A. oryzae*, *C. canescens*, *C. cocodes*, *C. gloeosporioides*, *C. orbiculare*, *C. destructans*, *F. oxysporum* f.sp. *cucmerinum*, *F. oxysporum* f.sp. *lycopersici*, and *S. sclerotiorum* at low concentrations of 4−16 μg/mL (Table 2).

In vivo, strevertenes A and B strongly inhibited Fusarium wilt development on tomato plants grown under greenhouse conditions at 1 μg/mL (Figure 4). Strevertenes A and B were as effective as benomyl within the same concentration range. The antibiotics ST-1 and ST-2 did not show any phytotoxicity toward tomato plants at a concentration of 500 μg/mL.

DISCUSSION

Because potent antifungal activity in vitro cannot always be replicated in planta, microbial metabolites with potential antifungal activity must be tested under greenhouse and field

Table 1. NMR Spectroscopic Data for Antibiotics ST-1 and ST-2 (^{1}H and ^{13}C at 500 MHz in DMSO-d_6)

	strevertene A (ST-1)		strevertene B (ST-2)	
carbon no.	^{13}C, δ	^{1}H, δ (m[a], J in Hz)	^{13}C, δ	^{1}H, δ (m[a], J in Hz)
1	174.30		174.40	
2	46.85	2.24 (qd, 7.1, 7.3)	54.00	2.23 (m)
2-Me	14.30	1.06 (d, 7.1)	22.10	1.43 (m), 1.74 (m)
3	72.40	3.65 (m)	71.38	3.61 (m)
4	42.01	1.24 (m)	42.10	1.25 (m)
5	71.92	3.75 (m)	71.89	3.80 (m)
6	43.38	1.24 (m)	43.42	1.25 (m)
7	71.70	3.50 (m)	71.68	3.52 (m)
8	39.27	1.09 (m)	39.30	1.09 (m)
9	22.92	1.70 (m), 0.88 (m)	22.90	1.73 (m), 0.90 (m)
10	38.64	0.89 (m), 1.21 (m)	38.62	0.92 (m), 1.23 (m)
11	69.90	3.43 (m)	69.87	3.44 (m)
12	41.09	1.49 (m), 1.53 (m)	41.12	1.47 (m), 1.55 (m)
13	67.22	3.38 (m)	67.22	3.37 (m)
14	58.15	2.50 (dd, 9.5)	58.20	2.49 (dd, 9.5)
14-COOH	174.46		173.80	
15	72.64	4.07 (t, 9.5)	72.50	4.07 (t, 9.5)
16	135.18	5.51 (dd 15.3, 9.5)	135.10	5.51 (dd 15.3, 9.5)
17	132.09	6.14 (dd, 15.3)	132.12	6.13 (dd, 15.3)
18	131.68	6.24	131.65	6.26
19	133.92	6.26−6.27 (overlap)	133.87	6.26−6.27 (overlap)
20	132.36	6.26−6.27 (overlap)	132.40	6.26−6.27 (overlap)
21	133.83	6.26−6.27 (overlap)	133.70	6.26−6.27 (overlap)
22	131.66	6.26−6.27 (overlap)	131.61	6.26−6.27 (overlap)
23	133.70	6.25	133.65	6.25
24	129.75	6.11 (dd, 15.6)	129.69	6.12 (dd, 15.6)
25	136.95	5.90 (dd, 15.6, 6.6)	137.01	5.94 (dd, 15.6, 6.6)
26	40.85	2.33 (qdd, 9.0, 6.9, 6.6)	40.83	2.34 (qdd, 9.0, 6.9, 6.6)
26-Me	16.24	0.99 (d, 6.9)	16.31	1.01 (d, 6.9)
27	74.20	4.63 (dd, 9.0, 6.3)	74.25	4.64 (dd, 9.0, 6.3)
28	19.34	1.16 (d, 6.3)	19.38	1.18 (d, 6.3)

[a] Abbrevations of signal multiplicity: d, doublet; t, triplet; m, multiplet; dd, doublet of doublets; qd, quartet of doublets; qdd, quartet of doublet of doublets.

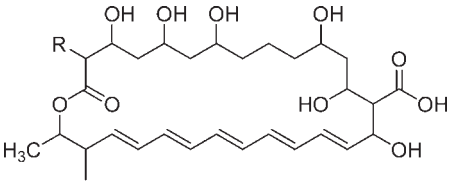

Figure 3. Structures of pentaene antibiotics ST-1 (strevertene A) and ST-2 (strevertene B).

conditions.[18] The culture extract of *S. psammoticus* strain KP1404 showed broad and potent antifungal activity against various plant pathogenic fungi in vitro as well as effective inhibitory activity against Fusarium wilt caused by *F. oxysporum* f.sp. *lycopersici* JCM 12575 (data not presented). Therefore, we decided to identify the agents in the culture extract of strain KP1404 that were responsible for impeding Fusarium wilt development. During the purification procedure, two major compounds, ST-1 and ST-2, were identified by HPLC analysis. No other fractions collected from peak or baseline fractions showed antifungal activity against *F. oxysporum* f.sp. *lycopersici*. A variety of spectrometric analyses revealed that the structures of ST-1 and ST-2 are pentaene macrolides strevertenes A and B, respectively, lacking hemiketal-tetrahydropyran and aminoglycoside moieties that are common to most pentaene antibiotics.

Since the first polyene antibiotic nystatin was discovered in the 1950s, over 200 polyenes, including amphotericin B, filipin, and rapamycin, have been isolated from *Streptomyces* spp., and most have been reported to have antifungal activities against various yeast and filamentous fungal species.[19−24] Despite their potent and broad-spectrum antifungal activities, however, the polyenes are not well recognized as fungicides for plant disease control because of their inherent structural vulnerability. The polyenes typically possess one or several potentially unstable structural functionalities, including hydrolyzable esters, acetals, and hemiacetals, as well as conjugated polyene systems, which are vulnerable to oxidation.[25] Thus, to a certain extent, polyene antifungals

Table 2. Minimum Inhibitory Concentrations (MICs) of Strevertenes A and B from *S. psammoticus* Strain KP1404 against Various Plant Pathogenic Fungi

	MIC[a] (μg/mL)	
plant pathogenic fungus	strevertene A	strevertene B
Alternaria brassicola	>64[b]	>64
Alternaria mali	16	16
Aspergillus oryzae	16	10
Cercospora canescens	16	32
Colletotrichum cocodes	8	8
Colletotrichum gloeosporioides	8	8
Colletotrichum orbiculare	8	4
Cylindrocarpon destructans	16	16
Diaporthe citri	32	32
Fusarium oxysporum f.sp. *cucumerinum*	16	8
Fusarium oxysporum f.sp. *lycopersici*	16	8
Sclerotinia sclerotiorum	8	8
Rhizoctonia solani	>64	>64

[a] The lowest concentration that completely inhibited the growth of the plant pathogenic fungi was determined after incubation for 1−3 days. [b] >64 indicates that growth of the test microorganism was not inhibited at concentrations above 64 μg/mL.

are subject to inactivation or degradation under the harsh conditions routinely encountered in crop-growing fields, such as elevated temperatures, atmospheric oxygen, and exposure to light.[25,26] For example, the polyene antibiotic compound natamycin (pimaricin) showed strong antifungal activity against a wide range of plant pathogenic fungi in vitro but, because of autoxidation, lost its antifungal activity within 3 h of application on cucumber leaves.[27] Despite the vulnerability of polyenes under crop-growing conditions, the potent and broad-spectrum antifungal activities of these compounds have continued to encourage the development of polyene-based fungicides. Recently, a number of novel polyenes and polyene formulations have been devised and examined for efficacy against plant diseases. Nystatin has antifungal activity against *Botrytis*, *Alternaria*, and *Fusarium* spp. causing the spotting of orchids, and candicidin D exhibits antifungal activity in vivo against *Cladosporium fulvum*.[28,29] Recently, a natamycin−lignosulfonate combination has been reported to inhibit the development of *Botrytis* on lily leaf tips.[30] Other combinations, such as natamycin−phosphate and natamycin−potassium phosphate, have been reported to inhibit *F. oxysporum* f.sp. *tulipae* and *Helminthosporium solani* in vivo, respectively.[30,31] Also, the polyene antibiotics produced by an endophytic *Streptomyces* sp. isolated from a *Rhododendron* sp. exhibit potent antifungal activity against *Phytophthora cinnamomi* in vivo, which demonstrated that polyene-producing microorganisms had potential as microbial fungicides.[32] As a result, there has been a revival of interest in polyene antibiotics, as well as in the strains that produce them, as agrichemicals and biological control agents.

The polyene antibiotic strevertenes were first discovered in cell extracts of *Streptoverticillium* LL-30F848 by Schlingmann et al.[17] Although well-known polyenes such as nystatin, amphotericin, and filipin are mainly produced by *Streptomyces* species,[33,34] to date no *Streptomyces* sp. have been reported to produce strevertenes. Strevertenes were known to inhibit the growth of some plant pathogenic fungi, such as *Botrytis cinerea*, *Cercospora beticola*, *Erysiphe graminis* f.sp. *tritici*, *Plasmopara viticola*, *Puccinia recondita* f.sp. *tritici*, *Pyrenophora teres*, *Uromyces fabae*, and *Venturia inaequalis*.[17] In the present study, we examined the antifungal activity of strevertenes against other plant pathogenic microorganisms such as *A. mali*, *A. oryzae*, *C. canescens*, *C. orbiculare*, *C. destructans*, *D. citri*, *F. oxysporum* f.sp. *lycopersici*, *S. sclerotiorum*, and *R. solani*. As with other polyene antibiotics, strevertenes appear to have a very broad antifungal spectrum against plant pathogenic fungi.

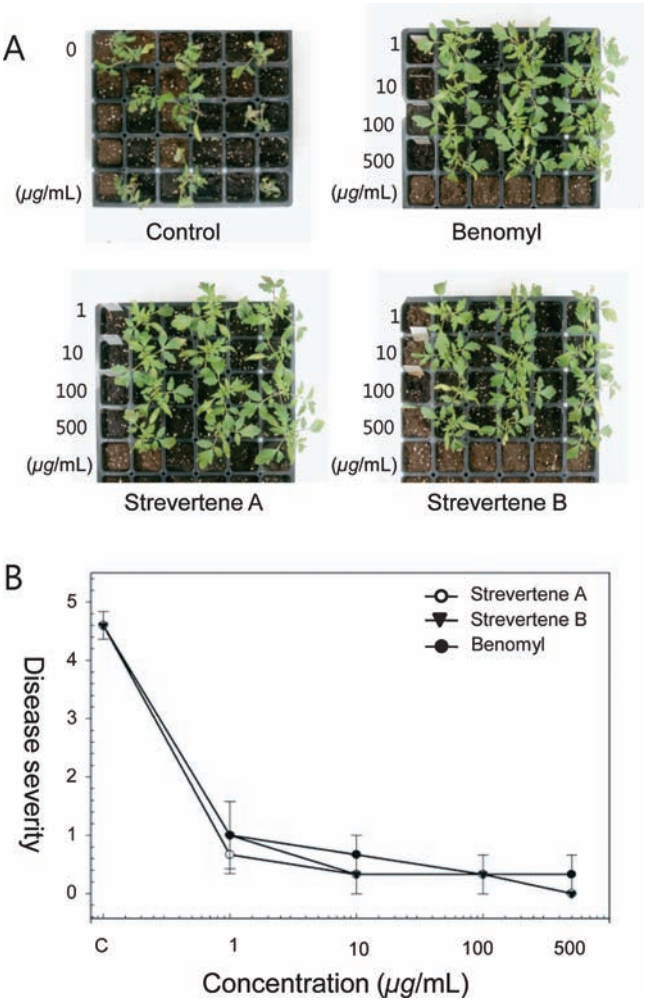

Figure 4. In vivo inhibitory activities of strevertenes and benomyl against Fusarium wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* JCM 12575 in tomato plants: (A) disease development observed in the tomato plants treated with different concentrations of strevertenes and benomyl; (B) disease severity based on visible symptoms (scale of 0−5) observed 10 days after inoculation with *F. oxisporum* f.sp. *lycopersici*. Error bars reperesent standard deviations of three replicates.

Despite the potent antifungal activity of strevertenes in vitro, their effectiveness as disease control agents against plant diseases has not been reported. One reason for this might be their inherent structural instability. However, in vivo assays revealed that strevertenes A and B had potent control activity against Fusarium wilt on tomato plants even when used at low concentrations and that they were as effective as the commercial fungicide benomyl. These results show that strevertenes A and B are major constituents of the culture filtrate of *S. psammoticus* strain KP1404 and that they have inhibitory activity against Fusarium wilt in tomato plants, establishing their potential as disease control agents. To

our knowledge, this is the first report of strevertenes produced by *Streptomyces* sp. and proposing their potential as disease control agents against Fusarium wilt in tomato plants.

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Funding Sources

This research has been supported in part by the Korea Ministry for Food, Agriculture, Forestry and Fisheries (IPET project) and K2 research fund raised by Kyung Nong Corp.

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