Isolation, Antifungal Activity, and Structure Elucidation of the Glutarimide Antibiotic, Streptimidone, Produced by *Micromonospora coerulea*

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The antibiotic Ao58A ,which showed strong antifungal activity against some plant pathogenic fungi, was purified from the culture broth and mycelial mats of *Micromonospora coerulea* strain Ao58 using various chromatographic procedures. The molecular formula of the antibiotic Ao58A was deduced to be $C_{16}H_{23}NO_4$ (M + H, *m/z* 294.1707) by high-resolution FAB mass spectroscopy. Analyses of ^{1}H NMR, ^{13}C NMR, and 2D NMR spectral data revealed that the antibiotic Ao58A is the glutarimide antibiotic streptimidone, 4-(2-hydroxy-5,7-dimethyl-4-oxo-6,8-nonadienyl)-2,6-piperidinedione. The antibiotic Ao58A was very effective in inhibiting growth of *Phytophthora capsici*, *Didymella bryoniae*, *Magnaporthe grisea*, and *Botrytis cinerea* in the range ∼3−10 μg mL^{-1} of MICs. In vivo evaluation of the antibiotic Ao58A under greenhouse condition showed strong control efficacies against the development of *P. capsici*, *B. cinerea*, and *M. grisea* on pepper, cucumber, and rice plants, respectively. The antibiotic Ao58A was equally as effective as metalaxyl, vinclozolin, and tricyclazole in the control of these plant diseases. However, it did not show any phytotoxicity on the plants even when treated with 500 μg mL^{-1}.

Keywords: *Micromonospora coerulea; streptimidone; antifungal activity; plant disease control*

INTRODUCTION

With the advances in fungicide chemistry and the improvements in availability of effective fungicides, developments of new fungicides from natural bioactive compounds for the efficient control of plant diseases are urgently required to minimize adverse effects of synthetic fungicides on agro-ecosystems and emergence of plant pathogens resistant to the currently used fungicides (Tanaka and Omura, 1993; Russell et al., 1995). Since blasticidin S was successfully used for the control of rice blast disease (Takeuchi et al., 1957), there has been an interest in microbial metabolites as fungicides for agricultural use, which might eliminate various deleterious effects caused by the use of synthetic compounds. Because of the rapid biodegradability of the microbial metabolites, they disappear readily from the soil environment after application to fields (Tanaka and Omura, 1993).

The antifungal metabolites of microbial origin contribute to the development of new agricultural fungicides. The antibiotics such as blasticidin S, polyoxin, kasugamycin, validamycin, and mildiomycin are being practically used as active ingredients for effective control of plant diseases (Godfrey, 1994; Knight et al., 1997). Several antifungal metabolites of simple structures have provided lead compounds for the chemical synthesis of fungicides. Recent examples of these compounds include fenpiclonil and fludioxonil derived from pyrrolnitrin (Fischer et al., 1992), and synthetic derivatives of the antibiotic strobilurins, such as the β-methoxyacrylates azoxystrobin and kresoxim-methyl (Anke et al., 1977; Godfrey, 1994). More recently, some antibiotic compounds such as gopalamicin (Nair et al., 1994), tubercidin (Hwang et al., 1994; Hwang and Kim, 1995), and a manumycin-type antibiotic (Hwang et al., 1996) were found to have excellent potent antifungal activity against plant fungal diseases.

The actinomycetes are noteworthy as antibiotic producers, making up three-fourths of all known antibiotics from natural products (Huck et al., 1991). In particular, *Streptomyces* are prolific producers of bioactive natural products from the viewpoint of diversity in chemical structures. However, the frequency of rediscovery of known compounds by *Streptomyces* spp. was fairly high. In an effort to isolate novel natural products, therefore, the use of rare actinomycete strains other than *Streptomyces* spp. has steadily increased (Tanaka and Omura, 1993). Recently, the genus *Micromonospora* has been recognized as one of the important sources of antimicrobial metabolites (Goodfellow, 1988). The *Micromonospora* spp. are widely distributed in soils of various geographical regions, but they are only a minor component of the actinomycete population (Hayakawa et al., 1988; Vobis, 1991). Since the discovery of the aminoglycoside antibacterial antibiotic gentamicin from *Micromonospora purpurea* and *Micromonospora echinospora* (Weinstein et al., 1964), the microorganisms in the genus *Micromonospora* have extensively been studied as a source of antimicrobial agents. As results, many aminoglycoside type antibiotics were isolated. Neomycin B is the first example of an aminoglycoside antibiotic produced by both *Streptomyces* species and *Micromonospora* species (Wagman et al., 1973). Macrolide type antibiotics such as megalomicins (Weinstein et al.,

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1969), rosaramicin (Wagman et al., 1972), and juvenimicins (Hatano et al., 1976), that showed strong antibacterial activity against Gram-positive and -negative bacteria were also produced by *Micromonospora* strains. Production of the macrolide erythromycin B outside of the genus *Streptomyces* was first demonstrated by *Micromonospora* species (Wagman, 1980). In addition, miscellaneous types of antibiotics such as iodinin (Gerber and Lechevalier, 1964), tetrenolin (Pagani et al., 1973), and bottromycins (Nakamura et al., 1965) have been found in the culture of various species of *Micromonospora*.

The antibiotics of the glutarimide group are structurally characterized by the presence of glutarimide ring bearing a side chain at the 4-position. To date, approximately 20 antibiotics of the glutarimide group have been isolated from various species of *Streptomyces* (Betina, 1994). In most members, the side chain terminates in a substituted carbocyclic ring, usually cyclohexyl or phenyl except for streptimidone for which the side chain is acyclic. The biogeneses of these glutarimide antibiotics are in general similar to each other, and proceeds basically in a polyketide fashion including the initiation of the carbon skeleton by a malonate unit, the presence of a branch completing the glutarimide ring, and the introduction of additional methyl substituents (Cudlin et al., 1969; Allen et al., 1976). The glutarimide antibiotics noted for their antifungal activity were found to inhibit protein synthesis in a wide range of eukaryotic cells (Kohberger et al., 1960; Grollman, 1966; Obrig et al., 1971) but neither in prokaryotes nor in mitochondria and chloroplasts (Ellis, 1969). The well-known glutarimide antibiotic cycloheximide, which has strongly inhibitory activity against yeasts and filamentous fungi, showed systemic control activity against some plant diseases (Ford et al., 1958). However, the toxicity on the host plants limited its use as a plant chemotherapeutic agent.

In our search program for microorganisms producing antifungal antibiotics useful for plant protection, we isolated an actinomycete strain Ao58 from sea-mud soils in Korea which was active against some plant pathogenic fungi (Kim and Hwang, 1997). In the present study, the antagonistic actinomycete strain Ao58 was identified as *Micromonospora coerulea*. The antibiotic substance Ao58A active against some plant pathogenic fungi was purified from the culture broth of *M. coerulea* using various chromatographic procedures. Using various spectral analyses, we identified the compound as 4-(2-hydroxy-5,7-dimethyl-4-oxo-6,8-nonadienyl)-2,6-piperidinedione, the glutarimide antibiotic streptimidone. In vitro antifungal activity and in vivo control efficacy of the antibiotic Ao58A against some plant diseases were also evaluated by comparison with those of the commercial fungicides. This is the first report of production of the glutarimide antibiotic by the genus *Micromonospora*.

EXPERIMENTAL PROCEDURES

Identification of Actinomycete Strain Ao58. The actinomycete strain Ao58 antagonistic to plant pathogenic fungi was isolated from the sea-mud soil at Young-Jong island in Korea using the screening procedures described by Kim and Hwang (1997). The organism was identified using the methods developed by Waksman (1961) and *Bergey's Manual of Systematic Bacteriology* (Williams et al., 1989).

The organism was cultured on oatmeal agar [20 g of oatmeal, 1 mL of trace salts solution (0.1 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $MnCl_2 \cdot 4H_2O$, and 0.1 g of $ZnSO_4 \cdot H_2O$, in 100 mL of distilled water), 18 g of agar, and 1 L of distilled water] for 21 days. Spore surface ornamentation of strain Ao58 was examined by low-temperature scanning electron microscopy (LT-SEM) and classified as described by Dietz and Mathews (1971). The specimens prepared for SEM were observed with a scanning electron microscope (JSM-5410 LV, JEOL, Japan) interfaced with a cryo-transfer system (CT 1500 Cryotrans, Oxford Instruments, Oxford, U.K.) at 20 kV.

For the chemotaxonomic identification, diaminopimelic acid (DAP) and whole cell sugar pattern of strain Ao58 were analyzed by the method of Schaal (1985) and classified based on the description of Lechevalier and Lechevalier (1970). Major menaquinones were determined using the procedures of Kroppenstedt (1985). The carbon utilization test was performed with D-arabinose, L-arabinose, D-fructose, D-galactose, glycerol, *myo*-inositol, β-lactose, D-mannitol, α-melibiose, raffinose, L-rhamnose, and D-ribose as described by Pridham and Gottlieb (1948). Other biochemical tests such as Gram staining, glycosidase activity, and nitrate reduction were performed as described in the Methods for General and Molecular Bacteriology (Smibert and Krieg, 1994).

Production and Purification of Antibiotics. The actinomycete strain Ao58 was precultured in a 1 L Erlenmeyer flask containing 500 mL of yeast−malt extract broth (YMEB, 10 g of malt extract, 4 g of yeast extract, 4 g of glucose, and 1 L of distilled water, at pH 7.3 before autoclaving) on a rotary shaker at 150 rpm at 28 °C for 6 days. A 5 mL aliquot of the culture broth was transferred into 500 mL of soluble starch broth (SSB, 10 g of soluble starch, 10 g of glycerol, 3 g of $(NH_4)_2SO_4$, 2 g of yeast extract, 1 g of $MgSO_4$, and 1 L of distilled water, at pH 7.0−7.2 before autoclaving) and 3% (w/v) Amberlite XAD-16 hydrophobic aromatic resin (Sigma) in a 1 L Erlenmeyer flask. The 180 inoculated flasks were incubated for 21 days at 28 °C on a rotary shaker at 150 rpm. Actinomycete mycelia and XAD-16 resin were separated from a total of 90 L of culture broth of strain Ao58 by centrifugation at 7000*g*. The mycelial mats and XAD resin were extracted with methanol−acetone (50:50, v:v, 10 L) and filtered using Whatman No. 2 filter paper. The filtrate was concentrated in vacuo to an aqueous solution (3 L) and then extracted with 5 L of 1-butanol. The organic layer was concentrated in vacuo to yield an oily material, which was dissolved in a small volume of 50% aqueous methanol.

The crude antibiotic extracts were purified by flash column chromatography using C18 and silica resins. The crude extracts were loaded on an open glass column (150 × 200 mm) packed with C18 resin (Lichroprep RP-18, 40−63 μm, Merck). The column was eluted with stepwise gradients of water and methanol (v:v, 100:0, 80:20, 60:40, 40:60, 20:80, and 0:100). Each fraction of the eluate (3 L) was concentrated in vacuo, and its antifungal activity was examined against *Phytophthora capsici* and *Colletotrichum gloeosporioides* using the paper disk method (Hwang and Kim, 1995). Each fraction obtained from chromatographic procedures was applied to sterile paper disks (8 mm in diameter). The paper disks treated with each fraction of the eluate were placed in the center of the *P. capsici* seeded agar plates. The clear inhibition zones of mycelial growth around the paper disks were measured after incubation for 3 days at 28 °C. The antifungal-active fractions Ao58A and Ao58B were concentrated in vacuo, respectively.

The crude antibiotic substances from the fraction Ao58A were dissolved in a small volume of chloroform−methanol (9:1, v:v) and further purified by silica gel flash chromatography (silica gel $60F_{254}$, particle size 63−200 μm, Merck). The column was eluted with stepwise gradients of chloroform and methanol (v:v; 100:0, 90:10, 70:30, 50:50, 30:70, 10:90, and 0:100). Each fraction was concentrated and then bioassayed using the paper disk method. The antifungal-active fractions were chromatographed on a Sephadex LH-20 column (26 × 950 mm, C26/100 column packed with Sephadex LH-20 resin, Pharmacia, Uppsala, Sweden). The Sephadex LH-20 column was eluted with methanol at 0.15 mL min^{-1} flow rate. The 3 mL fractions were collected using a fraction collector (Pharmacia RediFrac, Pharmacia). The antifungal activities of all the fractions

collected were examined for *P. capsici* using the paper disk method. The active fractions were pooled and concentrated in vacuo.

The antifungal-active substance Ao58A was further purified by preparative TLC on a silica gel (60 F_{254}, Merck) plate (20 × 20 × 0.2 cm). The bioautography of thin layer chromatograms was used for detecting antifungal substances in crude eluates (Homans and Fuchs, 1970; Lazarovits et al., 1982). The band of antifungal-active Ao58A (R_f ~0.58−0.63) developed with chloroform−methanol (9:1, v:v) was scraped off, extracted with methanol, and filtered through Whatman No. 5 paper. The antifungal activity against *P. capsici* was ascertained by paper disk bioassay. The antibiotic substance Ao58A was further purified by semipreparative HPLC with C18 reverse-phase column (SymmetryPrep C18, 7μm particle size, 7.8 × 300 mm, Waters). The chromatography was conducted with a Gilson HPLC system (Gilson, Middleton, WI) at a flow rate of 2 mL min^{-1} using a linear gradient solvent system from 30% methanol in H_2O to 70% methanol. The eluate of each peak was collected under the monitoring at an absorbance of 232 nm by a UV−vis detector (118UV/VIS detector, 0.2 mm cell path, Gilson). The fractions were bioassayed against *P. capsici* using the paper disk method. The purity of antifungal substance Ao58A was examined by analytical HPLC with a C18 reverse-phase column using various solvent systems.

The crude antibiotic substances from the fraction Ao58B were purified by silica gel flash column chromatography and Sephadex LH-20 column chromatography as described above. The substance Ao58B was further purified by using preparative HPLC with C18 reverse-phase column (SymmetryPrep C18, 7 μm particle size, 7.8 × 300 mm, Waters). The chromatography was conducted with a Gilson HPLC system (Gilson, Middleton, WI) at a flow rate of 2 mL min^{-1} using an isocratic elution of 70% methanol in H_2O. The eluate of each peak was collected under the monitoring at an absorbance of 350 nm by UV−vis detector (118UV/VIS detector, 0.2 mm cell path, Gilson). The fractions were bioassayed against *C. gloeosporioides* using the paper disk method.

Structure Elucidation of the Antibiotic Ao58A. Nuclear magnetic resonance (NMR) spectra of the purified antibiotic Ao58A were recorded on a Bruker AMX 500 NMR spectrometer (Billerica, MA). ^{1}H NMR spectra were recorded in $CDCl_3$. Chemical shifts are given in δ values (ppm) referenced to the proton of solvent at 7.25 ppm as internal standard ^{13}C NMR spectra were recorded in $CDCl_3$ using broad-band proton decoupling. DEPT NMR spectra were recorded in $CDCl_3$. Chemical shifts are given in δ values (ppm) referenced to the carbon of solvent at 77.0 ppm in $CDCl_3$. COSY, HMBC, HMQC, and ROESY spectroscopies were performed on a Bruker AMX 500 NMR spectrometer. Low- and high-resolution mass spectra were recorded on a JEOL JMS-HX110/110A tandem mass spectrometer (JEOL, Peabody, MA) using the fast atom bombardment method. The UV absorption spectrum was measured with a Beckman DU650 spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

Antimicrobial Bioassays. Minimum inhibitory concentrations (MICs) of the antibiotic Ao58A against plant pathogenic fungi, yeast, and bacteria were examined in vitro using a method modified from Nair et al. (1992). A 1 mL sample of potato dextrose broth (10 g of potato dextrose in 1 L of distilled water) was pipetted into each well of a 24-well microtiter dish (Cell Wells, Corning Glass Works, Corning, NY) containing the antibiotic Ao58A in the range from 0 to 50 μg mL^{-1}. The inocula used in this test were zoospore suspension (10^6 zoospores mL^{-1}) of *P. capsici*, mycelial suspension of *Rhizoctonia solani*, spore suspensions (10^6 spores mL^{-1}) of other plant pathogenic fungi (*Alternaria mali, Botrytis cinerea, Cladosporium cucumerinum, Colletotrichum gloeosporioides, Cylindrocarpon destructans, Didymella bryoniae, Fusarium oxysporum* f.sp. *cucumerinum, M. grisea*), yeast (10^4 cell mL^{-1}) (*Candida albicans, Saccharomyces cerevisiae*), and bacteria (10^4 cell mL^{-1}) (*Bacillus subtilis, Erwinia carotovora* pv. *carotovora, Pseudomonas solanacearum,* and *Xanthomonas campestris* pv. *vesicatoria*). The 10 μL of germ suspension was added per well. The inoculated well plates were incubated at 28 °C on a rotary shaker.

Evaluation of Antifungal Activity in vivo. The antibiotic Ao58A was evaluated for the ability to suppress *P. capsici* infection on pepper plants in a growth room. Pepper plants were raised in a growth room at 28 ± 2 °C with 5000 lux illumination for 16 h per day. The commercial fungicide metalaxyl was used to compare the antifungal activity with the antibiotic Ao58A. Antibiotic Ao58A and metalaxyl dissolved in methanol and acetone, respectively, were diluted with 0.05% Tween 20 solution to give concentrations of 10, 50, 100, and 500 μg mL^{-1}. Each of the solutions was sprayed on the surface of a pepper plant at the first branch stage 1 day before inoculation with *P. capsici*. The pepper plants treated with the antibiotic Ao58A and metalaxyl were wounded by making 1 cm longitudinal slits on the stems 1 cm from the soil surface. The sterile cotton dipped in zoospore suspension (1×10^5 zoospores mL^{-1}) was placed on the wounded sites of the stem. Disease severity on pepper plants was rated daily after inoculation based on a scale of 0−5: 0 = no visible disease symptoms; 1 = leaves slightly wilted with brownish lesions beginning to appear on stems; 2 = 30−50% of entire plant diseased; 3 = 50−70% of entire plant diseased; 4 = 70−90% of entire plant diseased; 5 = plant dead.

The efficacy of antibiotic Ao58A against *Botrytis cinerea* on cucumber plants was compared with that of the commercial fungicide vinclozolin in a growth room. Antibiotic Ao58A and vinclozolin were diluted with 0.05% Tween 20 solution to give the concentrations of 10, 50, 100, and 500 μg mL^{-1}. Each of the solutions was sprayed on the primary leaf of a cucumber plant at the third leaf stage 1 day before inoculation with *B. cinerea*. Suspensions of 10^5 spores mL^{-1} of *B. cinerea* in sterile 0.05% Tween 20 solution were sprayed on the leaves of cucumber plants. The diseased area on the primary leaf of each plant was evaluated 5 days after inoculation.

The antibiotic Ao58A was evaluated for the ability to suppress leaf blast development on rice plants under the greenhouse condition. The commercial fungicide tricyclazole was used to compare the antifungal activity with the antibiotic Ao58A. Antibiotic Ao58A and tricyclazole were diluted with 0.05% Tween 20 solution to give concentrations of 10, 50, 100, and 500 μg mL^{-1}. Each of the solutions was sprayed on the leaves of rice at the five-leaf stage 1 day before inoculation with *M. grisea*. The conidial suspension of 10^5 spores mL^{-1} of *M. grisea* was sprayed on the rice leaves. Lesions on the third leaf of each plant were counted 3−4 days after inoculation when typical lesions usually would appear on the leaves of plants.

RESULTS

Identification of Actinomycete Strain Ao58. The cultural characteristics of actinomycete strain Ao58 were examined on various International *Streptomyces* Project (ISP) media (no data shown). The strain Ao58 grew well on trypton−yeast extract agar (ISP1), yeast−malt extract agar (ISP2), oatmeal agar (ISP3), and peptone−yeast extract agar (ISP6) but poorly on inorganic salts starch agar (ISP4) and glycerol asparagine agar (ISP5). Aerial mycelia of strain Ao58 were scarcely produced on most of these media. The color of the vegetative mycelia was yellow to pale orange. In aging cultures, yellowish green or dark green pigments were produced around the colonies. Under the scanning electron microscope, strain Ao58 was ascertained to have nonmotile spores borne singly on short or long sporophores (Figure 1). Spores were spherical and ~0.9−1.2 μm in diameter. The spore surface was smooth. Special structures, such as synnemata, sclerotia, or sporangia were lacking.

The physiological characteristics of strain Ao58 are shown in Table 1. Chemical analysis of cell wall hy-

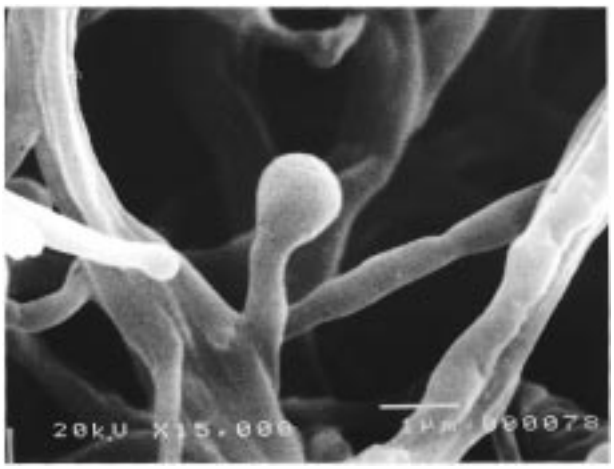

Figure 1. Scanning electron micrograph of spore morphology of the actinomycete strain Ao58 cultured on oatmeal agar for 21 days. Bar represents 1 μm.

drolysate revealed the presence of *meso*-diaminopimelic acid (DAP), as a component of the cell wall, and xylose and arabinose as diagnostic sugars. The major menaquinones were MK-10(H_4) and MK-10(H_6). Strain Ao58 utilized D-fructose, D-galactose, glycerol, β-lactose, D-mannitol, α-melibiose, and raffinose but not D-arabinose, L-arabinose, *myo*-inositol, L-rhamnose, and D-ribose. Cellulose, gelatin, and starch were degraded to make a clear zone around the colonies. Strain Ao58 possessed α-galactosidase and α-mannosidase activities but did not show any β-xylosidase activity. Nitrate reduction and denitrification were not found. Strain Ao58 tolerated well up to 3% NaCl in the basal medium.

In conclusion, the morphological and physiological characteristics of the strain Ao58 examined in this study indicated that the strain Ao58 was identical to *Micromonospora coerulea*, compared to data from Luedemann (1971) and *Bergey's Manual of Systematic Bacteriology* (Williams et al., 1989).

Production and Purification of Antibiotic Substances. Individual antibiotics active against plant pathogenic fungi were purified from the culture (90 L) of *M. coerulea* strain Ao58 using different chromatography procedures (data not shown). The antifungal activities against *P. capsici* and *C. gloeosporioides* were found in the crude butanol extracts of XAD resin and mycelial mats. The oily antifungal substances were obtained from C18 flash column chromatography of the butanol extracts. The antifungal-active fractions were divided into Ao58A and Ao58B. The active fractions Ao58A from the 20% and 40% methanol eluates were inhibitory to *P. capsici*. Antifungal activity against *C. gloeosporioides* was found in the fraction Ao58B from the 60% and 80% methanol eluates.

The fractions containing Ao58A were further chromatographed on a silica gel column. The fractions eluted with chloroform−methanol (v:v, 90:10) showed antifungal activity against *P. capsici*. The strongly antifungal-active eluates were further purified by gel filtration. The active fractions 81−101 were pooled and concentrated in vacuo.

The antifungal-active substance Ao58A was detected by bioautography on TLC plates. A clear inhibition zone appeared in the position of R_f 0.65 on the silica gel TLC plates developed by a solvent system chloroform−methanol (v:v, 9:1) (data not shown). Preparative TLC was performed with the same solvent system. Methanol

Table 1. Characteristics of Antagonistic *Micromonospora* Strain Ao58

characteristic	strain Ao58	*M. coerulea*[a]
formation of aerial mycelium	rare	−
diagnostic mycelial pigment	green	blue-green
diffusible pigment	−	−
spores	single	single
spore type	oval or spherical	spherical
spore motility	−	−
cell wall chemotype	II	II
whole cell sugar	xylose and arabinose	xylose and arabinose
major menaquinone	MK-10(H_4) MK-10(H_6)	MK-10(H_4) MK-10(H_6)
growth on		
Czapek−sucrose agar	−	−
potato slice	+	+
carbohydrate utilization		
D-arabinose	−	−
L-arabinose	−	−
D-fructose	+	+
D-galactose	+	+
glycerol	+	−
myo-inositol	−	−
β-lactose	+	+
D-mannitol	+	+
α-melibiose	+	+
raffinose	+	+
L-rhamnose	−	−
D-ribose	−	−
glycosidase activity		
α-galactosidase	+	+
α-mannosidase	+	+
β-xylosidase	−	−
nitrate reduction	−	−
degradation of		
cellulose	+	+
gelatin	+	+
starch	+	+
max NaCl tolerance(% w/v)	3%	1.5%
cell component		
3-hydroxy-diaminopimelate	−	−

[a] Data from Bergey's Manual of Systematic Bacteriology (Williams et al., 1989).

eluates of the active band scraped from preparative TLC plates were bioassayed against *P. capsici* to confirm the antifungal activity of Ao58A. The methanol eluates of the active band were further purified by semipreparative HPLC using a reverse-phase C18 column. The pure antibiotic Ao58A was obtained from a single peak at the retention time of 35.4 min at 232 nm. Finally, 402.7 mg of crystalline solid of the antibiotic Ao58A was obtained from 80 L of the culture extracts of *M. coerulea* strain Ao58. The purified antibiotic Ao58A was eluted as a single peak when chromatographed on analytical C18 column using various solvent systems. It was moderately soluble in acetone, chloroform, ethanol, ethyl acetate, and methanol, but insoluble in H_2O.

The Ao58B fractions were further chromatographed on a silica gel column. The fractions eluted with chloroform−methanol (7:3) and chloroform−methanol (5:5) showed antifungal activity against *C. gloeosporioides*. Antifungal activity was found in fractions 54−98 from the gel filtration of the eluates. The active fractions were further purified by semipreparative HPLC using C18 column, which were then eluted with 70% methanol in H_2O. The HPLC profile indicated that fractions Ao58B was composed of six different components (retention times: 25.1, 28.3, 33.1, 37.6, 42.0, and 45.2 min).

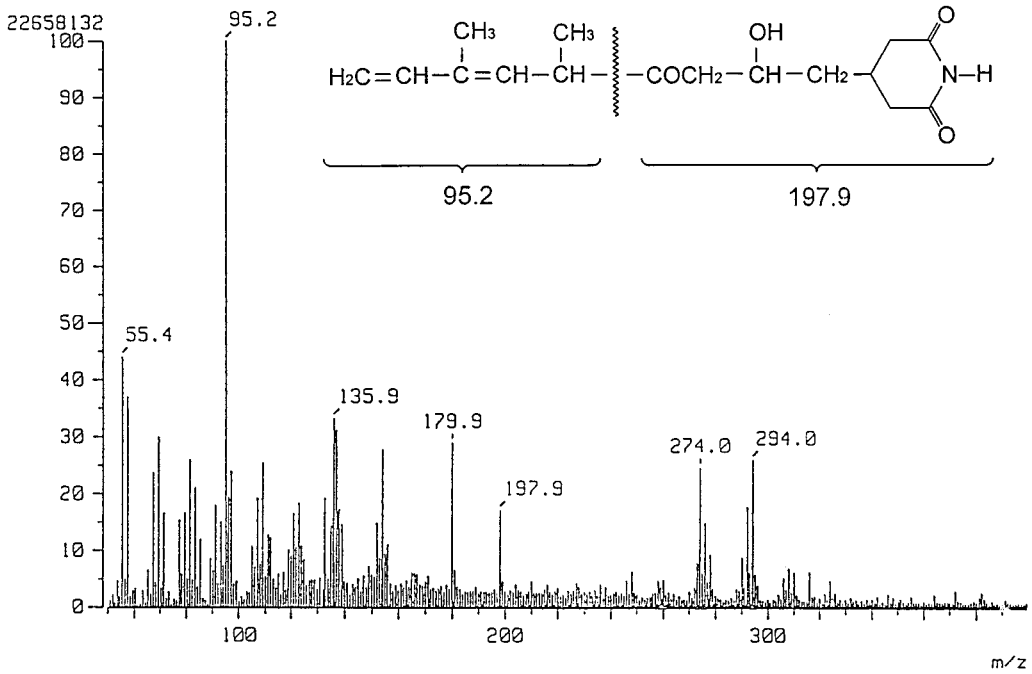

Figure 2. High-resolution FAB−mass spectrum of the antibiotic Ao58A.

Table 2. NMR Spectral Data of the Antibiotic Ao58A (^{1}H at 500 MHz and ^{13}C at 125 MHz in $CDCl_3$)

carbon no.	^{13}C, δ	^{1}H, δ (m,[f] J in Hz)[g]	HMBC[a]
1	172.6[b]	−	2
1′	172.7[b]	−	2′
NH	−	8.76 (s)	
2	37.0[c]	2.27 (m),[d] 2.70 (m)[e]	NH, 4, 2′
2′	38.2[c]	2.27 (m),[d] 2.70 (m)[e]	NH, 4, 2′
3	27.0	2.45 (m)	2, 2′, 4
4	40.8	1.32 (ddd, 14.0, 8.7, 3.0)	
		1.57 (ddd, 14.0, 10.3, 5.0)	
5	64.6	4.10 (m)	4, 6
5-OH	-	3.42 (br s)	
6	47.5	2.53 (dd, 17.9, 3.3)	
		2.61 (dd, 17.9, 8.4)	
7	211.9	−	6, 8, 8-Me
8	46.8	3.48 (dq, 9.7, 6.8)	8-Me
8-Me	16.0	1.15 (d, 6.8)	8, 9
9	130.1	5.31 (d, 9.7)	8, 8-Me, 10-Me, 11
10	136.5	−	8, 12, 10-Me, 11
10-Me	12.1	1.80 (d, 1.24)	9, 11
11	140.4	6.33 (dd, 17.4, 10.7)	9, 12, 10-Me
12	112.9	5.03 (d, 10.7), 5.18 (d, 17.4)	

[a] Correlation to proton number. [b] Interchangeable. [c] Interchangeable. [d] Overlapped. [e] Overlapped. [f] Abbreviations of signal multiplicity are as follows: s, singlet; d, doublet, dd, doublet of doublets; ddd, doublet of doublets of doublets; dq, doublet of quartets; and m, multiplet. [g] Saito et al. (1974) for the literature values of ^{1}H NMR data.

Each eluate of these components was effective against *C. gloeosporioides* but not against *P. capsici*. The UV spectral patterns of six components were same one another, having absorption maxima at 350, 332, and 317 nm in methanol, characteristic of polyene antibiotics. Purification procedures of these substances are in progress.

Structure Elucidation of Antibiotic Ao58A. The molecular formula $C_{16}H_{23}NO_4$ of the antibiotic Ao58A was established by high-resolution FAB mass spectroscopy (M + H, *m*/*z* 294.1707, calculated 294.1705) (Figure 2). The structure of the antibiotic Ao58A was fully elucidated by ^{1}H NMR, ^{13}C NMR spectroscopy, a DEPT experiment, and 2D NMR spectral studies (^{1}H−^{1}H COSY, HMQC, HMBC, and ROESY experiments). The

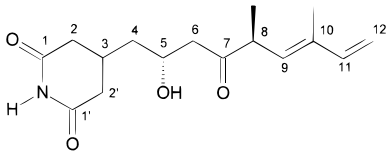

Figure 3. Structure of the antibiotic Ao58A isolated from *Micromonospora coerulea* strain Ao58.

proton and carbon counts from ^{1}H and ^{13}C NMR spectra and the DEPT experiment indicated the presence of 23 protons and 16 carbons (4 C, 5 CH, 5 CH_2, and 2 CH_3), thus supporting the molecular formula (Table 2). Correlation of protons from the COSY spectrum were observed as follow: protons on C-12 to H-11, H-9 to H-8 and allylically methyl protons at C-10, H-5 to two pairs of methylene protons on C-4 and C-6, protons on C-4 to H-3, and H-3 to two pairs of the overlapped methylene protons on C-2 and C-2′. The configuration of the double bond was determined to be *E*, since H-9 showed no NOE with the methyl protons at C-10 in the ROESY spectrum. The remaining carbonyl and quaternary carbons could be placed from the HMBC experiments with the following correlations: C-10 to the alkenic protons on C-11 and C-12, C-7 to H-8 and methylene protons on C-6, and C-1 and C-1′ to the exchangeable proton N−H and the overlapped methylene protons on C-2 and C-2′. The HMBC correlations are summarized with the NMR assignments for the antibiotic Ao58A in Table 2. In light of all the spectral data, the structure of the antibiotic Ao58A was determined to be 4-(2-hydroxy-5,7-dimethyl-4-oxo-6,8-nonadienyl)-2,6-piperidinedione (molecular formula $C_{16}H_{23}NO_4$) (Figure 3).

Antifungal Activity of the Antibiotic Ao58A. The minimum inhibitory concentrations of the antibiotic Ao58A against various microorganisms are shown in Table 3. Mycelial growths of the tested filamentous fungi, except *C. destructans* and *F. oxysporum* f. sp. *cucumerinum*, were greatly inhibited by the antibiotic Ao58A. In particular, the antibiotic Ao58A was very effective in inhibiting growth of *B. cinerea*, *D. bryoniae*,

Table 3. Minimum Inhibitory Concentrations (MICs) against Various Microorganisms of the Antibiotic Substance Ao58A from *Micromonospora coerulea* Strain Ao58

microorganism	minimum inhibitory concn (μg mL^{-1})[a]
Alternaria mali	50
Botrytis cinerea	10
Cladosporium cucumerinum	30
Colletotrichum gloeosporioides	50
Cylindrocarpon destructans	>50[b]
Didymella bryoniae	3
Fusarium oxysporum f. sp. *cucumerinum*	>50
Magnaporthe grisea	5
Phytophthora capsici	3
Rhizoctonia solani	50
Candida albicans	>50
Saccharomyces cerevisiae	0.3
Bacillus subtilis	>50
Erwinia carotovora pv. *carotovora*	>50
Pseudomonas solanacearum	>50
Xanthomonas campestris pv. *vesicatoria*	>50

[a] The lowest concentration that completely inhibits the growth of test microorganism was determined after incubation for 3∼5 days. [b] >50 represents that the growth of test microorganism was not inhibited at the concentrations up to 50 μg mL^{-1}.

M. grisea, and *P. capsici.* The growth of *S. cerevisiae* was inhibited even at the low concentration of 0.3 μg mL^{-1}. However, the antibiotic Ao58A did not affect *C. albicans.* No antibacterial activity of Ao58A against *B. subtilis*, *E. carotovora* pv. *carotovora*, *P. solanacearum*, and *X. campestris* pv. *vesicatoria* was observed, even at 50 μg mL^{-1}.

The antibiotic Ao58A and the commercial fungicide metalaxyl were compared for the control of Phytophthora blight in pepper plants under a greenhouse condition (Figure 4A). Treatment with 50 μg mL^{-1} of the two chemicals substantially inhibited disease development on pepper plants compared to the untreated control plants. Pepper plants treated with 500 μg mL^{-1} of each chemical did not show any disease symptom on the stems. The efficacy for Phytophthora control of the antibiotic Ao58A was in general somewhat less than that of metalaxyl on pepper plants. The antibiotic Ao58A did not induce any phytotoxic symptom on pepper plants even when treated with 500 μg mL^{-1}.

In vivo efficacy of the antibiotic Ao58A and the fungicide vinclozolin for control of *B. cinerea* on cucumber plants is presented in Figure 4B. Antibiotic Ao58A and vinclozolin began to show protective activity against *B. cinerea* infection at a concentration of 50 μg mL^{-1}. Treatment with 100 μg mL^{-1} of the antibiotic Ao58A and vinclozolin significantly reduced gray mold infection on cucumber leaves to show 20% diseased leaf area whereas the untreated leaves were entirely diseased. No disease symptoms were found on the cucumber leaves treated with 500 μg mL^{-1} of each chemical.

The antibiotic Ao58A was evaluated in a greenhouse for its ability to suppress blast development on rice leaves (Figure 4C). The typical symptoms of the rice blast began to appear on the rice leaves 3 days after inoculation. As the concentration of the antibiotic Ao58A and the commercial fungicide tricyclazole increased, *M. grisea* infection was inhibited on rice leaves. Treatment with 50 μg mL^{-1} of the antibiotic Ao58A and tricyclazole greatly reduced blast lesions on rice leaves, and the inhibition effect was more pronounced by use of Ao58A. No blast lesions were produced on the rice leaves treated with 500 μg mL^{-1} of both chemicals. The antibiotic Ao58A did not show any phytotoxic symptoms on rice leaves treated with 500 μg mL^{-1}.

DISCUSSION

The actinomycete strain Ao58 was isolated from sea mud soils, which has strong antifungal activity against *P. capsici*, *M. grisea*, *C. gloeosporioides*, and *R. solani* (Kim and Hwang, 1997). The organism was identified as *Micromonospora coerulea* by comparison to the taxonomic data of the type strain demonstrated by Luedemann (1971). The genus *Micromonospora* has been known to be widely distributed in a variety of soils, but it was only a minor component of the actinomycete population, called rare actinomycetes (Vobis, 1991). Recently, the genus *Micromonospora* has attracted interest as a new source of antibiotics, but little has been known of its ability to produce antifungal antibiotics which have potent activity as fungicides for agricultural uses (Goodfellow, 1988).

There is much evidence to show that the compounds with high antifungal activity in vitro often possess negligible fungicidal activity in vivo (Fawcett and Spencer, 1970). To realize the use of antifungal metabolites of microorganisms as a fungicide for plant protection, their control efficacy of plant diseases on host plants should be evaluated at the beginning of screening procedures. Besides the strong in vitro activity, the culture extracts of *M. coerulea* strain Ao58 showed marked potency in the control of Phytophthora development on pepper plants (no data presented).

The antibiotic Ao58A showing antifungal activity against *P. capsici* was purified from the culture extracts of *M. coerulea* strain Ao58 using various chromatographic procedures. During the purification procedures, six other antibiotic substances active against *C. gloeosporioides* were separated as minor compounds. The six individual antibiotics inhibited the mycelial growth of *C. gloeosporioides* at 10−25 μg mL^{-1}, but did not show antifungal activity against *P. capsici* even at a concentration of 50 μg mL^{-1}. The chemical structures of these antibiotics were not fully elucidated. On the basis of UV and proton NMR spectral data, however, they were confirmed to be polyene type antibiotics with similar chemical structures. The broad antifungal spectrum of the culture extracts of the strain Ao58, which showed strong antifungal activity against *C. gloeosporioides*, *F. oxysporum* f. sp. *cucumerium*, and *R. solani*, may be due to the production of various antibiotics by the strain Ao58. Most antibiotic-producing microorganisms usually produce a variety of substances of different natures. However, fermentation conditions often determine the amounts produced (Bérdy, 1974). In the fermentation of the strain Ao58, the production of polyene type antibiotics was enhanced by the increased addition of organic nitrogen sources (no data shown).

The assignments of our NMR spectral data indicated that the antibiotic Ao58A is a 4-substituted glutarimide antibiotic bearing a non-carbocyclic side chain. In most of the glutarimide antibiotics such as cycloheximide, naramycin B, isocycloheximide, inactone, and streptovitacins, the side chain is composed of a substituted carbocyclic ring, usually cyclohexyl (Becker and Rickards, 1976). In other glutarimide-type antibiotics, however, the side chains of streptimidone (Frohardt et al., 1959), protomycin (Sugawara, 1963), and the 9-methyl derivative of streptimidone (Saito et al., 1974; Allen et al., 1976) are non-carbocyclic. Protomycin was later

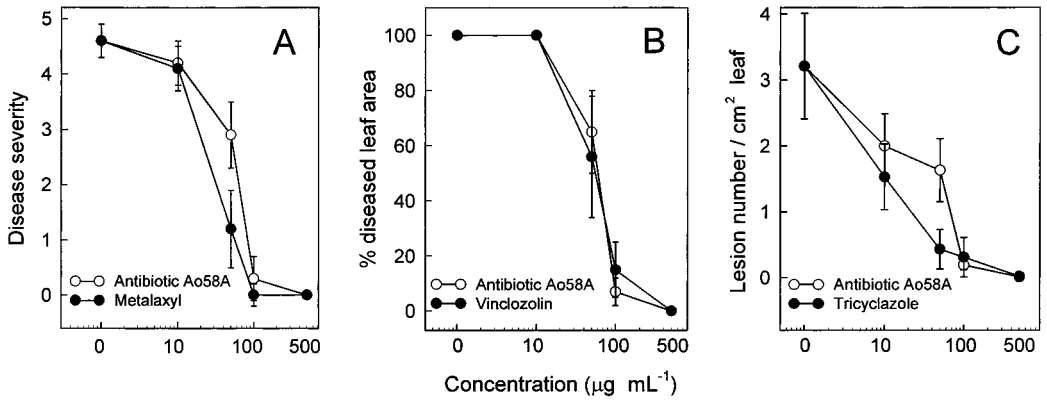

Figure 4. In vivo efficacy of the antibiotic Ao58A for the control of plant pathogenic fungi in host plants: (A) Effects of the antibiotic Ao58A and metalaxyl on Phytophthora blight in pepper plants caused by *P. capsici*; (B) Effects of the antibiotic Ao58A and vinclozolin on gray mold in cucumber leaves caused by *B. cinerea*; (C) Effects of the antibiotic Ao58A and tricyclazole on leaf blast of rice caused by *M. grisea*. Each compound was applied on the plants 1 day before inoculation. Disease development was rated daily after the appearance of disease symptom on untreated control plants. Vertical bars represent standard deviations.

found to have the same structure as streptimidone (Becker and Rickards, 1982).

In the EI mass spectrum of the antibiotic Ao58A, the signals of fragment ions at *m/e* 95 and 198 verified their assignments as the allylic ion $C_7H_{11}^+$ and the acylium ion $C_9H_{12}NO_4^+$, respectively, which originated from the cleavage of the molecular ion between the diene and carbonyl functional groups. By comparison of proton magnetic resonance, EI mass spectra (*m/e* 293, 198, 180, 95), optical rotation {$[\alpha]_D^{27}$ +243° (*c*, 0.5 in $CHCl_3$)} with those of previous reports (Saito et al., 1974; Johnson et al., 1965; Frohardt et al., 1959), Ao58A was recognized to be identical with that of the glutarimide antibiotic streptimidone. Since ^{13}C NMR chemical shift data for streptimidone was not available in the literatures, we have presented the assignments of all the carbon signals (Table 2).

A number of glutarimide antibiotics have been isolated from various species of streptomycetes (Grollman, 1966; Sisler and Siegel, 1967). Streptimidone was also isolated from *Streptomyces* species (Frohardt et al., 1959; Allen et al., 1976). However, we first revealed that the genus *Micromonospora* produces the glutarimide-type antibiotics including streptimidone. The genus *Micromonospora*, which belongs to the genera of actinoplanetes, taxonomically differs from the genera of streptomycetes (Stackebrandt et al., 1983; Vobis, 1991).

Streptimidone was demonstrated to have a strong antiyeast activity along with antifungal and antiprotozoal activity (Kohberger et al., 1960), of which the mode of action against eukaryotic cell involves the inhibition on protein synthesis such as peptide initiation and extension by an effect on the donor ribosomal site (Obrig et al., 1971). The compound has usually been examined in the interest of biochemical studies as an inhibitor of the protein synthesis on yeast, but little has been known about the efficacy of its antifungal activity against filamentous fungi.

In vitro bioassay revealed that the antibiotic Ao58A completely inhibited the growth of *S. cerevisiae* at the relatively low concentration of 0.3 μg mL^{-1}, but *C. albicans* was not affected even at a concentration of 50 μg mL^{-1}. Such a marked difference in inhibitory activity toward closely related organisms may be one of the interesting characteristics of the glutarimide family antibiotics (Sisler and Siegel, 1967). In the tests for antifungal spectrum of the antibiotic Ao58A, we observed marked antifungal activity against some plant pathogenic fungi, such as *P. capsici*, *M. grisea*, *D. bryoniae*, and *B. cinerea* (Table 3). In previous studies, streptimidone was demonstrated to have antifungal activity against *Phytophthora cinnamomi* and *Phytophthora dreschleri* in vitro (Allen et al., 1976). However, further evaluation for its efficacy in plant chemotherapy as a fungicide seemed to not have been performed because of an earlier report on some phytotoxicity of the compound (Fawcett and Spencer, 1970). In our in vivo tests for control efficacy of the antibiotic Ao58A against Phytophthora blight on pepper plants, gray mold on cucumber leaves, and leaf blast on rice leaves, the compound effectively inhibited the development of these plant diseases on the host plants at the concentration of 100 μg mL^{-1}, at which the commercial fungicides showed similar control efficacy against the diseases. However, we could not observe any phytotoxicity on pepper, cucumber, and rice plants even at high concentrations over 500 μg mL^{-1}.

From these results, we can conclude that the antibiotic Ao58A, streptimidone from *M. coerulea* strain Ao58, shows a good potent control efficacy against some plant diseases. For the practical use of the compound as an agricultural fungicide, the disease control efficacy should be further evaluated in the field. In addition, synthesis of analogues of streptimidone will be necessary to further evaluate the possibility of the use of streptimidone analogues as lead compounds for the development of new agricultural antibiotics.

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