Structure Elucidation and Antifungal Activity of an Anthracycline Antibiotic, Daunomycin, Isolated from *Actinomadura roseola*

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The actinomycete strain Ao108 producing antifungal metabolites active against some plant pathogenic fungi was identified as *Actinomadura roseola*, based on the analyses of morphological and physiological characteristics. The antibiotic Da2B that showed a strong antifungal activity was isolated from the culture broth and mycelial mats of *A. roseola* strain Ao108 using various chromatographic procedures. On the basis of ^{1}H NMR, ^{13}C NMR, and 2-D NMR correlation data, the antibiotic Da2B was confirmed to have the structure of an anthracycline antibiotic, daunomycin. In vitro antimicrobial spectrum tests showed that the antibiotic Da2B had substantial inhibitory activity (10 μg mL^{-1} of MICs) against mycelial growth of *Phytophthora capsici* and *Rhizoctonia solani*. The antibiotic also showed antiyeast activity against *Saccharomyces cerevisiae*, but the growth of *Candida albicans* was not affected. Antibacterial activity was found only against Gram-positive bacteria. In the further evaluation of in vivo efficacy, application of the antibiotic Da2B effectively inhibited the development of *Phytophthora* blight in pepper plants. However, the control efficacy of the antibiotic against *Phytophthora* infection was somewhat less than that of metalaxyl. The antibiotic Da2B did not show any phytotoxicity on pepper plants even at 500 μg mL^{-1}.

Keywords: *Actinomadura roseola; daunomycin; antifungal activity; plant disease control*

INTRODUCTION

Microbial secondary metabolites, represented by antibiotics, have been important sources of antifungal compounds for the development of fungicides. Antibiotics of microbial origin have promising characteristics as fungicides for control of plant diseases. The antibiotics from microorganisms would be likely to degrade rapidly and easily in biosphere, thus leading to low residue levels. Additionally, their versatility in biological activity and chemical structure provides highly specific modes of action (Tanaka and Omura, 1993). The merits of antimicrobial metabolites are expected to overcome the problems caused by synthetic fungicides, that is, occurrence of resistant strains of plant pathogens and harmful effects of the residual chemicals in soils (Porter, 1985).

The first microbial product commercialized as an agrochemical fungicide is the antibiotic blasticidin S, very effective in controlling rice blast caused by *Magnaporthe grisea* (Takeuchi et al., 1957). The success of the first application of blasticidin S encouraged the screening of various microbial metabolites for agricultural uses, which eventually brought out kasugamycin, validamycin A, polyoxins, and mildiomycin (Godfrey, 1994; Knight et al., 1997). They have practically been used as ingredients of fungicides for the control of various fungal diseases of fruit trees and vegetables (Worthington, 1988). The antifungal metabolites also have been provided as lead compounds for the synthesis of new and potent fungicides that have improved properties such as photochemical stability, low cytotoxicity and phytotoxicity. For examples, the β-methoxyacrylates azoxystrobin and kresoxim-methyl were developed from the antibiotics strobilurins (Anke et al., 1977; Godfrey, 1994). The microbial metabolites that were not evaluated in the previous screening procedure were often rediscovered for the potent antifungal activity against plant pathogenic fungi. Recently, the antibiotic compounds such as gopalamicin (Nair et al., 1994), tubercidin (Hwang et al., 1994; Hwang and Kim, 1995), and a manumycin-type antibiotics (Hwang et al., 1996) were found to have potent antifungal activity for the control of plant fungal diseases.

Actinomycetes have been the most prolific producers of various kinds of antimicrobial metabolites such as aminoglycoside, anthracyclines, tetracyclines, glycopeptide, β-lactams, macrolides, nucleosides, peptides, polyenes, and polyethers (Okami and Hotta, 1988). Among them, the genus *Streptomyces* has extensively been studied as a source of antifungal antibiotics as well as an important producer of microbial metabolites. In recent years, as the frequency of rediscovery of known compounds increased, however, the use of actinomycete isolates other than *Streptomyces* spp. has been noted for new sources of antifungal antibiotics (Tanaka and Omura, 1993).

During the screening procedure for potent antifungal metabolites as a plant chemotherapeutic agent, we isolated a number of actinomycete strains active against some plant pathogenic fungi from sea-mud soils in Korea (Kim and Hwang, 1997). Among the antifungal-active actinomycetes, the actinomycete strain Ao108 that showed a high level of antifungal activity was identified as *Actinomadura roseola*. The antibiotic Da2B

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was isolated from the culture broth of *A. roseola* strain Ao108 using various chromatographic procedures. The structure of the antibiotic Da2B was determined by analyzing various spectral data. In vitro antifungal activity of the antibiotic Da2B was evaluated against various plant pathogenic fungi. In vivo efficacy for the control of *Phytophthora* blight also was examined under the greenhouse condition.

EXPERIMENTAL PROCEDURES

Identification of Actinomycete Strain Ao108. The actinomycete strain Ao108 was isolated from a sea-mud soil sample collected at Young-Jong island in Korea during the course of a search for actinomycetes antagonistic to plant pathogenic fungi (Kim and Hwang, 1997).

Taxonomical studies of strain Ao108 were performed using the methods described by Waksman (1961) and Shirling and Gottlieb (1966) and *Bergey's Manual of Systematic Bacteriology* (Williams et al., 1989).

The cultural characteristics of the organism were examined on various International *Streptomyces* Project (ISP) media after incubation for 14 days at 28 °C. Spore chain morphology and surface ornament of strain Ao108 examined by light and scanning electron microscopies (SEM) were classified as described by Pridham et al. (1948) and Dietz and Mathews (1971). The organism was cultured for 14 days 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 $MnCl_2 \cdot 4H_2O$, 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]. The specimens for SEM prepared as described by Williams and Davies (1967) were examined using a Hitachi S-450 scanning electron microscope.

For the chemotaxonomic identification, diaminopimelic acid (DAP) in the cell wall of strain Ao108 was first analyzed by the methods of Becker et al. (1965) and Schaal (1985). Analysis of the whole cell sugar pattern was determined by the methods of Schaal (1985) and classified as described by Lechevalier and Lechevalier (1970) and Lechevalier et al. (1971). Major menaquinone was analyzed using the procedures of Tamaoka et al. (1983) and Kroppenstedt (1985). The carbon utilization test was performed with L-arabinose, D-fructose, D-galactose, glycerol, *myo*-inositol, β-lactose, D-mannitol, L-rhamnose, raffinose, sucrose, and D-xylose as described by Pridham and Gottlieb (1948). Gram-staining, hydrolysis tests, and nitrate reduction were tested as described in the Methods for General and Molecular Bacteriology (Gerhardt et al., 1994).

Production and Purification of Antibiotics. The actinomycete strain Ao108 was grown on yeast-malt extract agar (YMEA, 10 g of malt extract, 4 g of yeast extract, 4 g of glucose, 18 g of agar, 1 L of distilled water, pH 7.3 before autoclaving) for 6 days at 28 °C. Agar plugs from these plates were inoculated into 1 L Erlenmeyer flasks containing 500 mL of YME broth. The inoculated flasks were placed on a rotary shaker at 150 rpm at 30 °C for 3 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, 3.0 g of $(NH_4)_2SO_4$, 2.0 g of yeast extract, 1.0 g of $MgSO_4$, 1 L of distilled water, 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 inoculated flasks were incubated at 30 °C on a rotary shaker at 150 rpm for 13 days. After centrifugation of culture broth at 7000*g*, the XAD-16 resin and mycelia were extracted with 10 L of methanol and 4 L of acetone, respectively. The crude extracts were filtered through Whatman No. 2 filter paper, pooled, and evaporated under reduced pressure 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 gel as a packing material. The crude extracts were loaded onto an open glass column (150 × 200 mm) packed with C18 (Lichroprep RP-18, 40−63 μm, Merck). The column was eluted with stepwise gradients of water and methanol (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100, v/v/v). Each fraction of the eluate (2.5 L) was concentrated in vacuo, and its antifungal activity was examined against *Phytophthora capsici* using the paper disk method. The active fractions were dissolved in a small volume of ethyl acetate−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 ethyl acetate and methanol (100:0, 90:10, 70:30, 50:50, 30:70, 10:90, and 0:100, v/v). 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). 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 pooled active-fraction was further purified using a Gilson HPLC system (Gilson) with a gel filtration column (KW-8025, Shodex). The column was eluted with methanol at a flow rate of 2 mL min^{-1}. The separation was monitored at an absorbance of 232 nm by UV−vis detector (118 UV−vis detector, 0.2 mm cell path, Gilson). The antifungal active peaks were collected and further chromatographed on a C18 reversed-phase column (SymmetryPrep C18, 7 μm, 7.8 × 300 mm, Waters). The chromatography was conducted with a HPLC at a flow rate of 2 mL min^{-1} using a linear gradient solvent system from 10% acetonitrile in H_2O to 60% acetonitrile. The eluate of each peak was collected under the monitoring at an absorbance of 232 nm by UV−vis detector. The fractions were bioassayed against *P. capsici* using the paper disk method. The purity of antifungal substance was examined by analytical HPLC with a C18 reversed-phase column using various solvent systems.

Structure Elucidation of the Antibiotic Da2B. Nuclear magnetic resonance spectra of the purified antibiotic Da2B were recorded on a Bruker AMX 500 NMR spectrometer. ^1H NMR spectra (500 MHz) were recorded in CD_3OD. Chemical shifts are given in δ values (ppm) referenced to the proton of solvent at 3.30 ppm as internal standard, and coupling constants (J) are given in Hz. ^{13}C NMR spectra (125 MHz) were recorded in CD_3OD using the broad-band proton decoupling. DEPT and 2D-NMR spectroscopies such as $^1H-^1H$ correlation spectra (COSY), HMBC, HMQC, and ROESY were recorded on a Bruker AMX 500 NMR spectrometer. High-resolution mass spectra were recorded on a JEOL JMS-HX110/110A tandem mass spectrometer (JEOL) using the fast-atom bombardment method. The UV absorption spectrum was measured with a Beckman DU650 spectrophotometer (Beckman Instruments Inc.).

In Vitro and in Vivo Antimicrobial Bioassays. The antifungal active fractions during the purification procedures were confirmed by the paper disk bioassay method. Each fraction eluted from different chromatographies was applied to sterile paper disks (8 mm in diameter). The zoospore suspension of *P. capsici* (10^6 zoospores mL^{-1}) was added to molten potato dextrose agar at 40 °C. The seeded agar was poured into 9-cm Petri dishes. The treated paper disks were placed in the center of the 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 bioautographic technique on TLC plates was used (Homans and Fuchs, 1970) to confirm the active compound as a band on TLC plates. The pooled active eluates were spotted on silica gel TLC plates (60 F_{254}, 0.2 mm thickness, Merck) and developed with chloroform−methanol (75:25, v/v). After air-drying to remove solvent, the TLC plate was placed on the water agar plate (2% agar in a 15 cm-diameter Petri dish). Molten V8 juice agar seeded with the zoospores of *P. capsici* (10^6 zoospores mL^{-1}) was uniformly spread onto the TLC plate. After incubation for 3 days at 28 °C, the plate was stained with 0.1% naphthol blue black solution in 5% acetic acid for 2−3 min and then destained with 5% acetic acid for 2−3 h.

The R_f value of antifungal-active substance was confirmed by comparing the inhibition zone with the bands on TLC plates visualized by UV irradiation or anisaldehyde−sulfuric acid reaction (Jork et al., 1994).

In vitro minimum inhibitory concentration (MIC) of the antibiotic Da2B against plant pathogenic fungi, yeast, and bacteria was determined in a 24-well microtiter dish (Cell Wells, Corning Glass Works) using a method modified from Nair et al. (1992). The antibiotic Da2B was diluted to be the concentrations of the range from 0 to 50 μg mL^{-1} in the each well containing 1 mL of potato dextrose broth. 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 into each well. The inoculated well plates were incubated at 28 °C on a rotary shaker at 120 rpm. The inhibitory effects of the antibiotic Da2B on the growth of test microorganisms were evaluated after incubation for 2−4 days. The lowest concentration that completely inhibited the growth of the microorganisms was considered to be a minimum inhibitory concentration.

The protective activity of antibiotic Da2B on pepper plants against *P. capsici* was evaluated in a growth room. Pepper seeds (*Capsicum annuum* cv. Hanbyul) were grown in a plastic tray (55 × 35 × 15 cm) containing steam-sterilized soil mix (peat moss, perlite and vermiculite, 5:3:2, v/v/v), sand, and loam soil (1:1:1, v/v/v). Six seedlings at four-leaf stage were transplanted into a plastic pot (5 × 15 × 10 cm) containing the soil mix described previously. 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 Da2B. Antibiotic Da2B and metalaxyl dissolved in methanol and acetone, respectively, was diluted to give the concentrations of 1, 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 each chemical were wounded by making 1 cm longitudinal slits on the stems 1 cm from the soil surface. The zoospore suspension was prepared to give the concentration of 1 × 10^5 zoospores mL^{-1}. The sterile cotton soaked with zoospore suspension was placed on the wounded sites of the stem. The inoculated sites were covered with plastic tape to maintain a moist condition. Disease severity on pepper plants was rated daily after inoculation based on a scale 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, and 5 = plant dead. Data are the means of 10 plants per treatment.

RESULTS

Identification of Actinomycete Strain Ao108. The cultural characteristics of the actinomycete strain Ao108 were examined on various ISP media (Table 1). The strain Ao108 grew well on trypton−yeast extract agar (TYEA, ISP1), yeast−malt extract agar (YMEA, ISP2), oatmeal agar (OA, ISP3), and peptone−yeast extract agar (PYEA, ISP6), but poorly on inorganic salts starch agar (ISSA, ISP4) and glycerol asparagine agar (GAA, ISP5). Aerial mycelia, which formed farinaceous surfaces of colonies, were produced on all the media used in this test, except for ISSA and GAA. The colors of aerial mycelia were white to pale pink. The reverse side colors of colonies were pink to brownish red, but diffusible pigments were not produced in all test media.

Table 1. Morphological and Biochemical Characteristics of the Actinomycete Strain Ao108

characteristic	strain Ao108	*A. roseola*[a]
morphological character		
fragmentation of substrate mycelium	−[b]	−
spore chain	straight or slightly bent	straight
spores per chain	8−10 spores	short or long spore chain
inter spore pad	−	−
spore morphology	elliptical, warty	elliptical, folded
cultural character		
ISP2 AM[c]	pink	pink
SM	brown-red	brown-red
SP	−	−
ISP3 AM	pink-violet	pink-violet
SM	red-brown	red-brown
SP	−	−
ISP4 AM	−	−
SM	red, scare	−
SP	−	−
physiological character		
diagnostic amino acid	*meso*-DAP	*meso*-DAP
characteristic sugar	madurose	madurose
mycolic acids	ND	
predominant menaquinone	MK-9[d](H_4,H_6,H_8)	MK-9(H_4,H_6,H_8)
reduction of nitrate	+	+
hydrolysis of casein	−	−
DNA	ND	
esculin	+	+
gelatin	+	+
hypoxanthine	+	+
starch	+	−
testosterone	+	+
tyrosine	+	+
xanthine	−	−

[a] Data were obtained from *Bergey's Manual of Systematic Bacteriology* (Williams et al., 1989). [b] Symbols: +, present; −, absent; ND, not determined. [c] Abbreviations are as follows: AM, aerial mycelium; SM, substrate mycelium; and SP, soluble pigment. [d] MK-9(H_4) means menaquinones having four of the nine isoprene units hydrogenated.

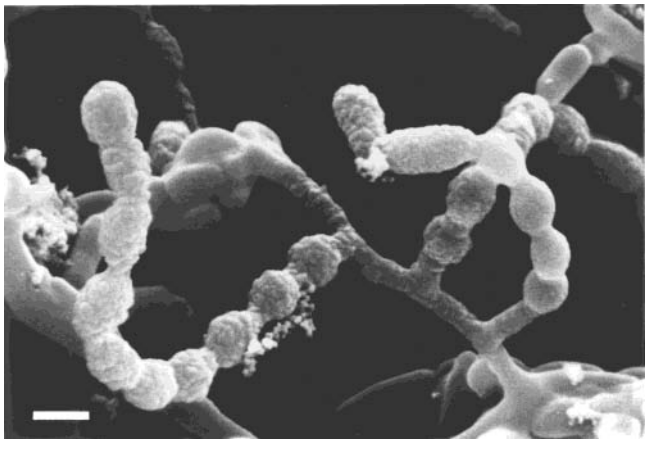

Figure 1. Scanning electron microscopy of spore chains of the actinomycete strain Ao108 cultured on oatmeal agar for 14 days. Bar represents 1 μm.

Spore chain morphology and surface ornament were examined under a scanning electron microscope (Figure 1). Eight or 10 spores of strain Ao108 formed straight or slightly bented chains, which were warty and elliptical (0.8−1.2 × 1.0−1.6 μm in size). Spore chains were

observed as a stalk or in cluster. Special structures, such as synnemata, sclerotia, or sporangia, were not observed.

The chemical and physiological characteristics of strain Ao108 are shown in Table 1. Chemical analysis of cell wall hydrolysates revealed the presence of *meso*-diaminopimelic acid (DAP) as a component of cell wall. Whole cell hydrolysates contained the sugar madurose (3-*O*-methyl-D-galactose). The MK-9 (H_4, H_6, H_8) types of menaquinones were predominantly detected. Strain Ao108 utilized D-fructose, D-galactose, glycerol, inositol, β-lactose, D-mannitol, L-rhamnose, raffinose, sucrose, and D-xylose, but not L-arabinose. Esculin, gelatin, hypoxanthine, starch, testosterone, and tyrosine were degraded to make a clear zone around the colonies. However, strain Ao108 did not degrade casein and xanthin. Nitrate reduction occurred in the medium amended with KNO_3.

On the basis of these morphological and physiological characteristics, the strain Ao108 was identified to be *Actinomadura roseola*, compared to data from *Bergey's Manual of Systematic Bacteriology* (Williams et al., 1989).

Purification of Antibiotic Substance. Antifungal activity against *P. capsici* was found in the extracts of mycelial mass and XAD-16 resin from the culture broth of *A. roseola* Ao108. The crude antibiotic substances were purified by various chromatographic procedures. The 60% and 80% methanol eluates from C18 flash column chromatography inhibited mycelial growth of *P. capsici*. The antibiotic substances in the eluates were monitored by bioautogram on a silica gel TLC plate. On the TLC plate developed with chloroform−methanol (75:25, v:v), two antifungal active bands were detected at R_f 0.26 (Da2B) and R_f 0.42 (Da2A), where clear inhibition zones against mycelial growth of *P. capsici* were produced. The antifungal-active methanol eluates from the C18 flash chromatogram were further purified using a silica gel column. Antifungal activity was detected in the fraction eluted with ethyl acetate-methanol (90:10, v:v), which was followed by Sephadex LH-20 gel filtration. The fraction nos. 67−85 showed antifungal activities.

The active fractions were purified by semipreparative HPLC using a gel filtration column and eluted with 100% methanol. The HPLC profile showed an antifungal active peak at 12.7 min. The active peak was collected and further purified by a HPLC system equipped with a C18 reversed-phase column. The antifungal substances Da2B and Da2A active against *P. capsici* were eluted at the retention time of 22.1 and 24.9 min, respectively. Finally, 7.3 mg of the pure antibiotic Da2B was yielded as red powder. The purity of antifungal substance Da2B was examined by analytical HPLC with a C18 reversed-phase column using various solvent systems. The UV spectral patterns of Da2B showed the absorption maxima at 232, 252, 290, 480, 495, and 532 nm in methanol. The antibiotic Da2A was obtained as a small amount of white powder, which could not be further characterized for its chemical structure.

Structure Elucidation of Antibiotic Da2B. The molecular formula for the compound Da2B was established as $C_{27}H_{29}NO_{10}$ (found 527.1782, calcd 527.1791) by high-resolution FAB mass spectroscopy (no data presented). Signals accounting for 23 unexchangeable protons and 27 carbons of the compound Da2B were observed in ^1H, ^{13}C, and DEPT NMR spectra in CD_3OD [carbons: 3 in CH_3, 3 in CH_2, 8 in CH, 13 in C (3 in C=O)] (Table 2). Analyses of $^1H-^1H$−COSY NMR experiment and confirmation by HMQC, HMBC, and ROESY spectral data revealed that partial structures of Da2B consist of a daunosamine moiety, a trisubstituted benzenoid moiety, a CH_2CCH_2CH unit, and a methoxy and an acetyl group (Figure 2). The presence of the acetyl group and two quinoid carbonyl carbons was confirmed by the carbonyl carbon signals at δ 213.6, 187.6, 188.0 ppm, respectively. A quinoid carbonyl carbon at δ 187.6 ppm showed correlation with the benzenoid proton at δ 7.80 ppm in the HMBC experiment. The methoxy group was connected to the benzenoid carbon at δ 162.4 ppm and the terminal protons of the CH_2CCH_2CH unit to the aromatic quaternary carbons at 135.7 and 135.9, respectively, based on the HMBC correlation. The coupling constants of H-1′ and

Table 2. NMR Spectral Data of the Antibiotic Da2B (^1H at 500 MHz and ^{13}C at 125 MHz in CD_3OD)

carbon no.	$^{13}C,\delta$	$^1H,\delta$ (m,[a] J inHz)	HMBC[b]
1	120.5	7.80 (d, 8.0)	3
2	137.2	7.75 (t, 8.0)	
3	120.3	7.49 (d, 7.6)	
4	162.4		OCH_3, 2
4-OCH_3	57.1	3.99 (s)	
4a	121.4		1, 3
5	188.0[c]		
5a	112.3[d]		
6	156.2[e]		
6a	135.9[f]		7, 8
7	71.7	4.99 (dd, 2.3, 4.9)	
8	36.9	2.29 (br d, 14.6), 2.12 (dd, 5.2, 14.6)	10
9	77.2		10
10	33.1	2.84 (d,18.4), 2.95 (dd, 1.4, 17.2)	8
10a	135.7[f]		10
11	157.4[e]		
11a	112.0[d]		
12	187.6[c]		1
12a	136.2		2
13	213.6		14
14	24.5	2.36 (s)	
1′	101.4	5.42 (br d of d, 3.9)	
2′	29.5	1.89 (br t of dd, 4.7, 12.8), 2.03 (dt, 3.9, 12.8)	4′
3′	48.6	3.58 (ddd, 2.9, 4.7,12.8)	1′, 4′
4′	67.9	3.68 (br s)	5′
5′	67.9	4.29 (m)	CH_3, 1′
5′-CH_3	17.0	1.29 (d, 6.5)	5′

[a] Abbreviations of signal multiplicity are as follows: s, singlet; d, doublet; dd, doublet of doublets; ddd, doublet of doublets of doublets; dt, doublet of triplets; br s, broad singlet; br d, broad doublet; br t, broad triplet. [b] Correlation to proton no. [c−f] Interchangeable.

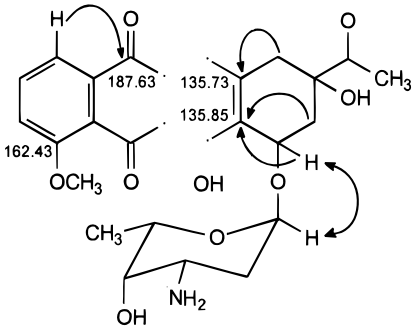

Figure 2. Partial structures and units with selected HMBC (H to C in a single-arrow) and ROESY (H to H in a double-arrow) correlations of the antibiotic Da2B.

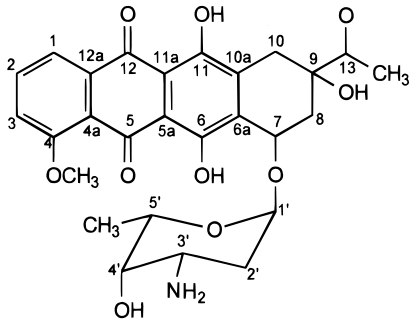

Figure 3. Structure of the antibiotic Da2B isolated from *A. roseola* strain Ao108.

Table 3. Minimum Inhibitory Concentrations (MICs) against Various Microorganisms of the Antibiotic Da2B from *A. roseola* Strain Ao108

microorganism	minimum inhibitory concentration (μg mL^{-1})[a]
Alternaria mali	>50[b]
Botrytis cinerea	30
Cladosporium cucumerinum	30
Colletotrichum gloeosporioides	>50
Cylindrocarpon destructans	30
Didymella bryoniae	30
Fusarium oxysporum f. sp. *cucumerinum*	50
Magnaporthe grisea	50
Phytophthora capsici	10
Rhizoctonia solani	10
Candida albicans	>50
Saccharomyces cerevisiae	7.5
Bacillus subtilis	10
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 above 50 μg mL^{-1}.

two H-2's (J = <1, 3.9 Hz), axial H-2′ and axial H-3′ (J = 12.8 Hz), and axial H-3′ and equatorial H-4′ (J = 2.9 Hz) indicated the conformation of the daunosamine as shown in Figure 3. It was confirmed by the ROESY data showing no NOE between H-1′ and H-5′ and NOE between H-3′ and H-5′. In addition, the connectivity of the daunosamine moiety to the aglycone was indicated in the ROESY experiment. Thus, the signal for H-7 of the aglycone correlated to the signal for the anomeric proton (H-1′) at δ 5.42 ppm. On the basis of the extensive 2-D NMR correlation studies, the structure of the antibiotic Da2B was identified as an anthracycline antibiotic daunomycin (Figure 3). The spectroscopic data were in good agreements with the literature (Iwamoto et al., 1968; Arnone et al., 1976) and with the authentic sample (Sigma Chemical Co.).

In Vitro and in Vivo Antifungal Activity of the Antibiotic Da2B. The in vitro antimicrobial spectrum of the antibiotic Da2B was determined by a serial dilution method in microtiter-well plates (Table 3). The antibiotic Da2B showed inhibitory effect against the mycelial growth of plant pathogenic fungi used in this test, except for *A. mali* and *C. gloeosporioides*. In particular, mycelial growth of *P. capsici* and *R. solani* was completely inhibited by treatment with 10 μg mL^{-1}. The growth of *C. albicans* was not affected even at the concentration of 50 μg mL^{-1}. However, treatment with

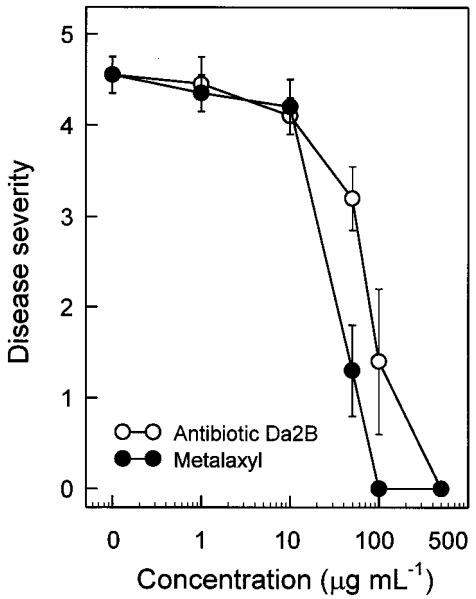

Figure 4. Effects of the antibiotic substance Da2B and metalaxyl at different concentrations on the disease development in pepper plants at the first branch stage inoculated with *Phytophthora capsici*. Disease severity is based on a 0−5 scale, where 0 = no visible symptom and 5 = plant dead. Vertical bars represent standard deviations.

7.5 μg mL^{-1} completely inhibited the growth of *S. cerevisiae*. Inhibitory activity of Da2B against gram-negative bacteria such as *E. carotovora* pv. *carotovora*, *P. solanacearum* and *X. campestris* pv. *vesicatoria* was not found even at 50 μg mL^{-1}. However, the growth of *B. subtilis* was inhibited at 10 μg mL^{-1}

In vivo efficacy of the antibiotic Da2B for the control of *Phytophthora* blight in pepper plants was evaluated under greenhouse conditions (Figure 4). Treatment with 50 μg mL^{-1} of Da2B and the commercial fungicide metalaxyl greatly inhibited disease development on pepper plants. However, the efficacy of Da2B for the control *Phytophthora* infection was somewhat less than that of metalaxyl on pepper plants. The pepper plants treated with 100 μg mL^{-1} of metalaxyl did not show any disease symptom. While, 500 μg mL^{-1} of the antibiotic Da2B was required to completely prevent *Phytophthora* blight on pepper plants. The antibiotic Da2B did not show any phytotoxicity on pepper plants at a concentration of 500 μg mL^{-1}.

DISCUSSION

The presence of *meso*-diaminopimelic acid and madurose in the strain Ao108 as characteristic whole-cell components and the formation of aerial mycelia with long spore chains were characteristic of the genus *Actinomadura* (Lechevalier and Lechevalier, 1970). The strain Ao108 was identified as *A. roseola* by comparison of data of the type strain demonstrated by Lavrova and Preobrazhenskaya (1975).

The culture extracts of the strain Ao108 showed not only in vitro antifungal activity but also effective control efficacy against *Phytophthora* infection in pepper plants (no data presented). In the screening procedures for the development of fungicides, in vivo bioassay is prerequisite at the beginning of the screening, since there is much evidence that the antimicrobial metabolites having strong antifungal activity in vitro often have no

control efficacy of plant diseases in host plants (Fawcett and Spencer, 1970).

From the culture extracts of *A. roseola* strain Ao108, a red-pigmented antibiotic Da2B showing antifungal activity against some plant pathogenic fungi were purified using various chromatographic procedures. The data from mass and UV−vis spectroscopy of the antibiotic Da2B indicated the presence of the hydroxyanthraquinone chromophore in the compound. The glycoside moiety of the compound was identified as an aminoglycoside, daunosamine, on the basis of various spectroscopic data. These chemical characteristics were similar to those of anthracycline antibiotics such as daunomycin (Iwamoto et al., 1968), adriamycin (Arcamone et al., 1969), and carminomycins (Pettit et al., 1975), which have anthracycline aglycon and aminoglycoside daunosamine moiety. The extensive NMR spectral examination revealed that the antibiotic Da2B has the same chemical structure as daunomycin. The complete structure of the antibiotic Da2B was confirmed by the comparisons of its chemical and spectral properties with that of the authentic compound.

Since daunomycin was discovered from the culture broth of *S. peuceticus* by DiMarco et al. (1964) and D'bost et al. (1963), several *Streptomyces* strains have been found to produce the antibiotic daunomycin (Strohl et al., 1989; White and Stroshane, 1984). However, other anthracycline antibiotics similar to daunomycin in chemical structures were isolated from various genera of actinomycetes. *Streptomyces peucetius* subsp. *caesius*, which is a mutant of daunomycin-producing strain (Arcamone et al., 1969), was described to produce 14-hydroxidaunomycin. Carminomycin having the structure of desmethyl daunomycin was also isolated from *Actinomadura carminata* sp. nov. (Pettit et al., 1975). Other carminomycin-related antibiotics were detected from *A. roseoviolacea* (Nakagawa et al., 1983). *Streptosporangium* sp. is also known to produce carminomycin I (Baldwin et al., 1975). Other daunomycin-related anthracyclines such as 11-deoxidaunorubicin, 11-deoxydoxorubicin, 11-deoxy-13-dihydrodaunorubicin, and 11-deoxy-13-deoxodaunor-ubicin were isolated from *Micromonospora peucetica* (Arcamone et al., 1980). Although the anthracycline antibiotics structurally related to daunomycin were isolated from various genera of actinomycetes, *Actinomadura* was not demonstrated to produce daunomycin in earlier studies.

The biological activity of daunomycin was mainly studied for the clinical uses as an anticancer agent. Daunomycin has been used primarily to treat adult myelogenous leukemia, whereas 14-hydroxidaunomycin is widely used to treat a variety of neoplasias (Arcamone, 1984). Their specificity between chemical structure and biological activity enables these types of antitumor drugs to be extensively studied (White and Stroshane, 1984). The biological activities in microbial systems were also performed for clinical use, which showed the marked inhibitory effect of daunomycin against the virus causing herpetic and vaccinial kertitis on HeLa cells (Cohen et al., 1969). Antimicrobial activity against some Gram positive bacteria such as *Staphylococcus aureus* (DiMarco et al., 1964) and *Streptococcus faecalis* (Pittillo and Hunt, 1968) was detected by in vitro bioassay. Nevertheless, the anthracycline daunomycin was first isolated from the *Streptomyces* strain selected during an antibacterial and antifungal screening procedures (Grein, 1987).

The antibiotic Da2B showed substantial antifungal activity against *P. capsici* and *R. solani* and antiyeast activity against *S. cerevisiae*. We also observed antibacterial activity against Gram positive bacteria and weak antifungal activity against other plant pathogenic fungi such as *B. cinerea*, *C. cucumerinum*, *C. destructans*, and *D. bryoniae*. Such an antifungal activity of daunomycin has not been demonstrated in earlier studies.

In vivo assay of the antibiotics on plants is the most accurate means of predicting the potential antifungal activity in fields, which reflect not only the intrinsic potency of the antibiotics but also its chemical and physical properties such as stability in agricultural environment and distribution in host plants. The antibiotic Da2B showed not only strong inhibitory effect on the mycelial growth of *P. capsici* but also suppressed the Phytophthora development on pepper plants. Although its in vivo fungicidal efficiency was somewhat less effective than that of the commercial fungicide metalaxyl, the pepper plants were completely protected from Phytophthora infection by treatment with 500 μg mL^{-1} of the antibiotic Da2B. In view of antifungal activity of the antibiotic Da2B, the analogues of the compound may be worthwhile to examine their antifungal activity against various plant pathogenic fungi.

From these results, we could conclude that the antibiotic Da2B, designated daunomycin, from *A. roseola* strain Ao108 has potent antifungal activity against some plant pathogenic fungi and in vivo control efficacy against Phytophthora development on pepper plants. This is the first report of isolation of daunomycin-producing strain of *Actinomadura*, and the antifungal activity of daunomycin.

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