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A Peptide Antibiotic AMRSA1 Active against Multidrug-resistant *Staphylococcus aureus* Produced by *Streptomyces* sp. HW-003

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The antibiotic-producing strain HW-003 was screened from soil and found to be effective against the multidrug-resistant *Staphylococcus aureus*. The spore chain of HW-003 was retinaculiaperti, and the spore surface was spiny. Strain HW-003 has a LL-diaminopimelic acid isoform in the cell wall. The aerial mass color of the strain was gray, and the reverse side was yellow-brown. The strain produced melanin, but did not produce soluble pigments. According to the Taxon program, HW-003 showed best match with *Streptomyces cyaneus*. Antibiotic production reached a maximum after 72-h cultivation. The antibiotic was purified with silica gel column chromatography, octadecylsilyl column chromatography, and HPLC. The purified antibiotic, AMRSA1, showed strong inhibitory activity against multidrug-resistant *Staphylococcus aureus* and gram-positive bacteria. The molecular weight of AMRSA1 was about 1,100. AMRSA1 was a peptide antibiotic containing alanine and serine.

A lot of antibiotics has been developed and used for the treatment of bacterial infections. However, unfortunately the evolution and spread of antibiotic resistant bacteria were followed after clinical use. A great threat to successful antibiotic coverage is the evolution and the spread of antibiotic-resistant bacteria. The number of hospital infections with gram-positive cocci has increased significantly (10, 18). As recent outgrowth of the AIDS epidemic, as well as the increasing prevalence of aggressive cancer chemotherapy and organ transplantation, subsequent opportunistic infections have become significant health problems (16). Particularly, methicillin-resistant *Staphylococcus aureus* (MRSA) is known to be a pathogen responsible for the serious infections during the clinical treatment of immune system-debilitated patients (2).

Methicillin has proved to be a very useful β-lactam antibiotic in the clinical treatments of infections by β-lactamase-producing bacteria, as it is not destroyed by β-lactamase. The β-lactam antibiotics have a binding affinity for the penicillin-binding protein (PBP) which has peptidyl transferase activity for the synthesis of peptidoglycan in the bacterial cell wall. Methicillin resistance was not the result of destruction of the antibiotic by β-lactamase but was involved as an intrinsic factor. MRSA usually has a *mec* gene coding for penicillin binding protein 2a (PBP 2a) which has a low affinity to all of the β-lactam antibiotics used clinically. As a result, MRSA shows a resistance to β-lactam antibiotics. Notably, the *mec* gene has a sequence region in which various antibiotic-resistant genes can be inserted (8, 9, 11). In this respect, most of the MRSA recently isolated from patients in Korea have multidrug-resistant.

Vancomycin and teicoplanin are useful antibiotics against MRSA in the clinical treatments. However, resistant enterococci and coagulase-negative staphylococci for these antibiotics have been reported recently, and these glycopeptides are relatively expensive (5). Ciprofloxacin and pefloxacin, novel quinolone antibiotics, are also highly active against these pathogens. However, quinolone-resistant mutants are rapidly starting to appear (3). Thus, the need for new antibiotics effective against MRSA is increasing.

In this paper, we report on the screening and identification of an antibiotic-producing strain, and characteristics of the antibiotic active against multidrug-resistant *S. aureus*.

MATERIALS AND METHODS

Selection of Multidrug-resistant *Staphylococcus aureus* (MDRSA)

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The MDRSAs were isolated from patients in Wonju Christian Hospital. The clinical samples were cultivated on a blood agar at 37°C for 24 h, and a golden pigment-producing strain was subcultured. The strains were tested for methicillin resistance, mannitol fermentation, DNase activity, catalase activity, and hemolysis according to *Methods for General and Molecular Bacteriology* (7). The strains which showed positive results for all the tests were selected as MRSA. The antibiotic resistance of 150 MRSA strains was examined by a 2-fold serial agar dilution susceptibility test (14). The antibiotics used for the resistance test were penicillin, methicillin, cephalothin, cefazolin, tobramycin, amikacin, gentamicin, chloramphenicol and vancomycin. About 75% of 150 MRSAs showed resistance against more than 5 antibiotics. This may indicate that most of the MRSAs isolated from patients in Korea are multidrug-resistant. Ten resistant-strains against more than 7 antibiotics were chosen as MDRSA for the antimicrobial activity test.

Screening of Antibiotic-producing Microorganisms

The actinomycetes were isolated from soil on the isolation medium containing 1.0% soluble starch, 0.2% peptone, 0.1% NaCl, 0.1% K_2HPO_4, and 0.05% $MgSO_4$ at pH 7.0. The isolated strains were streaked on to tryptic soy agar plates (Difco), and cultivated at 30°C for 5 days. Next, molten agar containing *S. aureus* 6538P was poured to the plates, and incubation was continued at 37°C for 20 h. The strains which made an inhibition zone around the colony were selected as antibiotic-producing strains. A loop of the strains was inoculated into a 250-ml Erlenmeyer flasks containing 50-ml tryptic soy broth, and was cultivated with shaking at 30°C for 5 days. The antibacterial activity of filtrates against 10 MDRSA strains was measured by the paper disc diffusion method (14). During the experiments, the antibiotic-producing strain was transferred to the isolation medium every 4 weeks, and maintained in a refrigerator.

Identification of Antibiotic-producing Strain

The antibiotic-producing strain HW-003 was examined for morphological and cultural characteristics, according to the method of Shirling and Gottlieb (15). The strain was investigated for physiological characteristics and diaminopimelic acid isoform in the cell wall, according to standard methods (1, 7, 21). For the identification of strain HW-003, all of the results were analyzed using the Taxon program (19).

Determination of Antibacterial Activity and Minimum Inhibitory Concentration (MIC)

Antibacterial activity was measured by the paper disc diffusion method on Mueller-Hinton agar (14), and *S. aureus* 6538P was used for the antibiotic assay. *S. aureus* 6538P does not show resistance against any antibiotic. For the investigation of antimicrobial spectrum, MIC was measured by a 2-fold serial agar dilution susceptibility test using Mueller-Hinton agar for the bacteria after 24 h incubation at 37°C and using Sabouraud dextrose agar (Difco) for the yeast and fungi after 72 h incubation at 28°C (14). The fresh microorganisms were used for the inoculum. For the measurement of cell growth, the cultured cells were washed with distilled water, dried at 105°C for 24 h, and then cell growth was determined as dry cell weight (DCW).

Production of Antibiotic

For the preparation of inoculum, the strain was cultivated with shaking in 500-ml Erlenmeyer flask containing 50-ml tryptic soy broth at 30°C for 2 days. The medium of 1.5 liters in 3-liter jar fermenter was autoclaved at 121°C for 10 min. The medium for antibiotic production was composed of 0.75% soluble starch, 0.75% glucose, 0.15% peptone, 0.15% tryptone, 0.1% $MgSO_4 \cdot 7H_2O$, 0.05% K_2HPO_4, 0.1% serine, 0.1%, alanine and 0.05% phenylalanine at pH 8.0. The inoculum size was 5%. And it was cultivated at 200 rpm agitation and 1.5 vvm aeration at 32°C for 4 days. The production of antibiotic was examined every 12 h over the cultivation period. The residual reducing sugars in the culture broth were measured by the dinitrosalicylic acid (DNS) method (4).

Purification of Antibiotic

The antibiotic was extracted in a 4-liter culture filtrate using chloroform, and then the chloroform extract was evaporated. The residue was chromatographed on a silica gel column with chloroform-ethanol (97 : 3 v/v). The active fraction was evaporated, and then chromatographed on a octadecylsilyl (ODS) column with water-methanol (55 : 45 v/v). After the active fraction was evaporated *in vacuo*, the crude antibiotic was chromatographed on a sepadex LH-20 column with methanol. The active fraction was evaporated, and the crude antibiotic was purified by preparative HPLC. Preparative ODS-HPLC was performed with acetonitrile-water (6 : 4 v/v) at flow rate 7 ml/min. After the active fraction was evaporated, the preparative silica gel HPLC was performed with acetonitrile-chloroform (6 : 4 v/v) at flow rate 7 ml/min. A single peak of antibiotic was obtained on the silica gel HPLC chromatogram. During HPLC, the antibiotic was detected at 254 nm. For the purity test of antibiotic, thin layer chromatography was performed with several solvents on the silica gel plates. One spot appeared on the all of chromatogram. Finally 4.1 mg white powder was gained as a purified antibiotic, and it was tentatively named as AMRSA1. The purification steps are summarized in Fig. 1.

Physicochemical Properties of the Antibiotic

For ultraviolet (UV) spectrometric analysis, 20 μg antibiotic was dissolved in 2-ml methanol, and then the UV spectrum was scanned from 200 to 400 nm. KBr pellets of the antibiotic was used to measure IR absorption.

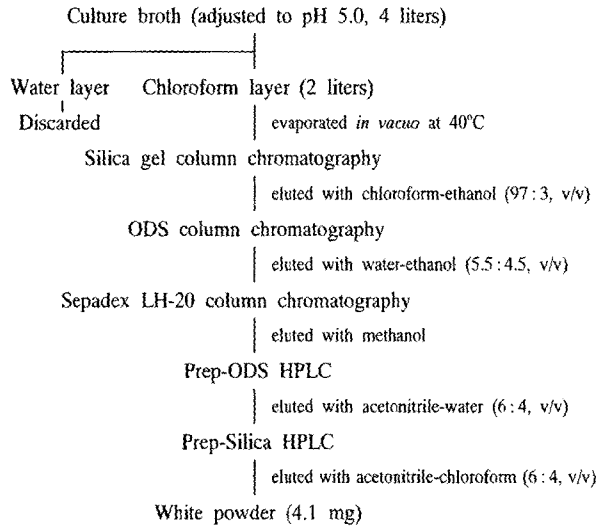

Fig. 1. Purification steps of the antibiotic.

The molecular weight of AMRSA1 was measured by FAB mass spectrometry (VG-VSEQ, VG analytical, UK) under conditions that the matrix was 3-nitrobenzyl alcohol and the FAB source was 35 KeV. A solution of 10 mg antibiotic dissolved in CD_3OD was put into 5 mm NMR tube, and the NMR spectrum was analyzed with 600 MHz NMR spectrometer (Bruker Model 600).

RESULTS AND DISCUSSION

Screening of Antibiotic-producing Strain

About 2,000 strains of actinomycetes were isolated from soil collected in the province of Wonju, Korea. The 51 strains which made an inhibition zone around the colony on agar plates were selected. After the selected strains were cultivated on tryptic soy broth, the antibacterial activities of the culture filtrates against 10 MDRSA strains were measured using the paper disc diffusion method. Among them, the strain HW-003 was selected, because the strain produced the most active antibiotic for all the tested MDRSA.

Identification of Strain HW-003

The aerial mycelium grown on the oat meal agar for 2 weeks was woolly in form and branching with a tree type structure, and one spiral turn at the end. The spore chain of HW-003 was retinaculiaperti, and the spore surface was spiny as shown in Fig. 2. The aerial mass color was gray, and the reverse-side color was yellow-brown. The strain produced melanin on the peptone-yeast extract agar (ISP-6 medium) and tyrosine agar (ISP-7 medium), but it did not produce soluble pigments on the International Streptomyces Project (ISP) media as shown in Table 1. The strain has an LL-diaminopimelic acid isoform in the cell wall.

The strain utilized glucose, fructose, rhamnose and mannitol, but did not utilize xylose, inositol, sucrose, raffinose and cellobiose (Table 2). The strain hydrolyzed α-aminobutyric acid, histidine and 4-hydroxyproline. The strain showed lecithinase activity and nitrate reductase activity, but did not show pectinase activity. The strain showed antibiosis against *Bacillus subtilis*, but did not show antibiosis against *Streptomyces murinus* and *Aspergillus niger*. The strain degraded xanthine, but did not degrade allantoin and arbutin. The strain did not have resistance for neomycin and rifampicin. The strain did

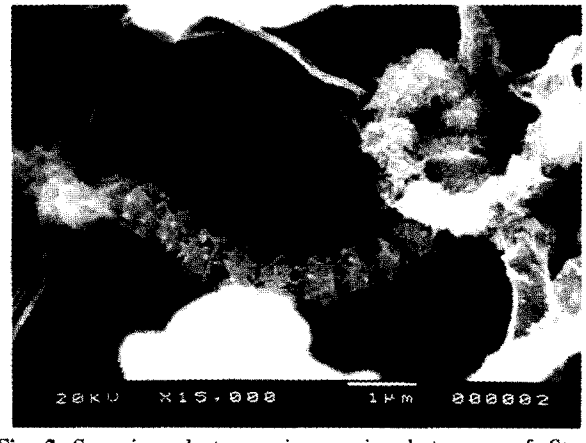

Fig. 2. Scanning electron microscopic photogram of *Streptomyces* sp. HW-003.

After the strain HW-003 was incubated on the oat meal agar at 28°C for 14 days, the sheet of the cell was cut down to 1×1 cm. The cell was coated in gold, and then examined by scanning electron microscope.

Table 1. The cultural characteristics of *Streptomyces* sp. HW-003.

ISP Media	Aerial mass color	Reverse-side color	Soluble pigment	Sporulation
Yeast extract-malt extract agar	Dark gray	Brown	None	Excellent
Oat meal agar	Gray	Yellow	None	Moderate
Inorganic salt-starch agar	Gray	Pale yellow	None	Good
Glycerol-asparagine agar	Pale gray	Yellow	None	Good
Peptone-yeast extract agar	Gray	Brown	None	Poor
Tyrosine agar	Dark gray	Black	None	Excellent

The strain HW-003 was cultured at 28°C for 3 weeks.

Table 2. The characteristics of strain HW-003.

Characteristics	Strain HW-003
Spore chain	Retinaculiaperti
Spores chain ornamentation	Rugosa
Color of aerial spore mass	Gray
Reverse color	Yellow/brown
Melanin production on tyrosine agar	+
Fragmentation of mycelium	−
Lecithinase activity on egg-york medium	+
Pectin hydrolysis	−
Nitrate reduction	+
H_2S production	−
Bacillus subtilis antibiosis	+
Streptomyces murinus antibiosis	−
Aspergillus niger antibiosis	−
Degradation of xanthine	+
allantoin	−
arbutin	−
Resistance to neomycin (50 μg/ml)	−
rifampicin (50 μg/ml)	−
Growth at 45°C	+
NaCl (7 w/v)	−
sodium azide (0.01 w/v)	−
phenol (0.1 w/v)	−
DL-aminobutyric acid	+
L-histidine	+
L-hydroxyproline	+
glucose	+
D-xylose	−
meso-inositol	−
mannitol	+
D-fructose	+
L-rhamonose	+
raffinose	−
inulin	−
adonitol	+
sucrose	−
cellobiose	−

+, Positive; −, Negative.

not grow in 7% NaCl, 0.01% sodium azide, and 0.1% phenol. The characteristics of strain HW-003 are summarized in Table 2.

According to our search of the Taxon program, the strain HW-003 did best match with major cluster 18 (*Streptomyces cyaneus*). The degree of the relationship was the Tax distance 0.5819, 95% Taxon radius 0.4276%, Probability of strain further away 0.0000, SSM value 73%, and Willcox probability 0.748018. Willcox probability required more than 0.850000 for the identification using Taxon program, but this strain has Willcox probability 0.748018. Also, this strain has low further away. Therefore, strain HW-003 did best match with *Streptomyces cyaneus*, but it was not identified clearly according to Taxon program.

Production of Antibiotic

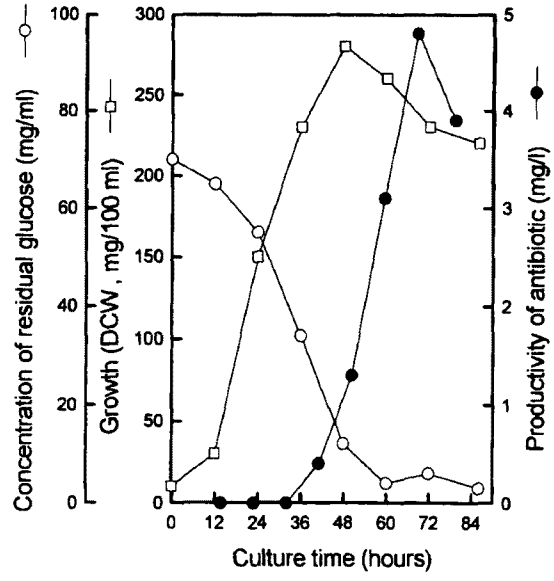

Fig. 3. Time course on the production of antibiotic in a jar fermenter.

The antibiotic production medium was composed of 0.75% starch, 0.75% glucose, 0.15% peptone, 0.15% tryptone, 0.1% $MgSO_4 \cdot 7H_2O$, 0.05% K_2HPO_4, 0.1% serine, 0.1%, alanine and 0.05% phenylalanine at pH 8.0. The culture conditions were 200 rpm agitation speed and 1.5 vvm aeration at 32°C. The cell growth was expressed as dried cell weight (DCW, mg/100 ml). The productivity was estimated as purified antibiotic activity.

Cell growth reached a maximum after 48 h, whereas antibiotic production reached a maximum after 72 h when the strain was cultivated in a 3-liter jar fermenter. The maximum productivity of the antibiotic was 4.8 mg/l and the product formation pattern was of a non-growth associated type as shown in Fig. 3. The reducing sugar of the broth was gradually decreased throughout the culture time. Most of the reducing sugar was consumed after the strain HW-003 was cultivated for 48 h. After complete consumption of sugar, antibiotic production was rapidly increased.

Antimicrobial Spectrum of AMRSA1

The antimicrobial activities of AMRSA1 were investigated using the 2-fold serial agar dilution suceptibility test (14). AMASA1 showed strong inhibitory activity against *Micrococcus luteus*, *S. aureus* ATCC 6538P, *Corynebacterium diphtheriae* and *Staphylococcus epidermidis* with the MICs less than 0.05 μg/ml. It also showed moderate inhibitory activity against *B. subtillis*, *Bacillus anthracis* and *Bacillus cereus* with MICs of less than 4 μg/ml. It showed weak inhibitory activity against *Escherichia coli* ATCC 25922 and *Citrobacter freundii* with MICs of about 30 μg/ml. However, the antibiotic did not show any antibacterial activity against *Streptococcus mutans*, *Pseudomonas aeruginosa*, *Shigella son-*

nei, Enterobacter cloacae, Enterobacter aerogenes, Proteus vulgaris or *Salmonella typhi*. It did not show antifungal activity against *Saccharomyces cerevisiae, Candida albicans, Aspergillus niger* and *Penicillum notatum*, either (Table 3). The antimicrobial spectrum of AMRSA1 showed similarity with pyrroindomycins and eremomycin. However, there are different antimicrobial spectrum between AMRSA1 and those antibiotics. Pyrroindomycins from *Streptomyces rugosporus* has antibacterial activity against vancomycin-resistant enterococci and eremomycin has antimicrobial activity against streptococci (6, 13). Technological refinements are likely to bring rapid diagnostic methods and lead to effective dosing with narrow-spectrum agents in the future (16). Given the specific antibacterial spectrum against staphylococci, it may be expected that AMRSA1 could be useful in the treatment of MDRSA infections avoiding antibiotics abuse. It is reported that vancomycin, teicoplanin, LY264826 and LY191145 exhibit 2 μg/ml, 0.5 μg/ml, 0.25 μg/ml and 0.25 μg/ml of MICs against methicillin-susceptible *S. aureus*, respectively (12). The purified antibiotic AMRSA1 was typically 20 to 200 times more potent than these antibiotics. AMRSA1 showed a strong inhibitory activity against MDRSA with MIC of 0.01~0.1 μg/ml. This is compared to the report that vancomycin, teicoplanin, LY264826 and LY191145 exhibit MICs of 2 μg/ml, 1 μg/ml, 1 μg/ml and 0.5 μg/ml against MRSA, respectively (12). AMRSA1 was typically 10 to 50 times more potent than these antibiotics. Therefore, a strong activity against MRSA may be expected and AMRSA1 could be useful in the treatments of MRSA infections.

Table 3. The antimicrobial spectrum of the purified antibiotic.

Strains	MIC (μg/ml)
Micrococcus luteus	0.05
Staphylococcus aureus ATCC 6538P	0.01
Staphylococcus epidermidis	0.01
Bacillus subtilis	1
Bacillus anthracis	4
Bacillus cereus	0.1
Corynebacterium diphtheriae	0.05
Streptococcus mutans	> 100
Pseudomonas aeruginosa	> 100
Shigella sonnei	> 100
Citrobacter freundii	30
Enterobacter cloacae	> 100
Enterobacter aerogenes	> 100
Proteus vulgaris	> 100
Escherichia coli ATCC 25922	30
Salmonella typhi	> 100
Saccharomyces cerevisiae	> 100
Candida albicans	> 100
Aspergillus niger	> 100
Penicillium notatum	> 100
Staphylococcus aureus clinical isolate 10	0.01
Staphylococcus aureus clinical isolate 12	0.05
Staphylococcus aureus clinical isolate 39	0.01
Staphylococcus aureus clinical isolate 48	0.01
Staphylococcus aureus clinical isolate 67	0.10
Staphylococcus aureus clinical isolate 92	0.05
Staphylococcus aureus clinical isolate 104	0.05
Staphylococcus aureus clinical isolate 113	0.10
Staphylococcus aureus clinical isolate 129	0.01
Staphylococcus aureus clinical isolate 133	0.05

MIC was measured by 2-fold serial agar dilution method using Mueller-Hinton media for bacteria after 24 h incubation at 37°C and using Sabouraud dextrose media for yeast and fungi after 72 h incubation at 28°C.

Physicochemical Properties of AMRSA1

The antibiotic was soluble in methanol, ethanol,

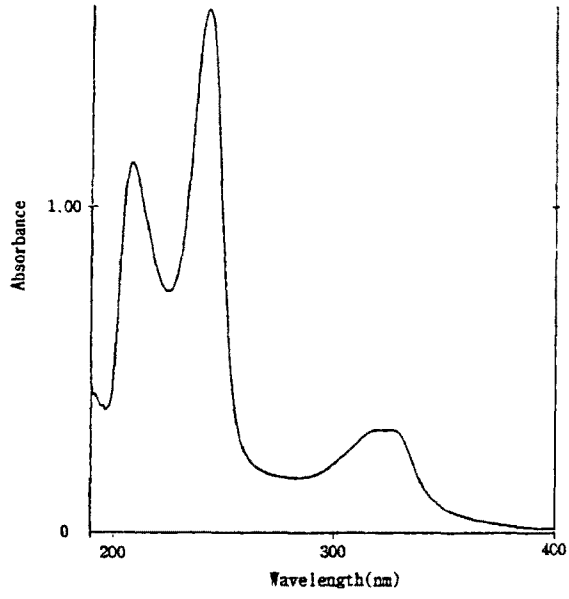

Fig. 4. UV spectrum of AMRSA1.
The purified antibiotic of 20 μg was dissolved in 2 ml methanol.

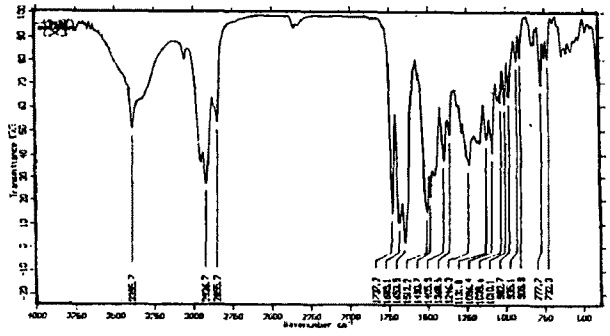

Fig. 5. IR-spectrum of AMRSA1.
The purified antibiotic of 1 mg and KBr was mixed, and the KBr pellet was made under 700 kg/cm^2.

acetone, butanol, ethyl acetate, and chloroform, but insoluble in water and benzene. The antibiotic did not react with Biuret reagent, Molish's reagent, diphenylamine-anilin and phenol-sulfuric acid, while it reacted with $KMnO_4$, H_2SO_4, and Emerson's reagent. This indicate that the antibiotic has many double bonds. AMRSA1 did not react with ninhydrin, but the acid hydrolysate of AMRSA1 which was hydrolyzed by 6 N trifluoroacetic acid at 120°C for 2 h reacted with ninhydrin. This result may indicate that the antibiotic is a cyclic peptide. The antibiotic showed no optical rotation, and decomposed at 190°C without melting.

Three UV absorption maxima of the antibiotic were observed at 208, 241 and 315~325 nm as shown in Fig. 4. The antibiotic showed strong IR absorption at 3395, 2926, 2855, 1737, 1689, 1653, 1512, 1490, 1405 and

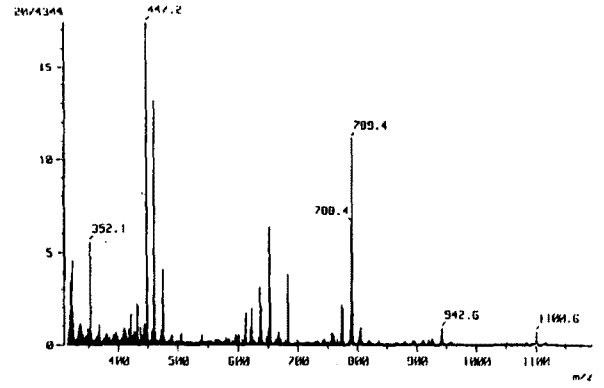

Fig. 6. FAB mass spectrum of AMRSA1.
The molecular weight of AMRSA1 was measured by FAB mass spectrometry (VG-VSEQ, VG analytical, UK). The matrix was 3-nitrobenzyl alcohol, and FAB source was 35 KeV.

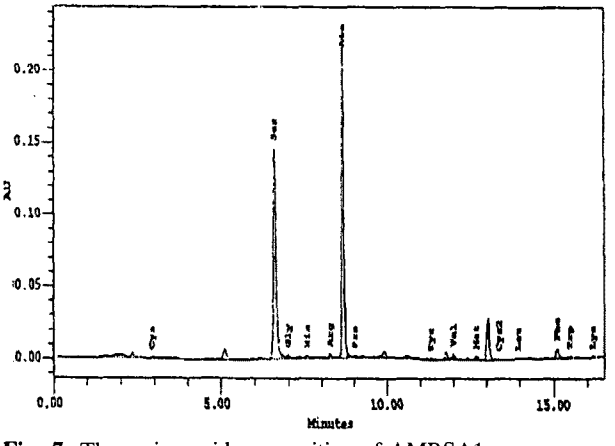

Fig. 7. The amino acid composition of AMRSA1.
The antibiotic was dissolved in 0.8 ml HCl (6 N). The air of the tube was exchanged by N_2 gas, and then was hydrolyzed at 110°C for 24 h. The hydrolysate was analyzed by amino acid analyzer with C_{18} column (Pico-Tag system, 4 μm, 0.4×15 cm).

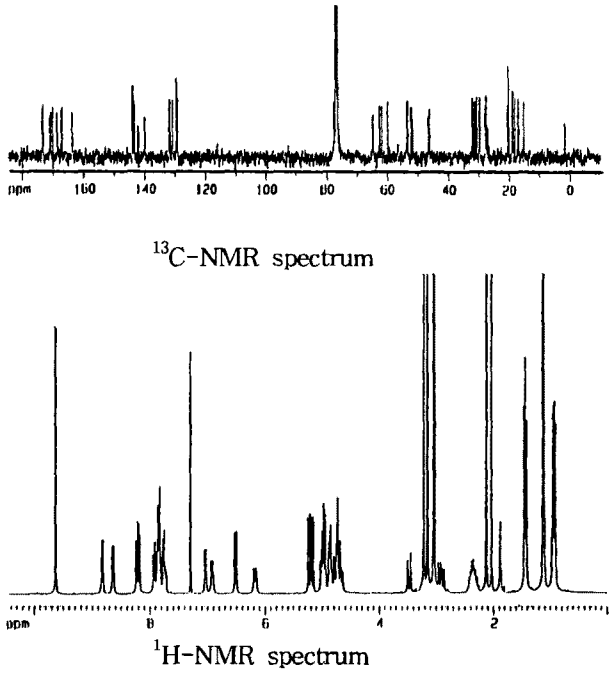

Fig. 8. ^{13}C-NMR spectrum and ^{1}H-NMR spectrum of AMRSA1.
The purified antibiotic of 10 mg was dissolved in CD_3OD, and then the solution put into 5 mm NMR tube. The NMR spectrum was measured by 600 MHz NMR Spectroscopy.

Table 4. The physicochemical properties of AMRSA1.

Properties	Description
Nature	White powder
Molecular weight	1100
Melting point	190°C (Decomposed temperature)
Polarity in methanol $[\alpha]_D^{25}$	0° (1 mg/ml)
Solubility	Soluble: methanol, ethanol, acetone, butanol, ethyl acetate, chloroform Insoluble: water and benzene
Color reactivity	Positive: $KMnO_4$, H_2SO_4, ninhydrin (hydrolysate), iodine, Emerson's test Negative: Biuret test, Molish's test, ninhydrin, diphenylamine-anilin, phenol-sulfuric acid
UV λ_{max} (MeOH, nm)	208, 241, 315~325
IR (cm^{-1})	3395, 2926, 2855, 1737, 1689, 1653

1368 cm^{-1} as shown in Fig. 5. It is believed that the peptide bond appeared at 3395 cm^{-1}, the alkene group appeared at 2926, and 2855 cm^{-1}, and the carbonyl and amide group appeared at 1737, 1689 and 1653 cm^{-1}. The molecular weight of the antibiotic was about 1,100 on the FAB mass spectrum as shown in Fig. 6. The antibiotic contains alanine and serine (molar ratio 1 : 1) as the major amino acids according to the amino acid analysis as shown in Fig. 7. The physicochemical properties of the purified antibiotic are summarized in Table 4.

The analyses of ^{13}C-NMR spectrum of AMRSA1 revealed that the beta-methyls of alanine were observed at 15.21~20.34 ppm. The alpha-methins of serine and alanine were observed at 46.21~62.66 ppm. The beta-CH_2(OH) of serine was assigned at 64.78 and 64.97 ppm, and several -CO-NH- were observed at 163.85~173.51 ppm (Fig. 8). In conclusion, all of these results suggest that AMRSA1 is probably a peptide antibiotic. We are now investigating the structural elucidation of the antibiotic.

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