A New Anti-MRSA Antibiotic Complex, WAP-8294A

I. Taxonomy, Isolation and Biological Activities

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WAP-8294A, produced by *Lysobacter* sp., is a complex consisting of water soluble depsipeptide antibiotics. It was further purified by column chromatographies and HPLC, and 19 components were obtained. WAP-8294A2, a major component, and minor components A1, A4, Ax8, Ax9 and Ax13 were active against Gram-positive bacteria, in particular, methicillin-resistant *Staphylococcus aureus* (MRSA) *in vitro*. WAP-8294A2 was highly active *in vivo* in mice against the systemic infection of MRSA.

During the past decade, nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals have become a serious clinical problem¹⁾. The glycopeptide antibiotic, vancomycin, has been used for the treatment of infections due to MRSA. Recently, MRSA strains with intermediate resistance to vancomycin (MIC, 8 µg/ml) have been reported in clinical isolates²⁾. Therefore, a new anti-MRSA antibiotic, which differs from vancomycin in mode of action, is clinically of interest.

In the course of our screening system for new anti-MRSA antibiotics, a strain No. WAP-8294 belonging to the genus *Lysobacter* was found to produce a new peptide antibiotic which showed inhibitory activity against MRSA. It was isolated as a complex of WAP-8294A from the fermentation broth by chromatography. In the HPLC analysis of WAP-8294A complex, a major component WAP-8294A2 and 18 minor components were observed (Fig. 1). Of those components, A1, A4, A2, Ax8, Ax9 and Ax13 were isolated (Fig. 2). The structure of WAP-8294A2 had been reported in the previous communication³⁾. The structure elucidation of the isolated components will be reported in detail in the subsequent paper⁴⁾. The present paper deals with the taxonomy of the producing organism, fermentation,

isolation and biological activities of WAP-8294A1, A2, A4, Ax8, Ax9 and Ax13.

Materials and Methods

Microorganism

The producing strain No. WAP-8294 was isolated from a soil sample collected in Shimoda City, Shizuoka Prefecture, Japan. This strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as *Lysobacter* sp. WAP-8294 with the accession number FERM BP-4990.

Activity *In Vitro*

MIC values were determined by the broth micro-dilution technique with 2-fold serial dilution of the test compounds. MRSA strains were tested on Mueller-Hinton Broth (Difco) supplement with 2% NaCl. Other bacteria were tested in Mueller-Hinton Broth. All tests were done with medium only and plus 10% fetal calf serum. Micro-titer plates were inoculated with 10⁴ cfu/ml, and were incubated at 37°C for 24 hours.

Fig. 1. HPLC chromatogram of WAP-8294A.

Column: YMC-Pack ODS-A (6 × 150 mm), flow rate: 1 ml/min, detection: UV at 214 nm.

Fig. 2. Structure of WAP-8294A.

Activity *In Vivo*

The *in vivo* antibacterial activity of WAP-8294A2 was determined by experimental infection of mice using nine strains of MRSA. The male mice strains ICR-MCH (Nippon Clea) were challenged by intraperitoneal administration of 0.5 ml of 0.1% cyclophosphamide 3 days before the infection. The mice were then infected by intraperitoneal injection of 0.5 ml of MRSA. Eight dose levels of WAP-8294A2 were subcutaneously administered (0.2 ml) after 1 hour of infection. Vancomycin was tested as the reference antibiotic. To the control groups, 0.2 ml of 0.9% saline was subcutaneously administered. Each dose group had eight animals. The effective dose (ED₅₀) was estimated from the survival ratios by computerized Probit Analysis.

Bactericidal Activity

MRSA was grown overnight in Mueller-Hinton broth at 28°C. The culture was washed and diluted to 1.0×10^6 CFU/ml. The cells were separately exposed to different concentrations of WAP-8294A and vancomycin. At the indicated times, 0.1 ml of the solution was removed and plated onto Mueller-Hinton agar. After 24 hours at 35°C, the colonies were enumerated to determine viable cell counts⁵⁾.

Cytotoxicity

The L1210 mouse leukemia cell line was cultured with RPMI 1640 medium (Nissui) containing 10% fetal bovine serum, 50 mM 2-mercaptoethanol and 0.1 mg/ml kanamycin. The cells (5×10^4 cells/ml) were incubated with different concentrations of WAP-8294A and vancomycin at 37°C for 72 hours in an atmosphere of 5% CO₂ in air. The growth of the cell line was measured at 540 nm by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The IC₅₀ values were determined by plotting the logarithms of the drug

concentrations *versus* growth rates of the treated cells⁶⁾.

Results

Taxonomy

The strain WAP-8294 was cultured at 25°C for 3 days on bouillon-agar plates, observed under a microscope and found to be a rod-like cell ($0.4 \sim 0.6 \times 3.2 \sim 4.2 \mu\text{m}$) as shown in Fig. 3. It is an aerobic Gram-negative bacterium, free of flagellum, but shows a gliding movement, and does not form any spores. Colonies are slimy and brownish-yellow. The temperature range and pH range for growth were 15 to 37°C and 5 to 8, respectively. Other physiological characteristics are shown in Table 1. Acid formation was observed from D-glucose, D-mannose, D-fructose, D-galactose, maltose, sucrose, lactose and trehalose. No gas formation was observed from the above carbohydrates. Tests for indole, methyl red and the Voges-Proscauer reaction were all negative. The mol% of guanine and cytosine in the strain WAP-8294 DNA was 68.3%. The characteristics of the strain were compared with those of bacteria registered in BERGEY's Manual of Systematic Bacteriology Volume 3.

Fig. 3. Scanning electron micrograph of *Lysobacter* sp. WAP-8294.

Table 1. Physiological characteristics of strain WAP-8294.

Properties	Results	Properties	Results
Catalase test	+	Phosphatase test	+
Oxidase test	+	Deoxyribonuclease test	+
Urease test	—	Voges-Proscauer test	—
OF-Test	non degradation	Methyl red test	—
Starch hydrolysis	—	Denitrification	—
Nitrate reduction	—	Optimum temperature (°C)	15~37
Indole production	—	Optimum pH	5~8
H ₂ S production	—	Mole % of G + C of DNA	68.3
Tween 80 esterase test	+		

Fig. 4. Isolation procedures for WAP-8294A.

This strain belongs to genus *Lysobacter*⁷⁾. According to further comparison with those of species belonging to the same genus, the strain is most closely related to *Lysobacter enzymogenes*, but it is not in agreement with the latter in specific details such as the ability of assimilating citric acid. Therefore, the strain was named *Lysobacter* sp. WAP-8294.

Fermentation and Isolation

A well-grown slant culture of strain WAP-8294 was inoculated into a 500 ml Erlenmeyer flask containing 100 ml of seed medium consisting of glucose 2.5%, defatted soybean flour 2%, soybean oil 0.4%, NaCl 0.25% and CaCO₃ 0.5%, pH 7.2 and incubated at 30°C for 1 day on a rotary shaker (180 rpm). A 2 ml portion of the seed culture was transferred into a 500 ml Erlenmeyer flask containing 100 ml of the same medium. Fermentation was done at 30°C for 3 day on a rotary shaker (180 rpm). The antibiotic production in the fermentation broth was monitored by HPLC analysis and paper disc assay.

An example of the typical isolation is illustrated in

Fig. 4. The fermentation broth (5 liters) was mixed with equal volumes of ethanol (5 liters), and the mixture was adjusted to pH 2.5 with 6N HCl. The mixture was centrifuged and the cell mass was discarded. The supernatant was passed through a column of Diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan). The column was washed with water, 80% acetone (pH 11.0) and 80% acetone, and then eluted with 80% acetone containing 0.05% trifluoroacetic acid (TFA). The eluate was concentrated and freeze-dried to give the WAP-8294A complex (3.4 g). The complex was chromatographed on a Chromatorex ODS (Fuji Silysia Chemical, Kasugai, Japan) column [30% acetonitrile containing 0.05% TFA]. The column was washed with 30% acetonitrile containing 0.05% TFA and then eluted with 40% acetonitrile containing 0.05% TFA and 45% acetonitrile containing 0.05% TFA. The fractions containing WAP-8294Ax1 ~ 13 were concentrated and separated on a preparative HPLC column of YMC pack SH-343-5 (20 × 250 nm, YMC) using a mobile phase of 37% acetonitrile containing 0.05% TFA. The eluate was concentrated and freeze-dried to give WAP-8294Ax8

(5.7 mg), WAP-8294Ax9 (3.3 mg) and WAP-8294Ax13 (1.6 mg) as trifluoroacetate. The A1 fraction and A4 fraction were concentrated and freeze-dried to give WAP-8294A1 (206 mg) and WAP-8294A4 (123 mg) as trifluoroacetate. The A2 fraction was applied to a column of SP-TOYOPEARL 650C (Tosoh, Tokyo). The column was washed with water and eluted in a stepwise manner with 0.1 M NaCl to 0.8 M NaCl. The active eluate was further purified by Chromatorex ODS (45% acetonitrile containing 0.05% TFA) to give 693 mg of pure A2 trifluoroacetate salt.

Physico-chemical Properties

The physico-chemical properties of WAP-8294A1, A2, A4, Ax8, Ax9 and Ax13 are quite similar. They are soluble in water, methanol and dimethyl sulfoxide, and insoluble in acetone, ethyl acetate and chloroform. They are positive to the ninhydrin reaction. In the IR spectra, they showed dominant absorptions at 1636 and 1541 cm^{-1} due to peptide bonds. Molecular formulas of WAP-8294A1, A2, A4, Ax8, Ax9 and Ax13 were determined by high resolution FAB mass spectrometry. The

Table 2. High resolution mass spectrometry of WAP-8294A1, A2, A4, Ax8, Ax9 and Ax13.

WAP-	Found (M+H) ⁺	Calcd. (M+H) ⁺	Molecular formula
A1	1548.8088	1548.8063	C ₇₂ H ₁₀₉ N ₁₇ O ₂₁
A2	1562.8224	1562.8219	C ₇₃ H ₁₁₁ N ₁₇ O ₂₁
A4	1576.8363	1576.8375	C ₇₄ H ₁₁₃ N ₁₇ O ₂₁
Ax8	1548.8079	1548.8063	C ₇₂ H ₁₀₉ N ₁₇ O ₂₁
Ax9	1548.8065	1548.8063	C ₇₂ H ₁₀₉ N ₁₇ O ₂₁
Ax13	1576.8324	1576.8375	C ₇₄ H ₁₁₃ N ₁₇ O ₂₁

Table 3. Antimicrobial activity of WAP-8294A1, A2, A4, Ax8, Ax9 and Ax13.

Test organism	MIC ($\mu\text{g/ml}$)					
	A1	A2	A4	Ax8	Ax9	Ax13
<i>Staphylococcus aureus</i> JCM 8702 (MRSA)	0.39	0.78	0.78	n.t.	n.t.	n.t.
+ 10% FCS	<0.1	<0.1	<0.1	n.t.	n.t.	n.t.
<i>S. aureus</i> No. 1 ^a (MRSA)	0.39	0.78	0.78	3.13	3.13	3.13
+ 10% FCS	<0.1	<0.1	<0.1	3.13	3.13	3.13
<i>S. aureus</i> No. 11 ^b (MRSA)	0.39	0.78	0.78	n.t.	n.t.	n.t.
+ 10% FCS	<0.1	<0.1	<0.1	n.t.	n.t.	n.t.
<i>S. aureus</i> ATCC 25923	0.39	0.78	0.78	3.13	3.13	3.13
+ 10% FCS	<0.1	<0.1	<0.1	1.56	1.56	1.56
<i>S. epidermidis</i> ATCC 12228	0.39	0.78	0.78	1.56	1.56	1.56
+ 10% FCS	<0.1	<0.1	<0.1	0.78	0.78	0.78
<i>Bacillus subtilis</i> ATCC 6633	0.78	0.78	0.78	1.56	3.13	3.13
+ 10% FCS	<0.1	<0.1	<0.1	0.78	1.56	1.56
<i>Enterococcus faecium</i> CIP 103510 (VRE)	n.t.	6.25	n.t.	n.t.	n.t.	n.t.
+ 10% FCS	n.t.	6.25	n.t.	n.t.	n.t.	n.t.
<i>Streptococcus pyogenes</i> ATCC 19615	6.25	6.25	6.25	>100	>100	>100
+ 10% FCS	25	25	25	>100	>100	>100
<i>Escherichia coli</i> ATCC 25922	>100	>100	>100	>100	>100	>100
+ 10% FCS	>100	>100	>100	>100	>100	>100
<i>Pseudomonas aeruginosa</i> ATCC 9027	>100	>100	>100	>100	>100	>100
+ 10% FCS	>100	>100	>100	>100	>100	>100
<i>Candida albicans</i> TIMM 0239	>100	>100	>100	>100	>100	>100
+ 10% FCS	>100	>100	>100	>100	>100	>100
<i>Aspergillus fumigatus</i> IAM 2004	>100	>100	>100	>100	>100	>100
+ 10% FCS	>100	>100	>100	>100	>100	>100

^a Clinical isolate.

FCS: Fetal calf serum, n.t.: not tested.

results are shown in Table 2. The properties of the main component WAP-8294A2 have been described in a previous communication³⁾.

Antimicrobial Activity

The antimicrobial spectra of WAP-8294A1, A2, A4, Ax8, Ax9 and Ax13 are shown in Table 3. The antibiotics were active against Gram-positive bacteria, including

MRSA but inactive against Gram-negative bacteria, some fungi and yeasts. The activity of these antibiotics was highly enhanced by the addition of 10% human serum (about 2-to 8-fold). Moreover, WAP-8294A2 was active against vancomycin-resistant Enterococci (VRE) (MIC 6.25 µg/ml).

Cytotoxicity

WAP-8294A2 showed a weak cytotoxic effect against L1210 cells (IC₅₀ 34 µg/ml).

Acute Toxicity

The acute toxicity of WAP-8294A2 was tested by oral (po), intravenous (iv) and intraperitoneal (ip) administration to mice. It was not toxic at 200 mg/kg (po), 50 mg/kg (iv) and 100 mg/kg (ip).

Mode of Action

The bactericidal action of WAP-8294A2 was compared with vancomycin. As shown in Fig. 5, WAP-8294A2 showed a clear bactericidal activity within 2 hours. On the contrary, vancomycin showed only a little bactericidal activity even after 6 hours. In addition, the activity of WAP-8294A2 was not inhibited by diacetyl-L-lysyl-D-alanyl-D-alanine which was a vancomycin antagonist, suggesting that the mode of action of WAP-8294A2 is different from that of vancomycin⁸⁾. In the disc diffusion test, the anti-MRSA activity of WAP-8294A2 was inhibited by the addition of phosphatidylglycerol or cardiolipin to the disc (Table 4). These results suggest that WAP-8294A2 interacts selectively with phospholipids in the target cell membrane resulting in its membrane damage.

Fig. 5. Bactericidal activity of WAP-8294A2 and vancomycin against MRSA JCM 8702.

Table 4. Effect of phospholipids on anti-MRSA activity of WAP-8294A2.

Phospholipid	Inhibition zone (mm)	
	WAP-8294A2 (10 µg/disc)	Vancomycin (5 µg/disc)
None	16.5	27.0
Phosphatidylethanolamine (PE)	12.5	27.0
Phosphatidylcholine (PC)	12.0	27.0
Phosphatidylinositol (PI)	9.5	27.0
Phosphatidyl-DL-glycerol (PG)	8.0	27.0
Cardiolipin (CL)	7.7	27.0

The assay organism was MRSA JCM 8702.

PE, PG, PC: 200 µg/disc, PI: 140 µg/disc, CL: 100 µg/disc.

Table 5. Comparison of WAP-8294A2 and vancomycin for treatment of systemic MRSA infection in mice.

Antibiotic	The mean ED ₅₀
Vancomycin	5.3 mg/kg
WAP-8294A2	0.38 mg/kg

In Vivo Activity

In vivo efficacies of WAP-8294A2 and vancomycin were assessed in the experimental systemic MRSA infection of mice. The mean ED₅₀ values of WAP-8294A2 and vancomycin against nine MRSA strains were 0.38 and 5.3 mg/kg, respectively (Table 5). These results indicated that WAP-8294A2 is 14 times more active than vancomycin.

Discussion

A new antibiotic complex WAP-8294A was isolated from the fermentation broth of *Lysobacter* sp. WAP-8294. Each component of the antibiotic complex is composed of 12 residues of amino acids and one residue of a 3-hydroxy fatty acid. The structure elucidation of WAP-8294A will be discussed in the subsequent paper⁴⁾. There are several antibiotics produced by bacteria: lysobactin⁹⁾ isolated from *Lysobacter* sp. and katanosins A and B^{10,11)} isolated from *Cytophaga* sp. Although these antibiotics are cyclic peptides containing a lactone linkage, WAP-8294A is clearly different from them with respect to molecular formula and constituent amino acids. Through the study of the mode of action, it was indicated that WAP-8284A2 interacts selectively with phospholipids in the target cell membrane, resulting in its membrane damage.

WAP-8294A2 showed strong activity against Gram-positive bacteria including MRSA and VRE *in vitro*. Moreover, WAP-8284A2 showed strong activity against MRSA *in vivo*. WAP-8294A2 exhibited a more potent activity against MRSA than the clinically useful anti-

MRSA antibiotic, vancomycin, and it is expected to be clinically useful for an anti-MRSA agent.

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