Micacocidin A, B and C, Novel Antimycoplasma Agents from *Pseudomonas* sp.

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Biological Activities

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A Gram-negative bacterium was found to produce a new zinc-containing antibiotics, micacocidin A and related compounds containing Cu or Fe, micacocidin B and C, respectively. These antibiotics were isolated by column chromatography on silica gel, and then separated by preparative TLC and HPLC. These new antibiotics exhibited an excellent activity against *Mycoplasma* species. The producing bacterium was characterized and ascribed to the genus *Pseudomonas*.

In the course of our screening work for new antibiotics from bacterial strains, a strain numbered No. 57-250 was found to produce antibiotics, which showed strong inhibitory activity against *Mycoplasma gallisepticum*. It was isolated by ethyl acetate extraction, and purified by silica gel chromatography, preparative TLC and HPLC into three components. They were shown to be new antibiotics and named micacocidin A, B and C. These structure will be presented in the succeeding paper[1]. In this paper, the taxonomy of the producing strain, the production and isolation of the antibiotics, as well as the physico-chemical and biological properties are presented.

Materials and Methods

Taxonomic Studies

A bacterial strain No.57-250 showing inhibitory activity against *M. gallisepticum* was isolated from a soil sample collected in Kouno, Fukui Prefecture, Japan. Taxonomic studies of the strain No. 57-250 were done according to the method proposed in BERGY's Manual of Systematic Bacteriology Vol. 1[2]. Nutrient medium (meat extract 1.0%, polypeptone 1.0%, NaCl 0.5%, agar 1.0%) was used for the identification of the bacterial strain.

Fermentation

Strain No. 57-250 was inoculated from an agar slant into a 500-ml Sakaguchi flask containing 100 ml of seed medium made up of Heart Infusion broth (Difco).

The flask was shaken on a reciprocal shaker at 28°C for 48 hours. All of the seed culture was added to a 10 liter jar fermentor containing 5 liter of production medium made up of 1.0% glucose, 0.5% yeast extract, 0.3% $CaCO_3$ and 0.01% polypropyleneglycol No. 2000. The pH of the medium was adjusted to 7.0 before sterilization. Fermentation was carried out at 28°C for 48 hours under aeration of 2.5 liter per minute and agitation of 400 rpm.

Isolation of micacocidin A, B and C

The culture broth adjusted to pH 7.0 with HCl, an equal volume of EtOAc was added, and liquid-liquid partitioning extraction was performed. The extract was concentrated to dryness using a rotary evaporator under reduced pressure.

Isolation was accomplished using silica gel (70~230 mesh, No. 7734, Merck) and Sephadex LH-20 (Pharmacia) chromatography of the concentrate, followed by preparative TLC (Kieselgel 60 F_{254}; Art. No. 5744, Merck). Preparative HPLC was carried out on a PREP-ODSm column (20 by 250 mm, Shimadzu Techno Research) and guard column (20 by 50 mm, Shimadzu Techno Research), using 75% aq. MeOH at 9 ml/minute

for elution. Detection was by UV absorption at 255 nm (SPD-6AV, Shimadzu).

Physico-chemical Studies

UV spectra taken in methanol were recorded on a Hitachi U-3200 spectrophoto-meter and IR spectra were recorded on a Nicolet 20S XB FTIR spectrometer. Optical rotation was recorded on a Perkin-Elmer 241 polarometer.

Evaluation of Biological Activity

The minimal inhibitory concentration (MIC) of micacocidin A and its related analogues (B and C) against test organisms were determined by the agar or liquid dilution method designated by the Society of Chemotherapy in Japan[3].

The liquid dilution method was used for determining MIC for *Mycoplasma*[4]. PPLO medium (Difco) containing 12% horse serum and 1% glucose was used for *M. gallisepticum*; Hanks Liquid medium (Nissui) with 0.5% lactoalbumin, 1% glucose, 10% horse serum and 1.25% yeast extract for *M. hyopneumonia*; Frey medium (Gibco) with 1% glucose, 10% swine serum and 0.01% β-NAD for *M. synoviae*; PPLO medium supplemented with 20% horse serum, 2.5% yeast extract and 1% glucose for *M. pneumoniae*. These media also contained 0.025% phenol red in order to detect the growth of the organisms according to color change. MIC values were determined after culturing at 37°C for 120~168 hours.

Mueller Hinton agar (MHA, Difco) containing 5% horse serum was used for *Streptococcus agalactiae* and MHA containing 0.05% β-NAD for *Actinobacillus pleuropneumoniae*. These bacteria were cultured at 37°C for 24~48 hours before determination of MIC. Anaerobic culture was performed using Gas-Pak method[5] with trypticase soy agar (BBL) containing 5% defibrinated sheep blood for *Serpulina hyodysenteriae* and GAM medium (Nissui) for *Clostridium perfringens*.

MICs were determined after culturing *S. hyodysenteriae* at 37°C for 96~120 hours and *C. perfringens* at 37°C for 24 hours. *Pasteurella piscicida* and *Streptococcus* sp. were cultured at 25°C for 20~24 hours using Brain Heart Infusion agar (Difco) containing 2% NaCl and sensitivity-disc N medium (Nissui), respectively. Other bacteria were cultured at 37°C for 20~24 hours using MHA.

Results and Discussion

Taxonomic Studies

Strain No.57-250 was an aerobic Gram-negative, non-sporulating rod (0.5~0.6×2.0~3.0 μm) with rounded ends. It was motile by polar multitrichous flagellation. It exhibited good growth at 28~37°C, and colonies on nutrient agar medium were moderate in growth, translucent, yellowish-white small dot, thereafter growing opaque and yellowish-white round form with or round swelling.

Soluble pigment was not diffused around colony. Poly-β-hydroxybutyrate was not accumulated as an intracellular carbon reserve. Other physiological properties are shown in Table 1. Acid formation was observed from D-glucose, D-galactose and D-xylose, but not from D-fructose, maltose, D-mannitol, lactose, sucrose, D-arabinose, D-sorbitol, D-mannose and D-trehalose. No gas formation was observed from the above carbohydrates. From comparisons of these properties with those of bacteria registered in the BERGEY's Manual

Table 1. Physiological characteristics of strain No. 57-250.

Properties observed	Results	Properties observed	Results
Catalase test	+	Lysine decarboxylase test	−
Oxidase test	+	Ornithine decarboxylase test	−
OF-test	Oxidative	β-Galactosidase test	−
Peptonization of milk	−	Urease test	+
Coagulation of milk	−	Tween 80 esterage test	+
Gelatin liquefaction	−	Voges-Proskauer test	−
Starch hydrolysis	−	Methyl red test	−
Escrine hydrolysis	−	Nitrate reduction	−
Indole production	−	Denitrification	−
H_2S production	−	Citrate utilization	+
Arginine dehydroxylase test	−	Fluorescent pigment	−

Table 2. Physico-chemical properties of micacocidin A, B and C.

	micacocidin A	micacocidin B	micacocidin C
Appearance	White crystalline	Green crystalline	Red-brown powder
MP(°C)	226~228	251~253	—
$[\alpha]_D^{24}$	+53.2 ± 0.9° (c 0.990, MeOH)	-1369.2 ± 136° (c 0.104, MeOH)	—
UV: λ_{max}^{MeOH} nm (ε)	238(19900) 264(sh ,12900) 336(5530)	223, 275, 375	218, 239
IR_{max}^{KBr} cm^{-1}	1589, 1648, 2927, 2962	1588, 2926	1586, 1664, 2927, 2961
TLC: R f *	0.47	0.34	0.65
Solubility			
Soluble:	MeOH, $CHCl_3$, DMSO	MeOH, $CHCl_3$, DMSO	MeOH, $CHCl_3$, DMSO
Slightly soluble:	EtOAc	EtOAc	EtOAc
Insoluble:	Water	Water	Water

* Silica gel $60F_{254}$, E. Merck , No.5744 , $CHCl_3$ - MeOH (9: 1)

of Systematic Bacteriology Vol. 1, strain No. 57-250 should be ascribed to the genus *Pseudomonas*.

No strain, however, was very similar to the properties of the strain No. 57-250 in the registered species of the genus. From the above, the strain No. 57-250 was designated as *Pseudomonas* sp. No. 57-250, and deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM P-14235.

Isolation and Purification

The total production of micacocidin A and its related analogues (micacocidin B and C) reached a maximum after 2 days of fermentation. The fermentation broth (8.5 liters) was adjusted to pH 7.0 with HCl, and extracted with an equal volume of EtOAc followed by liquid-liquid partitioning. The extract was fractionated by silica gel column chromatography. The active fractions were eluted stepwise with CH_2Cl_2 - MeOH (98 : 2). Micacocidin A was eluted with CH_2Cl_2 - MeOH (95 : 5) to give semipurified material (11 mg), following by purification using preparative TLC.

Further purification was conducted by Sephadex LH-20 column chromatography (15 by 870 mm) eluting with MeOH. A fraction containing 6.6 mg of micacocidin A was recrystallized with a mixture of MeOH/EtOAc to obtain 3.6 mg of colorless rhombic crystals, mp: 226~228°C (decomp.). Micacocidin A was soluble in MeOH, $CHCl_3$, DMSO and slightly soluble in EtOAc, but insoluble in water.

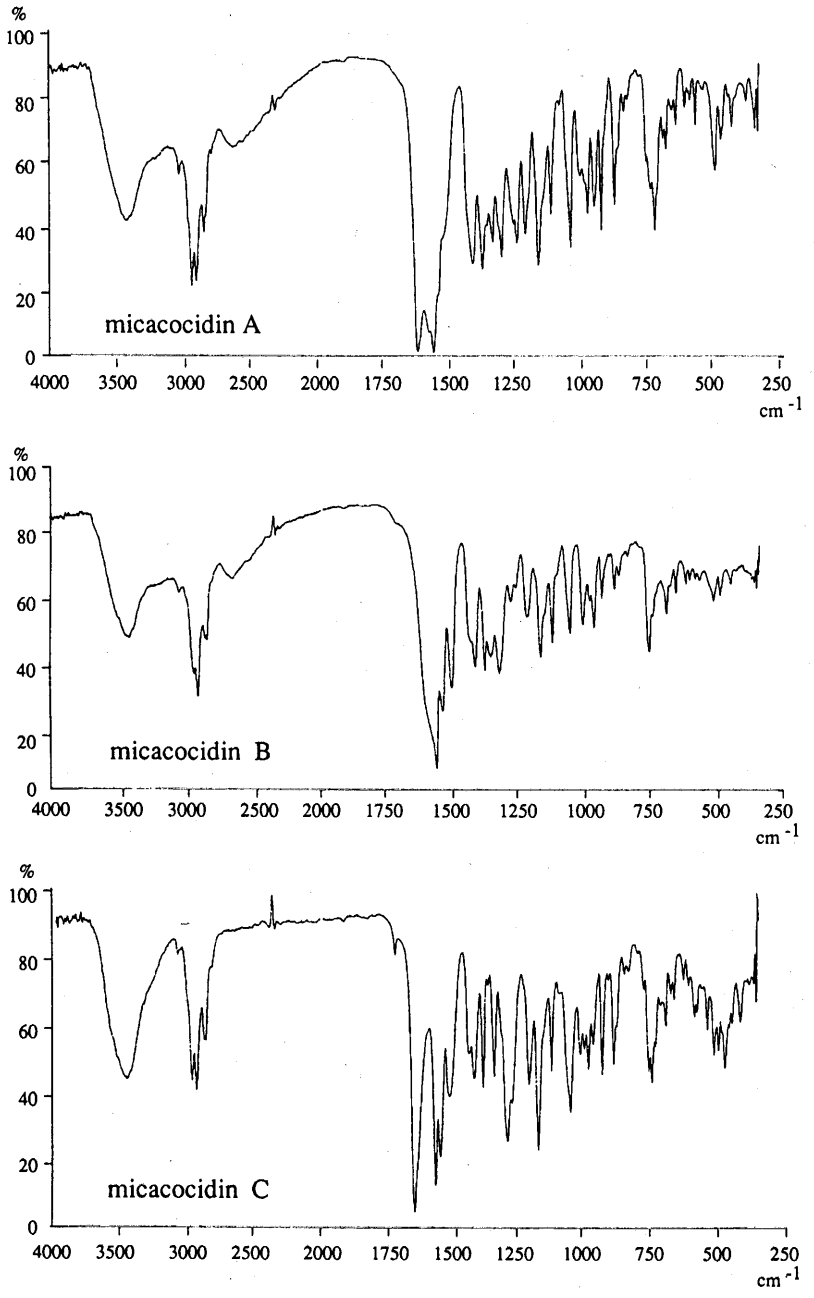
After eluting micacocidin A as described above, micacocidin B and C were eluted with CH_2Cl_2 - MeOH (98 : 2 ~ 9 : 1) from the silica gel column.

The fraction containing micacocidin B and C was further purified by preparative TLC with $CHCl_3$ - MeOH (9 : 1) developed for 15 cm. Each band containing micacocidin B and C was collected. Subsequent purification was performed by the preparative HPLC, resulting in 0.4 mg of micacocidin B and 1.3 mg of micacocidin C as amorphous solids. The physico-chemical properties of micacocidin A, B and C were given in Table 2. IR spectra of these compounds were shown in Fig. 1. The structural elucidation will be described in the following paper.

Antimicrobial Activities

For the determination of antibacterial activities, the antibiotics were dissolved in MeOH at a concentration of 1000 μg/ml, followed by a serial twofold dilutions using each medium for the test organisms. The liquid

Fig. 1. IR spectra of micacocidin A, B and C in KBr.



dilution method was used for determining MIC for *Mycoplasma*. MIC of micacocidin A, B and C against various organisms are shown in Table 3. The compounds had a strong activity against *M. gallisepticum*, *M. synoviae*, *M. hyopneumoniae* and *M. pneumoniae*, and an excellent activity against *M. pneumoniae*. These antibiotics, however, have poorer activity against the other bacteria tested.

Mycoplasmas have attracted attention as causative agents of pneumonia and upper respiratory infection, and recently the relationship between infection of Mycoplasmas and AIDS, has been reported[6~8]. Further examinations are necessary to clarify the mechanism of action micacocidins against Mycoplasmas.

Table 3. Antimicrobial spectrum of micacocidin A, B and C.

Organism	MIC(μg/ml)		
	micacocidin A	micacocidin B	micacocidin C
Mycoplasma pneumoniae Mac	≤ 0.00625	≤ 0.00625	≤ 0.00625
M. hyopneumoniae ST-11	0.025	0.05	0.1
M. gallisepticum S6	0.1	0.1	0.2
M. gallisepticum T-7T (Tylosin resistant)	0.2	0.2	0.2
M. synoviae 1853	0.78	NT	1.56
Staphylococcus aureus FDA 209P JC-1	25	>25	>25
Pasteurella multocida D-6	25	>25	>25
P. piscida No.2	>25	NT	25
Bordetella bronchiseptica H-16	25	>25	>25
Escherichia coli NIHJ JC-2	>25	>25	>25
Salmonella Enteritidis 8966(PT34)	>25	>25	>25
Klebsiella pneumoniae ATCC 27736	>25	>25	>25
Streptococcus sp. SN86119	6.25	>25	25
S. agalactiae ATCC 9925	>25	>25	>25
Actinobacillus pleuropneumoniae NB-001(1)	6.25 ~ 12.5	3.13	12.5
Clostridium perfringens ATCC 13124	6.25	6.25	12.5
Serpulina hyodysenteriae ATCC 27164	12.5	12.5	12.5

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