NEW ANALOGUES OF ROSARAMICIN ISOLATED FROM A *MICROMONOSPORA* STRAIN

I. TAXONOMY, FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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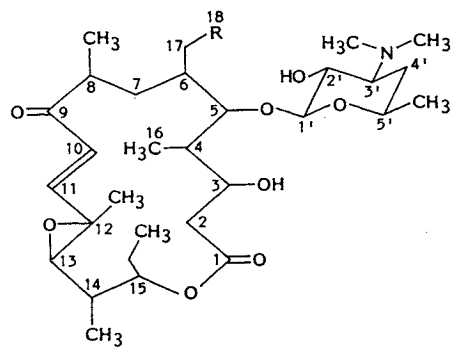
Antibiotics 6108 A_1, B, C and D, a new series of analogues of rosaramicin, were found together with rosaramicin, juvenimicin A_4 and M-4365 A_1 from the cultured broth of strain BA06108 which was assigned to be a new species of *Micromonospora*. 6108 A_1 and C showed inhibitory activity against Gram-positive and some Gram-negative bacteria as potent as rosaramicin and exhibited low acute toxicity in mice. However, 6108 B showed less potent antimicrobial activity and 6108 D showed higher toxicity than those two antibiotics.

In the course of our screening program for a new macrolide antibiotic which is active against both Gram-positive and Gram-negative bacteria, a strain BA06108 was found to produce macrolide antibiotics consisting of seven active components, designated 6108 $A_1 \sim A_4$, B, C and D. 6108 A_1, 6108 B, 6108 C and 6108 D were found to be novel analogues of rosaramicin which are differentiated at the C-18 position, while 6108 A_2, A_3 and A_4 were identical with juvenimicin A_4[1,2], rosaramicin[3,4], and M-4365 A_1[5,6],

Fig. 1. Structures of macrolide antibiotic isolated from strain BA06108.



6108 A_1	R = $-CH=N-NH-CO-CH_3$
6108 B	R = $-COOH$
6108 C	R = $-CH(OH)-S-CH_2-CH(NH_2)-COOH$
6108 D	R = $-CH=CH-CO-CH_3$
6108 A_2 (juvenimicin A_4)	R = $-CH_2OH$
6108 A_3 (rosaramicin)	R = $-CHO$
6108 A_4 (M-4365 A_1)	R = $-CH_3$

respectively (Fig. 1). This paper describes the taxonomy of the producing strain, fermentation, isolation, and physico-chemical and biological properties of components A_1, B, C and D. The structural elucidation of these compounds are described in an accompanying paper[7].

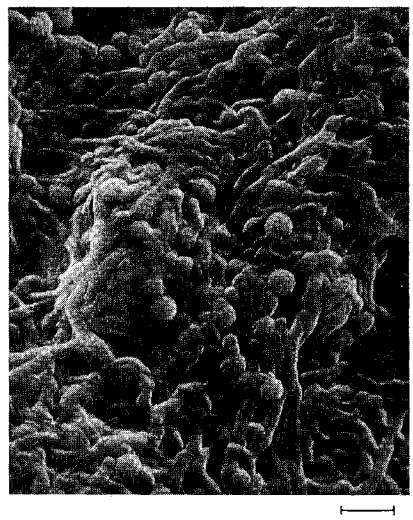
Taxonomy of Producing Organism

Strain No. BA06108 was isolated from a soil sample collected in Toyota City, Aichi Prefecture, Japan.

Vegetative mycelia were well developed and branched on agar medium, but aerial mycelium was not formed. No fragmentation of substrate mycelium was observed on agar medium. A single spore was produced on the tip of a short sporophore branching from the vegetative mycelia. This spore was $0.8 \sim 1.2$ μm i.d. and spherical to oval in shape. The spore surface showed a warty-like structure (Fig. 2). The abundant spore layer was formed on organic agar media.

Fig. 2. Scanning electron micrograph of spore-bearing substrate mycelium of strain BA06108 on ISP-2 medium.

Bar represents: 2 μm.



The cultural characteristics of strain BA06108 on various media are given in Table 1. The strain showed good or moderate growth on the organic media, but poor or very poor growth on the synthetic media. The vegetative mycelium is pale orange to pale brown on the organic media and a wine red soluble pigment was observed on some of the organic media. Physiological characteristics and utilization

Table 1. Cultural characteristics of strain BA06108.

Yeast extract-malt extract agar (ISP medium No. 2)	G:	Good, pale orange
	R:	Pale orange
	S:	Abundant, black
	SP:	Blackish red
Oatmeal agar (ISP medium No. 3)	G:	Moderate, pale orange
	R:	Pale orange
	S:	Abundant, black
	SP:	Pale purplish red
Inorganic salts-starch agar (ISP medium No. 4)	G:	Poor, pale yellowish orange
	R:	Pale yellowish orange
	S:	Very scant
	SP:	None
Glycerol-asparagine agar (ISP medium No. 5)	G:	Very poor
	R:	Colorless
	S:	Very scant
	SP:	None
Peptone-yeast extract-iron agar (ISP medium No. 6)	G:	Poor, pale orange
	R:	Pale orange
	S:	Abundant, black
	SP:	Blackish red
Tyrosine agar (ISP medium No. 7)	G:	Very poor
	R:	Colorless
	S:	Very scant
	SP:	None
Sucrose-nitrate agar	G:	Very poor
	R:	Colorless
	S:	Very scant
	SP:	None
Nutrient agar	G:	Moderate, pale orange
	R:	Pale orange
	S:	Moderate, black
	SP:	None
Glucose-asparagine agar	G:	Very poor
	R:	Colorless
	S:	Very scant
	SP:	None
Potato-glucose agar	G:	Moderate, pale brown
	R:	Pale brown
	S:	Abundant, black
	SP:	Pale purplish red

Abbreviations: G, Growth of vegetative mycelium; R, reverse; S, spore layer; Sp, soluble pigment.

of carbon sources of strain BA06108 are given in Tables 2 and 3, respectively. Only glucose was utilized well for growth, and the other carbon sources were not utilized.

Chemical analysis of cell wall revealed the presence of 3-hydroxy-*meso*-diaminopimelic acid and glycine, indicating cell-wall type II. Whole-cell sugar analysis revealed the presence of arabinose and xylose, indicating sugar type D. The GC content was 72.4%. The strain had phospholipids of type PII and MK-10 (H_4) as the major menaquinone.

The morphology and cell-wall analysis described above indicate that strain BA06108 belongs to the genus *Micromonospora* Orskov 1923. According to LEUDEMANN's classification described in BERGEY's Manual[8], the aerobic *Micromonospora* can be divided into major groups on the basis of carbon utilization, and strain BA06108 resembles *Micromonospora purpurea*[9], *Micromonospora olivasterospora*[10] and *Micromonospora griseorubida*[11], in non utilization of α-melibiose, L-rhamnose, raffinose and D-mannitol. However, strain BA06108 was clearly differentiated from *M. purpurea* and *M. olivasterospora* in sporulation patterns, colors of vegetative growth, soluble pigment and utilization of D-xylose, L-arabinose, D-fructose and sucrose, and also differentiated from *M. griseorubida* in colors of vegetative growth and utilization of L-arabinose, D-fructose and sucrose. Furthermore, strain BA06108 was distinguished from rosaramicin-producing *Micromonospora rosaria*[12], *Micromonospora capillata*[5] and *Micromonospora chalcea* subsp. *izumensis*[3], in carbohydrate utilization pattern as shown in Table 3. Strain BA06108 is characterized in the utilization of only glucose as carbon source. *M. rosaria* contains galactose in hydrolyzed whole cells, while strain BA06108 did not contain galactose in the cells. *M. chalcea* subsp. *izumensis* forms smooth spore, while strain BA06108 forms warty spore. *M. capillata* as well as two species described above does not produce wine red soluble pigment, while strain BA06108 produces the pigment.

Table 2. Physiological properties of strain BA06108.

Coagulation of milk	−
Peptonization of milk	−
Liquefaction of gelatin	−
Melanin formation	−
Hydrolysis of starch	+
Hydrolysis of cellulose	+
NaCl tolerance	4.0%
Temperature range	20~40°C

+: Positive, −: negative.

Table 3. Comparison of carbohydrate utilization pattern of strain BA06108 with *Micromonospora rosaria*, *M. capillata* and *M. chalcea* subsp. *izumensis*.

Carbohydrate	Strain BA06108	*M. rosaria*	*M. capillata*	*M. chalcea* subsp. *izumensis*
D-Xylose	−	+	+	+
L-Arabinose	−	+	+	−
D-Galactose	−	±	±	+
D-Glucose	+	+	+	+
D-Fructose	−	+	±	±
L-Rhamnose	−	+	+	−
D-Melibiose	−	±	±	+
Sucrose	−	+	+	+
Raffinose	−	−	−	+
Glycerol	−	−	−	−
i-Inositol	−	NT	NT	NT
D-Mannitol	−	+	−	−
Salicin	−	NT	NT	NT

The data were obtained from comparative experiments with type strains (*i.e. M. rosaria* NRRL 3718, *M. capillata* FERM-P2598, *M. chalcea* subsp. *izumensis* IFO 12988).
NT: Not tested.

Considering the results, we can recognize strain BA06108 as a new species of *Micromonospora*. We propose to designate it *Micromonospora fastidiosa* sp. nov. due to the fastidiousness on its carbon utilization. The strain BA06108 has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with the accession No. of FERM P-9897.

Table 4. Production media of 6108.

BE-2	%	ST-7	%
Glycerol	2.0	Glucose	0.1
Maltose syrup	1.0	Soluble starch	1.0
Yeast extract	0.1	Dried yeast	0.4
Pharma media	0.5	Yeast extract	0.1
Wheat germ meal	0.5	Skim milk	2.0
Meat extract	0.2	K_2HPO_4	0.05
$(NH_4)_2SO_4$	0.1	NaCl	0.1
KH_2PO_4	0.1	$MgSO_4 \cdot 7H_2O$	0.05
NaCl	0.2	$CaCl_2 \cdot 2H_2O$	0.05
$MgSO_4 \cdot 7H_2O$	0.2	MTE solution[21]	1.0
$CaCO_3$	0.2		
$ZnSO_4 \cdot 7H_2O$	0.001		
$FeSO_4 \cdot 7H_2O$	0.001		

Fermentation

The production ratio of new analogues during fermentation differed markedly depending upon the medium. For example, cultivation in BE-2 medium listed in Table 4 produced 6108 A_1 and B, while in the ST-7 medium 6108 B, C and D were produced. Production of 6108 complex was carried out in a 200-liter fermentation tank. The titer of each component in the fermentation broths was determined by HPLC analysis.

Isolation

Components A_1, B, A_2, A_3 and A_4 were isolated from the fermentation broth (110 liters) cultured in BE-2 medium. The cultured broth was mixed with filter aid and adjusted to pH 9.0 and filtered. The broth filtrate was adsorbed on Diaion HP-20 resin and after the resin was washed first with water, then with 30% aqueous methanol, the active components were eluted with 80% aqueous acetone. The eluate was concentrated *in vacuo*, adjusted to pH 9.5, and then extracted with ethyl acetate. The ethyl acetate extract containing components of 6108 A group was concentrated *in vacuo* to dryness. The residue was subjected to silica gel column chromatography and was separated into four components, A_1, A_2, A_3 and A_4. Each fraction was concentrated *in vacuo* to dryness and was purified by preparative reverse phase HPLC. Component B contained in the water layer of ethyl acetate extraction was extracted with butanol and then purified by silica gel column chromatography, Toyopearl HW-40 column chromatography and preparative reverse phase HPLC. Thirty six mg of component A_1, 141 mg of B, 274 mg of A_2, 155 mg of A_3 and 44 mg of A_4 were obtained, respectively.

Components C and D were isolated from the fermentation broth (110 liters) cultured in ST-7 medium. 6108 A group and D were extracted with ethyl acetate, and after back-extraction with hydrochloric acid (pH 3), active substances were reextracted with ethyl acetate at pH 9.5. Component D was purified by silica gel column chromatography and preparative reverse phase HPLC, to yield 51 mg of component D. Components B and C from aqueous layer of ethyl acetate extraction were extracted with butanol and subjected to silica gel column chromatography. The isolation of component C from B was performed by preparative reverse phase HPLC and provided 81 mg of C.

Components A_2, A_3 and A_4 were identical with juvenimicin A_4[1,2], rosaramicin[3,4] and M-4365 A_1[5,6], respectively, by direct comparison with authentic samples by HPLC and TLC.

Physico-chemical Properties

Each component was obtained as white amorphous powder. Physico-chemical properties of components A_1, B, C and D are summarized in Table 5 compared with those of rosaramicin. It is noteworthy

Table 5. Physico-chemical properties of 6108 A_1, 6108 B, 6108 C, 6108 D and rosaramicin.

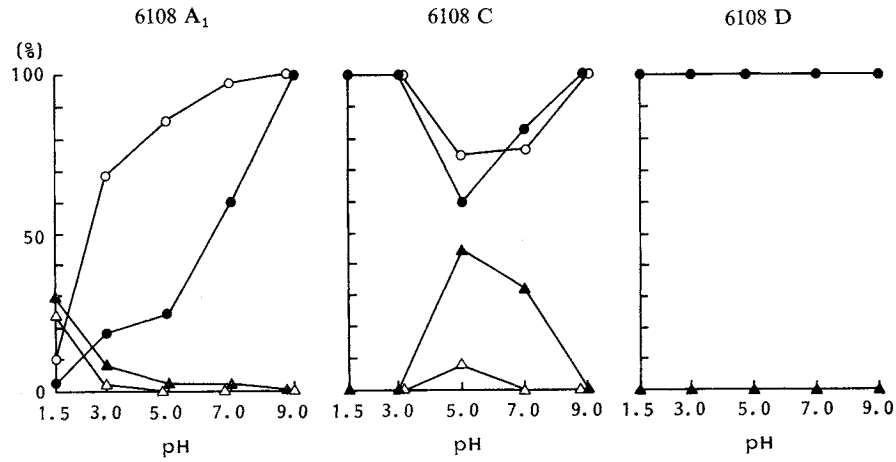
	6108 A_1	6108 B	6108 C	6108 D	Rosaramicin
Appearance	Colorless powder	Colorless powder	Colorless powder	Colorless powder	Colorless powder
Formula	$C_{33}H_{55}O_9N_3$	$C_{31}H_{51}O_{10}N$	$C_{34}H_{58}O_{11}N_2S$	$C_{34}H_{55}O_9N$	$C_{31}H_{51}O_9N$
MW (MS)	637	597	702	621	581
MP (°C)	108~112 (dec)	138~141 (dec)	168~169 (dec)	95~98 (dec)	119~122 (dec)
$[\alpha]_D^{20}$ (MeOH)	−39.0° (*c* 1.0)	−11.9° (*c* 1.0)	−97.4° (*c* 0.2)	−37.5° (*c* 1.0)	−35° (*c* 0.37)
UV λ_{max}^{MeOH} nm (ε)	234 (21,500)	240 (9,980)	239 (14,600)	232 (21,550)	240
TLC (Rf)[a]					
Solvent system A:	0.25	0.00	0.00	0.78	0.74
Solvent system B:	0.30	0.08	0.11	0.38	0.41
Solvent system C:	0.28	0.34	0.18	0.60	0.37
HPLC (Rt, minutes)[b]	39.2	22.9	24.1	46.4	44.0
Solubility					
Soluble:	Benzene, EtOAc, $CHCl_3$, EtOH, MeOH	BuOH, EtOH, MeOH, H_2O	BuOH, EtOH, MeOH, H_2O	Benzene, EtOAc, $CHCl_3$, EtOH, MeOH	Benzene, EtOAc, $CHCl_3$, EtOH, MeOH
Slightly soluble:		EtOAc, $CHCl_3$	EtOAc, $CHCl_3$		
Insoluble:	*n*-Hexane, H_2O	*n*-Hexane, benzene	*n*-Hexane, benzene	*n*-Hexane, H_2O	*n*-Hexane, H_2O

[a] Pre-coated Silica gel $60F_{254}$ plates (Merck). Solvent system A: $CHCl_3$ - MeOH - 5% NH_4OH (40 : 12 : 10) lower phase, solvent system B: $CHCl_3$ - MeOH (1 : 1), solvent system C: CH_3CN - AcOH - H_2O (4 : 1 : 1).

[b] See Experimental, HPLC analysis of 6108 components in a fermentation broth.

Fig. 3. Stabilities of 6108 A_1, C and D in various pH solution.

●, ○: Remained 6108 A_1, C or D, ▲, △: produced rosaramicin, open symbol: under coexistence of acetohydrazide or cysteine.



that components B and C are soluble in water, different from usual macrolides including rosaramicin.

Stabilities of components A_1, C and D were examined in various pH solutions at 37°C (Fig. 3). Compound A_1 was labile at pH's below 7.0, especially at pH 1.5, and was decomposed gradually into rosaramicin and acetohydrazide. Compound C was also decomposed at pH 5.0 producing some amount of rosaramicin and cysteine. Coexistence of ten equimolar acetohydrazide or cysteine improved the stability of compound A_1 or C, respectively.

Compound D was stable under conditions tested.

Biological Properties

The antimicrobial activity (MIC) of components A_1, B, C and D compared with rosaramicin and erythromycin A is shown in Table 6. Compounds A_1 and C were equally active to rosaramicin against Gram-positive bacteria and Gram-negative bacteria and showed higher activity than erythromycin A against Gram-negative bacteria and *Campylobacter coli*. Compound D was two to four times less active than rosaramicin. Compound B showed weak antimicrobial activity.

The acute toxicity of components A_1, C and D as well as rosaramicin and erythromycin A was tested by intraperitoneal and oral administration to mice (Table 7). Compounds A_1 and C were less toxic than rosaramicin and erythromycin A. On the other hand, the toxicity of D was higher than those of rosaramicin and erythromycin A.

Results of mouse protective effect of components A_1 and C against *Staphylococcus aureus* 4970 infection comparing rosaramicin, josamycin and erythromycin A are shown in Table 8. Compound A_1 was as active as rosaramicin, josamycin and erythromycin A. However, compound C was significantly less active than rosaramicin, josamycin and erythromycin A.

Discussion

S. ŌMURA suggested that the aldehyde at C-17 is essential for showing antimicrobial activity in 16-membered macrolide antibiotics[13]. However, the components A_1 and C possessing acetylhydrazide and cysteine moieties, respectively, as well as carbo-aldehyde moiety (rosaramicin) at C-18 position showed

Table 6. Antimicrobial spectra of 6108 A_1, 6108 B, 6108 C, 6108 D, rosaramicin (ROM) and erythromycin A (EM-A).

Test organism	MIC (μg/ml)					
	6108 A_1	6108 B	6108 C	6108 D	ROM	EM-A
Staphylococcus aureus FAD 209P	0.1	25	0.1	0.39	<0.1	<0.1
S. aureus Smith	0.39	50	0.2	0.39	0.2	0.2
S. aureus MS12739(C)Mac-R	>100	>100	>100	>100	>100	>100
S. epidermidis IAM 12012	0.2	50	0.39	0.78	0.2	0.39
Streptococcus pyogenes JCM 5674	0.2	1.56	0.1	0.39	0.1	0.1
Enterococcus faecalis	0.39	100	0.39	0.78	0.2	0.1
Bacillus subtilis ATCC 6633	<0.1	50	<0.1	0.39	<0.1	0.1
Micrococcus luteus ATCC 9341	<0.1	12.5	<0.1	<0.2	<0.1	<0.1
Corynebacterium diphtheriae JCM 1310	0.2	1.56	0.2	0.39	0.1	0.1
Escherichia coli K-12 C600 Mac-S Rms213	0.39	100	0.2	3.13	0.2	12.5
E. coli K-12 C600	3.13	>100	1.56	12.5	1.56	12.5
E. coli NIHJ JC-2	12.5	>100	12.5	50	6.25	50
Klebsiella pneumoniae 42	12.5	>100	25	50	12.5	25
K. pneumoniae IFO 3319	50	>100	25	>100	25	100
Proteus vulgaris OX 19	50	>100	25	>100	25	100
P. mirabilis IFO 3849	100	>100	50	>100	50	>100
Morganella morganii IFO 3848	12.5	>100	12.5	100	12.5	50
Serratia marcescens IAM 1223	12.5	>100	12.5	100	6.25	50
Pseudomonas aeruginosa IFO 3445	50	>100	50	>100	50	100
Salmonella paratyphi NIHJ	25	>100	25	100	12.5	50
S. typhimurium 901	12.5	>100	12.5	50	12.5	25
Shigella flexneri IMC B-0862	6.25	>100	3.13	25	3.13	12.5
S. dysenteriae IMC B-0860	0.39	12.5	0.39	0.78	0.2	1.56
Citrobacter freundii GN346	25	>100	25	50	12.5	50
Enterobacter cloacae NeK39	100	>100	50	>100	50	>100
Neisseria gonorrhoeae JCM 2884	0.2	100	0.39	0.78	0.2	0.1
N. meningitidis JCM 2887	0.39	100	0.39	0.78	0.2	0.1
Campylobacter coli NCTC 11366	0.39	>100	0.39	NT	0.39	3.13
C. jejuni ATCC 29428	<0.1	100	<0.1	NT	<0.1	0.1
C. fetus subsp. *fetus* ATCC 27374	0.39	100	0.39	NT	0.39	0.78
C. pylori NCTC 11637	<0.1	100	<0.1	NT	<0.1	0.2

NT: Not tested.

Table 7. Acute toxicity of 6108 A_1, 6108 C, 6108 D, rosaramicin and erythromycin A in mice.

Antibiotic	LD_{50} (mg/kg)	
	ip	po
6108 A_1	350	>4,000
6108 C	490	>5,000
6108 D	NT	800
Rosaramicin	280	2,500
Erythromycin A	280	4,000

NT: Not tested.

Table 8. Protective effect of 6108 A_1 and 6108 C against *Staphylococcus aureus* 4970 in mice.

Antibioic	ED_{50} (mg/kg)	MIC (μg/ml)
6108 A_1	104	0.2
6108 C	325	0.39
Rosaramicin	66.5	0.2
Josamycin	123	0.78

the strongest antimicrobial activity among 5 compounds tested. Compound B having a carboxy moiety showed weak activity. Both juvenimicin A_4 having a hydroxymethyl moiety and M-4365 A_1 carrying methyl moiety displayed intermediate activity.

It is likely that components of A_1 and C show antimicrobial activity by conversion to rosaramicin, because they easily dissociate to rosaramicin depending on pH. However, the acute toxicity and the mouse

protection effect of components A_1 and C showed different values from those of rosaramicin. These differences may depend on their conversion rate to rosaramicin in mice.

Experimental

General Method

UV and IR spectra were recorded on a Shimadzu UV-250 spectrophotometer and a Jasco IR-810 IR spectrophotometer, respectively. MP's were measured with a Shibata Model 5227-01. Optical rotations were measured with a Jasco DIP-360 polarimeter. MS spectra were taken on a Nihon Denshi JMS-DX 300/JMA-DA 500 mass spectrometer. NMR spectra were recorded on a Varian VXR 300 NMR spectrometer for ^1H NMR at 300 MHz and ^{13}C NMR at 75 MHz.

Taxonomic Studies of the Producing Organism

Most of the procedures used in the study were in accordance with the methods adopted by the International Streptomyces Project (ISP)[14]. Additional media recommended by WAKSMAN[15] were also used. All the various media were inoculated with a washed spore suspension grown for 7 days at 27°C in a yeast extract-malt extract agar medium. Color names were based on the Color Standard of Nihon Shikisai, Co., Ltd.†. Utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB medium containing 1.0% each carbon source. Spore and mycelial morphologies of the cultures were observed by both light microscopy and scanning electron microscopy. Whole cells were analyzed by the procedures of LECHEVALIER and LECHEVALIER[16], and KAWAMOTO *et al.*[17]. GC content, phospholipids and menaquinones were analyzed by the methods of KOMAGATA *et al.*[18], LECHEVALIER *et al.*[19] and COLLINS *et al.*, respectively.

Fermentation

A loopful of spore suspension of *M. fastidiosus* sp. nov. from agar slant culture was inoculated into 1 liter of a seed culture medium BE-2 in a 3-liter Erlenmeyer flask. The inoculated flask was shaken on a rotary shaker at 27°C for 72 hours. The seed culture was transferred to a 200-liter fermenter containing 130 liters of medium BE-2 or ST-7, and was incubated at 27°C under aeration (130 liters/minute) with agitation (240 rpm) for 70~94 hours.

HPLC Analysis of 6108 Components in a Fermentation Broth

A cultured broth (100 ml) was extracted with BuOH (100 ml) and the extract was concentrated under reduced pressure to dryness. The residue was dissolved in MeOH (1.0 ml). 0.2 ml of MeOH solution was loaded onto a HPLC column.

HPLC conditions: Column, Develosil ODS-5 10/250; mobile phase, (A) 0.01 M borate buffer (pH 7.0); (B) 80% CH_3CN, step-wise linear gradient elution,

minutes	0	10	20	40	45	65	70	85
(B) conc	15	25	40	80	100	100	15	(next injection)

flow rate, 4.0 ml/minute, temperature, 40°C; detection, UV 200~400 nm. Apparatus: HPLC system, TRI ROTAR-SR2 (Jasco); detector, MCPD-350 (Otsuka Electronics Co., Ltd.).

Isolation and Purification of Compounds A_1 and B

The fermentation broth (110 liters) cultured in BE-2 medium was treated with Hyflo Super-Cel (Johns-Manville Sales Co.) (3 kg), adjusted to pH 9.5 with 10% NaOH and then filtered. Diaion HP-20 (Mitsubishi Chemical Industries Ltd.) (4 liters) was added to the broth filtrate (100-liter) which was stirred for 2 hours, collected by filtration and packed into the column. The column was washed with water (18 liters) and 25% aqueous MeOH (8 liters). The active components were eluted with 80% aqueous MeOH. The eluate was concentrated *in vacuo* and the aqueous residue was adjusted to pH 9.5 with 5% NaOH and extracted twice with equal volume of EtOAc. The extract was evaporated *in vacuo* to give an oily residue. The residue was dissolved in a small amount of $CHCl_3$ and applied to a silica gel (C-300, Wako Pure Chemical Industries, Ltd.) column (4.0 cm i.d. × 75 cm) packed with $CHCl_3$. The column was developed with linear gradient system composed of $CHCl_3$ (4.0 liters) and $CHCl_3$-MeOH, 3:2 (4.0 liters). The

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components of 6108 A group were successively eluted in the order of 6108 A_4, A_3, A_2 and A_1. Each fraction was concentrated to give the crude powder. On the other hand, component B contained in the aqueous layer of the EtOAc extraction was extracted with an equal volume of BuOH and the extract was concentrated to give an oily residue. The oily residue was subjected to silica gel chromatography (Wako C-300, column: 3 cm i.d. × 30 cm). The column was developed with linear gradient system composed of $CHCl_3$-MeOH, 5:1 (1.0 liter) and $CHCl_3$-MeOH, 3:2 (1.0 liter). The active eluate was concentrated to give crude powder which was loaded on a column (3 cm i.d. × 30 cm) packed with Toyopearl HW-40 (Tosoh Co., Ltd.) and developed with MeOH. The active fraction was concentrated *in vacuo* to give crude B. The purification of each crude powder was achieved by preparative HPLC using a packed column (Develosil ODS-10, 20/250, Nomura Chemical Co., Ltd.) and TRI-ROTAR-V system (Jasco) with 0.01 M borate buffer (pH 7.0) as solvent A and 80% CH_3CN as solvent B at a flow rate of 16 ml/minute. The solvent system A-B (55:45), (45:55) and (60:40) were applied for the separation of 6108 A_1 and A_2, A_3 and A_4, and B, respectively. The UV absorbance of the eluate was monitored at 240 nm using a spectrophotometer (UVIDEC-100-V, Jasco) equipped with a preparative cell (Jasco).

Isolation and Purification of Compounds C and D

From the fermentation broth cultured with ST-7 medium, the EtOAc extract containing components A_2, A_3, A_4 and D was obtained by the same procedures described for the extraction of compound A_1. The EtOAc extract (26 liters) was back extracted with dil HCl (13 liters, pH 3.0) and the aqueous layer adjusted to pH 9.5 was reextracted with EtOAc (13 liters). The washed extract was concentrated to give an oily residue. Component D was purified from the oily residue by the same procedure described for compound A_3. Component C in the aqueous layer of the initial EtOAc extraction was purified by the same procedures as those of compound B except Toyopearl HW-40 chromatography.

Measurement of MIC

MIC of the antibiotics was determined by the serial agar dilution method on Mueller-Hinton agar (MH) for bacteria except *Campylobacter* at 37°C for 18 hours, and on 5% horse blood-supplemented MH for *Campylobacter* sp. at 37°C for 48 hours.

Acute Toxicity in Mice

LD_{50} values of antibiotics were determined by intraperitoneal or oral administration in female 5 weeks-old ICR mice purchased from Charles River Japan (Kanagawa). Each antibiotic was dissolved in DMSO and diluted to 5% of DMSO as a final concentration with 0.6% Tween-80 in saline. In the case of oral administration, antibiotics were suspended in 0.5% carboxymethyl cellulose in saline. Three mice per group were administrated intraperitoneally or orally at a volume 100 μl/10 g body weight and observed for a week. LD_{50} values were determined by LITCHFIELD and WILCOXON method.

Protective Effect in Mice

In vivo antimicrobial activity was studied against experimental infection in 4-weeks-old *dd*Y male mice (weight, 20±1 g). Ten mice in groups were intraperitoneally infected with 1,000 times LD_{50} (3.1×10^6 cfu/mouse) of *S. aureus* 4970 suspended in 0.5 ml of sterile saline containing 5% hog gastric mucin (Wako Pure Chemical, Osaka). One hour after challenge, mice were orally given a single administration of serial 2-fold doses of the test antibiotics which were suspended in 0.4% carboxymethyl cellulose containing 50 mM phosphate buffer (pH 7.7). The ED_{50} (mg/kg) was determined by the probit method from survival rate on day 5 after infection.

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