Epoxyquinomicins A, B, C and D, New Antibiotics from *Amycolatopsis*

I. Taxonomy, Fermentation, Isolation and Antimicrobial Activities

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A new structural class of the antibiotic, epoxyquinomicins A, B, C and D were isolated from the culture broth of the strain MK299-95F4, which was related to *Amycolatopsis sulphurea*. Antimicrobial activity of epoxyquinomicins A and B were weak against Gram-positive bacteria, and epoxyquinomicins C and D showed almost no antimicrobial activity and no cytotoxicity. All these antibiotics showed improvement of collagen induced arthritis *in vivo*.

In the previous paper[1], we described briefly the production, isolation, physico-chemical properties, structure determination and antimicrobial activities of epoxyquinomicins A (**1**) and B (**2**) from the culture broth of *Amycolatopsis* sp. MK299-95F4, which was isolated from soil sample collected at Sendai City, Miyagi prefecture, Japan. We found the other metabolites epoxyquinomicins C (**3**) and D (**4**) from the same culture broth. Compounds **3** and **4** possess a hydroxyl group at C-1 position instead of keto group in **2** and **1**, respectively (Fig. 1).

Compounds **1** and **2** showed weak antimicrobial activity against Gram-positive bacteria. While **3** and **4** showed almost no antimicrobial activity and no cytotoxicity. Epoxyquinomicins had a potent inhibitory effect on type II collagen-induced arthritis which is different from those of NSAIDs. Furthermore, no side effect have been found with **3** and **4**.

In this paper, we describe the taxonomy of the producing strain, fermentation, isolation and antimicrobial activities of **1**, **2**, **3** and **4** in detail.

Materials and Methods

Microorganisms

Amycolatopsis sulphurea IMC A-0170 (IFO 13270^T) and *A. orientalis* IMC A-0161 (ISP 5040^T) were used as reference strains.

Morphological Characteristics

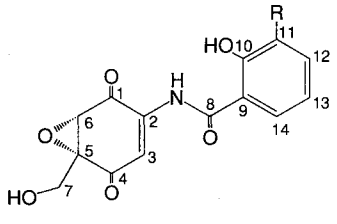
Morphological observations were made with a light microscope on cultures grown on yeast extract-malt extract agar (ISP med. 2)[3] and sucrose-nitrate agar[4] at 27°C for 1 to 4 weeks.

Spore morphology was studied with a scanning electron microscope (model Hitachi S-570).

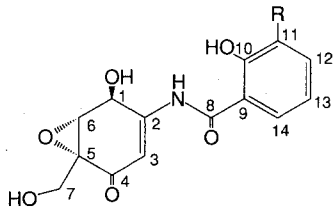
Cultural and Physiological Characteristics

The media and procedures used for cultural and physiological characteristics of strain MK299-95F4 were those described by SHIRLING and GOTTLIEB[3] and by WAKSMAN[4]. Cultures were incubated at 27°C for 1 to 4 weeks. Color determination was made by comparing the

Fig. 1. Structures of epoxyquinomicins A, B, C and D.



Epoxyquinomicin A (**1**): R = Cl
Epoxyquinomicin B (**2**): R = H



Epoxyquinomicin C (**3**): R = H
Epoxyquinomicin D (**4**): R = Cl

cultures with color chips from the Color Harmony Manual (Container Corporation of America). The temperature range for growth was determined on glucose-asparagine agar[4]. Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB[5].

Cell Chemistry

The strain MK299-95F4 was grown in YD medium (yeast extract 1.0 %, glucose 1.0 %, pH7.2) at 27°C for 3 days on a rotary shaker. The mycelia were centrifuged and were washed with distilled water. The washed and packed mycelia were then freeze-dried as a whole-cell preparation. Diaminopimelic acid isomers in the whole-cell hydrolysates were analyzed according to the methods of BECKER *et al.*[6], which were modified by STANECK and ROBERTS[7] for separation on thin layer plates.

Cell walls were prepared as described by KAWAMOTO *et al.*[8] except for treating them with pronase AS (Kaken Chemicals Co.). Cell wall sugars and whole-cell sugars were determined by the methods of LECHEVALIER and LECHEVALIER[9], and MIKAMI and ISHIDA[10]. The glycolate test was examined by the procedures of UCHIDA[11]. Phospholipids and mycolic acids were analyzed by the procedures of MINNIKIN *et al.*[12] and MINNIKIN *et al.*[13], respectively. Menaquinones were extracted and purified by the method of COLLINS *et al.*[14] and were analyzed by HPLC and mass spectrometry as described by TAMAOKA *et al.*[15], using a CAPCELL PAK C_{18} AG120 column (4.6 by 250 mm, Shiseido) with a solvent system of methanol - isopropyl alcohol (2 : 1, v/v) as mobile phase at a flow rate of 1 ml/minute. The fatty acid methyl esters were obtained by means of whole-cell methanolysate[16] and were determined with a SHIMADZU model GC-17A gas chromatograph, a flame ionization detector, and SHIMADZU CHROMATOPAC model C-R6A. A Megabore DB-1 column was used (15 m by 0.53 mm, film 1.5 μm, J & W Scientific).

Preparation and Hybridization Analysis of DNA

After incubation in YD medium supplemented with 0.5% glycine at 27°C for 3 to 5 days on a rotary shaker, mycelia were centrifuged and washed with a buffer (25 mM Tris-HCl - 25 mM EDTA-25 mM NaCl, pH 7.4). Total DNAs were extracted from the washed mycelia by the method described by HAMADA *et al.*[17] and dissolved in 1 × TE (10 mM Tris-HCl - 1 mM EDTA, pH 7.6).

DNA homologies between strains were determined fluorometrically by the method of EZAKI *et al.*[18]. Fluorescence intensity in the wells was measured with a microplate reader MTP-32 (Corona Electric).

Measurement of Antimicrobial Activity

The minimum inhibitory concentrations (MIC) of epoxyquinomicins were examined by serial agar dilution method using Mueller-Hinton agar (Difco) for antibacterial test which was incubated at 37°C for 18 hours and a nutrient agar containing 1 % glycerol for antimycobacterial test which was incubated at 37°C for 42 hours.

Anititumor Activity

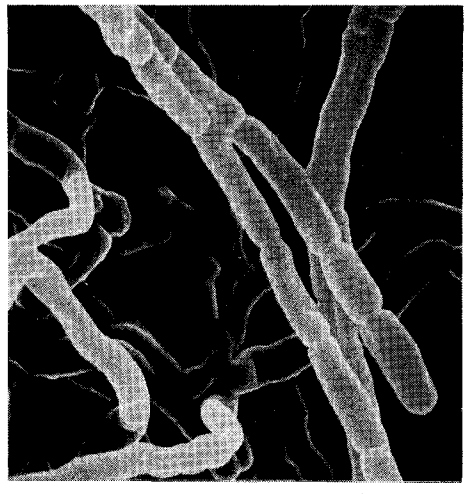
Tumor cells were incubated in 96-well plate for 24 hours prior to the addition of epoxyquinomicins into culture well at varied concentrations. After 2 to 3 days incubation at 37°C, MTT reagent was added and further incubated for 4 hours. Growth inhibition activity was determined according to the standard MTT assay method[19] and IC_{50} was calculated.

Results and Discussion

Taxonomic Studies

Strain MK299-95F4 had a branched vegetative hyphae which was a slight tendency to zig-zag-shaped, and which tended to break down into squarish subunits. The aerial hyphae produced cylindrical conidia in straight to flexuous chains. The spores were 0.4 to 0.6 by 1.1 to 1.6 μm in size with smooth surface. No sporangia, motile spores or synnemata were observed (Fig. 2). The cultural characteristics of strain MK299-95F4 were summarized

Fig. 2. Scanning electron micrograph of spore chains of *Amycolatopsis* sp. MK299-95F4.



Bar represents 1.76 μm

Table 1. Cultural characteristics of strain MK299-95F4.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Colorless	Thin, white	None
Yeast extract-malt extract agar (ISP No. 2)	Pale yellowish brown (3ic, Lt Amber)	Thin, white	None
Oatmeal agar (ISP No. 3)	Colorless~Pale yellow (1 1/2 ca, Cream)	Thin, white	None
Inorganic salts-starch agar (ISP No. 4)	Colorless	Thin, white	None
Glycerol-asparagine agar (ISP No. 5)	Pale yellowish brown (3ie, Camel~3le, Cinnamon)	White	Yellowish brown
Tyrosine agar (ISP No. 7)	Pale yellowish brown (2lg, Mustard Tan)~Grayish yellow brown (3lg, Adobe Brown)	White	Pale yellowish brown
Glucose-asparagine agar	Pale yellow (2ea, Lt Wheat~2gc, Bamboo)	White	Yellowish
Nutrient agar	Pale yellow (2ea, Lt Wheat)	Thin, white	None
Starch agar	Colorless	Thin, white	None
Calcium malate agar	Colorless	Thin, white	None

in Table 1. The physiological characteristics and carbohydrate utilization of strain MK299-95F4 were shown in Table 2.

The whole-cell hydrolysate contained *meso*-2,6-diaminopimelic acid, arabinose and galactose. The cell wall hydrolysate contained arabinose and galactose. These data indicated that strain MK299-95F4 has a type IV cell wall and a type A whole-cell sugar pattern.

From the results of glycolate test, it was acetyl type. A type PII phospholipid pattern (phosphatidylethanolamine present, phosphatidylcholine and glucosamine-containing phospholipids absent) was found. Mycolic acids were absent. The predominant menaquinone was MK-9 (H_4). The strain contained major amounts of hexadecanoic acid (16:0), 13-methyltetradecanoic acid (*iso*-15:0), 14-methylpentadecanoic acid (*iso*-16:0), *cis*-hexadecenoic acid (16:1), heptadecanoic acid (17:0) and other minor components.

Based on these characteristics, strain MK299-95F4 was placed in the genus *Amycolatopsis*[20~23]. Among the genus *Amycolatopsis*, *A. sulphurea*[23] was similar to strain MK299-95F4.

As shown in Table 3, strain MK299-95F4 resembled *A. sulphurea* with reference to the fatty acids composition, the color of growth and the utilization of inositol. DNA hybridization tests were used to identifying the strain MK299-95F4 by comparison with *A. sulphurea* and *A. orientalis*. The latter organism is the type species of genus *Amycolatopsis*. Strain MK299-95F4 showed low homology with *A. sulphurea* and *A. orientalis* as shown in Table 4. Therefore, we considered that the strain MK299-95F4 was different from *A. sulphurea* and was designated *Amycolatopsis* sp. MK299-95F4. This strain has been deposited in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-15243.

Table 2. Physiological characteristics of strain MK299-95F4.

Temperature range for growth (°C)	20~37
Optimum temperature (°C)	27
Formation of melanoid pigment	Negative
Hydrolysis of starch	Negative
Reduction of nitrate	Negative
Liquefaction of gelatin	Weakly positive
Coagulation of milk (30°C)	Negative
Peptonization of milk (30°C)	Weakly positive
Utilization of	
L-Arabinose	−
D-Xylose	±
D-Glucose	+
D-Fructose	+
Rhamnose	−
Sucrose	−
Raffinose	−
Inositol	+
D-Mannitol	+

+, Positive utilization; ±, doubtful utilization; −, no utilization.

Fermentation

A slant culture of the epoxyquinomicins-producing organism was inoculated into a 500 ml Erlenmeyer flask containing 110 ml of a seed medium consisting of glycerol 0.5%, sucrose 2%, soybean meal 1%, dried yeast 1%, corn steep liquor 0.5% and $CoCl_2$ 0.001% (adjusted to

Table 3. Comparison of strain MK299-95F4 with *Amycolatopsis sulphurea*.

	Strain MK299-95F4	*Amycolatopsis sulphurea* IMC A-0170 (IFO 13270^T)
Fatty acids[a]	16:0, *i*-15:0, *i*-16:0, 16:1, 17:0	16:0, *i*-16:0, 17:0
Aerial mass color	White	White
Color of growth	Colorless ~ pale yellowish brown	Colorless ~ dull yellow
Soluble pigment	None ~ yellowish brown	None ~ yellow
Coagulation of milk	Negative	Negative
Peptonization of milk	Weakly positive	Negative
Utilization of[b]		
D-Xylose	±	−
D-Glucose	+	+
Inositol	+	−

[a] 16:0, hexadecanoic acid; *i*-15:0, 13-methyltetradecanoic acid; *i*-16:0, 14-methylpentadecanoic acid; 16:1, *cis*-hexadecenoic acid; 17:0, heptadecanoic acid.
[b] +, Positive utilization; ±, doubtful utilization; −, no utilization.

Table 4. DNA homologies among strain MK299-95F4, *Amycolatopsis sulphurea* and *A. orientalis*.

Plate-bound DNA	% Hybridization with photobiotin labeled DNA		
	Strain MK299-95F4	*Amycolatopsis sulphurea*	*A. orientalis*
Strain MK299-95F4	100	18	11
Amycolatopsis sulphurea	13	100	8
A. orientalis	9	8	100

pH 7.0 before sterilization). The inoculated medium was incubated at 30°C for 5 days on a rotary shaker (180 rpm). Two ml aliquots of this seed culture were transferred to eighteen of 500 ml Erlenmeyer flasks each containing 110 ml of a producing medium consisting of glycerol 2%, dextrin 2%, Bacto-soytone (Difco) 1%, yeast extract 0.3%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2% and one drop of silicon oil, adjusted to pH 7.4 before sterilization. The fermentation was carried out at 27°C for 4 days on rotary shaker.

Isolation and Purification

The fermentation broth was centrifuged, and the supernatant (1800 ml) was extracted with butyl acetate (1800 ml) at pH 2.0. The butyl acetate layer was concentrated under reduced pressure (980 mg of brown oil). The crude material was chromatographed on a silica gel column (Merck Kieselgel 60, 70 ~ 230 mesh, 120 ml) and eluted stepwise with toluene-acetone (10:1, 5:1, 3:1) to give **1** (18 mg), **2** (19 mg) and mixture of **3** and **4** (170 mg). One portion of the mixture of **3** and **4** (51 mg) was further purified by preparative TLC (Merck Art 1.05715, $CHCl_3$-10% aq. MeOH, developed 3 times) providing **3** (13 mg) and **4** (23 mg) (Fig. 3).

Antimicrobial Activities

Antimicrobial activities of epoxyquinomicins were shown in Table 5. The antimicrobial activities of **1** and **2** were moderate against Gram-positive bacteria and several strains of *Pasteurella piscicida*. On the other hand, **3** and **4** showed almost no antimicrobial activity.

As shown in Table 6, compounds **1** and **2** showed cytotoxicity at the concentration of 2 ~ 20 μg/ml against various tumor cells *in vitro*, but compounds **3** and **4** showed no cytotoxicity even at the concentration of 100 μg/ml.

Compounds **1** and **2** showed acute toxicity at a dose of 25 and 50 mg/kg, respectively. Compounds **3** and **4** showed no sign of acute toxicity even at a dose of 100 mg/kg when administrated once to mice intraperitoneally.

The other biological activities[2] and structure elucida-

Fig. 3. Isolation procedure of epoxyquinomicins A, B, C and D.

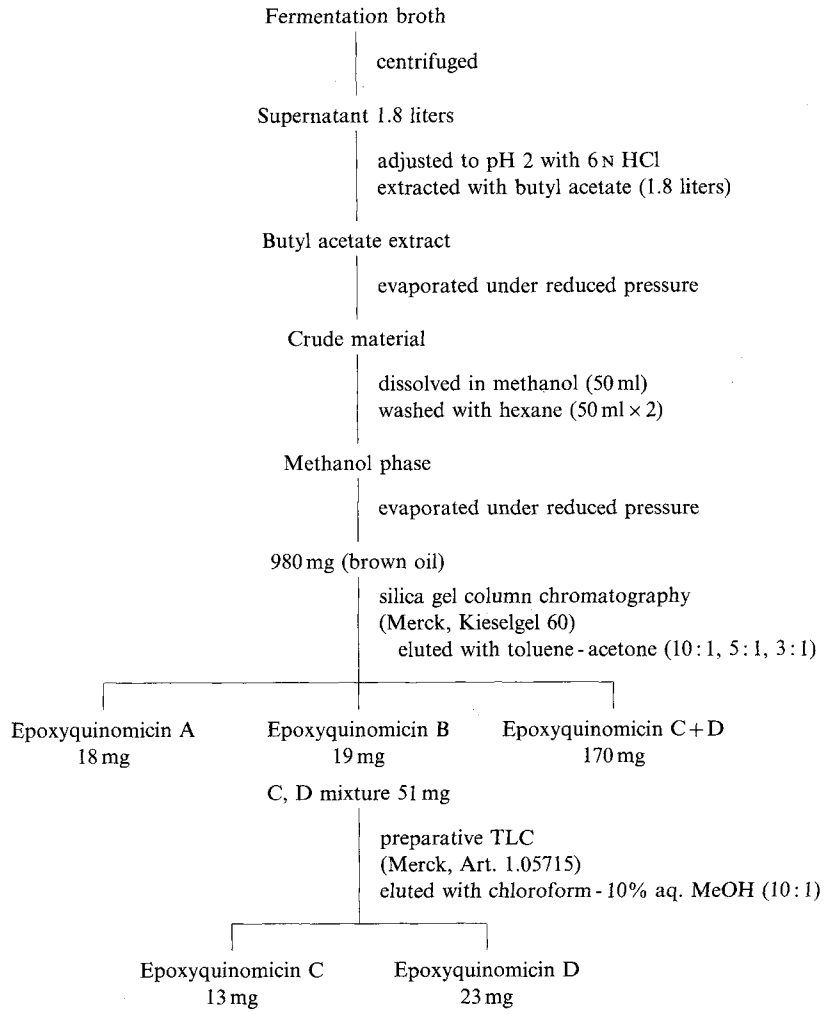


Table 5. Antimicrobial activities of epoxyquinomicins A, B, C and D.

Test organism	MIC (μg/ml)			
	A	B	C	D
Staphylococcus aureus FDA209P	12.5	12.5	>100	>100
S. aureus Smith	12.5	12.5	>100	>100
S. aureus MS9610	50	25	>100	>100
S. aureus MS16526	25	25	>100	>100
Micrococcus luteus IFO3333	3.12	6.25	>100	>100
M. luteus PCI1001	6.25	6.25	>100	>100
Bacillus subtilis PCI219	12.5	6.25	>100	>100
Escherichia coli NIHJ	50	50	>100	>100
Shigella dysenteriae JS11910	50	50	>100	>100
Salmonella typhi T-63	100	100	>100	>100
Providencia rettgeri GN311	100	100	>100	>100
Serratia marcescens	100	100	>100	>100
Pseudomonas aeruginosa GN315	100	100	>100	>100
Klebsiella pneumoniae PCI602	100	100	>100	>100
Mycobacterium smegmatis ATCC607	>100	>100	>100	>100
Candida albicans 3147	100	100	>100	>100
Pasteurella piscicida sp. 6395	12.5	12.5	>100	>100
P. piscicida p-3347	3.12	12.5	>100	>100
P. piscicida p-3353	6.25	12.5	>100	>100
Enterococcus seriolicida 4038	100	>100	>100	>100

Table 6. Cytotoxicity of epoxyquinomicins A, B, C and D.

Cell	IC_{50} (μg/ml)			
	A	B	C	D
L1210	2.64	16.3	>100	>100
IMC ca.	9.67	17.9	>100	>100
Ehrlich	NT	6.58	>100	>100
B16	7.97	NT	>100	>100
S 180	7.67	NT	NT	NT

NT, not tested.

tion study[24] of epoxyquinomicins will be reported in the accompanying papers.

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