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Kosinostatin, a Quinocycline Antibiotic with Antitumor Activity from *Micromonospora* sp. TP-A0468

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Kosinostatin, a quinocycline antibiotic was isolated from the culture broth of an actinomycete strain TP-A0468 along with isoquinocycline B. The producing strain was isolated from the seawater sample collected in Toyama Bay and identified as *Micromonospora* sp. based on the taxonomic study. Kosinostatin was obtained from the culture fluid by solvent extraction and ODS column chromatography. Kosinostatin inhibited the growth of Gram-positive bacteria strongly (MIC=0.039 μg/ml) and Gram-negative bacteria and yeasts moderately (MIC=1.56~12.5 μg/ml). It showed cytotoxicity against various cancer cell lines with the IC_{50} of 0.02~0.6 μM and inhibited human DNA topoisomerase IIα with the IC_{50} of 3~10 μM.

In the course of screening for new antibiotics from rare actinomycetes, kosinostatin was found in the fermentation broth of an actinomycete strain TP-A0468. The producing strain was isolated from the seawater collected at 321 meters in depth in Toyama Bay, Japan and identified as *Micromonospora* sp. Structural study revealed that kosinostatin (Fig. 1) is a quinocycline[1~3] antibiotic, presumably identical with quinocycline B, as described in the accompanying paper.[4]

In this paper, we report on the taxonomy and fermentation of the kosinostatin-producing strain, and isolation and biological properties of kosinostatin.

Materials and Methods

Microorganism

Strain TP-A0468, kosinostatin-producer, was isolated from the seawater sample collected at 2,600 meters off the shore and 321 meters in depth at Namerikawa, Toyama, Japan. The spores of the strain were trapped by the membrane filter method and cultured on an agar plate. A pure culture of strain TP-A0468 was preserved in 20% glycerol at −80°C. It was also maintained at 10°C for laboratory use as a slant on Bennett's agar.

Taxonomy

Taxonomic characteristics of strain TP-A0468 were determined by cultivation on various media described by SHIRLING and GOTTLIEB[5], WAKSMAN[6] and ARAI[7]. Morphological characteristics were observed after incubation of the culture at 30°C for 14 days on oatmeal agar (ISP med. 3) supplemented with 0.2% yeast extract. Cultural and physiological characteristics were determined after growth at 30°C for 14 days. The color names and hue numbers were assigned using the Manual of Color Names (Japan Color Enterprises Co., Ltd., 1987). Temperature range for growth was determined using a temperature gradient incubator TN-2148 (Advantec Toyo Co.). The carbon utilization was determined by the method of

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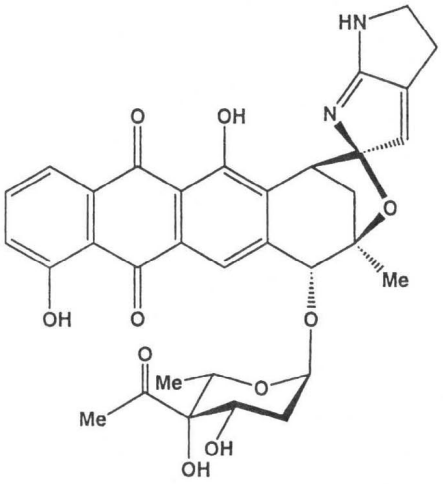
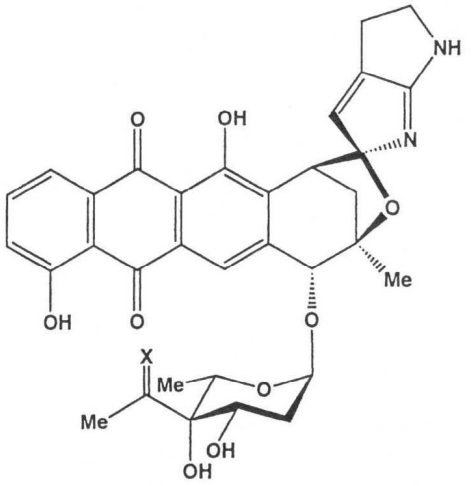
Fig. 1. Structure of kosinostatin and isoquinocyclines A and B.



Kosinostatin



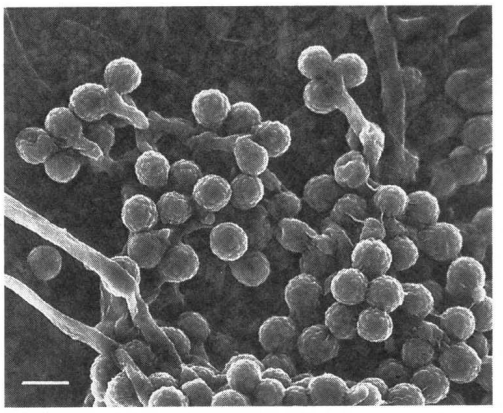
Isoquinocycline A X=H and OH
B X=O

SHIRLING and GOTTLIEB[5]. Cell wall composition was analyzed by the method of LECHEVALIER and LECHEVALIER[8], using thin layer chromatography plates as described by STANECK *et al.*[9]. Phospholipids compositions was determined by the methods of LECHEVALIER[10] and SUZUKI *et al.*[11], respectively.

Biological Assay

Antibiotic acitivity in fermentation broths and purification samples were evaluated by the conventional paper disc assay method using *Bacillus subtilis* ATCC6633 as a test strain. MIC values were determined by the conventional serial two-fold dilution method. Cytotoxicity against human myeloid leukemia U937 cells was determined by MTT method according to the supplier's protocol (Promega Corp.). IC_{50} values against other cancer cell lines were determined by colorimetric analysis of the cells stained with sulforhodamine B.

Fig. 2. Scanning electron micrograph of *Micromonospora* sp. TP-A0468.



Bar represents 1 μm.

Strain TP-A0468 grown on oatmeal agar supplemented with 0.2% yeast extract at 30°C for 10 days, showing the formation of single spores with warty surface on substrate mycelium.

Results and Discussion

Taxonomy of the Producing Strain

On observation with light microscope, spores were singly formed on substrate mycelium. By scanning electron microscope, the spore was oval in shape and 0.8~1.2 μm in size (Fig. 2). The cultural characteristics are summarized in Table 1. Color of colony was light orange to olive gray. Color of reverse side was light orange to dark gray. Aerial mycelium and diffusible pigments were not formed. As summarized in Table 2, starch hydrolysis and milk coagulation and peptonization gave a positive reaction. The temperature range for growth was 13~41°C and the

Table 1. Cultural characteristics of strain TP-A0468.

Medium	Vegetative mycelium	Reverse side	Aerial mycelium	Diffusible pigment
Sucrose – nitrate agar (Waksman med. 1)	Yellowish white (120) ~ grayish brown (120), poor	Grayish white (390) ~ grayish brown (117)	None	None
Glucose – nitrate agar	Beige white (392) ~ light grayish brown (110), poor	White (389)	None	None
Glucose – asparagine agar (Waksman med. 2)	Light orange (64) ~ light orange (62), poor	Light orange (64) ~ light orange (62)	None	None
Nutrient agar (Waksman med. 14)	Yellowish brown (101), good	Yellowish brown (97)	None	None
Bennett's agar (Waksman med. 30)	Light orange (66) ~ dark gray (417), good	Light orange (64) ~ dark gray (417)	None	None
Yeast extract - malt extract agar (ISP med. 2)	Soft orange (83) ~ olive gray (411), good	Light yellowish brown (92) ~ medium gray (406)	None	None
Oatmeal agar (ISP med. 3)	Light brown (90) ~ Grayish brown (114), good	Light yellowish brown (92) ~ grayish brown (117)	None	None
Inorganic salts - starch agar (ISP med. 4)	Dark brown (104) ~ dark yellowish brown (106), good	Dark yellow brown (106)	None	None
Glycerol asparagine agar (ISP med. 5)	White (389), poor	White (389)	None	None
Tyrosine agar (ISP med. 7)	Beige gray (401) ~ light yellowish brown (92), poor	Grayish white (390) ~ yellowish brown (101)	None	Good

Table 2. Physiological characteristics of strain TP-A0468.

Test	Results
Starch hydrolysis (on ISP med. 4)	Positive
Nitrate reduction	Negative
Milk (Difco, 10% skimmed milk)	
Coagulation	Positive
Peptonization	Positive
Cellulose decomposition (sucrose nitrate solution with a paper strip as the sole carbon source)	Negative (Growth: good)
Gelatin liquefaction	Positive
Melanine formation (on ISP med. 7)	Positive
Temperature range for growth (on Yeast starch agar)	13~41°C
Optimum temperature (on Yeast starch agar)	25~39°C
pH range for growth	6~10
Optimum pH	7~8
NaCl tolerance (on ISP med. 2)	<4%

optimum temperature for growth was 25~39°C. D-Glucose, sucrose, maltose, L-rhamnose, D-mannose, D-fructose, L-arabinose and D-galactose were utilized by strain TP-A0468 for growth. Inositol, D-mannitol, raffinose and D-xylose were not utilized (Table 3).

Whole cell hydrolysates contained *meso*-diaminopimelic acid and glycine, and galactose, xylose, arabinose and glucose as constituent amino acids and sugars, respectively. Strain TP-A0468 has type PII phospholipid (presence of phosphatidylethanolamine and phosphatidylinositol).

The taxonomic results of this strain described above coincided with the characteristics of the genus *Micromonospora* Orskov 1923. Thus it is concluded that the culture TP-A0468 represents a species of

Micromonospora.

Fermentation

A loopful of a mature slant culture of *Micromonospora* sp. TP-A0468 was inoculated into a 500-ml K-1 flask containing 100 ml of the seed medium consisting of soluble starch 1%, glucose 0.5%, NZ-case (Humco Scheffield Chemical Co.) 0.3%, yeast extract (Difco Laboratories) 0.2%, tryptone (Difco Laboratories) 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0). The flask was incubated at 30°C for 4 days on a rotary shaker (200 rpm). Three-ml aliquots of the seed culture were transferred into thirty 500-ml K-1 flasks each containing 100 ml of the production medium consisting of lactose 4.0%, Pharmamedia (Trader's Protein) 2.0% and HP-20 (Mitsubishi Chemical Co.) 1.0%. Fermentation was carried out for 5 days at 30°C on a rotary shaker (200 rpm).

Table 3. Carbon utilization of strain TP-A0468.

Carbon source	Utilization
D-Glucose	+
Sucrose	++
Maltose	++
Inositol	−
L-Rammnose	+
D-Mannitol	−
D-Raffinose	−
D-Mannose	+
D-Fructose	+
D-Xylose	−
L-Arabinose	+
D-Galactose	+
None	−

−: negative, +: positive (32°C, 4 weeks)

Isolation

The fermented whole broth (3 liters) was centrifuged (6,000 rpm, 10 minutes) to separate into the mycelia and supernatant. The mycelial cake was extracted with acetone (3 liters) twice and the combined filtrates were evaporated *in vacuo* to give aqueous solution. The aqueous layer was combined with the supernatant and extracted with ethyl acetate (1.5 liters) three times. The extract was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a brown extract (3.47 g). The oily extract was dissolved in methanol (100 ml), defatted by partitioning with *n*-hexane (100 ml) eight times and the resultant methanol solution was concentrated *in vacuo* to dryness (1.75 g). The dark yellow residue was subjected to a column of YMC gel (200×40 mm, i.d., ODS-AM 120-S50, YMC Co., Ltd.). The column was eluted with 20~40% acetonitrile in 0.15% KH_2PO_4 buffer (pH 3.5) to give two active fractions each containing kosinostatin and isoquinocycline B. The pooled fractions were evaporated and the resultant aqueous solution was adjusted to pH 7 with $NaHCO_3$ solution and extracted with ethyl acetate. The extract was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to dryness. After repeating this chromatographic operation several

Table 4. *In vitro* antibacterial activities of kosinostatin (**1**) and its aglycon (**2**), isoquinocycline B (**3**) and its aglycon (**4**), and aclacinomycin A.

Organism	MIC (μg/ml)				
	1	**2**	**3**	**4**	Aclacinomycin A
Staphylococcus aureus 209P JC-1	0.039	6.25	0.78	12.5	4.2
Bacillus subtilis ATCC6633	0.039	3.125	1.56	25	4.2
Escherichia coli NIHJ J-2	12.5	25	>100	>100	>16.7
Pseudomonas aeruginosa A3	12.5	>100	>100	>100	>16.7
Proteus mirabillis ATCC2110	3.125	50	50	>100	>16.7
Proteus vulgaris IFO3851	1.56	12.5	50	50	>16.7
Saccharomyces cerevisiae S-100	6.25	>100	100	>100	2.1
Candida albicans A9540	100	>100	>100	>100	4.2
Candida glabrata IFO0622	3.125	12.5	25	>100	1.0
Aspergillus fumigatus IFO8866	>100	>100	>100	>100	>16.7

times, kosinostatin (60 mg) and isoquinocycline B (18 mg) were obtained. These compounds could be obtained as HCl salt by the extraction of the organic layer with 0.01 N HCl and the subsequent lyophilization. The HCl salts were not stable for long storage even at −20°C.

Biological Properties

Kosinostatin (**1**) is the stereoisomer of isoquinocycline B and considered to be identical with quinocycline B as described in the accompanying paper[4]. These compounds belong to quinocycline antibiotics which were originally isolated from the fermentation broth of *Streptomyces aureofaciens* as an antituberculosis agent[1,2]. **1** possesses the anthracyclinone-like aglycon coupled perpendicularly to the unusual pyrrolopyrrole ring, and was expected to show different biological profile from other anthracycline antibiotics. In this study, the biological activity of **1** was evaluated in the antibacterial and cytotoxic assays in comparison with its aglycon (**2**), isoquinocycline B (**3**) and its aglycon (**4**), and aclacinomycin A.

The antimicrobial activity of **1** and related compounds is summarized in Table 4. **1** inhibited the growth of Gram-positive bacteria, *Staphylococcus aureus* 209P JC-1 and *Bacillus subtilis* ATCC6633, with the MIC of 0.039 μg/ml. It also showed the modest growth inhibition against Gram-negative bacteria and some yeasts. The antibacterial activity of **2** was *ca.* 100-fold less potent probably due to the poor solubility. **3**, the stereoisomer of **1**, showed 10-fold weaker activity than **1** against the tested bacterial strains and **4** showed less potent antibacterial activity. In contrast, aclacinomycin A exhibited a little more potent antifungal acitivity than **1** but 100-fold weaker than **1** against Gram-positive bacteria. These results likely indicate that **1** has the higher permeability into the cell and affinity to the bacterial DNA.

The cytotoxicity of **1** was initially tested against U937 cells comparing with the related compounds (Table 5). The IC_{50} of **1** was 0.09 μM, comparable to that of aclacinomycin A. The potency of **2**, **3** and **4** relative to **1** was in the same order as found in the antibacterial activity, implying the common mode of action. **1** was further tested against a panel of 39 human cancer cell lines (Table 6). The growth inhibition was observed with the IC_{50} of less than 0.1 μM against 21 cell lines. The mean graph pattern using COMPARE analysis[12] showed the similarity to that of other DNA intercalating agents such as doxorubicin. In addition, **1** inhibited the human DNA topoisomerases I and IIα with the IC_{50} of 10~30 μM and 3~10 μM respectively. These results suggest that the mechanism of growth inhibition is based on the interaction with DNA molecules. Further study is necessary to elucidate the effect of the

Table 5. *In vitro* cytotoxicity of kosinostatin (**1**) and its aglycon (**2**), isoquinocycline B (**3**) and its aglycon (**4**), and aclacinomycin A against U937 cells.

IC_{50} (μM)				
1	**2**	**3**	**4**	Aclacinomycin A
0.09	0.77	0.68	1.9	0.03

Table 6. *In vitro* cytotoxicity of kosinostatin against a panel of 39 human cancer cell lines.

Cell line	IC_{50} (μM)	Cell line	IC_{50} (μM)
Breast		Melanoma	
HBC-4	0.02	LOX-IMVI	0.03
BSY-1	0.06		
HBC-5	0.10	Ovary	
MCF-7	0.05	OVCAR-3	0.09
MDA-MB-231	0.21	OVCAR-4	0.10
		OVCAR-5	0.05
Central nervous system		OVCAR-8	0.06
U251	0.12	SK-OV-3	0.25
SF-268	0.08		
SF-295	0.12	Kidney	
SF-539	0.04	RXF-631L	0.18
SNB-75	0.55	ACHIN	0.11
SNB-78	0.15		
		Stomach	
Colon		St-4	0.15
HCC2998	0.05	MKN1	0.04
KM-12	0.09	MKN7	0.04
HT-29	0.07	MKN28	0.05
HCT-15	0.98	MKN45	0.02
HCT-116	0.03	MKN74	0.03
Lung		Prostate	
NCI-H23	0.13	DU-145	0.11
NCI-H226	0.02	PC-3	0.11
NCI-H522	0.04		
NCI-H460	0.04		
A549	0.09		
DMS273	0.40		
DMS114	0.10		

pyrrolopyrrole ring moiety on the biological activity.

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