A NEW BROAD-SPECTRUM AMINOGLYCOSIDE ANTIBIOTIC COMPLEX, SPORARICIN*

I. FERMENTATION, ISOLATION AND CHARACTERIZATION

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Two new aminoglycoside antibiotics, sporaricins A ($C_{17}H_{35}N_5O_5$) and B ($C_{15}H_{32}N_4O_4$) produced by a rare actinomycetales, *Saccharopolyspora hirsuta* subsp. *kobensis* have been isolated by column chromatography on a cation-exchange resin. Sporaricin A is highly active against Gram-positive and Gram-negative bacteria including aminoglycoside-resistant strains.

In the course of our screening program of new antibiotics, it has been found that two antimicrobial agents are produced by an unusual actinomycetales, designated as *Saccharopolyspora hirsuta* subsp. *kobensis*[1]. The organism has been isolated from a soil sample collected at Kobe City, Hyogo Prefecture, Japan. The organism shows streptomyces-like morphology but differs from streptomyces in cell wall composition[1].

A new water-soluble basic antibiotic complex named sporaricin was extracted from the broth filtrate by a cation-exchange resin process. Sporaricins A and B were separated and purified by column chromatography. As reported in a succeeding paper[2], sporaricins A and B have a unique aminocyclitol moiety and 6-*epi*-purpurosamine B.

Sporaricin A inhibits the growth of various Gram-positive and Gram-negative bacteria including mycobacteria and aminoglycoside-resistant strains.

In this paper, the fermentation, isolation and characterization of sporaricins A and B are reported.

Fermentation

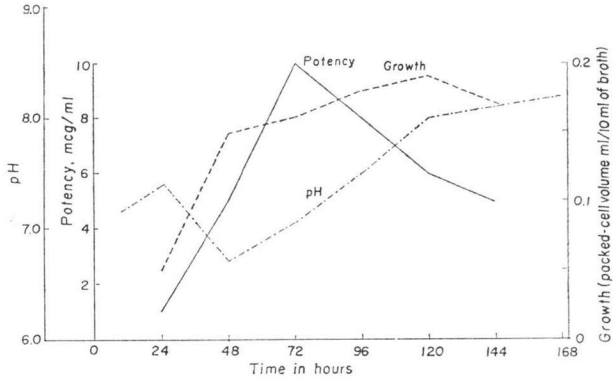
Saccharopolyspora hirsuta subsp. *kobensis*, strain KC-6606, was cultured in an Erlenmeyer-flask which contained 100 ml of a medium composed of 3.0% starch, 1.5% soybean meal, 0.5% corn steep liquor, 0.2% yeast extract and a mixture of small amount of inorganic salts including 0.3% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.3% $CaCO_3$ and 0.001% $CoCl_2 \cdot 6H_2O$ (pH was adjusted to 7.0~7.2 before sterilization) on a rotary shaker at 27°C for 50 hours.

One hundred ml of the culture broth was inoculated into 100 liters of the above-mentioned medium with addition of 1.0% cotton seed oil using a 200-liter fermentor. The fermentation was conducted at 27°C under aeration of 50 liters/minute, agitation of 225 rpm and inner pressure of 0.5 kg/cm^2. The potency of the cultured broth was estimated by a disc-plate method against *Bacillus subtilis* ATCC 6633 and *Mycobacterium smegmatis* ATCC 607 using pure sporaricin A as an assay standard. After

* This antibiotic was initially designated as KA-6606

72-hour incubation, the antibiotic concentration reached the maximum (10 mcg/ml), as shown in Fig. 1.

Fig. 1. Fermentation of sporaricin.
Mycelium was expressed as packed-cell volume of the culture broth by centrifugation at 3,000 rpm for 15 minutes.



Isolation

The 72-hour cultured broth was filtered at pH 2.0 (adjusted with diluted sulfuric acid) by using Dicalite (Dicalite Orient Co., Ltd., Japan) as filter aid, and the filtrate was filtered again at pH 7.0 (adjusted with 4 N NaOH). Sporaricin in the filtrate (80 liters, pH 7.0, activity 8 mcg/ml) was adsorbed on a column (5 × 100 cm) of Amberlite IRC-50 (NH_4^+) resin. The column was thoroughly washed with deionized water (100 liters) and then eluted with 1 N NH_4OH (20 liters). Active fractions were combined, concentrated and lyophilized to give a pale brown powder of the sporaricin complex (8.5 g).

The complex (8.0 g) was dissolved in deionized water (2.8 liters) and charged on a column (3 × 150 cm) of Amberlite CG-50 (NH_4^+). After washing with 0.05 N NH_4OH (1.5 liters), the column was eluted with aqueous ammonia with a concentration gradient from 0.05 N (5 liters) to 0.5 N (5 liters) at a flow rate of 150 ml/hour. The fractions (25 ml each) were monitored by bioactivity against *Bacillus subtilis* ATCC 6633 and were detected by ascending paper chromatography developed with a lower phase of $CHCl_3$ - MeOH - 17% NH_4OH (2: 1: 1, v/v). Sporaricin A (fraction Nos. 235~310) was eluted first, followed by sporaricin B (fraction Nos. 340~380). Each fraction of sporaricins A and B was concentrated and lyophilized to give a white crude powder (component A: 660 mg, B: 280 mg). They were further purified by column chromatography on cellulose column and on CM-Sephadex C-25. The crude powder (660 mg) of sporaricin A was charged on a column (2 × 100 cm) of cellulose (Whatman CF-11) and developed with a lower phase of $CHCl_3$ - MeOH - 17% NH_4OH (2: 1: 1, v/v). The eluate was cut into 5-ml fractions and each fraction was monitored by bioactivity and paper chromatography. Fraction Nos. 121~153 that showed Rf 0.6 on tlc with $CHCl_3$ - MeOH - 17% NH_4OH (1: 8: 3, v/v) were collected and concentrated to a small volume and then diluted with deionized water. The diluted solution was charged on a column (1.5 × 80 cm) of CM-Sephadex C-25 (NH_4^+). The column was washed with deionized water and then eluted by gradient elution between 0.1 N NH_4OH (500 ml) and 0.5 N NH_4OH (500 ml), and the eluate was cut into 10-ml fractions. Active fractions (Nos. 53~68) were combined and lyophilized to afford a colorless solid (98 mg) of pure sporaricin A.

The crude powder (280 mg) of sporaricin B was chromatographed on a cellulose column (Fraction Nos. 73~95 showed Rf 0.52 on tlc) and on CM-Sephadex C-25 column in the similar manner described above to give a colorless solid (72 mg) of pure sporaricin B.

Physico-chemical Properties

Physico-chemical properties of sporaricins A and B are listed in Table 1. Sporaricins A and B

Table 1. Physico-chemical properties of sporaricins A and B.

	Sporaricin A		Sporaricin B	
Nature	Basic, colorless solid		Basic, colorless solid	
$[\alpha]_D^{25}$ (*c* 1, H_2O)	+104°		+139.5°	
UV	End absorption		End absorption	
Elementary analysis	$C_{17}H_{35}N_5O_5$		$C_{15}H_{32}N_4O_4 \cdot H_2O$	
(%)	Found	Calcd.	Found	Calcd.
C	52.03	52.42	51.89	51.40
H	8.90	9.06	9.38	9.78
N	17.50	17.98	15.81	15.99
MW (Mass)	389		332	
IR (KBr) cm^{-1}	3400, 2940, 1628, 1580		3400, 2940, 1575	
^1H NMR (D_2O)* ppm				
C-CH_3	1.50		1.49	
N-CH_3	3.52		2.81	
O-CH_3	3.88		3.86	
-NCH_2CO-	4.01		—	
anomeric H	5.42		5.39	

* TMS as external reference.

Table 2. pH and temperature stabilities of sporaricins A and B.

		pH 10.0	pH 7.0	pH 2.0
Sporaricin A	24°C	80	100	90
	60°C	70	95	90
Sporaricin B	24°C	100	100	100
	60°C	100	100	95

One mg/ml solution of sample was kept for 4 hours in the conditions indicated. Residual activities assayed by the paper disc method on *Bacillus subtilis* plate are shown as relative ratio to pH 7.0 at 24°C for 4 hours.

Table 3. Comparison of paper chromatography of sporaricins A and B with other aminoglycoside antibiotics.

Antibiotics	Rf
Sporaricin A	0.53
Sporaricin B	0.86
Gentamicin C_1	0.69
Gentamicin C_2	0.35
Gentamicin C_{1a}	0.12
Sagamicin	0.49
Sisomicin	0.12
Verdamicin	0.35
Antibiotic G-52	0.49
Fortimicin A	0.32
Fortimicin B	0.89
Fortimicin C	0.11
Others*	0.00~0.04

* Neomycins, kanamycins, paromomycin, lividomycins, ribostamycin, apramycin, tobramycin, *etc.*

Whatman No. 1 (1×40 cm)
Solvent system: $CHCl_3$-MeOH-17% NH_4OH (2: 1: 1, v/v) lower layer
Detection: Bioautographed on *Bacillus subtilis* plate

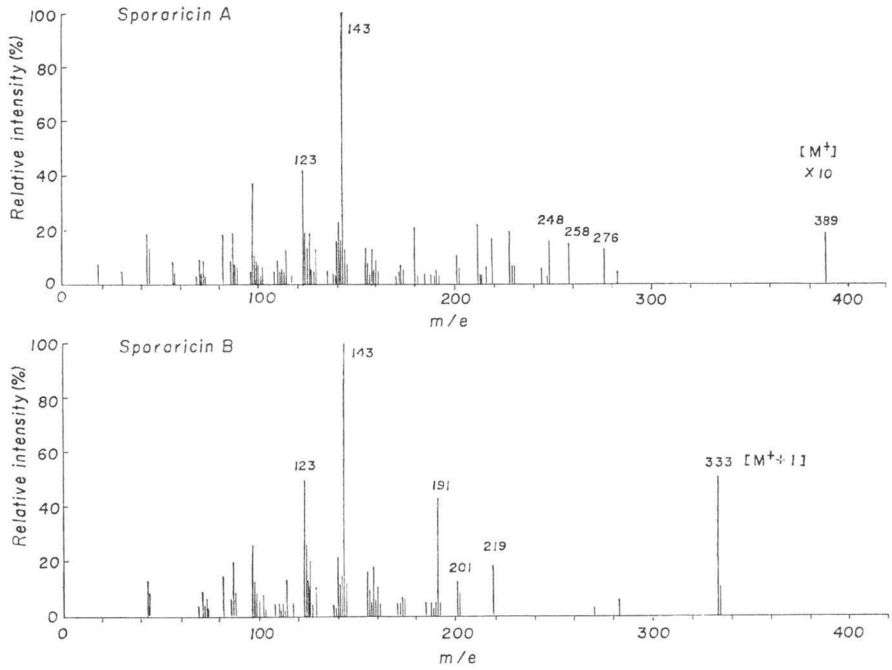
Table 4. Comparison of thin-layer chromatography of sporaricins A and B with other aminoglycoside antibiotics.

Antibiotics	Rf	
	Solvent I	Solvent II
Sporaricin A	0.60	0.56
Sporaricin B	0.52	0.57
Gentamicin C_1	0.40	0.52
Gentamicin C_2	0.44	0.51
Gentamicin C_{1a}	0.34	0.43
Sagamicin	0.32	0.45
Fortimicin A	0.56	0.53
Fortimicin B	0.70	0.60
Fortimicin C	0.56	0.52

Solvent I : $CHCl_3$ - MeOH-17% NH_4OH (1: 8: 3, v/v)
II: *n*-BuOH - EtOH - $CHCl_3$ - 17% NH_4OH (4: 5: 2: 5, v/v)

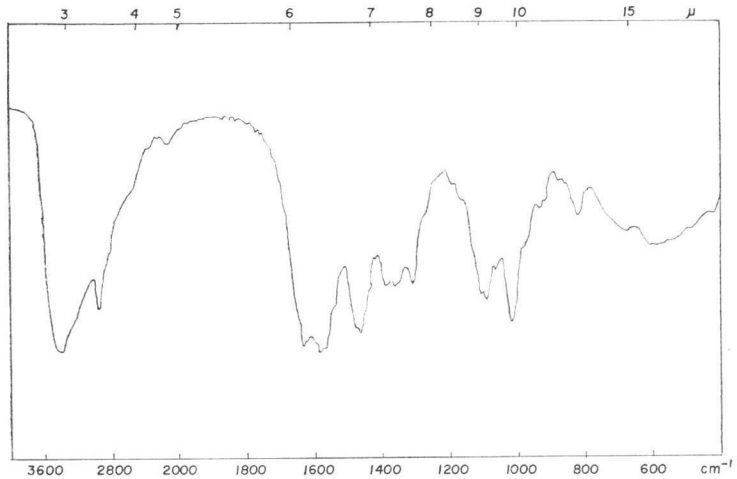
Thin-layer chromatography using tlc aluminium sheets silica gel 60 F_{254} pre-coated
Detection: ninhydrin

Fig. 2. Mass spectra of sporaricins A and B.



showed no definite melting or decomposition point. They are readily soluble in water and methanol, slightly soluble in ethanol and practically insoluble in other organic solvents such as chloroform, ether and benzene. Sporaricins A and B give positive ninhydrin and RYDON-SMITH but negative ELSON-MORGAN, FEHLING, biuret and SAKAGUCHI reactions. With ninhydrin reaction on tlc plate, sporaricin B gives purple color but A yellowish purple color.

Fig. 3. IR spectrum of sporaricin A.



Temperature and pH stability of sporaricins A and B are shown in Table 2. Sporaricin B was stable at all conditions tested but A was unstable at alkaline pH.

Molecular ion peak of mass spectra (Fig. 2) and the analytical data for sporaricins A and B agreed with the molecular formula of $C_{17}H_{35}N_5O_5$ (389) and $C_{15}H_{32}N_4O_4$ (332), respectively. The IR spectra of sporaricins A and B in KBr tablets are demonstrated in Figs. 3 and 4, respectively. In the spectrum of sporaricin A, absorption of amide carbonyl was observed at 1628 cm^{-1}, while it was

not observed in B. The 100 MHz ^1H NMR spectrum of sporaricin A (Fig. 5) indicated one anomeric proton (5.42 ppm) and three methyl groups assigned to C-CH_3 (1.50 ppm), N-CH_3 (3.52 ppm) and O-CH_3 (3.88 ppm). A signal of the N-methyl protons of sporaricin A shifts 0.71 to lower field compared with that of B (Fig. 6). Under high-voltage paper electrophoresis at 3,000 V for 20 minutes in formic acid - acetic acid - water (25 : 75 : 900, v/v), sporaricins A and B move to cathode with an Rm (relative mobility against alanine) of 2.16 and 2.10, respectively. Sporaricins A and B were clearly differentiated from known aminoglycoside antibiotics by paper and thin-layer chromatographies as shown in Tables 3 and 4.

Fig. 4. IR spectrum of sporaricin B.

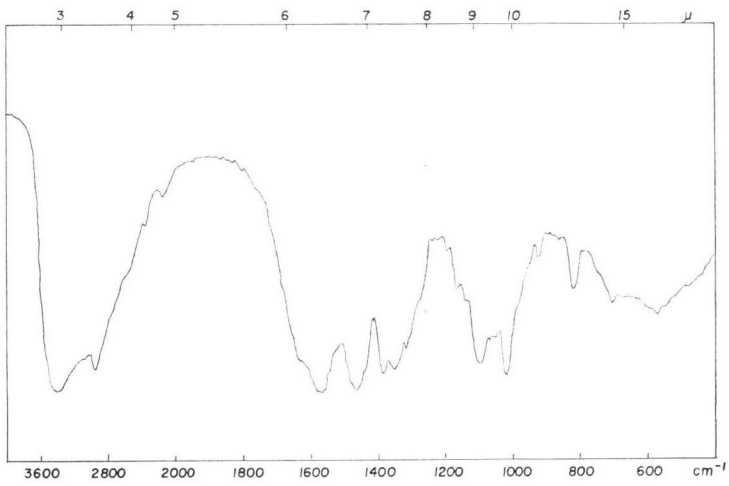
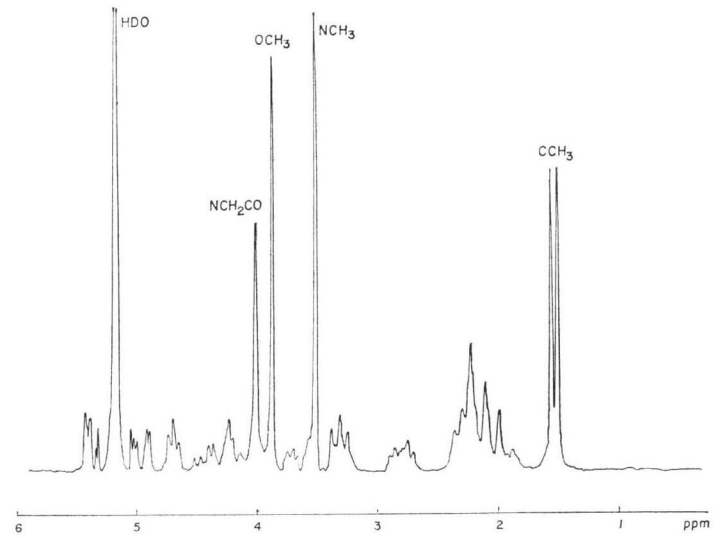


Fig. 5. 100 MHz PMR spectrum of sporaricin A in D_2O.



Biological Properties

The minimal inhibitory concentrations of sporaricins A and B against Gram-positive and Gram-negative bacteria were determined in a nutrient agar (Eiken Chemical Co., Ltd., Japan) by the two-fold serial dilution method and compared with those of amikacin[3] and tobramycin[4]. These results are shown in Table 5. The antibacterial activity of A was 30~60 times greater than that of B. Sporaricin A is highly active against Gram-positive and Gram-negative organisms including various aminoglycoside-resistant strains producing aminoglycoside 3′-phosphotransferases I and II [APH(3′)-I, APH(3′)-II], 2″-nucleotidyltransferase [AAD (2″)], 6′-acetyltransferase [AAC(6′)] and 2′-acetyltransferase [AAC(2′)]. It is very interesting that sporaricin A is highly active against organisms producing AAC(6′) and AAC(2′) in spite of having amino groups at 2′- and 6′-positions. On the other hand, sporaricin A is not active against organisms producing aminoglycoside 3-acetyltransferase [AAC(3)]. As reported in a succeeding paper[2],

Fig. 6. 100 MHz PMR spectrum of sporaricin B in D_2O.

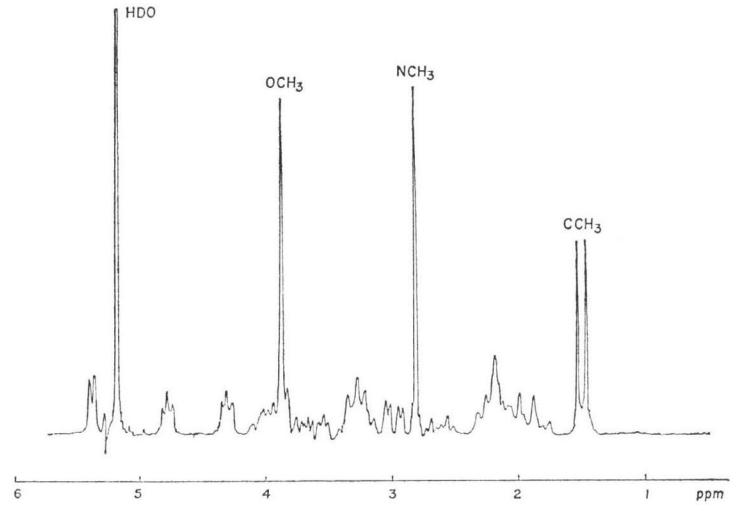


Table 5. Antimicrobial spectra of sporaricins A, B, amikacin and tobramycin.

Test organisms	M.I.C. (mcg/ml)			
	Sporaricin A	Sporaricin B	Amikacin	Tobramycin
Staphylococcus aureus FDA 209P	0.2	12.5	0.39	0.2
Staphylococcus aureus SMITH	0.1	6.25	0.2	0.1
Bacillus anthracis	0.2	6.25	0.2	0.2
Bacillus cereus	0.78	6.25	0.78	0.78
Bacillus subtilis ATCC 6633	0.2	6.25	0.39	0.1
Streptococcus faecalis	12.5	>100	50	6.25
Escherichia coli NIHJ	1.56	50	3.13	0.78
Escherichia coli K-12 ML1410	1.56	100	1.56	1.56
Escherichia coli K-12 ML1410 R-81[a]	1.56	>100	1.56	1.56
Escherichia coli K-12 ML1410 R-83[b]	1.56	>100	1.56	1.56
Escherichia coli K-12 ML1410 R-101[e]	1.56	>100	3.13	25
Proteus vulgaris OX-19	0.78	>100	0.78	0.39
Proteus inconstans[d]	0.78	>100	1.56	12.5
Klebsiella pneumoniae PCI 602	0.78	>100	0.78	0.39
Pseudomonas aeruginosa SHIBATA	1.56	>100	0.39	0.2
Pseudomonas aeruginosa No. 12	0.39	>100	0.39	0.2
Pseudomonas aeruginosa No. 99[e]	>100	>100	0.78	0.39
Pseudomonas aeruginosa GN315[f]	6.25	>100	25	100
Serratia sp.	0.78	>100	1.56	3.13
Mycobacterium smegmatis ATCC 607	0.2	>100	0.39	0.39

Medium: nutrient agar (Eiken Chemical Co., Ltd., Japan)
a) APH(3′)-I b) APH(3′)-II c) AAD(2″) d) AAC(2′) e) AAC(3)-I f) AAC(6′)

sporaricin A is a glycyl derivative of B, so that it is very interesting that the glycine moiety increased antibiotic activity and the relationship coincides with that of fortimicins A and B[5].

Sporaricin A and amikacin were administered subcutaneously 1 hour after intraperitoneal infec-

Table 6. The activity of sporaricin A and amikacin on the experimental bacterial infections in mice.

Organisms	Challenge dose (cells/mouse)	ED_{50} (mg/kg×1)*	
		Sporaricin A	Amikacin
Staphylococcus aureus SMITH	1.2×10^{2}	0.51	1.02
Escherichia coli GN 2411	9.8×10^{4}	3.08	4.06
Klebsiella pneumoniae No. 14	1.2×10^{5}	0.19	0.34
Proteus mirabilis No. 5	4.8×10^{4}	1.00	2.68
Serratia marcescens No. 2	1.2×10^{5}	14.1	21.4

* The ED_{50} is expressed as mg/kg in one subcutaneous dose (1 hour post-infection).

tion with representative Gram-positive and Gram-negative pathogenic bacteria in mice. As shown in Table 6, sporaricin A is more active than amikacin against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Serratia marcescens*.

The acute toxicity of sporaricins A and B were determined by intravenous and subcutaneous injections using male ICR-JCL mice. As shown in Table 7, acute toxicity of sporaricin A was approximately equal to that of tobramycin.

Table 7. Acute toxicities of sporaricins A and B in mice.

Route	LD_{50} (mg/kg)	
	Sporaricin A	Sporaricin B
Intravenous	73	>400
Subcutaneous	310	>800

mice: ICR - JCL ♂

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References

1) IWASAKI, A.; H. ITOH & T. MORI: A new broad-spectrum aminoglycoside antibiotic complex, sporaricin. II. Taxonomic studies on the sporaricin producing strain *Saccharopolyspora hirsuta* subsp. *kobensis* nov. subsp. J. Antibiotics 32: 180~186, 1979
2) DEUSHI, T.; M. NAKAYAMA, I. WATANABE, T. MORI, H. NAGANAWA & H. UMEZAWA: A new broad-spectrum aminoglycoside antibiotic complex, sporaricin. III. The structures of sporaricins A and B. J. Antibiotics 32: 187~192, 1979
3) KAWAGUCHI, H.; T. NAITO, S. NAKAGAWA & K. FUJISAWA: BB-K8, a new semisynthetic aminoglycoside antibiotics. J. Antibiotics 25: 695~708, 1972
4) NARA, T.; M. YAMAMOTO, I. KAWAMOTO, K. TAKAYAMA, R. OKACHI, S. TAKASAWA, T. SATO & S. SATO: Fortimicins A and B, new aminoglycoside antibiotics. I. Producing organism, fermentation and biological properties of fortimicins. J. Antibiotics 30: 533~540, 1977
5) THOMSPON, R. Q. & E. A. PRESTI: Nebramycin, a new broad-spectrum antibiotic complex. III. Isolation and chemical-physical properties. Antimicr. Agents & Chemoth.-1967: 332~340, 1968