A NEW AMINOGLYCOSIDE ANTIBIOTIC COMPLEX—THE SELDOMYCINS

I. TAXONOMY, FERMENTATION AND ANTIBACTERIAL PROPERTIES

TAKASHI NARA, MITSUYOSHI YAMAMOTO, SEIGO TAKASAWA, SEIJI SATO, TOMOYASU SATO, ISAO KAWAMOTO, RYO OKACHI, ITARU TAKAHASHI* and ATSUKO MORIKAWA

Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Ltd., 3-6-6 Asahimachi, Machidashi, Tokyo, Japan

*Fuji Factory, Kyowa Hakko Kogyo Co., Ltd., Suntogun, Shizuokaken, Japan

(Received for publication August 12, 1976)

A soil isolate named *Streptomyces hofunensis* sp. nov. was found to produce seldomycin factors 1, 2, 3 and 5, new aminoglycoside antibiotics. Taxonomy of the producing organism, a study of cultural conditions for seldomycin production, and antibacterial activity of seldomycins are reported. Seldomycin factor 5 was the most active both *in vitro* and *in vivo* against gram-positive and negative bacteria.

During the course of screening for new antibiotics, we encountered a *Streptomyces* species capable of producing new aminoglycoside antibiotics, seldomycin factors 1, 2, 3 and 5, which were previously designated XK-88-1, XK-88-2, XK-88-3 and XK-88-5, respectively.

This report will deal with the taxonomy of this culture, fermentation studies and antibacterial properties of the seldomycins. The isolation, identification and physicochemical properties of seldomycins and structure elucidation studies will be reported in the following three papers.[1,2,3]

1. Taxonomy of the Producing Organism

Streptomyces sp. MK-88, which produced new antibiotics seldomycins previously designated

Table 1. Cultural characteristics of strain MK-88

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	poor: no color~cream	poor: white	none
Glycerol-asparagine agar	poor: no color~cream	none	none
Oat meal agar	moderate: brown	none	none
Starch agar	moderate: brown	poor: white	none
Tyrosine agar	poor: no color~brown	none	none
Yeast ext.-malt ext. agar	moderate: brown	poor: white	none
Glycerol-asparagine agar	poor: no color~cream	none	none

XK-88s, was isolated from a soil sample collected at Hofu city, Yamaguchi Prefecture, Japan. Its taxonomy was studied mainly according to the method of SHIRLING and GOTTLIEB.[4] Cultural characteristics of MK-88 were observed on 8 kinds of media recommended by SHIRLING and GOTTLIEB,[4] and also by WAKSMAN[5] (Table 1). They were incubated at 30°C for 2 weeks. Strain MK-88 grows with no distinctive reverse color except cream or brown and does not produce any soluble pigment. White aerial mycelium is formed on sucrose-nitrate agar, starch agar and yeast extract-malt extract agar. The color of the white aerial mass does not change on prolonged cultivation. The formation of aerial mycelium is poor on the other media. The morphology of the spore chains was observed with a light microscope. The aerial mycelium is developed from the substrate mycelium with simple branching, the top of which forms straight or flexuous spore chains (Fig. 1). Mature spore chains usually consist of more than ten spores. The warty spore surface was observed with a scanning electron microscope (Fig. 2). Physiological properties are summarized in Table 2. No soluble pigment is produced on tyrosine agar medium and peptone-yeast extract iron agar medium, and MK-88 is considered non-chromogenic.

The main characteristics of MK-88 are summarized as follows:

1. simple branching and no whorls.
2. no distinctive reverse color.
3. white aerial mycelium.
4. straight or flexuous spore chains.
5. warty spore surface.
6. non-chromogenic.

These charateristics of MK-88 were compared with those of *Streptomyces* species described

Fig. 1. Sporophores of MK-88 by a light microscope (sucrose-nitrate agar medium, 30°C, 14 days).

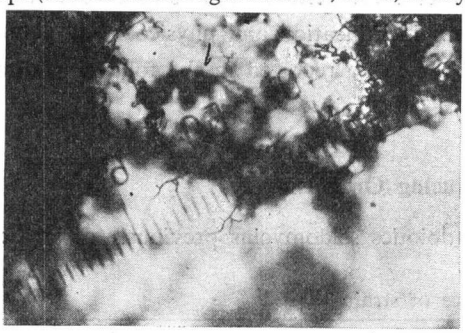


Fig. 2. Electron micrograph of spores of strain MK-88 (starch agar medium, 30°C, 14 days).

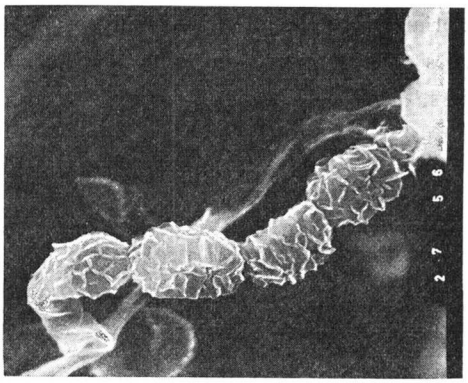


Table 2. Physiological properties of MK-88.

Growth temperature:	26°C~43°C
Liquefaction of gelatin:	negative
Hydrolysis of starch:	positive
Milk peptonization and coagulation:	negative
Chromogenecity:	no soluble pigment on tyrosine agar and peptone-yeast extract iron agar medium
Utilization of carbon sources:	++ glucose, sucrose, mannitol, xylose, rhamnose
	± arabinose
	− fructose, *i*-inositol, raffinose

Table 3. Comparison of MK-88 with related *Streptomyces*

	MK-88	*S. griseoplanus*	*S. candidus*
Aerial mass color	white	gray	white
Spore chain	Rectiflexibiles	Spirals	Rectiflexibiles
Spore surface	warty	warty	smooth
Carbon utilization	sucrose + mannitol + rhamnose − raffinose − fructose −	sucrose ± mannitol ± rhamnose ± raffinose + fructose +	sucrose ±

in "The Actinomycetes Vol. II" by WAKSMAN[5] and the ISP reports by SHIRING and GOTTLIEB.[6,7,8,9] *Streptomyces griseoplanus* and *S. candidus* were selected as related *Streptomyces*. As shown in Table 3, *S. griseoplanus* has, however, gray aerial mass color and the spore chains make a spiral, whereas MK-88 has white aerial mass color and the spore chains make no spirals. There are, also, many differences in carbon utilization between these two *Streptomyces*. On the other hand, *S. candidus* resembled MK-88 more closely. The greatest difference between them is the spore surface morphology. The spore surfaces of MK-88 and *S. candidus* are "warty" and "smooth", respectively. Generally speaking, there are few *Streptomyces* species whose spore surfaces are warty. All of them in ISP reports produce aerial mycelium of colors other than white. The characteristics of white aerial mass color and warty spore surface of MK-88 are very stable, and reliable as keys for classification and identification of this *Streptomyces*. In the group with gray aerial mass color, there are a few species of warty spore surface, but all of them produce spiral spore chains. MK-88 produces straight or flexuous spore chains and in our experience has not produced spirals. So, even in the "gray" group, we can not find species identical with MK-88.

Based on the above study, MK-88 is considered a new species and named *Streptomyces hofunensis* nov. sp. YAMAMOTO *et* NARA. A culture of the new species has been deposited in the American Type Culture Collection with accession number ATCC 21970.

2. Fermentative Production

Seed medium, S-1, consists of soluble starch 2%, yeast extract 0.1%, Polypeptone (casein hydrolysate) 0.5% and $CaCO_3$ 0.1% in tap water adjusted to pH 7.0 before autoclaving. A few loops of MK-88 spore on a slant were inoculated into 10 ml of the seed medium in a large test tube. The inoculum for seldomycins fermentation was prepared with cultivation at 30°C for 3 days on a reciprocating shaker. Then 10% by volume of the inoculum was transferred to 10 ml of fermentation medium in a large test tube or 30 ml in an Erlenmeyer flask. Fermentation was carried out at 30°C for 4~6 days on a shaker. Every day during the fermentation period, two or three tubes or flasks were removed from the shaker for assay. All the data shown are average values of these tubes or flasks. The figures in the tables represent the maximum observed during the fermentation period. Total antibiotic yield was estimated as seldomycin factor 5 by diffusion assay with *Bacillus subtilis* KY 4273, and expressed as arbitrary units. Growth was estimated by naked eye observation. F-1, the initial fermentation medium, consists of soluble starch 4%, soy bean meal 2%, meat extract 0.5%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.03% and $CaCO_3$ 0.3% in deionized water

adjusted to pH 7.0 before autoclaving. All media were sterilized by autoclaving at 120°C for 15 minutes.

The effect of carbon and nitrogen sources in the fermentation medium was tested in large

Table 4. Effect of carbon sources on production of seldomycins.

C-Source	pH	Growth[a]	Yield	C-Source	pH	Growth	Yield[b]
D-Raffinose	8.5	M	35	D-Xylose	6.2	M	n.d.
Maltose	7.7	M	n.d.	Glycerol	8.4	M	4.7
Sucrose	7.5	M	3.6	Sorbitol	8.4	M	32
Lactose	7.5	M	n.d.	Mannitol	7.9	M	18
D-Galactose	6.9	M	n.d.	Inositol	8.3	M	n.d.
Glucose	7.4	M	n.d.	s-Starch	8.5	M	110
L-Rhamnose	6.8	M	n.d.	Dextrin	7.6	M	94
D-Fructose	8.3	M	43	Lard oil	8.4	P	29
D-Mannose	7.3	M	n.d.	Soy bean oil	6.6	M	n.d.

Basal medium; carbon source 4 %, soy bean meal 2 %, meat ext. 0.5 %, K_2HPO_4 0.05 %, $MgSO_4 \cdot 7H_2O$ 0.05 %, KCl 0.03 %, $CaCO_3$ 0.3 %.
Fermentation in a large test tube.
a) By visual estimation. M: moderate, P: poor.
b) Total antibiotic yield as seldomycin factor 5 with diffusion assay with *Bacillus subtilis* KY4273, expressed by arbitrary units. n.d.: not detected.

Table 5. Effect of nitrogen sources on production of seldomycins.

N-Source	pH	Growth[a]	Yield	N-Source	pH	Growth	Yield[b]
S.B.M.	8.0	M	2.7	Ebios	6.6	M	n.d.
Meat ext.	8.0	P	n.d.	N-Z-amine A	8.1	M	n.d.
Polypepton	7.9	M	16	Tripticase	7.9	M	7.2
Yeast ext.	6.9	P	n.d.	Soy bean flour	8.1	M	n.d.
Malt ext.	8.1	G	n.d.	S.B.M. 2 %+	8.2	M	110
Oat meal	7.3	M	n.d.	Meat ext. 0.5 %			

Basal medium; s-starch 4 %, N-source 2.5 %, K_2HPO_4 0.05 %, $MgSO_4 \cdot 7H_2O$ 0.05 %, KCl 0.03 %, $CaCO_3$ 0.3 %.
Fermentation in a large test tube
a) The same as Table 4. G:good
b) The same as Table 4.
S.B.M.: Soy bean meal

Table 6. Combination effect of nitrogen sources on production of seldomycins.

N-Sources	pH	Growth[a]	Yield[b]
Soy bean meal 2 %+Meat ext. 0.5 %	8.3	##	54
Polypepton 2 %+Meat ext. 0.5 %	8.7	#	70
Trypticase 2 %+Meat ext. 0.5 %	8.9	#	88
Soy bean meal 2 %+ Yeast ext. 0.5 %	8.5	##	50
Polypepton 2 %+ Yeast ext. 0.5 %	8.5	##	250
Trypticase 2 %+ Yeast ext. 0.5 %	8.6	+	3.1

Basal medium; s-starch 4 %, N-sources 2.5 %, K_2HPO_4 0.05 %, $MgSO_4 \cdot 7H_2O$ 0.05 %, KCl 0.03 %, $CaCO_3$ 0.3 %.
Fermentation in a large test tube.
a) By visual estimation. ##: very good growth #: good growth, +: rather poor growth.
b) The same as Table 4.

Table 7. Effect of S-2 medium as a seed medium on production of seldomycins in 300-ml Erlenmeyer flask.

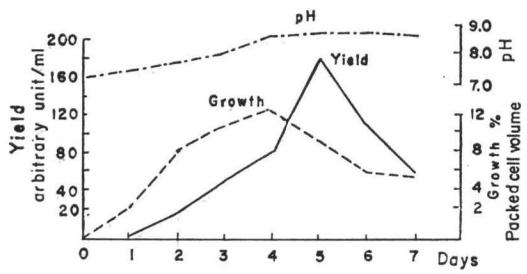
Fermentation medium	pH	S-2 medium		S-1 medium		
		Growth	Yield	pH	Growth[a]	Yield[b]
F-1 medium	8.7	++	34	8.3	+	21
F-2 medium	8.5	++	210	8.4	+	5

Fermentation vessel: 30 ml/300 ml Erlenmeyer flask
S-1 medium; s-starch 2 %, yeast ext. 0.1 %, Polypepton 0.5 %, $CaCO_3$ 0.1 %.
S-2 medium; s-starch 2 %, soy bean meal 0.5 %, meat ext. 0.1 %, $CaCO_3$ 0.1 %.
F-1 medium; s-starch 4 %, soy bean meal 2 %, meat ext. 0.5 %, K_2HPO_4 0.05 %, $MgSO_4 \cdot 7H_2O$ 0.05 %, KCl 0.03 %, $CaCO_3$ 0.3 %.
F-2 medium; s-starch 4 %, Polypepton 2 %, yeast ext. 0.5 %, K_2HPO_4 0.05 %, $MgSO_4 \cdot 7H_2O$ 0.05 %, KCl 0.03 %, $CaCO_3$ 0.3 %.
a) The same as Table 6.
b) The same as Table 4.

test tubes (Table 4). Though there was no great difference in growth among carbon sources tested, soluble starch, followed by dextrin, gave the best antibiotic production. Other sugars and oils were quite inferior to these carbohydrates in their effect on antibiotic production.

None of the nitrogen sources added singly to the basal fermentation medium showed a better antibiotic production than the control (soy bean meal 2 % plus meat extract 0.5 %) (Table 5). Though the addition of soy bean meal or meat extract singly showed almost no effect, the simultaneous addition of these two nitrogen sources was very effective. Many kinds of combinations of two nitrogen sources were then set up at random to be tested for the effect on antibiotic production. Some examples are shown in Table 6. The combination of Polypeptone 2 % and yeast extract 0.5 % gave the best antibiotic yields (4 to 5 times higher than control (250:54)).

Fig. 3. Time course of seldomycins fermentation.
Seed medium: s-starch 2 %, soy bean meal 0.5 %, meat extract 0.1 % and $CaCO_3$ 0.1 % in tap water, pH 7.0 before autoclaving.
Fermentation medium: s-starch 3 %, Polypepton 3 %, yeast extract 0.4 %, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCl_2 \cdot 2H_2O$ 0.2 %, in deionized water, pH 7.0 before autoclaving. Ten ml of the seed in a large test tube was cultivated at 30°C for 3 days; 10 % of the inoculum by volume was then transferred to 30 ml of the fermentation medium in a 300-ml Erlenmeyer flask and fermented at 30°C. Growth; Volume ratio of mycelia in the broth centrifuged. Yield; The same as Table 4.



The soy bean meal 2 % and meat extract 0.5 % in the F-1 medium were replaced by Polypeptone 2 % and yeast extract 0.5 %. However, as shown in Table 7, the effect of the new fermentation medium, F-2, initially observed in large test tubes was not seen immediately in shaken flasks. When S-1 medium was used as a seed medium, antibiotic yields in F-1 and F-2 medium were 21 u/ml and 5 u/ml, respectively. As the growth in this experiment was poorer than in the large test tubes, we developed a better seed medium, S-2. Using S-2 seed medium, the effect of F-2 medium appeared again (210 u/ml). S-2 medium consisted of soluble starch 2 %, soy bean meal 0.5 %, meat extract 0.1 % and $CaCO_3$ 0.1 % in tap water adjusted

to pH 7.0 before autoclaving. The study for the optimum concentration of each component in F-2 medium resulted in F-3 medium, the final fermentation medium. F-3 medium consisted of soluble starch 3 %, Polypeptone 3 %, yeast extract 0.4 %, $MgSO_4 \cdot 7H_2O$ 0.05 % and $CaCl_2 \cdot 2H_2O$ 0.2 % in deionized water adjusted to pH 7.0 before autoclaving. K_2HPO_4 and KCl were omitted and $CaCO_3$ was replaced by $CaCl_2 \cdot 2H_2O$.

The time course of seldomycin fermentation under the best conditions was then studied (Fig. 3). The pH of the broth was about 7.0 at the beginning of the fermentation and approached 9.0 towards the end of the fermentation. Growth reached its peak on the 4th day and decreased thereafter, possibly due to autolysis. The yields of seldomycin reached a maximum on the 5th day, closely following the growth peak, and decreased suddenly.

3. Biological Activities of Seldomycins

Each component of the seldomycins was isolated and purified as described in the succeeding

Table 8-1. *In vitro* antibacterial activities of seldomycins.

Test organism	M.I.C. (mcg/ml-free base)			
	SLD-1	SLD-2	SLD-3	SLD-5
Streptococcus faecalis ATCC10541	>416.5	>83.3	>83.3	10.5
Bacillus subtilis KY4273	52.1	0.35	2.7	0.04
B. cereus ATCC9634	6.6	2.7	2.7	1.31
B. cereus var. *mycoides* ATCC9463	3.3	0.7	2.7	0.66
Staphylococcus aureus ATCC6538p	6.6	0.18	0.18	0.07
S. aureus KY8942 (R-KM, PM, SM, GM, NBM)	52.1	1.4	1.4	0.66
S. aureus KY8950 (R-SM, TC, PC, SA)	13.1	0.35	0.35	0.17
S. aureus KY8956 (R-SM, PM, TC, KM, NBM, TBM, EM)	208.5	0.09	5.3	<0.02
S. aureus KY8957 (R-CM, SM, KM, PM, NBM, TBM, TC)	104.2	0.09	5.3	0.04
S. aureus KY8953 (R-SM, KM, PM, NM, TC, EM)	>416.5	0.35	>83.3	0.17

R-; resistant to, KM; kanamycin, PM; paromomycin, SM; streptomycin, GM; gentamicin, NBM; nebramycin, TC; tetracycline, PC; penicillin G, SA; sulfonamide, EM; erythromycin, CM; chloramphenicol, TBM; tobramycin. Medium used for the agar dilution assay consisted of 0.3 % tryptone, 0.3 % beef extract, 0.1 % glucose, 0.1 % yeast extract and 1.6 % agar.

Table 8-2. *In vitro* antibacterial activities of seldomycins.

Test organism	M.I.C. (mcg/ml-free base)			
	SLD-1	SLD-2	SLD-3	SLD-5
Klebsiella pneumoniae ATCC10031	13.1	0.18	0.05	<0.05
Escherichia coli ATCC26	26.1	0.35	0.18	0.08
E. coli KY8310(R-CM, GM, KM, SM, PM, NBM, TBM, SPC, TC)	>416.5	>83.3	>83.3	>41.6
E. coli KY8314 (R-SM)	52.1	1.4	0.35	0.33
E. coli KY8331 (R-KM, RBM, NM, PM, LVM, NBM)	>416.5	41.7	41.7	20.8
E. coli KY8334 (R-KM, TBM)	1.65	5.3	5.3	0.66
Proteus vulgaris ATCC6897	416.5	1.4	1.4	0.53
Pseudomonas aeruginosa KY8517	>416.5	10.5	>416.5	8.3
Shigella sonnei ATCC9290	52.1	1.4	0.35	0.14
Salmonella typhosa ATCC9992	26.1	0.7	0.18	0.09

LVM; lividomycin, SPC; spectinomycin.

paper[1].

The *in vitro* antibacterial activities of seldomycin factors 1, 2, 3 and 5 are shown in Table 8. Factor 5 shows the strongest activity followed by factor 2, and factor 1, the weakest. Factor 5 has a broad antibacterial spectrum against gram-positive and negative bacteria. It is effective against all the kinds of resistant *Staphylococcus aureus* shown in the table, and against two kinds of *Escherichia coli*, KY 8314 and KY 8334, resistant to streptomycin and to kanamycin and tobramycin, respectively. The activities of these factors can be compared with one another in terms of the structures, which will be reported by McALPINE, EGAN, SINCLAIR and others in following papers.[2,3]

From the available evidence, admittedly scanty, factor 3 appears to differ from factor 1 only in containing neamine instead of paromamine, and by analogy it should be more active than factor 1. Factor 2 consists of deoxystreptamine with one sugar attached, while factor 5 consists of factor 2 plus an aminopentopyranoside. Therefore the attachment of this aminopentopyranoside appears to enhance the bioactivity, since factor 5 is much more active than factor 2.

The *in vitro* activities of seldomycin factor 2 and factor 5 were compared with those of kanamycin A and gentamicin C (Table 9). Gentamicin C shows the strongest activities against all test organisms. Seldomycin factor 5 is almost comparable to or a little inferior to kanamycin in activity, and is similar in antibacterial spectrum to kanamycin.

Table 9. Comparison with other aminoglycoside antibiotics.

Test organism	M.I.C. mcg/ml-free base			
	SLD-2	SLD-5	KM	GM
Bacillus subtilis KY4273	0.49	0.1	0.02	0.003
Staphylococcus aureus ATCC6538p	0.12	0.03	0.03	0.003
Streptococcus faecalis ATCC10541	122	50	18	6.6
Escherichia coli ATCC26	0.95	0.1	0.14	0.03
Klebsiella pneumoniae ATCC10031	0.49	0.1	0.04	0.64
Proteus vulgaris ATCC6897	3.8	0.78	0.55	0.09
Pseudomonas aeruginosa KY8517	31	12.5	8.9	1.4
Salmonella typhosa ATCC9992	0.95	0.2	0.14	0.03
Shigella sonnei ATCC9290	1.91	0.39	0.28	0.09

SLD-2; Seldomycin factor 2, SLD-5; Seldomycin factor 5, KM; Kanamycin A, GM; Gentamicin C. Medium used for the agar dilution assay consisted of 0.15 % beef extract, 0.3 % yeast extract, 0.6 % peptone and 2.0 % agar.

Table 10. Acute toxicity and *in vivo* activity of seldomycins.

Antibiotic	LD_{50}[a] mg/kg	ED_{50}[b] mg/kg
Seldomycin factor 1 (free base)	>1,000	>1,000
Seldomycin factor 2 (sulfate)	470	30~60
Seldomycin factor 5 (sulfate)	365	7.5~15
Kanamycin A (free base)	350	7.5~15

a) An intravenous acute LD_{50} in mice.

b) The mice infected intraperitoneally with *Escherichia coli* GN2411-5 were treated intravenously with each antibiotic.

Acute toxicities and *in vivo* activities of the seldomycins were tested in comparison with kanamycin A (Table 10). Seldomycin factor 1 shows very low acute toxicity but its *in vivo* activity is quite weak. Factor 2 has rather low toxicity but its *in vivo* activity is also low. Seldomycin factor 1 and kanamycin A were tested as free base preparations, but factor 2 and factor 5, as sulfates with purities of 55 % and 70 %, respectively. Seldomycin factor 5 appears to be a little more toxic than kanamycin A but a little more active against *Escherichia coli* GN 2411-5 *in vivo*.

Acknowledgement

The authors are very grateful to Dr. A. C. SINCLAIR and his associates in Abbott Laboratories, U.S.A. for their kind advice and encouragement.

References

1) SATO, S.; S. TAKASAWA, T. SATO, M. YAMAMOTO, R. OKACHI, I. KAWAMOTO, T. IIDA, A. MORIKAWA & T. NARA: A new aminoglycoside antibiotic complex—The seldomycins. II. Isolation, physicochemical and chromatographic properties, J. Antibiotics 30: 25~30, 1977
2) EGAN, R. S.; A. C. SINCLAIR, R. L. DEVAULT, J. B. MCALPINE, S. L. MUELLER, P. C. GOODLEY, R. S. STANASZEK, M. CIROVIC, R. J. MAURITZ, L. A. MITSCHER, K. SHIRAHATA, S. SATO & T. IIDA: A new aminoglycoside antibiotic complex—The seldomycins. III. The structures of seldomycin factors 1 and 2. J. Antibiotics 30: 31~38, 1977
3) MCALPINE, J. B.; A. C. SINCLAIR, R. S. EGAN, R. L. DEVAULT, R. S. STANASZEK, M. CIROVIC, S. L. MUELLER, P. C. GOODLEY, R. J. MAURITZ, N. E. WIDEBURG, L. A. MITSCHER, K. SHIRAHATA, M. MATSUSHIMA, S. SATO & T. IIDA: A new aminoglycoside antibiotic complex—The seldomycins. IV. The structure of seldomycin factor 5. J. Antibiotics 30: 39~49, 1977
4) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Internat. J. Syst. Bacteriol. 16: 313~340, 1966
5) WAKSMAN, S. A.: The actinomycetes. Vol. II. Classification, identification and description of genera and species. The Williams & Wilkins Co., Baltimore, 1961
6) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. Internat. J. Syst. Bacteriol. 18; 69~189, 1968
7) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Internat. J. Syst. Bacteriol. 18: 279~392, 1968
8) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second, and third fourth studies. Internat. J. Syst. Bacteriol. 19: 391~512, 1969
9) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type strains of *Streptomyces*. V. Additional descriptions. Internat. J. Syst. Bacteriol. 22: 265~394, 1972