NEW ANTIBIOTIC XK-62-2 (SAGAMICIN). II

TAXONOMY OF THE PRODUCING ORGANISM, FERMENTATIVE PRODUCTION AND CHARACTERIZATION OF SAGAMICIN

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

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

(Received for publication May 10, 1974)

Cultures of *Micromonospora* species, strains MK 65 and MK 62, were found to produce a new antibiotic XK-62-2 (Sagamicin). Antibacterial and paperchromatographic data on an eluate from IRC-50 treatment of fermentation beers indicated that XK-62-2 is a new antibiotic with broad spectrum, basic, and water-soluble properties, probably closely related to the gentamicin C group of antibiotics.

In the course of screening for new antibiotics, a complex of antibacterial antibiotics was obtained from the culture broths of 2 strains of *Micromonospora*, MK-62 and MK-65. This antibiotic complex, XK 62, contained two main components designated as XK-62-1 and XK-62-2, both of which exhibited potent antibacterial activities against a broad range of gram-positive and gram-negative bacteria. Subsequent studies showed that the two antibiotics appear to belong to the gentamicin-group of antibiotics.[1] Although one component, XK-62-1, was identical to gentamicin C_{1a},[2,3] the other component, XK-62-2, was found to be a new antibiotic different from any gentamicin component so far reported. The new antibiotic (sagamicin) was shown to be 6′-N-methyl gentamicin C_{1a}, as reported in other papers describing isolation and characterization and structure determination.[4,5] This report describes the taxonomy of the producing organisms, fermentative production and some characterization studies of sagamicin.

1. Taxonomy

Strain MK 62 was isolated from a soil sample collected in North Chicago, Illinois, U.S.A., and strain MK 65 from a forest soil sample collected in Sagamihara city, Kanagawa, Japan, respectively. These two strains have been deposited at the American Type Culture Collection, Rockville, Maryland, U.S.A. and have been assigned accession numbers 21803 and 21826, respectively.

Most of the taxonomic studies of the two cultures were carried out in accordance with methods adopted by the International Streptomyces Project (ISP)[6]. Additional media recommended by WAKSMAN[7] were also used. The various media were inoculated with the washed mycelial suspension from a broth culture shaken at 30°C for 72 hours in a liquid medium (1.0% dextrin, 1.0% glucose, 0.5% yeast extract, 0.5% Polypeptone and 0.1% $CaCO_3$).

Plate 1. Photomicrograph of strain MK 65
(Yeast extract—malt extract agar ×1,500)

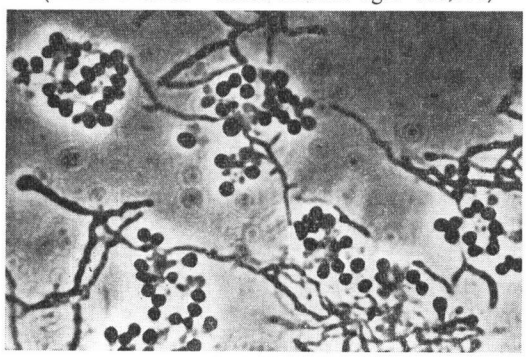
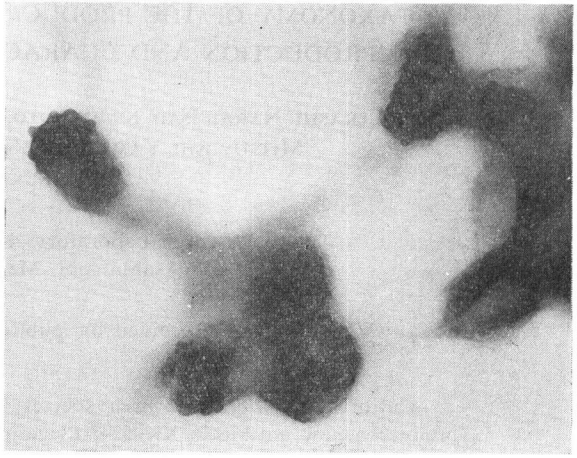


Plate 2. Electron micrograph of spores of strain MK 65
(Yeast extract—malt extract agar ×12,500)



Morphological Characteristics

Strain MK 65 showed good growth on the following medium: 1.0% glucose, 0.5% yeast extract, 0.5% tryptone, 1.0% malt extract and 2.0% agar (pH 7.3 before autoclaving). On this agar medium colonies were orange in the earlier stages of growth, and gradually turned brown or black. Many spores were found in the black colonies, but formation of true aerial mycelium was not observed on any of the agar media. Microscopic observation showed the substrate mycelia to be well developed and branched. The hyphae had a diameter of approximately 0.5 μ. The spores were borne singly from the substrate mycelia, usually on short sporophores but sometimes sessile. Electron microscopy revealed the mature spores were about

Table 1. Cultural characteristics of strain MK 65

Medium	Growth	Color*	Diffusible pigment
CZAPEK's agar (WAKSMAN No. 1)	moderate, flat	apricot (4ga) to dark brown (4nl)	none
Glucose—asparagine agar (WAKSMAN No. 2)	poor to moderate, flat	tile red (5ne)	none
Inorganic salts—starch agar (ISP No. 4)	moderate, granular	deep brown (4pl)	none
Egg albumin agar (WAKSMAN No. 25)	poor to moderate, flat	cocoa brown (51g)	none
Nutrient agar	poor to moderate, flat	light gold (2ic)	none
Malt extract—yeast extract agar (ISP No. 2)	moderate, granular	chestnut brown (4ni)	none
Oat meal agar (ISP No. 3)	poor, flat	orange (4la)	none
1% Glucose—3% NZ amine type A agar	moderate, flat	bright yellow (3la)	none
BENNETT's agar	moderate, flat	oak brown (4pi)	none
EMERSON's agar	poor to moderate, granular	orange (4la)	none
Glucose—yeast extract agar	moderate, granular	russet orange (4nc)	none
Tyrosine agar[9]	poor	beige brown (3ig)	none

* Color designation from Color Harmony Manual.

Table 2. Physiological properties of strain MK 65

Liquefaction of gelatin		weakly positive
Peptonization of milk		weakly positive
Coagulation of milk		negative
Decomposition of cellulose		weakly positive
Hydrolysis of starch		positive
Reduction of nitrate		positive
Formation of tyrosinase		negative
Formation of melanoid pigment		negative
Utilization of carbohydrate	good growth	D-glucose, D-xylose
	moderate growth	D-galactose, D-fructose, mannose, sucrose
	poor growth	D-arabinose, glycerol, D-lactose, L-inositol, D-mannitol, D-raffinose, L-rhamnose
pH range for growth*		6.6~9.0 (opt. pH 7.2~8.0)
Temperature range*		16.2°~40.0°C (opt. Temp. 30.0°~38.0°C)

* Medium used consisted of 1% glucose, 1% dextrin, 0.5% Polypeptone, 0.5% yeast extract and 0.1% $CaCO_3$.

1.0 μ in diameter, and spherical or oval in shape with a rough surface. (Plates 1 and 2).

Appearance on Various Media

The cultural characteristics of strain MK 65 shown in Table I were observed after two weeks of incubation at 27°C on the designated media. The number in parentheses corresponds to the hue number used in "Color Harmony Mannual"[8].

Physiological Characteristics

The physiological properties of strain MK 65 are shown in Table 2. The test for utilization of carbohydrates was performed using the basal medium consisting of 0.5% yeast extract and 0.1% reagent grade $CaCO_3$ because the growth of this strain was not enough for a visual estimate on the ISP medium.[6] The temperature range for growth was observed after cultivation for 5 days and the action upon milk and decomposition of cellulose was observed after one month. All other observations were made after two weeks.

Characteristics for strain MK 62 agree with those for strain MK 65 in all the characteristics shown in Tables 1 and 2 except that strain MK 62 fails to reduce nitrate or to peptonize milk.

Microscopic and cultural studies of strain MK 65 indicate that this isolate belongs to the genus *Micromonospora* ØRSKOV 1923. The systematics of the genus *Micromonospora* have been discussed by several authors.[10,11,12] Strain MK 65 was compared with the species described by these authors.

Most micromonosporae produce yellow-orange mycelial pigments, but some strains produce other diagnostic pigments. The nature of mycelial and diffusible pigments might be used as a criterion for characterization of species of genus *Micromonospora*. Strain MK 65 produces purple pigment in liquid media under certain conditions but not on most of agar media used. The pigment is red in the acid range and blue in the basic range, like that produced by *Micromonospora echinospora* subsp. *echinospora* LUEDEMANN *et* BRODSKY,[13] *M. echinospora* subsp. *ferruginea* LUEDEMANN *et* BRODSKY[13] and *M. purpurea* LUEDEMANN *et* BRODSKY, but unlike that

of *M. rosaria* WAGMAN *et al.*

Spore morphology, and especially spore surface morphology, are considered stable characters for description of streptomycetes, and have been used in describing micromonosporae. Spores with blunt spines have been reported for *M. echinospora* LUEDEMANN *et* BRODSKY,[13] *M. inyoensis* WEINSTEIN *et al.*[15] and *M. grisea* WEINSTEIN *et al.*[14] Strain MK 65 appears to belong to the group forming spores with blunt spines.

LUEDEMANN[12] considered that carbohydrate utilization was useful for characterizing strains and species of *Micromonospora*. Strain MK 65 does not utilize D-arabinose, glycerol, L-inositol, D-mannitol, D-raffinose and L-rhamnose for growth. The same carbohydrate utilization pattern is found for *M. carbonacea* LUEDEMANN *et* BRODSKY, *M. grisea* WEINSTEIN *et al.*, *M. inyoensis* WEINTEIN *et al.*, *M. megalomicea* LUEDEMANN *et* BRODSKY, *M. melanosporea* BALDACCI *et* LOCCI, *M. narashino* AIISO *et* ARAI, *M. parva* JENSEN, and *M. purpurea* LUEDEMANN *et* BRODSKY. Utilization of L-rhamnose by *M. echinospora* and its two subspecies appears to be a significant characteristic.[12]

Accordingly, species of *Micromonospora* which closely resemble strain MK 65 are *M. echinospora*, *M. grisea*, *M. inyoensis* and *M. purpurea*. *Micromonospora echinospora* and *M. purpurea* produce gentamicins, *M. inyoensis* sisomicin, and *M. grisea* antibiotic G-148, verdamicins, sisomicin and gentamicin A. These antibiotics are closely related in chemical structure.

Cultural comparison between resembling species was made as follow. Strain MK 65 resembles *M. echinospora* subsp. *echinospora* NRRL 2985[13] and *M. echinospora* subsp. *ferruginea* NRRL 2995[13] with respect to spore surface and pigments, but differs in failing to utilize L-rhamnose for growth. The strain is similar to *M. echinospora* subsp. *pallida* NRRL 2996 in respect of spore surface character, but differs in mycelial pigments and utilization of L-rhamnose. The isolate is similar to *M. grisea* NRRL 3800[14] and *M. inyoensis* NRRL 3292[15] in spore-morphology and carbohydrate utilization pattern, but differs in mycelial pigments. *Micromonospora grisea* NRRL 3800 appears grayish green on some agar media. *Micromonospora inyoensis* NRRL 3292 does not produce diagnostic mycelial pigments, but does produce reddish brown diffusible pigments in both tyrosine agar[9] and a medium consisting of 3% NZ Amine type A, 1% glucose and 1.5% agar. Strain MK 65 is similar to *M. purpurea* NRRL 2953 in mycelial pigments and carbohydrate utilization pattern, but differs in the morphology of its spores, because *M. purpurea* is an abortive or non-sporulating organism,[13] while strain MK 65 shows good sporulation on various agar media and the surface of its spores is blunt spiny.

Considering the above-mentioned information strain MK 65 resembles each of the cultures which produce gentamicin type antibiotics, but is not identical with any of them, nor with other species of *Micromonospora*.

2. Fermentation

A number of carbohydrate and nitrogen sources were investigated for their effect on growth of *M. icromonospora* sp. MK 62 and on antibiotic XK 62 complex production. Basal medium used contained 3% soybean meal and 0.1% $CaCO_3$. Maltose, dextrin, soluble starch and potato starch were excellent carbon sources for production of antibiotic XK 62. Although lucose supported abundant growth of the organism, this sugar was a poor carbon source for

Table 3. Effect of carbohydrate source on production of antibiotic XK 62 complex*

Carbohydrate	pH	Growth**	Antibiotic XK 62 produced*** (arbitrary units/ml)
D-Xylose	7.4	+	trace
D-Galactose	7.0	+	trace
D-Glucose	7.7	++	16
D-Fructose	7.7	+	8.6
Maltose	7.5	++	100
Sucrose	7.6	++	83
Dextrin	8.2	++	100
Soluble starch	8.0	++	120
Potato starch	8.0	++	160

* Basal medium; 3% soybean meal and 0.4% $CaCO_3$ (pH 7.4)

All carbohydrates (3% final concentration) were sterilized separately, and added to the basal medium just before inoculation. Three ml of seed grown at 30°C for 3 days was transferred into 30 ml of fermentation medium in 250 ml Erlenmeyer flasks. Fermentation was carried out at 30°C for 6 days.

pH, growth and titer of antibiotic XK 62 shown in Table are values of 6 day beer.

** Growth by visual estimation: +moderate, ++ good

*** Antibiotic XK 62 bioassay using *Escherichia coli* KY 8301 (cup, agar diffusion assay)

Table 4. Effect of nitrogen source on production of antibiotic XK 62 complex*

Nitrogen source	pH	Growth**	Antibiotic XK 62 produced*** (arbitrary units/ml)
Polypeptone			
1.0%	8.3	+	trace
2.0%	8.3	+	1.0
NZ Amine			
type A 1.0%	8.5	+	1.4
2.0%	8.8	++	4.5
Bacto tryptone			
1.0%	7.3	++	trace
2.0%	8.5	++	3.0
Wilson peptone			
1.0%	8.2	+	5.5
2.0%	8.0	+	4.3
Polypeptone S			
1.0%	8.5	++	7.0
2.0%	8.5	++	14
Casamino acid			
1.0%	8.3	+	6.0
2.0%	8.3	+	6.5
Meat extract			
1.0%	8.0	++	trace
2.0%	8.4	+++	6.0
Beef extract			
1.0%	8.3	++	4.6
2.0%	8.2	+++	4.5
Soybean meal			
3.0%	8.6	+++	100

* Basal medium; 3% soluble starch and 0.4% $CaCO_3$ (pH 7.4 before autoclaving). Three ml of seed grown at 30°C for 3 days in a medium containing 1% glucose, 1% dextrin, 0.5% yeast extract, 0.5% Polypeptone and 0.1% $CaCO_3$ (pH 7.2) transferred to 30 ml of fermentation medium in 250 ml Erlenmeyer flask. Fermentation was carried out at 30°C for 6 days. pH, growth and titer of antibiotic XK 62 shown in Table are values of 6 day beer.

** Growth as measured by dried cell weight; +1~4 mg/ml; ++ 4~8 mg/ml; +++ 8 mg/ml.

*** Antibiotic XK 62 bioassay using *Echerichia coli* KY 8301 (cup, agar diffusion assay)

production. Additions of D-xylose and D-galactose at 3% level (not 1%) reduce the growth, and therefore resulted in poor formation of the antibiotic (Table 3). Among the nitrogen sources shown in Table 4, a variety of protein hydrolyzates were not good nutrient sources for production of the antibiotic. Meat and beef extracts were good nitrogen sources for growth. Soybean meal was a suitable source for growth and production. Polypeptone S, a hydrolyzate of soybean meal, was a better source than casein hydrolyzate. Soybean meal might contain certain effective components for the production of antibiotic XK 62.

Based on the above results, one fermentation process adequate for antibiotic XK 62 production is as follows:

A loopful spores of *M. icromonospora* sp. MK-62 was inoculated into 30 ml of seed medium in a 250 ml Erlenmeyer flask. The seed medium consisted of 1% dextrin, 1% glucose, 0.5% peptone, 0.5% yeast extract and 0.1% $CaCO_3$ (pH 7.2 before autoclaving). After being shaken at 30°C for 5 days, 30 ml of the culture was inoculated into 300 ml of the same medium in a 2-liter Erlenmeyer flask. After shaking at 30°C for 2 days, 1.5 liter of the culture was transferred to 15 liters of the same medium in a 30-liter jar fermentor. The fermentation was conducted with aeration (15 liters/min.) and agitation (250 rpm) at 30°C for 2 days. Fifteen

Fig. 1. Chemical changes during antibiotic XK 62 complex fermentation

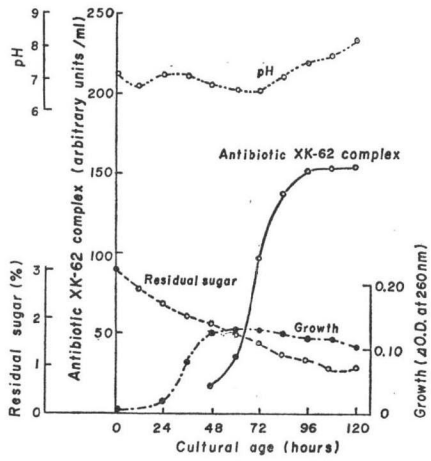


Table 5. Antimicrobial spectrum of antibiotic XK 62 complex*

Test organisms	Dilution units**	
	Assay at pH 7.0	Assay at pH 8.0
Streptococcus faecalis KY4280	< 10	40
Staphylococcus aureus KY4279	320	5,000
Bacillus subtilis KY4273	640	10,000
Escherichia coli KY4271	40	2,500
Proteus vulgaris KY4227	20	1,280
Shigella sonnei KY4281	10	640
Salmonella typhosa KY4278	160	2,500
Klebsiella pneumoniae KY4275	320	5,000
Pseudomonas aeruginosa KY4276	10	160

Medium used for agar dilution assay consisted of 0.3% tryptone, 0.3% beef extract, 0.1% glucose, 0.1% yeast extract and 1.6% agar.

* Sample for assay was an eluate obtained from IRC-50 (NH_4^+) treatment of 120-hour fermentation beer.

** Dilution units are shown as the highest dilution of the sample which competely inhibited growth.

liters of the culture then was transferred into 60 liters of seed medium in a 300-liter fermentor. Cultivation at the fourth stage was carried out with aeration (60 liters/min.) and agitation (150 rpm) at 30°C for 2 days. Finally, 60 liters of the culture was inoculated into 600 liters of a fermentation medium in a 1,000-liter tank fermentor. The fermentation medium consisted of 3% soluble starch, 3% soybean meal and 0.4% $CaCO_3$ (pH 7.2 before sterilization).

The fermentation was run with aeration (500 liters/min.) and agitation (150 rpm) at 30°C. The antibiotic titer reached a maximum at about 4~5 days. Chemical changes during this fermentation are shown in Fig. 1. It can be seen that antibiotic production began after growth had essentially finished. That is, antibiotic XK 62 synthesis did not occur during trophophase but began during idiophase, similar to most other antibiotics.

4. Antibacterial Activities

Because of the basic properties of the two antibiotics, they were adsorbed on an IRC 50 (NH_4^+) ion-exchange resin column and eluted with diluted NH_4OH. The eluate was concentrated *in vacuo*, examined for antibacterial activity and bioautographed using *Bacillus subtilis* KY 4373. The spectrum of activity given in Table 5 shows a broad range against various gram-positive and gram-negative bacteria. These activities were enhanced when bioassayed at pH 8 rather than at pH 7 or less, consistent with the basic property of antibiotic XK-62.

It was also found by use of agar dilution and agar diffusion assays that *Escherichia coli* KY 8348, whose R factor carrying inactivating enzyme was found in our enzyme experiments to be gentamicin acetyltransferase type I[16], was resistant to antibiotic XK 62 and the gentamicin C complex, but sensitive to other aminoglycosides *e.g.*, kanamycin, tobramycin and neomycin. These results suggest that antibiotic XK-62 resembles the gentamicin C group antibiotics.

As shown in Table 6, paperchromatograms obtained with five solvent systems indicate that

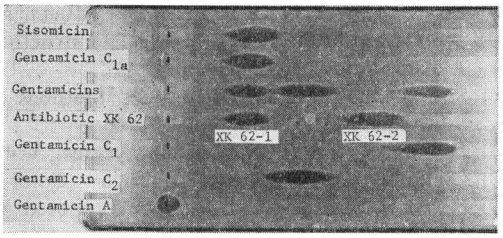
Table 6. Ascending paper chromatography of antibiotic XK 62 complex*

Solvent system	Rf value**	Developing period(hour)
20% (w/v) Ammonium chloride	0.98	3
Water-saturated *n*-butanol	0.00	15
n-Butanol-acetic acid-water (3:1:1)	0.06	15
Water-saturated ethyl-acetate	0.00	4
Water-saturated *n*-butanol containing 2% (w/v) of *p*-toluene sulfonic acid and 2% (v/v) of piperidine	0.03	15

* Sample for assay was an eluate obtained from IRC-50 (NH_4^+) treatment of 120-hour fermentation beer.

** Rf values were calculated from inhibition zones appearing on plates seeded with *Bacillus subtilis* KY4273.

Plate 3. Ascending paper chromatography of antibiotic XK62-1, antibiotic XK62-2 and antibiotics related to gentamicins



Solvent system; the lower layer of a mixture of chloroform, methanol and 17% aqueous ammonia. (2:1:1 by volume)
Development time; 12 hours.
Temperature; room temperature.
Test organism; *Bacillus subtilis* KY 4273.

the antibiotics are water-soluble and organic-solvent insoluble.

A paperchromatography system used for the separation of the gentamicin C components[2] gave the results shown in Plate 3. Antibiotic XK-62 was separated into two components. One component (XK-62-1) had the same Rf value as gentamicin C_{1a} and sisomicin, and the other component (XK-62-2) had an Rf value between that of gentamicin C_2 and C_1, different from that of any of the gentamicin components. All known aminoglycoside antibiotics except the gentamicin C complex, sisomicin and spectinomycin remained almost at the starting point with this paperchromatogram system.[4] Therefore, antibiotic XK-62-2 is a new antibiotic similar to the gentamicin C group of antibiotics. Further chemical work showed definitely that XK-62-2 is 6'-N-methyl gentamicin C_{1a}[4,5], while XK-62-1 was found to be identical with gentamicin C_{1a}. The trivial or common name for antibiotic XK-62-2 was designated as sagamicin.

Acknowledgements

The authors are grateful to Dr. A.C. SINCLAIR and his associates of Abbott Laboratories, North Chicago, Illinois, U.S.A. for their kind advice and encouragement. They are also thankful to Dr. G. H. WAGMAN, Schering Corp., Bloomfield, New Jersey, U. S. A., for the gift of gentamicins A, C complex, C_1, C_2, and C_{1a} and sisomicin, and to Drs. R. L. HAMILL and K. F. KOCH, Eli Lilly & Co., Indianapolis, Indiana, U.S.A., for the gift to tobramycin.

References

1) WEINSTEIN, M.J.; G.M. LUEDEMANN, E.M. ODEN & G.H. WAGMAN: Gentamicin, a new broad-spectrum antibiotic complex. Antimicr. Agents & Chemoth. -1963: 1~7, 1964
2) WAGMAN, G.H.; E.M. ODEN & M. J. WEINSTEIN: Differential chromatographic bioassay for the gentamicin complex. Appl. Microbiol. 16: 624~627, 1968
3) COOPER, D.J.; P.J.L. DANIELS, M.D. YUDIS, H.M. MARIGLIANO, R.D. GUTHRIE & S.T.K. BUKKARI: The gentamicin antibiotics. III. The gross structures of the gentamicin C components. J. Chem. Soc. (C) 1971: 3126~3129, 1971

4) OKACHI, R.; I. KAWAMOTO, S. TAKASAWA, M. YAMAMOTO, S. SATO, T. SATO & T. NARA: A new antibiotic XK-62-2. I. Isolation, physicochemical and antimicrobial properties. J. Antibiotics 27: 793~800, 1974
5) EGAN, R.S.; R.L. DEVAULT, S.L. MUELLER, M.I. LEVENBERG, A.C. SINCLAIR & R.S. STANASZEK: A new antibiotic XK-62-2. III. The structure of XK-62-2, a new gentamicin C complex antibiotic. J. Antibiotics 28: 29~34, 1975
6) SHIRLING, E.B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Intern. J. Syst. Bacteriol. 16: 313~340, 1966
7) WAKSMAN, S.A.: The Actinomycetes. Vol. II. The Williams & Wilkins Co., 1961
8) Container Corporation of America: Color Harmony Manual, 4th edition, Chicago, U.S.A., 1958
9) GORDON, R.E. & M.M. SMITH: Proposed group of characters for the separation of *Streptomyces* and *Nocardia*. J. Bact. 69: 147~150, 1955
10) SVESHNIKOVA, M.A., T.S. MAKSIMOVA & E.S. KUDRINA: The species of the genus *Micromonospora* ØRSKOV, 1923 and their taxonomy. Mikrobiologiya 38: 883~893, 1969
11) LUEDEMANN, G.M.: *Micromonospora* taxonomy. Adv. in Appl. Microbiol. 11: 101~133, 1970
12) LUEDEMANN, G.M.: Species concepts and criteria in the genus *Micromonospora*. Trans. Acade. Sci. 33: 207~218, 1971
13) LUEDEMANN, G. M. & B. C. BRODSKY: Taxonomy of gentamicin-producing *Micromonospora*. Antimicr. Agents & Chemoth. -1963: 116~124, 1964
14) WEINSTEIN, M.J.; G.H. WAGMAN, R. T. TESTA & J. A. MARQUEZ: Antibiotics G-418 and verdamicin I and methods for production thereof. Japan Kōkaikōfo (Patent Announce.), Showa 48-52991, July 25, 1973
15) WEINSTEIN, M. J.; G. M. LUEDEMAN & G. H. WAGMAN: Antibiotic 66—40 and methods for production thereof. Japan Patent, Showa 49-1559, Jan. 14, 1974
16) BENVENISTE, R. & J. DAVIES: Mechanism of antibiotic resistance in bacteria. Ann. Rev. Biochem. 42: 471~506, 1973