New Types of Liposidomycins that Inhibit Bacterial Peptidoglycan Synthesis and are Produced by *Streptomyces*

I. Producing Organism and Medium Components

KEN-ICHI KIMURA*, SHINOBU KAGAMI, YOSHIKAZU IKEDA, HIDETOSHI TAKAHASHI and MAKOTO YOSHIHAMA

Research Institute of Life Science, Snow Brand Milk Products Co., Ltd., Ishibashi-machi, Shimotsuga-gun, Tochigi 329-0512, Japan

HIROO KUSAKABE, HIROYUKI OSADA and KIYOSHI ISONO

The Institute of Physical and Chemical Research, RIKEN, Wako-shi, Saitama 351-0198, Japan

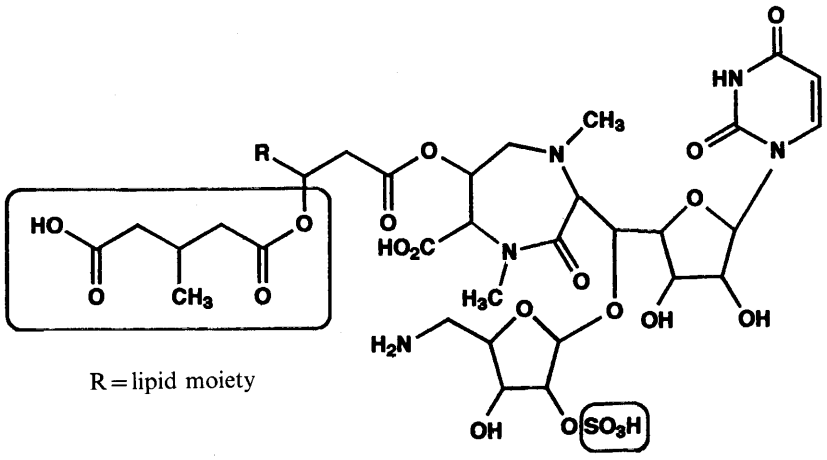
(Received for publication February 2, 1998)

Liposidomycins are atypical lipid-bearing nucleoside antibiotics that inhibit bacterial peptidoglycan synthesis. A producing strain was identified as a *Streptomyces* sp. from its cultural characteristics and physiological properties. It produced new types of liposidomycins that lacked sulfate and/or 3-methylglutaric acid moieties present in known liposidomycins by changing medium components. Sucrose and malt extract were particularly suitable sources for specific production of the new types of liposidomycins.

Many commercial antibiotics, including β-lactams, vancomycin *etc.*, inhibit the biosynthesis of peptidoglycan. Because of resistance to currently used antibiotics, there is a need to explore new targets for antibiotic action[1,2]. Liposidomycins were found by screening for peptidoglycan synthesis inhibitors and consisted of twelve compounds (A through L), at least on the basis of HPLC profiles[3]. Liposidomycins A, B and C possess the same 5'-substituted uridine, 5-amino-5-deoxyribose-2-sulfate and perhydro-1,4-diazepine moieties, and differ only in the structures of the lipid side chains[4,5]. The other components also probably differ only in the structures of lipid side chains. This has been demonstrated for liposidomycins G and H[5]. Liposidomycins A, B and C inhibit peptidoglycan synthesis in *Escherichia coli* at $IC_{50}=0.03\,\mu g/ml$ by means of a paper chromatographic method. The primary inhibition site of liposidomycin C was determined to be at phospho-*N*-acetylmuramoyl-pentapeptide-transferase (EC 2.7.8.13, translocase I), the first step in the lipid cycle of peptidoglycan synthesis[6]. Recently, the inhibition mechanism against translocase I was examined by comparison of liposidomycin B, tunicamycin[7] and mureidomycin A[8]. In this study, liposidomycin B and mureidomycin A were shown to be slow-binding inhibitors and tunicamycin to be a reversible inhibitor[9]. Moreover, liposiodmycin B inhibits formation of lipid intermediates in glycoconjugate biosynthesis at high concentrations compared with its activity against translocase I[10]. Contrary to the potent *in vitro* activity, liposidomycins A, B and C show only limited activity against Gram-positive and -negative bacteria. The most sensitive bacterium to liposidomycins is *Mycobacterium phlei* ($MIC=0.16\,\mu g/ml$)[3]. Transportation into cells might be limited by the hydrophilic ionic sulfate moiety. Thus we tried to control the putative sulfotransferase activity of the producing strain by changing medium components.

In this paper, we report the producing microorganism and the medium components for the fermentation of new types of liposidomycins that lack sulfate and/or 3-methylglutaric acid moieties (Fig. 1.). Details of the isolation, structure determination and biological properties of new types of liposidomycins will be reported in following papers[11,12].

Fig. 1. Structure of liposidomycins.



Materials and Methods

Microorganism

Streptomyces sp. RK-1061 which produced liposidomycins A through L was isolated from soil collected in Misaka-machi, Yamanashi prefecture, Japan. This and mutant strain *Streptomyces* sp. SN-1061M were maintained on SY slant agar (1% soluble starch, 0.5% yeast extract, 1.5% agar, pH 7.0). The strains have been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with accession numbers FERM P-8278 and FERM BP-5800, respectively.

Taxonomic Studies

Taxonomical characterization was carried out according to the method of International Streptomyces Project (ISP)[13]. The color of mycelia was identified by using the Color Harmony Manual (4th Ed., 1958, Container Corporation of America, Chicago, Illinois). Whole cell sugars were identified by the method of LECHEVALIER and LECHEVALIER[14] and diaminopimelic acid isomers were analyzed by the method of BECKER *et al.*[15].

Mutation

Spore cells from an SY slant culture of *Streptomyces* sp. RK-1061 were suspended in 10 ml of saline at 1×10^8 cells/ml and spread on Petri dishes. UV irradiation was performed at a level giving 1% survival by colony formation on SY agar. Sixty-six colonies were tested for antibiotic production, and strain SN-1061M, which revealed the highest antimicrobial activity against *Mycobacterium phlei*, was selected.

Medium and Fermentation

The seed medium and the production medium for the original liposidomycins, which have sulfate and 3-methylglutaric acid moieties were composed of glucose 2%, soluble starch 1%, meat extract 0.1%, dried yeast 0.4%, soybean flour 2.5%, NaCl 0.2% and K_2HPO_4 0.005%, adjusted to pH 6.7 (designated C4 medium)[3]. The effect of replacing glucose with alternative carbon sources was tested in C4 medium for growth and liposidomycin production by strain SN-1061M. A piece of SY slant culture was inoculated into 70 ml of each medium in 500 ml Erlenmeyer flasks. The flasks were incubated for 3 days at 27°C on a rotary shaker (200 rpm). Two ml of the culture was transferred into new medium and then incubated at 27°C on a rotary shaker (200 rpm). The culture was isolated from the flask at 3, 5 and 7 days, and centrifuged at 3000 rpm for 10 minutes. Afterward, mycelium volume, pH and the antimicrobial activity were measured. The butanol extract of the broth filtrate and the acetone extract of the mycelium were dissolved in methanol and used for TLC and HPLC analyses.

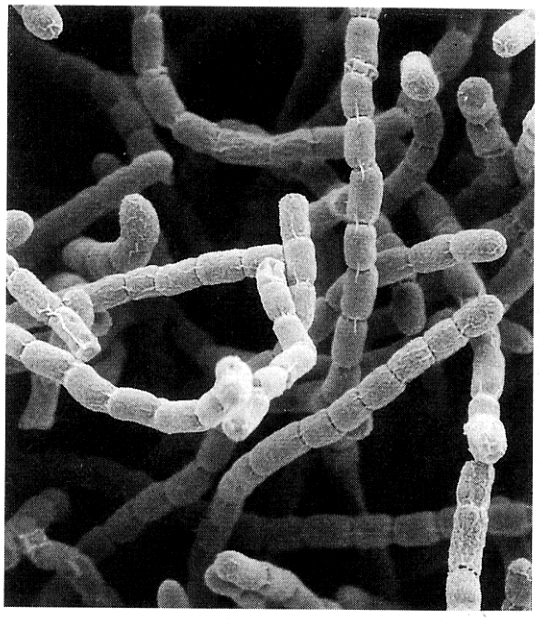
TLC and HPLC Analyses

Liposidomycins (sulfate type) and new types of liposidomycins (nonsulfate type) were analyzed by silica gel TLC (Merck, Art. 5715, 0.25 mm) with BuOH : AcOH : H_2O = 4 : 1 : 2. Sulfated and nonsulfated liposidomycins were distinguished by their Rf values of 0.35 and 0.41, respectively. All liposidomycins were analyzed in detail by HPLC (CAPCELL PAK, Shiseido Co., 4.6 i.d. × 250 mm, CH_3CN : 0.1% diethylamine-HCOOH (pH 4) = 45 : 55, 254 nm, 1.5 ml/minute).

Table 1. Cultural characteristics of *Streptomyces* sp. RK-1061.

Culture medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Sucrose-nitrate agar	Moderate	None	Pearl (2ba)	None
Glucose-asparagine agar	Moderate	None	Pearl (2ba)	None
Glycerol-asparagine agar(ISP No. 5)	Moderate	None	Pearl (2ba)	None
Inorganic salts-starch agar (ISP No. 4)	Good	None	Pearl (2ba)	None
Tyrosine agar (ISP No. 7)	Moderate	Scant, oyster white (b), cobalt brown (3ni), chesnut brown (4ni)	Dark brown (3pn)	Deep brown (3pl)
Nutrient agar	Poor	None	Yellow maple (3ng)	Yellow maple (3ng)
Yeast extract-malt extract agar (ISP No. 2)	Modearte	Excellent, gray (e)	Gold brown (3pi)	Dark brown (3pn)
Oatmeal agar (ISP No. 3)	Modearte	Moderate, rose wood (5ge)	Rose beige (4ge)	None
Peptone-yeast extract-iron agar	Poor	None	Pearl (2ba)	Dark brown (5pn)
Starch-yeast extract agar	Good	Excellent, rose beige (4ge), beaver(4li)	Rose beige (4ge)	Olive gray (1ih)

Fig. 2. Scanning electron micrograph of spore chains of *Streptomyces* sp. RK-1061.



The strain was cultured at 27°C for 14 days on starch-yeast extract medium.

Table 2. Physiological properties of *Streptomyces* sp. RK-1061.

Characteristics	*Streptomyces* sp. RK-1061
Temperature for growth	20~35°C
Liquefaction of gelatin	Negative
Hydrolysis of starch	Positive
Coagulation of milk	Positive
Peptonization of milk	Positive
Formation of melanin	Positive
Utilization of	
L-Arabinose	++
D-Xylose	+++
D-Glucose	++
D-Fructose	+
Sucrose	+
Inositol	+
L-Rhamnose	+
Raffinose	+
D-Mannitol	+
Lactose	+++
Melibiose	++

+++: Good utilization, ++: moderate utilization, +: poor utilization.

Results

Taxonomic Characterization of the Producing Strain

The cultural characteristics of strain RK-1061 grown on various media at 27°C for 21 days are shown in Table 1. The growth and amount of aerial mycelium were good on SY agar but poor on nutrient and peptone-yeast extract-iron agar media. The strain formed straight sporophores on the aerial mycelium and the spores were cylindrical, 0.5~0.7×0.5~1.0 μm in size, having a smooth surface with some wrinkles on SY agar medium

(Fig. 2.). No special morphological organs such as whirls, sclerotia, sporangia were observed on the media employed. Other physiological properties are shown in Table 2. Detection of LL-diaminopimelic acid in the whole cell hydrolysate of the culture indicated that this strain has a Type I cell wall[15]. Based on the taxonomic properties described above, strain RK-1061 is considered to be a member of the genus *Streptomyces*. On the basis of descriptions in BERGEY's Manual of Determinative Bacteriology, strain RK-1061 most resembles *Streptomyces griseosporeus*[16]. However, direct comparison has not yet been carried out. Though cultural characteristics and physiological properties of mutant strain SN-1061M strongly resemble those of RK-1061, antimicrobial acitity against *Mycobacterium phlei* was more potent than that of the parent strain (data not shown). Strain SN-1061M was used in the following experiments.

Medium Component

Streptomyces sp. SN-1061M also produced the original compounds (sulfate type) time dependently in C4 medium (Fig. 3.)[3]. This suggested to us that the putative sulfotransferase of *Streptomyces* sp. SN-1061M might transfer sulfate to the 2-position of 5-amino-5-deoxyribose of liposidomycins. Thus we have tried to control this sulfation specifically for production of nonsulfated liposidomycins. When glucose in the C4 medium was replaced with D-xylose, lactose, D-fructose, sucrose, inositol or D-mannitol, all replacements showed good production of nonsulfated liposidomycins. A typical TLC analytical pattern using the C4-sucrose medium is shown in Fig. 3. The same pattern was observed using the other five carbon sources as well. Table 3 shows that sucrose is a more suitable carbon source than the others,

Fig. 3. Analytical TLC patterns of the butanol extract of the C4, C4-sucrose and K1 media.

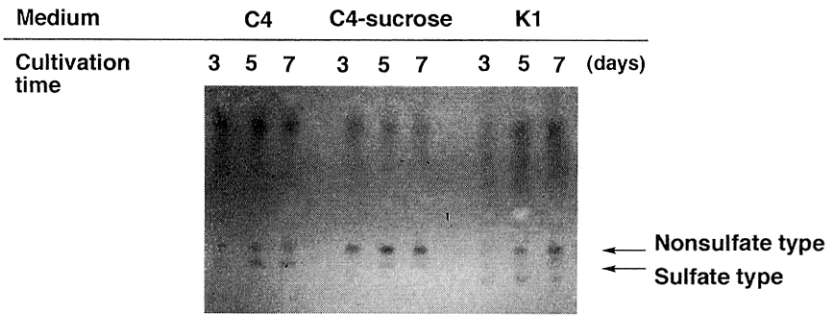


Table 3. Antimicrobial activity against *Mycobacterium phlei* of the broth of *Streptomyces* sp. SN-1061M in the various media.

Carbon source	Mycelium (wet vol (%))	pH	Inhibition zone (mm)			
			B[a]	M[a]	B[b]	M[b]
C4 medium (Glucose)	42	8.3	+	+	16.6	19.7
1. Maltose	41	7.9	+	0	13.9	17.7
2. D-Xylose	44	6.5	10.2	(12.1)	16.0	20.8
3. Lactose	45	7.5	11.9	11.4	18.9	22.0
4. D-Fructose	46	7.8	12.2	11.5	20.6	23.3
5. Sucrose	49	7.9	13.6	12.7	23.4	23.8
6. Inositol	35	7.9	13.1	10.7	20.2	20.0
7. L-Rhamnose	25	8.4	0	0	0	0
8. L-Arabinose	26	4.8	0	0	0	0
9. D-Mannitol	39	7.8	13.2	11.5	20.2	21.1
10. Raffinose	18	8.6	0	0	0	0
11. Salicine	13	8.6	0	0	0	0
12. L-Sorbose	20	8.6	0	0	0	0
13. D-Glucosamine	6	3.7	0	0	0	0

B: Broth filtrate, M: Mycelium extract.
[a] *Escherichia coli* BE 1186, [b] *Mycobacterium phlei* IFO 3158.

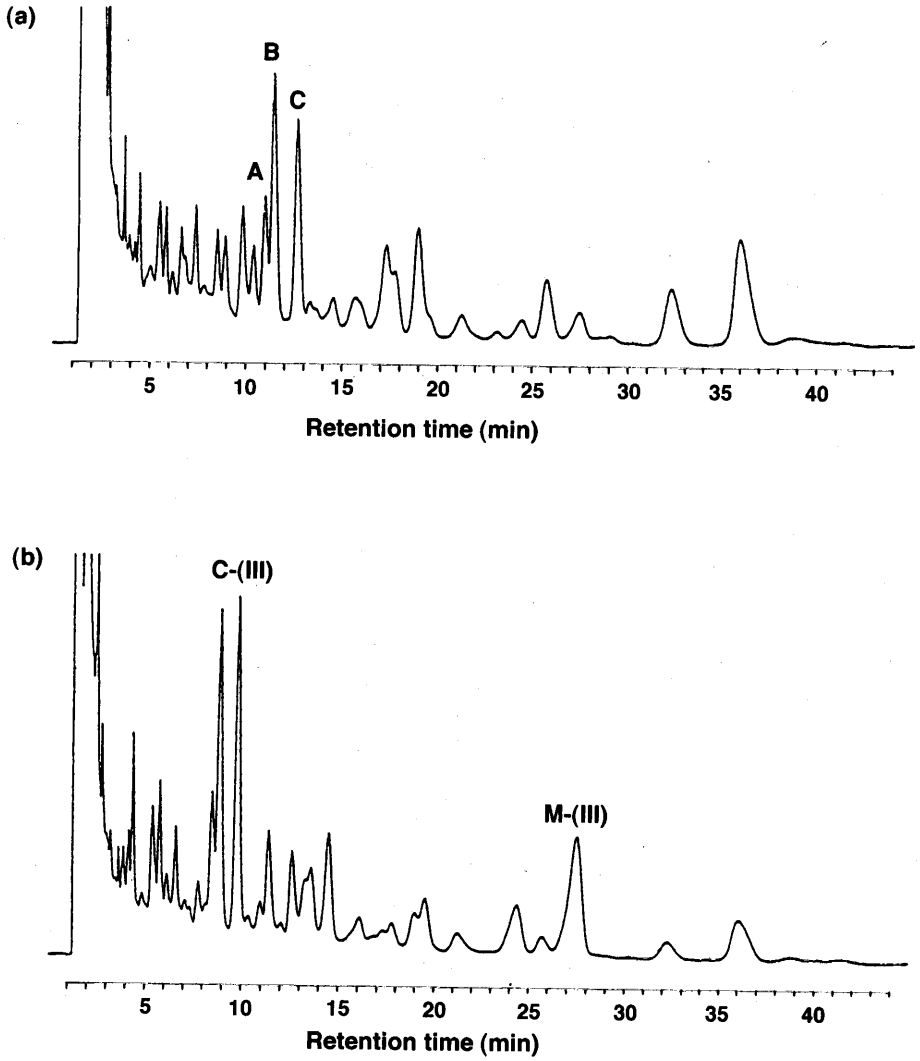
evaluating by antimicrobial activity. Liposidomycins were not produced using L-rhamnose, L-arabinose, raffinose, salicine, L-sorbose or D-glucosamine as carbon sources in C4 medium. Maltose gave the same profile as glucose in the production of liposidomycins.

Using sucrose as the basic carbon source, other combinations of medium components were tested. The most suitable medium composition for the production of the new types of liposidomycins was sucrose 4%, soybean flour 3%, wheat germ (or malt extract) 2% and NaCl 0.6%, pH adjusted to 7.0, designated as K1 medium. TLC analysis pattern using K1 medium is shown in Fig. 3. When wheat germ (or malt extract) was eliminated, liposidomycins were not produced at all, but other components were not so strictly required (data not shown).

Production of New Types of Liposidomycins

The time course of fermentation of strain SN-1061M in a 500 ml Erlenmeyer flask containing each the C4, C4-sucrose and K1 medium is shown in Table 4. At 5 days, activity of the broth filtrate and mycelium extract was almost the same in the C4 and C4-sucrose medium. Using the K1 medium, activity accumulated primarily in the mycelium. Nonsulfated liposidomycins were mainly produced in the C4-sucrose and the K1 media, suggesting depression of the activity of the putative sulfotransferase. Product analyses of the culture with C4 and K1 media by HPLC are shown in Fig. 4. New peaks were detected in the K1 medium in comparison to the C4 medium and were considered to be possible nonsulfated liposidomycins by TLC analysis. Isolation and structure elucidation of these compounds are reported the following paper[11].

Fig. 4. HPLC profiles of the butanol extract of the C4 (a) and K1 (b) media.



Column: CAPCELL PAK (4.6 i.d. × 250 mm), Solvent: CH_3CN - 0.1% diethylamine - HCOOH (pH 4) = 45 : 55, Detector: UV (254 nm), Flow rate: 1.5 ml/minute.

Table 4. A typical time course of fermentation by *Streptomyces* sp. SN-1061M in the C4, C4-sucrose and K1 media (in 500 ml volume Erlenmeyer flask).

		3 day	5 day	7 day
C4 medium	Myc. vol (%)	46	52	37
	pH	7.6	8.4	8.9
	B (mm)	13.0	19.4	18.3
	M (mm)	17.9	20.0	20.0
C4-sucrose medium	Myc. vol (%)	50	57	54
	pH	7.3	8.1	8.8
	B (mm)	16.6	25.1	24.3
	M (mm)	22.0	27.5	27.5
K1 medium	Myc. vol (%)	52	59	63
	pH	5.4	5.3	7.3
	B (mm)	0	11.6	16.9
	M (mm)	13.0	22.9	27.0

Myc. vol: Mycelium volume, B: Inhibition zone of broth filtrate against *Mycobacterium phlei* IFO 3158, M: Inhibition zone of mycelium extract against *Mycobacterium phlei* IFO 3158.

The same results were obtained when glucose in the C4 medium was replaced by other carbon sources such as D-xylose, lactose, D-fructose, sucrose, inositol and D-mannitol (data not shown).

Discussion

Sulfated biomolecules play important roles in many biological processes. Sulfation changes the polarity from hydrophobic to hydrophilic, and the hydrophilicity of a drug sometimes impedes drug permeability through cell membranes. As a consequence we tried to produce the nonsulfate type of liposidomycins from the same strain by changing the medium components. We found that sucrose was the preferable carbon source in the medium and that wheat germ (or malt extract) was essential for production. We established a suitable medium, designated as K1 medium, specifically for the production of nonsulfated liposidomycins. From the time course of production, we observed that nonsulfated compounds were produced followed by the sulfated production. As a result, we considered that sulfated compounds were derived from their nonsulfated by the putative sulfotransferase. We have tried to isolate inhibitors of the sulfotransferase from the K1 medium, but were not successful. Enhanced production of nonsulfated liposidomycins might depend on the growth rate of the producing strain or result from depression of the sulfotransferase in the K1 medium.

References

1) BUGG, T. D. H. & C. T. WALSH: Intracellular steps of bacterial cell wall peptidoglycan biosynthesis: Enzymology, antibiotics, and antibiotic resistance. Nat. Prod. Rep. 9: 199~215, 1992
2) BUGG, T. D. H. & P. E. BRANDISH: From peptidoglycan to glycoproteins: Common features of lipid-linked oligosaccharide biosynthesis; FEMS Microbiology Letters 119: 255~262, 1994
3) ISONO, K.; M. URAMOTO, H. KUSAKABE, K. KIMURA, K. IZAKI, C. C. NELSON & J. A. MCCLOSKEY: Liposidomycins: novel nucleoside antibiotics which inhibit bacterial peptidoglycan synthesis. J. Antibiotics 38: 1617~1621, 1985
4) UBUKATA, M.; K. ISONO, K. KIMURA, C. C. NELSON & J. A. MCCLOSKEY: The structure of liposidomycin B, an inhibitor of bacterial peptidoglycan synthesis. J. Am. Chem. Soc. 110: 4416~4417, 1988
5) UBUKATA, M.; K. KIMURA, K. ISONO, C. C. NELSON, J. M. GREGSON & J. A. MCCLOSKEY: Structure elucidation of liposidomycins, a class of complex lipid nucleoside antibiotics. J. Org. Chem. 57: 6392~6403, 1992
6) KIMURA, K.; N. MIYATA, G. KAWANISHI, Y. KAMIO, K. IZAKI & K. ISONO: Liposidomycin C inhibits phospho-*N*-acetylmuramyl-pentapeptide transferase in peptidoglycan synthesis. Agric. Biol. Chem. 53: 1811~1815, 1989
7) TAMURA, G.: "Tunicamycin", Japan Scientific Press, Tokyo, 1982
8) INUKAI, M.; F. ISONO & A. TAKATSUKI: Selective inhibition of the bacterial translocase reaction in peptidoglycan synthesis by mureidomycins. Antimicrob. Agents. Chemother. 37: 980~983, 1993
9) BRANDISH, P. E.; K. KIMURA, M. INUKAI, R. SOUTHGATE, J. T. LONSDALE & T. D. H. BUGG: Modes of action of tunicamycin, liposidomycin B, and mureidomycin A: inhibition of phospho-*N*-acetylmuramyl-pentapeptide translocase from *Escherichia coli*. Antimicrob. Agents Chemother. 40: 1640~1644, 1996
10) MUROI, M.; K. KIMURA, H. OSADA, M. INUKAI & A. TAKATSUKI: Liposidomycin B inhibits *in vitro* formation of polyprenyl(pyro)phosphate *N*-acetylglucosamine, an intermediate in glycoconjugate biosynthesis. J. Antibiotics 50: 103~104, 1997
11) KIMURA, K.; Y. IKEDA, S. KAGAMI, M. YOSHIHAMA, M. UBUKATA, Y. ESUMI, H. OSADA & K. ISONO: New types of liposidomycins that inhibit bacterial peptidoglycan synthesis and are produced by *Streptomyces*. II. Isolation and structure elucidation. J. Antibiotics 51: 647~659, 1998
12) KIMURA, K.; Y. IKEDA, S. KAGAMI, M. YOSHIHAMA, K. SUZUKI, H. OSADA & K. ISONO: Selective inhibition of the bacterial peptidoglycan biosynthesis by the new types of liposidomycins. J. Antibiotics to be submitted
13) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
14) LECHEVALIER, M. P. & H. A. LECHEVALIER: Chemical

composition as a criteria in the classification of aerobic actinomycetes. Int. J. Syst. Bacteriol. 20: 435~443, 1970

15) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964

16) PRIDHAM, T. G. & H. D. TRESNER: Family VII. Streptomycetaceae Waksman and Henrici 1943, 339. In Bergey's Manual of Determinative Bacteriology, 8th Ed., *Eds.*, R. E. BUCHANAN and N. E. GIBBONS, pp. 747~845, Williams and Wilkins Co., 1974