New Types of Liposidomycins Which Inhibit Bacterial Peptidoglycan Synthesis Produced by *Streptomyces*

Shinobu Kagami, Ken-ichi Kimura*, Yoshikazu Ikeda, Hidetoshi Takahashi,
Hiroo Kusakabe[†], Hiroyuki Osada[†], Kiyoshi Isono[†]
and Makoto Yoshihama

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

(Received Aug. 21, 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. Liposidomycins which have no sulfate moiety, exhibited more potent antimicrobial activity. Thus liposidomycins are the unique antibiotics showing highly specific inhibition toward bacterial peptidoglycan synthesis and could be a useful clinical antibiotic agent.

Introduction

Many commercial antibiotics including β -lactams, vancomycin etc. inhibit the biosynthesis of the cell wall peptidoglycan. The resistance to current antibiotics has caused clinical trouble and need to explore new targets for the antibiotic action^{1,2)}. Liposidomycins were found by screening for peptidoglycan synthesis inhibitors in 1985 and consisted of twelve compounds (A through L), at least on the basis of HPLC profiles³⁾. Liposidomycins A, B and C possess the same 5'-substituted uridine, 5-amino-5-deoxy-ribose-2-sulfate and perhydro-1,4-diazepine moieties, and differ only in the structures of the lipid side chains (Fig. 1.)^{4,5)}. They had been found as unique structure that might mimic the UDP-MurNAc-pentapeptide-PP-lipid of intermediate in cell wall peptidoglycan biosynthesis. The other components also probably differ only

Fig. 1. Structure of liposidomycins.

in the structures of lipid side chains. This has been demonstrated for liposidomycins G and H⁵⁾. Thus liposidomycins A, B and C inhibited peptidoglycan synthesis of *Escherichia coli* at IC_{50} = 0.03 μ g/ml by means of the paper chromatogra-

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

[†] To whom correspondence should be addressed. Tel: +81-285-52-1320, Fax: +81-285-53-1324, E-mail: fvbd7043@infoweb.or.jp

phic method³⁾ and primary inhibition site was determined to be at phospho-*N*-acetylmuramoyl pentapeptide transferase (EC 2.7.8.13, designated as translocase I), the first step in the lipid cycle of peptidoglycan synthesis (Fig. 2)⁶⁾. This target remains unexploited for therapeutic antibiotics.

Recently, inhibition mechanism against translocase I was examined in details by comparison of liposidomycin B, tunicamycin and mureidomycin A⁷⁾. In this study, liposidomycin B and mureidomycin A are shown as slow-binding inhibitor and tunicamycin has reversible inhibition mechanism. Moreover liposidomycin B inhibited formation of lipid intermediates in glycoconju-

gate biosynthesis at high concentrations compared with its active against translocase I⁸⁾. Though liposidomycins are specific inhibitors of translocase I, they did not reveal high anti-microbial activities³⁾. It was considered that permeability into cell membrane of liposidomycin would be limited due to its hydrophilic ionic sulfate moiety.

In this time, when we changed medium components, the mutant strain *Streptomyces* sp. SN-1061M mainly produced new types of liposidomycins which have a structure without sulfate moiety and they had shown more potent antimicrobial activity than the original types of liposidomycins containing sulfate moiety.

Fig. 2. Schematic of peptidoglycan synthesis and site of inhibition of known compounds.

Producing Microorganism⁹⁾

Streptomyces sp. RK-1061 which produced liposidomycins A through L was isolated from soil collected in Misaka-machi, Yamanashi prefecture, Japan. Mutant strain *Streptomyces* sp. SN-1061M which revealed the highest antimicrobial activity against *Mycobacterium phlei* was obtained by UV irradiation. They 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.

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 medium but poor on nutrient and peptone-yeast extract agar media. The strain formed straight sporophores on the aerial mycelium and the spores were cylindrical, 0.5 ~ 0.7 x 0.5 ~ 1.0 μ m in size, having a smooth surface with some wrinkles on SY agar medium (Fig. 3.). No special morphological organs such as whirles, sclerotia, sporangia were observed on the media employed. Detection of LL-diaminopimelic acid in the whole cell hydrolysate of the culture indicated that this strain has a Type I cell wall¹⁰⁾. Based on the taxonomic and other physiological properties, strain RK-1061 is considered to be a member of the genus *Streptomyces*^{11,12)}. On the basis of descriptions in Bergey's Manual of

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)	Moderate	Excellent, gray(e)	Gold brown(3pi)	Dark brown(3pn)
Oatmeal agar (ISP No.3)	Moderate	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. 3. 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 agar medium.

Determinative Bacteriology, strain RK-1061 most resembles *Streptomyces griseosporus* ¹³⁾. However, direct comparison has not yet been carried out.

Production Medium⁹⁾

Streptomyces sp. SN-1061M also produced the original compounds (sulfate type) time dependently in C4 medium (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). From the time course of production, we observed that non-sulfated compounds were produced followed by the sulfated production (Fig. 4.). This suggested to us that the putative sulfotransferase of *Streptomyces* sp. SN-1061M might transfer sulfate to the 2-position of 5-amino-5-deoxy-ribose of liposidomycins¹⁴⁾. 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. 4. The same pattern was observed using the other five carbon sources as well. Table 2 shows that sucrose is a more suitable carbon source than the others, evaluating by antimicrobial activity. Liposidomycins were not produced using L-rhamnose, L-arabinose, raffinose, salicine, L-sorbose or D-

Fig. 4. Analytical TLC patterns of the butanol extract of the C4, C4-sucrose and K1 media.

Table 2. Antimicrobial activity of the broth of *Streptomyces* sp. SN-1061M in the various media.

Carbon source	Mycelium (wet vol (%))	pH	Inhibition zone (mm)			
			B *	M *	B **	M **
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,

* : *Escherichia coli* BE 1186, ** : *Mycobacterium phlei* IFO 3158

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. 4. When wheat germ (or malt extract) was eliminated, liposidomycins were not produced at all, but other components were not so strictly required.

Production and Isolation ¹⁵⁾

The time course of fermentation of strain SN-1061M in a 500 ml Erlenmeyer flask containing each the C4, C4-sucrose and K1 media was examined. 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. New peaks were detected in the K1 medium in comparison to the C4 medium by HPLC (Fig. 5) and were considered to be possible nonsulfated liposido-

mycins by TLC analysis. 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. The flow diagram for isolation of liposidomycins from the broth of K1 medium is shown in Fig. 6. We have isolated 24 compounds including the 5 known compounds. Liposidomycins C-(III) and M-(III) were main components and the others were obtained as minor components. The physico-chemical properties of the new types of liposidomycins were almost identical with those of liposidomycins A, B and C³⁾. They are white amorphous powder and have UV maxima at 260 ~ 262 nm in 50 % aq. MeOH. Rf values on silica gel TLC (MERK, Art 5215) using the solvent (BuOH : AcOH : H₂O = 4 : 1 : 2) are different in compounds with (0.35) or without (0.41) the sulfate moiety. The solubilities of nonsulfated liposidomycins in water are less than those of sulfated liposidomycins. The molecular weights and formulae of liposidomycins were determined by FAB-MS and HRFAB-MS. All liposidomycins, including the known compounds (A, B, C, G and H) are listed in Table 3.

Structure¹⁵⁾

Almost all of the ¹H NMR signals of 19 new liposidomycins were identical with those of liposidomycins A, B and C except for the signals of sulfate and/or 3-methylglutaric acid moieties. The FAB mass spectra (positive and negative modes) revealed the characteristic fragment patterns of liposidomycins⁵⁾. These spectroscopic analyses indicated the following four types of structures: the A-(I) type which has sulfate and 3-methylglutaric acid moieties, the A-(II) type which has a sulfate moiety but no 3-methylglutaric acid moiety, the A-(III) type which has a 3-methylglutaric acid moiety but does not have a sulfate moiety, and the A-(IV) type which does not have either of these moieties, as shown in Fig. 7.

Fig. 5. HPLC profiles of the butanol extract of the C4 (a) and K1 (b) media. Column: CAPCELL PAK (4.6 ϕ x250 mm), Detector: UV (254 nm), Flow rate: 1.5 ml/min, CH₃CN-0.1% DEA-HCOOH (pH 4) = 45:55

Fig. 6. Isolation procedure for liposidomycins.

Table 3. Molecular formulae and weight of liposidomycins.

Compounds	Molecular formula	R (unsaturation)	Molecular weight
Type (I)			
① Z-(I)	$C_{42}H_{65}N_5O_{21}S$	$C_{11}H_{21}$ (1)	1007
* ② B-(I)	$C_{42}H_{67}N_5O_{21}S$	$C_{11}H_{23}$ (0)	1009
* ③ C-(I)	$C_{42}H_{67}N_5O_{21}S$	$C_{11}H_{23}$ (0)	1009
* ④ H-(I)	$C_{43}H_{69}N_5O_{21}S$	$C_{12}H_{25}$ (0)	1023
* ⑤ A-(I)	$C_{44}H_{67}N_5O_{21}S$	$C_{13}H_{23}$ (2)	1033
* ⑥ G-(I)	$C_{44}H_{69}N_5O_{21}S$	$C_{13}H_{25}$ (1)	1035
⑦ L-(I)	$C_{44}H_{71}N_5O_{21}S$	$C_{13}H_{27}$ (0)	1037
⑧ M-(I)	$C_{44}H_{71}N_5O_{21}S$	$C_{13}H_{27}$ (0)	1037
⑨ K-(I)	$C_{46}H_{71}N_5O_{21}S$	$C_{15}H_{27}$ (2)	1061
⑩ N-(I)	$C_{46}H_{73}N_5O_{21}S$	$C_{15}H_{29}$ (1)	1063
Type (II)			
① A-(II)	$C_{38}H_{57}N_5O_{17}S$	$C_{13}H_{23}$ (2)	887
② C-(II)	$C_{36}H_{57}N_5O_{17}S$	$C_{11}H_{23}$ (0)	863
Type (III)			
① X-(III)	$C_{41}H_{65}N_5O_{18}$	$C_{10}H_{21}$ (0)	915
② Y-(III)	$C_{42}H_{63}N_5O_{18}$	$C_{11}H_{19}$ (2)	925
③ Z-(III)	$C_{42}H_{65}N_5O_{18}$	$C_{11}H_{21}$ (1)	927
④ C-(III)	$C_{42}H_{67}N_5O_{18}$	$C_{11}H_{23}$ (0)	929
⑤ V-(III)	$C_{44}H_{65}N_5O_{18}$	$C_{13}H_{21}$ (3)	951
⑥ A-(III)	$C_{44}H_{67}N_5O_{18}$	$C_{13}H_{23}$ (2)	953
⑦ G-(III)	$C_{44}H_{69}N_5O_{18}$	$C_{31}H_{25}$ (1)	955
⑧ M-(III)	$C_{44}H_{71}N_5O_{18}$	$C_{13}H_{27}$ (0)	957
⑨ K-(III)	$C_{46}H_{71}N_5O_{18}$	$C_{15}H_{27}$ (2)	981
⑩ N-(III)	$C_{46}H_{73}N_5O_{18}$	$C_{15}H_{29}$ (1)	983
Type (IV)			
① A-(IV)	$C_{38}H_{57}N_5O_{14}$	$C_{13}H_{23}$ (2)	807
② C-(IV)	$C_{36}H_{57}N_5O_{14}$	$C_{11}H_{23}$ (0)	783

* : Already reported compounds ^{3, 5)}.

1. Type (I)

3. Type (III)

2. Type (II)

4. Type (IV)

Fig. 7. Structures of four types of liposidomycins.

Table 4. Antimicrobial activities of the sulfated and nonsulfated liposidomycins.

Microorganism	Inhibition zone (20 µg/disc, mm)											
	A	A-(III)	C	C-(III)	M	M-(III)	K	K-(III)	N	N-(III)	Z	Z-(III)
<i>Escherichia coli</i> AB 1157	0	0	0	0	0	0	0	0	0	0	0	0
<i>Escherichia coli</i> BE 1186	+	17.8	0	14.3	0	(+)	(+)	13.5	0	15.2	-	18.8
<i>Staphylococcus aureus</i> IFO 12732	0	0	0	0	0	0	0	0	0	0	0	0
<i>Bacillus subtilis</i> IFO 3513	0	21.2	0	17.8	+	14.8	(+)	13.0	(+)	12.9	0	22.2
<i>Mycobacterium phlei</i> IFO 3158	18.9	24.8	22.1	30.3	27.2	25.7	28.6	23.3	23.1	20.8	-	26.3
<i>Candida albicans</i> IFO 5994	0	0	0	0	0	0	0	0	0	0	0	0

+ : < 10 mm, () : Partial inhibition zone, - : Not tested.

Biological Activities¹⁶⁾

Antimicrobial activities of sulfated (Type I)) and nonsulfated (Type III)) liposidomycins against some Gram-positive and -negative bacteria are listed in Table 4. Most of the nonsulfated liposidomycins have more potent antimicrobial activity against *Mycobacterium phlei*, *Escherichia coli* BE and *Bacillus subtilis* than the sulfated compounds.

Peptidoglycan inhibition potencies were recognized as A-(I) > A-(III) > A-(IV) > A-(II). Inhibition potencies of those liposidomycins were 30 ~ 500 times more potent than that of tunicamycin¹⁷⁾. 3-Methylglutaric acid moiety had more important role against peptidoglycan inhibition.

Dol-PP-GlcNAc biosynthesis inhibition potencies were recognized as A-(III) = A-(IV) > A-(II)

= A-(I). Inhibition potencies were 30~500 times less than that of tunicamycin¹⁷⁾. Contrary to the case of peptidoglycan inhibition, sulfate moiety in the molecule might have important role against Dol-PP-GlcNAc biosynthesis. All types of liposidomycins did not inhibit the growth of BALB/3T3 cells even at 25 µg/ml and IC₅₀ of tunicamycin was 0.05 µg/ml.

Though type (III) and (IV) had almost the same potency as Type (I) and (II) against peptidoglycan synthesis *in vitro*, they had the improved antimicrobial activity against bacteria, especially *Mycobacterium phlei*. This indicated that type (III) and (IV) might be able to penetrate through the cell membrane and inhibit the translocase I located in inner membrane^{14,16)}. Those results indicated that nonsulfated liposidomycin was highly potent and selective inhibitor of translocase I and could be a useful clinical antibiotic agent.

REFERENCES

- 1) Bugg, T.D.H. and 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. and 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 and 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 and 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 and 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 and K. Isono : Liposidomycin C inhibits phospho-N-acetylmuramyl-pentapeptide transferase in peptidoglycan synthesis. *Agric. Biol. Chem.* 53 : 1811-1815, 1989
- 7) Brandish, P.E. ; K. Kimura, M. Inukai, R. Southgate, J.T. Lonsdale and 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
- 8) Muroi, M. ; K. Kimura, H. Osada, M. Inukai and 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
- 9) Kimura, K. ; S. Kagami, Y. Ikeda, H. Takahashi, M. Yoshihama, H. Kusakabe, H. Osada and K. Isono : New types of liposidomycins that inhibit bacterial peptidoglycan synthesis and are produced by *Streptomyces*. I. Producing Organism and Medium Components. *J. Antibiotics* 51 : 640-646, 1998
- 10) Becker, B. ; M.P. Lechevalier, R.E. Gordon and H.A. Lechevalier : Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. *Appl. Microbiol.* 12 : 421-423, 1964
- 11) Shirling, E.B. and D. Gottlieb : Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* 16 : 313-340, 1966
- 12) Lechevalier, M.P. and H.A. Lechevalier : Chemical composition as a criteria in the classification of aerobic actinomycetes. *Int. J. Syst. Bacteriol.* 20 : 435-443, 1970
- 13) Pridham, T.G. and 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
- 14) Kimura, K. : "Sulfation of antibiotic and its biological activity", *Bioscience and Industry* (in Japanese), 56, 331-332, 1998
- 15) Kimura, K. ; Y. Ikeda, S. Kagami, M. Yoshihama, M. Ubukata, Y. Esumi, H. Osada and K. Isono : New types of liposidomycins that inhibit bacterial peptidoglycan synthesis and are produced by *Streptomyces*. II. Isolation and Structure Elucidation. *J. Antibiotics* 51 : 647-654, 1998
- 16) Kimura, K. ; Y. Ikeda, S. Kagami, M. Yoshihama, K. Suzuki, H. Osada and K. Isono : Selective

inhibition of the bacterial peptidoglycan biosynthesis by the new types of liposidomycins. J. Antibiotics. in press.

17) Tamura, G. : "Tunicamycin", Japan Scientific Press, Tokyo, 1982