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

II. Isolation and Structure Elucidation

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Various new liposidomycins were isolated from a culture of the strain *Streptomyces* sp. SN-1061M by changing medium components and they were classified into four types (I~IV) based on their structures. They were purified by butanol extraction, silica gel and LH-20 column chromatographies, and high performance liquid chromatography on ODS columns. Type (I) has the original structure which has sulfate and 3-methylglutaric acid moieties. Type (II) has no 3-methylglutaric acid moiety and type (III) has no sulfate moiety. Type (IV) has neither moiety. Type (III) and (IV) compounds, which have no sulfate moiety, exhibited more potent antimicrobial activity.

It has been reported that liposidomycins are produced by *Streptomyces* sp. RK-1061 and consist of at least twelve components (A through L) on the basis of HPLC profiles[1]. They have a common core structure that possess 5'-substituted uridine, 5-amino-5-deoxyribose-2-sulfate, and perhydro-1,4-diazepine moieties, and only differ in the structures of the lipid side chains[2,3]. In the previous paper, we reported that when we changed medium components, the mutant strain *Streptomyces* sp. SN-1061M mainly produced new types of liposidomycins[4].

In this paper, we report isolation and the structure elucidation of new types of liposidomycins that lack sulfate and/or 3-methylglutaric acid moieties found in the original liposidomycins.

Materials and Methods

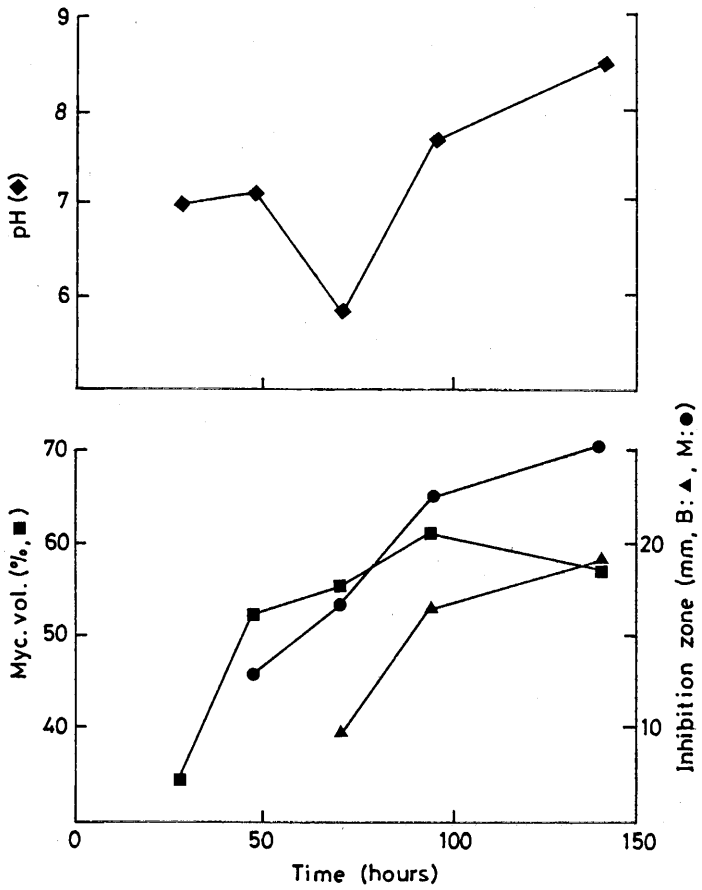
Microorganism

The mutant strain of *Streptomyces* sp. RK-1061, designated SN-1061M, was used in this experiment.

Fermentation

The seed medium and the production medium for the production of new types of liposidomycins composed of sucrose 4%, soybean flour 3%, wheat germ 2%, and NaCl 0.6%, pH 7.0 (designated K1 medium). A piece of an SY agar slant culture was inoculated into 70 ml of the medium in a 500 ml Erlenmeyer flask. The flask was 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 for 3 days at 27°C on a rotary shaker. After the incubation, 140 ml of seed culture was transferred into a 30-liter jar fermentor con-

Fig. 1. A typical time course of fermentation by *Streptomyces* sp. SN-1061M in K1 medium (in 500 ml Erlenmeyer flask).



Myc.vol.: Mycelium volume, B: Broth filtrate, M: Mycelium extract.
Inhibition zone shows the antimicrobial acrivity against *Mycobacterium phlei*.

taining 18 liters of the medium. The fermentor was operated for 96 hours at 27°C with agitation at 180 rpm and aeration of 18 liters/minute. A typical time course for the fermentation of strain SN-1061M in a 500 ml Erlenmeyer flask containing the K1 medium is shown in Fig. 1.

Antimicrobial Activity

Antimicrobial activity was estimated by the paper disc agar diffusion method, comparing sulfated and non-sulfated liposidomycins.

Physico-chemical Properties

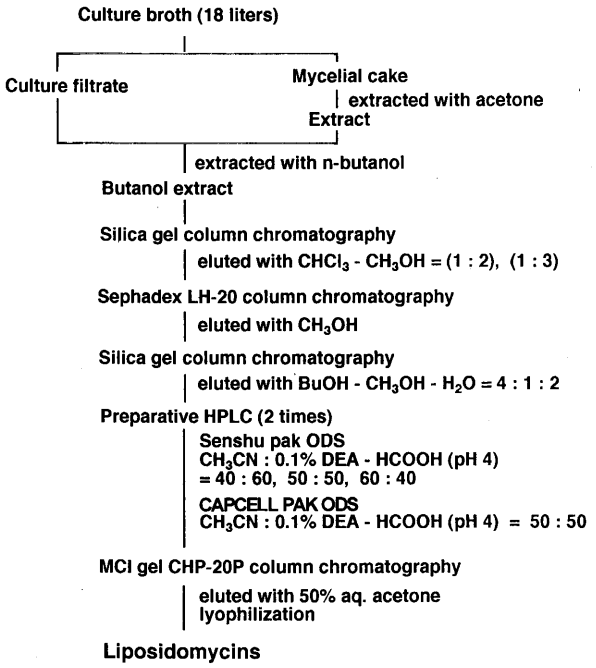
FAB-MS and HRFAB-MS were recorded on a JEOL model JMS HX-110 mass spectrometer. The UV spectrum was measured on an HITACHI model U-3210 spectrophotometer. The ^1H NMR spectrum was measured on a JEOL α500 instrument.

Results

Isolation of New Types of Liposidomycins

The flow diagram for isolation of new types of liposidomycins is shown in Fig. 2. The culture broth (18 liters) was centifuged and the mycelial cake (2.9 kg) was obtained. From the acetone extract of the mycelial cake combined with the culture filtrate, the active material was extracted with *n*-butanol and concentrated to a crude complex (60.7 g). This was suspended in $CHCl_3$: MeOH (3 : 1) and chromatographed on a silica gel column (8 × 21 cm), washing with $CHCl_3$: MeOH (2 : 1 and 1 : 1) and eluting with $CHCl_3$: MeOH (1 : 2 and 1 : 3). The active fraction was concentrated *in vacuo* and lyophilized. The crude powder (14.3 g) was dissolved in a small amount of MeOH and loaded onto a Sephadex LH-20 column (3 × 79 cm) using MeOH as the eluent. The active fraction was concentrated *in vacuo* and lyophilized (10.3 g). This was dissolved in BuOH : MeOH : H_2O

Fig. 2. Isolation procedure for liposidomycins.



(4 : 1 : 2) and rechromatographed on a silica gel column (8 × 21 cm) with the same solvent. The crude powder (6.5 g) was dissolved in a small amount of MeOH, and final purification was carried out by preparative HPLC using Senshu pak ODS (20 × 250 mm) and CAPCELL PAK ODS (20 × 250 mm) with successive elution using 40%, 50% and 60% CH_3CN-0.1% diethylamine-HCOOH (pH 4). After evaporating the solvent, active fractions were charged on an MCI GEL CHP-20P column, washing with water and eluting the active compound with 50% aq. Me_2CO. After removal of Me_2CO, lyophilization gave white powders. We have isolated 24 compounds including the 5 known compounds. About 5 mg of liposidomycins C-(III) and M-(III), the main components, were obtained, and 0.1 ~ 2 mg of the others were obtained as minor components.

Physico-chemical Properties of the New Types of Liposidomycins

The physico-chemical properties of the new types of liposidomycins were almost identical with those of liposidomycins A, B and C[1]. 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 ($BuOH : AcOH : H_2O = 4 : 1 : 2$) are different in compounds with or without 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 1. An HPLC profile of the typical four types of liposidomycins (A-(I) ~ A-(IV)) is shown in Fig. 3.

Structure Elucidation

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. Type A-(I) was the original compound (liposidomycin A) which had sulfate and 3-methylglutaric acid moieties. The ^1H NMR spectra of A-(II) and A-(IV) did not show the signals of 3-methylglutaric acid, 2b (δ 2.43, 2.15), 3b (δ 2.37, m), 3b-CH_3 (δ 1.0, d, $J = 7$ Hz) and 4b (δ 2.25, 2.19) present in that of A-(I)[3]. Additionally, the 2a and 3a signals of the lipid side chain in A-(II) and A-(IV) were shifted to δ 5.87 (d, $J = 15.1$ Hz) and δ 7.00 (dt, $J = 10.8$, 15.1 Hz), respectively. A conjugated double bond could be formed by the elimination of 3-methylglutaric acid from the 3a position. In the spectra of A-(III) and A-(IV), the 1″ (δ 5.52, br s) and 2″ (δ 4.67, d, $J = 5.4$ Hz) signals of the 5-amino-5-deoxyribose moiety in A-(I), which has a sulfate moiety at 2″-position, shifted to δ 5.15 and δ 4.26, respectively. This upfield shift of proton signals at the 1″- and 2″-positions and the molecular formulae suggested that the sulfated hydroxyl group at the 2″-position in liposidomycin A-(I) was replaced by a hydroxyl group in A-(III) and A-(IV).

The FAB mass spectrum (negative mode) of A-(II) showed an m/z 886 $(M-H)^-$ ion and the abundant fragment ion m/z 592 $(M-(\text{lipid}+CO_2))^-$. These ions originate from the negatively charged sulfate as observed for liposidomycin A[3]. While the FAB mass spectrum (positive mode) of A-(III) showed m/z 954 $(M+H)^+$ and m/z 823 $(M-(\text{amino sugar})+H)^+$ ions. Other sequential fragment ions were also observed: m/z 711 $(M-(\text{amino sugar}+\text{uracil})+H)^+$, m/z 579 $(M-(\text{amino sugar}+\text{uracil}+\text{ribose})+H)^+$ and m/z 427 $(M-(\text{lipid}+\text{amino sugar})+H)^+$. The FAB mass spectrum (positive mode) of A-(IV) showed m/z 808 $(M+H)^+$ and m/z 677 $(M-(\text{amino sugar})+H)^+$ ions as reported previously (Fig. 4.)[3].

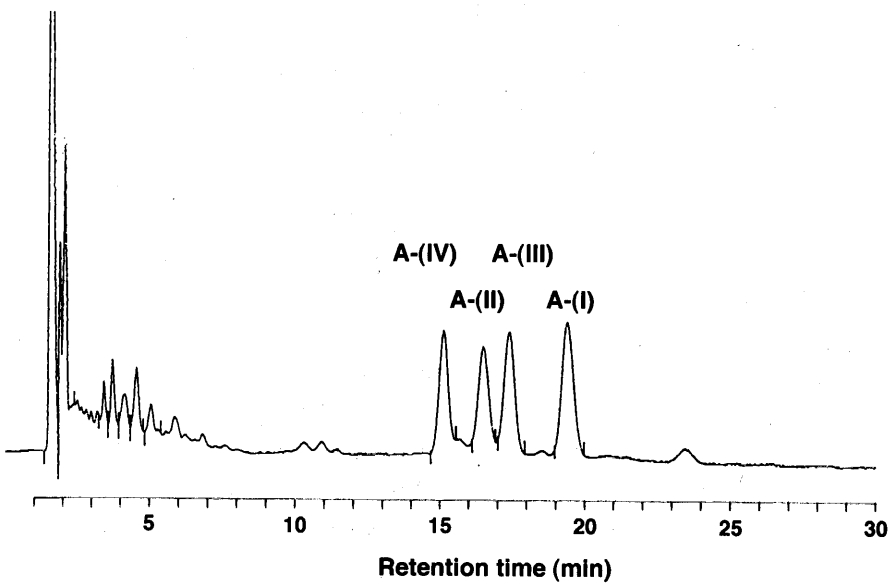
These spectroscopic analyses indicated the following four types of structures: the A-(I) type which has sulfate and 3-methylglutaric acid moieties, the A-(II) type which has a sulfate moiety but no 3-methylglutaric acid moiety,

Table 1. Molecular formulae and weights 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)[a]	$C_{42}H_{67}N_5O_{21}S$	$C_{11}H_{23}$ (0)	1009
③ C-(I)[a]	$C_{42}H_{67}N_5O_{21}S$	$C_{11}H_{23}$ (0)	1009
④ H-(I)[a]	$C_{43}H_{69}N_5O_{21}S$	$C_{12}H_{25}$ (0)	1023
⑤ A-(I)[a]	$C_{44}H_{67}N_5O_{21}S$	$C_{13}H_{23}$ (2)	1033
⑥ G-(I)[a]	$C_{44}H_{69}N_5O_{21}S$	$C_{13}H_{25}$ (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)	$C_{44}H_{69}N_5O_{18}$	$C_{13}H_{25}$ (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

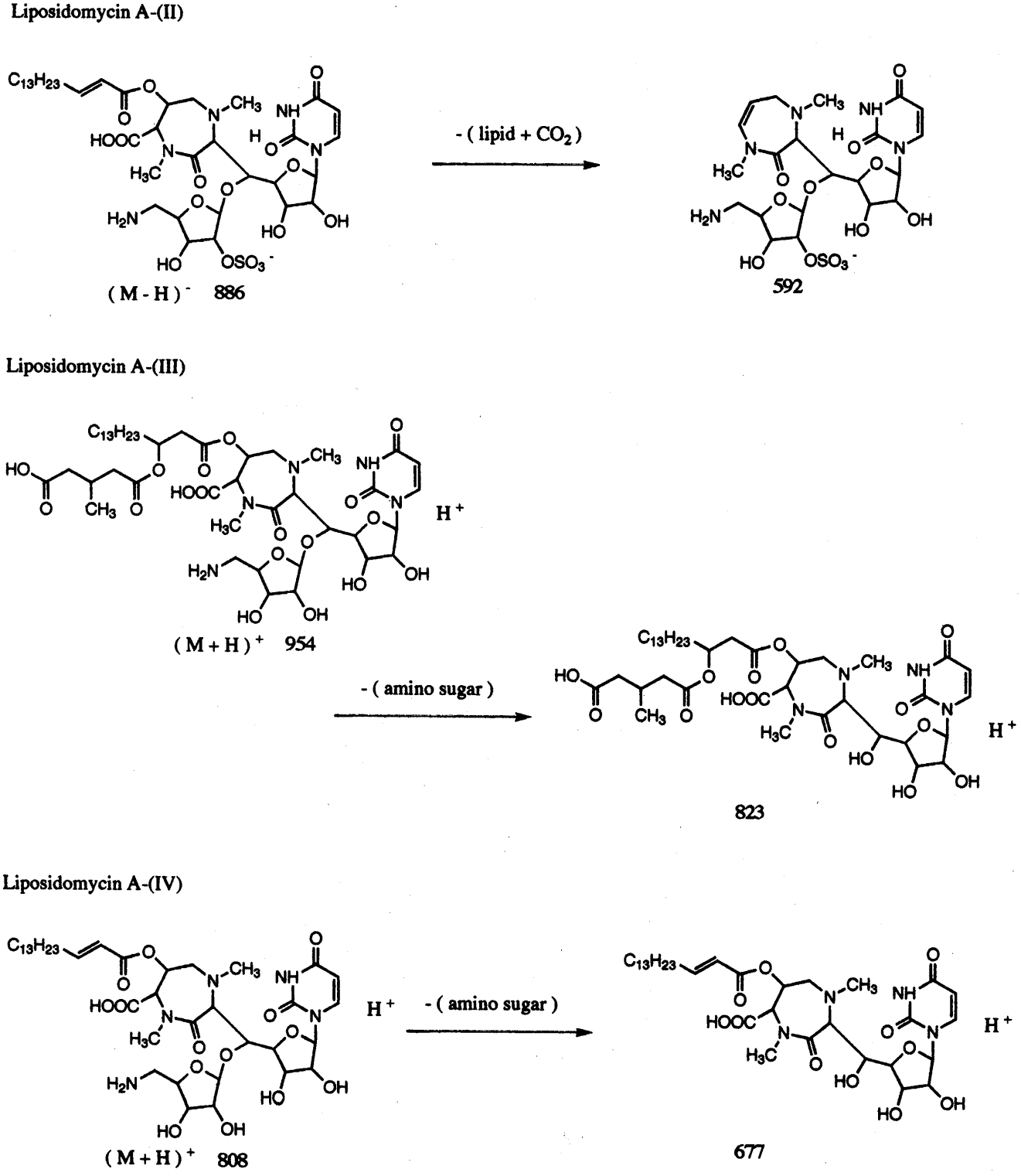
[a] Already reported compounds[3].

Fig. 3. HPLC analysis pattern of the typical four types of liposidomycins (A-(I) ~ A-(IV)).



Column: CAPCELL PAK (4.6 i.d. × 250 mm), Solvent: CH_3CN-0.1% diethylamine-HCOOH (pH 4) = 40 : 60, Detector: UV (260 nm), Flow rate: 1.5 ml/minute.

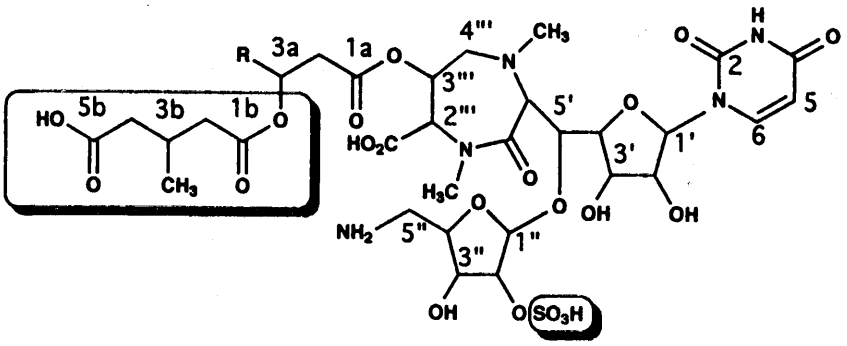
Fig. 4. FAB-MS analysis of liposidomycins A-(II), A-(III) and A-(IV).



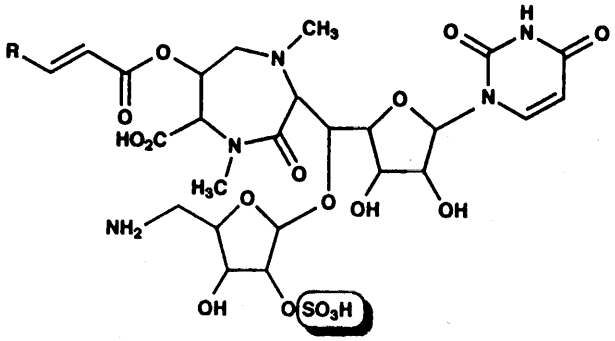
the A-(III) type which has a 3-methylglutaric acid moiety but does not have a sulfate moiety, and the A-(IV) type which does not have either of these moieties, as shown in Fig. 5. Other liposidomycins were also classified into the four types in a similar manner. Liposidomycins A-(I)~A-(IV) have the same lipid moiety (3-(3'-methylglutaryl)-7,10-hexadecadienoic acid) which shows signals in the ^1H NMR spectra characteristic of a methylene-interrupted diene at δ 2.77 (9a, t, $J=6.6$ Hz)[3]. The structures of the lipid moieties of all liposidomycins

Fig. 5. Structures of four types of liposidomycins.

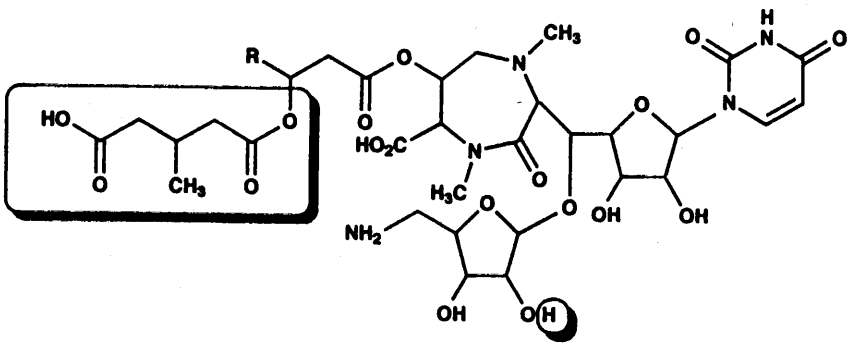
1. Type (I)



2. Type (II)



3. Type (III)



4. Type (IV)

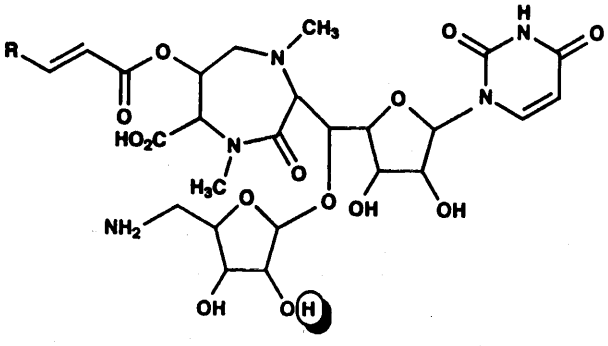


Table 2. Antimicrobial activities of the sulfated and nonsulfated liposidomycins.

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)
Escherichia coli AB 1157	0	0	0	0	0	0	0	0	0	0	0	0
Escherichia coli BE 1186	+	17.8	0	14.3	0	(+)	(+)	13.5	0	15.2	−	18.8
Staphylococcus aureus IFO 12732	0	0	0	0	0	0	0	0	0	0	0	0
Bacillus subtilis IFO 3513	0	21.2	0	17.8	+	14.8	(+)	13.0	(+)	12.9	0	22.2
Mycobacterium phlei IFO 3158	18.9	24.8	22.1	30.3	27.2	25.7	28.6	23.3	23.1	20.8	−	26.3
Candida albicans IFO 5994	0	0	0	0	0	0	0	0	0	0	0	0

+: <10 mm, −: not tested.
( ): Partial inhibition zone.

are now under investigation using a tandem mass spectral (MS/MS/MS) technique and will be reported elsewhere.

Antimicrobial Activities of New Types of Liposidomycins

Antimicrobial activities of sulfated (Type (I)) and nonsulfated (Type (III)) liposdiomycins against some Gram-positive and -negative bacteria are listed in Table 2. Most of the nonsulfated liposidomycins have more potent antimicrobial activity against *Mycobacterium phlei*, *Escherichia coli* BE and *Bacillus subtilis* than the sulfated compounds. Biological activities of the four types of liposidomycins will be reported in detail elsewhere[5].

Discussion

As reported in the previous paper, activity primarily accumulated in the mycelium when K1 medium was used, and we have mainly isolated new types of liposidomycins that lack sulfate and/or 3-methylglutaric acid moieties present in the previously reported structures. However, liposidomycins that lack the 3-methylglutaric acid moiety (Type (II) and (IV)) were minor components. In this case, we were able to isolate A-(II) and A-(IV), but the amounts of C-(II) and C-(IV) were insufficient for isolation. They could be detected only by FAB-MS. Under the culture conditions, nonsulfated types were found as main products, and this strain produced liposidomycins containing unbranched C_{14} and C_{16} fatty acid moieties, especially liposidomycins C-(III) and M-(III), abundantly. However, in a 200-liter tank fermenation containing 120 liters of the same medium, B-(III) and L-(III), which have iso-type lipid moieties were produced. We have designated the original liposidomycins alphabetically correspondiong to the HPLC elution order[1]. Other types of liposidomycins ((II)~(IV)) were named depending on the molecular formula of the lipid moiety corresponding to that of the original type ((I)). By changing the medium components and the fermentation conditions (especially carbon sources, aeration conditions and incubation time), it may be possible to isolate all four types of all the liposidomycins. Thus a nomenclature system such as the one devised for the tunicamycins may be needed[6].

The antimicrobial activity of type (I) compounds (M, K and N) against *Mycobacterium phlei* in Table 2 was a little stronger than that of the type (III) compounds. When same experiment was performed at 2 μg/disc of K and K-(III), the result was reversed. Detailed structure-activity relationship will be possible when the synthesis of liposidomycin is accomplished.

Acknowledgments

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References

1) 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
2) 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
3) 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

4) KIMURA, K.; S. KAGAMI, Y. IKEDA, H. TAKAHASHI, M. YOSHIHAMA, H. KUSAKABE, H. OSADA & 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

5) KIMURA, K.; Y. IKEDA, S. KAGAMI, M. YOSHIHAMA, K. SUZUKI, H. OSADA & K. ISONO: Selective inhibition of the bacrerial peptidoglycan biosynthesis by the new types of liposidomycins. J. Antibiotics to be submitted.

6) ECKARDT, K.: Tunicamycins, streptovirudins, and corynetoxins, a special subclass of nucleoside antibiotics. J. Nat. Prod. 46: 544～550, 1983