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Control of Liposidomycin Production through Precursor-directed Biosynthesis

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Liposidomycin, a potent inhibitor of bacterial peptidoglycan biosynthesis, represents the main component of a group of over 24 closely related, fatty acyl nucleoside antibiotics produced by *Streptomyces*. Exogeneously supplied myristic acid or palmitic acid resulted in the almost exclusive production of liposidomycin C-(III) and/or M-(III). Exogeneously supplied [1-^{13}C]-palmitic acid was incorporated directly into liposidomycin M-(III) as judged by the FAB-MS and ^{13}C NMR spectra.

Liposidomycin is a complex of antibiotics produced by *Streptomyces griseosporeus*, having a common core structure that possesses 5′-substituted uridine, 5-amino-5-deoxyribose-2-sulfate, perhydro-1,4-diazepine moieties and different fatty acid side chains[1~3]. Recently, four new types of liposidomycins that lack sulfate and/or 3-methylglutaric acid moieties were isolated by changing medium composition[4,5]. Among them, non-sulfate type liposidomycins (type (III) and (IV) compounds) have potent antimicrobial activity. Major forms are liposidomycins C-(III) and M-(III) which contain C14 and C16 fatty acid side chains, respectively[6]. The primary target for liposidomycin C was determined to be the *mraY* gene product (Phospho-*N*-acetylmuramoyl pentapeptide transferase (EC 2.7.8.13), designated as translocase I), which catalyzes the first step in the lipid cycle of peptidoglycan biosynthesis[7,8]. While *mraY* is essential for growth in *Escherichia coli*, this target remains unexploited for therapeutic antibiotics[9]. The natural occurrence of the myristoyl and palmitoyl factors, liposidomycins C-(III) and M-(III) has been too low to permit isolation in sufficient quantity for industrial development under standard fermentation conditions.

Precursor-directed biosynthesis is the derivatization of a secondary metabolite by incorporating biosynthetic precursor analogues into the fermentation broth of the producing organism. Such an approach is worth applying to the field of industrial antibiotic production[10]. In the lipopeptide antibiotic complex, A21978 and A54145, precursor (fatty acid) directed biosynthesis has already succeeded[11,12]. In this paper, we report the successful incorporation of foreign fatty acids into the nucleoside antibiotic, liposidomycin, leading to increased production.

Materials and Methods

Strain

Streptomyces griseosporeus strain SN-1061M is a high producing mutant obtained following UV treatment of the parent (*S. griseosporeus* RK-1061) as noted previously[4].

Cultivation

The spore and mycelial-suspension used for inoculation was produced by *S. griseosporeus* SN-1061M grown for 14 days at 27°C on SY agar medium consisting of soluble starch (10 g), yeast extract (5 g) and agar (15 g) per liter of

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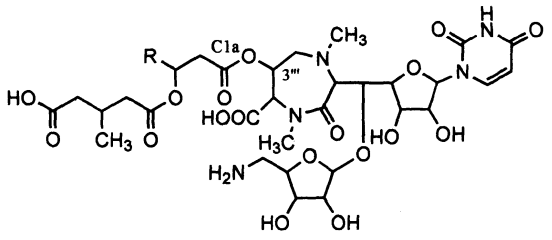
distilled water (pH 7.0). A portion of the slant culture was inoculated into 70 ml of a medium consisting of sucrose (40 g), soybean flour (30 g), malt extract (20 g) and NaCl (6 g) per liter (pH 7.0) in 500 ml Elrenmeyer flasks. After incubation for 3 days at 27°C, 1 ml of the fermentation broth was added to 70 ml of the same medium containing an aliquot of myristic or palmitic acid dissolved in 1 ml of DMSO in 500 ml Erlenmeyer flasks which were then incubated for 120~160 hours at 27°C on a rotary shaker (200 rpm).

Fig. 1. Structures of liposidomycins C-(III) and M-(III).



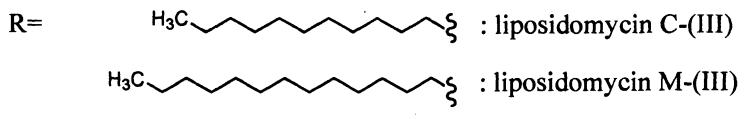


Table 1. Production of liposidomycins C-(III) and M-(III) in fermentation broth supplemented with myristic acid (a) or palmitic acid (b).

(a)

Myristic acid (mg/70 ml)	Liposidomycin (mg/l)		Relative ratio	
	C-(III)	M-(III)	C-(III)	M-(III)
0	39.2	34.4	1.0	1.0
100	88.9	28.6	2.3	0.8
300	126.2	22.1	3.2	0.6
1000	145.2	17.9	3.7	0.5
3000	78.5	16.7	2.0	0.5
10000	14.2	16.5	0.4	0.5

(b)

Palmitic acid (mg/70 ml)	Liposidomycin (mg/l)		Relative ratio	
	C-(III)	M-(III)	C-(III)	M-(III)
0	39.2	34.4	1.0	1.0
100	46.9	66.3	1.2	1.9
300	92.3	115.6	2.4	3.4
1000	140.7	175.1	3.6	5.1
3000	150.7	168.7	3.8	4.9
10000	11.7	16.7	0.3	0.5

Quantification is based on peak area following HPLC.

Analytical Methods

Fermentation broth was separated by centrifugation. The mycelial cake was homogenized with an equal volume of MeOH and concentrated *in vacuo* to remove the MeOH. The combined filtrates (each 1 ml portion) were extracted with 1 vol of BuOH and the solvent was evaporated. The extract was dissolved in 100 μl of MeOH and 10 μl was subjected to HPLC analysis in order to quantify liposidomycin. HPLC conditions have been described previously[4,5].

Isolation

Liposidomycins C-(III) and M-(III) were isolated directly from ten pooled flasks consisting of 70 ml each of the fermentation broth containing 1 g of myristic or palmitic acids as described previously[5]. Identification of liposidomycin was done by HR-FABMS, ^{1}H NMR and the retention time on HPLC. [1-^{13}C]-palmitic acid was purchasd from Nihon Sanso Co. Ltd. Other chemicals were reagent grade. FAB-MS and NMR measurements were performed using JEOL JMS-HX110 mass and GSX-α500 NMR spectrometers. The structures of liposidomycins C-(III) and M-(III) are shown in Fig. 1.

Assay of Antimicrobial Activity

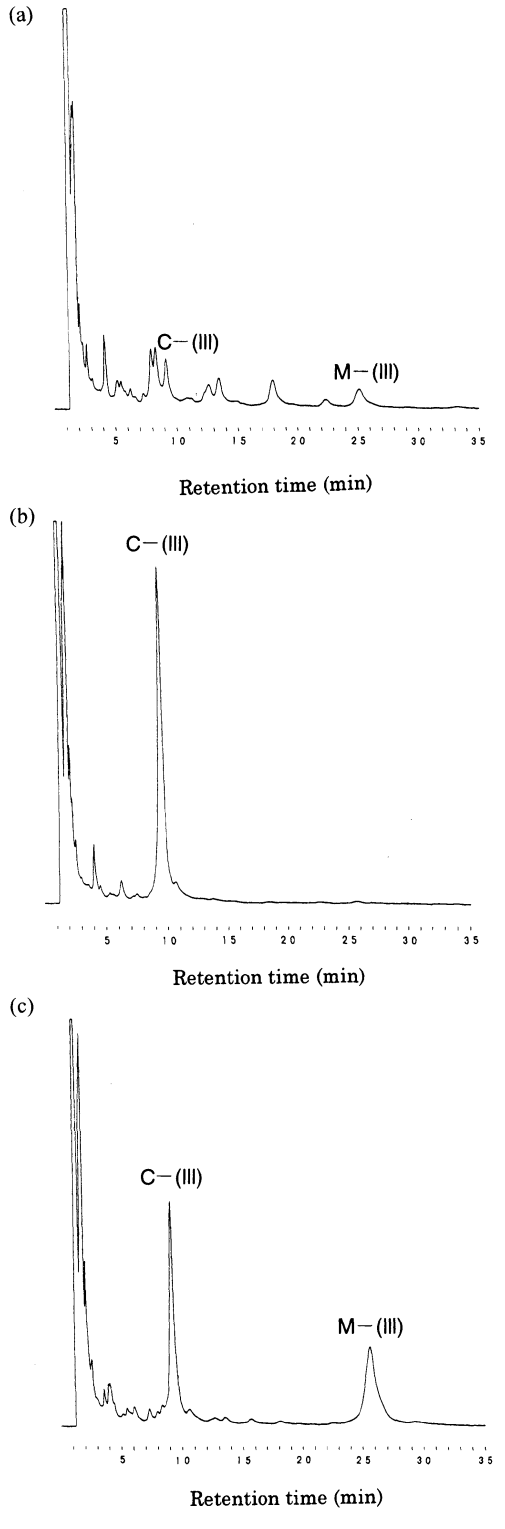
Antimicrobial activity against *Mycobacterium phlei* was measured by the paper disc method for 2 days at 37°C on the medium consisting of glycerin (30 g), Meat extract (5 g), Peptone (10 g), NaCl (3 g) and agar (15 g) per liter (pH 6.8).

Results and Discussion

The effect of myristic acid and palmitic acid on the fermentation production of liposidomycins C-(III) and M-(III) having the constituent fatty acids at position 3‴ was investigated. Addition of myristate to the fermentation increased liposidomycin C-(III) production in a dose-dependent manner up to 1000 mg/70 ml. At doses higher than 1000 mg/70 ml, the fatty acid was inhibitory (Table 1(a)). The yield of liposidomycin C-(III) with myristate added at 1000 mg/70 ml was 3.7 times higher than that obtained with no addition (Table 1, Fig. 2(a), 2(b)). On the other hand, myristate had no significant effect on liposidomycin M-(III) production.

Addition of palmitate to the fermentation increased liposidomycin M-(III) production in a dose-dependent manner up to 1000 mg/70 ml. At higher doses, the fatty acid was inhibitory (Table 1(b)). Maximum production of

Fig. 2. HPLC profiles of liposidomycins.



(a) Natural fermentation, (b) Myristic acid-supplemented fermentation, (c) Palmitic acid-supplemented fermentation.

liposidomycin M-(III) (at 1000 mg/70 ml) was about 5.1 times higher than when no palmitic acid was added (Table 1, Fig. 2(a), 2(c)). Palmitate addition not only increased liposidomycin M-(III) production but also improved the yield of liposidomycin C-(III) which contains a C14 acyl chain. The yield was 3.8 times higher than the control when 3000 mg/70 ml of myristate was added to the fermentation. In this case the exogenously added palmitate is presumably being incorporated into the C-(III) component of liposidomycin after losing a two-carbon unit by β-oxidation (Fig. 2(c)).

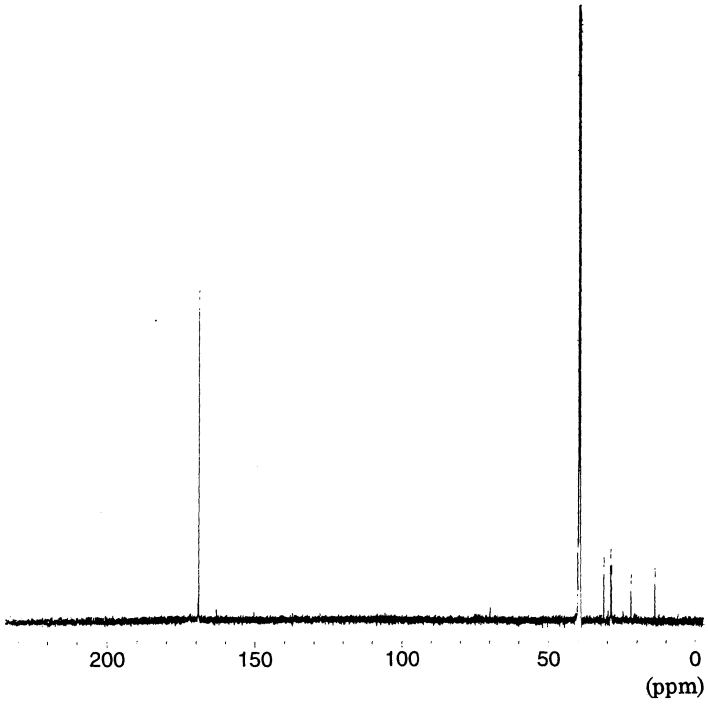
The yields of liposidomycins C-(III) and M-(III) from the fermentation broths are shown in Table 2. With 1 g per 70 ml of the medium addition of palmitate, 4.4 and 2.6 times more liposidomycins C-(III) and M-(III), respectively were obtained as compared with the control. Increase in the yield of liposidomycins componenets by the presumed incorporation of exogenous myristate and palmitate suggested the feasibility of feeding other lipids to produce new derivatives with novel acyl chain analogues. However, a number of lipid precursors tested (*n*-capronic acid [C6], capric acid [C10] and behenic acid [C22]) were not successful due to their toxicity or insolubility in the medium at 1 g/70 ml (data not shown).

In order to determine if the increase in liposidomycin production was due to the direct incorporation of added

Table 2. Liposidomycins C-(III) and M-(III) isolated from fermentation broths.

	Liposidomycin C-(III) (mg/l (relative ratio))	Liposidomycin M-(III) (mg/l (relative ratio))
Control	3.4 (1.0)	4.1 (1.0)
Palmitic acid (1g/70 ml)	15.1 (4.4)	10.6 (2.6)

Fig. 3. ^{13}C NMR spectrum of liposidomycin M-(III) from the producing strain fed [1-^{13}C]-palmitic acid (125 MHz, DMSO-d_6).



fatty acid, isotopic [1-^{13}C]-palmitic acid was added to the fermentation broth. After fermentation, liposidomycin M-(III) was isolated by HPLC and analyzed by HR-FABMS and ^{13}C NMR. The ^{13}C NMR spectrum of the isolated liposidomycin M-(III) indicated enrichment at 169.1 ppm, the signal assigned to the C1a carbonyl carbon (Fig. 3). The molecular formula was determined by HR-FABMS to be $^{12}C_{43}{}^{13}CH_{71}N_5O_{18}$ (Found: $(M+H)^+$, *m/z* 959.4908; calculated: $(M+H)^+$, *m/z* 959.4906), which was one mass unit larger than the molecular formula of the natural liposidomycin M-(III) ($C_{44}H_{71}N_5O_{18}$). The one mass unit increase in the ^{13}C-labeled liposidomycin M-(III) demonstrates that [1-^{13}C]-palmitic acid was directly incorporated into liposidomycin M-(III). On the other hand, liposidomycin C-(III) isolated from the same incorporation study was shown to have a molecular ion $(M+H)^+=930$, which is the same as the natural liposidomycin C-(III) ($C_{42}H_{69}N_5O_8$). This result suggests that [1-^{13}C]-palmitic acid had lost a $-CH_2-{}^{13}COOH$ unit by β-oxidation and the resulting non-isotopic C14 acyl group (myristic acid) was then incorporated into the liposidomycin C-(III) molecule.

The results from the isotope-labelling experiments show that the added myristic and palmitic acids were directly incorporated into the nucleoside antibiotics, liposidomycins C-(III) and/or M-(III) and feeding of these acids to the fermentation had permitted facile isolation of these antibiotics in sufficient quantity.

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