STUDIES ON THE BIOSYNTHESIS OF BASIC 16-MEMBERED MACROLIDE ANTIBIOTICS, PLATENOMYCINS. II

PRODUCTION, ISOLATION AND STRUCTURES OF 3-O-PROPIONYL-5-O-MYCAMINOSYL PLATENOLIDES I AND II, 9-DEHYDRO DEMYCAROSYL PLATENOMYCIN AND DEMYCAROSYL PLATENOMYCIN

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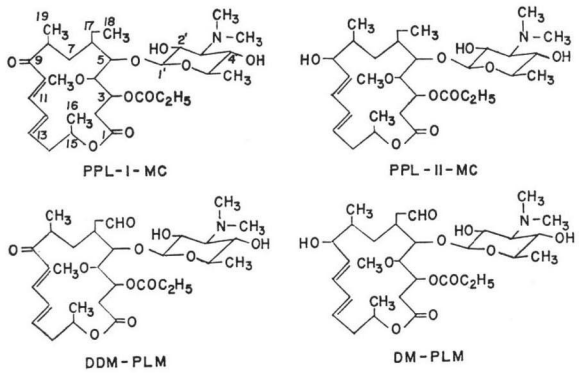
Four basic glycosides have been isolated from the fermentation broth of the blocked mutants of *Streptomyces platensis* subsp. *malvinus* MCRL 0388. These compounds isolated and purified by solvent extraction and column chromatography were identified as 3-O-propionyl-5-O-mycaminosyl platenolides I (PPL-I-MC) and II (PPL-II-MC), 9-dehydro demycarosyl platenomycin (DDM-PLM) and demycarosyl platenomycin (DM-PLM).

In the preceding paper,[1] it was reported that blocked mutants of a platenomycin-producing strain (*Streptomyces platensis* subsp. *malvinus* MCRL 0388), especially those belonging to groups A and B may produce some biosynthetic intermediate(s) of platenomycin (PLM). To prove this possibility, isolation and characterization of the metabolite(s) produced by blocked mutants of group A was first attempted. These blocked mutants produce at least three major and one minor glycosidic compounds in common and they were finally determined to be 3-O-propionyl-5-O-mycarosyl platenolides I (PPL-I-MC*) and II (PPL-II-MC*), 9-dehydro demycarosyl platenomycin (DDM-PLM) and demycarosyl platenomycin (DM-PLM) (Fig. 1). Among these compounds, DDM-PLM and DM-PLM showed weak activity against Gram-positive bacteria, while PPL-I-MC and PPL-II-MC which contained a methyl residue instead of the aldehyde function present in DDM-PLM and DM-PLM respectively lacked any antibiotic activity. In this paper, the production, isolation and structure elucidation of intermediates PPL-I-MC, PPL-II-MC, DDM-PLM and DM-PLM are reported. The compounds produced by blocked mutants belonging to group B will be dealt in a succeeding paper.

Fig. 1. Structures of PPL-I-MC, PPL-II-MC DDM-PLM and DM-PLM.



Materials and Methods

Organisms and media

The strains N-33, N-90 and U-253

* PPL-I-MC and PPL-II-MC were previously communicated as PL-I-MC and PL-II-MC, respectively.[5]

used in the present study were already reported[1] and are derived from *Streptomyces platensis* subsp. *malvinus* MCRL 0388,[2] a platenomycin-producing organism. They were blocked mutants belonging to group A. They were maintained on a BENNETT's agar slant at 27°C. To prepare the vegetative inoculum for liquid culture, SC medium (seed culture medium) was used of which composition was previously reported.[1] For the production of metabolite(s), in shaking or submerged culture, P medium (production medium) was used which was composed of corn starch (1.5 %), glucose (0.5 %), soy bean meal (1.8 %), gluten feed (Nihon Shokuhin Kako Co., Ltd., 0.15 %), NaCl (0.5 %) and $CaCO_3$ (0.2 %), pH being adjusted to 7.0 before sterilization at 120°C for 30 minutes. As needed, Silicon KM-75 (Shinetsu Chemical Co., Ltd.) was used as an antiform agent.

Fermentation

Mature spores from a slant were inoculated into a 500-ml Erlenmeyer flask containing 100 ml of SC medium and cultivated at 28°C for 48 hours on a rotary shaker. Then, the resultant vegetative inoculum was transferred to 100 ml of P medium prepared in 500-ml SAKAGUCHI flasks (inoculum size: 2~3 %) and fermented at 26°C for 96 hours on a reciprocal shaker.

In the case of submerged fermentation in a 30-liter jar fermentor, 200 ml of inoculum were transferred to a jar fermentor containing 15 liters of P medium.

Assay of antibiotic activity

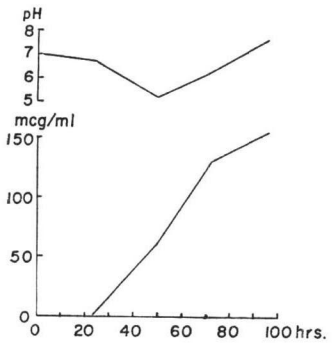
Antimicrobial activity in the fermentation broth due to DDM-PLM and DM-PLM was assayed by a cylinder plate method as applied to platenomycin (YL-704),[2] using *Bacillus subtilis* ATCC 6633 as a test organism and DM-PLM as a standard material, and the activity was demonstrated as DM-PLM.

Results and Discussion

Fermentation

In shaken culture the strains N-33 and N-90 did not produce PLM at all, but produced three major basic glycosides and at least six minor compounds. Similarly strain U-253 produced 20~30 mcg/ml of PLM in addition to basic glycosidic compounds. Therefore, in this strain it was not possible to assay for the production of DM-PLM. As it was found that the strain N-90 was superior to N-33 with respect to the production of DM-PLM (productivity (mcg/ml): 110 by N-33, 150 by N-90), fermentation in a jar fermentor was carried out with the strain N-90. A typical pattern of production of DM-PLM is shown in Fig. 2.

Fig. 2. A typical time course of DM-PLM production in a 30-liter jar fermentor.
Strain: The blocked mutant strain N-90
Medium: 15 liters
Temperature: 24~26°C
Aeration: 6~7 liters/minute
Agitation: 250 r.p.m.
Internal pressure: 0.5 kg/cm².



Isolation

The isolation of glycoside complex was carried out by an usual procedure for a lipophilic basic substance. As shown in Fig. 3, 10.5 g of the crude complex was recovered from 50 liters of filtered broth. The complex was then separated into each component by silica gel chromatography followed by Sephadex LH-20 chromatography. Thus, 0.33 g of PPL-I-MC, 0.97 g of PPL-II-MC, 0.04 g of DDM-PLM and 3.4 g of DM-PLM were obtained as homogeneous substance judging by

Fig. 3. Isolation and purification of PPL-I-MC, PPL-II-MC, DDM-PLM and DM-PLM.

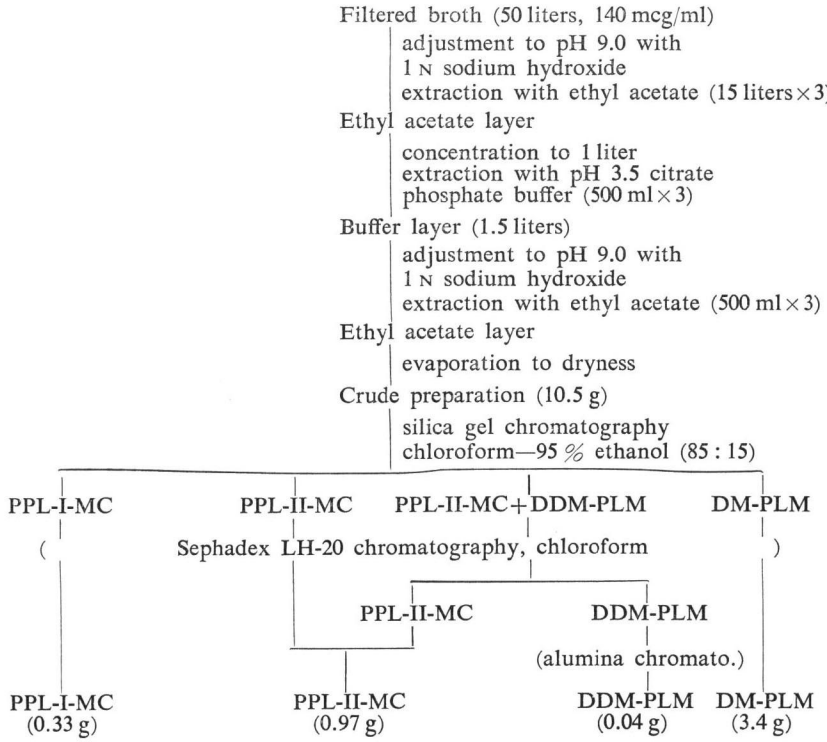


Fig. 4. ^{1}H-NMR spectra of PPL-I-MC, PPL-II-MC, DDM-PLM and DM-PLM in $CDCl_3$.

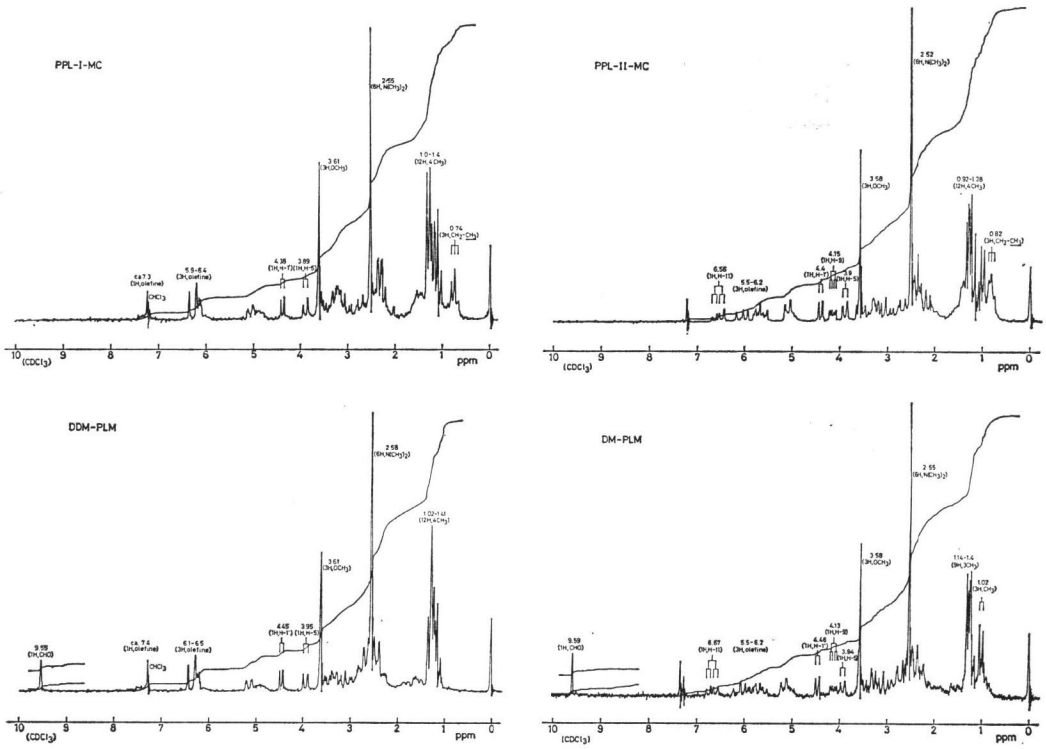


Table 1. Physicochemical properties of PPL-I-MC, PPL-II-MC, DDM-PLM and DM-PLM.

Component	PPL-I-MC	PPL-II-MC	DDM-PLM	DM-PLM
Appearance	colorless needles	colorless needles	colorless prisms	colorless prisms
m.p.	110~113°C	114~115°C	108~110°C	113~115°C
Formula	$C_{31}H_{51}NO_{10}$	$C_{31}H_{53}NO_{10}$	$C_{31}H_{49}NO_{11}$	$C_{31}H_{51}NO_{11}$
Mol. Wt. (MS)	597 (M^+)	599 (M^+)	611 (M^+)	613 (M^+)
Elem. Anal. (%)	Obsd. Calcd.	Obsd. Calcd.	Obsd. Calcd.	Obsd. Calcd.
	C 62.70 62.29	C 62.47 62.08	C 60.58 60.88	C 60.63 60.69
	H 8.24 8.60	H 8.79 8.91	H 8.14 8.02	H 8.19 8.32
	N 2.29 2.34	N 2.20 2.33	N 2.29 2.29	N 2.15 2.28
UV λ_{max}^{EtOH} (nm) (log ε)	279.5 (4.33)	232 (4.46)	279.5 (4.31)	232 (4.44)
$[\alpha]_D^{25}$ ($CHCl_3$)	+54.6° (*c* 0.4)	+31.5° (*c* 0.4)	+34.0° (*c* 0.5)	−8.0° (*c* 0.5)
IR (nujol) (cm^{-1})	3445, 1745, 1680, 1635, 1595, 1300, 1280, 1255, 1195, 1165, 1145, 1125, 1100, 1075, 1060, 1005, 980, 960, 940, 890, 865, 850, 835, 805.	3430, 1735, 1665, 1640, 1300, 1275, 1265, 1240, 1190, 1170, 1150, 1125, 1080, 1055, 1010, 995, 955, 900, 890, 865, 840, 805.	3440, 2720, 1740, 1690, 1640, 1600, 1305, 1260, 1190, 1170, 1145, 1130, 1090, 1060, 1005, 985, 940, 895, 870, 840, 810.	3420, 2715, 1730, 1650, 1630, 1300, 1280, 1265, 1245, 1190, 1170, 1125, 1110, 1080, 1055, 1000, 950, 940, 900, 890, 865, 835, 805.
Rf values*				
Silica gel GF I	0.65	0.50	0.35	0.20
Silica gel GF sheet I (Woelm)	0.90	0.71	0.62	0.34
Alumina-Kieserguhr II	0.68	0.40	0.24	0.12
(6 : 1) III	0.47	0.34	0.24	0.11

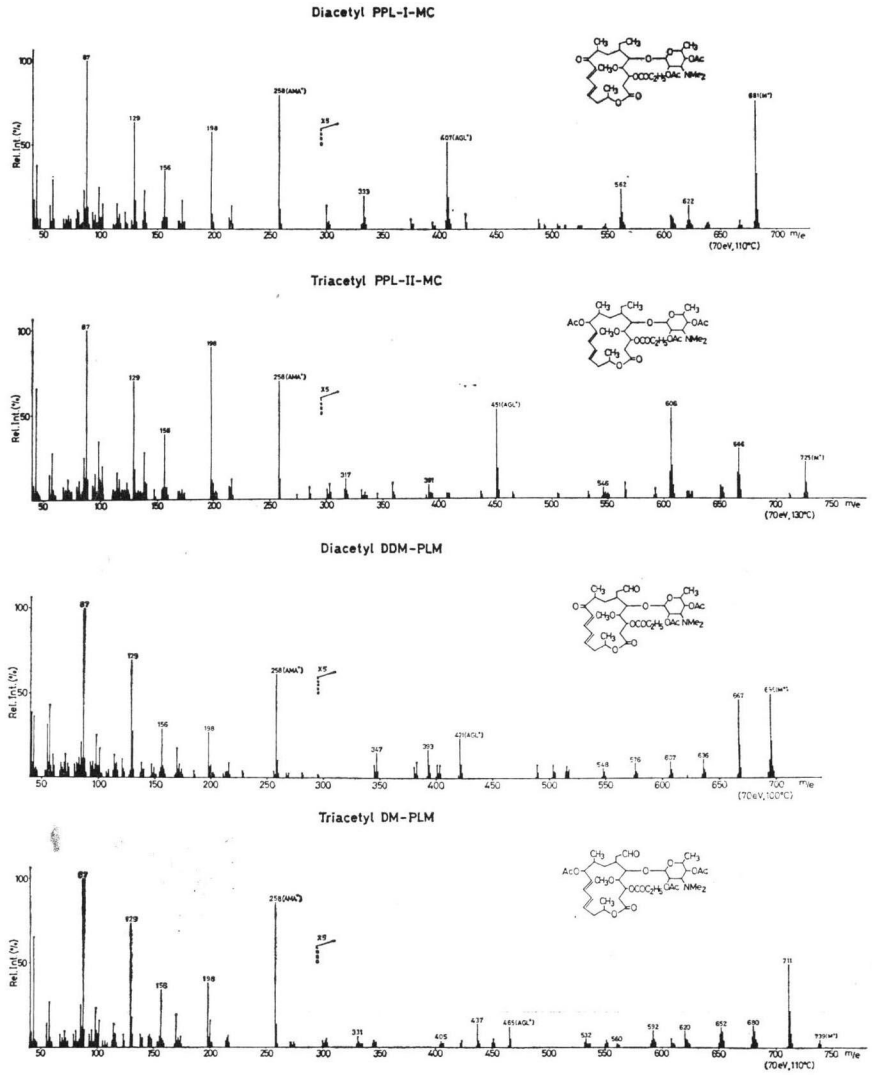
* Solvent system: I: Chloroform - methanol - 7% ammonia water (40 : 12 : 20,)
II: Benzene - acetone (7 : 3),
III: Ethyl acetate - acetone (8 : 2),

their behavior on thin-layer chromatography (TLC). They were further purified through recrystallization from benzene - *n*-hexane.

Physicochemical Properties and Structures

Four glycosides were differentiated from each other as they were visualized on a TLC plate as yellow (PPL-I-MC), brown (PPL-II-MC), green turning to grayish brown (DDM-PLM) or dark purpule (DM-PLM) spots after spraying 40 % sulfuric acid followed by heating. Rf values and physicochemical properties are illustrated in Table 1. Their ^{1}H-NMR spectra were shown in Fig. 4. PPL-I-MC and DDM-PLM showing a strong UV maximum at 279.5 nm possess an $\alpha, \beta, \gamma, \delta$-dienone chromophore as in PLM-W_1,[3] and PPL-II-MC and DM-PLM with a UV maximum at 232 nm have an $\alpha, \beta, \gamma, \delta$-diene alcohol chromophore like PLM-A_1.[4] IR bands also suggested the presence of a ketonic group (1690~1680 cm^{-1}) in PPL-I-MC and DDM-PLM.

Fig. 5. Mass spectra of diacetyl PPL-I-MC, triacetyl PPL-II-MC, diacetyl DDM-PLM and triacetyl DM-PLM.



Moreover, IR adsorption bands at 2720 cm^{-1} and 2715 cm^{-1} which are assigned to an aldehyde function were observed respectively in DDM-PLM and DM-PLM, but not in PPL-I-MC and PPL-II-MC. PPL-I-MC and DDM-PLM gave a diacetate derivative, and PPL-II-MC and DM-PLM gave a triacetate with an usual acetylation procedure. The IR spectra of these acetates did not indicate the presence of a tertiary hydroxyl group in these compounds. The mass spectra and the ^{1}H-NMR spectra of the parent compounds and their acetyl derivatives indicated that PPL-I-MC and DDM-PLM had two hydroxyl groups, and PPL-II-MC and DM-PLM had three hydroxyl groups. In Fig. 5, mass spectra of the acetyl derivatives are shown. Diacetyl PPL-I-MC gave abundant peaks at *m/e* 681(M^+),* 622($M^+-CH_3COO\cdot$), 562($M^+-CH_3COO\cdot-CH_3COOH$), 407($AGL^{+**}$), 333($AGL^+-C_2H_5COOH$), 258, 198, 156, 129 and 87; triacetyl PPL-II-MC at *m/e* 725(M^+), 666)$M^+-CH_3COO\cdot$), 606($M^+-CH_3COO\cdot-CH_3COOH$), 451($AGL^+$), 391 ($AGL^+-CH_3COOH$), 317($AGL^+-CH_3COOH-C_2H_5COOH$), 258, 198, 156, 129 and 87; diacetyl DDM-PLM at *m/e* 695(M^+), 667(M^+-CO), 421(AGL^+), 393(AGL^+-CO), 347($AGL^+-C_2H_5COOH$), 258, 198, 156, 129 and 87; and triacetyl DM-PLM at *m/e* 739(M^+) 711(M^+-CO), 465(AGL^+), 437(AGL^+-CO), 405(AGL^+-CH_3COOH), 331($AGL^+-CH_3COOH-C_2H_5COOH$), 258, 198, 156, 129 and 87. The five intense fragments at *m/e* 258, 198, 156, 129 and 87 which were common to the four acetyl derivatives were ascribed to the acetyl mycaminose moiety. However, fragments due to the acyl mycarose moiety were not observed in these mass spectra. The differences between the four compounds were also noticeable in their NMR spectra. DDM-PLM and DM-PLM showed an aldehyde proton signal at δ 9.55 and δ 9.59 respectively, while PPL-I-MC and PPL-II-MC showed, instead of an aldehyde proton, a triplet methyl signal at δ 0.74 and δ 0.82 ppm respectively. PPL-II-MC and DM-PLM showed a H-9 proton signal as a double-doublet respectively at δ 4.15(J=9.3, 4.5 Hz) and δ 4.13(J=9.1, 4.5 Hz). From the above physicochemical data together with the molecular formulae, PPL-I-MC appears to be a

Table 2. Antimicrobial activities of PPL-I-MC, PPL-II-MC, DDM-PLM and DM-PLM (Serial agar dilution method).

Test organism	M.I.C. (mcg/ml)				
	PPL-I-MC	PPL-II-MC	DDM-PLM	DM-PLM	PLM-A_1
Staphylococcus aureus 209PJC-2	>100	>100	6.25	6.25	0.78
S. aureus Terajima	>100	>100	6.25	6.25	0.78
S. aureus Smith	>100	>100	6.25	6.25	0.39
S. aureus T-88*	>100	>100	>100	>100	>100
Sarcina lutea PCI 1001	>100	>100	3.12	6.25	0.05
Bacillus subtilis ATCC 6633	>100	>100	3.12	6.25	0.19
Escherichia coli NIHJ JC-2	>100	>100	>100	>100	>100
Salmonella typhimurium	>100	>100	>100	>100	>100
Klebsiella pneumoniae	>100	>100	50	50	25
Pseudomonas aeruginosa A_3	>100	>100	>100	>100	>100
Proteus vulgaris	>100	>100	>100	>100	>100

* SM, TC, PC and macrolide resistant strain.
Medium: Heart infusion agar.

* M^+: Molecular ion.
** AGL^+: Aglycone ion.

9-dehydro derivative of PPL-II-MC, and DDM-PLM is a 9-dehydro derivative of DM-PLM. The differences between DDM-PLM/DM-PLM group and the PPL-I-MC/PPL-II-MC group were also evident in their NMR and IR spectra. In the former group an aldehyde function was found, while in the latter group an aldehyde function was not observed. Instead, a methyl residue was indicated. PPL-I-MC, PPL-II-MC and DDM-PLM are new compounds, while DM-PLM is identical to demycarosyl YL-704-I[5] which was obtained by the hydrolysis of YL-704-A_1(PLM-A_1). On the basis of these findings, the structures of these glycosidic compounds are proposed as shown in Fig. 1.

Antimicrobial Activity

As shown in Table 2, DDM-PLM and DM-PLM are weakly active against Gram-positive bacteria and *Klebsiella pneumoniae*, while PPL-I-MC and PPL-II-MC are devoid of antibiotic activity. Therefore, as in the case of other basic 16-membered macrolides,[7,8,9] an aldehyde function in PLM family seems to be required for biological activity.

Experimental

Isolation of PPL-I-MC, PPL-II-MC, DDM-PLM and DM-PLM

After 96 hours of fermentation of the strain N-90 in a jar fermentor, the broth was mixed with Celite 545 and filtered, after which the filtrate was treated as shown in Fig. 2. Thus, starting from 50 liters of filtered broth 10.5 g of a crude product was obtained. This material was roughly fractionated into components by silica gel column chromatography prepared in chloroform - 95% ethanol (9 : 1). The same solvent was used for elution. Further separation and purification of PPL-I-MC, PPL-II-MC, DDM-PLM and DM-PLM was carried out by Sephadex LH-20 chromatography using chloroform. DDM-PLM was further purified by alumina (activity IV) column chromatography using benzene - ethyl acetate (9 : 1) as a developing solvent. Fractions which showed a single spot on TLC were combined and evaporated to dryness. PPL-I-MC (0.33 g), PPL-II-MC (0.97 g), DDM-PLM (0.04 g) and DM-PLM (3.4 g) thus obtained were recrystallized from benzene - *n*-hexane.

From the cultured broth (10 liters) of the strain N-33, PPL-I-MC (0.03 g), PPL-II-MC (0.16 g) and DM-PLM (0.53 g) were isolated by the same procedure mentioned above. DDM-PLM was only detected on TLC.

By treating the cultured broth (12 liters) of the strain U-253 as above, crude PLM complex (0.28 g), PPL-I-MC (0.7 g), PPL-II-MC (0.18 g) and DM-PLM (0.38 g) were obtained. The presence of DDM-PLM was only detected on TLC. IR, UV and mass spectra of the compounds produced by the strains N-33 and U-253 showed a good agreement with those of PPL-I-MC, PPL-II-MC and DM-PLM produced by the strain N-90.

Diacetyl PPL-I-MC

PPL-I-MC (100 mg) was dissolved in 1 ml of dry pyridine and treated with 1 ml of acetic anhydride. The reaction mixture was kept at room temperature overnight and then poured onto cracked ice. The solution was extracted with chloroform (30 ml×2) at pH 8.0. The extract was washed with water (30 ml) and dried over anhydrous sodium sulfate, and then concentrated *in vacuo*. Recrystallization of the residue from benzene - *n*-hexane gave needle crystals (80 mg) of diacetyl PPL-I-MC. m.p.: 204~205°C. MW: 681 (M^+, *m/e*), Anal. Calcd. for $C_{35}H_{55}NO_{12}$: C 61.67, H 8.07, N 2.06. Found: C 61.73, H 8.10, N 2.05. UV(EtOH): 279.5 nm (log ε 4.33). IR (nujol): 1745, 1715, 1685, 1640, 1600, 1575, 1305, 1230 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 2.04 (6H, s, 2×CH_3CO–).

Triacetyl PPL-II-MC

PPL-II-MC (100 mg) was acetylated as above. Triacetyl PPL-II-MC obtained was recrystallized from benzene - *n*-hexane to afford colorless needles (71 mg). m.p.: 128~130°C, MW: 725

(M^+, *m/e*). Anal. Calcd. for $C_{37}H_{59}NO_{13}$: C 61.24, H 8.13, N 1.93. Found: C 61.19, H 8.06, N 1.89. UV (EtOH): 232 nm ($\log \varepsilon$ 4.47). IR (nujol): 1760, 1740, 1665, 1640, 1300 1230 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 2.00 (6H, s, 2×CH_3CO–), δ 2.04 (3H, s, CH_3CO–).

Diacetyl DDM-PLM

DDM-PLM (30 mg) dissolved in 0.3 ml of dry pyridine was acetylated with 0.3 ml of acetic anhydride, and treated as above. Diacetyl DDM-PLM was recrystallized from benzene - *n*-hexane to give colorless needles (26 mg). m.p.: 204~206°C. MW: 695 (M^+, *m/e*). Anal. Calcd. for $C_{35}H_{53}NO_{13}$: C 60.43, H 7.63, N 2.01. Found: C 60.25, H 7.70, N 2.17. UV (EtOH): 297.5 nm ($\log \varepsilon$ 4.34). IR (nujol): 2715, 1745, 1715, 1680, 1600, 1305, 1220, 1200 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 2.06 (3H, s, CH_3CO–), δ 2.08 (3H, s, CH_3CO–).

Triacetyl DM-PLM

DM-PLM (100 mg) was acetylated as done with PPL-I-MC, and triacetyl DM-PLM (65 mg) was obtained as colorless needles. m.p.: 115~117°C. MW: 739 (M^+, *m/e*). Anal. Calcd. for $C_{37}H_{57}NO_{14}$: C 60.08, H 7.71, N 1.89. Found: C 59.93, H 7.63, N 1.68. UV (EtOH): 232 nm (log 4.45), IR (nujol): 2710, 1755, 1740, 1730, 1660, 1625, 1300, 1230, 1170 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 2.02 (6H, s, 2×CH_3CO–), δ 2.06 (3H, s, CH_3CO–).

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