NOVEL MICROBIAL METABOLITES OF THE PHOSLACTOMYCINS FAMILY INDUCE PRODUCTION OF COLONY-STIMULATING FACTORS BY BONE MARROW STROMAL CELLS

II. ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE DETERMINATION

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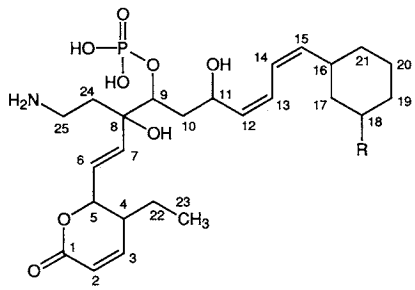
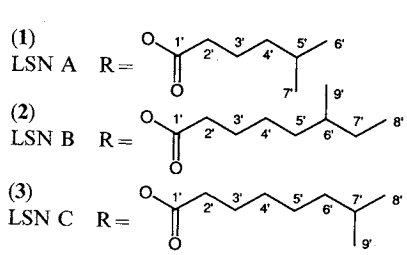
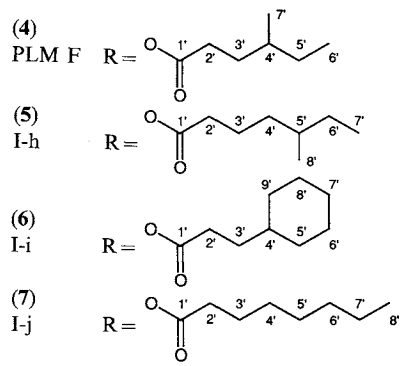
Leustroducsins (LSNs) A, B and C, novel inducers of colony-stimulating factors (CSFs), were isolated from culture broth of *Streptomyces platensis* SANK 60191 mainly by ethyl acetate extraction and preparative reverse-phase HPLC. The molecular weights and molecular formulae of LSNs A, B and C are 641: $C_{32}H_{52}O_{10}NP$, 669: $C_{34}H_{56}O_{10}NP$ and 669: $C_{34}H_{56}O_{10}NP$, respectively. The structure elucidation revealed that they belong to the phoslactomycin group antibiotics, and their structures contain an α,β-unsaturated δ-lactone, an amino group, a phosphate ester and a cyclohexane ring moiety. The structures differ only at the substituent bound to the cyclohexane ring.

Leustroducsins (LSNs) A, B and C (Fig. 1) which induce production of colony-stimulating factors (CSFs) by bone marrow stromal cells. These compounds were isolated from the culture broth of an

Fig. 1. Structures of the leustroducsins and their related compounds.





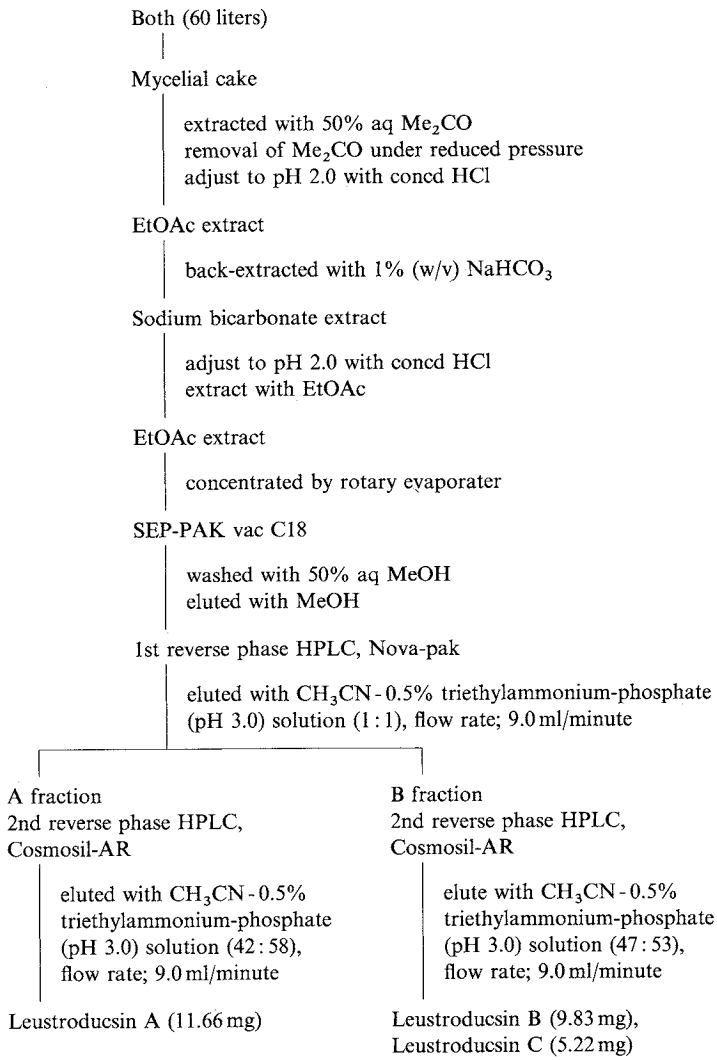


actinomycete strain identified as *Streptomyces platensis* SANK 60191. Screening method, taxonomy of the producing strain, fermentation and biological activities of LSNs have been reported in the preceding papers[1,2]. In this paper we report the purification, physico-chemical properties of these metabolites, structural elucidation based on spectroscopic analyses and degradation studies.

Purification of LSNs

The flow diagram for the purification for LSNs is shown in Scheme 1. Because the activity was mainly present in mycelia, the mycelial cake was extracted with 50% aq Me_2CO. The active substances were purified by sequential extraction with EtOAc, 1% (w/v) $NaHCO_3$ and EtOAc. The extract was concentrated approximately 100-fold, to which MeOH was added to yield a MeOH solution. The MeOH solution was diluted with an equal volume of water and absorbed on SEP-PAC vac C18 (Waters). After washing, the active substances were eluted with MeOH and concentrated to dryness. The residue was dissolved in

Scheme 1. Purification procedure for leustroducsins.



MeOH and subjected to reverse phase preparative HPLC. LSN A(**1**) and a mixture of LSNs B (**2**) and C (**3**) were eluted at 13 (A fraction) and 24 minutes (B fraction) after injection, respectively. Active fractions were collected and concentrated to a small volume, and then subjected to another reverse phase preparative HPLC. To desalt, the active fractions were adsorbed on SEP-PAK vac C18 and eluted with MeOH after washing with water. The eluates were concentrated *in vauco* to give **1** (11.66 mg), **2** (9.83 mg) and **3** (5.22 mg) as yellow sticky oil. LSNs were isolated together with four other active compounds. These compounds were identified as phoslactomysin F (PLM F) (**4**)[3,4] and its homologues I-h (**5**), I-i (**6**) and I-j (**7**) (Fig. 1)[5] as described below. The HPLC profile of these compounds is shown in Fig. 2.

Physico-chemical Properties of LSNs

LSNs are amphoteric yellow sticky oils. They are soluble in alcohol, EtOAc and alkaline water, but are insoluble in hexane. Their UV (234 nm in MeOH), IR, ^{1}H and ^{13}C NMR spectra resemble each other very closely (Tables 2 and 3). Thus, LSNs are thought to be structural analogs. They reacted positively on TLC to iodine, sulfuric acid, ninhydrin and ammonium molybdate-perchloric acid reagents, indicating the presence of amino and phosphorus groups.

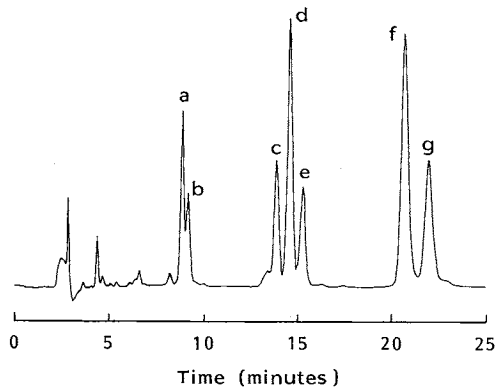
Structure Determination

The MWs and molecular formulae of the seven isolated active compounds shown in Table 1 were established by high resolution liquid secondary ion mass spectrometry (HRLSI-MS). Compounds **4**, **5**, **6** and **7** were identified as the previously reported antifungal antibiotics PLM F[3,4] and its homologues I-h, I-i and I-j[5], respectively (Fig. 1), by a detailed analysis of the NMR data. On the other hand, compounds **1**, **2** and **3** are considered to be a new isomer and two homologues of the phoslactomycin-group antibiotics[3~9], because their NMR spectra closely resemble the other members reported in this group. The structures of these three new compounds were determined as follows.

The compound **1** (LSN A) is an isomer of **4** (PLM F), because their molecular formulae are

Fig. 2. HPLC elution profile of the mixture of LSNs and related compounds.

a; PLMF, b; LSN A, c; I-h, d; I-i, e; I-j, f: LSN B, g; LSN C.



Column conditions: Column; Cosmosil-AR 4.6 i.d. × 250 mm, solvent; acetonitrile - 0.5% triethylammonium phosphate buffer pH 3.0 (45 : 55), flow rate; 1.0 ml/minute, detector; UV (230 nm).

Table 1. HRLSI-MS data, MWs, and molecular formulae of the isolated compounds.

	HRLSI-MS data of $(M+H)^+$		MW	Formula
	Obsd.	Calcd.		
1 (LSN A)	642.3403	642.3407	641	$C_{32}H_{52}NO_{10}P$
2 (LSN B)	670.3732	670.3720	669	$C_{34}H_{56}NO_{10}P$
3 (LSN C)	670.3733	670.3720	669	$C_{34}H_{56}NO_{10}P$
4 (PLM F)	642.3430	642.3407	641	$C_{32}H_{52}NO_{10}P$
5 (I-h)	656.3574	656.3564	655	$C_{33}H_{54}NO_{10}P$
6 (I-i)	668.3565	668.3564	667	$C_{34}H_{54}NO_{10}P$
7 (I-j)	656.3576	656.3564	655	$C_{33}H_{54}NO_{10}P$

Table 2. ^{1}H NMR spectral data for LSN's A, B and C (in CD_3OD).

δ (ppm)	n[a]	m[b]	J (Hz)	Assignment	δ (ppm)	n[a]	m[b]	J (Hz)	Assignment
Leustroducsin A					2.93~3.15	2	m		25-H
7.80	1	dd	9.8, 4.9	3-H	2.50~2.71	2	m		4-H, 16-H
6.21~6.35	1	m		13,14-H	2.27	2	t	7.3	2′-H
6.06	1	dd	15.6, 6.1	6-H	2.17	1	m		24-Ha
6.02	1	dd	9.8, 1.4	2-H	1.00~2.01	22	m		10-H, 17-H,
5.94	1	d	15.6	7-H					19~22-H,
5.46	1	m		12-H					24-Hb,
5.31	1	m		15-H					3′~7′-H
5.10	1	dd	6.1, 4.4	5-H	0.95	3	t	7.5	23-H
4.94	1	m		11-H	0.87	3	t	6.8	8′-H
4.72	1	m		18-H	0.86	3	d	6.6	9′-H
4.29	1	td	10.1, 10.1, 2.4	9-H	Leustroducsin C				
2.93~3.15	2	m		25-H	7.08	1	dd	9.8, 4.9	3-H
2.50~2.71	2	m		4-H, 16-H	6.21~6.35	2	m		13-H, 14-H
2.25	2	t	7.6	2′-H	6.07	1	dd	15.6, 6.1	6-H
2.16	1	m		24-Ha	6.02	1	dd	9.8, 1.5	2-H
0.99~2.01	18	m		10-H, 17-H,	5.94	1	d	15.6	7-H
				19~22-H,	5.46	1	m		12-H
				24-Hb,	5.31	1	m		15-H
				3′~5′-H	5.10	1	dd	6.1, 4.9	5-H
0.95	3	t	7.6	23-H	4.94	1	m		11-H
0.89	6	d	6.8	6′-H, 7′-H	4.72	1	m		18-H
Leustroducsin B					4.28	1	td	10.1, 10.1, 2.4	9-H
7.09	1	dd	9.8, 4.9	3-H	2.93~3.15	2	m		25-H
6.21~6.35	2	m		13-H, 14-H	2.50~2.71	2	m		4-H, 16-H
6.07	1	dd	15.6, 6.1	6-H	2.27	2	t	7.3	2′-H
6.02	1	dd	9.8, 1.5	2-H	2.16	1	m		24-Ha
5.94	1	d	15.6	7-H	1.00~2.01	22	m		10-H, 17-H,
5.46	1	m		12-H					19~22-H,
5.31	1	m		15-H					24-Hb,
5.10	1	dd	6.1, 4.4	5-H					3′~7′-H
4.94	1	m		11-H	0.95	3	t	7.3	23-H
4.72	1	m		18-H	0.88	6	d	6.3	8′-H, 9′-H
4.29	1	td	9.9, 9.9, 2.6	9-H					

[a] Number of protons.
[b] Multiplicity.

Table 3. ^{13}C chemical shifts for LSN's A, B and C (in CD_3OD).

Assignment	δ (ppm) LSN			Assignment	δ (ppm) LSN		
	A	B	C		A	B	C
C-1	166.33	166.35	166.36	C-18	73.86	73.90	73.88
C-2	121.06	121.01	121.05	C-19	32.43	32.42	32.43
C-3	152.65	152.66	152.70	C-20	24.68	24.66	24.67
C-4	40.58	40.56	40.55	C-21	33.06	33.06	33.07
C-5	82.33	82.34	82.32	C-22	22.72	22.70	22.74
C-6	127.66	127.67	127.65	C-23	11.40	11.37	11.41
C-7	137.27	137.39	137.34	C-24	34.27	34.14	34.15
C-8	77.75	77.78	77.76	C-25	37.12	37.12	37.10
C-9	78.51	78.45	78.43	C-1′	174.97	175.05	175.03
C-10	40.58	40.56	40.55	C-2′	35.65	35.46	35.44
C-11	64.65	64.65	64.63	C-3′	24.02	26.44	26.16
C-12	135.26	135.23	135.24	C-4′	39.48	27.57	30.42
C-13	124.26	124.25	124.26	C-5′	28.94	37.32	28.17
C-14	123.71	123.70	123.71	C-6′	22.89	35.54	40.02
C-15	138.20	138.18	138.19	C-7′	22.89	30.49	29.10
C-16	36.14	36.13	36.14	C-8′	—	11.70	23.06
C-17	39.39	39.37	39.38	C-9′	—	19.58	23.06

Fig. 3. Negative ion LSI-MS/MS spectra and fragmentations (insets) from deprotonated molecules of (a) phoslactomycin F and (b) leustroducsin A.

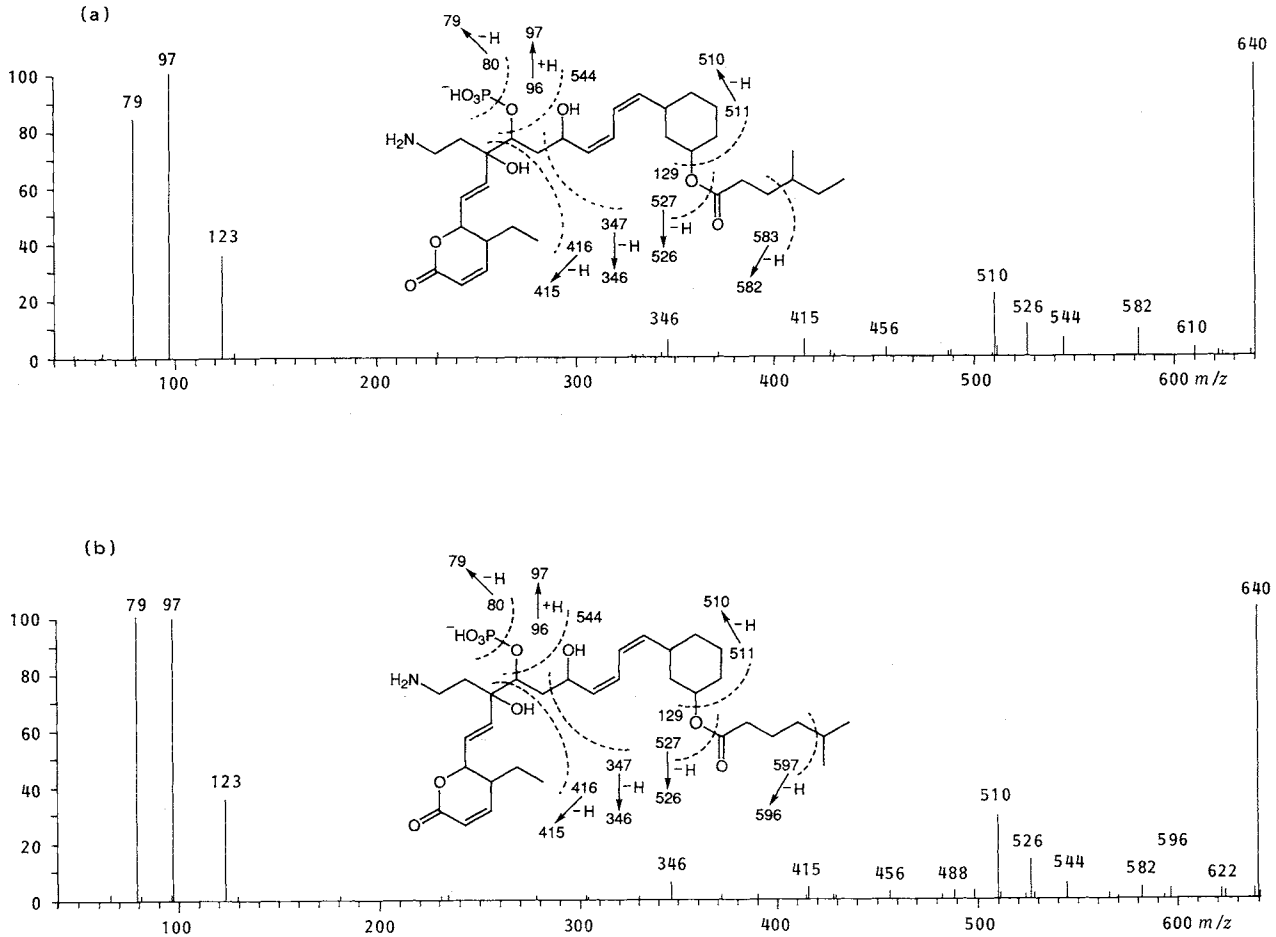
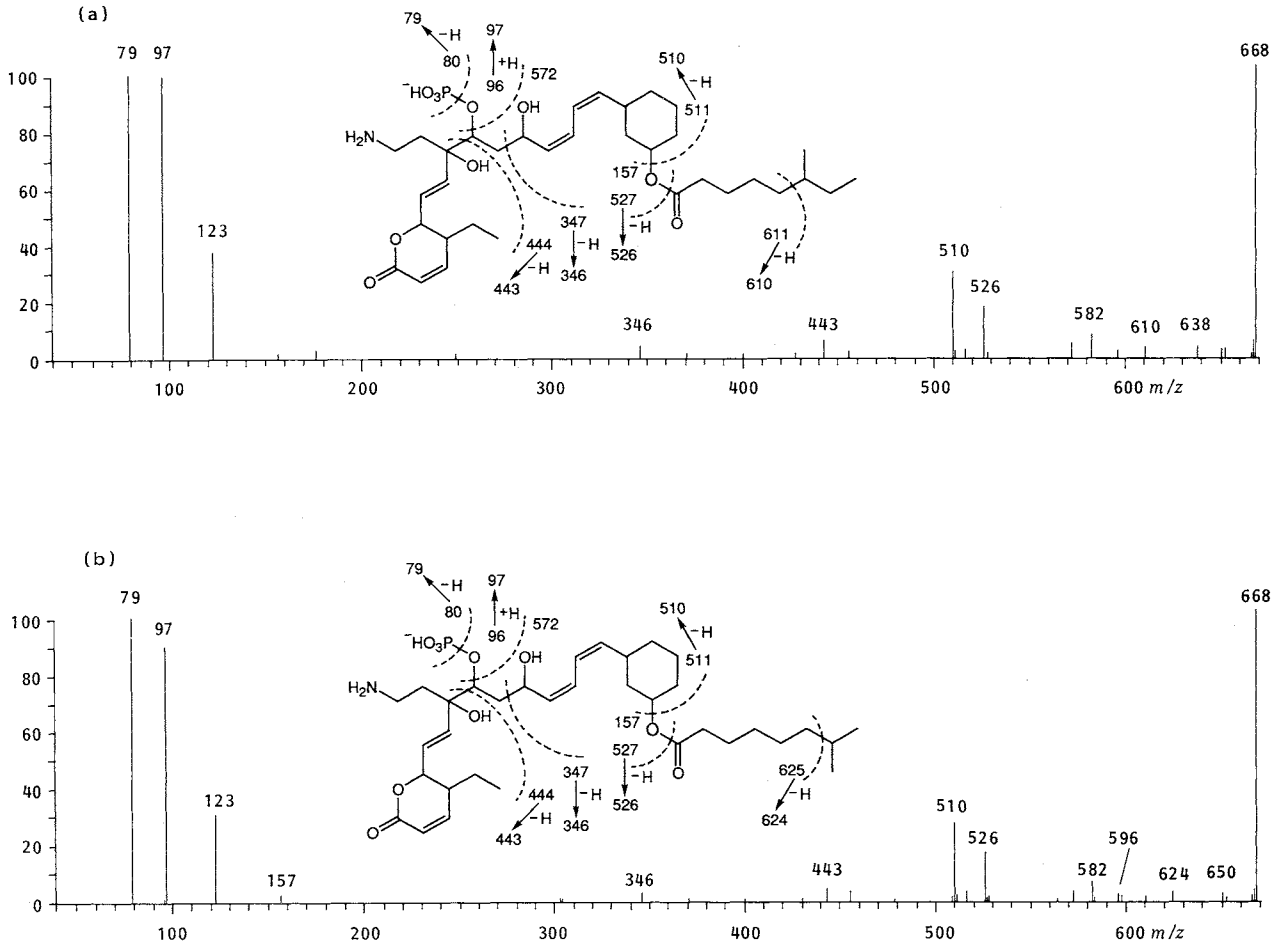


Fig. 4. Negative ion LSI-MS/MS spectra and fragmentations (insets) from deprotonated molecules of (a) leustroducsin B and (b) leustroducsin C.



identical. Although the downfield regions of the ^{1}H and ^{13}C NMR spectra of **1** are in close agreement with those of **4**, there are some striking differences in the upfield regions (Tables 2 and 3): The 6′ triplet-methyl signal of **4**[3] disappears at 0.88 ppm and an additional doublet-methyl signal appears at 0.89 ppm in the ^{1}H NMR spectrum of **1** compared with **4** (Table 2). These spectra suggested that the 4-methylhexanoyl group in **4** is displaced by 5-methylhexanoyl group in **1**. ^{13}C NMR spectra (Table 3) and MS/MS analysis of these two compounds supported the displacement of the acyl groups. The charge-remote fragmentations[10~12] observed in the negative ion LSI-tandem mass (MS/MS) spectra of these two compounds clearly showed different branching positions in the acyl groups (Fig. 3). A moderate relative intensity of *m/z* 582 and the absence of *m/z* 596 in the LSI-MS/MS spectrum of **4** (Fig. 3a) show the branching at the 4′ position, whereas a moderate relative intensity of *m/z* 596 and the absence of *m/z* 610 in the spectrum of **1** (Fig. 3b) show the branching at the 5′ position. Thus the structure of **1** was determined as shown in Fig. 1.

The molecular formulae of the compounds **2** (LSN B) and **3** (LSN C) are identical and contain an additional C_2H_4 compared with PLM F or **1**. The negative ion LSI-MS/MS spectra of **2** and **3** (Fig. 4) closely resemble those of **4** and **1** and show common product ions below *m/z* 526, the mass corresponding to a product ion formed *via* a releasing of acyl groups. Accordingly, it was suggested that all four of these compounds have a same structure except at the acyl groups. This assumption was supported by the ^{1}H and ^{13}C NMR data (Tables 2 and 3).

The upfield region of the ^{1}H NMR spectrum of **2** closely resembles that of **4**. The presence of a triplet methyl signal at 0.87 ppm and a doublet methyl signal at 0.86 ppm in the ^{1}H NMR spectrum of **2** suggested methyl-branching in the aliphatic chain. The branching position was deduced to be at the 6′ position of the acyl group on the basis of the LSI-MS/MS analysis. A moderate relative intensity of the product ion at *m/z* 610 and the absence of *m/z* 624 in the negative ion LSI-MS/MS spectrum of the compound (Fig. 4a) clearly showed the methyl-branching at the 6′- position. Consequently, the structure of **2** was determined as shown in Fig. 1.

In contrast with **2**, the upfield region of the ^{1}H NMR spectrum of **3** closely resembled that of **1**. The two overlapped doublet-methyl signal at 0.88 ppm suggested terminal methylbranching in the acyl group. This was supported by the negative ion LSI-MS/MS spectrum of the compound (Fig. 4b). The moderate relative intensity of the product ion at *m/z* 624 and the absence of *m/z* 638 confirmed the methyl-branching at the 7′ position of the acyl group. Thus the structure of **3** was determined as shown in Fig. 1.

Experimental

The ^{1}H and ^{13}C NMR spectra were recorded on a Jeol JNM-GX270 or a Jeol JNM-GX400. The chemical shifts are given downfield from internal TMS.

The LSI-MS and LSI-MS/MS spectra were obtained using a VG 70-4SE tandem mass spectrometer (EBEB geometry) with a 3-nitrobenzyl alcohol matrix. A mixture of polyethylene glycol was used as the reference material for the accurate mass measurement. A 4-keV laboratory frame collision energy and a helium target gas were used for the MS/MS experiments.

Preparative HPLC was carried out on a Shimadzu SCL-10A apparatus using a Radial-PAK 25 × 10 Nova-pak column (Waters) and a Cosmosil-AR 20 × 250 column (Nakarai tesque). A Cosmosil-AR 4.6 × 250 column (Nakarai tesque) was used for analytical HPLC.

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