BUTYROLACTOLS A AND B†, NEW ANTIFUNGAL ANTIBIOTICS

TAXONOMY, ISOLATION, PHYSICO-CHEMICAL PROPERTIES, STRUCTURE AND BIOLOGICAL ACTIVITY

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New antifungal antibiotics, butyrolactols A and B, have been isolated from the culture broth of *Streptomyces rochei* S785-16. They are novel type of molecules containing a common 2,3-dihydroxybutyrolactone nucleus substituted with a different long hydroxyalkyl side chain. Butyrolactol A showed good antifungal activity against *Aspergillus fumigatus* and *Trichophyton mentagrophytes*, and moderately inhibited the growth of yeasts.

In our systematic search for microbial metabolites effective against pathogenic fungi, an actinomycete strain S785-16, was found to produce a complex of novel antibiotics, butyrolactol. They were extracted with organic solvent and purified by solvent partition and column chromatography to afford the major component butyrolactol A and the minor butyrolactol B. Spectrometric analysis and partial degradation experiments established that they were unusual 4-(pentahydroxyalkyl)-2,3-dihydroxy-γ-lactones differing each other in their alkyl chains. They showed moderate *in vitro* activity against a wide variety of fungi and yeasts. This paper describes the taxonomy of the producing organism, isolation, physico-chemical properties, structure determination and biological activity of butyrolactols A and B.

Taxonomy

Source of Strain S785-16

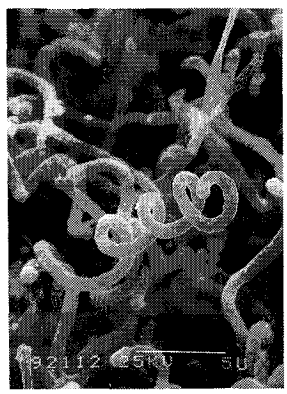
A soil sample collected in the Philippines.

Morphology

Aerial mycelia developed from substrate mycelia. Spore-chains were born monopodially on the aerial mycelium, and were open irregular spiral, hook or flexuous in shape. The spore chain morphology appeared to belong to either *Retinaculiaperti* or *Spirales*. Most chains of mature spores contained 10 to 30 or more spores per a chain. Scanning electron micrography showed that the spores were oval with smooth surface and

Fig. 1. Scanning electron micrograph of *Streptomyces rochei* S785-16 grown on ISP 4 medium for 2 weeks at 28°C.

Bar represents 5.0 μm.



† Butyrolactol was originally called as BU-4408.

Table 1. Cultural characteristics of strain S785-16.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Sucrose-nitrate agar (CZAPEK-DOX agar)	Poor	Scant; light gray	Colorless	None
Tryptone-yeast extract broth (ISP No. 1)	Moderate; pellicle, not turbid	Poor; white	Colorless	None
Yeast extract-malt extract agar (ISP No. 2)	Good	Moderate; light brownish gray (63) to medium gray	Moderate yellowish brown (77)	Dark orange yellow (72)
Oatmeal agar (ISP No. 3)	Moderate	No or scant; whitish	Grayish yellow (90)	None
Inorganic salts-starch agar (ISP No. 4)	Moderate	Poor; light gray	Dark grayish yellow (91)	None
Glycerol-asparagine agar (ISP No. 5)	Moderate	Poor; light gray	Light yellowish brown (78)	None
Peptone-yeast extract-iron agar (ISP No. 6)	Moderate	None	Colorless	None
Tyrosine agar (ISP No. 7)	Moderate	Moderate; yellowish white (92)	Light to deep yellowish brown (76, 75)	None
Glucose-asparagine agar	No or scant	None	Colorless	None
BENNETT's agar	Moderate	None	Colorless	None

Observation after incubation at 28°C for 2 weeks.
Color name, used: ISCC-NBS color-name charts.

$0.7 \times 0.9 \sim 1.2\,\mu$m in size (Fig. 1). The substrate mycelia in agar media were not fragmented. Sporangia, motile spores and sclerotia were not observed.

Table 2. Carbohydrate utilization of strain S785-16.

Glycerol	+	Cellobiose	+(w)
D(−)-Arabinose	−	Melibiose	+
L(+)-Arabinose	+	Trehalose	+
D-Xylose	+	Raffinose	+
D-Ribose	+	D(+)-Melezitose	−
L-Rhamnose	+	Soluble starch	+
D-Glucose	+(w)	Cellulose	−
D-Galactose	+	Dulcitol	−
D-Fructose	+	Inositol	+
D-Mannose	+	D-Mannitol	+
L(−)-Sorbose	−	D-Sorbitol	−
Sucrose	+(w)	Salicine	+
Lactose	+		

Observation after incubation at 28°C for 2 weeks.
Basal medium: PRIDHAM-GOTTLIEB's inorganic medium (ISP medium No. 9).
Abbreviation: +, positive utilization; +(w), weakly positive utilization; −, negative utilization.

Cultural and Physiological Characteristics

The color of mature spores in aerial mass was gray (Gray-color series). The substrate mycelium was colorless or yellowish brown. Melanin and other characteristic pigments were not produced. Gelatin and starch were hydrolyzed. Milk in 10% skimmed milk was coagulated and peptonized. Nitrate was reduced to nitrite in both CZAPEK's inorganic broth and peptone broth. Tyrosinase was not produced. Growth occurred on 13% NaCl but not on 15%. Growth temperature ranged between 14°C and 45°C. No growth was seen at 10°C and 48°C. The cultural characteristics and the carbon utilization of strain S785-16 are shown in Tables 1 and 2, respectively.

Cell Chemistry

The diamino acid in the whole cell hydrolysate was determined to be L,L-diaminopimelic acid by the method of LECHEVALIER[1].

Taxonomic Position

A comparison of the strain was made with the published descriptions of various *Streptomyces* species. The result, indicated that strain S785-16 closely resembled *Streptomyces rochei* Berger, Jampolsky and Goldberg 1953[2]. They are different only in that the former had smooth spore surface but the latter smooth to warty spore surface. In spite of the minor difference, the two strains showed numerous similarities. Thus, strain S785-16 was identified as a strain of *Streptomyces rochei*.

Fermentation

The spores of *S. rochei* S785-16 was inoculated into a 500-ml Erlenmeyer flask which containing 100 ml of seed medium consisting of soluble starch 3%, lactose 1%, fish meal 1%, $CaSO_4$ 0.6% and $CaCO_3$ 0.5% (pH 7.0 before sterilization). The seed flasks were incubated at 32°C for 4 days on a rotary shaker (200 rpm), and 2 liters of the seed culture were transferred to a 200-liter fermenter containing 120 liters of the production medium composed of the same ingredients as the above seed medium. Fermentation was carried out at 28°C under aeration of 120 liters/minute and agitation of 250 rpm. The antibiotic production was monitored by the broth dilution assay method using *Candida albicans* A9540 as the test organism in yeast nitrogen broth containing 1% glucose. After 45 hours of cultivation, the broth produced 10 μg/ml of butyrolactol mixture.

Isolation and Purification

The fermentation broth (200 liters) was extracted with BuOH (100 liters). The extract was concentrated *in vacuo* to 3.8 liters which was added into *n*-hexane (36 liters). The resulting precipitates were triturated with 24 liters of EtOAc - water (2 : 1) mixture, and the EtOAc layer was evaporated *in vacuo* to 1.3 liters. The concentrate was allowed to stand for 16 hours at 5°C and the precipitates deposited were collected by filtration. The crude complex of butyrolactol (21 g) obtained was adsorbed on the top of a dry-silica gel column (Wako-gel C-200, 100 g). The column was developed with EtOAc - MeOH - water (20 : 4 : 1) mixture and eluate monitored by iodine vapor on TLC plate (silica gel; chloroform - MeOH - water (65 : 35 : 10) lower phase). The iodine-positive fractions were combined (2.8 liters) and concentrated *in vacuo* to give a semi-pure mixture of butyrolactols A and B (4.3 g). It was dissolved in DMSO (50 ml) and subjected to reversed phase column chromatography (ODS-A60, 5 liters, YMC Co., Ltd.). Upon elution with acetonitrile (CH_3CN) - 0.15% KH_2PO_4, pH 3.5 (45 : 55) and then with 60% CH_3CN - phosphate buffer, pH 3.5, the eluate was analyzed by HPLC (Column: YMC-gel, A301-3, 4.6 mm i.d. × 100 mm, 3 μm, YMC Co., Ltd. Mobile phase: CH_3CN - 0.15% KH_2PO_4, pH 3.5 (55 : 45). Flow rate: 1.0 ml/minute. Detection: UV absorption at 230 nm). Butyrolactol B is eluted first followed by butyrolactol A. The fractions containing butyrolactol A or B were pooled and concentrated *in vacuo* to an aqueous solution. The each concentrate was extracted with EtOAc and the extract was concentrated to afford semi-pure butyrolactols A (970 mg) and B (110 mg). The semi-pure solid were desalted by Sephadex LH-20 (750 ml) chromatography eluting with 90% aqueous tetrahydrofuran to yield pure butyrolactols A (430 mg) and B (40 mg).

Physico-chemical Properties

Butyrolactols A and B were neutral lipophilic antibiotics. They are soluble in DMSO and pyridine, slightly soluble in MeOH, and practically insoluble in *n*-hexane, chloroform and water. The antibiotics showed positive responses to iodine and conc H_2SO_4, but were negative to ninhydrin, ferric chloride and 2,3,5-triphenyltetrazolium chloride tests. The physico-chemical properties of butyrolactols A and B are

Table 3. Physico-chemical properties of butyrolactols A and B.

	Butyrolactol A	Butyrolactol B
Nature	Neutral white amorphous powder	
MP (dec)	139.4~139.8°C	Not tested
$[\alpha]^{26°}$ (c 2.0, DMSO)	−25.5°	Not tested
MS FAB-MS (positive) m/z	527 $(M+H)^+$	513 $(M+H)^+$
FAB-MS (negative) m/z	525 $(M-H)^-$	511 $(M-H)^-$
Molecular weight	526	512
Elemental analysis	$C_{28}H_{46}O_9 \cdot \frac{1}{2}H_2O$	$C_{27}H_{44}O_9 \cdot H_2O$
	Calcd Found	Calcd Found
	C 62.78 62.78	C 61.11 61.22
	H 8.84 8.70	H 8.74 8.50
UV λ_{max} nm (ε)		
in MeOH	228 (sh, 18,700), 236 (19,800)	229 (sh, 18,900), 236 (19,900)
in 0.01 N HCl-MeOH	229 (sh, 21,100), 236 (21,800)	229 (sh, 21,400), 236 (21,900)
IR (KBr) cm^{-1}	3392, 3016, 2955, 2870, 1774, 1617, 1407, 1365, 1083, 1008, 949	3390, 3010, 2950, 1765, 1620~1640 (broad), 1410, 1385, 1080, 1010, 950

Table 4. NMR data of butyrolactol A (DMSO-d_6).

Carbon No.	^1H	^{13}C	Carbon No.	^1H	^{13}C
1		174.8	11a	2.45 (1H, m)	35.9
2	4.25 (1H, dd, J=9.0, 6.4)	74.2	11b	1.86 (1H, dt, J=13.7, 8.9)	
2-OH	6.04 (1H, d, J=6.4)		12	5.58 (1H, dt, J=14.5, 7.3)	131.4
3	4.16 (1H, dt like, J=6.0, 8.5)	72.5	13	5.98 (1H, m)	131.4
3-OH	5.70 (1H, d, J=5.9)		14	6.02 (1H, m)	130.8
4	4.34 (1H, d, J=8.1)	79.5	15	5.55 (1H, dt, J=14.5, 6.8)	130.8
5	3.64 (1H, d, J=8.1)	66.4	16	2.10 (2H, dt, J=7.2, 6.0)	32.1
5-OH	4.84 (1H, d, J=7.7)		17	2.21 (2H, dt, J=7.3, 7.7)	27.0
6	3.71 (1H, t, J=7.7)	68.3	18	5.27 (1H, dt, J=10.7, 7.7)	128.6
6-OH	4.31 (1H, d, J=7.7)		19	5.93 (1H, t, J=11.1)	129.0
7	3.73 (1H, t, J=8.9)	68.4	20	6.31 (1H, ddd, J=15.0, 11.9, 1.9)	125.2
7-OH	4.00 (1H, d, J=8.1)		21	5.66 (1H, dt, J=15.0, 6.8)	135.8
8	3.48 (1H, t, J=8.1)	69.2	22	2.04 (2H, dt, J=9.3, 7.3)	27.6
8-OH	3.95 (1H, d, J=8.1)		23	1.24 (2H, m)	43.2
9	3.34 (1H, d, J=9.0)	72.6	24		30.1
9-OH	3.92 (1H, d, J=8.2)		25~27	0.87 (9H, s)	29.2
10	1.69 (1H, m)	35.7	28	0.77 (3H, d, J=6.8)	15.7

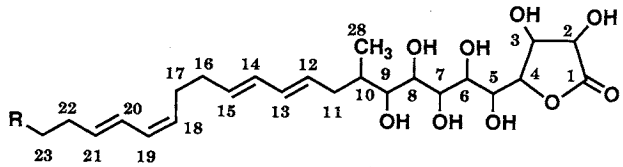
summarized in Table 3.

The molecular formulae of butyrolactols A and B were established as $C_{28}H_{46}O_9$ and $C_{27}H_{44}O_9$, respectively, based on the elemental analyses, FAB-MS and ^1H NMR spectra data. Their UV spectra showed a characteristic absorption maximum at 236 nm. The IR spectra of butyrolactols A and B exhibited polyhydroxyl at 3392 and carbonyl at 1774 cm^{-1}. The ^1H NMR spectrum of butyrolactol A (Table 4) in DMSO-d_6 revealed the presence of eight olefinic (δ 5.27, 5.55, 5.58, 5.66, 5.93, 5.98, 6.02 and 6.31) and seven hydroxy protons (δ 3.92, 3.95, 4.00, 4.31, 4.84, 5.70 and 6.04) and four methyls. Its ^{13}C NMR spectrum (Table 4) exhibited 28 carbons which were analyzed as $4\times CH_3$, $5\times CH_2$, $9\times CH$, $8\times CH=$, $1\times >C<$ and $1\times C=O$ by DEPT experiment.

Structural Studies

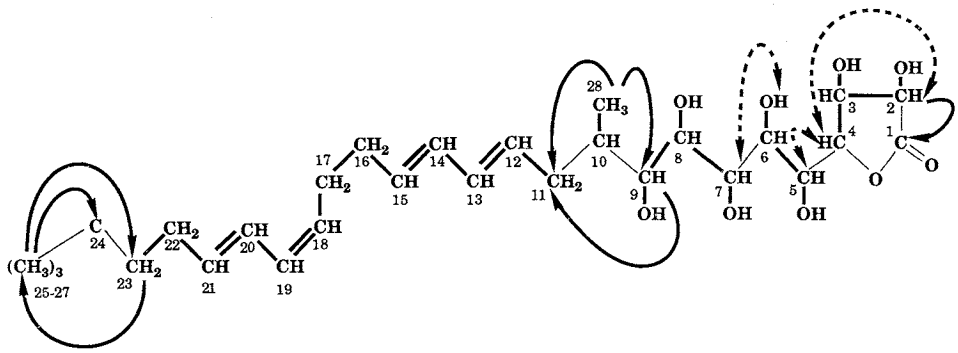
The structural studies were carried out first for butyrolactol A (**1a**, Fig. 2), the major component of

Fig. 2. Structures of butyrolactols A and B.



	R	
Butyrolactol A	$(CH_3)_3C-$	(**1a**)
Butyrolactol B	$(CH_3)_2CH-$	(**1b**)

Fig. 3. 2D COSY experiment of butyrolactol A.



—— ^1H-^1H COSY, ⟶ ^1H-^{13}C long range COSY, ----→ relayed ^1H-^1H COSY.

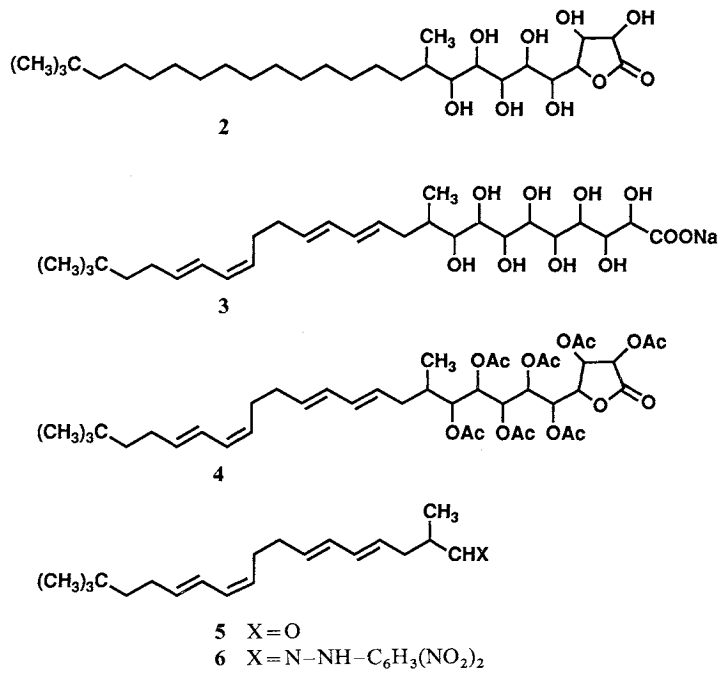
the complex, and the structure of butyrolactol B (**1b**) was subsequently determined by comparing its spectral data with those of **1a**. Molecular formula ($C_{28}H_{46}O_9$) of **1a** indicated six unsaturation, five of which were attributable to four carbon-carbon double bonds (δ 135.8, 131.4, 131.4, 130.8, 130.8, 129.0, 128.6 and 125.2) and one carbonyl (δ 174.8) group based on ^{13}C NMR. Hydrogenation of **1a** over palladium charcol gave an octahydro derivative (**2**): FAB-MS *m/z* 535 $(M+H)^+$: UV, end absorption.

The residual one unsaturation was assigned to be a γ-lactone ring, which had been indicated by a characteristic IR absorption band at 1774 cm^{-1}. **1a** gave an acidic hydrolysis product (**3**) by treatment with dil NaOH. Its IR spectrum showed no absorption band at 1774 cm^{-1} but strong band at 1590 cm^{-1}. Upon treatment with *p*-toluenesulfonic acid, **3** was re-cyclized to yield **1a** which was confirmed by the IR and HPLC.

Two of the nine oxygen atoms in **1a** were assigned to the lactone, and the remaining seven to the hydroxy groups by ^1H NMR. Treatment of **1a** with acetic anhydride in pyridine gave a heptaacetyl derivative **4**: FAB-MS *m/z* 821 $(M+H)^+$; IR ν (KBr) 1750, 1810 cm^{-1}.

The connectivities of most of the carbons were established by ^1H-^1H 2D COSY as shown in Fig. 3. The relayed 2D COSY revealed the linkages between 4-H (δ 4.34) and 5-H (δ 3.64), and 6-OH (δ 4.31) and 7-H (δ 3.73) which could not be determined by ^1H-^1H 2D COSY experiment. On the long-range HETCOR, the ester carbonyl carbon (C-1, δ 174.8) showed a connectivity to 2-H (δ 4.25). In addition, the experiment exhibited cross peaks between C-11 and 28-H (δ 0.77), C-11 and 9-H (δ 3.34), establishing

Fig. 4. The structures of derivatives and degradation products of butyrolactol A.



the linkages from C-9 to C-11 in the side chain. The quarternary carbon at δ 30.1 was assigned as C-24, since long-range couplings were observed between 23-H (δ 1.24) and C-25~27, and 25~27-H and C-23 (δ 43.2), and C-24 (δ 30.1). The downfield chemical resonance of 4-H (δ 4.34) indicated that the γ-lactone ring was between C-1 to C-4.

For the confirmation of the assigned structure, **1a** was treated with $NaIO_4$ in tetrahydrofuran-water. The produced aldehyde (**5**: EI-MS, *m/z* 288 $(M)^+$), was reacted with 2,4-dinitrophenylhydrazine to afford a yellow 2,4-DNP derivative **6**, whose EI-MS (*m/z* 468 $(M)^+$) and ^1H NMR were identical with the expected structure.

The geometry of the two dienes were determined as *E*(C12-13), *E*(C14-15), *Z*(C18-19), *E*(C20-21) on the basis of their coupling constants in the ^1H NMR ($J_{12,13}=14.5$, $J_{14,15}=14.5$, $J_{18,19}=10.7$, $J_{20,21}=15.0$ Hz). Thus, the complete structure of **1a** was determined as Fig. 2 and those of the degradation products as Fig. 4.

The molecular formula of butyrolactol B (**1b**, $C_{27}H_{44}O_9$) is a CH_2 lower than that of **1a**. In the ^1H NMR spectrum of **1b**, the three singlet methyl signals (δ 0.87) of **1a** were missing, and a new methine at δ 1.53 (1H, m) and two doublet methyl signals (δ 0.87) were observed instead. Therefore, the *C*-terminal of **1b** was determined to be an isopropyl group in place of the *t*-butyl group of **1a**. (Fig. 2)

Biological Activity

Antifungal activity of butyrolactol A was compared with that of nystatin by the agar dilution method on yeast morphology agar buffered with 1/15 M phosphate, pH 7.0. A 5-μl suspension containing 2×10^6 cells/ml (2×10^7 cells/ml for *Trichophyton mentagrophytes* 4329) was spotted on the surface of the agar plates containing serial dilutions of the antibiotic and the plates were incubated at 28°C for 40 hours. Table 5 summarizes MICs for butyrolactol A and nystatin. Butyrolactol A showed good activity against

Table 5. *In vitro* antifungal activity of butyrolactol A.

Organism		MIC (μg/ml)	
		Butyrolactol A	Nystatin
Saccharomyces cerevisiae	ATCC 9763	25	3.1
Candida albicans	IAM 4888	6.3	3.1
C. albicans	A 9540	3.1	3.1
C. albicans	ATCC 38247	1.6	50
C. albicans	ATCC 32354 (B311)	25	3.1
C. albicans	83-2-14 (Juntendo)	12.5	3.1
Candida tropicalis	85-8 (Kitasato)	25	6.3
C. tropicalis	IFO 10241	>100	3.1
Cryptococcus neoformans	D49	3.1	3.1
C. neoformans	IAM 4514	3.1	3.1
Aspergillus fumigatus	IAM 2034	1.6	3.1
Trichophyton mentagrophytes	4329	0.4	12.5

MICs determined on yeast morphology agar buffered with 1/15 M phosphate, pH 7.0.

strains of *Aspergillus fumigatus* and *T. mentagrophytes* and moderate activity against yeasts. The MICs against polyene-resistant *C. albicans* (ATCC 38247) were 1.6 and 50 μg/ml for butyrolactol A and nystatin, respectively, indicating no cross resistance between the two antibiotics. Butyrolactol B was as active as butyrolactol A against *C. albicans* A9540 by the broth dilution method in yeast nitrogen broth containing glucose.

Butyrolactol A was inactive against Gram-positive and Gram-negative bacteria at 50 μg/ml when tested by the serial agar dilution method on nutrient agar at 32°C. It had no cytotoxic activity against cultured cells of HCT116 at 50 μg/ml. No lethal toxicity was observed in mice after intraperitoneal administration of 100 mg per kg body weight of butyrolactol A.

Discussion

Butyrolactols A and B are new class of antifungal antibiotics containing 2,3-dihydroxybutyrolactone and a long polyhydroxylalkyl side chain.

They are structurally somewhat related to but are distinct from 1233A[3,4] (antifungal and HMG CoA inhibitory activity) which contains a β-lactone substituted with a long conjugated diene. KOISO *et al.*[5] reported that some sugar γ-lactones (D-arabinoro-1,4-lactone, L-rhamnoro-1,4-lactone *etc.*) isolated from the rice plant promoted the conidial anastomosis of *Gerlachia oryzae*. Butyrolactol A lost its antifungal activity by acetylation, periodate oxidation or catalystic hydrogenation indicating that the hydroxyls and two diene moieties are essential for the activity of butyrolactol.

The absolute configuration of butyrolactols A and B were not known, but it is interesting how butyrolactols A and B can be related with the antifungal activity.

Experimental

Thin layer chromatography (TLC) was performed on precoated silica gel plates (Kieselgel $60F_{254}$, Merck). The IR spectra were determined on a Jasco IR-810 IR spectrometer and the UV spectra on a Jasco UVIDEC-610C spectrometer. Electron impact and fast atom bombartment mass spectra (EI-MS and FAB-MS) were measured on a Jeol JMS-AX 505H mass spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Jeol JML-GX400 spectrometer operated in the Fourier transfer mode. Optical rotation was measured with a Jasco DIP-140 digital polarimeter.

Hydrogenation of **1a**

1a (10 mg) in tetrahydrofuran (THF) - water (5 : 2) was hydrogenated under atmospheric pressure in the presence of 20% Pd-C (20 mg) for 20 hours. The mixture was filtrated and evaporated *in vacuo* to yield an oily residue. This residue was chromatographed on Sephadex LH-20 column (40 ml) using THF - water (20 : 1) to afford a white amorphous powder (**2**, 9.5 mg). **2**: UV (MeOH) end absorption; IR ν (KBr) 3400, 2920, 2850, 1085, 1010 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 0.79 (3H, d, J=6.4 Hz), 0.84 (9H, s), 1.24 (26H, m), 1.61 (2H, m), 3.48 (1H, t, J=8.5 Hz), 3.63 (1H, t, J=8.0 Hz), 3.71 (1H, t, J=8.6 Hz), 3.82 (1H, d, J=8.1 Hz), 3.88 (1H, d, J=8.6 Hz), 3.99 (1H, d, J=8.1 Hz), 4.16 (1H, dt, J=6.4 and 8.4 Hz), 4.26 (1H, dd, J=9.0 and 6.4 Hz), 4.31 (1H, d, J=7.7 Hz), 4.33 (1H, d, J=9.0 Hz), 4.84 (1H, d, J=7.7 Hz), 5.69 (1H, d, J=6.0 Hz), 6.03 (1H, d, J=6.4 Hz); FAB-MS m/z 535 $(M+H)^+$.

Alkaline Hydrolysis of **1a** to **3**

To a solution of **1a** (4 mg) in 3.6 ml of CH_3CN - water (3 : 1) was added 0.1 N NaOH 400 μl. The precipitate deposited was washed with acetone and ether to afford **3** (3.8 mg) which was extremely insoluble in water and organic solvents. **3**: IR ν (KBr) 3350, 3280, 1590, 1420, 1100, 1020 cm^{-1}. To a suspension of **3** (3.5 mg) in DMF (0.8 ml) was added *p*-toluenesulfonic acid, and the mixture was allowed to stand at room temperature for 15 hours. The reaction solution was diluted with water and extracted with EtOAc. The EtOAc layer was concentrated to give a pale yellow solid (3.0 mg, 77%), whose physico-chemical and spectral properties were identical with those of **1**.

Acetylation of **1a**

1a (15 mg) was stirred with acetic anhydride (5.2 ml) and dry pyridine (2.2 ml) for 20 hours at 4°C. The reaction mixture was diluted with water (20 ml) and extracted with EtOAc (15 ml). The extract was purified by preparative HPLC system (YMC-ODS-5, 250 mm i.d. × 20.0 mm, 5 μm, YMC Co., Ltd.) developed with 90% aq CH_3CN followed by Sephadex LH-20 column (40 ml) using methylene chrolide - MeOH (1 : 1) to afford a homogeneous white amorphous powder (2.1 mg) of heptaacetyl derivative (**4**).

4: UV λ_{max} (MeOH), 236 nm (ε 12,500); IR ν (KBr) 1810, 1750, 1370, 1210, 1030 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.89 (9H, s), 0.91 (3H, d, J=6.4 Hz), 1.27 (2H, m), 1.62 (1H, m), 1.81 (1H, dt-like, J=14.1 and 8.5 Hz), 2.08 (3H, s), 2.09 (6H, s), 2.11 (3H, s), 2.12 (3H, s), 2.16 (3H, s), 2.18 (3H, s), 2.25 (2H, dt-like, J=7.7 and 6.9 Hz), 4.54 (1H, dd, J=6.9 and 3.8 Hz), 4.74 (1H, d, J=9.0 Hz), 5.20 (1H, d, J=6.8 Hz), 5.24 (1H, d, J=6.4 Hz), 5.25 (1H, d, J=6.8 Hz), 5.27 (1H, dt, J=11.2 and 6.8 Hz), 5.36 (1H, s), 5.39 (1H, t, J=6.8 Hz), 5.41 (1H, dt, J=14.5 and 6.8 Hz), 5.51 (1H, d, J=6.8 Hz), 5.58 (1H, dt, J=14.6 and 6.8 Hz), 5.67 (1H, dt, J=15.3 and 6.8 Hz), 5.95 (1H, t, J=11.1 Hz), 6.1 (2H, m), 6.27 (1H, ddd, J=15.1, 11.1 and 1.3 Hz). FAB-MS m/z 821 $(M+H)^+$.

Periodic Acid Oxidation of **1a**

To a solution **1a** (10 mg) in 1 ml of THF - water (9 : 1) was added a solution of potassium periodate (75 mg) in 1 N sulfuric acid (2.5 ml). The mixture was stirred for 10 minutes at 40°C, cooled to 15°C, diluted with 1.5 ml of water and extracted with ethyl ether (4 ml). After evaporation of the extract, the residual solid was purified by preparative TLC (silica gel, hexane - Me_2CO (10 : 1)) followed by Sephadex LH-20 chromatography (40 ml) using MeOH to afford 4.1 mg of **5**. **5**: Colorless amorphous powder; UV λ_{max} (MeOH) 235 nm (ε 59,900); IR ν (KBr) 3020, 2960, 2870, 2710, 1730, 1465, 1365, 1260 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.89 (9H, s), 1.10 (3H, d, J=7.3 Hz), 1.27 (2H, m), 2.06 (2H, dt, J=8.5 and 7.3 Hz), 2.16 (3H, m), 2.26 (2H, dt-like, J=7.7 and 6.2 Hz), 2.44 (1H, m), 2.48 (1H, dd, J=6.8 and 6.4 Hz), 5.28 (1H, dt, J=10.7 and 7.3 Hz), 5.51 (1H, dt, J=14.6 and 7.3 Hz), 5.62 (1H, dt, J=15.1 and 6.8 Hz), 5.67 (1H, dt, J=15.1 and 6.8 Hz), 5.96 (1H, t, J=11.3 Hz), 6.04 (2H, m), 6.29 (1H, ddd, J=15.4, 10.6 and 0.9 Hz), 9.65 (1H, d, J=11.7 Hz). EI-MS m/z 288 $(M)^+$. A solution of **5** in ethyl acetate (1 ml) was reacted with 2,4-dinitrophenylhydradine. The mixture was developed on preparative TLC (silica gel, hexane - Me_2CO (5 : 1)), and the DNP-derivative (**6**, 6.2 mg) obtained was desalinated by Sephadex LH-20 chromatography (40 ml) using dichloromethane - MeOH (1 : 1).

6: UV λ_{max} (MeOH) 233 nm (ε 23,600), 272 (sh, 500), 359 (1,000); IR ν (KBr) 2940, 1620, 1590, 1515, 1130 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.89 (9H, s), 1.20 (3H, d, J=6.8 Hz), 2.06 (2H, dt, J=8.5 and

7.7 Hz), 2.16 (3H, m), 2.26 (2H, dt, J=7.7 and 6.9 Hz), 2.38 (1H, m), 2.65 (1H, m), 5.28 (1H, dt, J=10.7 and 7.3 Hz), 5.56 (1H, dt, J=14.1 and 4.1 Hz), 5.64 (1H, dt, J=14.1 and 6.8 Hz), 5.68 (1H, dt, J=15.3 and 6.4 Hz), 5.96 (1H, t, J=11.5 Hz), 6.07 (2H, m), 6.28 (1H, ddd, J=15.4, 11.1 and 1.3 Hz), 7.45 (1H, d, J=15.1 Hz), 7.92 (1H, d, J=9.4 Hz), 8.29 (1H, ddd, J=9.4 , 2.5 and 0.9 Hz), 9.12 (1H, d, J=2.5 Hz). EI-MS m/z 468 $(M)^+$.

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