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AMPHIRIONINS-3 AND -6, NEW POLYKETIDES FROM THE CULTURED MARINE DINOFLAGELLATE *AMPHIDINIUM* SPECIES

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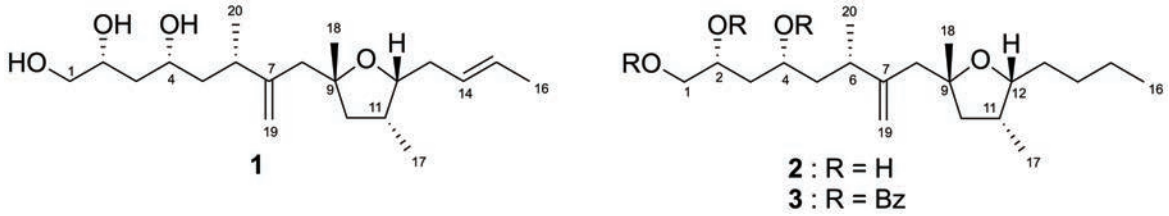
Abstract – Amphirionins-3 (**1**) and -6 (**2**) have been isolated from the marine dinoflagellate *Amphidinium* species. The structures were elucidated by detail analyses of the spectroscopic data, and chemical conversions. Compounds **1** and **2** exhibited moderate cytotoxic activity against human cervix adenocarcinoma HeLa cells.

Marine dinoflagellates of the genus *Amphidinium* are well known to produce polyketide-like metabolites.[1,2] Cytotoxic macrolides[3,4] and polyhydroxy long-chain polyketide compounds[5,6] have been isolated from the marine symbiotic and free-swimming *Amphidinium* species. On the other hand, certain low-polarity polyketide compounds with a relatively-small molecule size, represented by amphidinins[7,8] and amphidinoketides,[9] constitute the third class of *Amphidinium* dinoflagellates metabolites. Recently, we have also discovered a series of new polyketides, amphirionins-2,[10] -4,[11] and -5,[12,13] from the marine benthic dinoflagellate *Amphidinium* species collected off Iriomote Island, Okinawa Prefecture, Japan.

Our continuing search for new polyketides from laboratory-cultured marine dinoflagellate *Amphidinium* species[14-16] resulted in the isolation of two new linear polyketides, amphirionins-3 (**1**) and -6 (**2**), from the benthic dinoflagellate *Amphidinium* species (strains KCA09057 and KCA09056). The structures were elucidated by detailed analyses of the spectroscopic data and chemical conversions. The absolute

configuration was assigned on the basis of the CD chirality method with a tribenzoate derivative (**3**). In this paper, we describe the isolation and structure elucidation of **1** and **2** (Scheme 1).



Scheme 1. Structures of amphirionins-3 (**1**) and -6 (**2**)

The dinoflagellate *Amphidinium* species (strain KCA09057) was monoclonally separated from benthic sea sand samples collected off Iriomote Island, Japan, and cultured unialgally in a 1% Provasoli's enriched seawater (PES) medium with a light and dark cycle of 16 h and 8 h, respectively, for 2 weeks at 27–29 °C. The cultured algal cells (53.54 g, dry weight) of the strain obtained from 2000 L of the medium were extracted with MeOH–toluene (3:1). The toluene-soluble materials of the extract were subjected to a SiO_2 gel, C_{18}, and amino silica gel column chromatography followed by C_{18} HPLC to afford amphirionin-3 (**1**, 0.002% from dry weight). Meanwhile, amphirionin-6 (**2**) was isolated in 0.028% yield from the toluene-soluble materials of the algal cells of the *Amphidinium* strain KCA09056.

Amphirionin-3 (**1**) was obtained as a colorless oil and was optically active $\{[\alpha]_D^{20}$ -26 (*c* 0.1, $CHCl_3)\}$. The molecular formula of **1** was established to be $C_{20}H_{36}O_4$ by HRESIMS (*m/z* 363.25026 $[M+Na]^+$, Δ -0.87 mmu). The ^1H and ^{13}C NMR data (Table 1) of **1** in $CDCl_3$ and C_6D_6 presented a total of 20 carbon resonances, arising from an sp^2 quaternary carbon, sp^2 methylene, oxygenated sp^3 quaternary carbon, two sp^2 methines, five sp^3 methines, six sp^3 methylenes, and four methyls. As two of the three degrees of unsaturation were accounted for, **1** was inferred to possess a single ring system.

Interpretation of ^1H-^1H COSY and TOCSY spectra revealed one isolated methylene (H_2-8) and two proton-proton networks from H_2-1 to H-6 and H_3-20 and from H_2-10 to H_3-16 and H_3-17 (Figure. 1). The disubstituted C-14–C-15 double bond was assigned to be *E*, as suggested by the *J*(H-14/H-15) value (15.2 Hz). The presence of the exomethylene unit at C-7 was suggested by HMBC correlations for H_2-8 (δ_H 2.26 and 2.24)/C-7 (δ_C 150.8), H_3-20 (δ_H 1.00)/C-7, H_2-19 (δ_H 4.85 and 4.84)/C-6 (δ_C 34.4), and H_2-19/C-8 (δ_C 50.5). HMBC correlations from a singlet of methyl protons (H_3-18; δ_H 1.17) to C-8, C-9 (δ_C 82.6), and C-10 (δ_C 47.7) indicated that the oxygenated quaternary carbon at C-9 connects to C-8, C-10, and C-18. The NOESY correlation for H-12 (δ_H 3.97)/H_3-18 suggested that C-9 was involved in an ether linkage with C-12. Thus, the planar structure of **1** was assigned to be a linear polyketide triol associated with a tetrahydrofuran ring and four one-carbon branches.

Table 1. ^{1}H and ^{13}C NMR data of amphirionin-3 (**1**) in $CDCl_3$ and C_6D_6

	$CDCl_3$ [a]				C_6D_6			
positn.	^{13}C		^{1}H		^{13}C		^{1}H	
1	67.0	CH_2	3.60 3.48	m m	67.2	CH_2	3.67 3.58	dd, 10.8, 4.0 dd, 10.8, 5.5
2	72.6	CH	3.94	m	72.8	CH	4.02	m
3	39.1	CH_2	1.62 1.51	m m	39.8	CH_2	1.75 1.40	m m
4	69.1	CH	4.00	m	69.4	CH	4.10	m
5	44.3	CH_2	1.56 1.45	m m	44.6	CH_2	1.49[b]	m
6	34.4	CH	2.65	m	34.5	CH	2.80	m
7	150.8	C			151.0	C		
8	50.5	CH_2	2.26 2.24	d, 12.9 d, 12.9	50.5	CH_2	2.15 2.05	d, 13.0 d, 13.0
9	82.6	C			82.4	C		
10	47.7	CH_2	1.99 1.49	dd, 12.3, 7.3 m	47.4	CH_2	1.57 1.15	dd, 12.3, 7.4 dd, 12.3, 7.6
11	35.1	CH	2.39	m	35.1	CH	2.01	m
12	81.5	CH	3.97	m	81.5	CH	3.72	m
13	34.5	CH_2	2.13[b]	brt, 6.8	34.8	CH_2	2.00[b]	m
14	127.3	CH_2	5.35	dtq, 15.2, 6.8, 1.5	127.6	CH	5.38	m
15	127.8	CH_2	5.49	brdq, 15.2, 6.3	127.9	CH	5.42	m
16	18.2	Me	1.65[c]	dd, 6.3, 1.5	18.2	Me	1.70[c]	brd, 4.3
17	14.8	Me	0.99[c]	d, 7.0	14.5	Me	0.74[c]	d, 6.5
18	25.8	Me	1.17[c]	s	25.5	Me	1.05[c]	s
19	111.9	CH_2	4.85 4.84	s s	111.9	CH_2	4.87 4.82	s s
20	24.0	Me	1.00[c]	d, 6.8	24.1	Me	1.06[c]	d, 6.8

[a]OH signals were observed at δ_H 4.88 (1H, t, J = 2.2 Hz), 4.76 (1H, brs), and 2.36 (1H, brs). [b]2H. [c]3H.

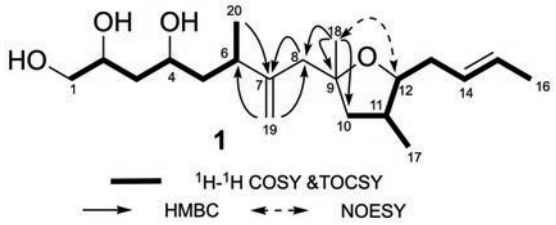


Figure 1. Selected 2D NMR correlations for amphirionin-3 (**1**)

The gross structure of **1** was reminiscent of the amphidinin A[7,17] (**4**) structure. Therefore, the elucidation of the relative stereochemistry of **1** was performed by comparing the ^{1}H and ^{13}C NMR chemical shifts for **1** with those obtained for amphidinin A (**4**). Figure 2 illustrates ^{1}H and ^{13}C chemical shift differences [$\Delta\delta$ (in ppm) = δ for **1** – δ for **4**] for C-1–C-12 and the four branched C_1 moieties at C-6, C-7, C-9, and C-11. The ^{13}C chemical shift differences for C-1–C-7 and the C-20 methyl were negligible (≤0.3 ppm),

indicating that the relative stereochemistry for C-2, C-4, and C-6 of **1** were *R**, *R**, and *S**-configurations, respectively, i.e., the same as those of **4**.[17] The relative configuration for the tetrahydrofuran ring at C-9–C-12 was elucidated by analysis of the NOESY correlations observed for **1** in $CDCl_3$ (Figure. 3). NOESY correlations for H-6/H-8a, H-8a/H_3-20, and H-8b/H_3-18 suggested the 6*S**- and 9*R**-relations. Thus, total six stereocenters in **1** were established to have the same relative configurations as those of **4**.

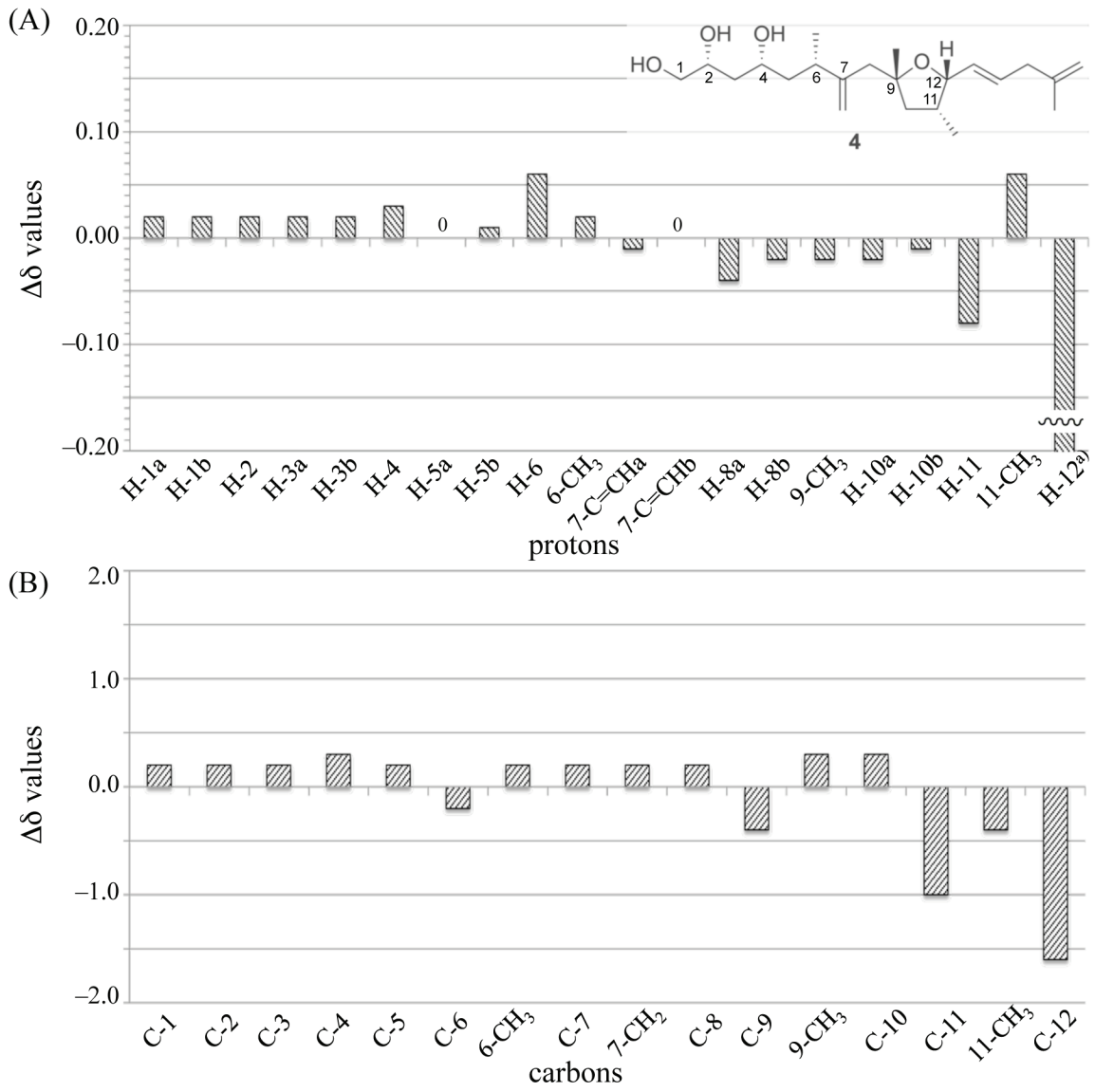


Figure 2. Differences between NMR chemical shifts of amphirionins-3 (**1**) and amphidinin A (**4**) in $CDCl_3$. (A) $\Delta\delta_H$ (in ppm) = δ_H of **1** − δ_H of **4**. (B) $\Delta\delta_C$ (in ppm) = δ_C of **1** − δ_C of **4**. "a" and "b" for germinal proton pairs denoted low-and high-field resonances, respectively. a) $\Delta\delta_H$ values for H-12 was -0.43.

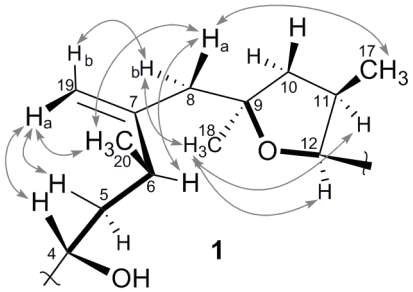


Figure 3. Stereostructure for the C-4–C-12 portion in amphirionin-3 (**1**).
Arrows show the NOESY correlations in $CDCl_3$.

The molecular formula, $C_{20}H_{38}O_4$, of amphirionin-6 (**2**) was revealed by [ESIMS *m/z* 365.26675 $(M+Na)^+$, Δ -0.03 mmu]. The ^{13}C NMR data of **2** in C_6D_6 presented a total of 20 carbon resonances, arising from an sp^2 quaternary carbon, sp^2 methylene, oxygenated sp^3 quaternary carbon, five sp^3 methines, eight sp^3 methylenes, and four methyls. The planar structure of **2** elucidated on the basis of detailed NMR studies in C_6D_6 was established to be a 14,15-dihydro derivative of amphirionin-3 (**1**). The reduction of **1** using palladium–charcoal under hydrogen atmosphere afforded **2**, thus indicating that **2** possessed identical stereochemistry to that of **1**. The absolute stereochemistry of C-4 in **2** was assigned as *R* on the basis of the CD spectrum of the tribenzoate derivative (**3**) of **2**, where the negative and positive Cotton effects were detected at 233 (Δε -5.61) and 220 (+2.60) nm, respectively.[18] Therefore, the absolute configurations of the six chiral centers of **1** and **2** were concluded to be 2*R*, 4*R*, 6*S*, 9*R*, 11*R*, and 12*R*.

Two new low-polarity polyketide compounds, amphirionins-3 (**1**) and -6 (**2**), are congeners of amphidinins A[7] and G.[8] Compounds **1** and **2** exhibited medium growth inhibition against human cervix adenocarcinoma HeLa cells with equal IC_{50} values of 30 μM, indicating that the double bond at C-14 do not affect the cytotoxic activity.

EXPERIMENTAL

General information. The optical rotations were measured on a JASCO DIP-370 and P-2300 polarimeters, IR spectra were recorded on a JASCO FT/IR-5300 spectrophotometer. The CD spectrum was obtained on a JASCO J-1500 spectropolarimeter. NMR data for **1** were recorded on an Agilent NMR400WB spectrometer equipped with a PFG-HX or XH nanoprobe. NMR spectra of **2** were measured on a Bruker AMX-500 spectrometer using 2.5–mm microcells (Shigemi Co. Ltd.). The ^1H and ^{13}C chemical shifts in $CDCl_3$ were reported in ppm relative to the residual signals of $CDCl_3$ (δ_H 7.16 and δ_C 77.16). Chemical shifts in C_6D_6 are reported in ppm with reference to the residual proton and carbon signals (δ_H 7.20 and δ_C 128.0) of the solvent. ESIMS spectra were obtained on an LTQ Orbitrap XL and Exactive spectrometers (ThermoFisher Scientific Inc.).

Cultivation and Isolation. A seawater medium was added to benthic sea sand samples collected off Iriomote Island, Japan, and the mixture was incubated for several weeks. Two dinoflagellate strain KCA09056 and KCA09057 were isolated monoclonally by micropipetting. The voucher specimens were deposited at the Center for Advanced Marine Core Research, Kochi University. The KCA09057 strain was cultured at 27–29 °C for two weeks in seawater medium enriched with 1% PES supplement, with light and dark cycles of 16 h and 8 h, respectively. The dried algal cells (53.54 g from 2,000 L culture) were extracted with MeOH–toluene (3:1), and partitioned between toluene and H_2O. The toluene-soluble materials (2.94 g) were subjected to silica gel column chromatography using a gradient elution of 0–5% MeOH in $CHCl_3$. The fraction eluted with $CHCl_3$–MeOH (95:5) was then chromatographed on C_{18} (MeCN–H_2O, 7:3) and amino silica gel (hexane–EtOAc, 1:1–1:4) columns. The fraction eluted with hexane–EtOAc (1:4) was separated by C_{18} HPLC [YMC-Pack Pro C_{18}, 10 mm × 250 mm; eluent, MeCN–H_2O (70:30); flow rate, 2 mL/min; UV detection at 230 nm] to afford amphirionin-3 (**1**, 1.0 mg, 0.002%). The strain KCA09056 was cultured under the above-described conditions. The harvested cells (15.3 g, from 400 L culture) were extracted with MeOH–toluene (3:1), and the extract was partitioned between toluene and water. The toluene-soluble fractions (2 g) were subjected to SiO_2 column chromatography using a stepwise elution of $CHCl_3$ and $CHCl_3$–MeOH (98:2). The fraction eluted with ($CHCl_3$–MeOH, 98:2) was chromatographed successively on a C_{18} column (MeCN–H_2O, 7:3) followed by C_{18} HPLC [YMC-Pack Pro C_{18}, 5 μm, YMC Co., Ltd., 10 x 250 mm; eluent, MeCN–H_2O (60:40); flow rate, 2 mL/min; UV detection at 210 nm] to afford amphirionin-6 (**2**, 4.3 mg, 0.028%).

Amphrionin-3 (1): colorless oil; $[\alpha]_D^{20}$ -26 (*c* 0.1, $CHCl_3$); IR (neat) ν_{max} 3450 (broad) and 2980 cm^{-1}; ^1H and ^{13}C NMR data ($CDCl_3$ and C_6D_6, see Table 1); HRESIMS *m/z* 363.25026 [calcd for $C_{20}H_{36}O_4Na$, $(M+Na)^+$: 363.25113].

Amphirionin-6 (2): colorless oil; $[\alpha]_D^{20}$ -34 (*c* 0.08, $CHCl_3$); $[\alpha]_D^{20}$ -15 (*c* 0.05, C_6H_6); $[\alpha]_D^{20}$ -29 (*c* 0.05, MeOH); IR (neat) ν_{max} 3446 (broad) and 2920 cm^{-1}; ^1H NMR (C_6D_6) δ 0.76 (3H, d, *J* = 6.8 Hz, H_3-17), 0.94 (3H, t, *J* = 6.7 Hz, H_3-16), 1.07 (3H, d, *J* = 7.0 Hz, H_3-20), 1.09 (3H, s, H_3-18), 1.18 (1H, dd, *J* = 12.2 and 6.8 Hz, H-10b), 1.24 (2H, m, H-14b and H-15b), 1.26 (1H, m, H-13b), 1.30 (1H, m, H-15a), 1.38 (2H, m, H-3b and H-14a), 1.39 (1H, m, H-13a), 1.50 (2H, m, H_2-5), 1.62 (1H, dd, *J* = 12.2 and 7.2 Hz, H-10a), 1.75 (1H, dt, *J* = 14.7 and 10.8 Hz, H-3a), 1.97 (1H, m, H-11), 2.07 (1H, d, *J* = 12.9 Hz, H-8b), 2.20 (1H, d, *J* = 12.9 Hz, H-8a), 2.82 (1H, m, H-6), 3.57 (1H, dd, *J* = 10.5 and 5.5 Hz, H-1b), 3.66 (1H, m, H-1a), 3.67 (1H, m, H-12), 4.01 (1H, m, H-2), 4.13 (1H, m, H-4), 4.83 (1H, s, H-19b), and 4.87 (1H, s, H-19a); ^{13}C NMR (C_6D_6) δ 14.2 (CH_3, C-16), 14.6 (CH_3, C-17), 23.1 (CH_2, C-15), 24.0 (CH_3, C-20), 25.4 (CH_3, C-18), 28.6 (CH_2, C-14), 30.6 (CH_2, C-13), 34.7 (CH, C-6), 35.2 (CH, C-11), 39.9 (CH_2, C-3), 44.5 (CH_2, C-5), 47.7 (CH_2, C-10), 50.6 (CH_2, C-8), 67.2 (CH_2, C-1), 69.3 (CH, C-4), 72.8

(CH, C-2), 81.6 (CH, C-12), 82.1 (C, C-9), 112.0 (CH_3, C-19), and 150.9 (C, C-7); HRESIMS *m/z* 365.26675 [calcd for $C_{20}H_{38}O_4Na$, $(M+Na)^+$: 365.26678].

Reduction of Amphirionin-3 (1). Amphirionin-3 (**1**, 0.3 mg) was dissolved in EtOAc (200 μL), and treated with 10% palladium-charcoal (1 mg) under hydrogen atmosphere at room temperature for 1 h. After filtration and evaporation of the solvent, the residue was purified by silica gel column chromatography (hexane–EtOAc, 2:1) to afford **2** (0.1 mg). **2**: colorless oil; $[\alpha]_D^{20}$ -40 (*c* 0.05, $CHCl_3$); ^{1}H NMR (C_6D_6) δ 0.76 (3H, d, *J* = 6.7 Hz), 0.95 (3H, t, *J* = 6.7 Hz), 1.07 (3H, d, *J* = 6.9 Hz), 1.09 (3H, s), 1.13 ~ 1.30 (5H, m), 1.35 ~ 1.42 (3H, m), 1.50 (2H, m), 1.62 (1H, dd, *J* = 12.4 and 7.4 Hz), 1.76 (1H, brdt, *J* = 14.7 and 10.8 Hz), 1.97 (1H, m), 2.07 (1H, d, *J* = 13.0 Hz), 2.20 (1H, d, *J* = 13.0 Hz), 2.82 (1H, m), 3.57 (1H, dd, *J* = 10.5 and 5.5 Hz), 3.62 ~ 3.69 (2H, m), 4.02 (1H, m), 4.14 (1H, m), 4.84 (1H, s), and 4.87 (1H, s); HRESIMS *m/z* 365.26573 [calcd for $C_{20}H_{38}O_4Na$, $(M+Na)^+$: 365.26678].

1,2,4-Tris-*O*-benzoyl Ester (3) of amphirionin-6 (2). To a solution of amphirionin-6 (**2**, 1 mg) in pyridine (40 μL) was added benzoyl chloride (6 μL), and the mixture was stirred at room temperature for 2 h. After addition of water, the mixture was extracted with EtOAc, and the combined extract was washed with 1 M aqueous HCl, saturated aqueous $NaHCO_3$, and water. After removal of the solvent, the residue was purified by silica gel column chromatography (hexane–Et_2O, 9:1) to afford the 1,2,4-tris-*O*-benzoyl ester (**3**, 0.61 mg) of **2**. **3**: CD (MeOH) λ_{ext} 233 (Δε -5.61) and 220 nm (+2.60) nm; ^{1}H NMR ($CDCl_3$) δ 0.84 (3H, t, *J* = 7.0 Hz), 0.87 (3H, d, *J* = 7.0 Hz), 1.04 (3H, s), 1.06 (3H, d, *J* = 7.0 Hz), 1.18 ~ 1.35 (4H, m), 1.46 (1H, m), 1.48 ~ 1.60 (2H, m), 1.73 (1H, m), 1.76 ~ 1.90 (2H, m), 2.21 (1H, d, *J* = 14.1 Hz), 2.25 (1H, d, *J* = 14.1 Hz), 2.25 (1H, m), 2.28 (1H, m), 2.34 (1H, m), 2.47 (1H, m), 3.73 (1H, m), 4.50 (1H, d, *J* = 6.1 and 12.2 Hz, H-1), 4.64 (1H, dd, *J* = 2.9, and 12.2 Hz, H-1), 4.75 (1H, s, H-18), 4.79 (1H, brs, H-18), 5.34 (1H, m), 5.64 (1H, m), 7.39 (6H, m), 7.52 (3H, m), and 7.98 (6H, m); HRESIMS *m/z* 677.34426 [calcd for $C_{41}H_{50}O_7Na$, $(M+Na)^+$: 677.34542].

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