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IRIOMOTEOLIDES-4A AND -5A, HYDROPHILIC MACROLIDES FROM MARINE DINOFLAGELLATE *AMPHIDINIUM* SPECIES

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Abstract – Two new macrolides, iriomoteolides-4a (**1**) and -5a (**2**), have been isolated from a marine benthic dinoflagellate *Amphidinium* sp. (strain HYA024), and the structures were assigned by detailed analyses of 2D NMR data. Iriomotelide-4a (**1**) is a 16-membered macrolide with five hydroxyl groups on the lactone ring and an isoprenoid-like side chain, while iriomoteloide-5a (**2**) is a 20-membered macrolide with five hydroxyl groups and three vicinally-located C₁ branches. Iriomoteolides-4a (**1**) and -5a (**2**) exhibited moderate cytotoxic activity against antitumor cells.

Amphidinium dinoflagellates have been known as producers of polyketide-like metabolites with unique structural features.^{1,2} We have screened numerous *Amphidinium* strains by using genetic analyses,³ cytotoxic screening, and metabonomics analyses, and found an *Amphidinium* strain, named HYA024, which produced unknown cytotoxic macrolides. Three new cytotoxic 20-membered macrolides, iriomoteolides-1a, 1b, and 1c, and a 15-membered macrolide, iriomoteolide-3a, have been isolated from the strain.⁴⁻⁶ Further examination of the extract led to the isolation of two new 16- and 20-membered macrolides, iriomoteolides-4a (**1**) and -5a (**2**). Herein we describe the isolation and structure elucidation of **1** and **2**.

The *Amphidinium* strain, HYA024 was monoclonally separated from sea sand collected off Iriomote Island, Japan. The cultured algal cells (15 g, dry weight) obtained from 400 L of the medium were extracted with the MeOH/toluene solvent system. The toluene soluble materials of the extract were subjected to SiO₂ gel, C₁₈, and NH₂-SiO₂ columns followed by C₁₈ HPLC to afford iriomoteolides-4a (**1**, 0.001 %) and -5a (**2**, 0.001%). Known iriomoteolides were obtained from a less-polar fraction of the SiO₂ gel column. The structures of **1** and **2** were shown in Figure 1.

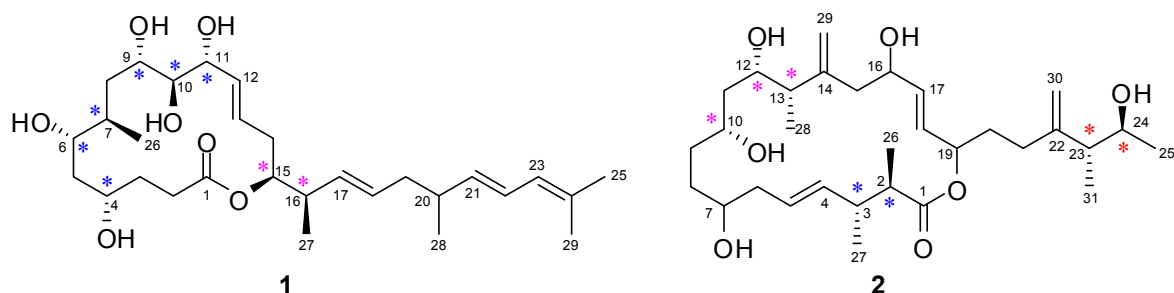


Figure 1. The structures of Iriomoteolide-4a (**1**) and Iriomoteolide-5a (**2**)

C-4, C-5, C-7, C-9, C-10, and C-11 and C-15 and C-16 of **1** were independently relative.

C-2 and C-3, C-10, C-12, and C-13, and C-23 and C-24 for **2** were independently relative.

Iriomoteolide-4a (**1**) showed the pseudomolecular ion peaks at m/z 531 ($M+Na$)⁺ in the positive-mode ESIMS spectra, respectively. The molecular formula, C₂₉H₄₈O₇, of **1** was established by HRESIMS data [m/z 531.3336 ($M+Na$)⁺, Δ +0.1 mmu]. ¹H and ¹³C NMR data (Table 1) in CDCl₃ assigned by using HMQC spectrum disclosed the presence of a total of 29 carbon signals due to an ester carbonyl, an sp² quaternary carbon, seven sp² methines, nine sp³ methines including six oxygenated ones, six sp³ methylenes, and five methyls.

Table 1. ¹H and ¹³C NMR data of Iriomoteolide-4a (**1**) in CDCl₃

position	¹³ C	¹ H (<i>J</i> ind Hz)
1	174.5 C	
2	30.0 CH ₂	2.51 ^a m
3	31.1 CH ₂	1.99 m
4	71.1 CH	1.86 m
5	36.6 CH ₂	4.03 brs
6	73.6 CH	1.57 m
7	35.5 CH	1.45 m
8	35.4 CH ₂	3.93 brd, 10.0
9	68.5 CH	1.96 m
10	77.1 CH	1.66 m
11	73.0 CH	1.59 m
12	130.8 CH	3.73 m
13	128.3 CH	3.57 dd, 2.0, 4.5
14	33.6 CH ₂	4.33 dd, 2.0, 6.3
15	78.0 CH	5.69 dd, 6.3, 15.6
16	40.4 CH	5.73 m
		2.41 ddd, 2.0, 6.3, 14.5
		2.32 ddd, 6.3, 8.0, 14.5
		4.79 dt, 2.0, 8.0
		2.45 m

17	132.1	CH	5.28	dd, 8.1, 15.6
18	130.4	CH	5.44	m
19	40.3	CH ₂	2.05	m
			2.02	m
20	37.1	CH ₂	2.23	m
21	137.0	CH	5.45	m
22	125.1	CH	6.18	dd, 10.9, 15.1
23	125.1	CH	5.77	brd, 10.9
24	133.2	C		
25	18.2	CH ₃	1.73 ^b	brs
26	15.2	CH ₃	1.03 ^b	d, 6.6
27	17.1	CH ₃	0.97 ^b	d, 6.6
28	20.1	CH ₃	0.99 ^b	d, 6.6
29	25.9	CH ₃	1.75 ^b	brs

^a2H. ^b3H.

The planar structure of **1** was elucidated on the basis of 2D NMR data measured in CDCl₃. Analyses of ¹H-¹H COSY and TOCSY spectra revealed a continued spin network from H₂-2 to H-23, H₃-26, H₃-27 and H₃-28 (Figure 2). Three disubstituted *E*-double bonds at C-12, C-17 and C-21 were indicated by *J*(H-12/H-13) (15.6 Hz), *J*(H-17/H-18) (15.6 Hz) and *J*(H-21/H-22) values (15.1 Hz). Both of two singlet methyl signals (H₃-25; δ_H 1.73, H₃-29; δ_H 1.75) showed HMBC correlations for an sp² quaternary and an sp² methine carbons (C-23; δ_C 125.1, C-24; δ_C 133.2, respectively), suggesting the presence of an isobutene terminus. The HMBC correlation for H₂-2 (δ_H 2.51, 2H)/C-1 (δ_C 174.5) implied an ester carbonyl was attached to C-2, and the relative low field resonance for H-15 (δ_H 4.79) suggested that C-15 was involved in an ester linkage with C-1. Thus, the planar structure of iriomoteolide-4a was revealed to be a 16-membered macrolide associated with five hydroxyl groups and four olefins.

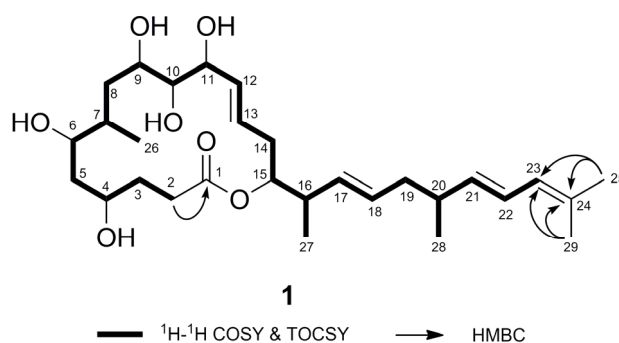


Figure 2. Selected 2D NMR correlations for iriomoteolide-4a (**1**)

The relative stereochemistry of nine chiral centers in the macrocyclic ring was assignable from analyses of sp³-sp³ bond rotations based on ¹H-¹H coupling constants and NOESY data (Figure 3). Magnitudes of ¹H-¹H coupling constants were estimated by selective population transfer (SPT) experiments and intensities of correlations observed for ¹H-¹H COSY spectrum, when signals were overlapped with other signals or had multiple couplings. Nevertheless, the ⁿJ_{CH} were not obtained due to the small amount of samples. Compound **1** is difficult to get by re-cultivation of this dinoflagellate from the reason why the macrolide producing ability may decrease significantly or be lost.

The 1,3-*syn* relation for 4-OH and 6-OH was inferred by relatively large ^1H - ^1H couplings for H-4/H-5a and H-5a/H-6, and rather small ones for H-4/H-5b and H-5b/H-6 and NOESY correlations for H-4/H-6 and H-5b/H-7 (Figures 3a, 3b, and 3i). Bond rotation analyses as shown in Figure 3c was suggested to be *erythro* for C-6–C-7. The 1,3-*anti* relation for the 7-methyl (C-26) and the 9-hydroxyl groups was deduced from coupling magnitudes of H-7/H₂-8 and H₂-8/H-9 and NOESY correlations as shown in Figures 3d and 3e. Both *erythro* configurations for C-9–C-10 and C-10–C-11 were assigned by ^1H - ^1H coupling constants for H-9/H-10, H-10/H-11, and H-11/H-12 (4.5, 2.8 and 6.3 Hz, respectively) and NOESY correlations for H-8a/H-12 and H-8b/H-10 (Figure 3f, 3g, and 3j). Thus the relative configuration was concluded to 4*S**, 6*S**, 7*R**, 9*R**, 10*R**, and 11*S**. On the other hand, the C-15–C-16 bond (Figure 3h) was elucidated to be *erythro* by NOESY correlations for H-14a/H₃-27, H-14b/H-15, H-15/H-17, and H-15/H₃-27 as well as ^1H - ^1H coupling constants for H-15/H-16 (8.0 Hz). Nevertheless,

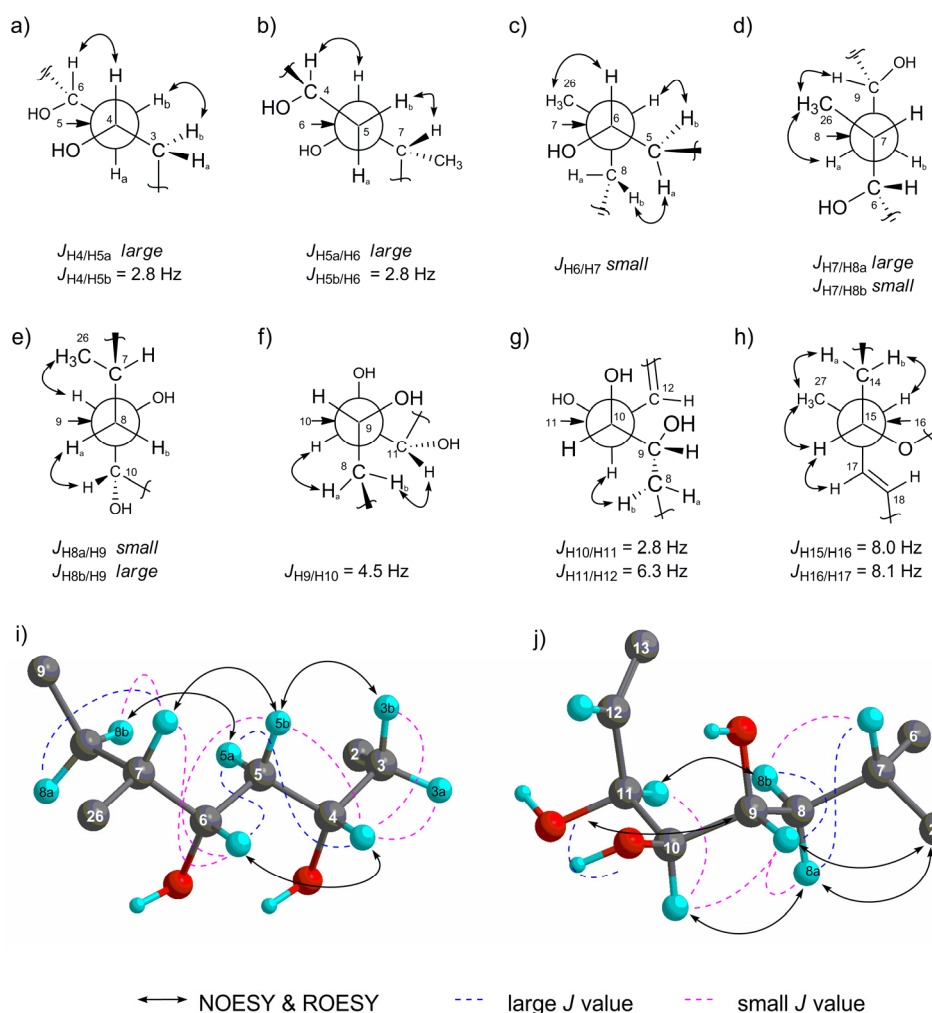


Figure 3. Bond rotations for (a) C-4–C-5, (b) C-5–C-6, (c) C-6–C-7, (d) C-7–C-8, (e) C-8–C-9, (f) C-9–C-10, (g) C-10–C-11 and (h) C-15–C-16 and perspective drawings for (i) C-2–C-8 and (j) C-6–C-13 portions in iriomoteolide-4a (1).

the relation between C-11 and C-15 through four carbon–carbon bonds is not assignable unambiguously, because transannular NOE was not observed.

HRESIMS data [m/z 531.3336 (M+Na)⁺, Δ +0.1 mmu] of iriomoteolide-5a (**2**) established the molecular formula C₃₁H₅₂O₇. ¹H and ¹³C NMR data (Table 2) in CDCl₃ assigned by using HMQC spectrum disclosed the presence of a total of 31 carbon signals due to an ester carbonyl, two sp² quaternary carbons, four sp² methines, two sp² methylenes, ten sp³ methines including six oxygenated ones, seven sp³ methylenes, and five methyls.

Table 2. ¹H and ¹³C NMR Data of Iriomoteolide-5a (**2**) in CDCl₃

position	¹³ C		¹ H (<i>J</i> in Hz)	
1	175.7	C		
2	45.8	CH	2.33	m
3	40.4	CH	5.32	m
4	137.6	CH	5.52	dd, 6.9, 15.4
5	126.0	CH	5.42	ddd, 5.5, 8.8, 15.4
6	39.9	CH ₂	2.36	m
			1.96	m
7	69.8	CH	3.60	m
8	32.0	CH ₂	1.64	m
			1.45	m
9	31.6	CH ₂	1.61	m
			1.59	m
10	69.2	CH	3.97	m
11	38.0	CH ₂	1.88	m
			1.66	m
12	69.8	CH	4.04	m
13	43.8	CH	2.23	m
14	148.5	C		
15	43.6	CH ₂	2.36	m
			2.15	m
16	70.3	CH	4.26	m
17	129.4	CH	5.72	dd, 5.7, 15.8
18	135.1	CH	5.69	dd, 5.6, 15.8
19	73.3	CH	5.30	dt, 13.3, 5.6
20	32.6	CH ₂	1.85	m
			1.82	m
21	29.4	CH ₂	2.12	m
			2.05	m
22	151.1	C		
23	48.9	CH	2.10	m
24	69.9	CH	3.65	m
25	20.3	CH ₃	1.20 ^b	d, 6.8

26	16.3	CH ₃	1.17 ^b	d, 6.8
27	18.4	CH ₃	1.07 ^b	d, 7.0
28	14.1	CH ₃	1.15 ^b	d, 6.8
29	113.8	CH ₂	5.07	s
			5.01	s
30	110.8	CH ₂	4.93	s
31	14.1	CH ₃	0.99	d, 6.8

^a2H. ^b3H.

Detailed analyses of ¹H-¹H COSY and TOCSY spectra revealed three spin systems from H-2 to H-13, H₃-26, H₃-27 and H₃-28, from H₂-15 to H₂-21, and from H-23 to H₃-25 and H₃-31 (Figure 4). *J*(H-4/H-5) and *J*(H-17/H-18) values (15.4 and 15.8 Hz, respectively) were suggestive of both *E*-geometries for two disubstituted double bonds at C-4 and C-17. HMBC correlations for H₃-28 (δ_{H} 1.15)/C-14 (δ_{C} 148.5), H₂-29 (δ_{H} 5.07 and 5.01)/C-14, and H₂-29/C-15 (δ_{C} 43.6) indicated that C-13 was attached to C-15 through an exomethylene unit (C-14–C-29). Connection of C-21 and C-23 via another exomethylene unit (C-22–C-30) was deduced from HMBC correlations for H₂-30 (δ_{H} 4.93, 2H)/C-21 (δ_{C} 29.4), H₂-30/C-23 (δ_{C} 48.9) and H₃-31 (δ_{H} 0.99)/C-22 (δ_{C} 151.1). The methyl proton on C-2 (H₃-26, δ_{H} 1.17) showed an HMBC correlation to the carbonyl carbon (C-1, δ_{C} 175.7), indicating the attachment of the ester carbonyl to C-2. The relatively low-field resonance of H-19 (δ_{H} 5.30) suggested the existence of 20-membered macrolactone ring with an ester linkage between C-1 and C-19. Thus, the planar structure of iriomoteolide-5a was concluded to be **2**.

Although iriomoteolide-5a (**2**) possessed ten chiral centers, it was limiting for 1,2- and 1,3-relations to elucidate the stereochemistry from bond rotation analyses. The *erythro* relation for C-2–C-3 bond (Figure 5a) was suggested by NOESY correlations for H-4/H₃-27 and H₃-26/H₃-27 and the small coupling magnitude for H-2/H-3. The *J*(H-10/H-11a), *J*(H-10/H-11b), *J*(H-11a/H-12), and *J*(H-11b/H-12) values (4.0, 6.1, 7.3, and 4.3 Hz, respectively) implied *gauche* for H-10–H-11a and H-11b–H-12 and *anti* for H-10–H-11b and H-11a–H-12 (Figure 5b and 5c). Both *gauche* relations for C-9–C-12 and C-10–C-13 were elucidated from NOESY correlations for H-9b/H-12, H-10/H-13, and H-10/H₃-28, thus indicating the 1,3-*anti* relation for two hydroxyl groups at C-10 and C-12. The C-12–C-13 and C-23–C-24 bonds were concluded to be *threo* and *erythro*, respectively, from ¹H-¹H

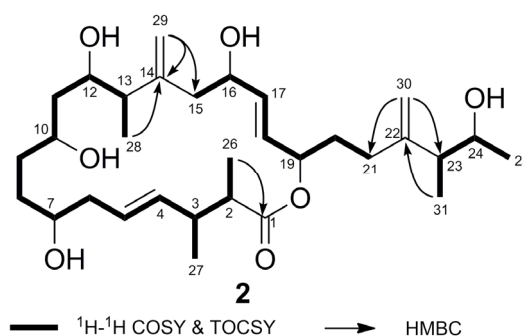


Figure 4. Selected 2D NMR correlations for iriomoteolide-5a (**2**)

coupling data and NOESY correlations as shown in Figures 5d and 5e. Although relatively long-range NOE's for H-5/H-7, H-13/H-16 and H-15a/H-18 were observed, the relative stereochemistries for these three units and three isolated chiral centers of C-7, C-16, and C-19 remained unknown.

Iriomoteolide-4a (**1**) is a novel 16-membered macrolide having a unique carbon skeleton associated with five hydroxyl groups and an isoprene-like side chain. **1** is the second example with a 16-membered macrocyclic ring in *Amphidinium* macrolides.⁷ The isoprene-like side chain may be generated from a polyketide chain with C₁ branches derived from C-2 of acetate.⁸ Iriomoteolide-5a (**2**) is a novel 20-membered macrolide

possessing 5-hydroxyl groups and three portions of vicinally locating C₁ branches. Although two classes of 20-membered macrolides such as amphidinolides A and U had been isolated from the symbiotic dinoflagellate *Amphidinium* species,^{9,10} the carbon chain length and C₁-branched and oxygen-substituted positions for **2** are quite different from those of these known 20-membered macrolides. Our preliminary in vitro screening on antitumor activity showed that iriomoteolide-4a and -5a exhibited moderate cytotoxicity against human B lymphocyte DG-75 (IC₅₀: 0.8 and 1.0 μg/mL, respectively).

EXPERIMENTAL

General. Optical rotations were measured on a JASCO DIP-370 polarimeter. IR spectra were recorded on a JASCO FT/IR-5300 spectrophotometer. ¹H, ¹³C, and 2D NMR spectra were measured on a Bruker AMX-500 spectrometer using 2.5 mm micro cells for CDCl₃ (Shigemi Co., Ltd.). Chemical shifts in CDCl₃ are reported in ppm with reference to the solvent residual proton and carbon signals (δ_H 7.26 and δ_C 77.0). ¹H-¹H Coupling constants were estimated by SPT analyses and magnitudes of ¹H-¹H COSY correlations. ESIMS spectra were obtained on a JEOL JMS 700-TZ spectrometer at -80 V as a focus voltage using a sample dissolved in MeOH with flow rate of 200 μL/min.

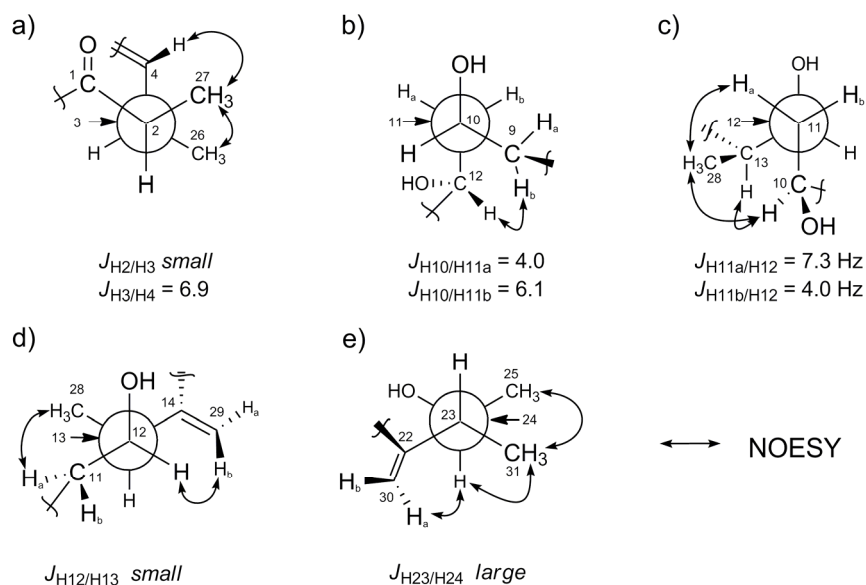


Figure 5. Bond rotations for (a) C-2-C-3, (b) C-10-C-11, (c) C-11-C-12, (d) C-12-C-13 and (e) C-23-C-24 in iriomoteolide-5a (**2**).

Isolation. Cultivation and extraction were described previously.⁴ The toluene-soluble fractions (2 g) obtained from the harvested HYA024 cells (15.3 g, from 400 L of culture) were subjected to SiO₂ column chromatography (40 x 200 mm) using a stepwise elution of CHCl₃ (200 mL) and CHCl₃/MeOH (98:2, 200 mL and then 95:5, 200 mL). The fraction eluted with (CHCl₃/MeOH, 95:5) was chromatographed successively by using a C₁₈ (MeCN/H₂O, 7:3) and then NH₂-SiO₂ columns (n-hexane/EtOAc, 2:1). A macrolide-containing fraction was separated by C₁₈ HPLC [YMC-Pack Pro C₁₈, 5 μm, YMC Co., Ltd., 10 x 250 mm; eluent, MeCN/H₂O (60:40); flow rate, 2 mL/min; UV detection at 210 nm] to afford iriomoteolides-4a (**1**, 0.001%) and 5a (**2**, 0.001%).

Iriomoteolide-4a (1). Colorless amorphous; $[\alpha]_D^{20}$ -20 (*c* 0.02, CHCl₃); IR (neat) ν_{\max} 3300 (broad) and 1721 cm⁻¹; ¹H and ¹³C NMR data (Table 1); ESIMS (pos.) *m/z* 531 (M+Na)⁺; ESIMS (neg.) *m/z* 543 and 545 [ca. 3:1, (M+Cl)⁻]; HRESIMS *m/z* 531.3336 [calcd for C₂₉H₄₈O₇Na, (M+Na)⁺: 531.3336].

Iriomoteolide-5a (2). Colorless amorphous; $[\alpha]_D^{22}$ +65 (*c* 0.02, CHCl₃); IR (neat) ν_{\max} 3420 (broad) and 1718 cm⁻¹; ¹H and ¹³C NMR data (Table 2); ESIMS (pos.) *m/z* 559 (M+Na)⁺; ESIMS (neg.) *m/z* 571 and 573 [ca. 3:1, (M+Cl)⁻]; HRESIMS *m/z* 559.3615 [calcd for C₃₁H₅₂O₇Na, (M+Na)⁺: 559.3615].

Cytotoxic Assay. Human B lymphocyte DG-75 cells were seeded at a density of 5000 cells per well into 96-well plates in culture medium containing 10% FBS. After 72 h, the number of viable cells was counted using Cell Counting Kit 8 (Dojindo Co., Kumamoto, Japan) according to the manufacturer's instructions. The assay reagent is a tetrazolium compound (WST-8) that is reduced by live cells into a colored formazan product measured at 450 nm using a microplate reader (Bio-Rad, USA). The viability of the treated groups was estimated as a percentage of control groups. The cytotoxicity was shown as the concentration causing a 50% reduction of cell growth (IC₅₀). Doxorubicin and 5-fluorouridine (IC₅₀: 0.04 and 1.2 μg/mL, respectively) were used as authentic samples, and the experiments were repeated in triplicate wells.

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