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

Keiko Kumagai,[1] Masashi Tsuda,[2]* Eri Fukushi,[3] and Jun Kawabata[3]

[1] Science Research Center, Kochi University, Nankoku, Kochi 783-8506, Japan. [2] Center for Advanced Marine Core Research, Kochi University, Nankoku, Kochi 783-8502, Japan. [3] Research Faculty of Agriculture, Hokkaido University, Sapporo, Hokkaido 060-0812, Japan. E-mail: mtsuda@ kochi-u.ac.jp

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 C_1 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 SiO_2 gel, C_{18}, and NH_2-SiO_2 columns followed by C_{18} HPLC to afford iriomoteolides-4a (**1**, 0.001 %) and -5a (**2**, 0.001%). Known iriomoteolides were obtained from a less-polar fraction of the SiO_2 gel column. The structures of **1** and **2** were shown in Figure 1.

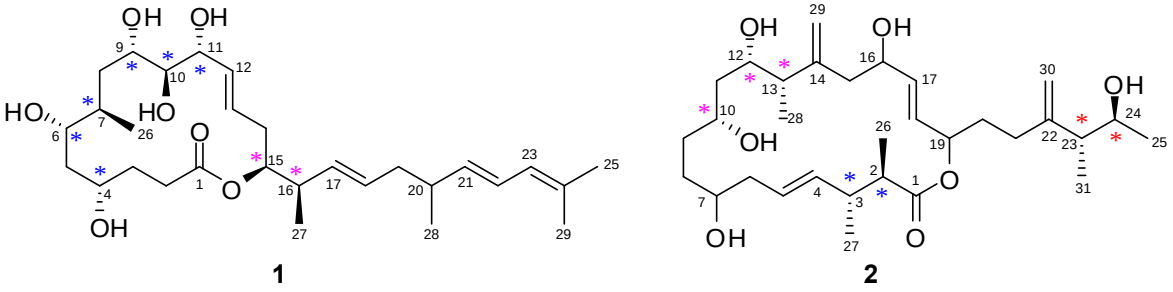

Figure 1. The structures of Iriomoteolide-4a (**1**) and Iriomoteolide-5a (**2**)
C-4, C-5, C-7, C-9, C-10, and C-11 and C-15 and C-16 of **1** were independently relative. C-2 and C-3, C-10, C-12, and C-13, and C-23 and C-24 for **2** were independently relative.

Iriomoteolide-4a (**1**) showed the pseudomolecular ion peaks at *m/z* 531 $(M+Na)^+$ in the positive-mode ESIMS spectra, respectively. The molecular formula, $C_{29}H_{48}O_7$, of **1** was established by HRESIMS data [*m/z* 531.3336 $(M+Na)^+$, Δ +0.1 mmu]. ^1H and ^{13}C NMR data (Table 1) in $CDCl_3$ assigned by using HMQC spectrum disclosed the presence of a total of 29 carbon signals due to an ester carbonyl, an sp^2 quaternary carbon, seven sp^2 methines, nine sp^3 methines including six oxygenated ones, six sp^3 methylenes, and five methyls.

Table 1. ^1H and ^{13}C NMR data of Iriomoteolide-4a (**1**) in $CDCl_3$

position	**^{13}C**		**^1H** (*J* ind Hz)	
1	174.5	C		
2	30.0	CH_2	2.51[a]	m
3	31.1	CH_2	1.99	m
			1.86	m
4	71.1	CH	4.03	brs
5	36.6	CH_2	1.57	m
			1.45	m
6	73.6	CH	3.93	brd, 10.0
7	35.5	CH	1.96	m
8	35.4	CH_2	1.66	m
			1.59	m
9	68.5	CH	3.73	m
10	77.1	CH	3.57	dd, 2.0, 4.5
11	73.0	CH	4.33	dd, 2.0, 6.3
12	130.8	CH	5.69	dd, 6.3, 15.6
13	128.3	CH	5.73	m
14	33.6	CH_2	2.41	ddd, 2.0, 6.3, 14.5
			2.32	ddd, 6.3, 8.0, 14.5
15	78.0	CH	4.79	dt, 2.0, 8.0

16	40.4	CH	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

[a]2H. [b]3H.

The planar structure of **1** was elucidated on the basis of 2D NMR data measured in $CDCl_3$. Analyses of ^{1}H-^{1}H COSY and TOCSY spectra revealed a continued spin network from H_2-2 to H-23, H_3-26, H_3-27 and H_3-28 (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 (H_3-25; δ_H 1.73, H_3-29; δ_H 1.75) showed HMBC correlations for an sp^2 quaternary and an sp^2 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 H_2-2 (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.

The relative stereochemistry of nine chiral centers in the macrocyclic ring was assignable from analyses of sp^3-sp^3 bond rotations based on ^{1}H-^{1}H coupling constants and NOESY data (Figure 3). Magnitudes of ^{1}H-^{1}H coupling constants were estimated by selective population transfer (SPT) experiments and intensities of correlations observed for ^{1}H-^{1}H 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.

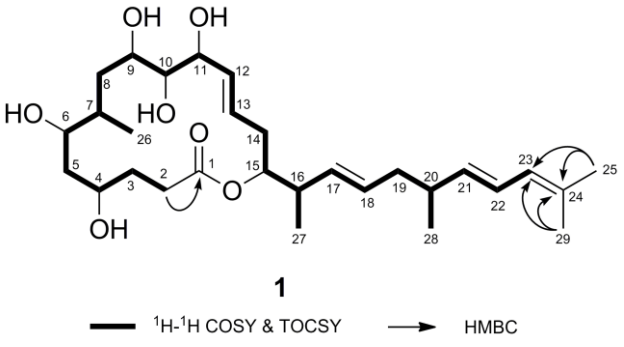

Figure 2. Selected 2D NMR correlations for iriomoteolide-4a (**1**)

The 1,3-*syn* relation for 4-OH and 6-OH was inferred by relatively large ^{1}H-^{1}H 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/H_2-8 and H_2-8/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 ^{1}H-^{1}H 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/H_3-27, H-14b/H-15, H-15/H-17, and H-15/H_3-27 as well as ^{1}H-^{1}H coupling constants for H-15/H-16 (8.0 Hz). Nevertheless,

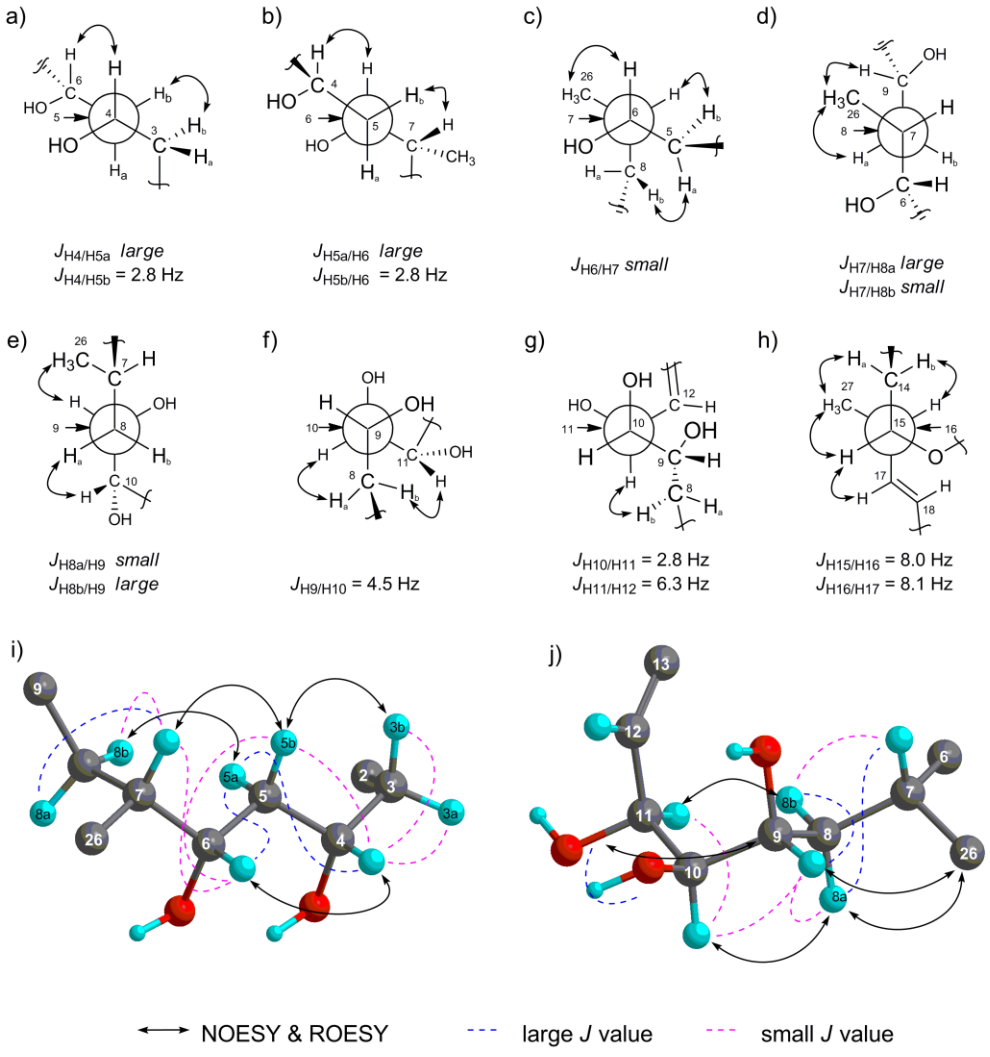

Figure 3. Bond rotations for (a) C-4–C-5, (b) C-5–C-6, (c) C-6–C-7, (d) C-7–C-8, (e) C-8–C-9, (f) C-9–C-10, (g) C-10–C-11 and (h) C-15–C-16 and perspective drawings for (i) C-2–C-8 and (j) C-6–C-13 portions in iriomoteolide-4a (**1**).

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 $C_{31}H_{52}O_7$. ^1H and ^{13}C NMR data (Table 2) in $CDCl_3$ assigned by using HMQC spectrum disclosed the presence of a total of 31 carbon signals due to an ester carbonyl, two sp^2 quaternary carbons, four sp^2 methines, two sp^2 methylenes, ten sp^3 methines including six oxygenated ones, seven sp^3 methylenes, and five methyls.

Table 2. ^1H and ^{13}C NMR Data of Iriomoteolide-5a (**2**) in $CDCl_3$

position	**^{13}C**		**^1H** (*J* 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

[a]2H. [b]3H.

Detailed analyses of ^{1}H-^{1}H COSY and TOCSY spectra revealed three spin systems from H-2 to H-13, H_3-26, H_3-27 and H_3-28, from H_2-15 to H_2-21, and from H-23 to H_3-25 and H_3-31 (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 H_3-28 (δ_H 1.15)/C-14 (δ_C 148.5), H_2-29 (δ_H 5.07 and 5.01)/C-14, and H_2-29/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 H_2-30 (δ_H 4.93, 2H)/C-21 (δ_C 29.4), H_2-30/C-23 (δ_C 48.9) and H_3-31 (δ_H 0.99)/C-22 (δ_C 151.1). The methyl proton on C-2 (H_3-26, 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/H_3-27 and H_3-26/H_3-27 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/H_3-28, 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 ^{1}H-^{1}H

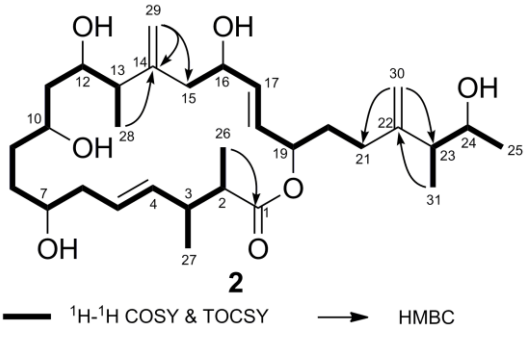

Figure 4. Selected 2D NMR correlations for iriomoteolide-5a (**2**)

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 C_1 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 C_1 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 C_1-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 (IC_{50}: 0.8 and 1.0 μg/mL, respectively).

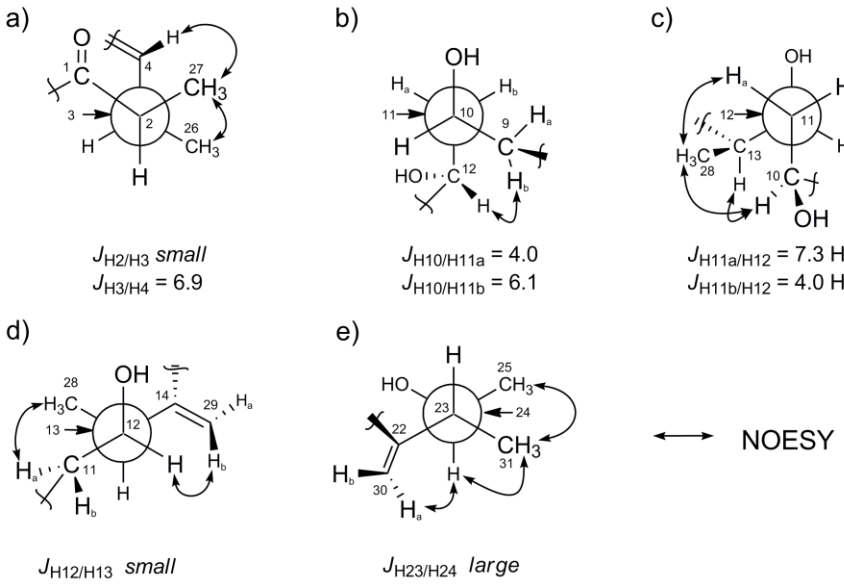

Figure 5. Bond rotations for (a) C-2–C-3, (b) C-10–C-11, (c) C-11–C-12, (d) C-12–C-13 and (e) C-23–C-24 in iriomoteolide-5a (**2**).

EXPERIMENTAL

General. Optical rotations were measured on a JASCO DIP-370 polarimeter. IR spectra were recorded on a JASCO FT/IR-5300 spectrophotometer. ^1H, ^{13}C, and 2D NMR spectra were measured on a Bruker AMX-500 spectrometer using 2.5 mm micro cells for $CDCl_3$ (Shigemi Co., Ltd.). Chemical shifts in $CDCl_3$ 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.

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 SiO_2 column chromatography (40 x 200 mm) using a stepwise elution of $CHCl_3$ (200 mL) and $CHCl_3$/MeOH (98:2, 200 mL and then 95:5, 200 mL). The fraction eluted with ($CHCl_3$/MeOH, 95:5) was chromatographed successively by using a C_{18} (MeCN/H_2O, 7:3) and then NH_2-SiO_2 columns (n-hexane/EtOAc, 2:1). A macrolide-containing fraction was separated 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 iriomoteolides-4a (**1**, 0.001%) and 5a (**2**, 0.001%).

Iriomoteolide-4a (1). Colorless amorphous; $[\alpha]_D^{20}$ -20 (*c* 0.02, $CHCl_3$); IR (neat) ν_{max} 3300 (broad) and 1721 cm^{-1}; ^1H and ^{13}C 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 $C_{29}H_{48}O_7Na$, $(M+Na)^+$: 531.3336].

Iriomoteolide-5a (2). Colorless amorphous; $[\alpha]_D^{22}$ +65 (*c* 0.02, $CHCl_3$); IR (neat) ν_{max} 3420 (broad) and 1718 cm^{-1}; ^1H and ^{13}C 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 $C_{31}H_{52}O_7Na$, $(M+Na)^+$: 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 (IC_{50}). Doxorubicin and 5-fluorouridine (IC_{50}: 0.04 and 1.2 μg/mL, respectively) were used as authentic samples, and the experiments were repeated in triplicate wells.

ACKNOWLEDGEMENTS

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