Iriomoteolides-1b and -1c, 20-Membered Macrolides from a Marine Dinoflagellate *Amphidinium* Species

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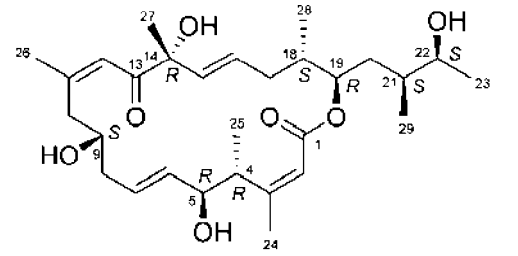
Two 20-membered macrolides, iriomoteolides-1b (**1**) and -1c (**2**), have been isolated from a marine dinoflagellate *Amphidinium* sp. (strain HYA024), and the structures were elucidated on the basis of detailed analyses of 2D NMR data and chemical correlation.

Marine dinoflagellates have been proven to be important sources of structurally unique metabolites with significant biological activities.[1] From symbiotic dinoflagellate *Amphidinium* species, a series of cytotoxic macrolides, designated amphidinolides, have been isolated.[2,3] These macrolides have a variety of backbone skeletons and different sizes of macrocyclic lactone rings (12–29-membered rings). Some of the amphidinolides contain biosynthetically unique partial structures such as vicinally located C_1 branches. Recently, we have screened numerous *Amphidinium* strains by using genetic analyses, cytotoxic screening, and metabolomics analyses and found the *Amphidinium* strain HYA024, which was monoclonally separated from sea sand collected off Iriomote Island, Japan. Iriomoteolide-1a (**3**), a new type of potent cytotoxic 20-membered macrolide, has been isolated from the strain HYA024.[4] Further investigation of the constituents of this strain led to the isolation of two new 20-membered macrolides, iriomoteolides-1b (**1**) and -1c (**2**), structurally related to iriomoteolide-1a (**3**). Herein we describe the isolation and structure elucidation of **1** and **2**.

The dinoflagellate *Amphidinium* sp. (HYA024 strain) was mass-cultured unialgally at 23 °C for 2 weeks in a 2% Provasoli's enriched seawater (PES) medium including 3 mM $NaHCO_3$. The algal cells (15.3 g, dry weight) obtained from 400 L of the medium were extracted with MeOH–toluene (3:1). The toluene-soluble materials of the extract were subjected to SiO_2 gel, C_{18}, and NH_2–SiO_2 gel columns followed by C_{18} HPLC to afford iriomoteolide-1b (**1**, 0.007% from dry weight). A less polar fraction of the first SiO_2 gel column was separated successively by C_{18} and NH_2–SiO_2 gel columns, C_{18} HPLC, and then SiO_2 gel column to yield iriomoteolide-1c (**2**, 0.002%) together with iriomoteolide-1a (**3**).

Iriomoteolide-1b {**1**, $[\alpha]_D^{20}$ −140 (*c* 0.1, $CHCl_3$)} had the same molecular formula, $C_{29}H_{46}O_7$, as that of iriomoteolide-1a (**3**), as revealed by HRESIMS data [*m/z* 529.3148 $(M + Na)^+$, Δ +0.7 mmu]. The ^{13}C NMR data (Table 1) in $CDCl_3$ disclosed a total of 29 carbon signals due to a ketone (δ_C 200.8), an ester carbonyl (δ_C 166.7), two quaternary sp^2 carbons (δ_C 160.8 and 157.5), six sp^2 methines (δ_C 133.2, 132.4, 130.4, 129.6, 121.8, and 116.2), a quaternary sp^3 carbon (δ_C 77.9), seven sp^3 methines (δ_C 74.7, 72.8, 72.2, 68.3, 48.7, 37.2, and 36.5), four sp^3 methylenes (δ_C 48.6, 40.8, 34.7, and 32.4), and seven methyls (δ_C 25.2, 20.4, 20.0, 19.7, 14.8, 14.6, and 11.2). Because six out of seven degrees of unsaturation were accounted for, iriomoteolide-1b (**1**) was inferred to have a monocyclic ring system.



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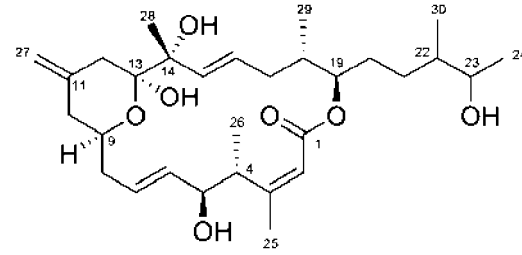

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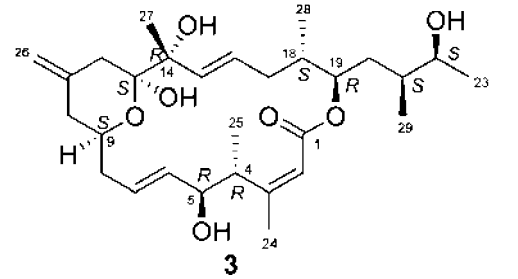

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The structure of **1** was elucidated by detailed analyses of ^1H–^1H COSY, TOCSY, HMQC, and HMBC spectra measured in $CDCl_3$ (Figure 1). ^1H–^1H COSY and TOCSY spectra revealed two proton networks from H-4 to H_2-10 and H_3-25 and from H-15 to H_3-23, H_3-28, and H_3-29. ^1H–^1H COSY cross-peaks due to allyl couplings for H-2 (δ_H 5.63)/H_3-24 (δ_H 2.20) and H-12 (δ_H 6.18)/H_3-26 (δ_H 2.17) were attributed to the presence of two trisubstituted double bonds (C-2–C-3 and C-11–C-12). HMBC correlations were observed for H-2/C-1 (δ_C 166.7), H-2/C-3 (δ_C 160.8), H_3-24/C-4 (δ_C 48.7), and H_3-25 (δ_H 1.18)/C-3, indicating that the trisubstituted double bond at C-2–C-3 was adjacent to an ester carbonyl carbon (C-1) and C-4. On the other hand, HMBC correlations for H_3-26/C-10 (δ_C 48.6), H_2-10 (δ_H 2.28 and 2.23)/C-11 (δ_C 157.5), and H-12/C-13 (δ_C 200.8) suggested that the other trisubstituted double

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Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of Iriomoteolides-1b (**1**) and -1c (**2**) (500 MHz, $CDCl_3$)

	1				**2**			
position	δ_C		δ_H		δ_C		δ_H	
1	166.7	C			167.2	C		
2	116.2	CH	5.63	br s	115.7	CH	5.71	br s
3	160.8	C			161.5	C		
4	48.7	CH	2.62	m	47.9	CH	2.43	m
5	72.8	CH	4.46	m	72.3	CH	4.23	m
6	132.4	CH	5.56	m	131.7	CH	5.58	dd, 3.8, 15.7
7	129.6	CH	5.56	m	127.7	CH	5.68	m
8	40.8	CH_2	2.30	m	39.5	CH_2	2.18[a]	m
			2.08	m				
9	68.3	CH	3.72	m	71.0	CH	3.81	tt, 3.4, 9.5
10	48.6	CH_2	2.28	m	40.7	CH_2	2.22	br d, 12.9
			2.23	m			1.91	br t, 12.9
11	157.5	C			141.7	C		
12	121.8	CH	6.18	s	36.8	CH_2	2.40	m
							2.23	m
13	200.8	C			99.6	C	3.52[b]	br s
14	77.9	C	4.33[c]	br s	77.0[d]	C		
15	133.2	CH	5.58	d, 15.6	134.9	CH	5.67	d, 15.5
16	130.4	CH	5.74	ddd, 4.0, 10.0, 15.6	128.9	CH	5.76	ddd, 3.0, 10.0, 15.5
17	34.7	CH_2	2.24	m	38.0	CH_2	2.16	m
			1.75	m			1.97	dt, 14.8, 10.4
18	37.2	CH	1.72	m	36.3	CH	1.80	m
19	74.7	CH	4.92	dt, 8.2, 3.3	70.9	CH	5.10	m
20	32.4	CH_2	1.76	m	32.4	CH_2	1.63	m
			1.13	m			1.21	m
21	36.5	CH	1.45	m	26.5	CH_2	1.50	m
							1.37	m
22	72.2	CH	3.61	m	39.8	CH	1.32	m
23	19.7	CH_3	1.13[e]	d, 6.3	70.5	CH	3.90	quint, 6.3
24	20.4	CH_3	2.20[e]	s	22.7	CH_3	1.11[e]	d, 6.6
25	11.2	CH_3	1.18[e]	d, 7.3	23.8	CH_3	2.21[e]	s
26	20.0	CH_3	2.17[e]	s	15.8	CH_3	1.23[e]	d, 7.3
27	25.2	CH_3	1.44[e]	s	110.7	CH_2	4.80[a]	s
28	14.6	CH_3	0.90[e]	d, 6.8	22.6	CH_3	1.22[e]	s
29	14.8	CH_3	0.87[e]	d, 6.7	14.1[f]	CH_3	0.95[e]	d, 6.7
30					14.0[f]	CH_3	0.91[e]	d, 6.7

[a] 2H. [b] 13-OH. [c] 14-OH. [d] This signal was overlapped with $CDCl_3$. [e] 3H. [f] These signals were interchangeable.

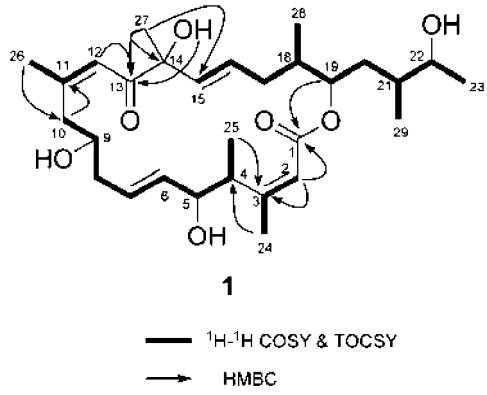

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Figure 1. Selected 2D NMR correlations for iriomoteolide-1b (**1**).

bond (C-11–C-12) was attached to C-10 and a ketone carbonyl carbon (C-13). Connection between C-13 and C-15 via an oxygenated quaternary sp^3 carbon (C-14) was deduced from HMBC correlations for H_3-27 (δ_H 1.44)/C-13, H_3-27/C-14 (δ_C 77.9), and H_3-27/C-15 (δ_C 133.2). The HMBC correlation for 14-OH (δ_H 4.33)/C-14 was suggestive of attachment of a hydroxyl group at C-14. The ester linkage between C-1 and C-19 was established by the relatively lower field chemical shift for H-19 (δ_H 4.92) and the HMBC correlation for H-19/C-1. Thus, the carbon skeleton of iriomoteolide-1b (**1**) was shown to be the same as that of iriomoteolide-1a (**3**).

The C-15–C-16 double bond was elucidated to have *E*-geometry by the *J*(H-15/H-16) value (15.6 Hz). Although the *J*(H-5/H-6) value was not determined due to overlapping of signals for H-5 and H-6, *E*-geometry of the C-5–C-6 double bond was inferred from the similarity of the chemical shifts of C-5 (δ_C 72.8), C-6 (δ_C 132.4), C-7 (δ_C 129.6), and C-8 (δ_C 40.8) for **1** to those of the corresponding carbons [C-5 (δ_C 72.3), C-6 (δ_C 132.0), C-7 (δ_C 126.8), and C-8 (δ_C 39.5)] for **3**. Both of the trisubstituted double bonds were indicated to have *Z*-geometry by the chemical shifts of C-24 (δ_C 20.4) and C-26 (δ_C 20.0). Thus, the planar structure of iriomoteolide-1b was concluded to be **1**.

Treatment of **3** with triethylamine in CH_2Cl_2 for 168 h afforded a polar product (30% yield), of which the ^{1}H NMR and $[\alpha]_D$ data were identical with those of natural **1**. Evidence of the stereochemistry of the nine chiral centers (4*R*, 5*R*, 9*S*, 13*S*, 14*R*, 18*S*, 19*R*, 21*S*, and 22*S*) in **1** was therefore provided, since the absolute configuration of **1** has been already elucidated.

The pseudomolecular ion peak of iriomoteolide-1c {**2**, $[\alpha]_D^{20}$ +24 (*c* 0.01, $CHCl_3$)} was observed at *m/z* 543 $(M + Na)^+$ by ESIMS, which was larger than that of iriomoteolide-1a (**3**) by 14 amu. HRESIMS data [*m/z* 543.3286 $(M + Na)^+$, Δ −1.2 mmu] of **2** established the molecular formula $C_{30}H_{48}O_7$. The ^{1}H and ^{13}C NMR (Table 1) spectra were similar to those of iriomoteolide-1a (**3**) except for the presence of an additional methylene signal (C-21: δ_C 26.5, δ_H 1.50 and 1.37) as well as the chemical shift differences for two methine signals between **2** (C-22: δ_C 39.8, δ_H 1.32 and C-23: δ_C 70.5, δ_H 3.90) and **3** (C-21: δ_C 36.5, δ_H 1.40 and C-22: δ_C 72.2, δ_H 3.58). Interpretation of ^{1}H and ^{13}C NMR and ^{1}H–^{1}H COSY, TOCSY, and HSQC data revealed that **2** possessed the same macrocyclic portion (C-1–C-19 and five C_1 branches) as that of **3**. The existence of a 4-hydroxy-3-methylpentyl side chain (C-20–C-24 and C-30) was revealed by analyses of ^{1}H–^{1}H COSY and

TOCSY spectra, while **3** had a 3-hydroxy-2-methylbutyl side chain (C-20–C-23 and C-29). Thus the planar structure of iriomoteolide-1c was assigned as **2**.

Comparing ROESY data and ^{1}H–^{1}H coupling constants of **2** with those of **3**, the relative stereochemistry of the macrocyclic ring portion (C-1–C-19 and five C_1 branches) for **2** was considered to be the same as that for **3**. However, relations for C-19–C-22 and C-22–C-23 were not determined, since HETLOC correlations were not obtained due to the limited amount of sample.

Iriomoteolides-1b (**1**) and -1c (**2**) are new 20-membered macrolides structurally related to iriomoteolide-1a (**3**). Iriomoteolide-1b (**1**) has a hydroxyl group at C-9 and a ketone at C-13 conjugated with an *E*-double bond at C-11–C-12, while the corresponding part for **3** is a six-membered hemiacetal ring and an exomethylene group. Although iriomoteolide-1b (**1**) might be an artifact generated from **3**, interchange between **1** and **3** in solvents such as $CHCl_3$ or MeOH was not observed. On the other hand, iriomoteolide-1c (**2**) is a homologue of **3** with a 4-hydroxy-3-methylpentyl side chain instead of a 3-hydroxy-2-methylbutyl side chain. Co-isolation of C_1-homologues such as **2** and **3** from an individual strain is rare, although amphidinolides T1[5] and its C_1-lengthened homologue, amphidinolide T2,[6] have been isolated from the *Amphidinium* Y-56 strain.

Iriomoteolide-1c (**2**) exhibited potent cytotoxicity against human B lymphocyte DG-75 cells (IC_{50} 0.002 μg/mL) and Epstein–Barr virus (EBV)-infected human B lymphocyte Raji cells (IC_{50} 0.004 μg/mL), which was almost the same as those of iriomoteolide-1a. (**3**). However, the IC_{50} value (0.9 μg/mL) of iriomoteolide-1b (**1**) against DG-75 cells is 450 times less potent than those of **2** and **3**. This result suggests that the presence of the six-membered hemiacetal ring (C-9–C-13) and/or the exomethylene unit (C-11–C-26) in iriomoteolide-1a (**3**) is important for potent cytotoxicity.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-370 polarimeter. IR and UV spectra were recorded on a JASCO FT/IR-5300 and a Shimadzu UV-2550PC spectrophotometer, respectively. ^{1}H, ^{13}C, and 2D NMR spectra were measured on a Bruker AMX-500 spectrometer using 2.5 mm microcells 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 77.0). 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.

Material. The dinoflagellate *Amphidinium* sp. (strain number HYA024) was separated monoclonally from benthic sea sands collected off Iriomote Island, Japan. The culture was maintained in sterilized seawater medium enriched with 1% Provasoli's enriched seawater supplement at 23 °C under an illumination of about 30 μmol photons m^{-2} s^{-1} with 16:8 light/dark cycle. The small subunit rRNA gene (SSU rDNA) was amplified from a single cell using the primer pairs described previously,[7] and both the coding and noncoding strands were sequenced using an Applied Biosystems thermal cycler GeneAmp PCR system 9700 DNA sequencer. The amplification of the SSU rDNA from a single cell was carried out three times. The DNA sequence was compared with those of the SSU rDNA in the databases using BLAST SEARCH, and the SSU rDNAs of *Amphidinium gibossum*[8] (as *A. belauense*, strain 324, accession no. L13719), which was originally described as a symbiont of a flatworm, *Haplodiscus* sp.,[9] and *Amphidinium* sp.[10] (strain Y-42, accession no. AB107845), separated from an acoel flatworm *Amphiscolops* sp., were found to be the closest relative (>99% identity). The voucher specimen and the SSU rDNA gene were deposited at the Center for Advanced Marine Core Research, Kochi University.

Isolation. Cultivation and extraction of the dinoflagellate were described previously.[1] The toluene-soluble fractions (2 g) of the extract were subjected to SiO_2 gel column chromatography (40 × 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 95% $CHCl_3$–MeOH was chromatographed successively by using C_{18} (CH_3CN–H_2O, 7:3) and then NH_2–SiO_2 gel 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 × 250 mm; eluent, CH_3CN–H_2O (60:40); flow rate, 2 mL/min; UV detection at 210 nm] to afford iriomoteolide-1b (**1**, 1.0 mg, 0.007% from dry weight). The fraction eluted with 98% $CHCl_3$–MeOH from the first SiO_2 gel column was separated by C_{18} (CH_3CN–H_2O, 7:3) and NH_2–SiO_2 gel columns (*n*-hexane–EtOAc, 2:1) followed by C_{18} HPLC (the same conditions described above). Finally, iriomoteolide-1c (**2**, 0.2 mg, 0.002%) was purified by a SiO_2 gel column (*n*-hexane–acetone, 4:1).

Iriomoteolide-1b (1): colorless, amorphous solid; $[\alpha]^{20}_D$ −140 (*c* 0.1, $CHCl_3$); IR (neat) ν_{max} 3439 (broad), 2921 and 1703 cm^{-1}; UV (EtOH) λ_{max} 208 nm (ε 8000); ^{1}H and ^{13}C NMR data (Table 1); ESIMS (pos.) *m/z* 529 $(M + Na)^+$; ESIMS (neg.) *m/z* 541 and 543 $(M + Cl)^-$; HRESIMS *m/z* 529.3148 [calcd for $C_{29}H_{46}O_7Na$, $(M + Na)^+$, 529.3141].

Iriomoteolide-1c (2): colorless, amorphous solid; $[\alpha]^{20}_D$ +24 (*c* 0.01, $CHCl_3$); IR (neat) ν_{max} 3420 (broad), 2918 and 1693 cm^{-1}; UV (EtOH) λ_{max} 208 nm (ε 4000); ^{1}H and ^{13}C NMR data (Table 1); ESIMS (pos.) *m/z* 543 $(M + Na)^+$; ESIMS (neg.) *m/z* 555 and 557 $(M + Cl)^-$; HRESIMS *m/z* 543.3286 [calcd for $C_{30}H_{48}O_7Na$, $(M + Na)^+$, 543.3298].

Treatment of Iriomoteolide-1a (3) with Et_3N. To a solution of iriomoteolide-1a (**3**, 0.50 mg) in CH_2Cl_2 (100 μL) was added Et_3N (10 μL), and the mixture was stirred at 4 °C for 168 h. After evaporation of the solvent *in vacuo* the residue was subjected to a SiO_2 gel column (*n*-hexane–acetone, 9:1 to 2:1) to afford iriomoteolide-1b (**1**, 0.15 mg, 30% yield). Iriomoteolide-1b (**1**) was identified by ^{1}H NMR, TLC (*n*-hexane–acetone, 2:1, R_f 0.03) and the $[\alpha]_D$ value.

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Supporting Information Available: ^{1}H and ^{13}C NMR data for compound **1**. This information is available free of charge via the Internet at http://pubs.acs.org.

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