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Symbiodinolide, a novel polyol macrolide that activates N-type Ca^{2+} channel, from the symbiotic marine dinoflagellate *Symbiodinium* sp.

Masaki Kita,[a] Nao Ohishi,[b] Kaori Konishi,[b] Mikiko Kondo,[b] Tomoyuki Koyama,[b,†] Makoto Kitamura,[b] Kaoru Yamada[b] and Daisuke Uemura[b,*]

[a]*Research Center for Materials Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa, Nagoya 464-8602, Japan*
[b]*Department of Chemistry, Graduate School of Science, Nagoya University, Furo-cho, Chikusa, Nagoya 464-8602, Japan*

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Abstract—A 62-membered novel polyol macrolide with a molecular weight of 2859 mu, symbiodinolide, was isolated from the symbiotic dinoflagellate *Symbiodinium* sp. Symbiodinolide exhibited a potent voltage-dependent N-type Ca^{2+} channel-opening activity at 7 nM and immediately ruptured the tissue surface of the acoel flatworm *Amphiscolops* sp. at 2.5 μM. The planar structure of symbiodinolide was elucidated by spectroscopic analysis and chemical degradations including hydrolysis and ethenolysis using the second-generation Grubbs' catalyst. Symbiodinolide was found to be a structural congener of zooxanthellatoxins. The relative stereochemistries of C26–C32, C44–C51, and C64–C66 parts, and the absolute stereochemistries of C69–73, C83–C103, and C3′–C18′ parts in symbiodinolide were established.

1. Introduction

Various biologically and physiologically active secondary metabolites have been isolated from marine origin.[1–3] In particular, large polyol and polyether compounds, such as palytoxin and halichondrin, are some of the most attractive and unusual molecules for these sources. These compounds are composed of a long carbon backbone that are highly functionalized by oxygen, and are referred to as 'super-carbon-chain compounds (SCC)'.[1] The true origin of secondary metabolites isolated from marine invertebrates such as sponges, ascidians, nudibranchs, has been suggested to be mostly microorganisms, i.e., microalgae, bacteria, and fungi, which accumulate in the host animals through a symbiotic relationship or a food chain. Thus, marine dinoflagellates can be considered rich resources of bioactive compounds. Indeed, several kinds of SCC have been isolated from symbiotic dinoflagellates, such as karatungiols[4] and amphidinols[5] from *Amphidinium* sp., zooxanthellatoxins (ZTs)[6,7] and zooxanthellamides[8–10] from *Symbiodinium* sp., and durinskiols[11] (Fig. 1) from *Durinskia* sp. However, the true physiological functions or roles of SCCs in the ecosystem or symbiotic relationship have rarely been clarified.[12–14] Based on their structural, biological, and conformational diversity and uniqueness, various potential abilities of SCC can be considered, i.e., chemical communication with host animals, defense materials, or nutrient sources. To establish their roles in symbiotic organisms, we have investigated polyol compounds and related unique secondary metabolites from symbiotic dinoflagellates.

The symbiotic marine dinoflagellate *Symbiodinium* sp., which is a type of zooxanthellae, is found in a wide range of marine invertebrates.[15–17] From this dinoflagellate species, we previously isolated two unique amphoteric iminium compounds, symbioimine and neosymbioimine.[18–21] In our continuing search for biologically active compounds from symbiotic dinoflagellates, we isolated symbiodinolide (**1**), a polyol macrolide that activates N-type Ca^{2+} channels (Fig. 2). We describe here the isolation, structure elucidation, and biological activities of **1**.

2. Results and discussion

2.1. Isolation of symbiodinolide

The cultured dinoflagellate *Symbiodinium* sp. (88 g wet wt), isolated from the marine acoel flatworm *Amphiscolops* sp., was extracted with 80% aqueous ethanol. The concentrated

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* Corresponding author. Tel./fax: +81 52 789 3654; e-mail: uemura@chem3.chem.nagoya-u.ac.jp
† Present address: Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan.

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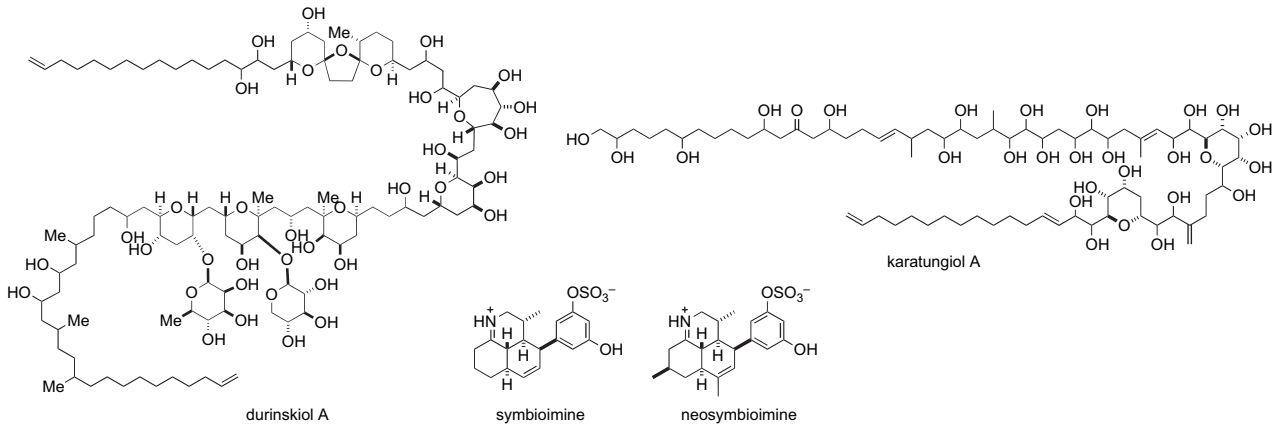


Figure 1. Bioactive secondary metabolites isolated from symbiotic marine dinoflagellates.

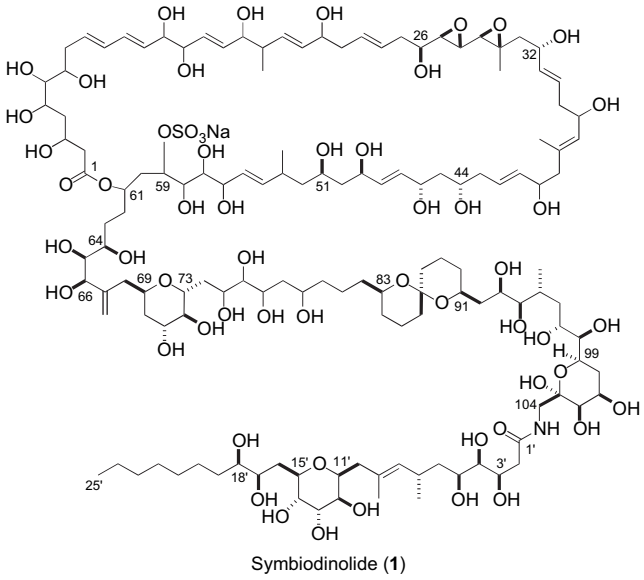


Figure 2. Structure of symbiodinolide (**1**). The absolute configurations of the C69–C73, C83–C103, and C3′–C18′ parts were established (see text for detail). Relative stereochemistries are shown for other parts.

extract was partitioned with ethyl acetate and water, and the aqueous layer was chromatographed on TSK G-3000S polystyrene gel and DEAE-Sephadex. Final purification was achieved by reversed-phase HPLC to give symbiodinolide (**1**) (9.3 mg), along with two amphoteric iminium compounds, symbioimine and neosymbioimine.[19]

2.2. Biological activity of symbiodinolide

Symbiodinolide (**1**) caused a significant increase in the intracellular free Ca^{2+} concentration at 7 nM against differentiated IMR-32 neuroblastoma cells in the presence of niphedipine (L-type Ca^{2+} channel blocker). This result revealed that symbiodinolide (**1**) possessed significant voltage-dependent N-type Ca^{2+} channel-opening activity. In contrast, **1** showed relatively weak acute toxicity against mice (LD_{99} ~5 mg/kg, ip injection). Of the various enzymatic profiling screening assays tested, symbiodinolide (**1**) showed a significant cyclooxygenase-1 (COX-1) inhibitory effect at 2 μM (65% inhibition). Meanwhile, no potent inhibitory effects were observed against other kinds of enzymes at the same concentration, such as protein serine/threonine kinase, protein tyrosine kinase, protein tyrosine phosphatase, acetylcholinesterase, α-D-glucosidase, H^+/K^+- or Na^+/K^+-ATPase, COX-2, monoamine oxidase (MAO-A and B), nitric oxide synthase (eNOS, iNOS, nNOS), peptidases (angiotensin converting enzyme, cathepsins 1, B, D, chymotrypsin, tissue plasminogen activator, trypsin, renin, thrombin, factor Xa, and factor VIIa), or phosphodiesterase (PDE 3–6). Thus, the inhibitory effect of **1** toward COX-1 was quite specific.

Furthermore, to clarify the role of SCC in the symbiotic relationships between microalga and their host animals, we examined the effects of these compounds on host animals that possess symbiotic dinoflagellates. Notably, symbiodinolide (**1**) caused immediate rupture of the tissue surface of the flatworm *Amphiscolops* sp. at 2.5 μM (Fig. 3, panels B and D). Meanwhile, the dinoflagellates released from the host animals seemed to suffer no significant damage, though they hardly moved. It is largely unknown how much SCC including symbiodinolide (**1**) is accumulated in a flatworm. Still, our preliminary results suggest that symbiodinolide (**1**) may act as a defense substance, which prevents digestion of their host animal. Further studies on its mode of action are currently underway.

2.3. Planar structure of symbiodinolide

The molecular weight of symbiodinolide (**1**) was estimated to be 2859 mu [*m*/*z* 1452.2 for $(M+2Na)^{2+}$; 975.8 for $(M+3Na)^{3+}$] by positive ESI-MS analysis. A strong ion peak that corresponded to the same molecular weight [1426.8 $(M-Na-H+H_2O)^{2-}$] was also observed in the negative ESI-MS analysis, which suggested the presence of a sulfate group. Representative signals in the ^1H NMR spectrum of **1** (Fig. 4) are summarized as follows: three doublet and a triplet methyl protons (δ_H 0.90–1.03); a singlet and two vinyl methyl protons (δ_H 1.45, 1.70, 1.71); several oxymethyne protons (δ_H 2.66–4.69); *exo*-methylene protons (δ_H 5.12 and 5.19); and two internal protons in a conjugated olefin (δ_H 6.14 and 6.25). ^{13}C and DEPT NMR spectra showed the presence of 137 carbon signals in **1**, including 8 methyls, 42 methylenes, 4 methines, 55 oxymethines, 2 acetal carbons (δ_C 97.0 and 99.4), 24 olefin carbons including an

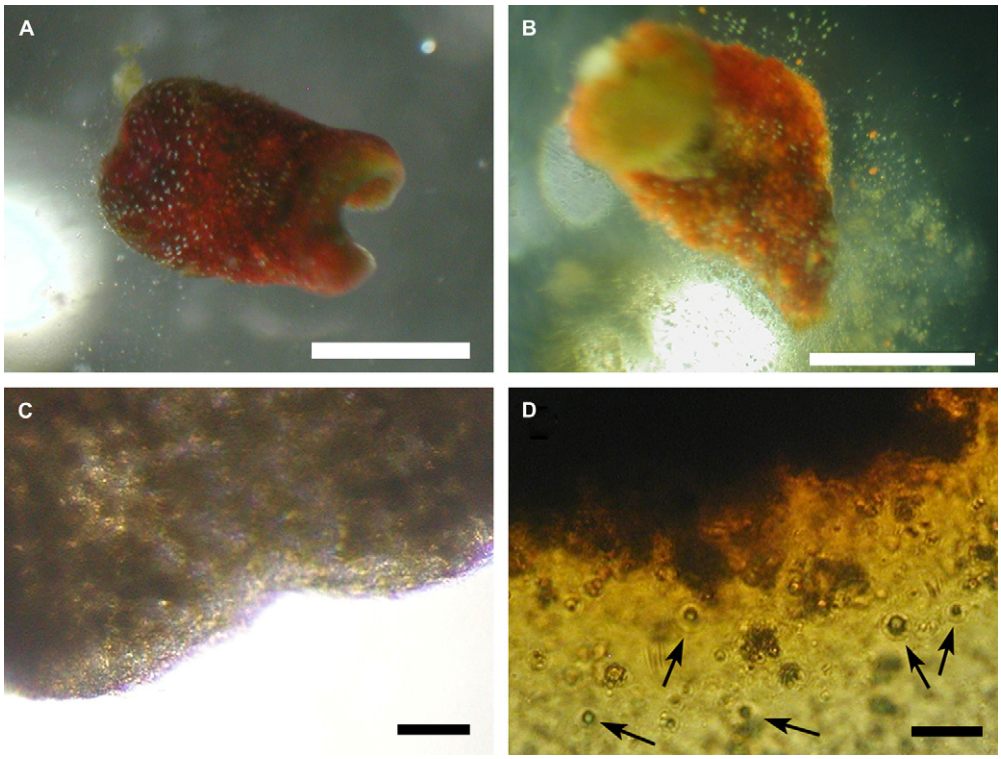


Figure 3. Bioassay using symbiodinolide (**1**) against host animals (acoel flatworm *Amphiscolops* sp.). A and C, control, B and D, treatment with **1** (2.5 μM) after 10 min. Panels A and B were taken with an optical microscope (scale bar, 1 mm). Panels C and D were taken with an inverted microscope (scale bar, 20 μm). Arrows in panel D indicate dinoflagellates liberated from host animals.

exo-olefin moiety (δ_C 115.0 and 147.6), and 2 carbonyl carbons (δ_C 173.4 and 175.3).

An extensive analysis of DQF-COSY, TOCSY, and HMQC spectra in CD_3OD allowed us to construct nine partial structures of **1**: C2 to C37 and a methyl group; C39 to C58 and a methyl group; C59 to C66; C68 to C80; C82 to C86; C88 to C102 and a methyl group; C2′ to C7′ and a methyl group; C10′ to C19′, and C23′ to C25′ (Fig. 5 and Table 1). HMBC correlations concerning the carbonyl carbons (H2/C1, H61/C1, H104/C1′, and H2′/C1′) suggested the presence of a 62-membered macrolide structure and an amide structure in **1**. Based on the ^{13}C NMR data (δ_C 46.4), the methylene carbon C104 may connect to a nitrogen atom, not an oxygen atom. Two acetal carbons were placed at C87 and C103, based on the HMBC correlations H86/C87, H88/C87, H91/C87, and H104/C103. Connectivities around the four quaternary carbons (C30, C38, C67, and C8′) and between the oxymethine carbons (C58 and C59) were also established by HMBC correlations. The presence of two tetrahydropyran moieties on C69 and C11′ was confirmed by the HMBC correlations H69/H73 and H11′/C15′. Additionally, a key NOE H102/H104b supported carbon–carbon bond connectivities between C102 and C104. Combining these data, the partial planar structure of symbiodinolide (**1**) was established except for the two carbon–carbon connectivities: C80 to C82 and C19′ to C23′.

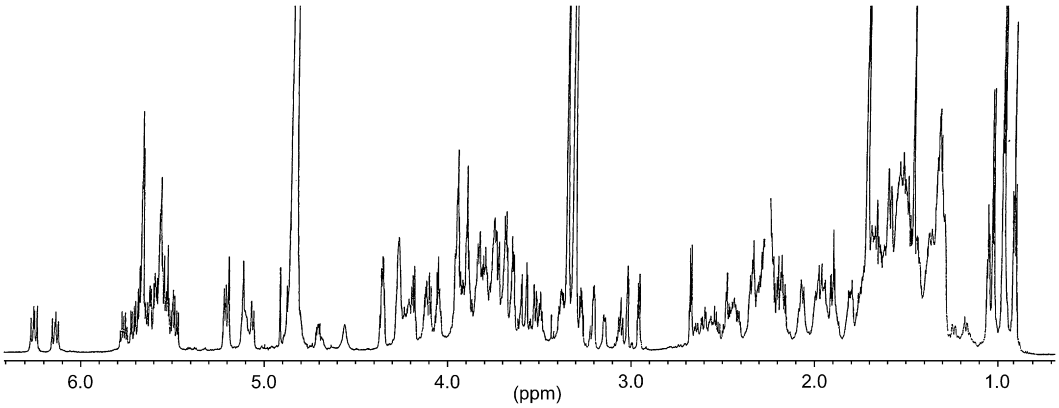


Figure 4. ^1H NMR spectrum of symbiodinolide (**1**) (800 MHz, CD_3OD).

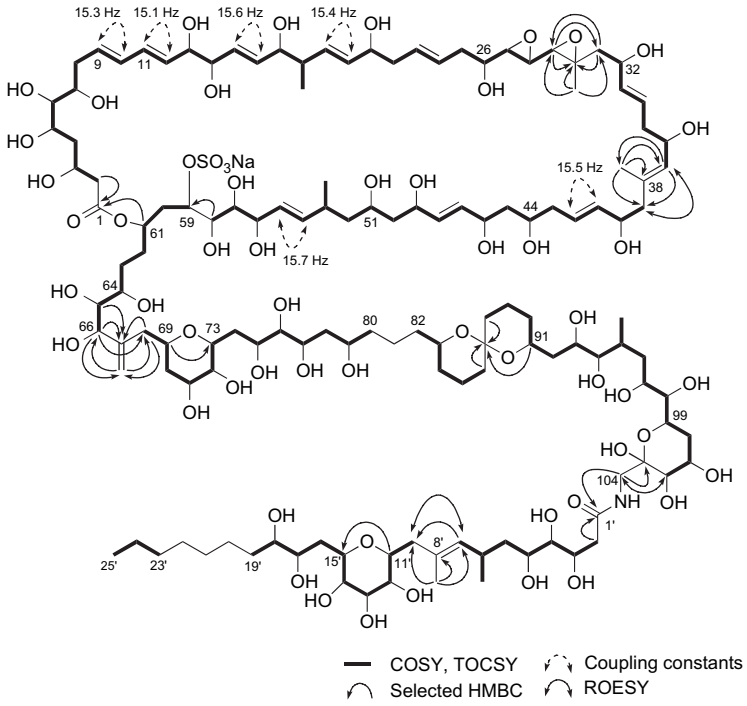


Figure 5. Planar structure of **1** determined by 2D-NMR spectroscopy.

To distinguish the ethereal or esterified carbons with hydroxyl group-connected carbons in **1**, a deuterium shift analysis was performed in CD_3OH/CD_3OD (1:1) (Fig. 6). In addition to the four carbon signals of the diepoxide moiety (C27–C30), 10 carbon signals (C59, C61, C69, C73, C83, C87, C91, C99, C11′, and C15′) were observed as single peaks. Meanwhile, a pair of split signals was observed for C103, which corresponded to a hemiacetal carbon. The chemical shifts (δ_C 97.0 and 99.4) suggested that both acetal carbons were derived not from five-membered ether rings, but from six-membered rings. Thus, a 6,6-spiroacetal and a six-membered hemiacetal structures were established. A relatively low chemical shift value (δ_H 4.69) indicated that the remaining oxymethine carbon atom (C59) was sulfated. Thus, the molecular formula of symbiodinolide (**1**) was elucidated to be $C_{137}H_{232}NNaO_{57}S$, with an overall C_{129} carbon-chain skeleton and 43 hydroxyl groups.

To confirm the entire planar structure of symbiodinolide (**1**), degradation reactions were carried out. Two of the most frequently used degradation reactions for natural products, especially SCCs, are ozonolysis and periodate oxidation. Indeed, in the case of ZTs, $NaIO_4$ degradation was examined[22,23] and the absolute stereochemistries of the six-membered ether part (C71–C75), the spiroacetal moiety (C81–C94), and the side chain part (C3′–C7′ and C11′–C18′) of ZT-A were determined by synthesis of its degraded fragments.[24–27] Meanwhile, to obtain fragments of the macrocyclic part of symbiodinolide (**1**) without redundant modification, we examined the ethenolysis of **1** using olefin metathesis catalyst (Scheme 1).[28–32] To overcome the fairly poor solubility of **1** in CH_2Cl_2 or toluene and of the second-generation Grubbs' catalyst in MeOH, a mixed solvent ($MeOH/CH_2Cl_2$=3:1) was chosen. Even though we had only a small amount (~0.2 mg), the degraded fragment **2** that corresponded to the C24–C33 part was obtained,[33] and its structure was confirmed by the analyses of 2D-NMR spectra and HR-ESIMS [*m/z* 263.1284, Δ +2.5 mmu for $(M+Na)^+$].

Furthermore, alkaline hydrolysis of symbiodinolide (**1**) gave the side chain C1′–C25′ fragment **3** and a seco acid C1–C104 fragment **4**. The molecular formula of **3** was found to be $C_{27}H_{50}O_{11}$ [*m/z* 573.3250, Δ +0.7 mmu for $(M+Na)^+$] by HR-ESIMS, which confirmed the length of the terminal alkyl chain in **3**. Based on the ESI-MS data [*m/z* 1117.1 for $(M+2Na-3H_2O)^{2+}$] and its ninhydrin-positive feature, **4** was confirmed to be a desulfated seco acid with a terminal amino group. Thus, the planar structure of symbiodinolide (**1**) was confirmed, as shown in Figure 2.

As a result, symbiodinolide (**1**) was determined to be a structural congener of ZTs, and had a similar 62-membered mono-sulfated macrolactone moiety, bis-epoxide moiety, 6,6-spiroacetal, and hemiacetal rings. The molecular weight of **1** was 36 mu smaller than that of ZT-A[6] and 6 mu larger than that of ZT-B,[7] and the lengths of the C104 and C25 linear carbon-chain in **1** were identical to those of ZT-B and two carbons smaller than those in ZT-A. Meanwhile, the terminal carbon-chain moiety (C21′–C25′) in **1** was saturated, while it is a conjugated diene moiety in ZTs. Other differences between **1** and ZTs were the presence or absence of three hydroxyl groups on C3, C76, and C100.

To date, two seco acid congeners of ZTs, zooxanthellamides (ZADs) A and B, and 63- to 66-membered macrolides, ZAD-Cs, have been isolated from the same dinoflagellate species.[8–10] Notably, vasoconstrictive activity was only seen with the macrolactone congeners ZTs and ZAD-Cs, which indicated that the huge macrolactone structures were

Table 1. NMR data for symbiodinolide (**1**) in CD_3OD

Position	δ_C (multiplicity)[a]		δ_H (multiplicity, J in Hz)[b]			Position	δ_C (multiplicity)[a]		δ_H (multiplicity, J in Hz)[b]		
1	173.4	s				65	75.7	d	3.49	dd	(5.5, 7.4)
2a	43.6	t	2.51	dd	(8.3, 15.4)	66	78.5	d	4.09	d	(7.4)
2b			2.59	dd	(5.1, 15.4)	67	147.6	s			
3	68.4	d	4.24	m		68a	34.0	t	2.33	m	
4	41.0	t	1.78	m	2H	68b			2.63	m	
5	72.4	d	3.91	m		69	71.4	d	4.27	m	
6	75.6	d	3.33	m		70a	42.0	t	1.64	m	
7	73.3	d	3.74	m		70b			1.72	m	
8a	38.0	t	2.32	m		71	70.0	d	3.80	m	
8b			2.40	m		72	77.0	d	3.04	t	(8.3)
9	132.0	d	5.76	td	(7.6, 15.2)	73	71.4	d	3.69	m	
10	133.4	d	6.14	dd	(10.4, 15.2)	74a	37.4	t	1.62	m	
11	133.7	d	6.25	dd	(10.4, 15.3)	74b			2.07	m	
12	134.0	d	5.59	ddd	(1.3, 7.8, 15.3)	75	69.6	d	3.89	m	
13	76.6	d	3.95	m		76	78.5	d	3.18	m	
14	76.6	d	3.95	m		77	70.5	d	3.95	m	
15	131.6	d	5.61	ddd	(1.1, 6.8, 15.6)	78a	42.0	t	1.53	m	
16	134.2	d	5.68	m		78b			1.67	m	
17	77.1	d	3.89	m		79	70.3	d	3.84	m	
18	43.6	d	2.24	m		80	34.0	t	1.51	m	2H
19	134.0	d	5.57	m		81	23.1	t	1.3-1.6	m	2H
20	134.2	d	5.48	ddd	(1.4, 7.0, 15.4)	82	36.5	t	1.59	m	2H
21	73.2	d	4.05	m		83	70.3	d	3.75	m	
22	41.7	t	2.24	m	2H	84a	32.4	t	1.16	m	
23	128.9	d	5.57	m		84b			1.59	m	
24	128.9	d	5.57	m		85a	20.1	t	1.54	m	
25	38.7	t	2.30	m	2H	85b			1.87	m	
26	70.0	d	3.64	m		86a	36.5	t	1.38	m	
27	58.7	d	3.01	dd	(2.0, 4.3)	86b			1.59	m	
28	54.3	d	2.94	dd	(2.0, 6.6)	87	97.0	s			
29	64.3	d	2.66	d	(6.6)	88a	36.5	t	1.38	m	
30	60.6	s				88b			1.58	m	
31a	46.9	t	1.49	m		89	19.9	t	1.94	m	2H
31b			1.89	dd	(4.1, 14.1)	90a	32.8	t	1.25	br d	(14.0)
32	70.5	d	4.20	m		90b			1.59	m	
33	136.6	d	5.55	m		91	67.1	d	3.95	m	
34	128.4	d	5.63	m		92a	46.4	t	1.53	m	
35a	41.4	t	2.20	m		92b			1.70	m	
35b			2.26	m		93	68.7	d	4.05	m	
36	68.7	d	4.36	m		94	80.9	d	3.12	dd	(1.8, 7.8)
37	131.3	d	5.21	d	(8.3)	95	33.5	d	2.06	m	
38	135.6	s				96	38.7	t	1.45	m	2H
39a	48.7	t	2.17	m		97	70.5	d	3.84	m	
39b			2.24	m		98	78.5	d	3.18	m	
40	71.4	d	4.19	m		99	68.8	d	4.19	m	
41	136.4	d	5.51	dd	(7.0, 15.5)	100a	30.7	t	1.49	m	
42	128.0	d	5.68	m		100b			1.95	q	(12.4)
43	41.5	t	2.23	m	2H	101	67.1	d	4.12	m	
44	70.8	d	3.75	m		102	70.3	d	3.59	d	(3.0)
45	42.0	t	1.66	m	2H	103	99.4	s			
46	71.4	d	4.27	m		104a	46.4	t	3.28	d	(14.0)
47	134.2	d	5.68	m		104b			3.68	m	
48	134.5	d	5.67	m		C18-Me	16.3	q	0.95	d	(6.8) 3H
49	71.4	d	4.26	m		C30-Me	17.9	q	1.45	s	3H
50a	42.0	t	1.59	m		C38-Me	17.1	q	1.70	d	(1.7) 3H
50b			1.72	m		C53-Me	21.8	q	1.01	d	(6.8) 3H
51	70.5	d	3.77	m		C67-CH_2a	115.0	t	5.12	br s	
52a	45.9	t	1.37	m		C67-CH_2b			5.19	br s	
52b			1.45	m		C95-Me	19.0	q	1.03	d	(6.8) 3H
53	34.3	d	2.43	dd	(8.5, 15.1)	1′	175.3	s			
54	137.6	d	5.55	m		2′a	41.3	t	2.45	m	
55	131.4	d	5.72	ddd	(1.2, 6.6, 15.7)	2′b			2.49	m	
56	72.0	d	4.36	m		3′	71.3	d	4.13	m	
57	74.0	d	3.63	dd	(1.6, 9.3)	4′	75.6	d	3.26	dd	(3.1, 5.1)
58	74.9	d	3.82	m		5′	70.4	d	3.67	m	
59	76.3	d	4.69	t	(7.2)	6′a	41.8	t	1.43	m	
60	36.3	t	2.23	m	2H	6′b			1.68	m	
61	73.0	d	5.10	m		7′	29.6	d	2.54	m	
62a	41.7	t	1.70	m		8′	134.0	d	5.06	d	(9.6)
62b			1.94	m		9′	132.6	s			
63	38.0	t	1.52	m	2H	10′a	41.6	t	2.18	m	
64	74.2	d	3.69	m		10′b			2.26	m	

(*continued*)

Table 1. (*continued*)

Position	δ_C (multiplicity)[a]		δ_H (multiplicity, J in Hz)[b]			Position	δ_C (multiplicity)[a]		δ_H (multiplicity, J in Hz)[b]		
11′	75.0	d	3.82	m		19′b			1.52	m	
12′	73.0	d	3.56	d	(3.0)	20′	36.9	t	2.01	m	2H
13′	72.2	d	3.90	m		21′	36.5	t	1.42	m	2H
14′	69.9	d	3.51	dd	(3.5, 9.9)	22′	32.8	t	1.62	m	2H
15′	73.7	d	3.73	m		23′	32.9	t	1.28	m	2H
16′a	36.8	t	1.49	m		24′a	23.4	t	1.31	m	
16′b			1.97	dd	(10.2, 12.3)	24′b			1.51	m	
17′	73.6	d	3.73	m		25′	14.4	q	0.90	t	(7.4) 3H
18′	75.8	d	3.37	m		C7′-Me	21.4	q	0.95	d	(6.8) 3H
19′a	34.0	t	1.44	m		C9′-Me	17.1	q	1.71	d	(1.6) 3H

[a] Recorded at 150 MHz. Multiplicity was determined based on HMQC and DEPT spectra.
[b] Recorded at 800 MHz. Higher-field methylene signals are labeled as 'a' and lower-field signals as 'b'.

important for their activity. It has also been shown that ZT-A caused aggregation in rabbit washed platelets, accompanied by an increase in the cytosolic Ca^{2+} concentration.[34] Although the vasoconstrictive activity of symbiodinolide (**1**) has not been examined, its potent voltage-dependent Ca^{2+} channel-opening activity may be a common feature of symbiodinolide and ZTs, and may be intimately involved in such constrictive activity.

2.4. Relative stereochemistry of the C26–C32 part

The partial relative stereochemistry of symbiodinolide (**1**) was elucidated using NOESY, ROESY, NOEDF, and homonuclear *J*-resolved NMR spectra (Fig. 7). Regarding the C24–C33 part, the chemical shifts and *J* values in the ^1H NMR spectrum of the degraded fragment **2** closely agreed with those of **1**. Thus, the C24–C33 part of **1** and fragment **2** may have similar conformations in which the carbon chains are lengthened in a zigzag manner. The small magnitude of $J_{27,28}$ (2.3 Hz) and the key NOE H28/C30-CH_3 indicated that stereochemistries of the both C27 and C29 epoxides were trans. The magnitudes of $J_{26,27}$ (4.4 Hz) and $J_{28,29}$ (6.3 Hz) suggested that the oxymethine protons H26 and H27 were oriented in a *gauche* arrangement, while H28 and H29 were in an *anti* arrangement. Key NOEs H26/H28 and H27/H29 supported this conformation. The magnitudes of $J_{31a,32}$ (9.9 Hz) and $J_{31b,32}$ (3.9 Hz) and the strong NOE C30-CH_3/H31b suggested that the methyl group on C30 and two protons H31b and H32 were located in the β-face on the carbon-chain. Furthermore, the intensive NOE H27/C30-CH_3 strongly supported that the C26–C31 part was slightly twisted from the linear carbon-chain and that both the bis-epoxide oxygen atoms were located in the α-face on such carbon-chain.[35] Thus, the relative stereochemistry at the bis-epoxide moiety was confirmed to be 26*S**, 27*R**, 28*S**, 29*R**, 30*R**, and 32*S**.

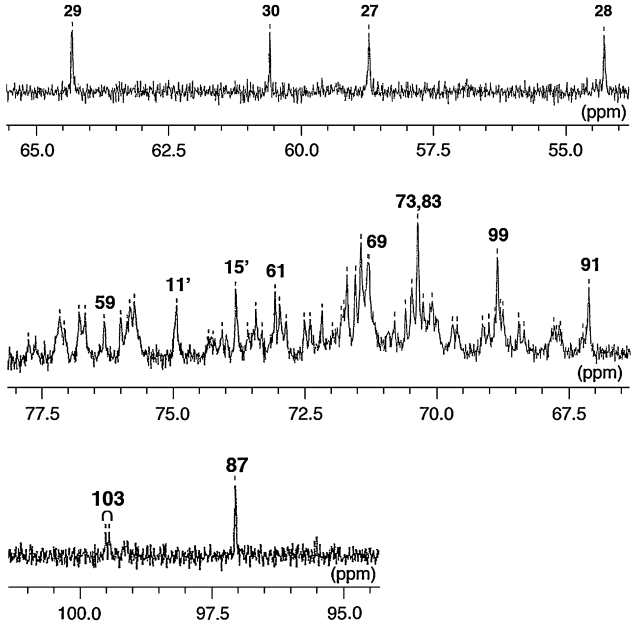


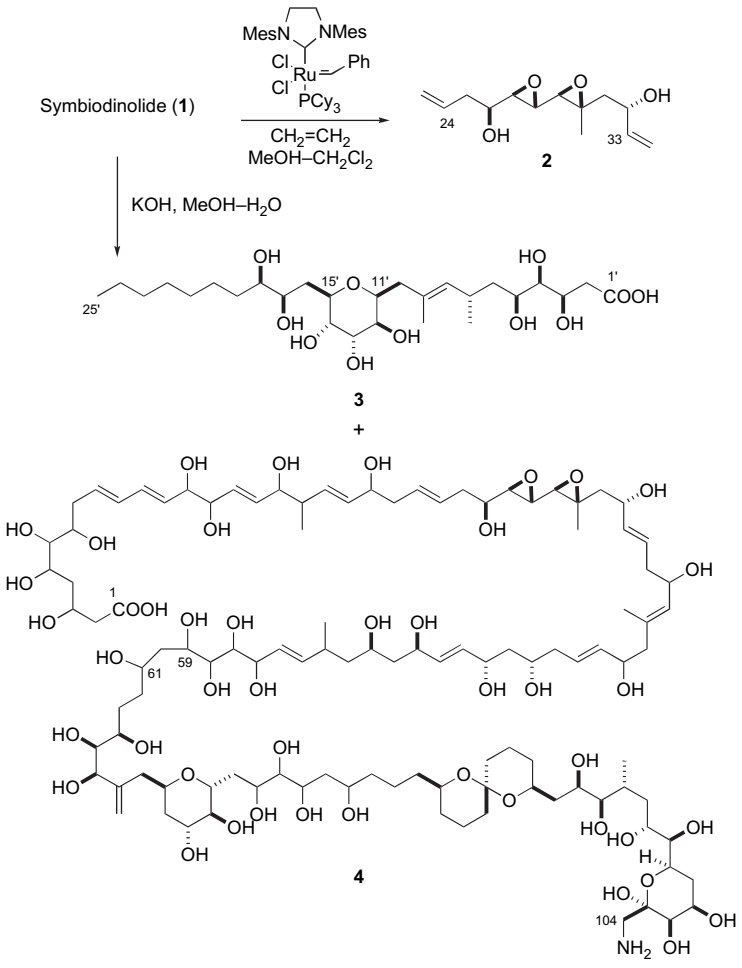
Figure 6. Deuterium exchange experiment for **1** (150 MHz, CD_3OD/CD_3OH=1:1). Bold numbers correspond to ethereal or sulfated carbons. A pair of split signals was observed for C103 (a hemiacetal carbon).

2.5. Relative and absolute stereochemistries of the C69–C73 part

Based on the NOEs observed for H68b/H71/H73 and H70a/H72, the ether ring on C69 was elucidated to be a *trans*-2,6-dialkyltetrahydropyran. The large magnitudes of $J_{71,72}$ and $J_{72,73}$ (8.3 Hz) suggested that the three protons H71, H72, and H73 were oriented in axial conformations. The *trans*-2,6-dialkyl-3,4-dihydroxytetrahydropyran moiety in **1** is also seen in ZTs. Based on a detailed spectroscopic analysis, the relative stereochemistry of the C69–C73 part of symbiodinolide (**1**) was confirmed to be identical to that of the C71–C75 part of ZT-A.[25] As mentioned previously, the biogenesis of such polyol compounds and the enzymes involved may have common features, and thus the absolute configuration of the common part in **1** might be the same as that of ZTs. Thus, the stereochemistry of the C69–C73 part of **1** was established to be 69*R*, 71*R*, 72*R*, and 73*S*.

2.6. Relative and absolute stereochemistries of the C83–C103 part

The large magnitudes of $J_{99,100a}$ and $J_{100a,101}$ (12.4 Hz) indicated that the three protons H99, H100a, and H101 may be oriented in axial conformations with respect to the six-membered hemiacetal ring with a chair conformation. Similarly, the small value of $J_{101,102}$ (3.0 Hz) and obvious NOEs H99/H101 and H102/H104b suggested that the hydroxyl group on C102 was oriented in axial conformation. Based on the stability of six-membered hemiacetal ring system, it was



Scheme 1.

suggested that the hydroxyl group on C103 was also oriented in axial conformation. The characteristic NOE H83/H91 indicated that both oxymethine protons H83 and H91 faced each other and were oriented in axial conformations with respect to the six-membered ether rings due to anomeric effects in the axial C–O conformations in a spiro[6.6] system. This conformation may also be reasonable from the viewpoint of steric repulsions between the equatorial substituents. Thus, the relative stereochemistry of the spiroacetal moiety was confirmed to be 83*R**, 87*R**, and 91*S**.

As for the carbon-chain part (C91–C99), the large magnitudes of $J_{94,95}$ and $J_{97,98}$ (7.8 Hz) and the obvious relayed NOEs H83/H93/C95-CH_3/H97, H92a/H94, and H98/H100b strongly suggested that the three hydroxyl groups on C93, C97, and C98 were oriented in a pseudoaxial conformation, whereas the hydroxyl and methyl groups on C94 and C95 were oriented in a pseudoequatorial conformation with a zigzag configuration from C90 to C101. Thus, the relative stereochemistry of the C83–C104 part of **1** has been established. In the case of ZTs, the relative stereochemistry of the carbon-chain moiety including the hemiacetal ring part, i.e., C94–C106 in ZT-A, has not been determined. However, the absolute configurations of the two oxymethine carbons of the spiroacetal moiety in ZT-A were reported as 85*R* and 93*S*.[26] Thus, absolute stereochemistry of the C83–C104 part of **1**, the common amino-terminal carbon-chain part of symbiodinolide (**1**) and ZTs, was established to be 83*R*, 87*R*, 91*S*, 93*R*, 94*R*, 95*R*, 97*R*, 98*R*, 99*R*, 101*R*, 102*R*, and 103*R*.

2.7. Relative and absolute stereochemistries of the C1′–C25′ part

As for the side chain part (C1′–C25′) of symbiodinolide (**1**), the triol and diol moieties on C3′ and C17′ and the 3,4,5-trihydroxy-2,6-dialkyltetrahydropyran moiety were similar to those in ZTs, and their absolute stereochemistries have been reported.[24,27] The relative stereochemistry of the C3′–C7′ part of ZTs was also established by using the *J*-based configuration analysis method.[36] Against this background, we reconsidered the stereochemistry of the C1′–C25′ part of symbiodinolide (**1**).

Based on the small magnitudes of $J_{3',4'}$ (4.4 Hz) and $J_{4',5'}$ (2.8 Hz), C3′–C5′ was confirmed to have a *syn*,*syn*-1,2,3-triol configuration. The moderate magnitudes of $J_{5',6'a}$, $J_{5',6'b}$, and $J_{6b',7'}$ (6.7 Hz) indicated that the sp^3–sp^3 and sp^3–sp^2 bonds in C5–C8 were twisted to some degree from the stretched zigzag carbon-chain due to steric repulsion between the two methyl groups at C7′ and C9′. The large magnitude of $J_{6'a,7'}$ (8.8 Hz) can be explained by

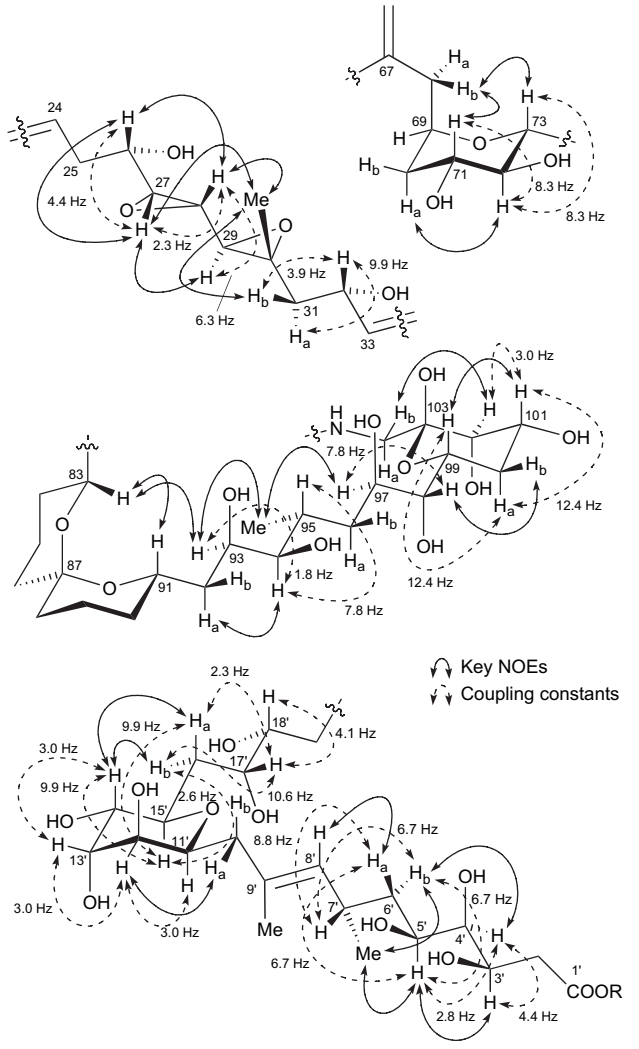


Figure 7. Relative stereochemistry of the partial structures for **1**. Coupling constants in the C24–C33, C1′–C7′, and C11′–C18′ moieties were taken from the NMR data of degraded fragments **2** or **3**. Other *J* values and NOE data were taken from those of **1**.

a synperiplanar conformation between H6′a and H7′, and the C7′ methyl group may be oriented in the α-face of the carbon chain. Key NOEs H3′/H5′, H4′/H6′b, H6′a/H8′, H5′/C7-CH_3, and H27/H29 supported this conformation.

The large magnitude of $J_{14',15'}$ and $J_{15',16'}$ (9.9 Hz) and the small magnitude of $J_{11',12'}$, $J_{12',13'}$, and $J_{13',14'}$ (2.8 Hz) suggested that three oxymethine protons H11′, H14′, and H15′, and two hydroxyl groups on C12′ and C13′ were oriented in axial conformations with respect to the tetrahydropyran ring with a chair conformation. The intensive NOE H10′a/H12′ supported the axial conformation of both H11′ and the hydroxyl group on C12′. The small magnitude of $J_{17',18'}$ (4.1 Hz) suggested that the C17′/C18′ diol moiety in **1** had a *threo* configuration. Furthermore, the magnitude of $J_{16'a,17'}$ (2.3 Hz) and $J_{16'b,17'}$ (10.6 Hz) and the key NOEs H14′/H16′a and H14′/H16′b indicated that the oxymethine proton H17′ was oriented in a pseudoequatorial conformation with a zigzag conformation from carbon atoms C13′ to C18′. Thus, the relative stereochemistries of the C1′–C7′ and C11′–C18′ parts of symbiodinolide (**1**) were confirmed, and these coincided with those of ZTs. Thus, the absolute stereochemistry of the side-chain part of **1** was established to be 3′*R*, 4′*R*, 5′*S*, 7′*S*, 11′*S*, 12′*S*, 13′*S*, 14′*S*, 15′*R*, 17′*R*, and 18′*R*.

2.8. Relative stereochemistries of the C44–C51, C64–C66, and other parts

The relative stereochemistry of the continuous olefinic 1,3-diol moieties (C44–C51) in **1** was determined by using a universal NMR database established by Kishi and his co-workers.[37–41] The chemical shifts of oxymethine carbons in acyclic 1,3-diol and 1,3,5-triol moieties mostly depended on their own relative configurations, and a ca. 2 ppm downfield-shift of the oxymethine carbons in the *anti*-isomer was observed compared with the *syn*-isomer. Based on a comparison of the carbon chemical shifts in **1** with those of the *syn*- and *anti*-**5**, [37] both the C44 and C49 diol moieties had *syn* configurations (Fig. 8). Notably, the ^{1}H and ^{13}C NMR data of C43–C47 were mostly identical to those of C52–C48 in **1**, which indicated that the 1,3-diol moieties were arrayed symmetrically on a zigzag carbon-chain. All of the four protons H46–H49 and olefinic carbons C47 and C48 may be located in the same plane due to minimization of steric repulsions. A molecular modeling study using a Merck molecular force field (MMFF) for the C43–C52 tetraol moiety **6** also supported such conformation (Fig. 9). Under the assumption that the C43–C52 part in **1** was fully stretched even in the 62-membered macrolactone ring, its relative stereochemistry was estimated to be 44*S**, 46*S**, 49*R**, and 51*R**.

As for the other parts, a *threo*/*erythro* configuration of the C64/C65/C66 triol moiety was established based on the magnitudes of $J_{64,65}$ (5.5 Hz) and $J_{65,66}$ (7.4 Hz). Stereochemistries of the di-substituted olefins on C9, C11, C15, C19, C41, and C54 and tri-substituted olefins C37 and C8′ were confirmed to be *E* based on the *J* values and ROESY spectrum data (Fig. 5). Thus, the relative stereochemistry

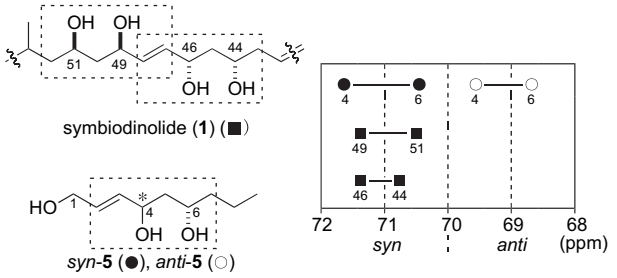


Figure 8. Chemical shift distribution of the oxymethine carbons of 1,3-diol moiety (C44–C51) in symbiodinolide (**1**) and *syn*- and *anti*-**5** in CD_3OD. Indicated numbers below the circle and square marks correspond to the C-atom numbers of each compound.

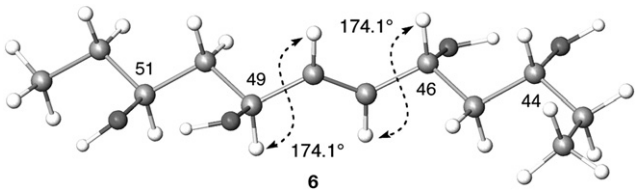


Figure 9. Three-dimensional model structure of the C43–C52 tetraol moiety **6**, which was energetically minimized via iterative application of the MMFF (Macromodel 9.0). Substituent groups on C43 and C52 were replaced with methyl groups.

of symbiodinolide (**1**), including partial absolute configurations, was elucidated, as shown in Figure 2.

3. Conclusion

In conclusion, a novel macrocyclic polyol compound with a molecular weight of 2859 mu, symbiodinolide (**1**), was isolated from the culture of the symbiotic marine dinoflagellate *Symbiodinium* sp. Its planar and partial stereochemistries were determined by 2D-NMR analysis and chemical degradations. Compound **1** showed potent Ca^{2+} channel-opening activity and immediately ruptured the tissue surface of flatworms. Further studies on the biological activities of symbiodinolide (**1**) and a complete elucidation of the stereostructure are currently underway.

4. Experimental

4.1. General

Optical rotations were measured with a JASCO DIP-1000 polarimeter. IR spectra were recorded on a JASCO FT/IR-230 spectrometer using a KBr pellet. NMR spectra were recorded on a JEOL JNM-A600 spectrometer (600 MHz for ^1H and 150 MHz for ^{13}C) or a JEOL JNM-ECA800 spectrometer (800 MHz for ^1H and 201 MHz for ^{13}C). Chemical shifts are reported in parts per million (ppm) with coupling constants (*J*) in hertz relative to the solvent peaks; δ_H 3.30 (residual C*H*D_2OD) and δ_C 49.0 for CD_3OD, respectively. The number of data points in 2D-NMR was 1024×512, and this was finally zero-filled to 2048×1024. High-resolution electrospray ionization mass spectra (HR-ESIMS) were obtained on a PE Biosystems QSTAR mass spectrometer. Fuji Silysia silica gel BW-820MH and Nacalai Tesque Cosmosil 75C_{18}-OPN were used for column chromatography. Merck precoated silica gel 60 F_{254} plates were used for thin layer chromatography (TLC).

4.2. Isolation of symbiodinolide (1)

The dinoflagellate *Symbiodinium* sp. was separated from interior cells of the marine acoel flatworm *Amphiscolops* sp. (three individuals), which was collected from the reef of Sesoko Island, Okinawa, Japan. The dinoflagellates were cultivated at 23 °C and 70% RH for 60 days in sterilized seawater medium enriched with 2% Provasoli's Ert-Schreiber (ES) supplement, under a 10 h/14 h light/dark cycle. The harvested cells (88 g wet weight from 78 L of culture) were extracted with 80% aqueous ethanol (300 mL) for three days. The extract was filtered, and the residue was boiled with 80% aqueous ethanol (300 mL) for 2 min and then extracted at room temperature for one day. The combined extracts were concentrated, and the residue (4.5 g) was partitioned with ethyl acetate (3×300 mL) and water (300 mL). The aqueous layer was loaded on a TSK G-3000S polystyrene gel column (ϕ 30×50 mm, Tosoh Co., Osaka, Japan) and eluted with 0, 25, 50, 75, and 100% aqueous ethanol (150 mL each). The concentrated 50% aqueous ethanol fraction (195 mg) was loaded on a DEAE-Sephadex column (ϕ 17×170 mm, Amersham Bioscience), and eluted with 20 mM and 200 mM phosphate (pH 6.9), and 2 M NaCl (50 mL each). The concentrated 20 mM phosphate fraction (101 mg) was applied four times to a Develosil TMS UG-5 reversed-phase HPLC column (ϕ 20×250 mm, Nomura Chemical Co., Aichi, Japan). A linear gradient of 20–40% aqueous acetonitrile was applied for 210 min at a flow rate of 5 mL/min, with monitoring at 215 nm, to give symbiodinolide (**1**) (9.3 mg, 0.011% based on wet wt, t_R=101.3 min); **1**: a colorless oil; $[\alpha]_D^{20}$ +9.6 (*c* 0.40, MeOH); IR (KBr) 3384 (br), 1725, 1661, 1442, 1073, 988 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HR-ESIMS *m/z* 1452.2350 $(M+2Na)^{2+}$ (calcd for $C_{68.5}H_{116}N_{0.5}Na_{1.5}O_{28.5}S_{0.5}$, Δ +0.1 mmu).

4.3. N-type Ca^{2+} channel-opening activity assay

Human IMR-32 neuroblastoma cells were incubated at 37 °C in a humidified atmosphere (5% CO_2/95% air) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% heat-inactivated fetal calf serum.[42] The cells were differentiated by treatment with 1 mM N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt and 2.5 μM 5-bromo-2′-deoxyuridine in DMEM with 10% NU-SERUM™V (BD Bioscience) for seven days. The cells were then incubated with DMEM with 10% NU-SERUM™V (medium A) containing 10 μM Fura-2/acetoxy methyl (AM)[43] for 30 min at 37 °C, then were incubated with medium A for 20 min at 37 °C. After being washed with Krebs-Ringer HEPES (KRH) buffer, Fura-2-loaded cells were prepared at a concentration of 1.1×10^6 cells/mL, and each 400 μL cell suspension was used for fluorometry. Fluorescence was measured with a JASCO CAF-110 spectrofluorometer that detected 500 nm fluorescence emitted upon excitation at 340 nm (F_{340}) and 380 nm (F_{380}) alternately at a frequency of 128 Hz, and the F_{340}/F_{380} ratio from successive excitation periods was calculated. After cells were treated with 0.5 μM niphedipine for 1 min, samples were added. KCl of 20 μM was used as a positive control, and a response of >50% of the maximum response was considered meaningful.

4.4. Acute toxicity against mice

Lethality was tested by intraperitoneal (ip) injection into male ddY mice weighing between 9.9 and 10.1 g (Japan SLC, Hamamatsu, Japan) as described previously.[44]

4.5. Bioassay using flatworms

Symbiodinolide (**1**) in MeOH (5 μL, 0.01–10 mM) was dropped in a 96-well plate and then dried up. Flatworms were arrayed in the plate as above, one specimen per well with 200 μL of seawater (final concentration of **1** was 0.25–250 μM), and then stored in an incubator at 25 °C under a 12 h/12 h light/dark cycle. Digital images of flatworms were acquired using an attached Olympus C-3030 digital camera under an Olympus CKX-40 inverted microscope or a Nikon SMZ optical microscope.

4.6. Ethenolysis of symbiodinolide (1)

A solution of symbiodinolide (**1**) (3.6 mg, 1.2 μmol) in MeOH (0.6 mL) was degassed three times and then replaced with an ethylene gas atmosphere. To the above stirred solution was added the second-generation Grubbs' catalyst

(5.1 mg, 6.0 μmol) in CH_2Cl_2 (0.2 mL), and the mixture was stirred for 12 h at room temperature in the dark. After dilution with ethyl vinyl ether (0.2 mL), the reaction mixture was stirred for an additional 2 h and then concentrated in vacuo. The residual oil was purified by SiO_2 gel column chromatography ($CHCl_3$/MeOH=19:1–1:1), and ODS column chromatography (aqueous 80% MeOH), and then applied four times to a Develosil HG-5 reversed-phase HPLC column (ϕ 4.6×250 mm). A linear gradient of 20–60% aqueous MeCN was applied for 60 min at a flow rate of 0.5 mL/min, with monitoring at 215 nm, to give the C24–C33 fragment **2** (0.2 mg, 69%). Compound **2**: ^{1}H NMR (800 MHz, CD_3OD) δ 5.07 (m, 1H, C24-CH_2a), 5.14 (td, J=1.5, 17.3 Hz, 1H, C24-CH_2b), 5.90 (tdd, J=6.8, 10.4, 17.2 Hz, 1H, H24), 2.28 (m, 1H, H25a), 2.37 (m, 1H, H25b), 3.64 (m, 1H, H26), 2.97 (dd, J=2.3, 4.6 Hz, 1H, H27), 2.93 (dd, J=2.3, 6.2 Hz, 1H, H28), 1.46 (s, 3H, C28-CH_3), 2.65 (d, J=6.2 Hz, 1H, H29), 1.43 (dd, J=9.8, 13.9 Hz, 1H, H31), 1.91 (dd, J=3.7, 3.9 Hz, 1H, H31b), 4.24 (m, 1H, H32), 5.87 (ddd, J=5.7, 10.5, 17.3 Hz, 1H, H33), 5.07 (m, 1H, C32-CH_2a), 5.24 (td, J=1.5, 17.3 Hz, C32-CH_2b); HRMS (ESI) m/z 263.1284 (M+Na)$^+$ (calcd for $C_{13}H_{20}NaO_4$, Δ +2.5 mmu); HPTLC, R_f 0.35 ($CHCl_3$/MeOH=19:1).

4.7. Hydrolysis of symbiodinolide (1)

To a stirred solution of symbiodinolide (**1**) (8.0 mg, 2.7 μmol) in MeOH (0.5 mL) was added aqueous 3 M KOH (0.5 mL). After being stirred for 7.5 h at room temperature, the reaction mixture was neutralized with satd aq NH_4Cl, and desalted with a TSK G-3000S polystyrene gel column (ϕ 10×25 mm). The aqueous 25% EtOH fraction was purified by an ODS column (MeOH) and reversed-phase HPLC [Develosil HG-5 column (ϕ 4.6×250 mm), 20–70% aqueous MeCN] to give the side chain C1′–C25′ fragment **3** (1.0 mg, 67%) and the seco acid C1–C104 fragment **4** (0.2 mg, ∼3%). Compound **3**: a colorless oil; $[\alpha]_D^{20}$ +10.6 (*c* 0.05, MeOH); IR (KBr) 3270 (br), 1685 cm^{-1}; ^{13}C NMR (150 MHz, CD_3OD, most signals were determined by HMQC and HMBC spectra) δ 180.2 (s, C-1′), 42.1 (t, C-2′), 71.5 (d, C-3′), 76.0 (d, C-4′), 71.4 (d, C-5′), 41.3 (t, C-6′), 29.7 (d, C-7′), 134.9 (d, C-8′), 131.7 (s, C-9′), 41.5 (t, C-10′), 73.6 (d, C-11′), 71.9 (d, C-12′), 72.0 (d, C-13′), 69.9 (d, C-14′), 74.0 (d, C-15′), 36.9 (t, C-16′), 71.2 (d, C-17′), 75.5 (d, C-18′), 33.9 (t, C-19′), 33.7 (t, C-20′), 30.5 (t, C-21′), 30.1 (t, C-22′), 33.0 (t, C-23′), 23.3 (t, C-24′), 14.2 (q, C-25′), 21.4 (q, C7′-*Me*), 16.2 (q, C9′-*Me*); ^{1}H NMR (800 MHz, CD_3OD) δ 2.40 (d, J=6.4 Hz, 2H, H-2′), 4.03 (dt, J=4.4, 6.4 Hz, 1H, H-3′), 3.25 (dd, J=2.8, 4.4 Hz, 1H, H-4′), 3.68 (dt, J=2.8, 6.7 Hz, 1H, H-5′), 1.42 (ddd, J=6.7, 8.8, 13.4 Hz, 1H, H-6′a), 1.63 (td, J=6.7, 13.4 Hz, 1H, H-6′b), 2.56 (m, 1H, H-7′), 5.07 (br d, J=9.1 Hz, 1H, H-8′), 2.21 (dd, J=7.1, 13.6 Hz, 1H, H-10′a), 2.25 (dd, J=7.1, 13.6 Hz, 1H, H-10′b), 3.88 (dt, J=3.0, 7.1 Hz, 1H, H-11′), 3.56 (t, J=3.0 Hz, 1H, H-12′), 3.89 (t, J=3.0 Hz, 1H, H-13′), 3.51 (dd, J=3.0, 9.9 Hz, 1H, H-14′), 3.73 (dt, J=2.6, 9.9 Hz, 1H, H-15′), 1.50 (ddd, J=2.3, 9.9, 14.1 Hz, 1H, H-16′a), 1.95 (ddd, J=2.6, 10.6, 14.1 Hz, 1H, H-16′b), 3.70 (ddd, J=2.3, 4.1, 10.6 Hz, 1H, H-17′), 3.37 (m, 1H, H-18′), 1.42 (m, 2H, H-19′), 1.50 (m, 2H, H-20′), 1.33 (m, 2H, H-21′), 1.31 (m, 2H, H-22′), 1.30 (m, 2H, H-23′), 1.29 (m, 2H, H-24′), 0.89 (t, J=6.9 Hz, 3H, H-25′), 0.95 (d, J=6.4 Hz, 3H, C7′-CH_3), 1.67 (s, 3H, C9′-CH_3); HRMS (ESI) m/z 573.3250 (M+Na)$^+$ (calcd for $C_{27}H_{50}NaO_{11}$, Δ +0.7 mmu), m/z 549.3274 (M−H)$^-$ (calcd for $C_{27}H_{49}O_{11}$, Δ −0.7 mmu). Compound **4**: a colorless oil; MS (ESI) m/z 1117.1 (M+2Na−3H_2O)$^{2+}$.

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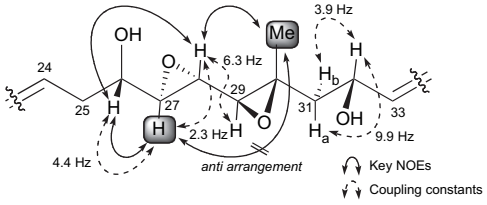

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