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Carteraol E, a potent polyhydroxyl ichthyotoxin from the dinoflagellate *Amphidinium carterae*

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ABSTRACT

Carteraol E (**1**), $C_{74}H_{126}O_{24}$, a polyhydroxyl ichthyotoxin, was isolated from the lab-cultured marine dinoflagellate *Amphidinium carterae*. Carteraol E possessed three tetrahydropyrans, and 19 hydroxyl groups on a C69-linear aliphatic chain with a ketone moiety, an *exo*-methylene, and three methyl branches. The structure was elucidated by extensive analyses of 2D NMR spectra. Cateraol E exhibited potent ichthyotoxicity with LD_{50} value of 0.28 μM, and antifungal activity against *Aspergillus niger*.

From species of the dinoflagellate *Amphidinium*, a group of polyhydroxy-polyene antifungal and hemolytic agents, amphidinol and its congeners, have been reported.[1] In addition to this polyether skeleton, bioactive polyhydroxy compounds, such as luteophanols,[2] colopsinols,[3] lingshuiols,[4] karatungiols,[5] amphezonol A,[6] and karlotoxins[7] were also isolated from various species or strains of *Amphidinium* and *Karlodinium veneficum*. Some strains of symbiotic *Amphidinium* sp. not only produced the polyhydroxy substances, but also capably generated the complex macrolides, which showed strong cytotoxic activities against tumor cell lines.[8] Thus, the genus of *Amphidinium* became a rich source for a variety of chemically interesting and biologically significant natural products. In our continuing search for bioactive substances from marine microalgae, we made a collection of the dinoflagellates off Taiwan waters and maintained as unialgal cultures in the laboratory. Bioactive species and fractions were identified by the mouse, brine shrimp, and cancer cell lines toxicity assay. On separation of the *n*-butanol soluble fraction of a methanol extract of the dinoflagellate *Amphidinium carterae*, we isolated a polyhydroxy-polyenes ichthyotoxic compound, carteraol E (**1**) (Fig. 1), consisting of C69-linear aliphatic chain possessing three tetrahydropyran rings. This Letter describes the isolation and structure elucidation of **1**.

From the wash-off epiphytes of seaweeds, collected from the southern coast of Taiwan, we isolated a strain of *A. carterae* AC021117009 in 2002. The strain was grown unialgally in sterilized seawater enriched with the K medium at 25 ± 2 °C for 4 weeks under 16 h and 8 h light/dark cycle. When the cell density reached 5–6 × 10^5 cells/mL, the algae were harvested by continuing centrifugation at 8000 rpm and extracted with methanol. The methanol-soluble extract was fractionated by solvent partitioning (CH_2Cl_2/60%MeOH(aq) followed by *n*-BuOH/H_2O), and the *n*-BuOH layer (2.7 g) was chromatographed on Sephadex LH-20 (MeOH) giving a partially purified bioactive mixture. Final purification was achieved by reversed-phase HPLC (CH_3CN/H_2O = 4:6) and pure carteraol E (**1**, 28.5 mg) was collected.

Cateraol E (**1**) was obtained as a white amorphous solid: $[\alpha]_D^{23}$ +4.9 (*c* 0.71, MeOH); IR (BaF_2) λ_{max} 3362, 2929, 1645 cm^{-1}; UV λ_{max} (MeOH) 259 (ε 35,700), 269 (ε 43,300), 280 nm (ε 34,700). Electrospray ionization mass spectroscopy (ESIMS) of **1** showed a pseudomolecular ion peak at *m*/*z* 1421 $[M+Na]^+$, 722 $[M+2Na]^{2+}$, and at *m*/*z* 1397 $[M-H]^-$. The molecular formula was inferred as $C_{74}H_{126}O_{24}$ from the HRESIMS data (*m*/*z* 722.4269 $[M+2Na]^{2+}$, Δ +5.7 mmu, calcd for 1/2 × $C_{74}H_{126}O_{24}Na_2$ 722.4212). The ^1H and ^{13}C NMR data suggested that **1** contained a ketone group (δ_C 210.6), two sp^2 quaternary carbons, twelve sp^2 methines, two sp^2 methylenes, twenty-seven sp^3 methines, of which twenty-five are oxymethines, twenty-six sp^3 methylenes including one oxymethylene, and four methyl groups. Nine of twelve elements of unsaturation implied by the molecular formula were accounted for carbon–carbon or carbon–oxygen double bonds. Therefore, **1** was inferred to possess three rings.

The extensive 2D NMR experiments, including DQF-COSY, ROESY, editing-HSQC, H2BC, and HMBC spectra were carried out in CD_3OD and/or CD_3OD–C_5D_5N (2:1) solvents for structure elucidation of **1**. Detailed analysis of DQF-COSY, editing-HSQC, and H2BC spectral data of **1** led to assignments of the following four partial structures: (a) from C-1 to C-12 (Fig. 2a), (b) from C-14 to C-33, C-70, and C-71 (Fig. 2b), (c) from C-35 to C-45 and C-72 (Fig. 2c), and (d) from C-47 to C-69 (Fig. 2d).

For substructure (a), the DQF-COSY spectrum revealed connectivities of H-1 to H-3 and H-6 to H-12. The connectivities from C-3

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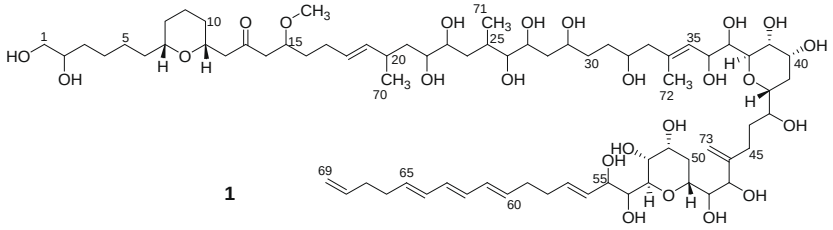

Figure 1. Structure of carteraol E (**1**). Stereochemistry of tetrahydropyran rings is relative.

to C-6 were assigned by H2BC, which showed cross-peaks due to $^{2}J_{CH}$ couplings of protonated carbons.[9] The two-bond correlations from H-2 and H-4, H-3 and H-5, H-4 and H-6, H-5 and H-7 to C-3, C-4, C-5, and C-6 were clearly observed, respectively. For the substructure (b), connectivities from H-14 to H-22, H-23 to H-30, and H-31 to H-33 were deduced from DQF-COSY cross-peaks. These fragments were connected by the H2BC spectrum, which showed cross-peaks for H-21/C-22, H-23/C-22, H-22/C-23, H-24/C-23, H-29/C-30, H-31/C-30, H-30/C-31, and H-32/C-31. The HMBC correlation between C-15 and protons of the methoxy group[10] (δ_H 3.30, δ_C 57.2) revealed adjacency to the methoxy group and C-15. The disubstituted double bond at C-18 was determined to have an E geometry based on $^{1}H–^{1}H$ coupling constant ($J_{18,19}$ = 13.5 Hz).

The DQF-COSY correlations suggested the proton connectivities from H-35 to H-45 in substructure (c). The assignments of H-38/H-39 and H-42/H-43, which were closed to the diagonal projection, were further confirmed by cross-peaks, H-39/C-38, H-38/C-39, H-43/C-42, and H-42/C-43 observed in the H2BC spectrum. The geometry of the trisubstituted double bond at C-34 was also confirmed to be E as revealed by the carbon chemical shift of the C-72 vinyl methyl group (δ_C 17.5) and the NOESY correlation between H_2-33 and H-35. The fragments from C-47 to C-51, C-52 to C-58, C-60 to C-66, and C-67 to C-69 in substructure (d) were deduced from the DQF-COSY correlations. The overlap signals of H-61, H-62, H-63, and H-64, in conjunction with its DQF-COSY correlations to H-60 and H-65, and the UV absorption at 269 nm (ε 43,300) suggested that C-60-C-65 forms a conjugated triene. The connectivities of these four fragments in substructure (d) were assigned by the H2BC correlations, H-51/C-52, H-52/C-51, H-58/C-59, H-59/C-58, H-66/C-67, and H-67/C-66. The geometry of Δ^{56} was assigned as E by the carbon chemical shift of the allylic carbon C-58 (δ_C 33.6). The ^{1}H and ^{13}C NMR data (Table 1) from C-58 to C-66 agreed quite well with those from C-44 to C-52 of amphidinol (AM-7), which indicated that the geometries of the conjugated trienes are all E-form.

Four partial structures from (a) to (d) were assembled on the basis of HMBC data. HMBC correlations H-12/C-13 and H-14/C-13 suggested that substructures (a) and (b) were connected through a C-13 ketone group. Similarly, HMBC correlations, H-72/C-33, H-72/C-34 and H-72/C-35 indicated that C-33 and C-35 were connected to a quaternary carbon C-34. HMBC correlations, H-73/C-46, H-45/C-46, and H-47/C-46 indicated that C-45 and C-47 were connected to *exo*-olefin carbon C-46. According to the data-mentioned above, the whole carbon backbone was able to be assembled, leaving the position of hydroxyl groups and ether linkages to be determined. The positions of hydroxyl groups and three tetrahydropyran rings were deduced from deuterium-induced shift analysis[11] of the oxymethine carbon signals in the ^{13}C NMR spectra of **1** in CD_3OD and CD_3OH, respectively. It turned out that seven oxymethine signals for C-7 (δ_C 79.2), C-11 (δ_C 75.5), C-15 (δ_C 77.6), C-38 (δ_C 79.0), C-42 (δ_C 75.6), C-49 (δ_C 70.3), and C-53 (δ_C 80.4) did not exhibit deuterium-induced shift. C-15 was connected to the methoxyl carbon through an ether linkage based on aforementioned HMBC correlation. Thus, it was suggested that C-7 and C-11, C-38 and C-42, as well as C-49 and C-53 have connectivities with each other through an ether linkage, respectively. Therefore, the gross structure of carteraol E was determined to be as shown in **1**. A summary of the assignments of all the protons and carbons are shown in Table 1.

The relative stereochemistries of the three tetrahydropyran rings (C-7–C-11, C-38–C-42, and C-49–C-53) in **1** were determined by $^{1}H–^{1}H$ coupling constants[12] and ROESY correlations (Fig. 3). The ROE correlations H-7/H-9, H-9/H-11 and H-7/H-11, and $^{3}J_{7,8a}$ = 10.0 Hz, $^{3}J_{10a,11}$ = 10.9 Hz suggested that the tetrahydropyran ring of C-7–C-11 had a chair conformation, and H7 and H11 are syn configuration. The ROE correlations H-37/H-40, H-37/H-42, H-36/H-42, and H-40/H-42 suggested that the tetrahydropyran ring of C-38–C-42 had a chair conformation and H-40 and H-42 were in a 1,3-diaxial orientation with each other. Meanwhile, the $^{1}H–^{1}H$ coupling constants (J_{H38H39} = 2.2 Hz, J_{H39H40} = 3.2 Hz, $J_{H40H41b}$ = 4.7 Hz, $J_{H40H41a}$ = 11.1 Hz, $J_{H41bH42}$ < 3 Hz, and $J_{H41aH42}$ = 9.7 Hz) suggested that H-38 and H-39 were oriented in an equatorial conformation. The tetrahydropyran ring of C-49–C-53 was also suggested to exhibit a chair conformation based on

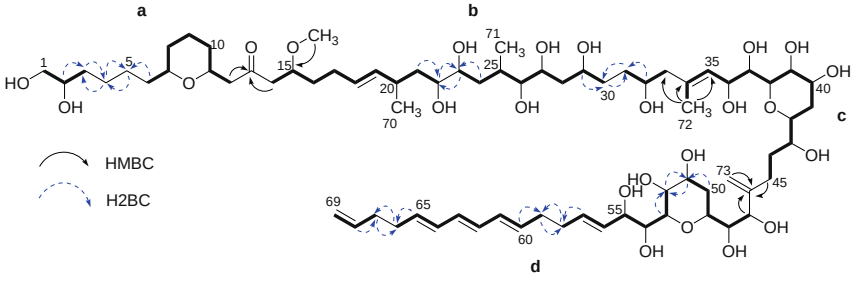

Figure 2. Gross structure of carteraol E (**1**). Heavy lines indicate the connectivities assigned on the basis of DQF-COSY and HSQC. Arrows/dashed arrows denote the correlation protons (tail) and carbon (head) observed in the HMBC/H2BC.

the large coupling constants ($J_{H49-H50a}$ = 12.0 Hz, $J_{H50a-H51}$ = 9.7 Hz), small coupling constants ($J_{H49-H50b}$ = 1.9 Hz, $J_{H50b-H51}$ = 1.0 Hz, $J_{H51-H52}$ = 3.0 Hz, and $J_{H52-H53}$ = 2.1 Hz) and the ROE correlations of H-49/ H-55 and H-50b/H-51.

Carteraol E (**1**) was isolated from a free-living marine dinoflagellate *A. carterae*. Using spectroscopic analysis, the structure of **1** was determined to be a novel polyhydroxy-polyene compound. Carteraol E was a amphidinol analog and structurally similar to karatungiol A (**2**),[5] which was isolated from a symbiotic *Amphidinium* species and lingshuiol (**3**),[4] (Fig. 4). Particularly, **1** has a conjugated triene and a terminal carbon–carbon double bond in the polyene C14-chain portion in one end of the molecule, as in amphidinol 2 (**4**), 4, 6, 7, 11, 12,[1] whereas karatungiols and lingshuiol possess a saturated chain without conjugated trienes. The central region (C-34–C-57 moiety) of **1** was structurally common to those of amphidinols,[1] karatungiols[5], and lingshuiol.[4] The polyhydroxy end of **1** (C-1–C-33) is quite different from those of amphidinols[1] and luteophanols.[2] Based on the results of biosynthetic studies of AM-2 and AM-4[13], the polyhydroxy end of **1** was divided into three regions (Fig. 4). The C10 region is quite conserved among lingshuiol type of amphidinol analogues, although **1**, **3**, and **4** were isolated from Taiwaese, Indonesian, and southern Chinese *Amphidinium*, respectively. The oxidative positions were quite diverse in C8 portion of the C8 + C2 region among **1**, **3**, and **4**. Interestingly, The C8 portion of the C8 + C2 region of **1** was exactly the same with the C8 region of AM-2 (**4**). Further labeling experiments of **1** may provide new insights into the polyketide biosynthesis of *Amphidinium*. Carteraol E (**1**) exhibited potent ichthyotoxicity with LD_{50} value of 0.28 μM, and antifungal activity against *Aspergillus niger* at 15 μg/disk. This is the first report that carteraol E (**1**) showed ichthyotoxic potency as an amphidinol derivative.[14] Carteraol E showed neither cytotoxicity against CCRF-CEM and DLD-1 cancer cell lines in vitro (LD_{50} >40 μg/mL), nor antimicrobial activity against Gram-positive bacterium *Staphylococcus aureus* (>50 μg/disk), although lingshuiol (**3**) possessed powerful cytotoxic activity, and luteophanol A exhibited weak antimicrobial activity.[15] Further studies on the structures of carteraols[16] as well as the labeling experiments are in progress.

Table 1
^{1}H and ^{13}C NMR spectral data of carteraol E (**1**)[a]

No.	In CD_3OD		In CD_3OD/C_5D_5N (2:1)	
	δ_C (mult.)	δ_h	δ_c (mult.)	δ_h
1	67.4 (t)	3.41, 3.45	67.5 (t)	3.52
2	73.2 (d)	3.56	73.1 (d)	3.64
3	34.4 (t)	1.35, 1.46	34.5 (t)	1.32, 1.39
4	26.7 (t)	1.36	26.7 (t)	1.33
5	26.8 (t)	1.35, 1.42	26.7 (t)	1.42
6	37.4 (t)	1.38, 1.44	37.4 (t)	1.32, 1.39
7	79.2 (d)	3.30	78.9 (d)	3.20
8	32.5 (t)	1.14, 1.57	32.3 (t)	1.07, 1.45
9	24.5 (t)	1.56, 1.82	24.5 (t)	1.45, 1.72
10	32.6 (t)	1.18, 1.59	32.5 (t)	1.10, 1.51
11	75.5 (d)	3.77	75.3 (d)	3.73
12	51.2 (t)	2.47, 2.61	51.2 (t)	2.44, 2.62
13	210.6 (s)		209.9 (s)	
14	48.8 (t)	2.57, 2.74	48.7 (t)	2.55, 2.74
15	77.6 (d)	3.70	77.5 (d)	3.69
16	35.0 (t)	1.54	35.0 (t)	1.52
17	29.3 (t)	2.05	29.2 (t)	2.02
18	130.1 (d)	5.45	129.9 (d)	5.44
19	137.4 (d)	5.26	136.4 (d)	5.26
20	34.7 (d)	2.37	34.7 (d)	2.44
21	41.5 (t)	1.36, 1.46	41.6 (t)	1.42, 1.51
22	73.4 (d)	3.43	73.4 (d)	3.54
23	73.0 (d)	3.50	73.0 (d)	3.62
24	38.2 (t)	1.35, 1.66	38.5 (t)	1.48, 1.81
25	31.1 (d)	2.14	31.2 (d)	2.32
26	77.0 (d)	3.35	77.0 (d)	3.51
27	72.4 (d)	3.67	72.7 (d)	3.80
28	41.8 (t)	1.36, 1.46	42.0 (t)	1.62, 2.10
29	72.0 (d)	3.88	72.1 (d)	3.98
30	34.3 (t)	1.61	34.7 (t)	1.67
31	33.8 (t)	1.53, 1.59	34.0 (t)	1.62
32	70.4 (d)	3.78	70.3 (d)	3.81
33	49.0 (t)	2.18	49.0 (t)	2.18
34	136.8 (s)		136.5 (s)	
35	128.6 (d)	5.50	129.0 (d)	5.63
36	67.7 (d)	4.56	67.8 (d)	4.70
37	72.2 (d)	3.69	72.4 (d)	3.82
38	79.0 (d)	3.95	79.1 (d)	4.17
39	68.7 (d)	4.04	68.9 (d)	4.24
40	67.3 (d)	3.97	67.4 (d)	4.10
41	30.3 (t)	1.78	30.6 (t)	1.90, 1.95
42	75.6 (d)	3.48	75.7 (d)	3.58
43	74.4 (d)	3.60	74.5 (d)	3.69
44	32.4 (t)	1.56, 1.95	32.5 (t)	1.65, 2.04
45	27.8 (t)	2.09, 2.40	27.9 (t)	2.20, 2.56
46	151.2 (s)		151.7 (s)	
47	76.6 (d)	4.18	76.6 (d)	4.34
48	75.0 (d)	3.34	75.2 (d)	3.47
49	70.3 (d)	4.03	70.5 (d)	4.17
50	31.5 (t)	1.55, 2.08	31.7 (t)	1.62, 2.26
51	67.2 (d)	4.05	67.3 (d)	4.15
52	68.5 (d)	4.03	68.7 (d)	4.25
53	80.4 (d)	3.74	80.6 (d)	3.94
54	71.8 (d)	3.97	72.1 (d)	4.14
55	74.0 (d)	4.36	74.1 (d)	4.55
56	129.0 (d)	5.62	129.5 (d)	5.73
57	135.0 (d)	5.80	134.4 (d)	5.82
58	33.6 (t)	2.19	33.5 (t)	2.08
59	33.6 (t)	2.19	33.5 (t)	2.08
60	134.3 (d)	5.70	134.4 (d)	5.65
61	132.3 (d)	6.07	132.2 (d)	6.04
62	132.3 (d)	6.09	132.2 (d)	6.06
63	132.3 (d)	6.09	132.3 (d)	6.06
64	132.2 (d)	6.05	132.1 (d)	6.00
65	134.2 (d)	5.66	134.2 (d)	5.61
66	33.3 (t)	2.15	33.2 (t)	2.10
67	34.7 (t)	2.13	34.6 (t)	2.07
68	139.3 (d)	5.81	139.2 (d)	5.75
69	115.3 (t)	4.49, 5.01	115.3 (t)	4.91, 4.97
70	22.6 (q)	1.01	22.7 (q)	1.00
71	13.9 (q)	0.92	14.1 (q)	0.97
72	17.5 (q)	1.76	17.6 (q)	1.76
73	113.1 (t)	4.98, 5.07	112.9 (t)	4.99, 5.12
–OMe	57.2 (q)	3.30	57.2 (q)	3.26

[a] Reference to residual solvent CD_3OD signals at δ_h 3.3 and δ_c 49.0 and measured at 25C, 400 MHz for ^{1}H and 100 MHz for ^{13}C. ^{13}C multiplicities were assigned from DEPT experiments.

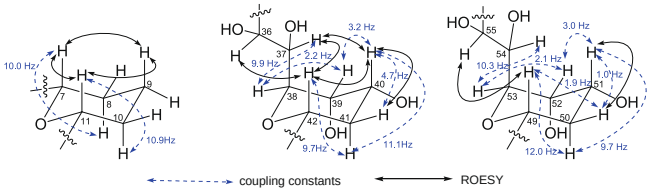

Figure 3. Relative stereochemistries of three tetrahydropyran rings of carteraol E (**1**). Dashed arrows denoted coupling constants and plain arrows denoted ROESY.

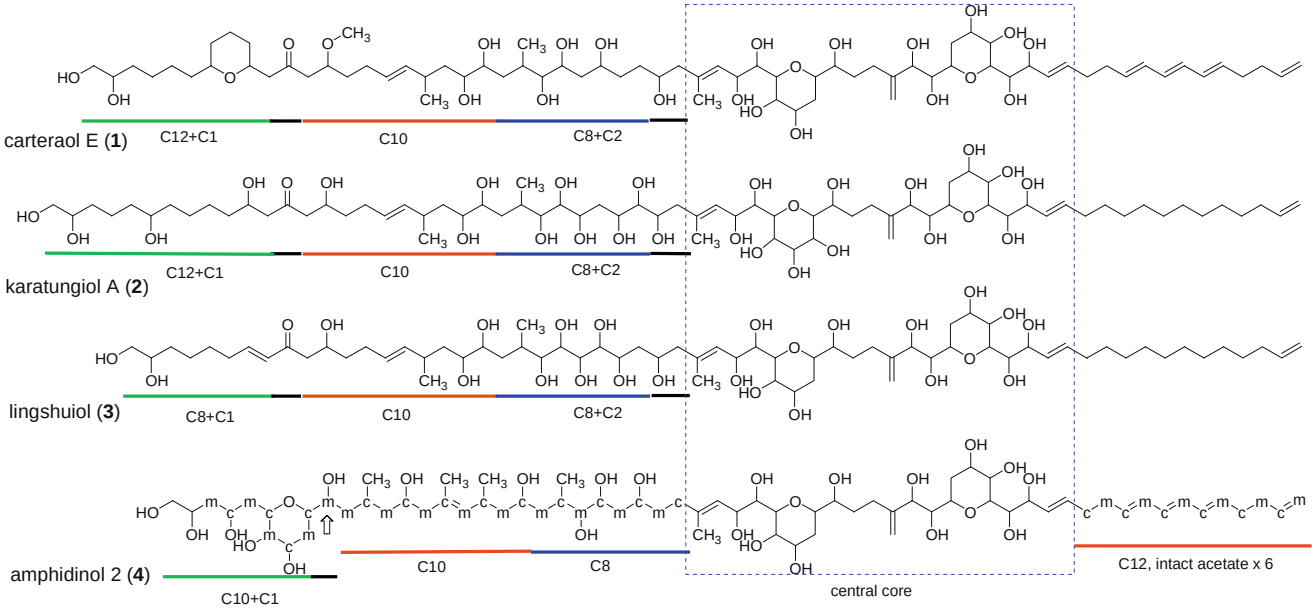

Figure 4. Structures of carteraol E (**1**), karatungiol A (**2**), lingshuiol (**3**), and amphidinol 2 (**4**).

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10. A methoxy group has not so far occurred in amphidinol family. The two double doublets of 14b signal in ^{1}H NMR (CD_3OD) may come from 2 diastereomers with respect to C-15. This suggested that the methoxy group may come from Michael addition of MeOH to α,β-unsaturated ketone.
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12. The $^{3}J_{H,H}$ coupling constants were either directly measured from ^{1}H NMR spectra in CD_3OD and CD_3OD–C_5D_5N (2:1) or extracted from the E. COSY and DQF-COSY experiments.
13. Houdai, T.; Matsuoka, S.; Murata, M.; Satake, M.; Ota, S.; Oshima, Y.; Rhodes, L. L. *Tetrahedron* **2001**, *57*, 5551–5555.
14. Although karlotoxins (KmTxs), produced by *Karlodinium veneficum*, were reported to kill fish causing damage to sensitive gill epithelial tissues and the structures of KmTx-1 and -2 were published, the ichthyotoxic potencies were not reported: Washida, K.; Koyama, T.; Yamada, K.; Kita, M.; Uemura, D. *Tetrahedraon Lett.* **2006**, *47*, 2521–2525. and references cited therein..
15. Lingshuiol showed the IC_{50} of 0.21 and 0.23 μM against A-549 and HL-60 cell lines in vitro, respectively. Luteophanol A exhibited weak antimicrobial activity against *Staphlococcus aureus* with MIC values of 33 μg/mL.
16. Carteraols A-D, C60 to C72 polyhydroxy-polyene compounds, possessed a penta-ene side chain and a rather phydrophilic polyol terminus with respect to carteraol E. Carteraols A (C66) and B (C66) are geometrical isomers. Carteraol C (C72) is a glycoside of carteraol A, and carteraol D possessed a C57 linear aliphatic chain and a sulfate group. The ichthyotoxicities of carteraols A-D, which we isolated from of *Amphidinium carterae* strain AC021117006 were arranged from 0.54 to 6.0 μM (LD_{50}).