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Formosalides A and B, cytotoxic 17-membered ring macrolides from a marine dinoflagellate *Prorocentrum* sp.

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ABSTRACT

Formosalides A (**1**) and B (**2**), two novel cytotoxic 17-membered ring macrolides with all-*cis* tetraenes, a tetrahydropyran ring, and a tetrahydrofuran ring, were isolated from the cultured marine dinoflagellate *Prorocentrum* sp. Their gross structures, including local relative stereostructures, were elucidated by extensive spectroscopic studies.

Marine dinoflagellates have been proven to be one of the rich resources for a variety of chemically interesting and biologically significant natural products, such as okadaic acid, brevetoxins, and amphidinolides. Okadaic acid, a linear polyether, is potent phosphatase PP1 and PP2A inhibitors produced by species of the genus *Prorocentrum*.[1] Brevetoxins, the ladder-frame polyethers, are sodium channel activators which were produced by *Karenia brevis* (*Gymnodinium brevis*).[2] Amphidinolides, complex macrolides, have been isolated from *Amphidinium* species, and many have shown strong cytotoxic activities against tumor cell lines.[3] During our search for bioactive substances produced by marine dinoflagellates of the genus *Prorocentrum*, we have examined extracts of the laboratory-cultured marine dinoflagellate *Prorocentrum lima* (strain PL021117001) and isolated a new type of linear polyether.[4] Here we describe the isolation and structure elucidation of the novel 17-membered ring macrolides, formosalides A (**1**) and B (**2**), possessing all-*cis* tetraenes, a tetrahydropyran ring, and a tetrahydrofuran ring moieties from another okadic acid-producing dinoflagellate, *Prorocentrum* sp., strain PL040104002.

The dinoflagellate, isolated from the wash-off epiphytes of seaweeds at South Bay, southern Taiwan, was mass cultured in a seawater medium enriched with K nutrients[5] at 25 ± 2 °C for 4 weeks. The harvested cells (1.24 kg wet weight, from 600L culture) were extracted with methanol. After partial solvent evaporation, the aqueous methanol was fractionated into *n*-hexane, dichloromethane and *n*-butanol-soluble fractions. The dichloromethane-soluble material was subjected to silica gel flash column chromatography (CH_2C1_2/MeOH, stepwise), followed by LH-20 (MeOH) gel filtration chromatography. Final purification was achieved by reversed-phase HPLC (CH_3CN/H_2O = 7:3) to afford formosalide A (**1**, 35.9 mg) and B (**2**, 7.7 mg).

Formosalide A, $[\alpha]_D^{25}$ +17.3 (c 0.01, MeOH), was obtained as a colorless amorphous solid. High resolution mass spectroscopy (HRESIMS) suggested a molecular formula of $C_{32}H_{50}O_9$ (observed $[M+Na]^+$, *m/z* 601.3381; calcd for $C_{32}H_{50}O_9Na$, *m/z* 601.3353, Δ +2.8 mmu), which was consistent with the carbon and hydrogen numbers observed in the NMR spectral data. UV absorption at 246 nm (log ε 4.13) was indicative of conjugated diene(s), and IR absorptions at 3362 and 1708 cm^{-1} were attributed to hydroxy and ester carbonyl (or lactone) groups, respectively. The ^1H and ^{13}C NMR data (Table 1) for **1** revealed the presence of an ester or lactone carbonyl, four olefinic bonds, a hemiketal, eight methine carbons, twelve methylene, and two methyl groups. The molecular formula suggested the presence of a total of eight unsaturation equivalents in the molecule. Thus, the remaining three unsaturation equivalents were from rings. Detailed analyses of NMR spectra, including ^1H–^1H COSY, selective TOCSY and HSQC spectra, of **1** led to assignments of proton connectivities for three partial structures of (a) C2–C7, (b) C9–C22, and C31, (c) C24–C30, which are designated by bold lines in Figure. 1. The connectivities of three partial structures (a–c) were assigned on the basis of HMBC and NOESY NMR data. HMBC cross-peaks for H21/C23, H24/C23, H32/C23, H32/C22, and H32/C24 revealed connectivities of C22 to C24. HMBC NMR cross-peaks were observed for H9/C8 and H7b/C8, thereby revealing that the hemiketal carbon was adjacent to C7 and C9. This assignment was further supported by a NOESY NMR cross-peak for H7a/H9. The carbon chemical shift of C8 (δ_C 98.1) was in agreement with the values reported for those (δ_C 96.3–99.7) of the six-membered hemiketal ring.[6–9] An ester carbonyl carbon (δ 173.9, C1) showed a HMBC correlation for H2 and H16 as well as the low-field resonance of H16 (δ_H 5.02) indicating that the ester linkage was located between C1 and C16 to

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Table 1
^{1}H NMR and ^{13}C NMR data of formosalides A (1) and B (2) ($CDCl_3$)[a]

Atom number	1		2	
	δ_C (mult.)	δ_H (mult. *J* in Hz)	Sc (mult.)	δ_H (mult. *J* in Hz)
1	173.9 (s)		173.4 (s)	
2	77.4 (d)	4.58 (dd, 8.1,3.5)	77.6 (d)	4.55 (dd, 8.1,2.1)
3	27.8 (t)	2.16; 2.26 (m)	26.3 (t)	1.95; 2.33 (m)
4	32.2 (t)	1.43; 2.10 (m)	31.7 (t)	1.32; 2.08 (m)
5	79.7 (d)	4.15 (m)	76.6 (d)	4.02 (m)
6	29.5 (t)	1.70; 1.84 (m)	27.9 (t)	1.70; 2.19 (m)
7	34.8 (t)	1.61; 2.24 (m)	33.9 (t)	1.55; 1.80(m)
8	98.1 (s)		100.7 (s)	
9	77.3 (d)	3.06 (d, 9.0)	74.2 (d)	3.28 (d, 9.0)
10	75.2 (d)	3.40 (dd, 9.0, 9.5)	74.9 (d)	3.42 (dd, 9.0, 9.7)
11	42.3 (d)	1.35 (m)	42.2 (d)	1.34 (m)
12	70.3 (d)	3.51 (t, 10.6)	72.8 (d)	3.13 (t, 9.1)
13	30.9 (t)	1.33; 1.45 (m)	30.4 (t)	1.55; 1.83 (m)
14	20.0 (t)	1.43 (m)	19.6 (t)	1.28; 2.01 (m)
15	33.9 (t)	1.46, 1.60 (m)	31.1 (t)	1.27; 1.50 (m)
16	73.1(d)	5.02 (br t, 10.3)	70.1 (d)	5.20 (br t, 10.1)
17	42.5 (t)	1.56; 1.74 (m)	43.0 (t)	1.57; 1.67 (m)
18	67.3 (d)	3.54 (m)	67.2 (d)	3.50 (m)
19	35.1 (t)	2.33 (m)	35.0 (t)	2.32 (m)
20	126.0 (d)	5.36 (m)	125.6 (d)	5.40 (m)
21	126.1 (d)	6.29 (t, 11.0)	126.3 (d)	6.30 (t, 11.0)
22	120.7 (d)	6.05 (d, 11.0)	120.8 (d)	6.06 (d, 11.0)
23	138.1 (s)		137.9 (s)	
24	30.6 (t)	3.0 (d, 7.7)	30.6 (t)	3.01 (d, 7.5)
25	129.6 (d)	5.40 (m)	129.7 (d)	5.39 (m)
26	124.2 (d)	6.29 (t, 11.0)	124.2 (d)	6.31 (t, 11.0)
27	125.4 (d)	6.46 (dd, 11.0, 10.3)	126.1 (d)	6.46 (dd, 11.0, 10.4)
28	127.9 (d)	5.50 (td, 7.3, 10.3)	127.9 (d)	5.49 (td, 7.3, 10.4)
29	31.0 (t)	2.44 (td, 6.6, 7.3)	31.0 (t)	2.45 (td, 6.5, 7.3)
30	62.1 (t)	3.65 (t, 6.6)	62.2 (t)	3.66 (t, 6.5)
31	13.0 (q)	0.92 (d, 6.4)	12.7 (q)	0.94 (d, 6.5)
32	24.1 (q)	1.76 (s)	24.1 (q)	1.76 (s)
33			47.5 (q)	3.20 (s)

[a] Reference to residual solvent $CDCl_3$ signals at δ_H 7.24 and δ_C 77.0 and measured at 25 °C, 400 MHz for ^{1}H and 100 MHz for ^{13}C. ^{13}C multiplicities were assigned from DEPT experiments.

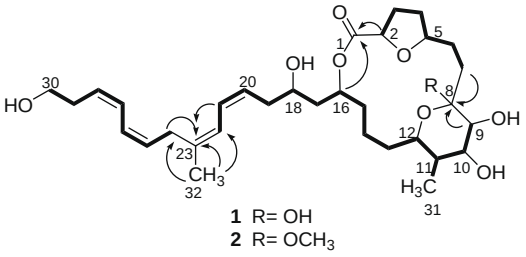


Figure 1. Connectivities established by ^{1}H–^{1}H COSY, HSQC and HMBC. Heavy lines indicate the connectivities assigned on the basis of ^{1}H–^{1}H COSY and HSQC. Arrows denote the correlations between protons (tail) and carbons (head) around the quaternary carbons observed in the HMBC.

form a 17-membered lactone ring. According to the data above, the whole carbon backbone was able to be assembled, leaving the position of hydroxyl groups and ether linkages to be determined. The deuterium-induced upfield ^{13}C chemical shift was observed upon replacing the NMR solvent with CD_3OD (originally CD_3OH), resulting in the identification of hydroxyl-bearing carbons.[10] Significant shifts (0.09–0.12 ppm) were observed for C8, C9, C10, C18 and C30, while the remaining four signals (C2, C5, C12 and C16) were superimposed on each other within 0.03 ppm. These oxycarbons were arranged to form the macrolide ester bond, a tetrahydrofuran and a tetrahydropyran rings. Therefore, a planar structure of formosalide A (**1**) was elucidated. A summary of the assignments of all the protons and carbons mentioned above are shown in Table 1.

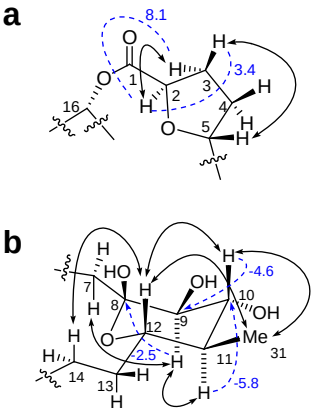
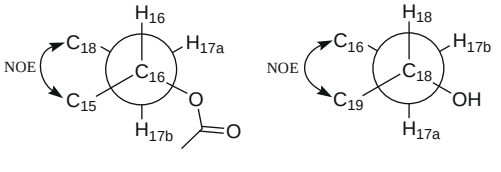


Figure 2. The relative stereochemistry for (a) C1–C5 and (b) C7–C14. The arrows show NOESY correlations, and the dashed lines show the coupling constants. $^{2}J_{CH}$ coupling constants are indicative in the dashed arrow.

The presence of *cis*-dienes at $\Delta^{20,21}$, $\Delta^{22,23}$, $\Delta^{25,26}$ and $\Delta^{27,28}$ were deduced from the ^{1}H–^{1}H coupling constant ($J_{20,21}$ = 11.0 Hz, $J_{25,26}$ = 11.0 Hz and $J_{27,28}$ = 10.3 Hz) as well as the ^{13}C chemical shifts. The carbon chemical shift of the C32 vinyl methyl group (δ_C 24.1) suggested that the trisubstituted $\Delta^{22,23}$ double bond possessed *Z* configuration.[11] The all *Z*-geometries were also supported by the NOESY data (H19/H22, H20/H21, H24/H21, H24/H27, H25/H26, H26/H29 and H27/H28). The relative stereochemistry of macrolide **1** was elucidated by detailed analysis of $^{3}J_{H,H}$, long-range $J_{C,H}$, and NOESY correlations[12,13], as shown in Figures 2 and 3. The $^{3}J_{H,H}$ coupling constants were extracted from the E. COSY experiment,[14] and the measurement of heteronuclear coupling constants ($^{2,3}J_{C,H}$) relied on analysis of G-BIRD$_{R,X}$-HSQMBC.[15] For the C2–C3 bond, the values of $^{3}J_{H2,H3a}$ = 8.1 Hz and $^{3}J_{H2,H3b}$ = 3.5 Hz inferred that H2/H3a was vicinal cis and H2/H3b was vicinal trans.[16] NOESY correlations were observed for H2/H3a and H5/H3b, indicating that the relative stereochemistry between H2 and H5 was *anti*-oriented. This *anti*-oriented configuration was further proved by the lack of NOE between H2/H5. A chair form of the tetrahydropyran ring was assignable from NOESY cross-peaks for H9/H11, H9/H7a, H10/H12, H10/H31, and H12/H31. The $^{3}J_{H,H}$ and $^{2}J_{C,H}$ coupling constants for the tetrahydropyran ring were as follows: $^{3}J_{H9,H10}$ = 9.0 Hz, $^{3}J_{H10,H11}$ = 9.6 Hz, $^{3}J_{H11,H12}$ = 10.6 Hz, $^{2}J_{C8,H9}$ = −2.5 Hz, $^{2}J_{C9,H10}$ = −4.6 Hz and $^{2}J_{C10,H11}$ = −5.8 Hz. To determine the stereochemical configuration between C16 and C18, the diastereotopic methylene protons of C17 were assigned. As shown in Figure 3, $^{3}J_{H16,17b}$ (10.0 Hz) and $^{3}J_{17a,H18}$ (9.7 Hz) revealed the values that are typical of *anti*-orientation of a 1,3-methine system.[17] On the other hand, the coupling constants of $^{3}J_{H16,17a}$ (3.4 Hz) and $^{3}J_{17b,H18}$ (3.1 Hz) indicate that H16/H17a and H18/H17b are both gauche interactions in the 1,3-methine system. Regarding the C16–C17 bond, the values for



$^{3}J_{H16H17a}$ = 3.4 Hz
$^{3}J_{H16H17b}$ = 9.9 Hz
$^{2}J_{C16H17a}$ = -5.4 Hz
$^{2}J_{C16H17b}$ = -5.9 Hz
$^{3}J_{C15H17a}$ = 6.1 Hz
$^{3}J_{C15H17b}$ = 3.2 Hz

$^{3}J_{H17aH18}$ = 9.7 Hz
$^{3}J_{H17bH18}$ = 3.1 Hz
$^{2}J_{C18H17a}$ = -5.8 Hz
$^{2}J_{C18H17b}$ = -5.8 Hz
$^{3}J_{C19H17a}$ = 3.5 Hz
$^{3}J_{C19H17b}$ = 7.6 Hz

Figure 3. The relative configuration for C16–C18.

$^2J_{C16,H17a}$ (−5.4 Hz), $^2J_{C16,H17b}$ (−5.9 Hz) and $^3J_{C15,H17a}$ (6.1 Hz) suggest that H17a is H17b is gauche to C16–O– and H17a is *anti* to C15. With respect to C17–C18 bond, the values of $^2J_{C18,H17a}$, $^2J_{C18,H17b}$ and $^3J_{C19,H17b}$ were measured to be −5.8, −5.8 and 7.6 Hz, respectively. These indicate that H17a and H17b are gauche to C18-OH and H17b is *anti* to C19. Assembling these two diastereomeric relationships unambiguously established the *anti*-C16/C18 configuration. NOESY correlations were observed for H15/H18 and H16/H19, which also supported the relative assignment as showed in Figure 3.

Formosalide B (**2**) was obtained as a colorless amorphous solid; $[\alpha]_D^{24}$ +18.8 (c 0.04, MeOH); UV_{max} (MeOH) 247 nm ($\log\varepsilon$ 4.18); IR (KBr) 3389, 2924, and 1709 cm^{-1}. The molecular formula of **2** was determined as $C_{33}H_{52}O_9$ by HRESIMS (observed $[M+Na]^+$, *m/z* 615.3515, calcd for $C_{33}H_{52}O_9Na$, *m/z* 615.3504, Δ +1.1 mmu), which corresponds to the formula of **1** in which hydrogen is replaced by a methyl group. The spectral data of **2** were almost analogous to those of compound **1** except for the following observations: the ^{13}C NMR spectrum of **2** showed an additional methoxy carbon (δ_C 47.5, C33), and a ^1H NMR signal for the methoxy group was observed at 3.20 ppm (3H, s). The chemical shifts differences for two methine signals between **2** (C9: δ_C 74.2, δ_H 3.28 and C12: δ_C72.8, δ_H 3.13) and **1** (C9: δ_C 77.3, δ_H 3.06 and C12: δ_C70.3, δ_H 3.51) were found. In addition, a HMBC NMR cross peak was observed between H33 and C8. From these findings, formosalide B was suggested to possess the structure **2**: the hydroxy group of hemiketal C8 of formosalide A (**1**) was substituted by a methoxy group. The relative configurations of the chiral centers were inferred as the same as those of **l** from the ^1H NMR coupling constants and NOESY correlation data.

Formosalides A (**1**) and B (**2**) represent a new class of 17-membered ring macrolides from dinoflagellate of the genus *Prorocentrum*. The compounds possess all-*cis* tetraenes, a tetrahydropyran ring, a tetrahydrofuran ring, two branched methyls and a C14 linear side-chain. Interestingly, **1** and **2** are structurally similar to the macrolides from *Amphidinium* species,[3,7–9] a phylogenetically different group of dinoflagellates. Formosalides A and B (**1**, **2**) exhibited cytotoxicity against CCRF-CEM human T-cell acute lymphoblastic leukemia cells and/or DLD-1 human colon adenocarcinoma cells in vitro (LD_{50} values: **1**, 0.54 and >40 μg/mL, respectively; **2**, 0.43 and 2.73 μg/mL, respectively). Although, **1** and **2** shared the same six-membered hemiketal ring and/or tetrahydrofuran ring moieties with caribenolide I and amphidinolide N, the cytotoxicity of **1** and **2** against cancer cells was much less potent than these two macrolides.[18] Formosalides A and B possessed a smaller lactone ring (17- to 26-membered) and the longer side-chain (C14 to C4) than those of caribenolide I and amphidinolide N.

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