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Prorocentin, a New Polyketide from the Marine Dinoflagellate *Prorocentrum lima*

Chung-Kuang Lu,*,†,‡ Hong-Nong Chou,§ Ching-Kuo Lee,⊥ and Tzong-Huei Lee⊥

National Museum of Marine Biology and Aquarium, Pingtung 944, Taiwan, Institute of Marine Biotechnology, National Dong Hwa University, Pingtung 944, Taiwan, Institute of Fisheries Science, National Taiwan University, Taipei, 106, Taiwan, and Graduate Institute of Pharmacognosy, Taipei Medical University, Taipei, 110, Taiwan

cklu@nmmba.gov.tw

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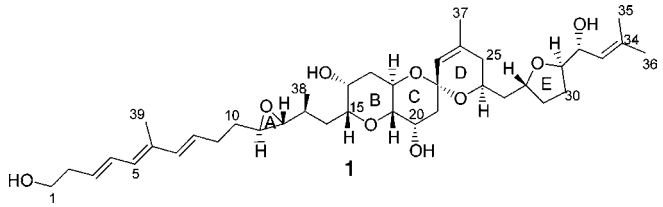
ABSTRACT



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Prorocentin (1), isolated from an okadaic acid-producing organism, *Prorocentrum lima*, possessed all-trans trienes, an epoxide, as well as the 6/6/6-trans-fused/spiro-linked polyether ring moieties. The unique structure supports the proposed cyclization mechanism, polyene formation, epoxidation, and cyclization, of marine polyether toxins. The relative stereostructure was determined on the basis of spectral data.

Marine dinoflagellates of the genus *Prorocentrum* have been reported to produce novel bioactive secondary metabolites of entirely different skeletons such as linear polyether toxins[1] and macrolides.[2] Okadaic acid and its analogues are some of the most attractive substances in marine natural products chemistry.[1] In particular, okadaic acid has been shown to be a highly selective inhibitor of protien phosphatases,[3] to be mainly responsible for diarrhetic shellfish poisoning,[4] and to be biosynthesized via an unusual route involving both the carbon backbone formation[5] and the cyclization mechanism.[6] This irregularity of the carbon backbone synthesis has also been reported in other marine dinoflagellate polyketides, e.g., brevetoxins,[7] goniodomin A,[7] amphidinolides,[7] yessotoxins,[8] and amphidinols.[9] Among those, only the okadaic acid skeleton and brevetoxins went through the cyclization mechanism to form spiro-linked and/or trans-fused polyether

† National Museum of Marine Biology and Aquarium.
‡ National Dong Hwa University.
§ National Taiwan University.
⊥ Taipei Medical University.

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rings. The enzyme-mediated epoxidation at the double bonds, followed by nucleophilic addition upon cyclization, were suggested as the mechanisms of formation of ether rings.[6,7c] However, none of the natural products bearing the serial functional groups of cyclization cascade had been isolated from dinoflagellates. In our project, searching for new biologically active substances from marine microalgae, we isolated prorocentin (**1**) from the okadaic acid-producing organism *Prorocentrum lima* clone PL021117001.[10] In this paper, we report the structure elucidation of prorocentin (**1**), which possesses the functional groups, all-trans trienes, an epoxide, and the tricyclic ether rings, of the proposed cyclization mechanism for marine polyether toxins. In addition, the possible biosynthesis pathways of its tricyclic ether ring moiety are proposed.

The methylene chloride extracts of lab-cultured *P. lima* were fractionated by a series of chromatography and RP-HPLC.[11] Prorocentin (**1**, 3 mg), an amorphous solid had $[\alpha]^{25}_{D}$ −12.7° (*c* 0.2, MeOH), UV_{max} (MeOH) 274 nm (ϵ 25 300), and IR υ_{max} (KBr) 3426, 2924, 1438, 1058, 1027 cm^{-1}. HR-FTMS and the total number of carbons determined by ^{13}C NMR spectra, measured in $CDCl_3$ and CD_3OD, suggested a possible molecule of $C_{39}H_{60}O_9$ (observed $[M + Na]^+$, *m*/*z* 695.4137; calcd for $[M + Na]^+$, *m*/*z* 695.4135), which may contain 10 sites of unsaturation. The resonances of seven olefinic methines (δ122.2, 123.9, 128.3, 129.2, 129.7, 130.2, 135.4) and three olefinic quateric carbons (δ 133.8, 135.4, 137.7) in the ^{13}C spectrum ($CDCl_3$) accounted for 5 of the 10 sites of unsaturation. The remaining five sites of unsaturation had to be accounted for by ring structures. Since HSQC and DEPTs data showed that 56 of the 60 hydrogen atoms were attached to carbons (5 methyl groups, 11 methylenes, 19 methines), there should be 4 hydroxyl groups in **1**.

The proton connectivities were elucidated by detailed analysis of two-dimensional NMR experiments, including $^1H-^1H$ COSY, HSQC, HMBC, and NOESY. Long-range $^4J_{H-H}$ couplings via sp^2 carbons such as H-5/H_3-39, H-23/H_2-25, H-23/H_3-37, H-33/H_3-35, and H-33/H_3-36 were clearly indicated by cross-peaks. Four partial structures (I, C1−C6; II, C7−C15; III, C16−C21; IV, C23−C36) were obtained as shown in Figure 1. The absence of coupling between H-15 (δ3.55) and H-16 (δ3.80) was caused by a dihedral angle near 90°. The connectivity between C15 and C16 was further deduced from the NOESY cross-peak. The terminal quaternary carbons (C6, C22, C24, and C34) were linked to the fragments via HMBC correlations (Figure 1). 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

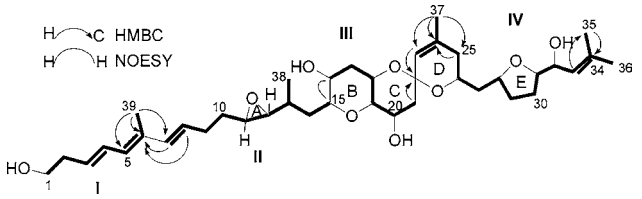

Figure 1. Connectivities established by $^1H-^1H$ COSY, HSQC, HMBC, and NOESY. Heavy lines indicate the connectivities assigned on the basis of $^1H-^1H$ COSY and HSQC. Arrows denote the correlations between protons (tail) and carbons (head) around the quaternary carbons observed in the HMBC. The arc (H-15/H-16) denoted the correlation of NOESY.

replacing the NMR solvent with CD_3OD (originally CD_3-OH), resulting in the identification of hydroxyl-bearing carbons.[12] Significant shifts (0.09−0.12 ppm) were observed for C1, C16, C20, and C32, while the remaining nine signals were superimposed on each other within 0.03 ppm. These oxycarbons were arranged to form an epoxide, a furan and three pyran rings. Therefore, a planar structure of **1** was elucidated. A summary of the assignments of all the protons and carbons mentioned above are shown in Table 1 (see also Supporting Information Table S1).

Table 1. ^1H NMR and ^{13}C NMR Data of **1** ($CDCl_3$)[a]

No.	δ_C (mult.)	δ_H (mult. *J* in Hz)	No.	δ_C (mult.)	δ_H (mult. *J* in Hz)
1	62.0 (t)	3.66 (t, 6.2)	$\mathbf{21}_{ax}$ $\mathbf{21}_{eq}$	40.0 (t)	1.76 (m) 2.05 (dd, 14.3, 3.4)
2	36.6 (t)	2.38 (q, 6.4)	**22**	96.2 (s)	
3	130.2 (d)	5.65 (m)	**23**	122.2 (d)	5.27 (s)
4	129.7 (d)	6.44(dd, 15.1, 11.1)	**24**	137.7 (s)	
5	129.2 (d)	5.96 (d, 11.1)	$\mathbf{25}_{ax}$ $\mathbf{25}_{eq}$	35.2 (t)	1.79 (m) 1.97 (m)
6	133.8 (s)		$\mathbf{26}_{ax}$	68.1 (d)	4.01(m)
7	135.4 (d)	6.10 (d, 15.5)	**27**	40.8 (t)	1.75 (m)
8	128.3 (d)	5.56 (m)	**28**	79.5 (d)	4.06 (m)
9	29.3 (t)	2.25 (m)	$\mathbf{29}_a$ $\mathbf{29}_b$	33.8 (t)	1.49 (m) 1.98 (m)
10	32.2 (t)	1.62 (m)	$\mathbf{30}_a$ $\mathbf{30}_b$	28.5 (t)	1.34 (m) 1.81 (m)
11	57.8 (d)	2.72 (td, 5.6, 2.1)	**31**	85.5 (d)	3.93 (dt, 7.0, 8.4)
12	63.2 (d)	2.46 (dd, 7.0, 2.1)	**32**	72.1 (d)	4.10 (dd, 7.0, 8.9)
13	32.2 (d)	1.62 (m)	**33**	123.9 (d)	5.02 (d, 8.9)
$\mathbf{14}_a$ $\mathbf{14}_b$	39.2 (t)	1.42 (m) 1.65 (m)	**34**	135.4 (s)	
$\mathbf{15}_{ax}$	73.6 (d)	3.55 (m)	**35**	18.9 (q)	1.70 (s)
$\mathbf{16}_{eq}$	70.5 (d)	3.80 (m)	**36**	25.9 (q)	1.69 (s)
$\mathbf{17}_{ax}$ $\mathbf{17}_{eq}$	39.4 (t)	1.4 1 (m) 2.00 (m)	**37**	22.8 (q)	1.71 (s)
$\mathbf{18}_{ax}$	70.9 (d)	3.81 (m)	**38**	17.5 (q)	0.92 (d, 6.4)
$\mathbf{19}_{ax}$	76.1 (d)	3.00 (dd, 2.9, 9.3)	**39**	12.8 (q)	1.81 (s)
$\mathbf{20}_{eq}$	66.4 (d)	4.02 (m)			

[a] Reference to residual solvent $CDCl_3$ signals at δ_H 7.24 and δ^{13}_C 77.0 and measured at 25 °C, 500 MHz for ^1H, and 125 MHz for ^{13}C. ^{13}C multiplicities were assigned from DEPT experiments.

The proposed structure was further supported by positive ion ESI Q-TOF tandem mass studies (Supporting Information). An electron spray ionization MS/MS experiment was

(10) Dinoflagellate *P. lima* PL021117001 was isolated from the northern coast of Taiwan in 2002 and cultured in seawater enriched with K nutrient at 25 °C with an 8/16 dark/light photoperoid cycle for 4 weeks.

(11) Algal cells (8.3×10^9 cells), harvested from 450 L of the cultures, were extracted exhaustively with methanol. Purification of the CH_2Cl_2 solubles by successive chromatography with the silica gel 60 (9:1 CH_2Cl_2/MeOH), Sephdex LH-20 (MeOH), and HPLC (Biosil Pro-ODS 5U, 55% CH_3CN/H_2O) gave 3 mg of pure prorocentin.

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carried out using ions with positive charge (cations), and $[M + Na]^+$ ion (*m*/*z* 695.5) was chosen as a precursor ion. The prominent product ions at *m*/*z* 497, 301, and 221 confirmed the structure of 6/6/6-trans-fused/spiro-linked tricyclic ethers.[13]

The relative stereochemistry of the tricyclic ether rings (C15−C26) and the tetrahydrofuran ring (C28−C31) were deduced from NOESY correlations and $^1H-^1H$ coupling constants as shown in Figure 2. The typical axial−axial

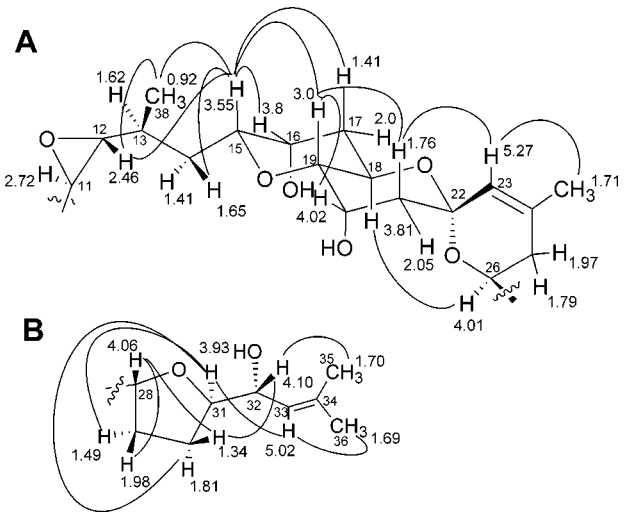
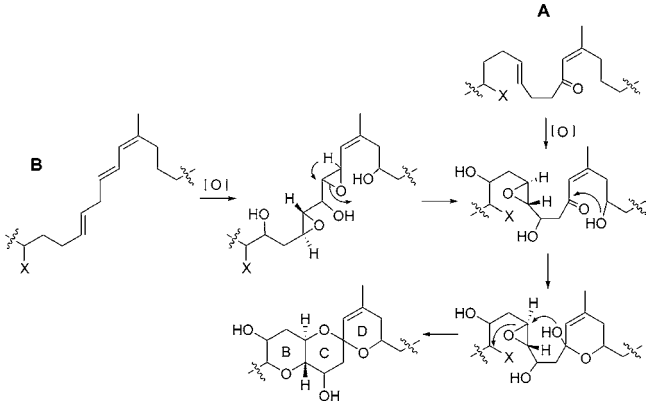


Figure 2. Relative stereochemistry of substructures A (C15−C26) and B (C28−C36) in **1**. The arc shows NOESY correlations.

coupling constant of 9.3 Hz between H-18_{ax} and H-19_{ax}[14] and NOEs, H-15_{ax}/H-16_{eq}, H-15_{ax}/H-17_{ax}, H-19_{ax}/H-15_{ax}, H-19_{ax}/H-17_{ax}, H-19_{ax}/H-20_{eq}, H-19_{ax}/H-21_{ax}, demonstrated that the B/C rings were both chair conformations and trans-fused. In addition, the small coupling constants observed between H-16_{eq}/H-15_{ax} and between H-20_{eq}/H-19_{ax} indicated axial orientations for both 16-OH and 20-OH. The NOESY spectrum gave strong NOE cross-peaks for H-18_{ax}/H-26_{ax} and H-21_{ax}/H-23 defined the relative configuration at the spiroketal carbon (C22). The cross-peaks of H_3-38/H-12, H_3-38/H-15, H-12/H-15, and H14_b/H15 in NOESY contributed to the relative configuration of the C12 to C15 carbon chain (Figure 2A). The lack of NOE between H-28/H-31 was indicative of the anti arrangement for H-28 to H-31 in the tetrahydrofuran ring. This anti-oriented configuration was further proved by the clear NOE cross-peaks of H-28/H-29_b, H-28/H-30_a, H-31/H-29_a, and H-31/H-30_b. The coupling constants of H-31/H-32 (7.0 Hz) and H-32/H-33 (8.9 Hz), and NOEs, H-31/H33, H-33/H_3-36, H-32/H-30_a, and H-32/H_3-35, indicated rotational constraints along the C31−C32 and C32−C33 bonds, allowing us to interrelate the relative configuration of C31 to C36 (Figure 2B). The epoxide ring at C11−C12 was elucidated as trans by proton−proton coupling constant ($J_{11,12}$ = 2.1 Hz).[15] The $\Delta^{3,4}$, $\Delta^{5,6}$, and $\Delta^{7,8}$ olefins were indicated to have all (*E*)-geometries on the basis of NOESY data (H_2-2/H-4, H-4/H_3-39, H-8/H_3-39, H-3/H-5, and H-5/H-7) and the $^1H-^1H$ coupling constants ($J_{3,4}$ = 15.1 Hz, and $J_{7,8}$ = 15.5 Hz). All the data above allowed us to assign the relative stereostructure of **1**.

Prorocentin, a C35 polyketide chain with four pendant methyl groups, possessed an all-trans triene moiety, an epoxide, a furan ring, and the 6/6/6-trans-fused/spiro-linked tricyclic ether rings. This new marine dinoflagellate polyketide supported the proposed cyclization mechanism, polyene formation, epoxidation, and cyclization, of marine polyether toxins such as okadaic acid and brevetoxins. Both **1** and okadaic acid found in the PL021117001 strain of *P. lima* suggested that they might share part of their biosynthetic pathway. The fact that both spiro-linked ethers (C/D rings) of **1** and okadaic acid[16] (A/B rings) shared the same backbone might be explained by a plausible biosynthetic pathway as shown in Scheme 1A. An alternative route (Scheme 1B) was

Scheme 1



proposed according to the conjugated dienes moiety in **1**. Toward further disclosure of this polyketide biosynthesis, labeling experiments are currently under the way. Prorocentin (**1**) exhibited inhibitory activity against human colon adenocarcinoma DLD-1 and human malignant melanoma RPMI7951 with IC_{50} values of 16.7 and 83.6 μg/mL, respectively. The antimicrobial activity against *Staphylococcus aureus* BRBC 12154 was negative at a dose of 100 μg/mL.

(13) These bond cleavages at the sites characteristic of ether rings were reported on dinoflagellate toxins of ciguatoxins (Yasumoto, T.; Igarashi, T.; Legrand, A.-M.; Cruchet, P.; Chinain, M.; Fujita, T.; Naoki, H. *J. Am. Chem. Soc.* **2000**, *122*, 4988−4989), yessotoxin (Naoki, H.; Murata, M.; Yasumoto, T. *Rapid Comm. Mass Spectrom.* **1993**, *7*, 179−182), and amphidinol (Satake, M.; Murata, M.; Fujita, T.; Naoki, H.; Yasumoto, T. *J. Am. Chem. Soc.* **1991**, *113*, 9859−9861).

(14) Coupling constants of anguar protons in the case of brevetoxins and ciguatoxin (Murata, M.; Legarand, A. M.; Ishibashi, Y.; Fukui, M.; Yasumoto, T. *J. Am. Chem. Soc.* **1990**, *112*, 4380−4386) were ca. 9 Hz.

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(16) The high incorporation ratio at O(4), the oxygen atom of the A ring in okadaic acid, from [$^{18}O_2$]acetate has been reported: Murata, M.; Izumikawa, M.; Tachibana, K.; Fujita, T.; Naoki, H. *J. Am. Chem. Soc.* **1998**, *120*, 147−151. Needham, J.; Hu, T.; McLachlan, J. L. *J. Chem. Soc., Chem. Commun.* **1995**, 1623−1624.

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Supporting Information Available: Experimental details, as well as one- and two-dimensional NMR and Q-TOF MS/MS data for prorocentin. This material is available free of charge via the Internet at http://pubs.acs.org.

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