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Three new okadaic acid derivatives isolated from a benthic dinoflagellate *Prorocetrum lima*

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Abstract Toxins produced by marine toxigenic algae have garnered growing attention due to their detrimental impacts on marine ecosystem, aquaculture, and human health. Among these, diarrhetic shellfish poisoning (DSP) toxins, such as okadaic acid (OA), are of particular concern. In this study, we report the successful isolation and structural elucidation of three new derivatives of OA from the marine dinoflagellate *Prorocentrum lima*. These newly identified compounds, OA-2Me-C7, OA-2-Me-C8, and OA-1-Me-C8, were characterized through a comprehensive series of NMR experiments, combined with structural comparisons to the well-known OA. The identification of these derivatives contributes to the expanding knowledge of DSP toxin diversity and provides new insights into the structural variations of these harmful algal toxins.

Keywords 1D and 2D NMR, *Prorocentrum lima*, Okadaic acid, Okadaic acid diol esters, DSP isolation

Introduction

Harmful algal blooms (HAB) are increasingly prevalent in marine environments, posing significant threats to marine ecosystem, aquaculture, and human health.[1] A key concern in these blooms is the production of various toxins by marine algae, including diarrhetic shellfish poison (DSP) toxins, which have been detected in offshore waters worldwide. Consumption of shellfish contaminated by DSP toxins can lead to gastrointestinal issues such as diarrhea, nausea, vomiting, and other severe symptoms.[2] The primary causative organisms responsible for DSP toxins production are dinoflagellates, especially species within the genera *Prorocentrum* spp, and *Dinophysis* spp., including *D. acuminata*, *D. acuta*, *P. lima*, *P. concavum*, and *P. minimum*.[3-5] Over the past decades, substantial research has been dedicated to identifying and characterizing DSP toxins and their derivatives produced by these harmful marine algae.[6-8] To dates, two major DSP toxins, okadaic acid (OA) and dinophysistoxin-1 (DTX-1), have been successfully isolated from cultured *P. lima*. In addition to OA and DTX-1, several derivatives of these DSP toxins have been discovered in cultures of *Prorocentrum* species.[9-10] However, the environmental impact of wide range of DSP-related toxins remains underexplored, and risk assessments are hindered by lack of comprehensive data on these compounds. To fully understand the ecological and health risks posed by DSP toxins, systematic research into their complete characterization and environmental occurrence is crucial.

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In this study, we aimed to assess the regional risks associated with DSP toxins by monitoring marine dinoflagellates along the offshore coasts of Korean islands. Among the species identified, *Prorocentrum lima*, a known producer of OA and DTX-1, was selected for large-scale cultivation in the laboratory. From a 450 L culture, we successively isolated three new derivatives of OA.

This paper presents the isolation and structural elucidation of these new compounds (compounds **1**–**3**) from *P. lima*. Through a series of NMR experiments and structural comparisons with OA, we provide detailed insights into the molecular structure of these new DSP toxin derivatives.

Experimental Methods

Extraction and isolation - The *Prorocentrum lima* strain used in this study was originally isolated from the macroalga *Sargassum fulvellum*, collected by divers at a depth of approximately 3 m off the coast of Geomundo Island, Korea, in November 2012. The biomass was subsequently cultured to a volume of 450 L using a method previously reported. When the cell concentration reached approximately 10,000–12,000 cells mL^{-1}, the *P. lima* cells were harvested by centrifugation at 15,000 rpm for 10 hours. The harvested cells were extracted with MeOH over a period of two days. The resulting methanolic extract was partitioned with n-BuOH and H_2O solvents. The organic fraction was repartitioned between 85% aqueous MeOH and hexane. Subsequently, the aqueous layer underwent reversed-phase vacuum flash chromatography, employing stepwise gradients of MeOH in H_2O (50%, 60%, 70%, 80%, 90%, and 100%). Among these fractions, the 100% MeOH fraction exhibited promising signals in the down-field region of the ^1H NMR spectrum and was subjected to further separation. This fraction was separated into five subfraction (M1–M5) using Sephadex LH20 open column chromatography to facilitate the isolation of specific compounds. From these subfractions, the toxin-rich M2 fraction (165 mg) was selected for further separation via reversed-phase HPLC, using a Phenomenex C8 column (250 mm × 10 mm) and a Waters UV-486 detector. The solvent system used ranged from H_2O : ACN = 60 : 40 to 100% ACN over 40 min, leading to the separation of mixed compounds. The HPLC peak obtained at a retention time of 35 min was further purified, yielding compound **1** (2 mg) and **3** (2.6 mg). The final purification step involved reversed-phase HPLC using a Phenomenex C6-phenyl column (250 mm × 10 mm) and a Waters RI-410 detector, with a solvent mixture of 75% MeOH and 25% H_2O. Similarly, compound **2** (1 mg) was isolated through a re-purification process of the HPLC peak at a retention time of 38 min.

NMR experiment - The 1D and 2D NMR spectra were acquired using a Varian VNMRS system operating at 500 MHz for proton and 125 MHz for carbon nuclei. Chemical shifts for ^1H and ^{13}C NMR spectra were referenced to CD_3OD at 3.30 and 49.0 ppm, respectively. Throughout all experiments, the temperature was maintained at a constant 297 K.

Specific parameters for the 2D NMR experiments were as follows: Gradient COSY spectra were gathered within a spectral width of 2567 Hz using a 512(t1) × 1024 (t2) matrix, employing a 1 ms pulse gradient with a strength 10 G/m. These spectra were processed using a sinebell function for optimal results. For the gradient HSQC spectra, measurements were conducted in a 128(t1) × 1024(t2) matrix, utilizing J_{CH}=140 Hz and processed in a 256 (t1) × 1024 (t2) matrix through a linear prediction method to achieve higher resolution.

The gradient HMBC experiment was fine-tuned for a long-range coupling constant of 8 Hz. Additionally, the NOESY experiment involved a mixing time of 250 ms to capture pertinent data for analysis.

Results and Discussion

The toxin-rich M2 fraction, obtained from LH20 open column chromatography, led to the successful isolation of three compounds (**1**–**3**), which were identified new derivatives of okadaic acid (Fig. 1).

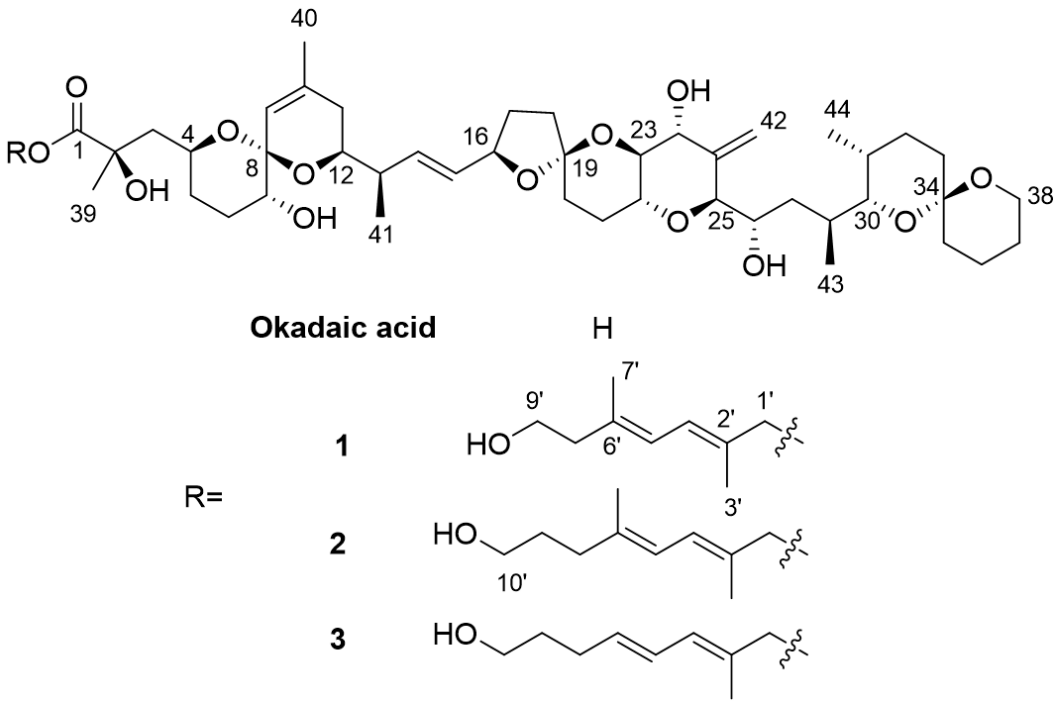

Figure 1. Three OA derivatives isolated from the marine dinoflagellate *Prorocentrum lima*.

The structures of these compounds shared significant similarities with each other, but distinct differences were also observed. The structural elucidation of compounds **1**–**3** began with a detailed comparison of their ^{1}H and ^{13}C NMR spectra with those of okadaic acid, the parent compound. This comparison highlighted the key structural motifs present in okadaic acid while revealing new, additional signals that were absent in the original compound. These new signals provided crucial evidence for the modifications present in the derivatives. A comprehensive analysis combining NMR and MS experiments was employed to determine the newly introduced moieties. This integrative approach enabled to identify compounds **1**–**3** as new okadaic acid derivatives.

Compound **1** was isolated as a colorless solid, with a molecular formula of $C_{53}H_{82}O_{14}$ determined from its ammonium-adducted ion peak ($[M + NH_4]^+$ *m/z* = 960.6006, Δ=3.8 ppm) in the HR-ESI MS spectrum and its ^{13}C NMR spectrum. The ^{1}H and ^{13}C NMR spectra, measured in CD_3OD, closely resembled those of okadaic acid (OA) isolated from *Prorocentrum lima*. The carbon chemical shifts for the two compounds, listed in Table 1, highlight their similarities. The structure of **1** was established through the identification of the additional resonances associated with the $C_8H_{14}O$ moiety. In the ^{1}H NMR spectrum, two additional olefinic methyl groups (δ_H 1.76 and 1.79) and two olefinic protons (δ_H 6.09 and 6.31) were clearly observed. The ^{13}C and HSQC spectra revealed two non-protonated olefinic carbons (δ_C 14.5 and 16.9), two oxymethylene carbons (δ_C 61.5 and 71.6), four olefinic carbons (δ_C 123.0, 125.2, 131.1, and 137.7), and a shielded carbon (δ_C 44.3). Compared to the carbon chemical shifts of okadaic acid, the resonances of C-1 (δ_C 177.0), C-2 (δ_C 75.8), and C-4 (δ_C 68.4) in **1** were shielded, while C-3 (δ_C 46.1) was deshielded, with other resonances showing strong similarity to those of okadaic acid. As shown in Fig. 2, in addition to the COSY correlations for okadaic acid protons, new COSY correlations were observed between H-4’ and H-5’, as well as H-8’ and H-9’. These two fragments were connected by HMBC

Table 1. Spectral Data for Okadaic Acid (OA) and OA part of compound **1** in CD_3OD (500 MHz, ^1H).

	δ_H (**1**)	δ_C (**1**)	δ_C (**OA**)
1		177.0, C	179.6
2		75.8, C	76.3
3	1.82 (m); 1.87 (m)	46.1, CH_2	45.4
4	4.00 (t, 10.3)	68.4, CH	69.6
5	1.35 (m); 1.72 (m)	33.4, CH_2	33.4
6	1.65 (m); 1.96 (m)	28.0, CH_2	28.8
7	3.35 (m)	73.0, CH	73.0
8		97.6, C	97.7
9	5.26 (br s)	123.4, CH	123.0
10		139.6, C	140.2
11	1.84 (m); 1.93 (m)	33.9, CH_2	34.0
12	3.68 (m)	72.2, CH	72.0
13	2.34, m	43.0, CH	43.4
14	5.78 (dd, 15.2, 8.4)	136.6, CH	137.5
15	5.53 (dd, 15.2, 7.7)	132.4, CH	132.5
16	4.57 (m)	80.4, CH	80.5
17	1.59 (m); 2.20 (m)	31.6, CH_2	31.6
18	1.85 (m); 2.00 (m)	38.0, CH_2	38.1
19		107.1, C	107.1
20	1.84 (m); 1.88 (m)	34.1, CH_2	34.1
21	1.77 (m); 1.89 (m)	27.7, CH_2	27.7
22	3.63 (m)	71.2, CH	71.3
23	3.37 (t, 10.0)	78.3, CH_2	78.1
24	4.09 (d, 10.0)	71.8, CH	72.1
25		147.0, C	147.3
26	3.93 (d, 8.9)	86.4, CH	86.3
27	4.10 (m)	66.1, CH	66.2
28	0.93 (m); 1.35 (m)	36.8, CH_2	36.8
29	1.87 (m)	32.3, CH	32.3
30	3.23 (dd, 10.2, 2.0)	76.8, CH	76.8
31	1.78 (m)	28.8, CH	28.8
32	1.41 (m); 1.98 (m)	27.5, CH_2	27.5
33	1.13 (m); 1.94 (m)	31.2, CH_2	31.2
34		97.0, C	97.0
35	1.48 (m)	37.0, CH_2	37.0
36	1.45 (m); 1.62 (m)	19.8, CH_2	19.8
37	1.51 (m); 1.62 (m)	26.5, CH_2	26.5
38	3.48 (m); 3.68 (m)	61.3, CH_2	61.3
39	1.40 (s)	26.2, CH_3	27.8
40	1.74 (s)	23.1, CH_3	23.2
41	1.04 (d, 6.7)	16.5, CH_3	16.7
42	5.02 (br s); 5.33 (br s)	112.5, CH_2	112.3
43	1.03 (d, 6.4)	16.7, CH_3	16.6
44	0.92 (d, 7.3)	11.1, CH_3	11.1

correlations from H_3-3' to C-1', C-2', and C-4', and from H_3-7' to C-5', C-6', and C-8'. The hydroxy group was attached to C-9' based on the molecular formula and the carbon chemical shift. The protons attached to the oxymethylene C-1' showed HMBC correlations with carbonyl carbon, C-1, in okadaic acid portion, indicating that the $C_8H_{14}O$ moiety is connected to okadaic acid via an ester bond. The downfield shift of the H-1' proton further supported the presence of this ester bond. Finally, the two double bonds in the moiety were determined to have *E*-geometry based on NOE correlations between H_3-3' and H-5', and H_3-7' and H-4'. Consequently, compound **1** was identified as a 1,4- dimethyl C9 diol ester of okadaic acid (OA-2-Me-C7).

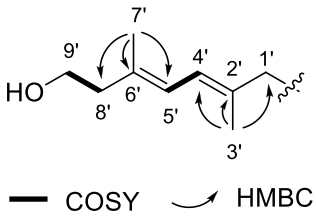

Figure 2. COSY correlations and Key HMBC correlations in the partial structure of **1**.

Compound **2** was obtained as a colorless solid, and its molecular formula determined to be $C_{54}H_{84}O_{14}$ based on the ammonium-adducted ion peak ($[M + NH_4]^+$ *m/z* = 974.6199, Δ=1.7 ppm) in the HR-ESI MS spectrum. Compared to compound **1**, compound **2** lacks a CH_2 group. The ^1H spectra of **1** and **2** showed the difference in the chemical shift ranges of 2.1 – 2.3 ppm and 3.5 – 3.7 ppm. Additionally, two olefinic methyl protons near 1.77 ppm were observed, though the HSQC spectrum indicated that a methyl signal at δ_H 1.77 was an overlap of two methyl groups. Careful analysis of 1D and 2D NMR spectra of **2** revealed a close similarity to **1**, except for differences in the resonances of H-8', H-9', H-10', C-7', C-8', C-9', and C-10'. The proton at H-8' (δ_H 2.16), assigned via HMBC correlation with H_3-7', showed COSY correlations with H-9' (δ_H 1.66), which in turn correlated with a neighboring proton H-10' (δ_H 3.65). Thus, compound **2** was identified as a 1, 4-dimethyl C10 diol ester of okadaic acid (OA-2-Me-C8).

The molecular formula of compound **3** was determined to be $C_{53}H_{82}O_{14}$, as evidenced by the ammonium-adducted ion peak ($[M + NH_4]^+$ *m*/*z* = 960.6023, Δ=2.1 ppm) in the HR-ESI MS spectrum. Notably, although the molecular formula of **3** matched that of **1**, distinct differences emerged in the ^{1}H NMR spectrum, specifically, the olefinic methyl groups at around δ_H 1.77 ppm, along with a new proton resonance at δ_H 5.73. While compound **1** displayed three olefinic methyl groups, **3** exhibited only two. The proton signal at δ_H 5.73 ppm was found to be coupled with H-8' (δ_H 2.20), which in turn was coupled to a shielded proton H-9' (δ_H 1.63). Further analysis revealed that H-9' was correlated with H-10' in the COSY spectrum, and these correlations were further validated by the TOCSY spectrum. These spectral observations pointed to the loss of the methyl group at C-5' in **2**, leading to the structural determination of compound **3**. Based on these finding, **3** was confidently identified as a 1-methyl C9 diol ester of OA (OA-1-Me-C8).

This study represents the first successful isolation and identification of OA-2-Me-C7, OA-2-Me-C8, and OA-1-Me-C8 from cultured *Prorocentrum lima*. A thorough review of the literature revealed that OA-2-Me-C7 closely resembled the 1,4-dimethyl C9 diol ester of DTX-1, with the key distinction being the absence of a methyl group at the C-35 position.[11] Furthermore, OA-1-Me-C8 was identified as a compound with one fewer methylene group in its ester fragment compared to the previously related analogs.[12]

Table 2. Spectral Data for the diol part of compounds 1–3.

	1		**2**		**3**	
	δ_H (mult, Hz)	δ_C	δ_H (mult, Hz)	δ_C	δ_H (mult, Hz)	δ_C
1'	4.55 (d, 12.5) 4.65 (d, 12.5)	71.6, CH_2	4.55 (d, 12.5) 4.64 (d, 12.5)	71.6, CH_2	4.51 (d, 12.6) 4.60 (d, 12.6)	71.3, CH_2
2'		131.1, C		130.7, C		131.0, C
3'	1.77, s	16.9, CH_3	1.77, s	14.4, CH_3	1.77, s	14.5, CH_3
4'	6.31 (d, 11.1)	125.2, CH	6.30 (d, 11.3)	125.4, CH	6.05 (d, 10.9)	129.3, CH
5'	6.09 (d, 11.1)	123.0, CH	6.07 (d, 11.3)	121.5, CH	6.31 (dd, 15.0, 10.9)	127.3, CH
6'		137.7, C		140.6, C	5.74 (dt, 15.0, 7.0)	136.4, C
7'	1.79, s	14.5, CH_3	1.77, s	16.9, CH_3		
8'	2.32, m	44.3, CH_2	2.16 (t, 8.3)	37.5, CH_2	2.20, m	30.2, CH_2
9'	3.65 (t, 6.6)	61.5, CH_2	1.66, m	32.0, CH_2	1.63, m	33.3, CH_2
10'			3.54 (t, 6.6)	62.5, CH_2	3.55 (t, 6.5)	62.3, CH_2

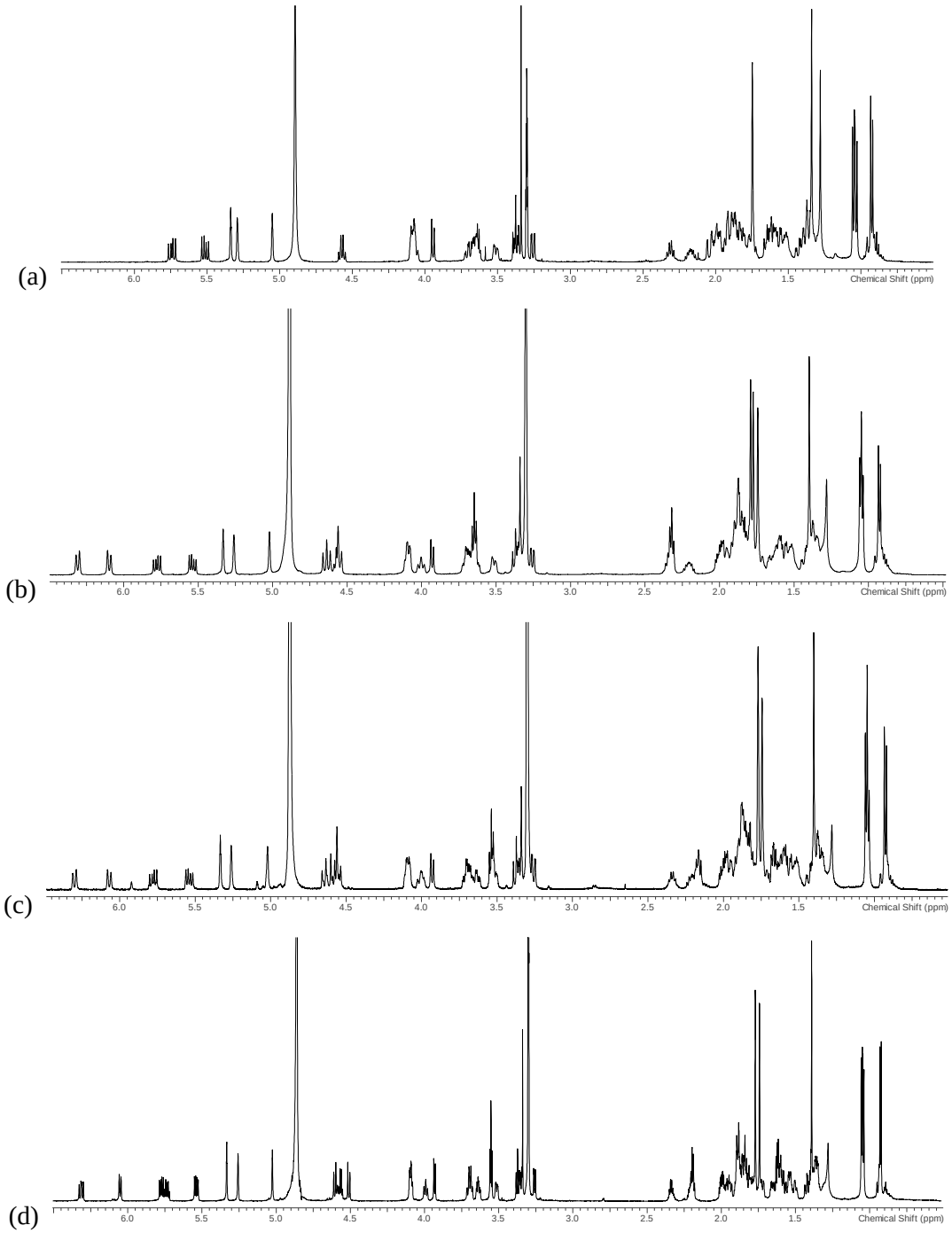

Figure 3. ^{1}H NMR spectra of (a) okadaic acid, (b) **1**, (c) **2**, and (d) **3** in CD_3OD.

Acknowledgements

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