Identification of New Okadaic Acid Derivatives from Laboratory Cultures of *Prorocentrum lima*

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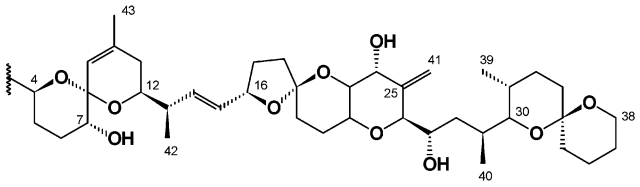
The dinoflagellate *Prorocentrum lima* produces toxins involved in the red tide phenomenon known as diarrhetic shellfish poisoning (DSP). This paper reports the isolation and spectroscopic structural elucidation of new compounds related to DSP toxins, isolated from a laboratory culture of strain PLV2. Their structures were established from their spectroscopic data.

Diarrhetic shellfish poisoning (DSP) typically causes diarrhea, nausea, vomiting, and, in some cases, abdominal pain in human beings. Although no fatalities have been ascribed to DSP, the illness can be deeply debilitating for several days at a time, putting a great strain on public health and economic resources worldwide. The toxins held mainly responsible are okadaic acid (**1**) (OA) and its derivatives, produced by dinoflagellates of the genera *Dinophysis* and *Prorocentrum*.[1] Many of these toxins are present as OA ester derivatives, predominantly diol esters, and have been obtained from different species of *Prorocentrum* cultures, and their structures determined.[2,3] The composition and total production of these toxins show marked variations depending on the strain and range from 2 to 28.6 pg/cell.[4] The development and implementation of liquid chromatography−mass spectrometry (LC-MS) and LC-MS/MS techniques have been crucial in expanding knowledge of DSP production and analyzing new derivatives that cannot be detected directly by the usual Lee's procedure.[5] Taking into account the necessity of reference material, structural research into novel derivatives is a key step toward controlling red tides using the new equipment available for marine biotoxin monitoring.

Recently, we published a study of a new OA derivative, DTX-6 (**2**), obtained from artificial cultures of the strain PLV2 of *P. lima*.[6] Complementing this work, we are now reporting the isolation of OA methyl ester (**3**) and norokadanone (**4**) as natural metabolites, as well as a new compound, **5**, from cultures of the same strain. These structures were determined on the basis of their spectroscopic data.

The first compound was easily identified as okadaic acid methyl ester (**3**) by comparing the spectral data of OA (**1**). The amount of compound **3** obtained and the fact that it was not found in similar previous procedures suggest that it may be an algal metabolite.[7]

Norokadanone (**4**) was isolated as a white amorphous powder; $[\alpha]^{25}_{D}$ +9.8 (*c* 0.06, $CHCl_3$). Its molecular formula was established as $C_{43}H_{66}O_{11}$ from the HRMS ion at *m*/*z* 758.4610 (calcd, 758.4605), which agrees with the presence of an OA nor derivative. The absence of the carboxyl function and the presence of a ketone moiety in the molecule were suggested by a strong band at 1715 cm^{-1} in the IR spectrum and confirmed by the ^{13}C NMR spectrum (Table 1) at carbon C-2 (δ_C 207.2) on the following considerations. Comparison of the ^1H NMR for this compound with **3** showed the presence of a methyl group centered at δ_H 2.15 (H_3-1) and no signal for H_3-44. Also, the signals for protons H_2-3 and H-4 were remarkably different, in **3** centered at δ_H 1.68/2.05 and 3.93, but in compound **4** at δ_H 2.42/2.62 and 4.25, respectively. Furthermore, in the HMBC experiment, the quaternary carbon centered at δ_C 207.2 (C-2) was correlated with those proton signals, defining the structure for the fragment C-1→C-4 as CH_3−CO−CH_2−HCO−. This compound has been chemically semisynthesized from OA, but, as far as we are aware, this is the first report of its natural occurrence.[8]



During the biosynthetic studies of the oxygen source of okadaic acid, Murata et al.[9,10] had observed prominent incorporation from $^{18}O_2$ at C-2, but not from $H_2{}^{18}O$, while C-1-O_2 was enriched from $H_2{}^{18}O$ as expected. The isolation of norokadanone (**4**) reinforces this hypothesis, suggesting a possible biosynthetic pathway. According to Figure 1, oxygen is probably introduced by P450 enzymes at C-2 and norokadanone (**4**) is an intermediate in the biosynthesis of okadaic acid (**1**).

Compound **5**, $[\alpha]^{25}_{D}$ −15 (*c* 0.02, $CHCl_3$), proved to be an OA diol ester derivative closely related with one previously reported in our laboratory.[3,6,11] In addition to the proton signals for OA (**1**), the NMR data for **5** showed the presence of three methylene groups. The moiety showed signals for an A−B system centered at δ_H 4.72 and 4.83 (J = 13.0 Hz), an olefinic methylene at δ_H 5.19 and 5.25, and, finally, a peak at δ_H 4.15 due to a methylene branched to a hydroxy

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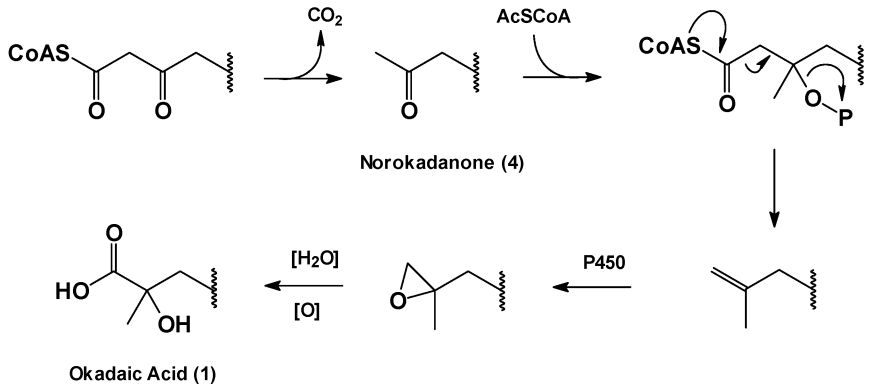

Figure 1. Norokadanone (**4**) as possible biosynthetic intermediate of OA (**1**).

Table 1. ^{13}C and ^{1}H NMR Chemical Shift Data ($CDCl_3$) for Compounds **3**, **4**, and **5**

	OA-methyl ester (**3**)		norokadanone (**4**)		**5**	
carbon	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H
1	176.9		30.9	2.15	176.0	
2	75.9		207.2		75.8	
3	43.9	1.68/2.05	49.1	2.42/2.62	44.2	1.71/2.02
4	68.7	3.93	65.8	4.25	68.7	3.97
5	31.6	1.37/1.70	30.2	1.25/1.36	31.9	1.36/1.75
6	27.3	1.82/1.97	27.3	1.73/1.87	27.2	1.85/2.02
7	71.5	3.36	71.7	3.39	72.1	3.38
8	96.0		95.8		95.9	
9	121.6	5.31	122.9	5.34	122.0	5.32
10	138.6		138.7		138.5	
11	32.9	1.85/1.88	33.0	1.87/1.92	32.9	1.86/1.89
12	70.9	3.57	71.2	3.69	70.8	3.64
13	41.8	2.26	41.9	2.31	42.0	2.30
14	135.4	5.56	136.9	5.76	135.9	5.58
15	130.5	5.47	131.5	5.51	131.2	5.48
16	78.9	4.46	79.7	4.52	79.2	4.48
17	30.6	1.58/2.13	30.7	1.63/2.19	30.2	1.58/2.17
18	37.1	1.86/2.04	37.5	1.88/2.01	37.3	1.86/2.03
19	105.7		105.5		106.0	
20	31.5	1.28/1.42	32.2	1.36/1.53	32.0	1.26/1.40
21	26.4	1.80/1.87	26.9	1.84/1.90	26.5	1.82/1.89
22	69.5	3.58	70.3	3.57	69.4	3.59
23	75.5	3.39	77.1	3.45	76.8	3.42
24	70.8	4.10	70.7	4.11	71.2	4.11
25	143.9		143.1		143.8	
26	84.8	3.92	85.1	3.91	85.0	3.94
27	64.5	4.06	64.7	4.07	64.9	4.06
28	35.2	0.97/1.43	35.6	0.98/1.32	35.2	0.96/1.31
29	31.0	1.91	31.1	1.96	31.3	1.95
30	74.9	3.27	75.4	3.28	75.2	3.27
31	27.2	1.79	27.6	1.80	27.4	1.81
32	27.3	1.91/1.97	26.5	1.82/1.92	26.0	1.92/1.97
33	30.3	1.34/1.52	30.6	1.34/1.52	30.2	1.36/1.56
34	95.5		95.5		96.0	
35	35.8	1.31/1.48	36.4	1.31/1.46	36.0	1.31/1.47
36	18.7	1.39/1.61	23.5	1.48/1.65	20.2	1.45/1.65
37	25.4	1.44/1.51	25.0	1.58/1.82	25.2	1.51/1.60
38	60.3	3.54/3.64	61.0	3.56/3.66	60.3	3.56/3.66
39	10.6	0.90	10.8	0.92	11.0	0.91
40	16.1	1.04	16.2	1.06	16.1	1.05
41	112.4	5.04/5.38	113.0	5.02/5.35	112.4	5.04/5.35
42	15.8	1.01	16.2	1.05	16.1	1.03
43	22.9	1.71	22.6	1.75	23.0	1.73
44	27.6	1.34			27.3	1.39
1′	52.7	3.79			75.6	4.72/4.83
2′					142.1	
3′					63.9	4.15
4′					114.6	5.19/5.25

group. These proton signals were correlated in the HSQC experiment with the carbons centered at δ_C 75.6 (C-1′), 114.6 (C-4′), and 63.9 (C-3′), respectively. The structure for the ester chain in compound **5** was completed by an HMBC experiment whereby carbon C-1 (δ_C 176.0) was correlated with protons H-1′ at δ_H 4.72 and 4.83, and a new quaternary carbon centered at δ_C 142.1 (C-2′) showed correlations

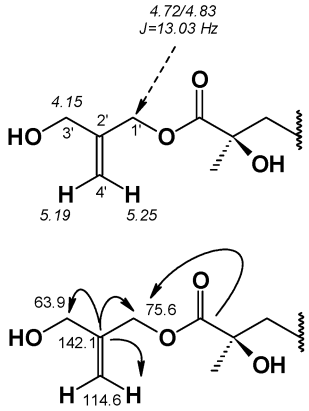

Figure 2. HMBC correlations and chemical shifts for the ester chain in compound **5.**

with all the protons present in the ester chain (Figure 2). The correlations observed in the ROESY spectrum for **5** are, moreover, in agreement with a stereochemistry identical to OA.

Experimental Section

General Experimental Procedures. Optical rotation was determined on a Perkin-Elmer 241 polarimeter. The IR spectrum was measured on a Bruker IFS55 spectrometer. The NMR spectra were obtained with a Bruker Avance 500 MHz instrument. Chemical shifts are reported relative to TMS, and coupling constants are given in Hz. HRMS was taken on a VG AutoSpec Fison spectrometer. HPLC was carried out with a Shimadzu system equipped with a differential diffractometer detector. Si gel CC and TLC were performed on Si gel Merck 60 G. TLC plates were visualized by spraying with phosphomolibdic acid 20 wt % in ethanol solution and heating.

Culture. Cultures of the PLV2 strain of the dinoflagellate *Prorocentrum lima* were obtained by inoculating 18 × 80 L tanks, each of which contained 40 L of Guillard K medium, with 5 L of *P. lima* culture grown and incubated under constant white fluorescent illumination at 25 °C for three weeks.

Extraction and Isolation. *P. lima* cells were harvested by continuous centrifugation at 7000 rpm, sonicated, and extracted with acetone. The solvent was evaporated, and the resultant extract was chromatographed by gel filtration on a Sephadex LH-20 column eluted with a mixture of $CHCl_3$–MeOH–*n*-Hex (1:1:2) and over a medium-pressure reversed-phase Lobar LiChroprep RP-18 column with MeOH–H_2O (85:15). Final purification of the compounds was achieved on a μ-Bondapak C18 HPLC column using isocratic elution [MeOH–H_2O (85:15)] to afford the pure substances **3** (5.6 mg), **4** (1.7 mg), and **5** (0.9 mg).

OA Methyl ester (**3**)**:** white amorphous powder; $[\alpha]^{25}_{D}$ +10.0° (*c* 0.27, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3444, 2934, 1739, 1681, 1454, 1382, 1236, 1182, and 1077 cm^{-1}; ^1H and ^{13}C NMR (Table 1); FAB HRMS *m*/*z* 841.4709 (calcd for $C_{45}H_{70}O_{13}$ + Na, 841.4714).

Norokadanone (**4**)**:** white amorphous powder; $[\alpha]^{25}_{D}$ +9.8° (*c* 0.06, $CHCl_3$); UV (EtOH) λ_{max} 242 and 270 nm; IR ($CHCl_3$) ν_{max} 3453, 2929, 1715, 1455, 1381, 1236, 1182, 1076, and 1045 cm^{-1}; ^1H and ^{13}C NMR (Table 1); FAB HRMS *m*/*z* 758.4610 (calcd for $C_{43}H_{66}O_{11}$, 758.4605).

Compound 5: white amorphous powder; $[\alpha]^{25}_{D}$ −15° (*c* 0.02, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3428, 2924, 2852, 2360, 2341, 1739, 1462, 1379, 1261, 1236, 1212, 1182, 1159, 1112, 1078, and 1045 cm^{-1}; ^{13}C and ^1H NMR (Table 1); FAB HRMS *m*/*z* 874.5156 (calcd for $C_{48}H_{74}O_{14}$, 874.5078).

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