New Targets in Diarrhetic Shellfish Poisoning Control

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The toxic profile of dinoflagellates varies even among identical species, raising an interesting question about the mechanism of toxin regulation and production. In consequence, it also poses a considerable problem in their control methods. In this paper, we report on the isolation and structural elucidation of several new ester derivatives of okadaic acid (OA) from artificial cultures of the genus *Prorocentrum*. These new compounds enlarge the range of target molecules that must be considered in the monitoring programs.

Since 1982, when the diarrhetic shellfish poisoning (DSP) toxins were first described,[1] they have been the focus of scientists' attention, not only for their public health and economic impacts but also for establishing the true origin of toxin production. Structure elucidation of minor new toxins is important in understanding the molecular basis of their mechanism, which is critical to their eventual therapeutic application. It also aids in detection in contaminated samples and in determination of their biosynthetic pathway.[2−4] Obtaining these toxins from artificial sources will expand their use in pharmacology. By using cultures of the dinoflagellates *Prorocentrum lima* and *Prorocentrum maculosum*, interesting biosynthetic experiments have been carried out and several new water-soluble or diol-ester derivatives have been isolated.[5]

Monitoring of DSP toxins needs to be revised in the light of the new metabolites. HPLC fluorometric analysis cannot detect directly the ester derivatives of OA; tandem HPLC-MS seems to be more effective. The identification of new structural variations is fundamental in the development and optimization of this technique.[6,7]

Recently, we described from *P. lima*, besides OA (**1**) and DTX1 (**2**), the isolation and identification of compounds **3**−**8** (Figure 1).[8−11] This paper reports on the isolation from artificial laboratory cultures, together with the OA diol-ester **9** previously obtained by Hu et al.[12] and Suzuki et al.,[7] of the new toxins **10**−**12** of *P. lima*, as well as OA (**1**) and the new metabolite **13** from *P. belizeanum*. Their structures were determined by the interpretation of their spectroscopic data.

Artificial cultures of microalgae were carried out by inoculating 80 L tanks, each containing 40 L of Guillard K medium with 5 L of *P. lima* (PL2V strain) or *P. belizeanum* culture grown and incubated under white fluorescent illumination at 25 °C for 3 weeks up to final volume of 700 L for *P. lima* and 560 L for *P. belizeanum*. Cells were harvested by centrifugation, sonicated, and extracted with acetone. The resultant extracts were successively chromatographed as described in the Experimental Section, yielding the new pure compounds **10**−**12** from *P. lima* and **13** from *P. belizeanum*.

Comparison of the spectroscopic data for compound **10** with those of the previously reported **5** showed some differences at the side chain, where new signals are exhibited for a double bond centered at δ_H 5.90 (d, *J* = 16.2
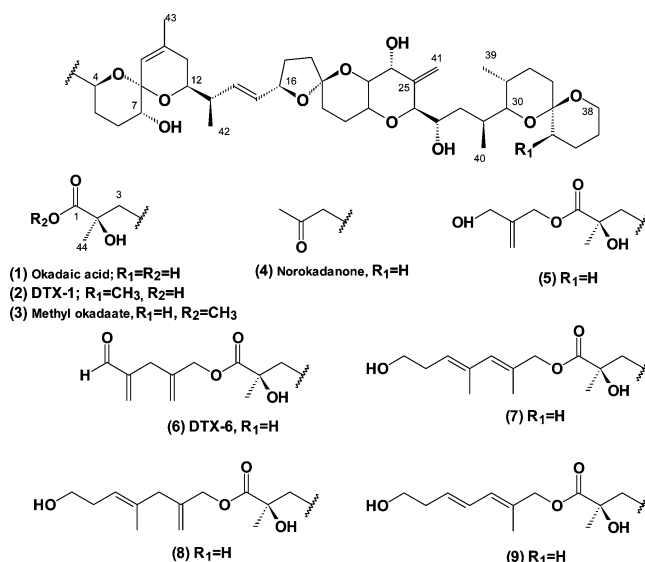
Figure 1. OA derivatives previously isolated from dinoflagellates of the genus *Prorocentrum*.

Hz) and 5.85 (dd, *J* = 4.5 and 16.2 Hz), which were correlated in the HSQC experiment with the carbon signals centered at δ_C 130.3 and 132.8. These signals were assigned to C-3′ and C-4′ in accordance with the following correlations. The protons H$_2$-1′ at δ_H 5.09 and 4.54, previously identified by their HMBC correlations with the ester carbon signal C-1 (δ_C 176.1), were also correlated in this experiment with the olefinic methylene group C-6′ (δ_C 115.7) and the quaternary olefin carbon C-2′ (δ_C 149.9), which, in turn, was correlated with the new proton signal at δ_H 5.90 (H-3′). These correlations define a conjugated diene moiety that ended at an allylic hydroxy-methylene group (Figure 2). The stereochemistry of the C-3′−C-4′ double bond was proposed as *E* on the basis of the coupling constants and the lack of correlation in the ROESY experiment between the respective protons.

Compound **11** showed an ester side chain closely related with that of DTX6 (**6**). Thus, a detailed comparative analysis of their proton and carbon NMR spectra showed that fragment C-1′→C-4′ was identical in both compounds, while the α,β-unsaturated aldehyde in **6** was substituted by an allylic hydroxyl methine centered at δ_H 4.36 (H-5′) in **11**. This proton was correlated with two methylenes centered at δ_H 1.82/1.61 and 3.84/3.78 through its selective irradiation in the TOCSY experiment, thus defining a
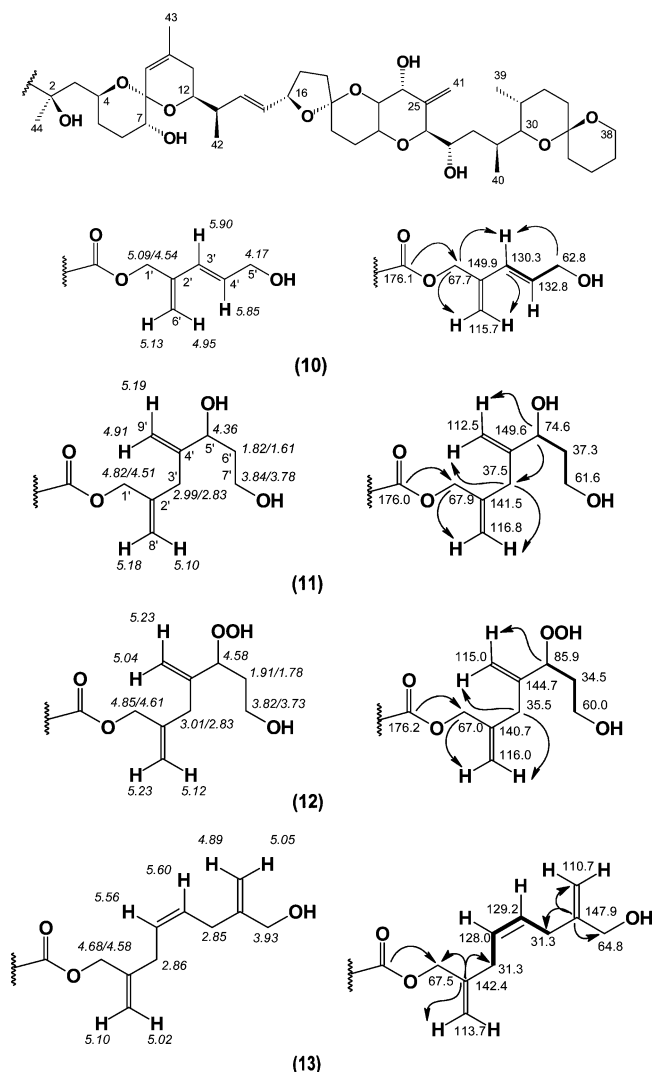
Figure 2. Chemical shift data and significant HMBC correlations (arrows) for the ester side chain in compounds **10**, **11**, and **12** from *P. lima* and **13** from *P. belizeanum*. Fragments obtained from COSY/TOCSY [bold lines].

−CH(OH)−CH₂−CH₂OH fragment. The most significant HMBC correlations are shown in Figure 2, emphasizing those between the carbon C-5′ (δ_C 74.6) and the protons H₂-3′ (δ_H 2.99 and 2.83) and H₂-9′ (δ_H 5.19 and 4.91) that fixed the position of the new fragment in the ester chain.

The comparison of NMR spectral data of compound **11** with those of the new compound **12** showed that the differences between them were located around carbon C-5′. Thus, in the ¹H NMR spectrum of compound **11**, the proton signals H-5′ and H₂-6′ were centered at δ_H 4.36 and 1.82/1.61, while in that of compound **12** these signals were centered at δ_H 4.58 and 1.91/1.78, respectively. Moreover, in the ¹³C NMR spectrum a significant change was observed in the chemical shift of the carbon bearing an oxygen atom at C-5′, which shifted from δ_C 74.6 in compound **11** to δ_C 85.9 in **12**. These data in conjunction with the molecular ion observed in its HRMS suggested the presence of a hydroperoxyl group at carbon C-5′ in compound **12**, as shown in Figure 2.

DTX6 (**6**) and compounds **11** and **12** could be considered to be biogenetically derived from the OA diol-ester **8**, previously isolated from this strain of *P. lima*. Thus, compound **8** is converted into the hydroperoxide derivative **12** by oxidation at C-5′. This intermediate could evolve to
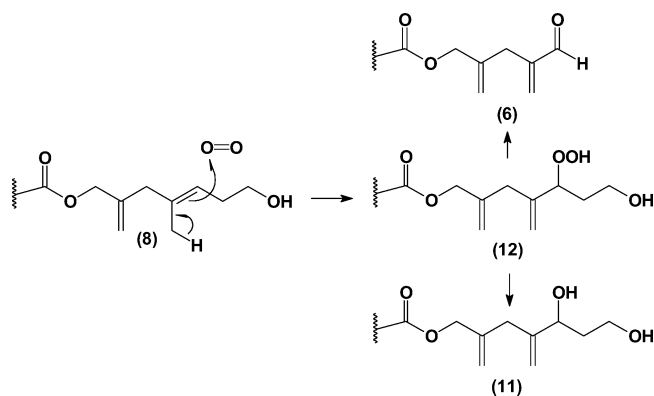
Figure 3. Proposed biogenetic relationship between OA derivatives in *P. lima* (PL2V strain).

the formation of DTX6 (**6**) by oxidative fragmentation or to the diol **11** by reduction, as is shown in Figure 3.

From the acetonic extract of *P. belizeanum* cultures was isolated, together with a significant amount of OA, the new diol-ester **13**. This toxin was isolated as an amorphous white solid, and its molecular formula was established as $C_{54}H_{82}O_{14}$ on the basis of the FABMS spectrum. The ¹H NMR spectrum clearly resembled those of the previous esters of OA identified in *P. lima*, showing, in addition to the proton signals corresponding to the OA backbone, 14 new signals, which were correlated with the corresponding carbon signals in the HSQC experiment. There were two olefinic signals at δ_H 5.56 (δ_C 128.0) and 5.60 (129.2) for a Z double bond (*J* = 8.0 Hz); an AB system centered at δ_H 4.68 and 4.58 (δ_C 67.5), together with a broad singlet at δ_H 3.93 (δ_C 64.8) both due to methylene groups branched to an oxygen atom; two isolated signals at δ_H 2.86 (δ_C 31.3) and 2.85 (δ_C 31.3) assignable to two allylic methylenes; and finally, two olefinic methylenes centered at δ_H 5.10/5.02 and 5.05/4.89 (δ_C 113.7 and 110.7). Using the HMBC correlations shown in Figure 2, those signals were identified as the olefinic methines H-4′ and H-5′ and the methylenes H₂-1′, H₂-8′, H₂-3′, H₂-6′, H₂-9′, and H₂-10′, respectively, thus establishing the structure of the ester chain fragment.

The number of structures of toxin derivatives involved in the DSP syndrome has markedly increased in the past decade, and the need to prevent or minimize this public health hazard implies the necessity to have an effective monitoring system whose efficiency will be based on real knowledge of the target toxins. In this case, since MS became the most valuable technique for seafood control, drastically reducing the number of samples required for analysis and avoiding the use of animals, the elucidation of the new structures involved in the toxin mixtures constitutes considerable progress in the determination of the substances involved in a specific toxic event.

Experimental Section

Table 1. ^{13}C and ^1H NMR Chemical Shift Data (CDCl$_3$) for Compounds **10**−**13**

carbon	10 δ ^{13}C	10 δ ^1H	11 δ ^{13}C	11 δ ^1H	12 δ ^{13}C	12 δ ^1H	13 δ ^{13}C	13 δ ^1H
1	176.1		176.0		176.2		176.3	
2	76.0		75.5		75.6		75.8	
3	44.0	2.10/1.70	43.9	2.06/1.69	44.0	2.07/1.68	44.2	2.03/1.70
4	68.4	3.98	69.0	3.97	68.6	3.99	68.7	3.99
5	32.0	1.75/1.38	31.9	1.71/1.34	31.7	1.73/1.34	32.0	1.72/1.35
6	26.5	2.10/1.85	27.3	1.83/1.68	26.4	2.01/1.86	27.7	1.82/1.78
7	71.8	3.39	71.6	3.39	71.8	3.39	72.0	3.39
8	96.1		95.9		96.5		71.6	
9	121.8	5.38	122.1	5.32	121.8	5.33	122.3	5.34
10	139.0		138.7		138.8		138.7	
11	32.9	1.88/1.86	33.1	1.94/1.89	33.0	1.90/1.85	33.3	1.88/1.86
12	71.1	3.53	71.6	3.49	71.0	3.50	71.2	3.57
13	42.2	2.26	42.2	2.27	42.0	2.28	42.3	2.28
14	136.0	5.52	136.6	5.50	135.9	5.51	135.7	5.54
15	131.3	5.49	131.3	5.47	131.1	5.47	131.3	5.51
16	79.1	4.50	79.4	4.47	79.1	4.43	79.5	4.48
17	30.5	2.22/1.58	30.8	2.17/1.60	30.8	2.17/1.59	31.0	2.16/1.60
18	37.1	2.04/1.88	37.7	2.09/1.87	37.3	2.05/1.87	37.3	2.05/1.87
19	106.0		105.2		105.8		106.0	
20	32.2	1.42/1.38	32.2	1.41/1.32	31.8	1.35/1.26	30.0	1.42/1.37
21	26.8	1.85/1.76	27.0	1.89/1.82	27.3	1.83/1.78	28.0	1.81/1.74
22	69.9	3.59	70.2	3.59	69.8	3.56	70.3	3.60
23	76.2	3.42	76.9	3.43	76.7	3.43	77.4	3.43
24	71.2	4.11	71.3	4.11	71.0	4.15	71.3	4.12
25	144.0		144.8		144.5		143.9	
26	84.7	3.95	85.1	3.95	84.8	3.96	85.3	3.94
27	64.4	4.06	64.9	4.07	64.6	4.08	66.3	4.05
28	35.1	1.35/0.98	35.3	1.30/0.98	35.1	1.32/0.97	35.5	1.33/0.97
29	31.2	1.91	31.8	1.97	31.2	1.95	31.5	1.94
30	75.0	3.29	75.1	3.29	75.1	3.30	75.2	3.30
31	27.2	1.81	27.6	1.79	27.5	1.80	28.0	1.80
32	26.2	1.99/1.86	26.8	2.00/1.90	26.4	2.01/1.86	27.0	2.02/1.87
33	30.4	1.53/1.38	30.7	1.53/1.39	30.3	1.57/1.40	31.0	1.54/1.40
34	95.6		95.0		95.4		96.3	
35	35.9	1.65/1.46	35.9	1.63/1.46	36.0	1.65/1.44	36.2	1.65/1.45
36	18.7	1.89/1.54	19.0	1.91/1.53	18.8	1.90/1.58	19.0	1.88/1.55
37	25.6	1.57/1.50	25.0	1.57/1.43	25.3	1.56/1.49	25.3	1.56/1.53
38	60.7	3.66/3.56	60.5	3.66/3.56	60.3	3.66/3.57	60.7	3.68/3.55
39	10.9	0.92	10.9	0.92	10.8	0.93	11.0	0.92
40	16.1	1.05	16.1	1.05	16.2	1.07	16.5	1.06
41	112.7	5.35/5.06	113.4	5.32/5.05	112.4	5.35/5.07	112.7	5.37/5.05
42	15.8	1.01	15.9	1.05	15.8	1.02	16.2	1.02
43	23.0	1.74	23.5	1.74	23.1	1.75	23.2	1.73
44	28.1	1.41	27.9	1.38	27.6	1.40	27.8	1.41
1′	67.7	5.09/4.54	67.9	4.82/4.51	67.0	4.85/4.61	67.5	4.68/4.58
2′	149.9		141.5		140.7		142.4	
3′	130.3	5.90	37.5	2.99/2.83	35.5	3.01/2.83	31.3	2.86
4′	132.8	5.85	149.6		144.7		128.0	5.56
5′	62.8	4.17(2H)	74.6	4.36	85.9	4.58	129.2	5.60
6′	115.7	5.13/4.95	37.3	1.82/1.61	34.5	1.91/1.78	31.3	2.85
7′			61.6	3.84/3.78	60.0	3.82/3.73	147.9	
8′			116.8	5.18/5.10	116.0	5.23/5.12	64.8	3.93(2H)
9′			112.5	5.19/4.91	115.0	5.23/5.04	113.7	5.10/5.02
10′							110.7	5.05/4.89

diffractometer detector. TLC were performed on Si gel Merck 60 G. TLC plates were visualized by spraying with H$_2$SO$_4$/H$_2$O/AcOH (1:4:20) and heating.

Culture. Cultures of the dinoflagellate *P. lima* (PL2V) and *P. belizeanum* were carried out by inoculating 80 L tanks, each containing 40 L of a Guillard K medium with 5 L of the respective microalgal culture grown and incubated under constant white fluorescent illumination at 25 °C for 3 weeks up to final volumes of 700 and 560 L, respectively.

Extraction and Isolation. Both batch culture cells were separately harvested by continuous centrifugation at 7000 rpm, sonicated, and extracted with acetone. After solvent evaporation, the resultant extracts were both successively chromatographed under identical conditions by gel filtration on a Sephadex LH-20 column eluted with a mixture of CHCl$_3$/MeOH/*n*-hexane (1:1:2) and over a medium-pressure reversed-phase Lobar LiChroprep RP-18 column with MeOH/H$_2$O (17:3). Final purification of compounds was achieved on a

μ-Bondapak C-18 HPLC column using a gradient solvent system, A = CH$_3$CN/H$_2$O/AcOH (50:50:0.1), B = CH$_3$CN/AcOH (100:0.1), 1 h, followed by an isocratic elution, MeOH/H$_2$O (17:3), with *P. lima* yielding OA (**1**) (80.0 mg), DTX1 (**2**) (6.0 mg), **3** (2.7 mg), norokadanone (**4**) (0.6 mg), **5** (0.2 mg), DTX6 (**6**) (1.3 mg), **7** (14.0 mg), **8** (0.6 mg), **9** (1.2 mg), and pure new substances **10** (0.4 mg), **11** (1.0 mg), and **12** (0.6 mg) and *P. belizeanum* affording OA (**1**) (14.1 mg) and **13** (3.2 mg).

Compound 10: white amorphous powder; [α]^{25}$_D$ +26.6° (*c* 0,04, CHCl$_3$); UV (EtOH) λ_{max} (log ϵ) 205 nm (4.19); IR (CHCl$_3$) ν_{max} 3418, 2956, 2854, 2360, 2341, 1732, 1670, 1455, 1380, 1236, 1215 cm^{-1}; ^1H and ^{13}C NMR (Table 1); FABHRMS *m/z* 923.5071 (calcd for C$_{50}$H$_{76}$O$_{14}$+Na, 923.5132).

Compound 11: white amorphous powder; [α]^{25}$_D$ +10° (*c* 0,10, CHCl$_3$); IR (CHCl$_3$) ν_{max} 3427, 2932, 1737, 1651, 1455, 1382, 1236, 1160, 1077 cm^{-1}; ^1H and ^{13}C NMR (Table 1); FABHRMS *m/z* 981.5489 (calcd for C$_{53}$H$_{82}$O$_{15}$+Na, 981.5551).

Compound 12: white amorphous powder; $[\alpha]^{25}_{D}$ +10° (c 0,06, CHCl₃); IR (CHCl₃) ν_{max} 3417, 2923, 2851, 2360, 2341, 1738, 1531, 1379, 1235 cm⁻¹; ¹H and ¹³C NMR (Table 1); FABHRMS m/z 997.5438 (calcd for $C_{53}H_{82}O_{16}$+Na, 997.5500).

Compound 13: white amorphous powder; $[\alpha]^{25}_{D}$ +8.4° (c 0.24, CHCl₃); IR (CHCl₃) ν_{max} 3443, 2924, 2360, 1732, 1682, 1456, 1076 cm⁻¹; ¹H and ¹³C NMR (Table 1); FAB HRMS m/z 977.5541 (calcd for 977.5602 $C_{54}H_{82}O_{14}$+Na).

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