Identification of DTX-4, a New Water-soluble Phosphatase Inhibitor from the Toxic Dinoflagellate *Prorocentrum lima*

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A water-soluble DSP toxin Dinophysistoxin-4 (DTX-4, 1) is isolated from a butanol-soluble fraction of a *P. lima* strain, and its structure characterized on the basis of spectral analysis and chemical reactions.

Some dinoflagellates belonging to the genera *Prorocentrum* and *Dinophysis* produce lipid-soluble polyether compounds responsible for diarrhetic shellfish poisoning (DSP).[1–5] Sporadic occurrence of these toxins has affected the shellfish industry globally[6] and interest in the toxins was further heightened by the discovery that they are phosphatase inhibitors affecting many cellular functions.[7] Related diol esters have also been isolated from DSP toxin-producing strains of *Prorocentrum*, but these compounds are not phosphatase inhibitors, suggesting that the free carboxy group is essential for this activity.[8]

An investigation of DSP toxins in various *Prorocentrum* species revealed that the butanol-soluble fraction of one *P. lima* strain[9] (isolated from Mahone Bay, Nova Scotia, Canada) gave positive results in the mouse bioassay and led to the isolation of a polar, water-soluble compound DTX-4 **1**, structurally related to the DSP toxin family, that was toxic in the mouse bioassay (LD_{50} 610 μg kg^{-1}, i.p.), and inhibited the phosphatases PP1 and PP2A.

Cultures of *P. lima* (200 g wet weight) were extracted with aqueous methanol and this extract was partitioned successively against hexane, diethyl ether and butanol. The known DSP toxins were identified in the ether fraction and traces of these lipid-soluble DSP toxins were also present in the butanol fraction. However, the DSP activity of the butanol fraction in the mouse bioassay was more than could be accounted for by known DSP toxins. Purification by a combination of normal phase and gel permeation chromatography yielded the purified toxin **1**, (14.0 mg) as a white solid.† The molecular formula $C_{66}H_{104}O_{30}S_3$ derived from HR-LSIMS experiments was supported by the observation of 66 carbon resonances in the ^{13}C NMR spectra. The presence of sulfate was suggested by the strong IR band at 1235 cm^{-1}, and the sequential loss of three sulfate groups in the LSIMS spectrum of **1**. The positive LSIMS spectrum of a sample saturated with Na^+ ions displayed peaks‡ at 1561 $(M - 3H + 4Na)^+$, 1459 $(M - 2H - SO_3 + 3 Na)^+$, 1357 $(M - H - 2SO_3 + 2 Na)^+$, and 1255 $(M - 3SO_3 + Na)^+$, corresponding to the sequential elimination of three sulfated groups in the sodium salt form. When the sample was saturated with K^+ ions, the LSIMS spectrum showed peaks at 1625 $(M - 3H + 4K)^+$, 1507 $(M - 2H - SO_3 + 3K)^+$, 1389 $(M - H - 2SO_3 + 2K)^+$, and 1271 $(M - 3SO_3 + K)^+$ corresponding to the potassium salt form. The negative ion LSIMS spectrum (without added metal ions) showed two major clusters of peaks at m/z 1509 $(M - 2H + K)^-$ and 1311 $(M - H - 2SO_3)$.$^-$.

The ^1H and ^{13}C NMR data (Table 1) of DTX-4 **1** were similar to the corresponding NMR data of the co-metabolites[8] okadaic acid **2** and its diol ester **3**. Indeed, alkaline hydrolysis (0.1 mol dm^{-3} NaOH) of **1** yielded okadaic acid **2** and methanolysis (0.1 mol dm^{-3} HCl in absolute MeOH) yielded the diol ester **3**. Comparison of the ^1H and ^{13}C NMR spectroscopic data of **1** with those of **3** showed fourteen extra carbons in **1**, of which thirteen were protonated. The TOCSY spectrum indicated that all protons attached to these thirteen protonated carbons were in the same spin system, and the connectivity of these fourteen carbons was obtained through analysis of the COSY and HMBC spectra (Fig. 1). The ester linkage of this additional 14 carbon moiety to the diol ester of okadaic acid was revealed by the shift of H-51 from δ 3.69 in **3** to δ 4.12 in **1** and was confirmed by the HMBC correlation between H-51 (δ 4.12) and C53 (δ 173.8). The *Z* configuration of the double bond at C55–C56 was established from the NOESY correlations between H-54 (δ 3.12) and H-57 (δ 2.20, 2.13) as well as between H-55 (δ 5.52) and H-56 (δ 5.59) (Fig. 1).

From the NMR data and solvolysis reactions it was concluded that the three sulfate groups of **1** were located on the additional fourteen-carbon moiety. Information on their position was obtained from deuterium-induced upfield ^{13}C chemical shifts upon changing the NMR solvent from CD_3OH to CD_3OD. Such shifts are found for carbons bearing an hydroxy group[10–12] and these were observed for all hydroxy-bearing carbons in the diol ester portion of **1** ($\Delta\delta_C$ *ca* 0.1 ppm). In the additional acyl portion of the molecule, the upfield shift of the C59 resonance ($\Delta\delta_C$ 0.116 ppm) was consistent with a hydroxy group at this position, whereas the C64 resonance was

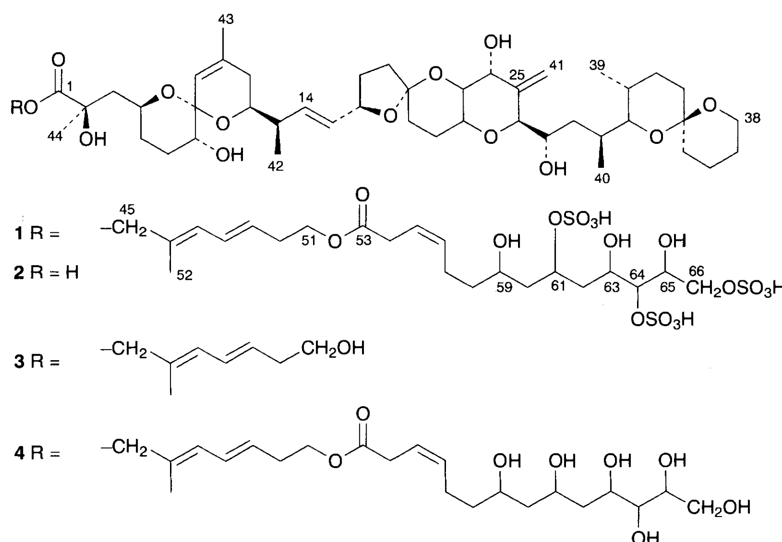

Table 1 Selected NMR data for DTX-4 **1**[a]

C	δC	δH	C	δC	δH	C	δC	δH
45	71.1 (t)	4.56	53	173.8 (s)		61	76.2 (d)	4.77
46	132.1 (s)		54	33.8 (t)	3.12	62	39.6 (t)	1.78 2.13
47	128.8 (d)	6.07	55	122.4 (d)	5.52	63	68.5 (d)	4.34
48	129.4 (d)	6.39	56	134.0 (d)	5.59	64	82.0 (d)	4.37
49	131.7 (d)	5.70	57	24.7 (t)	2.20 2.13	65	71.7 (d)	4.17
50	33.3 (t)	2.47	58	38.3 (t)	1.47 1.60	66	70.4 (t)	4.23 4.43
51	65.1 (d)	4.12	59	69.1 (d)	3.78			
52	14.6 (q)	1.78	60	44.4 (t)	1.74 2.01			

[a] For δ_C ppm, (s) = C, (d) = CH, (t) = CH_2, (q) = CH_3. Sample in CD_3OD. The other NMR data for positions 1–44 are essentially identical with those for okadaic acid recorded in CD_3OD.

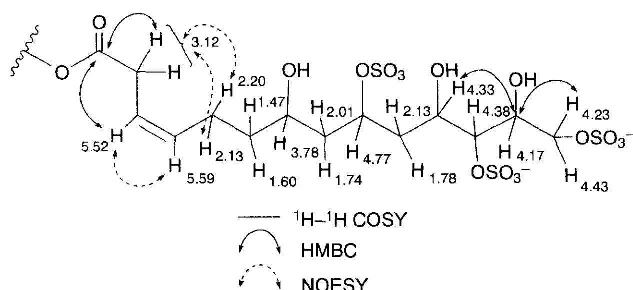

Fig. 1 Partial structure of DTX-4, showing ¹H–¹H COSY, HMBC and NOESY correlations

unchanged and that for C61 virtually unperturbed ($\Delta\delta_C$ 0.007 ppm), indicating that both these positions were sulfated. The location of the third sulfate group on one of the remaining three contiguous oxygen bearing positions C63, C65, and C66, could not be determined by this approach, due to the magnitude and similarity of the isotope shifts ($\Delta\delta_C$ 0.063, 0.045 and 0.066 ppm, respectively). However, comparison of the chemical shift data (δ_H 4.23 and 4.43; δ_C 70.4) for position 66 with known similar compounds,[13–15] strongly suggested that the terminal secondary alcohol group was sulfated. Further proof was obtained from analysis of the desulfated product **4**.§ The positive LSIMS and IR data confirmed that all three sulfate groups had been removed. In the ^{13}C NMR spectrum of **4**, resonances of all the previously sulfated positions C61, C64 and C66, were shifted upfield ($\Delta\delta_C$ 7.7, 6.5 and 5.3 ppm, respectively) compared with those of **1**, consistent with loss of sulfate at these positions.[16] Moreover, a slight downfield shift was observed for C59 and C65 ($\Delta\delta_C$ 1.63 and 1.70 ppm), as well as a very small shielding effect for C63 ($\Delta\delta_C$ 0.2 ppm). Similar comparison of the ^1H NMR data revealed shielding of H-61, H-64 and H-66 ($\Delta\delta_H$ 0.76, 1.06 and 0.65 ppm, respectively) in **4**, once again consistent with loss of sulfate at these positions,[13] and weaker secondary shielding effect on H-59, H-63 and H-65 ($\Delta\delta_H$ 0.01, 0.24 and 0.49 ppm, respectively). These combined data led us to assign the three sulfate groups to C61, C64 and C66.

The discovery of DTX-4 **1** represents the first polar DSP toxin that is only sparingly soluble in chloroform when partitioned between chloroform and water. Consequently, it is not possible to accurately quantify **1** under the usual DSP extraction and chemical analytical procedures which use chloroform and the ADAM fluorescent derivative.[17] Additional procedures will have to be developed for the analysis of **1** in microalgae and shellfish.

It is surprising that **1** acts as a phosphatase inhibitor at all. Previous work had indicated that the free carboxyl group in **2** is necessary for phosphatase inhibition activity, and that the ester **3** is inactive in the enzyme assay.[8] Both **1** and the desulfated product **4**, which are built upon the diol ester structure, are toxic in the mouse bioassay and like okadaic acid both inhibit PP2A more effectively than PP1, though they are 10–50 fold less potent than the free acid.

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Footnotes

† Compound **1**, mp >300 °C (decomp.); $[\alpha]_D^{26}$ −12.0 (*c* 0.10, MeOH); UV λ_{max}/nm (MeCN) 230; IR υ_{max}/cm^{-1} 3440, 2938, 1737, 1235, 1078, 999; HR-LSIMS $C_{66}H_{104}O_{30}S_3$ [$(MNa_4 - 3H)^+$ 1561.5085, calc. 1561.5131]. The presence of phosphate was eliminated by the failure to observe ^{31}P resonances in NMR spectra.

‡ LSIMS numbers refer to the *m/z* value of the first isotope in each cluster.

§ Desulfation reaction: DTX-4 **1** (1.0 mg) was heated at 120 °C in dioxane/pyridine for 2.5 h. The reaction mixture was dried in a stream of nitrogen, the residue redissolved in methanol, and purified by HPLC (CSC, ODS2 column, eluting with methanol–water 82 : 18, UV det. 210 nm) to give the desulfated product **4**: IR υ_{max}/cm^{-1} 3391, 2937, 1736, 1076, 1003 (the strong peak of 1235 cm^{-1} in **1** disappeared); positive LSIMS, 1233 and 1255; ^{13}C NMR (CD_3OD, 125 MHz, reported for carbons from C53 to C66 only) δ 173.8 (C), 33.7 (CH_2), 122.3 (CH), 134.0 (CH), 24.5 (CH_2), 38.1 (CH_2), 70.7 (CH), 45.9 (CH_2), 68.5 (CH), 42.5 (CH_2), 68.3 (CH), 75.5 (CH), 73.3 (CH) and 65.1 (CH_2); ^1H NMR (CD_3OD, 500 MHz) δ 3.12 (2H, H-54), 5.52 (1H, H-55), 5.58 (1H, H-56), 2.17 (2H, H-57), 1.47, 1.55 (2H, H-58), 3.78 (1H, H-59), 1.60, 1.64 (2H, H-60), 4.01 (1H, H-61), 1.46, 1.82 (2H, H-62), 4.10 (1H, H-63), 3.31 (1H, H-64), 3.68 (1H, H-65), 3.59, 3.78 (2H, H-66).

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