Isolation of a New Diarrhetic Shellfish Poison from Irish Mussels

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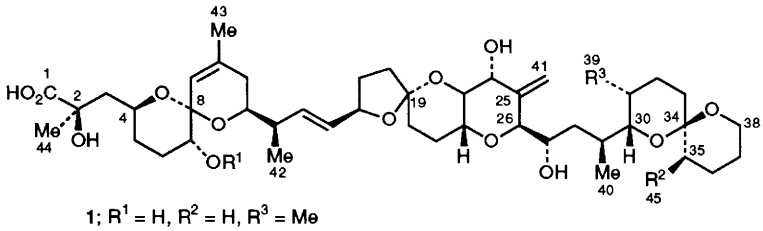
A new marine toxin dinophysistoxin-2 (DTX-2) **4**, isolated from toxic Irish mussels and biogenetically related to the toxins okadaic acid **1** and dinophysistoxin-1 (DTX-1) **2**, the principal agents responsible for diarrhetic shellfish poisoning (DSP), is reported.

Diarrhetic shellfish poisoning (DSP) has had a disastrous effect upon the shellfish industry in many parts of the world.[1] The DSP toxins are known to comprise a group of polyether metabolites **1**–**3**, produced by marine dinoflagellates belonging to the genera *Dinophysis* and *Prorocentrum*, that accumulate in the digestive glands of shellfish.[2] Recently, following the discovery that they are potent inhibitors of type 1 and type 2A protein phosphatases[3] scientific interest in these compounds has grown. During the course of routine monitoring for DSPs in mussels from Bantry Bay, Ireland, where *Dinophysis* spp. have occurred, DSP-activity in the standard rat bioassay[4] was observed. Reversed-phase HPLC–fluorescence detection analysis of the ADAM (9-anthranyldiazomethane)-derivatised[5] samples revealed a signal for okadaic



1; R^1 = H, R^2 = H, R^3 = Me
2; R^1 = H, R^2 = Me, R^3 = Me
3; R^1 = acyl, R^2 = Me, R^3 = Me
4; R^1 = H, R^2 = Me, R^3 = H

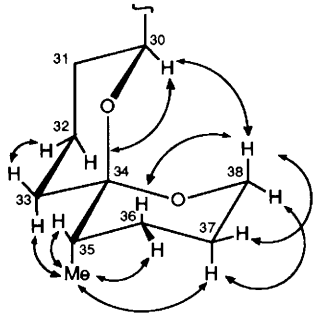


Fig. 1 Selected NOE connectivities for **4**

acid **1** (11.3 min), and another, unidentified, closely eluting peak (12.3 min) that did not correspond to DTX-1. Furthermore, the DSP activity in the rat bioassay was more than could be accounted for by okadaic acid alone. Examination of an underivatised extract of mussel digestive gland by a newly developed ion spray LC–MS method[6] indicated that this additional compound was an isomer of okadaic acid, and detailed NMR spectroscopic analysis of the isolated product has shown this to possess structure **4**. In keeping with the established nomenclature for these marine toxins we propose the name dinophysistoxin-2 (DTX-2) for this new addition to the group.† To facilitate comparison of structural and spectral data among the DSPs, we have adopted the numbering system from the X-ray study of **1**.[1]

Dinophysistoxin **2** was isolated as a colourless solid, m.p. 128–130 °C, $[\alpha]_D^{20}$ +15.49° ($CHCl_3$, *c* 0.213), from the digestive glands of toxic mussels (4.2 kg wet weight, yield: 8×10^{-5}%), after exhaustive extraction with methanol and acetone, followed by a combination of adsorption, size exclusion and reversed-phase chromatography. The high resolution fast atom bombardment (FAB)-MS data were consistent with an elemental composition of $C_{44}H_{68}O_{13}$ (MH^+ 805.4709, calc. 805.4738) confirming the compound to be an isomer of **1** with different $[\alpha]_D$ {**1**: $[\alpha]_D^{20}$ + 53.3° ($CHCl_3$, *c* 0.182)}.

The ^1H and ^{13}C NMR spectroscopic data for DTX-2 **4** revealed a strong resemblance to okadaic acid **1** and DTX-1 **2**. For example, the ^1H and ^{13}C chemical shifts for positions 1 to 26 in **4** were almost identical to the corresponding shifts in **1** and **2** (δ_H within 0.05 ppm, δ_C within 0.7 ppm). From position 27 to 30 some differences in chemical shifts were observed, however spin–spin coupling connectivity (^1H–^1H and ^1H–^{13}C) was the same in **1**, **2** and **4** up to C-30. Comparison of ^1H multiplets for these positions also confirmed that spin–spin coupling constants in the vicinity of chiral centres were identical up to C-30, and so the combined data established that **4** has the same molecular structure including relative stereochemistry, from C-1 to C-30, as **1** and **2**.

Table 1 Partial NMR chemical shift data for **1**, **2** and **4**[a]

	δ_C (ppm)			δ_H (ppm)		
Position	**1**	**2**	**4**	**1**	**2**	**4**
26	84.90	84.94	84.85	3.93	3.95	3.98
27	64.67	64.60	66.03	4.09	4.08	4.03
28	35.29	35.20	36.67	1.01	1.01	1.42
				1.32	1.32	1.35
29	31.11	31.18	34.62	1.93	1.91	1.87
30	76.08	74.79	73.30	3.28[b]	3.28[b]	3.47[b]
31	27.44	27.41	26.78	1.80	1.77	1.24
						1.50
32	26.38	26.45	18.98	1.38	1.41	1.59
				2.03	2.01	1.73
33	30.37	25.97	32.43	1.40	1.15	1.17
				1.58	1.92	1.69
34	95.62	97.86	98.08			
35	35.92	39.05	35.86	1.43	1.50	1.64
				1.65		
36	18.78	27.47	25.74	1.54	1.62	1.29
				1.88	1.42	2.13
37	25.46	26.36	19.96	1.53	1.50	1.25
					1.62	1.77
38	60.34	59.94	60.44	3.66	3.62	3.65
				3.57	3.53	3.54
39	10.72	10.74		0.93	0.91	
40	16.21	16.04	15.18	1.05[c]	1.01[c]	0.93[c]
45		16.78	14.40		0.92[d]	0.98[d]

[a] All samples dissolved in $CDCl_3$; ^1H NMR spectra recorded at 500 MHz; ^{13}C NMR spectra at 75.5 MHz or 125.8 MHz. Complete and satisfactory spectra were obtained for all compounds. Assignments were made by COSY, TOCSY, HETCOR, HMBC, NOESY and ROESY. [b] NOE to C-29 methyl, H_{eq}-31, H_{ax}-32 and H_{ax}-38. [c] NOE to H-27, H-29 and H-30; NOE observed to H_{ax}-38 in **1** and **2** only. [d] NOE to H_{eq}-33, H-35, H_{eq}-36 and H_{ax}-37.

The TOCSY spectra of **1**, **2** and **4** each demonstrated five distinct ^1H spin systems, corresponding to protons at positions 3 to 7; 9 to 18 plus 42 and 43; 20 to 24 plus 41; 26 to 33 plus 39 and 40 (in **1** and **2**); and 35 to 38 plus 45 (in **2**, **3** and **4**). In the spin system that includes positions 26 to 33, the methyl group attached to C-31 was missing in **4**. Instead, the 2D COSY data showed that H-30 was coupled to a methylene group resonating at δ 1.24 and 1.50 and to a methine proton at δ 1.87. Furthermore, the HMBC data of **1** and **2** revealed a long range ^1H–^{13}C coupling between the protons of the C-39 methyl group and C-30 that is not present in the HMBC data of **4**.

Significantly, the remaining spin system (positions 35 to 38) of **4** displayed features that resembled the comparable system in **2** (Table 1). Furthermore, a common feature of the spectra of all three compounds is the characteristic low field methylene signals assigned to position 38, and from here it was

† The term DTX-2 was originally used by Professor Yasumoto to describe an unknown toxin from mussel tissue. Later this compound was identified as yessotoxin which is not a DSP derivative. Consequently, we propose, with Professor Yasumoto's approval, to utilise the term for this new compound.

possible, using the COSY and TOCSY data, to establish that the spin system in **4** contained a methyl group at C-35. The positioning of the methyl group was confirmed by the HMBC data which revealed a long range $^{1}H-^{13}C$ coupling from the C-45 methyl protons to the deshielded quaternary carbon at C-34, which resonates at δ 97.86 and 98.08 in **2** and **4**, respectively, but at δ 95.62 in **1**.

The NOESY data support the published relative configurations for **1** and **2**, and indicate the same relative stereochemistry in **4** at all the common chiral centres. In addition, data to determine the chirality at C-34 and C-35 of **4** were sought from the 2D NOESY and ROESY experiments with **1**, **2** and **4** (Fig. 1). In all cases, cross peaks between H-30 and the axial proton (δ 3.62) of the characteristic H-38 resonances, as well as between H-30 and the axial H-32 (δ 1.73), confirmed that the configuration of the spiro junction at position 34 and the conformation of both rings was the same in **1**, **2** and **4**. This result is in accord with the X-ray structure for **1**.[1] In DTX-2, the axial proton H-38 also shows a 1,3 diaxial NOE connectivity to the H-36 resonance at δ 2.13, which in turn shows an NOE with the neighbouring equatorial proton H-35 (δ 1.64). This establishes the axial orientation of the C-35-methyl group which was confirmed by NOE to both the equatorial H-36 (δ 1.29) and the axial H-37 (δ 1.77). Proof of the latter orientation was provided by an NOE connectivity to the equatorial proton H-38 (δ 3.54). Hence DTX-2 has the (35*S*) configuration which is the same as that reported for DTX-1.[1]

The discovery of **4** completes the suite of compounds bearing a methyl group at either C-31 and/or C-35, and suggests a biogenesis in which these fragments arise from an acetate unit (no *C*-methyl present) or a propionate unit (*C*-methyl present). Interestingly, we have observed trace amounts of **4** in cultures of *Prorocentrum lima*, and it would not be surprising to discover other biogenetic relatives in cultures of related toxic dinoflagellates.

There is considerable interest in the mode of action of DSP toxins. Current evidence indicates that a free carboxyl group is essential for inhibition of phosphatase activity,[7] and that **2** is as potent an inhibitor as okadaic acid **1**. Significantly, **4** is as active as **1** and **2** in this bioassay and has comparable activity in the rat bioassay. Thus, **4** must be added to the list of diarrhetic shellfish poisoning toxins.

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