Pergamon

Tetrahedron Letters 40 (1999) 3981–3984

TETRAHEDRON LETTERS

Oxidative Transformation of a Naturally Occurring Okadaic Acid Diol Ester by the Diatom *Thalassiosira weissflogii*

Tingmo Hu,[a] Ian Burton,[a] Jonathan M. Curtis,[a] Michael A. Quilliam,[a] John A. Walter,[a] Anthony J. Windust[b] and Jeffrey L.C. Wright*[a]

[a]*Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford Street, Halifax Nova Scotia, Canada B3H 3Z1*

[b]*Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4J1*

Received 12 February 1999; revised 2 March 1999; accepted 3 March 1999

Abstract: The diarrhetic shellfish poisoning (DSP) toxin okadaic acid (**1**) is found in extracts of the dinoflagellate *Prorocentrum lima* together with a suite of diol esters such as **2**. When **2** was added to the culture medium of the centric diatom *Thalassiosira weissflogii*, it was transformed into a range of products within three days. Three of these products, **3**, **4** and **5**, were isolated from the medium and identified as oxidation products of **2**, suggesting that the diatom produces DSP metabolites of greater polarity as a means of detoxification. In addition, another diol ester product **6** was identified as a minor impurity of **2**.

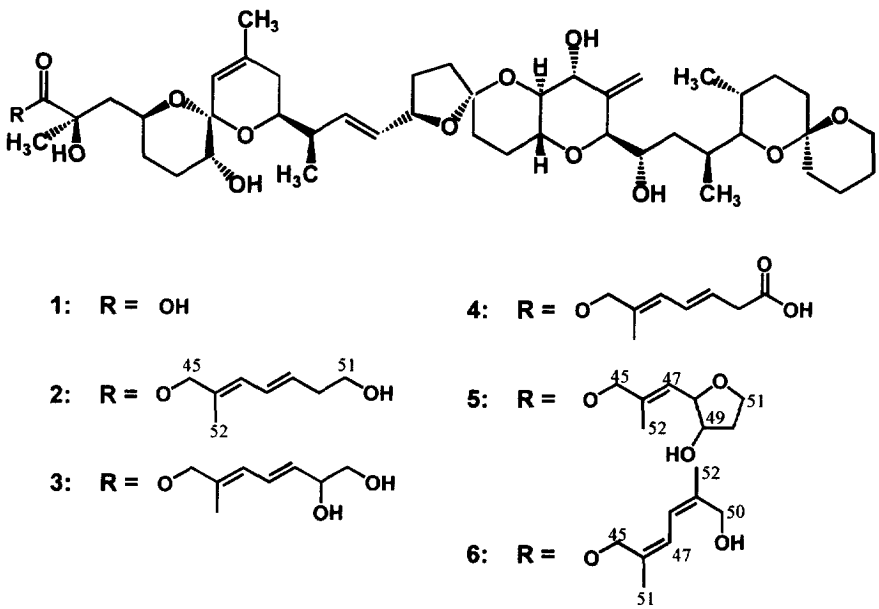
Key words: Diarrhetic shellfish poison, DSP, okadaic acid, dinoflagellate, *Prorocentrum lima*, diatom, *Thalassiosira weissflogii*.

Diarrhetic shellfish poisoning (DSP) toxins are powerful allelopaths[1] and potent inhibitors of the serine/threonine phosphatases PP1 and PP2A.[2] Intracellular storage of the toxins as the less active sulfates may provide an effective self-protection mechanism for the DSP-producing cell.[3] However, it is known that following cell rupture, sulfated DSP toxins are immediately hydrolyzed to the diol esters of okadaic acid or DTX-1.[4] This raises questions regarding the latent toxicity of these lipophilic esters to other eukaryotic cells, as the diol esters could readily traverse cell membranes and then be hydrolyzed to yield the active toxins okadaic acid or DTX-1.[5] To investigate this, cultures of the centric diatom *Thalassiosira weissflogii* (20 x 100 ml in f/2 medium) were supplied with a total of 4.0 mg of okadaic acid diol ester (**2**, MW 928), obtained from cultures of *Prorocentrum lima*.[6] Based on previous data[5] this concentration would be expected to cause partial inhibition of growth. The levels of **2** and its metabolites were monitored using LC-UV and LC-MS. Three days after its introduction, **2** was found to have been completely metabolized to unknown compounds in the medium, all of considerably greater polarity based on their LC profile.[7] Here we report the structural elucidation of the principal transformation products.

The cultures of *T. weissflogii* were combined and extracted with methylene chloride. The lipid-soluble extract of the media (2 L) was subjected to flash silica gel chromatography. Guided by TLC analysis (Merck silica gel 60 plate, toluene/acetone/methanol as developing solvent), fractions containing diol esters of okadaic acid were combined and further purified by reversed-phase HPLC (column: Zorbax RX-C8; solvent system: MeCN/H_2O with 1.0 mM NH_4Ac; detection: UV at 210 nm), yielding four compounds (**3 - 6**) in order of elution **4** (most polar), **5**, **3**, then **6** (similar polarity to **2**). All four compounds showed ^1H NMR signals and MS fragments characteristic of okadaic acid **1** suggesting that they were derivatives of **1**. This was confirmed by the detection of **1** following mild hydrolysis of each compound using porcine esterase.†

UV (238 nm) data for the major metabolite **3** suggested the presence of conjugated double bonds and IR (1737 cm^{-1}) indicated an ester linkage. High resolution liquid secondary ion mass spectrometry (LSIMS, positive ions) indicated a molecular formula $C_{52}H_{81}O_{15}$ ($[M+Na]^+$, 967.5369 ± 2.9 mDa (n = 4), Δ = 2.5 mDa), which upon comparison with **1** corresponds to an additional $C_8H_{13}O_2$ moiety.

0040-4039/99/$ - see front matter.
PII: S0040-4039(99)00516-X



The ^{1}H-^{1}H connectivity of the alcohol moiety of **3** was obtained by analysis of 2D TOCSY and COSY NMR spectra, and ^{13}C chemical shifts (Table 1) were correlated to those of directly-bonded and longer-range protons by 2D HMQC and HMBC data respectively. The low-field proton resonances at δ 4.52 and 4.64 (AB quartet) linked to a carbon at δ_C 70.9 ppm were characteristic of a methylene group (H45) forming an ester link with okadaic acid, as in **2** and in DTX-4.[6] Long-range coupling was observed in the COSY spectrum of **3** between the olefinic proton H47 (δ 6.11) and an olefinic methyl proton H52 (δ 1.81). As the latter also showed long range ^{1}H-^{1}H coupling to the methylene group at C45, it indicated that C45 to C47, plus C52, formed an isobutenic partial structure, as in **2**. The remainder of this spin system in **3** showed successive coupling from H47 (δ6.11, olefinic) to H48 (δ 6.58, olefinic), from H48 to H49 (δ 5.74, olefinic), from H49 to H50 (δ 4.18, methine), and from H50 to H51 (δ 3.48, methylene). The chemical shifts for C50 (δ_C 74.0, δ_H 4.15, methine) and C51 (δ_C 67.2, δ_H 3.48, methylene) indicated that both bore hydroxyl groups, in accordance with the molecular formula. Therefore, the planar structure of this major metabolite was established as **3**. The E, E geometry of the diene system in **3** was established by the NOESY correlations between H45 and H47, H47 and H49, H48 and H50, and between H48 and H52.

The molecular formula of compound **4** was determined by high resolution LSIMS (negative ion) to be $C_{52}H_{78}O_{15}$ ($[M-H]^-$ 941.5257 ± 0.7 mDa (n = 2), Δ = 0.5 mDa). The MS fragmentation data indicated the presence of a $C_8H_{11}O_2$ fragment in addition to an okadaic acid moiety, which was also clearly identifiable in the ^{1}H NMR spectrum. UV absorption at 282 and 240 nm indicated a conjugated diene system and characteristic peaks in the IR spectra at 3444 (br), 3385, 2928, 2856, 1733, 1719, 1560, 1077 cm^{-1} suggested the presence of a free carboxylic acid group and an ester group. Characteristic signals observed in the ^{1}H spectrum of **4** (Table 1): H45 (δ_H 4.51, 4.62, AB methylene), H52 (δ 1.78, methyl), H47 (δ 6.09, olefinic), H48 (δ 6.38, olefinic), H49 (δ 5.85, olefinic), were determined from COSY spectra to have the same coupling connectivity as **3**, implying the same partial structure C45-C49 plus C52.

The structure was further supported by HMBC's of C45 to H52, C46 to H52 and C47 to H52. Additional ^{1}H-^{1}H coupling of H49 to H50 (δ 3.08, methylene, δ_C 41.8) completed the ^{1}H spin system, the partial structure to this point needing only a COOH group to account for the molecular formula. The chemical shift of H50 was consistent with attachment of such a group to C-50. The conjugated diene system in **4** was found to have E, E geometry as shown by the ROESY correlations between H45 and H47, H47 and H49, H52 and H48, and H48 and H50.

Table 1, NMR Spectral Data for C45 - C52 of Compounds **3** - **6**.*

	3		**4**		**5**		**6**	
C	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
45	70.9 (t)	4.52, 4.64	71.0 (t)	4.51, 4.62	70.4 (t)	4.50, 4.58	71.3 (t)	4.54, 4.67
46	**		131.5 (s)		135.5 (s)		**	
47	128.3 (d)	6.11	128.9 (d)	6.09	127.8 (d)	5.42	120.5 (d)	6.30
48	127.4 (d)	6.58	128.5 (d)	6.38	83.5 (d)	4.43	124.4 (d)	6.34
49	135.2 (d)	5.74	130.9 (d)	5.85	77.8	4.03	**	
50	74.0 (d)	4.18	41.8 (t)	3.08	**	1.87, 2.12	68.3 (t)	4.02
51	67.2 (t)	3.48	178.5 (s)		67.2 (t)	3.94	14.3 (q)	1.80
52	14.8 (q)	1.81	14.4 (q)	1.78	14.4 (q)	1.78	14.1 (q)	1.76

* (s) = C, (d) = CH, (t) = CH_2, (q) = CH_3. Spectra were recorded at 500.13 MHz (^{1}H) and 125.77 MHz (^{13}C) using CD_3OD as solvent. Chemical shifts δ_C and δ_H (ppm) were referred to CHD_2OD = 3.30 ppm (^{1}H), CD_3OD = 49.0 ppm (^{13}C). Shifts for positions 1 to 44 were virtually identical to those for the corresponding positions in DTX-4.[6] ^{1}H resonances were correlated to those of directly-bonded carbons via HMQC or gradient HSQC spectra.

** Data not available.

The third metabolite **5**, having the same molecular formula as **3** (negative ion high resolution LSIMS, [M-H]- 943.5411 ± 2.7 mDa (n=5), Δ = 0.8 mDa), exhibited only end absorption in the UV spectrum, indicating that it did not contain a conjugated diene system. The TOCSY spectra showed a single spin system within the unidentified moiety ($C_8H_{13}O_2$) that contained all the protons of **5** shown in Table 1. As in **2**, **3** and **4**, weak coupling (*J* 1.6 Hz) was observed from one of the of the characteristic H45 methylene protons (δ 4.50) to the olefinic methyl group H52 (δ_H 1.78), which was also weakly coupled (*J* 1.6 Hz) to the olefinic methine H47 (δ_H 5.43). The HMBC of H52 to C45, C47 and the quaternary carbon C46 (δ_C 135.5) confirmed the partial structure from C45 to C47 plus C52 to be the same as in **2**, **3** and **4**. The COSY spectrum showed further successive coupling from H47 (δ_H 5.42) to H48 (δ_H 4.43), H49 (δ_H 4.02), H50 (δ_H 2.12, 1.87) and H51 (δ_H 3.94). The δ_H and δ_C for positions 48, 49 and 50 (Table 1) are consistent with bonding to oxygen at these positions, and the remaining degree of unsaturation in this compound implied a five-membered ring as depicted in structure **5**. The NOESY correlations between H47 and H45, and between H52 and H48, indicated an E configuration for the C46-C47 double bond.

The molecular weight of compound **6** could not be unambiguously determined by LSIMS in either the positive or negative ion modes, which both gave fragment ions indicative of the okadaic acid portion and only weak signals at higher mass. However, the base peak in the positive ion nano-electrospray mass spectrum was the $[M+Na]^+$ ion at m/z 951, and exact mass measurements gave the molecular formula $C_{52}H_{80}O_{14}$, ($[M+Na]^-$ 951.5448 ± 0.6 mDa (n=6), Δ = 0.2 mDa). Thus compound **6** is an isomer of **2**, containing a $C_8H_{13}O$ moiety attached to okadaic acid. The TOCSY spectrum revealed that the protons associated with this moiety formed a single spin system. Once again the characteristic ^{1}H resonances for H45 (δ_H 4.54, 4.67, AB system), and for the H51 methyl group (δ_H 1.80), long-range

coupled (*J* 1.4 Hz) to the olefinic methine H47 (δ_H 6.30), showed that **6** contained the same partial structure C45 to C47, plus C52, as seen for **2** to **5**. The resonances of H47 (δ 6.30) and H48 (δ 6.34) formed an AB coupled system (*J* 12.4 Hz), indicating that they were at the centre of a conjugated diene system, the presence of which was confirmed by UV absorption at 242nm. H48 was also long-range coupled (*J* 1.4 Hz) to a second methyl group H52 (δ 1.76) located on the second double bond. The remaining two-proton ^{1}H resonance at δ 4.02 was uncoupled, consistent with the hydroxymethyl group H50. In the final structure **6**, the double bond configurations were defined as Z, Z, based on the ROESY correlations between H48 and H45, H52, and between H47 and H50, H51. Resonances of carbons bearing ^{1}H were established by HMQC, but there was insufficient material for ^{13}C or HMBC spectra. Compound **6,** which has not been characterized previously, was later observed by HPLC-UV in the original sample of **2**, and hence was introduced in the *T. weissflogii* culture as a minor ester product of *P. lima.*

Three days after addition of the diol ester **2** to cultures of *T. weissflogii*, it was completely converted to a mixture of okadaic acid and a series of oxidized transformation products such as compounds **3**, **4** and **5**.[7] Plants (and animals) generally use oxidative enzymes such as the cytochrome P450s to modify exogenous compounds or xenobiotics.[8] This mechanism of coping with foreign compounds is well documented and there have been many reports of macroalgae and microalgae, such as *Chlorella* spp., that utilize the P450 family of enzymes to oxidize foreign compounds.[9] The oxidized compounds produced from the diol ester **2** represent examples of allylic oxidation (**3**), primary alcohol oxidation (**4**), and double bond epoxidation and internal cyclization (**5**). As the oxidation products are more polar than the parent ester, and are found in the culture medium, the process of oxidation could be a means of depurating DSP toxicity to the aqueous medium.[7] It is noteworthy that no evidence of any modification of the okadaic acid core of the ester molecule was found in the LC-MS analysis. All the transformation occurred on the short linear diol ester chain, and may reflect the substrate specificity of the oxidizing enzyme(s).[10]

Acknowledgements:

We thank Mr. Gordon MacPherson, Dalhousie University and NRC Institute for Marine Biosciences, Halifax, for isolation and preparation of the starting okadaic acid diolester **2**, and Dr John Coleman, NRC Institute for Marine Biosciences, for helpful comments. Issued as NRCC publication number 42289.

Footnotes and References

† Esterase (Sigma EC3.1.1.1, Lot 64H7200) and pH 8.0 buffer solutions (50 μl each) were transferred into diol ester solution (100 μl 30% DMSO solution), and the mixture was kept at 25°C for two hours. Okadaic acid was detected from the reaction products of all four diol esters using reversed phase HPLC (CSC ODS2 column, solvent system: MeCN-H_2O 60:40).

1. A. Windust, J.L. McLachlan, J.L.C. Wright, *Mar. Biol.* 1996, **126**, 19.
2. P. Cohen, C.F.B. Holmes, Y. Tsukitani, *Trends Biochem. Sci.* 1990, **15**, 98.
3. T. Hu, J.M. Curtis, J.A.Walter, J.L. McLachlan and J.L.C. Wright, *Tetrahedron Lett.* 1995, **36** (51), 9273.
4. M.A. Quilliam and N.W. Ross, in Biochemical and Biotechnological Applications of Electrospray Ionization Mass Spectrometry, ed. A.P. Snyder, American Chemical Society, Washington DC, 1996, pp. 351.
5. A.J. Windust, M.A. Quilliam, J.L.C. Wright and J.L. McLachlan, *Toxicon* 1997, **35**, 1591.
6. T. Hu, J.M. Curtis, J.A.Walter and J.L.C. Wright, *J. Chem. Soc., Chem. Commun.* 1995, 597.
7. A.J. Windust, M.A. Quilliam, J.L.C. Wright, T.Hu and J.L. McLachlan, *J. Phycol.*, in press.
8. S. Pflugmacher and H. Sanderman Jnr., *Plant Physiol.* 1998, **117**, 123 (and references therein).
9. F. Theis, T. Backhaus, B. Bossman, and L. Horst Grimme, *Plant Physiol.* 1996, **112**, 361.
10. Y. Kobayashi, X. Fang, G. D. Szklarz and J. R. Halpert, *Biochemistry* 1998, **37**, 6679.