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First identification of a C9-diol-ester of okadaic acid in *Dinophysis acuta* from Galician Rías Baixas (NW Spain)

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Abstract :

Dinoflagellates of the genus *Dinophysis* produce okadaic acid (OA) and analogues. Cultures of a strain of *Dinophysis acuta* isolated from the Galician Rías contained high levels of pectenotoxins but little OA. Isomeric forms of the latter were suspected as the concentration of OA increased after alkaline hydrolysis. High resolution mass spectra of candidate compounds suggest the presence of an OA-C9-diol ester, reported for the first time in *Dinophysis acuta*.

Highlights

► OA-C9-diol ester is reported for the first time in *Dinophysis acuta*. ► Use of HRMS combined with in-house database allowed to determine a largest toxin profile. ► A fragmentation pathway of OA-C9-diol ester is proposed.

Keywords : Okadaic acid, *Dinophysis*, Diol ester, Target analysis, HAMS

16 *Dinophysis* species are associated with Diarrhetic Shellfish Poisoning (DSP) toxins above regulatory
17 levels in the Galician Rías, northwest Spain. *Dinophysis acuta* populations may occur in that region
18 during late summer-early autumn (Reguera et al., 1995). Several polyether toxins are responsible for
19 DSP: okadaic acid (OA), OA congeners known as dinophysistoxins (DTXs= DTX1, DTX2) and their
20 esterified forms detected in algae (diol esters, DTX4, DTX5, DTX6) and in shellfish (DTX3) (Yasumoto
21 et al., 1985; Hu et al. 1992, 1995; Quilliam et al.1996; McNabb, 2008; Dominguez et al., 2010).
22 Pectenotoxins (PTX), originally included in the DSP group, were first isolated from the Japanese
23 scallop *Patinopecten yessoensis* (Yasumoto et al., 1985) but have not been confirmed to cause
24 diarrhea in humans. Initially, picked cells of *D. acuta* from the Galician Rías were reported to contain
25 only OA (9.4 pg.cell⁻¹) (Lee et al., 1987), however, it should be noted that DTX2 had not yet been

identified as an isomer of OA. Several years later, DTX2 was described in field samples where *D. acuta* was the dominant species of *Dinophysis*, and in picked cells (Fernández et al., 2000). In a separate study, analyses of picked cells revealed cells with an unusual profile composed just by PTX2 (Fernández et al., 2006). More recently, during an intense *D. acuta* bloom in November 2005, new toxins (PTX11, OA-D8) were reported from the same area (Pizarro et al., 2008a; 2008b). Suzuki et al. (2004) identified the OA trans-C8-diol ester in *D. acuta* field samples from Japan. Trans- and cis-C8-diol esters of OA were also found in European strains of *D. acuta* (Miles et al., 2004b; 2006; Pizarro et al., 2008). Similarly, Hackett et al., 2009, described free DTX1 and a C8-diol ester of OA in a cultured strain of *D. acuminata* isolated from North American waters. In addition, DTX3 was found to be a family of DTX1 derivatives which possess an ester with a fatty acid in their C7-OH position (Yasumoto et al., 1985). Lee et al. (1989) proposed that these forms may occur within bivalves resulting from acylation. Therefore, to quantify the total amount of DSP toxins in samples, presence of DTX3 (fatty acyl esters produced by shellfish metabolism), DTX4 and DTX5 (diol esters produced by the algae themselves) may be checked after hydrolysis (Fernández et al., 1996; Yasumoto et al., 1989).

Dinophysis acuta (VGO1065) was isolated from Ría de Pontevedra in October 2010 and was grown with diluted L1-Si (1:20) medium (Guillard and Hargraves, 1993), at 16°C with a salinity of 32 and fed with *Mesodinium rubrum* (ratio 1:10, *Dinophysis*:*Mesodinium*). Twelve samples were collected during the exponential growth phase in Falcon tubes of 15 or 50 ml and centrifuged (Sigma 3-18K, Fisher Bioblock Scientific, France). Pellets containing an average of ~22,000-40,000 cells were extracted twice with 0.5 mL methanol (MeOH), vortexed and sonicated at 45 KHz for 15 min. Then, 200 mg of glass beads were added and the mixture placed in a Mixer Mill MM 400 (Retsch GmbH, Germany) for 30 min to disrupt the cells, centrifuged at 12000 g and 4°C for 10 min and the supernatant (500 µl) filtered using 1.5 mL Eppendorf tubes with a 0.2 µm mesh filter insert. Basic hydrolysis of the *D. acuta* extracts was carried out to convert toxin esters (OA, DTXs) into their parent toxins (free toxins) (Mountfort et al., 2001). For this purpose, filtered MeOH extracts (250 µL) were mixed with 30 µL of 1 molar NaOH in hermetic-closing opaque-glass tubes and heated at 75°C for 40 min. Samples were

then left to cool down, their volumes verified and 30 μL of 1 molar HCl was added before being vortexed and filtered through a 0.2 μm microfiltering 1.5 mL Eppendorf tubes, centrifuged (12000 g , 10 min, 4 $^{\circ}\text{C}$) and transferred to 2 mL amber HPLC vials before injection. Analyses were carried out with a UHPLC system (1290 Infinity II, Agilent technologies, CA, USA) coupled to a high resolution time-of-flight mass spectrometer (Q-ToF 6550 iFunnel, Agilent technologies, CA, USA) equipped with a Dual Jet Stream[®] electrospray (LC-HRMS) ionization (ESI) interface operating in both negative and positive mode in separate runs. Chromatographic separation was carried out on a reversed-phase C₁₈ Kinetex column (100 $\text{\AA}$, 2.6 μm , 50 $\times$ 2.1 mm, Phenomenex, LePecq, France) at 40 $^{\circ}\text{C}$ using a mobile phase composed of water (A) and 95% acetonitrile/water (B) both containing 5 mM ammonium formate and 50 mM formic acid. The flow rate was set to 0.4 mL min^{-1} and the injection volume was 3 μL . Separation was achieved using the following mobile phase gradient: from 10% to 50% B in 2 min, to 90% B over the next 3 min, held for 5 min before return to the initial condition (10% B) in 0.5 min and a re-equilibration period (10% B) of 5.0 min. Mass spectral detection was carried out in full scan and targeted MS/MS mode in negative (ESI⁻) and positive (ESI⁺) ion acquisition. The full scan acquisition operated at a mass resolution of 40 000 Full Width at Half Maximum (FWHM) over a mass-to-charge ratio (m/z) range from 100 to 1700 with a scan rate of 2 spectra s^{-1} . The targeted MS/MS mode was performed in a Collision Induced Dissociation (CID) cell using a mass resolving power of 40 000 FWHM over the scan range m/z from 50 to 1700 with an MS scan rate of 10 spectra s^{-1} and an MS/MS scan rate of 3 spectra s^{-1} . Three different collision energies (i.e. 30, 50 and 70 eV) were applied to the precursor ions to obtain adequate fragmentation. The conditions of the ESI source were set as follows: source temperature, 200 $^{\circ}\text{C}$; drying and sheath gas was N₂; drying gas flow rate 11 mL min^{-1} ; sheath gas temperature 350 $^{\circ}\text{C}$; sheath gas flow rate 11 mL min^{-1} ; nebulizer 45 psig; capillary voltage 3.5 kV; nozzle voltage 500 V. A calibration check was carried out continuously over the entire run time using reference masses m/z 121.0509 (purine) and m/z 922.0099 (hexakis phosphazine). Acquisition was controlled by MassHunter software (Agilent technologies, CA, USA). Raw data were processed using the Find by Formula (FbF) algorithm in the Agilent MassHunter

Qualitative Analysis software (version B.07.00, service pack 1) using a Personal Compound Database and Library (PCDL) created by Phycotoxins laboratory as the source for formulae. The in-house database is composed of 90 OA, DTXs group and 18 PTXs group and is available as a download¹.

Twenty four samples were analyzed in ESI⁺ and ESI⁻ full scan mode: twelve original extracts of *D. acuta* culture and twelve hydrolyzed extracts. The toxin profile for all non-hydrolyzed samples in ESI⁺ (data not shown) was composed of nine major compounds against only five in ESI⁻ (an example is given in Fig. 1, Table 1). The most abundant components were easily identified as OA (3), DTX2 (4), PTX2 (5) and PTX2b (6) compared to certified reference materials (NRC-CRM, Halifax, Canada) and accurate mass data. After hydrolysis, only OA, DTX2 and peaks (1, 2) were present. The complete disappearance of peaks 5 to 9 is coherent with the instability of PTXs (peaks 5 and 6) in alkaline hydrolysis (Villar-González et al, 2011) and the potential presence of esters of toxins (peaks 7 – 9). Screening with the database permitted comparison of compounds detected by mass spectrometry with literature. In ESI⁺ mode, nine compounds were tentatively identified as OA analogs (peaks 1 and 2), OA (3), DTX2 (4), PTX2 (5), PTX2b (6), OA-C9-diol ester (7) and OA metabolites (8 and 9) with a score >87% and a relatively small mass error (<5ppm). Although, in ESI⁻ six common compounds (peaks 1 to 5 and peak 9) were found with a score up to 96% and a very small mass error (< 2ppm). The FbF-algorithm compared the exact value of the neutral mono-isotopic mass of each compound with the measured mass calculated from m/z values of all specified adducts within a ppm mass error. Compound (7) has a very good match with the compound reported by Hu et al., 1992, as 7-OH-2,4-dimethyl-2E,4E-heptadienyl okadaic acid ester from *P. lima* (0.21 ppm mass error). Several other observations led us to believe that the compound was a diol-ester: (i) compound (7) was present in raw (unhydrolyzed) extracts but not in hydrolyzed extracts suggesting esterification and (ii) compound (7) was detectable in positive ionization mode but not in negative, which is consistent with the chemical masking of the carboxylic acid group of OA via esterification. However, this workflow only provided the molecular formula of the compound and is not sufficient to

¹ [Website for list](#)

unequivocally identify a compound. Therefore, MS/MS spectral acquisition was necessary to confirm the nature of the compound present. As mentioned before, OA, DTX2, PTX2 and PTX2b were confirmed by accurate mass, match score and retention time compared to certified standards. Targeted MS/MS analyses were carried out for five peaks: (1) and (2) at 3.2 and 3.3 min on m/z 803.4587, 805.4733 and 822.4998, (7) at 4.2 min on m/z 943.5777 and 960.6043, (8) at 6.0 min on m/z 975.5676 and 992.5941, (9) at 6.3 min on m/z 813.481, 833.5046 and 850.5311. While abundance was not sufficient to confirm peaks (1) and (2) as OA analogues. Fragmentation of peaks (8) and (9) also did not correspond to OA analogues. While the most abundant ion for the putative C9 diol ester (7) was the $[M+Na]^+$ ion (Fig. 2), fragmentation of this adduct does not usually result in sensible spectra. Hence, further fragmentation was carried out on the ammoniated ion. A structure and fragmentation pathway for putative OA-C9-diol ester is proposed (Fig. 2B). The High Resolution MS/MS spectra obtained at 4.19 min for m/z 960.6043 showed peaks corresponding to consecutive water losses from $[M+NH_4]^+$ at m/z 943.5788 $[M+H]^+$, 925.5687 $[M-H_2O+H]^+$, 907.5580 $[M-2H_2O+H]^+$ and 889.5472 $[M-3H_2O+H]^+$. A series of characteristic fragments of OA such as m/z 805.4743 followed by losses of up to 5 water molecules at m/z 787.4644, 769.4539, 751.4434, 733.4318 and 715.4209 were also observed with a very small mass error (<3 ppm). Furthermore, the formation of protonated ions fragments at m/z 429.2300, 305.2114, 223.1697, and 169.1221, was consistent with previous studies on OA and its diol esters (Quilliam MA, 1995, Suzuki et al, 2004, Suzuki et al, 2011). To resume, the complete disappearance of peak (7) after alkaline hydrolysis, the very good score of accurate mass (>98%, 0.21ppm) and the correspondence with OA of 10 fragments ions (< 3 ppm), confirm the presence of an OA-C9-diol-ester in *D. acuta*.

In conclusion, full scan HRMS analysis of samples extracts of cultivated *D. acuta* were carried out to screen against an in-house phycotoxins database. This workflow allowed to identify five candidates of OA analogues or derivatives with a score of at least 87% (and up to 99%) and a relatively small mass error (< 5ppm). In addition, HRMS/MS spectra were acquired and product ions were compared to characteristic fragment ions of OA to confirm its analogy. This approach allowed to

reveal the presence of an OA-C9-diol ester, a toxin not previously described in *D. acuta*. The fact that a compound of identical molecular formula had previously been identified in *P. lima* suggests further similarity of biosynthetic pathways in these two genera. This finding thus strengthens the hypothesis that these two species may serve as model species for homologous gene expression studies to reveal biosynthetic pathways of okadaic acid and related compounds.

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Figure 1. LC-MS chromatograms acquired in positive (A, B) and negative (C, D) ionization full scan mode, represented an overview of the major peaks obtained in the original (A, C) and hydrolyzed (B, D) extracts of *D. acuta* with database search. OA, DTX2 and PTX2 are the dominant compounds and the tentative identification of the other peaks are listed in tab.1.

Table 1. High resolution LC-MS data of the retained candidates found in *D. acuta* extract through database search.

Peak	RT (min)	Compound identification	Molecular Formula	Mass (Da)	ESI +				ESI -			
					Measured (Da)	Error (ppm)	Score (%)	m/z (largest ion)	Measured (Da)	Error (ppm)	Score (%)	m/z (largest ion)
1	3.19	OA analogue	C44H68O13	804.464	804.4634	-3.23	95.89	827.4552 [M+Na] ⁺	804.4647	-1.57	97.17	803.458 [M-H] ⁻
2	3.27	OA analogue	C44H68O13	804.464					804.4644	-1.94	96.55	803.457 [M-H] ⁻
3	3.49	OA	C44H68O13	804.464	804.4668	0.97	87.03	822.5002 [M+NH ₄] ⁺	804.4664	0.56	98.85	803.459 [M-H] ⁻
4	3.67	DTX2	C44H68O13	804.464	804.4663	0.37	92.3	805.4734 [M+H] ⁺	804.4662	0.27	98.83	803.459 [M-H] ⁻
5	3.8	PTX2	C47H70O14	858.4766	858.4766	1.15	99.18	897.4399 [M+NH ₄] ⁺	858.4760	-0.71	98.4	857.469 [M-H] ⁻
6	4.3	PTX2b	C47H70O14	858.4766	858.4772	0.8	97.39	881.4661 [M+Na] ⁺			nd*	
7	4.19	C9-diol OA**	C53H82O14	942.5705	942.5707	0.21	98.77	965.5601 [M+Na] ⁺			nd	
8	5.94	OA metabolite 10	C53H82O16	974.5603	974.5560	-4.43	91.85	957.5544 [M+NH ₄ -H ₂ O] ⁺			nd	
9	6.29	DTX1 methyl ester or OA ethyl ester	C46H72O13	832.4973	832.4997	2.88	88.52	837.4776 [M+NH ₄ -H ₂ O] ⁺	832.4983	1.23	98.53	813.481 [M-H-H ₂ O] ⁻

*nd not detected.

**, possibly the compound reported by Hu et al., 1992, as 7-OH-2,4-dimethyl-2E,4E-heptadienyl okadaic acid ester from *P. lima*, CAS [157376-74-4]

Figure 2: Positive High resolution LC-MS spectra of putative C9-diol ester OA detected in *D. acuta* at 4.18 min (A) and a proposed MS/MS fragmentation of putative C9-diol ester OA (B), related to HR MS/MS spectra of $[M+NH_4]^+$ (m/z 960.6043) obtained in positive targeted MS/MS mode with an average of 3 collision energy (30, 50 and 70 eV). Characteristic fragments of OA (as per Quilliam, 1995; Suzuki et al., 2004), were reported in red in both the fragmentation pathway and in the mass spectrum (with mass errors given as delta ppm in brackets).

Highlights for TOXICON

Short note

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- Use of HRMS combined with in-house database allowed to determine a largest toxin profile
- A fragmentation pathway of OA-C9-diol ester is proposed