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Characterization of ovatoxin-h, a new ovatoxin analog, and evaluation of chromatographic columns for ovatoxin analysis and purification

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ABSTRACT

The presence of *Ostreopsis* cf. *ovata* on the Mediterranean coast represents a serious concern to human health due to production of toxins – putative palytoxin and ovatoxins (ovatoxin-a, -b, -c, -d, -e, -f and -g). However, purified ovatoxins are not widely available and their toxicities are still unknown. In the present study, we report on HR LC–MS/MS analysis of a French *O.* cf. *ovata* strain (IFR-OST-0.3V) collected at Villefranche-sur-Mer (France) during a bloom in 2011. Investigation of this strain of *O.* cf. *ovata* cultivated in our laboratory by ultra-high performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS) confirmed the production of ovatoxins-a to -e and revealed the presence of a new ovatoxin analog, named ovatoxin-h. *O.* cf. *ovata* extracts were pre-purified by Sephadex LH-20 to obtain a concentrated fraction of ovatoxins (OVTXs). This method provided a recovery of about 85% of OVTXs and a cleanup efficiency of 93%. Different stationary phases were tested with this fraction of interest to elucidate the structure of the new OVTX congener and to obtain purified ovatoxins. Eight reversed phase sorbents were evaluated for their capacity to separate and purify ovatoxins. Among them Kinetex C_{18}, Kinetex PFP and Uptisphere C_{18}-TF allowed for best separations almost achieving baseline resolution. Kinetex C_{18} is able to sufficiently separate these toxins, allowing us to identify the toxins present in the extract purified by Sephadex LH-20, and to partly elucidate the structure of the new ovatoxin congener. This toxin possesses one oxygen atom less and two hydrogens more than ovatoxin-a. Investigations using liquid chromatography coupled to high resolution tandem mass spectrometry suggest that the part of the molecule where ovatoxin-h differs from ovatoxin-a is situated between C42 and C49. Uptisphere C_{18}-TF was proposed as a first step preparative chromatography as it is able to separate a higher number of ovatoxins (especially ovatoxin-d and ovatoxin-e) and because it separates ovatoxins from unknown compounds, identified using full scan single quadrupole mass spectrometry. After pre-purification with Sephadex LH-20, purification and separation of individual ovatoxins was attempted using an Uptisphere C_{18}-TF column. During recovery of purified toxins, problems of stability of OVTXs were observed, leading us to investigate experimental conditions responsible for this degradation.

1. Introduction

Benthic dinoflagellates of the genus *Ostreopsis* are common in tropical and subtropical areas, but have recently been observed in increasing intensity and frequency in temperate seas [1,2]. Over the last decade, *Ostreopsis* sp. produced significant blooms during summer around the Mediterranean basin [3–8]. *Ostreopsis* bloom events may have important environmental and health consequences. Indeed, the occurrence of potentially toxic dinoflagellates in the ecosystem can have impact at several levels. Palytoxins can enter the food web and accumulate in marine organisms, and then can lead to food intoxications in seafood consumers. Moreover, *Ostreopsis* sp. was also involved in intoxication *via* inhalation [9]; irritations by direct contact, mainly skin irritations [10]; and mass mortalities of invertebrates [1,7,11,12].

Along the Mediterranean coasts of Europe, North Africa and the Atlantic coast of Portugal, blooms of *Ostreopsis* confer (cf.) *ovata* and less frequently of *Ostreopsis* cf. *siamensis* have been occurring over the last two decades [3,6,8,13]. In France, only blooms of *Ostreopsis* cf. *ovata* have been observed to date. This dinoflagellate produces

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Table 1
Structural information concerning PLTX and OVTXs: elementary formulae, elemental composition of A- and B-moieties resulting from cleavage between C8 and C9 and an additional water loss, molecular weight [15–17,19].

Toxins	Elementary formulae	A moiety $[M-B-H_2O]^+$ (m/z)	B moiety	MW Da
Palytoxin/p-PLTX[a]	$C_{129}H_{223}N_3O_{54}$	$C_{16}H_{26}N_2O_5$ (327.3)	$C_{113}H_{195}NO_{48}$	2680.14
Ovatoxin-a	$C_{129}H_{223}N_3O_{52}$	$C_{16}H_{26}N_2O_5$ (327.3)	$C_{113}H_{195}NO_{46}$	2648.14
Ovatoxin-b	$C_{131}H_{227}N_3O_{53}$	$C_{18}H_{30}N_2O_6$ (371.3)	$C_{113}H_{195}NO_{46}$	2692.20
Ovatoxin-c	$C_{131}H_{227}N_3O_{54}$	$C_{18}H_{30}N_2O_6$ (371.3)	$C_{113}H_{195}NO_{47}$	2708.20
Ovatoxin-d	$C_{129}H_{223}N_3O_{53}$	$C_{16}H_{26}N_2O_5$ (327.3)	$C_{113}H_{195}NO_{47}$	2664.14
Ovatoxin-e	$C_{129}H_{223}N_3O_{53}$	$C_{16}H_{26}N_2O_6$ (343.3)	$C_{113}H_{195}NO_{46}$	2664.14
Ovatoxin-f	$C_{131}H_{227}N_3O_{52}$	$C_{16}H_{26}N_2O_5$ (327.3)	$C_{115}H_{199}NO_{46}$	2676.20
Ovatoxin-g	$C_{129}H_{223}N_3O_{51}$	$C_{16}H_{26}N_2O_5$ (327.3)	$C_{113}H_{195}NO_{45}$	2632.14

[a] Palytoxin and putative palytoxin are chromatographically separated yet present the same mass spectral characteristics.

putative palytoxin (p-PLTX) and ovatoxins (OVTXs), a class of palytoxin analogs that have recently been identified in both field and cultured samples. Seven OVTXs have been described OVTX-a, -b, -c, -d, -e, -f [14–16] and OVTX-g, a novel ovatoxin isolated very recently in the South of Catalonia (NW Mediterranean Sea) [17]. Among them, only the structure of OVTX-a was elucidated by both MS^n and NMR [18]; the other ones only being structurally characterized by their high resolution mass spectrum (HRMS) and/or by MS^n data, in comparison with OVTX-a and PLTX (Table 1).

Palytoxin presents a long and highly functionalized chain with both hydrophilic and lipophilic parts. The molecule consists of a long partially unsaturated aliphatic backbone containing 2 amide groups, 1 amine function, 42 hydroxyl groups, 7 ether rings, ketal/hemiketal rings and 8 double bonds [20]. In comparison with PLTX, OVTX-a possesses an extra hydroxyl group at the 42-position and a lack of three hydroxyl groups at the 17-, 44-, and 64-positions [17] (Fig. 1) [18]. The fragmentation pattern of palytoxin, with informative cleavages all along the backbone of the molecule could provide direct strategy to get structural information on uncharacterized palytoxin congeners, available in quantities too small to be studied by NMR.

Production of different analogs depends on the strain of *Ostreopsis*. Both in the field and in culture, the toxin profile of *O.* cf. *ovata* is generally dominated by OVTX-a, followed by OVTX-b, OVTX-d/e, OVTX-c and p-PLTX [15,21,22]. Recently, a strain of *O.* cf. *ovata* was found to produce 50% of OVTX-f [16]. However, due to a lack of calibration standards for ovatoxins, LC–MS results are typically expressed as palytoxin equivalents (PLTX-equiv.), assuming that toxins of the palytoxin group possess the same molecular response factor in MS detection [14]. Hence, ovatoxins need to be purified and isolated for a better understanding of the molecular bases of their bioactivity.

Most authors have used reversed phase chromatography to analyze ovatoxins, mostly with C_{18} [23] and particularly Gemini C_{18} [9,15,16,24,25], C_8 sorbents [26] or Hydrophilic Interaction Liquid Chromatography (HILIC) [27,28]. These columns were suitable for OVTX identification and quantification, in association with MS detection, but not sufficiently efficient for complete separation and purification of OVTXs.

For purification of PLTX-analogs, several protocols were described in literature either from *Palythoa* sp. [29,30] or from *Ostreopsis* sp. [18,23,31]. Among these protocols, liquid–liquid extraction, solid phase extraction (SPE) or flash chromatography, and finally preparative chromatography were generally used.

In the case of *O.* cf. *ovata*, as ovatoxins possess very close chemical and physical properties, purification of these toxins into individual toxins remains difficult in spite of the complex protocols described in the literature. Several purification steps were reported, including partitioning [23], solid phase extraction (SPE) with C_{18} or ion-exchange sorbents [32,33], and flash chromatography [18]. Sometimes, several methods were combined [34]. Among the purification methods starting from *O.* cf. *ovata* cells, Hwang et al. [35] extracted ostreol A, a new cytotoxic compound from *O.* cf. *ovata*. For this purpose they used liquid–liquid partitioning with butanol followed by purification *via* flash chromatography with silica gel, then Sephadex LH-20 and, finally, preparative C_{18} chromatography [35]. Uchida et al. [34] isolated OVTX analogs in purified extracts of *O.* cf. *ovata* from Japanese IK2 strain using liquid–liquid partition with dichloromethane followed by purification through SPE cartridge (OASIS HLB). Ciminiello et al. [18] succeeded in isolating OVTX-a using an *O.* cf. *ovata* strain which produced 77% of OVTX-a, and which did not produce any OVTX-b and OVTX-c (OVTXs eluting very close to OVTX-a). The cell extract was first partitioned with dichloromethane followed by flash chromatography and preparative chromatography both with C_{18} stationary phase, and finally purified on a Kinetex 2.6 μm HPLC column [18].

In the present study, we report on HR LC–MS/MS analysis of a French *O.* cf. *ovata* strain collected at Villefranche-sur-Mer (France) during a bloom in 2011. This strain (IFR-OST-0.3V) revealed a toxin profile qualitatively different to those previously reported, with a new OVTX congener. Different columns were tested to elucidate the structure of this new OVTX congener and to obtain purified ovatoxins. After pre-purification with Sephadex LH-20 [22], purification and separation of individual OVTXs was attempted using an Uptisphere C_{18}-TF column. During recovery of purified toxins, problems of stability of OVTXs were observed, leading us to investigate the experimental conditions responsible for this degradation.

2. Experimental

2.1. Chemicals

Acetonitrile (ACN) for LC–MS/MS analysis and methanol (MeOH) were obtained as HPLC grade solvents (JT Baker) from Atlantic Labo (Bruges, France). Milli-Q water used for mobile phase and extraction was supplied by a Milli-Q integral 3 system (Millipore). Formic acid (Puriss quality), ammonium formate (Purity for MS), and acetic acid (99% purity) were from Sigma Aldrich (Saint Quentin Fallavier, France). PLTX standard for LC–MS/MS analysis was purchased from Wako Chemicals GmbH (Neuss, Germany). Sephadex LH-20 was purchased from VWR (Strasbourg, France). Acetonitrile and water used for LC-HR MS^n analysis were optima purity from Fisher Scientific (Illkirch, France).

2.2. O. cf. ovata cultures

Purification of ovatoxins was carried out from cells of cultured *O.* cf. *ovata*. Cells were originally isolated by capillary pipet from field water collected in the bay of Villefranche-sur-Mer in summer 2011, during a bloom of *O.* cf. *ovata*.

After initial growth in microplates, the cells were cultured in 350 mL flasks at 22 °C under 16L:8D cycle (420 μmol m^{-2} s^{-1}). Culture conditions were previously optimized [36] and were