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New palytoxin-like molecules in Mediterranean *Ostreopsis* cf. *ovata* (dinoflagellates) and in *Palythoa tuberculosa* detected by liquid chromatography-electrospray ionization time-of-flight mass spectrometry

Rachele Rossi [a], Vincenzo Castellano [a], Eleonora Scalco [b], Luigi Serpe [a], Adriana Zingone [b], Vittorio Soprano [a,*]

[a] *Istituto Zooprofilattico Sperimentale del Mezzogiorno, Via Salute 2, 80055 Portici, Italy*
[b] *Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Napoli, Italy*

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ABSTRACT

A rapid, high resolution liquid chromatography coupled with ElectroSpray Ionization Time-Of-Flight Mass Spectrometry (ESI/TOF/MS) method was developed for the determination of the toxin pattern in cultured cells of *Ostreopsis* cf. *ovata* from the Mediterranean Sea. The samples were separated on a Phenomenex Luna 3μ HILIC 200A (150 × 2.00 mm) and analyzed by LC/TOF/MS with electrospray ionization (ESI) interface in positive ion mode. The method developed here provides the capability for a fully automated analysis, which requires relatively easy sample preparation and gives clean and simple chromatograms. The method was successfully applied to the determination of ovatoxin-a, mascarenotoxin-a and four new palytoxins in *O.* cf. *ovata*. Another new palytoxin was detected in the standard material from *Palythoa tuberculosa* provided by Wako Chemicals.

1. Introduction

Ostreopsis ovata is a dinoflagellate that colonises macroalgae, seagrasses and benthic animals or grows directly on the substrate. In recent years, massive blooms of this species have become a threat along the Mediterranean coasts, where the toxins produced by the species can affect human health through aerosol (Mangialajo et al., 2008), render seafood toxic (Aligizaki et al., 2008) and affect benthic animals (Sansoni et al., 2003; Simoni et al., 2003). The Atlantic/Mediterranean populations of *O. ovata* actually differ from the Indo-Pacific populations in both molecular and morphological characteristics (Penna et al., 2005, 2010), but no formal change has been proposed as to the taxonomic status of the species as yet. For this reason, the use of the name *Ostreopsis* cf. *ovata* is preferable at this stage.

Species of the genus *Ostreopsis* are known to produce toxic substances including palytoxin (PlTX), one of the most potent non-protein marine toxins (Fig. 1), which was firstly isolated from the marine zoanthids *Palythoa* spp. (Moore and Scheuer, 1971). Recently, a putative PlTX and a new PlTX-like compound named ovatoxin-a (OvTX-a) have been identified in *Ostreopsis* cf. *ovata* from the Mediterranean Sea (Ciminiello et al., 2008). Numerous other PlTX-like substances have been described from various marine organisms (Deeds and Schwartz, 2010; Panagiota, 2007).

These toxins can enter the food web through feeding interactions and accumulate in higher trophic levels. Based on the limited toxicological database of the PlTX-group

* Corresponding author. Tel.: +39 0817865 214; fax: +39 0817865 239.
E-mail addresses: rachele.rossi@izsmportici.it (R. Rossi), vincenzo.castellano@cert.izsmportici.it (V. Castellano), eleonora.scalco@szn.it (E. Scalco), luigi.serpe@cert.izsmportici.it (L. Serpe), zingone@szn.it (A. Zingone), v.soprano@izsmportici.it (V. Soprano).

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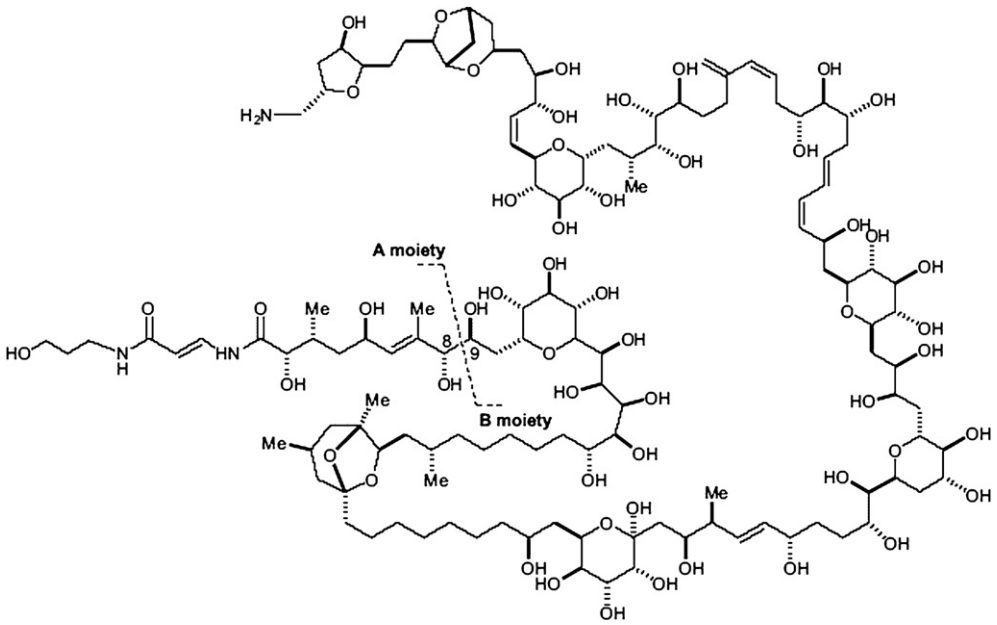

Fig. 1. Palytoxin structure.

toxins, a regulatory level of 30 μg of the sum of palytoxins and ostreocin-D per kg of body tissues has been proposed for the harvesting and consumption of shellfish resources by EFSA (European Food Safety Authority, 2009).

High performance liquid chromatography (HPLC) with precolumn derivatization and fluorescence detection (Riobó et al., 2006) or coupled with mass spectrometry (LC-MS) (Ciminiello et al., 2006) are among the methods used for the determination of PlTX in cells and shellfish tissues. In this study, we set up and tested a new method in LC/TOF/MS for the determination of the PlTXs in *O.* cf. *ovata* cultures. The method proved to be sensitive and provided a high resolution allowing for the detection of five new palytoxin-like compounds.

2. Materials and methods

2.1. Isolation and cultivation methods

The strain D483 of *O.* cf. *ovata* was obtained from a sample collected at the station Gaiola in the Gulf of Naples (40°47′33″N, 14°11′18″E) in September 2008. Macroalgae of the species *Asparagopsis taxiformis* (Rhodophyta) were sampled by scuba diving. In the laboratory, the macroalgae were shaken in a plastic jar with seawater from the same site and the liquid sample containing free microalgal cells was collected. Cultures of *O.* cf. *ovata* were established from a single cell isolated with a pipette under the light microscope. Cultures were maintained in K/2 medium without the addition of silicates (Keller et al., 1987) at 18 °C with a photoperiod of 12:12 dark:light conditions and an irradiance of 50 μmol photon m^{-2} s^{-1}. An aliquot of ca 5 ml of culture was transferred to fresh medium approximately every two weeks.

For morphological identification, cells of strain D483 were observed in light microscopy with a Zeiss microscope Axiovert. Cells were also observed in scanning electron microscopy.

For toxin analysis, three plastic flasks (a, b, and c) containing 1 l of K/2 medium were incubated with 1000–2000 cells ml^{-1} and allowed to grow in the same conditions as above. At different growth phases, the flasks were shaken and aliquots of 10 ml were sampled to estimate cell concentrations. The aliquots were treated with Na-EDTA solution at a final concentration of 0.01 M to dissolve mucous aggregates. 1 ml was placed in a Sedgewick-Rafter Counting Cell Slide and counted in duplicates in the light microscope (ZEISS AXIOPHOT). After 23 days the cultures were centrifuged at 4000 rpm for 10 min and the pellet was stored in a freezer until toxins extraction.

2.2. Chemicals

Analytical standard of PlTX was purchased from Wako Chemicals GmbH (Neuss, Germany). Acetonitrile, methanol and water were HPLC grade. Formic acid was obtained from Rudi Pont.

2.3. Sample extraction

For palytoxin analysis, the pellet was sonicated for 15 min, resuspended in 1 ml of MeOH/W (1:1) and vortexed for 3 min. Cells were lyzed by sonication again for 15 min and centrifuged at 4000 rpm for 5 min. The clear supernatant was transferred into a glass test tube without disturbing the pellet. The pellet was resuspended in 1 ml of MeOH/W (1:1) and vortexed for 3 min. All the steps above were repeated 3 times. Then the supernatant was evaporated and the residue was resuspended in 500 μl of