Toxicon 60 (2012) 810–820

Contents lists available at SciVerse ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

A sensitive assay for palytoxins, ovatoxins and ostreocins using LC-MS/MS analysis of cleavage fragments from micro-scale oxidation

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ARTICLE INFO

Article history:
Received 13 March 2012
Received in revised form 24 May 2012
Accepted 24 May 2012
Available online 31 May 2012

Keywords:
Palytoxin
Ostreocin-D
Ovatoxin
Seafood poisoning
Periodate oxidation

ABSTRACT

Palytoxin is a highly toxic non-proteinaceous marine natural product that can pass through the food chain and result in human illnesses. A recent review by the European Food Safety Authority concluded that palytoxin requires regulation in seafood and a limit of 30 μg kg^{-1} for shellfish flesh was suggested. Current methods based on LC-MS detection of intact palytoxins do not have sufficient sensitivity to enforce this limit for palytoxin. To improve sensitivity for trace analysis, a novel screen approach has been developed that uses LC-MS/MS analysis of substructures generated by oxidative cleavage of vicinal diol groups present in the intact toxin. Oxidation of palytoxins, ovatoxins or ostreocins using periodic acid generates two nitrogen-containing aldehyde fragments; an amino aldehyde common to these toxins, and an amide aldehyde that may vary depending on toxin type. Conditions for micro-scale oxidation of palytoxin were optimised, which include a novel SPE cleanup and on-column oxidation step. Rapid analysis of cleavage fragments was established using LC-MS/MS. Linear calibrations were established for the amino aldehyde from a palytoxin reference standard, which is suitable for all known palytoxin-like compounds, and for the confirmatory amide aldehydes of palytoxin and ostreocin-D. Palytoxin recoveries (at 10 μg kg^{-1}) from shellfish and fish tissues were 114–119% (as amine aldehyde) and 90–115% (as amide aldehyde) with RSDs for both of ≤18% (all tissues, $n = 12$). The method LOD was determined to be approximately 1 ng mL^{-1} and the LOQ 4 ng mL^{-1}, which corresponds to 10 μg kg^{-1} in tissue (flesh of shellfish or fish). The method has potential for use in research and is sufficiently sensitive for regulatory testing, should it be required.

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1. Introduction

Palytoxin (Fig. 1, $C_{129}H_{223}N_3O_{54}$) is one of the most complex and toxic non-proteinaceous natural products known with an LD_{50} (mouse, acute i.p.) of less than 1 μg kg^{-1} (Munday, 2011). Palytoxin was originally isolated from the Hawaiian soft coral *Palythoa toxica* (Moore and Bartolini, 1981) and subsequently a range of palytoxin analogues have been isolated from *Palythoa* species or species of *Ostreopsis*, a benthic dinoflagellate genus. The number of known palytoxin-like analogues now approaches 20, including the structurally related ostreocin-D, mascarenotoxins and ovatoxins (Rossi et al., 2010; Ciminiello et al., 2011a, 2012a, 2012b), although definitive structures are sometimes lacking (Ciminiello et al., 2009). Transfer of palytoxin through the marine food chain (Taniyama et al., 2003) and human illnesses associated with consumption of seafood containing palytoxin (Onuma et al., 1999) have been documented in tropical regions. Strong interest in the environmental toxicology of palytoxin and analogues was generated by a major bloom of *Ostreopsis ovata* in the Eastern Mediterranean in 2005 (Mangialajo et al., 2011), which was associated with human respiratory disorders and also some dermal and ocular

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0041-0101/$ – see front matter © 2012 Published by Elsevier Ltd.
10.1016/j.toxicon.2012.05.024

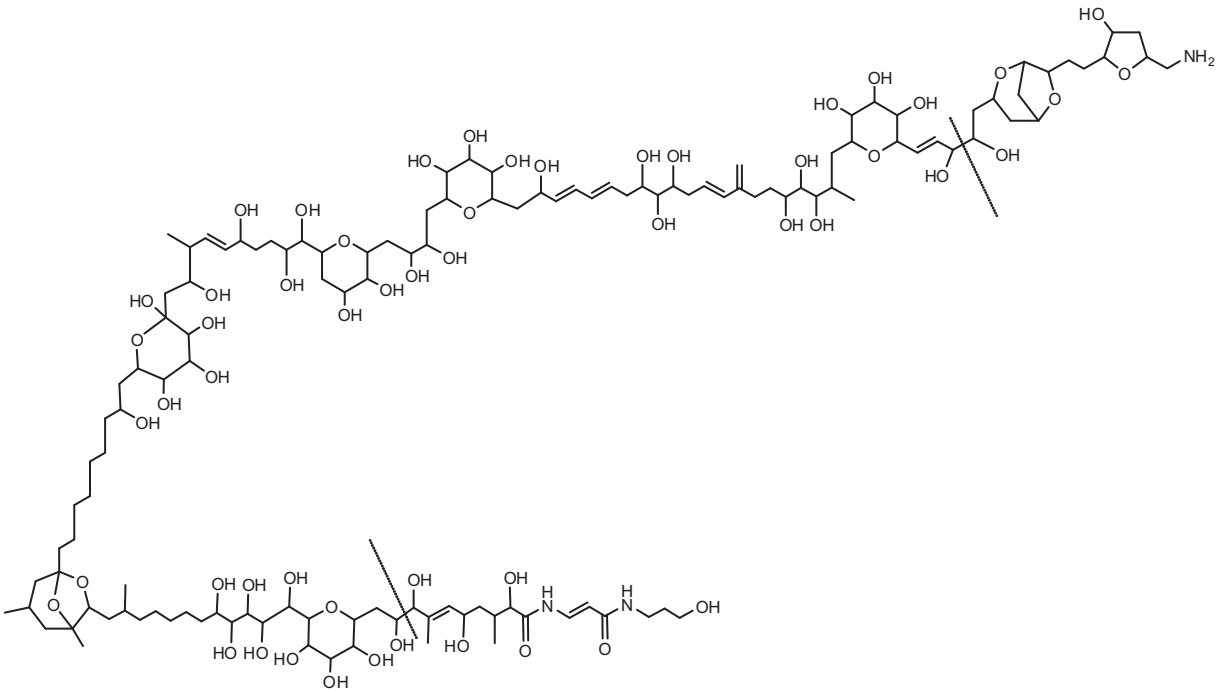

Fig. 1. Structure of palytoxin (PLTX). The two lines through the macrostructure indicate the location of the vicinal diols closest to either terminus.

effects (Deeds and Schwartz, 2010). Although the oral toxicity of palytoxin and 45-hydroxy palytoxins are about 1000-fold less than that observed by intraperitoneal injection (Munday, 2011; Sosa et al., 2009; Tubaro et al., 2011), a regulatory limit of 30 μg kg^{-1} has been proposed for shellfish flesh (EFSA, 2009). *Ostreopsis* has a world-wide occurrence (Rhodes, 2011) and the increased frequency and intensity of *Ostreopsis* blooms, particularly in coastal waters of the Mediterranean (Mangialajo et al., 2011), have raised global concern about the causes and consequences of these phenomena which have been reviewed in a recent monograph (Rossini, 2011).

Analytical methods are integral to understanding the production, distribution, uptake and metabolism of marine biotoxins. A variety of techniques have proved suitable for characterising palytoxin and related analogues, and their biological activities (Riobó and Franco, 2011). Mass spectrometric methods are powerful, particularly coupled with liquid chromatography-mass spectrometry (LC-MS) and have provided structural data on new analogues (Rossi et al., 2010; Ciminiello et al., 2011b, 2011c) as well as some information on levels in algae. However, quantitative analysis of intact palytoxins by LC-MS with electrospray ionisation is complicated by the complex spectra with the ionisation spread across multiple charge states, mixed cationised species (H^+, Na^+, K^+, NH_4^+, Ca^{2+}) and the large ^{13}C isotope contributions (Ciminiello et al., 2006, 2012c). This leads to ambiguities in identification with low and variable sensitivity and specificity for trace detection using selected ion recording (SIR) or multiple reaction monitoring (MRM) techniques, especially in biological extracts. There is an urgent need for sensitive, cost-effective, quantitative methods for trace determination of palytoxin and related analogues. The potential regulation of palytoxin in seafood, and the relative insensitivity of established methods that monitor intact palytoxin structures, heightens the need for such an assay (EFSA, 2009). In this study we describe a quantitative screening method for palytoxins, ovatoxins and ostreocins in shellfish and fish flesh using LC-MS/MS analysis of substructures generated by periodate oxidation, which incidentally represents the reaction used as part of the original structural elucidation of palytoxins (Moore and Bartolini, 1981; Usami et al., 1995) and ostreocin-D (Ukena et al., 2001).

2. Materials and methods

2.1. Chemicals and reagents

High purity methanol was obtained from Burdick & Jackson (MI, USA). Proanalysis grade ethanol, ethyl acetate, acetic acid, periodic acid, Lichrosolv grade acetone, acetonitrile, and Suprapur grade formic acid were from Merck (Darmstaldt, Germany). Purified water was produced with a Milli-Q system (Millipore, Nepean, ON, Canada). Purified palytoxin standard was from Wako (Japan), and the ostreocin-D standard was a gift from Associate Professor Masayuki Satake (University of Tokyo, Japan). Stock standard solutions of palytoxin and ostreocin-D were prepared at 10–25 μg mL^{-1} in methanol–water (1:1 v/v). Serial dilutions were made in methanol–water (2:3 v/v) and stored at −20 °C.

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