RESEARCH ARTICLE

High Resolution LC-MSⁿ Fragmentation Pattern of Palytoxin as Template to Gain New Insights into Ovatoxin-a Structure. The Key Role of Calcium in MS Behavior of Palytoxins

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Abstract

Palytoxin is a potent marine toxin and one of the most complex natural compounds ever described. A number of compounds identified as palytoxin congeners (e.g., ovatoxins, mascarenotoxins, ostreocins, etc.) have not been yet structurally elucidated due to lack of pure material in quantities sufficient to an NMR-based structural investigation. In this study, the complex fragmentation pattern of palytoxin in its positive high resolution liquid chromatography tandem mass spectra (HR LC-MSⁿ) was interpreted. Under the used conditions, the molecule underwent fragmentation at many sites of its backbone, and a large number of diagnostic fragment ions were identified. The natural product itself was used with no need for derivatization. Interestingly, most of the fragments contained calcium in their elemental formula. Evidence for palytoxin tendency to form adduct ions with calcium and other divalent cations in its mass spectra was obtained. Fragmentation pattern of palytoxin was used as template to gain detailed structural information on ovatoxin-a, the main toxin produced by *Ostreopsis ovata*, (observe correct font) a benthic dinoflagellate that currently represents the major harmful algal bloom threat in the Mediterranean area. Either the regions or the specific sites where ovatoxin-a and palytoxin structurally differ have been identified.

Key words: HR LC-MSⁿ, Palytoxin, Ovatoxin-a, Calcium adducts

Introduction

Palytoxin (Figure 1) is one of the most potent non-protein marine toxins so far known as well as one of the most complex non-polymeric natural compounds ever described: it presents a long and highly functionalized alkyl chain containing, among others, 42 hydroxyl groups and seven

ether rings, with no repeating units [1–4]. A number of palytoxin analogues have been so far isolated either from soft corals (*Palythoa* genus) or *dinoflagellates* (*Ostreopsis* spp.): homo-, bishomo-, neo-, and 73-deoxy-palytoxin [5], ostreocin-D (namely 42-hydroxy-3,26-didemethyl-19,44-dideoxypalytoxin) [6], and 42-hydroxy-palytoxin [7]. Structure elucidation of these compounds was carried out by extensive NMR analyses and in most cases through the use of degradation/derivatization reactions [1, 5, 8]. This kind of study requires relatively high amounts of pure material and, therefore, structures of a number of minor congeners of palytoxin so far identified were not elucidated due to lack of

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Figure 1. Structure of palytoxin and HR LC-MSⁿ-based structural hypothesis for ovatoxin-a. An NMR study [20] on pure ovatoxin-a demonstrated that ovatoxin-a is the 42-hydroxy-17,44,64-trideoxy-palytoxin. Cleavages resulting from various HR CID MSⁿ spectra are reported in the structure and in the scheme below. All cleavages in palytoxin occurred also in ovatoxin-a apart from those marked as white spots in black cells

pure material in quantities sufficient to structural investigation. For this purpose, mass spectrometry (MS) could be a useful tool as it is both sensitive and selective even when analytes are contained at trace levels in quite complex matrices. However, structural complexity of palytoxins is reflected in equally complex mass spectra, and this hampers a straightaway interpretation of their fragmentation patterns [9]. Significant efforts in this direction were made by Ukena et al. [10], who used negative ion fast atom bombardment (FAB) collision induced dissociation (CID) tandem MS to confirm the structure of ostreocin-D. However, this study

was not carried out on ostreocin-D itself but on a derivatized sample of ostreocin-D obtained by reaction with 2-sulfo-benzoic acid cyclic anhydride; this resulted in introduction of a negative charge in the molecule that facilitated the charge-remote fragmentations.

In the present study, we fully interpreted the positive ion high resolution (HR) electrospray (ESI) MSⁿ spectra of palytoxin; the natural product itself was used with no need for derivatization. In the spectra obtained, besides the abundant and uninformative ions due to water losses, a large number of diagnostic fragment ions were present;

interestingly, most of them contained calcium in their elemental formula. Evidence for palytoxin tendency to form adduct ions with calcium and other divalent cations in its ESI MS and MSⁿ spectra was obtained.

Fragmentation pattern of palytoxin, full of informative cleavages all along the entire backbone of the molecule, could provide a direct strategy to get structural information on uncharacterized palytoxin congeners, available in quantities too small to be studied by NMR. So, the results obtained were used for structural investigation of the main toxin produced by *Ostreopsis ovata*. This benthic dinoflagellate currently represents the major threat in the Mediterranean area both from an environmental and a public health perspective [11–13]. The most relevant episode associated to *O. ovata* occurred in 2005 along the Ligurian coasts (Italy) when hundreds of people required medical attention after exposure to marine aerosols [14, 15]. *O. ovata* blooms have repeatedly occurred in subsequent years in Italy as well as along the Mediterranean coasts of Spain, France, and Greece [11]. Putative palytoxin and several new palytoxin-like compounds, named ovatoxin-a, -b, -c, -d, and -e, were identified by our group in both natural and cultured *O. ovata* samples based on comparison of their HR LC-MS and MS² behavior with that of palytoxin [9, 16–18]. In these early studies, elemental composition of ovatoxins was provided together with preliminary information on their structures, which allowed only including ovatoxins within the palytoxin group of toxins.

In the present study, interpretation of the fragmentation pattern of palytoxin was used as rational basis for structural investigation of ovatoxin-a, the major component of *O. ovata* toxin profile [17, 18], and this allowed to identify the regions or even the specific sites of difference between palytoxin and ovatoxin-a.

Materials and Methods

Chemicals and Materials

All organic solvents and glacial acetic acid (Laboratory grade) were by Carlo Erba (Milan, Italy). Calcium, magnesium, strontium, zinc, potassium, sodium, lithium acetate, and disodium EDTA were purchased from Sigma Aldrich (Milan, Italy). Palytoxin was purchased from Wako Chemicals GmbH (Neuss, Germany) and dissolved in methanol/water (1:1, vol/vol) to a concentration of 10 µg/mL. A crude extract of *O. ovata* [18] containing ovatoxin-a at levels of 6 µg/mL was used.

HR LC-MS and MSⁿ

All analyses were performed on an Agilent 1100 LC binary system (Palo Alto, CA, USA) coupled to a hybrid linear ion trap LTQ Orbitrap XL Fourier transform mass spectrometer (FTMS) equipped with an ESI ION MAX source (ThermoFisher, San José, CA, USA). A 3 µm Gemini C18 column

(150×2.00 mm; Phenomenex, Torrance, CA, USA) was eluted at 0.2 mL/min with water (eluent A) and 95% acetonitrile/water (eluent B), both containing 30 mM acetic acid (control conditions). A gradient elution (20%–50% B over 20 min, 50%–80% B over 10 min, 80%–100% B in 1 min, and hold 5 min) was used. Injection volume was 5 µL. In adduct formation experiments, 100 nM calcium, magnesium, strontium, zinc, potassium, sodium, and lithium acetate were separately added to mobile phase. Disodium EDTA was added to mobile phase to a final concentration of 0.1, 1, 2, and 4 mM.

HR full MS experiments (positive ions) were acquired either in the range m/z 800–1400 or m/z 2000–3000 at resolution settings 15,000 to 100,000. The following source settings were used: spray voltage = 4 kV, capillary temperature = 290 °C, capillary voltage = 45 V, sheath gas = 35, and auxiliary gas = 1 (arbitrary units), tube lens voltage = 165 V (m/z 800–1400) or 250 V (m/z 2000–3000).

HR collision induced dissociation (CID) MS² experiments were acquired on $[M + H + Ca]^{3+}$, $[M + 2H - H_2O]^{2+}$, and $[M + H]^+$ ions of palytoxin (m/z 906.8, 1331.7, and 2680.4, respectively) and ovatoxin-a (m/z 896.2, 1315.7, and 2648.5, respectively) at collision energies (CE) = 25%, 21%, and 65%, respectively. HR CID MS³ experiments were carried out on palytoxin (m/z 906.8>743.4, 906.8>507.3, and 906.8>327.1) and ovatoxin-a (m/z 896.2>727.4, 896.2>507.3, and 896.2>327.1) at CE = 25%. HR CID MS⁴ experiments were carried out on palytoxin (m/z 906.8>327.1>309.1) and ovatoxin-a (m/z 896.2>327.1>309.1) at CE = 20%. HR pulsed Q collision induced dissociation (PQD) MS² experiments were acquired on m/z 906.8 and 1331.7 of palytoxin at CE = 37% and 25%, respectively. HR high energy collision dissociation (HCD) MS² experiments were acquired on m/z 906.8, 1331.7 and 2680.4 of palytoxin at CE = 48%, 25%, and 48%, respectively. A 60,000 resolving power, an activation Q of 0.250, and an activation time of 30 ms were used in all cases.

Calculation of elemental formulae was performed by using the monoisotopic ion peak of each ion cluster. A mass tolerance of 5 ppm was used and the isotopic pattern of each ion cluster was considered.

Results and Discussion

HR LC-MSⁿ spectra of palytoxin were obtained under various fragmentation modes (namely CID, PQD, and HCD) using as precursors the most abundant triply-, doubly-, and mono-charged ions of the full MS spectra (SI Figure S1a, b) of m/z 906.8, 1331.7, and 2680.4, respectively. The most informative results were obtained by interpretation of MSⁿ spectra of the triply-charged ion of m/z 906.8; this ion, previously assigned to $[M + 2H + K]^{3+}$ [18], had to be reassigned since most of the fragments contained in its MSⁿ spectra were not consistent with any reasonable cleavage of palytoxin molecule, including the precursor formula C₁₂₉H₂₂₅KN₃O₅₄ in the element con-

strains (errors >5 ppm in most cases) [16–18]. A more thorough examination revealed that the alternative formula $C_{129}H_{224}CaN_3O_{54}$ could be assigned to the precursor, that is to say the ion of m/z 906.8 could be a palytoxin calcium adduct in place of a potassium adduct; in this way, the unassigned ions of the MSⁿ spectra could all be interpreted as calcium-containing fragments deriving from reasonable cleavages of palytoxin molecule. Confident option between the two alternative formulae for the precursor was not immediate as the monoisotopic ion of m/z 906.4851 could be assigned either to $[M + 2H + K]^{3+}$ or $[M + H + Ca]^{3+}$ ion ($\Delta = -0.737$ ppm and 2.551 ppm, respectively), which have undistinguishable m/z values (at resolution 100,000) and the same isotopic pattern (SI Figure S1c). Definitive support for the calcium adduct option was gained by investigating formation of adduct ions of palytoxin in its MS spectra in the presence of various mono- and divalent cations, as described below.

Palytoxin Adducts with Mono- and Divalent Cations

HR full MS spectra of palytoxin were acquired in the mass range m/z 800–1400 after addition of various monovalent (K^+ , Na^+ , Li^+) or divalent (Ca^{2+} , Mg^{2+} , Sr^{2+} , Zn^{2+}) cations to the mobile phase, and they were compared with the spectrum of palytoxin obtained under control conditions (Figure 2a).

Addition of K^+ acetate to mobile phase (Figure 2b) resulted in a significant increase in intensity of the doubly-charged $[M + H + K]^{2+}$ ion of palytoxin of m/z 1359.7 (monoisotopic m/z 1359.2259, $C_{129}H_{224}KN_3O_{54}$, $\Delta = 0.674$ ppm), whereas the appearance of the mass region containing triply-charged ions (m/z 850–920) remained almost unchanged with respect to control, with the ion of m/z 906.8 still dominating the spectrum. Conversely, addition of Ca^{2+} acetate to mobile phase (Figure 2c) dramatically changed the appearance of palytoxin full MS spectrum, which contained only the triply-charged ion of m/z 906.8, while doubly-charged ions of palytoxin in the mass range m/z 1300–1370 were barely detectable. Such results supported involvement of calcium in the formation of the ion of m/z 906.8. To unravel any doubt, increasing concentrations of the divalent ion chelating agent EDTA [19] (0.1–4 mM) were added to the mobile phase: intensity of the ion of m/z 906.8, as well as of all the triply-charged ions of palytoxin, decreased as EDTA concentration increased, with no triply-charged ion of palytoxin being present in the spectrum at EDTA concentrations ≥ 4 mM (Figure 2d). These results clearly indicated that in full MS spectrum of palytoxin, the ion of m/z 906.8 was actually a calcium adduct, namely $[M + H + Ca]^{3+}$.

Figure 2. HR full MS spectra of palytoxin (m/z 800–1400) obtained under control conditions (a) and after addition of various monovalent (b), (e), (g) and divalent cations (c), (f), (h), (i), (j) or disodium EDTA (d) to mobile phase. The most abundant peak of each ion cluster is shown

A further interesting result of the EDTA experiment was that the triply-charged ion of palytoxin of m/z 901.5 (Figure 2a), previously assigned to a palytoxin adduct with a monovalent cation (sodium), was actually a palytoxin adduct with a divalent cation, as it disappeared after EDTA addition. The most appropriate candidate was magnesium as the monoisotopic ion peak of m/z 901.1581 could be assigned either to $[M + 2H + Na]^{3+}$ or $[M + H + Mg]^{3+}$ ions ($\Delta = -3.354$ and 1.290 ppm, respectively) which, once again, present undistinguishable m/z values and the same isotopic pattern under the experimental resolution setting (SI Figure S1d). The Mg adduct option was definitively proved by HR full MS spectra of palytoxin acquired adding Na^+ and Mg^{2+} salts separately to the mobile phase; the results obtained (Figure 2e, f) paralleled those obtained in the K^+ and Ca^{2+} experiments, respectively; particularly, the doubly-charged ion of m/z 1351.7 (monoisotopic m/z 1351.2368, $C_{129}H_{224}NaN_3O_{54}$, $\Delta = -0.921$ ppm) increased after addition of the monovalent cation (Na^+), while the triply-charged ion of m/z 901.5 was the base peak after addition of the divalent cation (Mg^{2+}).

In order to have a wider view of palytoxin behavior in the presence of mono- and di-valent cations, individual addition of Li^+ , Sr^{2+} and Zn^{2+} acetate to the mobile phase was also carried out (Figure 2g, h, i). As expected, addition of Sr^{2+} and Zn^{2+} resulted into abundant triply-charged ions of m/z 922.7977 (monoisotopic m/z 922.4641, $C_{129}H_{224}N_3O_{54}Sr$, $\Delta = 0.330$ ppm) and m/z 914.8047 (monoisotopic m/z 914.4716, $C_{129}H_{224}N_3O_{54}Zn$, $\Delta = -0.043$ ppm), respectively, and into barely detectable doubly-charged ions. On the contrary Li^+ addition resulted into a quite abundant $[M + H + Li]^{2+}$ ion of m/z 1343.7505 (monoisotopic m/z 1343.2499, $C_{129}H_{225}LiN_3O_{54}$, $\Delta = -0.940$ ppm) while no triply-charged lithium adduct was observed.

Finally, when equimolar amounts of Ca^{2+} , Mg^{2+} , and Sr^{2+} acetate were concurrently added to mobile phase (Figure 2j), some selectivity among the tested divalent cations in formation of palytoxin triply-charged adducts emerged, with the base peak of m/z 906.8 being the palytoxin calcium adduct.

In conclusion, the above results indicated that in the presence of divalent cations, triply-charged adduct ions of palytoxin dominate its full MS spectrum, while, in the presence of monovalent cations, formation of doubly-charged adducts of palytoxin is favored, and that palytoxin presents the highest affinity for calcium among the tested divalent cations.

Fragmentation Pattern of Palytoxin

HR CID MS² of m/z 906.8

Following reassignment of the triply-charged ion of m/z 906.8 as $[M + H + Ca]^{3+}$, the whole fragmentation pattern of palytoxin contained in its HR CID MS² spectrum (Figure 3) was interpreted, including calcium in the element constraints. It has to be noted that correct ion assignment and

cleavage identification was complicated by two factors: (1) the ions representative of each cleavage were always followed by a number of ions due to subsequent water losses; this resulted in a very crowded spectrum and, in some cases, in extensive overlap of ion clusters; (2) a number of elemental formulae were possible for each ion within a mass tolerance of 5 ppm; fortunately, some alternative formulae could be ruled out on the basis of structural features of palytoxin, including degree of insaturations (RDB) and location of the three nitrogen atoms in the molecule; 2 N atoms are at the A-side terminal and 1 N atom is at the B-side terminal.

HR CID MS² spectrum of m/z 906.8 contained a number of diagnostic ions; a few of them, namely the mono-charged ion of m/z 327.1908 and the doubly-charged ion of m/z 1187.6228 with associated water losses, derived from cleavage between C-8 and C-9 of palytoxin molecule (Table 1, Figure 1) and they had been assigned to A- and B-side fragments of cleavage #4, respectively [18]. A very limited number of mono-charged ions could be assigned to protonated B-side fragments deriving from cleavage #15, #16, #17, #19, and #21 (Table 1, Figure 1), whereas all the remaining ions were calcium-containing fragments.

Only A-side fragments were observed arising from cleavages #11, #22, #23, #24, #25, #26, #27, and #28, while cleavage #18 led to only B-side fragment (Table 1, Figure 1). On the contrary, several other cleavages (#4, #12, #13, #14, #15, #16, #17, #19, and #21) generated both A-side and B-side fragments (Table 1, Figure 1). A few ions could not be assigned to any fragment containing either the A-side or the B-side terminal of palytoxin molecule. All of them were found to be internal fragments due to a combination of two cleavages; they contained no nitrogen atom in their elemental formula and originated from the inner portion of the molecule stretching from C-9 to C-48 (Table 2, Figure 1). Such fragments can be divided in two groups: the first includes fragments resulting from the B-side fragment of cleavage #4 after further fragmentation at cleavage sites #12, #13, #15, and #16; the second group includes fragments resulting from the A-side fragment of cleavage #12 after further fragmentation at new cleavage sites labeled as #5, #7, #8, and #9 (Table 2, Figure 1).

HR CID MS³ and MS⁴

Some HR CID MS³ and MS⁴ experiments were carried out by fragmenting the most abundant ions of the HR CID MS² spectrum of m/z 906.8. Useful information was obtained through fragmentation of the internal fragment ions of m/z 743.4 and 507.3, relevant to part structures C-9 to C-41 (cleavage #4+#12) and C-18 to C-41 (cleavage #8+#12), respectively. Most of the ions contained in the MS³ of m/z 743.4 were relevant to internal fragments already assigned in the MS² of m/z 906.8 (cleavages #5+#12; #7+#12, and #8+#12) (Table 2, Figure 1); in addition, two further fragment ions of m/z 567.3 and 477.3 were contained in the spectrum, relevant to

Figure 3. HR CID MS² spectrum of the $[M + H + Ca]^{3+}$ ion of palytoxin of m/z 906.8. The most abundant peak of each ion cluster is shown together with cleavage numbers (#1–28) each fragment is relevant to. Ion assignment is reported in Tables 1 and 2, while cleavages are shown in Figure 1. In mass expansions of selected regions of the spectrum, ions due to different cleavages are displayed in different colors. It has to be noted that in most cases, each fragment ion was followed by several ions because of subsequent water losses; their assignment is reported in Table S1

part structures ranging from C-16 to C-41 (cleavage #6+#12) and from C-19 to C-41 (cleavage #9+#12), respectively. The latter ion was also contained in the MS³ spectrum of m/z 507.3 together with an additional fragment of m/z 447.3 relevant to part structure C-20 to C-41, due to cleavage #10+#12. Such results, confirmed assignment of the internal fragment ions contained in MS² of m/z 906.8 to the inner portion of palytoxin molecule ranging from C-9 to C-41 (Table 2), and provided further structural details on this region of the molecule, where some palytoxin-like compounds (ostreocin-D) present structural differences with palytoxin.

The A-side fragment of cleavage #4 was also fragmented within MS³ (precursor m/z 906.8>327.1) and MS⁴ (precursor m/z 906.8>327.1>309.1) experiments. The fragments obtained due to cleavages #1+#4, #2, and #3 (Table 2, Figure 1) provided information on the part structure ranging from C-8 to the A-side terminal of palytoxin which is a further region of the molecule where structural differences among palytoxin and most of its congeners (ostreocin-D, homo, bishomo-, neo-palytoxin, ovatoxin-b, -c, and -e) occur [5, 6, 10, 18].

HR CID MS² of m/z 2680.4 and 1331.7

HR CID MS² spectra of both $[M + H]^+$ of m/z 2680.4 and $[M + 2H - H_2O]^{2+}$ of m/z 1331.7 were acquired; while the spectrum of the former precursor was dominated by ions due to water losses and contained only two diagnostic fragments due to cleavage #16 and #19, the spectrum of the m/z 1331.7 confirmed several already observed cleavages and contained the additional cleavage #20 (Table 1, Figure 1).

HR PQD and HCD MSⁿ of m/z 2680.4, 1331.7 and 906.8

Alternative fragmentation modes, namely pulsed Q collision induced dissociation (PQD) and high energy collision dissociation (HCD) were also investigated. None of these experiments were as informative as HR CID MS² spectra reported above since they contained only some ions due to the observed cleavages (SI Table S2).

Fragmentation Pattern of Ovatoxin-a

Previous studies on ovatoxin-a allowed to assign its elemental formula C₁₂₉H₂₂₃N₃O₅₂, thus ascertaining that it presents two oxygen atoms less than palytoxin (C₁₂₉H₂₂₃N₃O₅₄). The elemental composition difference between ovatoxin-a and palytoxin was found to occur in the wide region ranging from position 9 to 115, on the basis of elemental formulae of A- and B-side fragments of cleavage #4 [17, 18]. So it was suggested that ovatoxin-a and palytoxin share the region ranging from position 8 to the A-side terminal of the molecule, at least in its elemental composition.

The approach proposed in the present study for palytoxin was used for ion assignment of all mono-, doubly-, and

triply-charged ions (Tables 1 and 2) contained in HR CID MSⁿ spectra of ovatoxin-a. Fragmentation pattern of ovatoxin-a closely paralleled that of palytoxin: all fragments contained in its HR CID MS² (Figure 4), MS³, and MS⁴ spectra were due to the same cleavages observed for palytoxin (Figure 1) with the exception of only four out of the 32 cleavages (cleavage #14, #20, #28, and #6+#12) that were lacking in ovatoxin-a. This pointed to a close structural similarity between the molecules, suggesting that they share the same backbone; however, there were some clues in the spectra that indicated the regions and, in some cases, the specific sites where structural differences between the two compounds occur. Such structural information could be drawn by comparison between elemental formulae of fragments of palytoxin and ovatoxin-a for each cleavage (Tables 1 and 2), the most salient details being reported below.

The regions near the A- and the B-side terminal were deduced the same in both palytoxin and ovatoxin-a, whereas structural differences between palytoxin and ovatoxin-a had to be comprised in the core of the molecules ranging from C-9 to C-78, where cleavages generated fragments with elemental composition having either 1 or 2 oxygen atoms less in ovatoxin-a than in palytoxin.

Particularly, fragmentation of ovatoxin-a between C-78 and C-79 (cleavage #19) generated a B-side fragment with the same elemental composition as the relevant fragment in palytoxin spectrum suggesting that the two molecules share the part of the molecule stretching from C-79 to C-115. This was further corroborated by the A-side fragments generated by cleavage #19, #21, #22, #23, #24, #25, #26, and #27 in ovatoxin-a: all of them presented two oxygen atoms less than the relevant fragments in palytoxin. Absence of one of the two oxygens was found to occur in the region ranging from C-53 to C-78, as the B-side fragment generated by cleavage between C-52 and C-53 (cleavage #18) in ovatoxin-a presented only one oxygen atom less than the relevant fragment in palytoxin. Unfortunately, no fragmentation occurred in the region from C-53 to C-78, so the exact site of difference between the molecules could not be identified.

Moving toward A-side terminal up to C-46, all A- and B-side fragments generated by cleavage #17, #16, and #15 in ovatoxin-a still contained one oxygen atom less than the relevant fragments in palytoxin. This suggested that the lack of a further oxygen atom should occur in the region stretching from C-45 to the A-side terminal and, thus, ovatoxin-a should parallel palytoxin structure in the region from position 46 to 52.

Cleavage #13 in ovatoxin-a originated a B-side fragment containing two oxygen atoms less than the relevant fragment in palytoxin and an A-side fragment with the same elemental composition as in palytoxin. This suggested that compared with palytoxin, ovatoxin-a lacks of an hydroxyl group either at C-44 or C-45; no choice could be made between such positions, as cleavage #14 did not occur in ovatoxin-a.

Table 1. Assignment of Fragments Contained in HR CID MS² Spectra of Palytoxin and Ovatoxin-a to Relevant Cleavages (Clv). Elemental Formulae of the Mono-Isotopic Ion Peaks (*m/z*) are Reported with Ion Charge State (1+, 2+, or 3+), Relative Double Bonds (RDB), and Errors (Δ , ppm)

	Palytoxin		Ovatoxin-a		
	Clv	A side <i>m/z</i> (1+,2+,3+) Formula RDB, Δ	B side <i>m/z</i> (1+,2+,3+) Formula RDB, Δ	A side <i>m/z</i> (1+,2+,3+) Formula RDB, Δ	B side <i>m/z</i> (1+,2+,3+) Formula RDB, Δ
	#4 ^a	327.1908 (1+) C ₁₆ H ₂₇ O ₅ N ₂ 4.5, -1.982	1187.1212 (2+) C ₁₁₃ H ₁₉₅ O ₄₈ N _{Ca} 17.0, -1.664 791.7502 (3+) C ₁₁₃ H ₁₉₆ O ₄₈ N _{Ca} 16.5, -1.274	327.1908 (1+) C ₁₆ H ₂₇ O ₅ N ₂ 4.5, -1.982	1171.1261 (2+) C ₁₁₃ H ₁₉₅ O ₄₈ N _{Ca} 17.0, -1.845 781.0867 (3+) C ₁₁₃ H ₁₉₆ O ₄₈ N _{Ca} 16.5, -1.578
	#11 ^a	446.2214 (2+) C ₄₀ H ₇₇ O ₁₇ N ₂ Ca 6.0, -2.009		438.2239 (2+) C ₄₀ H ₇₇ O ₁₆ N ₂ Ca 6.0, -2.143	
	#12 ^a	544.2943 (2+) C ₅₂ H ₉₂ O ₁₉ N ₂ Ca 8.0, -2.134	807.8892 (2+) C ₇₃ H ₁₂₅ O ₃₂ N _{Ca} 16.0, -0.968	536.2967 (2+) C ₅₂ H ₉₂ O ₁₈ N ₂ Ca 8.0, -2.431	799.8912 (2+) C ₇₃ H ₁₂₅ O ₃₁ N _{Ca} 16.0, -1.656
	#13 ^a	566.3074 (2+) C ₅₄ H ₉₆ O ₂₀ N ₂ Ca 8.0, -2.064	803.8868 (2+) C ₇₅ H ₁₂₅ O ₃₃ N _{Ca} 14.0, -0.795	566.3074 (2+) C ₅₄ H ₉₆ O ₂₀ N ₂ Ca 8.0, -2.064	787.8919 (2+) C ₇₅ H ₁₂₅ O ₃₁ N _{Ca} 14.0, -0.793
	#14 ^a	572.3074 (2+) C ₅₅ H ₉₆ O ₂₀ N ₂ Ca 9.0, -2.042	797.8864 (2+) C ₇₄ H ₁₂₅ O ₃₃ N _{Ca} 13.0, -1.303	nd ^c	nd ^c
	#15 ^a	596.3183 (2+) C ₅₆ H ₁₀₀ O ₂₂ N ₂ Ca 8.0, -1.397	782.8815 (2+) C ₇₃ H ₁₂₅ O ₃₂ N _{Ca} 13.0, -0.839 1526.8081 (1+) C ₇₃ H ₁₂₄ O ₃₂ N 12.5, -1.308	588.3204 (2+) C ₅₆ H ₁₀₀ O ₂₁ N ₂ Ca 8.0, -2.169	774.8833 (2+) C ₇₃ H ₁₂₅ O ₃₁ N _{Ca} 13.0, -1.807 1510.8129 (1+) C ₇₃ H ₁₂₄ O ₃₁ N 12.5, -1.510
	#16 ^a	633.3363 (2+) C ₅₉ H ₁₀₆ O ₂₄ N ₂ Ca 8.0, -1.931	745.8630 (2+) C ₇₀ H ₁₁₇ O ₃₀ N _{Ca} 13.0, -1.029 1452.7717 (1+) C ₇₀ H ₁₁₈ O ₃₀ N 12.5, -1.113	625.3387 (2+) C ₅₉ H ₁₀₆ O ₂₃ N ₂ Ca 8.0, -2.184	737.8650 (2+) C ₇₀ H ₁₁₇ O ₂₉ N _{Ca} 13.0, -1.776 1436.7765 (1+) C ₇₀ H ₁₁₈ O ₂₉ N 12.5, -1.324
	#17 ^a	647.3343 (2+) C ₆₀ H ₁₀₆ O ₂₅ N ₂ Ca 9.0, -1.051	1406.7657 (1+) C ₆₀ H ₁₁₆ O ₂₈ N 12.5, -1.520	639.3361 (2+) C ₆₀ H ₁₀₆ O ₂₄ N ₂ Ca 9.0, -2.226	1390.7711 (1+) C ₆₀ H ₁₁₆ O ₂₇ N 12.5, -1.311
	#18 ^a		694.8289 (2+) C ₆₅ H ₁₀₇ O ₂₈ N _{Ca} 13.0, -1.191		686.8319 (2+) C ₆₅ H ₁₀₇ O ₂₇ N _{Ca} 13.0, -0.539
		#19 ^a	948.4986 (2+) C ₉₀ H ₁₅₆ O ₃₇ N ₂ Ca 14.0, -1.575	804.4369 (1+) C ₃₀ H ₄₆ O ₁₆ N 7.5, -0.884	932.5036 (2+) C ₉₀ H ₁₅₆ O ₃₅ N ₂ Ca 14.0, -1.693
#20 ^b			744.4136 (1+) C ₃₇ H ₆₂ O ₁₄ N 8.0, -3.871	nd ^c	nd ^c
#21 ^a		1129.5956 (2+) C ₁₀₇ H ₁₈₆ O ₄₃ N ₂ Ca 16.0, -1.352	406.2218 (1+) C ₂₂ H ₃₂ O ₆ N 7.5, -1.512	1113.6007 (2+) C ₁₀₇ H ₁₈₆ O ₄₃ N ₂ Ca 16.0, -1.359	406.2215 (1+) C ₂₂ H ₃₂ O ₆ N 7.5, -2.250
#22 ^a		1144.6023 (2+) C ₁₀₈ H ₁₈₈ O ₄₆ N ₂ Ca 16.0, -0.096		1128.6057 (2+) C ₁₀₈ H ₁₈₈ O ₄₄ N ₂ Ca 16.0, -1.591	
#23 ^a		1174.6132 (2+) C ₁₁₀ H ₁₉₂ O ₄₈ N ₂ Ca 16.0, -0.192		1158.6154 (2+) C ₁₁₀ H ₁₉₂ O ₄₆ N ₂ Ca 16.0, -2.296	
#24 ^a		1215.6328 (2+) C ₁₁₅ H ₁₉₈ O ₄₉ N ₂ Ca 18.0, -0.911		1199.6333 (2+) C ₁₁₅ H ₁₉₈ O ₄₇ N ₂ Ca 18.0, -4.745	
#25 ^a		1222.6416 (2+) C ₁₁₆ H ₂₀₀ O ₄₉ N ₂ Ca 18.0, -0.108		1206.6488 (2+) C ₁₁₆ H ₂₀₀ O ₄₇ N ₂ Ca 18.0, 1.643	
#26 ^a		1235.6482 (2+) C ₁₁₈ H ₂₀₂ O ₄₉ N ₂ Ca 19.0, -1.098		1219.6512 (2+) C ₁₁₈ H ₂₀₂ O ₄₇ N ₂ Ca 19.0, -2.822	
#27 ^a		1236.6328 (2+) C ₁₁₇ H ₂₀₀ O ₅₀ N ₂ Ca 19.0, -5.167		1220.6386 (2+) C ₁₁₇ H ₂₀₀ O ₄₈ N ₂ Ca 19.0, -4.649	
#28 ^a		1321.6819 (2+) C ₁₂₆ H ₂₁₄ O ₅₃ N ₂ Ca 21.0, -3.357		nd ^c	

^a Ions in the HR CID MS² spectra of the [M + H + Ca]³⁺ ion of palytoxin (*m/z* 906.8) and ovatoxin-a (*m/z* 896.2), respectively.^b Ions in the HR CID MS² spectra of the [M + 2H - H₂O]²⁺ ion of palytoxin (*m/z* 1331.7) and ovatoxin-a (*m/z* 1315.7), respectively.^c nd = not detected.

Table 2. Assignment of the internal Fragments Contained in HR CID MS² and of the Fragments Contained in HR CID MS³ and MS⁴ Spectra of Palytoxin and Ovatoxin-a to Relevant Cleavages (Clv). Elemental Formulae of the Monoisotopic Ion Peaks (*m/z*) are Reported Together with Ion Charge State (1+, 2+, or 3+), Relative Double Bonds (RDB), and Error (Δ , ppm)

	Clv	Palytoxin <i>m/z</i> (1+,2+,3+) Formula RDB, Δ	Ovatoxin-a <i>m/z</i> (1+,2+,3+) Formula RDB, Δ
	#1+#4 ^{a,b}	234.1127 (1+) C ₁₃ H ₁₆ O ₃ N 6.5, 0.983	234.1123 (1+) C ₁₃ H ₁₆ O ₃ N 6.5, -0.726
	#2 ^b	127.0867 (1+) C ₆ H ₁₁ ON ₂ 2.5, 0.869	127.0866 (1+) C ₆ H ₁₁ ON ₂ 2.5, 0.082
	#3 ^{a,b}	281.1861 (1+) C ₁₅ H ₂₅ O ₃ N ₂ 4.5, 0.465	281.1862 (1+) C ₁₅ H ₂₅ O ₃ N ₂ 4.5, 0.821
	#4+#12 ^c	372.1975 (2+) C ₃₆ H ₆₄ O ₁₃ Ca 5.0, -1.593	364.2001 (2+) C ₃₆ H ₆₄ O ₁₂ Ca 5.0, -1.471
		743.3878 (1+) C ₃₆ H ₆₃ O ₁₃ Ca 5.5, -1.492	727.3927 (1+) C ₃₆ H ₆₃ O ₁₂ Ca 5.5, -1.780
	#4+#13 ^c	394.2105 (2+) C ₃₈ H ₆₈ O ₁₄ Ca 5.0, -1.777	394.2104 (2+) C ₃₈ H ₆₈ O ₁₄ Ca 5.0, -2.030
	#4+ #15 ^c	424.2211 (2+) C ₄₀ H ₇₂ O ₁₆ Ca 5.0, -1.568	416.2233 (2+) C ₄₀ H ₇₂ O ₁₅ Ca 5.0, -2.421
	#4+ #16 ^c	461.2389 (2+) C ₄₃ H ₇₈ O ₁₈ Ca 5.0, -2.720	453.2417 (2+) C ₄₃ H ₇₈ O ₁₇ Ca 5.0, -2.201
	#5+#12 ^{c,d}	657.3514 (1+) C ₃₂ H ₅₇ O ₁₁ Ca 4.5, -1.111	641.3559 (1+) C ₃₂ H ₅₇ O ₁₀ Ca 4.5, -2.051
	#6+#12 ^d	567.3189 (1+) C ₂₉ H ₅₁ O ₈ Ca 4.5, -2.708	nd
	#7+#12 ^{c,d}	537.3075 (1+) C ₂₈ H ₄₉ O ₇ Ca 4.5, -4.414	521.3137 (1+) C ₂₈ H ₄₉ O ₆ Ca 4.5, -2.411
	#8+#12 ^{c,d}	507.2982 (1+) C ₂₇ H ₄₇ O ₆ Ca 4.5, -2.182	507.2981 (1+) C ₂₇ H ₄₇ O ₆ Ca 4.5, -2.379
	#9+#12 ^{c,d,e}	477.2878 (1+) C ₂₆ H ₄₅ O ₅ Ca 4.5, -1.974	477.2868 (1+) C ₂₆ H ₄₅ O ₅ Ca 4.5, -4.069
	#10+#12 ^e	447.2777 (1+) C ₂₅ H ₄₃ O ₄ Ca 4.5, -1.068	447.2762 (1+) C ₂₅ H ₄₃ O ₄ Ca 4.5, -4.421

^a Ions in the HR CID MS³ spectra of palytoxin (*m/z* 906.8>327.1) and ovatoxin-a (*m/z* 896.2>327.1).

^b Ions in the HR CID MS⁴ spectra of palytoxin (*m/z* 906.8>327.1>309.1) and ovatoxin-a (*m/z* 896.2>327.1>309.1).

^c Ions in the HR CID MS² spectra of the [M + H + Ca]³⁺ ion of palytoxin (*m/z* 906.8) and ovatoxin-a (*m/z* 896.2).

^d Ions in the HR CID MS³ spectra of palytoxin (*m/z* 906.8>743.4) and ovatoxin-a (*m/z* 896.2>727.4).

^e Ions in the HR CID MS³ spectra of palytoxin (*m/z* 906.8>507.3) and ovatoxin-a (*m/z* 896.2>507.3).

Although elemental formula of the A-side fragment of cleavage #13 was the same for both ovatoxin-a and palytoxin, structural details in the region stretching from C-43 to A-side terminal cannot be assumed to be the same for the two molecules, as fragment ions obtained by cleavages within this region were different between ovatoxin-a and palytoxin. Cleavage #12, between C-41 and C-42, unlike cleavage #13, generated A- and B-side fragments, both lacking one oxygen atom in ovatoxin-a compared with palytoxin. This indicated that in ovatoxin-a, an additional hydroxyl group was likely present at C-42 and that a further oxygen atom must be missing in the region from C-41 to the A-side terminal. The A-side fragment of cleavage #11, containing one oxygen atom less in ovatoxin-a than in palytoxin, allowed locating the missing oxygen in the more restricted region from C-30 to the A-side terminal, and extended structural similarity between palytoxin and ovatoxin-a to the region from C-41 to C-31.

A comparative analysis of the internal fragments due to double cleavages (Table 2) in ovatoxin-a and palytoxin suggested that the former lacks the hydroxyl group at C-17. This was deduced by comparing fragments generated by two subsequent double cleavages, namely cleavage #8+#12 and #7+#12, relevant to part structures ranging from C-18 to C-41 and from C-17 to C-41, respectively; while the fragment due to cleavage #8+#12 had the same elemental composition in both molecules, the fragment due to cleavage #7+#12 presented one oxygen atom less in ovatoxin-a than in palytoxin.

Finally, comparison between ovatoxin-a and palytoxin of fragments due to cleavages #10+#12, #9+#12, #5+#12, #4+#12, #3, #2, and #1+#4 (Table 2, Figure 1) suggested that the regions ranging from C-30 to C-18 and from C-16 to the A-side terminal were the same in both molecules.

Summing-up, on the basis of the above results, ovatoxin-a, compared with palytoxin, (1) lacks three hydroxyl groups, one at C-17, one at C-44 or C-45, and one in the region C-53 to C-78, respectively; (2) presents an additional hydroxyl group at C-42, similarly to 42-hydroxy palytoxin and ostreocin-D [6, 7]; (3) presents the same structural features as palytoxin in the regions ranging from the A-side terminal to C-16, from C-18 to C-41, from C-46 to C-52, and from C-79 to the B-side terminal.

A concurrent NMR study we carried out on pure ovatoxin-a [20] confirmed the presence in the molecule of the hydroxyl group at C-42, the absence of the hydroxyl group at C-17, and most importantly allowed to exactly locate the missing hydroxyl groups at C-44 and C-64, thus demonstrating that ovatoxin-a is the 42-hydroxy-17,44,64-trideoxy derivative of palytoxin.

Conclusion

Interpretation of the complex fragmentation pattern of palytoxin demonstrated that much structural information can be obtained from the diagnostic fragment ions contained

in positive HR MSⁿ spectra of palytoxin, as the molecule undergoes fragmentation at many sites of its backbone with the only exception of the regions containing methylene chains and the region ranging from C-53 to C-78.

The fragmentation pattern of palytoxin was used as a template to gain detailed structural information on the major component of *O. ovata* toxin profile, ovatoxin-a, directly in the algal extract. A concurrent NMR study that fully elucidated the structure of ovatoxin-a [20] proved validity of the HR LC-MSⁿ-based approach in confirming all the structural hypotheses made.

Although the observed fragmentation was not as complete as that reported by Ukena et al. [10] for the 2-sulfobenzoic acid derivative of ostreocin-D, the present approach required no derivatization. Therefore, HR LC-MSⁿ spectra of palytoxin and ovatoxin-a could be used as fingerprints for a confident identification of these molecules in crude extracts. Since natural extracts are usually contaminated by a complex mixture of palytoxin-like compounds (mascarenotoxins, ovatoxins, ostreocins, etc.) [10, 17, 18, 21], this approach could also be used for a preliminary structural characterization of the unknown minor components contained in the extracts at levels too low for NMR studies.

The present study also showed the key role played by divalent cations, and particularly by calcium, to form adduct ions of palytoxin in ESI MS spectra. This is a quite peculiar behavior that should be taken into account in future MS studies on palytoxin congeners for correct assignment of triply-charged ions in their full MS spectra and for interpreting their MSⁿ spectra. Palytoxin affinity for divalent cations could represent an interesting aspect to address studies on the molecular mechanism of action of palytoxin-like compounds [22].

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