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Gas-Phase Fragmentation Reactions of Protonated Pumiliotoxin (+)-251D and Allopumiliotoxin (+)-267A in ESI-MS/MS

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In recent years, there has been great interest in understanding the chemistry of natural products from different organisms and their role in ecological processes. Some species of frogs sequester and metabolize dietary alkaloids obtained primarily from mites and ants as chemical defense. Most of these studies have employed gas chromatography techniques coupled with mass spectrometry, which restricts the possibility of observing more polar metabolites. In the case of pumiliotoxin (+)-251D and allopumiliotoxin (+)-267A, the fragmentation mechanisms in electrospray ionization systems with collision-induced fragmentation are undescribed. The present study aims to elucidate the fragmentation pathways of these two toxins. For this purpose, we used direct infusion of toxins in a time-of-flight hyphenated quadrupole electrospray ionization tandem mass spectrometry (ESI-MS/MS) system. Different collision energies were applied, and the data rationalized from the concepts of reaction mechanisms. The joint analysis allowed to present a robust map of fragmentation opening perspectives for their application in studies of the occurrence of new polar analogues.

Keywords: allopumiliotoxin (+)-267A, alkaloid, fragmentation mechanism, mass spectrometry, pumiliotoxin (+)-251D

Introduction

Amphibians have evolved a large arsenal of chemical signals, including volatile and nonvolatile pheromones for sexual communication[1,2] and toxins to deter predation,[3-5] parasitization,[6,7] and pathogenic infections.[8-10] Although most amphibian defensive chemicals are endogenous, controlled experiments involving the oral administration of lipophilic alkaloids have revealed that so-called "poison frogs" (a polyphyletic collection of diurnal, brightly colored, microphagous frogs distributed in the families Bufonidae,[3] Dendrobatidae,[11,12] Eleutherodactylidae,[13] Mantellidae[14] and Myobatrachidae)[3] sequester their defenses[15-17] from dietary arthropods, primarily ants and mites.[10,16,18-20]

Alkaloids accumulate in the skin and are detected in the liver in higher concentrations as well as muscles and kidney in lower amounts.[21,22] The accumulation occurs within a couple of hours.[15,22-25] Further, biomodifications of sequestered alkaloids are known, including hydroxylation of the pumiliotoxin (PTX) (+)-251D to the allopumiliotoxin (aPTX) (+)-267A in the dendrobatids *Oophaga pumilio*, *Adelphobates galactonotus*, *Dendrobates auratus* and *Dendrobates tinctorius*,[10,15,25] *N*-methylation in decahydroquinoline (DHQ) in *Adelphobates galactonotus*,[24] and enzymatic reduction and/or hydroxylation of dietary PTX 307A into PTXs of molecular weights 309, 323, and 325 in species of the myobatrachid *Pseudophryne*.[26]

To date classical gas chromatography combined with electron ionization mass spectrometry (GC-MS) has been the preferred tool for chemical analysis and structural elucidation.[22,24,27-32] Although GC-MS is still one of the best strategies for thermally stable and volatile compounds,[12,33] recent technological advances have allowed a more complete analysis of chemical signals at various molecular levels by different sources configurations in mass spectrometry.[34] For example, liquid chromatography coupled to mass spectrometry with an electrospray (ESI) source is a powerful tool in the analysis and identification of a greater number of substances since charge transfer is a lighter process and allows analyzing from medium polarity compounds to polar substances.[35] Recently, the investigation of biomodification of PTX (+)-251D into the more potent aPTX (+)-267A used for the first time

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high performance liquid cromatography technique coupled mass spectrometry with electrospray ionization (HPLC-ESI-MS).[25] Although, this more sensitive technique lacks commercially available internal standards which can present a challenge for structural confirmation and quantitative analysis.[36] The authors describe ions related to metabolites with two additional hydroxylation structures that would not be possible to observe in GC-MS, and they also report problems in detecting one of the substances that could result from its rapid metabolization or ionization problems. Reactions at the source can lead to unexpected ions such as radical cations,[37,38] coordination with metals other than sodium,[39] in addition to all ionic suppression phenomena,[40,41] indicating the need for additional techniques to characterize new PTX, aPTX, and homopumiliotoxin (hPTX) derivatives. With this in mind, the objective of this study was to carry out a systematic analysis of the fragmentation reactions in ESI in order to characterize the structure of all fragment ions in MS/MS experiments of PTX (+)-251D and aPTX (+)-267A for application in metabolization studies.

Experimental

Chemicals and reagents

High performance liquid chromatography (HPLC) grade methanol and P.A. grade acetic acid were purchased from JTBaker (Phillipsburg, NJ, USA). The pumiliotoxin (PTX (+)-251D) used for feeding the frogs as for the ultra performance liquid chromatography (UPLC) analysis was obtained from Ralph Saporito.[42]

Animals: *Adelphobates galactonotus* toxin administration

The carcass of *Adelphobates galactonotus* frogs was used as source of the aPTX (+)-267A. The carcass was extracted with methanol following the article published by Saporito *et al.*[43] and Jeckel *et al.*[31] The sampling authorization was obtained with the number SISBIO: 54640-1b and the research was registered and approved by the Comissão de Ética no Uso de Animais under No. 402/2023 - CEUDo Institute of Biosciences of the University of São Paulo. The samples were stored at the Laboratório de Anfíbos da Universidade de São Paulo.

Mass spectrometry

High-resolution electrospray ionization (ESI) mass spectrometry analyses were performed on a micrOTOF-QII mass spectrometer (Bruker Daltonics, USA) fitted with time-of-flight analyzer (TOF). The spectrometer was operated in the positive ionization mode and the capillary voltage was fixed at 3.5 kV. For accurate mass analysis, the TOF analyzer was calibrate with sodium trifluoroacetic acid TFA–Na^+ as the internal standard. For MS/MS spectra, protonated molecule was selected and fragmented at different laboratory frame collision energies, Elab, (ranging from 5 to 50 eV). N_2 was employed as collision gas. Energy-resolved plots were obtained from ion intensities variation for each Elab applied. Finally, samples were inserted by direct infusion in a syringe pump.

Results and Discussion

Interpretation of the product ion spectrum showed stability of the protonated molecule, and only the water elimination fragment could be observed up to collision energies greater than 30 eV (Figure 1). Some natural products present this difficulty, being a characteristic directly related to the physical chemistry properties of the analyte and requiring a careful strategy in data analysis.[44,45] Figure 1 clearly demonstrates the need for data acquisition, the structural determination of analogues, using energies higher than 45 eV. Thus, as with some natural products containing fused rings, all other observed fragment ions are formed from $[M - 18]^+$.[44,45]

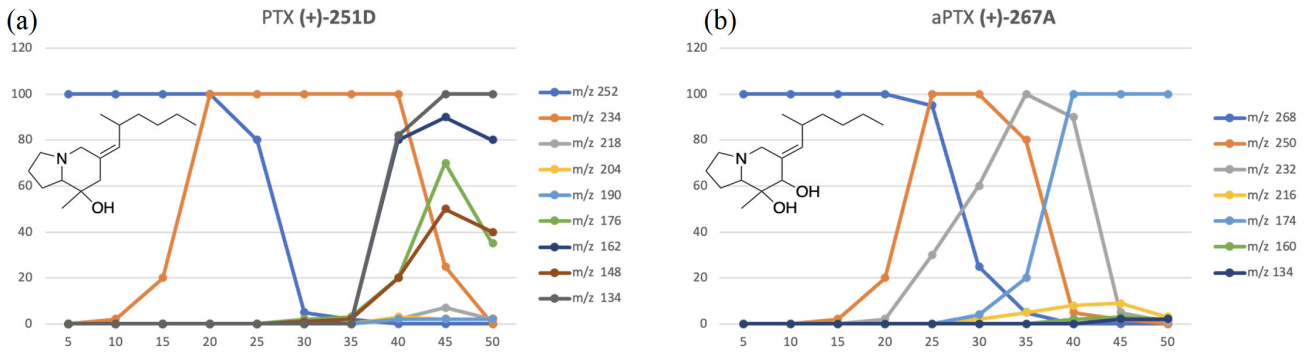

Figure 1. Energy-resolved plots from the ESI-MS/MS spectra of pumiliotoxin (+)-251D (a) and allopumiliotoxin (+)-267A (b). All the mass spectra and energy plots are available at Supplementary Information section.

As commented above, the main ion for the beginning of the fragmentation is the elimination of water. This elimination can occur by two mechanisms, charge retention fragmentations (CRF) or charge migration fragmentations (CMF).[46] In the comparison between the amine and the alcohol, protonation is more likely to occur at the N atom (which favors the CRF mechanism), as previously described for similar models of six- and five-membered cycles containing N.[47] Furthermore, CMF reactions pass through a stage of carbocation formation, which would be unfavorable compared to a possible CRF reaction, like a remote hydrogen rearrangement.[46] An additional gain of stability can occur by the formation of a fragment where the double bonds stay conjugated, confirming the mechanism.[46]

From the $[M - 18]^+$ (Figure 2), a series of other fragments is formed by the elimination of alkanes and alkenes (Figure 2). All these CRF reactions allowed the ions at *m/z* 204, 190, and 176 resulting from the elimination of alkanes, which require a higher transition energy, but they are viable in the gas phase, as previously observed in other cyclic alkaloids.[48]

Finally, in the second collection, the ions were observed at low intensity. These ions can be obtained after an open ring process lead by a conjugate carbocation formation. The new ion can be in equilibrium returning to the exactly $[M - 18]^+$ or the electrons form the N-atom can attack in the opposite side leavening a new cycle formation (Figure 1), which can eliminate the side chain by a CMF mechanism resulting in the minor ion at *m/z* = 148.[46] Another two ions can occur after ring opening via an initial Grob-Watter gas phase reaction[44,45] that yielded the ion at *m/z* = 218, which will form the ion at *m/z* = 162 after the CRF reaction.[46]

The next step was the analysis of the gas phase fragmentation reaction of the aPTX in electrospray source ionization with quadrupole time of flight (ESI-QTof). As expected, the introduction of a second hydroxyl group at alpha position of the oxo-ring double bound and near by the previous hydroxyl group does not increase the number of observed ions or the intensity of fragmentation. This fact can be explained by two restrictions effects for water elimination.[46] Then, to form an equilibrium between CMF and CRF mechanisms it requires two steps. An initial 1,4 water removal (Figure 3) followed by a similar E2 mechanism leading to a stable conjugate system.[46] The comparison of

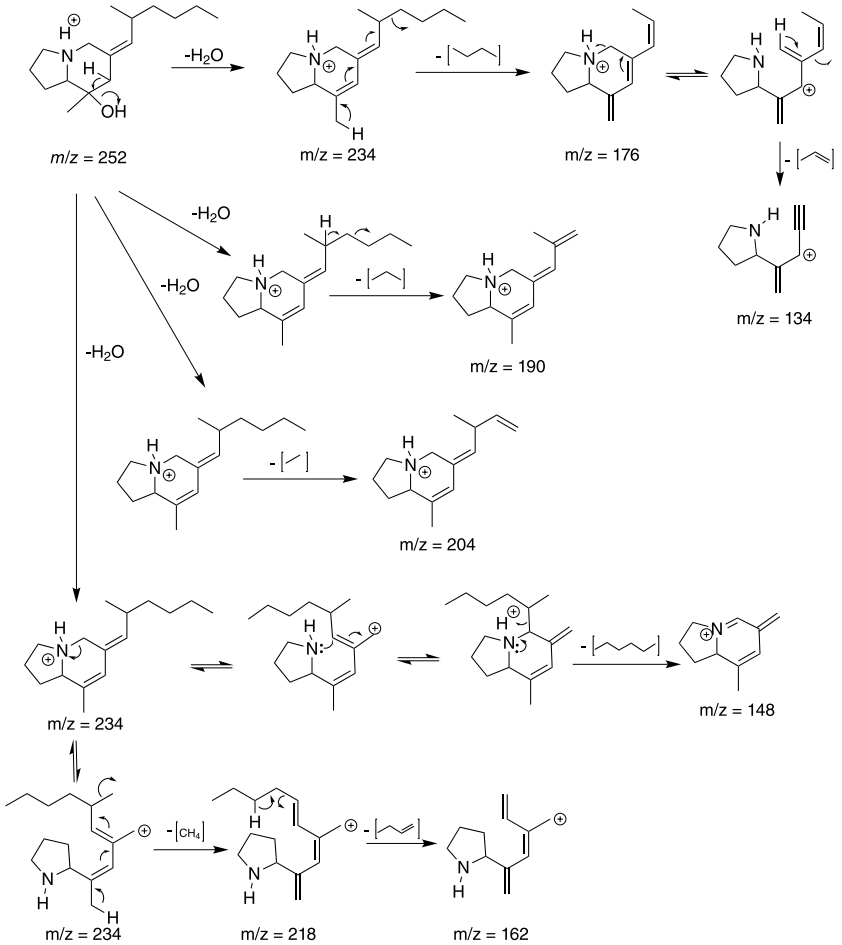

Figure 2. Fragmentation mechanism proposed for PTX (+)-251D.

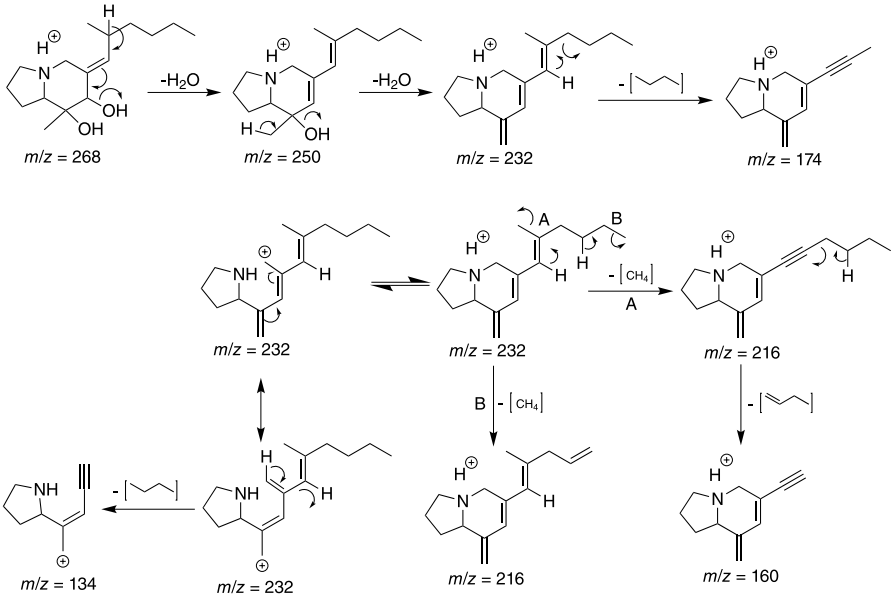

Figure 3. Fragmentation mechanism proposed for aPTX (+)-267A.

the MS/MS of both toxins suggest that the ions after loss of water do not show a collection of neutral eliminations of alkanes and alkenes, at least with some intensity. Again, the strong stability is responsible for the inhibition, and only the ion at *m/*z = 174 has some significance. The other signal at *m/*z 216, 134, and 160 cannot be observed at more than 10% in all applied energies (see Figure 1 and Supplementary Information section). Finally, our results open the perspective for biologists to investigate more possible alkaloid metabolites through liquid chromatography coupled tandem mass spectrometer (LC-MS/MS) instead of GC-MS, which cannot detect the more polar compounds.

Conclusions

Our results show that the structural determination of new analogues is possible, since the first elimination of water opens three different consecutive fragmentation pathways, and that substitutions in different positions, as with aPTX, can alter this balance. Further, the different pathways are eliminated from different parts of the skeleton, allowing us to better understand the location of any new functionalization.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

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