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## Indolizidine 239Q and Quinolizidine 275I. Major alkaloids in two Argentinian bufonid toads (*Melanophryniscus*)

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### Abstract

Alkaloid profiles in skin of poison frogs/toads (Dendrobatidae, Mantellidae, Bufonidae, and Myobatrachidae) are highly dependent on diet and hence on the nature of habitat. Extracts of the two species of toads (*Melanophryniscus klappenbachi* and *M. cupreuscapularis*) from similar habitats in the Corrientes/Chaco Provinces of Argentina have similar profiles of alkaloids, which differ considerably from profiles from other *Melanophryniscus* species from Brazil, Uruguay and Argentina. Structures of two major alkaloids **239Q** (**1**) and **275I** (**2**) were determined by mass, FTIR, and NMR spectral analysis as 5Z,9Z-3-(1-hydroxybutyl)-5-propylindolizidine and 6Z,10E-4,6-di (pent-4-enyl) quinolizidine, respectively. A third alkaloid, **249F** (**3**), is postulated to be a homopumiliotoxin with an unprecedented conjugated exocyclic diene moiety.

### Keywords

Alkaloids; ants; bufonidae; dietary arthropods; mass spectrometry; mites

### 1. Introduction

Alkaloids in skins of anurans were first discovered for a Colombian poison-dart frog (*Phylllobates aurotaenia*) of the neotropical family Dendrobatidae (Tokuyama *et al.*, 1969).

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Following this discovery, numerous other alkaloids of diverse structural classes were subsequently identified from other species of dendrobatid frogs, and by 1978 they were collectively referred to as “dendrobatid alkaloids” (Daly *et al.*, 1978). Then in 1984, such dendrobatid alkaloids were reported from a bufonid toad from South America (*Melanophryniscus moreirae*), two mantellid frogs from Madagascar, and a myobatrachid toadlet from Australia (Daly *et al.*, 1984). Subsequently, a diverse array of alkaloids was found to be present in skin extracts of other species of *Melanophryniscus* toads (Garraffo *et al.*, 1993; Mebs *et al.*, 2005; Daly *et al.*, 2007). It now appears that all the skin alkaloids of the dendrobatid, mantellid and bufonid anurans are sequestered from dietary alkaloid-containing arthropods (Daly *et al.*, 2002; Jones *et al.*, 1999; Saporito *et al.*, 2004, 2006, 2007a, b; Takada *et al.*, 2005), while the skin pseudophrynamines of the myobatrachid frogs (*Pseudophryne*) are produced by the frogs (Smith *et al.*, 2002). Both dietary specialization and microphagy have been proposed as significant components in the ecology and evolution of sequestered defenses in poison frogs, and in dendrobatid poison frogs, multiple origins of dietary specialization have been proposed (see Toft, 1995; Caldwell, 1996; Vences *et al.*, 1998; Santos *et al.*, 2003; Darst *et al.*, 2005). Recently, the over 800 alkaloids detected in amphibian skin extracts were summarized along with their occurrence in the four above-mentioned anuran families and, for some, the discovery of putative dietary arthropod sources (Daly *et al.*, 2005). The present report documents the occurrence of alkaloids in two bufonid species (*Melanophryniscus klappenbachi* and *M. cupreuscapularis*) from neighboring locales in the Corrientes/Chaco Provinces of Argentina. Remarkably, three of the major alkaloids in these extracts had not been previously detected in any extracts of anuran skin and represent new alkaloids from poison frogs. Structures for the 3,5-disubstituted indolizidine **239Q** (**1**) and the 4,6-disubstituted quinolizidine **275I** (**2**) are proposed based on mass spectral and FTIR spectral analysis and in the case of **239Q**, NMR spectral analysis. The structure of the third major alkaloid **249F** remains uncertain, but is postulated to be that of an unusual homopumiliotoxin diene (**3**). Structures of **1**, **2**, and **3** and three other minor alkaloids (**4**, **5**, **6**) found in extracts of skins of these two bufonid species are shown in Figure 1.

## 2. Materials and Methods

### 2.1. Bufonid toads (*Melanophryniscus*)

Specimens of *Melanophryniscus klappenbachi* were collected in February 1999 in fields near the Aeropuerto Viejo, Resistencia, Chaco, Argentina. The eleven skins (3.3 g wet weight) were placed in methanol. Specimens of *Melanophryniscus cupreuscapularis* were collected also in February 1999 near Perichón, Corrientes, Argentina. The eleven skins (1.3 g wet weight) were placed in methanol.

The *Melanophryniscus* specimens were dissected and prey items were classified by taxon, to family or ordinal level, and life stage, using a binocular microscope. Items that could not be identified under any order but which were clearly of animal origin were classed only as individuals. Abundance of each of the various prey items was estimated from digestive tract contents. For each taxon, the frequency of occurrence (number of digestive tracts containing that particular taxon divided by the total number of digestive tracts analyzed) was calculated according to the formula of Lescure (1971).

### 2.2. Instrumentation

Mass spectral data [EI-MS and CI-MS (NH<sub>3</sub>)] were obtained with a Finnigan GCQ instrument, having a Restek RTX-5MS capillary column (30 m, 0.25 mm i.d.) programmed from 100° to 280° C at 10° C per min. GC-FTIR and EI-MS spectra were obtained in series with a Hewlett-Packard model 5890 gas chromatograph, having an HP-5 fused silica-bonded capillary column (30 m, 0.32 mm i.d.) programmed from 100° to 280° C at a rate of 10° C per min and interfaced

with a Hewlett-Packard model 5971 Mass Selective Detector and a Model 5965B IRD with a narrow band ( $4000\text{-}750\text{ cm}^{-1}$ ) detector. A Hewlett-Packard ChemStation was used to generate EI-MS and FTIR spectra. High resolution GC-MS data was generated with a Waters GCT instrument. The  $^1\text{H-NMR}$  spectra were measured with a Varian VXR-500S spectrometer. An Agilent Model 1100 LC with a binary pump and UV detection at 260nm was used for the alkaloid work and purification. This LC with a DAD detector (Thermo-Finnigan UV6000LP) was used interfaced with a Thermo-Finnigan LCQ mass spectrometer in the APCI mode for the bufadienolide / cardenolide work. Typically a vaporizer heater at  $480^\circ\text{C}$  and capillary heater at  $150^\circ\text{C}$  and a flow rate of  $0.5\text{ mL / min}$  was used.

### 2.3. Isolation and Analysis

The methanol extracts were subjected to acid-base partitioning as described (Garraffo *et al.*, 1993). The resultant alkaloid fractions were investigated by GC-MS and GC-FTIR spectral analysis (see Results). About one-half of the alkaloid fraction from *Melanophryniscus klappenbachi* in methanol was concentrated to  $125\text{ }\mu\text{L}$  and  $25\text{ }\mu\text{L}$  portions were subjected to HPLC fractionation. A reversed-phase column (Phenomenex column AQUA-125A, C18,  $250\text{ mm} \times 4.6\text{ mm i.d.}$  with particle size  $5\text{ }\mu\text{m}$ ) was used with  $\text{CH}_3\text{CN}$  (0.1% HOAc)- $\text{H}_2\text{O}$  (0.1% HOAc) and a gradient from 10:90 to 90:10 over a 30 min period with a flow rate of  $0.5\text{ mL / min}$ . Thirty fractions of  $0.5\text{ mL}$  were collected, and alkaloid content assayed by GC-MS. The fractions containing nearly pure **239Q** were combined and used for NMR spectral analyses. APCI mass spectrometry with a Thermoquest-Finnigan LCQ LC-MS and the above solvent system and program yielded purified **239Q** with a protonated molecular ion of  $m/z$  240 and a single fragment showing loss of water from  $m/z$  240.

## 3. Results

The alkaloids detected and identified by GC-MS and GC-FTIR spectral analysis are documented in Table 1.

The GC-MS chromatograms are shown in Figure 2A and 2B. The FTIR spectra for the three major new alkaloids (**239Q**, **275I**, and **249F**) are shown in Figure 3A, 3B and 3C. The spectral data are as follows:

### 239Q (1)

The major GC peak at  $rt$  10.70 min had a MW of 239 as determined with CI-MS ( $\text{NH}_3$ ) and had one exchangeable hydrogen as indicated by  $m/z$  242 using  $\text{ND}_3$ . The molecular formula was  $\text{C}_{15}\text{H}_{29}\text{NO}$  by HR-MS (mass measured on  $m/z$  238 (M-H)) with a base peak at  $m/z$  166, mass measured as  $\text{C}_{11}\text{H}_{20}\text{N}$  indicating a  $\text{C}_4\text{H}_9\text{O}$  loss. The exchangeable hydrogen is in this fragment as CIMS ( $\text{ND}_3$ ) still had a detectible EI fragment (22%) at  $m/z$  166. A minor mass fragment at  $m/z$  196 (ca. 10%) by HR-MS fits  $\text{C}_{12}\text{H}_{22}\text{NO}$ , indicating a propyl fragment is cleaved. In the structure we propose for **239Q** (see Fig. 1), this propyl loss can occur from both the 5- position by  $\alpha$ -cleavage and from the 3-(1-hydroxybutyl) group by cleavage at the 1'- position. There was also a significant (10%) fragment ion at  $m/z$  70. LC-MS with APCI indicated a major ion at  $m/z$  240 with only a loss of 18 amu ( $\text{H}_2\text{O}$ ) producing an ion at  $m/z$  222 (6%). The presence of a hydroxyl group was confirmed by GC-FTIR where the vapor phase infrared spectrum indicated a very broad absorption, centered at  $3531\text{ cm}^{-1}$  suggesting a hydrogen-bonded hydroxyl group ( $\nu_{\text{O-H}}$ ). Another absorption at  $1209\text{ cm}^{-1}$  ( $\nu_{\text{C-O}}$ ) is also seen. The FTIR spectrum with a moderate Bohlmann band at  $2798\text{ cm}^{-1}$  (see Fig. 3A) was typical of a 3,5-disubstituted indolizidine with the 5Z, 9Z configuration. Quantitation by GC-MS with a standard of another 3,5-disubstituted indolizidine, **239AB** ( $3\text{ }\mu\text{g}/\mu\text{L}$ ) indicated **239Q** was present in the *M. klappenbachi* extract at a concentration of  $16.5\text{ }\mu\text{g}/\mu\text{L}$ .

Thus the mass and IR spectral data indicated an indolizidine ring structure with  $\alpha$ -propyl and  $\alpha$ -hydroxybutyl side-chains and the three  $\alpha$ -hydrogens being on the same molecular face. Frog skin alkaloid **239AB** has a 4'-hydroxybutyl substituent at C-3 and a propyl substituent at the C-5 position. We assumed *ab initio* that the propyl and butyl side-chains in **239Q** would likewise be at C-5 and C-3 respectively, as usually (but not invariably) the odd-carbon side-chain is at C-5 in frog skin or arthropod indolizidines, and the even-carbon side-chain is at C-3. The position of the hydroxyl group in the butyl side-chain of **239Q** remained unassigned as did conclusive proof of the ring position of the side-chains. The IR spectrum indicated the hydroxyl group was hydrogen-bonded to nitrogen and consequently was likely at either the 1' or 2' position of the butyl group and not the terminal position as seen previously with **239AB** (Daly *et al.* 2005). Another naturally occurring regioisomer, **239CD**, also had a terminal hydroxyl in the propyl group.

An  $^1\text{H-NMR}$  spectrum on HPLC purified **239Q** was surprisingly complex. The deuteriochloride in  $\text{d}_6$ -acetone revealed two methyl triplets ( $\delta$  0.93, 0.89), not in the expected 1:1 ratio, but rather in *ca.* a 2:1 ratio, respectively. The hydrogen on the carbon bearing the hydroxyl group is likewise seen as a pair of doublets ( $\delta$  4.19, 4.20) however in *ca.* a 3:2 ratio. Three downfield signals ( $\delta$  3.1–3.8) are assigned to the  $\alpha\text{-CH-ND}^{\oplus}$  protons and  $\delta$  4.2 to the  $\text{CH-OD}$  proton. Proton H-3 shows up as a single dd signal at  $\delta$  3.73 ( $J = 10.4, 2.6$  Hz). This was shown by a clear NOESY cross peak to be coupled with the downfield  $\text{CH-OD}$  (H-10) signal, even though no H-3—H-10 scalar coupling is detected. The broad triplet signal assigned to H-5 shows no such cross peak but only methylene hydrogen cross peaks ( $\delta$  1.7–2.5) indicating that the hydroxybutyl group is at C-3 as anticipated. Molecular modeling and energy-minimized structures with an imposed hydrogen bond ( $^{\oplus}\text{N-D}\cdots\text{O(D)-C-10}$ ) was consistent with the hydroxyl group being adjacent to the five-membered ring (i.e. the 1'-butyl position) and having a dihedral angle ( $\theta_{3-10}$ ) approaching  $100^\circ$  and  $J \approx 0$  Hz if the  $\text{R}^*$  configuration is assigned to C-10. Consequently H-3 shows only coupling with two hydrogens with large and small coupling constants assigned to  $2\beta$ - and  $2\alpha$ -ring protons, respectively. On 1D irradiation, the H-10 proton showed coupling only with multiplets at  $\delta$  1.37 and 1.50, assigned to side-chain H-11 protons. Other 1D- and 2D H-H (TOCSY and Relay-H) couplings are reported in Table 2 as well as several relevant NOESY cross peaks.

The complexity of the NMR spectrum, we think, arises from a conformational equilibrium between the conformers A and B, in which the six-membered ring is significantly in a boat conformation to minimize the butyl-propyl side-chain steric interactions resulting from the  $\text{R}^*$  configuration at C-10. If the six-membered ring exists in a 5:3 boat:chair proportion and if the terminal methyls (C-13, C-16) overlap in the chair but not in the boat, where the terminal methyl (C-16) of the propyl group is shielded and slightly upfield, then a 2:1 overall proportion of methyls at C-13 and C-16 arises. Also the two signals observed for HO in approximately a 3:2 ratio, most clearly and directly seen in the back-exchanged spectrum discussed below (but also for H-10 in the initial spectra), are explained (63% A : 37%B). See Figure with Table 2. The five-membered ring is locked into the envelope conformation in both A and B by the H-bond. Because H-3 is a simple, uncomplicated dd signal, we do not ascribe the complexity of H-10 to two different hydroxyl C-10 diastereomers for the two resulting dihedral angles of  $\text{H}_3\text{-H}_{10}$  cannot both have  $J$ s of zero. Furthermore, the GC chromatogram of **239Q** shows only a single homogeneous peak, not two peaks as typically seen with a diastereomeric pair. Invoking however a conformational equilibrium between 5 parts A and 3 parts B does explain the observed  $^1\text{H-NMR}$  spectrum. The  $\alpha$ -proton at H-5 is a complex multiplet resembling a triplet with two large  $J$ s and several small or medium  $J$ s. We think this reflects conformational equilibria involving the six-membered ring only. Minimized energies are 36.5 and 29.8 kcal/mol for A and B, respectively.

Additional complications arose when the sample was reexamined after standing in a capped tube for one week and several new signals appeared. These were ascribed to a slow exchange occurring with the OD and ND groups when ambient H<sub>2</sub>O in the vapor above the acetone in the NMR tube exchanged for those deuteriums in **239Q**•DCl in d<sub>6</sub>-acetone. The signals for H-3, H-5 and H-9 had all become more complex as additional coupling with <sup>⊕</sup>N-H now exists. New signals at δ 5.78 and 5.80 (3:2 parts respectively) now are seen which we assign to the HO-C(10) signals, A  $J \approx 5$  Hz is seen with the H-10—OH doublet in each of the two conformers. The new N-H signal is seen as a very broad singlet at δ 10.7 and on irradiation, the H-3, H-5 and H-9 signals all simplify as a *trans* coupling with <sup>⊕</sup>NH is removed. On irradiation of the HO signals at C-10, H-10 becomes a narrower but still complex multiplet, indicating that the H-10 signals are a composite now of HOCH- and the original CH(10)—CH(11) couplings. Irradiation of H-11 at δ 1.37 removes the  $J_{10-11}$  coupling and with higher power, collapses H-10 to a singlet and also the upfield methyl triplet at δ 0.89 becomes a doublet indicating that all the additional complications seen in the <sup>1</sup>H-NMR on standing one week can all be explained by a back-exchange. Irradiation of H-10 removes a small  $J$  (*ca.* 1 Hz) from HO coupling with H-10, leaving a doublet of *ca.* 5 Hz. On reexamination of the NMR sample by GC-MS, no change was seen in the mass spectrum or retention time, indicating all the NMR changes are due to proton back-exchange. On additional standing, signals for H<sub>2</sub>O (δ 2.85) and acetone (δ 2.05) are observed. Reexamination of 1D irradiations affecting the H-3, H-5, H-9 and H-10 protons in the back-exchanged spectra were unchanged from the original irradiations indicating the structure was unchanged.

Because of the complexity of the proton NMR spectrum, we found 2D spectra less useful than 1D irradiations. We focused mostly on those involving the four downfield signals and the upfield methyls. Irradiation at δ 1.35–1.37 (H-11) perturbs both the doublets at H-10 to a nearly overlapping pair of signals approximating broad singlets and collapses the upfield methyl triplet (H-16, δ 0.89) to a doublet. Here one of the H-15 protons in the boat configuration is affected as well as H-11 in both conformers A and B. Molecular modeling indicates that one H<sub>10</sub>–H<sub>11</sub> has a  $J$  approaching zero. This proton at H-11 in A/B is shown to fall at δ 1.50 on irradiation of the pair of doublets of H-10 (δ 4.19) where a change is seen at both δ 1.50 and δ 1.36–1.40. Irradiation at δ 1.60 changes the downfield triplet at H-5 to a doublet-like signal. We think that the signal for H-5 (a broad triplet) is complex because of the conformational equilibrium and this is reflected in the couplings of H-5 with protons at δ 1.77, 1.90–1.93, 2.26–2.35. Additional assignments in Table 2 reflect these 1D-irradiations. The <sup>1</sup>H-NMR data for **239Q**•DCl is tabulated in Table 2 below.

Thus, the most likely structure for **239Q** is that of a 3-(1-hydroxybutyl)-5-propyl indolizidine (**1**) (Figure 1), but because of certain anomalies in the NMR spectra, further studies are warranted if additional pure compound can be isolated. The postulation of the conformational equilibrium would, for example, be tested by spectra taken at various temperatures.

## 2751 (2)

The proposed structure of major alkaloid 2751 (**2**), shown in Figure 1, is based on MS and FTIR spectral analyses. High resolution MS indicated an empirical formula of C<sub>19</sub>H<sub>33</sub>N. The mass spectrum was dominated by loss of C<sub>5</sub>H<sub>9</sub> to afford a base peak at  $m/z$  206. A McLafferty rearrangement of this ion with concerted loss of C<sub>5</sub>H<sub>8</sub> afforded a diagnostic fragment at  $m/z$  138 (20) that was mass measured and fits C<sub>9</sub>H<sub>16</sub>N. A fragment ion at  $m/z$  84 (5) indicated a quinolizidine rather than an indolizidine, where  $m/z$  70 would be expected instead. No exchangeable hydrogens were detected using CIMS (ND<sub>3</sub>). On LC-MS with an APCI interface, an ion at  $m/z$  276 was seen with no other significant fragments. The FTIR spectrum also indicated a quinolizidine with a weak Bohlmann band at 2813 cm<sup>-1</sup> (Figure 3B). That the side-

chain double bonds are terminal is indicated by a C=CH<sub>2</sub> absorption at 3084 cm<sup>-1</sup>, a ν<sub>C=C</sub> at 1641 cm<sup>-1</sup> and absorptions at 993 and 912 cm<sup>-1</sup> in the FTIR spectrum.

Thus, the most likely structure is that of a 4,6-dipent-4-enyl quinolizidine (**2**) with the relative configuration shown being based on that of another 4,6-disubstituted quinolizidine, **195C**, from amphibian skin, whose Bohlmann bands are similar to those of **275I** (Jones *et al.*, 1999). However, NMR spectral data on isolated **275I** will be required to confirm the proposed relative configuration.

### 249F (3)

A major alkaloid, **249F**, in *Melanophryniscus cupreuscapularis* did not exhibit a mass spectral fragmentation diagnostic for any of the structural classes of alkaloids from amphibian skin and it was left unclassified in Daly *et al.*, 2005. The empirical formula is C<sub>16</sub>H<sub>27</sub>NO. There is a significant loss of ethyl to yield a fragment at *m/z* 220, while the base peak is at *m/z* 84. The FTIR spectrum has a moderate, broad Bohlmann band at 2755 cm<sup>-1</sup> and an absorption at 3550 cm<sup>-1</sup> indicating a hydrogen-bonded OH (Figure 3C) falling in the characteristic range of the tertiary hydroxyl absorption in homopumiliotoxins. A possible structure for **249F** (**3**), shown in Figure 1, is that of an unusual homopumiliotoxin with a conjugated exocyclic diene. The FTIR spectrum shows a weak absorption at 1650 cm<sup>-1</sup> (likely ν<sub>conj. C=C</sub>) not seen in homopumiliotoxins but a fingerprint region nearly identical with another homopumiliotoxin **265N**. Integration of the ν<sub>CH asym</sub> region for methyls and methylenes indicates two methyl groups are present (Garraffo, unpublished results) indicating that the usual side-chain allylic methyl of the pumiliotoxins and homopumiliotoxins is missing and is replaced by a double bond. That structure must be considered tentative until isolated compound can be subjected to NMR spectral analysis.

The gastrointestinal contents for *M. klappenbachi* and *M. cupreuscapularis* are presented in Table 3 and Table 4. Ants represented the major components that were detected.

## 4. Discussion

Bufonid toads of the genus *Melanophryniscus* now have been found to have over 80 different alkaloids in skin extracts (Daly *et al.*, 2005). The amounts and profiles differ considerable in different species. The Brazilian species *Melanophryniscus moreirae*, collected in 1979 from a cloud forest on Serra da Mantiquerira, 19 km NW of Itatiaia in the state of Rio de Janeiro, contained pumiliotoxin **267C** as a major alkaloid and allopumiliotoxin **323B** as a minor alkaloid (Daly *et al.*, 1984). Subsequently, *Melanophryniscus montevidensis* collected from La Coronilla, Depto. Rocha, Uruguay was also shown to contain pumiliotoxin **267C** as the major alkaloid and allopumiliotoxin **323B** as a trace alkaloid (Garraffo *et al.*, 1993). Recently, pumiliotoxin **251D** was reported as a major alkaloid in some, but not all populations of *Melanophryniscus montevidensis* from Depto. Rocha, Uruguay (Mebs *et al.*, 2005). Other pumiliotoxins were reported to be present, along with some unidentified indolizidines.

Two populations of Argentinian *Melanophryniscus stelzneri* collected in Tanti, Córdoba in May 1989 and Las Alpacas, Córdoba in November, 1987 from montane fields with spring-fed streams contained similar profiles of alkaloids (Garraffo *et al.*, 1993). Major alkaloids included decahydroquinolines, 3,5-disubstituted indolizidines, 3,5-disubstituted pyrrolizidines and a 5,6,8-trisubstituted indolizidine. All of these alkaloids are suspected to have an ant or mite dietary origin (Daly *et al.*, 2005; Takada *et al.*, 2005; Saporito *et al.*, 2007a, b). The two populations come from similar habitats and 21 of the 27 alkaloids were shared by both populations. In a recent study, the alkaloid profile from the 1989 collection of *M. stelzneri* was compared to the alkaloid profile for a 1999 collection from the same site (Daly *et al.*, 2007). The profiles were similar except that the decahydroquinolines of the 1989 collection were all

absent from the 1999 collection. Such decahydroquinolines are presumed to be obtained from dietary ants (Jones, *et al.*, 1999).

Four populations of Argentinian *Melanophryniscus rubriventris*, collected in the provinces of Salta and Jujuy, all contained pumiliotoxins as major alkaloids (Daly *et al.*, 2007). One population also contained the tricyclic alkaloid precocinelline (**193C**) as a major alkaloid. The pumiliotoxins are highly toxic (Daly and Myers, 1967; Daly *et al.*, 2003) and were proposed to be of mite origin. The precocinelline probably originates from coccinellid beetles, but a mite source is also possible (Takada *et al.*, 2005; Saporito *et al.*, 2007b). The major pumiliotoxins in the *M. rubriventris* toads varied by population, but included PTXs **251D**, **291G**, **307G** and **323A** (Daly *et al.*, 2007).

The current study on two species of *Melanophryniscus* from similar habitats in fields near the cities of Corrientes, Corrientes and Resistencia, Chaco, Argentina, documents the presence of alkaloids in both species. Only three alkaloids were present as major or minor alkaloids in both species: 3,5-disubstituted indolizidine **239Q** (**1**), 4,6-disubstituted quinolizidine 275I (**2**), and an alkaloid, **249F** (**3**) postulated to be a homopumiliotoxin (Table 1 and Figure 1). However, while pumiliotoxin **251D** (**5**) was present as a minor alkaloid in *M. klappenbachi*, it was absent in *M. cupreuscapularis*. Alkaloid **207S**, tentatively proposed to have an indolizidine structure (Daly *et al.*, 2005), is present as a trace alkaloid in *M. klappenbachi* and as a minor alkaloid in *M. cupreuscapularis*. It has an FTIR indicating a terminal double bond ( $\nu_{\text{HC}=\text{C}}$ , 3082  $\text{cm}^{-1}$ ) very likely disubstituted (significant 1642, 893  $\text{cm}^{-1}$ ; overtone 1790  $\text{cm}^{-1}$ ) and an intense Bohlmann band at 2791  $\text{cm}^{-1}$  typical of an indolizidine with three *trans* anti-parallel hydrogens. The structure is still under investigation, and will probably require revision. There are 30 other alkaloids detected in trace amounts, 24 of which were found in only one of the two species.

Even though both *Melanophryniscus klappenbachi* and *M. cupreuscapularis* are distributed exclusively in the Gran Chaco, their geographic ranges do not overlap. The former species is broadly distributed in the eastern Chacoan region, but also in parts of the western or dry Chaco. The latter species is restricted to the north-western province of Corrientes, on the eastern shore of the Paraná river, where it inhabits an environment known as “levee and northeastern subconcave plain” (Carnevali, 1994).

*Melanophryniscus klappenbachi* is slightly larger (SVL (mm) mean = 27.7, range = 22.2–32.4, n = 76, Baldo, personal obs.) than *M. cupreuscapularis* (SVL mean = 23.9, 15 range = 21.825.8, n = 15, Baldo, personal obs.). The specimens included in this study were collected in localities quite close to each other, with similar climatic regimes and biotic compositions. The specimens of *M. cupreuscapularis* were collected in seasonally flooded grassy savannahs, where the tree *Schinopsis balansae* is the originally dominant element (Carnevali, 1994). Specimens of *M. klappenbachi* were collected in temporary marshes in remnants of deciduous xerophilous forest, with a predominance of grasses, cacti, and terrestrial bromeliads (Cabrera, 1976; Cabrera & Willink, 1980).

The first observations on the diet composition of species of *Melanophryniscus* extend back to the end of the 19<sup>th</sup> and the beginning of the 20<sup>th</sup> century, when earlier workers noticed the predominance of dietary ants in species of *Melanophryniscus* (Jiménez de la Espada, 1875 on an unidentified species of the genus; Fernández, 1926 on *M. stelzneri stelzneri*). Subsequently, more detailed studies corroborated a predominance of ants (mainly of the genus *Crematogaster*), and mites (mostly Oribatidae) in the diet of *M. moreirae* from the planalto of Itatiaia, in the state of Rio de Janeiro, Brazil (Bokerman, 1967). Filipello and Crespo (1992) indicate a preponderance of ants (no taxonomic information provided), followed by mites and termites in adults of *M. stelzneri* from San Luis, Argentina, whereas in the juveniles, the diet

has a preponderance of Collembolans. A non-quantitative analysis of feces of *M. montevidensis* revealed the presence of ants (*Pheidole* sp.) and mites (Mebs *et al.*, 2005). Skin alkaloids and stomach contents of four populations of *M. rubriventris* from NW Argentina were recently reported (Daly *et al.*, 2007). Two of the populations had ants (38 and 41%) and mites (36 and 40%) as the major prey items, while one had ants (79%) as the only major prey items; the fourth had hemipterans (39.2%) followed by collembolans (22%) and mites (20%) as the major prey items. Pumiliotoxins, now presumed to be of mite origin (Saporito *et al.*, 2006; Takada *et al.*, 2005) were the major alkaloids from all four populations. Indeed, a putative ant alkaloid, namely the 3,5-disubstituted indolizidine **223AB**, was detected only from one population and then only in trace amounts.

The present analysis of contents of the intestinal tracts of *M. cupreuscapularis* and *M. klappenbachi* revealed that both contained a preponderance of ants (Table 3 and Table 4) of two subfamilies, Ecitoninae and Myrmicinae; these myrmicines were collectively much more abundant in terms of frequency of occurrence. Ant remains were followed in preponderance by unidentified mites (order Opilioacarina), in *M. klappenbachi*, that were undetected in *M. cupreuscapularis*. In *M. klappenbachi*, the most frequently detected prey items were immature instars of *Acromyrmex hispidus* (61 %) followed by *Wasmannia auropunctata* (9 %), *Pheidole subaberrans* (7 %), *Crematogaster quadriformis* (6%), *Labidus praedator*, *Pheidole* sp., and *Solenopsis* sp. (see Table 3 for quantitative data). Of these genera, only the *Solenopsis* are known to contain alkaloids (T.H. Jones, personal communication).

In *M. cupreuscapularis* the most frequently detected prey items were *Crematogaster quadriformis* (69 %) followed by *Pheidole subaberrans* (8 %), *Acromyrmex ambiguus* (4 %), *Eciton* sp, *Labidus praedator*, and *Wasmannia auropunctata* (see Table 4 for quantitative data).

It now appears that mites will prove to be the dietary source of the pumiliotoxins and of the izidines, containing branch points in their carbon skeletons (Saporito *et al.*, 2007b; Takada *et al.*, 2005). However, mites were detected in only 20% and 33% of gastrointestinal tracts of *M. klappenbachi* and *M. cupreuscapularis*, respectively (Table 3 and Table 4). To our knowledge, mites of the order Opilioacarida have not been examined for alkaloids. Certain pumiliotoxins and related homopumiliotoxins were either minor (**251D** and **249F** in *M. klappenbachi*) or major (**249F** in *M. cupreuscapularis*) alkaloid constituents (Table 1). There were other pumiliotoxin/ homo-pumiliotoxins and branched chain izidines (**207A**, **223D**, **219G**, **249L**, **259E**), but all in only trace amounts. It should be noted that pumiliotoxins **307A** and **323A** have been reported in extracts of two species of formicine ants of the genera *Brachymyrmex* and *Paratrechina* from Panama (Saporito *et al.*, 2004). The unprecedented occurrence of such alkaloids in a formicine ant must perhaps be reconsidered in view of the detection of pumiliotoxins in mites and the possibility that mites have been prey items for these ants. Myrmicine ants have been well accepted as the probable source of the decahydroquinolines and izidines with linear carbonchain skeletons (Jones *et al.*, 1999). Decahydroquinolines (four in *M. klappenbachi* and one in *M. cupreuscapularis*) were detected only in trace amounts (Table 1). None of these decahydroquinolines have been reported from an arthropod, although closely related alkaloids of this class have been found in ants of the *Solenopsis*, subgenus *Diplorhoptrum* (Jones *et al.*, 1999). The 3,5-disubstituted indolizidines **195B** and **223AB** found in the present *Melanophriniscus* species as trace alkaloids also are well known from myrmicine ants, *Monomorium pharaonis* and *Solenopsis* (*Diplorhoptrum*) species, respectively. One of the major alkaloids **239Q** of both *Melanophriniscus* species is a 3,5-disubstituted indolizidine, which is unusual in having a side-chain hydroxyl group. Such an indolizidine has not been detected in an ant, but an isomeric 3,5-disubstituted indolizidine with a ring hydroxyl group has recently been reported from a myrmicine ant *Myrmecaria melanogaster* from Brunei in the South Pacific (Jones *et al.*, 2007). The other major alkaloid of *M. klappenbachi* is the 4,6-disubstituted quinolizidine **275I**. It is minor in *M. cupreuscapularis*. The only 4,6-disubstituted

quinolizidine as yet reported from an arthropod is **195C** from a myrmicine ant *Solenopsis* (*Diplorhoptrum*) species, *picea* group (Jones *et al.*, 1999). Although ants, including immature instars of the myrmicine ant *Acromyrmex hispidus* represent major prey items, none are of genera known to contain decahydroquinoline or izidine alkaloids. Ants were collected at the *M. klappenbachi* site in November, 2001. These were *Solenopsis tridens*, a *Pheidole* species (perhaps containing some *Solenopsis*), and *Crematogaster quadriformis*. Methanol extracts of the *Solenopsis* and *Pheidole* confirmed several 2-methyl-6-substituted piperidines (data not shown), alkaloids known to occur in fire ants of the genus *Solenopsis* (MacConnell *et al.*, 1971). Such alkaloids were not detected in the *M. klappenbachi* or *M. cupreuscapularis*, nor have they been found in prior studies of alkaloids of *Melanophryniscus*. The *Crematogaster* extract had no alkaloids.

Further studies on other species of *Melanophryniscus* toads are in progress and should provide additional evidence that habitat and thus availability of dietary alkaloid-containing prey are major factors in defining profiles of alkaloids in such bufonid toads. It appears almost certain that all of the alkaloids found in skin extracts from *Melanophryniscus* species are sequestered without change from dietary arthropods. Thus, it is not surprising that toads from distant locales share few alkaloids, while toads from close and similar habitats share a large number of alkaloids.

### Bioactive molecules and phylogenetic relationships of Bufonids

*Melanophryniscus* is a very distinctive group of toads that currently comprises twenty described species. Several non-described species are also known. So far only 5 of these 20 species are known to have been screened for alkaloids, all with positive results. No lipophilic alkaloids have so far been reported in any of the other remaining bufonids.

Until 1980, toxic bufadienolides were known to occur in amphibians only in the widespread bufonid toads of the genus *Bufo*. Bufadienolides (Steyn and van Heerden, 1998) have an unusual C<sub>17</sub> steroidal nucleus with an  $\alpha$ -pyrone ring at the C-17 position and usually A–B and C–D *cis*-fused rings with 3 $\beta$ , 14 $\beta$  hydroxyl groups and often additional oxygenation, such as epoxide, ketone, acetoxyl or more hydroxyl groups. These are neutral lipophilic materials. If the C-3-alcohol is esterified with a C<sub>2</sub>–C<sub>8</sub> dicarboxylic acid and this in turn has the other carboxyl group in an amide link with an amino acid such as arginine, the hydrophilic (polar) bufotoxin class results. If the lactone at C-17 is a butenolide, then the cardenolide class results with the equivalent conjugate at C-3 being referred to as a cardenobufotoxin. All four classes have been found in bufonids but bufadienolides and bufotoxins greatly predominate. Polar 3-*O* sulfates of bufadienolides and cardenolides have also been reported in bufonids. In plants, there are conjugates of bufadienolides and cardenolides with sugars.

In 1980 bufadienolides were reported in skin extracts of *Atelopus* with telocinobufagin and bufotalin being tentatively identified in extracts of *A. ignescens* (Flier *et al.*, 1980). In addition, polar bufotoxin-like substances were detected in skin extracts of *Melanophryniscus moreirae* and *Dendrophryniscus minutus* (Flier *et al.*, 1980).

Our own unpublished work (TFS and JWD) indicates that the aqueous methanol fraction, remaining after extraction of alkaloids and other lipophilic compounds with chloroform from *Melanophryniscus moreirae*, after storage in a freezer for 14 years, now shows by HPLC-UV-MS, at least 5 bufadienolide-like (UV  $\lambda_{\max} \approx 300$  nm) and three cardenolide-like (UV  $\lambda_{\max} \approx 220$  nm) non-polar substances with 1–2 exchangeable hydrogens in hydroxyl groups, one of which is acetylatable but in only one bufadienolide. These appear, from the MWs (354–384 amu), to be in the range of cardiotonic steroids. All are neutral, forming both  $[M + H]^{\oplus}$ , and  $[M + NH_4]^{\oplus}$  ions with APCI mass spectrometry and HPLC-acetonitrile-water solvent systems. Alkaloids typically form only  $[M + H]^{\oplus}$  ions under these conditions. One or two losses of

H<sub>2</sub>O and frequently one CO are seen from [M+H]<sup>⊕</sup> for both classes while CO<sub>2</sub> losses are seen only with the bufadienolides. The ammonium ion adducts are base peaks with the cardenolides but significantly weaker with bufadienolides. Additional work is underway to obtain more structural information on these presumed elimination products that have lost functionality at C-3. The structures of the polar bufadienolide-like toxins have, as yet, likewise not been elucidated.

Recently, bufadienolides were identified in skin extracts of *Ansonia inthanon* (arenobufagin, hellebrigenin, telocinobufagin, marinobufagin and two unidentified bufadienolides), *Leptophryne borbonica* (two unidentified bufadienolides), and *Pedostibes hossi* (arenobufagin, cinobufotalin, gamabufotalin, hellebrigenin, telocinobufagin and one unidentified bufadienolide (Daly *et al.*, 2004). The bufadienolides were shown to be synthesized by the toad in the case of *Rhinella schneideri* (formerly *Bufo paracnemis*) (Porto and Gros, 1971), *R. arenarum* (formerly *Bufo arenarum*) (Garraffo and Gros, 1986) and appear to be synthesized by the toad *Atelopus varius* (Daly *et al.*, 1997). The genus *Bufo* is now undergoing partition into several genera (see review of literature by Frost *et al.*, 2006). This has added further genera, all of which were formerly *Bufo*, to those that have been reported to have bufadienolides. The former *Bufo* group that have been reported to contain bufadienolides are as follows: *Amietophrynus regularis*; *Anaxyrus americanus*, *A. fowlerii*, *A. terrestris*, *A. woodhousii*; *Bufo bankorensis*, *B. bufo*, *B. gargarizans*, *B. japonicus*; *Cranopsis alvarius*, *C. valliceps*; *Duttaphrynus melanostictus*; *Ingerophrynus biporcatus*, *I. parvus*; *Peltophryne peltoccephala*; *Phrynooidis asper*; *Pseudoepidalea raddei*, *P. viridis*; *Rhaebo blombergi*; *Rhinella arenarum*, *R. crucifer*, *R. granulosa*, *R. icterica*, *R. marina*, *R. schneideri*, *R. rubescens*, *R. spinulosa*.

The taxonomic distribution of bufadienolides within bufonids also requires further research, but current data indicates the presence of bufadienolides, in assays of exemplars of the following genera: *Amietophrynus* (Flier *et al.*, 1980), *Anaxyrus* (Shimada and Nambara, 1980), *Ansonia* (Daly *et al.*, 2004), *Atelopus* (Flier *et al.*, 1980), *Bufo* (Schröter *et al.*, 1958), *Dendrophryniscus* (Flier *et al.*, 1980), *Duttaphrynus* (Iseli *et al.*, 1964), *Ingerophrynus* (Daly *et al.*, 2004), *Leptophryne* (Daly *et al.*, 2004), *Melanophryniscus* (Flier *et al.*, 1980; see above), *Mertensophryne* (Daly, unpublished results), *Phrynooides* (Shimada, *et al.*, 1985; Daly *et al.*, 2004), *Pseudoepidalea* (Shimada & Nambara, 1986), *Peltophryne* (Barbier *et al.*, 1961), *Ollotis* (Barbier *et al.*, 1961), *Rhaebo* (Barbier *et al.* 1961) and *Rhinella*, formerly *Chaunus*, *Bufo* (Schröter *et al.*, 1958; Flier *et al.*, 1980). This taxonomic distribution suggests that the presence of bufadienolides is a synapomorphy of Bufonidae.

Various phylogenetic studies with disparate taxonomic samplings have suggested that *Melanophryniscus* is the sister taxon of all remaining bufonids (Graybeal, 1997; Darst & Cannatella, 2004; Faivovich *et al.*, 2005; Frost *et al.*, 2006; Grant *et al.*, 2006) or in a similar position forming a clade with a few other bufonid genera (Pramuk, 2006). Although the taxonomic distribution of sequestration within *Melanophryniscus* requires further studies, in the context of the phylogenetic hypothesis of Frost *et al.* (2006), the ability to sequester dietary alkaloids clearly optimizes as a synapomorphy of *Melanophryniscus*.

Alkaloids have so far not been reported in any of the bufonids except *Melanophryniscus*. Such lipophilic alkaloids have not been detected in the following bufonid genera: *Amietophrynus*, *Ansonia*, *Atelopus*, *Dendrophryniscus*, *Ingerophrynus*, *Leptophryne*, *Mertensophryne*, *Pedostibes*, *Phrynooidis*, *Rhaebo*, and *Rhinella* (Daly *et al.*, 2004 and unpublished results). Related to this is the interesting observation of two types of skin glands in *Melanophryniscus cupreuscapularis* (Delfino, *et al.* 1998); one similar to the secretory glands of bufonids, the other thought to secrete "proteinaceous products."

Other putatively defensive molecules present in bufonids include biogenic amines (Ceï *et al.*, 1968, 1972) and the tetrodotoxins (Daly *et al.*, 1994, 1997, Kim *et al.*, 1975, Mebs *et al.*, 1995) and the related chiriquitoxin (Yotsu, *et al.*, 1990) and zetekitoxin (Yotsu-Yamashita *et al.*, 2004). The tetrodotoxins occur in at least four families of frogs including most species of *Atelopus* in Bufonidae (Daly, 2004), however they are not present in all *Atelopus* species. The fact that no tetrodotoxin-like inhibitory activity of [<sup>3</sup>H] saxitoxin binding test or presence of tetrodotoxin was recorded in ethanolic extracts of several other bufonids (Daly *et al.*, 1994; Mebs *et al.*, 1995; Daly, 2004) suggests that the ability to incorporate tetrodotoxins is likely a synapomorphy of at least some species of *Atelopus*. Other anurans known to have tetrodotoxins are (1) at least three of the eleven species of *Brachycephalus* (family Brachycephalidae, Sebben *et al.*, 1986; Pires *et al.*, 2005), (2) *Colostethus inguinalis* (family Dendrobatidae, Daly *et al.*, 1994), and (3) an unidentified species of *Polypedates* (family Rhacophoridae, Tanu *et al.*, 2001). These three are quite distant phylogenetically from bufonids (Frost *et al.*, 2006) so the most parsimonious explanation is that 23 during the evolutionary history of anurans there have been at least four independent origins of the presence of tetrodotoxin, regardless of its specific mechanism of sequestration.

Bufadienolide-like compounds and biogenic amines (Ceï *et al.*, 1968) are present in *Melanophryniscus* (Flier *et al.*, 1980). A recent study (Mebs *et al.*, 2007) did not detect bufadienolides in extracts of *M. atroluteus*, *M. devincenzii*, and *M. montevidensis*, but the possible presence of the more polar bufadienolide-like toxins reported to be present in *M. moreirae* by Flier *et al.* (1980) was not unambiguously excluded from these three toads since no bioassays were reported. Current studies indicate that the polar bufadienolide-like compounds, present in extracts of *Melanophryniscus moreirae* hydrolyze over time to bufadienolides and cardenolides (unpublished results, TFS and JWD, see above) and it seems likely that polar bufadienolides are also present in *M. montevidensis* and make a major contribution to the toxicity of all toads of the genus *Melanophryniscus*. Indeed, in terms of interaction with the target Na/K ATPase responsible for toxicity, the levels of bufadienolide-like compounds in *Melanophryniscus moreirae* exceeds the levels of bufadienolides in *Amietophrynus regularis*, *Rhinella granulosa* and *R. marina* (Flier *et al.*, 1980).

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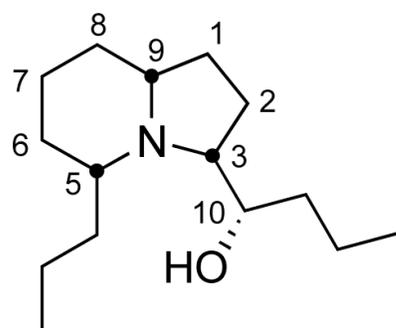
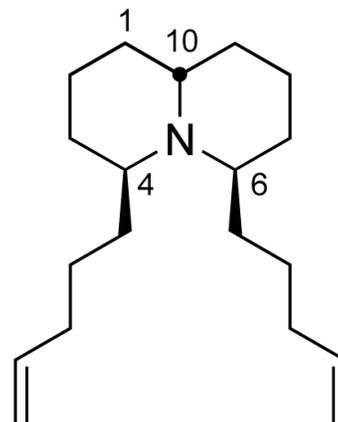
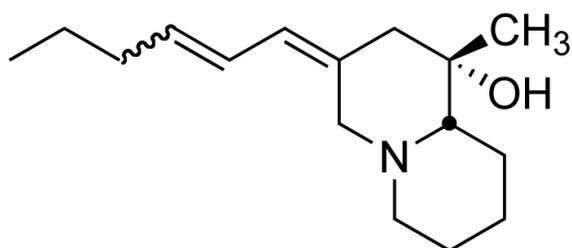
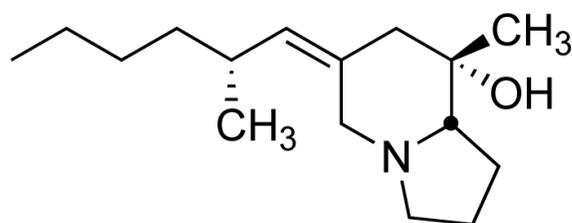
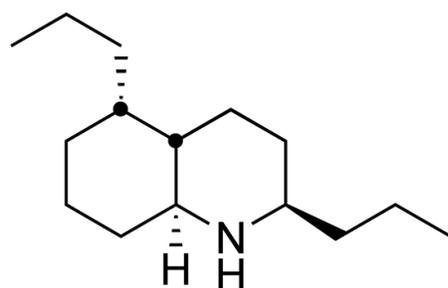
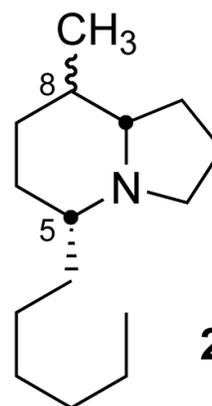
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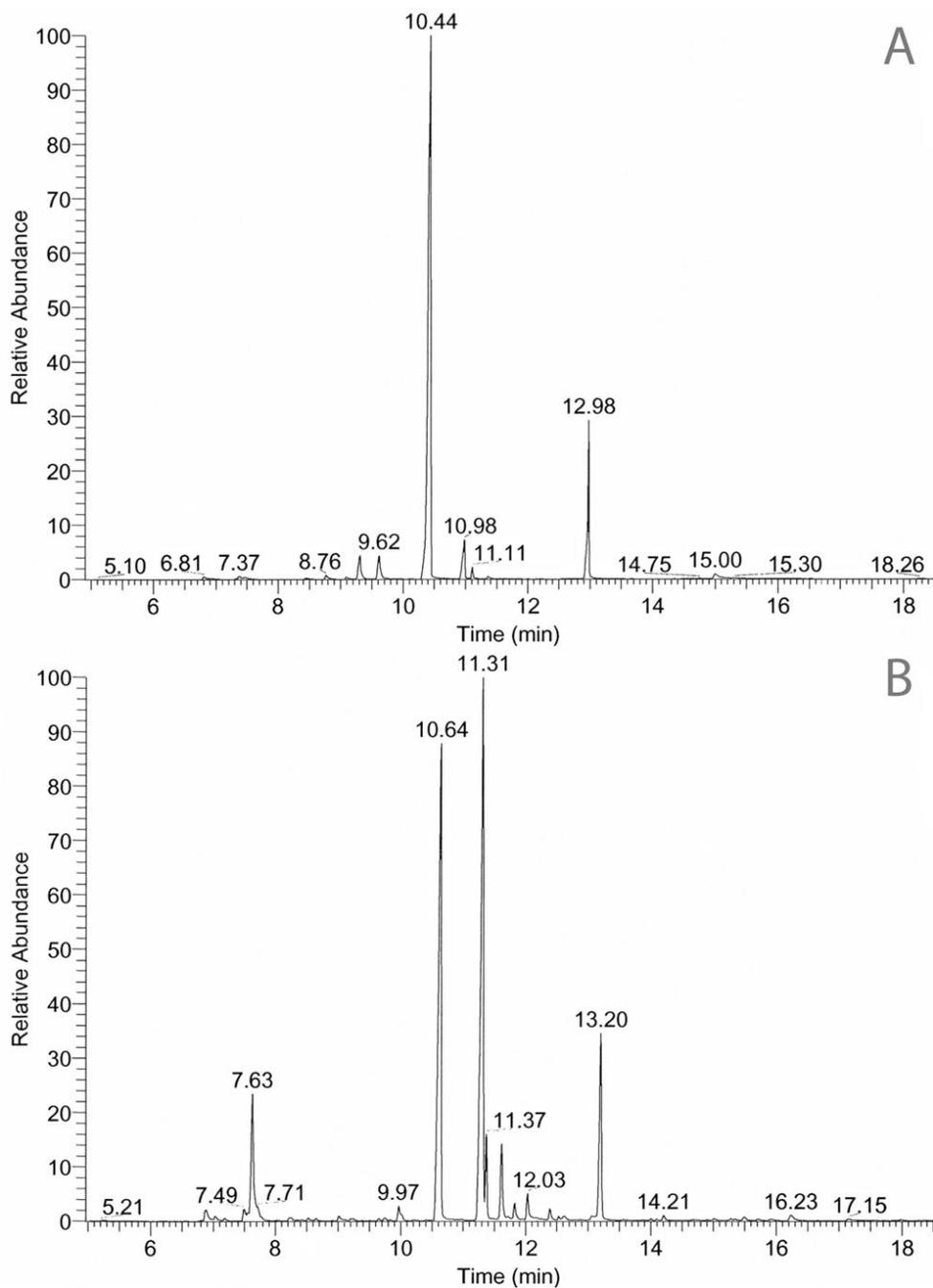
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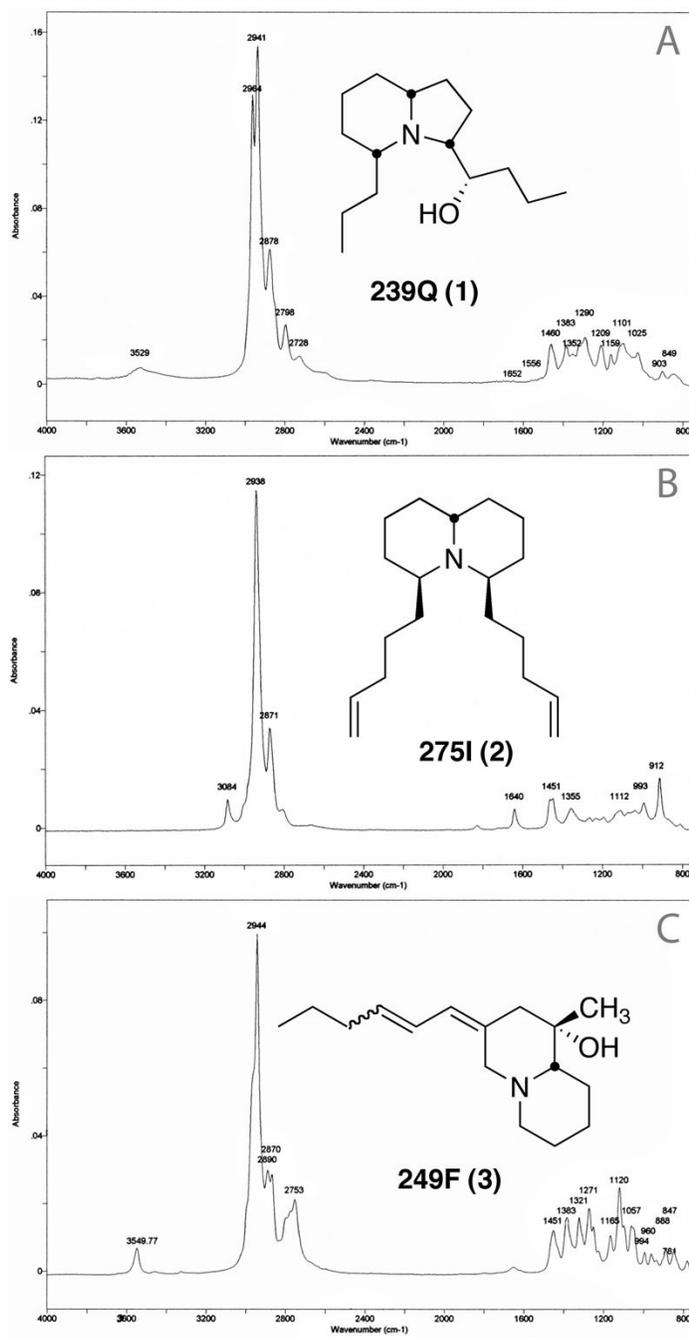
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**239Q (1)****275I (2)****249F (3)****251D (4)****trans-223F (5)****223D (6)**

**Figure 1.**  
Structures of major and minor alkaloids detected from *M. klappenbachi* and *M. cupreuscapularis*.



**Figure 2.** GC-MS chromatograms for A) *Melanophryniscus klappenbachi* and B) *Melanophryniscus cupreuscapularis*. Alkaloids were identified from retention times (Rt) and spectral data that are documented in Daly *et al.*, 2005. Major and minor alkaloids are as follows: **A:** **223F** (5), 9.20 min; **223D** (6), 9.62 min; **239Q** (1), 10.44 min; **249F** (3), 10.98 min; **251D** (4), 11.11 min; **275I** (2), 12.98 min. **B:** **207S**, 7.63 min, (see text); **235S**, 9.97 min; **239Q** (1), 10.64 min; **249F** (3), three isomers, 11.31, 11.83, 12.03 min; **259B**, two isomers, 11.37, 11.62 min; **275I** (2), 13.20 min. Rts for **1**, **2**, and **3** differ between **A** and **B**, as the analyses were made several years apart. The x's indicate non-alkaloid impurities.



**Figure 3.** FTIR spectra for **A)** 3,5-disubstituted indolizidine **239Q (1)**; **B)** 4,6-disubstituted quinolizidine **275I (2)**; and **C)** the putative homopumiliotoxin diene **249F (3)**.

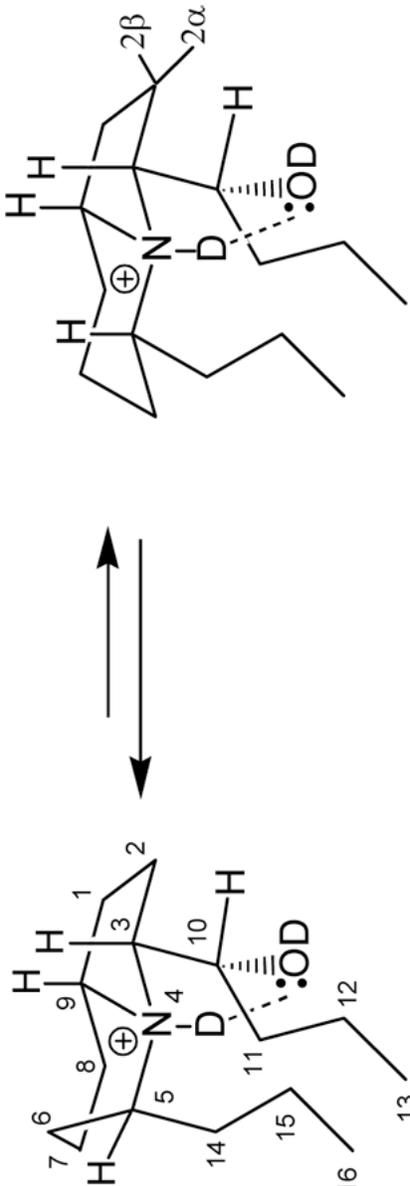
**Table 1**Occurrence of alkaloids in skin extracts of two Argentinian species of *Melanophryniscus*.<sup>a</sup>

Alkaloid	<i>M. klappenbachi</i>	<i>M. cupreuscapularis</i>
Pumiliotoxins		
251D (4)	++	---
277B	---	+
307F	---	+,+
307G	---	+
323A	+	---
Homopumiliotoxins		
249F (3)	+,+,+	+,+,+,+
265N	+	---
Decahydroquinolines		
223F (5)	+,+,+	---
269AB	+	+
269B	+	---
271D	+	---
3,5-Disubstituted Indolizidines		
195B	+	---
5Z,9Z-223AB	+	+
239Q (1)	+++	+++
5,8-Disubstituted Indolizidines		
207A	+	---
223D (6)	++	---
259B	+	+,+,+
Dehydro-5,8-Disubstituted Indolizidines		
219G	+	---
249L	+	---
1,4-Disubstituted Quinolizidines		
259E	---	+
4,6-Disubstituted Quinolizidines		
275I (2)	+++	++
279H	---	+
Izidines		
211C	---	+
Tricyclics		
193C	---	+
205H	---	+
205I	+	+
235M	---	+
Unclassified		
193K	---	+
207S	+	++
209R	---	+

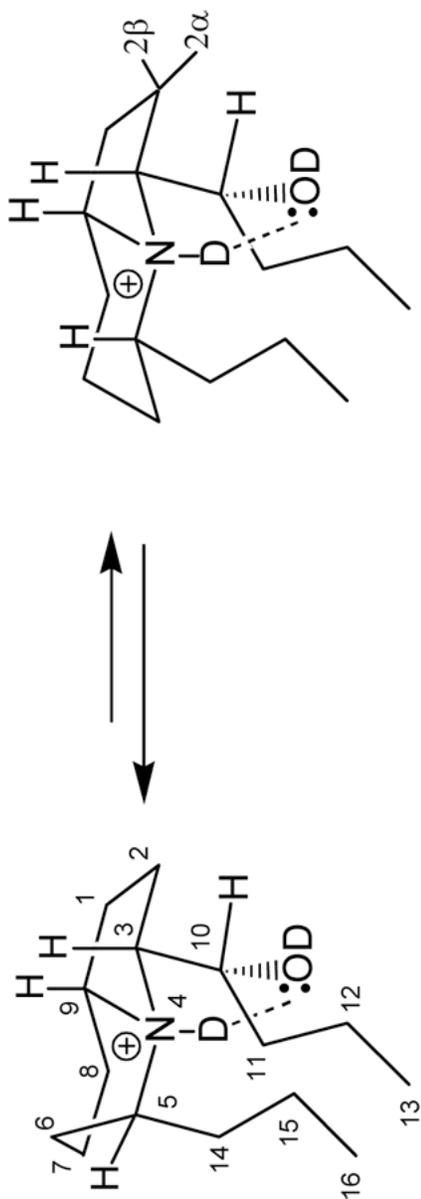
Alkaloid	<i>M. klappenbachi</i>	<i>M. cupreuscapularis</i>
<b>223EE</b>	+	----
<b>223T</b>	+	+
<b>235S</b>	+	++
<b>293H</b>	----	+
<b>305H</b>	----	+
<b>307J</b>	+	----

<sup>a</sup>Major (+++, > 50 µg/100 mg skin); Minor (++, <50 and >5 µg per 100 mg skin); Trace (+, <5 µg per 100 mg skin). Two or more entries for an alkaloid in any species indicate isomers. Other trace alkaloids were present, but data was not sufficient to assign a code number and letter. For structures of some of the above alkaloids, including tentative structures, see Figure 1 and Daly *et al.*, 2005. The postulated structure of **259B** requires revision (Nelson *et al.*, 2008), as does that of **207S**.

Table 2

<sup>1</sup>H-NMR Assignments for 239Q (1) in d<sub>6</sub>-acetone.


H#	$\delta$ (m, $J$ (Hz)) <sup>a</sup>	ID-irradiation at H# affects signal at $\delta$	ID-irradiation at $\delta$ affects signal at H#	TOCSY Cross-peaks	NOESY Cross-peaks
1	2.32 $J=lg$ .				
2	1.78 $J=lg$ ; 2.32, $J=sm$ . (2 $\beta$ , 2 $\alpha$ respectively)				
3	3.73 dd (10.4, 2.6)	1.83, 1.88–1.93; 2.16–2.21 2.32 (sm. $J$ removed)	1.52–1.56; 1.77, 1.88–1.89; 2.16–2.23; 2.32	1.9 [1.95, 2.0, 2.2, 2.32 3.13]	H <sub>3</sub> -H <sub>6</sub> ; H <sub>3</sub> -H <sub>10</sub>
4 NH, 6d	10.7 broad s				
5	3.12 dddd (12.0, 10.8, 3.0, 1.5) appears as br. t	2.26–2.35 removes lg. $J$ (t→d), 1.91, 1.74–1.82 (removes lg. $J$ )	1.75–1.77, 1.89, 2.16–2.23.	2.32 [1.6, 1.8, 1.9, 1.95, 2.0, 2.2]	
6	1.78, 2.32	1.91–1.94; 2.28–2.34	1.89; 2.16–2.23	1.8, 2.2	
7/8	2.32			1.95, 2.20	
9	3.25 dddd (11.6, 11.3, 3.0, 2.8)	2.21–2.26, sm. $J$ with H-3 removed at $\delta$ 2.17–2.21 [dd→d; <sup>13</sup> C-type coupling?]; 2.32 two lg. $J$ s removed.	1.76–1.81; 1.92; 2.17–2.23;	1.95, 2.22 [1.6, 1.9, 2.04, 2.32]	H <sub>9</sub> -H <sub>3</sub>
10	4.19, 4.2 (3:2, d, $J=8.5$ )	1.33–1.38 (→s), 1.48–1.5 (dd→d)	1.33–1.38; 1.46–1.52;	1.4 [1.5]	H <sub>10</sub> -H <sub>3</sub>
11	1.36–1.4; 1.49–1.5		1.48–1.52		
12	1.48–1.52 (A) 1.28–1.31 (B)				
13	0.93 (A, B); $J=7.3$	1.52–1.55	1.52–1.55	1.55	



H-#	$\delta$ (m, $J$ (Hz)) <sup>a</sup>	1D-irradiation at H-# affects signal at $\delta$	TOCSY Cross-peaks	NOESY Cross-peaks
15	1.40			
16	0.89 (A); 0.93 (B) 1:2, t, $J=7.3$	1.37–1.38	1.5, 1.78, 2.12	
OH, 6 d	5.75; 5.8 3:2 ( $J=5.1$ )	1.47–1.52		

<sup>a</sup> s, singlet; d, doublet; t, triplet; br., broad; 6d, spectra on back-exchanged alkaloid after 6 days; lg., large  $J$ ; sm., small  $J$ ; tocsy data in brackets are for the longest mixing time (0.05 sec); other data is for 0.002 sec.

**Table 3**Main prey taxa encountered in gastrointestinal tracts of *Melanophryniscus klappenbachi*<sup>a</sup>

PREY TYPES	n	f (%)	f <sub>a</sub>	FO (%)
ARTHROPODA				
INSECTA				
HYMENOPTERA				
Apidae				
<i>Apis mellifera</i>	2	0.17	2	5.9
Formicidae				
Ecitoninae				
<i>Labidus praedator</i>	27	2.3	10	29.4
Myrmicinae				
<i>Crematogaster quadriformis</i>	67	5.8	21	61.8
<i>Wasmannia auropunctata</i>	101	8.7	21	61.8
<i>Pheidole</i> sp.	22	1.9	7	20.6
<i>Pheidole subaberrans</i>	78	6.7	14	41.2
<i>Solenopsis</i> sp.	14	1.2	6	17.6
<i>Acromyrmex hispidus</i> (immature instars)	778	67.2	27	79.4
DIPTERA				
Culicidae	2	0.17	2	5.9
Ephedridae	1	0.08	1	2.9
COLLEMBOLA	7	0.60	6	17.6
ARACHNIDA				
ARANAE				
Araneomorphae				
Thomisidae	7	0.60	5	14.7
(n.i)	4	0.34	4	11.8
ACARI, Opilioacarida	11	0.94	7	20.6
SCORPIONIDAE (immature instar)				
Buthidae				
<i>Tityus trivittatus</i>	4	0.34	3	8.8
PLANTS				
ANGIOSPERMS				
Poaceae				
seeds	17	1.46	7	20.6
leaf	9	0.77	6	17.6
Polygonaceae (seeds)	1	0.08	1	2.9
Compositae				
<i>Taraxum officinale</i> (seeds)	6	0.51	5	14.7
ANIMAL FRACTION (n.i)	X	X	26	76.5
TOTAL NUMBER OF PREY	1158			

<sup>a</sup> 35 Individual toads, were examined including the 11 analyzed for alkaloids). n = Number of individuals per taxon, f (%) = percent of the total number of prey accounted for by that particular prey type, f<sub>a</sub> = number of gastrointestinal tracts with that prey category and FO (%) = frequency of occurrence.

X = not quantified, n.i = not identified. In addition, many items, presumably of animal origin, occurred in 26 of the 35 gastrointestinal tracts but could not be identified.

**Table 4**Main prey items encountered in gastrointestinal tracts of *Melanophryniscus cupreuscapularis*<sup>a</sup>

PREY TYPES	n	f (%)	f <sub>a</sub>	FO (%)
ARTHROPODA				
INSECTA				
HYMENOPTERA				
Formicidae				
Ecitoninae				
<i>Labidus praedator</i>	1	0.41	1	8.3
<i>Eciton</i> sp.	5	2.1	2	16.6
Myrmicinae				
<i>Crematogaster quadriformis</i>	168	69.4	12	100
<i>Wasmannia auropunctata</i>	1	0.41	1	8.3
<i>Pheidole subaberrans</i>	20	8.3	4	33.3
<i>Acromyrmex ambiguus</i>	10	4.1	2	16.6
COLEPTERA				
Melodidae	2	0.82	1	8.3
Crysolmelidae	1	0.41	1	8.3
COLLEMBOLA	2	0.82	1	8.3
PROTURA	11	4.5	3	25
ARACHNIDA				
ARANAE				
Araneomorphae	17	7.0	4	33.3
SCORPIONIDAE (immature instar)				
Buthidae				
<i>Tityus trivittatus</i>	2	0.82	2	8.3
PLANTS				
POACEAE				
leaf	1	0.41	1	0.83
ANIMAL FRACTION (n.i)	X	X	8	66.6
TOTAL NUMBER OF PREY	242			

<sup>a</sup>Twelve toads, were examined including the 11 analyzed for alkaloids. n = Number of individuals per taxon, f (%) = percent of the total number of prey accounted for by the particular prey type, f<sub>a</sub> = number of gastrointestinal tracts with the prey category and FO (%) = frequency of occurrence. X = not quantified, n.i = not identified. In addition, many items, presumably of animal origin, occurred in 8 of the 12 gastrointestinal tracts, but could not be identified. It should be noted that the prey detected in the present study and others do not represent the long term prey items that over the course of the toad's life have given rise to the alkaloids stored in the skin glands