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Alkaloids in the Mite *Scheloribates laevigatus*: Further Alkaloids Common to Oribatid Mites and Poison Frogs

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Abstract Poison frogs are chemically defended from predators by diverse alkaloids, almost all of which are sequestered unchanged from alkaloid-containing arthropods in the frog diet. Oribatid mites recently have been proposed as a major dietary source of poison frog alkaloids. Here, we report on alkaloids common to an oribatid mite and poison frogs. Gas chromatographic-mass spectrometric analysis of methanol extracts of adult *Scheloribates laevigatus* (Oribatida: Scheloribatidae) revealed nine alkaloids. Five of these have been detected previously in the skin glands of poison frogs: two isomers of the pumiliotoxin **291G**, two isomers of the 5,6,8-trisubstituted indolizidine **209C**, and the 5,6,8-trisubstituted indolizidine **195G**. The other four alkaloids, a pumiliotoxin, a tricyclic (coccinelline-like), and two isomers of an izidine, were not previously known, but are similar in structure to alkaloids found in poison frogs. Alkaloids were not detected in immature *S. laevigatus*, suggesting that they are adult-specific and possibly the result of mite biosynthesis. Although most of the alkaloids detected in *S. laevigatus* are common to poison frogs, the geographic distributions of these organisms are not sympatric. The findings of this study indicate that oribatid mites, and in particular, members of the genus *Scheloribates*, represent a relatively unexplored arthropod repository for alkaloids and a significant dietary source of alkaloids in poison frogs.

Key Words Chemical defense · Dendrobatids · Indolizidines · Oil glands · Opisthonotal glands · Pumiliotoxins · Scheloribatidae · Tricyclic alkaloids · Poison frog

Introduction

The term ‘poison frog’ refers to the four known evolutionary lineages of anurans characterized by the ability to sequester alkaloid-based chemical defenses from dietary arthropods. They include certain dendrobatids from Central and South America, mantellids from Madagascar (*Mantella*), bufonids from South America (*Melanophryniscus*), and myobatrachids from Australia (*Pseudophryne*). Over the past 40 years, more than 850 lipophilic alkaloids, organized into over 20 structural classes, have been detected in the skin glands of poison frogs, apparently reflecting the diversity of alkaloids present in arthropod prey (Daly et al., 2005; Saporito et al., 2009). Most of these alkaloids are pumiliotoxins, indolizidines, and coccinelline-like tricyclics (Saporito et al., 2009), and have molecular weights of less than 400 atomic mass units (Daly et al., 2005). A large number of alkaloids have been identified in arthropods (Jones and Blum, 1983; Braekman et al., 1998), and insofar as is currently known, most poison frog alkaloids are sequestered from alkaloid-containing mites, ants, beetles, and millipedes in the diet (Saporito et al., 2009).

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Oribatid mites are among the most abundant and diverse arthropods in soil and leaf-litter, both in temperate and tropical regions (Maraun and Scheu, 2000; Franklin et al., 2004), but they also are found frequently in arboreal microhabitats, both on exposed surfaces and in "suspended soil" (Lindo and Winchester, 2006). Their food generally consists of decaying higher plant material and saprophytic fungi (Schneider and Maraun, 2005), although stable isotope studies indicate that necrophagy or predation on small invertebrates also is common, especially in tropical soils (Illig et al., 2005). Consistent with low rates of secondary production and relatively long lives (1–5 years or more), these mites have multiple defenses, especially as adults (Norton, 1994). These defenses include cuticular sclerotization or mineralization, specialized cuticular outgrowths, special defensive body forms and behaviors, and defensive chemicals (Sanders and Norton, 2004; Schneider and Maraun, 2009). Chemical defenses probably are derived from paired exocrine glands (Raspotnig et al., 2003) called opisthonotal or oil glands. These glands secrete a wide range of organic compounds including monoterpenes, sesquiterpenes, aromatics, aliphatic aldehydes, a ketone, fatty acids, fatty acid esters, an alkyl formate, and hydrocarbons (Kuwuhara, 2004; Raspotnig, 2009).

Recently, alkaloids have been identified in oribatid mites, representing a repository of these natural products in animals as well as a major dietary source for these compounds in poison frogs (Takada et al., 2005; Saporito et al., 2007a, 2009). More than 80 alkaloids, representing 11 structural classes, have been identified in oribatids, including pumiliotoxins, a homopumiliotoxin, 5,8-disubstituted- and 5,6,8-trisubstituted indolizidines, dehydro-5,8-disubstituted indolizidines, coccinelline-like tricyclics, a 1,4-disubstituted quinolizidine, a 4,6-disubstituted quinolizidine, a 3,5-disubstituted indolizidine, two pyrrolidines, a spiropyrrolizidine, and several that could not be assigned to a specific structural class (Saporito et al., 2009). These alkaloids have been reported in the scheloribatid mites *Scheloribates azumaensis* and *Scheloribates* sp. from Japan (Takada et al., 2005), and in oribatid mites from Costa Rica and Panama, including numerous scheloribatid mites (Saporito et al., 2007a). The full extent of the diversity and taxonomic distribution of alkaloids in oribatid mites is unknown, but initial studies indicate that they are common in the widespread family Scheloribatidae.

As part of a large, ongoing study on the taxonomic distribution of alkaloids in oribatid mites, we analyzed extracts from adult and immature specimens of the soil mite *Scheloribates laevigatus* (C. Koch) (Acari: Oribatida: Scheloribatidae). This mite is a generalist feeder on fungi, algae, and litter (Woodring and Cook, 1962; Hubert et al., 2001) and is common and abundant in Europe and in anthropogenic habitats in North America, where it appears to be an introduced species (Marshall et al., 1987; Weigmann, 2006; R. Norton, unpublished). Here, we report the presence of pumiliotoxins, indolizidines, and a coccinelline-like tricyclic alkaloid in adults of this species. Most of these alkaloids have been detected previously in the skins of poison frogs, lending support to suggestions that scheloribatid mites are an important dietary source of alkaloids in poison frogs.

Methods and Materials

Mite Collection and Sample Preparation Adult mites were extracted with Berlese-funnels from soil and leaf-litter samples collected near Syracuse, NY, USA. Individuals were maintained at room temperature in small glass jars containing a plaster-charcoal substrate, and were provided with alga-covered tree bark and small amounts of leaf-litter and woody debris. Reproduction in the cultures provided a source of immature mites. For chemical analysis, adult or immature mites (10–25 individuals) were extracted for 24 hr in glass vials with 20 µl of methanol.

Alkaloid Identification When possible, alkaloids were identified by comparison of mass spectra, vapor-phase FTIR spectra, and GC retention times to those of previously reported poison frog alkaloids (Daly et al., 2005). Poison frog alkaloids are assigned code names consisting of a bold-faced number corresponding to the nominal mass (molecular weight) and a bold-faced letter to distinguish alkaloids with the same nominal mass (Daly et al., 2005).

Alkaloids in *S. laevigatus* were quantitated by extracting 25 adult mites with 20 µl of methanol containing 100 ng of nicotine [(-)-nicotine ≥99%, Sigma-Aldrich, Milwaukee, WI, USA] as an internal standard. Nicotine was used because its retention time is outside of the typical values for most mite alkaloids. All alkaloids were quantitated by comparing peak areas to that of the nicotine internal standard in the total ion current (TIC) gas-chromatogram (see *Chemical Analysis*) with the ICIS peak detection function in Xcalibur © version 1.4 SR1 (Thermo Electron Corporation, 1998–2003). Although a flame ionization detector (FID) may provide better dynamic range linearity, the moderate precision reported in this paper can be achieved with either TIC or FID if an internal standard is used to account for losses due to instrument behavior and sample manipulation. A similar analysis was done for immature mites, but without the internal standard, which could interfere with the determination of low levels of alkaloids. The analyses were repeated on three independent extracts.

Chemical Analysis GC-MS data were obtained on a Thermo-Electron Polaris-Q instrument coupled to a Focus GC with a 30 m×0.25 mm i.d. Restek-5MS fused silica column. GC separation of alkaloids was achieved using a temperature program from 100 to 280°C at a rate of 10°C per min with He as carrier gas (flow rate: 1 ml/min.). Each extract was analyzed with both electron impact-mass spectrometry (EI-MS) and chemical ionization-mass spectrometry (CI-MS) with NH_3 as the reagent gas. High resolution GC-MS analysis was obtained using a Waters GCT instrument coupled to a Hewlett-Packard model 6890 GC, with a column, temperature program, and carrier gas conditions identical to those listed above. GC-FTIR data were obtained with a Hewlett-Packard model 5890 gas chromatograph fitted with a 30 m×0.32 mm i.d. Phenomenex Zebron ZB-5 capillary column (same temperature program as above), interfaced with a Hewlett-Packard model 5971 Mass Selective Detector and a Model 5965B IRD with a narrow range (4000–750 cm^{-1}) infrared detector.

Results and Discussion

GC-MS analysis of adult *Scheloribates laevigatus* extracts detected nine alkaloids (including isomers), representing five structural classes (Figs. 1 and 2a; Table 1). Five of these have been identified previously in the skin of poison frogs: the 5,6,8-trisubstituted indolizidines (5,6,8-I) **195G** and **209C** (two isomers) and pumiliotoxin (PTX) **291G** (two isomers). The 5,6,8-I **195G** also has been previously detected in an unidentified oribatid mite species in the family Scheloribatidae from Bocas del Toro, Panama

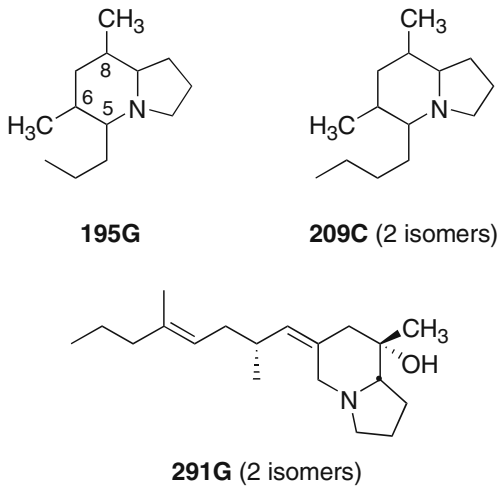

Fig. 1 Structures of alkaloids identified in *Scheloribates laevigatus* that have been previously identified from the skins of certain poison frogs

(Saporito et al., 2007a). The remaining four alkaloids have not been observed previously, but are similar in structure to other alkaloids identified in poison frogs: a pumiliotoxin of molecular weight (MW) 307, a tricyclic (Tri) of MW 247, and an izidine (two isomers) of MW 275 (Table 1; see Supplementary Material for vapor-phase FTIR). Tricyclics represent a large alkaloid group of varied structural types, characterized by mass spectra with many intense peaks, often homologous, but without any dominant alpha-substituent cleavages, probably having three aliphatic rings, and are typified by the coccinelline alkaloids (Daly et al., 2005). Izidines also are a large alkaloid group of varied structural types that are generally characterized as bicyclic izidines with either one or two substituents readily lost by alpha-cleavage during mass spectrometric analysis, but without fragmentation patterns corresponding to any of the currently known pyrrolizidine, indolizidine, or quinolizidine classes (Daly et al., 2005). The chemical properties (MW, empirical formulae, GC retention times, mass spectral data, vapor-phase FTIR spectral data, and other data) of these previously unreported alkaloids are found in Table 1. Additional physicochemical characterizations (e.g., ^{1}H and ^{13}C NMR) will be necessary in order to assign definitive structures to these alkaloids.

Nine frog-skin alkaloids of MW 275 are currently known with the empirical formula assigned to the two new MW 275 mite alkaloids. They are assigned codes of **275A-G**, **275I**, and **275K** and are found in dendrobatid, mantellid, or bufonid species. None has a mass spectrum similar to the ones from the two mite MW 275 isomers. Nine dendrobatid or mantellid frog alkaloids, **247B-247J,** have MW 247 and the same empirical formula calculated for the mite MW 247 alkaloid. Only one of the frog compounds is a tricyclic, and it has a significantly different mass spectrum from that of the mite alkaloid.

Individual *S. laevigatus* adults (average wet mass 29 μg) contained approximately 17±2 ng (mean ± SD) of alkaloids, about 0.06% of the mite's body weight. The most abundant alkaloid was PTX **291G** (two isomers), accounting for approximately 9 ng (50%) of the total alkaloid quantity per mite. Takada et al. (2005) reported that individual *S. azumaensis* contained about 10 ng of PTX **251D**. The next most abundant alkaloids in *S. laevigatus* were the novel alkaloids PTX of MW 307, Tri of MW 247, and the two izidines of MW 275 (all of them present in approximately equal amounts), followed by the 5,6,8-Is **195G** and **209C** (two isomers). Figure 2a is a representative chromatogram illustrating the relative alkaloid quantities for adult *S. laevigatus*.

All of the alkaloids identified in *S. laevigatus* contain branch points in their carbon skeleton (Fig. 1), which appears to be the most common alkaloid structural characteristic seen in oribatid mites (Saporito et al., 2009).

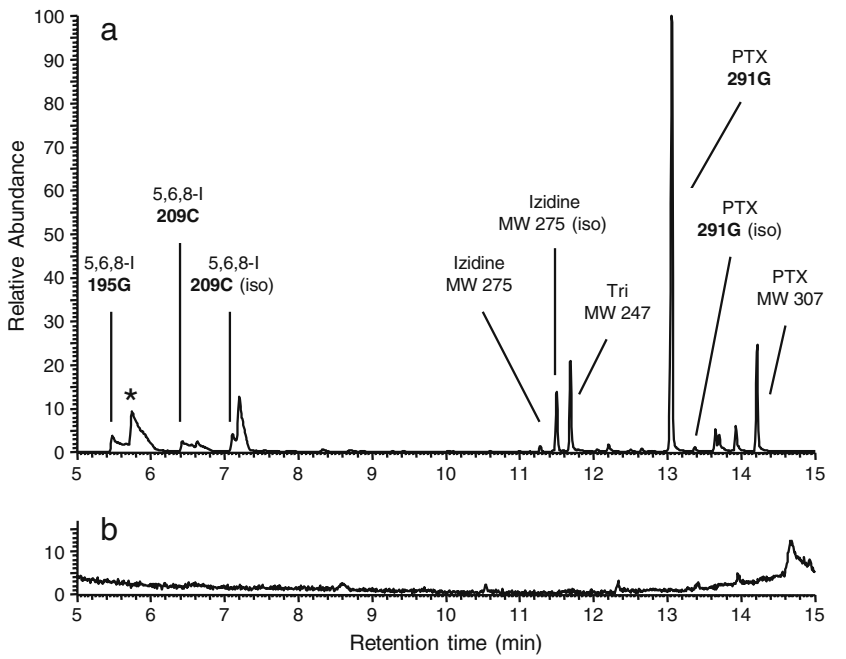

Fig. 2 Gas chromatogram of *Scheloribates laevigatus* extracts in adults (**a**) and immature mites (**b**). The unlabeled peaks in chromatogram a and b are not alkaloids. 5,6,8-I=5,6,8-trisubstituted indolizidine; PTX = pumiliotoxin; Tri = coccinelline-like tricyclic. An *asterisk* (*) indicates the nicotine internal standard in the adult extracts. The term (*iso*) refers to an alkaloid isomer

Numerous branched-chain alkaloids (including pumiliotoxins, 5,6,8-trisubstituted indolizidines, and tricyclics) have been identified previously in oribatid mites, including other members of the genus *Scheloribates* (Saporito et al., 2009). Takada et al. (2005) identified the branched-chain alkaloids PTX **251D**, Tri **193C** (precoccinelline), and an unidentified Tri in *Scheloribates azumaensis*, and the branched-chain PTX **237A**, deoxy-pumiliotoxin **193H**, 5,6,8-I **223A**, and the 1,4-disubstituted quinolizidine (1,4-Q) **231A** in an unidentified *Scheloribates* sp. from Japan. Saporito et al. (2007a) identified mainly branched-chain alkaloids in several different species of oribatid mites (including *Scheloribates*) from Costa Rica and Panama, which included pumiliotoxins, a homopumiliotoxin, 5,6,8-trisubstituted indolizidines, 5,8-disubstituted indolizidines (5,8-Is), dehydro-5,8-disubstituted indolizidines, a 1,4-disubstituted quinolizidine, tricyclics (including precoccinelline), and a spiropyrrolizidine (see Tables 1 and 2 in Saporito et al. 2007a, 2008). A smaller number of unbranched alkaloids also have been identified in oribatid mites (Saporito et al., 2007a). Branched-chain alkaloids also represent the most common alkaloid structural type found in poison frogs worldwide, accounting for approximately 70% of all classified alkaloids, the majority of which are pumiliotoxins, 5,6,8-Is, 5,8-Is, and tricyclics (Saporito et al., 2009).

GC-MS analysis indicated that immature *S. laevigatus* contained no detectable alkaloids (Fig. 2b), which suggests that alkaloids are adult-specific and possibly biosynthesized by the mites. Under culture conditions, both adults and immature mites had the same food choices, and therefore it is unlikely that adult *S. laevigatus* acquire alkaloids or precursor(s) from a dietary source. The culture substrates provided to the mites—tree bark with *Protococcus* alga and secondary fungal growth—are not known to contain the alkaloids in question. However, it remains possible that: (1) adults contained alkaloids that were sequestered from a natural diet, whereas immature mites, which bred in culture, did not have an opportunity to sequester such alkaloids from a natural diet; (2) adults and immature mites had unobserved dietary differences within the cultures; and/or (3) immature mites lack an ability to acquire alkaloids or precursor(s) from their diet. Takada et al. (2005) similarly

Table 1 Chemical properties of previously unreported alkaloids from oribatid mites. These alkaloids have not been found in poison frogs

247. Tri. $C_{17}H_{29}N$. Rt 12.24. MS: *m/z* 247 (18), 232 (5), 218 (12), 204 (22), 190 (100), 176 (78), 162 (52), 148 (36), 137 (50), 136 (35), 124 (16), 96 (22), 70 (20). Vapor phase FTIR (cm^{-1}): 3015 (cis double bond), 2968, 2938 (s), 2866, 2791 (strong Bohlmann band), 984. DBE = 4.

275. Izidine. $C_{19}H_{33}N$. Rt 11.84. MS: *m/z* 275 (22), 246 (5), 190 (48), 178 (22), 150 (100), 136 (20), 70 (8). DBE = 4.

275. Izidine (Isomer). $C_{19}H_{33}N$. Rt 12.06. MS: *m/z* 275 (20), 246 (5), 190 (55), 178 (24), 150 (100), 136 (18), 70 (10). Vapor phase FTIR (cm^{-1}): 2967(s), 2901, 2789. DBE = 4.

307. PTX. $C_{19}H_{33}NO_2$. Rt 14.78. MS: *m/z* 307 (<1), 291 (15), 206 (38), 194 (12), 193 (14), 176 (16), 166 (100), 70 (35). Vapor phase FTIR (cm^{-1}): 3543 (H-bonded OH), 2969 (s), 2888, 2799 (strong Bohlmann band), complex fingerprint region typical of PTXs. DBE = 4.

The tabulation of alkaloids and format of properties, including the Rt values which were proportionally corrected to the known value, follow those of the Appendix in Supporting Information of Daly et al. (2005)

reported that alkaloids occur only in adults of *S. azumaensis* and *Scheloribates* sp., and suggested that alkaloids may be biosynthesized by adult oribatid mites. Our study supports the hypothesis that adult oribatids synthesize alkaloids, but the possibility of a microsymbiont cannot be excluded. Microsymbionts are the source of chemical defenses and/or natural products in a variety of organisms, including vertebrates and invertebrates (Piel, 2002; Daly, 2004).

Some of the alkaloids (and alkaloid classes) found in oribatid mites have been identified in other arthropods. These include some pumiliotoxins, which have been detected in formicine ants of the genera *Brachymyrmex* and *Paratrechina*; some tricyclics (including precoccinelline), detected in coccinellid beetles; a spiropyrrolizidine, detected in polyzoniid millipedes in the genera *Rhinotus* and *Kiusiozonium*; and a number of unbranched-chain alkaloids that were originally considered to be exclusively of myrmicine ant origin: a 3,5-disubstituted indolizidine, two 4,6-disubsituted quinolizidines, and two pyrrolidines (Saporito et al., 2009).

The occurrence of identical alkaloids in both oribatids and other arthropods raises the possibility that some may be transferred among different arthropod groups, possibly through trophic interactions (Takada et al., 2005; Saporito et al., 2009). Certain myrmicine ants in the genera *Myrmecina*, *Pheidole*, and *Oligomyrmex*, and several families of small beetles (Ptiliidae, Staphylinidae, and especially Scydmaenidae) are predators of oribatid mites (Ito and Takaku, 1994; Sanders and Norton, 2004; Wilson, 2005). Furthermore, oribatid mites can be found in ant nests as specialized or unspecialized myrmecophiles (Ito and Takaku, 1994). Although it is not known if alkaloids are present in arthropod predators of oribatid mites, it is conceivable that predation and sequestration could explain the presence of identical alkaloids in at least some of these evolutionarily distinct arthropod lineages. However, it is also possible that different lineages of arthropods synthesize identical alkaloids, and/or that there is a yet unknown, widespread alkaloid-containing microsymbiont present among different arthropod groups.

The biological function of alkaloids in *S. laevigatus* and other oribatid mites is unknown. However, the pumiliotoxins, which comprise the most abundant alkaloids in *S. laevigatus*, are highly toxic (Weldon et al., 2006) and may defend oribatid mites against predation. In poison frogs, alkaloids serve as a chemical defense against potential invertebrate and vertebrate predators (Saporito et al., 2007b), microorganisms (Macfoy et al., 2005), and possibly ectoparasites (Weldon et al., 2006). The functions of other compounds identified in oribatid mites have not been fully studied, but include alarm signals and chemical defenses (Shimano et al., 2002; Raspotnig et al., 2003). In the better-studied and closely related mite group Astigmata, organic compounds also function as aggregation signals and sex pheromones (Kuwuhara, 2004). Alkaloids most likely comprise part of the defensive "tool box" of adult *S. laevigatus*, similar to the non-alkaloid secretions present in immature mites of this species and in many other oribatid mites (Raspotnig, 2009). Research is needed to identify the specific role of alkaloids in oribatids, and to determine why there is such a marked ontogenetic shift in scheloribatid mite gland chemistry, if indeed the alkaloids are housed there.

It has been established from analysis of stomach contents that mites constitute a major prey item for many poison frogs (Simon and Toft, 1991; Valderrama-Vernaza et al., 2009). It is evident that some oribatid mites contain a relatively high diversity and quantity of alkaloids, and that many of these are in the skin of poison frogs. We think oribatid mites may constitute the most important dietary source of alkaloids in poison frogs, more so than ants, which have long been considered to be the principal source (Saporito et al., 2004). Furthermore, since members of the widespread genus *Scheloribates* contain the greatest diversity of alkaloids known from oribatids to date, scheloribatids may be an especially important dietary item for poison frogs worldwide. Additional studies of poison frog diet through stomach content analysis, in which mites are taxonomically identified, coupled with information on the distribution and availability of scheloribatids, will be necessary to understand the importance of scheloribatid mites to the alkaloid defenses of poison frogs.

Although oribatids are a significant proximate source, the ultimate biological source of these alkaloids remains unknown. To more conclusively understand the origin of alkaloids in mites (and therefore in poison frogs), we propose three general research themes: (1) a series of feeding experiments in which cultured mites, raised through multiple generations, are provided a diet with and without alkaloids and/or precursors; (2) identification of the location of alkaloids within adult mites (e.g., are they located in the opisthonotal glands?); and (3) an attempt to culture the microbes (e.g., bacteria, fungi, etc.) present in mites in search for a possible alkaloid-producing microsymbiont.

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