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A SIPHONOTID MILLIPEDE (*RHINOTUS*) AS THE SOURCE OF SPIROPYRROLIZIDINE OXIMES OF DENDROBATID FROGS

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Abstract—Poison frogs of the neotropical family Dendrobatidae contain a wide variety of lipophilic alkaloids, which are accumulated from alkaloid-containing arthropods. A small millipede, *Rhinotus purpureus* (Siphonotidae), occurs microsympatrically with the dendrobatid frog *Dendrobates pumilio* on Isla Bastimentos, Bocas del Toro Province, Panamá. Methanol extracts of this millipede contain the spiropyrrolizidine *O*-methyloxime **236**, an alkaloid previously known only from skin extracts of poison frogs, including populations of *D. pumilio*. Thus, *R. purpureus* represents a likely dietary source of such alkaloids in dendrobatid frogs.

Key Words—Alkaloids, arthropods, frogs, dendrobatids, millipedes, and pyrrolizidines.

INTRODUCTION

Millipedes produce a wide range of allomones, which presumably act to deter potential predators. Millipede allomones include benzoquinones, phenols, benzaldehyde/hydrogen cyanide, and alkaloids (Eisner et al., 1978 and references therein). Three groups of alkaloids

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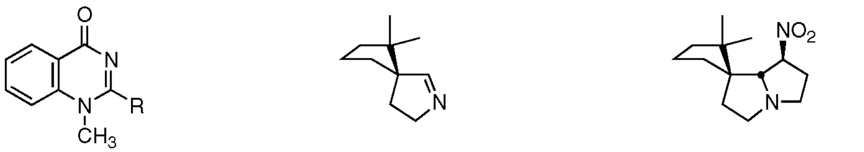
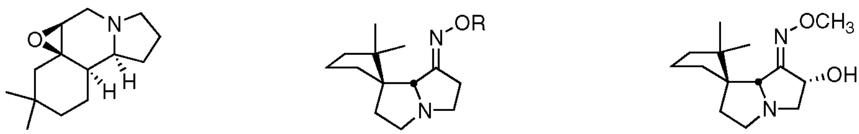


Ia R = CH_3 **Glomerin** II **Polyzonimine** III **Nitropolyzonamine**

Ib R = C_2H_5 **Homoglomerin**



IV **Buzonamine** Va R = H **222** VI **252A**

Vb R = CH_3 **236**

FIG. 1. Millipede alkaloids.

are known from millipedes as follows: (1) the glomerins (Ia & b) from millipedes of the genera *Glomeris* and *Loboglomeris* (Order: Glomerida, Family: Glomeridae) (Schildknecht et al., 1967 and reference therein), (2) the terpenoid alkaloids polyzonimine (II) and nitropolyzonamine (III) from the millipede *Polyzonium rosalbum* [now *Petaserpes cryptocephalus*] (Order: Polyzoniida, Family: Polyzoniidae) (Meinwald et al., 1975 and reference therein), and (3) the terpenoid alkaloid buzonamine (IV) from a millipede of the genus *Buzonium* (Order: Polyzoniida, Family: Polyzoniidae) (Wood et al., 2000) (Figure 1). Certain poison frogs/toads of the families Dendrobatidae, Mantellidae, and Bufonidae contain spiropyrrolizidine alkaloids (Figure 1; Va & b and VI) (Daly et al., 1994 and references therein) with carbon skeletons identical to those of the millipede alkaloid nitropolyzonamine. These spiropyrrolizidine oximes have been assigned 'bold type' code names (**222**, **236**, and **252A**), which consist of a boldfaced number corresponding to the nominal mass and a boldfaced letter for identification of individual alkaloids. Such frogs appear to obtain their skin alkaloids from dietary sources (Daly et al., 1994, 2000), and, therefore, it seemed likely that a polyzoniid millipede would prove to be a dietary source of spiropyrrolizidine oximes.

Spiropyrrolizidine **236** and nitropolyzonamine have been detected in extracts of leaf-litter arthropods collected with Berlese Funnels from Cerro Ancón, Panamá (Daly et al., 1994) and in mixed leaf-litter samples from Isla Bastimentos, Panamá (Daly et al., 2002), however, the specific arthropod source was not determined. In an effort to taxonomically identify arthropod sources for the frog skin alkaloids, a series of leaf-litter arthropod samples, separated by taxa (including millipedes), were again collected and analyzed from

Isla Bastimentos, Panamá. From these collections, the spiropyrrolizidine oxime **236** was detected in samples containing only millipedes. We now report on the identification of a small millipede, *Rhinotus purpureus* (Pocock, 1894) (Order: Polyzoniida, Family: Siphonotidae), that contains the spiropyrrolizidine oxime **236** and, thus, represents a dietary source for the same alkaloid found in the skin extracts of the dendrobatid frogs (*Dendrobates pumilio*) from the same location on Isla Bastimentos. In addition, trace amounts of nitropolyzonamine were detected in the millipede extract. Based on stomach content analysis, the diet of *D. pumilio* consists of a variety of small leaf-litter arthropods, including small millipedes (Donnelly unpublished data; Saporito et al., unpublished data).

METHODS AND MATERIALS

A variety of arthropods were collected from leaf-litter and separated by taxa at several different sites on Isla Bastimentos, Bocas del Toro Province, Panamá. Arthropod collections were made from February 2–8, 2003, from the following 8 locations: Site 1: N 9°21.618'; W 82°12.074'. Site 2: N 9°21.250'; W 82°12.519'. Site 3: N 9°21.169'; W 82°12.627'. Site 4: N 9°21.123'; W 82°12.620'. Site 5: N 9°20.996'; W 82°12.726'. Site 6: N 9°20.364'; W 82°10.807'. Site 7: N 9°21.021'; W 82°12.704'. Site 8: N 9°20.490'; W 82°10.486'. Several of these sites were at the same location where mixed arthropod collections were previously collected (Daly et al., 2002). All arthropod specimens were collected with forceps, from leaf-litter placed on white cloth, and placed in plastic 1.5 ml vials according to individual taxa. Only small arthropods (less than 10 mm), suitable as prey for the dendrobatid frog *D. pumilio* (average snout-vent length 20 mm), were collected from these sites.

Gas chromatographic mass spectral analyses (GC-MS) were conducted with a Finnigan GCQ instrument with a 25 m × 0.25 mm i.d. Rtx-5 Amine fused silica column, using a temperature program from 100 to 280°C at a rate of 10°C/min. Arthropod extracts were screened for the presence of alkaloids by injecting a 4 μl mixture, which consisted of 1 μl portions from each of 4 different vials (arthropod samples). All mixtures were based on specific taxonomic groups (i.e., millipede samples were only combined with other millipede samples, etc.). If an alkaloid was detected in a mixture, then each of the four vials was analyzed separately to determine the source of the alkaloid. Identification of alkaloids was based on gas chromatographic mass spectra and comparison to authentic spiropyrrolizidine **236** or nitropolyzonamine.

RESULTS AND DISCUSSION

The spiropyrrolizidine alkaloid **236** was detected in two separate millipede samples, both of which were from site 7 located on Isla Bastimentos. Both samples contained one individual of *Rhinotus purpureus* (Pocock, 1894). In addition, trace amounts of nitropolyzonamine were also found in these samples.

Alkaloids were not detected in extracts from the following other millipede samples: (1) Order: Polyxenida; 1 individual. (2) Order: Stemmiulidae; Family: Stemmiulidae; Species: *Prostemmiulus* cf. *cincinnatus* (Loomis); 3 individuals. (3) Order: Spirobolida; Family: Spirobolellidae; Species: *Spirobolellus trifasciatus* (Loomis); 3 individuals. (4) Order: Polydesmida; Family: Aphelidesmidae; Genus: *Aphelidesmus*; 1 adult and 1 immature. (5) Order: Polydesmida; Family: Chelodesmidae; Genus: *Trichomorpha*; 1 individual. (6) Order: Polydesmida; Family: Cyrtodesmidae; Genus: *Cyrtodesmus*; 1 individual. (7) Order: Polydesmida; Family: Fuhrmannodesmidae; 2 individuals. (8) Order: Polydesmida; 3 immature individuals.

Spiropyrrolizidine **236** was not detected in any of the other arthropod extracts examined in this study, however, a decahydroquinoline and a 5,6,8-trisubstituted indolizidine were detected in certain ant extracts. These results will be reported separately. Decahydroquinolines have been reported from myrmicine ants (Daly et al., 2000, 2002 and references therein). However, until now, there have been no known reports of branched chain izidine alkaloids from ant extracts, although a branched chain 5,8-disubstituted indolizidine was detected in extracts from mixed arthropod samples from Isla Bastimentos (Daly et al., 2002). In the present study, the frogs from the site at which the millipedes had the spiropyrrolizidine **236** were also shown to have that alkaloid in skin extracts (Daly et al., 2002 and unpublished data). Nitropolyzonamine was not detected in the skin extracts from *D. pumilio* on Isla Bastimentos. However, nitropolyzonamine, as well as the spiropyrrolzidine oximes **236** and **252B**, (not shown, see below) have been detected as trace amounts in *Dendrobates auratus* from Cerro Ancón, Panamá (Daly et al., 1994).

The spiropyrrolizidine oxime **236** has been detected in frog skin extracts from several species of dendrobatid frogs from a variety of locations throughout Central and South America (Table 1). From these locations, it is interesting to note the absence of **236** from some of the frog populations in Panamá during initial visits (Table 1). The appearance of **236** in later visits to the same Panamanian sites may suggest an introduction and, therefore, availability of *R. purpureus* to frogs within these populations.

The millipede *R. purpureus* has a relatively wide distribution throughout the tropics; therefore, it seems probable that other *Rhinotus* species may serve as a source for spiropyrrolizidine oximes found in dendrobatid frogs of South America. Spiropyrrolizidine oxime **236** has been detected from an Argentinean bufonid toad (Garraffo et al., 1993) and from Madagascan mantellid frogs (Daly et al., 1996). In addition, one extract from an Australian myobatrachid frog had spiropyrrolizidine oxime **252B**, an isomer of **252A** in which the hydroxyl group is in a different position (Daly et al., 1990). It is likely that such spiropyrrolizidine oximes will be found to have a deterrent effect on potential millipede predators,

TABLE 1. OCCURRENCE OF SPIROPYRROLIZIDINE **236** IN FROG/TOAD SKIN EXTRACTS: AN INDICATION OF AVAILABILITY OF *RHINOTUS* MILLIPEDES?

Family: *Species*	**Location**	**Year**	**236**
Dendrobatidae:			
Dendrobates pumilio	Rumpala, Bocas Province, Panamá	1981	Present
	Isla Bastimentos, Bocas Province, Panamá	1972	Absent†
		1980, 1981, 1987, 1993, 2002*	Present
	La Gruta, Isla Colón, Bocas Province, Panamá	1983, 1986	Absent†
		1992	Present
	Río Sand Box, Limón Province, Costa Rica	1989	Present
	Río Sarapiqui, Heredia Province, Costa Rica	1989	Present
	28 Millas, Limón Province, Costa Rica	1990, 1995	Present
	Siquirres, Limón Province, Costa Rica	1995	Present
	Pocora, Limón Province, Costa Rica	1995	Present
Dendrobates auratus	Isla Tobago, Panamá	1974	Absent†
		1993	Present
	Cerro Ancón, Panamá	1993	Present
Dendrobates tinctorius	Pet Trade	1985	Present
Epipedobates macero	Parque Nacional Manu, Perú	1989	Present
Epipedobates cf. pictus	Parque Nacional Manu, Perú	1989	Present
Epipedobates pulchripectus	Serra de Navio, Amapa, Brazil	1992	Present
Epipedobates tricolor	Pasaje, El Oro, Ecuador	1987	Present
Epipedobates trivittatus	Pet Trade	1985	Present
Mantellidae:			
Mantella baroni	Sahavondrana, Madagascar	1993	Present
Mantella betsileo	Mahambo, Madagascar	1998	Present
Bufonidae:			
Melanophryniscus stelzneri	Tanti, Córdoba, Argentina	1989	Present

*see Daly et al. 2002.
†not detected.

such as ants, however, such studies have yet to be conducted. The oxime **236** does exert potent blocking action at mammalian nicotinic receptors, particularly those of the ganglionic subtype (Badio et al., 1996).

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