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Detoxification of pyrrolizidine alkaloids by the harvestman *Mitopus morio* (Phalangidae) a predator of alkaloid defended leaf beetles

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Summary. The harvestman *Mitopus morio* (Phalangidae) is a generalist predator. It is known to prey on larvae of the chrysomelid leaf beetle *Oreina cacaliae* defended by plant acquired pyrrolizidine alkaloids (PAs). Tracer feeding experiments were performed to determine how harvestmen tolerate protoxic PAs. Minced meat containing either [^{14}C]senecionine or [^{14}C]senecionine *N*-oxide was fed to *M. morio* and subsequently feces and bodies were analyzed. Labeled alkaloid *N*-oxide remained stable and was eliminated almost unaltered with the feces; only 10% was recovered as tertiary PA. In contrast, approximately 80% of labeled tertiary alkaloid (senecionine) ingested with the diet was *N*-oxidized and eliminated; the remaining 20% consisted of unchanged senecionine and a polar metabolite of unknown structure. Harvestmen process their diet by excreting digestive juice, indicated by bleaching of the meat color. Analysis of the processed diet revealed some *N*-oxidation of [^{14}C]senecionine, suggesting the gut as the site of *N*-oxidation. Analysis of the bodies of harvestmen 80 hours after the tracer feeding pulse revealed only trace amounts of the polar metabolite. Neither senecionine nor its *N*-oxide could be detected in the body extracts. The results are discussed in relation to the strategies of PA adapted insects to avoid accumulation of tertiary PAs in living tissues.

Key words. Alkaloid sequestration – Detoxification – Chemical defense – *Mitopus morio* – *Oreina* leaf beetles – Predation – Pyrrolizidine alkaloids

Introduction

Leaf beetles of the genus *Oreina* are chemically protected by defensive secretions released from exocrine glands located in the elytra and pronotum (Pasteels *et al.* 1988; Pasteels *et al.* 1994). This chemical defense is primarily autogenous by cardenolides synthesized from plant acquired sterols (van Oycke *et al.* 1987; Dobler *et al.* 1996; Pasteels *et al.* 1996). Only a few *Oreina* species (e.g. *O. cacaliae* and *O. speciosissima*) feeding on plants belonging to the Asteraceae, sequester pyrrolizidine alkaloids (PAs) from their host plants, e.g. *Adenostyles alliariae* and *Senecio nemorensis* (Pasteels *et al.* 1996; Hartmann *et al.* 1997). The PA concentrations in the defensive secretions of alkaloid sequestering *Oreina* species may reach 0.3 mol × l^{-1} (Rowell-Rahier *et al.* 1991; Hartmann *et al.* 1997). Besides accumulation in adults of sequestered PAs in the defensive secretions, adult beetles and larvae are capable of storing PAs in their hemolymph and tissues, preferentially the hemolymph (Ehmke *et al.* 1991; Rowell-Rahier *et al.* 1991; Pasteels *et al.* 1992; Dobler & Rowell-Rahier 1994; Pasteels *et al.* 1995; Hartmann *et al.* 1997; Ehmke *et al.* 1999). Although PA concentrations in the body-tissue are much lower than in the defensive secretions of adults, most of the alkaloids found in an individual are localized in the body compartment. Total PAs in the body of larvae and adults may reach 70 –130 mg per individual (i.e., 1 – 2 mg per g fresh weight) in comparison to around 4 µg PAs in the total secretions of an adult (i.e., ca 50 mg per g secretion) (Hartmann *et al.* 1997).

Host-plant acquired PAs are assumed to provide the leaf beetles with an efficient defense against predators. In fact, it has been shown that PAs in *Oreina* leaf beetles provide better protection against predation by birds than cardenolides (Rowell-Rahier *et al.* 1995). The value of PAs for protection in insects was also shown in specialist Lepidoptera. Egg batches of the arctiid moth *Utetheisa* are efficiently protected by PAs against predation by coccinellid beetles and ants (Hare & Eisner 1993). The giant tropical orb spider, *Nephila clavipes*, is an important predator of butterflies. PA-protected lepidopterans such as the moths *Utetheisa* (Eisner 1982) and *Hyalurga* (Trigo *et al.* 1993) and various Ithomiinae butterflies (Brown 1984; Masters 1990) are rejected by the spider; they are cut out of the web and liberated unharmed.

The harvestman *Mitopus morio* (Arachnida, Opioliones, Phalangidae) is the major predator of *O. cacaliae* larvae in their natural environment (Labeyrie & Rahier 2003). Opilionids are considered to be generalist predators. Predation of beetle larvae by opilionids is poorly documented (Drummoud *et al.* 1990). Nothing is known about the mechanisms that enable harvestmen to prey on *Oreina* larvae defended by protoxic PAs. Here we show that *Mitopus morio* possesses efficient biochemical mechanisms to detoxify, stabilize and eliminate PAs ingested with their prey.

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Material and methods

Animals

Adult *Mitopus morio* harvestmen were sampled at the Swiss field sites where *Oreina* is found (Labeyrie & Rahier 2003) and in the vicinity around Neuchâtel (Switzerland). Their body length (without legs) ranged from 4 to 7 mm. They were kept individually in plastic boxes (5 × 7 cm) with plaster bottom that was kept moist to maintain a high humidity level.

Tracer feeding studies

[^{14}C]Senecionine and [^{14}C]senecionine *N*-oxide (each 1.07 GBq × mmol^{-1}) were prepared biosynthetically from [1,4-^{14}C]putrescine (4.4 GBq × mmol^{-1}; Amersham Biosciences, Freiburg, Germany) using root cultures of *Senecio vulgaris* (Hartmann 1994). Tracer-labeled diet was prepared by adding 80 µl of the aqueous tracer solution to 220 mg of thoroughly minced beef meat. The tracer solutions were prepared as follows: (i) [^{14}C]senecionine - 80 µg non-labeled senecionine containing approx. 10^6 cpm labeled senecionine was dissolved in 10 µl methanol and diluted with 70 µl water; (ii) [^{14}C]senecionine *N*-oxide - 80 µg non-labeled *N*-oxide containing approx. 10^6 cpm labeled *N*-oxide was directly dissolved in 80 µl water. After addition of the tracer solutions the meat samples were thoroughly homogenized in a 500 µl Eppendorf vial using a plastic rod. In each series the labeled diet was portioned and placed into plastic vials (23 mm i.d.; 50 mm height) equipped with a moist filter paper and sealed with a pierced cap. Each vial received approx. 17 mg diet; the precise weight of the diet was determined. Then in each series ten harvestmen which had been starved for at least 24 hours were added individually to the vials; three vials were treated as controls. The vials were kept at 15°C and the harvestman were allowed to feed for ca 4 hours and were then transferred into new vials containing fresh untreated minced meat. Feces was collected at intervals as indicated. In addition the remains of the tracer-treated diet was separated from the filter paper, weighed, and both the meat-remains and the filter paper were analyzed for radioactivity.

Total radioactivity evaluated for the collected feces fractions as well as animal extracts was related to the amount of "ingested tracer" (= 100%). Ingested tracer is defined as total radioactivity offered minus total radioactivity recovered from the diet-remains and the filter paper on which the diet had been offered.

Feces samples, meat-remains and animals were extracted twice with 2 ml methanol each. After centrifugation total radioactivity was determined by scintillation counting (Rialuma, Baker). Separation of the labeled extracts to localize ^{14}C-labeled senecionine, senecionine *N*-oxide and their metabolites was achieved by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) according to Ehmke *et al.* (1991) and Hartmann and Dierich (1998). Radioactively labeled compounds were located by means of a TLC multichannel analyzer (Rita-32a, Raytest) and a HPLC radioactivity monitor LB-506D (Berthold).

Results

Metabolism and elimination of radioactively labeled PAs by harvestmen

Preliminary experiments revealed that harvestmen readily feed on minced beef-meat. Therefore a minced meat diet was applied to feed harvestmen individuals with ^{14}C-labeled senecionine and its *N*-oxide. Each individual was supplied with ca 17 mg minced meat containing ca 6 µg alkaloid (with a total radioactivity of ca 7.5 × 10^5 cpm). The harvestmen were fed for 4 h and then transferred to fresh untreated food; the feces was collected in regular intervals over a period of 80 h. At the end of the tracer feeding period (4 h) 72% (senecionine *N*-oxide) and 73% (senecionine) of the respective diets had been consumed by the harvestmen. Pharmacokinetics of the elimination of total radioactivity with the feces is illustrated in Fig. 1. The ingested radioactivity was rapidly eliminated; the two first feces samples collected after 23 and 30 hours contained 75 to 80% of total radioactivity; only trace amounts were found in feces samples taken after 57 and 80 hours. Analysis of the body extracts (after 80 h) revealed a residual radioactivity of 5% ± 2 in the senecionine fed animals and less than 1% in the *N*-oxide fed individuals (Fig. 1).

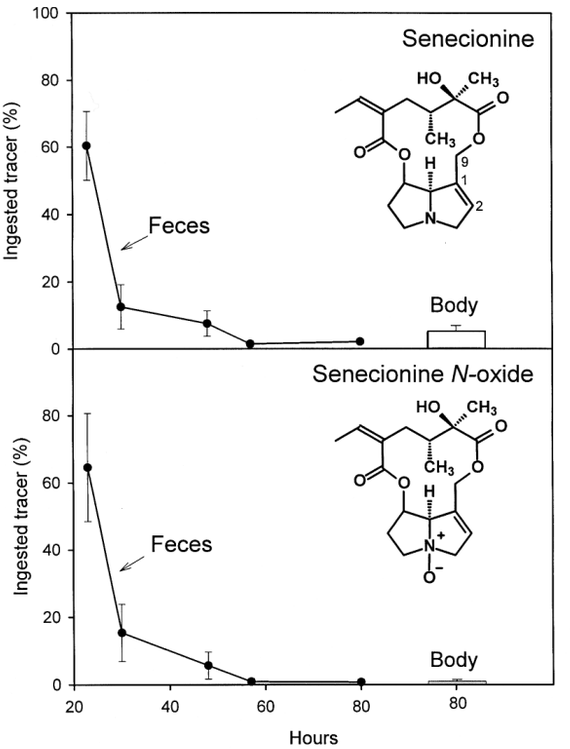

Fig. 1 Elimination of radioactivity with the feces from harvestmen fed for 4 hours with minced meat containing [^{14}C]senecionine and [^{14}C]senecionine *N*-oxide, respectively. After termination of the experiments (80 h) the harvestmen bodies were extracted and analyzed for residual radioactivity. n = 10 each set

TLC analysis of the first feces samples collected after 23 hours revealed that after feeding on either tracer senecionine *N*-oxide was the major compound (77% and 89%, respectively) (Fig. 2). In both experiments only around 10% of radioactivity was recovered as senecionine. In the feeding experiment with senecionine a polar metabolite (Fig. 2, compound X) accounting for 10% of radioactivity was detected; this compound was absent when senecionine *N*-oxide was fed as tracer. The structure of compound X is unknown; it shows almost no migration on the TLC plate, indicating a very polar metabolite. The same polar metabolite was detected in body extracts of harvestmen previously fed on [^{14}C]senecionine. Extracts prepared from bodies 80 days after the

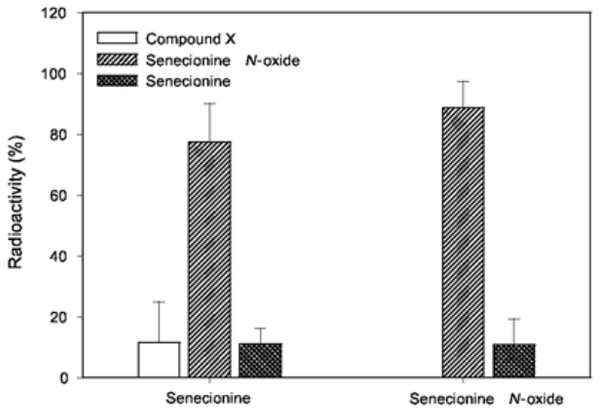

Fig. 2 Analysis (TLC radio-monitoring) of radioactivity eliminated with the feces (collected cumulatively after 23 h) from harvestmen fed with [^{14}C]senecionine and [^{14}C]senecionine *N*-oxide, respectively. Total radioactivity recovered from the feces was set 100%

tracer feeding pulse which still contained 5% of ingested radioactivity (Fig. 1) contained the polar compound X as the only labeled compound; neither senecionine nor its *N*-oxide was detected.

During feeding, harvestmen apparently condition their diet indicated by a rapid change of the color of the meat from red to white. Most likely this process is based on external fermentation by excretion of digestive juice. To see whether this external fermentation affects the added alkaloids the remains of the meat were analyzed immediately after termination of the tracer feeding pulse. The results are summarized in Table 1. As in the respective controls, radioactively labeled senecionine *N*-oxide remains unaltered in the diet processed by the harvestmen. In contrast, the tertiary alkaloid is less stable, although the results display great variation between individual samples. In some samples varying amounts of senecionine *N*-oxide and compound X appeared, whereas senecionine was no longer detectable; in other samples only minor changes occurred. At least the results indicate the potential of the digestive juice to transform senecionine into its *N*-oxide and compound X. The respective controls behave stably within the time limits of the experiments.

Discussion

PAs exist in two molecular forms, the free base (tertiary alkaloid) and its *N*-oxide. Members of the plant families Asteraceae, Boraginaceae and Fabaceae, which represent the major sources of PA containing plant species, synthesize and store PAs exclusively as *N*-oxides (Hartmann & Witte 1995; Hartmann 1999). Most PA-sequestering insects also handle and accumulate the alkaloids as *N*-oxides, including Lepidoptera such as several arctiid moths (Ehmke *et al.* 1990; Hartmann *et al.* 1990; Nickisch-Rosenegk *et al.* 1990; Nickisch-Rosenegk & Wink 1993) and ithomiine butterflies (Trigo *et al.* 1996), Orthoptera such as the aposematic grasshopper *Zonocerus* (Biller *et al.* 1994) and leaf-beetles of the genus *Oreina* (Pasteels *et al.* 1996; Hartmann *et al.* 1997; Hartmann *et al.* 1999). Tertiary PAs with certain structural features (e.g., 1–2 double bond and esterification of the allylic hydroxyl group at C-9) are potentially toxic. They are easily bioactivated by microsomal cytochrome P-450 enzymes into unstable pyrrolic intermediates which are highly reactive alkylating agents (Winter & Segall 1989). In vertebrates this bioactivation is responsible for the well understood liver toxicity of PAs (Mattocks 1986; Cheeke 1994, 1998). Insects which possess a similar xenobiotic metabolism with microsomal cytochrome P-450 enzymes (Hodgson 1985; Brattsten 1992) should be affected by potentially toxic PAs in the same way as vertebrates. In fact, genotoxic effects of PAs in the *Drosophila* wing test have been demonstrated (Frei *et al.* 1992). Non-adapted herbivores feeding on a PA plant ingest the alkaloid *N*-oxides which are easily reduced in the gut (Mattocks 1986) and are taken up passively as lipophilic tertiary alkaloid (Lindigkeit *et al.* 1997). This explains the toxicity of plant PAs in spite of their stable storage as *N*-oxides.

Specialized insects that utilize plant acquired PAs for their own defense must prevent accumulation of detrimental concentrations of PAs in the toxic tertiary state. So far three major strategies are known: (i) PA sequestering arctiids (Lepidoptera) have in their hemolymph a flavin-dependent soluble mixed function senecionine *N*-oxygenase which specifically *N*-oxidizes the passively absorbed tertiary PAs (Lindigkeit *et al.* 1997; Naumann *et al.* 2002). The grasshopper *Zonocerus variegatus* (Orthoptera) employs the same strategy. (ii) *Oreina* leaf beetles prevent reduction of ingested PA *N*-oxides in the gut and absorb the alkaloids directly as *N*-oxides; any absorbed tertiary PA is detoxified by glucosylation (Hartmann *et al.* 1999). (iii) Leaf beetles of the neotropical genus *Platyphora* which are taxonomically closely related to *Oreina,* absorb host plant acquired PAs only in the tertiary state (Pasteels *et al.* 2001). They prevent an accumulation of detrimental concentrations of toxic tertiary PAs by efficient transport of the absorbed alkaloid from the hemolymph into the defensive glands. Unlike *Oreina* they do not store PAs in the body outside the glands (Hartmann *et al.* 2001).

Mitopus morio tolerates the defensive alkaloids of its prey by avoiding bioactivation. Ingested tertiary PAs are efficiently detoxified by *N*-oxidation; the *N*-oxides are rapidly eliminated with the feces. PA *N*-oxide ingested with the prey is not reduced in the gut but eliminated unaltered. Probably the *N*-oxidation takes place in the gut and not in the hemolymph. A strong argument for this assumption is the *N*-oxidizing activity in the digestive juice exuded by the harvestmen to process their diet.

M. morio is the first example of a non-sequestering arthropod which detoxifies PAs by *N*-oxidation. So far, the only known enzyme which catalyzes PA *N*-oxidation is mammalian microsomal multisubstrate flavin monooxygenase (EC 1.14.13.8). In some vertebrates such as guinea pigs, the PA *N*-oxidation catalyzed by this enzyme by far exceeds the cytochrome-P-450-dependent bioactivation of PAs. This explains the high resistance of guinea pigs to toxic effects of PAs (Miranda *et al.* 1991). Multisubstrate flavin

Table 1 Feeding of radioactively labeled senecionine (A) and its N-oxide (B) to harvestmen. TLC analysis of the diet remains at the end of tracer feeding pulse (4 h). Diet remains (minced meat) and the filter paper on which the diet was offered were analyzed separately.

		Relative abundance of radioactivity (%)					
		Diet remains (4 h)			Filter paper (4 h)		
	Diet remains weight (%)	"X"	S *N*-ox	Sen	"X"	S *N*-ox	Sen
A [^{14}C] senecionine							
Control (0 h)[1]	100	–	–	100	–	–	100
Control (4 h)[2]	100	< 3	< 3	96	7	< 3	90
Individual A7	14	100	–	–	44	11	45
Individual A8	46	14	–	86	3	–	97
Individual A10	47	84	16	–	12	55	33
Individuals A1-A3	9	–	20	80	10	30	60
B [^{14}C] senecionine *N*-oxide							
Control (0 h)[1]	100	–	100	–	–	100	–
Control (4 h)[2]	100	–	93	7	–	100	–
Individual B6	50	–	94	6	–	100	–
Individual B9	63	–	100	–	–	95	5
Individuals B1-B5	26	–	94	6	< 2	98	< 2

[1]Control (tracer plus meat) at the beginning of the feeding period
[2]Control (tracer plus meat) at the end of the 4 hour feeding period
"X" = polar metabolite; S *N*-ox = senecionine *N*-oxide; Sen = senecionine; – = not detectable

monooxygenase is not known to occur in arthropods (Brattsten 1992). Further studies are needed to characterize the PA *N*-oxidizing activity in the digestive juice of harvestmen. The only other PA *N*-oxygenases known so far are a particulate senecionine *N*-oxygenase isolated from seedlings of a PA producing plant (i.e. *Crotalaria scassellatii,* Fabaceae) (Chang & Hartmann 1998) and a soluble senecionine *N*-oxygenase characterized and cloned from the hemolymph of PA sequestering arctiid larvae (Lindigkeit *et al.* 1997; Naumann *et al.* 2002). Both the plant and the insect enzymes, specifically *N*-oxidize potentially toxic PAs; in this respect they are clearly different from the multisubstrate monooxygenase.

Harvestmen appear to be well adapted to cope with the defensive toxins of their prey. Further studies must show whether this adaptation is a general taxonomic feature of predator species of the Opiliones or a specific adaptation of *Mitopus morio.*

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