

Biochemical strategy of sequestration of pyrrolizidine alkaloids by adults and larvae of chrysomelid leaf beetles

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Abstract

Tracer feeding experiments with ¹⁴C-labeled senecionine and senecionine *N*-oxide were carried out to identify the biochemical mechanisms of pyrrolizidine alkaloid sequestration in the alkaloid-adapted leaf beetle *Oreina cacaliae* (Chrysomelidae). The taxonomically closely related mint beetle (*Chrysolina coeruleans*) which in its life history never faces pyrrolizidine alkaloids was chosen as a 'biochemically naive' control. In *C. coeruleans* ingestion of the two tracers resulted in a transient occurrence of low levels of radioactivity in the hemolymph (1–5% of radioactivity fed). With both tracers, up to 90% of the radioactivity recovered from the hemolymph was senecionine. This indicates reduction of the alkaloid *N*-oxide in the gut. Adults and larvae of *O. cacaliae* sequester ingested senecionine *N*-oxide almost unchanged in their bodies (up to 95% of sequestered total radioactivity), whereas the tertiary alkaloid is converted into a polar metabolite (up to 90% of total sequestered radioactivity). This polar metabolite, which accumulates in the hemolymph and body, was identified by LC/MS analysis as an alkaloid glycoside, most likely senecionine *O*-glucoside. The following mechanism of alkaloid sequestration in *O. cacaliae* is suggested to have developed during the evolutionary adaptation of *O. cacaliae* to its alkaloid containing host plant: (i) suppression of the gut specific reduction of the alkaloid *N*-oxides, (ii) efficient uptake of the alkaloid *N*-oxides, and (iii) detoxification of the tertiary alkaloids by *O*-glucosylation. The biochemical mechanisms of sequestration of pyrrolizidine alkaloid *N*-oxides in Chrysomelidae leaf beetles and Lepidoptera are compared with respect to toxicity, safe storage and defensive role of the alkaloids.

Keywords: Chrysomelidae; Pyrrolizidine alkaloid *N*-oxide; Pyrrolizidine alkaloid glycoside; Sequestration; Chemical defense

1. Introduction

Plant pyrrolizidine alkaloids with certain structural features such as the presence of a 1–2 double bond, esterification of the allylic hydroxyl group and a free or esterified second hydroxyl group (Fig. 1) are potentially toxic (Mattocks, 1986; Cheeke, 1989). If these alkaloids are ingested by a herbivore they are passively absorbed as tertiary alkaloids and are converted into reactive pyrrolic metabolites by multisubstrate cytochrome P450 oxidases. These pyrrolic intermediates easily react with

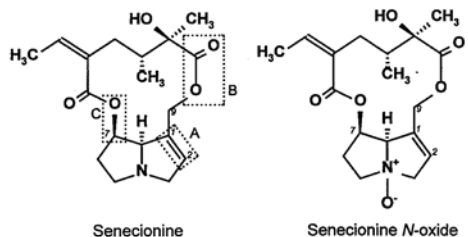


Fig. 1. Senecionine a potentially toxic tertiary pyrrolizidine alkaloid and its *N*-oxide. The structural features of senecionine which are essential for cytochrome P450 catalyzed bioactivation of pyrrolizidine alkaloids: (A) 1–2 double bond; (B) esterification of the allylic hydroxyl group at C9; and (C) a free or esterified hydroxyl group at C7.

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biological nucleophiles, with the consequence of detrimental cytotoxic and genotoxic effects. In vertebrates this bioactivation is catalyzed by microsomal cytochrome P450 enzymes (EC 1.14.14.1) (Winter and Segall, 1989). These enzymes are mainly localized in liver and lung tissues. They are components of the xenobiotic metabolism and transform absorbed foreign compounds (xenobiotics) into excretable metabolites. In the case of pyrrolizidine alkaloids per se non-toxic compounds are converted into toxic metabolites which are responsible for the hepatotoxicity and pneumotoxicity of pyrrolizidine alkaloids in vertebrates (Mattocks, 1986; Cheeke, 1989). Insects which have a similar xenobiotic metabolism with microsomal cytochrome P450 enzymes (Hodgson, 1985; Brattsten, 1992) should be affected by pyrrolizidine alkaloids similarly as vertebrates. In fact, pyrrolizidine alkaloids were shown to be genotoxic in the *Drosophila* wing spot test (Frei et al., 1992).

A number of insect species from unrelated taxa have developed adaptations to sequester pyrrolizidine alkaloids from their host plants and utilize them against their own predators. Pyrrolizidine alkaloid sequestering species are found among Lepidoptera (many moths or butterflies of the Arctiidae, Danainae and Ithomiinae) (Boppré, 1986; Schneider, 1987; Hartmann, 1999; Hartmann and Witte, 1995) and Coleoptera such as leaf beetles (Chrysomelidae) of the genus *Oreina* (Pasteels et al. 1988b, 1994; Hartmann et al., 1997). In addition isolated occurrences of alkaloid sequestering species are known from the Orthoptera, i.e. the African grasshopper *Zonocerus* (Bernays et al., 1977; Fischer and Boppré, 1997) and Homoptera, i.e. *Aphis jacobaeae*, a specialist phloem-feeder on *Senecio* species (Witte et al., 1990).

The question is, how pyrrolizidine alkaloid sequestering insects protect themselves against alkaloid toxicity. Arctiids (Ehmke et al., 1990; Hartmann et al., 1990) and *Zonocerus* (Biller et al., 1994) store plant-derived pyrrolizidine alkaloids exclusively in the *N*-oxide state. *N*-oxidation converts the tertiary alkaloid into a derivative which cannot be bioactivated. Some vertebrates detoxify potentially toxic tertiary alkaloids by *N*-oxidation (Cheeke, 1994). For example, guinea pigs possess a microsomal multisubstrate flavin monooxygenase (EC 1.14.13.8) which efficiently converts ingested pyrrolizidine alkaloids into their *N*-oxides. In guinea pig liver *N*-oxygenation by far exceeds cytochrome P450 mediated bioactivation (Miranda et al., 1991). This explains the high resistance of guinea pigs to the toxic effects of pyrrolizidine alkaloids. Similarly, Arctiids store sequestered pyrrolizidine alkaloids in the safe *N*-oxide state (Lindigkeit et al., 1997).

Pyrrolizidine alkaloid sequestration in leaf beetles differs from sequestration in lepidopterans. Pyrrolizidine alkaloid sequestering leaf beetles have two alkaloid storage sites: (i) exocrine defensive glands located in the elytra and the pronotum of adult beetles from which the

defensive secretion is actively released upon attack (Pasteels et al. 1988a, 1989); (ii) the whole body in which adults (Ehmke et al., 1991; Rowell-Rahier et al., 1991; Pasteels et al., 1992) and larvae (Dobler and Rowell-Rahier, 1994; Ehmke et al., 1999) store alkaloids acquired from the host plant. The major storage site in the body is the hemolymph. Like lepidopterans, *Oreina* larvae and adults store the alkaloids as *N*-oxides. However, in contrast to lepidopterans they are not able to efficiently convert the tertiary alkaloids into the respective *N*-oxides (Ehmke et al., 1991).

The purpose of this study was to elucidate the mechanisms by which pyrrolizidine alkaloid sequestering leaf beetles, e.g. *O. cacaliae*, handle tertiary alkaloids and protect themselves against pyrrolizidine alkaloid toxicity. *Chrysolina coeruleans* was chosen as a non-adapted control. The results provide some general insight into the mechanisms and strategies adopted by leaf beetles during their evolutionary adaptation to host plant-derived chemical defense. These mechanisms will be compared with the those adopted by lepidopteran species utilizing host-derived pyrrolizidine alkaloids as defensive compounds.

2. Material and methods

2.1. Insects

Adult beetles of *C. coeruleans* (Scriba) were collected in May and June in the Medicinal Plant Garden of the Institute of Pharmaceutical Biology, Technical University Braunschweig. The beetles fed on *Mentha spicata* L. (Lamiaceae). Adults of *Oreina cacaliae* (Schrank) were collected in May in the Val Ferret (Valais, Switzerland) and kept on their food plant *Adenostyles alliariae* (Gouan) Kern. (Asteraceae) at room temperature or in a cooling-chamber at 8°C until use. The offspring was raised on *A. alliariae*; 2nd and 3rd instar larvae were used in the experiments. All feeding experiments were carried out at room temperature.

2.2. Tracer feeding experiments

[¹⁴C]Senecionine (1.07-1.34 GBq×mmol⁻¹) and its *N*-oxide were prepared biosynthetically from [1,4-¹⁴C]putrescine (4.1 GBq×mmol⁻¹; Amersham Buchler, Braunschweig) using root cultures of *Senecio vulgaris* L. according to Hartmann (1994).

2.2.1. *C. coeruleans*

Tracer feeding of adult beetles was successfully achieved using small rooted mint plants in pots. All leaves except one pair were removed. Tracers were applied as aqueous solutions (ca 10 µl) on the lower surface of the leaves (1.5×10⁵ cpm per plant). The *N*-oxide was directly

dissolved in water, whereas the tertiary alkaloid was dissolved in a small volume of methanol and then diluted with water; the final methanol concentration did not exceed 5%. After evaporation of the solvent one beetle was placed on each plant. Host plant and beetle were caged in a plastic tube (85×25 mm) with its bottom pressed into the earth and its top covered with gauze. The beetles (two individuals per group) were allowed to feed for 4, 24 or 45 hr. In pulse feeding experiments the beetles were transferred to untreated host plants after termination of the feeding-pulse and were allowed to feed till the end of the experiment.

2.2.2. *O. cacaliae*

Tracer feeding experiments with adult beetles and larvae of *O. cacaliae* were performed in Petri dishes as described previously (Ehmke et al. 1991, 1999). Five larvae (2nd and 3rd instar; individual larval weight between 15 to 22 mg) were placed in a Petri dish and were fed with 1.2×10^5 cpm of [^{14}C]senecionine or 1.2×10^5 cpm of [^{14}C]senecionine *N*-oxide, respectively. Fifteen Petri dishes were prepared with each tracer. Tracers were painted as aqueous solutions on the upper surface of fresh leaf discs (10 mm \varnothing) of *Adenostyles alliariae*, one disc per dish. Larvae were allowed to feed for 17 hr, then a total of 25 larvae from five dishes of each treatment were preserved in methanol and the larvae from the remaining 10 dishes of each set were transferred to fresh untreated host plant leaves and preserved in methanol after an additional feeding period of 24 and 48 hr, respectively.

Experiments with adult beetles were arranged in the same way as described above for larvae. However, only one individual was placed in each Petri dish and the duration of tracer feeding was 13 hr. After termination of the experiment and weighing of the beetles the hemolymph was recovered. Hemolymph and the carcasses were preserved separately in methanol until analysis.

2.3. Sampling of hemolymph

2.3.1. Centrifugation method

The legs of a beetle were cut off at the tibia and the beetle was placed head-down on the top of a pre-weighed 200 μl Eppendorf micro-vial. Hemolymph was recovered by centrifugation at 3500 rpm for 15 min (Sigma 101M microcentrifuge). The vial was re-weighed and the amount of hemolymph calculated. During centrifugation the beetle remained stuck to the top of the conical micro-vial and the body fluid was collected at the bottom of the vial. The hemolymph obtained by this method was a clear fluid.

2.3.2. Capillary method

One leg or antenna of a beetle was cut off at the basal segment and the extruding hemolymph was collected with a capillary and immediately preserved in methanol.

2.4. Extraction procedure

Individual beetles or 5 larvae were ground in a mortar with ca 500 μl methanol and washed with another 500 μl methanol into a 2 ml Eppendorf vial. After centrifugation (Sigma 101 M microcentrifuge) at 15 000 rpm for 10 min, the supernatant was recovered and the pellet extracted with another 300 μl methanol. The supernatants were combined and directly used for analysis of total radioactivity by scintillation counting or chromatographic analysis. Isolated hemolymph (between 3 and 25 mg) was mixed with 200 μl methanol and the precipitate removed by centrifugation. The supernatant was analyzed in the same way as the extracts.

2.5. Chromatographic methods

2.5.1. Thin-layer chromatography (TLC)

Crude methanol extracts of larvae, adult beetles, hemolymph or tracer-treated food plant remains were separated by TLC and evaluated quantitatively by means of a multichannel radioactivity detector (Rita-32a, Raytest). Separation was achieved on silica gel 60 (Merck) using the solvent system: dichloromethane:methanol: NH_4OH (25%) (84:14:2, by volume).

2.5.2. High pressure liquid chromatography (HPLC)

Crude methanol extracts were separated isocratically on an RP-18 column (25 cm×0.4 cm i.d.; Nucleosil; Macherey and Nagel). Solvent: 15% acetonitrile: 85% 0.01 M potassium phosphate, pH 2.2. Flow: 1 ml×min⁻¹. *R_t* (min): presumed senecionine glucoside, 7.53; senecionine, 11.00; senecionine *N*-oxide, 12.30. Compounds were detected by (a) UV 209 nm; (b) radioactivity monitor LB-506D (Berthold) equipped with a 2 ml flow-cell and a split-mixer LB-5034; Rialuma (Baker) was used as liquid scintillator.

2.6. Alkaloid hydrolysis

To isolate the radioactively labeled necine base moiety (retronecine) of [^{14}C]senecionine the labeled alkaloid was subjected to alkaline hydrolysis in 5% NaOH at 55°C for 3 hr. The necine base was recovered from the hydrolysis solution by extraction with diethyl ether.

2.7. Isolation and identification of pyrrolizidine alkaloid *O*-glycosides

2.7.1. Seneciphylline feeding

Adults of *C. cacaliae* (99 specimens) were kept in Petri dishes (5 beetles per dish) on fresh leaf discs (15 mm \varnothing) of *A. alliariae* each painted with 20 μl of an aqueous solution of 1 mM non-labeled seneciphylline, which previously had been isolated and purified from young sprouts of *A. alliariae*. The beetles were allowed

to feed ad libitum on seneciophylline for 48 hr; consumed alkaloid-treated leaf discs were immediately replaced by new ones. After termination of the experiment the beetles were kept frozen (-18°C) until analysis.

2.7.2. Extraction

The frozen beetles were ground in a mortar and extracted twice with methanol. After centrifugation the solvent was evaporated at room temperature, the residue dissolved in a small volume of methanol and subjected to TLC on silica gel (PSC plates, 1 mm, Merck). The assumed alkaloid glycoside was localized by co-chromatography in the same lane with a radioactively labeled sample of the assumed senecionine glycoside which should migrate with the same R_f -value as the respective seneciophylline derivative. The marked zone was scraped off the plate and extracted with methanol. The methanol extract was subjected to HPLC using the same procedure as described above, but using 15% acetonitrile: 85% trifluoroacetic acid as solvent. Promising fractions localized by co-chromatography with the radioactively labeled assumed glycoside were collected, the solvent evaporated and the fractions directly applied to LC-MS analysis.

2.7.3. LC-MS analysis

The positive ion electrospray (ES) spectra and the ES-MS/MS measurements were obtained with a Finnigan MAT TSQ 7000 instrument (electrospray voltage, 4.5 kV; heated capillary temperature, 220°C ; sheath gas, nitrogen) coupled with a Micro-Tech Ultra-Plus Micro-LC system equipped with a RP18-column ($4\ \mu\text{m}$, $1\times 100\ \text{mm}$, SEPSERV). Separation was achieved with the following solvent gradient: water:acetonitrile (9:1), each containing 0.2% acetic acid, to water:acetonitrile (1:1) within 10 min, then holding the 1:1 ratio for 10 min. The flow rate was $70\ \mu\text{l}\ \text{min}^{-1}$. Collision-induced dissociation (CID) mass spectra were generated during the HPLC run under the following conditions: collision energy (collision cell) $-40\ \text{eV}$ (seneciophylline) and $-50\ \text{eV}$ (seneciophylline *O*-glucoside); collision gas, argon; collision pressure, $1.8\times 10^{-3}\ \text{Torr}$. All mass spectra are averaged and corrected for background.

3. Results

3.1. Uptake and metabolism of [^{14}C]senecionine and its *N*-oxide by *Chrysolina coeruleans*

Preliminary experiments revealed that it is possible to feed *C. coeruleans* on mint leaves painted with small quantities ($<1\ \mu\text{g}$) of radioactively labeled pyrrolizidine alkaloids without refusal reactions of the beetles.

Adults of *O. coeruleans* were fed with [^{14}C]senecionine *N*-oxide and [^{14}C]senecionine for 4 to 45 hours. At inter-

vals some beetles were sacrificed, their hemolymph recovered, and analyzed for total radioactivity and its metabolite composition. The results are summarized in Table 1. With both tracers a small proportion of radioactivity (1 to 5% of total radioactivity offered) was detected in the hemolymph. Their presence there was temporary. Beetles which were transferred from the radioactive source to a non-labeled food leaf lost almost all of the radioactivity from their bodies within 24 hours. TLC analysis of the radioactivity recovered from the hemolymph showed that the two compounds are metabolized in a characteristic manner. Upon feeding of [^{14}C]senecionine *N*-oxide, 80–90% of the radioactivity recovered from the hemolymph was tertiary senecionine. In addition, a small proportion of non-metabolized [^{14}C]senecionine *N*-oxide (up to 12%) was found. The latter result was confirmed with two different methods of hemolymph sampling (i.e. centrifugation and the capillary collection). Thus, contamination of hemolymph samples with external tracer can be excluded. The radioactivity recovered from the hemolymph upon feeding of [^{14}C]senecionine was mainly ($>60\%$) non-metabolized senecionine, the remainder of radioactivity was associated with a polar metabolite (PM in Table 1). Because the amount present was small it was not possible to perform HPLC and to compare this metabolite with the alkaloid glycoside found in *O. cacaliae* (see below). In none of the feeding experiments with labeled senecionine, could labeled *N*-oxide be detected in the hemolymph.

3.2. Uptake and metabolism of [^{14}C]senecionine and its *N*-oxide by *Oreina cacaliae*

The same types of experiment as described above for the non-adapted *C. coeruleans* were carried out with adults and larvae of *O. cacaliae*. Tables 2 and 3 show the total radioactivity found in the hemolymph, in the carcasses of adults and in larval bodies, respectively, upon feeding of [^{14}C]senecionine and its *N*-oxide. Recovery of radioactivity (expressed as percentage of total tracer offered on the food-leaf) in adult beetles was 16 to 34% (senecionine *N*-oxide feeding) and 15 to 22% (senecionine feeding). With larvae, the respective values were 13 to 18% (*N*-oxide) and 6 to 12% (tertiary alkaloid). The percentages of accumulated radioactivity were evaluated at the end of the tracer feeding period (13 and 17 hours, respectively) and 24 and 48 hours after the insects had been transferred from tracer containing to normal diet. No effort was made to correct accumulation for the non-consumed radioactivity on the food-leaf remains. This explains the result that total radioactivity appeared to increase with time after an initial pulse feeding of ^{14}C -labeled tracer (e.g. senecionine in the hemolymph; see Table 2). The data just indicate that the 24/48 hour groups consumed more labeled leaf-mass

Table 1
Uptake and metabolism of radioactively labeled senecionine *N*-oxide (Sen-Nox) and senecionine (Sen) by the non-adapted leaf beetle *Chrysolina coeruleans*^f

Tracer	Time (hours)	Total radioactivity in hemolymph (% of applied) ^a	Radioactivity (relative abundance, %)		
			PM ^b	Sen-Nox	Sen
Exp. I					
Adults					
Sen-Nox	45	2	18	<3	81
Sen	45	1	38	nd	62
Food-leaf ^c					
Sen-Nox	45			>85	9
Sen	45			nd	<75 ^d
Exp. II					
Adults					
Sen-Nox	4	1.7	nd	12	88
Sen-Nox	24	5.1	10	<3	89
Sen-Nox	24 + 24 ^e	<0.5			

^a A total of 1.5×10^5 cpm (100%) of the respective tracer were painted on two leaves of a small mint plant and offered to one individual; n = 2 per group.

^b Polar metabolite.

^c Analysis of the food leaf remains at the end of the feeding period (n = 4).

^d Ca 25% loss of sen by unspecific decay, i.e. several labeled spots of unknown identity on the TLC plate.

^e 24 hr pulse feeding of tracer, followed by 24 hr feeding on non-treated leaves.

^f Hemolymph extracts were separated by TLC and analyzed by radioactivity detection. nd = not detected.

Table 2
Uptake of [¹⁴C]senecionine *N*-oxide and [¹⁴C]senecionine by adults of *Oreina cacaliae*^a

Tracer	Time (hours)	Uptake cpm ($\times 10^5$) \times g ⁻¹ fresh weight	
		In the hemolymph	In the remaining body
Senecionine <i>N</i> -oxide	0	19.73 \pm 10.83	7.19 \pm 3.16
	24	15.24 \pm 4.02	5.35 \pm 1.62
	48	8.50 \pm 3.23	2.13 \pm 0.51
Senecionine	0	4.42 \pm 1.68	1.58 \pm 0.65
	24	7.32 \pm 1.94	2.27 \pm 0.43
	48	8.20 \pm 1.78	2.75 \pm 0.84

^a n = 5 per group; means \pm standard error. Individuals were allowed to feed on host plant leaves treated with the respective tracer for 13 hours, then one group was analyzed (hour 0). Two other groups were transferred to untreated host plant leaves and fed for a further 24 hours (24) and 48 hours (48), respectively.

than the zero-group. Moreover, we know from previous studies that during the period after an initial pulse feeding of labeled alkaloid there is no (adults) or only a minor loss (larvae) of total accumulated radioactivity from the bodies (Pasteels et al., 1992; Ehmke et al., 1999). The main goal of the following experiments was a comparative analysis of the metabolite patterns of the accumulated radioactivity and not the comparison of the absorbed total amounts.

The results summarized in Tables 2 and 3 show that:

Table 3
Uptake of radioactively labeled senecionine *N*-oxide and senecionine by larvae (2nd and 3rd instar) of *Oreina cacaliae*^a

Tracer	Time (hours)	Uptake cpm $\times 10^5 \times$ g ⁻¹ fresh weight
Senecionine <i>N</i> -oxide	0	14.69 \pm 1.70
	24	2.61 \pm 0.60
	48	2.84 \pm 1.18
Senecionine	0	2.41 \pm 0.58
	24	0.90 \pm 0.20
	48	1.51 \pm 0.45

^a n = 5 per treatment; mean \pm standard error. Larvae were fed on host plant leaves treated with the respective tracer for 17 hours, then one group was analyzed (hour 0). The two other groups were transferred to untreated host plant leaves and allowed to feed for a further 24 hours (24) and 48 hours (48), respectively.

(i) the two tracers are taken up in comparable amounts; (ii) the sequestered radioactivity is retained by adult beetles and by larvae over two days following the tracer feeding-pulse; (iii) in adult beetles, hemolymph and remaining-body contain almost equal concentrations of sequestered radioactivity.

All extracts were analyzed by TLC and HPLC to establish the respective metabolite patterns. The results of the TLC analysis are illustrated in Fig. 2. When the *N*-oxide was fed as tracer more than 95% of the radioactivity found in the hemolymph of adult beetles was

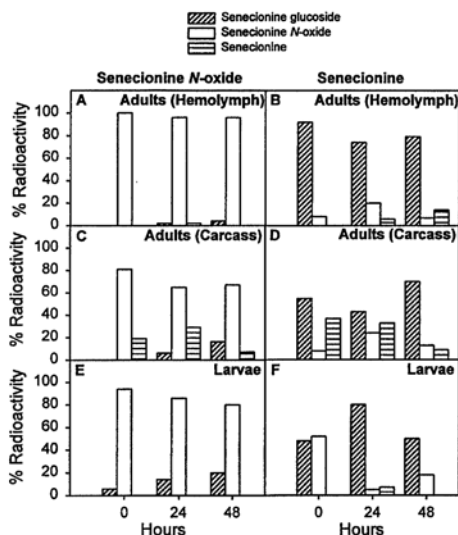


Fig. 2. Fate of [^{14}C]senecionine *N*-oxide (A, C, E) and [^{14}C]senecionine (B, D, F) ingested by adults (A-D) and larvae (E, F) of *Oreina cacialae*. Individuals were allowed to feed on pieces of host plant leaves treated with the respective tracer for 13 hours (adults) or 17 hours (larvae). Samples of each group each were then analyzed (hour 0). Others were transferred to untreated host plant leaf material and allowed to feed for a further 24 hours and 48 hours, respectively. A and B = adults, methanol extracts of hemolymph; C and D = adults, methanol extracts of carcasses; E and F = methanol extracts of larvae. For uptake and tissue distribution of total radioactivity see Tables 2 and 3.

unaltered *N*-oxide (Figs. 2A and 3A). Body extracts contained 65 to 80% unaltered senecionine *N*-oxide, but in addition also [^{14}C]senecionine and a very polar compound with a low R_f value (Fig. 3B). The polar compound was later identified as an alkaloid glycoside (most likely the glucoside). In the larval extracts the recovery of senecionine *N*-oxide accounted for 80 to 95% of total radioactivity; it was always accompanied by the glycoside which appeared to increase slightly with time (Fig. 2E).

A completely different result was obtained when adults or larvae were fed with [^{14}C]senecionine instead of the *N*-oxide (Fig. 2B, D and F). Hemolymph extracts from adults (Figs. 2B and 3C) contained senecionine glucoside (75 to 92% of total activity) as major metabolite, accompanied by small proportions of senecionine *N*-oxide (7 to 20%) and less than 15% of residual senecionine. Body extracts revealed a more complex composition, but in any case the alkaloid glycoside was the predominating metabolite (Figs. 2D and 3B). Senecionine *N*-oxide was always present (8 to 24%). The relative amounts of

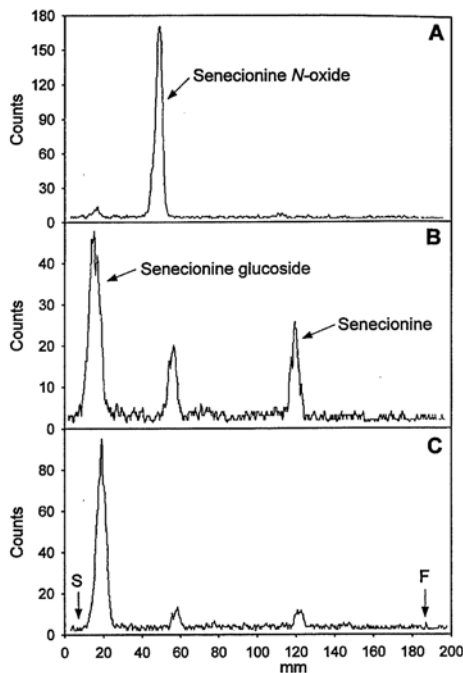


Fig. 3. [^{14}C]Senecionine *N*-oxide and [^{14}C]senecionine fed to adults of *O. cacialae*. TLC separation of crude methanol extracts. (A) hemolymph, tracer senecionine *N*-oxide (24 hours); (B) body extract, tracer senecionine (24 hours); (C) hemolymph, tracer senecionine (48 hours). S = Start, F = Front. See legend of Fig. 2 for details.

residual senecionine were higher in the carcass than in the hemolymph and decreased with time. TLC separation of the larval extracts revealed a composition similar to that of adult body extracts (Fig. 2F). Two points were remarkable: in comparison to adults, residual senecionine appears to be lower or absent, whereas senecionine *N*-oxide was always present in adults and larvae.

3.3. Identification of pyrrolizidine alkaloid *O*-glycosides

HPLC analysis of hemolymph extracts from a feeding experiment with [^{14}C]senecionine confirmed that the polar metabolite eluted as a single peak. Alkaline hydrolysis of this compound yielded one labeled product with the same chromatographic behavior as authentic retronecine. This confirmed that the polar metabolite still contains the intact necine base moiety. To obtain sufficient amount of the polar metabolite for reliable analysis by liquid chromatography/mass spectrometry

(LC/MS), adults of *O. cacaliae* were fed with non-labeled seneciophylline. Seneciophylline was chosen instead of the closely related senecionine because it is the major alkaloid found in the host plant *A. alliariae*. Positive ion electrospray (ES) mass spectrometry revealed a compound with a prominent $[M + H]^+$ ion at m/z 496 and using skimmer CID a further ion showed up at m/z 334 as base peak (Fig. 4A). The loss of 162 u formally corresponds to the elimination of a hexose unit at formation of the protonated alkaloid. This result was confirmed by a collision induced (CID) mass spectrum of compound m/z 496 (Fig. 4B) in comparison to the respective spectrum of authentic seneciophylline ($[M + H]^+$, m/z 334) (Fig. 4C). The two mass spectra show almost identical

fragment ions (e.g. at m/z 306, 151, 138, 120, and 94) in the mass range between m/z 50 to 350. From these data we conclude, that the compound with a $[M + H]^+$ at m/z 496 represents an *O*-glycoside composed of a hexose and seneciophylline. It is reasonable to assume that the hexose is a glucose and thus the alkaloid glycoside most likely is seneciophylline *O*-glucoside (Fig. 4A). In the same extract an additional compound with a $[M + H]^+$ ion at m/z 498 could be recorded by ES-MS. Using skimmer CID a further ion at m/z 336 (presumably representing senecionine) was detected as base peak. Although the concentrations were too low to record the respective CID spectra, the compound with a $[M + H]^+$ at m/z 498 most likely represents senecionine *O*-glucoside.

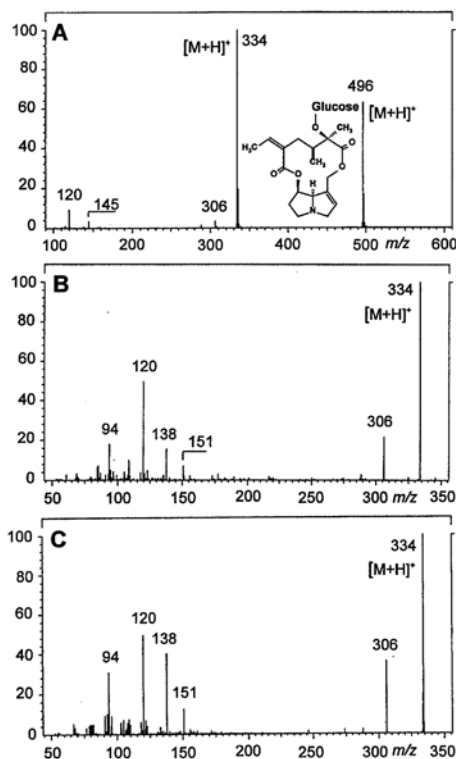


Fig. 4. Identification of presumed seneciophylline *O*-glucoside by LC-MS. (A) positive ion electrospray (ES) mass spectrum of a compound obtained by LS-MS (R_t 7.35 min), showing a prominent $[M + H]^+$ ion at m/z 496 and using skimmer CID a ion at m/z 334 as base peak. (B) collision induced dissociation (CID) mass spectrum of compound m/z 496. (C) CID mass spectrum of reference seneciophylline ($[M + H]^+$, m/z 334).

4. Discussion

The genera *Chrysolina* and *Oreina* are taxonomically closely related (Daccordi, 1994; Hsiao and Pasteels, 1999). *C. coeruleans*, which in his life history never faces pyrrolizidine alkaloids, was chosen as a 'biochemically naive' control. It should help to detect any biochemical adaptation of *O. cacaliae* that enables this leaf beetle to cope with and safely store host-plant-derived pyrrolizidine alkaloids. The following conclusions can be drawn from the results of the tracer-feeding experiments with *C. coeruleans*: (i) as expected, *C. coeruleans* does not sequester pyrrolizidine alkaloids; (ii) during feeding on both labeled senecionine and labeled senecionine *N*-oxide, low levels of radioactivity are temporarily detected in the hemolymph; (iii) [^{14}C]senecionine *N*-oxide appears to be efficiently reduced in the gut, since upon feeding of either of the two alkaloidal forms, 60 to 80% of the radioactivity recovered in the hemolymph is associated with tertiary senecionine; (iv) since after feeding of labeled senecionine *N*-oxide, a small proportion of senecionine *N*-oxide could be detected in the hemolymph, it appears that *C. coeruleans* is able to absorb the alkaloid *N*-oxide; (v) As no *N*-oxide was found after feeding of senecionine *C. coeruleans* is apparently unable to *N*-oxidize the alkaloid; (vi) although *C. coeruleans* is not specifically adapted to pyrrolizidine alkaloids, it is able to excrete efficiently the absorbed alkaloids and their metabolites (Fig. 5A).

The reduction of ingested pyrrolizidine alkaloid *N*-oxides is in agreement with the general observation that pyrrolizidine alkaloid *N*-oxides are easily reduced in the gut. All vertebrates so far studied show it including humans (Mattocks, 1986; Winter and Segall, 1989) as well as various arctiids (Lepidoptera) and the grasshopper *Zonocerus variegatus* (Lindigkeit et al., 1997). Liver toxicity of pyrrolizidine alkaloids in humans and domestic animals (Mattocks, 1986; Huxtable, 1992) is initiated in the digestive tract by reduction of the

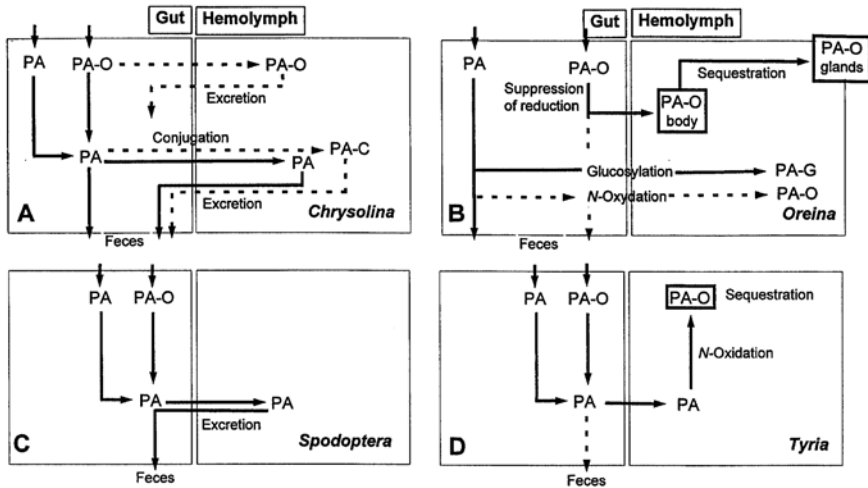


Fig. 5. Strategies of uptake, metabolism and maintenance of tertiary pyrrolizidine alkaloid (PA) and its *N*-oxide (PA-O) in (A) *Chrysolina coerulans* (non-adapted, no sequestration; 'biochemical control'); (B) *Oreina cacaliae* (adapted, sequestration), (C) *Spodoptera littoralis* (tolerating alkaloids, no sequestration); (D) *Tyria jacobaeae* (adapted, sequestration). Solid arrows: main pathways; dotted arrows: minor pathways. PA, tertiary pyrrolizidine alkaloid; PA-O, pyrrolizidine alkaloid *N*-oxide; PA-C, pyrrolizidine alkaloid conjugate; PA-G, pyrrolizidine alkaloid glucoside.

ingested alkaloid *N*-oxide followed by nonspecific passive absorption of the lipophilic tertiary alkaloid. The alkaloid *N*-oxides are easily reduced even in presence of weak reducing agents such as cysteine (Hartmann and Toppel, 1987). It has been suggested that the intestinal flora plays a major role in *N*-oxide reduction (Mattocks, 1971; Winter and Segall, 1989), detailed studies, however, are still missing.

In comparison to the non-adapted *C. coerulans*, absorption and metabolism of pyrrolizidine alkaloids is completely different in alkaloid sequestering larvae and adults of *O. cacaliae*. The most striking difference is that the *N*-oxide is not reduced in the gut but taken up as such and maintained unchanged in the body. By contrast, ingested tertiary alkaloid is efficiently converted into the respective *O*-glycoside, most likely the *O*-glucoside. Both adults and larvae are capable of *N*-oxidizing senecionine. However, in comparison to glycosylation, *N*-oxidation seems less efficient, and it was rather variable in different experiments. It is not clear whether glycosylation and *N*-oxidation take place in the gut or in the hemolymph. The increase of the relative proportion of the alkaloid glucoside in the course of the pulse feeding experiments (e.g. *N*-oxide feeding Figs. 2A–E) may suggest that the hemolymph is the site of glycosylation, but conclusive evidence is lacking. This is the first report on the formation of alkaloid *O*-glycosides from pyrrolizidine alkaloids.

Assuming that the reduction of pyrrolizidine alkaloid

N-oxides generally takes place in the gut of non-adapted leaf beetles such as *C. coerulans*, we infer that the alkaloid sequestering specialist in the course of its adaptation to plants containing pyrrolizidine alkaloids has lost the ability to reduce the alkaloid *N*-oxides in the gut. This is a prerequisite for the efficient uptake of pyrrolizidine alkaloid *N*-oxides (Fig. 5B). The direct uptake of the alkaloid *N*-oxide and its storage in the body of larvae or adult beetles has been demonstrated in this and previous studies (Ehmke et al. 1991, 1999; Rowell-Rahier et al., 1991; Hartmann et al., 1997). The uptake of the alkaloid *N*-oxide from the gut into the hemolymph is difficult to explain without postulating a carrier-mediated process. In the case of pyrrolizidine alkaloid sequestering larvae of Lepidoptera it has been convincingly demonstrated that the polar, salt-like alkaloid *N*-oxides are unable to permeate the membrane barrier between gut and hemolymph (Lindigkeit et al., 1997), although it had been claimed previously that such a carrier for *N*-oxides does exist (Wink and Schneider, 1988). In plants containing pyrrolizidine alkaloids a specific membrane carrier facilitates the transfer of the alkaloid *N*-oxides through the tonoplast into the cell vacuole (Ehmke et al., 1988; Hartmann et al., 1989). We know that in *O. cacaliae* the transport of pyrrolizidine alkaloid *N*-oxides through the gut epithelia into the hemolymph is a rather unspecific process. A number of host-plant derived pyrrolizidine alkaloid *N*-oxides as well as several other pyrrolizidine alkaloid *N*-oxides which do not occur in the beetle's host

plants, are absorbed into the hemolymph (Hartmann et al., 1997). By contrast, the transfer of pyrrolizidine alkaloid *N*-oxides from the hemolymph into the defensive glands (Fig. 5B), which takes place in adult beetles (Pasteels et al., 1992), is much more selective. Only certain alkaloid *N*-oxides taken up from the host plants are transported into the defensive glands (Rowell-Rahier et al., 1991; Ehmke et al., 1991; Hartmann et al., 1997). Selective alkaloid transport into the defensive glands and maintenance of a high concentration gradient (>150:1) between the secretion of the gland and the hemolymph argues for a specific energy-driven transport. With respect to the less specific uptake of the pyrrolizidine alkaloid *N*-oxides in *C. coeruleans* we noted a weak but unequivocal absorption of pyrrolizidine alkaloid *N*-oxide from the gut into the hemolymph. It is unlikely that *C. coeruleans* possesses a specific carrier for these alkaloids, but it is possible that a nonspecific carrier system involved in xenobiotic transport and elimination is responsible for the temporary presence of the *N*-oxide in the hemolymph. We hypothesize that the development of such a carrier system for the uptake of the pyrrolizidine alkaloid *N*-oxides was an important step in the evolutionary adaptation of *O. cacialiae* resulting in a host-mediated defense mechanism. Moreover, *N*-oxide uptake in *O. cacialiae* is favored by the suppression of *N*-oxide reduction in the gut. In *C. coeruleans* *N*-oxide uptake is low because it is counteracted by the efficient *N*-oxide reduction.

Small proportions of tertiary alkaloids are formed from the *N*-oxide in the gut of *Oreina* (see Fig. 2C and E) but a more efficient mechanism converts tertiary pyrrolizidine alkaloids into hydrophilic glycosides. These conjugates seem to remain in the body and are not excreted or transferred into the defensive glands of adults (Ehmke et al., 1991). Most likely the alkaloid glycoside is a detoxification product. In comparison to the efficient detoxification of tertiary pyrrolizidine alkaloids by conjugation, *N*-oxidation seems to play only a minor role, if any, in alkaloid inactivation.

By contrast to pyrrolizidine alkaloid sequestering leaf beetles, alkaloid sequestering arctiid larvae completely reduce ingested pyrrolizidine alkaloid *N*-oxide in the gut and passively absorb the tertiary alkaloid into the hemolymph. Such a mechanism is also found in larvae of the lepidopteran *Spodoptera littoralis* (Noctuidae) which tolerates pyrrolizidine alkaloids because excretion is very efficient (Fig. 5C). In arctiids the toxic tertiary alkaloids are efficiently *N*-oxidized by a soluble mixed function *N*-oxygenase (senecionine *N*-oxygenase) localized in the hemolymph (Lindigkeit et al., 1997). The enzyme is specific for toxic pyrrolizidine alkaloids. Interestingly, an enzyme with almost the same properties as the insect enzyme has recently been isolated and characterized from a plant synthesizing pyrrolizidine alkaloids (Chang and Hartmann, 1998).

Sequestration of pyrrolizidine alkaloids shows common elements in specialized arctiids and specialized leaf beetles although the two unrelated taxa use quite different biochemical strategies (Fig. 5B and D). Both of them store pyrrolizidine alkaloids in the form of *N*-oxides and both specifically prevent the accumulation of potentially toxic tertiary alkaloids. The specific mechanism for alkaloid sequestration in arctiids is *N*-oxidation of the passively absorbed tertiary alkaloid by senecionine *N*-oxygenase in the hemolymph (Fig. 5D). The specific mechanism for alkaloid sequestration in leaf beetles is suppression of *N*-oxide reduction in the gut and efficient uptake of the *N*-oxide. The specificity of *N*-oxide uptake needs further clarification. In arctiids and specialized leaf beetles the ability to excrete the stored alkaloid *N*-oxide is greatly reduced. This is a further specific prerequisite for sequestration. *O. bifrons* (Ehmke et al., 1991) and *Spodoptera* (Lindigkeit et al., 1997) which do not sequester pyrrolizidine alkaloids but excrete senecionine *N*-oxide injected into the hemolymph almost completely within 24 hours. *O. cacialiae* detoxify tertiary alkaloids more efficiently by conjugation than by *N*-oxidation (Fig. 5B).

To maintain sequestered pyrrolizidine alkaloids in the *N*-oxide state seems to be a prerequisite for alkaloid sequestration in specialized insects. It is reasonable to assume that this mechanism has been selected during the adaptation of insects to plants containing pyrrolizidine alkaloids to keep the alkaloids in a nontoxic and metabolically safe state. The success of the pyrrolizidine alkaloids as defensive compounds in plants and alkaloid sequestering insects is, that these alkaloids can exist in two easily interchangeable forms: the nontoxic *N*-oxide and the potentially toxic tertiary alkaloid (Lindigkeit et al., 1997; Hartmann, 1999). In herbivores feeding on plants containing pyrrolizidine alkaloids, or in predators of insects loaded with alkaloid *N*-oxides, the *N*-oxides are easily reduced in the gut and absorbed passively as tertiary alkaloids. The tertiary alkaloids are biotransformed into toxic metabolites in organisms with accessible microsomal multisubstrate cytochromes P450. The biochemical strategies of pyrrolizidine alkaloid sequestration developed by arctiids and chrysomelid leaf beetles are in accordance with this general idea.

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