The two faces of pyrrolizidine alkaloids: the role of the tertiary amine and its N-oxide in chemical defense of insects with acquired plant alkaloids

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Larvae of Creatonotos transiens (Lepidoptera, Arctiidae) and Zonocerus variegatus (Orthoptera, Pyrgomorphidae) ingest "C-labeled senecionine and its N-oxide with the same efficiency but sequester the two tracers exclusively as N-oxide. Larvae of the non-sequestering Spodoptera littoralis eliminate efficiently the ingested alkaloids. During feeding on the two alkaloidal forms transient levels of senecionine (but not of the N-oxide) are built up in the haemolymph of S. littoralis larvae. Based on these results, senecionine [14O]N-oxide was fed to C. transiens larvae and Z. variegatus adults. The senecionine N-oxide recovered from the haemolymph of the two insects shows an almost complete loss of 14O label, indicating reduction of the orally fed N-oxide in the guts, uptake of the tertiary alkaloid and its re-N-oxidation in the haemolymph. The enzyme responsible for N-oxidation is a soluble mixed function oxidase. It was isolated from the haemolymph of the sequestering arctic Tyria jacobaeae and purified to electrophoretic homogeneity. The enzyme is a flavoprotein with a native M, of 200 000 and a subunit M, of 51 000. It shows a pH optimum at 7.0, has its maximal activity at a temperature of 40–45°C and an isoelectric point at pH 4.9. The reaction is strictly NADPH-dependent (Km 1.3 μM). From 20 pyrrolizidine alkaloids so far tested as substrates, the enzyme N-oxidizes only alkaloids with structural elements which are essential for hepatotoxic and genotoxic pyrrolizidine alkaloids (i.e. 1,2-double bond, esterification of the allylic hydroxyl group, presence of a second free or esterified hydroxyl group at carbon 7). A great variety of related alkaloids and xenobiotics were tested as substrate, none was accepted. The Km values of senecionine, monocrotaline and helenin, representing the three main types of pyrrolizidine alkaloids, are 1.3 μM, 12.5 μM and 290 μM, respectively. The novel enzyme was named senecionine N-oxygenase (SNO). The enzyme was partially purified from two other arctids. The three SNOs show the same general substrate specificity but differ in their affinities towards the main structural types of pyrrolizidine alkaloids. The enzymes from the two generalists (Creatonotos transiens and Arctia caja) display a broader substrate affinity than the enzyme from the specialist (Tyria jacobaeae). The two molecular forms of pyrrolizidine alkaloids, the lipophilic protox toxic tertiary amine and its hydrophilic nontoxic N-oxide are discussed in respect to their bioactivation and detoxification in mammals and their role as defensive chemicals in specialized insects. Pyrrolizidine-alkaloid-sequestering insects store the alkaloids as nontoxic N-oxides which are reduced in the guts of any potential insectivore. The lipophilic tertiary alkaloid is absorbed passively and then bioactivated by cytochrome P-450 oxidase.

Keywords: Tyria jacobaeae (Lepidoptera, Arctiidae); pyrrolizidine alkaloid; alkaloid uptake; senecionine N-oxygenase; chemical defense.

Pyrrolizidine alkaloids are unique among the some 20 000 plant alkaloids in respect to their attractiveness for specialized insects. A number of species from diverse taxa have evolved adaptations to sequester and utilize plant pyrrolizidine alkaloids against insectivores (Hartmann, 1991; Hartmann and Witte, 1995). Larvae of the European cinnabar moth Tyria jacobaeae store pyrrolizidine alkaloids from their host plant Senecio jacobaeae and retain the alkaloids during all stages of metamorphosis (Aplin et al., 1968; Aplin and Rothschild, 1972). In the arctic moth Utetheisa ornatrix, which also sequesters pyrrolizidine alkaloids from its larval food plant, both parents provide pyrrolizidine alkaloids for egg protection. Females receive the alkaloids from males during copulation and transmit these pyrrolizidine alkaloids together with their own load to the eggs (Dussourd et al., 1988). The transmission process is governed by the pyrrolizidine-alkaloid-derived male courtship pheromone hydroxydandial which signals the male's systemic load of pyrrolizidine alkaloids to the female and thus stimulates its behavior (Dussourd et al., 1991). Similar biparental contributions to egg defense are known from the arcticid Creatonotos transiens.
Gilippus participate in the transformation of lipophilic foreign compounds potentially toxic pyrrolizidine alkaloids in the same way as vertebrate pyrrolizidine alkaloids from their host plants and accumulate into excretable metabolites. In the case of the pyrrolizidine alcalozymes (Hodgson, 1985; Brattsten, 1992) should be affected by exceeding a 0.5 M concentration (Pasteels et al., 1988; Hartmann), toxic to vertebrates (Mattocks, 1986; Cheeke, 1989) and genotoxic to insects (Frei et al., 1992). Since the pioneering discoveries of Mattocks and his collaborators (Butler et al., 1970), who first suggested a pyrrolic metabolite as responsible for pyrrolizidine alkaloid toxicity, the mode of action of hepatotoxic pyrrolizidine alkaloids has been completely unraveled (Mattocks, 1986; Winter and Segall, 1989). In vertebrates microsomal cytochrome P-450 oxidases catalyze the conversion of pyrrolizidine alkaloids into unstable pyrroles which are highly reactive alkylating agents. The microsomal P-450 enzymes are part of xenobiotic-metabolism, mainly localized in liver and lung tissue, and participate in the transformation of lipophilic foreign compounds into excretable metabolites. In the case of the pyrrolizidine alkaloids, however, a per se nontoxic compound is converted into a toxic metabolite (bioactivation). Insects which have a similar xenobiotic metabolism with microsomal cytochrome P-450 enzymes (Hodgson, 1985; Brattsten, 1992) should be affected by potentially toxic pyrrolizidine alkaloids in the same way as vertebrates. Besides bioactivation, two other pathways of pyrrolizidine alkaloid metabolism have been established: hydrolysis of the ester bonds and, more important, N-oxidation. The two reactions are believed to be detoxification mechanisms (Cheeke, 1994). N-oxidation converts the potentially toxic tertiary alkaloid into a derivative which no longer can be transformed into a pyrrolic toxin. For example, guinea pigs possess a reactive microsomal multisubstrate flavin monooxygenase (FMO) which catalyzes efficiently the conversion of ingested pyrrolizidine alkaloids into their N-oxides. This explains the high resistance of guinea pigs to toxic effects of pyrrolizidine alkaloids. The N-oxidation by far exceeds the cytochrome-P-450-dependent bioactivation (Miranda et al., 1991). On the contrary, rats which are highly susceptible to pyrrolizidine alkaloid intoxication show only very low N-oxidation activity (Williams et al., 1989).

In this context, it is notable that organisms adapted to deal with pyrrolizidine alkaloids generally keep these alkaloids in the N-oxide state. In plants such as Senecio species pyrrolizidine alkaloids are synthesized in the roots as N-oxides (Hartmann and Toppel, 1987). The N-oxides are specifically translocated via the phloem path into the shoots where they are stored in the cell vacuoles (Hartmann et al., 1989). A membrane carrier has been identified which selectively mediates the translocation of the polar N-oxides into the vacuole (Ehmke et al., 1988). Adapted insects store plant acquired pyrrolizidine alkaloids also preferentially as N-oxides (Mattocks, 1971). More than 80% of the pyrrolizidine alkaloids associated with Utetheisa eggs were found in the N-oxide state (Dussourd et al., 1988). Recent investigations revealed that in a number of alkaloid-sequestering species pyrrolizidine alkaloids are exclusively stored as N-oxides, e.g. Arctiidae (Ehmke et al., 1990; Hartmann et al., 1990; Trigo et al., 1993), Ithomiinae (Trigo et al., 1996), Orthoptera (Billar et al., 1994), chrysomelid leaf beetles (Pasteels et al., 1988, 1996; Hartmann et al., 1997). These findings indicate that storage of pyrrolizidine alkaloids as N-oxides must be advantageous to the sequestering species.

The present study was undertaken to understand the role of the N-oxide state of pyrrolizidine alkaloids in alkaloid sequestering insects. Particularly the mechanisms of resorption of orally ingested tertiary pyrrolizidine alkaloids or their N-oxides from the guts and their fate in the insect’s body were investigated.

**MATERIALS AND METHODS**

**Experimental insects.** Larvae of Creatonotos transiens Walker (Lepidoptera, Arctiidae) were from a laboratory culture originated from females collected in Bali (Indonesia), larvae of Arctia intercallaris L. (Arctiidae) were obtained from a private breeder (Mr Kreuzer, Wackersdorf). The two species were maintained on artificial pyrrolizidine-alkaloid-free diet (Bergomaz and Boppré, 1986). Arctia caja L. larvae were obtained from a private breeder (Mr Sage, Niedergottsau) and reared on leaves of Taraxacum officinale. A total of =8000 late instar larvae of Tyria jacobaeae L. (Arctiidae) were collected in the field (Meijendel dunes, Leiden, The Netherlands) in July 1993 and July 1994, transported to the laboratory and kept in cages on their food plant Senecio jacobaea until haemolymph collection. Spodoptera littoralis Boisdval (Noctuidae) larvae were from a laboratory colony and reared on artificial diet (Bowling, 1967). Zonocerus variegatus L. (Orthoptera, Pygromorphidae) originated from a population collected in the Republic of Bénin (West Africa); specimens were reared from eggs on pyrrolizidine-alkaloid-free plants.

**Preparation of [14C]Senecionine and [14C]Senecionine N-oxide.** The two tracers were obtained biosynthetically by feeding of [1,4-14C]putrescine (109 mCi mmol-1; Amersham Buchler) to root cultures of Senecio vulgaris as described by Hartmann (1994).

**Feeding experiments with [14C]Senecionine and [14C]Senecionine N-oxide.** Late instar larvae of Spodoptera or Creatonotos and adults of Zonocerus were fed individually with 1 mg senecionine or senecionine N-oxide containing 5×10⁶ cpm of the respective tracer. The tracers were incorporated into small pieces of the respective artificial diet (Spodoptera, Creatonotos) or applied on glass-fiber discs (diameter 10 mm) (Zonocerus) and offered to the insects which had previously been starved for 12 h. After consumption of the labeled diet the insects were allowed to continue feeding on untreated diet or Chinese cabbage (Zonocerus) for 48 h. If required (Spodoptera) the excrement was collected at intervals. After termination of the experiment the individuals were crushed in liquid nitrogen and extracted with 10 ml methanol/25% HCl (100: 1, by vol.) for 10 min. After centrifugation, the pellet was extracted twice in the same manner. Aliquots were taken to estimate total radioactivity by scintillation counting and for TLC separation of labeled senecionine and its N-oxide. The alkaloids were separated on silica gel 60 (Merck) with the solvent system methylene chloride/methanol/25% ammonium hydroxide (80:20:5, by vol.). Labelled senecionine (Rt = 0.87) and its N-oxide (Rt = 0.49) were localized by radiodetection using the TLC multichannel analyzer RITA (Raytest).

In injection experiments (Spodoptera) 2 µl Ringer’s solution containing 3.5 µg alkaloid with 5×10⁶ cpm of the respective tracer was injected into the haemolymph of late instar larvae with a 10-µl microsyringe (Hamilton). Exuding body fluid was removed with a piece of filter paper. Larvae were kept and further treated as described for oral feeding.

For analysis of the transient alkaloid concentration in the haemolymph of Spodoptera, larvae which were actively feeding on their diet containing the respective tracer were collected and the haemolymph was sampled immediately as described below.
in the $^{18}$O experiment and frozen in liquid nitrogen. After lyophili-
ization, the dry haemolymph samples were dissolved in 50–
200 µl methanol and analyzed for total radioactivity and $^{14}$C-
labeled senecionine and senecionine N-oxide as described above.

**Preparation of $[^{14}C]senecionine N-[{^{18}O}]oxide.** A gas-tight
all-glass apparatus was applied, consisting of a 100-ml round
flask connected to the $^{18}$O reservoir and a vacuum pump; it was
sealed with a screw cap containing a teflon septum. $[^{14}C]Sen-
cionine (8.2 mg; 4.2X10^6 cpm) was added to the reaction flask
and mixed with 25 µl of a solution of desalted crude sene-
cionine N-oxide (SNO) in reaction buffer (10 mM potas-
sium phosphate pH 7.0, 2 mM dithioerythritol). The apparatus
was sealed and flushed/evacuated 10 times with >99.9% $^{18}$O.
After one flash with $^{18}$O, the apparatus was filled with $^{18}$O$_2$
(98–99%: Linde) and the reaction was started by injecting
50 mg NADPH in 1 ml helium-flushed reaction buffer. The
reaction was allowed to proceed for 24 h at 30°C and was then
terminated by injection of 2.5 ml 25% HCl; precipitating protein
was removed by centrifugation. The aqueous supernatant
dried under vacuum, the residue dissolved in 3 ml methanol and
in the supernatant, the dry haemolymph samples were dissolved in
the all-glass apparatus was applied, consisting of a 100-ml round
flask with a 10-µl microsyringe (Hamilton). Exuding body
fluid was removed with a piece of filter paper. With $[^{14}C]Sen-
ecionine and particularly small amounts of contaminating seneci-
phylline N-[{^{18}O}]oxide which has the same $M_r$ (i.e. 351) as un-
labeled senecionine N-oxide. HPLC was performed using a
RP-18 column ($Nucleosil 7 µm, 250 mm long; 25 mm i.d.;
Macherey & Nagel). The sample was redissolved in 800 µl water/trichloroacetic acid (1000:2, by vol.), centrifuged and ap-
plied onto the column via a Rheodyne rotary valve with a 500-
µ1 loop. Separation was achieved isocratically using acetonitrile/
water/trichloroacetic acid (150:850:2, by vol.) at a flow rate of
9 ml · min$^{-1}$. Detection was by absorbance at 254 nm. The reten-
tion times ($t_R$) for the pyrrolizidine alkaloids are: seneciphylline,
27 min; senecionine N-oxide, 33 min; senecionine, 39 min;
senecionine N-oxide, 47 min. The fractions containing senec-
ionine N-oxide were recovered, the solvent removed by evapora-
tion and the residue extracted with toluene at pH 11 (adjusted
with NH$_4$OH) to remove any traces of non-N-oxidized seneci-
onine. Three samples were prepared. The $^{18}$O content was deter-
mined by fast-atom-bombardment mass spectrometry (FAB-
MS); all measurements were corrected for natural abundance of
$^{13}$C. The specific radioactivity was determined by scintillation
counting and quantitative GC analysis of the Zn/H$^+$-reduced
sample. Sample A: 86% $^{18}$O in N-oxide oxygen (specific radio-
activity, 1.3X10$^{8}$ cpm/mmol); sample B: 97% $^{18}$O in N-oxide
oxygen (specific radioactivity, 1.3X10$^{8}$ cpm/mmol); sample C:
98% $^{18}$O in N-oxide oxygen (specific radioactivity, 1.5X10$^{8}$
cpm/mmol).

**Oral feeding of $[^{14}C]senecionine N-[{^{18}O}]oxide. Creato-
notus** final instar larvae and Zonocerus adults were fed individu-
ally with 1.3 mg tracer each applied on glass-fiber discs (diamete-
ter 5–10 mm). *Arctia* larvae were treated in the same way but
the tracer was offered with artificial diet. All insects had been
starved for 12 h. After consumption of the entire sample, the
arctid larvae were allowed to feed on untreated diet and Zono-
cerus on chinese cabbage for 48 h.

**Injection of $[^{14}C]senecionine N-[{^{18}O}]oxide.** The tracer
(0.7 mg/3 µl Ringer’s solution) was injected into the haemo-
ymph of *C. transiens* last instar larvae between the 3rd and 4th
segment with a 10-µl microsyringe (Hamilton). Exuding body
fluid was removed with a piece of filter paper. With *Z. variega-
tus* adults the injection was directly into the abdominal cavity
between the 2nd and 3rd abdominal segment. After injection
the insects were kept on their normal diets for 72 h.

**Preparation of haemolymph and alkaloid analysis.** Haemolymph of cooled (5 min at $-20^\circ$C) individuals was col-
lected with a calibrated glass capillary after cutting off a pseudo-
pod (arctiid larvae) or leg (Z. variegatus). After recording the
total volume the haemolymph of each individual was immedi-
ately added to 0.6 ml methanol/25% HCl (6:1, by vol.). The
precipitated protein was removed by centrifugation and the
supernatant evaporated under an air stream. Labeled senecionine
N-oxide was purified by HPLC and further analyzed as given
for preparation of $[^{14}C]senecionine N-[{^{18}O}]oxide.

**Capillary gas chromatography.** Sample preparation, separa-
tion and quantitative analysis of pyrrolizidine alkaloids as ter-
tary alkaloids were performed according to the standard meth-
ods described by Witte et al. (1993).

**Enzymatic assay of senecionine N-oxygenation (tracer
assays).** Enzyme activity was assayed by determining the for-
mation of radioactively labeled senecionine N-oxide from $[^{14}C]-
senecionine either by radioactive thin-layer chromatography
(TLC method) or by determination of the remaining substrate
senecionine due to its selective solubility in toluene (toluene
method). The reaction mixture contains in a total volume of
300 µl: 10 mM potassium phosphate pH 7.0 plus 2 mM dithio-
erythritol (standard buffer), 0.2 mM $[^{14}C]senecionine (2–3X10^4$
cpm/assay), 1.2 mM NADPH and enzyme in standard buffer.
The reaction was started by addition of NADPH and generally
allowed to proceed 30 min at 37°C. The reaction was termin-
ated by addition of 100 µl 2 M HCl.

**TLC method.** The reaction mixture was evaporated under air
flow, redissolved in 20 µl methanol and subjected to radioactive
TLC analysis. Enzyme activity was calculated from the substrate
(senecionine)/product (senecionine N-oxide) ratio.

**Toluene method.** The enzyme assay as described above was
performed in a scintillation vial (4 ml). The reaction was termi-
inated by addition of 100 µl 2 M HCl, then 1 ml of a mixture
of toluene/Lipo-Luma (Baker) (1:1, by vol.) was added. After
addition of 100 µl 5 M NaOH and thorough shaking, the mix-
ture was centrifuged and, without separation, was directly analy-
ed in a scintillation counter. The labeled polar alkaloid N-oxide
remains quantitatively in the aqueous layer and does not interfere
with scintillation counting. Generally an addition of 20000 cpm
labeled substrate/assay was sufficient to give reliable results.
Controls without addition of NADPH were treated in the same
way.

**Enzymatic assay of senecionine N-oxygenation (photo-
metric assay).** The enzyme assay was as given above. The re-
action was started by the addition of the substrate and followed
continuously by the decrease of the absorption (NADPH) at
334 nm (Eppendorf photometer) or 340 nm (Pye Unicum UV/
VIS) at 37°C.

**Enzyme purification.** The haemolymph was collected from
*Tyria jacobaeae* late instar larvae and kept on ice until a total
volume of ~8 ml had been obtained (~80 individuals). Then
40 ml cooled saturated ammonium sulfate in 0.1 M potassium
phosphate pH 7.0 plus 2 mM dithioerythritol (standard buffer)
was slowly added. The suspension was stirred for 60 min, the
precipitate recovered by centrifugation (20 min at 20000 · g)
and stored at $-20^\circ$C. In this way crude enzyme preparations
could be preserved without significant loss of activity for more
than one year.

**Step 1 (Phosphocellulose, Sigma).** Phosphocellulose medium
(mesh Sigma) was cycled following the manufacturer’s instruc-
tions. The preserved ammonium sulfate precipitate of the
haemolymph from 80 individuals was dissolved in 20 ml stand-
ard buffer and desalted via Sephacel G-25 (PD-10 columns,
Pharmacia). The desalted solution was applied to a phosphocel-
lulose column (bed volume 40 ml; 75 mm length, 26 mm i.d.)
previously equilibrated with 150 ml standard buffer. The column
was washed with 80 ml buffer at a flow rate of 0.5 µl · min$^{-1}$.
Protein was fractionated by linear gradient elution of 0–1 M potassium phosphate pH 7.0 (100 ml). Fractions of 2.5 ml were collected, enzyme activity eluted in fractions 14–20.

*Step 2 (Hydroxyapatite Bio-Gel HT, Bio-Rad).* The desalted fractions with enzyme activity from step 1 were applied to a hydroxyapatite column (bed volume 4 ml; 50 mm length, 10 mm i.d.) previously equilibrated with 40 ml standard buffer. The column was eluted with 20 ml buffer followed by a 70-ml linear gradient of 0–0.5 M potassium phosphate pH 7.0. The flow rate was 0.5 ml min⁻¹; 1.5-ml fractions with enzyme activity were eluted between 125–250 mM potassium phosphate.

*Step 3 (Mono Q 5/5 HR, Pharmacia).* Active protein obtained from step 2 was applied to an FPLC-Mono Q column, which had previously been equilibrated with 20 ml standard buffer. Protein was eluted with a 100-ml 0–0.1 M KCl gradient followed by a 60-ml 0.1–0.3 M KCl gradient. Fractions of 0.5 ml were collected; enzyme activity was found in fractions 23–30.

**Determination of $M_r$.* For the determination of the native $M_r$, the purified enzyme was applied to a Superdex 200 HR (10/30) column (Pharmacia) and was eluted with standard buffer at a flow rate of 0.5 ml min⁻¹. As reference proteins, the kit MS II (Serva) was used. The $M_r$ of the denatured enzyme was obtained by SDS/PAGE (Laemmli, 1970). A protein ladder of 12-kDa steps between 10–120 kDa (Life Technologies) was used as molecular marker. The purified samples (Mono Q eluate) were desalted on PD 10 columns and concentrated on Centricon-30 concentrators (Amicon) if necessary.

**Determination of pH.* The pH was determined on an Ampholine® PAGPlate pH 3.5–9.5; length 110 mm (Pharmacia) using the horizontal electrophoresis system Multiphor II (Pharmacia). The protein reference mixtures V (Merk) (pH 4.75–10.65) and Serva (pH 3.50–10.65) were used for calibration. The enzyme was localized by silver staining (Heukeshoven and Dernick, 1988). In addition, one gel was cut into 10-mm slices and protein was eluted with standard buffer. Subsequently SNO activity was localized by applying the TLC method.

**Pyrrolizidine alkaloids applied as enzyme substrates.* Seneciphylline and retorsine were obtained from Roth, monocrotaline from Aldrich, heliotrine from Corkwood Enterprises. The other pyrrolizidine alkaloids were isolated and purified from respective plant sources: senecionine, senecivernine and senkirvine from *Senecio vernalis* (Inofice) according to Hartmann and Zimmer (1988); axillaridine and axillarine from seeds of *Creatonotos scassellati* according to Wiedenfeld et al. (1985); triangularine and sarracine were isolated from *S. sylvaticus*, indicine was isolated from flowers and lycopasamine from seeds of *Heliotropium indicum*, tindelaine from flowers of *Eupatorion laevigatum* and phalaenopsine from flowers of *Phalaenopsis* hybrids. Purity and identity of the structures were verified by GC/MS and, if necessary, NMR analysis. All pyrrolizidine alkaloids were purified as tertiary alkaloids. The respective N-oxides were prepared according to Craig and Purushothaman (1970). Retrocine and heliotridine were prepared by alkaline hydrolysis of monocrotaline and heliotrine, respectively (Hartmann et al., 1990). [14C]Labeled pyrrolizidine alkaloids were isolated from the respective root cultures after feeding of labeled putrescine as biosynthetic precursor (Hartmann, 1994).

**RESULTS**

**Table 1. Sequestration of pyrrolizidine alkaloids by *Creatonotos* larvae and adults of *Zonocerus* after feeding on [14C]senecionine and [14C]senecionine N-oxide.** Alkaloid (1 mg = 100%) was fed per individual; after consumption of the labeled alkaloid, individuals were kept on untreated diet for 48 h prior to analysis. Mean ± SEM is given; $n = 5$ (*Creatonotos*); $n = 4$ (*Zonocerus*).

<table>
<thead>
<tr>
<th>Species</th>
<th>Senecionine N-oxide sequestered</th>
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<tr>
<td></td>
<td>senecionine fed</td>
</tr>
<tr>
<td><em>Creatonotos transiens</em></td>
<td>$7.7 \pm 2.3$</td>
</tr>
<tr>
<td><em>Zonocerus variegatus</em></td>
<td>$28.9 \pm 6.9$</td>
</tr>
</tbody>
</table>

The results (Table 1) give two main answers: (a) exclusively [14C]senecionine N-oxide could be detected in extracts of insects which had fed on either of the two alkaloid forms; (b) there is no significant difference in the amount of sequestered alkaloid N-oxide between insects having fed on senecionine and senecionine N-oxide.

**Uptake and elimination of pyrrolizidine alkaloids by the noctuid *Spodoptera.* Larvae of *Spodoptera* easily feed on pyrrolizidine-alkaloid-containing plants but do not sequester the alkaloids (Aplin and Rothschild, 1972). Feeding experiments with labeled senecionine and its N-oxide show that with both tracers the radioactivity is almost completely eliminated with the excretion within the first 24 h; less than 2% of the radioactivity offered with the alkaloids is retained in larval bodies after 48 h (Fig. 1A, B). The tracers are even more rapidly eliminated if they are internally applied, i.e. injected into the haemolymph of larvae (Fig. 1C, D). Considerable amounts (40–80%) of [14C]senecionine could be recovered from methanolic excrement extracts after oral and internal application of the two tracers, whereas [14C]senecionine N-oxide was detectable in traces only (<2%) in excrement extracts after oral application of the two alkaloidal tracers. In the injection experiments, labeled N-oxide was not detectable in excrement extracts. Thus, *Spodoptera* efficiently eliminate orally or internally administered senecionine and its N-oxide. The alkaloids are preferentially excreted in the reduced state (i.e. as tertiary alkaloid).

A more detailed experiment revealed that, during oral uptake of the two labeled alkaloids, considerable amounts of senecionine were transiently detectable in the haemolymph of larvae (Table 2). [14C]Senecionine was the only labeled metabolite detectable in the haemolymph, not even traces of the N-oxide were found. [14C]Senecionine N-oxide injected into the haemolymph is efficiently excreted but not even traces of tertiary alkaloid could transiently be detected in the haemolymph. In conclusion, these results strongly favor the following fate of orally ingested pyrrolizidine alkaloids by *Spodoptera*: (a) the alkaloid N-oxide is easily reduced in the guts; (b) the tertiary alkaloid is passively absorbed into the haemolymph and again eliminated by efficient excretion.

**Mechanism of pyrrolizidine alkaloid absorption in alkaloid-sequestering arctids and *Zonocerus.* For pyrrolizidine alkaloid absorption in alkaloid-storing insects, two possible mechanisms have to be taken into consideration: (a) the existence of a specific carrier which particularly favors the uptake of the salt-like polar alkaloid N-oxides as suggested by Wink and Schneider (1988); (b) reduction of pyrrolizidine alkaloid N-oxide in the guts, uptake of the lipophilic tertiary alkaloids in their unprotonated form by simple diffusion followed by rapid re-N-oxygena-
Table 2. Transient concentration of $[^{14}C]$seconionine in the haemolymph of late instar larvae of *Spodoptera littoralis* feeding on labeled seconionine and its N-oxide. Larvae ($n = 4$, seconionine; $n = 8$, seconionine N-oxide) were fed on artificial diet containing the labeled alkaloids in the concentration indicated; actively feeding larvae were removed and the haemolymph was immediately collected. Mean ± SEM is given; n.d. = not detectable.

<table>
<thead>
<tr>
<th>Alkaloid fed</th>
<th>Concentration fed</th>
<th>Feeding time</th>
<th>Concentration of $[^{14}C]$seconionine</th>
<th>Concen-</th>
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<td></td>
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<tr>
<td>$[^{14}C]$seconionine</td>
<td>6.27</td>
<td>20–33</td>
<td>157 ± 161</td>
<td>n.d.</td>
</tr>
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with the second mechanism the $^{18}O$ should be completely replaced by $^{16}O$.

Double-labeled $[^{14}C]$seconionine $[^{18}O]$N-oxide was prepared and fed orally to larvae of the two arctids *Creatonotos* and *Arctia* and to adults of the grasshopper *Zonocerus*. To prevent any complications with background levels of pyrrolizidine-alkaloids the insects had been reared on pyrrolizidine-alkaloid-free diets. After sequestration of the labeled alkaloid, the haemolymph was collected, the seconionine N-oxide extracted and purified by HPLC. Identity and purity of the isolated compound was verified: (a) by comparison of its specific radioactivity with that of the tracer applied (Table 3); (b) after reduction by GC/MS. The purified N-oxide was analyzed for its $^{18}O$ content by means of FAB-MS. The result of one measurement is illustrated in Fig. 2. It shows that, in comparison to the control, the $^{18}O$ label in the seconionine N-oxide recovered from the haemolymph is almost completely exchanged for $^{16}O$. With the three insects 86–99%
of the $^{18}$O label was lost during sequestration of orally fed $^{18}$O-labeled senecionine N-oxide (Table 3). If the $^{18}$O-labeled N-oxide is directly injected into the haemolymph, the label is retained completely stable for at least 72 h. This excludes any significant turnover of the N-oxide oxygen in the haemolymph and proves that the reduction of the orally ingested alkald N-oxide must have been taken place in the guts. The significant loss of 12% of $^{18}$O label in the experiment with Creatonotos, which was not observed with Zonocerus, can be explained by the larvae’s licking their wounds. After injection small droplets of haemolymph were observed to ooze out the wound. These droplets were ingested by licking and any labeled N-oxide contained in the wound droplets would loose its label during the gut passage.

**Detection of senecionine N-oxygenating enzyme activity in pyrrolizidine-alkaloid-storing insects.** The tracer experiments with the $^{18}$O-labeled senecionine N-oxide indicate the presence of efficient N-oxidizing enzyme activity in the insects’ bodies. In preliminary studies, isolated haemolymph of a number of pyrrolizidine-alkaloid-sequestering species was tested for the ability to N-oxidize [C]senecionine. Significant in vitro enzyme activity could be detected in the following species and metamorphic stages: family Arctiidae, Tyria jacobaeae (larvae, pupae); Callimorpha dominula (larvae); Creatonotos transiens (larvae); Arctia caja, A. intercallaris (larvae); Amerila phaedra (adults) and family Danainae, Idea leuconoe (pupae). In the grasshopper Zonocerus the N-oxygenating enzyme activity is associated with the fat body and not with the haemolymph. As expected, no enzyme activity was detected in Spodoptera which does not sequester pyrrolizidine alkaloids.

**Purification of senecionine N-oxygenase (SNO) from haemolymph of T. jacobaeae.** Due to their easy availability larvae of the European cinnabar moth, Tyria jacobaeae, were chosen as enzyme source. A soluble NADPH-dependent mixed-function monoxygenase was detected in crude haemolymph preparations. In ammonium sulfate precipitates of freshly collected haemolymph the enzyme activity could be preserved for months without significant loss of activity. Purification to electrophoretic purity (Fig. 3) was achieved in a three-step procedure by combination of phosphocellulose chromatography, hydroxyapatite chromatography and anion-exchange chromatography (Mono Q) (Table 4). The purification via phosphocellulose was not cation-exchange but phosphate-affinity chromatography since elution of enzyme activity was only possible with potassium phosphate but not potassium chloride. The matrix-fixed phosphate binds to proteins known to react with phosphate esters (Sternbach, 1991).

**General and molecular properties of SNO.** The purified enzyme from Tyria haemolymph shows a pH optimum of 7.0 in standard buffer. It has its maximal activity at a temperature of 40–45°C; the activation energy ($E_a$) of senecionine N-oxygenation was determined over a temperature range of 10–35°C and was found to be 53.1 kJ/mol. The reaction is strictly dependent on molecular oxygen. The reaction is not dependent on cytochrome P-450, as indicated by the following lines of evidence: (a) it is a soluble enzyme; (b) it is not carbon-monoxide-sensitive and the carbon monoxide difference spectrum of the dithionite-reduced enzyme did not show the typical carboxy ferrochrome absorption maximum at 450 nm (Hodgson, 1985); (b) typical cytochrome P-450 inhibitors (e.g. metyrapone, SKF 525A, n-octylamine and guanethidine) did not significantly reduce enzyme activity. Rather, the oxygenase is a flavoprotein. An absolute spectrum of purified SNO in the presence of oxygen

![Image of SDS/PAGE](image-url)

**Fig. 3. SDS/PAGE of purified SNO from Tyria jacobaeae.** The two bands of SNO (left) migrate slightly above the 50-kDa protein marker. The molecular mass markers (right) represent a protein ladder of 12-kDa steps between 10 kDa (bottom) and 120 kDa. The top band represents a 200-kDa fragment. Proteins were stained with Coomassie brilliant blue.

![Image of Ultraviolet/visible spectrum](image-url)

**Fig. 4. Ultraviolet/visible spectrum of purified SNO from Tyria jacobaeae.** The spectrum was recorded with 131 μg protein in the oxidized state of the enzyme, i.e. in presence of substrate (senecionine) and O₂ and absence of NADPH.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>228</td>
<td>24.2</td>
<td>0.11</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>4.5</td>
<td>8.2</td>
<td>1.81</td>
<td>34</td>
<td>17</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>0.619</td>
<td>5.6</td>
<td>8.98</td>
<td>23</td>
<td>85</td>
</tr>
<tr>
<td>Mono Q</td>
<td>0.045</td>
<td>3.5</td>
<td>77.56</td>
<td>14</td>
<td>731</td>
</tr>
</tbody>
</table>

### Table 4. Purification of senecionine N-oxygenase (SNO) from haemolymph of Tyria jacobaeae larvae. Crude extract: desalted ammonium sulfate precipitate of ~8 ml haemolymph (corresponding to 80 late-instar larvae).
Table 5. Substrate specificity of seneconine N-oxygenase from the haemolymph of three arctiids.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Activity with SNO from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tyria</td>
</tr>
<tr>
<td>Senecione type</td>
<td>%</td>
</tr>
<tr>
<td>Senecione</td>
<td>100</td>
</tr>
<tr>
<td>Seneciphylidine</td>
<td>95</td>
</tr>
<tr>
<td>Retrorsine</td>
<td>97</td>
</tr>
<tr>
<td>Senecivernine</td>
<td>81</td>
</tr>
<tr>
<td>Senkirkine</td>
<td>0</td>
</tr>
<tr>
<td>Triangularine type</td>
<td>%</td>
</tr>
<tr>
<td>Triangularine</td>
<td>60</td>
</tr>
<tr>
<td>Sarricine</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Monocrotaline type</td>
<td>%</td>
</tr>
<tr>
<td>Monocrotaline</td>
<td>92</td>
</tr>
<tr>
<td>Axillarine</td>
<td>74</td>
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<tr>
<td>Axillaridine</td>
<td>76</td>
</tr>
<tr>
<td>Lycopsamine type</td>
<td>%</td>
</tr>
<tr>
<td>Lycopsamine</td>
<td>20</td>
</tr>
<tr>
<td>Rinderine</td>
<td>23</td>
</tr>
<tr>
<td>Indicine</td>
<td>35</td>
</tr>
<tr>
<td>Heliotrine</td>
<td>25</td>
</tr>
<tr>
<td>Supinine</td>
<td>0</td>
</tr>
<tr>
<td>Phalaenopsine type</td>
<td>%</td>
</tr>
<tr>
<td>Phalaenopsine</td>
<td>0</td>
</tr>
<tr>
<td>Necines bases</td>
<td>%</td>
</tr>
<tr>
<td>Retromecine</td>
<td>0</td>
</tr>
<tr>
<td>Heliotridine</td>
<td>0</td>
</tr>
<tr>
<td>Supinine</td>
<td>0</td>
</tr>
<tr>
<td>Isoretromecanol</td>
<td>0</td>
</tr>
</tbody>
</table>

and its substrate seneconine and in the absence of NADPH is shown in Fig. 4. The spectrum exhibits the typical absorption pattern for an oxidized flavoprotein, i.e. absorption maxima at 375 and 456 nm with a ratio A_{375}/A_{456} of 1.5 (Dawson et al., 1986). Basing on a subunit $M_r$ of 51 000 of SNO and a molar absorption coefficient for FAD of 11 300 $M^{-1} \cdot cm^{-1}$, a molar apoenzyme/FAD ratio of 0.4 was estimated. Assuming that one mole of each apoenzyme subunit binds one mole of FAD, the ratio should be 1. Due the small quantity of pure enzyme available and the low absorption values, a considerable error of calculations has to be taken into account. However the estimated ratio is well within the expected order of magnitude. This strongly supports the assumption that SNO is a flavoprotein.

The isoelectric point of the homogenous enzyme is at pH 4.9 ± 0.1. The native $M_r$ of the enzyme determined by gel permeation chromatography with a Superdex 200 HR column was found to be 200 000 ± 10 000. SDS/PAGE always shows two sharp bands of almost identical migration and intensity (Fig. 3) with an $M_r$ of 51 000 ± 1000, which indicates that the active enzyme is a tetramer composed of subunits of nearly identical size.

Substrate specificity and substrate kinetics of SNO from three arctiids. The purified enzyme from Tyria and partially purified enzyme preparations obtained from the haemolymph of Creatonotos and Arctia caja larvae were chosen for comparative studies on substrate specificity and substrate kinetics of larval SNO. In Table 5 all available pyrrolizidine alkaloids are listed which were tested as substrates for SNO. They comprise examples from the main structural types of pyrrolizidine alkaloids according to the classification given by Hartmann and Witte (1995) (Fig. 5). The three enzymes show the same substrate specificity but differ in the relative efficiency for individual substrates. Not all pyrrolizidine alkaloids are N-oxidized. Ester alkaloids lacking an hydroxyl group at carbon seven (e.g. supinine and phalaenopsine), 1,2-dihydro-alkaloids such as sarracine and phalaenopsine and unesterified necine bases are not accepted as substrates. Senkirkine the otonecine analogue of seneconine, is also not N-oxidized. This, however, is not surprising as the necine derivatives are the only plant pyrrolizidine alkaloids which are never found as N-oxides.

In addition to pyrrolizidine alkaloids a large number of related structures and potential substrates of multisubstrate FMOs were tested as substrates of SNO. None of these compounds, which include tropane alkaloids, quinolizidine alkaloids, nicotine, indole alkaloids, caffeine, isoquinolines and synthetic heterocyclic tertiary amines, were accepted as substrate by SNO from Tyria.

The SNO-catalyzed reaction is strictly NADPH-dependent; NADH cannot replace NADPH as cosubstrate. The $K_m$ values for NADPH from the enzymes from Tyria and Creatonotos are 1.3 μM and 2.2 μM, respectively.

The substrate kinetics were established for three selected substrates representing the major classes of pyrrolizidine alkaloids (Table 6). With an apparent $K_m$ value of 1.4 mM the Tyria enzyme has a high affinity for its substrate seneconine which represents the type of pyrrolizidine alkaloids of its exclusive host plant Senecio jacobaea. It displays a considerably lower affinity toward other structural types such as the monocrotaline (10-fold lower) and particularly the monoester heliotrine (200-fold lower), which are not present in S. jacobaea. These differences are less expressed with the enzymes from the two other arctiids which, in contrast to T. jacobaeae, are generalists feeding on a variety of alkaloidal food plants with different pyrrolizidine alkaloid patterns.

DISCUSSION

Uptake behavior of the two forms of pyrrolizidine alkaloids (i.e. tertiary alkaloid and its N-oxide) was studied with Spodoptera littoralis, a species which tolerates but does not sequester pyrrolizidine alkaloids and several pyrrolizidine-alkaloid-sequestering species. For both non-sequestering and sequestering species only the tertiary was taken up into the body, irrespectively of whether the tertiary alkaloid or its N-oxide had been fed. The ingested alkaloid N-oxide has to be reduced in the gut prior to absorption. This was directly proved in the case of Spodoptera by analysis of transient levels of tertiary alkaloid in the haemolymph. With the sequestering species, which in their bodies store only the N-oxide form, feeding of [14C]-N-oxide labeled alkaloid revealed that the alkaloid N-oxide recovered from the haemolymph had lost all of its 14C label. Since [14C]-N-oxide directly injected into the haemolymph completely retained its 14C label, the reduction must have occurred in the gut. It has long been known that in mammals any pyrrolizidine alkaloid N-oxide ingested with food is easily reduced in the gut and the lipophilic tertiary alkaloid is passively absorbed (Matsocks, 1986). The same happens in insects. In Spodoptera alkaloid diffuses passively into the haemolymph creating a transient
Fig. 5. The structures of pyrrolizidine alkaloids applied as potential substrates of SNO.

level of tertiary alkaloid. Spodoptera larvae which are adapted to feed on plants containing pyrrolizidine alkaloids obviously prevent deleterious poisoning by rapid and efficient excretion of the absorbed tertiary alkaloid. Even the alkaloid N-oxide, which never enters the haemolymph via the guts, is rapidly excreted if administered internally. As demonstrated with the ¹⁵O-labeled senecionine N-oxide, pyrrolizidine alkaloid uptake in alkaloid-sequestering lepidopterans and the taxonomically unrelated grasshopper Zonocerus proceeds exactly in the same way as in Spodoptera, i.e. reduction in the guts followed by uptake of the tertiary amine into the haemolymph. Two mechanistic features facilitate alkaloid accumulation in pyrrolizidine-alkaloid-sequestering species: efficient re-N-oxidation and lack of N-oxide excretion (metabolic trapping). The ¹⁵O experiment clearly disproves a specific carrier-mediated uptake of senecionine N-oxide as recently claimed for Creatonotos transiens (Wink and Schneider, 1988). We cannot exclude a carrier-mediated uptake for tertiary alkaloids in pyrrolizidine-alkaloid-sequestering insects. However, uptake of tertiary pyrrolizidine alkaloids by passive

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$</th>
<th>$V$</th>
<th>$V/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tyria jacobaeae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Senecionine</td>
<td>1.4</td>
<td>202</td>
<td>100</td>
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<tr>
<td>Monocrotaline</td>
<td>12.5</td>
<td>155</td>
<td>9</td>
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<tr>
<td>Heliotrine</td>
<td>284.0</td>
<td>124</td>
<td>&lt;1</td>
</tr>
<tr>
<td><strong>Creatonotos transiens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Senecionine</td>
<td>3.1</td>
<td>554</td>
<td>100</td>
</tr>
<tr>
<td>Monocrotaline</td>
<td>2.9</td>
<td>344</td>
<td>67</td>
</tr>
<tr>
<td>Heliotrine</td>
<td>22.9</td>
<td>628</td>
<td>15</td>
</tr>
<tr>
<td><strong>Artia caja</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Senecionine</td>
<td>4.8</td>
<td>198</td>
<td>100</td>
</tr>
<tr>
<td>Monocrotaline</td>
<td>5.1</td>
<td>168</td>
<td>81</td>
</tr>
<tr>
<td>Heliotrine</td>
<td>18.6</td>
<td>222</td>
<td>29</td>
</tr>
</tbody>
</table>
diffusion as demonstrated for the non-sequestering species Spodoptera and well known from vertebrates (Mattocks, 1986) makes the existence of such a carrier unlikely.

In the pyrrolizidine-alkaloid-sequestering arctiids the N-oxidation of the passively absorbed tertiary alkaloid is catalyzed by a specific monooxygenase (SNO). Purified SNO shares its flavin dependency and ability to N-oxidize pyrrolizidine alkaloids with microsomal multisubstrate flavine monooxygenase (FMO) but is completely different with respect to its substrate specificity. FMO has been purified from mammalian liver, lung and kidney (Ziegler and Mitchell, 1972; Tynes and Hodgson, 1985; Gasser et al., 1990). The enzyme shows an extraordinarily broad substrate specificity and oxidizes a variety of nucleophilic organic nitrogen (i.e. lipophilic alicyclic and cyclic aliphatic amines) and sulfur compounds (Ziegler, 1993). Multisubstrate FMO is not known in insects (Brattsten, 1992). Aside from FMO and two functionally related enzymes from bacteria (Ryerson et al., 1982) and Trypanosoma (Agosin and Ankley, 1987), SNO is the only characterized non-cytochrome-P-450 enzyme which converts an amine into its N-oxide. In contrast to FMO, it is not microsomal but soluble and specifically N-oxidizes only pyrrolizidine alkaloids which combine the following structural features: (a) presence of an 1,2-double bond (1,2-dehydropyrrolizidines); (b) an allylic esterified hydroxyl group (at C9); (c) a free or esterified second hydroxyl group at C7 (see Fig. 6). These are precisely the structural features of pyrrolizidine alkaloids found to be hepatotoxic in vertebrates (Winter and Segall, 1989) and genotoxic in the Drosophila wing spot test (Frei et al., 1992). The nontoxic free necine bases and 1,2-saturated alkaloids such as sarracine and phalaenopsine (see Fig. 5) are not N-oxidized by SNO. No other substrates outside pyrrolizidine alkaloids have been found to be accepted by the enzyme so far.

N-Oxidation seems to be a prerequisite for alkaloid sequestration in arctiids and Zonocerus and it is reasonable to assume that this mechanism has been selected to keep the alkaloid in the insect's body in a nontoxic and metabolically inert state. This view is substantially supported by the substrate specificity of SNO. It suggests that SNO must have been selected and optimized during the coevolutionary adaptation of sequestering insects to plants containing pyrrolizidine alkaloid. From the mechanistic point of view, this idea is further supported by a comparison of the substrate specificities of the enzymes from the three arctiids. Tyria jacobaee is a specialist, feeding almost exclusively on Senecio jacobaea which contains only pyrrolizidine alkaloids of the senecionine type (Hartmann and Witte, 1995). Thus, not unexpectedly, senecionine is the best substrate of Tyria SNO, whereas monocrotaline and heliotrine as representatives of the monocrotaline type and lycopsamine type, respectively, which are not found in Senecio, are less efficient substrates with 10-fold and 200-fold higher $K_m$ values, respectively (see Table 6). On the contrary, Creatonotos and Arctia are generalists with larvae feeding on a variety of plants containing pyrrolizidine alkaloid, which may possess quite different alkaloid patterns. Consequently the differences in the affinity of their SNOs towards the three substrates are less pronounced (Table 6). Further studies must show to what extent the relative substrate specificity of SNO is related to the alkaloidal type of the insect's preferential pyrrolizidine alkaloid sources. Preliminary experiments with Brazilian Ithomiinae butterflies which are adapted to sequester pyrrolizidine alkaloids of the lycopsamine type revealed that N-oxidation is more efficient with these monoesters than with macrocyclic senecionine (Trigo, J. R. and Hartmann, T., unpublished results). Sequence data of SNO should help us to answer mechanistic questions of structure/activity relationships with respect to substrate specificity as well as questions concerning the evolutionary origin of the enzyme.

Why are pyrrolizidine alkaloids so unique among alkaloids with regard to their role in insects? From the mechanistic point of view, our knowledge of the chemical and biological attributes of pyrrolizidine alkaloids is now substantial enough to address this question conclusively. The essential feature of pyrrolizidine alkaloids is that they can exist in two interchangeable forms:
the alkaloid N-oxide and the respective tertiary alkaloid (Fig. 7). Plants synthesize, translocate and store pyrrolizidine alkaloids as hydrophilic N-oxides (Hartmann et al., 1989). During the ingestion of food loaded with N-oxides by a herbivore, the N-oxides are reduced in the guts and absorbed passively as tertiary alkaloids. The tertiary alkaloid is deleterious for organisms or cells possessing a microsomal multisubstrate cytochrome P-450 and this accounts for its toxicity to perhaps any herbivore. Some organisms have evolved adaptations to overcome this potential toxicity by efficient N-oxidation (e.g. guinea pigs) or rapid excretion [e.g. Spodoptera as shown in this study or Melanoplus (Ehmke et al., 1989)]. Pyrrolizidine-alkaloid-sequestering insects are safe as long as they are able to keep the alkaloid in the N-oxide state. In lepidopterans and Zonocerus, SNO guarantees this safety. Leaf beetles of the genus Oreina (Chrysomelidae) sequester pyrrolizidine alkaloids in their defensive glands also exclusively as N-oxides, but they are not able to N-oxidize tertiary pyrrolizidine alkaloids. Contrary to lepidopteran species, they are able to take up directly the plant-derived pyrrolizidine alkaloid N-oxides and eliminate tertiary pyrrolizidine alkaloids (Ehmke et al., 1991; Rowell-Rahier et al., 1991).

In addition to aposeatically colored lepidopterans, leaf beetles provide the strongest arguments favoring the role of pyrrolizidine alkaloids as powerful defensive compounds. One species, O. cacalae, has even lost the ability to autogenously produce cardenolides for defense but instead accumulates pyrrolizidine alkaloid N-oxides acquired from its food plant in its defensive secretion (Pasteels et al., 1996). These beetles were found to be better protected against predation by birds than a closely related species which secretes autogenous cardenolides (Rowell-Rahier et al., 1995).

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