

Sequestration of plant pyrrolizidine alkaloids by chrysomelid beetles and selective transfer into the defensive secretions

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Summary

Oreina cacaliae and *O. speciosissima* (Coleoptera, Chrysomelidae) sequester in their elytral and pronotal defensive secretions pyrrolizidine alkaloids (PAs) as N-oxides (PA N-oxides). The PA N-oxide patterns found in the beetles and their host plants were evaluated qualitatively and quantitatively by capillary gas chromatography/mass spectrometry (GC-MS). Of the three host plants *Adenostyles alliariae* (Asteraceae) is the exclusive source for PA N-oxide sequestration in the defensive secretions of the beetles. With the exception of O-acetylseneciphylline the N-oxides of all PAs of *A. alliariae*, i.e. senecionine, seneciphylline, spartioidine, integerrimine, platyphylline and neoplatyphylline were identified in the secretion. PA N-oxides typical of *Senecio fuchsii* (Asteraceae) were detected in the bodies of the beetles but not in their secretion. No PAs were found in the leaves of the third host plant, *Petasites paradoxus* (Asteraceae). The results suggest the existence of two distinctive storage compartments for PA N-oxides in the beetle: (1) the defensive secretion, containing specifically PA N-oxides ac-

quired from *A. alliariae*; (2) the body of the beetle, sequestering additionally but less selectively PA N-oxides from other sources, e.g. *S. fuchsii* or monocrotaline N-oxide fed in the laboratory. The concentration of PA N-oxides in the defensive secretion is in the range of 0.1 to 0.3 mol/l, which is more than 2.5 orders of magnitude higher than that found in the body of the beetle. No significant differences exist in the ability of the two species of beetles to sequester PA N-oxides from *A. alliariae*, although *O. speciosissima*, but not *O. cacaliae*, produces autogenous cardenolides. A negative correlation seems to exist between the concentrations of plant-derived PA N-oxides and *de novo* synthesized cardenolides in the defensive secretion of *O. speciosissima*.

Key words

pyrrolizidine alkaloid N-oxides, alkaloid sequestration, defensive secretion, host plant, Coleoptera, Chrysomelidae, *Oreina*, Asteraceae, *Adenostyles alliariae*, *Senecio fuchsii*, *Petasites paradoxus*

Introduction

Pyrrolizidine alkaloids (PAs) are regarded as protective chemicals for plants which produce them, particularly against insect herbivores (Boppré 1986; Schneider 1987). Additionally, many of the insects specialized on PA-containing plants are able to sequester PAs for their own benefit. They are often aposematically coloured and are known to be avoided by potential predators. Well known examples are Lepidoptera of the families Arctiidae, Ctenuchiidae, Danainae, and Ithomiinae (Boppré 1990).

Recently, and for the first time, the sequestration of plant PAs in specialized exocrine glands of leaf beetles (Chrysomelidae) was demonstrated in *Oreina cacaliae* (Pasteels *et al.* 1988a). Leaf beetles are in many ways typical herbivores. Many chrysomelids are host plant specialists and pass all or most of their life cycle on their food plants.

Chemical defense is particularly well known in the Chrysomelinae. The defensive toxins are not generally distributed throughout the body but are confined to glands with characteristic morphology and distribution. These occur in the adults of 4 of the 19 chrysomelid subfamilies: in all the Criocerinae and Chrysomelinae, and in some Alticinae and Galerucinae (Deroc & Pasteels 1982; Pasteels *et al.* 1989). The chemical nature of the toxins present in the adult glands is well known only in European species of the tribe Chrysomelini in the subfamily Chrysomelinae. The diversity of defensive chemistry found in the tribe Chrysomelini is unique. Host-derived chemical defense is well known in the larvae of several species of Chrysomelinae (Pasteels *et al.* 1988b, 1990). To date, however, only one case of host plant influence is known: the above mentioned sequestration of PAs (mainly seneciphylline N-oxide) by the adults of *Oreina cacaliae* (Pasteels *et al.* 1988a).

Oreina is an extremely speciose genus of central Europe. The different species are difficult to tell apart without examining the male genitalia. *Oreina* feeds on Apiaceae and Asteraceae, both families containing numerous toxins (e.g. coumarins, sesquiterpene lactones, alkaloids and others) (Hegnauer 1964, 1989). The adults are defended by secretion oozing from dorsal glands. The larvae are not known to be chemically defended but this possibility is not excluded. *O. cacaliae* secretes PA N-oxides and is a specialized herbivore on asteraceous plants known to contain such compounds.

Here we present a detailed qualitative and quantitative analysis of the PAs found in *O. cacaliae* and the related *O. speciosissima* in relation to those found in their host plants. The secretion of *O. speciosissima* was previously only analyzed from populations feeding on *P. paradoxus*. In this case only cardenolides were found in the defensive secretions. Special emphasis is put on the following problems. (1) The ability to take up PAs from the host plant and to transfer them into the defensive secretion. (2) Comparison of *O. cacaliae* and *O. speciosissima* living sympatrically on both *A. alliariae* and *P. paradoxus*. (3) The role of the N-oxide form of PAs and the ability of *Oreina* to N-oxidize PAs. (4) The relationship between *de novo* synthesis of cardiac glycosides and sequestration of host-plant derived PA N-oxides by *O. speciosissima*.

Material and methods

Beetles and plants

Adults of *Oreina cacaliae* and *O. speciosissima* (Chrysomelidae) were collected in May and June 1989 and 1990 and kept at room temperature on their host plants *A. alliariae*, *Senecio fuchsii*, and *Petasites paradoxus* (Asteraceae). *O. cacaliae* originated from Wasserliesen (Vosges, France), Brülisau (Appenzell, Switzerland) and Zastler (Schwarzwald, Germany), *O. speciosissima* from Zastler and from La Lécherette (Vaud, Switzerland).

The defensive secretion from each individual beetle was collected in calibrated capillary glass and the volume of the secretion estimated. Thereafter the secretions were pooled and stored in MeOH. In some instances, whole beetles were stored in MeOH for PA analyses.

Host plant leaves were collected in the same locations as the beetles and lyophilized.

Feeding experiments in the laboratory

Adult beetles collected in the field were first "milked" to remove their defensive secretion and then fed for 8 days on various acceptable plants on which they are never found in nature (*Senecio vulgaris*, *S. vernalis*, *S. jacobaea*, *S. silvaticus*), and which originated from the vicinity of Braunschweig, Germany. Their secretion was subsequently collected a second time.

Alkaloid extraction

Plant material. Powdered, freeze-dried or air-dried leaves of *A. alliariae*, *P. paradoxus* or *S. fuchsii* (0.5 to 1.0 g each) were extracted in 25 ml 0.05 M H₂SO₄ for 3–4 min

(Ultra-turrax) and left to stand for 30 min. After centrifugation half of the supernatant was adjusted to pH 11 with NH₄OH and was extracted by liquid-solid extraction (Hartmann & Toppel 1987) using Chem Elut (Jct., Frankfurt), previously purified by exhaustive refluxing with acetone. The basic solution was applied to a column (1 ml/g Chem Elut) and PAs were eluted with CH₂Cl₂ (6 ml/g Chem Elut). This eluate contains the tertiary PAs. The remaining half of the acidic supernatant was adjusted to 0.25 M H₂SO₄ and mixed with Zn dust in excess. The mixture was stirred for 5 h. The solution was then made basic and was further processed as given above; it constitutes the fraction of total PAs (tertiary PAs + PA N-oxides). After evaporation of the solvent the residues were redissolved in MeOH for GC and GC-MS analyses.

Insect material. Defensive secretions preserved in excess MeOH were evaporated to dryness and the residue was redissolved in 5 ml of 1 M HCl. Aliquots of 2 ml each were treated as above to provide the fractions of tertiary PAs and total PAs (tertiary PAs + PA N-oxides). Beetles which had been preserved in MeOH or kept frozen were ground with acidic MeOH (1% HCl) and quartz sand in a mortar for 10 min. After centrifugation the supernatant was divided into two aliquots and the MeOH evaporated. One aliquot was directly dissolved in dilute NH₄OH, the second aliquot was redissolved in 0.1 M H₂SO₄ and reduced in the presence of Zn dust. Both samples were further treated as above to provide the fractions of tertiary PAs and total PAs.

Alkaloid analysis

PAs were separated and evaluated quantitatively by capillary GC on quartz columns (WCOT, 15 m × 0.25 mm; DB-1, J&W scientific CA) using a Perkin-Elmer, Sigma 2B apparatus (Toppel *et al.* 1987). Conditions: injector, 250°C; temp. progr.: 150–300°C, 6°C/min; split ratio: 1:20; injection vol.: 1–2 µl; carrier gas: He 0.75 bar. Detection: flame ionization and nitrogen detectors. Atropine was used as internal standard. The GC response factors of the major PAs analyzed in these experiments were shown to be almost identical. Retention indices (RIs) were calculated from cochromatographed hydrocarbon standards according to Kovats (1958). In order to separate sparteoidine and platyphylline the DB-1 capillary column was substituted by the more polar DB-5 column in some experiments.

GC-MS: A Carlo Erba Mega 5160 gas chromatograph, equipped with a quartz column (30 m × 0.32 mm) as specified above, was directly coupled to a quadrupole mass spectrometer Finnigan MAT 4515. GC conditions as specified above.

Quantitative analysis of cardenolides

Quantitative analysis of the cardenolide content expressed in ouabain (G-strophantidin) equivalents was performed by HPLC using a reverse-phase column (RP 18, 3 µ, 125 mm-Pharmacia). Conditions: solvents, A = H₂O, B = CH₃CN; gradient from B = 15% to 42% linearly in 35 min; flow: 0.45 ml/min; detection: UV 220 nm. The secretions analysed were obtained from samples of beetles feeding in the laboratory on different host plants under the same conditions as those used for PA analysis.

Results

PAs of the host plants

Adenostyles alliariae. Seven PAs were detected in *A. alliariae* leaves by GC (Table 1; Fig. 1). The identities of the structures were confirmed by their RIs, molecular ions and MS fragmentation patterns in comparison to reference compounds. The retronecine esters seneciophylline (major alkaloid) and senecionine, which are accompanied by small amounts of their E-isomers spartioidine and intergerrimine, respectively, as well as the platynecine (2,3-dihydroretronecine) esters platyphylline (Z-isomer) and neoplatyphylline (E-isomer) are well known PAs from other sources (von Borstel *et al.* 1989). The identity of O-acetyl-seneciophylline was further confirmed by comparison with authentic O-acetyl-seneciophyl-

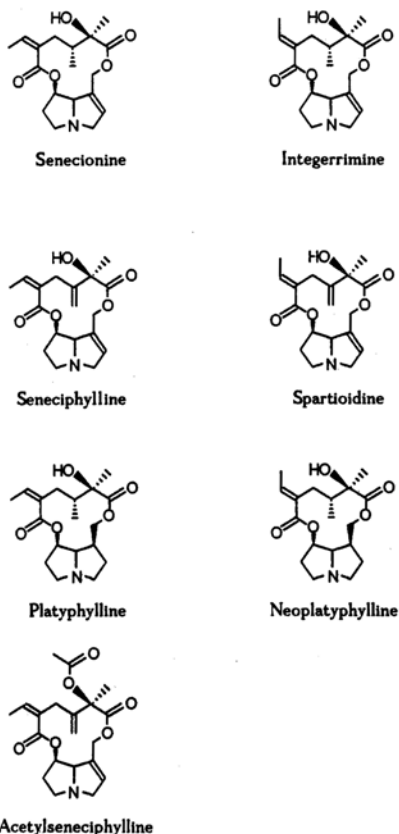


Fig. 1 PAs identified from *A. alliariae*. With the exception of O-acetyl-seneciophylline these alkaloids are also found in the defensive secretions of *O. cacialae* and *O. speciosissima* feeding on *A. alliariae*. In the plant as well as in the defensive secretions the PAs are genuinely present in the form of their N-oxides

Table 1 PA composition and PA content of leaves of *Adenostyles alliariae*

Alkaloids	RI	M ⁺ (m/z)	PA composition (%)	
			Leaves A ^a	Leaves B ^b
Senecionine	2290	335	1	1
Seneciophylline	2303	333	81	78
Spartioidine	2342	333	6	5
Platyphylline	2345	337	10	12
Integerrimine	2350	335	tr	tr
Neoplatyphylline	2400	337	1	1
O-Acetyl-seneciophylline	2460	375	1	3
Total PAs (mg/g dry wt):			15.4	22.3

^a from Zastler (Schwarzwald) June 9, 1989

^b from Wasserliesen (Vosges) May 1, 1989

tr = traces

line obtained by chemical acetylation of seneciophylline. The occurrence of senecionine, platyphylline as well as seneciophylline and its O-acetyl derivative in *A. alliariae* has been previously reported by Schmid *et al.* (1987).

More than 90% of PAs in the alkaloid extracts are present in the form of their N-oxides. The small proportion of tertiary alkaloid may be produced by spontaneous reduction of the N-oxides during sample preparation (Hartmann & Toppel 1987).

The PA patterns established for leaf samples of specimens from different areas are comparable. Seneciophylline was always found to be the major PA, although the concentration of total PAs in leaves varied considerably between different sources.

Petasites paradoxus. Several leaf samples of *P. paradoxus* were analyzed, but not even trace amounts of PAs could be found. Senkirkine, a typical PA reported for the closely related species *P. hybridus* and *P. albus* (Lüthy *et al.* 1983), could never be detected either in the plant or in beetles feeding on it.

Senecio fuchsii. Analysis of *S. fuchsii* leaves collected from *O. cacialae* host plants displayed complex patterns of 13 to 20 PA-N-oxides which are dominated by monoesters of retronecine/platyphylline with angelic/tiglic acid (RI 1787 to 1900) as well as open-chain diesters (RI 2375 to 2440), including isomeric triangularines and sarracines well known from *S. triangularis* (Roitman 1983) and *S. silvaticus* (Röder *et al.* 1986). These patterns are comparable to those described for *S. nemorensis* subsp. *fuchsii* (C. C. Gmelin) Celak. (= *S. fuchsii* C. C. Gmelin) (Schmid *et al.* 1987). PAs typical of *S. nemorensis* subsp. *nemorensis* (including *S. bulgaricus* Velen. and *S. jacquinianus* Reichenb.) (Tutin *et al.* 1976), such as nemorensine, bulgarsenine and retroisosenine (for refs. see Schmid *et al.* 1987) were never detected. Most importantly in the context of this work, there is no overlap between PAs occurring in *S. fuchsii* and PAs identified from *A. alliariae*. Senecionine, previously claimed to occur in *S. fuchsii* (Wiedenfeld & Röder 1979), was absent from all our samples.

PAs in the defensive secretions and bodies of *O. cacaliae* feeding on *A. alliariae*

In preliminary experiments secretions "milked" from *O. cacaliae* populations collected in the field from different food plants (*A. alliariae*, *P. paradoxus* or *S. fuchsii*) as well as from geographically separated populations (French Vosges; Schwarzwald, Germany; Vaud, Switzerland) were analyzed. The amounts of PAs found in the secretions were highly variable and depended on the host plant and the period of collection. No PAs, or only trace amounts of them, were detected in the secretion of *O. cacaliae* found on *P. paradoxus* and *S. fuchsii*, whereas high concentrations of PAs were found in the secretions of *O. cacaliae* feeding on *A. alliariae*. After hibernation and before feeding, there is almost no PA in the secretion. The same is true at the end of summer, when the beetles stop feeding and are rarer in the field. Furthermore, only PAs related to the PA pattern found in *A. alliariae* were detected in the secretions of *O. cacaliae* collected at various different places.

GC-MS analysis of the PAs found in the defensive secretions of an *O. cacaliae* population collected from *A. alliariae* is shown in Table 2. With the exception of O-acetylseneciphylline, all the PAs identified from *A. alliariae* leaves (see Table 1) were detected. Additional GC-signals indicate the presence of compounds that most probably originated from *A. alliariae* PAs by degradation, i.e. by hydrogenation and loss of water (compounds with $M^+ 319$) and hydrolysis of

the less stable allylic O²-ester bond of seneciphylline/spartioidine (retronecine O²-esters with $M^+ 351$). Further evidence for PA hydrolysis is the occurrence of considerable amounts of free retronecine. Since the polar retronecine is not quantitatively recovered by the PA extraction procedure employed, even small amounts of this necine base may indicate vigorous hydrolysis of the respective ester alkaloids.

The amounts of PAs stored in the bodies of previously milked beetles exceed considerably the amount of PAs recovered from the defensive secretions. The PA concentrations in the secretions, however, reach 0.12 to 0.13 mol/l and thus are two orders of magnitude or more higher than those estimated for the bodies (Table 2 "wild type"). The PA pattern is almost identical with the plant PA pattern, again with the exception of O-acetylseneciphylline, which never was found in beetle extracts. Since the PA degradation products found in the defensive secretions are virtually absent from beetle and plant extracts, both artificial formation during sample preparation and formation in the gut of the beetles can be excluded.

To see whether PAs stored in the beetle are translocated into the secretion, beetles collected from *A. alliariae* were "milked" and subsequently allowed to feed either on *A. alliariae* or *P. paradoxus*. Secretions were sampled again after 9 days and again after 16 days, and individually analyzed. The results are summarized in Table 2. Beetles that continued feeding on *A. alliariae* for a further 9 days showed

Table 2 PA patterns found in the defensive secretions (S) and beetle extracts (B; glands emptied) of *O. cacaliae* following feeding on *A. alliariae* and *P. paradoxus*. The beetles were collected in the field from *A. alliariae* (Appenzell/Switzerland; 6/1/1990). Secretions of all beetles were taken on 6/6/90 and were analyzed together with alkaloid extracts of 4 milked beetles ("wild population"). The remaining milked beetles fed separately on *A. alliariae* and *P. paradoxus*, respectively. Secretions and beetles were taken again twice on 6/15/90 ("First milking") and 6/22/90 ("Second milking"). Beetles were killed and stored in 5 ml MeOH, secretions in 400 µl MeOH until PA extraction and GC analysis

Alkaloids	RI	M^+ (m/z)	Alkaloid composition (rel. abundance, %)									
			Wild popu- lation		Adenostyles		First Milking (9 d)		Second Milking (16 d)		Petasites	
			S	B	S	B	S	B	S	B	S	B
Retronecine	1420	155	5	tr	1	-	13	-	-	-	-	-
PA	2233	335	5	-	8	-	-	-	-	-	-	-
PA	2262	319	3	-	7	-	-	-	-	-	-	-
PA	2270	319	3	-	7	-	-	-	-	-	-	-
PA	2285	319	1	-	4	-	6	-	-	-	-	-
Senecionine	2290	335	14	6	13	8	49	8	-	9	-	tr
Seneciphylline	2303	333	45	86	20	86	4	85	tr	84	-	100
Spartioidine	2342	333	6	tr	6	-	-	-	-	-	-	-
Platyphylline	2345	337	9	5	6	4	22	7	-	3	-	-
Integerrimine	2350	335	2	1	2	1	6	7	-	4	-	tr
PA	2358	351	2	-	4	-	-	-	-	-	-	-
Neoplatyphylline	2400	337	<1	<1	<1	-	-	-	-	-	-	-
Retronecine O ² -ester	2407	351	3	-	6	-	-	-	-	-	-	-
Retronecine O ² -ester	2417	351	3	-	7	-	4	-	-	-	-	-
Other PAs			<1	2	9	<1	8	<1	-	<1	-	-
Total PAs* (nmol/S or B)			10.2	49.8	8.7	55.6	0.3	19.8	tr	34.5	-	2.1
PA concentration* (mol · 10 ⁻² /l S)			12		13		0.6		tr		-	
(mol · 10 ⁻⁴ /kg B fresh weight)				6.1		7.5		2.7		4.6		0.3
n			34	4	13	3	9	3	2	3	7	3

* calculated on the basis of a mol weight of 333 (seneciphylline)
tr = traces

PA patterns and concentrations in secretions and bodies comparable to those of the "wild population". Beetles transferred to *P. paradoxus* for 9 days, however, displayed a reduced but still significant PA level in the bodies, whereas virtually no PAs could be detected in the secretions.

Sequestration of PAs in the defensive secretions and bodies of *O. cacialae* feeding on *S. fuchsii*

Only trace amounts of PAs were found in defensive secretions of *O. cacialae* feeding on *S. fuchsii*, and senecionine and platyphylline were the only PAs detectable (Table 3). Considerable amounts of PAs were, however, found in the bodies of the same beetles. A number of PAs which are known to occur in *S. fuchsii* leaves were identified in the PA pattern of the beetles. However, none of these PAs could be detected in the secretion. Senecionine and platyphylline, which are major PAs in the secretion, were not found in extracts of *S. fuchsii*. This result strongly indicates that *O. cacialae* is capable of sequestering *S. fuchsii* PAs in its body but is unable to translocate them via the gland cells into the secretion. It can not be excluded that the beetles previously had access to *A. alliarie*, which was present, but rare, at the field site. This may explain the trace amounts of *Adenostyles* PAs detected in the samples.

PAs in defensive secretions of *O. cacialae* and *O. speciosissima* living sympatrically

Populations of the two beetle species were found living sympatrically on their preferred host plants, *P. paradoxus* (*O. speciosissima*) and *A. alliarie* (*O. cacialae*), respectively. The two host plants grew equally abundantly with intertangled leaves. The defensive secretions of the beetles were collected and analyzed for their PA content and composition (Table 4). *O. cacialae* showed the typical *A. alliarie* pattern with a total PA concentration of 0.23 mol/l. Somewhat unexpectedly *O. speciosissima* was found to sequester PAs too. Although the amounts and concentrations found in the secretions were much lower, all PAs known from *A. alliarie* could be identified, with the exception of O-acetylseneciphylline. Considerable differences, however, were found in the relative abundance of the individual alkaloids, e.g. spartioidine, the geometric isomer of seneciphylline, which is a minor PA in both *A. alliarie* and *O. cacialae*, is the major alkaloid found in *O. speciosissima*.

Sequestration of PAs in the secretions of laboratory-fed beetles

The results of the PA analysis of the secretions collected from the two beetle species fed in the laboratory on their three "natural" host-plants are summarized in Table 5. The total amount of PAs found in the secretions shows clearly that both *O. cacialae* and *O. speciosissima* are able to sequester PAs with the same efficiency in the laboratory, when fed on *A. alliarie*. As in "field" samples, the PAs found in the secretions reflect those found in *A. alliarie*. Again, as observed in field-collected *O. speciosissima* (Table 4), spartioidine rather than seneciphylline is the dominant PA in this species, at least when fed on *A. alliarie*. Feeding the beetles on the other host plants greatly reduced the amount of detectable PAs in the secretions (Table 5). Since not even

Table 3 PA pattern found in defensive secretions (S; n=6) and beetles (B; glands emptied; n=6) collected from *S. fuchsii* (wild habitat; Vosges, May 7, 1990)

Alkaloids	RI	M ⁺ (m/z)	PA composition (%)		
			S	B	S.F. ^a
O ⁷ -Angeloylretronecine	1787	237	-	1	+
O ⁹ -Angeloylretronecine	1798	237	-	1	+
O ⁷ -Angeloylplatynecine	1818	239	-	6	+
O ⁹ -Angeloyl-2-hydroxy-pyrrolizidine ^b	1820	239	-	1	+
O ⁹ -Angeloylplatynecine	1850	239	-	12	+
PA	2045	?	-	1	-
Senecionine	2290	335	50	27	-
Seneciphylline	2303	333	-	tr	-
Platyphylline	2345	337	50	16	-
Integerrimine	2350	335	tr	3	-
Triangularine	2375	335	-	1	+
PA	2437	353	-	27	+
PA	2540	?	-	4	-
Total PAs (nmol/S or B)			<0.5	36.6	

^a + = PA signals which are also present in extracts of *S. fuchsii*

^b tentatively identified by its MS data in comparison to Hirschmann & Jacupovic (1988); tr = traces

Table 4 Composition of PAs found in the defense secretions of *O. cacialae* and *O. speciosissima* living as sympatric population on their host plants *A. alliarie* and *P. paradoxus*

Alkaloids	PA composition (%)	
	<i>O. cacialae</i>	<i>O. speciosissima</i>
Senecionine	25	tr
Seneciphylline	44	18
Spartioidine	9	63
Platyphylline	3	tr
Integerrimine	1	11
Neoplatyphylline	1	4
Other PAs ^a	17	4
Total PAs (nmol/secretion)	18.0	2.1
PA conc. (mol/l secretion)	0.23	0.05

^a degradation products as indicated in Table 2

traces of new PAs could be detected, it seems reasonable to assume that the PAs detected in these beetles are residual *A. alliarie* PAs.

We studied the ability of the two beetle species to sequester PAs in the secretions when fed on different non-host *Senecio* species. Not even traces of the most characteristic PA N-oxides known to occur as N-oxides in the species tested, i.e. *S. silvaticus* (sarracine, triangularine), *S. vernalis* (senecivernine, senkirkine) and *S. jacobaea* (jacobine, erucifoline) (Witte *et al.* 1990) were found in the beetle secretions. Only when fed on *S. vulgaris* does a higher percentage of senecionine N-oxide in comparison to seneciphylline N-oxide indicate accumulation of the former from *S. vulgaris* leaves. The results should be considered with caution, however, since feeding activity was generally reduced and some *Senecio* species seem toxic, e.g. *S. jacobaea*, *S. vernalis* and *S. silvaticus*. The mortality of the beetles on these species was 60%, 30% and 20%, respectively, after 1 week.

Table 5 PA pattern and concentrations found in defense secretions of *O. cacialae* and *O. speciosissima* fed on different host plants. *O. cacialae* were collected in the field from *S. fuchsii*, *O. speciosissima* from *P. paradoxus* (May 22, 1989), glands were emptied and the beetles fed the three food plants for 8 d. Then the secretions were collected and analyzed (n = 14 to 63)

Species Host plant	Alkaloid composition (%)			<i>O. speciosissima</i>		
	<i>O. cacialae</i> A. a.	S. f.	P. p.	A. a.	S. f.	P. p. ^a
Alkaloid						
Senecionine	8	25	39	2	–	3
Seneciophylline	44	38	9	19	–	81
Spartiodine	20	16	3	68	–	16
Platyphylline	3	8	10	tr	–	tr
Integerrimine	5	12	8	–	–	–
Neoplatyphylline	1	–	2	6	–	–
Other PAs ^b	19	tr	29	5	–	–
Total PAS (nmol/secretion)	65.5	0.6	6.9	50.8	–	2.4
PA conc. (mol/l secretion)	0.33	0.01	0.17	0.32	–	0.03

A. a. = *Adenostyles alliariae*; S. f. = *Senecio fuchsii*; P. p. = *Petasites paradoxus*. – = not detectable; tr = traces

^a analysis of defense secretions collected on May 22, 1989

^b degradation products as indicated in Table 2

Table 6 Sequestration of monocrotaline (MC) and monocrotaline N-oxide (MC N-ox) in defense secretions (S) and beetles (B; glands emptied) of *O. speciosissima* and *O. cacialae*. Beetles fed on leaf discs (*A. alliariae*) treated with 1 μ mol MC N-ox and MC, respectively. New leaf discs with the same amount of PA were offered every day. MC was offered for 5 days, MC N-ox for 4 days

Alkaloid fed	PA composition (%)		<i>O. cacialae</i>		MC	
	<i>O. speciosissima</i> MC N-ox	S	MC N-ox	B	S	B
Total alkaloids						
Monocrotaline	–	35	tr	34	tr	4
Senecionine	22	8	28	11	18	9
Seneciophylline	72	53	46	51	52	78
Spartiodine/Platyphylline	6	4	12	3	11	4
Integerrimine	–	tr	tr	–	–	–
Other PAs	<1	<1	1	1	19	5
Total PAs (nmol/S or B)	11	183	7	290	15	80
n	6	6	13	14	9	9

Ingestion of monocrotaline and its N-oxide

To see whether a "foreign" PA would be sequestered by the beetles when offered with the "natural" host plant, monocrotaline and its N-oxide were applied on *A. alliariae* leaf-discs. The results are shown in Table 6. Only traces of monocrotaline N-oxide or none at all could be detected in the secretions of either species, whereas considerable amounts were found in beetle extracts, i.e. 34% (*O. cacialae*) and 35% (*O. speciosissima*) of total PAs, when the alkaloid was applied in its N-oxide form. The trace amounts of monocrotaline N-oxide recovered from the secretions may be due to surface contamination of the beetles by the alkaloid N-oxide. PAs found in the beetle are predominantly (if not exclusively) present as N-oxides (> 80–90% of total PAs). The monocrotaline N-oxide found in the bodies of *O. cacialae* and *O. speciosissima* accounts for 14% and 9% respectively of the total monocrotaline N-oxide consumed by the beetles. Accumulation is greatly reduced if the alkaloid is offered in its tertiary form (Table 6, *O. cacialae*). In this case only 60% of total monocrotaline recovered from the beetles was present in its N-oxide form.

Free bases and N-oxides of monocrotaline and senecionine were offered at concentrations of 40 μ mol/leafdisc (diam. 16 mm) and no feeding deterrence was observed with either beetle species.

Quantitative analysis of cardiac glycosides in the secretion of *O. speciosissima* fed on different plants

The results of a quantitative analysis of cardenolides in the secretion of *O. speciosissima* are summarized in Table 7. The secretion usually contains more cardenolides

Table 7 Cardenolides and PAs found in the secretions of *O. speciosissima* fed on different host plants

Host plant	n	Cardenolides		PAs	
		μ g/s	mol/l	μ g/s	mol/l
<i>S. fuchsii</i>	15	2.6	0.099	0	0
<i>P. paradoxus</i>	8	5.7	0.111	0.4	0.02
<i>A. alliariae</i>	14	3.0	0.034	3.2	0.30
<i>P. paradoxus</i> / <i>A. alliariae</i>	9	5.2	0.114	0.7	0.05

than PAs, except when fed on *A. alliariae*. The lowest concentration of cardenolides in the secretion was found in beetles fed with *A. alliariae* and sequestering the highest amount of PAs. In this case the cardenolides are 3 times less concentrated than in the secretion of the beetles fed on food plants which do not supply PAs for the secretion, i.e. *S. fuchsii* and *P. paradoxus*.

Discussion

The PA patterns found in the *Oreina* species establish the existence of two separate compartments for PA storage, the body of the beetle and the defensive secretion. These two compartments are clearly distinguished by their specificity for particular PAs. The storage compartment of the body is less compound-specific, as shown by the large quantities of *S. fuchsii* PAs (Table 3) and monocrotaline N-oxide (Table 6) found in bodies of the beetles that had access to these compounds. The defensive secretion, in contrast, contained exclusively those PAs found in *A. alliariae*. Despite the relatively high concentrations of typical *S. fuchsii* PAs or monocrotaline N-oxide sequestered in the beetle, none of these PAs could be identified in the defensive secretion.

It should be recalled that in order to reach the defensive secretion plant-derived PAs have to pass at least two cellular barriers, the gut epithelium and the secretory gland cells. Obviously the resorption of PAs from the gut is less selective than their transfer into the gland cells. It must be emphasized that in both, the food plant and the insect, PAs are present as N-oxides. It is well established for PA-containing plants that the N-oxides are the specific alkaloid forms for long-distance translocation, cellular transport and vacuolar storage (Hartmann *et al.* 1989). Other PA-sequestering insects, such as the arctiids *Tyria jacobaeae* (Ehmke *et al.* 1990) and *Creatonotos transiens* (von Nickisch-Rosenegk *et al.* 1990; Hartmann *et al.* 1990), not only sequester PA N-oxides but are also able to N-oxidize the respective tertiary PAs. By contrast, the ability of *O. calacaliae* to N-oxidize tertiary PAs seems to be less pronounced, as indicated by the low percentage of monocrotaline sequestration if the alkaloid is offered in its tertiary form (see Table 6). Furthermore, only traces of ^{14}C -labelled senecionine N-oxide were recovered from the defensive secretions of *O. calacaliae* that had ingested [^{14}C] senecionine (Ehmke *et al.* 1991). This again emphasizes the physiological importance of the N-oxide form to both plants and insects. The polar salt-like PA N-oxides are not able to diffuse passively through cell-membranes unless a specific membrane carrier is present. A carrier system specific for PA N-oxides has been characterized in cells of *Senecio vulgaris* (Ehmke *et al.* 1988) and preliminary evidence for a carrier-mediated uptake of PA N-oxides from the midgut has been demonstrated for *Creatonotos transiens* (Wink & Schneider 1988).

A. alliariae seems to be the exclusive source of the PA N-oxides found in the defensive secretions of *O. calacaliae* and *O. speciosissima*. The beetles accumulate PA N-oxides in their defensive secretions at total concentrations as high as 0.1 to 0.3 mol/l. This indicates the specific ability of the beetle to concentrate PA N-oxides acquired from the host plant in the secretion. For comparison, the PA N-oxide concentrations of wild-caught larvae or pupae of *Tyria jacobaeae* range from 0.003 to 0.017 mol/kg fresh weight (Ehmke *et al.* 1990). In a typical PA-containing plant, such as *S. vulgaris*, the tis-

sue concentrations may reach levels of ca 0.01 mol/kg in the tubular florets and up to 0.02 mol/kg in the achenes (Hartmann & Zimmer 1986).

The PA pattern of the secretion reflects the plant pattern. O-Acetylseneciphylline is the only *A. alliariae* PA which was never found in the secretion. This is comparable to the situation in *Tyria jacobaeae* (Ehmke *et al.* 1990). Feeding experiments with radioactively labelled O-acetylseneciphylline revealed that *Tyria* hydrolyzes the ester PA in the gut and sequesters only seneciphylline (A. Biller, A. Ehmke, L. Witte, T. Hartmann, in prep.). Presumably the same is true for *Oreina*. On the other hand, degradation products of *Adenostyles* PAs were always detectable in secretions (Table 2). Since these degradation products were never found either in the body of the beetle or in the food plant, we assume that they are formed spontaneously in the secretion fluid. Furthermore, feeding experiments with [^{14}C] senecionine N-oxide revealed that the alkaloid is released into the secretion as a virtually pure compound (Ehmke *et al.* 1991). We assume that the secretion fluid itself is not optimized to keep the acquired PA N-oxides in a chemically stable state.

The puzzling observation that spartioidine N-oxide and not seneciphylline N-oxide was frequently found to be the major alkaloid (Table 4 and 5) in secretions collected from *O. speciosissima* cannot as yet be explained. An artificial Z/E-isomerization cannot be excluded.

It is interesting to note that, in the laboratory, both *O. calacaliae* and *O. speciosissima* will feed on various plants containing PAs. PAs which are not sequestered do not have a deterrent effect. Even monocrotaline and its N-oxide does not affect the quantity eaten by the beetles. This contrasts strongly with the deterrent effect of monocrotaline or senecionine N-oxide on *O. bifrons* (a non-sequestering, but closely related species). Only one of these beetles tested (respectively $n=10$; $n=5$) accepted leaves of their normal host (*Chaerophyllum hirsutum*; family Apiaceae) when these were painted with PAs (Ehmke *et al.* 1991). The same individual beetles fed amply thereafter when offered normal leaves.

In nature *O. calacaliae* is often found feeding on *A. alliariae*, and is therefore well defended. It is sometimes found on *P. paradoxus*, but only in early Spring. Indeed, *Petasites* leaves appear in the field before those of the other acceptable hosts (*Adenostyles* and *Senecio*). On *Petasites* the beetles are not protected by PAs, but they benefit from an earlier start. The situation of the populations of *O. calacaliae*, frequently found on *S. fuchsii* during the whole season, is puzzling, since this host plant does not allow the beetles to exploit fully their potential for sequestration of PAs as chemical defence. PAs typical of *S. fuchsii* are sequestered in the body but not in the secretion. High concentrations of PAs in the secretion rather than in the body might enable the beetle to survive predator attack better. Indeed, the deterrent secretion is immediately perceived by the attacker. The beetles on *S. fuchsii* might also be part of an automimicry complex, with those feeding *A. alliariae* acting as models and thus gain protection from predators in this way.

The leaves of *S. fuchsii* have a much higher nitrogen content than those of *Adenostyles* (for equivalent water and carbon content) and might therefore be nutritively

advantageous (B. Speiser, pers. comm.). Indeed, the relative growth rate of *O. calaciae* larvae is higher on *Senecio* than on *Adenostyles*.

In nature, *O. speciosissima* is found on *P. paradoxus* or in mixed stands of *P. paradoxus* and *A. alliariae*. Like *O. calaciae*, *O. speciosissima* is well adapted to sequester PA N-oxides, but it does not utilize this capacity in the field, which means that this species probably stays mostly on *Petasites* as a host plant even when *Adenostyles* is available. In laboratory choice experiments the adults prefer *A. alliariae*, but the larval relative growth rate is significantly higher on *P. paradoxus* than on *S. fuchsii* or on *A. alliariae*, where it is minimal. It remains open whether these beetles are still efficiently defended by cardenolides. The quantities and/or concentrations of cardenolides found in the secretions of *O. speciosissima* (i.e. 2.6–5.7 µg/secretion; 0.05–0.11 mol/l) are not very much lower than those found in species that are specifically defended by cardenolides: *O. gloriosa* (13.3–30 µg; 0.27 mol/l), *C. coerulans* (27.5 µg; 0.21 mol/l), *C. herbacea* (138 µg; 0.31 mol/l) (Pasteels *et al.* 1979; van Ooycke *et al.* 1988 and unpubl.). A dose of 75 µg of cardenolides sequestered by the monarch can be emetic for the blue jay although the emetic dose varies widely with the nature of the cardenolides (Brower & Fink 1985). It is possible that smaller doses are emetic in the beetles since cardenolides in the secretion can be easily absorbed because they are not bound in the tissue. On the other hand the concentration rather than the quantity of the cardenolides in the different species, including those of *O. speciosissima* on its natural host *P. paradoxus*, are similar (see above). At these concentrations the beetles must be very bitter to vertebrate predators. The synthesis of cardenolides seems to be negatively influenced by the sequestration of PAs (see Table 7). Since PAs are also very bitter, both defense chemicals, cardenolides and PAs, may participate additively in repellency.

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