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SELECTIVE SEQUESTRATION AND METABOLISM OF PLANT DERIVED PYRROLIZIDINE ALKALOIDS BY CHRYSOMELID LEAF BEETLES

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Key Word Index—*Oreina*; Chrysomelidae; leaf beetle; *Adenostyles*; *Senecio*; Asteraceae; pyrrolizidine alkaloid *N*-oxides; alkaloid sequestration; alkaloid functions.

Abstract—Pyrrolizidine alkaloids (PAs) are assumed to function as plant defence compounds against herbivory. A number of adapted insects are known to sequester plant derived PAs for their own benefit. Here we summarize the chemical interactions between leaf beetles of the genus *Oreina* (Coleoptera, Chrysomelidae) and their host plants *Adenostyles* spp., *Senecio nemorensis*, and *S. fuchsii* (Asteraceae, tribe Senecioneae). Seneciphylline *N*-oxide and senecionine *N*-oxide, the main PAs of *Adenostyles*, are sequestered in the bodies and exocrine defensive glands of the leaf beetles. The comparison with the PA patterns of the *Senecio* host plant indicates a selective PA uptake. The uptake into the body (hemolymph) is less specific, whereas the translocation into the defensive glands is highly specific. Only the *N*-oxides of macrocyclic retronecine esters of the senecionine type are found in significant amounts in the defensive secretions. Many other PAs such as monoesters and open-chain diesters as well as PAs of other structural types (e.g. monocrotaline *N*-oxide and senkirkine) are not transferred into the defensive glands. Leaf beetles sequester PAs exclusively as *N*-oxides. A novel PA not found in the food plants was detected in the defensive secretions of *Oreina elongata*; it was identified as 13,19-expoxisenecionine *N*-oxide (oreine), the epoxidation product of seneciphylline *N*-oxide. Besides this transformation, leaf beetles are able to catalyse further transformations such as the *O*-dealkylation of heliotrine *N*-oxide to rinderine *N*-oxide and the *O*-deacetylation of acetylseneciphylline *N*-oxide to seneciphylline *N*-oxide. The plant–beetle interactions are discussed in the functional context of PAs as powerful plant defensive chemicals.

INTRODUCTION

The pyrrolizidine alkaloids (PAs) represent a diverse class of *ca* 360 structures with restricted occurrence in certain higher plant taxa such as the genera *Senecio* (Asteraceae, tribe Senecioneae), *Eupatorium* (Asteraceae tribe Eupatorieae), *Heliotropium* (Boraginaceae), *Crotalaria* (Fabaceae) as well as solitary occurrences in several unrelated taxa (e.g. Orchidaceae, Ranunculaceae, Apocynaceae, Convolvulaceae) [1–3]. PAs are assumed to be powerful plant defence compounds. A number of insects from diverse taxa, e.g. Lepidoptera, Orthopthera, Homoptera and Coleoptera, have evolved adaptations to sequester and utilize PAs for their own benefit [3–6]. In the past, particularly the adaptations of various lepidopterans to PA-containing plants have been studied in detail [3–8].

More recently leaf beetles of the genus *Oreina* (Chrysomelinae) turned out to be another promising model system to study mechanistic and evolutionary aspects of sequestration of PAs for insect defence [9, 10]. Chemical defence is prominent in Chrysomelinae. The defensive compounds are released from specialized exocrine glands located in the elytra and pronotum [11]. Most species produce autogenously defence compounds such as cardenolides. The first example of host-derived defence in adult Chrysomelinae was documented for *Oreina cacaliae*. The adult leaf beetle sequesters in its defensive secretion seneciphylline *N*-oxide, a PA derived from its host plant *Adenostyles alliariae* (Asteraceae) [12]. Subsequent studies were mainly focused on the chemistry

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and biochemistry of PA sequestration [13, 14] and mechanistic and evolutionary aspects concerning autogenous and host-derived chemical defences in chrysomelid leaf beetles [15, 16].

In this paper we present some chemical and biochemical aspects of the relationships between *Oreina* leaf beetles and their host-plants, i.e. PA patterns of host-plants and beetles, structural discrimination in sequestration, transformation of plant-derived PAs by the beetles and the role of the *N*-oxide form in sequestration.

RESULTS

PA pattern in sequestrating leaf beetles and their host plants

Five PA-containing host plant species for *Oreina* leaf beetles have been identified so far. The respective PA patterns of the plant organs consumed by the beetles (i.e. leaves) were analysed by capillary GC and identified by GC-mass spectrometry (Table 1). In most cases unequivocal structure identification was possible on the basis of the respective R_i-values, the $[M]^+$ and characteristic mass spectrometry fragmentation patterns in comparison to authentic samples and reference data [17]. The PA patterns of the three *Adenostyles* species are very similar, and in accordance with earlier reports [13, 18, 19]. Seneciphylline (**18**) is always the dominant alkaloid accounting for >85% of total PAs. The two *Senecio* species, *S. fuchsii* C.C.Gmelin and *S. nemorensis* L., which are sometimes classified as subspecies (i.e. *S. nemorensis* ssp. *fuchsii* (C.C.Gmelin) Celak and *S. nemorensis* ssp. *jaquinianus* (Reichenbach) Celak, respectively) [20], can easily be distinguished by their completely different PA pattern. *S. fuchsii* contains monoesters and diesters of the triangularine type according to the classification given in ref. [3], with platynecine diesters (i.e. **11** and isomers) and retronecine diesters (i.e. **6** and isomers) as major PAs. The isomers of **6** and **11** were separated and identified by their GC data, as recently proposed [21]. On the contrary, *S. nemorensis* contains macrocylic PAs of the senecionine type [3]

Table 1. PA composition of the host plants of alkaloid sequestrating leaf beetles

			Relative abundance (%)				
Alkaloid*	R_i	m/z $[M^+]$	*S.f.*	*S. n.*	*A. a.*	*A. l.*	*A. g.*
1 7-Angeloylretronecine	1787	237	tr	–	–	–	–
2 9-Angeloylretronecine	1797	237	+	–	–	–	–
3 7-Angeloylplatynecine	1818	239	++	–	–	–	–
4 9-Angeloylplatynecine	1850	239	+++	–	–	–	–
5 7-Angeloyl-9-acetylplatynecine	1902	281	+	–	–	–	–
6 Triangularine	2375	335	+	–	–	–	–
7 Neotriangularine	2390	335	(+++)[1]	–	–	–	–
8 Triangularicine	2394	335	(+++)[1]	–	–	–	–
9 Sarracine	2398	337	(+++)[1]	–	–	–	–
10 Neotriangularicine	2406	335	(+++)[2]	–	–	–	–
11 Sarranicine	2410	337	(+++)[2]	–	–	–	–
12 Neosarracine	2418	337	+	–	–	–	–
13 Retroisosenine	2285	335	–	tr	–	–	–
14 Nemorensine	2267	337	–	tr	–	–	–
Nemorensine isomer	2320	337	–	1	–	–	–
15 Doronenine	2780	459	–	tr	–	–	–
16 Bulgarsenine	2350	337	–	92	–	–	–
17 Senecionine	2294	335	–	–	3	tr	tr
18 Seneciphylline	2303	333	–	–	87	97	95
19 Spartioidine	2343	333	–	–	(4)[3]	(5)[3]	(4)[3]
20 Platyphylline	2345	337	–	–	(4)[3]	(5)[3]	(4)[3]
21 Integerrimine	2350	335	–	–	tr	–	–
22 Neoplatypylline	2373	337	–	7	tr	–	–
23 Ac-seneciphylline	2460	375	–	–	6	–	tr
Total PAs (mg g dry wt^{-1})			nd	8	66	23	19

* PAs are present in the form of their *N*-oxides; see Fig. 1 for structures.

[1,2,3] The respective structures were identified by individual quantification was not possible due to their close R_is. *S. f.*, *Senecio fuchsii*; *S. n.*, *Senecio nemorensis*; *A. a.*, *Adenostyles alliariae*; *A. l.*, *A. leucophylla*; *A. g.*, *A. glabra*. In case of *S. fuchsii* quantitative integration of the signals was not possible; the number of + + indicate the relative abundance; tr, trace amounts.

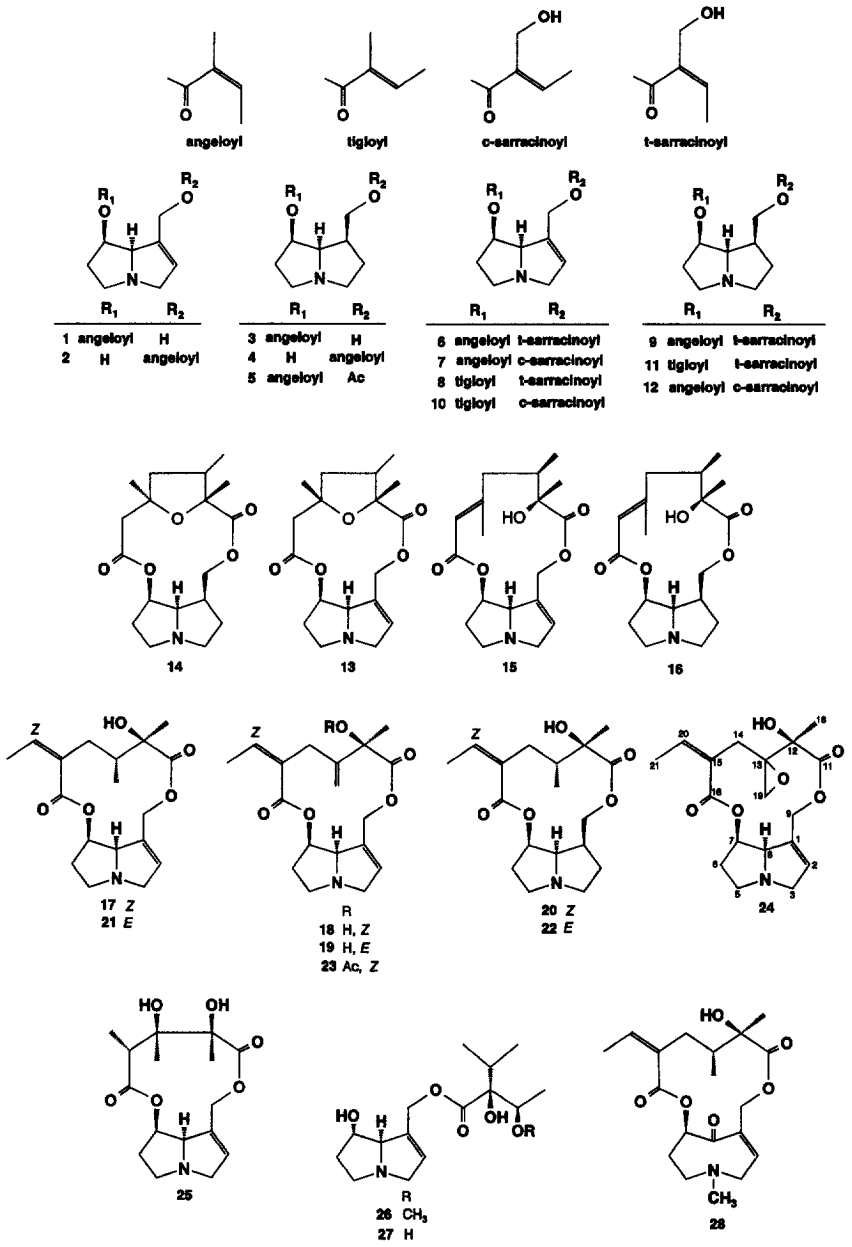

Fig. 1. Structures of pyrrolizidine alkaloids (PAs) mentioned. All structures are illustrated as tertiary bases.

belonging to the rather rare nemorensine group [3], with **16** as the major alkaloid. In leaf samples collected from the beetles' food-plants, we never observed the co-occurrence of macrocyclic PAs and diesters of the triangularine type in either species as reported elsewhere [22–25].

The PA patterns of adults of the four *Oreina* species, feeding on their respective PA-containing food plants, were established for the collected defensive secretions and total extracts of the remaining bodies. The results (Table 2) can be summarized as follows: (I) Defensive secretions of *O. cacaliae*, which previously had fed on *S. fuchsii*, are devoid of even traces of host-plant PAs (i.e. open chain monoesters or diesters), although these PAs are sequestered in the bodies of the beetle. Trace amounts of **17** and **20** which are detectable in the secretions indicate that the beetles in their past (possibly before hibernation) had fed on *A. alliariae*; residual *A. alliariae* PAs (i.e. **17**, **18**, **20**, **21**) can also be traced in the body extracts of the beetles. (II) Defensive secretions of *O. cacaliae* feeding on *S. nemorensis* contain only traces of the macrocyclic platynecine diester **16**, the major host plant PA. Instead **15** the respective retronecine diester, which is a trace component in the host-plant, has been sequestered as major PA; again the complete PA pattern of the host plant was detected in the body extracts. (III) *O. cacaliae* feeding on *A. alliariae* sequesters in its defensive secretions and body preferentially **18** the major PA of its host-plant, but also considerable amounts of the

Table 2. PA composition (relative abundance, %) in defensive secretions (S) and body (B) of leaf beetles sequestering alkaloids from their respective host-plants

Host-plant:	*S. fuchs.*		*S. nemor.*		*A. alliar.*		*A. alliariea/S. nemor.*				*A. leuco.*	
Leaf-feeding beetle:			*O. cacaliae*				*O. speci.*		*O. intric.*		*O. elong.*	
Alkaloid*	S	B	S	B	S	B	S	B	S	B	S	B
1 7-Angeloylretronecine	–	1	–	–	–	–	–	–	–	–	–	–
2 9-Angeloylretronecine	–	1	–	–	–	–	–	–	–	–	–	–
3 7-Angeloylplatynecine	–	6	–	–	–	–	–	–	–	–	–	–
4 9-Angeloylplatynecine	–	12	–	–	–	–	–	–	–	–	–	–
6 Triangularine	–	3	–	–	–	–	–	–	–	–	–	–
13 Retroisosenine	–	–	–	tr	tr	–	–	–	–	–	–	–
15 Doronenine	–	–	68	62	tr	tr	41	99	16	44	–	–
16 Bulgarsenine	–	–	tr	16	tr	–	tr	tr	–	tr	–	–
17 Senecionine	50	27	tr	tr	21	11	–	–	–	tr	56	11
18 Seneciphylline	–	tr	32	23	79	89	59	tr	84	56	15	41
19 Spartioidine	–	–	–	tr	tr	tr	tr	tr	tr	tr	tr	2
20 Platyphylline	50	16	–	tr	tr	tr	tr	tr	tr	tr	–	–
21 Integerrimine	tr	3	–	–	–	–	–	–	–	–	–	–
22 Neoplatyphylline	–	–	–	tr	–	–	–	–	–	–	–	–
24 Oreine	–	–	–	–	–	–	–	–	–	–	29	46
Total PAs (μg/S or B)	<0.2	12	4.5	74	4.2	102	8.3	25.3	7.4	25	7.0	247.3

* PAs are present in the form of their *N*-oxides; see Fig. 1 for structures.

minor component **17**. (IV) *O. speciosissima* feeds mostly on *Petasites albus* which in its leaves is devoid of PAs, but in mixed populations with *A. alliariae* and *S. nemorensis* they are frequently also found on the two PA plants. The PAs found in the defensive secretions and the bodies (i.e. **18** and **15**) indicate that the individual must have had a mixed diet of the two species. The same result was found with *O. intricata*, a species feeding on both *A. alliariae* and *S. nemorensis*. (V) *O. elongata* a beetle feeding either on *Cirsium spinosissimum*, an Asteraceae species devoid of PAs, or on *Adenostyles* spp. shows the typical PAs of the latter species in its defensive secretions and body. In addition, considerable amounts of a hitherto unknown PA *N*-oxide, named oreine, was detected in defensive secretions and beetle extracts (identification see below).

The total amount of PAs found in the residual bodies of adult beetles is always higher than that found in the respective defensive secretion (Table 2). It is comparable to total PAs sequestered by *Oreina* larvae feeding on the same host-plants (Table 3) and also comparable to the quantity reported for fourth instar larvae of *O. cacaliae* (i.e. 61 μg) [26]. *Oreina* larvae do not possess defensive glands. Comparison of the PA concentrations again shows comparable values for adult and larval bodies, but very high values for the fluid defensive secretion, i.e. about 40- to 75-fold higher levels in the examples given in Table 3 which correspond to PA concentrations of 0.17 mol l^{-1} and 0.21 mol l^{-1}, respectively. In additional experiments we directly measured the PA concentrations in isolated hemolymph and defensive secretations of two species (data not shown); the concentrations in the secretions were *ca* 65- to 180-fold higher than in the hemolymph.

Oreine a transformation product of seneciphylline N-*oxide in* O. elongata

The new PA oreine (**24**) was only detected in *O. elongata* adults feeding on their host plant *Adenostyles* (Table 2) which does not contain even trace-amounts of this alkaloid. The compound (R_i 2475; *m/z* $[M]^+$ 349) has a MS fragmentation pattern which closely resembles the 12,13-epoxy-PA erucifoline (R_i 2510; *m/z* $[M]^+$ 349). This led to the suggestion that **24** might be 13,19-epoxysenecionine the transformation product of **18** the major PA sequestered from the food plant (Table 2). Since there was no chance to isolate sufficient substance from the beetles for detailed structural studies, we synthesized 13,19-epoxysenecionine from **18** according to ref. [27]. The major product of synthesis was identical with **24** in respect to its GC and GC-mass spectrometry data. The structure of the synthetic product was further proved by ^1H and ^{13}C NMR (see Experimental). the ^1H NMR data closely resemble those given for 13,19-epoxysenecionine which previously was isolated from two *Senecio* species (i.e. *S. megaphyllus* from Costa Rica and *S. discolor* from Peru) [28]. Thus, there is convincing evidence for the structural identity of **24** with 13,19-epoxysenecionine, which might be formed by the beetle as a transformation product during sequestration. It should be mentioned that **24** was only detected in *O. elongata* ssp. *occidentalis* Ruffo and not in *O. elongata* ssp. *ruffoi* Kranz; other subspecies still await examination.

Table 3. Amounts and concentrations of major PAs in larvae (fourth instar) and adults (defensive secretions and bodies) of *Oreina cacaliae* populations on two different host-plants

Host-plant:	*Adenostyles alliariae*			*Senecio nemorensis/A. alliariae*[a]			
Alkaloids*	**17**	**18**	Total	**15**	**16**	**18**	Total
Larvae ($n = 19/22$)[b]							
μg/individual	4	65	69	50	27	—	77
mg/g fr. wt	0.10	1.63	1.73	1.28	0.69	—	1.97
Adults ($n = 9/9$)[b]							
I. Secretions							
μ/individual	0.9	3.3	4.2	3	—	1.5	4.4
mg/ml	12	45	57	48	—	23	71
II. Body							
μg/individual	8	123	131	42	8	25	75
mg/g fr. wt	0.09	1.36	1.44	0.52	0.01	0.31	0.93

* PAs are present in the form of the *N*-oxides.

[a] Larvae fed exclusively on *S. nemorensis*; adults moved apparently from *S. nemorensis* to *A. alliariae*.

[b] The first number refers to the population on *A. alliariae*, the second number to the population on *S. nemorensis*.

Sequestration and transformation of foreign PAs

Comparison of the PAs patterns in the defensive secretions of *Oreina* beetles with those of their respective host-plants indicates a rather unspecific uptake of host-plant PAs into the hemolymph of *Oreina* larvae and adults, but a highly selective sequestration of certain macrocyclic PAs into the defensive secretions (Tables 2 and 3). This behaviour was further tested by feeding selected PAs or PA mixtures to *O. cacaliae* adults and larvae. Acetylseneciphylline (**23**) which occurs in the host-plant was never found in beetle extracts. Feeding of a mixture of radioactively labelled **18** and **23** to adults clearly show that the acetylated derivative is not sequestered at all (Table 4). It occurs in the frass but not in beetle extracts or the defensive secretion. When pure labelled **23** was fed to the beetles, considerable amounts of **18** were recovered from beetle extracts (results not shown). This clearly proves that some deacylation must occur in the guts, which is also indicated by the change of the ratio between **18** and **23** in the frass extracts (Table 4). In further experiments three PAs which do not occur in the beetle's host plants were administered orally to the beetles. The result is summarized in Table 5. Monocrotaline (**25**) *N*-oxide an 11-membered macrocyclic alkaloid is easily taken up by larvae and adults and stored in the bodies; however, no transfer into the beetle's defensive glands was observed. Heliotrine (**26**) *N*-oxide a monoester PA of the lycopsamine type was also taken up by larvae. In addition to the applied alkaloid, considerable amounts of its demethylation product rinderine (**27**) *N*-oxide could be recovered from the larvae (Table 5). Finally, senkirkine (**28**) the otonecine analogue of **17**, which does not form an *N*-oxide, was not at all sequestered in the beetle's body.

Table 4. Uptake of a mixture of ^{14}C-labelled seneciphylline *N*-oxide (**18** *N*-ox) and acetylseneciphylline *N*-oxide (**23** *N*-ox) orally fed to adults of *O. cacaliae* (representative analysis out of eight replications)

	Rel. abundance (%)	
MeOH extracts	**18** *N*-ox	**23** *N*-ox
^{14}C-labelled PA mixture*	43	57
Defensive secretion (5 day)	100	—
Beetle's body (5 day)	100	—
Frass (24 hr)	66	34
Frass (48 hr)	67	33

* Beetles were allowed to ingest the labelled mixture (*ca* 10^5 cpm) for 24 hr with there food-leaves (*A. alliariae*) and were then transferred to untreated leaves and were analysed at the times indicated.

DISCUSSION

The importance of secondary metabolites in plant defence is still a matter of controversy [29, 30]. Sometimes the most convincing arguments in favour of a defensive function are provided by adapted herbivores (e.g. specialized insects) which actively sequester and use these compounds for their own benefit. In this respect the *Oreina* species adapted to PA-containing plants are an impressive example. The four species known to sequester plant PAs (Table 2) are all found to feed on species of the alpine genus *Adenostyles*, preferentially *A. alliariae*, although *Petasites albus* and *Cirsium spinosissimum* are the preferred food plant of *O. speciosissima* and certain populations of *O. elongata*, respectively, and *S. nemorensis* is the preferred food plant of *O. intricata* [16]. PA sequestration by adults can be described as a two-step mechanism. The first step is absorption of the ingested PAs from the guts into the hemolymph and the second step is the transfer into the highly specialized defensive glands [13–16]. The uptake into the hemolymph as

Table 5. Uptake sequestration and metabolism of foreign PAs by larvae or adults of *O. cacaliae*. The mixtures of foreign PAs were offered painted on *A. alliariae* leaves

Alkaloid fed orally	Alkaloid in B (extract) or S (secretion)	PA conc. μ/g fr.wt	% of total PAs
Exp. I*	Larvae (fourth instar)		
25 Monocrotaline *N*-oxide	**25** *N*-oxide	458	33
26 Heliotrine *N*-oxide	**26** *N*-oxides	198	14
	27 Rinderine *N*-oxide	213	16
Exp. II**	Adults		
25 Monocrotaline *N*-oxide	B: **25** *N*-oxide	428	12
	S: **25** *N*-oxide	tr	
Exp. III***	Adults		
28 Senkirkine	B: Senkirkine	tr	
	S: Senkirkine	n.d.	

* Exp. I: leaf discs treated with **25** *N*-oxide + **26** *N*-oxide (0.25 mg each) were offered to five larvae for 2 days; new discs with the same amount were offered every day; subsequently larvae were kept on untreated diet for another two days until extraction.

** Exp. II: leaf discs treated with 0.3 mg **25** *N*-oxide were offered to six individuals for 4 days; new discs with the same amount were offered every day.

*** Exp. III: leaf discs treated with 0.16 mg **28** were offered to 10 beetles for 4 days; new discs with the same amount were offered every day.

tr, trace amounts; n.d. not detectable.

also shown in this study is rather unspecific, even foreign PAs such as monocrotaline *N*-oxide and heliotrine *N*-oxide are taken up. In this respect larvae and adults behave similarly (Table 3) although the sequestration in larvae is somewhat transient. In larvae, but not adults [15], PAs are continuously lost with time but this loss seems to be compensated by the continuous food intake (A. Ehmke, M. Rowell-Rahier, J. M. Pasteels, T. Hartmann, unpubl. results).

The transfer of PAs from the hemolymph into the gland cells of adults seems to be restricted to a few macrocyclic retronecine esters of the senecionine type, i.e. **18** and **17** the major PAs of *Adenostyles* spp. and **15** as the only *Senecio nemorensis* PA found in significant amounts in the defensive glands of *Oreina* species feeding on this plant (see Table 2). The respective 1,2-saturated derivatives, i.e. the platynecine esters **20** or **16** are at best detectable in traces in the defensive secretions. However, the uptake of a PA into the gland cells is not only specified by the necine base moiety, but also by the structural type of the necic acid. Other retronecine derivatives such as O^9- or O^7-mono esters, open-chain O^9-, O^7-diesters or the 11-membered macrocyclic **25** are taken up into the hemolymph, but are not sequestered in the defensive secretions. PA sequestrating *Oreina* spp. seem to be well adapted to the PA patterns of their main host-plant, i.e. the genus *Adenostyles*, but not to the PA pattern of *S. nemorensis*.

The PAs sequestered in the defensive secretions of *Oreina* are exclusively present as *N*-oxides [12–14]. In this respect leaf beetles behave like PA sequestering lepidopterans [31, 32] and orthopterans (i.e. *Zonocerus*) [33] which also sequester PAs exclusively as *N*-oxides. However, in contrast to leaf beetles, lepidopterans possess a soluble, substrate-specific monooxygenase which efficiently converts tertiary PAs into the respective *N*-oxides [34]. In experiments with ^{18}O-labelled **17***N*-oxide it was conclusively shown that PA *N*-oxides are reduced in the guts and were taken up as tertiary PAs into the hemolymph [34]. The operation of a carrier-mediated uptake of the polar salt-like PA *N*-oxides in the guts of lepidopterans as previously postulated [35] could not be substantiated for lepidopterans, but may operate in leaf beetles. Adults of *Oreina* spec. are unable to *N*-oxidize tertiary PAs; only traces of labelled senecionine *N*-oxide were found in the defensive secretions of *O. cacaliae* which previously had fed on [^{14}C]senecionine [14]. To date, the only exception are larvae of the same species, which with its PA-based defence strategy (see below) is the most specialized *Oreina* species, are able to *N*-oxidize senecionine, whereas larvae of *O. speciosissima* are unable to do so (A. Ehmke, M. Rowell-Rahier, J. M. Pasteels, T. Hartmann, unpubl. results). If we postulate a carrier-mediated uptake of the PA *N*-oxides in *Oreina* we have to refer to two distinctive uptake-systems: (i) a rather unspecific carrier mediating the alkaloid uptake from the guts into the hemolymph; (ii) a highly specific carrier responsible for the selective PA *N*-oxide translocation from the hemolymph into the gland cells. This latter process requires an active transport component in order to create and maintain the high concentration gradient ($>150:1$) found to exist between defensive secretion and hemolymph.

The transformations observed with food-plant PAs (e.g. formation of **24** by epoxidation of **18** and deacyl-

ation of **23**) or with foreign PAs (e.g. the demethylation of **26** to **27**) are most likely catalyzed by toxicant-metabolizing enzymes well known to catalyse epoxidations, *O*-dealkylations and hydrolytic reactions as those observed here [36]. Oreine is the only transformation product in *Oreina* which is translocated into the defensive glands. Specific transformation products such as the insect PAs callimorphine or creatonotine synthesized by arctiid larvae from a necine base derived from a plant PA and necic acid produced by the insect [31, 32] are not found in leaf beetles.

To understand the role of PAs in the defence strategies of leaf beetles it is important to know that the species of the genus *Oreina* developed three kinds of defensive chemistry [10]. The defensive compounds released with the exocrine secretions may contain: (i) autogenously synthesized cardenolids; (ii) autogenous cardenolides + host-plant derived PAs (e.g. *O. elongata, O. intricata, O. speciosissima*); (iii) host-plant derived PAs (e.g. *O. cacaliae*). Phylogenies established of the genus *Oreina* strongly suggest that autogenous synthesis of cardenolides is most likely ancestral. The evolution of mixed chemical defence (i.e. cardenolides + PAs) may be followed by pure sequestration [10, 37] (T. H. Hsiao and J. M. Pasteels, unpubl. results). Although a return to pure autogenous defence from mixed chemical defence may have occurred in some species, the reverse evolution from pure PA sequestration to pure autogenous cardenolide synthesis is most unlikely.

The evolutionary scenario represents a strong argument for the role of PAs as efficient defensive compounds. In fact, it was shown that PAs provide leaf beetles with a better protection from predation by birds than do cardenolides [38]. Only 21% of *O. cacaliae* beetles with a total of 4.2 μg PAs at a concentration of 0.17 M in their defensive secretions (and *ca* 100 μg in the body) were eaten by wild-caught red-winged blackbirds (*Agelaius phoeniceus*); the number rose to 36% if the defensive secretions had been physically removed. On the contrary, 55% of *O. gloriosa* beetles with a total of 25 μg cardenolides at 0.27 M in their defensive secretions were eaten and this number increased to 95% when the secretion was removed. Thus, even a small quantity of highly concentrated PAs in the secretion produced at the surface of the beetle's body afforded substantial protection against avian predators. Many other fascinating examples of PA mediated defence in insects are known, especially from PA sequestrating lepidopterans [3–8]. However, direct evidence for their role in plant defence is still very scarce. One explanation is the difference in the defence strategies between insects and plants [39]. Whereas plants can afford to lose part of their organs (e.g. leaves or twigs), insects need total protection. Plants can regenerate lost tissues or organs, insects cannot. In comparison to plants, chemical defence in insects is much more active, they behave conspicuously and signal their unpalatability (e.g. by defensive secretion, aposematism etc.). In plant constitutive defence, chemicals such as PAs, just need to be accumulated at proper concentrations hidden in those tissues worth protection (e.g. inflorescences, epidermal tissues etc.) [3, 5, 40, 41]. In evolutionary terms PAs may have evolved and been shaped as plant defensive compounds under the selection pressure of a competing environment (e.g. herbivory). There is no better argument in favour of the great value of PAs as plant defensive compounds than those insects which, during coevolutionary adaptation, successfully recruited plant PAs as part of their own defence strategies.

EXPERIMENTAL

Plants and insects. Host-plants and leaf beetles feeding on them were collected in the field at the following localities (for details see ref. [16]): *Oreina cacaliae* (Schrank) (larvae and adults) on *Adenostyles alliariae* L. and adults on *Senecio nemorensis* L, Tschiertschen (Graubünden, Switzerland), on *Senecio fuchsii* C.C.Gmelin, Wasserliesen (Vosges, France); *O. speciosissima* (Scopoli) and *O. intricata* (Germar) on mostly *Petasites albus* L. (Gaertn.) and *S. nemorensis*, respectively, Tschiertschen (Graubünden, Switzerland); *O. elongata* (Suffrian) on *A. leucophylla* (Wild.) Richenb., Parc National du Mercantour (Vallée des Merveilles, France).

Host-plant leaves were lyophilized until analysis. The defensive secretions of the beetles were collected and kept in MeOH according to ref. [16], the residual beetles were kept frozen at −20° until analysis.

PA feeding experiments. Adults or larvae of *O. cacaliae* were placed individually or in groups of five in Petri dishes (5 cm diameter) and fed with *A. alliariae* leaf-discs (1 cm diameter) treated with 10 μl of a MeOH soln of the respective PA or PA mixt. After a feeding period of generally 24 hr the insects were transferred to fresh untreated host plant material until analysis.

Alkaloid analysis. PA extraction and analysis of insect and plant materials by GC was carried out as given in refs. [16, 17]. The identity of individual compounds were proved by GC-MS [17].

Chemicals and radioactively labelled tracers. Monocrotaline (**25**) was obtained from Aldrich-Chemie (Steinheim), **26** was a gift from Dr Boppré (Freiburg); **28** isolated from *S. vernalis* according to ref. [40]. Preparative *N*-oxidation of tertiary PAs and the preparation of ^{14}C-labelled **18** + **23** from root-cultures of *S. jacobaea* was performed as given in ref. [42].

Identification of 13,19-*epoxysenecionine* (*oreine*). Epoxidation of **18** was performed according to ref. [27]: 10 mg **18** were dissolved in 400 μl 99% HCO_2H and 1.2 ml of 35% H_2O_2 were slowly added to the soln. The mixt. was allowed to stand for 5 hr at room temp, then 2 N HCl was added to the reaction mixt. and the soln was reduced with zinc dust. After filtration the soln was made alkaline with 25% NH_4OH

and extracted ×3 with Et_2O. The identity of the product with 13,19-epoxysenecionine was proved by MS, ^1H NMR and ^{13}C NMR:

MS *m/z* (%): 349(3), 304(2), 288(1.2), 262(1.4), 260(1.4), 244(2), 220(5), 218(4), 190(2), 183(3), 167(3), 166(4), 165(8), 164(5), 152(3), 151(5), 138(31), 136(90), 120(100), 119(66), 108(14), 106(13), 95(32), 94(58), 93(59), 80(22), 43(20).

^1H-NMR($CDCl_3$/TMS): $\delta = 6.23$ (*br*; H-2), 6.00 (*q*, $J = 7.2$ Hz; H-20), 5.38 (*d*, $J = 11.7$ Mz; H-9a), 5.20 (*td*, $J = 3.9$, 1.6 Hz; H-7), 4.33 (*br m*; H-8), 4.12 (*dd*, $J = 11.7$, $\leqslant 0.6$ Hz; H-9b), 3.97 (*dm*, $J = 16.0$ Hz; H-3a), 3.43 (*ddd*, $J = 16.1$, 6.0, 1.9 Hz; H-3b), 3.32 (*br t*, $J = 8.4$ Hz; H-5a), 2.85 (*dd*, $J = 14.5$, $\leqslant 0.6$ Hz; H-14a), 2.77, 2.73 (AB, $J = 4.1$ Hz; H-19a, H-19b), 2.59 (*ddd*, $J = 11.9$, 9.3, 6.0 Hz; H-5b), 2.46 (*d*, $J = 14.5$ Hz; H-14b), 2.25 (*br dd*, J 13.8, 5.9 Hz; H-6a), 2.15 (*m*; H-6b), 1.87 (*d*, $J = 7.2$ Hz; H-21), 1.23 (*s*; H-18). The spectrum indicates the presence of the skeleton of **18**, in which the 13,19-double bond has been transformed into the oxirane moiety (i.e. $\delta_c = 60.2$ (*s*) and 49.7 (*t*); $\delta_H = 2.77$ and 2.73, AB spectrum with $J = 4.1$ Hz). These data closely resemble those given in ref. [27, 28].

^{13}C-NMR ($CDCl_3$, $\delta = 77.05$): $\delta = 176.1$ (*s*; C-11), 167.2 (*s*; C-16), 140.1 (*d*; C-20), 136.9 (*d*; C-2), 131.7 (*s*; C-1), 127.6 (*s*; C-15), 77.5 (*d*; C-8), 76.2 (*s*; C-12), 74.7 (*d*; C-7), 62.8 (*t*; C-3), 61.7 (*t*; C-9), 60.2 (*s*; C-13), 53.8 (*t*; C-5), 49.7 (*t*; C-19), 36.1 (C-14), 34.4 (C-6), 21.9 (*q*; C-18), 15.8 (*q*; C-21). Signal assignments were achieved by two-dimensional C,H-shift correlation, for C-2 vs C-20 and C-1 vs C-15 by selective proton decoupling.

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