Pyrrolizidine alkaloids as oviposition stimulants for the cinnabar moth, Tyria jacobaeae

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PYRROLIZIDINE ALKALOIDS AS OVIPOSITION STIMULANTS FOR THE CINNABAR MOTH, *Tyria jacobaeae*

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Abstract—In choice experiments with artificial leaves, we tested related pyrrolizidine alkaloids (PAs) for their stimulatory effects on the oviposition of the cinnabar moth, a specialist on the PA-containing plant *Senecio jacobaea*. The PAs from *S. jacobaea* that we tested stimulated oviposition. Monocrotaline also stimulated oviposition although this PA is not found in plants of the genus *Senecio*. The moths preferred ovipositing on filter paper with a PA mixture extracted from *S. jacobaea* to ovipositing on filter paper with single PAs. Senkirkine, heliotrine, and retrorsine did not stimulate oviposition. The nonactive retrorsine differs only in one OH group to the active senecionine, indicating that small structural differences alter the stimulatory activity of PAs. However, a PA mixture extracted from a nonhost plant, *Senecio inaequidens*, that consisted of 81% of the nonactive retrorsine did stimulate oviposition. Oviposition preferences between *Senecio* species seem to be determined by chemical compounds other than PAs.

Key Words—Arms-race hypothesis, plant–insect interactions, chemical diversity, oviposition, stimulants, *Senecio jacobaea*, pyrrolizidine alkaloids.

INTRODUCTION

The oviposition behavior of Lepidoptera is determined by a complex set of visual, olfactory, and chemical cues (Papaj and Rausher, 1983). After locating the potential host plant through visual and olfactory cues, acceptance of the host plant often depends on chemotactile stimuli (e.g., Myers, 1969; Miller and Stickler, 1984). Leaf allelochemicals can be important oviposition stimulants for specialist butterfly and moth species (Schoonhoven, 1972; Feeny et al., 1983; Honda, 1995). For example, glucosinolates are known to stimulate oviposition for *Pieris* species (van

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Loon et al., 1992; Renwick et al., 1992; Huang and Renwick, 1994); glycosides and flavonoids in *Citrus* species are oviposition stimulants for some *Papilio* species (Ohsugi et al., 1985; Honda, 1986; Honda, 1995 and references therein); and cnicin, a sesquiterpene lactone, stimulates oviposition for the specialist moth *Pterolonche inspersa* (Landau et al., 1994).

The stimulatory response of specialist lepidopteran species to allelochemicals in their host plants presents an important dilemma for the plant. Plant secondary metabolites may deter generalist herbivores while attracting specialist herbivores (Linhart, 1991; van der Meijden, 1996). Many plant species display a diversity of secondary metabolites, and often a variety of structurally related compounds can be found within a plant. The evolution of this staggering diversity of compounds is still poorly understood. One possible explanation for the diversity of structurally related secondary metabolites is that new compounds evolve in a continuous evolutionary arms race between a plant and its specialist insect herbivores. In such an arms race, a plant that synthesizes new compounds is able to escape herbivory and the insect herbivores, in turn, adapt to these compounds (Ehrlich and Raven, 1964). This coevolutionary model implies that structurally related compounds differ in their effects on specialist herbivores. Most emphasis has been on the toxicity of related compounds when discussing the arms-race hypothesis. However, for specialists, for which related compounds are mostly not toxic, as well as for generalist herbivores, for which related compounds are mostly all toxic, there is little evidence indicating differences in toxicity of structurally related compounds. Alternatively, related compounds may differ in their stimulatory effect on specialists. Thus, within the evolutionary arms race, plants may be able to (temporarily) overcome the dilemma of contrasting selection pressures of specialists and generalists if a new compound evolves that masks the plant for specialists and still deters generalist herbivores. Under this model, it is expected that structurally related compounds differ in the stimulation of oviposition of specialist lepidopteran species. The response to plant secondary metabolites by specialist lepidopteran species can be highly specific, acting only on specific compounds or combinations of compounds, or can be more general, acting on a certain group of compounds (Honda, 1995). Huang and Renwick (1994) found that all the aromatic glucosinolates they tested, except for glucosinalbin, stimulated oviposition by *Pieris rapae*, while the tested aliphatic glucosinolates did not stimulate oviposition. For another *Pieris* species, *P. napi oleracea*, structurally related glucosinolates differed in their stimulatory activity and there was no clear-cut relationship between activity and structure.

An excellent system for studying the evolution of related secondary metabolites are the various pyrrolizidine alkaloids (PAs) in *Senecio jacobaea*. This species can contain more than 10 structurally related pyrrolizidine alkaloids (Witte et al., 1992), and variation in PA composition in *S. jacobaea* is at least partly genetically determined (Vrieling et al., 1993). PAs can be deterrent to generalist herbivores (Van Dam et al., 1995; Hägele and Rowell-Rahier, 2000). In contrast, the specialist *Tyria jacobaeae* (cinnabar moth) sequesters PAs from its host plant *S. jacobaea*

(Aplin and Rotschild, 1972; Rotschild et al., 1979), and all life-stages of *T. jacobaeae* contain pyrrolizidine alkaloids (van Zoelen and van der Meijden, 1991). If specialist herbivores, like the cinnabar moth, have been selective forces in the evolution of different PAs, we expect that structurally related PAs differ in their effects on the cinnabar moth. Larval performance of *T. jacobaeae* did not differentiate between *S. jacobaea* plants with different PA composition (Macel et al., 2002). *T. jacobaeae* can detoxify PAs from *S. jacobaea* through N-oxidation by an enzyme that must have been recruited during the coevolutionary adaptation of *T. jacobaeae* to PAs (Lindigkeit et al., 1997; Naumann et al., 2002). Little is known about the importance of PAs as feeding or oviposition stimulants for the cinnabar moth. Adding PAs to the leaves of *S. jacobaea* did not increase oviposition (van der Meijden et al., 1989), suggesting that PA concentration on the leaf surface is not important as a proximate factor in the selection of plants for oviposition. It is not clear if PAs in themselves can stimulate oviposition.

In this study, we addressed the following questions: (1) Do PAs stimulate oviposition for the cinnabar moth? (2) If they do, is there a relationship between stimulatory activity and structure of the PAs? (3) Are specific (combinations of) PAs preferred over others? (4) Do PAs stimulate oviposition at a range of concentrations? We tested whether or not a PA mixture from *S. jacobaea* and a PA mixture extracted from a nonhost plant species (*Senecio inaequidens*) stimulated oviposition by the cinnabar moth. We also tested several single PAs from *S. jacobaea* as well as related and nonrelated plant species for stimulating effects on oviposition. To determine whether other chemical compounds can also play a role in the oviposition preference of *T. jacobaeae*, we tested whether leafsap from the preferred host plant, *S. jacobaea*, and from the nonhost plant, *S. inaequidens*, stimulated oviposition.

METHODS AND MATERIALS

Tyria jacobaeae. Female moths of *T. jacobaeae* used in the experiments were either captured in the field or reared in the lab. Female moths were captured in the dunes of Meijendel, The Netherlands, in the summer of 2000 and 2001. Moths reared in the lab were collected from Meijendel as fifth (last) instar caterpillars in the summer of 1999 and 2000. Each caterpillar was put into a glass tube without food until pupation. Pupae were stored for 10 months in a cold growth chamber (photoperiod L8:D16, 4°C, RH 70%) for hibernation. Following hibernation, pupae were placed into another growth chamber (L16:D8, 20:15°C, RH 70%) to emerge. Prior to the oviposition experiments, male and female moths were kept together for at least 1 week to mate. Lab-reared moths used for the experiments were 1–3 weeks old. The age of the moths collected in the field was not known.

Pyrrolizidine Alkaloids. Most of the PAs, we used belong to the structural group of senecionine type PAs, except for heliotrine, which is a lycopsamine type

PA, and monocrotaline, which is a monocrotaline type PA (Hartmann and Witte, 1995). Senecionine-type PAs are mostly 12-membered, macrocyclic diesters with a retronecine or otonecine base. Lycopsamine-type PAs are monoesters or diesters containing a hydroxylated 2-isopropylbutyric acid as a necic acid. Monocrotaline-type PAs are 11-membered, macrocyclic diesters with a retronecine base (Hartmann and Witte, 1995). The structures of the pyrrolizidine alkaloids we used are given in Table 1. The pyrrolizidine alkaloid mixtures were extracted from flowering plants of *S. jacobaea* and *S. inaequidens* using a modified version of the method developed by Koekemoer and Warren (1951) as described by Hol and van Veen (2002). The composition of the PA mixtures (Table 2) was determined by GC (HP 6890, 30 m $\times$0.25 μm, HP-1). GC conditions were, injector 250°C, temperature program 0-22-5-250, split mode 1–30, carrier gas N_2 at 0.9 ml/min, pressure 56 kPa, detector FID. All observerd peaks coincided with known PAs. The PA mixtures were 99% pure as determined by a color reaction modified after Mattocks (1967). Monocrotaline and retrorsine were obtained from Sigma; senecionine, seneciphylline, and senkirkine were obtained from Roth; and heliotrine was obtained from LATOXAN, France. In the plant, PAs mainly occur as N-oxides (Hartmann and Toppel, 1987). It is not known whether PAs on the leaf surface are free-base PAs or PA N-oxides. We tested free-base PAs to determine whether they can serve as oviposition stimuli.

Oviposition Choice Experiments. All tests were performed in a growth chamber (L16:D8, 20:15°C, RH 70%). In two-choice experiments, we tested the preference of *T. jacobaeae* for PAs using artificial "leaves" composed of filter paper. Two white filter paper leaves (Whatmann 91, 10 cm long and 2 cm wide) were attached to the bottom of a glass vial (2.5 cm diam.) with a small piece of tape (Figure 1). The vials were partly filled with sand to secure stability and covered with a white lid.

PAs were dissolved in methanol (5 mg PA per 1 ml methanol). We added 100 μl of test solution, PAs (25 μg/cm^2) or methanol, to the artificial leaves. Single PAs were all tested against the control (methanol). PA mixtures were tested against the control and against each other. We also tested the preference of *T. jacobaeae* for the sap of *S. jacobaea* versus the sap of *S. inaequidens*. Saps were made by squeezing leaves of the two *Senecio* species and collecting 100 μl of the sap that was added to the artificial leaves. Further tests were performed with diluted pure saps, by adding 50% of demi-water to each sap. A 1:1 mixture of both undiluted saps was also made. Again, 100 μl of diluted sap or mixture of saps were added to the artificial leaves.

One vial with test solutions and one female *T. jacobaeae* were placed in to a transparent plastic cylinder (30-cm diam. 50-cm height) with top and bottom covered with gauze. The vials were placed in such a way that direction of the filter paper leaves was arranged randomly. Every 24 hr, the number of eggs on each filter paper leaf as well as the number of eggs elsewhere in the cage (i.e.,

TABLE 1. STRUCTURES OF PYRROLIZIDINE ALKALOIDS USED IN THIS STUDY

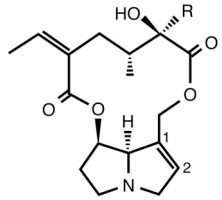
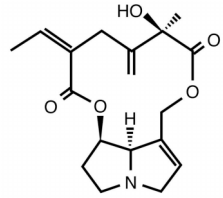
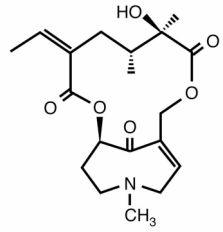
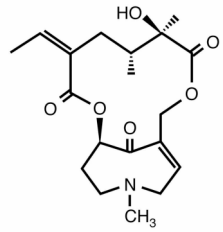
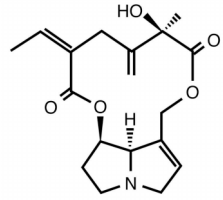
Name	Structure	Plant species
Senecionine: R=CH_3 Retrorsine: R=CH_2OH		*Senecio* sp., e.g., *S. jacobaea* (senecionine) *S. inaequidens* (senecionine + retrorsine)
Seneciphylline		*Senecio* sp., e.g., *S. jacobaea*
Senkirkine		*Senecio* sp., e.g., *S. vernalis*
Monocrotaline		*Crotalaria* sp.
Heliotrine		*Heliotropum* sp.

TABLE 2. PA COMPOSITION (% OF TOTAL PAS) OF MIXTURES EXTRACTED FROM *S. jacobaea* AND *S. inaequidens*

PA	S. jacobaea	S. inaequidens
Senecivernine		5.4
Senecionine	10.5	6.7
Integerrimine	4.6	3.6
Seneciphylline	17.4	
Jacobine	30	
Jacozine	3.6	
Jacoline	3.6	
Jaconine	25.2	
Retrorsine		81.3
Erucifoline	5.1	
Usaramine		1.9
Otosenine		1.1

other than on the paper leaves) was recorded. The cage covers an area of 50 × 70 cm, far greater than the surface area of the artificial leaves. Egg batches laid on the cage were considered neither a negative nor a positive response to our PA treatment since such egg batches could indicate that the moths had not encountered the artificial leaves. Each female was used only once. Experiments were replicated 15–21 times, depending on the number of female moths available.

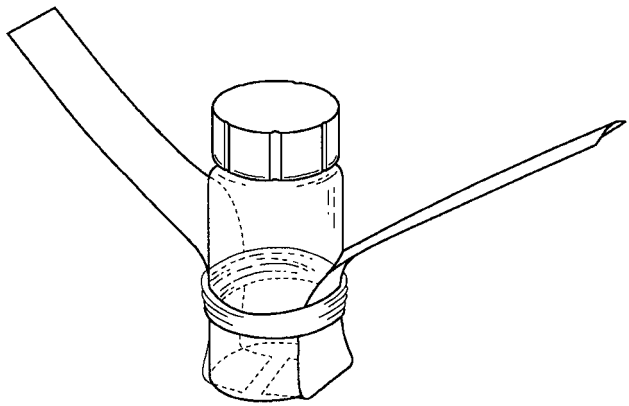


FIG. 1. Experimental design with artificial leaves. The glass vial is 6 cm in height and 2.5 cm in diameter, the filter paper leaves are Whatmann 91, 10 × 2 cm. The vial was placed in a transparent plastic cylinder with top and bottom of gauze. Test solutions were added to the artificial leaves.

RESULTS

On average (over all experiments), the moths laid 1.7 egg batches per female, and 23.9% of the moths did not oviposit at all. The percentage of females that did not lay any eggs did not differ among the experiments ($\chi^2 = 10.80$, $df = 14$, $P = 0.73$). Although egg batches were also found elsewhere in the cage, in most experiments more than 50% of the eggs were laid on the artificial leaves.

Oviposition Stimulation of PAs. Table 3 shows the results of the oviposition choice experiments using the different PAs against the control treatment. *T. jacobaeae* females strongly preferred to oviposit on filter paper with the PA

TABLE 3. OVIPOSITION CHOICE OF *T. jacobaeae* BETWEEN PAS AND CONTROL

Choice	No. of eggs	No. of egg batches	*P*	*N*	Lab/field
PAs *S. jacobaea*	1370	16	<0.001	18	Field
Control (methanol)	146	1			
Cage	160	8			
PAs *S. inaequidens*	374	13	0.002	17	Lab
Control	56	3			
Cage	164	10			
Senecionine	515	10	0.011	14	Field
Control	60	2			
Cage	111	6			
Retrorsine	438	7	0.705	11	Lab
Control	158	6			
Cage	189	8			
Seneciphylline[a]	363	13	0.024	11	Lab
Control	87	4			
Cage	175	6			
Senkirkine	356	10	0.102	9	Field
Control	64	2			
Cage	554	10			
Monocrotaline	939	11	0.009	10	Field
Control	0	0			
Cage	33	3			
Heliotrine	266	5	0.180	11	Lab
Control	117	2			
Cage	290	10			

Note: *P* values of Wilcoxon's signed-ranks matched-pairs test on number of egg batches on control and on PAs. The last column indicates if field or lab reared females were used. *N* = number of ovipositing females.

[a] 7.5 μg PA/cm^2 was applied instead of 25 μg/cm^2.

TABLE 4. OVIPOSITION CHOICE OF *T. jacobaeae* BETWEEN PA MIXTURES FROM *S. jacobaea*, *S. inaequidens* AND SINGLE PAS

Choice	No. of eggs	No. of egg batches	*P*	*N*	Lab/field
PAs *S. jacobaea*	351	11	0.180	14	Lab
PAs *S. inaequidens*	325	8			
Cage	143	8			
PAs *S. jacobaea*	654	17	0.020	12	Lab
Senecionine	162	7			
Cage	117	6			
PAs *S. jacobaea*	747	14	0.001	16	Lab
Monocrotaline	30	3			
Cage	38	6			

Note: *P* values of Wilcoxon's signed-ranks matched-pairs test on number of egg batches on PAs. The last column indicates if field or lab reared females were used. N = number of ovipositing females.

mixture from *S. jacobaea*, and also preferred the PA mixture from *S. inaequidens* to the control. In the experiments with single PAs, senecionine and seneciphylline had a stimulatory effect on oviposition. Both senecionine and seneciphylline are found in *S. jacobaea*. Monocrotaline was also preferred to the control. Interestingly, this PA is not found in *Senecio* species. Retrorsine, a PA found in *S. inaequidens* and other *Senecio* species but not found in *S. jacobaea*,did not stimulate oviposition of the cinnabar moth. Senkirkine and heliotrine also did not have a stimulatory effect on oviposition.

Preference for Specific PAs. Females did not distinguish between the two PA mixtures from *S. jacobaea* and *S. inaequidens* (Table 4). When females were given a choice between senecionine and the PA mixture of *S. jacobaea*, they preferred the mixture to the single senecionine. In addition, this PA mixture was also preferred to monocrotaline (Table 4).

Different Concentrations of PAs. The PA mixture from *S. jacobaea* stimulated oviposition at all three concentrations (Table 5).

Other Leaf Chemicals. Significantly more eggs and egg batches were laid on the filter paper with the sap of *S. jacobaea* than on the filter paper with sap of *S. inaequidens* (Table 6). The PA concentration in the *S. jacobaea* sap (223 μg/ml) was twofold higher than the PA concentration in the *S. inaequidens* sap (108 μg/ml), which might explain the oviposition difference. However, moths equally preferred the pure sap of *S. jacobaea* and a mixture of 50% *S. jacobaea* sap and 50% *S. inaequidens* sap, and preferred this mixture to the pure sap of *S. inaequidens* (Table 6). This is a clear indication that besides PAs other leaf chemicals in *S. jacobaea* also stimulate oviposition in the cinnabar moth.

TABLE 5. OVIPOSITION CHOICE OF *T. jacobaeae* BETWEEN THREE CONCENTRATIONS OF THE PA MIXTURE FROM *S. jacobaea* AND CONTROL

Choice	No. of eggs	No. of egg batches	*P*	*N*	Lab/field
PAs *S. jacobaea* 5 μg/cm^2	338	11	0.002	11	Lab
Control	0	0			
Cage	96	5			
PAs *S.jacobaea* 0.5 μg/cm^2	225	9	0.038	12	Lab
Control	22	2			
Cage	342	12			
PAs *S. jacobaea* 0.05 μg/cm^2	361	8	0.034	11	Lab
Control	6	1			
Cage	414	16			

Note: *P* values of Wilcoxon's signed-ranks matched-pairs test on number of egg batches on control and on PAs. The last column indicates if field or lab reared females were used. N = number of ovipositing females.

DISCUSSION

The attraction of Lepidoptera to PAs in food sources has been studied extensively (Pliske, 1975; Boppré, 1984, 1986; Schneider, 1987). To our knowledge, this is the second study that shows that PAs can also be oviposition stimuli for specialist lepidopteran species. Honda et al. (1997) showed that PAs from *Parsonia laevigata* stimulated oviposition by the danaid butterfly *Idea leuconoe*. In our study, the PAs from *S. jacobaea* stimulated oviposition by the cinnabar moth. Monocrotaline also stimulated oviposition even though this PA is not found in *S. jacobaea* or its relatives. Furthermore, the PA mixture from *S. inaequidens*, a nonhost plant, also stimulated oviposition. Our results show that the response of *T. jacobaeae* to PAs

TABLE 6. OVIPOSITION CHOICE OF *T. jacobaeae* BETWEEN SAPS OF *S. jacobaea* AND *S. inaequidens*

Choice	No. of eggs	No. of egg batches	*P*	*N*	Lab/field
S. jacobaea sap	1464	14	<0.001	14	Lab
S. inaquidens sap	2	1			
Cage	10	2			
50% *S. jacobaea* sap[a]	420	20	0.196	15	Field
Sap mixture	214	19			
50% *S. inaequidens* sap[a]	140	5	0.013	15	Field
Sap mixture	738	24			

Note: *P* values of Wilcoxon's signed-ranks matched-pairs test on number of egg batches on saps. The last column indicates if field or lab reared females were used. N = number of ovipositing females.
[a] In tests with sap mixtures no measurements were made of eggs on the cage.

is not specific, since nonhost PAs stimulated oviposition. Senkirkine, heliotrine, and retrorsine did not stimulate oviposition. Retrorsine differs only in one OH group at C-12 from senecionine, a PA that did stimulate oviposition. Remarkably, the PA mixture of *S. inaequidens*, which consists of 81% retrorsine (Table 1), did stimulate oviposition.The preference of *T. jacobaeae* for the *S. jacobaea* PA mixture over the single PAs senecionine and monocrotaline, and equal preference for both *S. jacobaea* and *S. inaequidens* PA mixtures, suggests that *T. jacobaeae* is most stimulated by (*Senecio*) PA mixtures irrespective of the composition of these mixtures.

It is unclear whether females of *T. jacobaeae* can detect PAs inside the leaf. The PA concentration we used in most of our experiments was comparable to the average concentration of 0.5% dry weight that is found inside the leaves of *S. jacobaea* plants (Vrieling et al., 1993). PAs are present on the leaf surface of *S. jacobaea* at an average concentration of 0.04 μg/cm^2 (Derridj and Vrieling, unpublished data). This concentration is comparable to the 1 μg (0.05 μg/cm^2) we used with different concentrations of PA mixture from *S. jacobaea* and which stimulated oviposition. It is not clear whether there is a threshold value for PA concentration or whether the stimulatory activity of PAs are concentration-dependent. The results of Van der Meijden et al. (1989) suggest that there is a threshold value for PA concentration since adding PAs to leaves of *S. jacobaea* did not increase oviposition. If there is a threshold, it must be at a concentration that is lower than 0.05 μg/cm^2 based on our results with different concentrations of the PA mixture of*S. jacobaea*.

In previous oviposition experiments with plants, we have shown that the cinnabar moth strongly preferred *S. jacobaea* to *S. inaequidens* (Macel et al., 2002). The cinnabar moth does not distinguish between the PA mixtures of *S. jacobaea* and *S. inaequidens* and, therefore, the oviposition preference of the cinnabar moth for *S. jacobaea* is not based on the PA composition of these plant species. The sap experiments suggest that the oviposition preference between these two *Senecio* species is determined by other chemical factors. A whole set of chemical cues likely determines the oviposition behavior of *T. jacobaeae*, of which PAs are only a part.

Although the attraction of the cinnabar moth to PAs in its host plant certainly presents an evolutionary dilemma for the plant, it is doubtful whether the cinnabar moth is a selective force in the evolution of the diversity of PAs in *S. jacobaea*. On one hand, small differences in structure can influence the stimulatory activity of PAs, as is shown by the active senecionine and non-active retrorsine. On the other hand, a nonhost plant PA of a different structure (monocrotaline) stimulated oviposition. Furthermore, the PA mixture of *S. inaequidens* with 81% of the non-active retrorsine still stimulated oviposition. By producing a new PA within its existing set of PAs, *S. jacobaea* might not be able to escape oviposition by the cinnabar moth.

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