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Alkaloidal oviposition stimulants for a danaid butterfly, *Ideopsis similis* L., from a host plant, *Tylophora tanakae* (Asclepiadaceae)

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Abstract. Chemicals releasing oviposition by an Asclepiadaceae feeder, *Ideopsis similis*, were identified from a host plant, *Tylophora tanakae*. A strong positive response was evoked by a methanolic extract of the plant, which proved to contain multiple stimulants. A mixture of two phenanthroindolizidine alkaloids, (+)-isotylocrebrine and (−)-7-demethyltylophorine, isolated from organic fractions, elicited significant ovipositional responses from females.
Key words. Oviposition stimulants; *Ideopsis similis*; Danaidae; *Tylophora tanakae*; Asclepiadaceae; (+)-isotylocrebrine; (−)-7-demethyltylophorine; alkaloids.

During the last decade investigations of phytochemicals involved in egg-laying by butterfly species of the Papilionidae, Pieridae and Nymphalidae have revealed an array of oviposition stimulants[1–16,20,21] and deterrents[17–21]. However, for danaid butterflies, which use various plants including Apocynaceae, Asclepiadaceae, Euphorbiaceae and Moraceae as hosts in nature, chemical substances that mediate host selection by ovipositing females have not yet been reported. Cardenolides[22,23] have often been implicated in chemical defense, and pyrrolizidine alkaloids (PAs)[24–27] in both chemical defense and pheromone production. Recent publications have documented that cardenolide content in *Asclepias* plants affects oviposition behavior of the monarch butterfly, *Danaus plexippus*; plants with a higher level of cardiac glycosides, irrespective of their structural types, were less preferred or rejected by the females[28,29].

Ideopsis similis, inhabiting South-western islands in Japan, chiefly infests an asclepiadaceous plant, *Tylophora tanakae*. We describe here oviposition stimulants for this species identified from *T. tanakae*.

Materials and methods

Females subjected to behavioral bioassays were obtained from laboratory stock cultures which originated from the population of Yaeyama islands. The larvae, fed with fresh leaves of *T. tanakae*, were reared at 22 °C with a 16 h light-8 h dark regime. Both sexes of newly emerged individuals were kept under quasi-natural condition until mating in a green house (7 × 10 m^2; height, 3.5 m) equipped with flowers and PA-containing plants. Thereafter, females were transferred to our laboratory, and were fed with 15% aqueous sucrose daily during the experiments. The females used for the behavioral assay were 7- to 20-day-old gravid individuals that responded positively to the foliage of *T. tanakae*.

Squares of yellowish-green paper (5.0 × 5.0 cm^2; reflectance, 78% at 510 nm), which had previously been soaked in methanol for a day to remove miscellaneous additives, were employed as an ovipositional substrate. Three to four pairs of females in a transparent plastic chamber (25 × 35 cm^2; height, 15 cm) were provided with a choice of four sheets of paper, two of which were treated with the same test sample, and others with solvent only (control). They were set horizontally about 2.5 cm apart from each other and 10 cm above the bottom of the chamber, and were sprayed with water prior to the experiments. The stimulatory activity of test materials at a dose of 90 μg/cm^2 was estimated by counting the number of eggs deposited on the substrate during a period of 3 h. Each experiment was replicated three times. Significance of differences between treatments and controls was assessed by a sign test.

Fresh leaves and stems of *T. tanakae* collected in the field were extracted with methanol (ca. 10 ml/g) at room temperature for a month. The methanolic extract was filtered and evaporated in vacuo below 50 °C to dryness. The residue, dispersed in water, was extracted with benzene and/or chloroform. Since preliminary experiments suggested that some of stimulatory substances are present in alkaloidal fractions (positive to Dragendorff reagent), active compounds were isolated from organic fractions by means of column chromatography with silica gel and preparative TLC. Identification of active compounds was based mainly on spectral evidence from ^{1}H- and ^{13}C-NMR, FAB-MS, UV and CD. The details of the procedure for isolation and identification will be published elsewhere[30].

Table 1. Ovipositional response of *Ideopsis similis* to extracts and fractions from the host plant, *Tylophora tanakae*.

Sample[a]	Number of eggs laid		p < (sign test)
	treated	control	
MeOH extract	45	3	0.0001
$CHCl_3$ Fr.	32	0	0.0001
H_2O Fr.	25	0	0.0001
Fr. 1[b]	0	0	
Fr. 2	27	2	0.0001
Fr. 3	0	1	
Fr. 4	2	1	NS[c]
Fr. 5	28	2	0.0001

[a]Quantity applied: 90 μg/cm^2; [b]refer to figure 1; [c]not significant.

Results

The ovipositing females of *I. similis* exhibited a characteristic positive response to the methanol extract of *T. tanakae*, rapidly drumming the surface of the substrate with their forelegs, curling the abdomen and bringing the ovipositor in contact with the underside of the substrate, and finally laying an egg (table 1). This undoubtedly implies that the methanol extract contains active substances responsible for egg-laying by this species.

In the first experiment, the residue of methanol extract was partitioned between chloroform and water, and the ovipositional response to the respective fractions was examined. The proportion of chloroform-soluble materials in the fresh weight of the foliage was estimated at ca. 0.78%, and that of the water-soluble, 4.1%. As table 1 clearly shows, either fraction induced significant oviposition behavior, thereby suggesting the involvement of multiple constituents in host recognition by the females. The aqueous fraction, however, was not pursued further in this study.

In the second experiment, the residue of methanol extract was extracted successively with benzene and chloroform. The subsequent procedure for fractionation is shown in figure 1. Benzene extract, dissolved in 10% aq. acetic acid, was extracted with ether to give an ethereal fraction (Fr. 1) and an aqueous one, which, after alkalination with aq. NH_3, was extracted again with chloroform to yield a chloroform-soluble fraction (Fr. 2). Chloroform extract was also treated with methanol, and the resulting precipitates (Fr. 5) were filtered off. The filtrate was concentrated and subsequently treated with acid and alkali in a similar manner to that of the benzene extract. Five fractions prepared in total (Frs 1–5) were evaluated for their oviposition-stimulatory activities (table 1). Neutral and acidic fractions (Frs 1 and 3) apparently exerted no stimulatory effect on oviposition, whereas Frs 2 and 5 were highly active in evoking egg-laying by the females. Although the influence of Fr. 4 is equivocal, Frs 2, 4 and 5 were combined and subjected to chromatography and TLC. In this way we isolated compounds **1**, **2** and several other alkaloids with the phenanthroindolizidine framework[30].

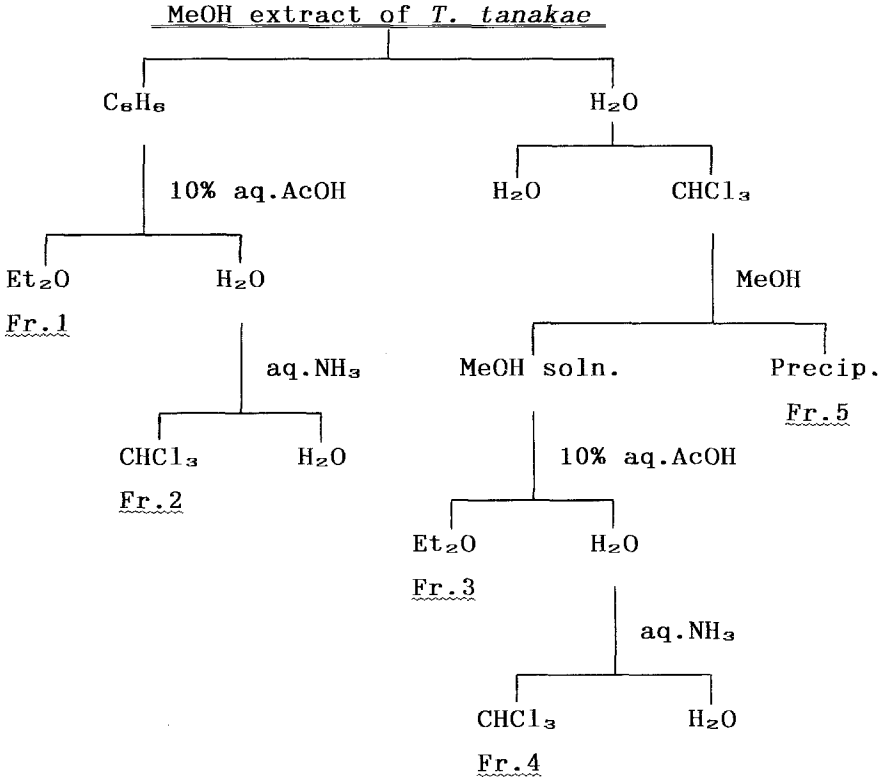

Figure 1. Schematic procedure for the separation of oviposition stimulants for *Ideopsis similis*.

Table 2. Ovipositional response of *Ideopsis similis* to alkaloidal components from the host plant, *Tylophora tanakae*.

Compound[a]	Number of eggs laid		p < (sign test)
	treated	control	
1[b]	0	0	
2[c]	0	0	
1 + 2	23	1	0.0001

[a]Quantity applied: 90 μg/cm² of each component; [b](+)-isotylocrebrine; [c](−)-7-demethyltylophorine.

Compound **1**: mp 198–203 °C (dec); $[\alpha]_D^{26}$ +20.2° ($CHCl_3$, c0.50); FAB-MS, $[M+H]^+$ at m/z 394.2019 giving the molecular formula of $C_{24}H_{27}NO_4$, was identified, based on the NMR spectra, as (+)-isotylocrebrine (3,4,6,7-tetramethoxyphenanthroindolizidine)[31]. Compound **2**: mp 250–260 °C (dec); $[\alpha]_D^{27}$ −49.3° ($CHCl_3$, c0.25); FAB-MS, $[M+H]^+$ at m/z 380.1865 (indicating $C_{23}H_{25}NO_4$), was presumed to be a 7-demethylated derivative of tylophorine[32] by comparison of fragmentation patterns of FAB-MS and UV spectra. Using this information, along with the NMR spectral data, we determined compound **2** to be (−)-7-demethyltylophorine (7-hydroxy-2,3,6-trimethoxyphenanthroindolizidine).

These compounds were entirely inactive when bioassayed alone, but turned out to be highly active in combination (table 2). Consequently, these two alkaloids can be regarded as oviposition stimulants for *I. similis* that function synergistically (fig. 2).

Discussion

These findings demonstrate that host recognition by *I. similis* adults is likely to be mediated principally by alkaloid constituents. The synergistic action of at least two related components, isotylocrebrine and 7-demethyltylophorine, seems to stimulate oviposition. Although the effect of other co-occurring alkaloids on oviposition remains unknown at present, it is very feasible that some of other constituent alkaloids sharing a phenanthroindolizidine moiety have analogous activities. By contrast, tylophorine, tylophorinine and pergularinine isolated from *T. asthmatica* have been reported to exhibit antifeedant activity against the larvae of a moth, *Spodoptera litura*[33]. The mechanism by which *I. similis* assesses host plants resembles that of the Papilionidae in that as far as is known, all these butterflies utilize more than one chemical to recognize host plants[4–6,8,10]. This contrasts with the mechanisms of *Pieris* (Pieridae)[12,14,15] and *Junonia* (Nymphalidae)[16] butterflies in which oviposition by females is usually induced by a single compound. However, the system in *I. similis* appears to be somewhat simpler than that of papilionid species, where multiple compounds of diverse chemical categories synergistically serve as stimulants. Some alkaloids also play an important role in host selection by *Papilio* species[3,5,6], but these compounds do not seem to be dominant factors regulating acceptance of the hosts. In this respect, the chemical cues on which *I. similis* relies to assess the suitability of plants are as specific as glucosinolates in *Pieris*[12,14,15] and iridoid glycosides in *Junonia*[16]. This finding provides a new insight into chemical mediation of host selection by herbivorous insects. Although both isotylocrebrine and 7-demethyltylophorine may not be specific to *T. tanakae*, phenanthroindolizidine alkaloids characterize the chemistry of *Tylophora* plants[30,32–36]. It is therefore reasonable that such chemicals, characteristic of the host plant, can play a role in releasing egg-laying by the adapted insect, *I. similis*. Further attempts to explain the function of other constituents are now in progress.

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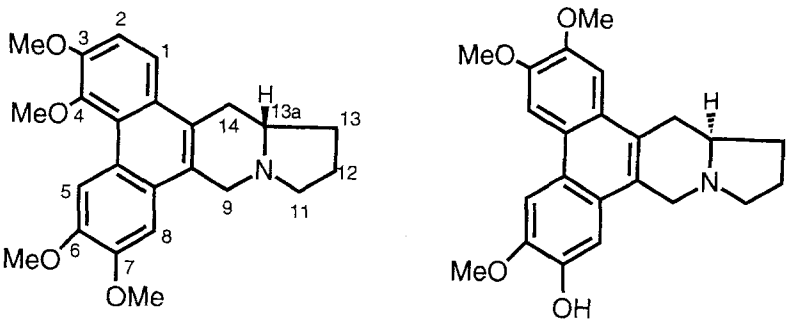

(+)-ISOTYLOCREBRINE (−)-7-DEMETHYLTYLOPHORINE

Figure 2. Structures of oviposition stimulants for *Ideopsis similis* identified from *Tylophora tanakae*.

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