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Oviposition stimulants of a *Citrus*-feeding swallowtail butterfly, *Papilio xuthus* L.

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Summary. A methanolic extract of *Citrus unshiu* induces oviposition by females of a *Citrus*-feeding swallowtail butterfly, *Papilio xuthus* L. The chemical factors responsible for stimulating oviposition were isolated and characterized as 5-hydroxy-*Nω*-methyltryptamine, adenosine, vicenin-2, narirutin, hesperidin and rutin. An artificial blend of these six components elicited significant oviposition behavior, apparently identical to that induced by contact with intact *Citrus* leaves.
Key words. Oviposition stimulant; host selection; butterfly; *Papilio xuthus; Citrus;* 5-hydroxy-*Nω*-methyltryptamine; adenosine; flavonoid.

The larvae of most lepidopterous insects feed on a limited number of closely related host plants. The choice of oviposition sites by the adult females is crucial to the survival of their offspring[1,2]. *Papilio xuthus* L. (Papilionidae) is a swallowtail butterfly whose larvae feed exclusively on plants in the family Rutaceae. More than seventy related species in the genus *Papilio* are strongly associated with rutaceous plants[3,4] and many of them are pests of *Citrus* crops. The females lay eggs with great precision on young leaves of their host plants. Alighting on the plants, females vigorously drum upon the leaf surface with their forelegs. After detecting, through their tarsal receptors[5], the specific oviposition stimulants contained in their host plants, the females then lay eggs[5]. The same oviposition behavior can be induced when female butterflies are brought into contact with a piece of filter paper treated with a methanolic extract of their host plants (fig. 1). The oviposition stimulant of *P. xuthus* appeared to be a mixture of highly polar non-volatile compounds[5,6]. We describe here how we were able to resolve the mixture and identify six compounds, the blend of which elicited oviposition behavior apparently identical to that elicited by extracts or intact leaves of *Citrus* plants.

Materials and methods. Leaves of *Citrus unshiu* Marc. (1.2 kg), one of the most common host plants of *P. xuthus*, were extracted with methanol (3 l, by soaking for three months at 5 °C). Gravid females of *P. xuthus* usually responded immediately to the standard methanol extract at a dose of 0.03 g leaf equivalents per 10 cm^2 of filter paper (g.l.e./f.p.) in the behavioral bioassay shown in figure 1. Each female butterfly (hand-paired within 2 days of emergence, 3–10 days old) was introduced into a bioassay chamber (50 × 50 × 50 cm^3) which is open in front and illuminated by a fluorescent lamp (15 W) at the rear. The test filter paper was brought into contact with the female's forelegs as much as possi-

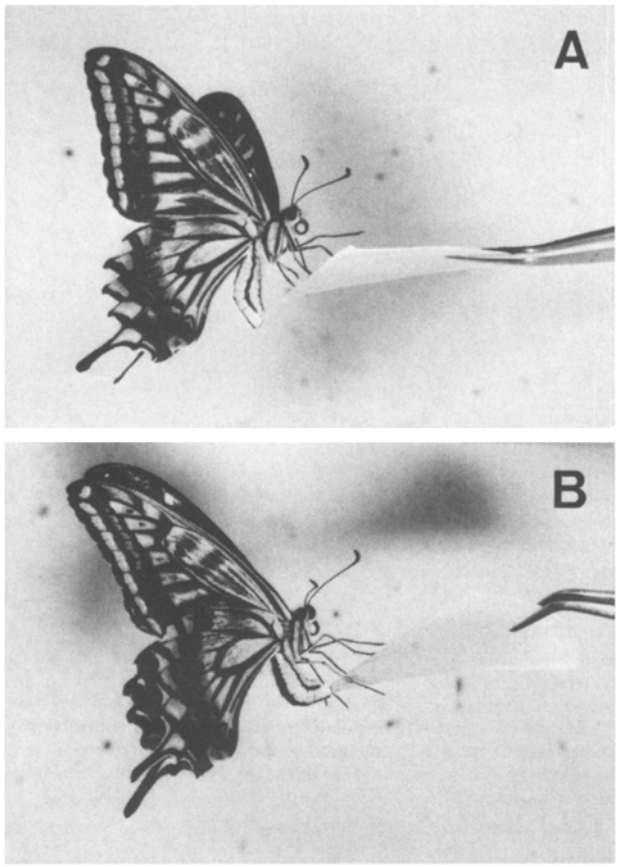

Figure 1. Oviposition response of a *Papilio xuthus* female to filter paper treated with the methanolic extract of *Citrus unshiu*. *A* The female is sensing the oviposition stimulants through her tarsal chemoreceptors by drumming upon the surface with her forelegs. *B* She lays an egg on the paper, curling her abdomen.

ble during 30 s so that she could detect the oviposition stimulants (fig. 1A). The females were scored for the abdomen-curling response (fig. 1B). Immediately before bioassay, each test paper was moistened by misting with distilled water.

The methanolic extract was fractionated into ether, ethyl acetate, butanol and water layers by solvent extraction. The oviposition activity was recovered mainly from the water layer, though weak activity was present also in the butanol layer. The aqueous layer was then chromatographed on a C_{18} reverse-phase column (ODS-W, micro bead silica gel 5D, 100–200 mesh, Fuji-Davison Chemical Ltd.), eluting with water alone (fraction A), 10% methanol (fraction B), 30% methanol (fraction C) and methanol (fraction D). The activity was found in fraction A (a 75% positive response at 0.1 g.l.e./f.p., N = 24) and fraction C (a 93% positive response at 0.1 g.l.e./f.p., N = 29). Fraction C was rechromatographed on the same reverse-phase column into four fractions designated CA (eluted with 1% acetic acid in water), CB (1% acetic acid plus 20% methanol), CC (1% acetic acid plus 40% methanol) and CD (1% acetic acid in methanol). Among these, only fraction CA was found to be active, although its activity was much lower than that of the original fraction C. However, the activity of fraction C was fully recovered when fraction CA was combined with the inactive fraction CB (table).

Results. Compound **1** (fig. 2) was isolated from fraction CA by high performance liquid chromatography (HPLC), monitoring the synergistic activity by mixing each eluate with fraction CB (YMC-pack, S-5 ODS, 5 µm, 300 mm × 8 mm i.d., eluting with a mixture of methanol, water and acetic acid (10:90:1) at 2 ml/min, *Rt* (retention time) = 8.6 min, yield: 10 µg/g.l.e.). Compound **1** proved to be inactive by itself, but distinct oviposition activity was generated when it was combined with fraction CB (table). Compound **1** was identified as 5-hydroxy-*Nω*-methyltryptamine by ultraviolet (UV), proton and carbon magnetic resonance (PMR and CMR) and mass spectra (CMR in deuterium oxide: δ (ppm) 151.4. 134.1, 129.6, 127.7, 115.4, 114.5, 110.7, 105.0, 51.6, 35.2 and 24.0). An additional compound **2** was also isolated from fraction CA by the same HPLC condition above (*Rt* = 11.6 min, yield: 5 µg/g.l.e.) and identified as adenosine by its diagnostic UV, PMR and CMR spectra, melting point (mp 235 °C), and optical rotation ($[\alpha]_D^{20} = -57°$ in water). In contrast to compound **1**, compound **2** was not active when combined with fraction CB, but a mixture with the flavonoid components (**3**+**4**+**5**+**6**) did exhibit significant activity as shown in the table (chi-square test: $p < 0.05$).

Four flavonoid compounds **3**, **4**, **5** and **6** were identified as stimulants through their synergistic effects on the activity of fraction CA. Compound **3** had been isolated previously from a fraction corresponding to CB and identified as vicenin-2 (6,8-di-*C*-β-D-glucopyranosylapigenin), a synergist of fraction CA[6]. Compounds **4**, **5**, and **6** were isolated from the butanol layer by using the same HPLC column employed for fraction CA, eluting with a mixture of methanol and water (1:1) at 1 ml/min (*Rt* = 22.0, 24.5 and 32.4 min; yields: 15 µg, 150 µg and 400 µg/g.l.e., respectively), and identified as narirutin (naringenin 7-*O*-β-rutinoside, mp 156–163 °C), hesperidin (hesperetin 7-*O*-β-rutinoside, mp 261–263 °C), rutin (quercetin 3-*O*-β-rutinoside, mp 188–190 °C) respectively (fig. 2). As shown in the table, each of these compounds was inert, but exhibited significant activity when mixed individually with fraction CA.

The activities of various blends of authentic samples, each at a dose of 10 µg, are also shown in the table. A mixture of **1**+**2** appeared to represent the weak activity of fraction CA alone. A mixture of the flavonoids **3**+**4**+**5**+**6** was also found to be only weakly active, but the activity was increased significantly by addition of either **1** or **2** to the mixture (chi-square test: $p < 0.05$). Finally, maximum oviposition activity was generated when all of the six components were mixed together. It is thus clear that the oviposition is stimulated by a mixture of components acting synergistically.

An artificial mixture of these six components in the actual ratio found in the methanolic extract exhibited a 80% ovi-

Oviposition responses of *Papilio xuthus* females to fractions and compounds isolated from a methanolic leaf extract of *Citrus unshiu*

Sample (dose/filter paper*)	% Response (number of females)
CA (0.3 g.l.e.)	0 (34)
CA (1 g.l.e.)	16 (34)
CB (0.3 g.l.e.)	0 (21)
CB (1 g.l.e.)	0 (13)
CA (0.3 g.l.e.) + CB (0.3 g.l.e.)	81 (21)
1 (10 µg)	0 (46)
2 (10 µg)	0 (67)
CB (1 g.l.e.) + **1** (10 µg)	100 (13)
CB (1 g.l.e.) + **2** (10 µg)	0 (16)
3 (30 µg)	0 (23)
4 (30 µg)	0 (23)
5 (30 µg)	0 (23)
6 (30 µg)	0 (23)
CA (0.3 g.l.e.) + **3** (30 µg)	83 (23)
CA (0.3 g.l.e.) + **4** (30 µg)	70 (23)
CA (0.3 g.l.e.) + **5** (30 µg)	73 (23)
CA (0.3 g.l.e.) + **6** (30 µg)	70 (23)
1 + **2** (10 µg each)	13 (30)
3 + **4** + **5** + **6** (10 µg each)	27 (30)
1 + **3** + **4** + **5** + **6** (10 µg each)	86 (49)
2 + **3** + **4** + **5** + **6** (10 µg each)	63 (30)
1 + **2** + **3** + **4** + **5** + **6** (10 µg each)	100 (30)

* Doses are expressed either as the amounts of the methanolic leaf extract applied in g leaf equivalents per 10 cm^2 of filter paper (g.l.e./f.p.) or as the absolute amount applied to the filter paper (µg/f.p.).

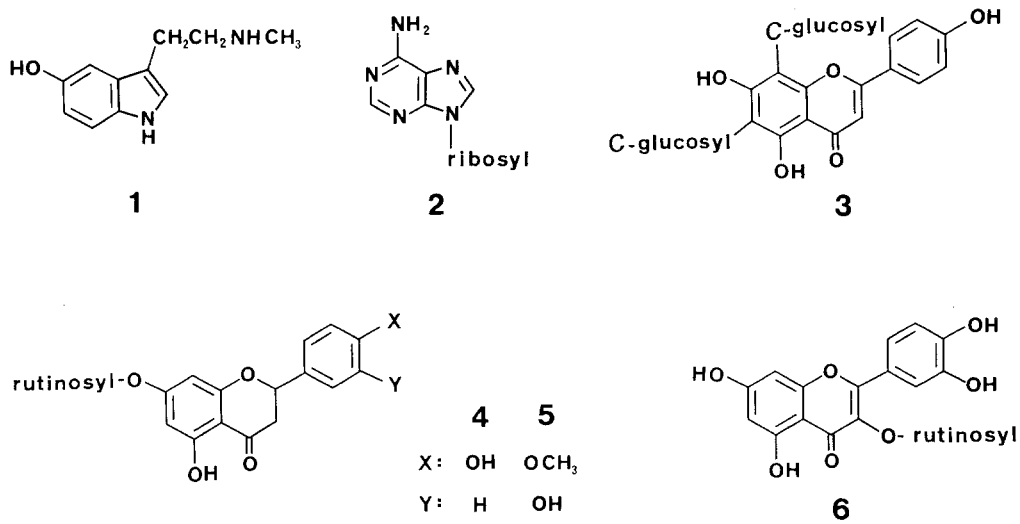

Figure 2. Oviposition stimulants for *Papilio xuthus* from leaves of its host plant *Citrus unshiu*. **1**: 5-Hydroxy-*N*ω-methyltryptamine, **2**: Adenosine, **3**: Vicenin-2, **4**: Narirutin, **5**: Hesperidin, **6**: Rutin.

position response (N = 20) at a dose of 0.1 g.l.e./f.p. (**1**:**2**:**3**:**4**:**5**:**6** = 1:0.5:30:1.5:15:40 µg/f.p., which was still lower than that of the total methanolic extract at the same dose (a 100% positive response, N = 20). The difference appeared to be mainly due to an active factor(s) present in fraction A, identification of which is currently in progress.

Discussion. Our knowledge of the distribution of the active compounds in plants is incomplete. Compound **1** has been isolated from few plant species[7,8], though adenosine (**2**) is an essential component of all living tissues[9]. Vicenin-2 (**3**) and rutin (**6**) are flavonoid compounds widely distributed in plants[10]. Both narirutin (**4**) and hesperidin (**5**) are flavanones associated with rutaceous plants, although **4** is known so far only from *Citrus*[11,12]. The simultaneous occurrence of these components in nature may be sufficient to account for the specificity of oviposition by *P. xuthus*.

Cabbage butterflies, *Pieris brassicae* are known to lay eggs in response to the presence of sinigrin, a component associated with their host-plants in the family Cruciferae[13]. More generally, however, host-plant specificity in oligophagous insects seems to be controlled by complex mixtures of plant chemicals, as shown in the oviposition stimulants of the carrot fly, *Psila rosae* (Diptera)[14] and the rice weevil, *Sitophilus zeamis* (Coleoptera)[15]. In addition to *P. xuthus*, females of several other Rutaceae-feeding swallowtail butterflies, namely *P. macilentus*[5], *P. protenor*[16,17] and *P. bianor*[17], are known to lay eggs in response to aqueous solutions of host-plant extracts, and Feeny et al.[18] found that oviposition stimulant(s) of *P. polyxenes*, a species that feeds exclusively on plants of the Umbelliferae family, were present in aqueous fractions of carrot leaf extracts. Interestingly, females of *P. polyxenes*[18] as well as another umbellifer-feeding species, *P. machaon hippocrates*[5] respond positively to extracts of rutaceous plants. Umbellifer-feeders are assumed to have evolved from Rutaceae-feeders[1,2]. A systematic study of the chemical ingredients of oviposition stimulants among these closely related species may provide a key to understanding the evolution of host specificity in herbivorous insects.

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