Rapid Paper

Oviposition Stimulant of *Papilio xuthus*, a *Citrus*-feeding Swallowtail ButterflyTakao OHSUGI, Ritsuo NISHIDA
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Methanol extracts of fresh leaves of *Citrus* plants induce oviposition behavior by females of the *Citrus*-feeding swallowtail butterfly, *Papilio xuthus* L. One of the oviposition stimulants was isolated from an extract of *Citrus unshiu* Marc. and identified as 6,8-di-*C*- β -D-glucopyranosylapigenin. The compound was inactive alone, but elicited oviposition behavior when mixed with another component which has not yet been identified.

Papilio xuthus L. is a swallowtail butterfly whose larvae feed exclusively on plants of *Citrus* and some other genera in the family Rutaceae. Females of *P. xuthus* lay eggs on young leaves of their host plants with great precision. Nishida (1977)¹⁾ demonstrated that the same ovipositional response was induced when female butterflies were brought into contact with filter paper that had been treated with methanolic extracts of their host plants (see Fig. 1). The oviposition stimulant(s) appeared to be highly polar and to stimulate chemoreceptors located on the tarsomeres of the female's forelegs.¹⁾ We report here the isolation and identification of one of the oviposition stimulants contained in the leaves of *Citrus unshiu*.

A methanol extract of *C. unshiu* was fractionated into ether, ethyl acetate, *n*-butanol and water layers by solvent extraction. The oviposition activity was found only in the aqueous layer, which was then subjected to C_{18} reverse-phase column chromatography. The activity separated into two different fractions,

corresponding to elution with water (fraction A) and with 30% methanol in water (fraction C). The less polar fraction C was rechromatographed on the same column, eluting successively with water, 20%- and 40%-aqueous methanol, and methanol, each of which contained acetic acid in a concentration of 1%. Fraction CA, eluted without retention, was found to be the only fraction to induce ovipositional behavior, but its activity appeared to be much lower than that of the original fraction C. However, the activity of fraction C was almost fully recovered when fraction CA was combined with the inactive fraction CB, which was eluted from the column with 1% acetic acid + 20% methanol in water. The synergistic compound I in fraction CB was separated by high performance liquid chromatography (HPLC), and then crystallized from methanol to yield a pale yellow amorphous solid (mp 266~268°C).

The ovipositional responses of *P. xuthus* females to filter papers treated with the pure compound I, with fraction CA, and with a mixture of both are shown in Table I. Each component was inactive by itself, but distinct oviposition activity was generated when both were mixed together. The response of females to fraction CA alone was found to be dosage-dependent. The dose chosen for the bioassay

FIG. 1. Ovipositional Response of a *Papilio xuthus* Female to a Piece of Filter Paper Treated with the Methanol Extract of *Citrus unshiu*.

The female curled her abdomen and laid one egg immediately after touching the filter paper with the tarsal chemoreceptors on her forelegs.

TABLE I. OVIPOSITIONAL RESPONSE OF
Papilio xuthus FEMALE TO
COMPOUND I AND
FRACTION CA.

Sample (dose/filter paper)	Response (N=23)
Compound I (vicenin-2) (30 µg)	0%
Fraction CA (0.3 g.l.e.)	0%
Fraction CA (0.3 g.l.e.) + Compound I (30 µg)	83%
Fraction CA (0.3 g.l.e.) + Compound I (10 µg)	70%

Vicenin-2 (I)

reported here was about the minimum that is usually required to elicit oviposition. By contrast with fraction CA, compound I alone failed to stimulate oviposition at any dose.

The molecular weight of compound I was determined to be 594, since three prominent peaks at m/z 595, 617 and 633, corresponding to $M+1$, $M+Na$ and $M+K$, respectively, were observed in its secondary ion mass spectrum (SIMS). Ultraviolet (UV), infrared (IR), and proton and carbon-13 nuclear magnetic resonance (PMR and CMR) spectra of compound I indicated that this compound was a 6,8-di- C -glycosyl derivative of apigenin.²⁾ Comparison of its optical rotatory dispersion (ORD) and circular dichroism (CD) spectra,³⁾ its HPLC retention time,⁴⁾ and of the spectra of its acetyl derivative⁵⁾ with those of an authentic sample permitted Dr. J. Chopin to confirm its identity as 6,8-di- C - β - D -glucopyranosylapigenin (vicenin-2). Vicenin-2 is a flavonoid compound widely distributed in plants,^{2,5~14)} and is already known to occur in the peels of lemon, *Citrus limon* Burm.¹⁴⁾

In addition to *P. xuthus*, there are several other *Papilio* species whose larvae feed exclusively on rutaceaeous plants in Japan. Females of three of these, namely *P. macilentus*,¹⁾ *P. protenor*^{15,16)} and *P. binor*,¹⁶⁾ are known to lay eggs in response to tarsal contact

with aqueous solutions of *Citrus* leaf extracts. Similarly, Feeny *et al.*¹⁷⁾ found that oviposition stimulant(s) of *P. polyxenes*, a species that attacks plants of the family Umbelliferae, were present in the aqueous layer of carrot leaf extracts. Among the authentic chemicals they tested, 7- O -glucosylapigenin was one of the few compounds that induced an apparent ovipositional response, although its activity was very low.¹⁷⁾ A C -glycosylapigenin has been isolated as one of the feeding stimulants for the larvae of the yellow butterfly, *Eurema hecabe mandarina* De L'Orza, from the leaves of its host plant *Lespedeza cuneata* G. Don.^{10,18)} It seems that glycosylflavonoids might have widespread significance as contact stimulants in the host-finding behavior of butterflies.

The isolation of other oviposition stimulants from fraction A and fraction CA in *C. unshiu* extracts is now in progress.

EXPERIMENTAL

SIMS was measured with a Hitachi M-80 mass spectrometer at 8 kV. Xenon was used to provide the primary beam of atoms, and compound I was measured as a suspension in glycerol on the stainless-steel sample stage. UV spectra were recorded with a Shimadzu UV-360 recording spectrophotometer, and the IR spectrum was measured with a Shimadzu IR-400 spectrometer. ORD and CD spectra were measured with a JASCO ORD Model J-5 spectropolarimeter, and with a JASCO J-20 spectrophotometer, respectively. PMR and CMR spectra were measured with a JEOL JNM FX-90Q spectrometer (90 MHz) using TMS as an internal standard. The letters s, d, t, q and m represent singlet, doublet, triplet, quartet and multiplet, respectively.

Bioassay. *P. xuthus* larvae were raised on leaves of *Poncirus trifoliata* (L.), *C. unshiu* or other *Citrus* plants at $24 \pm 2^\circ\text{C}$ (16:8 light:dark photoperiod). Females were hand-paired within 2 days of emergence, fed a 10% sucrose solution daily, and used for bioassay when 3~10 days old. Test samples were applied to a piece of filter paper (approximately 10 cm²) by cutting a round filter paper (Toyo Filter Paper, qualitative No. 2, 9 cm diameter) into 6 equal fan-shaped pieces. Aliquots of 100 µl of each concentration were applied to the filter paper and allowed to dry. Doses are expressed either as gram leaf equivalent per 10 cm² of filter paper (g.l.e./f.p.) or as the absolute amount applied to the filter paper (µg/f.p.). The bioassay chamber (50 × 50 × 50 cm³) was open in front, and its interior was illuminated by a fluorescent lamp (15W)

placed behind a screen at the rear. The inside walls were covered with white polystyrene-foam sheets. Within the chamber, test filter papers were misted with distilled water immediately before the bioassay. Each female butterfly was placed in turn on the test paper and kept in contact with it as much as possible during 30 seconds. Females usually responded immediately to the standard methanol extract of *C. unshiu* (0.03 g.l.e./f.p.), curling their abdomens and eventually laying eggs (see Fig. 1). Any females either giving a positive response to a moistened blank filter paper or giving no response to the standard methanol extract were rejected from the tests. The activity of test samples was evaluated by the percentage of females giving a positive response at various given doses. The bioassay was performed during daylight hours at $24 \pm 2^\circ\text{C}$.

Isolation of compound I (vicenin-2). Fresh leaves of *Citrus unshiu* (Sugiyama-unshiu, 1.2 kg) were collected in Osaka Prefecture on June, 1982, and extracted with methanol (3 liter). Evaporation of the extract gave a viscous brown oil (66.0 g), which was dissolved in water (1.2 liter) and washed with ether (0.8 liter $\times$ 3), followed by ethyl acetate (0.8 liter $\times$ 3) and *n*-butanol (0.8 liter $\times$ 3). Among these four fractions, the water layer (yield 51.0 g) was found to be active: an 85% positive response at 0.01 g.l.e./f.p. and 100% positive response at 0.03 g.l.e./f.p. when tested with 27 females. The water layer was chromatographed on a reverse-phase column (72 g of ODS-W, micro bead silica gel 5D, 100~200 mesh, Fuji-Davison Chemical Ltd., packed in distilled water, 180 mm $\times$ 35 mm i.d.), eluting with 400 ml of water (fraction A), 400 ml of 10% methanol in water (fraction B), 800 ml of 30% methanol in water (fraction C) and 1 liter of 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 in fraction C (a 93% positive response at 0.1 g.l.e./f.p., $N=29$). Fractions B and D appeared to be inactive. Fraction C was reapplied to the same column, which was eluted in sequence with 1 liter of 1% acetic acid in water (fraction CA), 1% acetic acid + 20% methanol in water (fraction CB), 1% acetic acid + 40% methanol in water (fraction CC) and 1% acetic acid in methanol (fraction CD). The synergistic fraction CB was then subjected to a series of preparative HPLC fractionations, monitoring the synergistic activity by mixing each eluate with fraction CA. First, portions of fraction CB were chromatographed on a column of ODS (S-343, 10 μm , YMC-pack, 250 mm $\times$ 20 mm i.d.), eluting with a mixture of methanol, water and acetic acid (30:70:1) at 5 ml/min. The synergistically active zone was found at around $t_R=20\sim22$ min. This fraction was then subjected to a column of ODS (S-5, 5 μm , YMC-pack, 300 mm $\times$ 8 mm i.d.), eluting with the same solvent system as above at a flow rate of 1.5 ml/min. The synergistic principle I gave $t_R=15.3$ min. This was further purified on another reverse phase column (Cosmosil 5-Phenyl, 5 μm , Nakarai Chemicals Ltd., 250 mm $\times$ 10 mm i.d.), again eluting

with the same solvent system. Compound I was isolated at $t_R=15.3$ min. The total yield of compound I from 1.2 kg of the leaves was approximately 20 mg. The combined eluate of compound I was purified further by crystallization from methanol to yield a pale yellow solid (mp $266\sim268^\circ\text{C}$). A portion of the pure compound I was used to determine the biological activity (Table I). SIMS m/z (%): 594 (6), 595 (100), 617 (58), 633 (45). UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (ϵ): 271 (11,800), 328 (13,500); $\lambda_{\text{max}}^{\text{MeOH} + \text{NaOMe}}$ nm (ϵ): 281 (13,300), 332 (9650), 397 (18,000). IR ν_{KBr} cm^{-1} : 3350, 1620, 1570, 1502, 1420, 1355, 1272, 1207, 1172, 1075, 1035, 1020, 925, 827. ORD ($c=0.01$, MeOH) $[\phi]^{20}$ (nm): $-47,500^\circ$ (330), $-17,800^\circ$ (295), $-19,600^\circ$ (265). CD ($c=0.003$, MeOH) $[\theta]_{\text{max}}^{20}$ (nm): $-17,620^\circ$ (231), -8710° (250), $+3565^\circ$ (273), -3170° (284), $+7130^\circ$ (303), $+3760^\circ$ (345). PMR (d_6 -DMSO) δ : 13.69 (1H, broad s), 8.01 (2H, d, $J=8$ Hz), 6.90 (2H, d, $J=8$ Hz), 6.79 (1H, s), 4.8 (2H, m, after D_2O exchange). CMR (d_6 -DMSO) δ (C-H coupling pattern): 182.2 (s), 164.0 (s), 161.1 (s), 160.7 (s), 158.4 (s), 154.9 (s), 128.7 (d), 121.3 (s), 115.8 (d), 107.2 (s), 105.1 (s), 103.8 (s), 102.5 (d), 81.6 (d), 80.7 (d), 78.8 (d), 77.7 (d), 74.0 (d), 73.3 (d), 73.1 (d), 71.8 (d), 70.6 (d), 69.1 (d), 61.2 (t), 59.8 (t).

Acetylation of compound I. Compound I (30 mg) was dissolved in a mixture of pyridine (1 ml) and acetic anhydride (1 ml), and maintained overnight at room temperature. The reaction mixture was mixed with ethyl acetate and washed successively with sat. NaHCO_3 , 1 N HCl and sat. NaCl. The organic layer was dried over anhyd. Na_2SO_4 , and purified through a short column of silica gel (5 g, Wako Gel C-200), eluting with 60% ethyl acetate in benzene, to yield a pale yellow syrup (23 mg). ORD ($c=0.01$, MeOH) $[\phi]^{20}$ (nm): $+4750^\circ$ (353), $-10,100^\circ$ (318), $-24,950^\circ$ (240). CD ($c=0.003$, MeOH) $[\theta]_{\text{max}}^{20}$ (nm): $+8215^\circ$ (255), $+9855^\circ$ (287), $+21,355^\circ$ (332). PMR (d_6 -DMSO) δ : 8.06 (2H, d, $J=9$ Hz), 7.40 (2H, d, $J=9$ Hz), 6.65 (1H, s), 6.0~3.7 (~14H, m), 2.54 (3H, s), 2.48 (3H, s), 2.34 (3H, s), 2.09 (3H, s), 2.06 (3H, s), 2.05 (3H, s), 2.02 (3H, s), 1.98 (3H, s), 1.91 (3H, s), 1.87 (3H, s), 1.75 (3H, s). CMR (d_6 -DMSO) δ : 175.9, 170.6, 170.2, 170.1, 169.6, 169.5, 168.7, 168.0, 167.3, 161.7, 156.9, 153.7, 153.2, 149.6, 128.7, 128.3, 127.8, 122.7, 118.9, 112.1, 108.8, 77.2, 76.9, 74.8, 74.4, 73.9, 72.8, 69.5, 69.1, 68.3, 61.9, 21.5, 21.1, 20.7, 20.6, 20.2.

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