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HAIRPENCIL PHEROMONE COMPONENTS OF MALE ORIENTAL FRUIT MOTHS, *Grapholitha molesta*[1,2]

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Abstract—Male *Grapholitha molesta* hairpencil components are ethyl *trans*-cinnamate, mellein, methyl jasmonate, and methyl 2-epijasmonate. The natural behavioral effect elicited by hairpencil-displaying males during courtship in attracting sex-pheromone-releasing females from several centimeters away can be duplicated by mixtures of ethyl *trans*-cinnamate in various combinations with the other components.

Key Words—Hairpencil, pheromone, *Grapholitha molesta*, Lipidoptera, Tortricidae, Oriental fruit moth, ethyl *trans*-cinnamate, mellein, methyl jasmonate, methyl 2-epijasmonate.

INTRODUCTION

Male Lepidoptera possess an unusually varied array of accessory scent-producing organs, usually groups of elongated, hairlike scales (hairpencils) that are bundled into special pouches, then everted and splayed in the vicinity of a female during courtship (Birch, 1974; Weatherston and Percy, 1977). Studies of most species have revealed, however, that the volatile chemicals from these scales exert a minimal observable effect on female behavior, and the lack of an overt female response has hindered the identification of behaviorally active constituents. In those cases where both the behavior and

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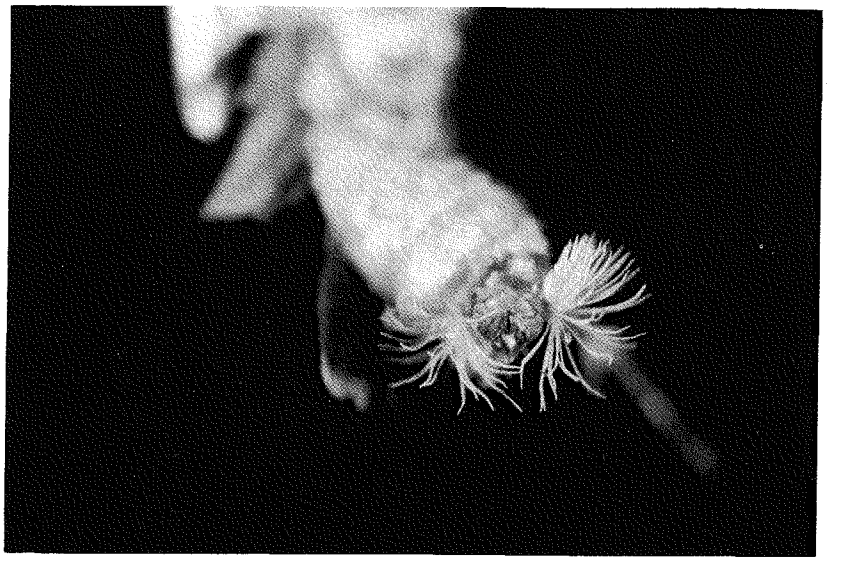


FIG. 1. Male *G. molesta* with extruded abdominal hairpencils.

chemistry have been worked out, the compounds have elicited female "acceptance" through inferred quiescence (Pliske and Eisner, 1969) or abdominal extension (Grula et al., 1980).

Courtship in *Grapholitha molesta* (Busck), the Oriental fruit moth, is thus far unique in the Lepidoptera, in that males attract females after they themselves have been attracted to the female's vicinity by her sex pheromone (Baker and Cardé, 1979). A few centimeters from the female, males turn away and repeatedly extrude and retract their abdominal hairpencils (Figure 1), propelling volatile chemicals over the female with wind generated from wing vibration. The female immediately walks toward the source of the odor and with her head touches the tip of the male's abdomen, evoking from him a copulatory attempt (Baker and Cardé, 1979). The overt movement of females toward displaying males provided a rare opportunity to define a lepidopteran courtship pheromone that attracts females (Baker et al., 1981).

METHODS AND MATERIALS

Crude Pheromone Extract. *G. molesta* males were reared on small green thinning apples on a 16:8 light–dark photoperiod regime at 25° C and variable humidity. Newly emerged males were held 5 days at 25° C (16:8 light–dark), and then each male's paired hairpencils plus claspers were excised and

immersed for several minutes in a vial containing Skellysolve B (Skelly B). The extract was removed from the residue and stored at −20° until use.

Behavioral Assays. Female *G. molesta* moths were held in isolation in 33 × 27 × 31-cm cages on a 16 : 8 light–dark photoperiod regime and transferred onto 3.8 × 3.8-cm sheet-metal platforms during their mating activity period beginning at 3 hr before darkness. The platforms were placed in a 25 × 30 × 5-cm cage in the wind flow beside a 25 × 25-cm sheet-metal observation arena for acclimation to the 20° C, 60–80% relative humidity, 700 lux light intensity, and 71 cm/sec wind. Individual 4 to 5-day-old females on their platforms then were placed onto the observation arena. A laminar wind field was created by cheesecloth baffles placed between the fan and arena.

Chemical treatments were prepared by placing 1 ng of authentic compound or 1 male equivalent (ME) of natural extract in 1 μl Skelly B followed by 5 μl of Skelly B onto a 5 × 7-mm filter paper (Whatman No. 1) skewered to a metal thumbtack. After solvent evaporation, the thumbtack was placed with forceps in the arena so that the treated filter paper hung just above the surface 2 cm upwind of a female. Females were scored as to whether they walked upwind and touched the paper; walked upwind to, but did not touch, the paper; began walking upwind; or did not move at all during a 10-sec exposure to the treatment. One filter paper preparation was used for two females and then discarded. Each day a treatment was tested against 1 ME crude extract and a 5-μl Skelly B blank. Females emitting sex pheromone ("calling") sometimes respond by walking to changes in wind flow (Baker and Cardé, 1979), such as produced by inserting a treatment, and so if crude extract responses were not significantly higher than the solvent blank on a particular day, the whole replicate was repeated. The arena surface and metal plates were washed with acetone between uses.

Electroantennogram Assays. EAG responses were obtained using the techniques previously described (Roelofs, 1977). Test samples (ca. 3 ME) were deposited in glass capillary tubes by evaporating 3 μl of a Skelly B solution in the tube. Samples were assayed by puffing 1 ml of air through the tube and into an airstream flowing over an antenna taken from a 3 to 5-day-old female *G. molesta* moth. A mean response amplitude to each fraction was obtained from nine replications of three repeated puffs each. Amplitudes were read from an oscilloscope or directly from digital display (Bjostad and Roelofs, 1980).

Chemical Analyses. Extracts of 1000 male equivalents were processed by removing the solvent under a stream of nitrogen and analyzing the residue by GLC. GLC was carried out using a 3% OV-101 glass column (methyl silicone on 100–120 mesh Gas-Chrom Q, 2 m × 4 mm ID) and a 3% XF-1150 glass column (cyanoethyl methyl silicone on 100–120 mesh Chromosorb W-AW-BWCS, 2 m × 2 mm ID). Proton magnetic resonance spectra were obtained with a Varian XL 100A (100 MHz) instrument using $CDCl_3$ as solvent and

tetramethylsilane as an internal standard. Mass spectra were recorded with an HP5985A GC-MS using a 30-m OV-101 capillary column.

Microhydrogenations were carried out by bubbling hydrogen vigorously through a solution of the sample (ca. 0.5 μg) in Skelly B(100 μl) containing a catalytic amount of platinum oxide at 0° C for 2 min. Ozonolyses were conducted by bubbling ozone through a syringe needle into a solution of sample (ca. 1 μg) in CS_2 (100 μl) for 30 sec. The reaction vial was held in a dry ice-acetone bath throughout the reaction. Excess triphenylphosphine was added and the reaction mixture concentrated under nitrogen for GC analysis. Epimerization of methyl 2-epijasmonate (IV) to methyl jasmonate (III) was effected by adding 2 μl of 1% *p*-toluenesulfonic acid in Skelly B to a solution of 20 ng of IV in Skelly B. The mixture was held at room temperature overnight, and then it was concentrated under nitrogen for GLC analysis.

Chemicals. Ethyl *trans*-cinnamate was obtained from Aldrich Chemical Co., and ethyl *cis*-cinnamate was prepared according to the procedure by Wittig and Haag (1955) to yield a 1:4.5 ratio of *cis–trans*. The mixture was separated by column chromotography using a 15% $AgNO_3$-Florisil column eluted with 10% ether in Skelly B. Ethyl 3-phenylpropanoate was obtained by Jones oxidation of 3-phenyl-1-propanal (Aldrich Chem. Co.) followed by ethylation with ethanol in the presence of a catalytic amount of sulfuric acid.

Samples of (+)- and (−)-mellein were obtained from Drs. D. Aldridge (ICI), N. Davis (Auburn U.), and J. Moore (U. North Alabama). (±)-Methyl jasmonate was provided by International Flavor and Fragrances, and methyl 2-epijasmonate was isolated from lemon peels (Nishida, Acree, and Roelofs, unpublished).

RESULTS

Isolation, Bioassay, and Identification of Hairpencil Components. Crude hairpencil extract from 5000 male *G. molesta* was processed and fractionated on the OV-101 GLC column (150° C) into 12 fractions (Figure 2). EAG assay (Figure 3) of these fractions showed female antennal response (0.6 mV ± 0.2 SD; $N = 9$) only for fraction 3, although the crude extract elicited a much higher response (1.25 mV ± 0.31 SD; $N = 9$).

Behavioral assays were used to test the fractions for their attractiveness to calling females in an arena in moving air. Only fraction 3 produced a significant amount (29%: $N = 35$) of upwind walking by females compared to the solvent blank (3%). After identification of the only component (compound I) found in fraction 3, synthetic I was added to the GLC fractions and various combinations thereof.

A blend of I (10 ng) with fractions 6 and 7 (3 ME each) gave significantly greater attraction of females (80% response; $N = 40$) than I (10 ng) alone

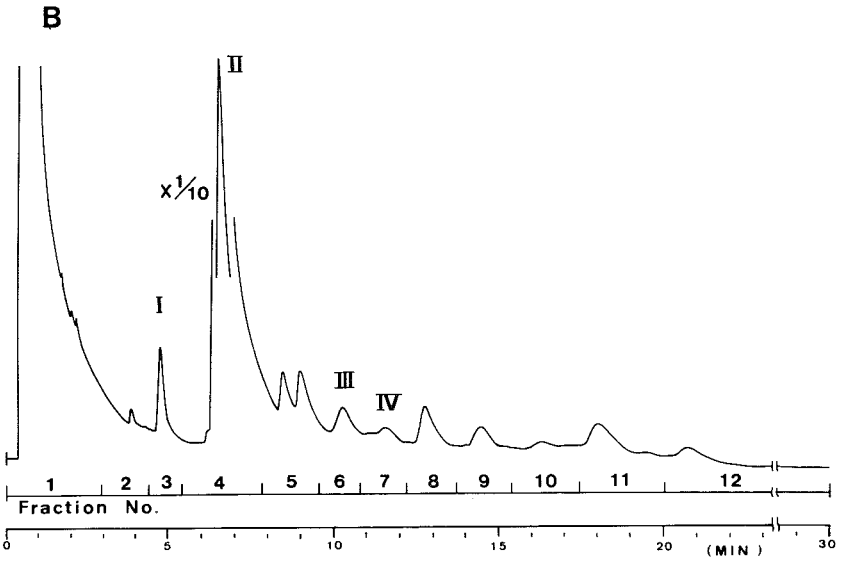


FIG. 2. GLC (OV-101) tracing of male *G. molesta* hairpencil extract.

(45%) (significantly different at the 5% level according to a χ^2 2×2 test of independence with Yates' correction). The response to a Skelly B blank ($N = 40$) was 13%. The components of fractions 6 and 7 were labeled compounds III and IV, respectively.

The predominant compound was found in fraction 4. This fraction was inactive by itself in bioassays, but this major hairpencil compound was labeled compound II and identified for testing in combination with the other active components.

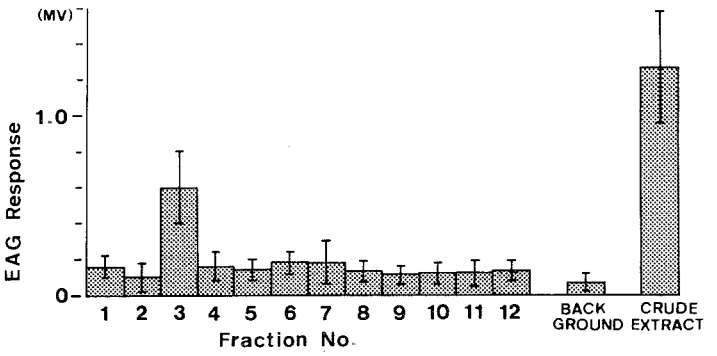
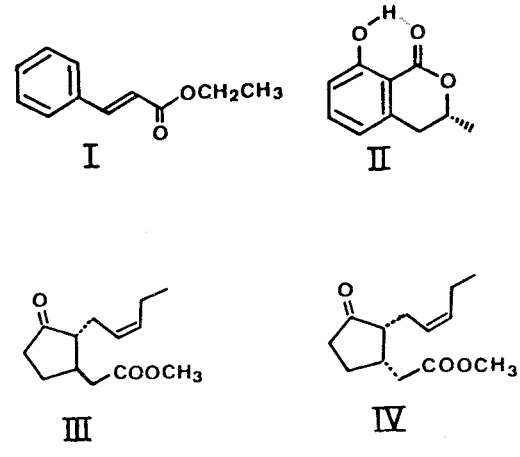


FIG. 3. Mean EAG response amplitudes to GLC fractions (Figure 2). $N = 27$; nine replicates of three responses to each fraciton in a randomized, complete-block design.

Compound I. The only observable compound in fraction 3 was present at a rate of 0.5 ng/male. The retention times on OV-101 and XF-1150 (4.8 and 3.9 min, respectively) were identical to those of ethyl *trans*-cinnamate, with no evidence of the *cis* isomer (3.3 and 2.9 min, respectively). The mass spectrum of compound I (Figure 5A) was identical to that of synthetic ethyl *trans*-cinnamate, as was the UV spectrum [λ_{max} 270 nm (ϵ 15,000) in Skelly B]. Additional evidence in identifying compound I to be ethyl *trans*-cinnamate (Figure 4) was that catalytic hydrogenation of compound I produced a product with GLC retention times identical to ethyl 3-phenylpropanoate on OV-101 and XF-1150.

Compound II. Compound II was isolated from fraction 4 at a yield of 20 ng/male. Removal of the solvent left a fine crystalline residue that was shown to be *R*-(−)-mellen (II, Figure 4) by instrumental analyses. A mass spectrum (Figure 5B) of compound II exhibited a molecular ion peak at 178, which was similar to that of authentic mellein (Brand et al., 1973). A UV spectrum of compound II in ethanol exhibited absorption bands at λ_{max} 210 nm (ϵ 20,000), 247 nm (ϵ 4000), and 313 nm (ϵ 5000), all of which showed a bathochromic shift in the presence of sodium hydroxide to λ_{max} 228 nm (ϵ 16,500), 252 nm (ϵ 4500), and 350 nm (ϵ 7000). The NMR spectrum was identical to that of an authentic sample. One proton singlet at δ 11.03 suggested the presence of a phenolic proton hydrogen-bonding with a carbonyl oxygen at an *ortho* position. Three aromatic protons were indicated in adjacent positions, since a proton at δ 7.41 (1H, double doublet, J = 7.3 and 8.4) was coupled with a proton at δ 6.90 (1H, doublet, J = 8.4) and at δ 6.70 (1H, doublet, J = 7.3). One methine proton at δ 4.73 (1H, sextet, J = 7) was indicated to be adjacent



I II III IV

FIG. 4. Structures of the four components isolated from *G. molesta* hairpencils (Figure 2).

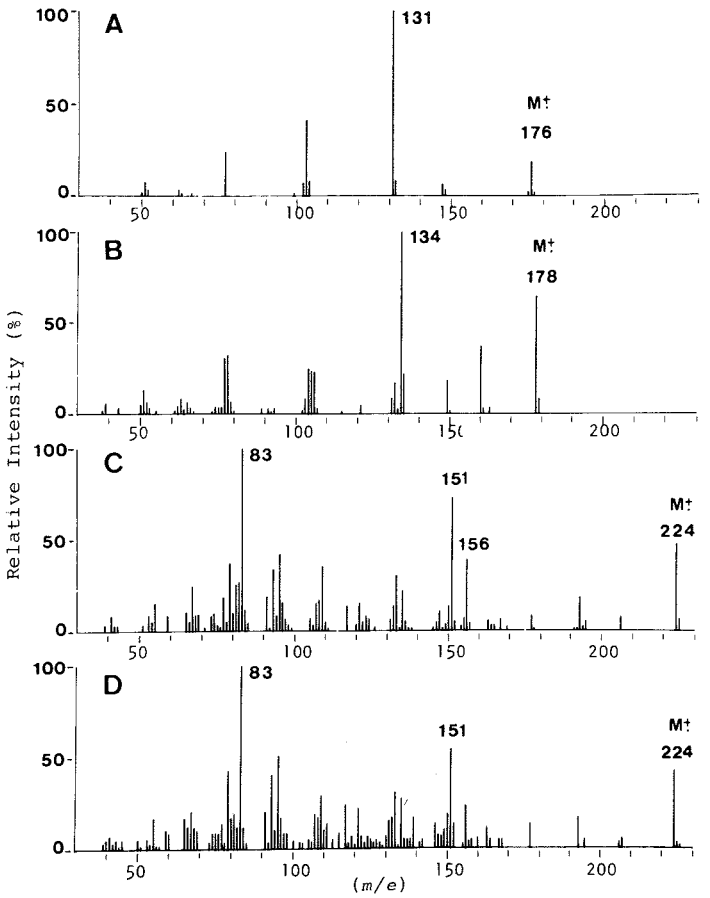


FIG. 5. Mass spectra of components isolated from *G. molesta* hairpencils.

to an oxygen atom and was coupled with secondary methyl protons at δ 1.53 (3H, doublet, $J = 6.3$) and benzylic protons at δ 2.93 (2H, doublet, $J = 7.0$). The optical rotation of II gave a negative sign $[\alpha] = -133°, c = 0.01$ in $CHCl_3$. This confirmed II to be *R*-(−)-mellein, since Arakawa et al. (1969) had determined that the absolute configuration of (−)-mellein was *R*.

Compounds III and IV. Fractions 6 and 7 (Figure 2) were characterized by a distinct herbal odor, similar to that obtained when the male *G. molesta* hairpencils are extruded. Compound III was isolated from fraction 6 (0.3 ng/male) by collection from the XF-1150 column and found to be methyl jasmonate (Figure 4, III) by the physical data and comparison to an authentic sample. The natural component was identical to that of an authentic sample of methyl jasmonate possessing a Z double bond (Table 1). The mass spectrum

TABLE 1. GLC (OV-101) RETENTION TIMES (MIN) OF HAIRPENCIL COMPONENTS III AND IV (FIGURE 2), THEIR DERIVATIVES, AND SOME SYNTHETICS

	III	Me jasmonate	*E* isomer	IV	Me 2-epijasmonate
Untreated	10.2	10.2	9.9	11.6	11.6
Hydrogenated	10.5	10.5		12.0	12.0
Ozonolysis-aldehyde	6.0	6.0			6.2
Epimerized				10.2	10.2

(Fig. 5C) exhibited a molecular ion at *m/e* 224 and was identical to that of the authentic sample. Ozonolysis of component III produced an aldehyde that was identical by GLC (Table 1) and mass spectrometry (M^+ 198) to that obtained by ozonolysis of the synthetic sample. Hydrogenation of component III produced a product whose mass spectrum (M^+ 226) and GLC retention time on OV-101 (Table 1) was identical to that of methyl dihydrojasmonate.

Compound IV was isolated from fraction 7 (0.01 ng/male) by collection from the XF-1150 column and identified to be methyl 2-epijasmonate. GLC retention times (Table 1) of the natural compound and its hydrogenated product were identical to those of authentic sample. Mass spectra of the natural sample and of its ozonolysis product were similar to those of the authentic samples, and compound IV was readily epimerized to III in the

TABLE 2. BEHAVIORAL RESPONSE OF *G. Molesta* FEMALES TO COMPOUNDS ISOLATED FROM MALE HAIRPENCILS[a]

	No. females walking upwind			No. females walking to source			No. females touching source		
Treatment	Treatment	Crude	Blank	Treatment	Crude	Blank	Treatment	Crude	Blank
I	11*	11*	4	7	10*	3	3	8	3
II	3	11	4	1	9	3	1	8	2
III	4	11*	2	1	10*	0	1	10*	0
IV	4	14*	3	4	10*	3	1	9	3
I+II	6	13*	3	4	12*	1	2	12*	1
I+III	5	8	2	3	6*	0	1	5	0
I+IV	16*	13*	2	12*	11*	2	10**	11*	2
I+II+III	12*	11*	0	7*	6*	0	6	3	0

[a]Treatments were 1 ng of each authentic compound and 1 ME for crude. $N = 20$ for each treatment. Responses are significantly different from the blank under the same behavior if followed by $^*(P < 0.05)$ according to a χ^2 2×2 test to independence with Yates' correction. Response under same behavior is significantly greater ($P < 0.05$) than to I alone if followed by **

presence of *p*-toluenesulfonic acid (Table 1) (Demole and Stall, 1962; Tanaka and Torii, 1975; Fukui et al., 1977). Mixtures of authentic III and IV had the characteristic odor of extruded hairpencils.

Biological Activity of Hairpencil Compounds. Authentic samples of compounds I–IV were tested alone and in combination for behavioral response activity with female *G. molesta* (Table 2). Compound I was the only synthetic to elicit significant female attraction, although it was less active than crude extract in behavioral responses of walking to the source and touching the source. Combinations of I + IV and of I + II + III were as active as crude extract in eliciting all behavioral responses.

DISCUSSION

This is the first report of characterized male moth hairpencil compounds that are behaviorally active in attracting females. Previous studies have identified the volatile chemicals extracted from hairpencils (Meinwald et al., 1966, 1969, 1971, 1974; Aplin and Birch, 1970; Culvenor and Edgard, 1972; Edgar et al., 1973, 1979; Grant et al, 1972; Jacobson et al., 1976; Bestmann et al., 1977; Petty et al., 1977) or other specialized scales (Lundgren and Bergstrom, 1975; Edgar et al., 1976; Honda, 1980) without knowledge of the behavior these compounds elicited. Other studies attempted to define the behavioral roles played by scent through ablation (Myers and Brower, 1969; Birch, 1970; Grant, 1974, 1976; Gothilf and Shorey, 1976; Hirai, 1977; Thibout, 1978; Ono, 1979; Rutowski, 1977, 1980), trapping experiments (Dahm et al., 1971; Finn and Payne, 1977), or observational inference (Clearwater, 1972; Pliske, 1975; Barrer and Hill, 1980).

Female EAG responses were helpful in determining the GLC area of activity later found to be a behaviorally active compound I, ethyl *trans*-cinnamate. Fraction 3 (I) was the only significantly EAG-active area from extracts (Figure 2). Female EAGs have been used in previous studies to measure responses to potentially behaviorally active male-produced compounds (Schneider and Seibt, 1969; Grant, 1970, 1971; Birch, 1971; Payne and Finn, 1977; Chow et al., 1980).

Although the behavioral activity of compounds I and IV is clear from the data, additional studies are needed to determine whether II and III further increase activity when added to the other two components. Both II and III were inactive alone or individually in combination with I, but in a trinary blend with I, elicited significant upwind movement from females.

The percentages of attraction of females might at first appear unduly low. However, even during normal courtship, many females do not respond to displaying males (Baker and Cardé, 1979). Also, a pulsed wind of 45–90 cm/sec did not accompany the chemical treatments as it does during a display; this additional wind significantly increases female locomotion (Baker and

Cardé, 1979). We do not know whether a pulsed pheromone emission pattern, most likely the case during display, would further increase the percentage of responding females.

The four identified compounds have been reported previously in the literature, but have heretofore not been found in the Lepidoptera. Ethyl *trans*-cinnamate is similar to 2-phenylethanol, which has been found in the hairpencils of a number of lepidopteran species (Aplin and Birch, 1970; Jacobson et al., 1976; Bestman et al., 1977; Edgar et al., 1979). Mellein is a known fungal metabolite (Nishikawa, 1933; Yabuta and Sumiki, 1933; Patterson et al., 1966; Aldridge et al., 1971; Cole et al., 1971) and has been found in a number of ant species (Brand et al., 1973). Methyl jasmonate is a constituent of jasmine oil and known in the perfume industry as the "queen of aroma." It closely resembles *cis*-jasmone, which was identified from male *Amauris ochlea* butterfly hairpencils (Petty et al., 1977).

Although methyl 2-epijasmonate can be isolated from lemon (Nishida, Acree, and Roelofs, unpublished) and methyl jasmonate from jasmine, and could possibly be present in fruits fed upon by *G. molesta* larvae, it is not known whether *G. molesta* males are dependent on their various fruit and nut tree host species for immediate precursors to the hairpencil compounds, which produce an herbal-like odor. Preliminary studies indicate *G. molesta* hairpencils from males reared on an artificial diet do not possess the characteristic herbal odor of hairpencils from males reared on the usual small green apples. Elimination of hairpencil extrusion prevents attraction of females (Baker and Cardé, 1979), and it is possible that a reduction of these compounds due to larval feeding on deficient hosts might affect male mating success. Danaidone, known to increase mating success in *Danaus gillippus* males (Pliske and Eisner, 1969) and apparently also a constituent of the hairpencil secretion of *D. chrysippus* (Meinwald et al., 1971), likely is metabolized from dihydropyrrolidizine alkaloids, which the latter adult males ingest when visiting dried *Heliotropium* plants (Edgar and Culvenor, 1975). Rigorous work would be needed to determine first whether the *G. molesta* courtship pheromone components are present in their fruit hosts and then whether they are ingested and sequestered through to adulthood and used as the odorous components that attract females.

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