A Fungal Metabolite as the Male Wing Gland Pheromone of the Bumble-Bee Wax Moth, *Aphomia sociella* L.

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3,4-Dihydro-9-hydroxy-3-methylisocoumarin [*R*(−)-mellein] was identified as the major component of the male wing gland pheromone of the bumble-bee wax moth, *Aphomia sociella* L. (Pyralidae, Galleriinae). The presence of *Aspergillus ochraceus* Wilhelm, a mellein producing microorganism, was detected in the intestines of last-instar larvae and in bumble-bee nests. Arguments for a new type of insect-fungus relationship are presented.

Most sex pheromones identified in female moths until now are straight-chain alcohols, acetates or aldehydes with 12–18 carbon atoms and one or two double bonds. A general biosynthetic pathway involving Δ^{11} desaturation and chain-shortening of hexadecanoic acid as key steps, which explains most structural features, has been proposed [1].

Slightly differing results have been obtained in the study of the biosynthesis of (*E*)-11-tetradecenal in the Eastern spruce budworm *Choristoneura fumiferana* [2]. Labelling studies suggest a *de novo* formation of long-chain aliphatic compounds from acetate *via* an alcoholic precursor. On the other hand, a similar investigation of the biosynthesis of (*Z*)-11-hexadecenal, a pheromone component of the rice stem borer, *Chilo suppressalis,* provided evidence for a pathway beginning with the reduction of hexadecanoic acid into the corresponding aldehyde followed by introduction of the double bond in position 11 [3].

Some of the above mentioned differences in the biosynthetic pathways of Lepidopterous sex pheromones may be due to different feeding techniques, and an unified biogenetic scheme for long-chain aliphatic pheromones, which constitute the most prevalent class of compounds, may exist.

We wish to report evidence for a totally different source of pheromones in Lepidoptera. In the course of our study of several Galleriinae species [4–6], we investigated the behaviour and chemical communication of *Aphomia sociella* L., which is a major parasite of useful, pollinating insects such as bumble-bees. The predatory effect is exerted by the caterpillar which not only destroys the waxy material of the nests but also feeds on eggs, larvae, and pupae of the host [7].

Female-emitted pheromones are well-known in Pyralid subfamilies such as the Phycitinae, Pyraustinae or Crambinae, but they do not occur in Galleriinae. In this subfamily, male pheromones emitted by wing glands [8] evoke searching behaviour by females [4–11].

Olfactometric tests indicated that the wing gland extracts of male *A. sociella* were active on females. A GC/MS analysis of the hexane extract of 120 wing glands revealed the presence of only two volatile compounds. The first one, a minor constituent (< 5%), showed a molecular peak at $m/z = 152$ and fragments at $m/z = 84$ as well as a base peak at $m/z = 69$. This compound could be identified, by comparison with an authentic sample, as (*Z*)-2,6-nonadien-4-olide (**1**) ($C_9H_{12}O_2$) previously isolated from *Aphomia gularis* Zeller by Y. Kuwahara [12].

The identity of the major constituent with (*R*)-mellein (ochracin) (**2**) was established by comparing its physical constants (GC-retention time, mass spectrum, and circular dichroism) with those of an authentic sample [13–14].

Pure (*R*)-mellein, tested (5 μg) alone on 2 days-old virgin females, evoked the same searching behaviour as the crude extract, thus confirming its role as the major component of the wing-gland pheromone of *A. sociella.*

Mellein (**2**) has previously been found in insect secretions on several occasions. For instance, a blend of volatile compounds including mellein (**2**) is released by the hair-pencil organs of the male Oriental fruit moth, *Grapholita molesta* Busck, which exerts a close-range attraction on calling females [15]. Mellein (**2**) has also been detected in the male mandibular gland secretions of some carpenter ant species (*e.g. Camponotus pennsylvanicus, C. herculeanus*

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and *C. noveboracensis* [16], and in the gaster of *Rhytidoponera metallica* [17].

In spite of the fact that mellein (**2**) has been found several times in various insect secretions, the presence in insects of a typical fungal metabolite [18] was intriguing, especially since an entomogenous strain of *Aspergillus flavus* has been isolated from pupae of the wax moth, *Galleria mellonella*, another Galleriinae species [19].

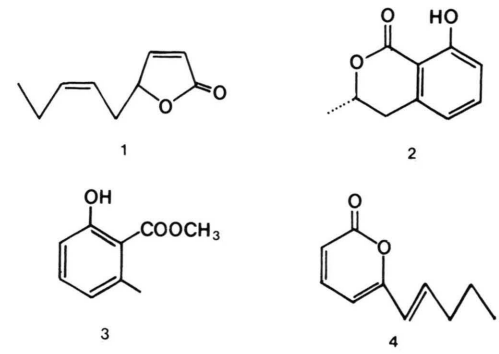
These observations prompted us to look for a possible exogenous origin of mellein (**2**) in *A. sociella*. A systematic search for mellein-producing microorganisms was undertaken at various instars and with adult males of *A. sociella*.

When wing glands of adult male moths were carefully clipped under sterile conditions and maintained in pure culture on Czapek's agar medium, no fungus could be detected. A parallel search for fungi in the intestines of adult males was equally unsuccessful. In contrast, several fungus strains could be isolated from the alimentary canals of last instar larvae. One of these strains was identified as *Asp. ochraceus* Wilhelm [20].

In order to check its capability to produce mellein (**2**), *Asp. ochraceus* was cultivated on Fries's modified liquid medium for one month [21]. Mellein (**2**) was identified by R_F on TLC, its characteristic bright fluorescence at 366 nm, GC-retention time, and its diagnostic mass spectral fragmentation.

The presence of a widespread mellein producing microorganism in the alimentary canals of last-instar larvae of *A. sociella* may suggest a biosynthesis of the wing gland pheromone by a fungus present in the intestines. The passage of exogenous chemicals through the gut walls of insects is not without precedent. Male *Danaus* butterflies obviously use dietary alkaloids to synthesize the chief volatile component of their scent organs [22].

Laboratory-reared *A. sociella* feed on a non-sterilized diet of pollen and wax. A sample of a bumble-bee nest was maintained in pure culture on Czapek's agar medium and a mellein-producing *Asp. ochraceus* was detected among several other fungal strains. It should be noted that the chromatographic analysis of a methylene chloride extract of a bumble-bee nest did not provide any evidence of mellein (**2**) in the food of larvae. Thus, it seems that a widespread microorganism *(Asp. ochraceus)*, which is introduced by bumble-bees into their honey-combs synthesizes the sex-pheromone of a parasite



(A. sociella), which feeds on infested pollen and wax. The exact mechanism by which one of the metabolites of the fungus ends up in the wing glands of male bumble-bee wax moths remains unknown.

The biosynthesis of insect sex pheromones by microorganisms will most probably remain a rare event. But the origin of mellein (**2**) and other fungal metabolites like (**3**) [23] and (**4**) [24] in insect secretions (particularly of social insects) deserves certainly reconsideration. The role of microorganisms in the synthesis of aggregation pheromones was thoroughly investigated. For example, *Bacillus cereus* isolated from *Ips paraconfusus* guts converts α-pinene to *cis*-verbenol, one component of the aggregation pheromone of this species [25]. Woods decayed by the fungus *Lenzites trabea* produce (*Z*)-3,(*E*,*E*)-6,8-dodecatrienol-1, which is also found to be used as a trail pheromone by the termite *Reticulitermes virginicus* [26]. A close examination of the habitat of many social insects may provide evidence for a new type of relationship between insects and fungi, in addition to the well explored use of "fungus gardens" for nutrition [27, 28].

Materials and Methods

Insects

Laboratory-reared *Bombus* nests were exposed outdoor until colonization by *A. sociella* larvae was observed. The caterpillars were fed with bumble-bee wax and pollen.

Histological investigations revealed that the male moths possess a gland located on the basal part of the forewing. These glands were carefully clipped and soaked in hexane for 2 h at room temperature. The resulting extract was then filtered and reduced to about 100 µl under N_2 flow.

Each female moth to be tested was placed inside a small cylindrical box (5 cm × 8 cm diameter). Crude extracts or synthetic compounds were blown over the female through a pasteur pipette. The female moth which responded positively to the stimulation had to climb up the inner walls of the box and escape in less than 2 min. This climbing behaviour is very similar to that of *Eldana saccharina* [4].

Analyses

Crude extracts and pure compounds were studied by gas chromatography (GC) (Fractovap 2900 Carlo Erba, capillary column 25 m, CPSil 5 CB, isothermally operated at 140 °C). Micropreparative GC was performed on a 2 m (⅛″) column packed with 3% SE-30 on GCQ at 150 °C (Perkin-Elmer 3920). Gas chromatography-mass spectrometry (GC-MS) analysis was accomplished on a Ribermag R-10-10C (Nermag) equipped with a Sidar data acquisition system.

The fungus cultures were separated from the medium by ultrafiltration. The medium was then extracted twice with ethyl acetate. The organic layer was dried over anhydrous $MgSO_4$ and evaporated after filtration. The crude extract obtained was then purified by preparative thin-layer chromatography (hexane : ethyl acetate = 9:1).

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