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Clarification of the role of 4-methylnonanol, female-produced sex pheromone of the yellow mealworm beetle, *Tenebrio molitor* (Coleoptera: Tenebrionidae)

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ABSTRACT

The objective of this study was to clarify the role of (4*R*)-(+)-4-methyl-1-nonanol (MNol), a female-produced sex pheromone, in mediating the mating behavior of male *Tenebrio molitor* L. beetles. The abilities of MNol and authentic female extract (FE) to attract virgin males and to elicit copulatory behavior (CB) from the males were determined by bioassay. Hollow glass rods were used to mimic females. The samples of interest were assayed by coating each either on the outside or on the inside surface of the hollow rods, in order to determine if the responses were due to volatile pheromones (which would be perceived whether coated on the inside or the outside of the rods) or to "contact" pheromones (which would be perceived only when coated on the outside of the rods). The responses of virgin male beetles (6–11 days post-emergence) to FE, MNol, or pentane (control) were compared at both high and low concentrations ($n = 10$ for each treatment). MNol and the FE showed a similar ability to attract males, at either high or low concentrations. At high concentrations, both MNol and the FE elicited CB from the males. However, unlike the FE, at low concentrations MNol was unable to elicit CB from the males. Thus the FE must contain at least one separate "copulation pheromone" that can elicit the CB at low concentrations. Furthermore, the observed "copulation pheromone" must be volatile, since no difference in response was observed whether the FE was coated on the inside or on the outside surface of the hollow rods. Thus the biological role of MNol is likely to attract males to the females and, once the male is in the immediate vicinity of the female, to act in concert with other pheromones (of which at least one is volatile) to facilitate copulation.

1. Introduction

The yellow mealworm beetle, *Tenebrio molitor* L., is a cosmopolitan stored-products pest, with infestations usually originating in poorly stored animal feed and stored grain (Sinha and Watters, 1985). The yellow mealworm produces several different types of pheromones, including both primer pheromones that stimulate reproductive maturation (Happ et al., 1970), and releaser pheromones that mediate aggregation (Weaver et al., 1989), mating (reviewed in Plarre and Vanderwel, 1999; Bryning et al., 2005), and epideictic (Weaver et al., 1989, 1990) behaviors. One of the female-produced sex pheromones has been identified as (4*R*)-(+)-4-methyl-1-nonanol (MNol) (Tanaka et al., 1986, 1989). There are several reports regarding the role and regulation of the female-produced "sex pheromone" in the yellow mealworm beetle (reviewed in Plarre and Vanderwel, 1999). In most of these studies pheromone production was quantified by bioassay, since they were conducted before Tanaka et al. (1986, 1989) identified MNol as a female-produced sex pheromone. Different studies used different behavioral responses for their bioassays, confounding unambiguous interpretation of the results. Most of the studies quantified sex pheromone in terms of the ability of the female or female extract to elicit copulatory behavior (CB) from the males (Tschinkel et al., 1967; TschinkeL, 1970; Menon, 1970; August, 1971; Menon and Nair, 1972, 1976; Hurd and Parry, 1991) or the ability either to attract the males or to elicit CB (Happ and Wheeler, 1969; Happ, 1970; Happ et al., 1970). A small proportion of the studies

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reported the ability of females or female-extracts to attract males, but not necessarily to elicit CB (Valentine, 1931; Happ, 1969; Tanaka et al., 1986). In their preliminary evaluation of the role of MNol, Tanaka et al. (1986) reported that MNol serves as a male attractant, with the ability to synergize the activity of a separate pheromone that elicits CB. Thus there is no direct evidence that the "sex pheromone" examined in the earlier studies was in fact MNol: rather, it is likely that the activities of at least two pheromones (an attractant and one that elicits CB) were studied.

The objective of this study was to clarify the role of MNol in mediating the mating behavior of the yellow mealworm, by comparing the abilities of MNol and authentic female extract (FE) to elicit CB from the males and/or to attract the males. MNol and FE were assayed at two concentrations: the concentration that would occur at a female beetle (one "female equivalent", or FEQ) and a lower concentration that would occur some distance from the female (0.07 FEQ, selected as it is roughly equivalent to the dose used in many earlier bioassay-based studies). Using a modification of the procedure of Tschinkel et al. (1967), hollow glass rods were used to mimic females. Samples of interest were assayed by coating each either on the outside or on the inside surface of the rods, in an attempt to determine if the responses were due to volatile or "contact" pheromones (which would not be directly accessible when placed inside the rod).

2. Methods

2.1. Chemicals

All solvents were purchased from Mallinckrodt (Kentucky, USA) and were of spectrophotometric grade or better. 2-Bromopropene (99%), pentanal (97%), trimethylorthoacetate (TMOA) (99%), palladium on charcoal (5% Pd), silica gel (Merck grade 60, 230–400 mesh, 60 Å) and lithium aluminum hydride (95%) were obtained from Aldrich (Milwaukee, Wisconsin, USA). Florisil (100–200 mesh) and all other reagents were obtained from BDH (Toronto, Ontario, Canada) or Mallinckrodt (Kentucky, USA), and were of ACS grade or better.

MNol was synthesized through a Claisen orthoester route according to the synthetic scheme outlined in Fig. 1. Intermediates were purified by column chromatography or distillation to provide a final product that was >99% pure as determined by gas chromatography (GC). GC-mass spectroscopy (chemical ionization), ^{1}H and ^{13}C nuclear magnetic resonance spectra corresponded with those of an authentic standard and with literature values (Tanaka et al., 1986, 1989; Carpita et al., 1989; Kitahara and Kang, 1994; Ismuratov et al., 2003).

2.2. Rearing of insects

T. molitor larvae were purchased from Northwest Scientific Supply Ltd. (Victoria, British Columbia, Canada). The insects were maintained in an incubator at 25 °C with a 12 h light/12 h dark reverse photoperiod and a humidity of 20–30%. Moist paper towels were spread over the colonies in order to provide moisture to the insects. The larvae were fed a diet of wheat bran, rolled oats, and wheat germ (4:2:1 by volume). Pupae were removed from the colonies and separated by sex by examining the developing genitalia behind the 7th visible abdominal sternite (Bhattacharya et al., 1970). Males and females were maintained separately, in order to ensure virginity. Mature virgin females and males 5–12 days post-emergence were used for experiments. Only individuals that appeared healthy, with no visible physical deformities, were selected for experiments.

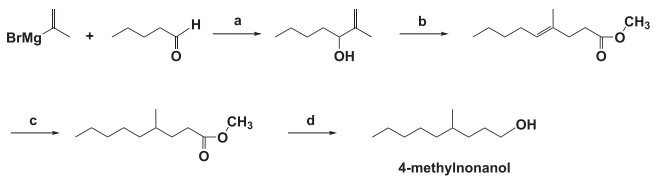

Fig. 1. Synthesis of 4-methylnonanol. (a) Grignard reaction in tetrahydrofuran (reflux); (b) 30 h reflux with trimethylorthoacetate and catalytic quantity of propanoic acid in a modified procedure of Johnson et al. (1970); (c) reduction with hydrogen gas over palladium on charcoal in ethanol; (d) reduction with lithium aluminum hydride in diethyl ether at 0 °C.

2.3. Quantification of MNol in female extracts

Four virgin females were placed in 2 mL pentane containing 200 ng of the internal standard 1-decanol. The contents were vortexed for 1 min and then allowed to stand at RT for 15 min, then vortexed again for 1 min. After removal of the beetles, the extract was carefully concentrated to about 5 μL (on ice in a glass conical vial) with a gentle stream of nitrogen gas. The sides of the vial were occasionally rinsed down with small amounts of pentane. The MNol in each sample was quantified relative to the internal standard using a Hewlett-Packard Gas Chromatography/Mass Selective Detector (5970 Series) equipped with an SP-1000 column (15 m × 0.25 mm ID, 0.25 μm film thickness). The sample was introduced through splitless injection, and separated with initial oven temperature of 50 °C, which was held for 1.0 min and then ramped to 180 °C at 5 °C/min, where the temperature was held for 15 min.

2.4. Sample preparation

Forty nanograms of MNol (10 μL of 4 μg/mL MNol in pentane) was used as equivalent to one female (1 FEQ) of MNol, while 2.8 ng MNol (10 μL of 0.28 μg/mL MNol in pentane) was used as equivalent to 0.07 females (0.07 FEQ).

To prepare each replicate of 0.07 FEQ FE, seven virgin female beetles were weighed and placed in 1.0 mL of pentane. The mixture was vortexed 30 s, left to stand for 1 min, then vortexed for 15 s every min for 4 min, left to stand for 15 min, and vortexed once more for 15 s before the extract was removed. Samples were either placed on ice for immediate use, or stored for a maximum of 24 h at − 20 °C. Ten microlitres of the extract was considered equivalent to 0.07 females.

To prepare each replicate of 1.0 FEQ FE, 10 female beetles were weighed and placed in 1.5 mL of pentane. The beetles were extracted as described above, then the extracts were placed into a conical tube on ice and carefully concentrated to 100 μL with a gentle stream of nitrogen gas (while washing down the sides of the tube). Ten microlitres of the concentrated extract was used as equivalent to one female.

The weights and ages (days post-emergence) of the females used to prepare the extracts were recorded.

2.5. Bioassays

Bioassays were performed under a dim red light, 6–11 h into the scotophase. The temperature and humidity at the time of each assay were recorded, and varied from 23 to 26 °C and 47–53%, respectively. All samples were coded so that the identities were unknown to the observers (so as to limit personal bias), and the samples were assayed in random order. The responses to solvent, FE and MNol were assayed in two experiments, one using 0.07 FEQ and the other using 1.0 FEQ (see below). Using a modification of the