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Anatomical localization and stereoisomeric composition of *Tribolium castaneum* aggregation pheromones

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Abstract We report that the abdominal epidermis and associated tissues are the predominant sources of male-produced pheromones in the red flour beetle, *Tribolium castaneum* and, for the first time, describe the stereoisomeric composition of the natural blend of isomers of the aggregation pheromone 4,8-dimethyldecanal (DMD) in this important pest species. Quantitative analyses via gas chromatography—mass spectrometry showed that the average amount of DMD released daily by single feeding males

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S. Tamogami Technical Research Institute, T. Hasegawa Co., Ltd., 29-7 Kariyado, Nakahara-ku, Kawasaki-shi, Kanagawa 211-0022, Japan of T. castaneum was 878±72 ng (SE). Analysis of different body parts identified the abdominal epidermis as the major source of aggregation pheromone; the thorax was a minor source, while no DMD was detectable in the head. No internal organs or obvious male-specific glands were associated with pheromone deposition. Complete separation of all four stereoisomers of DMD was achieved following oxidation to the corresponding acid, derivatization with (1R, 2R)- and (1S, 2S)-2-(anthracene-2,3-dicarboximido) cyclohexanol to diastereomeric esters, and their separation on reversed-phase high-performance liquid chromatography at -54°C. Analysis of the hexane eluate from Porapak-Ocollected volatiles from feeding males revealed the presence of all four isomers (4R,8R)/(4R,8S)/(4S,8R)/(4S,8S) at a ratio of approximately 4:4:1:1. A walking orientation bioassay in a wind tunnel with various blends of the four synthetic isomers further indicated that the attractive potency of the reconstituted natural blend of 4:4:1:1 was equivalent to that of the natural pheromone and greater than that of the 1:1 blend of (4R,8R)/(4R,8S) used in commercial lures.

Keywords Chirality 4,8-Dimethyldecanal · Insect · Red flour beetle · *Tribolium* · Chemical ecology · Stored grain

Introduction

The red flour beetle, *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae), is a major cosmopolitan pest of stored cereal grains, beans, nuts, and other durable agricultural products worldwide (Weston and Rattlingourd 2000; Campbell et al. 2010). The feeding adult male secretes an aggregation pheromone, which is attractive to both sexes. This pheromone was identified as 4,8-dimethyldecanal (DMD), which has two asymmetric carbons at C-4 and C-8 (Suzuki 1980; Suzuki et

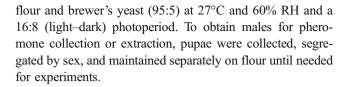


al. 1984). Suzuki (1980) synthesized a mixture (1:1:1:1) of all four stereoisomers of DMD and found it to be less active than the natural pheromone (Suzuki 1981; Suzuki et al. 1987). Based on bioassay of the four synthetic stereoisomers of DMD with *T. castaneum*, Suzuki and Mori (1983) inferred that the natural pheromone was the (4*R*,8*R*)-stereoisomer because it appeared to be as active as the natural pheromone. Levinson and Mori (1983) supported this claim after documenting responses to each of the four synthetic stereoisomers. Subsequently, a 4:1 mixture of (4*R*,8*R*)- and (4*R*,8*S*)-DMD was found to be ten times more active than (4*R*,8*R*)-DMD alone, although (4*R*,8*S*)-DMD itself was inactive at lower doses (Suzuki et al. 1984). However, the absolute configuration of 4,8-DMD produced by male *T. castaneum* was not further investigated and remained unknown until the present.

The chemical synthesis of the four stereoisomers of DMD at high enantiomeric purity and a derivatization and high-performance liquid chromatography (HPLC) separation method that gave baseline resolution of all four stereoisomers were recently described (Akasaka et al. 2011). The primary objective of the research reported here was to determine the stereoisomeric composition of naturally produced DMD from male beetles and to compare the attraction of natural and artificial blends to that of the synthetic pheromone blend used in commercial lures [a 1:1 mixture of (4R,8R)- and (4R,8S)-DMD)]. Although malespecific production of DMD has been demonstrated, there has been some confusion regarding the location of tissues or glands in males that might produce and/or release the pheromone. The male-specific exocrine glands beneath the setiferous patches on the ventral side of the profemura were considered the site of production by Faustini et al. (1981), but Bloch-Qazi et al. (1998) demonstrated that males with profemura surgically removed would continue to release pheromone. Olsson et al. (2006) provided evidence from the closely related species Tribolium confusum that attractive compounds were associated not only with the glands on the femurs but also with multiple locations around the body. In order to assist in future work on pheromone biosynthesis in T. castaneum, a second objective of this work was to identify the tissues responsible for DMD biosynthesis, deposition, and/or release.

Materials and methods

Insect cultures T. castaneum from the GA-1 strain (Haliscak and Beeman 1983), which originated nearly 30 years ago from a field site in the state of GA, USA, was used for pheromone collection and the KS-1 strain (Romero et al. 2009), recently collected from a commercial flour mill in Kansas, USA, was used for the wind tunnel walking bioassay. Beetles were reared on a mixture of whole-wheat



Collection of natural pheromone Adult males were placed in 7.5×2.75 cm cylindrical glass aeration chambers with 2.5 g of cracked wheat kernels (Triticum aestivum), and volatiles were collected according to the methods of Edde and Phillips (2006). Initial collections were made with individual beetles in aeration chambers. Subsequently, it was determined that the amount of pheromone produced per chamber could be maximized by aerating groups of five males. For remaining collections, groups of five 7-14-dayold males were used, with five aeration chamber systems running concurrently. Aerations were conducted at approximately 28°C and under 24 h of constant light, to elicit maximum pheromone production and release (Hussain 1993), provided by a 40-W incandescent light bulb. The incoming air flow rate was 200 ml/min, and the air was humidified by passing though a flask of distilled water. Volatiles were collected on small glass columns packed with Porapak-Q (Alltech Assoc., Deerfield, IL); columns were changed daily for 4 days. Columns were each eluted with approximately 500 µl of HPLC-grade hexane and the eluate spiked with 555 ng of n-dodecane in 5 µl of hexane (111 ng/µl) as an internal standard. Extracts were stored in 1.5 ml glass vials with Teflon-lined septum caps at -80°C pending analysis.

Analyses of tissues for pheromone content Male beetles used for tissue extracts were 7-14 days old. The virgin males were placed individually in glass vials with 0.5 g cracked wheat mixed with wheat flour for feeding until analyzed. Dissection protocols were modified from Olsson et al. (2006). Briefly, the beetles were sedated with CO₂ and then were mounted ventral-side-up onto double-stick cellophane tape affixed to the bottom of a glass Petri dish. Head, thorax, and abdomen were separated from the body with forceps, and groups of five for each body part were pooled for extraction. For each body region, five such pools were separately extracted in 1 ml HPLC-grade hexane for 30 min. An internal standard of 555 ng of *n*-dodecane was added directly to each extract before further processing. Extracts were concentrated to approximately 500 µl under a gentle stream of N2 at room temperature. After we confirmed that abdomens were the primary source of DMD, the abdomens of additional males (five groups of five abdomens each) were further dissected into two parts, namely the cuticle with any adhering tissues, and all other tissues. The latter included the digestive system (including the hindgut, posterior midgut, and Malpighian tubules),



reproductive system, and fat body. The two abdominal tissue groups were extracted as described above for the three body regions. All extracts were stored at -20°C for no longer than 48 h before chemical analyses.

Chemical analyses Samples from all aeration and tissue extraction experiments were subjected to quantitative analysis by coupled gas chromatography-mass spectrometry (GC-MS) with electron impact ionization (70 eV) using a Shimadzu GC-MS QP5050A (Kyoto, Japan) equipped with a J&W Scientific DB-1 capillary column (30 m× 0.25 mm×0.25 µm) in splitless mode, with helium as carrier gas. The injector oven was set at 250°C, and the heated transfer line to the MS was set at 250°C. Oven temperature was programmed at 40°C for 0.5 min, then increased 10°C/min to 200°C, and was held for 1 min, then increased to 240°C at 20°C/min, and held at 240°C for 1 min. Initial studies were conducted with the MS in the full scan mode, recording mass fragments from 35 to 350 amu. A 1:1 mixture of (4R,8R)- and (4R,8S)-DMD was analyzed for retention time and mass spectrum, which matched the spectrum published by Suzuki (1981). In order to maximize detection sensitivity for DMD in the experiments described above, the MS was subsequently operated in the multiple ion detection mode (MID) in which only the characteristic fragment ions m/z=41 and m/z=57 were detected. These fragment ions are common to both the internal standard dodecane and DMD. The quantity of DMD in each sample was determined by comparison of the peak area of the internal standard, representing 555 ng in the initial solution, and that of DMD from the MID chromatogram. Accuracy of our internal standard quantification was determined by analyzing a series of solutions with known amounts of synthetic DMD and dodecane over a range of concentrations. The total amount of pheromone produced by each beetle over the 6-day collection period was calculated, and differences among treatments were determined with analysis of variance (ANOVA) using SAS software. Gas chromatography (GC) and HPLC analyses of stereoisomeric composition, either with or without derivatization of the naturally collected pheromones, were conducted according to the methods of Akasaka et al. (2011).

Sample preparation procedure for analytical HPLC was as follows. To a pheromone sample solution in hexane (ca. 0.4 ml containing ca. 4 μg of DMD), about 1 ml of acetone and 0.5 mg of KMnO₄ were added. The mixture was stirred at room temperature for 1 h. After oxidation, 0.5 ml of 10% NaHSO₃ solution was added to the mixture and stirred for several minutes. The resulting clear and colorless solution was acidified with 2 ml of 15% citric acid solution (pH<3.5). The acid fraction was extracted with 2 ml of hexane three times. After drying the

hexane extract over Na_2SO_4 , the solvent was removed under reduced pressure. The residue was dissolved in 0.6 ml of toluene/acetonitrile (1:1, v/v). The solution was divided into two portions. One was used for derivatization with (1R,2R)-2-(2,3-anthracenedicarboximido)cyclohexanol, and the other was for derivatization with the (1S,2S)-reagent (see Akasaka et al. 2011, for details).

Walking orientation bioassay We used a wind tunnel built from the general design of Miller and Roelofs (1978) for behavioral bioassays of adult T. castaneum. The chamber consisted of an acrylic box, 100 in length and 40 cm in width and height with screening at each end. Air flow was generated by a centrifugal fan, with the air passing through an activated charcoal filter to cleanse it and a porous metal plate to generate a laminar flow of 0.35-0.4 m/s. Wind tunnel bioassays were conducted at 25°C and 60% RH. Mixed-sex adults were individually isolated in 5.0-ml opened glass shell vials and starved for 24 h prior to bioassay. The walking behavior of individual beetles in response to volatiles in moving air was observed on a sheet of 22×28 cm white photocopy paper placed on the floor of the wind tunnel at the upwind end, with paper changed between each replicate beetle. Test samples in a hexane solution were applied on Whatman no. 1 filter paper disks 2.0 cm in diameter placed on a circular metal disk, 2.0 cm in diameter and 2.0 mm thick above the floor. Twenty microliters of hexane alone (for controls) or hexane containing the test sample was applied to the filter paper circle and allowed to dry for 5 min in a fume hood before placing the metal disk with filter paper on the paper arena 1.0 cm from the middle of the upwind edge of the sheet of paper in the wind tunnel. Test beetles were released ~10 cm directly downwind of the source filter paper by inverting the glass vial over the paper and lifting it off as soon as the beetle was observed to be upright. Observations of beetles terminated when one of three events occurred: The beetle walked upwind and touched the metal disk (i.e., located the odor source); the beetle walked to the edge of sheet of the paper (i.e., did not respond to the source, and attempted to leave the observation area); or the beetle remained on the paper for 2 min without one of the other two events occurring.

Twenty mixed-sex adult beetles comprised a replicate and were tested individually in direct succession, with the odor source filter paper changed after every five beetles. Five replicates (i.e., five groups of 20 individual beetle observations) were utilized for each experimental treatment, with one replicate for each treatment and control blocked within a day. The hexane-only control bioassays were performed first in a given day, and the treatment order was randomized in successive groups in a given day.

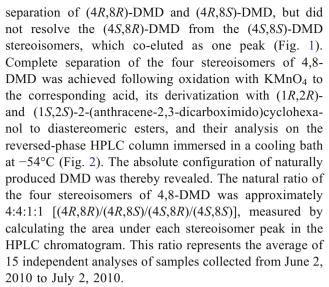


All four stereoisomers of DMD were synthesized as previously described (Akasaka et al. 2011); chemical purities were as follows: (4R,8R)-isomer at 92.2%, (4R,8S)-isomer at 95.3%, (4S,8R)-isomer at 91.7% and (4S,8S)-isomer at 94.4%; stereoisomeric purities of all four stereoisomers were 97% ee at C-4 and >99% ee at C-8. We compared the behavioral responses of individual beetles to various blends of DMD stereoisomers at a total DMD dosage of 0.1 ng in each bioassay. This very low dose represented a tiny fraction of the total amount released per day by a single "calling" male (see below), but was close to the response threshold and fell within the linear range in the dose-response curve (unpublished data). The following treatments were tested: a 1:1 blend of synthetic stereoisomers (4R,8R)- and (4R,8S)-DMD, which mimics the blend used in commercial pheromone lures; a 1:1:1:1 blend of all four synthetic stereoisomers (4R,8R)-, (4R,8S)-, (4S,8R)-, and (4S,8S)-DMD; a 4:4:1:1 blend of synthetic stereoisomers (4R,8R)-, (4R,8S)-, (4S,8R)-, and (4S,8S)-DMD, which mimics the natural blend of stereoisomers produced by male beetles (reported below); naturally collected DMD, which included other potential semiochemicals in the complete eluate collected on Porapak-Q during aeration of feeding males; and a solvent control. The percentage of beetles in a given test group contacting the odor source within 2 min (i.e., percentage of positive responders out of 20 beetles) was subjected to arcsine square root transformation to normalize distribution and then analyzed using ANOVA. Differences among means were determined using the Student-Newman-Keuls Test with SAS software (SAS Institute, 2001).

Results

Quantitative analysis of volatiles collected on Porapak-Q from aerations showed that the average amount of DMD released by a single feeding male was 878±72 (SE)ng per 24-h period (n=275 beetle-day-equivalents). No 4,8-DMD was found in extracts of the head or from internal organs and tissues. The amount of DMD in extracts of whole abdomens averaged 285±51 ng per beetle, which was almost fivefold greater than the 62±14 ng extracted from thoraces (ANOVA, $F_{2,25}=10.39$, p<0.01, SAS software). No DMD was detected in heads. In a separate experiment to examine localization in the abdomen, 100% of the DMD (159±25 ng per beetle) was localized in abdominal cuticle and epidermis, while none was found in the remainder of the abdomen. The results clearly indicated that the abdominal epidermis of male T. castaneum was the predominant source of aggregation pheromone.

Enantioselective GC analysis on a chiral stationary phase column (details in Akasaka et al. 2011) enabled good



The behavioral responses of T. castaneum to the four synthetic stereoisomers of 4,8-DMD at the naturally produced ratio ([(4R,8R)-/(4R,8S)-/(4S,8R)-DMD= 4:4:1:1]) and to the natural blend of pheromones and other semiochemicals collected from virgin feeding males were similar to each other, but were significantly greater than the response to the (4R,8R)-DMD:(4R,8S)-DMD=1:1 (as used in commercial pheromone lures) or to the (4R,8R)-/(4R,8S)-/(4S,8R)-/(4S,8S)-DMD=1:1:1:1 ratio treatment (Fig. 3). All treatments with stereoisomers of DMD elicited significantly higher responses than did the solvent controls in the walking bioassay (Fig. 3). The results confirmed that

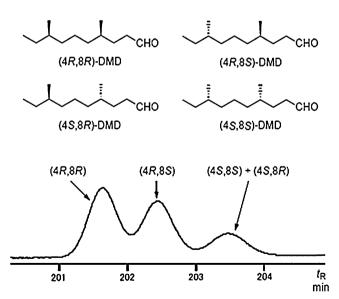


Fig. 1 Separation of naturally collected aggregation pheromone stereoisomers on a cyclodextrin-based chiral stationary phase column (50% MOMTBDMSGCD) via GC-flame ionization detection of volatiles from Porapak-Q aerations of feeding males. The *RR* and *RS* stereoisomers resolve but the *SS* and *SR* stereoisomers co-elute as one broad peak



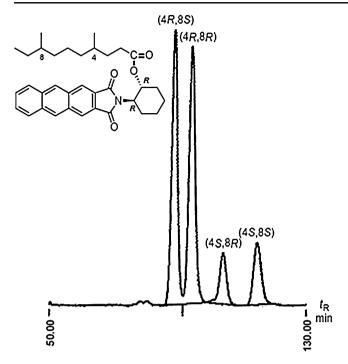


Fig. 2 HPLC separation at -54° C of the aggregation pheromone derivatives (structures shown) prepared from the naturally occurring pheromone from Porapak-Q aerations of feeding males. See Akasaka et al. (2011) for details. All four isomers were resolved. (4*R*,8*S*)-/(4*R*,8*R*)-/(4*S*,8*R*)-/(4*S*,8*S*)- isomers=4:4:1:1 (peak area)

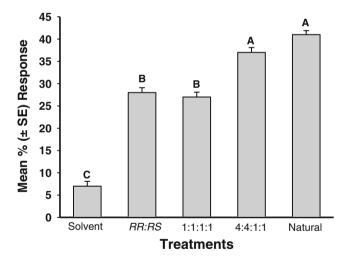


Fig. 3 Mean upwind walking responses (and SEs) of mixed-sex *T. castaneum* adults to different 4,8-DMD treatments in a wind tunnel bioassay. Solvent control was 20 μL hexane-only, RR/RS=1:1 was a 1:1 ratio of (4R,8R)-DMD/(4R,8S)-DMD; 1:1:1:1 was an equal mixture of (4R,8R)-DMD/(4R,8S)-DMD/(4S,8R)-DMD/(4S,8S)-DMD/(4S,8R)-DMD/(4S,8S)-DMD/(4S,8S)-DMD that mimics the naturally produced blend of pheromone stereoisomers; natural pheromone was the Porapak-Q hexane eluate collected from multiple groups of five feeding males with cracked wheat and wheat flour. Total amount of DMD in any given treatment was 0.1 ng on filter paper. Means with *different letters* are significantly different; ANOVA (F_{4,20}=118.57, P<0.001) followed by means comparison with the Student–Newman–Keuls (SNK) test (P<0.01, n=5)

a synthetic blend of DMD stereoisomers in the same ratio as that of the natural aggregation pheromones is more attractive to *T. castaneum* males and females than is the blend of stereoisomers currently used in commercial pheromone lures.

Discussion

The present study demonstrates that feeding *T. castaneum* males release DMD at rates similar to the 635 ng 24 h⁻¹ reported by Hussain (1993), but at sevenfold higher rates than those reported by Bloch-Qazi et al. (1998). These differences are most likely due to the fact that our aerations and those of Hussain (1993) were conducted under 24-h light conditions, while those of Bloch-Qazi et al. (1998) were conducted under photoperiodic regimens that incorporated alternating scotophase and photophase.

The pheromone production site in *T. castaneum* was originally believed to be associated with setiferous glands on the ventral side of the prothoracic femurs (Faustini et al. 1981). However, the study by Bloch-Qazi et al. (1998) clearly demonstrated that these patches and their associated glands were not the predominant sources of DMD biosynthesis or deposition in *T. castaneum*. Our findings show more conclusively that male pheromones are produced and/or deposited predominantly in the abdominal epidermis and, possibly to a lesser extent, in the thorax. The results are consistent with the report by Olsson et al. (2006) for *T. confusum*, who found that females were attracted not only to extracts of male legs but also to whole body extracts and extracts of male bodies without legs.

Chiral specificity in pheromones or allelochemicals as single isomers or in precise ratios of mixed isomers is essential for chemical communication in numerous insect species (Silverstein 1979; Mori 2007). The two asymmetric carbons of DMD potentially provide for four different stereoisomers, and it has been hypothesized that the isomeric composition may differ among species of *Tribolium* (Suzuki et al. 1984, 1987; Arnaud et al. 2002, Verheggen et al. 2007). Enantiomers typically are identical in various physical properties except the direction of optical rotation, such that they cannot be separated by conventional and achiral chromatographic methods. However, the method of Akasaka et al. (2011) enabled baseline separation of all four stereoisomers of DMD.

This is the first report of the natural composition of stereoisomers of DMD produced by male *T. castaneum*. Insects that use chiral pheromones typically produce and respond to either a single stereoisomer or to a species-specific blend of only some of the possible stereoisomers. Seldom does an insect produce and employ a non-racemic mixture of all possible stereoisomers of a pheromone



molecule, particularly for compounds with more than a single chiral carbon. In such cases, alternative stereoisomers are more often either not produced or are inactive (Mori 1997, Mori 2007). Here, we found that feeding T. castaneum males produce all four stereoisomers of DMD at a specific ratio of approximately 4:4:1:1 and that this blend has greater attractant activity than either racemic DMD or the commercial blend. Although (4R,8R)-+(4R,8S)-blends of stereoisomers used for commercial pheromone lures in traps have high biological activity, as does the pure (4R,8R)-stereoisomer alone (unpublished data), the natural blend of four stereoisomers reported here proved to be more active than any of the other blends tested. Despite the natural production of all four stereoisomers in a nonracemic blend by male beetles in our study, it is possible that not all four stereoisomers are required for normal biological activity. Future work will be needed to address this possibility. Furthermore, since the 0.1-ng DMD dosage used in each bioassay replicate refers to the total contribution of all DMD isomers in the given blend, the amount of the most active (4R,8R)-isomer differed by as much as twofold among the blends tested. Thus, the separate effects of dose versus blend ratio could not be unambiguously resolved in this experiment. Additional details of the effects of individual stereoisomers of DMD tested at a greater range of concentrations and ratios will be reported separately. We note also that the total amount of DMD required to elicit positive responses in our walking bioassays (0.1 ng per filter disk) was very small, approximately 1/10,000th the amount produced by a single male in 1 day. This fact points to the extremely high potency of DMD for short-distance orientation and attraction and raises the question of why calling males produce and release the attractant in such apparent excess.

The commonly used lure for commercially available traps to monitor pest populations of *Tribolium* beetles is a synthetic mixture of the (4R,8R)- and (4R,8S)-isomers at a 1:1 ratio, but the current work indicates that, at least in the case of the red flour beetle, the optimal ratio for use in traps may be that which mimics the naturally produced ratio for *T. castaneum*. The mixture of all four stereoisomers at 1:1:1:1, presumably the lowest cost to produce, was found to be similar in activity to the 1:1 mixture of (4R,8R)- and (4R,8S)-isomers and may be adequate for commercial use. Efficacy of the chiral derivatization and HPLC separation developed by Akasaka et al. (2011) will now allow for resolution of DMD stereoisomer ratios in additional populations of T. castaneum and in other species of Tribolium beetles. Future research on synthesis of DMD stereoisomers may lead to cost-effective production of improved pheromone lures for several species of pest Tribolium. Information from pheromone traps is increasingly critical for decision-making in integrated pest management of stored durable food products, as consumers and government regulatory agencies call for reduced use of chemical insecticides, adoption of biologically based pest management methods, and provision of safe, high-quality foods (Phillips and Throne 2010).

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