Chemical ecology of astigmatid mites LVIII.
2-Hydroxy-6-methylbenzaldehyde: Female sex pheromone of Cosmoglyphus hughesi Samšíňák (Acari: Acaridae)

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Abstract
2-Hydroxy-6-methylbenzaldehyde (2,6-HMB) was identified as the female sex pheromone from the acarid mite, Cosmoglyphus hughesi. Although the sex pheromone functioned to stimulate males to tap females and then mount, it was detectable as a major component of hexane extracts not only from females but also from males and protonymphs. Its contents were estimated to be 283.1±49.4 ng per female, 135.2±25.5 ng per male and 3.8±0.4 ng per protonymph. Sexual activity was induced in males by exposure of a filter paper impregnated with 100 ng of synthetic 2,6-HMB. Behavioral observation demonstrated that the male could not distinguish females from males upon attempt to mate.

Key words: Mite, Acari, Astigmata, 2-hydroxy-6-methylbenzaldehyde, female sex pheromone

INTRODUCTION
Astigmatid mites can be classified into two groups by their sex pheromone systems: one possessing both male and female sex pheromones, and the other consisting of only the female sex pheromone. The former case has been postulated for Acarus siro (Levinson et al., 1989), and the corresponding active compound(s) have been identified in the related species Ac. immobile as a mixture of hydrocarbons and 2-hydroxy-6-methylbenzaldehyde (hereafter abbreviated as 2,6-HMB), respectively (Sato et al., 1993). As examples of the latter case, female sex pheromones have been identified from the following five species: Sancassania (ÔCaloglyphus) polyphyllae (ß-acaridial [2(E)-(4-methyl-3-pentenyldiene)-butanedi], Leal et al., 1989a), Aleuroglyphus ovatus (2,6-HMB, Kuwahara et al., 1992), Sancassania rodriguezi (undecane, Mori et al., 1995), an unidentified Sancassania sp. (tentatively named as Sancassania sp. MJ) [(2R,3R)-epoxyneral [(2R,3R)-2,3-epoxy-3,7-dimethyl-6-octanal], Mori et al., 1996] and another unidentified Sancassania sp. (named as Sancassania sp. HP) (rosefuran, Mori et al., 1998b). Although all these pheromones function to trigger the male’s mounting behavior, the compounds were distributed over both females and males in various ratios (female/male). In Sancassania sp. HP (8.4/1), S. rodriguezi (6.3/1) and S. polyphyllae (3.4/1), differences in the amounts of female sex pheromone between sexes allow males to discriminate females from males, while Sancassania sp. MJ (1.4/1) cannot do this because of insufficient difference (Mori and Kuwahara, 2000). Moreover, each sex pheromone is found even in protonymphs of Sancassania sp. MJ and S. polyphyllae, while the pheromone is adult specific in S. rodriguezi and Sancassania sp. HP. To rationalize their unusual distribution as sex pheromones, we have postulated an evolutionary trend among four Sancassania species, from the primitive stage at which the sex pheromone is distributed in all stages (e.g. in S. polyphyllae and Sancassania sp. MJ) to the developed stage at which distribution biased towards females (e.g. in S. rodriguezi and Sancassania sp. HP) (Mori and Kuwahara, 2000).

To better understand the sex pheromone systems of Astigmata, it is desirable to accumulate more knowledge on sex pheromones from these mites. We report here identification of the female sex pheromone of Cosmoglyphus hughesi, which stimulates males sexually. This pheromone was distributed not only in females but also in males and
protonymphs, and its content was quantitatively determined. We evaluated the male ability to discriminate conspecific females from males, and also observed female behavior against males to detect the possible presence of the male sex pheromone.

**MATERIALS AND METHODS**

**Mites.** A population of *Cosmoglyphus hughesi* Samšišák 1966, was obtained from organic soils at Kyoto University. The mites were reared at 20–25°C by feeding on dried yeast on wet filter papers set in plastic petri dishes (90 mm i.d.×18 mm ht.). The petri dishes were kept in plastic zip-loc bags (240×170×0.04 mm) to maintain humidity.

**Extraction and purification.** One hundred females collected from a mass rearing colony were dipped into distilled hexane (100 μl) for 3 min. The extract was separated on a conventional SiO₂ column (500 mg, Wako-gel C-200), and the column was eluted in a stepwise manner with 4 ml each of the following solvents: hexane, ether and hexane mixtures (1%, 5%, 10%, 20% and 50% ether in hexane) and ether. Pheromone activities of all fractions was monitored by bioassay as described below.

For quantitative studies, a male, a female or a protonymph was separately transferred to a conical-bottomed glass tube (8 mm o.d.×30 mm ht.) with a needle. Both sexes were randomly selected from the stock culture and used without prior knowledge of their mating experiences. Each mite was soaked for 3 min in hexane (6 μl) containing hexadecane as an internal standard (10 ng/ml). Aliquots of 3 μl corresponding to 0.5 mite equivalents were subjected to gas liquid chromatography (GC).

**Bioassay method.** The method was designed to detect and quantify the activity of the sex pheromone. The bottom surface of the petri dishes (7 mm i.d.×5 mm ht.) was covered with a small piece of damp filter paper and small amounts of moistened dry yeast were added as a nutrient source. After introduction of a group of 10 males or 10 females, the chamber was closed with a cover glass to prevent mites from escaping and maintained for 1–3 h. A piece of filter paper (1×3 mm) impregnated with one of the candidate materials was then placed in the center of the chamber. The number of mounting attempts exhibited by males at room temperature or of any behavior observable by females was counted for 2 min under a binocular microscope. Hexane was used as the control and the assay was repeated 5 or 10 times to evaluate statistical significance.

**Observation method of hetero- and homo-sexual behavior.** To examine whether or not male mites were able to discriminate females from males, a behavioral assay was done as follows: One male was introduced into the same chamber as used for the bioassay method. The chamber was maintained for 1–3 h, and another mite (female or male) was then introduced with minimal disturbance. Initially, the numbers of chambers in which mounting behavior was observed within 13 min were compared between male or female introductions. In addition, in the case of responding pairs, the time required for initiation of mounting attempts was compared between groups. This trial was repeated 30 times for each male or female introduction. Observation of each case was stopped within 13 min, since nearly 90% of test males had copulated with introduced females by this time point.

**Data analyses.** The assay results were processed to evaluate significance of differences in the numbers by a Kruskal-Wallis test followed by Dunn’s multiple-comparison test. The numbers of responding and non-responding pairs in the two cases obtained by the behavioral observation method were statistically examined by a χ² test for independence. The times required for initiation mounting attempts were processed by a Mann-Whitney U test. All values are expressed as the mean±SEM.

**GC and GC/MS analyses.** GC was performed by a Hewlett Packard 5890 series II plus gas chromatograph equipped with a flame ionization detector, using an HP-5 capillary column (0.32 mm×30 m, 0.33 μm in film thickness) at a split less mode. The carrier gas was helium at 1.23 ml/min, and the oven temperature was programmed from 60°C to 290°C at 10°C/min with an initial 2 min hold. The chromatogram was processed with an HP 3396 series II Integrator. GC-mass spectrometry (GC/MS) was carried out on an HP 5989B mass spectrometer coupled with an HP 5890 series II plus gas chromatograph at a split less mode, using the same column and conditions as described above.
RESULTS

Chemical analysis

The hexane extracts of females showed the following four major peaks on GC; peak A: $t_R$ 5.37 min, peak B: $t_R$ 6.86 min, peak C: $t_R$ 8.27 min, peak D: $t_R$ 8.44 min, and a minor peak E: $t_R$ 10.83 min (Fig. 1). GC profiles from adults indicated no qualitative differences between the sexes, while the profile of protonymphs differed from those of adult extracts. Peak A was only a minor peak and peak C was never found in nymphal extracts.

GC/MS analysis of peak A ($t_R$ 5.37 min) gave $M^+$ ion and the base ion at $m/z (%)$ 150 (100) with the following diagnostic ions at $m/z (%)$ 135 (91), 107 (34), 95 (31), 91 (45), 79 (36) and 41 (25). Both mass spectrum and GC $t_R$ were identical to those of rosefuran (Leal et al., 1989b).

Mass spectrum of peak B ($t_R$ 6.86 min) indicated $M^+$ ion and the base ion at $m/z (%)$ 136 (100) with diagnostic ions at $m/z (%)$ 135 (95), 107 (13), 90 (22), 79 (20) and 77 (26). The component of peak B was identified as 2,6-HMB, based on GC/MS data and GC $t_R$, using the standard 2,6-HMB (Noguchi et al., 1997).

Peak C component ($t_R$ 8.27 min) showed the largest fragment ion at $m/z (%)$ 150 (60) and the base ion at $m/z (%)$ 121 (100) with the following ions at $m/z (%)$ 122 (49), 93 (39), 76 (12), 65 (28) and 63 (13) by GC/MS analysis. The structure of the component remains to be determined.

The component of peak D ($t_R$ 8.44 min) was determined to be tridecane from the following GC/MS data: $M^+$ ion at $m/z (%)$ 184 (12) and the base ion at $m/z (%)$ 57 (100) with diagnostic ions at $m/z (%)$ 113 (7), 99 (11), 85 (44), 71 (68), 43 (69). The authentic tridecane also indicated the same mass spectrum and GC retention time.

The peak E component ($t_R$ 10.83 min) indicated the largest fragment ion at $m/z (%)$ 180 (10.8) and the base ion at $m/z (%)$ 69 (100) with the following ions at $m/z (%)$ 134 (34), 119 (40), 91 (30), 83 (44), 55 (31) and 41 (58) by GC/MS analysis. The structure of the compound remains unknown.

Biological activity

When test males were exposed to the filter paper containing the female extract (corresponding to 0.3 female equivalents), mounting behaviors took place among males. Thus the presence of the female sex pheromone was confirmed. When test females were exposed to the male’s extract or to an introduced male, no peculiar behavior was observed among female groups. As a result, the possibility of a male sex pheromone was disclaimed.

Extract from 100 females was chromatographed on an SiO$_2$ column and eluted in a stepwise manner with hexane-ether mixtures. The 5% ether in hexane fraction indicated the same pheromone activity at 0.3 female equivalents as that of the female extract. The fraction consisted only of peak 2 by GC and the component was identified as 2,6-HMB by GC/MS. All other column eluates were inactive (Kruskal-Wallis test followed by the Dunn’s multiple-comparison test, $p<0.01$).

The dose-response relationship of the synthetic 2,6-HMB indicated a convex curve, and pheromone at 100 ng demonstrated the highest activity among the ranges tested (Fig. 2, Kruskal-Wallis test followed by the Dunn’s multiple-comparison test, $p<0.01$). Based on the quantity in females ($283.1\pm49.4$ ng per female, mentioned below, and active at 0.3 female equivalents) and the dose-response of synthetic 2,6-HMB (active at 100 ng), the female sex pheromone was concluded to be 2,6-HMB.

Quantitative study

The pheromone 2,6-HMB was a major component, not only in females but in males and protonymphs, as well. Relative compositions of 2,6-HMB in hexane extracts were $65.7\pm1.3\%$ (mean$\pm$SEM, $n=10$) for females, $60.4\pm1.0\%$ for males ($n=10$) and $52.1\pm3.3\%$ for nymphs ($n=5$).
Contents were determined to be 283.1±49.4 ng (mean±SEM, \( n=12 \)) for females, 135.2±25.5 ng (\( n=12 \)) for males and 3.8±0.4 ng (\( n=6 \)) for protonymphs. Significant differences in 2,6-HMB contents were observed between females and males (Mann-Whitney \( U \) test, \( p<0.01 \), each \( n=12 \)).

**Discrimination test**

After introduction of a female or a male to a conditioned male, the numbers of responding and non-responding pairs were 27 and 3 in the former case, and 24 and 6 in the latter case, respectively. No significant difference was detected in the numbers of mounting attempts between the female-to male and male-to male introductions (Fisher's exact probability test, \( p>0.05 \), each \( n=30 \)). Furthermore, frequency distributions of the lag time until mounting behavior took place showed no significant difference between the two cases [Fig. 3, Mann-Whitney \( U \) test, \( p>0.05 \), \( n=51 \) (27+24)]. As a result, we concluded that males had no ability to discriminate between conspecific males and females.

**DISCUSSION**

The existence of the female sex pheromone was first demonstrated in the genus *Cosmoglyphus*, based on the observation that female extract of the species stimulated the male sexually. The compound responsible for the activity was concluded to be 2,6-HMB. The activity occurs in response to a dose of 100 ng, which is roughly 0.3 female equivalents (84.9 ng) of the hexane extract. The compound indicated maximum activity at 100 ng with a convex dose-response curve. A similar phenomenon of dose-response is known in other sex pheromone studies of astigmatid mites (Leal et al., 1989a; Mori et al., 1995, 1996, 1998b).

The compound is widely distributed among astigmatid mites not only as a semiochemical but also as a component of the opisthonotal gland exudate with unknown functions (Kuwahara, 1995). Two species besides *C. hughesi*, *Al. ovatus* (Kuwahara et al., 1992) and *Ac. immobilis* (Sato et al., 1993), also use this compound as their female sex pheromone. The compound also functions as the alarm pheromone of *Tyrophagus putrescentiae* (Leal et al., 1988), and has been detected as a major or minor component with unknown functions from *Dermatophagoides farinac* (Kuwahara et al., 1990), *Ac. siro* (Curtis et al., 1981), *T. putrescentiae* (Leal et al., 1988) and *S. rodriguezi* (Ayorinde et al., 1984; Mori et al., 1995).

On the other hand, no evidence was obtained that suggested the presence of the male sex pheromone by bioassay and behavioral observations. Consequently, the mating process of the present species appears to be accomplished by involvement of the female sex pheromone, like the cases of the genus *Sancassania* (Mori et al., 1998b).
The compound functioning as the sex pheromone was a major component not only in females but also in males and protonymphs, as in *Sancassania* sp. MJ (Mori et al., 1996) and *S. polyphylla* (Leal et al., 1989a). Although, *C. hughesi* females contained 2,6-HMB at levels 2.1-fold higher than males, males appeared not to be able to discriminate between females and other males. It is, therefore, inferred that the pheromone system of *C. hughesi* is primitive, as in the case of *Sancassania* sp. MJ, where the quantitative difference between sexes is 1.4 fold (Mori et al., 1996). The other *Sancassania* sp. HP has been suggested to be more developed; the female/male ratio is 8.4/1, and males possess the ability to discriminate females from males (Mori and Kuwahara, 2000). This hypothesis has been supported by a molecular phylogeny study based on COX1 DNA analysis (Kuwahara et al., 1998). A similar evolutionary process might be revealed by DNA analysis among the genus *Cosmoglyphus*, if more pheromone studies are done and biological data such as relative abilities for male-female discrimination and their content ratio between sexes come available.

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