Chemical Ecology of Astigmatid Mites
XXXII. 2-Hydroxy-6-methyl-benzaldehyde, the Sex Pheromone
of the Brown-Legged Grain Mite *Aleuroglyphus ovatus*
(TROUPEAU) (Acarina: Acaridae)¹

Yasumasa KUWAHARA, Masashi SATO, Takako KOSHII
and Takahisa SUZUKI

*Institute of Applied Biochemistry, University of Tsukuba,
Tsukuba, Ibaraki 305, Japan*

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The female sex pheromone of the brown-legged grain mite *Aleuroglyphus ovatus* was identified as 2-hydroxy-6-methyl-benzaldehyde (2,6-HMBD), which sexually stimulated males to copulate. Neither the crude hexane extract nor the fractions partially separated by a silica gel column demonstrated activity; the sex pheromone was masked by the alarm pheromonal activity of citral present in the extract. The fact that 2,6-HMBD was the major component in the adult extracts, and a minor component in the nymphal stage extract, suggested its pheromonal role among adults. The activity was elicited when 2,6-HMBD was carefully isolated by TLC and/or LC. Synthetic 2,6-HMBD elicited the same sexual response as that demonstrated with the isolated natural pheromone.

Key words: sex pheromone, 2-hydroxy-6-methyl-benzaldehyde *Aleuroglyphus ovatus*, Brown-legged grain mite, Astigmata

INTRODUCTION

For almost two decades our knowledge of sex pheromones in Acari has been restricted to spider mites and ticks [reviewed by SONENSHINE (1985) and GOTHE (1987)], although observed evidence has suggested the presence of male and female sex pheromones among *Astigmata* (BOCZEK and GRIFFITHS, 1978). Only recently the occurrence of such semiochemicals in astigmatid mites came to light; LEVINSON et al. (1989) reasoned that the courtship ritual and mating of *Acarus siro* is mediated by two sex pheromones, and LEAL et al. (1989 a) demonstrated that male *Caloglyphus polyphyllae* exhibited sexually excited behavior upon exposure to the β -acaridial [2(*E*)-(4-methyl-3-pentenylidene)-butanedial] present in female mites. This sex pheromone was revealed to be emitted by the opisthonotal glands of females; the external scapular setae are the receptors. It is reasonable to assume that all bisexual astigmatid mites possess their sex pheromone(s) like insecta.

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The brown-legged grain mite *Aleuroglyphus ovatus* (TROPEAU) (Astigmata: Acaridae) is an economically important pest, which causes serious damage to stored products. The courtship behavior of this species has been observed in the culture medium. The male orientates towards the female, mounts her dorsum in a parallel position, turns his body and attaches it to the female's, after moving part of it beyond the female's opisthosoma. In that position the male massages the female's opisthosoma with his third pair of legs. We report here that orientation and mounting steps are mediated by 2-hydroxy-6-methylbenzaldehyde.

MATERIALS AND METHODS

The culture of *A. ovatus* was initially a strain maintained for more than 20 y by Dr. K. MATSUMOTO of Tokyo Women's Medical College (MATSUMOTO, 1965). Mites were removed from the culture medium by shaking in a separatory funnel with brine.

Large-scale extraction and purification. The extract of all stages was prepared by soaking collected mites in hexane for 3 min and filtering off mite bodies. One part of the concentrated extract was chromatographed on a SiO₂ (Wako-gel C-200) column and TLC (Silica gel 60 HF₂₅₆, E. Merck, 5 cm × 20 cm, 0.25 mm in thickness) to isolate and to identify each component. Biological activity was also monitored on each fraction. The other part of the extract was hydrolyzed by 3% alcoholic KOH at r.t. for overnight and the non-saponifiable was extracted by ether under basic condition. The extract was purified successively on a SiO₂ and florisil (Floridin Co.) column using mixtures of ether and hexane to give 2,6-HMBD.

Small-scale extraction. Fifty males and 50 females (virgin and mated, respectively) as a group were separately collected and transferred to a micro-vial (8 mm o.d. × 50 mm) with needles. Similar volume of deutonymphs, which had somehow escaped from the stock culture and aggregated in the crevice between the rearing vial and the cover were collected into the micro-vial. After addition of hexane (ca. 100 µl) to those mites, each of the resulting extracts was transferred to a conical-bottomed vial (hand-made from the micro-vial) using a micro-syringe (50 µl), and concentrated to ca. 1 µl under a gentle N₂ flow. The concentrate was then subjected to GLC analysis.

Bioassay method. Two assay systems were devised using a Petri dish (Petri dish method) or a glass tube (glass tube method). The former was conducted as follows; about 50 males and females were separately placed in several Petri dishes (16 mm i.d. × 13 mm) with a drop of moistened dry yeast. When all mites in a Petri dish settled motionless and (or) fed on the yeast at ca. 60% RH under ambient temperature and photo-regimen, a small filter paper (2 mm × 3 mm) impregnated by a test material was introduced with minimal disturbance. If mites were then sexually aroused to frequent male-to-male mountings within several min, the sample at its concentration was concluded to be active. The latter was done as follows; about 10–20 mites (males or females) were kept with moist food in a separable tube (15 mm o.d. × 30 mm) with conical ends (15 mm o.d. × 15 mm ht.), connected to capillary tubes (4 mm o.d. × 15 mm). One end, plugged with cotton, was connected to a syringe (5 ml vol.) to secure the tube against rolling. The other end was sealed with parafilm during conditioning and test material was introduced into the tube through the tip by a small glass. Mite behavior was subsequently observed under a binocular microscope and activities were scored.

Analysis method. GLC was done on a Hitachi 263-30 instrument equipped with a FID using a CP-Sil 19 CB capillary column (0.22 mm×25 m; Chrompack) and operated at temperatures programmed from 135°C to 250°C at 4°C/min. Peaks were processed by a SIC intelligent integrator model 7000A. GC/MS was measured by a Hitachi M-80B high resolution mass spectrometer coupled with a gas chromatograph model 367-0200, operated at 70 eV and low resolution mode, using an Al-Clad fused silica methylsilicone capillary column (0.22 mm×25 m; Quadrex) at a temperature programmed from 110°C to 250°C at 4°C/min. NMR was run on a Bruker 500 MHz with CDCl₃ solutions and TMS as internal standard.

RESULTS

The conventional hexane extract of mites containing all stages consisted of at least 11 peaks (peak A–K) by GLC, as indicated in Fig. 1. All of those compounds were identified in Table 1. Comparison of GLC profiles among extracts from males, females and nymphal stages (mainly deutonymph) revealed considerable quantitative differences for peak E (see Fig. 2), which gave M⁺ ion at m/z 136 and the base ion at m/z 135 and was tentatively identified as 2-hydroxy-6-methyl-benzaldehyde (2,6-HMBD). Although its content in males (average 16 ng/mite) and females (average 12 ng/mite) did not significantly vary, their relative abundances were much higher than those of nymphal extracts, as well as in the extract of all stages, when compared either by relative abundance based on tridecane or by the sum of neral and geranial (Table 2). The synthesized 2,6-HMBD were therefore presented to males and females using the Petri dish method. Upon exposure to 2,6-HMBD, only males started to move around and try to mount on anything they encountered, proving the compound to be a sexual excitant against males (active at 100 ppm by the Petri dish method), while no particular behavior was observed against females.

The conventional hexane extract from all stages was then chromatographed on a SiO₂ column eluting with a stepwise mixture of hexane and diethylether. The fractions eluted by hexane, hexane–ether (20:1, 10:1, 5:1 and 1:1) and ether were

Fig. 1. Typical gas liquid chromatogram of the mite extracts of all developmental stages, using a CP-Sil 19CB capillary column at a temperature programmed condition (135–250°C, 4°C/min). Alphabets in the chromatogram refer to Table 1.

Table 1. Compounds identified in the brown-legged grain mites, *Aleuroglyphus ovatus*, of all stages

Compound	Mark in GLC	GLC-tr (min)	Abundance (%) ^a	Identified methods	Reference
Perillene	A	1.17	trace	GLC, GC/MS	SUZUKI et al. (1986)
Tridecane	B	2.25	20.8	GLC, GC/MS	HOWARD et al. (1988)
Tetradecane	C	2.84	0.2	GLC, GC/MS	HOWARD et al. (1988)
Epoxyneral	D	2.96	1.6	GLC, GC/MS	LEAL et al. (1989b)
2,6-HMBD	E	3.06	3.3	GLC, GC/MS	LEAL et al. (1988a)
GC/FT-IR, NMR					
Neral	F	3.28	19.3	GLC, GC/MS	KUWAHARA et al. (1980)
Geranial	G	3.72	3.0	GLC, GC/MS	KUWAHARA et al. (1980)
α -Acaridial	H	4.45	0.5	GLC, GC/MS	LEAL et al. (1989c)
β -Acaridial	I	6.18	2.3	GLC, GC/MS	LEAL et al. (1989d)
Neryl myristate	J	30.04	37.6	GLC, GC/MS	LEAL et al. (1988b)
Squalene	K	38.57	11.4	GLC, GC/MS	KUWAHARA et al. (1988)

^a Data was obtained from a typical chromatogram given in Fig. 1.

Fig. 2. Examples of gas liquid chromatogram, derived from 50 females (1), 50 males (2) and (mainly) deutonymphs (3) of an uncertain quantity. GLC condition is given in the text. Letters in the chromatogram refer to Table 1.

Table 2. Relative abundance of 2,6-HMBD, tridecane and citral in extracts derived from a mixture of all stages, nymphal stages, and 50 males and 50 females, determined by GLC

		Tridecane	Citral ^a	2,6-HMBD
Nymphal stages	Exp. 1	58.0%	28.8%	13.2%
	2	49.8	36.9	13.3
50 Males	Exp. 1	37.2	7.3	55.5 (14.3 ng/male)
	2	35.2	4.8	60.0 (16.5 ng/male)
50 Females	Exp. 1	35.1	12.3	52.6 (15.5 ng/females)
	2	33.7	10.8	55.5 (9.8 ng/female)
Mixture of all stages	Exp. 1	45.6	50.7	3.7
	2	44.9	48.0	7.1

^a Sum of neral and geranial.

concentrated to ca. 1000 ppm and bioassayed. No sex pheromonal activity, however, was observed in any fractions. Even though 2,6-HMBD was detected in the hexane-ether (20:1) fraction by GLC and GC/MS along with citral and neryl myristate, the fraction merely indicated alarm pheromonal activity when concentrated.

Each component of the hexane-ether (20:1) fraction was, then, separated by a preparative TLC and bioassayed using the Petri dish method. No activity was found on neryl myristate, and citral demonstrated alarm pheromonal activity. On the other hand, 2,6-HMBD evoked the same response to males as the synthetic 2,6-HMBD. The hexane-ether (10:1 and 5:1) fractions contained a minute amount of β -acaridial, the sex pheromone of *C. polyphyllae*, and were re-examined at various concentrations; inactivity was confirmed.

In order to fully identify the male excitant sex-pheromone, a substantial amount of 2,6-HMBD was isolated. The conventional hexane extract (200 mg) from all stages of collected mites (40 g) was hydrolyzed. After usual work-up with ether, the neutral fraction was purified twice on a SiO_2 and a florisil column, both eluting with 5% ether-hexane, to give pure 2,6-HMBD (5 mg, active at 0.5 μg against males but inactive against females by the glass tube method). Not only the GC-mass spectrum, high-resolution NMR (500 MHz, CDCl_3) but also GC/FT-IR spectra of the natural compound were completely identical to those from the synthetic 2,6-HMBD; M^+ at m/z 136 and the base peak at m/z 135 (97%): 1,2,3-substituted aromatic protons at δ 7.38 (1H, *t*, $J=8.29$ and 7.57), at 6.82 (1H, *d*, $J=8.29$ Hz) and 6.72 (1H, *d*, $J=7.57$ Hz), along with at 11.9 (*s*, OH), 10.33 (*s*, CHO) and 2.62 (*s*, CH_3): GC/FT-IR λ_{max} at 1666(*s*), 1620 (shoulder), 1585 (shoulder), 1454 (*m*), 1327 (*m*), 1296 (*m*, shoulder), 1234 (*m*), 1192 (*m*), 1161 (*m*) and 725 (*m*). The synthetic 2,6-HMBD gave the same biological activity (active at 0.5 μg , by the glass tube method) as the isolated compound.

DISCUSSION

Astigmatid mites have a typical sex ratio (1:1) of bisexual non-parthenogenetic species (HEINEMANN and HUGHES, 1970, BOCZEK and GRIFFITHS, 1978). Presence of both female- and male-produced sex pheromones were postulated from evidence based on visual observation (BOCZEK and GRIFFITHS, 1978): when virgin pairs of *Acarus siro* or *Lardoglyphus kono*i are placed together, the female is often the first to become aware of her partner, and follows his trail. It is not until the female's opisthosoma is close to the male gnathosoma that the male become active. This indicates the release of an attractant stimulus by the female. Further evidence that such an attractant is released stems from the fact that when a female is introduced to a number of males, unsuccessful males will pair with each other. Although chemical structures are not identified, LEVINSON et al. (1989) reported the dose-response relationships between the male and the female extracts of *Acarus siro*, claiming the presence of two different sex pheromone systems in the species: a male-produced attractant and arrestant for virgin females, and a female-produced attractant and copulatory stimulant for unmated males. LEAL et al. (1989 a) identified β -acaridial as the sex pheromone found in female *Caloglyphus polyphyllae* (virgin and mated) attractive to adult males.

The compound which indicated the most peculiar quantitative difference between adult and nymphal stages of *A. ovatus* could be ascertained as 2,6-HMBD (with an unusually high content 13%, rather than the expected average of 5% from the con-

ventional extract) which was subsequently identified as the sexual excitant pheromone emitted from females. However, the fact that almost the same quantity of the compound is also present in males is intriguing. As observed by BOCZEK and GRIFFITHS (1978), copulatory attempts between males were quite characteristic and took place frequently, not only in the assaying container but even in the stock culture. Once males were sexually aroused, copulatory attempts lasted for a long time, possibly due to mutual discharge of the compound among males. On the other hand, females did not indicate any peculiar movement against extracts and chromatographic fractions. To identify the male-produced sex pheromone of the species, one should develop a bioassay method based on a new concept, such as developed by LEVINSON et al. (1989), or trail-following activity of females as suggested by BOCZEK and GRIFFITHS (1978).

GLC survey revealed that 2,6-HMBD was detected not only in mated adults, but also in unmated virgin males. This indicated biosynthetic origins playing still unknown functions in males of the species. Such a double role of a chemical in astigmatid mites is not novel; KUWAHARA et al. (1989) reported that β -acaridial, which is the sex pheromone of *C. polyphyllae*, is also a potent antifungal substance.

In marked contrast to the sex pheromones of insects, especially of Lepidoptera, the activity of mites' air-borne pheromones seem to be generally weak, requiring a high female-equivalent (FE) threshold to get male response as 0.5–5 FE in *C. polyphyllae* (LEAL et al., 1989 a). In this study, the activity was confirmed at 0.5 μ g (the glass tube method) and of 100 ppm (the Petri dish method), which roughly corresponded to 45 FE and 90 FE (if 10 μ l of the pheromone solution was assumed to be retained in the filter paper) of *A. ovatus*, respectively. In the case of the almond moth *Cadra cautella*, an example of lepidopteran sex pheromone, male response was evoked by presenting one drop of 10^{-3} – 10^{-4} FE pheromone solution (KUWAHARA et al., 1968). These mite sex pheromone are comparatively quite short-ranged.

It is also remarkable that related species of astigmatid mites use completely different pheromones. In ticks, various species use the same compound, 2,6-dichlorophenol, as their sex pheromone (reviewed by SONENSHINE (1985) and GÖTHE (1987)). *A. ovatus*, although possessing β -acaridial—the sex pheromone of another acarid—utilizes a different semiochemical.

No sex pheromone activity of 2,6-HMBD was demonstrated until the alarm pheromone component of citral was completely removed either by a preparative TLC or a column chromatography. This inactivity, therefore, is concluded to be due to the co-occurrence of the alarm pheromone, which may preclude sex pheromone activity. The following fact also supports this possibility; no alarm pheromones have ever been reported in *C. polyphyllae* (LEAL et al., 1989 a) or in *A. siro* (CURTIS et al., 1981), although the sex pheromone (β -acaridial) has been identified in the former and its possible presence (LEVINSON et al., 1989) has been demonstrated in the latter.

The diversity of roles for the same semiochemical in the group is also outstanding; *Tyrophagus perniciosus*—a mite belonging to the same subfamily acarinae—uses 2,6-HMBD as the alarm pheromone (LEAL et al., 1988 a). This compound has been also isolated from other species of the same family: *Acarus siro* (CURTIS et al., 1981) and *Caloglyphus rodriguezi* (AYORINDE et al., 1984), but its ecological role(s) remains obscure.

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