Chemical Ecology of Astigmatid mites LXXX. \( \gamma \)-Acaridial (3-Hydroxybenzene-1,2-dicarbaldehyde) as a Female Sex Pheromone from an Alarm-Pheromone-Emitting Unidentified \textit{Rhizoglyphus} Mite (Acari: Acaridae).

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Abstract

The presence of a female sex pheromone in the unidentified \textit{Rhizoglyphus} mite 'sapporo' was hypothesized, based on the fact that male's tapping behavior was observed among isolated males to the introduced female but not to a hexane-rinsed female. The same activity was, however, not reproducible neither by the female mite hexane extracts nor by any SiO\(_2\) column chromatographic fractions. Since \( \gamma \)-acaridial (3-hydroxybenzene-1,2-dicarbaldehyde), which was a major component in the extracts, has been known to be lost during purification process by chromatography and was actually not detected in any fractions, synthetic \( \gamma \)-acaridial was subjected to bioassay, and it was found that the compound was the active principle of the female sex pheromone at a dose of 1 - 10 ng with convex dose-response relationships. Its content was determined to be 39 ± 6.4 ng per female and 10 ± 4.1 ng per male.

Key words : sex pheromone; \( \gamma \)-acaridial; 3-hydroxybenzene-1,2-dicarbaldehyde; \textit{Rhizoglyphus} mite; Astigmata; mites

Introduction

Astigmatid mites contain monoterpene, hydrocarbons, sesquiterpenes, aromatics and other miscellaneous compounds as excretory products from a pair of opisthonotal glands. Parts of these compounds behave as three kinds of pheromones; alarm, aggregation and sex pheromones, as listed in Kuwahara (2004), whereas the function of most compounds remains obscure. It is natural to assume that each species of mite can handle only one pheromone function, otherwise conflicting behavior such as alarm (= escaping) and aggregation (= attraction) may possibly take place. However, we have recently realized that each species of mites can manage two combinations of pheromones depending upon the mite's conditions and circumstances around their habitat, such as alarm-sex, sex-aggregation, and aggregation-alarm combinations, by sharing one compound or by using two compounds each with a different pheromonal function (Kuwahara, 2004).

From the following three species that commonly contain neryl formate \( [(Z)-3,7\text{-dimethyl}-2,6\text{-octadienyl formate}] \) as alarm pheromone, two female sex pheromones have been identified; \( a \)-acaridial from \textit{Rhizoglyphus robini} and isorobinal from \textit{R. setosus} (Mizoguchi \textit{et al.}, 2003; 2005). In the unidentified \textit{Rhizoglyphus} mite 'sapporo' collected from organic soil on Hokkaido Island, neryl formate as well as \( a \)-acaridial \( [2\text{-}(2\text{-hydroxyethylidene})\text{-6-methylhept-5-enal} \) and citral (a mixture of neral and geraniol) have resulted in alarm pheromone activity, each at a level of 100 ng for this species (Akiyama \textit{et al.}, 1997), and the possible presence of a female sex pheromone has been suggested. Mites belonging to the genus \textit{Rhizoglyphus} are known as serious agricultural pests, attacking the root parts of various crops. In the present study, the chemical structure of the female sex pheromone in this unidentified \textit{Rhizoglyphus} mite was elucidated and identified without isolation of the compound.

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Materials and Methods

Mites.

The unidentified Rhizoglyphus mite "sapporo" (Astigmata: Acaridae) was a strain derived from organic soils in Sapporo City, Hokkaido Island. The species was maintained in our laboratory at 20°C asceptically on agar medium, as reported previously (Kuwahara, 1999). Its second internal transcribed spacer (ITS2) region consisted of 447 bps (code name RH 34, GenBank Accession Number: AB104969) as listed in Noge et al., (2005). Males of the species are dimorphic, like R. robini. However, not only the oval shape of the hypopus, but also the gas liquid chromatography (GC) profile of the hexane extract was different from those of R. robini.

Bioassay.

The sex pheromone activity was evaluated as reported previously (Mori et al., 1996), with minor modifications. Groups of 10 males or females were placed into each assay chamber of small culture dishes (7 mm i.d. × 5 mm in height) with a cover glass (18 mm × 18 mm). The bottom surface was covered with moistened filter paper. A small lump of moistened dry yeast was also placed on the paper as feed. After conditioning for at least one hour, a test sample [a living female, a female rinsed with hexane for 30 sec. (still alive after rinse), or a piece of filter paper (2 mm × 2 mm) impregnated with a candidate compound at each indicated dose (in 1 μl aliquot)] was introduced into an assay chamber with minimum disturbance, and the frequency of subsequent male-male mounting attempts was counted for three minutes. The assay was repeated 10 times, each using a new group of males under a binocular microscope. Living males and filter paper impregnated with hexane (1 μl) were used as control. Results were processed to evaluate the significance of differences with the Mann-Whitney U-test.

Instrumental analyses

Gas liquid chromatography coupled with mass spectrometry (GC-MS) was performed using a Hewlett Packard HP-5890 series II Plus with the same column under the same conditions as those used for GC-MS analysis.

Quantitative determination of \( \gamma \)-acaridial.

One mite (male or female) was dipped in hexane (2 μl, containing 10 ng of n-dodecane as the internal standard) for three minutes, and the extract was subjected to GC analysis as mentioned above, to determine the \( \gamma \)-acaridial content. The amount (ng per mite) of \( \gamma \)-acaridial was calculated by response factors relative to n-dodecane [peak area ratios (x) of \( \gamma \)-acaridial to n-dodecane] using the following equation;

\[
\text{y (ng of } \gamma \text{-acaridial)} = 1.69 \times \text{x.}
\]

The Mann-Whitney U-test was employed for statistical analysis.

Conventional extraction, purification and chemicals.

A total of 200 females were collected with a needle from the stock culture, and dipped into hexane (20 μl) for 3 min. The extract was, without concentration, subjected to a SiO2 column (200 mg, Wakogel C-200), and separated by eluting with 2 ml of each of the following solvents; hexane, ether in hexane mixture (1%, 3%, 10%, 30% and 50%), and ether. All fractions were subjected to a bioassay to locate the active fraction, the composition of which was then determined by GC-MS. n-Dodecane was available commercially. \( \gamma \)-Acaridial was prepared as reported by Sakata and Kuwahara (2001).

Results

GC-MS analysis.

The GC profile of the hexane extract from females was composed of the following 20 peaks (a - t), as shown in Fig. 1A. That of males was similar, but the relative intensity of the males’ profile was lower than that of females (Fig. 1B). Peaks b, d, e, f, g, j and s were major components in female extracts, while male extracts composed of four major peaks e, g, j and o. The distribution of peaks b and s were biased only to females (Fig. 1). All components were identified by comparing their GC-MS data with those of each reported and/or of authentic compounds, as follows: a, dehydrocineole (tR 5.62 min, Ayorinde et al., 1984); b, rosefuran (tR 7.30 min, Leal et al., 1989b); c, nerol (tR 9.28 min); d, neral (tR 9.48 min, Kuwahara et al., 1980); e, a mixture of \( \alpha \)-acaridial (tR 10.06 min, Leal et al., 1989a) and neryl formate (tR 10.06 min, Kuwahara et al., 1975); f, \( \gamma \)-acaridial (3-hydroxybenzene-1,2-
Sex Pheromone $\gamma$-Acaridial from Rhizoglyphus sp.

Fig. 1 Typical gas liquid chromatograms of extracts from 5 females (A) and 5 males (B) of the unidentified Rhizoglyphus mite 'sapporo'.

See text for conditions.

- a. dehydrocineole
- b. rosefuran
- c. nerol
- d. neral
- e. a mixture of $\alpha$-acaridial and neryl formate
- f. $\gamma$-acaridial, 3-hydroxybenzene-1,2-dicarbaldehyde
- g. tridecane
- h. unknown, M$^+$ at m/z 166 and the base ion at m/z 69
- i. isorobinal (4-isopropenyl-3-oxo-1-cyclohexene-1-carboxaldehyde)
- j. $\beta$-acaridial [(E)-2-(4-methyl-3-pentenylidene)-butanalid]
- k. $\beta$-Z-acaridial, (Z)-2-(4-methyl-3-pentenylidene)-butanalid
- l. 7-hydroxyphthalalde, 7-hydroxy isobenzofuranone
- m. tetradeacne
- n. robindal, 3-oxo-4-isopropylidene-1-cyclohexene-1-carbaldehyde
- o. $\alpha$-acarial, 2-(2-hydroxyethylidene)-6-methylhept-5-enal
- p. $\alpha$, $\alpha$-acariolide, 3-(4-methyl-3-pentenyl)-2(5H)-furanone
- q. pentadecane
- r. heptadecane
- s. hexyl rhizoglyphinate
- t. squalene

- dicarbaldehyde ($t_R$ 10.21 min, Sakata and Kuwahara, 2001)
- g. tridecane ($t_R$ 10.28 min, Howard et al. 1988)
- h. unknown, M$^+$ at m/z 166 and the base ion at m/z 69
- i. isorobinal (4-isopropenyl-3-oxo-1-cyclohexene-1-carboxaldehyde) ($t_R$ 10.99 min, Sakata et al. 1996)
- j. $\beta$-acaridial [(E)-2-(4-methyl-3-pentenylidene)-butanalid] ($t_R$ 11.04 min, Leal et al. 1989c)
- k. $\beta$-Z-acaridial, (Z)-2-(4-methyl-3-pentenylidene)-butanalid ($t_R$ 11.19 min, Shimizu et al. 2003)
- l. 7-hydroxyphthalalde (7-hydroxy isobenzofuranone) ($t_R$ 11.37 min, Shimizu and Kuwahara, 2001)
- m. tetradeacne ($t_R$ 11.61 min, Howard et al. 1988)
- n. robindal (3-oxo-4-isopropylidene-1-cyclohexene-1-carbaldehyde) ($t_R$ 12.03 min, Leal et al. 1990a)
- o. $\alpha$-acarial [2-(2-hydroxyethylidene)-6-methylhept-5-enal] ($t_R$ 12.08 min, Shimizu et al. 2003)
- p. $\alpha$, $\alpha$-acariolide [3-(4-methyl-3-pentenyl)-2(5H)-furanone] ($t_R$ 12.33 min, Tarui et al. 2002)
- q. pentadecane ($t_R$ 12.87 min, Howard et al. 1988)
- r. heptadecane ($t_R$ 15.30 min, Howard et al. 1988)
- s. hexyl rhizoglyphinate (hexyl 2-formyl-3-hydroxybenzoate) ($t_R$ 17.30 min, Leal et al. 1990b)
- t. squalene ($t_R$ 24.95 min, Kuwahara et al. 1988)

Demonstration of female sex pheromone activity.

As shown in Fig. 2, an introduced female aroused nearby males sexually to give 5.1 ± 0.7 counts of mounting attempts, while male introduction (1.4 ± 0.3) indicated no activity. A hexane-washed female body (1.6 ± 0.4) was also inactive, suggestive of the fact that the active principle(s) was lost by rinsing with hexane. There was a significant difference between the male and female introductions ($p < 0.01$, Mann-Whitney U-test), but not between the male and the rinsed female. This fact indicates that the active principle(s) are soluble in hexane, and is a female sex
Biological activity of y-acaridial. According to the present species, the sex pheromone activity was not demonstrated against the control (1.5 ± 0.3 count). As a result, the compound was recognized as the sex pheromone with a convex dose-response relationship (Fig. 4).

Quantitative determination of y-acaridial. Each female contained 38.9 ± 6.4 ng (average ± S.E., n = 4) of y-acaridial, and each of the males contained 10.1 ± 4.1 ng. The relative average female/male ratio was therefore 3.85. Consequently, 1/39 (less than 3%) of the y-acaridial content in a female is sufficient to elicit sex pheromone activity.

Discussion

From the unidentified Rhizoglyphus mite "sapporo", whose ITS2 region consisted of 447 bps (code name RH 34, GenBank Accession Number: AB104969), a female sex pheromone was demonstrated other than the previously identified alarm pheromone (Akiyama et al., 1997), and its active principle was identified as y-acaridial. This is the fourth example of the female sex pheromone identification from alarm pheromone emitting mites. The crude hexane extracts of three other species, R. robini, R. setosus and Tyrophagus putrescentiae, did not indicate any sex pheromone activity, whereas a fraction after removal of the alarm pheromone with SiO2 column chromatography restored the sex pheromone activity, and each active principle was identified (Mizoguchi et al., 2003; 2005). On the other hand, in the present species, sex pheromone activity was not demonstrated, not only in the crude hexane extracts but neither in any chromatographic fraction.

Based on the clear bioassay indicating the presence of a sex pheromone (Fig. 2), the active principle was
Sex Pheromone \(\gamma\)-Acaridial from *Rhizoglyphus* sp.

![Graph](image)

**Fig. 3** Sex pheromone activity of SiO\(_2\) column eluates, assayed at 0.1 female equivalent, together with the control (A) and a living female (B).

* : Significant differences \((p < 0.01)\) determined by the Mann-Whitney \(U\)-test relative to the control.

![Graph](image)

**Fig. 4** Dose-response relationships of synthetic \(\gamma\)-acaridial.

* : Significant differences \((p < 0.01)\) determined by Mann-Whitney \(U\)-test relative to the control.

identified using a rather unusual approach as the natural product chemistry. As reported by Sakata and Kuwahara (2001), \(\gamma\)-acaridial is usually not recovered from a SiO\(_2\) ( Wakogel C-200) column, possibly due to chelate formation with some metallic cations present in SiO\(_2\) as impurities. If the amount of the compound is available in sufficient quantities and applied to a SiO\(_2\) column, it can be recovered in Fr. 4 (10% E/H eluate).

In the present study, however, no trace of the compound was detected in any fractions. Known chromatographic behavior of \(\gamma\)-acaridial, and its actual vanishing from all fractions after column chromatography together with the lack of activity detected in either fraction, enabled us to investigate the pheromone activity of \(\gamma\)-acaridial, leading to the unanticipated discovery of \(\gamma\)-acaridial as the pheromone. It might be an example of a mite sex pheromone study, based on comprehensive analysis of mite components.

The dose response relationship of the pheromone was convex, like most cases of the sex pheromone identified among Astigmata (Kuwahara, 2004). The
compound was distributed not only in females (38.9 ± 6.4 ng in average), but also in males (10.1 ± 4.1 ng in average) with a female / male ratio of 3.85, and activities were demonstrated at a dose of 1 ng and 10 ng. These results did not contradict the 14 cases of sex pheromone identification on Astigmatid mites (Kuwahara, 2004). Furthermore, the pheromone content and activity were identical to the case of R. robini sex pheromone α-acaridial (Mizoguchi et al., 2003). Although the alarm pheromone content and its activity have not yet been strictly determined like in R. robini, a similar mechanism for the appropriate expression of the two pheromones (the alarm and the sex pheromones) (Mizoguchi et al., 2003) may also be conceivable in the present species.

γ-Acaridial is not only widely distributed among Astigmata as summarized in Kuwahara (2004), but also among Oribatida (Sakata et al., 2003). Although the compound has been detected in 34 of the 61 species of Astigmata examined (Kuwahara, 2004), this is the first species in which γ-acaridial has been demonstrated to function as female sex pheromone.

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