Chemical Ecology of Astigmatid Mites LXXIII. Neral as an Alarm Pheromone of the Acarid Mite, *Oulenzia* sp. (Astigmata: Winterschmidtidae)

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ABSTRACT

Hexane extracts derived from an unidentified *Oulenzia* sp. showed the alarm pheromone activity. The extract was composed of the following 10 compounds; tridecane, neral, pentadecane, dodecane, 3-hydroxybenzene-1,2-dicarbaldehyde, geranial, (Z)-7-pentadecene and 7-hydroxyphthalide in decreasing order together with two unknown compounds. After fractionation with a silica gel column, the active fraction consisted of a mixture of neral and geranial. Neral was found to show the activity, but geranial was not. Neral was, therefore, identified as the alarm pheromone of this species.

Key words: Astigmatid mite, Winterschmidtidae, alarm pheromone, *Oulenzia* sp., neral

INTRODUCTION

Alarm pheromones are known in 16 species belonging to six families from 52 species in nine families of Astigmata investigated. Active components of these pheromones are secreted from a pair of opisthonotal glands present in each species (Kuwahara et al., 1979 and 1980; Mizoguchi et al., 2003). Chemical structures of the pheromones so far known are classified into three groups; 1) monoterpenes, 2) aromatics and 3) hydrocarbons (Kuwahara, 1999). The monoterpenes consist of five compounds; neryl formate in six species [four species belong to the genus *Rhizoglyphus* (Akiyama et al., 1997), *Tyroborus lini* (Tomita et al., 2003), and *Tyrophagus putrescentiae* (Kuwahara et al., 1975)], neral in five species [*Carpoglyphus lactis* (Kuwahara and Suzuki, 1983), *Glycyphagus domesticus* (Kuwahara et al., 1991a), *Suidasia medanensis* (Leal et al., 1989), *Lardoglyphus konoii* (Kuwahara et al., 1980) and *Schwiebiaea elongata* (Kuwahara et al., 2001)], geranial in *Histiostoma laboratorum* (Kuwahara et al., 1991b), isopiperitenone in *Tyrophagus similis* (Kuwahara et al., 1987) and β -acaridial in *Tyrophagus longior* (Noguchi et al., 1998). The aromatic compound 2-hydroxy-6-methylbenzaldehyde functions as the pheromone in *Tyrophagus perniciosus* (Leal et al., 1988). A mixture of alkenes (C₁₃–C₁₅) in *Tyrophagus neiswanderi* (Kuwahara et al., 1989) and (Z,Z)-6,9-heptadecadiene in *Tortonia* sp. (Kuwahara et al., 1995), are also known as the alarm pheromones. No relationships have been recognized between the

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chemical structures of alarm pheromones and taxonomic status of Astigmata.

Other than alarm pheromones, sex and aggregation pheromones are also present as the same gland components of Astigmata (Kuwahara et al., 1980; Mizoguchi et al., 2003), and it is reasonable to understand that each species can handle a kind of pheromone function within the three. Recently, it has been realized that a species of mites can manipulate at least two kinds of pheromones (Kuwahara, 2001), such as found in *Rhizoglyphus robini* where a combination of the alarm and sex pheromones (neryl formate and α -acaridial, respectively) present in the same gland is manipulated to show each own function (Mizoguchi et al., 2003), and as found in *Schwiebia elongata* where neral functions not only as an alarm pheromone at a high dose but also as an attractant at a low dose, possibly an aggregation pheromone (Nishimura et al., 2002). Therefore, a new identification of the alarm pheromone from an unidentified mite *Oulenzia* sp. (Astigmata: Winterschmidtidae) may serve as a basic knowledge for further understanding of mite's multiple pheromone systems.

Oulenzia sp. is collected twice from banana and rambutan in the Philippines. Based on behavioral observations (Kuwahara et al., 1975), the presence of an alarm pheromone is also conceivable. A bioassay method (Tomita et al., 2003) originally devised for the present species was successfully applied for the study. We report here the identification of neral as the alarm pheromone of *Oulenzia* sp. triggering mite repellent behavior. This pheromone is distributed in both sexes and its content can be quantitatively determined. We evaluated the alarm pheromone activity using purified neral fraction and synthetic neral with a simple bioassay method.

MATERIALS AND METHODS

Mites. An unidentified species of the genus *Oulenzia* (Astigmata: Winterschmidtidae) whose ITS2 region consisted of 289 or 282 bps (code name OL 50, GenBank Accession Number: AB104992 and AB104993) was a population collected in June 1997 from the deteriorated parts of organic banana produced in the Philippines. Males exhibit guarding behavior for tritonymphs at resting period. A pair of opisthonotal glands is visible under binocular microscope in the opisthosoma as brown spots. The strain was fed on dry yeast at room temperature under 70% relative humidity in a plastic culture dish (85 mm in diameter, 20 mm depth) with the bottom surface covered with a sheet of filter paper. Each dish was kept in a zip-lock polyethylene bag (240×170 mm, 0.04 mm film thickness, Seisan-nihon Co. Ltd.) twice to maintain the humidity. When the mite number reaches a certain level in a stock culture, this species starts to escape naturally from the medium and to stay on the inner surface of the dish. Such a cover dish with an appropriate mite number was used for behavioral experiments.

Analytical methods. Gas liquid chromatography (GC) was conducted with a Hewlett Packard 5890 series II Plus equipped with FID and an HP-5 capillary column (0.25 mm in diameter×30 m, 0.25 μ m film thickness, Hewlett Packard, split-less mode) at a temperature programmed from 60°C (with an initial 2-min hold) to 290°C (with a 5-min hold) at 10°C/min. Gas liquid chromatography coupled with mass spectrometry (GC/MS) was carried out with a Hewlett Packard HP-5989B gas chromatograph-mass spectrometer operated at 70 eV

in the splitless mode and an HP-5 MS capillary column (0.25 mm×30 mm, 0.25 µm film thickness) under the same conditions as described above.

Bioassay method. The activity was evaluated as reported (Tomita et al., 2003) with minor modifications. A cover dish with an appropriate mite number as described above was placed upside down on a sheet of paper, on which many circles (6 mm in diameter) were previously printed. Selecting arbitrarily a resting mite (without differentiating sexes, and conditioning), the paper underneath was gently shifted to set the mite in the center of circle. A piece of filter paper (2×1 mm) impregnated with a candidate sample, such as 1 µl (about 3 female equivalent) of the whole-mite extract (1 ml) or 10 µl of its each separated fraction (10 ml), was placed within the circle with the least physical disturbance. The time (in seconds) required for the mite to be aroused and to leave the test circle was measured under a binocular microscope for 120 seconds after exposure to the alarm pheromone. Hexane was used as a control. This assay was repeated 10 times at room temperature under ambient humidity. Resulting data were processed to evaluate significant differences in the required time by a Mann-Whitney *U* test or a Kruskal-Wallis test followed by Dunn's multiple-comparison test.

Extraction and purification of the pheromone. Ten males or females were transferred to a conical-bottomed tube (handmade, 8 mm in outer diameter×30 mm in height) from the stock culture, and extracted with 4 µl of hexane for 3 min. The hexane extract was collected with a microsyringe, and subjected to GC/MS analysis without concentration to monitor compositions. To determine the active compound(s), mites (110 mg) including all developmental stages were separated from the culture medium by the conventional saline flotation method (Matsumoto, 1965), and dipped in hexane (1 ml) for 3 min. The resulting hexane extract, after evaporating the solvent, was applied to a silica gel column (1.0 g, Wako-gel C-200), and eluted with 10 ml each of the following solvents: hexane, hexane-ether (20:1), (10:1) and (5:1), and ether. The activity and composition of each eluate were monitored by bioassay, GC and GC/MS.

Quantitative determination of the pheromone. A male or a female was placed with a needle into a conical-bottomed tube. Hexane (3 µl) containing hexadecane (60 ng) as an internal standard was added to each tube for extraction. After 3 min, 1 µl of the extract corresponding to 1/3 male or female equivalent was subjected to GC analysis. The quantity of neral per male or female was determined by the relative ratio of the peak area to that of the internal standard from 10 replicates.

Preparation of neral and geranial. Neral (content of geranial less than 1%) and geranial (content of neral less than 1%) were each synthesized by the active MnO₂ oxidation of nerol and geraniol, respectively (Leal et al., 1989).

RESULTS

GC/MS analysis of the hexane extracts

The hexane extracts were composed of the following 10 compounds in decreasing order: tridecane, neral [(*Z*)-3,7-dimethyl-2,6-octadienal], pentadecane, dodecane, unknown peak (238/69, *t*_R: 13.43 min), 3-hydroxybenzene-1,2-dicarbaldehyde (γ-acaridial, Sakata and

Kuwahara, 2001), geranial [(*E*)-3,7-dimethyl-2,6-octadienal], (*Z*)-7-pentadecene, unknown peak (136/69, t_R : 9.31 min), 7-hydroxyphthalide (7-hydroxy-3*H*-isobenzofuran-1-one, Shimizu and Kuwahara, 2001), including two unknown peaks (M^+ ion/the base ion peak). The eight compounds identified are summarized in Table 1. No peculiar differences were observed in the chromatographic profiles of males and females.

Identification of the alarm pheromone

When a filter paper impregnated with the hexane extract (1 μ l) was introduced in the vicinity of a resting adult mite in the center of a circle, the mite began moving and left the circle. The time (9.4 ± 1.5 sec.) for a mite to leave of the test circle was significantly different from the control (36.6 ± 6.5 sec.) (Mann-Whitney *U* test, $p < 0.05$) (Fig. 1). We concluded an alarm pheromone was present in this species.

After fractionation of the mite extract with a silica gel column chromatography, the pheromone activity was recovered in a fraction eluted with a hexane-ether (10:1) mixture (Fig. 2). All the other fractions were entirely inactive. Significant differences were demonstrated

Table 1. GC/MS data for compounds obtained from the hexane extract of *Oulenzia* sp.

Compound	GC t_R (min)	Diagnostic ions m/z (intensity %)
Dodecane	8.85	43 (72), 57 (100), 71 (64), 85 (41), 99 (12), 113 (10), 127 (9), 141 (5), 170 (M^+ , 26)
Neral	9.53	41 (90), 69 (100), 84 (34), 94 (46), 109 (32), 119 (20), 134 (12), 137 (8), 152 (M^+ , 3)
Geranial	9.90	41 (69), 69 (100), 84 (30), 94 (22), 109 (10), 123 (9), 137 (13), 152 (M^+ , 9)
3-Hydroxybenzene-1,2-dicarbaldehyde	10.20	51 (5), 63 (9), 65 (22), 76 (7), 93 (33), 121 (100), 122 (55), 149 (9), 150 (M^+ , 93)
Tridecane	10.30	43 (66), 57 (100), 71 (73), 85 (49), 99 (14), 113 (9), 127 (9), 141 (7), 184 (M^+ , 24)
7-Hydroxyphthalide	11.40	51 (5), 65 (14), 77 (2), 93 (15), 105 (2), 121 (100), 122 (9), 149 (12), 150 (M^+ , 71)
<i>Z</i> -7-Pentadecene	12.78	41 (69), 55 (100), 69 (90), 83 (91), 97 (76), 111 (43), 125 (17), 137 (9), 210 (M^+ , 48)
Pentadecane	12.85	41 (30), 43 (68), 57 (100), 71 (72), 85 (44), 99 (16), 113 (9), 127 (6), 212 (M^+ , 11)

Fig. 1. Alarm pheromone activity of the hexane extract from *Oulenzia* sp. Significant difference ($p < 0.05$, each $n = 10$).

Fig. 2. Alarm pheromone activity of each silica gel column fraction (Hex: hexane; Et: diethyl ether). Significant difference (* $p < 0.05$, each $n = 10$).

Fig. 3. Alarm pheromone activity of synthetic neral and geranial. Significant difference (* $p < 0.05$, ** $p < 0.01$, each $n = 10$).

between the active fraction and the control (Kruskal-Wallis test followed by the Dunn's multiple-comparison test, $p < 0.01$). The active fraction monitored by GC/MS analyses consisted of two peaks (9.53 and 9.90 min) identified as neral (relative abundance; 95%) and geranial (relative abundance; 5%). Therefore, neral and (or) geranial are suggested as alarm pheromones.

Evaluation of the synthetic alarm pheromone

Synthetic neral showed the alarm pheromone activity at doses of 1–1000 ng, which were statistically significant at $p < 0.01$ or $p < 0.05$ (Kruskal-Wallis test followed by the Dunn's multiple-comparison test) (Fig. 3). There was no significant difference between a 0.1 ng dose of neral and the control. On the other hand, synthetic geranial indicated no alarm pheromone activity at doses of 0.1–100 ng, while geranial at a dose of 1000 ng was active (Fig. 3). As a result, neral was concluded as the alarm pheromone of this species. The quantity of neral determined by GC in the hexane extract was 1.05 ± 0.20 ng per male and 0.75 ± 0.13 ng per female (each an average of 10 replicates), respectively.

DISCUSSION

The alarm pheromone of this *Oulenzia* sp. was identified as neral active at 1–1000 ng (significant at $p < 0.05$ for 1 ng and at $p < 0.01$ at 10, 100 and 1000 ng). The dose-response relationship seemed to be sigmoidal, similar to other alarm pheromones (Nishimura et al., 2002). On the other hand, geranial (isomeric purity > 99%), the geometric isomer of neral, was only active at 1000 ng (significant at $p < 0.05$). This activity, however, can be explained by the presence of neral at a dose of 10 ng. Based on these results, we concluded that neral was the alarm pheromone of this species and geranial was inactive.

Neral is one of the most widely distributed monoterpene pheromone among astigmatid mites (in six species including the present study), together with neryl formates (distributed in six species) (Kuwahara, 1999, 2001). On the other hand, geranial has been shown as the alarm pheromone only in one species of Astigmata *H. laboratorium* (Kuwahara et al., 1991b) among 52 species examined.

The amount of neral in the mite (1.05 ± 0.20 ng for a male and 0.75 ± 0.13 ng for a female) is almost consistent with that to stimulate the alarm pheromone activity. This fact indicates that total discharge from either sex is necessary to release the alarm pheromone behavior among test mites. The same situation, total discharge of an active component required, has been evidenced in at least two other cases of *R. robini* (Mizoguchi et al., 2003) and *T. lini* (Tomita et al., 2003), and seems to be a common phenomenon in alarm pheromones. For better understanding of mite's multiple pheromone systems, further evidence must be accumulated to investigate the condition, in which alarm pheromones function in Astigmata.

In addition, the first example of the alarm pheromone in Oribatida has been reported from nymphs of *Nothrus palustris* (Shimano et al., 2002). Its active principle is geranial, and its presence is limited only in nymphal stages. Because oribatid species belonging to four cohorts among six cohorts are known to possess the same opisthonotal gland as Astigmata, and volatiles possibly functioning as defense compounds (Shimano, 2001) have been identified among 60 species examined (Raspotnig et al., 2002; Sakata and Norton, 2001; Sakata et al., 2003; Shimano et al., 2002), it is of no wonder to find further examples of alarm pheromones among Oribatida, like cases of Astigmata.

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摘要

コナダニ *Oulenzia* sp. の警報フェロモンとしてネラールの同定

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未同定種 *Oulenzia* sp. のヘキサン抽出物に警報フェロモン活性を認めた。ヘキサン抽出物は、2種の未知化合物を含め、トリデカン、ネラール、ペンタデカン、ドデカン、3-ヒドロキシベンゼン-1,2-ジカルバルデヒド、ゲラニール、(Z)-7-ペンタデセン、7-ヒドロキシフタライドの10化合物で構成されていた。抽出物をシリカゲルカラムで精製したところ、活性フラクションにはネラールとゲラニールが含まれていた。ネラール（1–1000 ngで活性）はゲラニール（1000 ngでのみ活性）よりも強い活性を示すことから、ネラールを本種ダニの警報フェロモンと同定した。