Chemical Ecology of Astigmatid Mites LIX Neral, the Alarm Pheromone of *Schwiebea elongata* (Banks) (Acari: Acaridae)¹

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ABSTRACT

The alarm pheromone activity was detected in the hexane rinse of *Schwiebea elongata*, the pool mite strain. The GC profile of the large scale extraction of mites revealed four components, and their chemical structures were identified by comparing their GC/MS spectra and GC retention times with those of authentic compounds, as follows; neral {3,7-dimethyl-(Z)-2,6-octadienal}, geranial {3,7-dimethyl-(E)-2,6-octadienal}, neryl formate {3,7-dimethyl-(Z)-2,6-octadienyl formate}, and *n*-tridecane. The alarm pheromone activity was only detected in fractions of 5% and 10% ether in hexane eluates from an SiO₂ column, and these fractions consisted only of a set of peaks, neral and geranial. Neral was the major component along with tridecane, and its percentage in citral (a mixture of neral and geranial) was differed among samples analyzed such as 94% in the small scale extraction, 90% in large scale extraction, and 79% in the active SiO₂ column eluate. The synthetic neral (purity 91% and yield 83%) was active at 10-100 ng-dose, while geranial (purity 98% and yield 32%) was hundred times less active than neral (active at ng-dose ppm), therefore neral was identified as the alarm pheromone.

Key words: alarm pheromone, *Schwiebea elongata*, neral, 3,7-dimethyl-(Z)-2,6-octadienal, the pool mite

INTRODUCTION

Alarm pheromones have been identified in total 16 species of Astigmata belonging to 7 families, and twelve compounds present in their opisthonotal glands are known to be responsible for their activities (Kuwahara, 1990, 1991, 1995; Akiyama *et al.*, 1997; Noguchi *et al.*, 1998). Neral {3,7-dimethyl-(Z)-2,6-octadienal} and neryl for-

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mate {3,7-dimethyl-(Z)-2,6-octadienyl formate} are the most widely distributed pheromones, and both compounds function in each 5 species (Kuwahara, 1990, 1991, 1995).

Neral has been recorded as the alarm pheromone from the unidentified *Schwiebea* species (actually *Schwiebea elongata* by the gas-liquid chromatographic profile) obtained in a soil of a garbage depot in Tsukuba city, Ibaraki-Prefecture, and the result has also been included in the above mentioned summary (Kuwahara, 1990, 1991, 1995), however, the strain has eventually been lost before identifying the species. Therefore, we tried to restore the species and collected a total of 4 strains of similar-looking *Schwiebea* sp. Those were identified as *Schwiebea elongata* (Banks, 1906).

All 4 strains were demonstrated to possess their alarm pheromones by the following observations; mites were noticed to start escape, when the needle tip on which the opisthonotal gland secretion had previously been smeared was presented in front of appropriate mites in the stock culture.

In order to confirm the identity of the species along with gas-liquid chromatographic profiles, the chemical structure of the alarm pheromone was re-examined, by purifying the pheromone using the extract of pool mite strain *S. elongata*, and also by monitoring its activity by bioassay against 4 strains.

MATERIALS AND METHODS

Mites: Schwiebea elongata (Acari: Acaridae) (pu-ru mizu konadani in Japanese), the pool mite strain, was mass-reared by feeding dried yeast upon a wet filter paper in a disposable Petrie dish (90 mm in diameter and 18 mm in depth). Mites were separated from the culture medium by two methods; the saturated NaCl flotation method for a large scale extraction, and sampling one by one on a needle for a small scale extraction, and both of the collected mites were dipped in hexane for 3 min to obtain extracts for chemical analysis.

The species is a medium size among astigmatid mites, weighing 55 μ g in average for female, which was originally collected from a heated swimming pool in Ibaraki prefecture in February 1992 (Tagami *et al.*, 1993) and possible allergenicities due to the species had been examined (Tagami, 1995). The strain is parthenogenetic, and its sex ratio (usually less than 1% for males) has not been affected by rearing conditions (Okabe, 1995).

The other three strains (1, 2 and 3), were also used as standards for the alarm pheromone bioassay. Strains 1 and 2, which were collected by us from a soil and litter at Kyoto University, and was kept in our laboratory, whereas 3 was bisexual in nature and newly collected from the same area. Gas liquid chromatography (GC) profiles derived from 4 strains were the same each other (Fig. 1). All components detected in the female extract were also found in the male extract and no sexual differences were recognized by GC.

Instrument: Gas liquid chromatography (GC) was carried out on an HP-5890 series

Fig. 1. GC profiles from 4 strains of *Schwiebia elongata* on a HP-5 capillary column.

See the text for analytical conditions.

1: Peak 1, 2: peak 2, 3: peak 3, 4: peak 4 and U: structure not yet determined.

II plus equipped with an FID using a HP-5 capillary column (0.32 mm $\times$ 30 m, 0.33 μ m in film thickness) at a temperature programmed condition from 60°C to 290°C at 10°C/min with an initial 2 min hold. Sample was analyzed at a split-less mode using He as a carrier gas, and the chromatogram was processed by an HP 3396 series II Integrator to obtain R_f 's and relative abundances (%) of each peak. GC-Mass spectrometry (GC/MS) were performed by a Hewlett Packard 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 those mentioned above.

Chemicals: Neral and geranial were each prepared by the active MnO_2 oxidation of nerol [3,7-dimethyl-(*Z*)-2,6-octadienol] and geraniol [3,7-dimethyl-(*E*)-2,6-octadienol], respectively. Alcohol (each, 1 g) was dissolved in freshly distilled acetone (15 ml) and stirred at r.t. for 48 hr by adding active MnO_2 (2.5 g) portionwise in three times. After removing MnO_2 by filtration and successive concentration under reduced pressure, the resulting oil was purified on a SiO_2 (20 g) column, eluting with a mixture of ether and hexane. Neral (purity 91% by GC and yield 83%) and geranial

(98% and 32%) were each obtained in the 10% ether in hexane eluate of the column. Because both neral and geranial tend to isomerize each other thermally and give an equilibrium mixture, both synthetic preparations were used without distillation. The solution was then diluted successively with hexane to 10,000, 1,000, 100, 10, and 1 $\mu\text{g}/\text{ml}$ for bioassay.

Bioassay method: Bioassay for the alarm pheromone activity was conducted by the modified method (Akiyama *et al.*, 1997), based on the absolute quantity of a candidate compound impregnated as 1 μl aliquot to a piece of filter paper (2 mm $\times$ 2 mm). Following four strains; the pool mite strain, strain-1, -2 and -3 were used as the test mites.

Small scale extraction of mites for GC and GC/MS, and quantitative determination of neral: Five alive females were collected by a needle into a small conical-bottomed tube (handmade, 8 mm o.d. $\times$ 30 mm ht) at a time. Four μl of hexane was added to the tube and the resulting hexane rinse was collected by a micro-syringe at 3 min (exact) after introduction of the solvent. Then all portions of the rinse was subjected to the analysis of GC/MS. To determine neral content, each female was rinsed with 2 ppm hexane solution (5 μl) of n-hexadecane for 3 min and 1 μl portion was analyzed by GC. The peak-area ratio of neral to n-hexadecane was correlated with the standard curve which had been prepared using known amounts of neral and n-hexadecane. The sampling and GC analyses were replicated 12 times and the result was indicated as the mean $\pm$ SD.

Large scale extraction and purification for bioassay and identification: *Schwiebia elongata*, the pool mite strain (2.25 g) collected from the culture medium were dipped in hexane (7.5 ml $\times$ 2) for three minutes, and then mite bodies were removed by filtration. The resulting hexane rinse, after confirming its alarm pheromone activity by bioassay, was concentrated to ca. 2 ml under gentle flow of N_2 gas, was transferred on an silica gel (Wako-gel C200, 0.4 g) column, and the column was eluted and fractioned with each 6 ml of hexane, ether-hexane mixture (1% ether in hexane, 3%-, 5%-, 10%-, 20%- and 50%-) and ether, respectively. All fractions were then subjected to bioassay as stated above.

RESULTS AND DISCUSSION

The hexane rinse (7.5 ml $\times$ 2) from *S. elongata* (2.25 g, pool mite strain) which indicated the alarm pheromone activity at 1–10 μl dose, gave the following four major peaks by GC; peak 1: R_t 9.51 min (44.0%), peak 2: 9.91 min (trace), peak 3: 10.05 min (16.2%) and peak 4: 10.29 min (39.7%).

GC/MS analysis of peak 1 (R_t 9.51 min) gave M^+ ion at m/z 152 (1%) and the base ion at m/z 69 (100%) with diagnostic ions at m/z 137 (7%), 134 (9%), 119 (18%), 109 (30%), 94 (43%), 84 (31%) and 41 (76%). The MS data and GC retention time were both identical to those derived from the standard neral.

The other peak 2 (R_t 9.91 min) gave M^+ ion at m/z 152 (10%) and the base ion at m/z 69 (100%) with diagnostic ions at m/z 137 (13%), 123 (13%), 109 (18%), 94

(20%), 84 (31%) and 41 (52%). The peak was identified as geranial., based on GC/MS data and GC retention times, using geranial as the standard.

Peak 3 (10.05 min) was obtained as a 3% ether in hexane fraction, suggestive of ester. The peak gave $M^+-HCOOH$ ion at m/z 136 (30%) and the base ion at m/z 69 (100%) with diagnostic ions at m/z 121 (21%), 107 (8%), 93 (51%), 80 (15%) and 41 (39%). These MS data was identical as those of authentic neryl formate. Retention time of the peak was also the same as that of neryl formate.

Although the GC profile of small scale extraction indicated an additional peak at 10.22 min as shown in Fig. 1, however, the peak was not detectable in the large scale extraction with unknown reasons. It gave M^+ ion at m/z 150 (70%) and the base ion at m/z 121 (100%) with diagnostic ions at m/z 122 (48%), 93 (37%) and 65 (33%), while the structure remained obscure.

The hexane eluate contained peak 4 (10.29 min), which gave M^+ ion at m/z 184 (22%) and the base ion at m/z 57 (100%) with diagnostic ions at m/z 127 (12%), 113 (12%), 99 (17%), 85 (54%), 71 (82%) and 43 (51%). The mass spectrum was identical as those of tridecane.

After concentrating the hexane rinse of mite bodies (2.55 g) to ca. 2 ml, the concentrate was chromatographed on a SiO_2 column and eluted with each 6 ml of indicated solvents. The alarm pheromone activity was recovered in the 5% and 10% ether in hexane eluates. The fraction consisted of a mixture of neral (peak 1, R_f 9.51 min, 79%) and geranial (peak 2, R_f 9.91 min, 21%).

Although the alarm pheromone activity was demonstrated in both synthetic neral and geranial, its activity level differed 100 times; neral (purity 91%, with geranial 9% as the other contaminant) was active at 10–100 ng/dose, whereas geranial (purities 98%, with neral 2%) was at 1,000–10,000 ng/dose. Thus we can conclude that neral is the pheromone. All 4 strains indicated also the same activity (Table 1). The hexane rinse (15 ml, active at 1–10 μ l dose) derived from mites (2.25 g) was calculated to contain neral 7.6 ng/ μ l (or 11.4 μ g/15 ml), so that the neral content in the active dose (1–10 μ l dose) corresponded to 7.6–76 ng. The activity was comparable to that of synthetic neral (active at 10–100 ng dose). Neral was, therefore, concluded as the only active compound in the hexane rinse of mite.

If we assume that collected mite bodies (2.25 g) consists of females, neral 11.4 μ g is corresponded to the total yield from 41,000 mites (2.25 g/55 μ g) and the average yield per mite is calculated to be 0.28 ng. On the other hand, the direct determination of neral per mite resulted to be 20.37 ± 11.74 ng (average $\pm$ SD), as a mixture of neral (94% on average) and geranial (6% on average). As a result, the recovery rate of neral from mite is calculated as low as 1.23%. It was also noticed that neral percentage in citral (a mixture of neral and geranial) was not always consistent among samples and tended to decrease such as 94% in the small scale extraction, 90% in large scale extraction, and 79% in the active SiO_2 column eluate, as reported on other species (Kizawa *et al.*, 1993).

It is not unusual that most of the alarm secretion have been lost during separation of mite bodies from culture medium by dipping into brine, because the present species

Table 1. Alarm pheromone activity of neral and geranial against 4 strains of *Schwiebea elongata*.

Species	Strain (habitat)	Neral* (ng)	Geranial** (ng)
<i>Schwiebea elongata</i>	pool mite (pool)	10–100	1,000–10,000
	1 (soil)	10–100	1,000–10,000
	2 (soil)	10–100	1,000–10,000
	3 (soil)	10–100	1,000–10,000

*; A mixture of neral (91%) and geranial (9%).

**; A mixture of geranial (98%) and neral (2%). Activity of the mixture corresponds to that of contaminated neral

is very sensitive to be handled; as demonstrated by its large variation among neral's quantitative determination, and by its strong emanation of neral flavour upon spraying water to the culture to keep wet condition.

Although *S. elongata*, the pool mite strain, has been collected from a heated swimming pool, its original habitat in nature remains obscure, and efforts to find its origin have been continued since then. In the present study, total three strains (1, 2 and 3) which were collected from litters at each different location and timing were turned out to be the same species as the pool mite, morphologically. Therefore, it may be the case that a species ordinarily living in litters have found a new habitat and have adapted to its nutritionally poor and oxygen insufficient environment.

Because all four strains were morphologically the same, it was reasonable that these strains gave the same GC profiles and all efforts to distinguish each other chemically among the three (the pool mite, and strains 1 and 2) have failed. Strains 1 and 2 produce a few males like the pool mite strain, while strain 3 indicated nearly 1 : 1 sex ratio.

Neral was, thus, identified as the alarm pheromone of *S. elongata*, other than the following five species; *Lardoglyphus konoii*, *Carpoglyphus lactis*, *Suidasia medanensis*, *Glyciphagus domesticus* and *Schwiebea* sp., among 31 species of astigmatid mites (Kuwahara, 1990, 1991 & 1995). Although the culture of the original *Schwiebea* sp whose pheromone had been identified as neral have been lost and it is not certain at present whether the species correspond to the present species or not, neral as the alarm pheromone was again confirmed in the present species *S. elongata*.

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摘 要

無気門亜目ダニの化学生態学 第59報. *Schwiebea elongata* Banks の警報フェロモンとして、ネラールを同定

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プールで見つかったプールミズコナダニ *Schwiebea elongata* Banks (プールダニ株) のヘキサン抽出液に警報フェロモン活性を認めた. ダニの大量抽出物を GC/MS 分析すると, 4 成分 (ネラール, ゲラニール, 蟻酸ネリル, トリデカン) を認めた. シリカゲル・カラムで分離精製すると, 活性は 5 % 及び 10 % エーテル/ヘキサン溶出部に認められた. 本分画はネラールを主成分 (他の成分はゲラニール) とするシトラールが得られ, 投与量 7.6–76 ng で活性を示した. シトラール中のネラール量は試料の調製法で変動する. ネラールとゲラニールをそれぞれ調製し生物試験すると, ネラールのみが投与量 10–100 ng で活性を示し, 警報フェロモンはネラールと同定した. 他に同種と同定され, 土壌から採取された 3 株はネラールに対してプールダニ株と同様の活性を示した.