Communication

DOI:10.13179/canchemtrans.2014.02.03.0101

Soldier-Specific Terpenoid Compounds of the Japanese Reticulitermes (Isoptera, Rhinotermitidae)

Tuan T. NGUYEN,1,2* Kenji KANAORI,3 Yoko TAKEMATSU,4 and Toshiharu AKINO2

1Department of Chemistry, College of Natural Sciences, Cantho University, Campus II, 3/2 street, Cantho city, Vietnam
2Laboratory of Chemical Ecology, Center for Bioresource Field Science, Kyoto Institute of Technology, Matsugasaki, Kyoto 606-8585, Japan
3Department of Biomolecular Engineering, Kyoto Institute of Technology, Matsugasaki, Kyoto 606-8585, Japan
4Faculty of Agriculture, Yamaguchi University, Yoshida 1677-1, Yamaguchi 753-8515, Japan

*Corresponding Author, E-mail: trongtuan@ctu.edu.vn, Phone: +84-918-858-131

Received: March 6, 2014 Revised: April 1, 2014 Accepted: April 5, 2014 Published: April 6, 2014

Abstract: Terpenoid secretions specific to termite soldiers were analyzed in Reticulitermes speratus speratus (Rss), R. speratus kyushuensis (Rsk), R. kanmonensis (Rk) and R. okinawanus (Ro), and 3 quantitatively major compounds were successfully identified. All these species possessed β-selinene whose content was high in soldiers of Rss and Rsk. While δ-cadinene was detected only in Ro, (S)-(+)-(E,E)-geranyllinalool was first detected in the other species. Both soldiers of Rk and Rsk contained a large amount of this compound. Except for Ro, the soldier-specific terpenoids were identical among Rss, Rsk and Rk.

Keywords: Reticulitermes; terpenoids; β-selinene; (S)-(+)-(E,E)-geranyllinalool; δ-cadinene

1. INTRODUCTION

In the termite society, the soldier caste comprises a small proportion of the population and is responsible for colony defense. The defensive secretions originating from the frontal gland of a soldier termite may contain different chemical components, including alkanes, alkenes, nitroalkanes, vinyl ketones, ketoaldehydes, mono-, sesqui-, di-, and ses-terpenes, and organic acids [1]. Such soldier-specific chemicals have been compared among several species of the subterranean Reticulitermes termites that are distributed in the temperate zones. Concerning volatile mono- and sesqui-terpene hydrocarbon, including α-pinene, β-pinene, limonene, carene, (E,E)-α-farnesene, β- and γ-selinene, germacrene A and C, and γ-cadinene, they serve as aggregation and alarm signals for the nestmate termites [2-6]. Some Reticulitermes species seem to use diterpene alcohol, e.g. geranyllinalool, instead of the terpenoid hydrocarbons to avoid ant attacks [7] or as an attractant and alarm pheromone in the colonies [5].
Japanese *Reticulitermes* species consisted of at least 7 species with 3 subspecies [8], which are distributed from the north (Hokkaido) to the south (the Ryukyu Islands). Our previous paper focused on *R. speratus* collected in Kyoto, which is taxonomically identified as *R. speratus speratus* [9], to identify the soldier-specific secretion as β-selinene, and we also discussed its ethological functions [10]. However, there are no comparative reports on the chemical composition of soldier secretions in the Japanese *Reticulitermes* species. This study starts investigating the soldier-specific terpenoid secretions from 4 Japanese *Reticulitermes* species and subspecies, i.e., *R. speratus speratus* (*Rss*), *R. speratus kyushuensis* (*Rsk*), *R. kanmonensis* (*Rk*), and *R. okinawanus* (*Ro*), which are relatively easier to collect the specimens than the others.

2. MATERIALS AND METHODS

We collected 9 colonies of *Rss* in Matsugasaki Mountains, Kyoto Prefecture, 7 colonies of *Rsk* in Fukiage beachside Park, Kagoshima Prefecture, 4 colonies of *Rk* in Suziyama Park, Yamaguchi Prefecture, and 4 colonies of *Ro* in the Urazoe and Iso Parks, in the Okinawa Prefecture from June to September, 2011. They were kept in plastic boxes (35 cm x 25.5 cm x 4.5 cm) with their nest wood retained as a food resource. The boxes were stored in an incubator at 27 ± 5°C, and water was supplied occasionally to maintain humidity.

The presence of terpenoid compounds was confirmed after separately soaking 10 soldier termites of *Rss, Rsk, Rk,* and *Ro* in 500 µL of hexane for 24 h. After the solvent had evaporated, the residues were fractionated using 0.15 g silica gel (230-400-mesh ASTM, Merck, Germany) packed in a Pasteur pipette as a chromatography column, eluted successively with 500 µL each of hexane; 5, 10, and 50% ether-in-hexane; and 100% ether. All the crude extracts and the fractions were used for gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses. Also, the whole bodies of 400 soldier termites of *Rsk* were immersed in 10 mL of hexane solvent 4 times for 24 h, and was then chromatographed on approximately 3 g of silica gel packed in a glass column (30 cm length and 1 cm ID), and successively eluted with 10 mL each of hexane; 5, 10, and 50% ether-in-hexane; and 100% ether. The 50% ether-in-hexane that contained a large amount of the compound was kept in a deep freezer until it was needed for nuclear magnetic resonance (NMR) analyses. To quantify the terpenoid compounds, 10 soldiers of each colony in each species and subspecies were immersed separately in 500 µL hexane solvent for 24 h. After evaporating fully the solvent and then re-dissolving in 100 µL hexane (total 24 extracts), 1 µL of each extract (0.1 soldier equivalent) was analyzed by GC. 100 ng of n-pentadecane or n-eicosane was also analyzed to quantify terpenoid compounds by analysis of the peak area.

All-trans-geranylgeranyldiphosphate (all-trans-GGPP) was purchased from Sigma-Aldrich Company, and commercial geranyl linalool was purchased from the Tokyo Chemical Industry Company. In a small glass vial, 20µL of all-trans-GGPP solution (1 µg/µL) was mixed with 1 mL of 3 mol dm⁻³ HCl. The mixture then was incubated at 30°C for 30 min in an incubator. After incubation, the aqueous solution was added to 500 µL of GC-grade hexane and mixed for 1 minute. After a short centrifugation, the upper organic phase was transferred to a new vial, rinsed 3 times with distilled water, and a small amount of sodium sulfate was added to eliminate the water. The organic phase was carefully removed and concentrated to as little as 100 µL under a stream of nitrogen. This solution was then analyzed by GC and GC-MS to confirm the presence of (E,E)-geranyllinalool.

GC was performed using a Shimadzu GC-14A equipped with a flame-ionization detector (FID), and either a capillary column DB1-HT (15 m in length x 0.25 mm ID; 0.1 µm in film thickness, J & W Scientific, USA) and DB-5 (30 mm in length x 0.25 mm ID; 0.25 µm in film thickness) or a capillary
Figure 1. Gas chromatograms of terpenoid components of crude hexane extract from 4 species: Ro (A); Rsk (B), Rss (C), and Rk (D). Each number indicates a compound. GC condition; column DB1-HT 15m x 0.25 mm (ID) x 0.25 μm film thickness, temperature program: 60°C (10 min) heated to 300°C at 20°C/min and kept at final temperature for 10 min. Injection was made at splitless mode (A), and split mode (B, C, D).
column DB-Wax (30 m in length x 0.25 mm ID; 0.25 μm in film thickness). Helium was used as the carrier gas, and the column head pressure was set at 100 kPa. Both the injection port and detector were set at 300°C for DB-1 and DB-5. The DB1-HT column oven temperature was set at 60°C for 10 min, heated to 300°C at 20°C /min. and then kept at the final temperature for 10 min, while DB-5 column was heated to 240°C at 3°C /min. For the DB-Wax column, both the injection port and detector were set at 200°C and the column oven was set at 60°C for 1 min, then increased to 220°C at 10°C /min, and kept at the final temperature for 10 min. Data were stored and analyzed using a Shimadzu C-R6A chromatopac integrator. The Kovats Retention indexes (KI) were calculated on the termite chemicals by injecting with authentic hydrocarbon mixtures from nC10 to nC20 for the compounds in hexane fraction or even-numbered hydrocarbons nC20 to nC26 for the compound in 50% ether-in-hexane as Kovats suggested [11]. GC-MS analyses of extracts and fractions were conducted using a Shimadzu QP-5000 MS system equipped with a DB1-HT capillary column as described above was used in the electron impact mode at 70 eV.

Optical rotation was taken on a SEPA-300 high-sensitive polarimeter (HORIBA). All NMR experiments were conducted at 27°C with a Bruker AV-600 spectrometer (600.19 MHz for 1H and 125.6 MHz for 13C). Standard TOPSPIN software (Bruker) was used to perform 2-dimensional double quantum filtered correlated spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), heteronuclear multiple quantum coherence spectroscopy (HMQC), and heteronuclear multiple bond coherence spectroscopy (HMBC) experiments. The signals due to the residual proton of CDCl3 and its carbon signal were used as internal standards (δ = 7.26 ppm for 1H and δ = 77.0 ppm for 13C).

3. RESULTS AND DISCUSSION

GC and GC-MS analyses revealed not only the similarities of the terpenoid composition among Rss, Rsk, and Rk soldier but also the dissimilarities in Ro soldiers (Figure 1). All these species possessed β-selinene (peak 1 in Figure 1, tR 12.1 min) that was eluted in a hexane fraction from a silica gel column when the soldiers’ crude extract was chromatographed. An additional compound (peak 2 in Figure 1A, tR 13.5 min.) detected only in Ro soldiers was eluted in the hexane fraction together with β-selinene. Another additional compound (peak 3 in Figures. 1B, C, D, tR 16.6 min.) was found in the soldiers of Rss, Rsk, and Rk, and it was eluted in a 50% ether-in-hexane fraction.

The compound 2 (KI of 1499 on DB1-HT and of 1526 on DB-5) presented an M⁺ at m/z 204 (relative intensity 36%), and a base ion peak at m/z 161 (100%), with the following fragment ions: 189 (13%), 176 (2%), 147 (7%), 134 (59%), 119 (77%), 105 (67%), 91 (37%), 81 (31%), and 41 (72%) (Fig. 2A). As it was eluted in the hexane fraction, we supposed the compound 2 was a sesquiterpene hydrocarbon. According to NIST Mass Spectral Library data, its mass spectrum was highly similar to that of δ-cadinene with 92% compatibility. It was known, however, that γ-cadinene was one of the more prevalent compounds in the genus Reticulitermes. When comparing the mass spectra between δ- and γ-cadinenes on the database, relative intensity of the fragment ion at m/z 189 was larger in δ-cadinene (approximately 13%), as well as in the compound 2, than in γ-cadinene (approximately 7%) [12]. Thus, the fragmentation pattern of the compound 2 appears more similar to that of δ-cadinene than γ-cadinene [13,14]. Furthermore, KI of compound 2(1526, DB-5) was much similar to that of δ-cadinene (1524, DB-5) than that of γ-cadinene (1513, DB-5) [15]. We, therefore, estimated the compound 2 to be δ-cadinene.

The compound 3 (KI of 2535 on DB-Wax) gave a molecular ion (M⁺) at m/z 290 (relative intensity 0.2%), and a base ion peak at m/z 69 (100%) (Figure 2B). The value of optical rotation [α]D+ 6.5 (c 0.002, CHCl3). The NMR spectral data of compound 3 (Supporting information – Figures S1A, B, C, D) were summarized as follows: ¹H-NMR (CDCl3): δ: 5.92 (1H, dd, J = 10.8, 17.4 Hz, H-2), 5.22 (1H, d, J = 17.4 Hz, H-1a), 5.14 (1H, t, J = 7.2 Hz, H-14), 5.10 (2H, H-6, H-10), 5.06 (1H, d, J = 10.8 Hz, H-
1b), 1.97-2.08 (10H, H-5,8,9,12,13), 1.68 (3H, s, H-16), 1.60 (3H, s, H-20), 1.60–1.59 (6H, s, H-18 and H-19), 1.57 (2H, H-4) 1.28 (3H, s, H-17). $^{13}$C-NMR (CDCl$_3$) δ: 145.0 (C-2), 135.7–135.1 (C-7 and C-11), 131.3 (C-15), 124.0 (C-6, C-10, and C-14), 111.6 (C-1), 73.5 (C-3), 42.2 (C-4), 27.9 (C-17), 25.6 (C-16), 23-27 (C-5,8,9,12,13) and 18-19 (C-18,19,20).

Figure 2. Mass spectra and chemical structure of compound 2 [$\delta$-cadinene (A)] of hexane fraction separating from crude hexane extract of Ro and compound 3 [(S)(-)-(E,E)-geranyllinalool (B)] of 50% ether-in-hexane fraction separating from crude hexane extract of Rsk, Rss, and Rk.

Comparing the fragmentation of the compound 3 with that of some diterpene alcohols identified in the other Reticulitermes species [12], we estimated it to be geranyllinalool. This estimation was supported by coincidences of the chemical shifts of respective vinyl protons and carbons between the compound 3 and geranyllinalool [12, 16-18]. The geometrical configuration of compound 3 was also confirmed based on the chemical shift of methyl groups at 1.60 ppm for H-18, -19, and -20, which indicated methyl groups on trans-substituted double bonds in acyclic isoprenoids, whereas those of cis-substituted double bonds appeared at 1.68 ppm [16]. In addition, the retention time and fragmentation patterns in the EI-MS data for this compound matched well with those of (E,E)-geranyllinalool, which was produced from all-trans-GGPP by acid hydrolysis[19,20]. Finally, the absolute configuration of compound 3 was identified based on the value of its optical rotation. Baker et al. 1982 [17] reported that terpenoid secretion of R. lucifugus soldiers contained R-(E,E)-geranyllinalool, while its enantiomer
was confirmed as S-(+)-geranyllinalool [18]. Compound 3 was therefore identified as (S)-(+-)(E,E)-geranyllinalool. This is the first report about absolute configuration of a diterpene alcohol (S)-(+-)(E,E)-geranyllinalool isolated from the genus *Reticulitermes* in Japan.

The average amounts of terpenoid compounds found in the 4 *Reticulitermes* species and subspecies are listed in Table 1. It was of interest that the sums of the average amounts of β-selinene and (S)-(+-)(E,E)-geranyllinalool were approximate for *Rss*, *Rsk*, *Rk*, and that there was no statistically significant difference between these species (*P > 0.05*, one-way ANOVA). However, the total amount of the terpenoids was apparently smaller in *Ro* than the other 3 species. Both *Rss* and *Rsk*, contained a significantly larger amount of β-selinene than (S)-(+-)(E,E)-geranyllinalool (*P < 0.05*, paired t-test), while there was no significant difference in the amount between two terpenoid compounds in *Rk*. The *Ro* soldiers had δ-cadinene instead of (S)-(+-)(E,E)-geranyllinalool, of which amount was almost equal to that of β-selinene (*P > 0.05*, paired t-test).

**Table 1.** Quantity (mean value ± SE) of terpenoid compounds for the soldiers of 4 *Reticulitermes* species and subspecies

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Amount (mean ± S.E) [µg/individual]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rss (N=9)</td>
</tr>
<tr>
<td></td>
<td>Rsk (N=7)</td>
</tr>
<tr>
<td></td>
<td>Rk (N=4)</td>
</tr>
<tr>
<td></td>
<td>Ro (N=4)</td>
</tr>
<tr>
<td>β-selinene</td>
<td>9.84 ± 1.0†</td>
</tr>
<tr>
<td></td>
<td>7.12 ± 0.8†</td>
</tr>
<tr>
<td></td>
<td>4.30 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>δ- cadinene</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>(S)-(+-)(E,E)-geranyllinalool</td>
<td>1.48 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>3.08 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>4.80 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>Total terpenoids</td>
<td>11.32 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>10.20 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>9.10 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>0.27 ± 0.04</td>
</tr>
</tbody>
</table>

†ND: not detected. (*): Statistically significant difference at *P < 0.05* (paired t-test, using SPSS version 16 software, SAS Institute, USA) between the quantity of β-selinene and (S)-(+-)(E,E)-geranyllinalool in the same species in the respective column.

Thus, the terpenoid compounds resembled well in *Rss*, *Rsk*, and *Rk* soldiers, but differed in *Ro*. Although the phylogenetic study suggests the similarity between *Rk* and *Ro* and between *Rss* and *Rsk* [21], soldier chemical comparison revealed clear differences between *Rk* and *Ro*. It is uncertain what causes such dissimilarity, but the habit of these termite species might influence to them. Since *Ro* distributes in the subtropical zone, the fauna of its natural enemies would differ from those of the other three species. It might influence to the compositions of soldier-specific terpenoids serving as defensive chemicals. Further consideration should be necessary to understand the reason of such dissimilarities.

This study confirmed β-selinene not only in *Rss* [10] but also in *Rsk*, *Rk*, and *Ro*. This suggests that β-selinene would be a characteristic terpenoid compound in the Japanese *Reticulitermes* species, and it might be a key component to discriminate the Japanese *Reticulitermes* species from the European and American *Reticulitermes*. According to Nelson *et al.* 2008 [22], the termites of many *Reticulitermes* species in California, USA contained some abundant sesquiterpene hydrocarbons such as germacrene A, and γ-cadinene. In contrast, Quintana *et al.* 2003 [12] reported that the European *Reticulitermes* species possessed geranyllinalool as a major component. To test our hypothesis on β-selinene, further comparisons of the soldier-specific chemicals would be necessary in additional 5 species and 1 subspecies in the Japanese *Reticulitermes* species.

Concerning the ethological functions of these soldier-specific chemicals, Several sesquiterpenes including cadinones, β- and γ-selinenes, and (E,E)-α-farnesene are involved in alarm communication of termites and in soldier differentiation [4-6,23], but there are no reports available on the ethological function of δ-cadinene. Some reports have also confirmed the biological activities of geranyllinalool such as germacrene A, and...
as being a deterrent and toxin against predators or competitors [7], functioning as an attractant and alarm pheromone in the colony [5], and enhancing presoldier formation when combined with JH III [23]. In those studies, however, what kind of stereochemical structure of geranyllinalool showing bioactivities was not mentioned. Therefore, the notions being mentioned above must be confirmed in future studies.

ACKNOWLEDGEMENTS

We especially send sincere thanks to Professor Christiane Gatz of Georg-August-University, Göttingen, Germany; Professor Dorothea Tholl of Virginia Polytechnic Institute and State University, Blacksburg, VA, USA; and Dr. Tobias G. Köllner of the Max-Planck-Institute, Göttingen, Germany, for their kind help and for providing us with a method for preparing (E,E)-geranyllinalool. We are grateful to Dr. Masaru K. Hojo and Dr. Masaru Hojo of the Faculty of Agriculture, University of the Ryukyus, Okinawa, Japan for collecting, confirming, and providing us with the Rokinawanus colony. Thanks send to Dr. Nobuhiro Shimizu of Kyoto Gakuen University, Kyoto, Japan for measurement of the optical rotation of the sample.

SUPPORTING INFORMATION

Figures S1A, S1B, S1C, S1D (NMR spectrum) are included in the supporting information

REFERENCES


The authors declare no conflict of interest

© 2014 By the Authors; Licensee Borderless Science Publishing, Canada. This is an open access article distributed under the terms and conditions of the Creative Commons Attribution license http://creativecommons.org/licenses/by/3.0/