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

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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 avoids ant attacks [7] or as an attractant and alarm pheromone in the colonies [5].

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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 ± 5ºC, 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 30ºC 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

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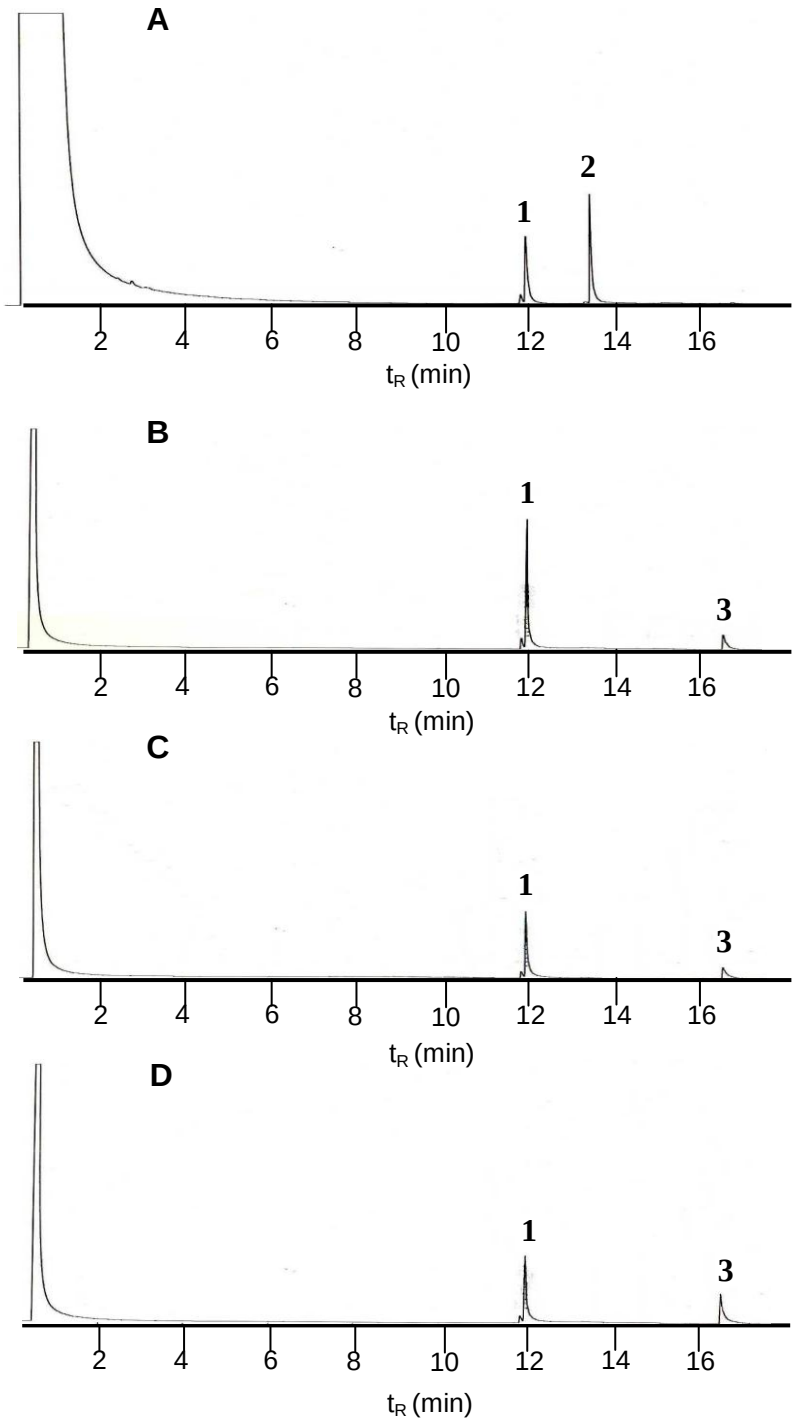

Figure 1. Gas chromatograms of terpenoid components of crude hexane extract from 4 species: *Ro* (**A**); *Rsk* (**B**), *Rss*(**C**), and *Rk* (**D**). Each number indicates for a compound. GC condition; column DB1-HT 15m x 0.25 mm (ID) x 0.25 µm film thickness, temperature program: 60°C (10 min) heated 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**).

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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 300ºC for DB-1 and DB-5. The DB1-HT column oven temperature was set at 60ºC for 10 min, heated to 300ºC at 20ºC /min. and then kept at the final temperature for 10 min, while DB-5 column was heated to 240ºC at 3ºC /min. For the DB-Wax column, both the injection port and detector were set at 200ºC and the column oven was set at 60ºC for 1 min, then increased to 220ºC at 10ºC /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 nC_{10} to nC_{20} for the compounds in hexane fraction or even-numbered hydrocarbons nC_{20} to nC_{26} 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 27ºC with a Bruker AV-600 spectrometer (600.19 MHz for ^{1}H and 125.6 MHz for ^{13}C). 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 $CDCl_3$ and its carbon signal were used as internal standards (δ = 7.26 ppm for ^{1}H and δ = 77.0 ppm for ^{13}C).

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, t_R 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, t_R 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, t_R 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 $M^{+\cdot}$ 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%), 55 (21%), 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 (approximately13%), 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 ($M^{+\cdot}$) 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 $[\alpha]_{20}^{D}$+ 6.5 (c 0.002, $CHCl_3$). The NMR spectral data of compound 3 (Supporting information – Figures S1A, B, C, D) were summarized as follows: ^{1}H-NMR ($CDCl_3$) 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-

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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).

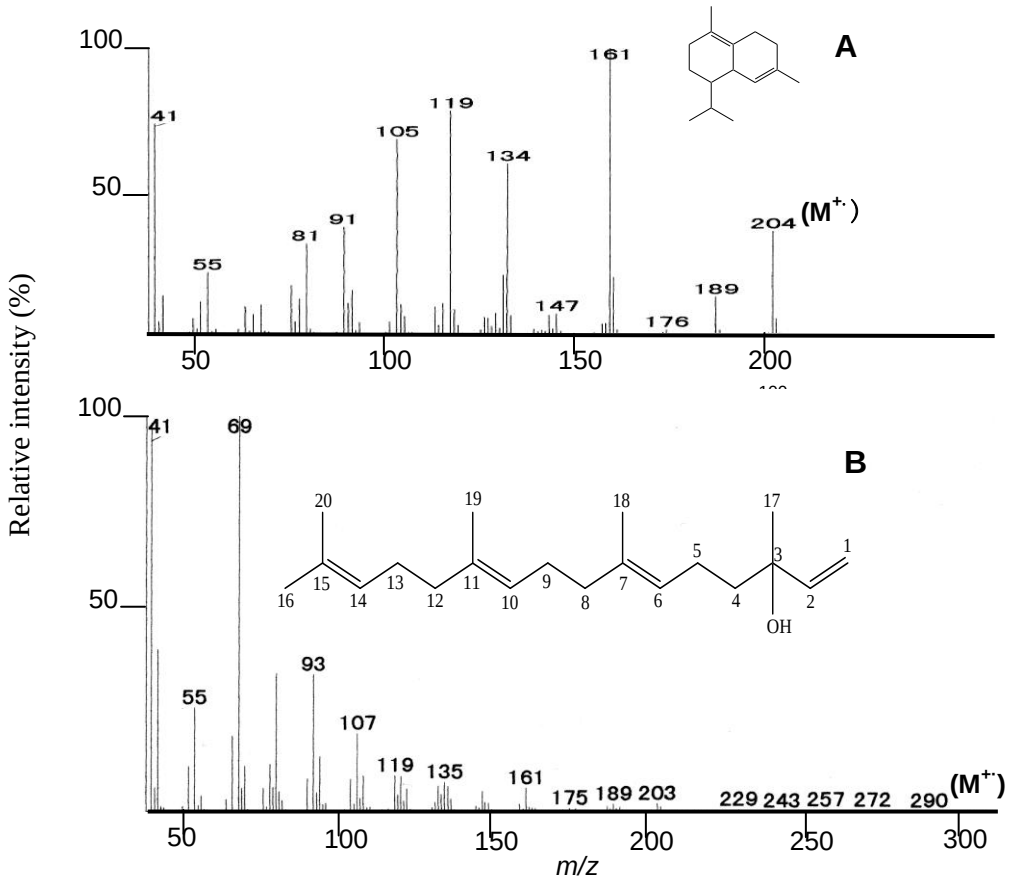

Figure 2. Mass spectra and chemical structure of compound 2 [*δ*-cadinene (**A**)] of hexane fraction separating from crude hexane extract of *Ro* and compound 3 [(S)-(+)-(*E*,*E*)-geranyllinalool (**B**)] of 50% ether-in-hexane fraction separating from crude hexane extract of *Rsk*, *Rss*, and *Rk*.

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

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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 *Reticulitemes* 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*-cardinene 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

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

†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 phylogenic 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 cadinenes, *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

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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.

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SUPPORTING INFORMATION

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

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The authors declare no conflict of interest

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