Research article

Identification of a potent termite repellent from the Bornean Dolichoderine ant *Dolichoderus sulcaticepus* (Hymenoptera: Formicidae)

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ABSTRACT. Ants are considered the most serious threat to termites. It is also well known that they utilize a number of chemicals closely related to their existence. If termites were able to detect and repel these chemicals, it is possible they could directly reduce the risk of predation. We investigated chemicals from the ant *Dolichoderus sulcaticepus* that act as a repellent for the Formosan subterranean termite *Coptotermes formosanus*. *C. formosanus* repelled filter paper treated with 0.1 ant-equivalent extract from the abdomen of *D. sulcaticepus* for the duration of 1 hour. The potent repellent from this ant was consequently purified by several chromatographic techniques. Gas chromatography, gas chromatography-mass spectrometry and nuclear magnetic resonance data revealed that the repellent *D. sulcaticepus* is *cis*, *trans*-dolichodial. Each worker-ant of this species possess approximately 60 μg of *cis*, *trans*-dolichodial in their pygidial gland. *C. formosanus* clearly repelled 6 μg of purified *cis*, *trans*-dolichodial for 225 min. These results suggest that the chemicals emitted by Dolichoderine ants has potential for application in termite control.

Key words:Coptotermes formosanus, Dolichoderus sulcaticepus, Dolichodial, termite control
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INTRODUCTION

Ants occupy almost every ecological niche on earth and are major predators of numerous insects (Vander Meer & Alonso, 1998). Of these insects, termites are constantly at risk from predation because their habitats overlap with those of ants; both live in the ground and in/on trees. To avoid predation by ants, termites have developed a soldier caste specialized in colony defense. Termite soldiers possess both chemical and physical defense systems; for example, some species use terpenoids as a defense substance against predators (Waller & LaFage, 1987; Deligne *et al.*, 1981), while the soldiers of *Reticulitermes speratus* utilize the head part of their bodies as a stopper in the entrance of their nests (Matsuura, 2002). Ants utilize chemicals mainly stored in the exocrine glands (Hölldobler & Wilson, 1990) for communication among nestmates, to keep the nest sanitary (Attygalle & Morgan, 1984). It has also been reported that some of these chemicals possess a repellent and/or insecticidal activity; for example, metapleural

gland secretions from the ant *Crematogaster deformis* include phenolic substances that possess repellent and insecticidal activity against heterospecific ants (Attygalle *et al.*, 1989). Since ants are the most serious natural enemy of termites, it is possible that termites possess strategies allowing them to escape predation. The chemicals utilized by ants are closely related to their existence, and therefore, if termites were able to sense and repel them, they could potentially escape predation. We therefore isolated the bioactive substances in ants that acts as the repellent to termites. In searching for a strong candidate, ant collection was conducted in Sabah, Malaysia, which has one of the most richest biodiversity in the world, with 524 known ant species living in the sampling area alone (Brühl *et al.*, 1998). The aim of the present study was to identify the potent repellent contained in ant extracts and evaluate its repellency. The potential applicability of ant chemicals in termite control is also discussed.

MATERIALS AND METHODS

Ants and termites

Foraging workers of *Dolichoderus sulcaticepus* were collected in Kinabalu National Park, Sabah, Malaysia (6° 05'N, 160° 33' E) in November 2003. All samples were temporarily soaked in 70% ethanol for transportation back to the laboratory where head and abdomen parts were separated by forceps and individually soaked in fresh 70% ethanol.

Formosan subterranean termites, *Coptotermes formosanus*, foraging in fallen wood were collected in Shirahama, Wakayama, Japan (33° 41'N, 135° 23'E) in August 2003 and reared in a plastic container (70 × 42 × 32 cm) at room temperature. Water was supplied daily with a sprayer.

Bioassay

About 300 *C. formosanus* workers were introduced into a circular plastic dish (60mm i.d., 32 mm height) then, after allowing a few hours for the termites to settle, a piece of filter paper (5 mm diameter, 1 mm thickness) treated with 0.1 ant-equivalent extract was placed in the center of the dish (Fig. 1). The numbers of termites that walked within 10 mm of the filter paper were then counted until the samples lose its repellency. When five termites consecutively crossed the filter paper without hesitation, the sample was considered to have lost its repellency. Filter paper treated with a solvent of each treatment was used as a control. The duration of repellency was used to evaluate samples, and those that possessed the longest durations were regarded as active fractions and applied to chromatography analysis.

Isolation of the termite repellent

About 600 *D. sulcaticepus* ants were used for isolation of the repellent. The head and abdomen parts of *D. sulcaticepus* ants were homogenized in 70% ethanol in water. When we conducted extraction of the substances from the ants in 70% ethanol in water, cuticular hydrocarbons of the ants were not dissolved into the samples. Consequently, we could obtain the samples that only contained chemical substances from the inside of the ants. Since most ant exocrine glands are located in the head and abdomen (Hölldobler & Wilson, 1990), the repellency of both parts was then checked by bioassay (as described above). For this purpose, 1 ml of active extract was added to 2 ml of saturated NaCl aq., 1 ml of *n*-hexane and 1 ml of ethyl acetate, mixed for 30 sec. with a vortex mixer and allowed to settle for 3 min. The resultant organic (mixture of *n*-hexane and ethyl acetate) and aqueous layers (saturated NaCl aq.) were then checked

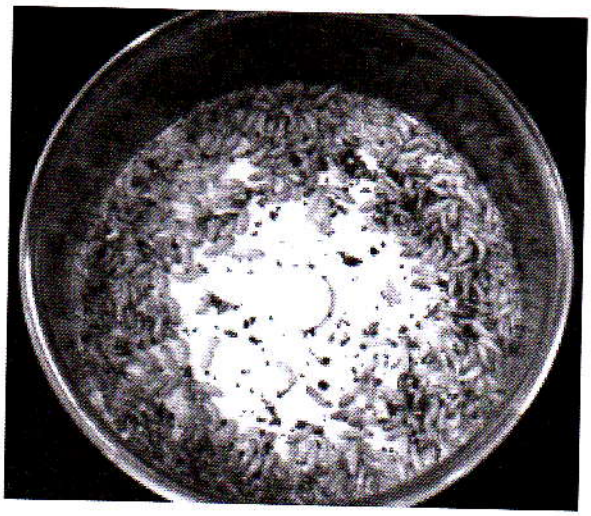

Figure 1. Workers of *C. formosanus* repelling filter paper treated with an abdominal extract of *D. sulcaticepus*. About 300 *C. formosanus* workers were placed into a plastic dish then a piece of filter paper treated with 0.1 ant-equivalent of extract was placed in the center. The duration of repellency was consequently observed.

for their repellency and active fractions were applied to silica-gel column chromatography. Approximately 1g of silica gel (230-400 mesh, Merck) was prepared for chromatography in advance. Constituents were successively eluted with 3 ml of *n*-hexane, 10, 30 and 50% diethyl ether in *n*-hexane, diethyl ether, ethyl acetate and methanol, resulting in a total of 7 fractions. The duration of repellency in 7 fractions and control were then examined by ANOVA and Fisher's protected least significance difference (PLSD) multiple comparison to assess the repellency of each fraction.

Chemical analysis

Active fractions were subjected to analysis on a Shimadzu GC-14A gas chromatographer equipped with an apolar capillary column (DB-1; 30 m x 2.5 mm i.d. x 0.25 ìm film thickness, J&W Scientific) and flame ionization detector. Helium was used as the carrier gas at a column head pressure of 200kPa. Injections were made in a split-less mode for 1 min at 280°C and the detector temperature was set at 300°C. The oven temperature was initially held at 60°C for 1 min then programmed to increase at 20°C/min up to 120°C, 5°C/min up to 160°C, and 20°C/min up to 280°C and held at this final temperature for 6 min. Identification of active fraction components was performed using Shimadzu QP-5000 gas chromatography-mass spectrometry (GC-MS) in EI mode at 70 eV. GC was operated under the same conditions as with GC analysis, but the column head pressure of the carrier gas was 100 Pa. After single peaks detected in the active fractions were collected by preparative GC, proton nuclear magnetic resonance (NMR) spectra of the active components were recorded on Brucker ARX-500 (*Fourier transform*) at 500 MHz. $CDCl_3$ was used as the solvent.

Glandular source of the repellent

Three major exocrine glands located in the abdomen, the Dufour's gland, pygidial gland and poison gland, were dissected from *D. sulcaticepus* under the microscope. Those exocrine glands were crushed in *n*-hexane. *n*-Hexane extracts of these exocrine glands were analyzed by GC-MS.

RESULTS

Separation of the termite repellent

Repellency was only detected with abdomen extracts, and after organic/aqueous separation, workers of *C. formosanus* only repelled filter paper treated with the organic layer. *C. formosanus* repelled filter paper treated with 0.1 ant-equivalent of organic layer for 76.5±46.2 min (mean±SD; n=50). Among the fractions obtained from silica-gel chromatography, only Fr. 3 (30% diethyl ether in *n*-hexane) possessed strong repellency against termites. When all of the data was analyzed, the duration of the repellency was found to be significantly different between resultant fractions. *C. formosanus* only repelled the filter paper treated with 0.1 ant equivalent of Fr. 3 significantly for 225±16.67 min (mean±SD; n=50) (Fisher's PLSD multiple comparison test, $p<0.05$).

Identification of the termite repellent

The components of active fraction Fr. 3 were consequently analyzed by gas chromatography, revealing only one peak (Fig. 2a). After collection by prep-GC, further bioassays using the collected compound were conducted. The repellency of Fr. 3 and the collected compounds were shown to be almost identical (data not shown). We therefore assumed this single peak was the active termite repellent (hereafter this peak is referred to as compound A) and used GC-MS analysis to reveal its mass spectrum (Fig. 2b). It was shown to have a molecular ion at m/z 166, and as diagnostic ions, m/z 151 (M-15), 165 (M-1), 137 (M-29) were also detected. These ions suggested that the structure of compound A includes a methyl lateral chain and formyl protons. ^{1}H-NMR signals for two aldehydes (9.45 [s-like] and 9.55 [s] ppm, respectively) protons, two terminal alkenes (6.15 [d] and 6.35 [d] ppm, respectively), and a secondary methyl group (1.15 [d] ppm) were confirmed. ^{1}H-^{1}H COSY data revealed the existence of a five member spin systems coupled with methyl and formyl protons, and a terminal alkene coupled with a formyl proton. Together with the structural information obtained, compound A was consequently identified as *cis, trans*-dolichodial ($C_{10}H_{14}O_2$) (Fig. 3).

Location of the repellent

Cis, trans-dolichodial was detected in extracts from the pygidial gland as the chief component and GC-MS analysis of this gland confirmed the presence of small amounts of four monoterpenoids (α-Pinene, β-Pinene, α-Copaene, and Limonene) (data not shown); mass spectrometry was used for their identification.

DISCUSSION

We isolated and identified Dolichodial, a dicarbonyl compound ($C_{10}H_{14}O_2$), in the Bornean dolichoderine ant *D. sulcaticepus* as a potent repellent against *C. formosanus*, a major destructive insect pest found worldwide. Dolichodial was also previously isolated from the mint plant *Teucrium marum* (Labiatae) (Eisner *et al.*, 2000) and was confirmed to show repellency toward the ant *Monomorium pharaonis*, cockroach *Periplaneta americana* and fly *Phormia regina*. Cornelius *et al.* (1995) also reported that the ant *Ochetellus glaber* possesses *cis, trans-* and *trans, cis*-isomers of dolichodial at a 3:1 ratio and checked its

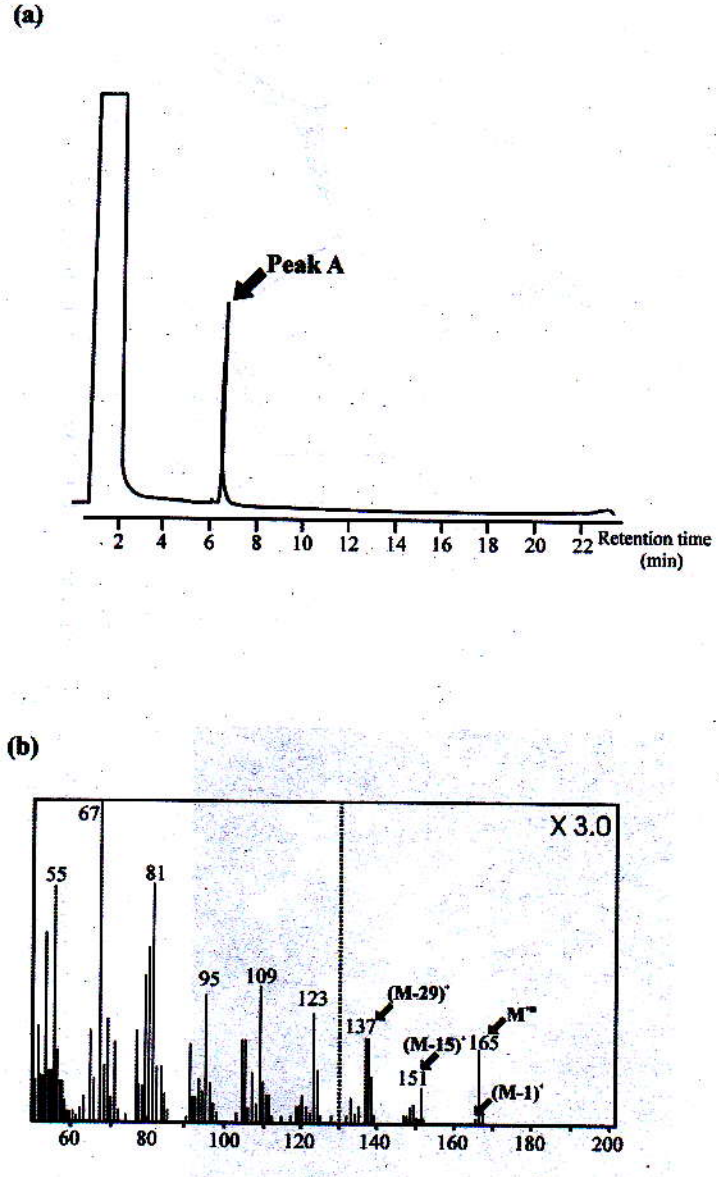

Figure 2. Active fraction Fr. 3 was analyzed by GC-MS. (a) The resultant gas chromatogram; only one peak was detected (peak A). (b) Mass spectrum of compound A, which had a molecular ion at m/z 166.

Figure 3. The structure of the identified repellent, *cis*, *trans*-dolichodial ($C_{10}H_{14}O_2$). White, red and turquoise balls represent hydrogen, oxygen and carbon atoms, respectively.

toxicity and repellency to *C. formosanus*. In the case of *D. sulcaticepus*, only a *cis, trans*-isomer of dolichodial was isolated and identified as the repellent of *C. formosanus*. Our data shows that not only a mixture of these isomers, but also a single *cis, trans*-isomer possesses potent repellency against *C. formosanus*.

Both *Ochetellus* and *Dolichoderus* are included in the subfamily Dolichoderine. The anal (pygidial) gland of ants belonging to this subfamily mainly stores terpenoids, some of which are known to have insecticidal activities (Cavill & Houghton 1974, Cavill *et al.*, 1976). Dolichodial is a cyclopentanoid monoterpene, and its analogues have also been isolated as repellents to other ant species (Scheffrahn *et al.*, 1984). How these cyclopentanoid monoterpenes affect other insects has been extensively studied. Generally, terpenoids are thought to be defense substances (Blum, 1981); however, their role in the existence of dolichoderine ants remains unknown. During our fieldwork in Sabah, the ants often sprayed chemicals on the forceps used for collection, and at this point the smell of dolichodial was clearly apparent. Simultaneously, almost all nearby nestmates rushed to the point at which the ant was caught. This reaction is probably a part of their alarm behaviors (Hölldobler & Wilson, 1990). It is suggested therefore that dolichodial acts as an alarm pheromone in this species; however, further bioassays demonstrating this alarm activity are necessary.

Recently, there has been great interest in naturally produced chemicals as alternatives to synthetic insecticides or repellents for use in pest control. In this study, we focused on the interaction between termites and ants, the most serious natural enemy of termites, and hypothesized that termites are able to detect the chemicals secreted by ants and consequently repel them. A termite repellent in the ant species *D. sulcaticepus* was identified, and the possibility that termites utilize chemicals extracted by these ants to reduce the risk of predation is apparent. Other reports have documented ant-termite interactions, but most were carried out under artificial conditions, with few long-term ecological observations (Higashi & Ito, 1989). Based on our findings, we concluded that naturally produced chemicals could be found through studies on the interactions between hetero-specific insect species, helping identify new natural products that could be applied to pest control.

Most semiochemicals from the ants from the tropics have not been studied well. And it will be expected to find new or much potent bioactive compounds from the ant exocrine glands. We have to store the information of both chemicals and its corresponding bioactivity, and also utilize the information that was brought from the rich biodiversity of tropics, effectively.

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