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Nonadecadienone, a New Termite Trail-Following Pheromone Identified in *Glossotermes oculatus* (Serritermitidae)

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Abstract

Within the multitude of chemical signals used by termites, the trail marking by means of pheromones is ubiquitous. Chemistry and biology of the trail-following communication have been described in more than 60 species from all families except for the Neotropical Serritermitidae. The chemical ecology of Serritermitidae is of special interest not only as a missing piece of knowledge on the diversity and evolution of isopteran pheromones but also because it may contribute to the debate on the phylogenetic position of this family, which is still unresolved. Therefore, we aimed in this study to identify the trail-following pheromone of the serritermitid *Glossotermes oculatus*. Based on a combined approach of analytical chemistry, electrophysiology, and behavioral bioassays, we propose (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one to be the trail-following pheromone of *G. oculatus*, secreted by the sternal gland of pseudergates. Thus, we report on a new termite trail-following pheromone of an unexpected chemical structure, a ketone with 19 carbons, contrasting with unsaturated alcohols containing 12 carbons as trail-following pheromones in other advanced termite families. In addition to this unique trail-following pheromone, we also describe the sternal gland in pseudergates as an organ of unusual shape, size, and structure when compared with other isopteran species. These results underline the peculiarity of the family Serritermitidae and prompt our interest in the chemistry of pheromones in the other genus of the family, *Serritermes*.

Key words: *Glossotermes*, Serritermitidae, sternal gland, termites, trail-following pheromone, (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one

Introduction

Termites, the eusocial "dwellers in the dark," represent an excellent example of the prime role of chemical communication in insect societies. Within the multitude of chemical signals used by termites, the trail marking by means of pheromones is ubiquitous, regardless of the differences in foraging and nesting habits of particular species (Bordereau and Pasteels 2011). The sole source of trail-following pheromones, the sternal gland, occurs as a homologous organ in all termite species and castes, despite the variability in its position, size, and ultrastructure among particular isopteran families (Noirot 1969; Ampion and Quennedey 1981; Quennedey et al. 2008). However, its use by foragers in trail marking is probably derived from its ancestral role in mate attraction of termite imagoes during dispersal (Traniello and Leuthold 2000). This is evidenced by the widespread use of the sternal gland in courtship behavior of imagoes as well as by the frequent use of the same pheromone for both mate attraction and trail marking, though in different amounts and in different contexts (Bordereau and Pasteels 2011).

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The chemistry of termite trail-following and sex pheromones is quite well known, due also to numerous recent contributions using modern methods of extraction and analysis, and has been very recently summarized by Bordereau and Pasteels (2011). Based on our current knowledge, 2 conclusions can be drawn regarding the trail-following pheromones. First, the trail-following pheromones usually consist of a single component; even though this may be due to omissions of tiny amounts of minor components, detected recently in several termite genera only by means of electrophysiology (e.g., see Sillam-Dussès et al. 2009). And second, the evolution of chemical diversity of trail-following pheromones appears to be quite conservative, with only 8 compounds being identified as trail-following pheromones in 6 families and more than 60 species studied up to date. Moreover, the same compounds may occur as trail-following pheromones in phylogenetically distant taxa with a very different foraging ecology. Nevertheless, the relative uniformity of trail-following pheromones within particular families or subfamilies may still be useful to infer various evolutionary conclusions at higher taxonomic levels (see Bordereau and Pasteels 2011).

The Neotropical family Serritermitidae, consisting of 2 genera, *Serritermes* and *Glossotermes* (Cancello and DeSouza 2005), remains the only isopteran family which has not yet been studied with respect to trail and sex pheromones. Its biology, including chemical ecology, is of special interest due to the debated phylogenetic relationships between Serritermitidae and basal lineages of Rhinotermitidae, with several alternative scenarios being recently proposed, that is, Serritermitidae + (Rhinotermitidae + Termitidae), Rhinotermitidae + (Serritermitidae + Termitidae), or Serritermitidae nested within Rhinotermitidae (see Lo et al. 2004; Ohkuma et al. 2004; Inward et al. 2007; Legendre et al. 2008; Engel et al. 2009).

The biology of the genus *Glossotermes* has long been unknown; only very recently, we described the caste pattern (Bourguignon et al. 2009) and soldier defensive adaptations (Šobotník, Bourguignon, et al. 2010). The genus was originally placed within Rhinotermitidae, as the sister group of *Psammotermes* (Emerson 1950) but was recently transferred to Serritermitidae (Cancello and DeSouza 2005). The monophyly of the family is supported by molecular data (e.g., Lo et al. 2004), morphology, and a unique defensive strategy of soldiers through suicidal dehiscence of the frontal gland (Costa-Leonardo and Kitayama 1991; Engel et al. 2009; Šobotník, Bourguignon, et al. 2010). In this study, we investigated the chemical identity of the trail-following pheromone in *Glossotermes oculatus*.

Materials and methods

Insects

Colonies of *G. oculatus* were collected near Petit Saut dam in French Guiana (05°04′N, 52°59′W) from wet rotten logs (Figure 1). Five fragments of colonies (500–2000 individuals each) were collected in January 2010 and transported to Prague in their original portions of wood. They were used for trail-following pheromone identification (histology, preliminary bioassays, chemical analysis, electrophysiology). Three additional colonies were collected in November 2010. They were used for trail-following pheromone confirmation and quantification by means of a set of bioassays, performed at Laboratoire Environnement Hydreco (French Guiana) and by means of gas chromatography–mass spectrometry (GC/MS) and electrophysiology, performed in Prague.

Anatomy of the sternal gland

Optical microscopy of the sternal gland of pseudergates was performed using the protocol and equipment described in Šobotník, Sillam-Dussès, et al. (2010).

Sternal gland extracts of pseudergates

Cold-immobilized pseudergates were carefully dissected and a portion of the ventral integument containing the sternal gland was submerged into hexane. The sternal glands (30–300 glands) were extracted for 6 h in hexane (1 µL/1 gland) at 4 °C. The extracts were used directly or stored at –18 °C. For preliminary bioassays, whole body extracts of pseudergates (WBE) were prepared following the same procedure.

Gas chromatography–mass spectrometry

Chemical identification was carried out using 2D gas chromatography with time-of-flight mass spectrometric detection (GC × GC/TOF-MS, Pegasus 3D; Leco); for details, see Hanus et al. (2009). The temperature program for the first column was 40 °C (1 min) to 320 °C (5 min) at 7 °C/min; the temperature of the second column was set 20 °C higher.

For quantification of nonadeca-10,13-dien-2-one, 3 different sternal gland extracts of pseudergates (SGE) were measured by means of GC/MS (quadrupole DSQ II; Thermo Scientific) with a DB-5 column (30 m, inner diameter 0.25 mm, 0.25 µm

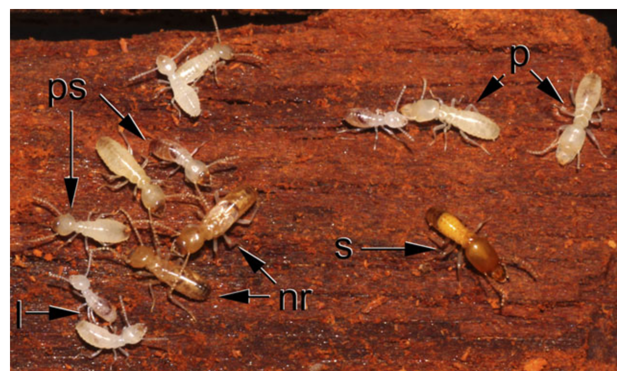

Figure 1 View of a colony of *Glossotermes oculatus* excavated from a rotten log: neotenic reproductives (nr), larvae of various stages (l), pseudergates (ps), presoldiers (p), soldier (s).

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phase). Temperature program: 80 °C (1 min) to 320 °C (3 min) at 15 °C/min. One gland equivalent (GEq) in 1 μL of hexane was injected in a splitless mode. Prior to injection, tricosan-12-one has been added as internal standard to the SGE at a concentration of 26 ng/μL.

Column chromatography and preparative gas chromatography

In order to localize the compound(s) responsible for the trail following in the complex blend detected by GC in the SGE, we fractionated the extract using column chromatography. Columns (Pasteur pipettes) were loaded with 2 g of silica gel and prewashed with 10 mL of pure hexane. One milliliter of SGE was applied on the top of the column and eluted with a series of solvents of increasing polarity (2 mL of hexane:ether from 10:0 to 0:10). Solvent drips were collected into glass vials and concentrated.

Selected fractions were further subjected to preparative gas chromatography. Preparation was performed with a gas chromatograph (AT 6890N; Agilent Technologies) linked to a Preparative Fraction Collector (Gerstel), equipped with a liquid nitrogen cooling system. For GC separation, a HP-5 column (30 m, id 0.53 mm, 0.88 μm phase) was used. The temperature program was 40 °C (1 min) to 300 °C (5 min) at 20 °C/min. The flow rate of carrier gas (helium) was 6 mL/min, the injection volume was 5 μL. The separation intervals of particular fractions were related to linear retention indexes (LRIs) of *n*-alkane standards (C7–C30).

Chemicals

(3*Z*,6*Z*,8*E*)-Dodeca-3,6,8-trien-1-ol (dodecatrienol) and (1*E*,5*E*,9*E*,12*R*)-1,5,9-trimethyl-12-(1-methylethenyl)-1,5,9-cyclotetradecatriene (neocembrene) were kindly supplied by Christian Bordereau (for details, see Sillam-Dussès et al. 2005). Methyl linoleate (purity ≥ 99%), *t*-butyl acetate (purity ≥ 99%), pure dimethyl disulphate, and other reagents were purchased from Sigma-Aldrich. Solvents were purchased from Penta.

Synthesis of (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one

The synthesis was analogous to that described in detail by Adams et al. (2010) and performed under an argon atmosphere. A solution of *n*-butyllithium in pentane (1.2 mL, 2.24 mmol, 3.3 equivalent) was added dropwise to a solution of diisopropylamine (300 μL, 2.24 mmol, 3.3 equivalent) in tetrahydrofuran (3 mL) cooled to –78 °C. The mixture was stirred at –78 °C for 15 min, warmed up to 0 °C for 5 min, and cooled down to –78 °C again. After cooling, *t*-butyl acetate (300 μL, 2.24 mmol, 3.3 equivalent) was added, and the mixture was maintained at –78 °C for 10 min. Methyl (9*Z*,12*Z*)-octadeca-(9,12)-dienoate (200 mg, 0.68 mmol, 1 equivalent) in tetrahydrofuran (1.2 mL) was then added, and the reaction mixture was stirred at –78 °C for 1.5 h. Finally, saturated aqueous ammonium chloride (8 mL) was added, and the mixture was warmed to room temperature.

The solution was diluted with water (50 mL) and extracted with ethyl acetate (50 mL). The aqueous phase was washed with ethyl acetate (3 × 20 mL). The organic extracts were combined and washed with aqueous 1 N hydrochloric acid (25 mL) and saturated sodium bicarbonate (50 mL). During this process the product, (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one, was formed. Finally, the organic phase was dried over magnesium sulfate and concentrated in vacuo to give a crude oil that was purified by silica gel chromatography (hexane:ether, 8:2). The pure compound (LRI = 2075) was obtained using preparative GC (the same conditions as described above), temperature program: 70 °C (1 min) to 110 °C at 10 °C/min then to 300 °C at 6 °C/min. The flow rate of helium was 6.5 mL/min, and the injection volume was 5 μL.

Preparation of dimethyl disulfide derivatives

Dimethyl disulfide (DMDS) derivatives were prepared from the synthetic (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one and the SGE of pseudergates. Fifty microliters of the sample in hexane (extract or standard) containing approximately 50 ng of the studied compound were mixed with 50 μL DMDS and 5 μL of iodine solution (60 mg/mL iodine in fresh distilled diethyl ether). The vial was shielded and shaken overnight at room temperature. The reaction was finished after 16 h with adding aqueous sodium thiosulfate (5% in water), the mixture was extracted twice with 200 μL of hexane, concentrated, and injected into GC × GC/TOF-MS.

Behavioral experiments

Five colony fragments were used to test the ability of pseudergates to follow the WBE, SGE, particular fractions of these extracts, and standards of termite trail-following pheromones. The ability of pseudergates to follow the WBE, SGE, and the synthetic (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one was confirmed in all 3 colonies collected in November 2010; all results reported in this paper (Tables 1 and 3, Figure 4, GC/MS quantification) were recorded on individuals from one colony, collected a few hours before the bioassays. The termites were removed from the wood only a few minutes prior to the experiment, which was carried out at 26 °C under reduced light intensity.

Trail-following bioassays were performed to test the orientation activity of WBE, SGE, neocembrene, dodecatrienol, and (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one. Solutions were tested with a "Y open field" bioassay on Whatman No. 1 filter paper (15 cm diameter), with a 120° angle between the branches. An artificial trail was drawn with 10 μL of the solution spread on the stem (3 cm) and one of the branches (7 cm). Ten microliters of hexane were deposited on the stem and the other branch. One termite was placed inside a holding chamber (55 mm Petri dish), with a 2 mm opening located at the base of the Y. The activity

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Table 1 Open field Y trail-following bioassays with whole body extract, sternal gland extract, and (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one

	Whole body extract (Geq/cm)		Sternal gland extract (Geq/cm)		Nonadecadienone (ng/cm)					*n*
	10^{-1}	1	10^{-1}	0.5	10^{-3}	10^{-2}	10^{-1}	1	10	
Pseudergates	7.8 ± 3.3	8.7 ± 2.8	9.1 ± 1.2	9.9 ± 0.4	1.6 ± 1.6	6.2 ± 4	9.6 ± 1.5	9.7 ± 0.8	9.9 ± 0.4	30
Control	0	0	0	0	<1.5	0	0	0	0	
Soldiers	—	9.3 ± 2	—	—	<1.5	5.6 ± 3.6	9 ± 2.7	—	—	15
Control	—	0	—	—	<1.5	0	0	—	—	

The distance traveled by one pseudergate or one soldier on trails made of whole body extract or sternal gland extract of pseudergates or a series of nonadecadienone concentrations was measured (mean in centimeter ± standard deviation). Hexane was used as a control. GEq, sternal gland equivalent; *n*, number of repetitions.

threshold was defined as the minimum concentration that elicited termites to travel a mean distance of more than 3 cm. Each experiment was performed with 30 pseudergates, some of them also with 15 soldiers.

With the same setup, choice tests evaluating the preference of pseudergates for SGE or (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one solutions were performed in order to estimate the quantity of the compound in one sternal gland. Each of the compared stimuli was deposited on the stem and on one of the Y branches. This test was repeated with 30 pseudergates; results were evaluated using χ^2-test.

Electrophysiology

The experimental setup is described in Sillam-Dussès et al. (2009). Gas chromatography coupled with electroantennographic detection (GC-EAD) was used to identify the physiologically active compounds in the SGE, using the antenna of a pseudergate. To confirm the identity of the pheromone, a solution of (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one was used. The times of antennal responses were related to the retention times of *n*-alkanes (C8–C22) in order to calculate their LRI (DB-5 column). Temperature program: 40 °C (2 min) to 270 °C (10 min) at 30 °C/min (70 °C/min in the second set of experiments), injector 220 °C, detector 250 °C.

Electroantennographic bioassays (EAG) were used to quantify the amount of the pheromone in one sternal gland. SGE and a series of concentrations of (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one (0.1–100 ng per stimulation) were tested in EAG on antennae of pseudergates, with hexane and air being used as controls.

Results

Source of the trail-following pheromone

In the trail-following bioassays, WBE elicited the trail-following behavior in pseudergates as well as in soldiers (Table 1). SGE elicited the trail following in pseudergates at both tested concentrations, 0.1 and 0.5 GEq/cm of the trail (Table 1). We concluded that the compound(s) responsible for the trail following is (are) secreted by the sternal gland.

Structure of the sternal gland

The sternal gland of *G. oculatus* pseudergates is located on the anterior half of the fifth abdominal sternite and is about 200 µm long. It is about 65 µm thick in the anterior part; the thickness decreases toward the posterior (see Figure 2A). The gland is formed by secretory cells: class 1, 2, and 3, class 1 cells being the most abundant. The extracellular reservoir (about 20 µm long and 10 µm wide) is located in the posterior part of the gland and penetrated by dendrites belonging to campaniform sensillae (see Figure 2B). The gland is entirely covered by the preceding sternite, thus forming a pouch in which the gland secretion is temporarily stored.

Chemical identity of the trail-following pheromone

As the first step, we performed trail-following bioassays with standards of trail-following pheromones of Rhinotermitidae and Termitidae (see Bordereau and Pasteels 2011), that is, dodecatrienol (10^{-4} to 10^{-1} ng/cm) and neocembrene (10^{-2} to 10 ng/cm). None of the tested concentrations was effective in eliciting trail-following behavior in pseudergates. The analysis of SGE using GC × GC/TOF-MS did not allow us to detect any of the known termite trail-following pheromones based on characteristic fragment ions of their mass spectra. We concluded that the trail-following pheromone of *G. oculatus* is a new compound, not described as yet in termites.

Subsequently, we performed GC-EAD experiments with SGE and the antennae of pseudergates to pinpoint physiologically active compounds, which would be possible candidates for the trail-following pheromone. Unfortunately, this first set of experiments (temperature rate 30 °C/min) did not reveal unambiguously any candidate compound. Therefore, we decided to localize the pheromonal component(s) in the rich blend by dividing the SGE of 300 pseudergates into fractions based on polarity, using column chromatography. Two of the resulting 11 fractions (8:2 and 9:1, hexane:ether)

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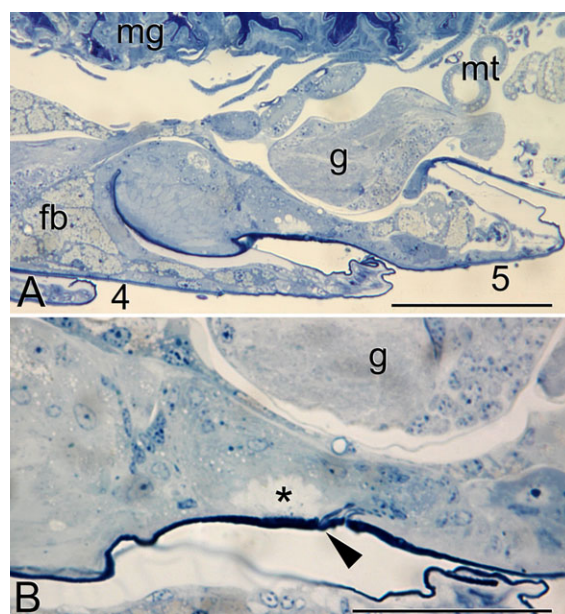

Figure 2 Structure of the sternal gland in pseudergates of *Glossotermes oculatus*. **(A)** Sagittal section of the sternal gland. Scale bar represents 100 μm. **(B)** Parasagittal section of the sternal gland showing the campaniform sensillae (marked by arrowhead) in the posterior part of the extracellular reservoir (marked by asterisk). Scale bar represents 50 μm. Abbreviations: 4, the fourth sternite; 5, the fifth sternite; fb, fat body; g, neural cord ganglion; mg, midgut; mt, Malpighian tubules.

elicited trail-following in pseudergates at 1 GEq/cm, the distance traveled being 6.8 ± 0.9 and 8.4 ± 0.87 cm, respectively. These 2 fractions were merged and further fractionated in 3 steps using preparative GC. At each step, all obtained fractions were tested with respect to their trail-following activity; the active fraction was further fractionated. The LRIs and trail-following activity of particular fractions are summarized in Table 2.

A single behaviorally active fraction F6.3.3 (LRI = 2060–2130) was obtained in the third step of fractionation. This fraction was subjected to a detailed GC × GC/TOF-MS analysis, revealing the presence of a prominent peak (LRI = 2075), corresponding to an unknown compound, a candidate for the trail-following pheromone (Figure 3). The electron ionization (EI) mass spectrum of this compound can be interpreted as follows. The primary losses of masses 15, 43, and 60 from the inferred molecular ion of *m*/*z* 278, as well as the base peak of *m*/*z* 43, indicated that the molecule contained a terminal acetyl group. Furthermore, the molecular mass and natural isotopic contributions suggested that the acetyl group was attached to a hydrocarbon ligand containing 2 double bonds ($C_{17}H_{31}$). These results led us to hypothesize that the molecule might be biosynthesized as an acetyl derivative of linoleic acid (linoleyl methyl ketone), and, indeed, the fragmentation pattern at lower masses is similar to those from linoleic acid and its derivatives. Thus, we tentatively designed a molecular

Table 2 LRIs of fractions obtained by means of preparative gas chromatography of the sternal gland extract of 300 pseudergates and their trail-following activity in an open field Y bioassay

	Fraction	LRI (DB-5)	Distance traveled (cm ± SD)	
			Fraction	Control
Step 1	F1	700–900	<1.5	<1.5
	F2	900–1100	<1.5	<1.5
	F3	1100–1300	<1.5	<1.5
	F4	1300–1500	<1.5	<1.5
	F5	1500–1700	<1.5	<1.5
	F6	**1700–3000**	**6.33** ± **1.07**	0
Step 2	F6.1	1700–1850	<1.5	<1.5
	F6.2	1850–1970	<1.5	<1.5
	F6.3	**1970–2180**	**6.6** ± **0.97**	0
	F6.4	2180–3000	<1.5	<1.5
Step 3	F6.3.1	1970–2020	<1.5	<1.5
	F6.3.2	2020–2060	<1.5	<1.5
	F6.3.3	**2060–2130**	**3.53** ± **0.61**	<1.5
	F6.3.4	2130–2180	<1.5	<1.5

The distance traveled by one pseudergate (mean in centimeter ± standard deviation [SD], *n* = 30) on trails made of particular fractions diluted at a concentration corresponding to approximately 0.1–1 sternal gland equivalent and hexane as control. Behaviorally active fractions in bold.

structure of the unknown compound to be (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one and proceeded to the synthesis of the compound.

Confirmation and quantification of the trail-following pheromone

Once the synthetic (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one was purified, we compared its chemical properties with those of the candidate compound from the SGE. The retention characteristics and EI mass spectra of both compounds matched perfectly (see Figure 3). In order to localize the positions of the double bonds, DMDS derivatives of the compounds were analyzed using GC × GC/TOF-MS. The derivatives of both compounds corresponded in their retention characteristics as well as in their EI mass spectra, containing the molecular ion *m*/*z* 404 and expected characteristic fragments *m*/*z* 357, 309, 225, 201, 203, 155, and others (see Figure 3). The fragments 225 $[M - C_7H_{15}S^{\cdot} - CH_3SH]^+$, 203 $[M - C_{11}H_{21}OS]^+$, 201 $[M - C_{10}H_{19}S_2^{\cdot}]^+$, and 155 $[M - C_{11}H_{21}OS^{\cdot} - CH_3SH]^+$ are indicative of the double bonds in positions 10 and 13 (e.g., see Vicenti et al. 1987).

Behavioral bioassays, summarized in Table 1, clearly show that (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one elicits the trail-following behavior in both pseudergates and soldiers, the activity threshold being 10^{-2} ng/cm of the trail for both

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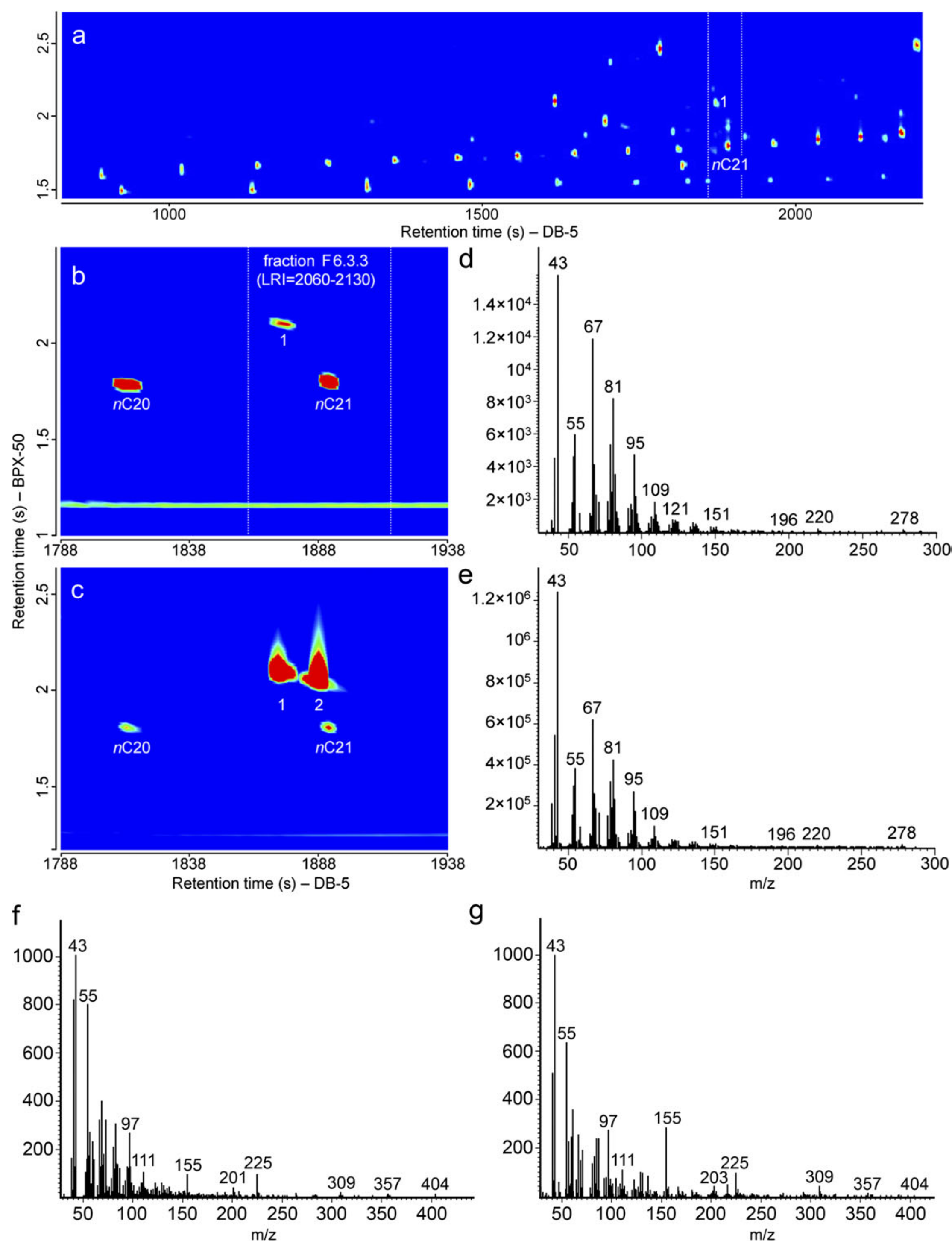

Figure 3 Two-dimensional chromatograms of **(a)** the extract of sternal glands of pseudergates before fractionation, with dashed lines indicating the LRI interval 2060–2130, **(b)** behaviorally active fraction F.6.3.3 of the sternal gland extract of pseudergates, and **(c)** a solution of synthetic (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one, after synthesis and before purification. All samples were coeluted with *n*-alkane standards (*n*C7–*n*C30). EI mass spectra of **(d)** the candidate compound from the sternal gland extract, **(e)** the synthetic (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one, **(f)** DMDS derivative of the synthetic (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one, and **(g)** DMDS derivative of the candidate compound. Numbering: 1, candidate compound (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one; 2, methyl (9*Z*,12*Z*)-octadeca-(9,12)-dienoate (linoleic acid methyl ester).

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castes. The distance traveled on the trail lengthened with increasing doses of the compound; we did not observe any decrease in the trail-following activity at high concentrations, up to 10 ng/cm of the trail in pseudergates.

In the subsequent GC-EAD experiment (rate 70 °C/min), we observed a consistent response of pseudergate antenna to the SGE at the retention time corresponding precisely to the LRI of (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one as well as a response to the synthetic (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one (9′09″, LRI = 2075).

From these evidences, we concluded that the only or at least the major component of the trail-following pheromone of *G. oculatus* is nonadeca-10,13-dien-2-one. This is congruent with our initial hypothesis on the biosynthesis of this compound from the naturally occurring linoleic acid with 2 double bonds in *cis* configuration. We proposed therefore (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one as *G. oculatus* trail-following pheromone.

From the choice trail-following experiment (see Table 3), we estimated the amount of the compound in a sternal gland of a pseudergate to be between 0.2 and 1 ng. The subsequent quantitative analysis of 3 independent SGEs by means of GC-MS estimated this quantity to be approximately 1.7 ± 0.45 ng per sternal gland. As the very last step, we attempted to quantify the amount of the pheromone by means of EAG. The approximate quantity estimated by this method was 1–20 ng (Figure 4).

Table 3 Two-choice trail-following bioassays with sternal gland extract and (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one presented to pseudergates at 2 concentrations

Sternal gland extract (GEq/cm)		Nonadecadienone (ng/cm)		*n*	*P*
10^{-1}	0.5	10^{-2}	10^{-1}		
29	—	1	—	30	$<10^{-3}$
3	—	—	27	30	$<10^{-3}$
	22	—	8	30	10^{-2}

Values indicate the number of choices made by one pseudergate when given a choice between a trail made of sternal gland extract or (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one. GEq, sternal gland equivalent; *n*, number of repetitions, *P*, *P* value (χ^2-test).

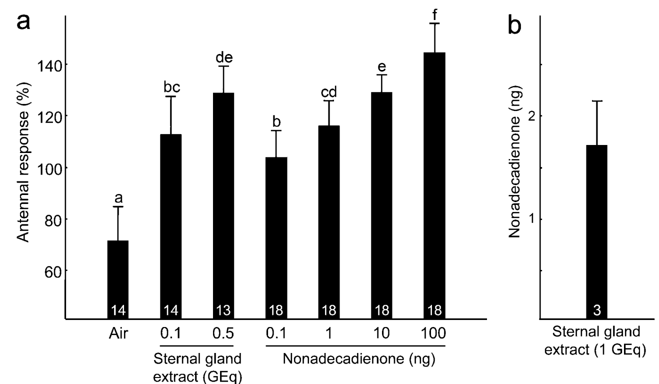

Figure 4 Estimated quantity of nonadeca-10,13-dien-2-one in the sternal gland of one *Glossotermes oculatus* pseudergate. **(a)** EAG responses of pseudergates to air control, sternal gland extract, and a series of concentrations of (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one (nonadecadienone) (mean ± standard deviation). Data were normalized to hexane stimulations. Data normality was controlled using Levene's test ($P = 0.12$). Bars marked with different letters indicate significant differences ($P < 0.05$) among treatments, calculated by means of analysis of variance ($F_{6,106} = 68.4$, $P < 10^{-4}$) with subsequent post hoc comparison using Tukey's Honestly Significant Difference technique test for unequal *n*. Numbers at the base of each bar represent the number of observations for each treatment. GEq, sternal gland equivalent. **(b)** GC/MS quantification of nonadeca-10,13-dien-2-one in 3 independent sternal gland extracts (mean ± standard deviation).

Discussion

The Neotropical family Serritermitidae remained the only isopteran family which had not been studied with respect to trail and sex pheromones. In the present study, we propose that (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one is the trail-following pheromone of the serritermitid *G. oculatus* or at least the major component of this pheromone. The activity threshold was estimated to be 10^{-2} ng of the compound per centimeter of the trail for both tested castes, pseudergates and soldiers. The quantity of the pheromone in the sternal gland of 1 pseudergate was estimated using 3 independent assays to range from hundreds of picograms to units of nanograms.

In addition to the identification of the trail-following pheromone, we have also described the anatomy of the sternal gland in *G. oculatus* pseudergates. The structure of the gland differs in several aspects from that observed in other isopteran species studied so far, including *Serritermes*, above all by the very posterior position of the extracellular reservoir. At the same time, the gland is relatively large, covering about a half of the fifth sternite length, while it is usually much smaller in other species (for comparison, see Quennedey et al. 2008).

In the early steps, we failed to detect a consistent physiological response to the sternal gland extract by means of GC-EAD, probably due to the loss of activity of the antennae at the late elution time of nonadeca-10,13-dien-2-one (23′10″) with the given temperature program. In consequence, the laborious methods of fractionation had to be used combined with trail-following bioassays. Indeed, at the faster temperature program, applied in the final experiment, the antennae of pseudergates responded well to nonadeca-10,13-dien-2-one contained in the sternal gland extract as well as to the synthetic compound (elution time 9′09″).

Our recent studies indicated that the presence of minor components in termite trail-following pheromones may be much more common than previously thought, the knowledge about them being limited due to their barely detectable quantities (Sillam-Dussès et al. 2009, 2010). The most frequent minor component of termite trail-following pheromones is dodecatrienol,

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which has a very strong physiological activity even at very low quantities (see also Hanus et al. 2009), and only units of picograms may be present in one forager (see Bordereau and Pasteels 2011). However, in the case of *Glossotermes*, we did not observe any trail-following activity of dodecatrienol, despite the broad range of tested concentrations. In conclusion, we did not identify any minor component of the trail-following pheromone, even though their presence cannot be excluded.

In proposing the molecular structure of nonadecadienone based on GC/MS, a question arised about the position and geometry of the 2 double bonds in the molecule. Using DMDS derivatization and GC $\times$ GC/MS, we situated the 2 double bonds in positions 10 and 13. Unfortunately, the geometry of these double bonds could not be empirically studied. Nevertheless, the most parsimonious and obvious hypothesis is a biosynthetic origin of nonadeca-10,13-dien-2-one as an acetyl derivative of naturally occurring and abundant linoleic acid (linoleyl methyl ketone), therefore with 2 *cis* double bonds. Moreover, it has been suggested previously that (3*Z*,6*Z*)-dodeca-3,6-dien-1-ol, the trail-following pheromone and sex pheromone of *Ancistrotermes pakistanicus*, is biosynthesized from a linoleic acid ligand (Robert et al. 2004). Thus, we conclude that the trail-following pheromone of *G. oculatus* is very probably (10*Z*,13*Z*)-nonadeca-10,13-dien-2-one.

The description of nonadeca-10,13-dien-2-one as the trail-following pheromone of *G. oculatus* is surprising in 2 aspects. Firstly, it is not in agreement with the major evolutionary trend inferred from available data by Bordereau and Pasteels (2011). The authors have noted a transition to unbranched and unsaturated alcohols with 12 carbon atoms and/or a diterpene (neocembrene or trinervitatriene) in the advanced group Kalotermitidae + Rhinotermitidae + Termitidae (within which Serritermitidae have undoubtedly branched); a phenomenon concurrent with the development of the sternal gland on the fifth sternite. Secondly, the occurrence of a C19 ketone is interesting from the functional point of view, as this compound has the lowest volatility and the highest molecular weight of all termite trail-following pheromones described so far. In this respect, it is worth noting that the effective physiological quantities of the pheromone (activity threshold and content in one sternal gland) are relatively high (compared with those observed for C12 alcohols and diterpenes), given the small body size of *Glossotermes*. These observations are in agreement with the unusual structure and large size of the sternal gland in pseudergates. Thus, not only a new compound but also a new and unexpected category of unsaturated ketone with 19 carbons secreted from an unusual sternal gland has to be added on the list of 8 identified termite trail-following pheromones.

In conclusion, the occurrence of nonadeca-10,13-dien-2-one as the trail-following pheromone of *G. oculatus* represents a unique peculiarity of this genus. None of the relevant scenarios of relationships among Rhinotermitidae, Serritermitidae, and Termitidae can explain the unexpected presence of a C19 ketone instead of a C12 alcohol, even though there are possible similarities in the biosynthesis of these alcohols and nonadeca-10,13-dien-2-one (Robert et al. 2004; Bordereau and Pasteels 2011). These results underline once again the special biology of the 2 serritermitid genera, *Serritermes* and *Glossotermes*, together with the unique defensive strategy of their soldiers (Costa-Leonardo and Kitayama 1991; Šobotník, Bourguignon, et al. 2010) and the lack of true workers in *Glossotermes* (Bourguignon et al. 2009). It would be of great interest to investigate the chemical ecology and caste system of *Serritermes* but also to identify the trail-following pheromone in *Termitogeton*, a poorly known rhinotermitid genus lacking true workers (Parmentier and Roisin 2003) and considered in some studies (e.g., Inward et al. 2007) as a sister clade to Serritermitidae.

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