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Studies on Trail Pheromones Isolated from the Japanese Rhinotermitid Termites

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INTRODUCTION

Termites which are predominantly distributed in the tropical and temperate zones of the world are social insects comprising the order Isoptera contains approximately 2500 species. Although they are commonly called "white ants" in Japan only because of their color and resemblance to ants in behavior, they are more closely related to cockroaches [Blattariae: Dictyoptera (in Neoptera)] than to the true ants (Formicidae: Hymenoptera) in taxonomy. They are occasionally called "social cockroaches" by biologists. Ants are social insects belonging to order Hymenoptera of Holometabola, while the Isopteran insect (*i.e.* termites) are Heterometabola which are relatively primitive in evolutional taxonomy. Thus, termites systematically differ from Hymenopteran social insects (*i.e.* ants, bees and wasps).

Sixteen termite species have been found in Japan so far. Of these, two subterranean termite species, *Reticulitermes speratus* (Kolbe) and *Coptotermes formosanus* Shiraki which belong to family Rhinotermitidae in lower termite are widely distributed and known as serious pests to wooden structures. The former species is distributed from the southern islands to the southern part of Hokkaido prefecture in Japan. They generally attack damp fungus-infected wood without any obvious nest and population size of a colony is relatively small (*ca.* 100,000). While the distribution of the latter species is relatively limited in the warm regions. They built rigid carton nest and can attack damp and dry wood very aggressively and the population size of a colony is much larger (*ca.* 1,000,000). In both species, observed the several castes : Reproductives (First-form reproductives: Queen and King), Pseudergate (false worker), Soldier, Nymph, Larva, Replacement reproductives are usually found in a colony, and the pseudergates have plenty of intestinal symbiotic protozoa, and the termites can attack cellulosic materials such as wood, paper, plants, etc. with the aid of such symbiotic microorganisms.

Economic losses caused by termite attacks are roughly estimated to be equivalent to those of fire in Japan where many wooden structures are available for the subterranean termites. In the U.S.A., the annual cost related to termite control probably exceeds 750 million dollars (Beal *et al.* 1983). Thus, termite control is very realistic and serious for the human life.

Prevention and control of termite attack has been primarily depend on chemical treatments so far. Organochlorines have been used as the most effective termiticides. Those were *aldrin, chlordane, dieldrin* and *heptachlor*. However, detrimental side effects which were released by the recent environmental survey and toxicological studies would not allow the use of organochlorine termiticides any more in the most of the world. Despite a lot of efforts to search for replacements for the termiticides, an effective and safe replacement has not been developed yet. Because in general insecticides are toxic to organisms and environmentally detrimental, it is necessary to develop a new control method from the different viewpoints. Apart from the development of novel low toxicity termiticides, it seems particularly important to investigate biology, physiology and ecology of termites in order to find a clue of the future termite control methods.

Termite life style is so highly socialized that we are apt to take it as if they had a certain intention when observing their behavior and activity. The individuals are differentiated into various morphological forms (*polymorphism*) or castes (*polyethism*) so that they share the roles performing different biological functions. Thus, they establish highly integrated units, the societies or colonies. With the increase of the observatory knowledge on termite behavior, the importance of chemical communication among termite individuals was recognized from the biological aspects.

Generally speaking, chemical signal substances are well recognized in the insect world, and a term "*semiochemicals*" is

now firmly established. Due to the recent development of the analytical instruments, the presence of the semiochemicals could have been precisely demonstrated. These chemicals are functionally divided into two groups : *allelochemicals* (*eg. kairomones, allomones*) if semiochemicals act interspecifically, and *pheromones* if act intraspecifically (Birch & Haynes 1982).

The term "*pheromone*" first appeared in the literature over 30 years ago to mean compounds which acted as intraspecific chemical messengers (Karlson & Lüscher 1959). The term was later defined as follows :

"Pheromones are chemical substances which are secreted to the outside by an individual and received by a second individual of the same species in which they release a specific reaction, for example, a definite behavior or developmental process" (Karlson & Luscher 1959).

The first identified insect pheromone, was "bombykol" extracted from virgin females of silkworm moth, *Bombyx mori* (Bombycidae : Lepidoptera) in Germany (Karlson & Butenandt 1959).

Pheromones which are involved in the behavior and physiological processes of insects resultantly induce the increased reproductive success. Insects use the pheromones for the intraspecific communication to locate food, habitats and mates as well as escaping from predation. Since Karlson and Lüscher (1959) initially defined the pheromones only 30 years ago, a plenty of pheromones have been isolated and identified by now. Pheromones are functionally divided into two groups, *i.e. primer pheromones* and *releaser pheromones* (Birch & Haynes 1982). The former relates to trigger long term and irreversible physiological changes. "Queen substance" of honeybees (Butler 1967 ; Gary 1974) which act as mediators of cast differentiation and molting is a good example of the primer pheromones. The latter releases rather immediate behavioral patterns as shown by sex attractants. Those are designated as *sex pheromone, aggregation pheromone, dispersal* or *spacing pheromone, alarm*

pheromone, *trail* or *recruit pheromone* and *maturation pheromone* (Birch & Haynes 1982).

Although in early days biochemical approaches were far behind other biological studies, the pace of biochemical investigators have been very much accelerated with the advance of accuracy in chemical analysis. Data obtained using laboratory colonies obviously showed that almost every aspect of termite behavior is mediated by chemical signals. Thus, it is particularly important to investigate physiological and ecological roles of termite pheromone from chemical viewpoints. While only a few termite pheromones have been isolated and chemically identified (Karlson *et al.* 1968; Matsumura *et al.* 1968; Birch *et al.* 1972). The effects will be directly or indirectly leading to the development of novel termite control methods with new concepts. Considering various functions and efficiency of the pheromones, "trail pheromone" seems worthy of examination as it is applicable for a bait-block technique as an attractant (Prestwich *et al.* 1982).

Trail pheromones are common in the social insects, especially true for the ants and termites. When food or water source is located near a frontier individual termite (worker or soldier), the individual usually lays down a pheromonal trail on the way back to the colony so that other members can follow the path to the source (MacFarlane 1983). Trail pheromones can also facilitate territorial behavior (*e.g.* migration) of the colony.

Following a few early observations suggesting that certain substances might induce trail-following behavior (Grasse and Noirot 1951; Lüscher and Müller 1960), termite trail pheromone was first demonstrated by Stuart (1961,1963,1969). Ants secrete trail pheromones from a several glandular sources, *e.g.* poison glands, tarsal glands, Dufour's glands, Pavan's gland and the hind gut, while termites do only from a sternal gland on the ventral surface of the abdominal sternite. According to Noirot and Timothee (1965), *Mastotermes darwiniensis* Froggatt (Mastotermitidae) possess three sternal glands on the middle

portion of the third, fourth, and fifth sternites. *Stolotermes*, *Porotermes* (Termopsidae) and *Hodotermes* (Hodotermitidae) have a single gland on the fourth sternite, and all other termites have one at the base of the fifth sternite. Sternal gland extracts of several these termites have been shown to possess trail-following activity (Lüscher & Müller 1960; Stuart 1963; Smythe & Coppel 1966; Moore 1970). Termites might secrete a trail pheromone from the gland continuously or intermittently if necessary. Much research has been made on the morphological and behavioral aspects of trail-following. In contrast, only a few trail pheromones have been identified chemically. Moore (1966, 1969) was the first to isolate a trail pheromone from whole body extracts of *Nasutitermes exitiosus* (Hill) (Nasutitermitinae: Termitidae) and identified it as the unsaturated diterpenoid hydrocarbon $C_{20}H_{32}$ (neocembrene-A: Birch *et al.* 1972). Karlson *et al.* (1968) also isolated a trail pheromone from whole body extracts of *Zootermopsis nevadensis* Hagen (Termopsidae), and identified as *n*-caproic acid (*n*-hexanoic acid). However its biological activity did not satisfy the species.

Trail pheromone studies of family Rhinotermitidae show a most interesting development. The termites are known to be attracted by food odors, particularly those associated with fungus-infected wood. Extracts from those materials show high trail-following activity that was indistinguishable from the termites extracts (Esenther *et al.* 1961; Smythe *et al.* 1965). Both the rhinotermitid termite *Reticulitermes virginicus* (Banks) and the brown rot fungus *Gloeophyllum trabeum* (Pers. ex Fr.) Murr. possess almost the similar trail-following substances, and the latter identified as (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (hereinafter referred to as DTE-OH) (Matsumura *et al.* 1968,1969).

DTE-OH successfully induced the trail-following of several termites (Traniello *et al.* 1982; Prestwich *et al.* 1983; McDowell & Oloo 1984; Hall & Traniello 1985) and termitophiles (Howard 1980). That was one of the first examples of a non-species

specific pheromone (Matsumura *et. al.* 1971). A variety of synthetic chemical analogues of DTE-OH was proved to elicit the trail-following response of rhinotermitids to some extent (Tai *et al.* 1971; Ritter *et al.* 1973,1975,1977; Prestwich *et al.* 1984).

As briefly reviewed above, if the same trail-following substance is obtained from both decayed wood and termites, it is not clear whether the trail pheromone is synthesized *in vivo* or is directly derived from the food source (decayed wood). Although purified sternal gland extracts appear to yield active substance that might be just trail pheromone, it still has not been exactly proven that termites biosynthesize DTE-OH in the sternal gland, as yet.

According to Howard *et. al.* (1976), five requirements must be met in the experimental testings in order to authenticate trail pheromones : (1) high biological activity, (2) localization in an expected biosynthesizing organ (= sternal gland), (3) active deposition on a substrate, (4) chemical identity of the deposited materials with the stored substances in the organ, and (5) biological identity of response to deposited and stored chemicals. They strongly insisted on the presence of authentic trail pheromones of *R. virginicus*, *R. flavipes*,, and *R. tibialis*, and suggested the possibility of such a pheromone in *C. formosanus* as well.

The indispensable step to fully understand the significance and importance of trail pheromones is to supply, accurate information on the biosynthesis of the pheromones. In this dissertation, the two predominant subterranean Rhinotermitid termite *R. speratus* and *C. formosanus* were served for demonstrating the presence of trail pheromones. PART I deals with the trail-following activity by bioassay. Isolation and identification of the trail pheromone was discussed in PART II. Trail pheromone precursors were examined for proving the biosynthesis of the trail pheromone by each termite in the final part.

PART I

BIOASSAY FOR EVALUATING TRAIL-FOLLOWING ACTIVITY

Chapter 1 : Modified Open-Field Bioassay

Chapter 2 : Modified Choice Bioassay

Chapter 1 : Modified Open-Field Bioassay

1.1 Introduction

Bioassay is a well known technique to examine the behavior of insects when a certain stimulus is given to them. Termite trail-following activity was assayed by Stuart (1963) approximately 30 years ago. Since then, a number of bioassay results has been published.

Because termite trail pheromone elicits a very obvious trail-following behavior even if the amount is very small, it is relatively easy to detect the pheromone in termite bioassays. Careful bioassays, therefore, can supply appropriate information on the follows : the threshold concentration (available quantity) followed, the range of concentrations followed, the percentage following, the speed of following, and the optimal concentration. As was pointed out earlier (Matsumura *et al.* 1972; Tai *et al.* 1971; Ritter *et al.* 1977; Prestwich *et al.* 1984), the similarity and difference between the true trail pheromone and their analogues could be demonstrated in the repeated bioassays in stages.

Open-Field bioassay was first developed by Smythe *et al.* in 1967 to detect a trail following substance of a fungus, *G. trabeum*. Matsumura *et al.* (1968,1969) who worked on the trail pheromone of *R. virginicus* could successfully apply in the course of isolation of their target substance, DTE-OH. Afterwards, the technique was modified by Howard *et al.* (1976) and Prestwich *et al.* (1984) in order to make the method more reliable.

In this experiment, newly modified Open-Field bioassay, which was rather similar to the Prestwich and other's, was employed to detect trail pheromones of Japanese subterranean termites (Tokoro *et al.* 1989,1990a,1991,1992a).

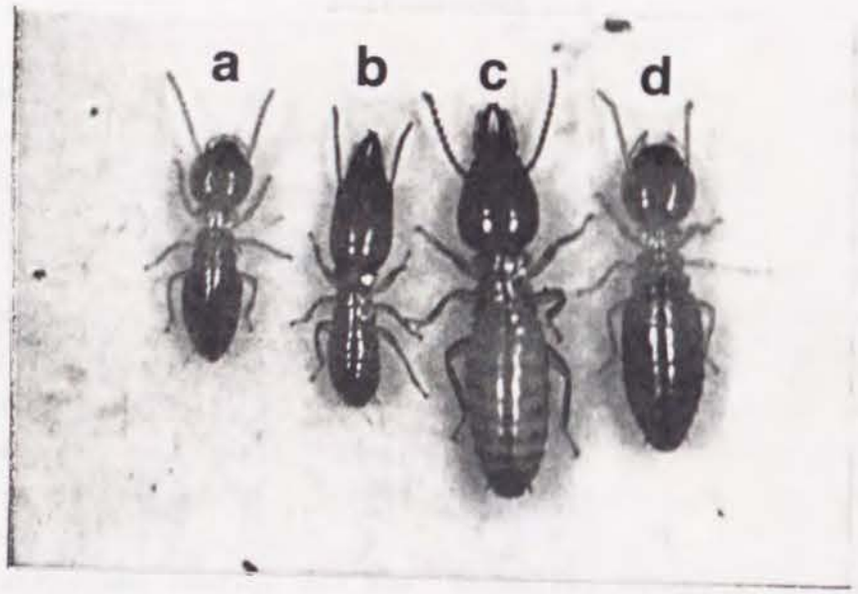


Fig. 1-1 Test termites
Reticulitermes speratus (Kolbe) - a: Worker, b: Soldier
Coptotermes formosanus Shiraki - c: Soldier, d: Worker

1.2. Materials and Methods

1.2.1 Preparation and maintenance of test termites

(1). *Reticulitermes speratus* (Kolbe)(Fig.1-1,(a) and (b))

Termites of *R. speratus* were obtained from five colonies (A-E). Two large colonies A and B were collected at the campus of Kyoto Institute of Technology in the northern part of Kyoto city, and two small colonies, C and D at the campus of Kyoto University

in Uji city, one small colony E at Iriomote island in Okinawa prefecture. Those were transported to the laboratory. Individual termites from two colonies A and B were carefully taken out of the timber for extraction in the laboratory. The remaining termites from colony B with the timber were kept at 20 ± 2°C and 50 ± 5% relative humidity (RH) under moistened condition in the laboratory over three months until they were subjected to modified "Open-Field" bioassay. The termites from colonies C, D and E with the timber were kept at 26 ± 2°C and *ca.* 60 ± 5% RH under moistened condition in the laboratory over a month until they were subjected to preliminary experiments and further bioassay "Y-test" which will be mentioned later. In this investigation, the test termites were undifferentiated pseudergates older than third instar as determined by the size of termite body (hereinafter referred to worker). Test individual workers from colonies A and B for whole body extraction (PART II, Chapter 3) had been fed with moistened filter paper as food source for about five hours to minimize food or nest odor contamination before extraction. The workers for bioassay were placed in a Petri dish with a moistened filter paper for one hour prior to bioassay.

Other worker termites (200 individuals) used for sternal gland excision were immobilized in a refrigerator at -20°C for ten hours. Two hundred abdominal fifth-sternites at which the sternal gland was positioned were carefully dissected from the bodies of worker termites under a binocular stereoscopic microscope. The dissected parts were extracted with *n*-hexane as sternal gland extract.

(2). *Coptotermes formosanus* Shiraki (Fig.1-1,(c) and (d))

Colonies of *C. formosanus* were collected in Wakayama city (Japan) and kept in the laboratory of Wood Research Institute, Kyoto University for about seven years (colony F), ten years

(colony G) and twelve years (colony H) with Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) as food source at 28±2°C and *ca.* 80% RH. Undifferentiated pseudergates (hereinafter referred to workers) and soldiers were selected for the present investigation. The workers were placed in a Petri dish with moistened filter paper for one hour prior to bioassay.

Test individual workers for whole body extraction (PART II, Chapter 4 and 7) had been fed with moistened filter paper as food source for about five days so that the materials in the termite intestine were replaced with filter paper before extraction. It was confirmed by microscopic observation that the intestinal materials were completely substituted with filter paper within five days.

Other test termites (*ca.* 20,000 individuals) collected from colony F used for sternal gland excision were fed only with moistened filter paper as food source for about one year (colony I). The dissection procedure was the same as in the case of *R. speratus*. Extraction methods of each sample will be detailed in PART II and III.

1.2.2. Authentic alcohols

Twelve authentic alcohols were also tested for their trail-following activity. Those were : DTE-OH and (*Z,E,E*)-3,6,8-dodecadien-1-ol ((*Z,E,E*)-DTE-OH) supplied by Dr. H. Yamamoto (Nagoya University); (*Z,Z*)-3,6-dodecadien-1-ol ((*Z,Z*)-3,6-DDE-OH) (*Z*)-3-dodecene-1-ol supplied by Dr. G. D. Prestwich (State University of New York at Stony Brook); Isomers of 6,8-dodecadien-1-ol (6,8-DDE-OH) supplied by Dr. T. Ando (Tokyo University of Agriculture and Technology) and others were purchased from Nacalai Tesque INC. (NTI; Kyoto, Japan). Chemical solutions were prepared with *n*-hexane and the test concentrations are shown in Table 1-1~3 together with bioassay results.

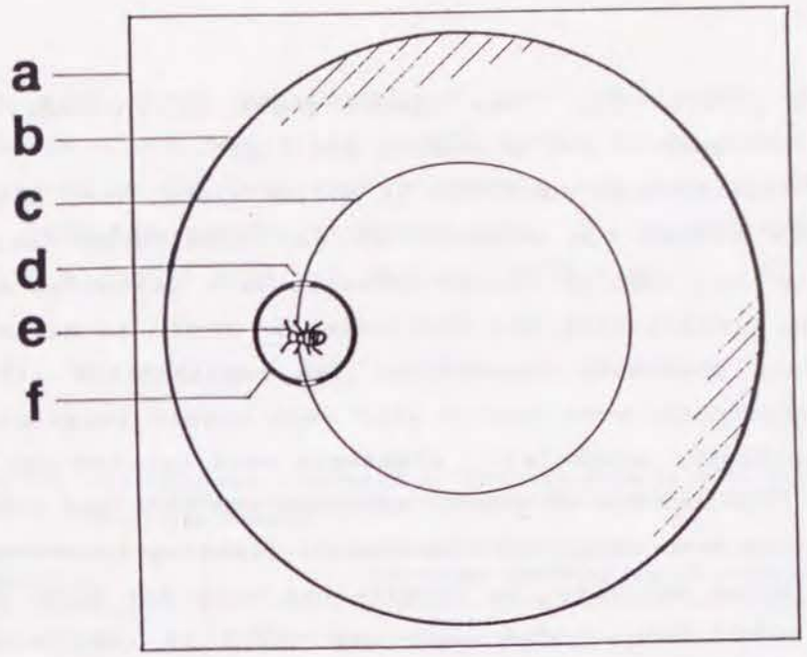


Fig. 1-2 Scheme of Modified Open-Field bioassay
a: Fine quality clay coated paper, b: Petri dish lid (1.5 x 9 cm i.d.), c: Pencil guide line (15 cm long), d: Opening, e: Test worker, f: Plastic cylinder (1 x 1.5 cm i.d.)

1.2.3. Modification of Open-Field bioassay

A modification of the Prestwich and others'*et al.*(1984) modified Open-Field bioassay (Howard *et al.* 1976) was used to establish threshold response levels for trail-following. As shown in Fig. 1-2, 2 μl of a dissolved sample was streaked along a circle with a diameter of 4.7 cm (*ca.* 15 cm long) of pencil guide line drawn on slight absorbent fine quality clay coated paper, OK coat 135K (Oji Paper Co. Ltd., Tokyo, Japan) with a 5 μl-micropipette. After evaporation of solvent (*ca.* 2 min later), a plastic cylinder (1 x 1.5 cm i.d.) was placed on the paper at a left side of the test arena. Two openings of the cylinder directed the termite toward the test arena. A single test termite was then introduced into the cylinder, and a red colored Petri

dish lid (1.5 x 9 cm i.d.) was placed above it in order to minimize the influence of air movements and light.

When a test termite succeeded in moving along the sample streaked circle within two minutes, it was considered that a "basic activity" was induced. Three termites were tested on each piece of paper, constituting one replicate, in order to minimize additional trail pheromone deposition. Ten replications (three termites per replicate) were tested with each sample (*e.g.* crude extracts or authentic alcohols). Bioassays were carried out at approximately 20°C and 50% RH for *R. speratus* and 26°C and 60% RH for *C. formosanus* both under the fluorescent lighting to estimate the trail-following activity. No termite was used for more than one bioassay in 24 hours. When over one third of test worker termites showed the complete trail-following action, the sample was considered to have elicited a positive response. Various levels of sample concentration were prepared to determine the "minimum effective worker equivalent" [MEWE (worker equivalent /15 cm-trail)].

1.3 Results and Discussion

Although Smythe *et al.* (1967) took a semicircular trail line and a group of termites at a test for evaluating trail-following activity in an original Open-Field bioassay, Prestwich *et al.* (1984) later used a single worker termite instead of a termite group to minimize the group effect and/or mutual following behavior. Preliminary trial with Japanese termites by the original method also proved that following behavior (tandem running) was sometimes induced when tested with a group. Therefore, a single termite worker was employed at a test in the present investigations. In addition, *C. formosanus* were found to exhibit a different movement in comparison with *R. speratus*. *R. speratus* often turned around and returned toward the starting

point even when the amount of trail following substance was available along the trail semicircular line. While a fewer *C. formosanus* would take such movements but some occasionally run away from the sample streaked line. In consideration of such cases a circular line was employed this time.

Table 1-1 Trail-following response of *R. speratus* workers to whole body extracts and sternal gland extracts and authentic alcohols evaluated in modified Open-Field bioassay

Substances	Percentage following (per 30 workers)									
Whole body ex.	3[a]	2	1	0	-1	5x-2	-2	5x-3	-3	-4
								(WE[b]/15 cm-trail)		
Colonies A+B	10.0 (7)[c]	80.0 (0)	90 (0)	93.3 (0)	63.3 (1)	26.7 (4)	10 (8)	6.7 (8)	0	0
Colony C	23.3 (4)	96.7 (0)	100 (0)	100 (0)	93.3 (0)	86.7 (0)	46.7 (2)	20 (5)	3.3 (9)	0
Sternal gland ex.	3[a]	2	1	0	-1	5x-2	-2	5x-3	-3	-4
								(WE[b]/15 cm-trail)		
Colony B	-	93.3 (0)	100 (0)	96.7 (0)	76.7 (0)	16.7 (6)	6.7 (8)	3.3 (9)	0	0
Authentic alcohol	0[a]	-1	-2	-3	-4	-5	-6	-7	-8	
								(μg/15 cm-trail)		
(*Z,Z,E*)-DTE-OH	0	13.3 (7)	86.7 (0)	100 (0)	100 (0)	93.3 (0)	76.7 (0)	43.4 (2)	0	
(*Z,E,E*)-DTE-OH	-	83.3 (0)	93.3 (0)	83.3 (0)	86.7 (0)	16.7 (6)	6.7 (8)	0	0	
(*Z,Z*)-3,6-DDE-1-ol	0	73.3 (0)	86.7 (0)	83.3 (0)	50 (1)	0	0	-	-	
(*Z,Z*)-6,8-DDE-1-ol	-	86.7 (0)	13.3 (7)	0	0					
(*E,E*)-6,8-DDE-1-ol	-	83.3 (0)	3.3 (9)	0	0					
(*E,E*)-8,10-DDE-1-ol	50 (2)	76.7 (1)	0	0	-					
(*Z*)-3-dodecen-1-ol	6.7 (8)	83.3 (0)	53.3 (2)	10 (8)	0					
1-dodecanol	33.3	56.7	0	0						
1-undecanol	66.7	6.7	0	0						
1-tridecanol	50	0	0	0						
1-tetradecanol	6.7	0	0	0						
1-pentadecanol	0									

a: The values -log quantity.
b: Worker equivalent.
c: Number of three termites' group (one replicate) that showed no trail following behavior is shown in the parentheses.

Trail-following activities of whole body extracts, sternal gland extracts and authentic trail pheromone analogues are shown in Tables 1-1~3. As shown in Table 1-1, the hexane extracts of whole bodies of *R. speratus* obtained from colony A and B exhibited remarkably high activities in the range from 100 to 0.1 WE/15 cm-trail. According to the definition of MEWE described in the section 1.2.3 MEWE was 0.1 WE/15 cm-trail. That was identical with that of sternal gland extract (Table 1-1), and statistical analysis (*t*-test) did not show any significance at 95% confidence limit. As for the test termites from colony C, MEWE was 0.01 WE/15 cm-trail, that was much lower than the above figure for colonies A and B. The results suggested that trail-following activity and the amount of pheromone available per individual worker would vary among colonies.

In regard to *C. formosanus*, the hexane extracts of whole bodies seemed have a wide, conspicuous range of activities in terms of concentrations (dilution) of extracts. The range was from 10 to the MEWE, 0.005 WE/15 cm-trail. Similarly to *R. speratus* the values were identical with those of sternal gland extracts (Table 1-2).

Both termite species readily reacted to the authentic DTE-OH and other related materials when examined in the modified Open-Field bioassay (Table 1-2 and 1-3). The phenomena was quite similar to that of other rhinotermitid termites (Tai *et al.* 1971; Matsumura *et al.* 1972; Ritter *et al.* 1975,1977; Kajiwara *et al.* 1978; Du *et al.* 1982; Kaib *et al.* 1982).

As briefly described above, the present results well supported the early finding that trail-following action was induced by the trail pheromone and its analogues within a limited range of quantity of active substances (Moore 1966; Ritter *et al.* 1969,1975; Howard *et al* 1976; Prestwich *et al.* 1984; Hall & Traniello 1985). When a high response was observed (over 70% trail-following), any of three worker termites as one replication generally could follow the sample-streaked circle line.

Table 1-2 Trail-following response of *C. formosanus* workers to whole body extracts and sternal gland extracts and authentic alcohols evaluated in modified Open-Field bioassay

Substances	Percentage following (per 30 workers)									
	3[a]	2	1	0	-1	5x-2	-2	5x-3	-3	-4
									(WE[b]/15 cm-trail)	
Whole body ex.	0	20 (6)[c]	83.3 (0)	96.7 (0)	100 (0)	90 (0)	86.7 (0)	73.3 (0)	6.7 (8)	0
Sternal gland ex.	-	-	66.7 (0)	96.7 (0)	100 (0)	96.7 (0)	93.3 (0)	83.3 (0)	16.7 (7)	0
Authentic alcohol	0[a]	-1	-2	-3	-4	-5	-6	-7	-8	
									(μg/15 cm-trail)	
(*Z,Z,E*)-DTE-1-ol	0	6.7 (8)	73.3 (0)	96.7 (0)	100 (0)	93.3 (0)	83.8 (0)	36.7 (3)	0	
(*Z,E,E*)-DTE-1-ol	-	96.7 (0)	96.7 (0)	93.3 (0)	86.7 (0)	6.7 (8)	3.3 (9)	0	0	
(*Z,Z*)-3,6-DDE-1-ol	0	46.7 (2)	83.3 (0)	83.3 (0)	33.3 (3)	10 (6)	0	-	-	
(*Z,Z*)-6,8-DDE-1-ol	-	66.7 (0)	73.3 (0)	13.3 (7)	0					
(*E,E*)-6,8-DDE-1-ol	-	83.3 (0)	6.7 (9)	0	0					
(*Z*)-3-dodecen-1-ol	6.7 (8)	66.7 (0)	83.3 (0)	50 (2)	0					
1-dodecanol	66.7	50	0	0						
1-undecanol	50	66.7	0	0						
1-tridecanol	83.3	23.4	0	0						
1-tetradecanol	56.7	0	0	0						
1-pentadecanol	0									

Captions are the same as in Table 1-1.

During the series of bioassay trials, two supplemental experiments were planned. In the first supplemental test, soldiers of *C. formosanus* were served for the modified Open-Field bioassay using whole body extracts of workers and some authentic alcohols. The soldiers produced a sensitive response to the samples as shown in Table 1-3.

Table 1-3 Trail-following response of *C. formosanus* soldiers to whole body extract and authentic alcohols evaluated in modified Open-Field bioassay

Substances	Percentage following (per 30 soldiers)									
	3[a]	2	1	0	-1	5x-2	-2	5x-3	-3	-4
								(WE[b]/15 cm-trail)		
Whole body ex.	0	6.7 (9)[c]	76.7 (0)	83.3 (0)	93.3 (0)	90 (0)	66.7 (1)	66.7 (0)	13.3 (7)	0
Authentic alcohol	0[a]	-1	-2	-3	-4	-5	-6	-7	-8	
								(μg/15 cm-trail)		
(Z,Z,E)-DTE-1-ol	0	0	16.7 (6)	93.3 (0)	96.7 (0)	100 (0)	96.7 (0)	66.7 (1)	6.7 (9)	
(Z,E,E)-DTE-1-ol	-	83.3 (0)	96.7 (0)	96.7 (0)	83.3 (0)	16.7 (7)	0			
(Z,Z)-3,6-DDE-1-ol	-	33.3 (4)	50 (2)	83.3 (0)	66.7 (0)	3.3 (9)	0			
(Z)-3-dodecen-1-ol	-	-	73.3 (0)	33.3 (3)	16.7 (6)	0				

Captions are the same as in Table 1-1.

Furthermore, whole body extracts of the two test termite species were tested for their trail-following activities using worker termites obtained from different colonies to compare the activity levels among colonies. Results in Table 1-4 indicated that sensitivity was varied with the combinations of the origin of extracts and tested individual worker termites, although trail-following behavior was unexceptionally induced in any case.

On the basis of the results here, "Open-Field" bioassay was considered to be a simple, and reproducible technique in regard to the evaluation of trail-following activity of test samples.

Table 1-4 Comparison of trail-following threshold values among different colonies

R. speratus	Colony source of extracts			
	A+B	C	D	E
Test colony	Threshold level[a] (MEWE[b]: WE[c]/15 cm-trail)			
A+B	0.1	-	-	-
C	0.05	0.01	0.05	0.01
D	0.05	0.01	0.025	0.005
E	0.1	0.025	0.05	0.01
C. formosanus	Colony source of extracts			
	F	G	H	I
Test colony	Threshold level (MEWE: WE/15 cm-trail)			
F	0.005	0.005	0.01	0.005
G	0.005	0.01	0.01	0.005
H	0.01	0.01	0.025	0.01
I	0.005	0.005	0.01	0.005

a: The activity was estimated by a modified Open-Field bioassay. When three or more of nine workers followed the 15 cm trail, the sample was designated positive. Tenfold dilution series and more highly diluted solutions of each sample were employed to determine the MEWE (WE/15 cm-trail).

b: Minimum effective worker equivalent.

c: Worker equivalent.

1.4 Summary

A modification of Prestwich and other's Open-Field bioassay was employed to the trail following activity of extracts from whole bodies and sternal glands and authentic alcohols. As demonstrated earlier, the level of trail-following activity of whole body extract was equivalent to that of sternal gland extract. In general, termites showed trail-following response to a certain range of concentration (= quantity available for trail-following). Applicability of the bioassay technique was confirmed for the assessment of trail-following activity of the test materials.

Chapter 2 : Modified "Choice bioassay"

2.1. Introduction

As demonstrated in Chapter 1, termite extracts and some authentic alcohols succeeded in eliciting trail-following behavior within a certain range of concentrations (quantity) tested. However, it still remains questionable whether test termites would show the equal preference when they are forced to choice either of the two substances with the equal trail-following activity proved in the modified Open-Field bioassay. Preferential differences might be dependent on species specificity. In order to determine species specificity and to make the data more authentic, Howard *et al.*(1976) first employed choice bioassay technique.

They demonstrated that each four rhinotermitid termite workers could distinguish a conspecific trail pheromone from an equally active heterospecific trail pheromone.

Although their results suggested the species specificity in terms of trail-following response, the problem has not fully solved due to the lack of chemical substantiation.

In this experiment, the modified Choice bioassay (referred to Y-test) was employed to elucidate the species specificity of trail pheromone using both *R. speratus* and *C. formosanus* (Tokoro et al. to be published).

2.2. Materials and Methods

2.2.1 Preparation of sternal gland extracts.

Preparation of sternal gland extract was done in the same way as described in Chapter 1 (section 1.2.1).

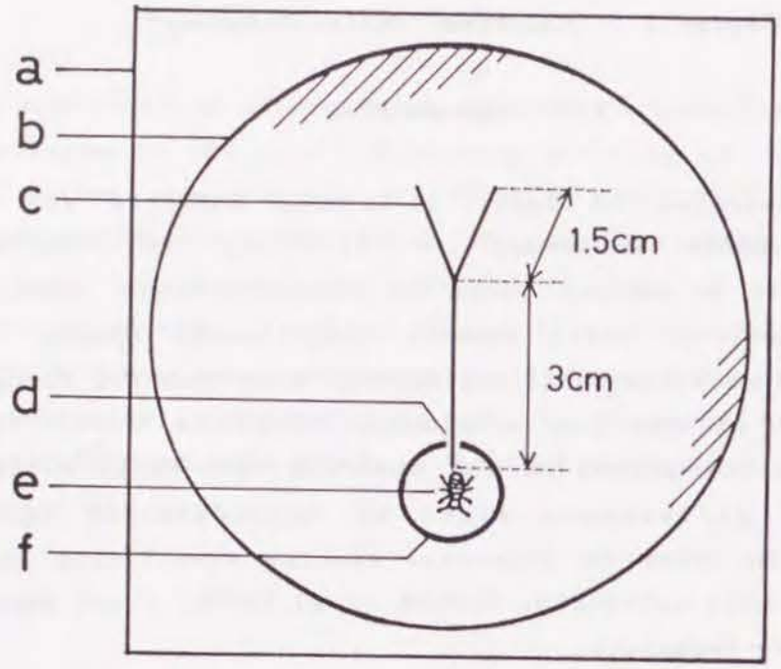


Fig. 2-1 Scheme of Modified Choice bioassay
a: Fine quality clay coated paper, b: Petri dish lid (1.5 x 9 cm i.d.), c: Pencil guide line, d: Opening, e: Test termite, f: Plastic cylinder

2.2.2 Choice bioassay (Y-test)

A modification of the Howard and other's Choice bioassay was used to examine the preferential difference between the two rhinotermitid termites in trail-following activity of some substances. As shown in Fig. 2-1, 1 μl each of one dissolved sample was streaked from the junction to the distal end of the branched line (1.5 cm long), and stem (3 cm long) another 1 μl was streaked from the junction to the end of basal point of Y-shaped pencil guide line (*ca.* 15 cm long and the angle formed by branches is 45°) and also 1 μl of another dissolved sample was streaked along another branch (1.5 cm long) and the same stem of the Y-shaped pencil guide line drawn on the clay coated paper with a 5 μl-micropipette. The same was done for the other

branched and basal lines with the other dissolved sample. One opening of the cylinder directed the termite toward the test arena. A worker termite was then introduced into the cylinder, and a red colored Petri dish lid (1 x 5.7 cm i.d.) was placed above it in order to minimize the influence of air movements and light.

When a worker termite succeeded in moving along the sample streaked Y-shaped line within one minute, it was considered that a "basic activity (2)" was induced. When the termite complete a trail-following and reached to one distal end of branched part of a Y-shaped line or deviated from the trail at least 2 cm, the termite was removed from the test stage immediately. Ten replicates of Y-shaped lines were prepared and ten termites (one each at a time) were tested each line. Numbers of test worker termites which selected either of the branched parts were recorded respectively. A given sample was considered to have elicited a threshold trail-following response if more than 50 % of hundred test termites showed a basic activity (2). Two fold dilution series of each sample were employed to determine the trail-following activity of the test materials. Bioassays were carried out at approximately 26°C and 60% RH under the fluorescent lighting to estimate the trail-following activity. No termite was used for more than one bioassay in 24 hours. All the data were served for a *chi*-square statistical analysis using the Yates Correction for Continuity (Yates, 1934).

2.3 Results and Discussion

Preliminary trails which employ the original bioassay technique of Howard *et al.* well supported that termites statistically showed equal response to the two chemicals of the same activity level generating 50/50 distribution (Table 2-1).

Table 2-1 Reexamination of Howard and others' method of choice bioassay with DTE-OH

Test workers (Quantity)	Number of termites choosing			Chi-square[a]
	Trial	Left	Right	
R. speratus (10^{-7}μg/cm)[c]	1	6 (0)[b]	9	0.27
	2	6 (0)	9	0.27
	3	8 (0)	7	0.00
	4	9 (1)	5	0.64
	5	5 (0)	10	1.07
	(Total)	34 (1)	40	0.34
C. formosanus (10^{-7}μg/cm)	1	6 (0)	9	0.27
	2	5 (0)	10	1.07
	3	8 (2)	5	0.31
	4	4 (0)	11	2.40
	5	6 (1)	8	0.07
	(Total)	29 (3)	43	2.35

a: Chi-square analysis made on the hypothesis that if the two sample trail is identical, a 1:1 distribution will result, and value of Chi-square will be less than 3.84 at the 95% level of significance.
b: Number of termites deviating.
c: Test concentration of DTE-OH was the threshold level estimated by modified Open-Field bioassay.

However, it was noticeable during the bioassay that a group of termites took tandem running and some termites seemed to disturb the natural behavior of the others through returning to the test "nest" especially at the junction and opening site of "exit tube". Therefore, a single worker (or soldier) termite was tested at a time instead of a group of 15 termites to rule out group effects. In addition, the shorter branched lines were considered to result in the evenness of the streaked quantity of test chemicals along the trail line.

Results obtained in the modified Choice bioassay are tabulated below (Tables 2-2~7). As expected two samples which were the same in Trail-following activity level generally induced a 50/50 distribution. The results suggested the applicability of the bioassay technique to examine the species specificity using two termite species. Each threshold quantity, which could induce the basic activity to more than 50% of test workers, of respective sample was estimated as follows : in case of *R. speratus* : DTE-OH 100 fg, (*Z,E,E*)-DTE-OH 50 pg/1.5 cm-trail, *R. speratus* sternal gland extract (R.s-SGE) 10^{-4} WE/1.5 cm-trail, *C. formosanus* sternal gland extract (C.f-SGE) $5x10^{-5}$ WE/1.5 cm-trail ; in case of *C. formosanus* worker : DTE-OH 100 fg, (*Z,E,E*)-DTE-OH 25 pg/1.5 cm-trail, R.s-SGE 10^{-4} WE/1.5 cm-trail, C.f-SGE $5x10^{-5}$ WE/1.5 cm-trail ; in case of *C. formosanus* soldier : DTE-OH 50 fg, (*Z,E,E*)-DTE-OH 25 pg/1.5 cm-trail, R.s-SGE $5x10^{-5}$ WE/1.5 cm-trail, C.f-SGE $2.5x10^{-5}$ WE/1.5 cm-trail.

Table 2-2 Distribution of termites in Y-test when a couple of the same DTE-OH concentrations or (*Z,E,E*)-DTE-OH concentrations applied to *R. speratus* workers

Test samples weight/1.5 cm	Number of termites choosing			Chi-square[a]
	Left		Right	
DTE-OH				
50 ng	8	(72)[b]	20	4.32
5 ng	46	(13)	41	0.18
500 pg	54	(0)	46	0.49
50 pg	48	(0)	52	0.09
5 pg	41	(6)	53	1.29
500 fg	54	(6)	40	1.80
250 fg	38	(12)	50	1.38
100 fg	46	(10)	44	0.00
50 fg	18	(66)	16	0.03
25 fg	15	(82)	3	6.72
5 fg	0	(100)	0	-
(*Z,E,E*)-DTE-OH				
50 ng	41	(2)	57	2.30
5 ng	48	(1)	51	0.04
500 pg	39	(4)	57	3.01
50 pg	41	(9)	50	0.70
25 pg	21	(65)	14	1.03
10 pg	8	(88)	6	0.07
5 pg	14	(78)	6	2.45
500 fg	1	(94)	5	1.50

a: Chi-square analysis made on the hypothesis that if the two sample trail is identical, a 1:1 distribution will result, and value of Chi-square will be less than 3.84 at the 95% level of significance.
b: Number of termites deviating.

Table 2-3 Distribution of termites in Y-test when a couple of the same DTE-OH concentrations or (*Z,E,E*)-DTE-OH concentrations applied to *C. formosanus* workers

Test samples weight/1.5 cm	Number of termites choosing			Chi-square[a]
	Left		Right	
DTE-OH				
50 ng	11	(83)[b]	6	0.94
5 ng	39	(30)	31	0.70
500 pg	55	(2)	43	0.23
50 pg	42	(3)	55	1.48
5 pg	46	(2)	52	0.26
500 fg	46	(7)	39	0.42
250 fg	47	(0)	33	2.11
100 fg	48	(22)	30	3.71
50 fg	24	(58)	18	0.60
25 fg	0	(96)	4	2.25
5 fg	0	(100)	0	-
(*Z,E,E*)-DTE-OH				
50 ng	54	(2)	57	2.30
5 ng	44	(1)	51	0.04
500 pg	58	(3)	39	3.34
50 pg	56	(6)	38	3.07
25 pg	40	(28)	32	0.68
10 pg	5	(92)	3	0.13
5 pg	2	(96)	2	0.25
500 fg	1	(99)	0	0.00

Captions are the same as in Table 2-2.

Table 2-4 Distributions of termites in Y-test when a couple of the same DTE-OH concentrations or (*Z,E,E*)-DTE-OH concentrations applied to *C. formosanus* soldiers

Test samples weight/1.5 cm	Number of termites choosing			Chi-square[a]
	Left		Right	
DTE-OH				
50 ng	3	(91)[b]	6	0.44
5 ng	10	(79)	11	0.00
500 pg	47	(6)	47	0.00
50 pg	41	(4)	55	1.76
5 pg	51	(3)	46	0.16
500 fg	51	(4)	45	0.26
250 fg	52	(14)	34	3.36
100 fg	44	(23)	33	1.30
50 fg	38	(12)	50	1.38
25 fg	12	(78)	10	0.05
5 fg	4	(95)	1	0.80
(*Z,E,E*)-DTE-OH				
50 ng	55	(5)	40	2.06
5 ng	46	(4)	46	0.16
500 pg	50	(4)	46	0.09
50 pg	54	(5)	41	1.52
25 pg	44	(16)	40	0.11
10 pg	10	(58)	32	10.50
5 pg	4	(94)	2	0.17
500 fg	0	(100)	0	-

Captions are the same as in Table 2-2.

Table 2-5 Distributions of termites in Y-test when a couple of the same sternal gland extracts applied to *R. speratus* workers

Test samples WE/1.5 cm	Number of termites choosing			Chi-square[a]
	Left		Right	
R.s-SGE[b]				
0.1	44	(6)[c]	50	0.27
0.01	46	(2)	52	0.26
0.005	50	(0)	50	0.01
0.001	42	(0)	58	1.40
0.0005	53	(8)	39	1.84
0.00025	48	(11)	41	0.40
0.0001	44	(13)	43	0.00
0.00005	18	(54)	28	1.76
0,000025	12	(72)	18	0.83
0.0000125	0	(96)	4	2.25
0.000005	0	(100)	0	-
C.f-SGE[d]				
0.1	50	(1)	49	0.00
0.01	52	(2)	46	0.26
0.005	59	(0)	41	2.89
0.001	44	(6)	50	0.27
0.0005	52	(0)	48	0.09
0.00025	43	(3)	54	1.03
0.0001	40	(6)	54	1.80
0.00005	28	(49)	23	0.31
0.000025	12	(76)	12	0.04
0.0000125	2	(98)	0	0.50
0.000005	0	(100)	0	-

a: Chi-square analysis made on the hypothesis that if the two sample trail is identical, a 1:1 distribution will result, and value of Chi-square will be less than 3.84 at the 95% level of significance.
b: Sternal gland extract of *R. speratus*.
c: Number of termites deviating in the parentheses.
d: Sternal gland extract of *C. formosanus*.

Table 2-6 Distributions of termites in Y-test when a couple of the same sternal gland extracts applied to *C. formosanus* workers

Test samples WE/1.5 cm	Number of termites choosing			Chi-square[a]
	Left		Right	
R.s-SGE[b]				
0.1	43	(8)[c]	49	0.27
0.01	47	(5)	48	0.00
0.005	44	(9)	47	0.04
0.001	45	(8)	47	0.01
0.0005	50	(2)	48	0.01
0.00025	48	(11)	41	0.40
0.0001	39	(18)	43	0.11
0.00005	21	(52)	27	0.52
0.000025	12	(78)	10	0.05
0.0000125	9	(87)	4	1.23
0.000005	4	(95)	1	-
C.f-SGE[d]				
0.1	51	(1)	48	0.04
0.01	48	(4)	48	0.01
0.005	48	(6)	46	0.01
0.001	45	(8)	47	0.01
0.0005	46	(10)	44	0.01
0.00025	44	(9)	47	0.04
0.0001	40	(8)	52	1.32
0.00005	33	(34)	33	0.02
0.000025	24	(57)	19	0.37
0.0000125	14	(68)	18	0.28
0.000005	11	(75)	14	0.16

Captions are the same as in Table 2-5.

Table 2-7 Distributions of termites in Y-test when a couple of the same sternal gland extracts applied to *C. formosanus* soldiers

Test samples WE/1.5 cm	Number of termites choosing			Chi-square[a]
	Left		Right	
R.s-SGE[b]				
0.1	41	(11)[c]	49	4.32
0.01	47	(3)	50	0.18
0.005	47	(7)	46	0.49
0.001	48	(8)	46	0.09
0.0005	44	(8)	48	1.29
0.00025	48	(6)	46	1.80
0.0001	49	(5)	46	1.38
0.00005	47	(12)	41	0.28
0.000025	29	(51)	20	1.31
0.0000125	16	(74)	10	6.72
0.000005	12	(82)	0	-
C.f-SGE[d]				
0.1	53	(2)	45	0.50
0.01	47	(3)	50	0.04
0.005	45	(4)	51	0.26
0.001	46	(3)	51	0.16
0.0005	45	(2)	53	0.50
0.00025	52	(2)	46	0.26
0.0001	46	(6)	48	0.01
0.00005	45	(5)	50	1.17
0.000025	40	(16)	44	0.11
0.0000125	20	(51)	29	1.31
0.000005	23	(61)	16	0.92

Captions are the same as in Table 2-5.

2.4 Summary

A modification of Howard and other's Choice bioassay was employed to assess the distribution patterns of termites when a couple of the same materials applied to the organisms. As expected in such choice test, the same test samples consistently resulted in a 50/50 distribution, which was well supported in statistical analysis . The results strongly encouraged to apply the method to determine species specificity in trail-following activity.

PART II

ISOLATION AND IDENTIFICATION OF TRAIL PHEROMONE

Chapter 3 : Isolation and Identification of Trail Pheromone from *Reticulitermes speratus* (Kolbe)

3.1 Introduction

Trail pheromone of rhinotermitid termite was first isolated from whole body extract of *R. virginicus* (Banks) (Rhinotermitidae) by Matsumura *et al.*(1968,1969). The amount of the isolated pheromone was too small to allow determination of the complete chemical structure of the pheromone. However, they found that a substance isolated from wood infected by a brown rot fungus, *Gloeophyllum trabeum* (Pers. ex. Fr.) Murr. was chemically similar to the trail pheromone of *R. virginicus*, and identified it as (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (DTE-OH). They finally concluded that the chemical was identical with the trail pheromone of *R. virginicus* (Tai *et al.* 1969).

It was later reported that DTE-OH elicited trail-following behavior for other species of the family Rhinotermitidae, *e.g. Reticulitermes flavipes* (Kollar) (Matsumura *et al.* 1972), *R. hesperus* Banks (Matsumura *et al.* 1972; Grace *et al.* 1987), *R. lucifugus santonensis* Feytaud (Ritter *et al.* 1975) , *R. tibialis* Banks (Matsumura *et al.* 1972) , *R. speratus* (Kolbe), as well as *Coptotermes formosanus* Shiraki (Matsumura *et al.* 1972; Howard *et al.* 1976). Since the trail-following substance shows a weak species specificity, DTE-OH appears to be common among the family Rhinotermitidae.

On the other hand, Tai *et al.* (1971), Ritter *et al.* (1973, 1975,1977), Prestwich *et al.* (1984) synthesized analogues of DTE-OH in order to asses the relation between the trail-following behavior and the stereochemical structure of such substances. They and others demonstrated that many of the analogues could induce trail-following behavior for some rhinotermitid termites (Matsumura *et al.* 1972; Ritter *et al.* 1975; Tai *et al.* 1971; Kaib *et al.* 1982; Prestwich *et al.* 1984).

The complete structure (positions and stereochemistry of the double bonds) of the trail pheromone DTE-OH was determined by comparison of active threshold level of synthetic compounds with that of the native pheromone (Tai *et al.* 1969). Since the analogues of pheromone compounds possibly induce trail-following activity and the threshold level of this activity varies as a function of multiple components of these active substances (Howard *et al.* 1976; Kaib *et al.* 1982; Traniello 1982), this approach remains questionable in the determination of the complete structure of the native trail pheromone. Moreover, Howard *et al.* (1976) evidenced that some of the rhinotermitid termites could recognize their own extract trail. The finding might suggest that this spices specificity could be caused by variation of trail pheromone, and suggested that small quantities of close chemical analogues may act in a multicomponent sense to confer specificity to the trail. It is, therefore, important to examine whether rhinotermitid termite actually secretes the DTE-OH or not.

Many methods have been developed for the chemical structure elucidation of highly unsaturated components such as DTE-OH, but most are limited to the determination of the positions of double bonds (Hummel & Karlson 1968; Karlson *et al.* 1968; Moore 1969; Stuart 1969). Considering the structure elucidation of highly unsaturated insect pheromones, the most difficult problem is the determination of the stereochemistry of these double bonds in minute amounts of sample.

In this Chapter, *R. speratus* was served for the isolation and identification of the trail pheromone, and then new methods were applied for the determination of the stereochemistry of each double bond in the trail pheromone. Furthermore, the coincidence in the chemical structure of the active substances extracted from both whole bodies and sternal glands was examined with the aid of new analytical techniques (Tokoro *et al.* 1990a,1991; Yamaoka *et al.* 1987).

3.2. Materials and Methods

3.2.1 Test insects and bioassay methods

Test insects and bioassay methods (Open-Field Bioassay) was described in the section of 1.2.1 in Chapter 1.

3.2.2 Chemicals

Authentic alcohols used were given in the section 1.2.2 in Chapter 1. All other reagents were purchased from Nacalai Tesque INC. (NTI; Kyoto, Japan).

3.2.3 Isolation

Isolation process of the trail pheromone is shown in Fig. 3-1.

(1) Extraction

Test individuals were placed on the moistened filter paper in a Petri dish for about five hours before extraction, and soaked in *n*-hexane (Hx) for three days (*ca.* 1000 ml per 100,000 individual workers). The extracts were then filtered and stored at -20°C until the next step. These operations were repeated to obtain extracts from approximately 146 g termite workers (*ca.* 97,000 in number).

On the other hand, dissected fifth-sternites as sternal glands were soaked in 500 μl of Hx for ten hours. As a control sample, one hundred of workers were soaked in 500 μl of Hx for ten hours. Both of the extracts were used for capillary GC-MS-HR-SIM analysis.

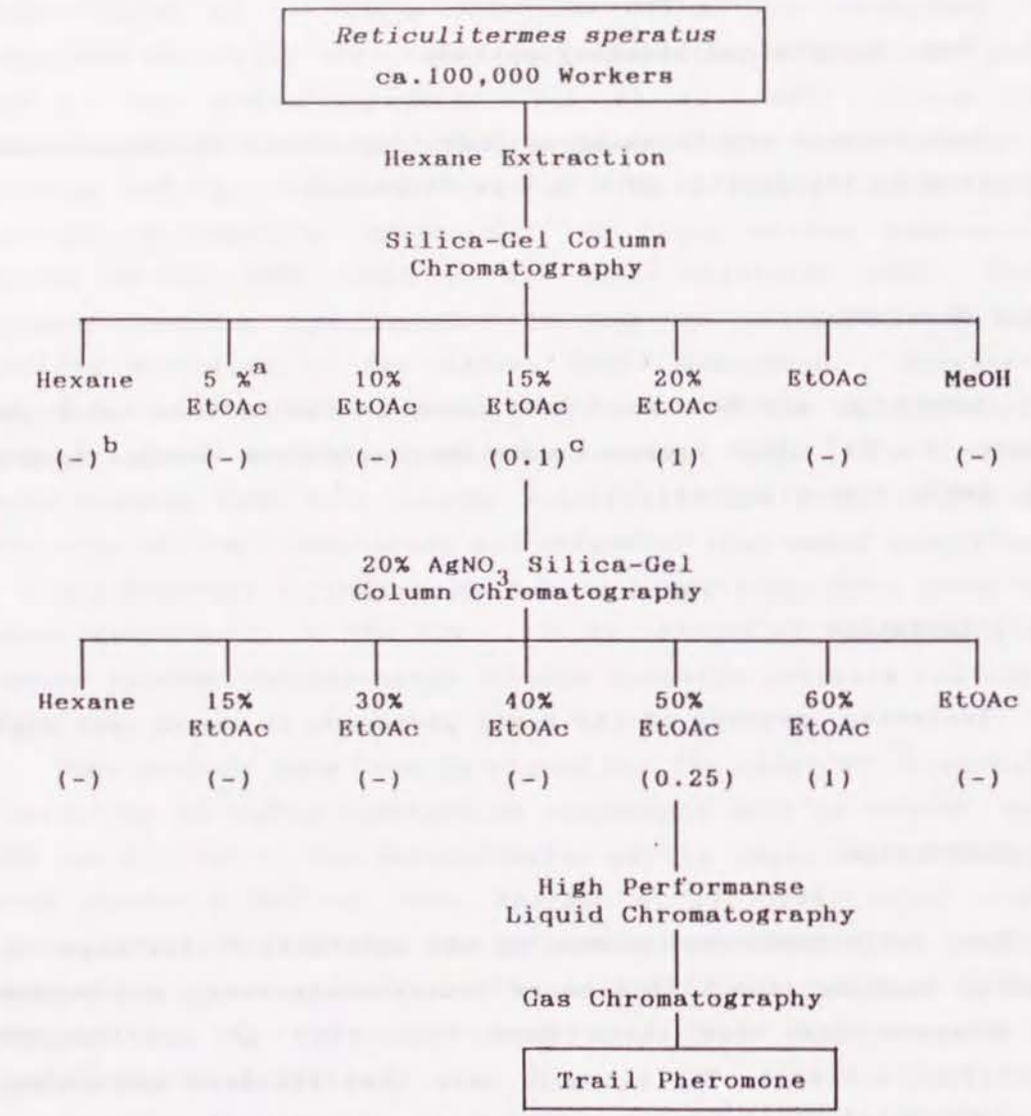


Fig. 3-1 Isolation process of the trail pheromone of *R. speratus*.
a: Percentage by volume of EtOAc / Hx (%) as eluants
b: No response or insufficient response for trail-following
c: Value means trail-following activity as minimum effective worker equivalent

(2) Silica-gel column chromatography

The whole body extracts were dried with anhydrous sodium sulfate for 1 hour, and concentrated by rotary vacuum evaporator until the yellowish crude lipid (*ca.* 3 g) was obtained. This crude lipid was first fractionated by silica-gel column chromatography with Hx / ethyl acetate (EtOAc) and methanol (MeOH) eluants. Silica-gel (200 g; Silica-gel 60, 70-230 mesh; NTI) was packed into a glass column (1 m x 5 cm i.d.) with the sample, and eluted with Hx/EtOAc, successively increasing the polarity, and with MeOH as shown in Fig. 3-1 and Table 3-1. Volume of eluants was one liter for each elution step, regardless of mixing ratios of Hx and EtOAc. It was finally separated into 7 fractions (22 subfractions).

(3) Argentation silica-gel column chromatography

The pooled active fractions were then fractionated by 20% (w/w) $AgNO_3$ silica-gel chromatography with Hx/EtOAc in the similar manner as above (20 cm x 7 mm i.d. glass column; $AgNO_3$ silica-gel 1.5 g). Volume of eluants was 6 ml for each elution step. The active fractions were finally separated into 7 fractions (22 subfractions).

(4) Normal phase high performance liquid chromatography (HPLC)

Active fractions were fractionated by HPLC with Hx/EtOAc (7/3) and detected by a UV detector at 234 nm. The HPLC was a Model LC-5A (Shimadzu, Kyoto, Japan) pump connected to a model UVIDEC-100I detector (Jasco, Tokyo, Japan). The analytical column was a normal phase Cosmosil 5SL (silica-gel, 25 cm x 4.6 mm i.d., particle size 5 μm; NTI) and guard column was a Cosmosil 10SL (5 cm x 4.6 cm i.d., silica-gel, particle size 10 μm; NTI). Flow rate of the eluent was 1.0 ml/min.

(5) Preparative gas chromatography (GC)

The final purification step was carried out by preparative GC using a nonpolar column, followed by a polar column. The preparative GC employed a YANACO Model G180PF (Yanaco, Kyoto, Japan) equipped with a flame ionization detector and a glass column (1.5 m x 3 mm i.d.) packed with 5% Carbowax 20M-W-HP (100/120 mesh) (polar column; Hewlett-Packard (HP), Pennsylvania, USA) or a wide bore capillary column (10 m x 0.53 mm i.d.) coated with methyl silicon (coating width 2.5 μm) (nonpolar column; HP). Analytical conditions for each column were as follows: nonpolar column, 140°C (injection 250°C), carrier gas N_2 (40 cm/sec); polar column, 180°C (injection 250°C), carrier gas N_2 (25 ml/min). In both analyses, the outlet gas for each peak was isolated and the trail-following activity in response to it was determined. To obtain GC fractions for analysis, a glass tube cooled with dry ice in the middle portion, was indirectly connected to the metal head of the FID detector with a Teflon tube. The glass tube was rapidly rinsed with diethyl ether to extract the effluent for the later evaluation of trail-following activity.

The isolation was repeated so that 4 μg of the trail pheromone was obtained to determine the complete structure of the pheromone.

3.2.4 Identification

(1) The analysis of primary structure of the isolated trail pheromone by capillary gas chromatography - mass spectrometry (GC-MS)

The active GC peak was analyzed by capillary gas chromatography-mass spectrometry (GC-MS). The GC-MS was performed

on a M-80B mass spectrometer to which a Model 0101 on-line data system (Hitachi, Naka, Ibaraki, Japan) was connected. The gas chromatograph was a Model 5970 (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a fused-silica capillary column (Shimadzu CBP 20M, 25 m x 0.25 mm i.d.). Ion-source temperature was 200°C, and the ionization voltage was 70 eV. The injection temperature was 250°C. Samples in 2 μl of hexane were injected with a Grob-type splitless injector. The oven temperature was kept at 80°C for the first minute and then raised at 20°C/min to 180°C. The carrier gas (helium) velocity was *ca.* 40 cm/sec.

Determination procedure of the geometric configuration of the isolated trail pheromone is shown in Fig. 3-2.

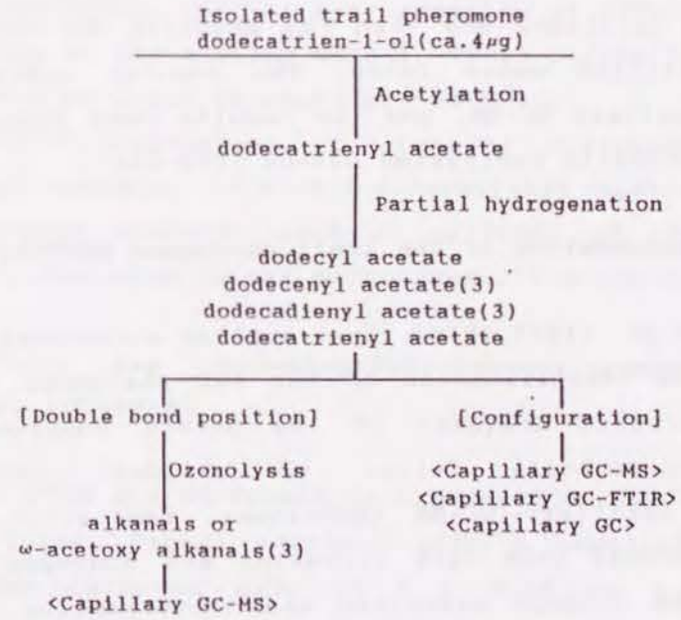


Fig. 3-2 Determination procedure of the geometric configuration of the isolated trail pheromone

(2) Acetylation of the trail pheromone

The isolated trail pheromone was first converted to the corresponding acetate. Approximately 5 μg of the purified trail pheromone was dissolved in 200 μl of benzene. Both 2 mg of acetic anhydride and 0.1 mg of pyridine were added to the solution, and the mixture was incubated at 60°C for three hours. Formation of the acetate was ascertained by analytical capillary GC. The analytical capillary gas chromatograph used was a Shimadzu Model 7A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a fused-silica capillary column described above. It was operated under the same conditions as for capillary GC-MS analysis, except that the temperature programming rate was 32°C/min. Two hundred microliter of benzene were added to the reacted solution, and then the solution was washed with 200 μl of distilled water twice. The reacted substance was analyzed by capillary GC-MS, and the results were compared with those of the authentic acetylated DTE-OH (DTE-OAc).

(3) Partial hydrogenation of the trail pheromone acetate

Yamaoka *et al.* (1976,1978) first applied micro-scale partial hydrogenation to identification of the sex pheromone [(*E,Z,Z*)-4,7,10-tridecatrienyl acetate] of the potato tuberworm moth, *Phthorimaea operculella* Zeller (Gelechiidae: Lepidoptera) combined with capillary GC-MS techniques. Hydrogenation of a carbon-carbon double bond with hydrazine and hydrogen peroxide usually proceeds without migration and isomerization and at a moderate rate (Aylward *et al.* 1962; Privett *et al.* 1966; Schofield *et al.* 1969). Theoretically, 2 mole each of hydrazine and hydrogen peroxide are needed. Practically, however, large excess of these reagents are required to accomplish the hydrogenation within an appropriate period. Preliminary trials using linolenyl acetate indicated that satisfactory yields of the

partially hydrogenated monoene and diene derivatives were obtained when the acetate (*ca.* 1 μg) in ethanol (200 μl) was treated with theoretical 2000- and 200-fold excess amounts of hydrazine and hydrogen peroxide respectively at 50-60°C for 4-5 hr. (Yamaoka *et al.* 1976).

About 4 μg of the trail pheromone acetate was partially hydrogenated under the following conditions. The stock solutions of hydrazine hydrate and hydrogen peroxide were respectively prepared by dissolving 30 μl of hydrazine hydrate or 4 μl of 30% hydrogen peroxide in 1 ml of ethyl alcohol. An 100 μl each of these stock solutions was added to a glass vial (9 cm x 15 mm i.d.) containing the trail pheromone acetate, and the mixture was incubated at 60°C for 4 hours. Distilled water was later added for quenching the reaction, and the mixture was thus extracted with *n*-hexane twice. Partial hydrogenation of DTE-OAc was carried out according to the similar manner. Those partially hydrogenated products were analyzed by capillary GC-MS.

Partial hydrogenation of reference compounds, (*Z,Z*)-3,6-dodecadienyl acetate, (*Z,E*)-6,8-dodecadienyl acetate and (*Z,Z*)-6,8-dodecadienyl acetate (each 10 μg)(Ando *et al.* 1985), were carried out according to the method described above.

(4) Capillary gas chromatography-Fourier-transform infrared spectroscopy (GC-FTIR)

The GC-FTIR was performed on a Model GCC-100GC/FT-IR (JEOL, Akishima, Tokyo, Japan) equipped with a capillary column (HP bonded phase Carbowax 20M, 25 m x 0.25 mm i.d.). A high-sensitivity MCT (mercury cadmium telluride) detector (DET-101) was used. The GC conditions were the same as for the GC-MS analysis.

(5) Ozonolysis of the partially hydrogenated trail pheromone acetate

A half aliquot of the partially hydrogenated substances was treated with ozone gas for several seconds so that alkanals and ω-acetoxy alkanals were obtained. Those ozonolysis products were also analyzed by capillary GC-MS to determine the positions of double bond in the trail pheromone.

(6) Determination of stereochemistry of each double bond of the trail pheromone

The stereochemistry of each double bond was determined by comparing retention time data of the partially hydrogenated trail pheromone acetate and reference acetates. Partially hydrogenated DTE-OAc and a mixture of (*E,Z*),(*Z,Z*) and (*E,E*)-6,8-dodecadienyl acetate were employed as reference compounds.

3.2.5 Capillary gas chromatography mass spectrometry High Resolution Selected Ion Monitoring (GC-MS-HR-SIM) analysis of the sternal gland extracts.

The extracted components from sternal glands and whole termite bodies were condensed by an evaporator and analyzed by Capillary GC-MS-HR-SIM. The GC-MS for HR-SIM was a Model 5890 GC (HP) combined with a Model JMS-DX303HF (JEOL Ltd., Tokyo, Japan) equipped with a Model JMA-DA5000 mass data system (JEOL). A fused-silica capillary column (CBP-20-S25-050, 25m x 0.33 mm i.d.; Shimadzu) was additionally used with this system. The ion source temperature was 180°C, and ionization voltage was 70 eV. Samples were injected with a splitless injector in 0.5 - 1 μl of EtOAc. The oven temperature increased at a rate of 30°C/min over the range of 120 - 210°C, and the injection temperature was

210°C. The resolution was approximately 5000. SIM monitors were set at *m/z* 180.151 and *m/z* 181.155, the former was precise molecular weight of DTE-OH and the latter was that of natural stable isotopes due to ^{13}C.

3.3 Results and Discussion

3.3.1 Isolation of the trail pheromone

(1) Results of bioassay with the crude hexane extracts

It is well known that sternal gland is the only secreting organ of the trail pheromone (Stuart 1961,1963,1969; Moore 1966; Howard *et al.* 1976; Smythe & Coppel 1966; Liang *et al.* 1979; Macfarlane *et al.* 1983). Although a trail pheromone should be isolated through extraction only from the sternal gland or its secretion (Tschinkel & Close 1973; McDowell & Oloo 1984; Smythe *et al.* 1966), it seems difficult to obtain a sufficient amount of the target components in a short time. Whole bodies of the insects were extracted by soaking in a solvent without homogenization in the present investigation.

Results of bioassay with the crude hexane extracts were already described in Chapter 1 (Table 1-1). The MEWE was determined as 0.1 WE/15cm. Its activity was comparable to that of sternal gland extracts. The corpses after extraction were homogenized with diethyl ether and served for the measurement of trail following activity. The activity was less than one-fiftieth of the primary hexane extracts.

Matsumura *et al.* (1968,1969) indicated that fungus-infected wood which was preferably ingested by termites because of the decayed wood contained the trail-following substance. Resultantly, it still remained unsolved whether the trail-following activity derived from the decayed wood which was

ingested into digestive organs of termites. However, the extracts of intestinal contents of test workers showed no trail following activity in the preliminary experiment this time.

Bromination and acetylation markedly lowered the trail-following activity of crude hexane extracts. These results suggested that the trail pheromone of the extracts might have double bonds and hydroxyl groups like as DTE-OH.

Table 3-1 Results of trail-following bioassay of the crude hexane extracts after silica-gel column chromatography

Test fraction			Quantity of lipid(mg)[a]	Activity as MEWB[b] (WE/15 cm-trail)
Crude hexane extracts			3000	0.1
Fr.No.	Eluants(volume(ml))			
1	Hx	1[c] (500)	132	-[d]
2		2 (500)	70	-
3	5 %[e]	1 (500)	2	-
4		2 (500)	912	-
5	10 %	1 (250)	407	-
6		2 (250)	33	-
7		3 (250)	12	-
8		4 (250)	11	-
9	15 %	1 (200)	4	-
10		2 (200)	11	1
11		3 (200)	51	0.1
12		4 (200)	54	1
13		5 (200)	80	5
14	20 %	1 (200)	76	1
15		2 (200)	80	10
16		3 (200)	97	-
17		4 (200)	84	-
18		5 (200)	68	-
19	EtOAc	1 (500)	283	-
20		2 (500)	122	-
21	MeOH	1 (500)	98	-
22		2 (500)	54	-

a: Weight of crude lipids.
b: The activity was estimated by a modified Open-Field bioassay. When three or more of nine workers followed the 15 cm trail, the sample was designated positive. Tenfold dilution series and more highly diluted solutions of each sample were employed to determine the MEWB (WE/15 cm-trail).
c: The number of subfraction.
d: No trail following response or insufficient response (*i.e.* the estimated value was more than 10WE/15cm-trail) for trail-following.
e: Percentage by volume of EtOAc/Hx (%) as eluants.

(2) Results of bioassay after silica-gel column chromatographies

The trail pheromone was eluted mainly in the range of 15 - 20% EtOAc/Hx on silica-gel column chromatography (Table 3-1, Fig. 3-1). Fractions with this property usually consist of alcohols, sterols, and fatty acids. This elution pattern was similar to that of earlier reports (Honda *et al.* 1975).

Table 3-2 Results of trail-following bioassay of the hydrolyzed products after $AgNO_3$ silica-gel column chromatography

Test fraction			Quantity of lipid(mg)[a]	Activity as MEWB[b] (WE/15 cm-trail)
Active fractions			62	0.1
Fr.No.	eluants(volume(ml))			
1	Hx	1[c] (3)	t[d]	-[e]
2		2 (3)	t	-
3	10 %[f]	1 (3)	t	-
4		2 (3)	t	-
5	20 %	1 (3)	t	-
6		2 (3)	21	-
7	30 %	1 (3)	16	-
8		2 (3)	14	-
9	40 %	1 (3)	8	-
10		2 (3)	6	-
11	50 %	1 (1.5)	t	-
12		2 (1.5)	1	1
13		3 (1.5)	t	0.5
14		4 (1.5)	t	1
15	60 %	1 (1.5)	t	1
16		2 (1.5)	t	1
17		3 (1.5)	t	10
18		4 (1.5)	t	-
19	100 %	1 (1.5)	t	-
20		2 (1.5)	t	-
21		3 (1.5)	t	-
22		4 (1.5)	t	-

a: Weight of crude lipids.
b: The activity was estimated by a modified Open-Field bioassay. When three or more of nine workers followed the 15 cm trail, the sample was designated positive. Tenfold dilution series and more highly diluted solutions of each sample were employed to determine the MEWB (WE/15 cm-trail).
c: The number of subfraction.
d: Trace amount.
e: No trail following response or insufficient response (*i.e.* the estimated value was more than 10WE/15cm-trail) for trail-following.
f: Percentage by volume of EtOAc/Hx (%) as eluants.

Based on the bioassay results of the trail pheromone following chromatography on 20% $AgNO_3$ silica-gel, an active substance was eluted mainly in the 50 - 60% EtOAc/Hx fractions (Table 3-2, Fig. 3-1).

(3) Results of HPLC preparation

The finding that the trail pheromone exhibited an absorption peak at 234 nm in UV spectroscopic analysis (Matsumura *et al.* 1969) would suggest the presence of conjugated double bonds at defined by UV detector at 234 nm in the present study. The trail pheromone showed high activity in a peak at the time of retention around 2.6 minute, confirming the presence of conjugated double bonds.

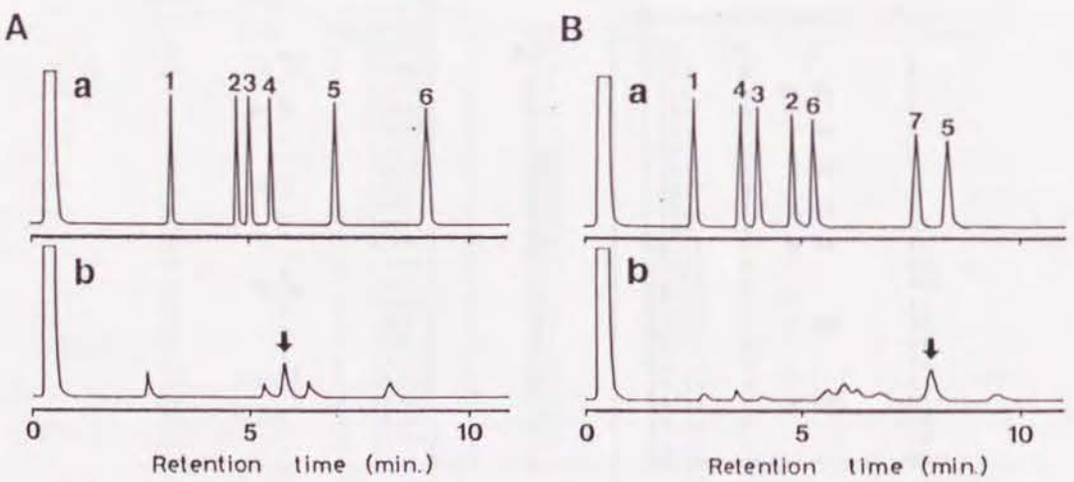


Fig. 3-3 Gas chromatograms using nonpolar column (A) and polar column (B)
a: Authentic alcohols [Retention times(min) in columns A and B]
1:$C_{11}H_{23}OH$(3.2, 2.5), 2:(*Z,Z*)-3,6-$C_{12}H_{21}OH$(4.7, 4.8),
3:(*Z*)-3-$C_{12}H_{23}OH$(5.0, 4.0), 4:$C_{12}H_{25}OH$(5.5, 3.6),
5:(*E,E*)-8.10-$C_{12}H_{21}OH$(7.0, 8.3), 6:$C_{13}H_{27}OH$(9.1, 5.3),
7:$C_{14}H_{29}OH$(7.6)
b: The isolated trail pheromone [Retention times(min) in columns A and B
Arrows indicate the active peak (5.8, 7.9)

(4) Results of preparative GC analyses

As shown in the results of GC analyses (Fig. 3-3), the retention time of the trail pheromone peak was 5.8 minutes for a non-polar column, nearly identical with that of 1-dodecanol, and 7.9 minutes for a polar column, slightly less than that of 1-tetradecanol. The two different GC analyses suggested that this pheromone could be a twelve-carbon alcohol with conjugated double bonds.

3.3.2 Identification of the trail pheromone

(1) The primary structure of the trail pheromone

Capillary GC-MS data are shown in Fig. 3-4. The total ion chromatogram (TIC) of the active fraction from HPLC gave the same pattern of the gas chromatogram. The electron impact (EI) mass spectrum of the trail pheromone showed the prominent molecular ion peak at *m/z* 180 ($M^{+\cdot}$; 35%). The result suggested that the series of characteristic fragment ion peaks at *m/z* 91 (100%), 105 (47%), 119 (26%), 133 (19%) might correspond to the increasing 14 mass units of the carbon skeleton of a trienyl alcohol following dehydration, which can be expressed as a general formula of C_nH_{2n-7}. These results thus showed that the substance had the typical pattern of a straight-chain primary alcohol with conjugated double bonds. The fact that alcohols with conjugated double bonds show prominent molecular ion peaks, while primary alcohols seldom show strong molecular ion peaks supports this conclusion. It was confirmed that the primary structure of the trail pheromone of *R. speratus* was straight-chained dodecatrien-1-ol containing conjugated double bonds. The amount of the trail pheromone per single worker termite was estimated as about 3 pg on the basis of external standard method of GC analysis.

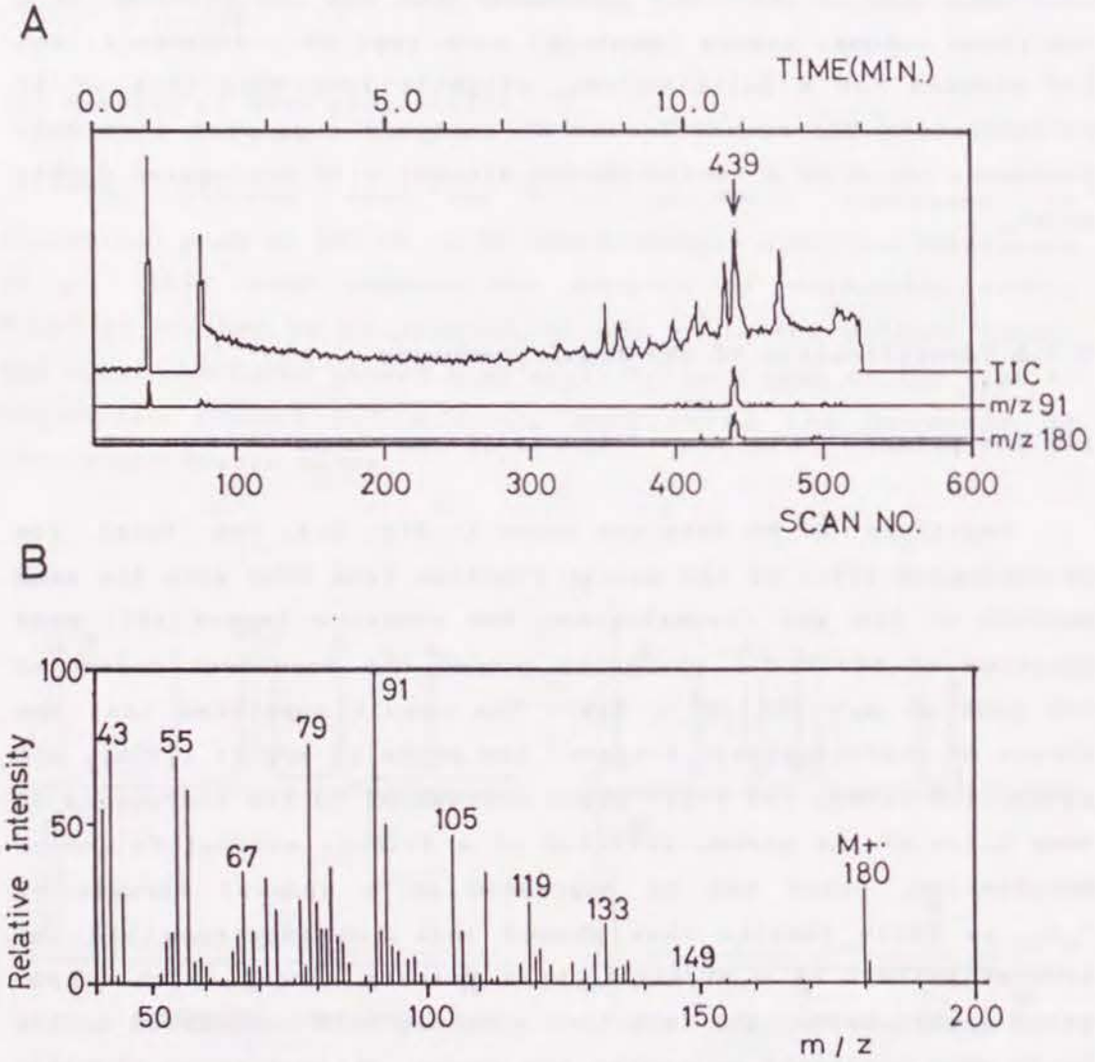


Fig. 3-4 Capillary GC-MS data of the isolated trail pheromone
A: Total ion chromatogram(TIC) and mass chromatograms(*m/z* 91, 180)
An arrow indicates the active peak(Scan No.439)
B: Mass spectrum of the active peak(Scan No.439)

(2) The complete chemical structure of the trail pheromone

(a) Capillary GC-MS analysis of the trail pheromone acetate

The EI mass spectrum of the acetylated pheromone shows a molecular ion at *m/z* 222 ($M^{+\cdot}$). It also showed a diagnostic ion at *m/z* 162 $(M-60)^{+\cdot}$ which came from McLafferty rearrangement, and the series of prominent characteristic fragment ions *i.e. m/z* 91, 105, 119, 133 were observed. These data indicate that the compound was corresponding to dodecatrienyl acetate with a conjugated double bond.

(b) Capillary GC-MS analysis of the partially hydrogenated trail pheromone acetate

Results of capillary GC-MS analysis of the partially hydrogenated products of the acetylated pheromone were shown Fig. 3-5. The total ion chromatogram of the products shows seven peaks: A (Scan No. 345), B (Scan No. 353), C+D (Scan No. 357), E (Scan No. 364), F (Scan No. 368), G (Scan No. 406) and H (Scan No. 420). The scan number and mass spectrum of peak A were identical with those of the reference dodecyl acetate. The mass spectrum shows diagnostic ions at *m/z* 168 $[(M-60)^{+\cdot}]$, 140 $[(M-60-28)^{+\cdot}]$ and 61 (CH_3COOH + H). The mass spectra of peaks B and C+D show ions at *m/z* 166 $[(M-60)^{+\cdot}]$ and 138 $[(M-88)^{+\cdot}]$ two mass units lower than those of dodecyl acetate. Thus, these peaks were considered to be due to dodecenyl acetates. The mass spectra of peaks E and F show a diagnostic ion at *m/z* 164 $[(M-60)^{+\cdot}]$ and other ions typical for dodecadienyl acetate without conjugated double bonds. The mass spectrum of peak G gives rise to important ions at *m/z* 164 (as for peaks E and F) and 224. The latter corresponds to the molecular ion of dodecadienyl acetate. Judging from the longer retention time of peak G on the polar column than those of peaks E and F, which exhibit the same ion at *m/z* 164

[$(M-60)^{+\cdot}$], peak G must have conjugated double bonds. Thus, it was considered to be due to dodecadienyl acetate with conjugated double bonds. The mass spectrum and scan number of peak H were identical with those of the original dodecatrienyl acetate.

The total ion chromatogram and mass chromatogram of the partially hydrogenated trail pheromone acetate are shown in Fig. 3-5. Mass chromatograms were recorded for the diagnostic ions, $(M-60)^{+\cdot}$ and/or $M^{+\cdot}$, of dodecyl acetate [$(M-60)^{+\cdot}$: *m/z* 168], dodecenyl acetate [$(M-60)^{+\cdot}$: *m/z* 166], dodecadienyl acetate [$(M-60)^{+\cdot}$: *m/z* 164 and $M^{+\cdot}$: *m/z* 224] and dodecatrienyl acetate [$(M-60)^{+\cdot}$: *m/z* 162 and $M^{+\cdot}$: *m/z* 222], respectively. These mass chromatograms also indicated that peaks B and C+D were monounsaturated. Partial hydrogenation of a triunsaturated compound should produce three monounsaturated isomers. Judging from the half-height width, peak C+D consists of two dodecenyl acetates. Peaks E, F and G represent dodecadienyl acetates of which G has conjugated double bonds.

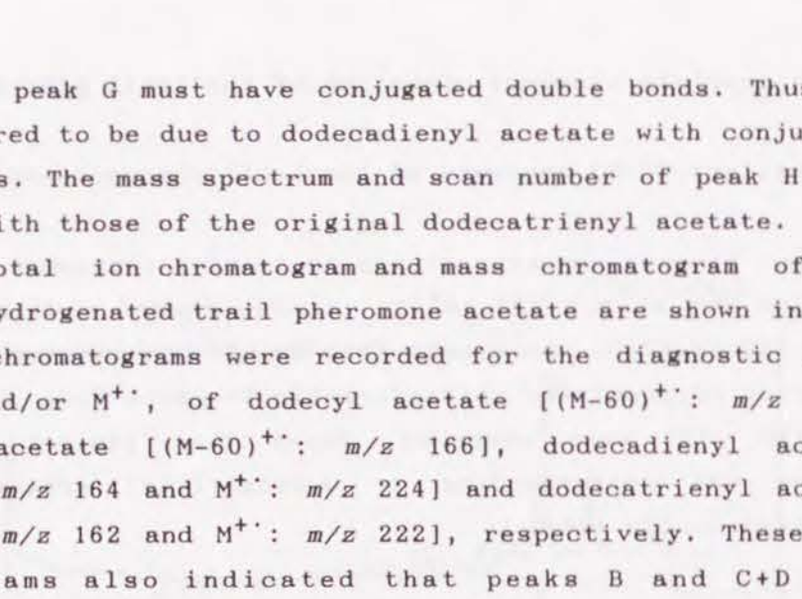


Fig. 3-5 Total ion chromatogram(TIC) and mass chromatograms of the partially hydrogenated trail pheromone acetate
A: $C_{12}H_{25}OAc$, B and C+D: $C_{12}H_{23}OAc$,
E, F and G: $C_{12}H_{21}OAc$, H: $C_{12}H_{19}OAc$

(c) Capillary GC-MS analysis of the partially hydrogenated trail pheromone acetate after ozonolysis

The total ion chromatogram of the ozonolysis products of the partially hydrogenated trail pheromone acetate showed major four peaks "W","X","Y" and "Z" (Scan No. 292, 149, 277, 397 respectively ; Fig. 3-6). Component of the peak "W" was identified as unreacted dodecyl acetate by the retention time and mass spectrum. Components of the peaks "X","Y","Z" were identified to 1-nonanal, 6-acetoxy hexanal and 8-acetoxy octanal by their retention times and mass spectra. Thus, the double bond positions of the trail pheromone were determined as 3, 6 and 8.

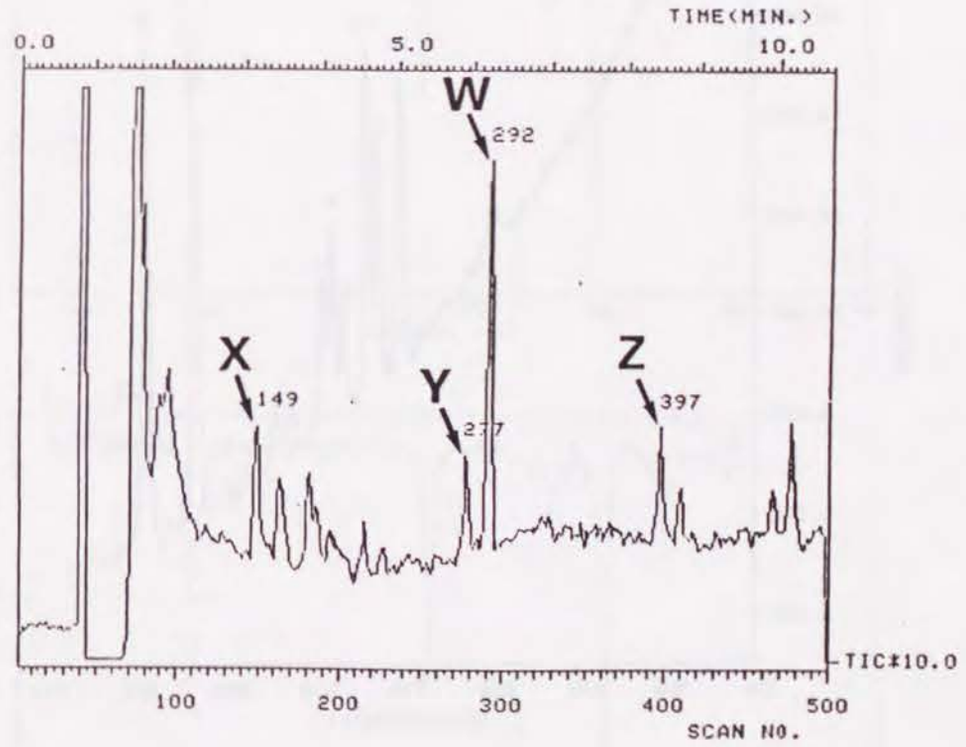


Fig. 3-6 Total ion chromatogram(TIC) of the ozonolysis products of the partially hydrogenated trail pheromone acetate
W: Dodecyl acetate(Scan No.292), X: 1-Nonanal(Scan No.149),
Y: 6-Acetoxy hexanal(Scan No.277), Z: 8-Acetoxy octanal(Scan No.397)

(d) Capillary GC-FTIR analysis of the partially hydrogenated trail pheromone acetate

Half of the partially hydrogenated trail pheromone acetate was analyzed by capillary GC-FTIR with a MCT detector. The total absorbance monitoring (TAM) of GC-FTIR showed eight peaks (Fig. 3-7). The IR spectrum of the vapor phase of all the peaks, except peak I, showed absorptions typical of acetate derivatives of primary alcohols, *i.e.*, C-H stretching around 2900 cm^{-1}, C=O stretching at 1762 cm^{-1}, C-O-R stretching at 1234 cm^{-1} (Wilmshust, 1957). The IR spectrum of peak I suggests an hydrocarbon, which may be derived from solvent impurity.

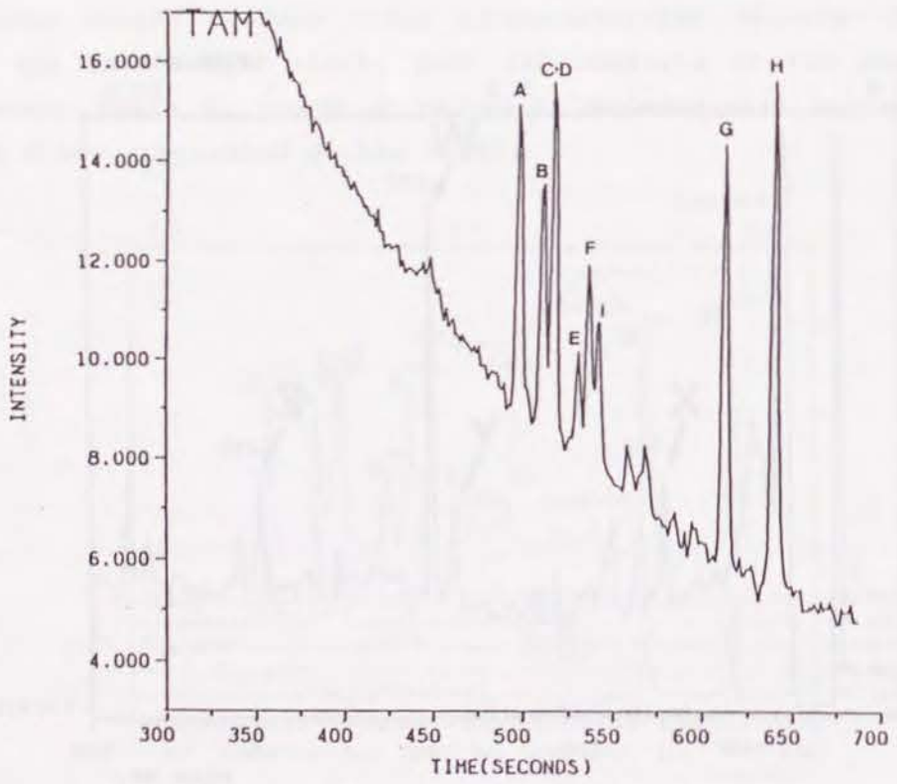
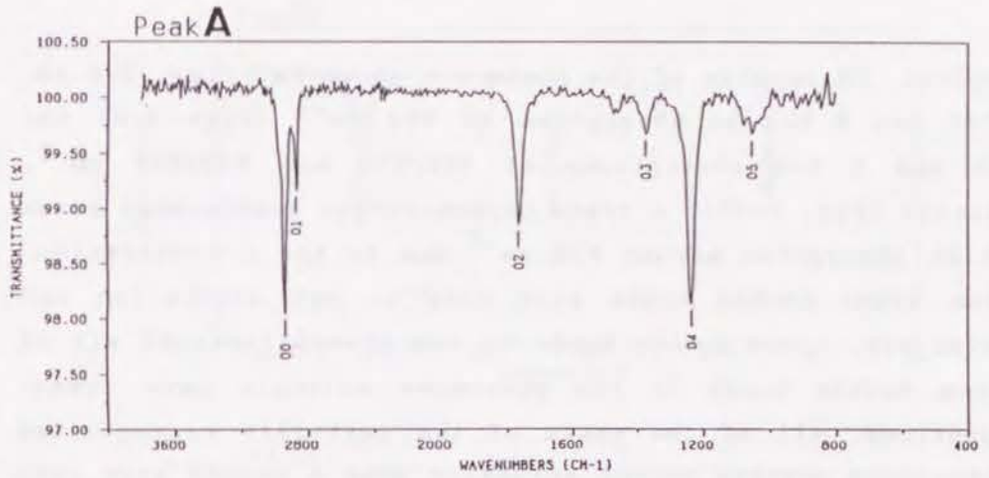
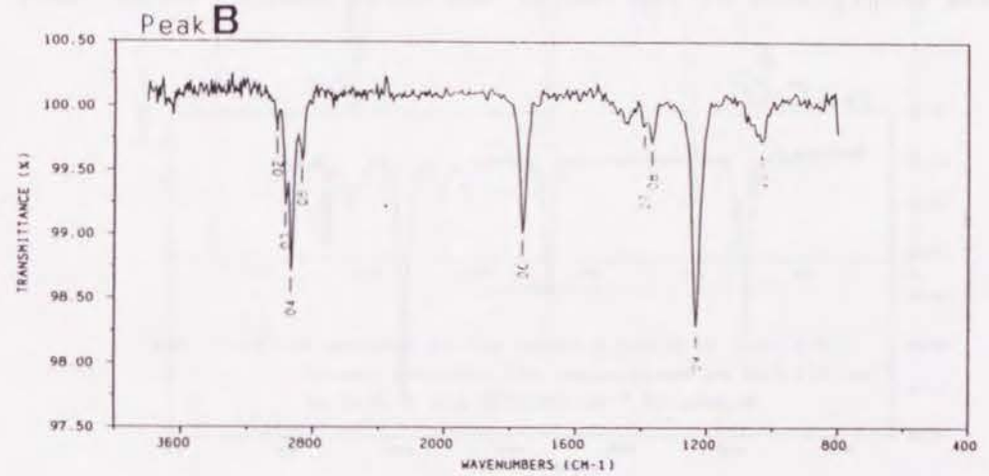


Fig. 3-7 Total absorbance monitoring (TAM) of the partially hydrogenated trail pheromone acetate





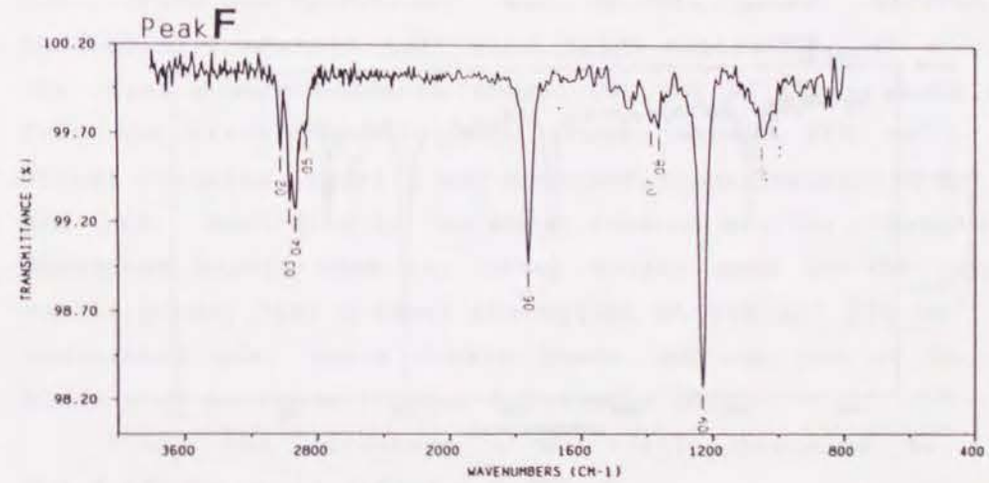


Fig. 3-8 Infrared(IR) spectra of the peaks A, B and F in Fig. 3-7

Typical IR spectra of the peaks are shown in Fig. 3-8~10. Peaks C+D and E had an absorption at 960 cm^{-1} (Fig. 3-9) and peaks G and H two absorptions at 949/976 and 949/980 cm^{-1}, respectively (Fig. 3-10). A *trans* carbon-carbon double bond gives rise to an absorption around 970 cm^{-1} due to the C-H vibration. Isolation *trans* double bonds give rise to one absorption and conjugated *cis, trans* double bonds to two absorptions. If all of the three double bonds in the pheromone molecule have *trans*-configurations, all of the peaks of the partially hydrogenated trail pheromone acetate except saturated peak A should give rise to *trans* absorption. If two out of the three double bonds have

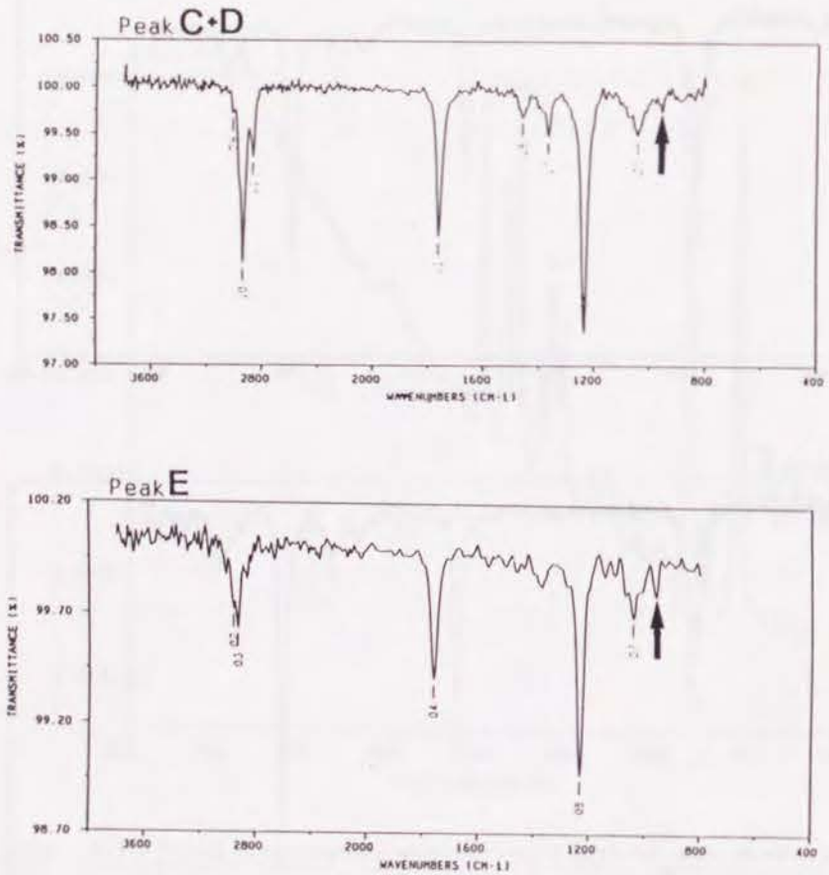


Fig. 3-9 IR spectra of the peaks C+D and E in Fig. 3-7
Arrows indicate the absorption at 960 cm^{-1}

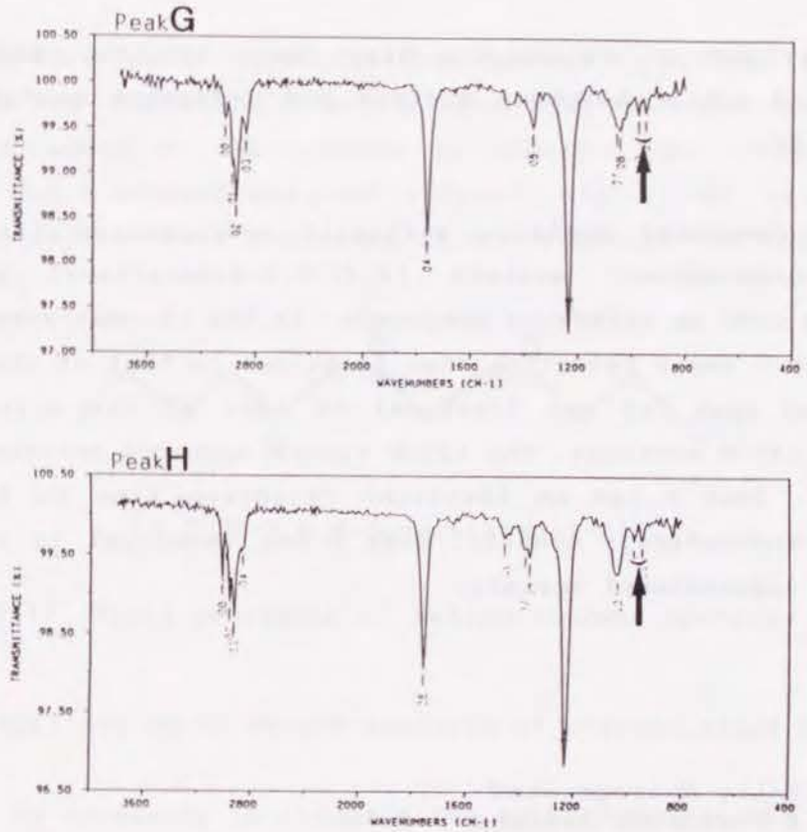


Fig. 3-10 IR spectra of the peaks G and H in Fig. 3-7
Arrows indicate the absorptions at 949/976 cm^{-1}
in peak G and 949/980 cm^{-1} in peak H

the *trans*-configuration, all of the peaks derived from dodecadienyl acetate must show *trans* absorption. If one out of the three double bonds is *trans*, two out of three peaks derived from the dienyl acetate must absorb around 970 cm^{-1}. Of the dienyl acetates, peaks E and G showed *trans* absorption but peak F did not. According to the above reasons as, the termite trail pheromone should have one *trans* double bond in the conjugated double bonds. Peak G shows absorption at 949 and 976 cm^{-1} due to conjugated *cis, trans* double bonds and one out of two double bonds must be *trans* (Clemer & Solomons 1953).

Thus, the structure of the trail pheromone is (*Z,E,Z*)-3,6,8-DTE-OH or (*Z,Z,E*)-3,6,8-DTE-OH.

(e) Comparison of retention time data of the partially hydrogenated trail pheromone acetate and reference acetates in capillary GC.

(*Z*)-3-dodecenyl acetate, partially hydrogenated (*Z*)-3, (*Z,E*)-6,8-dodecadienyl acetate (*Z,Z*)-6,8-dodecadienyl acetate (1:2) were used as reference compounds. In the chromatogram (Fig. 3-11), peak B had a retention time identical to that of the (*Z*)-3 acetate and peak C+D one identical to that of the mixture of (*Z*)-6 and (*E*)-8 acetates. The (*Z*)-8 isomer appeared between peaks C+D and E. Peak F had an identical retention time to that of (*Z,Z*)-3,6-dodecadienyl acetate, peak G one identical to that of (*Z,E*)-6,8-dodecadienyl acetate.

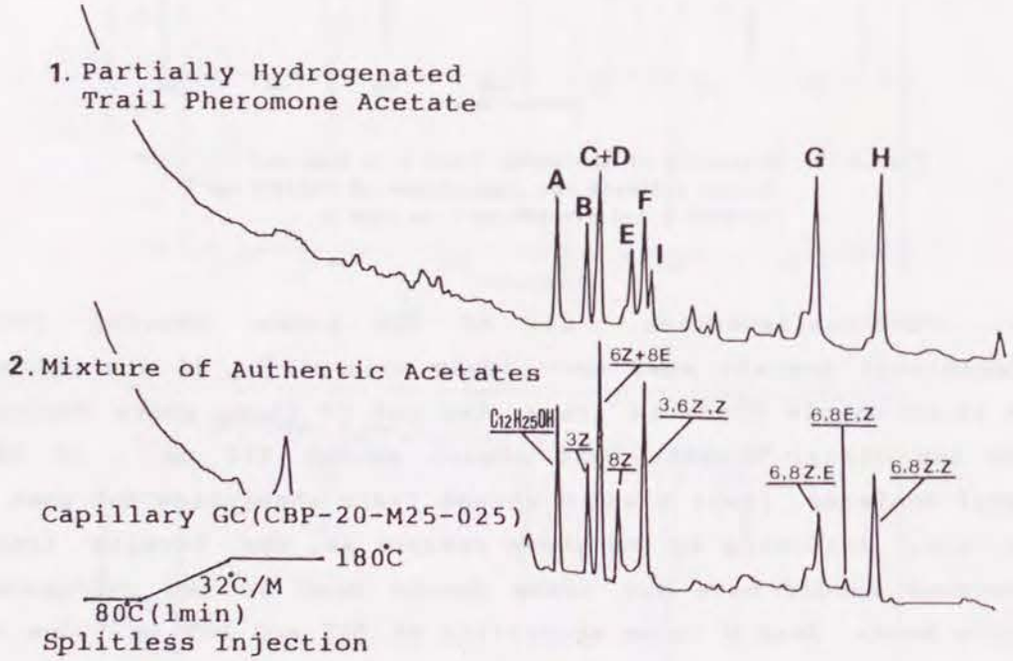
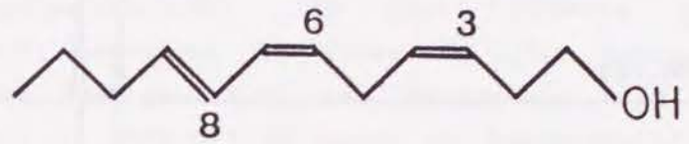


Fig. 3-11 Comparison of retention data of the partially hydrogenated trail pheromone acetate and reference acetates in capillary GC.

All the data supported that the configuration of the 8 position was *trans* and 3 and 6 were *cis*, and proved that the trail pheromone of the termite *R. speratus* was identical with (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (DTE-OH; Fig. 3-12) as the same compound of the trail pheromone of *R. virginicus* by Matsumura *et al.*(1968).



(*Z,Z,E*)-3,6,8-dodecatrien-1-ol

Fig. 3-12 Trail pheromone of *Reticulitermes speratus* (Kolbe)

3.3.3 Capillary GC-MS-HR-SIM analysis of sternal gland extract

It is necessary to evidence the coincidence in the chemical structure of the active substances from both whole body extracts and sternal gland extracts. Otherwise, it can not be concluded whether the trail pheromone is true trail pheromone of *R. speratus* or not. Since the molecular weight of the trail pheromone is known theoretically, it is possible to detect the target component high-sensitively and high-selectively by means of capillary GC-MS-HR-SIM analysis.

Results of GC-MS-HR-SIM analyses shown in Fig. 3-13 demonstrated that the extracted components from sternal glands and whole termite bodies could be detected as sharp peaks of HR-SIM at *m/z* 180.151 and *m/z* 181.155, and that both of them could be high-selectively isolated from the impurities. The retention times of the both components were the same as that of authentic DTE-OH. The component of the sternal gland extracts exactly corresponded to that of the whole body extracts, which was identical with DTE-OH.

On the basis of the present results of GC-MS-HR-SIM analysis, it was concluded that the isolated substance from whole body was definitely the true trail pheromone of *R. speratus*.

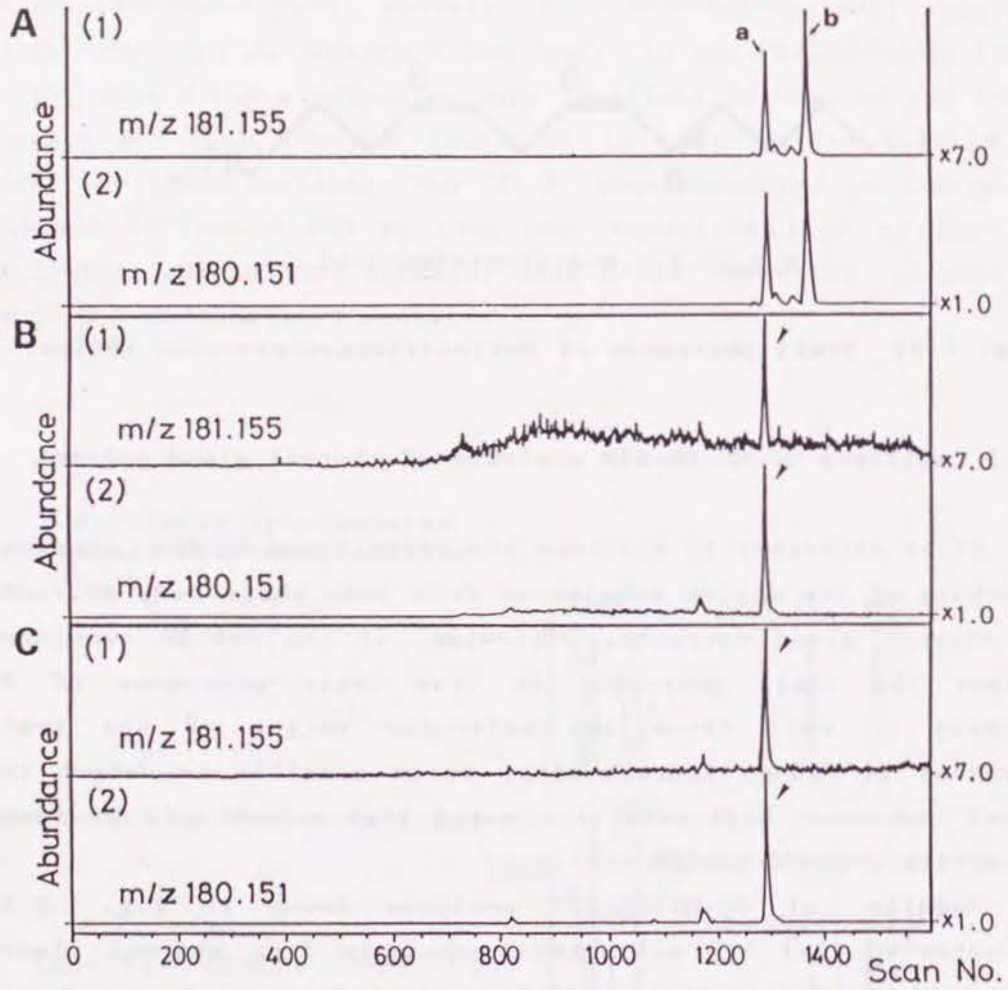


Fig. 3-13 Capillary GC-MS-HR-SIM data of the sternal gland extracts.
A: Authentic dodecatrienol isomers
a:(*Z,Z,E*)-DTE-OH; d:(*Z,E,E*)-DTE-OH
B: Whole body extracts
C: Sternal gland extracts
(1): HR-SIM chromatogram at m/z 181.155
(2): HR-SIM chromatogram at m/z 180.151
Arrow heads indicate the (*Z,Z,E*)-DTE-OH peaks

3.4 Summary

Approximately 100,000 workers of the termite, *R. speratus* (Kolbe) were extracted with *n*-hexane to isolate trail pheromone. The extract was purified by silica-gel column chromatography, argentation silica-gel column chromatography, normal phase HPLC and gas chromatography. Its trail-following activity was coincidentally examined by bioassays. The complete chemical structure of the pheromone was determined as (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (DTE-OH) by means of instrumental analyses in conjunction with several micro-chemical reactions. Sternal gland extracts also contained DTE-OH, when analyzed by capillary gas chromatography mass spectrometry high resolution selected ion monitoring (GC-MS-HR-SIM).

Chapter 4 : Isolation and identification of the Trail Pheromone of the termite, *Coptotermes formosanus* Shiraki

4.1. Introduction

The trail pheromone of the termite, *Reticulitermes speratus* (Kolbe) (Rhinotermitidae) was isolated and identified as (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (DTE-OH) which has exactly the same structure as that from *R. virginicus* (Matsumura *et al.* 1968,1969), as described in Chapter 3.

In this experiment, *C. formosanus* was served for the isolation of the trail pheromone and the determination of its complete chemical structure with a special reference to species specificity of the pheromone within the family Rhinotermitidae (Tokoro *et al.* 1989; 1992a).

4.2 Materials and Methods

Except for the termite species tested, bioassay methods and chemicals were the same as described in the sections of 3.2.1 and 2, as of this part.

4.2.1. Isolation of the trail pheromone

(1) Extraction

The test termites were soaked in *n*-hexane (*ca.* 300 ml per 20,000 individual termites) for three days without homogenization. The hexane extracts were obtained by filtration and stored at -20°C until the next step. These manipulations were repeated twenty times to obtain extracts from approximately 561g workers.

(2) Silica-gel column chromatography

The extracts combined with *n*-hexane were concentrated by a rotary vacuum evaporator until the yellowish crude oil was obtained (*ca.* 51 g). This crude oil was first divided into two parts, and each part was separately fractionated by silica-gel column chromatography with Hx/EtOAc eluants. Five hundred grams of silica-gel (70-230 mesh) was packed into glass column (1 m x 6 cm i.d.), with the halved crude oil placed on the top of the silica-gel. The column was eluted with Hx/EtOAc combination, successively increasing the polarity, and with methyl alcohol. Volume of eluants was three liters for each elution step, regardless of mixing ratios of *n*-hexane and EtOAc. It was finally separated into 21 fractions (one liter each).

(3) Argentation silica-gel column chromatography

The pooled active fractions were concentrated to obtain the crude material (*ca.* 350 mg), and then fractionated into 9 subfractions (45 ml each) by 20% (w/w) $AgNO_3$ silica-gel column chromatography with Hx/EtOAc in the similar manner described above ($AgNO_3$ silica-gel: *ca.* 7g ; glass column: 30 cm x 1 cm i.d.).

(4) Normal phase HPLC

The active fractions subdivided were further fractionated by normal phase HPLC with 10% EtOAc/Hx, and flow rate was 1 ml/min. The active substance was detected by UV detector at 234 nm and fractionated at each peak for bioassay. The HPLC was Model LC-6A (Shimadzu) pump connected with Model UVIDEC-100II detector (Jasco, Tokyo, Japan). The analytical column and guard column used were the same as described in the section of 3.2.4.(4) in Chapter 3.

(5) Preparative capillary GC

The next step of the purification was carried out by GC with a nonpolar column and consecutively by a polar column. Gas chromatography (GC) employed a Shimadzu-GC Model 15A (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a splitless injector and fused silica capillary columns : Shimadzu HiCap series CBP1-S25-050 (25 m x 0.33 mm i.d.: for analytical column), CBP1-W12-300 (12 m x 0.53 mm i.d.: for preparative column) <non polar columns, chemically bonded with methyl silicone>, CBP20-S25-050 (25 m x 0.33 mm i.d.: for analytical column), CBP20-W12-300 (12 m x 0.53 mm i.d.: for preparative column) <polar columns, chemically bonded with polyethylene glycol>. The conditions were as follows: on the nonpolar column, oven temperature increased at a rate of 20°C/min over the range of 80-300°C; injection temperature at 250°C and detector temperature at 350°C; carrier gas, helium at a velocity of approximately 40 cm/sec: on the polar column, oven temperature increased at a rate of 20°C/min over the range of 80-180°C; injection temperature at 250°C and detector temperature at 350°C; carrier gas, helium at a velocity of approximately 40 cm/sec. In both analyses using preparative wide bore capillary columns, the outlet gas was fractionated for each peak and the trail following activity was determined in the similar manner of Chapter 3.

4.2.2 Identification

(1) Capillary GC-MS analysis of the isolated trail pheromone

Finally, the active peak characterized by GC was analyzed by capillary GC-MS. The capillary GC-MS apparatus and the conditions were the same as described in the sections 3.2.4.(1) of chapter 3.

(2) Acetylation of the trail pheromone

The isolated trail pheromone was first converted to the corresponding acetate in the similar manner of Chapter 3. Approximately 10 μg of the purified trail pheromone was dissolved in 400 μl of benzene. Both 4 mg of acetic anhydride and 0.2 mg of pyridine were added to the solution, and the mixture was incubated at 80°C for three hours. Formation of the acetate was ascertained by capillary GC. The reacted solution was washed with 1N-HCl once and then with distilled water twice. The reacted substance was analyzed by capillary GC-MS, and the results were compared with those of the authentic acetylated DTE-OH (DTE-OAc).

(3) Partial hydrogenation of the trail pheromone acetate

About 8 μg of the trail pheromone acetate was partially hydrogenated, in the similar manner of Chapter 3, under the following conditions. The stock solutions of hydrazine hydrate and hydrogen peroxide were respectively prepared by dissolving 90 μl of hydrazine hydrate or 5 μl of 30% hydrogen peroxide in 1 ml of ethyl alcohol. An 100 μl each of these stock solutions was added to a glass vial containing the trail pheromone acetate, and the mixture was incubated at 60°C for four hours. Distilled water was later added for quenching the reaction, and the mixture was thus extracted with *n*-hexane twice. Partial hydrogenation of DTE-OAc was carried out according to the similar manner. Those partially hydrogenated products were analyzed by capillary GC-MS.

(4) Ozonolysis of the partially hydrogenated trail pheromone acetate

A half aliquot of the partially hydrogenated substances was ozonized with ozone gas, and those ozonolysis products were also analyzed by capillary GC-MS to determine the positions of double

bond in the trail pheromone, in the similar manner of Chapter 3.

(5) Determination of stereochemistry of each double bond of the trail pheromone

The stereochemistry of each double bond was determined by comparing retention time data of the partially hydrogenated trail pheromone acetate and reference acetates, in the similar manner of Chapter 3.

4.2.3 Capillary gas chromatography mass spectrometry high resolution selected ion monitoring (GC-MS-HR-SIM) analysis of the sternal gland extracts.

The dissected abdominal fifth-sternites and the whole termite bodies were soaked in 1 ml of *n*-hexane for ten hours respectively. The whole body extract were simply purified by flash silica-gel column chromatography. Both of the extracts were condensed by rotary vacuum evaporator and analyzed by capillary GC-MS-HR-SIM. The capillary GC-MS apparatus was a Model 5890 gas chromatograph (HP) combined with a Model M-80B mass spectrometer, which equipped with a model 0101 on-line data system (Hitachi, Ibaraki, Japan). A fused-silica WCOT capillary column (25 m x 0.25 mm i.d., liquid phase PEG-HT; GL Science Ltd. Tokyo, Japan) was used with this system.The analytical conditions were as follows. The ion source temperature was 180°C, and ionization energy was 70 eV. Samples were injected with a Grob type splitless injector in 1 μl of *n*-hexane. The oven temperature increased at a rate of 30°C/min over the range of 60 - 210°C, and the injection temperature was 210°C. The resolution was approximately 6000, and SIM monitor was set at *m/z* 180.1513, which was precise molecular weight of DTE-OH.

4.3. Results and Discussion

4.3.1 Isolation of the trail pheromone

Trail-following activities estimated by bioassay are shown in Fig. 4-1 along with the isolation procedures of the trail pheromone from *C. formosanus*.

(1) Results of bioassay with the crude hexane extracts

In this investigation all the termites employed were once fed by filter paper as described in the Tables 1-2,3 in Chapter 1, because it was requested to ascertain that the initial hexane extract did not contain any intestinal substance which is involved in trail-following behavior other than a true trail pheromone (Tschinkel & Close 1973). Detected trail-following activity definitely did not derive from the decayed wood ingested by termites as indicated by previous investigators (Matsumura *et al.* 1968,1969; Matsuo & Nishimoto 1974; Grace & Wilcox 1988).

Qualitative analysis of functional groups indicated that the trail following activity of crude hexane extracts was remarkably lowered by bromination and by acetylation. It suggested that the substance might have double bonds and hydroxyl groups as similar to the pheromone of *R. speratus* (DTE-OH).

(2) Results of bioassay after silica-gel column chromatographies

Based on the results of the silica-gel column chromatography, the trail pheromone was eluted mainly in the range of 15-20% EtOAc/Hx (Fig. 4-1). Fractions with this property usually consist of alcohols, sterols, and fatty acids. The fact that the trail pheromone of *R. speratus* is a kind of alcohol (DTE-OH), appears to support that the pheromone could be a similar alcohol compound.

Results of bioassay after 20% $AgNO_3$ silica-gel chromatography are shown in Fig. 4-1. Separation of compounds under these conditions depends on the number of double bonds and the geometrical configuration of isomers. This pheromone was mainly eluted in fractions of 40-60% of EtOAc/Hx.

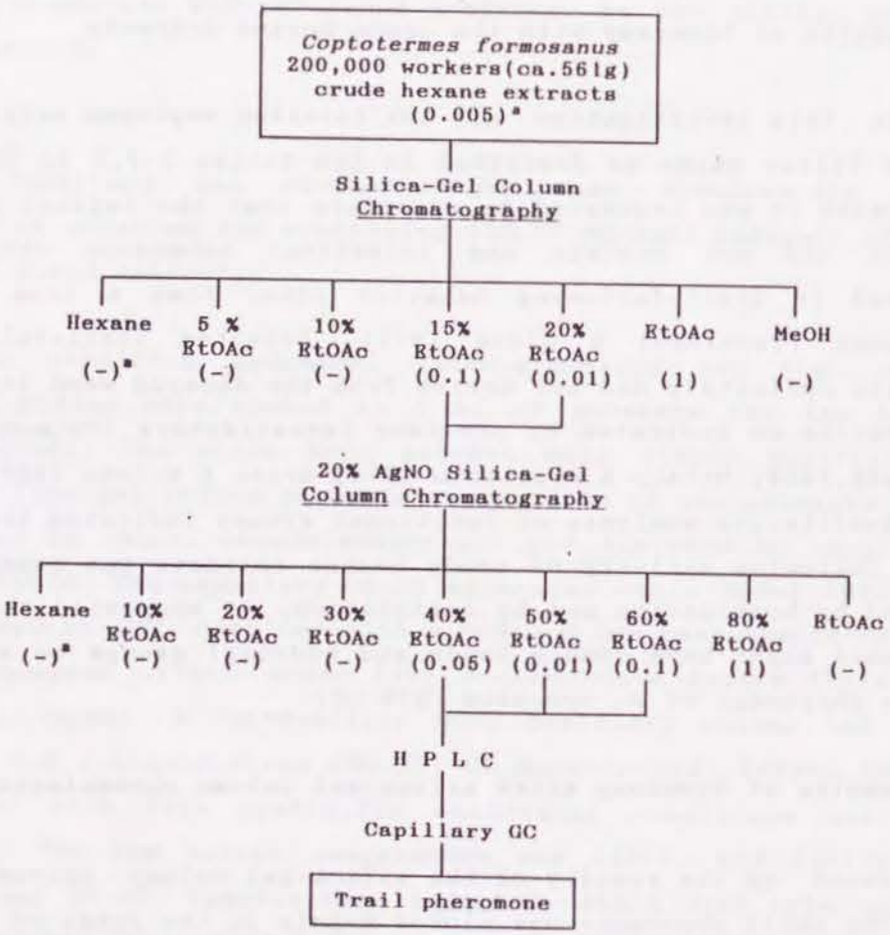


Fig. 4-1 Isolation process and trail-following activity of the trail pheromone of *C. formosanus*
a: Activity estimated by modified Open-Field bioassay (figures in parentheses mean minimum effective worker equivalent(MEWE) per 15cm-trail and "-" means > MEWE 10)

(3) Results of HPLC preparation

To indicate the presence of conjugated double bonds, the UV detector of the HPLC was set at 234 nm. This pheromone showed high activity in a peak at the retention time of *ca.* 14.5 min, confirming the presence of conjugated double bonds (Fig. 4-2).

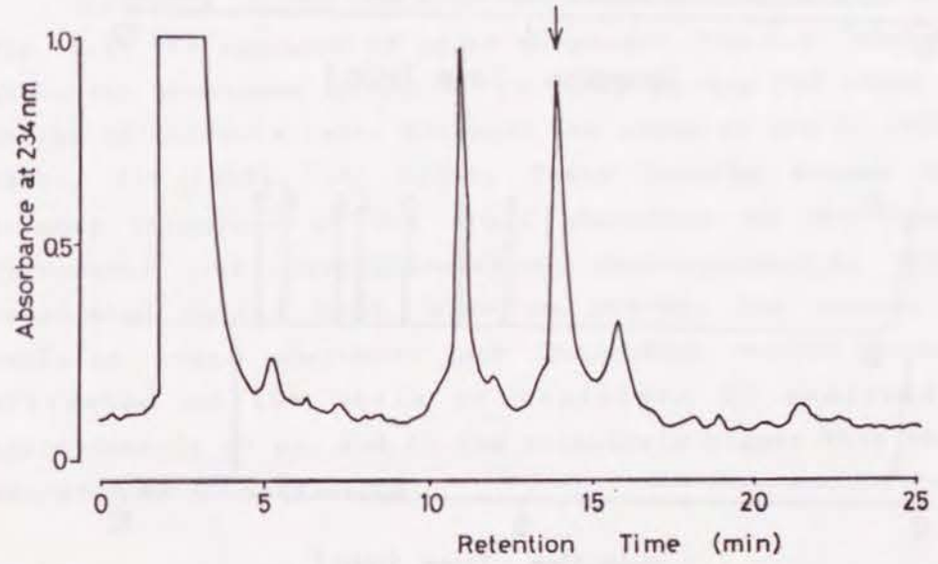


Fig. 4-2 HPLC chromatogram of the active fractions after fractionation by $AgNO_3$ silica-gel chromatography
An arrow indicates the active peak(tR. 14.5)

(4) Results of GC analyses

Results of GC are shown in Fig. 4-3. At first, on using a non-polar column, the retention time of the pheromone was *ca.* 6.46 min, which was nearly identical with that of 1-dodecanol.

Secondly, on using a polar column, the retention time was *ca.* 8.48 min, slightly more than that of 1-tetradecanol.

The two different GC analyses suggested that this pheromone could be a twelve-carbons alcohol with conjugated double bonds.

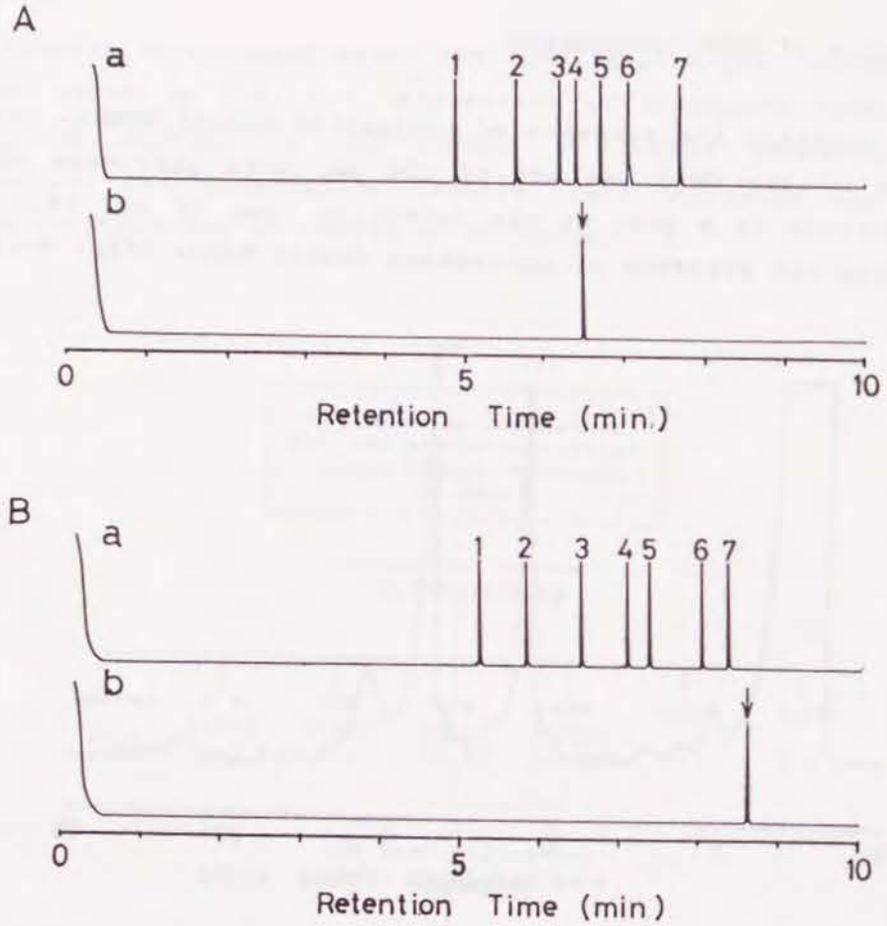


Fig. 4-3 Gas chromatograms of the trail pheromone from *C. formosanus*
A: Nonpolar column
a: Authentic alcohols[retention times(min)]
1:$C_{10}H_{21}OH$(4.9), 2:$C_{11}H_{23}OH$(5.7), 3:(*Z,Z*)-3,6-$C_{12}H_{21}OH$(6.2),
4:$C_{12}H_{25}OH$(6.4), 5:(*Z,E*)-6,8-$C_{12}H_{21}OH$(6.7), 6:$C_{13}H_{27}OH$(7.0),
7:$C_{14}H_{29}OH$(7.7)
b: The trail pheromone of *C. formosanus*.
An arrow indicates the active peak(tR. 6.5)
B: Polar column
a: Authentic alcohols[retention time(min)]
1:$C_{10}H_{21}OH$(5.3), 2:$C_{11}H_{23}OH$(5.8), 3:$C_{12}H_{25}OH$(6.5),
4:(*Z,Z*)-3,6-$C_{12}H_{21}OH$(7.1), 5:$C_{13}H_{27}OH$(7.4),
6:(*Z,E*)-6,8-$C_{12}H_{21}OH$(8.1), 7:$C_{14}H_{29}OH$(8.4)
b: Trail pheromone of *C. formosanus*,
An arrow indicates the active peak(tR. 8.5)

4.3.2 Identification of the trail pheromone

(1) The primary structure of the trail pheromone

(a) Major component

Capillary GC-MS data of the trail pheromone are shown in Fig. 4-4. The spectrum of major component (Fig 4-4; Scan No. 333) shows the prominent molecular ion peak at *m/z* 180 (30%) and the series of characteristic fragment ion peaks at *m/z* 91 (100%), 105 (58%), 119 (29%), 133 (13%). These results showed that the primary structure of the trail pheromone of the termite *C. formosanus* was straight-chained dodecatrien-1-ol containing conjugated double bond, same as DTE-OH. The amount of the isolated trail pheromone per individual worker termite was estimated on the basis of capillary GC analysis to be approximately 50 pg, and it was relatively bigger than that of *R. speratus* or *R. virginicus*.

(b) Minor component

In addition, I also isolated a minor component which showed the trail-following activity (Fig. 4-4; Scan No. 346). The mass spectrum of the component also showed a prominent molecular ion at *m/z* 180 and the series of diagnostic ions at *m/z* 91, 105, 119, 133 (C_nH_{2n-7}). These results indicated that the component was a isomer of DTE-OH. It was possible that the minor component was a species specific sub factor of the trail pheromone of *C. formosanus*. Amount of the minor substance per individual worker termite was to be approximately 5 pg from external standard technique of GC analysis. Further stereochemical analysis was described Chapter 5.

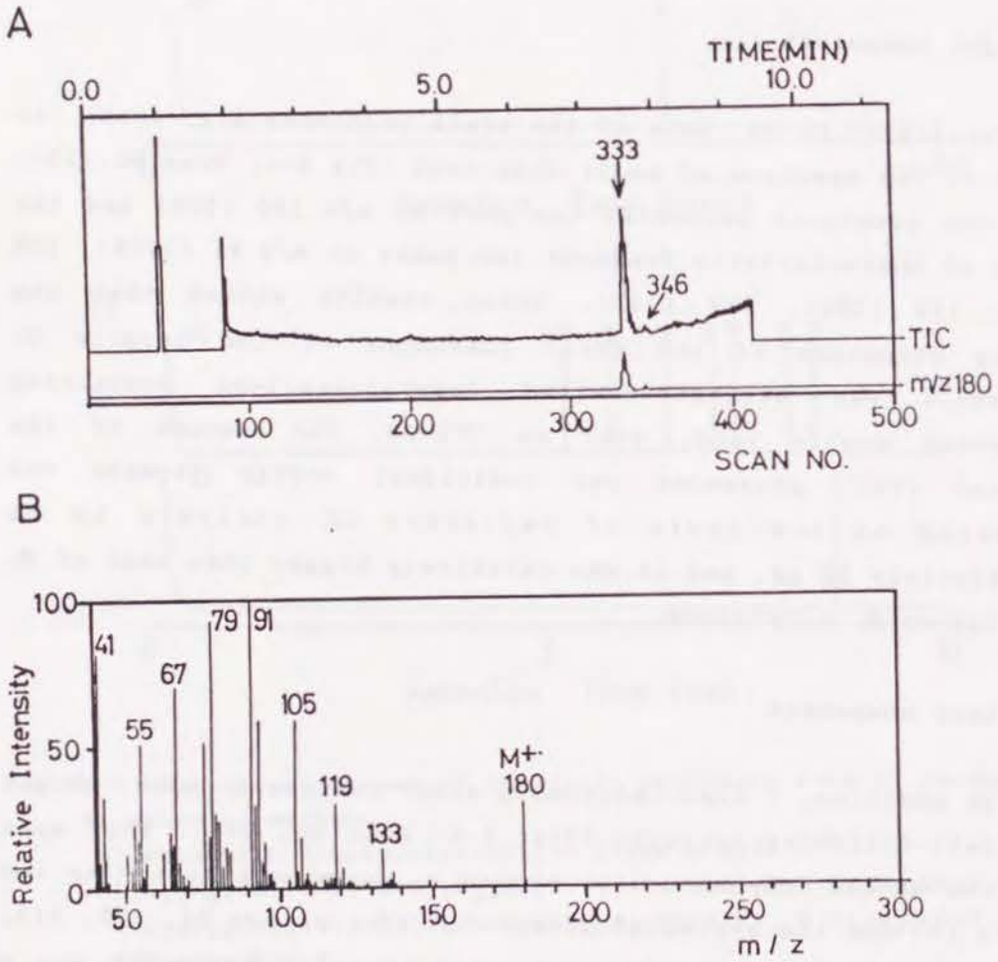


Fig. 4-4 GC-MS data of the isolated trail pheromone from *C. formosanus*.
A: Total ion chromatogram and mass chromatograms.
Arrows indicate the active peaks(Scan Nos.333 and 346)
B: Mass spectrum of the trail pheromone(Scan No.333).

(2) The detailed structure of the trail pheromone (major component).

(a) Capillary GC-MS analysis of the trail pheromone acetate

The electron impacted (EI) mass spectrum of the acetylated pheromone is shown in Fig. 4-5. The EI mass spectrum showed a molecular ion at *m/z* 222 ($M^{+\cdot}$; 9.2%) and base peak ion at *m/z* 43 ($CH_3C{\equiv}O^+$; 100%). It also showed diagnostic peak ion of the pheromone acetate at *m/z* 162 ($M\text{-}60^{+\cdot}$; 11.0%) which came from McLafferty rearrangement, and the prominent diagnostic fragment ions of the pheromone skeleton, *i.e.* *m/z* 91 (56%), 105 (27%), 119 (18%), 133 (25%) were also observed. The present analysis indicated that the substance was corresponded to dodecatrienyl acetate with a conjugated double bond.

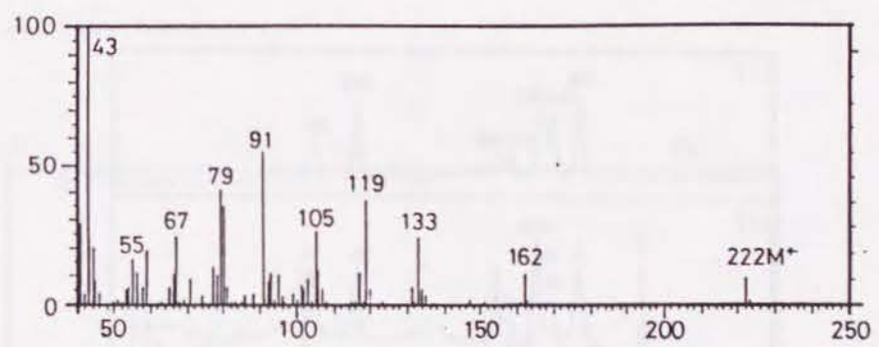


Fig. 4-5 Mass spectrum of the trail pheromone acetate

(b) Capillary GC-MS analysis of the partially hydrogenated trail pheromone acetate

The total ion chromatogram (TIC) and the mass chromatograms (MC) of the partially hydrogenated products were well corresponded to that of authentic DTE-OH, as shown in Fig. 4-6. The TIC showed seven peaks marked as "A","B","C","E","F","G" and

"H". Component of the peak "A" (Scan No. 339) showed the diagnostic peak of mass chromatogram at *m/z* 168 [$(M-60)^{+\cdot}$]. It was identical with dodecyl acetate. Components of the peaks "B" (Scan No. 344) and "C" (Scan No. 347) showed the diagnostic peaks of MC at *m/z* 166 [$(M-60)^{+\cdot}$]. Those were identical with dodecenyl acetates. Half height width of peak C suggested that it consisted of two monoenic compounds ("C" and "D"). Components of the peaks "E" (Scan No. 353) and "F" (Scan No. 355) showed the diagnostic peaks of MC at *m/z* 164 [$(M-60)^{+\cdot}$]. Those were identical with dodecadienyl acetates. Component of the peak "G" (Scan No. 382) showed the diagnostic peak of MC at *m/z* 224 ($M^{+\cdot}$), and also showed the peak of MC at *m/z* 164 [$(M-60)^{+\cdot}$]. It was identical with the conjugated dodecadienyl acetate. The mass spectrum and scan number (Scan No. 391) of the peak "H" were identical with those of the original dodecatrienyl acetate, and also showed the diagnostic peaks of MC at *m/z* 222 ($M^{+\cdot}$) and *m/z* 162 [$(M-60)^{+\cdot}$].

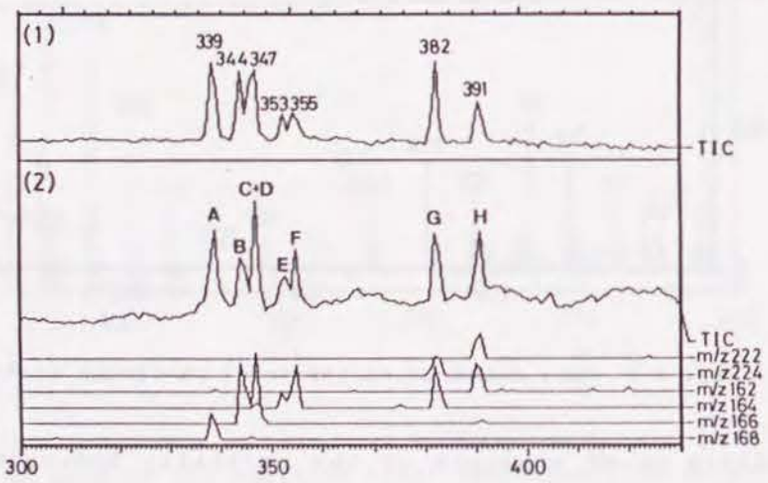


Fig. 4-6 Total ion chromatograms(TIC) and mass chromatograms(MC) of the partially hydrogenated trail pheromone acetates
(1): TIC of the partially hydrogenated authentic DTE-OAc
A(Scan No.339) = dodecanoic acetate
B(Scan No.344) and C+D(Scan No.347) = dodecenoic acetate,
E(Scan No.353) and F(Scan No.355) = dodecadienyl acetate,
G(Scan No.382) = conjugated dodecadienyl acetate,
H(Scan No.391) = dodecatrienyl acetate
(2): TIC and MC of the partially hydrogenated trail pheromone acetate.

(c) Capillary GC-MS analysis of the partially hydrogenated trail pheromone acetate after ozonolysis.

The TIC of the ozonolysis products of the partially hydrogenated trail pheromone acetate revealed prominent four peaks "W","X","Y" and "Z" (Fig. 4-7). Component of the peak "W" (Scan No. 340) was identified as unreacted dodecyl acetate on the basis of the retention time and mass spectrum.

The mass spectrum of the peak "X" (Scan No. 193) showed the diagnostic fragment ions at *m/z* 124 [$(M-H_2O)^{+\cdot}$], 114 [$(M-CH_2=CH_2)^{+\cdot}$], 99 [$(M-43)^{+}$], 98 [$(M-44)^{+\cdot}$], 70 [$(M-44-28)^{+\cdot}$], 44 (CH_2=CH-OH), 43 (CH_2=CH-O·). The mass spectrum and the scan number were identical with 1 -nonanal.

The mass spectrum of the peak "Y" (Scan No. 327) showed the diagnostic fragment ions at *m/z* 115 [$(M-43)^{+}$], 98 [$(M-60)^{+\cdot}$], 87 [$(M-43-28)^{+}$], 61 (CH_3COOH + H), 55 [$(M-60-43)^{+}$], 43 ($CH_3C{\equiv}O^{+}$ and CH_2=CH-O·). The mass spectrum and the scan number were identical with 6-acetoxy hexanal.

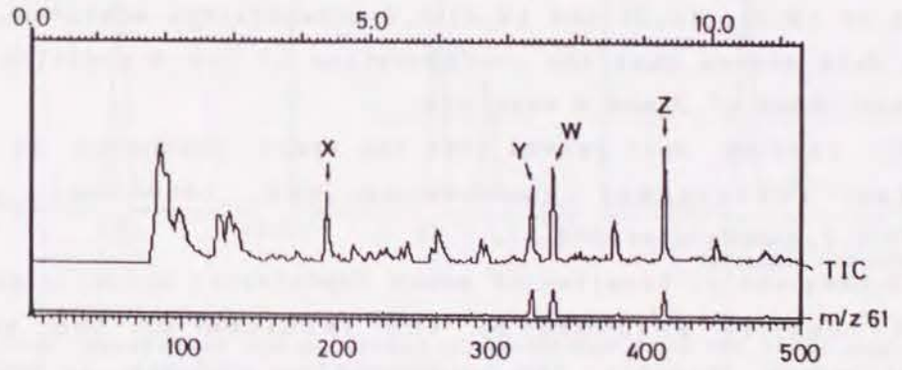


Fig. 4-7 Total ion chromatogram (TIC) and mass chromatogram (*m/z* 61) of the ozonolysis products of the partially hydrogenated trail pheromone acetate
W(Scan No.340) = dodecanoic acetate
X(Scan No.193) = 1-nonanal
Y(Scan No.327) = 6-acetoxy hexanal
Z(Scan No.412) = 8-acetoxy octanal

The mass spectrum of the peak "Z" (Scan No. 412) showed the diagnostic fragment ions at *m/z* 129, 115, 101, 87, 73 [(M-43-$(CH_2)_n)^+$], 98 [$(M-60-28)^{+\cdot}$], 83 [$(M-60-43)^+$], 61 (CH_3COOH + H), 43 ($CH_3C{\equiv}O^+$ and CH_2=CH-O·). The mass spectrum and the scan number were identical with 8-acetoxy octanal.

Those data agreed with that of previous experiment on sex pheromone of the potato tuberworm moth, *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae; Yamaoka 1978). Those data indicated that the positions of double bond in the trail pheromone were determined as *3, 6* and *8*, which were the same as those of *R. virginicus* and *R. speratus*.

(d) Comparison of retention time data of the partially hydrogenated trail pheromone acetate and reference acetates in capillary GC.

Retention time data of partially hydrogenated trail pheromone acetate exactly corresponded with that of partially hydrogenated DTE-OAc, and was not identical with that of the mixture of (*E,Z*), (*Z,Z*) and (*E,E*)-6,8-dodecadienyl acetates. All of the data showed that the configuration of the *8* position was *trans* and those of *3* and *6* were *cis*.

The results well proved that the trail pheromone of the termite, *Coptotermes formosanus* was identical with (*Z,Z,E*)-3,6,8-dodecatrien-1-ol.

Subsequently, termites of genus *Coptotermes* actually secrete DTE-OH that is produced by the termites of the genus *Reticulitermes*. Possibly, the interspecific confusion is brought about if the same trail pheromone works among different termite species. However, it is inferred that such confusion dose not happen normally in the natural environment, since the termites oppose to each other when they meet in the natural habitat. Termites may distinguish members of the same colony from others by detecting other agents such as surface hydrocarbon (wax)

instead of a trail pheromone. Mechanism of discrimination is worthy of further investigation so as to appreciate "wisdom" of termites, which has been created possibly as the result of the evolution of these kinds of animals.

4.3.3 Capillary GC-MS-HR-SIM analysis of sternal gland extracts

Results of capillary GC-MS-HR-SIM analyses demonstrated that the extracted components from sternal glands and whole bodies could be detected as a peak of HR-SIM at *m/z* 180.1513 and that those component could be high-selectively isolated from the impurities (Fig. 4-8).

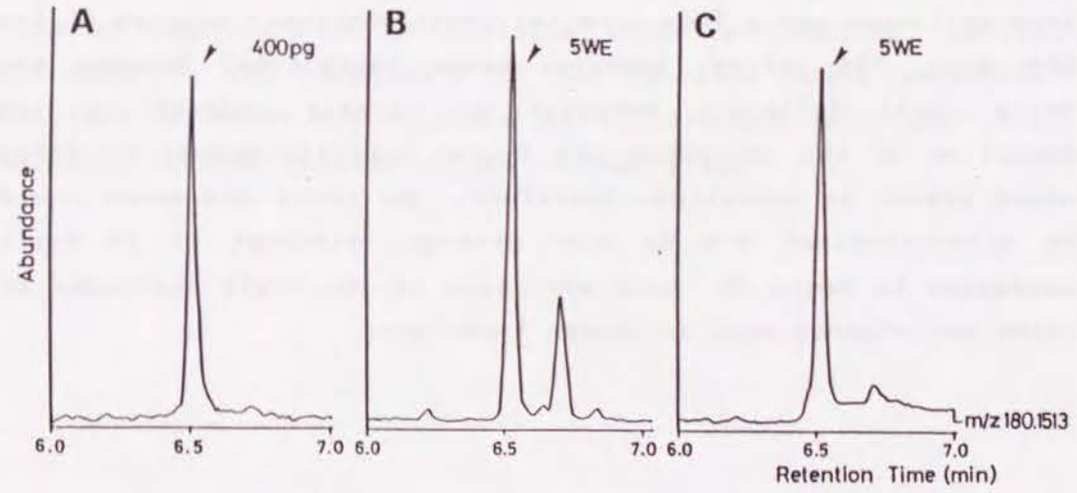


Fig. 4-8 Results of the capillary GC-MS-HR-SIM (*m/z* 180.1513) analyses
A: Authentic DTE-OH (400pg)
B: Whole body extracts (5 workers equivalent)
C: Sternal gland extracts (5 workers equivalent)
Arrow heads indicate the (*Z,Z,E*)-DTE-OH peaks

The components from sternal glands and whole termite bodies were absolutely identical with DTE-OH in the capillary GC-MS-HR-SIM analysis. The amount of the pheromone extracted from 100 whole bodies was estimated to be approximately 90 pg per a worker termite, it was relatively more than the isolated pheromone from 200,000 whole bodies. The weight difference might be due to the loss during the process of the latter's purification. On the basis of the present results of capillary GC-MS-HR-SIM analyses, we concluded that the isolated and identified trail pheromone was the true trail pheromone of *C. formosanus*.

As demonstrated here, the trail pheromone was certainly isolated from termite workers fed only with moistened filter paper which was pure cellulose for a year or longer. There are two possibilities to account for the fact : *de novo* synthesis from cellulose and a long term (at least one year) storage of the pheromone. The latter, however, seems impractical because the daily trail following behavior is closely related to the secretion of the pheromone and stored quantity sooner or later would result in depletion. Therefore, the trail pheromone could be biosynthesized *via de novo* pathway, although it is still necessary to prove *de novo* synthesis of the trail pheromone by other methodology such as tracer technique.

4.4 Summary

The trail pheromone was extracted from the whole bodies of *C. formosanus* Shiraki (*ca.* 200,000 in number), and purified by silica-gel column chromatography, argentation silica-gel column chromatography, high performance liquid chromatography (HPLC) and preparative capillary gas chromatography (GC). The trail-following activity of each fraction obtained by chromatography was examined by bioassay. The active GC peak was analyzed by capillary gas chromatography-mass spectrometry (GC-MS) to determine the primary structure. The primary structure of the pheromone was determined as normal chain dodecatrien-1-ol by capillary GC-MS analysis. Moreover, the complete structure of the trail pheromone was found to be (*Z,Z,E*)-3,6,8-dodecatrien-1-ol by means of capillary GC-MS analysis after acetylation, partial hydrogenation and ozonolysis of the isolated trail pheromone. Capillary GC-MS-HR-SIM analysis also confirmed that the trail pheromone exactly existed in the sternal gland.

Chapter 5 : Presence of a Minor Component of the Trail Pheromone from *Coptotermes formosanus* Shiraki

5.1 Introduction

Based on the results of the investigations on the trail pheromones from *R. speratus* and *C. formosanus* (Chapter 3 and 4), the pheromones proved common to both termite species. The chemical structure of the pheromone was exactly identical with (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (DTE-OH) which was previously obtained from *R. virginicus* (Matsumura *et al.* 1968,1969; Tai *et al.* 1969).

Moreover, in the course of the isolation and identification of trail pheromone from *C. formosanus*, a small amount of an isolated material which was not identical with DTE-OH chemically but showed the trail-following activity was obtained as described in Chapter 4. The mass spectrum of capillary GC-MS analysis for the material was diagnostic pattern of isomer of DTE-OH. It, therefore, seemed possible that the material was a rather minor component of the trail pheromone that was specific to *C. formosanus*.

The main purpose of this experiment was to identify the component and to determine whether the component is a minor component of trail pheromone of *C. formosanus* or not by means of new analytical technique. In addition, the species specificity of the minor component was examined in choice bioassay using the two termite species (Tokoro *et al.* 1992a; to be published).

5.2 Materials and Methods

Test termites, chemicals, apparatus, bioassay methods (Y-test), methods of isolation and capillary GC-MS analysis were the same as already described in chapters 1, 2, and 4.

5.2.1 Partial hydrogenation of the minor component

The minor component and authentic DTE-OH isomer were partially hydrogenated with hydrazine hydrate and hydrogen peroxide in a similar manner as mentioned in Chapter 3. The obtained products were analyzed by capillary GC-MS, and the results were compared with each other.

5.2.2 Authenticity of the minor component by capillary GC-MS-HR-SIM analysis.

Three analytical samples were prepared as follows:
sample (1) - Extract of 500 whole body workers was purified by silica-gel pipette flash column chromatography with EtOAc/Hx eluants by stages after soaking in *n*-hexane for 10 hours, and then condensed prior to filling up to 30 μl with *n*-hexane;
sample (2) - Extracting 50 whole body workers with diethyl ether for 5 minutes, the solvent was evaporated with nitrogen gas. The remaining extract was filled up to 5 μl with *n*-hexane;
sample (3) - After extracting one whole body worker with *n*-hexane for 40 minutes, the solvent was evaporated with nitrogen gas. The remaining extract was filled up to 1 μl with *n*-hexane.

The above three samples were prepared for both termite species. One μl each of the sample was served for analysis.

5.2.3 Interspecific Y-test for crude sternal gland extract and the authentic alcohol.

In order to determine the species specificity of trail pheromone, interspecific Y-tests were conducted. Preparation of sternal gland extract and authentic DTE-OH solutions and the Y-test method were described in Chapters 1 and 2, respectively.

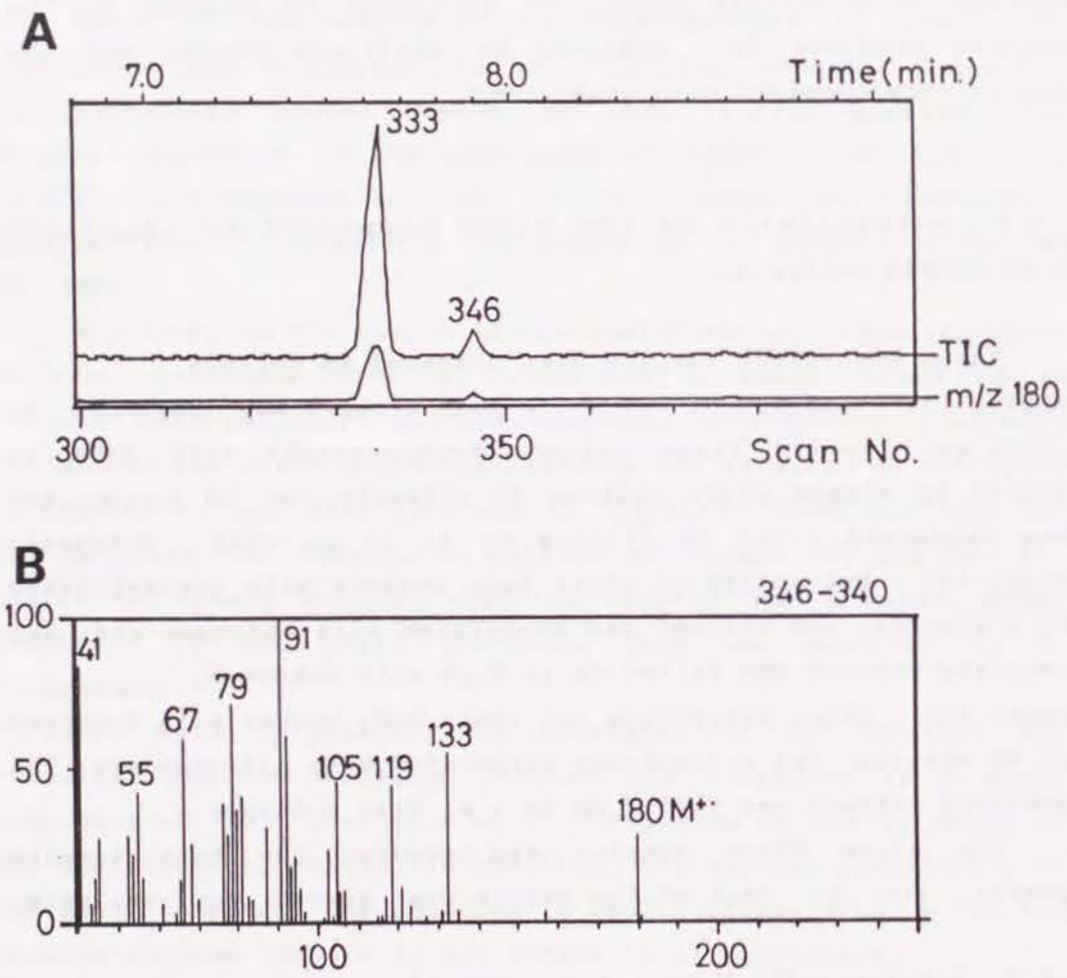


Fig. 5-1 Capillary GC-MS data of the trail pheromone and the minor component isolated from *Coptotermes formosanus*
A: Total ion chromatogram and mass chromatogram(*m/z* 180) of the trail pheromone(Scan No.333) and the minor component(Scan No.346)
B: Mass spectrum of the minor component(Scan No.346)

5.3 Results and Discussion

5.3.1 Chemical elucidation of the minor component

(1)Comparison of capillary GC-MS data between the minor component and isomer of authentic DTE-OH.

The electron impacted (EI) mass spectra of the minor component and authentic DTE-OH were shown in Fig. 5-1. The isolated minor component (Scan No. 346) showed the trail-following activity, and its mass spectrum also showed a prominent molecular ion at *m/z* 180 ($M^{+\cdot}$; 29%) and the series of diagnostic ions at *m/z* 91 (100%), 105 (46%), 119 (45%), 133 (55%) (C_nH_{2n-7}). These results indicate that the component was an isomer of DTE-OH. It was possible that the minor component was species specific in terms of the trail pheromone of *C. formosanus* as suggested for a sex pheromone of gelechiid moths in Roelofs *et al.* (1969). The mass spectrum and retention time were the same as an authentic DTE-OH isomer (*Z,E,E*)-3,6,8-dodecatrien-1-ol [hereinafter referred to (*Z,E,E*)-DTE-OH]. Amount of the minor substance per an individual worker termite was to be approximately 5 pg on the basis of GC analysis.

(2) Results of the partial hydrogenation of the minor component.

In order to ascertain the identity of the minor component with the authentic (*Z,E,E*)-DTE-OH, partially hydrogenated products were compared with each other in capillary GC-MS analysis. Results of partial hydrogenation of each component were shown in Fig. 5-2. Both products showed seven peaks marked as "A","B","C","E","F","G" and "H", and each peak was identified as A = *n*-dodecanol ; B = (*Z*)-3-dodecen-1-ol ; C = (*E*)-6-dodecen-1-ol and (*E*)-8-dodecen-1-ol ; D = (*Z,E*)-3,8-dodecadien-1-ol ; E = (*Z,E*)-3,6-dodecadien-1-ol ; F = (*E,E*)-6,8-dodecadien-1-ol and G =

(*Z,E,E*)-DTE-OH. Retention time data of the partially hydrogenated minor component exactly corresponded with that of partially hydrogenated (*Z,E,E*)-DTE-OH. Therefore, it was concluded that the minor component was identical with (*Z,E,E*)-DTE-OH (Fig.5-3).

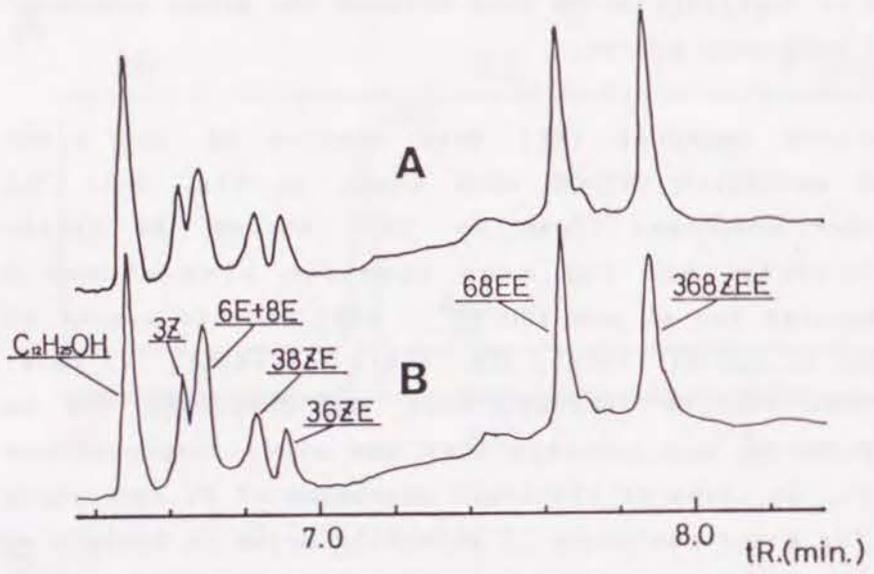
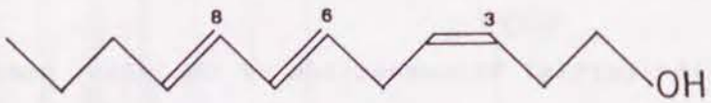


Fig. 5-2 Capillary GC data of the partially hydrogenated minor component and authentic (*Z,E,E*)-DTE-OH
A: Partially hydrogenated minor component
B: Partially hydrogenated (*Z,E,E*)-DTE-OH



(*Z,E,E*)-3,6,8-dodecatrien-1-ol

Fig. 5-3 Minor conponent of the trail pheromone of *Coptotermes formosanus* Shirki

5.3.2 Capillary GC-MS-HR-SIM analysis of sternal gland extracts

It was necessary to evidence whether the minor component was actually biosynthesized by termite (in sternal gland) or the

natural DTE-OH was isomerized into the minor component.

High-sensitive and high-selective analysis (capillary GC-MS-HR-SIM) can essentially facilitate the detection of such minor component as possible before isomerization occurs.

As the precise molecular weights of the minor component and trail pheromone are known theoretically, to the target component high-sensitively and high-selectively were detectable by means of capillary GC-MS-HR-SIM analysis.

Results of capillary GC-MS-HR-SIM analyses demonstrated that the DTE-OH isomers (*i.e.* trail pheromone isomers) from each test sample could be detected as a peak of HR-SIM at *m/z* 180.1513 and that those component could be high-selectively isolated from the impurities, although each two samples (2) and (3) of two species exhibited several impurity peaks due to the solvent impurity (samples (2)) or the background impurity from liquid phase (samples (3)). In case of *C. formosanus* (Fig. 5-4), all test samples exhibited the prominent (*Z,Z,E*)-DTE-OH peaks. The samples (1) and (2) also exhibited the clear peaks of (*Z,E,E*)-DTE-OH, which was equivalent of 35% (w/w) each of (*Z,Z,E*)-DTE-OH). These results suggested that the minor component, (*Z,E,E*)-DTE-OH was contained in the body of *C. formosanus*.

On the other hand, in case of *R. speratus* (Fig. 5-5), all test samples also exhibited the prominent (*Z,Z,E*)-DTE-OH peaks. However, the samples (1) and (2) obtained from *R. speratus* didn't exhibit (*Z,E,E*)-DTE-OH peaks. The minor component, (*Z,E,E,*)-DTE-OH, seemed species specific for *C. formosanus*. The amounts of detected trail pheromone and minor component for each termite species and analytical sample were determined by external standard technique. DTE-OH was *ca.* 15 ng, 142 pg and 46 pg in the samples (1),(2) and (3) respectively for *C. formosanus*. The minor component, (*Z,E,E*)-DTE-OH was 525 pg, 50 pg, in the samples (1) and (2) respectively for the same termite species. For *R. speratus*, DTE-OH was 313 pg, 301 pg and 32 pg in the samples (1),(2) and (3) respectively.

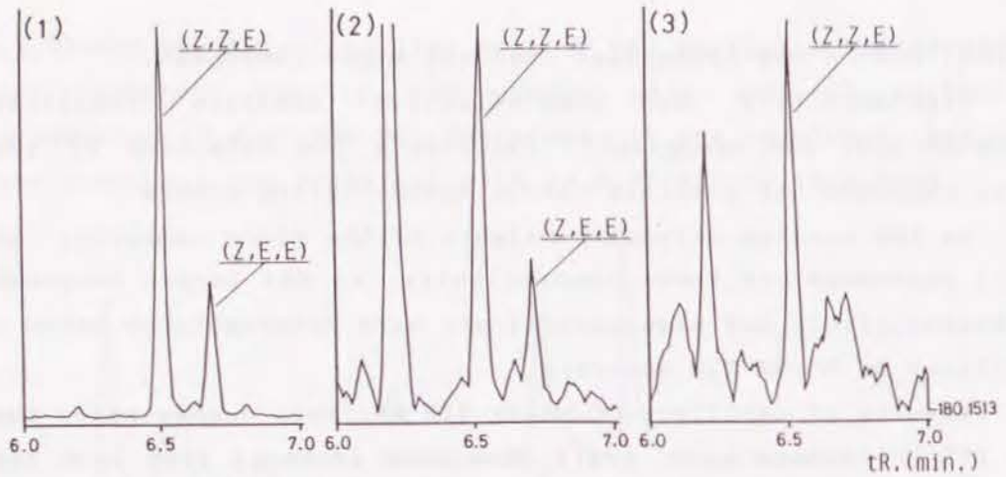


Fig. 5-4 Capillary GC-MS-HR-SIM data of the whole body extracts from *C. formosanus*
HR-SIM chromatograms at *m/z* 181.1513
Sample (1) : 16.7 WE/μl
Sample (2) : 10 WE/μl
Sample (3) : 1 WE/μl

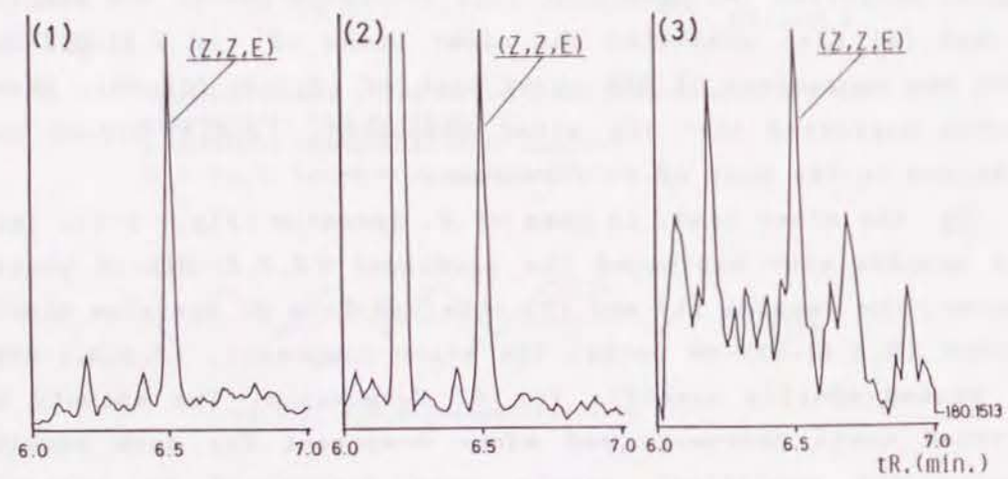


Fig. 5-5 Capillary GC-MS-HR-SIM data of the whole body extracts from *R. speratus*
HR-SIM chromatograms at *m/z* 181.1513
Sample (1) : 16.7 WE/μl
Sample (2) : 10 WE/μl
Sample (3) : 1 WE/μl

5.3.3 Results of Y-test using sternal gland extract and authentic DTE-OH

Although (*Z,E,E*)-DTE-OH was proved to exist in the body of *C. formosanus*, the significance of the component was not yet demonstrated. Since the active threshold level of (*Z,E,E*)-DTE-OH was approximately 100 times higher than that of DTE-OH, a behavioral effect of the component to the termite might be insignificant by itself. It, therefore, is necessary to evidence whether the (*Z,E,E*)-DTE-OH and DTE-OH synergically induce the workers of *C. formosanus* to species specific trail-following behavior.

Two kinds of solutions of DTE-OH were served for the Y-tests. Solution A was a hexane solution of DTE-OH as an artificial trail pheromone of *R. speratus*, and solution B was a mixture of DTE-OH and (*Z,E,E*)-DTE-OH (35% (W/W) of DTE-OH) as an artificial trail pheromone of *C. formosanus*.

Results of Y-test with solutions A and B were shown in Tables 5-1~3.

Table 5-1 Y-test of solution A (DTE-OH) trail versus solution B [DTE-OH + (*Z,E,E*)-DTE-OH] trail for *R. speratus* workers

Quantity of DTE-OH (weight/1.5 cm)	Number of termites choosing			Chi-square[a]
	Solution A		Solution B[b]	
5 ng	32	(20)[c]	48	2.81
500 pg	56	(4)	40	2.34
50 pg	47	(1)	52	0.16
5 pg	45	(1)	54	0.65
500 fg	48	(10)	42	0.28
100 fg	27	(32)	41	2.49

a: Chi-square analysis made on the hypothesis that if the two sample trail is identical, a 1:1 distribution will result, and value of Chi-square will be less than 3.84 at the 95% level of significance.
b: (*Z,E,E*)-DTE-OH was added to solution A at the rate of 35% of DTE-OH quantity.
c: Number of termites deviating.

Table 5-2 Y-test of solution A (DTE-OH) trail versus solution B [DTE-OH + (*Z,E,E*)-DTE-OH] trail for *C. formosanus* workers

Quantity of DTE-OH (weight/1.5 cm)	Number of termites choosing			Chi-square[a]
	Solution A		Solution B[b]	
5 ng	30	(30)[c]	40	1.16
500 pg	49	(7)	44	0.17
50 pg	44	(7)	49	0.17
5 pg	40	(6)	54	1.80
500 fg	36	(8)	55	3.56
100 fg	24	(36)	38	2.73

Captions are the same as in Table 5-1.

Table 5-3 Y-test of solution A (DTE-OH) trail versus solution B [DTE-OH + (*Z,E,E*)-DTE-OH] trail for *C. formosanus* soldiers

Quantity of DTE-OH (weight/1.5 cm)	Number of termites choosing			Chi-square[a]
	Solution A		Solution B[b]	
5 ng	12	(76)[c]	12	0.04
500 pg	48	(5)	47	0.00
50 pg	42	(2)	56	1.72
5 pg	44	(0)	56	1.21
500 fg	40	(5)	55	2.06
100 fg	33	(19)	52	3.81

Captions are the same as in Table 5-1.

In all cases, the observed preference of termites well corresponded to the 50/50 distribution at the 95% level of significance. These results indicated that *R. speratus* workers could not distinguish between solution A and solutions B. Contrary to expectation, could not discern the two solutions either, regardless of castes. Thus, the minor component (*Z,E,E*)-DTE-OH couldn't elicit a specific trail-following behavior to both *C. formosanus* and *R. speratus*.

Y-tests were concurrently conducted with the sternal gland extracts (as natural pheromone) of the two species to examine whether a termite species could recognize their own extracts from other extracts. As demonstrated in Tables 2-5~7 (Chapter 2), threshold trail-following levels of sternal gland extract from *C. formosanus* was two times higher than that from *R. speratus* regardless of termite species, and the soldiers' sensitivity of *C. formosanus* to sternal gland extract was two times higher than that of workers'. Therefore, test concentrations were selected based on the trail-following activities. In other wards, The concentrations which succeeded in inducing the same level of trail-following behavior to both termite species were streaked along the Y-shaped line in choice bioassay.

Results of Y-test of interspecific sternal gland extract were shown in Tables 5-4~9.

Table 5-4 Y-test of sternal gland extract from *R. speratus* versus that from *C. formosanus* using *R. speratus* workers

Test samples WE/1.5 cm (a) *R.s*-SGE[b]	(b) *C.f*-SGE[c]	Number of termites choosing (a)		(b)	Chi-square[a]
0.02	0.01	40	(0)[d]	60	3.61
0.002	0.001	38	(5)	57	3.41
0.0002	0.0001	43	(11)	46	0.04
0.0001	0.00005	42	(3)	55	1.48
0.03	0.01	57	(1)	42	1.98
0.003	0.001	76	(0)	24	26.01
0.0003	0.0001	57	(3)	39	3.01
0.01	0.01	12	(5)	83	51.58
0.001	0.001	30	(5)	65	12.17
0.0001	0.0001	16	(8)	76	37.84
0.01	0.02	13	(1)	86	52.36
0.001	0.002	12	(1)	87	55.31
0.0001	0.0002	10	(1)	89	61.45

a: Chi-square analysis made on the hypothesis that if the two sample trail is identical, a 1:1 distribution will result, and value of Chi-square will be less than 3.84 at the 95% level of significance.
b: Sternal gland extract of *R. speratus*.
c: Sternal gland extract of *C. formosanus*.
d: Number of termites deviating in the parentheses.

When the samples with the same trail-following activity were tested, any termite showed unselective behavior to the extracts from both species. Termites could not recognize their own sternal gland extract trail under such test conditions, but were rather more sensitive to quantitative differences.

Table 5-5 Y-test of sternal gland extract from *R. speratus* versus that from *C. formosanus* using *C. formosanus* workers

Test samples ex. WE/1.5 cm (a) *R.s*-SGE[b]	(b) *C.f*-SGE[c]	Number of termites choosing (a)		(b)	Chi-square[a]
0.02	0.01	39	(6)[d]	55	2.69
0.002	0.001	40	(2)	58	2.95
0.0002	0.0001	43	(10)	47	0.16
0.0001	0.00005	27	(30)	43	3.21
0.03	0.01	68	(6)	26	17.88
0.003	0.001	62	(2)	36	6.38
0.0003	0.0001	62	(11)	27	12.99
0.01	0.01	22	(10)	68	22.50
0.001	0.001	26	(4)	70	19.26
0.0001	0.0001	6	(6)	88	69.80
0.01	0.02	7	(3)	90	69.32
0.001	0.002	15	(4)	81	44.01
0.0001	0.0002	10	(6)	84	56.69

Captions are the same as Table 5-4.

Table 5-6 Y-test of sternal gland extract from *R. speratus* versus that from *C. formosanus* using *C. formosanus* soldiers

Test samples ex. WE/1.5 cm (a) *R.s*-SGE[b]	(b) *C.f*-SGE[c]	Number of termites choosing (a)		(b)	Chi-square[a]
0.02	0.01	48	(4)[d]	48	0.01
0.002	0.001	58	(2)	40	2.95
0.0002	0.0001	58	(3)	39	3.34
0.0001	0.00005	49	(13)	38	1.15
0.03	0.01	60	(2)	38	4.50
0.003	0.001	82	(0)	18	39.69
0.0003	0.0001	80	(2)	18	37.97
0.01	0.01	22	(2)	76	28.66
0.001	0.001	31	(1)	68	13.09
0.0001	0.0001	34	(7)	59	6.19
0.01	0.02	12	(4)	84	52.51
0.001	0.002	6	(2)	72	54.17
0.0001	0.0002	6	(0)	94	75.69

Captions are the same as Table 5-4.

These results were different from the data of the previous report by Howard *et al.* (1976) who worked on *R. virginicus*, *R. flavipes*, *R. tibialis* and *C. formosanus* and concluded that trail pheromones were species specific. This disagreement may be due to the different test methods using different test termites. It, however, is of great interest to point out that termites always chose the line with a sample of 2-3 times higher trail-following activity (Tables 5-7~9).

Such sensitive preference might be directly related to orientation activity as demonstrated with *R. hesperus* by Grace *et al.* (1988).

Table 5-8 Distribution of termites in Y-test when a couple of the same sternal gland extracts applied to *C. formosanus* workers

Test samples WE/1.5 cm	Number of termites choosing			Chi-square[a]
R.s-SGE[b]	x 2[c]		x 1[c]	
0.1	68	(6)[d]	26	17.88
0.01	65	(4)	31	11.34
0.005	82	(5)	13	48.67
0.001	58	(4)	38	3.76
0.0005	74	(9)	17	34.46
0.0001	64	(18)	18	24.70
0.00005	56	(18)	26	10.26
0.000025	22	(74)	4	11.12
R.s-SGE	x 3		x 1	
0.1	83	(7)	10	55.74
0.01	79	(3)	18	37.11
0.001	80	(9)	11	50.81
0.0001	84	(10)	6	65.88
C.f-SGE[e]	x 2		x 1	
0.1	78	(6)	16	39.59
0.01	71	(2)	27	18.87
0.005	60	(5)	35	6.06
0.001	70	(3)	27	18.19
0.0005	66	(15)	19	24.89
0.0001	58	(20)	22	8.00
0.00005	57	(18)	25	11.72
0.000025	62	(22)	16	25.96
C.f-SGE	x 3		x 1	
0.1	94	(2)	4	80.83
0.01	74	(8)	18	32.88
0.001	84	(4)	12	52.51
0.0001	76	(18)	6	58.06

Captions are the same as Table 5-7.

Table 5-9 Distribution of termites in Y-test when a couple of the same sternal gland extracts applied to *C. formosanus* soldiers

Test samples WE/1.5 cm	Number of termites choosing			Chi-square[a]
R.s-SGE[b]	x 2[c]		x 1[c]	
0.1	75	(7)[d]	18	33.72
0.01	52	(6)	32	4.30
0.005	85	(5)	10	57.64
0.001	70	(10)	20	26.68
0.0005	84	(2)	14	48.58
0.0001	76	(10)	14	41.34
0.00005	70	(13)	17	31.08
0.000025	52	(40)	8	30.82
R.s-SGE	x 3[c]		x 1[c]	
0.1	76	(6)	18	34.56
0.01	80	(5)	15	43.12
0.001	84	(8)	8	61.14
0.0001	88	(2)	10	60.50
C.f-SGE[d]	x 2		x 1	
0.1	88	(2)	10	60.50
0.01	70	(2)	28	17.15
0.005	68	(5)	27	16.84
0.001	64	(4)	32	10.01
0.0005	68	(8)	24	20.10
0.0001	78	(8)	14	8.00
0.00005	66	(20)	14	32.51
0.000025	59	(20)	21	17.11
C,f-SGE	x 3		x 1	
0.1	96	(4)	4	82.81
0.01	81	(2)	2	40.50
0.001	78	(2)	2	33.15
0.0001	90	(8)	2	82.27

Captions are the same as Table 5-7.

5.4 Summary

In the course of the elucidation of the primary structure of an isolated trail pheromone from *C. formosanus*, a minor component which had the same molecular weight of the major pheromone (DTE-OH) was detected in mass chromatogram of capillary GC-MS. Mass spectrum of the minor component exactly showed a prominent pattern of dedecatrienol. Comparative chemical studies with authentic DTE-OH isomer clearly demonstrated that the complete structure was (*Z,E,E*)-DTE-OH.

Furthermore, capillary GC-MS-HR-SIM analysis indicated that the component originated in termites, although any significance of the component was not noticeable in bioassay.

PART III

ISOLATION AND IDENTIFICATION OF TRAIL PHEROMONE PRECURSOR

Chapter 6 : Evidence for a Trail Pheromone Precursor in the Termite *Reticulitermes speratus* (Kolbe)

Chapter 7 : Evidence for a Trail Pheromone Precursor in the Termite *Coptotermes formosanus* Shiraki

Chapter 8 : Isolation and Identification of Trail Pheromone Precursors from *Coptotermes formosanus* Shiraki

Chapter 6 : Evidence for a Trail Pheromone Precursor in the Termite *Reticulitermes speratus* (Kolbe)

6.1 Introduction

Trail pheromone of *R. speratus* was successfully isolated and identified as DTE-OH which was exactly the same structure as that of *R. virginicus* (PART II. Chapter 3). On the basis of the small amount of trail pheromone extracted from the termites, each individual did not seem to induce trail-following behavior over a long distance in the natural habitat. Therefore, it was hypothesized that the pheromone might be stored in the body of the termite in the form of a precursor, and secreted as required following transformation from the precursor.

The main purpose of the present investigation was to find precursor candidate of the trail pheromone of *R.speratus* in the course of isolation by chemical techniques and bioassays for the better understanding on the origin or biosynthesis of the trail pheromone (Tokoro *et al.* 1990a,1990b)

6.2 Materials and Methods

6.2.1 Test termites, chemicals, apparatus and bioassay method

Test termite, chemicals, apparatus and bioassay method were same as before, described in PART I and II.

6.2.2 Isolation and activation of precursor candidates

Method of extraction and silica-gel column chromatography were described in Chapter 3.

Fractions which did not show any trail-following activity in the initial test were subjected to various kinds of chemical

reaction, because it was assumed that a precursor could exist. Active trail-following behavior was produced for the 5% and 10% EtOAc/Hx fractions by hydrolysis with 2% KOH/MeOH or by reduction.

The yellowish crude lipids (*ca.* 1.3 g) obtained from these activable fractions were dissolved in 2 ml of dry diethyl ether and hydrolyzed with 50 ml of 2% KOH / MeOH in a 100 ml glass flask for 10 hours at room temperature. After hydrolysis, the reaction mixtures were separated with 100 ml each of diethyl ether and NaCl-saturated water in a separatory funnel. The ether layer (added 100 ml diethyl ether) was washed once with 1N-HCl and twice with distilled water, and dried with anhydrous sodium sulfate.

Subsequently, this activated fraction (ether layer, 155 mg) was also fractionated by silica-gel column chromatography (20 cm x 7 mm i.d. glass column, 4.7 g silica-gel). The pooled active fractions were then fractionated into 22 subfractions by 20% (w/w) $AgNO_3$ silica-gel chromatography with EtOAc/Hx in the similar manner of natural trail pheromone. Separation of compounds under these conditions depends on the number of double bonds and the geometrical configuration of isomers. Active fractions were fractionated by normal phase HPLC with 10% EtOAc/Hx and detected using a UV detector set at 234 nm.

The final purification step was carried out by GC using a nonpolar column, followed by a polar column. The detail methods and conditions were described in Chapter 3.

6.2.3 Identification of the hydrolysis product

The active GC peak was analyzed by capillary GC-MS. Structure determination of the hydrolysis product was attempted by means of capillary GC-MS and capillary GC-FTIR analysis combined with microscale chemical reactions, as described in Chapter 3.

6.3 Results and Discussion

6.3.1 Results of bioassays with the crude hexane extracts

Results of trail following activity bioassays with crude hexane extracts are shown in Chapter 1.

As shown in Table 1.1 described in Chapter 1, the hexane extracts exhibited remarkable activities ranging from 100 to 0.1 WE/15 cm. The MEWE was thus identified as 0.1 WE/15 cm. In the preliminary experiments, the extracts of intestinal contents of test workers showed no trail-following activity. After extracting with hexane, the corpses were homogenized with diethyl ether and was subjected to the measurement of trail-following activity. The activity was less than one-fiftieth of the primary hexane extracts.

The result of the bioassays with the hexane extract indicated that a 0.1 WE extract induced trail-following over a 15 cm trail, so a worker termite could only produce a 1.5 m-long trail. Although the mechanism of secretion of the trail pheromone (Macfarlane 1983) was not clear in rhinotermitid termite (Grace *et al.* 1988), the quantity of trail pheromone in a worker seems to be very small. This phenomenon led to the assumption that the pheromone is stored in the body in the form of precursor that is secreted as required following conversion to the active form.

6.3.2 Silica-gel column chromatography and alkaline hydrolysis

The natural trail pheromone was eluted mainly in the range of 15-20% EtOAc/Hx on silica-gel column chromatography (Table 6-1). Preliminary experiments which were intended to investigate the possible existence in the extract of a pheromone precursor showed that fractions eluted with 5-10% EtOAc/Hx were activated both by hydrolysis with 2% KOH / MeOH and by reduction with

$LiAlH_4$. Since the fraction mainly contained fatty-acid esters, it was suspected that the precursor candidate would be stored as an esterified pheromone. The activity of the newly activated substances were about 20 times that of the natural pheromone by weight (Table 6-1).

Table 6-1 Results of trail-following bioassay of the crude hexane extracts after silica-gel column chromatography

Test fraction				Quantity of lipid(mg)[a]	Activity as MEWE[b] (WE/15 cm-trail)	
					Natural	After hydrolysis
Crude hexane extracts				3000	0.1	0.005
Fr.No.	Eluants(volume(ml))					
1	Hx	1[c]	(500)	132	-[d]	-
2		2	(500)	70	-	-
3	5 %[e]	1	(500)	2	-	-
4		2	(500)	912	-	0.005
5	10 %	1	(250)	407	-	0.01
6		2	(250)	33	-	0.1
7		3	(250)	12	-	0.1
8		4	(250)	11	-	1
9	15 %	1	(200)	4	-	10
10		2	(200)	11	1	(1)[f]
11		3	(200)	51	0.1	(0.1)
12		4	(200)	54	1	(1)
13		5	(200)	80	5	(5)
14	20 %	1	(200)	76	1	(1)
15		2	(200)	80	10	(10)
16		3	(200)	97	-	-
17		4	(200)	84	-	-
18		5	(200)	68	-	-
19	EtOAc	1	(500)	283	1	-
20		2	(500)	122	-	-
21	MeOH	1	(500)	98	1	-
22		2	(500)	54	1	-

a: Weight of crude lipids.
b: The activity was estimated by a modified Open-Field bioassay. When three or more of nine workers followed the 15 cm trail, the sample was designated positive. Tenfold dilution series and more highly diluted solutions of each sample were employed to determine the MEWE (WE/15 cm-trail).
c: The number of subfraction.
d: No trail following response or insufficient response (*i.e.* the estimated value was more than 10WE/15cm-trail) for trail-following.
e: Percentage by volume of EtOAc/Hx (%) as eluants.
f: The values in parentheses are natural activity.

Hydrolysis reaction was subsequently employed because it is the milder. After separation with diethyl ether and NaCl-saturated water, the ether layer showed high activity ($5x10^{-3}$ MEWE). When the water layer was acidified with 1N-HCl solution, a plenty of free fatty acids which were supposed to derive from triglyceride by alkaline hydrolysis was separated.

Bromination and acetylation markedly lowered the trail-following activity of activated substance after hydrolysis. It suggested that the substance might have double bonds and hydroxyl groups.

Table 6-2 Results of trail-following bioassay of the hydrolyzed products after silica-gel column chromatography

Test fraction				Quantity of lipid(mg)[a]	Activity as MEWE[b] (WE/15 cm-trail)
Hydrolyzed products				155	0.005
Fr.No.	Eluants(volume(ml))				
1	Hx	1[c]	(25)	0	-[d]
2	5 %[e]	1	(25)	50	-
3	10 %	1	(25)	21	-
4	15 %	1	(6)	14	-
5		2	(6)	20	0.01
6		3	(6)	18	0.006
7		4	(6)	3	0.01
8	20 %	1	(12.5)	11	0.01
9		2	(12.5)	4	0.1
10	100 %	1	(12.5)	54	1
11		2	(12.5)	80	10
12	MeOH	1	(25)	98	-

Captions are the same as in Table 6-1.

Table 6-3 Results of trail-following bioassay of the hydrolyzed products after $AgNO_3$ silica-gel column chromatography

Test fraction				Quantity of lipid(mg)[a]	Activity as MEWE[b] (WE/15 cm-trail)
Hydrolyzed products				70	0.005
Fr.No.	Eluants(volume(ml))				
1	Hx	1[c]	(6)	t[d]	-[e]
2		2	(6)	t	-
3	10 %[f]	1	(6)	t	-
4		2	(6)	1	-
5	20 %	1	(6)	12	-
6		2	(6)	28	-
7	30 %	1	(6)	12	-
8		2	(6)	9	-
9	40 %	1	(6)	t	-
10		2	(6)	t	-
11	50 %	1	(3)	t	-
12		2	(3)	t	0.1
13		3	(3)	t	0.01
14		4	(3)	t	0.1
15	60 %	1	(3)	t	0.1
16		2	(3)	t	0.01
17		3	(3)	t	0.01
18		4	(3)	t	1
19	100 %	1	(3)	t	1
20		2	(3)	t	10
21		3	(3)	t	10
22		4	(3)	t	-

a: Weight of crude lipids.
b: The activity was estimated by a modified Open-Field bioassay. When three or more of nine workers followed the 15 cm trail, the sample was designated positive. Tenfold dilution series and more highly diluted solutions of each sample were employed to determine the MEWE (WE/15 cm-trail).
c: The number of subfraction.
d: Trace amount.
e: No trail following response or insufficient response (*i.e.* the estimated value was more than 10WE/15cm-trail) for trail-following.
f: Percentage by volume of EtOAc/Hx (%) as eluants.

6.3.3 Isolation and identification of the hydrolyzed active products

Behavior of the newly activated substances on silica-gel chromatography closely corresponded to that of the natural pheromone (Table 6-2).

Behavior of the hydrolyzed product on subsequently chromatographies (*i.e.* $AgNO_3$ Silica-gel, HPLC, preparative GC) closely corresponded to that of the natural pheromone (Table 6-3 and Fig. 6-1~3).

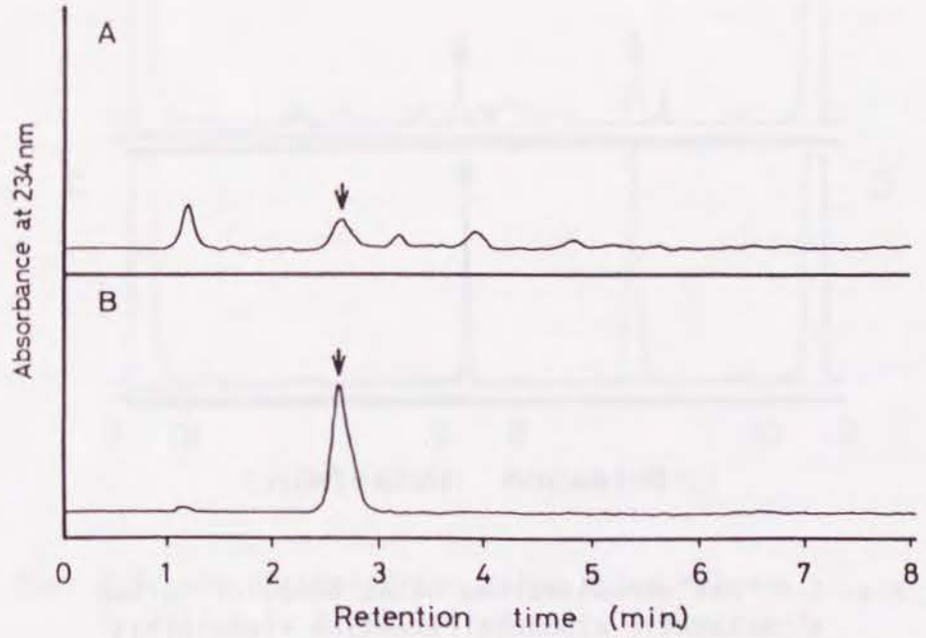


Fig. 6-1 HPLC chromatograms of the active fractions after fractionation $AgNO_3$ silica-gel column chromatography
A: The native active fractions(trail pheromone)
B: The newly activated fractions
Arrows indicate the active peak(tR. 2.6)

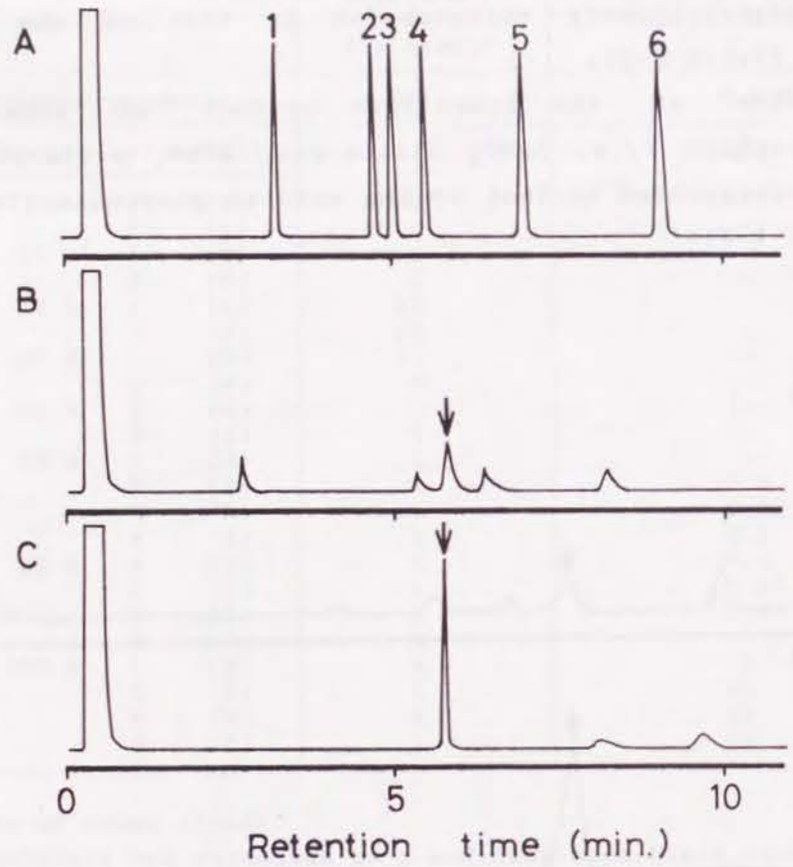


Fig. 6-2 Gas chromatograms using nonpolar column
A: Authentic alcohols[retention times(min)]
1:$C_{11}H_{23}OH$(3.2), 2:(*Z,Z*)-3,6-$C_{12}H_{21}OH$(4.7),
3:(*Z*)-3-$C_{12}H_{23}OH$(5.0), 4:$C_{12}H_{25}OH$(5.5),
5:(*E,E*)-8.10-$C_{12}H_{21}OH$(7.0), 6:$C_{13}H_{27}OH$(9.1),
B: The native trail pheromone
C: The hydrolyzed product
Arrows indicate the active peaks(tR.5.8)

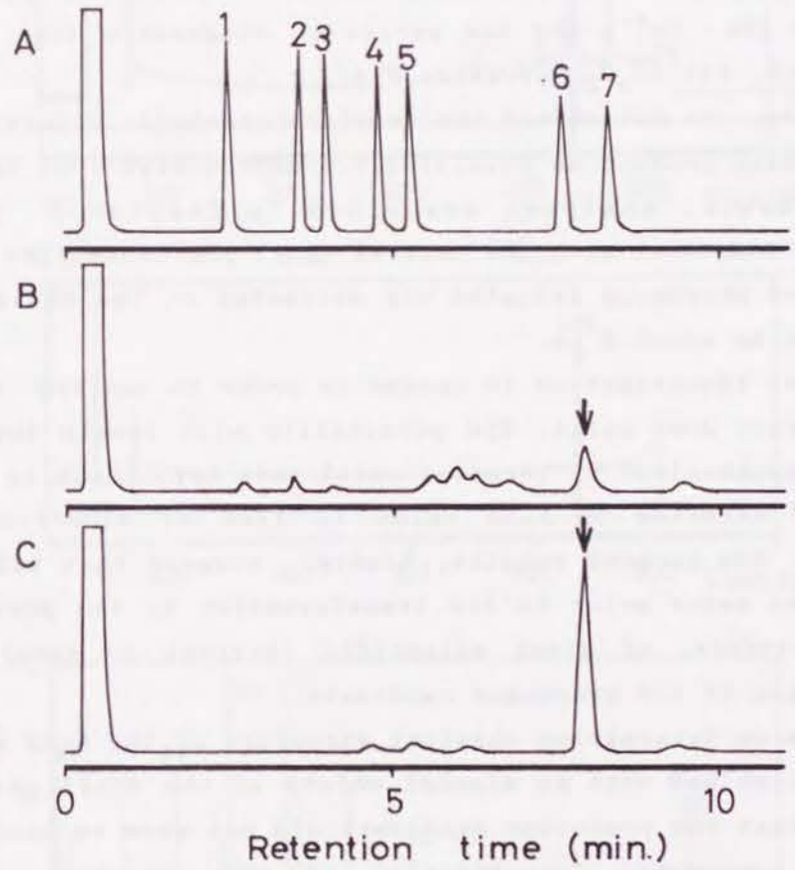


Fig. 6-3 Gas chromatograms using polar column
A: Authentic alcohols[retention times(min)]
1:$C_{11}H_{23}OH$(2.5), 2:$C_{12}H_{25}OH$(3.6),
3:(*Z*)-3-$C_{12}H_{23}OH$(4.0), 4: (*Z,Z*)-3,6-$C_{12}H_{21}OH$(4.8),
5:$C_{13}H_{27}OH$(5.3), 6:$C_{14}H_{29}OH$(7.6),
7:(*E,E*)-8.10-$C_{12}H_{21}OH$(8.3),
B: The native trail pheromone,
C: The hydrolyzed product
Arrows indicate the active peaks(tR.7.9)

In addition, the capillary GC-MS data of the hydrolyzed product coincided well with that of the natural pheromone, exhibiting the same retention time on capillary GC and the same pattern in the mass spectrum which showed a prominent molecular ion at *m/z* 180 ($M^{+\cdot}$) and the series of diagnostic ions at *m/z* 91, 105, 119, 133 (C_nH_{2n-7}) (Fig. 6-4).

Finally, we determined the complete chemical structure of the hydrolyzed product as (*Z,Z,E*)-3,6,8-dodecatrien-1-ol by means of instrumental analyses described in Chapter 3. It was definitely identical with the natural trail pheromone. The amount of activated pheromone isolated was estimated on the basis of GC analysis to be about 5 μg.

Further investigation is needed in order to confirm whether this precursor does exist. The possibility will remain that DTE-OH is not synthesized by termites until this hypothesis is tested by rearing termites on food which is free of microflora and microfauna. The present results, however, suggest that DTE-OH is stored as an ester prior to its transformation to the pheromone. It is, therefore, of great scientific interest to specify the storing organ of the precursor candidate.

Trials on determining chemical structure of the acid moiety, which was combined with an alcohol moiety of the trail pheromone suggested that the precursor candidate did not seem to consist of only one component. Investigation of the variation of the precursor candidate would increase the interest in relation to the taxonomy of family Rhinotermitidae, similar as defense compounds (Prestwich *et al.* 1984).

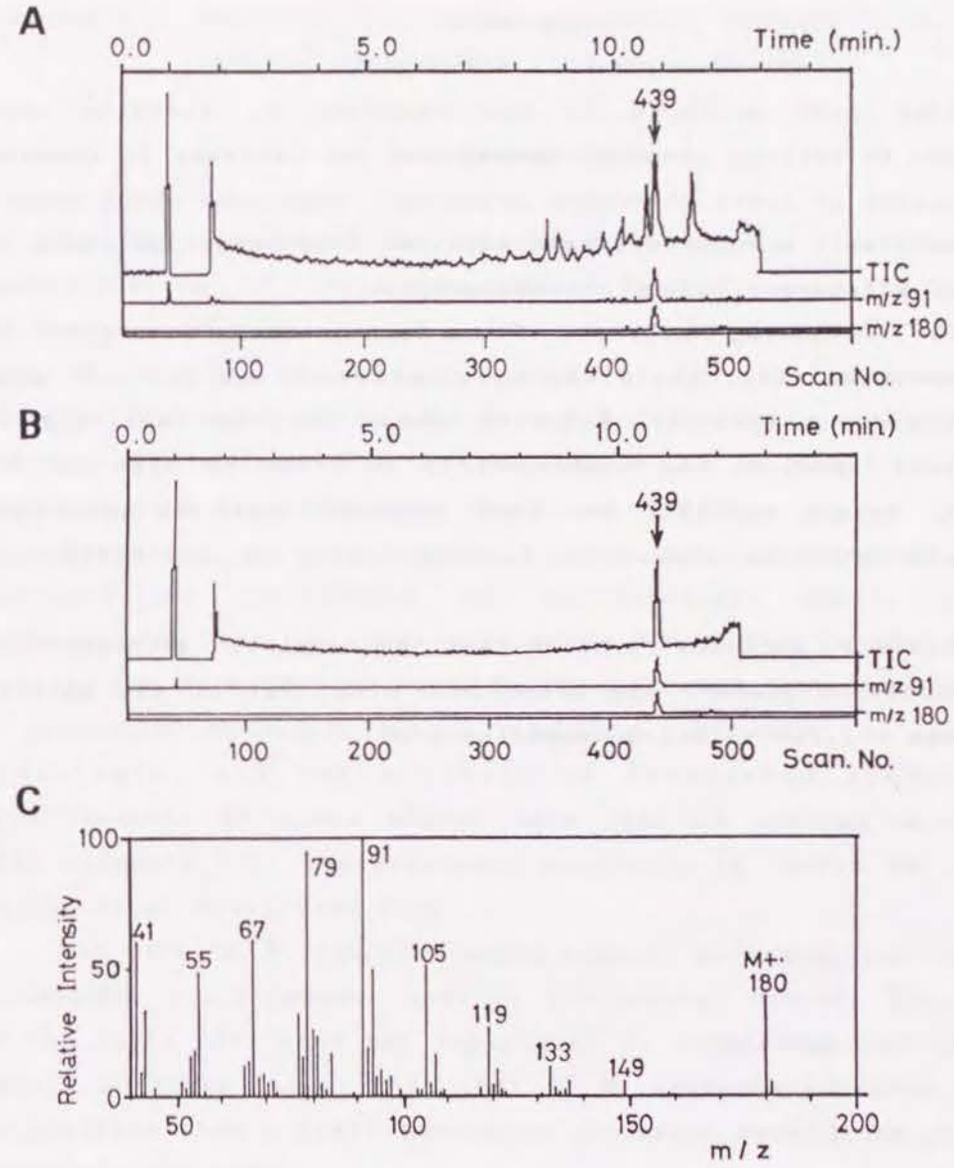


Fig. 6-4 Results of capillary GC-MS analyses
A, B: Total ion chromatograms (TIC) and mass chromatograms of the native active fractions (trail pheromone :A) and of the newly activated fraction(B) by hydrolysis
Arrows indicate the active peaks(Scan No.439)
C: Mass spectrum of the active peak(Scan No.439 in B)

6.4 Summary

Whole body extracts of the termite, *R. speratus* were subjected to various chemical operations and bioassay to examine the presence of trail pheromone precursor. Fractions which mainly contained fatty acid esters were obtained from hexane extracts by means of silica-gel column chromatography.

Trail-following activity of the fractions was activated by alkaline hydrolysis, while the original fractions did not show any conspicuous activity. Bioassay showed that the activity of hydrolyzed product was approximately 20 times as high as the original hexane extract. The fact suggests that the precursor candidate could be stored in termite bodies as an esterified form.

Chemical analyses revealed that the complete structure of the hydrolyzed product was coincident with that of the natural pheromone ((*Z,Z,E*)-3,6,8-dodecatrien-1-ol).

Chapter 7 : Evidence for trail pheromone precursors in the termite *Coptotermes formosanus* Shiraki

7.1 Introduction

In the course of the investigations on the isolation and identification of trail pheromones from *R. speratus* and *C. formosanus*, the pheromones were proved to be common to both termite species. The chemical structure of the pheromone was exactly identical with (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (DTE-OH) which was obtained for *R. virginicus* as a trail pheromone (Matsumura *et al.* 1968,1967; Tai *et al.* 1969).

Moreover, as the amount of the trail pheromone of *R. speratus* per individuals was extraordinary small, it was hypothesized that the pheromone might be stored in the termite body in the form of precursor. We demonstrated that there existed a precursor candidate which could be activated by alkaline hydrolysis, and the activity of hydrolyzed product was approximately 20 times higher than that of natural pheromone. This suggests that the precursor candidate is stored in termite bodies as an esterified form.

The work on *R. speratus* would support an assumption that *C. formosanus* could produce similar precursors. However the amount of the trail pheromone per individual *C. formosanus* was approximately 20 times larger than that of *R. speratus* (Chapter 3), it is possible that a trail pheromone precursor candidates could be stored in the body.

The main purpose of this Chapter was to find precursor candidate of the trail pheromone in *C. formosanus*, for the better understanding of the biosynthesis or supplementary system of the trail pheromone (Tokoro *et al.* 1992b).

7.2. Materials and Methods

7.2.1 Test termites, chemicals and bioassay methods

Test termites were prepared from colony F and I as described Chapter 1, and chemicals and bioassay methods (Open-Field bioassay) were the same as described in Chapter 1 and 3.

7.2.2 Apparatus

The HPLC was a model LC-9A (Shimadzu, Kyoto, Japan) pump connected in series to a model SPD-6AV (Shimadzu) UV-VIS spectrophotometric detector and to a model ERC-7511 (Erma, Tokyo, Japan) refractive index detector. All other apparatus and bioassay methods were the same as those in the previous Chapter.

7.2.3 Extraction of precursor candidates

The worker termites(colony F: *ca.* 938 g, *ca.* 310,000 in number) were soaked in *n*-hexane (*ca.* 300 ml per 20,000 worker termites) at -20°C for about ten days without homogenization. The extract was then tested for its trail following activity before and after alkaline hydrolysis and minimum effective worker equivalent (MEWE) was estimated by bioassay.

7.2.4 Silica-gel column chromatography and alkaline hydrolysis of the hexane extracts

The hexane extracts were filtered and concentrated using a rotary vacuum evaporator until the yellowish crude lipid (*ca.* 71 g) was obtained. This crude lipid was first fractionated by

silica-gel column chromatography with EtOAc/Hx and MeOH eluants.

Each fraction was subjected to alkaline hydrolysis to examine the presence of trail pheromone precursor in the same manner as previously described in Chapter 6. One-tenth each of the crude lipids obtained from these fractions were combined all together and hydrolyzed with 2% KOH/MeOH.

Since it remained questionable whether the fractions did not contain any natural trail-following substance, it was necessary to compare the activity levels (MEWE) of active substance between before and after hydrolysis in bioassay.

7.2.5 Isolation and identification of the hydrolysis products

Hydrolysis products of each fraction were isolated by silica-gel column chromatography, and followed by 20% (w/w) $AgNO_3$ silica-gel column chromatography, HPLC and capillary GC. Following isolation, those were served for bioassay in the same manner for the natural trail pheromones as described in Chapter 4.

The isolated components were identified by means of capillary GC-MS and capillary GC-FTIR analysis combined with microscale chemical reactions (acetylation, partial reduction, ozonolysis). Details should be referred to the Chapter 3.

7.2.6 Determination of the morphological storage site of the trail pheromone precursor candidates

Dissections were made under a binocular stereoscopic microscope to obtain the abdominal fifth-sternites where the sternal gland was located, because it was generally accepted that the organ of the Rhinotermitid termite excreted trail pheromone.

To examine the presence of trail pheromone precursor and its storage site, twenty worker termites (ten each from the two worker colonies F and I) were used for dissecting individual body five parts. The five test parts were head, digestive tube (from the foregut to the rectum), abdominal fifth-sternite, all other cuticular segments and remaining portion. A fused central portion (approximately 0.3 mm square) of the abdominal fifth-sternite and the abdominal fifth-sternite (hereinafter referred to "fifth-sternite") was dissected this time to avoid injuring a sternal gland. Each group of the dissected tissues was soaked in 500 µl of Hx for ten hours at -20°C.

Ten whole bodies of the worker termites (worker group F) were also soaked in the similar manner as a reference sample. Each extract was then tested for its trail following activity before and after alkaline hydrolysis, and MEWE was estimated.

7.3 Results and Discussion

7.3.1 Results of bioassays with the crude hexane extracts

Bioassay results of trail following activity of the crude hexane extracts indicated that a 0.005 WE extracts induced trail-following behavior over a 15 cm-trail: the MEWE was identified as 0.005 WE/15 cm. An experiment which was intended to examine the possible existence of a pheromone precursor showed that the extract was activated by hydrolysis with 2% KOH/MeOH as similar to the case of *R. speratus* (Chapter 6).

The activity of the newly activated extract was about 20 times higher than that of the extract before hydrolysis by weight, and the MEWE of the hydrolyzed extract was identified as 0.00025 WE/15 cm. It was also possible that the precursor candidate would be stored as an esterified pheromone in the body of *C. formosanus*.

After extracting with hexane, the corpses were homogenized with diethyl ether and was subjected to the measurement of trail-following activity before and after alkaline hydrolysis. Regardless of alkaline hydrolysis, the activities were less than one-fiftieth of the primary hexane extracts, extraction of the trail pheromone and the precursor candidates was considered to be done sufficiently.

On the basis of the bioassay, a worker termite of *C. formosanus* could produce a 30 m long trail. The length was about 20 times as long as that of *R. speratus*. This quantitative difference may be due to the higher daily behavior of a *C. formosanus* worker in comparison with *R. speratus*.

However the mechanism of secretion of the pheromone (MacFarlane 1983) was not clear in rhinotermitid termite, the daily trail following behavior of termite is closely related to the secretion of the pheromone, and the stored quantity sooner or later would result in depletion. Since the hydrolyzed hexane extract indicated that a 0.00025 WE extracts induced trail-following over a 15 cm trail, a worker termite could produce a 600 m available trail.

Therefore, there was sufficient quantity of trail pheromone stock in a worker's body of *C. formosanus* as a form of precursor. Thus, the esterified storage system of the trail pheromone may be advantageous for the termite living.

7.3.2 Silica-gel column chromatography and alkaline hydrolysis

Bioassay results of the hexane extracts after silica-gel column chromatographies are shown in Table 7-1 described in Chapter 1. The natural trail pheromone was eluted mainly in the range of 15-20% EtOAc/Hx in the silica-gel column chromatography, and the result was similar to that of previous experiment described in Chapter 4.

Table 7-1 Results of trail-following bioassay of the crude hexane extracts after silica-gel column chromatography

Test fraction			Quantity of lipid(mg)[a]	Activity as MEWE[b] (WE/15 cm-trail)	
				Natural	After hydrolysis
Crude hexane extracts			67979	0.005	0.00025
Fr.No.	Eluants(volume(l))				
1	Hx	1[c] (1.5)	467	-[d]	-
2		2 (1.5)	542	-	-
3	5 %[e]	1 (1.5)	18	-	-
4		2 (1.0)	9884	-	0.005 (CG-1)[f]
5		3 (1.0)	29067	-	0.002 (CG-1)
6	10 %	1 (1.5)	17160	-	0.05
7		2 (1.5)	1585	-	0.001 (CG-2)[f]
8		3 (1.5)	447	-	0.01 (CG-2)
9	15 %	1 (1.5)	285	-	0.002 (CG-2)
10		2 (1.0)	442	0.01	0.005 (CG-2)
11		3 (0.5)	347	0.025	0.01
12		4 (0.5)	142	0.1	0.002 (CG-3)[f]
13	20 %	1 (0.5)	197	0.1	0.001 (CG-3)
14		2 (1.5)	807	0.05	0.001 (CG-3)
15		3 (1.0)	227	-	0.005 (CG-3)
16		4 (1.0)	110	-	0.1
17	EtOAc	1 (1.5)	272	1	0.01
18		2 (1.5)	447	-	0.05
19	MeOH	(2.0)	1131	1	0.1

a: Weight of crude lipids.
b: The activity was estimated by a modified Open-Field bioassay. When three or more of nine workers followed the 15 cm trail, the sample was designated positive. Tenfold dilution series and more highly diluted solutions of each sample were employed to determine the MEWE (WE/15 cm-trail).
c: The number of subfraction.
d: No trail following response or insufficient response (*i.e.* the estimated value was more than 10WE/15cm-trail) for trail-following.
e: Percentage by volume of EtOAc/Hx (%) as eluants.
f: CG-1: component group 1, CG-2: component group 2, and CG-3: component group 3.

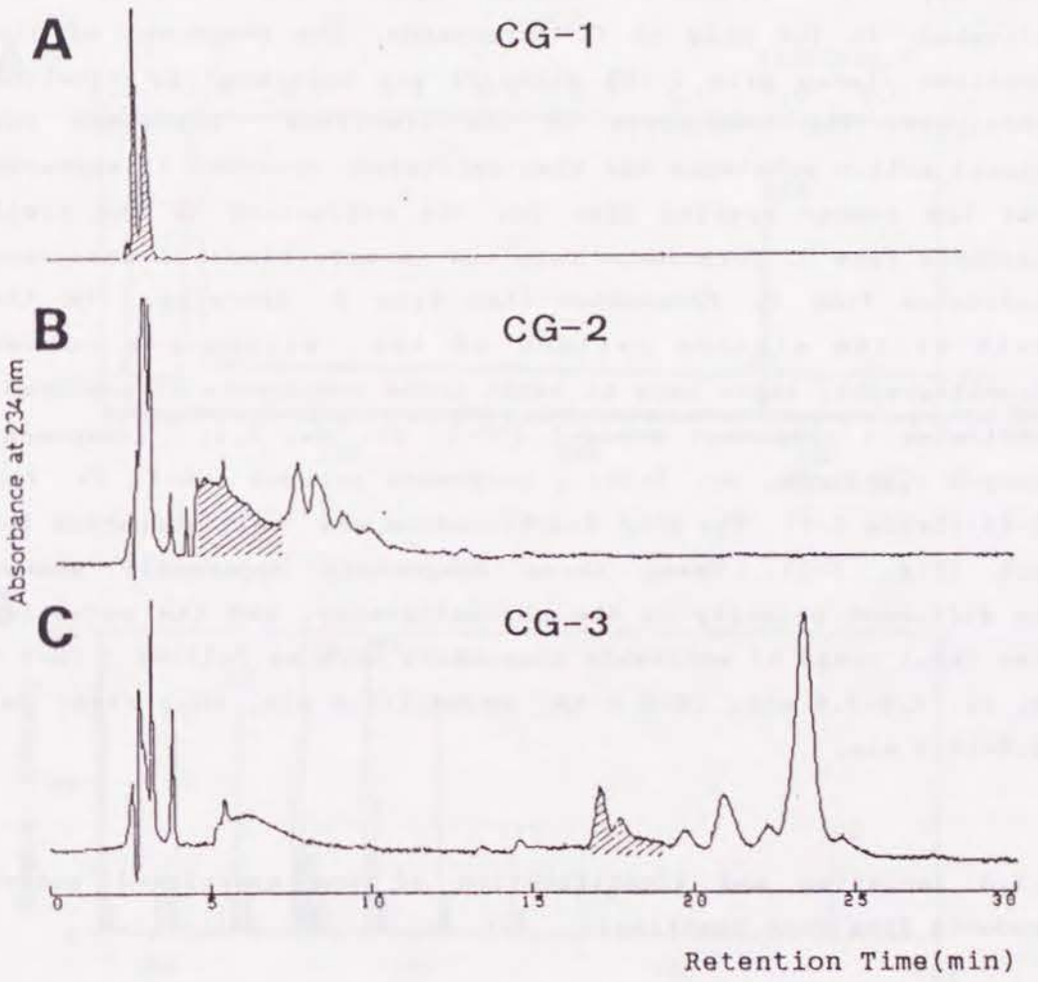


Fig. 7-1 Results of preparative HPLC of three activatable fractions from the silica gel column chromatography
A: Fraction number 4,5 (CG-1)
B: Fraction number 7,9,10 (CG-2)
C: Fraction number 12-15 (CG-3)
The shadow areas are the activable fractions.

Active trail-following behavior was produced by several fractions after hydrolysis in this experiment, although as for *R. speratus*, the fractions eluted with 5-10% EtOAc/Hx were activated. In the case of *C. formosanus*, the component of the fractions eluted with 5-10% EtOAc/Hx was activated by alkaline hydrolysis. The components of the fractions contained the natural active substance was also activated. However, It appeared that the longer soaking time for the extraction of the trail pheromone from *C. formosanus* resulted in more kinds of precursor candidates from *C. formosanus* than from *R. speratus*. On the basis of the elution pattern of the silica-gel column chromatography, there were at least three components of precursor candidates : component group-1 (CG-1; Fr. No. 4,5) ; component group-2 (CG-2; Fr. No. 7-10) ; component group-3 (CG-1; Fr. No. 12-15)(Table 7-1). The HPLC fractionation was well supported the fact (Fig. 7-1). These three components apparently showed the different polarity on the chromatography, and the retention time (tR.) range of activable components were as follows : CG-1 = tR. *ca.* 2.4-3.6 min; CG-2 = tR. *ca.* 4.7-7.5 min; CG-3 = tR. *ca.* 16.7-19.3 min.

7.3.3 Isolation and identification of the hydrolyzed active products from each fraction.

Behavior of the hydrolyzed products was closely related to that of the natural pheromone. The capillary GC-MS data of the hydrolyzed active product coincided well with that of the natural pheromone, exhibiting the same retention time on capillary GC and the same pattern in the mass spectrum (Fig. 7-2).

The complete chemical structure of the hydrolyzed active product was determined as (*Z,Z,E*)-3,6,8-dodecatrien-1-ol by means of instrumental analyses. It was definitely the same as the natural trail pheromone.

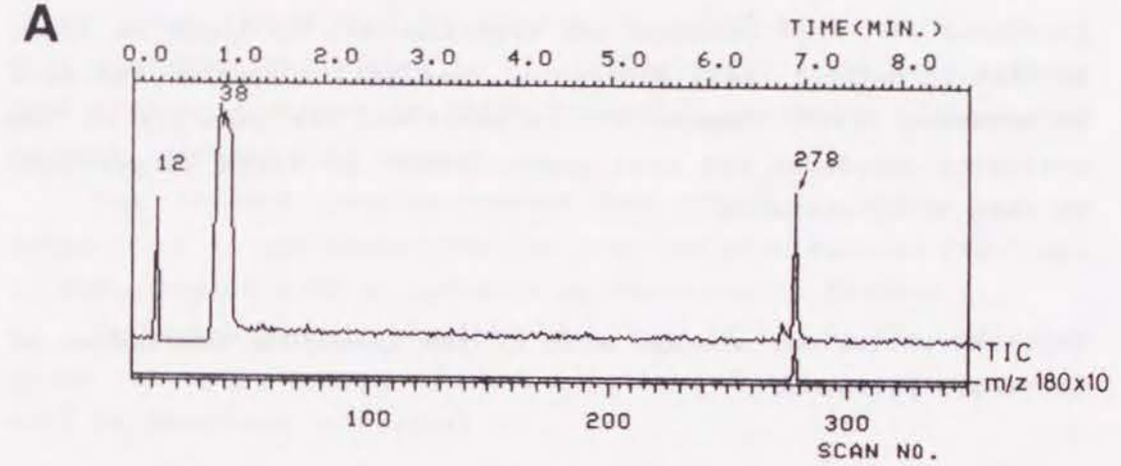


Fig. 7-2 Results of capillary GC-MS of the newly activated substance by alkaline hydrolysis
A: Total ion chromatogram(TIC) and the mass chromatogram(*m/z*:180) of the newly activated substance by alkaline hydrolysis
The arrow indicate the active peak(Scan No.278)
B: Mass spectrum of the active peak(Scan No.278)

Amount of the activated trail pheromone per individual worker termite was estimated to be about one nanogram on the basis of GC analysis. The quantity of the isolated activated pheromone of *C. formosanus* was approximately 20 times as large as that of natural trail pheromone, that well supported the data of bioassay (text, Chapter 3). In addition, the quantity of the activated pheromone was also approximately 20 times larger than as that of *R. speratus*.

7.3.4 Morphological storage site of the precursor candidates of the trail pheromone

Results of trail-following bioassay for each extract are shown in Table 7-2. Trail-following activity of the hexane extracts from whole bodies was highly activated by alkaline hydrolysis. The activities of the hydrolyzed hexane extracts (0.00025 MEWE) were about 20 times as high as that of the hexane extracts before alkaline hydrolysis (natural pheromone: 0.005 MEWE) as indicated in the bioassays. The results apparently suggested that the extracts prepared by soaking for a short time also contained a precursor candidates.

On the basis of the bioassay results for the five parts, the extracts from the fifth-sternites showed higher activity before (0.005 MEWE) and after hydrolysis (0.00025 MEWE) than those from other sections (more than 0.05 MEWE or no activity). The activity of the whole body extracts before and after hydrolysis was identical with that of the fifth-sternite extracts. The results clearly indicated that both pheromone and precursor candidates were stored in a sternal gland.

As any conspicuous difference was not observed between the two termite colonies (F and I), the precursor candidates were considered to be maintained at certain quantity level in a sternal gland for a year or longer even when the termite colony

was fed with cellulose and water. The fact suggested that the termite could prepare the precursor candidates from cellulose as a sole nutrient source. Further investigation (*e.g.* a experiment using tracer technique) is needed in order to confirm whether this precursor candidates is biosynthesized *via de novo* pathway from cellulose source or esterified with pheromone alcohol *via* salvage pathway like process.

The present results suggest that DTE-OH is stored as an ester prior to the transformation into the pheromone as previously demonstrated with *R. speratus* as described in Chapter 6.

Determination of chemical structure of the acidic moieties which is combined with alcohol moieties of the trail pheromone will be described in Chapter 8.

Table 7-2 Results of trail-following bioassay for the extracts from four morphological parts of *C. formosanus*

Test parts	Test worker termites			
	Group a[a]		Group b[b]	
	Activity[c]	Activity[d] after hydrolysis	Activity	Activity after hydrolysis
Whole body	0.005[e]	0.00025	0.005	0.00025
Head	-[f]	-	-	-
Digestive tube	1	1	1	1
Abdominal fifth-sternites	0.005	0.00025	0.005	0.00025
Other cuticular segment	0.5	0.5	0.5	0.5
Remaining portion	0.1	0.1	0.1	0.1

a: The worker newly collected from the laboratory nest.
b: The worker fed only with moistened filter paper as food source.
c: The trail-following activity as MEWE (WE/15 cm-trail).
d: The trail-following activity as MEWE (WE/15 cm-trail) after alkaline (2% KOH/MeOH) hydrolysis.
e: Activity as MEWE (WE/15 cm-trail).
f: No response or insufficient response for trail-following.

7.4 Summary

On the basis of the resent result of which trail pheromone precursors existed in *Reticulitermes speratus*, *Coptotermes formosanus* was served for examining the presence of the similar substances.

Whole body *n*-hexane extracts from 310,000 worker termites of *C. formosanus* were fractionated by silica-gel column chromatography. Trail-following activity was measured before and after the hydrolysis of the fractions with KOH/MeOH. Bioassay showed that the activity of hydrolyzed products was approximately 20 times higher than that of the original hexane extracts. Chemical analyses revealed that the complete structure of the hydrolyzed product was coincident with that of the natural pheromone, (*Z,Z,E*)-3,6,8-dodecatrien-1-ol.

As the trail pheromone was collected by alkaline hydrolysis of these different fractions, it was probable that the precursor candidates were stored in *Coptotermes* termite bodies as esterified forms.

Chapter 8 : Isolation and Identification of Trail Pheromone Precursors from *Coptotermes formosanus* Shiraki

8.1 Introduction

Whole body extracts of the termite, *C. formosanus* were served for examining the presence of trail pheromone precursor. The hydrolyzed substance of several fractions prepared by silica-gel column chromatography showed high trail-following activity, and was exactly the same as DTE-OH as described in Chapter 7. The fact suggest that the trail pheromone of *C. formosanus* could be stored in termite bodies as esterified forms, which is similar to the case of *R. speratus* (Chapter 6).

The main purpose of this Chapter, therefore, was to isolate the trail pheromone precursors from *C. formosanus* and to identify them (Tokoro *et al.* 1992c).

8.2 Materials and Methods

8.2.1 Test termites and bioassay methods.

Test termites and bioassay methods were already described in Chapter 7.

8.2.2 Chemicals.

Standard dodecanoic fatty acid esters were prepared by esterification reaction with *p*-toluenesulfonic acid as catalyst, and purified by the high-performance liquid chromatograph (HPLC). All other chemicals were the same as Chapters 1 and 3.

8.2.3 Apparatus.

One of the GC-MS was the same as described in Chapter 3, and another one was a model GC-14A gas chromatograph combined with a model QP-1000-EX mass spectrometer (Shimadzu, Kyoto, Japan). All other apparatus was the same as described in Chapter 7.

8.2.4 Confirmation of the precursor candidates

Since precursor candidates would not always show trail following activity, existence of precursor candidates during the purification procedures was examined after alkaline hydrolysis by means of bioassay. Methods of hydrolysis and bioassay were described previously.

8.2.5 Isolation of the precursor candidates.

Methods of extraction and silica-gel column chromatography were described in Chapter 7. On the basis of the elution pattern of the silica-gel column chromatography, there were at least three components of precursor candidates : component group-1 (CG-1: Fr. No. 4,5) ; component group-2 (CG-2: Fr. No. 7-10) ; component group-3 (CG-3: Fr. No. 12-15)(Chapter 7, Table 7-1). The CG-1 fractions were further purified by secondary silica-gel column chromatography in a similar manner (Fig. 8-1).

(A) Preparative HPLC

The active fractions after silica-gel column chromatography(ies) were further fractionated by HPLC. Normal phase HPLCs

were performed with Cosmosil 5SL analytical column and Cosmosil 10SL guard column, and reveres phase HPLCs were performed with Cosmosil 5C18-AR (ODS: 15 cm x 6 mm i.d., particle size 5μm; NTI) analytical column and Cosmosil 10C18-AR (ODS: 1 cm x 4.6 mm i.d., particle size 10 μm; NTI) guard column.

(a)CG-1 : HPLC (a)-1, normal phase with 2.5% diethyl ether (Er)/Hx eluants ; HPLC (a)-2, normal phase with 0.5% Er/Hx eluants

(b)CG-2 : HPLC (b)-1, normal phase with 15% Er/Hx eluants ; HPLC (b)-2, reverse phase with 50% acetonitrile (MeCN)/MeOH eluants.

(c)CG-3 : HPLC (c)-1, normal phase with 30% Er/Hx eluants ; HPLC (c)-2, reverse phase with 50% MeCN/MeOH eluants.

Flow rates of all HPLC preparation were 1.0 ml/min, and the active substance was detected by UV-VIS detector at 234 nm (It was depended on the conjugated double bond of the pheromone skeleton) and RI detector, and fractionated at each peak for modified Open-Field bioassay.

(B) Preparative wide bore capillary GC

All of precursor candidates after preparative HPLC were finally purified by capillary GC. A fused silica wide-bore capillary column was a CBP1-W12-300 (12 m x 0.53 mm i.d.: Shimadzu) coated with methyl silicon (coating width : 3 μm). Analytical condition was as follows : oven temperature increased at a rate of 20°C/min over the range of 180 - 300°C ; injection temperature at 320°C and detector temperature at 350°C ; carrier gas, helium at a velocity of approximately 60 cm/sec. The outlet gas was fractionated for each peak and the trail following activity after hydrolysis was determined.

8.2.6 Identification of the precursor candidates

(A) Capillary GC-MS

The active GC peak was analyzed by capillary GC-MS. The Cross-Linked Methyl Silicon fused silica capillary column (12.5 m x 0.2 mm i.d., 0.2 μm : HP) was used. The conditions were as follows. The oven temperature of GC increased at a rate of 15°C / min over the range of 200 - 300°C, and the injection temperature was 300°C, and the carrier gas was helium at a velocity of approximately 60 cm / sec. The ion source temperature was 200°C, and the ionization voltage of EI mass spectrometer was 70 eV. The CI mass spectrometry was performed with *iso*-buthane.

(B) Alkaline hydrolysis and methylation

A part of each component of precursor candidates were hydrolyzed with 2% KOH/MeOH as a similar manner of Chapter 6. Subsequently, each hydrolyzed product was methylated with 1-ethyl-3-*p*-tolyltriazene, subsequently, each product was washed with 1N-HCl and 1N-$NaHCO_3$ and distilled water. Each methylated product was also analyzed by capillary GC-MS as described above.

8.3 Results and Discussion

8.3.1 Results of isolation

In this experiment, it was examined to isolate the precursor candidate from the 5 % EtOAc/Hx fractions (CG-1). These fractions contained the fatty-acid esters including vast quantity of triglyceride. Subsequently, based on the results of the secondary silica-gel column chromatography, the precursor candidate was

eluted mainly from 2 % EtOAc/Hx (Fig. 8-1).

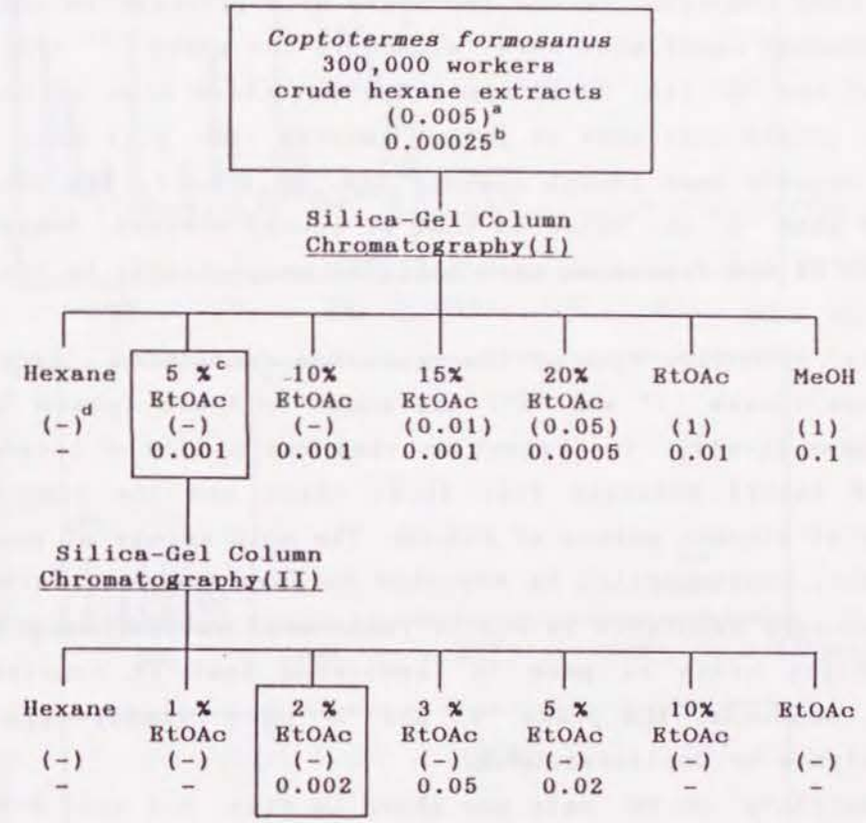


Fig. 8-1 Results of siliga-gel column chromatographies of the whole body extracts from *C. formosanus*
a: The signs and values (MEWE) in parentheses are the trail-following activity before hydrolysis
b: The signs and values are the trail-following activity (MEWE) after hydrolysis
c: Percentage by volume of EtOAc/Hx as eluants
d: Not effective (> MEWE 10)

On the results of HPLC preparation, the precursor candidates were eluted in a peak at the time of retention around 3 min on HPLC 1. It seemed that the retention time of the precursor candidates was earlier than that of the triglycerides contained in the same fraction. On the secondary HPLC preparation (HPLC 2), the precursor candidates were eluted in the peaks "1" (tR. 38-43 min) and the "2" (tR. 47-53 min). The retention time of the peak "1" was longer than that of lauryl laurate (tR. 22.3 min), and a little shorter than lauryl acetate (tR. 48.5 min). The retention time of peak "2" was close to that of lauryl acetate. Subsequently, both of the fractions were analyzed respectively by capillary GC.

The retention time of the precursor candidates from both reactions (peaks "1" and "2") was about 10.5 min (peaks "a" and "b", respectively). The retention time was a little longer than that of lauryl stearate (tR. 10.2) which had the same carbon numbers of alcohol moiety of DTE-OH. The acid moiety of precursor candidate, consequently, is expected to have eighteen carbons if the precursor candidate is one of fatty-acid esters. However, the half height width of peak "a" indicated that it consisted of plural compounds. The peaks "a" and "b" were finally served for the analysis by Capillary GC-MS.

Capillary GC-MS data are shown in Figs. 8-2 and 8-3. The components of the peak "a" were separated into two component peaks by the total ion chromatogram (TIC: peaks "X" and "Y" in Fig. 8-2,(A)). The electron impact (EI) mass spectrum of the peak "X" (Scan No. 361, Fig. 8-2(B)) showed a molecular ion at *m/z* 446 (*ca.* 6%). It also showed a diagnostic base peak ion at *m/z* 162 (M-284) which came from McLafferty rearrangement, (that is, ester cleavage of DTE-acetate) and the prominent diagnostic fragment ions of DTE-OH *m/z* 91, 105, 119, 133 were observed. The present analysis indicated that the component of peak "X" corresponded to dodecatrienyl stearate.

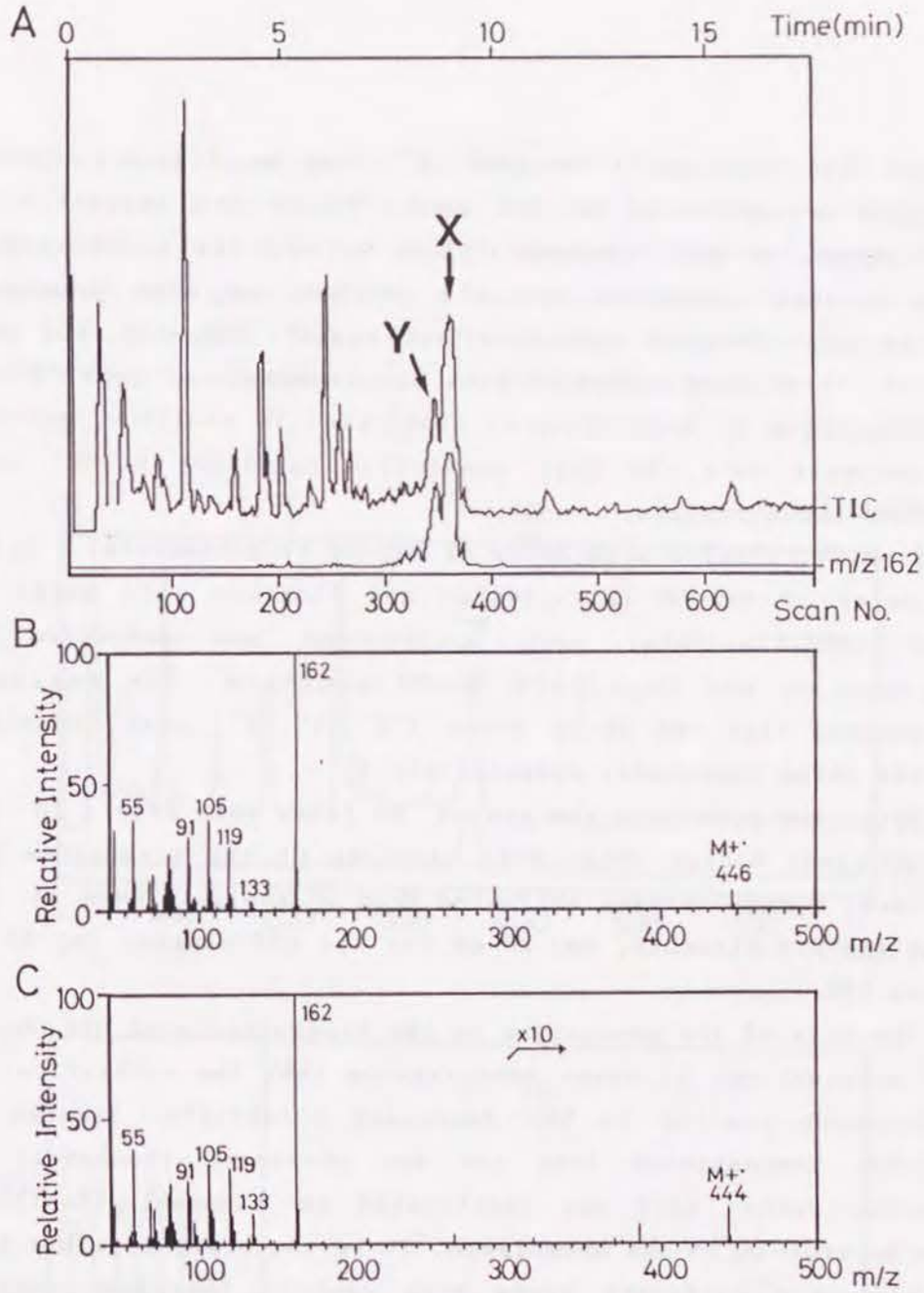


Fig. 8-2 : Results of capillary GC-MS analyses.
A: Total ion chromatogram(TIC) and mass chromatogram of the active fractions
Arrows indicate the active peaks (peak X : Scan No.361; peak Y : Scan No.346)
B: Mass spectrum of the peak X(Scan No.361)
C: Mass spectrum of the peak Y(Scan No.346)

The mass spectrum of the peak "Z" (Scan No. 353, Fig. 8-3), which was corresponding to the peak "b" of the capillary GC chromatogram, showed a molecular ion at *m/z* 442 (1.6%) and a diagnostic base peak ion *m/z* 162 (M-280) and also prominent characteristic fragment ions of DTE-OH *m/z* 91, 105, 119, 133 were observed. These data indicated that the component of peak "Z" is a corresponding to dodecatrienyl linoleate. In addition, precise mass analysis data (by high resolution capillary GC-MS) well supported these results.

Finally, stearic acid ester of DTE-OH (DTE-stearate), oleic acid ester of DTE-OH (DTE-oleate) and linoleic acid ester of DTE-OH (DTE-linoleate) were synthesized and subjected to capillary GC and capillary GC-MS analyses. The analyses demonstrated that the three peaks ("X","Y","Z") were identical with the three compounds, respectively.

Thus, the precursor are stored as fatty acid esters in the *C. formosanus* bodies (Fig. 8-4). Amounts of the precursors per individual worker termite estimated from GC analysis were *ca.* 100 pg for the DTE-stearate, *ca.* 25 pg for the DTE-oleate, *ca.* 25 pg for the DTE-linoleate.

The role of the precursors in the biosynthesis of DTE-OH is under examination. It seems advantageous that the esterification of pheromone results in the decreased volatility. Yamaoka et al.(1985) demonstrated that the sex pheromone (bombykol) of silkworm, *Bombyx mori* was inactivated as bombykol linolenate bound to protein in the haemolymph. It is therefore possible that the precursor candidate binds with protein. Although further investigation is needed in order to account for the significance of esterification of the pheromone, it may be related to the transportation of the pheromone in the termite bodies if the precursor binds a protein. Biosynthesizing organ of the precursors not specified in the present investigation because whole termite bodies were employed for extraction. Therefore, it is a great scientific interest to determine the organs for

The mass spectrum of the peak "Y" (Scan No. 346, Fig. 8-2,(C)) showed a molecular ion at *m/z* 444 (1.4%) and a diagnostic base peak ion at *m/z* 162 (M-282) derived from McLafferty rearrangement, and the prominent diagnostic fragment ions of DTE-OH were also present. These data indicated that the component of peak "Y" was corresponding to dodecatrienyl oleate.

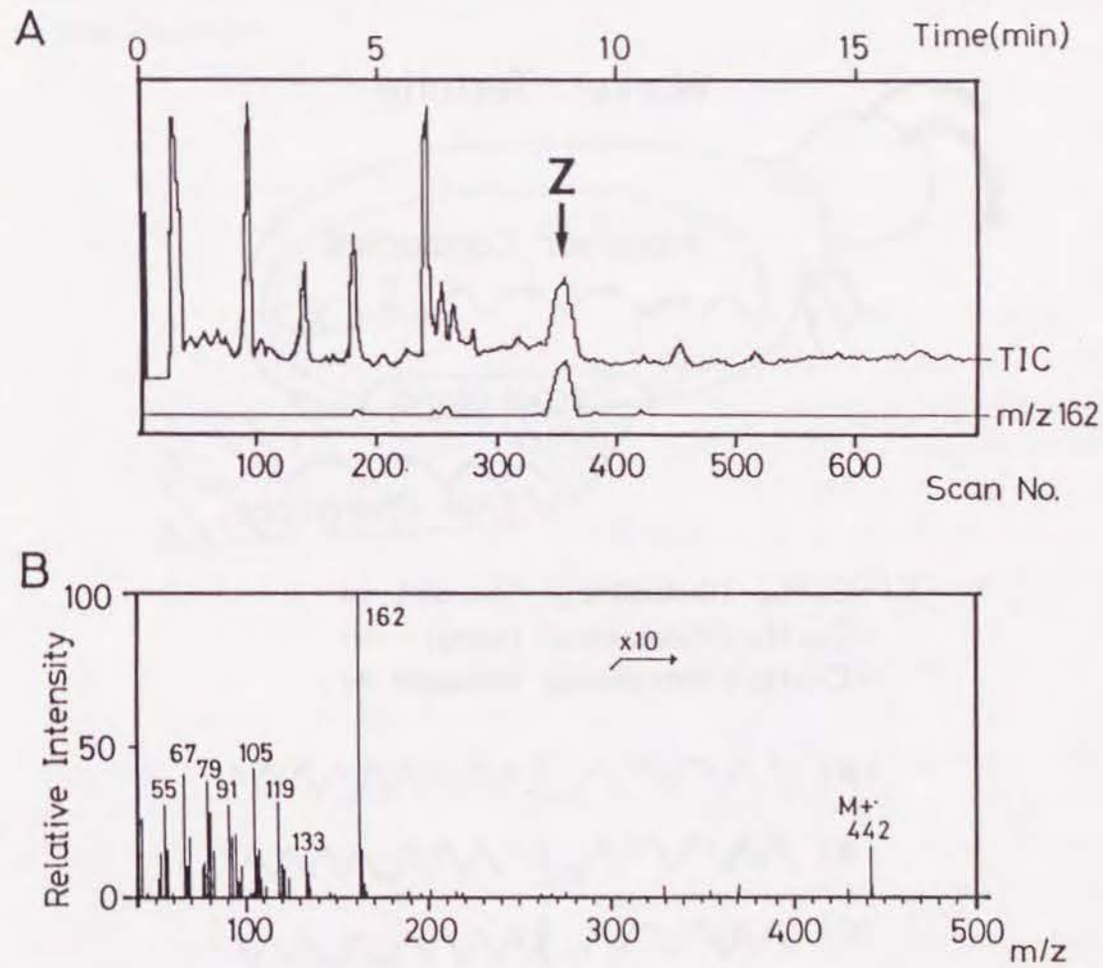
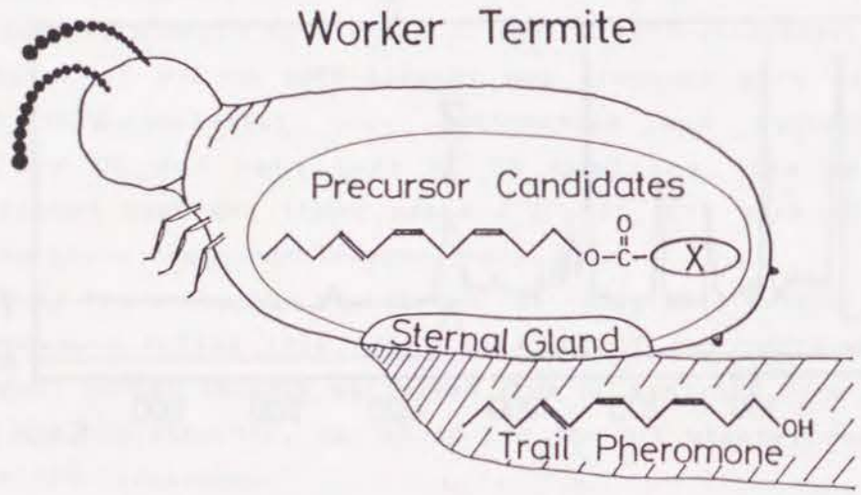


Fig. 8-3 : Results of capillary GC-MS analyses.
A: Total ion chromatogram and mass chromatogram of the active fraction
Arrows indicate the active peak (peak Z : Scan No.353)
B: Mass spectrum of the peak Z(Scan No.353)

biosynthesizing and storing the precursors.

Based on the results of the first silica-gel column chromatography and HPLC, it seems possible that there exist other kinds of precursor candidates of the trail pheromone in the hexane extracts, those are CG-2 and CG-3 (Chapter 7), it remains to be proved that the existence of other precursor candidates and entirely reveal the supplementary system of the trail pheromone.



X : $-C_{17}H_{35}$ (Dodecatrienyl Stearate) ··(A)
$-C_{17:1}H_{33}$ (Dodecatrienyl Oleate)·····(B)
$-C_{17:2}H_{31}$ (Dodecatrienyl Linoleate)··(C)

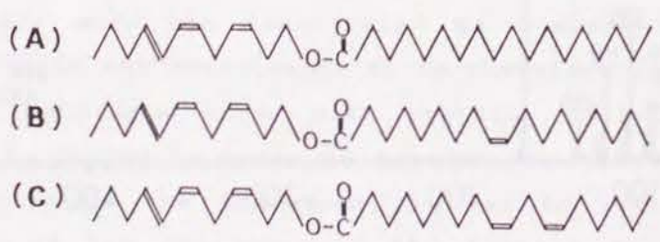


Fig. 8-4 : Storage of the trail pheromone precursors in the sternal gland

8.4 Summary

Whole body extracts of the termite, *Coptotermes formosanus* Shiraki were served for examining the presence of trail pheromone precursor(s). Three trail pheromone precursors were isolated using various chromatographic methods in conjunction with bioassay. Those were identified as dodecatrienyl stearate, dodecatrienyl oleate and dodecatrienyl linoleate by capillary GC-MS analyses.

CONCLUSION

With regard to the strategic approach to the mystery of termite biology, well-designed bioassay and precise chemical analysis are definitely indispensable for the assessing the authenticity of trail pheromones.

The presence of a termite trail pheromone was positively demonstrated in modified Open-Field bioassay and choice test in which the tested termites were carefully and uniformly conditioned prior to serving to the tests as described in PART I. Trail-following activities of the extracts from both whole body and sternal gland which is supposed to be a morphological organ to contain or excrete a trail pheromone were proven to be a equivalent in the bioassay. The results, therefore, appear to lead a conclusion that sternal gland is a region which is directly involved in trail-following behavior of the two Japanese rhinotermitid termites, *Reticulitermes speratus* (Kolbe) and *Coptotermes formosanus* Shiraki. In addition, modifications of the bioassay techniques were rational because behaving characteristics of the tested termite species were critically considered.

In PART II, trail pheromones were investigated mainly from chemical viewpoint in terms of isolation and identification. A great number of termites were collected and preconditioned with care prior to extraction and fractionation by various chemical operations. Novel combined chemical techniques, (*e.g.* capillary GC-MS and capillary GC-FTIR with the microchemical reactions of acetylation, partial hydrogenation, ozonolysis) enabled to elucidate the chemical structure of the trail pheromone directly from the extracts of termites. Biological tests were concurrently conducted to evaluate the trail-following activity levels of the candidates fractions. The results clearly

demonstrated that the isolated trail pheromone from both termite species was DTE-OH. Comparison of the present results with those of early workers suggested that DTE-OH was common to the members of family Rhinotermitidae.

A minor component which showed some trail-following activity was obtained from *C. formosanus* and chemically identified as (*Z,E,E*)-DTE-OH, whereas such component was undetectable from *R. speratus*. Species specificity of the trail pheromone was not conspicuously demonstrated in the choice tests using both termite species. However, it is worthy to note that termite can distinguish a small difference in concentrations of active substances even if one is 2-3 times higher in concentration than the other in the choice test since the ability seems to be related to orientation activity of termites through the concentration gradient of attractive materials.

In PART III, trail pheromone precursors were isolated from both termite species, and identified those chemical structures of *C. formosanus*. Fractions which were prepared from whole body extracts and mainly contained fatty acid esters showed the much increased trail-following activity after activation by alkaline hydrolysis. This strongly suggested that the precursor candidate could be stored in termite bodies as an esterified form. Precursor-holding system is advantageous to the termites as they can maintain its activity for a longer period of time and can utilize it at any time when necessary. As for *C. formosanus*, three groups of precursor candidates were recognized and three precursors of a group were identified as (*Z,Z,E*)-3,6,8-dodecatrienyl stearate, (*Z,Z,E*)-3,6,8-dodecatrienyl oleate and (*Z,Z,E*)-3,6,8-dodecatrienyl linoleate.

It seems necessary to chemically demonstrate that the substance deposited by termites is the same that contained in the sternal gland. With the development of analytical instruments such as capillary GC-MS-HR-SIM in these years, it will become relatively easy to analyze a small amount of sample under the crude conditions. Trail pheromone precursors were first isolated and identified by various instrumental means *e.g.* HPLC, capillary GC, capillary GC-MS and micro chemical reactions *e.g.* alkaline hydrolysis, methylation, in conjunction with biological tests. Further works will be giving us a clearer image of termite trail pheromone in the near future, although the present investigations could contribute to the addition of new knowledge to the trail pheromone of Japanese rhinotermitid termites.

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