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Isolation and Primary Structure of Trail Pheromone of the Termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae)[*1]

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Abstract——The trail pheromone of the termite, *Coptotermes formosanus* Shiraki was isolated using various chromatographic methods in conjunction with bioassay, and the primary structure of the pheromone was confirmed by capillary GC-MS analysis. Approximately 200,000 workers of the termite were extracted with *n*-hexane. The extract was fractionated by various chromatographic procedure so that a trace amount of the active substance could be isolated for identification. Capillary GC-MS analysis of this compound demonstrated that a primary chemical structure of the pheromone identified was similar to normal chain of dodecatrien-1-ol isolated from the termite of genus *Reticulitermes*.

Key words: termite, *Coptotermes formosanus* Shiraki, trail pheromone, isolation, dodecatrien-1-ol

1. Introduction

In Japan, two termites, (*Coptotermes formosanus* Shiraki and *Reticulitermes speratus* (Kolbe)) are the major serious wood pests. It is particularly interesting to investigate their physiological and ecological roles from chemical veiwpoints of termite pheromones.

Only two compounds so far have been identified as trail pheromones of termites[1~5]. Although Matsumura *et al.*[2,3] isolated a trail pheromone from whole body extracts of *Reticulitermes virginicus* (Banks) (Rhinotermitidae), they failed to determine the complete chemical structure because the paucity of the isolated pheromone in amounts. However, they found that a substance isolated from wood decayed by the fungus, *Gloeophyllum trabeum* (Pers. ex. Fr.) Murr., was chemically similar to the trail pheromone of *R. virginicus*, and identified it as *cis*-3, *cis*-6, *trans*

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-8-dodecatrien-1-ol[2]. They finally concluded that the compound was identical with the trail pheromone of *R. virginicus*[4].

Quite recently, we isolated the trail pheromone from another termite (*R. speratus* (Kolbe); Rhinotermitidae) and identified it as *cis*-3, *cis*-6, *trans*-8-dodecatrien-1-ol (DTE-OH) which has exactly the same structure as that from *R. virginicus*[6].

It was reported that, this pheromone, DTE-OH exhibits a trail-following activity to other species, such as *R. flavipes* (Kollar), *R. hesperus* Banks, *R. tibialis* Banks in the genus *Reticulitermes* and *C. formosanus* Shiraki in the genus *Coptotermes* which all belong to the family Rhinotermitidae[7~9]. These reports indicate that DTE-OH show broad species specificity. It was demonstrated that the analogues of DTE-OH also induce the high trail-following activity of these species. Howevr, we suspect that strength of the activity changes due to a possible effect of multi-components of trail pheromones[7,10~12]. It is, therefore, important to examine whether *C. formosanus* actually secrete the DTE-OH or not.

In this investigation, we tried to isolate and identify the trail pheromone from the termite *C. formosanus* for better understanding of nature of the trail pheromone occurring within the family of Rhinotermitidae.

2. Materials and Methods

2.1 Termite

Colonies of *C. formosanus* were collected in Wakayama city (Japan) and kept in our laboratory (in Uji city) for about five years with Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) as food source at 28±2°C and ca. 80% relative humidity. Only undifferertiated pseudergates of *C. formosanus* (workers; ca. 561 g, ca. 200,000 in number) were selected for this investigation.

2.2 Chemicals

Cis-3, *cis*-6-dodecadien-1-ol was supplied by professor Dr. G. D. Prestwich (State University of New York at Stony Brook). Isomers of 6,8-dodecadien-1-ol were supplied by Dr. T. Ando (Tokyo University of Agricurture and Technology). All other reagents were purchased from Nacalai Tesque. INC. (Kyoto, Japan).

2.3 Extraction

From 5,000 to 20,000 workers of the test termite were collected each time. Test individual workers had been fed with moistened filter paper as food source for about five days to allow them before extraction to replace the existing intestinal materials with filter peper. It was confirmed by microscopic observation that the intestinal materials of the termite were completely substituted with filter paper within five days. The termites were then 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 615 g, ca. 200,000 workers.

2.4 Bioassay

A madification of the Howard *et al.*[8] Open-Field bioassay was used to establish threshold response levels for trail-following. 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 fine quality paper with a 5 μl-micropipette. After evaporation of the solvent, a plastic cylinder (1 cm × 1.5 cm i.d.) was placed on the paper at a left side of the test arena. Two openings of the plastic cylinder directed the termite toward the test arena. A worker termite was then introduced into the cylinder, and red colored Petri dish lid 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 streak for two minutes, it was considered that a "basic activity" was induced. Nine replications were done for each sample. When three out of nine or more of the test worker termites showed the basic activity, the sample was evaluated to be active. Ten-fold dilution series of each sample were employed to determine the "minimum effective worker equivalent" (MEWE).

2.5 Isolation

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 *n*-hxane / ethyl acetate (EtOAc) eluants. Five hundred grams of silica gel (70∼230 mesh) was packed into glass column (1 m × 6 cm i.d.), with the halved crude oil placed on the top of the silica gel. The column was eluted with *n*-hexane / 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 hexane and EtOAc. It was finally separated into 21 fractions (1 liter each).

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 *n*-hexane / EtOAc in the similar manner ($AgNO_3$ silica gel: ca. 7 g; glass column: 30 cm × 1 cm i.d.).

The active fractions subdivided were further fractionated by HPLC with *n*-hexane/EtOAc (9/1), 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 next step of the purification was carried out by GC with a nonpolar column and consecutively by a polar column. 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 wide bore columns, the outlet gas was fractionated for each peak and the trail following activity was determined.

Finally, the active peak characterized by GC was analyzed by capillary GC-MS. The ion source temperature was 200°C, and the ionization voltage was 70 eV. Samples were injected with a Grob type splitless injector in 2 μl of *n*-hexane. The oven temperature increased at a rate of 20°C/min over the range of 80~180°C, and the injection and the detector temperature was 250°C, and the carrier gas, helium at a velocity of approximately 40 cm/sec.

2.6 Apparatus

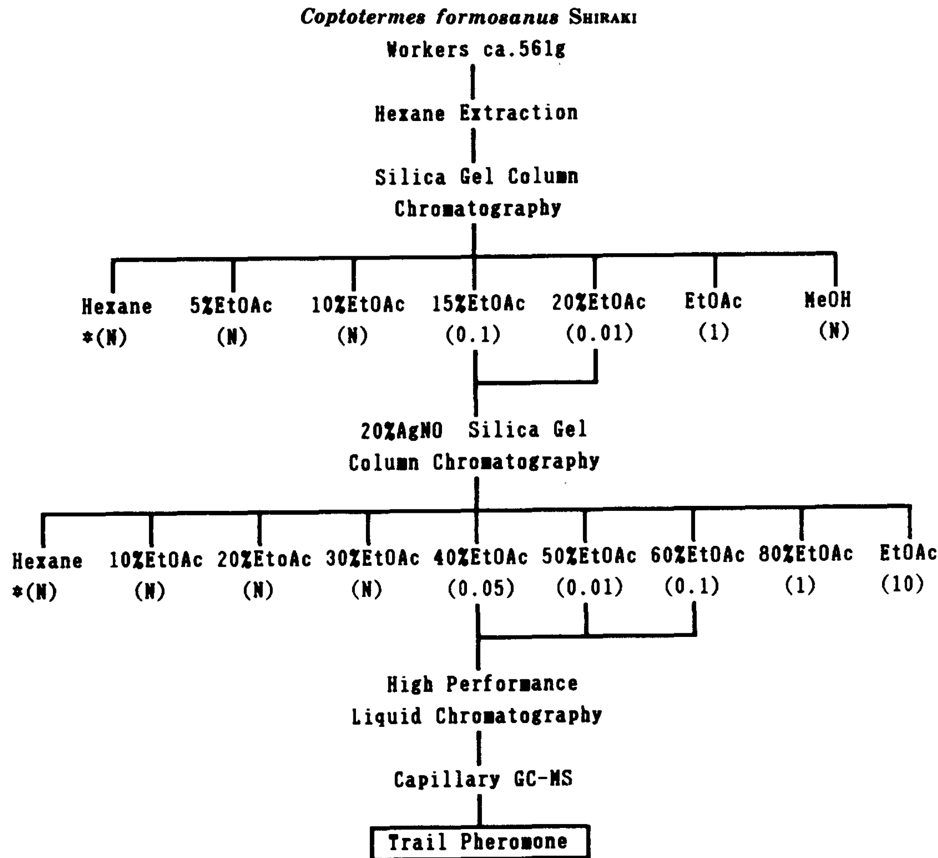
The high performance liquid chromatograph (HPLC) was Model LC-5A (Shimadzu) pump connected with Model UVIDEC-100II detector (Jasco, Tokyo, Japan). The analytical column used a Cosmosil 5SL (silica gel, 25 cm × 4.6 mm i.d., particle size 5 μm, Nacalai tesque) with a Cosmosil 10SL (5 cm × 4.6 mm i.d., particle size 10 μm) for the guard 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 CBP1-S25 (25 m × 0.33 mm i.d.), CBP1-W12 (12 m × 0.53 mm i.d.) <non polar columns, chemically bonded with methyl silicone>, CBP20-S25 (25 m × 0.33 mm i.d.), CBP20-W12 (12 m × 0.53 mm i.d.) <polar columns, chemically bonded with polyethylene glycol>.

The gas chromatograph-mass spectrometer (GC-MS) was a Model 5970 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 capillary column (Shimadzu CBP 20 M, 25 m × 0.25 mm i.d.) was used with this system.

3. Results and Discussion

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



Scheme 1. The isolation process and trail-following activity of the trail pheromone of *C.formosanus.*
*: activity estimated by bioassay (values in bracket mean minimum effective worker equivalent per 15 cm trail and, N means absence of activity.)

investigation all the termites employed were once fed by filter paper as described in Materials and Methods, 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[13]. Therefore, detected trail-following activity definitely did not derive from the decayed wood ingested by termites as indicated by previous investigators[2,3,14~16].

In addition, results of the trail following activity bioassays wich crude hexane extracts are shown in Table 1. The previous investigators have shown that trail-

Table 1. Results of Trail-following Bioassays against Crude Hexane Extracts

WE[a)]/15 cm	<	0.001	0.005	0.01	0.05	0.1	1	10	100	<
number of workers trail-following per 30 workers	0	2	22	26	27	30	29	25	6	0

a) Worker equivalent

following activity is induced by the trail pheromone within a limited range of "worker equivalents" (WE). Termites show little or no response outside of the range of WE[1,12]. *C. formosanus* was not exceptional to the rule.

As shown in Table 1, the hexane extracts exhibited marvelous activities ranging from 10 to 0.005 WE/15 cm. The MEWE was thus evaluated as 0.005 WE/15 cm. Then, other samples were assayed using nine workers each, and it was considered that basic activity was present if 3 or more individuals responded positively. The MEWE at which each sample elicited activity also was determined for the 10-fold dilution series. Consequently the relative strength of trail-following activity was compared on the basis of the MEWE.

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*.

Based on the results of the silica gel column chromatography, the trail pheromone was eluted mainly in the range of 15~20% EtOAc / *n*-hexane (Scheme 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 Scheme 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 / *n*-hexane.

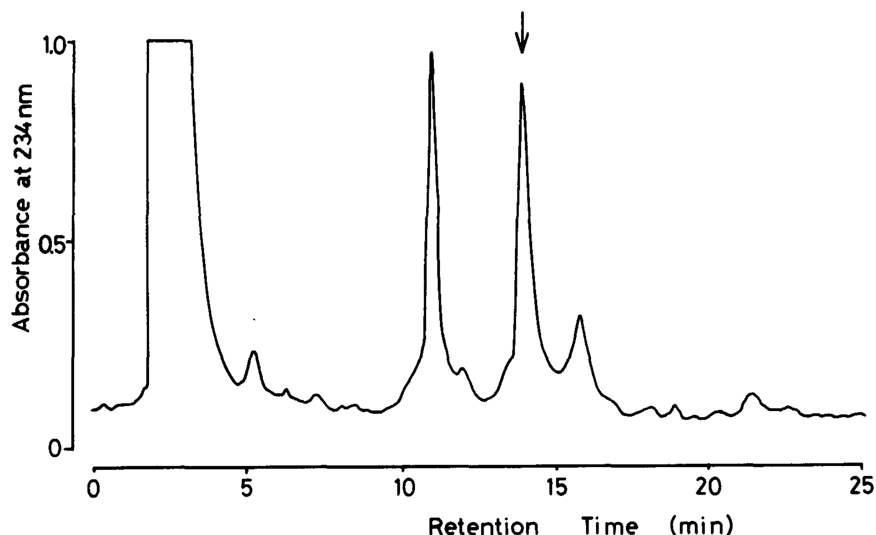


Fig. 1. HPLC chromatogram of the active fractions following $AgNO_3$ silica gel chromatography. (the arrow indicates the active peak: tR14.5)

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 ca. 14.5 min, confirming the presence of conjugated double bonds (Fig. 1).

Results of GC are shown in Fig. 2. 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 (Fig. 2, A). Relative retention time of standard normal alco-hols are in the order of carbon numbers, a little longer on the homologues with double bonds, and a little shorter on the homologues with conjugated double bonds. However, the variations by double bonds or conjugated double bonds are

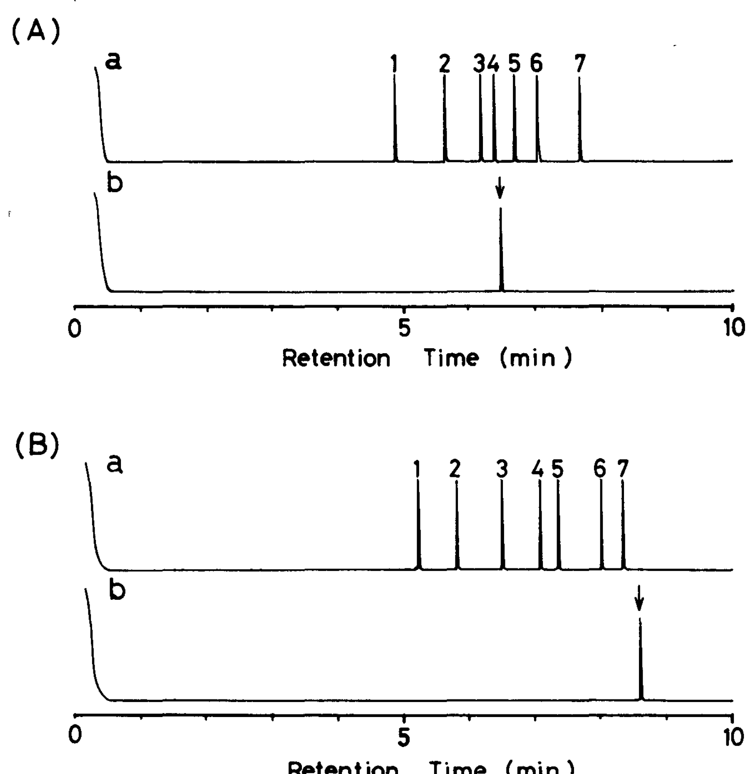


Fig. 2. Gas chromatograms of the trail pheromone from *C. formosanus*.
(A) Nonpolar column.
a. Authentic alcohols; 1: $C_{10}H_{21}OH$ (tR. 4.9), 2: $C_{11}H_{23}OH$ (tR. 5.7), 3: (3Z, 6Z)-$C_{12}H_{21}OH$ (tR. 6.2), 4: $C_{12}H_{25}OH$ (tR. 6.4), 5: (6Z, 8E)-$C_{12}H_{21}OH$ (tR. 6.7), 6: $C_{13}H_{27}OH$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

less than those caused by carbon numbers. If the pheromone is a kind of straight-chained normal alcohol, the pheromone has twelve carbons. Secondly, on using a polar column, the retention time was ca. 8.48 min, slightly more than that of 1-tetradecanol (Fig. 2, B). Relative retention time of standard normal alcohols are also influenced in the order of carbon numbers and a little longer on the homologues with double bonds, but rather longer on the homologues with conjugated double bonds. The latter variation are more than those by carbon numbers. The two different GC analyses suggested that this pheromone could be a twelvecarbons alcohol with conjugated double bonds.

Capillary GC-MS data are shown in Fig. 3. The spectrum shows the prominent molecular ion peak at *m/z* 180 and the series of characteristic fragment ion peaks at *m/z* 91, 105, 119, 133. These peaks might correspond to the increasing 14 mass units of the carbon skeleton of a trienyl alcohol following dehydration, which can be expressed as the 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

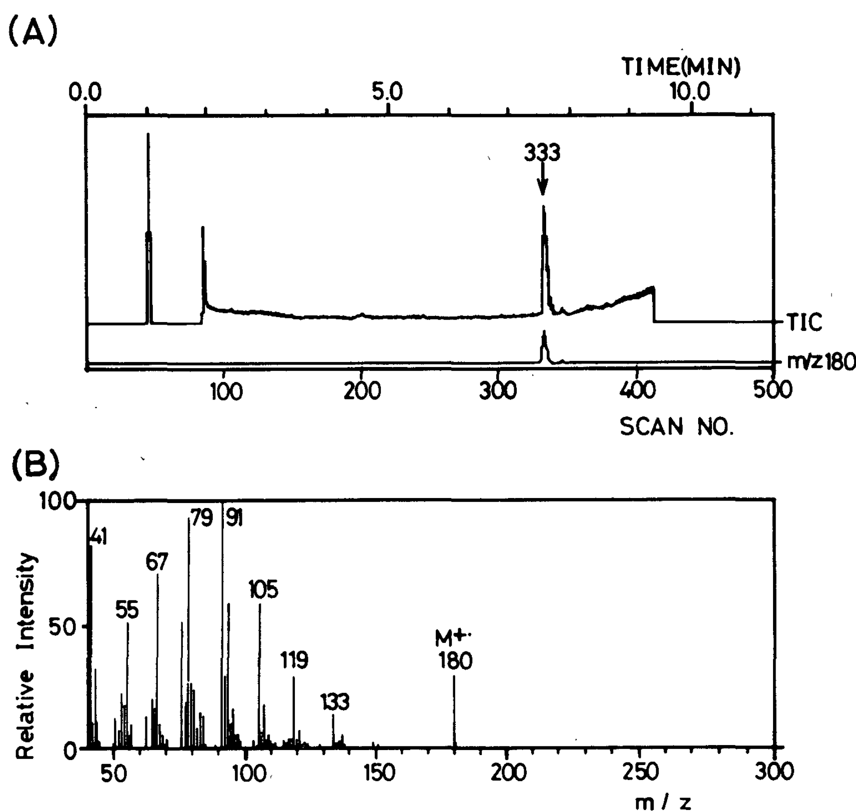


Fig. 3. GC-MS data of the trail pheromone from *C. formosanus*.
(A) Total ion chromatogram and mass chromatograms. (the arrow indicates the active peak: scan no. 333)
(B) Mass spectrum of the trail pheromone.

prominent molecular ion peaks, and primary alcohols seldom show strong molecular ion peaks supports this conclusion.

As discussed above, 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 pheromone isolated was estimated on the basis of capillary GC analysis to be approximately 10 μg.

Confirmation of the complete structure of the trail pheromone has been investigated in our laboratory by means of capillary GC-MS and capillary GC-FTIR analyses combined with micro chemical reactions, and the results obtained so far seem to demonstrate. That the trail pheromone is identical with DTE-OH.

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 pheromone 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.

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References

1) B.P. MOORE: *Nature,* **211**, 746-747 (1966).
2) F. MATSUMURA, H.C. COPPEL and A. TAI: *Nature*, **219**, 963 (1968).
3) F. MATSUMURA, H.C. COPPEL and A. TAI: *J. Econ. Entomol.*, **62**, 599-603 (1969).
4) A. TAI, F. MATSUMURA and H.C. COPPEL: *J. Org. Chem*, **34**, 2180-2182 (1969).
5) A.J. BIRCH, W.V. BROWN, J.E.T. CORRIE and B.P. MOORE: *J. Chem. Soc. Perkin. Trans. I*, **1**, 2653 (1972).
6) R. YAMAOKA, M. TOKORO and K. HAYASHIYA: *J. Chromatogr.*, **399**, 259-267 (1987).
7) F. MATSUMURA, D.M. JEWETT and H.C. COPPEL: *J. Econ. Entomol.*, **65**, 600-602 (1972).
8) R. HOWARD, F. MATSUMURA and H.C. COPPEL: *J. Chem. Ecol.*, **2**, 147-166 (1976).
9) J.K. GRACE, D.L. WOOD and G.W. FRANKIE: *J. Chem. Ecol.*, **14**, 653-667 (1988).
10) A. TAI, F. MATSUMURA and H.C. COPELL: *J. Insect. Physiol.*, **17**, 181-188 (1971).
11) M. KAIB, O. BRUINSMA and R.H. LEUTHHOLD: *J. Chem. Ecol.*, **8**, 1193-1205 (1982).
12) P. HALL and J.F.A. TRANIELLO: *J. Chem. Ecol.*, **11**, 1503-1513 (1985).

13) W.R. TSCHINKEL and P.G. CLOSE: *J. Insect. Physiol.* **19**, 707-through 721 (1973).
14) H. MATSUO and K. NISHIMOTO: *Material u. Organismen* **9**, 225-238 (1974).
15) H. MATSUO and K. NISHIMOTO: *Wood Research* No. 59/60, 40-48 (1976).
16) J.K. GRACE and W.W. WILCOX: *Pan-Pac. Entomol.* **64**, 243-249 (1988).