Mating Behavior of *Ascogaster reticulatus* WATANABE (Hymenoptera: Braconidae), an Egg-Larval Parasitoid of the Smaller Tea Tortrix, *Adoxophyes* sp. (Lepidoptera: Tortricidae) III. Identification of a Sex Pheromone

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A sex pheromone of *Ascogaster reticulatus* WATANABE (Hymenoptera: Braconidae) which is deposited on the substrate by females and elicits male antennal-searching response, was analyzed and characterized. The hexane extract of the virgin females was subjected to silica gel column chromatography. An active fraction (active at $3.3 \times 10^{-1}$–$10^8$ ng/line) was analyzed with gas chromatography (GLC) and GC-MS, and three major peaks were identified as citronellal (44%), hexadecanal (12%) and hexadecenal (43%). Double bond location at ninth position in hexadecenal was determined by GC-MS after dimethyl disulfide derivatization. Geometric isomers of 9-hexadecenal were both co-chromatographed on GLC and (Z)-9-hexadecenal was identical to the natural 9-hexadecenal. Bioassay with these authentic compounds showed that only (Z)-9-hexadecenal elicited pheromonal activity (active at $5 \times 10^{-4}$–5 ng/line). Therefore, (Z)-9-hexadecenal was identified as the sex pheromone of *A. reticulatus*.

Key words: *Ascogaster reticulatus*, *Adoxophyes* sp., parasitoid, sex pheromone, (Z)-9-hexadecenal

INTRODUCTION

In the case of solitary parasitoids, mate searching is an important aspect in their life history, which differs from gregarious or quasi-gregarious parasitoids (van den Assem, 1986). In the latter group, mates can easily be found in the area where they emerge.

*Ascogaster reticulatus* WATANABE is an egg-larval solitary endoparasitoid of the smaller tea tortrix, *Adoxophyes* sp. Takagi (1974) observed their behavior in the tea field and described that *A. reticulatus* mostly walks on the leaves and flies a short distance from leaf to leaf, which is different from another sympatric braconid *Apaneles adoxophyesi*.

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MINAMIKAWA that mostly flies between the ridges sometimes landing on leaves. Their habit of searching may reflect the function of the sex pheromone. The mating behavior of *A. reticulatus* was analyzed from behavioral observation, and the antennal response by male parasitoids was found to be elicited by the presence of a sex pheromone deposited on the substrate by walking females (KAMANO et al., 1989). The female hexane extract painted in a line on the bottom of a Petri dish caused males to follow along that line in antennal-searching behavior (KAMANO et al., 1989).

In this study, we purified the active compound with column chromatography and identified it by chemical analyses combined with bioassay, GLC, and GC-MS. The functions of the sex pheromone are also discussed in terms of the chemical properties of this compound.

MATERIALS AND METHODS

*Insects.* The parasitoids and their hosts were from our stock culture based on the rearing method by KAINOH and TAMAKI (1982) and KAINOH (1986). Emerged adults were sexed and 10 to 20 insects of each sex were kept in transparent plastic containers (15 cm dia., 12 cm ht.) in which honey and wet cotton were provided. The rearing of insects and the bioassay were conducted at 25 ± 1°C and 50 ± 20%, RH with 16L–8D photoperiod.

*Bioassay.* The bioassay of the extract or other purified samples was based on that of KAMANO et al. (1989). Five microliters of the sample was applied in a straight line (9 cm long) across the center of a 9-cm glass Petri dish. After the solvent was air-dried, a three- to four-day-old virgin male parasitoid was released into the dish that was then capped. The activity was scored based on the total walking distance within 3 min while responding with antennal-searching along the treated line as follows: Score 0: no response, 1: less than 9 cm, 2: more than 9 cm. Three to five males were used for each sample, and the average score was used to evaluate the activity.

*Purification of pheromone.* Virgin female parasitoids (1850) three- to ten-days old were anesthetized with CO₂ and dipped in hexane (200 µl/female) for a few minutes (less than 5 min) at room temperature. The extract was filtered with a glass filter and concentrated to 5 ml with a rotary evaporator. Concentrated virgin female extract [5 ml, 1850 female equivalent (FE)] was chromatographed on the 1st silica gel column (Wakogel C-200, 3 g, 12 mm ID × 5 cm in ht.) eluting in steps by a series of hexane/ether mixtures (100: 0, 95: 5, 90: 10, 75: 25, 50: 50, 0: 100 v/v, 10 ml each). Each fraction was diluted with hexane and subjected to bioassay (1.32 × 10⁻⁴ FE/line). The active fractions were combined and evaporated to dryness (1.3 mg) and diluted with 2 ml of hexane. An aliquot of 1 ml of the solution was further purified with the second silica gel column (Wakogel C-200) of the same size and eluted with 5 ml each of 1% ether/hexane. The activity of each eluate was monitored by bioassay (6.17 × 10⁻⁴ FE/line) after dilution with hexane and active fractions were analyzed with GLC as described below.

*Analysis.* The most active fraction (No. 6) from the second column chromatography was concentrated to 0.5 ml, 2 µl of which was injected into a purged-splitless GLC (Shimadzu GC-9A) fitted with a capillary column (fused silica, CP Wax 57 CB, 0.24 mm ID × 50 m, df=0.23 µm, Chrompack) at programmed conditions (100–200°C, 4°C/min). Mass spectra (MS) were obtained with a Hitachi M-80B gas chroma-
to graph-mass spectrometer at 70eV using an Al-Clad fused silica capillary column ('400' methyl silicone, 0.25 mm ID × 25 m, df=0.1 μm, Quadrex) at 30 ml/min He flow rate and at 100°C.

The double bond position of hexadecenal was determined by GC-MS analysis after dimethyl disulfide (DMDS) derivatization (Francis and Veland, 1981).

Dose response of (Z)- and (E)-9-hexadecenal. Each authentic compound of (Z)- and (E)-9-hexadecenal was diluted in a stepwise fashion with hexane and subjected to bioassay (5 × 10⁻⁵–5 ng/line).

Authentic chemicals. (Z)- and (E)-9-hexadecenal were kindly provided by Shin-Etsu Chemical Co., Ltd. and Dr. H. Sugie of National Institute of Agro-Environmental Sciences, respectively.

RESULTS

Dose response of the crude extract

The dose/response relationship of the crude pheromone extract is shown in Fig. 1. The activity appeared at 1.85 × 10⁻⁶ FE/line and became highest at 1.85 × 10⁻³ FE/line. Higher dosage decreased the activity until it ceased at 1.85 × 10⁻¹ FE/line and beyond.

Identification

In the 1st silica gel column chromatography, the antennal-searching activity of A. reticulatus was shown in the fractions No. 3 (avg score = 0.6) and No. 4 (avg score = 1.3). The other fractions had no activity.

The fraction No. 6 obtained from the 2nd silica gel column chromatography had the highest activity (avg score = 1.3) among the active fractions (No. 5–8) (avg score = 0.3–1.0) and gave three major peaks accompanied by several minor ones in GLC (Fig. 2). MS of peak 1 (Fig. 3-A) suggested that the structure is monoterpene [M⁺ m/z 154 (C₁₀H₁₈O), m/z 69, m/z 41 (base peak)]. Considering the chromatographic behavior on silica gel columns and on GLC, and diagnostic ions of MS, peak 1 was identified as citronellal. MS of peak 2 (tᵣ = 21.84 min) indicated a terpene-like pattern (highest ion m/z 222; base peak m/z 41, 69) but could not be characterized. MS of peak 3 (Fig. 3-B) showed a profile of n-alkene and gave the highest ion at m/z 222 (M−H₂O)⁺; which suggested hexadecanal (C₁₆H₃₂O). Commercially available hexadecanal (Wako Pure Chemical) was co-chromatographically identical to peak 3 (tᵣ = 25.23 min on CP wax

![Fig. 1. Dose/response curve of crude extract of A. reticulatus females.](image-url)
Fig. 2. Gas chromatogram of the purified fraction of a female *A. reticulatus* extract. Column: CP Wax 57 CB, fused silica; programmed temp: 100–200°C at 4°C/min. S: solvent. Peak numbers are referred to in the text.

Fig. 3. Mass spectra of major peaks in gas chromatogram (Fig. 2). A: peak 1, B: peak 3, C: peak 4

57). MS of peak 4 (Fig. 3-C) showed a profile of monounsaturated straight chain aldehyde [M** m/z 238 (C_{16}H_{32}O), (M–18)** m/z 220, (M–29)** m/z 209, (M–44)** m/z 194]. As to the double bond location, MS of the DMDS adducts showed molecular ion at m/z 332 and diagnostic ion peaks at m/z 145 and 187 indicative of the ninth position (Fig. 4). When each authentic isomer, (Z)- and (E)-9-hexadecenal, was cochromatographed with the combined active fraction (No. 3+4) from the 1st silica gel column chromatography, only (Z)-isomer enhanced the peak 4 (Z: *t_R* = 25.93 min and E: *t_R* = 25.83 min, respectively). The amount of the active compound was estimated to be 19 ng per female in GLC analyses.
Sex Pheromone of *Asagaster reticulatus*

![Figure 4](image1.png)

**Fig. 4.** Mass spectrum of DMDS adduct of peak 4.

![Figure 5](image2.png)

**Fig. 5.** Dose/response curves of (Z)- and (E)-9-hexadecenal.

**Evaluation of authentic chemicals as pheromone components**

Both citronellal and hexadecanal (5 × 10⁻⁵–5 ng/line) did not have any antennal-searching activity for males. Dose/response relationship of authentic (Z)- and (E)-9-hexadecenal showed only (Z)-isomer was active (Fig. 5). The activity of (Z)-isomer appeared at 5 × 10⁻⁴ ng/line and the highest activity was obtained in the range of 5 × 10⁻⁸ to 5 × 10⁻¹ ng/line. The activity decreased to 35% of the maximum response at 5 ng/line.

**DISCUSSION**

The sex pheromone of *A. reticulatus* was identified as (Z)-9-hexadecenal. Synthetic (Z)-9-hexadecenal has a relatively wide activity range (Fig. 5) compared with crude extract of virgin females (Fig. 1). This may be due to the impurities in the crude extract, which deter the pheromone activity. Synergistic effect of hexadecenal and/or citronellal on male response to (Z)-9-hexadecenal was not observed (data not shown), however, a possibility of other synergists (e.g., an unidentified peak 2 in Fig. 2) remained.

Citronellal identified from the female body extract in the present study was also found in the male body extract (data not shown). In our preliminary experiment, citronellal did not produce any behavioral activity in male adults. The biological function of the compound in this species is not certain, while in *Lasius spathetus* (Wheeler) (Hymenoptera: Formicidae), citronellal was identified as an alarm pheromone (Kistner and Blum, 1971).

(Z)-9-Hexadecenal is the common compound as a component of sex pheromone in some noctuids (*Heliothis* spp.) and some pyralids (e.g. *Chilo suppressalis*) (Sugie and
TAMAKI, 1987). In these moth species, sex pheromones are released from calling females and act as volatile cues. In contrast, in Hymenoptera, the Argentine ant, *Iridomyrmex humilis* (Mayr) (Formicidae) uses the same (Z)-9-hexadecenal as one of the trail pheromone components (Cavill et al., 1979; Cavill et al., 1980). In this case, (Z)-9-hexadecenal is regarded as an attractant, since the insects showed a positive response in an olfactometer. On the other hand, in *A. reticulatus*, sex pheromone is deposited on the substrate where the females walk around and it is likely that the males detect the pheromone as a contact chemical. In fact, males seemed to walk around randomly until they contacted the treated line of a sample in a Petri dish in bioassay. Also, we could not observe the attraction of males toward the odor source of females in a Y-tube olfactometer (Kainoh, unpublished).

When five females of three-day-old *A. reticulatus* were confined in a 9-cm Petri dish for 5 min, the antennal-searching activity of males in response to the dish decreased to 1/5 in 5 hr and ceased altogether in 24 hr (Kamano and Kainoh, unpublished). The relatively brief longevity of the chemical was similar to the trail pheromone of *I. humilis* (Van Vorhis Key et al., 1981). Since this pheromone may be easily dissipated, its effect as a signal for males does not persist for long, which is, in turn, effective for males to inform them of the presence of their mate in a close vicinity. Males of gregarious parasitoid, *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) mark the substrate at intervals; the markings are persistent and attractive to other males and virgin females (Van den Assem, 1986). The function of this marking pheromone to form a lek is similar to that of the sex pheromone of *A. reticulatus*.

*A. reticulatus* males did not react to (E)-9-hexadecenal, the geometric isomer of the sex pheromone (Fig. 5). This ability to discriminate was similar to the case of *I. humilis* (Van Vorhis Key and Baker, 1982).

Robacker and Hendry (1977) identified neral and geranial as components of sex pheromone of an ichneumonid *Hoplectris conquistor* (Say), which are the same unsaturated aldehydes as in the case of *A. reticulatus*. Another ichneumonid, *Syndicus rubiginosus* Walley has ethyl (Z)-9-hexadecenoate as a sex pheromone (Eller et al., 1984), which has the same position of double bond and chain length as in the case of *A. reticulatus*.

Even the high dosage of (Z)-9-hexadecenal or other identified compounds did not elicit other components of a sequence of mating behavior, such as wing vibration or mounting (data not shown). The lack of behavioral components may indicate that stimuli other than the sex pheromone are necessary to elicit the whole mating behavior. In a braconid parasitoid, *Apanteles glomeratus* L., the male parasitoids orientated and attempted copulation with a small piece of black paper in the presence of sex pheromone (Obara and Kitano, 1974; Kitano, 1975). In order to understand the whole sequence of mating behavior in *A. reticulatus*, further research is necessary especially on analyzing visual and/or chemical stimuli from females acting in close vicinity.

REFERENCES


Sex Pheromone of *Azogaster reticulatus*


