Journal of Chemical Ecology, Vol. 26, No. 12, 2000

PARASITIC WASP, *Dinarmus basalis*, UTILIZES OVIPOSITION-MARKING PHEROMONE OF HOST AZUKI BEAN WEEVILS AS HOST-RECOGNIZING KAIROMONE

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(Received November 16, 1999; accepted July 29, 2000)

Abstract—A host-recognizing kairomone responsible for the stinging behavior of the parasitic wasp, *Dinarmus basalis*, was studied. Fresh azuki beans coated with an acetone extract of the azuki beans, from which both emerged wasps and their host weevils were removed, elicited stinging behavior from female wasps. The kairomone is a mixture of saturated hydrocarbons and diacylglycerols, both of which are required for activity. The kairomone is composed of normal and methyl-branched hydrocarbons with carbon numbers ranging from 25 to 35, most of which are known as the hydrocarbon constituents of an oviposition-marking pheromone of the host azuki bean weevils, *Callosobruchus chinensis*. This indicates that *D. basalis* utilizes the oviposition-marking pheromone of its host weevils as a host-recognizing kairomone.

Key Words—Parasitic wasp, *Dinarmus basalis*, kairomone, host recognition, azuki bean weevil, *Vigna angularis*.

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INTRODUCTION

Dinarmus basalis (Rondani) (Hymenoptera: Pteromalidae) was first discovered in the Punjab region of India parasitizing its host, the azuki bean weevil, *Callosobruchus chinensis* (L.). The wasp was introduced into Japan in 1980. Females are ectoparasites on larvae and pupae of several bruchid weevils including azuki bean weevil, cowpea weevil, *C. maculatus* (F.), *C. analis*, Mexican bean weevil, *Zabrotes subfasciatus* Bohman, and maize weevil, *Sitophilus zeamais* (Motschulsky) (Fujii and Wai, 1990; Verma, 1990; Caubet and Jaisson, 1991). These and subsequent biological studies (Verma, 1991; Caubet et al., 1992; Islam, 1994; Nishimura and Johon, 1996; Nishimura, 1997) showed that the fourth-instar larvae of *C. chinensis* were the most suitable host for reproduction of next generation *D. basalis* and that both sexes of *D. basalis* emerged from eggs laid by mated females, whereas only males emerged from eggs laid by unmated females.

Female wasps are 2.5 mm in length with black abdomens, while males are ca. 2.0 mm in length with white abdomens. Thus, the sexes can easily be distinguished. When a female locates an azuki bean containing a host larva, she examines the surface of the bean by antennal tapping, bends her abdomen vertically, stings her ovipositor through the surface of the bean (stinging behavior), and lays an egg on the surface of the larva. Of the sequential behaviors described above, the stinging behavior is observed only when females locate beans containing host larvae (or pupae).

Several studies that utilized *D. basalis* as natural enemies against these legume bruchids have been made in South Asia (Islam and Kabir, 1995) and West Africa (Sanon et al., 1998), but little is known about the chemical ecology. We now report the characterization of the host-recognizing kairomone, from the azuki beans from which emerged wasps and host weevils were removed, that is responsible for the stinging behavior.

METHODS AND MATERIALS

Insects. Azuki bean weevils and *D. basalis* were obtained from original colonies kept at Fujii's laboratory (Tsukuba University). Several adult weevils (both sexes) were transferred to plastic Petri dishes (9 cm ID × 15 mm in height) packed to half the height with azuki beans, *Vigna angularis*. Weevils were reared at 30°C, 55 ~ 60% relative humidity, and 16L : 8D. After one week, adults were removed by sifting with a sieve. New weevils began to emerge after 24 days. *D. basalis* were reared as follows; several wasps (both sexes) were released into a plastic Petri dish containing azuki beans in which host larvae (16–17 days after laying, the fourth instar) were present. Wasps were also reared under the same environmental conditions as the bruchids. Wasps began to emerge 12 days after

being exposed to infested beans. The newly emerged wasps (not sexed) were transferred similarly to other dishes containing azuki beans with the fourth-instar larvae for reproduction. Mated females were used for bioassay after starvation of one or two days.

Bioassay. In preliminary studies, the active kairomone(s) eliciting the stinging behavior could be extracted with acetone but not with hexane, ether, or methanol. The behavior was observed when the extract was applied to a fresh azuki bean but not to a glass bead or a filter paper.

Based on these observations, the bioassay was conducted as follows: a fresh azuki bean treated with 5 μl [2.5 mg bean equivalent (BE)] of the test sample (0.5 g BE/ml) was covered with a glass container (18 mm ID $\times$ 13 mm in height; mini P, Tsukuba Flat Co.) after evaporation of the solvent. Similarly, a fresh azuki bean treated with 5 μl of the solvent was used as a control. A mated female wasp (2 ~ 3 days old) was released into the container, and her stinging behavior was observed for 15 min. One assay set was composed of eight pairs of tests. At least three sets (24 pairs) were repeated for each sample. The total number of females showing the stinging behavior at least once during 15 min was counted (N = 24, 32, or 40). The assay was done in a room controlled at 25°C and 60% R.H. The activity was evaluated by Fisher's exact test ($P < 0.05$).

Analytical Methods. Gas chromatographic (GC) analyses were carried out by using two capillary columns: fused silica CP-Sil 19 CB column (25 m $\times$ 0.25 mm ID, Chrompack, Middelburg, The Netherlands) with a Hewlett-Packard 5890 (Palo Alto, California) or a Hitachi 263-30 (Hitachi, Ltd., Tokyo, Japan) gas chromatograph, and an FFAP column (25 m $\times$ 0.25 mm ID, quadrex, New Haven) with a Yanaco G-180F (Yanagimoto Co. Ltd., Kyoto, Japan). Nitrogen was the carrier gas. The column temperatures were: 200–270°C at 4°C/min for analyses of hydrocarbons (HP 5890), isothermally at 230°C for measuring Kovats retention indices (Hitachi 263-30), and isothermally at 160°C for analyses of fatty acid methyl esters (Yanaco G-180 F). Mass spectra (MS) were measured with a Hitachi M-80B GC-mass spectrometer by using an Al-clad methylsilicone (nonpolar) capillary column (25 m $\times$ 0.22 mm ID, quadrex) (EI, 70 eV). Mass spectra of diacylglycerols were obtained by the direct inlet method, and those of other compounds were obtained by GC-MS. The column temperatures were: 200–270°C at 4°C/min for analyses of saturated hydrocarbons, 135–250°C at 4°C/min for analyses of fatty acid methyl esters, and 200–350°C at 4°C/min for analyses of dimethyl disulfide (DMDS) adducts of the methyl esters. Helium was used as the carrier gas. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM-500 (German) or a Jeol EX 270 (Jeol Ltd., Tokyo, Japan) spectrometer with TMS as an internal standard in $CDCl_3$. Thin-layer chromatography (TLC) was done with silica gel (SiO_2) $60F_{254}$ plates (E. Merck, Darmstadt, Germany; precoated plates, 5 $\times$ 20 cm, 0.2 mm thickness) and $AgNO_3$-impregnated SiO_2 plates made by dipping the plates

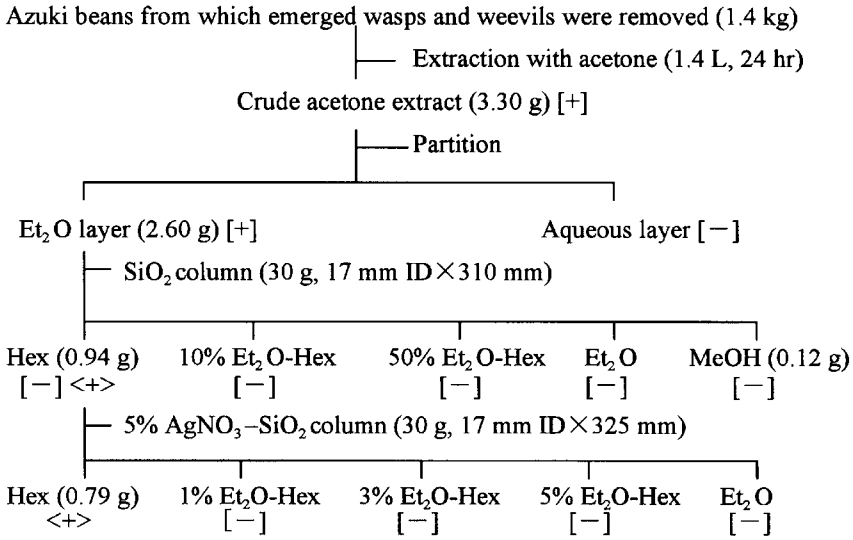

FIG. 1. Extraction procedure of the kairomones and purification of the active saturated hydrocarbons. The activity when used alone ([ ]) or mixed (< >) with the methanol fraction, respectively. −: inactive; +: active; Et_2O: ethyl ether; Hex: hexane; MeOH: methanol.

in 5% $AgNO_3$–acetonitrile solution and activation for 2 hr at 105°C. Spots were detected by UV (245 or 365 nm) or by exposing to iodine vapor the SiO_2 $60F_{254}$ plates and by spraying with 50% sulfuric acid and heating at 105°C or with 0.02% rhodamine 6G ethanol solution followed by UV (365 nm) (fluorescent spots) for $AgNO_3$-impregnated SiO_2 plates.

The double bond positions of unsaturated fatty acids (after methylation with diazomethane) were determined based on the diagnostic ions derived from their DMDS adducts (Francis and Veland, 1981; Vincenti et al., 1987).

Extraction and Purification of Kairomone(s). As described above, the kairomone(s) were extracted with acetone from the beans containing host larvae. Since female wasps also showed the stinging behavior for the azuki beans from which both emerged wasps and host weevils were removed, the extraction was conducted by using azuki beans (1.4 kg; ca. 70 mg/bean) from which emerged host weevils were removed (Figure 1). The acetone extract was filtered through cotton, and the filtrate was concentrated to dryness. The residue (3.30 g) was partitioned with ether and water, and the active ether layer (2.60 g) was purified as shown in Figure 1. No fraction showed any activity, but the activity was recovered by mixing the hexane and the methanol fractions. The hexane fraction (0.94 g) was fractionated by using a 5% $AgNO_3$-impregnated

SiO_2 column. Only the hexane eluate (0.79 g) comprising saturated hydrocarbons displayed the activity by mixing it with the methanol fraction. The saturated hydrocarbons were analyzed by GC, GC-MS, and Kovats retention indices.

The methanol (MeOH) fraction (0.12 g) was separated into acidic, neutral, and basic fractions, as follows: it was evaporated to dryness and dissolved in ether (50 ml). This was extracted successively with 2 NHCl (25 ml × 3), 2 N NaOH (25 ml × 3), and water (25 ml × 2) to give a neutral fraction (0.02 g). The acidic aqueous layer (75 ml) was treated with 2 N NaOH (80 ml) and extracted with ether (75 ml × 3). The ether layer (225 ml) was washed with water to yield the basic fraction (almost no residue). The aqueous basic layer (75 ml) was acidified with 2 N HCl (80 ml), extracted with ether (75 ml × 3), and washed with water to give the acidic fraction (0.19 g). Only the neutral fraction showed activity when mixed with the hexane fraction. However, the small amount of the neutral fraction prevented further chemical analyses. Therefore, the extraction was scaled up by using azuki beans (18 kg) from which emerged insects were removed, and the extract was treated by a method similar to the first extraction, except elution with 10% ether in hexane was omitted this time. The active methanol fraction [4.30 g; hereafter, showing kairomone activity when mixed with the hexane fraction (12.02 g)] was separated into acidic, neutral, and basic fractions as described above, in which only the neutral fraction (0.28 g) was active. This was submitted to SiO_2 column chromatography (the first SiO_2 column chromatography) according to the procedure shown in Figure 2.

Both 1% (17.25 mg) and 5% EtOAc–benzene (Bz) (5.55 mg) fractions obtained by the third SiO_2 column chromatography were active and further studied. The former fraction was less polar and gave two spsots (R_f = 0.63 and a tailing spot) in TLC (Bz–EtOAC 1 : 1, v/v). The latter spot was also observed in the Bz fraction and showed no activity. Therefore, the former spot (R_f = 0.63) was separated from the 1% EtOAc–Bz fraction by SiO_2 chromatography. The 5% EtOAc–Bz eluate (7.75 mg) yielded a single spot (R_f = 0.57, named EB-1). EB-1 was further separated into two spots by $AgNO_3$–SiO_2TLC ($CHCl_3$–MeOH 95 : 5). EB-1A (R_f = 0.61) and EB-1B (R_f = 0.53) were separated by preparative TLC by using $AgNO_3$-impregnated SiO_2 plates.

Another active 5% EtOAc-Bz fraction (5.55 mg) from the third SiO_2 column chromatography yielded one spot (R_f = 0.57; $CHCl_3$–MeOH 1 : 1). Preparative TLC with $AgNO_3$–SiO_2 plates of this spot gave the active compound (2.18 mg; EB-5). The chemical structures of EB-1 and EB-5 were analyzed by MS and NMR.

Analysis of Saturated Hydrocarbons of Azuki Beans and Larvae or Adult Weevils. Since we employed azuki beans from which both emerged insects were removed, it is possible that the source of the saturated hydrocarbons is derived from secretions (adults and larvae), excrement (larvae), and/or azuki beans themselves. To confirm the source of the saturated hydrocarbons, adult weevils (500

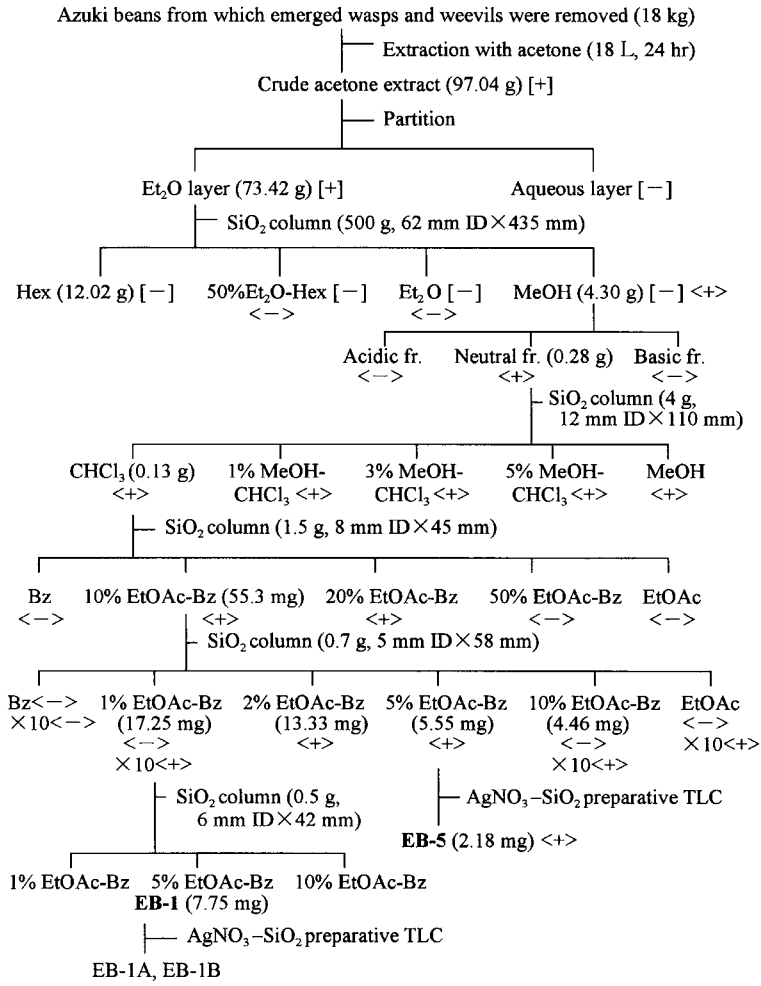

FIG. 2. Large scale extraction of the kairomones and purification of the active diacylglycerols. The activity when used alone ([ ]) or mixed (< >) with the hydrocarbon fraction, respectively. −: inactive; +: active; Et_2O: ethyl ether; Hex: hexane; EtOAc: ethyl acetate; Bz: benzene.

individuals) and their larvae (112 individuals) were extracted separately with hexane for 3 min. Each hexane extract was treated with SiO_2 chromatography followed by $AgNO_3$-impregnated SiO_2 column chromatography to give cuticular saturated hydrocarbons. Fresh azuki beans (300 g, crushed) were extracted with acetone for 24 hr, and upon evaporation of the solvent, the extract was par-

titioned with ether and water. The ether layer was treated by the same method to yield hydrocarbons.

Synthesis of Diacylglycerols. Several 1,2-diacylglycerols were prepared by the procedure of Mattson and Volpenhein (1961). Hydrolysis of triacylglycerols with pancreas lipase type II (EC 3.1.1.3.; Sigma, St. Louis, MO, USA) in the presence of bile salts (Difco bile salts No. 3) and $CaCl_2$ in Tris buffer solution (pH 8.0) at 40°C, yielded 2-acylglycerols. These were treated with desired, equimolar acyl chlorides to give the target 1,2-diacylglycerols. The corresponding 1,3-diacylglycerols were obtained as byproducts from isomerization of the above reactions. For example, 2-oleoyl-1-palmitoylglycerol (2O1P) and 1-oleoyl-3-palmitoyl-glycerol (1O3P) were prepared as follows: a mixture of trioleoylglycerol (1O2O3O; 3.02 g, 3.41 mmol, purity >99%; Wako Pure Chemical Industries, Ltd., Osaka, Japan), Tris buffer (1 M, 36 ml, pH 8.0), $CaCl_2$aq. solution (45%, 2 ml), and bile salt (1% aq. solution, 0.8 ml) wsa stirred at 40°C in a test tube, to which pancreas lipase (500 mg suspended in 4 ml of 1 M Tris buffer) was added. This mixture was vigorously stirred at 40°C for 45 min. The reaction mixture was treated with ethanol (40 ml) and 6 N-HCl (20 ml) to stop hydrolysis. After dilution with water, the 2-monooleoylglycerol (2O) formed was extracted with CH_2CL_2 (150 ml). The CH_2Cl_2 layer was washed successively with saturated $NaHCO_3$ (×4) and saturated NaCl and dried over Na_2SO_4.

The crude 2O was purified by SiO_2 column chromatography with stepwise elution of the ether–hexane solvent system. Pure 2O (380.7 mg, 1.06 mmol) was obtained from the ether eluate, which was treated with palmitoyl chloride (359.3 mg, 1.27 mmol; Nakalai Tesque, Inc., Kyoto, Japan) in the presence of pyridine (85 μl, 1.06 mmol) in anhyd. $CHCl_3$(10 ml) for three days at room temperature. The reaction mixture was washed successively with water, 2 N HCl, and water (×2), and dried over Na_2SO_4. Upon evaporation, the residue was purified by SiO_2 column chromatography. A mixture of 2O1P and 1O3P (191.4 mg, 3.22×10^{-1} mmol, yield 9.4%) was obtained from the 40% ether–hexane eluate. From a part of this mixture, pure 2O1P (5.90 mg) and 1O3P (6.76 mg) were obtained from further SiO_2 column chromatography. Similarly, 1-oleoyl-2-palmitolyglycerol (1O2P), 2-oleoyl-1-stearoylglycerol (2O1S), and 1-oleoyo-3-stearoyl-glycerol (1O3S) were prepared, but the yields were low, because solid tripalmitoylglycerol (1P2P3P, mp 66–67°C) and tristearoylglycerol (1S2S3S, mp 68°C) were not liquefied and barely hydrolyzed by lipase in the reaction mixture at 40°C, and large amounts of them were recovered. These synthetic diacylglycerols showed one spot in TLC (hexane–ether–formic acid 25 : 25 : 1, v/v).

The MS, ^{1}H NMR and ^{13}C NMR spectra of the synthetic diacylglycerols were measured. MS (relative abundance): 2O1P: 576($M^+ - H_2O$, 10), 339($M^+ - OCOC_{15}H_{31}$, 18), 313($M^+ - OCOC_{17}H_{34}$, 59), 264(30), 239($M^+ - COC_{15}H_{31}$, 25), 43(100); 1O3P: 576(9), 339(14), 313(44), 264(21), 239(19), 43(100); 1O2P: 576(13), 339(20), 313(66), 264(31), 239(28); 2O1S: 604($M^+ - H_2O$, 6), 341(M^+

– $OCOC_{17}H_{34}$, 36), 339(M^+ – $OCOC_{17}H_{35}$, 16), 267(M^+ – $COC_{17}H_{35}$, 14), 264(21), 43(100); 1O3S: 604(7), 341(33), 339(16), 267(14), 264(18), 43(100).

Kairomonal Activity of Candidate Diacylglycerols. The six candidate diacylglycerols, 1O2P, 2O1P, 2O1S, 1O3S, 1O3O, 1O3P; three related monoacylglycerols, 1-monopalmitoylglycerol (1P), 1-monooleoylglycerol (1O), and 1-monostearoylglycerol (1S); and four triacylglycerols, 1P2P3P, 1S2S3S, 1O2O3O, and triacetylglycerol (1A2A3A) were used for bioassay. The former four diacylglycerols were synthetic compounds, and the others were purchased from Wako Pure Chemical Industries, Ltd.). These acylglycerols were assayed by mixing with the saturated hydrocarbon fraction (the first extraction). A fixed quantity (2.5 BE = 1.41×10^{-3} mg) of hydrocarbons was used for all the sample mixtures. The quantities of the acylglycerols used were at doses of 3×10^{-7} or 3×10^{-6}, 3×10^{-8} and 3×10^{-9} mg. The dose of 3×10^{-7} mg was decided on as follows: the combined diacylglycerols (9.93 mg) of EB-1 (7.75 mg) and EB-5 (2.18 mg) were composed of five diacylglycerols [1O2P (or 2O1P) and 1O2S (or 2O1S) for EB-1 and 1O3P, 1O3S, and 1O3O for EB-5], and the mean content per diacylglycerol was 1.99 mg (obtained from 18 kg of the azuki beans). Thus, 1.1×10^{-7}(= $1.99/18 \times 10^6$) mg of the diacylglycerols were involved in 1 mg BE, and 2.5 mg BE were used in the bioassay corresponded to 2.78×10^{-7} (rounded up to 3×10^{-7}) mg.

RESULTS

Structural Determination of Saturated Hydrocarbon Fraction. Individual fractions were inactive, and the activity was recovered only when the hexane fraction was mixed with the methanol fraction (Table 1). The hexane fraction gave the same GC profiles (Figure 3) before and after passing through a $AgNO_3$-impregnated SiO_2 column, indicating that these fractions were composed of saturated hydrocarbons. This was confirmed by GC and GC-MS analysis as well as by the Kovats retention indices (CP-Sil column, 230°C, isothermal). The authentic saturated hydrocarbons (*n*-C_{24}–*n*-C_{34}) were used for measuring the indices. At least 29 saturated hydrocarbons, with carbon numbers ranging from 25 to 35, were detected from the hexane fraction in which the major peak (7) was identified as *n*-heptacosane (M^+*m*/*z* 380). 3-Methylalkanes yielded clear fragments at *m*/*z* M^+ – 29, along with the fragments at *m*/*z* M^+ – 15, and M^+ – 57, and the increment of Kovats retention index due to the 3-methyl position was ca. – 30 (Kojima, 1983) compared with the corresponding *n*-alkane. Similarly, internally branched methylalkanes gave fragments at *m*/*z* M^+ – 15 and ion doublets according to the positions of methyl-branching (Lockey, 1978; Suzuki et al., 1988). The next abundant peak (8) was determined as 11-methylheptacosane, as judged by the diagnostic ions at *m*/*z* 379 (M^+ – 15), 253, 252

TABLE 1. KAIROMONAL ACTIVITY OF EACH FRACTION AND THEIR MIXTURES (FIRST EXTRACTION)

Sample	Dose (mg BE)	No. of females responding[a]		*P*	Activity
		Sample	Control		
Acetone extract	2.5	11/24	0/24	<0.001	+
Ether layer	2.5	13/24	0/24	<0.001	+
Aqueous layer	2.5	0/24	0/24	1.000	–
Fractions and their mixtures separated by SiO_2 gel column chromatography					
Hex. fr. (1)	2.5	3/32	0/32	0.119	–
	25.0	0/24	0/24	1.000	–
10% Et_2O–Hex. fr. (2)	2.5	0/24	0/24	1.000	–
50% Et_2O–Hex. fr. (3)	2.5	0/24	0/24	1.000	–
Et_2O fr. (4)	2.5	0/24	0/24	1.000	–
MeOH fr. (5)	2.5	0/24	0/24	0.245	–
	25.0	3/24	1/24	0.305	–
Mixture of (1)–(5)	2.5	6/24	0/24	0.020	+
Mixture of (1) and (2)	2.5	0/24	0/24	1.000	–
Mixture of (1) and (3)	2.5	2/24	1/24	0.500	–
Mixture of (1) and (4)	2.5	1/24	0/24	0.500	–
Mixture of (1) and (5)	2.5	6/32	0/32	0.013	+
Mixture of (2) and (3)	2.5	0/24	0/24	1.000	–
Mixture of (2) and (4)	2.5	0/24	1/24	0.500	–
Mixture of (2) and (5)	2.5	1/24	1/24	0.756	–
Mixture of (3) and (4)	2.5	0/24	0/24	1.000	–
Mixture of (3) and (5)	2.5	0/24	0/24	1.000	–
Mixture of (4) and (5)	2.5	3/24	0/24	0.119	–
Mixtures of MeOH fr. (5) and each fraction separated by 5% $AgNO_3$–SiO_2 column chromatography					
Mixture of (5) and Hex. fr. (1′)	2.5	9/32	0/32	0.002	+
Mixture of (5) and 1% Et_2O–Hex. fr. (2′)	2.5	1/24	0/24	0.500	–
Mixture of (5) and 3% Et_2O–Hex. fr. (3′)	2.5	1/24	0/24	0.500	–
Mixture of (5) and 5% Et_2O–Hex. fr. (4′)	2.5	1/24	0/24	0.500	–
Mixture of (5) and Et_2O fr. (5′)	2.5	0/24	0/24	1.000	–
Mixture of (5) and (1′)–(5′)	2.5	7/24	0/32	0.003	+

[a] A total number of female wasps showing stinging behavior at least one time during 15 min (N = 24 or 32). BE: bean equivalent; Et_2O: ethyl ether; Hex.: hexane, MeOH: methanol.

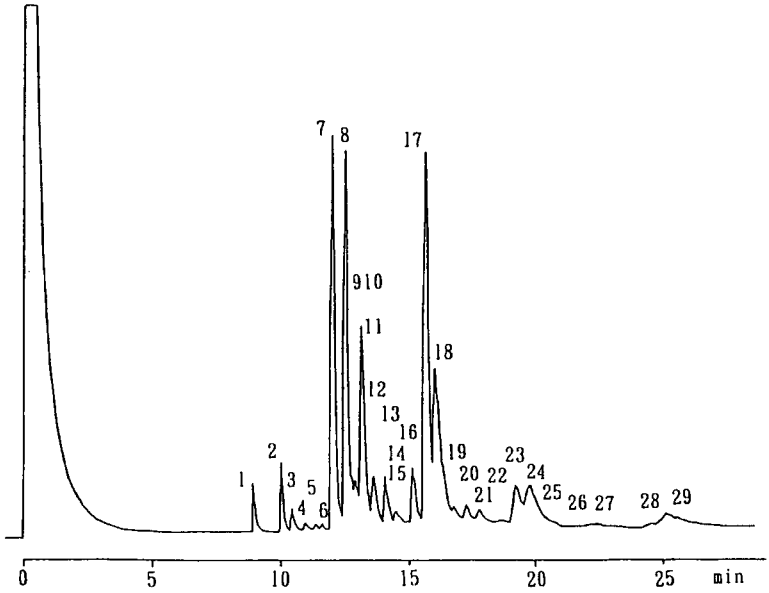

FIG. 3. Gas chromatogram of the active saturated hydrocarbons. Condition: CP-Sil 19 CB capillary column; 200–270°C, 4°C/min. Arabic figures indicate GC peak numbers.

(ion doublets), 169, and 168 (ion doublets), and the increment (−70). Dimethylalkanes afforded fragments at m/z $M^+ - 15$, and the increment was about −45. Similarly, 13 other hydrocarbons were determined by GC-MS and Kovats indices as shown in Table 2. Percent composition of each hydrocarbon component of the hexane fraction is shown in Table 3.

Chemical Analysis of EB-1 and EB-5 in Methanol Fraction. As with the first extraction, no kairomonal activity was observed when each primary fraction was tested alone, and the activity was recovered only after mixing the hexane and the methanol fractions. In addition, the hexane fractions from the first and second extractions gave almost identical GC profiles. Table 4 displays the bioassay results from the mixtures of the hexane fraction and the subfractions of the methanol fraction (the second extraction). The fixed quantity (2.5 BE = 1.67×10^{-3} mg) of the hexane fraction was used for all the test mixtures.

The active EB-5 (2.18 mg) and EB-1 (7.75 mg, a mixture of EB-1A and EB1B) were analyzed by ^{1}H NMR (500 MHz), ^{13}C NMR (125.7 MHz), and MS (direct inlet method).

EB-5: A ^{13}C NMR spectrum gave carbon signals as follows (ppm): 14.1 (CH_3), 22.7 (CH_2), 24.9 (2 CH_2), 25.0 (2 CH_2), 27.2 (2 CH_2), 29.1 (3 CH_2), 29.2 (CH_2), 29.3 (2 CH_2), 29.4 (CH_2), 29.5 (2 CH_2), 29.6 (CH_2), 29.7 (2 CH_2),

TABLE 2. SATURATED HYDROCARBON COMPONENTS IN HEXANE FRACTION

GC Peak[a]	Kovats retention index	Compound	MS diagnostic fragment and base peak (relative abundance)
1	2497	*n*-C_{25}	352(M^+, 0.5), 43(100)
2	2571	3-Me-C_{25}	351(M^+–15, 0.2), 337(5), 309(0.3), 57(100)
3	2597		
4	2631	Me-C_{26}	
5	2655	di-Me-C_{28}(?)	
6	2670	3-Me-C_{26}(?)	
7	2699	*n*-C_{27}	380(M^+, 0.5), 43(100)
8	2731	11-Me-C_{27}	379(M^+–15, 0.5) 253(8), 252(4), 169(4), 168(10), 57(100)
9	2747		
10	2756	di-Me-C_{28}	
11	2772	3-Me-C_{27}	379(M^+–15, 0.2), 365(6.5), 337(0.5), 57(100)
12	2800	*n*-C_{28}	394(M^+, 0.8), 57(100)
13	2829	10-Me-C_{28} + 11-Me-C_{28}	408(M^+, 0.2), 281(0.4), 280(1), 267(0.3), 266 (0.5), 169(0.4), 168(4), 155(6), 154(8), 57(100)
14	2855	di-Me-C_{29}(?)	
15			
16	2897	*n*-C_{29}	408(M^+, 0.3), 57(100)
17	2930	11-Me-C_{29} + 13-Me-C_{29}	407(M^+–15, 0.5), 281(0.7), 280(3), 253(1.7), 252(2), 197(0.8), 196(1.8), 169(4), 168(10), 57(100)
18	2955	9,13-di-Me-C_{29}	436(M^+, 0.1), 323(2), 295(0.3), 253(0.3), 252 (0.4), 211(4), 183(2), 141(5), 140(6.5), 57 (100)
19	2999	*n*-C_{30} (?)	
20	3027	11-Me-C_{30} + 12-Me-C_{30}	295(1.1), 294(0.6), 281(1), 280(0.7), 183(1), 182(4), 169(3.5), 168(6), 57(100)
21	3055	di-Me-C_{30}	435(M^+–15, 0.5), 57(100)
22	3100		
23	3129	11-Me-C_{31} + 13-Me-C_{31}	450(M^+, 0.1), 309(2), 308(2), 281(1.5), 280 (2), 197(2), 196(3), 169(4), 168(8), 57(100)
24	3155	9,13-di-Me-C_{31}	449(M^+–15, 0.2), 351(2), 323(0.5), 281 (0.7), 280(0.7), 211(4), 141(3), 140(8), 57 (100)
25	3196		
26			
27	3251		
28			
29	3349	11,15-di-Me-C_{33}	478(M^+, 0.1), 351(3), 281(2), 280(2), 239 (4), 169(4), 168(8), 57(100)

[a] GC peak number corresponds to that of Figure 3.

TABLE 3. COMPOSITION OF SATURATED HYDROCARBON COMPONENTS IN HEXANE FRACTION AND EXTRACTS FROM ADULTS AND LARVAE OF WEEVILS

GC peak[a]	Composition (%)		
	Hexane fraction	Adult	Larvae
1	1.74	0.44	1.80
2	2.13	1.53	0.66
3	0.94	0.77	1.87
4	0.29	0.40	
5	t	0.29	
6	t	0.37	
7	16.07	17.08	47.31
8	14.02	15.08	3.98
9	0.72	0.53	t
10	1.29	1.92	
11	8.89	14.38	3.51
12	2.73	1.94	5.22
13	2.24	1.90	1.15
14	0.67	0.84	
15	t	t	
16	3.01	2.90	12.22
17	19.31	11.96	11.93
18	9.17	9.43	4.97
19	0.82	0.91	t
20	1.14	0.98	t
21	1.00	0.93	t
22	0.42	0.49	1.76
23	4.41	2.27	3.62
24	4.37	4.25	
25	t	0.39	
26	t	t	
27	0.68	0.97	
28	t	t	
29	3.94	7.05	

[a] GC peak number corresponds to that of Table 2 and Figure 3. t: trace amount.

29.8 (CH_2), 31.9 (2 CH_2), 34.1 (2 CH_2), 34.3 (2 CH_2), 61.6. 62.0, 72.2, 129.7 (CH=), 130.1 (CH=), 173.4, and 173.8. TLC behavior and a ^{13}C NMR spectrum revealed that EB-5 was a 1,2-diacylglycerol. Two carbon signals at 173.4 and 173.8 ppm indicataed the presence of two ester carbonyl groups. Three carbon signals at 61.2, 62.0, and 72.1 ppm were assigned to two methylene and one methyne carbons connected with oxygen atoms of glycerol, respectively. The ^1H NMR spectrum yielded the proton signals (ppm): 0.88 (6H, t, J = 6.88 Hz), 1.27 (br.), 1.61 (2H, quintet, J = 6.97 Hz), 1.63 (2H, quintet, J = 6.97 Hz), 2.01 (4H, q, J = 6.50 Hz), 2.32 (2H, t, J = 7.54 Hz), 2.34 (2H, t, J = 7.56

TABLE 4. KAIROMONAL ACTIVITY OF MIXTURES OF HEXANE FRACTION AND SUBFRACTION OF METHANOL FRACTION (SECOND FRACTION)

Sample[b]	Dose (mg BE)	No. of females responding[a]		*P*	Activity
		Sample	Control		
Acetone extract	2.5	12/24	2/24	0.002	+
Mixtures of Hex. fr. (a) and three sub-frs. of MeOH fr.					
Acidic fr.	2.5	3/32	0/32	0.118	–
Neutral fr.	2.5	8/24	0/24	0.002	+
Basic fr.	2.5	2/24	0/24	0.245	–
Mixtures of (a) and each fraction separated by first SiO_2 column chromatography					
$CHCl_3$ fr.	2.5	8/24	0/24	0.002	+
1% MeOH–$CHCl_3$ fr.	2.5	8/24	2/24	0.037	+
3% MeOH–$CHCl_3$ fr.	2.5	7/32	0/32	0.005	+
5% MeOH–$CHCl_3$ fr.	2.5	7/24	1/24	0.024	+
MeOH fr.	2.5	5/24	0/24	0.025	+
Mixture of above five frs.	2.5	7/24	0/24	0.005	+
Mixtures of (a) and each fraction separated by the second SiO_2 column chromatography					
Bz fr.	2.5	6/32	2/32	0.129	–
10% EtOAc–Bz fr.	2.5	8/32	1/32	0.014	+
20% EtOAc–Bz fr.	2.5	5/32	0/32	0.025	+
50% EtOAc–Bz fr.	2.5	2/24	0/24	0.245	–
EtOAc fr.	2.5	1/24	0/24	0.500	–
Mixture of above five frs.	2.5	5/32	0/32	0.025	+
Mixtures of (a) and each fraction separated by the third SiO_2 column chromatography					
Bz fr.	2.5	0/24	0/24	1.000	–
	25.0	7/32	2/32	0.074	–
1% EtOAc–Bz fr.	2.5	3/32	0/32	0.120	–
	25.0	12/32	1/32	<0.001	+
2% EtOAc–Bz fr.	2.5	10/40	3/40	0.034	+
5% EtOAc–Bz fr.	2.5	7/32	1/32	0.027	+
10% EtOAc–Bz fr.	2.5	4/32	1/32	0.178	–
	25.0	10/24	2/24	0.009	+
Et_2O fr.	2.5	2/24	1/24	0.500	–
	25.0	7/32	2/32	0.074	–
Mixture of above six frs.	2.5	10/40	3/40	0.034	+
Mixtures of (a) and EB-5 separated by preparative TLC					
EB-5	2.5	5/32	0/32	0.027	+

[a] See Table 1 ($N = 24$, 32, or 40). BE: bean equivalent; Hex.: hexane; Et_2O: ethyl ether; MeOH: methanol; $CHCl_3$: chloroform; Bz: benzene; EtOAc: ethyl acetate.

[b] Fixed quantity (2.5 BE = 1.67×10^{-3} mg) of Hex. fr. (a) was used for all the sample mixtures.

Hz), 3.73 (2H, ddd, J = 5.54, 5.54, and 1.88 Hz), 4.24 (1H, dd, J = 11.89, and 5.66 Hz), 4.32 (2H, dd, J = 11.91, and 4.55 Hz), 5.08 (1H, dddd, J = 4.97, 4.97, 4.97, and 4.97 Hz), and 5.34 (2H, m). The four multiplets at 3.73, 4.24, 4.32, and 5.08 ppm were assigned to $C_{III}H_{\alpha}H_{\beta}$, C_1H_{β}, C_1H_{ω} and $C_{II}H$ protons attached to glycerol carbons (C_I–C_{III}), respectively, when compared with the authentic 1,2-dioleoylglycerol. The MS spectrum yielded the fragments at m/z 576 ($M^+ - H_2O$, 6%), 339 ($M^+ - OCOC_{15}H_{31}$, 6), 313 ($M^+ - OCOC_{17}H_{33}$, 22), 265 ($COC_{17}H_{33}$, 34), and 239 ($COC_{15}H_{31}$, 22), indicating that EB-5 was a mixture of 1,2-diacylglycerol connected with two fatty acids, octadecenoic, and hexadecanoic acids.

To determine the acids attached to position 1 of the 1,2-diacylglycerols, EB-5 was treated with lipase (Mattson and Volpenhein, 1961) to give a mixture of palmitic, stearic, and oleic acids (4.3 : 1.0 : 5.6). In addition, a spot of 1,3-diacylglycerol was detected in TLC (developing solvent: hexane–ether–formic acid 25 : 25 : 1) from the recovered acylglycerols. This indicated that the 1,2-diacylglycerols were partly isomerized to the 1,3-diacylglycerols during the hydrolysis by lipase. Hydrolysis of EB-5 with 5% KOH ethanol afforded a mixture of palmitic, stearic, and eleic acids in a ratio of 4.4 : 1.0 : 5.0. Based on these results, we conclude that EB-5 is a mixture of 1 (or 2)-oleoyl-2 (or 1)-palmitolylglycerol, and 1 (or 2)-oleolyl-2 (or 1)-stearoylglycerol (ratio = 4.4 : 1.0). Almost no difference was found between EB-5 and the synthetic 1-oleolyl-2-palmitolylglycerol or 2-oleoyl-1-palmitoylglycerol.

EB-1: EB-1 was thought to be 1,3-diacylglycerols, based on TLC behavior (less polar than EB-5) and a ^{13}C NMR spectrum showing two carbon signals at 65.1 and 66.5 ppm that were assigned to one methyne carbon and two methylene carbons attached to oxygen atoms of glycerol, respectively. Three multiple proton signals at 4.08, 4.13, and 4.19 ppm in the ^1H NMR spectrum were assigned to the protons in the glycerol skeleton, compared with the authentic 1,3-oleolyglycerol. Hydrolysis of EB-1 with 5% KOH ethanol gave a mixture of palmitic, stearic, and oleic acids (2.9 : 1.0 : 7.9). EB-1 was composed of at least two components, EB-1A (R_f = 0.61) and EB-1B (R_f = 0.53), as disclosed by $AgNO_3$–SiO_2 TLC, and MS. The MS spectrum gave fragments at m/z 602 ($M^+ - H_2O$, 0.7%), 576 ($M^+ - H_2O$, 8), 339 (22), 313 (25), 264 (16), and 239 (24). EB-1A and EB-1B were isolated by preparative TLC by using a $AgNO_3$–SiO_2 plate. The MS spectrum of EB-1B yielded fragments at m/z 602 ($M^+ - H_2O$, 6%), 339 ($M^+ - OCOC_{17}H_{34}$, 50), and 265 ($M^+ - COC_{17}H_{34}$, 28). EB-1A and EB-1B were separately hydrolyzed by alkali. EB-1A yielded a mixture of palmitic, stearic, and eleic acids in a ratio of 4.4 : 1.0 : 6.9. If EB-1A is composed of a mixture of 1-oleoyl-3-palmitoylglycerol and 1-oleoyl-3-stearoylglycerol in a ratio of 4.4 : 1.0, the theoretical ratio of palmitic, stearic, and oleic acids obtained by hydrolysis would be 4.4 : 1.0 : 5.4 (= 4.4 + 1.0). The analytical ratio of these acids was in acceptable agreement with the theoretical ratio. The

content of oleic acid was higher than that of the theoretical one, which may be due to contamination of EB-1B.

Hydrolysis of EB-1B with alkali afforded a mixture of oleic and palmitic acids in a ratio of 10.6 : 1.0. Detection of palmitic acid suggested the presence of 1-oleoyl-3-palmitoylglycerol or 1,3-dipalmitoylglycerol, but the R_f value of the former was 0.61 and the latter, >0.61. Since the R_f value of EB-1B was 0.53, palmitic acid may be a contaminant of EB-1A. Thus, we conclude EB-1B is primarily 1,3-dioleoylglycerol.

Based on these data, EB-1 was thought to be a mixture of 1-oleoyl-3-palmitoyl-glycerol, 1-oleoyl-3-stearoylglycerol, and 1,3-dioleoylglycerol in a ratio of approximately 2.9 : 1.0 : 2.0 [(7.9 − 2.9 − 1.0)/2], which was supported by MS analysis of EB-1; the fragments at m/z 576 and 602 were assigned to the dehydrated ions from the molecules of 1-oleoyl-2-palmitoylglycerol and 1,3-dioleolylglycerol, respectively. Fragments at m/z 239 ($COC_{15}H_{31}$), 265 ($COC_{17}H_{33}$), 313 ($M^+ - OCOC_{17}H_{33}$), and 339 ($M^+ - OCOC_{15}H_{31}$) were also observed.

Saturated Hydrocarbons from Azuki Beans. The saturated hydrocarbons (0.06 g) from the azuki bean extract showed no significant peaks in GC. In addition, no activity was found by mixing the hexane fraction with the methanol fraction (the first extraction), supporting the idea that the active saturated hydrocarbons were insect-derived.

Saturated Hydrocarbons from Adults and Larvae of Azuki Bean Weevils. The GC profile and the composition of the saturated hydrocarbons of the adult weevils were nearly identical to those of the first extraction (Table 3). Furthermore, the adult hydrocarbons displayed the kairomone activity when mixed with the methanol fraction (data not shown). On the other hand, the larval hydrocarbons contained higher amounts of *n*-heptacosane (ca. 50%) than that of adults (Table 3), and their GC profiles were qualitatively similar, although the constituents were present in different ratios.

Kairomonal Activity of Glycerols. Not only the separated diacylglycerols but also the six authentic diacylglycerols showed the kairomone activity in combination with the saturated hydrocarbons (Table 5). Of these diacylglycerols, 1O3S showed the activity at a lower dose (3×10^{-8} mg). Three monoacylglycerols were also tested, because other active fractions of neutral fraction, considerng their polarities, may be composed of monoacylglycerols (Figure 2 and Table 4). They were active. Triacylglycerols are known as components of the oviposition-marking pheromone of the host azuki bean weevils (Sakai et al., 1986). Four triacylglycerols that were easily commercially obtained were also assayed. All triacylglycerols showed kairomonal activity, and even triacetylglycerol (1A2A3A), a nonnatural compound, had activity. 1O2O3O was the most active of the triacylglycerols tested (3×10^{-8} mg). Therefore, mono- and triacylglycerols, as well as diacylglycerols, also showed kairomone activity.

TABLE 5. KAIROMONAL ACTIVITY OF CANDIDATE DIACYLGLYCEROLS AND RELATED MONO- AND TRIACYLGLYCEROLS

	No. of females responding[b]											
	3×10^{-6} mg			3×10^{-7} mg			3×10^{-8} mg			3×10^{-9} mg		
Sample[a]	S	C	*P*	S	C	*P*	S	C	*P*	S	C	*P*
1O				16/32	2/32	<0.001(+)	4/32	0/32	0.057(−)			
1P				7/32	1/32	0.027(+)	1/32	1/32	0.754(−)			
1S				13/32	1/32	<0.001(+)	4/32	0/32	0.057(−)			
1O2P				8/32	0/32	0.003(+)	3/32	0/32	0.120(−)			
2O1P	9/32	2/32	0.022(+)	2/32	0/32	0.247(−)						
2O1S				7/32	1/32	0.027(+)	1/32	0/32	0.500(−)			
1O3O				16/32	2/32	<0.001(+)	4/32	0/32	0.057(−)			
1O3P	12/32	1/32	<0.001(+)	3/32	0/32	0.120(−)						
1O3S				14/32	2/32	<0.001(+)	10/32	1/32	0.003(+)	4/32	1/32	0.178(−)
1A1A3A				11/32	1/32	0.002(+)	3/32	0/32	0.120(−)			
1P2P3P	9/32	0/32	0.002(+)	7/32	2/32	0.074(−)						
1O2O3O				15/32	0/32	<0.001(+)	7/32	0/32	0.006(+)	2/32	0/32	0.247(−)
1S2S3S				7/32	0/32	0.006(+)	5/32	1/32	0.0099(−)			

[a] Fixed quantity (2.5 BE = 1.41×10^{-3} mg) of the saturated hydrocarbons (the first extraction) was used for the all sample mixtures.
[b] See Table 1 ($N = 32$). IP: 1-monopalmitoylglycerol; 1O: 1-monooleoylglycerol; 1S: 1-monostearoylglycerol; 1O2P: 1-oleoyl-2-palmitoylglycerol; 2O1P: 2-oleoyl-1-palmitoylglycerol; 2O1S: 2-oleoyl-1-stearoylglycerol; 1O3O: 1,3-dioleoylglycerol; 1O3P: 1-oleoyl-3-palmitoylglycerol; 1O3S: 1-oleoyl-3-stearoylglycerol; 1A2A3A: triacetylglycerol; 1P2P3P: tripalmitoylglycerol; 1O2O3O: trioleoylglycerol; 1S2S3S: tripalmitoyl-glycerol; S: sample; C: control.

DISCUSSION

Host selection by parasitic wasps is separated into the following processes according to Doutt (1959): (1) host habitat finding; (2) host finding; (3) host acceptance; and (4) host suitability. Of these processes, the stinging behavior of *D. basalis* that was used in this study is thought to be involved in step 3. Vinson (1985) indicated that these processes may be mediated mainly by chemical stimuli and not by sight. In *D. basalis*, however, stinging behavior was observed only when the extract was applied to a fresh azuki bean but not to a glass bead or a filter paper, indicating the necessity of physical conditions as well as chemical stimuli. The encyrtid parasitoid, *Anicetus beneficus* Ishii et Yasumatsu, which attacks the scale, *Ceroplastes rubens* Maskell, also shows ovipositional behavior for a hemispherical shape but not for a filter paper treated with the scale extract (Takahashi and Takabayashi, 1984).

Azuki bean weevils secrete a copulation release pheromone, electin, which elicits males to extrude their genital organs and to attempt copulation (Tanaka et al., 1981), and they also secrete an oviposition-marking pheromone to prevent multiple ovipositions in a bean (Sakai et al., 1986). The former pheromone is composed of hydrocarbons and (*E*)-3,7-dimethyl-2-octene-1,8-dioic (callosobruchusic acid), which act synergistically. The GC profile of the hexane fraction of electin, although it was obtained by using a 1% OV-1 packed column, was similar to that of Figure 3 in the present study. In the hexane fraction of electin, mono-methylalkanes (3-Me-C_{25}, 11- and 3-Me-C_{27}, 11- and 15-Me-C_{29}, and 13-Me-C^{31}) and dimethylalkanes (11,15-di-Me-C_{29}, 9,13-di-Me-C_{31}, and 11,15-di-Me-C_{33}) had strong activity. The latter pheromone is a mixture of hydrocarbons, triacylglycerols, and fatty acids. A total of 17 straight and methyl-branched hydrocarbons (C_{26}–C_{35}), of which 11 hydrocarbons (3-Me-C_{25}, *n*-C_{27}, 11-Me-C_{27}, 3-Me-C_{27}, *n*-C_{28}, *n*-C_{29}, 11-Me-C_{29}, 13-Me-C_{29}, 13-Me-C31,9,13-di-Me-C_{31}, and 11,15-di-Me-C_{33}) were also determined in the present study, were characterized from the latter pheromone.

Both pheromones involve common saturated hydrocarbons. The host-recognizing kairomone responsible for the stinging behavior of *D. basalis* was demonstrated to be a mixture of saturated hydrocarbons (C_{25}–C_{35}, straight chain and methyl-branched) and diacylglycerols. The active hydrocarbon kairomone consisted of almost the same components as those of the pheromones and of the cuticular hydrocarbons of adult weevils. Nearly no adult weevils were present in the azuki beans employed for extracting the kairomones (but it was possible that some host larvae were present in the azuki beans). Thus, the active hydrocarbon kairomone may be derived from the hydrocarbon components of these two pheromones marked by host weevils. This suggests that *D. basalis* utilizes one of the oviposition-marking pheromones of its host azuki bean weevils as a host-recognizing kairomone. Thus, beans marked with the pheromone

by their host weevils suggest to female wasps the presence of host larvae inside beans.

n-Heptacosane (16.07%) was a major component of the active hydrocarbons, but mixtures of the authentic *n*-heptacosane or *n*-alkanes (*n*-C_{25}, *n*-C_{27}, and *n*-C_{29}, total 23.55%) and the methanol fraction were inactive (data not shown), suggesting the necessity of the branched hydrocarbons for specific recognition. However, it was not determined whether both groups of the hydrocarbons were required for bioactivity or whether only mono-methylalkanes showed the activity, due to lack of authentic methylalkanes. The content of mono-methylalkanes (total >47.73%, 11-Me-C_{27} was the main one) was higher than that of *n*-alkanes. 13-Methylhentriacontane has been identified from larvae of corn earworm, *Heliothis zea*, as a host-seeking stimulant for the parasitic wasp, *Microplitis croceipes* (Jones et al., 1971).

The amounts of the diacylglycerols obtained were small compared with those of the hydrocarbon fraction ($\sim 1 \times 10^{-6}$), but no activity was obtained from the hydrocarbons alone. Three diacylglycerols (1O3P, 1O3S, and 1O3O) were characterized, and 1(or 2) O2(or 1)P and 1(or 2)O2(or 1)S were also determined as the kairomonal components. The neutral fraction of the methanol fraction contained other active compounds (Figure 2 and Table 4). These fracations (1%, 3%, 5% and MeOH–$CHCl_3$ and MeOH fractions, etc., in the first SiO_2 column chromatography in Figure 2) may be composed of other di- or monoacylglycerols, considerng their polarities, but remain to be further studied. In fact, some monoacylglycerols induced the stinging behavior. Neither diacylglycerols nor monoacylglycerols have been described as components of the oviposition-marking pheromone (Sakai et al., 1986) or copulation release pheromone (Tanaka et al., 1981) of host azuki bean weevils. Triacylglycerols were also active (Table 5). Surprisingly, even triacetylglycerol (1A2A3A) showed the activity. This is a nonnatural glycerol with a short-chain fatty acid, which means that *D. basalis* responds to all types of glycerols. Azuki bean weevils secrete a large quantity of triacylglycerols as oviposition-marking pheromone, but individual triacylglycerol has not been identified from this marking pheromone. It is thought that triacylglycerols were eluted in the 10% Et_2O-Hex fraction (the first extraction, Figure 1) or in the 50% Et_2O-Hex fraction (the second extraction, figure 2), considering their polarities, but both of these were inactive. The reason is unclear and remains to be further studied.

The two components of the kairomone probably act as short-range attractants (hydrocarbons) and chemical contact stimuli (diacylglycerols) for *D. basalis* and may be useful in biological control of the weevils.

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