Identification of a sex pheromone component of the yellowish elongate chafer, *Heptophylla picea* Motschulsky (Coleoptera: Scarabaeidae)

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Abstract

A sex pheromone component was isolated from the hexane extract of females of the yellowish elongate chafer, *Heptophylla picea* Motschulsky by chromatography. The activity was examined by attraction in a glass tube and in the field. The isolated component was analyzed by GC-MS and NMR, and its chemical structure was estimated to be 7,15-hexadecadien-4-olide. The geometric isomerism was determined to be *Z* by comparative GC-MS and NMR analyses of the isolated component and synthetic *E*- and *Z*-isomers. Synthetic (*Z*)-7,15-hexadecadien-4-olide demonstrated a potent attractiveness equal to that of two virgin females in the field.

Key words: Identification, sex pheromone, *Heptophylla picea*, (*Z*)-7,15-hexadecadien-4-olide

INTRODUCTION

The yellowish elongate chafer, *Heptophylla picea*, is distributed in Japan (Nakane et al., 1963). The larvae of this species attack the root of the blueberried honeysuckle, *Lonicera caerulea* L. var. *emphyllocalyx* Nakai (Caprifoliaceae) (Kakizaki et al., 1991), the tea, *Thea sinensis* L. (Theaceae), and many other kinds of plants (Osakabe, 1986). Adults also inhabit in the soil during the daytime and thus it is difficult to determine the occurrence of this species. Adults are captured in a light trap (Kakizaki, unpublished data) but this can not be used in local fields without electricity. Sex pheromone traps would be useful to monitor its occurrence in the field. As sex pheromones have been reported in another member of the same family (Tamaki et al., 1985) and the existence of a sex pheromone in this species was expected, we initiated isolation and identification of the sex pheromone.

MATERIALS AND METHODS

Insects. Adults of *H. picea* were collected during June in 1990 from tea fields (Kanaya, Shizuoka Prefecture) and during July in 1990 from the berry fields (Naganuma, Hokkaido). Adults were sexed according to the difference in the shape of the antennae (Niijima et al., 1917). Males used for laboratory bioassay were fed on clover leaves in plastic pots (21 cm id, 12 cm high) at room temperature under natural light conditions. Sand was added to the pots for the males to be able to rest during the daytime.

Preparation of pheromone extract. Females were dipped in hexane (1 ml/female) for ca. 8 h at 4°C and filtered off. The extract was kept in a freezer at –20°C until use.

Laboratory bioassay. Attractiveness was investigated in a wind tunnel consisting of a glass tube (10 cm id × 1 m) according to the method of Tamaki (1984). Since males survived for more than two weeks in a preliminary laboratory experiment, they were used several times on

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different days for this bioassay. Males had been reported to emerge from the soil about 30 min before sunset and wait for females appearing from the soil for mating (Nishigaki et al., 1986). Therefore, males emerging from the sand in the evening were used for the bioassay. Ten males were released at the downwind end of the tube and a fan-shaped piece of filter paper (4.5 × 3 cm, Toyo, No. 2) impregnated with a sample was placed in the other end. The wind velocity was adjusted to 0.5 m/s. After 10 min, the number of males attracted to within 15 cm from the lure was counted. Bioassays were carried out between 17:00 h and 20:00 h under natural light conditions. The assay was repeated 3 times and the number of attracted males (X) was transformed to $\sqrt{X+0.5}$ and submitted to analysis of variance. Means were ranked by Tukey's test when a significant F -value was indicated.

Field bioassay. Field attraction tests were carried out using water pan traps (30 cm id and 6 cm in depth) at Naganuma (Hokkaido). A small amount of detergent was added to the water in the trap. Traps were placed at intervals of 10 m on the ground in berry fields and larch forests where the male adults were observed. Cotton ropes impregnated with a test sample (10 FE or 1 μ g of chemicals) or only with 100 μ l of hexane (blank) were set on each trap at around 17:00 h and the numbers of males caught were counted at around 10:00 h next morning. In order to evaluate the attractiveness of fractionated samples, field bioassays were carried out using one trap for each sample on 26, 28 June and 9, 10 July in 1991, and three blank traps on 26 June. Furthermore, on 24 and 28 June in 1994, the attractiveness of racemic (*Z*)-7,15-hexadecadien-4-olide was compared with that of 2 virgin females. The trap data (X) were transformed to $\sqrt{X+0.5}$ and submitted to analysis of variance. Means were ranked by Tukey's test when a significant F -value was indicated.

Liquid chromatography. Column chromatography on Florisil (100/200 mesh, 30 g) was carried out according to the class-separating method for lipids (Carroll, 1961). Compounds were successively eluted with hexane, 5%, 15%, 25%, and 50% ether in hexane and 2% methanol in ether. Silica gel impregnated with 16.7% silver nitrate was used for column chromatogra-

phy to purify chemicals by stepwise elution with an ether-hexane solvent system. High performance liquid chromatography (HPLC) was carried out with a Hewlett-Packard (HP) system (LC100) using a silica gel column (4.6 mm id × 25 cm, Inertsil SIL, GL Science Inc.). Solvents for elution were 15% ether in hexane (1 ml/min). Eluate was monitored by an RI detector (Shodex RI SE-11, Showa Denko Ltd.).

Gas chromatography (GC). GC (HP 5890) equipped with an FFAP column (0.53 mm id × 15 m × 1.0 μ m, J & W Scientific) was used under a programmed oven temperature: 120°C for 1 min and increased to 200°C at a rate of 6°C/min.

Gas chromatography-mass spectrometry (GC-MS). A gas chromatograph-mass spectrometer (JMS-SX102A, JEOL) was used for analyses with a DB-23 capillary column (0.25 mm id × 30 m × 0.25 μ m, J & W Scientific) and an OV-1 capillary column (0.25 mm id × 30 m × 0.25 μ m, HP). The temperature of the interface was 230°C. The oven temperature was programmed as follows: 80°C for 1 min and then an increase to 220°C at a rate of 5°C/min for the DB-23 column; 80°C for 1 min and then an increase to 300°C at a rate of 10°C/min for the OV-1 column. Samples were injected to a GC-MS in the splitless mode (purge delay time, 1 min). Helium was used as the carrier gas at a flow rate of 20 cm/s and isobutane was used as a reagent gas in the chemical ionization (CI) mode. High resolution mass spectra (HR-MS) were measured with the same spectrometer.

Nuclear magnetic resonance (NMR) analyses. ^1H NMR and ^{13}C NMR spectra were obtained with a JEOL A600 NMR (^1H NMR 600 MHz, ^{13}C NMR 150.8 MHz) in CDCl_3 . Protons and carbons were assigned using spectra obtained from the measurement by H,H-COSY, H,C-COSY and DEPT.

Chemical reactions. Hydrogen gas was bubbled into an ethanol solution of about 1 μ g sample for 30 s. Platinum black was added to the solution as a catalyst.

Derivatization with dimethyl disulfide (DMDS) was carried out according to the method of Vincenti et al. (1987) to identify the positions of the double bonds. Both 50 ml of DMDS and 0.3% iodine in carbon disulfide solution were

added to about 1 μ g of sample. The mixture was kept at 40°C for 8 h in a 1 ml sealed vial on a block heater. The reaction was quenched by adding 100 μ l of 3% sodium thiosulfate aqueous solution. The reaction products were extracted with hexane.

Chemicals. Racemic (*Z*)-7,15-hexadecadien-4-olide was synthesized by Shin-Etsu Chemical Co. Ltd. as follows (Fukumoto et al., 1997). (*Z*)-3,11-Dodecadienol was synthesized by Wittig reaction with 8-nonenal and phosphonium salt of 3-bromo propanol at a yield of 62%, and converted to (*Z*)-12-chloro-1,9-dodecadiene at a yield of 87%. This was reacted with methyl acetate and acetyl chloride by grignard reaction to synthesize methyl (*Z*)-4-oxo-7,15-hexadecadienoate. After reducing this compound by sodium borohydride, (*Z*)-7,15-hexadecadien-4-olide was obtained using *p*-toluenesulfonic acid at a yield of 61.3%. It was purified by column chromatography on silica gel impregnated with 16.7% silver nitrate and the isomeric purity was confirmed to be more than 99% on GC-MS. (*E*)-7,15-Hexadecadien-4-olide was also obtained in the preceding fractions from this chromatography. The chemical structure of synthesized (*Z*)-7,15-hexadecadien-4-olide was confirmed by a ¹H NMR spectrum obtained with a JEOL GSX-270 NMR (270 MHz) in CDCl₃. The coupling constant of the protons (*J*_{7,8}) at the double bond position was determined to be 10.7 Hz for *Z*-isomer and 15.6 Hz for *E*-isomer by decoupling the proton on the 6th carbon. By IR measurement (KBr), absorption at 960 cm⁻¹, which was expected from the *E* configuration, was observed only in the case of the *E*-isomer.

The other chemicals were purchased from Wako Chemical Co. Ltd. Solvents except carbon disulfide were distilled before use.

RESULTS AND DISCUSSION

A total of 10,000 female equivalents of the crude extract was accumulated. The attractiveness of the crude extract was evaluated using the laboratory bioassay (Table 1). The crude extract of 0.35 FE attracted 5.0 males but that of 0.035 FE showed weak attractiveness. Thereafter, 0.1 FE of fractionated samples was used for the bioassay. The crude extract was fractionated by column chromatography on Florisil (LC). The ac-

tivity of each LC fraction was evaluated by the laboratory bioassay. Attractiveness of the two fractions was evident: ca. 5.0 males were attracted by fractions eluted with 25% and 50% ether in hexane, which were significantly larger than those by the other fractions (Table 1). These two active LC fractions were combined and the attractiveness of the mixture was confirmed in the field bioassay (Table 2). The active mixture was further fractionated by HPLC using the silica gel column. The HPLC fractions of the retention time (*t*_R) of 12.5–19.0 min attracted 5.0 males by the laboratory bioassay, and this number was significantly larger than those of the other fractions (Table 1). Then, the attractiveness of this fraction was confirmed by a bioassay in the field (Table 2). This active

Table 1. Laboratory assay of the crude extract, fractions from Florisil, HPLC and preparative gas chromatography

Sample	No. of males attracted (mean $\pm$ SE)	
Crude extract		
3.5 FE	5.3 $\pm$ 0.3	a
3.5 $\times$ 10 ⁻¹ FE	5.0 $\pm$ 1.2	a
3.5 $\times$ 10 ⁻² FE	0.7 $\pm$ 0.3	b
3.5 $\times$ 10 ⁻³ FE	0	b
Florisil (0.1 FE)		
Hexane	0	a
5% ether/hexane	0	a
15% ether/hexane	0	a
25% ether/hexane	5.0 $\pm$ 0.6	b
50% ether/hexane	5.0 $\pm$ 1.7	b
2% methanol/ether	0	a
Blank	0	a
HPLC (0.1 FE)		
0–12.5 min	0	a
12.5–19 min	5.0 $\pm$ 0.6	b
19–25 min	0	a
Blank	0	a
Preparative GC (0.1 FE)		
0–14 min	0	a
14–15 min	4.7 $\pm$ 0.3	b
15–25 min	0	a
Blank	0	a

Thirty males were used for each test. Data (*X*) were transformed to $\sqrt{X+0.5}$ and submitted to analysis of variance. Means followed by the same letter in the same column are not significantly different at 5% level by Tukey's test.

Table 2. Field assay of fractions from Florisil LC, HPLC and preparative gas chromatography (1991, Naganuma, Hokkaido)

Sources	No. of males caught/ trap/day
26 June	
Florisil LC fractions	
25% + 50%	24
Others	0
HPLC fractions	
12.5–19 min	45
Others	0
All	32
2 Females	16
Blank	0.3 ^a
28 June, 9, 10 July	
GC fractions	
14–15 min	11.0 ± 0.7 ^b a
Others	0.3 ± 0.3 ^b b
2 Females	12.0 ± 0.3 ^b a
Blank	2.0 ± 0.7 ^b b

One water pan trap was used for each source. The amount of lure was 10 female equivalents. Data (X) were transformed to $\sqrt{X+0.5}$ and submitted to analysis of variance. Means followed by the same letter are not significantly different at 5% level by Tukey's test. ^aMean for three traps, ^bmean ± SE.

HPLC fraction was further purified by preparative GC. The GC fraction of $t_R=14-15$ min

attracted 4.7 males by the laboratory bioassay and this number was significantly larger than those of the other fractions (Table 1). The attractiveness of this GC fraction was also confirmed to be the same as that of females by the bioassay in the field (Table 2).

When the active fraction was injected into a GC-MS equipped with a DB-23 column, a single peak was observed at $t_R=31.18$ min. This isolated component showed the characteristic fragment ions by GC-MS analyses in the electron impact ionization (EI) mode; m/z (relative intensity, estimated composition from HR-MS): 80 (base peak), 85 (24%, $C_4H_5O_2$), 150 (93%, $C_{11}H_{18}$), 166 (8%, $C_{10}H_{14}O_2$), 177 (2%, $C_{13}H_{21}$), 190 (3%, $C_{14}H_{22}$), 250 (5%, $C_{16}H_{26}O_2$, M^+) (Fig. 1). The molecular ion was confirmed from the mass spectrum in the CI mode where the $M+1$ ion was observed at m/z 251 (base peak) (Fig. 1).

The isolated component was hydrogenated and injected into a GC-MS. A single product was observed at $t_R=30.02$ min and had the characteristic fragment ions; m/z (relative intensity): 85 (base peak), 218 (25%, $(M-2(H_2O))^+$), 236 (34%, $(M-H_2O)^+$) and 254 (8%, M^+) in the EI mode. This fragment ion pattern was typical of the γ -lactone (Beynon et al., 1968), which led us to speculate that the hydrogenated component was hexadecan-4-olide, that is, that the original

Fig. 1. Mass spectra of the pheromone component of *Heptophylla picea*.

component had two double bonds. Then it was treated with DMDS to determine the double bond positions and two mono-DMDS products were successfully detected by GC-MS analysis using the OV-1 column. One product observed at $t_R=22.63$ min was estimated to be 14,15-bis(methylthio) hexadecen-4-olide from the characteristic fragment ions in the EI mode; m/z (relative intensity, estimated composition from HR-MS): 283 (base peak, $C_{16}H_{27}O_2S$), 344 (28%, $C_{18}H_{32}O_2S_2$, M^+). The other product observed at $t_R=21.31$ min was estimated to be 7,8-bis(methylthio)hexadecen-4-olide; m/z 173 (base peak, $C_8H_{13}O_2S$), 171 (85%, $C_{10}H_{19}S$), 344 (3%, $C_{18}H_{32}O_2S_2$, M^+). From these data, the isolated component was judged to be 7,15-hexadecadien-4-olide.

In order to evaluate the geometric configuration, synthesized *Z*- and *E*-isomers of 7,15-hexadecadien-4-olides were used to compare with the isolated component in the GC-MS analyses. The mass spectra of these compounds were identical to that of the isolated component, while the t_R value for the isolated component (31.18 min) was the same as that for the *Z*-isomer but different from the *E*-isomer (31.65 min). Then the geometric configuration of the isolated component was determined to be *Z*, that is, the isolated component was identified as (*Z*)-7,15-hexadecadien-4-olide.

This result was confirmed by the NMR analyses of the isolated component; 1H NMR (600 MHz): δ : 1.22–1.44 (8H, m, (=CH₂-10–13)), 1.56–1.69 (1H, m, (=CH₂-5b)), 1.80 (1H, m, (=CH₂-5a)), 1.86 (1H, ddt, $J=7.7, 12.8, 9.5, 9.5$ Hz, (=CH₂-3b)), 1.94–2.09 (4H, m, (=CH₂-9, 14)), 2.11–2.23 (2H, m, (=CH₂-6)), 2.32 (1H, ddt, $J=7.7, 12.8, 6.6, 6.2$ Hz, (=CH₂-3a)), 2.47–2.56 (2H, m, (=CH₂-2)), 4.49 (1H, ddt, $J=5.1, 6.6, 7.9, 8.1$ Hz, (=CH-4)), 4.91 (1H, ddt, $J=2.2, 10.3, 1.1, 1.1$ Hz, (=CH₂-16b)), 4.99 (1H, ddt, $J=2.2, 17.0, 1.8$ Hz, (=CH₂-16a)), 5.33 (1H, dtt, $J=10.6, 7.2, 7.3, 1.5, 1.5$ Hz, (=CH-7)), 5.42 (1H, dtt, $J=10.6, 7.2, 7.3, 1.5, 1.5$ Hz, (=CH-8)), 8.80 (1H, ddt, $J=10.3, 16.6, 6.7, 6.8$ Hz, (=CH-15)); ^{13}C NMR (150.5 MHz): δ : 23.1 (=CH₂-6), 27.2 (=CH₂-9), 28.0 (=CH₂-3), 28.85 (=CH₂-2), {28.88, 29.0, 29.1, 29.6, (=CH₂-10–13)}, 33.8 (=CH₂-14), 35.6 (=CH₂-5), 80.4 (=CH-4), 114.2 (=CH₂-16), 127.7 (=CH-7), 131.5 (=CH-8), 139.2

Table 3. Field assay of racemic (*Z*)-7,15-hexadecadien-4-olide (24, 28 June, 1994, Naganuma, Hokkaido)

Sources	No. of males caught/trap/day (mean $\pm$ SE)
1 μ g (<i>Z</i>)-7,15-hexadecadien-4-olide	1.8 $\pm$ 0.4 a
2 Females	1.5 $\pm$ 0.1 a
Blank	0 b

Two traps were used for each source. Data (X) were transformed to $\sqrt{X+0.5}$ and submitted to analysis of variance. Means followed by the same letter are not significantly different at 5% level by Tukey's test.

(=CH-15), 177.2 (C=O). The geometric configuration of the isolated component was also confirmed to be *Z*, as its $J_{7,8}$ value (10.6 Hz) fit well with $J_{7,8}$ (10.7 Hz) of synthesized (*Z*)-7,15-hexadecadien-4-olide.

Synthetic (*Z*)-7,15-hexadecadien-4-olide had a potent attractiveness equal to that of two virgin females (Table 3). This compound was, therefore, confirmed to be a sex pheromone component of *Heptophylla picea*.

(*Z*)-7,15-hexadecadien-4-olide is a new compound. This compound would be useful for population monitoring, although the absolute configuration of the sex pheromone component still remains to be determined. Similar pheromonal compounds, (*R,Z*)-5-(–)-(oct-1-enyl) oxacyclopentan-2-one and (*R,Z*)-5-(–)-(dec-1-enyl) oxacyclopentan-2-one, were reported from *Anomala cuprea* (Leal, 1991), *Anomala albopilosa sakishimana* (Leal et al., 1994a) and *Anomala octiescostata* (Leal et al., 1994b) which are in the same family as *Heptophylla picea*, although, the pheromone of *Anomala rufocuprea* was reported to be methyl (*Z*)-5-tetradecenoate (Tamaki et al., 1985), which has a different chemical structure from γ -lactones. Thus far, it is not possible to relate the chemical structure of pheromones to their taxonomical position.

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