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(*R*,*Z*)-7,15-Hexadecadien-4-olide, Sex Pheromone of the Yellowish Elongate Chafer, *Heptophylla picea*

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Abstract—An active component of the sex pheromone system of the yellowish elongate chafer, *Heptophylla picea* was identified by GC–EAD. Mass spectral data and hydrogenation revealed that the active compound was a hexadecadien-4-olide. It was not possible to determine the double bond positions by direct DMDS derivatization of the pheromone, but partial hydrogenation (diimide) followed by DMDS derivatization showed that the double bonds were located in positions 7 and 15. FTIR (tracer) of the pheromone corroborated the lactone structure (1772 cm^{-1}) and showed a band characteristic of a terminal double bond at 3073 cm^{-1}, and one of a double bond in the *cis*-configuration at 3002 cm^{-1}. Chiral resolution of the pheromone, after hydrogenation, demonstrated that the natural lactone had the (*R*)-stereochemistry. Synthetic (*R*,*Z*)-7,15-hexadecadien-4-olide, prepared from L-malic acid in 14 steps, was identical to the natural product in MS, IR, retention times and biological activity. This is the first fatty acid derivative compound found as a sex pheromone of a Melolonthinae species and as far as biosynthesis is concerned this is the most complex pheromone constituent of a scarab species. Copyright © 1996 Elsevier Science Ltd

Introduction

The yellowish elongate chafer *H. picea* Motshulsky (Coleoptera, Scarabaeidae, Melolonthinae) is a very important agricultural pest in Japan, particularly in Shizuoka Prefecture and Hokkaido, where it causes tremendous losses in tea and flower productions, respectively. This prompted us to launch a joint project aimed at elucidating the pheromone chemistry of this species and exploiting the feasibility of using environmentally sound semiochemicals for this pest management. In addition, we were highly motivated by the fact that the knowledge of the pheromone chemistry of this melolonthine species would allow us to test the hypothesis on the evolution of sex pheromone communication in plant-feeding scarab beetles, that only the most evolved species, such as rutelines, utilize more 'sophisticated' fatty acid derivative compounds.[1]

Results and Discussion

Considering that laboratory bioassays showed that the whole-body extracts of *H. picea* females were as attractive as caged females, but the responses of males to female airborne volatiles were very weak, we decided to extract the active compound(s) by washing female beetles with hexane.

GC–EAD of the female whole-body extract, using a male antennae as a sensing element, revealed the occurrence of two EAD-active peaks (Fig. 1), one appearing at 18.26 min and the other of a longer retention time (ca. 18.45 min) being undetected by FID (Fig. 1, offset). Bioassay of the fractions from a preliminary separation of the crude whole-body extract on the silica gel column revealed that only the hexane:ether (80:20) fraction was active (60% wing raising, 80% attraction). GC–EAD of this fraction gave only one EAD-active peak at 18.25 min (Fig. 2). Since males displayed a full behavioral sequence (wing raising, attraction and precopulatory behavior) in response to the fraction containing only the major component and due to the fact that the scanty amounts of the minor constituent (undetected by FID) prevented its chemical characterization, we concentrated our effort on the identification of the major constituent. Many scarab species respond with a full behavioral sequence to one single compound, although their pheromone systems are probably comprised of more than one compound[2,3].

The molecular ion peak of the EAD-active compound was not detected by EI–MS [Fig. 3(a)], but the molecular weight was demonstrated to be 250 in a CI measurement ($[M+H]^+$ 251). Catalytic hydrogenation

Key words: (*R*)-4-hexadecanolide, (*S*)-4-hexadecanolide, (*S*)-7,10-hexadecadien-4-olide, GC–EAD, Scarabaeidae.

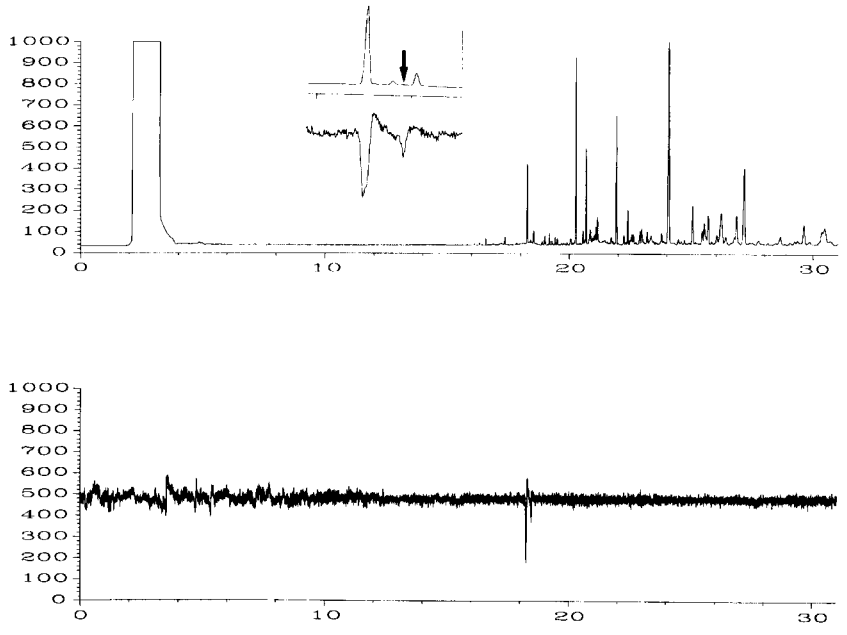

Figure 1. Parallel FID and EAD chromatograms obtained from the whole-body extract of female *H. picea* separated on an HP-5MS column using a male antenna as a sensing element. The expanded area of the EAD-active peaks is displayed on the top of the figure.

of the pheromone gave a spectrum [Fig. 3(b)] unambiguously identified as 4-hexadecanolide. This suggested that the pheromone was a related diene having the two double bonds in the alkyl moiety; because of the prominent peak at *m/z* 85 [Fig. 3(a)], the occurrence of any of these C=C double bonds in the oxacyclopentan-2-one ring was ruled out.

In order to determine the position of the two double bonds, we carried out DMDS derivatization, expecting to obtain one of the patterns suggested by Vincenti et al. for diene systems.[4] Surprisingly, the MS of the DMDS derivative did not show a meaningful pathway of fragmentation that could unambiguously lead us to determine the original positions of the two double bonds. GC–FTIR (Fig. 4) of the pheromone corroborated the occurrence of an oxacyclopentan-2-one ring (1772 cm^{-1}) and suggested that the compound had a terminal double bond (3073 and 1641 cm^{-1}) and that the other double bond was in a *cis* configuration (3002 cm^{-1}).

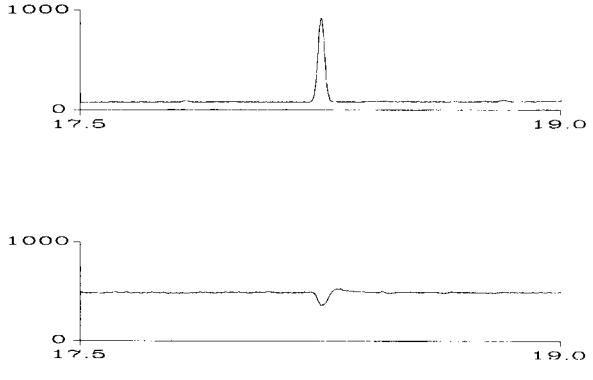

Figure 2. Coupled GC–EAD response of male *H. picea* to the biologically active fraction, showing only one EAD-active peak.

Partial hydrogenation with diimide gave two monoene compounds along with a small amount of the saturated lactone, 4-hexadecanolide (17.55 min). The major monoene [Fig. 5(a)] appeared at 17.15 min and gave the same retention time as 7-hexadecen-4-olide (as confirmed by comparison with an authentic sample), whereas the minor monoene appeared very close to the saturated compound at 17.49 min. Since diimide selectively reduces the terminal over internal double bonds,[5] the longer retention time monoene was found in small amounts in the reaction product, its retention time was very close to the saturated lactone and its MS had a base peak at *m/z* 85 [Fig. 5(b)], this was suggested to be 15-hexadecen-4-olide. A terminal double bond would also explain the occurrence of an outstanding peak at *m/z* 41 [Fig. 3(a)] in the MS of the sex pheromone.

In order to confirm this assignment, DMDS derivatization of the mixture was obtained. MS of the DMDS derivative of 7-hexadecen-4-olide gave a molecular ion peak at *m/z* 346 and a base peak at *m/z* 173, fragments that confirm the original location of the double bond in position 7 [Fig. 6(a)]. The minor monoene lactone gave the major fragments at *m/z* 285 and 61, which are in accordance with a terminal double bond [Fig. 6(b)]. In addition, the molecular peak appeared at *m/z* 346 and the fragment of the lactone ring appeared at *m/z* 85. Therefore, the structure of the pheromone was characterized as 7,15-hexadecadien-4-olide.

One way to expeditiously determine the stereochemistry of pheromones is to obtain enantiomeric

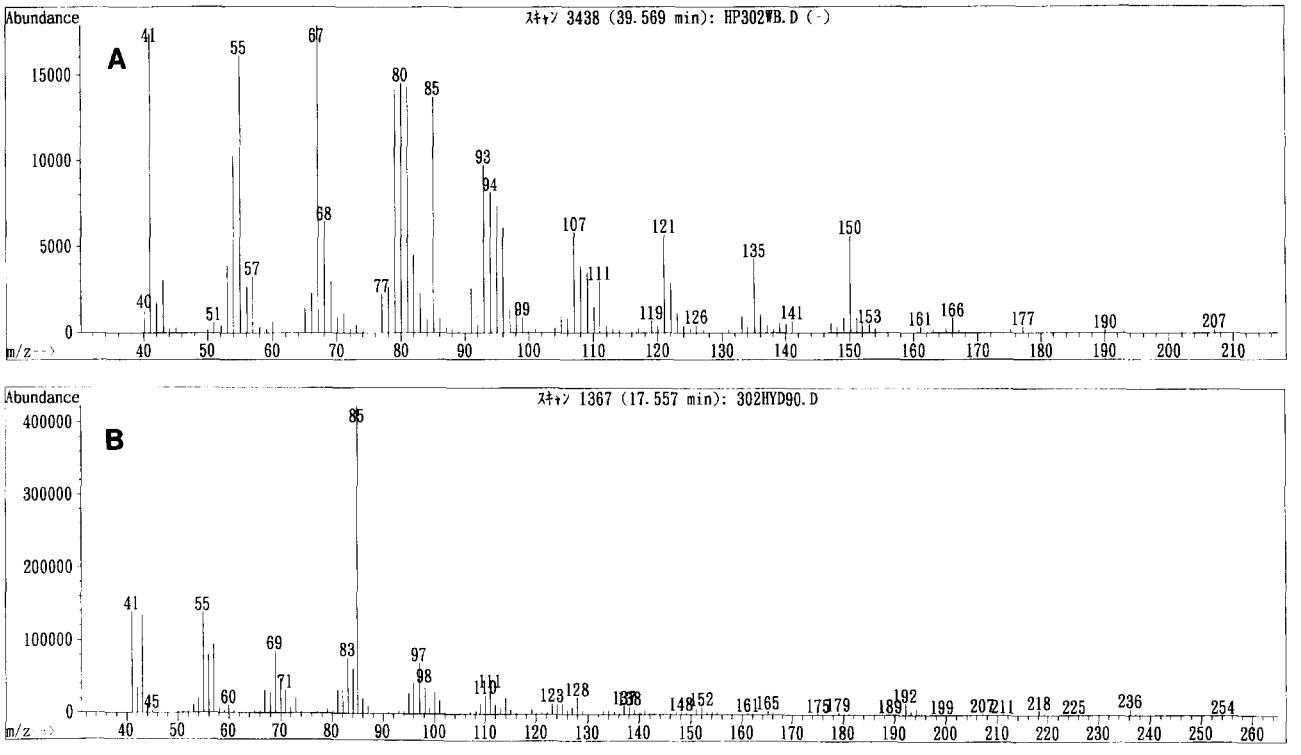

Figure 3. Mass spectral data: (A) sex pheromone and (B) its derivative, obtained by catalytic hydrogenation.

resolution on a chiral column. The assignments of the peaks can be carried out with samples of known stereochemistry and the absolute configuration of the natural product is determined by comparison of its retention time under the same conditions. Resolution of chiral lactones having a double bond adjacent to the lactone ring have been achieved for 5-dodecen- and 5-tetradecen-4-olides[6] on a capillary column having a trifluoroacetylated cyclodextrin phase, Chiraldex GTA, but for higher molecular weight lactones separation would not be possible because this would require higher temperatures than the maximal allowed for this type of column. Nevertheless, resolutions of the related saturated lactones are much easier to achieve even on columns where the unsaturated lactones cannot be separated (Leal, unpublished data).

Racemic 4-hexadecanolide and (*S*)-4-hexadecanolide were obtained by the hydrogenation of the corresponding racemic and (*S*)-7,10-hexadecadien-4-olide, which were synthesized for a different project. Almost baseline separation was achieved with the racemic sample on Chiraldex GTA [Fig. 7(a)]. The hydrogenated natural product gave one single peak

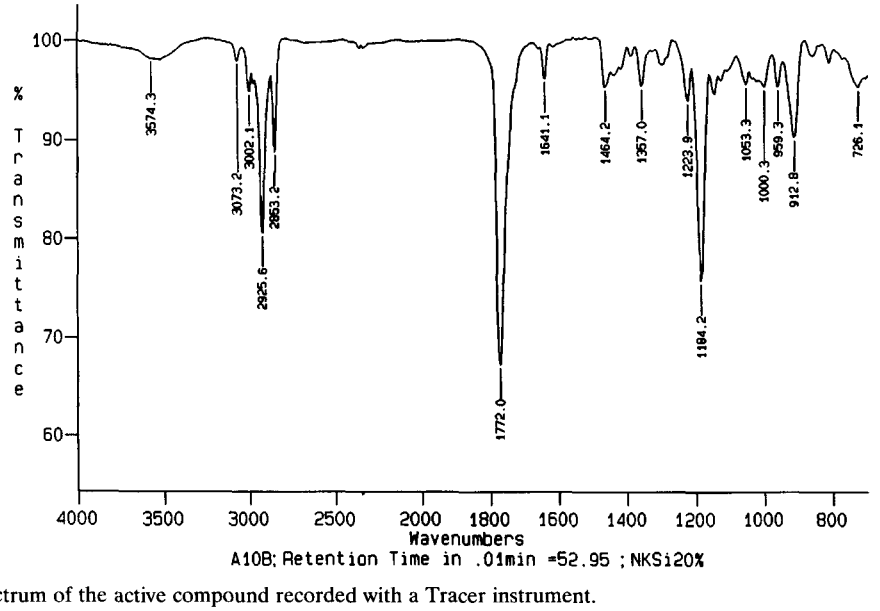

Figure 4. GC-FTIR spectrum of the active compound recorded with a Tracer instrument.

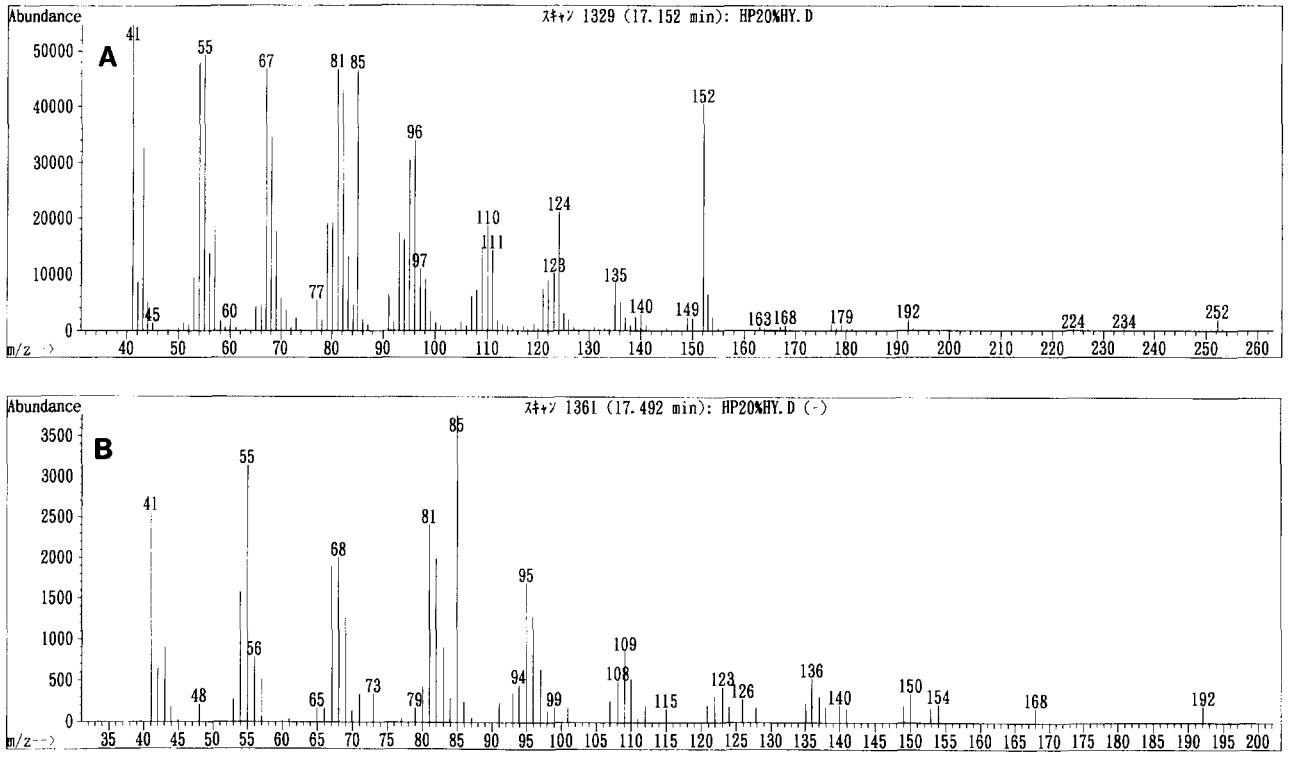

Figure 5. MS data of derivatives obtained by partial hydrogenation of the pheromone with diimide. (A) The major monoene product showing a fragmentation pattern that matched with 7-hexadecen-4-olide. (B) A monoene lactone obtained only in small amounts.

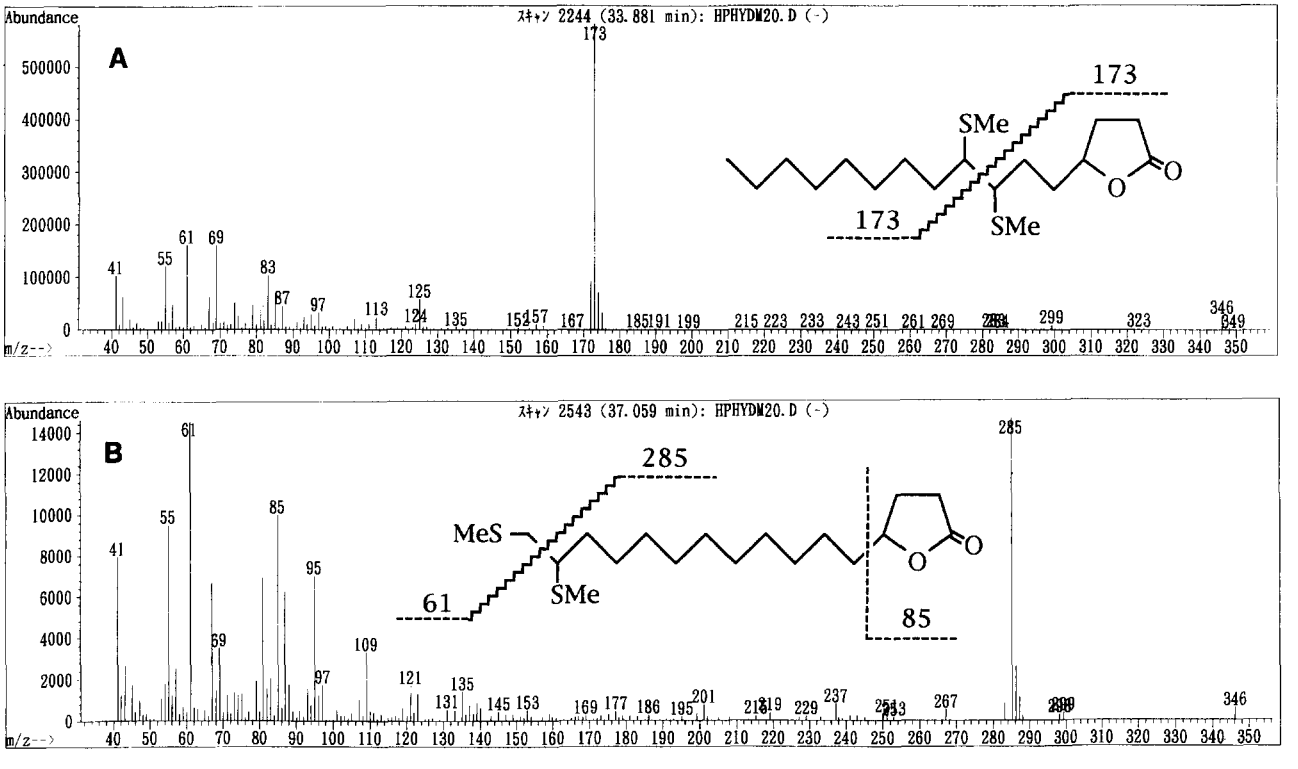

Figure 6. MS of the derivatives obtained by partial hydrogenation of the sex pheromone and subsequent DMDS reaction. (A) The major product gave a fragmentation pattern that confirmed the original location of the double bond in position 7. (B) The fragmentation pattern of the minor product confirmed that originally there was a terminal double bond.

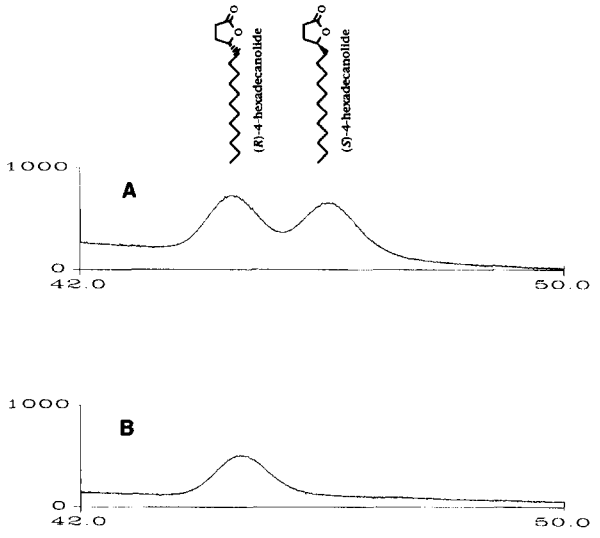

Figure 7. (A) Resolution of enantiomers of 4-hexadecanolide on a Chiraldex GTA column. Synthetic (*S*)-4-hexadecanolide had the same retention time as the second peak. (B) Lactone obtained by hydrogenation of the natural product.

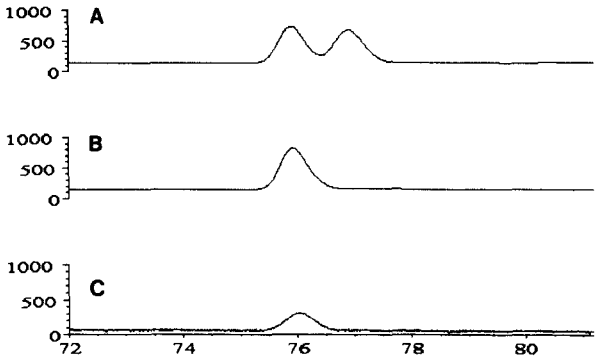

Figure 8. (A) Resolution of enantiomers of 4-hexadecanolide on a Chirasil-DEX CB column (75.9 and 76.9 min). (B) Synthetic (*R*,*Z*)-7,15-hexadecadien-4-olide, after catalytic hydrogenation (75.9 min). (C) Lactone obtained by hydrogenation of the sex pheromone (75.9 min).

[Fig. 7(b)] that corresponded to the peak of shorter retention time, 45.0 min. Synthetic (*S*)-4-hexadecanolide had the same retention time as the second peak, 46.5 min, and thus the two peaks were identified as (*R*) and (*S*), respectively (Fig. 7). The stereochemistry of the sex pheromone could be assigned as (*R*).

(*R*,*Z*)-7,15-Hexadecadien-4-olide was prepared from L-malic acid in 14 steps. Details of the synthesis will be described elsewhere.

The synthetic pheromone gave the same MS, IR, retention time and GC–EAD response as the natural product. A small amount of the (*E*) isomer (due to the Wittig coupling) gave a longer retention time than the pheromone, further corroborating that the double bond at position 7 had the *cis* configuration (as assigned by IR). Hydrogenation of the natural product and comparison on a chiral column, Chirasil-DEX CB, showed that both the synthetic and natural pheromone had the same (*R*) stereochemistry (Fig. 8). A detailed report on the biological activity of the synthetic sex pheromone will appear elsewhere.

Based on a handful of sex pheromones identified in scarab beetles and other evidence, one of us (WSL) hypothesized that the more primitive Melolonthinae species (compared with Rutelinae) use less complex compounds because sex pheromones in this group seem to have evolved from a primary defensive role.[1] In this book[1] we have an example that supports this hypothesis: the identification of (*Z*,*E*)-α-farnesene, a putative sex pheromone of *Maladera matrida* (Yarden et al.). The case of the yellowish elongate chafer, however, denies the hypothesis. As far as biosynthesis of sex pheromones of scarabaeids is concerned, the sex pheromone of *H. picea* is the most complex compound identified in this group to date and fills in the category of fatty acid derivative compounds that was supposed to be a characteristic only of rutelines.

Hitherto, the lactones identified as scarab sex pheromones (japonilure and buibuilactone) have a double bond adjacent to the lactone ring that are likely produced by two cycles of β-oxidation from oleic or palmitoleic acid, one allylic oxidation and cyclization.[1] Apart from one β-oxidation, the biosynthesis of (*R*,*Z*)-7,15-hexadecadien-4-olide from oleic acid would require the oxidation of a nonactivated carbon (as opposed to an allylic oxidation in the previous cases) and a terminal double bond formation, which would require the participation of different enzymes. By contrast, relatively few enzymes might be responsible for the great diversity of sex pheromones in the Lepidoptera. Roelofs and Brown[7] have hypothesized that one enzyme in particular, Δ-11-desaturase,[8] is the only one needed to explain most of the great variety of tortricid and noctuid pheromones. The major variable would be whether it acted before or after a chain-shortening step or a chain-elongation step, also enzyme-performed. Conjugated, di-unsaturated pheromone molecules would result from the Δ-11-desaturase acting both before and after chain-length alterations. For new pheromone signals to evolve, new enzymes would not need to come into play.

It is worth noting that the sex pheromone of *H. picea* has an absolute configuration different from the previously identified japonilure and buibuilactone.[1] Although the sex pheromone of the yellowish elongate chafer has different spatial arrangements in the chiral center, compared with japonilure and buibuilactone, they all have the (*R*)-stereochemistry because of the (*R*)/(*S*) conventions.

Experimental

Insects

H. picea beetles were collected in the tea field of the Shizuoka Tea Experiment Station and in the lily fields of Kyodo Trading Co. Ltd, Hokkaido. They were

transported to Tsukuba at low temperature and kept in the laboratory at 20 °C and under a 16 L:8 D photoperiod.

Collection of insect volatiles

Airborne volatiles from *H. picea* females produced during the scotophase were trapped on a Super Q (Alltech, Deerfield, IL) column set in all glass aeration apparatus. The column was washed with hexane and the extract concentrated to 0.1 female equivalent (FE)/μL. Whole-body extracts were obtained during the first 30 min of the dark period by immersing female beetles in hexane for 3 min. The extracts were filtered over Pasteur pipettes containing cotton plugs and 3 cm of anhydrous sodium sulfate and subsequently concentrated in vacuo to give a 0.1 FE/μL solution.

Bioassay

In the beginning of the scotophase (ca. 30 min after the lights went off), 10 males were placed in a plastic box (20 × 30 cm; 5 cm height). Two pieces of filter paper (2 × 2 cm), one loaded with the test material and the other with hexane (blank), were placed in opposite sites of the arena and the behavior of the insect was visually observed for 15 min. Males responded to active material by extending the antenna with the lamellae opened, raising the wings, gathering on the filter paper and attempting to copulate with each other by mounting and probing with their aedeagus extended. Male responses to whole-body extracts laden on filter paper were similar to their responses when caged females (three) were placed inside the arena.

Isolation of the active material

Whole-body extracts were subjected to flash column chromatography on silica gel columns; in the exploratory run, this was eluted with hexane:ether (100:0, 95:5, 90:10, 80:20, 50:50, 100:0) mixtures. Since the active compound was recovered (along with other impurities) in the hexane:ether (80:20) fraction, 200 FE were newly fractionated by eluting with hexane:ether (100:0, 97.5:2.5, 95:5, 92.5:7.5, 90:10, 87.5:12.5, 85:15, 82.5:17.5, 80:20, 70:30, 60:40) mixtures and collecting smaller fractions (1.5 mL × 3 for each mixture). The active compound was isolated (> 97% purity) in the hexane:ether (85:15) as well as in other partially contaminated fractions.

General analytical procedures

GC analyses were performed on HP 5890 Series II Plus gas chromatograph equipped with FID, electronic pressure control and an HP 3365 Series II Chemstation. The following capillary columns were used: 30 m × 0.25 m × 0.25 μm HP-5 MS (oven temperature was held at 70 °C for 1 min, increased to 270 °C at a rate of 10 °C/min, held at 270 °C for 20 min), 30 m × 0.25 mm × 0.25 μm HP-INNOWAX (oven temperature was held at 70 °C for 1 min, increased to 250 °C at a rate of 10 °C/min, held at this temperature for 20 min). Chiral separations were obtained on cyclodextrin-based capillary columns, Chiraldex GTA (20 m × 0.25 mm × 0.25 μm; Astec, Whippany, NJ) operated at 150 °C and 2.5 kg/cm^2 (119 cm/s; 9.42 mL/min) and Chirasil-DEX CB (25 m × 0.25 mm × 0.25 μm; Chrompack, The Netherlands) operated at 165 °C and 1.2 kg/cm^2 (47.5 cm/s, 2.54 mL/min). GC–MS was carried out in the following systems. Electron impact (EI) mass spectra were obtained either on a Hewlett Packard mass selective detector MSD 5972 Series coupled with an HP5890 Series II Plus gas chromatograph equipped either with an HP-5MS or HP-INNOWAX capillary column or on a Hewlett Packard gas chromatograph electron ionization detector GCD Series, equipped with an HP-5MS capillary column. Chemical ionization (CI) with isobutane was performed on an Automass System (JEOL, Japan). IR spectra were generated on a Tracer FTS-60A FTIR combined with an HP 5890 gas chromatograph and equipped with a 30 m × 0.25 mm × 0.25 μm DB-wax capillary column (J&W, Scientific, Folsom, CA). NMR spectra were recorded on a JEOL JNM-A500 spectrometer.

Gas chromatography coupled with an electroantennographic detector (GC–EAD)

GC–EAD was performed according to the method of Struble and Arn.[9] An HP 5890 Series II Plus gas chromatograph was modified to have the effluent from the capillary column split into EAD and FID (3:1 ratio). Beetle antennae were placed in a previously described acrylic stage;[10] this was set inside the glass transfer line (2 cm away from the GC outlet) and connected with gold wires to an amplifier (gain 5) and filtered through a passive filter (cut-off frequency 0.12 Hz). The signal was fed on an A/D 35900E Interface (Hewlett Packard, Palo Alto, CA). Simultaneous acquisitions of FID and EAD signals were obtained with the above mentioned chemstation.

Derivatizations

The diene was partially reduced by a mixture of hydrazine and hydrogen peroxide to generate diimide in situ.[11] The stock solutions were prepared as follows: hydrazine hydrate (3 mL) in EtOH (100 mL), and 30% H_2O_2 (0.4 mL) in EtOH (100 mL). A solution of the isolated pheromone (50 μL) was dried up and EtOH (50 μL) was added; this was mixed with 50 μL of each stock solution and the reaction mixture was heated at 60 °C for 30 min. After being allowed to cool to room temperature, it was acidified with 1 N HCl, extracted with hexane and concentrated. For hydrogenation, a solution of the isolated pheromone in hexane (50 μL), containing a very small amount of PtO_2, was stirred for 1 h under a hydrogen atmosphere. DMDS deriva-

tization was carried out according to the method of Vincenti et al.[4]

Synthesis

L-Malic acid was converted into (*S*)-2,2-diethyl-4-(2-iodoethyl)-1,3-dioxolane by a known procedure.[12] Alkylation of the iodide with 1,3-dithiane followed by deprotection of the acetal group gave (*S*)-2-(3,4-dihydroxybutyl)-1,3-dithiane, which was then converted into the corresponding epoxide *via* tosylation of the primary hydroxyl group. The epoxide was ring-opened with the anion of acetonitrile and the resulting alcohol was protected as its 1-ethoxyethyl ether. Reduction of the nitrile with diisobutylaluminum hydride gave an aldehyde, which was subjected to the Wittig reaction with (8-nonenylidene)triphenylphosphorane to give a (*Z*)-olefin.[13] Removal of the protective groups followed by oxidation gave (*R*)-7,15-hexadecadien-4-olide.

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