

**Study on the Sex Pheromones Produced by Female
Moths in the Subfamily Pyraustinae**

ノメイガ亜科雌蛾性フェロモンに関する研究

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**STUDY ON THE SEX PHEROMONES
PRODUCED BY FEMALE MOTHS IN THE
SUBFAMILY PYRAUSTINAE**

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CHAPTER I: PREFACE

There are millions of organisms living on the earth, and many kinds of behavior are observed between con-specific or hetero-specific species incessantly, such as predation, reproduction, migration and so on. What conduct the behavior? How do they communicate with each other?

When humans communicate with each other, we rely on acoustic sounds and signals, as well as words and body language. In the insect world, the communication among insects, like that of humans, has species-specific single- or multiple-component chemical messaging that convey the information. Many insect species recognize information coded like pheromones to recognize other individuals during social interactions (Leal, 2005). Pheromones are chemicals capable of acting outside the body of the secreting individual to impact the behavior of the receiving individual (Karlson and Lüscher, 1959). There are alarm pheromones, trail pheromones, sex pheromones, recognition pheromones and many others that affect behavior or physiology.

The sex pheromones of lepidopteran are generally produced by female moths to attract male moths for mating. In the late nineteenth century, Jean Henri Fabre first described the attraction of male moths by con-specific females (Fabre, 1879; Touhara, 2013), and then the first sex pheromone Bombykol, the sex pheromone of the silk moth *Bombyx mori*, was discovered by Butenandt in 1959 (Butenandt et al., 1959, Fig. I-1). Over the last 50 years, a great deal of progress has been made to understanding the chemical communication system in insect. Because of the great economic importance of moths and butterflies, the pheromones of lepidopteran is the best investigated so far (Ando 2014; El-Sayed 2014). Up to now, sex pheromones from more than 650 species were identified in this order and have been classified into groups of Type I (75%), Type II (15%), and others (10%) according to their chemical structures. Type I compounds, which are fatty alcohols with a C10-C18 straight

chain and their derivatives, have been widely identified from lepidopteran insects in many families, such as Crambidae and Pyralidae in the superfamily of Pyraloidea. Type II compounds, which are unsaturated hydrocarbons and their epoxy derivatives, have been widely identified from highly evolved families, such as Geometridae, Noctuidae, Arctiidae, etc. These Type II components commonly possess (Z)-double bonds or cis-epoxy rings at the 6,9 and 3,6,9-positions in a straight chain. Miscellaneous pheromone components are classified as neither Type I nor II, such as secondary alcohols, methyl-branched chemicals, or unsaturated ketones, etc. (Ando et al., 2004, Fig. I-2).

Many of lepidopteran sex pheromones were utilized in integrated pest management as a monitoring tool, or used in pest control by mating disruption and mass trapping. Different from the use of ordinal insecticides, the sex pheromone has high target selectivity, no toxicity to mammals, and is environmental friendly (Ando et al., 2004). The principal use of insect pheromones as a monitoring tool is to attract insects to traps for detection of temporal distribution. The synthetic pheromone (1 mg) is immersed in a small rubber septum (8 mm OD), and a trap baited with this lure can effectively catch males for at least one month (Yan et al., 2014). Sex pheromone can be used for disruption of mating, which is achieved by placing high concentrations of pheromone at regular intervals throughout the field. The high concentration of pheromone saturates the area resulting in males failing to find females, which produce very minute quantities of these chemicals, thus preventing mating and multiplication of the pest (Cardé and Minks, 1995; Ohtani et al., 2001). Mass trapping of insects is one concept of the direct application for the control of pest species (Ando et al., 2004).

The superfamily Pyraloidea comprise more than 15,576 described species worldwide, which includes many serious agriculture pest, such as Indian meal moth (*Plodia interpunctella*), European corn borer (*Ostrinia nubilalis*), rice moth (*Corcyra cephalonica*), etc (Nuss et al., 2011). However, only 90 species's sex pheromone had

been identified to date. Even so, for many species in this family, the lure baited with the synthetic pheromone has less attractive than female, such as *Hellula undalis*, *Diaphania indica*, *Omphisa anastomosalis*, etc (Arai et al., 1982; Sugie et al., 2003; Wakamura et al., 1998, 2010). It is necessary to clarify the communication system in Pyraloidea species, in order to establish a new insect pest control program base upon using sex pheromone as alternative to insecticide, along with resistant cultivars, mechanical and biological control methods, that was used in the integrated pest management (IPM) strategy for the control of pest. As the biggest subfamily of Pyraloidea, Pyraustinae include more than 340 species in Japan, this thesis focused on the sex pheromones of nine Pyraustinae species which mainly inhabiting in Asia.

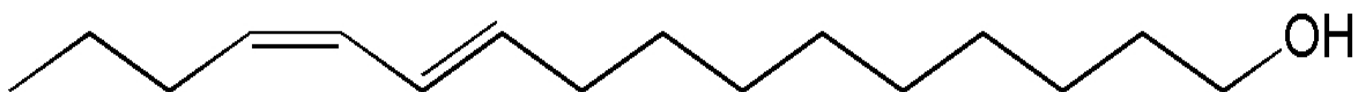
The *O. anastomosalis* and *Leucinodes orbonalis* are both borer moths, and serious pests in sweet potato and eggplant in Asia. The sex pheromone of *O. anastomosalis* was identified as (10*E*,14*E*)-10,14-hexadecadienal (E10,E14-16:Ald) as the major pheromone component, with hexadecanal, (*E*)-10-hexadecenal, and (*E*)-14-hexadecenal as minor components (Wakamura et al., 2010). The sex pheromone of *L. orbonalis* was identified as (*E*)-11-hexadecenyl acetate (E11-16:OAc), the major component, and (*E*)-11-hexadecen-1-ol (E11-16:OH) as the minor component (Zhu et al., 1987; Cork et al., 2001). However, for the two pests, traps baited with the synthetic lures were less effective in attracting males than those with virgin females on field trials. So the pheromone extracts of *O. anastomosalis* and *L. orbonalis* were reexamined, and a new component (3*Z*,6*Z*,9*Z*)-3,6,9-tricosatriene (Z3,Z6,Z9-23:H) was found in the two species, respectively. Followed field evaluation indicated that this tricosatriene plays a big role as a strong synergist in male attraction for both species. Which make up the Chapter II.

As the biggest genus in the subfamily Pyraustinae in Japan, *Herpetogramma* include 18 species and most of them inhabit Okinawa islands. Although they have a closed geographic distribution because island areas are not large, mechanism of their reproductive isolation is very interesting. Each species seems to have a strict sexual

communication system that prevents it from mating with different species. Based on this hypothesis, some researches on the sex pheromones of genus *Herpetogramma* were conducted in Chapter III. Sex pheromones of two *Herpetogramma* species (*H. submarginale* and *H. basale*) were examined by GC-EAD and GC-MS analyses. Mass spectra of the pheromone components and their derivatives with DMDS or MTAD indicated that females of *H. submarginale* and *H. basale* produced (Z)-13-hexadecenyl acetate (Z13-16:OAc) and (11Z,13E)-11,13-hexadecadienyl acetate (Z11,E13-16:OAc), respectively. Z13-16:OAc is a new compound identified as an insect pheromone. In the field test, Z13-16:OAc alone was attractive to *H. submarginale* males effectively. However, synthetic lures baited with Z11,E13-16:OAc were not attracted *H. basal* males. The unsuccessful field test of *H. basale* suggested that additional pheromone components may be present in the female moths.

Furthermore, sex pheromones of other five Pyraustinae species were examined in Chapter IV as follows: *Palpita nigopunctalis*, *Pleuroptya sabinusalis*, *P. inferior*, *Spoladea recurvalis*, and *Eurrhparodes accessalis*. In addition to the usual GC-EAD and GC-MS analyses, GC-FT/IR analysis was conducted in the experiments with pheromone extracts from the former two species. IR spectra confirmed the configuration of double bonds and the kind of functional groups at terminal positions. Although field evaluation of synthetic pheromones of these five species was unfortunately showed no good male attraction, results of the identification indicate diversity of pheromone structures produced by Pyraustinae species.

The sex pheromone communication is one of the chemical communications which are universal in nature. Further research on insect pheromone could help better understand chemical communication system, mechanisms of reproductive isolation and insect speciation of subfamily Pyraustinae species.



IUPAC name: (10*E*,12*Z*)-hexadeca-10,12-dien-1-ol, Bombykol

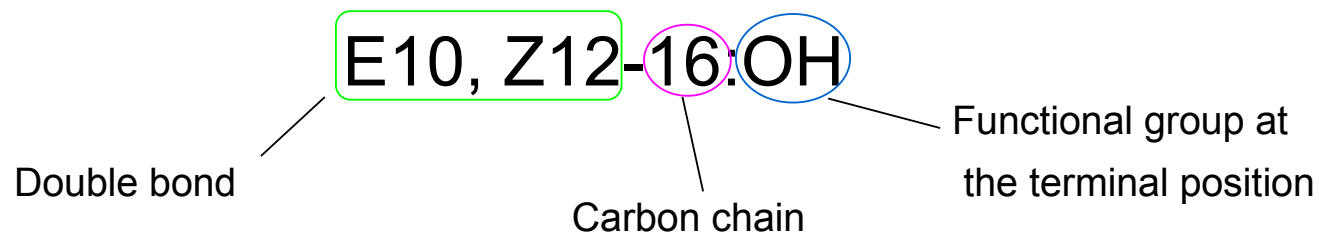


Fig. I-1 Naming rules of pheromone compounds

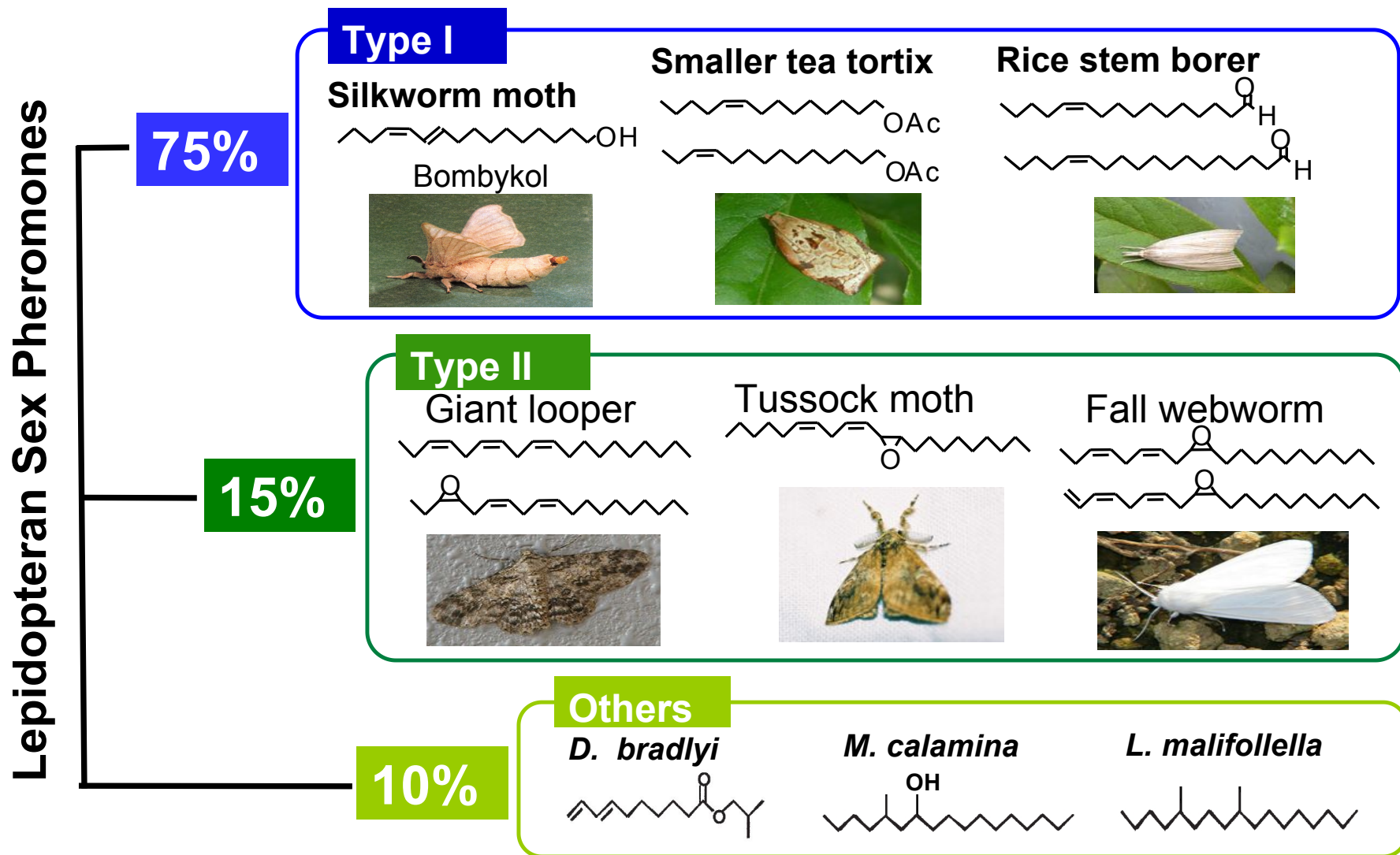


Fig. I-2. Classification of sex pheromone in Lepidopteran. (Photos from <http://www.jpmoth.org/>)

List of Abbreviations

EAG = Electroantennogram

EI = Electron impact ionization

FID = Flame ionization detector

GC-EAD = Gas chromatography combined with an EAG detector

GC-FT/IR = GC-Fourier transfer/infrared spectrometry

GC-MS = Gas chromatography combined with mass spectrometry

KI = Kovats retention index

NMR = Nuclear magnetic resonance spectrometry

RID = Refractive index detector

R_t = Retention time

TIC = Total ion chromatogram

DMDS = Dimethyl disulfide

DMF= *N,N*-dimethylformamide

DMSO = Dimethylsulfoxide

EtOAc = Ethyl acetate

EtOH = Ethanol

Hx = Hexane

HMPA= Hexamethylphosphoramide

MeOH = Methanol

MTAD = 4-Methyl-1,2,4-triazoline-3,5-dione

PCC = Pyridinium chlorochromate

THF = Tetrahydrofuran

CHAPTER II: Reexamination of the Female Sex Pheromones of *Omphisa anastomosalis* and *Leucinodes orbonalis*

II-1 Introduction

The sweet potato vine borer moth, *Omphisa anastomosalis* Guenée (Lepidoptera: Crambidae: Pyraustinae), is a serious pest of Convolvulaceae plants, especially the sweet potato, which is a reliable crop in cases of crop failure of other staple foods due to typhoon flooding in Southeast Asia. In previous work using a population from Okinawa, Japan, the sex pheromone of *O. anastomosalis* was identified as a novel dienyl component (10*E*,14*E*)-10,14-hexadecadienal (E10,E14-16:Ald) as the major pheromone component, with hexadecanal, (*E*)-10-hexadecenal, and (*E*)-14-hexadecenal as minor components (Wakamura et al., 2010). However, traps baited with the synthetic compounds were less effective at attracting males in the field than those baited with virgin females.

The eggplant fruit and shoot borer, *Leucinodes orbonalis* Guenée (Lepidoptera: Crambidae: Pyraustinae), is one of the most destructive pests on eggplant (*Solanum melongena* Linnaeus) in South and Southeast Asia. The larvae bore inside plant shoots and fruits adversely affecting plant growth, yield and fruit quality. Eggplant is one of the most economically important vegetables of tropics (Latif et al., 2010). However, effective control by insecticide sprays is just obtained in a short period when newly larvae are outside the plant tissue. The IPM strategy based on sex pheromone for managing the *L. orbonalis* should be expanded and can be beneficial in holistic manner. The major component of *L. orbonalis* sex pheromone was identified as (*E*)-11-hexadecenyl acetate (E11-16:OAc) (Zhu et al. 1987). Although E11-16:OAc was used alone attracted significantly numbers of male moths (Srinivasan and Babu, 2000), the synthetic pheromone was inferior to live virgin female moths (Mainali, 2014).

Therefore, we speculated that there must be some unknown pheromone components which were overlooked in preliminary analysis for the two pests. In order to find the additional important pheromone components, the pheromone extracts from

the two kinds female glands were reexamined in this chapter. The research work aimed to develop an effective tool for pest control on both species.

II-2 Methods and Materials

II-2-1 Insects and pheromone extracts

II-2-1-1 *Omphisa anastomosalis*

Sweet potato veins containing larvae of *O. anastomosalis* were collected from sweet potato fields in An Giang Province, Vietnam. The larvae were fed on young leaf pedicels of a sweet potato plant placed in transparent plastic boxes and kept in 12L-12D at 27–30°C. The pedicels were renewed every two days until the larvae pupated. Male and female pupae were distinguished by inspecting their sexual slot on the 8th and 9th abdominal segments, and each pupa was separately placed in a small glass tube. About fifty abdominal tips of 2- or 3-day old virgin females were excised 2 hr after the onset of the scotophase and soaked in hexane (10 µl/female) for 15 min to extract the pheromone components. The crude extract was used for structural analysis of pheromone components after filtration. In order to confirm the positions of the double bonds in the proposed pheromone components, after removal of hexane, crude extracted materials (45 female equivalents, FE) were treated with DMDS (50 µl) that included a diethyl ether solution of iodine (60 mg/ml, 5 µl) and were held at 40°C overnight (Buser et al., 1983; Vang et al., 2011). After adding a 10% sodium thiosulfate solution (0.5 ml), produced DMDS adducts were extracted with hexane and analyzed by GC-MS.

II-2-1-2 *Leucinodes orbonalis*

Eggplant fruits infected with larvae of *L. orbonalis* were collected from eggplant fields in Can Tho city, Vietnam and brought to Can Tho University (Can Tho city, Vietnam). In laboratory, the eggplant fruits and larvae were placed in transparent plastic boxes and kept in 12 L:12D at 27-30°C until the larvae pupated. Male and female pupae were distinguished by inspecting their sexual slot on the 8th abdominal segments, and each pupa was separately placed in a small glass tube. After eclosion, abdominal tips of 2- or 3-day old virgin females were excised 2-3 h after the onset of the scotophase and soaked in hexane (10 µl/female) for 15 min to extract pheromone

components. Otherwise, body including abdomen and thorax (head, wings and legs were removed) of the females were also extracted by the same manner doing for the abdominal tips. The crude extracts were filtrated before using for structural analysis.

II-2-2 Analytical Instruments

II-2-2-1 Gas Chromatography (GC) - Mass Spectrometry (MS)

GC-MS was conducted using electron impact ionization (EI, 70 eV) on an HP5975 mass spectrometer (quadrupole type, Agilent Technologies Inc., Palo Alto, CA, USA). The GC was equipped with a split/splitless injector and a capillary column, DB-225 (0.25 mm \times 30 m, 0.25 μ m, J & W Scientific, Folsom, CA, USA), or DB-23 (0.25 mm \times 30 m, 0.25 μ m, J & W Scientific), or HP-5 (0.25 mm ID, 30 m length, 0.25 μ m film thickness, J & W Scientific). The flow rate of the carrier gas (He) was 1.0 mL/min, and the GC-inlet temperature was 220°C. The oven temperature for analysis of crude pheromone extracts was maintained at 80°C for 1 min, programmed at 8°C/min to 210°C, and then held for 10 min. For analysis of DMDS derivatives, the HP-5 column was used and the oven temperature was maintained at 100°C for 2 min and then programmed at 15°C/min to 280°C.

II-2-2-2 Other analytical instruments

IR spectra were recorded as a thin film (neat liquid) with a Jasco FT/IR-350 (JASCO, Tokyo, Japan). ^1H and ^{13}C NMR spectra were recorded with an AL300 FT-NMR spectrometer (JEOL Ltd., Tokyo, Japan) at 300.53 and 75.57 MHz, respectively, in CDCl_3 solutions containing TMS as an internal standard.

II-2-3 Chemicals

II-2-3-1 (10E,14E)- and (10E,14Z)-Isomers of 10,14-Hexadecadienal (E10,E14-16: Ald and E10,Z14-16:Ald, Fig. II-1a)

One hydroxyl group of the commercially available C_9 diol **1** was brominated by a treatment with an aqueous HBr solution, and a methoxymethyl (MOM) ether of 9-bromononane-1-ol **2** was obtained by protection of another hydroxyl group with dimethoxymethane. After conversion into a THP-ether **4**, the terminal acetylene

compound **3** was coupled with **2** by butyllithium in a mixed solvent of dry THF and HMPA to prepare a 10-tetradecynyl compound **5**. The THP group of the key intermediate **5** was selectively removed by treatment with *p*-TsOH in EtOH, and then the produced acetylene alcohol was reduced to an unsaturated alcohol **6** by treatment with LiAlH₄ in diglyme (McElfresh et al., 2001). The *E* configuration of the double bond at the 10-position was confirmed by chemical shifts (28.9 and 32.5 ppm) of two allylic methylene carbons. By oxidation with pyridinium chlorochromate (PCC), the C₁₄ alcohol **6** was converted to the corresponding aldehyde **7**. The Wittig reaction between **7** and an ylide derived from ethyltriphenylphosphonium bromide furnished a 1:2 mixture of MOM ethers of (10*E*,14*E*)-10,14-hexadecadien-1-ol and the (10*E*,14*Z*)-isomer **8**. The mixing ratio was estimated by signal intensities of allylic methyl carbons at 17.9 ppm of the (10*E*,14*E*)-isomer and 12.8 ppm of the (10*E*,14*Z*)-isomer. Mixture **8** was treated with dry HCl in MeOH to yield two geometrical isomers of the C₁₆ dienyl alcohol, which were separated by column chromatography over silica gel impregnated with AgNO₃. Each pure isomer was treated with PCC in CH₂Cl₂, and E10,E14-16:Ald and E10,Z14-16:Ald were obtained. Experimental details are described in experiment part to this manuscript. IR data of E10,E14-16:Ald; (neat) ν_{\max} cm⁻¹: 2920 (s), 2855 (m), 2715 (w), 1717 (s, C=O), 1475 (m), 968 (s). NMR data of E10,E14-16:Ald; ¹H MR δ ppm: 1.29 (10H, broad s), 1.62 (2H, m, CH₂CH₂CHO), 1.64 (3H, d, *J* = 4.1 Hz, CH₃), 1.97 (2H, m, CH=CHCH₂CH₂), 2.03 (4H, broad s, CH=CHCH₂CH₂CH=CH), 2.42 (2H, td, *J* = 9.8, 2.4 Hz, CH₂CHO) 5.41 (4H, m, CH=CHCH₂CH₂CH=CH), 9.76 (1H, t, *J* = 2.4 Hz, CHO); ¹³C NMR δ : 17.9, 22.1, 29.0, 29.2, 29.28, 29.33, 29.5, 32.5, 32.69, 32.75, 43.9, 124.9, 129.7, 130.6, 131.0, 202.9. IR data of E10,Z14-16:Ald; (neat) ν_{\max} cm⁻¹: 3015 (w), 2919 (s), 2854 (m), 2715 (w), 1716 (s, C=O), 1475 (m), 968 (m), 725 (w). NMR data of E10,Z14-16:Ald; ¹H NMR δ : 1.29 (10H, broad s), 1.60 (3H, d, *J* = 5.4 Hz, CH₃), 1.62 (2H, m, CH₂CH₂CHO), 1.98 (2H, m, CH=CHCH₂CH₂), 2.06 (4H, m, CH=CHCH₂CH₂CH=CH), 2.42 (2H, td, *J* = 9.7, 2.4 Hz, CH₂CHO) 5.41 (4H, m, CH=CHCH₂CH₂CH=CH), 9.76 (1H, t, *J* = 2.4 Hz, CHO); ¹³C NMR δ : 12.8, 22.1, 27.0, 29.1, 29.2, 29.29, 29.34, 29.5, 32.5, 32.6, 43.9, 124.0, 129.8, 130.2, 130.7,

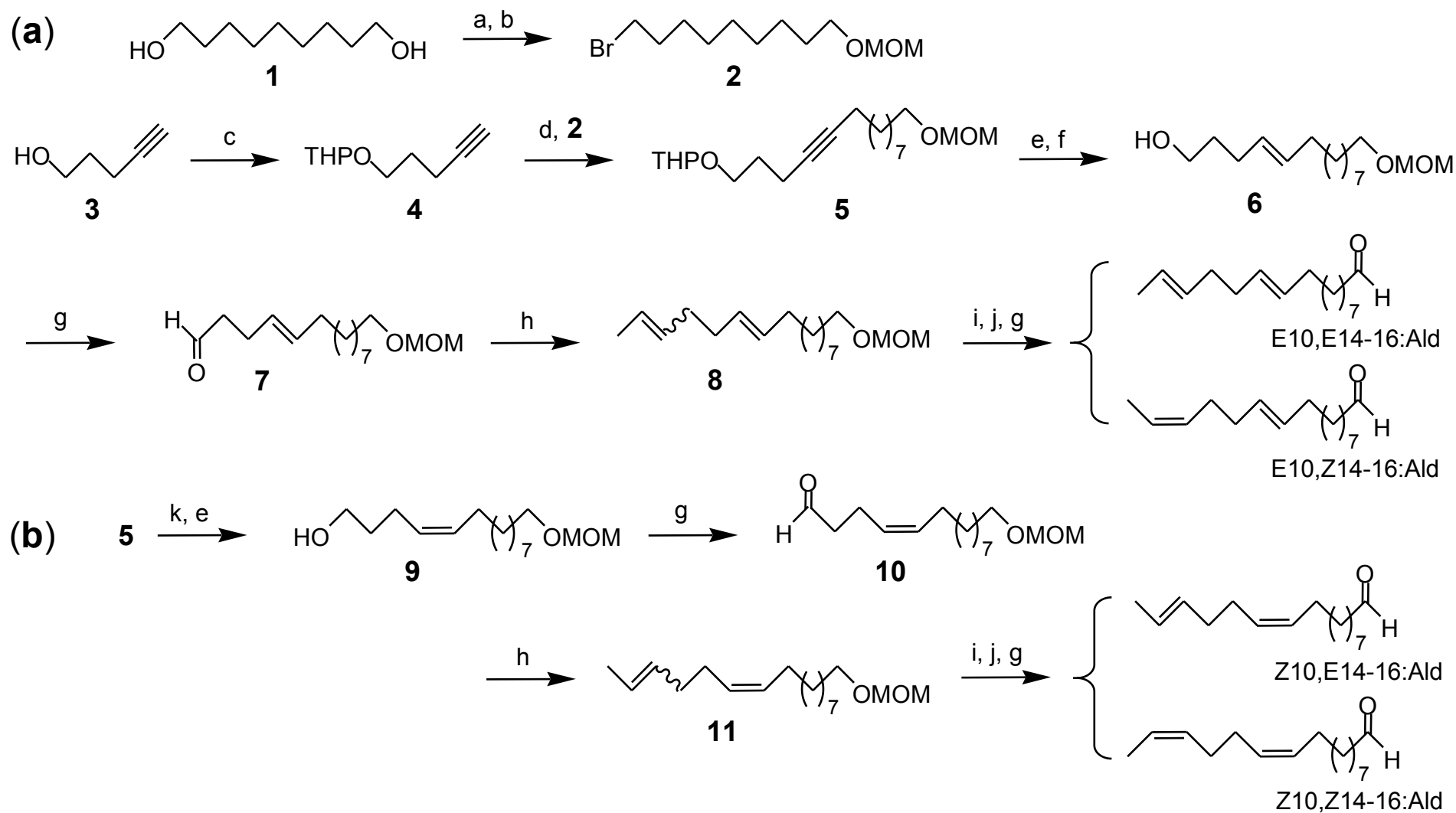


Fig. II-1. Synthetic schemes for 10,14-hexadecadienal; **(a)** (10*E*,14*E*)- and (10*E*,14*Z*)-isomers (E10,E14-16:Ald and E10,Z14-16:Ald), and **(b)** (10*Z*,14*E*)- and (10*Z*,14*Z*)-isomers (Z10,E14-16:Ald and Z10,Z14-16:Ald). Reagents: a, HBr (40%), toluene; b, dimethoxymethane (DMM), *p*-TsOH, LiBr; c, 3,4-dihydro-2*H*-pyran, *p*-TsOH, CH₂Cl₂; d, BuLi, THF, HMPA; e, *p*-TsOH, EtOH; f, LiAlH₄, diglyme; g, PCC, CH₂Cl₂; h, CH₃CH=PPh₃, THF; i, dry HCl, MeOH; j, column chromatography with SiO₂-AgNO₃; k, H₂, P-2 Ni, EtOH.

203.0.

II-2-3-2 (10*Z*,14*E*)- and (10*Z*,14*Z*)-Isomers of 10,14-Hexadecadienal (Z10,E14-16: Ald and Z10,Z14-16:Ald, Fig. II-1b)

Acetylene compound **5** was reduced by catalytic hydrogenation over P-2 nickel (Jain et al., 1983) and deprotected with *p*-TsOH in EtOH to yield unsaturated alcohol **9**. The *Z* configuration of the double bond at the 10-position was confirmed by chemical shifts (23.6 and 27.2 ppm) of two allylic methylene carbons. In the same manner as Scheme (a), the alcohol **9** was oxidized to aldehyde **10** and coupled with an ylide derived from ethyltriphenylphosphonium bromide to yield a 1:2 mixture of the (10*Z*,14*E*)- and (10*Z*,14*Z*)-isomers of a C₁₆ dienyl compound **11**. The mixing ratio was also estimated by signal intensities of allylic methyl carbons at 17.8 ppm of the (10*Z*,14*E*)-isomer and 12.8 ppm of the (10*Z*,14*Z*)-isomer. By deprotection with dry HCl, column chromatography over silica gel impregnated with AgNO₃, and PCC oxidation, Z10,E14-16:Ald and Z10,Z14-16:Ald were obtained. Experimental details are described in experiment part to this manuscript. IR data of Z10,E14-16:Ald; (neat) ν_{\max} cm⁻¹: 3005 (w), 2921 (s), 2854 (m), 2715 (w), 1715 (s, C=O), 1475 (m), 968 (m), 725 (w). NMR data of Z10,E14-16:Ald; ¹H MR δ ppm: 1.29 (10H, broad s), 1.62 (2H, m, CH₂CH₂CHO), 1.64 (3H, d, *J* = 4.0 Hz, CH₃), 2.03 (6H, m, CH=CHCH₂CH₂CH=CHCH₂), 2.42 (2H, td, *J* = 9.8, 2.3 Hz, CH₂CHO), 5.36 (2H, m, CH=CH), 5.43 (2H, m, CH=CH), 9.76 (1H, t, *J* = 2.3 Hz, CHO); ¹³C NMR δ : 17.9, 22.1, 27.2, 27.3, 29.17, 29.22, 29.3 (×2), 29.7, 32.7, 43.9, 125.0, 129.2, 130.2, 131.0, 203.0. IR data of Z10,Z14-16:Ald; (neat) ν_{\max} cm⁻¹: 3007 (m), 2917 (s), 2853 (m), 2715 (w), 1717 (s, C=O), 1474 (m), 725 (m). NMR data of Z10,Z14-16:Ald; ¹H NMR δ ppm: 1.30 (10H, broad s), 1.61 (3H, d, *J* = 5.4 Hz, CH₃), 1.62 (2H, m, CH₂CH₂CHO), 2.02 (2H, m, CH=CHCH₂CH₂), 2.09 (4H, m, CH=CHCH₂CH₂CH=CH), 2.43 (2H, td, *J* = 9.8, 2.4 Hz, CH₂CHO), 5.37 (2H, m, CH=CH), 5.43 (2H, m, CH=CH), 9.76 (1H, t, *J* = 2.4 Hz, CHO); ¹³C NMR δ : 12.8, 22.1, 27.0, 27.21, 27.23, 29.1, 29.2, 29.3 (×2), 29.7, 43.9, 124.1, 129.2, 130.2, 130.3, 202.9.

II-2-3-3 Other Chemicals

Monoenyl compounds E10-16:Ald and E14-16:Ald were synthesized by a selective reduction of the corresponding acetylene compounds, which were prepared *via* a THP ether of 10-undecyn-1-ol or 14-pentadecyn-1-ol. E11-16:OH was supplied by Shin-Etsu Chemical Co., Ltd., (Tokyo, Japan), its acetate and aldehyde were obtained by the acetylation and PCC oxidation. A C₂₃ 6,9-diene (Z6,Z9-23:H) and C₁₉–C₂₃ 3,6,9-trienes (Z3,Z6,Z9-19:H, Z3,Z6,Z9-21:H, Z3,Z6,Z9-22:H, and Z3,Z6,Z9-23:H) had been synthesized (Ando et al., 1993; 1995). A C₂₅ 3,6,9-triene (Z3,Z6,Z9-25:H) and C₂₃–C₂₅ 3,6,9,12,15-pentaenes (Z3,Z6,Z9,Z12,Z15-23:H and Z3,Z6,Z9,Z12,Z15-25:H) were synthesized using a similar method, starting from linolenic acid and ethyl 5,8,11,14,17-eicosapentaenoate, respectively.

Before using as synthetic standards or for field evaluation, these chemicals were purified by an open column packed with 15% silver nitrate (AgNO₃) in silica gel, and the purity which was checked by GC was >98%.

II-2-4 Synthesis Experiments

Tetrahydrofuran (THF) was distilled with CaH₂ and LiAlH₄, and stored under sodium threadlet. Reactions with air-sensitive and/or water-sensitive reagents were carried out in dried glasswares under argon (Ar) atmosphere. Each crude product was dried over anhydrous Na₂SO₄, concentrated by rotary evaporation under reduced pressure, and purified by column chromatography with silica gel (SiO₂, 63-210 μm; Kanto Chemical Co., Japan).

II-2-4-1 Synthesis of (10E,14E)- and (10E,14Z)-isomers of 10,14-hexadecadienal

MOM ether of 9-bromononan-1-ol (2) In a water reflux releasing device, HBr (40% in water, 6.0 ml) was added dropwise to a mixture of 1,9-nonanediol (**1**, 4.5 g, 28 mmol) and toluene (100 ml), and the solution was heated overnight at 110°C under a refluxing and stirring condition. After cooling, the mixture was quenched with water (50 ml). The product was extracted with hexane (60 ml ×3) and washed with a

saturated aqueous solution of NaHCO₃ (50 ml ×2) and brine (50 ml ×2). After usual workup, the crude product was chromatographed over SiO₂ (60 g) to give 9-bromononan-1-ol (4.9 g, 22 mmol, 78% yield). Next, the alcohol (4.0 g, 18 mmol) was dissolved in dimethoxymethane (65 ml) and treated with *p*-toluenesulfonic acid monohydrate (*p*-TsOH, 0.25 g, 1.5 mmol) and LiBr (0.45 g, 5.2 mmol). After stirring overnight at room temperature (r.t.), the mixture was poured into a saturated aqueous solution of NaHCO₃ (60 ml) and the product was extracted with hexane (60 ml ×3). After usual workup, the crude product was chromatographed over SiO₂ (50 g) to give **2** (4.5 g, 17 mmol, 95%). IR (neat) ν_{\max} cm⁻¹: 2929 (s), 2854 (m), 1385 (w), 1111 (m), 1047 (m), 920 (w). ¹H NMR δ : ~1.3 (10H, broad s), 1.59 (2H, m, CH₂CH₂O), 1.85 (2H, m, CH₂CH₂Br), 3.36 (3H, s, OCH₃), 3.40 (2H, t, *J* = 9.2Hz, CH₂Br), 3.52 (2H, t, *J* = 8.8Hz, CH₂CH₂O), 4.62 (2H, s, OCH₂O). ¹³C NMR δ : 26.2, 28.1, 28.7, 29.3, 29.4, 29.7, 32.8, 34.0, 55.1, 67.8, 96.4.

THP ether of 4-pentyn-1-ol (4) 3,4-Dihydro-2H-pyran (3.4 g, 40 mmol) dissolved in CH₂Cl₂ (20 ml) was added dropwise to 4-pentyn-1-ol (**3**, 3.0 g, 36 mmol) and *p*-TsOH (80 mg, 0.46 mmol) dissolved in CH₂Cl₂ (40 ml). The reaction mixture was stirred for 2.5 h at r.t., and was poured into a saturated aqueous solution of NaHCO₃ (50 ml). The product was extracted with hexane (60 ml ×3). After usual workup, the crude product was chromatographed over SiO₂ (60 g) to give **4** (5.8 g, 35 mmol, 97% yield). IR (neat) ν_{\max} cm⁻¹: 3296 (m), 2943 (s), 2871 (m), 1385 (m), 1138 (m), 1120 (s), 1035 (s), 636 (w). ¹H NMR δ : 1.51–1.85 (8H, m), 1.95 (1H, t, *J* = 3.4 Hz, C≡CH), 2.31 (2H, td, *J* = 9.5, 3.4 Hz, CH₂C≡CH), 3.47 (2H, m, OCHHCH₂ ×2), 3.82 (2H, m, OCHHCH₂ ×2), 4.59 (1H, t, *J* = 4.5Hz, OCHCH₂). ¹³C NMR δ : 15.3, 19.5, 25.5, 28.7, 30.7, 62.2, 65.8, 68.5, 84.0, 98.8.

MOM ether of 14-(tetrahydropyran-2-yloxy)-10-tetradecyn-1-ol (5) Butyllithium (BuLi, 2.7 M, 6.7 ml, 18 mmol) was added dropwise to a stirred mixture of **4** (2.5 g, 15 mmol), hexamethylphosphoramide (HMPA, 5.0 ml), and THF (50 ml) at -30°C under Ar. The mixture was warmed to -20°C and stirred for 1 h. Next, **2** (4.0 g, 15 mmol) dissolved in THF (15 ml) was added dropwise, the reaction mixture was warmed to r.t. After overnight stirring, the reaction mixture was quenched with

water (20 ml). The product was extracted with hexane (60 ml \times 3) and washed with a saturated aqueous solution of NH_4Cl (30 ml \times 2) and brine (30 ml \times 2). After usual workup, the crude product was chromatographed over SiO_2 (40 g) to give **5** (3.1 g, 8.7 mmol, 58% yield). IR (neat) ν_{max} cm^{-1} : 2929 (s), 2856 (m), 1384 (w), 1140 (m), 1117 (m), 1036 (s), 920 (w). ^1H NMR δ : \sim 1.3 (10H, broad s), 1.4–1.6 (8H, m), \sim 1.75 (4H, m, $\text{OCH}_2\text{CH}_2 \times 2$), 2.13 (2H, m, $\text{CH}_2\text{C}\equiv\text{C}$), 2.26 (2H, m, $\text{CH}_2\text{C}\equiv\text{C}$), 3.36 (3H, s, OCH_3), 3.45 (1H, m, OCHHCH_2), 3.51 (1H, m, OCHHCH_2), 3.52 (2H, t, $J = 6.7$ Hz, OCH_2CH_2), 3.82 (1H, m, OCHHCH_2), 3.88 (1H, m, OCHHCH_2), 4.60 (1H, t, $J = 4.5$ Hz, OCHCH_2), 4.62 (2H, s, OCH_2O). ^{13}C NMR δ : 15.7, 18.8, 19.5, 25.5, 26.2, 28.9, 29.1 ($\times 2$), 29.3, 29.4, 29.5, 29.8, 30.7, 55.1, 62.1, 66.1, 67.9, 79.4, 80.6, 96.4, 98.8.

(E)-14-hydroxy-10-tetradecenyl MOM ether (6) The THP ether **5** (0.75 g, 2.1 mmol) and *p*-TsOH (80 mg) dissolved in EtOH (20 ml) was stirred for 3 h at r.t. The mixture was poured into a saturated aqueous solution of NaHCO_3 (10 ml), and the product was extracted with hexane (30 ml \times 3). After usual workup, the crude product was chromatographed over SiO_2 (20 g) to give 14-hydroxy-10-tetradecynyl MOM ether (0.52 g, 1.9 mmol, 91% yield). ^1H NMR δ : \sim 1.3 (10H, broad s), 1.45 (2H, m, $\text{C}\equiv\text{CCH}_2\text{CH}_2$), 1.59 (2H, m, $\text{CH}_2\text{CH}_2\text{OCH}_2$), 1.73 (2H, m, $\text{CH}_2\text{CH}_2\text{OH}$), 2.28 (2H, m, $\text{C}\equiv\text{CCH}_2$), 2.13 (2H, m, $\text{C}\equiv\text{CCH}_2$), 3.36 (3H, s, OCH_3), 3.52 (2H, t, $J = 8.8$ Hz, $\text{CH}_2\text{CH}_2\text{OCH}_2$), 3.75 (2H, m, CH_2OH), 4.62 (2H, s, OCH_2O). ^{13}C NMR δ : 15.5, 18.7, 26.2, 28.8, 29.0, 29.1, 29.4, 29.5, 29.7, 31.6, 55.1, 62.0, 67.9, 79.3, 81.1, 96.4.

Next, LiAlH_4 (0.35 g, 9.3 mmol) was added in portions to an ice-cooled mixture of dry diglyme (6.0 ml) and THF (1.0 ml) under Ar. After vigorous foaming subsided, the MOM ether (0.5 g, 1.85 mmol) dissolved in diglyme (2.0 ml) was added to the thick slurry of LiAlH_4 and stirred for 20 min. Furthermore, the mixture was stirred for 20 h at 140°C , and then cooled to 0°C . After adding hexane (30 ml), the mixture was sequentially treated with water (1.0 ml), a 20% NaOH solution (0.80 ml), and water (3.0 ml) in a cautious dropwise manner, and was stirred for 30 min to allow precipitate to white gummy slurry. The hexane layer was decanted and the residue was rinsed with hexane (30 ml \times 3). The hexane extract

was washed with water (30 ml \times 3) and brine (30 ml \times 2). After usual workup, the crude product was chromatographed over SiO₂ (20 g) to give **6** (0.47 g, 1.7 mmol, 93% yield). IR (neat) ν_{max} cm⁻¹: 3410 (m), 2925 (vs), 2854 (s), 1466 (w), 1385 (m), 1145 (m), 1113 (m), 1047 (s), 968 (w), 920 (w). ¹H NMR δ : ~1.3 (12H, broad s), ~1.65 (4H, m, OCH₂CH₂ \times 2), 1.97 (2H, m, CH₂C=CH), 2.08 (2H, m, CH₂C=CH), 3.36 (3H, s, CH₃), 3.52 (2H, t, J = 6.7 Hz, CH₂CH₂OCH₂), 3.65 (2H, t, J = 6.5 Hz, CH₂CH₂OH), 4.62 (2H, s, OCH₂O), 5.42 (2H, m, CH=CH). ¹³C NMR δ : 26.2, 28.9, 29.1, 29.4 (\times 2), 29.5 (\times 2), 29.7, 32.5, 32.6, 55.1, 62.6, 67.9, 96.4, 129.4, 131.3.

MOM-ether of (E)-14-hydroxy-4-tetradecenal (7) A solution of **6** (0.24 g, 0.82 mmol) in CH₂Cl₂ (2 ml) was added dropwise to a mixture of pyridinium chlorochromate (PCC, 0.27 g, 1.2 mmol) and CH₂Cl₂ (20 ml). After stirring for 1 h at r.t., powdery MgSO₄ (0.50 g) was added and the mixture was stirred for 15 min. The solvent was decanted and the residue was washed with hexane (30 ml \times 3). The two organic layers were combined and washed with brine (20 ml \times 2). After usual workup, the crude product was chromatographed over SiO₂ (20 g) to give **7** (0.22 g, 0.80 mmol, 97% yield). IR (neat) ν_{max} cm⁻¹: 2925 (s), 2854 (s), 2719 (w), 1728 (s), 1466 (w), 1385 (m), 1145 (m), 1113 (ms), 1045 (s), 970 (w), 920 (w). ¹H NMR δ : ~1.3 (12H, broad s), 1.59 (2H, m, CH₂CH₂OCH₂), 1.97 (2H, m, CH₂CH=CH), 2.33 (2H, m, CH₂CH=CH), 2.49 (2H, td, J = 9.5, 2.2 Hz, CH₂CHO), 3.36 (3H, s, OCH₃), 3.52 (2H, t, J = 6.6 Hz, CH₂CH₂O), 4.62 (2H, s, OCH₂O), 5.43 (2H, m, CH=CH), 9.76 (1H, t, J = 2.2 Hz, CH₂CHO). ¹³C NMR δ : 25.2, 26.2, 29.1, 29.40, 29.43 (\times 2), 29.6, 29.8, 32.5, 43.6, 55.1, 67.9, 96.4, 127.6, 132.1, 202.5.

MOM-ether of (10E,14EZ)-10,14-hexadecadien-1-ol (8) BuLi (0.34 ml, 0.85 mmol) was added dropwise to suspension of ethyltriphenylphosphonium bromide (0.33 g, 0.88 mmol) in dry THF (8.0 ml) at 0°C under Ar. After stirring for 1 h at 0°C, a solution of **7** (0.20 g, 0.74 mmol) in THF (2.0 ml) was added dropwise to the produced ylide. The reaction mixture was stirred for 3 h at r.t. and poured into water (10 ml). The product was extracted with hexane (20 ml \times 3), washed with brine, 1 N HCl, and saturated NaHCO₃. After usual workup, the crude product was chromatographed over SiO₂ (15 g) to give **8** (0.18 g, 0.64 mmol, 87% yield).

GC-MS m/z (relative intensity): two peaks (about 1:3), Rt 18.01 min, 282 (0.2%, M^+), 250 (11%), 135 (22%), 121 (34%), 109 (42%), 95 (100%), 81 (86%), 67 (64%), 55 (90%); Rt 18.24 min, 282 (0.3%, M^+), 250 (13%), 135 (24%), 121 (37%), 109 (45%), 95 (100%), 81 (83%), 67 (65%), 55 (74%).

E10,E14-16:OH and E10,Z14-16:OH MOM ether **8** (0.18 g, 0.64 mmol) was dissolved in MeOH (7.0 ml) and treated with HCl (4 *N* solution in dioxane, 1.0 ml). The mixture was stirred overnight at r.t., and poured into water (50 ml). The product was extracted with hexane and washed with a saturated aqueous solution of NaHCO_3 . After usual workup, the crude product was analyzed by GC-MS. Since the analysis showed *E10,E14-16:OH* and *E10,Z14-16:OH* in a ratio of 1:2.3, the mixture was chromatographed over SiO_2 (15 g) impregnated with AgNO_3 (15%) to yield pure *E10,E14-16:OH* (29 mg, 0.12 mmol) and *E10,Z14-16:OH* (46 mg, 0.19 mmol). *E10,E14-16:OH*; IR (neat) ν_{max} cm^{-1} : 3329 (m), 3022 (w), 2925 (s), 2854 (m), 1454 (w), 1385 (m), 1055 (m), 964 (w). ^1H NMR δ : 1.28 (12H, broad s), 1.56 (2H, m, $\text{CH}_2\text{CH}_2\text{OH}$), 1.64 (3H, d, $J = 3.9$ Hz, $=\text{CHCH}_3$), 1.97 (2H, m, $=\text{CHCH}_2\text{CH}_2\text{CH}_2$), 2.03 (4H, broad s, $=\text{CHCH}_2\text{CH}_2\text{CH}=\text{CH}$), 3.64 (2H, t, $J = 8.8$ Hz, CH_2OH), 5.40 (4H, m, $\text{CH}=\text{CH} \times 2$). *E10,Z14-16:OH*; IR (neat) ν_{max} cm^{-1} : 3334 (m), 3014 (w), 2925 (s), 2854 (s), 1461 (w), 1385 (m), 1057 (m), 966 (w), 702 (w). ^1H NMR δ : 1.28 (12H, broad s), 1.57 (2H, m, $\text{CH}_2\text{CH}_2\text{OH}$), 1.60 (3H, d, $J = 5.3$ Hz, $=\text{CHCH}_3$), 1.98 (2H, m, $\text{CH}=\text{CHCH}_2\text{CH}_2\text{CH}_2$), 2.07 (4H, m, $=\text{CHCH}_2\text{CH}_2\text{CH}=\text{CH}$), 3.64 (2H, t, $J = 8.8$ Hz, CH_2OH), 5.41 (4H, m, $\text{CH}=\text{CH} \times 2$).

E10,E14-16:Ald *E10,E14-16:OH* (43 mg, 0.17 mmol) dissolved in CH_2Cl_2 (2.0 ml) was added dropwise to a solution of PCC (68 mg, 0.25 mmol) in CH_2Cl_2 (10 ml). The same procedure as the synthesis of **7** gave *E10,E14-16:Ald* (38 mg, 0.15 mmol, 88% yield). IR (neat) ν_{max} cm^{-1} : 2920 (s), 2855 (m), 2715 (w), 1717 (s, $\text{C}=\text{O}$), 1475 (m), 968 (s). ^1H NMR δ : 1.29 (10H, broad s), 1.62 (2H, m, $\text{CH}_2\text{CH}_2\text{CHO}$), 1.64 (3H, d, $J = 4.1$ Hz, $=\text{CHCH}_3$), 1.97 (2H, m, $\text{CH}=\text{CHCH}_2\text{CH}_2$), 2.03 (4H, broad s, $=\text{CHCH}_2\text{CH}_2\text{CH}=\text{CH}$), 2.42 (2H, td, $J = 9.8, 2.4$ Hz, CH_2CHO), 5.41 (4H, m, $\text{CH}=\text{CH} \times 2$), 9.76 (1H, t, $J = 2.4$ Hz, CH_2CHO).

E10,Z14-16:Ald The aldehyde were synthesized also in the same manner. IR

(neat) ν_{\max} cm^{-1} : 3015 (w), 2919 (s), 2854 (m), 2715 (w), 1716 (s, C=O), 1475 (m), 968 (m), 725 (w). ^1H NMR δ : 1.29 (10H, broad s), 1.60 (3H, d, $J = 5.4$ Hz, $=\text{CHCH}_3$), 1.62 (2H, m, $\text{CH}_2\text{CH}_2\text{CHO}$), 1.98 (2H, m, $\text{CH}=\text{CHCH}_2\text{CH}_2$), 2.06 (4H, m, $=\text{CHCH}_2\text{CH}_2\text{CH}=\text{CH}$), 2.42 (2H, td, $J = 9.7, 2.4$ Hz, CH_2CHO), 5.41 (4H, m, $\text{CH}=\text{CH} \times 2$), 9.76 (1H, t, $J = 2.4$ Hz, CH_2CHO).

II-2-4-2 Synthesis of (10Z,14E)- and (10Z,14Z)-isomers of 10,14-hexadecadienal

(Z)-14-hydroxy-10-tetradecenyl MOM ether (9) Nickel acetate tetrahydrate (0.30 g, 1.2 mmol) was dissolved in EtOH (99.5%, 50 ml) and the solution was stirred under H_2 gas. NaBH_4 (0.18 g, 4.8 mmol) dissolved in EtOH (20 ml) was injected to reduce nickel acetate to a P-2 nickel catalyst. After finish of gas evolution, the reactor was again purged with H_2 gas, and ethylenediamine (0.50 ml, 7.5 mmol) was mixed. After stirring for 10 min, alkyne **5** (1.1 g, 3.1 mmol) in EtOH (99.5%, 5.0 ml) was introduced into the reactor. Quantitative uptake of H_2 gas was finished within 1 h and the absence of **5** was confirmed by GC-MS analysis. The catalyst was filtered and most of EtOH was evaporated. The residue was poured into water (50 ml) and the product was extracted with hexane (50 ml $\times 3$). After usual workup, the crude product was chromatographed over SiO_2 (15 g) to give THP ether of **9** (1.1 g, 3.0 mmol, 95% yield). Next, a mixture of the THP ether of **9** (0.70 g, 2.0 mmol), *p*-TsOH (80 mg, 0.46 mmol), and EtOH (10 ml) was stirred for 3h at r.t. The mixture was poured into a saturated aqueous solution of NaHCO_3 (50 ml), and the product was extracted with hexane (50 ml $\times 3$). After usual workup, the crude product was chromatographed over SiO_2 (10 g) to give **9** (0.38 g, 1.4 mmol, 70 % yield). IR (neat) ν_{\max} cm^{-1} : 3400 (m), 3003 (w), 2925 (vs), 2854 (s), 1465 (w), 1384 (w), 1145 (m), 1111 (m), 1045 (s), 920 (w), 723 (w). ^1H NMR δ : ~ 1.3 (12H, broad s), ~ 1.65 (4H, m, $\text{OCH}_2\text{CH}_2 \times 2$), 2.03 (2H, m, $\text{CH}_2\text{C}=\text{CH}$), 2.12 (2H, m, $\text{CH}_2\text{C}=\text{CH}$), 3.36 (3H, s, CH_3), 3.52 (2H, t, $J = 6.6$ Hz, $\text{CH}_2\text{CH}_2\text{OCH}_2$), 3.66 (2H, t, $J = 6.5$ Hz, $\text{CH}_2\text{CH}_2\text{OH}$), 4.62 (2H, s, OCH_2O), 5.38 (2H, m, $\text{CH}=\text{CH}$). ^{13}C NMR δ : 23.6, 26.2, 27.2, 29.3, 29.4, 29.46, 29.54, 29.68, 29.73, 32.7, 55.1, 62.7, 67.9, 96.4, 128.9, 130.8.

MOM-ether of (Z)-14-hydroxy-4-tetradecenal (10) A mixture of PCC (0.43 g,

2.0 mmol) and CH_2Cl_2 (30 ml) was stirred for 5 min at r.t. Alcohol **9** (0.36g, 1.3 mmol) dissolved in CH_2Cl_2 (2 ml) was added dropwisely to the mixture. The same procedure as the synthesis of **7** gave the aldehyde **10** (0.28 g, 1.0 mmol, 78% yield). IR (neat) ν_{max} cm^{-1} : 3006 (w), 2925 (s), 2854 (s), 2719 (w), 1728 (m), 1462 (w), 1385 (m), 1146 (w), 1111 (m), 1045 (m), 918 (w), 719 (w). ^1H NMR δ : ~1.3 (12H, broad s), 1.58 (2H, m, $\text{CH}_2\text{CH}_2\text{OCH}_2$), 2.02 (2H, m, $\text{CH}_2\text{CH}=\text{CH}$), 2.38 (2H, m, $\text{CH}_2\text{CH}=\text{CH}$), 2.48 (2H, td, $J = 9.5, 2.1$ Hz, CH_2CHO), 3.36 (3H, s, OCH_3), 3.52 (2H, t, $J = 6.6$ Hz, $\text{CH}_2\text{CH}_2\text{O}$), 4.62 (2H, s, OCH_2O), 5.38 (2H, m, $\text{CH}=\text{CH}$), 9.77 (1H, t, $J = 2.1$ Hz, CH_2CHO). ^{13}C NMR δ : 20.1, 26.2, 27.2, 29.3, 29.4, 29.5, 29.6 ($\times 2$), 29.8, 43.9, 55.1, 67.9, 96.4, 127.1, 131.8, 202.3.

MOM-ether of (10Z,14EZ)-10,14-hexadecadien-1-ol (11) In the same manner as the synthesis of diene **8**, aldehyde **10** (0.28 g, 1.0 mmol) was coupled with a ylide derived from thyltriphenylphosphonium bromide (0.46g, 1.3 mmol) to give **11** (0.26 g, 0.92 mmol, 89% yield). GC-MS m/z (relative intensity): two peaks (about 1:3), Rt 17.94 min, 282 (0.2%, M^+), 250 (13%), 135 (24%), 121 (36%), 109 (44%), 95 (100%), 81 (90%), 67 (69%), 55 (96%); Rt 18.19 min, 282 (0.3%, M^+), 250 (13%), 135 (26%), 121 (39%), 109 (46%), 95 (100%), 81 (88%), 67 (67%), 55 (78%).

Z10,E14-16:OH and Z10,Z14-16:OH In the same manner as deprotection of MOM ether **8**, **11** (0.24 g, 0.85 mmol) was treated with HCl (4 N solution in dioxane, 1.0 ml) to give a mixture of Z10,E14-16:OH and Z10,Z14-16:OH in a ratio of 1 : 2.4 (0.18 g, 0.75 mmol, 88% yield). The mixture was chromatographed over SiO_2 (15 g) impregnated with AgNO_3 (15%) to yield pure Z10,E14-16:OH (31 mg) and Z10,Z14-16:OH (100 mg). Z10,E14-16:OH; IR (neat) ν_{max} cm^{-1} : 3329 (m), 3006 (w), 2925 (s), 2854 (s), 1454 (m), 1385 (m), 1057 (m), 964 (m), 721 (w). ^1H NMR δ : 1.29 (12H, broad s), 1.56 (2H, m, $\text{CH}_2\text{CH}_2\text{OH}$), 1.64 (3H, d, $J = 4.0$ Hz, $=\text{CHCH}_3$), 2.03 (6H, m, $=\text{CHCH}_2 \times 3$), 3.64 (2H, t, $J = 8.6$ Hz, CH_2OH), 5.36 (2H, m, $\text{CH}=\text{CH}$), 5.44 (2H, m, $\text{CH}=\text{CH}$). Z10,Z14-16:OH; IR (neat) ν_{max} cm^{-1} : 3323 (m), 3010 (m), 2925 (s), 2854 (s), 1657 (w), 1454 (m), 1385 (m), 1057 (m), 721 (m). ^1H NMR δ : 1.29 (12H, broad s), 1.56 (2H, m, $\text{CH}_2\text{CH}_2\text{OH}$), 1.61 (3H, d, $J = 5.6$ Hz, $=\text{CHCH}_3$), 2.04 (2H, m, $=\text{CHCH}_2\text{CH}_2\text{CH}_2$), 2.09 (4H, m, $=\text{CHCH}_2\text{CH}_2\text{CH}=\text{CH}$), 3.64 (2H, t, $J = 8.8$

Hz, CH₂OH), 5.41 (4H, m, CH=CH ×2).

Z10,E14-16:Ald and Z10,Z14-16:Ald These aldehydes were synthesized in the same manner as the synthesis of E10,E14-16:Ald. *Z10,E14-16:Ald*; IR (neat) ν_{\max} cm⁻¹: 3005 (w), 2921 (s), 2854 (m), 2715 (w), 1715 (s, C=O), 1475 (m), 968 (m), 725 (w). ¹H NMR δ : 1.29 (10H, broad s), 1.62 (2H, m, CH₂CH₂CHO), 1.64 (3H, d, J = 4.0 Hz, =CHCH₃), 2.03 (6H, m, =CHCH₂ ×3), 2.42 (2H, td, J = 9.8, 2.3 Hz, CH₂CHO), 5.36 (2H, m, CH=CH), 5.43 (2H, m, CH=CH), 9.76 (1H, t, J = 2.3 Hz, CH₂CHO). *Z10,Z14-16:Ald*; IR (neat) ν_{\max} cm⁻¹: 3007 (m), 2917 (s), 2853 (m), 2715 (w), 1717 (s, C=O), 1474 (m), 725 (m). ¹H NMR δ : 1.30 (10H, broad s), 1.61 (3H, d, J = 5.4 Hz, =CHCH₃), 1.62 (2H, m, CH₂CH₂CHO), 2.02 (2H, m, =CHCH₂CH₂CH₂), 2.09 (4H, m, =CHCH₂CH₂CH=), 2.42 (2H, td, J = 9.8, 2.4 Hz, CH₂CHO), 5.37 (2H, m, CH=CH), 5.43 (2H, m, CH=CH), 9.76 (1H, t, J = 2.4 Hz, CH₂CHO).

II-2-4-3 NMR data of monoenyl aldehydes and polyunsaturated hydrocarbons

(E)-10-Hexadecenal (E10-16:Ald): ¹H NMR δ : 0.89 (3H, t, J = 6.0 Hz, CH₂CH₃), 1.29 (16H, broad s), 1.61 (2H, m, CH₂CH₂CHO), 1.97 (4H, m, CH₂CH=C ×2), 2.43 (2H, td, J = 7.4, 1.8 Hz, CH₂CHO), 5.39 (2H, m, CH₂CH=C ×2), 9.77 (1H, t, J = 1.8 Hz, CH₂CHO). ¹³C NMR δ : 14.1, 22.1, 22.6, 29.1, 29.2, 29.28, 29.34 (×2), 29.6, 31.4, 32.6 (×2), 43.9, 130.3, 130.5, 203.1.

(Z)-10-Hexadecenal (Z10-16:Ald): ¹H NMR δ : 0.89 (3H, t, J = 6.0 Hz, CH₂CH₃), 1.30 (16H, broad s), 1.61 (2H, m, CH₂CH₂CHO), 2.02 (4H, m, CH₂CH=C ×2), 2.43 (2H, td, J = 7.4, 1.8 Hz, CH₂CHO), 5.36 (2H, t, J = 5.6 Hz, CH₂CH=C ×2), 9.77 (1H, t, J = 1.8 Hz, CH₂CHO). ¹³C NMR δ : 14.1, 22.1, 22.6, 27.2 (×2), 29.15, 29.20, 29.32, 29.34, 29.4, 29.7, 31.5, 43.9, 129.8, 130.0, 203.1.

(E)-14-Hexadecenal (E14-16:Ald): ¹H NMR δ : 1.26 (20H, broad s), 1.61–1.65 (5H, m, CH₂CH₂CHO, C=CHCH₃), 1.96 (2H, m, CH₂CH=CHCH₃), 2.43 (2H, td, J = 7.4, 1.8 Hz, CH₂CHO), 5.41 (2H, m, CH=CH), 9.77 (1H, t, J = 1.8 Hz, CH₂CHO). ¹³C NMR δ : 18.0, 22.1, 29.17, 29.21, 29.37, 29.43, 29.53, 29.58, 29.63 (×3), 32.6, 43.9, 124.5, 131.7, 203.1.

(*Z*)-14-Hexadecenal (*Z*14-16:Ald): ^1H NMR δ : 1.26 (20H, broad s), 1.59–1.65 (5H, m, $\text{CH}_2\text{CH}_2\text{CHO}$, $\text{C}=\text{CHCH}_3$), 2.01 (2H, m, $\text{CH}_2\text{CH}=\text{CHCH}_3$), 2.43 (2H, td, $J = 7.4, 1.8$ Hz, CH_2CHO), 5.40 (2H, m, $\text{CH}=\text{CH}$), 9.77 (1H, t, $J = 1.8$ Hz, CH_2CHO). ^{13}C NMR δ : 12.8, 22.1, 26.8, 29.2, 29.3, 29.36, 29.43, 29.57($\times 3$), 29.63 ($\times 2$), 43.9, 123.6, 130.9, 203.1.

(*3Z,6Z,9Z*)-3,6,9-Pentacosatriene (*Z*3,*Z*6,*Z*9-25:*H*): ^1H NMR δ : 0.88 (3H, t, $J = 6.4$ Hz, CH_2CH_3), 0.98 (3H, t, $J = 7.5$ Hz, $\text{CH}_3\text{CH}_2\text{CH}=\text{C}$), 1.26 (26H, broad s), 2.06 (4H, m, $\text{CH}_3\text{CH}_2\text{CH}=\text{C}$, $\text{CH}_2\text{CH}_2\text{CH}=\text{C}$), 2.81 (4H, m, $\text{C}=\text{CHCH}_2\text{CH}=\text{C} \times 2$), 5.36 (6H, m, $\text{CH}=\text{CH} \times 3$). ^{13}C NMR δ : 14.2, 14.3, 20.6, 22.7, 25.5, 25.6, 27.3, 29.3, 29.4, 29.6, 29.68 ($\times 3$), 29.72 ($\times 5$), 31.9, 127.1, 127.6, 128.2, 128.3, 130.4, 132.0.

(*3Z,6Z,9Z,12Z,15Z*)-3,6,9,12,15-Tricosapentaene (*Z*3,*Z*6,*Z*9,*Z*12,*Z*15-23:*H*): ^1H NMR δ : 0.88 (3H, t, $J = 6.6$ Hz, CH_2CH_3), 0.98 (3H, t, $J = 7.5$ Hz, $\text{CH}_3\text{CH}_2\text{CH}=\text{C}$), 1.27 (10H, broad s), 2.04–2.10 (4H, m, $\text{CH}_3\text{CH}_2\text{CH}=\text{C}$, $\text{CH}_2\text{CH}_2\text{CH}=\text{C}$), 2.84 (8H, m, $\text{C}=\text{CHCH}_2\text{CH}=\text{C} \times 4$), 5.40 (10H, m, $\text{CH}=\text{CH} \times 5$). ^{13}C NMR δ : 14.1, 14.3, 20.6, 22.7, 25.5, 25.6 ($\times 3$), 27.3, 29.26, 29.31, 29.7, 31.9, 127.0, 127.5, 127.89, 127.91, 128.17, 128.20, 128.55, 128.57, 130.5, 132.0.

(*3Z,6Z,9Z,12Z,15Z*)-3,6,9,12,15-Pentacosapentaene (*Z*3,*Z*6,*Z*9,*Z*12,*Z*15-25:*H*): ^1H NMR δ : 0.88 (3H, t, $J = 6.6$ Hz, CH_2CH_3), 0.98 (3H, t, $J = 7.5$ Hz, $\text{CH}_3\text{CH}_2\text{CH}=\text{C}$), 1.26 (14H, broad s), 2.02–2.10 (4H, m, $\text{CH}_3\text{CH}_2\text{CH}=\text{C}$, $\text{CH}_2\text{CH}_2\text{CH}=\text{C}$), 2.84 (8H, m, $\text{C}=\text{CHCH}_2\text{CH}=\text{C} \times 4$), 5.40 (10H, m, $\text{CH}=\text{CH} \times 5$). ^{13}C NMR δ : 14.2, 14.3, 20.6, 22.7, 25.5, 25.6 ($\times 3$), 27.3, 29.3 ($\times 2$), 29.59, 29.63, 29.68, 31.9, 127.0, 127.5, 127.89, 127.91, 128.17, 128.20, 128.55, 128.57, 130.5, 132.0.

II-2-5 Field Evaluation

The field experiments (OaA-OaD) of *O. anastomosalis* were conducted in sweet potato fields of An Giang Province, Vietnam (10.38°N, 104.99°E), in 2013. The field experiments (LoA-LoC) of *L. orbonalis* were conducted in eggplant fields of An Giang Province, Vietnam (10.03°N, 105.78°E), in 2014. The synthetic compounds (purity >98%) were dissolved in hexane (20 mg/ml) and applied to rubber septa (white rubber, 8 mm O.D., Sigma-Aldrich Inc., St. Louis, MO, USA) that were used as dispensers. Control septa were loaded with hexane (50 µl) only. Lures were placed at the center of sticky traps (SEtrap®, 30×27 cm bottom plate with a roof, Sankei Chemical Co., Tokyo, Japan), and were hung separately 1 m above ground level at intervals of 10 m. For virgin female baited traps, a 2 or 3 d-old female was contained in a wire-net cage supplied with wet cotton wool. Survival was checked every evening during the evaluation, and each female was renewed within 3 d. Three or four replicates for each treatment were tested, and the captured males were counted every week.

Data obtained in each field test were analyzed by one-way ANOVA, and pairwise comparisons among traps were performed with Tukey-Kramer Test with P-values adjusted for multiple comparisons. In order to homogenize the variance, means were transformed by using log (x+0.5) transformation. Treatments with zero catches were omitted from the ANOVA. All statistical analyses were performed with R version 3.0.1 (R Development Core Team 2014).

II-3 Results

II-3-1 Analyses of Pheromone Extracts

II-3-1-1 *Omphisa anastomosalis*

In the GC/MS analysis of a crude extract of pheromone glands of *O. anastomosalis* females (1 FE), the total ion chromatogram (TIC) showed three unsaturated aldehydes, Oa-I – Oa-III, in a ratio of approximately 7:3:100 (Fig. II-2a). The mass spectra of Oa-I (Rt 16.31 min) and Oa-II (Rt 16.59 min) with $[M-18]^+$ at m/z 220 indicated two C16 monoenyl aldehydes. Compound Oa-III (Rt 16.75 min) showed M^+ at m/z 236 and $[M-18]^+$ at m/z 218, indicating a C16 dienyl aldehyde (Fig. II-2b). In addition to the three Type I compounds that had been identified by Wakamura et al. (2010), one polyunsaturated hydrocarbon was found as Oa-IV (Rt 19.00 min). The mass spectrum of this showed M^+ at m/z 318 and characteristic fragment ions at m/z 262, 108, and 79, indicating a C23 3,6,9-triene (Fig. II-2c) (Ando and Yamakawa, 2011). The ratio of peak areas of compounds Oa-III and Oa-IV was approximately 100:6.

In order to confirm the positions of the double bonds in the proposed pheromone components, the crude pheromone extract (45 FE) was treated with DMDS. While a DMDS adduct of Oa-IV was not detected, three DMDS adducts corresponding to compounds Oa-I – Oa-III were recorded (Fig. II-3a). Characteristic fragment ions at m/z 201 and 131 of the DMDS adduct of Oa-I (Rt 13.99 min, M^+ at m/z 332) indicated its original double bond at the 10-position, and those m/z 257 and 75 of the DMDS adduct of Oa-II (Rt 14.64 min, M^+ at m/z 332) indicated its original double bond was at the 14-position (Buser et al., 1983). Since Oa-III included two methylene groups between two double bonds, it was derivatized to a five-membered cyclic thioether (Rt 16.93 min), which showed M^+ at m/z 362 and characteristic fragment ions at m/z 287, 239, 201, and 161 (Fig. II-3b) (Vincenti et al., 1987). The mass spectrum indicated there were two double bonds at 10 and 14-positions in the parent dienyl aldehyde.

Finally, structural determination of each component was accomplished by

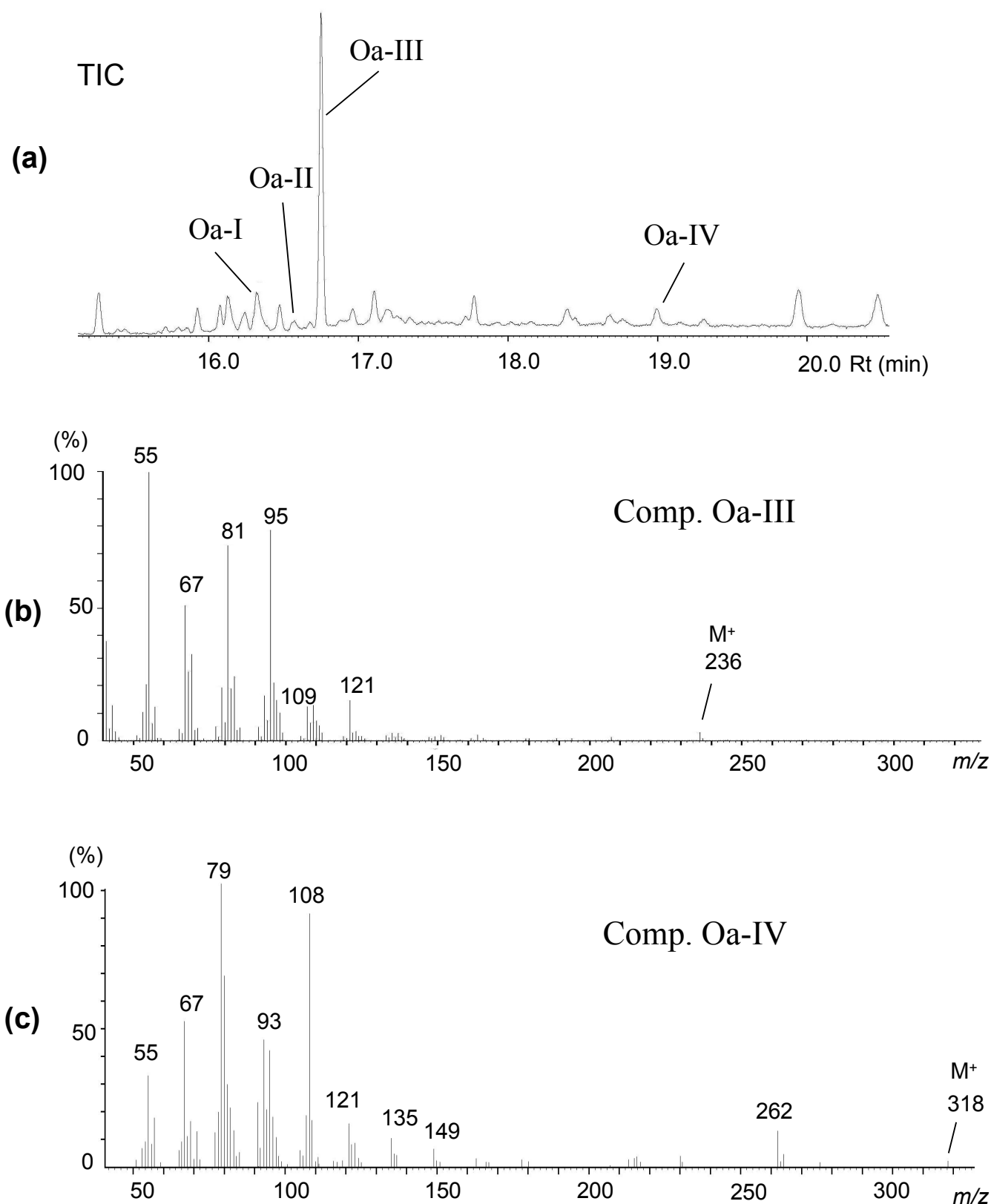


Fig. II-2. GC-MS analysis of a crude pheromone extract of the *O. anastomosalis* female (1 FE) on a DB-225 capillary column. **(a)** total ion chromatogram (TIC), **(b)** mass spectrum of Comp. Oa-III, and **(c)** mass spectrum of Comp. Oa-IV. Comp. Oa-I = E10-14:Ald, Comp. Oa-II = E14-16:Ald, Comp. Oa-III = E10,E14-16:Ald, and Comp. Oa-IV = Z3,Z6,Z9-23:H.

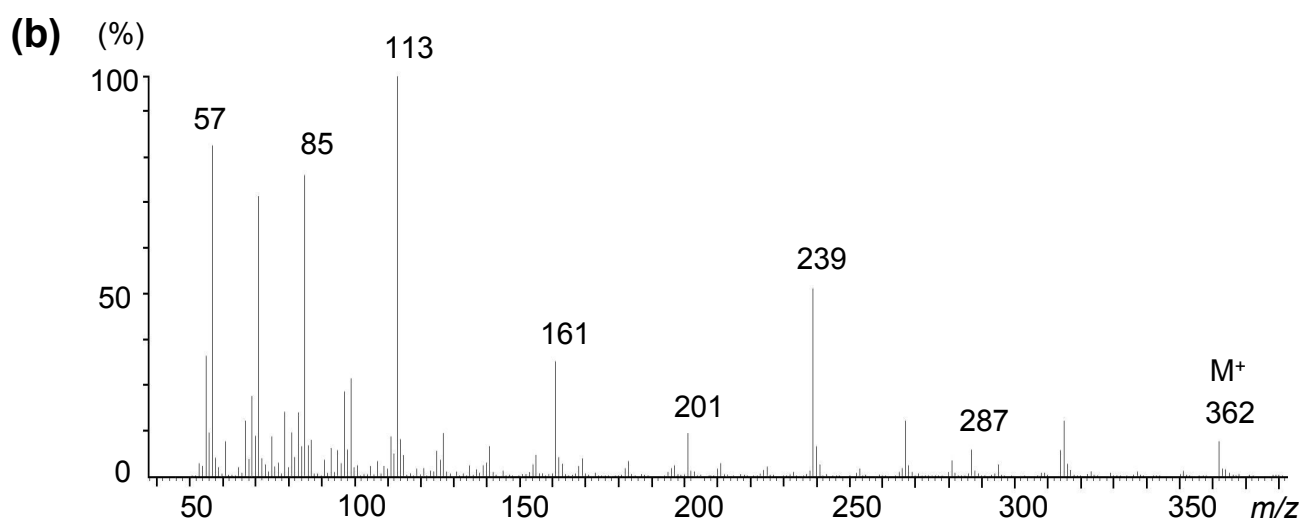
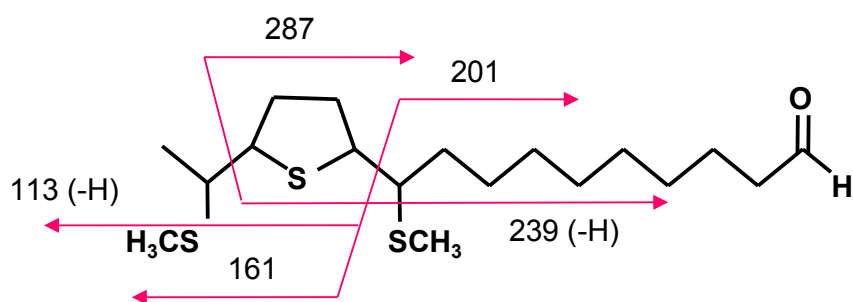
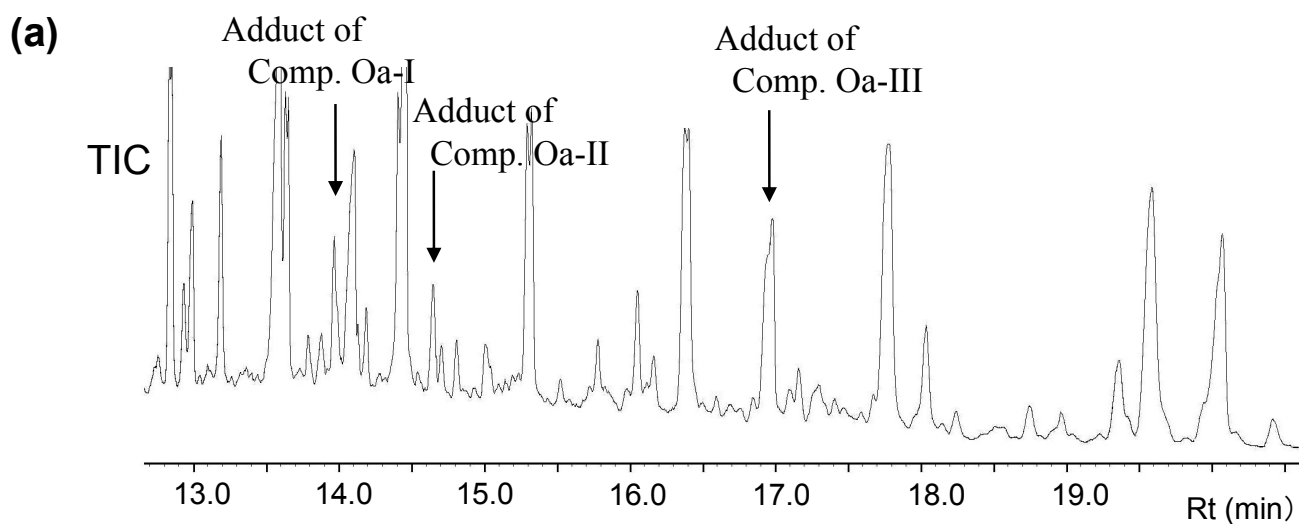


Fig. II-3. GC-MS analysis of a pheromone extract of *O. anastomosalis* females (45 FE) treated with dimethyl disulfide (DMDS). **(a)** TIC and **(b)** a mass spectrum of a DMDS-adduct of Comp. Oa-III.

comparing the GC/MS data with those of authentic samples. The analyses were conducted with two different columns, a polar column (DB-225) and a non-polar column (HP-5). In both C16 monoenyl and dienyl aldehydes, the configuration of the double bond at the 14-position more strongly affected elution order on GC than did that at the 10-position. Good separation of geometrical isomers was achieved on the DB-225 column. Table II-1 shows the retention times and KI values of natural components and synthetic standards. Essentially identical retention times were observed for Oa-I and E10-16:Ald, Oa-II and E14-16:Ald, and Oa-III and E10,E14-16:Ald. While only one geometrical isomer was available as a standard in the case of Oa-IV, its retention time coincided well with that of Z3,Z6,Z9-23:H.

II-3-1-2 *Leucinodes orbonalis*

In the GC/MS analysis of a crude extract of pheromone glands of *L. orbonalis* females (2 FE), as shown in TIC (Fig. II-4a), in addition to E11-16:OAc (Comp. Lo-I, 15.26 min) that had been identified by Zhu et al. (1987), two polyunsaturated hydrocarbons Z3,Z6,Z9-22:H (Comp. Lo-II, Rt 14.75 min) and Z3,Z6,Z9-23:H (Comp. Lo-III, Rt 15.65 min) were detected (Fig. II-4c). The mass spectrum of Lo-III showed M^+ at m/z 318 and characteristic fragment ions at m/z 262, 108, and 79, indicating a C23 3,6,9-triene (Fig. II-4e). Although M^+ at m/z 304 was not detected, characteristic fragment ions at m/z 248, 108, and 79, indicating a C22 3,6,9-triene (Fig. II-4d) (Ando and Yamakawa, 2011). In the GC/MS analysis of a crude extract of female body, the three components Lo-I, Lo-II, Lo-III were detected as well. But different with pheromone glands extracts, C23 3,6,9-triene seems present a high content in body extracts (Fig. II-4b).

II-3-2 Synthesis of Four Geometrical Isomers of 10,14-hexadecadienal

While E10,E14-16:Ald was previously synthesized by cis-trans isomerization of the (10Z,14Z)-isomer (Wakamura et al., 2010), the chemical data and synthesis of other geometrical isomers have not been reported. In a moderate yield, all four isomers were synthesized starting from 1,9-nonanediol **1** and 4-pentyn-1-ol **3** using

Table II-1. Chromatographic behavior of natural pheromone components of *O. anastomosalis* females and synthetic compounds on GC-capillary columns ^a

Compound	Rt and KI value			
	DB-225MS		HP-5MS	
	Rt (min)	KI	Rt (min)	KI
Comp. Oa-I	16.31	2161	15.45	1802
Comp. Oa-II	16.59	2186	15.69	1822
Comp. Oa-III	16.75	2201	15.51	1807
Comp. Oa-IV	19.00	2401	23.44	2273
E10-16:Ald	16.31	2161	15.45	1802
Z10-16:Ald	16.42	2171	15.43	1801
E14-16:Ald	16.58	2185	15.69	1822
Z14-16:Ald	16.90	2216	15.87	1837
E10,E14-16:Ald	16.76	2202	15.51	1807
E10,Z14-16:Ald	17.07	2233	15.68	1821
Z10,E14-16:Ald	16.89	2215	15.50	1807
Z10,Z14-16:Ald	17.21	2246	15.69	1822
Z3,Z6,Z9-23:H	18.99	2400	23.42	2272

^a Each compound was analyzed by GC-MS equipped with a DB-225MS column (0.25 mm ID × 30 m) and an HP-5MS column (0.25 mm ID × 30 m). The column temperature program was 80°C for 2 min, 8°C/min to 210°C, and 210°C for 10 min.

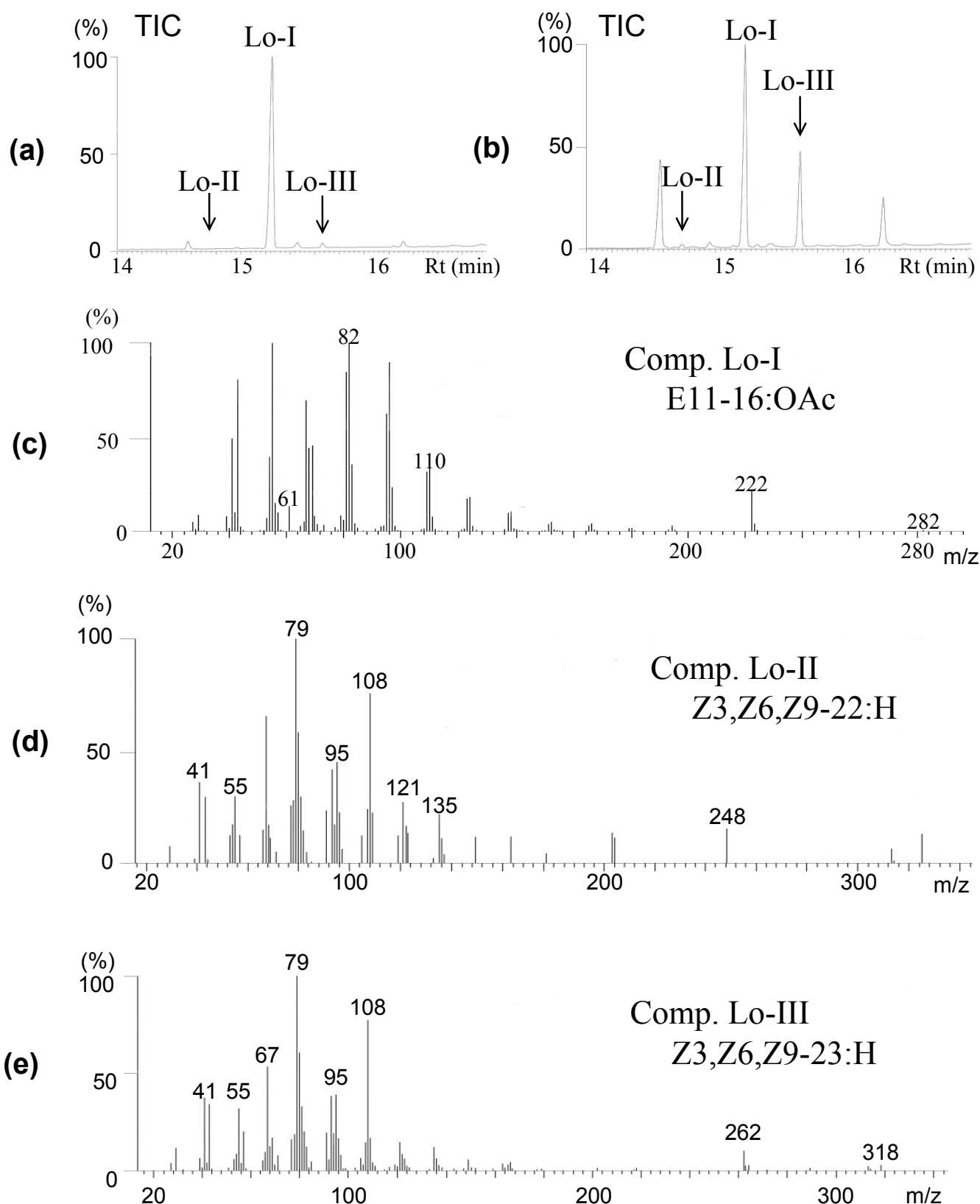


Fig. II-4. GC-MS analysis of a crude pheromone gland extract (2 FE) and body wax extract (3 FE) of the *Leucinodes orbonalis* female on a DB-23 capillary column. **(a)** total ion chromatogram (TIC) of pheromone gland extract, **(b)** TIC of body wax extract, **(c)** mass spectrum of Comp. Lo-I, **(d)** mass spectrum of Comp. Lo-II, and **(e)** mass spectrum of Comp. Lo-III. Comp. Lo-I = E11-16:OAc, Comp. Lo-II = Z3,Z6,Z9-22:H, Comp. Lo-III = Z3,Z6,Z9-23:H.

acetylene coupling and the Wittig reaction as shown in Fig. II-1. The (*E*) and (*Z*)-double bonds at the 10-position were selectively introduced by LiAlH₄ reduction and hydrogenation, respectively. The chemical structure of each isomer was confirmed by IR and NMR. In particular, the configuration of each double bond was confirmed by chemical shifts of allylic carbons. In addition to the parent alcohols, the ¹³C signals of the dienyl aldehydes were assigned and listed in Table II-2.

II-3-3 Male Attraction by Synthetic Lures

II-3-3-1 *Omphisa anastomosalis*

A preliminary field test conducted with several blends that included synthetic compounds Oa-I – Oa-IV during August of 2013 suggested that Oa-III (E10,E14-16:Ald) and Oa-IV (Z3,Z6,Z9-23:H) might play a role in attracting *O. anastomosalis* males (data not shown). Thus, the attractiveness of E10,E14-16:Ald (0.5 mg) mixed with different amounts of Z3,Z6,Z9-23:H (25–1,000 µg) was examined first (Experiment OaA). The result confirmed that traps baited with the binary blends captured more males than did the traps baited with the single components (Table II-3). The highest catches were obtained with ratios of E10,E14-16:Ald and Z3,Z6,Z9-23:H from 1:0.2 to 1:2, and traps baited with the 1:2 mixture caught ten times more males than those baited with E10,E14-16:Ald alone. Z3,Z6,Z9-23:H alone attracted six males, but this was fewer than the number captured in traps baited with E10,E14-16:Ald alone.

In Experiment OaB, the effects of addition of compounds Oa-I (E10-16:Ald) and Oa-II (E14-16:Ald) to the 1:2 mixture of E10,E14-16:Ald (0.5 mg) and Z3,Z6,Z9-23:H (1.0 mg) were examined. Based on the relative content of these minor components in the pheromone gland extract, E10-16:Ald (0.035 mg) and E14-16:Ald (0.015 mg) were mixed separately and together with the binary blend. In addition, blends with the monoenyl aldehydes at a 10-fold higher dose also were evaluated. As shown in Table II-4, none of the ternary or quaternary blends was more attractive than the binary blend. Addition of the two monoenyl aldehydes at the high dose decreased the number of males captured although none of the differences were significant in this

Table II-2. ^{13}C NMR data of 10,14-hexadecadien-1-ol and 10,14-hexadecadienal.

Position	Chemical shift (δ , ppm)							
	10,14-hexadecadien-1-ol (10,14-16:OH)				10,14-hexadecadien-1-ol (10,14-16:Ald)			
	E10,E14-	E10,Z14-	Z10,E14-	Z10,Z14-	E10,E14-	E10,Z14-	Z10,E14-	Z10,Z14-
1	63.1	63.1	63.1	63.1	202.9	203.0	203.0	202.9
2	32.8	32.8	32.8	32.8	43.9	43.9	43.9	43.9
3	25.8	25.7	25.8	25.8	22.1	22.1	22.1	22.1
4	29.1	29.1	29.3	29.3	29.0	29.1	29.2	29.2
5	29.5	29.4	29.4	29.4	29.2	29.2	29.2	29.2
6	29.5	29.5	29.5	29.5	29.3	29.3	29.3	29.3
7	29.6	29.6	29.6	29.6	29.3	29.3	29.3	29.3
8	29.6	29.6	29.7	29.7	29.5	29.5	29.7	29.7
9	32.6 ^a	32.6 ^c	27.3 ^e	27.3 ^g	32.5 ⁱ	32.6 ^k	27.3 ^m	27.2 ^o
10	129.7 ^b	129.7 ^d	129.2 ^f	129.2 ^h	129.7 ^j	129.8 ^l	129.2 ⁿ	129.2 ^p
11	130.7 ^b	130.8 ^d	130.2 ^f	130.4 ^h	130.6 ^j	130.8 ^l	130.2 ⁿ	130.3 ^p
12	32.7 ^a	32.5 ^c	27.3 ^e	27.2 ^g	32.7 ⁱ	32.5 ^k	27.2 ^m	27.2 ^o
13	32.8 ^a	27.0	32.7	27.1 ^g	32.7 ⁱ	27.0	32.7	27.0 ^o
14	131.0 ^b	130.2 ^d	131.1 ^f	130.2 ^h	131.0 ^j	130.2 ^l	131.0 ⁿ	130.2 ^p
15	124.9	124.0	125.0	124.1	124.9	124.0	125.0	124.1
16	17.9	12.8	17.9	12.8	17.9	12.8	17.9	12.8

^{a-p} Chemical shift values may be reversed.

experiment ($P>0.05$).

In order to confirm the 10*E*,14*E* configuration of Oa-III, four geometrical isomers of 10,14-hexadecadienal mixed with Z3,Z6,Z9-23:H in a 1:2 ratio were tested in Experiment OaC. As shown in Table II-5, almost all males were caught in traps baited with the lure containing the (10*E*,14*E*)-isomer. Only six males were captured by the lure with the (10*E*,14*Z*)-isomer, and none with the lures containing the (10*Z*,14*E*) or (10*Z*,14*Z*)-isomer. The synergistic effects of several polyunsaturated hydrocarbons structurally related to Z3,Z6,Z9-23:H were examined in Experiment OaD. E10,E14-16:Ald was mixed with one of the following diene, triene or pentaene hydrocarbons in a 1:2 ratio: (6*Z*,9*Z*)-6,9-tricosadiene (Z6,Z9-23:H), (3*Z*,6*Z*,9*Z*)-3,6,9-nonadecatriene (3Z,6Z,9Z-19:H), (3*Z*,6*Z*,9*Z*)-3,6,9-heneicosatriene (Z3,Z6,Z9-21:H), (3*Z*,6*Z*,9*Z*)-3,6,9-docosatriene (Z3,Z6,Z9-22:H), Z3,Z6,Z9-23:H, (3*Z*,6*Z*,9*Z*)-3,6,9-pentacosatriene (Z3,Z6,Z9-25:H), (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-3,6,9,12,15-tricosapentaene (Z3,Z6,Z9,Z12,Z15-23:H) and (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-3,6,9,12,15-pentacosapentaene (Z3,Z6,Z9,Z12,Z15-25:H). As shown in Table II-6, as many males were caught in traps baited with the binary lures including the C21 triene, C22 triene, or C23 pentaene as did those baited with the mixture with Z3,Z6,Z9-23:H. The synergistic effects of the C23 diene, C25 triene, and C25 pentaene were weaker than that of Z3,Z6,Z9-23:H. The C19 triene was the least active compound among the hydrocarbons tested. Traps baited with a virgin female moth were included in this experiment, but these caught fewer males than traps baited with the binary mixture of the synthetic pheromone components.

II-3-3-2 *Leucinodes orbonalis*

According to the results of field test of *O. anastomosalis*, Z3,Z6,Z9-23:H plays a key role in attracting *O. anastomosalis* males. Thus, the attractiveness of E11-16:OAc (1,000 µg) mixed with different amounts of Z3,Z6,Z9-23:H (50–1,000 µg) was examined first (Experiment LoA). The result confirmed that traps baited with the binary blends captured more males than did the traps baited with the single component (Table II-7). The highest catches ratio were obtained with mixtures of

Table II-3. Attraction of *Omphisa anastomosalis* males in a sweet potato field in An Giang Province (Vietnam) by lures baited with two synthetic pheromone components (Experiment OaA)

Lure component (mg/rubber septum) ^a			Captured males	
E10,E14-16:Ald (Comp. Oa-III)	Z3,Z6,Z9-23:H (Comp. Oa-IV)	Ratio	/trap/week ^b	(Total)
0.5	0.0	1:0	1.0 ± 0.4 d	(12)
0.5	0.025	20:1	2.8 ± 0.3 c	(34)
0.5	0.1	5:1	5.8 ± 1.1 b	(70)
0.5	0.5	1:1	8.8 ± 0.8 a	(105)
0.5	1.0	1:2	9.7 ± 0.5 a	(116)
0.0	0.5	0:1	0.5 ± 0.1 d	(6)
0.0	0.0		0.0	(0)

^a Tested with three traps of each lure from September 7 to October 4, 2013.

^b Mean ± SE. Values within each test followed by a different letter are significantly different at P<0.05 by Tukey-Kramer Test.

Table II-4. Attraction of *Omphisa anastomosalis* males in a sweet potato field in An Giang Province (Vietnam) by synthetic lures [E10,E14-16:Ald (Comp. **Oa-III**) and Z3,Z6,Z9-23:H (Comp. **Oa-IV**)] mixed with minor pheromone components, E10-16:Ald (Comp. **Oa-I**) and E14-16:Ald (Comp **Oa-II**) (Experiment OaB)

Lure (mg/rubber septum) ^a	Captured males	
	/trap/week ^b	(Total)
E10,E14-16:Ald (0.5) + Z3,Z6,Z9-23:H (1.0)		
+ none	5.4 ± 1.7 a	(65)
+ E10-16:Ald (0.035)	3.8 ± 0.6 a	(45)
+ E10-16:Ald (0.35)	3.9 ± 1.0 a	(47)
+ E14-16:Ald (0.015)	3.2 ± 1.1 a	(38)
+ E14-16:Ald (0.15)	3.2 ± 0.7 a	(38)
+ E10-16:Ald (0.035) + E14-16:Ald (0.015)	4.9 ± 1.2 a	(59)
+ E10-16:Ald (0.35) + E14-16:Ald (0.15)	1.2 ± 0.4 b	(14)
control	0.0	(0)

^a Tested with four traps of each lure from October 13 to November 3, 2013.

^b Mean ± SE. Values within each test followed by a different letter are significantly different at P<0.05 by Tukey-Kramer Test.

Table II-5. Attraction of *Omphisa anastomosalis* males in a sweet potato field in An Giang Province (Vietnam) by Z3,Z6,Z9-23:H mixed with E10,E14-16:Ald or the geometrical isomer (Experiment OaC)

Lure (mg/rubber septum) ^a	Captured males	
Z3,Z6,Z9-23:H (1.0)	/trap/week ^b	(Total)
+ E10,E14-16:Ald (0.5)	17.1 ± 4.0 a	(137)
+ E10,Z14-16:Ald (0.5)	0.8 ± 0.3 b	(6)
+ Z10,E14-16:Ald (0.5)	0.0	(0)
+ Z10,Z14-16:Ald (0.5)	0.0	(0)
control	0.0	(0)

^a Tested with four traps of each lure from November 3 to 17, 2013.

^b Mean ± SE. Values within each test followed by a different letter are significantly different at P<0.05 by Tukey-Kramer Test.

Table II-6. Attraction of *Omphisa anastomosalis* males in a sweet potato field in An Giang Province (Vietnam) by E10,E14-16:Ald mixed with an unsaturated hydrocarbon (Experiment OaD)

Lure (mg/rubber septum) ^a	Captured males	
E10,E14-16:Ald (0.3)	/trap/week ^b	(Total)
+ Z6,Z9-23:H (0.6)	3.1 ± 0.6 bc	(28)
+ Z3,Z6,Z9-19:H (0.6)	1.0 ± 0.5 d	(9)
+ Z3,Z6,Z9-21:H (0.6)	13.1 ± 0.9 a	(118)
+ Z3,Z6,Z9-22:H (0.6)	15.9 ± 1.9 a	(143)
+ Z3,Z6,Z9-23:H (0.6)	11.8 ± 4.0 a	(106)
+ Z3,Z6,Z9-25:H (0.6)	5.2 ± 0.6 b	(47)
+ Z3,Z6,Z9,Z12,Z15-23:H (0.6)	12.1 ± 2.1 a	(109)
+ Z3,Z6,Z9,Z12,Z15-25:H (0.6)	3.8 ± 0.6 bc	(34)
Virgin female	3.1 ± 0.5 c	(28)
control	0.0	(0)

^a Tested with three traps of each lure from December 5 to 26, 2013.

^b Mean ± SE. Values within each test followed by a different letter are significantly different at P<0.05 by Tukey-Kramer Test.

E11-16:OAc and Z3,Z6,Z9-23:H was 10:1, and traps baited with the 10:1 mixture caught four times more males than those baited with E11-16:OAc alone. Z3,Z6,Z9-23:H alone attracted seven males, but this was fewer than the number captured in traps baited with E11-16:OAc alone.

The synergistic effects of several polyunsaturated hydrocarbons structurally related to Z3,Z6,Z9-23:H were examined for *L. orbonalis* males as well (Experiment LoB). E11-16:OAc was mixed with one of the following diene, triene or pentaene hydrocarbons in a 10:1 ratio: Z3,Z6,Z9-20:H, Z3,Z6,Z9-21:H, Z3,Z6,Z9-22:H, Z3,Z6,Z9-23:H, Z3,Z6,Z9-25:H, Z3,Z6,Z9,Z12,Z15-23:H and Z3,Z6,Z9,Z12,Z15-25:H. As shown in Table II-8, more males were caught in traps baited with the binary lures including the C22 and C23 triene than did those baited with the mixture with other polyenes. The synergistic effect of the C20, C21, C25 triene, C23 and C25 pentaene seems weaker than that of C22 and C23 triene.

In Experiment LoC, the activity of E11-16:OH was examined. The E11-16:OH was added to the two kinds of mixtures, one was E11-16:OAc (0.5 mg) and Z3,Z6,Z9-22:H (1.0 mg), and the other was E11-16:OAc (0.5 mg) and Z3,Z6,Z9-23:H (1.0 mg). As shown in Table II-9, addition of E11-16:OH has no contribution to male attraction, and the increasing dose of E11-16:OH obviously decreased the number of males captured.

Table II-7. Field attraction of *Leucinodes orbonalis* males in an eggplant field in Can Tho City (Vietnam) by traps baited with lures containing E11-16:OAc and/or Z3,Z6,Z9-23:H (6 May – 3 June 2014, 3 replicates). (Experiment LoA)

Lure components (mg)		Captured males	
E11-16:OAc	Z3,Z6,Z9-23:H	/week ^a	Total
1.0	0.0	16.5 ± 2.2 b	198
1.0	0.05	42.3 ± 7.0 ab	507
1.0	0.1	62.2 ± 6.0 a	746
1.0	0.5	39.9 ± 8.8 ab	479
1.0	1.0	27.1 ± 3.6 ab	325
0.0	1.0	0.6 ± 0.6 c	7
0.0	0.0	0.0 ± 0.0	0

^a Mean ± SE. Values within each test followed by a different letter are significantly different at P<0.05 by Tukey-Kramer Test (one-way ANOVA, F=41.28; df=5,12; P<0.001)

Table II-8. Field attraction of *Leucinodes orbonalis* males in an eggplant field in Can Tho City (Vietnam) by traps baited with lures containing E11-16:OAc (1.0 mg) mixed with unsaturated hydrocarbons (each 0.1 mg) (17 June – 15 July 2014, 3 replicates). (Experiment LoB)

Lure (mg/rubber septum)	Captured males	
E11-16:OAc (1.0)	/week ^a	Total
+ None	10.6 ± 0.8 b	127
+ Z3,Z6,Z9-20:H (0.1)	11.9 ± 1.2 b	143
+ Z3,Z6,Z9-21:H (0.1)	16.1 ± 7.1 b	193
+ Z3,Z6,Z9-22:H (0.1)	64.3 ± 7.3 a	771
+ Z3,Z6,Z9-23:H (0.1)	44.8 ± 7.8 a	537
+ Z3,Z6,Z9-25:H (0.1)	16.1 ± 4.3 ab	193
+ Z3,Z6,Z9,Z12,Z15-23:H (0.1)	34.5 ± 10.1 ab	414
+ Z3,Z6,Z9,Z12,Z15-25:H (0.1)	14.8 ± 4.7 b	178
Unbaited control	0 ± 0.0	0

^a Mean ± SE. Values within each test followed by a different letter are significantly different at P<0.05 by Tukey-Kramer Test (one-way ANOVA, F=7.27; df=7,16; P<0.001)

Table II-9. Field attraction of *Leucinodes orbonalis* males in an eggplant field in Can Tho City (Vietnam) by traps baited with lures containing E11-16:OAc (1.0 mg) mixed with Z3,Z6,Z9-22:H, Z3,Z6,Z9-23:H and E11-16:OH, (30 July – 20 August 2014, 3 replicates). (Experiment LoC)

Lure (mg/rubber septum) E11-16:OAc (1.0)			Captured males	
Z3,Z6,Z9-22:H	Z3,Z6,Z9-23:H	E11-16:OH	/week ^a	Total
0.1	0.0	0.0	67.6 ± 10.7 a	608
0.1	0.0	0.1	26.7 ± 13.1 a	240
0.1	0.0	0.25	0.9 ± 0.4 b	8
0.1	0.0	0.5	1.1 ± 1.1 b	10
0.0	0.1	0.0	71.6 ± 14.0 a	644
0.0	0.1	0.1	14.6 ± 1.2 a	131
0.0	0.1	0.25	2.2 ± 0.6 b	20
0.0	0.1	0.5	1.8 ± 1.0 b	16
0.0	0.0	0.0	0.0 ± 0.0	0

^a Mean ± SE. Values within each test followed by a different letter are significantly different at P<0.05 by Tukey-Kramer Test (one-way ANOVA, F=22.06; df=7,16; P<0.001)

II-4 Discussion

In GC/MS analyses of a single crude extract of 50 pheromone glands of virgin female *O. anastomosalis* obtained from Vietnam, three unsaturated aldehydes, E10-14:Ald (Oa-I), E14-16:Ald (Oa-II), and E10,E14-16:Ald (Oa-III) were detected in a ratio of 7:3:100. These components previously have been identified in a Japanese population in a ratio of 3:0.7:100 by Wakamura et al. (2010), and while the ratios are slightly different, the dienyl aldehyde is the main component in both. In addition to these Type I compounds, the Z3,Z6,Z9-23:H (Oa-IV) was found in the pheromone gland extract of virgin females. In the pheromone gland extract of *L. orbonalis*, Z3,Z6,Z9-23:H (Lo-III) was detected with a low content as well, and another polyunsaturated hydrocarbon Z3,Z6,Z9-22:H (Lo-II) was also detected with a low content.

In field tests in Vietnam, traps baited with a mixture of synthetic E10,E14-16:Ald and Z3,Z6,Z9-23:H in ratios from 1:0.2 to 1:2 caught more *O. anastomosalis* males than those baited with E10,E14-16:Ald alone. For the field tests of *L. orbonalis*, the mixture of synthetic E11-16:OAc and Z3,Z6,Z9-23:H in a ratio of 10:1 has the best effective of male attraction. The optimal attraction of the two species indicating a strong synergistic effect of the Type II compound. Although Z3,Z6,Z9-23:H is a minor component in the pheromone extract of *O. anastomosalis*, the number of males captured increased with increase in relative amount of the triene up to the maximum tested. Furthermore, the best synthetic lure was more attractive to male moths than a virgin female. This may have been due to a higher overall release rate from the synthetic lure, or because the laboratory-reared female moths were not in optimum condition. However, it is interesting that lures containing the 1:2 blend were more attractive than those containing a 1:0.05 blend similar to that found in the pheromone gland extract of *O. anastomosalis*. Evaluation of the binary blend in Japan and other countries will be important for understanding whether all populations of this species utilize Z3,Z6,Z9-23:H in mating communication.

In field tests of *O. anastomosalis*, addition of the other minor pheromone

components, monoene aldehydes E10-16:Ald (Oa-I) or E14-16:Ald (Oa-II) to the binary blend of E10,E14-16:Ald and Z3,Z6,Z9-23:H individually or together did not increase the number of male moths captured, and there was a tendency to decrease catches, particularly at high dose. Combinations of Z3,Z6,Z9-23:H with the other three geometrical isomers of E10,E14-16:Ald attracted few males, substantiating the assignment of the 10*E*,14*E* configuration to the naturally-occurring dienyI component. For *L. orbonalis*, another minor pheromone components E11-16:OH obviously plays a role as an inhibitor in male attraction. As previously reported, E11-16:OH presented a low content relative to the major component E11-16:OAc in a pheromone extracts prepared from Indian and Taiwanese population, and blends containing between 1 and 10% E11-16:OH caught more males than E11-16:OAc alone in field trials (Cork et al., 2001). This is obviously contrary to our conclusion. Further field work with the pheromone traps in different areas will clarify the role of E11-16:OH and the ecological aspects of this species.

In field tests with traps baited with E10,E14-16:Ald mixed with other polyunsaturated hydrocarbons, two-component lures that included a C21 triene, a C22 triene, or a C23 pentaene, attracted as many males as did the mixture of E10,E14-16:Ald and Z3,Z6,Z9-23:H. In contrast to the strict recognition of the double-bond configuration of the Type I compound, this result indicated low specificity in recognition of the chain length and degree of unsaturation of the Type II compound, although C19 and C25 trienes and a C23 diene were less effective synergists. But for the males of *L. orbonalis*, it shows low specificity in recognition between C22 and C23 trienes. To the best of our knowledge, the specificity of this synergistic effect of Type II compounds has not been investigated in other species of Pyraloidea.

In the case of *L. orbonalis*, the trienes was also found in the body wax, and their content are higher than those in a pheromone gland extract, especially Z3,Z6,Z9-23:H. Z3,Z6,Z9-23:H was also found in the body wax of *Conogethes punctiferalis*, and the content was 10 times more than that in a pheromone gland extract (Xiao et al., 2012). While hydrocarbons in the body wax of *O. anastomosalis* have not yet been analyzed,

the large differences between the ratios of E10,E14-16:Ald and Z3,Z6,Z9-23:H in the pheromone gland and the optimum lure for male attraction suggest the possibility that the triene also is present and released from the cuticle of *O. anastomosalis* females.

A rubber septum dosed with synthetic pheromones attracted males for at least one month, indicating the potential of the lure as a monitoring tool for *O. anastomosalis* and *L. orbonalis*. Further study of the correlation between the frequency of damaged crops and the number of males captured by the synthetic pheromone will contribute to IPM program against *O. anastomosalis* and *L. orbonalis*.

CHAPTER III: Sex Pheromones of *Herpetogramma submarginale* and *H. basale* that Inhabit Okinawa Islands

III-1 Introduction

The subfamily Pyraustinae has more than 7,500 species worldwide. While the majority of this group occurs in tropical regions, there are 340 species in Japan which are divided into 132 genera (Yamanaka, 2013). As the biggest genus of Pyraustinae, *Herpetogramma* include 18 species and most of them inhabit Okinawa islands. Their host plants are different, but some species expect to be sympatric in the island and the females might come across males of other species. Although they have a closed geographic distribution because island areas are not large, they seem to have a strict sexual communication system that prevents them to mate with different species.

Since the sex pheromone of only one *Herpetogramma* species has been identified from *H. licarsisale* (Gibb et al., 2007), sex pheromones of other species have not yet been studied. In order to understand the mechanism of reproductive isolation of Okinawa species in the genus *Herpetogramma*, as a first step toward understanding interspecific similarities in pheromone molecular structures, sex pheromones of two *Herpetogramma* species (*H. submarginale* and *H. basale*) were examined by GC-EAD and GC-MS analyses. Mass spectra of the pheromone components and their derivatives with DMDS or MTAD indicated that females of *H. submarginale* and *H. basale* produced (Z)-13-hexadecenyl acetate (Z13-16:OAc) and (11Z,13E)-11,13-hexadecadienyl acetate (Z11,E13-16:OAc), respectively. Z13-16:OAc was first identified as a novel moth sex pheromone component in Lepidoptera. Field test revealed the Z13-16:OAc alone could strongly attract the *H. submarginale* male moths, while male attraction by the synthetic pheromone of *H. basale* was failed.

III-2 Methods and Materials

III-2-1 Insects and Pheromone Extract

The larvae of *H. submarginale* were collected on the seaside in Yaese Town, and the larvae of *H. basal* were collected on the seaside in Ohyama, Okinawa Prefecture from September to November in 2012. In the natural photoperiod, the larvae were reared using *Helianthus tuberosus* for fresh foods under in 14L-10D at 25 ± 2 °C before pupation. After eclosion, about forty abdominal tips of two kinds of virgin females were excised at the calling time (8-9.5 hrs from the start of scotophase of *H. submarginale*, and 5-8 hrs from the start of scotophase of *H. basal*) and soaked in hexane (10 μ l/female) for 10 min to extract the pheromone components. The crude extract was used for structural analysis of pheromone components after filtration.

In order to confirm the positions of the double bonds in the proposed pheromone components, after removal of hexane, crude extracted materials of *H. submarginale* (13 female equivalents, FE) were treated with DMDS (50 μ l) that included a diethyl ether solution of iodine (60 mg/ml, 5 μ l) and were held at 40°C overnight (Buser et al., 1983). After adding a 10% sodium thiosulfate solution (0.5 ml), produced DMDS adducts were extracted with hexane and analyzed by GC-MS. And a 25-FE aliquot of crude extract of *H. basal* was dissolved in CH_2Cl_2 (10 μ l), and treated with a CH_2Cl_2 solution of MTAD (10 mg/ml, 40 μ l) for 30 min at room temperature (Do et al., 2011). After changing the solvent to hexane, the MTAD adduct were analyzed by GC-MS.

III-2-2 Analytical Instruments

III-2-2-1 Gas Chromatography (GC) - Electroantennographic Detector (EAD)

Insect perceives odors by olfactory receptors which are most abundant on their antennae, and electroantennogram (EAG) is a chart records the potential changes between base and tip of an antenna during the stimulation of volatile compounds. The gas chromatograph (GC) is an apparatus used for separating and determining the

identity and relative abundance of compounds present in complex mixtures of volatile and or semi-volatile compounds. GC-EAD is coupled GC-electroantennographic detection (EAD), which takes the EAG by using the antenna of an insect as a detector for a capillary-column gas chromatograph. The electrical signals so-called EAG-response recorded as the result of specific olfactory stimulations on the antenna. This information can be used to discover potentially useful compounds, such as sex pheromones, that alter the behavior of insects (Arn et al., 1975).

For analyses with GC-EAD, an HP-5890 Series II gas chromatograph (Agilent Technologies Inc., Palo Alto, CA, USA) was equipped with a DB-23 capillary column (0.25 mm ID, 30 m length, 0.25 μ m film thickness, J & W Scientific, Folsom, CA, USA). The column effluent at a 1:1 split ratio was directed toward the FID and EAD with a male moth antenna (Shibasaki et al., 2013). The oven temperature was maintained at 80°C for 1 min and then increased by 8°C/min to 210°C.

III-2-2-1 Gas Chromatography (GC) - Mass Spectrometry (MS)

Analyses of pheromone gland extracts by GC coupled with mass spectrometry (GC/MS) employed a Hewlett-Packard 5973 mass selective detector (Agilent Technologies Inc.), which was equipped with a DB-23 or HP-5 (0.25 mm \times 30 m, 0.25 μ m; J & W Scientific, Folsom, CA, USA) capillary column and operated in electron impact ionization mode (70 eV). The oven temperature for the DB-23 column was programmed in the same manner as for GC-EAD analysis. The flow rate of helium carrier gas was 1.0 ml/min in all GC analyses, and the GC-inlet temperature was 220 °C. For analysis of DMDS and MTAD derivatives, the HP-5 column was used and the oven temperature was maintained at 100°C for 2 min and then programmed at 15°C/min to 280°C.

III-2-3 Chemicals

(*Z*)- and (*E*)-13-hexadecenol (Z13-16:OH and E13-16:OH) were synthesized by a selective reduction of the corresponding acetylene compounds, which were prepared *via* a tetrahydropyranyl (THP) ether of 13-tetradecyn-1-ol. The acetate and aldehyde derivatives were obtained by the acetylation and PCC oxidation. The four geometric isomers of 11,13-hexadecadienyl acetate were supplied from a stock library in our laboratory. Z13-16:OH, ¹H NMR δ : 0.95 (3H, t, J = 7.5 Hz, CH₃), 1.26 (18H, broad s), 1.56 (2H, m, CH₂CH₂OH), 2.02 (4H, m, CH₂CH=CHCH₂), 3.64 (2H, t, J = 6.6 Hz, CH₂OH), 5.33 (2H, m, CH=CH). ¹³C NMR δ : 14.4, 20.5, 22.7, 25.8, 27.1, 29.3, 29.5, 29.6 (\times 2), 29.7, 29.8, 31.6, 32.8, 63.1, 129.4, 131.5. E13-16:OH, ¹H NMR δ : 0.96 (3H, t, J = 7.5 Hz, CH₃), 1.26 (18H, broad s), 1.57 (2H, m, CH₂CH₂OH), 1.97 (4H, m, CH₂CH=CHCH₂), 3.64 (2H, t, J = 6.6 Hz, CH₂OH), 5.41 (2H, m, CH=CH). ¹³C NMR δ : 14.0, 22.7, 25.7, 29.2, 29.5, 29.6 (\times 3), 29.7, 31.1, 31.6, 32.6, 32.8, 63.1, 129.4, 131.9.

III-2-4 Bioassay and Field Trapping Tests

The bioassay of synthetic compounds on *H. submarginale* were measured by GC-EAD using the same conditions for the gland extracts. Four different male antennae were used for GC-EAD analyses of every dose. Data obtained in each group test were analyzed by one-way ANOVA, and pairwise comparisons among traps were performed with Tukey-Kramer Test with P-values adjusted for multiple comparisons.

The field experiments of *H. submarginale* were conducted at the seashores on Okinawa Island, Okinawa, Japan (within a 4 km radius from 26.7°N, 128.2°E) from 2013. The synthetic compounds (> 98% isomerically pure by GC analysis) were dissolved in hexane (20 mg/ml) and applied to rubber septa (white rubber, 8 mm O.D., Sigma-Aldrich Inc., St. Louis, MO, USA) that were used as dispensers. Control septa were loaded with hexane (50 μ l) only. Lures were placed at the center of sticky traps (SE trap[®], 30 \times 27 cm bottom plate with a roof, Sankei Chemical Co., Tokyo, Japan), and were hung separately 1 m above ground level at intervals of 10 m. Three or four replicates for each treatment were tested, and the captured males were counted every

week. The number of captured males in each field tests whose total was not zero were analyzed by using a generalized linear mixed model (GLMM) with Poisson distribution and Log link function, applying treatment as a fixed factor, and checked date and place of traps as random factors, and then significant explanatory variables were compared by Tukey's test. These analyses were performed with R version 3.1.2 (R Development Core Team, 2014).

III-3 Results

III-3-1 Analyses of Pheromone Extracts

III-3-1-1 *H. submarginale*

A pheromone gland extract (1 FE) of *H. submarginale* females showed only one candidate pheromone component (Comp. Hs-I, Rt. 17.28 min) that reproducibly elicited a response from male antennae in the GC-EAD analyses (Fig. III-1a). Next, the extract (1 FE) was analyzed by GC/MS (Fig. III-1b), the mass spectrum of Comp. Hs-I (Rt. 15.403 min) showed the diagnostic fragment ions at m/z 222 ($[M-60]^+$) and 61 ($CH_3COOH_2^+$) indicating a hexadecenyl acetate (Fig. III-1c) (Ando and Yamakawa, 2011). Interestingly, another component (Comp. Hs-II, Rt. 15.175 min) was found with a similar mass spectrum with Comp. Hs-I (Fig. III-1d). The ratio of peak areas of Comps. Hs-I and Hs-II was approximately 87:13. Although the mass spectrum of Hs-I and Hs-II are similar to those of synthetic 11-hexadecenyl acetate (11-16:OAc) and 12-hexadecenyl acetate (12-16:OAc), the abundance of fragment ions at m/z 68 are obviously different (Hs-I, 78%; Hs-II, 80%; 11-16:OAc, 42%; 12-16:OAc, 45%).

In order to confirm the positions of the double bond in Comps. Hs-I and Hs-II, the crude pheromone extract (13 FE) was treated with DMDS. GC-MS analysis of the DMDS-derivatives revealed two adducts of hexadecenyl acetate (Comp. Hs-I adduct, Rt 26.35 min; Comp. Hs-II adduct, Rt 26.45 min) as a ratio of 81:19 (Fig. III-2a), and they showed the same characteristic fragment ions at m/z 89 ($C_3H_6SCH_3^+$) and 287 ($CH_3COOC_{13}H_{25}SCH_3$) of the DMDS adducts (M^+ at m/z 376) indicated their original double bond at the 13-position (Fig. III-2b and 2c). Finally, the Comps. Hs-I and Hs-II were assigned as Z13-16:OAc and (*E*)-13-hexadecenyl acetate (E13-16:OAc) by comparisons with Rts of synthetic standards, respectively.

III-3-1-2 *H. basale*

A pheromone gland extract (0.1 FE) of *H. basale* females showed only one candidate pheromone component (Comp. Hb-I, 18.72 min) that reproducibly elicited a response from male antennae in the GC-EAD analyses repeated three times (Fig.

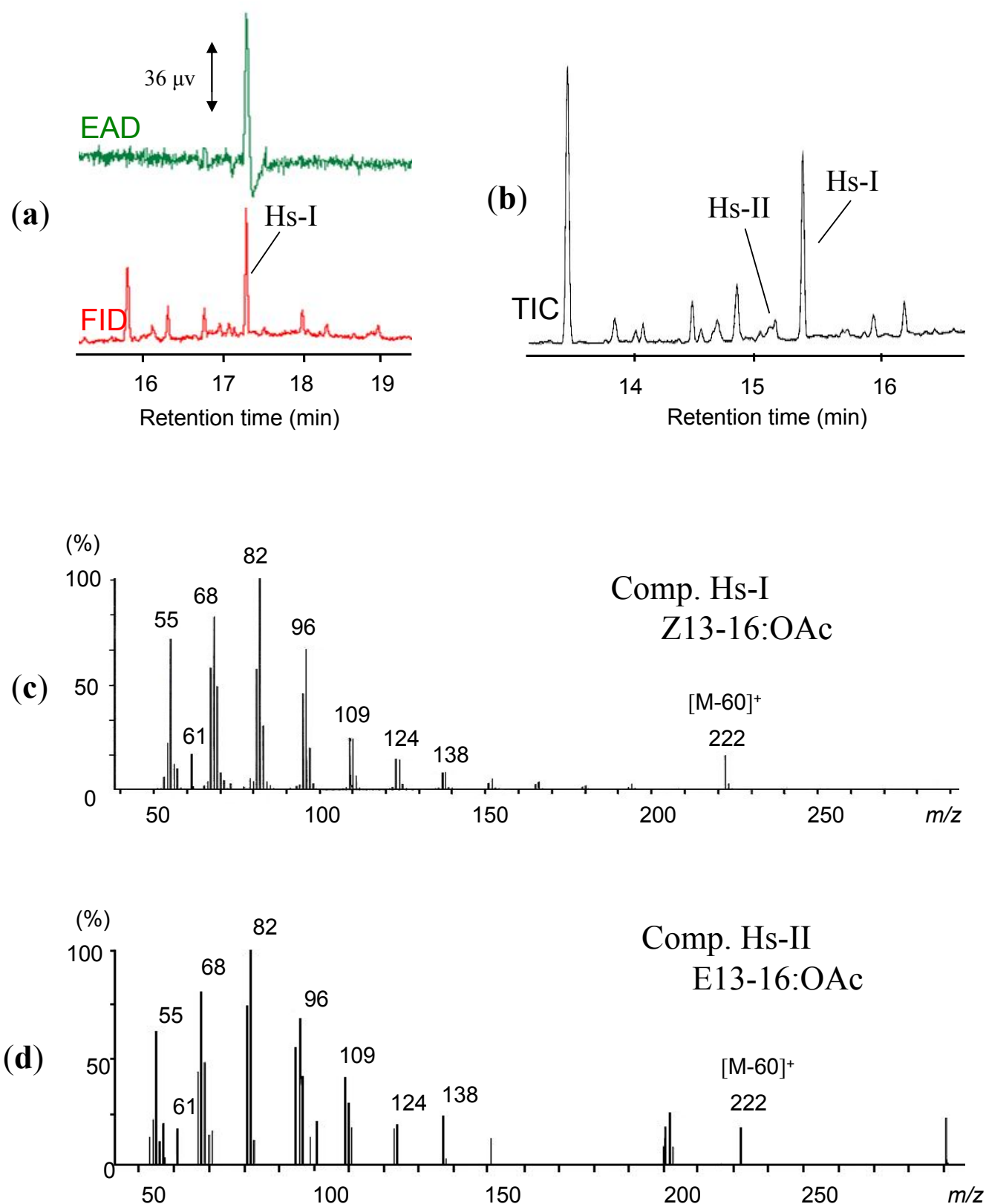


Fig. III-1. Analyses of the sex pheromone of *Herpetogramma submarginale* females by GC-EAD and GC/MS. **(a)** Chromatograms of the pheromone extract (1 FE) recorded by EAD and FID; **(b)** TIC of the pheromone extract (1 FE); **(c)** a mass spectrum of Comp. Hs-I (Rt 15.4 min, Z13-16:OAc) and **(d)** a mass spectrum of Comp. Hs-II (Rt 15.18 min, E13-16:OAc).

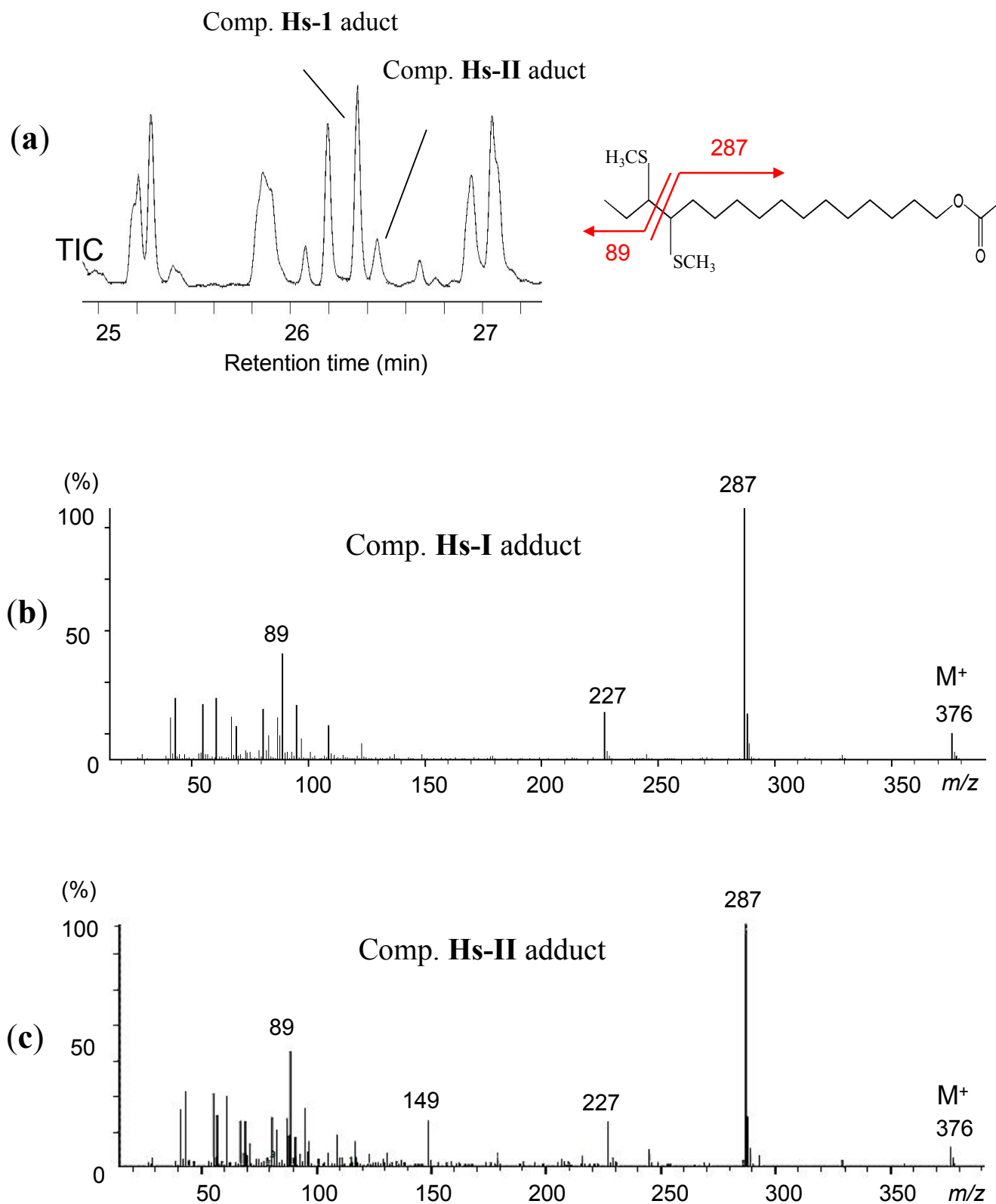


Fig. III-2. GC-MS analysis of a pheromone extract of *Herpetogramma submarginale* females (13 FE) treated with dimethyl disulfide (DMDS); (a) TIC; (b) a mass spectrum of a DMDS-adduct of Comp. **Hs-I** and (c) a mass spectrum of a DMDS-adduct of Comp. **Hs-II**.

III-3a). Next, the extract (0.5 FE) was analyzed by GC/MS (Fig. III-3b). The mass spectrum of Comp. Hb-I (Rt 16.63 min) showed the ions at m/z 280 (M^+), 220 ($[M-60]^+$) and 60 suggested a hexadecadienyl acetate (Fig. III-3c) (Ando and Yamakawa, 2011). The retention time of Comp. Hb-I on the DB-23 and HP-5 columns suggested a C16-conjugated dienyl acetate after a comparison with a number of C16-conjugated and C16-nonconjugated dienyl acetate standards.

The analysis of the MTAD-derivatized pheromone gland extract by GC-MS revealed an adduct with a molecular ion at m/z 393, and diagnostic ions at m/z 364 ($[C_{19}H_{30}N_3O_4]^+$) and 194 ($[C_9H_{12}N_3O_2]^+$) (Fig. III-4), indicative of a C16 dienyl acetate with the diene in the 11,13 position. Thus, Comp. Hb-I was identified as an 11,13-hexadecadienyl acetate. Finally, structural determination of geometrical configuration was accomplished by comparing the Rts and RI values with those of authentic samples. The analyses were conducted with two different columns, a polar column (DB-23) and a non-polar column (HP-5). Table III-1 shows the retention times and RI values of natural components and synthetic standards. Essentially identical retention times unambiguously confirmed Comp. Hb-I as Z11,E13-16:OAc.

III-3-2 Antennal Responses of *H. submarginale* to Synthetic Standards

By GC-EAD equipped with an *H. submarginale* male antenna, the EAG activity of each isomer of Z13-16:OAc and E13-16:OAc was measured at three different doses (Fig. III-5). The activity of Z13-16:OAc at each dose was higher than those of E13-16:OAc. The antenna was stimulated more strongly by Z13-16:OAc with a 1-ng dose than 0.1-ng dose and 0.01-ng dose. It has no significant difference between 0.1-ng and 0.01-ng dose.

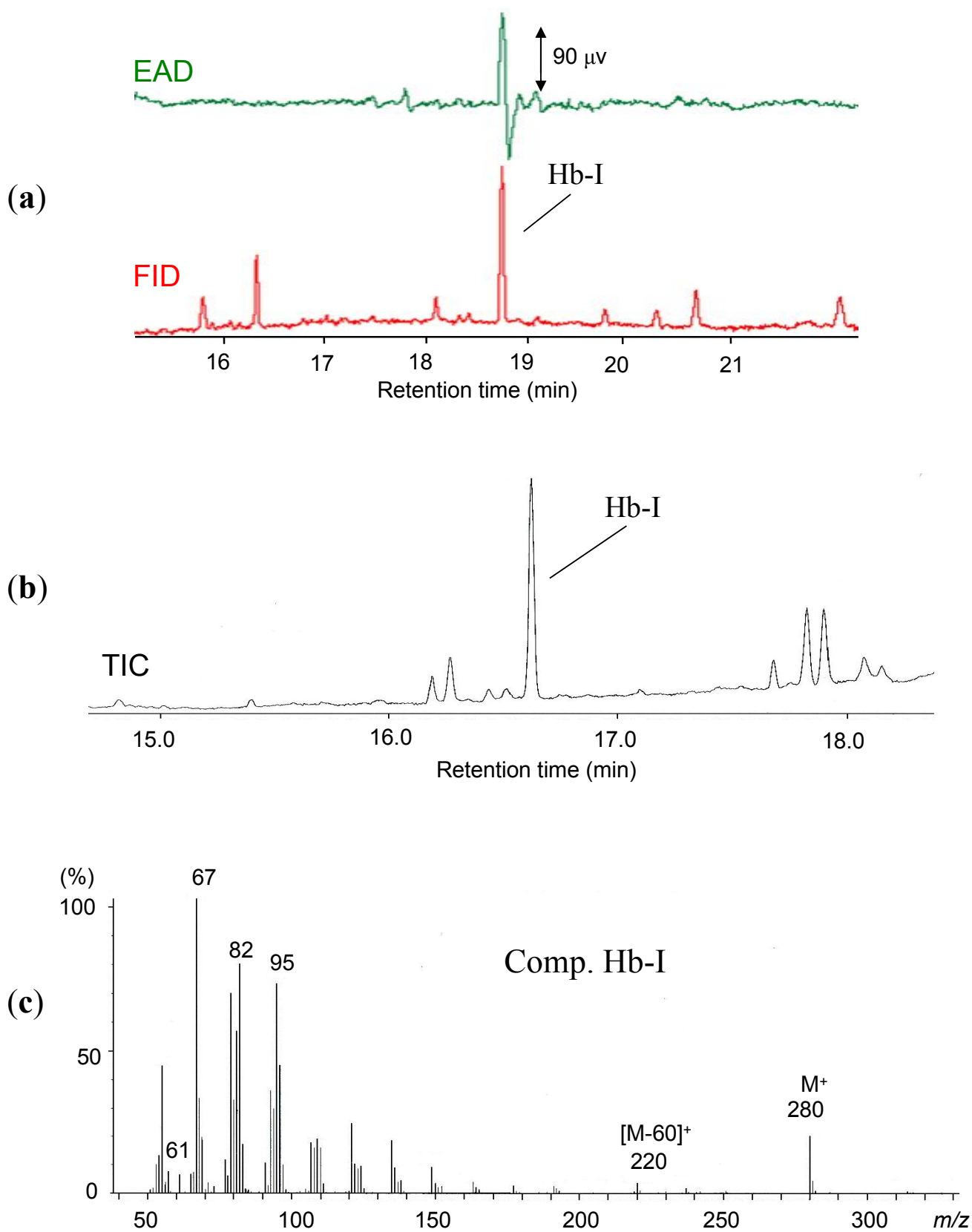


Fig. III-3. Analyses of the sex pheromone of *Herpetogramma basale* females by GC-EAD and GC/MS. (a) Chromatograms of the pheromone extract (0.1 FE) recorded by EAD and FID; (b) TIC of the pheromone extract (0.5 FE); (c) a mass spectrum of Comp. Hb-I (Rt 18.72 min, Z11,E13-16:OAc).

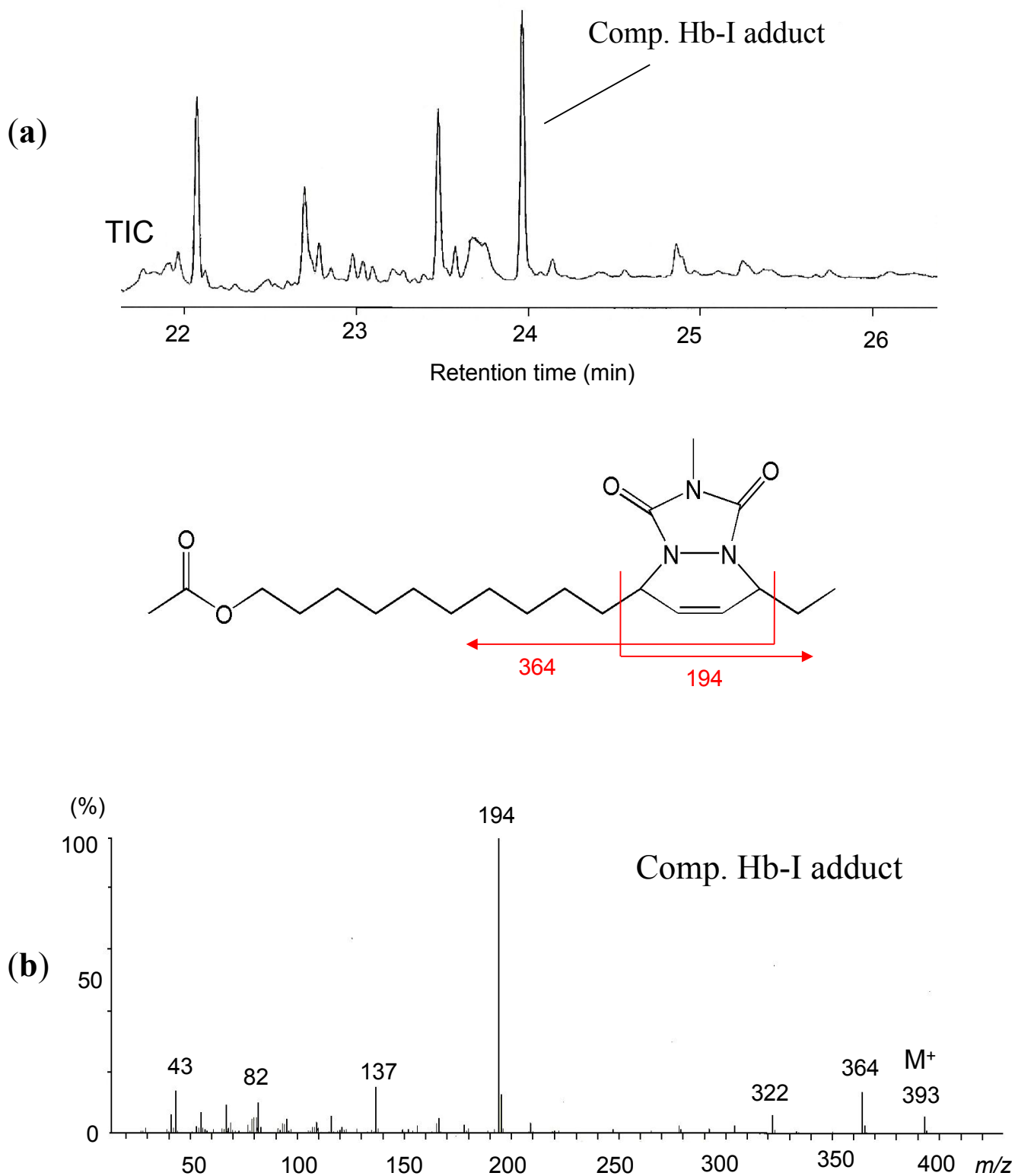


Fig. III-4. GC-MS analysis of a pheromone extract of *Herpetogramma basale* females (25 FE) treated with 4-methyl-1,2,4-triazoline-3,5-dione (MTAD); (a) TIC; (b) a mass spectrum of a MTAD-adduct of Comp. Hb-I.

Table III-1 Retention times (Rt) and retention indices (RI) of insect produced pheromones (Comp. Hb-III) and synthetic compounds^a

Compound	GC column ^b			
	DB-23		HP-5MS	
	Rt (min)	RI	Rt (min)	RI
Comp. III	16.63	2190	18.34	2026
E11,E13-16:OAc	16.78	2204	18.59	2043
Z11,E13-16:OAc	16.63	2190	18.30	2024
E11,Z13-16:OAc	16.72	2198	18.41	2031
Z11,Z13-16:OAc	16.75	2201	18.52	2038

^a Comp. Hb-III is (11Z,13E)-hexadecadienyl acetate of *Herpetogramma basale* in Fig. III-3.

^b Each compound was analyzed by GC-MS equipped with a DB-23 column (0.25 mm ID × 30 m) and an HP-5MS column (0.25 mm ID × 30 m). The column temperature program was 80°C for 2 min, 8°C/min to 210°C, and 210°C for 10 min.

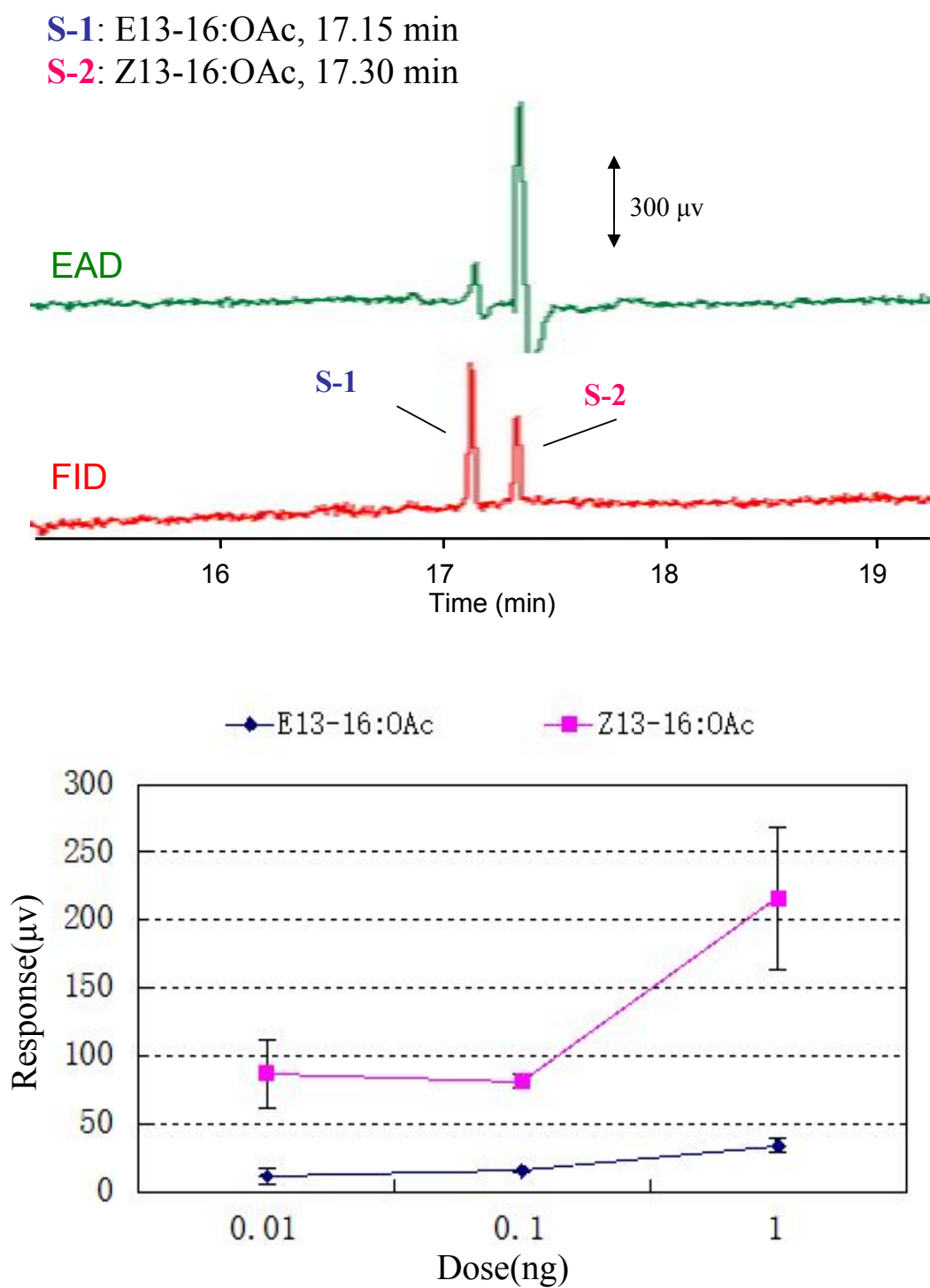


Fig. III-5. EAG responses of male *Herpetogramma submarginale* antennae stimulated by (E)- and (Z)-13-hexadecadienyl acetate. The responses (mean \pm SE) were calculated with data measured by GC-EAD using at least 4 antennae for each dose.

III-3-3 Male Attraction by Synthetic Compounds

For the field experiments of *H. submarginale*, the attractiveness of Z13-16:OAc mixed with E13-16:OAc in different ratios were examined as first (Table III-2, Tests A and B). Z13-16:OAc as a single component was attractive to males, none of the binary blends was more attractive than the Z13-16:OAc alone, whereas E13-16:OAc was unattractive alone. Based on this result, another test was designed to evaluate mixtures of Z13-16:OAc and in binary combinations with other functional derivatives (Z13-16:OH and Z13-16:Ald). Unfortunately, Z13-16:OH significantly reduced the number of captured males, and Z13-16:Ald have not improved the attractiveness of the lure (Table III-2, Tests C). Traps baited with a virgin female moth were included in this experiment, but these caught fewer males than traps baited with synthetic pheromone components (Table III-2, Test C).

The field experiment was carried out to attract *H. basale* males in a forest on Okinawa Main Island on November, 2012. The lures baited with synthetic Z11,E13-16:OAc were not attracted the *H. basal* males effectively, only 5 males were captured by the trap in total.

Table III-2. Attraction of *H. marginale* males by traps, which were baited with Z13-16:OAc unmixed and mixed with E13-16:OAc, Z13-16:Ald or Z13-16:OH in different ratios.

Compounds (mg)		<i>N</i>	<u>Captured males^d</u> (Mean ± SE)		
Test A ^a	Z13-16:OAc (1,000)	3	1.95 ± 0.35	a	
	Z13-16:OAc (900) + E13-16:OAc (100)	3	2.05 ± 0.52	a	
	Z13-16:OAc (500) + E13-16:OAc (500)	3	0.90 ± 0.17	b	
	Z13-16:OAc (100) + E13-16:OAc (900)	3	0.71 ± 0.31	b c	
	E13-16:OAc (1,000)	3	0		
	Control	3	0.05 ± 0.05	c	
Test B ^b	Z13-16:OAc (1,000)	3	6.03 ± 1.10	a	
	Z13-16:OAc (1,000) + E13-16:OAc (10)	3	3.49 ± 0.58	c	
	Z13-16:OAc (1,000) + E13-16:OAc (50)	3	4.08 ± 0.73	b c	
	Z13-16:OAc (1,000) + E13-16:OAc (100)	3	5.33 ± 1.46	a b	
	Z13-16:OAc (1,000) + E13-16:OAc (300)	3	4.69 ± 1.21	a b c	
	Z13-16:OAc (1,000) + E13-16:OAc (500)	3	4.69 ± 0.66	a c	
	Control	3	0.23 ± 0.16	d	
Test C ^c	Z13-16:OAc (1,000)	3	2.59 ± 0.81	a	
	Z13-16:OAc (1,000) + Z13-16:Ald (100)	3	2.33 ± 0.51	a b	
	Z13-16:OAc (1,000) + Z13-16:OH (100)	3	1.56 ± 0.37	b	
	Z13-16:OAc (1,000) + Z13-16:Ald (100) + Z13-16:OH (100)	3	1.95 ± 0.57	a b	
	Virgin female x 1	3	0.77 ± 0.35	c	
	Control	3	0		

^a Tested at three seashores in Kunigami Village and Ohgimi Village, Okinawa Prefecture, from 8 to 15 December 2013.

^b Tested at three seashores in Kunigami Village and Ohgimi Village, Okinawa Prefecture, from 7 to 27 October 2014.

^c Tested at three seashores in Kunigami Village and Ohgimi Village, Okinawa Prefecture, from 5 to 18 December 2014.

^d Males/trap/day. Values followed by a different letter are significantly different by the Tukey's test ($P < 0.05$) after GLMM (family = poisson, link = log).

III-4 Discussion

Chemical analyses and field test revealed that the female-produced sex pheromone of *H. submarginale* is Z13-16:OAc, a novel moth sex pheromone component in lepidopteran. A trace isomer E13-16:OAc also was detected in pheromone gland extracts (Fig. III-1b), but addition of this compound had not enhanced the attraction to Z13-16:OAc, even presented a weak inhibiting effect in male attraction (Table III-2; Tests A and B). Furthermore, the best synthetic lure was more attractive to male moths than a virgin female (Table III-2; Test C). The synthetic lure can be used as a monitoring tool to study ecological aspects of this species.

By mass spectral analyses of natural pheromone components and MTAD adducts, and retention index comparisons with synthetic standards, the sex pheromone of *H. basale* was identified as Z11,E13-16:OAc. The field test of the synthetic compound showed no good male attraction. The possible explanation for the unsuccessful attraction of *H. basale* may be due to setting traps in an area with low population density, or the lures lack of minor components that missed by chemical analyses. It is important to know whether or not *H. basale* have minor components that have some effects on the attraction of males.

11,13-hexadecadienyl compounds have been identified from *H. licarsisale* (Gibb et al., 2007) and some species in the families of Crambidae and Pyralidae (Ando, 2014), indicating participation of Δ 13-desaturase in their pheromone biosyntheses. Since Δ 11-desaturation is a common biosynthetic step of many moth species (Liu et al., 2004, Fujii et al., 2011) and no 13-hexadecenyl compounds have been found, the dienyl compounds have been estimated to be produced by the Δ 11-desaturation as an initial step. The finding of Z13-16:OAc, however, indicates another possibility that Δ 13-desaturation of palmitoyl-CoA proceeds before the Δ 11-desaturation. While 13-octadecenyl pheromone compounds have been known, they might be produced chain-elongation of an 11-hexadecenyl intermediate. The *H. submarginale* is one of the best species to characterize an interesting new desaturase, and the analysis of genes expressed in the pheromone gland may clarify the new

desaturase.

In seashores of Okinawa Island, two closely related species, *H. basale* and *H. cynarale*, systematically distribute and appear all seasons the same as *H. submarginale* (Yamanaka, 2013). While the field evaluation of the synthetic lures of *H. submarginale* are limited, no males of *H. basale* and *H. cynarale* were attracted by the synthetic lures indicating high specificity of Z13-16:OAc at least in Okinawa Island. It is necessary to conduct field tests with 13-hexadecenyl compounds in the main island of Japan in order to make clear the specificity. Further research on chemical communication of species in genus *Herpetogramma* could help better understand mechanisms of sympatric speciation and reproductive isolation by sex pheromones.

CHAPTER IV: Identification of Sex Pheromones Secreted by Other Five Pyraustinae Species

IV-1 Introduction

Sex pheromone identification is still difficult because the chemical structure of many minor components present in small amounts in mixtures of similar molecules has to be elucidated. Many lepidopteran sex pheromones are straight-chain compounds, their structures are determined by clarification of the functional group, degree of unsaturation, carbon chain length, and position and geometry of the double bond(s). To determine chemical structures of pheromone components, GC-EAD and GC-MS are usually used. GC-EAD and GC-MS analyses on the pheromone extract can generally give us the information about functional group, carbon chain length, and the number and position of the double bond(s) of pheromone components. An IR spectrum can show a reliable information about the functional group and double-bond configuration. But because the species-specific pheromones are usually composed of multiple components, which are produced around ng level (Ando et al., 2004; Komoda et al., 2000), the IR analysis rarely has been utilized for pheromone identifications.

Recently, solid-phase GC-Fourier transfer infrared spectrometry (GC-FT/IR) has been utilized for structure determination of sex pheromones (Zarbin et al., 2012; Soldi et al., 2012; Shibasaki et al., 2013). GC-FT/IR is combination between GC and FT/IR machines, which utilized the high separation of capillary GC and high sensitivity of FT/IR. This machine equipped with a zinc selenide disk cooled at -30 °C to -40°C. When the disk turning slowly, compounds eluting from a capillary column are fixed on the disk in discrete positions, and FT/IR spectra of compounds are measured continuously (Fig. IV-1). Because of the zinc selenide disk shows high transparency of IR rays, the IR spectra are similar to familiarized spectra measured by KBr or liquid-film methods (Shibasaki et al., 2013). This increased analytical sensitivity is facilitate application of GC-FT/IR to pheromone studies of lepidopteran species, particularly for determination of double-bond configurations.

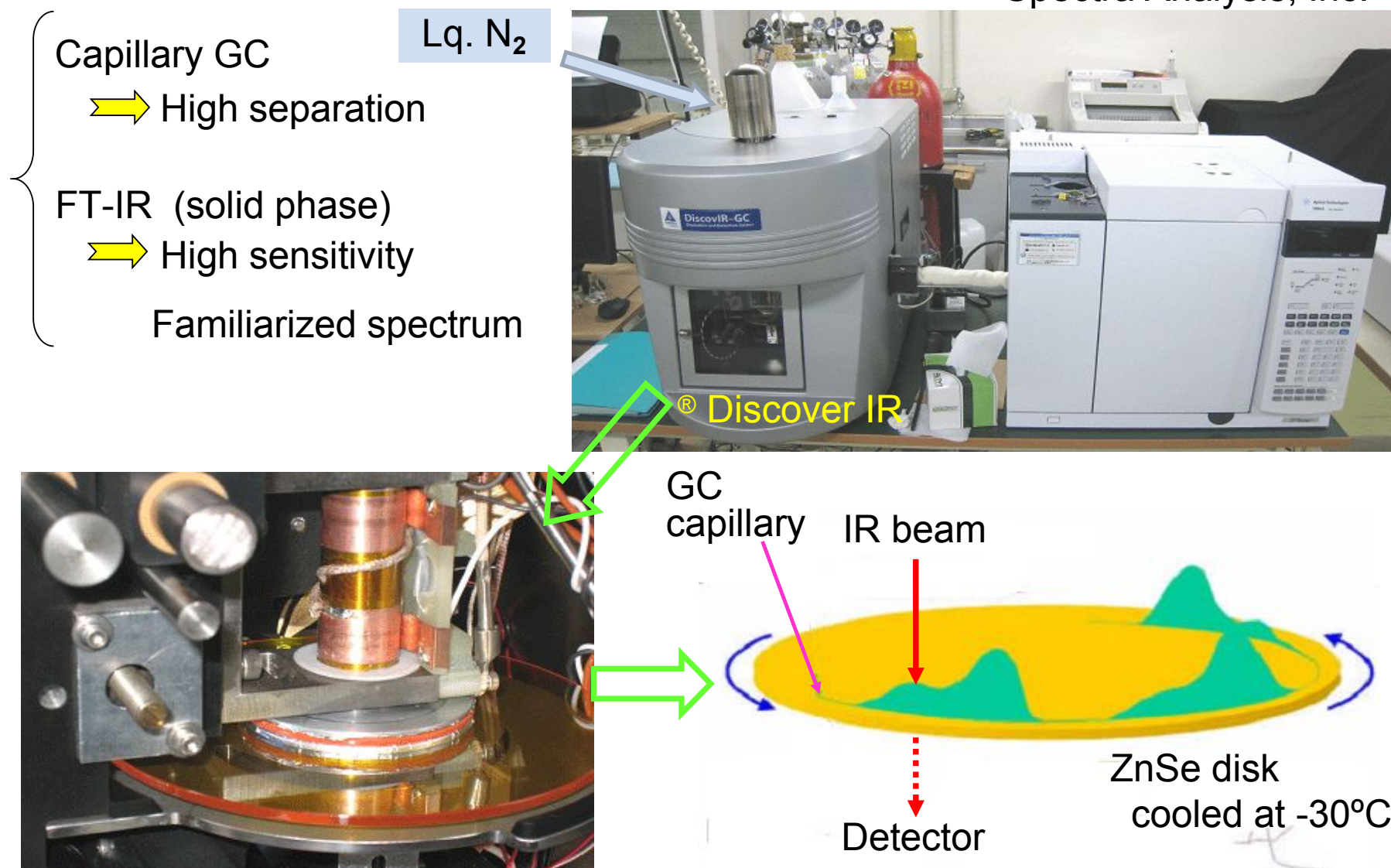


Fig. IV-1. Solid-phase Gas chromatography-Fourier transfer infrared spectrometry (GC-FT/IR)

In this chapter, the sex pheromones of five Pyraustinae species, *Palpita nigropunctalis* Bremer, *Pleuroptya sabinusalis* Walker, *P. inferior* Hampson, *Spoladea recurvalis* Fabricius, and *Eurrhyarodes accessalis* Walker have been studied by usual GC-EAD and GC-MS analyses. In addition to GC-EAD and GC-MS analyses, GC/FT-IR analysis was conducted in the experiments with pheromone extracts from *P. nigropunctalis* and *P. sabinusalis*.

Moreover, the *P. nigropunctalis* is a common pest of evergreen and deciduous Oleaceae, and mainly distributes in eastern Asia; *S. recurvalis* is a pest of many leafy vegetables (such as Spinach, Beet, etc.), and widely distributes in the tropical and subtropical regions. Therefore, the study on their sex pheromones can help to develop an efficient strategies for monitoring and control of the two pests in fields.

IV-2 Methods and Materials

IV-2-1 Insects and Pheromone Extracts

IV-2-1-1 *Palpita nigropunctalis*

The larvae were collected from *Ligustrum obtusifolium* trees in November 2011 at Tottori, Japan. Collected larvae were reared using fresh leaves of *L. obtusifolium* (14L:10D, 24-26°C). When larvae were matured, they were collected and moved into a cup with only some tissue papers until the larvae pupated. Male and female pupae were distinguished by inspecting the sexual slot on the 8th and 9th abdominal segments, and each pupa was placed separately as the same conditions as larvae. After eclosion, a non-alcohol beverage, Pocari Sweat (Ohtsuka Pharmaceuticals Co. Ltd., Tokyo) was supplied to adults at libitum. About twenty abdominal tips of 2-day old virgin females were excised at the calling time (4-7 hrs. from the start of scotophase) and soaked in hexane (10 µl/female) for 15 min to extract the pheromone components. The crude extract was used for structural analysis of pheromone components after filtration. In order to confirm the positions of the double bonds in the proposed pheromone components, after removal of hexane, crude extracted materials (8 female equivalents, FE) were treated with dimethyl disulfide (DMDS, 50 µl) and analyzed by GC-MS as the same method with *O. anastomosalis* in chapter II.

IV-2-1-2 *Pleuroptya sabinusalis*

The larvae were collected from various places in Tottori Prefecture in 2012. Collected larvae were reared using fresh leaves of *Boehmeria nivea* (16L:8D, 24-26°C). Other processing steps and methods were the same with *P. nigropunctalis*.

IV-2-1-3 *Pleuroptya inferior*

The larvae were collected from various places in Tottori Prefecture in 2014. Collected larvae were reared using fresh leaves of *Rubus parvifolius*, *R. palmatus* and *R. buergeri* (18L:6D, 24-26°C). Other processing steps and methods were the

same with *P. nigropunctalis*.

IV-2-1-4 *Spoladea recurvalis*

The larvae were collected from various places in Tottori Prefecture from late August to the middle of October, 2011-2014. Collected larvae were reared using fresh leaves of *Amaranthus blitum* (16L:8D, 24-26°C). Other processing steps and methods were the same with *P. nigropunctalis*.

IV-2-1-5 *Eurhypharodes accessalis*

The larvae were collected from various places in Tottori Prefecture in 2013. Collected larvae were reared using fresh leaves of *Achyranthes bidentata* (18L:6D, 24-26°C). Other processing steps and methods were the same with *P. nigropunctalis*.

IV-2-2 Analytical Instruments

IV-2-2-1 GC-EAD and GC-MS

The analyses with GC-EAD and GC-MS were conducted as the same with chapter III.

IV-2-2-2 GC - Fourier transfer infrared spectrometry (FT/IR)

IR spectra were recorded on an FT/IR (DiscoverIR; Spectra Analytics, Marlborough, MA, USA) instrument coupled to an Agilent Technologies 7980C GC, equipped with an HP-5 capillary column (0.25 mm ID, 30 m length, 0.25 μ m film thickness, J & W Scientific). A liquid nitrogen-cooled photo-conductive mercury-cadmium-telluride detector was used with a FT/IR resolution of 8 cm^{-1} . Compounds eluting from the capillary column were solidified on a zinc selenide disc at $-30\text{ }^{\circ}\text{C}$ and rotated at 3 mm/min. The distance between the column end and disc was 5 mm. The oven temperature for all GC analyses was set initially at $50\text{ }^{\circ}\text{C}$ for 2 min, and then programmed at $10\text{ }^{\circ}\text{C}/\text{min}$ to $160\text{ }^{\circ}\text{C}$, and $4\text{ }^{\circ}\text{C}/\text{min}$ to $220\text{ }^{\circ}\text{C}$. The flow rate of the carrier gas (He) was 1.0 ml/min, and the GC inlet temperature was $220\text{ }^{\circ}\text{C}$.

IV-2-3 Chemicals

E11-16:OH and Z11-16:OH was supplied by Shin-Etsu Chemical Co., Ltd., (Tokyo, Japan), and their acetate (E/Z11-16:OAc) or aldehyde (E/Z11-16:Ald) derivatives were obtained by the acetylation or PCC oxidation. Z3,Z6,Z9-23:H, the four geometric isomers of 11,13-hexadecadienyl acetate, and the four geometric isomers of 10,12-hexadecadienal were supplied from a stock library in our laboratory.

IV-2-4 Field Evaluation

The field experiments of five species were conducted in a forest of Tottori Prefecture from 2013. All the field experiments of other factors and data statistical analyses were conducted as the same with Chapter III.

IV-3 Results

IV-3-1 Analyses of Pheromone Extracts

IV-3-1-1 *Palpita nigropunctalis*

A crude pheromone extract of the females was examined by GC-EAD and GC-MS. The GC-EAD analysis indicated three EAG-active components (Pn-I – Pn-III) (Fig. IV-2a). The GC trace by FID indicated a mixing ratio of about 1:0.2 of Pn-I and Pn-II, (Fig. IV-2b). Mass spectra of Pn-I and Pn-II were successfully recorded (Fig. IV-2c, 2d). Ions at m/z 238 (M^+) and 220 ($[M-18]^+$) detected for Pn-I indicated a hexadecenal. While M^+ was not detected for Pn-II, ions at m/z 222 ($[M-60]^+$) and 61 ($[AcOH+1]^+$) suggested Pn-II was a hexadecenyl acetate. In addition to the two Type I components, one polyunsaturated hydrocarbon was found as Pn-IV (Rt 15.58 min, Fig. IV-2b). The mass spectrum of Pn-IV showed M^+ at m/z 318 and characteristic fragment ions at m/z 262, 108, and 79 (base peak), indicating a Z3,Z6,Z9-23:H (Fig. IV-2e).

GC-MS analyses of the extract treated with DMDS showed mass spectra of two adducts derived from Pn-I and Pn-II (Fig. IV-3). Ions at m/z 332, 215, and 117 for the adduct of Pn-I and ions at m/z 376, 259, and 117 for the adduct of Pn-II revealed their double bonds at the same 11-position.

Furthermore, the pheromone extract was examined by GC-FT/IR (Fig. IV-4a). An IR spectrum of Pn-I showed characteristic absorption at 1716 and 966 cm^{-1} , which indicated a formyl group and *E* configuration of the double bond, respectively (Fig. IV-4b). In the case of Pn-II, absorption at 1745 and 968 cm^{-1} indicated an ester carbonyl and *E* configuration (Fig. IV-4c). As facilitated by comparison with authentic standards in infrared band chromatograms recorded with GC-FT/IR data of a pheromone extract (Fig. IV-5, a), synthetic C16-chain compounds with the (*E*)-11-double bond (Fig. IV-5, b), and those with the (*Z*)-11-double bond (Fig. IV-5, c). Above all, the Comp. Pn-I, Pn-II and Pn-IV were concluded E11-16:Ald, E11-16:OAc and Z3,Z6,Z9-23:H, respectively, and Pn-III was speculated as E11-16:OH.

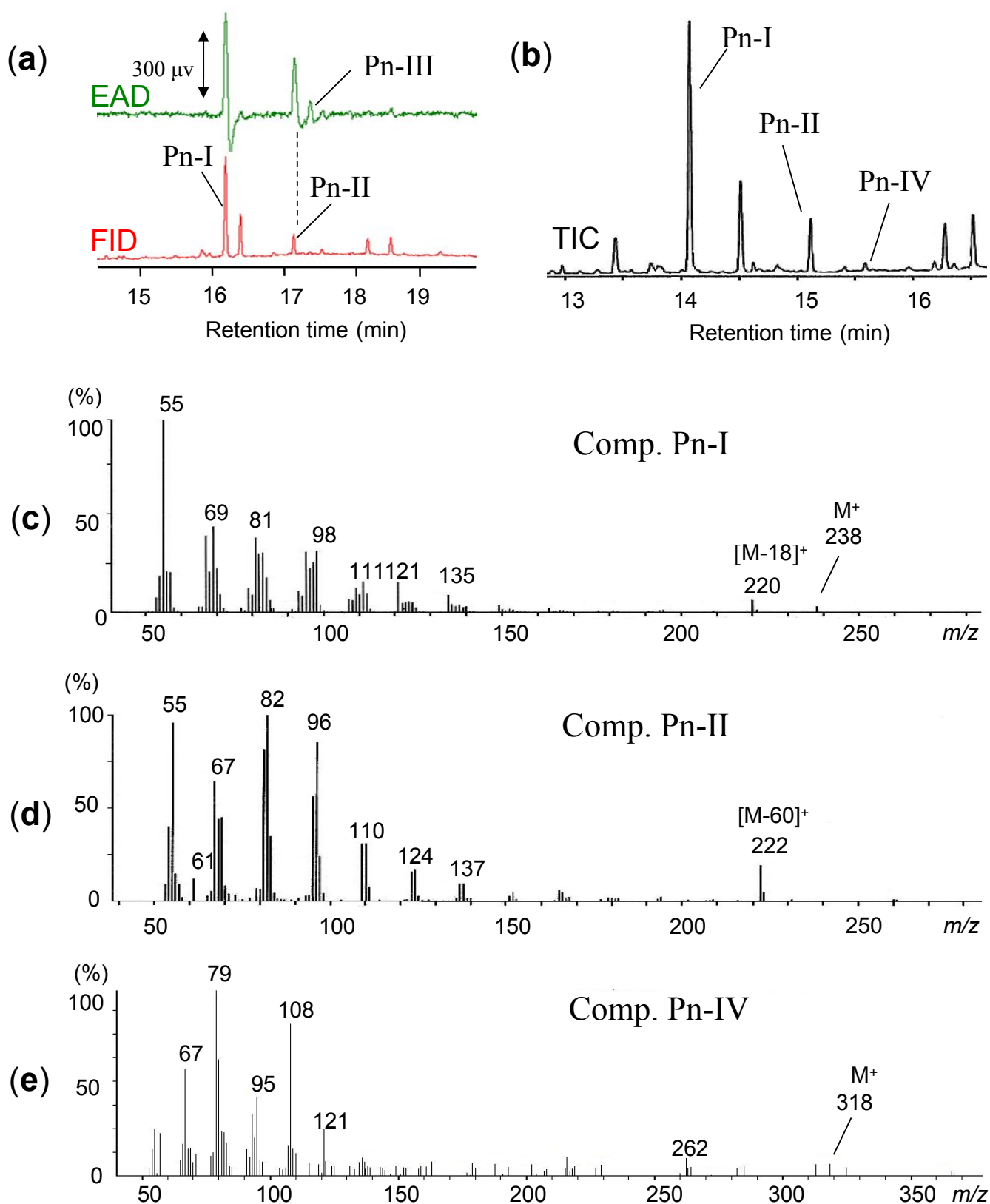


Fig. IV-2. Analyses of the sex pheromone of *Palpita nigropunctalis* females by GC-EAD and GC/MS. **(a)** Chromatograms of the pheromone extract (1 FE) recorded by EAD and FID; **(b)** TIC of the pheromone extract (1 FE); **(c)** a mass spectrum of Comp. Pn-I (Rt 14.08 min, E11-16:Ald); **(d)** a mass spectrum of Comp. Pn-II (Rt 15.12 min, E11-16:OAc) and **(e)** a mass spectrum of Comp. Pn-IV (Rt 15.58 min, Z3,Z6,Z9-23:H).

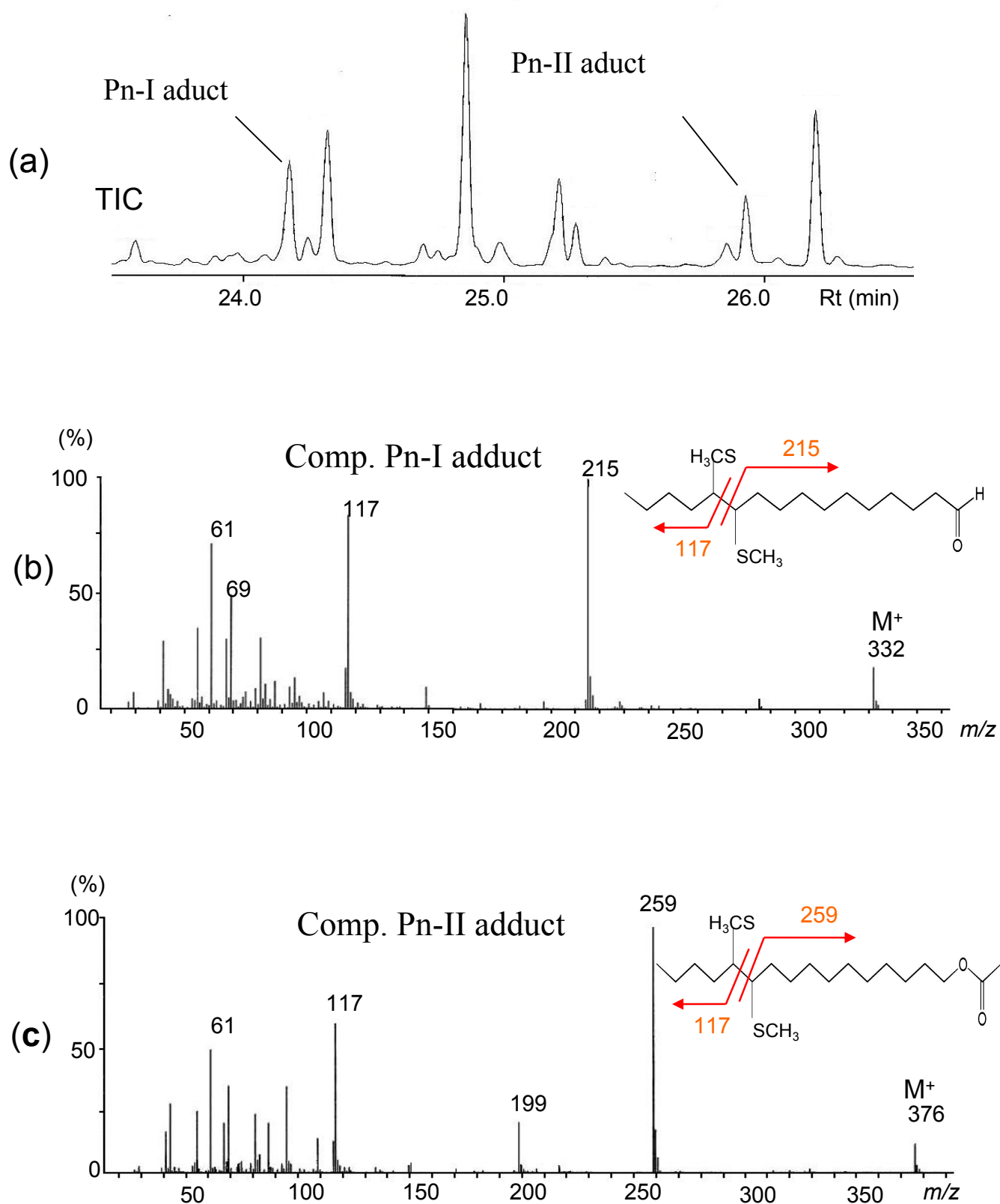


Fig. IV-3. GC-MS analysis of a pheromone extract of *Palpita nigropunctalis* females (2 FE) treated with dimethyl disulfide (DMDS); (a) TIC; (b) a mass spectrum of a DMDS-adduct of Comp. Pn-I and (c) a mass spectrum of a DMDS-adduct of Comp. Pn-II.

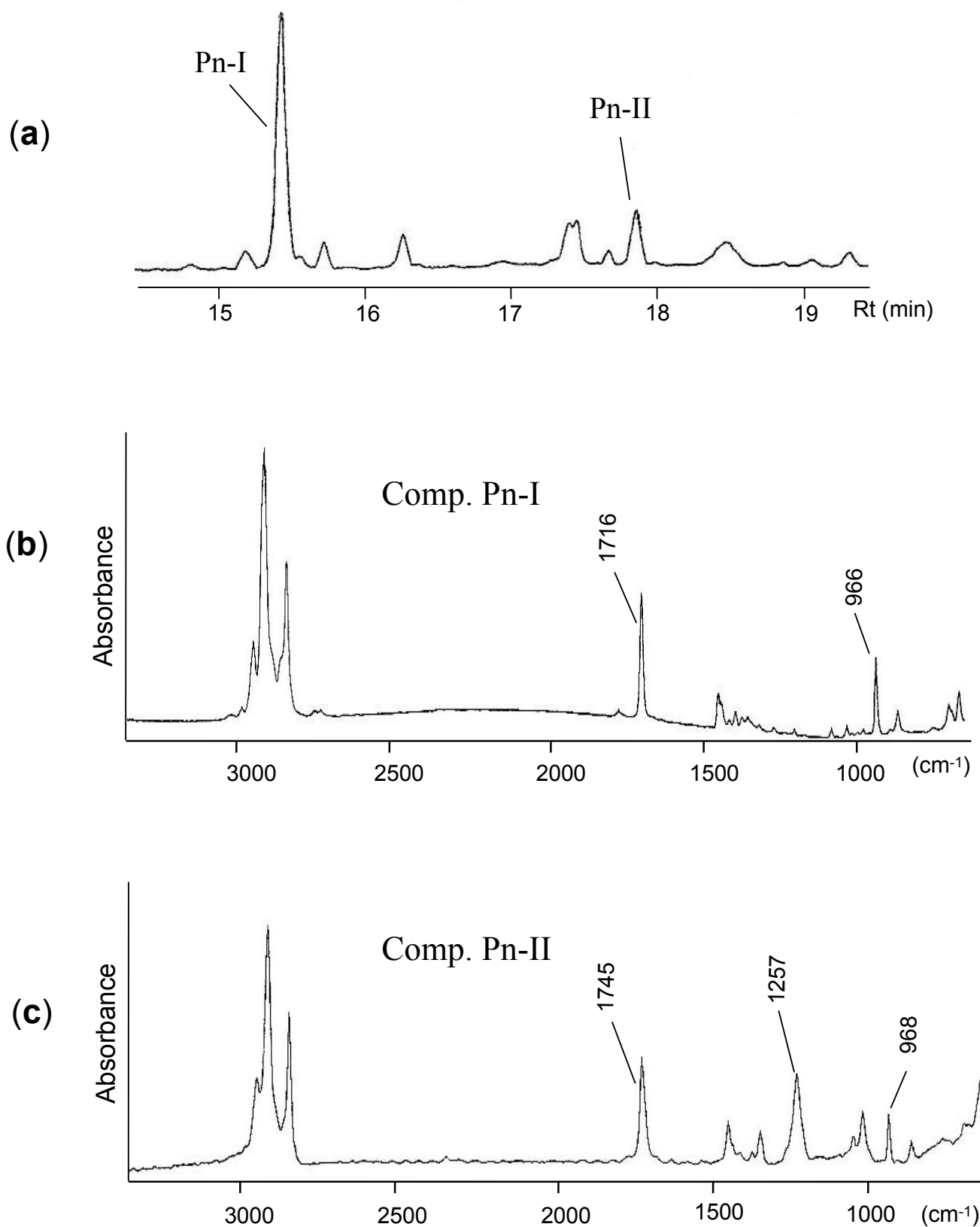


Fig. IV-4. Infrared spectra, obtained by gas chromatography/Fourier transform infrared spectroscopy of pheromone components of *Palpita nigropunctalis* females; (a) Infrared spectroscopy absorption of pheromone extracts (6 FE); (b) Comp. Pn-I (E11-16:Ald); (c) Comp. Pn-II (E11-16:OAc).

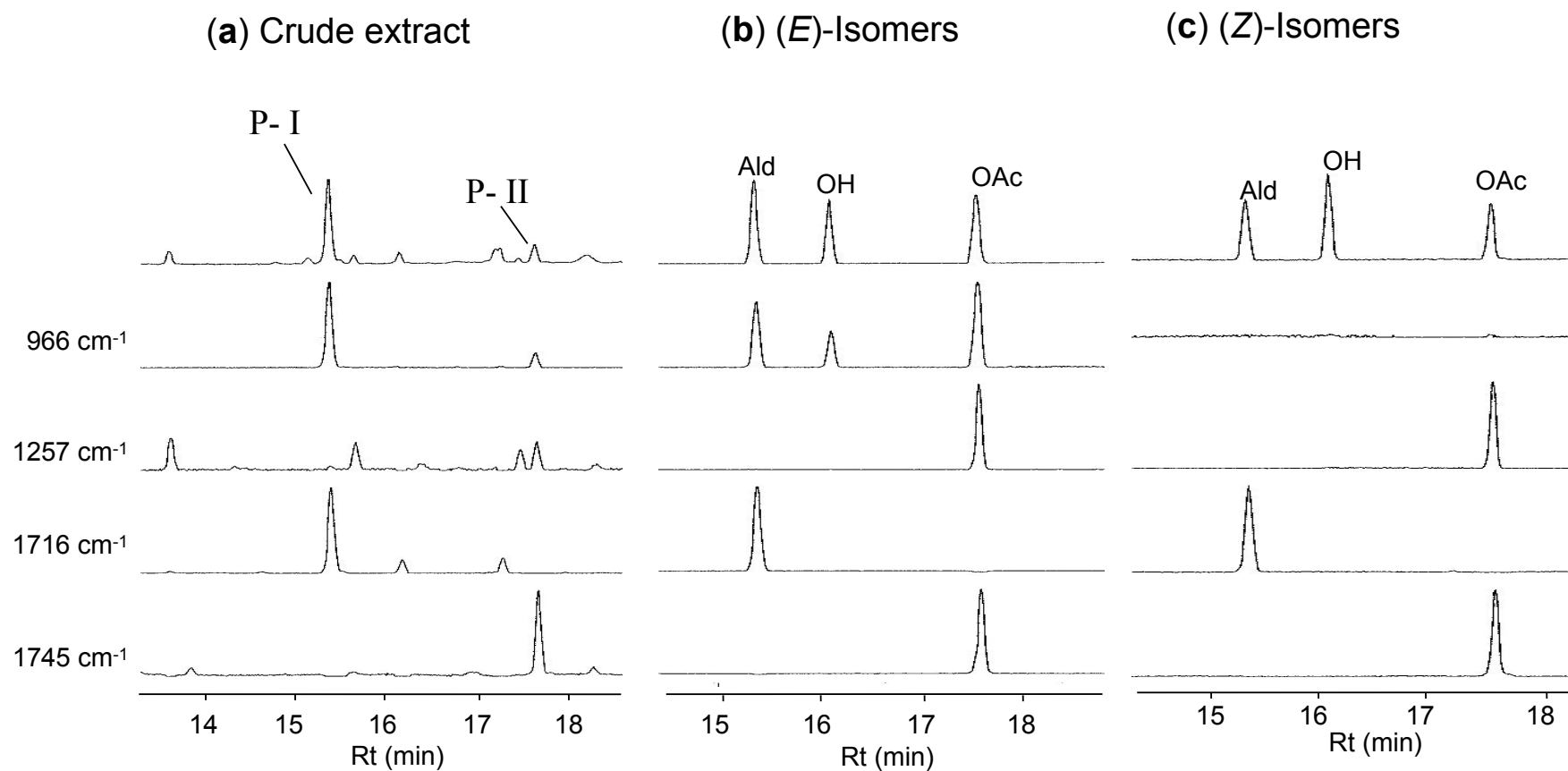


Fig. IV-5. Infrared band chromatograms at 966, 1257, 1716 and 1745 cm⁻¹ from gas chromatography/Fourier Transform infrared spectroscopy analyses of pheromone extracts of *Palpita nigropunctalis* and synthetic compounds: (a) *P. nigropunctalis* (20 female equivalents); (b) E11-16:Ald, 40 ng and (c) E11-16:OAc, 40 ng.

IV-3-1-2 *Pleuroptya sabinusalis*

The GC-EAD analysis of a crude pheromone extract (0.5 FE) from the *P. sabinusalis* females indicated two EAG-active components (Ps-I and Ps-II) (Fig. IV-6a). GC-MS analyses on pheromone gland extracts (0.5 FE) indicated the presence of the two pheromone components Ps-I and Ps-II with a mixing ratio of 1:1.6 (Fig. IV-6b). Comps. Ps-I and Ps-II showed the same mass spectra (Fig. IV-6c, 6d), ions m/z at 238 (M^+) and 220 ($[M-18]^+$) detected for Ps-I and Ps-II indicated the structure of a hexadecenal. GC-MS analysis of the extract treated with DMDS showed mass spectra of the adducts derived from Ps-I and Ps-II (Fig. IV-7). Ions at m/z 332, 215, and 117 for the adducts of Ps-I and Ps-II revealed their double bonds at the same 11-position.

Furthermore, the pheromone extract was examined by GC-FT/IR (Fig. IV-8a). An IR spectrum of Ps-I showed characteristic absorption at 1715 and 966 cm^{-1} , which indicated a formyl group and *E* configuration of the double bond, respectively (Fig. IV-8b). In the case of Ps-II, absorption at 1715 indicated a formyl group, and the lack of absorption around 960 cm^{-1} indicated the *Z* configuration of the double bond (Fig. IV-8c). Therefore, Comps. Ps-I and Ps-I were concluded E11-16:Ald and Z11-16:Ald, respectively.

IV-3-1-3 *Pleuroptya inferior*

The GC-EAD analysis a crude pheromone extract of the females indicated three EAG-active components (Fig. IV-9a). But just the mass spectra of Comp. Pi-I and Pi-II were successfully recorded, and a GC trace by FID indicated a mixing ratio about 1:0.7 of Pi-I and Pi-II (Fig. IV-9b). Comp. Pi-I shows a same mass spectra with Pn-I (Fig. IV-9c). In the case of Comp. Pi-II, it shows a similar mass spectra with 10,12-hexadecadienal (Ando et al., 1988) (Fig. IV-9d). Finally, Comps. Pi-I and Pi-II were assigned as E11-16:Ald and E10,E12-16:Ald by comparisons with Rts of synthetic standards, respectively.

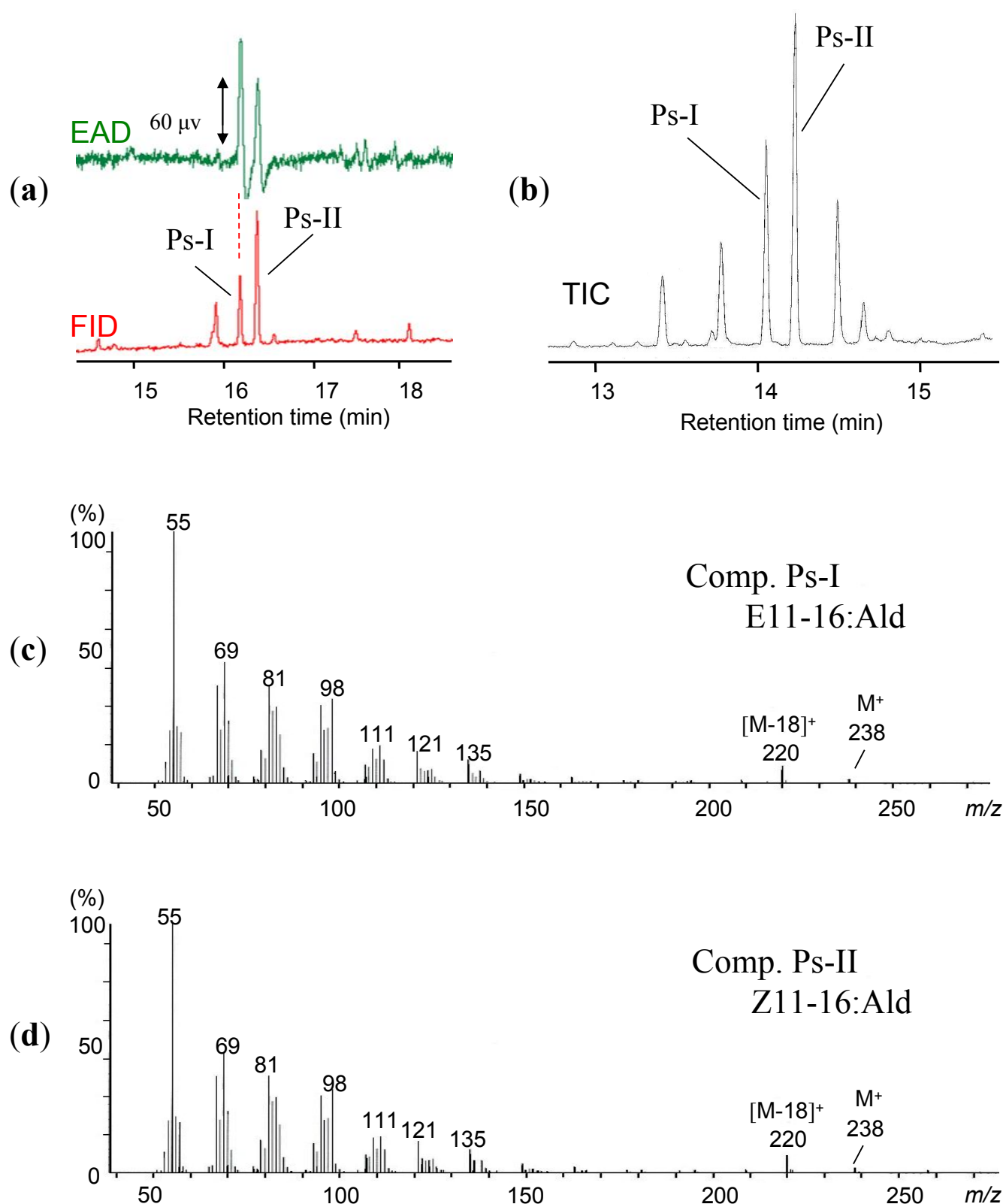


Fig. IV-6. Analyses of the sex pheromone of *Pleuroptya sabinusalis* females by GC-EAD and GC/MS. (a) Chromatograms of the pheromone extract (0.5 FE) recorded by EAD and FID; (b) TIC of the pheromone extract (0.5 FE); (c) a mass spectrum of Comp. Ps-I (Rt 14.07 min, E11-16:Ald) and (d) a mass spectrum of Comp. Ps-II (Rt 14.25 min, Z11-16:Ald).

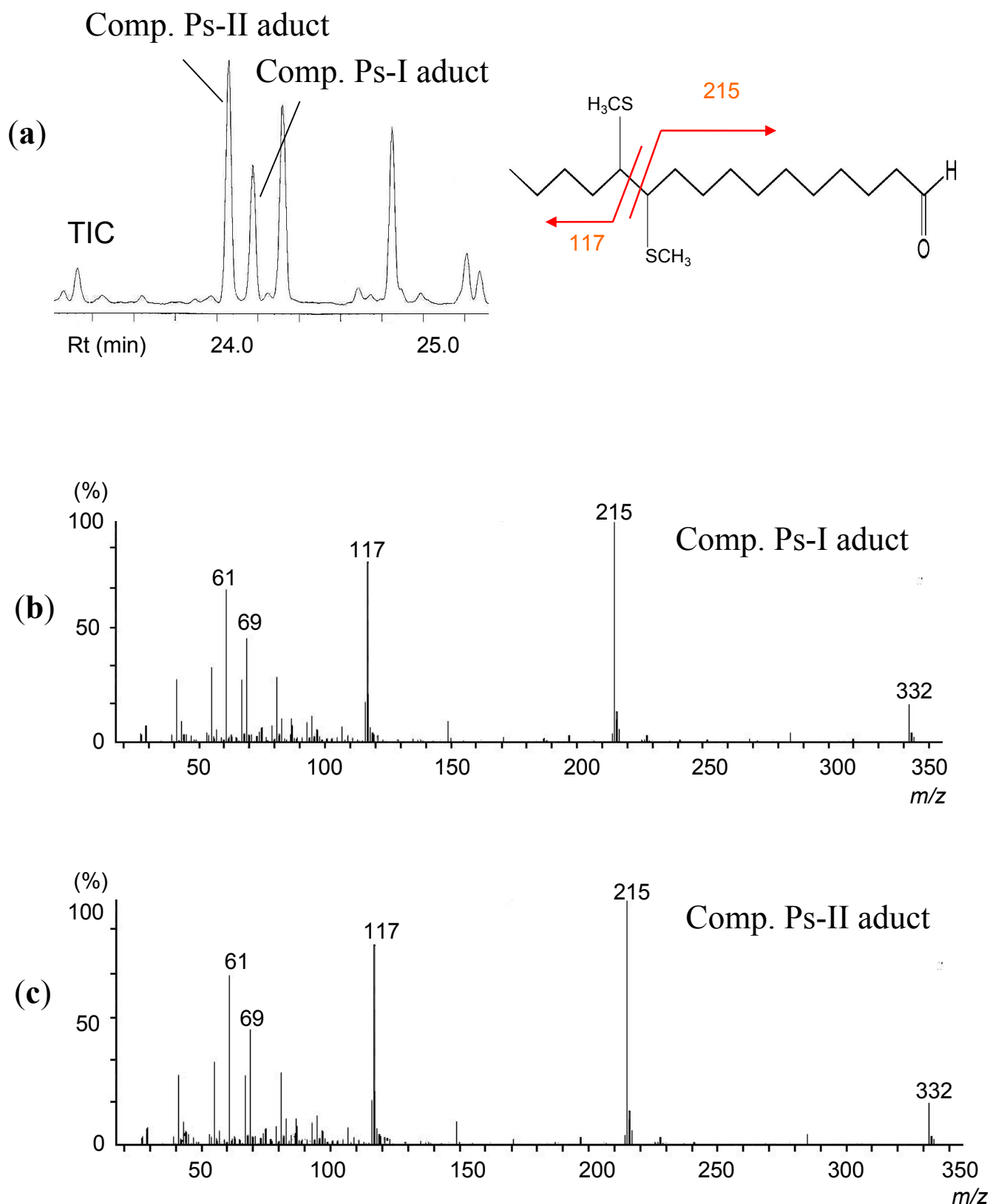


Fig. IV-7. GC-MS analysis of a pheromone extract of *Pleuroptya sabinusalis* females (3 FE) treated with dimethyl disulfide (DMDS); (a) TIC; (b) a mass spectrum of a DMDS-adduct of Comp. Ps-I and (c) a mass spectrum of a DMDS-adduct of Comp. Ps-II.

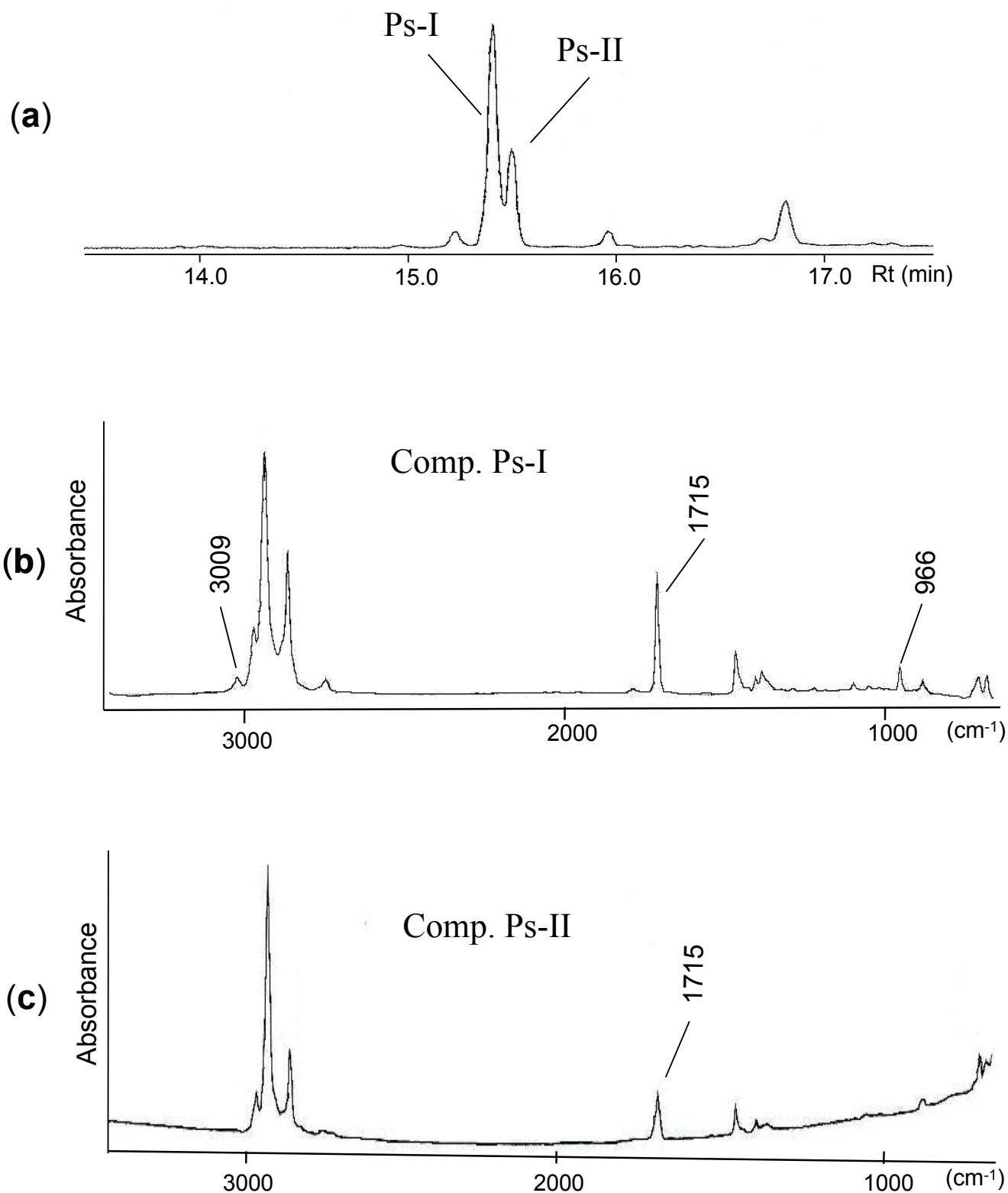


Fig. IV-8. Infrared spectra, obtained by gas chromatography/Fourier transform infrared spectroscopy of pheromone components of *Pleuroptya sabinusalis* females; (a) Infrared spectroscopy absorption of pheromone extracts (15 FE); (b) Comp. Ps-I (15.42 min, E11-16:Ald); (c) Comp. Ps-II (15.54 min, Z11-16:Ald).

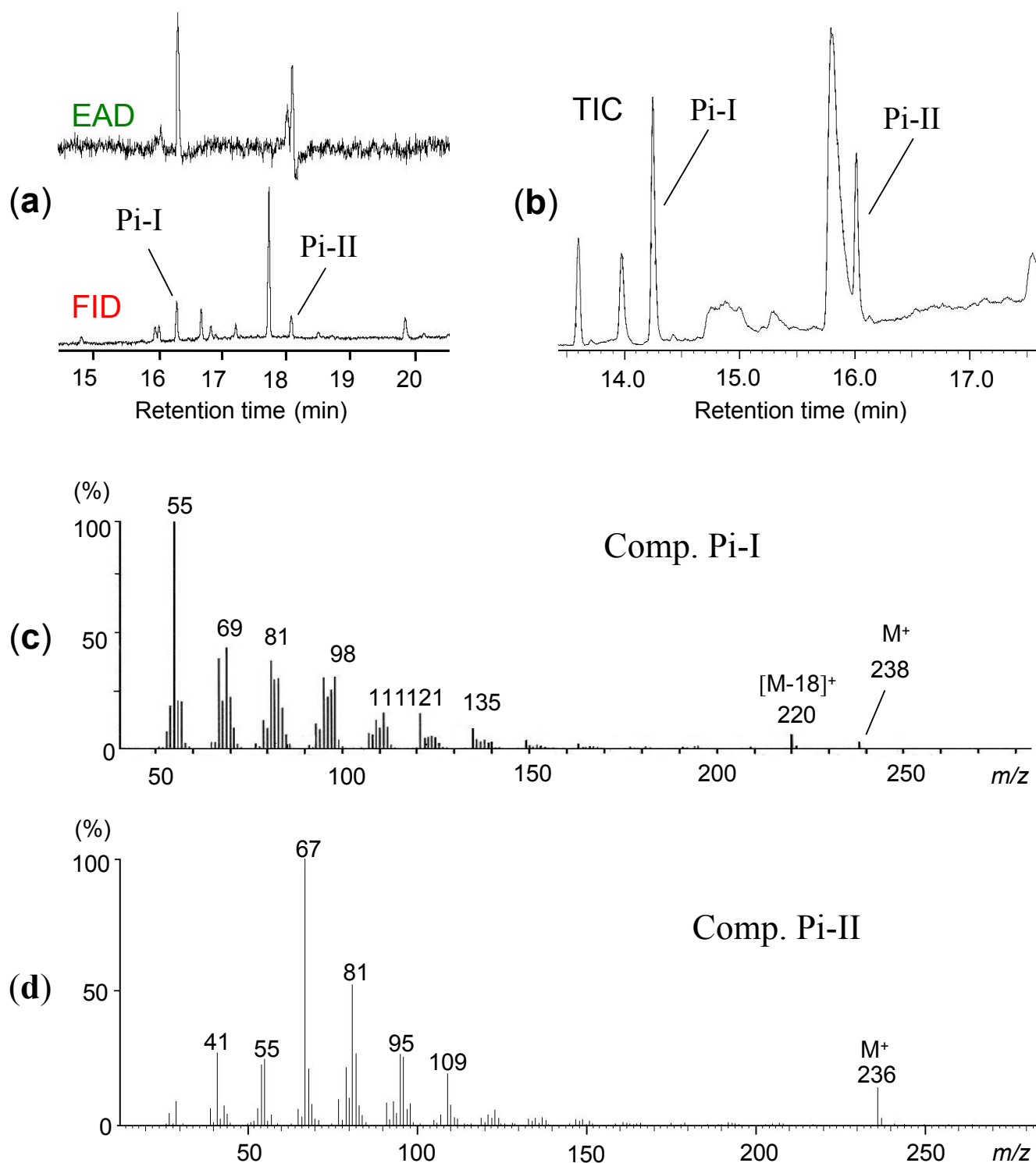


Fig. IV-9. Analyses of the sex pheromone of *Pleuroptya inferior* females by GC-EAD and GC/MS. (a) Chromatograms of the pheromone extract (0.5 FE) recorded by EAD and FID; (b) TIC of the pheromone extract (1 FE); (c) a mass spectrum of Comp. Pi-I (Rt 14.25 min, E11-16:Ald); (d) a mass spectrum of Comp. Pi-II (Rt 16.02 min, 10,12-16:Ald)

IV-3-1-4 *Spoladea recurvalis*

A crude pheromone extract of the females was examined by GC-EAD and GC-MS. The GC-EAD analyses indicated three EAG-active components (Sr-I – Sr-III) (Fig. IV-10a). GC-MS analyses indicated the presence of the two pheromone components Sr-I and Sr-II with a mixing ratio about 1:1.3 (Fig. IV-10b). The mass spectra of Sr-I and Sr-II are same with those of *P. nigropunctalis* (Fig. IV-10c, 10d). Thus, Comps. Sr-I and Sr-II were concluded E11-16:Ald and E11-16:OAc, respectively, and Sr-III was speculated as E11-16:OH.

IV-3-1-5 *Eurhypharodes accessalis*

A crude pheromone extract of the females was examined by GC-EAD (1 FE) and GC-MS (2 FE). The GC-EAD analysis indicated six EAG-active components (Fig. IV-11a). But just the mass spectra of Comps. Ea-I and Ea-II were successfully recorded, and a GC trace by FID indicated a mixing ratio of about 1:0.3 of Ea-I and Ea-II (Fig. IV-11b). Comp. Ea-I shows the same mass spectra with Pn-II (Fig. IV-11c). In the case of Comp. Ea-II, it shows a similar mass spectra with Hb-I (Fig. III-3c, Fig. IV-11d). Finally, the Comps. Ea-I and Ea-II were assigned as E11-16:OAc and Z11,E13-16:OAc by comparisons with Rts of synthetic standards, respectively.

IV-3-2 Male attraction by synthetic lures

The field tests were firstly conducted with several blends that included synthetic compounds E11-16:Ald, OAc and OH during September of 2012 (Experiment Sr-A). As shown in Table IV-1, it suggested that no *P. nigropunctalis* males were captured by the lures, only two or three *S. recurvalis* males were captured by the lure with the ratio 1:1 or 83:17 of E11-16:Ald and OAc. Then, the attractiveness of E11-16:Ald and OAc with a ratio of 9:1 mixed with two different amounts of Z3,Z6,Z9-23:H (25 and 250 µg) were examined (Experiment Sr-B). As shown in Table IV-2, a number of *S. recurvalis* males were captured by the ternary blends, it suggested that Z3,Z6,Z9-23:H might play a synergistic role in attracting *S. recurvalis* males. But still no *P. nigropunctalis* males were captured as before in

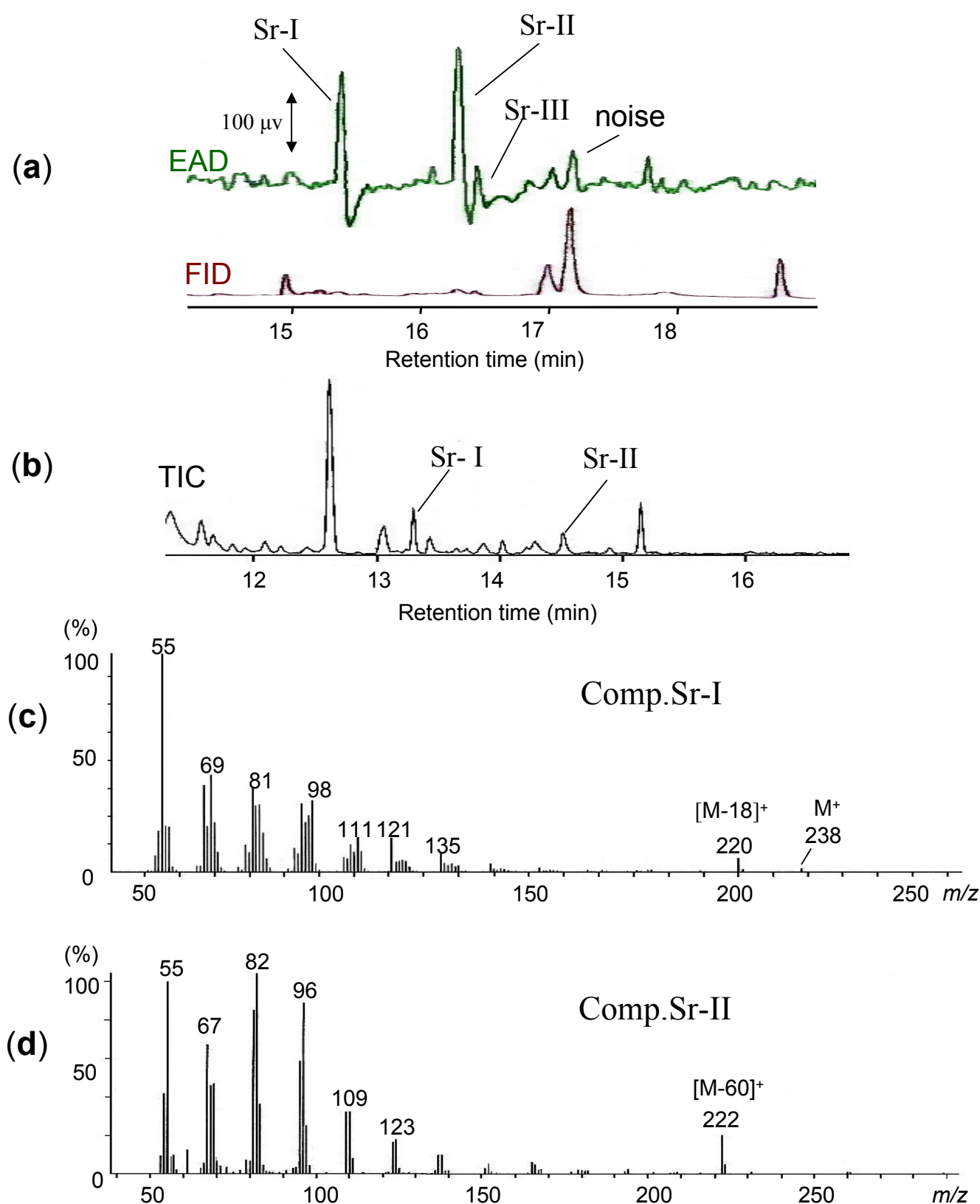


Fig. IV-10. Analyses of the sex pheromone of *Spoladea recurvali* females by GC-EAD and GC/MS. **(a)** Chromatograms of the pheromone extract (0.5 FE) recorded by EAD and FID; **(b)** TIC of the pheromone extract (1 FE); **(c)** a mass spectrum of Comp. Sr-I (Rt 13.41 min, E11-16:Ald) and **(d)** a mass spectrum of Comp. Sr-II (Rt 14.51 min, E11-16:OAc).

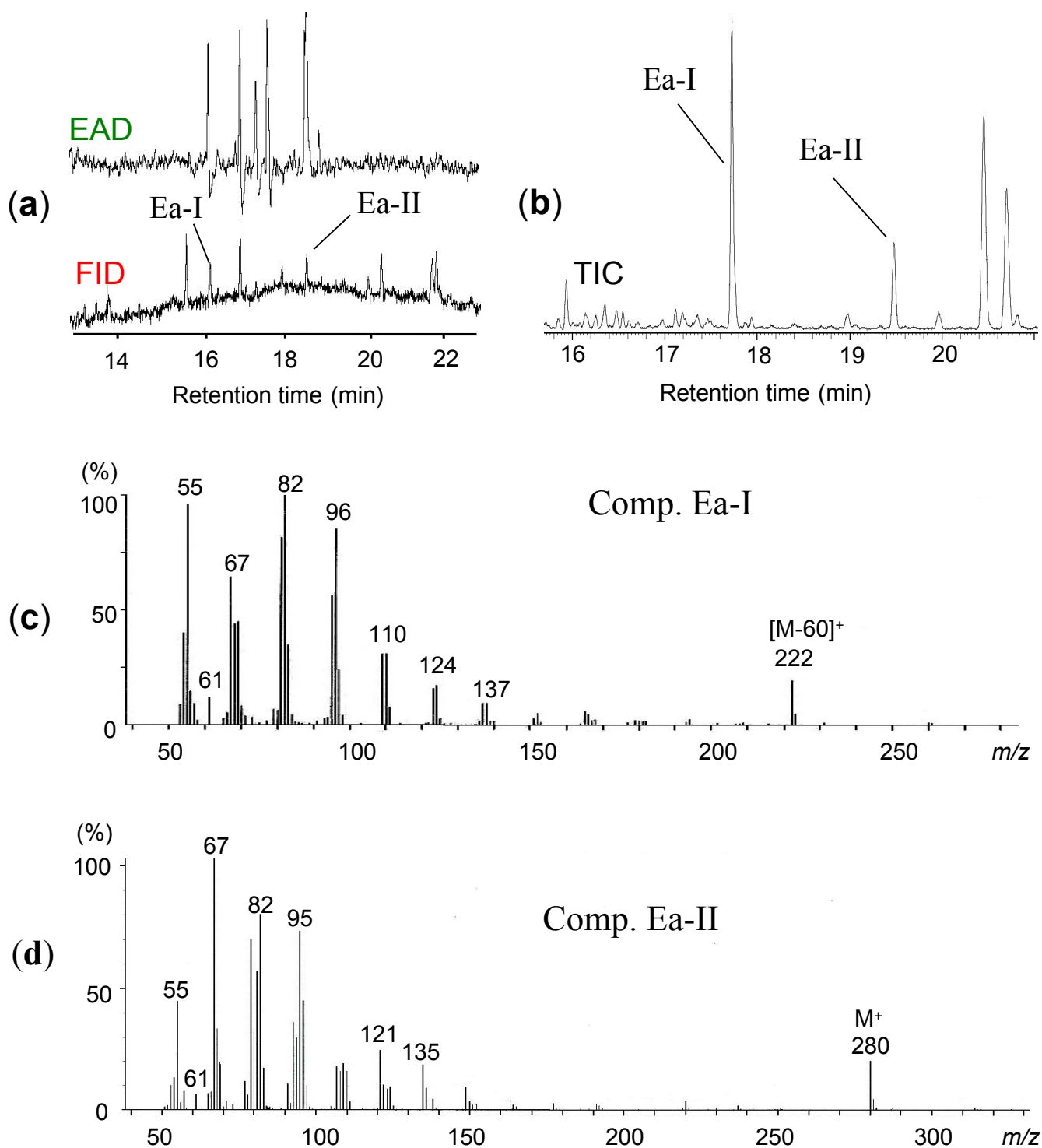


Fig. IV-11. Analyses of the sex pheromone of *Eurrhynchos accessalis* females by GC-EAD (DB-23 column) and GC/MS (DB-225ms column).
 (a) Chromatograms of the pheromone extract (1 FE) recorded by EAD and FID; (b) TIC of the pheromone extract (2 FE); (c) a mass spectrum of Comp. Ea-I (Rt 17.73 min, E11-16:OAc); (d) a mass spectrum of Comp. Ea-II (Rt 19.48 min, Z11,E13-16:OAc).

experiment Sr-B. At last, the synergistic effects of several polyunsaturated hydrocarbons structurally related to Z3,Z6,Z9-23:H were examined in Experiment Sr-C, and traps baited with virgin female moths were included in this experiment. The ternary blends contained Z3,Z6,Z9-23:H captured more males than other blends, and two kinds of does of Z3,Z6,Z9-23:H are no significantly difference. However, all the traps baited with the synthetic lure were less effective in attracting males than those with virgin females (Table IV-3).

The preliminary field tests of synthetic pheromones of other four species was unfortunately showed no good male attraction as well (data not shown), further field evaluation will be conducted in future.

Table IV-1 Catches of *Spoladea recurvali* and *Palpita nigropunctalis* males in Tottori Prefecture in traps baited with synthetic compounds. (3 replicates; 12 – 29 September 2012) (Experiment Sr-A)

Lure components (mg/septum)			Total capture (males)	
E11-16:Ald	E11-16:OAc	E11-16:OH	<i>S. recurvali</i>	<i>P. nigropunctalis</i>
1.0	0	0	0	0
0.9	0.1	0	0	0
0.6	0.4	0	0	0
0.5	0.5	0	2	0
0.83	0.17	0	3	0
0.83	0.17	0.1	0	0
0	0	0	0	0

Table IV-2 Catches of *Spoladea recurvali* and *Palpita nigropunctalis* males in Tottori Prefecture in traps baited with synthetic compounds. (3 replicates; 1 – 15 August 2013) (Experiment Sr-B)

Lure components (µg/septum)			Total capture (males)	
E11-16:Ald	E11-16:OAc	Z3,Z6,Z9-23:H	<i>S. recurvali</i>	<i>P. nigropunctalis</i>
450	50	0	0	0
450	50	25	28	0
450	50	250	15	0
0	0	0	0	0

Table IV-3 Attraction of *Spoladea recurvali* males in Tottori Prefecture (Japan) in traps baited with synthetic compounds by E11-16:Ald and E11-16:OAc (9:1) mixed with an unsaturated hydrocarbon. (Experiment Sr-C)

Lure (μg/rubber septum) ^a	Captured males	
E11-16:Ald and E11-16:OAc (450:50)	/trap/week ^b	Total
+ none	3.7 ± 1.1 de	11
+ Z3,Z6,Z9-23:H (25)	13.3 ± 0.5 c	40
+ Z3,Z6,Z9-23:H (250)	9.7 ± 0.9 cd	29
+ Z3,Z6,Z9,Z12,Z15-23:H (25)	4.0 ± 0.8 de	12
+ Z3,Z6,Z9,Z12,Z15-25:H (25)	6.0 ± 0.6 cde	18
Virgin female×1	35 ± 6.0 b	105
Virgin female×2	71 ± 18.9 a	213
control	0	0

^a Tested with three traps of each lure from September 21 to October 10, 2013.

^b Mean ± SE. Values within each test followed by a different letter are significantly different at P<0.05 by Tukey-Kramer Test.

IV-4 Discussion

Solid-phase GC-FT/IR has been utilized for structure determination of male-produced sex pheromones of Heteroptera, including for methyl 4,8,12-trimethylpentadecanoate in *Edessa meditabunda* (Zarbin et al., 2012) and 5,9,17-trimethylhenicosane in *Phthia picta* (Soldi et al., 2012). The first use of solid-phase GC-FT/IR for the identification of lepidopteran pheromones in *Monema flavescens* (Shibasaki et al., 2013). We analyzed sex pheromones of *P. nigropunctalis* and *P. sabinusalis* by GC-FT/IR here and obtained a reliable data of natural sex pheromone compounds, especially the geometry of the double bond(s). In the GC-FT/IR analysis of the natural pheromone, the band chromatogram around 960 cm^{-1} was useful, based on the characteristic absorption, the *E* configuration of the monoenyl Comps. Pn-I, Pn-II and Ps-I to be assigned (Fig. IV-4b,4c, Fig. IV-8b). The IR spectra of the absorptions around 1715 and 1740 cm^{-1} also showed a reliable information about the functional group of an carbonyl group and ester group, respectively (Fig. IV-4, Fig. IV-8).

As usual, a pheromone extract which contains about 40 ng pheromone component was required for a efficient IR spectrum. For other four species, because of a low level of pheromone components in the pheromone glands, the GC-FT/IR analyses of this species were not measured. GC-FT/IR analysis could be an important tool for obtaining structural information unable to be defined by GC-MS. It is also necessary to analyze synthetic pheromones of many lepidopteran species by GC-FT/IR to obtain a database to aid in the identification of natural sex pheromone compounds.

Our results suggested that *P. nigropunctalis* and *S. recurvalis* share the same pheromone components as E11-16:Ald, OAc and OH, which belong to the Type I pheromones. Another Type II component Z3,Z6,Z9-23:H was also found in pheromone extracts of *P. nigropunctalis*. In followed field test, none *P. nigropunctalis* males were attracted by the synthetic pheromone components suggested that some unknown pheromone components should be overlooked in preliminary analysis, or

the sexual communication of this species follow a strict ratio of pheromone components. Although, addition of Z3,Z6,Z9-23:H increased the *S. recurvalis* males captured, the attractiveness of *S. recurvalis* was much weaker than virgin female moths. It is suggested two points, one is that it is possible that Z3,Z6,Z9-23:H exist in pheromone glands with a low content; the other is that some unknown pheromone components of *S. recurvalis* may be overlooked. The unsuccessful male attraction in the preliminary field tests of other four species suggested that it is necessary to reexamine the female sex pheromone of all the five species.

On the other hand, it is clearly to see that the five species using different pheromone components, or share the same pheromone components with different ratio in their sexual communication system (Table IV-4). Although field tests of synthetic pheromones of these five species was unfortunately showed no good male attraction, results of the identification indicate diversity of pheromone structures produced by Pyraustinae species.

Table IV-4 Sex Pheromones secreted by five Pyraustinae species inhabit Japan

Sex pheromone components	Ratio (* trace)				
	<i>Palpita nigopunctalis</i>	<i>Pleuroptya sabinusalis</i>	<i>Pleuroptya inferior</i>	<i>Spoladea recurvalis</i>	<i>Eurrhyarodes accessalis</i>
E11-16:Ald	1	1	1	1	
E11-16:OAc	0.2			1.3	1
E11-16:OH	*			*	
Z11-16:Ald		1.6			
Z11,E13-16:OAc					0.3
E10,E12-16:Ald			0.7		
Z3,Z6,Z9-23:H	*				

CHAPTER V: GENERAL DISCUSSION

Up to now, sex pheromones of more than 30 species have been identified in subfamily Pyraustinae (Ando 2014; El-Sayed 2014). Most of the pheromone components are monoene or diene and their derivatives with a terminal functional group and 14-, 16-, or 18-carbon chain, and some of them are always shared by different species. For example, E10,E12-16:Ald was used as the major component in more than five species (*Agathodes ostentalis*, *Fumibotys fumalis*, *Maruca vitrata*, etc.). E11-16:Ald and its derivatives were used as the major components in more than six species. Nevertheless, after a long period of evolution, every species has a strict sexual communication system that prevents them to mate with different species, multiple components with different match or ratio of various common components should be critical for species specificity and reproductive isolation in Pyraustinae.

Compared with lepidopteran insects in highly evolved groups, such as Geometridae, Arctiidae, Lymantriidae, and Noctuidae, that produced Type II pheromones; or some species secrete miscellaneous pheromone components that include at least one chiral center (Fig. I-2), the chemical structure of most major pheromone components in Pyraustinae species is not difficult to clarify just by usual instrument analyses. But in field attraction of many Pyraustinae species, the communication system seems convoluted, particularly for multiple components, most minor components may play a key role in male attraction. According to our data of nine species, the major pheromone components were easily identified, but due to a very low level in the pheromone glands for most species, various minor components were not always detected successfully by GC-MS. A case study of *E. accessalis*, six EAG-active components were recorded by GC-EAD analysis, but only two components (Ea-I and Ea-II) were successfully detected by GC-MS (Fig. IV-6a). On the other hand, some minor components were easily overlooked because of their very weak responses elicited from male antennae. As a potential minor component of *P. nigropunctalis*, Z3,Z6,Z9-23:H was detected in a GC-MS analysis of the pheromone

extracts, but it elicited no responses from male antennae (Fig. IV-6a).

For a particular species, the blend components of sex pheromone usually belong to only one of two general structural classes (Type I and Type II). However, recent studies have revealed that some species use a combination of Type I and Type II pheromone compounds for mate attraction. In addition to *O. anastomosalis*, *L. orbonalis* and *P. nigropunctalis*, Z3,Z6,Z9-23:H has been identified in pheromone gland extracts of other four Crambidae species, *Neoleucinodes elegantalis* (Cabrera et al., 2001), *Conogethes pluto* (El-Sayed et al. 2013), *Conogethes punctiferalis* (Xiao et al., 2012), and *Deanolis sublimbalis* (Gibb et al., 2007). Furthermore, other two pentaenes Z3,Z6,Z9,Z12,Z15-23:H and Z3,Z6,Z9,Z12,Z15-25:H have been found in five other Pyralidae species, *Amyelois transitella* (Leal et al., 2005; Kanno et al., 2010; Kuenen et al., 2010), *Pyralis farinalis* (Leal et al., 2005; Kuenen et al., 2010), *Dioryctria abietella* (Löfstedt et al., 2012), *Dioryctria abietivorella* (Millar et al., 2005; Strong et al., 2008) and *Dioryctria amatella* (Miller et al., 2010) (Table V-1). While the Type II pheromones have been identified from insects in rather limited Pyraustinae species and play an important role as a synergist in male attraction, after the first identification of Z3,Z6,Z9-23:H from *N. elegantalis* by Cabrera et al. (2001), the discovery of moth species that use both Type I and Type II pheromone should be significant developments of the past fifteen years in lepidopteran pheromone research. In the case of *P. nigropunctalis*, the addition of Z3,Z6,Z9-23:H to lures does not enhance trap efficiency in field attraction. This further indicated the complexity of sexual communication of this species. Although except *O. anastomosalis*, *L. orbonalis* and *P. nigropunctalis*, we have not found the triene in pheromone glands of other six Pyraustinae species yet, it is possible that some polyunsaturated hydrocarbons may exist in their pheromone glands with a very low level content.

Another point worth noting is that the polyunsaturated hydrocarbons were easily overlooked in preliminary analysis because of their low content and the weak responses eliciting from antennae in GC-EAD analyses. In the pheromone gland

Table V-1 Statistic data of insect sex pheromone in superfamily Pyraloidea.

Family	Species	Sex pheromone	Reference
Pyralidae	<i>Pyralis farinalis</i> Linnaeus	Z11,Z13-16:Ald	Landolt, 1982
	Meal moth	Z3,Z6,Z9,Z12,Z15-23:H	Leal, 2005
		Z3,Z6,Z9,Z12,Z15-25:H	Kuenen, 2010
	<i>Amyelois transitella</i> Walker	Z11,Z13-16:Ald, OH	Coffelt, 1979
	Navel orangeworm	Z3,Z6,Z9,Z12,Z15-23:H	Leal, 2005
		Z3,Z6,Z9,Z12,Z15-25:H	Kuenen, 2010
	<i>Dioryctria abietella</i> Denis & Schiffmüller	Z9,E11-14:OAc	Löfstedt, 2012
	Spruce coneworm	Z3,Z6,Z9,Z12,Z15-25:H	
	<i>Dioryctria abietivorella</i> Grote	Z9,E11-14:OAc	Millar, 2005
	Fir coneworm	Z3,Z6,Z9,Z12,Z15-25:H	Strong, 2008
	<i>Dioryctria amatella</i> Hulst	Z11-16:OAc	Hanula, 1984
	Coneworm	Z3,Z6,Z9,Z12,Z15-25:H	Meyer, 1986 Miller, 2010
Crambidae	<i>Neoleucinodes elegantalis</i> Guenée	E11-16:OH	Cabrera, 2001
	Tomato fruit borer	Z3,Z6,Z9-23:H	
	<i>Deanolis sublimbalis</i> Snellen	Z11-16:Ald	Gibb, 2007
	Red banded mango	Z3,Z6,Z9-23:H	
	<i>Conogethes punctiferalis</i> Guenée	E10-16:Ald,	Konno, 1982
	Yellow Peach Moth	Z10-16:Ald Z3,Z6,Z9-23:H	Xiao, 2012
	<i>Conogethes pluto</i> Butler	E10-16:Ald,OH E10,E12-16:Ald Z3,Z6,Z9-23:H	El-Sayed, 2013
	<i>Omphisa anastomosalis</i> Guenée	E10,E14-16:Ald	Wakamura,2010
	Sweetpotato vine borer	Z3,Z6,Z9-23:H	Yan, 2014

extract of *O. anastomosalis*, the content of Z3,Z6,Z9-23:H and E10,E14-16:Ald (the major component) was approximately 100:6 (Fig. II-2a), similarly to the case of *L. orbonalis*(Fig. II-4a). Although the EAG response elicited by Z3,Z6,Z9-23:H to male antennae of *O. anastomosalis* and *L. orbonalis* were not conducted because of a limited number of larvae collected in field, Z3,Z6,Z9,Z12,Z15-25:H elicited very weak responses from male antennae of *D. abietivorella* in GC-EAD analyses, but the pentaene clearly obtain the optimal behavioral responses in field test (Millar et al., 2005).

According to Table V-1, it seems that Crambidae species prefer to use Z3,Z6,Z9-23:H as one of their pheromone components, but the family Pyralidae species prefer to Z3,Z6,Z9,Z12,Z15-23:H or Z3,Z6,Z9,Z12,Z15-25:H. In many classifications, the Crambidae have been treated as a subfamily of the Pyralidae. The latest review by Munroe and Solis, in Kristensen (1999) retains the Crambidae as a full family. More and more findings on sex pheromone of superfamily Pyraloidea may help to better understand that Crambidae and Pyralidae are two independent families.

The sex pheromone communication is one of the chemical communications which are universal in nature. Most lepidopteran moths rely on the species-specific sex pheromone communication for mate finding and reproductive isolation between species. In addition to applying in pest control, further research on insect pheromone could help better understand chemical communication system, mechanisms of reproductive isolation and insect speciation of subfamily Pyraustinae species.

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ABSTRACT

Pyraustinae is a large subfamily of the lepidopteran family Crambidae. It includes about 1,400 species in the world, and many of them are serious agricultural pests because the larvae bore into stems and fruit of plants. The female sex pheromones of more than 40 Pyraustinae species had been identified up to date. Against males in this subfamily, however, lures baited with the synthetic pheromones often show less attractive activity than virgin female moths do. In order to make clear the communication systems in Pyraustinae species in detail and apply them for plant protection, the sex pheromones of nine Pyraustinae species have been studied.

The *Omphisa anastomosalis* and *Leucinodes orbonalis* are serious pests of sweet potatoes and eggplants, respectively, in South and Southeast Asia. While major sex pheromone components had been identified as (10*E*,14*E*)-10,14-hexadecadienal (E10,E14-16:Ald) for *O. anastomosalis* and (*E*)-11-hexadecenyl acetate (E11-16:OAc) for *L. orbonalis*, it had been reported that male attraction of the traps baited with their synthetic lures were insufficient in the field. Therefore, we reexamined their pheromone extracts by GC-MS analyses, and found a new component, (3*Z*,6*Z*,9*Z*)-3,6,9-tricosatriene (Z3,Z6,Z9-23:H). Field evaluation of new lures mixed with Z3,Z6,Z9-23:H successfully revealed its strong synergistic effect on the known pheromone components, indicating improved synthetic lures for monitoring of male moths.

There are 18 Japanese species in the genus *Herpetogramma* and most of them distribute in Okinawa islands. Their host plants are different but they concurrently inhabit a closed area. Since the mechanism of their reproductive isolation is interesting, sex pheromones of two *Herpetogramma* species (*H. submarginale* and *H. basale*) were examined by GC-EAD and GC-MS analyses. Mass spectra of the pheromone components and their derivatives with DMDS or MTAD indicated that females of *H. submarginale* and *H. basale* produced (*Z*)-13-hexadecenyl acetate

(Z13-16:OAc) and (11Z,13E)-11,13-hexadecadienyl acetate (Z11,E13-16:OAc), respectively. Z13-16:OAc is a new compound identified as an insect pheromone and the synthetic pheromone attracted the males in the field, while male attraction by the synthetic pheromone of *H. basale* was failed.

Furthermore, sex pheromones of other five Pyraustinae species were examined as follows: *Palpita nigopunctalis*, *Pleuroptya sabinusalis*, *P. inferior*, *Spoladea recurvalis*, and *Eurrhynchos accessalis*. In addition to the usual GC-EAD and GC-MS analyses, GC-FT/IR analysis was conducted in the experiments with pheromone extracts from the former two species. IR spectra confirmed the configuration of double bonds and the kind of functional groups at terminal positions. Although field evaluation of synthetic pheromones of these five species was unfortunately showed no good male attraction, results of the identification indicate diversity of pheromone structures produced by Pyraustinae species.

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