oxidation of the side chain of cholesterol could occur before any modification of the ring system, as already suggested by Mitropoulos and Myant[4]. The present in vivo experiment shows that the hamster liver can cleave the side chain of 3β-hydroxycholest-5-en-26-oic acid, which could logically be expected to be derived from 26-hydroxycholesterol, giving 3β-hydroxychol-5-en-24-oic acid, together with lithocholic and chenodeoxycholic acids. As previously reported[2,3,10], 3β-hydroxychol-5-en-24-oic acid is converted to lithocholic acid and chenodeoxycholic acid. In addition, in hamsters[3,6] but not in rabbits[3], lithocholic acid is metabolized into chenodeoxycholic acid.

It has been shown that 3β,7α-dihydroxychol-5-en-24-oic acid can be metabolized to chenodeoxycholic acid[11,12]. The present studies also permit the conclusion that 7 alpha hydroxylation of the monohydroxy C-27 bile acid can also provide a pathway for the production of chenodeoxycholic acid.

Although 26-hydroxycholesterol is not an important precursor of cholic acid in the rat[1,13], it can provide sufficient amounts of the acid in the hamster[1], rabbit[5], and human[14] to be considered a possible precursor along with 7α-hydroxycholesterol. However, the current findings in hamsters strongly suggest that 7 alpha hydroxylation of 26-hydroxycholesterol rather than further oxidation of the side chain must occur to account for significant amounts of cholic acid.

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Biosynthesis of a monoene and a conjugated diene sex pheromone component of the lightbrown apple moth by Δ11 desaturation

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Summary. Fatty acyl moieties present in the female sex pheromone gland of the lightbrown apple moth, *Epiphyas postvittana*, include the analogues of the two sex pheromone components, (*E*)-11-tetradecenyl acetate and (*E,E*)-9,11-tetradecadienyl acetate. Application of deuterium-labelled fatty acids followed by analysis by gas chromatography-mass spectrometry showed that biosynthesis of the two pheromone components involved initial Δ11-desaturation of myristic and palmitic acids respectively.
Key words. Sex pheromone; biosynthesis; Lepidoptera; *Epiphyas postvittana*; deuterium-labelling; (*E*)-11-tetradecenyl acetate; (*E,E*)-9,11-tetradecadienyl acetate.

Over the last 10 years, the biosynthetic pathways of sex pheromone components have been studied in a number of species of Lepidoptera[1]. These sex pheromone components are biosynthesized by a modified fatty acid synthesis occuring in the sex pheromone gland of females. Two important processes involved in these biosyntheses are desaturation and 2 carbon chain-shortening through limited β-oxidation[1]. The different desaturases and the action of these two processes at different stages of pheromone biosynthesis are largely responsible for the structural diversity of common pheromone components found in families such as the Tortricidae and Noctuidae[2].

In the biosynthesis of lepidopteran sex pheromone components the most common type of desaturation is apparently Δ11[1], although Δ9[3] and Δ10[4] are also known. For the biosynthesis of monoenic components a single desaturation step occurs[1]. However, somewhat surprisingly, a single desaturation step has also been found to be involved in the biosynthesis of conjugated dienic compo-

nents. The fatty acyl groups present in the sex pheromone glands of female *Bombyx mori* include the putative pheromone precursors, (*E,Z*)-10,12-hexadecadienoate and (*Z,E*)-10,12-hexadecadienoate, as well as (*Z*)-11-hexadecenoate[5,6]. Biosynthesis of the pheromone component, (*E,Z*)-10,12-hexadecadienol, from (*Z*)-11-hexadecenoate has been demonstrated using deuterium-labelling combined with analysis by gas chromatography-mass spectrometry (GC-MS)[6], which indicated that the conjugated dienic bond is formed by initial Δ11-desaturation followed by the oxidative removal of two hydrogen atoms on the two carbons adjacent to the olefinic bond. Likewise in *Cydia pomonella*, the pheromone component (*E,E*)-8,10-dodecadienol is formed by initial Δ9-desaturation of laurate followed by the oxidative removal of two hydrogens from carbons adjacent to the olefinic bond[3].

In contrast to the type of pathway found for the biosynthesis of the dienic components in these two species, analysis of the fatty acyl moieties in the sex pheromone glands of female *Spodoptera littoralis* suggested that the pheromone component (*Z,E*)-9,11-tetradecenyl acetate is biosynthesized by two cycles of Δ11-desaturation separated by a 2 carbon chain-shortening step (from Δ11 to Δ9)[7].

The lightbrown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae) is an indigenous Australian leafroller moth that is also found in New Zealand[8]. Its sex pheromone has been identified as a mixture of (*E*)-11-tetradecenyl acetate (E11–14:OAc) and (*E,E*)-9,11-tetradecadienyl acetate (E,E-9,11–14:OAc) present in the female gland in a ratio of approximately 20:1 respectively[9]. The Δ11-unsaturated monoenic component of the pheromone suggested to us that there was Δ11-desaturase activity in the pheromone gland of this species. Therefore it appeared likely that the dienic component was biosynthesized through two Δ11-desaturation steps (separated by a 2 carbon chain-shortening limited β-oxidation step), rather than by a pathway similar to that observed in *B. mori*[5,6] and *C. pomonella*[7]. We report here that biosynthesis of both E11–14:OAc and E,E-9,11–14:OAc in *E. postvittana* involves Δ11-desaturation.

Materials and methods

E. postvittana were used from a laboratory colony maintained at Entomology Division, D.S.I.R. The larvae were reared on synthetic diet[10]. Female pupae were separated from male pupae, and either used in New Zealand or shipped to New York by air. Female moths were used 3 days after eclosion, unless they had emerged during shipment to New York, whereupon they were used immediately upon arrival at the laboratory.

Omega-labelled (18-D_3)-stearic acid (D_3-18:COOH) and (16-D_3)-palmitic acid (D_3-16:COOH) were purchased from KOR Isotopes, Cambridge, Massachusetts, and (14-D_3)-myristic acid (D_3-14:COOH) was purchased from ICON Services Inc., Summit, New Jersey. All three labelled acids were greater than 98% isotopically pure.

Reference synthetic acetates and common saturated and Δ9-unsaturated fatty acid methyl esters (methyl esters purchased from Sigma Chemical Co., St Louis, Missouri), were available in both laboratories. Methyl esters of uncommon fatty acids were synthesized from the corresponding alcohols by oxidation with pyridinium dichromate[11], followed by acid methanolysis using hot (100 °C) methanol:sulphuric acid:benzene (30:1:15) solution[12].

The sex pheromone glands of female *E. postvittana* were extruded at the beginning of the scotophase using an alligator clip placed on the lower abdomen of the insect. The labelled acids were applied topically to the gland as dimethyl sulphoxide solutions (ca 10 μg/μl) as described[13]. The glands were dissected some 2–5 h later and extracted as follows. For analyses of the pheromone components and other acetates, 5–15 glands were extracted in ca 10–20 μl of Skelly B for ca 20 h at ambient temperature. However, in order to analyse the glands for fatty acyl moieties, the excised glands (5–15) were extracted with ca 20 μl of dichloromethane for ca 1 h at ambient temperature; the extract was decanted away from the glands, the dichloromethane evaporated using a stream of dry nitrogen, and the fatty acyl groups present in the residue were converted to methyl esters by base methanolysis[14].

Methyl esters of fatty acyl groups present in the gland were analysed by capillary gas chromatography (GC) and GC-MS. Methyl esters were analysed initially using a Varian 3500 gas chromatograph equipped with a splitless injector and a flame ionisation detector. Nitrogen at a linear flow velocity of 10 cm s^{-1} was the carrier gas. Two capillary columns were used; a Quadrex Corp. (New Haven, Connecticut) 50 m × 0.25 mm i.d. Silar 10C (programmed from 60 to 140 °C at 8 °C min^{-1} following an initial delay of 1 min, then to 180 °C at 2 °C min^{-1}) and a Quadrex Corp. 25 m × 0.32 mm i.d. bonded phase Carbowax 20M (programme, as above except the final temperature was 200 °C, and the final rate 2.5 °C min^{-1}). Electron ionisation mass spectra of the fatty acid methyl esters were recorded using a Hewlett Packard 5890-VG Instruments 7070 gas chromatograph-mass spectrometer system. For these analyses, a Supelco Inc., (Bellefonte, Philadelphia) 30 m × 0.25 mm i.d. SP2340 capillary column was used, temperature-programmed as for the Carbowax 20M column above. Helium at a linear flow velocity of 25 cm s^{-1} was the carrier gas.

The incorporation of deuterium label from the various acids into acetates and fatty acid methyl esters was determined by GC-MS[1]. Selected ions characteristic of the unlabelled and labelled compounds, were recorded on two mass spectrometers. In Geneva, a Hewlett Packard 5985 quadrupole mass spectrometer interfaced with a Hewlett Packard 5840 gas chromatograph was used. Ion

intensities were recorded using chemical ionisation, with isobutane as the reactant gas. Under these conditions, the most intense ion of the spectrum of a long chain acetate or fatty acid methyl ester is the molecular ion plus one mass unit $[(M+1)^+]$. Therefore in order to observe whether the D_3-label from the various saturated acids was incorporated into particular compounds, the ion corresponding to the $(M+4)^+$ ion of a particular unlabelled compound was recorded. Generally, the ions corresponding to the $(M+2)^+$ and $(M+3)^+$ ions of the unlabelled compound were also recorded in order to observe the dimunition of the ion intensity of a particular compound. The gas chromatograph was fitted with a 30 m × 0.25 mm i.d. Supelcowax 10 capillary column (Supelco Inc.), temperature-programmed from 80 to 200 °C at 4 °C min^{-1} following an initial delay of 3 min. In New Zealand, ion intensities were recorded using the Hewlett Packard 5890-VG Instruments 7070 system (see above). All gas chromatograph conditions (including column) were as above. Using electron ionisation, the characteristic $(M-60)^+$ ion (loss of acetic acid) of the unlabelled long chain acetate, along with the ions corresponding to the loss of acetic acid from D_3-, D_2-, and D-labelled isotopomers were recorded as above. For the methyl esters the parent ion and corresponding labelled D_3 ions (as above) were recorded. For the diagrams showing the results of this work, the recorded characteristic ions of a particular unlabelled compound from both chemical ionisation and electron ionisation recordings have been combined and shown as 'M', while the recorded ions characteristic of a particular labelled compound are represented as 'M+3'.

Results

On both columns, GC analyses of base-methanolysed extracts of female *E. postvittana* pheromone glands gave tentative identifications, based on retention times, of methyl esters of the following fatty acyl moieties: laurate, myristate (14:Acyl), (*E*)-9-tetradecenoate (*E*)-11-tetradecenoate (E11–14:Acyl), (*Z*)-9-tetradecenoate, (*Z*)-11-tetradecenoate (Z11–14:Acyl), palmitate, (*E*)-11-hexadecenoate (E11–16:Acyl), (*Z*)-9-hexadecenoate (Z9–16:Acyl), stearate, oleate, linoleate and linolenate (fig. 1). The combination of very polar (Silar 10C) and polar (Carbowax 20 M) columns enabled us to unequivocally assign the above 14-carbon and 16-carbon chain methyl esters with regard to all other straight chain 14-carbon and 16-carbon methyl esters. The above assignments of the 14- and 16-carbon methyl esters were confirmed by comparing the electron ionisation mass spectra of the compounds found in the base-methanolysed extracts with the mass spectra of authentic samples of the various compounds. In addition to the above compounds, a compound with retention times on both columns exactly matching those of the methyl ester of (*E,E*)-9,11-tetradecadienoate (E,E-9,11–14:Acyl) was observed. The mass spectrum of this compound was indistinguishable from the mass spectrum of methyl (E,E-9,11–14:Acyl). Although our data do not allow us to unequivocally assign this compound as methyl (E,E-9,11–14:Acyl) we assume that the pattern of other compounds found in the pheromone gland, especially the pheromone component E,E-9,11–14:OAc, strongly supports our assignment.

Application of D_3-16:COOH to pheromone glands of female *E. postvittana* and subsequent GC-MS analysis of

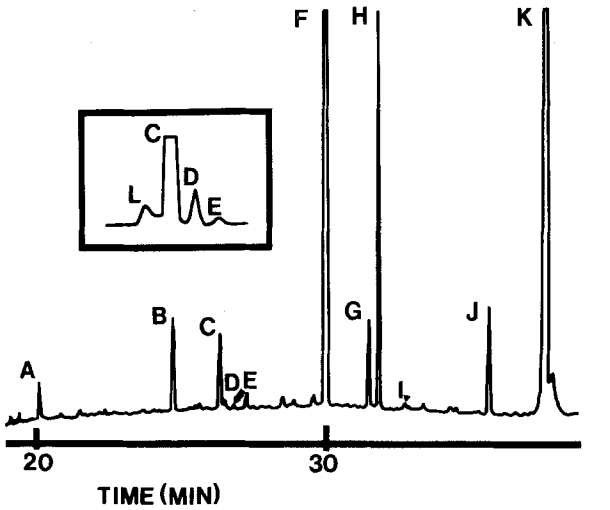

Figure 1. Gas chromatogram (50 m × 0.25 mm i.d. Silar 10 C column) of a base methanolysed sex pheromone gland extract from female *Epiphyas postvittana*. A = methyl laurate, B = methyl myristate, C = methyl (*E*)-11-tetradecenoate, D = methyl (*Z*)-9-tetradecenoate, E = methyl (*Z*)-11-tetradecenoate, F = methyl palmitate, G = methyl (*E*)-11-hexadecenoate, H = methyl (*Z*)-9-hexadecenoate, I = methyl (*E,E*)-9,11-tetradecadienoate, J = methyl stearate, K = methyl oleate, L = methyl (*E*)-9-tetradecenoate (in inset).

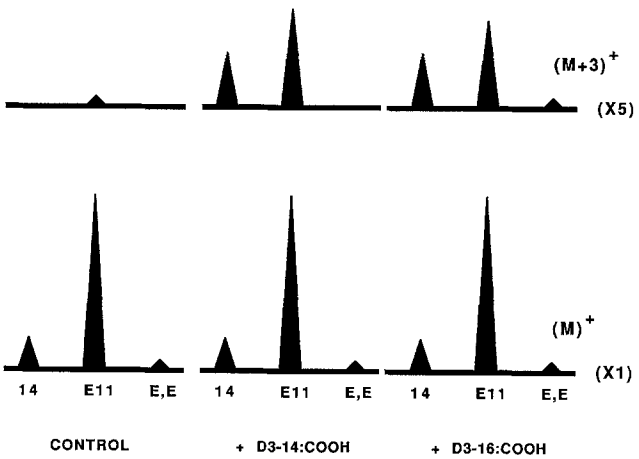

Figure 2. Relative intensities of characteristic ions of tetradecyl acetate (14), (*E*)-11-tetradecenyl acetate (E11) and (*E,E*)-9,11-tetradecadienyl acetate (E,E), extracted from sex pheromone glands of female *Epiphyas postvittana* treated with, (14-D_3)-myristic acid (D3–14:COOH) (mean of 3 experiments) or (16-D_3)-palmitic acid (D3–16:COOH) (mean of 2 experiments), and untreated (CONTROL) (1 experiment). $(M)^+$ and $(M+3)^+$ = ions characteristic of the unlabelled and D_3-labelled compounds respectively. The ion intensities in each of the treatments have been normalised to the most intense peak.

a Skelly B extract of the glands (containing pheromone components and other acetates), showed incorporation of the D_3-label into both pheromone components, E11–14:OAc (10.0% incorporation of label) and E,E-9,11–14:OAc (11.1% incorporation), and also into tetradecyl acetate (14:OAc) (18.8% incorporation) (fig. 2). However, in three separate experiments when D_3-14:COOH was applied to glands, and Skelly B extracts of the glands analysed, the D_3-label was found to be incorporated only into E11–14:OAc (11.9% incorporation) and 14:OAc (22.9% incorporation), and not into the diene, E,E-9,11–14:OAc (fig. 2).

Base methanolysed glands, previously treated with either D_3-18:COOH or D_3-16:COOH were found to have incorporated label into the methyl esters of the putative precursors of all three of the above acetates, namely 14:Acyl (3.6% incorporation), E11–14:Acyl (2.7% incorporation) and E,E-9,11–14:Acyl (2.4% incorporation (fig. 3). The D_3-label was also found in the methyl ester of E11–16:Acyl (approximately 2% incorporation), but not in the methyl ester of E9–14:Acyl. In the latter case, the relatively low amount of methyl (E9–14:Acyl) found in the gland and the relatively small percentage incorporation of label into the various fatty acyl moieties probably precluded detection of any incorporation of label into E9–14:Acyl. Although both fatty acyl moieties were in relatively high abundance (particularly Z9–16:Acyl), we did not detect the incorporation of the label into the methyl esters of Z9–14:Acyl and Z9–16:Acyl (fig. 2).

Discussion

Our data show that the major sex pheromone component of *E. postvittana*, E11–14:OAc, can be biosynthesized from either palmitic or myristic acids. Given the relatively high abundance of the putative precursor, E11–14:Acyl, in the sex pheromone gland of female *E. postvittana*, it appears highly likely that E11–14:OAc is biosynthesized through Δ11-desaturation of myristate as observed in other tortricid species[1,2] (fig. 4). The other pheromone component, E,E-9,11–14:OAc, can also be biosynthesized from palmitic acid but cannot be biosynthesized from myristic acid. The fatty acyl moieties found in the pheromone gland of female *E. postvittana*, and the pattern of incorporation of D_3-label from either palmitic or stearic acids into these moieties, strongly suggests that E,E-9,11–14:OAc is also biosynthesized via Δ11-desaturation. The following pathway is the most consistent with our data (see also fig. 4). A Δ11-desaturase acts on palmitate to produce E11–16:Acyl, which is chain-shortened to E9–14:Acyl; Δ11 desaturation of E9–14:Acyl produces the putative precursor, E,E-9,11–14:Acyl, which is reduced and acetylated to form the pheromone component.

A conceivable alternative biosynthetic pathway is one similar to those found in the biosynthesis of dienic compounds in *B. mori*[5,6] and *C. pomonella*[7]. However, if E,E-9,11–14:OAc was biosynthesized in *E. postvittana* via this pathway then we would expect to observe a Δ10-tetradecenoate intermediate in the pheromone gland. We found no evidence for such an intermediate, and additionally found that E,E-9,11–14:OAc was not biosynthesized from myristic acid.

In a study on the biosynthesis of (*Z,E*)-9,11-tetradecenyl acetate in *Spodoptera littoralis* a very large amount of the presumed intermediate, (*Z*)-9-tetradecenoate was observed in the female sex pheromone gland[7]. In contrast, we observed only very small amounts of the analogous

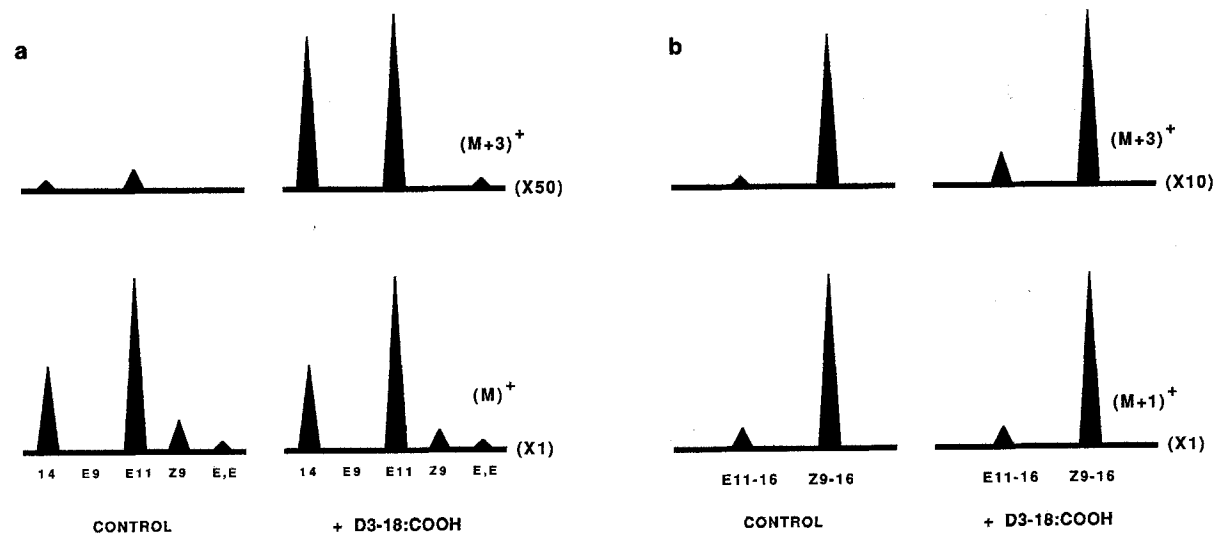

Figure 3. Relative intensities of characteristic ions of methyl esters of (*a*) tetradecanoate (14), (*E*)-9-tetradecenoate (E9), (*E*)-11-tetradecenoate (E11), (*Z*)-9-tetradecenoate (Z9), (*E,E*)-9,11-tetradecadienoate (E,E), and (*b*) (*E*)-11-hexadecenoate (E11–16), (*Z*)-9-hexadecenoate (Z9–16), extracted from base methanolysed sex pheromone glands of female *Epiphyas postvittana* treated with (18-D_3)-stearic acid (D3–18:COOH) (mean of 2 experiments) and untreated (CONTROL) (1 experiment). $(M)^+$, $(M+1)^+$, and $(M+3)^+$ = ions characteristic of the unlabelled, unlabelled plus one mass unit and D_3-labelled compounds respectively. $(M+1)^+$ ions are shown in (*b*) due to the large quantity of Z9–16 in the extract and the resultant overloading of the detection system. The ion intensities have been normalised to the most intense peak.

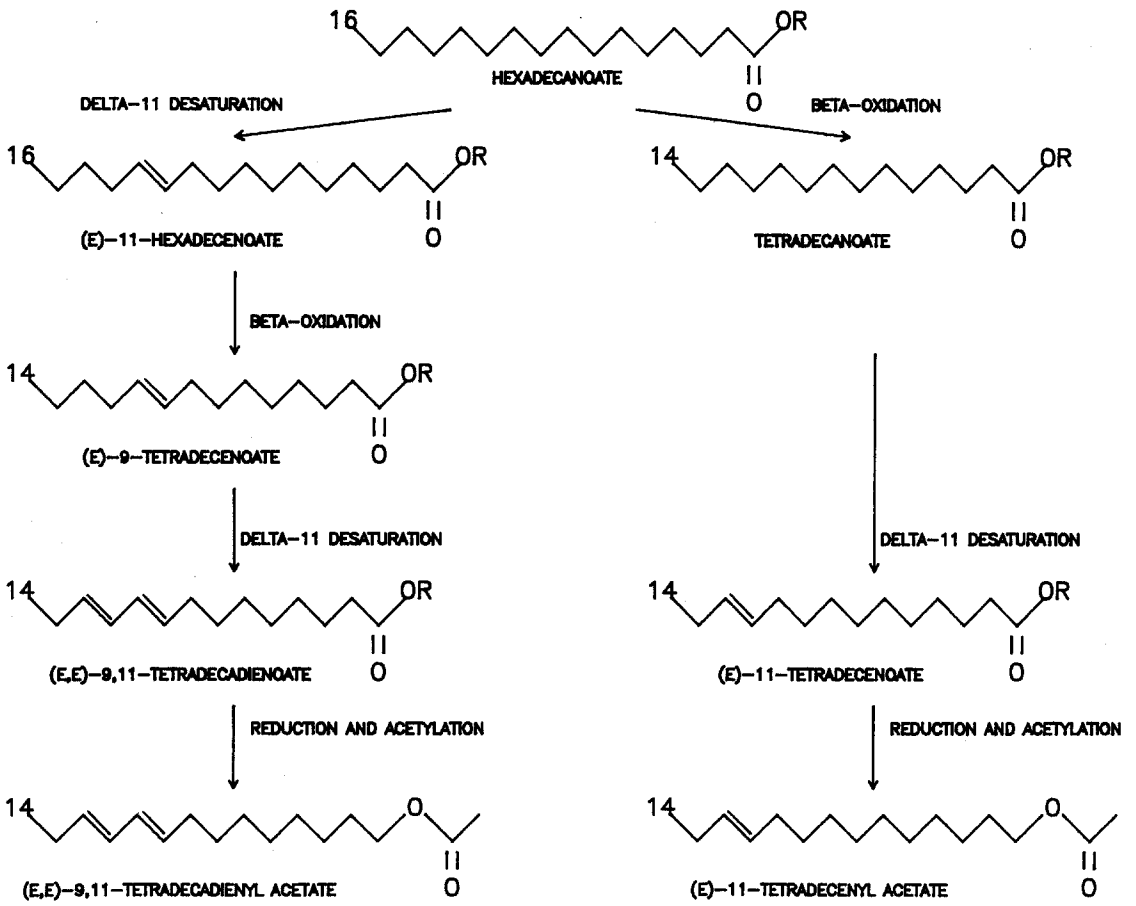

Figure 4. Proposed biosynthetic pathways for the two pheromone components, (*E*)-11-tetradecenyl acetate and (*E,E*)-9,11-tetradecadienyl acetate in *Epiphyas postvittana*.

intermediate in the biosynthesis of E,E-9,11-14:OAc, E9-14:Acyl. Clearly in *E. postvittana*, Δ11-desaturation of tetradecanoate, and reduction and acetylation of the resultant unsaturated fatty acyl moiety occurs readily, as evidenced by the large amounts of E11-14:Acyl and E11-14:OAc found in the pheromone gland. Perhaps the low amount of E9-14:Acyl found in the pheromone gland of *E. postvittana* is due to the relatively rapid (*E*)-11-desaturation, and subsequent reduction and acetylation of this moiety in comparison with the relatively slow β-oxidation of E11-16:Acyl to E9-14:Acyl. We hope to conduct kinetic studies on these processes to test this hypothesis, and investigate the biosynthesis of the two sex pheromone components of *E. postvittana* in more detail.

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