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Biosynthetic pathway for producing the sex pheromone component (*Z*,*E*)-9,12-tetradecadienyl acetate in moths involves a Δ12 desaturase

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Abstract. Sex pheromones are used by insects as a form of chemical communication for the purpose of attracting conspecific mates. Female moths *Cadra cautella* and *Spodoptera exigua* use the diene (*Z*,*E*)-9,12-tetradecadienyl acetate as the major pheromone component. Biosynthesis of this pheromone component was demonstrated to occur through Δ11 desaturation of hexadecanoic acid (palmitate) to produce (*Z*)-11-hexadecenoic acid which is then chain-shortened to (*Z*)-9-tetradecenoic acid. A unique Δ12 desaturase uses the (*Z*)-9-tetradecenoic acid to produce (*Z*,*E*)-9,12-tetradecenoic acid which is reduced and acetylated to form the acetate ester pheromone component. Both moths also use a pheromonotropic peptide to stimulate pheromone biosynthesis.
Key words. Lepidoptera; *Cadra cautella*; *Spodoptera exigua*; pheromone biosynthesis; Δ12 desaturase; PBAN.

Sex pheromones are produced by an individual to attract the opposite sex. Among moths it is usually the female that releases a blend of pheromone compounds to attract the male for mating. These pheromone components are biosynthesized in the pheromone gland which is usually located near the ovipositor at the posterior end of the abdomen. In this gland several different compounds are biosynthesized from fatty acid precursors, resulting in a species-specific pheromone blend with variations in chain length, degree of unsaturation, functional group and total number of compounds [1].

The biosynthetic pathway of several different moths has been determined since the original work of Bjostad, Wolf, and Roelofs in 1981 [2, 3]. These pathways were determined through identification of possible fatty acid intermediates and the use of labelled precursor fatty acids. Usually, biosynthesis of sex pheromones in moths begins with the production of palmitic (16:acid) and stearic (18:acid) fatty acids. The fate of these fatty acids depends on the chain length, degree of unsaturation and functional group of the pheromone blend for each species of moths. For example, the pheromone of the redbanded leafroller moth, *Argyrotaenia velutinana*, consists of a blend of acetate esters ((*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, tetradecanyl acetate, (*Z*)-9-dodecenyl acetate, (*E*)-9-dodecenyl acetate, 11-dodecenyl acetate, and dodecanyl acetate). These are produced by chain-shortening 16:acid to 14:acid which then undergoes Δ11 desaturation. The Z and E 11-tetradecenoic acids are selectively chain-shortened to Z and E 9-dodecenoic acids [2, 4]. These fatty acids are converted into alcohols and then acetylated by a reductase and acetyl-CoA: fatty alcohol acetyltransferase respectively [5].

The key enzymes involved in pheromone biosynthetic pathways thus are a) acetyl-CoA carboxylase and fatty acid synthetase, to make 16 and 18 carbon fatty acids; b) specific desaturases to make mono- and diunsaturated fatty acids; c) specific chain-shortening enzymes to make the right chain length fatty acid; d) and, depending upon the functional group of a particular species' pheromone, a reductase, an acetyltransferase, or an oxidase is used, sometimes in combination, to make the final pheromone product. The order in which these enzymes are used determines the final pheromone components.

In the present paper the production of a diene pheromone component (*Z*,*E*)-9,12-tetradecadienyl acetate (Z9,E12-14:OAc) in two different moths was investigated. Both the almond moth, *Cadra cautella*, and the beet armyworm, *Spodoptera exigua*, use this acetate ester as one of the major pheromone components. The other component is (*Z*)-9-tetradecenyl acetate (Z9-14:OAc). Stable isotope-labelled fatty acid precursors were used to demonstrate the biosynthetic pathway. My work indicates that a unique Δ12 desaturase is present in pheromone glands of these moths. Regulation of the pheromone biosynthetic pathway by a pheromonotropic peptide was also demonstrated.

Materials and methods

Cadra cautella were reared on a stored-products diet. *Spodoptera exigua* were obtained from the USDA Integrated Pest Management Research laboratory (Mississippi State, MS, USA) as pupae. Pupae were sexed, separated and two- to four-day-old virgin females used throughout this study. Pupae and adults were kept at 14:10 L:D and 25 °C. Pheromone amounts were deter-

mined by removing the pheromone gland, extracting in hexane for 5 min and then subjecting the extract to GC analysis, as described below. In the case of *C. cautella* one-day-old moths were decapitated in order to lower pheromone amounts. Both moths were used during the photophase. Isolated abdomen incubations were performed as described in Jurenka et al. [6]. A pheromonotropic peptide with the sequence KYRQD-PEQIDSRTRYFSPRL-NH_2 was purchased from Chiron (San Diego, CA, USA).

Deuterium (^{2}H)-labelled compounds [14,14,14-$^2H_3$]tetradecanoic acid ($^2H_3$-14:acid) and [16,16,16-$^2H_3$]hexadecanoic acid ($^2H_3$-16:acid) were purchased from ICON Services Inc., Summit, NJ, USA. The other two ^{2}H compounds, (*Z*)-11-[13,13,14,14,15,15,16,16,16-$^2H_9$]hexadecenoic acid ($^2H_9$-Z11-16:acid) and (*Z*)-9-[13,14-$^2H_2$]tetradecenoic acid ($^2H_2$-Z9-14:acid) were made as described in Jurenka et al. [7]. The ^{2}H-labelled compounds were topically applied to the pheromone gland in dimethyl sulphoxide (1 μg/0.1 μl) and isolated abdomens were immediately placed on 5 μl saline with or without the pheromonotropic peptide. After a 1 h incubation groups of five glands were removed and extracted together in hexane. Incorporation of ^{2}H-labelled compounds into pheromone precursors and pheromone components was determined by mass spectrometry. A Hewlett-Packard 5972 series mass selective detector was used in the single ion mode to detect unlabelled and labelled pheromone components and precursors. The ions monitored for acetate esters were monounsaturated C14: 194 and diunsaturated C14: 192. ^{2}H-labelled compound incorporation into acetate esters was monitored at ions 192 and 199 plus the number of ^{2}H in the applied substrate (+2, +3, or +9). A 30 m × 0.32 mm DB-225 column (J&W Scientific, Folsom, CA, USA) temperature programmed (60 °C for 1 min and then 10 °C/min to 200 °C) in a Hewlett-Packard 5890 GC was used to separate compounds in the labelling experiments.

In all other experiments where pheromone amounts were analysed a Hewlett-Packard 5890 GC equipped with a flame ionization detector, an autoinjector and an oven (temperature programmed at 60 °C for 1 min and then 10 °C/min to 250 °C) was used. A SE-30 or Carbowax capillary column was used (both 30 m × 0.25 mm, from Alltech Associates, Inc., Deerfield, IL, USA). Peak areas were integrated and pheromone amounts determined using tridecanyl acetate as an internal standard.

Results and discussion

Pheromone can be found in *C. cautella* pheromone glands throughout the photoperiod [8], indicating continuous production of pheromone. Decapitation decreased the amount of pheromone and treatment of decapitated insects with a synthetic pheromonotropic peptide stimulated pheromone production (fig. 1). Incubation of isolated abdomens with 0.01 pmol peptide increased pheromone production, with maximum production occurring at 2 pmols of peptide. These results indicate that the natural source of a pheromonotropic peptide resides in the head, in the subesophageal ganglion, as has been demonstrated for a number of other moths [9, 10]. Pheromone production was also stimulated in *S. exigua* with a pheromonotropic peptide during the photophase (control = 0.32 ± 0.05 ng/gland, n = 8, 5 pmol peptide = 2.53 ± 0.55 ng/gland, n = 9). *Spodoptera exigua* normally produces pheromone only during the scotophase, but pheromone production can be stimulated during the photophase when incubated with a pheromonotropic peptide.

Using stable isotope labelled fatty acid precursors and stimulation with a pheromonotropic peptide it was demonstrated that the pheromone component Z9,E12-14:OAc of *C. cautella* and *S. exigua* is produced through the following pathway (fig. 2). Palmitate (16:acid) is desaturated at the Δ11 position to produce Z11-16:acid which is then chain-shortened by two carbons to Z9-14:acid. This fatty acid can be reduced to the alcohol and then acetylated to form Z9-14:OAc, or it can be desaturated at the Δ12 position to form Z9,E12-14:acid. The diene fatty acid is then reduced and acetylated to form Z9,E12-14:OAc.

The evidence for this pathway is presented in figure 3, which shows the data obtained with *C. cautella*. When $^2H_3$-14:acid was applied to pheromone glands of *C. cautella* it did not label Z9,E12-14:OAc or Z9-14:OAc but did label another C14 monounsaturated acetate ester. This acetate ester was tentatively identified as E11-14:OAc based on retention times with known standards. As indicated in figure 3, the presumptive E11-14:OAc elutes just prior to Z9-14:OAc on the DB-225 capillary column. Relatively large amounts of labelled

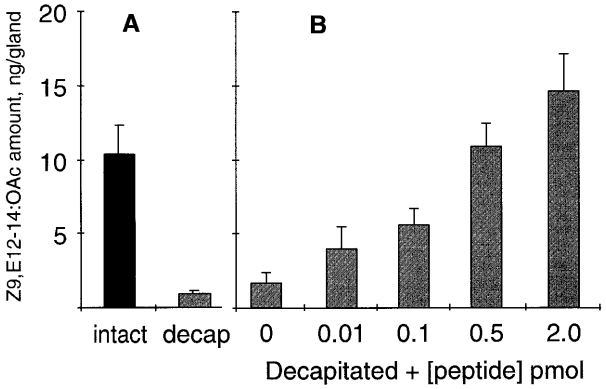

Figure 1. (*A*) Levels of pheromone in females of *C. cautella* that were either left intact or decapitated (n = 8 and 10, respectively). 24 h later pheromone levels were determined as described in Materials and methods. (*B*) Levels of pheromone in isolated abdomens, taken from decapitated females, after a 3 h incubation with the indicated concentrations of pheromonotropic peptide (n = 8–10).

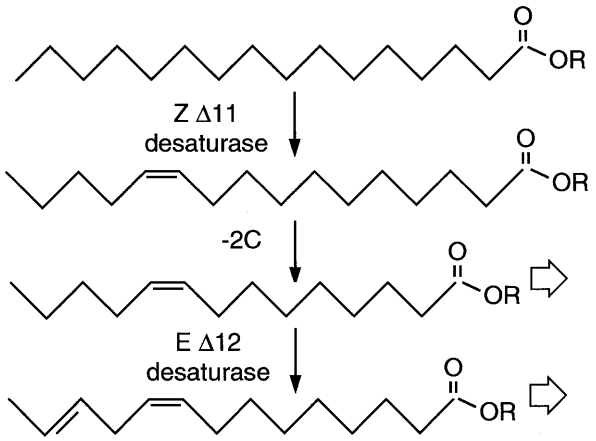

Figure 2. Proposed pathway for biosynthesis of the two pheromone components in *C. cautella* and *S. exigua*. Open arrows indicate reduction and acetylation to form the acetate ester pheromone components.

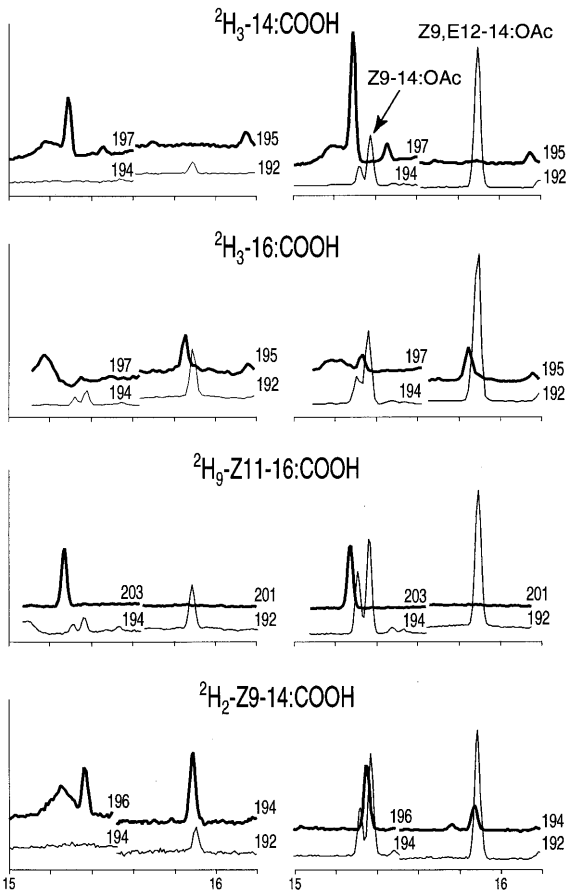

Figure 3. Partial GC/MS chromatograms of pheromone gland extracts obtained from female *C. cautella* after topical application of the indicated deuterium-labelled fatty acid. Single ions were monitored corresponding to (M+)-60 of 14 carbon monoene and diene acetate esters. Bold tracings are single ions monitored corresponding to the deuterium enrichment of the topically applied fatty acid. Chromatograms on the left were from saline controls, those on the right were from 1 pmol pheromonotropic peptide treatments. Both sets of chromatograms were scaled to the same relative ion intensity. The peak eluting just before Z9-14:OAc was tentatively identified as E11-14:OAc.

E11-14:OAc were also found in both saline and pheromonotropic peptide treated insects (table 1 and fig. 3), but only with $^2H_3$-14:acid as a precursor. This indicates that E11-14:OAc is probably biosynthesize through a ΔE11 desaturase using 14:CoA as a substrate, and that decapitation did not prevent incorporation of label, indicating that the enzymes are active in the absence of a pheromonotropic peptide.

As shown in figure 3, when $^2H_3$-16:acid was applied to pheromone glands both Z9-14:OAc and Z9,E12-14:OAc were labelled. However, the other C14 monounsaturated acetate ester (E11-14:OAc) was not labelled. This indicates that 16:acid is the starting fatty acid in the biosynthetic pathway, and also that a Δ11 desaturase acts on 16:acid to form Z11-16:acid which is chain-shortened to Z9-14:acid. The chain-shortening of Z11-16:acid was confirmed by applying $^2H_9$-Z11-16:acid to pheromone glands. This precursor was chain-shortened to Z9-14:acid and then converted to pheromone, but it was not further desaturated to the diene. Further desaturation did not occur because the ^{2}H-labelled precursor contained nine ^{2}H atoms producing an isotope effect that interfered with the Δ12 desaturase. The presence of a Δ12 desaturase was demonstrated by applying $^2H_2$-Z9-14:acid to pheromone glands. This precursor fatty acid was converted into the diene pheromone component, Z9,E12-14:OAc. I therefore conclude that the major pheromone components Z9,E12-14:OAc and Z9-14:OAc were produced through a biosynthetic pathway as shown in figure 2.

Using the same labelling procedure with *S. exigua* pheromone glands, as described for *C. cautella*, I postulated use of the same biosynthetic pathway. Application of $^2H_3$-16:acid to pheromone glands resulted in label incorporation into Z9-14:OAc but not Z9,E12-14:OAc. However application of $^2H_2$-Z9-14:acid to pheromone glands resulted in the label being detected in Z9,E12-14:OAc (results not shown). These results indicate that *S. exigua* does use the same biosynthetic pathway as *C. cautella*. Other moths producing Z9,E12-14:OAc will probably also use this same pathway.

Identification of biosynthetic precursors as fatty acid methyl esters has also been used to help identify possible biosynthetic pathways. However, at least in *C. cautella*, the amount of C14 precursors was very low (data not shown). The analysis of glands by GC-MS indicated that tetradecanoic acid was found at about 1% of the total fatty acid composition, while Z9-14:acid was found at about 0.1%. No evidence of Z9,E12-14:acid was found. However the precursor Z11-16:acid was present in pheromone glands (5.5% of total fatty acid composition).

Most moths use a unique desaturase in their pheromone biosynthetic pathway. The desaturases indicated in

Table 1. Incorporation of deuterium-labelled fatty acids into the pheromone of *C. cautella*. Labelled fatty acids were topically applied to isolated abdomens taken from decapitated females and immediately placed on 5 µl saline with or without 1 pmol of the pheromonotropic peptide. After a 1 h incubation the amount of labelled pheromone was determined by GC/MS as described in Materials and methods. n = 3 or 4. No significant differences were found between saline and 1 pmol peptide treatments for any of the precursors (Student's *t*-test, $p < 0.05$).

		Amount labelled, ng/gland ±SEM	
Precursor	Treatment	Z9-14:OAc	Z9,E12-14:OAc
$^2H_3$-14:COOH	saline	3.93 ± 1.70*	nd
	1 pmol	4.46 ± 0.90*	nd
$^2H_3$-16:COOH	saline	0.09 ± 0.06	0.78 ± 0.13
	1 pmol	0.18 ± 0.08	1.22 ± 0.13
$^2H_9$-Z11-16:COOH	saline	0.65 ± 0.31	nd
	1 pmol	0.52 ± 0.07	nd
$^2H_2$-Z9-14:COOH	saline	1.06 ± 0.24	1.72 ± 0.35
	1 pmol	1.80 ± 0.04	0.56 ± 0.02

*These values represent the amount incorporated into the C14 monounsaturated acetate ester tentatively identified as E11-14:OAc.
nd = not detected.

moths so far are Δ5 [11], Δ9 [12, 13], Δ10 [14], Δ11 [2, 15], Δ13 [16], and Δ14 [17]. To this list we can now add a Δ12 desaturase. Most of these desaturases are unique in that they are found only in pheromone glands, and they use specific precursors. Thus the Δ12 desaturase found in pheromone glands of *C. cautella* and *S. exigua* will probably use only Z9-14:CoA as a substrate and produce only the E isomer. The other Δ12 desaturase present in some insects (but not Lepidoptera) uses Z9-18:CoA as a substrate to produce Z9,Z12-18:CoA (linoleic acid) and is thought to be found in fat body and epidermal tissue throughout the body [18, 19]. This desaturase also produces only the Z isomer, whereas the one in pheromone glands produces the E isomer. It will be interesting to determine homology amongst these various desaturases as they are characterized.

The incorporation of deuterium-labelled compounds into both monoene and diene pheromone components was quantified to determine where in the biosynthetic pathway the pheromonotropic peptide acts. The data in table 1 and figure 3 indicate that there is little difference between saline and peptide treatment in the amount of label incorporation. Whereas labelled precursors were incorporated into pheromone components in both treatments, only unlabelled pheromone amounts increased in the presence of the pheromonotropic peptide. These results indicate that application of precursor fatty acids to pheromone glands in the absence of peptide will result in reduction and acetylation to pheromone. Therefore it appears that the pheromonotropic peptide acts at a step prior to reduction, probably at the level of fatty acid synthesis. It has been demonstrated that pheromonotropic peptides act on a step in fatty acid synthesis in several moths including *Helicoverpa zea* [20], *Mamestra brassicae* [21], and *Agrotis segetum* [22], although in *A. segetum* a significant increase in incorporation of $^2H_9$-Z11-16:COOH into the chain-shortened 14, 12, and 10 carbon pheromone components was observed. In contrast, other studies with *Spodoptera littoralis* [13], *Thaumetopoea pityocampa* [23], and *Bombyx mori* [24] indicate that the reduction of fatty acids is regulated by pheromonotropic peptides. It appears that the control point for pheromonotropic peptides in moths is either the biosynthesis of fatty acids or the reduction of fatty acids.

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