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Pheromone Biosynthetic Pathways in the Moths *Heliothis subflexa* and *Heliothis virescens*

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Sex pheromones of many moth species have relatively simple structures consisting of a hydrocarbon chain with a functional group and one to several double bonds. These sex pheromones are derived from fatty acids through specific biosynthetic pathways. We investigated the incorporation of deuterium-labeled tetradecanoic, hexadecanoic, and octadecanoic acid precursors into pheromone components of *Heliothis subflexa* and *Heliothis virescens*. The two species utilize (Z)11-hexadecenal as the major pheromone component, which is produced by Δ11 desaturation of hexadecanoic acid. *H. subflexa* also produced (Z)11-hexadecanol and (Z)-11-hexadecenyl acetate via Δ11 desaturation. In *H. subflexa*, octadecanoic acid was used to biosynthesize the minor pheromone components (Z)9-hexadecenal, (Z)9-hexadecenol, and (Z)9-hexadecenyl acetate. These minor components are produced by Δ11 desaturation of octadecanoic acid followed by one round of chain-shortening. In contrast, *H. virescens* used hexadecanoic acid as a substrate to form (Z)11-hexadecenal and (Z)11-hexadecenol and hexadecenal. *H. virescens* also produced (Z)9-tetradecenal by Δ11 desaturation of the hexadecanoic acid followed by one round of chain-shortening and reduction. Tetradecanoic acid was not utilized as a precursor to form Z9-14:Ald in *H. virescens*. This labeling pattern indicates that the Δ11 desaturase is the only active desaturase present in the pheromone gland cells of both species. Arch. Insect Biochem. Physiol. 59:53–58, 2005.

KEYWORDS: pheromone; biosynthesis; PBAN; desaturases; Lepidoptera; Noctuidae

INTRODUCTION

Sex pheromones of many moths are linear, fatty acid-derived compounds, 12–18 carbons in chain length with an oxygenated functional group and one to three double bonds. Specific enzymes found in pheromone glands of female moths are involved in key steps of the pheromone biosynthetic pathway. The pathway generally includes fatty acid synthetases, desaturases, limited chain-shortening enzymes, and functional modification of the carbonyl carbon to make species-specific pheromone blends. Biosynthetic pathways in a variety of moths have been elucidated since the first pathway was determined in *Trichoplusia ni* (Bjostad and Roelofs, 1981; Jurenka, 2003). Pheromone biosynthesis was demonstrated by monitoring the incorporation of labeled precursors into fatty acid intermediates and pheromone components. Specific desaturases play a predominant role in producing unsaturated intermediates from saturated fatty acids of various chain lengths. Limited chain-shortening of longer chain-length intermediates will produce additional fatty acid intermediates. The concerted action of these enzymes will produce the species-specific pheromone blends (Jurenka, 2003).

In the *Heliothis* and *Helicoverpa* genera, all species investigated so far use (Z)-11-hexadecenal (Z11-16:Ald) as their major sex pheromone component, except *Helicoverpa assulta* (Cork et al., 1992) and *Helicoverpa gelotopoeon* (Cork and Lobos, 2003), which utilize (Z)-9-hexadecenal (Z9-16:Ald) as the major

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sex pheromone component. Minor components found in *Heliothis subflexa* are Z9-16:Ald, (Z)-11-hexadecenyl acetate (Z11-16:OAc), (Z)-9-hexadecenyl acetate (Z9-16:OAc), (Z)-11-hexadecen-1-ol (Z11-16:OH), and (Z)-9-hexadecen-1-ol (Z9-16:OH) (Klun et al., 1982). The minor components of *Heliothis virescens* include, Z9-16:Ald, Z11-16:OH, (Z)-9-tetradecenal (Z9-14:Ald), and hexadecenal (16:Ald) (Klun et al., 1980). The major and minor components are most likely made from precursors of 16- or 18-carbon saturated fatty acids and have similar biosynthetic steps to formation of the Z11-16:Ald as shown in *H. zea* (Jurenka et al., 1991; Teal and Tumlinson, 1986).

In the present study, we determined which precursor fatty acid is utilized to produce major and minor pheromone components in pheromone gland cells of *H. subflexa* and *H. virescens* by monitoring deuterium-labeled precursor incorporation. We were especially interested in determining how the minor pheromone components were formed in both species. It appears that the Z9-16 components found in *H. subflexa* are produced through Δ11 desaturation of 18:Acid followed by chain shortening. The Z9-14:Ald found in *H. virescens* is produced by chain shortening Z11-16:acid followed by reduction to the aldehyde.

MATERIALS AND METHODS

Insects

Colonies of *H. subflexa* and *H. virescens* were maintained at North Carolina State University and pupae shipped to Iowa State University. Pupae were sexed and allowed to emerge separately. A sucrose solution (10%) was provided to adults. Virgin female adults in their third or forth photophase were used throughout this study.

Materials

The deuterium-labeled fatty acids: (14, 14, 14-D_3)-tetradecanoic acid (D_3-14:Acid), (16,16,16-D_3)-hexadecanoic acid (D_3-16:Acid) (ICON Services Inc., Summit, NJ), and (18,18,18-D_3)-octadecanoic acid (D_3-18:Acid) (CDN Isotopes Inc., Pointe-Claire, Quebec) were dissolved in dimethyl sulfoxide (DMSO). Synthetic *H. zea* PBAN (Peninsula Laboratories, Belmont, CA) was dissolved in an insect saline (21 mM KCl, 12 mM NaCl, 3 mM $CaCl_2$, 18 mM $MgCl_2$, 170 mM glucose, and 5 mM pipes buffer at pH 6.6 using KOH).

Incorporation Procedure

The pheromone precursors, D_3-14:Acid, D_3-16:Acid, and D_3-18:Acid, were used to monitor the biosynthetic pathway by topical application directly to the gland. Deuterium-labeled compounds dissolved in DMSO (10 μg/gland) were applied to the surface of the pheromone gland, which was extruded using soft forceps. The gland was extruded for 10 min and then allowed to resume its normal position. A saline solution with or without PBAN (10 pmol) was then injected through abdominal intersegmental membranes into the hemocoel. Insects were left for 1 h at room temperature, and then the pheromone glands were removed, extracted, and analyzed for pheromones as described previously (Choi et al., 2002).

Analyses by GC-MS

Glands were extracted with hexane (100 μl) containing 100 ng *Z*-9 tetradecenal (Z9-14:Ald) as an internal standard for 10 min. The extracts were analyzed by single ion monitoring using gas chromatography and mass spectroscopy (GC-MS). Incorporation of the labeled precursors into pheromones was determined by GC-MS using a Hewlett-Packard (Corvallis, OR) 5890 II mass selective detector coupled with a Hewlett-Packard 5890 GC equipped with 30-m capillary columns, DB-5 (30 m × 0.25 mm; J&W Scientific, Folsom, CA) or EC-wax (30 m × 0.25 mm; Alltech, Deerfield, IL). The oven was temperature programmed at 60°C for 1 min, then 10°C/min to 300°C and held for 5 min when using the DB-5 column and programmed at 80°C for 1 min, then 10°C/min to 220°C and held for 15 min when using the EC-wax column. The mass selective detector was used in the single ion monitoring mode

to detect the following characteristic ions: 192 for Z9-14:Ald, 220 for Z9- and Z11-16:Ald, and 222 for Z9- and Z11-16:OH, and Z9- and Z11-16:OAc. Labeled compounds were detected using the characteristic ion +3 when D_3-14:Acid, D_3-16:Acid, and D_3-18:Acid were administered to the gland.

RESULTS AND DISCUSSION

Pheromone amounts in heliothines are typically cyclic with low amounts during the photophase and increasing amounts during the scotophase coinciding with the calling period. Production of pheromone is regulated in part by the neuropeptide PBAN and injection during the photophase will stimulate pheromone production (Teal et al., 1993). In order to ensure maximum amount of label incorporated after topical application to the pheromone gland, PBAN was injected into females during the photophase. Although pheromone titers were not measured in this study, significant increases in amounts occurred in response to PBAN treatment. A detailed study using *H. virescens* and *H. subflexa* has indicated that PBAN treatment results in a similar pheromone profile to that of untreated females during the scotophase (Groot et al., 2005). Therefore, PBAN treatment provides an efficient method to increase pheromone amounts during labeling studies.

The illustrations in Figures 1 and 2 obtained from GC-MS analysis of pheromone gland extracts indicate typical results found with the labeling studies. As expected, when D_3-16:Acid was applied, the most abundantly labeled aldehyde was Z11-16:Ald, which is the major pheromone component of both species. The minor pheromone components Z11-16:OH and Z11-16:OAc were also labeled when D_3-16:Acid was applied to pheromone glands. These results indicate that a Δ11 desaturase introduces the double bond into 16:Acid to produce the intermediate Z11-16:Acid, which is then modified at the carbonyl carbon to yield a pheromone component. Previous labeling studies conducted with *Helicoverpa zea* (Choi et al., 2002; Jurenka et al., 1991) and *H. virescens* (Foster, 2004) demonstrated the same results. In fact, Δ11 desaturation in pheromone biosynthetic pathways of moths is a common occurrence (Jurenka, 2003).

Labeling patterns of the minor pheromone components are of more interest because they

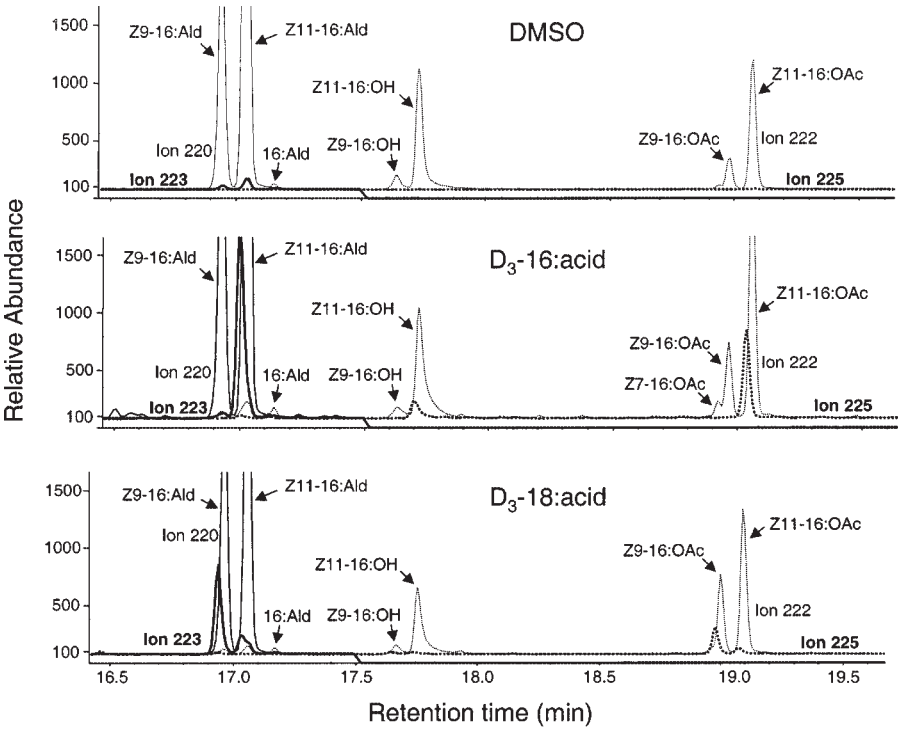

Fig. 1. Representative partial chromatograms obtained from GC/MS analysis of hexane extracts of pheromone glands of *H. subflexa* after topical application of 1 µl DMSO (DMSO) or 1 µl DMSO containing 10 µg D_3-16:Acid (D_3-16:Acid) or 1 µl DMSO containing 10 µg D_3-18:Acid (D_3-18:Acid). The GC/MS was set in the selective ion mode and the ions 220, 223, 222, and 225 are shown. Ions 223 and 225 are shown in bold and represent the label incorporation from the deuterium-labeled substrates. The chromatograms were produced using a DB-5 capillary column.

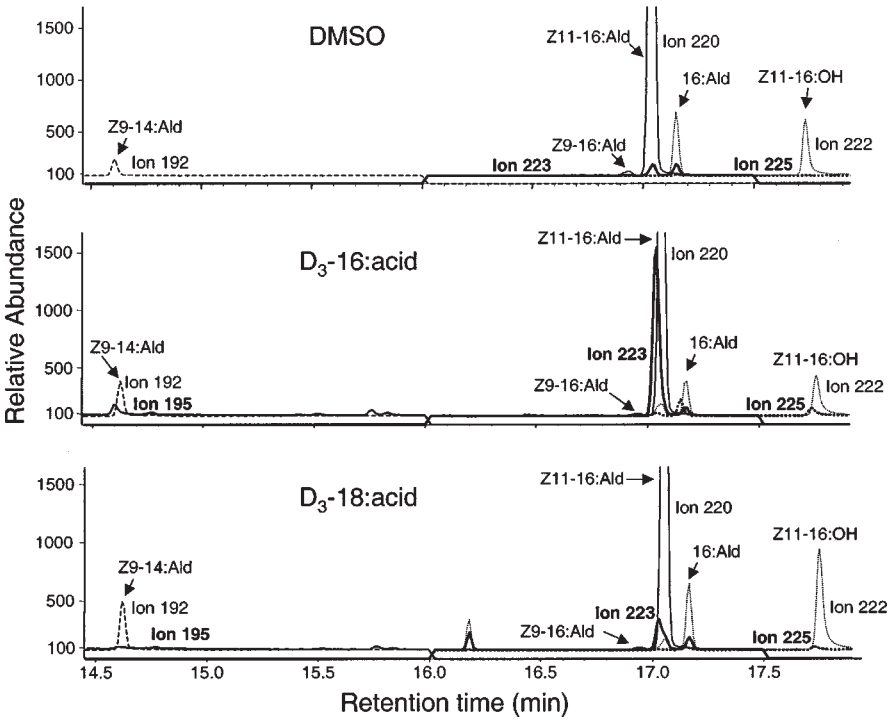

Fig. 2. Representative partial chromatograms obtained from GC/MS analysis of hexane extracts of pheromone glands of *H. virescens* after topical application as described in Figure 1. The GC/MS was set in the selective ion mode and the ions 192, 195, 220, 223, 222, and 225 are shown. Ions 195, 223, and 225 are shown in bold and represent the label incorporation from the deuterium-labeled substrates. The chromatograms were produced using a DB-5 capillary column.

could be produced through two possible pathways. The Z9 compounds could be produced directly through the action of a Δ9 desaturase or they could be produced by chain shortening a Δ11 desaturated longer chain fatty acid. Both pathways are known to occur in the biosynthesis of various sex pheromone components in moths (Jurenka, 2003). To help determine which pathway is occurring, the labeling pattern between D_3-16:Acid and D_3-18:Acid can be compared. This is best observed with *H. subflexa* due to the greater abundance of the Z9 components (Fig. 1). As expected, D_3-16:Acid was incorporated into all three Z11-16 compounds but no label incorporation was detected in the Z9-16 compounds. In contrast, D_3-18:Acid was incorporated into Z9-16:Ald, Z9-16:OAc, and a minor amount into Z9-16:OH. Some incorporation of D_3-18:Acid was observed in the Z11-16 compounds indicating that 18:Acid could be chain shortened to 16:Acid with subsequent Δ11 desaturation. This labeling pattern favors a pathway in which the Z9 compounds are produced through chain shortening a Z11-18:Acid precursor (Fig. 3).

Another interpretation of this labeling pattern is that the D_3-18:Acid could be chain shortened to 16:Acid with subsequent Δ9 desaturation. If this were the case, we would expect a significant amount of labeling when D_3-16:Acid was utilized. Therefore, we believe that a Δ9 desaturase is not active in producing the 16-carbon components in *H. subflexa*. On the other hand, labeling of the Z9-14:Ald, detected in pheromone glands of *H. virescens*, when D_3-16:Acid was utilized, may indicate that a Δ9 desaturase is present (Fig. 2). This incorporation could occur through direct Δ9 desaturation of 14:Acid or through chain shortening of Z11-16:Acid. To help determine which pathway occurs in *H. virescens*, D_3-14:Acid was applied to glands and analyzed using the EC-Wax column. This acid was not incorporated into any pheromone component (data not shown). These results indicate that Z9-14:Ald is made by chain-shortening Z11-16:Acid to Z9-14:Acid with subsequent aldehyde formation.

A series of desaturase-encoding genes have been identified from a number of moths (Knipple and Roelofs, 2003). There appears to be some desaturases that are specific to the pheromone gland and are involved in pheromone biosynthesis. An interesting finding of these studies is that addi-

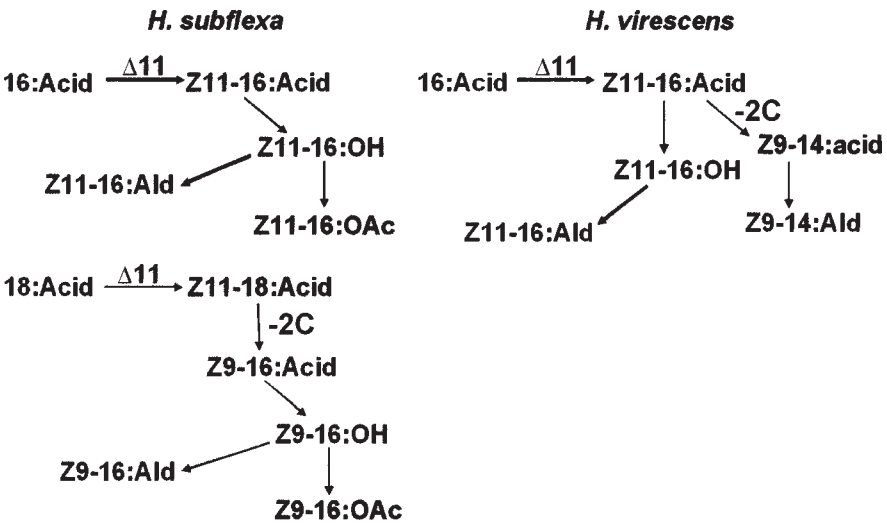

Fig. 3. Proposed pathways for the biosynthesis of the major pheromone components produced by females of *H. subflexa* and *H. virescens*. Δ11 = Δ11 desaturase; -2C = chain-shortening by 2 carbons.

tional desaturases are found in pheromone glands than are apparently needed to biosynthesize pheromone. The most ubiquitous is a Δ9 desaturase that is similar but different from the Δ9 desaturase found in the fat body. It is unknown whether or not these additional desaturases are expressed and functional in pheromone glands. It is apparent that in the case of most heliothines, a functional Δ9 desaturase is not involved in pheromone production (Choi et al., 2002). However, a functional Δ11 desaturase is found in pheromone glands and this desaturase has been cloned from *H. zea* (Rosenfield et al., 2001). Presumably a similar desaturase would be found in *H. virescens* and *H. subflexa*.

Two other minor components, Z7-16:OAc and Z7-16:Ald, are found in pheromone glands of *H. subflexa* and are produced at very low levels (Klun et al., 1982). In the present study, we could not determine how these components are produced due to the lack of incorporation from any deuterium labeled precursor. However, the Z7-16:OAc and Ald are probably produced by chain-shortening the ubiquitous Z9-18:Acid to Z7-16:acid followed by carbonyl carbon modification. This biosynthetic pathway for producing Z7-16:Ald as minor component of *H. zea* has been demonstrated (Choi et al., 2002).

In conclusion, *H. subflexa* females utilize a 16-carbon fatty acid to produce Z11-16:Ald, Z11-16:OH, and Z11-16:OAc, and an 18-carbon fatty acid to produce Z9-16:Ald, Z9-16:OH, and Z9-16:OAc. A Δ11 desaturase is likely the only active enzyme for desaturation and will utilize both 16- and 18- carbon fatty acids as substrates to produce all pheromone components in this species. *Heliothis virescens* utilizes a 16-carbon fatty acid only to produce the pheromone components, Z11-16:Ald, Z11-16:OH, and Z9-14:Ald, indicating that a similar Δ11 desaturase is active in the gland. In both females, a chain-shortening enzyme is active to shorten long-chain fatty acids to form specific pheromone precursors.

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REFERENCES

Bjostad LB, Roelofs WL. 1981. Sex pheromone biosynthesis from radiolabeled fatty acids in the redbanded leafroller moth. J Biol Chem 256:7936–7940.

Choi M-Y, Han KS, Boo KS, Jurenka R. 2002. Pheromone biosynthetic pathway in *Helicoverpa zea* and *Helicoverpa assulta*. Insect Biochem Mol Biol 32:1353–1359.

Cork A, Lobos E. 2003. Female sex pheromone components of *Helicoverpa gelotopoeon*: first heliothine pheromone without (Z)-11-hexadecenal. Entomol Exp Appl 107:201–206.

Cork A, Boo KS, Dunkelblum E, Hall DR, Jee-Rajunga K, Kehat M, Jie EK, Park KC, Tepgidagarn P, Xun L. 1992. Female sex pheromone of Oriental tobacco budworm, *Helicoverpa assulta* (Guenee) (Lepidoptera: Noctuidae): identification and field testing. J Chem Ecol 18:403–418.

Foster SP. 2004. The effects of topical application of various fatty acids on pheromone and glandular lipid biosynthesis in the moth *Heliothis virescens*. Insect Biochem Mol Biol 34:467–475.

Groot A, Fan Y, Brownie C, Jurenka R, Gould F, Schal C. 2005. Effect of PBAN on the sex pheromone profile in mated *Heliothis virescens* and *Heliothis subflexa* females. J Chem Ecol 31:15–28.

Jurenka RA. 2003. Biochemistry of female moth sex pheromones. In: Blomquist G, Vogt R, Blomquist G, Vogt R, editors. Insect pheromone biochemistry and molecular biology. New York: Academic Press; p 53–80.

Jurenka RA, Jacquin E, Roelofs WL. 1991. Control of the pheromone biosynthetic pathway in *Helicoverpa zea* by the pheromone biosynthesis activating neuropeptide. Arch Insect Biochem Physiol 17:81–91.

Klun JA, Bierl-Leonhardt BA, Plimmer JR, Sparks AN, Primiani M, Chapman OL, Lepone G, Lee GH. 1980. Sex pheromone chemistry of the female tobacco budworm moth, *Heliothis virescens*. J Chem Ecol 6:177–183.

Klun JA, Leonhardt BA, Lopez JD, Lachance LE. 1982. Female *Heliothis subflexa* (Lepidoptera, Noctuidae) sex pheromone: chemistry and congeneric comparisons. Environ Entomol 11:1084–1090.

Knipple DC, Roelofs WL. 2003. Molecular biological investigations of pheromone desaturases. In: Blomquist GJ, Vogt RG, Blomquist GJ, Vogt RG, editors. Insect pheromone biochemistry and molecular biology. New York: Academic Press; p 81–106.

Rosenfield C, You KM, Marsella-Herrick P, Roelofs WL, Knipple DC. 2001. Structural and functional conservation and divergence among acyl-CoA desaturases of two noctuid species, the corn earworm, *Helicoverpa zea*, and the cabbage looper, *Trichoplusia ni*. Insect Biochem Mol Biol 31:949–964.

Teal PEA, Tumlinson JH. 1986. Terminal steps in pheromone biosynthesis by *Heliothis virescens* and *H. zea*. J Chem Ecol 12:353–366.

Teal PEA, Oostendorp A, Tumlinson JH. 1993. Induction of pheromone production in females of *Heliothis virescens* (F.) and *H. subflexa* (GN.) (Lepidoptera: Noctuidae) during photophase. Can Entomol 125:355–366.