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SUBSTRATE SPECIFICITY OF ACETYLTRANSFERASE AND REDUCTASE ENZYME SYSTEMS USED IN PHEROMONE BIOSYNTHESIS BY ASIAN CORN BORER, *Ostrinia furnacalis*[1]

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Abstract—The substrate specificity of the acetyltransferase and the reductase enzyme systems used by *Ostrinia furnacalis* (Lepidoptera: Pyralidae) in pheromone biosynthesis was studied in vivo by topical application of precursors to pheromone glands. Each of the tetradecenols, varying in double bond position (from 7 to 13) and geometry of the double bond, was converted to the corresponding acetate by the acetyltransferase. The similarity in the conversion rates of all tested fatty alcohols indicated that the acetyltransferase has a low substrate specificity. Most of the corresponding tetradecenoic acids could also be converted to the respective acetates. However, very different conversion rates among the tested fatty acids demonstrated that the reductase system has a higher substrate specificity than the acetyltransferase. The conversion rates of most *E* isomers were higher than those of the corresponding *Z* isomers, except for the (Δ)-11-tetradecenoic acids, in which much more *Z* isomer was converted to the product. Saturated tetradecanoic acid was converted to the corresponding acetate at a high rate; the shorter homolog, tridecanoic acid, was converted at a lower rate (56%), and conversion to the respective acetates of the longer homolog, pentadecanoic and hexadecanoic acids, was insignificant (<5%). The results from the present study showed that specificity of pheromone production is to a large extent controlled by the pheromone gland reductase system.

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INTRODUCTION

Many moths use *E/Z* isomers of monounsaturated acetates in specific ratios in their sex pheromones. The sex pheromone of *Ostrinia furnacalis* Guenée (Lepidoptera: Pyralidae), consists of (*E*)- and (*Z*)-12-tetradecenyl acetate (*E*12- and *Z*12-14:OAc) in a 47:53 ratio (Cheng et al., 1981). Two pheromone strains of the European corn borer, *Ostrinia nubilalis* Hübner, both have (*E*)- and (*Z*)-11-tetradecenyl acetate (*E*11- and *Z*11-14:OAc) as pheromone components but in different ratios: an *E/Z* ratio of 99:1 was found in *E* strain, whereas the same compounds were produced in a 3:97 ratio in the *Z* strain (Kochansky et al., 1975; Klun and Cooperators, 1975). Studies of the pheromone biosynthesis in both species showed a difference between isomeric ratios of acetates and the corresponding fatty acid precursors in the pheromone glands. In *O. furnacalis*, the ratio of (*E*)- and (*Z*)-12-tetradecenoate (*E*- and *Z*12-14:OAcyl) is about 12:88 (Zhao et al., 1990). Both the *E* and *Z* strains of *O. nubilalis* have a 70:30 *E/Z* ratio of Δ11-tetradecenoate (Δ11-14:Acyl) (Wolf and Roelofs, 1987; Jurenka and Roelofs, 1989). The specific *E/Z* ratio of acetates in *O. nubilalis* and *O. furnacalis* may thus be produced from the immediate fatty acid precursors by the selectivity of the postulated last two steps in the pheromone biosynthetic pathway: reduction and acetylation (Wolf and Roelofs, 1987; Zhao et al., 1990).

Recently, in vivo experiments demonstrated that the acetylation step has a very low substrate specificity in three noctuid species (Teal and Tumlinson, 1987; Bestmann et al., 1987; Dunkelblum et al., 1989). In contrast, an in vitro enzyme assay showed that the acetyltransferase exhibited some selectivity for the *Z* isomer of Δ11-tetradecenol (Δ11-14:OH) in *Argyrotaenia velutinana* Walker and three other species of tortricid moths, but not in *E*, *Z* and hybrid strains of *O. nubilalis*. Thus, in the later case, the *E/Z* acetate ratios produced may be due to the selectivity of the reduction step, whereas in *A. velutinana* the specific *E/Z* ratio may be controlled by the coupling of the reductase and the acetyltransferase (Jurenka and Roelofs, 1989). In general, the reductase system should be very important for the production of a specific ratio of acetates in many moth species, including tortricid moths (Morse and Meighen, 1987; Jurenka and Roelofs, 1989). However, so far the substrate specificity of the reductase in moth pheromone biosynthesis systems has not been experimentally investigated.

In the present study, we investigated the selectivity of the acetyltransferase and the reductase enzymes in *O. furnacalis* based on in vivo experiments using

a series of tetradecenols (Δ14 : OH) and tetradecenoic acids (Δ14 : Acid) as substrates.

METHODS AND MATERIALS

Chemicals. Tetradecenols, varying in double bond position from 7 to 13 as well as in geometry of the double bond, were purchased from the Research Institute for Plant Protection (Wageningen, The Netherlands). Gas chromatographic analyses using a BP-20 capillary column showed that the purity of the alcohols ranged from 96.3 to 99.4% and that the corresponding geometric isomer in each compound was less than 1%. Tetradecanol (14 : OH) and tridecanoic acid (13 : Acid) were available in our laboratory. [14,14,14-$^2H_3$]Tetradecanoic acid ([14-D_3]14 : Acid), [15,15,15-$^2H_3$]pentadecanoic acid ([15-D_3]15 : Acid), and [16,16,16-$^2H_3$]hexadecanoic acid ([16-D_3]16 : Acid) were purchased from Larodan Fine Chemicals, Malmö, Sweden.

Each of the tetradecenoic acids was prepared from the corresponding alcohol by a method modified from Corey and Smidt (1979): 45 μl of an alcohol, 600 mg pyridinium dichromate (PDC), and 2 ml dry dimethylformamide (DMF) were added to an 8-ml vial with a Teflon-lined screw cap. After 12 hr at 25°C without stirring, the reaction was stopped and the rest of the steps were performed as described by Corey and Smidt (1979).

The products were purified by centrifugal chromatography. Petroleum ether (60–90°)–ether–acetic acid (90 : 10 : 1) was used as the developing solvent at 3 ml/min flow rate. The fractions containing tetradecenoic acids were combined, and the solvent was removed with a stream of nitrogen. Capillary GC analyses of the fatty acids after conversion to methyl esters indicated that all were at least 96% pure and no corresponding alcohol or aldehyde was detected (the limit of detection was 0.1%). The yield for the different acids varied between 50 and 70%.

Deuterium-labeled pheromone precursors were synthesized by the procedures described below. High-resolution mass spectra of final products were recorded on a Jeol SX-102 mass spectrometer. ^1H NMR spectra were recorded on a Varian XL-300 spectrometer in $CDCl_3$ solutions with Me_4Si as the internal reference. Products were purified by flash chromatography (Taber, 1982) on TLC-Silica gel 60 H supplied by Merck and argentation liquid chromatography (Houx et al., 1974). DMPU was purchased from Fluka AB. Immediately before use, it was distilled over CaH_2 at reduced pressure and kept over 4 Å molecular sieves under an argon atmosphere.

(*Z*)-[14,14,14-$^2H_3$]12-Tetradecenoic acid (**1**) ([14-D_3]*Z*12–14 : Acid) (Figure 1) was prepared from 1-(2-tetrahydropyranyloxy)-dodecyne (6.0 g, 21 mmol) (**3**) (Smith and Beumel, 1974; Rossi et al., 1980) *n*-butyl lithium (11 ml, 2.09

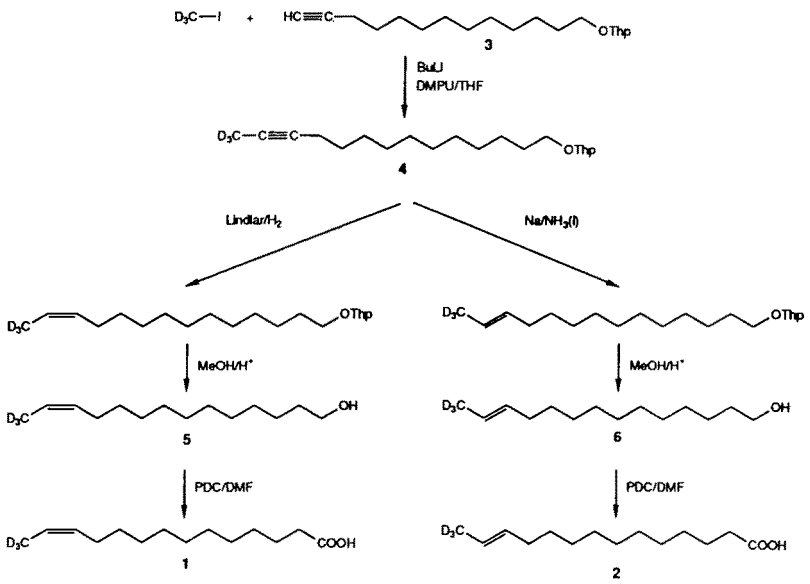

FIG. 1. Scheme for the synthesis of (*Z*)- and (*E*)-[14,14,14-$^2H_3$]12-tetradecenoic acid.

M in hexane) in dry THF (21 ml) and [$^2H_3$]methyl iodide (4.1 g, 34 mmol) in DMPU (36 ml) according to a method previously described for similar systems (Bengtsson and Liljefors, 1988) affording 5.6 g (88%) of the product (**4**) (Figure 1) after flash chromatography. Reduction with Lindlar catalyst (Leznoff et al., 1977; Wong et al., 1984) gave the *Z* monoene, which was converted to the corresponding alcohol (**5**) by treatment with *p*-toluenesulfonic acid in methanol. The fatty alcohol (**5**) was oxidized by the same method used in the synthesis of Δ14:Acid and purified with liquid chromatography.

The final product (**1**) was obtained in 60% overall yield. *m/z* 229 (M^+, 11%), 211(31), 169(17), 137(13), 124(20), 110(31), 97(49), 83(60), 69(100), 58(77), 55(97), 41(51). High-resolution mass spectra: $[M]^+_{calc}$ = 229.2121, $[M]^+_{obs}$ = 229.2114. ^1H NMR (300 MHz); δ 1.27–1.35 (m, 14H, CH_2CH_2), 1.58–1.65 (m, 2H, CH_2—C—COOH), 1.99–2.05 (m, 2H, CH_2—C=C), 2.35 (t, 2H, CH_2—COOH), 5.36–5.41 (m, 2H, CH=CH). Capillary GC analyses showed that the purity of (*Z*) [14,14,14-$^2H_3$]12-tetradecenol ([14-D_3]*Z*12-14:OH) was 97.4%, and no *E* isomer was detected (the limit of detection was 0.1%). The purity of [14-D_3]*Z*12-14:Acid was 97.4%, with no corresponding alcohol detected.

(*E*)-[14,14,14-$^2H_3$]12-Tetradecenoic acid (**2**) ([14-D_3]*E*12-14:Acid) was prepared as described above for the corresponding *Z* isomer (**1**), except that the

reduction of the triple bond was instead performed with sodium in liquid ammonia (Warthen and Jacobson, 1973) to give the desired *E* monoene. *m/z* 229 (M^+, 11%), 211(27), 193(4), 169(15), 138(12), 123(19), 110(31), 97(46), 83(57), 69(88), 58(90), 55(100), 43(42), 41(59). High-resolution mass spectra: $[M]^+_{calc}$ = 229.2121, $[M]^+_{obs}$ = 229.2115. ^1H NMR (300 MHz); δ 1.23–1.28 (m, 14H, CH_2CH_2), 1.60–1.65 (m, 2H, CH_2—C—COOH), 1.92–1.98 (m, 2H, CH_2—C=), 2.32 (t, 2H, CH_2—COOH), 5.39–5.42 (m, 2H, CH=CH). Capillary GC analyses showed that the purity of (*E*)-[14,14,14-$^2H_3$]12-tetradecenol ([14-D_3]*E*12-14:OH) was 94.0%, and the corresponding *Z* isomer was 0.9%. The purity of [14-D_3]*E*12-14:Acid was 95.7%, and no corresponding alcohol was detected.

Insect Source, Application of Fatty Alcohol or Fatty Acid to Pheromone Glands. Insects were reared as previously described (Zhao et al., 1990). Pupae were sexed and then kept under a 16:8 hr light–dark cycle. The application procedure was as follows, unless otherwise specified: 5–8 hr after dark, 24 to 48 hr old female moths were used for topical application (Bjostad and Roelofs, 1981; Zhao et al., 1990) of a tested compound. (*E*)-11-Tetradecenol (*E*11-14:OH) and (*E*)-11-tetradecenoic acid (*E*11-14:Acid) were chosen as metabolic standards for studying substrate specificity of the acetyltransferase and the reductase, respectively. The glands were exposed by gently squeezing the female abdomen. To each pheromone gland we applied 0.05 μl dimethyl sulfoxide (DMSO) containing 500 ng of a mixture of a standard and the compound to be tested in an approximate 1:1 ratio. About 15 min after application, the pheromone glands (5 glands/batch for the acetyltransferase study and 10 glands/batch for the reductase study) were extracted with hexane containing 6 ng of pentadecyl acetate as an internal standard for about 1 hr.

Chain length specificity of the reductase was investigated in two separate experiments. In the first experiment, a mixture of 13:Acid, [14-D_3]14:Acid, and [15-D_3]15:Acid was applied to glands of 3–9 females. Conversion rates in each batch (replicate) were calculated relative to that of [14-D_3]14:Acid. In the second experiment, a mixture of 13:Acid and [16-D_3]16:Acid was applied to glands of 5–10 females.

Gas Chromatography and Combined Gas Chromatography–Mass Spectrometry. Gas chromatographic (GC) analyses were performed on a Pye Unicam 204 GC equipped with a splitless capillary injector, a flame ionization detector, and an HP 3394 integrator. The linear velocity of the carrier gas (hydrogen) was 50 cm/sec and the purge valve was opened 0.25 min after injection. The following columns were used for the separation of various positional and geometric isomers of tetradecenyl acetates in the extracts: (A) 25 m × 0.22 mm ID BP-20 (Pye Unicam), temperature program: 1 min at 80°C and then 3°/min to 200°C; (B) 25 m × 0.22 mm ID BP-5 (SGE), temperature program: 1 min at 80°C and then 4°/min to 200°C; (C) 50 m × 0.22 mm ID BP-20 (SGE), temperature program: 1 min at 80°C and then 2°/min to 200°C; and (D) 51 m

× 0.22 mm ID CPSIL 88 (Chrompack), temperature program: 1 min at 80°C and then 2°/min to 200°C.

Gas chromatographic–mass spectrometric analyses (GC-MS) were performed using an Hewlett-Packard 5970B GC-MS system (electron impact, 70 eV) equipped with a 59970B computer system and interfaced with an Hewlett-Packard model 5890 GC. The diagnostic ions *m/z* 194.20 and *m/z* 197.20 were selected in an acquisition program to monitor unlabeled and D_3-labeled tetradecenyl acetates, respectively. Other acetates were also monitored at ions corresponding to [M-60].

Relative Conversion Factors. To define substrate specificity, a relative conversion factor was calculated for each compound tested. The relative conversion factor (RCF) for a specific compound was calculated as:

$$RCF = (\text{Test:OAc}/E11\text{–}14\text{:OAc}) \times (\text{Met stand/test compound})$$

Test:OAc/*E*11–14:OAc is the ratio between the acetate generated from the test compound and *E*11–14:OAc generated from the metabolic standard in gland extracts after incubation as determined by GC or GC-MS analyses. Met stand/test compound is the GC-determined ratio between the amounts of the compound to be tested and the metabolic standard in the solution that was applied to the gland (this ratio was always close to 1 but varied slightly between mixtures, which made this correction necessary).

Synthetic samples of the naturally occurring pheromone precursors, (*E*)- and (*Z*)-12-tetradecenol (*E*12- and *Z*12–14:OH), were initially used to determine the relative conversion factors for these isomers. The amount of *E*12- or *Z*12–14:OAc from the corresponding applied precursor was calculated by subtraction of the average amount of natural pheromone in a control group (no acids applied). The amount of tetradecanyl acetate (14:OAc) produced from applied 14:OH was calculated in a similar way (a normal ratio between native 14:OAc and one of the pheromone components was determined in control groups); thus, the relative conversion factor of 14:OH was calculated. The deuterated precursors [14-D_3]*E*12- and [14-D_3]*Z*12–14:OH were synthesized in the course of the study, and the relative conversion factors of *E* and *Z*12–14:OH were confirmed by the application of these compounds.

The relative conversion factors of (*E*)- and (*Z*)-12-tetradecenoic acid (*E*12- and *Z*12–14:Acid) were also determined with both unlabeled and labeled acids ([14-D_3]*E*- and [14-D_3]*Z*12–14:Acid, compounds **2** and **1** and Figure 1), using the same approach as in the acetyltransferase study.

RESULTS

Dosage for Topical Application. In an initial experiment, 100 ng of (*Z*)-11-tetradecenoic acid (*Z*11–14:Acid) was applied to the pheromone glands. One

batch of 10 female glands was incubated for 15 min, and another one was incubated for 30 min. Gas chromatographic analyses showed that the average recovered amount of Z11-14:OAc was significantly larger after 15 min of incubation than after an incubation time of 30 min (3.03 and 1.16 ng, respectively; t test, $P \leq 0.05$, $N = 5$). Thus, 15 min of incubation was used in all of the following experiments. Glands incubated with larger amounts of Z11-14:Acid produced slightly more Z11-14:OAc. It was evident from this experiment that the amount of the product may vary considerably between replicates (Figure 2). We subsequently used a dosage of 250 ng of a tested fatty acid or a tested fatty alcohol throughout the study.

Acetyltransferase Specificity. In the study of the acetyltransferase specificity, each of the tetradecenols tested, as well as the saturated 14:OH, was converted to the corresponding acetate with similar relative conversion factors (Figure 3) with one exception. The exception was (Z)-7-tetradecenol (Z7-14:OH), for which the smallest relative conversion factor (0.56) was determined. The relative conversion factors for the other fatty alcohols were in the range of 0.72 to 1.01. For each pair of geometric isomers the E isomer was normally converted at a higher rate than the Z isomer. A typical mass chromatogram showed that [14-D_3]E12-14:OH and E11-14:OH were converted to the corresponding acetates in approximately equal amounts (Figure 4A). Very similar relative conversion factors of E- and Z12-14:OH were obtained from both unlabeled and labeled precursors (Figure 3).

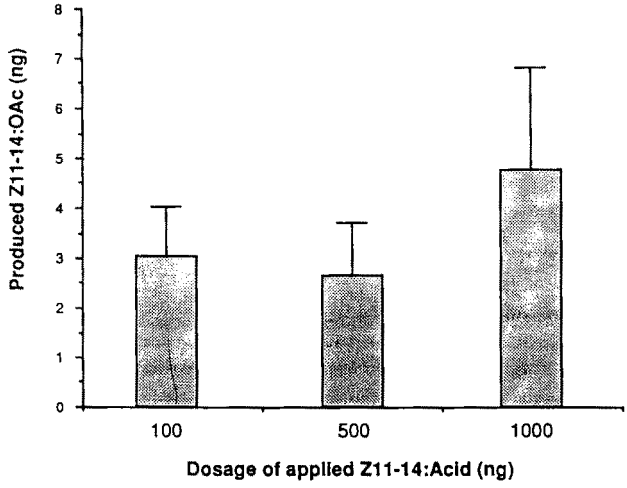

FIG. 2. Titer of Z11-14:OAc in pheromone glands of *O. furnacalis* as determined by GC analysis after topical application of different amounts of Z11-14:Acid and 15 min of incubation ($N = 5$, each batch consisted of 10 female glands).

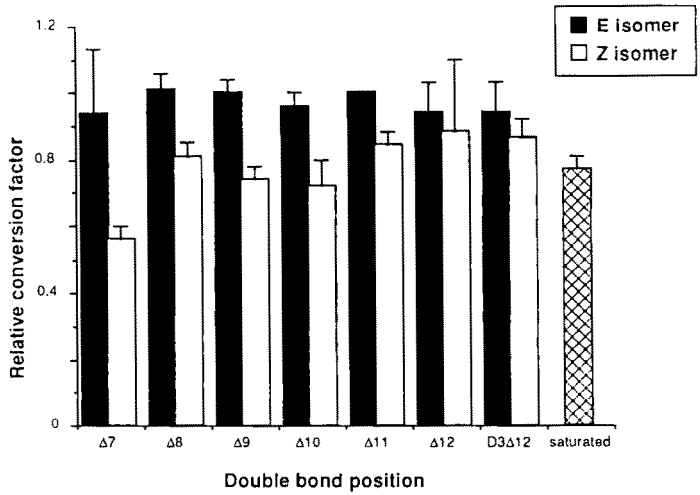

FIG. 3. Substrate preference of the acetyltransferase in *O. furnacalis* for 14-carbon alcohols. All conversion factors are normalized to that of *E*11–14:OH, which thus becomes 1.0 ± 0.0 by definition. The relative conversion factors of Δ7, Δ11, Δ12, and saturated 14:OH were determined by GC analyses of gland extracts from five females ($N = 6$). The relative conversion factors of Δ8, Δ9, Δ10, and [14-D_3]Δ12–14:OH (D3Δ12) were determined by GC-MS analyses of individual females ($N = 4$). Vertical bars indicate standard error of the mean.

Reductase Specificity. In the study of the reductase specifity, most of the tetradecenoic acids varying in double bond position (from 7 to 13) and geometry of the double bond, as well as saturated [14-D_3]14:Acid, were converted to the corresponding acetates, but with very different conversion factors (Figure 5).

The relative conversion factors of the *E* isomers of the tested Δ14:Acids were larger than those of the corresponding *Z* isomers, except for Δ11–14:Acid, in which the relative conversion factor of the *Z* isomer is much larger than that of the *E* isomer (Figure 5). The smallest conversion factor was found for the (*Z*)-7- and (*Z*)-8-tetradecenoic acids, from which the corresponding acetates could not be detected by GC upon incubation with pheromone glands. A typical mass chromatogram showed that [14-D_3]*E*12–14:Acid and *E*11–14:Acid were converted to the corresponding acetates at different rates (Figure 4B). The relative conversion factor for [14-D_3]*E*12–14:Acid was 2.6 ± 0.2, while the factor for [14-D_3]*Z*12–14:Acid was 1.4 ± 0.1. Thus, the relative conversion factor for the *E* isomer pheromone precursor was almost two times larger than that of the *Z* isomer. This relation was also found for unlabeled pheromone precursors (Figure 5).

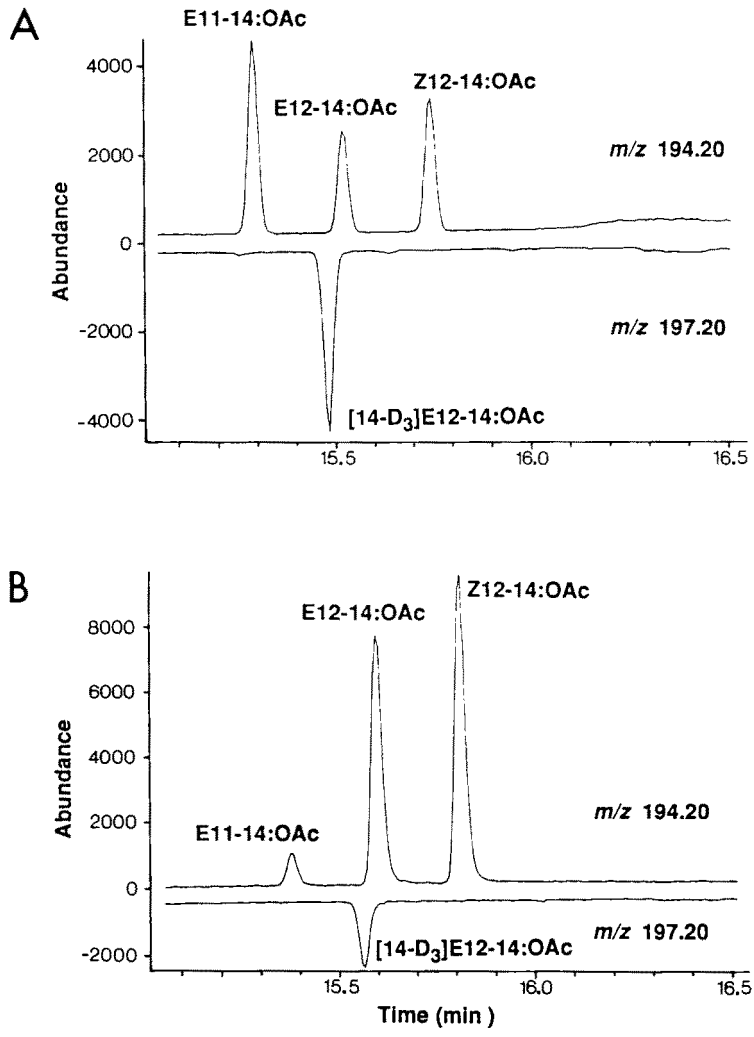

FIG. 4. Mass chromatograms from analyses of the pheromone gland extracts of *O. furnacalis* showing labeled and native pheromone components after application of labeled pheromone precursors. (A) The acetates produced from the extract of a gland after application of [14-D_3]E12-14:OH and *E*11-14:OH. (B) The acetates produced from the extract of two glands after application of [14-D_3]*E*12-14:Acid and *E*11-14:Acid.

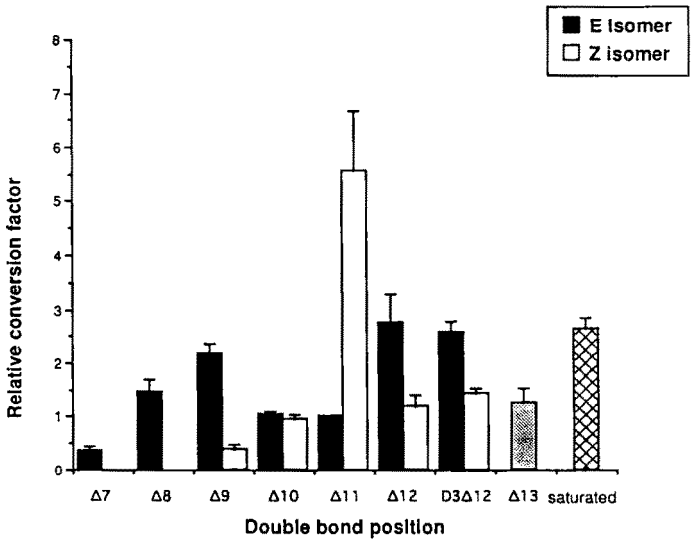

FIG. 5. Substrate preference of the fatty acid reductase in *O. furnacalis* for 14-carbon acids. All conversion factors are normalized to that of *E*11-14:Acid, which thus becomes 1.0 ± 0.0 by definition. The relative conversion factors of [14-D_3]Δ12-14:Acid (D3Δ12) were determined by GC-MS analyses of gland extracts from two females (N = 6). The relative conversion factors of other fatty acids including [14-D_3]14:Acid (saturated) were determined by GC analyses of pooled gland extracts from 10 females (N = 6). The relative conversion factors of *Z*7- and *Z*8-14:Acid were below the limit of detection. Vertical bars indicate standard error of the mean.

Conversion of 13:Acid to the corresponding acetate was less than 60% of that obtained with [14-D_3]14:Acid (Table 1). For the longer homolog, [15-D_3]15:Acid, conversion to acetate was less than 5% of that observed with the 14-carbon substrate, and with [16-D_3]16:Acid the production of acetate was below the limit of detection.

Production of Specific Ratios. *E/Z* ratios of the pheromone precursors and components obtained by GC-MS analyses of methanolized and acetylated gland extracts were compiled from two earlier investigations (Zhao and Wang, 1990; Zhao et al., 1990). It is apparent that an approximate 50:50 ratio of *E*12- and *Z*12-14:OAc is produced by the reductase and acetyltransferase from a very different ratio of *E*12- and *Z*12-14:Acyl found in the pheromone gland (15:85 and 24:76, in the two investigations, respectively). Furthermore, the ratio of *E*12- and *Z*12-14:Acyl was produced from another ratio of (*E*)- and (*Z*)-14-

TABLE 1. CONVERSION OF TOPICALLY APPLIED SATURATED FATTY ACIDS TO ACETATES IN PHERMONE GLANDS OF *O. furnacalis*

Substrate	Relative conversion	*N*
13 : Acid	56[a]	6
[14-D_3]14 : Acid	100[b]	6
[15-D_3]15 : Acid	< 5[c]	6
[16-D_3]16 : Acid	0[d]	4

[a] Range of observed values was 47.0–60.7.
[b] Conversion rates were calculated relative to that of [14-D_3]14 : Acid, which thus always became 100 by definition.
[c] Observed values were below the limit of quantification, but were always less than 5% of [14-D_3]14 : OAc.
[d] [16-D_3]16 : OAc was below the limit of detection in all experiments.

hexadecenoate (*E*14- and *Z*14–16 : Acyl, ca. 75 : 25 in the two studies) by a β-oxidase for chain shortening (Figure 6). When a 13 : 87 ratio of [14-D_3]*E*- and [14-D_3]*Z*12–14 : Acid (the ratio is similar to that found in the gland) was applied to the glands, an average ratio 28 : 72 $\pm$ 8.5 of [14-D_3]*E*- and [14-D_3]*Z*12 : OAc was obtained from the gland extracts ($N = 6$). The results implied that an approximate 50 : 50 ratio of *E*12- and *Z*12–14 : OAc cannot be produced by the reductase from the ratio of *E*12- and *Z*12–14 : Acyl found in the gland (Figures 5 and 6).

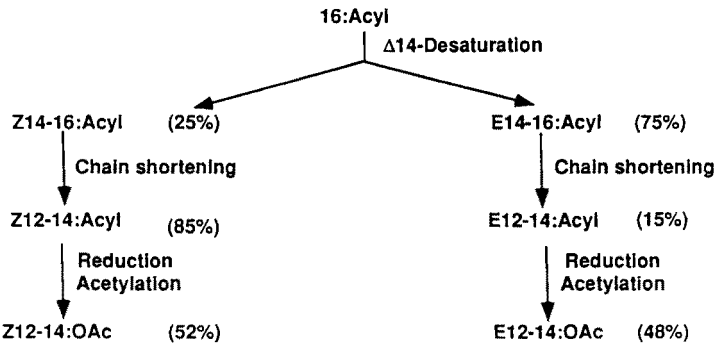

FIG. 6. Biosynthetic pathways for the production of pheromone components in *O. furnacalis* (after Zhao et al., 1990) with data added on the isomeric composition at each level.

DISCUSSION

The production of specific ratios between pheromone components in moths may be controlled by the selectivity of several postulated biosynthetic reactions. In the current study we have demonstrated a high chain-length specificity for the conversion of exogenously applied fatty acids to corresponding acetates in *O. furnacalis*. A highly selective conversion of positional and geometric isomers of tetradecenoic acid to the respective acetates was also shown. In contrast, the postulated final step in the pheromone biosynthesis, the acetylation of alcohols, is essentially nonselective with respect to the geometry and position of the double bond in the tetradecenol substrates. Our interpretation of these results is that the specificity of one or several reaction steps involved in the reduction of fatty acid precursors to alcohols is responsible for the production of a specific ratio between the pheromone components *E*12- and *Z*12-14:OAc.

Ideally it should be possible to study pheromone biosynthesis in individual insects to minimize the need for insect material and labeled precursors and to allow investigations of the biosynthetic basis for individual variation in pheromone composition. In the present study, we made some efforts to optimize the protocol for topical application and in vivo studies of the metabolism of pheromone precursors. We found that in *O. furnacalis*, variation of the dosage of the applied fatty acid between 100 and 1000 ng had no significant influence on the amount of product formed. This result is similar to what was found in *A. velutinana* for the application of tetradecanoic acid. When Bjostad and Roelofs (1986) increased the amount of saturated precursor from 100 to 4000 ng, the production of labeled (*E*)- and (*Z*)-11-tetradecenoate as well as labeled pheromone components increased only slightly. We subsequently used a standard dosage of 250 ng of both acid and alcohol precursors throughout our studies. Obviously, only a small percentage of the topically applied precursor is converted into pheromone components, and this fraction was even lower after 30 than after 15 min of incubation. The 15-min incubation time used in our studies of the acetyltransferase and the reductase in *O. furnacalis* was much shorter than that reported for the study of the actylation of alcohols in *Chrysodeixis chalcites* (Esper) (Dunkelblum et al., 1989) and *Hydraecia micacea* (Esper) (Teal and Tumlinson, 1987), but similar to what was used in the study of acetylation in *Mamestra brassicae* (L.) (Bestmann et al., 1987). In *M. brassicae*, the acetate could be found 5 min after the corresponding alcohol was applied to the pheromone gland. We believe that metabolism of precursors is generally very fast and 15 min of incorporation should normally be sufficient for in vivo studies of moth pheromone production. The decrease in titer of the products after longer periods of incubation could be due to release of pheromone by calling females or to recycling of labeled products, possible explanations that were not further investigated.

The calculation of a relative conversion factor in the present study is similar to the approach previously used by Dunkelblum et al. (1989). *E*11-14:OH and *E*11-14:Acid were chosen as metabolic standards for the acetyltransferase and the reductase study, respectively. These compounds are not naturally present in the gland but they are readily metabolized. The high variation between replicates in the formation of product, whether due to the performance of the insects or to the procedures used, makes the use of a metabolic standard important for every quantitative study of pheromone biosynthesis.

The results from the acetyltransferase study demonstrated low selectivity for tetradecenols with different position and geometry of the double bond. Low substrate specificity of the acetyltransferase was also observed in *H. micacea* (Teal and Tumlinson, 1987), *M. brassicae* (Bestmann et al., 1987), and *C. chalcites* (Dunkelblum et al., 1989). In all these noctuid species, in vivo experiments demonstrated that the acetylation was not specific with respect to carbon chain length, degree of unsaturation, or geometry of double bonds. In vitro experiments showed that 1.5–2.0 times more *Z* isomer of Δ11-14:OH was converted to the corresponding acetate by the acetyltransferase in *A. velutinana*, and similar results were reported for three other species of Tortricidae (Jurenka and Roelofs, 1989). However, in the same study no significant substrate selectivity with respect to *E*11- and *Z*11-14:OH was found in the *E*, *Z*, and hybrid strains of *O. nubilalis*, similar to what we report for *O. furnacalis*. We therefore conclude that the observed differences in substrate specificity among species are not due to the use of different techniques, but rather reflect real differences in insect biochemistry.

In contrast to the low selectivity of the acetyltransferase, high selectivity of the reductase system with respect to chain length and to double bond position and geometry was demonstrated by the experiments with different acid precursors. Because the acetyltransferase has low specificity for the series of corresponding tetradecenols as demonstrated above, the differences in relative conversion factors of the tested fatty acids should be due to high specificity of the reductase or other enzymes involved in the reduction of acyl precursors. To date little is known about the actual pathways for reduction of acids to aldehydes and alcohols in insects (Morse and Meighen, 1987). The fatty acid reductase elucidated in luminescent bacteria is part of a multienzyme complex (500 kDa), the so-called fatty acid reductase complex (for a review, see Meighen, 1993). In moth pheromone biosynthesis, the fatty acid reductase should generally be coupled to an aldehyde reductase, as fatty alcohols and not aldehydes are the normal product of the reduction (Morse and Meighen, 1987). Thus any one or several of the reaction steps taking place in this reductase complex could be responsible for the observed selectivity, In experiments with the European corn borer, *O. nubilalis*, we actually found that reduction of labeled aldehydes, postulated transient intermediates in sex pheromone biosynthesis, showed almost

the same selectivity as the overall conversion of free fatty acids to pheromone components, suggesting that the postulated aldehyde reductase alone may account for the selectivity (Zhu et al., 1995).

Moths do not normally make pheromones from exogenously applied precursors and the fatty acid precursors occur as acyl derivatives rather than free acids in the pheromone glands. Thus the observed selective conversion of precursors to acetates could theoretically be due to differences in uptake between different compounds, selective transport between cell compartments, or selective hydrolysis. However, the current study clearly demonstrates that hexadecanoic acid is not converted to the corresponding acetate 16:OAc, although earlier experiments with *O. furnacalis* (Zhao, et al. 1990) showed that topically applied hexadecanoic acid is readily desaturated, chain-shortened, and finally incorporated into the pheromone components. Thus we find it very unlikely that differences in uptake account for the pattern observed in the current study and favor an explanation involving the selectivity of the reductase complex or enzymes operating in close connection with this. The final proof of this hypothesis awaits the isolation and characterization of the different enzymes involved.

As mentioned above, hexadecanoic acid was not at all converted to the corresponding acetate. Neither was Z7–14:Acid, which is especially interesting as native (*Z*)-7-tetradecenoate is present in gland extracts at a titer comparable to that of (*E*)-12-tetradecenoate, whereas neither (*Z*)-7-tetradecenol nor Z7–14:OAc is (Zhao and Wang, 1990).

The reductase system had a preference for the *E* isomer of most of the Δ14:Acids tested in the present study. The exception from this pattern was Z11–14:Acid, which had the largest relative conversion factor among all the fatty acids tested. The efficient reduction of a fatty acid that does not naturally occur in *O. furnacalis* was surprising to us, but it may indicate a close relationship between this species and the *Z* strain of *O. nubilalis* or another corn borer species in which Z11–14:OAc is a major pheromone component. The productive interaction with Z11–14:Acid may be an evolutionary "memory-effect," an ability that is still there just because it has not been selected against.

The difference in *E/Z* ratios between the pheromone components and precursors can not be entirely accounted for by the twofold higher relative conversion factor for *E*12–14:Acid compared to the *Z* isomer. In *A. velutinana*, it was suggested that the fatty acyl precursors for reduction and acetylation are selected from a fresh supply of phospholipid acyl groups, whereas the unused portion is incorporated into triacylglycerols (Bjostad et al., 1987). More recently, it was proposed that phospholipids are not directly involved in pheromone biosynthesis, but that the biosynthetic enzymes interact with CoA derivatives. Once the intermediates are incorporated into a lipid class, they are no longer available for conversion to a pheromone component (Jurenka and Roelofs, 1993; Russell Jurenka, personal communication). Similarily, one may hypothesize that in

O. furnacalis, the pheromone is biosynthesized from a pool of fatty acids in which the *E/Z* ratio of Δ12–14 : Acyl is different from that found in total lipid extracts of the gland. Unfortunately, it seems as if pheromone biosynthesis in the gland proceeds so fast that the *E/Z* isomeric ratio of Δ12–14 : Acyl from the "fresh pool" can not be determined by in vivo experiments. The titer of deuterium-labeled pheromone was high 10 min after the application of deuterium-labeled palmitic acid to the glands. In *O. furnacalis*, the *E/Z* ratio of Δ12–14 : Acyl available for pheromone production is not only influenced by the geometric specificity of the desaturase, but also by the selectivity of the β-oxidase responsible for chain shortening from a Δ14–16 : Acyl (Figure 6). Therefore, in *O. furnacalis*, the specific *E/Z* ratio of the pheromone is influenced in concert by at least three different enzyme systems in the pheromone biosynthesis.

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