Journal of Chemical Ecology, Vol. 13, No. 5, 1987

REINVESTIGATION CONFIRMS ACTION OF Δ11-DESATURASES IN SPRUCE BUDWORM MOTH SEX PHEROMONE BIOSYNTHESIS

WALTER A. WOLF and WENDELL L. ROELOFS

Department of Entomology
New York State Agricultural Experiment Station
Geneva, New York 14456

(Received March 27, 1986; accepted June 13, 1986)

Abstract—The biosynthesis of a large number of sex pheromone components of various moth species has been shown to start with common fatty acids and involve chain shortening by two carbons and introduction of a double bond at the 11–12 position. A recent report indicates that one of these common components, (*E*)-11-tetradecenyl acetate, is present in the eastern spruce budworm, *Choristoneura fumiferana*, but is not made by this pathway. Reinvestigation of this insect using in vivo and in vitro techniques indicates that the acetate indeed is made by a sequence of reactions similar to that used in other leafroller moths. In fact, evidence was found for the presence of several Δ11-desaturase systems in spruce budworm. One produced a large quantity of (*Z*)-11-hexadecanoic acid, and another produced (*E*)-11-tetradecanoic acid. It is not known if the small amount of (*Z*)-11-tetradecanoic acid is produced by either of those two systems or by a third system. A comparison with other species showed that cabbage looper moths have only the first system, red-banded leafroller moths use the last two systems, and European corn borer moths have all three.

Key Words—Spruce budworm, *Choristoneura fumiferana*, sex pheromone biosynthesis, Δ11-desaturase, *Trichoplusia ni*, *Argyrotaenia velutinana*, *Ostrinia nubilalis*, Lepidoptera, Tortricidae, Noctuidae, Pyralidae.

INTRODUCTION

The biosynthesis of many lepidopterous sex pheromone components has been shown to involve the reaction of common fatty acids with two key enzyme systems (Roelofs and Bjostad, 1984). These include microsomal β-oxidation to give limited chain shortening by two carbons, and a Δ11-desaturase to yield

0098-0331/87/0500-1019$05.00/0

acids with unsaturation at the 11–12 position. A variety of compounds is produced by varying (1) the length of the starting fatty acids (normally between 12 and 18 carbons), (2) the order in which the chain shortening and desaturation steps occur, (3) the number of times chain shortening occurs, (4) the stereochemistry of the unsaturated products, and (5) the functionality (alcohol, acetate, aldehyde) of the pheromone components, which are finally produced by reducing the acid precursors.

A good example of these key steps is given by the biosynthetic routes for the cabbage looper moth (CL), *Trichoplusia ni*, pheromone components. In this species the Δ11-desaturase system produces large quantities of Z11–16 : Acid[1] and Z11–18 : Acid from palmitic and stearic acid, respectively. These are chain shortened to give pheromone precursor acids Z9–14 : Acid and Z7–12 : Acid from the former, and Z7–14 : Acid and Z5–12 : Acid from the latter (Bjostad and Roelofs, 1983). The Δ11-desaturase enzyme from CL was partially purified from the microsomal fraction and found to have a substrate specificity for 16- and 18-carbon acids (Wolf and Roelofs, 1986). However, in the redbanded leafroller moth (RBLR), *Argyrotaenia velutinana*, the Δ11-desaturase enzyme produces unsaturated 14-carbon acids from myristic acid in an *E*/*Z* ratio of ca. 3/2 (Bjostad and Roelofs, 1981). These are reduced to give a specific 8 : 92 ratio of the pheromone components, *E*11– and Z11–14 : OAcs.

Recently, Morse and Meighen (1984, 1986) indicated that the eastern spruce budworm (SBW), *Choristoneura fumiferana*, does not utilize the Δ11-desaturase enzyme system for production of the *E*11– and Z11–14 : OAcs found in their pheromone glands as precursors to the corresponding aldehyde pheromone components. Their data did not support a specific desaturation of myristic acid to give Δ11–14 : Acid, and so they suggested that the pheromone was produced by some unspecified pathway, perhaps starting directly from acetate.

We found it surprising that there were two different biosynthetic pathways for *E*11– and Z11–14 : OAcs in two leafroller moth species. It was difficult to accept an alternate route in the case of SBW also because Dunkleblum et al. (1985) had shown that the SBW pheromone glands contain both 14- and 16-carbon Δ11-unsaturated fatty acyl moieties, indicating the presence of a Δ11-desaturase in their pheromone gland. Thus we decided to reinvestigate the biosynthesis of the *E*11– and Z11–14 : OAcs in SBW by both in vivo and in vitro techniques.

Since the SBW apparently desaturated both 14- and 16-carbon acids, we also conducted a comparative study with the Δ11-desaturase system of other species, such as the CL moth (specific to 16- and 18-carbon acids), the RBLR moth (specific to 14-carbon acids), and two strains of the European corn borer

[1] Specific compounds will be referred to by an abbreviated naming system, where a letter (*Z* or *E*) indicates the stereochemistry, the first number gives the site of unsaturation, the second number the chain length, and symbols indicate the oxygen function. Thus Z9–18 : Acid is the abbreviation for oleic acid.

moth (ECB), *Ostrinia nubilalis*, that use *E*11- and Z11-14:OAc pheromone components (Roelofs et al., 1985) but produce Δ11 products of both 16- and 14-carbon acids. We tested the ability of pheromone gland extracts of each of these species to desaturate 14- and 16-carbon fatty acyl coenzyme A derivatives.

METHODS AND MATERIALS

Female SBW pupae were obtained from Dale Grisdale, Forest Pest Management Institute, Sault Ste. Marie, Canada, and maintained in our laboratory on a 16:8 light–dark regime. Experiments (gland removal or compound topical application) were conducted on 2-day-old (24–48 hr) insects approximately 1 hr before the onset of scotophase. CL, RBLR, and ECB pupae were obtained from cultures raised in our laboratory and were treated in the same manner.

[1-^{14}C]Palmitoyl-coenzyme A (59 mCi/mmol), [1-^{14}C]myristoyl-coenzyme A (31 mCi/mmol), [U-^{14}C]palmitic acid (403 mCi/mmol), [1-^{14}C]lauric acid (36 mCi/mmol), [1-^{14}C]myristic acid (38 mCi/mmol), and [1-^{14}C]-palmitic acid (57 mCi/mmol) were obtained from Amersham Corporation, Arlington Heights, Illinois. Solutions of the acids in dimethyl sulfoxide were prepared, with each containing aproximately 100,000 dpm/μl.

Extracts were prepared by adding the whole glands or the cell-free reaction mixture to 3 ml of 2:1 chloroform–methanol. After 15 min, 1 ml water was added, the organic layer was removed, and the water extracted with chloroform. The organic layers were combined and concentrated (Folch et al., 1957).

Fatty acids were analyzed as their methyl esters. The extracts were concentrated and placed in a solution containing 2 ml methanol, 1 ml benzene, and 0.1 ml sulfuric acid. The mixture was heated for 1 hr at 85°, cooled, and 1 ml water added. The organic layer was removed, washed three times with water and dried. The transmethylated extract was concentrated and 0.2 ml of acetyl chloride added to regenerate the acetates from the pheromone alcohols. After 10 min, the residual acetyl chloride was removed with N_2.

Gas–liquid chromatography (GLC) separation of the mixtures into fractions of differing chain length (C_{12}, C_{14}, and C_{16}) was performed on a column containing 3% OV-1 (methyl silicone) on 100–120 mesh Gas Chrom Q, with fractions being collected in 30-cm capillary tubes when appropriate. Further separation of *E* and *Z* isomers was accomplished on a column containing 10% XF-1150 (50% cyanoethyl, methyl silicone) on 100- to 120-mesh Chromosorb W-AW-DMCS. Capillary GLC was carried out using a 30-m Supelcowax column, 0.25 mm ID, on a Hewlett-Packard 5890 gas chromatograph.

Thin-layer chromatography (TLC) was performed on Whatman K5 plates that had been sprayed with a solution of 10 g silver nitrate in 65 ml 80% ethanol until damp. The plates were activated for 1 hr at 110° before use. The plates

were developed with a 9:1 mixture of Skelly B and ether, and the areas of radioactivity located by autoradiography. Areas of the plate were scraped into vials, scintillation fluid added, and radioactivity determined in a Packard Tri-Carb scintillation counter.

Ozonolysis was accomplished by adding a solution of the compound in CS_2 to a solution of ozone in CS_2 at $-70°C$. The reaction was allowed to slowly warm to room temperature and was concentrated and directly subjected to GLC analysis.

Topical application was performed in a manner similar to that previously reported (Bjostad and Roelofs, 1983). Female insects were anesthetized with carbon dioxide and their pheromone glands held everted by means of smooth-jawed alligator clips. A droplet of radiolabeled material dissolved in DMSO was applied to the glands. After 1 hr, the insects were removed from the clips and maintained in the dark for 3 hr. At this time they were cooled to 0° for 10 min and the glands removed and extracted as above.

Cell-free reactions were conducted similarly to that reported in Wolf and Roelofs (1986). Pheromone glands were removed and placed in a cold buffer containing 0.10 M phosphate, 0.35 M sucrose, and 5 mM dithiothreitol, pH 7.6. The glands were homogenized in a Potter Elvehjem tissue grinder and centrifuged at 12,000*g* for 15 min at 4°C. The supernatent was combined with 0.1 μCi of labeled fatty acid CoA ester and cofactors in a total volume of 0.20 ml. Cofactor concentrations were: NADH, 5 mM; 5 mM; NADPH, 1 mM; BSA, 1 mg/ml. After 1 hr at 25°C, the assays were extracted as above.

For capillary GLC, five glands were excised, extracted, and transmethylated. An aliquot was subjected to capillary GLC and compared to standards. The rest of the material was subjected to GLC on OV-1, and the relevant peaks subjected to ozonolysis to confirm the position of the double bond.

For in vivo experiments, 3.0 μl of radiolabeled fatty acid in DMSO was topically applied to the glands of 10 insects. After extraction and transmethylation, the products were subjected to GLC on OV-1. Trapped fractions were counted or subjected to further GLC on XF-1150 or ozonized.

In vitro experiments were conducted by preparing the cell-free extract, adding 0.1 μCi of labeled fatty acid, and subsequent extraction and transmethylation. The resultant mixture was analyzed by TLC, GLC, and ozonolysis.

RESULTS

Five SBW glands were extracted, transmethylated, and analyzed by capillary GLC as well as by ozonolysis of the compounds collected from nonpolar and polar GLC columns. This confirmed the presence of Δ11-unsaturated acids in SBW extracts (Table 1). Both the *E* and *Z* isomers of the 14-carbon acid were found, but only the *Z* isomer of the 16-carbon acid was detected.

TABLE 1. FATTY ACIDS IN SBW EXTRACTS[a]

Chain length (carbons)	Saturated	Δ-9	*E*11	*Z*11
14	1.81	0.53	1.13	0.19
16	26.17	1.19	[b]	0.90

[a] As percent of total fatty acids.
[b] Not found.

In Vivo Experiments. The results of topical application of radiolabeled saturated fatty acids are presented in Table 2. Using uniformly labeled palmitic acid as the starting material, significant radioactivity was obtained in both the 14-carbon acid and 14-carbon acetate fractions. When palmitic acid labeled in only the 1-carbon was used, the amount of label found in the 14-carbon acid fraction decreased more than 60%, and very little radioactivity was detected in the unsaturated 14-carbon acetates. Similarly, lauric acid labeled in the 1-position gave rise to some labeled 14-carbon acids, but no radioactivity was detected in the acetate fraction.

When myristic acid labeled in the 1-carbon was topically applied, the greatest amount of incorporated radioactivity was detected in the 14-carbon acetates. The 14-carbon acid fraction in this reaction was not counted directly, but was collected from the XF-1150 GLC column to separate the saturated acid (starting material) and the isomers of the Δ11-unsaturated acids. Typical results, presented as percent of initial radioactivity incorporated into the fraction, were: saturated, 0.52%; *E* isomer, 0.54%; *Z* isomer, 0.07%. The ratio of *E*/*Z*

TABLE 2. INCORPORATION OF RADIOLABELED COMPOUNDS USING TOPICAL APPLICATION TO SBW PHEROMONE GLANDS

Starting material	Product[a]	
	14 : Acid[b]	*E*/*Z*11-14 : OAc
[U-^{14}C]16 : Acid	0.65	0.36
[1-^{14}C]16 : Acid	0.24	0.03
[1-^{14}C]12 : Acid	0.34	[c]
[1-^{14}C]14 : Acid	[d]	0.48

[a] As percent of starting material.
[b] Total (saturated and unsaturated).
[c] Not found.
[d] Further separated; see text.

TABLE 3. INCORPORATION OF RADIOLABELED COMPOUNDS USING CELL-FREE EXTRACTS PREPARED FROM SBW

Starting material	Product[a]	
	*E*11 acid	*Z*11 acid
[1-^{14}C]14:CoA	6.25	0.72
[1-^{14}C]16:CoA	0.35	1.82

[a] As percent of starting material.

incorporation is very close to that found for the total amounts of the acids present (Table 1).

In Vitro Experiments. Pheromone glands from SBW were excised, homogenized in buffer, centrifuged, and the resulting extracts reacted with radiolabeled CoA esters. After transmethylation and TLC separation, the isomers of the desaturated acids were located by autoradiography and quantified. The results are given in Table 3. Both 14- and 16-carbon acids were desaturated. The 14-carbon acids showed a high reactivity (over 7% of the starting saturated acid was isolated in the products), and the *E*/*Z* ratio was similar to that observed in the topical application experiments. The 16-carbon acids, however, were much less reactive in this system (just over 2% total) and gave predominantly the *Z* isomer.

These same experiments were repeated using extracts prepared from other species. As shown in Table 4, the results show a pattern similar to the acid

TABLE 4. INCORPORATION OF RADIOLABELED COMPOUNDS USING CELL-FREE EXTRACTS PREPARED FROM SEVERAL INSECTS

Species	Product (pmol)[a]			
	11–14:Acid		11–16:Acid	
	E	*Z*	*E*	*Z*
CL	[b]	4	[b]	67
RBLR	17	25	[b]	[b]
SBW	200	24	6	31
ECB (*Z* strain)	114	45	1	189
ECB (*E* strain)	41	18	<1	142

[a] Products were *E*- and *Z*11 isomers of the 14- and 16-carbon acids. Starting materials were 14:CoA and 16:CoA, respectively.
[b] Not found.

precursors in each species. For CL only the *Z* isomer of the 11-desaturated product was observed, predominantly from the 16-carbon acid. In RBLR, only the 14-carbon acid underwent desaturation, but both the *E* and *Z* isomers of the product were labeled. In two strains of ECB, one having a predominantly *E*11 pheromone and the other having mostly *Z*11, the pattern of unsaturated products was similar. In both strains, the 14-carbon starting material gave about 70% *E* product, whereas the 16-carbon acid produced less than 1% *E* product. Also in both, the 16-carbon substrate was preferred (11.2% incorporation total compared to 5.0% for the 14-carbon acid in the *Z* strain).

DISCUSSION

Investigations of the biosynthesis of sex pheromones in moths have involved techniques such as analysis of the fatty acid content of the pheromone gland and in vivo and in vitro incorporation of radiolabeled precursors to gain insight into possible pheromone intermediates or precursors (Lofstedt and Roelofs, 1985; Roelofs and Bjostad, 1984). When we applied these techniques to the SBW, we obtained strong evidence that this insect uses the previously proposed (Roelofs and Bjostad, 1984) biosynthetic pathway involving chain-shortening of palmitic to myristic acid, followed by Δ11-desaturation and then reduction to *E*11- and *Z*11-14:OAcs.

First, we confirmed the presence of 11-12 unsaturated 14- and 16-carbon acids in the gland (Dunkelblum et al., 1985). Since previous studies with moth sex pheromone glands demonstrated that acids unsaturated in this position are made directly by a Δ11-desaturase (Wolf and Roelofs, 1986), our findings indicate the presence of such an enzyme in SBW.

We then topically applied several radiolabeled fatty acids to SBW sex pheromone glands. If the *E*/*Z*11-14:OAcs were being made directly from acetate, we would expect similar results from all of the starting materials, as they each would be degraded to this common intermediate. As can be seen in Table 2, this was not the case. Both lauric and palmitic acids labeled in the 1-carbon gave rise to little or no labeled *E*/*Z*11-14:OAcs. This is the expected result if the first step in the biosynthetic pathway involves chain shortening of palmitic acid, resulting in the loss of the first two carbons, including the labeled one. Lauric acid cannot participate in this reaction. Note that in both cases some activity is incorporated in the myristic acid fraction, perhaps by degradation and recycling through the fatty acid cycle.

Radiolabel from uniformly labeled palmitic acid and from myristic acid labeled in the 1-carbon is incorporated into the *E*/*Z*11-14:OAcs. In addition, the myristate gives rise to labeled *E* and *Z*11-14:Acids in a ratio close to that of the total Δ11-14:Acid found in the gland. Again, this supports the pathway involving chain shortening to myristic acid followed by 11-desaturation.

More direct evidence for the existence of a Δ11-desaturase was provided by in vitro experiments. Cell-free preparations from SBW sex pheromone glands were capable of introducing an 11–12 double bond into CoA esters of myristic and palmitic acids. No radioactivity was found in any compound other than the starting material and this product, indicating that degradation–resynthesis was not occurring under these conditions.

These data demonstrate that the biosynthesis of the *E*/*Z*11–14:OAcs in SBW follows a pathway similar to that in other moths, involving chain shortening and Δ11-desaturation. It differs only in that in SBW these acetates, which are found as major components in the pheromone gland, are converted to the corresponding aldehydes when emitted as a pheromone components.

While collecting the data to prove the pheromone biosynthetic pathway for SBW, we were surprised at the results found in Table 3 showing the stereoselective production of *Z*11–16:Acid. This had not been found in RBLR, which also produces an *E*/*Z* mixture of Δ11–14:Acids. We conducted studies similar to those of Table 3 with other species and found evidence for the presence of several Δ11-desaturase enzyme systems. The results in Table 4 show that there is a system that produces the *Z* isomer and is specific to 16-carbon chain acids. This system is found in all the insects studied except RBLR. Another system is specified to 14-carbon chain acids and produces the *E* isomer. This is found in all the species except CL. There also may be a third enzyme system that is specific to 14-carbon chain acids and produces the *Z* isomer. Although some of the *Z*11–14:Acid could be produced by the enzyme specific to 16:Acid, such as in CL, this does not appear to be the case with RBLR or SBW. The presence of an isomerase to yield the *E*/*Z* mixtures of 14:Acids has been ruled out by previous studies using radiolabeled Δ11–14:Acids (Bjostad and Roelofs, 1981). Further purification and characterization of these Δ11-desaturase systems will be needed to clarify the number of variations involved.

Acknowledgments—We gratefully acknowledge Kathy Poole and Marlene Campbell for rearing the insects used in these studies. This research was supported by National Science Foundation grant DCB-8406348.

REFERENCES

BJOSTAD, L.B., and ROELOFS, W.L. 1981. Sex pheromone biosynthesis from radiolabelled fatty acids in the redbanded leafroller moth. *J. Biol. Chem.* 256:7936–7940.

BJOSTAD, L.B., and ROELOFS, W.L. 1983. Sex pheromone biosynthesis in *Trichoplusia ni*: Key steps involve delta-11 desaturation and chain shortening. *Science* 220:1387–1389.

DUNKELBLUM, E., TAN, S.H., and SILK, P.J. 1985. Double-bond location in monounsaturated fatty acids by dimethyl disulfide derivatization and mass spectrometry. *J. Chem. Ecol.* 11:265–277.

FOLCH, J., LEES, M., and SLOANE STANLEY, G.H. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* 226:497–509.

LOFSTEDT, C., and ROELOFS, W.L. 1985. Sex pheromone precursors in two primitive New Zealand tortricid moth species. *Insect Biochem.* 15:729–734.

MORSE, D., and MEIGHEN, E. 1984. Aldehyde pheromones in Lepidoptera: Evidence for an acetate ester precursor in *Choristoneura fumiferana. Science* 226:1434–1436.

MORSE, D., and MEIGHEN, E. 1986. Pheromone biosynthesis and role of functional groups in pheromone specificity. *J. Chem. Ecol.* 12:335–351.

ROELOFS, W.L., and BJOSTAD, L.B. 1984. Biosynthesis of Lepidopteran pheromones. *Bioorg. Chem.* 12:279–298.

ROELOFS, W.L., DU, J.-W., TANG, X.-H., ROBBINS, P.S., and ECKENRODE, C.J. 1985. Three European corn borer populations in New York based on sex pheromones and voltinism. *J. Chem. Ecol.* 11:829–836.

WOLF, W.A., and ROELOFS, W.L. 1986. Properties of the delta-11-desaturase enzyme used in cabbage looper moth sex pheromone biosynthesis. *Arch. Insect Biochem. Physiol.* 3:45–52.