Control of the biosynthetic pathway of *Sesamia nonagrioides* sex pheromone by the pheromone biosynthesis activating neuropeptide

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Abstract

(Z)-11-Hexadecenyl acetate, the main pheromone component of *Sesamia nonagrioides* sex pheromone, is biosynthesized from palmitic acid by Δ¹¹-desaturation followed by reduction and acetylation. Production of (Z)-11-hexadecenyl acetate is regulated by the Pheromone Biosynthesis Activating Neuropeptide (PBAN). Transformation of (Z)-11-hexadecen-1-ol into the corresponding acetate is a target step for PBAN in the regulation of this biosynthetic sequence, thus being the first example of a PBAN-activated acetylation. The production of the minor component (Z)-11-hexadecenal is also stimulated by PBAN. The usefulness of pentafluorobenzylxime-derivatives for the analysis of aldehyde pheromone constituents by gas chromatography coupled to mass spectrometry is also reported. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Biosynthesis of lepidopteran sex pheromones occurs from fatty acids by the combined action of desaturases, chain-shortening enzymes, reductases, acetyltransferases and oxidases (Roelofs and Bjostad, 1984). Whereas chain-shortening enzymes and desaturases are involved in modification of length and degree of unsaturation of the alkyl chain, respectively, reductases, acetyltransferases and oxidases are responsible for the type of functionality of the pheromone component.

Sex pheromone biosynthesis in many moth species is regulated by PBAN (Raina, 1993), a peptide hormone produced in the sub-esophageal ganglion complex. This hormone has been isolated and sequenced from brains of *Helicoverpa zea* (Raina et al., 1989), *Bombyx mori* (Kitamura et al. 1989, 1990) and *Lymantria dispar* (Masler et al., 1994), and its sequence has been deduced from the encoding cDNA in *Agrotis ipsilon* (Duportets et al., 1998), *H. assulta* (Choi et al., 1998) and *Mamestra brassicae* (Jacquin-Joly et al., 1998). In all these species, PBAN is a 33 or 34 amino acid peptide with an amidated C-terminus, in which the pheromonotropic activity lies in the C-terminal pentapeptide fragment FSPLR-NH₂ (Raina and Kempe, 1990). Other peptides sharing this C-terminal sequence and thereby exhibiting pheromontropic activity have also been isolated from *Pseudaletia separata* (Matsumoto et al. 1990, 1992), *B. mori* (Imai et al., 1991) and cockroaches (Schoofs et al., 1993), in which they regulate coloration in phase polymorphism, induction of embryonic diapause and muscle contraction, respectively.

The effect of PBAN on the different enzymes involved in moth sex pheromone biosynthesis has only been studied in a few species. Thus, in *Argyrotaenia velutinana* (Tang et al., 1989), *H. zea* (Jurenka et al., 1991) and *M. brassicae* (Jacquin et al., 1994) the peptide controls a step in or prior to fatty acid synthesis. However, in *Spodoptera littoralis* (Fabriàs et al., 1994; Martínez et al., 1990), *B. mori* (Ozawa et al., 1993), *Thaumetopoea pierrei* (Jacquin et al., 1994) and *Cypa pomonella* (Marche et al., 1994) the peptide controls a step in or prior to fatty acid synthesis. However, in *Spodoptera littoralis* (Fabriàs et al., 1994; Martínez et al., 1990), *B. mori* (Ozawa et al., 1993), *Thaumetopoea pierrei* (Jacquin et al., 1994) and *Cypa pomonella* (Marche et al., 1994) the peptide controls a step in or prior to fatty acid synthesis.

Abbreviations: GC–MS, gas chromatography coupled to mass spectrometry; SCAN–GC–MS, gas chromatography coupled to mass spectrometry under SCAN; Z11-16:AL, (Z)-11-hexadecenal; Z11-16:OL, (Z)-11-hexadecen-1-ol; Z11-16:OAc, (Z)-11-hexadecenyl acetate; 16:Acid, hexadecanoic acid; d₁₆:Acid, (16,16,16-²H₃) hexadecanoic acid; d₂Z11-16:Acid, (13,13,14,14,15,15,16,16,16-²H₉) (Z)-11-hexadecenoic acid; d₂Z11-16:OAc, (13,13,14,14,15,15,16,16,16-²H₉) (Z)-11-hexadecenyl acetate; d₂Z11-16:OAc, (13,13,14,14,15,15,16,16,16-²H₉) (Z)-11-hexadecenyl acetate; PBAN, pheromone biosynthesis activating neuropeptide; SIM, selected ion monitoring; 13:OAc, tridecyl acetate.

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topoea pityocampa (Fabrias et al., 1995) and Manduca sexta (Fang et al., 1995) PBAN appears to act on the reduction of fatty acyl moieties.

The main component of Sesamia nonagrioides female sex pheromone is (Z)-11-hexadecenyl acetate (Z11-16:OAc), with minor amounts of the corresponding alcohol, (Z)-11-hexadecen-1-ol (Z11-16:OL) and aldehyde, (Z)-11-hexadecenal (Z11-16:AL) in 92:5:3 ratio (Sreng et al., 1985). Mazomenos also reported that dodecyl acetate was present in the pheromone gland extracts (Mazomenos, 1989). From these structures, we anticipated that Z11-16:OAc would arise from Δ11-desaturation of palmitic acid (16:Acid) followed by further reduction and acetylation. In this article we confirm this proposed biosynthetic sequence and provide data indicating that PBAN regulates this biosynthetic pathway with probable activation of the final acetylation step.

2. Experimental procedures

2.1. Insects

S. nonagrioides were supplied by the Centre UdL-IRTA, Lleida (Spain) as pupae. Pupae were sexed and kept under a 16-h light/8-h dark photoperiod at 25°C. The adults were provided with a 10% sucrose solution. Only virgin females (2-days old) were used in the experiments.

2.2. Chemicals

Bom-PBAN was obtained from Peninsula laboratories (Belmont, CA). Dimethylsulfoxide was obtained from Sigma (St Louis, MO) and pentafluorobenzylhydroxylamine hydrochloride was purchased from Aldrich (Milwaukee, WI). (16,16,16-2H3) Hexadecanoic acid (d16:Acid) was supplied by IC Chemikalien (Munich, Federal Republic of Germany) and it was 99% pure. (13,13,14,14,15,15,16,16,16-2H9) (Z)-11-Hexadecenoic acid (d9,Z11-16:Acid) was synthesized in our laboratory following reported procedures (Navarro et al., 1997) and it was 99% pure (gas chromatography analysis of the methyl ester).

2.3. Treatments

To monitor the effect of PBAN on pheromone production, 3 h before the beginning of darkness, insects were injected into the abdomen with 20 μl of Meyer and Miller’s saline containing the amounts of PBAN indicated. Pheromone glands were dissected two hours after PBAN injection and the pheromone gland was extracted as described in the following section.

In the deuterium-labeling experiments, 4 h before the onset of the scotophase females were immobilized under netting and putative precursors were topically applied to the gland as dimethylsulfoxide solutions (0.1 μl, 10 μg/μl). Controls received 0.1 μl of dimethylsulfoxide. One hour after the application, insects were freed and 20 μl of a PBAN solution (2.5 pmol/μl) in Meyer and Miller’s saline (Martinez and Camps, 1988) were injected into the abdomen. Controls were injected with 20 μl of saline. Pheromone glands were excised two hours after PBAN injection and the pheromone blend was extracted as described below.

2.4. Pheromone gland extraction

Unless otherwise stated, individual pheromone glands were extracted with a 30 μl hexane solution of tridecyl acetate (13:OAc, 1 ng/μl) for 1 h at 25°C.

2.5. Aldehyde derivatization

Groups of 5–7 pheromone glands were extracted with 100 μl of hexane. After 1 h at 25°C, hexane extracts were carefully evaporated almost to dryness under a gentle stream of nitrogen and the resulting residue was treated with a solution of pentafluorobenzylhydroxylamine hydrochloride (5 μl, 1 mg/ml) in methanol. After 1.5 h at 25°C, the whole sample was injected into the chromatograph. Derivatization reactions with synthetic Z11-16:Al showed that more than 90% conversions were achieved with 1–50 ng samples using this protocol, as determined by gas chromatography coupled to mass spectrometry (GC–MS) analysis of samples using hexadecane as internal standard.

2.6. Analytical methods

To determine the effect of PBAN on pheromone production, the extracts were analyzed by capillary gas chromatography using a Fisons gas chromatograph (8000 series) equipped with a non-polar Hewlett Packard HP-1 capillary column (30 m×0.20 mm I.D.) The following temperature program was used: from 100 to 220°C at 5°C/min and then to 260°C at 10°C/min after an initial delay of 1 min.

Pentafluorobenzoxime-derivatives were analyzed by GC–MS using a Fisons gas chromatograph (8000 series) coupled to a Fisons MD-800 mass selective detector. The system was equipped with a non-polar Hewlett Packard HP-1 capillary column (30 m×0.20 mm I.D.) using the following temperature program: from 100 to 250°C at 5°C/min and then to 280°C at 10°C/min after an initial delay of 2 min. Analyses were conducted under the SCAN mode. The same procedure was used to analyze Z11-16:AL in pheromone extracts.

In the mass-labeling experiments, extracts were analyzed by GC–MS using the same equipment and conditions, but analyses were performed under selected ion
monitoring (SIM) mode. The ions selected were: 13:OAc, 168 (M-60); Z11-16:OAc, 222 (M-60); (16,16,16-2H3) (Z)-11-hexadecenyl acetate (d3Z11-16:OAc), 225 (M-60); (13,13,14,14,15,15,16,16,16-2H9) (Z)-11-hexadecenyl acetate (d9Z11-16:OAc), 231 (M-60).

3. Results and discussion

Pheromone titers of S. nonagrioides fluctuate diurnally with the highest titers occurring at the sixth hour of the scotophase (Babilis and Mazomenos, 1992). No pheromone is produced during the photophase (Babilis and Mazomenos, 1992). This photoperiodicity of pheromone production is probably controlled by a brain factor similar to PBAN. In agreement with this assumption, PBAN injection into whole S. nonagrioides females stimulates sex pheromone production in the photophase. Furthermore, as shown in Fig. 1, this effect was dose-dependent. The minimum dose tested that elicited a significant response was 12.5 pmol. Although maximum stimulation was achieved with 200 pmols/gland, there was not statistical difference in the amounts of pheromones produced with 50, 100 and 200 pmol. A PBAN dose of 100 pmol/insect was considered suitable for further experiments.

To determine the effects of PBAN on the production of Z11-16:AL, 12–15 pheromone glands from insects that had been injected with either saline or PBAN in the photophase were pooled and the GC–MS analysis of these extracts were performed under SCAN (SCAN–GC–MS). Whereas no Z11-16:AL was detected in extracts from insects injected with saline, both the retention time and mass spectrum of putative Z11-16:AL present in extracts from PBAN-injected females were identical to those of a synthetic sample of Z11-16:AL (data not shown). The identity of Z11-16:AL was also confirmed by SCAN–GC–MS analysis of samples obtained from pools of 5–7 glands derivatized with pentafluorobenzyl hydroxylamine hydrochloride (van Kuijk et al., 1986). In our search for aldehyde derivatives having characteristic and intense fragments for the efficient and reliable identification of pheromone aldehydes, we found that the pentafluorobenzyl oximes (van Kuijk et al., 1986) were very suitable, since they are easily prepared and they give abundant and characteristic fragments in their mass spectra. These fragments arise from cleavage at the benzylic position to give both, the tropilium ion (m/z 181, base peak) and ions corresponding to R–CH=N–O+ and R–CH=N+. These last fragments are of diagnostic value for structural characterization of the parent aldehyde, R–CHO. As shown in Fig. 2, the mass spectra of both natural and synthetic materials were identical. Diagnostic ions are those at m/z 252 and 236, corresponding to fragments C6H5–CH=CH–(CH2)9–CH=N–O+ and C6H5–CH=CH–(CH2)9–CH=N+, respectively.

The last objective of this work was to investigate the effect of PBAN on the sex pheromone biosynthetic path-

![Fig. 1](image1.png)

![Fig. 2](image2.png)
Fig. 3. Proposed biosynthetic pathway of S. nonagrioides sex pheromone and step activated by PBAN.

way of S. nonagrioides sex pheromone. As previously reported in other species (Jacquin et al., 1994; Jurenka et al., 1991), the mass labeling experiments reported in this article show that Z11-16:OAc, the main component of S. nonagrioides sex pheromone, is biosynthesized from palmitic acid by Δ11-desaturation followed by reduction and acetylation (Fig. 3). To investigate the step(s) activated by PBAN in this species, incorporation of label from a series of tracers into Z11-16:OAc was monitored in the photophase. When d3 16:Acid was administered, labeled Z11-16:OAc was only detected in females that had been injected with PBAN (Table 1). Similar results were also found in treatments with d9 Z11-16:Acid and d9 Z11-16:OL, which gave rise to labeled d9 Z11-16:OAc only in females treated with PBAN. This last result is in contrast with those reported in other species (Bestmann et al., 1987; Dunkelblum et al., 1989; Jurenka and Roelofs, 1989; Teal and Tumlinson, 1987; Zhao et al., 1995), in which alcohol acetylation is rather unspecific and occurs throughout the photoperiodic cycle. Additionally, this result indicates that in S. nonagrioides, PBAN stimulation of the biosynthesis occurs, at least in part, by activation of the acetyl transferase. This is the first example of activation of acetyl transferase by PBAN in a sex pheromone biosynthetic pathway. In the other species so far investigated, PBAN appears to act on a step prior to fatty acid synthesis (Jurenka et al., 1991; Tang et al., 1989) or by activation of the reduction step(s) activated by PBAN in this species, incorporation of label from a series of tracers into Z11-16:OAc was monitored in the photophase. When d3 16:Acid was administered, labeled Z11-16:OAc was only detected in females that had been injected with PBAN (Table 1). Similar results were also found in treatments with d9 Z11-16:Acid and d9 Z11-16:OL, which gave rise to labeled d9 Z11-16:OAc only in females treated with PBAN. This last result is in contrast with those reported in other species (Bestmann et al., 1987; Dunkelblum et al., 1989; Jurenka and Roelofs, 1989; Teal and Tumlinson, 1987; Zhao et al., 1995), in which alcohol acetylation is rather unspecific and occurs throughout the photoperiodic cycle. Additionally, this result indicates that in S. nonagrioides, PBAN stimulation of the biosynthesis occurs, at least in part, by activation of the acetyl transferase. This is the first example of activation of acetyl transferase by PBAN in a sex pheromone biosynthetic pathway. In the other species so far investigated, PBAN appears to act on a step prior to fatty acid synthesis (Jurenka et al., 1991; Tang et al., 1989) or by activation of the reduction of the last acyl precursor (Fabrias et al., 1994; Fang et al., 1995; Ozawa et al., 1993).

Although mass-labeling experiments were not performed, it is reasonable to assume that Z11-16:AL is biosynthesized from Z11-16:Acid, either by direct reduction of Z11-16:Acid or by oxidation of Z11-16:OL. As for the first possibility, fatty acyl reductases that give rise to aldehydes have been reported in plants (Vioque and Kolattukudy, 1997), although such enzymes are heretofore unprecedented in insect pheromone glands. Several articles have reported on the biosynthesis of pheromone aldehydes from the corresponding alcohols (Morse and Meighen, 1984; Teal and Tumlinson, 1987), or even from the acetates (Morse and Meighen, 1984). Therefore, in the light of the existing literature, the biosynthesis of Z11-16:AL by oxidation of Z11-16:OH seems more plausible. This assumption is currently under investigation in our laboratories using the pentafluorobenzyloxime-derivative.

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