J. Pestic. Sci., **33**(1), 17–20 (2008)
DOI: 10.1584/jpestics.R07-06

Commentary

(Special Topic)

Epoxyalkenyl sex pheromones produced by female moths in highly evolved groups: biosynthesis and its endocrine regulation

Tetsu ANDO,* Takeshi KAWAI and Kanae MATSUOKA

Graduate School of Bio-Applications and Systems Engineering (BASE), Tokyo University of Agriculture and Technology, Koganei, Tokyo 184–8588, Japan

(Received June 20, 2007; Accepted September 13, 2007)

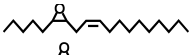
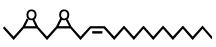
Polyunsaturated hydrocarbons with a C_{17}-C_{23} straight chain and the epoxy derivatives constitute a second major class of lepidopteran sex pheromones and are referred to as Type II pheromones. While regionspecific epoxidation proceeds in a pheromone gland, the hydrocarbons are biosynthesized from dietary polyunsaturated fatty acids outside the pheromone gland and transported into the gland after association with lipophorin. *In vivo* as well as *in vitro* experiments using Japanese giant looper (*Ascotis selenaria cretacea*, Geometridae) demonstrated that pheromone biosynthesis-activating neuropeptide (PBAN) accelerated precursor uptake by the gland but not the biosynthetic step, which was contrast with the biosynthesis of Type I pheromones. The neuropeptide of *A. s. cretacea*, Assc-PBAN, was characterized to clarify its mode of unique action.

Keywords: lepidopteran sex pheromone, pheromone biosynthesis, lipophorin, neuropeptide hormone, cDNA of PBAN.

Introduction

While many classical pheromones, such as bombykol of the silkworm moth (*Bombyx mori*), have been structurally elucidated as C_{10}-C_{18} unsaturated fatty alcohols and their derivatives (now referred to as Type I pheromones), Hill *et al*. identified *cis*-9,10-epoxy-(*Z,Z*)-3,6-henicosadiene from *Estigmene acrea* (Arctiidae) in 1981.[1] This epoxyalkene is the first example of Type II pheromones with a characteristic unsaturated straight chain and differentiated from the structural feature of dispalure, which is a well-known epoxy pheromone produced by the gipsy moth (*Lymantria dispar*, Lymantriidae) but has a methyl-branched saturated skeleton. Since then, similar epoxyalkenyl pheromones with a C_{17}-C_{23} straight chain and/or the parent polyunsaturated hydrocarbons have been reported from about 100 lepidopteran species in some highly evolved groups, such as the families of Arctiidae and Geometridae, and constitute a second major class of lepidopteran sex pheromones.[2,3] In Japan, the chemical structures of the Type II pheromones of several species, including *Biston robustum* (Geometridae),[4] *Milionia basalis* (Geometridae),[5] and *Perina nuda* (Lymantriidae),[6] have been determined. Type II pheromones are composed of (*Z,Z*)-6,9-dienes, (*Z,Z,Z*)-3,6,9-trienes, and their epoxy derivatives, mainly mono *cis*-epoxides, which are abbreviated as shown in Table 1. These compounds lack a terminal functional group and are easily differentiated from Type I pheromones. Diversity of an insect species indicates further structural modification, such

Table 1. Type II sex pheromones identified from female moths inhabiting Japan

Family Species	Sex pheromone	Abbreviation
Geometridae		
Biston robustum		epo6,Z9-19:H
		Z3,epo6,Z9-19:H
Milionia basalis		epo3,Z6,Z9-19:H
Lymantriidae		
Perina nuda		Z3,epo6,Z9-21:H
		epo3,epo6,Z9-21:H

* To whom correspondence should be addressed.
E-mail: antetsu@cc.tuat.ac.jp

as the introduction of an extra double bond, and the following compounds were recently identified: (*E*,*Z*,*Z*)-4,6,9-trienes, (*Z*,*Z*,*Z*)-1,3,6,9- and (*Z*,*Z*,*Z*,*E*)-3,6,9,11-tetraenes, (*Z*,*Z*,*Z*,*Z*,*Z*)-3,6,9,12,15-pentaenes, and their epoxy derivatives.[2,3,7]

Biosynthetic Pathways of Epoxyalkenyl Pheromones

The variation of the lepidopteran pheromones results from differences in both their biosynthetic starting material and enzyme systems. Type I pheromones are biosynthesized from saturated fatty acids produced by *de novo* synthesis starting from acetyl-CoA. For example, (*Z*)-7-dodecenyl acetate is produced in the female pheromone glands of a Noctuidae species *via* the following successive reactions: desaturation of palmitic acid, chain shorting by β-oxidation, reduction of an acyl group, and acetylation.[8] In contrast, Type II pheromones are expected to be produced from linoleic and linolenic acids [(*Z*,*Z*)-9,12-octadecadienoic and (*Z*,*Z*,*Z*)-9,12,15-octadecatrienoic acids] because these dietary acids are unsaturated at the same positions of the pheromones if the positions of double bonds are counted from terminal methyl groups. For example, *cis*-3,4-exoxy-(*Z*,*Z*)-6,9-nonadecadiene (epo3,Z6,Z9-19:H), a major pheromone component of *A. s. cretacea*,[9,10] might be produced by chain elongation of linolenic acid to C_{20} trienoic acid, decarboxylation to C_{19} (*Z*,*Z*,*Z*)-3,6,9-triene (Z3,Z6,Z9-19:H, a minor pheromone component), and epoxidation of the double bond at the 3-position.

In a late scotophase, the virgin female of *A. s. cretacea* takes a calling pause while extruding the ovipositor, which is the terminal abdomen from 8th to 9th segments. The ovipositor is unusually long to lay eggs in deep bark, and a pheromone gland is localized on the terminal side of the elongated intersegmental membrane between the two segments.[11] GC-MS analysis of an extract of the glands treated with deuterium labeled Z3,Z6,Z9-19:H showed its specific conversion into the pheromonal 3,4-epoxide, confirming that the C_{19} triene is a biosynthetic precursor of the epoxy pheromone and that epoxidation proceeded in the gland. Moreover, in order to examine the substrate specificity of the enzyme catalyzing this epoxidation step, several unsaturated hydrocarbons not occurring in the gland were applied. Not only (*Z*,*Z*,*Z*)-3,6,9-trienes with varying chain lengths (C_{17}, C_{18}, and etc.) but also (*Z*,*Z*)-3,6-dienes (C_{17}, C_{19}, and etc.) were converted into the corresponding 3,4-epoxides in remarkable yield, while no 6,7- and 9,10-epoxides could be detected. (*Z*)-3-Nonadecene was also changed to the epoxide, but (*E*)-3-, (*Z*)-2-, and (*Z*)-4-double bonds in the C_{19} chain were not oxidized. These *in vivo* experiments revealed that the epoxidase regiospecifically attacked the (*Z*)-3-double bond of straight chain hydrocarbons regardless of their length and unsaturated degree.[12]

The low substrate specificity indicated that the production of a species-specific pheromone might be caused essentially by strict production of an unsaturated hydrocarbon. *In vivo* experiments with another geometrid species, *Hemerophila atrilineata*, secreting *cis*-9,10-exoxy-(*Z*,*Z*)-3,6-octadecadiene (Z3,Z6,epo9-18:H), also clarified the epoxidation step,[13] however, characterization of the epoxygenase has not been accomplished, and the pathway of triene biosynthesis has not been confirmed. We synthesized a deuterated C_{20} trienoic acid and topically applied it to the pheromone gland of *A. s. cretacea*, but its conversion to the triene could not be detected.

Transportation of Biosynthetic Precursors by Lipophorin

The biosynthesis of cuticle hydrocarbons in oenocytes suggests that pheromonal hydrocarbons are also produced there and transported into the pheromone gland after association with lipophorin, as shown in Fig. 1. This estimate was experimentally demonstrated with *A. s. cretacea* and *H. atrilineata* females. GC-MS analysis of a solvent extract from the hemolymph showed the occurrence of the trienyl precursor, and the deuterated epoxy pheromones were yielded from the pheromone glands of females that had been injected with deuterated trienes in their abdomen.[13] Furthermore, high-density lipophorin (HDLp) in the *A. s. cretacea* females showing two bands (apoLp I with ca. 250 kDa and apoLp II with ca. 80 kDa) on SDS-PAGE was purified by KBr equilibrium density-gradient ultracentrifugation, and the association of the triene was confirmed by analysis of an extract from the isolated protein.[14] While saturated hydrocarbons with a long chain, such as C_{23} and C_{25}, are commonly present in the hemolymph of the adult female, male, and larva, the triene is specifically associated to lipophorin in the adult female hemolymph. The

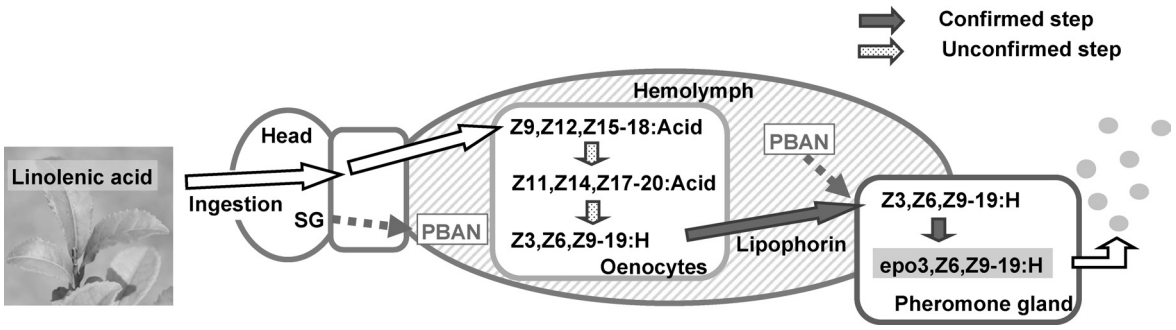


Fig. 1. Biosynthesis of an epoxyalkenyl sex pheromone in the *A. s. cretacea* female. The pheromone and biosynthetic precursors are abbreviated as shown in Table 1.

total of the associated triene in one female is about 1 μg.

The topical application of a mixture including the deuterated triene and two other related hydrocarbons showed equal amounts of association by HDLp of the *A. s. cretacea* females but selective delivery of the precursor to pheromone glands.[14] The adult females did not develop HDLp specialized in the transport of the triene, but selective supply of the precursor to the gland seems to play an important role in the biosynthesis of the species-specific pheromone.

Endocrine Regulation of Biosynthesis

The level of the epoxy pheromone in the *A. s. cretacea* females reaches a peak during the late scotophase, when female moths frequently display a calling behavior.[11] The titer rapidly decreases after lights-on. The biosynthesis of lepidopteran pheromones is usually activated at calling time, in synchronicity with an environmental cue by an endocrine system; the neuropeptide hormone named pheromone biosynthesis-activating neuropeptide (PBAN) is produced in the subesophageal ganglion (SG) under a circadian rhythm that is entrained by the photoperiodic cue.[15] In the geometrid species, the females also lose the epoxy pheromone after decapitation and produce it again by the injection of a head extract or synthetic PBAN of *B. mori* (Bom-PBAN), indicating that the neuropeptide controls the pheromone biosynthesis of *A. s. cretacea*, activating the epoxidation of the trienyl precursor in the pheromone gland.

To confirm the above prediction, two experiments were carried out using deuterated triene and Bom-PBAN.[16] When the precursor was injected into the abdomens of decapitated females, the titers of both unlabeled and labeled epoxy pheromones were dose-dependently increased by Bom-PBAN injection; however, when the trienyl precursor was topically applied to the isolated pheromone gland, the triene was epoxidized without Bom-PBAN. This result showed that the neuropeptide was not necessary for the epoxidation in the *A. s. cretacea* females, and we concluded that Assc-PBAN accelerated triene uptake by the pheromone gland. Injected triene was associated with lipophorin, and Assc-PBAN was necessary to carry the triene into the pheromone gland. In the case of topical application, the triene dissolved in DMSO probably entered the pheromone gland with the help of the solvent. In female moths secreting Type I pheromones, PBAN usually activates a biosynthetic step. Assc-PBAN showed unique action (Fig. 1).

Identification of Assc-PBAN

As the first step to clarify the details of the activation process, we identified Assc-PBAN. Applying the degenerate primers, a PCR product was obtained in the experiment with cDNA from brain-SG complexes of *A. s. cretacea*, and Assc-PBAN cDNA was isolated by using 5′- and 3′-rapid amplification of cDNA end strategies. The cDNA encodes 181 amino acids, including a PBAN homologue and four other putative peptides: a diapause hormone homologue, α-SG neuropeptide (SGNP), β-SGNP, and γ-SGNP, all of which shared an FXPR(K)L motif on their C-termini.[17] Although PBANs with 30–35 amino acids have been characterized, the Assc-PBAN homologue (Assc-PBAN I) consisted of 28 amino acids and showed lower homology (<46%) than the others, as shown in Fig. 2. Assc-β-SGNP with 8 amino acids was also shorter than the other β-SGNPs (16–22 amino acids). Furthermore, all of the known PBAN cDNAs encode a GRR sequence between β-SGNP and PBAN as a cleavage site,[18] but the Assc-PBAN cDNA showed an unusual GR sequence at the corresponding position, indicating the possibility of non-cleavage

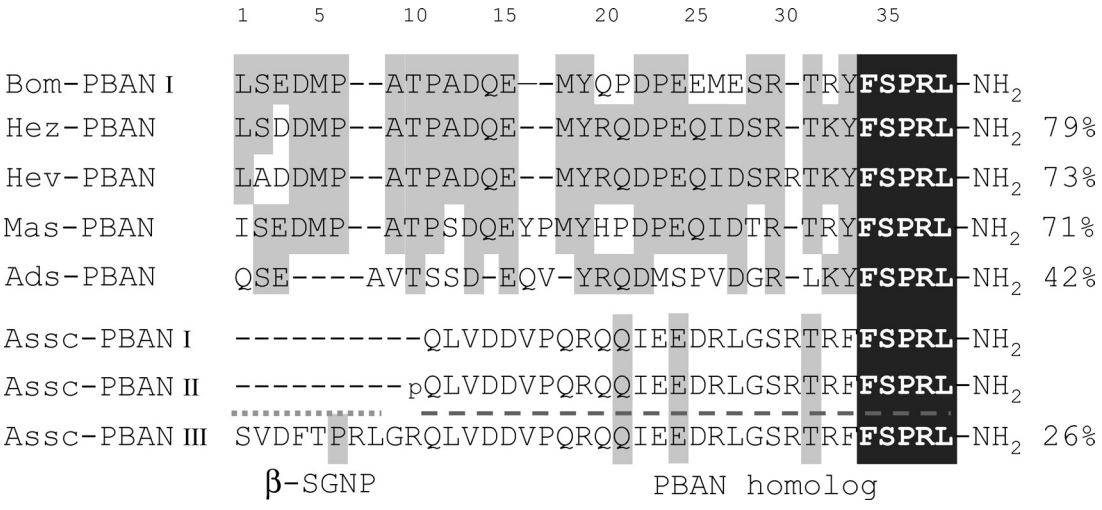


Fig. 2. Homology comparison of PBAN of *Ascotis selenaria cretacea*, Assc-PBANs I-III (AB308061), to those of other lepidopteran species (GenBank accession numbers). Bom, *Bombyx mori* (S50045); Hez, *Helicoverpa zea* (U08109); Hev, *Heliothis virescens* (AY173075); Mas, *Manduca sexta* (AY172672); Ads, *Adoxophyes* sp. (AF395670). Percentages on the right represent amino acid identities, which are compared to the sequence of Bom-PBAN I. Assc-PBAN III, which included the sequences of two FXPRL peptides (β-SGNP and PBAN homolog), showed strongest activity among three candidates (I–III) and its secretion was estimated.

between the β-SGNP and PBAN; i.e., secretion of an unusually long peptide with 38 amino acids (Assc-PBAN III). When the GR sequence was a cleavage site, the question arose of whether or not the glutamine residue at the N-terminus of the Assc-PBAN homologue was cyclized and the neuropeptide including a pyroglutamine (Assc-PBAN II) was secreted.

We synthesized Assc-PBANs I-III (Fig. 2) with the predicted sequences by using solid-phase methodology with Fmoc-strategy using an automated peptide synthesizer and observed the highest activity of Assc-PBAN III among the three candidates.[17] Furthermore, to identify the sequence of the Assc-PBAN actually produced, the brain-SG extract was fractionated by HPLC referring to the synthetic peptides, and pheromonotropic activity of each fraction was measured. Chromatographic behavior of the natural pheromonotropic peptide revealed a unique structure, including β-SGNP (Assc-PBAN III).[17] By RT-PCR screening, specific high expression of Assc-PBAN mRNA was detected in the brain-SG complexes of the female and male adults and, interestingly, weak expression was observed in thoracic ganglions.

In previous studies, PBAN cDNAs have been reported from 15 other lepidopteran species, eight Noctuidae, two Bombycidae, one Sphingidae, and so on. These insects exclusively produce Type I pheromones; therefore, our study deals with the first identification of PBAN cDNA from the species secreting a Type II pheromone. The phylogenic tree indicates the homology of these PBAN cDNAs, which correlated well with their taxonomical aspects. However, our knowledge of PBAN genes is still limited, and further studies with other species, particularly those producing the Type II pheromone, will be necessary.

Next Challenges

Future studies will seek the site for production of polyunsaturated hydrocarbons, the precursors of epoxyalkenyl sex pheromones. The most likely organ is the oenocytes, although fat bodies are another possibility. While complete separation of the two organs is not easy in a moth, we would like to compare the titers of the precursors at several stages to differentiate biosynthetic and stockpiling sites. We will also try to identify the long-chain fatty acids occurring there and examine the conversion of the labeled acids into polyunsaturated hydrocarbons to present a full picture of Type II pheromone biosynthesis. In the case of Type I pheromones, several desaturases with regiospecificity have been characterized. The epoxygenases working in Type II pheromone biosynthesis also have regiospecificity and are important enzymes to understand the mechanism whereby species-specific pheromones are produced. As an extension of the studies on Assc-PBAN, the receptor structure of the neuropeptide hormone and physiological functions of other FXPRL amides, DH homologue and three SGNPs found in the cDNA of *A. s. cretacea*, are interesting subjects. In addition to pheromone production in females, modern techniques of molecular biology are unraveling the construction of the male antennae and mechanism of odorant perception. The diversity of the mating communication systems mediating with sex pheromones is owing to the combination of biosynthetic development in females and synchronized changes of male perception. Recently, receptors of bombykol and some Type I pheromones were identified. Recognition specificity in Type II pheromones should also be understood in order to apply this knowledge to pest control in the near future.

References

1) A. S. Hill and W. L. Roelofs: *J. Chem. Ecol*. **7**, 655–668 (1981).
2) http://www.tuat.ac.jp/~antetsu/LepiPheroList.htm
3) T. Ando, S. Inomata and M. Yamamoto: *Topics Curr. Chem*. **239**, 51–96 (2004).
4) M. Yamamoto, M. Kiso, H. Yamazawa, J. Takeuchi and T. Ando: *J. Chem. Ecol*. **26**, 2579–2590 (2000).
5) H. Yasui, S. Wakamura, N. Arakaki, H. Irei, C. Kiyuna, H. Ono, H. Yamazawa and T. Ando: *J. Chem. Ecol*. **31**, 647–656 (2005).
6) S. Wakamura, N. Arakaki, H. Yamazawa, N. Nakajima, M. Yamamoto and T. Ando: *J. Chem. Ecol*. **28**, 449–467 (2002).
7) S. Wakamura, N. Arakaki, M. Yamamoto, S. Hiradate, H. Yasui, T. Yasuda and T. Ando: *Tetrahedron Lett*. **42**, 687–689 (2001).
8) M. Komoda, S. Inomata, A. Ono, H. Watanabe and T. Ando: *Biosci. Biotechnol. Biochem*. **64**, 2145–2151 (2000).
9) T. Ando, K. Ohtani, M. Yamamoto, T. Miyamoto, X.-R. Qin and Witjaksono: *J. Chem. Ecol*. **23**, 2413–2423 (1997).
10) Witjaksono, K. Ohtani, M. Yamamoto, T. Miyamoto and T. Ando: *J. Chem. Ecol*. **25**, 1633–1642 (1999).
11) T. Fujii, M. G. Suzuki, T. Kawai, K. Tsuneizumi, A. Ohnishi, M. Kurihara, S. Matsumoto and T. Ando: *J. Insect Physiol*. **53**, 312–318 (2007).
12) T. Miyamoto, M.Yamamoto, A. Ono, K.Ohtani and T. Ando: *Insect Biochem. Mol. Biol*. **29**, 63–69 (1999).
13) W. Wei, T. Miyamoto, M. Endo, T. Murakawa, G.-Q. Pu and T. Ando: *Insect Biochem. Mol. Biol*. **33**, 397–405 (2003).
14) K. Matsuoka, H. Tabunoki, T. Kawai, S. Ishikawa, M. Yamamoto, R. Sato and T. Ando: *Insect Biochem. Mol. Biol*. **36**, 576–583 (2006).
15) A. K. Raina: *Annu. Rev. Entomol*. **38**, 329–349 (1993).
16) W. Wei, M. Yamamoto, T. Asato, T. Fujii, G.-Q. Pu and T. Ando: *Insect Biochem. Mol. Biol*. **34**, 1215–1224 (2004).
17) T. Kawai, A. Ohnishi, M. G. Suzuki, T. Fujii, K. Matsuoka, I. Kato, S. Matsumoto and T. Ando: *Insect Biochem. Mol. Biol*. **37**, 330–337 (2007).
18) J. A. Veenstra: *Arch. Insect Biochem. Physiol.* **43**, 49–63 (2000).