Identification of Three Sex Pheromone Components of the Female Saturniid Moth *Antheraea pernyi* (Lepidoptera: Saturniidae)*

Hans-Jürgen Bestmann, Athula B. Attygalle, Thorolf Brosche, Joachim Erler, Hans Platz, Jürgen Schwarz, Otto Vostrowsky, and Wu Cai-Hong

Institut für Organische Chemie, Universität Erlangen-Nürnberg, Henkestraße 42, D-8520 Erlangen, Bundesrepublik Deutschland

Karl Ernst Kaissling

Max Planck Institut für Verhaltensphysiologie, D-8131 Seewiesen, Bundesrepublik Deutschland

and

Chen Te-Ming

Department of Biology, Beijing University, Beijing, PR China

Z. Naturforsch. **42c,** 631–636 (1987); received December 12, 1986

Sex Pheromone, *Antheraea pernyi,* Saturniidae, Lepidoptera

By means of electroantennography and single cell recordings, GC and GCMS analyses and GC analysis with EAG detection (6*E*,11*Z*)-6,11-hexadecadienal, (6*E*,11*Z*)-6,11-hexadecadienyl acetate and (4*E*,9*Z*)-4,9-tetradecadienyl acetate were identified as the primary components of the sex pheromone of female *Antheraea pernyi* (Lepidoptera: Saturniidae).

The composition of two female saturniid moth sex pheromones has been reported to date, (*Z*)-5-decenyl isovalerate as the attractant of the pine emperor moth, *Nudaurelia cytheraea* (F.) [2], and a mixture of (6*E*,11*Z*)-6,11-hexadecadienyl acetate and (6*E*,11*Z*)-6,11-hexadecadienal as the pheromone of the wild silkworm moth, *Antheraea polyphemus* (Cramer) [3].

In the course of electrophysiological studies on interspecific relationships of pheromone perception in saturniid moths [4], we were interested in the composition of the pheromone blend of another silkworm moth, *Antheraea pernyi* (Saturniidae). This species appears in the north-eastern parts of China.

* Pheromones 59 [1].

Abbreviations: LC, liquid chromatography; GC, gas chromatography; FSCC, fused silica capillary column; GCMS, GC combined mass spectrometry; EAG, electroantennogram; EAD, electroantennographic detector; FID, flame ionisation detector; E6Z11-16:Al, (6*E*,11*Z*)-6,11-hexadecadienal; E6Z11-16:Ac, (6*E*,11*Z*)-hexadecadienyl acetate; E4Z9-14:Ac, (4*E*,9*Z*)-4,9-tetradecadienyl acetate; E6-16:Ac, (*E*)-6-hexadecenyl acetate; Z11-16:Ac, (*Z*)-11-hexadecenyl acetate; 16:Ac, hexadecyl acetate; TCD, thermal conductivity detector.

Reprint requests to Prof. Dr. H.-J. Bestmann.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/0500–0631 $ 01.30/0

Material and Methods

Insect material

Pupae of *Antheraea pernyi* were provided by the Institute of Silkworm Science of Liaoning Province, PR China. The insects were raised in the laboratory on wild oak foliage, two generations each year. The first generation pupae, raised between March and July, dit not diapause. The second generation cocoons were maintained in underground holes, at 5 °C during the winter time. After diapause, raising the temperature to 20–25 °C caused emergence three to four weeks later.

The pupae were sexed and kept at 22 °C in plastic boxes lined with moistened filter paper. A reversed 16:8 h light:dark cycle was maintained throughout the study. Eclosed adults were collected daily. To observe the calling behaviour, the insects were examined under a red light during the scotophase.

Pheromone extraction

Abdominal tips of individual virgin females were excised and stored in 1 ml hexane at −20 °C. The hexane extract was filtered through glass wool and concentrated by blowing a fine stream of nitrogen over it.

LC fractionation

LC fractionation was performed on a 30 × 1.5 cm silica gel column (0.06–0.2 mm) by an eluent system of increasing polarity [5]. Hexane extracts of abdominal tips were separated into 15 fractions [5] and the fractions were reconcentrated before use.

GC fractionation

A 2 m × 0.25″ steel GC column, packed with 10% SE30 on Chromosorb W, 100/120 mesh, mounted in a HP 5750 gas chromatograph equipped with a thermal conductivity detector was used.

Five µl each of crude hexane extracts were injected (inj. temp. 250 °C, oven 180 °C, det. 250 °C) and the effluent, after passing the TCD, was condensed in cooled (−78 °C) pasteur pipettes. The pipettes were exchanged at two-minute intervals and each condensate was dissolved in 150 µl hexane each. The solutions were subsequently used for electrophysiological testing.

Encapsulation of pheromone glands

The abdominal tips of 3-day-old female moths were excised during the period of maximum calling. The intersegmental membrane between the segments VIII and IX containing the pheromone components was excised under a binocular microscope according to Attygalle *et al.* [6]. The samples were sealed in soda glass capillaries (2 cm × 2 mm) and used for immediate analysis or stored at −20 °C.

Gas chromatography with solid sample injection

Capillary gas chromatography with flame ionization detection was performed with a Packard United-Technologies 438A instrument equipped with a splitless injector and a Shimadzu Chromatopac C-R3A data system. FSCC (25 m × 0.22 mm) SP-2340, 0.21 µ film, 60 °C for 2 min, 60–195 °C at 4 °C/min. Samples sealed in glass capillaries were chromatographed by a solid sample injection technique [6].

Gas chromatography with EAG detection

The effluent of a CPSil 19 FSCC (25 m × 0.22 mm, mounted in a Packard United-Technologies gas chromatograph) was split and one part of it directed over a male insect antenna [5, 7]. By means of implanted capillary electrodes on the isolated antenna the retention times of electrophysiologically active compounds present in the column effluent were determined (electroantennographic detection, EAD).

GC peak trapping of the tetradecadienyl acetate

The glandular tissues of 5 calling females were extracted into 10 µl of hexane. The extract was reduced to 5 µl and injected onto a 4% OV-17 GC column (glass, 80–100 mesh Gas Chrom Q, 2 m × 4 mm, 210 °C isothermal, Hewlett-Packard 5750 G gas chromatograph). The effluent was split 99:1 (trap: FID, all glass splitter). The effluent volume corresponding to that of C_{14}-acetates was trapped in a glass tube cooled in dry ice.

Microozonolysis

Dry ozone was passed through the glass tube containing the sample to be ozonised, *via* a fused silica tube (0.4 mm OD) for 20 sec, until O_3 eluted from the other end and turned a piece wet starch-KI paper blue. The tube was immediately sealed and chromatographed by a solid sample injection technique.

Results and Discussion

Electrophysiological studies

Single sensillum recording performed with sensory hairs of male antennae of *A. pernyi* resulted in the detection of three types of receptor cells. Two of these cells were most effectively excited by the two compounds, (6*E*,11*Z*)-6,11-hexadecadienal and (6*E*,11*Z*)-6,11-hexadecadienyl acetate, already known as the pheromone components of the saturniid moth *A. polyphemus* [3]. This result indicated that these two substances can also be regarded as candidates for pheromone components of *A. pernyi*. This was supported by the fact that male *A. pernyi* antennae showed a complete electroantennogram reaction to gland volatiles of female *Antheraea polyphemus* [8] and have two types of receptor cells responding very sensitively to these two compounds [9].

Furthermore, morphological investigations showed that the sensory hairs of male antennae of *A. pernyi* have rarely a third receptor cell [10]. In *A. polyphemus*, nerve impulses of a third cell have been recorded [9] suggesting the existence of a third pheromone component. One (or more) pheromone components other than E6Z11-16:Al and E6Z11-

16:Ac were predicted for *A. pernyi* based on comparisons with other saturniid species [9].

From abdominal tips of 15 female moths a hexane extract was prepared. The extract was gas chromatographically fractionated, and each fraction was tested by the electroantennogram technique (EAG) as well as with single cell recording of single sensory hairs of male *Antheraea* antennae. EAG recordings performed with *A. pernyi* male antennae gave significantly high response amplitudes to those fractions having the retention times corresponding to those of E6Z11-16:Al and E6Z11-16:Ac, respectively. Similarly, single cell recordings of the GC fractions were performed with sensory hairs of males of the related saturniid *A. polyphemus*. The sensilla trichodea of this species possess two specialized types of receptor cells, one maximally responding to (6*E*,11*Z*)-6,11-hexadecadienal (cell type A in Fig. 1) and the other one to (6*E*,11*Z*)-6,11-hexadecadienyl acetate (type B). Since *A. polyphemus* antennae produce larger response amplitudes to the acetate, and those of *A. pernyi* are more responsive to the aldehyde, the respective antennae can be employed as biological detectors for these substances.

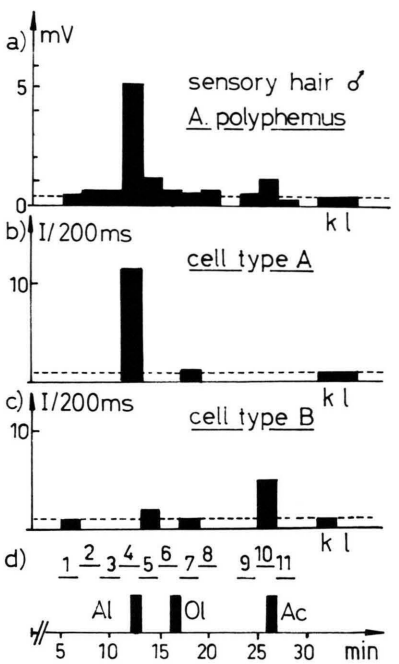

Fig. 1. Electrophysiological testing on sensory hairs of male *A. polyphemus* (single cell recording, electrosensillogram, ESG) of eleven GC fractions of a hexane extract from 15 *A. pernyi* glands. a) Receptor potential [mV], k, l = blanks; b) action potentials [impulse rate I/200 msec], aldehyde cell type A; c) action potentials, acetate cell type B; d) fractions no. 1–11, blanks k, l, retention times of E6Z11-16:Al (Al), E6Z11-16:Ol (OH) and E6Z11-16:Ac (Ac).

With the aid of these detectors, the presence of E6Z11-16:Al as well as E6Z11-16:Ac in certain gas chromatographic fractions of hexane extracts of *A. pernyi* glands was established. These fractions had the same retention times as those of authentic samples of E6Z11-16:Al and E6Z11-16:Ac, respectively.

A quantification of the relative amounts of these two pheromone components could be made by comparing the impulse rates of sensory cells of male *A. polyphemus* antennae given by the respective fractions of female *A. pernyi* and *A. polyphemus* extracts, with those from known quantities of E6Z11-16:Al and E6,Z11-16:Ac (Table I). While for the *polyphemus* pheromone a 1:10 (aldehyde:acetate) ratio could be determined in agreement with ref. [3], for *pernyi* a 5:1 ratio was found. This opposite ratio may explain why no cross attraction occurs between these two species in field tests. Also, traps baited with the lure for *A. polyphemus* did not attract any *A. pernyi* males [3].

Table I. Female equivalents [μg] of E6Z11-16:Al and E6Z11-16:Ac, in extract fractions of abdominal tips of *A. pernyi* (N = 15) and *A. polyphemus* (N = 5) as determined by single cell recording (ESG) technique. The quantification was performed by a comparison of responses with those from known quantities of authentic compounds.

	Male insect antenna of *A. polyphemus*	
GC fractions of an extract from females of	Cell type A aldehyde	B acetate
A. pernyi	1×10^{-3} μg	2×10^{-4} μg
fraction no.	4	10
A. polyphemus	1×10^{-4} μg	1×10^{-3} μg
fraction no.	4	10

Gas chromatography with EAG detection

Crude pheromone extracts, as well as EAG active fractions from column chromatography of abdominal extracts of female *pernyi* moths, were gas chromatographed on capillary columns and the col-

umn effluent was monitored by insect antennae (EAG-detector [5, 7]). When an *A. pernyi* antenna was used as the biological detector, in most cases, only one signal with a retention time corresponding to that of E6Z11-16:Al was obtained. With an *A. polyphemus* antenna, which is more responsive to E6Z11-16:Ac than to the corresponding aldehyde, always two signals were detected. The retention times of these two signals were same as those of the hexadecadienal, E6Z11-16:Al, and the hexadecadienyl acetate, E6Z11-16:Ac, respectively. The same results were also achieved by the solid sampling GC technique [6] described below, using *A. pernyi* and *A. polyphemus* antennae, respectively, as the GC detectors.

GC and GCMS analysis by a solid sample injection technique

In the later stages of this work, GC and GCMS analyses were done by a solid sample injection technique [6]. Under our laboratory conditions, *A. pernyi* females showed maximum calling activity *ca.* 4 h after the onset of the scotophase. During the calling period, the abdominal segments containing the pheromone gland were excised, encapsulated in soda glass capillaries, and chromatographed. The polar GC phase SP2340, used in this investigation, is able to resolve most of the positional and geometrical isomers of unsaturated compounds [11] found as lepidopteran pheromones, and it also resolved the four geometrical isomers of the *Antheraea* pheromone (for the synthesis of these compounds see ref. [12]).

Fig. 2 shows a gas chromatogram of a GCMS recording of the volatiles from one pheromone gland of female *A. pernyi*. In addition to the expected E6Z11-16:Ac, (*E*)-6-hexadecenyl acetate (E6-16:Ac), (*Z*)-11-hexadecenyl acetate (Z11-16:Ac), hexadecyl acetate (16:Ac), an octadecenyl acetate, a hexadecadienol and a tetradecadienyl acetate were also identified (Table II). The double bond position of the monounsaturated acetates was established by comparing their mass spectra by the method of Horiike and Hirano [13] with those from a series of candidate compounds. The configuration of the double bonds was determined by comparing the retention times with those from authentic material. The double bond positions of the tetradecadienyl acetate were determined by microozonolysis as described below.

In addition to these "pheromone-like" substances, acetamide and a series of hydrocarbons were also found in the abdominal tips of the female moths. These compounds were considered as non-specific tissue components, since they are frequently found in most pheromone analyses [1, 6, 14] by this technique, regardless of the species.

As seen from Fig. 2, a peak corresponding to E6,Z11-16:Al was not in the chromatogram, when a mass spectrometer was used as the detector as opposed to a biological antennal detector. This may be due to either the aldehyde being biosynthesized just before release, or some limitation of the method, which makes it not being suitable for such an analysis. Similar observations have been made with an analysis of *Bombyx mori* glands [6].

However, the aldehyde E6Z11-16:Al must be considered as the most effective constituent of *A. pernyi* pheromone, because of the evidence discussed in the section before. Furthermore, the substances 16:Ac, Z11-16:Ac, hexadecadienol and octadecenyl acetate were ruled out as primary pheromone components because of the lack of any significant physiological activity in the EAG and EAD-GC experiments.

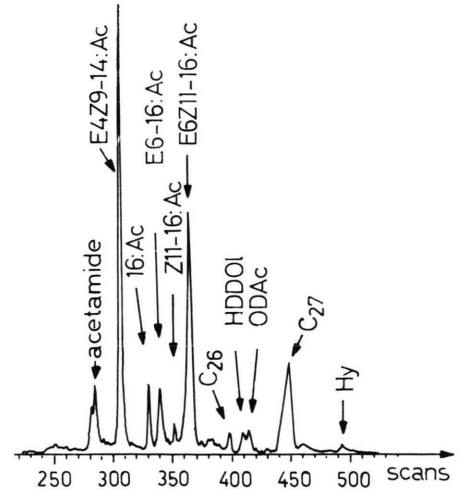

Fig. 2. GCMS analysis of the volatiles from one pheromone gland of a female *A. pernyi* moth (solid sample injection). (Abbr.: 16:Ac = hexadecyl acetate, E6-16:Ac = (*E*)-6-hexadecenyl acetate, Z11-16:Ac = (*Z*)-11-hexadecenyl acetate, E4Z9-14:Ac = (4*E*,9*Z*)-4,9-tetradecadienyl acetate, E6Z11-16:Ac = (6*E*,11*Z*)-6,11-hexadecadienyl acetate, HDDOl = hexadecadienol, ODAc = octadecenyl acetate, Hy, C_{26}, C_{27} = hydrocarbons.)

Table II. A list of compounds identified from the volatiles of a pheromone gland of female *A. pernyi*, their relative amounts and method of identification.

Compound[a]	Rel. amount[b] (E6Z11-16:Al = 100)	Method of identification[c]
E6Z11-16:Al	100	EAG ESG EAD GC-ESG
E6Z11-16:Ac	20	EAG ESG EAD GC-ESC GC GCMS
E4Z9-14:Ac	40	ESG GC GCMS OZ
16:Ac	4	GC GCMS
E6-16:Ac	4	GC GCMS
Z11-16:Ac	1	GC GCMS
HDDOl		GCMS
ODAc		GCMS

[a] Abbreviations see Fig. 2.
[b] Only pheromone-like substances considered.
[c] EAG, electroantennogram; ESG, single cell recording; EAD, electroantennogram GC-detection; GC-ESG, single cell recording of GC-fractions; GC, gas chromatography; GCMS, GC combined mass spectrometry; OZ, ozonolysis.

(4E,9Z)-4,9-Tetradecadienyl acetate, the third pheromone component of A. pernyi

As GCMS analysis revealed the presence of a tetradecadienyl acetate in significant amounts (Fig. 2) in the gland, microozonolysis was performed to determine the locations of the double bonds in this compound. Among the ozonolysis products pentanal and 4-acetoxybutanal were identified by gas chromatography, thus deducing the structure as a 4,9-tetradecadienyl acetate. The double bond geometries were established as (E4,Z9) by comparison of the retention time with those of authentic geometrical isomers of the tetradecadienyl acetate (for synthesis see [12]).

Once the complete structure of the tetradecadienyl acetate was known, a thorough electrosensillographic survey (ESG) was carried out using E6Z11-16:Al, E6Z11-16:Ac and E4Z9-14:Ac as stimulus sources. This survey revealed that E4Z9-14:Ac is an essential component of the pheromone complex of *A. pernyi*. All the short olfactory hairs investigated (N = 31) contained a cell for E6Z11-16:Ac with small spike amplitude (100%). A cell for E6Z11-16:Al firing large spikes was found in 97% of the sensilla, whereas a cell for E4Z9-14:Ac, which elicits large spikes also, was detected only in 55% of the hairs studied [15].

The proof for the presence of (4*E*,9*Z*)-4,9-tetradecadienyl acetate in the pheromone gland volatiles, corroborated by the evidence of sensory cells for this substance in male antennae, justified the consideration of this compound as a primary pheromone constituent of female *A. pernyi*. This compound has, so far, not been detected in the glands of *A. polyphemus*, although the third receptor cell type of *polyphemus* males (small nerve impulses, [9]) responds to this compound very sensitively [15].

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft, the Stiftung Volkswagenwerk and the Alexander von Humboldt-Stiftung (A.B.A.).

[1] Pheromones 58: A. B. Attygalle, Wu Cai-Hong, J. Schwarz, O. Vostrowsky, I. Hasenfuß, and H. J. Bestmann, J. Chem. Ecol., in press (1987).
[2] H. E. Henderson, F. L. Warren, O. P. N. Augustyn, B. V. Burger, D. F. Schneider, P. R. Boshoff, H. S. C. Spies, and H. Geertsema, J. Chem. Soc., Chem. Comm. **1972,** 686.
[3] J. Kochansky, J. Tette, E. F. Taschenberg, R. T. Cardé, K. E. Kaissling, and W. L. Roelofs, J. Insect Physiol. **21,** 1977 (1975).
[4] H. J. Bestmann, Wu Cai-Hong, B. Döhla, Li-Kedong, and K. E. Kaissling, Z. Naturforsch. **42c,** 435 (1987).
[5] O. Vostrowsky and H. J. Bestmann, Mitt. dtsch. Ges. allg. angew. Ent. **1,** 152 (1978).
[6] A. B. Attygalle, M. Herrig, O. Vostrowsky, and H. J. Bestmann, J. Chem. Ecol. **13,** 1299 (1987).
[7] D. L. Struble and H. Arn, in: Techniques in Pheromone Research (H. E. Hummel and T. A. Miller, eds.), p. 161, Springer Verlag, Berlin 1984.
[8] E. Priesner, Z. vergl. Physiol. **61,** 263 (1968).
[9] K. E. Kaissling, in: Chemical Ecology: Odour Communication in Animals (F. J. Ritter, ed.), p. 43, Elsevier, North-Holland, Biomed. Press 1979.
[10] T. A. Keil, Zoomorphology **104,** 147 (1984).
[11] R. R. Heath and J. H. Tumlinson, in: Techniques in Pheromone Research (H. E. Hummel and T. A. Miller, eds.), p. 287, Springer Verlag, Berlin 1984.
[12] H. J. Bestmann, B. Döhla, Li-Kedong, and T. Zeibig, in preparation.
[13] M. Horiike and C. Hirano, Agric. Biol. Chem. **46,** 2667 (1982).
[14] A. B. Attygalle, J. Schwarz, O. Vostrowsky, and H. J. Bestmann, Z. Naturforsch. **41c,** 1077 (1986).
[15] Wu Cai-Hong, H. J. Bestmann, C. Meng, and K. E. Kaissling, in preparation.