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0014-4754/92/060606-05$1.50 + 0.20/0

Pheromone components of the female elephant hawk-moth, *Deilephila elpenor*, and the silver-striped hawk-moth, *Hippotion celerio* [1]

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Received 27 March 1991; accepted 29 November 1991

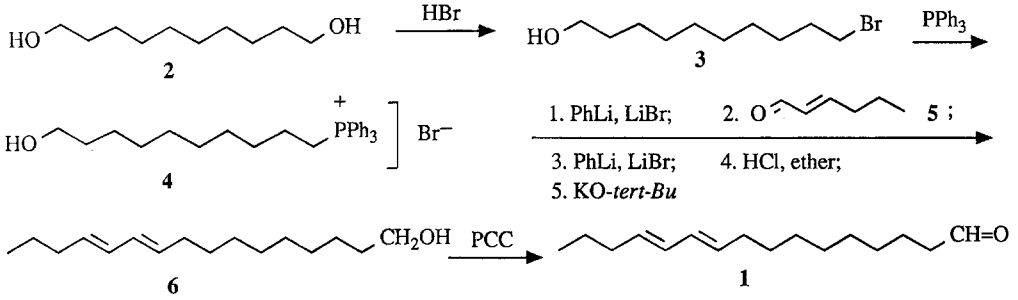
Abstract. By means of gas chromatographic and mass spectroscopic methods, and combined GC-electroantennogram and electrosensillogram techniques, (*E*)-11-hexadecenal and (10*E*, 12*E*)-10,12-hexadecadienal [(*E*,*E*)-bombykal] were identified as components of the sex pheromone of the female sphingid moth *Deilephila elpenor*. The (*E*,*E*)-bombykal is also the main constituent of the pheromone of the silver-striped hawk-moth *Hippotion celerio*. The biological activity of the substances was demonstrated with electroantennogram and single cell recording, and the physiological efficacy of the different hexadecadienal isomers compared.
Key words. Deilephila elpenor; Hippotion celerio; Sphingidae; pheromone components; (*E*,*E*)-bombykal; (*E*)-11-hexadecenal.

The elephant hawk-moth (German: Mittlerer Weinschwärmer), *Deilephila elpenor* L. (Lepidoptera, Sphingidae), is one of the most common sphingid species of Central Europe, with a distribution throughout the palearctic region. The silver-striped hawk-moth (German: Grosser Weinschwärmer), *Hippotion celerio* L. (Sphingidae), is a sphingid species of the tropics and subtropics of the old world, occasionally migrating to Central Europe.

Already in 1979 Starrat et al. described the identification of (10*E*, 12*Z*)-10,12-hexadecadienal [bombykal] as an active component of the sex attractant of the female tobacco hornworm moth, *Manduca sexta* L. (Sphingidae), using an electroantennogram biosasay [2]. Bombykal was originally found as a sex pheromone component in the silkworm moth *Bombyx mori* L. (Bombycidae) [3]. A more detailed analysis of solvent rinses of pheromone glands of *M. sexta* revealed the presence of a series of twelve saturated, mono-, di- and triunsaturated C_{16}- and C_{18}-aldehydes [4]; (10*E*, 12*Z*)-10,12-hexadecadienal [bombykal] and (10*E*, 12*E*, 14*Z*)-10,12,14-hexadecatrienal represented the active principle required to stimulate a complete behavioral sequence [4]. This is, to the best of our knowledge, the only pheromone from any member of the sphingid family of Lepidoptera of which the composition is known.

Materials and methods

Insect rearing. D. elpenor L. were caught at Erlangen (FRG), and subsequent generations reared on willowherb *Epilobium* (Onagraceae) in the laboratory. *H. celerio* L. were collected on the Canary Island "la Gomera" and also reared on *Epilobium* species in summer and on vine *Parthenocissus* (Vitaceae) in winter, under a 14 : 10 h light : dark regime. Pupae were sexed and separated a few days before hatching.



Synthesis of (10*E*, 12*E*)-10,12-hexadecadienal **1** [*E,E*-bombykal].

Isolation and identification. From newly-hatched calling virgin females the eight and ninth abdominal segments with the corresponding intersegmental membrane, the pheromone gland, were excised under a stereo microscope, and each preparation extracted with 15 µl hexane or CS_2 for GC analysis. In addition separated glands were sealed in glass capillaries and analyzed gas-chromatographically using the solid sampling technique[5].

The GC analyses were performed on a HP model 5890 A gas chromatograph equipped with a splitless injector, a flame ionization detector [FID] and a solid sampler[5]. The volatiles were chromatographed on a 25-m FSCC SE 54 capillary column (0.25 mm ID). Injection port and detector temperatures were 200 °C and 240 °C, respectively, and the column was programmed from 80 °C initial temperature (3 min) to 230 °C final temperature at 4 °C/min. Carrier gas (N_2) flow rate was 1 ml/min. GCMS analyses were conducted with a Finnigan MAT 90 GC-mass spectrometer in electron impact [EI] mode, 70 eV, with splitless injection, 25 m FSCC SP 2340 (0.25 mm ID), injector 200 °C, carrier gas He, 2 ml/min.

Electrophysiological bioassays. Electroantennograms [EAG] were recorded with separated male antennae, using filter paper loaded with test chemicals as stimulus source. For the electrosensillograms [ESG], single-cell recording was performed according to Kaissling's method[6] by cutting off the tip of a sensillum trichodeum and inserting it into the "different" electrode, the "indifferent" one being inserted into the base of the antenna. GLC-coupled EAG and ESG recordings were made using a Packard United Technologies A 49 model with a 1 : 1 effluent splitter which partitioned the column effluent to FID and electroantennogram detector. Injector 240 °C, FID detector 260 °C, column 25 m FSCC SP 2340 (0.25 mm ID), temperature program 5 min at 70 °C, 70–195 °C at 4 °C/min, carrier gas N_2.

Synthesis of E,E-bombykal. (10*E*, 12*E*)-10,12-Hexadecadienal **1** [*E,E*-bombykal] was synthesized according to Schlosser's method of (*E*)-stereoselective carbonyl olefination[7], starting from 1,10-decandiol **2**. Reaction with HBr and subsequent with triphenyl phosphane yielded the 10-hydroxydecyl triphenylphosphonium bromide **4**, which was converted into the corresponding ylide with PhLi and olefinated with (*E*)-2-hexenal **5**. Treatment of the reaction mixture with additional PhLi and LiBr, HCl and KO-*tert*-Bu gave (10*E*, 12*E*)-10,12-hexadecadien-1-ol **6** [36%, Kp 115 °C/0.01 Torr (bath temp.), IR (film): 3350, 1670, 980 cm^{-1}; ^{1}H-NMR ($CDCl_3$): 0.90–2.40 (23 aliph. H), 3.15 (s, OH, H-D), 3.48 (t, J = 6 Hz, CH_2O) and 4.89–6.44 (mc, 4 CH=) ppm; ^{13}C-NMR ($CDCl_3$): 13.95 (CH_3), 22.45, 25.60, 27.54, 29.08, 29.20, 29.25, 29.31, 31.32, 32.60, 32.75 (10 CH_2), 62.67 (CH_2O), 125.57, 128.47, 129.98 and 134.36 (4 CH) ppm; MS (70 eV): 238 (M^+), 220 ($-H_2O$), 67 (100)], from which *E,E*-bombykal **1** [33%, Kp 115 °C/0.01 (bath temp.); IR (film): 1740, 1670, 980 cm^{-1}; ^{1}H-NMR ($CDCl_3$): 0.90–2.40 (25 aliph. H), 4.90–6.35 (mc, 4 CH=) 9.93 (t, 2 Hz, CHO); ^{13}C-NMR ($CDCl_3$): 13.81 (CH_3), 22.63, 23.95, 26.91, 28.80, 29.02, 29.31, 29.44, 37.82, 43.66 (all CH_2), 126.03, 128.51, 129.11, 132.3 (4 CH) and 204.22 (CHO) ppm; MS (70 eV): 236 (6, M^+), 192 (1), 67 (100)] was obtained by pyridinium chlorochromate [PCC] oxidation (formula).

10,12-Hexadecadienal isomers as GC standards. (10*E*, 12*Z*)-10,12-Hexadecadienal [bombykal] and (10*Z*, 12*E*)-10,12-hexadecadienal [*Z,E*-bombykal] were available in our laboratory from previous work[8]. (10*Z*, 12*Z*)-10,12-Hexadecadienal [*Z,Z*-bombykal] was obtained as a minor isomeric reaction product only, resulting from an attempt to synthesize it starting from commercially available (*Z*)-2-hexenol. The mixture, mainly comprising (*Z,E*)-bombykal, still contained enough of the (*Z,Z*)-isomer for determination of GC retention and comparison.

Results and discussion

About 8 h after onset of the scotophase female *D. elpenor* exhibited calling behavior, that is, they visibly protruded their ovipositors. From groups of two or three female insects, the 8th and 9th abdominal segments together with the common intersegmental membrane were dissected and extracted with solvent. In initial attempts to identify pheromonally active compounds in the gland extract, the extract equivalent to two females was analyzed by gas chromatography with synchronous EAG-detection [EAD][9], using a male antenna. These are most sensitive towards the conspecific sex pheromone. Two physiologically active compounds (a and c in fig. 1 A) were found to

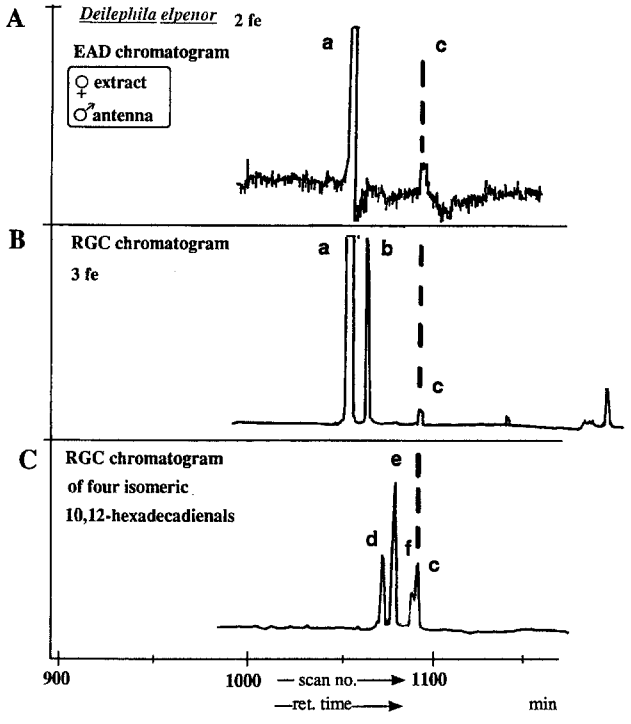


Figure 1. Structure elucidation of female *D. elpenor* sex pheromone components. *A* Gas chromatogram with electroantennogram detection [EAD] of volatiles of two female gland equivalents [fe]; *B* GCMS reconstructed gas chromatogram [RGC] of three insects' glands [fe]; a) (*E*)-11-hexadecenal, b) hexadecanal, c) (10*E*, 12*E*)-10,12-hexadecadienal; *C* RGC chromatogram of the four synthetic isomers of 10,12-hexadecadienal: d) (10*Z*, 12*E*)-10,12-hexadecadienal, e) (10*E*, 12*Z*)-10,12-hexadecadienal, f) (10*Z*, 12*Z*)-10,12-hexadecadienal and c) (10*E*, 12*E*)-10,12-hexadecadienal.

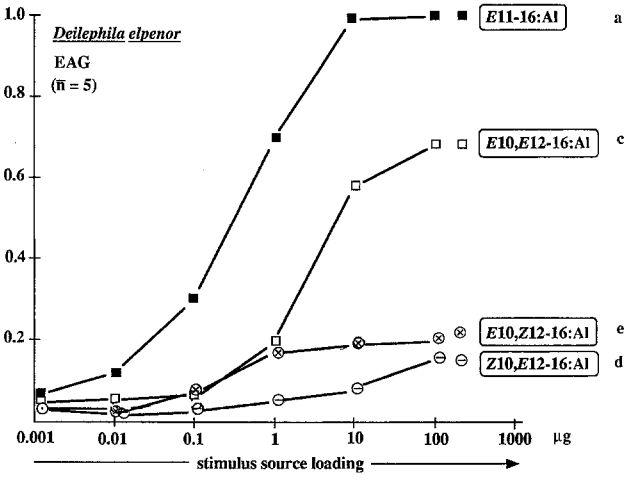


Figure 2. Normalized electroantennogram [EAG] dose response curves of (*E*)-11-hexadecenal [*E*11–16:Al a (= 1.0)], (10*E*, 12*E*)-10,12-hexadecadienal [*E*10 *E*12–16:Al c] and the 10,12-hexadecadienal isomers *Z*10 *E*12–16:Al d and *E*10 *Z*12–16:Al e obtained from male *D. elpenor* antennae (mean n of 5 recordings). Stimulus loading is given in µg.

have the retention time of oxygenated C_{16}-compounds. Recording the GC mass spectra, in the corresponding elution range the chromatogram of the gland extract of three female insects revealed three peaks (a, b and c in fig. 1 B). The two which eluted first were identified as (*E*)-11-hexadecenal (a) and hexadecanal (b) by comparing their mass spectrum and retention time with those of authentic compounds. The spectrum of c was almost identical with that of bombykal, but c had a slightly longer retention time. Because of this retention difference, substance c was thought to be an isomer of bombykal. Since originally only (10*E*, 12*Z*)- [bombykal] and (10*Z*, 12*E*)-10,12-hexadecadienal [*Z,E*-bombykal] were available in our laboratory, and the retention time of the (10*Z*, 12*E*)-isomer also differed from that of c, (10*E*, 12*E*)-10,12-hexadecadienal **1** [*E,E*-bombykal] had to be prepared according to the formula scheme.

The mixture of the four geometrical isomers of 10,12-hexadecadienal was separated gas-chromatographically under the same GC conditions, and the retention sequence determined with (10*Z*, 12*E*) (peak d in the figure), (10*E*, 12*Z*) (e), (10*Z*, 12*Z*) (f) and (10*E*, 12*E*) (c), as shown in figure 1 C. The mass spectrum and the retention time of compound c were in full agreement with those of **1**, defining the natural hexadecadienal of *D. elpenor* as (10*E*, 12*E*)-10,12-hexadecadienal **1**.

Both synthetic substances, (*E*)-11-hexadecenal a and (10*E*, 12*E*)-10,12-hexadecadienal c (**1**), were cochromatographed with an extract equivalent on a polar SP 2340 column with EAD detection for final identification. Additionally, comparative EAGs were recorded with (*E*)-11-hexadecenal a together with the hexadecadienal isomers c–e. The (*E,E*)-bombykal c was about ten times less active than the monounsaturated aldehyde a, both giving typically sigmoid dose response curves (fig. 2). The other bombykal isomers d and e revealed no significant electrophysiological activity at all. The receptor potential responses to a and c recorded by single cell measurements (ESG) reflected similar *dose response* types like the EAG experiments.

Since for the analysis of the female *H. celerio* pheromone an even smaller number of moths was available, the identification had to be based on more specific electrophysiological techniques. An extract of pheromone glands was produced by similar methods to those used before, gas-chromatographed, and monitored with a male antenna as a species-specific detector. The chromatogram of the extract revealed the presence of one physiologically active compound only. Its retention time was the same as that of (10*E*, 12*E*)-10,12-hexadecadienal c (**1**), as it had already been determined for the sex pheromone component of *D. elpenor*. To ascertain this identification, (*E,E*)-bombykal (**1**, c) was monitored with a male moth antenna, and gave a high response signal (fig. 3 A). A *sensillum trichodeum*, the pheromone-specific olfactory hair of a male antenna, was then prepared for an electrosensillogram (ESG, single cell recording)[10], and this most specific detector used in the GC analysis of the pheromone extract. With this ESG detector, only one type of spike was observed, reflecting the occurrence of only one physiologically active component in the

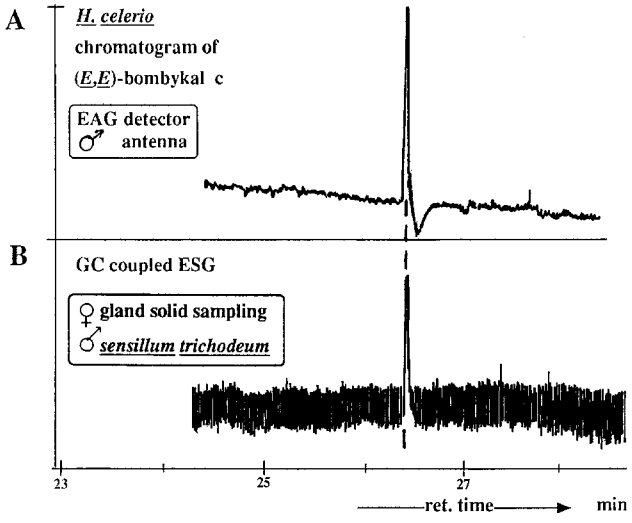


Figure 3. Pheromone identification of female *H. celerio* moths. *A* Gas chromatogram of (*E,E*)-bombykal **c** monitored with a male insect antenna; *B* chromatogram of the pheromone extract monitored by single cell recording (ESG) (receptor potential) with a male olfactory hair.

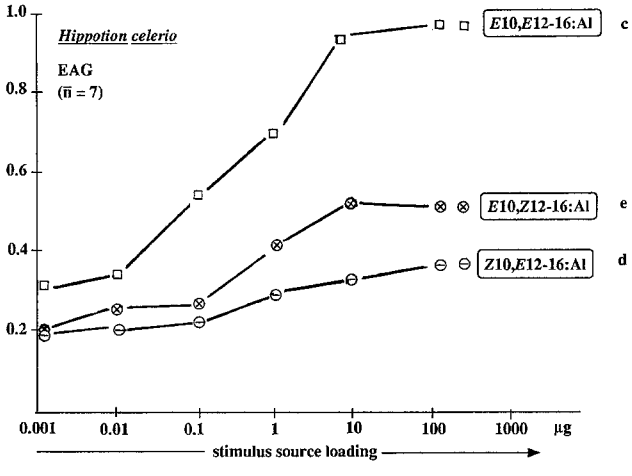


Figure 4. Normalized dose response curves of EAG tests obtained from male *H. celerio* antenna and the bombykal isomers *E*10 *E*12–16 : Al (c), *Z*10 *E*12–16 : Al (d) and *E*10 *Z*12–16 : Al (e) (mean n of 7 recordings), stimulus source loading in µg.

pheromone extract. The spike signal could be correlated with one significant receptor response in the extract (fig. 3 B), again at the retention time of (10*E*, 12*E*)-10,12-hexadecadienal **1**. Finally, the mass spectrum was recorded and the exact retention time determined, and the spectrum as well as the retention time of the physiologically active compound were found to be identical with those of authentic (*E,E*)-bombykal **1**.

In additional electrophysiological experiments, the same spike type of response was obtained from a male *H. celerio* antenna with (10*E*, 12*E*)-10,12-hexadecadienal **1** by separate ESG recordings. The spike frequency reflected a typical dose-response dependence. Comparing the electrophysiological activities of the isomeric bombykals using the electroantennogram test (EAG), the highest antennal responses were found (fig. 4) for (*E,E*)-bombykal **1**.

Acknowledgment. We want to thank Martin Grund, Claudia Obermeier and Regina Walther for their help in rearing the caterpillars. We thank the Deutsche Forschungsgemeinschaft and the Volkswagen-Stiftung for financial assistance. V. M. is indebted to the Deutscher Akademischer Austauschdienst for awarding a fellowship, D. S. for one from the Studienstiftung des Deutschen Volkes.

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