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Identification of a New Lepidopteran Sex Pheromone in Picogram Quantities using an Antennal Biodetector: (8*E*,10*Z*)-Tetradeca-8,10-dienal from *Cameraria ohridella*

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Abstract: A major sex attractant released by the virgin female of the horse-chestnut leafminer *Cameraria ohridella* Deschka et Dimić (Lepidoptera: Gracillariidae) which devastates horse-chestnut trees in Europe, was identified in picogram quantities as (8*E*,10*Z*)-tetradeca-8,10-dienal without using spectral methods. The identification solely relied on gas chromatography with electroantennographic detection (GC-EAD), calculation of Kovats' indices of the active principle on different GC phases, and construction of antennal response spectra (EAG response profiles) to C_{12} and C_{14} saturated and unsaturated standards with different functional groups. The dienal was prepared by a stereospecific synthesis and shown to be highly active for conspecific males in pg amounts and fully comparable to the natural substance.

Keywords: Pheromone; Dienals; Chromatography; Stereocontrol.

Many species of Lepidoptera have developed a remarkably sensitive communication system between sexes whereby males can respond to minute amounts of a pheromone produced by virgin females.[1] Although the structures of Lepidopteran sex pheromones are usually rather simple (straight-chain acetates, alcohols, aldehydes or hydrocarbons),[2] small amounts of the active compounds, usually found in a complex matrix, make the identification of their chemical structure a challenge. The recent development of spectroscopic techniques (GC/MS^n, GC/FTIR) and micro-chemical reactions provides us with methods for the identification of polyunsaturated sex pheromones in nano-gram amounts.[3] However, if the amount of the active principle is lower than 10 pg we reach a detection limit of the most sensitive MS instruments (ion traps) and an alternative approach must be sought. Recently, we described chemical communication in the horse-chestnut leafminer *Cameraria ohridella* Deschka et Dimić (Lepidoptera: Gracillariidae).[4] This pest, which devastates horse chestnut tree foliage, was described[5] in Macedonia in 1985 and it has colonized most of Europe since that time.[6] At present, measures against this pest are rather limited (raking of the damaged leaves, spraying with insecticides). The sex pheromone can be a powerful element in designing an integrated pest management (IPM) program to combat this pest.

The preliminary examination of hexane extracts of the calling females using GC-EAD,[7] where male antennae were used as a biological detector, showed pronounced antennal activity on the EAD trace, but no corresponding GC peak was detected by FID detector (Fig. 1A). Furthermore, when ~ 100 female equivalents (FE) were injected on a GC/MS (ion trap) no reliable mass spectrum was obtained

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from the EAD-active area. Clearly, the only analytical tools available were: 1) retention behavior of EAD peak on different GC phases, 2) an examination of antennal specificity to libraries of pheromone-like synthetic compounds (EAG response profiles), and 3) micro-derivatizations of gland extracts combined with EAG.

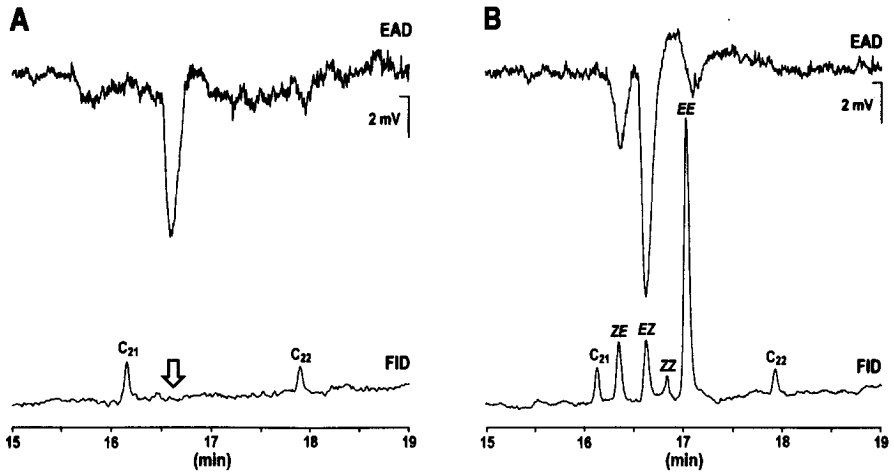


Fig. 1 Sections of GC-FID-EAD traces; **A:** a hexane extract of *C. ohridella* calling females (~ 5 FE); **B:** a synthetic mixture of 8,10-tetradecadienal (7) isomers[11] (100 ng) on DB-WAX phase, both co-injected with hydrocarbon standards (C_{21}, 5 ng).

Kovats' indices (KIs) of the EAD peak (Fig. 1A) were determined using several GC phases of increasing polarity and the measured values were compared with KIs of straight-chain aldehydes, alcohols and acetates (Table 1).

Table 1. A comparison of Kovats' indices[a] of EAD peak in *C. ohridella* female extracts with some synthetic compounds

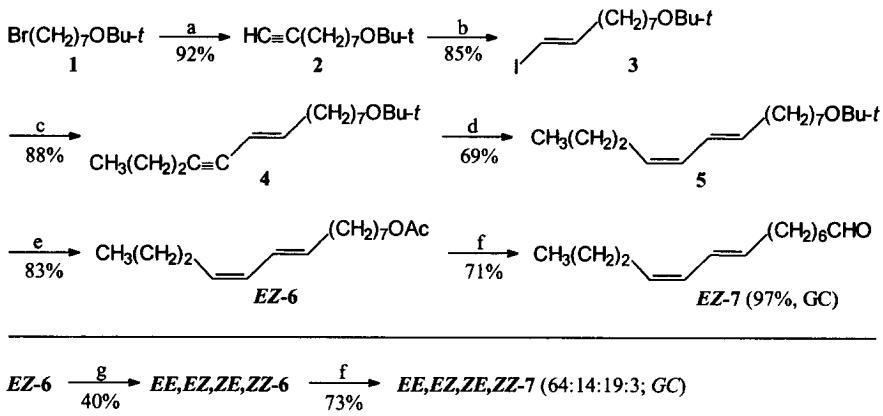
GC phase[b]	Female extract[c]	Compounds			
	ca 5 FE	12:Ac	14:Ald	14:OH	***EZ*-7**[c]
DB-1[d]	1623.3	-	-	-	1623.9
DB-5[d]	1674.4	1605.9	1610.8	1675.4	1674.3
DB-WAX[e]	2031.2	-	-	-	2031.8

[a] based on saturated hydrocarbons; [b] J & W Scientific, 30m × 0.25 mm, film thickness 0.25 mm; [c] for EAD trace; [d] 170 °C; [e] 140 - 240 °C @ 5 °C / min

Based on these measurements, a series[8] of all geometric isomers of dodecen-1-yl acetates, tetradecen-1-ols and tetradecenals and their saturated congeners (1 μg) were tested on the EAG preparation.[4] Both KIs and the EAG profiles obtained clearly showed that the pheromone should bear the aldehyde functionality and that unsaturation must be situated near the C-9 atom. The aldehydic nature of

the pheromone was confirmed by micro-derivatization experiments where the hexane extracts, treated with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride in methanol solution,[9] were tested both on EAG and in a wind tunnel. The pheromonal activity diminished after this derivatization. Unfortunately (*E*)-tetradec-9-enal which had the highest EAG potential (from EAG profiles) showed low behavioral activity (wind tunnel bioassay) and a different KI (1601.8 on DB-5) in comparison to the natural extract. Therefore, it was not considered to be the sex pheromone.

From comparison of KIs on DB-1 and DB-5 phases it seems reasonable to speculate that the pheromone should have more double bonds. Its KI on the DB-5 phase is much higher than the respective KI on a DB-1 phase, which usually points towards conjugation.[10] Based on this consideration, and on the measured EAG profiles, we prepared mixtures of all geometric isomers of 7,9-, 8,10-, and 9,11-tetradecadienals using a general strategy shown for the tetradeca-8,10-dienal (**7**) isomers:



Scheme 1. a) HC≡CLi.EDA / DMSO; b) 1. $AlH(i\text{-}Bu)_2$ / hexane, 2. I_2 / THF; c) 1-pentyne, $Pd(Ph_3P)_4$, $BuNH_2$, CuI / benzene; d) 1. Dicyclohexylborane, 2. AcOH , 3. pH 10 (NaOH) / H_2O_2; e) $FeCl_3$ / ether / Ac_2O; f) 1. NaOH / MeOH, 2. PDC / CH_2Cl_2; g) PhSH / benzene, 80°C.

The EAG examination of the mixtures of geometric isomers showed that only an isomeric mixture of **7** displays the highest antennal activity. Geometric isomers of the aldehyde **7** were reasonably separated on GC capillary columns and we were able to obtain GC-EAD of the individual isomers.[11] Although males' antennae were, to some extent, sensitive to more than one geometric isomer in the mixture we could clearly eliminate the ***ZE*-7** and ***EE*-7** isomers. The ***EZ*-7** isomer showed higher EAD activity than ***ZZ*-7** isomer (Fig. 1B). When ***EZ*-7** was measured on GC-EAD using several GC phases the corresponding EAG activity showed identical retention behavior (at sub-ng amounts) to hexane-extracted female abdomens (Table 1). In wind tunnel behavior assay 1 - 0.1 pg of the ***EZ*-7** isomer displayed high attractiveness, which was comparable to 3 FE of gland extract (100% of all tested males were activated,

took off and 80% of them localized the odor source and tried to copulate with it). In contrast, the pure ***ZZ*-7** displayed a different KI to the natural extract and its behavioral activity was negligible. In preliminary field experiments sticky traps baited with 5 ng of ***EZ*-7** isomer (loaded on BBL Taxo paper disc, 1/2 inch dia) were, similarly to virgin females, highly attractive for *C. ohridella* males. All the presented data confirm that ***EZ*-7** isomer is the main component of *C. ohridella* sex pheromone.

Insect antenna is shown here as a powerful analytical tool when spectroscopic techniques reach their sensitivity limits. It is extremely sensitive to pheromone components *via* specialized pheromone receptors, however, at elevated concentrations it, to a certain extent, responds to pheromone analogs. Consequently, by screening of libraries of the analogs at different concentrations we can direct our structural information towards the pheromone structure.

(8*E*,10*Z*)-Tetradeca-8,10-dienal (***EZ*-7**) is, to the best of our knowledge,[2] a newly discovered sex pheromone and the first identified sex pheromone in genus *Cameraria*. Other isomers of 7 have been described as attractants for males of other Lepidopteran species; ***EE*-7** for *Acrocercops* sp.[12] and ***ZZ*-7** for *Phyllonorycter* sp.[13] The ***EZ*** conjugated double bond system is quite common, found for example in bombykol, the first described sex pheromone. The high activity of ***EZ*-7** would be advantageous for establishing of an IPM system based on mating disruption techniques.[14]

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