Female Sex Pheromone of *Cameraria ohridella* Desch. and Dim. (Lepidoptera: Gracillariidae): Structure Confirmation, Synthesis and Biological Activity of (8*E*,10*Z*)-8,10-tetradecadienal and Some Analogues

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Cameraria ohridella, Sex Pheromone, Tetradecadienal

Mass spectrometric investigations confirmed the structure of the female produced sex pheromone of the horse-chestnut leafminer *Cameraria ohridella* Desch. and Dim. to be (8*E*,10*Z*)-8,10-tetradecadienal. Pure samples, prepared in a straightforward synthesis, were highly attractive in field tests and proved to be suitable for monitoring of flight activities and population dynamics. In mixtures with the synthetic pheromone, analogues like 9-tridecynal and 7-dodecynyl formate were shown to reduce trap catches. In electroantennographic experiments, pheromone analogues were less active than the pheromone. 9-Tridecynal was the most EAG active analogue tested, followed by 7-dodecyn-1-yl formate and 7-undecyn-1-yl formate.

Introduction

The horse-chestnut leafminer *Cameraria ohridella* Desch. and Dim. (Lepidoptera: Gracillariidae) was first mentioned by Simova-Tosic and Filev (1985) who reported about an unknown pest attacking horse chestnut in Macedonia, near the lake of Ohrid. Later, the insect was described as a new species, *Cameraria ohridella* (Deschka and Dimic 1986). The species was spreading rapidly around Europe and until 1999 was found in northern Italy, Switzerland, Hungary, Austria, Czech Republic, Slovak Republic, southern and central Germany, southern Poland (Skuhravy, 1999), Bulgaria (Pelov *et al.*, 1993), and Belgium (De Prins and Puplesiene, 2000).

Svatoš *et al.* (1999a) described the calling behaviour of *C. ohridella* females and the same group as well as Khosbayar and Szöcs (1998) demonstrated attraction of males to conspecific females. The Czech group identified the sex pheromone to be (8*E*,10*Z*)-8,10-tetradecadienal (8E10Z-14Al) (Svatoš *et al.*, 1999b, Hoskovec *et al.*, 2000). Since structure assignment of the natural product could not be based on spectroscopic data, its confirmation by an independent way is necessary despite high attractivity of synthetic samples.

Here we report about unambiguous structure confirmation of the pheromone of *C. ohridella* by mass spectrometry as well as a new synthesis of 8E10Z-14Al. In addition, results of electrophysiological investigations and field bioassays using synthetic E8Z10–14Al and some analogues are presented.

Materials and Methods

Extracts

Leaves with *Cameraria ohridella* mines were collected from infested horse chestnut trees in

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Sofia in 1998 and stored for overwintering under natural conditions at the Institute of Zoology, Bulgarian Academy of Sciences. In the following February the overwintered caterpillars were collected from the leaves, separated by sex and kept at 5 °C. Every day, 60 female pupae were warmed up to room temperature for hatching so as to have freshly emerged moths regularly over a longer period. The pupae were put in test tubes in groups of 20 and after emerging into new tubes by 3.

The day after emergence, calling females were freeze killed during 30 min, and whole bodies were extracted for 10 min with pentane. A batch of whole-body extracts of about 600 females was prepared and concentrated (Klimetzek *et al.*, 1989). For pheromone extraction and GC-EAD measurements in the Budapest laboratory, experimental insects were obtained from leaves collected during 1997–1999 near Budapest. For the preparation of extracts, females showing calling posture were immediately extracted in pentane for 10 min. During fall 2000 infested leaves were collected in Prague. After emergence, males kept in the refrigerator at 5 °C were used for EAG experiments.

Electrophysiological investigations

Natural samples

For determining the retention time of antennal response, extracts were analysed using coupled gas chromatography-electroantennography (GC-EAD). Samples (1.5 female equivalents) were run on a HP5890 gas chromatograph equipped with a Supelco SP2340 fused silica (30 m × 0.32 mm, film thickness: 0.2 μm) (splitless injection) and a HP Ultra-1 crosslinked methyl silicone column (25 m × 0.2 mm, film thickness: 0.33 μm (on-column injection), using helium as a carrier gas, and the following conditions: 60 °C for 1 min, increase of 10 °C/min to 150 °C, followed by increase of 5 °C/min to 220 °C. A male *C. ohridella* antenna, amputated at the base segment and with the very tip removed was suspended between two capillar electrodes filled with Ringer-Beadle-Ephrussi solution, and placed *ca.* 0.5 cm from the opening of the EAD outlet of the GC. The measuring electrode was linked to a high impedance DC amplifier *via* a connection interface box (Syntech, Hilversum). The FID and the EAD signals were simultaneously monitored and analysed on a personal computer using GC-EAD software (Chromperfect, Justice Inc.).

EAG recordings and stimulation

The method of EAG recordings from *C. ohridella* was already described in Svatoš *et al.* (1999b). Briefly: the whole animal preparation and Ag/AgCl electrodes were used. The recording apparatus consisted of a CyberAmp 320 amplifier (Axon Instruments) equipped with a AI402 probe (Axon Instruments), a 486X PC with A/D board (Metrabyte DAS-16) and a Stand-Alone Enhanced Data Acquisition System (Version 3.0, RUN Technologies, CA).

The pheromone and analogues were dissolved in decadic steps in hexane (0.1 ng – 1 μg/μl. Aliquots of 10 μl were pipetted on a filter paper disc (1 cm dia). The solvent was allowed to evaporate. The loaded discs were placed individually into Pasteur pipettes, sealed with Parafilm®, and stored in a freezer until use. During the stimulation a solenoid activated odour delivery system was used to switch a continuous clean air stream blowing over the antennal preparation (charcoal filtered and humidified, velocity 1 l/min) through the loaded Pasteur pipette (stimulus duration −0.5 s). The maximal negative deflections of antennal potentials were evaluated. Successive stimuli were separated by 2–15 min intervals to allow the antennae to recover from the previous stimulation. The antennal sensitivity and its possible changes during the experiments were checked by standard stimulation (10 ng of E8Z10–14Al). The antennal responses were normalised relative to the standard. Stimuli were applied to the antenna from lower to higher doses to prevent an antennal adaptation. Each dose response series was tested on 6 individuals. One-way ANOVA statistic was used to evaluate data.

Chemical analysis

Concentrated pentane extracts were analysed by coupled gas chromatography-mass spectrometry: A Hewlett-Packard gas chromatograph 5890 (Palo Alto, US) was linked to a double focussing mass spectrometer VG 70/250 SE (Vacuum Generators, Manchester, UK) which was operated at 70 eV. Using helium as the carrier gas, separations were achieved with a 50 m × 0.25 mm id, 0.2 μm film

CPSil 88 fused silica column (Chrompack, Middleburg, The Netherlands) under the following conditions: injector temperature 250 °C, 1 min splitless, 3 min 60 °C, then programmed to 240 °C at a rate of 3 °C/min. Hexane for residual analysis (Fluka) was used for dilution series.

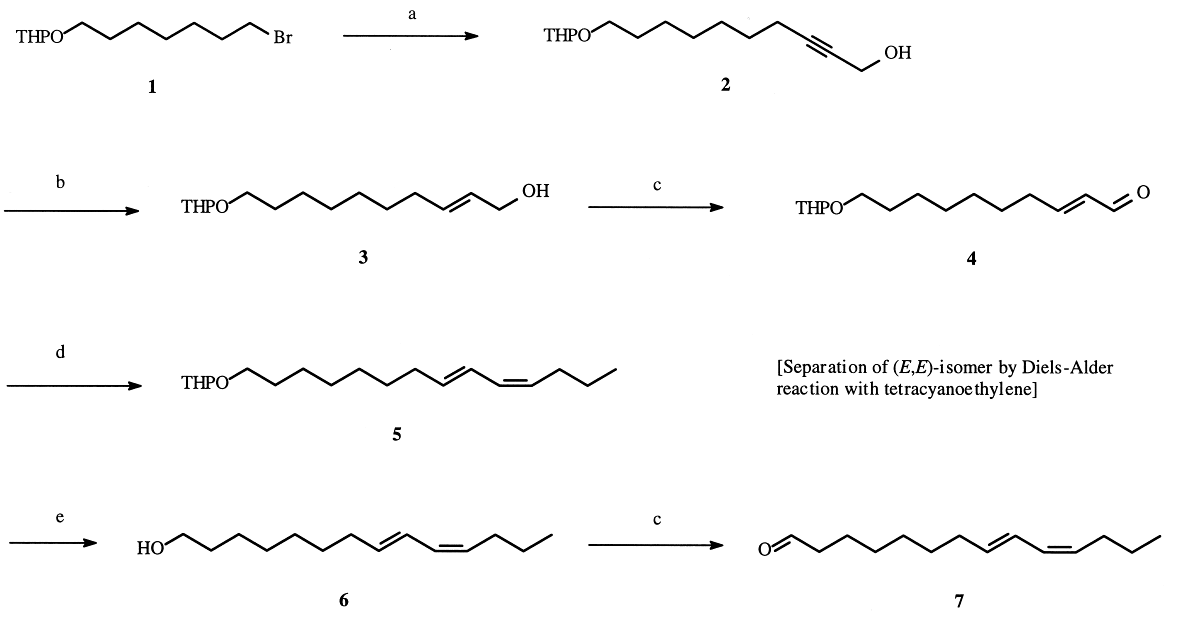
Syntheses

Syntheses involving air-sensitive reagents were carried out under argon in flame-dried glassware. All solvents were distilled prior to use and dried according to standard procedures. For column chromatography, Merck silica (0.040–0.063 mm) was used, with hexane–ethyl acetate mixtures as the eluent. NMR spectra were recorded with a Bruker AMX400; chemical shifts δ are given in [ppm] relative to tetramethylsilane (internal standard, 0 ppm), coupling constants *J* are given in [Hz]. Solvents and fine chemicals, purchased from Aldrich and Merck, were of highest purity available. ω-Halogen-alkanols were prepared from commercially available diols according to Tellier (1990).

1) (8*E*,10*Z*)-8,10-tetradecadienal [8E10Z-14Al] (**7**) (Fig. 1)

7-Bromoheptanol was protected with 3,4-dihydro-2*H*-pyran (Miyashita, 1977) to yield **1** which was coupled to 2-propyn-1-ol according to laboratory standards (Brandsma, 1971). The resulting 10-(2′-tetrahydropyranyloxy)-2-decyn-1-ol (**2**) was reduced to the corresponding mono-protected diol **3** showing a *trans*-configurated double bond (Rossi and Carpita, 1977). Swern oxidation (Omura and Swern, 1978) of this allylic alcohol produced **4** which was chain elongated by Wittig-reaction following Bestmann's method (Bestmann *et al.*, 1976, Vinczer *et al.*, 1988) to furnish the protected (*E*,*Z*)-dienol **5**. Small amounts of the corresponding (*E*,*E*)-isomer and other impurities were removed by Diels-Alder-reaction with tetracyanoethylene (Goto *et al.*, 1975) and column chromatography on silica gel. Deprotection of pure **5** and Swern oxidation of the resulting dienol **6** followed by column chromatography yielded the final product **7** in an overall yield of 30%.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 9.77 (t, *J* = 1.5, 1H, H-1), 6.30 (ddd, *J* = 15.3, 10.7, 1.0, 1H, H-9),



a: [2-propyne-1-ol / $LiNH_2$], fl. NH_3 **b:** $LiAlH_4$, Et_2O, RT **c:** 1. [$(COCl)_2$ / DMSO] 2. NEt_3, CH_2Cl_2, -78 °C **d:** [Butyl-triphenylphosphoniumbromide / $NaN(SiMe_3)_2$], THF, -20 °C **e:** p-TsOH, MeOH, RT

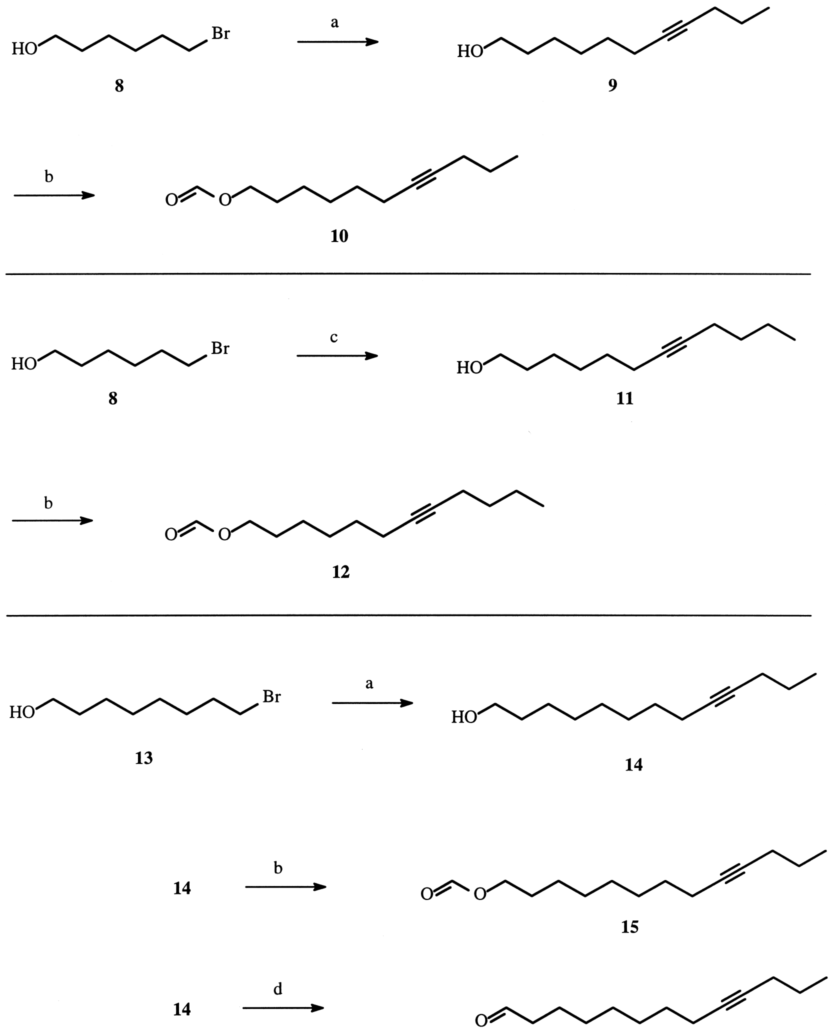
Fig. 1. Synthesis of (8*E*,10*Z*)-8,10-tetradecadienal.

5.95 (dd, *J* = 10.7, 10.7, 1H, H-10), 5.64 (dt, *J* = 15.3, 7.6, 1H, H-8), 5.31 (dt, *J* = 10.7, 7.6, 1H, H-11), 2.42 (dt, *J* = 7.6, 1.5, 2H, H-2), 2.18–2.06 (m, 4H, H-7, 12), 1.44–1.30 (m, 10H, H-3, 4, 5, 6, 13), 0.92 (t, *J* = 7.6, 3H, H-14). – ^{13}C-NMR (100.6 MHz, $CDCl_3$): δ = 202.9 (C-1), 134.3 (C-8), 130.0 (C-11), 128.7 (C-10), 125.8 (C-9), 43.9 (C-2), 32.7, 29.8, 29.1, 29.0, 28.9, 22.9, 22.0, 13.8 (C-14).

2) 7-Undecynyl formate [7Y-11Fo] (**10**) (Fig. 2)

6-Bromohexanol (**8**) was converted to 7-undecyn-1-ol (**9**) (Brandsma, 1971) which was further transformed to the corresponding formate (Iranpoor *et al.*, 1998). The final product **10** was purified by chromatography on silica gel.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 8.06 (s, 1H), 4.17 (t, *J* = 6.6, 2H, H-1), 2.18–2.10 (m, 4H, H-6, 9), 1.71–1.64 (m, 2H, H-2), 1.54–1.35 (m, 8H, H-3, 4, 5, 10), 0.97 (t, *J* = 7.6, 3H, H-11). – ^{13}C-



a: [1-pentyne / $LiNH_2$], fl. NH_3 **b:** HCOOEt, Cu(II), reflux **c:** [1-hexyne / $LiNH_2$], fl. NH_3 **d:** 1. [$(COCl)_2$ / DMSO] 2. NEt_3, CH_2Cl_2, -78 °C

Fig. 2. Syntheses of pheromone analogues, 7Y-11Fo (**10**), 7Y-12Fo (**12**), 9Y-13Fo (**15**), and 9Y-13Al (**16**).

NMR (100.6 MHz, $CDCl_3$): δ = 161.2, 80.3, 80.0, 64.0, 29.0, 28.44, 28.35, 25.4, 22.6, 20.8, 18.7, 13.5.

3) 7-Dodecynyl formate [7Y-12Fo] (**12**) (Fig. 2)

6-Bromohexanol (**8**) was converted to 7-dodecyn-1-ol (**11**) (Brandsma, 1971) which was further transformed to the corresponding formate **12** (Iranpoor *et al.*, 1998). The final product was purified by chromatography on silica gel.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 8.05 (s, 1H), 4.17 (t, *J* = 6.6, 2H, H-1), 2.18–2.11 (m, 4H, H-6, 9), 1.72–1.64 (m, 2H, H-2), 1.51–1.25 (m, 10H, H-3, 4, 5, 10, 11), 0.91 (t, *J* = 7.1, 3H, H-12). – ^{13}C-NMR (100.6 MHz, $CDCl_3$): δ = 161.2, 80.4, 79.9, 64.0, 29.0, 28.5, 28.4, 26.9, 25.4, 21.9, 18.7, 18.4, 13.6.

4) 9-Tridecynyl formate [9Y-13Fo] (**15**) and 9-tridecynal [9Y-13Al] (**16**) (Fig. 2)

8-Bromooctanol (**13**) was converted to 9-tridecyn-1-ol (**14**) which was further transformed to the corresponding formate **15** (Brandsma, 1971). The final product was purified by chromatography on silica gel.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 8.06 (s, 1H), 4.14 (t, *J* = 6.6, 2H, H-1), 2.18–2.09 (m, 4H, H-8, 11), 1.70–1.61 (m, 2H, H-2), 1.58–1.25 (m, 12H, H-3, 4, 5, 6, 7, 12), 0.96 (t, *J* = 7.1, 3H, H-13). – ^{13}C-NMR (100.6 MHz, $CDCl_3$): δ = 161.2, 80.3, 80.1, 64.1, 29.10, 29.07, 29.0, 28.7, 28.5, 25.8, 22.6, 20.8, 18.7, 13.5.

Swern oxidation (Omura and Swern, 1978) of the alcohol **14** yielded the corresponding aldehyde **16**. The final product was purified by chromatography on silica gel.

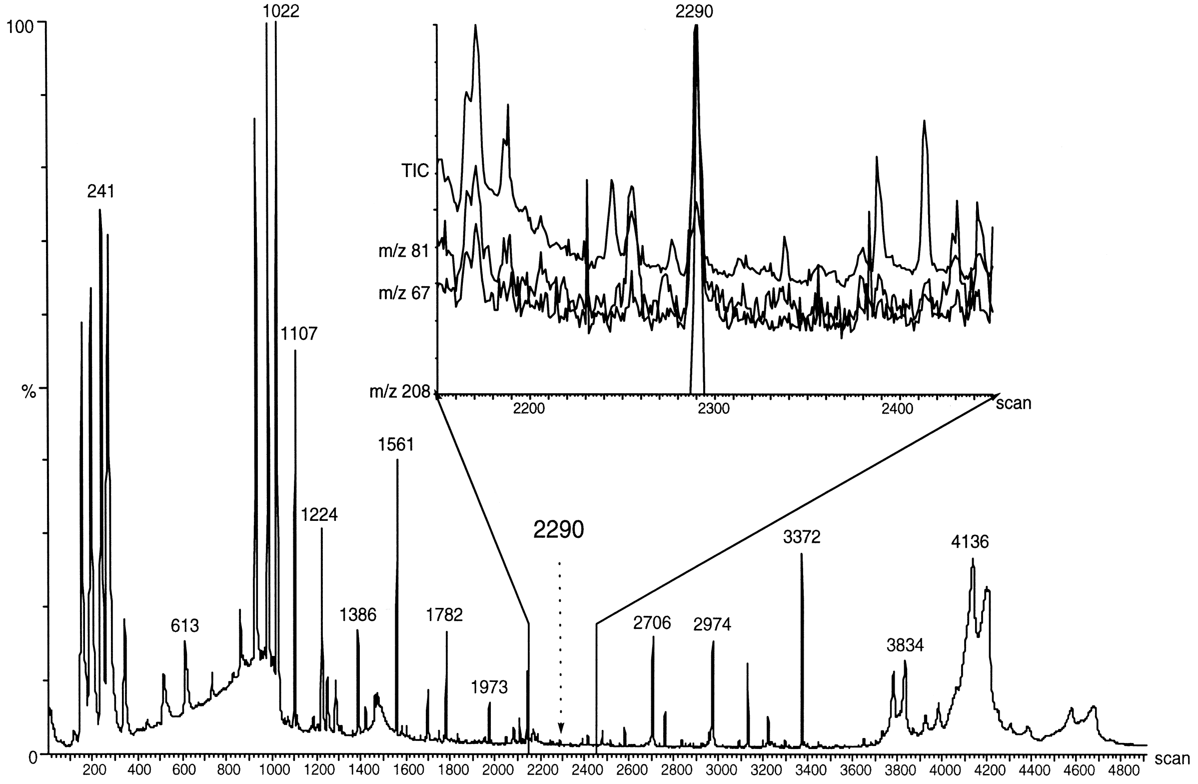


Fig. 3. Gas chromatogram of a crude extract of female *C. ohridella*; insert shows crucial area of retention time, with the target compound eluting at scan no 2290. Selected ions are *m/z* 67, *m/z* 81 and *m/z* 208. For conditions see text.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 9.77 (t, *J* = 2.0, 1H, H-1), 2.43 (dt, *J* = 7.1, 2.0, 2H, H-2), 2.17–2.09 (m, 4H, H-8, 11), 1.67–1.59 (m, 2H, H-3), 1.55–1.30 (m, 10H, H-4, 5, 6, 7, 12), 0.97 (t, *J* = 7.1, 3H, H-13). – ^{13}C-NMR (100.6 MHz, $CDCl_3$): δ = 202.9 (C-1), 80.20, 80.16, 43.9 (C-2), 29.1, 28.9, 28.6, 22.6, 22.0, 21.9, 20.8, 18.7, 13.5 (C-13).

Field tests

For preparing baits, compounds were dissolved in hexane and administered on rubber dispensers (MSZ 9691/6 in Hungary and penicillium vial caps in Bulgaria). Sticky delta traps were used in both countries. The traps within a replicate were placed in a distance of *ca.* 3 m from each other, while replicates were separated by *ca.* 50 m. Traps were hung on branches of horse-chestnut trees at a height of *ca.* 2 m above the ground. Since fast saturation of traps with captured moths decisively influenced the results, traps were inspected and captures recorded daily, or when neccessary in intervals of one or two hours. In recording the diurnal flight activity of *C. ohridella*, traps were inspected hourly between 6 a.m. and 2 p.m. (Bulgaria), or between 6 a.m. and 6 p.m. (Hungary), then again on the following day during the same period.

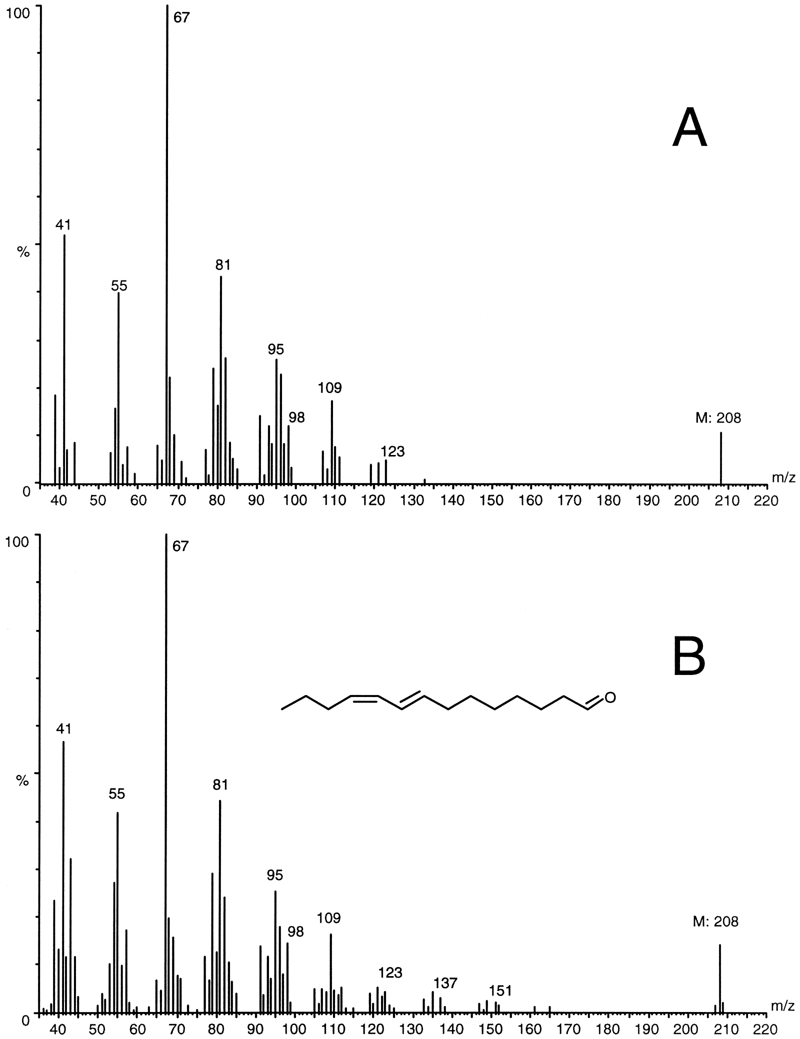


Fig. 4. 70 eV-mass spectra of natural pheromone (A) and synthetic (8*E*,10*Z*)-8,10-tetradecadienal (B).

In performing statistical evaluations, catches recorded at each inspection were regarded as replicates. Capture data were statistically analyzed after transformation to $\sqrt{(x + 0.5)}$ by a one way ANOVA. If the F value was significant, mean catches were separated using the Duncan's New Multiple Range Test using SuperANOVA® (Abacus Concepts Inc.).

Results

Electrophysiological investigations of natural extracts

One strong antennal response was detected in GC-EAD analyses of natural extracts. The compound eliciting this antennal response eluted at 13.90 min on the SP2340 column between eicosene (10.20 min) and tetracosene (14.00 min). On the Ultra-1 column the antennal response was observed at 23.20 min, between hexadecane (22.53 min) and heptadecane (24.81 min).

Chemical investigations

A concentrated pentane extract representing *ca.* 300 female equivalents was submitted to GC/MS-analysis. The corresponding largely overcrowded chromatogram (Fig. 3) showed a lot of contamination, however, at the retention time, known for the EAG-active compound, an extremely small peak, interferring with an impurity became visible. Selected ion traces indicated the presence of an aliphatic compound with a relatively abundant molecular ion at M = 208. Careful subtraction procedures and processing of what could be a relevant spectrum, suggested a tetradecadienal with a conjugated double bond system for the candidate compound (Ando *et al.*, 1988). In fact, the obtained mass spectrum (Fig. 4A) matched that of synthetic 8E10Z-14Al (Fig. 4B).

Our new synthesis of 8E10Z-14Al involving alkyne coupling, stereoselective reduction of the triple bond and stereoselective Wittig reaction is straight forward in the establishment of the conjugated double bond system. Yields and purity of the final product are good. The same is true for the analogues.

EAG activity of pheromone analogues

EAG dose response revealed that 8E10Z-14Al was *ca.* 3 orders more active than 9Y-13Al, the most active analogue tested. The second best analogue was 7Y-11Fo followed by 7Y-12Fo. 9Y-13Fo was almost inactive (Fig. 5). EAG threshold responses (significantly higher EAG than to hexane) were observed at doses of 0.1, 1 and 10 μg for 9Y-13Al, 7Y-11Fo or 7Y-12Fo and 9Y-13Fo, respectively (One-way Anova, Dunnett's Multiple Comparison Test, P = 5). When the pheromone and analogues were compared at equipotent doses (10 ng for 8E10Z-14Al, 10 μg for analogues), 7Y-11Fo and 7Y-12Fo exhibited considerably longer recovery of EAG responses after the stimulus offset than 9Y-13Al, 9Y-13Fo and 8E10Z-14Al (Fig. 6).

Field activity of 8E10Z-14Al

8E10Z-14Al showed very high attractivity towards conspecific males. In a dose-response test in Hungary, traps baited with as little as 0.1 μg 8E10Z-14Al captured significantly more males than the unbaited control (Fig. 7). Catches in traps baited with 0.3, 1, 3, 10 and 30 μg increased gradually. So many moths were trapped between in-

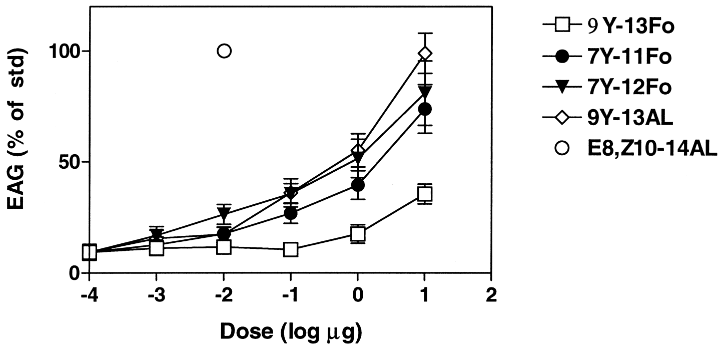


Fig. 5. Dose response curves of 7Y-11Fo (full circle), 7Y-12Fo (full triangle), 9Y-13Fo (open square), and 9Y-13Al (open diamond). EAG responses were normalized to standard (10 ng of E8Z10–14Al = 100%, open circle). Each dot represents a mean of six independent recordings on antennae of *C. ohridella* males, error bars represents standard error of the mean value (SEM).

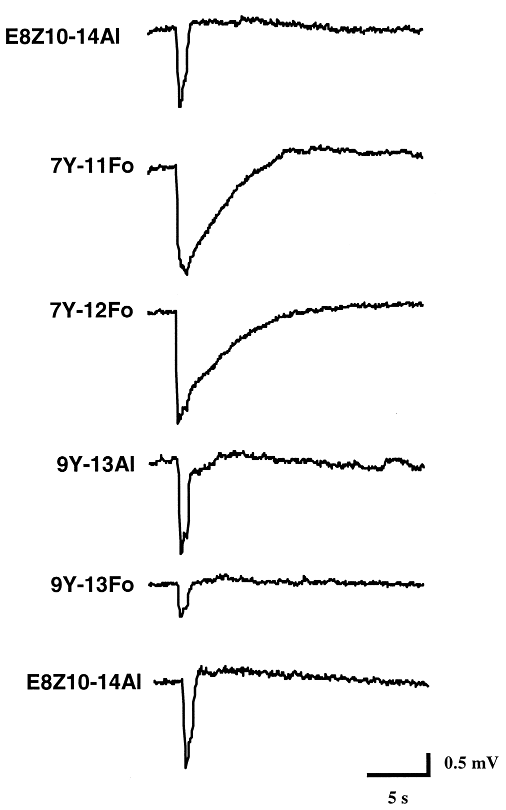


Fig. 6. Typical EAG responses to equipotent doses of pheromone (10 ng) and respective analogues (10 µg) measured on an antenna of *C. ohridella* male and plotted in the same scale. Bars in the right corner represent duration of the response and its amplitude.

spections (see materials and methods) in traps baited with either 10 or 30 µg that the sticky layers were nearly saturated. In Bulgaria, high captures were recorded at 10 µg, while captures at lower doses were not significantly higher than those of the unbaited control (Fig. 7). At 100 µg significantly more males were caught than at 10 µg. No other lepidopterous species were caught in the tests in both countries.

Hourly inspections showed that most males were caught in traps baited with E8Z10–14Al in both Hungary and Bulgaria between 6 a.m. and 12 a.m., with a peak between 7 a.m. and 11 a.m. (Fig. 8). Observations during every 15 minutes after 5 a.m. in Bulgaria revealed the flight to start at 6.15 a.m. just after sunrise.

Effect of pheromone analogues on trap captures

Captures by the analogues *per se* did not differ significantly from that of unbaited controls in both Hungary and Bulgaria (Table I). In Hungary, traps baited with a blend of 7Y-12Fo and 8E10Z-14Al (100 + 100 µg) caught males in significantly lower numbers than 8E10Z-14Al on its own (Table I, Fig. 9). This was corroborated by the results in Bulgaria, where mixtures of 8E10Z-14Al and either 7Y-11Fo or 9Y-13Fo or 9Y-13Al proved to be less attractive than pure 8E10Z-14Al (Fig. 7). In both countries, 7Y-12Fo reduced captures drastically. Additional tests at a ten fold lower dose level confirmed the inhibitory effect of especially 7Y-12Fo and 9Y-13Al in Hungary and in Bulgaria (Table II, Fig. 10). In tests in Bulgaria, mixtures of 10 µg of 8E10Z-14Al with 10 µg and 20 µg of

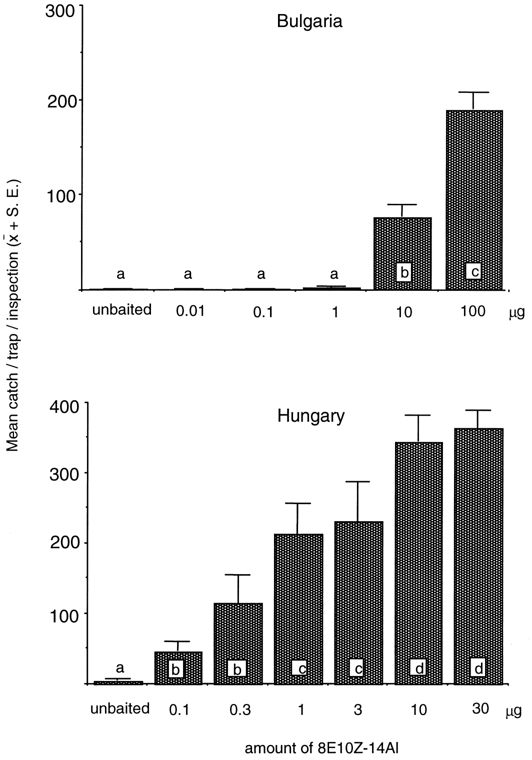


Fig. 7. Catches of *C. ohridella* males in traps baited with various doses of 8E10Z-14Al. Bulgaria: August 2–9, 2000 daily checks, Sofia, 4 traps/treatments. Hungary: June 19–July 3, 2000, Nagykovácsi, Pest county, 2 traps/treatments. Catches with the same letter are not significantly different at P = 5% by ANOVA followed by Duncan's NMRT.

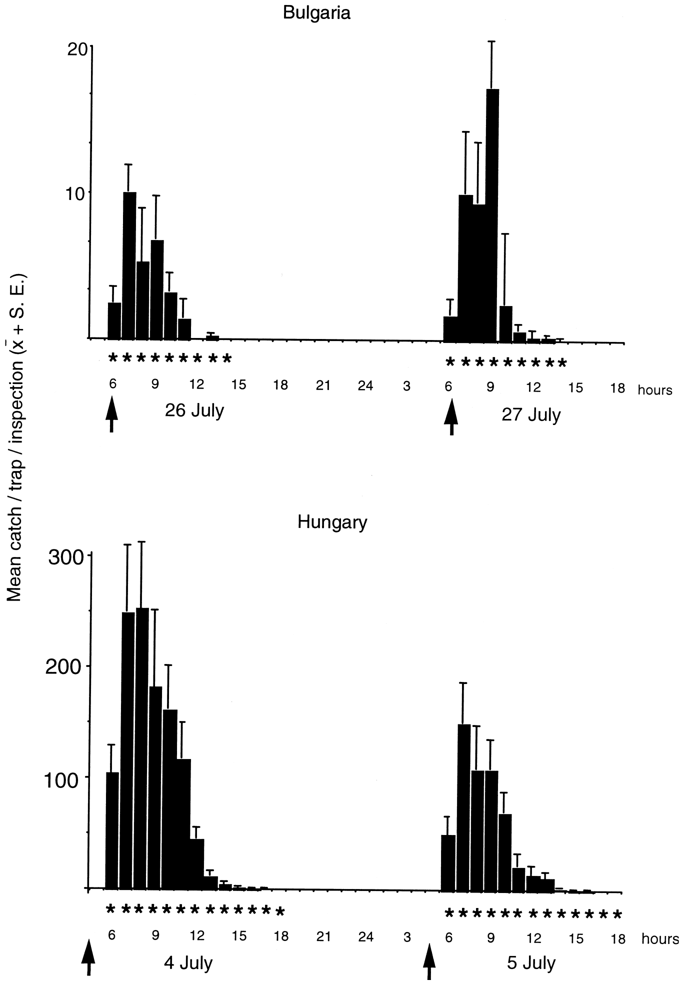


Fig. 8. Hourly catches of *C. ohridella* captures in sticky traps baited with 8E10Z-14Al. Hungary: July 4–5, 2000 Nagykovácsi, Pest county, 6 traps/treatments; Bulgaria: July 26–27, 2000, Sofia, 4 traps/treatments. Arrows show sunrise. Asterisks means inspections of captures.

7Y-12Fo, or with 20 μg of 9Y-13Al showed significantly reduced trap catches (Fig. 11). However, no significant reduction in captures was detected with 15 μg of 1:2 mixtures of the analogues and the synthetic pheromone or with 20 μg of a 1:1 mixture of 9Y-13Al and 8E10Z-14Al, although in this latter case captures were numerically lower.

Discussion

The female sex pheromone of *C. ohridella* is present in picogram amounts per individuum, only (Svatoš *et al.*, 1999a; Svatoš *et al.*, 1999b). Though pronounced EAD-activity was recorded from five female equivalents, no corresponding FID-signal could be detected. In these investigations, even 100 female equivalents did not yield consistent mass spectral data, and monitoring of the active principle had to rely on signals obtained with coupled gas chromatography electroantennography (GC-EAD). Since no spectroscopic methods could be applied in the identification of the pheromone, structure elucidation of E8Z10–14Al had to be based solely on calculations of Kovats indices of the active principle on three different GC-phases, on microreactions, and on EAG-response profiles, obtained with synthetic straight chain reference compounds (Svatoš *et al.*, 1999a; Svatoš *et al.*, 1999b).

In a new approach, using 300 female equivalents, we obtained a good mass spectrum of the natural pheromone (Fig. 4A) that matches that of

Table I. Catches of *C. ohridella* males in traps baited with E8Z10–14Al and with mixtures of E8Z10–14Al and Y7–11Fo, Y7–12Fo, Y9–13Fo, and Y9–13Al. April 25 and 27, 2000, checks in 9.00 and 14.00, Érd-Elvira, Hungary, 2 replicates; May 3–7, 2000, daily checks, Forest Institute, BAS, Bulgaria, 4 replicates. May 8 and 9, 2000, hourly checks (7.00–17.00), Forest Institute, BAS, Bulgaria, 4 replicates. Catches with same letter are not significantly different at P = 5% by Duncan's NMRT within one test.

Compound [μg]					Total number of moths caught		
E8Z10–14AL	Y7–11Fo	Y7–12Fo	Y9–13Fo	Y9–13Al	I	II	III
100	–	–	–	–	330ab	13901a	18757a
–	100	–	–	–	5c	355c	95e
–	–	100	–	–	3c	302c	38e
–	–	–	100	–	12c	965b	102e
–	–	–	–	100	3c	374c	88e
100	100	–	–	–	480a	13870a	8754b
100	–	100	–	–	108b	9795a	1774d
100	–	–	100	–	381ab	14067a	14538a
100	–	–	–	100	412ab	12690a	5057c
–	–	–	–	–	–	48d	50e

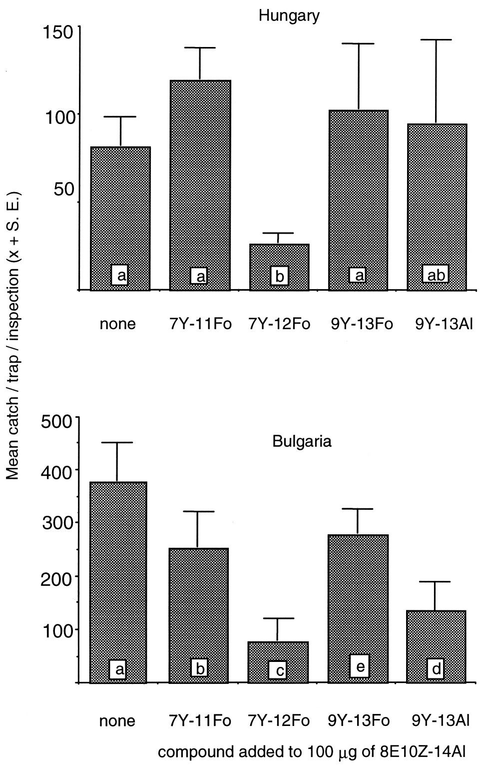


Fig. 9. Catches of *C. ohridella* males in traps baited with 100 µg of 8E10Z-14Al on its own or its 1:1 mixtures with 7Y-11Fo, 7Y-12Fo, 9Y-13Fo, or 9Y-13Al. Hungary: April 25–27, 2000, Érd-Elvira, Pest county, 2 traps/treatments. Bulgaria: May 8–9, 2000, Sofia, 4 traps/treatments. Significance: see Fig. 7.

synthetic E8Z10–14:Al perfectly well (Fig. 4B). The synthetic compound is EAG-active and coelutes with the EAG-active natural product, thus, unambiguously confirming the earlier structure assignment (Svatoš *et al.*, 1999b). In field tests, the biological activity of the synthetic pheromone proved to be high. We recorded significant trap captures when the rubber dispenser was loaded with as little as 0.1 µg of the synthetic pheromone. This shows that *C. ohridella* males respond to extremely low amounts of the pheromone. The gypsy moth, *Lymantria dispar* L., another species known to produce and respond to tiny amounts of pheromone, was reported to be captured in traps charged with 1 µg of its synthetic pheromone (Bierl *et al.*, 1970). In the present study with *C. ohridella,* dose-capture experiments show that an effective trap should be loaded with amounts of 1–100 µg of pheromone (depending on the type of the dispenser). In contrast, previous results obtained with pheromones of some other Gracillariids show that recommended loads of dispensers for traps are generally much higher than in the case of *C. ohridella* [5 mg for *Phyllonorycter ringoniella* Matsumura (Boo and Jung, 1996); 1.2 mg for *Conopomorpha cramerella* Snellen (Beevor *et al.*, 1986); 1 mg for *Caloptilia theivora* Walsingham (Ando *et al.*, 1985); 1–0.2 mg for *Ph. ulmifoliella* Hbn. (Mozuraitis *et al.*, 1997); only for *Ph. crataegella* Clemens (Ferrao *et al.*, 1998) the small amount of 10 µg has been recommended].

Table 2. Catches of *C. ohridella* males in traps baited with E8Z10–14Al and with mixtures of E8Z10–14Al and Y7–11Fo, Y7–12Fo, Y9–13Fo, and Y9–13Al. May 22–30, 2000, daily checks, Forest Institute, BAS, Bulgaria, 4 replicates; July 4–5, hourly checks (6.00–17.00), Érd-Elvira, Hungary, 6 replicates. Catches with same letter are not significantly different at P = 5% by Duncan's NMRT within one test.

Compound [μg]					Total number of moths caught	
E8Z10–14Al	Y7–11Fo	Y7–12Fo	Y9–13Fo	Y9–13Al	Bulgaria	Hungary
10	–	–	–	–	574a	10055a
10	10	–	–	–	584a	–
10	–	10	–	–	26c	1650b
10	–	–	10	–	485a	–
10	–	–	–	10	141b	2255b
–	–	–	–	–	–	630c

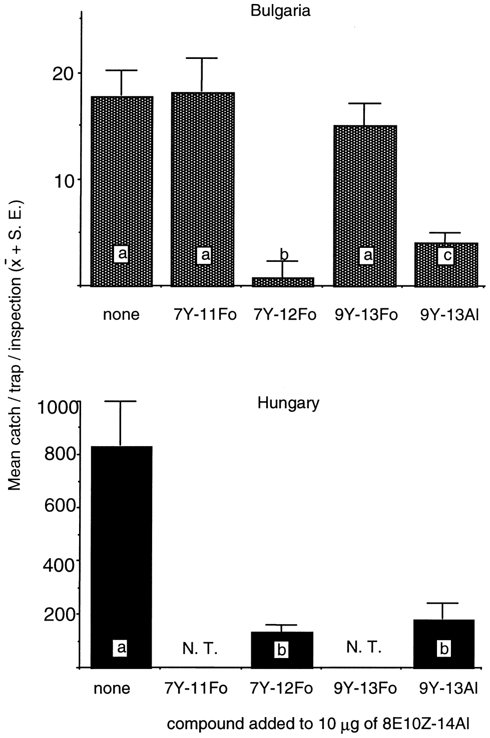


Fig. 10. Catches of *C. ohridella* males in traps baited with 10 µg of 8E10Z-14Al on its own or its 1:1 mixtures with 7Y-11Fo, 7Y-12Fo, 9Y-13Fo, or 9Y-13Al. Bulgaria: May 22–30, 2000, Sofia, 6 traps/treatments; Hungary: July 4–5, 2000, Nagykovácsi, Pest county, 2 traps/treatments. N. T. means that particular treatment was not tested. Significance: see Fig. 7.

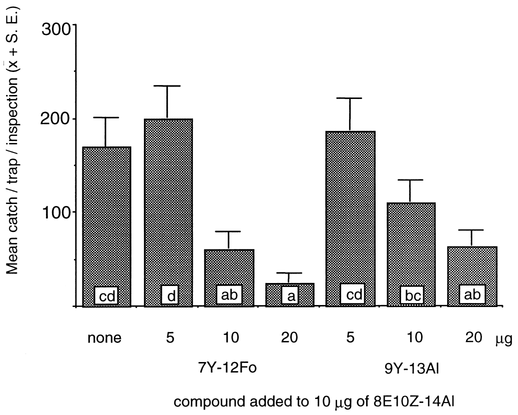


Fig. 11. Catches of *C. ohridella* males in traps baited with 10 µg of 8E10Z-14Al on its own or in 10:5, 10:10, and 10:20 mixtures with either 7Y-12Fo or 9Y-13Al. June 16–25, 2000 daily checks, Sofia, 4 traps/treatments. Significance: see Fig. 7.

In the structures of pheromone analogues investigated in this paper, the following modifications of 8E10Z-Al were made in order to obtain stable and simple compounds:

- the conjugated double bond system of 8E10Z-14Al was replaced by one triple bond positioned at the area of highest π-electron density of the natural product (reports on the field activity of acetylenic pheromone analogues are scarce, see Brückner *et al.*, 1988; Schwarz *et al.*, 1990; Millar and Rice, 1996)
- the distance between the carbonyl group and the area affected by π-electrons was kept and also the length of the alkyl tail attached to the π-system at the end of the chain (see Priesner *et al.*, 1975; Bestmann and Vostrowsky, 1991; Norinder *et al.*, 1997)
- the α-methylene group of 8E10Z-14Al was replaced by an oxygen, i.e. the aldehyde structure of the natural product was changed into a formic acid ester (see Priesner *et al.*, 1975; Bestmann *et al.*, 1987; Mori *et al.*, 1990).

The addition of equal amounts of either 7Y-12Fo or 9Y-13Al to 8E10Z-14Al significantly inhibits trap catches, which indicates that these compounds interfere with the communication system of *C. ohridella* at the receptor level.

EAG is supposed to be a summation of receptor potentials of antennal olfactory receptor cells activated by a given stimulus (Kaissling, 1987). The prolonged EAG recovery may indicate insufficient inactivation of the semiochemical (Kaissling, 1987). Such an insufficient inactivation of 7Y-11Fo and 7Y-12Fo might represent a basis as to how these analogues may disturb the pheromone signal and, thus, influence male behaviour. However, 7Y-12Fo (but not 7Y-11Fo) and 9Y-13Al (with dynamic EAG characteristics similar to those found for 8E10Z-14Al) reduced the attractiveness of the synthetic pheromone in field tests. These data suggest that the mechanism by which pheromone analogues 7Y-12Fo and 9Y-13Al inhibit pheromone attractivity is complex and not fully understood. Nevertheless, several compounds with similar structural replacements inhibiting pheromone attraction in various species have been reported. Upwind flights of males of the European corn borer *Ostrinia nubilalis* Hbn. to sources of the female pheromone, (*E*)-/(*Z*)-11-tetradecenyl acetate, were strongly inhibited by 11-dodecynyl acetate (Schwarz *et al.*, 1990). In field tests with the peach tree borer *Anarsia lineatella* Zeller, 5-decyn-1-yl acetate proved to be a powerful antagonist of the natural pheromone which is a 4:1 blend of (*E*)-5-decenyl acetate and (*E*)-5-decenol (Millar and Rice, 1996). In contrast, introducing a triple bond instead of the *trans*-configurated double bond in (7*E*,9*Z*)-7,9-dodecadienyl acetate, the pheromone of the European grapevine moth, *Lobesia botrana* (Denis and Schiffermüller), yielded an electrophysiologically active and highly attractive compound (Brückner *et al.*, 1988). Formates are known as mimics of aldehyde pheromones: For *Heliothis virescens F.,* a mixture of the non-natural (9*Z*)-tetradecenyl formate and (9*Z*)-tetradecenal was shown to be as attractive as its natural pheromone, a 16:1 blend of (11*Z*)-hexadecenal and (9*Z*)-tetradecenal (Mitchell *et al.*, 1978), however, (9*Z*)-tetradecenyl formate had been earlier reported to be highly disruptive in field tests (Mitchell *et al.*, 1975). The pheromone of *Dichocrocis punctiferalis* Guenée, (10*E*)- and (10*Z*)-hexadecenal is mimiced by (8*E*)- and (8*Z*)-tetradecenyl formate (Mori *et al.*, 1990), while (7*Z,9E*)-7,9,11-dodecatrienyl formate mimics (9*Z*,11*E*)-9,11,13-tetradecatrienal, the major sex pheromone component of *Ectomyelois ceratoniae* Z. (Todd *et al.*, 1992). In the case of 7Y-12Fo and 9Y-13Al, replacement of the conjugated double bond system by a triple bond may largely account for the antagonistic effect of these compounds. Further studies in structure activity relationships of analogues of 8E10Z-14Al would require detailed calculations on the structures of model compounds as well as investigation of basic physiology of their perception.

According to the results obtained with 7Y-12Fo and 9Y-13Al, 9-tetradecynal should provide a good structure for a biologically active candidate: it shows the same distance between the carbonyl group and the triple bond as 7Y-12Fo and 9Y-13Al and has the same chain length as the natural pheromone.

Using traps baited with 8E10Z-14Al we showed that the diurnal flight of *C. ohridella* males towards a source of sex pheromone coincides well with the diurnal calling behavior of the females. This starts at the beginning of the photophase and lasts about four and a half hours according to Svatos *et al.* (1999a) or even more than 8 hours (Subchev *et al.*, unpublished data). Our investigations also confirm the results of Khosbayar and Szöcs (1998) who found attraction between 7 a.m. and 2 p.m. in July in Hungary by using alive females as baits. With respect to recommendations for practical application, 10–100 μg of 8E10Z-14Al applied on a rubber dispenser provide an effective trap bait for *C. ohridella*, which may be used as a highly sensitive and species specific tool for detection and monitoring of the pest.

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