Pheromonal Production of and Response to Optically Active Epoxydienes in Some Geometrid Moths (Lepidoptera: Geometridae)

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In pheromone extracts of calling female *Chiasma clathrata* L. (Lepidoptera: Geometridae), a defoliator pest of alfalfa, (*Z,Z,Z*)-3,6,9-heptadecatriene and (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene was identified. Chiral gas chromatography using a modified cyclodextrin and synthetic reference samples proved the natural epoxide to show (3*R*,4*S*)-configuration. In field trapping tests, only the pure (3*R*,4*S*)-enantiomer of the epoxide attracted males. The addition of the triene component was synergistic. Males of the sympatric species *Tephrina arenacearia* Hbn. (Lepidoptera: Geometridae) were caught only in traps with baits containing the (3*S*,4*R*)-enantiomer [together with a previously described minor component, (*Z,Z*)-3,9-*cis*-6,7-epoxyheptadecadiene]. In trapping tests conducted in a different biotope, *Abraxas grossulariata* L. (Lepidoptera: Geometridae) males were attracted by the (3*S*,4*R*)-enantiomer, whereas the (3*R*,4*S*)-enantiomer attracted a close relative, *Abraxas sylvata* Scop. (Lepidoptera: Geometridae). The present results suggest that one of the key mechanisms responsible for pheromone specificity among both the two alfalfa geometrids and the two *Abraxas* species in their respective biotops, may be the use of different enantiomers of the same polyene-derived epoxide as a sex pheromone component. It is probable that this discrimination mechanism is widespread among moth species utilizing epoxide pheromone components.

Chiasma clathrata L. (Lepidoptera: Geometridae) is one of several defoliator pests of alfalfa, the population dynamics of which is regularly monitored by the Plant Protection Service of the Ministry of Agriculture and Food (Budapest, Hungary), based on light-trap captures. Pheromone traps would be a much more selective and easy-to-use forecasting method for this pest. The present studies were started to elucidate the chemical structure of the sex pheromone of *C. clathrata* with the final aim of developing a selective sex attractant for the pest. Data on selective attraction of related species to epoxide enantiomers are also presented.

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Materials and Methods

Insects and pheromone extraction

Laboratory cultures of *C. clathrata* were maintained on fresh alfalfa leaves during the summer seasons in Hungary. Adults were kept separate and under a reversed 18/6 light/dark photoperiod in the laboratory for behavioral observations. Insects used in electrophysiological studies in Lund were transferred as pupae to Sweden. Pheromone extracts were prepared by excising the terminal segments of the abdomens of calling unmated fe-

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males. Batches of excised segments were extracted in a minimal amount (*ca.* 2 µl/female) of redistilled hexane or pentane for 10 min.

Analyses

Gas chromatography with combined flame ionization and electroantennographic detection (GC-FID/EAD) was conducted in Lund (Sweden), according to methods earlier described (Tóth *et al.*, 1991). Samples were separated on DB-1 and DB-wax 30 m×0.25 mm i.d. fused silica capillary columns (J&W Scientific, Folsom, CA, U.S.A.).

Combined gas chromatography/mass spectrometry (GC/MS), and synthesis of pheromone compounds was carried out in Hamburg (Germany). Structure elucidation of the target compounds was performed by GC/MS under the same conditions as described earlier (Hansson *et al.*, 1990). Mass spectra and gas chromatographic retention times (coinjection) of racemic samples served as references. Chemical purity of synthetic (*Z,Z*)-6,9-cis-3,4-epoxyheptadecadienes was checked on a 25 m×0.25 mm i.d. fused silica column held for 1 min at 80 °C, then programmed to 140 °C at a rate of 20 °C/min and held at this temperature.

Enantiomeric separation of chiral epoxides was achieved by gas chromatography using a 1:1 mixture of heptakis-(2,6-di-O-methyl-3-O-pentyl)-β-cyclodextrin and OV 1701 as the stationary phase and hydrogen (2 ml/min) as the carrier gas. Synthesis of the cyclodextrin as well as preparation of our tailor-made columns have been described in detail (König *et al.*, 1992; Pietruszka *et al.*, 1992). Under the carefully optimized conditions mentioned above, the enantiomers showed an α-value *i.e.* rt(3*R*,4*S*)– : rt(3*S*,4*R*)– of 1.02. Assignment of absolute configuration of naturally occurring epoxydienes was performed by chiral gas chromatography using synthetic optically active reference compounds.

Synthesis

Synthetic samples of the target compounds used for structure confirmation and bioassays were prepared according to the procedure which we already described for the bishomologue C19-epoxydienes (Szőcs *et al.*, 1993). Our compounds showed chemical purities higher than 98%, and optical purities of 94% ee (enantiomeric excess); optical rotations were found to be $[\alpha]_D^{20}$ – 3.3 (C = 2, 0, CH_2Cl_2) for the (3*R*,4*S*)-enantiomer and $[\alpha]_D^{21}$ + 3.4 (C = 2, 0, CH_2Cl_2) for the (3*S*,4*R*)-enantiomer.

Trapping

Dispensers for the trapping tests were prepared by using 1×0.5 cm pieces of rubber tubing (Taurus, Budapest, Hungary; No. MSZ 9691/6; extracted 3 times in boiling ethanol for 10 min, then also 3 times in methylene chloride overnight, prior to usage). For making up the baits the required amounts of compounds were administered to the surface of the dispensers in hexane (Merck AG) solutions. Prepared dispensers were stored at –65 °C until use.

Field tests were conducted at several sites in Hungary. Traps used in the tests were similar in shape and size to those described earlier (Arn *et al.*, 1979), but were made from polyethylene sheets. Traps were suspended from wooden poles in alfalfa fields at a height of 0.5 m above ground. Traps containing different baits were set up in rectangular blocks. The distance of traps within a block was 4–5 m. The distance between blocks ranged between 100 and 1000 m. Traps were moved one position forward within a block at each occasion when the traps were inspected. At the same time, captured males were recorded and sticky inserts were replaced by new ones. Capture data were transformed to log(x+1) and differences between means were tested for significance by ANOVA followed by Duncan's New Multiple Range Test (DNMRT).

Results and Discussion

Emerging females called very intensively from the second day after emergence, and during the hour following lights-on. Extracts prepared from calling females evoked one EAD-active peak when subjected to GC-FID/EAD analysis on a DB-1 column, whereas analyses on a DB-wax column consistently produced two active peaks (Fig. 1). Upon comparison of mass spectra and retention times on different columns, their structures proved to be (*Z,Z,Z*)-3,6,9-heptadecatriene (Z3Z6Z9-17Hy) and (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene (Z6Z9-3,4-epo-17Hy) (Tóth *et al.*, 1991). In one analysis on the DB-wax column,

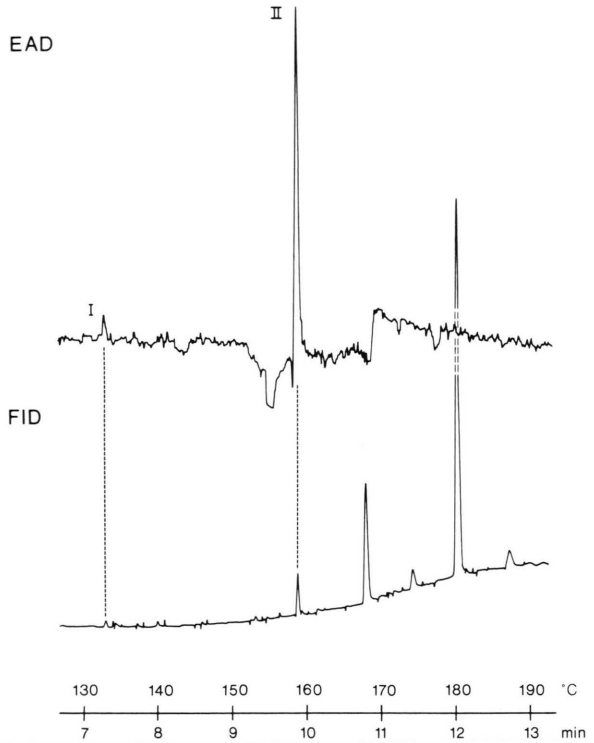

Fig. 1. Gas chromatogram from analysis of pheromone gland extracts of female *C. clathrata* with simultaneous flame ionization (FID) and electroantennographic detection (EAD); I = Z3Z6Z9-17Hy, II = (3*R*,4*S*)-Z6Z9-3,4-epo-17Hy.

EAD peaks were also produced by two peaks which could be identified as tricosane and tetracosane.

In preliminary field trapping tests conducted with racemic Z6Z9-3,4-epo-17Hy and various mixtures with the triene, no male *C. clathrata* were caught. However, when the pure enantiomers of Z6Z9-3,4-epo-17Hy were tested, large numbers of *C. clathrata* males were captured in traps baited with the (3*R*,4*S*)-enantiomer (Table I). When Z3Z6Z9-17Hy was added to the active enantiomer of the epoxide, a significant increase in catches was observed at the 3:1 epoxide/triene blend (Table II). The synergistic activity of the triene was further corroborated when several dosages of the blend and the epoxide alone were compared (Fig. 2). After chiral stationary phases, useful in enantiomeric separation of chiral epoxydienes, had become available, we carefully analyzed female abdominal tip extracts. As shown in Fig. 3, the insects produce an enantiomerically pure component, the absolute configuration of

Table I. Captures of male *C. clathrata* in traps baited with pure enantiomers of Z6Z9-3,4-epo-17Hy in Hungary (Gyöngyös, Heves county: July 25–August 15, 1991; Érd-Elvira major, Pest county: July 17–August 4, 1991; Julianna major, Budapest, August 4–19, 1991; at each site 2 traps were set up of each bait variation).

Z6Z9-3,4-epo-17Hy 3*S*,4*R* [µg]	Z6Z9-3,4-epo-17Hy 3*R*,4*S*	Julianna major	Total catch Gyöngyös	Érd-Elvira major
100	–	0	0	0
–	100	83	139	71
100	100	2	1	0

Table II. Captures of male *C. clathrata* in traps baited with the (3*R*,4*S*)-enantiomer of Z6Z9-3,4-epo-17Hy and with its blends with Z3Z6Z9-17Hy in Hungary (Julianna major, Budapest, July 17–24, 1991; inspected daily; from each bait variation 10 traps were set up. Means followed by same letter are not significantly different at p = 5% by Duncan's New Multiple Range Test).

Z6Z9-3,4-epo-17Hy 3*R*,4*S* [µg]	Z3Z6Z9-17 Hy	No. of males captured (mean/trap/inspection)
30	0.3	1.48 b
30	1	2.50 ab
30	3	2.78 ab
30	10	3.33 a
30	30	1.78 b
30	–	1.86 b

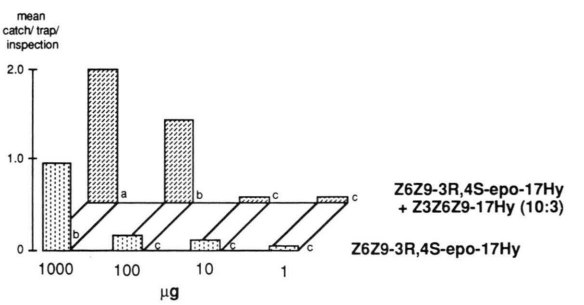

Fig. 2. Captures of male *C. clathrata* in traps baited with different dosages of Z6Z9-3,4-epo-17Hy and its 3:1 blend with Z3Z6Z9-17Hy in Hungary (Julianna major, Budapest, July 24–August 2, 1991; from each bait variation 10 traps were set up. Means followed by same letter are not significantly different at p = 5% by Duncan's New Multiple Range Test).

which was established to be (3*R*,4*S*)–. No traces of the other enantiomer were found in the natural sex pheromone. Consequently, the results show that the female sex pheromone of *C. clathrata* consists of the binary mixture of (3*R*,4*S*)– Z6Z9-3,4-epo-17Hy and the corresponding hydrocarbon Z3Z6Z9-17Hy. For monitoring purposes 100 to 1000 µg of a 3:1 epoxide:hydrocarbon mixture is proposed.

Singly or in combination, Z6Z9-3,4-epo-17Hy and Z3Z6Z9-17Hy were present in pheromone extracts or were attractive for males of several geometrids (Tóth *et al.*, 1991, 1992; Zheng-Ming Li *et al.*, 1988; Millar *et al.*, 1987, 1990a, 1990b; Gries *et al.*, 1993); binary epoxide/hydrocarbon mixtures showed improved field activity in 2 of the 4 spp. where both components were identified (Tóth *et al.*, 1991, 1992; Millar *et al.*, 1987; Gries *et al.*, 1993).

In the course of the tests with pure enantiomers of Z6Z9-3,4-epo-17Hy, we were surprised that no catches of the sympatric *Tephrina arenacearia* Hbn. (Lepidoptera: Geometridae) were recorded. Earlier we reported that males of this species were attracted to racemic Z6Z9-3,4-epo-17Hy (Tóth *et al.*, 1991). Since the synthetic sample used in these earlier tests contained *ca.* 2% of racemic (*Z,Z*)-3,9-*cis*-6,7-epoxyheptadecadiene (Z3Z9-6,7-epo-17Hy), which is a minor component in the pheromone of *T. arenacearia* (Tóth *et al.*, 1991), in the following trappings we tested mixtures of pure enantiomers of Z6Z9-3,4-epo-17Hy and racemic Z3Z9-6,7-epo-17Hy (Table III). Males of *T. arenacearia* were caught exclusively into traps which contained the (3*S*,4*R*)-enantiomer of Z6Z9-3,4-epo-17Hy together with 5% of racemic Z3Z9-6,7-epo-17Hy. The presence of the (3*R*,4*S*)-enantiomer of Z6Z9-3,4-epo-17Hy significantly decreased, but did not eliminate catches. Although at present it is not known whether female *T. arenacearia* produces pure enantiomers, the behavioral activity of the main pheromone component is clearly connected to the (3*S*,4*R*)-configuration.

In a test with pure enantiomers of Z6Z9-3,4-epo-17Hy conducted at the flood area of the Danube (mixed bushes and poplar/willow forest; an atypical site for alfalfa geometrids), males of two *Abraxas* species (Lepidoptera: Geometridae) were captured: *A. grossulariata* L. came into traps with the (3*S*,4*R*)-enantiomer, while *A. sylvata* Scop. was attracted to traps with the (3*R*,4*S*)-enantiomer (Fig. 4). The presence of the opposite enantiomer did not influence catches by *A. grossulariata,* while it was inhibitory in *A. sylvata.* The presence of Z6Z9-3,4-epo-17Hy as the main component of the sex pheromone of *A. grossulariata* has been reported (Tóth *et al.*, 1992), however, it is not known whether the female produces only the (3*S*,4*R*)-enantiomer in the pheromone. This is the first report concerning male attraction of *A. sylvata.* The present results suggest that in both the two alfalfa geometrids and the two *Abraxas* species occurring in their respective biotopes, one

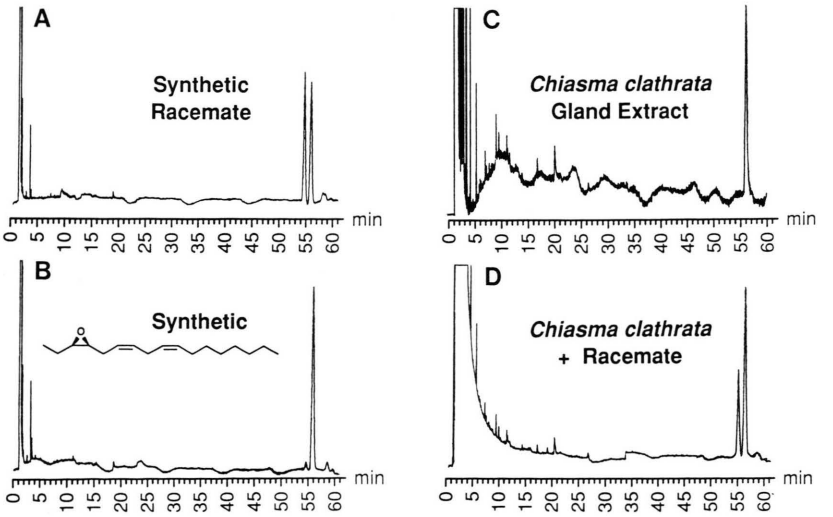

Fig. 3. Determination of the absolute configuration of Z6Z9-3,4-epo-17Hy in the female-produced pheromone of *C. clathrata* by chiral gas chromatography (stationary phase: mixture of 2,6-dimethyl-3-pentyl-β-cyclodextrin + OV1701; for conditions see Materials and Methods). A = synthetic racemate; B = synthetic (3*R*,4*S*)-enantiomer; C = natural extract; D = coinjection of natural extract + synthetic racemate.

Table III. Captures of *T. arenacearia* males in traps baited with enantiomers of Z6Z9-3,4-epo-17Hy and with its blends with Z3Z9-6,7-epo-17 Hy (Julianna major, Budapest, August 8–19, 1991; inspected daily; from each bait variation 5 traps were set up. Means followed by same letter are not significantly different at $p = 5\%$ by Duncan's New Multiple Range Test.

Z6Z9-3,4-epo-17Hy 3*S*,4*R*	racemic [μg]	3*R*,4*S*	Z3Z9-6,7-epo-17Hy racemic	No. of males captured (mean/trap/inspection)
100	–	100	–	0.0 b
–	–	100	–	0.0 b
100	–	–	–	0.0 b
100	–	100	5	1.4 a
–	–	100	5	0.1 b
100	–	–	5	2.5 c
–	100*	–	2*	1.0 a

* Synthetic sample used in tests reported in Tóth *et al.* (1991).

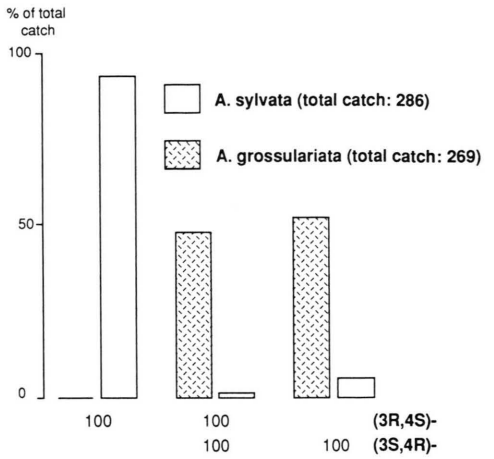

Fig. 4. Captures of male *Abraxas* spp. in traps baited with enantiomers of Z6Z9-3,4-epo-17Hy in Hungary (Adony, Fejér county, June 12–August 3, 1992; from each bait variation 4 traps were set up).

of the key mechanisms for maintaining pheromone specificity may be the use of different enantiomers of the same polyene-derived epoxide in the sex pheromone.

While the biological importance of enantiomeric discrimination is well represented among several other insect groups, *i.e.* bark beetles (Coleoptera, Scolytidae) (Vité *et al.*, 1978; Borden *et al.*, 1980); and references therein, so far only few examples of similar isolation mechanisms have been described in Lepidoptera due to the difficulties in the preparation of pure enantiomers, and in the analysis of the enantiomeric composition of epoxides. Very recently we found evidence for similar cases of pheromone specificity in a group of late autumn/early spring-flying geometrids (Szőcs *et al.*, 1993), supporting earlier indications of studies on geometrid epoxide pheromone and attractant components (Millar *et al.*, 1990b; Gries *et al.*, 1993). In the case of the gipsy moth and the nun moth (*Lymantria dispar* L., *L. monacha* L.) (Lepidoptera: Lymantriidae), it is known that the former is attracted to the (7*S*,8*R*)-enantiomer of 7,8-epoxy-2-methyloctadecane (disparlure), while the (7*R*,8*S*)-enantiomer is active on the latter species (Hansen, 1984). It is probable that the present discrimination mechanism is widespread among moth species utilizing epoxide pheromone components.

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