Epoxyheptadecadienes Identified as Sex Pheromone Components of *Tephrina arenacearia* Hbn. (Lepidoptera: Geometridae)

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(*Z,Z*)-3,9-*cis*-6,7-Epoxyheptadecadiene, and (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene were identified by gas chromatography/flame ionization detection–electroantennographic detection (GC/FID-EAD) and coupled gas chromatography–mass spectrometry (GC-MS) from abdominal tip extracts of *Tephrina arenacearia* (Lepidoptera: Geometridae), an alfalfa pest. In gas chromatography/flame ionization–single sensillum detection (GC/FID-SC) analyses, specific olfactory receptor cells were found for (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene, and tricosane. Synthetic samples of racemic (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene attracted large numbers of male *T. arenacearia* into traps. Best catches were observed at 100–1000 µg dosages. The addition of (*Z,Z,Z*)-3,6,9-heptadecatriene did not influence catches.

(*Z,Z,Z*)-3,6,9-Heptadecatriene and (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene were also identified in the pheromone gland extract of *Chiasma clathrata,* another geometrid pest of alfalfa.

Tephrina arenacearia Hbn. (Lepidoptera: Geometridae) is a known defoliator pest of alfalfa, causing damage mainly in Central and Eastern Europe [1–3]. For the past 30 years it has been on the list of species for which regular forecast should be given by the Plant Protection Service of Ministry of Agriculture and Food (Budapest, Hungary) [4]. At present, forecasts of the service are based almost exclusively on light trap data, although pheromone traps would be much more suitable for this purpose. The present project was started with the principal aim of identifying the compounds involved in sex attraction of *T. arenacearia*. This knowledge can be used later in the development of a pheromone trap for detection and monitoring of the species.

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Materials and Methods

Insects and extraction

Laboratory cultures of *T. arenacearia* were started from larvae collected from the field in Hungary, and were reared on fresh alfalfa leaves. Adults were held at 18/6 light/dark photoperiod and at 25 °C in the lab. Insects used in electrophysiological studies in Lund were transferred to Sweden from Hungary as pupae. Emerging females readily called from the 2nd day onwards after eclosion, at the end of the dark period. Pheromone extracts were prepared by excising the terminal segments of the abdomens of calling unmated females. Batches of excised segments were extracted in a minimal amount (approximate volume 2 µl per female) of distilled hexane for 10 min.

Gas chromatography/electrophysiology

For parallel flame ionization/electroantennographic detection (GC/FID-EAD) [5] two to four µl of an extract were injected on a DB wax (30 m × 0.25 mm i.d.) capillary column (J&W Scientific, U.S.A.) with a split outlet to allow simulta-

neous FID and EAD. The outlet for the EAD was made through a heated tube into a purified air stream flowing over the antennal preparation at a speed of 0.5 m/s. Carrier gas: hydrogen; make-up gas: nitrogen; initial temperature: 80 °C, programme rate: 10 °C/min; final temperature: 230 °C; HP 5830 gas chromatograph.

In analyses of extracts by simultaneous flame ionization/single sensillum detection (GC/FID-SC) [6, 7], single cell responses were recorded by the tip recording technique [8]. Except for the program rate, which was 15 °C/min, conditions of chromatography were the same as for GC/FID-EAD.

The equivalent chain lengths of the active compounds were calculated relative to a homologous series of straight chain acetates.

Mass spectrometry

For structure determinations, a GC/MS coupling system was used consisting of a VG 70-250-SE mass spectrometer (70 eV) linked to a HP 5890 gas chromatograph. Gas chromatographic separation was achieved on a fused silica DB5 column (30 m × 0,25 mm i.d.; J&W Scientific, U.S.A.), maintained for 3 min at an initial temperature of 60 °C and then programmed to 300 °C at a rate of 4 °C/min.

Synthesis

The synthesis of the target compounds followed the standard procedure outlined in Fig. 1. Protected 2,5-octadiyne-1-ol was prepared from 1-bromo-2-pentyne [9] and the reaction product of protected propargyl alcohol with ethyl Grignard in tetrahydrofuran at 50 °C [10]. After transformation to 1-bromo-2,5-octadiyne [11], and coupling to the reaction product of 1-nonyne with ethyl Grignard 3,6,9-heptadecatriyne was obtained. Subsequent hydrogenation over "P-2-nickel" in ethanol at room temperature [12] produced (*Z,Z,Z*)-3,6,9-heptadecatriene in high yield. Epoxidation with m-chloroperbenzoic acid (m-CPBA) in dichloromethane at −20 °C yielded a mixture of the three possible racemic epoxydienes which could be separated by liquid chromatography on silica (230–400 mesh ASTM, Merck, Germany) using hexane + 3% diethyl ether as the mobile phase. Mass spectra of the epoxyheptadecadienes showed patterns which closely resembled those of the respective epoxynonadecadienes [13]. Detailed NMR-analyses of the hydrogen resonances of the three epoxides were obtained from 2-D, H,H-COSY spectra [14] (see Table I).

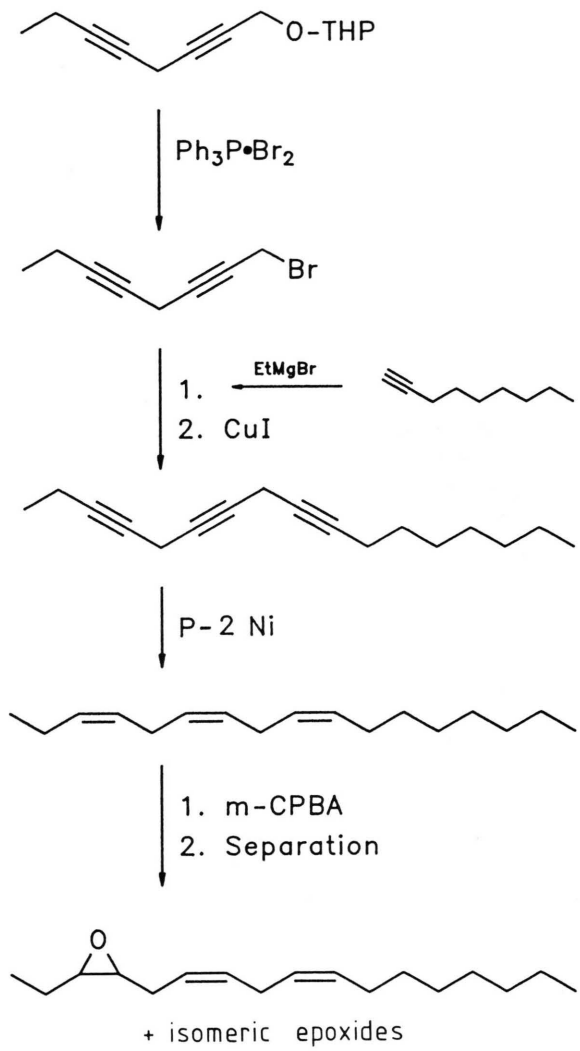


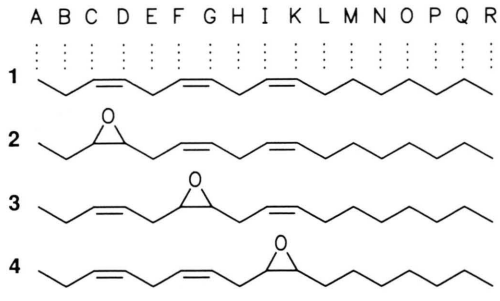
Fig. 1. Synthesis of (*Z,Z,Z*)-3,6,9-heptadecatriene and (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene.

Field tests

Field tests were conducted in alfalfa fields at Rákospalota, Budapest, and Gyöngyös, Heves county, Hungary, using sticky traps similar in shape and size to the ones described by Arn *et al.* [15].

Table I. ^{1}H-NMR-Data [ppm] ($CDCl_3$). Bruker WM 400 at 400 MHz, of (*Z,Z,Z*)-3,6,9-heptadecatriene (Comp. **1**). (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene (Comp. **2**). (*Z,Z*)-3,9-*cis*-6,7-epoxyheptadecadiene (Comp. **3**) and (*Z,Z*)-3,6-*cis*-9,10-epoxyheptadecadiene (Comp. **4**). Letters refer to positions as indicated in the drawing.

Pos.	A	B	C	D	E	F	G	H	I	K	L	M	N
Protons	3	2	1	1	2	1	1	2	1	1	2	10	3
Comp.													
	0.97	2.06	5.26–5.42	5.26–5.42	2.81	5.26–5.42	5.26–5.42	2.81	5.26–5.42	5.26–5.42	2.06	1.24–1.38	0.89
	t: $J = 7.2$ Hz				dd: $J = 6.0$ Hz			dd: $J = 6.0$ Hz					t: $J = 6.8$ Hz
2	1.08	1.50–1.66	2.92	2.99	2.25 2.43	5.63	5.63	2.83	5.53	5.53	2.07	1.26–1.42	0.91
	t: $J = 7.6$ Hz							dd: $J = 7.0$ Hz			dt: $J = 6.8$ Hz		t: $J = 6.8$ Hz
3	0.99	2.07	5.53	5.42	2.22 2.42	2.95	2.95	2.22 2.42	5.42	5.53	2.07	1.25–1.45	0.89
	t: $J = 7.6$ Hz												t: $J = 6.8$ Hz
4	0.97	2.07	5.40	5.30	2.80	5.40–5.55	5.40–5.65	2.22 2.40	2.93	2.93	1.45–1.55	1.24–1.40	0.89
	t: $J = 7.2$ Hz				dd: $J = 6.8$ Hz								t: $J = 6.8$ Hz



Trapping methods were the same as already described for *Peribatodes rhomboidaria* Den. & Schiff. [16].

Results and Discussion

Gas chromatography/electrophysiology

In parallel GC/FID-EAD analyses three FID peaks evoked responses from the antennae of male *T. arenacearia* (Fig. 2). Component **I, II,** and **III** had retention indices of 1414, 1467 and 1481, respectively. The main component **III** reached *ca.* 4 ng/female, and ratios of **I, II,** and **III** were 3:7:100.

In three GC/FID-SC analyses good responses were evoked from a cell firing with large amplitude in the sensilla stimulated by component **III** (Fig. 3). A small amplitude cell in the sensilla was

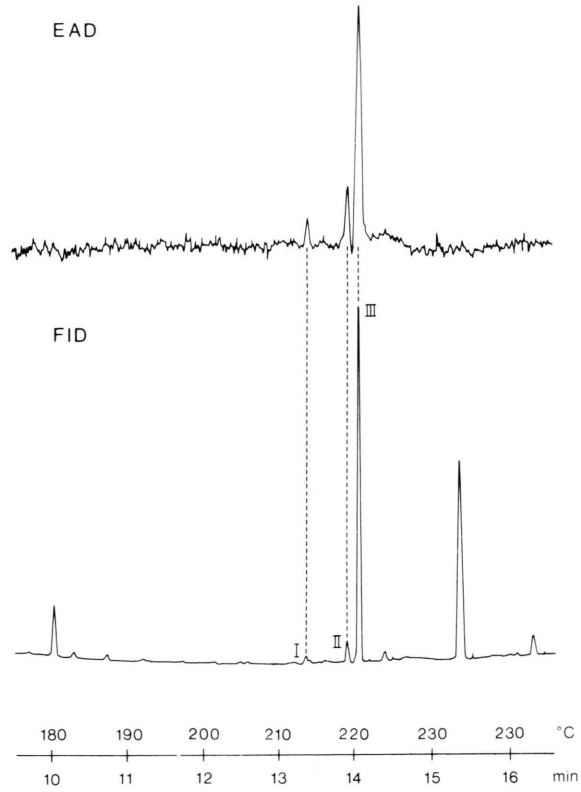


Fig. 2. Typical gas chromatogram of a *T. arenacearia* female abdominal extract, with simultaneous flame ionization/electroantennographic detection (GC/FID-EAD). I = component I, unidentified. II = (*Z,Z*)-3,9-*cis*-epoxyheptadecene-6,7-epoxyheptadecadiene. III = (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene.

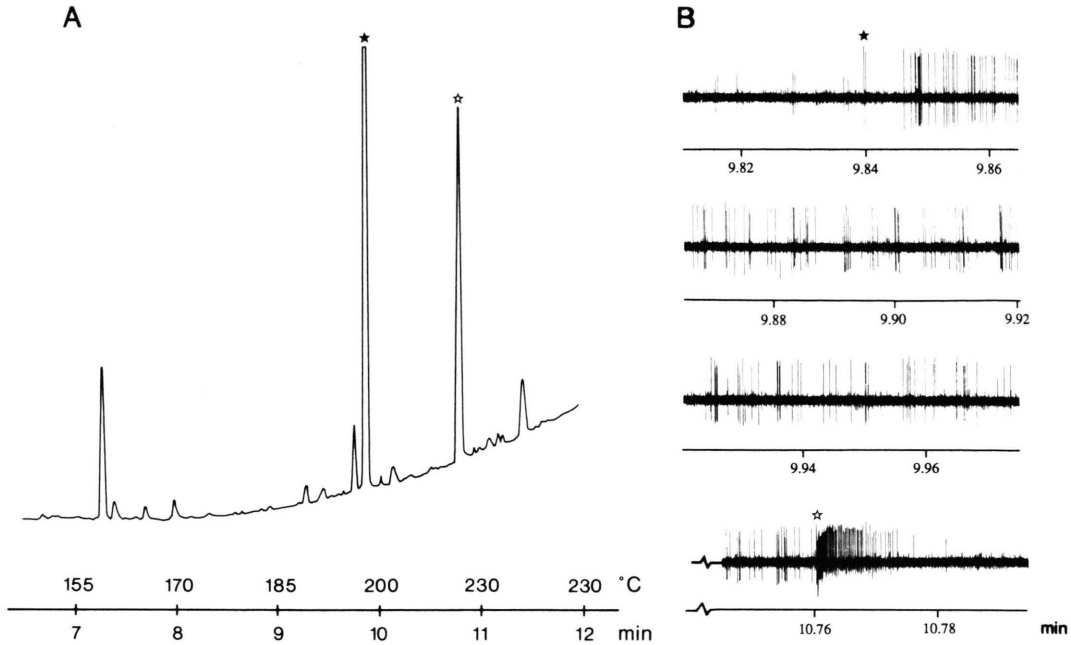


Fig. 3. GC-FID chromatogram from analysis of a female pheromone gland extract (A), and simultaneously recorded single sensillum response from a male olfactory sensillum in *T. arenacearia* (B). The retention time of (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene and the simultaneous onset of the stimulation of the single sensillum preparation is shown by a black star. The retention time of, and stimulation by tricosane is shown by an unfilled star.

stimulated by a component eluting later than the three EAD active peaks. The retention time and EI mass spectrum of this compound were identical with those of synthetic tricosane. The amount of tricosane was found to be *ca.* 2.4 ng/female in the extract. With respect to its biological activity, no further investigations have yet been carried out.

No cells responding to component **I** or **II** were found.

Identification

Apart from the three EAD active components, combined GC/MS analyses showed an earlier eluting component having a molecular weight of M = 234, while the molecular ions of the other three components could not be detected.

When compared with available synthetic substances, the retention time and mass spectrum of the first compound matched (*Z,Z,Z*)-3,6,9-heptadecatriene. In comparison to this hydrocarbon, relative retention times as well as mass spectra of components **II** and **III** suggested structures of epoxyheptadecadienes, since differences in relative retention times as well as fragmentation patterns were similar to a respective set of C 19 compounds, which we recently identified in another geometrid moth, *Erannis defoliaria* Clerck [13]. In fact, synthetic (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene and (*Z,Z*)-3,9-*cis*-6,7-epoxyheptadecadiene entirely matched the natural products **III** and **II,** respectively.

The mass spectrum of component **I** indicated the structure of an epoxyheptadecene. Though the spectral data were insufficient for an unambiguous interpretation, they strongly suggested the structure of (*Z*)-9-*cis*-6,7-epoxyheptadecene for component **I**. A respective synthetic sample matched the gas chromatographic retention time of the natural product.

Mass spectra of the heptadecatriene and the three possible *cis*-epoxides, which may be produced from it, are shown in Fig. 4. The mass spectra of the epoxides can easily be distinguished from each other; the spectrum of (*Z,Z*)-3,9-*cis*-6,7-epoxyheptadecadiene is the least specific one.

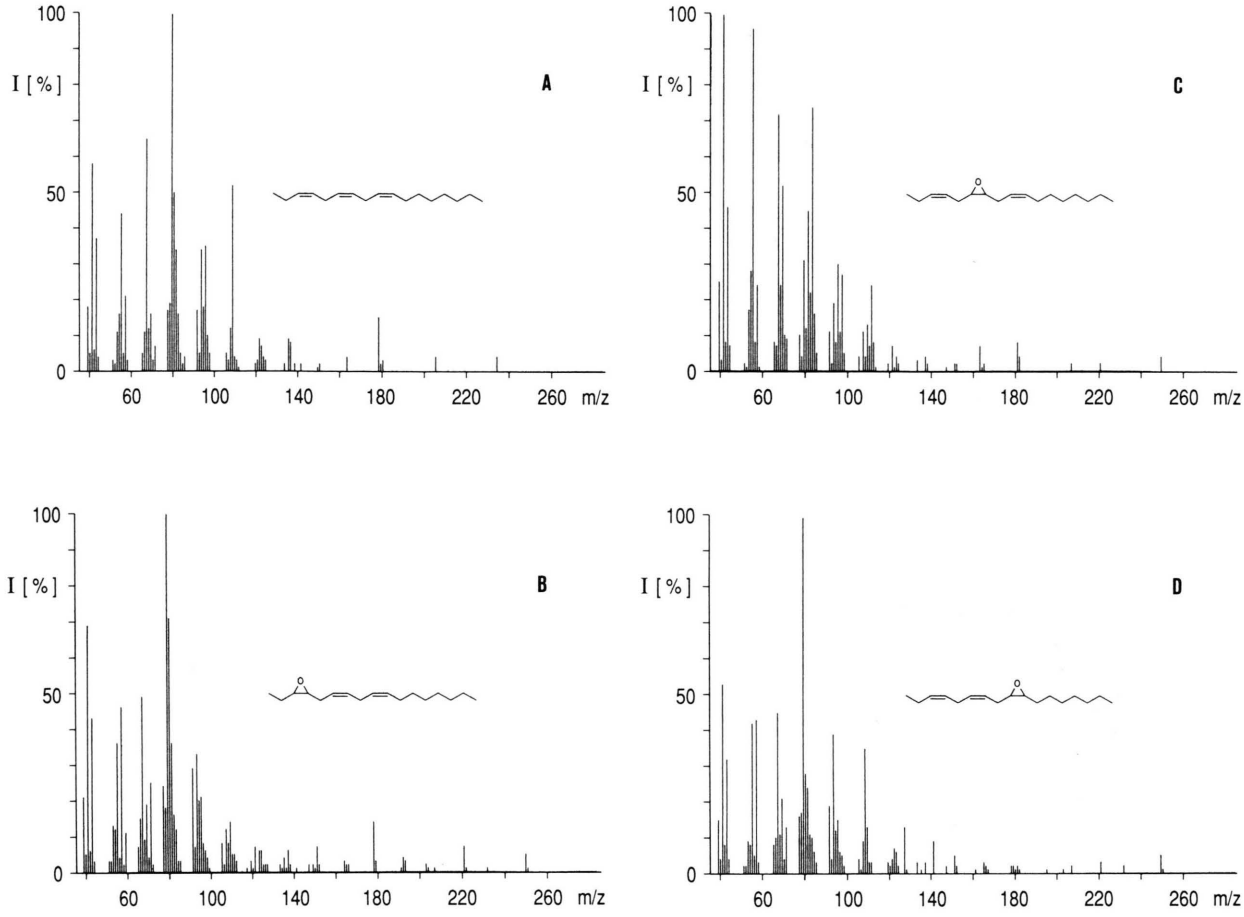


Fig. 4. EI mass spectra [VG 70-250-SE instrument, 70 eV] of A: (*Z,Z,Z*)-3,6,9-heptadecatriene, B: (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene, C: (*Z,Z*)-3,9-*cis*-6,7-epoxyheptadecadiene and D: (*Z,Z*)-3,6-*cis*-9,10-epoxyheptadecadiene.

Field tests

Preliminary field trapping experiments performed in Hungary showed substantial trap catches of male *T. arenacearia* by (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene and by mixtures of the epoxide and (*Z,Z,Z*)-3,6,9-heptadecatriene (Table II). The presence of the triene in the different blends did not influence catches at any of the test sites. Unbaited traps caught no males.

At the time of the trappings, no synthetic (*Z,Z*)-3,9-*cis*-6,7-epoxyheptadecadiene was available for testing its influence on catches by (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene.

In a dosage test, 1000 and 100 µg of (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene caught most males (Table III). Some males were still caught at 10 µg, while 1 µg was almost inactive.

No other moth species was caught in the tests.

Concluding remarks

T. arenacearia seems to belong to the group of geometrids, whose pheromones consist of both polyunsaturated hydrocarbons and corresponding unsaturated epoxides or ketones. Earlier examples include *P. rhomboidaria* [17], *Boarmia selenaria* Schiff. [18] and *Erannis defoliaria* Clerck, which all have pheromone components with 19 carbon atoms in the chain.

Only few data on C17 compounds have been published so far. Investigations of the abdominal

Table II. Catches of male *T. arenacearia* in traps baited with (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene and its mixtures with (*Z,Z,Z*)-3,6,9-heptadecatriene at two sites in Hungary (July 13–19, 1989).

(*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene [µg]	(*Z,Z,Z*)-3,6,9-heptadecatriene [µg]	Mean No. of male *T. arenacearia* caught Rákospalota	Gyöngyös
100	1	10.00a	7.73a
100	3	10.20a	6.93a
100	10	7.73a	8.00a
100	–	11.33a	7.87a
–	–	0.00	0.00

From each variation 5 traps were operated on each site. Captures in a trap when checked were observed as a replicate. Means show catch/trap/check; traps were checked each second day. Means with same letters not significantly different within one column at P = 5% by Duncan's NMRT, performed on $log(x + 1)$ transformed capture data.

Table III. Catches of male *T. arenacearia* in traps baited with different doses of (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene in Hungary (July 16–26, 1989; Gyöngyös).

(*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene [µg]	Mean No. of male *T. arenacearia* caught
1	0.38c
10	2.69b
100	9.69a
1000	13.38a

From each dosage 3 traps were operated. For other methods and test of significance see Table II.

tip extracts of four *Semiothisa* species from Canada indicated the presence of (*Z,Z,Z*)-3,6,9-heptadecatriene, as well as (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene, and (*Z,Z*)-3,9-*cis*-6,7-epoxyheptadecadiene, respectively, in some of the species [19]. The triene was also found in another geometrid moth [20]. Field tests carried out in Canada using various mixtures showed good captures for some species [19, 20].

In a preliminary report, the presence of the heptadecatriene and of (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene in a Chinese *Semiothisa* species [21] was also mentioned.

In the case of *T. arenacearia,* in contrast to earlier examples, more than one epoxide could be found in the pheromone. Both epoxides can evidently be derived through epoxidation of a triply unsaturated precursor.

Supplementary GC/FID-EAD and GC/MS analyses, carried out during our investigations, proved (*Z,Z,Z*)-3,6,9-heptadecatriene and (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene to be also present in female pheromone gland extracts of *Chiasma clathrata* L. (Lepidoptera: Geometridae). This species is also regarded as an alfalfa pest [1]. Results of more detailed studies on its pheromone composition and field trapping will be published elsewhere.

The vast majority of sex pheromones of female Lepidoptera originates from the acetate pool. It is interesting to note that most of the pheromone structures in Geometridae show uneven numbers of carbon atoms along an unbranched chain [22]. This is in contrast to families utilizing pheromones with a functional group at one end of the chain, where even chain length compounds prevail [22]. Both types of pheromones may be produced from even numbered acyl precursors, which in the case of geometrid pheromones are decarboxylated and loose one carbon atom, while in the other case a simple transformation of the functional group takes place which means that the former acyl carbon is kept [23].

In the present study the racemate of the major component of the sex pheromone of *T. arenacearia,* (*Z,Z*)-6,9-*cis*-3,4-epoxyheptadecadiene on its own was enough to achieve impressive trap catch-

es of males. Its performance seems to be satisfactory for direct utilization as a bait in monitoring traps. The structure elucidation of the EAD active minor components **I,** the possible effect of other compounds identified from the pheromone, and the determination of the absolute configurations of the epoxides will be subjects of further studies.

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