Sex Attractant of the Rosy Russian Gypsy Moth (*Lymantria mathura* Moore)*

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We report identification of the sex attractant of the rosy Russian gypsy moth, *Lymantria mathura* Moore. Two compounds, *Z,Z,Z*-3,6,9-nonadecatriene **1** and its monoepoxide *Z,Z*-(9*S*,10*R*)-9,10-epoxy-3,6-nonadecadiene **4a**, have been identified from abdominal tip extracts of female moths based on coupled gas chromatography/electroantennogram detector responses and dose response curves. Single cell recordings showed that only one of the monoepoxide enantiomers (*S,R*) was active. In field tests, both the (*S,R*)-monoepoxide and the racemate were active. This type of pheromone system, unusual for a Lymantriid, is more typical of those found in the families Arctiidae, Noctuidae and Geometridae.

Introduction

The Russian Far East Lymantriid Monitoring Program, a cooperative undertaking by the U. S. Forest Service, the U. S. Animal and Plant Health Inspection Service, the Federal Forest Service of Russia, and the State Plant Quarantine Inspection Service of the Russian Federation (Mastro, 1995) was initiated following an inadvertent introduction of the Asian gypsy moth into several sites in North America in the early 1990's (Bogdanowicz *et al.,* 1993, Mudge and Johnson, 1992.). Three lymantriids, the Asian gypsy moth (*Lymantria dispar* (L.)), the nun moth (*Lymantria monacha* (L.)), and the rosy gypsy moth (*Lymantria mathura* Moore), are of concern by virtue of their opportunities to deposit egg masses on Russian ships and/or shipping containers ultimately bound for North America (Mastro, 1995, Mudge and Johnson, 1992). Careful monitoring of Russian ships and port areas, and of possible U. S. introduction sites are an important part of this program.

Traps baited with pheromones or other attractants can play an important role in monitoring populations and introduction sites. Males of both *L. dispar* and *L. monacha* respond to (+)-disparlure [(7*R*,8*S*)-7,8-epoxy-2-methyloctadecane], although the latter are more efficiently trapped by a pheromone mixture that also contains a closely related epoxide, (7*R*,8*S*)-7,8-epoxyoctadecane (Gries *et al.,* 1996). The pheromone of *L. mathura* has not previously been investigated, but the moth is not trapped by disparlure (G. I. Yurchenko and V. C. Mastro, unpublished observations; results of an earlier experiment tentatively reporting capture of *L. mathura* in disparlure-baited traps (Odell *et al.,* 1992) have not been found to be reproducible). Likewise, male *L. mathura* do not respond to disparlure in electroantennogram assays (Dickens *et al.,* unpublished). Identification of an attractant usable in baited traps would greatly enhance monitoring efforts for *L. mathura*.

We here report the gas chromatography-electroantennogram detection (GC–EAD) of two components of female *L. mathura* abdominal tip extracts, their identification by gas chromatography-mass spectrometry (GC–MS), and the con-

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firmation of structures and biological activity by synthesis, and GC–EAD, GC–MS, and field trapping experiments with the synthetic materials.

Materials and Methods

Insects

L. mathura were reared in culture for six generations in the quarantine facilites at Otis Methods Development Center (Otis MA) from egg masses collected from Russian ships arriving in port areas of the Pacific Northwest, and from egg masses collected from different areas in the Russian Far East. Larvae were reared at 25 °C (photoperiod 14:10 L:D.) and approximately 50% R. H. in 6 oz. plastic cups covered with paper lids on the artificial diet of Bell *et al.* (1981). At pupation, male and female pupae were separated and transferred into 16 oz. paper cups covered with clear plastic lids for eclosion, and kept in different rooms for emergence. Abdominal tips of two-day-old or older females were extracted with hexane for pheromone collection six hours after the onset of scotophase (the time most mating was observed). Male *L. mathura* were shipped to Beltsville as pupae and maintained under both normal and reversed photophases (14:10, 70–80% relative humidity, 21 °C dark, 26 °C light). Electrophysiological studies were performed on males within 2–5 days postemergence.

Identification and synthesis

Gas chromatographic analyses were performed with Shimadzu model 9A or 14A instruments equipped with flame ionization detectors and fitted with either a 30 m DB-1 column (J & W Scientific, Folsom, CA) or with a 30 m chiral BDM Chiraldex column (ASTEC, Inc., Whippany, NJ). Mass spectra were recorded on a Finnigan Incos 50 GC–MS fitted with a 60 m DB-1 column (J & W Scientific, Folsom, CA); electron ionization spectra were collected at 70 eV and a source block temperature of 150 °C. Octyllithium was purchased from FMC Corporation, Lithium Division, Gastonia, North Carolina. Mention of a proprietary product or company does not imply endorsement by the U. S. Department of Agriculture.

Synthetic compounds were prepared according to published procedures: *Z,Z,Z*-3,6,9-nonadecatriene **1**, Heath *et al.* (1983); racemic 3,4-, 6,7-, and 9,10-monoepoxides of 3,6,9-nonadecatriene **2a**, **2b**, and **2c**, Hansson *et al.* (1990); *Z,Z*-(9*S*,10*R*)-9,10-epoxy–3,6-nonadecadiene **4a** and *Z,Z*-(9*R*,10*S*)-9,10-epoxy-3,6-nonadecadiene **4b**, Mori and Takeuchi (1989), Wong, *et al.* (1985). The mixture of racemic monoepoxides **2a**, **2b**, and **2c** was first separated from unreacted **1** and di- and triepoxides by flash chromatography on silica gel, eluting the column successively with hexanes followed by 2.5, and 5% ethyl acetate in hexanes. The monoepoxide fraction was then flash chromatographed on silver nitrate-impregnated silica gel, eluting with cyclohexane-benzene mixtures and continuing with 100% benzene. The 6,7-monoepoxide **2b** was the earliest eluting, and a pure sample was obtained. Later fractions containing **2a** and **2c** were combined and rechromatographed on silica gel with increasing portions of benzene in hexanes; the 9,10-epoxide **2c** eluted earlier than the 3,4-isomer **2a**, and essentially pure samples of each were obtained. The isomers were easily differentiated by comparison of their mass spectra to those published by Hansson *et al.* (1990).

Electrophysiology

Electroantennograms (EAG's) were recorded from adult males using a modification of an earlier technique (Schneider, 1957; Dickens et al, 1993). An excised antenna was fixed between two glass capillary electrodes filled with 0.1 N NaCl. Ag–AgCl wires in the glass capillaries connected the preparation to the recording instruments: a Grass P-16 preamplifier, a Tektronix 5111A analog storage oscilloscope, and a Alpkem 310 stripchart recorder. Coupled GC/EAG studies used a Tracor model 240 gas chromatograph equipped with a 30 m DB-5 capillary column. Initially, responses of the flame ionization detector and insect preparations were recorded using a dual channel stripchart recorder. One μl of a hexane extract of abdominal tips of female moths was injected into the GC which was held at 120 deg. for 2 min, then programmed at 30 deg./min to 240 deg., and held for several minutes. For subsequent electrophysiological studies, experimental odorants were serially diluted in hexane and were delivered as 5 μl

aliquots to a piece of Whatman #1 filter paper (8 × 18 mm). The filter paper was inserted into a glass tube (80 mm by 5 mm id) and oriented toward the preparation with the outlet 1 cm from the antenna. The solvent was allowed to evaporate for 15 seconds prior to use of the odor stimulation. Each stimulation lasted 1 second. Air flow was 1 l/min as measured by a flow meter. The interstimulus time interval was 2–3 minutes. Five μl of hexane served as a control after evaporating 15 sec from the filter paper. Hexyl acetate (100 μg stimulus load) was used as a standard in order that responses from different preparations might be compared. Hexyl acetate was selected from several green leaf volatiles (Visser *et al.,* 1979) since responses to it at this stimulus load were reliable. Stimulation with the standard preceded or followed response to each experimental odorant. Responses were represented as percent responses of the mean of the two nearest responses to the standard. Responses to equal stimulus loads of enantiomers of potential pheromone components were submitted for analysis of variance (Ostle, 1969) and compared by Duncan's multiple range test (Duncan, 1955).

For single cell studies, electrical activities of neurons associated with sexually dimorphic sensilla trichodea of *L. mathura* males were recorded using tungsten electrodes electrolytically sharpened to a tip diameter of less than 1 μm (Dickens, 1979). The recording electrode was positioned under optical control (600 X) to a point at or just above the base of the targeted sensillum. Action potentials were recorded and analyzed on a minicomputer equipped with neuroscience software (Autospike™, Syntech, Hilversum, The Netherlands).

Field trials

Synthetic compounds were tested for attractiveness in 1998 in the Vladivostok area of the Primorsky, Krai. Russia. (Preliminary studies in 1997 near Barabash, Eastern Russia, had provided information that aided in trap design and targeting loading rates for dispensers of synthetic compounds.) Populations in the 1998 test were characterized as very low density by sampling immature stages. Six sites were sampled for immature stages of *Lymantria* species by loosely wrapping the boles of 40 host trees with burlap bands (40 cm width). These bands provide a resting site for larvae of *L. mathura* and *L. dispar.* Bands were checked seven times throughout the larval development period. A total of 11 immature *L. mathura* were found for all sites and sampling times. Three trapping sites were selected based on these larval surveys. For testing, synthetic compounds (see Table I) and 1 to 2 day old virgin females were placed in wing traps (Pherocon-1C®, TRÉCÉ, Inc.®, Salinas CA). Traps were modified to provide approximately 9 cm spacing between the top and bottom halves. In addition, the top of the trap, which is usually not coated with adhesive, was replaced with an adhesive-coated bottom. These modifications were made to provide easier entry for males, to accommodate the plastic mesh (7 mm × 7 mm, cages 7 cm diameter × 6 cm) used for female moths, and to provide more sticky surface area for male captures.

Synthetic candidate compounds were dispensed in hexane solutions on cotton dental rolls (9 mm dia. X 9 mm high). Dispensers and caged females were suspended in traps so that they were not in contact with the adhesive surface. Treatments were tested in a randomized complete block design. Blocks consisted of traps hung from branches one to two meters high and spaced 50 to 100 m apart along straight lines. Two to three lines (replicates) were placed 200–300 m apart at the three sites which were separated by 4 to 7 km. A total of 8 complete replicates were checked daily during the 11 days of testing. The warm spring and early summer resulted in adult flight earlier than anticipated. Before the testing wa initiated, we believed flight was more than half over. Note: captures were recorded after the 11th day of trapping. Daily high and low temperatures during the trapping period were respectively 20–23 °C and 15–16 °C with frequent rain or drizzle.

Results

Two electrophysiological responses were obtained from crude abdominal tip extracts upon gas chromatography-electroantennogram detection. The first of these was a C_{19} triunsaturated hydrocarbon, whose mass spectrum matched a published spectrum of (*Z,Z,Z*)-3,6,9-nonadecatriene **1** (Becker *et al.,* 1983). A sample of **1** was synthe-

sized (Heath *et al.*, 1983) and its GC retention time and mass spectrum matched those of the unknown.

The second EAG-active component had a GC retention time consistent with an epoxide of **1**, and its mass spectrum matched that of the published spectrum of the 9,10-monoepoxide of 3,6,9-nonadecatriene **2c** (Hansson *et al.*, 1990). A sample of triene **1** was epoxidized with about 1 equivalent of *m*-chloroperoxybenzoic acid (Hansson, *et al.*, 1990) (Fig. 1). The mixture of three racemic monoepoxides (**2a** + **2b** + **2c**) was separated from unreacted **1** and di- and triepoxides by flash chromatography. Dose-response curves constructed from responses of male antennae to these fractions showed activity of the monoepoxide mixture to exceed that of either the triene or diepoxide mixture (Fig. 2A). Additional flash chromatography on silica gel and silver nitrate-impregnated silica gel provided small samples of the individual (racemic) positionally isomeric monoepoxides **2a**, **2b**, and **2c**. Structural assignments were made by comparing mass spectra of the purified isomers to those previously published for the same three epoxides (Hansson, *et al.*, 1990). Activity of the racemic 9,10-epoxide **2c** exceeded that of either of the two other racemic monoepoxides (Fig. 2B).

Recordings of the electrical activity from neurons associated with the sexually-dimorphic trichoid sensilla of males revealed that both (*Z,Z,Z*)-3,6,9-nonadecatriene **1** and the monoepoxide mixture stimulated a neuron with a large amplitude spike (Fig. 3A, 3B). A second neuron with a small amplitude spike was not activated by any other compound tested. Activity of the racemic 9,10 epoxide **2c** was substantially greater than that of triene **1** or of the other positional isomers **2a** and **2b** (Fig. 2B). Upon determination that the 9,10-monoepoxide was the most active isomer, its individual enantiomers were synthesized. Of the two enantiomeric 9,10-epoxides, only the (9*S*,10*R*) enantiomer **4a** reliably elicited appreciable activity from this neuron (Fig. 3C, 3D). Dose response curves constructed from EAG responses to the racemic 9,10-epoxide **2c**, and to its individual enantiomers **4a** and **4b** are consistent with receptors only for the (9*S*,10*R*) enantiomer **4a** (Fig. 3E). Responses to the (9*R*,10*S*)-enantiomer may be accounted for by the ca. 5% impurity of the (9*S*,10*R*) enantiomer in this sample.

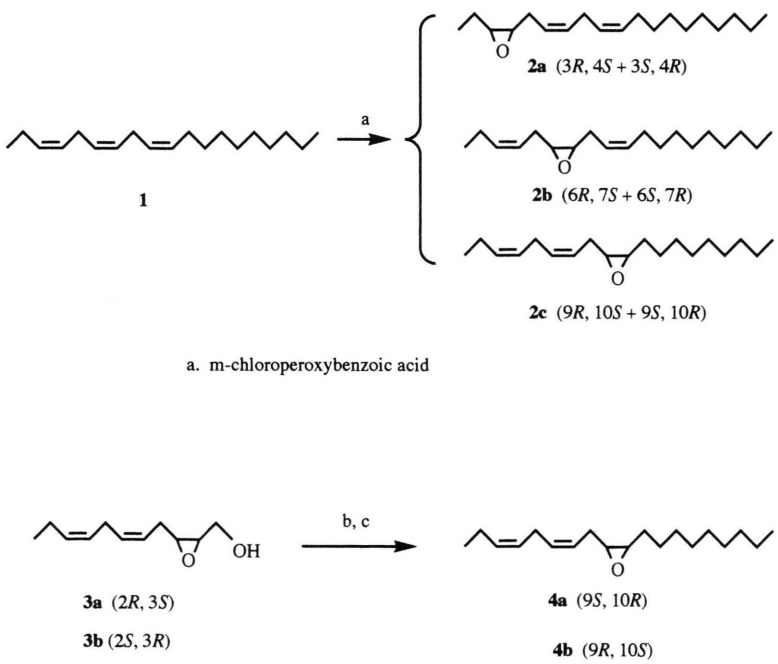

Fig. 1. Structures and synthetic scheme for *Lymantria mathura* compounds.

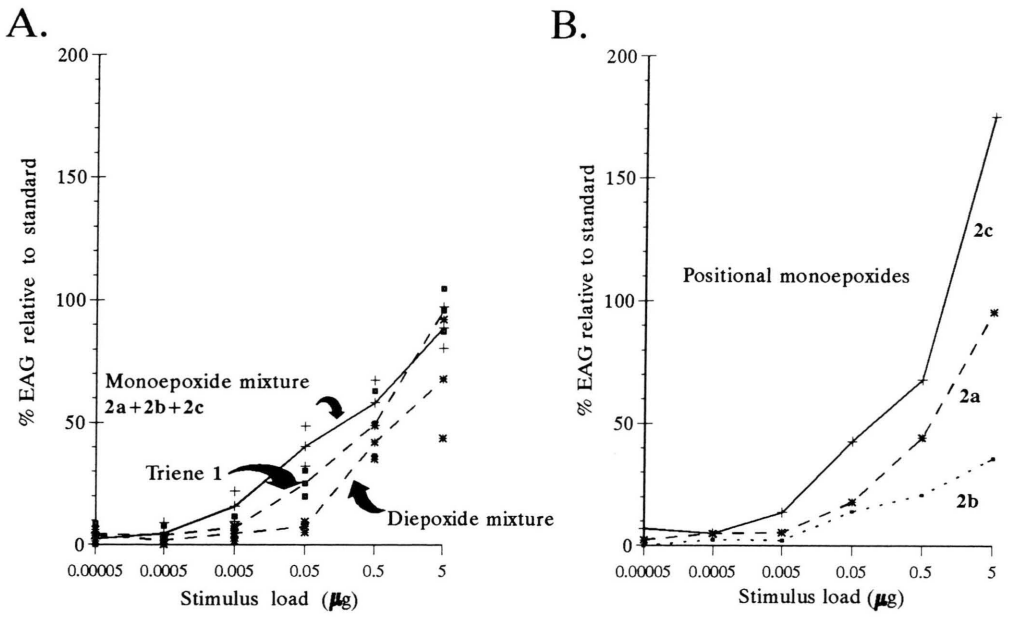

Fig. 2. A. Dose–response curves constructed from electroantennograms (EAGs) of *Lymantria mathura* males to serial dilutions of *Z,Z,Z*-3,6,9-nonadecatriene **1**, a mixture of monoepoxiedes of the triene, **2a** + **2b** + **2c**, and a mixture of diepoxides of the same triene. Corresponding symbols represent mean and standard errors. B. Dose–response curves constructed from EAGs of *Lymantria mathura* males to serial dilutions of three the racemic monoepoxides **2a** + **2b** + **2c**.

Field tests during August of 1997, conducted in the region of v. Barabash, were hampered by low populations of *L. mathura*. Several male moths were captured in traps baited with a ca. 91:9 mixture of enantiomers **4a** and **4b**, an encouraging but not statistically significant finding. During August of 1998, a somewhat higher population of *L. mathura* was present, and males were captured in traps baited with the enantiomer **4a** and also with a mixture of all three racemic isomers **2a**, **2b**, and **2c** (Table I). Although population densities in the region were still too low for ideal statistical evaluations, the highest applications of the racemic mixture (30 mg of **2a**+ **2b**+ **2c,** which contains roughly 5 mg of **4a**) resulted in catches of male *L. mathura* as high as those achieved with caged virgin females. From these preliminary results, it appears that racemic epoxide is as effective as the single (9*S*,10*R*) enantiomer, and that triene **1** is not required for attraction.

Discussion

(*Z,Z,Z*)-3,6,9-Nonadecatriene **1** is a familiar lepidopteran pheromone component. A search conducted in July, 1997 of Chemical Abstracts STN files revealed 41 publications or patents addressing this compound; all related, directly or indirectly, to the occurrence and/or application of the material as a pheromone component. Additionally, analogous skipped trienes (chain lengths ranging from C_{17} to C_{22}) also occur frequently (Wong *et al.,* 1985; Ando *et al.,* 1993 and 1995, and references therein). To date, nearly all these occurrences seem to have been reported for the families Arctiidae, Noctuidae and Geometridae; we are aware of only one other report of a skipped triene system from Lymantriidae [Gries *et al.,* (1997) have reported a mixture of two monoepoxides and two diepoxides of *Z,Z,Z*-3,6,9-heneicosatriene as pheromonal components of the satin moth, *Leucoma salicis* L.].

Previous work with pheromone systems including triene-monoepoxides suggests that the absolute configuration of the monoepoxide may play an important role in pheromone activity, but does not predict which enantiomer, or what enantiomeric ratio, should be expected. An entire range of results have been reported for the effects of the

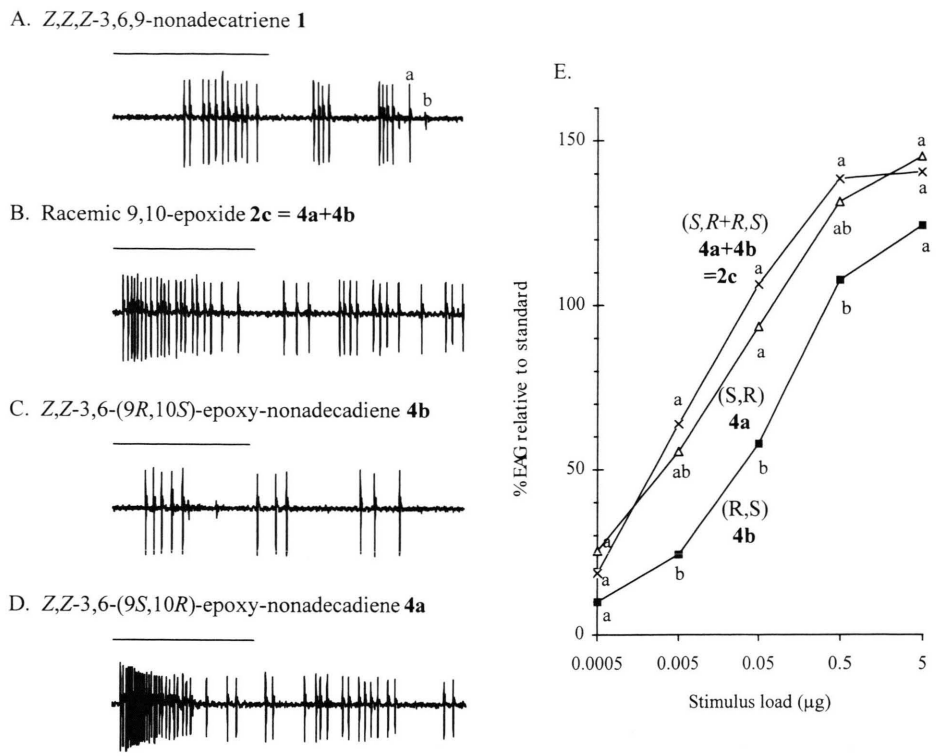

Fig. 3. Electrical responses of neurons associated with a sexually-dimorphic trichoid sensillum of a *Lymantria mathura* male to volatiles emanating from 500 ng on filter paper of: A. triene **1** (note the presence of two action potential types, labeled a and b in A, with differing amplitudes.) B. racemic 9,10-monoepoxide **2c**, C. the (9*R*,10*S*)-monoepoxide **4b**, D. the (9*S*,10*R*)-monoepoxide **4a**. Horizontal bar above each trace represents duration of stimulus + 500 msec. E. Dose-response curves constructed from electroantennogram responses of *Lymantria mathura* males to serial dilutions of racemic 9,10-monoepoxide **2c**, the (9*R*,10*S*)-monoepoxide **4b**, and the (9*S*,10*R*)-monoepoxide **4a**. EAG responses for different stimulus loads with different letters are significantly different ($P<0.05$, Duncan's multiple range test).

Table I. Number of *Lymantria mathura* males captured in traps baited with virgin females and synthetic compounds. Means followed by the same letter are not significantly different at the 5% level using the LSD test in the SAS General Linear Models procedure.

Treatment	Mean number + S. E. of males captured in traps (total
Z,Z,Z-3,6,9-nonadecatriene, **1**,1 mg	0.0 **d**
Z,Z-(9*S*,10*R*)-9,10-epoxy-3,6,nonadecadiene, **4a**, 0.1 mg	0.6 ± 0.22 (6) **cd**
Z,Z-(9*S*,10*R*)-9,10-epoxy-3,6,nonadecadiene, **4a**, 1 mg	0.8 ± 0.49 (8) **cd**
Z,Z,Z-3,6,9-nonadecatriene, **1**,1 mg + *Z,Z*-(9*S*,10*R*)-9,10-epoxy-3,6,nonadecadiene, **4a**, 1 mg	0.7 ± 0.26 (7) **cd**
Racemic monoepoxide mixture **2a** + **2b** + **2c**, 6 mg	1.9 ± 0.78 (19) **bc**
Racemic monoepoxide mixture **2a** + **2b** + **2c**, 30 mg	4.9 ± 2.86 (39) **ab**
Virgin females	4.0 ± 0.77 (20) **a**

"opposite" enantiomer (Szöcs *et al.*, 1993; Millar *et al.*, 1991, and references within) including cases where the "opposite" enantiomer was inert, was antagonistic, or was required for optimum performance. Similarly, the role of the triene can vary--it may serve as a pheromone component, or be essentially inert, presumably acting just as a pheromone precursor.

The individual enantiomers **4a** and **4b** (Fig. 1), as well as several homologs, have previously been synthesized by Wong *et al.* (1985) by creating both enantiomers **3a** and **3b** of the key epoxyalcohol and coupling their tosylates with the appropriate lithium dialkylcuprate. Mori and Ebata (1981, 1986), and Mori and Takeuchi (1989) have reported syntheses of the C_{21} analogs of **4a**, **4b** using the same coupling reaction and lithium di(n-decyl)cuprate. They exploited the crystallinity of the 3,5-dinitrobenzoates of **3a** and **3b** to achieve purification and chiral enhancement of those key intermediates, and thus to provide coupled epoxides of high optical purity. We coupled **3a** and **3b** with lithium dioctylcuprate to give **4a** and **4b**, respectively, of at least 95% optical purity (judged by gas chromatographic analysis of the acetates of alcohols **3a** and **3b** on a ChiraldexTM BDM column. Partial, but incomplete resolution of **4a** and **4b** was also achieved on this column).

As stated above, only the (9*S*,10*R*) enantiomer **4a** evoked appreciable single cell activity and EAG responses. The electrophysiological responses to **4a** and the relative lack of response to **4b** (the weak apparent response to **4b** may have resulted from the small amount of **4a** present as an impurity) are consistent with the requirement for **4a** for behavioral activity and imply that **4b** should neither be required for, nor inhibit, insect response to **4a** (consistent with results of our trapping experiments--*vide infra*). Alternatively, significant EAGs elicited by **4b** in dose response studies (Fig. 3D) may not be totally accounted for by the relatively small percentage of **4a** present in **4b**. If this were the case, then separate receptor sites may exist on neurons responsive to **4b**, or separate receptor neurons specialized for **4b** may occur in sensilla on the antenna not sampled in our study. Our data do not favor these latter interpretations since the existence of additional receptors for **4b** would be indicated by a higher saturation level for the dose response curve for the **4a** + **4b** blend relative to **4a** or **4b** alone (Fig. 4D); this was not observed.

Although two spikes of differing amplitude were generally present in recordings of electrical activity from single trichoid sensilla of males, only the (9*S*,10*R*) enantiomer **4a** evoked appreciable activity, and then only in the large amplitude spike. The presence of two odor receptor neurons within sexually dimorphic trichoid sensilla of male lymantriid has been previously reported for the gypsy moth, *L. dispar* L., and the nun moth, *L. monacha* L. (Hansen, 1984). Similar to *L. mathura*, activation of the large amplitude spike by (+)-disparlure in *L. dispar*, and both the large and small amplitude spikes in *L. monacha*, correlated with attraction of both species. However, the small amplitude spike in *L. dispar* was activated by (−)-disparlure (Hansen, 1984) and the presence of (−)-disparlure resulted in a decrease in attractiveness of the (+)-enantiomer (Vité *et al.,* 1976). Limited activation of the large amplitude action potential by the triene **1** is similar to activity of the olefin precursor for disparlure in studies with the gypsy moth, *L. dispar* L. and the nun moth, *L. monacha* L. (Schneider et al, 1977).

Trapping tests in the summer of 1998 supported preliminary results obtained in 1997. The highest application of a mixture of **2a**, **2b**, and **2c** resulted in catches as high as, or slightly higher than, those from live females (Table I). The successful use of this mixture may be of considerable practical value, since a mixture of **2a**, **2b**, and **2c** can be relatively easily and economically prepared from linolenic acid , in contrast to the more expensive and lengthy stereospecific synthesis of **4a** from shorter-chain precursors. Triene **1**, although EAG-active, appears to contribute little or nothing to the attractancy of the epoxides [as was shown for the analogous olefin in field studies with *L. dispar* and *L. monacha* (Schneider *et al.,* 1974)]

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