Pheromones 98*

Olfactory Electroantennogram Responses of the Formicine Ants *Lasius niger* and *Formica* Species (Hymenoptera: Formicidae) to 3,4-Dihydroisocoumarins

Friedrich Kern and Hans Jürgen Bestmann

Institut für Organische Chemie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Henkestraße 42, D-91054 Erlangen, Bundesrepublik Deutschland

Z. Naturforsch. **49c,** 865–870 (1994); received April 27/July 21, 1994

Formicidae, Trail Pheromone, Hindgut, Rectal Bladder, Electroantennogram (EAG)

Electroantennogram (EAG) studies of the formicine genera *Lasius* and *Formica* (Hymenoptera, Formicidae) were carried out with 3,4-dihydroisocoumarins, recently identified as trail pheromones within this ant subfamily. The here investigated formicine species *Lasius niger, Formica rufa, F. fusca* and *F. sanguinea* were all responsive towards stimuli of synthetic 3,4-dihydroisocoumarins. Synthesized samples of natural 3,4-dihydroisocoumarins, found in the hindgut of these species evoke the highest electrophysiological responses while structure analogues were less efficient. Number and position of the methyl groups of test substances were important factors for structure activity in EAG. *F. fusca* and *L. niger* showed a stereoisomeric differentiation in EAG responses between (*R*)- and (*S*)-isomers of 8-hydroxy-3,5,7-trimethyldihydroisocoumarin, a component in the hindgut of both species. Higher electrophysiological activity for (*R*)-8-hydroxy-3,5,7-trimethyl-3,4-dihydroisocoumarin in comparison to the (*S*)-isomer agrees with better behavior responses of previous trail following tests in the case of *Lasius niger.*

Introduction

In formicine ants such as *Lasius* and *Formica* species the workers lay chemical trails with glandular secretion from a food site back to the nest so that other nestmates can follow by odour orientation. The anatomical source of trail pheromones of formicine ants is the rectal sac which is a part of the hindgut in the abdomen (Blum and Wilson, 1964). In biotests an artificial trail of formicine rectal bladder secretion is able to elicit trail following behavior in worker ants of the same species (Hangartner, 1967).

The chemical structures of trail pheromones from few ants species of the subfamilies Myrmicinae, Dolichoderinae and Ponerinae are already known (Attygalle and Morgan, 1985; Attygalle *et al.,* 1988; Hölldobler and Wilson, 1990). The formicine subfamily received only little attention although these ants are very common in Europe and occur in different habitats. Recently we could identify 3,4-dihydroisocoumarins as a new class of trail pheromones of formicine workers in the rectal bladders of *Lasius* and *Formica* species (Bestmann *et al.,* 1992).

The purpose of the study presented here was to investigate by means of electroantennogram recordings (EAGs) the structure-activity relationships of 3,4-dihydroisocoumarins in the formicine species *Lasius niger* L., *Formica rufa* L., *Formica fusca* L. and *Formica sanguinea* Latr. Synthesized 3,4-dihydroisocoumarins found as natural compounds in the rectal bladders were tested as well as structure analogues of related species. The relationship of the recorded EAG responses in comparison to trail following behavior is discussed.

Materials and Methods

For EAG studies worker ants of *Lasius niger* L., *Formica fusca* L. (subgenus *Serviformica* Forel), *Formica rufa* L. (subgenus *Formica* L.) and *Formica sanguinea* Latr. (subgenus *Raptiformica* Forel) were used. A queenright colony of *Lasius niger* was found in a garden in Erlangen and trans-

* Pheromones 97: Bestmann H. J., Janssen E., Kern F., Schäfer D., Vostrowsky O. (1994), Der Sexualpheromonkomplex des weiblichen Blutbär *Thyria jacobaeae* (Lepidoptera, Arctiidae). Z. Naturforsch. **49c,** 276–279.

Reprint requests to Prof. Dr. H. J. Bestmann.
Telefax: (0 91 31) 85 68 64.

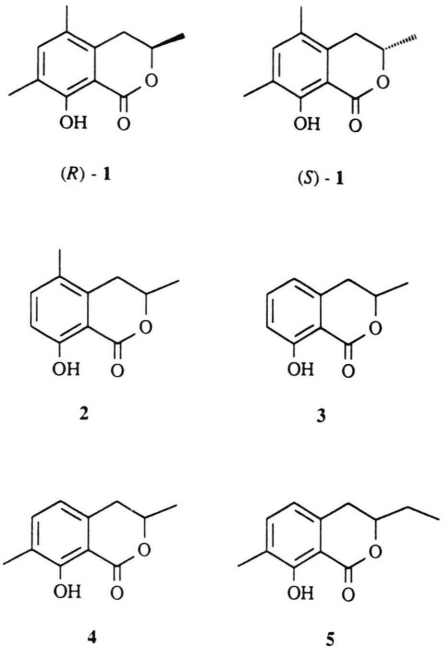
0939–5075/94/1100–0865 $ 06.00

ferred to our laboratory. Only a small number of *Formica rufa, Formica fusca* and *Formica sanguinea* workers were taken from nest sites of forests near Erlangen. The ants were kept in the laboratory at 80% relative humidity and at temperature of 25 ± 2 °C. Freshly killed insects such as larvae of *Tenebrio molitor* (Coleoptera) and nymphs of *Nauphoeta* spp. (Blattoidea) were served as food as well as honey in an aqueous solution.

Compounds **1, 2, 4, 5** (Formula 1) were previously synthesized at our institute for comparative GC-MS analysis (GC purity > 98%), behavioral tests and EAG measurements (Bestmann *et al.*, 1992). Both enantiomers of 8-hydroxy-3,5,7-trimethyl-3,4-dihydroisocoumarin **1** were available by stereoselective synthesis (*ee* > 95%). Furthermore synthetic samples of the structure analogues (3*R,S*)-8-hydroxy-3,5-dimethyl-3,4-dihydroisocoumarin **2,** (3*R,S*)-8-hydroxy-3,7-dimethyl-3,4-dihydroisocoumarin **4** as well as (3*R,S*)-8-hydroxy-3-ethyl-7-methyl-3,4-dihydroisocoumarin **5** were used for EAG measurements. (3*R,S*)-8-hydroxy-3-methyl-3,4-dihydroisocoumarin **3** (mellein) was synthesized for these measurements by a previous described route (Mali *et al.*, 1992).

The number of test substances for one row of EAG measurements is limited by the living duration of the antenna preparation. Therefore, preliminary EAG studies were carried out to select the most active test compounds (4 maximum) among (*R/S*)-**1,** (*R*)-**1,** (*S*)-**1,** (*R/S*)-**2,** (*R/S*)-**3,** (*R/S*)-**4** and (*R/S*)-**5** for dose response curves.

Methodology for (*R/S*)-EAG recording of an ant antenna is basically similar to that of a lepidopteran which is well established in the literature (Schneider, 1957). However, some modifications of the above EAG techniques have been described later for antennae of other insects (Payne, 1975; Payne *et al.*, 1975; Dickens, 1984). For electrophysiological recordings Ag–AgCl glass capillary electrodes filled with insect Ringer solution ($CaCl_2 \times H_2O$ 2 mmol, KCl 5 mmol, $NaHCO_3$ 2 mmol, NaCl 130 mmol) were used. The recording electrode was inserted into the distal segment of the antenna and the indifferent electrode was inserted in the head capsule near the base of the antenna both after puncture by a sharpened tungsten needle. The inserting point of the indifferent electrode was sealed with vaseline in order



Formula 1. **1,** 8-hydroxy-3,5,7-trimethyl-3,4-dihydroisocoumarin; **2,** 8-hydroxy-3,5-dimethyl-3,4-dihydroisocoumarin; **3,** 8-hydroxy-3-methyl-3,4-dihydroisocoumarin (mellein); **4,** 8-hydroxy-3,7-dimethyl-3,4-dihydroisocoumarin; **5,** 8-hydroxy-3-ethyl-7-methyl-3,4-dihydroisocoumarin.

to prevent leakage of the hemolymph and thus early dehydration of the preparation.

Serial dilutions of the test substances (in the 0.01–100 µg range) were delivered as 20 µl aliquots in hexane placed on filter-paper strips (7×20 mm) which were inserted into small glass cartridges the nozzles of which were oriented toward the antenna from a distance of 1 cm. Control cartridges were loaded with pure hexane on filter-paper. Molecules evaporating from the filter-paper were carried over the preparation by a filtered stream of pure air. Serial dilutions were delivered from the lowest (0.01 µg) to the highest (100 µg) concentration. This concentration range was selected out of results from previous experiments showing small responses at 0.01 µg and a saturation effect at about 100 µg. Stimulus duration was set at 1 s at an air flow of 0.5 l/min. A stream of filtered air continuously flushed the antenna between stimuli which were separated by intervals of at least 4 min. A vacuum nozzle was positioned approximately 10 cm behind the preparation to re-

move pheromone contaminated air from the area surrounding the ant antenna.

In order to compare the efficacy of the different test substances, the values of each test series was divided by the maximum value of that series (EAG/EAG_{max}). The largest potential difference observed for the given concentration range was between 1.0 and 1.5 mV. Normalized values of the recordings were used to obtain the mean value (MV) and standard errors (SE) were calculated. Student's t-test was used to find out the significant differences ($\alpha < 0.05$) among test substances.

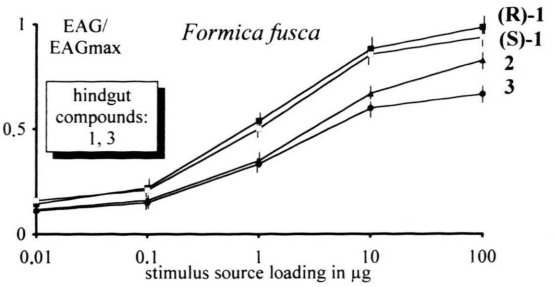


Fig. 2. Dosage-EAG-response curves of *Formica fusca* worker antennae to stimulation with 3,4-dihydroisocoumarins. Each point represents a mean of 20 replicates. Vertical bars represent standard errors.

Results

Trail pheromone 8-hydroxy-3,5,7-trimethyl-3,4-dihydroisocoumarin **1,** previous identified in the hindgut of *Lasius niger* showed the highest electrophysiological responses among the test substances **1, 2** and **3** (Fig. 1). Worker ants of this species gave weaker EAG responses with 8-hydroxy-3,5-dimethyl-3,4-dihydroisocoumarin **2** and 8-hydroxy-3-methyl-3,4-dihydroisocoumarin **3** (mellein). We could observe stereoselective differentiation in olfactory reception of both enantiomers of **1.** (*R*)-**1** in comparison to its (*S*)-enantiomer elicited higher responses in EAG. Significancy ($\alpha = 0.01$) of the differences for (*R*)-**1** in opposite to the (*S*)-**1** enantiomer was calculated at high doses (100 µg).

In *Formica fusca,* synthetic compounds of **1, 2** and **3** were used for EAG studies. Previous analysis showed **1** and **3** (mellein) as natural components in the hindgut of this species. In EAG with workers antennae of *F. fusca* the trimethylated compound **1** was the most efficient 3,4-dihydroisocoumarin among the synthetic test substances (Fig. 2). Compound **2** evoke only weaker electrophysiological responses, whereas the monomethylated **3** had the smallest electrophysiological activity. Significant differences between (*R*)-**1** and (*S*)-**1** at high doses (100 µg) point out that *F. fusca* workers are also able to discriminate between these two enantiomers ($\alpha = 0.05$).

The hindgut of the red wood ant *Formica rufa* contains **3** (mellein) which was previously shown as the main component of the trail pheromone. Fig. 3 shows the results of EAG studies with workers antennae towards stimuli of **1, 2** and **3,** all compounds as racemic mixtures. Trail pheromone **3** of this species elicits the highest EAG responses within the test group of 3,4-dihydroisocoumarins. Dimethylated **2** had less electrophysiological activity whereas trimethylated **1** elicited only very weak responses. The olfactory discrimination in this species such as between **3** and **2** is obvious since the calculated significancy in EAG responses

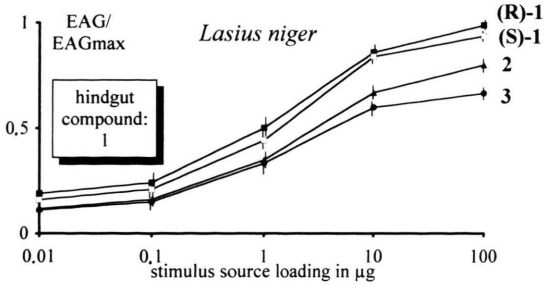


Fig. 1. Dosage-EAG-response curves of *Lasius niger* worker antennae to stimulation with 3,4-dihydroisocoumarins. Each point represents a mean of 7 replicates. Vertical bars represent standard errors.

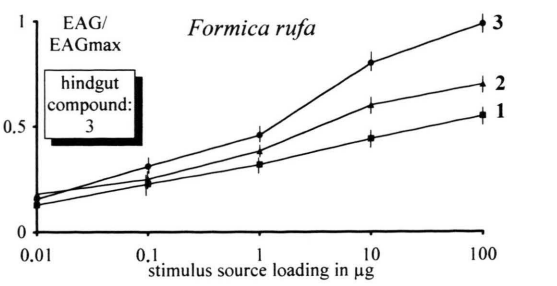


Fig. 3. Dosage-EAG-response curves of *Formica rufa* worker antennae to stimulation with 3,4-dihydroisocoumarins. Each point represents a mean of 10 replicates. Vertical bars represent standard errors.

between the hindgut compound **3** and **2** is α = 0.002 (100 µg).

For EAG with *Formica sanguinea* worker ants, stimuli of synthetic **1, 3,** 8-hydroxy-3,7-dimethyl-3,4-dihydroisocoumarin **4** and 8-hydroxy-3-ethyl-7-methyl-3,4-dihydroisocoumarin **5** were given. The two latter compounds were previously analyzed as natural ingredients of the *F. sanguinea* hindgut. Dose-response curves drawn from EAG measurements of this species are shown in Fig. 4. Here, **4** and **5** were found to be the most active compounds with no significant differences, whereas the electrophysiological activities of **1** were weaker. Stimuli with **3** evoke only very low electrophysiological responses on the *F. sanguinea* antenna.

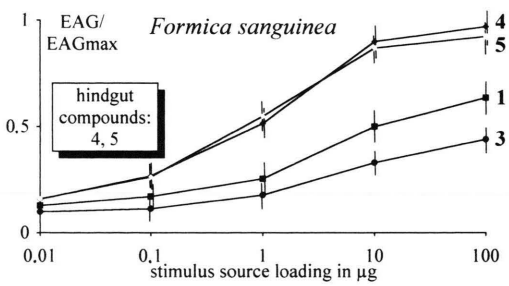


Fig. 4. Dosage-EAG-response curves of *Formica sanguinea* worker antennae to stimulation with 3,4-dihydroisocoumarins. Each point represents a mean of 20 replicates. Vertical bars represent standard errors.

Discussion

EAG results of this study show the antennal olfactory sensitivity of the formicine ants *Lasius niger, Formica fusca, F. rufa* and *F. sanguinea* towards 3,4-dihydroisocoumarins. These aromatic compounds were previously identified as a new class of trail pheromones in the hindgut of formicine ants (Bestmann *et al.,* 1992). In most cases synthetic samples of the species specific natural hindgut compounds evoke the highest EAG responses on the antennae of this species in comparison to structure analogues. The electrophysiological activity of the test substances which differed in number and position of added methyl groups was distinct, indicating that methyl groups play an important role for the chemoreception of these 3,4-dihydroisocoumarins.

In *Lasius niger* and *Formica fusca* the trimethylated **1** had the highest electrophysiological activity in opposite to dimethylated (**2**) and monomethyl analogues (**3**) which indicates that EAG activity of 3,4-dihydroisocoumarins decreases for this species with loss of methyl groups of the aromatic compound. Further with **1,** a stereospecific differentiation in EAG could been observed in both species, *L. niger* and *F. fusca,* where (*R*)-**1** elicited higher electrophysiological responses than the (*S*)-enantiomer. In this respect, the EAG results of *L. niger* and *F. fusca* are quite similar, although the hindgut of both species contains not the same ingredients, the latter both, **1** and **3,** the former one only **1** as natural components.

In the case of *L. niger* these EAG results agree with previous behavior results, because trail following responses of *L. niger* workers are significant better for synthetic (*R*)-**1** in comparison to (*S*)-**1** (Bestmann *et al.,* 1992). Structure analogues which had weak electrophysiological activity were unable to elicit trail following behavior.

Mellein **3** elicited the highest responses in EAG with *F. rufa* workers among the test substances **1, 2** and **3.** In opposite to *L. niger* and *F. fusca,* a decrease of EAG activity could be observed for 3,4-dihydroisocoumarins with increasing number of methyl groups of the 3,4-dihydroisocoumarins in the aromatic ring. Dimethylated **2** and trimethylated **1** had only low electrophysiological activity and are unable to elicit trail following behavior.

In EAG measurements with *F. sanguinea,* synthetic samples of the hindgut ingredients **4** and **5** had the highest activity. The comparison between **3** and **4** shows that the absence of the methyl group at carbon atom C-7 (**3**) lowers the EAG response drastically. An additional methyl group at C-5 position (**1**) also leads to weaker electrophysiological responses, compared to **4,** whereas substitution of a methyl (**4**) by an ethyl group (**5**) at carbon atom C-3 has not very much influence.

The presented EAG results show that 3,4-dihydroisocoumarins with various number of methyl and ethyl groups have different activities in the EAG with the investigated *Formica* and *Lasius* species. This lead to the consumption that the olfactory differentiation of 3,4-dihydroisocoumarins takes place already on the receptor level on the ant antenna. We suppose different receptors which respond distinct for various number of methyl

groups of 3,4-dihydroisocoumarins, identified as formicine pheromones. On that basis one can understand that in previous biotests for example *L. niger* and *F. rufa* worker ants recognized and followed only artificial trails of 3,4-dihydroisocoumarins with the same number of methyl groups as their species specific hindgut components. The comparative results of electrophysiology and behavior in formicine ants are in agreement with previous investigations of the ponerine *Leptogenys diminuta* where both stereoisomers and structure analogues of the pheromone had some activity in EAG, whereas in biotest only stereoisomers were able to elicit reduced behavior responses (Kern and Bestmann, 1993).

Although in previous tests trail following behavior could been observed only with species specific gland secretions, it has also been reported that hungry *Formica* species follow in some cases also odour trails of related formicine species (Horstmann, 1982; Gösswald, 1984) showing also the ability of olfactory orientation on foreign ant trails. However, there is no doubt that the antenna of some ant species must be extremely specialized for the species specific pheromone because of their ability to discriminate between stereoisomers of, for instance trail pheromones (Attygalle *et al.*, 1988; Bestmann *et al.*, 1992; Steghaus-Kovac *et al.*, 1992).

Mellein **3** was already found in several natural products such as in the secretion of the termite *Cornitermes* spp. (Blum, 1982), as a metabolite in the fungi species *Aspergillus melleus* (Turner, 1971), volatile constituent of the ponerine *Rhytidoponera metallica* (Brophy *et al.*, 1981) and as a metapleural bladder product in the myrmicine ant *Crematogaster deformis* (Attygalle *et al.*, 1989). It has also been identified in mandibular glands of formicine ants *Camponotus herculeanus* L. (Brand *et al.*, 1973) and *Polyrhachis (Cyrtomyrma) ?doddi* (Bellas and Hölldobler, 1985). Payne *et al.* (1975) recorded chemoreceptor EAG responses from all castes of *Camponotus herculeanus* to stimuli of the sex pheromone mellein and other volatile components of the mandibular gland of this species.

The origin of natural 3,4-dihydroisocoumarin production found in formicine hindguts is yet unknown. However, it is interesting that one group of chemical substances is able to release two different behaviors such as sex and trail pheromones within the same subfamily of ants.

Acknowledgements

We thank M. C. Witschel (Institute of Organic Chemistry, F.A. University of Erlangen-Nürnberg) for synthesis of 3,4-dihydroisocoumarins, Dr. D. Schäfer (Institute of Physiological Chemistry, F.A. University of Erlangen-Nürnberg) for GC-MS analysis and Prof. I. Hasenfuß (Institute of Zoology, F.A. University of Erlangen-Nürnberg) for the determination of the ant species. We also thank Dr. A. B. Attygalle (Cornell University, N.Y.) for his friendly advice. This work was supported by the Deutsche Forschungsgemeinschaft.

Attygalle A. B. and Morgan E. D. (1985), Ant trail pheromones. Adv. Insect Physiol. **18,** 1–30.

Attygalle A. B., Vostrowsky O., Bestmann H. J., Steghaus-Kovac S. and Maschwitz U. (1988), (3*S*,4*S*)-4-methyl-3-heptanol, the trail pheromone of the ant *Leptogenys diminuta* Smith (Formicidae, Ponerinae). Naturwissenschaften **75,** 315–317.

Attygalle A. B., Siegel B., Vostrowsky O., Bestmann H. J. and Maschwitz U. (1989), Chemical composition and function of the metapleural gland secretion of the ant *Crematogaster deformis* Smith (Hymenoptera: Myrmicinae). J. Chem. Ecol. **15,** 317–329.

Bellas T. and Hölldobler B. (1985), Constituents of mandibular and Dufour's glands of an Australian *Polyrhachis* weaver ant. J. Chem. Ecol. **11,** 525–538.

Bestmann H. J., Kern F., Schäfer D. and Witschel M. C. (1992), 3,4-Dihydroisocoumarins, a new class of ant trail pheromones. Angew. Chem. **104,** 757–758; Angew. Chem., Int. Ed. Engl. **31,** 757–758.

Bestmann H. J., Janssen E., Kern F., Schäfer D. and Vostrowsky O. (1994), Der Sexualpheromonkomplex des weiblichen Blutbär *Thyria jacobaeae* (Lepidoptera, Arctiidae). Z. Naturforsch. **49c,** 276–279.

Blum M. S. and Wilson E. O. (1964), The anatomical source of trail substances of formicine ants. Psyche **71,** 28–31.

Blum M. S. (1982), Pheromonal bases of insect sociality: communications, conundrums and caveats. Les Colloques de l'Institut National de la Recherche Agronomique **7,** 149–162.

Boyd N. D. and Martin M. M. (1975), Faecal proteinases of the fungus-growing ant, *Atta texana:* their fungal origin and ecological significance. J. Insect Physiol. **21,** 1815–1820.

Brand J. M., Fales H. M., Sokoloski F. A., MacConnel J. G., Blum M. S. and Dufield R. M. (1973), Identification of mellein in the mandibular gland secretions of carpenter ants. Life Sci. **13,** 201–211.

Brophy J. J., Cavill G. W. K. and Plant W. D. (1981), Volatile constituents of the Australian ponerine ant *Rhytidoponera metallica.* Insect Biochem. **11,** 307–310.

Burton H. S. (1950), Antibiotics from *Aspergillus melleus.* Nature **165,** 274–275.

Dickens J. C. (1984), Olfaction in the boll weevil, *Anthonomous grandis* Boh. (Coleoptera: Curculionidae): Electroantennogram studies. J. Chem. Ecol. **10,** 1759–1785.

Gösswald K. (1984), Die Waldameise. Aula-Verlag, Wiesbaden.

Hangartner W. (1964), Über die Geruchsspur von *Lasius fuliginosus* zwischen Nest und Futterquelle. Experientia **20,** 392–393.

Hangartner W. (1967), Spezifität und Inaktivierung des Spurpheromons von *Lasius fuliginosus* (Latr.) und Orientierung der Arbeiterinnen im Duftfeld. Z. Vergl. Physiol. **57,** 103–136.

Hölldobler B. and Wilson E. O. (1990), The Ants. Belknap Press of Harvard University.

Horstmann K. (1982), Spurorientierung bei Waldameisen (*Formica polyctena* Förster). Z. Naturforsch. **37c,** 348–349.

Kern F. and Bestmann H. J. (1993), Antennal electrophysiological responsiveness of the ponerine ant *Leptogenys diminuta* to trail and recruitment and its structure analogues. Naturwissenschaften **80,** 424–427.

Mali R. S., Jagtap P. G., Patil S. R. and Pawar P. N. (1992), Novel Al_3Cl-catalyzed syntheses of naturally occurring (±)-8-hydroxy-3-methyl-3,4-dihydroisocoumarins. J. Chem. Soc. Chem. Commun. 883–884.

Payne T. L. (1975), Bark beetle olfaction. III. Antennal olfactory responsiveness of *Dendroctonus frontalis* Zimmermann and *D. brevicomis* Le Conte (Coleoptera: Scolytidae) to aggregation pheromones and host tree terpene hydrocarbons. J. Chem. Ecol. **1,** 233–242.

Payne T. L., Blum M. S. and Duffield R. M. (1975), Chemoreceptor responses of all castes of a carpenter ant to male-derived pheromones. Ann. Entomol. Soc. Am. **68,** 385–386.

Schneider D. (1957), Elektrophysiologische Untersuchung von Chemo- und Mechanorezeptoren der Antenne des Seidenspinners *Bombyx mori* L. Z. Vergl. Physiol. **40,** 8–41.

Steghaus-Kovac S., Maschwitz U., Attygalle A. B., Frighetto R. T. S., Frighetto N., Vostrowsky O. and Bestmann H. J. (1992), Trail-following responses of *Leptogenys diminuta* to stereoisomers of 4-methyl-3-heptanol. Experientia **48,** 690–694.

Turner W. B. (1971), Fungal Metabolites. Academic Press, London, New York, pp. 118–123.