The Sex Pheromones of Two Pine Sawfly Species, *Gilpinia frutetorum* and *Gilpinia socia*: Chemical Identification, Synthesis and Biological Activity

Erik Hedenström[a,*], Helene Edlund[a], Ann-Britt Wassgren[b], Gunnar Bergström[b], Olle Anderbrant[c], Fredrik Östrand[c], Andrzej Sierpinski[d], Marie-Anne Auger-Rozenberg[e], Annette Herz[f,g], Werner Heitland[f,g], and Martti Varama[h]

[a] Chemistry, Department of Natural Sciences, Technology and Mathematics, Mid Sweden University, SE-851 70 Sundsvall, Sweden. Fax: +46 60 14 88 02. E-mail: erik.hedenstrom@miun.se
[b] Chemical Ecology, Göteborg University, Carl Skottsbergs Gata 22, SE-413 19 Göteborg, Sweden
[c] Department of Ecology, Lund University, Sölvegatan 37, SE-223 62 Lund, Sweden
[d] Forest Research Institute, Bitwy Warszawskiej 1920 R. No. 3, Box 61, 00-973 Warsaw, Poland
[e] Institut National de la Recherche Agronomique, Centre de Recherches d'Orleans, Ardon, F-45160 Olivet, France
[f] Lehrstuhl für Angewandte Zoologie, Forstwissenschaftliche Fakultät, Technische Universität München, Am Hochanger 13, D-85343 Freising, Germany
[g] Present address: Federal Research Centre for Cultivated Plants – Julius Kuehn-Institute, Institute for Biological Control, Heinrichstraße 243, D-64287 Darmstadt, Germany
[h] The Finnish Forest Research Institute, Vantaa Research Centre, P. O. Box 18, FIN-01301 Vantaa, Finland

* Author for correspondence and reprint requests

Z. Naturforsch. **64c**, 733–742 (2009); received May 20/June 24, 2009

3,7-Dimethylpentadecan-2-ol and 3-methylpentadecan-2-ol were identified in female whole body extracts from the two pine sawfly species *Gilpinia frutetorum* and *Gilpinia socia*. This is the first observation of 3-methylpentadecan-2-ol in extracts of a female pine sawfly species. Synthetic and highly pure stereoisomers of 3,7-dimethylpentadecan-2-ol and 3-methylpentadecan-2-ol were used to verify that the (2*S*,3*R*,7*R*)-isomer of 3,7-dimethylpentadecan-2-ol and (2*S*,3*R*)-3-methylpentadecan-2-ol were present in the extracts. The four stereoisomers of 3-methylpentadecan-2-ol and their biologically active esters were produced via chemoenzymatic methods and the synthesis is described in detail. Male *G. socia* antennae responded strongly in EAG recordings to the (2*S*,3*R*)-isomer of the acetate and propionate of 3-methylpentadecan-2-ol. Male antennae of both *G. frutetorum* and *G. socia* also responded to the (2*S*,3*R*,7*R*)- and (2*S*,3*R*,7*S*)-acetates of 3,7-dimethylpentadecan-2-ol.

Key words: Sex Pheromone, *Gilpinia frutetorum*, *Gilpinia socia*

Introduction

Several sawfly species within the family Diprionidae (Hymenoptera: Symphyta) are economically important defoliators of pines and other conifers in the northern temperate region (Smith, 1993). Most of these species belong to the genera *Diprion* or *Neodiprion*, but also members of other genera cause damage. *Gilpinia* is one such genus with around 30 described species native to Europe and northern Asia. In comparison to *Diprion* and *Neodiprion* only few studies on the chemical communication in *Gilpinia* have been performed (Anderbrant, 1993, 1999). The only thoroughly investigated species from this point of view is *G. pallida* (Klug) (Hedenström *et al.*, 2006). Females were found to produce and males to respond most strongly to the acetate or propionate of (2*S*,3*R*,7*R*)-3,7-dimethyltetradecan-2-ol. This compound is a one carbon atom longer homologue with the same stereoisomeric properties as the main pheromone component of *D. pini* L. (Bergström *et al.*, 1995; Anderbrant *et al.*, 1995). Two other and closely related species, *G. frutetorum* F. and *G. socia* Klug, can occasionally cause severe damage on pine trees (Pschorn-Walcher, 1982). *Gilpinia socia* is found only in Europe, lays

eggs in clusters and the larvae feed in groups. It may potentially be bivoltine and sometimes it occurs in outbreaks with *D. pini* (Pschorn-Walcher, 1982). No studies on its chemical communication have been reported. See Fig. 1 for sex pheromone precursor structures that up to now have been identified in pine sawfly species.

Gilpinia frutetorum occurs in Europe, western and northern Asia and has been introduced to eastern North America. It can also have two generations per year, but lays eggs singly and the larvae feed solitary (Pschorn-Walcher, 1982). Only one test on the chemical communication of this species has earlier been presented in which acetates and propionates of various isomers of the first identified pine sawfly pheromone, 3,7-dimethylpentadecan-2-ol (diprionol), were tested. This test revealed attraction to the acetate of the (2*S*,3*R*,7*R*/*S*)-isomer blend at a location in Wisconsin, USA (Kikukawa *et al.*, 1982). We present here our results from investigations aimed to identify the pheromones of *G. socia* and *G. frutetorum*. In addition to analyses of female extracts and syntheses of new substances found in these, we performed electroantennography (EAG) on male antennae to verify the biological activity. To our knowledge, no outbreaks of these species have occurred after our chemical and electrophysiological investigations and, therefore, no confirming field trials were possible.

Material and Methods

Insects

Extracts of female *G. socia* were prepared from insects of Finnish origin. Larvae were collected at a small outbreak on ornamenal *Pinus mugo* in Pori, southwestern Finland, during 1995–1998, and then reared until pupation outdoors in net cages in the FFRI experimental forest near Vantaa, Finland. Male *G. socia*, used for electrophysiological recordings came from a laboratory culture kept at INRA, Orleans, France, originating from insects collected between Byrgocz and Gdansk, Poland, in 2000. The *G. frutetorum* used in this study came from a laboratory culture kept at TU Munich, Germany (Herz and Heitland, 2002) originating

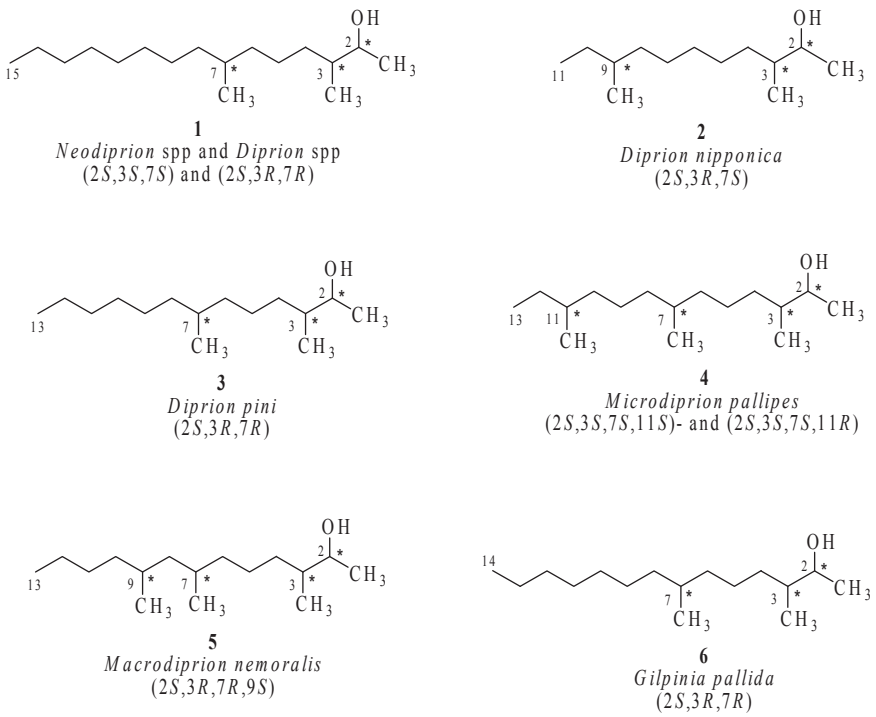


Fig. 1. Methyl-branched long-chain 3-methylalkan-2-ols that, during the years, have been identified as sex pheromone precursors in females of pine sawfly species.

from cocoons collected in Poland and eastern part of northern Germany during 1996.

Extraction

Whole bodies of varying numbers, from 40 to about 100 females, were extracted for 72 h with ethyl acetate. Extracts were prepared from insects of different ages, from freshly hatched to three-day-old. Extracts were cleaned by liquid chromatography according to Bergström *et al.* (1995), and fractions 7 and 8 containing diprionol and related alcohols were used in the chemical analyses.

Chemical analyses

The identification and quantification were performed by combined gas chromatography-mass spectrometry (GC-MS) using a Hewlett-Packard 5890 gas chromatograph equipped with a Finnigan TSQ 700 mass spectrometer in the electron impact mode (EI) (Method A). For chromatographic separations, a 25 m × 0.25 mm, 0.20 μm film thickness, Chrompack CP-Wax-58 fused silica column coated with nitroterephthalic acid-modified, chemically bound polyethylene glycol as stationary phase was used. The oven program was kept isothermal at 50 °C for 3 min, then programmed at 8 °C/min to 220 °C, and finally kept isothermal at 220 °C for 10 min. Helium was used as carrier gas at a linear velocity of 24 cm/s. The injection mode was splitless at an injector temperature of 220 °C. To quantify the amounts of the pheromone precursor alcohols in the extract 300 ng of 3,11-dimethyltetradecan-2-ol as internal standard were added to every extract before evaporation.

Stereochemical analyses

Determination of the exact stereoisomers was carried out by two different gas chromatographic methods. The first one, Method B, involved derivatization of the alcohols to isopropylisocyanate derivatives. The derivatives were then analyzed by a coupled nitrogen/phosphorus detector. A Hewlett-Packard 5880 gas chromatograph, equipped with a chiral fused silica column, 50 m × 0.23 mm I.D., coated with XE-60-(*S*)-valine-(*S*)-2-phenylethylamide (ChromPack), was coupled to the nitrogen/phosphorus detector. The conditions were as follows: The sample was injected by splitless mode into the injector at 195 °C; carrier gas was N_2 with a pressure of 97 kPa; the oven temperature was linearly programmed from 120 °C to 185 °C at 2 °C/min.

The second method, Method C, was a derivatization of the pheromone precursor alcohols to pentafluorobenzoates followed by gas chromatography using electron capture detection (ECD). In this case a Hewlett-Packard 6890 gas chromatograph was equipped with a Chrompack fused silica capillary column (50 m × 0.25 mm I.D., film thickness 0.2 μm) coated with the stationary phase Sil-88. The derivatives were injected at 220 °C by pulsed splitless technique supplied with electronic pneumatic control (EPC) using N_2 as carrier gas at a pressure of 103 kPa. Oven conditions were as follows: Isothermal at 50 °C for 3 min, then at 5 °C/min to 195 °C, isothermal for 30 min. The radioactive detector ^{63}Ni was heated to 240 °C.

Electrophysiology

The males of *G. frutetorum* and *G. socia* used for the EAG recordings were at most 3 and 4 days old, respectively. One antenna from each male was cut off and placed between two glass capillary electrodes filled with Ringer solution. The antennal base was in contact with one grounded platinum electrode, and the signal from the electrode holding the antennal tip was connected to a high-impedance amplifier. The antenna was continuously flushed with a charcoal-filtered, moistened airstream of 0.5 m/s with the outlet of the tube 1 cm from the antenna. The stimulus was injected into the air stream through an opening in the glass tube 20 cm upstream of the antenna. A stimulus was created by a stimulation device (Murphy Developments, Hilversum, The Netherlands), which delivered the stimulus in a 0.5-s puff at a flow rate of 5 ml/s. The stimulus was made of a Pasteur pipette containing a 5 × 20 mm piece of filter paper. The appropriate amount of synthetic sample, diluted in cyclohexane, was added to the filter paper and the solvent allowed to evaporate. During the recordings, stimulation with a standard stimulus was done every third time, including the first and the last one. As standard stimulus for the *G. frutetorum* recordings 0.01 μg of the acetate of (2*S*,3*R*,7*R*)-3,7-dimethyltridecan-2-ol was used, and for the *G. socia* recordings 1 μg of the acetate of (2*S*,3*R*,7*S*)-3,7-dimethylpentadecan-2-ol was used. EAG recordings were analyzed using "EAG version 2.2a" software (Syntech, Hilver-

sum, The Netherlands, 1993). The standardized EAG response of a stimulus was calculated by dividing the recorded response by the average of the responses of the standard puffed before and after. Mean responses were compared with ANOVA followed by Tukey's test at $P < 0.05$ using SYSTAT or SPSS software. If necessary the data were log-transformed before analysis to improve homogeneity among variances.

EAG recordings were performed at different occasions with different series of synthetic compounds. First, before the chemical analysis of the females had been completed, a number of earlier known diprionid pheromone isomers were tested on *G. frutetorum* males. Later, esters of several identified and available alkanol isomers were used as stimuli for *G. socia* antennae. Unfortunately no *G. frutetorum* was available at this time. All compounds tested are listed in Table I, with purities indicated.

Synthesis

Commercially available chemicals were used without further purification. The *cis*- and *trans*-epoxybutanes were obtained from Aldrich and analyzed by GC to be of >99.9% diastereoisomeric purity. Amano PS was obtained from Amano Pharmaceutical Co. Ltd., Nagoya, Japan. The lipase was stored at 4 °C over silica gel. Dry diethyl ether was distilled from $LiAlH_4$, and the alkyl halides were distilled prior to use and stored under argon. In the coupling reactions the solvents were degassed by argon for about 1 h prior to use. Li (s) was washed with *n*-heptane and was flattened by hammering and cut in very thin pieces prior to use. Preparative liquid chromatography (LC) was performed on normal phase silica gel (Merck 60, 230–400 mesh, 0.040–0.063 mm, Merck, Germany) employing a gradient technique using an increasing content of distilled ethyl acetate in distilled cyclohexane (0 → 100%) as eluent. To monitor the progress of the reactions, thin layer chromatography (TLC)

Table I. The purity and reference of synthesis of chemicals used in EAG recordings with *G. socia* and *G. frutetorum*.

Compound and stereoisomer	Chemical purity (%)	Stereochemical purity (%)	Contaminating isomer(s)	Reference
Acetate of 3,7-dimethyltridecan-2-ol				
(2*S*,3*R*,7*R*)	>97	>99.5	SRS, RSR	Bergström *et al.* (1995)
Acetate of 3,7-dimethylpentadecan-2-ol				
(2*S*,3*S*,7*S*)	>99	>97.5	SSR, SRS, RSS, RRS	Högberg *et al.* (1990)
(2*S*,3*R*,7*R*)	>98	>97.5	SRS, RSR, SSR, RRR	Högberg *et al.* (1990)
(2*S*,3*R*,7*S*)	>98	>97.0	SRR, SSS, RRS, RSS	Högberg *et al.* (1990)
Propionate of 3,7-dimethylpentadecan-2-ol				
(2*S*,3*S*,7*S*)	>99	>97.5	SSR, SRS, RSS, RRS	Högberg *et al.*, 1990
(2*S*,3*R*,7*R*)	>98	>97.5	SRS, RSR, SSR, RRR	Högberg *et al.* (1990)
(2*S*,3*R*,7*S*)	>98	>97.0	SRR, SSS, RRS, RSS	Högberg *et al.* (1990)
Acetate of 3-methylpentadecan-2-ol				
(2*S*,3*S*)	>99	>99.4	RR	This work
(2*R*,3*R*)	>99	>99.0	SS	This work
(2*S*,3*R*)	>99	>99.4	RS	This work
(2*R*,3*S*)	>99	>99.6	SR	This work
Propionate of 3-methylpentadecan-2-ol				
(2*S*,3*S*)	>99	>99.4	RR	This work
(2*R*,3*R*)	>99	>99.0	SS	This work
(2*S*,3*R*)	>99	>99.4	RS	This work
(2*R*,3*S*)	>99	>99.6	SR	This work

was performed on silica gel plates (Merck 60 F_{254}, pre-coated alumina foil) eluted with ethyl acetate (20–40% ethyl acetate in cyclohexane) and developed by spraying with vanillin in sulfuric acid and heated at 120 °C. NMR spectra were recorded on a Bruker DMX 250 (250 MHz ^{1}H and 62.9 MHz ^{13}C) spectrometer using $CDCl_3$ as solvent and TMS as internal reference. Optical rotations were measured on a Perkin Elmer 241 polarimeter using an 1-dm cell. Mass spectra were recorded on a Saturn 2000 instrument, operating in the EI mode, coupled to a Varian 3800 GC instrument with a 30 m × 0.25 mm I.D. capillary column coated with DB-1 (Durabond), d_f = 0.25 μm, carrier gas N_2, 83 kPa, split ratio 1:50. IR spectra were recorded neat between NaCl plates using a Perkin Elmer 782 infrared spectrometer. Conversions and purity were monitored by a 30 m × 0.32 mm I.D. capillary column coated with EC-1 (Varian), d_f = 0.25 μm, carrier gas N_2, 76 kPa, split ratio 1:50.

(2R,3R*)-3-Methylpentadecan-2-ol [(2R*,3R*)-**7**]*

The title compound was prepared using the method of Hedenström *et al*. (2002) to yield the racemic alcohol (2*R**,3*R**)-**7** >99% pure (GC). The analytical and spectroscopic data were similar to those published (Hedenström *et al*., 1992).

(2R,3S*)-3-Methylpentadecan-2-ol [(2R*,3S*)-**7**]*

As in Hedenström *et al*. (2002) the racemic alcohol (2*R**,3*S**)-**7** was prepared >99% pure (GC). The analytical and spectroscopic data were similar to those published (Magnusson, 1978).

General method for the enantioselective lipase-catalyzed acylation of the alcohols (2R,3R*)- and (2R*,3S*)-3-methylpentadecan-2-ol*

Racemic (2*R**,3*R**)-**7** [or (2*R**,3*S**)-**7**], *n*-heptane (4.3 ml/mmol alcohol), lipase (160 mg/mmol alcohol), and molecular sieve (3 Å) were stirred for 2 h in a sealed flask. Vinyl ester (4.8 mmol/mmol alcohol) was then added to start the reaction. The conversion was monitored by periodic withdrawal of samples. When the reaction reached the desired conversion, the mixture was filtered and the solid remaining on the filter was washed with several portions of *n*-pentane. The solvent was evaporated and the product 2*R*-ester and the remaining substrate 2*S*-alkanol were separated by LC. The esters and the alcohols were in this way obtained chemically pure by GC without any trace of the remaining alcohol or the product ester, respectively. The stereoisomeric purity of the obtained compounds was analyzed as formate esters as below, see Table II.

Analysis of the remaining substrate 2S-3-methylpentadecan-2-ol and the product 2R-esters from the above lipase-catalyzed acylation sequence for (2R,3S*)-**7** and (2R*,3R*)-**7** as formate ester on a β-dex 325 GC column*

The product 2*R*-propionate or 2*R*-acetate (30–40 mg) was reduced by $LiAlH_4$ in diethyl ether according to the standard method. The obtained 2*R*-alcohol or the 2*S*-substrate alcohol (10 mg) was then dissolved in formic acid and stirred at room temperature overnight. The formic acid was evaporated and the residue was purified by LC; the stereoisomeric purity of the obtained formate was determined using a Varian 3300 gas chromatograph equipped with a 30 m × 0.25 mm I.D. capillary column coated with β-dex 325 (Supelco Inc.), d_f = 0.25 μm, carrier gas He, 103 kPa, split ratio 1:100. (2*R**,3*S**)-**7**: GC programme: 150 ºC isothermal. Retention time (min): 90.3 (2*S*,3*R*-**7**), 103.4 (2*R*,3*S*-**7**). (2*R**,3*R**)-**7**: GC programme: 155 °C (40 min), 0.5 °C/min, 180 °C. Retention time (min): 68.1 (2*S*,3*S*-**7**), 69.8 (2*R*,3*R*-**7**). For the stereoisomeric purity see Table II.

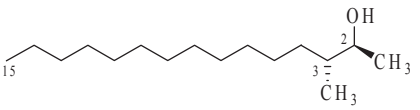
Table II. The stereoisomeric purity of the four synthetic stereoisomers of 3-methylpentadecan-2-ol.

Isomer	Stereoisomeric purity (%)				
	(2*S*,3*S*)	(2*S*,3*R*)	(2*R*,3*S*)	(2*R*,3*R*)	*Ee* of main stereoisomer
(2*S*,3*S*)-**7** Ac/Pr	99.4	<0.1	<0.1	0.70	98.7
(2*R*,3*R*)-**7** Ac/Pr	1.0	<0.1	<0.1	99.0	98.0
(2*R*,3*S*)-**7** Ac/Pr	<0.1	0.4	99.6	<0.1	99.2
(2*S*,3*R*)-**7** Ac/Pr	<0.1	99.4	0.5	<0.1	98.9

Results and Discussion

Chemical analysis of extracts from G. frutetorum and G. socia females

The structures of the unknown compounds isolated from extracts of female *G. frutetorum* and *G. socia* were established by comparison of their mass spectra and GC retention times with those of the synthesized compounds. In the extracts of both species we could identify (Method A) one new pheromone precursor, 3-methylpentadecan-2-ol (see Fig. 2), which is an analogue to the known biologically active compound (as acetate) 3,7-dimethylpentadecan-2-ol (diprionol) found in *Neodiprion sertifer*. With Method B the streoisomeric composition of this new pheromone precursor was identified as (2*R**,3*S**)-3-methylpentadecan-2-ol in both *G. frutetorum* and *G. socia*. The substance found occurs in quantities of approx. 2 ng/female in *G. frutetorum* (see Table III) and 4 ng/female in *G. socia* (see Table IV). In *G. socia*, the amounts of (2*R**,3*S**)-3-methylpentadecan-2-



(2*S*,3*R*)-**7**

Fig. 2. A new long-chain 3-methylalkan-2-ol, 3-methylpentadecan-2-ol, that has been found in females of the pine sawfly species *G. frutetorum* and *G. socia*, respectively.

Table III. The stereoisomeric composition (Methods B and C) and the amounts of different compounds (Method A) identified from whole-body extracts of unmated female *G. frutetorum*.

Compound identified and quantified in extract[a]	*G. frutetorum* of German and Polish origin	
	Freshly emerged 40 ♀ (1996)	Different age 80 ♀ (1997)
(2*S*,3*S*,7*R**)-3,7-Dimethylpentadecan-2-ol[b]	2 ng/♀	1–2 ng/♀
(2*R**,3*R**)-3-Methylpentadecan-2-ol[b]	100 pg/♀	200 pg/♀
(2*S*,3*R*)-3-Methylpentadecan-2-ol[c]	1–2 ng/♀	1–2 ng/♀

[a] Identification and quantification were performed by GC-MS, using a Chrompack CP-Wax-58 column (Method A).
[b] The pheromone precursors in the extract were also derivatized to pentafluorobenzoates and analyzed on a Chrompack fused silica capillary column coated with stationary phase Sil-88 (Method C).
[c] (2*S*,3*R*)-3-Methylpentadecan-2-ol and (2*S*,3*R*,7*R*)-3,7-dimethylpentadecan-2-ol, were also identified when the extracts were analyzed as isopropylisocyanate derivatives on a chiral XE-60-(*S*)-valine-(*S*)-2-phenylethylamide column (Method B).

Table IV. The stereoisomeric composition (Methods B and C) and the amounts of different compounds (Method A) identified from whole-body extracts of unmated female *G. socia*.

Compound identified and quantified in extract[a]	*G. socia* of Finnish origin (1997)		
	<24 h, 50 ♀♀	≥24 h, 65 ♀♀	72 h, 50 ♀♀
(2*R**,3*S**,7*R*/*S*)-3,7-Dimethylpentadecan-2-ol[b,c]	1 ng/♀	8 ng/♀	
(2*R**,3*R**,7*R*/*S*)-3,7-Dimethylpentadecan-2-ol[b]		2 ng/♀	
(2*R**,3*S**)-3-Methylpentadecan-2-ol[b,c]	0.7 ng/♀	4 ng/♀	1 ng/♀
(2*R**,3*R**)-3-Methylpentadecan-2-ol[b]		1 ng/♀	

[a] Identification and quantification were performed by GC-MS, using a Chrompack CP-Wax-58 column (Method A).
[b] The pheromone precursors in the extract were also derivatized to pentafluorobenzoates and analyzed on a Chrompack fused silica capillary column coated with stationary phase Sil-88 (Method C).
[c] (2*S*,3*R*)-3-Methylpentadecan-2-ol and (2*S*,3*R*,7*R*)-3,7-dimethylpentadecan-2-ol were also identified when the extracts were analyzed as isopropylisocyanate derivatives on a chiral XE-60-(*S*)-valine-(*S*)-2-phenylethylamide column (Method B).

ol varied between 0.7 to 4 ng per female in different batches of females of different age.

(2*R**,3*R**)-3-Methylpentadecan-2-ol was also found in both *G. frutetorum* and *G. socia* in quantities of 1 ng or less (see Tables III and IV, Methods A and C).

To identify which of the enantiomers, (2*R*,3*S*)- or (2*S*,3*R*)-3-methylpentadecan-2-ol, was present in the extracts, a method using a chiral GC column was applied (Method B). Using this method the stereoisomer (2*S*,3*R*)-3-methylpentadecan-2-ol was identified as the only isomer in female extracts from both species.

(2*R**,3*R**,7*R*/*S*)-/(2*R**,3*S**,7*R*/*S*)-Diprionol was also found (Method C) in both species (see Tables III and IV) and a variation in the amounts of this compound was also depending on the age of the insects extracted (Method A).

Fig. 3 shows a gas chromatogram with ECD of pentafluoro derivatives of an extract of *G. frutetorum*. The individuals analyzed only contained (2*R**,3*S**)-3-methylpentadecan-2-ol, no traces of other analogues, *i.e.* (2*R**,3*R**)-3-methylpentadecan-2-ol, (2*R**,3*S**,7*R**)-diprionol or (2*R**,3*R**,7*R**)-diprionol, were visible in this example with Method C.

Synthesis

Employing essentially the same synthetic methodology previously used by us for the synthesis of (2*R**,3*R**,7*R*/*S*)-3,7-dimethylpentadecan-2-ol (Hedenström and Högberg, 1994; Hedenström *et al.*, 2002), the desired (2*R**,3*S**)- and (2*R**,3*R**)-3-methylpentadecan-2-ols were obtained in good yields as a racemic mixture from the ring-opening of *cis*- or *trans*-epoxybutane, respectively, using the higher-order cyanocuprate prepared from 1-chlorododecane, as shown in Scheme 1.

Lipases have been used by us and others as catalysts in several successful resolutions (Hedenström *et al.*, 2002; Kamezawa *et al.*, 1994; Bergström *et al.*, 1992) and diastereoselections (Hedenström

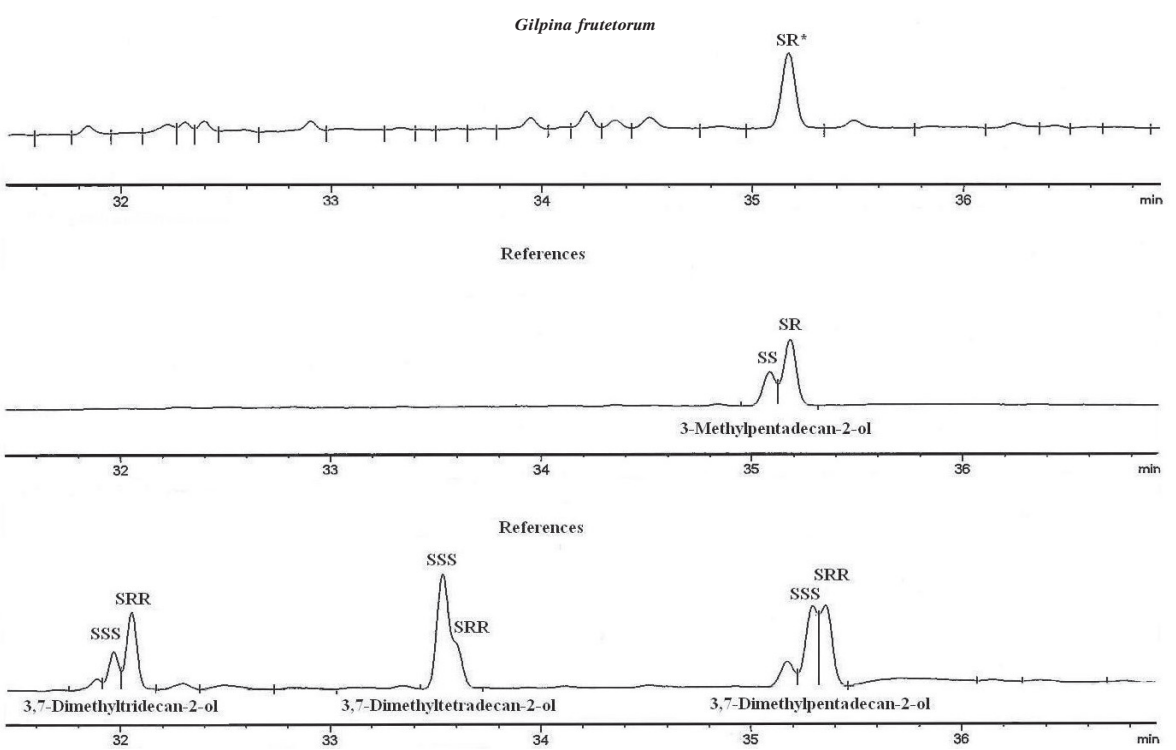
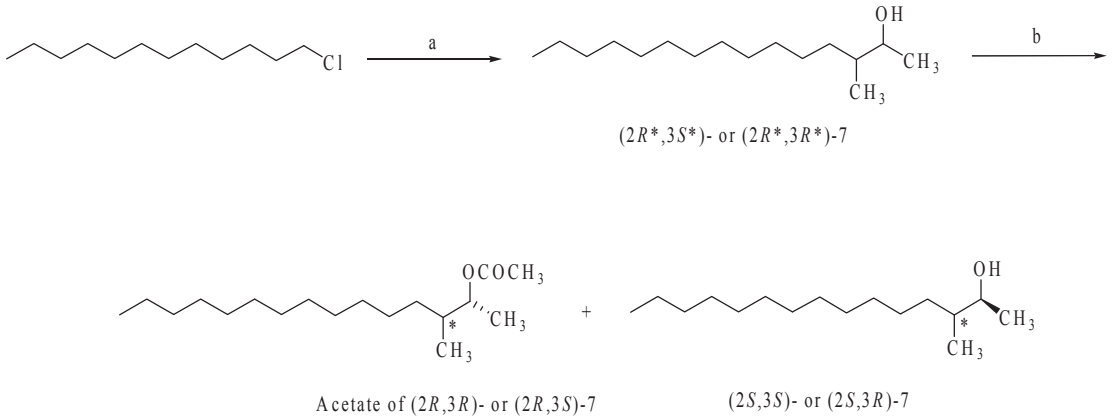


Fig. 3. An example of a gas chromatogram using a Chrompack fused silica capillary column coated with stationary phase Sil-88 analyzing pentafluoro derivatives of an extract of *G. frutetorum* (Method C). *When using Method A [isopropylisocyanate derivatives on a chiral XE-60-(*S*)-valine-(*S*)-2-phenylethylamide column] it was confirmed that the individuals analyzed only contained (2*S*,3*R*)-3-methylpentadecan-2-ol.



Scheme 1. Synthesis of all four pure stereoisomers of 3-methylpentadecan-2-ol. a) 1. Li, hexane. 2. Add to CuCN, Et_2O, –78 °C. b) Add *cis*- or racemic *trans*-epoxybutane. c) Amano PS, vinyl ester, heptane, 3 Å.

et al., 2002; Nakamura and Mori, 1999; Ohtsuka *et al*., 1997; Morgan *et al*., 1997; Lundh *et al*., 1996) of 2-alkanols and 3-methyl-2-alkanols. We have earlier shown that lipase efficiently catalyzes the stereoselective acylation of (2*R**,3*R**,7*R*/*S*)- and (2*R**,3*S**,7*R*/*S*)-3,7-dimethylpentadecan-2-ol (Hedenström *et al*., 2002, 2006; Lundh *et al*., 1996). Consequently, we applied this strategy in order to produce stereoisomerically highly pure sex pheromones of the pine sawfly species *G. frutetorum* and *G. socia*.

When using different acyl donors, *e.g*. vinyl acetate, vinyl propionate or vinyl butyrate, to acylate the alcohols very small differences were noted. Consequently, as the enantiomeric ratios *E* are high in both cases and the fact that the reaction rates also are similar, the preferred acyl donor to use is the one that produces the biologically active ester in the pine sawfly species of interest. Amano PS, used crude as received from the supplier, showed very high 2*R*-preference, *i.e.* enantiomeric ratio *E*, when using the alcohols (2*R**,3*S**)-**7** (*E* = 160) and (2*R**,3*R**)-**7** (*E* > 300), respectively, as substrates.

Consequently, the racemic substrate (2*R**,3*S**)-**7** or (2*R**,3*R**)-**7** was subjected to lipase-catalyzed acylation at a water activity (a_w) of ~ 0 (molecular sieves 3 Å) in the presence of vinyl propionate, *n*-heptane and Amano PS at room temperature. When starting with (2*R**,3*S**)-**7** the reaction was stopped after 32 h at 34% conversion by filtering off the lipase (see Scheme 1). The product ester of (2*R,*3*S*)-**7** was reduced, after LC separation of the remaining (2*S,*3*R*)-**7** alcohol, with $LiAlH_4$ and the resulting alcohol (2*R,*3*S*)-**7** was obtained in 99.2% ee (GC as formate). The enatiomerically enriched substrate alcohol (2*S,*3*R*)-**7** (51.4% ee, GC as formate) was once more subjected to lipase-catalyzed acylation at a_w ~0 (molecular sieves 3 Å) in the presence of vinyl propionate, *n*-heptane and Amano PS at room temperature. Thus, we obtained (2*S,*3*R*)-**7** with 98.9% ee when interrupting the reaction after 192 h and now at 27% conversion.

When (2*R**,3*R**)-**7** was used as substrate the reaction was stopped after 45 h at 37% conversion by filtering off the lipase (see Scheme 1). The product ester of (2*R,*3*R*)-**7** was reduced, after LC separation of the remaining (2*S,*3*S*)-**7** alcohol, with $LiAlH_4$ and the resulting alcohol (2*R,*3*R*)-**7** was obtained in 98% ee (GC as formate). The enatiomerically enriched substrate alcohol (2*S,*3*S*)-**7** (56% ee, GC as formate) was once more subjected to lipase-catalyzed acylation at a_w ~0 (molecular sieves 3 Å) in the presence of vinyl propionate, *n*-heptane and Amano PS at room temperature. Thus, we obtained (2*S,*3*S*)-**7** with 98.9% ee when interrupting the reaction after 167 h now at 28% conversion.

Electrophysiology

The screening of the response of male *G. frutetorum* to previously known pine sawfly pheromone isomers included the esters of acetic and propionic acid with (2*S*,3*S*,7*S*)-, (2*S*,3*R*,7*S*)-, and (2*S*,3*R*,7*R*)-3,7-dimethylpentadecan-2-ol (diprio-

nol). Three doses of each, 0.001, 0.01 and 0.1 μg, were used, *i.e.* 18 different stimuli. Only four of these elicited a response significantly stronger than the solvent control: The strongest dose of the (2*S*,3*R*,7*S*)- and (2*S*,3*R*,7*R*)-acetates and the two strongest doses of (2*S*,3*S*,7*S*)- propionate. However, the last isomer gave a stronger response for the intermediate dose compared to the strongest dose, which suggests that this result should be interpreted with caution.

Two different tests were done with *G. socia*. First, one dose, 1 μg, of each of 13 compounds/isomers was used to screen the activity of substances identified in the females. This screening revealed that three of eight stereoisomer/ester combinations of 3-methylpentadecan-2-ol elicited antennal response (Fig. 4). Both the acetate and propionate of the (2*S*,3*R*)-isomer and the acetate of the (2*S*,3*S*)-isomer stimulated the antennae. In addition, the acetate and propionate of (2*S*,3*R*,7*S*)-diprionol and the acetate of (2*S*,3*R*,7*R*)-diprionol were active (Fig. 4). This shows, as with other species, that only isomers with 2*S*-configuration seem active in diprionids. In the species *Gilpinia pallida* we recently found in the extract of females that the precursor 3,7-dimethyltridecan-2-ol occurred as 2*R*-form, and this has not been reported previously in pine sawfly species (Hedenström *et al.*, 2006). In the second test, three doses of the four most active substances were used as stimuli. All four showed a clear dose-response relationship, with the acetate of (2*S*,3*R*)-3-methylpentadecan-2-ol being the most active (Fig. 5).

In conclusion, the EAG recordings showed that both species respond to acetate and propionate of methyl-branched pentadecan-2-ols of the (2*S*,3*R*)-configuration. Whether the compounds found for the first time in this study, 3-methylpentadecan-2-ol or diprionol, are the pheromone precursors cannot be determined without future behavioural or field studies. However, the distinct response to esters of (2*S*,3*R*)-3-methylpentadecan-2-ol points towards this substance being a pheromone component of *G. socia*.

Acknowledgements

The research was supported by the Swedish Natural Science Research Council (NFR), Swedish Council for Forestry and Agricultural Research (SJFR), Carl Trygger's Foundation for Scientific Research and the Commission of the European Communities, Agriculture and Fisheries (FAIR), specific RTD programme, contract No. FAIR1-CT95–0339, "Pine sawfly pheromones for sustainable management of European

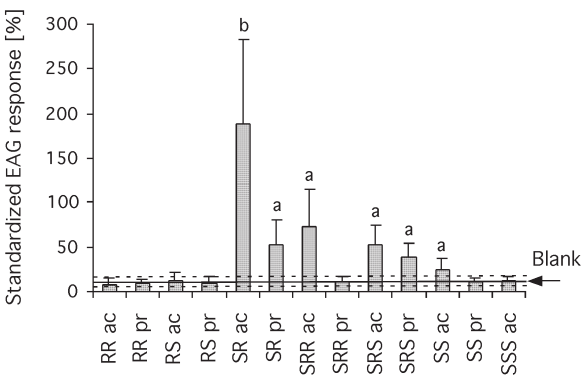


Fig. 4. Mean standardized electroantennography (EAG) response (+ SD) of antennae from male *Gilpinia socia*, expressed as percentage of standard stimulus (see Materials and Methods – Electrophysiology), to different stereoisomers and esters (ac, acetate; pr, propionate) of 3-methylpentadecan-2-ol and 3,7-dimethylpentadecan-2-ol, n = 8. The response to the solvent blank, (10 ± 6)%, is indicated by lines. Mean values different from the blank are labelled with letters; different letters indicate significantly different means (P = 0.05, Tukey's test on log-transformed data).

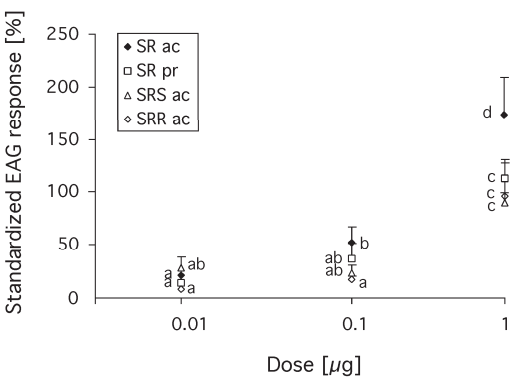


Fig. 5. Mean standardized electroantennography (EAG) response (+ or – SD) of antennae from male *Gilpinia socia*, expressed as percentage of standard stimulus (see Materials and Methods – Electrophysiology), to acetate and propionate of 3-methyl-pentadecan-2-ol and to the acetates of (2*S*,3*R*,7*S*)- and (2*S*,3*R*,7*R*)-3,7-dimethylpentadecan-2-ol, n = 6. Data points with the same letter are not significantly different from each other (P = 0.05, Tukey's test). At the lowest dose, data points have been spaced apart.

forests“. (This study does not necessarily reflect the Commission’s view and in no way anticipates its future policies in this area.) We are also grateful for the financial support (for EH) from EU (European Regional Development Fund) and from Länsstyrelsen i Västernorrlands län.

Anderbrant O. (1993), Pheromone biology of sawflies. In: Sawfly Life History Adaptations to Woody Plants (Wagner M. R. and Raffa K. F., eds.). Academic Press, New York, pp. 119–154.

Anderbrant O. (1999), Sawflies and seed wasps. In: Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants (Hardie J. and Minks A. K., eds.). CAB International, Wallingford, pp. 199–126.

Anderbrant O., Hansson B. S., Hallberg E., Geri C., Varama M., Hedenström E., Högberg H.-E., Fägerhag J., Edlund H., Wassgren A.-B., Bergström G., and Löfqvist J. (1995), Electrophysiological and morphological characteristics of pheromone receptors in male pine sawflies, *Diprion pini* (Hymenoptera: Diprionidae), and behavioural response to some compounds. J. Insect. Physiol. **41**, 395–401.

Bergström G., Wassgren A.-B., Högberg H.-E., Hedenström E., Hefetz A., Simon D., Ohlsson T., and Löfqvist J. (1992), Species-specific, two-component, volatile signals in two sympatric ant-lion species: *Synclysis baetica* and *Acanthaclisis occitanica* (Neuroptera, Myrmeleontidae). J. Chem. Ecol. **18**, 1177–1188.

Bergström G., Wassgren A.-B., Anderbrant O., Hedenström E., Högberg H.-E., Fägerhag J., Edlund H., Geri C., Auger M. A., Varama M., Hansson B. S., and Löfqvist J. (1995), Sex pheromone of the pine sawfly *Diprion pini* (Hymenoptera: Diprionidae): chemical identification, synthesis and biological activity. Experientia **51**, 370–380.

Hedenström E. and Högberg H.-E. (1994), Efficient opening of *trans*-2,3-epoxybutane by a higher order cuprate: Synthesis of *erythro*-3,7-dimethylpentadecan-2-yl acetate, pheromone of pine sawflies. Tetrahedron **50**, 5225–5232.

Hedenström E., Högberg H.-E., Wassgren A.-B., Bergström G., Löfqvist J., Hansson B., and Anderbrant O. (1992), Sex pheromone of pine sawflies. Chiral synthesis of some active minor components isolated from *Neodiprion sertifer* and some chiral analogues of diprionyl acetate. Tetrahedron **48**, 3139–3146.

Hedenström E., Edlund H., Lund S., Abersten M., and Persson D. (2002), Synthesis and lipase catalysed stereoselective acylation of some 3-methylalkan-2-ols, identified as sex pheromone precursors in females of pine sawfly species. J. Chem. Soc. Perkin Trans. 1, 1810–1817.

Hedenström E., Edlund H., Wassgren A.-B., Bergström G., Anderbrant O., Östrand F., Sierpinski A., Auger-Rozenberg M.-A., Herz A., Heitland W., and Varama M. (2006), The sex pheromone of the pine sawfly *Gilpinia pallida* (Hymenoptera: Diprionidae): chemical identification, synthesis and biological activity. J. Chem. Ecol. **32**, 2525–2541.

Herz A. and Heitland W. (2002), Comparison of the fat allocation patterns in female pine sawflies (Hymenoptera: Diprionidae). Eur. J. Entomol. **99**, 117–120.

Högberg H.-E., Hedenström E., Wassgren A.-B., Hjalmarsson M., Bergström G., Löfqvist J., and Norin T. (1990), Synthesis and gas chromatographic separation of the eight stereoisomers of diprionol and their acetates, components of the sex pheromone of pine sawflies. Tetrahedron **46**, 3027–3018.

Kamezawa M., Tachibana H., Ohtani Y., and Naoshima Y. (1994), Biocatalytic synthesis of (*S*)-2-tridecanyl acetate and (*S*)-2-pentadecanyl acetate, aggregation pheromone components of *Drosophila mulleri* and *D. busckii*, by enantioselective hydrolysis with lipase. J. Chem. Ecol. **20**, 1057–1061.

Kikukawa T., Matsumura F., Kraemer M., Coppel H. C., and Tai A. (1982), Field attractiveness of chirally defined synthetic attractants to males of *Diprion similis* and *Gilpinia frutetorum*. J. Chem. Ecol. **8**, 301–314.

Lundh M., Smitt O., and Hedenström E. (1996), Sex pheromone of pine sawflies: Enantioselective lipase catalysed transesterification of *erythro*-3,7-dimethylpentadecan-2-ol, diprionol. Tetrahedron: Asymmetry **7**, 3277–3284.

Magnusson G. (1978), Pheromone synthesis. II. Preparation of pine sawfly (Hymenoptera; Diprionidae) sex attractants and analogs with possible biological activity. Tetrahedron **34**, 1385–1388.

Morgan B., Stockwell B. R., Dodds D. R., Andrews D. R., Sudhakar A. R., Nielsen C. M., Mergelsberg I., and Zumbach A. (1997), Chemoenzymatic approaches to SCH 56592, a new azole antifungal. J. Am. Oil Chem. Soc. **74**, 1361–1370.

Nakamura Y. and Mori K. (1999), Pheromone synthesis. Part 198. Synthesis of (1*S*,2*S*,6*S*,10*R*)- and (1*S*,2*R*,6*R*,10*R*)-1,2,6,10-tetramethyldodecyl propanoate, the components of the sex pheromone of the pine sawfly, *Microdiprion pallipes*. Eur. J. Org. Chem., 2175–2182.

Ohtsuka Y., Katoh O., Sugai T., and Ohta H. (1997), Preparation of sulfur-containing optically active secondary alcohols based on *Pichia farinosa*-catalyzed anti-Prelog-rule reduction as the key step. Bull Chem. Soc. Jpn. **70**, 483–491.

Pschorn-Walcher H. (1982), Unterordnung Symphyta, Pflanzenwespen. In: Die Forstschädlinge Europas, Bd. 4 (Schwenke W., ed.). Paul-Parey Verlag, Hamburg, pp. 4–234.

Smith D. R. (1993), Systematics, life history, and distribution of sawflies. In: Sawfly Life History Adaptations to Woody Plants (Wagner M. R. and Raffa K. F., eds.). Academic Press, New York, pp. 3–32.