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Absolute Configuration of Chiral Terpenes in Marking Pheromones of Bumblebees and Cuckoo Bumblebees

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ABSTRACT The absolute configurations of citronellol, 2,3-dihydrofarnesol, and 2,3-dihydrofarnesal in male marking pheromones of seven species of bumblebees and cuckoo bumblebees were determined by enantioselective gas chromatography on a capillary column coated with 60% heptakis(2,3-di-*O*-acetyl-6-*O*-TBDMS)-β-cyclodextrin in polysiloxane PS 268. Pure (−)-*S*-enantiomers of all three terpenes were found in the labial glands of all investigated specimens of the following species: *Bombus (Bombus) terrestris, B. (Bombus) lucorum, B. (Pyrobombus) pratorum, B. (Pyrobombus) pyrenaeus, B. (Pyrobombus) jonellus, B. (Pyrobombus) impatiens*, and the cuckoo bumblebee *B. (Ashtonipsithyrus) bohemicus*. Within species, specimens were collected at different localities and in different years. Except for 2,3-dihydrofarnesol in *B. terrestris*, this is the first report on the absolute configuration of terpenes in marking pheromones of bumblebees. *Chirality 16:228–233, 2004.* © 2004 Wiley-Liss, Inc.

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Many insect semiochemicals are chiral compounds and the biological activity of both enantiomers often differ.[1] Usually, only one of the enantiomers is bioactive; interactions between receptor proteins in insect antenna with one of the enantiomers of a pheromone constituent were shown in some Coleoptera,[2] Lepidoptera,[3] and Hymenoptera.[4] However, the latest studies on chiral GC-single cell recordings show that these receptors respond strongly to one enantiomer but also more weakly to the other enantiomer (e.g., germacrene D in several heliothinae moths[5] or limonene in the pine weevil *Hylobius abietis*[6]). Sometimes, both enantiomers are biologically active and they may even show a different type of activity.[1] Thus, the "unnatural enantiomer" (i.e., not produced in the insect) can be equally active, less active (but enhancing the activity of the "natural enantiomer"), or it inhibits the activity of the natural pheromone.[1] Some insect species use pure enantiomers as pheromones, while others produce a racemic mixture or a mixture of enantiomers in a species-specific proportion.[1] In this way, the organisms possess an enormous diversity in communication systems.

Chiral acyclic terpene alcohols play an important role in the chemical communication of bumblebees.[7] They are components of the male marking pheromone in several species of bumblebees and cuckoo bumblebees.[8,9] The marking pheromone is secreted by the cephalic part of the labial gland. During the summer season, the bumblebee males scent-mark spots on the vegetation they survey by perching near the spot[10] or by patrolling from spot to spot.[11,12] Marking pheromone functions primarily as an attractant and arrestant for virgin females[7] and, moreover, as a short-term aphrodisiac for males themselves.[13]

Citronellol (**1**), 2,3-dihydrofarnesol (**2**), geranylcitronellol (**4**), and the corresponding aldehydes are often found in the male labial gland secretions of bumblebees and cuckoo bumblebees. Since the secretions are species-specific and the specificity is mostly reached by different proportions of components, knowledge of the enantiomeric purity of the chiral pheromone components is

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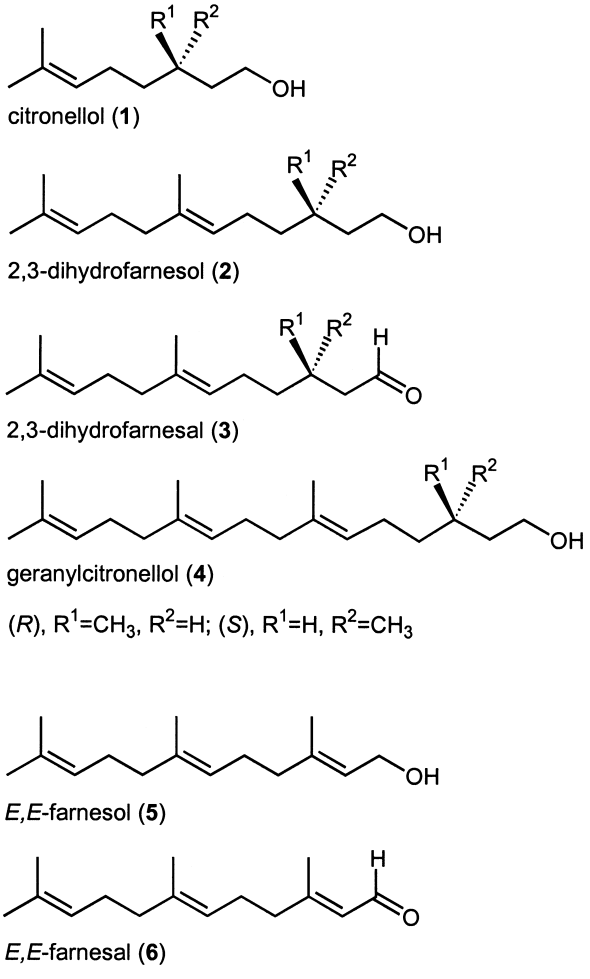

needed for full characterization of individual compounds and for further studies of their ecological importance.

The absolute configuration of 2,3-dihydrofarnesol (**2**, terrestrol) in the male marking pheromone of *Bombus terrestris* was determined earlier by a chemical derivatization method. Ställberg-Stenhagen[14] oxidized terrestrol to the corresponding aldehyde and prepared an acetal with (*R*)-propane-1,2-diol. The same derivatives were prepared from synthetic enantiomers of 2,3-dihydrofarnesol and the diastereoisomers formed were resolved by chromatography. Thus, the (*S*)-(−)-configuration was proven for terrestrol, (*S*)-**2.** This method could be applied to *B. terrestris* samples, where 2,3-dihydrofarnesol is the main component and can be obtained in tens of micrograms. The absolute configuration of other chiral compounds in the bumblebee pheromones of other species has not been reported previously. Novel techniques are needed to determine the configuration of the minute amounts of chiral compounds present in the glands of the other bumblebee species.

In this article we report the enantiomeric purity of citronellol (**1**), 2,3-dihydrofarnesol (**2**), and 2,3-dihydrofarnesal (**3**) forming major or minor components of the male marking pheromones of six bumblebee and one cuckoo bumblebee species using a two-dimensional gas chromatography (2D-GC) technique.[15]

EXPERIMENTAL

Insects

Male bumblebees of the following species were collected and used for analyses: *Bombus (Bombus) terrestris* L., *B. (Bombus) lucorum* (L.), *B. (Pyrobombus) pratorum* (L.), *B. (Pyrobombus) pyrenaeus* Pérez, *B. (Pyrobombus)* jonellus (Kirby), *B. (Pyrobombus) impatiens* Cresson, and the cuckoo bumblebee *B. (Ashtonipsithyrus) bohemicus* Seidl. Data on collection of specimens are summarized in Table 1. *Bombus terrestris* and some *B. lucorum* specimens were obtained from laboratory colonies established using mated queens from the previous year collected in South Moravia, Czech Republic.

Preparation of the Labial Gland Extracts

Labial glands (LG) were dissected from the bumblebee heads. Compounds were extracted from tissues with hex-

TABLE 1. Origin of bumblebees; contents and enantiomeric purities of chiral terpenes in their marking pheromones

		Relative abundance according to GC integration (nonchiral; chiral[b])		
Species	Origin of specimens[a]	Citronellol	2,3-Dihydrofarnesol	2,3-Dihydrofarnesal
B. terrestris	Brno (Czech Rep.), 1999 (lab. colony, 4 specimens)	—	28–32%; >97.9% (*S*)	1–3%; >99.3% (*S*)
B. pratorum	Horní Albeřice (East Bohemia, Czech Rep.), 1995	0.2%; >98.1% (*S*)	0.02%; not determined	—
	Horní Albeřice (East Bohemia, Czech Rep.), 1997	0.3%; >98.1% (*S*)	0.2%; not determined	—
B. lucorum	Brno (South Moravia, Czech Rep.) 1999 (lab. colony)	—	0.2%; >97.9% (*S*)	—
	Pavlov (South Moravia, Czech Rep.), 1998	—	0.05%; >97.9% (*S*)	—
B. jonellus	Zonhoven (Belgium) 2001	—	84%; >97.9% (*S*)	13%; >99.3%(*S*)
	Brdy-Jordán (Central Bohemia, Czech Rep.), 1995	—	16%; >97.9% (*S*)	41%; >99.3% (*S*)
B. bohemicus	East Pyrenees (France), 2001	0.2%; >98.1% (*S*)	—	—
B. impatiens	Montreal (Quebec, Canada), 2002	—	61%; >97.9% (*S*)	0.02%; >99.3% (*S*)
B. pyrenaeus	East Pyrenees (France), 2001	—	0.3%; >97.9% (*S*)	—

[a]One sample per species and locality was analyzed if not stated otherwise.
[b]Maximum contamination by the (*R*)-enantiomer was calculated from chromatographic parameters (difference in retention times, peak height, and peak tailing).

ane (50 μl per gland, Merck, Darmstadt, Germany, grade p.a. for residue analysis) or dichloromethane (200 μl per gland, Aldrich, Milwaukee, WI, capillary GC grade). After 2 h shaking at room temperature the extracts were filtered and stored at −18°C before analyses.

Chemicals

Enantiomers of citronellol (**1**, purity 98% for (*R*)- and 99% for (*S*)-enantiomers) were purchased from Aldrich. The racemate was made by mixing equimolar amounts of both enantiomers. *E,E*-Farnesal (**6**) was obtained by oxidation of *E,E*-farnesol (**5**, 96% purity, Sigma, St. Louis, MO) with MnO_2 in dichloromethane. Racemic 2,3-dihydrofarnesal (**3**) was prepared from *E,E*-farnesal (**6**) using palladium-catalyzed conjugate reduction with diphenyl silane and zinc chloride cocatalyst.[16] Racemic 2,3-dihydrofarnesol (**2**) was obtained by reduction of racemic 2,3-dihydrofarnesal (**3**) with $LiAlH_4$.

(*S*)-2,3-Dihydrofarnesol [(*S*)-**2**] was prepared from prenyl iodide (Aldrich) via a three-step synthetic procedure developed previously in our laboratory.[17] The synthesis represents an efficient [3 + 1 + 6] extension procedure on prenyl iodide, starting from methyl hydrogen (*S*)-(+)-3-methylglutarate (Aldrich). This compound was selectively reduced to hydroxy ester from which methyl (3*S*)-5-iodo-3-methylpentanoate was prepared. Prenyl iodide was extended by reaction with allene dianion and further with trimethylallane. The resulting (1*E*)-1-iodo-2,6-dimethylhepta-1,5-diene reacted then in a palladium-catalyzed cross-coupling with zinciodide prepared from methyl (3*S*)-5-iodo-3-methylpentanoate (see above) to afford (*S*)-2,3-dihydrofarnesol [(*S*)-**2**].[17]

(*S*)-2,3-Dihydrofarnesal [(*S*)-**3**] was obtained by oxidation of (*S*)-2,3-dihydrofarnesol [(*S*)-**2**] with pyridinium chlorochromate (Aldrich).[18]

Analyses

The constituents of the bumblebee LG were identified by GC with mass spectrometric detection. A Varian 3400 GC with Finnigan SSQ7000 MS was equipped with a DB-WAX column (polyethylene glycol; 30 m × 0.25 mm, film thickness 0.25 μm, J&W Scientific, Folsom, CA). The temperature program started at 50°C (2 min), then 4°C/min to 120°C (0.1 min), then 5°C/min to 220°C (20 min). Split injector temperature was 230°C. Some samples were analyzed in parallel on a GC CE 8000 with a splitless injector (220°C) and a mass detector (Fisons MD 800) in electron impact ionization mode. A DB-5 column (5% phenyl methyl silicone; 30 m × 0.25 mm, film thickness 0.25 μm, J&W Scientific) was programmed from 70°C (3 min), then 40°C/min to 140°C, then 2°C/min to 240°C, and 5°C/min to 300°C. The identification of compounds was based principally on their mass spectra compared with those in the National Institute of Standards and Technology Library (NIST, USA) and on co-chromatography with synthetic (2,3-dihydrofarnesol, 2,3-dihydrofarnesal) or commercially available (citronellol) standards.

Citronellol (**1**), mass spectrum, m/z (%): M^+ 156 (4), 138 (8), 123 (16), 95 (32), 81 (43), 69 (82), 55 (54), 41 (100). 2,3-Dihydrofarnesol (**2**), mass spectrum, m/z (%): $M^{+\cdot}$ 224 (1), 209 (1), 181 (21), 163 (14), 123 (54), 109 (19), 99 (20), 95 (50), 81 (80), 69 (100), 67 (33), 55 (26), 41 (63). 2,3-Dihydrofarnesal (**3**), mass spectrum, m/z (%): $M^{+\cdot}$ 222 (1), 204 (1), 179 (12), 161 (7), 123 (14), 109 (18), 93 (12), 81 (13), 69 (100), 55 (14), 41 (56).

Prefractionation of Extracts

Preparative TLC was done on plates DC-Fertigplatten (2.5 cm × 7.5 cm) with Kiesegel 60 (Merck). Terpene standards were co-chromatographed with the samples. A mixture of hexane:diethyl ether (7:3) was used as a mobile phase, detection was done by spraying with Rhodamine 6G (Sigma, 0.05% solution in ethanol) and UV light. The terpene zones were eluted from silica gel with diethyl ether (3 ml). The eluate was concentrated to a volume of 50 μl and injected into the 2D-GC.

Enantioselective Separations

A 2D-GC technique was used for separations of the linear terpene enantiomers. Two Varian 3400 gas chromatographs were connected with a heated interface and two

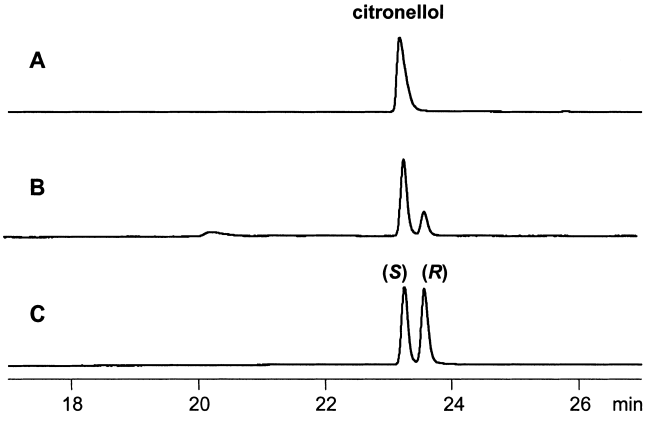

Fig. 1. Parts of chromatograms of citronellol enantiomers on the chiral column. **A:** Citronellol from *B. pratorum*. **B:** Coinjection of *B. pratorum* sample with racemic citronellol. **C:** Racemic citronellol.

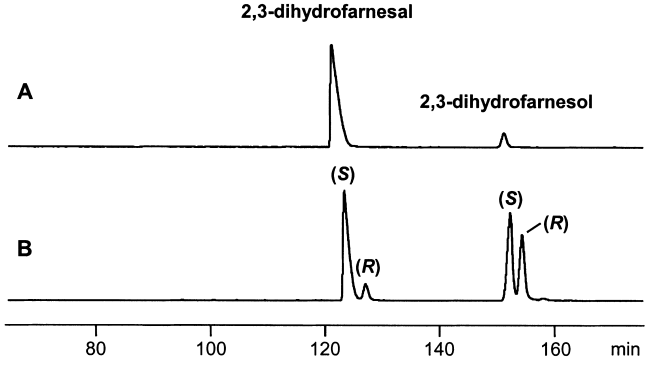

Fig. 2. Parts of chromatograms of 2,3-dihydrofarnesal and 2,3-dihydrofarnesol enantiomers on the chiral column. **A:** 2,3-Dihydrofarnesal and 2,3-dihydrofarnesol from *B. jonellus*. **B:** Coinjection of *B. jonellus* sample with racemic 2,3-dihydrofarnesal and 2,3-dihydrofarnesol.

Valco microvalves (1/32") were installed each in one GC oven as described in the literature.[15] Deactivated fused silica capillaries were used as retention gaps and for connections of the valves with each other and with the detectors. The valve in the first GC was time-programmed to switch between the FID and the interface. Thus, only compounds **1, 2,** or **3** were selected from the labial gland extracts to be separated on the chiral column installed in the second GC.[15]

In the first GC, a DB-WAX column (see above) was applied for nonchiral separations of terpene constituents in the extract samples. The temperature program started at 40°C (1 min), rose at 4°C/min to 120°C, then 5°C/min to 225°C (20 min). For separation of 2,3-dihydrofarnesol, the method started at 50°C and continued in the same way. The FID detector temperature was 250°C.

The stationary phase in the chiral column was 60% heptakis(2,3-di-*O*-acetyl-6-*O*-TBDMS)-β-cyclodextrin in polysiloxane PS 268 (Ref.[19]). A good separation of enantiomers of citronellol (**1**) was obtained isothermally at 115°C. 2,3-Dihydrofarnesol (**2**) and 2,3-dihydrofarnesal (**3**) enantiomers were isothermally separated at 100°C. A higher flow of carrier gas (linear velocity 32 cm/s) was used to shorten the retention times of the two sesquiterpenes. The elution order was determined from chromatograms of synthetic standards of pure (*S*)-enantiomers and the racemates. The absolute configuration in biological samples was assigned not only from retention times but also from coinjection with racemic standards and enhancement of the peaks of (*S*)-enantiomers (Figs. 1, 2).

RESULTS AND DISCUSSION

Enantiomeric pairs of citronellol (**1**), 2,3-dihydrofarnesol (**2**), and 2,3-dihydrofarnesal (**3**) separated well on a 60% heptakis(2,3-di-*O*-acetyl-6-*O*-TBDMS)-β-cyclodextrin in polysiloxane PS 268 column.[20] A baseline separation was obtained at 115°C for citronellol (Fig. 1) and at 100°C for both 2,3-dihydrofarnesol and 2,3-dihydrofarnesal (Fig. 2, Table 2). Analyses times were quite long for both sesquiterpenes; however, with higher temperatures the baseline separation of enantiomers was not obtained.

Some of the chiral terpenes are major components of the labial gland secretions (2,3-dihydrofarnesol, 84% in *B. jonellus*, 61% in *B. impatiens*, 30% in *B. terrestris*), some species, on the other hand, produce these compounds in minor amounts (2,3-dihydrofarnesol, 0.02% in *B. lucorum*, 0.3% in *B. pyrenaeus*; citronellol, 0.3% in *B. pratorum*, 0.2% in *B. bohemicus*) (Table 1). Considering that one gland extract contains ~50 μg of the secretion (mixture of up to 100 components), it is impossible to use classical methods (separation and measuring optical rotation) or chemical derivatization for determination of the enantiomeric purity, especially for minor compounds. The use of enantioselective GC in a 2D system[15] simplified the analyses and enabled the determination of the enantiomeric ratio of terpenes in amounts as small as 10 ng in complex mixtures with high accuracy.

All three terpenes, citronellol, 2,3-dihydrofarnesol, and 2,3-dihydrofarnesal, were found to be pure (*S*)-enantiomers (within detection limits) in all species studied (Table 1). No traces of (*R*)-enantiomers were detected in any of the three terpenes. In *B. terrestris*, our result was in agreement with a previous report on the enantiomeric purity of 2,3-dihydrofarnesol (terrestrol).[14] Despite the fact that the species studied belong to three different subgenera, and thus are not closely related, the enzyme systems producing terpene pheromone components must be very similar in all bumblebee species.

Citronellol (**1**) was previously reported in marking pheromone of *Bombus (Alpinobombus) hyperboreus*,[21] *B. (Pyrobombus) pratorum*,[11] and *B. (Ashtoniopsithyrus) bohemicus*.[11,22] (*S*)-Citronellol is a common constituent of essential oils, e.g., rose and geranium oils, while (*R*)-citronellol is found in high amounts in the family Rutaceae.[23] A difference in the perception of citronellol enantiomers was demonstrated in the honeybee, *Apis mellifera*.[4] This may be connected to the ability of the bee foragers to find rich food sources. There are numerous reports on the presence of citronellol in plant material and its role in communication among insects and between insects and plants needs to be investigated further.

2,3-Dihydrofarnesol (**2**) occurs both in animals and in plants. The compound is found in the floral fragrance of different plants, i.e., *Cyclamen*,[24] *Viola odorata*,[25] different orchids,[26] or *Lonicera japonica*.[27] The absolute configuration of the compound in fragrance was not reported. Several species of bumblebees were reported to contain 2,3-dihydrofarnesol in the marking pheromones. Thus, *Bombus (Alpinobombus*) hyperboreus,[21] *B. (Pyrobombus) huntii*,[28] *B. (Pyrobombus) jonellus, B. (Pyrobombus) cingulatus*,[21] and *B. (Bombus) terrestris*[29] males produce 2,3-dihydrofarnesol in substantial amounts. In *B. (Bombus)*

TABLE 2. Chromatographic properties of chiral terpenes on the chiral column (60% heptakis(2,3-di-*O*-acetyl-6-*O*-TBDMS)-β-cyclodextrin in polysiloxane PS-268)

	2,3-Dihydrofarnesol, racemic		2,3-Dihydrofarnesal, racemic		Citronellol, racemic	
Parameter/Enantiomer	(*S*)-(−)	(*R*)-(+)	(*S*)-(−)	(*R*)-(+)	(*S*)-(−)	(*R*)-(+)
Temperature (isothermal) [°C]	100		100		115	
Retention time [min]	152.2	155.5	126.8	130.7	23.2	23.6
Column efficiency, N/L [m^{-1}]	1.70×10^6	1.58×10^6	1.62×10^3	1.28×10^3	3.24×10^7	2.76×10^7
Resolution, R	1.05		1.65		1.40	
Column selectivity, α	1.019		1.033		1.006	

lucorum,[30] *B. (Metapsithyrus) campestris*, and *B. (Allopsithyrus) maxillosus*[31] male labial glands, 2,3-dihydrofarnesol is a minor or trace component. The role of 2,3-dihydrofarnesol in Dufour's gland of *B. terrestris* workers[32] has not been explained.

It is known that enantiomerically pure (*S*)-2,3-dihydrofarnesol is the main component of *B. terrestris* pheromone.[14] This is the only report available on the chirality of the marking pheromone components of bumblebees and cuckoo bumblebees. (*S*)-2,3-Dihydrofarnesol elicited responses on the female antennae of *B. terrestris* (Kalinová and Valterová, unpubl. results, and Ref. 7). We now report on the presence of the same pure enantiomer in four other bumblebee species.

There are also several reports on the presence of 2,3-dihydrofarnesol in glandular extracts of different animals. In insects, dihydrofarnesol is a semiochemical of *Lasius* ants[33] and traces were detected in Dufour's gland of army ants, *Dorylus (Anomma) molestus*.[34] The African elephant (*Loxodonta africana*) produces 2,3-dihydrofarnesol and related sesquiterpenes in its temporal glands that may play a role in chemical communication.[35] Furthermore, a series of 2,3-dihydrofarnesyl and citronellyl esters were found in the paracloacal gland secretion of the brown caiman (*Caiman crocodilus fuscus*).[36] The paracloacal gland secretion is thought to be involved in mate attraction or nest-site marking.[37] The absolute configuration has not been determined either in the elephant or in the caiman samples.

2,3-Dihydrofarnesol is a valuable compound used in perfumery.[38] The (*S*)-enantiomer was found to have a long-lasting graceful odor, in contrast to the (*R*)-enantiomer, which had a weak fragrance with a metallic side note. Similarly, animals including bumblebees are also likely to perceive the enantiomers with different sensitivity.

Compared to 2,3-dihydrofarnesol (**2**), 2,3-dihydrofarnesal (**3**) is much less common in natural sources. It was described in the scent of the orchid *Aerides jarckianum*, in the blossom fragrance of *Citrus limon*,[20] and the enantiomeric composition in these two samples was similar, 85:15 in favor of the (*S*)-enantiomer. Fresh juniper needles contain about 3% of 2,3-dihydrofarnesal.[39] Besides bumblebees, 2,3-dihydrofarnesal was reported as a component of a sex pheromone in *Ascogaster quadridentata*, a parasitoid of the codling moth, *Cydia pomonella*.[40] In bumblebees, 2,3-dihydrofarnesal was previously reported in *Bombus (Alpinobombus) hyperboreus*,[21] *B. (Pyrobombus) jonellus*, and *B. (Pyrobombus) cingulatus*.[41] We have now detected (*S*)-2,3-dihydrofarnesal in three species: *B. terrestris, B. jonellus*, and *B. impatiens* (Table 1). 2,3-Dihydrofarnesal usually accompanies 2,3-dihydrofarnesol in smaller amounts. Thus, their biosynthetic relatedness is obvious.

Biosynthesis of terpene insect pheromones has been thoroughly investigated in the order *Coleoptera*. It was shown in *Ips* species that acyclic monoterpene alcohols arise from monoterpene hydrocarbons by oxidation.[42] Monoterpenes can be built either by the classical isoprenoid pathway starting from mevalonate or by modification of the host dietary components. In the case of terpenes in the marking pheromones of bumblebees, we assume de novo biosynthesis starting from mevalonate, as the dietary conditions vary from colony to colony and from locality to locality and also depend on season. The final steps of the pathway are probably hydrolysis of diphosphate group in geranyldiphosphate or farnesyldiphosphate to alcohols followed by the enantioselective reduction of the double bond in position 2(3).[43] The formation of only one enantiomer, i.e., (*S*)-citronellol, (*S*)-dihydrofarnesol, and (*S*)-dihydrofarnesal, suggests the presence of only one highly selective oxidoreductase in the enzymatic system of bumblebees.

The importance of chirality is now well established in the chemical communication of organisms. Progress was made possible by advances in both analytical and synthetic organic chemistry. The development of chiral stationary phases for GC[44,45] simplifies the determination of the enantiomeric compositions of chiral compounds, especially when applied in a 2D GC-system for separating enantiomers in complex mixtures.[15,46–48] The absolute configuration of trace components of pheromones can now be determined and this knowledge used for further biological studies.

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