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(6*R*, 10*R*)-6,10,14-Trimethylpentadecan-2-one, a Dominant and Behaviorally Active Component in Male Orchid Bee Fragrances

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Abstract 6,10,14-Trimethylpentadecan-2-one (Hexahydrofarnesyl acetone; HHA) previously has been found to be a major component in tibial fragrances of male orchid bees, *Euglossa* spp. HHA is a chiral molecule with four possible stereoisomers, (6*R,* 10*R*)-, (6*R,* 10*S*)-, (6*S,* 10*R*)-, and (6*S,* 10*S*)-6,10,14-trimethylpentadecan-2-one. In the present study, we characterized HHA extracted from *Euglossa* as the pure enantiomer (6*R*, 10*R*)-6,10,14-trimethylpentadecan-2-one. During bioassays in Mexico and Panama, the synthetic *RR*-isomer attracted males of six species of orchid bees, including three that were known to contain HHA in their tibial fragrances. Possible sources of HHA for wild bees are flowers of euglossophilous orchids and aroids. With a molecular weight of 268, HHA is the largest natural molecule known to attract male orchid bees in pure form. Its attractiveness to males suggests that low-volatility compounds have a function in male signals, e.g., serve as a "base note" in complex odor bouquets.

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Introduction

Male orchid bees (Apinae, Euglossini) of the Neotropics collect volatile chemicals in their forest environment and combine them to complex blends in specialized hind tibial pouches (Vogel, 1966; Dodson et al., 1969; Eltz et al., 1999). These fragrances are exposed later by the males during lengthy courtship displays, presumably as signals to conspecific females (Eltz et al., 2005b). Although there is still no direct proof of a female response to the perfumes, the chemical specificity of male perfumes strongly suggests a pheromone-like recognition function (Eltz et al., 2005a; Ramírez et al., 2010). Across males of fifteen species of Panamanian *Euglossa*, the chemical composition of perfume blends consistently was more similar within than among species, with specific blends being characterized both by certain exclusive major compounds and characteristic compound proportions (Zimmermann et al., 2009). Many compounds were shared by several species, but normally they were quantitatively dominant in only a few. The prominent exception was 6,10,14-trimethylpentadecan-2-one, also known as hexahydrofarnesyl acetone (HHA), which was present in the perfume blends of all fifteen species and was a major component of nine species (Zimmermann et al., 2009). HHA is a derivative of the diterpene alcohol phytol, a widespread secondary compound of plants that is esterified to chlorophyll a (Willstätter and Stoll, 1913). HHA is found in floral scents of orchid species that are pollinated by male euglossines [R. Kaiser and M.

Whitten (Givaudan, Switzerland) personal communication], suggesting that it may be a behaviorally active compound that is collected actively by males. This has remained speculative, though, since HHA failed to lure male orchid bees in previous bioassays (M. Whitten and G. Gerlach personal communication; T. Eltz unpublished data.). However, HHA is a chiral compound, and the tested synthetic product was a mix of unknown isomeric composition (R. Kaiser personal communication), leaving room for further investigation. In the present study, we determined the stereochemistry of HHA in tibial extracts of three species of *Euglossa*. Furthermore, we synthesized an optically pure RR-HHA [(6*R*, 10*R*)-6,10,14-trimethylpentadecan-2-one] in addition to a mixture of the four possible optical isomers of HHA. Bioassays were conducted in southern Mexico and Panama to test behavioral attractiveness to male orchid bees.

Methods

Identification and Stereochemistry of 6,10,14-Trimethylpentadecan-2-one in Extracts of Euglossa Species Hexane extracts of pairs of hind legs of individual male *Euglossa imperialis*, *E. allosticta,* and *E. crassipunctata* (one each) were purified on a 500 mg Strata SI-1 Silica Teflon coated solid phase column with stepwise gradient elution using 1–11% ethyl acetate in pentane in steps of 1%. An elution and collection volume of 750 µl for each fraction was used, with twice the volume for fractions 4–6. These fractions, containing the ketone, were combined and evaporated to dryness under a stream of argon at room temperature, and then were redissolved in 1 ml of pentane and washed with 200 µl of 0.2 M KOH. The organic layer was collected and dried with sodium sulfate before it was evaporated to dryness under a stream of argon at room temperature and finally redissolved in 50 µl cyclohexane.

The ketone in the purified extract was reduced to alcohol with lithium aluminum hydride according to standard method, and then was derivatized with (R)-(+)-trans-chrysanthemoyl chloride (Brooks et al., 1973). The diastereomers were analysed with a Hewlett-Packard 6890N gas chromatograph (GC) with a polar Varian factorFOUR VF-23ms column (30 m×0.25 mm i.d., d_f=0.25 µm) and an HP 5973 mass spectrometer (GC-MS) in SIM mode (m/z=123, 124, 153, and 168). The carrier gas (1 ml/min) was helium; 1 µl of the sample was injected splitless, the injector temperature was 250°C, and the aux temperature was 280°C. The column temperature was increased from 50°C by 10°C/min up to 110°C, from 110°C by 0.01°C/min up to 115°C, and from 115°C by 10°C/min up to 230°C.

Identification was made by comparing the retention times and mass spectra of the unknown alcohol derivatives with a reduced and derivatized synthetic reference mixture of all four stereoisomers of 6,10,14-trimethylpentadecan-2-one and also with a derivatized synthetic reference mixture of (2*S*,6*R*,10*R*)- and (2*R*,6*R*,10*R*)-6,10,14-trimethylpentadecan-2-ol. Reduction of the ketone to the alcohol creates a new stereogenic center at the carbon with the formed hydroxyl group, which is disregarded when the stereochemistry of the original ketone is determined. Identification also was made by comparing the GC/infra red (GC/FT-IR) spectrum of the ketone in the extract with that of the synthetic ketone.

Chemicals Commercially available chemicals were used without further purification. Stereoisomerically pure (*R*,*R*)-phytol was purchased from TCI America. Preparative liquid chromatography was performed on normal-phase silica gel (Merck 60, 230–400 mesh, 0.040–0.063 mm, 10–50 g/g of product mixture) employing a gradient technique with an increasing concentration (0–10%) of distilled ethyl acetate in distilled cyclohexane. Progress of the reaction was monitored with thin layer chromatography on silica gel plates (Merck 60, precoated aluminium foil) using ethyl acetate (40%) in cyclohexane as an eluent. Plates were visualized with ultraviolet irradiation and/or by spraying with vanillin in sulfuric acid and heating at 120°C. Purity of the product was checked by GC on a Varian 3300 GC instrument using a capillary column (EC-1, 30 m×0.32 mm i.d, d_f=0.25 µm, with nitrogen as carrier gas and a split ratio of 1:20). The temperature was programmed for 2 min at 100°C followed by 10°C /min up to 300°C. GC-MS analyses were carried out on a Saturn 2000 GC/MS/MS instrument with a Varian 3800 GC instrument, using a capillary column (CP-Sil 5 CB, 30 m×0.32 mm i.d, d_f=0.25 µm, carrier gas helium, column flow = 1 ml/min, split ratio 1:20) and the temperature program described above. GC/FT-IR analysis of the natural and synthetic compounds was carried out on a Thermo Scientific Nicolet 6700 FT-IR spectrophotometer, coupled via an GC/FT-IR interface to an Agilent 7890A GC with a polar Varian factorFOUR VF-23ms column (30 m×0.25 mm i.d., d_f=0.25 µm). The carrier gas (1 ml/min) was helium; 1 µl of the sample was injected splitless, the injector temperature was 250°C, and the transfer line and flow cell temperature were both set at 250°C. The column temperature was increased from 50°C by 10°C/min up to 230°C, and kept at 230°C for 10 min. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 (500 MHz ^{1}H, 125.8 MHz ^{13}C) spectrometer using $CDCl_3$ as solvent and TMS as internal standard.

Oxidizing phytol with $NaIO_4$ and a catalytic amount of $RuCl_3$ (Sasaerila et al., 2003) gave pure (6*R*/*S*,10*R*/*S*)-6,10,14-trimethylpentadecan-2-one in near quantitative yield. Synthesis of (6*R*, 10*R*)-6,10,14-trimethylpentadecan-2-one was performed using the same method from stereoisomerically pure (*R*,*R*)-phytol.

Bioassays The *RR*-HHA and the 1:1:1:1 stereoisomeric mixture of HHA were dissolved at 100 mg/ml in pentane (Uvasol, Merck, Germany). Bioassays were conducted on 2 days in two different forest localities (near Palenque and near Lancanja, Chiapas) in southern Mexico in September 2008, and on 3 days in one forest locality in Central Panama (Barro Colorado Island) in May 2009. Aliquots of 50 μl of test solutions and solvent controls were pipetted onto filter papers (Whatman 1, 2.5 cm) pinned to trees at breast height. Filter papers were observed between 0900 to 1200 and refreshed once or twice during the morning with additional aliquots of 50 μl of test solution or solvent. We routinely exposed other synthetic chemical bait compounds (e.g., 1,8-cineole, methyl salicylate, *p*-dimethoxybenzene) in the vicinity. Those were of much higher absolute concentration than the HHA lures and at least 5 m away from them. Males were only counted as having visited a HHA lure when they had landed on the respective filter paper and performed characteristic volatile collecting behavior.

Results

HHA in hind leg pouches of male *Euglossa imperialis*, *E. allosticta*, and *E. crassipunctata* is optically pure (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-one, based on comparison with synthetic reference mixtures of known stereoisomeric composition (Figs. 1 and 2).

Isomerically pure *R*,*R*-phytol and a 1:1:1:1-mixture of phytol stereoisomers, respectively, were converted to stereoisomerically pure (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-one and a 1:1:1:1-mixture (6*R*/*S*,10*R*/*S*)-6,10,14-trimethylpentadecan-2-one, both in yields of 98% and purities of >99%. All analytical data were in accordance with those previously reported (Suga et al., 1989; Nam et al., 2007; Zhao et al., 2007; Kalinová et al., 2009).

In bioassays both the (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-one and the stereoisomeric 1:1:1:1-mixture attracted male euglossine bees in southern Mexico and Panama (Table 1), whereas solvent controls were not attractive. Overall, the (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-one attracted more individuals and species than the 1:1:1:1-mix. Generally, the HHA lures attracted relatively few bees in comparison with other synthetic compounds that were exposed at the same time (data not shown). On several occasions, a male *E. imperialis* first was attracted to a 1,8-cineole bait and subsequently visited a HHA filter paper.

Discussion

Our analytical results demonstrate that HHA in male *Euglossa* hindleg pouches is (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-one. This is in agreement with HHA being chemically derived from phytol [(2E,7*R*,11*R*)-3,7,11,15-tetramethyl-2-hexadecen-1-ol] from source plants. We further show that (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-

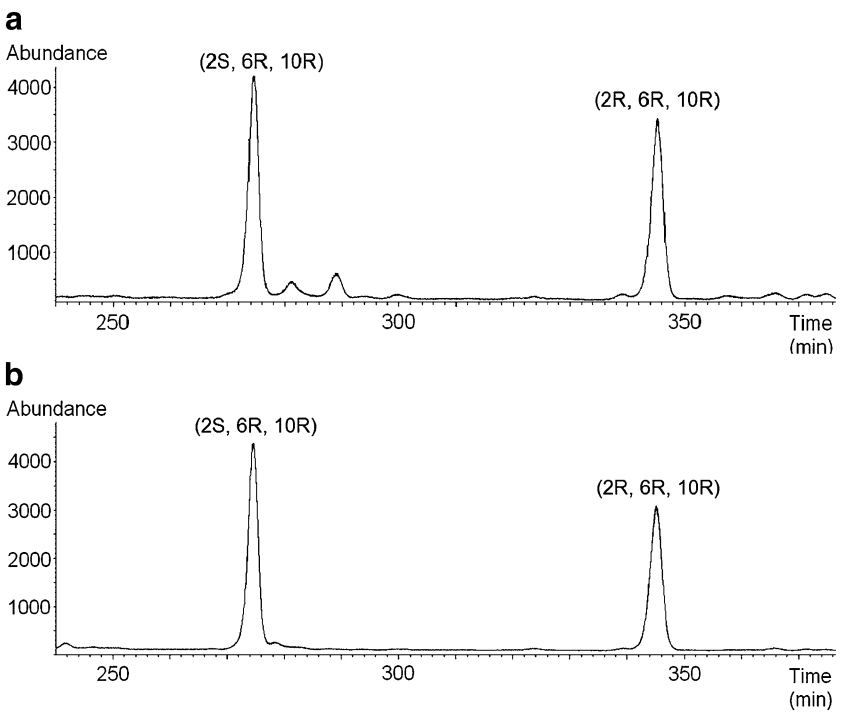

Fig. 1 **a** GC-MS chromatogram of a derivatized synthetic mixture of (2*S*,6*R*,10*R*)- and (2*R*,6*R*,10*R*)-6,10,14-trimethylpentadecan-2-ol. **b** GC-MS chromatogram of a reduced and derivatized extract from *Euglossa crassipunctata*. Two peaks appear due to an extra stereogenic centre created by the reduction of the ketone to alcohol. Extracts from *E. imperialis* and *E. allosticta* show the same result

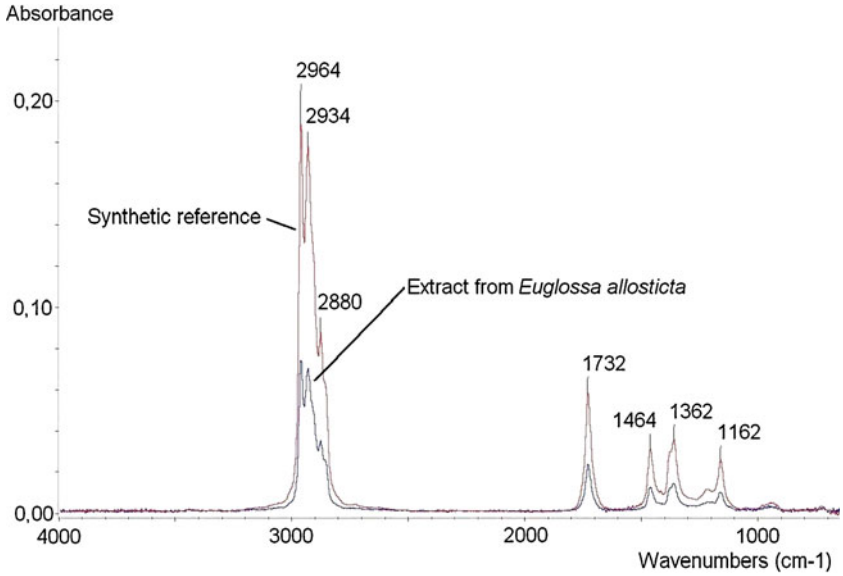

Fig. 2 GC/FT-IR spectra of synthetic (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-one and extract of *Euglossa allosticta*

one is a behaviorally active component for males of at least six species of euglossine bees that actively approached and collected the compound. Probably, extended bioassays would further extend the list of responsive taxa, especially with regard to rarer euglossine species. However, it should be noted also that our bioassays in Panama failed to attract *Euglossa despecta*, a species that has large quantities of HHA in its hindlegs (Zimmermann et al., 2009) and that was common at other chemical baits at the time of the study. Generally, HHA did not attract as many male bees as did some of the traditional synthetic bait compounds such as 1,8-cineole, methyl salicylate, or *p*-dimentoxybenzene. This may in part be explained by the relatively lower concentration of HHA in our bioassays, but probably also by its low volatility. HHA has a molecular weight of 268, whereas the mentioned other attractants have molecular weights around 150. In fact, HHA is the largest natural molecule known to date that attracts male orchid bees in pure form (Williams and Whitten, 1983; Ramírez et al., 2002). Its attractiveness to males, along with the recent discovery of other male-attracting semivolatiles in euglossine fragrances (Eltz et al., 2008; Ramírez et al., 2010), suggests that these larger and less volatile compounds are functionally important in the male signal. On one hand, they might act as a matrix or fixative for more volatile components such as mono- and sesquiterpenes (Eltz et al., 2007). On the other hand, they might represent the characteristic core of the fragrance signal, which is ornamented with more volatile but ubiquitous additives. In other words, the large and heavy components may represent the "base note" of a complex and multi-layered male odor.

Table 1 Number of males of different species of orchid bees visiting filter papers impregnated with either (6*R*,10*R*)-6,10,14-trimethylpentadecan-2-one or a 1:1:1:1-mixture of (6*R*/*S*,10*R*/*S*)-6,10,14-trimethylpentadecane-2-one at equal (overall) concentration. Solvent (pentane) controls attracted no bees at all. Data were pooled across localities/dates for Mexican and Panamanian bioassays

	RR-isomer	1:1:1:1-mixture
Mexico		
Euglossa imperialis	22	13
Euglossa hemichlora	1	
Euglossa obtusa	1	
Panama		
Euglossa imperialis	22	3
Euglossa hemichlora	4	
Euglossa igniventris	1	
Exaerete frontalis	1	
Eufriesea corusca	1	

Little is known about the natural source(s) of HHA for male euglossine bees. Among the many floral scents and essential oils analyzed by R. Kaiser (personal communication), HHA is relatively widespread, but occurs normally only as a minor component. Notable exceptions are a small number of euglossophilous orchids, in which HHA is the dominant compound found in the floral headspace (>50% of peak area): *Polycycnis ornata, Kegeliella kupperi*, *Acineta alticola*, and *Soterosanthus shepheardii* (R. Kaiser and M. Whitten personal communication). However, it is doubtful whether any of these orchids would be present in sufficient population densities to represent a significant HHA source for orchid bees. The outstanding abundance of HHA in male fragrances (Zimmermann et al., 2009) suggests rather that HHA sources are highly available, i.e., not orchids. Euglossine-pollinated aroids seem more likely candidates. HHA represented about half of the

volatiles in the headspace of inflorescences of *Anthurium thrinax*, an euglossophilous aroid from French Guyana (Hentrich et al., 2010). Among the male *Euglossa* visiting this plant for fragrance collection, the majority were *Euglossa hemichlora* (Hentrich et al., 2010), a widespread species that also collected HHA in southern Mexico and Panama (Zimmermann et al., 2009, this paper). *Anthurium thrinax* is restricted to the Guianas; other euglossophilous congeners occur in Central America (Dressler, 1968), but their floral scent has not been investigated.

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