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Determination of the Absolute Configuration of Female-Produced Contact Sex Pheromone Components of the Longhorned Beetle, *Neoclytus acuminatus acuminatus* (F)

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Abstract Cuticular hydrocarbons play important roles in contact chemical communication in insects. Many of these compounds are methyl-branched hydrocarbons with one or more chiral centers, which can exist in two or more stereoisomeric forms. Although the importance of chirality for the volatile semiochemicals that insects use for long-range communication is well established, almost nothing is known about the role of chirality in insect contact chemoreception. Here, we used reverse phase high performance liquid chromatography (RP-HPLC) and digital polarimetry to isolate and determine the absolute configuration of a component of the female-produced contact sex pheromone of the cerambycid beetle, *Neoclytus acuminatus acuminatus* (F.). The pheromone consists of 7-methylpentacosane (7-MeC_{25}), 7-methylheptacosane (7-MeC_{27}), and 9-methylheptacosane (9-MeC_{27}). The absolute configuration of the most abundant pheromone component, 7-MeC_{25}, was found to be (*R*). We then utilized enantiomerically pure synthetic pheromone components to test the hypothesis that males would respond more strongly to (*R*)- than to (*S*)-enantiomers of the three pheromone components. We also tested blends of (*R*)-7-MeC_{27}, the most bioactive component, with the (*S*)-enantiomers of the minor components and vice versa to determine if unnatural stereoisomers might decrease behavioral responses. Males responded most strongly to solvent-washed females treated with the blend of (*R*)-pheromone components, and to a lesser extent to (*R*)-7-MeC_{27} alone. A blend of (*R*)-7-MeC_{27} with the (*S*)-minor components elicited an intermediate response. Together, these findings suggest that the insects can discriminate the absolute configuration of the major and minor pheromone components, and that the configuration of all three components is likely to be (*R*).

Keywords Cuticular hydrocarbon · Contact sex pheromone · Stereochemistry · Chirality · Coleoptera · Cerambycidae

Introduction

The wax layer on the surface of the insect cuticle is composed of a complex mixture of involatile organic compounds, including long-chain alkanes, alkenes, and minor polar components such as aldehydes, aliphatic alcohols, fatty acids, ketones, and triglycerides, which function primarily to protect the insect from desiccation (Gibbs 1998). Cuticular hydrocarbons (CHCs) also can act as chemical signals that have diverse functions in insects (Howard and Blomquist 2005). Hydrocarbon contact sex pheromones are known from several insect orders including Diptera (Carlson et al. 1998a; Stoffolano et al. 1997; Wicker-Thomas 2007), Hymenoptera (Böröczky et al. 2009; Kühbandner et al. 2013; Steiner et al. 2006; Syvertsen et al. 1995), and Coleoptera (Geiselhardt et al. 2009; Ginzel 2010; Silk et al. 2009; Sugeno et al. 2006). Many of these contact semiochemicals contain methyl branches, which form chiral centers. Thus, each of these compounds can exist in two or more stereoisomeric forms, depending on the number and position(s) of these branch points. The absolute configurations of these compounds have been determined in only a few cases (e.g., Bello et al. 2015), despite the large number of

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methyl-branched hydrocarbons that have been identified in CHC extracts of insects.

Although there is an extensive literature on the critical importance of chirality for volatile insect pheromones (Ando and Yamakawa 2015; Birch et al. 1980; Levinson and Mori 1983; Mori 2007), few studies have investigated the effect of chirality in contact chemoreception. In one of the few examples reported to date, male *Tetropium fuscum* (F.) responded more strongly to solvent-washed female carcasses treated with (*S*)-11-methylheptacosane ((*S*)-11-MeC_{27}), the major contact sex pheromone component of females, than to the (*R*)-enantiomer, whereas both (*S*)-11-MeC_{27} and (*Z*)-9-heptacosene were required to elicit a full mating response from male *Tetropium cinnamopterum* Kirby (Silk et al. 2011). In the parasitic wasp *Lariophagus distinguendus* Förster, males did not respond when 3-methylheptacosane (3-MeC_{27}) was presented alone on a solvent-washed cadaver, but rather, the entire suite of CHCs was required in conjunction with the pheromone to elicit a response (Kühbandner et al. 2012). Moreover, males responded to both (*R*)- and (*S*)-3-MeC_{27} when applied to cadavers with the other CHCs (Kühbandner et al. 2012, 2013), suggesting that both enantiomers were being perceived, and both elicited similar behavioral responses. Likewise, male *Psacothea hilaris* Pascoe responded as strongly to both (8*Z*, 21*R*)- and (8*Z*,21*S*)-21-methylpentatriacont-8-ene, demonstrating no preference for either enantiomer, although they clearly distinguished the (*E*)- from the (*Z*)-stereoisomers (Fukaya et al. 1997). (3*S*,11*S*)-3,11-Dimethylnonacosan-2-one, the contact sex pheromone of female German cockroaches, *Blatella germanica* (L.), stimulated courtship behaviors in males (Eliyahu et al. 2004), but unexpectedly, at physiological doses the natural (*S*,*S*)-enantiomer was the least effective of the four possible stereoisomers at eliciting courtship behaviors. Overall, these and other studies suggest that the influence of chirality on the bioactivity of contact sex pheromones varies among insect species, and that combinations of CHCs may be necessary to stimulate courtship and mating behaviors.

Here, we investigated the effect of chirality on the responses of males of the cerambycid beetle *Neoclytus acuminatus acuminatus* (F.) to CHC components of the contact sex pheromone of females. Mate location in *N. a. acuminatus* consists of mutual attraction of both sexes to host plant volatiles and a volatile, male-produced aggregation pheromone, and at close range, mate recognition by males via a contact sex pheromone on the cuticle of females (Ginzel and Hanks 2005; Lacey et al. 2004, 2008). The volatile aggregation pheromone, produced from glands in the prothorax of males, has been identified as (2*S*,3*S*)-2,3-hexanediol (Lacey et al. 2004). The (2*R*,3*R*)-enantiomer was not attractive, nor did it inhibit responses to (2*S*,3*S*)-2,3-hexanediol when the racemic mixture was used as a trap bait. However, a blend of all four stereoisomers was significantly less attractive, indicating that one or both of the diastereomeric (2*R*,3*S*)- or (2*S*,3*R*)-2,3-hexanediols inhibit attraction (Lacey et al. 2004). Thus, these beetles are clearly capable of discriminating among the stereoisomers of their volatile aggregation pheromone.

The contact sex pheromone of female *N. a. acuminatus* consists of three chiral CHCs: 7-methylpentacosane (7-MeC_{25}), 7-methylheptacosane (7-MeC_{27}), and 9-methylheptacosane (9-MeC_{27}) (Lacey et al. 2008). In this species, the most abundant pheromone component is 7-MeC_{25}, but the most bioactive component appears to be the much less abundant 7-MeC_{27} — henceforth referred to as the major component. In the study by Lacey et al. (2008), which used racemic compounds, only 10 % of tested males attempted to mate with solvent-washed female cadavers treated with the racemate of the major component, 7-MeC_{27} (Lacey et al. 2008). However, 40 % of males attempted to mate with solvent-washed cadavers treated with a blend of the three racemic compounds (Lacey et al. 2008), indicating additive or synergistic effects between the components. The relatively low level of responses obtained even with the blend, in contrast to responses to freeze-killed females, suggested that the unnatural enantiomers present in the racemic synthetic pheromone components might have inhibited the responses of males to solvent-washed female cadavers treated with mixtures of these compounds. However, the lack of efficient syntheses of the pure enantiomers of methyl-branched hydrocarbons, and the difficulty in determining the configurations of the stereoisomers present in insect CHCs, hindered further investigation into the role of chirality in the functions of these contact pheromones. Methodology recently has been developed that allows the determination of the absolute configurations of at least the most abundant methyl-branched components in CHC extracts (Bello et al. 2015). This study found that 36 methyl-branched CHCs isolated from 20 different insect species all possessed the (*R*)-configuration, suggesting that these compounds are produced via biosynthetic pathways that are highly conserved among insects. In the present study, we exploited this newly developed methodology to isolate the most abundant methyl-branched hydrocarbon component of the contact sex pheromone of female *N. a. acuminatus*, and determined its absolute configuration to be (*R*). We also synthesized and tested the biological activities of each of the two enantiomers of the three methyl-branched hydrocarbon components of the contact pheromone in bioassays with male *N. a. acuminatus*.

Methods and Materials

Source of Beetles Live *Neoclytus a. acuminatus* were collected from flight intercept panel traps (Alpha Scents Inc., West Linn, OR, USA) baited with 5 × 7.5 cm resealable polyethylene

bags (Fisher Scientific, Waltham, MA, USA) containing 50 mg of racemic *syn*-2,3-hexanediol (synthesized as described in Lacey et al. 2004) in 1 ml of 2-propanol. Traps were deployed at Martell Forest, a mixed hardwood forest in Tippecanoe Co., IN, USA and mixed hardwood forests in Champaign, IL, USA during July–September 2013 and July–August 2014. *Neoclytus a. acuminatus* infest a wide variety of hardwoods, but most commonly attack ash (*Fraxinus* spp.), oak, hickory, persimmon, and common hackberry (Solomon 1995). Additional beetles were reared from infested common hackberry logs (*Celtis occidentalis* L.) throughout both summers as described by Browne (1972). Beetles from Illinois were washed in hexane, and the resulting extracts were sent to J.E.B. and J.G.M. for isolation of individual CHCs and polarimetric analysis. Beetles captured or reared in Indiana were held in a greenhouse, in individual cylindrical screen cages with 9-cm glass Petri dishes covering the top and bottom. Each beetle was provided a feeder of 10 % sucrose solution in an 8-ml vial with a 4-cm long cotton dental wick (Patterson Dental, St. Paul, MN, USA). Feeders were replaced every 2–3 d. Only visually healthy and active beetles were used in bioassays.

Authentic Standards of Methyl-Branched CHC Enantiomers The enantiomers of the methyl-branched hydrocarbon pheromone components of *N. a. acuminatus* were synthesized as described in Bello and Millar (2013). All compounds were estimated to be >99 % enantiomerically pure because they were prepared from intermediates that were at least 99 % enantiomerically pure (98 % enantiomeric excess, by chiral stationary phase GC), with no chance of epimerization in subsequent steps leading to the final products. Furthermore, the final hydrocarbon products were purified by recrystallization to reduce further the amounts of enantiomeric impurities present.

Extraction of CHCs from Female *N. a. acuminatus* To remove CHCs from the cuticle, each female *N. a. acuminatus* was subjected to two successive washes in hexane. For each wash, the female was placed in a 4-ml vial and immersed in 2 ml of hexane (Avantor Performance Materials, Center Valley, PA, USA), vortexed for 2 min and placed in a sonic bath for an additional 2 min (Ginzel et al. 2003). The female was removed from the solvent while the vial was in the sonication bath to reduce the amount of hydrocarbons adhering to the cuticle. The first wash was used for analysis of CHCs (see below). Analysis by GC/MS showed that the second wash contained a minimal amount of CHCs, so it was discarded.

Preparation, Fractionation, and Analysis of Cuticular Extracts A composite extract of 170 *N. a. acuminatus* females in hexane was prepared and filtered to remove particulates, concentrated to dryness, and then reconstituted in 1 ml hexane (Optima grade, Fisher Scientific). The extract was loaded onto a liquid chromatography column containing 500 mg of $AgNO_3$ impregnated silica gel (10 % wt/wt, +230 mesh; Aldrich Chemical Co., Milwaukee, WI, USA), preconditioned by eluting with hexane. The column was eluted with 4 ml of hexane to recover alkanes, followed by 4 ml of 5 % (vol/vol) cyclohexene in hexane to elute alkenes, and finally 4 ml of diethyl ether to elute more polar components (Bello et al. 2015). Fractions were analyzed using a Hewlett-Packard (HP) 6890 gas chromatograph coupled to an HP5973 mass selective detector (Hewlett-Packard, now Agilent Technologies, Santa Clara, CA, USA). The GC/MS was equipped with a DB-17MS capillary column (25 m × 0.2 mm × 0.33 μm film thickness, J&W Scientific, Folsom, CA, USA) and run in full-scan mode with electron impact ionization (70 eV). Samples were analyzed with splitless injection at 280 °C, and a temperature program of 100 °C for 1 min, then at 5 °C/min to 280 °C, and held for 20 min. Compounds were identified from their mass spectra by the enhanced diagnostic fragments from cleavages on either side of methyl groups, and by retention indices relative to straight chain alkane standards (Carlson et al. 1998b).

The alkane fraction was concentrated to dryness, reconstituted in 10 ml of isooctane, and 4 g of 5 Å powdered, activated molecular sieves were added (100 mg of sieves per mg of sample). The resulting slurry was stirred overnight, then centrifuged, and the supernatant was removed. The remaining pellet was resuspended in 10 ml of isooctane and recentrifuged. The resulting supernatants were combined and filtered through a glass wool plug into a tared vial, and concentrated to obtain a fraction containing only methyl-branched hydrocarbons. This fraction was dissolved in 500 μl of ethyl acetate and fractionated on an Infinity 1220 HPLC coupled to a Model 380 evaporative light scattering detector (ELSD; Agilent Technologies). The HPLC was equipped with an Eclipse XDB-C18 reverse phase column (5 μm particles, 4.6 mm i.d. × 250 mm length; Agilent Technologies) and a 100-μl sample loop. Twenty 5-μl injections were made sequentially. The column oven was set to 50 °C, the ELSD nebulizer was set to 40 °C, and the evaporation temperature was set to 70 °C. The nitrogen gas flow through the ELSD evaporation chamber was set to 1.2 standard liters per min (SLM). The column was eluted with EtOAc/MeOH (35:65) at a flow rate of 1 ml/min. The eluent was split 80:20 between the fraction collector and detector, and fractions containing the most abundant component, 7-MeC_{25}, were collected between 10.80 min and 11.25 min. Each isolated fraction then was analyzed by GC/MS to determine purity. Fractions from sequential analyses were pooled in a tared vial, and concentrated for polarimetric analysis. The other two pheromone components, 7-MeC_{27} and 9-MeC_{27}, could not be isolated in sufficient purity and quantity for polarimetric analysis.

The specific rotation of the isolated 7-MeC_{25} was measured with an Autopol IV digital polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA) operated in high

accuracy specific rotation mode. The temperature of the polarimetric chamber was set to 25 °C, and the light source to 589 nm. 7-Methylpentacosane isolated from female *N. a. acuminatus* (0.8 mg, c = 0.32 g/100 ml) was dissolved in 250 μl of dichloromethane and transferred to a T-32 small volume optical sample cell (2.5 mm i.d. × 50 mm length; 250 μl volume; Rudolph Research Analytical.) Specific rotation values were measured 10 times and averaged. Specific rotations of the synthetic standards were taken in the same manner. The results of the polarimetric analysis are shown in Table 1.

Bioassays The mating behavior of many cerambycid beetles, including *N. a. acuminatus*, can be divided into four sequential steps (Ginzel et al. 2003; Ginzel and Hanks 2005; Lacey et al. 2008). Upon antennal contact with a female, a male will 1) stop walking, 2) align his body with the female, 3) mount the female, and 4) bend his abdomen to couple the genitalia. Bioassays measuring the mating responses of males to possible contact sex pheromone components were conducted during the normal diel activity period of the beetle between 1000 and 1800 h, in the laboratory at room temperature under fluorescent lighting. A female *N. a. acuminatus* was presented to a male in a covered 9-cm Petri dish arena lined with Whatman filter paper (GE Healthcare UK Limited, Buckinghamshire, UK). The male was allowed 10 min to respond to the female. If the male did not display any mating response (steps 1–4), he was excluded from the study. A female was presented to no more than 5 males on the same day, each in his own Petri dish arena. The female then was freeze-killed, and after thawing at room temperature for 10 min, was presented to each male again for 10 min or until step 4 mating behavior was observed. The strength of the response of the male to the female carcass was recorded (steps 1–4, or 0 for no response), and served as the positive control for each male's response in a subsequent bioassay testing a particular treatment.

The CHCs of the female were then removed as described above, and the carcass was left to air dry for 1 h, to allow the solvent to evaporate. The female carcass was presented to the same males individually for 10 min each, and the mating response was recorded. Lack of response by males indicated that signals responsible for mate recognition had been removed by the extraction treatment. If any male displayed mating behavior, the female was washed a third time, allowed to air dry, and presented again to the male. A third wash was necessary for six females. Response to the solvent-washed female carcass served as a negative control.

The enantiomers of each of the synthetic standards of the female contact sex pheromone components were mixed in 100 μl of hexane in amounts equivalent to one female equivalent (FE) of the individual components found on the cuticle of female beetles, i.e., 12 μg of 7-MeC_{25}, 3 μg of 7-MeC_{27}, and 6 μg of 9-MeC_{27} (Lacey et al. 2008). We tested the bioactivity of the individual enantiomers of the major pheromone component, 7-MeC_{27}, and then as mixtures with either the (*R*)- or (*S*)-enantiomers of the minor components (Table 2). We did not assay individual minor components or pairwise mixtures of the major component with a single minor component, because Lacey et al. (2008) found that racemic blends of these mixtures resulted in mating responses that were significantly weaker than those toward unwashed cadavers. Each mixture of 1 FE was gradually applied with a pipet over the entire surface of the solvent-extracted carcass. A full FE was used because *N. a. acuminatus* males responded to treated female carcasses after application of an average of 0.73 ± 0.18 FE in a previous study (Lacey et al. 2008). The solvent was allowed to evaporate for 1 h, after which the treated carcass was presented to individual males, and the graded response of each male to the treated carcass was recorded.

Over the course of the study, 157 males were tested against 42 females. Because these data are dependent and do not meet the assumptions of normality, a Wilcoxon signed-rank test was used to compare the strength of the response of males towards the freeze-killed, solvent washed, and synthetically treated females in Statistica version 12 (Statsoft, Inc. 2013). Separate analyses were performed for each synthetic treatment group and corresponding controls (six groups total; Table 2). Two planned comparisons were performed with a Wilcoxon signed-rank test: 1) the response of males to freeze-killed females (positive control) was compared to that elicited by synthetic pheromone treatment, and 2) the response of males to the solvent-washed female carcass (negative control) was compared to that elicited by synthetic pheromone treatment. A Bonferroni correction was applied, resulting in a significance level set at $P < 0.025$. For those treatments that were

Table 1 Specific rotations of 7-methylpentacosane (7-MeC_{25}) isolated from *Neoclytus acuminatus acuminatus* and of authentic standards of the two synthetic enantiomers. The negative specific rotation of the isolated pheromone component closely matches the specific rotation of the (*R*)-7-MeC_{25} standard

Compound	Amount (mg)	Concentration (g/100 mL)	Specific Rotation $[\alpha]_D^{25}$ ± SD (N = 10)
Isolated 7-MeC_{25}	0.8	0.32	- 0.29 ± 0.03
Synthetic (*R*)-7-MeC_{25}	8.9	3.56	- 0.26 ± 0.01
Synthetic (*S*)-7-MeC_{25}	9.2	3.71	+ 0.24 ± 0.01

Table 2 Behavioral responses of male *Neoclytus acuminatus acuminatus* toward solvent-washed carcasses of females treated with enantiomers of components of the contact pheromone, as individual components or blends

Treatment(s)	*N*	% of males responding per step in the behavioral sequence[a]			
		Step 1	Step 2	Step 3	Step 4[b]
(*R*)-7-MeC$_{27}$	26	38	23	23	23* b
(*R*)-7-MeC$_{27}$; (*R*)-7-MeC$_{25}$; (*R*)-9-MeC$_{27}$	30	77	73	73	73* a
(*R*)-7-MeC$_{27}$; (*S*)-7-MeC$_{25}$; (*S*)-9-MeC$_{27}$	26	62	62	62	54* ab
(*S*)-7-MeC$_{27}$	26	12	8	8	8
(*S*)-7-MeC$_{27}$; (*S*)-7-MeC$_{25}$; (*S*)-9-MeC$_{27}$	24	25	13	13	13
(*S*)-7-MeC$_{27}$; (*R*)-7-MeC$_{25}$; (*R*)-9-MeC$_{27}$	25	16	12	12	12

[a] Males display a sequence of behaviors upon antennal contact with a female: 1) the male stops walking, 2) aligns his body with the female, 3) mounts the female, and 4) bends his abdomen to couple the genitalia. Displayed as percentage of males responding to treated females.

[b] Response of males to females treated with synthetic compounds versus solvent-washed controls were first compared using Wilcoxon signed-rank test (*$P < 0.001$). Responses to bioactive treatments were then compared using the Kruskal-Wallis test followed by multiple comparisons of mean ranks; entries marked with different letters are significantly different; $P < 0.025$

significantly different from the solvent-washed control, a Kruskal-Wallis test followed by multiple comparisons were used to identify treatments that elicited a significant response from males, again applying a Bonferroni correction and a significance level of $P < 0.025$. (Statsoft, Inc. 2013). Bioassays were conducted from 15 July–10 September 2013 and 30 July–23 August 2014.

Results

Isolation and Polarimetric Analysis of the Most Abundant Pheromone Component of *N. a. acuminatus* The methyl-branched fraction of the cuticular hydrocarbon extract was fractionated by reverse phase high performance liquid chromatography (RP-HPLC) eluting with a completely nonaqueous solvent system of ethyl acetate/methanol (35:65), yielding 0.8 mg of the most abundant methyl-branched pheromone component, 7-MeC$_{25}$, in >96 % purity (Figs. 1A-D). The optical rotation of the isolated 7-MeC$_{25}$ was measured by digital polarimetry, giving a specific rotation of -0.29 ± 0.03. Comparison with the specific rotations of authentic standards of (*R*)-7-MeC$_{25}$ and (*S*)-7-MeC$_{25}$ confirmed that the 7-MeC$_{25}$ isolated from *N. a. acuminatus* was the (*R*)-enantiomer (Table 1).

Bioassays

We recorded the responses of male *N. a. acuminatus* toward 1) freeze-killed females (positive control), 2) solvent-washed cadavers (negative control), and 3) solvent-washed cadavers treated with various synthetic pheromone components. More males responded to the freeze-killed female controls than to any combination of synthetic pheromone components (Wilcoxon signed-rank test, $P > 0.025$, data not shown), indicating that males do not respond as strongly to the synthetic contact pheromone components as they do toward the unaltered profile of female beetles. No males responded to the solvent-washed female controls, so this negative control served as a baseline to test the extent to which males responded to the synthetic blends. Males did not respond significantly to any of the three treatments that included (*S*)-7-MeC$_{27}$ (Table 2). However, more males responded to (*R*)-7-MeC$_{27}$ whether alone or in combination with either (*R*)- or (*S*)-minor components than toward the solvent-washed controls (Wilcoxon signed-rank test, $P < 0.025$). Moreover, males displayed a greater response toward solvent-washed cadavers treated with (*R*)-7-MeC$_{27}$ in combination with the (*R*)-minor components than toward the major component alone (Table 2, $P < 0.05$). Solvent-washed female cadavers treated with (*R*)-major component combined with (*S*)-minor components did not differ from either (*R*)-7-MeC$_{27}$ alone ($P = 0.18$) or the combination of (*R*)-7-MeC$_{27}$ with (*R*)-minor components ($P = 0.71$).

Discussion

Our analytical and bioassay results show that the contact pheromone of female *N. a. acuminatus* is composed of the (*R*)-enantiomer of the most abundant component, 7-MeC$_{25}$, and our bioassay results suggest that the two other pheromone components, 7-MeC$_{27}$ and 9-MeC$_{27}$, also have the (*R*)-configuration. The bioassays also confirmed the roles of the two minor components, because the combination of all three components was more active than the major component alone. However, contrary to our expectations, the (*S*)-enantiomers were not entirely inactive or inhibitory; males displayed copulatory behaviors toward female carcasses treated with (*R*)-7-MeC$_{27}$ and the (*S*)-minor components comparable to those

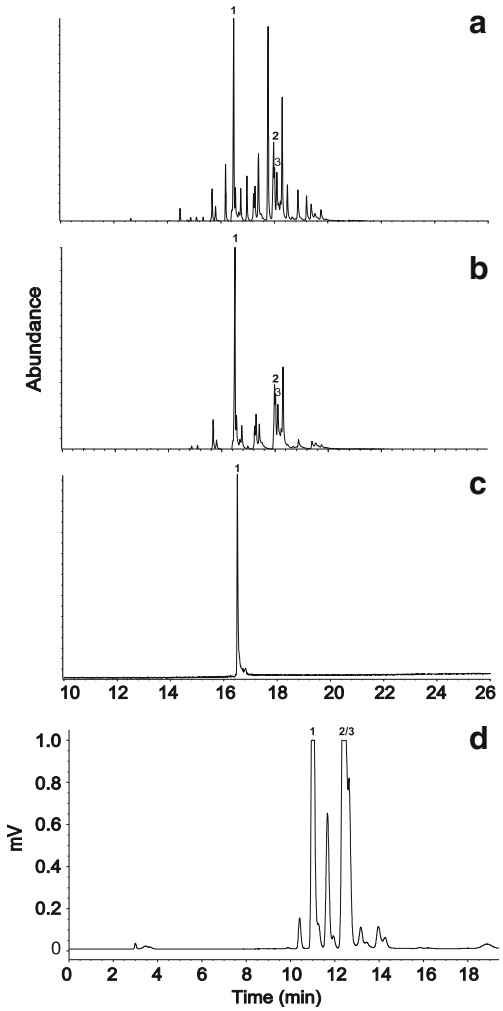

Fig. 1 Isolation of 7-MeC_{25} from CHC extracts of female *Neoclytus acuminatus acuminatus*. GC chromatograms of (*A*) the alkane fraction, (*B*) the methyl-branched alkane fraction after removal of *n*-alkanes with 5 Å molecular sieves, (*C*) isolated 7-MeC_{25} (96 % pure with a minor 3-MeC_{25} impurity); (*D*) HPLC-evaporative light scattering detection chromatogram of the methyl-branched alkanes fraction during the isolation procedure. The three contact pheromone components of *N. a. acuminatus*, 7-Me-C_{25}, 9-Me-C_{27}, 7-Me-C_{27} are labeled as compounds 1, 2, and 3 respectively on each chromatogram

displayed to the all-(*R*) blend (Table 2). The fact that the (*S*)-enantiomers were not inhibitory also explains the results from a previous study in which female carcasses treated with racemic blends of the pheromone components did indeed elicit copulatory responses from males, albeit at lower levels than the responses to freeze-killed females (Lacey et al. 2008). Overall, our results provide evidence that males of this species can distinguish the differences in the three-dimensional structures of the two enantiomeric forms of each pheromone component. The enantiomeric discrimination is most important for the major bioactive component of the contact pheromone, 7-MeC_{27}, because the unnatural (*S*)-7-MeC_{27} in a blend with the (*R*)-enantiomers of the two minor components did not elicit significant responses from males. Stereochemical variation in the minor components, 7-MeC_{25} and 9-MeC_{27}, also had an effect because activity of the blend with the (*S*)-enantiomers of both of the minor components was intermediate between all (*R*)-enantiomers together and the (*R*)-major component alone (Table 2). The addition of minor components to the (*S*)-major component did not affect its activity, regardless of the stereochemistry of the minor components.

None of the synthetic blend treatments in our study yielded a mating response from males as strong as that toward freeze-killed females (positive controls), and this reduced response may be an indication that other minor components are necessary for a complete and consistent mating response. In addition to the contact pheromone components, there are at least four other female-specific methyl-branched hydrocarbons in hexane extracts of female *N. a. acuminatus* (Lacey et al. 2008), and one or more of these compounds may produce further enhancements in the copulatory responses of males. Moreover, compounds that are not specific to females may also contribute to the activity of the contact pheromone. It also must be noted that many insects respond less strongly to a solvent-washed cadaver treated with crude cuticular extracts or synthetic compounds than toward an unextracted insect, suggesting that not only the composition, but also the arrangement and distribution of cuticular lipids may influence behavioral responses (Ginzel et al. 2006; Hughes et al. 2011; Rutledge et al. 2014; Silk et al. 2011). The arrangement of CHCs may be stratified within the cuticular wax layer of some cerambycids, with compounds that function as semiochemicals concentrated on the surface (Hughes et al. 2011). Solvent extraction and reapplication of the cuticular wax layer may scramble the CHCs, such that contact pheromones that are naturally abundant on the surface of the insect may become masked by other components of the wax layer, diminishing their bioactivity. This rearrangement of CHCs may explain the reduced response of males to solvent-washed females treated with cuticular extract, but it is unclear how stratification might affect male response to synthetic compounds. The hypothesis that the insect cuticular wax layer is stratified has yet to be empirically tested, and warrants further investigation.

To date, although there is evidence of contact chemoreception in over 20 species of longhorned beetles (reviewed in Ginzel 2010; Silk et al. 2011; Spikes et al. 2010), contact pheromone components have been identified for only a handful of species. The stereochemistry of contact pheromone components has been determined for only three other species: *T. fuscum* and *T. cinnamopterum* (Silk et al. 2011), and *Xylotrechus colonus* (Fabricius) (Bello et al. 2015). (*S*)-11-Methylheptacosane was reported to be a contact pheromone component in both *Tetropium* species, in contrast to the (*R*)-configurations of the pheromone components of *N. a. acuminatus* and *X. colonus*, and from the configurations of 36 insect-produced methyl-branched CHCs for which

absolute configurations have been determined, all of which were (*R*) (Bello et al. 2015).

In summary, the analytical and bioassay data from this study support the hypothesis that a large majority of insect-produced methyl-branched CHCs have the (*R*)-configuration (Bello et al. 2015). The bioassay data also suggest that male *N. a. acuminatus* can distinguish between the stereoisomers of methyl-branched CHCs, but that the unnatural (*S*)-enantiomers are not inhibitory, and can to some extent substitute for the natural (*R*)-enantiomers. It remains to be seen whether this will be a general phenomenon, or whether different species will exhibit different sensitivities and responses to the enantiomers of methyl-branched CHCs that have signaling functions as contact pheromone components.

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