DOI: 10.1111/j.1570-7458.2006.00508.x

Male-produced aggregation pheromone of the cerambycid beetle *Neoclytus mucronatus mucronatus*

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Accepted: 12 October 2006

Key words: sex pheromone, sex-specific prothoracic gland, (*R*)-3-hydroxyhexan-2-one, longhorned beetle, wood-boring insect, Coleoptera, Cerambycidae

Abstract

Adult male *Neoclytus mucronatus mucronatus* (F.) (Coleoptera: Cerambycidae: Cerambycinae) were observed to display behaviors identical to calling behaviors of the congener *N. acuminatus acuminatus* F., males of which produce an aggregation pheromone. Odors collected from male *N. m. mucronatus* contained one major male-specific compound, identified as (*R*)-3-hydroxyhexan-2-one. Bioassays determined that both sexes were weakly attracted to racemic 3-hydroxy-2-hexanone. Further field trials determined that enantiomerically enriched (*R*)-3-hydroxyhexan-2-one (94% ee) attracted more beetles of both sexes than did the racemic blend. This aggregation pheromone is produced by glands that discharge through pores lying within shallow cuticular depressions in the pronotum of male *N. m. mucronatus*.

Introduction

There is a growing body of evidence that male longhorned beetles of the subfamily Cerambycinae produce volatile pheromones (summarized in Lacey et al., 2004). In some species, the pheromones act as sex pheromones, specifically attracting females, whereas in other species, the pheromones have an aggregative function (Lacey et al., 2004). The pheromones are produced by specialized glands in the prothorax that connect to pores located in depressions in the cuticle (reviewed by Ray et al., 2006).

Release of pheromone by males of the cerambycine *Neoclytus acuminatus acuminatus* F. (Coleoptera: Cerambycidae) is associated with a characteristic calling behavior: males fully extend their front legs, elevating the head and thorax, and remain motionless for extended periods (ES Lacey, unpubl. data). Field and laboratory observations of the congener *Neoclytus mucronatus mucronatus* (F.) revealed that males often assumed an identical posture, suggesting that they also produce a volatile pheromone (Ginzel & Hanks, 2005). Moreover, the fact that both sexes of adult *N. m. mucronatus* commonly occur on larval hosts in mixed-sex groups (ES Lacey, pers. obs.) further suggests that an aggregation pheromone is involved. We report here on the identification of (*R*)-3-hydroxyhexan-2-one as the aggregation pheromone produced by male *N. m. mucronatus*, and also identify the location of pheromone-producing glands.

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Materials and methods

Natural history

Neoclytus mucronatus mucronatus is endemic to North America. The larvae infest woody tissues from stressed, moribund, and dead trees of numerous hardwood tree species (Linsley, 1964). Adults are present from mid-summer through autumn and are diurnal, being active from 12:00 until 17:00 hours (all behavioral information, ES Lacey, pers. obs.). Adults of both sexes mate soon after emergence, aggregating on larval hosts in groups that may exceed 40 individuals. Females begin ovipositing immediately after mating. Adults apparently do not feed, and usually live for fewer than 16 days in the laboratory.

Source of insects

Adult *N. m. mucronatus* used in laboratory bioassays and microscopy studies were collected from natural aggregations on larval hosts at the arboretum of the University of Illinois

at Urbana-Champaign (UIUC; Champaign Co., Urbana, IL, USA). Adults were caged individually in 0.3 m high cylindrical cages of aluminum window screen with plastic Petri dishes at top and bottom under ambient laboratory conditions (L12:D12, 20 °C, and 50% r.h.). Beetles used in bioassays were active and apparently healthy.

Testing for attractant pheromones

We tested for attractants using a horizontal glass Y-tube olfactometer (6 cm in diameter, main tube 26 cm long, arm length 22 cm, 70° angle between arms). Bioassays were conducted outdoors in partial shade because beetles were either sedentary or appeared agitated in the olfactometer under laboratory conditions. We bioassayed beetles between 13:00 and 20:00 hours on 14, 15, and 18 August 2004 (skies clear, air temperatures ~30 °C). We attached to each arm of the Y-tube a 2-l plastic chamber containing a cylinder of aluminum screen for a perch. One chamber held six males, and the other held six females. Ambient air was pulled through the olfactometer (air speed 2.5 m s^{-1}) with a 1.0 horsepower vacuum cleaner connected to a variable power supply. A beetle was released at the base of the Y-tube and allowed 10 min to respond to an odor source by crossing a line 18 cm down one arm. Beetles that did not respond within 10 min were recorded as 'no response'. Chambers were alternated between Y-tube arms every three trials to control for positional bias. Chambers and olfactometer were washed (Alconox® powder, Alconox, Inc., New York, NY, USA) and rinsed with acetone each day. We bioassayed 20 individuals of each sex and numbers of beetles responding to treatments were compared with a χ^2 goodness-of-fit test corrected for continuity (Sokal & Rohlf, 1995).

Identification of pheromone

We collected volatile compounds produced by adult *N. m. mucronatus* by placing five adult females and males in separate glass vacuum traps (0.3 l, custom manufactured by the glass shop, School of Chemical Sciences, UIUC) that were lined with aluminum screen to provide perches. A glass tube (6 cm long × 9.5 mm o.d. × 4 mm i.d.) containing 100 mg of 80/100 mesh SuperQ® (Alltech Associates, Deerfield, IL, USA) was attached to one nipple of each chamber with an 8-cm long section of Teflon® tubing (i.d. 9.5 mm; Nalge Nunc International, Rochester, NY, USA), and charcoal-purified air was pulled through the apparatus with a water aspirator at a rate of 1 l per min. Males and females were aerated simultaneously on a laboratory windowsill from 12:00 to 16:00 hours on 24 and 25 August 2004. We selected this time period for collecting volatiles from beetles because it was the only time during the day that males displayed the posture that appeared to be associated with release of pheromones (see Introduction). Collectors were eluted into silanized glass vials (Cat. no. 27114, Supelco®, Bellefonte, PA, USA) with three 0.5-ml aliquots of dichloromethane (CH_2Cl_2) and the resulting extracts were initially analyzed at UIUC with a Hewlett-Packard® (Sunnyvale, CA, USA) 6890 gas chromatograph (GC) interfaced to an HP 5973 mass spectrometer (MS), using an HP-5MS column (30 m × 0.25 mm × 0.25 µm film; J&W Scientific, Folsom, CA, USA) in splitless mode with helium-carrier gas. Injection port temperature was 250 °C and the oven was programmed from 40 °C for min, 10 °C per min to 300 °C, and held for 5 min. Gas chromatograph–mass spectrometer (GC-MS) analyses at University of California, Riverside, California initially were carried out with an HP-6890 GC fitted with a DB5-MS column (30 m × 0.25 mm, 25 µm film thickness; J&W Scientific) and programmed from 40 °C for min, 10 °C per min to 250 °C, held for 15 min, with an injector temperature of 250 °C. Injections were made in splitless mode. When it became clear that one or more of the compounds in the extract might be thermally unstable, the injector temperature was lowered to 100 °C, and the temperature program was changed to 20 °C for 2 min, 10 °C per min to 250 °C, held 15 min.

The absolute configuration of the insect-produced compound was determined by analysis of extract aliquots on a Cyclodex-B GC column (30 m × 0.25 mm, 0.25 micron film thickness, J&W Scientific) with the GC programmed from 50 °C for min, 5 °C per min to 200 °C, injector 100 °C, detector 200 °C. Identifications of peaks were confirmed by co-injections of extracts with authentic standards.

To determine the amount of pheromone produced by *N. m. mucronatus*, volatiles were collected from live male beetles as described above. The total weight of solvent in each extract was determined by transferring aeration extracts into tared vials and reweighing the vials. Total volume of each extract was then determined by dividing the mass by the density of the solvent. One microliter aliquots of each extract were analyzed by GC (as described above), and the total amount of pheromone in each extract was calculated by comparison with an external standard curve (2.5–250 µg ml^{-1}, regression $r^2 = 0.935$).

Synthesis of pheromone components

Tetrahydrofuran (THF) was distilled from sodium/benzophenone under argon atmosphere. ^1H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded with a Varian INOVA-400 (400 and 100 MHz, respectively) spectrometer, as $CDCl_3$ solutions. Chemical shifts are expressed in p.p.m. relative to $CDCl_3$ (7.26 and 77.23 p.p.m. for ^1H and ^{13}C NMR, respectively). Mass spectra were obtained with an HP 5890 GC interfaced to an HP

5970 mass selective detector, in electron ionization (EI) mode (70 eV) with helium carrier gas. The GC was equipped with a DB5-MS column (25 m × 0.20 mm i.d. × 0.33 μm film; J&W Scientific). Products were purified by flash or vacuum flash chromatography using silica gel (230–400 mesh, EM Science, Gibbstown, NJ, USA). Reactions with air- or water-sensitive reagents were carried out in oven-dried glassware under argon. Unless otherwise specified, solutions were dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation under reduced pressure. Lipase Amano PS was purchased from Amano Pharmaceutical Co. (Nagoya, Japan) and Lipase Amano AK (from *Pseudomonas fluorescens*) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Absolute configurations were determined using a chiral stationary phase GC column as described above.

Synthesis of racemic 3-hydroxyhexan-2-one

Racemic 3-hydroxyhexan-2-one was prepared in 74% yield from 2-methyl-1,3-dithiane by the method of Schröder et al. (1994), with the modifications that the crude final product was concentrated by removal of the solvent by fractional distillation with a Vigreux column rather than rotary evaporation because of the volatility of the hydroxyketone, and then purified by distillation (1.8 mm Hg, 36–39 °C) rather than by column chromatography. ^{1}H NMR, ^{13}C NMR, and EI mass spectra were in agreement with published spectra (Schröder et al., 1994).

Synthesis of (*R*)-3-hydroxyhexan-2-one

A mixture of racemic 3-hydroxyhexan-2-one (5.10 g, 43.9 mmol), vinyl acetate (11.34 g, 131.7 mmol), lipase Amano AK (1.275 g), and powdered 4Å molecular sieve (1.275 g) in pentane (90 ml) was stirred at 4 °C under argon. The reaction was periodically analyzed by chiral GC (Cyclodex B, see above) and stopped after 62 h, when the ee of the (*S*)-3-acetoxyhexan-2-one product started decreasing. The reaction mixture was filtered through Celite, which was rinsed with pentane. The solvent was distilled off through a Vigreux column and the residue was purified by vacuum flash chromatography, eluting with pentane/Et_2O – 9:1 until all the acetate had eluted, and then pentane/Et_2O – 2:1 to recover the unreacted hydroxyketone. Solvent was removed from fractions by distillation through a Vigreux column, and the products were Kugelrohr distilled [unreacted (*R*)-3-hydroxyhexan-2-one: bp 34–38 °C at 1.3 mm Hg, 1.62 g, 94% ee; 3-acetoxyhexan-2-one: bp 44–50 °C at 1.0 mm Hg, 3.94 g].

Synthesis of (*S*)-3-hydroxyhexan-2-one

Lipase Amano AK (0.76 g) was added to a well-stirred mixture of 3-acetoxyhexan-2-one (3.86 g) in phosphate buffer (0.1 M, pH 7.0, 114 ml) at room temperature under argon, following the reaction periodically on the Cyclodex B column. After 22.5 h, when the ee of the (*S*)-hydroxyhexan-2-one product started decreasing, the mixture was extracted with pentane/Et_2O – 1:1 (4 × 100 ml). The combined organic layers were washed with saturated sodium hydrogen carbonate and brine, dried, and the solvent was distilled off through a Vigreux column. The residue was purified by vacuum flash chromatography followed by Kugelrohr distillation as described above, yielding 2.02 g (*S*)-3-hydroxyhexan-2-one (93.8% ee) and 0.54 g recovered 3-acetoxyhexan-2-one.

Synthesis of a mixture of (*S*)-3-hydroxyhexan-2-one and (*S*)-2-hydroxyhexan-3-one standards

A solution of (2*S*,3*S*)-hexanediol (5 mg, 0.042 mmol) in dry CH_2Cl_2 (0.1 ml) was added dropwise to a stirred suspension of pyridinium chlorochromate (9 mg, 0.042 mmol) and powdered 4Å molecular sieve (9 mg) in CH_2Cl_2 (0.5 ml) at room temperature. After 3 h, dry Et_2O (3 ml) was added and the mixture was filtered through a pad of Celite and silica (1 cm of each, Celite on the bottom). The filtrate, containing a mixture of (*S*)-3-hydroxyhexan-2-one and (*S*)-2-hydroxyhexan-3-one, was analyzed by chiral GC. The same procedure was repeated for (2*R*,3*R*)-hexanediol to produce standards of (*R*)-3-hydroxyhexan-2-one and (*R*)-2-hydroxyhexan-3-one.

Testing release rates of pheromone components

To estimate the field longevity of cotton dental wicks (4 cm × 1 cm Ø; Patterson Dental Supply, Inc., St. Paul, MN, USA) that were used as release devices for the synthesized pheromone components, we prepared a mixture of (2*R**,3*R**)-hexanediol and racemic 3-hydroxyhexan-2-one (50 mg ml^{-1} of each in EtOAc). We included in this study racemic 2,3-hexanediol (available from previous work; Lacey et al., 2004) because such diols, and the hydroxyketones, apparently are common pheromones of cerambycine beetles (Lacey et al., 2004) and are likely to be bioassayed in future research. For each of the racemic materials, we placed 12 cotton wicks into individual one dram vials, and the wicks were loaded with 100 μl of the solution. Nine of the vials were placed outside on a sunny day, temperature 27 °C, underneath a cardboard top to avoid direct sunlight. The remaining three wicks served as controls and were kept in individual vials in a freezer at –20 °C (representing 0 h of exposure). Cotton wicks were retrieved (three at a time) after 4, 8, and 24 h, placed individually into 3 ml polypropylene syringe barrels and extracted with 10 ml of EtOAc containing 0.2 mg ml^{-1} of an internal standard (1-hexanol). The syringe plunger was used to express as much liquid as possible from the wicks after the solvent had drained through. The control wicks

were extracted in analogous fashion. The resulting extracts were analyzed by GC (50 °C for 2 min, 15 °C per min to 250 °C, hold at 250 °C for 5 min). For quantitation, it was assumed that the internal standard and the analyte produced equal responses from the flame ionization detector. Differences between treatments in amount of synthetic material remaining were tested by analysis of variance (ANOVA). To test differences between pairs of treatment means, we compared 95% confidence intervals (CI; lack of interval overlap is a conservative test of statistical significance; Payton et al., 2003).

Field bioassays of synthetic pheromone

We conducted a preliminary field bioassay of racemic 3-hydroxy-2-hexanone in an area adjacent to the UIUC arboretum and in proximity to a pile of freshly cut wood containing sectioned trunks and main branches of several tree species. Logs of hackberry, *Celtis occidentalis* L., and black walnut, *Juglans nigra* L., harbored a population of at least 30 adult *N. m. mucronatus* during the bioassay. We stapled sticky card traps ('mouse glue trap' cards, 12.6 × 22 cm baited, Victor® Pest Control Products, Lititz, PA, USA) to 1.5 m tall wooden stakes, with the middle of the card 1 m above the ground. A pair of cards was positioned 2 m apart at distances of 3 and 12 m upwind of the logs (n = 4 cards). One card of each pair was baited with a cotton dental wick that was attached to the middle of the card and loaded with 16 mg of 3-hydroxy-2-hexanone in CH_2Cl_2; the other card was baited with a control wick loaded with CH_2Cl_2 (treatment assigned randomly). We conducted the bioassay between 14:00 and 18:00 hours on 2 and 3 September 2004; skies clear, air temperature 27–30 °C, wind speed 8–20 kph. Traps and lures were replaced on the second day, with positions re-randomized. We compared total numbers of beetles responding to the treatment and control with the χ^2 goodness-of-fit test. Because these trials revealed that adult *N. m. mucronatus* could escape from sticky cards, in subsequent bioassays we used cross-vane flight-intercept panel traps (black, 80 × 30 cm, Intercept™, model PTBB, IPM Tech, Inc., Portland, OR, USA) that were more efficient at capturing beetles. We conducted bioassays at three sites to determine the optimum dose of synthetic pheromone for capturing adult *N. m. mucronatus*: the arboretum on the UIUC campus, a private tree-care company 9 km northeast of campus (adjacent to a pile of cut wood of various species), and Trelease Woods, a 25-ha mixed hardwood forest 11 km northeast of campus. At each site, we positioned five panel traps 10 m apart in a straight line and approximately perpendicular to the prevailing wind. Test chemicals were applied to cotton dental wicks in uncapped one dram vials that were hung in the open central slot of each trap. The bioassay included five dosage treatments that were randomly assigned to traps: a solvent control (500 µl of CH_2Cl_2), and 0.25, 1, 4, and 16 mg of racemic 3-hydroxy-2-hexanone in 500 µl of CH_2Cl_2. The bioassay was replicated 13 times between 12 and 19 August 2005; daily air temperatures 18–35 °C, variable cloud cover, negligible precipitation. For each replication, traps were set up at 14:00 hours, captured beetles were removed from traps the following morning, and the number and sex responding to treatments were recorded. Traps then were cleaned with glass cleaner (Windex®, S. C. Johnson and Sons, Inc., Racine, WI, USA) and treatments were re-randomized before each trial. Differences between treatments in total numbers of adult beetles captured were tested with the non-parametric Kruskal–Wallis test (PROC NPAR1WAY; SAS Institute, 2001) because zero values for some means violated assumptions of ANOVA (Sokal & Rohlf, 1995). To test differences between pairs of treatment means, we compared 95% CI. Replicated trials that captured fewer than two beetles in total were excluded from the statistical analysis (four of 13 replicates). Differences between numbers of female and male *N. m. mucronatus* captured were tested with the χ^2 goodness-of-fit test.

To determine whether the responses of adult *N. m. mucronatus* were affected by the 'unnatural' enantiomer of the pheromone, we conducted field bioassays with an experimental design identical to the previous bioassay, and at the same field sites, but with three traps in each replication. Treatments included a solvent control (500 µl of CH_2Cl_2), racemic 3-hydroxy-2-hexanone (8 mg), and enantiomerically enriched (*R*)-3-hydroxyhexan-2-one (4 mg, 94% ee). The bioassay was replicated eight times between 23 and 28 August 2005; daily temperatures 22–35 °C with variable cloud cover, negligible precipitation. Data were analyzed as described above. One replicated trial that captured no beetles was excluded from the analysis.

Location of pheromone glands

To confirm that pheromone glands were present in the prothorax of male *N. m. mucronatus* (see Introduction), we imaged the prothorax of one adult of each sex by scanning electron microscopy (SEM). Specimens were prepared for SEM by soaking overnight in a 4% Triton X100 solution (Sigma-Aldrich) to remove dirt and lipids. Carcasses then were dehydrated by sequential immersion in 70% and 95% EtOH for 5 min each. Residues of lipids were stripped by soaking specimens in mixed hexanes (Fisher Scientific, Fairlawn, NJ, USA) for 2 h and sonicating in hexanes for 30 s. Specimens were allowed to air dry. We attached specimens to an aluminum mount (25.4 mm diameter) with a double-sided adhesive carbon disc

(25 mm) and secured them with Flash-Dry™ silver paint (all materials from Structure Probe, Inc., West Chester, PA, USA). Specimens were coated with 6 μm of gold-palladium (Desk II TSC turbo sputter coater, Denton Vacuum, Moorestown, NJ, USA). Mounts were stored in a desiccator. We imaged specimens with a Philips XL30 environmental scanning electron microscope equipped with a field-emission electron gun (FEI Company, Hillsboro, OR, USA) at 5.0 kV. We examined the pronotum of each specimen for gland pores.

To confirm that pores in the prothorax of males (see Results) were associated with pheromone glands, we sectioned prothoraces of both sexes for light microscopy. One male and one female *N. m. mucronatus* were anesthetized with carbon dioxide and the entire prothorax was excised. Prothoraces were immediately transferred to cold fixative (0.5% paraformaldehyde and 2.5% gluteraldehyde in buffer of 0.1 M sodium cacodylate, 0.18 mM $CaCl_2$, and 0.58 mM sucrose, pH 7.3). Tissues in fixative were refrigerated for 3 h at 5 °C, rinsed in buffer, transferred to a 2% solution of osmium tetroxide in buffer, and refrigerated overnight. Fixed tissues were rinsed with buffer and dehydrated with serial dilutions of ethanol (50–100% in 10% steps, 15 min each), then subjected to three changes of propylene oxide followed by a 50:50 solution of propylene oxide and Medcast resin (Ted Pella Inc., Redding, CA, USA), then pure Medcast resin. After agitating overnight, tissues were oriented in molds along the midline of the body. Sections for light microscopy (1 μm) were cut from the Medcast block with a Reichert 0MU2 ultramicrotome (Reichert Microscope Services, Depew, NY, USA), mounted on glass slides, and stained with an aqueous solution of 1.0% sodium borate and 0.5% toluidine blue.

Voucher specimens of *N. m. mucronatus* have been submitted to the Insect Collection of the Illinois Natural History Survey (Champaign, IL, USA).

Results

Testing for attractant pheromones

In olfactometer bioassays, 17 females were attracted to odors emitted by live males, compared to only two that responded to odors of live females ($\chi^2 = 10.3$, d.f. = 1,19, $P<0.001$). Males showed a similar response, with 16 males responding to odors of live males, compared to only one responding to odors from live females ($\chi^2 = 11.5$, d.f. = 1,17, $P<0.001$). These findings confirmed that males produced a volatile compound that attracted both sexes.

Identification of pheromone

Initial GC-MS analysis of volatiles produced by male *N. m. mucronatus* using a GC injector temperature of 250 °C revealed a single major peak with a shoulder in the total ion chromatogram. This peak was absent in analogous samples from females. The mass spectrum of the peak was rather simple, with a base peak at m/z 55, and other fragments at m/z 43 (78% of base peak), 73 (53%), and 45 (26%). There were no other fragments above 15% of the base peak, and there was no obvious molecular ion. The compound was tentatively identified as 3-hydroxyhexan-2-one by comparison with the spectrum of an authentic standard from our library of cerambycine pheromone standards, and confirmed by matching retention times with the authentic standard. The shoulder on the major peak was identified as 2-hydroxyhexan-3-one, which has almost the same retention time on a DB5-MS column under the conditions used, but a markedly different mass spectrum, characterized by a base peak at m/z 43, and other large fragments at m/z 45 (87%), 71 (64%), 55 (59%), and 73 (36%). Because these two compounds are known to interconvert at higher temperatures (Leal et al., 1995), GC-MS analyses were repeated with a lower injector temperature and initial oven temperature (see Materials and methods). The reanalysis resulted in the virtual disappearance of the shoulder, indicating that males produced essentially pure 3-hydroxyhexan-2-one.

The absolute configuration of the insect-produced compound was determined to be (3*R*) by analysis on a chiral stationary phase Cyclodex-B column. The enantiomers were resolved to baseline, with the (3*R*)-enantiomer eluting first (see below). Furthermore, because the 3-hydroxyhexan-2-one and 2-hydroxyhexan-3-one enantiomers were all separated from each other on this column (see below), we were able to confirm that the extracts from male beetles contained almost exclusively (*R*)-3-hydroxyhexan-2-one, with only traces of both of the 2-hydroxyhexan-3-ones (<5% total), again suggesting that the latter compounds were artifacts generated either during collection and shipping of the extracts, or during analysis. In one extract with a particularly large amount of male-produced compounds, we also detected trace amounts of 2,3-hexanedione, and threo- and erythro-2,3-hexanediols, all at <1% of the (*R*)-3-hydroxyhexan-2-one peak. The identities of these compounds were confirmed by comparison of their retention times and mass spectra with those of authentic standards.

Under laboratory conditions, male *N. m. mucronatus* produced 120 ± 63 μg of (*R*)-3-hydroxyhexan-2-one during 4-h aeration periods (mean ± SD, range 32–166 μg perday, n = 4). This amount of pheromone probably was close to the total amount produced per day because pheromone collections had been conducted during the period (12:00–16:00 hours) when males adopted the posture associated with release of pheromone.

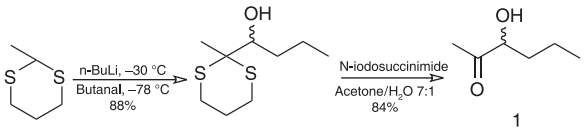

Figure 1 Synthesis of 3-hydroxyhexan-2-one **1**.

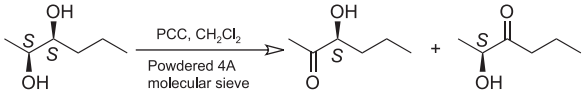

Figure 3 Synthesis of (*S*)-3-hydroxyhexan-2-one and (*S*)-2-hydroxyhexan-3-one.

Synthesis of pheromone components

Racemic 3-hydroxyhexan-2-one **1** was synthesized as previously described (Schröder et al., 1994, Figure 1). Briefly, 2-methyl-1,3-dithiane was deprotonated with n-BuLi in THF at –30 °C, followed by addition of butanal at –78 °C to give 2-(1-hydroxybutyl)-2-methyl-1,3-dithiane (88%). The dithiane protecting group was removed with N-iodosuccinimide to give racemic 3-hydroxyhexan-2-one **1** in 84% yield. Racemic 3-hydroxyhexan-2-one was then resolved by kinetic resolution with lipase enzymes, under both transesterification (Wood et al., 1999) and hydrolytic conditions (Ohtani et al., 1998). We tested two commercially available lipases (Amano PS and AK), for the first transesterification step. The enantioselectivity of the two lipases was similar, but the reaction with lipase AK was approximately three times faster. Thus, transacetylation of racemic 3-hydroxyhexan-2-one **1** with vinyl acetate and lipase AK in pentane at 4 °C gave a 64% yield (based on the *R* enantiomer) of unreacted (*R*)-3-hydroxyhexan-2-one in 94% ee (Figure 2). The enantio-enriched esterified product then was subjected to enzymatic hydrolysis using lipase Amano AK in 0.1 M phosphate buffer at room temperature and pH 7.0, giving (*S*)-3-hydroxyhexan-2-one in 79% yield (based on the *S* enantiomer) with 93.8% ee (Figure 2). To establish the absolute configuration of the kinetically resolved 3-hydroxyhexan-2-ones (2*S*,3*S*)-hexanediol and (2*R*,3*R*)-hexanediol, obtained by asymmetric dihydroxylation of (*E*)-2-hexene (Lacey et al., 2004), were partially oxidized with pyridinium chlorochromate to furnish the corresponding mixtures of (*S*)-3-hydroxyhexan-2-one/(*S*)-2-hydroxyhexan-3-one and (*R*)-3-hydroxyhexan-2-one/(*R*)-2-hydroxyhexan-3-one (Figure 3), respectively, for use as GC standards with the Cyclodex B column. The elution order for the four compounds was (*R*)-2-hydroxyhexan-3-one (retention time = 10.49 min), (*R*)-3-hydroxyhexan-2-one (10.56 min), (*S*)-2-hydroxyhexan-3-one (10.67 min), and (*S*)-3-hydroxyhexan-2-one (11.10 min) under the conditions used for the analyses.

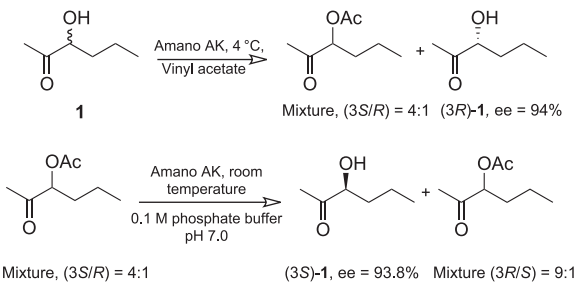

Figure 2 Enzymatic resolution of 3-hydroxy-hexan-2-one **1**.

Testing release rates of pheromone components

In the study to estimate the field longevity of cotton dental wicks that were used as release devices, the 3-hydroxyhexan-2-one was released very quickly, with >75% released in the first 4 h of exposure, and only 3% remaining after 24 h (Kruskal–Wallis test: $\chi^2 = 10.4$, d.f. = 3,4, P<0.5; Figure 4). In contrast, 2,3-hexanediol was released much more slowly, with 66 and 29% being recovered after 4 and 24 h, respectively (Kruskal–Wallis test: $\chi^2 = 9.4$, d.f. = 3,4, P<0.5; Figure 4).

Field bioassays of synthetic pheromone

In the first bioassay, sticky traps baited with the racemic 3-hydroxy-2-hexanone captured seven female and six male *N. m. mucronatus* over a 2-day period, whereas no beetles were caught on control traps (treatments significantly different; sexes combined: $\chi^2 = 11.1$, d.f. = 1,13, P<0.001; sex ratio not different from 1:1, $\chi^2 = 0.04$, d.f. = 1,13, P>0.05). Beetles of the wild population responded quickly

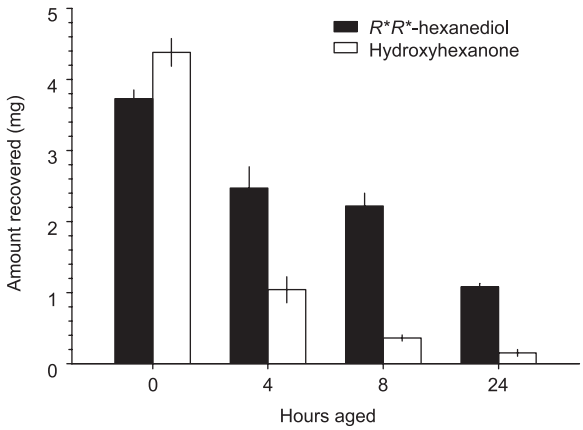

Figure 4 Amounts of (2*R**,3*R**)-hexanediol and 3-hydroxyhexan-2-one recovered from field-aged cotton wick release devices. Error bars represent 95% CI (n = 24).

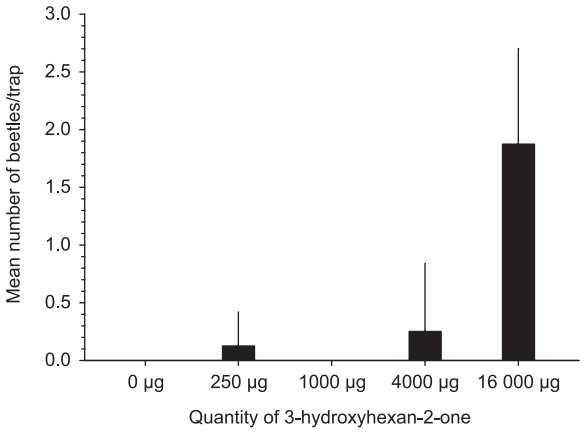

Figure 5 Relationship between mean number of adult *Neoclytus mucronatus mucronatus* caught in cross-vane panel traps (sexes combined) and dose of racemic 3-hydroxyhexan-2-one. Error bars represent 95% CI (n = 19).

to baited traps, with the first beetle arriving within 5 min of setting up the experiment.

In the subsequent test to determine optimal dosage, treatments differed significantly in the numbers of beetles captured with panel traps (sexes combined, Kruskal–Wallis: $\chi^2 = 21.9$, d.f. = 5,19, P<0.001; Figure 5), with the 16 000 µg treatment capturing more beetles than other treatments (indicated by lack of overlap of 95% CI with 0),

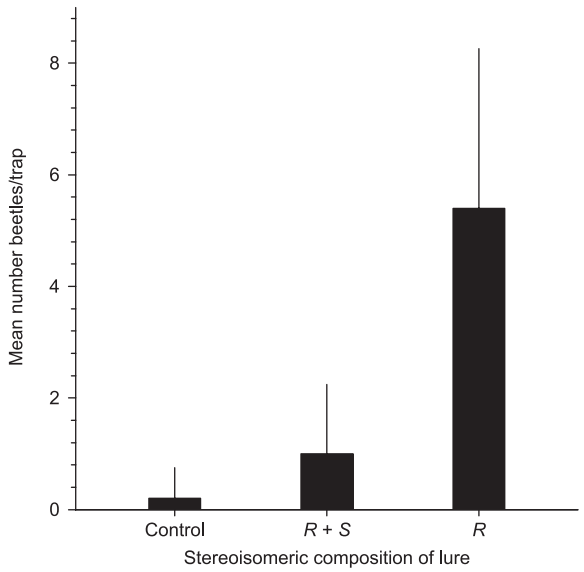

Figure 6 Relationship between mean number of adult *Neoclytus mucronatus mucronatus* caught in cross-vane panel traps (sexes combined) and stereoisomeric composition of the lure: solvent control, racemic 3-hydroxyhexan-2-one (*R* + *S*), and (*R*)-3-hydroxyhexan-2-one (*R*). Error bars represent 95% CI (n = 32).

whereas the 4000 µg dose was not different from the control. Traps captured 12 female and seven male *N. m. mucronatus* (sex ratio not different from 1:1, $\chi^2 = 1.36$, d.f. = 1,19, P>0.05).

In the bioassay comparing the attraction of beetles to racemic 3-hydroxyhexan-2-one vs. the (*R*)-enantiomer, traps baited with the enantiomerically enriched pheromone caught nearly five times as many beetles as did the racemic compound (treatments significantly different, Kruskal–Wallis: $\chi^2 = 10.8$, d.f. = 2,32, P<0.005; Figure 6). In this trial, and at the dosages tested, the racemic treatment was not different from the control. Traps captured 20 females and 12 males (sex ratio not different from 1:1, $\chi^2 = 1.54$, d.f. = 1,32, P>0.05).

Location of pheromone glands

Pores were present in cuticular depressions that were confined to sinuses on the pronotum of male *N. m. mucronatus* (Figure 7, top). Female *N. m. mucronatus* lacked sinuses on the pronotum, but had similar cuticular depressions that each contained a single central seta and no pores (Figure 7, bottom).

Histological sectioning of prothoraces yielded poor quality sections due to separation of cuticle from underlying tissues (not shown). Nevertheless, examination of sections revealed that prothoraces of males had clusters of cells underlying the cuticle of the pronotum that resembled the type III gland cells associated with pheromone glands of *N. a. acuminatus* (ES Lacey, unpubl. data). These cell clusters were absent in prothoracic sections from females.

Discussion

The attraction of both sexes of *N. m. mucronatus* to odors produced by live males in olfactometer bioassays provided the first evidence that males produce an aggregation pheromone. The fact that this was an aggregation pheromone and not a sex pheromone was confirmed during field bioassays with synthetic pheromone, in which statistically equivalent numbers of males and females were attracted to baited traps. To date, *N. m. mucronatus* and its congener *N. a. acuminatus* (Lacey et al., 2004) are the only cerambycid species definitely known to produce aggregation pheromones, although there is some evidence of attraction of beetles of both sexes by male-produced pheromones of a few other cerambycine species (see Iwabuchi et al., 1985; Nakamuta et al., 1997; Rhainds et al., 2001).

Location of mates by *N. m. mucronatus* apparently involves three sequential stages (see Ginzel & Hanks, 2005). First, both sexes are brought into the same habitat by their mutual attraction to volatiles emanating from larval hosts (Ginzel & Hanks, 2005). Second, the sexes are

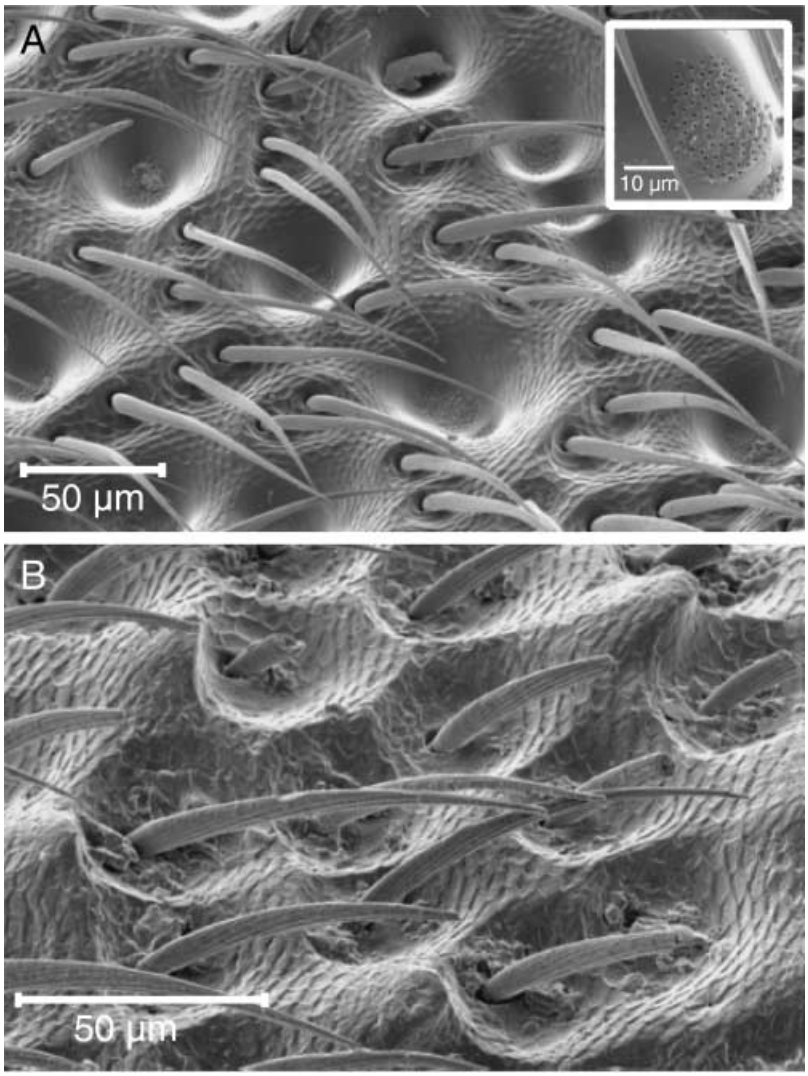

Figure 7 Scanning electron micrograph of the pronotum of adult *Neoclytus mucronatus mucronatus*. (A) male (inset, closeup of pore cluster); (B) female.

brought into proximity by the male-produced aggregation pheromone (*R*)-3-hydroxyhexan-2-one. Third, males recognize females by a contact pheromone in the cuticular wax layer (Ginzel & Hanks, 2003).

The structure of the pheromone of *N. m. mucronatus*, (*R*)-3-hydroxyhexan-2-one, is consistent with the diol/hydroxyketone structural motif of pheromones reported from another eight species of the Cerambycinae, with hydroxyl or carbonyl groups at C_2 and C_3 of six, eight, or 10 carbon chains (reviewed by Lacey et al., 2004). However, *N. m. mucronatus* and *N. a. acuminatus* are the only cerambycine species reported to date with pheromones comprised of a single component (Lacey et al., 2004). As with *N. a. acuminatus*, adult *N. m. mucronatus* responded most strongly to relatively large doses of synthetic pheromone in field bioassays. However, unlike *N. a. acuminatus*, which responded equally well to the naturally produced enantiomer or the racemic mixture of synthetic enantiomers (Lacey et al., 2004), adult *N. m. mucronatus* responded much more strongly to the natural (3*R*)-enantiomer of its pheromone than to the racemic mixture, indicating that the (3*S*)-enantiomer inhibited attraction.

The structures of glands and associated pores within cuticular depressions on the prothorax of male *N. m. mucronatus* were consistent with the pheromone glands of its congener *N. a. acuminatus* (ES Lacey, unpubl. data). Furthermore, a recent morphological survey of cerambycine species revealed analogous male-specific pores and glands in many species (Ray et al., 2006). In addition, the presence of single setae instead of pores within similar cuticular depressions on females is also consistent with findings from *N. a. acuminatus* and other cerambycines (Ray et al., 2006; ES Lacey, unpubl. data). These results strongly suggest that the sex-specific prothoracic glands

of *N. m. mucronatus* are indeed the source of the male-specific pheromone. Gland pores in *N. m. mucronatus* were confined to sinuses on the pronotum, whereas those of *N. a. acuminatus* were distributed more evenly over the entire surface of the prothorax (ES Lacey, unpubl. data). This morphological difference between such closely related species is another example of the variability in the pheromone-producing structures among cerambycine beetles (see Ray et al., 2006).

The stereotyped calling behavior exhibited by both *Neoclytus* species apparently serves to maximize dissemination rates of pheromone by positioning the pheromone-releasing area of the cuticle into convective air layers (ES Lacey, unpubl. data). To date, we have observed this behavior in males, but not females, of a number of other cerambycine species, including several species from which we have collected male-specific volatiles that probably are pheromone components (ES Lacey, unpubl. data). Thus, simple behavioral observations of male cerambycids may provide a reliable indicator as to whether or not a particular species uses volatile, male-specific pheromones.

Acknowledgements

We thank S. J. Robinson for help with SEM work, L. A. Miller for assistance with histological sectioning, and the UIUC Committee on Natural Areas and Doeble Tree Service, Urbana, IL, USA for access to research sites. The SEM research was made possible through collaboration with Bugscope, The Imaging Technology Group, Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign (http://bugscope.beckman.uiuc.edu/). We appreciate support from the Exotic/Invasive Pests and Diseases Research Program, University of California, under USDA-CSREES Grant no. 2004-34439-14691, and LMH gratefully acknowledges support from the Alphawood Foundation of Chicago, from the US Forest Service, agreement #03-JV-11231300-091. This work also was supported by Hatch Project #CA-R*ENT-5181H to JGM.

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