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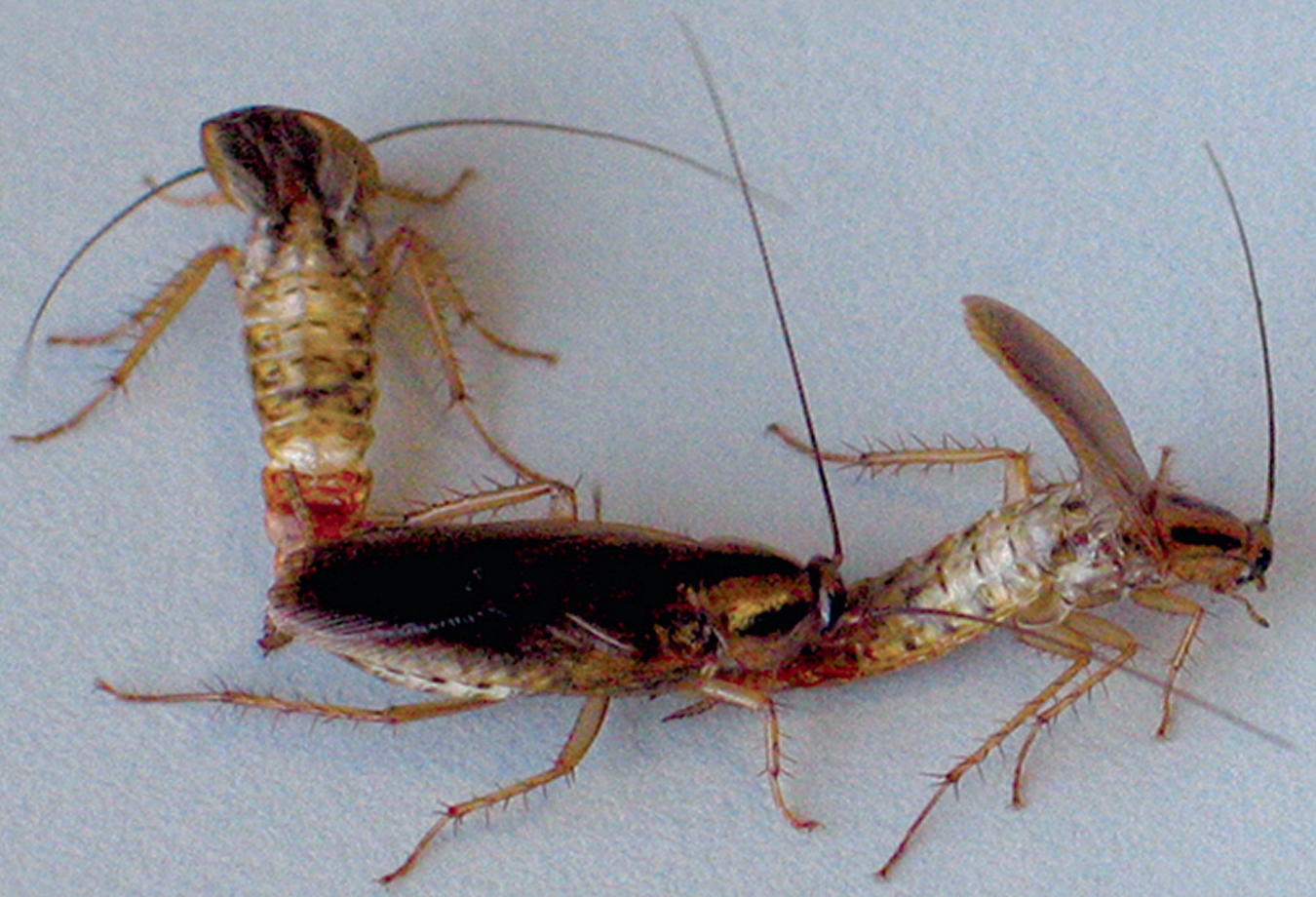

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ORIGINAL PAPER

Identification of cuticular lipids eliciting interspecific courtship in the German cockroach, *Blattella germanica*

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Abstract The cuticular surface of sexually mature females of the German cockroach contains a sex pheromone that, upon contact with the male's antennae, elicits a characteristic species-specific courtship behavior. This female-specific pheromone is a blend of several long-chain methyl ketones, alcohols and aldehydes, all derived from prominent cuticular hydrocarbons found in all life stages of this cockroach. We found that contact with the antennae of 5 out of 20 assayed cockroach species elicited courtship behavior in German cockroach males. The heterospecific courtship-eliciting compounds were isolated by behaviorally guided fractionation of the active crude extracts and compared to the native sex pheromone components. We identified two active compounds from the cuticular extract of the Oriental cockroach, *Blatta orientalis*—11-methylheptacosan-2-one and 27-oxo-11-methylheptacosan-2-one; the former compound was confirmed by synthesis and proved to independently stimulate courtship in German cockroach males. These compounds share common features with, but are distinct from, any of the known contact sex pheromone components. This suggests that sex pheromone reception in the male German cockroach is unusually promiscuous, accepting a wide range of compounds that share certain features with its native pheromone, thus resulting in a broad spectrum of behavioral response to other species. We propose that several characteristics of their mating system—chiefly, absence of closely related species in the anthropogenic environment, resulting in relaxation of selection on sexual communication, and a highly male-biased operational sex ratio—have driven males to respond with extremely low thresholds to a wide spectrum of related compounds.

Keywords *Blatta orientalis* · Contact pheromone · Methyl ketone · 11-Methylheptacosan-2-one · 27-Oxo-11-methylheptacosan-2-one

Introduction

Behavioral reproductive isolation—the species-specific reproductive signals that maximize attraction of conspecifics and minimize attraction of closely related heterospecific individuals—is important in initiating speciation, as well as maintaining it (Coyne and Orr 2004). Qualitative differences in species-specific sex pheromones and quantitative differences in blend ratios can form and reinforce such reproductive behavioral isolation (Löfstedt 1993; Groot et al. 2006). In cockroaches, it has been suggested that volatile pheromone blends contribute to reproductive isolation, especially among species of *Periplaneta* and related blattids that use several periplanone pheromone components in different blends (review: Gemeno and Schal 2004). Few other volatile sex pheromones have been identified in other cockroach species, but two other

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cockroaches, *Supella longipalpa* and *Blattella germanica*, appear to use single-component pheromones (Charlton et al. 1993; Nojima et al. 2005). This is in variance with the typical insect pattern—especially in Lepidoptera—and may be related to minor or no interaction with closely related species, relaxation from such interaction, and allopatry brought about by their anthropogenic habits, or possibly lack of concerted efforts by researchers to identify secondary (minor) constituents of sex pheromones.

The German cockroach, *B. germanica*, an important commensal pest of humans and domestic animals, also uses a highly effective contact pheromone to mediate sexual interactions, commonly in aggregations. The pheromone is produced by sexually receptive females and it elicits a typical species-specific courtship response in the male; without this pheromone, there can be no mating. Upon contact of the male's antennae with the female's cuticular surface, the male executes a turn while at the same time raising his wings; he maintains this position momentarily, thereby exposing specialized tergal glands and their nutritious secretion. Sugars and phospholipids in the secretions synergistically serve as phagostimulants (Nojima et al. 1999), and as the female mounts the male to feed on the glandular provisions, she is appropriately positioned for copulation (Roth and Willis 1952). Importantly, contact of the male's antennae with the female's antennae alone is sufficient to elicit courtship.

The contact sex pheromone components are derived from the dimethylalkanes 3,11-dimethylheptacosane and 3,11-dimethylnonacosane and are listed below from most to least abundant in hexane extracts of the epicuticular surface: 3,11-dimethylnonacosan-2-one; 3,11-dimethylheptacosan-2-one; 29-hydroxy-3,11-dimethylnonacosan-2-one; 27-hydroxy-3,11-dimethylheptacosan-2-one; 29-oxo-3,11-dimethylnonacosan-2-one; and 27-oxo-3,11-dimethylheptacosan-2-one (Nishida and Fukami 1983; Schal et al. 1990; Eliyahu 2007; Eliyahu et al. 2008). Each of these pheromone components can release courtship independently and there is no evidence for synergism among them (Schal et al. 1990; Eliyahu et al. 2008).

Interestingly, Nishida and Fukami (1983) found that detached antennae from different species of cockroaches and even from unrelated insects could elicit courtship in the German cockroach, suggesting that these antennae contain pheromone-like compounds. We similarly found in a previous study that detached antennae of male *S. longipalpa* can release courtship in German cockroach males (Eliyahu et al. 2004). In light of these findings, we propose two competing hypotheses. First, courtship-eliciting compounds on heterospecifics might be identical to one or more contact sex pheromone components of the German cockroach. This scenario could emerge, for example, from common metabolic pathways that give rise to convergent pheromone products. Alternatively, the heterospecific compounds might differ from the native contact sex pheromone components but retain sufficient common features to act as pheromone analogs. This hypothesis also invokes the notion that the male German cockroach responds to a broad spectrum of pheromone analogs.

To differentiate these two hypotheses, we first tested the capacity of detached antennae from 20 species of cockroaches to elicit courtship when stroked against the antennae of male German cockroaches. The compounds responsible for releasing this behavior were isolated from the cuticular extract of the Oriental cockroach and identified. We propose evolutionary scenarios and selection forces that might maintain heterospecific sexual signaling.

Materials and methods

Insects B. germanica cockroaches were maintained in groups of 500–1,000 at 27°C under 12:12 light–dark photoperiod with access to dry LabDiet rat chow (#5001; PMI Nutrition International, Brentwood, MO, USA) and water. Newly emerged adult males and females (50–100 of each sex) were separated daily from collectively reared nymphs.

The cockroach species listed in Table 1 in order of phylogenetic distance from *B. germanica* were also kept in groups at 27°C under 12:12 light–dark photoperiod and provisioned with dry rat or dog chow and water. Newly emerged adults of species whose antennae elicited courtship response in *B. germanica* males were separated by sex at eclosion.

Behavioral assay Male courtship response was tested using a modification of the assay developed by Roth and Willis (1952). An antenna from different species was excised, attached to a glass Pasteur pipette, and used immediately to test the responses of at least three groups of ten *B. germanica* 14–21-day- old males that were individually housed in 9×9×7.5 cm plastic cages. All assays were conducted during mid-scotophase, avoiding the first and last 2 h of the scotophase. A positive response was recorded when the male executed a courtship response, rotating his body relative to the stimulus and raising his wings within 30 s of being stroked by the test antenna. This is an unmistakable response that occurs only in a sexual context and is readily elicited by male test antennae fortified with female pheromone but never by normal male antennae or male antennae treated only with hexane.

For testing the behavioral response to fractions of cuticular extracts from courtship-eliciting species, an

Table 1 Species tested for eliciting courtship in adult male German cockroach: antennae, cuticular extract, and flash chromatography fractions of cuticular extracts (n=30 males assayed with antennae, each extract or each fraction)

	% males responding to whole antennae of		% males responding to crude extract of adult females and its flash chromatography fractions[a]										
	Female	Male	crude female extract	hexane	1% ether	2% ether	5% ether	10% ether	20% ether	40% ether	ether	EtOAc	MeOH
Blattellidae													
Blattella germanica	100	0	100	0	0	100	17	0	100	5	95	0	0
Parcoblatta lata	0	0											
P. pennsylvanica	0	0											
Supella longipalpa[d]	100	100	100	0	0	0	0	90	20	40	0	0	0
Blaberidae													
Blaberus atropos	0	0											
Blaberus craniifer	0	0											
Blaberus giganteus	0	0											
Blaptica dubia[b]	100	100	100	0	0	0	20	100	80	40	20	0	0
Diploptera punctata	0	0											
Eublaberus posticus	0	0											
Gromphadorhina portentosa[b]	100	0	0										
Leucophaea maderae	0	0											
Nauphoeta cinerea	0	0											
Pycnoscelus indicus	0	0											
Pycnoscelus surinamensis	0	0											
Schultesia lampyridiformes	0	0											
Blattidae													
Periplaneta australasiae[c]	100	100	100	0	0	0	0	87	80	0	0	0	0
P. americana	0	0											
P. fuliginosa	0	0											
Blatta orientalis[c]	100	100	100	0	0	96	88	100	64	4	0	0	0

[a] Extracts of species whose antennae did not elicit any response were not tested nor fractionated
[b] 0.06 female equivalents were applied onto the test antenna
[c] 0.1 female equivalents were applied onto the test antenna
[d] One female equivalents were applied onto the test antenna

antenna of a 14–21-day-old adult male *B. germanica* was excised, attached to a glass Pasteur pipette, and extracted briefly in hexane to remove male cuticular lipids. A 3-μl hexane solution of a test fraction was then applied to the distal 1 cm of the test antenna. Based on the size of the insect, 0.06 to 1.0 insect equivalents were used. The hexane was allowed to evaporate and the antenna was used immediately in the manner described above.

In a similar manner, serially diluted synthetic 11-methylheptacosan-2-one was applied on the test antenna

and its activity was compared to that of synthetic 3,11-dimethylheptacosan-2-one, a pheromone component of *B. germanica*.

Extraction and fractionation Same-sex individuals (ranging in numbers from 2 to 10, depending on size) were extracted in a 20-ml vial with ~6 ml hexane for 1 min. Assuming the courtship-eliciting compounds found on the antennae are also found throughout the surface of the cuticle, as in *B. germanica*, we conducted whole body extracts. The extract was decanted to a clean vial and slowly reduced under a stream of N_2 to ~100 μl. The extract was then fractionated by flash column chromatography over 200 mg of silica gel (100–200 mesh, Fisher Scientific) activated at 110°C for 30 min and washed with ~1 ml of hexane prior to application of the extract. The extract was eluted sequentially with 4 ml hexane, 2 ml each of 1%, 2%, 5%, 10%, 20%, and 40% diethyl ether, followed by 2 ml of each diethyl ether, ethyl acetate, and methanol. Each fraction was tested in the courtship bioassay. Fractions that elicited behavioral responses were further fractionated on a normal-phase high-performance liquid chromatography (HPLC) column (Econosphere silica 250×4.6 mm, 5 μm; Alltech, Deerfield, IL, USA) on an HP1050 HPLC (Hewlett-Packard, Palo Alto, CA, USA). Supellapyrone (Charlton et al. 1993) was added (400 ng) as internal standard and monitored at 296 nm with an HP1050 diode array detector; the *B. germanica* sex pheromone components have no ultraviolet absorption and their retention times were standardized to that of supellapyrone. Samples were eluted isocratically at 1 ml min^{-1} with 99% hexane and 1% 2-propanol. One-minute fractions were collected and bioassayed on at least 30 males.

Preparative gas chromatography HPLC fractions that elicited behavioral responses were further fractionated by preparative gas chromatography (GC). The HPLC fraction was reduced under N_2 to ~1 μl and injected into a splitless inlet coupled to a nonpolar EC5 Megabore column (30 m× 0.53 mm ID, 1.0-μm film thickness; Alltech, Deerfield, IL, USA) in a modified HP5890II GC. Injector and detector temperatures were set at 280°C, oven temperature increased from 60°C to 300°C at 15°C min^{-1} after an initial delay of 2 min. Detector B was custom-modified to accommodate a 20-cm section of Megabore column onto which the eluted compounds were trapped. The column trap could be quickly withdrawn and replaced with a new 20-cm trap. Collected GC fractions were eluted from each 20-cm Megabore column trap with 100 μl hexane into a conical vial, using a syringe and a GC column connector, for behavioral and chemical analyses.

Microchemical reactions 1,1-Dimethylhydrazine (DMH, 98%, Sigma-Aldrich, St. Louis, MO, USA) derivatization was used to stabilize the thermally unstable oxo-methyl-ketones for GC–mass spectrometry (MS) analysis. The behaviorally active HPLC fraction was reduced under N_2 to ~50 μl in a conical reaction vial and 5 μl dimethylhydrazine were added. The vial was incubated in a 60°C bead bath for 30 min.

A modified Wolff–Kishner reduction was performed on the active fraction from preparative GC to determine the methyl branch position: the fraction was combined with 10 μg 14-heptacosanone (as internal standard) and dried under N_2 in a 0.1-ml conical reaction vial to ~1–2 μl. Twenty microliter of 10% hydrazine hydrate in ethanol with a trace amount of formic acid were added and the mixture was allowed to stand for ~45 min at room temperature. The solvent was then evaporated and 20 μl of 10% KOH in diethylene glycol were added. The vial was warmed up to 200°C for 30 min, after which its contents were diluted with water and extracted with hexane for analysis by GC–MS.

Chemical analysis An Agilent 5975 mass selective detector, operated in electron impact ionization mode and coupled to an Agilent 6890 GC (Agilent, Santa Clara, CA, USA), was used for chemical structure determinations in active fractions. The GC was operated in splitless-injection mode and fitted with a 30 m×0.25 mm ID HP-5MS column (Agilent). The oven was programmed from 60°C to 300°C at 15°C min^{-1} after an initial delay of 2 min and held at 300°C for 20 min. Injector temperature was 280°C, MS quad 150°C, MS source 230°C, and transfer line 250°C.

Synthesis The scheme of the synthesis of 11-methylheptacosan-2-one is described in the Electronic Supplementary Material.

Statistical analysis For dose–response studies, the probit and logistic procedures were used to estimate the dose to which 50% of the males would respond (RD_{50}) and to compare the RD_{50} values for two compounds. The analysis was performed with Statistical Analysis System (SAS Institute 2003).

Results

Heterospecific cockroaches release courtship in German cockroach males The species tested and the capacity of their isolated antennae—taken from males and females—to elicit courtship in German cockroach males, are listed in Table 1 in order of phylogenetic relatedness. Antennae of 5

of the 20 species tested released courtship behavior, but there was no obvious phylogenetic pattern because the five species represented all three Blattoidea families that we tested. Nevertheless, we did not test other *Blattella* species and it is likely that such a pattern might emerge at the genus level. In four species, antennae of males and females elicited courtship, but *Gromphadorhina portentosa* male antennae, which are highly sexually dimorphic, did not release courtship.

Sexually mature adult females of the five species were extracted in hexane and bioassayed. Surprisingly, extracts of *G. portentosa* females failed to release courtship in any of the tested males. The extracts of the other four species were fractionated by flash column chromatography, and each fraction was tested in the courtship assay (Table 1). All four species whose extracts elicited courtship contained activity in the 10% and 20% ether fractions. Two *B. germanica* pheromone components, the aldehydes 29-oxo-3,11-dimethylnonacosan-2-one and 27-oxo-3,11-dimethylheptacosan-2-one, normally elute in these fractions. Only in *Blaptica dubia* was minor activity found in the 100% ether fraction, where 29-hydroxy-3,11-dimethylnonacosan-2-one and 27-hydroxy-3,11-dimethylheptacosan-2-one normally elute. Of the four fractionated extracts, only *Blatta orientalis* showed activity in the 2% ether fraction (which carried over to the 5% ether fraction), where the two *B. germanica* dimethylketone pheromone components, 3,11-dimethylnonacosan-2-one and 3,11-dimethylheptacosan-2-one, normally elute (Eliyahu 2007; Eliyahu et al. 2008).

Identification of the courtship-eliciting compounds of B. orientalis The 2% and 5% ether fractions from *B. orientalis* were combined and further fractionated by normal-phase HPLC, yielding a single 1-ml fraction that elicited courtship responses (not shown), which was in turn fractionated by preparative GC (Fig. 1a). Two separate behaviorally active fractions were collected in preparative GC: A highly active fraction at R_t 27.00–27.67 min and a much less active fraction at R_t 27.67–28.00 min (Fig. 1b). The first fraction consisted of a major peak in GC–MS analysis, with M^+=408 (as confirmed by chemical ionization–MS; Fig. 1c) that also constituted the largest peak in the 2% ether fraction (Fig. 1a). The major peak in the hydrocarbon fraction of the cuticular extracts of *B. orientalis* contains 11- and 13-methylheptacosane (Lockey and Dularay 1986). Based on the biosynthetic pathway of the contact sex pheromone in the German cockroach (Chase et al. 1992), we predicted that the largest peak in the 2% ether fraction (ketone fraction) of *B. orientalis* would contain 11- and/or 13-methylheptacosan-2-one or 15- and 17-methylheptacosan-2-one (depending on which side of the molecule the carbonyl group is located). MS data provided some support for this prediction: *m/z* 408 indicates the molecular weight of a 27 carbon chain with a single methyl branch, *m/z* 390 (M^+-18) indicates the loss of H_2O, a typical fragment in carbonyl-containing compounds.

A carbonyl group next to a terminal carbon was suggested by a prominent peak at *m/z* 58 which results from McLafferty rearrangement on C-2 without adjacent methyl branches. To conclusively determine the methyl branch position of the behaviorally active compound, we reduced the active fraction from preparative GC with the Wolff–Kishner reduction (Fig. 1d). A new M^+ of 394 and diagnostic fragments at *m/z* 379 (M^+-15), 168/169 ($C_{12}H_{25}$) and 252/253 ($C_{18}H_{37}$) indicated that the reduction product was 11-methylheptacosane. Final confirmation of the structure was obtained by comparison of MS fragmentation patterns to synthetic 11-methylheptacosan-2-one (not shown).

The combined 10% and 20% ether fractions of *B. orientalis* was fractionated on normal-phase HPLC, yielding a single 1-min fraction that elicited courtship in males. Because of its behavior in flash column chromatography and co-elution with *B. germanica* ketoaldehydes, the proposed biosynthetic pathway of ketoaldehydes in the German cockroach, and the fact that the ketone fraction contained 11-methylheptacosan-2-one, we speculated that this fraction would also contain 27-oxo-11-methylheptacosan-2-one. The fraction was derivatized with DMH and analyzed by GC–MS in selected ion monitoring mode (86 [aldehyde *N,N*-dimethylhadrazones McLafferty rearrangement], 464 [expected DMH product], 420 [M^+-dimethylamine], and 393 [M^+-(CH=N–N[CH_3]$_2$)]; Fig. 2). The molecule, which has two carbonyl groups could be derivatized both on C-2 and C-27, which would result in an additional DMH product of *m/z* 506; however, ketones do not derivatize as efficiently as aldehydes, and considering the small amount of the suspected 27-oxo-11-methylheptacosan-2-one, it is not surprising that we did not detect this product. The retention time of the peak with the appropriate relative abundance of the selected ion fragments was ~3 min earlier than that of DMH-derivatized 27-oxo-3,11-dimethylheptacosan-2-one. In addition, the active HPLC fraction was reduced using the modified Wolff–Kishner, and 11-methylheptacosane was detected by GC–MS. Combined, these data provide strong evidence that the active compound in the 10% and 20% ether fractions of *B. orientalis* is 27-oxo-11-methylheptacosan-2-one, but confirmation of this identification will need to await synthesis and bioassay of 27-oxo-11-methylheptacosan-2-one.

Behavioral response to synthetic 11-methylheptacosan-2-one The courtship response to synthetic 11-methylheptacosan-2-one was lower compared to synthetic 3,11-dimethylhep-

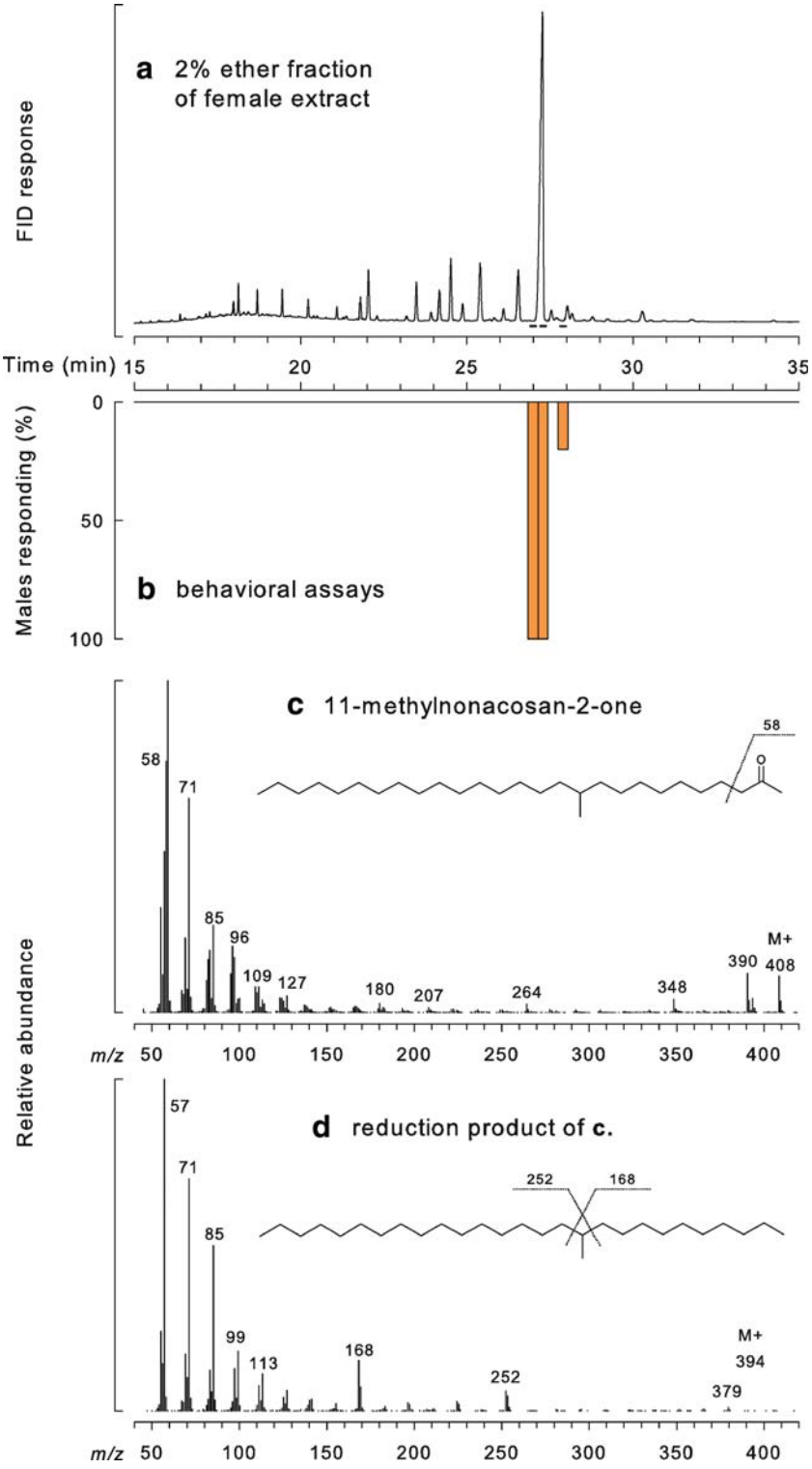

Fig. 1 Identification of the active component in the 2% ether fraction from flash column chromatography of *B. orientalis* cuticular extract. **a** GC-FID trace of the 2% ether fraction (30 female equivalents). **b** Behavioral activity of 0.33-min fractions from preparative GC (n=30 males per fraction). None of the 30 *B. germanica* males responded to fractions where no *bar* is shown. **c** Mass spectrum of the major peak found in the active fractions from preparative GC, and **d** mass spectrum of the active fractions from preparative GC after Wolff–Kishner reduction (see "Materials and methods")

tacosan-2-one (Fig. 3). The estimated dose to elicit courtship in 50% of the males, 25.33 ng, was significantly higher than that of the dimethyl ketone pheromone component (Table 2).

Discussion

Our studies, like other recent investigations, have relied upon a behavioral assay wherein an isolated *B. germanica*

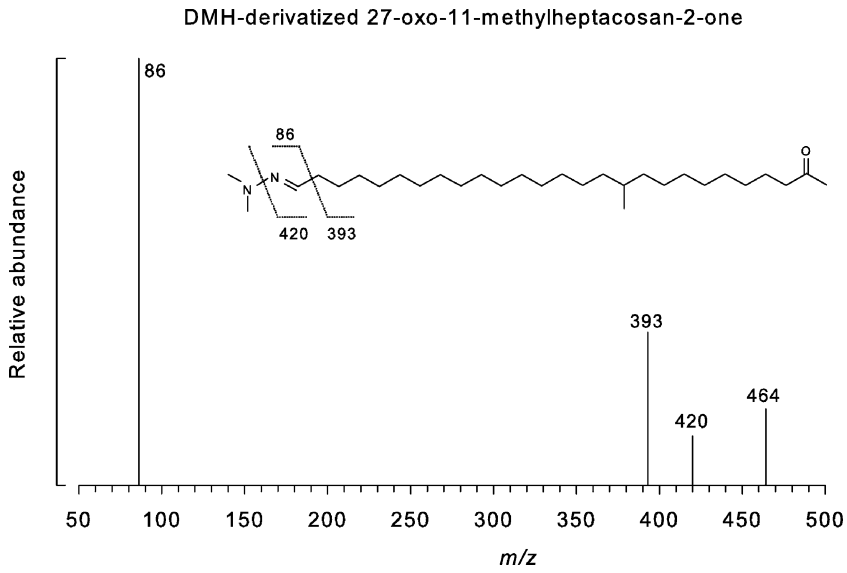

Fig. 2 Selected ion monitoring mass spectrum assigning 27-oxo-11-methylheptacosan-2-one to the courtship-eliciting fraction. *B. orientalis* cuticular extract (ten female equivalents) was fractionated by flash column chromatography, the 10% and 20% ether fractions were found to be behaviorally active, combined, and fractionated further by HPLC. The active HPLC fraction was derivatized with DMH and analyzed by SIM–MS

male antenna is first extracted (hence, made neutral) and then augmented with test fractions or compounds. This antenna is then manually stroked over the antennae of live *B. germanica* males to elicit the courtship response. Nishida and Fukami (1983) recognized, however, that male courtship is stimulated by a combination of chemosensory and tactile signals. Unlike most other contact pheromones which are active on any surface—for example, the Asian longhorned beetle courts plastic vials coated with female contact pheromone (see Zhang et al. 2003)—extracts of sexually mature *B. germanica* females, or synthetic pheromone components, fail to release courtship when loaded onto some artificial surfaces such as filaments or human hair. Remarkably, the pheromone also fails to elicit courtship when loaded on various insect antennae that appear to differ substantially in fine morphological structure from those of the German cockroach (Nishida and Fukami 1983). All the evidence to date indicates, however, that specific courtship-eliciting compounds must be present to stimulate the behavior, and some insect antennae that do not normally elicit courtship in male *B. germanica* can be made to do so when augmented with pheromone. An interesting case, revealed in the present study, is that the antennae of *G. portentosa* females can stimulate courtship in male *B. germanica* but antennae of male *G. portentosa* do not. It will be interesting to learn whether this disparity

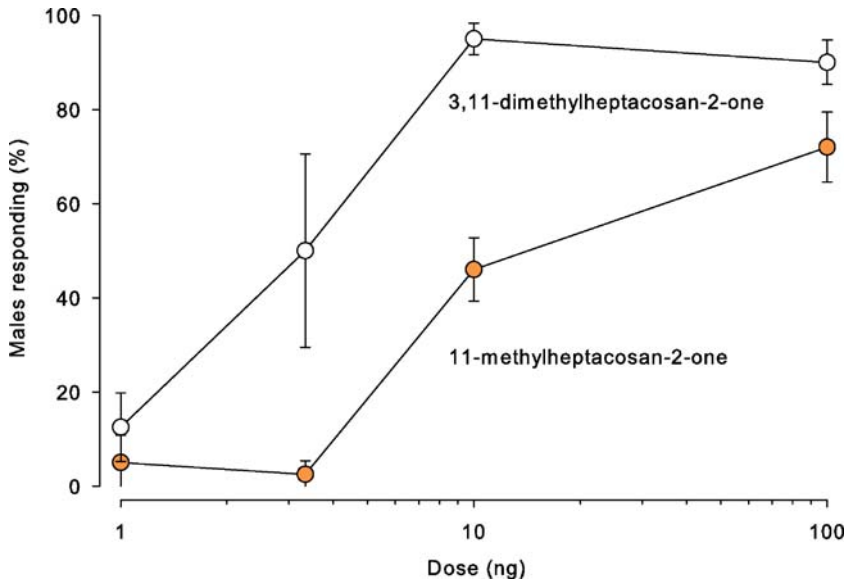

Fig. 3 Dose–response curves of the *B. germanica* sex pheromone analog identified from *B. orientalis* and the native *B. germanica* sex pheromone component, showing mean (±SE) percentage of males responding to 3,11-dimethylheptacosan-2-one and 11-methylheptacosan-2-one. (N=40–50 per compound–dose combination)

Table 2 Probit analysis of dose–response studies of the authentic *B. germanica* pheromone-like compound in cuticular extracts of *B. orientalis* and the *B. germanica* dimethyl ketone pheromone component

Pheromone, or pheromone-like compound	Slope	Intercept	RD_{50} (ng)[a]	95% fiducial limits
3,11-dimethylheptacosan-2-one	0.716	−0.777	3.60 a	1.08, 8.84
11-methylheptacosan-2-one	0.829	−1.693	25.33 b	14.28, 54.19

[a] Estimated dose to which 50% of males respond. Values followed by different letters are significantly different ($P<0.05$, Probit and logistic procedures, SAS Institute 2003).

is caused by sex—and stage—differences in cuticular chemicals or by the obvious sexual dimorphism in antennal morphology in this species. This species also was peculiar because extracts of behaviorally active whole insects failed to elicit courtship behavior. One possibility is that the courtship-eliciting compounds are concentrated on the antennae and therefore are diluted in extracts of the whole body. However, from our experience with contact pheromones, it is more likely that highly abundant cuticular lipids masked the active compounds.

This study, to identify compounds that mediate interspecific courtship in male *B. germanica*, was motivated by two competing hypotheses: (a) that some other species share one or more compounds with the contact sex pheromone of the female German cockroach; or (b) that the heterospecific compounds share certain common features with one or more *B. germanica* contact sex pheromone component and therefore act as pheromone analogs. First, we showed that German cockroach males court 5 of 20 species of cockroaches in three families: Blattellidae (to which *B. germanica* belongs), Blaberidae, and the more distant Blattidae. These results complement the report by Nishida and Fukami (1983) that *B. germanica* males court the antennae of the cockroaches *Blattella nipponica* and *B. orientalis* but not *Periplaneta fuliginosa* or *Periplaneta americana*. Interestingly, while we confirmed the lack of responses to the two *Periplaneta* species, we also showed that the closely related *Periplaneta australasiae* did elicit the courtship response.

We extended earlier observations of courtship toward antennae of *B. orientalis* (Nishida and Fukami 1983), showed that males, females, and nymphs of this species can elicit courtship, and used extracts of this evolutionarily distant species from *B. germanica* to address our two hypotheses. The results compel us to reject the first hypothesis and favor the second, that *B. orientalis* compounds act as *B. germanica* contact sex pheromone mimics because they share several common features that release the courtship response in males.

Coarse tuning of pheromone reception and interspecific courtship in males In most sex pheromones, slight changes in pheromone structure, such as changes in chirality or modifications in blend ratios, render the pheromone inactive or even behaviorally antagonistic or repellent (Baker et al. 1998; Mori 1998). It is important to note, however, that most of these examples come from attractant pheromones, which are better studied than contact pheromones. The contact sex pheromone of the German cockroach possesses several unusual features that distinguish it from most of the studied pheromones. First, each of six components that comprise the pheromone blend can independently elicit the full sexual response, albeit at different concentrations. Second, the most abundant pheromone components are not the most effective at eliciting courtship. Third, this contact sex pheromone includes apparently redundant features that can accommodate substantial structural modifications while retaining overall activity. For example, the C-2 carbonyl is indispensable for activity of 3,11-dimethylnonacosan-2-one, but its elimination from 29-hydroxy-3,11-dimethylnonacosan-2-one only reduces but does not eliminate behavioral activity (Nishida and Fukami 1983). However, reduction of the carbonyl to a hydroxy group significantly enhances activity of both the methyl ketone and hydroxy-methyl ketone components. Nishida and Fukami (1983) similarly showed that adding bulkiness to either the C-2 or C-29 substituents decreased activity of pheromone analogs. Although a 3,11-methyl branching pattern is essential for activity, 3-monomethyl ketone and 11-monomethyl ketone of the appropriate chain length also elicit lower activity; behavioral activity is also diminished but rarely eliminated as the methyl groups are shifted away from these two preferred positions. And finally, an alkyl chain of 29 carbons is most effective, but it can be lengthened or shortened with only a gradual, incremental loss of activity (Nishida and Fukami 1983).

Given this rather broad tuning of pheromone reception in *B. germanica* males, it is not surprising that 11-methylheptacosan-2-one and 27-oxo-11-methylheptacosan-2-one from *B. orientalis* can stimulate German cockroach males to engage in courtship behavior. As expected, 11-methylheptacosan-2-one stimulated significantly fewer males than the native pheromone component 3,11-dimethylheptacosan-2-one, in agreement with the results of structure–activity studies performed by Nishida and Fukami (1983), who found that both 3-methylnonacosan-2-one and 11-methyl-

nonacosan-2-one pheromone analogs had lower activity than the most abundant pheromone component 3,11-dimethylnonacosan-2-one. However, the relatively high abundance of the pheromone analog on the cuticle of *B. orientalis* may compensate for its lower behavioral activity.

Features of German cockroach ecology that promote broad-spectrum tuning of male sexual response Why, then, did we observe such a promiscuous courtship response in the German cockroach in our experiments? First, we cannot reject the notion that the contact sex pheromone of female *B. germanica* might consist of many more structurally related, yet diverse components, including the C_{27} compounds identified from *B. orientalis*. The female contact sex pheromone consists of three 29-carbon components and a homologous series of 27-carbon components (Nishida and Fukami 1983; Schal et al. 1990; Eliyahu et al. 2008). The 29-carbon dimethyl ketone (and probably the 27-carbon homolog as well) is derived from insertion of a C-2 carbonyl into the preformed 3,11-dimethylalkane (Chase et al. 1992), and a similar mechanism might give rise to the 29-hydroxy-, 29-oxo-, 27-hydroxy-, and 27-oxo- pheromone components. The epicuticular surface of *B. germanica* contains numerous methyl-branched alkanes in homologous series of 27- and 29-carbon chains (Augustynowicz et al. 1987; Carlson and Brenner 1988; Jurenka et al. 1989). It is possible that enzymes that catalyze the hydroxylation and oxidation reactions might recognize related hydrocarbons as substrates. Based on the systematic structure–activity studies of Nishida and Fukami (1983), almost all the potential methyl ketone derivatives of the cuticular hydrocarbons—especially of 3-methyl- and 11-methyl-alkanes—would elicit courtship responses in males. If so, the broad tuning of the male courtship response may be a natural consequence of an unusually promiscuous cytochrome P-450 enzyme system that produces a diverse contact sex pheromone blend in the female.

Sexually receptive *B. germanica* females also emit a volatile pheromone, blattellaquinone (Nojima et al. 2005), that could add species-specificity to the mate-finding process. This pheromone might diminish the need to fine-tune contact sexual signaling and signal reception at close range. This idea is probably much underappreciated in chemical ecology, as examples emerge of effective, volatile sex pheromone-mediated species isolation in the field that may be circumvented at close range (in cages) and result in interspecific mating (e.g., Groot et al. 2006).

Another factor affecting the reception spectrum of the German cockroach male is the male-biased operational sex ration in this species. Although the sex ratio in adult German cockroach populations is 1:1, the operational sex ratio in this mating system is highly male-biased. Females carry an egg case (ootheca) for the ~20-day duration of embryonic development, during which they are sexually unreceptive and are only potentially receptive for a short period of 2–3 days during each ~25-day ovarian cycle. Moreover, females effectively store sperm and only a fraction of the adult female population mates more than once. Hence, the probability of mating is low for most males, and it is possible that they evolved low response thresholds to the contact sex pheromone components to efficiently detect females within very large aggregations.

There are many cases in which unrelated species—even from different classes (e.g., elephants and moths)—have converged on similar or identical chemicals for sexual communication. Given the relatively limited chemical moieties of long-chain cuticular lipids it is perhaps not surprising that unrelated insect species would independently evolve similar cuticular lipids to serve physiological or behavioral functions, or both. As long as species with overlapping sexual communication signals remain separated by geography, space (microhabitat), or time, there is little imperative to diverge their shared sexual signals.

In our case, it could be that the absence of closely related species in the anthropogenic environment has relaxed selection for fine-tuned species-specific close-range sexual communication in *B. germanica*. In sympatry, species uniqueness is selected upon and maintained through reproductive character displacement, resulting in divergent sexual signals and behavioral and morphological differences (Wyatt 2005). The evolutionary history of cockroaches is poorly understood. Nevertheless, the fossil record and centers of extant species diversity suggest that while the genus *Blattella* probably originated in southeast Asia (Roth 1985), the five species that can elicit courtship in *B. germanica* probably originated in east and west Africa (Cornwell 1968). Some species (*B. orientalis*, *S. longipalpa*, and *P. australasiae*) are commensal with humans and domestic animals (Cornwell 1968), whereas other species (*B. dubia* and *G. portentosa*) are free living and limited to tropical climates; the distribution of the latter species (hissing cockroach) is limited to Madagascar. But even for the commensal species, interspecific encounters would be infrequent because they have different microhabitat preferences and mixed infestations of these species are rarely seen. Moreover, they are distant relatives and hybridization cannot occur between the German cockroach and any of the other species. Even closely related *Blattella* species, such as *Blattella asahinai*, which not only release courtship behavior in *B. germanica* but can hybridize, do not overlap in their spatial distributions. It thus appears that male *B. germanica* might have broadened their sexual behavioral response spectrum in the artificial, single-species "monoculture" structural environment in which they have lived for at least several thousand years. It would be fascinating to know

through comparative studies of congeners if broadening of the male antennal response spectrum has in turn resulted in novel female-produced pheromone components, and vice versa. The human-built ecological context might not only free *B. germanica* from interaction with closely related species and broaden its communication channel but it could also facilitate shifts in pheromone blend composition away from the ancestral blend and toward metabolically less costly blends.

Identification of the courtship-eliciting compounds of other cockroaches might shed light on the range of compounds that the *B. germanica* pheromone receptor(s) will accept and thus will enrich our understanding of the structure–activity relationship of pheromone reception in the German cockroach. It will also begin to address the question of whether these cockroaches share similar biochemical mechanisms that sequentially metabolize behaviorally inactive hydrocarbons to oxygenated active derivatives. Finally, these investigations should elucidate the functions that long-chain, cuticular methyl ketones, keto-alcohols, and ketoaldehydes serve in various insects.

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References

Ahn K-C, Jung J-C, Park O-S (2006) A simple and cost effective synthesis of 3,11-dimethylnonacosan-2-one, a female sex pheromone of the German cockroach. Molecules 11:751–757

Augustynowicz M, Malinski E, Warnke Z, Szafranek J, Nawrot J (1987) Cuticular hydrocarbons of the German cockroach, *Blattella germanica* L. Comp Biochem Physiol B 86:519–523

Baker TC, Fadamiro HY, Cossé AA (1998) Moth uses fine tuning for odour resolution. Nature 393:530

Carlson DA, Brenner RJ (1988) Hydrocarbon-based discrimination of three North American *Blattella* cockroach species (Orthoptera: Blattellidae) using gas chromatography. Ann Entomol Soc Am 81:711–723

Charlton RE, Webster FX, Zhang A, Schal C, Liang D, Sreng I, Roelofs WL (1993) Sex pheromone for the brownbanded cockroach is an unusual dialkyl-substituted α-pyrone. Proc Natl Acad Sci USA 90:10202–10205

Chase J, Touhara K, Prestwich GD, Schal C, Blomquist GJ (1992) Biosynthesis and endocrine control of the production of the German cockroach sex pheromone 3,11-dimethylnonacosan-2-one. Proc Natl Acad Sci USA 89:6050–6054

Cornwell PB (1968) The cockroach, vol. 1. Hutchinson, London

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Sunderland

Eliyahu D (2007) Chemical communication in the German Cockroach: pheromones and heterospecific courtship eliciting compounds. Ph.D. dissertation, North Carolina State University, Raleigh

Eliyahu D, Mori K, Takikawa H, Leal WS, Schal C (2004) Behavioral activity of stereoisomers and a new component of the contact sex pheromone of female German cockroach, *Blattella germanica*. J Chem Ecol 30:1839–1848

Eliyahu D, Nojima S, Mori K, Schal C (2008) New contact sex pheromone components of the German cockroach, *Blattella germanica*, predicted from the proposed biosynthetic pathway. J Chem Ecol (in press). DOI 10.1007/s10886-007-9409-8

Gemeno C, Schal C (2004) Sex pheromones of cockroaches. In: Cardé RT, Millar JG (eds) Advances in insect chemical ecology. Cambridge University Press, Cambridge, pp 179–247

Groot AT, Horovitz JL, Hamilton J, Santangelo RG, Schal C, Gould F (2006) Experimental evidence for interspecific directional selection on moth pheromone communication. Proc Natl Acad Sci USA 103:5858–5863

Jurenka RA, Schal C, Burns E, Chase J, Blomquist GJ (1989) Structural correlation between cuticular hydrocarbons and female contact sex pheromone of German cockroach *Blattella germanica* (L.). J Chem Ecol 15:939–949

Lockey KH, Dularay B (1986) Cuticular methylalkanes of adult cockroaches, *Blatta orientalis* and *Periplaneta americana*. Comp Biochem Physiol B 85:567–572

Löfstedt C (1993) Moth pheromone genetics and evolution. Phil Trans R Soc Lund B 340:167–177

Mori K (1998) Chirality and insect pheromones. Chirality 10:578–586

Mullins DE, Keil CB, White RH (1992) Maternal and paternal nitrogen investment in *Blattella germanica* (L) (Dictyoptera, Blattellidae). J Exp Biol 162:55–72

Nishida R, Fukami H (1983) Female sex pheromone of the German cockroach, *Blattella germanica*. Mem Coll Agric Kyoto Univ 122:1–24

Nojima S, Nishida R, Kuwahara Y, Sakuma M (1999) Nuptial feeding stimulants: a male courtship pheromone of the German cockroach, *Blattella germanica* (L.) (Dictyoptera: Blattellidae). Naturwissenschaften 86:193–196

Nojima S, Schal C, Webster FX, Santangelo RG, Roelofs WL (2005) Identification of the sex pheromone of the German cockroach, *Blattella germanica*. Science 307:1104–1106

Roth LM (1985) A taxonomic revision of the genus *Blattella* Caudell (Dictyoptera, Blattaria: Blattellidae). Entomol Scan Suppl 22:1–221

Roth LM, Willis ER (1952) A study of cockroach behavior. Am Midl Nat 47:66–129

Schal C, Burns EL, Jurenka RA, Blomquist GJ (1990) A new component of the female sex pheromone of *Blattella germanica* (L.) (Dictyoptera: Blattellidae) and interaction with other pheromone components. J Chem Ecol 16:1997–2008

Wyatt TD (2005) Pheromones: convergence and contrasts in insects and vertebrates. In: Mason RT, LeMaster MP, Müller-Schwarze D (eds) Chemical signals in vertebrates, vol. 10. Springer, New York, pp 7–20

Zhang A, Oliver JE, Chauhan K, Zhao B, Xia L, Xu Z (2003) Evidence for contact sex recognition pheromone of the Asian longhorned beetle, *Anoplophora glabripennis* (Coleoptera: Cerambycidae). Naturwissenschaften 90:410–413

Electronic Supplementary Material

Identification of cuticular lipids eliciting interspecific courtship in the German cockroach, *Blattella germanica*

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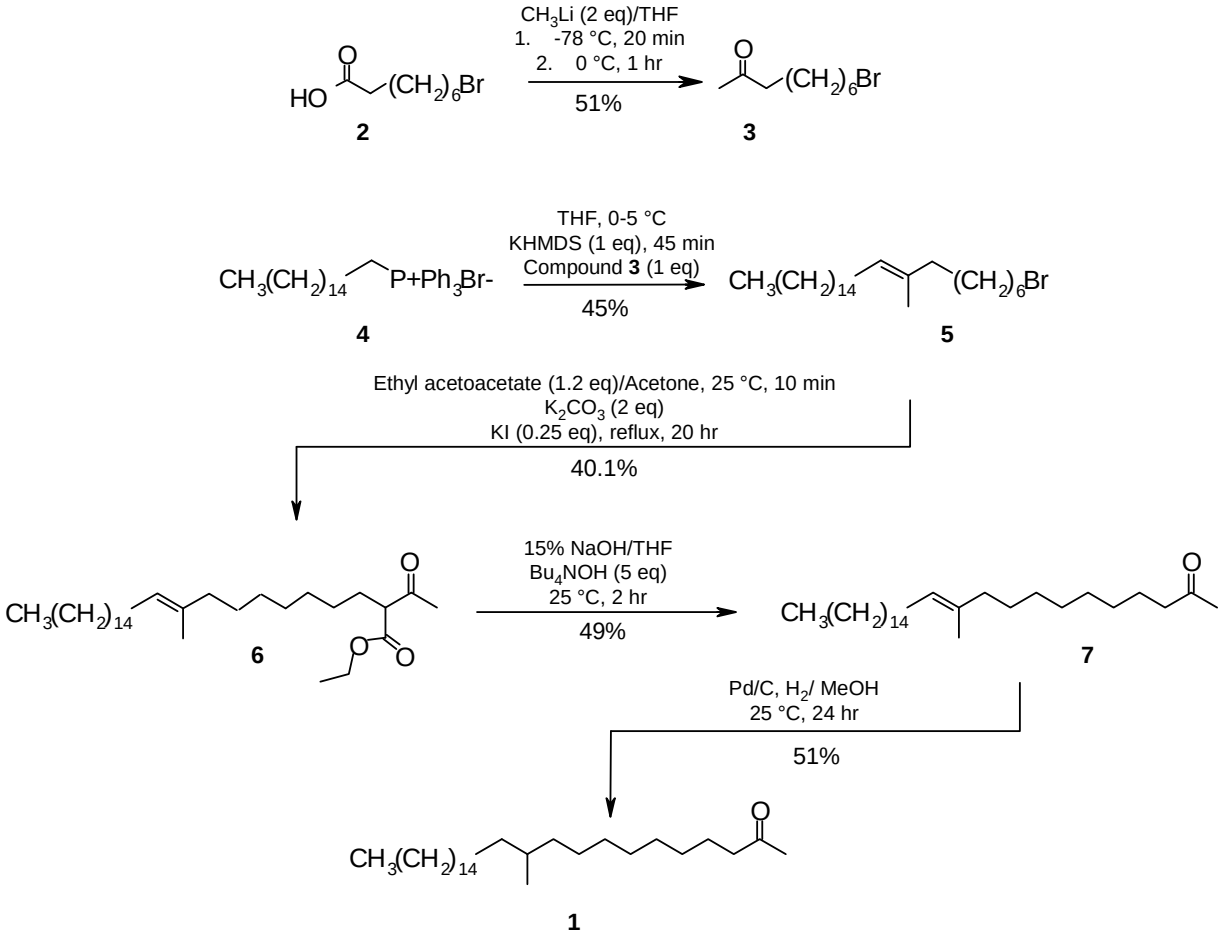
Experimentals:

All glassware used in the following experiments was flame-dried and flushed with argon. Tetrahydrofuran (THF), distilled from sodium and benzophenone, and methanol, distilled over CaH_2, were used. Thin layer chromatography (TLC) was performed on precoated silica gel plates and visualized with 254-nm UV light, or potassium permanganate staining. Flash chromatography was carried out on silica gel (Fisher 100-200 mesh). ^{1}H-NMR and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a varian instrument operating at 400 MHz (^{1}H) and 100 MHz (^{13}C). The chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane. Electron impact GC-mass spectra were recorded using an Agilent 6890 GC coupled to an Agilent 5975 mass selective detector (Agilent, Palo Alto, CA). High resolution mass spectra (HRMS) were measured using an electric field scanning and electron ionization with a JEOL (Tokyo, Japan) HX110HF mass spectrometer. The resolving power of the mass spectrometer was 10,000, the accelerating voltage 10 keV, and the ion source temperature 165 °C. The perfluorokerosene ions in the appropriate mass range were used as a reference standard and were analyzed simultaneously with the sample. The accuracy of the instrument was determined experimentally with a confidence level of 99.7%.

Compounds **2** and **4** were purchased from Alfa Aesar (MA) and used without further purification.

Scheme 1 A scheme of the synthesis of 11-methylheptacosan-2-one. A Wittig reaction was used in step 2 to create intermediate **5**, and Ethyl acetoacetic reaction was performed in step 3 to create intermediate **6**.



11-methylheptacosan-2-one (**1**) was prepared as follows:

9-bromononan-2-one (**3**)

A stirred solution of compound **2** (5.0 g, 22.4 mmol) in 50 mL dry THF was cooled to -78 °C and methyllithium (28.0 mL, 44.8 mmol) was added slowly (dropwise). The reaction mixture was allowed to warm to 0 °C for 1 hr, quenched with saturated aqueous NH_4Cl solution (10.0 mL), and the mixture was extracted with ether (3×10 mL). The organic layer was separated, dried over anhydrous $MgSO_4$, filtered and concentrated. The residue was purified by distillation

to give **3** (2.52 g, 51%) as a colorless oil. The procedure and product are described in Ahn et al. (2006). GC-MS m/z (relative abundance) 58 (1), 71 (0.15), 123 (0.06), 141 (0.10), 162 and 164 (0.04 each), 205 (0.006), 220 (0.0001), 222 (M^+, 0.0004).

1-bromo-8-methyltetracos-8-ene (**5**)

A stirred solution of *n*-hexadecyltriphenylphosphonium bromide (**4,** 2.5 g, 4.5 mmol) in 25.0 mL dry THF was cooled to 0–5 °C. KHMDS (9 mL, 0.5M, 4.5 mmol) was added dropwise with a syringe. Upon complete addition of the base, the solution turned red. After stirring the reaction for 45 min, compound **3** was added dropwise. The reaction mixture was then warmed to room temperature after 10 min at 0–5 °C and stirred for 48 hrs. The reaction was quenched with water (15.0 mL) and extracted with hexane (4×20.0 mL). The organic layer was washed with water 2 × 15.0 mL) and brine, dried with $MgSO_4$, filtered and concentrated. The product was purified by radial PLC (hexane), to give **5** (0.87 g, 45%) as a colorless oil. GC-MS m/z (relative abundance) 55 (1), 69 (0.86), 83 (0.85), 97 (0.70), 111 (0.36), 125 (0.16), 210 (0.05), 238 (0.08), 251 (0.07), 266 (0.12), 428 (M^+, 0.06).

11-methyl 3-ethoxycarbonylnonacosan-2-one (**6**)

To a solution of ethyl acetoacetate (0.16 mL, 1.22 mmol, Alfa Aesar) in acetone (5.0 mL) was added K_2CO_3 (360.0 mg, 2.61 mmol) and KI (57.0 mg, 0.35 mmol). The mixture was stirred for 10 min at room temperature before the addition of **5** (500 mg, 1.16 mmol). The reaction was brought to reflux in a heated sand bath for 20 hrs. The resultant mixture was cooled to room temperature, diluted with ether (25.0 mL) and filtered. The filtrate was washed with a saturated solution of aqueous NH_4Cl (20.0 mL) and then brine (20.0 mL). The organic layer was separated,

and the aqueous layer was extracted with ether (2×20.0 mL). The combined organic extracts were dried over anhydrous $MgSO_4$, filtered and concentrated. The product was purified by radial PLC (5% ethyl acetate in hexane) to give acetate **6** (223.0 mg, 40.1%) as a colorless oil. GC-MS 55 (1), 69 (0.80), 83 (0.74), 97 (0.63), 111 (0.41), 125 (0.54), 141 (0.19), 163 (0.36), 266 (0.13), 306 (0.03), 388 (0.04). 406 (0.9), 478 (M^+, 0.0008).

11-methyl 11-heptacosen-2-one (**7**)

To a stirred solution of 11-methyl 3-ethoxycarbonylheptacosen-2-one (**6**, 223.0 mg, 0.47 mmol) in 5 mL dry THF was added 15% aqueous NaOH solution (3.0 mL), followed by addition of tetrabutylammonium hydroxide solution (2.3 mL, 2.3 mmol, 1M in water). The mixture was stirred at room temperature for 2 hr, then diluted with ethyl acetate (7.5 mL) and washed with saturated aqueous NH_4Cl solution (5.0 mL) and then brine (5.0 mL). The organic layer was separated, and the aqueous phase was extracted with ethyl acetate (3.0 mL). The combined organic phases were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The product was purified by radial PLC (5% ethyl acetate in hexane) to give **7** as a white solid (0.0937 g, 49%). GC-MS 55 (1), 69 (0.86), 83 (0.84), 97 (0.75), 111, (0.52), 125 (0.73), 141 (0.27), 163 (0.51), 238 (0.07), 250 (0.07), 266 (0.23), 306 (0.06), 388 (0.08), 406 (M^+, 0.17).

11-methylheptacosan-2-one (**1**)

To a flask containing a stirred solution of 10% palladium on carbon (24.5 mg, 0.023 mmol) in methanol (5.0 mL) was added a solution of **7** (93.5 mg, 0.23 mmol) in methanol (2.0 mL). Air was evacuated from the flask and then it was back-filled with hydrogen. After 24 hrs under balloon pressure of hydrogen, the reaction was diluted with hexane (2.0 mL) and filtered. The

filtrate was washed with brine (2.0 mL) and water (2.0 mL), followed by extraction of the aqueous phase with hexane (2.0 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated. The product was purified by radial PLC (2% ethyl acetate in hexane) to give **1** (31.0 mg, 47%) as a white solid. ^{1}H-NMR δ 2.430-2.392 (t, 2H), 2.129 (s, 3H), 1.587 (s, 1H), 1.562-1.546 (d, *J* = 6.4 Hz, 2H), 1.252-1.268 (m, 42H), 1.075-1.059 (m, 3H), 0.893-0.859 (t, 3H); ^{13}C-NMR δ 209.649, 44.055, 37.314-37.291, 32.961, 32.142, 30.262-29.405, 27.312-27.267, 24.090, 22.914, 19.927, 14.346; HRMS calculated for $C_{28}H_{56}O$ (M^{+}) 408.4331, found 408.4341, ppm 2.4.