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New Contact Sex Pheromone Components of the German Cockroach, *Blattella germanica*, Predicted from the Proposed Biosynthetic Pathway

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Abstract Upon contacting the cuticle of a sexually mature female, a male German cockroach exhibits a characteristic courtship behavior: he turns away from the female and raises his wings, thereby exposing tergal glands. The glandular secretion stimulates the female to mount the male and feed, thus positioning her appropriately for copulation. A multicomponent contact sex pheromone produced by females is responsible for eliciting courtship behavior. The most abundant pheromone components are 3,11-dimethylnonacosan-2-one and 3,11-dimethylheptacosan-2-one, oxidation products of the abundant hydrocarbon analogs 3,11-dimethylnonacosane and 3,11-dimethylheptacosane, respectively. The C_{29}-dimethyl ketone is thought to be further metabolized to two less abundant pheromone components, 29-hydroxy-3,11-dimethylnonacosan-2-one and 29-oxo-3,11-dimethylnonacosan-2-one. Based on this proposed biosynthetic pathway of pheromone production, we hypothesized that 3,11-dimethylheptacosan-2-one also would be oxidized to give two candidate pheromone components, 27-hydroxy-3,11-dimethylheptacosan-2-one, and 27-oxo-3,11-dimethylheptacosan-2-one. By using bioassay-guided fractionation and chemical analyses of cuticular extracts of virgin females and synthesis of the (3*S*,11*S*)-isomer of each of the two predicted pheromone components, we showed that the epicuticle of the German cockroach does indeed contain these two compounds. The contact sex pheromone of the female German cockroach, thus may consist of at least six biosynthetically related components.

Keywords 27-Oxo-3,11-dimethylheptacosan-2-one · 27-Hydroxy-3,11-dimethylheptacosan-2-one · Sex pheromone · *Blattella germanica* · German cockroach · Methyl ketone · Alcohol · Aldehyde

Introduction

Sex pheromones of insects are most often produced and emitted as blends of related chemicals rather than single components. Furthermore, in recent years, it has become apparent that many pheromone molecules are produced from the action of tissue-specific enzymes on intermediates of fatty acid metabolic pathways, giving rise, among other compounds, to related hydrocarbons, alcohols, ketones, epoxides, acetates, and aldehydes (reviewed by Howard and Blomquist 2005). In many cases, each of the pheromone components alone is relatively ineffective, but a blend of two or more components may act as a "minimal blend" that stimulates attraction and/or courtship behavior (McNeil 1991). Unlike the vast majority of volatile sex pheromones, however, each of the four known contact sex pheromone components of the German cockroach, *Blattella germanica*, can independently elicit courtship, and some "minor" less abundant components are far more active than the major most abundant components (reviewed by Gemeno and Schal 2004). Therefore, chemical communication in *B. germanica* is of interest not only because this species is a pest of medical and veterinary importance but also because the chemistry, biochemistry, and behavioral

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ecology of its sexual communication system are relatively well understood.

The German cockroach lives in aggregations. Nevertheless, sexually receptive females release a volatile pheromone, blattellaquinone (Nojima et al. 2005), that attracts males from a distance. Upon contact with the antennae and mouth parts, the male perceives the female's contact sex pheromone on her cuticle and performs a characteristic courtship display. This behavior includes a rotation of the male's body, turning away from the female so as to orient his abdominal tip toward the female's head. Thus positioned, he raises the wings to almost 90°, exposing specialized glands on the seventh and eighth tergites. These glands secrete a mixture of lipids, proteins, and sugars that synergistically serve as feeding stimulants (Nojima et al. 1999a, b, 2002; Kugimiya et al. 2002). The female then mounts the male to feed on the tergal secretion, and this places her in the correct alignment for copulation (Roth and Willis 1952). Hence, the contact sex pheromone blend encodes sex- and species-specific information, and it triggers and mediates close-range sexual behavior. It is, therefore, imperative for mating.

The most abundant component of the female contact sex pheromone is (3*S*,11*S*)-dimethylnonacosan-2-one (**1**, Fig. 1; Nishida et al. 1974), which is derived through hydroxylation and subsequent oxidation of the abundant cuticular hydrocarbon 3,11-dimethylnonacosane (Chase et al. 1992). Based on the well-established biochemical scheme of conversion of hydrocarbons to methyl ketone pheromones in the housefly (reviewed by Blomquist 2003) and the presence of the hydrocarbon 3,11-dimethylheptacosane on the cuticular surface of females, we predicted and later confirmed through synthesis that 3,11-dimethylheptacosan-2-one (**4**) also served as a sex pheromone component in this cockroach (Jurenka et al. 1989; Schal et al. 1990; Eliyahu et al. 2004). This component is less abundant on the female's cuticular surface, and its biological activity is significantly lower in dose-response studies than that of its C_{29} homolog.

Two less abundant sex pheromone components have also been identified: the alcohol 29-hydroxy-3,11-dimethylnonacosan-2-one (**2**) and the aldehyde 29-oxo-3,11-dimethylnonacosan-2-one (**3**). Interestingly, the alcohol has been shown to be about tenfold more effective at eliciting behavioral responses than the C_{29}-dimethyl ketone (reviewed by Nishida and Fukami 1983). The biochemical pathway that gives rise to the alcohol and aldehyde components has not been elucidated, but Chase et al. (1992) proposed that female-specific hydroxylation and subsequent oxidation reactions that result in the dimethyl ketones might act at the C-29-position of the dimethyl ketone to produce the 29-hydroxy- and 29-oxo-dimethyl ketone pheromone components.

Although this hypothesis has yet to be tested with labeled precursors, it predicts that if the same mechanism converts 3,11-dimethylheptacosane to the corresponding dimethyl ketone pheromone, then its two oxidation analogs, 27-hydroxy- and 27-oxo-3,11-dimethylheptacosan-2-one, might also be present as pheromone components in the female German cockroach. We now report behavioral and analytical results that show that 27-hydroxy-3,11-dimethylheptacosan-2-one and 27-oxo-3,11-dimethylheptacosan-2-one (**5** and **6**, respectively, Fig. 1) are found on the cuticular surface of adult females, and we confirm their pheromonal activity by synthesis and bioassay of the synthetic compounds.

Methods and Materials

Insects Blattella germanica cockroaches, representing an insecticide-susceptible strain originally obtained from American Cyanamid in 1989, were kept in groups at 27°C under a 12:12 L/D photoperiod with continuous access to dry LabDiet rat chow (#5001; PMI Nutrition International, Brentwood, MO, USA) and water. Nymphs that hatched within 2–3 d were reared in synchronous cohorts, and newly emerged adult males and females were separated daily from cages containing late instar nymphs.

Behavioral Assay Male sexual response was tested by using a modification of the assay developed by Roth and Willis (1952). An antenna of a 14- to 21-d-old adult male *B. germanica* was excised, inserted into a small mass of

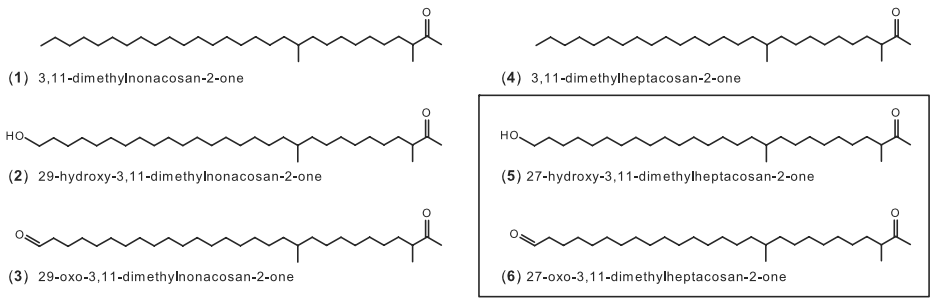

Fig. 1 Components of the contact sex pheromone of female *B. germanica*. Compounds **5** and **6**, *within the box*, are identified as new pheromone components in this paper

modeling clay at the end of a glass Pasteur pipette, and extracted for 1–2 sec in hexane to remove cuticular lipids. A test fraction or standard solution was applied with a 10-µl syringe (Hamilton, Reno, NV, USA) in 3 µl hexane to the distal 1 cm of the test antenna. The hexane was allowed to evaporate, and the antenna was used immediately to test the responses of at least 3 groups of 10 males, 14- to 21-d-old, that were housed individually in 9×9×7.5-cm plastic cages supplied with rat chow and water. All assays were conducted during mid-scotophase, avoiding the first and last 2 hr of the scotophase. The antennae of each male were gently stroked with the test antenna, and 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 sec. This is an unmistakable response that occurs only in a sexual context and is never elicited by male test antennae either unfortified with female pheromone or treated with hexane alone.

This behavioral assay was used to identify chromatographic fractions that elicited behavioral responses, to conduct dose-response studies with synthetic compounds, and to test for synergistic interactions among the three C_{27} pheromone components.

Extraction and Fractionation Sexually mature virgin females, 6- to 7-d-old, were freeze-killed and extracted in groups of 50 females in a 20-ml vial with ~6 ml hexane (Optima; Fisher Scientific, Waltham, MA, USA) for 1 min. The extract was transferred to a clean vial, and the hexane was slowly reduced to 100 µl under a gentle stream of N_2. The extract was fractionated by flash column chromatography: 14.5-cm glass Pasteur pipettes loaded with 200 mg of silica gel (100–200 mesh; Fisher Scientific) were activated at 110°C for 30 min and washed with ~1 ml hexane. The extract was loaded and eluted successively with 4 ml hexane, 2 ml each of 1, 2, 5, 10, 20, and 40% diethyl ether (Optima; Fisher Scientific) in hexane, 2 ml diethyl ether, 2 ml ethyl acetate (Optima; Fisher Scientific), and 2 ml methanol (HPLC grade; Fisher Scientific). Each fraction was tested in the courtship behavioral assay, and active fractions were further fractionated on a normal phase

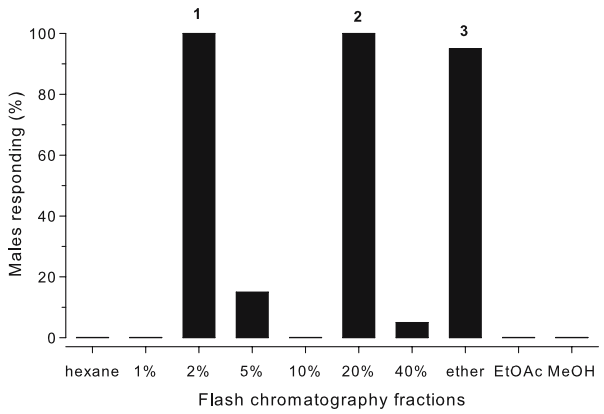

Fig. 2 Percentage of males (N>90 per fraction) responding to flash column chromatography fractions of female cuticular extract (0.05 female equivalents). The fractions in which synthetic compounds **1** (3,11-dimethylnonacosan-2-one), **2** (29-hydroxy-3,11-dimethylnonacosan-2-one), and **3** (29-oxo-3,11-dimethylnonacosan-2-one) would elute are indicated. Numbers (fractions) between hexane and ether represent percent ether in hexane. *EtOAc* Ethyl acetate, *MeOH* methanol

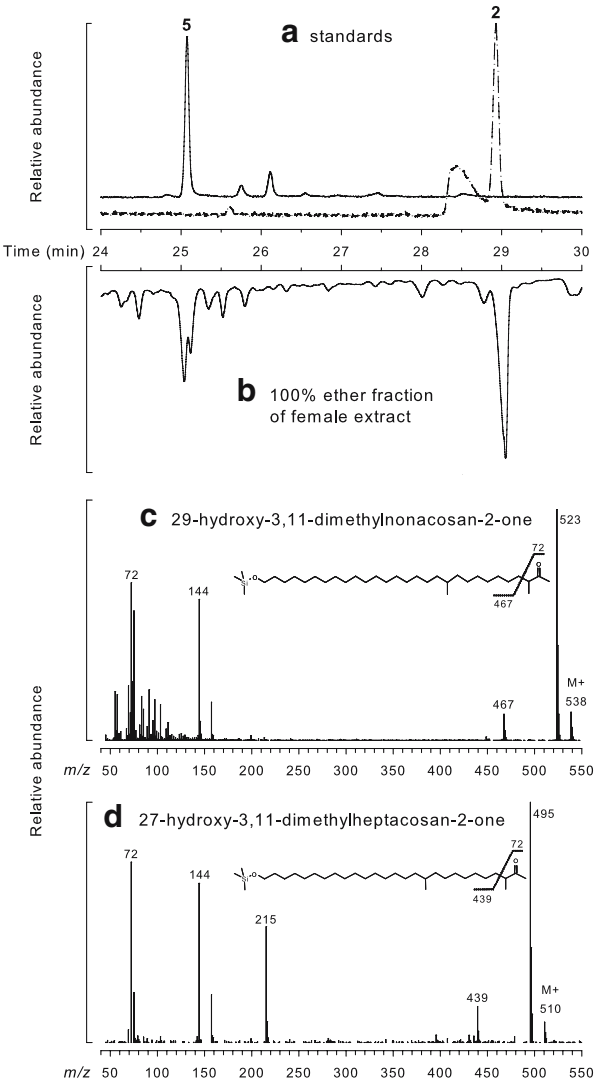

Fig. 3 Gas chromatograms and mass spectra of synthetic and natural alcohols. **a** Two separate total ion chromatograms of 160 ng of the silylated synthetic compound **2**, 29-hydroxy-3,11-dimethylnonacosan-2-one (*dotted line*) and 100 ng of the silylated synthetic compound **5**, 27-hydroxy-3,11-dimethylheptacosan-2-one (*solid line*). **b** The silylated 100% ethyl ether fraction of a composite extract of 120 females fractionated by flash column chromatography. **c** and **d** The 70-eV electron impact mass spectra of peaks in the silylated extract of females with retention times corresponding with those of compounds **2** and **5**, respectively. The fragment at *m*/*z* 72 is the result of McLafferty rearrangement of the 3-methyl-2-one-moiety

high performance liquid chromatography (HPLC) column (Econosphere, 5 μm silica, 250×4.6 mm; Alltech, Deerfield, IL, USA) on an HP1050 HPLC (Hewlett-Packard, Palo Alto, CA, USA). Supellapyrone (Charlton et al. 1993) was added as internal standard and monitored at 296 nm with an HP1050 diode array detector; the *B. germanica* sex pheromone components have no UV absorption. The sample was eluted isocratically at 1 ml min^{-1} with 99% hexane and 1% 2-propanol (HPLC grade; Fisher Scientific). One-minute fractions were collected and tested in behavioral bioassays on at least 30 males.

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 of compounds in active fractions. The GC was operated in splitless injection mode and fitted with a 30-m×0.25-mm ID DB-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 quadrupole 150°C, MS source 230°C, and transfer line 250°C.

Microchemical Reactions 1,1-Dimethylhydrazine (DMH, 98% pure; Sigma-Aldrich, St. Louis, MO, USA) derivatization was used to stabilize the thermally unstable oxo-dimethyl ketones (Nishida and Fukami 1983). The biologically active HPLC fraction was concentrated under N_2 to ~50 μl in a conical reaction vial, DMH (5 μl) was added, the vial was capped and incubated in a 60°C glass bead bath for 30 min, and the resulting solution immediately subjected to gas chromatography–mass spectrometry (GC-MS) analysis.

Silylation of the hydroxyl group was conducted before GC-MS analysis of the hydroxy-dimethyl ketones. The biologically active 100% ether fraction from flash chromatography of 120 females was concentrated under N_2 in a conical reaction vial, 80 μl *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA, Alltech) were added, the reaction mixture was incubated in an 80°C bead bath for 30 min, and the mixture was analyzed immediately by GC-MS.

Synthesis of 27-Hydroxy- and 27-Oxo-3,11-dimethylheptacosan-2-one (3*S*,11*S*)-27-Hydroxy-3,11-dimethylheptacosan-2-one and its oxidation product (3*S*,11*S*)-27-oxo-3,11-dimethylheptacosan-2-one were synthesized by K.M. The synthesis was executed based on an improved version of the previous synthesis of (3*S*,11*S*)-29-hydroxy-3,11-dimethylnonacosan-2-one (Mori et al. 1981). Oxidation of the alcohol to the aldehyde was carried out with Dess–Martin periodinane to avoid racemization at C-3. Details of the synthesis will be published separately by K.M. We used an enantioselective synthesis because the natural isomer of compound **1** is (3*S*,11*S*). We surmise that the natural isomers of compounds **4**, **5,** and **6** also have the (3*S*,11*S*) configuration, although this has not been shown analytically.

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 the 3 components. The analysis was performed with SAS (SAS Institute 2003).

Results

Identification of New Components Three flash chromatography fractions (2, 20, and 100% ether fractions) elicited sexual responses from male cockroaches in behavioral bioassays (Fig. 2). These fractions corresponded to the synthetic pheromone components 3,11-dimethylnonacosan-2-one (**1**, Fig. 1), 29-oxo-3,11-dimethylnonacosan-2-one (**3**, Fig. 1), and 29-hydroxy-3,11-dimethylnonacosan-2-one (**2**, Fig. 1), respectively. The elution pattern of the components corresponded to that reported by Nishida and Fukami (1983).

Table 1 Amounts and ratios of 6 sex pheromone components on the cuticular surface of adult female *B. germanica*

Pheromone Component	C_{27} Components[d]		C_{29} Components[d]	
	Amount (ng)	Ratio	Amount (ng)	Ratio
Dimethylketone[a]	97	100	470	100
Hydroxy-dimethylketone[b]	5.3	5.5	25.6	5.4
Oxo-dimethylketone[c]	0.15	0.15	0.5	0.11

[a] 3,11-Dimethylheptacosan-2-one and 3,11-dimethylnonacosan-2-one, respectively
[b] 27-Hydroxy-3,11-dimethylheptacosan-2-one and 29-hydroxy-3,11-dimethylnonacosan-2-one, respectively
[c] 27-Oxo-3,11-dimethylheptacosan-2-one and 29-oxo-3,11-dimethylnonacosan-2-one, respectively
[d] Amounts and ratios of sex pheromone components determined from GC analyses of a composite extract of the cuticular surface of 100 virgin adult females, adjusted to nanogram per female. Ratios relative to 100% of the respective dimethyl ketone

To determine if the 100% ether fraction contained the predicted 27-hydroxy-3,11-dimethylheptacosan-2-one, hydroxylic components in this fraction were converted to silyl ethers and subjected to GC-MS analysis. The silyl ether of compound **2**, 29-hydroxy-(3*S*,11*S*)-dimethylnonacosan-2-one (Fig. 3a) was found at retention time 29.04 min, and its identity was confirmed by comparing the GC retention time and MS fragmentation pattern with those of the authentic compound (Fig. 3c). The silyl ether of the predicted candidate 27-hydroxy-3,11-dimethylheptacosan-2-one was found at retention time 25.14 min (Fig. 3b) by careful examination of the fragmentation patterns of all components. The mass spectrum of compound **5** was somewhat noisy because of its low abundance in the extract and other components eluting very close to it. However, it showed diagnostic fragments at *m*/*z* 510 (expected molecular weight, 0.08 intensity), *m*/*z* 495 (base peak, loss of a methyl group), an intense *m*/*z* 72 from McLafferty rearrangement of the 3-methyl-2-one-moiety, supporting the structure of a methylketone with a methyl group in the alpha position (Fig. 3d). Silylation also produced a fragment at *m*/*z* 144 in compounds **2** and **5**. The intense fragment at *m*/*z* 215 in the MS of the silylated heptacosanone derivative appears to be an artifact resulting from compounds that co-elute with **5**. The identity of compound **5** was confirmed by comparing both its GC retention time and MS fragmentation pattern with those of the authentic compound.

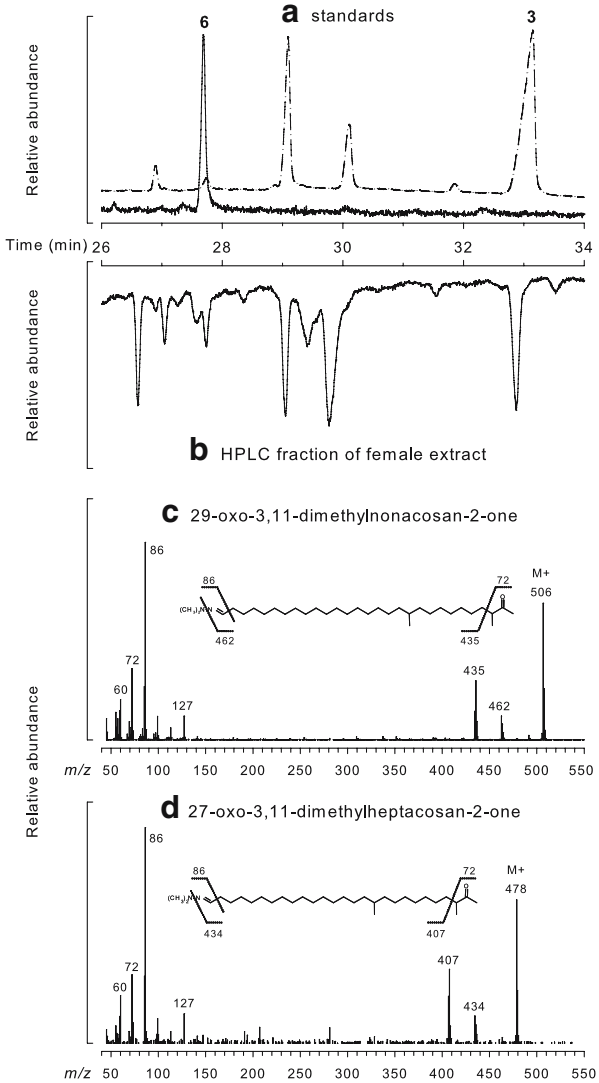

Fig. 4 Gas chromatograms and mass spectra of synthetic and natural aldehydes. **a** Two separate total ion chromatograms of 300 ng of the derivatized synthetic compound **3**, 29-oxo-3,11-dimethylnonacosan-2-one (*dotted line*), and 100 ng of the derivatized synthetic compound **6**, 27-oxo-3,11-dimethylheptacosan-2-one (*solid line*). **b** The similarly derivatized active HPLC fraction of the combined 20% ethyl ether fractions from a composite extract of 120 females fractionated by flash chromatography. **c** and **d** The electron impact mass spectra of peaks in the extract of females with retention times that corresponded with those of compounds **3** and **6**, respectively. The fragment at *m*/*z* 72 is the result of McLafferty rearrangement of the 3-methyl-2-one-moiety, and the intense fragment at *m*/*z* 86 represents McLafferty rearrangement of the 1,1-dimethylhydrazone moiety

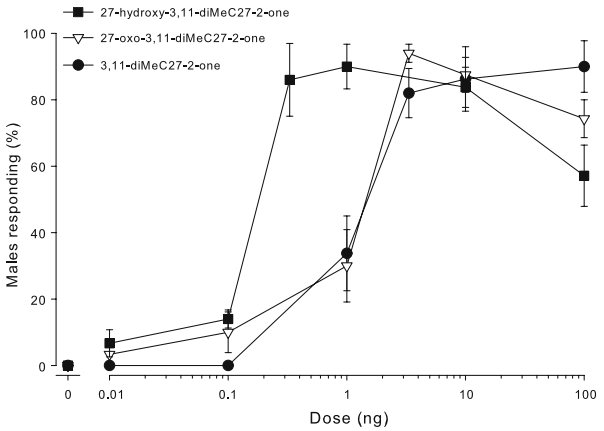

Fig. 5 Dose–response curves of 3 C_{27} sex pheromone components showing mean (±SE) percentage of males responding to 3,11-dimethylheptacosan-2-one (**4**), 27-hydroxy-3,11-dimethylheptacosan-2-one (**5**), and 27-oxo-3,11-dimethylheptacosan-2-one (**6**). (*N*=30–80 per compound/dose combination)

The amount of 27-hydroxy-3,11-dimethylheptacosan-2-one was estimated by comparison to authentic compound **2** to be ~5.3 ng per female, which is about fivefold less than the amount of 29-hydroxy-3,11-dimethylnonacosan-2-one found on the cuticular surface of sexually receptive females. Each of these alcohols is about 18-fold less abundant than the equivalent chain length dimethyl ketone from which the alcohol presumably arises (Table 1).

The 20% ether fraction was subjected to further bioassay-guided fractionation by HPLC. Two discrete fractions that elicited behavioral responses were obtained: the first 1-min fraction co-eluted with synthetic compound **3**, 29-oxo-3,11-dimethylnonacosan-2-one, whereas the second fraction

Table 2 Probit analysis of dose-response studies of 3 sex pheromone components

Sex Pheromone Component	Slope	Intercept	RD_{50} (ng)[a]	95% Fiducial Limits
3,11-Dimethylheptacosan-2-one (**4**)	0.590	−0.376	1.67 a	0.814, 2.818
27-Hydroxy-3,11-dimethylheptacosan-2-one (**5**)	0.763	0.664	0.18 b	0.042, 0.456
27-Oxo-3,11-dimethylheptacosan-2-one (**6**)	1.105	−0.093	1.18 a	0.567, 2.199

[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).

eluted several minutes later (data not shown). To determine if the first fraction also contained the predicted 27-oxo-3,11-dimethylheptacosan-2-one, aldehyde components in this fraction were reacted with 1,1-dimethylhydrazine and subjected to GC-MS analysis. The hydrazone derivative of compound **3** (Fig. 4a) was found at retention time 32.87 min, and the identity was confirmed by comparing the GC retention time and MS fragmentation pattern with those of the authentic compound (Fig. 4b). The hydrazone derivative of the predicted candidate 27-oxo-3,11-dimethylheptacosan-2-one was found at retention time 27.74 min (Fig. 4a) by screening of the fragmentation patterns of all components. Compound **6** showed diagnostic fragments at *m/z* 72 (0.32 intensity relative to base peak) and 86, indicating McLafferty rearrangements of alpha-methyl ketone and 1,1-dimethylhydrazone moieties, $–CH_2–CH(CH_3)–CO–CH_3$ and $–CH=N–N(CH_3)_2$, respectively. In addition, it showed diagnostic fragments of the 1,1-dimethylhydrazone derivative at *m/z* 407, 434, and 478, from M^+-44 and M^+-86, and the expected molecular ion of the derivative, respectively (Fig. 4c). The identity of compound **6** was confirmed by comparing the GC retention time and MS fragmentation pattern with those of the authentic compound. Although DMH reacts with carbonyl groups in general, the proximity of the ketone group to the methyl branch in compounds **3** and **6** appears to hinder its reaction with the carbonyl group at position 2. Accordingly, derivatizations of compound **1** did not result in a hydrazone (data not shown), and we did not find products with 2-hydrazone moieties.

The amount of 27-oxo-3,11-dimethylheptacosan-2-one, estimated by comparison with known amounts of 29-oxo-3,11-dimethylnonacosan-2-one, was ~0.15 ng per female. Thus, the ratio of the C_{29} aldehyde to its C_{27} homolog is about 4:1. These oxo-dimethyl ketone components were about 40-fold less abundant than the respective alcohols (compounds **2** and **5**, Table 1).

The 20% ether fraction from flash column chromatography also contained a second active fraction that eluted several minutes after the 27-oxo- and 29-oxo-dimethyl ketones in normal phase HPLC. It may represent additional sex pheromone components whose compositions are currently under investigation.

Biological Activity of the Synthetic New Pheromone Components The biological activities of 3,11-dimethylheptacosan-2-one, 27-hydroxy-3,11-dimethylheptacosan-2-one, and 27-oxo-3,11-dimethylheptacosan-2-one were tested at various doses on 3–8 groups of 10 individually housed males. 27-Hydroxy-3,11-dimethylheptacosan-2-one (**5**) was about tenfold more active than the other two C_{27} components (Fig. 5). The estimated doses to which 50% of the males responded for each component (RD_{50}) are presented in Table 2. The RD_{50} values for compounds **4** and **6** were similar, but significantly higher than for compound **5**, as demonstrated by both logistic analysis and non-overlapping 95% fiducial limits.

The activity of 3,11-dimethylheptacosan-2-one was compared in a dose-response study to the activity of a blend of 3,11-dimethylheptacosan-2-one, 27-hydroxy-3,11-dimethylheptacosan-2-one, and 27-oxo-3,11-dimethylheptacosan-2-one in a 100:10:1 ratio. No synergism between the different C_{27} compounds was observed: the activity of a mixture of 100:10:1 methyl ketone/alcohol/aldehyde was not significantly different from that of the methyl ketone alone (Fig. 6).

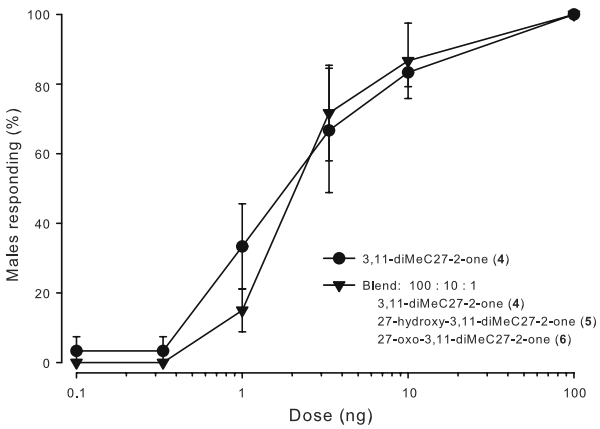

Fig. 6 Results of behavioral assays showing interaction among C_{27} sex pheromone components. 3,11-Dimethylheptacosan-2-one (**4**) was compared to a blend of 3,11-dimethylheptacosan-2-one, 27-hydroxy-3,11-dimethylheptacosan-2-one (**5**), and 27-oxo-3,11-dimethylheptacosan-2-one (**6**) in a ratio of 100:10:1, respectively. Data show mean percentage of males responding (±SE; N=30–60 per compound/dose combination)

Discussion

Semiochemicals are generally identified by the well-established approach of bioassay-guided sequential fractionation of extracts followed by chemical analysis, structure elucidation, and confirmation of biological activity with synthesized authentic compounds. A complementary approach predicts possible pheromone components based on an understanding of the chemistry and biochemistry of pheromone production coupled with knowledge of the evolutionary relationships among taxa. For example, Bjostad et al. (1984) predicted the structures of sex pheromone components of the cabbage looper, *Trichoplusia ni*, based on knowledge of biosynthetic pathways and fatty acid precursors. The pheromone gland of this noctuid moth utilizes acetate to produce octadecanoic and hexadecanoic acids which undergo Δ11-desaturation to produce Z11-18: acid and Z11-16:acid. However, the major component of the sex pheromone is (*Z*)-7-dodecen-1-yl acetate (Z7-12:OAc). When labeled Z11-16:acid was applied to glands, it was incorporated into both Z7-12:acid and the corresponding acetate ester pheromone. Because the main pheromone component was produced through a Δ11-desaturation followed by chain shortening, reduction, and acetylation to form the acetate ester, Bjostad et al. (1984) reasoned that other pheromone components would be produced from intermediates in the chain shortening sequence. Indeed, other acetate esters, including Z9-14:OAc, Z7-14:OAc, and Z5-12:OAc, were found to be pheromone components, the first derived from chain shortening of Z11-16:acid and the latter two from Z11-18:acid. In a similar manner, pheromone components of the spruce budworm (*Choristoneura fumiferana*) were predicted based on identification of fatty acids in the pheromone gland and their temporal variation in relation to pheromone production (Silk and Kuenen 1986), although in this case, the proposed components were not shown conclusively to be biologically active. Overall, few pheromone components have been identified from predictions based on biosynthetic pathways. In this paper, we confirmed that candidate compounds, predicted based on the biosynthetic pathways of related contact sex pheromone components, do indeed serve as pheromone components in the German cockroach.

In insects, cuticular hydrocarbons and hydrocarbon pheromones are formed through fatty acid elongation followed by decarboxylation (reviewed by Howard and Blomquist 2005). For example, in the housefly, the sex pheromone component (*Z*)-9-tricosene is first formed, and this alkene is then metabolized by a cytochrome-P450 enzyme to the corresponding epoxide and ketone, which also serve as sex pheromone components (reviewed by Blomquist 2003). This conversion of hydrocarbons to methyl ketone and epoxide pheromone components in the housefly served as a guide for our research on pheromone biosynthesis in the German cockroach.

The major cuticular lipids in all life stages of the German cockroach consist of an isomeric mixture of 3,7-, 3,9-, and 3,11-dimethylnonacosane (Jurenka et al. 1989). However, the dimethyl ketone fraction of cuticular extracts of adult females, which contains the pheromone 3,11-dimethylnonacosan-2-one, comprises only the 3,11-isomer. This led Jurenka et al. (1989) to propose a biosynthetic relationship between the hydrocarbon and its dimethyl ketone pheromone analog. Using radiolabeled compounds coupled with radio-GC, Chase et al. (1992) determined that the dimethyl ketone sex pheromone arises from the insertion of an oxygen into the preformed 3,11-dimethyl alkane. Radioactivity from the alkane was detected in both 3,11-dimethylnonacosan-2-ol and 3,11-dimethylnonacosan-2-one, but only in adult females, whereas when tritiated 3,11-dimethylnonacosan-2-ol was applied to the cuticle, it was readily and highly efficiently converted to the corresponding dimethyl ketone pheromone not only in females but also in males. These results confirmed that the dimethyl ketone sex pheromone of *B. germanica* arises via a female-specific hydroxylation of 3,11-dimethylnonacosane and a subsequent non-sex-specific oxidation to 3,11-dimethylnonacosan-2-one.

The same mechanism presumably converts 3,11-dimethylheptacosane, a component of the cuticular hydrocarbon profile of the German cockroach, to 3,11-dimethylheptacosan-2-one. Dose-response studies with both natural and synthetic 3,11-dimethylheptacosan-2-one confirmed its biological activity, although it was found to be approximately tenfold less active than its C_{29} homolog (Schal et al. 1990; Eliyahu et al. 2004).

Chase et al. (1992) previously proposed that a similar hydroxylation and oxidation at the C-29 position of 3,11-dimethylnonacosan-2-one might give rise to 29-hydroxy- and 29-oxo-3,11-dimethylnonacosan-2-one, the other components of the contact sex pheromone blend, and that the same mechanism might convert 3,11-dimethylheptacosan-2-one to its 27-hydroxy- and 27-oxo- analogs.

Identification of the C_{27} alcohol and aldehyde as sex pheromone components was fraught with difficulties, and it is not surprising that earlier studies with cuticular extracts of more than 200,000 females did not reveal these compounds. The two active homologs in each class (dimethyl ketones, alcohols, aldehydes) differ only in chain length and can only be separated by high performance fractionation methods, such as preparative GC. Furthermore, the aldehydes are thermally unstable, and derivatization is advisable before GC analysis. Also, silylation of the hydroxy group in the alcohols renders the analysis easier because the derivatives are less polar, and hence, less susceptible to adsorption by active sites on the column or in the injector. Moreover, both the C_{27} alcohol and aldehyde

occur in minute amounts that are largely obscured by other compounds in the extracts. Therefore, predictions based on the biosynthetic pathway of the C_{29} components were crucial for the location, recognition, and identification of the three C_{27} components.

Our finding of the predicted C_{27} compounds in the cuticular pheromone blend, and in a similar blend ratio to that of the C_{29} components, further corroborates the biosynthetic pathway proposed and partly demonstrated by Chase et al. (1992; reviewed by Schal et al. 2003). Furthermore, our results provide motivation for a reexamination of other hydrocarbons that might serve as precursors for methyl ketones and their oxidation derivatives that might also mediate behavioral responses. A prime candidate for consideration is 3,11-dimethylhentriacontane, which occurs on the cuticular surface in tiny amounts (Carlson and Brenner 1988). Structure–activity studies on 3,11-dimethylnonacosan-2-one indicate that the apparently promiscuous pheromone receptor of *B. germanica* males accepts chain lengths that are slightly shorter or longer than the optimal 29-carbon alkyl chain (Nishida and Fukami, 1983). Because the C_{27} homolog elicited behavioral responses, we predict that the C_{31} homolog will also show biological activity.

Although the 3,11-dimethyl structure is important for eliciting behavioral response, structure–activity studies have shown that both the 3-monomethyl and 11-monomethyl compounds are also biologically active, albeit at much higher doses (Nishida and Fukami 1983). The epicuticular lipids of *B. germanica* contain 11-methylheptacosane, 11-methylnonacosane, 11-methyltriacontane, 3-methylheptacosane, and 3-methylnonacosane, as well as dimethylalkanes that include an 11-methyl branch, including 11,15-dimethylheptacosane, 11,15-dimethylnonacosane, 5,11-dimethylheptacosane, and 5,11-dimethylnonacosane (Augustynowicz et al. 1987; Carlson and Brenner 1988; Jurenka et al. 1989). It is possible that the same mechanism that inserts a C-2 carbonyl into the preformed dimethylalkane might do the same with monomethyl alkanes of the proper chain length. We recently discovered that oxidation metabolites of 11-methylheptacosane are indeed biologically active. Nishida and Fukami (1983) found that when antennae of the Oriental cockroach, *Blatta orientalis*, stroked against the antennae of male German cockroaches, courtship was elicited in the latter species. Through fractionation and bioassays, we have recently identified two more active compounds: 11-methylheptacosan-2-one and 27-oxo-11-methylheptacosan-2-one (Eliyahu et al. 2008). 11-Methylheptacosane is abundant on the cuticular surface of the Oriental cockroach (Lockey and Dularay 1986), and it is likely that in this cockroach too, the methyl ketone and aldehyde are oxidation products of the hydrocarbon. 3-Methyl and 11-methyl hydrocarbons of the appropriate chain length are present in the cuticular lipids of German cockroach females, and they similarly could be converted to biologically active methyl ketones, alcohols, and aldehydes.

Our dose-response results with synthetic C_{27} components are in agreement with similar results from the C_{29} components (Nishida and Fukami 1983). Both the C_{29} and C_{27} primary alcohols were about tenfold more active than the respective methyl ketones, whereas the aldehydes were not significantly more active than the respective methyl ketones of the same chain length. Unlike most insect pheromone blends, especially in the case of volatile pheromones, each of the 6 contact sex pheromone components can independently elicit the complete repertoire of sexual responses. Schal et al. (1990) found that the 2 methyl ketone components of the pheromone interacted additively, not synergistically. We show here that there is also no synergism between different component classes. Therefore, it appears that the total semiochemical activity might be achieved by additive effects, with low amounts of the more polar compounds being compensated for by their lower thresholds for eliciting courtship responses.

Further studies are necessary to determine the definitive number of components in the contact sex pheromone blend of the female German cockroach. Two points of caution are worth mentioning. First, the sex pheromone reception system of the male German cockroach exhibits a highly promiscuous response, responding to compounds that deviate significantly from the native pheromone. Indeed, it represents one of the few cases where the native pheromone can be significantly improved upon, for example by changing the C-2 carbonyl to a C-2 hydroxy group. This might explain in part the frequent observations of interspecific courtship in cockroaches, as described above. It also highlights the important point that for a pheromone component to be considered as such, it must not only elicit a response from the receiver but also must be present on the producer.

Second, the relative behavioral value to the male of each female pheromone component must be considered. The dimethyl ketone generally constitutes 10% of the abundance of its precursor hydrocarbon analog (Chase et al. 1992), and the alcohol and aldehyde occur at ~5 and ~0.1% of the respective parent dimethyl ketone (Table 1). Assuming that other, yet to be identified, semiochemicals occur at similar ratios, it seems that if they arise from relatively poorly represented hydrocarbons, they might occur in subnanogram amounts. Moreover, these compounds are presumably spread over the total cuticular surface and possibly buried within hundreds of micrograms of cuticular hydrocarbons. It remains to be determined how much of each component is "operationally functional", that is, perceived by the male when he briefly strokes the female's antennae. We previously determined that each female antenna contains about 1 ng of 3,11-dimethylnonacosan-2-one and 0.4 ng of

3,11-dimethylheptacosan-2-one, closely matching the minimal amounts required to elicit courtship behavior from males as determined from dose-response studies (Eliyahu et al. 2004). It is conceivable that many minor components that could potentially act as pheromones at appropriate doses are represented on the female's cuticle and antennae at amounts substantially below the male's threshold of detection, and thus, they may represent "noise" arising from biosynthetic processes that are not completely selective. We suggest that such compounds should only be labeled as pheromone components if they elicit responses in dose ranges within which they are naturally found on the producer.

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