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BEHAVIORAL ACTIVITY OF STEREOISOMERS AND A NEW COMPONENT OF THE CONTACT SEX PHEROMONE OF FEMALE GERMAN COCKROACH, *Blattella germanica*

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Abstract—(3*S*,11*S*)-3,11-Dimethylnonacosan-2-one is a major component of the courtship stimulating, contact sex pheromone of the female German cockroach. Although the four synthetic stereoisomers of this compound have been tested in behavioral assays, their relative activity remains unresolved. Using isolated male antennae dosed with synthetic test compounds to assay male behavior, we found that at high doses all four stereoisomers elicited responses from 100% of the males. However, at physiologically relevant doses similar to those found on the female antenna, the (3*S*,11*S*)-isomer was the least effective of the four stereoisomers at eliciting courtship responses in males. This is the first example of a natural stereoisomer having less bioactivity than related stereoisomers that do not occur naturally. Another component of the sex pheromone blend, 3,11-dimethylheptacosan-2-one, was previously purified from the female's epicuticule and behaviorally assayed, but its activity was not confirmed through synthesis. We now confirm that synthetic (3*S*,11*S*)-3,11-dimethylheptacosan-2-one elicits behavioral responses, but less so than its C_{29} homolog.

Key Words—Contact sex pheromone, stereoisomers, German cockroach, behavioral assay, 3,11-dimethylnonacosan-2-one, 3,11-dimethylheptacosan-2-one.

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INTRODUCTION

Upon contact with a mature female, sexually mature males of the German cockroach, *Blattella germanica* (L.), exhibit characteristic courtship behavior that includes an unmistakable raising of the wings and turning away from the female (Roth and Willis, 1952; Nishida et al., 1974; review: Gemeno and Schal, 2004). This act exposes specialized tergal glands on the 7th and 8th abdominal segments of the male, which in turn attract the female to mount the male's abdomen. As the female feeds on nutrients within reservoirs of the tergal glands, she is properly aligned in a precopulatory position (Nojima et al., 1999). The elicitor of this behavioral sequence is a contact sex pheromone blend composed of several oxygenated derivatives of methyl-branched cuticular hydrocarbons. The most abundant component is 3,11-dimethylnonacosan-2-one (3,11-diMeC_{29}-2-one), while two other components, 29-hydroxy-3,11-dimethylnonacosan-2-one and 29-oxo-3,11-dimethylnonacosan-2-one (review: Nishida and Fukami, 1983) are presumably derived from it. The stereochemistry of the natural pheromone was suggested as 3*S*, by comparison of its optical rotation to that of a methyl ketone with an authentic *S*-configuration, together with its NMR measurement in the presence of a chiral shift reagent (Nishida et al., 1974). Its 11*S*-stereochemistry could be rigorously determined only after the synthesis of all four stereoisomers of 3,11-dimethylnonacosan-2-one, followed by comparison of their infrared spectra (as crystals in KBr disks, not as solutions), specific rotations, and melting points to those of the natural pheromone. 500 MHz ^{1}H or 125 MHz ^{13}C-NMR comparisons were useless, all the isomers having shown identical spectra. The final proof of the (3*S*,11*S*)-stereochemistry of the natural pheromone rested on the observed lack of melting point depression (mp 44–45°C) in a mixed melting point determination of the (3*S*,11*S*)-isomer (mp 44–44.5°C) with the natural pheromone (mp 45–46°C), whereas the mixtures of each of the remaining three stereoisomers with the natural pheromone melted at the range between 33.5–37.5°C (Mori et al., 1981).

Synthesis of the four stereoisomers of 3,11-diMeC_{29}-2-one (Mori et al., 1981) enabled behavioral assays of their relative activity. Unlike the high degree of stereospecificity demonstrated in the antennae of many insects, in which unnatural stereoisomers are usually less active, or even have an antagonistic effect, the four stereoisomers of 3,11-diMeC_{29}-2-one were shown to be equally active (review: Nishida and Fukami, 1983). However, Abed et al. (1993) reported preliminary observations (data were not presented) that at low doses the (3*S*,11*S*)-isomer was more active than the others. The latter assays used higher purity stereoisomers synthesized by Mori and Takikawa (1990). In the present work we conducted dose-response behavioral assays with the four stereoisomers of 3,11-diMeC_{29}-2-one of Mori and Takikawa (1990), in an effort to resolve this discrepancy.

A putative fourth pheromone component, 3,11-dimethylheptacosan-2-one (3,11-diMeC_{27}-2-one), was purified by gas chromatography (GC) from female

cuticular lipids and shown to be behaviorally active (Jurenka et al., 1989; Schal et al., 1990). Its gross structure was confirmed by synthesis and mass spectral comparison (Takikawa et al., 1997). We now report on the biological activity of synthetic (3*S*,11*S*)-3,11-diMeC_{27}-2-one (Takikawa et al., 1997) and confirm its activity as a component of the sex pheromone.

METHODS AND MATERIALS

Insects. *Blattella germanica* cockroaches were kept in groups at 27°C under 12:12 light–dark photoperiod and fed dry Purina rat chow and water. Newly emerged adult males and females were separated daily from collectively reared nymphs. Wild cockroaches were collected with a modified vacuum cleaner from an infested commercial pig farm in Warsaw, NC. Adult males were isolated from the collection at least 3 d prior to using them in behavioral assays.

Chemicals and Bioassays. The four stereoisomers of 3,11-diMeC_{29}-2-one were synthesized by Mori and Takikawa (1990) and had >99% diastereomeric excess and ≈100% enantiomeric excess. (3*S*,11*S*)-3,11-Dimethylheptacosan-2-one was synthesized by Takikawa et al. (1997). Each compound was dissolved in hexane, and the concentration of each stock solution was confirmed by GC (HP5890II, HP-5 column 30 m × 0.25 mm × 0.25 μm, splitless injection) relative to *n*-hexacosane as internal standard.

Male behavioral responses were tested using a modification of the "antenna on a stick" assay developed by Roth and Willis (1952). An antenna of a 14–21 d-old adult male *B. germanica* was excised, attached to a glass Pasteur pipette, and either extracted briefly in hexane to remove male cuticular lipids prior to application of the test compound, or used fresh. A 3-μl hexane solution of a test compound was then applied 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 30 males or several groups of 10 males 14–21 d old that were housed individually in 8-cm-ID × 8-cm-deep 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. Each individual male was tested sequentially. The antennae of each male were gently stroked with the test antenna for up to 1 min, 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 1 min. This is an unmistakable response that occurs only in a sexual context and is never elicited by male test antennae unfortified with female pheromone or treated with hexane alone.

Amount of Pheromone on Female Antennae. Antennae from ten 5-d-old females were extracted in hexane containing 100 ng of internal standard (heptacosan-14-one). The base of each antenna was prevented from contact with hexane so no

internal lipids were extracted. Five groups of 10 paired antennae were extracted. The extracts were reduced under N_2 to 1 μl and injected into a capillary GC column, as above. The GC oven temperature was kept at 70°C for 1 min, then elevated 30°C per min to 150°C, and 10°C per min to 300°C. The amount of pheromone per antenna was calculated by comparison of the area of the pheromone peak to that of the internal standard.

Statistical Analysis. Dose response assays were analyzed using chi-square analysis to find a discriminating dose and pairwise chi-square comparison with Fisher's exact test within the discriminating dose. ANOVA was used to find differences in responses to various compounds at a single dose. All statistical analysis was performed with SAS (SAS Institute, 2000).

RESULTS AND DISCUSSION

Amount of Pheromone on Female Antennae. Males generally orient to the female's antennae before performing courtship. GC-FID analyses indicated that each antenna of 5-d-old adult females contained 0.99 $\pm$ 0.12 ng (SEM, $N = 5$) of 3,11-diMeC_{29}-2-one, the major component of the contact sex pheromone, and 0.409 $\pm$ 0.004 ng of 3,11-diMeC_{27}-2-one. It is important to note, however, that female antennae also contain minute amounts of other, more active pheromone components, including the 29-oxo- and 29-hydroxy-analogs of 3,11-diMeC_{29}-2-one. Moreover, by touching other parts of the female body with his antennae, a male would be exposed to as much as 250 μg and 100 μg of 3,11-diMeC_{29}-2-one and 3,11-diMeC_{27}-2-one, respectively (Schal et al., 1990).

(3S,11S)-3,11-Dimethylheptacosan-2-one. The synthetic (3*S*,11*S*)-3,11-diMeC_{27}-2-one elicited little response from males at 1 ng, but 100% of tested males exhibited courtship responses at doses $\geq$10 ng (Figure 1A). This result confirms that 3,11-diMeC_{27}-2-one is indeed a component of the sex pheromone of *B. germanica* and corroborates previous results showing that natural 3,11-diMeC_{27}-2-one was less active than the C_{29} methyl ketone (Schal et al., 1990). However, these results indicate that the amount of 3,11-diMeC_{27}-2-one on the female antennae is insufficient by itself to elicit the full sexual response in males. Rather, they indicate that for contact with the antennae alone to elicit the full courtship response, 3,11-diMeC_{27}-2-one would need to operate in concert with the C_{29} methyl ketone pheromone, and possibly other components.

3,11-DiMeC_{29}-2-one is derived from the major cuticular hydrocarbon 3,11-dimethylnonacosane (Chase et al., 1990; Schal et al., 2003). The other pheromone components, 29-oxo- and 29-hydroxy-C_{29}, are probably derived, in turn, from 3,11-diMeC_{29}-2-one. Because 3,11-dimethylheptacosane is also a component of the cuticular lipids, it is probable that 3,11-diMeC_{27}-2-one is derived from it. If

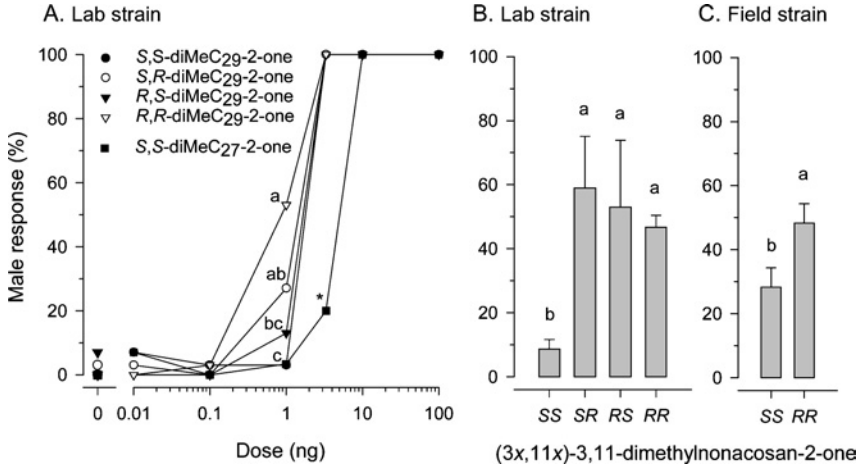

FIG. 1. Dose-behavioral response assays of the sexual responses of *Blattella germanica* adult males to four stereoisomers of 3,11-dimethylnonacosan-2-one and to (3*S*,11*S*)-3,11-dimethylheptacosan-2-one. In A, 30 males from a lab culture were assayed at each dose, whereas in B, a discriminating dose of 1 ng was used to assay three groups of 30 males each. In C, six groups of 10 males each, collected from a field population, were assayed with two of the stereoisomers. In A, different letters indicate significant differences based on χ^2 2×2 test of independence with Fisher's exact test; at all other doses there were no significant differences among treatments. Asterisk (*) indicates significantly higher responses to (3*S*,11*S*)-3,11-dimethylnonacosan-2-one than to (3*S*,11*S*)-3,11-dimethylheptacosan-2-one, at 3 ng. In B and C, different letters indicate significant differences based on ANOVA; SEM is shown for each mean.

so, it is possible that the C_{27} methyl ketone also gives rise to oxidation derivatives that might be present in the pheromone mixture. Because 29-hydroxy-3,11-diMeC$_{29}$-2-one is ~10-fold more active than the methyl ketone (Nishida et al., 1976), and 29-oxo-3,11-diMeC$_{29}$-2-one has intermediate activity between these two components (Nishida and Fukami, 1983), it is reasonable to expect that the C_{27} derivatives, if found, will show structure-activity patterns similar to the C_{29} pheromone components.

The stereochemistry of the naturally occurring 3,11-diMeC$_{27}$-2-one has not been established, and because no stereoselectivity was found in the male German cockroach for the major pheromone component, 3,11-diMeC$_{29}$-2-one (Figure 1A), bioactivity of the *S*,*S*-isomer does not necessarily demonstrate that it is the natural isomer. Nevertheless, it is reasonable to infer based on the (3*S*,11*S*) configuration of the other pheromone components that the 3*S*,11*S* configuration is most probable for 3,11-diMeC$_{27}$-2-one as well (Takikawa et al., 1997).

Stereoisomers of 3,11-Dimethylnonacosan-2-one. All four stereoisomers of 3,11-diMeC$_{29}$-2-one exhibited sharp dose-response curves ranging from background responses to 0.1 ng that were no different from responses to the solvent control, to 100% male response to 3 ng (Figure 1A). Surprisingly, however, these dose-response bioassays with carefully calibrated solutions showed that the *S*,*S*-isomer of 3,11-diMeC$_{29}$-2-one was significantly less active than the other three isomers (Figure 1A). This was unexpected because the *S*,*S*-isomer represents the natural configuration of 3,11-diMeC$_{29}$-2-one. Nevertheless, this observation was further confirmed with independent assays using freshly calibrated standard solutions and a discriminating dose of 1 ng loaded on the test antenna; again, 3*S*,11*S*-diMeC$_{29}$-2-one was significantly less active than the other three isomers (Figure 1B). This discriminating dose, interestingly, is similar to what is naturally found on a female antenna; and yet, a female antenna rarely fails to elicit courtship behavior in mature males. Therefore, either other pheromonal components are crucial for eliciting this high response, or the female antenna possesses textural/mechanical features that elicit higher sexual responses.

A third independent confirmation was obtained when activity of two of the synthetic isomers, *R*,*R*- and *S*,*S*-, was compared to the natural 3*S*,11*S*-diMeC$_{29}$-2-one that was extracted and purified from females by Nishida et al. (1974). The results are shown in Table 1. Male responses to the natural and synthetic 3*S*,11*S*-diMeC$_{29}$-one were similar, but significantly lower (ANOVA, $P < 0.05$) than to synthetic 3*R*,11*R*-diMeC$_{29}$-2-one.

It is possible that these unexpected results were an artifact of working with a cockroach colony that has been maintained in the laboratory for several decades. Therefore, we tested the *S*,*S*- and *R*,*R*-isomers of 3,11-diMeC$_{29}$-2-one on males that were freshly collected from an infestation in the field (Figure 1C). The results of this fourth assay were consistent with our previous findings that the natural stereoisomer was significantly less bioactive than one of the unnatural isomers, *R*,*R*.

In contrast, previous assays showed no significant differences in the doses of the four stereoisomers that were required to elicit courtship responses in males (Nishida et al., 1979; Nishida and Fukami, 1983). These differences might be

TABLE 1. MALE SEXUAL RESPONSES TO MALE ANTENNAE LOADED WITH NATURAL OR SYNTHETIC FEMALE SEX PHEROMONE

Compound tested (2 ng)	Males responding (%)[a]
Natural (3*S*,11*S*)-3,11-dimethylnonacosan-2-one	20.7 ± 4.7 a
Synthetic (3*S*,11*S*)-3,11-dimethylnonacosan-2-one	22.0 ± 3.3 a
Synthetic (3*R*,11*R*)-3,11-dimethylnonacosan-2-one	45.0 ± 7.2 b

[a] Fourteen groups of 10 males each were tested. SEM is shown for each mean. Different letters indicate significant differences ($P < 0.05$) based on ANOVA.

due to methodological differences. For example, the enantiomeric purity of the starting material [(*R*)-citronellic acid] used to synthesize the four stereoisomers in the early studies was ≈92% e.e. Our bioassays used stereoisomers from a more recent synthesis that used enantiomerically pure (*R*)-citronellol, which resulted in exceptionally pure stereoisomers, especially after careful recrystallization of both the intermediates and the final products (Mori and Takikawa, 1990). Also, Nishida and colleagues used antennae of the cockroach *Supella longipalpa* as a substrate for testing pheromone isomers and analogs, whereas we used antennae from conspecific *B. germanica* males. We observed that *S. longipalpa* antennae have some, albeit low, endogenous courtship-stimulating activity on *B. germanica* males (D. Eliyahu, preliminary data).

It is also possible that *B. germanica* male antennae have a compound(s) that masks or inhibits the response to the female pheromone. For example, Nishida and Fukami (1983) found that certain fatty acids inhibit the sexual responses of males to the contact pheromone. In *Nauphoeta cinerea*, the lobster cockroach, a male-specific pheromone (nauphoetin, octadecyl (*Z*)-9-tetracosenoate) elicits aggressive antennal fencing among males, but also serves as a courtship depressant (Fukui and Takahashi, 1983). Our results might, therefore, be explained if such a compound occurs in *B. germanica*, and if it more specifically inhibits the courtship response to the natural isomer than to the other isomers. To test this hypothesis, dose-response experiments were conducted with male test antennae that were extracted briefly in hexane prior to application of the test compound. These antennae were compared to fresh antennae that were not extracted before application (Figure 2). Although pre-extracting the antennae tended to reduce male responses, these depressions were not statistically significant at either low (0.1 and 1 ng) or high doses (10 and 100 ng). This general tendency was likely due to the stiffer and more brittle nature of the test antenna after hexane extraction. However, at a dose of 3.3 ng, extracted antennae that were loaded with the *S*,*R*- and *R*,*S*-pheromone stereoisomers stimulated courtship in significantly fewer males than fresh antennae loaded with the same stereoisomers, respectively. Although the same pattern was evident with the *R*,*R*- and *S*,*S*-stereoisomers, these minor differences were not statistically significant. At any rate, the dose-response studies showed, in a fifth independent test, that a synthetic stereoisomer of the natural pheromone (3*S*,11*S*) was the least bioactive of the four synthetic stereoisomers (Figure 2).

Interestingly, Abed et al. (1993) used the same high-purity stereoisomers that were synthesized by Mori and Takikawa (1990), but they suggested that 3*S*,11*S*-diMeC$_{29}$-2-one was more active than other stereoisomers. Resolution of this discrepancy will have to await publication of the methods and data in support of the brief mention of these preliminary results by Abed et al. (1993).

The importance of stereochemistry in olfactory communication is well known. Pheromones may occur as stereoisomeric mixtures, in which case each isomer may be independently active or the mixture may be most active (Mori,

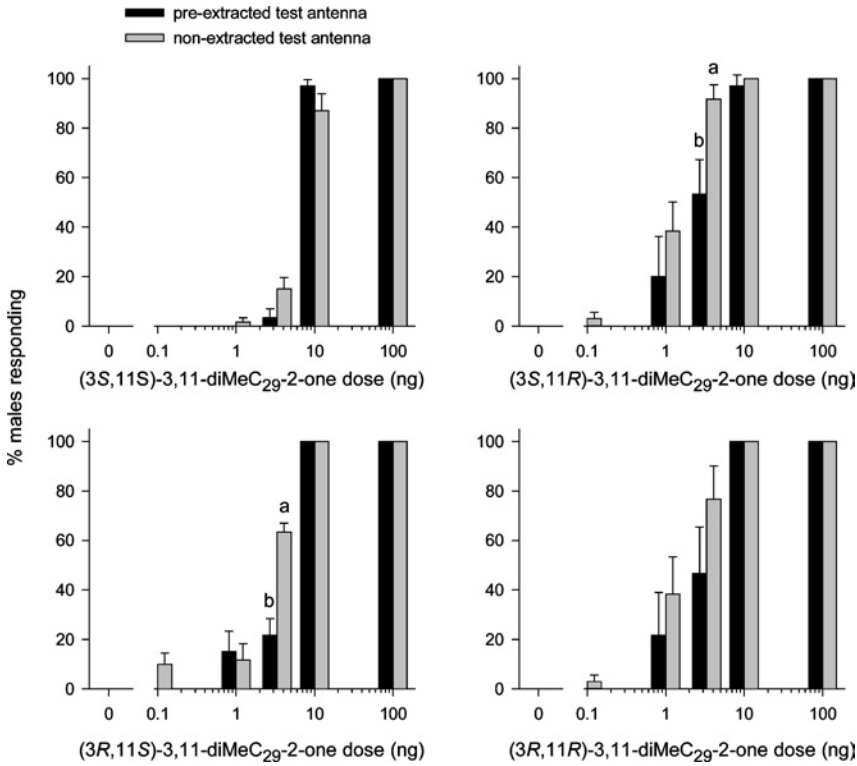

FIG. 2. Dose behavioral response assays of the sexual responses of *Blattella germanica* adult males to four stereoisomers of 3,11-dimethylnonacosan-2-one, applied onto extracted, or fresh nonextracted male antennae. For the doses of 1 and 3.3 ng, six groups of 10 males each were tested with each antennal treatment for responses to each of the four stereoisomers; three groups of 10 males each were tested with the other doses. SEM is shown for each mean. Different letters indicate significant differences based on ANOVA.

1998). In most cases, however, production of the pheromone is stereospecific, and so is its reception in the opposite sex, with the natural pheromone isomer being the most bioactive. Indeed, in closely related, sympatrically occurring species, the nonnatural isomer may have antagonistic effects on sexual orientation, as a mechanism of precopulatory reproductive isolation (Millar, 2000). Nevertheless, insects exhibit a wide range of relations between pheromone stereochemistry and pheromone activity, as reviewed by Mori (2002, 2004).

However, to our knowledge, this is the first example where unnatural stereoisomers are more active than the natural stereoisomer. Nevertheless, this observation will require further substantiation with studies of the interaction of

each stereoisomer with other pheromone components. For example, activity of the natural stereoisomer may be enhanced by other female pheromone components, whereas activity of the unnatural stereoisomers may not.

To resolve methodological uncertainties, it would be most informative to reexamine the behavior of the previously investigated cockroaches in Japan with the high-purity stereoisomers, to determine whether different populations of the German cockroach produce and respond to different stereoisomers of the contact sex pheromone.

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