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Olfactory Discrimination among Sex Pheromone Stereoisomers: Chirality Recognition by Pink Hibiscus Mealybug Males

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Abstract

Our previous field studies suggested that the two chiral centers in the sex pheromone of pink hibiscus mealybug, *Maconellicoccus hirsutus*, could elicit different male responses. The chiral center in the acid moiety of the pheromone seemed to be more critical than the alcohol portion of the pheromone molecule for attractiveness. The objective of the current study was to test this hypothesis by deploying stereoisomeric blends in pheromone traps. Captures of male *M. hirsutus* showed that pheromone with the naturally occurring (*R*)-maconelliyl (*S*)-2-methylbutanoate and (*R*)-lavandulyl (*S*)-2-methylbutanoate [*R-S* configuration] was most attractive and that pheromone with the unnatural *S-S* configuration was less attractive. In addition, the *RS-R* blend (containing *R-R* and *S-R* stereoisomers) yielded captures of male *M. hirsutus* that were comparable to blank controls, and an inhibitory effect was observed when *R-R* and *S-R* were combined with naturally occurring *R-S* blend. These results suggest a unique chirality recognition mechanism; olfactory discrimination among different pheromone stereoisomers depends upon both asymmetric centers. The *S* configuration on the acid moiety elicits attraction, whereas the *R* configuration induces inhibition. However, the attractive activity shows some degree of tolerance toward chirality change in the alcohol portion of the pheromone molecules.

Key words: antagonistic effect, chirality recognition, pink hibiscus mealybug, sex pheromone, stereoisomer

Introduction

The pink hibiscus mealybug, *Maconellicoccus hirsutus* (Green), is a highly polyphagous, invasive species from Southern Asia or Australia (Williams, 1996) and is a serious new threat to US agriculture, forestry, and the nursery industry, involving more than 200 plant genera in 70 families (USDA-APHIS, 2003). It was first reported in the Western Hemisphere (excluding Hawaii) in Grenada in 1994 (Matile-Ferrero and Eitienne, 1996; Etienne *et al.*, 1998) and has subsequently spread rapidly through the Caribbean Islands to Southern California (Carter-Lane and Redding, 1999) and Florida (USDA-APHIS, 2002). It has the potential to infest areas across the entire southern United States as far north as Virginia, Tennessee, and Northern California (USDA-APHIS, 2005). Potential cost to the United States of $750 million per annum has been estimated if the insect is not controlled (Carter-Lane and Redding, 1999).

Identification of the sex pheromone of female pink hibiscus mealybug (Zhang *et al.*, 2004a), a binary blend containing

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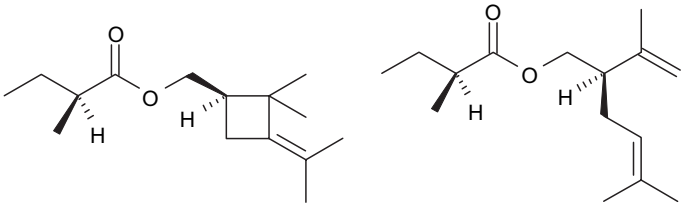
two isomeric components, (*R*)-maconelliyl (*S*)-2-methylbutanoate (**1**) and (*R*)-lavandulyl (*S*)-2-methylbutanoate (**2**) in a ratio of 5:1 [*R-S*], has provided an economical, convenient, and useful infestation detection and population-monitoring tool (Zhang and Amalin, 2005).



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The pink hibiscus mealybug pheromone is unique among scale insect and mealybug pheromones because two asymmetric centers are located separately in acid and alcohol moieties in both pheromone components. Trapping experiments (Zhang and Amalin, 2005) showed that *M. hirsutus* males responded most strongly to the *R-S* blend of pheromone. These experiments also showed that an unnatural (*S*)-maconelliyl (*S*)-2-methylbutanoate and (*S*)-lavandulyl (*S*)-2-methylbutanoate [*S-S*] blend lured a significant number of male *M. hirsutus* into traps, whereas captures in traps baited with unnatural (*S*)-maconelliyl (*R*)-2-methylbutanoate and (*S*)-lavandulyl (*R*)-2-methylbutanoate [*S-R*] and (*R*)-maconelliyl (*R*)-2-methylbutanoate and (*R*)-lavandulyl (*R*)-2-methylbutanoate [*R-R*] blends were not different from those in blank control traps. These results led us to hypothesize that chirality recognition by male *M. hirsutus* is essentially dependent upon the asymmetric center in the acid moiety of the ester but shows some degree of tolerance toward the chirality of the alcohol portion of the pheromone molecules. This chiral-sense mechanism seems to be unusual among mealybug and scale insects (Dunkelblum, 1999).

To test our hypothesis, this paper reports further trapping experiments using three synthetic pheromone blends containing partially racemic moieties, (*R*)-maconelliyl (*RS*)-2-methylbutanoate and (*R*)-lavandulyl (*RS*)-2-methylbutanoate [*R-RS*], (*RS*)-maconelliyl (*S*)-2-methylbutanoate and (*RS*)-lavandulyl (*S*)-2-methylbutanoate [*RS-S*], (*RS*)-maconelliyl (*R*)-2-methylbutanoate and (*RS*)-lavandulyl (*R*)-2-methylbutanoate [*RS-R*], and one completely racemic blend on both asymmetric centers, (*RS*)-maconelliyl (*RS*)-2-methylbutanoate and (*RS*)-lavandulyl (*RS*)-2-methylbutanoate [*RS-RS*], shown in Figure 1. The responses of male pink hibiscus mealybug to traps baited with these stimuli were compared with traps baited with optically pure *R-S* (naturally occurring configuration) and *S-S* (unnatural configuration) blends.

Materials and methods

Chemicals and instrumentation

Racemic and (*S*)-(+)-2-methylbutyric acids are commercially available (Aldrich, Milwaukee, WI). Racemic lavandulol was purchased from TCI (Portland, OR). (*R*)-(−)-2-Methylbutyric acid, (*R*)- and (*S*)-maconelliols, (*R*)- and (*S*)-lavandulols, and corresponding esters were synthesized, respectively, according to methods reported previously (Cardillo *et al.*, 1988; Shirali and Zhang, 2004; Zhang *et al.*, 2004b; Zhang and Nie, 2005), and their identities were confirmed individually by gas chromatography-mass spectrometer (GC-MS) and nuclear magnetic resonance data. The chemical purities were >95%, as determined by a Hewlett Packard (HP) 6890 GC with flame ionization detector (FID) using a 60-m × 0.25-mm ID, 0.25-μm film-thickness DB-WAXETR capillary column (J&W Scientific Inc., Folsom, CA) in the splitless mode with hydrogen as carrier (80°C for 2 min, then programmed to 250°C at 15°C per min and held for 15 min), and optical purities were >97% enantiomeric excess, as determined by an HP 6890 GC with FID using a 30-m × 0.25-mm ID, 0.25-μm film-thickness β-DEX 120 capillary column (Supelco, Inc., Bellefonte, PA) in the split mode (100:1) with hydrogen as carrier (55 cm/s, 100°C isothermal).

Pheromone formulation

All binary blends were formulated in a ratio of 5:1 (maconelliyl 2-methylbutanoate:lavandulyl 2-methylbutanoate, the natural ratio found in female effluvial collections) with hexane (1 μg/μl). Gray halo-butyl rubber septa (5 mm, West Pharmaceutical Services, Kearney, NE) were soxhlet

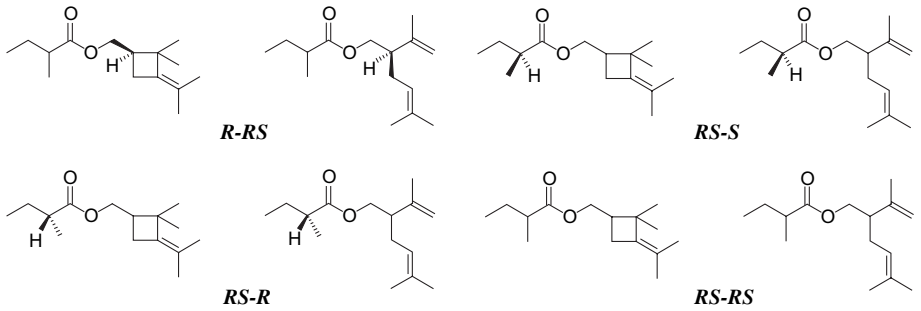

Figure 1 Chemical structure of stereoisomeric blends used in field bioassays.

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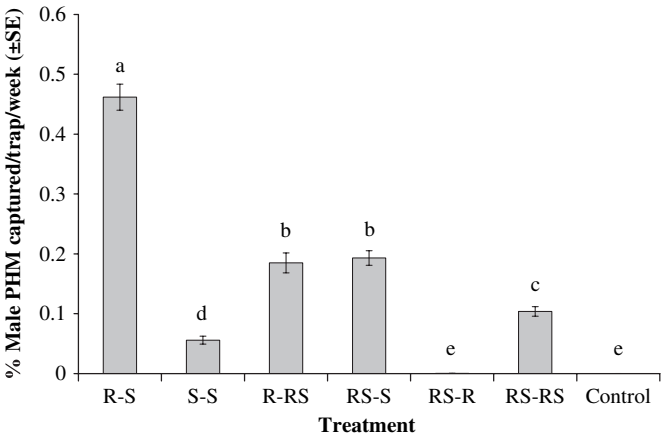

Figure 2 Mean percentages of captures of male *M. hirsutus* (±SEM) in delta traps baited with various binary stereoisomeric blends (1 μg dose/septum) and a blank control. The test was conducted from 11 July to 15 August 2005, and traps were replaced weekly. The total number of male *M. hirsutus* captured was 13,021. Means followed by the different letters are significantly different at $\alpha = 0.05$ [$n = 20$, $F(6,133) = 293.02$, $P < 0.00001$].

extracted with acetone for 48 h and dried in a fume hood before use. For Experiment 1, the extracted septa were loaded with 1 μg of the optically pure blends *R-S* and *S-S*; the partially racemic blends *R-RS*, *RS-S*, and *RS-R*; and a completely racemic blend *RS-RS*. For Experiment 2, the septa were loaded with 1 μg of the optically pure blends *R-S* and *S-S*; 2 μg of the partially racemic blends *R-RS*, *RS-S*, and *RS-R*; and 4 μg of the completely racemic blend *RS-RS*. The same amount of hexane used in the completely racemic blend (4 μl) was loaded onto septa for the blank controls. After loading, the solvent was allowed to evaporate in a fume hood for 30 min. Lures were then separately placed into zipped plastic bags, sealed, and immediately shipped to Florida by express carrier. Upon arrival, the lures were kept in a refrigerator at 4°C until used in the field.

Field bioassays

All field bioassays were conducted at the USDA, Subtropical Horticulture Research Station in Miami, FL, between 11 July and 13 October 2005, using green delta traps (10 × 18–cm panels, Scentry Biological Inc., Billings, MT) with a sticky surface on the bottom and one side of the panel. Lures were affixed to the trap by piercing and wrapping the narrow end of the rubber septum with 22-gauge galvanized wire, then looping the other end of the wire through the top holes, closing the trap, and suspending the wide end of the septum 3 cm above the trap bottom. Traps were placed on stands constructed of 1-inch × 2-inch × 4-ft (2.54 cm × 5.08 cm × 1.22 m) lumber placed in a 14-inch-wide × 6-inch-deep (35.56 cm wide × 15.24 cm deep) plastic pot, securing the lumber vertically with approximately 15 lb (6.8 kg) of concrete mix. A 6 × 8–inch (15.24 × 20.32 cm) metal shelf bracket was secured to the top of the lumber by wood screws, positioning the 8-inch (20.32 cm) side outward. Traps were hung from stands by twist ties placed through holes in the top of traps and at end of the shelf bracket, orienting the trap openings parallel to the prevailing wind direction in south Florida. Four lines of traps were deployed in areas on the research station with known pink hibiscus mealybug infestations. Trap lines were separated by 100 m and oriented perpendicular to the prevailing wind direction. Each pheromone treatment was represented once in each line of traps, and traps were spaced at 5-m intervals within each row. At the outset of each experiment, the pheromone treatments were randomly assigned to positions within each row of traps. Traps were collected weekly, the septa were transferred to new traps, and the traps were rotated among positions within each row. Collected traps were covered with transparent plastic wrap and brought to the laboratory, and the male pink hibiscus mealybugs captured were counted using a dissecting microscope.

Two separate experiments were conducted. In Experiment 1, we used 1-μg dose of *R-S*, *S-S*, *R-RS*, *RS-S*, *RS-R*, and *RS-RS* isomeric blends/septum and a blank control, and the traps were deployed from 11 July through 15 August 2005. In Experiment 2, we used 2-μg dose of *R-RS*, *RS-S*, and *RS-R*, 4-μg dose of *RS-RS*/septum, and a blank control, and the traps were deployed from 24 August through 13 October 2005.

Data analysis

The trap capture data were converted to proportion, then transformed by the standard variance stabilizing transformation for proportions (arcsin $\sqrt{p}$, where p is the original proportion), and analyzed using variances for analysis of variance (ANOVA). Means were compared by one-way ANOVA followed by Ryan–Einot–Gabriel–Welsch range test (SPSS 10.0 for Windows, George and Mallery, 2002) for significance at $\alpha = 0.05$ level.

Results

In Experiment 1, all traps baited with the *R-S* isomeric blend captured significantly more *M. hirsutus* males than those traps baited with the other blends [$F(6,133) = 293.02$, $P < 0.00001$] (Figure 2), confirming our previous results (Zhang and Amalin, 2005) that the *R-S* binary blend (the stereoisomers produced by *M. hirsutus*) is most effective for capturing males. Also as in previous studies (Zhang and Amalin, 2005), all traps baited with the unnatural *S-S* isomeric blends captured fewer males than the *R-S* blend but were still significantly more attractive than blank controls. Traps baited with the *R-RS* blend containing equal amounts of two isomeric blends, natural *R-S* and unnatural *R-R*, at 0.5 μg/septum, were significantly more attractive than the *S-S* blend. Traps baited with the *RS-S* blend containing equal amounts of natural *R-S* and unnatural (less active) *S-S* blends at

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0.5 μg/septum captured significantly lower numbers of male mealybugs compared with traps baited with a 1-μg dose of the *R-S* blend. Conversely, all traps baited with the *RS-R* isomeric blend containing equal amounts of two unnatural isomeric blends, *R-R* and *S-R*, at 0.5 μg/septum, yielded captures of male *M. hirsutus* that were comparable to the blank control. In addition, all traps baited with a 1-μg dose of the completely racemic *RS-RS* blend, containing equal amounts of all four isomeric blends, *R-S*, *S-S*, *R-R*, and *S-R*, at 0.25 μg/septum also captured significantly lower numbers of male mealybugs compared with traps baited with a 1-μg dose of the *R-S* blend, but they still captured significantly higher numbers of male mealybugs compared with traps baited with a 1-μg dose of the *RS-R* isomeric blend and the blank control.

Results from Experiment 1 also indicated that the captures in traps containing three blends of racemic isomers, *R-RS*, *RS-S*, and *RS-RS*, seemed to correlate with the amount of natural *R-S* isomers existing in those lures. To address this question, a second test was conducted. Three partially racemic blends, *R-RS*, *RS-S*, and *RS-R*, were loaded at a rate of 2 μg/septum (concentration of each isomeric blend increased two times) and a completely racemic blend, *RS-RS*, was loaded at a rate of 4 μg/septum (concentration of each isomeric blend increased four times). Therefore, each individual isomeric blend was held constant at 1 μg/septum. The results from Experiment 2 demonstrated that increasing the amounts of the partially racemic blend *RS-S* (by twofold) significantly improved the attractiveness of the lures; however, increasing the amount of the partially racemic blend *R-RS* (by twofold) and the completely racemic blend *RS-RS* (by fourfold) did not significantly improve the attractiveness of the lures compared with the *R-S* blend [$F(6,189) = 223.73$, $P < 0.00001$] (Figure 3).

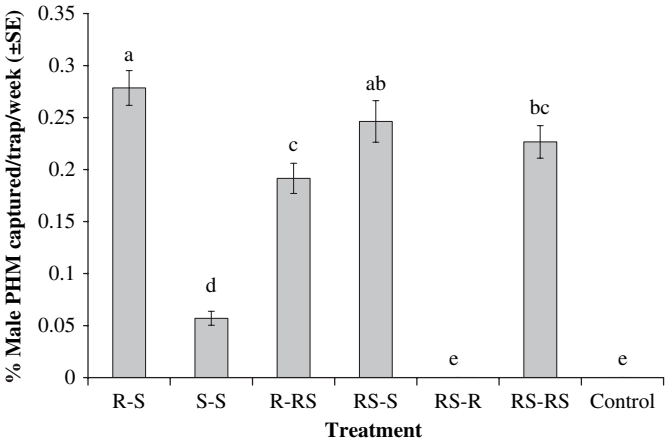

Figure 3 Mean percentages of captures of male *M. hirsutus* (±SEM) in delta traps baited with various binary stereoisomeric blends (1-μg dose for *R-S* and *S-S*; 2-μg dose for *R-RS*, *RS-S*, and *RS-R*; and 4-μg dose for *RS-RS*/septum) and a blank control. The test was conducted from 24 August to 13 October 2005, and traps were replaced weekly. The total number of male *M. hirsutus* captured was 8779. Means followed by the different letters are significantly different at $\alpha = 0.05$ [$n = 28$, $F(6,189) = 223.73$, $P < 0.00001$].

Despite large differences in the numbers of male *M. hirsutus* captured between Experiment 1 (total trap catches of 13,021) and Experiment 2 (total trap catches of 8779), due to a population decrease from July to October, a fairly consistent feature of the data was that the *RS-R* blend yielded trap catches of male *M. hirsutus* comparable to the blank control, despite the fact that the loading was increased by twofold.

Discussion

These results are consistent with those from previous pheromone stereoisomer comparisons reported in Zhang *et al.* (2004a). The substantial differences in the attraction of the stereoisomers clearly showed that the olfactory receptor of *M. hirsutus* is able to differentiate between the *R-S* and *S-S* configurations. The fact that, in both tests, captures of male pink hibiscus mealybugs were significantly higher in traps baited with the unnatural *S-S* blend than in the blank controls but were significantly lower than in traps baited with the naturally occurring *R-S* blend demonstrated that some degree of chirality change in the alcohol portion of the pheromone molecules could be tolerated for attraction. Considering that the *RS-R* blend, containing both *R-R*,and *S-R* isomers, yielded trap catches of male *M. hirsutus* comparable to the blank control in both experiments, we conclude that the pheromone blend interacts with male *M. hirsutus* olfactory receptors, with the chiral sense of their acid moiety (corresponding to that of *S* configuration) eliciting attraction. In other words, species specificity in the pheromone signal seemed to be essentially affected by the chirality of the acid portion of molecule.

In our previous studies, we found that when a pair of enantiomers was present, antipodes, *S-R* and *R-R*, significantly reduced the attraction to the naturally occurring *R-S* and unnatural *S-S* blends in *M. hirsutus*, respectively (Zhang and Amalin, 2005). The antagonistic effect of unnatural diastereoisomers to the naturally occurring *R-S* isomer was also observed in the present studies. The stereoisomer, *R-R*, in a partially racemic blend *R-RS* significantly reduced captures by the naturally occurring *R-S* blend. Similarly, those unnatural isomers (*R-R* and *S-R*) in a completely racemic blend *RS-RS* also significantly reduced attraction to the naturally occurring *R-S* blend (Figure 3).

Sex pheromones of mealybug and scale insects usually contain two or three pheromone components (Dunkelblum, 1999). The chiral center is only located in the alcohol portion, each component is active by itself, and other unnatural enantiomers or diastereomers usually had neither inhibitory nor synergistic influence on insect attraction (Bierl-Leonhardt *et al.*, 1980, 1981; Einhorn *et al.*, 1998; Millar *et al.*, 2002; Arai *et al.*, 2003). In contrast, *M. hirsutus* seems to be a unique insect that requires two separated chiral centers in the pheromone; one is in the acid moiety and the second is in the alcohol portion in both pheromone

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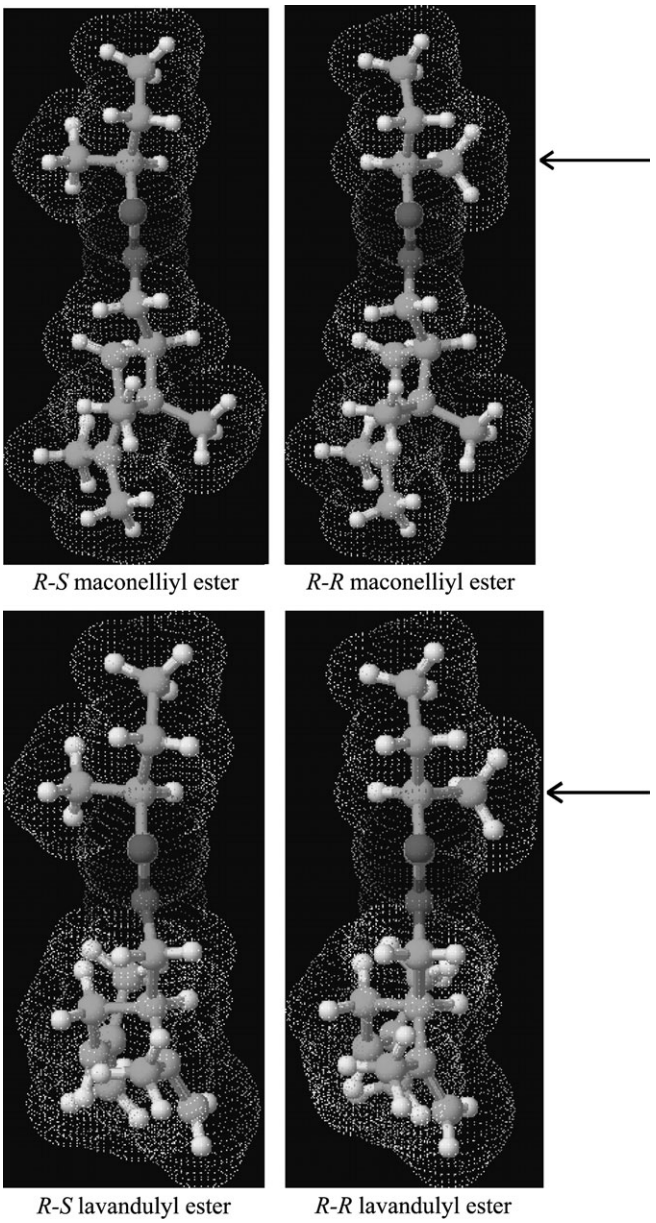

Figure 4 The behaviorally attractive *R-S* and inhibitory *R-R* configurations of maconelliyl esters and lavandulyl esters, with the methyl groups proposed to cause inhibitory interaction with olfactory receptors on male attraction indicated.

components, with correct configurations required for highest attractiveness. However, these two chiral centers displayed different perception specificity during chirality recognition. One chiral center located in the acid moiety with naturally occurring *S* configuration is absolutely essential for attractiveness. However, the same chiral center with *R* configuration will display antagonistic activity. The methyl group with *R* configuration in the acid moiety of the ester in the pheromone molecules is proposed to cause inhibitory interaction with olfactory receptors on male attraction in the field (Figure 4). Whereas the second chiral center located in the alcohol portion is less specific for attractiveness, with naturally occurring *R* configuration for highest attractiveness and with *S* configuration for low attractiveness. To the best of our knowledge, this is the first report of such pheromone system, and therefore, these studies have yielded novel and valuable information on the pheromone chemistry of mealybug and scale insects and will facilitate the development and improvement of pheromone-based monitoring and management tactics for *M. hirsutus*.

In conclusion, we found that synthetic sex pheromone of *M. hirsutus* with different steric configurations in two separated chiral centers evoked different behavioral responses in the recipient. The antagonistic effect of pheromone stereoisomers was suggested to be due to inhibitory interactions between the methyl groups with *R* configuration in the acid moiety of the ester in the pheromone molecules and olfactory receptors. These results provide strong evidence for our hypothesis that olfactory discrimination by *M. hirsutus* males among different pheromone stereoisomers depends upon both asymmetric centers. The *S* configuration on the acid moiety elicits attraction, whereas the *R* configuration induces inhibition. However, the attractive activity shows some degree of tolerance toward chirality change in the alcohol portion of the pheromone molecules, which is unusual in insect semiochemical communication systems.

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