Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 12112–12115, October 1996
Chemistry

Chemical communication in scarab beetles: Reciprocal behavioral agonist-antagonist activities of chiral pheromones

(*Anomala osakana*/*Popillia japonica*/*Anomala rufocuprea*/Scarabaeidae/japonilure)

WALTER SOARES LEAL

Laboratory of Chemical Prospecting, National Institute of Sericultural and Entomological Science, 1-2 Ohwashi, Tsukuba 305, Japan

Communicated by Jerrold Meinwald, Cornell University, Ithaca, NY, August 12, 1996 (received for review November 10, 1995)

ABSTRACT **A novel mechanism of reciprocal behavioral agonist-antagonist activities of enantiomeric pheromones plays a pivotal role in overcoming the signal-to-noise problem derived from the use of a single-constituent pheromone system in scarab beetles. Female *Anomala osakana* produce (*S, Z*)-5-(+)-(1-decenyl)oxacyclopentan-2-one, which is highly attractive to males; the response is completely inhibited even by 5% of its antipode. These two enantiomers have reverse roles in the *Popillia japonica* sex pheromone system. Chiral GC-electroantennographic detector experiments suggest that *A. osakana* and *P. japonica* have both *R* and *S* receptors that are responsible for behavioral agonist and antagonist responses.**

To achieve chemical communication in an environment rich in potential chemical cues, insects must overcome the signal-to-noise problem by filtering out a large amount of background noise. In moths, filtering is achieved by using blends of similar compounds, where the specific signature represented by the whole mixture is *sine qua non* to elicit sexual behavior (1). In marked contrast, some species of Coleoptera (2, 3) have sex pheromone systems composed of a single constituent; such communication channels seem *a priori* highly prone to noise interference from the environment. Nevertheless, these semiochemicals have by and large at least one chiral center, and chirality plays a major role in determining pheromone specificity. I report here that two scarab beetle species (Coleoptera: Scarabaeidae) achieve single-component chemical communication by a novel mechanism based on the reciprocal behavioral agonist–antagonist activities of the two enantiomers of a single chiral compound. The Osaka beetle, *Anomala osakana* Sawada, produces and responds only to (*S, Z*)-5-(+)-(1-decenyl)oxacyclopentan-2-one, the activity of which is completely inhibited by the presence of its enantiomer. On the other hand, it has been previously reported (4) that the Japanese beetle, *Popillia japonica*, produces and responds only to the (*R*)-stereoisomer of this lactonic pheromone, with the activity highly inhibited by its antipode.

MATERIALS AND METHODS

Analytical Procedures. GC was carried out on a Hewlett–Packard 5890 II Plus instrument equipped with a split/splitless injector, an electronic pressure control, a flame ionization detector, and an HP 3365 Series II Chemstation. High-resolution GC analyses were performed with polar and non-polar capillary columns, HP-Innowax and HP-5MS (30 m × 0.25 mm × 0.25 μm), respectively. These columns were operated at 70°C for 1 min, increased to 230°C at a rate of 10°C/min, and held at this temperature for 10 min. Chiral resolution was achieved on a capillary column having a trifluoroacetylated γ-cyclodextrin phase, Chiraldex GTA (20 m × 0.25 mm: 0.125 μm; Astec, Whippany, NJ), operated at 150°C and with a (helium) head pressure of 2.5 kg/cm^2 (9.42 ml/min; 119 cm/s). Low-resolution MS was carried out with an HP 5890 II Plus gas chromatograph linked to a mass selective detector (Hewlett–Packard; model MSD 5972). GC with electroantennographic detection (EAD) was performed according to the method of Struble and Arn (5). An HP 5890 Series II Plus gas chromatograph was modified to have the effluent from the capillary column split into EAD and flame ionization detector (3:1 ratio). Beetle antennae were placed in a previously described acrylic stage (6); this was set inside the glass transfer line (2 cm away from the GC outlet) and connected with gold wires to an amplifier (gain 5) and filtered through a passive filter (cutoff frequency = 0.12 Hz). The signal was fed into an A/D 35900E interface (Hewlett–Packard). The flame ionization detector, and EAD signals were acquired with the HP Chemstation. Chiral GC-EAD was performed with an active 2nd order Butterworth high-pass filter as described (7).

Extraction and Purification of the Natural Pheromone. Airborne volatiles of 100 field-collected female beetles were trapped with Super Q in an all-glass aeration apparatus, extracted with hexane, and concentrated to 1 female-equivalent/μl. Crude airborne volatiles of females were subjected to flash column chromatography on a silica column [eluant, ether (0–100%) in hexane], and the activity was monitored by GC-EAD. The sex pheromone was recovered along with a phthalate ester contaminant in the hexane/ether (80:20) fraction.

Syntheses. (*S*)- and (*R*)-Japonilure were obtained from L-and D-glutamate, respectively, according to a previously reported method (4). The optical purity of the two enantiomers was >97% enantiomeric excess, as established by chiral GC.

Bioassays and Field Tests. Laboratory bioassays were performed in plastic cages (20 × 30 cm; 5 cm high); after 10 males were transferred to the arena, pieces of filter paper (2 × 2 cm) loaded with the synthetic lactone or solvent only (control) were set inside the arena, and behavior was observed for 30 min. Gathering on the pheromone source and/or attempting copulation were the two criteria for male positive responses. After each test, new cages were used and males were randomly redistributed in the new arenas. Field tests were carried out in the Hamamatsu Sea Side Golf Club (Iwata, Shizuoka, Japan) on June 29–30, 1995. Chemicals (1 mg) were incorporated in plastic pellets (4–5 mm in diameter) made of polyethylene-vinyl acetate, which gave a release rate of 950 ng/h at 25°C (determined by trapping the released pheromone on a Super Q column). The pellets were loaded on sticky plates traps (20 × 20 cm), and tests were run with the traps distributed in randomized complete blocks with four replicates. Field experiments for evaluation of the effect of other species' pheromones on the capture of the soybean beetle (*Anomala rufocuprea*) were carried out in Tsukuba (July 20 to August 28, 1995) with funnel traps distributed in randomized complete blocks with four replicates. In one area, traps were baited with

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: EAD, electroanntenographic detector.

methyl (*Z*)-5-tetradecenoate (6 mg), (*R, Z*)-5-(—)-(1-octenyl)-oxacyclopentan-2-one (1 mg), and a combination of both compounds. In another area, (*R, Z*)-5-(—)-(1-octenyl)-oxacyclopentan-2-one was replaced by (*R*)-japonilure. Capture data were transformed to log (x + 1) before differences among means were tested for significance by ANOVA with JMP software, Version 2 (SAS Institute, Cary, NC). Treatments followed by the same letters are not significantly different at the 5% level in the Tukey–Kramer honestly significant difference test.

RESULTS AND DISCUSSION

Utilizing male antenna of *A. osakana* as the sensing element, the GC-EAD profile of the airborne volatiles of females showed the occurrence of a single EAD-active peak at 16.46 min, which was almost undetected by the flame ionization detector (Fig. 1).

By GC-MS, this compound was found to be indistinguishable from (*R, Z*)-5-(1-decenyl)oxacyclopentan-2-one [(*R*)-japonilure]. However, in a laboratory bioassay, males did not show any behavioral response to synthetic (*R*)-japonilure. This dilemma was solved when it was determined that males were highly attracted to the (*S*)-enantiomer of this lactone (Fig. 2*A*), whereas the (*R*)-stereoisomer was found to be a behavioral antagonist (Fig. 2*B*).

Chromatography of the isolated natural product on a chiral capillary column, Chiraldex GTA, revealed that females produce only the (*S*)-enantiomer. The natural product gave the same retention time (18.2 min) as the synthetic (*S*)-stereoisomer [the (*R*)-enantiomer appeared at 15.1 min]. Fortunately, the phthalate ester contaminant did not interfere with the stereochemistry assignment.

Field tests showed that catches of males in sticky traps baited with the (*S*)-stereoisomer were completely inhibited by the addition of only 5% of the (*R*)-enantiomer. Response to the (*R*)-enantiomer alone did not differ significantly from that of unbaited control traps (Fig. 3).

It has been demonstrated by single cell recording experiments that pheromone-specific receptors in scarab beetles respond only to the naturally occurring enantiomer of the relevant pheromone, and that the nonnatural stereoisomer remained undetected by these receptors (8). The existence of putative receptors specific to the nonnatural enantiomer has been suggested by the fact that electroantennogram (EAG) responses (summed recording of the activity of many receptors) are elicited by the nonnatural pheromone. However, in EAG experiments alone there is an element of ambiguity resulting from the fact that no synthetic chiral compound is 100% enantiomerically pure. Japonilure, for example, has not been obtained in optical purity higher than 99%; such a sample, when submitted to an antennal preparation, may stimulate receptors tuned to the minor antipode, if they exist. One way to expose an antenna to one enantiomer completely devoid of its antipode is by use of a recently developed chiral GC-EAD system (7), which takes advantage of the power of chiral resolution to generate highly purified enantiomers in the gas phase. In chiral GC-EAD, male antennae of both the Japanese and Osaka beetles responded to the two enantiomers of japonilure with peaks of different intensities but similar shapes (Fig. 4). If the behavioral antagonist were merely a mimic of the agonist, peaks of different shapes would be expected due to the different affinities that the two ligands would be expected to have for the pheromone-binding protein [for a comparison, see the response of *Supella longipalpa* to its natural sex pheromone, (*R, R*)-supellapyrone and to an epimeric mimic, the (*S, R*)-stereoisomer (7)]. These findings suggest that male antennae of the Osaka and Japanese beetles have different receptors tuned to each stereoisomer. It is worth noting that males of the two species responded more strongly to the stereoisomer produced by conspecific females (Fig. 4).

It is not known how these two species, which share a common habitat in Japan, have evolved to produce a single-enantiomer pheromonal system and have receptors tuned to both stereoisomers. Clearly, males would benefit from adaptations that help to avoid wasting mating effort pursuing females of the wrong species. Inhibition by the other species' pheromone, whether it is an enantiomer or even a different molecular structure, would be a likely solution to this problem. As a matter of fact, I have evidence that the sex pheromone of one scarab beetle species elicits a negative behavioral response in allospecific receivers. Such is the case with the soybean beetle, *A. rufocuprea*, that co-occurs with the cupreous chafer, *Anomala cuprea*, and the Japanese beetle, *P. japonica*. Field

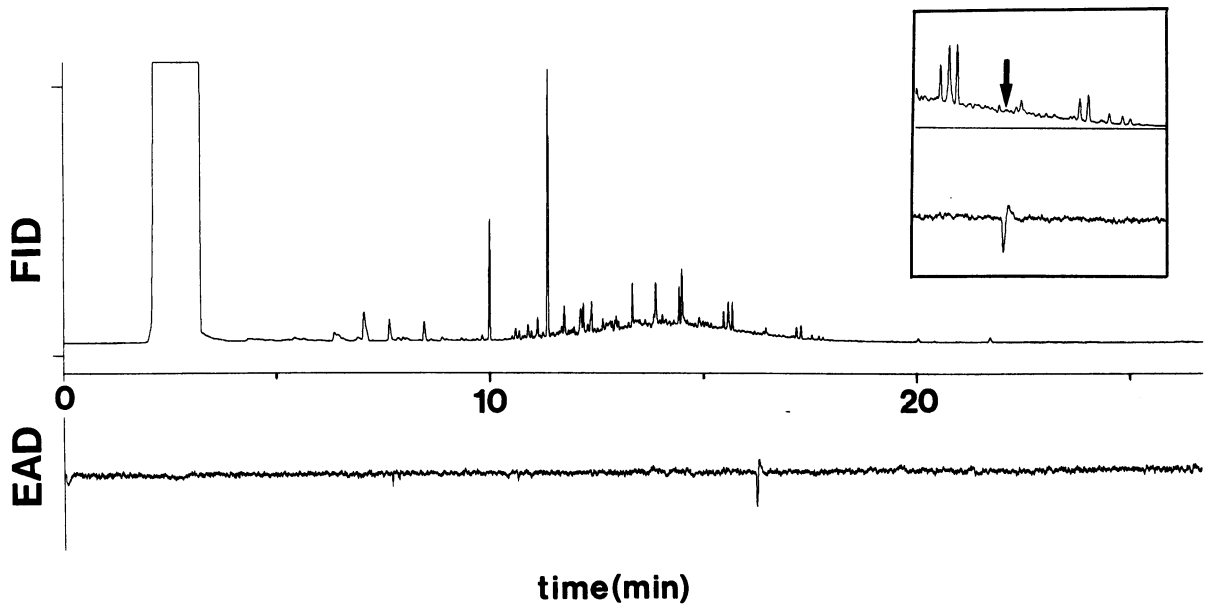

FIG. 1. Parallel flame ionization detector and EAD chromatograms obtained from airborne volatiles of *A. osakana* females (1 female-equivalent/day) separated on an HP-5MS capillary column. A male antenna was used as the sensing element. The expanded area of the EAD-active peak is displayed on the top of the figure.

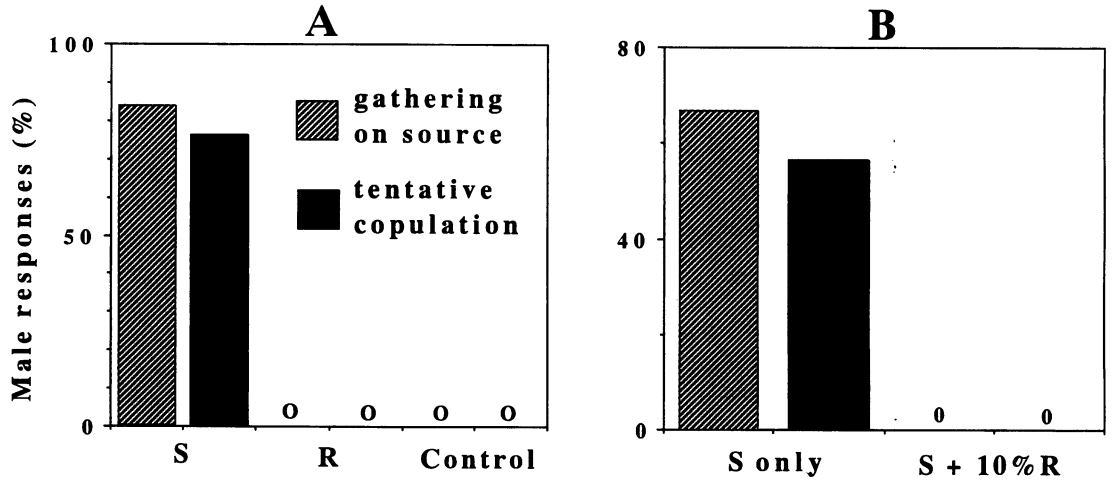

FIG. 2. Male average ($n = 5$) responses (gathering on the source and tentative copulation) in a laboratory bioassay. (*A*) Comparative responses of (*S*)-, (*R*)-japonilure and control. (*B*) Effect of the addition of 10% of the (*R*)-enantiomer on the activity of the (*S*)-stereoisomer.

experiments showed that the captures of *A. rufocuprea* males in traps baited with its synthetic sex pheromone (identified in ref. 9), methyl (*Z*)-5-tetradecenoate (34.3 ± 16.6 beetles per trap per day), were dramatically higher than catches in traps baited with a combination of its pheromone plus (*R, Z*)-5-(—)-(1-octenyl)oxacyclopentan-2-one, the major constituent of *A. cuprea* sex pheromone system (1 ± 0.9 males per trap per day) (10). Also, I observed that (*R*)-japonilure blocked male response in the soybean beetle.

It is also possible that there may have been selection pressure on females of an ancestral species to shut off the production of one enantiomer that would attract males of the wrong species and the present behavioral antagonism is a remnant of a past interaction. Evidence has been found in moths suggesting that a component that at one time could have been part of a pheromone blend has switched from agonist to behavioral antagonist (11). Also, species that can produce a specific mixture of enantiomers have been previously found among moths (12–14) and bark beetles (15). However, there is sometimes a lack of receptors tuned to one of the enantiomers (12, 13) and only one of the antipodes is behavioral antagonist to allospecific receivers. By contrast, the Osaka and the Japanese beetle have receptors tuned to both stereoisomers, each species produces only one enantiomer and is inhibited by minute amounts of its antipode.

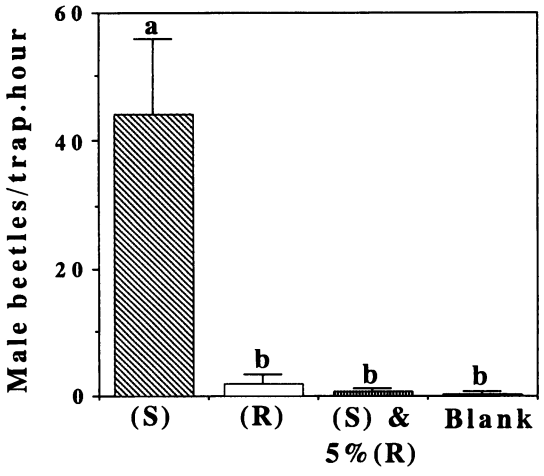

FIG. 3. Catches of *A. osakana* males in traps baited with enantiomerically pure (*S*)-japonilure. Responses to the (*R*)-stereoisomer and to the (*S*)-enantiomer contaminated with its antipode (5%) were not significantly different from the control.

From an anthropomorphic perspective, stereochemical discrimination may be considered the ultimate refinement in chemical communication (for a review, see ref. 16). The case of the Osaka and Japanese beetles is the first one where its importance in a reciprocal behavioral agonist-antagonist context is clearly demonstrated. Nevertheless, there is evidence in the literature to suggest that this phenomenon may occur in other species of Coleoptera (17–19) and Lepidoptera (20). The pheromone of the Western corn rootworm, *Diabrotica virgifera*, has been identified as 8-methyl-2-decyl propanoate (17). When the four possible stereoisomers were tested in the field, it was observed that *Diabrotica barberi* was attracted to the (2*R*, 8*R*)-isomer and inhibited by the (2*S*, 8*R*) (18), while *Diabrotica longicornis* was attracted by the (2*S*, 8*R*)- and inhibited by the (2*R*, 8*R*)-stereoisomer (19). However, the natural pheromones of the latter two species have never been isolated and characterized. On the other hand, enantiomers of (3*Z*, 9*Z*)-*cis*-6,7-epoxynonadecadiene have been identified as one of the sex pheromone constituents of two moth species, *Colotois pennaria* and *Erannis defoliaria* (20). The addition of the antipode in equal proportion to the naturally occurring enantiomer decreased, but did not completely inhibit male response to the

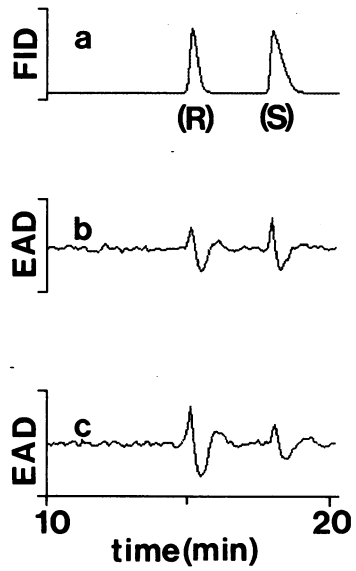

FIG. 4. Enantiomeric resolution of (*R*)- and (*S*)-japonilure on a chiral capillary column (*a*). Parallel EAD responses of male antennae of *A. osakana* (*b*) and *P. japonica* (*c*) to the two enantiomers.

enantiomer produced by conspecific females. This is probably due to the fact that in these species, the chiral compound is just one constituent of a multicomponent pheromone system. Thus, an antipode antagonist effect might be better tolerated than in a single-constituent sex pheromone system.

Although the agonist and antagonist roles of enantiomers in the Osaka and Japanese beetles provides a species-specific chemical signal with the use of a single chiral structure, temporal isolation also plays an important role in partitioning the sexual communication channel between these species. The Japanese and Osaka beetles share the same habitats in parts of the mainland of the Japanese archipelago, but the flight activity of the latter species occurs primarily during the hour after sunset whereas the former is active mainly during daylight hours.

I thank Bruce D. Hammock, Coby Schal, Wendell L. Roelofs, Pierre Escoubas, Kenneth F. Haynes, Jose Soares Andrade, Jr., Radoje Belusevic, Stuart Krasnoff, Klaus Jaffé, Robert H. DeWit, and Wittko Francke for critical review of a previous version of the manuscript. I am also grateful to the personnel of Toho Green Maintenance and S. Tada for their help in the field experiments. This research was supported by a special coordination fund for promoting science and technology (SCF) by the Science and Technology Agency, Japan. Chiral GC-EAD was developed in Jerrold Meinwald's lab (Cornell University) during W.S.L.'s sabbatical leave.

1. Baker, T. C. (1989) *Experientia* **45,** 248–262.
2. Leal, W. S. (1996) in *Pheromone Research: New Direction*, eds. Cardé, R. T. & Minks, A. K. (Chapman & Hall, New York), in press.
3. Mayer, M. S. & McLaughlin, J. R. (1991) *Handbook of Insect Pheromones and Sex Attractants* (CRC, Boca Raton, FL), pp. 25–953.
4. Tumlinson, J. H., Klein, M. G., Doolittle, R. E., Ladd, T. L. & Proveaux, A. T. (1977) *Science* **197,** 789–792.
5. Struble, D. L. & Arn, H. (1984) in *Techniques in Pheromone Research*, eds. Hummel, H. E. & Miller, T. A. (Springer, New York), pp. 161–178.
6. Leal, W. S., Mochizuki, F., Wakamura, S. & Yasuda, T. (1992) *Appl. Entomol. Zool.* **27,** 289–291.
7. Leal, W. S., Shi, X., Liang, D., Schal, C. & Meinwald, J. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 1033–1037.
8. Leal, W. S. & Mochizuki, F. (1993) *Naturwissenschaften* **80,** 278–281.
9. Tamaki, Y., Sugie, H. & Noguchi, H. (1985) *Appl. Entomol. Zool.* **20,** 359–361.
10. Leal, W. S. (1991) *Naturwissenschaften* **78,** 521–523.
11. Roelofs, W. L. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 44–49.
12. Hansen, K., Schneider, D. & Boppré, M. (1983) *Naturwissenschaften* **70,** 466–467.
13. Hansen, K. (1976) *Physiol. Entomol.* **9,** 9–18.
14. Klimetzek, D., Loskant, G., Vité, J. P. & Mori, K. (1976) *Naturwissenschaften* **63,** 581–582.
15. Lanier, G. N., Classon, A., Stewart, T., Piston, J. J. & Silverstein, R. M. (1980) *J. Chem. Ecol.* **6,** 677–687.
16. Seybold, S. J. (1993) *J. Chem. Ecol.* **19,** 1809–1831.
17. Guss, P. L., Tumlinson, J. H., Sonnet, P. E. & Proveaux, A. T. (1982) *J. Chem. Ecol.* **8,** 545–556.
18. Guss, P. L., Sonnet, P. E., Carney, R. L., Tumlinson, J. H. & Wilkin, P. J. (1985) *J. Chem. Ecol.* **11,** 21–26.
19. Krysan, J. L., Wilkin, P. H., Tumlinson, J. H., Sonnet, P. E., Carney, R. L. & Guss, P. L. (1986) *Ann. Entomol. Soc. Am.* **79,** 742–746.
20. Szöcs, G., Tóth, M., Francke, W., Schmidt, F., Philipp, P., König, W. A., Mori, K., Hansson, B. S. & Löfstedt, C. (1993) *J. Chem. Ecol.* **19,** 2721–2735.