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Chemistry

Application of chiral gas chromatography with electroantennographic detection to the determination of the stereochemistry of a cockroach sex pheromone

[*Supella longipalpa*/(2′*R*,4′*R*)-supellapyrone/chiral resolution]

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ABSTRACT **The coupling of an electroantennographic detector to a chiral capillary gas chromatographic system provides a highly sensitive technique for the determination of pheromone stereochemistry. Electronic modification of the usual electroantennographic detector enables the detector to respond to the relatively broad peaks produced by chiral gas chromatographic systems. By using this methodology, supellapyrone, a female sex pheromone of the brownbanded cockroach *Supella longipalpa* (Orthoptera: Blattellidae), is shown to be 5-(2′*R*,4′*R*-dimethylheptanyl)-3-methyl-2*H*-pyran-2-one.**

Stereochemical discrimination may be considered the ultimate refinement of chemical communication in nature. Its importance, with respect to *E*- and *Z*-isomerism, was recognized with the characterization of bombykol more than three decades ago (1), and the significance of *R*/*S* discrimination was established several years thereafter (2).

Despite the sophistication of contemporary analytical techniques, elucidation of the stereochemistry of a naturally occurring semiochemical remains one of the most difficult tasks in pheromone research (3). Most often, the stereochemistry of pheromones is not in fact analytically determined but is inferred from comparative bioassays conducted with all possible synthetic stereoisomers. This approach is unambiguous when one of the isomers is an antagonist to the natural pheromone. An example of this is provided by the Japanese beetle *Popillia japonica* whose sex pheromone has been identified as 5-(*Z*)-(−)-(dec-1-enyl)oxacyclopentan-2-one, the stereochemistry of which could be assigned as *R* because trap catches were reduced dramatically by the addition of the *S*-enantiomer (4). However, for a pheromone whose stereoisomers show similar bioactivity, the bioassay gives no information on the stereochemistry of the natural product. As an example, the final clue to the absolute configuration of the German cockroach contact sex pheromone was a classical mixture melting point determination, since all four possible stereoisomers proved to be equally bioactive (5).

Recently, a sex pheromone of the brownbanded cockroach *Supella longipalpa* (Orthoptera: Blattellidae) has been identified as 5-(2′,4′-dimethylheptanyl)-3-methyl-2*H*-pyran-2-one, supellapyrone; the stereochemistry of this pheromone was not elucidated, but it was demonstrated that the pheromone consists of, at most, two enantiomers (6). Our interest in developing synthetic methodologies for the direct joining of a side chain to pyrones led us to use a Pd-catalyzed coupling technique to elucidate the stereochemistry of supellapyrone. However, the fact that the nonnatural stereoisomers were already known neither to appreciably synergize nor to strongly inhibit male response (6) suggested that the synthetic approach alone would be unlikely to succeed. We now report the application of GC (using achiral and chiral columns), coupled with electroantennographic detection (EAD), to establish that natural supellapyrone has the 2′*R*,4′*R* configuration.

MATERIALS AND METHODS

Analytical Procedures. GC was carried out on a Hewlett–Packard 5890 II instrument equipped with a split/splitless injector, a flame ionization detector (FID), and a Hewlett–Packard 3396A integrator. High-resolution GC analyses were performed with polar and nonpolar capillary columns, DB-Wax (30 m × 0.25 mm; 0.25 μm; J & W Scientific, Folsom, CA) and CP-Sil 5 CB (25 m × 0.32 mm; 0.12 μm; Chrompack, Raritan, NJ), respectively. Unless otherwise mentioned, the DB-Wax column was operated at 60°C for 2 min, programmed at 10°C/min to 220°C, and held at this temperature for 20 min, whereas the CP-Sil 5CB column was operated at 50°C for 1 min, programmed at 10°C/min to 250°C, 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 142°C, using helium as a carrier gas at a head pressure of 2 kg/cm^2 (flow rate, 1.6 ml/min). Low-resolution MS was carried out with a Hewlett–Packard 5890 gas chromatograph linked to a Finnigan–MAT (San Jose, CA) ion-trap detector 800. GC separations were performed on a DB-1 capillary column (30 m × 0.25 mm; 0.25 μm; J & W Scientific) operated under the conditions described above. ^{1}H NMR spectra were recorded on a Varian XL-400 instrument.

GC–EAD. Initially, the responses of *S. longipalpa* male antennae were recorded with an amplifier and a passive high-pass filter, according to a reported method (7). The antenna was set on an acrylic stage (8) and placed inside the transfer tube 2 cm from the GC effluent. To overcome the difficulties derived from the broad peaks typical of chiral chromatography, an amplifier system with an active second-order Butterworth high-pass filter was designed (9) (Fig. 1). This filter has a pass-band gain of 1.6 and a cut-off frequency of 0.016 Hz. The values of the capacitors (*C*) and the resistors (*R*) in the filter, which are related to the cut-off frequency (f_c) by the equation $f_c = 1/(2\pi RC)$, were experimentally determined by examining the best detector response to a synthetic blend of the sex pheromone isomers. Since the amplifier was operated with gain 5, the system had a total gain of 8. The signal was recorded on a Fisher recordal series 5000 pen recorder set at 10 mV (full scale) and synchronized with the GC integrator.

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Abbreviations: EAD, electroantennographic detector; FID, flame ionization detector.

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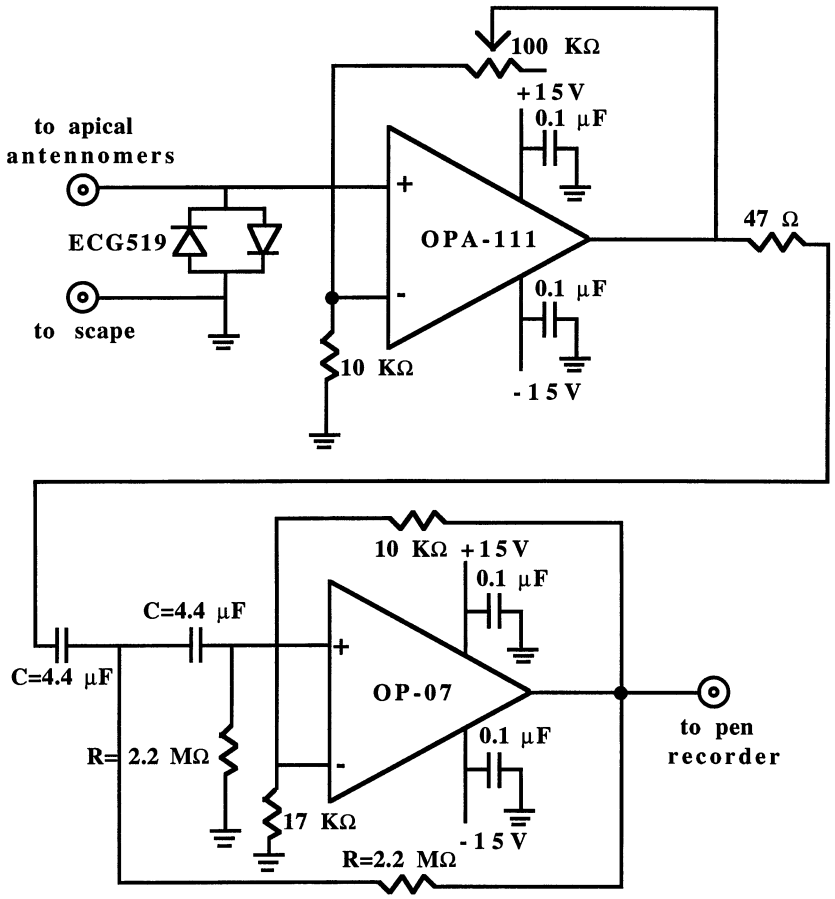


FIG. 1. Circuit diagram of an EAD with an active second-order Butterworth high-pass filter used for GC-EAD measurements with a capillary chiral column.

Purification of the Natural Pheromone. The fourth and fifth abdominal tergites of 200 female cockroaches were dissected and extracted as described (6). The crude extract was purified on a Pasteur pipette filled with silica gel 60 (230–400 mesh ASTM; EM Science) that was washed successively with solutions of ether in hexane (0, 5, 10, 20, 50, and 100%); material was collected in small fractions (1 ml) that were monitored by GC–MS.

RESULTS

Preliminary experiments with the GC–EAD system using an achiral capillary column (CP-Sil 5 CB) and an *S. longipalpa* male antenna as the sensing element revealed that amounts as small as 0.02 female-equivalent of the natural sex pheromone were detected by EAD. This was estimated to be ≈10 pg, far below the detection limit of state-of-the-art analytical instrumentation. The EAD technique, therefore, minimizes the amount of sample required for elucidation of pheromone stereochemistry, since the GC–FID peaks of the synthetic stereoisomers can be compared with the GC–EAD peak(s) of the natural product.

The first step toward the elucidation of supellapyrone's configuration was the synthesis of a racemic sample of predominantly *syn*-supellapyrone. As described in detail elsewhere (10), this was achieved by the palladium (II)-catalyzed coupling of 5-bromo-3-methyl-2*H*-pyran-2-one with the organozinc reagent prepared from (2*R**,4*R**)-1-bromo-2,4-dimethylheptane containing a small amount of (2*R**,4*S**)-stereoisomer (11), which yielded a mixture of (2′*R**,4′*R**)-supellapyrone (91%) and its (2′*R**,4′*S**)-diastereomer (9%).

These diastereomers were readily separated on both a polar GC column (DB-Wax: *syn* at 26.26 min and *anti* at 26.52 min) and a nonpolar column (CP-Sil 5CB: *syn* at 13.34 min and *anti*

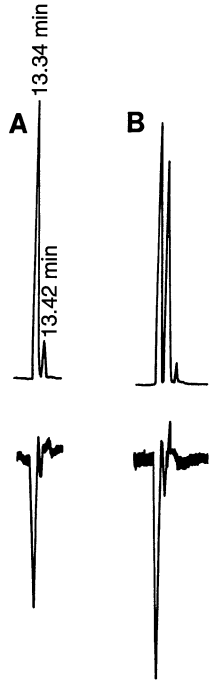


FIG. 2. Coupled GC–EAD responses of male *S. longipalpa* to a predominantly *syn*-supellapyrone (*A*) and to a *syn*/*anti* 1:1 mixture (*B*).

at 13.42 min). GC–EAD response to the *syn*-isomer was very clear (Fig. 2*A*), but it was unclear whether the small response to the *anti*-diastereomer was due to adaptation of the antenna to the first peak or to the smaller amount of the second peak. Upon injection into the GC column of a synthetic mixture of isomers containing roughly equal amounts of the *syn* and *anti* forms, obtained as reported (6), nearly the same EAD profile was obtained. To minimize the possible effect of adaptation on the EAD response to *anti*-supellapyrone, a better resolution of the two peaks was sought. This was achieved by operating the oven temperature at 80°C for 1 min, programming at 4°C/min to 170°C, and holding at this temperature for 20 min. Under these conditions, the *syn*- and *anti*-diastereomers appear at 18.32 and 18.57 min, respectively (Fig. 3*A*); however, the EAD profile did not change appreciably. To rule out the possibility that the weak response to the *anti*-isomer was due to sensory adaptation, two samples were injected successively so that the *syn*-isomer from the second injection coeluted with the *anti*-isomer from the first injection. As demonstrated by the EAD response (Fig. 3*B*), some adaptation may have occurred, but the antenna was able to show at least 85% of its original response when presented with the second peak of a mixture of *syn*- and *anti*-isomers. Most importantly, under the same conditions, the natural product gave only a single EAD peak corresponding to the retention time of the racemic *syn*-isomer, and thus we conclude that the stereochemistry of supellapyrone is (2′*R*,4′*R*), (2′*S*,4′*S*), or both. We conclude further that the natural pheromone does not contain the *anti*-isomer, despite the fact that at this stage we had not seen the peak of the natural product in the FID chromatogram. Further support for these conclusions was provided by the observation that the high-resolution ^{1}H NMR spectrum of our synthetic *syn*-isomer (10) matched that of the natural product (6).

To determine the absolute configuration of the pheromone, we synthesized a 1:1 mixture of (2′*R*,4′*R*)- and (2′*R*,4′*S*)-supellapyrone starting from (*S*)-3-bromo-2-methylpropanol (Aldrich; 99% enantiomeric excess) (10). Only one of the two product GC peaks, the peak corresponding to the (2′*R*,4′*R*)-isomer, proved to be EAD-active. We also synthesized a 1:1 mixture of (2′*S*,4′*S*)- and (2′*S*,4′*R*)-supellapyrone, starting from (*R*)-3-bromo-2-methylpropanol (Aldrich; 88% enantiomeric excess). This also gave an EAD-active peak with the same retention time as the *syn*-isomer. While this result can be attributed to contamination (≈12%) by the (2′*R*,4′*R*)-isomer, it prevented the elucidation of the natural pheromone's stereochemistry based on antennal response to these synthetic samples alone.

The absolute configuration of supellapyrone was finally established by the use of chiral chromatography to separate all four isomers, coupled with EAD detection of the active peak(s). Dramatic resolution of supellapyrone and its stereoisomers was achieved on a trifluoroacetylated γ-cyclodextrin phase, Chiraldex GTA, which gave baseline separation of the following four isomers: I, 23.85 min (k' = 33.07); II, 25.69 min (k' = 35.7); III, 26.46 min (k' = 36.8); IV, 27.82 min (k' = 38.74) (Fig. 4). Cochromatography of the random mixture of stereoisomers with the synthetic (91:9) *syn*-supellapyrone demonstrated that peaks I and III corresponded to the individual *syn*-enantiomers, whereas peaks II and IV were the *anti*-enantiomers. The 1:1 (2′*R*,4′*R*)/(2′*R*,4′*S*)-diastereomeric

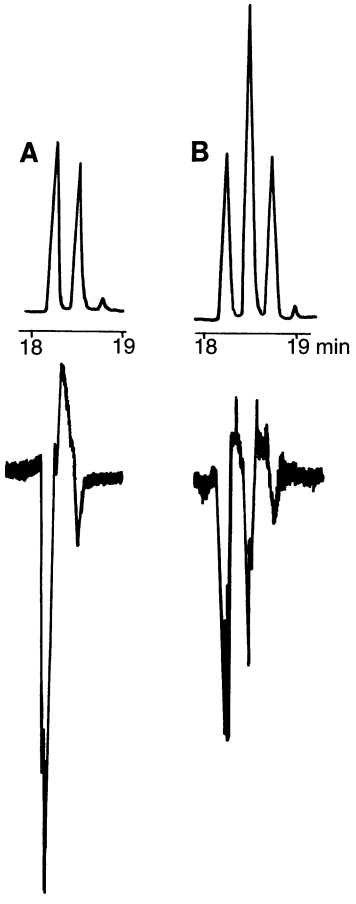


FIG. 3. Parallel FID and EAD chromatograms obtained from a mixture of the four isomers of supellapyrone (*A*) separated on a CP-Sil 5CB column. A male *S. longipalpa* antenna was used as the sensing element. (*B*) Response to the former sample injected twice so that the *syn* component from the second injection coelutes with the *anti* component from the first injection.

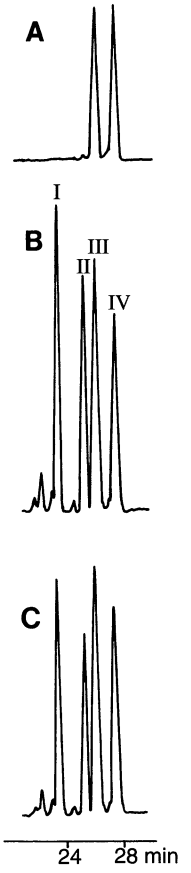


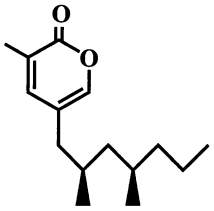
FIG. 4. Resolution of the following four isomers of supellapyrone on a chiral column: I, (2′*S*,4′*S*); II, (2′*S*,4′*R*); III, (2′*R*,4′*R*); IV, (2′*R*,4′*S*). (*A*) Mixture of the (2′*R*,4′*R*)- and (2′*R*,4′*S*)-diastereomers. (*B*) Resolution of all stereoisomers. (*C*) Coinjection of material used in *A* and *B*.

mixture gave two peaks with the same retention times as peaks III and IV (Fig. 4*A*). Coinjection with a mixture of all four isomers (Fig. 4*B*) corroborated that peaks III and IV were the (2′*R*,4′*R*)- and (2′*R*,4′*S*)-isomers, respectively (Fig. 4*C*). Consequently, the four peaks were characterized as follows: I, (2′*S*,4′*S*); II, (2′*S*,4′*R*); III, (2′*R*,4′*R*); IV, (2′*R*,4′*S*).

Despite the remarkable separation and unambiguous identification of these stereoisomers, our first attempts to use coupled chiral GC–EAD were unrewarding. In marked contrast to achiral capillary GC–EAD, which provides a sharp pulse of the eluted compound at the antenna, chiral capillary GC gave rise to broad peaks similar to those obtained with packed GC columns. This difficulty had been overcome in earlier studies with packed columns by accumulating column effluent in a reservoir for 15 sec and then flushing the collected sample over an electroantennogram preparation with nitrogen (12). In an alternative approach, we retained our continuous flow system and invested in the improvement of our electrophysiological instrumentation by designing an amplifier with an active second-order Butterworth high-pass filter. Clear-cut responses (Fig. 5) were achieved with a cut-off frequency of 0.016 Hz (R = 2.2 MΩ). To our surprise, however, two EAD peaks were detected, one to the *anti*-(2′*S*,4′*R*)-isomer and the other to the *syn*-(2′*R*,4′*R*)-isomer. We hypothesize that the earlier eluting (2′*S*,4′*R*)-isomer mimics the natural product well enough to elicit an EAD response and that adaptation of the antenna to this mimic prevents a better response to (2′*R*,4′*R*)-supellapyrone, which appears from the results described above to be the natural stereoisomer. This hypothesis was supported by the fact that there was no EAD response to (2′*S*,4′*S*)-isomer, which, as the earliest eluting component, was free of any adaptation-related complication. By having ruled out the (2′*S*,4′*S*)-stereochemistry, we sought confirmation that the natural product was (2′*R*,4′*R*).

This was achieved by isolating the natural sex pheromone from an extract of 200 virgin females. The isolated supellapyrone gave a single peak (16.19 min) on a DB-1 column, whose identity was confirmed by GC–MS analysis. These data confirmed that the natural pheromone has no *anti*-configuration component, since under the same GC–MS conditions a *syn*/*anti* mixture gave two peaks at 16.19 and 16.25 min.

The isolated natural pheromone also gave a single peak on the chiral column (Fig. 6*A*) and it was EAD-active (Fig. 6*B*). Coinjection of a mixture of the four stereoisomers with the isolated sex pheromone confirmed that the natural product coeluted with (2′*R*,4′*R*)-supellapyrone (Fig. 6*C*). Natural supellapyrone is, therefore, unambiguously shown to be 5-(2′*R*,4′*R*)-(dimethylheptanyl)-3-methyl-2*H*-pyran-2-one.



DISCUSSION

The application of GC–EAD in combination with stereochemically controlled synthesis is a valuable addition to the techniques available for determination of absolute configuration of insect sex pheromones. Since technical problems resulting from relatively broad GC peaks were overcome, this approach should be widely applicable in studies of insect chemical communication where chiral semiochemicals are involved.

It should be noted that when a nonnatural stereoisomer acts as an antagonist to a natural pheromone, this isomer will also elicit an electroantennogram response. This is the case with the scarab beetle *Anomala cuprea*, which utilizes (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one as the major sex pheromone (8), but whose males also have inhibitor-sensitive sensilla (13). Therefore, it is important to emphasize that ultimately stereochemistry can only be unambiguously assigned by comparison of retention times and EAD responses of the natural and synthetic materials. Assignment of stereochemistry based only on an EAD response to synthetic isomers may be misleading, as demonstrated by the response of an *S. longipalpa* male antenna to the nonnatural (2′*S*,4′*R*)-isomer.

Finally, based on the fact that behavioral responses of male *S. longipalpa* in a two-choice olfactometer were slightly lower to a 4-fold larger amount of a synthetic mixture of the four isomers than to the natural sex pheromone and that, on the other hand, electroantennogram responses were slightly greater to the synthetic mixture, it is conceivable that the

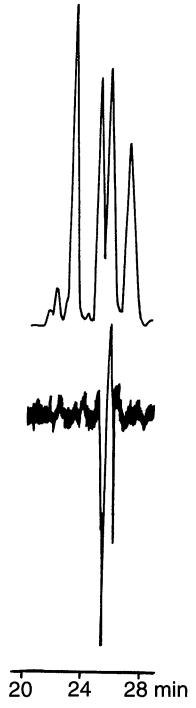


FIG. 5. Response of a male *S. longipalpa* antenna to synthetic isomers separated on a chiral column. The lower response to the (2′*R*,4′*R*)-isomer is attributed to adaptation of the antenna to the previously eluting nonnatural (2′*S*,4′*R*)-stereoisomer.

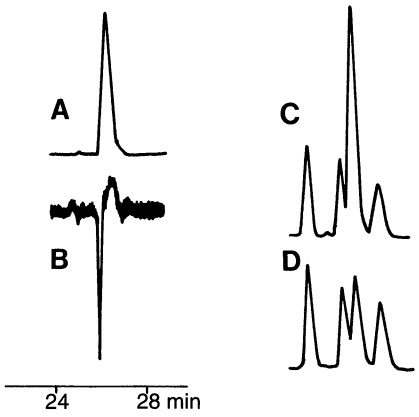


FIG. 6. Parallel FID (*A*) and EAD (*B*) chromatograms of the isolated natural sex pheromone. A male *S. longipalpa* antenna was used as the sensing element. Resolution of the four isomers with (*C*) and without (*D*) coinjecting the natural product is also shown.

(2′*S*,4′*R*)-isomer is an antagonist to the natural (2′*R*,4′*R*)-supellapyrone.

In conclusion, we have used the chiral GC–EAD technique to determine the stereochemistry of natural supellapyrone; enantiomerically pure sex pheromone can now be synthesized for use in the management of the brownbanded cockroach.

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