SEX PHEROMONE OF THE SCARAB BEETLE *Phyllophaga elenans* AND SOME INTRIGUING MINOR COMPONENTS

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Abstract—Three amino acid-derived compounds were identified in extracts from the pheromone glands of the scarab beetle *Phyllophaga elenans*, i.e., L-isoleucine methyl ester (LIME), *N*-formyl L-isoleucine methyl ester (For-LIME), and *N*-acetyl L-isoleucine methyl ester (Ac-LIME). The compounds were characterized from their spectral data (MS and IR), confirmed by synthesis, and their absolute configurations were assigned by gas chromatography with a chiral phase column. The amount of LIME in calling females was ca. 2 μg/gland, whereas only traces of For-LIME and Ac-LIME (0.005% of LIME) were detected in fresh extracts. Regardless of the storage temperature, the amounts of the minor constituents in the extracts increased over time. Field tests showed that traps baited with For-LIME captured more beetles than control traps. Ac-LIME *per se* was not attractive, and it did not increase trap catches when combined with For-LIME. Traps baited with LIME caught ca. 150 beetles/trap/day, but catches

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did not increase with the addition of For-LIME and/or Ac-LIME in binary or tertiary blends.

INTRODUCTION

We have been studying chemical communication in scarab beetles (Coleoptera: Scarabaeidae), which utilize a wide variety of chemical compounds as pheromones (Leal, 1998a, 1999; Ruther et al., 2001). While pheromones of rutelines (subfamily Rutelinae) arise largely by transformation of fatty acids (Leal et al., 1999), melolonthine (Melolonthinae) pheromones are derived from fatty acids and other precursors, including isoprenoids and even amino acids (Leal, 1999). An amino acid-derived pheromone, L-isoleucine methyl ester (LIME), was first isolated and identified in the large black chafer, *Holotrichia parallela* (Motschulsky) (Leal et al., 1992, 1993). Later, L-isoleucine and L-valine methyl esters were identified in the cranberry white grub, *Phyllophaga anxia* (Zhang et al., 1997).

The genera *Holotrichia* and *Phyllophaga* belong to the same tribe Melolonthini; their separation is based on a geographical division where species from Asia are placed in the former and species from South, Central, and North America in the latter (Mila Coca Abia, personal communication). We envisaged that other *Holotrichia* species would utilize amino acid-derived pheromones, but to our surprise the pheromone of a species native to India, *H. consanguinea* Blanchard, was identified as anisole (Leal et al., 1996). This finding prompted us to analyze the pheromone chemistry of *Phyllophaga* species from South and Central America to compare their pheromones. Here, we report the identification of the sex pheromone of *P. elenans* (Saylor), one of the most damaging pests of grain crops and sugarcane in Central America (King, 1984).

METHODS AND MATERIALS

Pheromone Extraction. Insects were collected in Turrialba and Guanacaste, Costa Rica, or obtained from laboratory cultures maintained in Turrialba. Abdominal tips of calling females were excised with forceps, and the material was cleaned to remove tissues other than the ball-shaped sac housing the pheromone glands (Kim and Leal, 1999). Glands were extracted for 3 min either with diethyl ether or methanol. After removing the pheromone glands, samples were transferred to glass ampoules, sealed, and shipped to Tsukuba, Japan, or Davis, California. Diethyl ether was distilled in an all-glass distillation apparatus, and methanol was HPLC grade (Merck). Distilled diethyl ether was sent to Costa Rica

in flame-sealed ampoules; some ampoules were returned with the samples for blank analyses. A whole body extract of a scarab beetle, *Cyclocephala amazonica* (Dynastinae: Cetoniinae), also was obtained so as to detect possible contaminants of the extraction procedure. In order to investigate possible artifacts in the samples shipped from Costa Rica, two series of experiments were conducted there (ChemTica Lab). First, calling females were collected in the field, brought to the lab, and extracted within 2 hr of collection. Extracts were made with diethyl ether (Merck analytical grade), flame-sealed, and kept at $-20°C$ (hereafter referred to as the $-20°C$ protocol). Aliquots were analyzed after 30 hr and 7 days. In another procedure, glands were extracted with diethyl ether at $-78°C$ (referred to as the $-78°C$ protocol), warmed to room temperature for 2 min, and then an aliquot was taken for analysis. Samples were stored at $-20°C$ and analyzed again after 3 days. The stability of LIME in diethyl ether was studied by preparing fresh samples (10 μg/ml) and analyzing them by GC-MS soon after their preparations and every other day up to 15 days. In addition, gland extracts from calling females of the large black chafer were also obtained in Tsukuba. The extracts were kept at room temperature and analyzed immediately after extraction and 3 days later.

Analytical Procedures. Low-resolution electron impact mass spectrometry (EI-MS) was carried out with an HP 6890 gas chromatograph (GC; Agilent, formerly Hewlett-Packard, Palo Alto, California, USA) linked to a mass selective detector (MSD 5973; Agilent). Chromatographic resolution was done on an HP-5MS column (30 m × 0.25 mm; 0.25 μm; Agilent) that was operated at $70°C$ for 1 min, increased to $230°C$ at a rate of $10°C$/min, and finally held at this temperature for 10 min. GC-MS analyses in Costa Rica were performed on a similar instrument equipped with a DB-5 column (30 m × 0.25 mm; 0.25 μm; J&W Scientific, Folsom, California, USA). The oven was operated at $50°C$, increased to $70°C$ at a rate of $25°C$/min, held at $70°C$ for 2 min, increased again at $10°C$/min to $200°C$, and finally held at this temperature for 2 min. Helium was used as the carrier gas at 1 ml/min (analyses in Japan and the USA) and 0.7 ml/min (analyses in Costa Rica). GC analyses with a chiral stationary phase were performed on an HP6890 gas chromatograph with a CP-Chirasil-DEX CB column (30 m × 0.25 mm; 0.25 μm; Chrompack, Middelburg, The Netherlands). The oven temperature was programmed initially $75°C$, held for 25 min, increased at a rate of $1°C$/min to $85°C$, held for 25 min, and increased again at $1°C$/min to $95°C$, held for 25 min, and finally increased at a rate of $10°C$/min to $120°C$, and held at this final temperature for 10 min. Vapor-phase Fourier transform infrared (FTIR) spectra were recorded with an HP 6890 GC coupled to a FTIR system equipped with a light pipe interface, FTS-40A, GC/C32 (BioRad, Cambridge, Massachusetts, USA). The GC was equipped with an HP-5 column (30 m × 0.32 mm; 0.25 μm) operated at $70°C$ for 1 min, increased to $250°C$ at a rate of $10°C$/min, and finally held at this temperature for 10 min. The light pipe and the transfer line were set at $230°C$ and $250°C$, respectively. Helium was used as carrier gas for GC-FTIR at 2 ml/min.

Syntheses. LIME was obtained as previously described (Leal et al., 1993). *N*-Acetyl- and *N*-formyl-isoleucine methyl ester were prepared by acetylation and formylation with acetic anhydride and formic acid, respectively. Acetic anhydride (3 ml) was added to a solution of LIME (0.5 g, 3.45 mmol) in dry ether (10 ml) at 40°C. After stirring for 15 min, diethyl ether was added (15 ml), and the mixture was washed with cold water and then sodium bicarbonate (10%). The organic phase was dried (anhydrous magnesium sulfate), concentrated, and purified by flash chromatography with Wakogel C-200 (Wako, Tokyo, Japan) to give *N*-acetyl L-isoleucine methyl ester (484 mg, 75%). mp 54°C. MS data:128 (100%), 41 (21), 43 (54), 44 (27), 57 (30), 60 (13), 69 (13.1), 74 (8.3), 86 (85), 88 (53), 89 (23), 99 (62), and 131 (13). Vapor-phase IR data (cm^{-1}): νN-H, 3456; νC-H, 2974; νC $=$ O (methyl ester) 1755; νC $=$ O, 1717 (acetate); νC-O (acetate), 1492; νC-O (ester) 1202. Similarly, *N*-formyl L-isoleucine methyl ester was prepared from LIME (0.3 g, 2.1 mmol) and formic acid (5 ml, 90%). The reaction mixture was refluxed for 15 min before extraction and washed with a saturated solution of sodium bicarbonate. After drying, concentration, and chromatographic purification, *N*-formyl L-isoleucine methyl ester was obtained (243 mg, 67%). bp 112°C/mm Hg. MS data: 85 (100%), 41 (24), 46 (19), 57 (16), 69 (28), 88(8), 96 (3), 114 (67), 117 (20), 128 (10). Vapor phase IR data (cm^{-1}): νN-H, 3447; νC-H, 2974; νC-H (aldehydic), 2851, νC $=$ O (methyl ester) 1755; νC $=$ O, 1728 (formate); νC-O (formate), 1487; νC-O (ester) 1186. Similar procedures were done for the synthesis of the other stereoisomers starting with the synthesis of the methyl esters of D-, L-*allo*, and D-*allo*-isoleucine methyl esters.

Field Experiments. Tests were conducted in a sugarcane plantation in Guanacaste, Costa Rica. Traps were 20-liter white plastic buckets with crossed white plastic vanes (75 × 35 cm). Detergent-laced water was added to the bottom to retain insects. Test compounds were released from membrane devices at a rate of ca. 1 mg/day. Traps were placed in a randomized block, with the intertrap distance of 35 m. Five replicates were placed in the field, and the treatments were rerandomized after daily capture counts. Capture data were transformed to log ($x + 0.5$) and analyzed by ANOVA.

RESULTS AND DISCUSSION

Analyses of the gland extracts from samples collected in Costa Rica and shipped to Japan showed three major peaks (Figure 1A). Other longer retention time peaks were ruled out on the basis of the profiles obtained with a blank (solvent) and a whole-body extract from *C. amazonica*, particularly hydrocarbons and phthalate esters. The mass spectrum profile of peak 1 (data not shown) was indistinguishable from LIME, previously identified from *H. parallela* (Leal et al., 1992, 1993). The base peak at *m/z* 86 is due to fragmentation
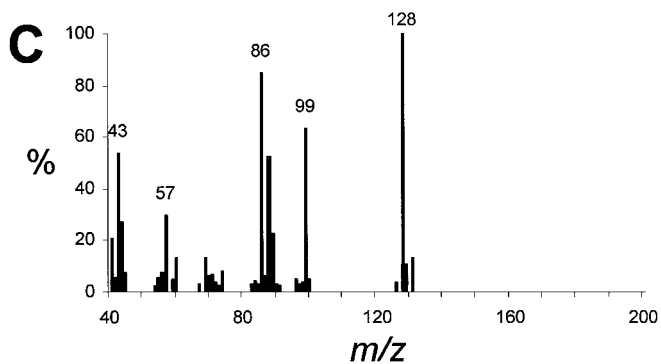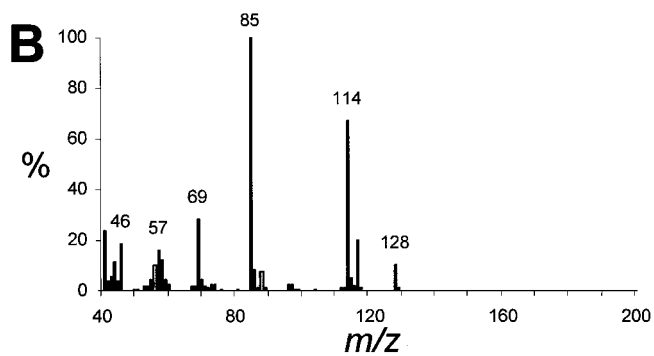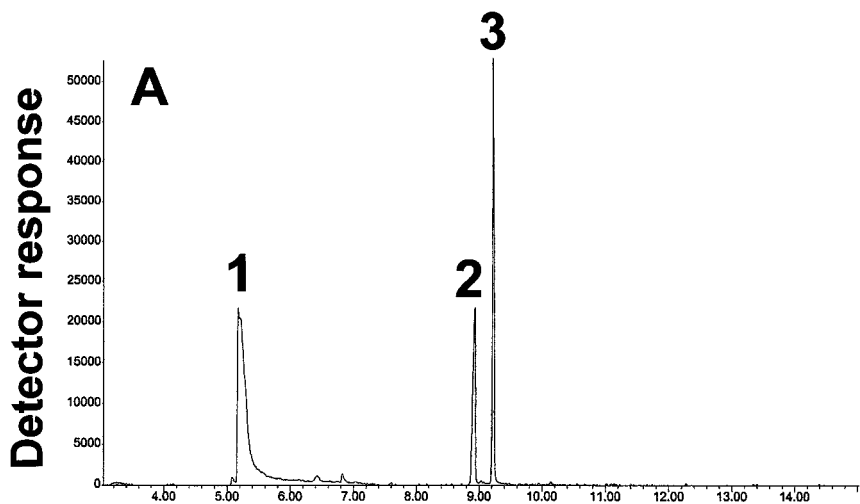
FIG. 1. GC-MS profile of a gland extract from *P. elenans* analyzed on a nonpolar HP-5MS column (A). The mass spectrum profile of compound 1 was identical to that of L-isoleucine methyl esters. Compounds 2 and 3 generated the MS displayed in B and C.
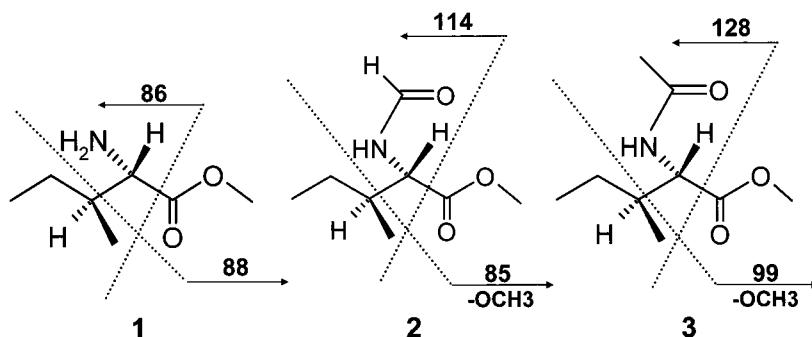
FIG. 2. Structures of compounds **1**, **2**, and **3** and proposed fragmentation patterns. The stereochemistry of displayed structures is consistent with the assignment made by chromatography with a chiral stationary phase.

of the C-2–C-3 bond. The vapor phase IR spectrum of 1 (data not shown) and its retention time (t_R = 5.14 min) were indistinguishable from those of authentic LIME. The mass spectral data for peaks 2 and 3 (Figure 1B and C, respectively) indicated that they were derived from LIME. Fragmentations of the C-1–C-2 bonds in the two compounds (Figure 2) give rise to the peaks at m/z 114 and 128 in the MS of compounds 2 and 3, respectively. Fragmentation of the C-2–C-3 bond seems to be followed by loss of OCH_3 to generate peaks at m/z 85 and 99 from compounds 2 and 3, respectively. Vapor phase IR spectra of these compounds (Figure 3) confirmed that 2 and 3 were formyl and acetyl derivatives of LIME, respectively. First, the two carbonyl bands were observed in the IR spectrum of each compound. A carbonyl band of the methyl ester moiety appeared at 1755 cm^{-1}, whereas the carbonyl bands of the formyl and acetyl moieties appeared at 1728 and 1717 cm^{-1}, respectively (Leal, 1998b). In addition, N–H stretching bands appeared at 3447 and 3456 cm^{-1} along with stretching bands of the formyl and acetyl groups at 1487 and 1492 cm^{-1}, respectively. Furthermore, an aldehydic stretching band at 2851 cm^{-1} confirmed the presence of a formyl group (Leal, 1998b).

Synthetic *N*-formyl L-isoleucine methyl ester (hereafter referred to as For-LIME) and *N*-acetyl L-isoleucine methyl ester (hereafter referred to as Ac-LIME) gave retention times identical to the natural compounds (For-LIME, t_R = 8.93 min; Ac-LIME, t_R = 9.23 min). Next, we investigated the stereochemistry of the formyl and acetyl derivatives of LIME. Chiral resolution of the four possible stereoisomers was obtained with a cyclodextrin-based column, CP-Chirasil-DEX CB. Separation of the four isomers of For-LIME was achieved (Figure 4D), with *N*-formyl D-allo-, L-allo-, D-, and L-isoleucine methyl ester appearing at 108.1, 109.3, 110.0, and 112.0 min, respectively. The diastereomers of Ac-LIME were well separated, but the enantiomers were not baseline separated (Figure 4B). The four stereoisomers, *N*-acetyl D-allo-, L-allo-, D-, and L-isoleucine methyl ester, appeared at 87.4, 88.1,
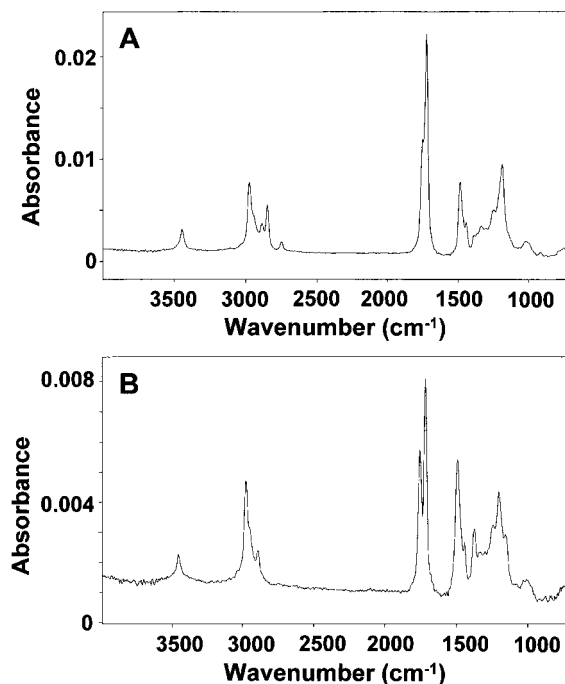
FIG. 3. Vapor-phase IR spectrum of compounds 2 (A) and 3 (B), showing two carbonyl bands, C-O and N-H stretching bands, and other identifying features. A major difference between the two spectra is the aldehydic C-H stretching band at 2851 cm^{-1} (A) in the formyl compound.

93.6, and 94.4 min, respectively. Injection of the natural products under the same conditions showed that peaks 2 and 3 eluted with the same retention times as *N*-formyl L-isoleucine methyl ester and *N*-acetyl L-isoleucine methyl ester at $t_R = 94.4$ min and 111.9 min, respectively (Figure 4A,C). The stereochemistry of the two compounds was, therefore, unambiguously identified as L, i.e., in the two compounds the carbons at positions 2 and 3 have the *S* configuration as in the natural amino acid isoleucine.

Initial attempts to determine the ratio of the two compounds in the pheromone glands failed, as the ratio varied dramatically from sample to sample, with some being even completely devoid of LIME (peak 1). We suspected that extraction artifacts could account for this variation. A series of experiments was conducted to clarify this. Analysis of gland extracts ($-20°C$ protocol) showed that LIME was the major constituent of fresh samples (Figure 5A). When analyzed 7 days later (stored at $-20°C$) (Figure 5B), the profile changed dramatically with content of LIME decreasing and For-LIME and Ac-LIME increasing.
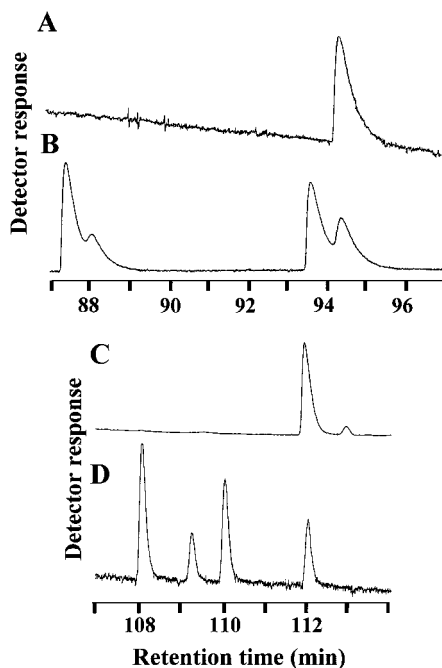
FIG. 4. Chiral resolution of the stereoisomers of the two compounds derived from LIME. Analysis of gland extracts (A and C). The *N*-acetyl isoleucine methyl esters showed shorter retention times (B) than the corresponding formyl compounds (D). Furthermore, the four peaks of the stereoisomers of *N*-formyl isoleucine methyl esters had higher resolution. The four stereoisomers for the two compounds appeared in the following order: D-*allo*, L-*allo*, D, and L.

We made an in-house comparison by analyzing fresh ether extracts from the pheromone glands of calling females of the large black chafer, *H. parallela*. Although trace amounts of For-LIME and Ac-LIME were detected in these fresh extracts (obtained with freshly distilled ether), there was no significant change in their contents over time. We observed, however, that the contents of LIME decreased dramatically over time when old ether was used for gland extracts, but this decrease was not associated with an increase in the trace amounts of For-LIME and Ac-LIME. Degradation of synthetic LIME in old ether solutions (10 μg/ml) was also observed, but this could not be correlated with formation of the acetyl and formyl derivatives.

Finally, analyses of fresh gland extracts obtained at low temperature ($-78°$C protocol) showed (Figure 5C) that LIME is the major constituent of the pheromone gland (ca. 2 μg/female gland), whereas For-LIME and Ac-LIME were detected in trace amounts (0.005% of LIME). These trace constituents increased over time
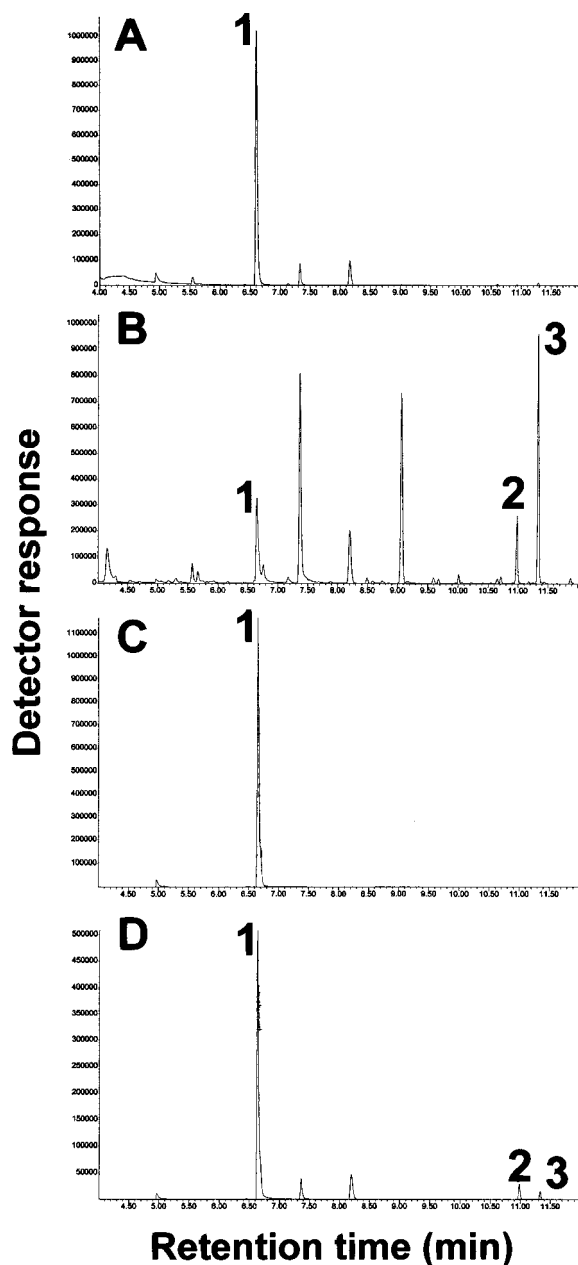
FIG. 5. Chemical analyses confirming the occurrence of trace amount of For-LIME (peak 2) and Ac-LIME (peak 3) in the glands of *P. elenans*. Sample obtained by the −20°C protocol analyzed soon after extraction (A) and 7 days later (B). Sample prepared by the −78°C protocol showed trace amounts of For-LIME and Ac-LIME (0.005% of LIME) (C); the amounts increases over time even in samples kept at −20°C (D).
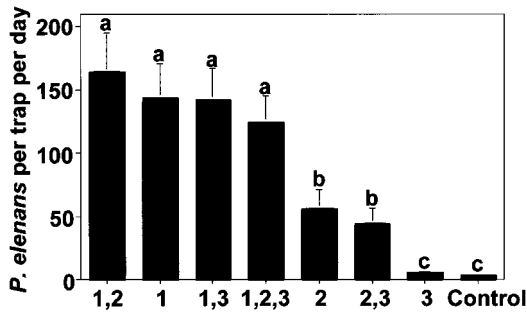
FIG. 6. Results of field tests conducted in a sugarcane plantation in Costa Rica. There is no difference in catches by traps baited with L-isoleucine methyl ester (1) alone, or in combination with For-LIME (2) and Ac-LIME (3). ANOVA on log $(x + 5)$ transformed data gave $F = 31.35, df = 7.63, P < 0.05$. Means followed by different letters are different by Bonferonni t test ($P > 0.95$).

(Figure 5D). We, therefore, conclude that the putative pheromone system of *P. elenans* comprises a major constituent (LIME) and two secondary compounds (For-LIME and Ac-LIME).

Field tests showed that traps baited with For-LIME alone caught more beetles than control traps, whereas captures in Ac-LIME traps were not different from the control (Figure 6). On the other hand, LIME–baited traps captured over 150 beetles/trap/day. There was no significant increase in trap catches when the secondary constituents, For-LIME and Ac-LIME, were added. However, catches varied with trap design and location within field (Oehschlager et al., 2003). In conclusion, LIME has been identified as a sex pheromone of *P. elenans*. Two minor constituents of the pheromone gland, For-LIME and Ac-LIME, are not essential for practical applications.

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