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Female sex pheromone of the longhorn beetle *Migdolus fryanus* Westwood: N-(2'S)-methylbutanoyl 2-methylbutylamine

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Abstract. The first known long-range female-released sex pheromone for the family Cerambycidae is reported from *Migdolus fryanus*, a sugarcane pest in South America. Although two female-specific compounds, namely, N-(2'S)-methylbutanoyl 2-methylbutylamine and N-formyl L-isoleucine methyl esters were identified, field tests with synthetic chemicals revealed that only the amide was active and that the amino acid derivative neither increased or decreased trap catches by the amide. This is the first identification of an amide as a sex pheromone.
Key words. *Migdolus fryanus*; Coleoptera; Cerambycidae; N-2'-methylbutanoyl 2-methylbutylamine; N-formyl L-isoleucine methyl ester; sex pheromone; chiral resolution; dose-response.

Long-range sex pheromones have been identified in various groups of insects, but their role is still poorly understood in some families as, for example, in cerambycid (longhorn) beetles (Coleoptera: Cerambycidae). Since a large number of longhorn beetles gather on host plants for feeding, it appears likely that they would rely more on contact sex pheromones for mate recognition, and plant volatiles for attraction rather than on long-range sex pheromones. Even for species that do not feed in the adult stage, only a short-range sex pheromone has been identified thus far[1,2]. Although it has been demonstrated a long time ago that male cerambycid beetles were attracted over long distances to tethered females[3], no female-released long-range sex pheromones have been identified in this group. Results of recent field tests in Brazil supported the fact that mate-finding in *Migdolus fryanus* Westwood, an economically important pest of sugarcane in South America, is mediated by a female sex pheromone[4] and this prompted us to identify the active chemicals.

Materials and methods

Beetles were captured in Olímpia, São Paulo, Brazil, at the beginning of the flight season of 1993. In order to compare the gas chromatogram profiles of males and females, volatiles from live insects and whole body extracts were collected. Extractions were done by washing the whole insect with dichloromethane for 3 min and volatiles from live insects were collected on Tenax TA® columns for 8 h. The columns were sealed and shipped to Japan where the volatiles were washed out, or they were washed in Brazil either with hexane or ether, and then the extracts sealed in ampules were air mailed to Japan.

Samples were analyzed by gas chromatography (GC) on DB-wax (30 m × 0.254 mm; 0.25 μm) or HP-1 (12 m × 0.2 mm; 0.33 μm) columns operated at 50 °C for 1 min, programmed at 4 °C/min to 180 °C, held at this temperature for 1 min and programmed again at 10 °C/min to 230 °C, and finally held at this temperature for 30 min, in short, 50(1)-180(1)/4-230(30)/10. Chiral resolution was done on a CP-Cyclodextrin β-236-M-19 (50 m × 0.25 mm; 0.25 μm; Chrompack) column operated at 50(1)-150(40)/1 (He at 0.9 ml/min; inlet pressure of 1 bar). Resolution was also attempted on Chiraldex GTA (20 m × 0.25 mm; 0.125 μm; Astec) column operated at 60, 70, 80, 90, 100, or 120 °C. Mass spectra were recorded on a Hewlett-Packard 5891 mass selective detector using a DB-wax capillary column operated under the same conditions as for GC. Vapor phase Fourier transform infrared (FTIR) was done on a Hewlett-Packard 5965B equipped with a DB-wax capillary column operated at 70(1)-150(1)/5-240(10)/10. The light pipe was operated at 250 °C and the transfer line at 270 °C.

N-Formyl L-isoleucine methyl ester was prepared by formylation of L-isoleucine methyl ester with formic acid and acetic anhydride in dry dichloromethane. The following amides were synthesized in small scale by the reaction of appropriate anhydrides or acid chlorides with amines: N-2'-methylbutanoyl 3-methylbutylamine [57(100), 87(85), 41(75)], N-2'-methylbutanoyl 2-methylbutylamine (data as for the natural product), N-3'-methylbutanoyl 3-methylbutylamine [73(100), 129(80), 85(65)], N-3'-methylbutanoyl n-pentylamine [114(100), 57(90), 41(85)], N-3'-methylbutanoyl 1-methylbutylamine [44(100), 41(56), 57(51)], N-2',2'-dimethylpropyl-3-methylbutanamide [58(100), 102(81), 41(40)], N-3'-methylbutyl-4-methylbutanamide [73(100), 85(71),

129(70)], N-2′-methylbutanoyl 1-methylbutylamine [44(100), 57(90), 41(68)], N-2′-methylbutanoyl n-pentylamine [57(100), 114(80), 41(75)], and N-2′,2′-dimethylpropyl-2-methylbutanamide [58(100), 102(91), 72(44)]. For the field tests, N-(2′S)-methylbutanoyl (2S)-methylbutylamine was prepared in dry dichloromethane and catalyzed by pyridine.

In the field experiments, funnel JT traps (Japan Tobacco Co., Tokyo, Japan) were placed 22 m apart, buried so that the lip of the funnel was positioned at the surface. They were randomly placed (and rerandomized every day) in lines at the board of the plantation and the corridors for allowing the access of trucks to the field. This design was adopted because under natural conditions male beetles search for females in these corridors rather than inside the plantation. The synthetic sex pheromone blend was incorporated into plastic pellets, placed into pellet-holders and attached to the traps 2 cm above the trap lip. Virgin females were placed inside small plastic bottles provided with the traps and set 7 cm above the trap lip. Capture data were transformed to log $(x + 1)$ before difference between means were tested for significance by the Tukey-Kramer HSD (honestly significant difference) test, or Student's t-test at a 5% level. In the figures, means of untransformed data are shown along with 1 SE for the error bars and treatments labeled with the same letters are not significantly different.

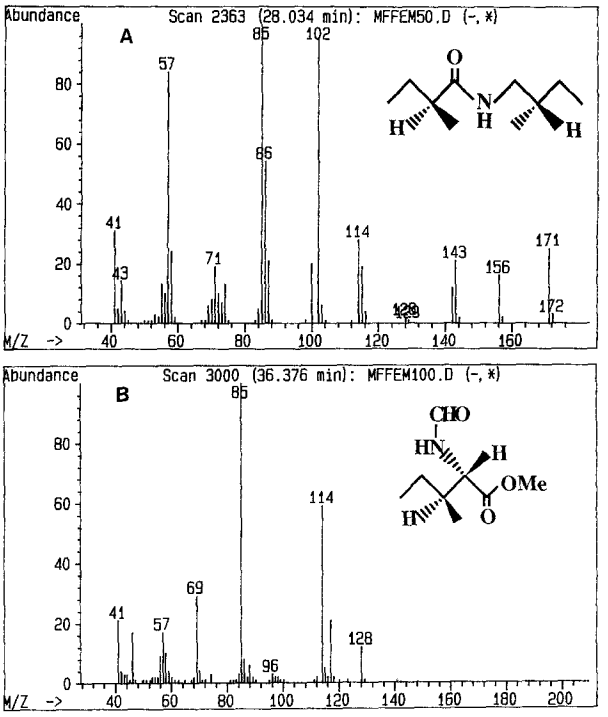

Figure 1. Mass spectral data of *M. fryanus* female-specific compounds and their chemical structures: *A*) N-(2′S)-methylbutanoyl (2S)-methylbutylamine; *B*) N-formyl L-isoleucine methyl ester.

Results and discussion

Comparative GC analyses of the samples from male and female beetles revealed that two peaks (ratio 7:1) were female-specific and that they were detected not only in the airborne volatiles, but also in the whole-body extracts. On a DB-wax capillary column, the major peak appeared at t_R 26.85 min whereas the minor gave a retention time of 35.06 min. On an HP-1 column, the two female-specific peaks appeared at t_R 23.64 and 23.20 min, respectively. The difference of their retention indices (ΔI) on these two columns was 631 ($I_{DB\text{-}wax}$ 1916; $I_{HP\text{-}1}$ 1285) and 987 ($I_{DB\text{-}wax}$ 2262; $I_{HP\text{-}1}$ 1275), respectively.

The samples of the volatiles collected from female beetles were combined and separated on a silica gel column by successively eluting with hexane-ether mixtures (100:0; 95:5; 90:10; 80:20; 50:50; 0:100). The major component, which was recovered into the 50% fraction, gave an MS with the base peak at m/z 85 and M^+ 171 (fig. 1A), for which no match was found in a library search (Wiley, Hewlett-Packard). The minor component eluted in the ether fraction and had an MS having the base peak at m/z 85 and a characteristic peak at m/z 114 (fig. 1B), which matched closely with the spectrum of N-formyl-isoleucine methyl ester (Wiley library). In addition, its retention index on the non-polar column was very close to previously reported data[5] ($I_{DB\text{-}1}$ 1276). Vapor-phase FTIR showed the two carbonyl bands only partially separated (1728 cm^{-1}) along with aldehydic CH and NH stretching bands at 2753 and 3444 cm^{-1}, respectively. Synthetic N-formyl L-isoleucine methyl ester gave the same retention times on the two columns and an MS identical to that of the natural product. Given the fact that L-isoleucine methyl ester has been previously identified[6,7] as the major component of the sex pheromone of the scarab beetle *Holotrichia parallela*, the stereochemistry of the N-formyl related compound was considered to be the same since these chemicals might be derived from the naturally occurring L-amino acid.

Vapor-phase FTIR of the major component showed the profile of a secondary amide, with the amide bands I and II at 1704 (νCO) and 1496 cm^{-1} (δNH + νCN of C–N–H; weak band at 1192 cm^{-1}), respectively. An N–H stretching band appeared at 3472 cm^{-1}. The MS fragmentation pattern (fig. 1A) suggested that both acid and hydrocarbon moieties had five carbons, probably branched. Fragmentation of the C–N bond would give rise to $[NHC_5H_{11}]^+$ at m/z 86 and $[C_4H_9CO]^+$ at m/z 85 and the peaks at m/z 57 and 114 would be due to $[C_4H_9]^+$ and M-C_4H_9, respectively.

All possible isomers were synthesized, but only N-2′-methylbutanoyl 2-methylbutylamine showed the same retention times on the two capillary columns as the natural product and gave an identical MS.

Chromatographic separation of the four possible optical isomers was tried on chiral columns. Although no

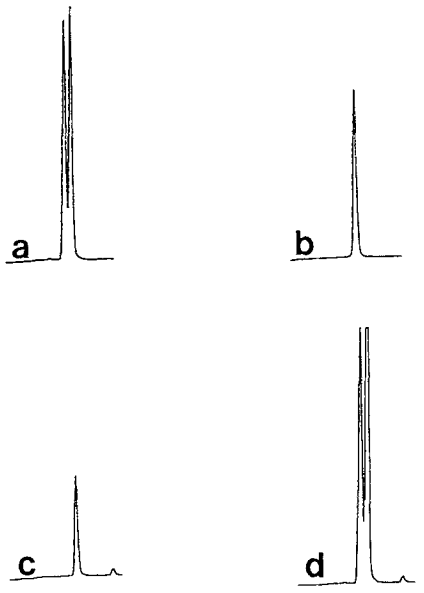

Figure 2. Chromatograms obtained on CP-Cyclodextrin β-236-M-19 chiral column: *a*) N-(2′R/S)-methylbutanoyl (2S)-methylbutylamine; *b*) N-(2′S)-methylbutanoyl (2R/S)-methylbutylamine; *c*) the natural product from female *M. fryanus* isolated in the 50% fraction, and *d*) co-injection of the natural product with N-(2′R/S)-methylbutanoyl (2S)-methylbutylamine.

resolution was obtained on Chiraldex GTA, partial separation of the isomers was achieved on the CP-Cyclodextrin column. Separation of the enantiomers of the acid moiety was almost baseline (t_R 102.619 and 102.939 min; $k'_1 = 15.79$, $k'_2 = 15.85$; $\alpha = 1.004$), as shown by the GC trace for N-(2′R/S)-methylbutanoyl (2S)-methylbutylamine (fig. 2a). N-(2′S)-Methylbutanoyl (2R/S)-methylbutylamine gave a single peak (corresponding to the peak of longer t_R in fig. 2a), so there was no resolution of the enantiomers of the hydrocarbon moiety (fig. 2b), probably because the active carbon is separated from the functional group (N) by a methylene. The natural product (fig. 2c) gave a single peak in that region, which had the same t_R as the amide having the acid moiety with the (S)-stereochemistry (longer t_R). Co-injection of the natural product with N-(2′R/S)-methylbutanoyl (2S)-methylbutylamine corroborated that the female-specific amide from *M. fryanus* co-eluted with N-(2′S)-methylbutanoyl 2-methylbutylamine (fig 2d). Based on biosynthetic reasoning, the natural product was identified as N-(2′S)-methylbutanoyl (2S)-methylbutylamine, although the stereochemistry of the hydrocarbon moiety remains to be analytically determined.

Field tests conducted in Olímpia (Feb. 7–12, 1994) during the flight season of *M. fryanus* demonstrated that traps baited with a synthetic mixture of N-(2′S)-methylbutanoyl (2S)-methylbutylamine and N-formyl L-isoleucine methyl ester (same natural ratio of 7:1, 1 mg) captured significantly more beetles (×2.7) than traps baited with two virgin females renewed daily; female-baited traps captured significantly more than control (fig. 3).

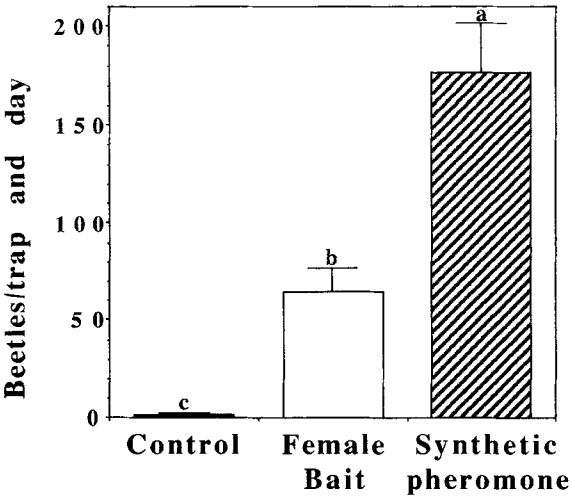

Figure 3. Captures of *M. fryanus* males in traps baited with synthetic sex pheromone, or 2 virgin females and control traps.

In a separate experiment, the effect of the chirality of the hydrocarbon moiety on the catches of the beetles was examined. Traps were baited either with N-(2′S)-methylbutanoyl (2S)-methylbutylamine ((S)-enantiomer), or N-(2′S)-methylbutanoyl (2R/S)-methylbutylamine (racemic) plus N-formyl L-isoleucine methyl ester in the same 7:1 ratio (dose 1 mg). Captures of *M. fryanus* male beetles were not significantly different (Student's t-test, 5% level). In the traps baited with the racemic sex pheromone 95.67 ± 13.60 beetles were caught whereas the (S)-enantiomer traps captured 110.33 ± 14.14 beetles.

The fact that N-formyl L-isoleucine methyl ester did not act synergistically with the major component was demonstrated in a field test comparing the captures in traps baited with three different ratios (3.5:1; 7:1; 14:1), the major, or the minor component alone (fig. 4).

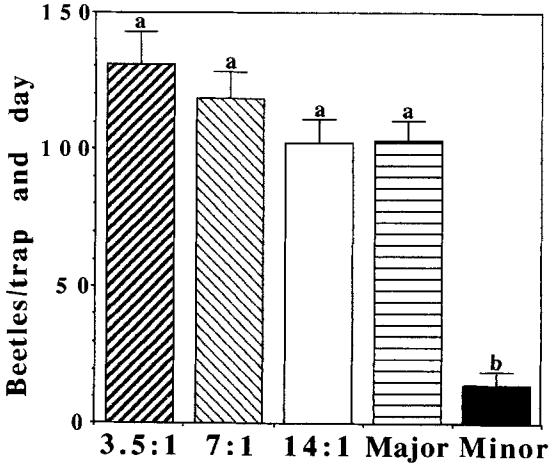

Figure 4. The effect of N-formyl L-isoleucine methyl ester on the attractancy of N-(2′S)-methylbutanoyl (2S)-methylbutylamine for males *M. fryanus*.

There was no significant difference in catches between the blends of the two compounds and N-(2'S)-methylbutanoyl 2-methylbutylamine alone. Although the amino acid derivative seemed to capture more than control traps, its role as a minor component of the sex pheromone system of *M. fryanus* was ruled out because its addition to the amide had no effect on the attraction. Some beetles of different family (like Scarabaeidae) may respond to a single component of their sex pheromone system, but the addition of a minor constituent usually dramatically increases the catches.

The flight season of *M. fryanus* is so short (<2 weeks) that all the experiments were previously planned and carried out almost simultaneously. It was, therefore, not possible to conduct follow-up experiments based on the results of previous tests. For this reason, even though results showed that N-formyl L-isoleucine methyl ester was not an effective minor component, the effect of the amount of pheromone on the catches of the beetles was investigated using a 7:1 blend of the two compounds. There was no significant difference in the catches with 1 mg (65.89 ± 11.52), 10 mg (68.57 ± 11.03), or 100 mg (70.67 ± 11.68) of the blend. Given the fact that this range (1–100 mg) of dosage represents a saturation level, captures of *M. fryanus* may be even higher at lower dosages. This hypothesis will be tested in future field experiments.

As recently proposed[8], cerambycids utilize three distinct reproductive strategies: both sexes congregate on the larval host where they mate (congregating strategy); adults both feed and mate on the adult host plant, but females leave to oviposit alone on the branches, trunk or root (solitary strategy), and some species do not feed in the adult stage (nonfeeding strategy). *M. fryanus* (Anoploderminae) uses the nonfeeding strategy and adult males, which live for 2 or 3 days (as opposed to the wingless females life span of over 30 days), rely on the female sex pheromone for reproduction. On the other hand, it has been reported that the non-feeding grape borer *Xylotrechus pyrroderus* Bates (Cerambycinae) utilizes a short-range male-released sex pheromone[1,2]. The reason for the evolution of different sex pheromone-mediated mating systems in the same family is not yet known, but as it has been pointed out[8], it is now clear that the pheromone-producing sex is more sedentary than the responding sex.

To the best of our knowledge, no amide has been previously identified as a sex pheromone, although N-isovaleroyl nonenylamine has been identified from thorax and abdomen extracts of ant workers in the species *Mesoponera castanea* and *M. castaneicolor*[9].

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1 Iwabuchi, K., Appl. Ent. Zool. *17* (1982) 494.
2 Sakai, T., Nakagawa, Y., Takahashi, J., Iwabuchi, K., and Ishii, K., Chem. Lett. (1984) 263.
3 Plavilstshikov, N. N., Akademia mauk S.S.S.R. Zoologicheskii institut-Fauma SSSR (Institute of Zoology of the Academy of Science of USSR) *21* (1936) 1.
4 Bento, J. M. S., Albino, F. E., Della Lucia, T. M. C., and Vilela, E. F., J. chem. Ecol. *18* (1992) 245.
5 Surburg, H., Güntert, M., and Schwarze, B., J. ess. Oil Res. *2* (1990) 307.
6 Leal, W. S., Matsuyama, S., Kuwahara, Y., Wakamura, S., and Hasegawa, M., Naturwissenschaften *79* (1992) 184.
7 Leal, W. S., Sawada, M., Matsuyama, S., Kuwahara, Y, Hasegawa, M., J. chem. Ecol. *19* (1993) 1381.
8 Hanks, L. M., Millar, J. G., and Paine, T. D. Insect Behav., (1994) in press.
9 Fales, H. M., Blum, M. S., Bian, Z., Jones, T. H., and Don, A. W., J. chem. Ecol. *10* (1984) 651.