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Male-Produced Sex Pheromone of the Stink Bug *Edessa meditabunda*

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Abstract *Edessa meditabunda* is a secondary pest within the piercing-sucking stink bug complex that attacks soybean crops in Brazil. The behavioral responses of males and females to aeration extracts from conspecifics suggested the presence of a male-produced sex pheromone. Gas chromatographic (GC) analysis of male and female aeration extracts revealed the presence of two male-specific compounds in a ratio of 92:8. Gas chromatographic - electroantennographic detection (GC-EAD) assays indicated that the major component is bioactive for females, supporting the behavioral data. Analysis of the mass and infrared spectra of the male-specific compounds suggested that they were both methyl-branched long-chain methyl esters. On the basis of the mass spectra of the respective hydrocarbons obtained by micro derivatizations, the structures of these methyl esters were proposed to be methyl 4,8,12-trimethylpentadecanoate (major) and methyl 4,8,12-trimethyltetradecanoate (minor). An 11 step synthetic route that was based on a sequence of Grignard reactions, starting from cyclopropyl methyl ketone, was developed to obtain synthetic standards with a 7.9 % overall yield for the major compound and a 9.9 % yield for the minor. The synthetic standards co-eluted with the natural pheromones on three different GC stationary phases. Y-tube olfactometer assays showed that the synthetic standards, including the major compound alone and a mixture of the major and minor compounds in the proportion found in natural extracts, were strongly attractive to females.

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Introduction

Soybeans (*Glycine max*) one of the most profitable and widespread crops in the Brazil (Conab, 2011), are strongly attacked by a complex of Pentatomidae (Panizzi and Slansky, 1985; Zarbin et al., 2009). The main pentatomid soybean pests are *Dichelops furcatus, D. melacanthus, Euschistus heros, Nezara viridula,* and *Piezodorus guildinii* (subfamily Pentatominae), and *Edessa meditabunda* (subfamily Edessinae) (Corrêa-Ferreira and Panizzi, 1999; Lourenção et al., 1999; Souza, 2007; Zarbin et al., 2009). In the central western region of Brazil, the Neotropical pentatomid, *E. meditabunda*, is one of the most important and abundant species detected in soybean fields (Souza, 2007).

Every year, millions of liters of insecticides are used to control stink bugs in Brazilian soybean crops (Corrêa-Ferreira and Moscardi, 1996). New methods for controlling these insects need to be developed as insecticide treatments are not efficient, and insecticide residues are health hazards and serious obstacles to exportation. The use of semiochemical methods, especially aggregation and sex pheromones, is a more environmentally benign management approach (Borges et al., 2007).

Out of all of the pentatomid species that attack soybeans in Brazil, attractant pheromones have been identified for three species: *E. heros* (Borges et al., 1998, 1999), *N. viridula* (Aldrich et al., 1987, 1989; Borges et al., 1987; Tillman et al., 2010), and *P. guildinii* (Borges et al., 2007). The genus *Edessa* is the largest in the Pentatomidae, containing approximately 260 described species (Silva et al., 2006); however,

information about the communication system in these species is sparse. Recently, Silva et al. (2012) described the mating behavior and vibratory communication of *E. meditabunda*, but information on the chemical communication of these species is lacking.

The goal of this research was to identify and synthesize the attractant pheromone of *E. meditabunda*, and to test the attractiveness of the synthesized pheromone candidates in laboratory experiments. We are describing herein the first identification of a sex pheromone for an *Edessa* species.

Methods and Materials

Insects A colony of *E. meditabunda* was started with insects collected at the EMBRAPA soybean fields, Londrina, Paraná, Brazil (23° 11′S, 51° 11′W). Adults were sexed and separated from nymphs, and maintained in plastic cages (35×20×20 cm) at 25±2°C, 70±5 % humidity and L14: D10 photoperiod. The colony was reared on soybean seeds (*Glycine max*), green beans (*Phaseolus vulgaris*), peanuts (*Arachis hypogaea*), and glossy privet fruits (*Ligustrum lucidum*). The food was replaced every 3 days.

Collection of Volatiles Volatiles were collected via the aeration method (Zarbin et al., 1999). Five males and 5 females were placed separately in glass chambers (33×3.5 cm ID) containing privet fruits. The collecting apparatus was maintained at the same temperature and photoperiod as the colony. A continuous 1 L/min flow of humidified and charcoal-filtered air was pulled through each chamber, carrying the volatiles to glass traps (11×1 cm ID) containing 60 mg of the adsorbent polymer Super-Q (Alltech Associates Inc., Deerfield, IL, USA). The adsorbed volatiles were eluted from the polymer once a day with double-distilled hexane (2 ml) and concentrated with argon to 50 μl (10 μl per insect). Extracts were stored at -20°C for chemical analyses and bioassays. To determine the day cycle, collections were made every 12 h. Additionally, collections were made every 2 h, over two consecutive days, during a photoperiod of higher pheromone release.

Chemical Analyses Initial analyses were carried out using a Shimadzu GC2010 gas chromatograph (GC) equipped with an FID detector and an RTX-5 (Restek, 30 m×0.25 m×0.25 μm film thickness) capillary column with helium as the carrier gas. The GC was operated in splitless mode (250°C). The temperature program was 50°C for 1 min, increasing at 7° C per min to 250°C with a 10-min hold. For the analyses determining the Kovats indices (Lubeck and Sutton, 1983) and coinjection of the natural product with the synthetic standard, two other GC columns were employed: RTX-WAX (Restek, 30 m×0.25 m×0.25 μm film thickness) and HP-1 (Agilent, 30 m×0.25 m×0.25 μm film thickness) capillary columns.

GC-mass spectrometry (MS) data were acquired using a Shimadzu QP2010-Plus electron ionization mass detector. The GC was equipped with an RTX-5 (30 m×0.25 m×0.25 μm) capillary column. The injector mode and temperature program were the same as those described above.

Extracts also were analyzed by GC-Fourier transform infrared spectroscopy (FTIR) with a Shimadzu GC2010 coupled to a DiscovIR-GC (Spectra Analysis, Marlborough, MA, USA) infrared detector (4000–750 cm^{-1}, resolution 8 cm^{-1}). The capillary column, injector mode and temperature program were the same as those used for the analyses described above.

^{1}H- and ^{13}C-NMR spectra of the synthetic compounds were recorded on a Bruker ARX-200 spectrometer (200 and 50 MHz, respectively) as $CDCl_3$ solutions. Chemical shifts are expressed in ppm relative to TMS.

GC-electroantennographic detection (GC-EAD) analysis data were acquired using a Shimadzu GC2010 coupled to a Syntech® electroantennographic detector (Hilversum, Netherlands). The GC was equipped with an RTX-5 capillary column (30 m×0.25 mm×0.25 μm), and operated in splitless mode (250°C) with a temperature program starting at 100°C for 1 min and increasing 7°C per min to 250°C. The column effluent was split in a 3:1, with one part going to the FID (270°C) and three parts going through the heated transfer line into humidified airstream (200 ml/min) directed to the electrodes. The antennae was detached from the scape base along with some muscular tissue and fixed between the two stainless electrodes (Syntech probe) using conductive gel (Signa gel, Parker Labs, USA). The electroantennograms were recorded using the Syntech GC-EAD32 program (version 4.6) (Ambrogi et al., 2012).

Micro-derivatizations

Lithium Aluminum Hydride ($LiAlH_4$) Reduction Aeration extract (50 μl) from males was treated with 5 μl of a dry ethereal solution of $LiAlH_4$ (~5 mg/ml) for approximately 1 min. A few μl (~10) of water were added, followed by extraction with ether and drying with anhydrous Na_2SO_4 (Attygalle, 1998).

Reduction to the Carbon Skeleton The resulting $LiAlH_4$ reduction product (50 μl) was converted to the respective mesylate by treatment with 400 μl of a 1 % solution of methanesulfonyl chloride in pyridine. After 24 h, the excess was treated with methanol and the solvent was evaporated under argon. The residue was dissolved in ethyl ether and reduced with a dry ether solution of $LiAlH_4$ (~5 mg/ml) for 3 h at room temperature. A few μl (~10) of water were

added, followed by extraction with ether and drying with anhydrous Na_2SO_4 (Attygalle, 1998).

Syntheses

^1H, ^{13}C-NMR, MS, and FTIR data of all the synthetic compounds are presented as supplementary material, available online.

2-cyclopropylpentan-2-ol (4) The Grignard reagent was prepared by the slow addition of bromopropane (2.45 g, 20 mmol) to a suspension of Mg^0 (266 mg, 22 mmol) in anhydrous ethyl ether (10 ml) under an inert atmosphere at room temperature. After observing the formation of the Grignard reagent, a solution of cyclopropylmethylketone **(3)** (2.02 g, 24 mmol) in diethyl ether (5 ml) was added dropwise. The reaction mixture was stirred overnight, and quenched by adding ice (2 g) followed by the addition of a saturated solution of NH_4Cl (5 ml). The aqueous layer was extracted with ether (3×15 ml), the organic layer was washed with brine, and dried over Na_2SO_4. The pure product was obtained by flash chromatography (hexane/ethyl acetate: 9/1), giving compound **4** at a 96 % yield (2.46 g, 19.2 mmol).

2-cyclopropylbutan-2-ol (5) This compound was made with a method analogous to that used to prepare compound **4,** at a 92 % yield (2.59 g, 22.7 mmol), from bromoethane (2.69 g, 24.7 mmol).

1-bromo-4-methylhept-3-ene (6) HBr 48 % (9.7 ml) was added to a strongly stirred solution of the alcohol **4** (2.4 g, 18.8 mmol) at 0°C. The mixture was stirred for 15 min between 0 and 5°C. The reaction was extracted with hexane, washed with a saturated solution of $NaHCO_3$, and dried with anhydrous Na_2SO_4. The crude product was purified by flash column chromatography (hexane), resulting in compound **6** at a 99 % yield (3.61 g, 18.6 mmol

1-bromo-4-methylhex-3-ene (7) This compound was made with a method analogous to that used to prepare compound **6,** at a 98 % yield (3.87 g, 21.5 mmol) starting from compound **5** (2.5 g, 21.9 mmol).

2-cyclopropyl-6-methylnon-5-en-2-ol (8) This compound was made with a method analogous to that used to prepare compound **4,** at a 60 % yield (2.00 g, 10.2 mmol) starting from compound **6** (3.30 g, 17 mmol).

2-cyclopropyl-6-methyloct-5-en-2-ol (9) This compound was made with a method analogous to that used to prepare compound **4,** at a 62 % yield (2.23 g, 12.4 mmol) starting from compound **7** (3.6 g, 20.0 mmol).

1-bromo-4,8-dimethylundeca-3,7-diene (10) HBr 48 % (3.7 ml) was added to a strongly stirred solution of the alcohol **8** (1.80 g, 9.17 mmol) at 0°C. The mixture was stirred for 30 min and held between 0 and 5°C. The reaction was extracted with hexane, washed with saturated solutions of NaCl and $NaHCO_3$, and dried with anhydrous Na_2SO_4. The crude product was purified by flash column chromatography (hexane), resulting in compound **10** with an 84 % yield (1.99 g, 7.70 mmol).

1-bromo-4,8-dimethyldeca-3,7-diene (11) This compound was made with a method analogous to that used to prepare compound **10,** at a 91 % yield (2.45 g, 10 mmol) starting from compound **9** (2.0 g, 10 mmol).

2-cyclopropyl-6,10-dimethyltrideca-5,9-dien-2-ol (12) This compound was made with a method analogous to that used to prepare compound **4,** with a 60 % yield (1.08 g, 4.09 mmol) starting from compound **10** (1.80 g, 6.81 mmol).

2-cyclopropyl-6,10-dimethyldodeca-5,9-dien-2-ol (13) This compound was made with a method analogous to that used to prepare compound **4,** at a 62 % yield (1.39 g, 5.57 mmol) starting from compound **11** (2.20 g, 8.98 mmol).

1-bromo-4,8,12-trimethylpentadeca-3,7,11-triene (14) HBr 48 % (610 μL) was added to a strongly stirred solution of alcohol **12** (0.90 g, 3.41 mmol) at 0°C. The reaction was extracted with hexane, washed with saturated solutions of NaCl and $NaHCO_3$, dried with anhydrous Na_2SO_4, and the solvent was evaporated. The crude product was purified by flash chromatography (hexane), resulting in compound **14** with a 63 % yield (0.70 g, 2.15 mmol).

1-bromo-4,8,12-trimethyltetradeca-3,7,11-triene (15) This compound was made with a method analogous to that of compound **14,** at a 70 % yield (0.84 g, 2.68 mmol) starting from compound **13** (1.2 g, 3.83 mmol).

4,8,12-trimethylpentadeca-3,7,11-trien-1-yl acetate (16) Anhydrous HMPA (2 ml) was added to a round-bottom flask containing 175 mg (2.3 mmol) of sodium acetate while stirring. The mixture was cooled to 0°C and 0.60 g (1.83 mmol) of the bromide **14** was added dropwise. After 12 h, the reaction was extracted with hexane (3×15 ml), washed with brine, rinsed several times with distilled water, and dried with Na_2SO_4. Compound **16** was purified by flash chromatography (hexane/EtOAc: 9/1) with a 77 % yield (0.43 g, 1.41 mmol).

4,8,12-trimethyltetradeca-3,7,11-trien-1-yl acetate (17) This compound was made with a method analogous to that

used to prepare compound **16,** at a 78 % yield (0.49 g, 1.67 mmol) starting from compound **15**.

4,8,12-trimethylpentadecyl acetate (18) Compound **16** (0.4 g, 1.31 mmol) in 5 ml of hexane was hydrogenated over Pd/C (10 %, 5.0 mg) at room temperature under a hydrogen atmosphere (25 psi) in a Parr® apparatus for 2 h. The mixture was filtered, and the filtrate was evaporated at reduced pressure to obtain the acetate **18** at a 89 % yield (0.37 g, 1.17 mmol).

4,8,12-trimethyltetradecenyl acetate (19) This compound was made with a method analogous to that used to prepare compound **18,** at an 83 % yield starting from compound **17**.

4,8,12-trimethylpentadecan-1-ol (20) A dry THF solution (1 ml) of the acetate **18** (0.3 g, 0.96 mmol) was slowly added to a suspension of $LiAlH_4$ (77 mg, 2 mmol) in THF (5 ml) cooled to 0°C. The mixture was stirred for 5 h at room temperature, then cooled to 0°C, hydrolyzed with an NaOH aqueous solution (15 %, 0.5 ml), and extracted several times with ethyl ether. The combined ether extracts were washed with water, dried over Na_2SO_4 and concentrated. The crude product was purified by flash chromatography (hexane/ethyl acetate: 8/2), resulting in the alcohol **20**, with an 80 % yield (0.21 g, 0.77 mmol).

4,8,12-trimethyltetradecan-1-ol (21) This compound was made with a method analogous to that used to prepare compound **20,** at an 82 % yield (0.21 g, 0.82 mmol) starting from compound **19** (0.3 g, 1.00 mmol).

Methyl 4,8,12-trimethylpentadecanoate (1) Jones reagent (~1 ml) was added to a solution of compound **20** (0.18 g, 0.67 mmol) in acetone at room temperature with magnetic stirring until the orange color remained. After 12 h, the excess CrO_3 was consumed by adding methanol. The mixture was concentrated under vacuum, the residue was diluted with water, and extracted with ethyl ether. The ethereal layer was washed with brine, dried with anhydrous $NaSO_4$, and evaporated under vacuum. The residue obtained was diluted in hexane (5 ml), and an ethereal solution of diazomethane was added at 0°C until the resulting solution shared the yellow color of the ethereal solution. The mixture was stirred at room temperature for 30 min, and concentrated under reduced pressure. The pure product was obtained by column chromatography (hexane/ethyl acetate : 9/1), resulting in compound **1** at an 80 % yield in two steps (0.16 g, 0.54 mmol).

Methyl 4,8,12-trimethyltetradecanoate (2) This compound was made with a method analogous to that used to prepare compound **1,** at an 85 % yield (0.17 g, 0.60 mmol) starting from compound **21** (0.18 g, 0.70 mmol).

Olfactometer Bioassays The behavioral responses of *E. meditabunda* to natural and synthetic compounds were tested in a Y-tube olfactometer using humidified, charcoal-filtered air at 2.5 L/min (Zarbin et al. 2007). The olfactometer consisted of a Y-shaped glass tube (4×40 cm) with two 20 cm arms. The odor sources, which consisted of pieces of filter paper (2×2 cm) impregnated with the synthetic compounds, natural extracts or hexane (control), were placed at the ends of the arms. An insect was introduced into the base of the olfactometer, and the behavior of the insect was observed over 20 min. An insect walking upwind toward the odor source for more than 5 cm in any of the arms, and remaining in the arm for more than 2 min, was considered a positive response. An insect remaining in the main tube was considered a negative response. Each insect was tested only once, representing one replicate experiment. The experiments were performed at the time of day of highest pheromone release, and the odor source was replaced after each test. Insects that did not choose either of the arms were excluded from statistical analysis. The data were analyzed using a *Chi-square* test with the BioEstat program (version 5.0) (Ayres et al., 2003).

Initially, four bioassay experiments were performed; the responses of males to odors from males, males to odors from females, females to odors from males, and females to odors from females. Because only females were attracted to the odors from males, males were not used in subsequent experiments.

Two experiments were conducted to determine the biological activity of the synthetic major and minor male-specific compounds. Females were tested with the synthetic major male-specific compound (methyl 4,8,12-trimethylpentadecanoate **1**, 50 μg), and with a blend of the two male-specific compounds (methyl 4,8,12-trimethylpentadecanoate **1**, 50 μg plus methyl 4,8,12-trimethyltetradecanoate **2**, 4.4 μg) at a ratio of 92:8.

Results

Evidence of a Sex Pheromone Comparison of the chromatograms of aeration extracts collected from male and female *E. meditabunda* adults showed the presence of two male-specific compounds (compounds **1** and **2**) along with other compounds that were common to both sexes (Fig. 1). The ratio between compounds **1** and **2** was calculated to be 92:8, respectively, based on the areas of the GC peaks detected by FID. The respective Kovats Indices were calculated on three different columns and are shown in Fig. 1.

Y-tube olfactometer bioassays employing natural extracts showed that aeration extracts of males are significantly more attractive to females than the solvent control [treatment 15 (68 %), control 8 (32 %), $N=22$, $P<0.05$]. The same extract,

Kovats Index			
	RTX-5	RTX-WAX	HP-1
Major (1)	1934	2212	1952
Minor (2)	1883	2124	1867

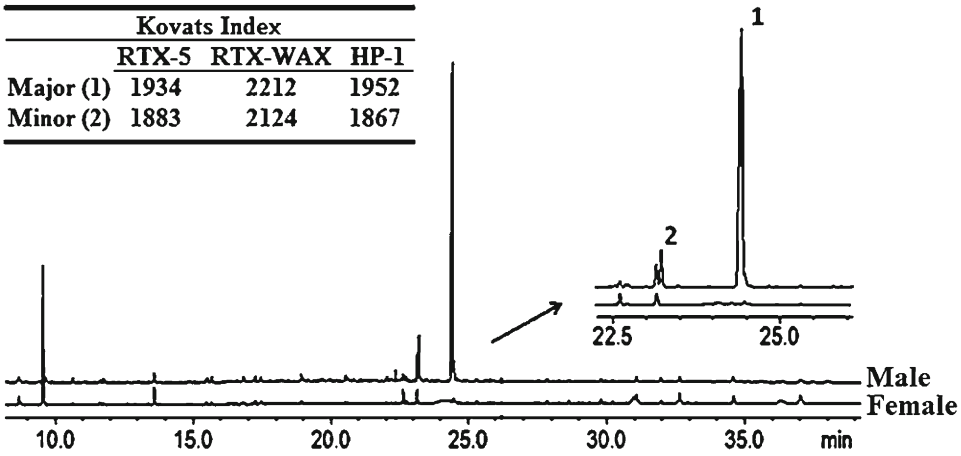


Fig. 1 Gas chromatograms of volatiles produced by *Edessa meditabunda* adults and Kovats Indices of the two male-specific compounds analyzed on RTX-5, RTX-WAX and HP-1 capillary columns. Chromatograms obtained by using a RTX-5 capillary column

however, was not attractive to conspecific males. On the other hand, the experiments carried out with volatiles from females did not exhibit attractiveness to either sex, and most of the insects in these experiments made no choice, remaining in the main olfactometer arm. These results are consistent with the GC profile of the extracts, and suggest that males produce a sex pheromone, as previously reported for other stink bugs such as *Thyanta pallidovirens* (Millar, 1997) and *Acrosternum hilare* (McBrien et al., 2001), and not an aggregation pheromone that attracts both sexes, as is the case for *N. viridula* (Harris and Todd, 1980b) and *P. hybneri* (Leal et al., 1998).

GC-EAD assays employing aeration extracts from males and the antennae from both sexes were conducted, and only antennae of females exhibited activity for the major male-specific volatile **1** (Fig. 2), thus confirming the evidence of a male-produced sex pheromone mediating the behavior of *E. meditabunda*. However, these electrophysiological responses were not detected for the minor component **2**, even after several analyses.

Dynamics of Production of the Male-Specific Components The production of the major male-specific compound was dependent on light. Although the compounds were detected throughout all monitoring periods, approximately 71 % of the daily release occurred during the 12 h of photophase (Fig. 3a). There were practically no differences between release during the six 2-h periods evaluated during photophase. Only production during the first period of photophase was significantly different from production during the other photophase periods (Fig. 3b).

Structural Identification The GC-FTIR spectrum of compound **1**, and GC-MS spectra obtained from compounds **1** and **2** are shown in Fig. 4. The infrared spectra of compound **1**, which is similar to that of compound **2** (not shown), shows two bands at 1175 cm^{-1} and 1196 cm^{-1}, indicating a C-O single bond; a C = O stretching band characteristic of esters at 1744 cm^{-1} (Silverstein et al., 2005) is also present. The mass spectra of compounds **1** and **2** are similar, exhibiting the same base peak at *m/z* 87, and molecular ion at *m/z* 298 for **1** (Fig. 4a) and *m/z* 284 for **2** (Fig. 4b). The base peak at *m/z* 87 suggests a branch close to the ester function. The base peak and the high relative intensities of the fragments at *m/z* 157 and 241 for compound **1**, and at *m/z* 157 and 227 for compound **2**, indicates a methyl branched structure. The difference of 14 mass units between the molecular ions and the fragments at *m/z* 227 and 241, suggest that compound **2** lacks a CH_2 in the main carbon chain relative to compound **1**.

To determine the structures of these compounds, several micro-derivatizations were carried out with the natural products. First, to determine what kind of ester was present, a reduction with $LiAlH_4$ was employed. A fragment at *m/z*

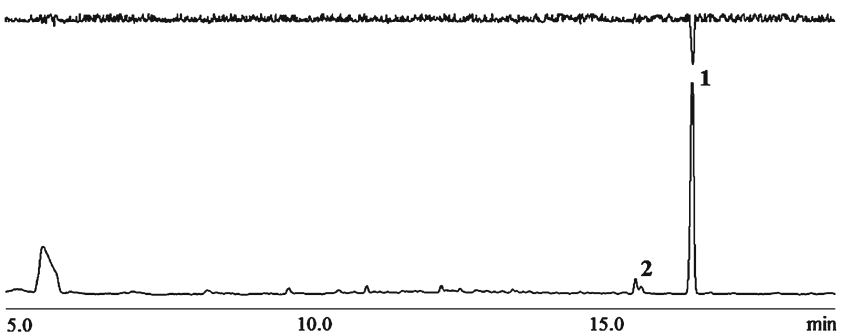


Fig. 2 Electroantennogram response in females (top) to an aeration extract of *Edessa meditabunda* males

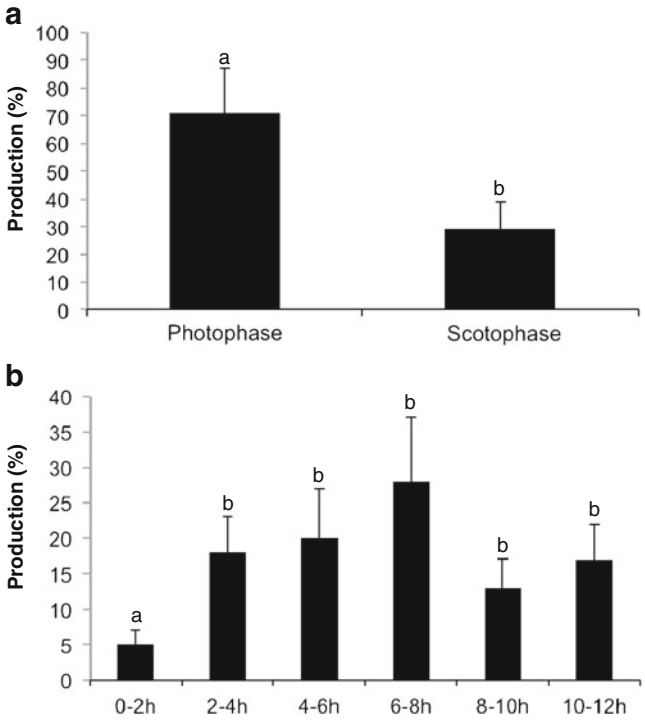


Fig. 3 Comparison of the amounts of the major component **1** collected from the aeration of *Edessa meditabunda* males: **a** between the photophase and scotophase (ANOVA + Tukey test, $P<0.01$, $P=0.005$); **b** during different times of the 12 h photophase (ANOVA + Tukey test, $P<0.05$). Mean values followed by the same letter are not significantly different ($N=3$)

252 related to the loss of water (M^+ - 18) was observed in the mass spectrum of the resulting alcohol, indicating a molecular ion at *m/z* 270, for a difference of 28 units from the parent compound (M^+ at *m/z* 298). This difference indicates a reduction of a methyl ester present on the natural compound **1**. The same pattern was observed in the minor derivative, also suggesting a methyl ester structure. The alcohol structure of the derivative was confirmed by the presence of a broad O–H stretching band at 3252 cm^{-1} in the FTIR spectrum. On the basis of these data, compounds **1** and **2** may have the molecular formulae $C_{19}H_{38}O_2$ and $C_{18}H_{36}O_2$, respectively.

To determine the number and positions of the possible methyl branches on the main carbon chain, the alcohol derivative was converted to a mesylate and reduced with $LiAlH_4$ to the corresponding hydrocarbon (Scheme 1A). The same procedure was performed using $LiAlD_4$ instead the $LiAlH_4$ to produce the hydrocarbon with 3 deuterons in the first carbon of the main chain (Scheme 1B).

The mass spectrum of the hydrocarbon derivative of the major compound exhibited high relative intensities of the fragments at *m/z* 71, 141 and 211, diagnostic fragments for the methyl branches at positions 4, 8, and 12 of the carbon chain (Fig. 5a). Slight increases in the relative intensities of these fragments were observed due to the symmetry of the resulting hydrocarbon. These methyl branch positions were corroborated by analyzing the mass spectrum of the $LiAlD_4$ hydrocarbon product; in the deuterated molecule, the symmetry was broken, and the fragments at *m/z* 74, 144, and 214 also had slightly elevated intensities (Fig. 5b). Considering that the two male-specific compounds are known to be methyl esters, and that they contain three methyl branches, methyl 4,8,12-trimethylpentadecanoate was proposed as the identity of the major compound **(1)**, and methyl 4,8,12-trimethyltetradecanoate was proposed as the identity of the minor compound **(2)** (Fig. 6).

Synthesis of Methyl 4,8,12-trimethylpentadecanoate (1) and Methyl 4,8,12-trimethyltetradecanoate (2) A synthetic methodology to obtain **1** and **2** was developed to confirm the proposed structures of the pheromones, with Julia olefination as a key step (Scheme 2), in a route similar to that proposed by Mori and Murata (1994) to synthesize the sex pheromones of the stink bugs *E. heros* and *E. obscurus*.

According to Scheme 2, 2-cyclopropylpentan-2-ol **(4)** was prepared at a 96 % yield by adding cyclopropylmethylketone **(3)** to a solution containing propylmagnesium bromide. The alcohol **4** formed was subjected to a Julia olefination with HBr 48 %, causing the opening of the cyclopropyl ring, yielding bromide **6** at 99 % (Mori and Murata, 1994). The stereoisomeric mixture had an *E*- to *Z*-**6** ratio of 3:1 observed by GC, as described previously (Biernacki and Gdula, 1979).

Preparing the Grignard reagent derived from compound **6** (Zarbin et al. 1998), adding it to cyclopropylmethylketone **(3),** and performing Julia olefination on the resulting alcohol **8**, gave bromide **10** with a 50 % yield over the two steps. The triene **14**, a direct precursor of the pheromone, was synthesized as a mixture of the eight stereoisomers by performing the same Grignard reaction and Julia olefination sequence using bromide **10**, with a 38 % yield over the two steps. The direct acetylation of the compound **14** using sodium acetate in HMPA (Larock, 1974) gave the ester **16** at a 77 % yield. Ester **16** was submitted to a catalytic hydrogenation under Pd/C, resulting in the hydrogenated acetate **18** with an 89 % yield (Zarbin et al., 2000). The reduction of the carboxyl group of the acetate **18** with $LiAlH_4$ resulted in the alcohol **20** with an 80 % yield (Vidal et al., 2010). The Jones oxidation (Zarbin et al., 2000) of the alcohol **20** resulted in the corresponding carboxylic acid, which was directly treated with an ethereal solution of diazomethane (Furniss et al., 1989), affording the desired ester **1** with a 57 % yield over the two steps. The overall yield of methyl 4,8,12-trimethylpentadecanoate **(1)** was 7.9 % from compound **3** after 11 steps.

The minor component, methyl 4,8,12-trimethyltetradecanoate **(2)**, was synthesized based on the same route starting from bromoethane, with an overall yield of 9.9 % from compound **3** after 11 steps.

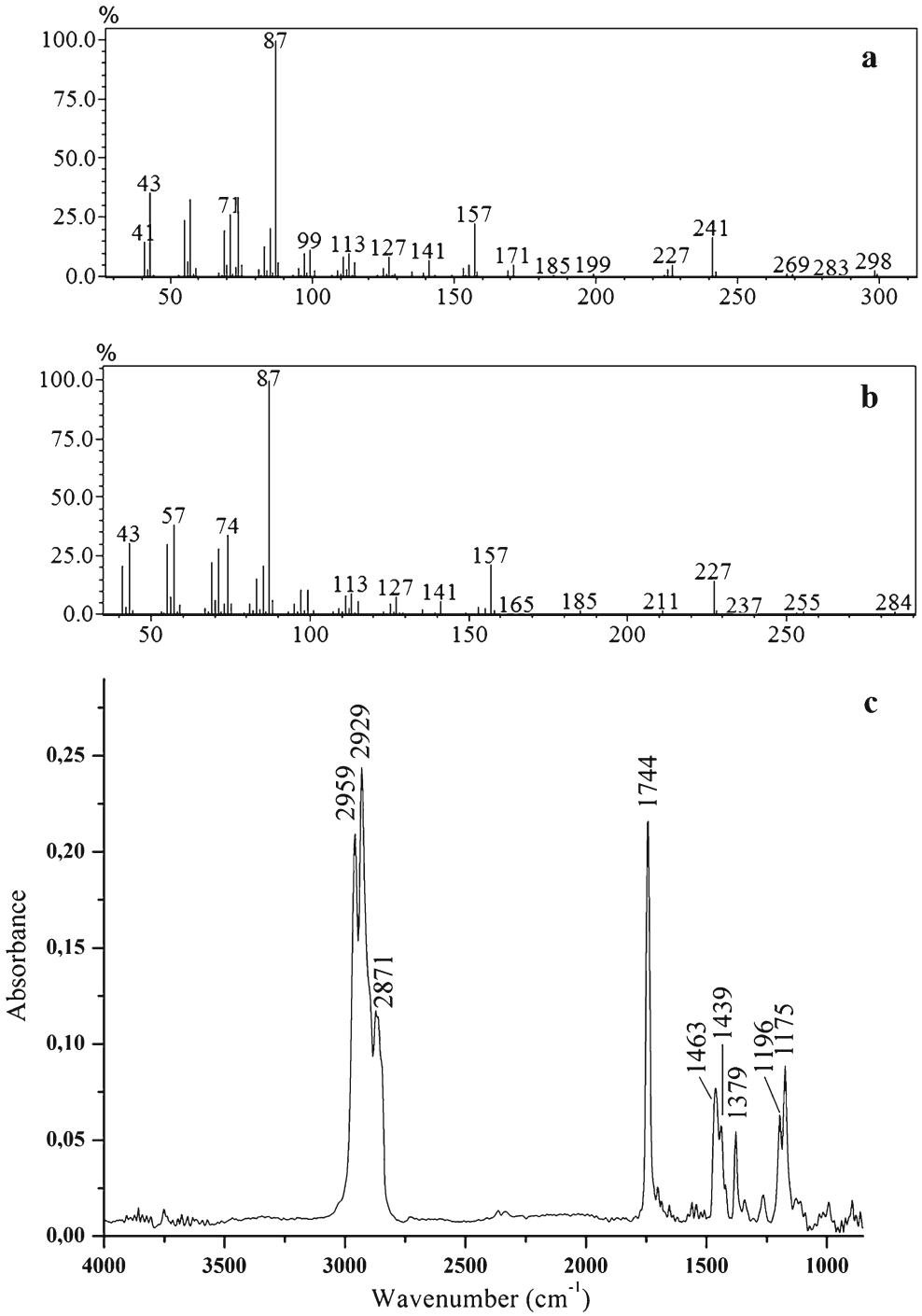
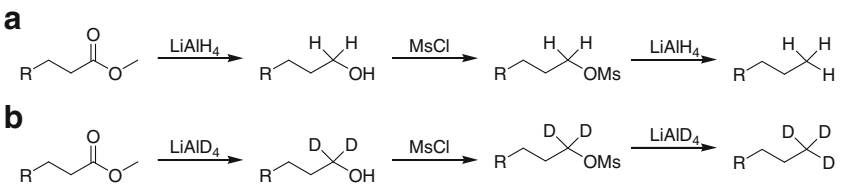


Fig. 4 Electron impact mass spectra of **1** (**a**) and **2** (**b**), and the infrared spectrum of **1** (**c**)

The two synthetic compounds were co-injected with the natural extracts of males, and they co-eluted on all three different GC columns tested (RTX-5, HP-1 and RTX-WAX). The mass and infrared spectra of the synthetic pheromones did not differ from the spectra of the natural compounds.



Scheme 1 Derivatization sequences with male volatile extracts to obtain the carbon skeletons of the major male-specific compounds of *Edessa meditabunda* (**A**: $LiAlH_4$ derivative; **B**: $LiAlD_4$ derivative

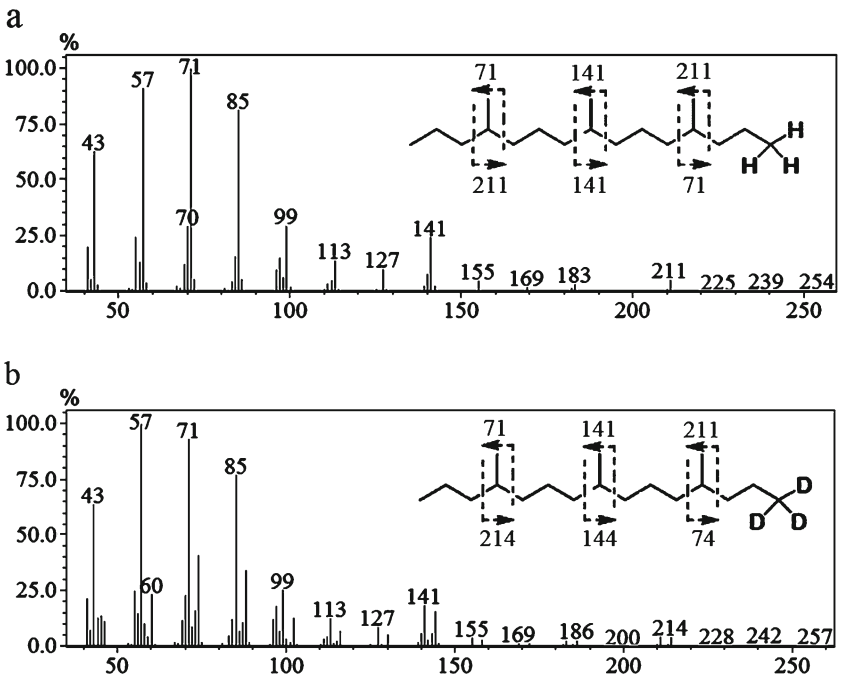


Fig. 5 Mass spectra of $LiAlH_4$ (**a**) and $LiAlD_4$ (**b**) hydrocarbon derivatives from the natural male-specific compound **1**

Y-tube Bioassays Employing the Synthetic Pheromones A Y-tube olfactometer was used to test the biological activities of the synthetic male-specific compounds (Fig. 7). The first experiment consisted of testing only the major male-specific compound (**1**) against the control (only solvent); females were significantly attracted to this compound [treatment 15 (75 %), control 5 (25 %), N=20, P=0.025]. The second experiment was performed with a mixture of the two synthetic compounds at the same ratio as found in volatile extracts of males against the control. Female attraction to the mixture was highly significant [treatment 16 (80 %), control 4 (20 %), N=20, P=0.007].

Discussion

Pheromone Production In the Pentatomidae, single bugs or small groups sometimes release more male-specific compounds during aeration than do larger groups (Ho et al., 2005; Zahn et al., 2008). For example, when the stink bug, *Murgantia histrionica*, was aerated individually or in groups of five insects, the males released higher amounts of sex-specific compounds than when they were aerated in groups of ten or more. It has been suggested that males can detect the presence of other males, which influences rates of pheromone release (Zahn et al., 2008). The use of five insects per aeration chamber for our *Edessa* aerations seemed appropriate, as the analyses produced clean chromatograms with small amounts of defensive compounds and large amounts of male-specific compounds.

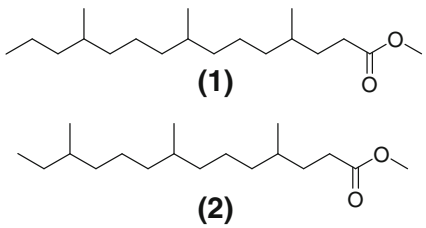
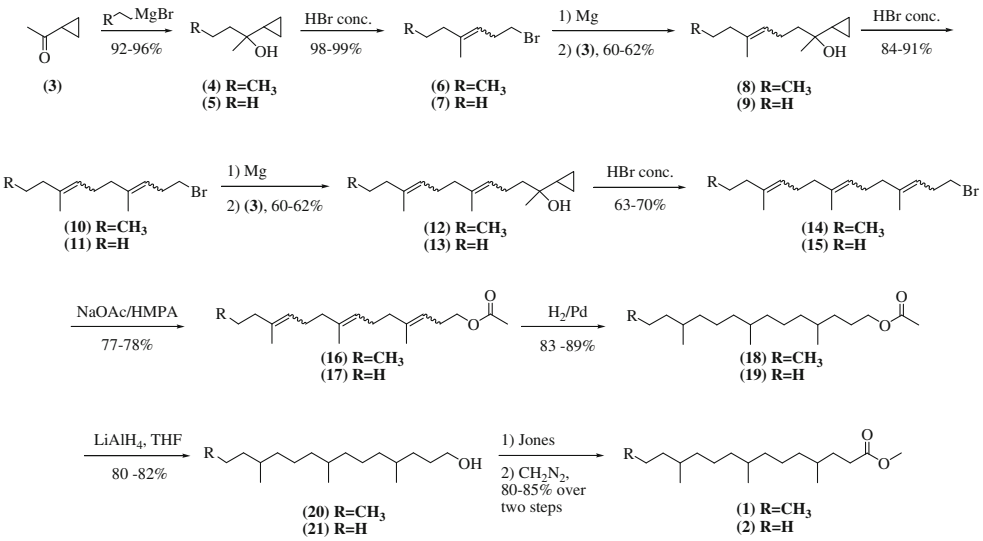


Fig. 6 Proposed structures for the major (**a**) and minor (**b**) male-specific compounds released by *Edessa meditabunda*

In addition, *M. histrionica* males release pheromone primarily during photophase (Zahn et al., 2008), as does *E. meditabunda*. However, most other phytophagous pentatomids studied engage in reproductive activity occurring during the late afternoon to early evening [e.g., the *Chlorochroa* genus (Fish and Alcock, 1973; Ho and Millar, 2001a, c), *N. viridula* (Harris and Todd, 1980a), *E. heros* (Borges et al., 1998) and *T. pallidovirens* (Wang and Millar, 1997)].

Thus far, all studies with aggregation or sex pheromones produced by Pentatomidae have shown that the males are responsible for attractant pheromone production (Moraes et al., 2008). Once on the same plant, the insects of both sexes emit substrate-borne vibratory signals that are used as a directional short-distance cues to locate the mate (Çokl, 2008). Some explanations for why males are responsible for aggregation and sex pheromone release have been proposed but, until now, none has been proven. One of these hypotheses is that

Scheme 2 Synthetic route for the male-specific compounds released by *Edessa meditabunda*



the male bugs are responsible for the recolonization process characteristic of many heteropteran species, such as the stink bugs *Podisus maculiventris,* in which males often search for food first and then call females to the new habitat using pheromones (Aldrich et al., 1984), and *E. servus*, in which sexually mature males migrate to corn before females (Herbert and Toews, 2011). Another suggestion is that because stink bug pheromones are used by parasitoids and predators to locate their prey (Aldrich, 1995; Powell, 1999), males have evolved to be smaller by developing more quickly, assuming the risk of discovery by natural enemies as the pheromone producers for the potential pay-off of mating with more females (Ho and Millar, 2001a).

Chemical Identification Methyl 4,8,12-trimethylpentadecanoate **(1)** and methyl 4,8,12-trimethyltetradecanoate **(2)** are novel molecules in pheromone chemistry, but the structures of these compounds are analogous to the sex pheromones produced by *E. heros* (Aldrich et al., 1991) and *E. obscurus* (Borges and Aldrich, 1994), methyl 2,6,10-trimethyltridecanoate and methyl 2,6,10-trimethyldodecanoate.

The Pentatomidae is a group that demonstrates structural chemical patterns of attractive pheromone components for some genera where several species have been studied (Moraes et al., 2008). In particular, the genus *Euchistus*, for which the pheromones of seven species have been identified, the pheromones of all include methyl (*E*,*Z*)-2,4-decadienoate as an aggregation pheromone component (Aldrich et al., 1991, 1994; Borges et al., 1998; Krupke et al., 2001). Although the genus *Edessa* is the largest of the Pentatomidae genera (Silva et al., 2006), heretofore only defensive compounds have been identified (Howard and Wiemer, 1983; Borges and Aldrich, 1992).

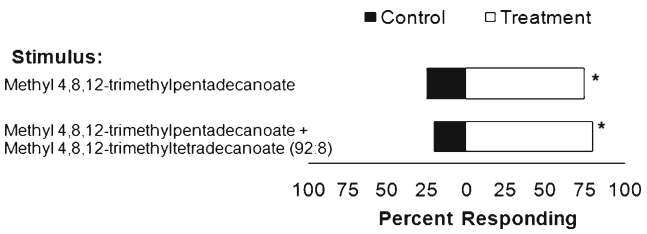


Fig. 7 Results of Y-tube bioassays testing the attraction of *Edessa meditabunda* females to the major synthetic compound, methyl 4,8,12-trimethylpentadecanoate **(1)**, or to a blend of the two synthetic compounds found only in male volatiles, methyl 4,8,12-trimethylpentadecanoate **(1)** and methyl 4,8,12-trimethyltetradecanoate **(2)** (92:8). *Chi-square* test, $N=20$, * $P<0.05$

Y-tube Bioassays The major male-specific compound, methyl 4,8,12-trimethylpentadecanoate **(1)**, and extracts of conspecific males showed biological activity on *E. meditabunda* females, inducing a behavioral response. The presence of the minor male-specific compound, methyl 4,8,12-trimethyltetradecanoate **(2)**, which was not EAD-active**,** did not improve attraction or act as a repellent; therefore, the significance of this compound in the pheromone blend remains unclear.

These data corroborate previous research on stink bug pheromones (Leal et al., 1998; Ho and Millar, 2001c; McBrien et al., 2001), where laboratory tests showed the function of the major compound as a pheromone but did not identify the roles of minor components (Ho and Millar, 2001b). However, field bioassays are still needed to determine whether the main pheromone compound alone may be sufficient for use as an attractant, and whether the minor compound has a function, such as attractiveness improvement or blend specificity.

In summary, the existence of a male-produced sex pheromone released by the stink bug *E. meditabunda* was determined. Two male-specific compounds were identified, methyl 4,8,12-trimethylpentadecanoate (**1**) and methyl 4,8,12-trimethyltetradecanoate (**2**); however, addition of **2** to the main component **1** did not significantly improve attraction of females in laboratory bioassays These novel compounds were synthesized as a mixture of all stereoisomers, and their biological activities were confirmed in laboratory tests. We are now working in a stereoselective synthesis of all the eight possible stereoisomers of compounds **1** and **2**, in order to determine the influence of chirality on behavior, as well as to determine the absolute configuration of these natural molecules. Field experiments employing the synthetic pheromones are underway, and the results will appear in due course.

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