JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Subscriber access provided by RUTGERS UNIVERSITY

Agricultural and Environmental Chemistry

Sex pheromone of the invasive mealybug citrus pest, *Delottococcus aberiae* (Hemiptera:Pseudococcidae). A new monoterpenoid with a necrodane skeleton

Sandra Vacas, Ismael Navarro Fuertes, Javier Marzo, Vicente Navarro-Llopis, and Jaime Primo

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.9b01443 • Publication Date (Web): 05 Aug 2019

Downloaded from pubs.acs.org on August 8, 2019

Just Accepted

"Just Accepted" manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides "Just Accepted" as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. "Just Accepted" manuscripts appear in full in PDF format accompanied by an HTML abstract. "Just Accepted" manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). "Just Accepted" is an optional service offered to authors. Therefore, the "Just Accepted" Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the "Just Accepted" Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these "Just Accepted" manuscripts.

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Published by American Chemical Society. Copyright © American Chemical Society. However, no copyright claim is made to original U.S. Government works, or works produced by employees of any Commonwealth realm Crown government in the course of their duties.

Sex pheromone of the invasive mealybug citrus pest, *Delottococcus aberiae* (Hemiptera:Pseudococcidae). A new monoterpenoid with a necrodane skeleton

Sandra Vacas,[1] Ismael Navarro,[2] Javier Marzo,[2] Vicente Navarro-Llopis,[1] Jaime Primo[1]

[1]CEQA-Instituto Agroforestal del Mediterráneo, Universitat Politècnica de València, Camino de Vera s/n, edificio 6C-5ª planta, 46022 Valencia (Valencia), Spain.

[2]Ecología y Protección Agrícola SL, Pol. Ind. Ciutat de Carlet, 46240 Carlet (Valencia), Spain.

Correspondence to: Sandra Vacas. CEQA-Instituto Agroforestal del Mediterráneo, Universitat Politècnica de València. Camino de Vera s/n, edificio 6C-5ª planta, 46022-Valencia, Spain. Email: sanvagon@ceqa.upv.es; phone: +34963879058; fax: +34963879059

ABSTRACT

Native to sub-Saharan Africa, *Delottococcus aberiae* De Lotto (Hemiptera: Pseudoccidae) is an invasive mealybug that has been recently reported in Europe seriously damaging citrus production in eastern Spain. In this study, we isolated and determined the structure of the sex pheromone of *D. aberiae*, to provide a highly specific and effective lure for detecting, monitoring and potentially controlling this pest. The volatile profile of *D. aberiae* virgin and mated females was studied by aeration and collection of effluvia in Porapak-Q. The resulting extracts were analyzed by gas chromatography coupled to mass spectrometry (GC-MS), revealing a candidate compound specific of virgin females. GC-MS and nuclear magnetic resonance spectroscopy data evidenced a new compound, (4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate, with an unusual β-necrodol skeleton. This compound was synthesized and shown to be attractive to male *D. aberiae* in both laboratory and field experiments. A GC analysis using an enantioselective stationary phase and polarimetry analyses of the synthetic enantiomers showed the natural compound emitted by virgin females to be the (-)-enantiomer.

KEYWORDS

semiochemical; chemical ecology; attractant; lure; volatile

INTRODUCTION

Delottococcus aberiae (De Lotto) (Hemiptera: Pseudococcidae) is an invasive mealybug that has been recently reported to cause severe damage to citrus in the Mediterranean area.[1] Native to sub-Saharan Africa,[2] the presence of *D. aberiae* has been reported in several countries in central and southern Africa, such as Kenya, Mozambique, Swaziland, Tanzania and Zimbabwe.[3] It is commonly found in South Africa on wild olive trees, the roots of the flowering shrub *Chrysanthemoides monilifera* (L.) T. Norl., and is irregularly distributed in citrus orchards north of the country.[4] Its recent introduction into eastern Spain has been demonstrated to match mealybug populations on citrus from the Limpopo Province (South Africa) by molecular techniques,[5] and has probably come via the international trade of citrus plants or fruits, which is the main pathway to disperse scale insects to Europe.[6] Like most mealybugs, *D. aberiae* is multivoltine and completes several generations per year under Mediterranean conditions and remains active even in winter.[7] After hatching, individuals undergo two nymphal stages (first and second) and then, females and males start to develop differently. Males had two pupal stages (pre-pupa and pupa) inside a cottony cocoon before developing into winged adult males. Females have one more nymphal stage to become adult females, and changes very slightly in appearance (wingless) except the size they grow to.

The population trends of *D. aberiae* in citrus have been recently investigated in Spain by visual examination of plant material, sampling developmental stages in corrugated cardboard band traps and detecting male flights in adapted sticky traps.[7,8] The seasonal trend revealed that *D. aberiae* density increases in spring, and reaches its first significant maximum in May and June, to coincide with fruit development in the Mediterranean Region, before developing on fruits until the end of August, and then

decreasing and remaining at low levels for the rest of the year.[7] In that work, visual examinations and samplings of developmental stages revealed several overlapping generations (two to four). Nonetheless, monitoring mealybugs by visual inspections is a time-consuming process because it is necessary to count the live insects present on plant material. However, two main male peak flights were also well-defined by using adapted sticky traps baited with virgin females, which confirmed the two main generations detected by inspections. Thus, Martinez-Blay et al.[7] provided evidence for a substance emitted by *D. aberiae* virgin females to attract males. The identification of the female sex pheromone would allow pheromone traps to be used to detect and monitor *D. aberiae* populations, and to develop control methods to be included in Integrated Pest Management strategies.

In the present work, we isolated and characterized the sex pheromone from the airborne volatiles emitted by *D. aberiae* virgin females by gas chromatography coupled to mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) spectrometry. Having elucidated the structure, the candidate compound was synthesized to confirm its structure and activity in both laboratory and field tests.

MATERIALS AND METHODS

Mealybug stock colony

The colony of *D. aberiae* was established in our facilities at the Universitat Politècnica de València (UPV, Valencia, Spain) using specimens from the Laboratory of Entomology, based at Instituto Valenciano de Investigaciones Agrarias (IVIA, Valencia, Spain). Mealybugs were reared on organic green lemons, which were previously covered with paraffin around the mid-section to delay their desiccation. Starting from

gravid females and ovisacs deposited on lemons, newly hatched individuals establish on the surface of fruit and follow the developmental cycle. Mated females are allowed to oviposit. When ovisacs are laid, they are gently transferred with an entomological needle to new lemons. Insects were maintained in a rearing chamber, in the dark at 24±2 ºC, with 40-60% relative humidity.

After the second instar stage, males produce a distinguishable cottony cocoon that covers their bodies to pupate and transform into winged adults. Lemons were visually inspected every 3-4 days for the presence of cocoons, which were manually removed with an entomological needle to leave lemons infested only with virgin females for volatile collection and profiling purposes. Mated females were sampled on the lemons from the main stock colony after checking for the presence of ovisacs.

Collection of volatiles

Five to six lemons infested with approx. 200 *D. aberiae* females (virgin or mated separately) were placed in 5-L glass reactors: 25-cm high × 17.5-cm diameter flask, with a 10-cm open mouth and a ground glass flange to fit the cover with a clamp. The cover had a 29/32 neck on top to fit the head of a gas washing bottle and to connect downstream a glass cartridge to trap effluents in 3 g Porapak-Q (Supelco Inc., Torrance, CA, USA) adsorbent. Samples were collected continuously for 9 days under 14:10 (L:D) light-darkness conditions by using an ultrapurified-air stream, provided by an air compressor (Jun-air Intl. A/S, Norresundby, Denmark) coupled with an AZ 2020 air purifier system (Claind Srl, Lenno, Italy) to provide ultrapure air (amount of total hydrocarbons < 0.1 ppm). In front of each glass reactor, an ELL-FLOW digital flowmeter (Bronkhorst High-Tech BV, Ruurlo, The Netherlands) was fitted to provide an air push flow of 400 mL/min during sampling. Trapped volatiles were then extracted

with 20 mL pentane (Chromasolv®, Sigma-Aldrich, Madrid, Spain) and extracts were concentrated to 500 µL under a nitrogen stream prior to the chromatographic analysis. Fifty rounds of virgin female collections were performed to obtain approximately 90,000 female-day equivalents (FDE), a sufficient quantity for the nuclear magnetic resonance (NMR) analysis.

All the resulting pentane extracts were analyzed by GC-MS in a Clarus 600 GC-MS (PerkinElmer Inc, Waltham, MA). The GC was equipped with a ZB-5MS fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 µm; Phenomenex Inc., Torrance, CA) and the oven was held at 40 °C for 2 min and then programmed at 5 °C/min to 180 °C, before being raised to 280 °C at 10 °C/min and maintained at 280 °C for 1 min. Helium was used as the carrier gas at a flow of 1 mL/min. Detection was performed in the EI mode (70 eV) with the ionization source set at 180ºC. Spectrum acquisition was carried out in full scan mode (mass range *m/z* 35-500) and chromatograms and spectra were recorded by means of GC-MS Turbomass software v. 5.4 (PerkinElmer Inc., Waltham, MA).

Isolation of the candidate compound

Having compared the GC-MS volatile profiles of the virgin and mated samples (lemons infested with virgin and mated females, respectively), the first step to isolate the virgin-specific compound was the fractionation of the Porapak-Q pentane extracts by gravity column (300 mm × 15 mm i.d.). Composite samples of ca. 30,000 FDE in 1 mL pentane were loaded in each column and fractionation was performed using 9.5 g of silica gel as the stationary phase (40-60 µm) and 20 mL mixtures of pentane:diethyl ether as the eluents (100:0, 95:5, 80:20, 0:100). Thirty fractions were collected and they were all analyzed by GC-MS to identify those that contained the candidate compound, by the methods described in the previous section. These enriched fractions were gathered to

finally isolate the pheromonal compound by preparative-GC, using a modified Clarus 500 GC (Perkin Elmer Inc., Wellesley, PA) equipped with a flame ionization detector (FID) that was set at 250ºC and a TRB-1 fused-silica capillary column (30 m × 0.53 mm i.d. × 0.5 μm; Teknokroma Analítica SA, Sant Cugat del Vallès, Barcelona, Spain). The GC oven was programmed at 40 °C for 2 min and then raised at 3 °C/min to 100 °C and, when reached, raised to 280 °C at 30 °C/min and maintained at 280 °C for 12 min. The end of the column was connected to a 1/16-inch stainless steel tube (10 cm long) which was then connected to a T-splitter to divide the column effluent towards the FID detector (a 180 cm long empty fused-silica capillary line) and a handmade fraction collection system. The collector consisted of a 12-gauge stainless steel needle (30 cm long) connected to the T-splitter on one side, while the opposite passed through an insulated thermostatted port set at 270º C (Syntech temperature controller TC-02, Ockenfels SYNTECH GmbH, Kirchzarten, Germany) to cross the GC left-side panel leaving an accessible luer-lock connector. Fractions were collected in 100 μL stainless steel HPLC loops connected to the aforementioned luer-lock connector and cooled by a dry ice–acetone bath for volatile condensation purposes. Compounds were then eluted from the loop with 2 × 100 μL pentane or deuterobenzene (C_6D_6). Thirty injections, 5 μL/each, were performed to collect the required quantity for the NMR analysis (ca. 30 μg). Then, the ^{1}H NMR spectrum of the natural isolated compound was recorded by a Bruker 600 Ultrashield Plus spectrometer (Bruker, Billerica, MA) at a frequency of 600 MHz, using C_6D_6 as the solvent with tetramethylsilane (TMS) as the internal standard.

Microreaction: hydrolysis of the natural (4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate (1)

The pentane extract of an aeration sample of ca. 1,000 FDE (~ 300 ng of pheromone) was hydrolyzed following related procedures.[9] The extract was dried under a gentle nitrogen stream in a 2-mL GC glass vial. The residue was treated with a 0.5 M solution of KOH in methanol (150 µL) and stirred for 45 min at room temperature. After this time, water (0.5 mL) was added and the solution was extracted with hexane (0.5 mL twice). The combined organic phases were concentrated under nitrogen to ca. 100 µL volume before being submitted to GC-MS analysis.

Synthesis of (4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate (1) (Figure 1)

General procedures. The ^{1}H and ^{13}C spectra were recorded on a Bruker AC-300 spectrometer (Bruker, Billerica, MA) using deuterated chloroform ($CDCl_3$) or C_6D_6 as the solvents and TMS as the internal standard. Chemical shift values are reported in δ (ppm) relative to chloroform (^{1}H NMR: 7.26 ppm and ^{13}C NMR: 77.0 ppm) or benzene (^{1}H NMR: 7.16 ppm and ^{13}C NMR: 128.4 ppm). High resolution mass spectra (ESI-HRMS) were measured on a Waters Xevo Q-TOF spectrometer (Waters Corp., Milford, MA) coupled with an Acquity UPLC-PDA system (Waters Corp., Milford, MA) using ionization by electrospray (ESI). The ESI source operated in the positive ionization mode using leucine-enkephalin as the reference mass ($[M+H]^+$ ion *m/z* 556.2771). The sample (2 µL) was injected into a Waters Acquity BEH column (50 x 2.1 mm i.d., 1.7 µm) using MeOH as isocratic eluent. The GC-MS analyses were performed with the aforementioned equipment (apparatus and column) and the following oven temperature program: 55 °C for 3 minutes, raised at 15 °C/min up to 180 °C, and then at 35 °C/min up to 280 °C, held for 6 minutes. A helium flow of 1 mL/min and an injection volume

of 1 μL were employed. Detection and spectra acquisition were performed as indicated above.

All the reagents and solvents (reagent grade) were purchased from Sigma-Aldrich (Madrid, Spain) and employed with not further purification unless otherwise stated. All the organic solvents employed in these experiments were dried with appropriate drying agents and distilled before use. Unless otherwise stated, all the reactions sensitive to moisture and/or air were carried out under nitrogen atmosphere using anhydrous solvents. The solvent extracts of the reaction mixtures were dried over $MgSO_4$ and concentrated by rotary evaporation under reduced pressure. Crude products were purified by column flash chromatography using silica gel Merck 9385 (230-400 mesh). Thin-layer chromatography (TLC) was performed using Macherey-Nagel silica gel 60 F_{254} plates with a fluorescent indicator and UV light of 254 nm wavelength as the visualizing agent. Ceric ammonium molybdate and *p*-anisaldehyde were used as stains.

3,4,4-trimethylcyclopent-2-en-1-one (**3**). Isobutyl crotonate **2** (40 g, 0.28 mol) was slowly added to polyphosphoric acid (200 g) at 95 ºC for 3 h. After continuous stirring at this temperature for 3 h, the solution was cooled down and poured over water (350 mL) with stirring. The mixture was extracted with diethyl ether (2 × 120 mL) and the organic layers were successively washed with $NaHCO_3$ sat. (50 mL), brine (50 mL), dried over Mg SO_4 and concentrated under reduced pressure. The crude material was distilled under reduced pressure to give 21 g of **3** as yellow oil. The spectroscopic data were fully coincident with those described in the literature.[10]

3-(bromomethyl)-4,4-dimethylcyclopent-2-en-1-one (**4**)**.** A 1-L Pyrex round-bottom flask containing a solution of **3** (5 g, 0.04 mol) and NBS (11.4 g, 0.064 mol, 1.6 eq.) in DCM (200 mL) was placed inside an irradiation chamber equipped with a 400 W visible lamp (HPI Plus, Koninklijke Philips N.V., Amsterdam, The Netherlands) and an air

cooling system. The flask was irradiated with stirring for 4.5 h, and during this time, the chamber's temperature remained below 30 ºC. After this period, the solution was poured in hexane (200 mL) and filtered. The organic solution was concentrated under vacuum and the crude residue (ca. 6.0 g) was used in the next step with no further purification. A small portion of the crude (200 mg) product was purified by flash column chromatography (silica gel, 10% Et_2O/hexane) to give 140 mg of 4 (65 %) as a yellow oil. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.19 (1H, s), 4.17 (s, 2H), 2.41 (s, 2H), 1.30 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) 206.4, 181.0, 132.5, 52.6, 43.2, 27.4 (2C), 24.3. EM (70 eV) *m/z*: 41 (40), 67 (100), 79 (78), 95 (44), 109 (32), 123 (23), 187 (30), 189 (28), 202 (17), 204 (M+, 18).

(5,5-dimethyl-3-oxocyclopent-1-en-1-yl)methyl acetate (**5**). KOAc (39 mmol, 3.83 g, 1.5 eq.) was added in portions during the 45-min period to a solution of crude **4** (ca. 6 g, 26 mmol) in DMSO (40 mL). The suspension was stirred for an additional 20-min period and poured into water (60 mL). The mixture was extracted with AcOEt (2 × 70 mL) and the combined organic layers were successively washed with brine (2 × 30 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (silica gel, 10% Et_2O/hexane) to give 4.45 g of **5** (60% starting from ketone **3**) as a yellow oil. ^{1}H NMR ($CDCl_3$, 300 MHz) δ_H 5.99 (1 H, t, *J* 1.8 Hz), 4.91 (2 H, d, *J* 1.8 Hz), 2.36 (2 H, s), 2.15 (3 H, s), 1.28 (6 H, s).^{13}C NMR (75 MHz, $CDCl_3$) δ 206.7, 181.2, 170.5, 127.6, 60.3, 52.2, 41.9, 27.4 (2C), 20.8. EM (70 eV) *m/z*: 43 (100), 67 (23), 79 (42), 95 (12), 110 (18), 125 (63), 140 (52), 167 (10), 182 (12, M+).

(4,5,5-trimethyl-3-oxocyclopent-1-en-1-yl)methyl acetate (**6**). A solution of **5** (3.0 g, 16.4 mmol) in THF (15 ml) was dropwise added over a solution of LDA (47.6 ml, 0.5 M, 23.8 mmol) at -78 ºC. After 20 min, the solution was warmed to -30 ºC and MeI (85

mmol, 5.3 mL, 5 eq.) was added. The mixture was kept at this temperature for 1 h and then, gradually warmed to room temperature. The reaction was quenched with NH_4Cl sat. (6 ml), poured into water (15 mL) and extracted with AcOEt (2 × 60 mL). The combined organic layers were successively washed with HCl (1M, 25 mL), $NaHCO_3$ sat. (30 mL), brine (30 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (silica gel, 5% Et_2O/hexane) to give 1.4 g of **6** (43 %) as a yellow oil. ^{1}H NMR (C_6D_6, 300 MHz) δ $_H$ 6.11 (1 H, s), 4.70 – 4.55 (2 H, m), 1.97 (1 H, qt, *J* 7.1, 2.5 Hz), 1.72 (3 H, s), 1.03 (3 H, d, *J* 7.1 Hz), 0.75 (3 H, s), 0.68 (3 H, s). ^{13}C NMR (75 MHz, C_6D_6) δ 206.8, 178.5, 169.5, 126.4, 60.2, 53.6, 44.3, 25.6, 23.4, 20.1, 9.7. EM (70 eV) m/z: 43 (100), 55 (23), 67 (33), 77 (14), 93 (38), 108 (20), 121 (14), 139 (57), 154 (32), 181 (22), 196 (8, M+). HRMS (ESI) *m/z* calculated for $C_{11}H_{16}O_3$ ([M^+ +H]) 197.1172, found 197.1170.

(4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate (**1**). Methyl magnesium chloride (8.1 mL, 3 M, 24.3 mmol) was added over a suspension of bis(cyclopentadienyl)titanium(IV) dichloride (3.03 g, 12.2 mmol) in toluene (110 mL) at 0 ºC. After 20 min of continuous stirring, the solution was gradually warmed to room temperature and a solution of ketone (1.2 g, 6.1 mmol) in dry toluene was added. The solution was warmed to 60 ºC for 24 h and was then cooled down to room temperature and poured into water (25 mL). The mixture was extracted with Et_2O (2 × 40 mL). The combined organic layers were successively washed with $NaHCO_3$ sat. (20 mL), brine (2 × 20 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (silica gel, 5% Et_2O/hexane) to give 615 mg of **1** (52 %) as a yellow oil. ^{1}H NMR (300 MHz, Benzene-d_6) δ $_H$ 6.06 (1 H, s), 4.94 (1 H, d, *J* 2.5 Hz), 4.74 (1 H, d, *J* 2.4 Hz), 4.70 – 4.55 (2 H, m), 2.36 (1 H, qt, *J* 7.1, 2.5 Hz), 1.69 (3 H, s), 0.93 (3 H, d, *J* 7.1 Hz), 0.88 (3 H, s), 0.75 (3 H, s).^{13}C

NMR (75 MHz, C_6D_6) δ 169.8, 156.7, 153.4, 129.2, 103.1, 60.8, 49.4, 47.5, 25.9, 22.1, 20.4, 12.4. EM (70 eV) *m/z*: 43 (72), 53 (12), 65 (14), 77 (21), 79 (19), 91 (40), 105 (20), 119 (100), 121 (72), 134 (22), 194 (10, M+). HRMS (ESI) m/z calculated for $C_{12}H_{18}O_2$ ([M^+ +H]) 195.1380, found 195.1375.

Assignment of enantiomeric series to the natural compound

The enantiomers contained in the synthetic sample of deacetylated **1,** following a standard procedure (Figure S2), were separated by means of preparative chiral liquid chromatography (LC) using a Sigma LP-1100 preparative system (VWR International, Radnor, PE), equipped with a DAICEL 19335 Chiralpak AD-H (1 × 25 cm) chiral column (Chiral Technologies Europe SAS, Illkirch, France). A hexane:isopropanol (99:1) solvent mixture was used as the eluent. Six injections, each containing 1 mg each of the racemic material dissolved in 0.5 ml of a 10:1 hexane:isopropanol mixture, were carried out. The pure fractions of each enantiomer were collected, and ca. 1.3 mg were recovered of each one after the solvent underwent rotary evaporation. The assignment of the specific optical rotation to each enantiomer was done after re-acetylation of the obtained alcohols under standard conditions using a polarimeter (Perkin Elmer Inc. Model 343) equipped with a sodium lamp (linea D, 589 nm) and a 1-dm glass cell at 20 ºC, using chloroform as the solvent (approx. 0.13 g/100 ml). Then, assigned levo (-) and dextro (+) enantiomers were injected into a InertCap CHIRAMIX chiral capillary column (30 m × 0.25 mm i.d. × 0.25 μm; GL Sciences Inc., Tokyo, Japan) installed in a Clarus 500 GC (Perkin Elmer Inc., Wellesley, PA) equipped with FID detector set at 250 ºC. The oven temperature was raised at 0.6 ºC/min from 50 ºC to 115ºC, and then at 25 ºC/min to 150ºC, which were finally held for 10 min. The assignment of the

285 enantiomeric series to the natural compound was done according to the matching retention times and coeluted samples (Figure S1).

Laboratory bioassay

The activity of the Porapak-Q extracts of the volatile collections containing the 290 pheromone or samples of the synthetic 4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate enantiomers were tested by the methodology described by Arai et al.[11] In each test, two pieces of 1 cm^2 filter paper were placed in a 30 cm-diameter glass Petri dish. One was treated with the extract or the enantiomers and the other was taken as the reference filter paper treated with the control extract or solvent (pentane). The control 295 extract refers to Porapak-Q pentane extract of the volatile sampling of lemons infested with immature instars (not containing adult females). The filter paper pieces were placed opposite one another in the Petri dish and 5-7 *D. aberie* males were released at the center of the dish. Ten minutes later, the number of males that had come into contact with each paper was revised. The filter paper pieces were loaded with 20 µL of pentane 300 solutions containing the substances to be tested. Twenty-µL of the crude pentane extract of the volatile sampling means approximately 1.25 female-hour equivalents (FHE), whereas 10 ng of a synthetic sample represents ca. 1.5 FHE. The reference filter paper pieces were loaded with the same volume of solvent (20 µL pentane). The position of the stimulus was rotated and the glass Petri dish was washed after each test.

305 The employed males were collected from the above-described stock colony. Recently formed cocoons were gently transferred from lemons using an entomological needle to the filter paper pieces inside the plastic Petri dishes, and remained there until emergence. After leaving the cocoons, emerged males were observed under a stereomicroscope to check their fitness (no missing legs or waxy caudal filaments, and

unfolded undamaged wings) before transferring them to the above-described glass Petri dishes for testing. All the assays were conducted under laboratory conditions, with artificial light, at 25 ± 2 °C and with 40-50% relative humidity. After each test, insects were discarded, so each test employed different males.

The null hypothesis that *D. aberiae* showed no preference for either treated filter paper (response equal to 50:50 for stimulus:solvent) was analyzed with a Chi-square goodness of fit test with the SPSS 16.0.1 statistical package (SPSS Inc., Chicago, IL).

Field experiments

The male *D. aberiae* response to synthetic 4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate was also tested under field conditions. Substances were emitted from rubber septa (Ecología y Protección Agrícola SL, Carlet, Spain), which were loaded by impregnation with a hexane solution of the separated (-) or (+) enantiomers (100 µg/each), or 100 µg of the racemate and allowing the solvent to evaporate. The traps employed were 95 × 150 mm white sticky boards (Ecología y Protección Agrícola SL, Valencia, Spain). Trials were carried out in a *Citrus reticulata* Blanco orchard var. Marisol, located in the municipality of Sagunto (Valencia, Spain) (coordinates: 39.689765, -0.289564). Four blocks of four traps were installed to test the attractiveness of the synthetic samples, also including a blank trap (only with the solvent employed for rubber septa impregnation). Traps were placed in the field in October 2018, rotated within each block and revised 6 times (every 7-15 days). Traps were hung at a height of 1.5 m and were spaced 20 m apart, with each block at least 50 m apart. The number of captured males was counted under a stereomicroscope (Stemi 508; Zeiss, Oberkochen, Germany) at 50X magnification.

The total number of *D. aberiae* males captured per trap was accumulated after one complete trap rotation inside each block, in such a way that two periods were used to avoid the dependence of catches as a factor of time. Then, one-way analysis of variance (ANOVA) was applied to these data without transformation, as they fulfilled the homocedasticity requirements and the residuals of the ANOVA fitted a normal distribution. The Fisher's least significant difference [LSD] test at $P < 0.05$ was employed for the multiple comparisons among the attractants. These analyses were conducted using the Statgraphics Centurion XVI package (StatPoint Technologies, Warrenton, VA).

RESULTS

Chemical analysis and structure elucidation

The chromatographic volatile profiles of the samples of lemons infested with either virgin or mated *D. aberiae* females showed a virgin-specific compound at 24.24 min (KI = 1322 on the ZB-5 column) (Figure 2). By sampling 90,000 FDE, ca. 30 µg of this compound were collected, which allowed us to calculate that a single *D. aberiae* female emitted approximately 0.3 ng on average. The mass spectrum of this compound had two main peaks at *m/z* 119 and 121, and significant fragments at *m/z* 134, 105, 93, 91 and 43, assuming the peak at *m/z* 194 to be the molecular ion (Figure 3). The fragmentation pattern suggested a monoterpenoid structure with an acetate ester indicated mainly by the characteristic loss of 60 amu to give *m/z* 134 and also supported by the presence of the ion at *m/z* 43. After performing the alkaline hydrolysis of one sample, the former sex pheromone peak completely disappeared, supporting the presence of an ester. The molecular ion at 194, and the demonstrated presence of an acetate, would lead to a

possible molecular formula of $C_{12}H_{18}O_2$, which in turn would require 4 sites of unsaturation in total.

The ^{1}H NMR spectrum (Figure 4) of the pure collected **1** by preparative GC (ca. 30 μg) showed some defined signals such as 6.07, 4.95 (d, *J*=2.4 Hz) and 4.75 (d, *J*=2.4 Hz) ppm. Given the small coupling constant shown, these three signals were initially assigned to a conjugated vinylic proton and two geminal methylenic protons respectively. The signal centered at 4.64 ppm was compatible with the presence of two isolated protons coupled to one another (AB system). The strong downfield chemical shift of the AB system suggested the vicinity of the former with the acetate moiety, which was, in turn, consistent with the singlet present at 1.68 ppm. Finally, the signals at 0.935, 0.87 and 0.75 ppm were tentatively assigned to three methyl groups. The first methyl group, a doublet with a coupling constant of *J*=7.1 Hz, was clearly coupled to the multiplet centered at 2.35 ppm according to the ^{1}H-^{1}H COSY spectrum, whereas the signals at 0.87 and 0.75 ppm probably corresponded to a geminal dimethyl group or two quaternary carbons. The three vinylic signals observed in ^{1}H NMR spectra probably indicated a combination of two double bounds and a ring to fulfill the aforementioned three unsaturations. This allowed us to suggest a necrodol carbon framework with an extra unsaturation as a plausible structure for this pheromone. In particular, a β-necrodol skeleton was compatible with the exo-methylenic group observed in the ^{1}H NMR spectra. Although the precise positions of the geminal and coupled methyl groups, as well as that of the acetate moiety, were not deducible *a priori* with the data in hand, (4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate **1** was tentatively proposed to be the target structure based on biosynthetic precedents of this core. Then, the synthesis was carried out to confirm the identity of the sex pheromone.

Synthesis and identification of the pheromone

(4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate was synthesized in the laboratory (Figure 1). The synthesis commences with the cyclization of isobutyl (E)-but-2-enoate I polyphosphoric acid to afford cyclopentyl enone **3**, under the previous conditions reported by Conia and Leviredend.[10] The functionalization of the enone methyl group *via* the allylic bromination of ketone **3** using NBS in DCM under irradiation,[12,13] gave bromo-ketone **4** as the main product with a 65 % yield (in some experiments, the α-keto di brominated product was also identified by the GC-MS analysis in variable amounts as a byproduct, which proved unstable after isolation by column chromatography, and decomposed in a few hours). The crude bromo-ketone **4** was treated with potassium acetate in DMSO[14,15] to afford acetate **5** with an overall 60 % yield starting from ketone **3**. The alkylation of the allylic acetate, with methyl iodide using LDA and THF as base[16], provided ketone **6** with a low yield of 43 %. Any attempt to change the base to HMDSLi or solvent did not improve the result, and was probably due to the presence of the acetate group. It was possible to finish the structure with the methylenation of ketone **6,** which was initially attempted under Wittig conditions, but in our hands, no conversion of the product was observed. The Lombardo methylenation using titanium tetrachloride and zinc metal, resulted in a total sample decomposition. Finally, a modest yield of 52 % of **1** was obtained by using the Petasis reagent[17] *in situ* generated with bis(cyclopentadienyl)titanium(IV) dichloride and methyl magnesium chloride in toluene.

The ^{1}H NMR analysis of the sample in C_6D_6 fully coincided with the signals observed in the natural sample (Figure 4). The identification of the pheromone was also fully supported by the coincidence of the GC retention time (24.24 min; Figure 2) and the MS

fragmentation pattern which was virtually identical to that of the natural virgin female volatile sampling.

Unfortunately, the direct separation of enantiomers of racemic **1** by employing preparative chiral LC was not possible under any of the assayed conditions. However, the separation of the corresponding racemic alcohol of **1,** after deacetylation with potassium carbonate in methanol,[18] by chiral LC afforded a small amount of pure enantiomers. The specific optic rotation of each enantiomer of (4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate **1** was then measured after re-acetylation under standard conditions by using triethyl amine as a base and acetic anhydride in DCM.[19] The retention times obtained by chiral GC and the co-injection of samples demonstrated that the natural (4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate emitted by *D. aberie* virgin females matched the previously assigned (-)-enantiomer (Figure S1). Given the small amount initially obtained of each enantiomer, no experiments were carried out to determine the absolute configuration of the pheromone and the enantiomeric purity of both enantiomers could not be accurately measured.

Laboratory and field assays

In the laboratory assays, the pentane extracts of virgin female volatile sampling had a significant effect on *D. aberie* males' behavior, as they significantly preferred (χ^2 = 20.57, $P < 0.0001$) the filter paper treated with this sample to a pentane extract of the volatile sampling of lemons infested with immature instars (Figure 5A).

Having synthesized the candidate compound and obtained the separated enantiomers, the laboratory tests showed that males significantly moved directly towards the filter

paper treated either with the (-)-enantiomer (Figure 5B; $\chi^2 = 31.17$, $P < 0.0001$) or the (+)-enantiomer (Figure 5C; $\chi^2 = 7.60$, $P = 0.006$), when they were separately presented against a blank filter paper piece loaded with pentane. Males significantly preferred the (-)-enantiomer when simultaneously offered both substances (Figure 5D; $\chi^2 = 18.90$, $P < 0.0001$).

In the field, blank traps captured only one male throughout the trial, whereas 1,644 and 931 males were captured in the traps baited with the (-) and (+) enantiomers, respectively, and 1,164 males were recorded with the racemate. Accordingly, trapping efficacy of the (-)-enantiomer obtained significantly more captures than the unnatural (+)-enantiomer ($F_{3,28} = 17.61$; $P < 0.0001$; Table 1), which confirmed the preference observed in the laboratory assays and, consequently, the identity of the natural sex pheromone compound previously determined by the analytical procedures. Even though the attractiveness observed for the unnatural enantiomer is not uncommon in this insect family,[20] a small contamination of the natural enantiomer in the unnatural one cannot be discarded, which might be responsible for the activity recorded. Racemate was also effective in trapping *D. aberiae* males but their trap counts were significantly lower than those of the (-)-enantiomer (Table 1).

DISCUSSION

Necrodols having 1,1,2,2-pentamethyl cyclopentane (necrodane) as core structural unit, have rarely been found in the plant kingdom[21] and only a few examples are reported in insects. Eisner and Meinwald[22] described the isolation of two novel irregular monoterpenoids, α and β-necrodols, from the defensive spray of the red line carrion beetle *Necrodes surinamensis* (Fabricius) (Coleoptera: Silphidae). Later, Figadere et

al.[23] identified the female-produced sex pheromone of the grape mealybug *Pseudococcus maritimus* (Ehrhorn) (Hemiptera: Pseudococcidae) as trans-α-necrodyl isobutyrate. Given its very uncommon irregular monoterpenoid structure, only a few synthetic routes have been developed for trans-necrodol and its isomers. As far as we know, the biosynthesis of this class of monoterpenes has not yet been studied. As suggested by Figadere et al.,[23] the necrodol skeleton could be generated from geranyl diphosphate that comes from the typical head-to-tail 1'-4 linkage of DMAPP and IPP (Figure 6) *via* the isocamphane skeleton, but assembling two isoprenoids units *via* 2-3' 2'-4 cyclization was also hypothesized. The sex pheromones of other mealybug species are mainly irregular terpenoids that display non head-to-tail 1'-2 linkages between the isoprene units. Of these, lavandulyl esters are certainly the simplest structural examples. Based on this fact, we suggest an alternative biosynthetic pathway to the necrodol skeleton *via* lavandulyl cation (Figure 7), followed by cyclization and the usual methyl rearrangement.

The diversity of relations between the chirality and bioactivity of pheromones has been thoroughly reviewed by Mori,[20] including many examples in which the unnatural stereoisomer(s) showed bioactivity. This can be stated of some pheromones belonging to the Coccoidea species. The primary component of the maritime pine scale (*Matsucoccus feytaudi* Ducasse (Hemiptera: Margarodidae)) pheromone, (8*E*,10*E*)-3,7,9-trimethyl-8,10-dodecadien-6-one, was determined as (3S,7R); however, the (3R,7R) isomer showed similar activity and, albeit weakly, males also responded to the others (3R,7S) and (3S,7S).[24] A closest example can be found in pink hibiscus mealybug (*Maconellicoccus hirsutus* (Green) (Hemiptera: Pseudococcidae) males, which slightly responded to the 1:5 mixture of the SS esters of the natural pheromone described as a 1:5 mixture of (R)-lavandulyl (S)-2-methylbutanoate and (R)-maconelliyl

(S)-2-methylbutanoate.[9] In other cases, and according to the classification of Mori,[20] only a single enantiomer of the pheromone is bioactive but the presence of other enantiomers in a mixture does not inhibit the response, which is the most common case in species of the family Pseudococcidae; e.g. the vine mealybug (*Planococcus ficus* Signoret),[25] Comstock mealybug (*Pseudococcus comstocki* Kuwana)[26] or citrus mealybug (*Pseudococcus cryptus* Hempel),[27] etc. Thus, mealybugs appear to generally insensitive or not negatively affected by the presence of stereoisomers or other analogs of their pheromones. This has been hypothetically attributed by Millar et al.[28] to the unique irregular terpenoid structures of the mealybug pheromones that create singular communication channels, free of any possible interference with, or competing for, the pheromone channel. Nonetheless, many examples can be found for the importance of enantiomeric specificity for different insect orders, and indeed the opposite enantiomer of the sex pheromone could be a strong behavioral antagonist in some cases.[20] As shown in the results of the field trial, enantiomeric purity was not crucial for *D. aberiae* attractiveness given that the racemate was also active. The substantial field attractiveness observed for the unnatural (+)-enantiomer reinforces this statement but, being these species strongly sensitive to small pheromone amounts, an effect of a small contamination with the natural enantiomer cannot be disregarded. Although absolute configuration of **1** needs further confirmation, trapping efficacy of the synthetic racemate is advantageous to further implement this sex pheromone as a management tool.

The convenient monitoring of *D. aberiae* with pheromone-baited traps will help growers to know pest pressure and when to apply insecticide treatments or to release natural enemies to optimize the efficacy of control methods. This species causes direct damage involving marked fruit distortions and size reduction, which are similar to those

symptoms caused by infestations of a close mealybug species *Delottococcus elisabethae* (Brain) (Hemiptera: Pseudoccidae), which is reported only in South Africa.[29] In this case, *D. elisabethae* is a quarantine pest in USA, Israel and South Korea[1] and it is, therefore, quite likely that *D. aberiae* can become a quarantine pest in the near future. In line with this, the availability of the *D. aberiae* pheromone will provide an unambiguous detection method to identify new infested areas, or to even detect infested fruits as part of shipments. The identification of this new pheromone opens up a new possibility to develop control methods that are already being used to fight against other Pseudococcidae species, such as mating disruption for *Planococcus ficus* (Signoret),[30-31] mass trapping or lure and kill.[32]

ACKNOWLEDGEMENTS

Authors are grateful to Martina Palomino-Schätzlein and Ruben Lamas (Servicio de Resonancia Magnética Nuclear, Centro de Investigación Príncipe Felipe, Valencia, Spain) for NMR support. Thanks to Helen L. Warburton for language revision and editing.

FUNDING SOURCES

This work received funding from Universitat Politècnica de València (post-doctoral contract for SV) and Conselleria de Agricultura, Medio Ambiente, Cambio Climático y Desarrollo Rural (Generalitat Valenciana) under grant agreement (S8456000).

SUPPORTING INFORMATION

Figure S1. Chiral GC chromatogram of single (-) and (+)-enantiomers of (4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate, natural pheromone and coeluted samples ((+)-enantiomer and natural pheromone).

Figure S2. Deacetylation of **1**.

Figure S3. Chiral HPLC chromatogram of synthetic alcohol **1a**.

Figure S4. (A) ^{1}H and (B) ^{13}C NMR spectra of **4.**

Figure S5. (A) ^{1}H and (B) ^{13}C NMR spectra of **5.**

Figure S6. (A) ^{1}H and (B) ^{13}C NMR spectra of **6.**

Figure S7. (A) ^{1}H and (B) ^{13}C NMR spectra of **1.**

Figure S8. ^{1}H-^{1}H COSY NMR spectrum of natural **1**.

REFERENCES

(1) Beltrà, A.; Garcia-Marí, A.; Soto, A. El cotonet de Les Valls, *Delottococcus aberiae*, new citrus pest. *Levante Agrícola* **2013**, *419*, 348-352 (in Spanish).

(2) Miller, D. R.; Giliomee, J. H. Systematic revision of the mealybug genus *Delottococcus* Cox & Ben-Dov (Hemiptera: Pseudococcidae). *African Entomol*. **2011**, *19*, 614-640.

(3) García-Morales, M.; Denno, B, D.; Miller, D.R.; Miller, G. L.; Ben-Dov, Y.; Hardy, N. B. ScaleNet: A literature-based model of scale insect biology and systematics. **2016**. URL (http://scalenet.info/catalogue/Delottococcus%20aberiae/) (26 February 2019).

(4) Martínez-Blay, V. Biology and management, by application of classical biological control, of the invasive mealybug *Delottococcus aberiae* (Hemiptera: Pseudococcidae) in citrus orchards in Spain. PhD dissertation **2018**, 148 pp.

(5) Beltrà, A.; Addison, P.; Ávalos, J. A.; Crochard, D.; Garcia-Marí, F.; Guerrieri, E.; Giliomee, J. H.; Malausa ,T.; Navarro-Campos, C.; Palero, F.;Soto, A. Guiding classical biological control of an invasive mealybug using integrative taxonomy. *PloS One* **2015**, *10(6)*, e0128685.

(6) Pellizzari, G., Porcelli, F. Alien scale insects (Hemiptera Coccoidea) in European and Mediterranean countries: the fate of new and old introductions. *Phytoparasitica* **2014**, 42, 713-721.

(7) Martínez-Blay, V.; Pérez-Rodríguez, J.; Tena, A.; Soto, A. Density and phenology of the invasive mealybug *Delottococcus aberiae* on citrus: implications for integrated pest management. *J. Pest Sci.* **2018**, *91*, 625-637.

(8) Martínez-Blay, V.; Pérez-Rodríguez, J.; Tena, A.; & Soto, A. (2018). Seasonal distribution and movement of the invasive pest *Delottococcus aberiae* (Hemiptera: Pseudococcidae) within citrus tree: Implications for its integrated management. *J. Econ. Entomol.* **2018**, *111*, 2684-2692.

(9) Zhang, A.; Amalin, D.; Shirali, S.; Serrano, M. S.; Franqui, R. A.; Oliver, J. E.; Klun, J. A.; Aldrich, J. R.; Meyerdirk, D. E.; Lapointe, S. L. Sex pheromone of the pink hibiscus mealybug, *Maconellicoccus hirsutus*, contains an unusual cyclobutanoid monoterpene. *PNAS* **2004**, *101*, 9601-9606.

(10) Conia, J.M.; Leriverend, M.L. Sur la préparation de cyclopenténones par action de l'acide polyphosphorique sur les esters d'acides éthyléniques. 1er Partie: Aspects pratiques. *Bull. Soc. Chim. France* **1970**, *8-9*, 2981-2991.

(11) Arai, T. The existence of sex pheromone of *Pseudococcus cryptus* Hempel (Homoptera: Pseudococcidae) and a simple bioassay. *Appl. Entomol. Zool.* **2000**, *35*, 525-528.

(12) Dessolin, J.; Biot, C.; Davioud-Charvet, E. Bromination studies of the 2, 3-dimethylnaphthazarin core allowing easy access to naphthazarin derivatives. *J. Org. Chem.* **2001**, 66, 5616-5619.

(13) Futamura, S.; Zhi-Min, Z. Photobromination of side-chain methyl groups on arenes with N-bromosuccinimide– Convenient and selective syntheses of bis (bromomethyl)-and (bromomethyl) methylarenes. *Bull. Chem. Soc. Japan* **1992**, 65, 345-348.

(14) Ardashov, O. V.; Khaid, E. V.; Mikhal'chenko, O. S.; Korchagina, D. V.; Volcho, K. P.; Salakhutdinov, N. F. First synthesis of p-mentha-1, 8-diene triol. *Russian Chem. Bull.* **2013**, 62, 171-174.

(15) Ciocarlan, A.; Aricu, A.; Ungur, N.; Biriiar, A.; Coltsa, M.; Nicolescu, A.; Deleanu, C.; Vornicu, N. Formal synthesis of (−)-pereniporin B and (−)-cinnamosmolide. *Natural Prod. Res.* **2014**, 28, 1619-1625.

(16) Kraft, P.; Kasim, P. New Musk Odorants:(3E)-4-(2′-Alkyl-5′, 5′-dimethylcyclopent-1′-enyl) but-3-en-2-ones and (3E)-1-Acetyl-3-alkylidene-4, 4-dimethylcyclohexenes. *Eur. J. Org. Chem.* **2008**, 2008, 4806-4814.

(17) Petasis, N. A.; Bzowej, E. I. Titanium-mediated carbonyl olefinations. 1. Methylenations of carbonyl compounds with dimethyltitanocene. *J. Am. Chem. Soc.* **1990**, 112, 6392-6394.

(18) Shimizu, N.; Mizoguchi, A.; Murakami, K.; Noge, K.; Mori, N.; Nishida, R.; Kuwahara, Y. Synthesis of (+)-(S)-isorobinal together with its antipod, a cyclic

monoterpene functioning as the sex pheromone of *Rhizoglyphus setosus* and its distribution among Astigmata. *J. Pestic. Sci.* **2006**, 31, 311-315.

(19) Mehta, G,; Kabirul, I. Enantioselective total synthesis of (−)-epoxyquinols A and B. Novel, convenient access to chiral epoxyquinone building blocks through enzymatic desymmetrization. *Tetrahedron Lett.* **2004**, 45, 3611-3615.

(20) Mori, K. Significance of chirality in pheromone science. *Bioorg. Med. Chem.* **2007**, *15*, 7505-7523.

(21) Garcia-Vallejo, M. C. M. I.; Sanz, J.; Bernabe, M.; Velasco-Negueruela, A. Necrodane (1, 2, 2, 3, 4-pentamethylcyclopentane) derivatives in *Lavandula luisieri*, new compounds to the plant kingdom. *Phytochemistry* **1994**, *36*, 43-45.

(22) Eisner, T.; Meinwald, J. Defensive spray mechanism of a silphid beetle (Necrodes surinamensis). *Psyche: J. Entomol.* **1982**, *89*, 357-367.

(23) Figadere, B. A.; McElfresh, J. S.; Borchardt, D.; Daane, K. M.; Bentley, W.; Millar, J. G. trans-α-Necrodyl isobutyrate, the sex pheromone of the grape mealybug, *Pseudococcus maritimus*. *Tetrahedron Lett.* **2007**, *48*, 8434-8437.

(24) Jactel, H.; Menassieu, P.; Lettere, M.; Mori, K.; Einhorn, J. Field response of maritime pine scale, *Matsucoccus feytaudi* Duc.(Homoptera: Margarodidae), to synthetic sex pheromone stereoisomers. *J. Chem. Ecol.* **1994**, *20*, 2159-2170.

(25) Hinkens, D. M.; McElfresh, J. S.; Millar, J. G. Identification and synthesis of the sex pheromone of the vine mealybug, *Planococcus ficus*. *Tetrahedron Lett.* **2001**, *42*, 1619-1621.

(26) Mori, K.; Ueda, H. Synthesis of the optically active forms of 2, 6-dimethyl-1, 5-heptadien-3-ol acetate, the pheromone of the comstock mealybug. *Tetrahedron* **1981**, *37*, 2581-2583.

(27) Nakahata, T.; Itagaki, N.; Arai, T.; Sugie, H.; Kuwahara, S. Synthesis of the sex pheromone of the citrus mealybug, *Pseudococcus cryptus*. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 2627-2631.

(28) Millar, J. G.; Midland, S. L.; McElfresh, J. S.; Daane, K. M. (2, 3, 4, 4-Tetramethylcyclopentyl) methyl acetate, a sex pheromone from the obscure mealybug: first example of a new structural class of monoterpenes. *J. Chem. Ecol*. **2005**, *3*1, 2999-3005.

(29) García-Morales, M.; Denno, B, D.; Miller, D.R.; Miller, G. L.; Ben-Dov, Y.; Hardy, N. B. ScaleNet: A literature-based model of scale insect biology and systematics. **2016**. URL (http://scalenet.info/catalogue/Delottococcus%20elisabethae/) (2 May 2019)

(30) Walton, V. M.; Daane, K. M.; Bentley, W. J.; Millar, J. G.; Larsen, T. E.; Malakar-Kuenen, R. Pheromone-based mating disruption of Planococcus ficus (Hemiptera: Pseudococcidae) in California vineyards. *J. Econ. Entomol.* **2006**, *99*, 1280-1290.

(31) Lucchi, A.; Suma, P.; Ladurner, E.; Iodice, A.; Savino, F.; Ricciardi, R.; Cosci, F.; Marchesini, E.; Conte, G.; Benelli, G. Managing the vine mealybug, *Planococcus ficus*, through pheromone-mediated mating disruption. *Environ. Sci. Pollut. Res.* **2019**, DOI: https://doi.org/10.1007/s11356-019-04530-6.

(32) Silva, E. B.; Mouco, J.; Antunes, R.; Mendel, Z.; Franco, J. C. Mate location and sexual maturity of adult male mealybugs: narrow window of opportunity in a short lifetime. *IOBC wrps Bull.* **2009**, *41*, 3-9.

Table 1. Total number of *D. aberiae* males captured during the field trial with each enantiomer of (4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate and the racemate.

period*	block	(-)-enantiomer	(+)-enantiomer	racemate	blank
1	1	285	193	281	0
	2	302	153	180	1
	3	161	68	101	0
	4	175	83	111	0
2	1	296	130	149	0
	2	192	163	179	0
	3	86	54	74	0
	4	147	87	89	0
mean ± SE**		**205.5 ± 30.2 a**	**116.4 ± 19.0 b**	**145.5 ± 25.6 b**	**0.1 ± 0.1 c**

645 * Each period accumulates total captures of one complete trap rotation.
** Mean number of male catches ± standard error. Values with different letters are significantly different according to Fisher's least significant difference [LSD] test ($F_{3,28} = 17.61$; $P < 0.0001$).

650

FIGURE CAPTIONS

Figure 1. Synthesis of (4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate **1:** a) Polyphosphoric acid, 95 ºC, 60 %; b) NBS, DCM, hv, 65 %; c) DMSO, KOAc, 95 %; d) LDA, MeI, THF, 43 %; d) Petasis reagent, PhMe, 55 ºC, 55 %.

Figure 2. GC-MS chromatogram of the volatile collections of lemons infested with virgin (A) and mated (B) *Delottococcus aberiae* females, showing a virgin-specific peak at 24.24 min, which matches the synthetic sample of (4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate (C).

Figure 3. Mass spectrum of (4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate.

Figure 4. ^{1}H-NMR (600 MHz) spectrum of the natural pheromone sample (ca. 30 µg) isolated from the virgin females volatile sampling and of the synthetic sample.

Figure 5. Behavioral response of male *Delottococcus aberiae* in the two-choice Petri dish laboratory bioassays: (A) pentane extract of the volatile sampling of lemons infested with virgin females (VF) vs. those infested with immature instars (I) (n = 36); (B) (-)-enantiomer vs. pentane (n = 34); (C) (+)-enantiomer vs. pentane (n = 36); (D) (-) vs. (+)-enantiomer (n = 42). Percentages were calculated according the total number of males employed in the bioassays. ***For each pair, differences were significant by Chi-square goodness of fit test ($P < 0.001$).

Figure 6. Proposed biosynthetic pathways from the regular monoterpenoid linkage and direct cyclization.

Figure 7. Alternative biosynthetic pathway from the irregular monoterpenoid linkage.

FIGURE GRAPHICS

675 Figure 1

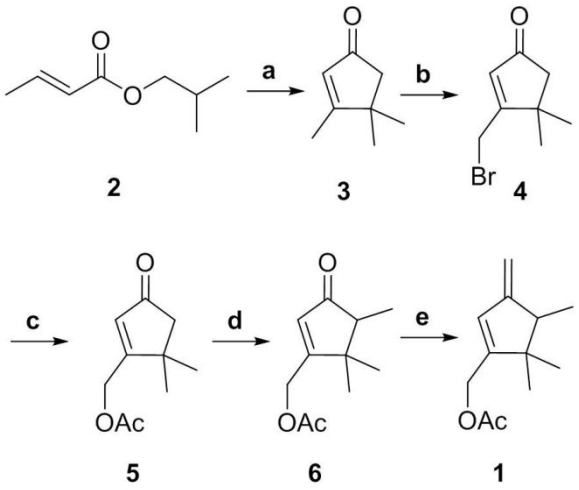
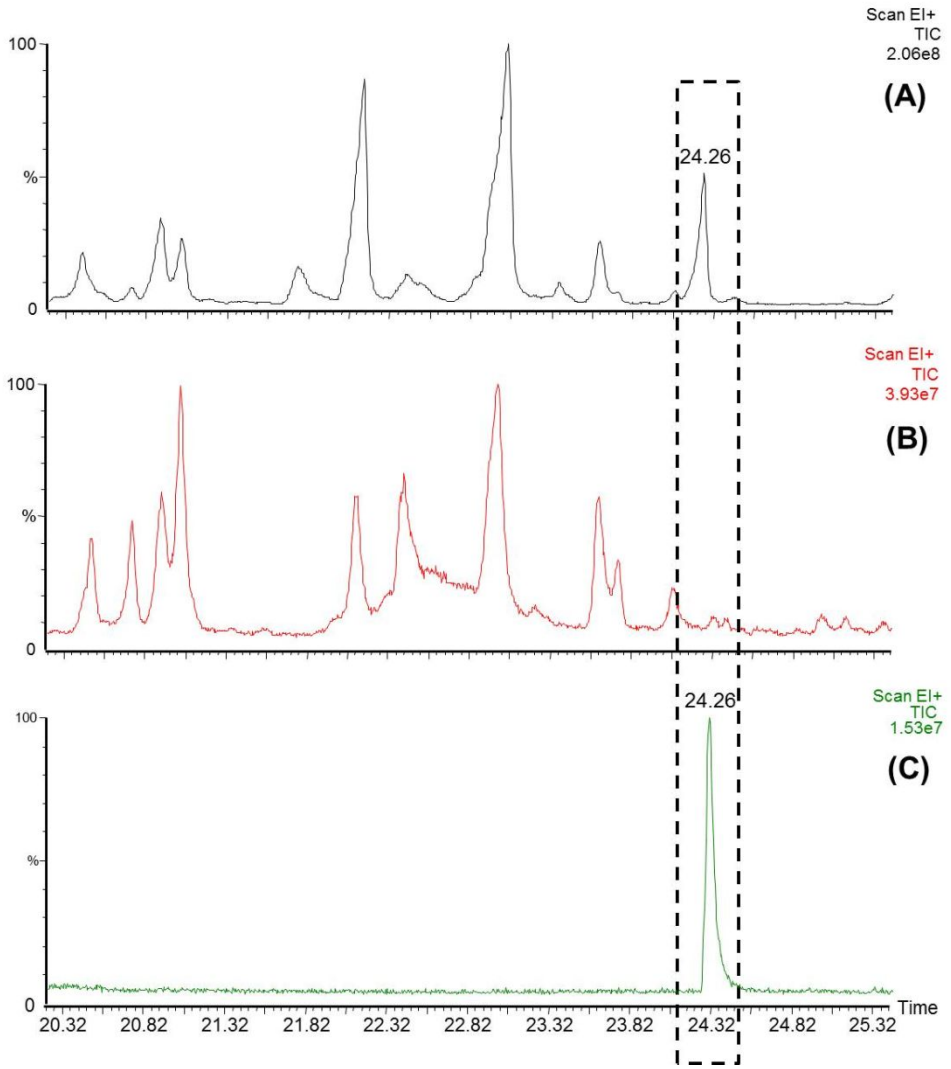


Figure 2


Scan EI+
TIC
2.06e8
(A)
24.26
100
%
0
Scan EI+
TIC
3.93e7
(B)
100
%
0
24.26
Scan EI+
TIC
1.53e7
(C)
100
%
0
Time
20.32
20.82
21.32
21.82
22.32
22.82
23.32
23.82
24.32
24.82
25.32

680

Figure 3

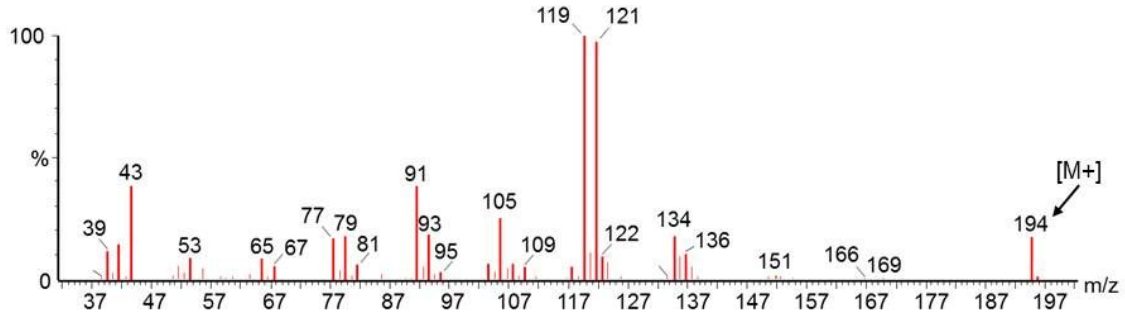
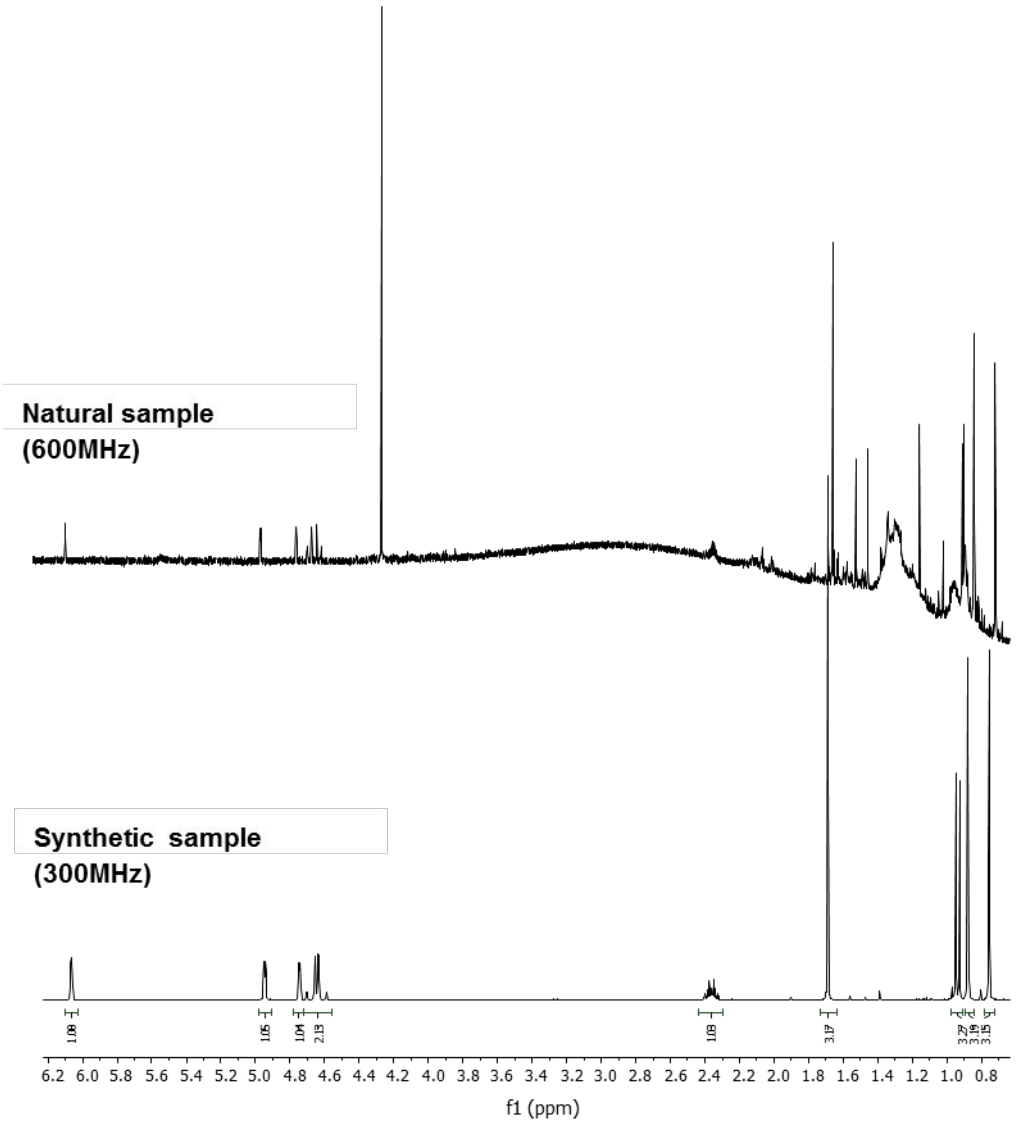
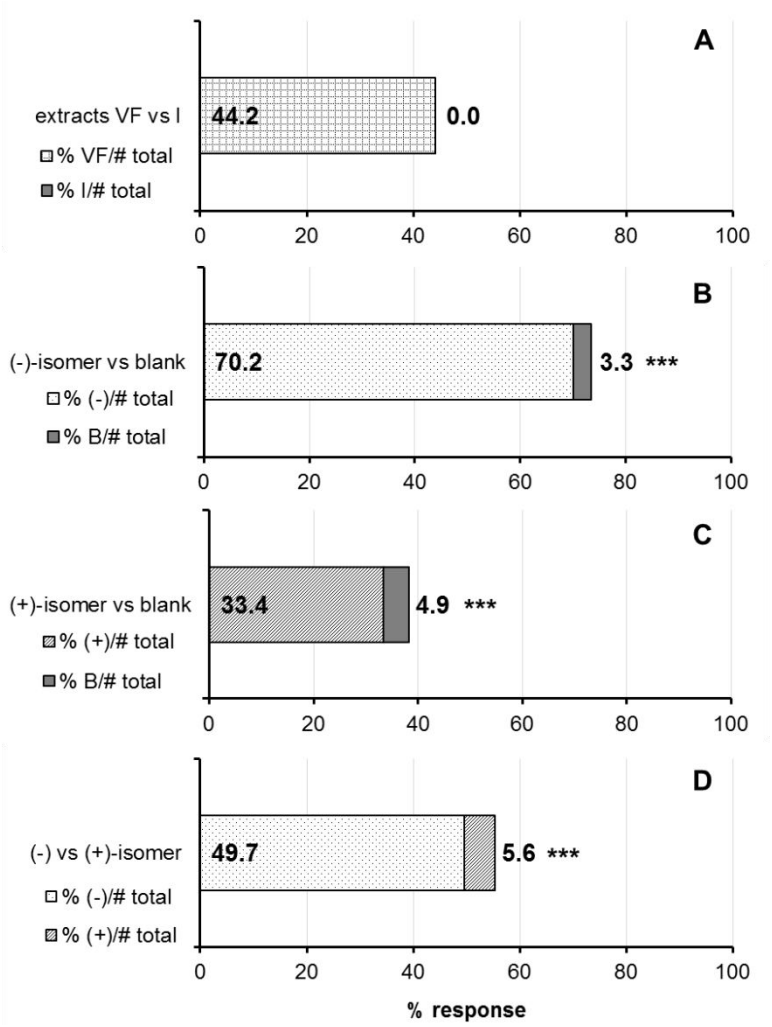

100
%
0
119
121
43
91
105
39
53
65
67
77
79
81
93
95
109
122
134
136
151
166
169
194
[M+]
m/z
37
47
57
67
77
87
97
107
117
127
137
147
157
167
177
187
197

Figure 4


Natural sample
(600MHz)
Synthetic sample
(300MHz)
1.08
1.05
1.04
2.13
1.08
3.17
3.27
3.19
3.15
6.2 6.0 5.8 5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8
f1 (ppm)

685

Figure 5


A
extracts VF vs I
44.2
0.0
% VF/# total
% I/# total
0
20
40
60
80
100
B
(-)-isomer vs blank
70.2
3.3 ***
% (-)/# total
% B/# total
C
(+)-isomer vs blank
33.4
4.9 ***
% (+)/# total
% B/# total
D
(-) vs (+)-isomer
49.7
5.6 ***
% (-)/# total
% (+)/# total
% response

690 Figure 6

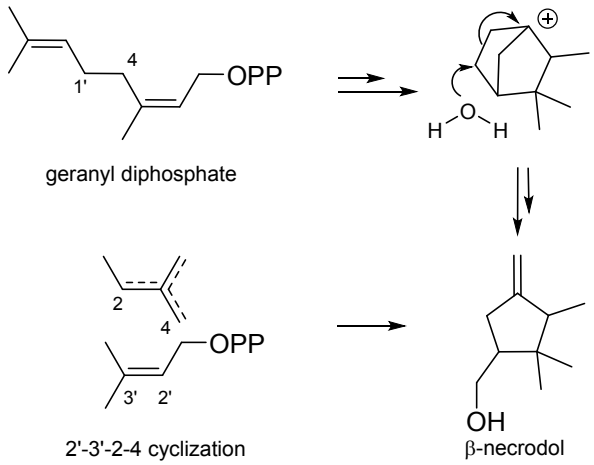
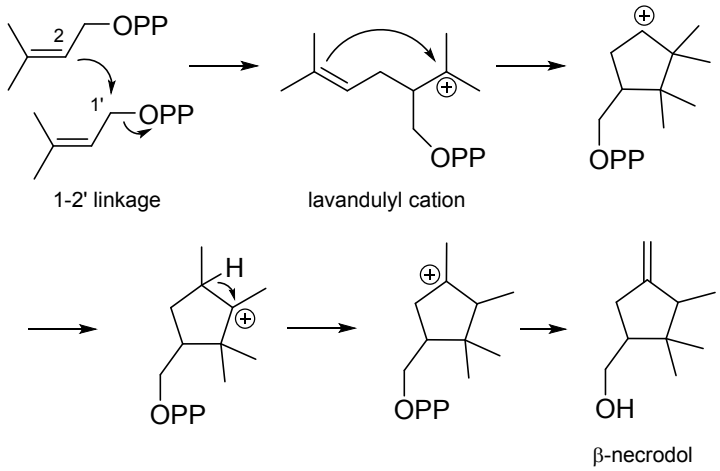

4
1'
OPP
geranyl diphosphate
H
O
H
2
4
OPP
3'
2'
2'-3'-2-4 cyclization
OH
β-necrodol

Figure 7


2
OPP
1'
OPP
1-2' linkage
OPP
lavandulyl cation
OPP
H
OPP
OPP
OH
β-necrodol

695

GRAPHIC FOR TABLE OF CONTENTS