

Semiochemicals of the Scarabaeinae: VIII. Identification of active constituents of the abdominal sex-attracting secretion of the male dung beetle, *Kheper bonellii*, using gas chromatography with flame ionization and electroantennographic detection in parallel

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Abstract

Using gas chromatography with flame ionization detection and electroantennographic detection in parallel (GC-FID/EAD), the active constituents of the sex attractant of male dung beetles of *Kheper bonellii* were located in the gas chromatogram of an extract of the secretion. These constituents were identified as propanoic acid, butanoic acid, indole, 3-methylindole (skatole) and methyl *cis*-cascaillate (methyl *cis*-2-2'-hexylcyclopropylacetate) by, *inter alia*, GC-MS, ¹H and ¹³C NMR analysis, and synthesis. These compounds elicited EAD responses in male as well as female antennae. Racemic methyl *cis*-cascaillate was synthesized for comparison with the natural methyl ester. Enantioselective GC-FID/EAD using a capillary column coated with OV-1701-OH containing 10% heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)- β -cyclodextrin showed that the natural compound co-eluted with the first-eluting enantiomer of the racemic methyl *cis*-cascaillate, which was the only enantiomer that elicited EAD responses in the antennae of male and female *K. bonellii*. The absolute configuration of this enantiomer was established by a stereoselective synthesis, which gave methyl (*R,R*)-cascaillate [methyl (1'*R*,2'*R*)-2'-2'-hexylcyclopropylacetate] in an enantiomeric excess of 69%.

Keywords: Electroantennography; EAD; Dung beetle; Sex attractant; Insect semiochemicals; Enantioselective GC; Enantioselective synthesis.

Introduction

The coprophagous fauna associated with the mammals of Africa plays an important role in the recycling of nutrients and the destruction of the habitat of many dung-breeding fly species [1]. The ecological importance of dung beetles was dramatically illustrated by the deterioration of pastures [2] and the uncontrolled increase in fly populations [3] that followed the introduction of cattle into Australia in the eighteenth century without their associated insect fauna. In Africa, on the other hand, where there are more than 4000 dung beetle species, dung breeding flies have not become such serious pests because the dung beetles destroy much of their habitat by burying fresh dung within a day or two.

Based on their nesting behaviour, dung beetles of the subfamily Scarabaeinae can be subdivided into three groups [4] and [5]: the endocoprids that excavate a chamber in the dung itself and form brood balls within this chamber; the paracoprids that excavate tunnels in which the dung is packed; and the telecoprids, for example species belonging to the genus *Kheper*, that detach a portion of the dung and roll it a distance from the dung before burying it. Adult *Kheper* beetles have a lifespan of about 3 years. The females produce one egg or, if the summer is long enough, two eggs per year, and she remains underground for several months to care for her offspring. Finding a partner with which to mate is clearly of the utmost importance for the survival of *Kheper* species. Nature has provided a mechanism that can be used by the male if he has not succeeded in finding a mate among the hundreds or even thousands of dung beetles belonging to the many different species in dung deposited by large herbivores such as elephants or rhinoceros.

To attract a female the male beetle assumes a characteristic stance, with the head lowered and the abdomen raised. White proteinaceous material is extruded through several hundred pores on both sides of the first abdominal sternite [6]. This substance quickly solidifies. During release of the secretion, the hind legs are retracted inwards towards the sides of the body and then simultaneously extended. This movement results in the brushes on the tibiae on the hind legs dispersing the secretion into the air as barely visible white puffs of particles [6]. These protein particles each contain a complex mixture of more than 150 organic compounds of which only a limited number are common to all three *Kheper* species investigated thus far.

Quite remarkably, the abdominal secretions of these different *Kheper* species each consist of only two proteins. The molecular masses are ca. 17 and 31 kDa, and the protein sequences are similar but not identical [7]. If a male has lost one of his hind legs, the secretion accumulates on that side and can be collected periodically with forceps. Care must be taken not to disturb the beetle otherwise it immediately disappears underground.

The majority of the volatile organic compounds present in the proteinaceous carrier material are long-chain hydrocarbons, carboxylic acids and alcohols. In combination with the proteins, this lipid material presumably plays a part in the controlled-release dissemination of the volatile sex pheromones of these species [7]. For example, in GC analyses using flame ionization detection and electroantennographic detection in parallel (GC-FID/EAD) only butanoic acid, 3-methylindole (skatole), and (*E*)-2,6-dimethyl-6-octen-2-ol (subaeneol) of the abdominal sex-attracting secretion of male *K. subaeneus* beetles, reproducibly elicited strong electrical responses in male as well as female antennae. Subaeneol, although giving a strong EAD response, was present in an extract of the abdominal secretion in such a low concentration that it gave a barely detectable FID signal. The major volatile constituent of the secretion of *K. subaeneus* males, (*S*)-(+)-3,6-dimethyl-5-heptenoic acid, elicited EAD responses in male antennae in only a few instances [8].

In spite of the ecological importance of dung beetles, little information is available on the semiochemical communication of these insects. In this paper we report on the identification of the EAD-active volatile constituents of the male abdominal secretion of *K. bonellii*.

Experimental

Collection of material

Adult *K. bonellii* dung beetles were collected each year from cattle dung pats in the vicinity of Elandsbaai on the south-western coast of the Cape Province (South Africa) during September, transported to Stellenbosch, and kept in greenhouses on horse dung. The glands underlying the abdominal pores on both sides of the first abdominal sternite of the males were carefully removed after the gut of the insects had been removed. The glandular tissue was sonicated with dichloromethane (Residue Analysis Grade) (Merck, Darmstadt, Germany) in a Reacti-Vial for about 5 min. The resulting emulsion was then centrifuged for 5 min at 3000 rpm, the dichloromethane extract was removed from the glandular tissue with a 250- μ l syringe, and the extract stored in a Reacti-Vial at -20 °C until used for analysis.

Instrumentation and methods

Gas chromatographic analyses were carried out on a Carlo Erba 4160 gas chromatograph (Carlo Erba, Milan, Italy) with FID and EAD in parallel. The instrument was equipped with a glass capillary column (40 m \times 0.3 mm) coated with OV-1701-OH (Ohio Valley Specialty, Marietta, OH, USA) at a film thickness of 0.38 μ m (column A). Helium was used as carrier gas at a linear velocity of 28.5 cm/s at 40 °C and a temperature programme of 2 or 4 °C from 40 to 250 °C was employed.

Enantioselective GC analyses were carried out using a glass capillary column (30 m \times 0.3 mm I.D.) coated with 0.25 μ m of the stationary phase OV-1701-OH containing 10% heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)- β -cyclodextrin as chiral selector (column B), using hydrogen as carrier gas at a linear velocity of 50 cm/s at 40 °C. The oven was ramped from 40 to 90 °C at 10 °C/min and held for 61 min, ramped to 120 °C at 10 °C/min and held for 10 min, and then ramped to 160 °C at 10 °C/min.

For the preparative isolation of one of the EAD-active components of the secretion the EAD set-up described below was adapted for the collection of fractions by bubbling the column effluent through CDCl_3 . Removable fraction collection vessels that could be connected in a press-fit fashion to the fused silica tip protruding from the EAD interface were used. A 30 m \times 0.6 mm I.D. glass column coated with 1.2 μ m of the apolar phase PS-255 (Petrarch Systems, Bristol, PA, USA) was used for preparative work. All capillary columns were drawn and coated in our laboratory.

Low-resolution electron impact (EI) mass spectra were obtained at 70 eV on a Carlo Erba QMD 1000 GC-MS instrument using the above columns. Helium was used as carrier gas at a linear velocity of 28.6 cm/s at 40 °C. The GC-MS interface temperature was set at 250 °C and the ion source temperature at 180 °C. The pressure in the source housing was ca. 2×10^{-5} Torr, at a column temperature of 40 °C, decreasing to ca. 1×10^{-5} Torr towards the end of the temperature programme. A scan rate of 0.9 s/scan with an interval of 0.1 s between scans was employed. GC-MS analyses were done with column A using helium as carrier gas at an average flow velocity of 31.0 cm/s at 40 °C. The oven was ramped from 40 to 250 °C at 4 °C/min.

^1H and ^{13}C NMR spectra were recorded on a Varian VXR-300 NMR spectrometer at 25 °C in CDCl_3 as solvent. Tetramethylsilane (TMS) was used as internal reference. The set-up used for FID and EAD recordings in parallel is illustrated in Fig. 1.

The column effluent was diluted with helium at a flow rate of 15 ml/min and then, using a glass 4-way junction, the diluted effluent was split in a 1:1 ratio between the FID and EAD by using deactivated fused silica capillaries of approximately equal lengths: one leading to the FID and the other into a stream of humidified air flowing through a glass tube (5 mm I.D.) at a linear velocity of 50 cm/s. The air was purified and humidified by passing it through a column of activated charcoal and bubbling it through a wash bottle with distilled water, respectively. After a while the evaporative cooling of the water in the wash bottle reached a steady state, which gave air with a relative humidity of 80% at 22 °C flowing over the preparation. In order to prevent condensation of the heavier analytes in the tip of this capillary the effluent was delivered into the air stream via a resistively heated aluminium interface.

The interface consisted of a cylindrical aluminium block (190 mm \times 45 mm O.D.), one end of which was positioned in the oven of the GC using the port provided for a GC–MS interface. This end of the aluminium cylinder had a short threaded tubular extension (10 mm \times 5 mm O.D. \times 3 mm I.D.). Using a graphite ferrule and ferrule-retaining nut, the effluent-conducting capillary was kept in a fixed position in the interface. The other end of the aluminium cylinder was provided with a short extension (6 mm) that ended in a sharply tapered tip. A hole was drilled through the centre of the interface to the tapered tip. This hole had an I.D. of 3 mm along most of its length, but the hole through the tapered extension was just wide enough to accommodate a 0.32 mm I.D. fused silica capillary. The effluent-conducting capillary was installed in the interface with its tip protruding about 1 mm from the tapered tip of the heated interface. The part of the aluminium cylinder exposed outside the GC was encapsulated in a thermally insulated stainless steel box (120 mm \times 80 mm \times 80 mm).

The interface, fitted with two thermocouples and temperature gauges, was normally used at temperatures between 200 and 250 °C. The tip of the capillary was inserted through a small hole in the wall of the glass tube carrying the humidified air stream at a position about 75 mm upstream from the preparation. This arrangement ensured that the effluent-delivering capillary was kept at a high temperature to prevent the condensation of high-boiling analytes in its tip, while very little heat was conducted to the glass tube because it was only touched by the sharp tip of the aluminium interface. The set-up was installed in an earthed Faraday cage.

Because dung beetles are too strong to tether, the insects' antennae were removed and used instead of using the live insects for EAD recording. Thus, an antenna of a male or female *K. bonellii* was carefully removed with a sharp scalpel and mounted between two pipettes filled with saline solution, as shown in Fig. 1. The preparation was inserted lengthwise into the glass air duct mentioned above, to a position about 10 mm from the outlet end of the tube and 75 mm from the point at which the column effluent was introduced into the humidified air stream. This arrangement gave a less noisy baseline than when the antenna was positioned outside the air duct, at right angles to the flow of the air. The antenna was protected against heat radiation from the gas chromatograph by a thin sandwich heat shield cooled by passage of water at 18 °C. Electrical responses in the antenna were recorded via Ag/AgCl electrodes placed in pipettes filled with saline solution containing NaCl (7.5 g/l), CaCl_2 (0.21 g/l), KCl (0.35 g/l), and NaHCO_3 (0.2 g/l) [9], as well as polyvinylpyrrolidone K90 (40 g/l) (Fluka, Buchs, Switzerland) to increase its viscosity [10]. Antennal responses (EAD) were amplified with a Murphy Developments AMS-025 amplifier (Hilversum, The Netherlands), using a time constant of 12 s. FID and EAD chromatograms were recorded on synchronized strip chart recorders.

To make provision for EAG recording with the same set-up, a section of the air duct between the tip of the capillary column and the antenna could be replaced with a T-piece (Fig. 1). This made it possible to record the EAG responses of an antenna to the whole secretion before, during or after the FID/EAD analysis. This was done by pipetting a sample of the glandular extract onto a strip of paper, allowing the solvent to evaporate, installing the paper strip in the side-arm of the T-piece, and flushing the volatile constituents of the pheromone into the humidified air stream using a pulse of air from a syringe, as illustrated in Fig. 1.

Reference compounds

Compounds required for comparison with constituents of the abdominal secretion that were not commercially available were synthesized from authentic starting materials.

Racemic methyl cis-cascarillate 4

Racemic methyl *cis*-cascarillate (methyl *cis*-2-2'-hexylcyclopropylacetate) **4** was synthesized according to the reaction scheme shown in Fig. 2. The starting compound, (*Z*)-3-decen-1-ol **7**, was prepared from 3-butyne-1-ol (Sigma–Aldrich, Cape Town) according to an appropriately adapted protocol described for the synthesis of (*Z*)-4-decen-1-ol [11]. A suspension of Zn/Cu couple (3.92 g; 60 mmol) in ether (20 ml) was treated with a few millilitres of diiodomethane under gentle heating to initiate the reaction [12]. The stirred suspension was then treated with a mixture of (*Z*)-3-decen-1-ol **7** (6.0 g; 38.38 mmol) and diiodomethane (14.4 g; 50 mmol) at such a rate that gentle refluxing was sustained. After 2 h only 45% of the unsaturated alcohol had been consumed (GC). The reaction mixture was stirred for a further 28 h. During this time, further quantities of Zn/Cu couple (5.4 and 2.7 g) were added at 10 and 28 h, respectively, but the percentage conversion of the starting compound **7** did not improve.

The liquid was decanted from the remaining metal and then washed consecutively with ice-cold dilute hydrochloric acid (1 M) and water, and dried on anhydrous K₂CO₃. Chromatography on silica gel and bulb-to-bulb distillation yielded a light yellow oil containing 61.7% (GC–MS) of *cis*-2-2'-hexylcyclopropylethanol **8**, which was purified by column chromatography on silver nitrate impregnated silica gel and elution with hexane/ethyl acetate (1:2) to give 1.2 g (18.4%) of the target compound. EI-MS *m/z* (%): 155 (0.3), 152 (1), 124 (5), 110 (6), 109 (8), 96 (15), 95 (24), 82 (41), 81 (52), 70 (34), 69 (55), 68 (69), 67 (66), 57 (40), 56 (61), 55 (100), 54 (36), 43 (48), 42 (30), 41 (70), 29 (25). ¹³C NMR (300 MHz, CDCl₃): 63.53 (C-1), 31.93 (C-8), 31.78 (C-2), 30.04 (C-7), 29.3 (C-5), 28.86 (C-6), 22.68 (C-9), 15.18 (C-4), 14.11 (C-10), 12.14 (C-3), 10.57 (C-11) (numbering as in Fig. 2).

The alcohol **8** (1.2 g; 7.1 mmol) in acetone (40 ml) was added over 6 h to a solution of CrO₃ (2.66 g; 26.6 mmol) in sulphuric acid (1.5 M; 25 ml) at a temperature between 5 and 10 °C. The reaction mixture was stirred for a further 6 h and then diluted with benzene (40 ml). The organic layer was removed and exhaustively extracted with dilute sodium hydroxide (1 M). The combined basic extracts were acidified with sulphuric acid (6 M) and extracted with benzene. Conventional work-up procedures yielded *cis*-2-2'-hexylcyclopropylacetic acid **9** (0.9 g; 69%), which was used without further purification in the next step of the synthesis.

A solution of the acid **9** (0.9 g; 4.9 mmol) and methanol (4 g; 125 mmol) in benzene (40 ml) was refluxed with Amberlite IR 120 for 24 h, using a Dean–Stark water separator. Removal of the ion-exchange resin and the solvent, followed by bulb-to-bulb distillation, yielded racemic methyl *cis*-2-2'-hexylcyclopropylacetate **4** (0.53 g; 55%) as a colourless oil in 92.3% purity. GC–EI-MS *m/z* (%): 183 (0.2), 166 (3), 155 (1), 141 (1), 137 (2), 124 (29), 101 (45), 96 (24), 87 (23), 83 (36), 82 (28), 81 (28), 74 (70), 69 (59), 68 (30), 67 (50), 59 (100), 56 (41), 55 (88), 54 (31), 43 (47), 41 (82), 39 (30), 29 (34).

Methyl (R,R)-cascarillate 4a

Methyl (*R,R*)-cascarillate [methyl (1'*R*,2'*R*)-2-2'-hexylcyclopropylacetate] **4a** was prepared as outlined in Fig. 3. A solution of (*E*)-1,3-octadiene **10** (6.2 g; 56 mmol), prepared according to a protocol of Alder and Vogt [13], and the enantioselective catalyst bis[(1*S*,9*S*)-5-cyano-1,9-bis(1-hydroxy-1-methylethyl)semicorrinato]Cu(II) **11**, prepared according to Fritschi et al. [14], in 1,2-dichloroethane (10 ml), was treated in an argon atmosphere with a portion of a solution of (1*S*,3*S*,4*R*)-menthyl diazoacetate **12** [15] (5.2 g; 23 mmol) in 1,2-dichloroethane (20 ml), and the resulting reaction mixture stirred and heated at 85 °C until the catalyst was completely dissolved and a brisk gas evolution had started (ca. 10 min). The reaction mixture was then cooled to room temperature and the remaining diazotate solution added to the stirred reaction mixture, using a syringe pump, over 24 h.

Stirring was continued for a further 4 h, then the resultant reaction mixture was diluted with hexane, and filtered. Column chromatography on silica gel and elution with hexane/ethyl acetate (60:1) yielded a mixture of (1*S*,3*S*,4*R*)-menthyl *cis*- and *trans*-2-hex-1'-enylcyclopropanecarboxylates **13** and **14**, both with (*S*)-configuration at C-1 of the cyclopropane ring, in a ratio of, respectively, 40:60 (GC–MS), and possibly a small, undetermined quantity of the corresponding menthyl esters with (*R*)-configuration at this position, as a colourless oil (3.8 g; 52%). Data of **13**: EI-MS *m/z* (%): 291 (0.3), 263 (0.2), 210 (0.5), 196 (1.5), 168 (47), 150 (10), 138 (16), 126 (18), 123 (38), 122 (33), 122 (28), 108 (30), 97 (50), 96 (54), 95 (72), 83 (100), 81 (86), 79 (40), 69 (60), 67 (57), 57 (23), 55 (77), 43 (33), 41 (62). The *trans*-isomer **14** has a practically identical MS.

Hydrogenation of a solution of the mixture of the unsaturated cyclopropane derivatives **13** and **14** (500 mg; 1.6 mmol) in benzene (10 ml) was carried out in the presence of Wilkinson's catalyst [tris(triphenylphosphine)rhodium(I) chloride] (Fluka, Buchs, Switzerland) [16] (100 mg; 0.27 mmol) at a hydrogen pressure of 550 kPa for 24 h. Removal of the solvent on a rotary evaporator and filtration of the residue through silica gel with hexane/ethyl acetate (60:1) yielded a mixture of products (500 mg) as a colourless oil, which contained, respectively, 21% and 49% of the diastereomeric *cis*- and *trans*-menthyl esters **15** and **16**, both with (*S*)-configuration at C-1, 30% of menthyl 3-methyldecanoate produced by

catalytic opening of the cyclopropane ring, and possibly a small undetermined quantity of the corresponding esters with (*R*)-configuration at C-1. Data of **15**: EI-MS *m/z* (%): 293 (0.1), 265 (0.1), 195 (0.5), 170 (13), 153 (18), 152 (18), 139 (23), 138 (57), 134 (18), 123 (23), 111 (16), 110 (16), 97 (24), 95 (52), 83 (100), 81 (45), 69 (64), 67 (18), 57 (30), 55 (83), 43 (26), 41 (34). The *trans*-isomer **16** has a practically identical MS.

The cyclopropanecarboxylates **15** and **16** were homologated to the corresponding menthyl 2-2'-hexylcyclopropylacetates according to the method of Kowalski et al. [17]. A solution of *n*-BuLi in hexane (15%; 3.5 ml; 5.6 mmol) was added to a solution of 2,2,6,6-tetramethylpiperidine (850 mg; 6.0 mmol) in THF (3.5 ml) in an argon atmosphere at 0 °C. The resulting reagent was added dropwise to a solution of dibromomethane (950 mg; 5.5 mmol) in THF at -90 °C. After 5 min the mixture of products from the previous step in this synthesis, containing the saturated cyclopropanecarboxylates **15** and **16** (500 mg) in THF (5 ml), was added dropwise to this mixture and the reaction mixture stirred for 10 min. After a further volume of *n*-BuLi solution (15%; 1.6 ml; 2.6 mmol) was added the cooling bath was removed and then the reaction mixture was slowly added to a solution of acetyl chloride (3.3 ml) in methanol (25 ml).

The resulting reaction mixture was diluted with ether (100 ml) and washed twice with dilute H₂SO₄ (10%), twice with saturated NaHCO₃, and once with saturated NaCl. Further work-up and column chromatography on silica gel, using hexane/ethyl acetate (20:1) as eluent, yielded a colourless oil (110 mg; 35%) containing the *cis*- and *trans*-esters **4** and **17**, and methyl 3-methyldodecanoate, the acyclic ester, as the three major products. Separation of these products by preparative HPLC on a reversed phase C₁₈ column (gradient methanol/water 60:40–100:0, 30 min, 1 ml/min, 27 °C) yielded a small sample of the *cis*-ester **4**. EI-MS *m/z* (%): 183 (0.5), 166 (4), 155 (1), 141 (1), 137 (3), 124 (33), 101 (37), 96 (25), 87 (19), 83 (38), 82 (27), 81 (22), 74 (57), 69 (48), 68 (24), 67 (33), 59 (100), 56 (44), 55 (82), 54 (28), 43 (47), 41 (70). GC analysis of this material on the enantioselective column B showed that the isolated methyl *cis*-cascarillate contained methyl (1'*R*,2'*R*)-2-2'-hexylcyclopropylacetate **4a** and the corresponding (1'*S*,2'*S*)-enantiomer **4b** in a ratio of ca. 84:16. The major, first-eluting enantiomer co-eluted with the natural compound present in the abdominal secretion of male *K. bonellii*.

Results and discussion

In contrast to other *Kheper* species, *K. bonellii* males hardly ever produced secretion in captivity. The volatile components of the secretion were therefore extracted from abdominal glandular tissue. As in the other *Kheper* species investigated in our previous studies, this extract consists mainly of constituents of relatively high molecular mass (Fig. 4), such as, for example, branched and unbranched long-chain fatty acids, alcohols and esters, which did not elicit EAD responses in the antennae of *K. bonellii*. The investigation was therefore focused on the more volatile constituents of the glandular extract.

Optimization of the FID/EAD set-up

Due to the high background noise that was mostly present in the EAD traces it proved difficult to obtain reproducible results in the GC-FID/EAD analyses of the abdominal volatiles of *Kheper* beetles. Although the excised antennae of these beetles remained viable for at least 18 h, their club-like lamellate antennae appeared to be much more sensitive to air turbulence, heat radiation, and fluctuations in air temperature and humidity than those of, for example, the Lepidoptera. Variation of the speed of the air flowing over the antennae in EAD experiments and/or changes in the temperature and humidity of the air could thus have been responsible for the noisy baseline. The influence of these parameters on EAD recordings was not investigated in detail.

However, it was found that the signal-to-noise ratio of the EAD signal could be improved by positioning the antenna inside the humidified air duct to avoid the turbulent mixing of the cooled and humidified air with ambient air on the surface of the antenna. Better results were also obtained when turbulence in the air stream was avoided by using a straight air tube without an upstream bend near the preparation and by protecting the preparation from infrared radiation with a heat shield.

In GC-FID/EAD experiments with other *Kheper* beetles we have found that fatty acid pheromones of medium chain length, which elute as fronting peaks from apolar columns, sometimes give only weak EAD responses or were not detected. This could possibly be ascribed to habituation of the sensillae on the antennae before the threshold of the detection system is reached. A column with a somewhat thicker than normal film of OV-1701-OH, from which underivatized fatty acids are eluted as sharp peaks, was therefore used in the present investigation. Reproducible results were obtained in GC-FID/EAD analyses of the extract of the abdominal glands of male *K. bonellii* using the set-up depicted in Fig. 1. Both male and female antennae responded to the same constituents of the extract. This is not an unexpected result for a sex attractant secreted by the male insect [18].

Structural elucidation

An example of a GC-FID/EAD analysis of the sex attractant of this species, using a male antenna, is shown in Fig. 5. Constituent **1**, eluting at 5.50 min, was identified as 2-butanone. Male and female *K. nigroaeneus* antennae also responded to 2-butanone [19] and it was subsequently found that this ketone was a trace solvent impurity that had been enriched by the concentration of the sample for GC and GC-MS analyses. This result is not altogether unexpected, because 2-hexanone and 3-hexen-2-one, constituents of the abdominal secretion of male *K. lamarcki*, elicited EAD responses in some male antennae of that species [20]. Constituents **2** and **3**, eluting at 6.27 and 13.62 min, were identified as propanoic acid and butanoic acid, respectively, and constituents **5** and **6**, eluting at 35.62 and 38.25 min as indole and 3-methylindole (skatole), respectively. These constituents and constituent **4** reproducibly gave EAD responses in male as well as female antennae. 3-Methylindole elicited EAD responses in the antennae of the other three *Kheper* species investigated thus far [8], [19] and [20].

Although the presence of a reasonably abundant ion at m/z 74 in the mass spectrum of constituent **4** (Fig. 6), eluting at 33.65 min, suggested the presence of a methyl ester moiety, the ions at m/z 87, 129, and 143 in the mass spectra of saturated methyl esters are uncharacteristically weak in this spectrum. It seemed unlikely that the ion at m/z 183 could be the molecular ion of this constituent. However, this ion could be attributed to the loss of a methyl group and the ion at m/z 124 to the elimination of methyl acetate by a McLafferty rearrangement of the molecular ion at m/z 198. The ion at m/z 166 was invoked as further evidence in favour of the presence of a methoxycarbonyl moiety in the compound.

Preparative GC of the material extracted from about 200 beetles gave the compound under discussion in sufficient quantity and purity that allowed the recording of ^1H and ^{13}C NMR (Table 1 and Table 2, respectively), APT, and COSY spectra, the results of which confirmed the presence of a methoxycarbonyl moiety. The absence of olefinic carbon atoms and the presence of a multiplet at $\delta - 0.137$ in the ^1H NMR spectrum were interpreted as evidence that the compound contains a cyclopropane ring instead of a double bond. The coupling constants between the hydrogen atoms on the cyclopropane ring (Table 1) are typical for a *cis*-disubstituted cyclopropane ring. The NMR spectral data are in accordance with data for unbranched substituents on the cyclopropane ring.

The position of the cyclopropane ring in such an unbranched carbon chain was determined by simulation of the ^{13}C NMR spectra [21] for the eight positional isomers of C_{11} carboxylic acids containing a *cis*-disubstituted cyclopropane ring. Although no perfect fit was found, the most likely structure appeared to be methyl *cis*-2-2'-hexylcyclopropylacetate. ^1H NMR spectra of both the *cis*- and *trans*-isomers of this compound, named methyl cascarillate, have been reported in the literature by Wilson and Prodan [22]. The published ^1H NMR spectrum of methyl *trans*-cascarillate, which contains a *trans*-substituted cyclopropane ring, does not contain any resonances with negative δ values, confirming the assignment of the *cis*-configuration of the cyclopropane ring in the EAD-active constituent of the dung beetle's abdominal secretion.

Methyl *cis*-cascarillate was synthesized according to the reaction scheme shown in Fig. 2. The conversion of (*Z*)-3-decen-1-ol **7** to *cis*-2-2'-hexylcyclopropylethanol **8** proceeded in low yield, with the consumption of only 45% of the starting material. The ^1H NMR spectrum of the alcohol **8** showed a multiplet at $\delta - 0.23$, confirming the *cis*-configuration of the substituents on the cyclopropane ring. This compound was oxidized with chromic acid and the resulting acid **9** converted to the methyl *cis*-cascarillate **4**. The spectral data of the synthetic product were identical in all respects to those of the natural compound.

GC separation of the two enantiomers of the synthetic ester **4** on a column coated with a solution of heptakis(2,3,6-tri-*O*-methyl)- β -cyclodextrin in the stationary phase OV-1701-OH (column B) and co-injection of the synthetic and natural compounds showed that the natural compound elutes together with the first-eluting enantiomer of the synthetic compound (Fig. 7). In a GC-EAD/FID analysis of the synthetic ester on column B, only the first eluting enantiomer elicited EAD responses in male and female *K. bonellii* antennae (Fig. 8).

The absolute configuration of this isomer was determined by preparing the (*1'R,2'R*)- and (*1'S,2'S*)-enantiomers of the compound, as outlined in Fig. 3, for retention time comparison with the natural compound on a chiral GC column. The synthesis was based on the enantioselective cyclopropanation of 1,3-octadiene **10** with (1*S*,3*S*,4*R*)-menthyl diazoacetate **12** [15] catalysed by bis[(1*S*,9*S*)-5-cyano-1,9-bis(1-hydroxy-1-methylethyl)semicorrinato]copper(II) **11** [14] to give a mixture of (1*S*,3*S*,4*R*)-menthyl 2-hex-1'-enylcyclopropanecarboxylate **13** and its *trans*-diastereomer **14**, both of which, according to Fritschi et al. [15], have (*S*)-configuration at C-1 of the cyclopropane ring. The catalytic hydrogenation of the double bond in **13** and **14** with Pt(C) as well as Pd(C) resulted in opening of the cyclopropane ring, apparently with some selectivity for the *cis*-isomer, which was present in a lower proportion in the hydrogenated product than in the starting material.

Better results were obtained with the homogeneous Wilkinson's catalyst [16], which gave the (1*S*,3*S*,4*R*)-menthyl *cis*-(1*S*)-2-hexylcyclopropanecarboxylate **15** and its *trans*-diastereomer **16** in a ratio of 30:70. Homologation [17] of the cyclopropanecarboxylates **15** and **16** followed by esterification gave the required methyl *cis*-casca^rillate, (methyl 2-2'-hexylcyclopropylacetate), **4** and its *trans*-diastereomer **17** in low yield. Repeated purification by preparative HPLC gave a small sample of the *cis*-diastereomer **4**.

Analysis of the product on the chiral capillary column B showed the presence of both the *cis*-enantiomers, with the target compound, methyl (*R,R*)-casca^rillate [methyl (1'*R*,2'*R*)-2-2'-hexylcyclopropylacetate] **4a**, in an enantiomeric excess (ee) of 69%. Apparently, either the condensation step either did not proceed with the expected high stereoselectivity or otherwise some base-catalysed racemisation took place at C-1 of the cyclopropanecarboxylate during the homologation step. Nevertheless, the major component methyl (*R,R*)-casca^rillate **4a** eluted first from this column, it co-eluted with the first-eluting enantiomer of the racemic methyl *cis*-casca^rillate and it co-eluted with the natural methyl casca^rillate. Hence it can be said that constituent **4** of the sex attractant of *K. bonellii* is methyl (*R,R*)-casca^rillate.

An attempt was made to simplify the synthesis by the enantioselective cyclopropanation of 1-octene, which would have obviated the catalytic hydrogenation step. Unfortunately, as expected [15], a low yield of 20%, and containing only 10% of the required *cis*-compound, was obtained.

To date it has not been possible to confirm the sex attracting action of the secretion. In the absence of herbivore dung, which is the primary attractant, neither the insect's secretion nor the synthetic components attracted any *K. bonellii*. Because, once mated, the females remain underground, the above-ground sex ratio of the dung beetle population constantly changes in favour of the males. Statistical analysis has shown that an apparently higher ratio of females to males in beetles trapped with dung plus the attractant, compared to beetles trapped with dung alone, was not significant.

Concluding remarks

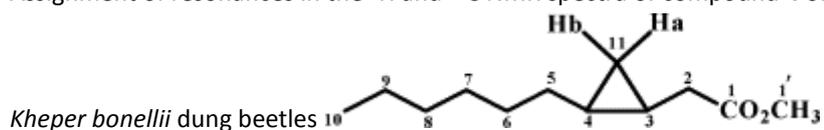
In the research reported on in this paper, the isolation of semiochemicals by repeated gas chromatographic fractionations in conjunction with behavioural assays in the field, an approach that can be described as a response-guided strategy, could not be employed because the active constituents of a sex attractant of the dung beetle are secreted in a complex matrix of organic compounds and also because the attractant does not elicit any response in the opposite sex in the absence of a feeding attractant. This situation is a typical example of one in which GC-FID/EAD is the only technique that can be used to successfully locate the semiochemical in the gas chromatogram of an insect's semiochemical secretion. In comparison with relatively unproblematic projects that have been carried out in our laboratory on the semiochemicals of Lepidoptera, and one on a dung beetle feeding kairomone, satisfactory FID/EAD analysis of the sex attracting secretion of *Kheper* dung beetles proved difficult, mainly because of the high EAD noise levels. The first acceptable results were only obtained after numerous experiments, extending over eight flight seasons.

This is our last report on the semiochemicals of dung beetles of the genus *Kheper*. We trust that the experimental details given in this paper and in our previous publications on the subject will serve to further research into the semiochemicals of dung beetles and the important role that they play in sustaining a sound ecological balance in large parts of Africa and other continents.

Tables

Table 1

Assignment of resonances in the ^1H and ^{13}C NMR spectra of compound **4** of the abdominal glandular extract of male



^1H	δ_{H} , J and multiplicity
1	3.70 (s)
2a	2.38 (dd); $^2J_{2a,2b} = 16.1$ Hz; $^3J_{2a,3} = 7.1$ Hz
2b	2.26 (dd); $^2J_{2b,2a} = 16.1$ Hz; $^3J_{2b,3} = 7.6$ Hz
3	1.1 (m)
4	0.81 (m)
5–9	1.42–1.24 (m)
10	0.89 (t); $^3J_{10,9} = 6.8$ Hz
11a	0.73 (dt); $^3J_{11a,3} = ^3J_{11a,4} = 8.4$ Hz; $^2J_{11a,11b} = -4.4$ Hz
11b	-0.14 (dt); $^3J_{11b,4} = ^3J_{11b,3} = 5.4$ Hz; $^2J_{11b,11a} = -4.4$ Hz

Table 2

Assignment of resonances in the ^{13}C NMR spectrum of compound **4** of the abdominal glandular extract of male *K. bonellii* dung beetles^a

^{13}C	δ_{C} and multiplicity
1	174.24 (s)
2	33.78 (t)
3	11.49 (d)
4	15.55 (d)
5	29.25 (t)
6	28.82 (t)
7	29.91 (t)
8	31.91 (t)
9	22.69 (t)
10	14.11 (q)
11	10.89 (t)
1'	51.57 (q)

^a Numbering of the carbon atoms as in Table 1.

Figures

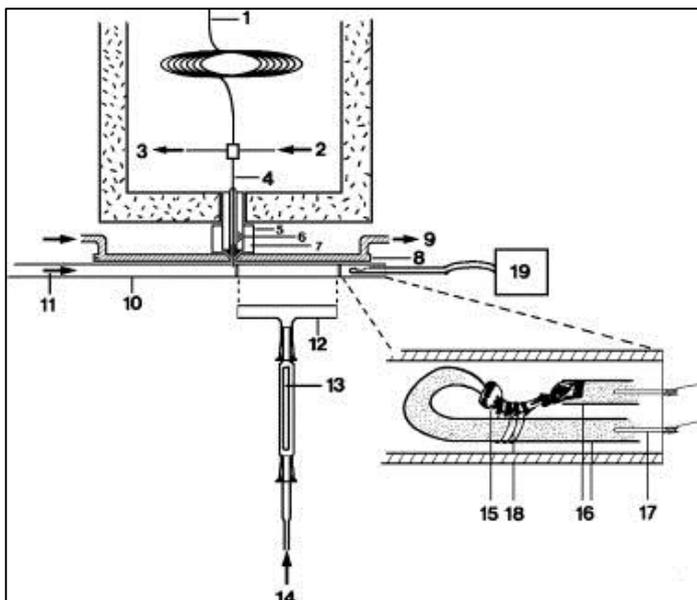


Fig. 1. Top view of a GC fitted with an effluent splitter for flame ionization detection (FID) and electroantennographic detection (EAD) in parallel: (1) capillary column; (2) make-up carrier gas at 15 ml/min; (3) FID; (4) fused silica capillary; (5) aluminium block; (6) heater; (7) insulation; (8) heat shield; (9) water at 18 °C; (10) 5 mm I.D. glass tube; (11) humidified air at 15 cm/s; (12) T-piece for recording of EAGs; (13) paper strip impregnated with sample; (14) air puffed over paper strip and antenna; (15) antenna; (16) glass capillaries containing saline solution; (17) Ag/AgCl electrodes; (18) thin cotton thread; (19) amplifier.

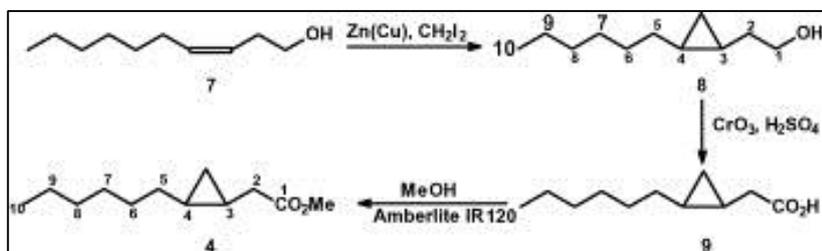


Fig. 2. Reaction scheme for the synthesis of racemic methyl cis-cascarillate (methyl cis-2'-hexylcyclopropylacetate) 4. The structures of compounds 4, 8 and 9 are formulated to show cis-substitution on the cyclopropane ring and not to convey information on the absolute configuration of the chiral carbon atoms.

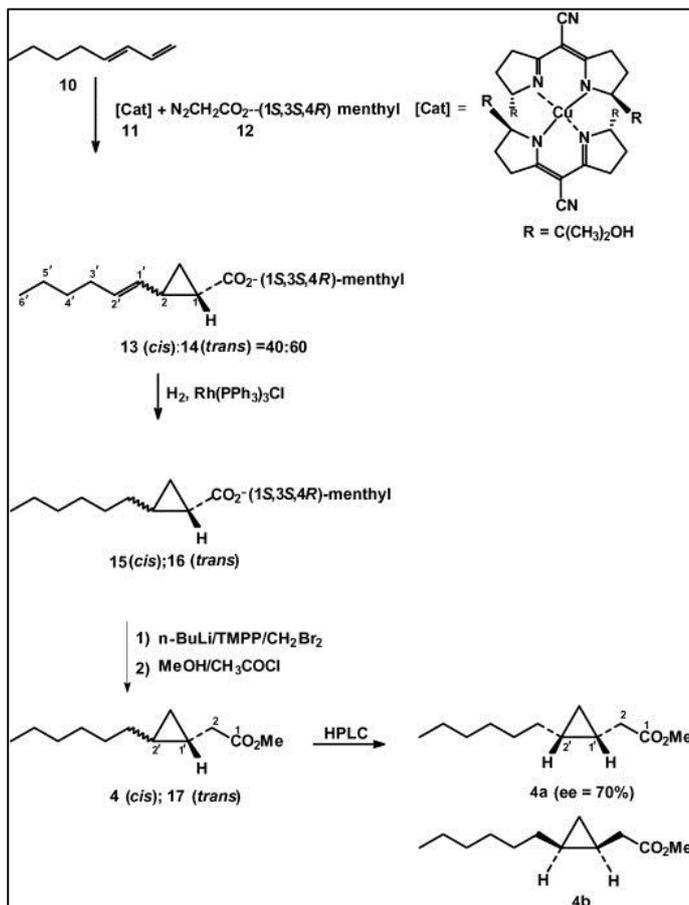


Fig. 3. Reaction scheme for the synthesis of methyl (R,R)-casarillate [methyl (1'R,2'R)-2'-hexylcyclopropylacetate] **4a**.

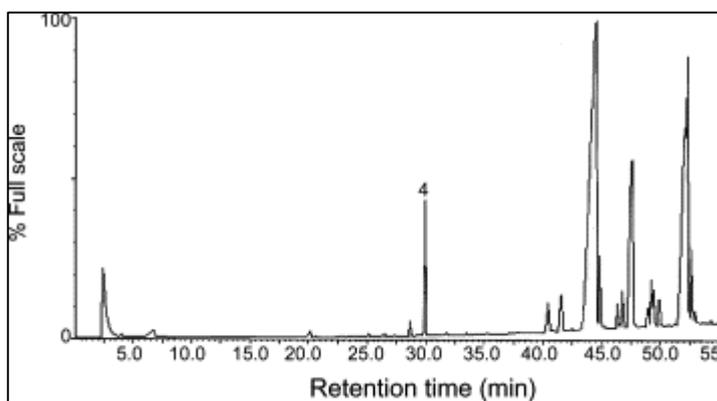


Fig. 4. Total ion chromatogram (TIC) of an extract of the abdominal sex pheromone glands of the dung beetle, *Kheper bonellii*. Gas chromatographic conditions are given in Section 2.2.

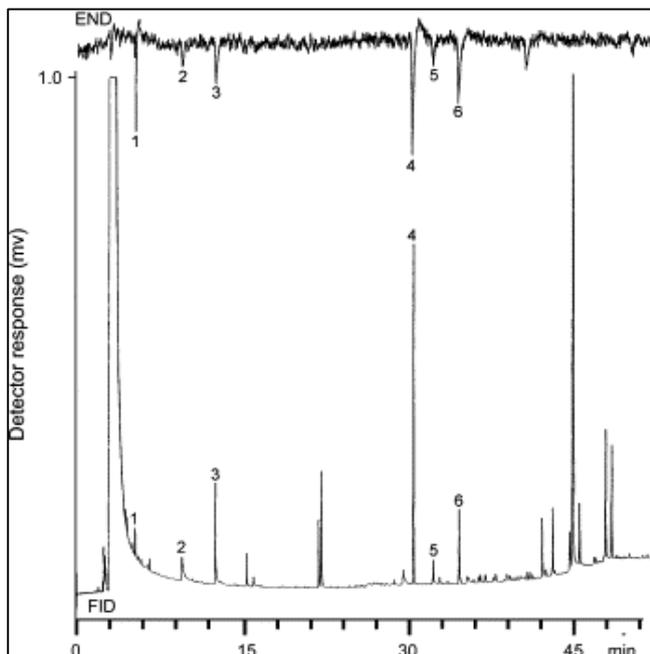


Fig. 5. Gas chromatographic analysis with FID and EAD recording in parallel of an extract of the abdominal sex pheromone glands of the dung beetle, *K. bonellii*. Gas chromatographic conditions are given in Section 2.2. Peaks are numbered to indicate simultaneous detector responses.

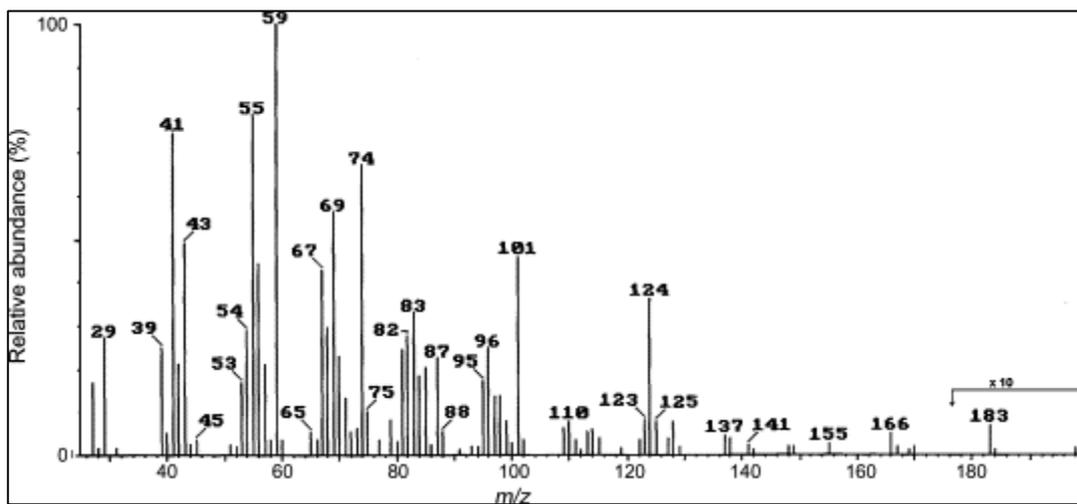


Fig. 6. EI-MS of component 4. The position of this component in the TIC is indicated in Fig. 4.

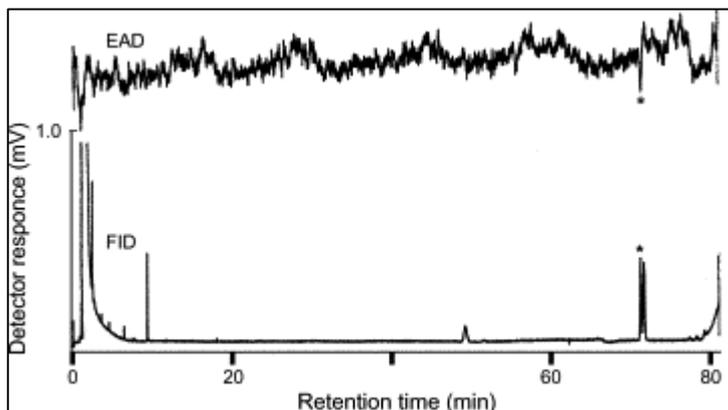


Fig. 7. Enantioselective GC analysis with FID and EAD recording in parallel of synthetic racemic methyl *cis*-cascarillate. Gas chromatographic conditions are given in Section 2.2. Component **4** (methyl *cis*-cascarillate) was eluted towards the end of the isothermal period at 120 °C. The coinciding responses of methyl (*R,R*)-cascarillate in the FID and EAD traces are marked with an asterisk.

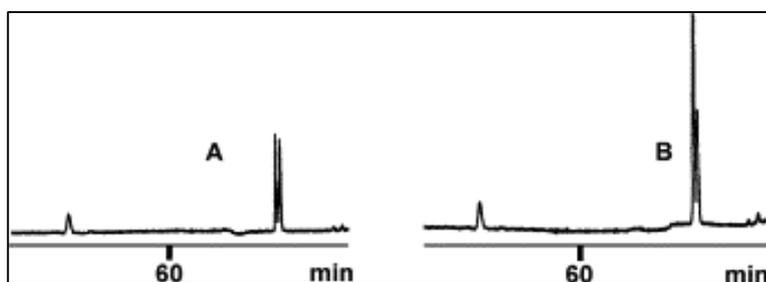


Fig. 8. Enantioselective GC analyses: (A) Synthetic racemic methyl *cis*-cascarillate. (B) Synthetic racemic methyl *cis*-cascarillate enriched with the natural methyl *cis*-cascarillate that was isolated by preparative gas chromatography from the extract of the abdominal sex pheromone glands of the dung beetle, *K. bonellii*. The experimental conditions are identical to those in Fig. 7.

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