SEMIOCHEMICALS OF THE SCARABAEINAE: VI. IDENTIFICATION OF EAD-ACTIVE CONSTITUENTS OF ABDOMINAL SECRETION OF MALE DUNG BEETLE, *Kheper nigroaeneus*

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Abstract—Using gas chromatography with flame ionization detection (FID) and electroantennographic detection (EAD) in parallel, and employing chiral and achiral capillary columns, three constituents of the abdominal sex-attracting secretion of male *Kheper nigroaeneus* dung beetles were found to elicit repro- ducible EAD responses in male and female *K. nigroaeneus* antennae. One of these constituents is present in the secretion in such a small quantity that it could not be detected by FID, and it was not identified. The other constituents were identified as 3-methylindole (skatole) and (*R*)-(+)-3-methylheptanoic acid.

Key Words—Scarabaeinae, *Kheper nigroaeneus*, dung beetle, sex attractant, sex pheromone, insect semiochemicals, EAD, electroantennographic detection.

INTRODUCTION

The coprophagous fauna associated with the mammals of Africa plays an important role in the recycling of nutrients and in the destruction of the habitat of many dung breeding flies (Heinrich and Bartholomew, 1979). During the summer months when dung beetle activity reaches its peak, hardly any flies are found in game reserves with normal herbivore populations. In contrast, areas a few kilometers outside these reserves, where the cultivation of land or other farming practices have resulted in a decrease in the dung beetle population, the indigenous people are often plagued by swarms of flies. Similar problems are experienced in Australia,

where cattle were introduced without the associated insect fauna, resulting in the deterioration of pastures (Waterhouse, 1974) and the uncontrolled increase of the fly population (Hughes, 1970).

Based on their nesting behavior, dung beetles of the subfamily Scarabaeinae can be divided into three groups (Halffter and Matthews, 1966; Bornemissza, 1969). The paracoprids construct their nests under the dung pad by excavating tunnels in which dung is packed, the endocoprids excavate a chamber in the dung pad itself, forming brood-balls within this chamber, and the telecoprids detach a portion of dung from the pad, rolling it some distance from the dung source before burying it. The majority of species of dung beetles found in southern Africa are crepuscular paracoprids (87%), although the diurnal telecoprids are also numerous, even if represented by fewer species (12%) (Ferreira, 1972). The telecoprid genus *Kheper* is confined mainly to the hot Lowveld areas of Natal and the Transvaal, extending into tropical Africa.

Telecoprid behavior is considered to have evolved from competition for dung, as dung is the meeting place of the sexes and is vital for mating success. Dung beetles, are attracted to dung and arrive by the hundreds at fresh dung where they either immediately start feeding or burrow into the dung. Within a few minutes, they distinguish between the sexes, determine the state of maturity of the opposite sex, and enlist the cooperation of the selected partner in molding and rolling a brood-ball. If the male has been unsuccessful in finding a female in or on the dung pad, he might fly away and start secreting a sex attractant or start constructing a dung ball on his own. The male *K. nigroaeneus* first buries a brood-ball before assuming the typical secretion-producing stance at the entrance to the burrow in which the ball has been buried, or even when he is not in possession of a dung ball (Edwards and Aschenborn, 1988). Supporting his body on the front two pairs of legs, the male raises his abdomen and lowers his head so that his body is at a relatively steep angle. Repeated rapid extension of the hind legs results in the extrusion of a white proteinaceous material from tiny openings in a depression on either side of the first abdominal sternite. The secretion is supplied from a glandular complex underlying the depressions. The movement of the hind legs results in the secretion being broken up into tiny particles between brushes on the tibia and comblike structures on the abdomen of the male. Small puffs of secretion are, thus, released into the air. Amputation of one of the hind legs of a beetle results in thin-strand fibers resembling cotton wool being formed on that side of its abdomen. The tibial brushes are rudimentary in the females. When the presence of an attracted female is detected by a calling male, he challenges the female by raising his forelegs and advancing on her. Her reaction is a submissive lowering of the forelegs, followed by antennal contact and acceptance by the male. If the male has not already prepared and, in the case of *K. nigroaeneus*, buried a dung ball, the male detaches a portion of dung from the pad and proceeds to roll both the dung and the female clinging to it and compacting the dung, away from the dung pad.

Once the dung ball is buried, copulation occurs below the soil, the male returning to the surface after a few days, while the female remains below to lay an egg and to brood the developing larva (Tribe, 1975, 1976).

The abdominal secretions produced by *Kheper* species consist of a visible proteinaceous material that acts as a pheromone-disseminating carrier material for minute quantities of a complex mixture of volatile organic compounds. A comparison of the secretions of *K. lamarcki*, *K. nigroaeneus*, and *K. subaeneus* revealed that similar, but not identical, proteins with molecular masses of ca. 15 kDa are the major constituents of the proteinaceous fractions of each of these secretions (Burger et al., 1990). The major volatile constituents of the secretion of *K. lamarcki* were found to be hexadecanoic acid, 2,6-dimethyl-5-heptenoic acid, and (*E*)-nerolidol (Burger et al., 1983). The volatile fractions of the secretions of these *Kheper* species are, in fact, complex mixtures, each containing at least 150 constituents such as, among others, hydrocarbons, straight- and branched-chain fatty acids and methyl and ethyl esters of these, and other fatty acids. The volatile compounds present in the secretions of these three species are similar, but only a few are common to two or all three of the species. The secretions of these insects contain many chiral long-chain compounds. Because the enantiomers of these compounds cannot be separated with the current technology, it was decided to suspend attempts to identify all the volatile constituents of the secretions in favor of the identification of the EAD-active constituents. In this paper, identification of two EAD-active constituents of the abdominal secretion of male *K. nigroaeneus* is presented.

METHODS AND MATERIALS

Collection and Purification of Carrier Material. The pentane, dichloromethane, and chloroform used for extraction purposes were of residue analysis grade (Merck). All Pyrex glassware used in the handling of the material was heated to 500°C in an annealing oven to remove any traces of organic material. Spatulas, syringes, etc., used in handling the secretion, were cleaned by rinsing with the dichloromethane specified above.

Adult *K. nigroaeneus* dung beetles were trapped in Mkuzi Game Reserve in Kwazulu Natal, South Africa, during the early summer months by baiting pitfall traps (Tribe, 1976) with fresh horse dung. On arrival in Stellenbosch, one hind leg was removed from all male beetles. A small percentage of the beetles died within a week or two after removal of a leg. This was, however, the best method to prevent dispersal of the secretion that, if not brushed off, collects at one side of the abdomen. The male and female beetles were kept at subtropical temperatures in greenhouses, the floors of which were covered with about 15 cm of moist sandy soil. Permits were granted for the collection and removal from the game reserve of 150 males and a small number of females annually for the duration of this project.

When disturbed, male dung beetles immediately stop secreting the attractant to investigate, and they may even disappear underground together with a female. Males observed to assume the attractant secreting posture were, therefore, fenced off from other beetles with aluminum strips. In some cases, the production of secretion continued for up to 2 hr. Without touching the body of the secreting male, the secreted material was removed periodically from its incapacitated side with ophthalmic forceps to prevent contamination with dust particles and the evaporation of the pheromone from the carrier material. In the meantime, the vial with material already collected was kept cool in an icebox. Tiny dust and dung particles picked up from the abdomen of the insects by the emerging secretion were carefully removed from the secretion under a microscope. Only about 5–10% of the males produced secretion in captivity, and in most instances, secretion production lasted only a short while and yielded too little material for gas chromatographic analysis of the secretion from individual insects. Small samples, therefore, had to be pooled for analysis. However, when secretion production was kept up for longer periods, it was possible to analyze the secretion from individuals. In a typical sample preparation, 3 mg of the secretion was sonicated for 1 min with 50 μl of chloroform in a Reacti-Vial, after which the suspension was centrifuged at 3000 rpm for 10 min. The white carrier material was concentrated in the upper layers of the solvent, while the remaining dust particles were precipitated. The solvent, containing soluble organic material, was carefully removed from between these two layers of solid material with a 100-μl syringe. The process was repeated a number of times, and extracts were pooled and concentrated for analysis of the volatile components of the secretion.

Analytical Methods. Gas chromatographic determinations were carried out with a Carlo Erba 4160 gas chromatograph with parallel flame ionization and electroantennographic detection (FID/EAD). The instrument was equipped with a 40 m × 0.3 mm glass capillary column coated with OV-1701-OH at a film thickness of 0.4 μm. Helium was used as carrier gas at a linear velocity of 28.5 cm/sec at 40°C, and a temperature program of 2°C/min from 40°C to 250°C was employed. Chiral gas chromatographic analyses were done on a glass column (25 m × 0.3 mm) coated with 10% heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin in OV-1701-OH at a film thickness of 0.25 μm, using H_2 as carrier gas at a linear velocity of 50 cm/sec. The instrumentation for gas chromatographic analysis of dung beetle secretions with FID and EAD recording in parallel, with the use of dung beetle antennae as sensing elements has been described by Burger and Petersen (1991). In short, an antenna was inserted lengthwise into the air duct, as this arrangement produced a less noisy baseline than positioning the antenna sideways in the airstream, a position in which the club of the antenna is subjected to the effect of the turbulent mixing of the effluent and ambient air. The antenna was protected against heat radiation from the gas chromatograph by a heat shield cooled with water at 18°C. Electrical responses in the antenna were recorded via Ag–AgCl electrodes placed in

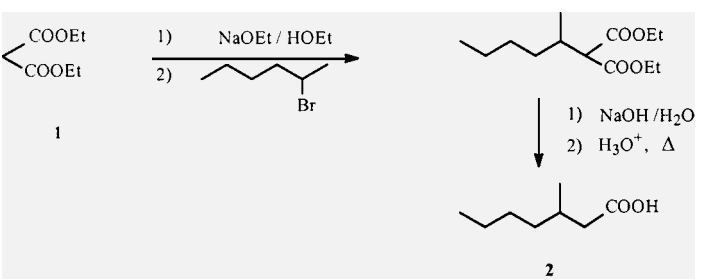
pipets filled with saline solution containing NaCl (7.5 g/liter), $CaCl_2$ (0.21 g/liter), KCl (0.35 g/liter), and $NaHCO_3$ (0.2 g/liter), as well as polyvinylpyrrolidone K90 (Fluka, 49 g/liter) to increase its viscosity (Van der Pers, 1980). Antennal responses were amplified with a Murphy Developments AMS-025 amplifier and were recorded on an Omniscribe recorder.

Mass spectra were recorded on a Carlo Erba QMD 1000 quadrupole mass spectrometer with the column and gas chromatographic conditions specified above. ^1H and ^{13}C NMR spectra of synthetic compounds and intermediates were recorded at 299.905 MHz and 75.42 MHz, respectively, at 25° C on a Varian VXR 300 NMR spectrometer.

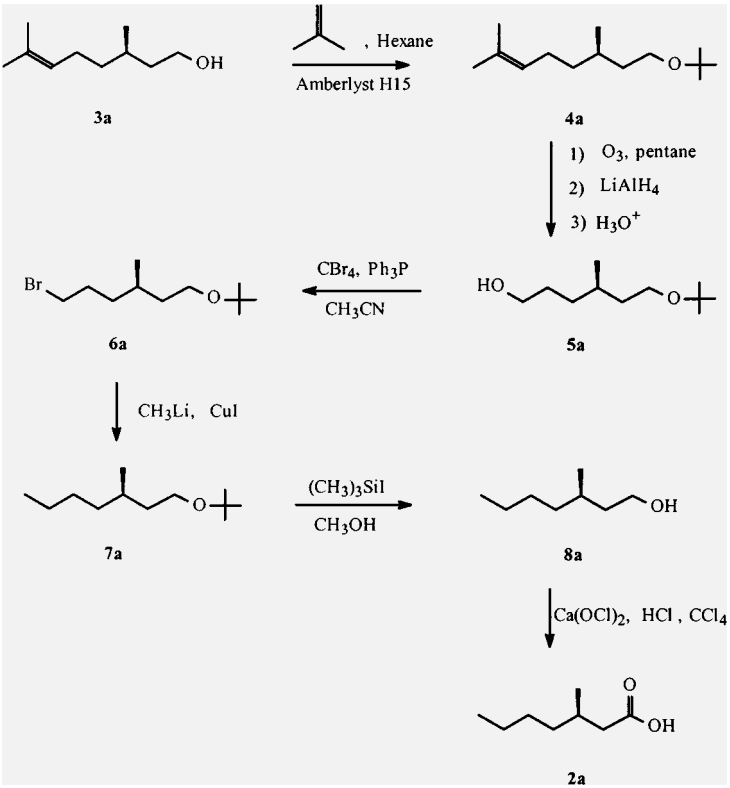
3-Methylheptanoic acid was isolated from extracts of the abdominal secretion by preparative gas chromatography on a glass column (40 m × 0.6 mm) coated with the apolar phase PS-255 at a film thickness of 1.0 μm. A 1:10 effluent splitter was used, and fractions were collected in $CDCl_3$ for NMR analysis. ^1H NMR ($CDCl_3$): δ = 2.357 (dd, 1H, C**H**H-2, $^2J_{2a,2b}$ = 15.0 Hz, $^3J_{2a,3}$ = 5.9 Hz); 2.153 (dd,1H, CH**H**-2, $^2J_{2b,2a}$ = 15.0 Hz, $^3J_{2b,3}$ = 8.0 Hz); 1.96 (m, 1H, C**H**-3); 1.45–1.15 (m, 6H, C$\mathbf{H}_2$-4,5,6); 0.894 (t, 3H, C$\mathbf{H}_3$-7, $^3J_{7,6}$ = 6,7 Hz); 0.970 (d, 3H, C$\mathbf{H}_3$-8, $^3J_{8,3}$ = 6.6 Hz).

Reference Compounds. 3-Methylindole and starting compounds were purchased from Sigma-Aldrich (Cape Town, South Africa).

Racemic 3-methylheptanoic acid (**2**) was synthesized from diethyl malonate according to Scheme 1. Diethyl malonate (**1**) (23.22 g; 145 mmol) was deprotonated with a solution of sodium ethoxide prepared from sodium (3.22 g; 140 mmol) and absolute ethanol (100 ml), and then alkylated with 2-bromohexane (23.11 g; 140 mmol) (Vogel, 1978). The resulting diethyl ester was saponified by refluxing it with a solution of potassium hydroxide (20 g) in water (30 ml), after which the reaction mixture was acidified and refluxed until the evolution of carbon dioxide had ceased. The conventional isolation procedures gave *rac*-3-methylheptanoic acid (**2**) as a colorless oil (12.94 g; 64%); ^1H NMR ($CDCl_3$): δ = 2.356 (dd, 1H, C**H**H-2, $^2J_{2a,2b}$ = 14.9 Hz, $^3J_{2a,3}$ = 6.0 Hz); 2.145 (dd, 1H, CH**H**-2, $^2J_{2b,2a}$ = 14.9 Hz, $^3J_{2b,3}$ = 8.1 Hz); 1.96 (m, 1H, C**H**-3); 1.44–1.14 (m, 6H,



SCHEME 1. Synthesis of racemic 3-heptadecanoic acid.



SCHEME 2. Synthesis of (*R*)-(+)-3-methylheptanoic acid.

C**H**$_2$-4,5,6); 0.894 (t, 3H, C**H**$_3$-7, $^3J_{7,6}$ = 6.6 Hz); 0.969 (d, 3H, C**H**$_3$-8, $^3J_{8,3}$ = 6.6 Hz).

(*R*)-(+)-*3-Methylheptanoic acid* (**2a**) was synthesized from (*R*)-(+)-3,7-dimethyl-6-octen-1-ol [(*R*)-(+)-β-citronellol] according to Scheme 2. Isobutene was slowly introduced into a suspension of Amberlyst H-15 (2.88 g) in a solution of (*R*)-(+)-3,7-dimethyl-6-octen-1-ol (**3a**) [9.91 g; 63.5 mmol, 98% pure, $[\alpha]_D^{19}$ + 5.3° (neat)] in hexane (100 ml) at room temperature for 12 hr (Alexakis et al., 1988). Removal of the catalyst and solvent gave a colorless oil (13.47 g) containing 99.9% (GC-MS) of (*R*)-(+)-8-*tert*-butoxy-2,6-dimethyl-2-octene (**4a**); $[\alpha]_D^{25}$ + 3.26° (neat). MS: *m*/*z*(%) = 156(6), 138(4), 137(5), 123(6), 109(7), 95(28), 81(67), 69(97), 57(100), 55(48), 43(35), 41(94). ^{1}H NMR ($CDCl_3$): δ = 1.599 (d, 3H, C**H**$_3$-1, $^4J_{1,3}$ = 1.4 Hz); 5.102 (m, 1H, =C**H**-3, $^3J_{3,4}$ = 7.1 Hz, $^4J_{1,3}$ = $^4J_{3,9}$ = 1.4 Hz); 1.980 (m, 2H, C**H**$_2$-4); 1.46–1.21 (m, 2H, C**H**$_2$-5); 1.62–1.47 (m, 1H, C**H**-6); 1.62–1.47 (m, 1H, C**H**H-7); 1.46–1.21 (m, 1H, CH**H**-7); 3.33 (m, 1H,

C**H**H-8); 3.38 (m, 1H, CH**H**-8); 1.679 (d, 3H, C**H**$_3$-9, $^4J_{9,3}$ = 1.4 Hz); 0.890 (d, 3H, C**H**$_3$-10, $^3J_{10,6}$ = 6.5 Hz); 1.185 (s, 9H, (C**H**$_3$)$_3$). ^{13}C NMR ($CDCl_3$): δ = 25.72 (q, C-1), 131.01 (s, C-2), 124.98 (d, C-3), 25.51 (t, C-4), 37.30 (t, C-5), 29.66 (d, C-6), 37.77 (t, C-7), 59.80 (t, C-8), 17.62 (q, **C**H$_3$-9), 19.72 (q, **C**H$_3$-10), 72.51 (s, C-1^1), 27.62 (q, 3**C**H$_3$).

Ozone was passed through a solution of the unsaturated ether (**4a**) (13.42 g; 63.3 mmol) in pentane (100 ml) until a sharp color change from colorless to blue was observed (Marvel and Nichols, 1941). Reduction of the ozonide with lithium aluminum hydride at –10°C, the usual work-up, and column chromatography on silica gel gave (*R*)-(+)-6-*tert*-butoxy-4-methyl-1-hexanol (**5a**) (7.02 g, 59%) in 99.2% purity (GC-MS); $[\alpha]_D^{25}$ + 1.95° (neat). MS: *m*/*z*(%) = 173(2), 132(6), 131(15), 115(28), 97(49), 69(40), 59(38), 57(100), 55(78), 43(23), 41(55). ^{1}H NMR ($CDCl_3$); δ = 3.627 (t, 2H, OC**H**$_2$-1, $^3J_{1,2}$ = 6.6 Hz); 1.72–1.47 (m, 2H, C**H**$_2$-2); 1.47–1.26 (m, 2H, C**H**$_2$-3); 1.72–1.47 (m, 1H, C**H**-4); 1.72–1.47 (m, 1H, C**H**H-5); 1.47–1.26 (m, 1H, CH**H**-5); 3.382 (qd, 1H, C**H**H-6, $^2J_{6a,6b}$ = 14.7 Hz, $^3J_{6a,5a}$ = 8.6 Hz, $^3J_{6a,5b}$ = 6.7 Hz); 3.360 (qd, 1H, CH**H**-6, $^2J_{6b,6a}$ = 14.6 Hz, $^3J_{6b,5a}$ = 8.6 Hz, $^3J_{6b,5b}$ = 7.0 Hz); 0.938 (d, 3H, C**H**$_3$-7); 1.185 [s, 9H, (C**H**$_3$)$_3$]. ^{13}C NMR ($CDCl_3$): δ = 63.37 (t, C-1), 30.20 (t, C-2), 33.05 (t, C-3), 29.72 (d, C-4), 37.67 (t, C-5), 59.53 (t, C-6), 19.72 (q, **C**H$_3$-7), 72.52 (s, C-1^1), 27.61 (q, 3**C**H$_3$).

Bromination of the alcohol (**5a**) (6.80 g; 36.20 mmol) with tetrabromomethane (14.37 g; 43.33 mmol) and triphenylphosphine (10.42 g; 39.72 mmol;) in acetonitrile (40 ml) (Christol et al., 1978; Slagle et al., 1981) gave (*R*)-(+)-1-bromo-6-*tert*-butoxy-4-methylhexane (**6a**) as a colorless oil (8.74 g, 96.4%) in 86% purity (GC-MS); $[\alpha]_D^{25}$ + 3.46° (neat). MS: *m*/*z* (%) = 237(3), 235(3), 179(9), 177(10), 150(2), 148(2), 137(9), 135(10), 123(6), 121(7), 109(6), 107(7), 97(92), 69(85), 59(88), 57(100), 56(77), 55(92), 43(86), 41(95). ^{1}H NMR ($CDCl_3$): δ = 3.354 (t, 2H, C**H**$_2$-1, $^3J_{1,2}$ = 7.0 Hz); 1.98–1.76 (m, 2H, C**H**$_2$-2); 1.49–1.21 (m, 2H, C**H**$_2$-3); 1.72–1.42 (m, 1H, C**H**-4); 1.69–1.50 (m, 2H, C**H**$_2$-5); 3.396 (qd, 1H, C**H**H-6, $^2J_{6a,6b}$ = 14.3 Hz, $^3J_{6a,5a}$ = 8.9 Hz, $^3J_{6a,5b}$ = 6.9 Hz); 3.354 (qd, 1H, CH**H**-6, $^2J_{6b,6a}$ = 14.9 Hz, $^3J_{6b,5a}$ = 8.8 Hz, $^3J_{6b,5b}$ = 6.9 Hz); 0.900 (d, 3H, C**H**$_3$-7, $^3J_{7,4}$ = 6.6 Hz); 1.85 [s, 9H, (C**H**$_3$)$_3$]. ^{13}C NMR ($CDCl_3$): δ = 34.29 (t, C-1), 30,41 (t, C-2), 35.57 (t, C-3), 29.35 (d, C-4), 37.87 (t, C-5), 59.53 (t, C-6), 19.72 (q, **C**H$_3$-7), 72.49 (s, C-1^1), 27.62 (q, 3**C**H$_3$).

Treatment of the bromide (**6a**) (7.35 g; 29.3 mmol) with lithium dimethylcuprate in ether at 0°C (Johnson and Dutra, 1973) and isolation of the product in the usual manner gave (*R*)-1-*tert*-butoxy-3-methylheptane (**7a**) as a colorless oil (4.04 g, 74%) in 85% purity (GC-MS). MS: *m*/*z* (%) = 171(2), 113(3), 84(3), 71(28), 69(7), 59(25), 57(100), 56(18), 55(17), 43(35), 41(43). ^{1}H NMR ($CDCl_3$): δ = 3.370 (qd, 1H, C**H**H-1, $^2J_{1a,1b}$ = 14.9 Hz, $^3J_{1a,2a}$ = 8.8 Hz, $^3J_{1a,2b}$ = 6.0 Hz); 3.344 (qd, 1H, CH**H**-1, $^2J_{1b,1a}$ = 14.9 Hz, $^3J_{1b,2a}$ = 8.7 Hz, $^3J_{1b,2b}$ = 6.5 Hz); 1.62–1.46 (m, 2H, C**H**$_2$-2); 1.70–1.46 (m, 2H, C**H**-3); 1.46–1.05 (m, 6H, C**H**$_2$-4,5,6); 0.899 (t, 3H, C**H**$_3$-7, $^3J_{7,6}$ = 6.6 Hz); 0.871 (d, 3H, C**H**$_3$-8,

$^3J_{8,3}$ = 6.6 Hz); 1.185 [s, 9 H, (C**H**$_3$)$_3$]. ^{13}C NMR ($CDCl_3$): δ = 59.88 (t, C-1), 37.87 (t, C-2), 25.65 (d, C-3), 36.88 (t, C-4), 29.21 (t, C-5), 23.01 (t, C-6), 14.17 (q, C-7), 19.78 (q, **C**H$_3$-8), 72.42 (s, C-1[1]), 27. 63 (q, 3**C**H$_3$).

Treatment of the *tert*-butoxy ether (**7a**) (3.21 g; 17.2 mmol) with iodotrimethylsilane (6.18 g; 30.91 mmol) in dichloromethane (50 ml) (Jung and Lyster, 1977) gave (*R*)-3-methyl-1-heptanol (**8a**) (2.24 g) in quantitative yield and 86% purity (GC-MS). MS: *m*/*z*(%) = 112(2), 97(3), 84(52), 83(25), 70(77), 69(37), 57(38), 56(67), 55(98), 43(100), 44(38), 41(87). ^{1}H NMR ($CDCl_3$): δ = 3.654 (t,2H, OC**H**$_2$-1, $^3J_{1,2}$ = 6.6 Hz); 1.66–1.48 (m,2H, C**H**$_2$-2); 1.69–1.48 (m,1H, C**H**-3); 1.47–1.11 (m,6H, C**H**$_2$-4,5,6); 0.896 (t, 3H, C**H**$_3$-7, $^3J_{7,6}$ = 6.7 Hz); 0.894 (d, 3H, C**H**$_3$-8). ^{13}C NMR ($CDCl_3$): δ = 61.28 (t, C-1), 40.01 (t, C-2), 27.35 (d, C-3), 36.82 (t, C-4), 29.20 (t, C-5), 22. 97 (t, C-6), 14.12 (q, C-7), 19.66 (q, **C**H$_3$-8).

Oxidation of the alcohol (**8a**) with various reagents such as chromic acid and permanganate gave the acid (**2a**) in very poor yields. The best results were obtained with calcium hypochlorite as oxidizing agent (Kabalka et al., 1990). Concentrated hydrochloric acid (7 ml) was, thus, added dropwise to a stirred suspension of hypochlorite (8.8 g; 61.5 mmol) in water (40 ml) giving a bright yellow solution. A mixture of *tert*-butanol (30 ml) and tetrachloromethane (20 ml) was added, followed by the addition of (*R*)-3-methyl-1-heptanol (**8a**) (1.87 g; 14.4 mmol) in *tert*-butanol (10 ml) over a period of 4 min. The reaction mixture was stirred for 5 hr, and the organic material extracted exhaustively with tetrachloromethane. The combined extracts were washed with a solution of sodium metabisulfite until it was colorless. The organic acid was extracted with sodium carbonate, and this basic solution was extracted thoroughly with ether to remove all non-acidic products. The water layer was carefully acidified with hydrochloric acid and the organic acid extracted with ether. The usual work-up procedures gave (*R*)-(+)-3-methylheptanoic acid (**2a**) as a colorless oil (1.14 g, 55%) in 99.3% purity (GC-MS); $[\alpha]_D^{25}$ + 4.94° (neat); 96.2% ee by gas chromatography on heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin. MS: *m*/*z* (%) = 144(3), 129(8), 115(3), 101(7), 87(55), 85(28), 84(33), 73(20), 69(22), 61(26), 60(100), 56(20), 55(38), 45(27), 43(75), 42(26), 41(65). ^{1}H NMR ($CDCl_3$): δ = 2.355 (dd,1H, C**H**H-2, $^2J_{2a,2b}$ = 14.9 Hz, $^3J_{2a,3}$ = 5.9 Hz); 2.144 (dd, 1H, CH**H**-2, $^2J_{2b,2a,}$ = 14.9 Hz, $^3J_{2b,3}$ = 8.1 Hz); 1.96 (m, 1H, C**H**-3); 1.46–1.11 (m, 6H, C**H**$_2$-4,5,6); 0.894 (t, 3H, C**H**$_3$-7, $^3J_{7,6}$ = 6.7 Hz); 0.968 (d, 3H, C**H**$_3$-8, $^3J_{8,3}$ = 6.6 Hz). ^{13}C NMR ($CDCl_3$): δ = 190.40 (s, C-1), 41.56 (t, C-2), 30.16 (d, C-3), 36.37 (t, C-4), 29.11 (t, C-5), 22.78 (t, C-6), 14.07 (q, C-7), 19.72 (q, **C**H$_3$-8).

(*S*)-(−)-*3-methylheptanoic acid* was synthesized in an analogous manner from (*S*)-(−)-β-citronellol in 51% yield, 99.2% purity (GC-MS), and 96.8% ee; MS: *m*/*z*(%) = 144(3), 129(9), 115(10), 101(7), 87(55), 85(38), 84(33), 73(20), 69(21), 61(25), 60(100), 56(19), 55(36), 45(26), 43(73), 42(25), 41(65), 39(30).

RESULTS AND DISCUSSION

A typical FID gas chromatogram of an extract of the abdominal secretion of the male *K. nigroaeneus* is shown in Figure 1. A faster programming rate and the injection of more material were found to produce a better signal-to-noise ratio in the EAD recording. Dung beetle antennae survived in EAD experiments for more than 12 hours. Figure 2 shows the first part of a gas chromatographic analysis with EAD and FID recording in parallel at a programming rate of 4°C/min. A female antenna was used as the EAD-sensing element in this analysis. Although male and female antennae mostly produced reproducible and qualitatively similar results, a few examples of male and female antennae responding reproducibly to a few other long-chain constituents of the secretion were encountered over a period of several years. Because these EAD analyses could not be reproduced with other antennae, identification of these compounds could not be verified by EAD analyses of the synthetic compounds. However, they were always long-chain fatty acids such as hexadecanoic acid and/or the methyl or ethyl esters of long-chain acids. It is difficult to explain these results, and this phenomenon was not investigated in more detail.

One explanation for the presence of a large number of long-chain alkanes, alcohols, fatty acids, etc., in the abdominal secretion could be that these compounds augment the pheromone disseminating capacity of the proteinaceous carrier material. However, the fact that the antennae of the male and female dung beetles on occasion seemed to respond to some of these compounds could indicate that, depending on circumstances, they possibly have a semiochemical function.

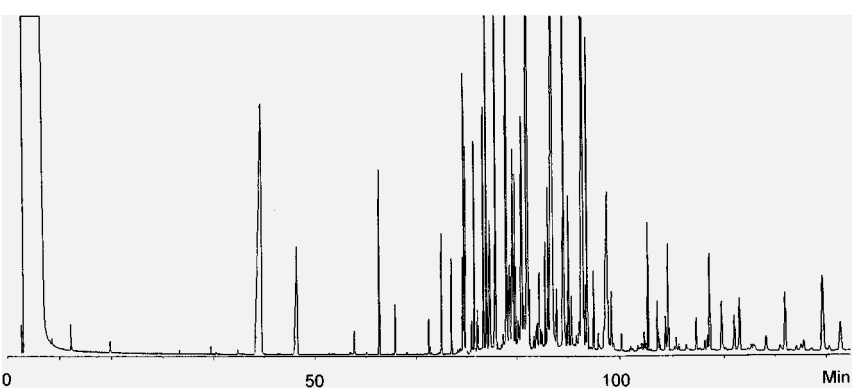


FIG. 1. Total ion chromatogram (GC-MS) of an extract of the male abdominal secretion of the dung beetle Kheper nigroaeneus. Gas chromatographic conditions: 40 m × 0.3 mm-ID glass column coated with OV-1701-OH at a film thickness of 0.375 µm and programmed at 2◦/min from 40◦ to 250◦C (hold); injector temperature 220◦C; FID 250◦C.

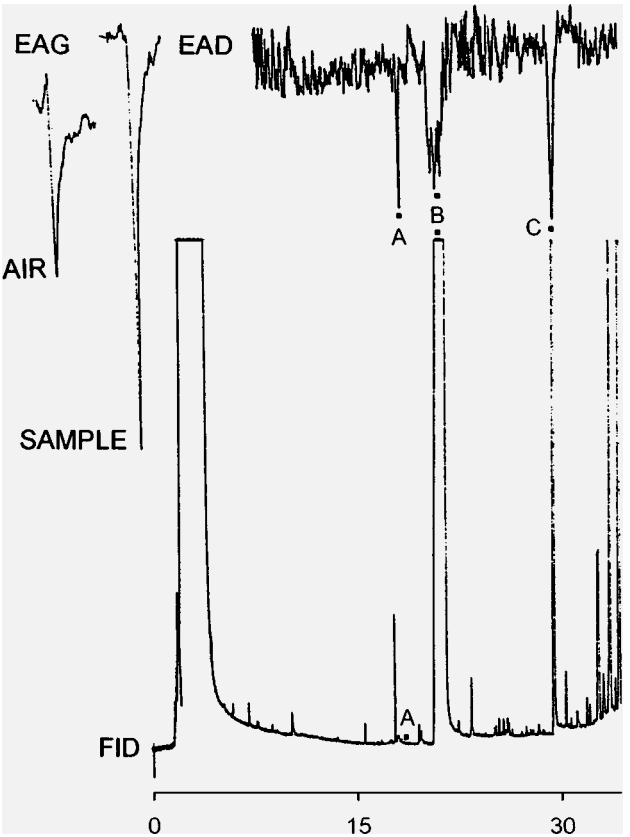


FIG. 2. First part of a gas chromatogram of an extract of the male abdominal secretion of the dung beetle *Kheper nigroaeneus* obtained by EAD/FID recording in parallel using a female antenna as detector element. Corresponding peaks in the EAD and FID chromatograms are indicated with a dot. Gas chromatographic conditions as in Figure 1 but with a programming rate of 4°C/min from 40° to 250°C (hold); EAD interface line 250°C; antenna and humidified air at 22°C. Assignment of EAD-active constituents: A = unidentified; B = (*R*)-(+)-3-methylheptanoic acid; C = skatole.

Although some of these long-chain compounds are common to the four *Kheper* species that were investigated, it is noteworthy that there were clearly large qualitative and quantitative differences in their secretions. This could be construed as evidence that the volatile constituents of the secretions are involved in the transmission of chemical signals. It is also possible, however, that the EAD and gas chromatographic techniques used in this study were not suitable for the detection of such long-chain compounds.

Constituent A, eluting at 18.55 min in the gas chromatographic analysis of the volatile organic fraction of the abdominal secretion shown in Figure 2, gave reproducible EAD responses, but was invisible in FID gas chromatograms despite relatively large samples having been used for the analysis. The low concentration in which this compound is present in the secretion and the fact that it was impossible to collect large enough quantities of abdominal secretion so far has precluded its identification.

Constituent B, eluting at 20.92 min, was initially identified as 5-methylheptanoic acid. However, it was found that the retention time and mass spectra of synthetic 5-methylheptanoic acid are different from those of the natural compound. 5-Methylheptanoic acid, furthermore, did not elicit any EAD response in the antennae of male or female *K. nigroaeneus*. All the available abdominal secretions and extracts of the secretion accumulated over more than eight years were combined for preparative gas chromatography, and enough material was isolated for ^{1}H NMR analysis of this acid, which was identified as 3-methylheptanoic acid.

Racemic 3-methylheptanoic acid (**2**) was synthesized from diethyl malonate (**1**) and 2-bromohexane according to Scheme 1. (*R*)-(+)-3-Methylheptanoic acid (**2a**) was synthesized from (*R*)-(+)-3,7-dimethyl-6-octen-1-ol [(*R*)-(+)-β-citronellol] (**3a**) according to Scheme 2 and was found to have mass and ^{1}H NMR spectra identical to those of the natural compound. This synthetic enantiomer was found to be EAD active. The *S* enantiomer was synthesized from (*S*)-(−)-β-citronellol according to an analogous synthetic route and was found to be EAD inactive. The two enantiomers are separable on a capillary column coated with a mixture of the stationary phase OV-1701-OH and heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin, the *S* enantiomer eluting first from this column. Finally, a gas chromatographic analysis with FID and EAD detection in parallel and using this chiral column confirmed the EAD activity of the second eluting *R* enantiomer.

The third EAD-active constituent of the secretion, C, eluting at 29.21 min in the gas chromatogram shown in Figure 2, was identified as 3-methylindole (skatole), which has also been identified as a constituent of the abdominal secretion of *K. lamarcki*. Synthetic 3-methylindole was found to be EAD active.

One of the goals of this research was the development of a pheromone-based monitoring system for some of the *Kheper* species as a possible method to evaluate certain aspects of the ecological status of areas where these dung beetles are found. So far, all attempts at devising a field test with which the constituents of the secretion could be evaluated have been unsuccessful. The primary source of attraction is dung, and in tests with constituents of the secretion of *K. lamarcki*, for example, no dung beetles were attracted to pitfall traps baited with hexadecanoic acid, 2,6-dimethyl-5-heptenoic acid, (*E*)-nerolidol, and skatole. In the case of *K. lamarcki*, initial indications that more females than males are attracted to pitfall traps baited with dung plus these compounds than to traps baited only with dung proved to be statistically insignificant. In this regard, however, it must also be taken

into consideration that the ratio of males to females constantly changes due to the fact that the females stay underground for a while after mating. The sex ratio could also be influenced by other factors, such as the localized availability of dung and differences in the texture and moisture content of the soil in different parts of the area where the tests were done.

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