Composition of the Heterogeneous Sex Attracting Secretion of the Dung Beetle, *Kheper lamarcki*

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The white flocculent sex attracting secretion produced by the male dung beetle, *Kheper lamarcki* MacLeay, was found to consist of tubular polypeptide fibres containing hexadecanoic acid, 2,6-dimethyl-5-heptenoic acid and (*E*)-nerolidol as major components, while a trace of skatole is responsible for the unpleasant odour of the secretion.

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 [1].

The failure of this process has been recorded in Australia where cattle were introduced without the associated insect fauna, resulting in the deterioration of the pastures [2] and the uncontrolled increase of the fly population [3].

Dung beetles of the subfamily Scarabaeinae can, on the basis of their nesting behaviour, be divided into three groups [4, 5]. 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 in southern Africa are found in the mainly crepuscular paracoprids (87%), although the diurnal telecoprids are also numerous, even if represented by fewer species (12%) [6]. The genus *Kheper* is confined mainly to the hot Lowveld areas of Natal and the Transvaal, extending into tropical Africa.

Telecoprid behaviour is considered to have evolved from competition for dung, the dung pad also being the meeting place of the sexes. Within minutes a beetle has to effectively distinguish

between sexes, determine the state of maturity of the opposite sex and engage the co-operation of the partner in moulding and rolling a ball. This is achieved by pheromonal and behavioural means. The male *Kheper* beetle releases pheromone only when in possession of dung and assumes a characteristic stance with the head lowered and abdomen raised. The pheromone emerges in the form of a white flocculent substance from the sides of the first abdominal sternite (Fig. 1) and is brushed into the air by distinct brushes on the tibiae of the hind legs. These brushes are usually rudimentary in the females. The secretion site resembles a sieve of

Fig. 1. Pheromone carrier emerging from the incapacitated side of a male *Kheper lamarcki* after removal of a leg to prevent the tibial brushes from assisting in the dispersal of the carrier.

several hundred pores supplied by a large gland complex immediately underlying the depression in which the sieve is located [7].

The male *Kheper* assumes the highest position on the dung pad when releasing the pheromone and an approaching female is challenged by the male when her presence is detected, by raising his forelegs and advancing on the female. Her reaction is a submissive lowering of the forelegs, followed by antennal contact and acceptance by the male. The male then detaches a portion of dung from the pad which is rounded by the female and proceeds to roll both the ball and the female clinging to it, away from the dung pad. Once the dung ball is buried, copulation occurs below the soil, the male returning to the surface after four days while the female remains below to lay an egg in the ball and to brood the developing larva.

Visible secretions are produced by several insects such as the tenebrionid *Cryptoglossa verrucosa* [8] and the butterfly larva *Epipyrops anomala* [9], but these are very different in function to that of the pheromone carrier of *Kheper*. No attempts have been made to investigate the composition of the sex attractant of dung beetles. This paper deals with the isolation and identification of the major constituents of the sex attracting secretion of one of the telecoprid species, *Kheper lamarcki*.

Materials and Methods

General

Dichloromethane (Merck, Residue Analysis Grade) and pentane (Merck, Spectroscopic Grade) were analysed gas chromatographically and found to be pure enough for extraction purposes when used in small quantities. 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, NMR sample tubes, etc., that were used to collect or handle material, were cleaned by rinsing with the dichloromethane specified above. Analytical gas chromatographic separations were carried out on a Carlo Erba Fractovap 2100 gas chromatograph, using helium as carrier gas. A glass capillary column (40 m × 0.3 mm), coated with Carbowax 20 M, was used for analytical separations and gc-ms analyses of the volatile constituents of the sex attracting secretion. Gc-ms

analyses were carried out on a Varian MAT 311A mass spectrometer interfaced to a Varian Aerograph 2700 gas chromatograph by coupling the capillary column directly to the mass spectrometer. This gc-ms system was used in combination with a Varian MAT Spectro-System 100 MS data system. For gas chromatographic and gc-ms analyses of derivatized amino acids, a glass capillary column (40 m × 0.3 mm) coated with SE-30, was employed. The gc-ms analysis of the derivatized amino acids was carried out on a Finnigan 4510 mass spectrometer. ^1H and ^{13}C NMR spectra were recorded on a Varian FT80 NMR spectrometer, using 99.98% isotopically pure deuterochloroform, distilled from molecular sieve (3 Å), as solvent. Energy dispersive X-ray analyses (EDX analyses) were carried out on an ISI-100A scanning electron microscope with an EDAX 9100 accessory.

Collection of the material

Adult male *K. lamarcki* were selected from dung beetles trapped in the Mkuzi Game Reserve, Natal, South Africa, during November 1980, by baiting pitfall traps [10] with fresh horse dung. A few males were also collected from rhinoceros dung middens. A total of 17 male *K. lamarcki* were transported to Stellenbosch by air in containers with moist soil. The dung beetles were kept in an 80 × 50 cm galvanized iron container with 6 cm of moist soil in the bottom and were provided with fresh horse dung every morning. To prevent the dung beetles from escaping, the container was covered with metal gauze. As soon as a beetle was observed to assume the attractant secreting posture, the hind leg on the right hand side was removed to prevent the dispersal of the secreted material on that side. The beetle was then placed in another similar metal container in which a maximum of three or four dung beetles were kept, as a beetle immediately stops secreting the attractant when it is disturbed by another beetle.

A beetle from which a hind leg had been removed, usually disappeared underground. Some of them reappeared after an hour or more and resumed secreting the sex attractant, while others, although apparently behaving normally, did not produce any further secretion. Some of the beetles died within a week or two after removal of a leg, while others survived to the end of the active

season towards the middle of January. In captivity, production of the sex attractant was found to be sporadic and, contrary to observations in nature, captive beetles produced the secretion even when not in possession of fresh dung. They had therefore to be kept under constant observation. Although the beetles frequently went through the initial stages of the production of the secretion, they usually stopped before any visible quantity of material had accumulated. In other cases the production of secretion continued for periods of up to 45 min, resulting in the accumulation of a substantial quantity of material. If, after having secreted some material, a beetle lowered its abdomen and prepared to go underground, it was taken firmly between the fingers and the secretion transferred to a Reacti-Vial (Pierce Chemical Co.) with a clean spatula. A total quantity of 650 µg of secretion was collected on ten occasions during the 1980–81 season.

Isolation and identification

Under a microscope tiny dust and dung particles were visible in the collected material. These impurities were most likely picked up from the abdomen by the emerging secretion. After it had been established in preliminary small-scale experiments that extraction of the carrier material with different solvents, such as pentane, dichloromethane and acetone, yielded extracts which gave virtually identical gas chromatographic analyses, the following procedure was employed to remove the impurities and to extract the soluble components from the white carrier material.

Secretion (450 µg) collected from four dung beetles was stirred in a 1 ml Reacti-Vial with 100 µl of pentane to give a suspension of the white insoluble carrier material in the solvent. To precipitate the carrier material together with any other insoluble matter, the suspension was centrifuged in the Reacti-Vial at 3000 rpm for 30 min. The supernatant pentane solution was carefully removed with a syringe. The extraction was repeated twice in the same manner. Combination of the pentane extracts and slow evaporation of the solvent by leaving the cap of the Reacti-Vial partially unscrewed, gave a concentrate (Fraction 1) which was used for gc and gc-ms analyses of the volatile constituents of the sex attracting secretion.

To remove dung particles from the carrier material from which the soluble constituents had been extracted, the residue in the Reacti-Vial was suspended in a mixture of pentane and dichloromethane from which the carrier material could be precipitated by centrifuging at 3000 rpm, leaving the dung particles suspended in the solvent. The solvent with the suspended impurities was removed with a finely drawn out Pasteur pipette, whereafter the process was repeated with pentane/dichloromethane mixtures having increasing densities until finally the carrier material remained in suspension and could be removed with a pipette from the heavier dust particles. The carrier material was precipitated from the suspension by dilution with pentane and centrifuging at 3000 rpm. Removal of the solvent with a syringe and drying the residue under reduced pressure at room temperature, yielded 400 µg (approx. 90% of the collected secretion) of pure white carrier material (Fraction 2).

Bioassay

Field tests were carried out to determine the biological activity of synthetic samples of the volatile compounds identified in the sex attracting secretion. For this purpose French chalk, which had been heated at 500 °C to remove any volatile compounds, was employed as inert carrier material. Hexadecanoic acid, (*E*)-nerolidol and skatole were obtained commercially. The unsaturated acid, 2,6-dimethyl-5-heptenoic acid, was prepared from 6-methyl-5-heptenone-2 by the method of Oldenziel *et al.* [11]. The purified French chalk (22.2 g) was impregnated with the synthetic compounds by dissolving hexadecanoic acid (1.6 g), 2,6-dimethyl-5-heptenoic acid (0.9 g), (*E*)-nerolidol (0.3 g) and skatole (0.01 g) in light petroleum, adding the chalk and removing the solvent on a rotary evaporator. To facilitate the handling of small quantities of the impregnated chalk in the field, samples of this material (0.5–1.0 g) were rubbed into thin squares (5 × 5 cm) of cottonwool, which in turn were enclosed between metal gauze squares.

Pitfall traps were constructed similarly to those described by Tribe [10]. Ten traps, five of which were used as controls, were set out at 50 m intervals along a straight line at right angles to the prevailing wind direction. The horse dung used as bait was suspended approx. 20 cm above the traps in metal gauze cylinders (5 × 20 cm). To bait a trap with the synthetic attractant plus horse dung, one of the cottonwool squares, prepared as described above,

was suspended above the trap together with a gauze cylinder filled with horse dung.

The traps were set out every morning at about 08.00 h for a period of four hours, whereafter the *K. lamarcki* were removed from the traps, sexed and counted. As it was impossible to count the enormous numbers of dung beetles of other species found in the traps, a rough estimate of the relative attractiveness of the test and control traps was made by measuring the volume of the dung beetles in each of the traps. All the dung beetles were set free several kilometres down-wind from the area where the field tests were carried out.

Results and Discussion

Gas chromatographic analysis of the pentane extract, Fraction 1, produced a gas chromatogram which showed the presence of only three major components in the ratio 9:33:58. After Fraction 1 had been stored overnight at −30 °C, the pentane solution contained only traces of the third (58%) component and it was found that two small crystals had grown on the bottom of the Reacti-Vial. The solvent was carefully removed from the crystals with a syringe and transferred to another Reacti-Vial (Fraction 3). The crystals were washed with cold pentane, dried under reduced pressure and dissolved in 13 µl of deuterochloroform for the recording of ^{1}H and ^{13}C NMR spectra. After the NMR

spectra had been recorded, a gas chromatographic analysis of the deuterochloroform solution showed that it contained only one major component in a purity of 94%. The mass spectrum of this component has prominent peaks at *m/e* 43, 60, 73, 85 and 115. A possible molecular ion at *m/e* 256 and fragments at *m/e* 129 and 185 characteristic of long chain aliphatic carboxylic acids, suggested that this component is hexadecanoic acid. An unbranched structure is confirmed by its ^{1}H NMR spectrum which shows the presence of only one methyl group at δ0.9 ppm. The ^{13}C NMR spectrum of the isolated compound (Table I) and comparison of its gas

Table I. Assignment of resonances (δ_c in ppm from TMS) in the ^{13}C NMR spectrum of the major component in Fraction 1.

Carbon atom	Chemical shift[a]
1	179.60
2	34.00
3	24.72
4–13	29.68
	29.46
	29.37
	29.25
	29.10
14	31.96
15	22.71
16	14.10

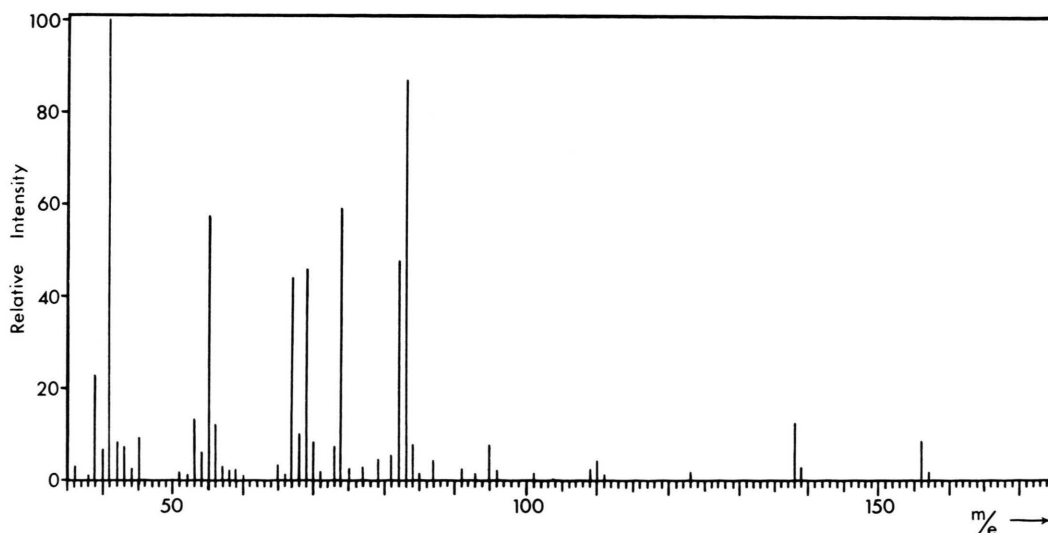[a] CDCl$_3$ with δ_c 77.03 ppm from TMS as internal reference.

Fig. 2. Mass spectrum of the second most abundant component in the soluble fraction of the sex attracting secretion.
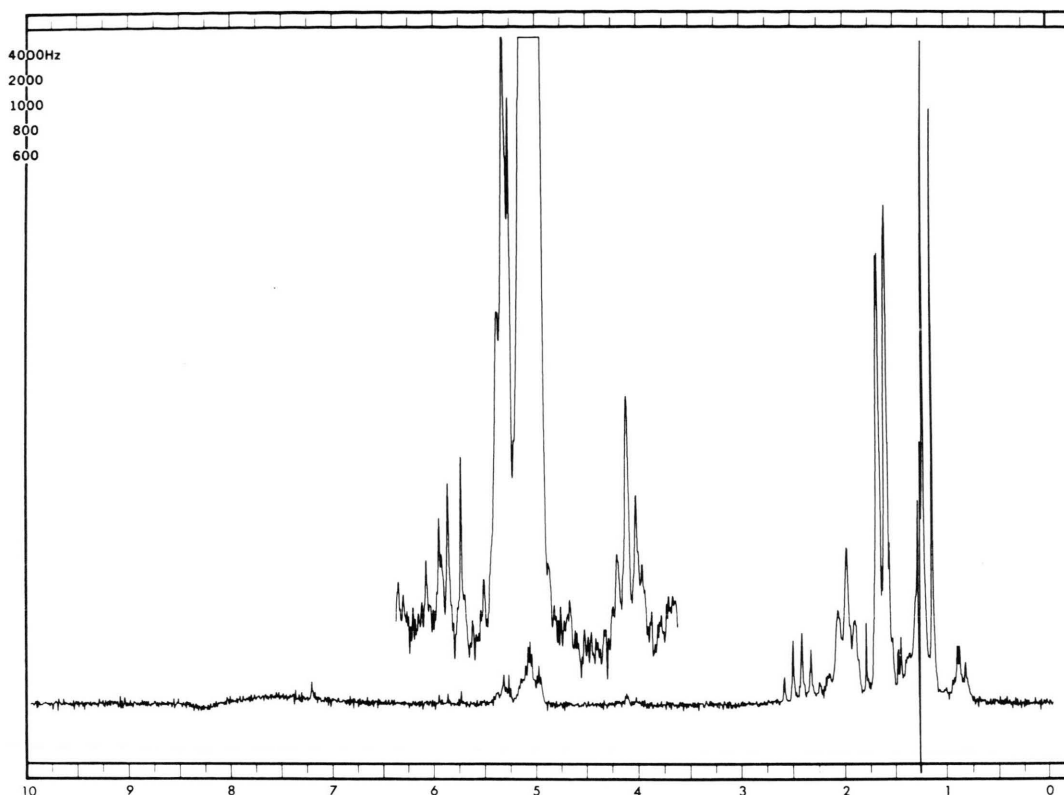
Fig. 3. ^{1}H FT NMR spectrum (80 MHz) of the unsaturated acid, after the spectrum of hexadecanoic acid which was present in the sample as an impurity, had been subtracted. The resonance at $\delta 5.94$ ppm is due to a further minor impurity, subsequently identified as (*E*)-nerolidol.

chromatographic retention time with that of a synthetic sample, furnished final confirmation of the identification of this major component of the secretion as hexadecanoic acid.
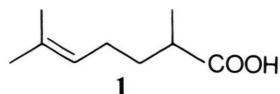
A mass spectrum (Fig. 2) of the second most abundant component in the material extracted from the secretion was obtained by gc-ms analysis of Fraction 3, in which this particular component was the major constituent. This spectrum has a molecular ion at *m/e* 156 corresponding to the molecular formula $C_9H_{16}O_2$ of a mono-unsaturated acid. The presence of a fragment at *m/e* 74 instead of one at *m/e* 60 is indicative of a 2-methyl-substituted structure. Further information on the structure of this unsaturated acid was obtained from its ^{1}H and ^{13}C NMR spectra.

As the loss of any material would have forfeited the possibility of obtaining a ^{13}C NMR spectrum of the unsaturated acid, its preparative isolation was not attempted. Fraction 3 was thus used without

further purification for the recording of ^{1}H and ^{13}C NMR spectra. The other components in this fraction were present in such small quantities that their resonances vanished in the base line noise of the ^{13}C NMR spectrum, leaving clearly discernable resonances for eight of the nine carbon atoms of the unsaturated acid. A reasonably clean and easily interpretable ^{1}H NMR spectrum (Fig. 3) of this compound was obtained by subtraction of the ^{1}H NMR spectrum of hexadecanoic acid from the spectrum of Fraction 3. In this spectrum the doublet at $\delta 1.17$ ppm (3H, $J = 7.0$ Hz) confirms the 2-methyl-substituted structure of the acid, the two lines at $\delta 1.58$ and $\delta 1.66$ ppm can be ascribed to two geminal methyl groups on an unsaturated carbon atom and the multiplet at δ approx. 2.4 ppm to the proton on the α-carbon atom which is coupled to a methyl and a methylene group. The remaining resonances at $\delta 1.95$ and δ approx. 5.1 ppm can be assigned to an allylic methylene group and an

olefinic proton respectively. These assignments can only be interpreted in terms of the terpenoid structure, 2,6-dimethyl-5-heptenoic acid (**1**).


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This structure for the second most abundant component in Fraction 1 is further substantiated by its ^{13}C NMR spectrum (Table II).

Final confirmation of the structure proposed for the unsaturated acid was afforded by spectroscopic and gas chromatographic comparison of the isolated material with a synthetic sample prepared for this purpose and for biological evaluation from 6-methyl-5-heptenone-2 according to the procedure of Oldenziel *et al.* [11].

The mass spectrum of the third most abundant component in Fraction 1, obtained by gc-ms analysis of Fraction 3, showed prominent ions typical for certain sesquiterpenes or sesquiterpene alcohols, at m/e 41, 55, 69, 93, 107, 119, 121, 136, 161 and 204. Comparison of this spectrum with mass spectra available on magnetic tape [12], as well as with spectra obtained in previous research projects, revealed similarities between this spectrum and that of (*E*)-*β*-farnesene isolated from the dorsal exudate of the antelope *Antidorcas marsupialis* [13]. However, gas chromatographic retention time comparison proved that the compound in the dung beetle secretion was neither one of the *β*-farnesene isomers, nor *α*-farnesene. Although retention time

comparison also ruled out farnesol as a possibility, the retention time of the component from the dung beetle secretion was only slightly shorter than that of (*Z, Z*)-farnesol, an observation which led to the conclusion that the compound must be a secondary or tertiary sesquiterpene alcohol. In the ^1H NMR spectrum (Fig. 3) of Fraction 3 a weak but typical vinyl proton doublet of doublets is observed at $\delta 5.94$ ppm. For isolated and conjugated vinyl groups this resonance is expected at δ approx. 5.8 and δ approx. 6.3 ppm respectively, whereas a resonance at $\delta 5.94$ ppm may be interpreted in terms of a sesquiterpene alcohol containing a vinyl carbinol moiety. By comparison of its spectroscopic properties and retention times with *inter alia* (*E*)- and (*Z*)-nerolidol, this component was identified as (*E*)-nerolidol (**2**).
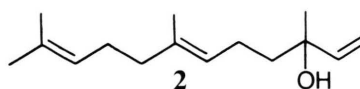
The presence of skatole was established unequivocally by gc-ms analysis and by mass spectral and retention time comparison with an authentic synthetic sample.

It is interesting to note that hexadecanoic acid was absent from extracts obtained from the excised pheromone producing glands of six *Kheper* species [14]. In these extracts a number of methyl and ethyl esters of long chain fatty acids constituted the major components. Furthermore skatole was present in considerable concentrations in these extracts whereas it appears to be present only in a minute concentration in the secretion produced by *Kheper lamarcki*.

In attempts to find a suitable solvent that could be used in experiments to determine the composition of the carrier material, the effect of various solvents on a small quantity of the material was observed under a microscope. For this purpose a few fragments of the carrier material was suspended in a droplet of one of the solvents on a clean microscope slide. However, none of the solvents, including various alcohols, ethers, ketones and esters, as well as pyridine, dimethyl sulphoxide and water, produced a positive test, *i.e.* the formation of an opaque surface on evaporation of the solvent. Although glycerol was not included in this investigation, the failure of attempts to obtain a mass spectrum of the material by fast atom bombard-

Table II. Assignment of resonances (δ_c in ppm from TMS) in the ^{13}C NMR spectrum of the second most abundant component in Fraction 1.

Carbon atom[a]	Chemical shift[b]
1	180.10
2	38.82
3	33.58
4	25.64
5	123.51
6	–
7	25.64
8	17.65
9	16.86

[a] Numbering as shown in Formula **1**.
[b] CDCl$_3$ with δ_c 77.02 ppm from TMS as internal reference.

ment ionization, can be attributed to the total insolubility of the carrier material in this solvent.

In an attempted melting point determination, the material slowly turned black at temperatures above 360 °C, without melting. Evidence that it contains sulphur was obtained by energy dispersive X-ray analysis, while ignition of a small sample of the material produced the characteristic smell of singed hair. As a working hypothesis it was therefore assumed that the carrier material may have a polypeptide structure. This assumption was confirmed by hydrolysis of the carrier material with constant boiling hydrochloric acid [15], followed by identification and quantitative determination of the amino acids in the hydrolysate as their heptafluorobutyryl-isobutyl ester derivatives [16]. A typical gas chromatographic separation of these derivatives on a capillary column coated with SE-30, is presented in Fig. 4.

As far as the major components are concerned, the sex attracting secretion of *K. lamarcki* therefore has the following composition:

Polypeptide carrier material	88.8%
Hexadecanoic acid	6.4%
2,6-Dimethyl-5-heptenoic acid	3.7%
(*E*)-Nerolidol	1.0%
Skatole	≪ 0.1%

A synthetic attractant containing the four volatile components in the ratio given above, was subjected to biological evaluation. As the nature of the carrier material was still unknown at that stage of the research, French chalk was chosen as an inert and insoluble support. In initial tests not a single dung beetle was lured into traps baited with the synthetic attractant alone, whereas large numbers of dung beetles of a variety of species were caught in the control traps baited with horse dung. As males only produce the secretion when they are in the possession of dung, this result is not totally unexpected. Apparently certain volatile components present in dung play an essential part in the attraction process. In further field tests the attractiveness of a combination of dung and synthetic attractant was therefore compared with that of dung in the control traps. Quite surprisingly, it was found that instead of increasing the numbers of dung beetles caught in the traps, the synthetic attractant actually decreased the attractiveness of dung significantly by approximately 50%. Furthermore, only male *K. lamarcki* were found in both the test and control traps.

The following tentative explanations can be put forward for these somewhat unexpected results. Firstly, the absence of female *K. lamarcki* from the traps can be explained in terms of the fact that the
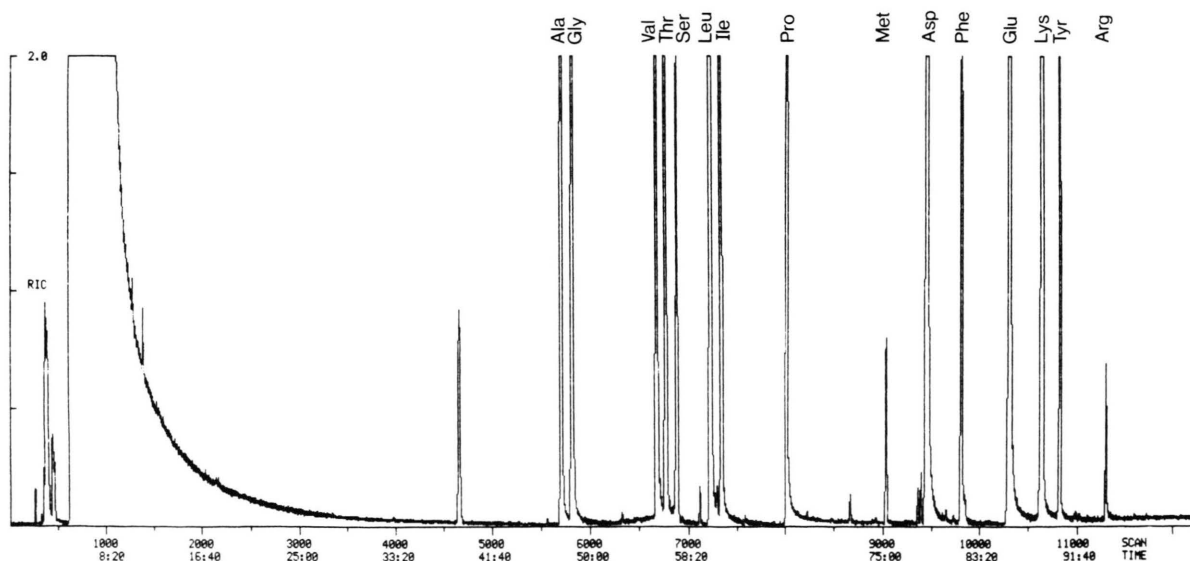

Fig. 4. Gc-ms analysis of the heptafluorobutyryl-isobutyl ester derivatives of the amino acids obtained by acid hydrolysis of the carrier material. Separation on SE-30 (glass, 40 m × 0.3 mm); programmed 40–230 °C at 2 °C/min.

field tests were carried out very late during the active season of this species when the females had already gone underground and were busy with brood caring. Secondly, the apparent deterrent effect of the synthetic material may be due to a confusingly high concentration of the synthetic material being released by the French chalk. The polypeptide employed by the dung beetle as carrier is expected to release the volatile components of the secretion in a more controlled manner. Furthermore, racemic synthetic samples of the unsaturated acid **1** and of (*E*)-nerolidol **2** were used in the formulation of the synthetic attractant, whereas only one of the enantiomers of each of these two compounds is expected to be produced by the insect. The presence of the other enantiomers in the synthetic formulation may have been responsible for the observed deterrent effect. As yet, due to the small quantity of the secretion available for structural elucidation, it had not been possible to establish the configurational identity of these two compounds in the secretion.

However, it is also possible that the function of the natural secretion is indeed to simultaneously attract females and deter males. Such a dual function may be essential for a male to elicit the assistance of a female in the construction and rolling of a dung ball and at the same time to retain possession of his dung or dung ball. Although it was impossible to identify and sex the thousands of dung beetles of other species found in the traps, it was, from the smaller numbers of beetles caught in traps baited with dung plus synthetic mixture, clear that the synthetic material also had a deterrent effect on other dung beetle species.

A gas chromatographic comparison of the secretions produced by other dung beetle species, the identification of the minor components present in these secretions and an investigation of the compounds responsible for the attraction of dung beetles to fresh dung are currently being undertaken.

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