*Journal of Chemical Ecology, Vol. 25, No. 9, 1999*

# MAMMALIAN EXOCRINE SECRETIONS. XIV: CONSTITUENTS OF PREORBITAL SECRETION OF STEENBOK, *Raphicerus campestris*

B. V. BURGER,* J. GREYLING, and H. S. C. SPIES

*Laboratory for Ecological Chemistry*
*Department of Chemistry*
*University of Stellenbosch*
*Stellenbosch 7600, South Africa*

(Received May 5, 1998; accepted May 4, 1999)

**Abstract**—In a study aimed primarily at qualitative comparison of the organic constituents of the preorbital secretion of the steenbok, *Raphicerus campestris*, with those previously found in the preorbital secretion of the grysbok, *R. melanotis*, 109 compounds were identified in the secretion of the steenbok. Although the secretions from the two antelope are similar in that they are mostly long-chain, unbranched, saturated and unsaturated alcohols and various derivatives of these alcohols, only 22 of the identified compounds are present in both secretions. This is a small percentage of the more than 260 compounds present in the secretion of the steenbok, which is much more complex than that of the grysbok.

**Key Words**—*Raphicerus campestris*, mammalian semiochemicals, mammalian pheromones, exocrine secretion, preorbital secretion, territorial marking.

## INTRODUCTION

Following identification of 34 constituents of the preorbital secretion of the grysbok, *Raphicerus melanotis*, in an early study of the chemical basis of the territorial marking behavior of this antelope (Le Roux, 1980; Burger et al., 1981a), behavioral tests and electrophysiological experiments were done. The secretions of conspecific animals as well as individual synthetic compounds were used in an attempt to find out whether some of the constituents have specific semiochemical functions. These experiments did not supply clear answers to any of the ques-

*To whom correspondence should be addressed.



0098-0331/99/0900-2099/$16.00/0 

tions that arose from observation of the animals' territorial behavior and from the results of the chemical characterization of the secretion (Le Roux, 1980). It is still not clear whether the small quantitative differences between male and female secretions have any semiochemical significance, especially because the quantitative differences between male and female secretions are almost exclusively observed in the very high molecular mass range. Although small quantitative differences were observed between the secretions of individual females, these differences probably have no semiochemical significance as far as the territorial behavior of the grysbok is concerned, because territorial marking with preorbital secretion has not been observed in female grysbok. Since information on the composition of the preorbital secretion of a related species could possibly shed light on the function of the preorbital secretions of these animals in their territorial behavior, an exploratory investigation of the qualitative composition of the preorbital secretion of the steenbok, *Raphicerus campestris*, was undertaken.

The genus *Raphicerus* comprises three species, the two mentioned above, and *R. sharpei* (Walker, 1968). The colloquial name of the steenbok is derived from Afrikaans for brick or stone, referring to the animal's even, rufous–brown to rufous–fawn color. The steenbok occurs on the African continent in two discrete areas, one in East Africa and the other in the southern parts of Africa with extensions of distribution into Angola and Zambia (Skinner and Smithers, 1990). Steenbok inhabit open grasslands, which must nevertheless provide some cover in the form of stands of tall grass, scattered bushes, or shrub. They do not occur in forests or thick woodland. Steenbok lead solitary lives except when a female has a lamb or when she is in estrus and is attended by a male. They are generally diurnal, but have some nocturnal activity, especially in areas where they are subject to disturbance (Skinner and Smithers, 1990).

Steenbok establish well-defined territories that both sexes will defend against trespassers, with established resting places, latrines, and preferred feeding places. Defense of these territories takes the form of displays rather than actual combat, and when fighting ensues between adults, it is only half-hearted and does not end in serious damage to the combatants. They have preorbital glands that show as dark marks just in front of the eyes, pedal glands between the hooves on the front and back feet, and a throat gland, all of which are presumed to be used for territorial marking (Skinner and Smithers, 1990).

## METHODS AND MATERIALS

The black viscous preorbital secretion was collected, the organic material extracted with dichloromethane, and the constituents identified by the procedures and instrumentation described in detail by Burger et al. (1996).

*Reference Compounds.* Some of the compounds present in the preorbital secretion are commercially available, while others were available from previous

research projects in this series. Certain compounds were synthesized during the present investigation according to published procedures.

Alkyl and alkenyl formates were synthesized by the uncatalyzed reaction of the respective long-chain alcohols with an excess of formic acid (Burger et al., 1996). The final products generally contained less than 1% (GC) of the unchanged alcohols.

Alkyl acetates were synthesized by the esterification of the appropriate long-chain alcohols with acetic anhydride in the presence of a catalytic quantity of perchloric acid (Ongley, 1973). The final products contained only traces (GC) of the unreacted starting materials.

Isopropyl hexadecanoate was prepared similarly by heating isopropyl alcohol (1.92 g, 32 mmol), hexadecanoic acid (10 g, 39 mmol), and perchloric acid (5 drops) at 100°C for 4 hr. The reaction mixture was cooled to room temperature, diluted with ether, and the resulting solution washed free of acid with water. Isolation of the reaction product gave pure (GC) isopropyl hexadecanoate (9.51 g, 99.74%). The product was not distilled.

Hydroxyesters were prepared by the $Al_2O_3$-catalyzed reaction of long-chain 1,2-epoxyalkanes with the appropriate carboxylic acids as described by Burger et al. (1999).

Cyclohexadecanone was prepared by two routes. In the first, cyclopentadecanone was used as starting material and the carbocyclic ring was expanded by one carbon atom (Taguchi et al., 1974a,b). The final product was obtained in an acceptable yield, but it contained the starting material (cyclopentadecanone) and cyclohexadecanone in a 1 : 1 ratio.

The second synthesis consisted of selective hydrogenation of the double bond of 8-cyclohexadecenone (Aldrich, Milwaukee, Wisconsin). A solution of 8-cyclohexadecenone (3.92 g, 17 mmol) in glacial acetic acid (50 ml) containing a catalytic quantity of Pt on activated charcoal (10%) was hydrogenated until the theoretical volume of hydrogen had been consumed. The reaction mixture was diluted with water, the catalyst filtered off, and the reaction product extracted from the filtrate with pentane (100 ml). Work-up procedures and Kugelrohr distillation gave cyclohexadecanone (3.66 g, 90.46%) containing 4.6% (GC) of impurities. $^{13}C$: $\delta(CDCl_3)$ 212,27 (s, C-1), 42.04 (2C, t, C-2 and 16), 26.54-27.68 (13C, t, C-3 to 15).

## RESULTS AND DISCUSSION

Male and female secretions were collected once a month for 13 months to find out whether seasonal qualitative and quantitative changes could be observed in the composition of the volatile organic fraction of their secretions. The secretions of the male and female were found to be qualitatively identical regardless of the reproductive state of the animals. The glands of both male and female

appeared to be slightly and possibly insignificantly more productive while the female was in estrus. Identified compounds are listed in Table 1, and a typical total ion chromatogram of an extract of the preorbital secretion of a male steenbok is shown in Figure 1.

The constituents of the preorbital secretion were tentatively identified by comparison of their mass spectra with those in NBS and Wiley spectra libraries and a library of the mass spectra of compounds previously identified in mammalian secretions.

Early in the investigation it became clear that the preorbital secretion of the steenbok was the most complex preorbital secretion so far analyzed by the Laboratory for Ecological Chemistry and that it would not be possible to identify all of the constituents by GC and GC-MS retention time comparisons. Identification of the alkanes, alkanols, alkanals, alkanoic acids, alkyl formates, and alkyl acetates presented no problems, as authentic synthetic samples of almost all of these compounds were available for retention time comparison. In previous studies (Mo et al., 1995; Burger et al., 1996) the position of double bonds in unsaturated constituents of relatively complex mixtures was determined by GC-MS analysis of the reaction products obtained by treating whole extracts of secretions with dimethyl disulfide (Buser et al., 1983; Vincenti et al., 1987). The preorbital secretion of the steenbok, however, contains such a large number of unsaturated and doubly unsaturated compounds, and the total ion chromatogram of the mixture of DMDS derivatives was so complex that this technique did not supply unequivocal information on the position of the double bonds in the unsaturated constituents. Retention time comparison with synthetic compounds was, therefore, the only means of identifying some of these unsaturated compounds.

The long-chain 2-alken-1-ols have typical mass spectra with the ion at $m/z$ 57 as the base peak. The configuration of the double bond was established by retention time comparison with two of these (*E*)-2-alken-1-ols. With a similar approach, a number of (*Z*)-6-alken-1-ols, four (*E*)-2-alken-1-yl methanoates, and two (*Z*)-6-alken-1-yl methanoates were identified in the secretion. The alcohols are not necessarily accompanied by their formic and acetic acid esters. These esters, therefore, do not appear to have been formed by simple esterification of the corresponding alcohols in the secretion.

The cycloalkanones present in the secretion have relatively prominent molecular ions, a base peak at $m/z$ 55 and an abundant rearrangement ion at $m/z$ 58. Cyclohexadecanone was synthesized as representative of this group of cyclic ketones for MS and retention-time comparison. The identification of three of the seven monounsaturated carboxylic acids present in the secretion as (*Z*)-9-alkenoic acids was confirmed by comparison with authentic synthetic material. With reasonable certainty the other two homologs can be assumed to be (*Z*)-9-dodecenoic acid and (*Z*)-9-tridecenoic acid.

It was relatively easy to characterize several long-chain hydroxyesters in

TABLE 1. CONSTITUENTS OF PREORBITAL SECRETION OF STEENBOK

| No. in Figure 1 | Compound | Remarks |
|---|---|---|
| 2 | Octane | *a,b* |
| 8 | Nonane | *a,b* |
| 10 | Decane | *a,b* |
| 16 | Dodecane | *a,b* |
| 6 | 5-Methyl-3-hexanol | *a,b* |
| 12 | 1-Octanol | *a,b* |
| 14 | 1-Nonanol | *a,b* |
| 18 | 1-Decanol | *a,b* |
| 23 | 1-Undecanol | *a,b,d* |
| 29 | 1-Dodecanol | *a,b,d* |
| 35 | 1-Tridecanol | *a,b,d* |
| 42 | 1-Tetradecanol | *a,b,d* |
| 50 | 1-Pentadecanol | *a,b,d* |
| 52 | 1-Hexadecanol | *a,b* |
| 70 | 1-Icosanol | *a,b* |
| 85 | 1-Tricosanol | *a* |
| 91 | 1-Tetracosanol | *a,b* |
| 97 | 1-Pentacosanol | *a,b* |
| 101 | 1-Hexacosanol | *a,b* |
| 105 | 1-Heptacosanol | *a,b* |
| 22 | (*E*)-2-Undecen-1-ol | *a* |
| 28 | (*E*)-2-Dodecen-1-ol | *a,b* |
| 34 | (*E*)-2-Tridecen-1-ol | *a* |
| 41 | (*E*)-2-Tetradecen-1-ol | *a,b* |
| 45 | (*E*)-2-Pentadecen-1-ol | *a* |
| 9 | (*Z*)-6-Hepten-1-ol | *a,c* |
| 17 | (*Z*)-6-Decen-1-ol | *a,c* |
| 21 | (*Z*)-6-Undecen-1-ol | *a,c* |
| 27 | (*Z*)-6-Dodecen-1-ol | *a,b,d* |
| 61 | (6*Z*,9*Z*)-6,9-Heptadecadien-1-ol | *a,c* |
| 1 | Hexanal | *a,b* |
| 7 | Heptanal | *a,b* |
| 13 | Nonanal | *a,b* |
| 19 | (2*Z*,4*Z*)-2,4-Decadienal | *a,b* |
| 20 | (2*E*,4*E*)-2,4-Decadienal | *a,b* |
| 53 | Cyclohexadecanone | *a,b* |
| 58 | Cycloheptadecanone | *a* |
| 62 | Cyclooctadecanone | *a* |
| 67 | Cyclononadecanone | *a* |
| 71 | Cycloicosanone | *a* |
| 77 | Cyclohenicosanone | *a* |
| 3 | Butanoic acid | *a,b* |
| 4 | 3-Methylbutanoic acid | *a,b* |
| 5 | 2-Methylbutanoic acid | *a,b* |
| 15 | Octanoic acid | *a,b* |

TABLE 1. CONTINUED

| No. in Figure 1 | Compound | Remarks |
|---|---|---|
| 24 | Decanoic acid | *a,b* |
| 36 | Dodecanoic acid | *a,b* |
| 47 | Tetradecanoic acid | *a,b,d* |
| 51 | Pentadecanoic acid | *a,b,d* |
| 57 | Hexadecanoic acid | *a,b,d* |
| 66 | Octadecanoic acid | *a,b,d* |
| 75 | Icosanoic acid | *a,b,d* |
| 33 | (*Z*)-9-Dodecenoic acid | *a,c* |
| 40 | (*Z*)-9-Tridecenoic acid | *a,c* |
| 46 | (*Z*)-9-Tetradecenoic acid | *a,b* |
| 56 | (*Z*)-9-Hexadecenoic acid | *a,b* |
| 64 | (*Z*)-9-Octadecenoic acid | *a,b* |
| 63 | (9Z,12Z)-9,12-Octadecadienoic acid | *a,b* |
| 26 | 1-Undecyl formate | *a,b,d* |
| 32 | 1-Dodecyl formate | *a,b,d* |
| 38 | 1-Tridecyl formate | *a,b,d* |
| 72 | 1-Icosyl formate | *a,b,d* |
| 78 | 1-Henicosyl formate | *a,b,d* |
| 81 | 1-Docosyl formate | *a,b,d* |
| 87 | 1-Tricosyl formate | *a,d* |
| 93 | 1-Tetracosyl formate | *a,b,d* |
| 99 | 1-Pentacosyl formate | *a,b,d* |
| 103 | 1-Hexacosyl formate | *a,b* |
| 106 | 1-Heptacosyl formate | *a,b* |
| 110 | 1-Octacosyl formate | *a,b* |
| 25 | (*E*)-2-Undecen-1-yl formate | *a,c* |
| 31 | (*E*)-2-Dodecen-1-yl formate | *a,b* |
| 37 | (*E*)-2-Tridecen-1-yl formate | *a,c* |
| 48 | (*E*)-2-Pentadecen-1-yl formate | *a,c* |
| 30 | (*Z*)-6-Dodecen-1-yl formate | *a,b* |
| 39 | (*Z*)-6-Tetradecen-1-yl formate | *a,c* |
| 44 | 1-Tridecyl acetate | *a,b* |
| 55 | 1-Pentadecyl acetate | *a,b* |
| 59 | 1-Hexadecyl acetate | *a,b* |
| 43 | Unidentified tridecen-1-yl acetate | *a* |
| 54 | Unidentified pentadecen-1-yl acetate | *a* |
| 68 | Unidentified nonadecen-1-yl acetate | *a* |
| 79 | Unidentified henicosen-1-yl acetate | *a* |
| 86 | Unidentified tricosen-1-yl acetate | *a* |
| 88 | Unidentified tetracosen-1-yl acetate | *a* |
| 49 | Isopropyl tetradecanoate | *a,b* |
| 60 | Isopropyl hexadecanoate | *a,b* |
| 69 | 2-Hydroxyheptadec-1-yl acetate | *a,b* |
| 74 | 1-Hydroxyoctadec-2-yl acetate | *a* |
| 76 | 2-Hydroxyoctadec-1-yl acetate | *a,d* |

TABLE 1. CONTINUED

| No. in Figure 1 | Compound | Remarks |
|---|---|---|
| 83 | 1-Hydroxyicos-2-yl acetate | *a,b* |
| 84 | 2-Hydroxyicos-1-yl acetate | *a,b,d* |
| 89 | 1-Hydroxyhenicos-2-yl acetate | *a* |
| 90 | 2-Hydroxyhenicos-1-yl acetate | *a* |
| 95 | 1-Hydroxydocos-2-yl acetate | *a* |
| 96 | 2-Hydroxydocos-1-yl acetate | *a* |
| 100 | 2-Hydroxytricos-1-yl acetate | *a* |
| 65 | 2-Hydroxytetradec-1-yl butyrate | *a,b* |
| 73 | 2-Hydroxyhexadec-1-yl butyrate | *a* |
| 80 | 1-Hydroxyoctadec-2-yl butyrate | *a,b* |
| 82 | 2-Hydroxyoctadec-1-yl butyrate | *a,b* |
| 92 | 1-Hydroxyicos-2-yl butyrate | *a,b* |
| 94 | 2-Hydroxyicos-1-yl butyrate | *a,b* |
| 102 | 1-Hydroxydocos-2-yl butyrate | *a* |
| 104 | 2-Hydroxydocos-1-yl butyrate | *a* |
| 11 | Limonene | *a,b* |
| 98 | Squalene | *a,b* |
| 107 | Cholesterol | *a,b* |
| 108 | α-Tocopherol | *a,b* |
| 109 | Unidentified steroid | *a* |

[a]Low-resolution mass spectrum.
[b]Retention time comparison.
[c]Position and configuration of double bonds, although not determined, are possibly as given.
[d]Also present in preorbital secretion of *Raphicerus melanotis*.

the secretion. The interpretation of the mass spectra of this compound type has been discussed by Burger et al. (1981b) and Le Roux (1980). The unbranched structures of the hydroxyesters were established by coinjection of the secretion and a number of authentic synthetic samples of representative compounds and by taking the retention time increments expected for the unbranched structures into consideration. It is possible that the hydroxyesters are formed by nucleophilic ring opening of long-chain oxiranes by ethanoic acid and butanoic acid. However, the difference in the ratios in which the 1-hydroxyalk-2-yl esters and 2-hydroxyalk-1-yl esters are formed cannot be explained in terms of such a route. 2-Hydroxyoctadec-1-yl and 1-hydroxyoctadec-2-yl butanoate are, for example, present in the secretion in a 1 : 1 ratio, whereas the 2-hydroxyicos-1-yl and 1-hydroxyicos-2-yl acetates are present in a ratio of 1 : 4. The stereochemistry of the hydroxyesters in this and several other mammalian exocrine secretions needs to be investigated further. The constituents of the secretion identified during this study are listed in Table 1.

In contrast to the preorbital secretion of the grysbok, *R. melanotis*, which has been almost fully characterized (Burger et al., 1996), the secretion of the

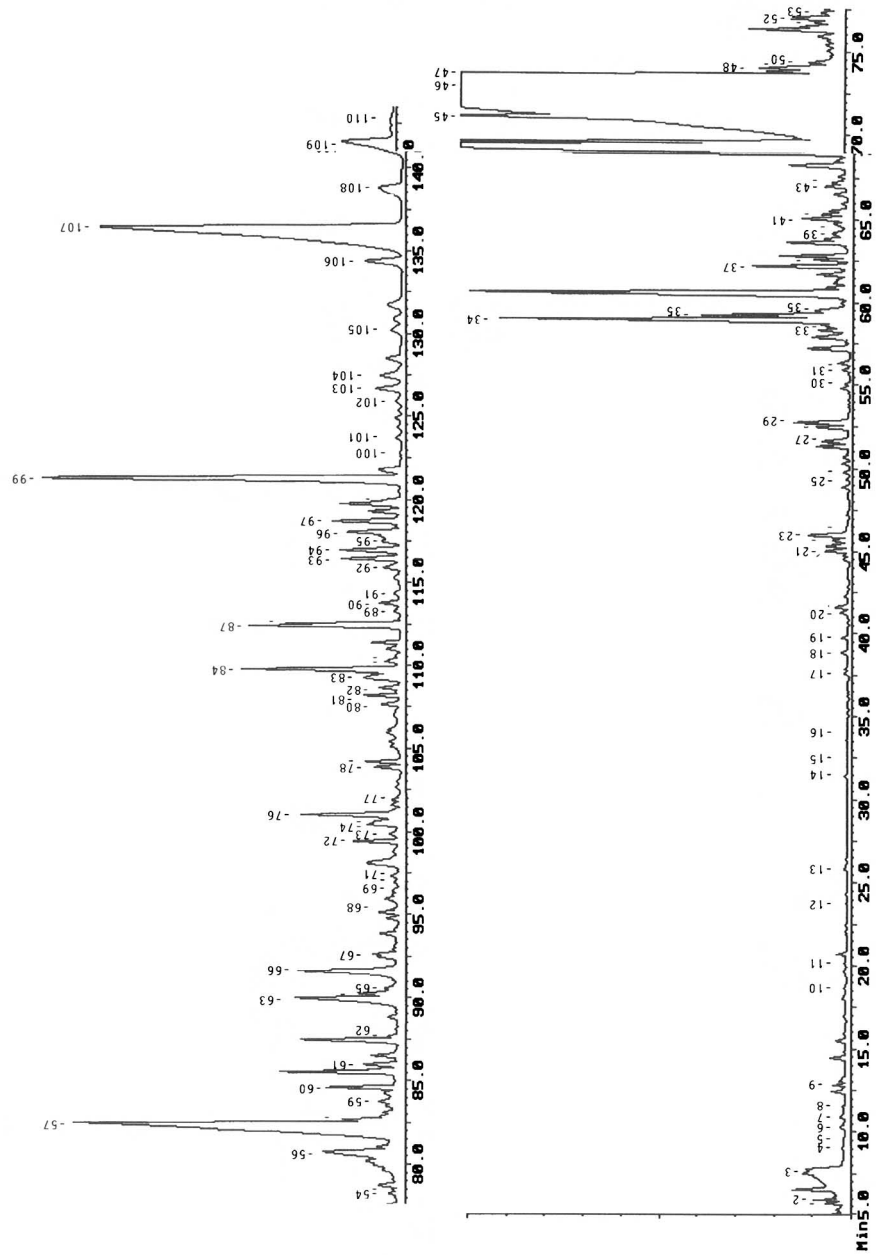

FIG. 1. Total ion chromatogram of an extract of the preorbital secretion of the steenbok. Glass capillary column coated with a 0.25-μm film of the apolar stationary phase PS-089-OH (95% dimethyl–5% diphenylsiloxane copolymer), programmed at 2°C/min from 40°C to 260°C (hold).

steenbok is much more complex. More than about 50% of an estimated 260 constituents remained unidentified in this study. This is largely due to the uninformative mass spectra of the long-chain compounds present in the secretion and the small concentrations in which they are present, which makes it impossible to isolate them for NMR and/or the determination of double bond positions. It is, however, clear that the unidentified compounds are of a similar long-chain unbranched type as those present in the grysbok and the steenbok secretions. Although the two secretions contain similar compounds, only 22 constituents are common to both secretions.

It is clearly not feasible to do field tests with individual constituents of the two secretions. However, the grysbok secretions contain certain lactones that are not present in the steenbok secretion. The steenbok secretion, on the other hand, contains a number of cyclic ketones not present in the grysbok secretion. There are also other differences as far as compound type is concerned. As a first approach to the semiochemical evaluation of the results presented here, field tests are planned in which the reaction of steenbok to their own secretion spiked with, for example, the lactones found in the grysbok secretion will be studied.

*Acknowledgments*—Support by the University of Stellenbosch and the Foundation for Research Development, Pretoria, of research reported in this paper is gratefully acknowledged. The authors are indebted to Mr. John Spence, Director of the Tygerberg Zoo, Cape Town, for permission to collect secretions from steenbok in the zoo.

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