Mammalian Pheromone Studies, VII*. Identification of Thiazole Derivatives in the Preorbital Gland Secretions of the Grey Duiker, *Sylvicapra grimmia,* and the Red Duiker, *Cephalophus natalensis*

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Z. Naturforsch. **43c,** 731–736 (1988); received March 28, 1988

Mammalian Pheromones, Semiochemicals, Mass Spectrometry, ^{13}C and ^{1}H NMR, Thiazole Derivatives

2-Isobutyl-1,3-thiazole and its 4,5-dihydro derivative were identified in the preorbital gland secretions of the grey duiker, *Sylvicapra grimmia*, and the red duiker, *Cephalophus natalensis*, but are absent from the preorbital secretion of the blue duiker, *C. monticola*. These two compounds which are present in high, but varying concentrations in the secretions of male grey duikers, are present in low concentrations in the secretions of females. This seems to be the only consistent significant difference between the secretions of male and female grey duikers and together with the fact that only males mark out their territories, was construed as evidence in favour of these two compounds playing a significant role in the territorial behaviour of male grey duikers.

Introduction

Two of the three duiker species found in South Africa, the blue duiker, *Cephalophus monticola,* and the red duiker, *C. natalensis*, inhabit forests in the eastern sub-tropical parts of the country, while the grey duiker, *Sylvicapra grimmia*, prefers the more exposed savanna bushveld and is normally not found in the dense indigenous forests [1]. All these duiker species are strongly territorial and mark their territories by smearing secretions from the preorbital glands on branches, trunks, rocks and other landmarks [1]. As part of a comprehensive study of the semiochemistry of the preorbital gland secretions of South African antelope species, a comparison of the composition of the secretions of these three duiker species was undertaken. One of the most significant observations in this regard is that, although the red and grey duikers belong to different genera, their preorbital secretions each contain two major constituents that are absent from the preorbital secretion of the blue duiker [2].

In addition to the possible taxonomic implications of this observation, the fact that these constituents were found to contain sulphur and nitrogen as hetero atoms, elicited special attention, since the constituents of the preorbital secretions studied so far are almost exclusively oxygenated hydrocarbons, the only exception being S-methyl phenylthioacetate, which is present in minute concentrations in the preorbital secretions of blue duikers [2]. In this communication the identification and synthesis of these two compounds are described.

Results and Discussion

According to GC and GC-MS-analyses carried out on crude, untreated preorbital gland secretions of the grey, red and blue duikers, as well as on extracts of these secretions, a major constituent present in the preorbital secretions of the first two species, is absent from material secreted by the blue duiker. The mass spectrum of this constituent (constituent 3208, Fig. 1) has a base peak at *m/z* 101 and other prominent peaks at *m/z* 55, 60, 73, 128, and 143. Chemical ionization mass spectrometry confirmed the *m/z* 143 ion to be the molecular ion of the compound. The presence of the abundant ion at *m/z* 101 suggested, as one possibility, a substituted dihydro-1,3-thiazole structure for the compound [3] and from the high-resolution data given in Table I, the substituent was presumed to be a butyl group. The for-

* For the preceding paper in this series see B. V. Burger and P. J. Pretorius, Z. Naturforsch. **42c,** 1355 (1983).

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/88/0900–0731 $ 01.30/0

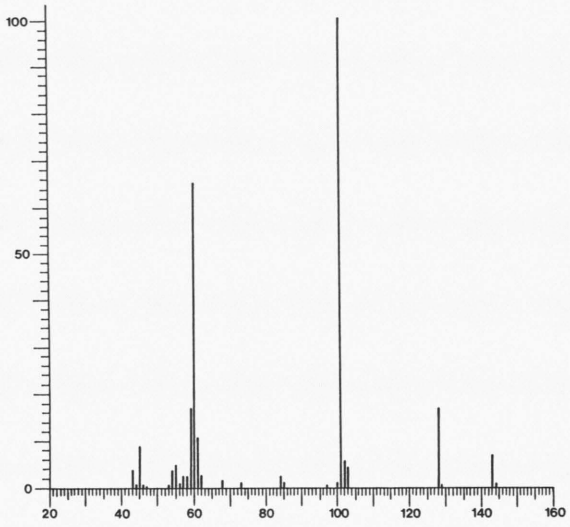
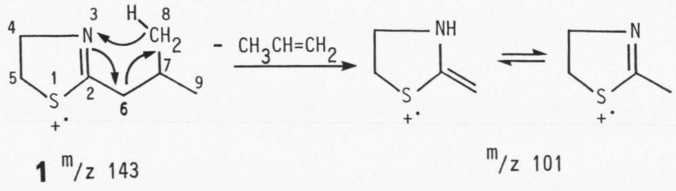


Fig. 1. Mass spectrum of constituent 3208 (2-isobutyl-4,5-dihydro-1,3-thiazole).

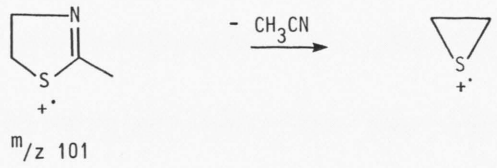
Table I. High-resolution MS data for the thiazole derivative **1**.

Measured mass *(m/z)*	Elemental composition of ion	Difference (millimass units)
60.0060	C_2H_4S	2.58
101.0311	C_4H_7NS	1.22
128.0533	$C_6H_{10}NS$	−0.11
143.0741	$C_7H_{13}NS$	−2.72

mation of the *m/z* 101 ion requires the loss of the elements of propene from the molecular ion with concomitant hydrogen transfer to, for example, the nitrogen atom of the dihydrothiazole ring. Assuming the substituent to be an isobutyl group in position 2 of a 4,5-dihydro-1,3-thiazole ring, this rearrangement can be formulated as follows:



The formation of the C_2H_4S ion at *m/z* 60 can be rationalized by invoking a cross-ring cleavage, in terms of which the fragmentation of similar heterocyclic systems has been interpreted [3–6].



With the substituent in positions 4 or 5 of the heterocyclic ring, the formation of a *m/z* 101 ion is still feasible, but the *m/z* 60 ion would then presumably be absent from the spectrum. Since the mass spectrum of the constituent under investigation does not contain information on which the substituent in position 2 could be unequivocally characterized, the most likely candidate, having an isobutyl group in this position, was synthesized from N-(2-hydroxyethyl)isovaleramide and phosphorus pentasulfide [7]. By mass spectral and gas chromatographic retention time comparison with this authentic material, constituent 3208 was finally identified as 2-isobutyl-4,5-dihydro-1,3-thiazole (**1**). The second constituent of interest (constituent 2652) has a mass spectrum (Fig. 2) which has abundant ions at *m/z* 141, 126, 99 and 58, *i.e.* at two mass units lower than the prominent ions in the spectrum of the dihydrothiazole **1**. The obvious conclusion, that this constituent could

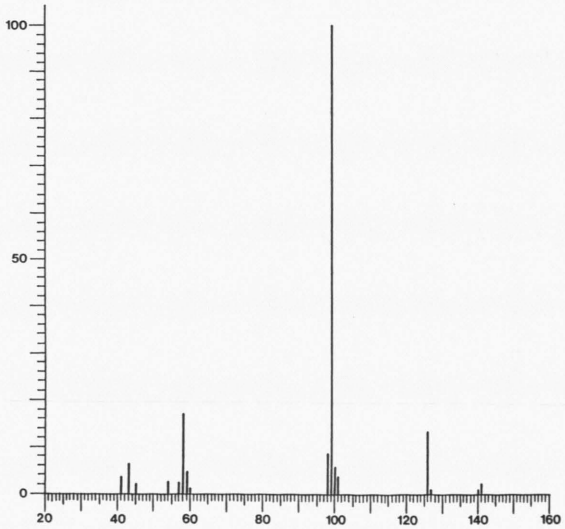
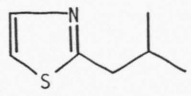


Fig. 2. Mass spectrum of constituent 2652 (2-isobutyl-1,3-thiazole).



2

be 2-isobutyl-1,3-thiazole (**2**), was confirmed by comparison of its mass spectral and gas chromatographic retention time data with those of authentic synthetic material, prepared according to the method of Dubs and Pesaro [8].

2-Isobutyl-1,3-thiazole (**2**) with a threshold to the human nose of 3.5 ppb [9] has first been identified as a natural flavour in the volatiles of fresh tomatoes by Viani *et al.* [10]. It has a strong green odour resembling that of tomato leaves [10] and is also responsible for the characteristic flavour of certain tomato varieties [11]. It has been synthesized by Dubs and Pesaro [8] by dehydrogenation of 2-isobutyl-2,5-dihydro-1,3-thiazole with chloranil, which gave better results than the dehydrogenating agents, such as iron(III)chloride and hydrogen peroxide, originally used for this purpose [12, 13]. To the best of our knowledge this compound or similar 2-substituted 1,3-thiazoles have not been found in insect or mammalian semiochemical secretions. Two dihydrothiazole derivatives, 2-isopropyl-4,5-dihydro-1,3-thiazole and 2-*s*-butyl-4,5-dihydro-1,3-thiazole, which are both closely related to the dihydrothiazole **1** were, however, identified as major constituents of the urine of male mice. They are either absent from the urine of females or present only in trace amounts [14] and their presence in male urine was shown to be androgen dependent [15, 16]. The urine of intact adult male mice elicits more investigatory sniffing from female mice than the urine of castrated males. Females exposed to castrate urine to which these two compounds have been added, displayed more frequent oestrus cycles [15]. Castrate urine reconstituted by the addition of the two dihydrothiazoles furthermore provoked male aggression which was quantitatively and qualitatively comparable to that elicited by intact male urine [17].

So far in the present investigation it has been impossible to reach a final conclusion as to the possible semiochemical function of these compounds. However, in spite of the problems involved in the collection of preorbital secretion from live duikers, the comparison of the quantitative composition of a small number of individual grey duikers did bring some interesting observations to light. The preorbital secretions of duikers and other antelope are heterogeneous suspensions, containing water, mucus, heavy waxes and small quantities of volatile organic material in varying proportions. Quantitation using an internal standard therefore gives inconsistent results. However, the secretions of both male and female red duikers contain hexadecanoic acid as a major constituent and this compound was therefore used as a quasi-internal standard by normalizing the quantitative data with respect to an arbitrary concentration of 100% for this compound. Captured duikers are extremely aggressive when handled and often die from stress. Quantitative data could therefore only be obtained from two males in captivity and all other data were obtained from culled animals. From the results presented in Table II it is nevertheless clear that the two thiazole derivatives **1** and **2** are present in high concentrations in the secretions of male animals. In the case of the male M2, for instance, these two compounds are by far the two most abundant constituents of the secretion. In the secre-

Table II. Concentration of 2-isobutyl-4,5-dihydro-1,3-thiazole (**1**) and 2-isobutyl-1,3-thiazole (**2**) in the preorbital gland secretions of male and female grey duikers normalized relative to an arbitrary concentration of 100% for the hexadecanoic acid present in the secretions.

Compounds	Relative concentration									
	Males			Females						
	M1[a]	M2[b]	M3[c]	F1[c]	F2[c]	F3[c]	F4[c]	F5[c]	F6[c]	F7[c]
1	81	151	52	5	2	4	7	2	9	8
2	29	106	28	3	1	1	7	1	3	5

[a] Material scooped from the glands of a captive male with a Reacti-Vial.
[b] Material deposited by a captive male on clean aluminium foil.
[c] Material scooped with Reacti-Vials from the glands of culled animals.

tions of females, on the other hand, they are present in low concentrations. It is interesting to note in this regard, that it is only the males that use their preorbital secretions for territorial marking. It furthermore seems unlikely that the variations in the concentration of these compounds are diet-related, since they are found in material collected from free ranging animals as well as animals fed on a diet of game pellets.

Although the concentration of other constituents of the secretion also vary in both male and female secretions, no consistent trend was observed and it is unlikely that these relatively small variations as well as the small differences between the concentrations of these constituents in male and female secretions could play more than a supporting semiochemical role.

Due to the risk of losing experimental animals when collecting material, a similar quantitative comparison of the secretions of individual animals was not undertaken at this relatively early stage in the research on the red duiker.

Materials and Methods

General

Dichloromethane (Merck, Residue Analysis Grade) was analyzed 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 preorbital secretions and extracts was heated to 500 °C in an annealing oven to remove traces of organic material. Syringes, stainless-steel needles, *etc.* that were used to handle material, were cleaned by rinsing with the dichloromethane specified above.

Gas chromatographic analyses were carried out with a Carlo Erba 5300 (Mega) gas chromatograph equipped with a flame ionization detector. Separation of the grey duiker preorbital volatiles was performed on a glass capillary column (40 m × 0.3 mm) coated with the OH-terminated apolar phase PS-089-OH (95% methyl, 5% phenyl) at a film thickness of 1.0 μm. A similar glass capillary column coated with 1.0 μm of cross-linked PS-255 (100% methyl) was used for analyses of the red duiker preorbital volatiles. Helium was used as carrier gas at a linear velocity of 28.6 cm sec^{-1} and both columns were temperature programmed at 2 °C min^{-1} from 40 °C to 250 °C. GC-MS analyses were carried out with a Finnigan MAT 4510 quadrupole mass spectrometer using the capillary columns and gas chromatographic parameters given above. High resolution data were obtained with a Varian MAT 311A mass spectrometer used in combination with a Kratos DS 90 data handling system. ^{1}H and ^{13}C NMR spectra were recorded with a Varian VXR 300/NMR spectrometer, using $CDCl_3$ as solvent.

Collection of material and sample preparation

Preorbital gland secretion was collected individually from netted or culled sexually mature male and female animals by scooping the exudate from the row of pores, through which the secretion of the underlying preorbital gland is released, with Reacti-Vials (Pierce Chemical Co., Rockford, Ill., U.S.A.). To avoid the handling of the very sensitive duikers, material was also collected from territorial marks, freshly deposited on pre-cleaned aluminium foil. The organic volatiles were extracted by vigorously shaking each collected sample (0.4–1.0 g) with dichloromethane (0.5–1.2 ml) in a mechanical flask shaker for 6 h, centrifuging the resulting emulsion for 2 h at 3000 r.p.m., removing the dichloromethane extract from underneath the supernatant water and mucus layer with a 1000-μl syringe and transferring it to a clean Reacti-Vial. These extracts were further concentrated in an inert, purified (activated charcoal) nitrogen atmosphere for GC-MS analysis. For quantitative comparison of the secretions of individual grey duikers, these extracts were diluted with dichloromethane to approximately the original volume of the collected material. Thus, an extract was diluted to, for instance, 430 μl if 430 mg of material had initially been collected from an animal. The extracts obtained by this procedure were used without further concentration to obtain quantitative gas chromatographic data. The extracts of the red duiker preorbital secretions which were obtained in this manner, contained large amounts of heavy waxy material and for GC-MS analysis these extracts were therefore subjected to preliminary preparative gas chromatographic purification. A Perkin Elmer 900 gas chromatograph, equipped with an effluent splitter and flame ionization detector, was used for preparative sample clean-up. Glass wool packed loosely in the injector liner of the instrument, served as support to retain the heavy oily residue in the injector and prevented this material from contaminating the metal parts of the injector or the column. Separation

was performed on a glass column (1.0 m × 9 mm) packed with 5% SE-30 on 60–80 mesh Chromosorb WAW-DMCS with hydrogen as carrier gas (35 ml min^{-1}) and temperature programmed at 12 °C min^{-1} from 40 °C to 240 °C, injector 200 °C, detector manifold 250 °C. The extract (150 µl) of the preorbital gland secretion of one red duiker was injected in a single batch and the effluent organic material was collected up to a retention time corresponding to that of octadecanoic acid. The effluent organic material was precipitated in a stainless-steel needle (Luer, Inox No. 18, 250 × 0.8 mm ID) by employing alternate cold (−50 °C) and hot (150 °C) zones along the middle 70 mm section of the needle. The collected material was rinsed from the needle into a Reacti-Vial with 100 µl of dichloromethane and the resulting solution concentrated to 50 µl in a purified nitrogen atmosphere.

Synthesis

2-Isobutyl-4,5-dihydro-1,3-thiazole (**1**)

The title compound was synthesized according to the procedure described by Wenker [7] for the preparation of 2-methyl-4,5-dihydro-1,3-thiazole. A mixture of isovaleric acid (1.2 g, 100 mmol) and 2-aminoethanol (6.1 g, 100 mmol) was heated in an open flask until the mass of the water lost from the flask corresponded to 100 mmol. The reaction producing the amide, N-(2-hydroxyethyl)isovaleramide, set in at 148 °C and was complete at 175 °C. The reaction product was used without further purification in the next step of the preparation of **1**.

Phosphorus pentasulfide (8.9 g, 20 mmol) and the N-(2-hydroxyethyl)isovaleramide prepared above (14.5 g, 100 mmol), were mixed in a distilling flask fitted to an efficient cooler. Only a slight warming of the reaction mixture was observed, in contrast to the strongly exothermic reaction reported by Wenker in the preparation of the 2-methyl derivative. The reaction mixture was therefore heated in an oil bath at 180 °C, at which temperature the reaction took place and the required reaction product distilled from the flask at reduced pressure (110 Torr). The crude product was dried on KOH and distilled to give the pure 2-isobutyl-4,5-dihydro-1,3-thiazole (**1**) in a yield of 28%, b.p. 79 °C/10 Torr (lit. [18], b.p. 70–73 °C/10 Torr); ^{1}H NMR ($CDCl_3$): δ 0.94 (d, 6H), 1.90–2.17 (m, 1H), 2.40 (d, 2H), 3.28 (t, 2H), 4.22 (t, 2H). ^{13}C NMR ($CDCl_3$): δ 22.32 (q, C-8 + C-9), 27.52 (d, C-7), 33.65 (t, C-5), 43.16 (t, C-6), 64.14 (t, C-4), 171.29 (s, C-2).

2-Isobutyl-1,3-thiazole (**2**)

2-Isobutyl-2,5-dihydro-1,3-thiazole, prepared from mercaptoacetaldehyde dimer, isobutyraldehyde and gaseous ammonia, was dehydrogenated to 2-isobutyl-1,3-thiazole (**2**) according to the procedure described by Dubs and Pesaro [8]. The reaction product was distilled to give the title compound **2** in a yield of 59%, b.p. 60–62 °C/14 Torr (lit. [8], b.p. 66 °C/10 Torr). ^{1}H NMR ($CDCl_3$): δ 0.98 (d, 6H), 2.02–2.21 (m, 1H), 2.88 (d, 2H), 7.16 (d, 1H), 7.66 (d, 1H). ^{13}C NMR ($CDCl_3$): δ 22.04 (q, C-8 + C-9), 29.52 (d, C-7), 41.96 (t, C-6), 117.71 (d, C-5), 142.00 (d, C-4), 169.80 (s, C-2).

Acknowledgements

Support by the University of Stellenbosch and the Foundation for Research Development, Pretoria, of the research reported in this paper is gratefully acknowledged. The authors are indebted to the Natal Parks, Game and Fish Preservation Board for a gift of red duikers for the research and to Mr. J. Spence of the Tygerberg Zoo and Prof. R. C. Bigalke of the Department of Nature Conservation of the University of Stellenbosch for assistance with the rearing of animals and the collection of preorbital secretion.

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