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0014-4754/84/020215-03$1.50 + 0.20/0

Identification of a testosterone-dependent unique volatile constituent of male mouse urine: 7-*exo*-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]-3-octene

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Summary. Investigations regarding the chemical composition of the volatiles in male mouse urine have recently enabled the structural elucidation of a hitherto unreported urinary component, 7-*exo*-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]-3-octene. This compound's uniqueness to mouse urine and its dependence on testosterone levels in the male suggest its probable role as a mouse pheromone or pheromone adjuvant.

Chemical messengers appear to play an important role in the overall social behavior and reproductive function of *Mus musculus*, the common house mouse[4-7]. Although the mouse has a variety of known and potential sources of chemical messengers, such as various glands, urine has been the most extensively studied source of primer pheromones and other intraspecific chemical signals. Estrus synchronization[8,9], puberty acceleration[10], pregnancy block[11-13], group and individual recognitions[7], sexual attraction[4,11,15], aggression[4,5,16], fear and stress[17,18], histocompatibility-related mating preference[19], etc., now appear to be well documented and traced to urine as a source.

Production of the primer pheromones in the male mouse is under androgen control. Because of certain similarities in the 3 primer effects, i.e., estrus synchronization, puberty acceleration, and the pregnancy block, Bronson[4] suggested that they could be mediated by the same substance(s). However, some disagreement about volatility of these messengers exists. Transport of estrus-synchronizing activity (Whitten effect) through a wind tunnel[20] and detection of the pregnancy-blocking activity (Bruce effect) in a condensed urine vapor by Hoppe[21] support the thesis of pheromone's volatility, whereas Marchlewska-Koj[22] associates both types of activity with larger (non-volatile) urinary constituents. Vandenbergh et al.[23,24] also demonstrated that the puberty-accelerating principle was due to a non-volatile fraction of the male mouse urine.

Studies in our laboratory[25] seem to indicate that the results of the above investigators may not be as contradictory as they appear to some. Further separation and chemical characterization[25] of the fraction responsible for puberty acceleration in female mice revealed that a) the active fraction contains non-volatile peptides, as suggested by Vandenbergh et al.[23]; and, b) it is associated with smaller molecules and retains the 'mousy' odor. Thus, the possibility of a volatile pheromone associating with a larger non-volatile adjuvant molecule needs to be considered.

While investigating volatile urinary profiles of the male mice, we have identified structurally a hitherto unreported constituent, 7-*exo*-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]-3-octene. This compound is unique to mouse urine[26] and is contained in the fraction active for puberty acceleration[25]; it displays a strong dependence on testosterone levels in the male mouse as shown in figure 1. The properties suggest a probable role as a pheromone or pheromone adjuvant.

The structure shown in figure 2 was deduced from chromatographic studies and 3 sources of spectral data and verified by synthesis.

Male mice, 1-6 months old, of BALB/cWT strain were maintained on a standard diet. Urine collections were performed with groups of 4-5 animals at the same time, using plastic metabolism cages. The urine collection vessels were cooled by dry ice, so that the specimens were immediately frozen. Collections from the same animals were obtained before and after castration, and for 20 days following the testosterone implantation (4 mm plastic tubes containing the hormone[27] grafted under the back skin).

Spectral information

Technique	Data
GCMS (quadrupole)	Integral mass (relative intensity)
	154 (12), 136 (8), 125 (26), 121 (8),
	111 (52), 97 (21), 96 (29), 95 (48), 83 (26),
	81 (23), 57 (32), 43 (100), 41 (22)
GC-HRMS	Exact mass (elemental composition)
(double focusing)	154.0986 ($C_9H_{14}O_2$)
	136.0914 ($C_9H_{12}O$)
	121.0685 (C_8H_9O)
	111.0817 ($C_7H_{11}O$)
	97.0620 (C_6H_9O)
	96.0537 (C_6H_8O)
	95.0466 (C_6H_7O)
	83.0473 (C_5H_7O)
	81.0322 (C_5H_5O)
GC-FTIR (vapor phase)	Major IR band frequency
	3051 cm^{-1}
	2970
	1454
	1394
	1254
	1196
	1045
	1026
	972
	860

1-ml aliquots of urine samples were purged at room temperature with purified helium gas onto a trap containing a small amount of porous polymer; the main methodological aspects of this sampling and concentration technique were previously described[28]. Capillary gas chromatography, using a 50 m × 0.25 mm i.d. glass column coated with a polypropylene glycol stationary phase, was used to resolve complex volatile fractions of mouse urine desorbed form the porous polymer preconcentrator. The sample complexity and overall difference in the urinary volatile profiles of intact, castrated and testosterone-treated males were shown in a previous report[25].

Presence of the compound with mol.wt 154 was ascertained through combined gas chromatography/mass spectrometry. Its relative concentrations in the urine of normal and castrated animals are indicated in figure 1 together with the values measured for 20 days following the hormone implantation (average of 5 individual measurements). While castration substantially decreases the concentration of this compound, testosterone recovers it to the normal value, indicating that the testes are not the site of biosynthesis.

Positive identification of the substance has been made using a combination of sensitive analytical techniques. Presence of either sulfur or nitrogen atoms in the molecule was ruled out from the lack of response to selective gas-chromatographic ionization detectors. A combined gas chromatograph/quadrupole mass spectrometer provided medium-resolution spectral data which allowed the fragmentation pattern to be scrutinized. A nominal mol.wt of 154 was indicated, but the fragmentation pattern appeared quite uncharacteristic.

When the glass capillary column was attached to a high-resolution mass spectrometer[29], the exact mass measurement yielded the value of 154.0986, corresponding to the elemental composition of $C_9H_{14}O_2$. The use of capillary gas chromatography/Fourier-transform IR spectroscopy added the information that neither oxygen atom was a part of a carbonyl or a hydroxyl group, while rendering unlikely the presence of an epoxide or vinylic ether moiety[30]. Similarities to the published spectra of ketals were noted. The spectral information from all 3 ancillary techniques is summarized in the table; their combination suggested the urinary compound to be 7-*exo*-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]-3-octene. It was then realized that the proposed structure was identical, except for the double bond, to that of the pine bark beetle pheromone *exo*-brevicomin, identified by Silverstein et al.[31]. Thus, the compound could also be named *exo*-3,4-dehydrobrevicomin.

Subsequently, a synthetic effort was undertaken to verify the above structural hypothesis. The synthesis (fig. 2) was patterned after a published work[32] on *exo*-brevicomin. Briefly, the epoxide from the tosylate of cis-3-hexen-1-ol was converted to the corresponding epoxy iodide, which was used to alkylate the enolate from phenylsulfenylacetone in the manner described by Coates et al.[33]. The main product of this alkylation was not the expected phenylsulfenyl epoxy ketone, but a dihydropyran resulting from base-promoted cyclization. This was converted by boron trifluoride etherate[34] into the corresponding phenylsulfenyl ketal, having the desired carbon-oxygen skeleton. The double bond was formed by thermolysis of the sulfoxide made by controlled oxidation of the phenylsulfenyl ketal.

The final product yields a single GC peak, suggesting that *endo* and *exo* diastereomers are not both present. The use

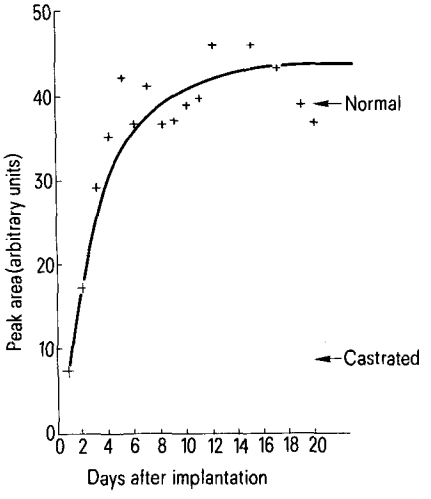

Figure 1. Testosterone dependence of *exo*-3,4-dehydro-brevicomin in male mouse urine. Castrated males were implanted with testosterone pellets and the volatile urinary components monitored by capillary GC (described in text).

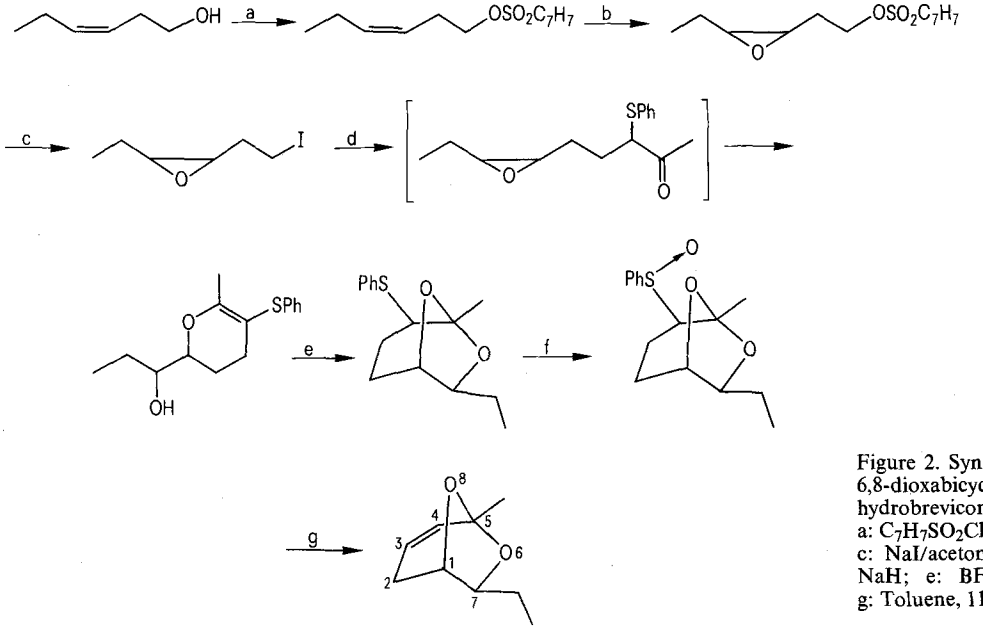

Figure 2. Synthesis of 7-*exo*-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]-3-octene (*exo*-3,4-dehydrobrevicomin).
a: $C_7H_7SO_2Cl$, pyridine; b: *m*-$ClC_6H_4CO_3H$; c: NaI/acetone; d: $PhSCH_2COCH_3$, excess NaH; e: $BF_3 \cdot Et_2O$; f: *m*-$ClC_6H_4CO_3H$; g: Toluene, 110°.

of boron trifluoride etherate in the ketalization step is expected to favor the *exo*-isomer[34]; indeed, hydrogenation of the synthetic material yielded a product identical in mass spectrum and GC retention time with known *exo*-brevicomin[32]. No attempt at resolution of the enantiomers of the synthetic material has been made.

The mass spectra and IR spectra of the purified final product and the urinary constituent show excellent agreement. Importantly, the synthetic compound co-elutes with the urinary constituent in capillary gas chromatograms; with the high resolving power of capillary columns it is nearly impossible that some related isomer with an identical spectrum would appear with precisely the same retention time.

Synthesis on a larger scale is currently being pursued to prepare *exo*-3,4-dehydrobrevicomin in the amounts sufficient for testing the primer pheromone activity as well as various behavioral effects in mice. These biological evaluations could be a long-term undertaking due to the frequently acknowledged complexities of mammalian olfactory communication. It is, however, of some interest that 2 additional ketals (multistriatin and frontalin), structurally related to *exo*-brevicomin, were previously identified[31] as insect pheromones. The structural similarity of *exo*-3,4-dehydrobrevicomin to compounds of demonstrated pheromonal activity has encouraged our efforts to pursue the biological testing of this compound. Our recent results of behavioral testing indicate that the compound is one of the aggression-promoting principles of male mice.

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30 We are grateful to Dr A. Fehl and the Procter and Gamble Company, Cincinnati, Ohio, for allowing us to use their capillary GC/FTIR spectrometer.
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35 We are grateful to Dr R.M. Silverstein, College of Environmental Sciences and Forestry, State University of New York, Syracuse, New York, for the generous gift of a quantity of *exo*-brevicomin.

0014-4754/84/020217-03$1.50 + 0.20/0

Identification of the nerve bundle in the tractus olfactorius of the tench, *Tinca tinca* L., which conducts the nervous excitation elicited by the alarm substance

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Summary. In a total of 29 tench (*Tinca tinca* L., Cyprinidae, Ostariophysi, Pisces) we operatively impaired either 1 or 2 of the 3 nerve bundles of both tractus olfactorii. Through behavioral experiments, using the fright reaction as a test, we found out which nerve bundle of the tractus olfactorius conducts the excitation elicited by the alarm substance. According to the results the conducting bundles are exclusively the lateral bundles in both tractus olfactorii.

Each of the 2 tractus olfactorii in the tench (*Tinca tinca* L.) consists of several nerve bundles, as it does in many species of fish studied[1]. In the tench each tractus olfactorius consists of 3 bundles, one lying laterally, one centrally, and one medially[2]. The action potentials of each tractus olfactorius are in accordance with this structure[3-5]. By means of