Characterization of urinary volatiles in Swiss male mice (*Mus musculus*): bioassay of identified compounds

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The present study was carried out to investigate the chemical nature of the urine of male mice and to assess its bioactivity. Urine of mature male mice was extracted with dichloromethane (1 : 1 ratio v/v) and analysed by gas-chromatography linked mass-spectrometry (GC-MS). Ten different compounds such as alkanes, alcohols, etc. were detected in the urine. Among the ten, five compounds are specific to males, namely 3-cyclohexene-1-methanol (I), 3-amino-s-triazole (II), 4-ethyl phenol (III), 3-ethyl-2,7-dimethyl octane (IV) and 1-iodoundecane (V). The compound, 4-ethylphenol, has been previously reported in several strains of male mice. Furthermore, the compounds (II) and (IV) are similar to 2-*sec*-butylthiazole and dehydro-exo-brevicomin compounds which have already been reported in male mice. Bioassay revealed that compounds (II), (III) and (IV) were responsible for attracting females and in inducing aggression towards males, as compared to the other compounds, i.e. (I) and (V). The results indicate that these three volatiles (II, III and IV) of male mice appear to act as attractants of the opposite sex.

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1. Introduction

Pheromonal communication plays an important role in mammalian reproductive and social behaviour including sexual attraction, mounting (Rekwot *et al* 2001; Kannan and Archunan 2001a), territorial marking (Balakrishnan and Alexander 1985; Prakash *et al* 1998) mother-young interactions (Leon 1983), and individual identification (Poddar-Sarkar and Brahmachary 1999). Urine is known to be a major source of mammalian chemosignals involved in this pheromonal communication. For example, male mouse urine is reported to have remarkable primer pheromonal effect on female mice (Dominic 1991; Brennan 1999). Exposure of female mice to male mice urine results in induction of estrus in grouped-house mice (Whitten 1956), acceleration of puberty (Vandenbergh 1967) and pregnancy block in newly inseminated mice (Bruce 1959). In mammals, the two primary sites of reception for pheromonal chemical signals are the main olfactory system and vomeronasal system. And, based on the physiology, the recipients use these specific olfactory pathways in the perception of chemical signals (Ben-Ari 1998).

It is well known that urine odours of rodents contain information about the species, sex, sexual state (estrus), dominance status, maternal state and individual identity of each animal (Brown and Schellinck 1992). Different types of information in the urinary chemosignals may have different basis. Species, sex and individual odours, for example, are likely to have a genetic basis, while odours of dominance status, sexual and maternal state depend upon hormonal changes (Brown *et al* 1996). Information about diet is also available in guinea pigs (Beauchamp 1976) and mice (Schellinck *et al* 1992), a

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Abbreviations used: DCM, Dichloromethane; GC-MS, gas-chromatography linked mass-spectrometry; MUP, major urinary protein.

change in diet can alter urine odours. Urinary steroid metabolites have been quantitatively altered during the starvation and re-feeding experiments for mice with different environmental background (Alasandro *et al* 1984). The natural circadian rhythm has also been reported to affect the release of pheromonal signals in mice (Marchlewska-Koj and Kruczek 1986). Urinary pheromones from a male of a different strain block pregnancy in newly inseminated female mice (Brennan 1999). Some of these factors, such as genetic difference, are constant over time. Odours related to bacteria and hormonal levels may also be similarly consistent, but other cues such as those provided by diet may change over time. It has been hypothesized that the constant cues provide an odour of individuality (Brown 1979; Halpin 1986, 1991). In mice and rats, the major histocompatability complex (MHC) is recognized as a genetic locus, which is important in determining the individuality of unique odours (Brown *et al* 1990; Beauchamp *et al* 1990).

Several substances have been identified in the urine of adult male mice, which are dependent upon high levels of testosterone (Novotny *et al* 1984). Some of these substances are volatiles such as 2-*sec*-butylthiazole (Liebich *et al* 1977), 2,3-dehydro-exo-brevicomin and 4-ethyl phenol (Novotny *et al* 1985a). The male originated 2-*sec*-butylthiazole and dehydro-exo-brevicomin were found to be involved in eliciting inter-male aggression (Novotny *et al* 1985a) and attractiveness to females (Jemiolo *et al* 1985). The compound 2,5-dimethyl pyrazine delays sexual maturation in young female mice and is a characteristic urinary volatile compound of grouped adult females (Drickamer and McIntosh 1980; Novotny *et al* 1985b). The puberty-accelerating pheromone in male mice urine is a protein or protein related substance (Marchlewska-Koj 1977; Mucignat-Caretta *et al* 1995; Novotny *et al* 1999). In contrast, Nishimura *et al* (1989) identified the testosterone dependent isobutyl and isoamyl amines as puberty accelerating pheromones in the urine of male mice.

Despite extensive research, it is still unclear what exactly is the precise chemical nature of the olfactory signals that can influence behaviour. In the present report GC-MS analysis on male mice urine was carried out to identify chemical odorants, and then behavioural experiments were undertaken to determine the effects of these chemicals on behaviour. Findings of the present study were compared with earlier reports.

2. Materials and methods

Male and female mice of the Swiss strain (fifteen regularly cycling virgin females [24 ± 03·6 g (± SD)] which were 8–10 weeks old and twenty intact males [27 ± 06·0 g (± SD)] which were 10–12 weeks old having scrotal testes) were housed separately in polyprophylene cages (40 × 25 × 15 cm), with 2 cm of rice husk lining the bottom as bedding material. The bedding material was changed before every odour preference test.

2.1 *Sample collection*

Both sexes of mice used in the present investigation were housed under laboratory conditions and reared on pelleted food (Hindustan Lever Ltd., Bangalore) and water *ad libitum*. Fifteen adult male mice were used as donors; the urine was collected by gentle abdominal massages while the mouse was held over a watch glass. The samples were pooled and screened through cheese cloth or nylon mesh (16–120 μm) at the time of collection. Immediately after screening, the samples were stored at – 20°C and analysed within a week.

2.2 *Solvent selection*

In a preliminary study nine organic solvents, viz. n-hexane, acetone, methanol, ethanol, petroleum ether, diethyl ether, chloroform, dichloromethane and benzene were used to extract the compounds from pooled urine. The maximum response was obtained when using dichloromethane (DCM) for extraction. So DCM was used as the solvent in the present study. The supernatant was filtered through a silica gel column (60–120 mesh) and concentrated under vacuum (at < 30°C) for fractionation and chemical identification by gas chromatography-mass spectrometry.

2.3 *Gas-chromatography linked mass-spectrometry analysis*

The gas-chromatography linked mass-spectrometry (GC-MS) analyses were done using a Shimatdzu QP-500 (Japan) under computer control at 70 eV. Chemical ionization was performed using ammonia as the reagent gas at 95 eV (Ramesh Kumar *et al* 2000). The identified compounds were then compared with the standard run under the same conditions. These data were already stored in a compact library of chemical substances (NIST 6221B).

2.4 *Separation of compounds by column chromatography*

As per the results obtained by GC-MS analysis, the identified urinary compounds were separated by column chromatography (Kannan and Archunan 2001a). For this, fresh urine (5 ml) was used with the solvent DCM at a 1 : 1 ratio. The supernatant was discarded and the remaining compound mixture was used for separating the compounds on the basis of retention time shown in

GC-MS analysis. The samples were distilled for 30 min at room temperature under a vacuum of 0·2 torr. The distillate was reduced and condensed to one fifth of the original volume by cooling with nitrogen. The volatiles from the distilled fractions were subjected to gas chromatography for cross checking and confirmation.

2.5 *Odour preference test*

The test animals (male and estrus female), i.e. the responders, were divided into two different sets. Four individuals randomly taken from a pool of 50 mice (male and female colonies maintained separately) were used in each set of behaviour analysis, and the experiment for each set was repeated thrice on different days. Fresh purified samples (compounds) were used for each trial. The behaviour study was carried out using a Y-maze apparatus made of tin sheet; the lateral sides were closed with glass plates and the top portion covered with wire mesh. This apparatus had the facility to provide food and water *ad libitum*. The Y-maze apparatus contains three arms: in the middle common arm, the responder was released into the apparatus; the purified compound(s) was placed in the experimental arm; and pure solvent (DCM; control) was placed in the control arm. The size of the middle arm is about 80 cm long and 15 cm wide. The remaining two choice arms are each 75 cm long. The frequency of the visits made by each responder towards the urine sample and solvent was recorded; responders were of the same and opposite sex. The odour preference test was assessed for 5 min with identified compounds (experimental) and the solvent (control).

The data were analysed using factorial design with the variable (number of visits) and two factors namely compound and sex (bi-variate ANOVA; Zar 1984). A post-hoc Duncan test was carried out to compare the behaviour of males and females in response to exposure to the different compounds.

3. Results

The extract prepared from the urine of male Swiss mice contained more than 40 different fractions, of which, the following ten chemical substances constituted the major portion of the urine extract. These were 3-cyclohexene-1-methanol (1), 3-amino-s-triazole (2), 4-ethyl phenol (3), 3-ethyl-2-methyl hexane (4), 2,7-dimethyl octane (5), undecane (6), 1-iodoundecane (7), 3-methyl undecane (8), 2-bromo-6-methyl heptane (9), 3-ethyl-2,7-dimethyl octane (10) (table 1). Among the ten, five compounds are specific to male, namely 3-cyclohexene-1-methanol (I), 3-amino triazole (II), 4-ethyl phenol (III), 3-ethyl-2,7-dimethyl octane (IV) and 1-iodoundecane (V), and these are not present in the urine of castrated males, adult females and prepubertals (unpublished data). The gas-chromatography analysis clearly showed that the major compounds fall between the retention time of 5–30 min (figure 1). Figures 2 and 3 show the mass spectrum and the chemical structure of the identified compounds respectively. The computer-matched data of the identified compounds showed above 95% similarity with the compound identified from the urine extracts.

Male and female mice differed in their responses as indicated by a significant main effect of sex [F (1, 50) = 32·061, $P < 0·001$; table 2]. In contrast to the males (2·46), females (4·00) showed more preference towards the identified compounds (t, 58 = 3·252, $P < 0·05$; table 2). The average number of visits does not differ significantly between the compounds [F (4, 50) = 2·098, $P > 0·05$; table 2]. The interactions between the compound and sex were significant [F (4, 50) = 29·371, $P < 0·001$; table 2]. The male mice exhibited significant difference between different compounds [F (4, 25) = 9·434, $P < 0·001$; table 2]. Male mice more frequently visited the compound I, 3-cyclohexene-1-methanol than all the other compounds. Post-hoc analysis also revealed that male mice preferred compound I, as compared to other compounds (refer Duncan test in table 2). Similarly, the

Table 1. List of compounds identified in male mice urine.

P. No	Retention time (min)	Mol. wt	Molecular formula	Compound name
1	7·017	112	$C_7H_{12}O$	3-cyclohexene-1-methanol
2	8·133	84	$C_2H_4N_4$	3-amino-s-triazole
3	11·067	122	$C_8H_{10}O$	4-ethyl phenol
4	16·400	128	C_9H_{20}	3-ethyl-2-methylhexane
5	17·983	142	$C_{10}H_{22}$	2,7-dimethyloctane
6	19·583	156	$C_{11}H_{24}$	Undecane
7	21·233	282	$C_{11}H_{23}I$	1-iodoundecane
8	22·967	170	$C_{12}H_{26}$	3-methyl undecane
9	24·383	192	$C_8H_{17}Br$	2-bromo-6-methyl heptane
10	24·933	170	$C_{12}H_{26}$	3-ethyl-2,7-dimethyl octane

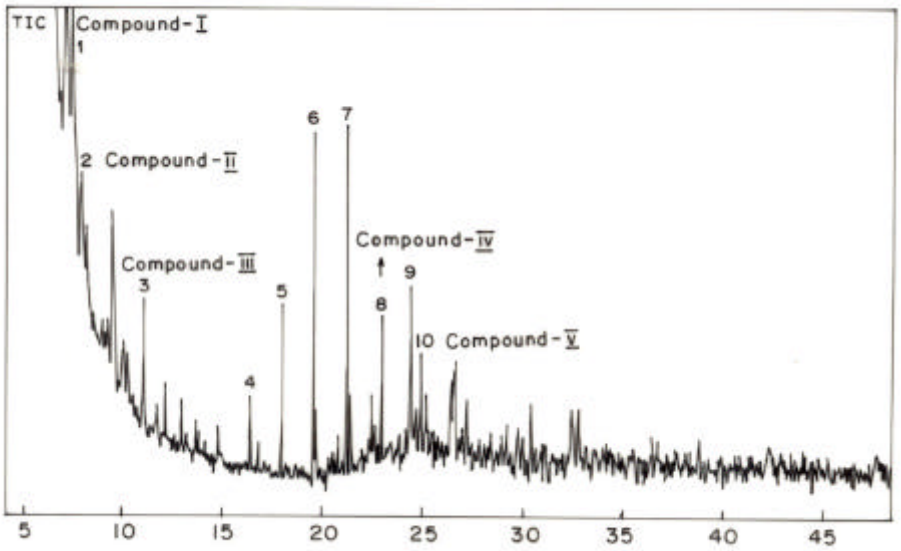

Figure 1. Gas-chromatographic profiles of the urine of male.

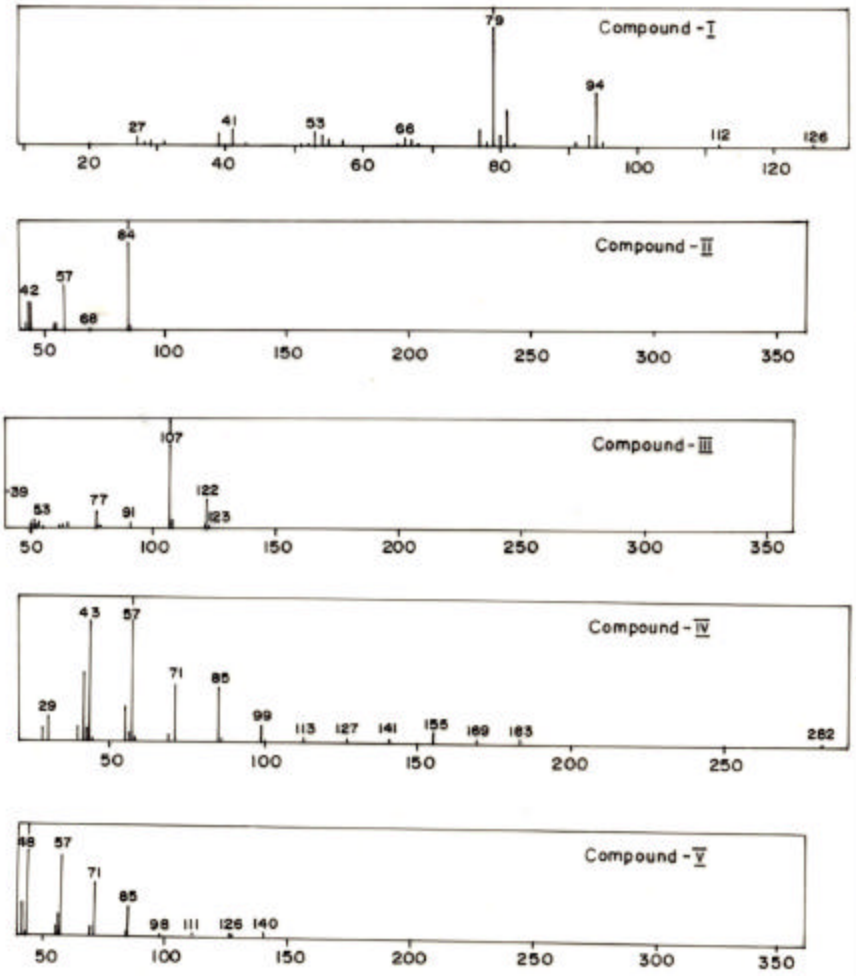

Figure 2. Mass spectra of the identified compounds of male laboratory mice urine.

female responders also showed a significant difference in their preference for the five compounds [F (4, 25) = 24·286, $P < 0·001$; table 2]. The female responder frequently visited the slides containing the compounds II, III and IV. Post-hoc analysis further revealed the statistical significance of preference by females for the compounds II, III and IV, rather than the compounds I and V (refer Duncan test in table 2). The overall bioassay data revealed that the compound IV, 3-ethyl-2,7-dimethyl octane, attracted the females while it repelled the males (table 2). Leaving the arm in the Y-maze apparatus within a few seconds is evidence of the averse behaviour of male mice.

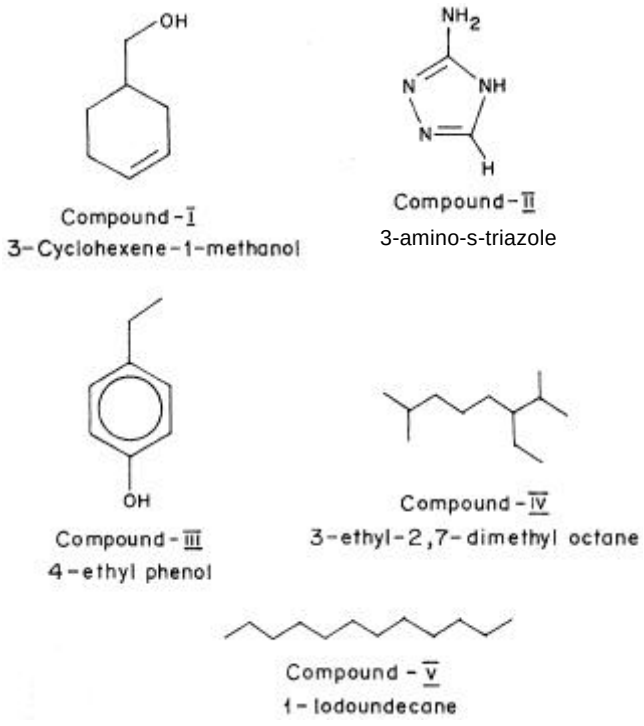

Figure 3. Chemical structure of the identified compounds of male mice urine.

4. Discussion

Chemical messages appear to play an important role in overall social behaviour and reproductive function in mice (Novotny *et al* 1984). Despite the large number of compounds characterized in house mouse urine (Schwende *et al* 1986), only fewer constituents were encountered in this strain. Further, only 10 detectable peaks were seen in the present study, with most featuring a chemical makeup which differs from the earlier report (Schwende *et al* 1986). The house mouse features a set of unique male-specific compounds, such as 2-*sec*-butyl dehydro thiazole, dehydrobrevicomin and 4-ethyl phenol (Novotny *et al* 1985a; Cavaggioni and Mucignat-Caretta 2000). In the present investigation, five male-specific compounds have been identified in which three compounds are characteristically shown as sex attractants. Further, findings reported earlier and the present one show that the 4-ethyl phenol acts as an attractant for the opposite sex and creates aversion towards the same sex. Strikingly, the compounds identified in the present study do not correlate with the chemical compounds identified in the urine of ICR strain male mice (Nishimura *et al* 1989). It therefore appears that the chemicals involved in pheromonal communication differ according to their genetic background.

Table 2. Number of visits made by male and female mice when exposed towards identified compounds of male mice urine (5 min test).

Exposures	Male	Female
3-cyclohexene-1-methanol (I)	$4·66 \pm 0·42^{a}$	$1·83 \pm 0·30^{c}$
Control	$0·83 \pm 0·30$	$1·16 \pm 0·30$
3-amino-s-triazole (II)	$2·33 \pm 0·33^{bc}$	$4·33 \pm 0·42^{b}$
Control	$1·00 \pm 0·36$	$0·66 \pm 0·33$
4-ethyl phenol (III)	$1·33 \pm 0·49^{c}$	$5·50 \pm 0·42^{a}$
Control	$0·50 \pm 0·34$	$0·33 \pm 0·21$
3,ethyl-2,7-dimethyl octane (IV)	$1·16 \pm 0·40^{c}$	$6·00 \pm 0·25^{a}$
Control	$1·00 \pm 0·36$	$1·66 \pm 0·21$
1-iodoundecane (V)	$3·00 \pm 0·51^{b}$	$2·16 \pm 0·47^{c}$
Control	$0·66 \pm 0·33$	$1·33 \pm 0·33$

Values are expressed in Mean ± SE of six observations.
Those means in the same vertical column that are not marked with the same superscript letters i.e. ^{a}, ^{b}, and ^{c} are significantly different at $\alpha = 0·5$ level (Duncan's test; Snedecor and Cochran 1967).

The compound IV, 3-ethyl-2,7-dimethyl octane found in male mice urine has not been reported in previous studies. Many different chemicals have been reported as pheromones, i.e. alcohols, aldehydes, saturated or unsaturated acids, aliphatic or aromatic compounds from non-polar molecules such as alkanes and alkenes, to very polar compounds which may be acidic (acids, phenols) or basic (amines) (Dominic 1991; Gassett *et al* 1996; Kannan *et al* 1998; Kannan and Archunan 1999; Ramesh Kumar *et al* 2000; Kannan and Archunan 2001a, b; Selvaraj and Archunan 2002). Indeed, the compound IV also belongs to an alkane group like that of brevicomin [brevicomin is also called 7-exo-5-methyl-6,8-dicarboxcylic [3·2·1] octane (Novotny *et al* 1984)]. However, the present compound, 3-ethyl-2,7-dimethyl octane lacks the carboxylic group with modifications in ethyl and methyl bonding. The odour preference test clearly indicate that compound IV, 3-ethyl-2,7-dimethyl octane has more attraction towards the estrus females and aversion towards the males and confirm its bioactivity in chemosignalling.

It is also interesting to note that the compound III, the 3-amino-s-triazole, has a close similarity with thiazoline, which is reported in male mice urine (Liebech *et al* 1977; Novotny *et al* 1985a). The compound 3-amino-s-triazole contains a pentagonal structure, along with a nitrogen compound similar to thiazoline (Liebech *et al* 1977). In fact, the compound III has an aromatic nature but lacks sulphur, while thiazoline contains sulphur without an aromatic nature. Since 3-amino-s-triazole is aromatic, it is possible to suggest that this compound may act as a sex attractant. The behaviour study also confirms that this compound acts as an opposite sex attractant. Even though both the compounds (III and IV) have a close similarity, as stated in previous reports and in the present study, these cannot exactly be considered as similar compounds as far as the structure is concerned. Minor modification in the chemical structure and nature of identified compounds would make it possible to suggest that certainly some factors such as environment and diet may be involved in the production of pheromone signals.

The odour preference tests demonstrate that compounds II, III and IV (4-ethyl phenol, 3-ethyl-2,7-dimethyl octane, and 3-amino-s-triazole) act individually as an attractant for opposite sex of the same species. This result leads to the conclusion that the single volatile compound present in the male mice urine is involved in sexual attraction. Therefore, the present finding is consistent with earlier reports that the single volatile compound namely hydroperoxide in the rat (Selvaraj and Archunan 2002) and (Z) 7-dodecynyl acetate in elephant (Rassmussen *et al* 1997) act as sexual attractants to the opposite sex. Even though each fraction has a unique extent of attraction on individuals of the same species, for further behavioural activities such as sniffing, licking and mounting a mixture of compounds may be involved. The present finding provides additional evidence in favour of previous reports related to pheromone identification and its biological significance in mice (Nishimura *et al* 1989), rat (Selvaraj and Archunan 2002; Kannan and Archunan 1999, 2001a) and bovine (Ramesh Kumar *et al* 2000). Studies have also indicated that either single or a mixture of volatile compounds is involved in conveying specific signals regarding reproductive and social behaviour (Kannan and Archunan 2001b). Similarly, in the present investigation, five different male specific compounds have been identified and shown to act as chemo-signals. Hence, the present study lends support to the concept that urine contains a large array of compounds which are involved in chemical communication.

Benzaldehyde is a ubiquitous constituent of mammalian urine, viz. house mouse (Schwende *et al* 1986), California mouse (Jemiolo *et al* 1994), deer mouse (Ma *et al* 1999), wolf (Raymer *et al* 1986), and the red fox (Jorgenson *et al* 1978). In the present work, however, benzaldehyde has not been identified and the reason for its absence is not known at present. The compound (V) 1-iodoundecane identified in Swiss mice urine is also reported in estrus bovine urine and believed to be a sex attractant towards the bulls (Ramesh Kumar *et al* 2000). The behaviour study convincingly demonstrated that 1-iodoundecane is an attracting compound irrespective of sex. In contrast, the compound 3-cyclohexene-1-methanol is a same sex attractant belonging to aliphatic unsaturated alcohol. The post-hoc analysis clearly indicated that 3-ethyl-2,7-dimethyl octane is more attractive towards the female, whereas, 3-cyclohexene-1-methanol is more attractive towards male. Hence, the present investigation provides evidence that the male mice urine contains both same and opposite sex attractants.

Urinary compounds that we identified as unique to the male mouse had molecular weights less than 300 and carbon atoms less than 20. Air-borne pheromones usually contain 5–20 carbon atoms and must be volatile to reach the receiver; the molecular weights of pheromones are less than 300 (Dominic 1991). In the cow, two specific volatiles that have molecular weights less than 300 (Ramesh Kumar *et al* 2000) occur exclusively in the urine during the heat period. Female Asian elephants release a urinary pheromone with 13 carbons and a molecular weight of 300 to attract males (Rasmussen *et al* 1997). The compounds identified in the present study have the physical properties necessary for consideration of pheromones. The bioassay provides additional support for considering the identified compounds as pheromones.

Another distinguishing characteristic of house mouse urine is the excretion of a large amount (typically 5 mg/ml) of proteinous material, the so-called major urinary protein (MUP) (Finlayson *et al* 1963; Cavaggioni

and Mucignat-Caretta 2000). It is now generally believed that all volatiles with distinct male or female pheromone activity bind strongly to MUP (Novotny *et al* 1999). The compounds 2-*sec*-butylthiazole and dehydro-exo-brevicomin are reported to bind along with the MUP (Cavaggioni and Mucignat-Caretta 2000). Our prospective experiments are directed toward an investigation of the interaction of the three male specific compounds, 4-ethyl phenol, 3-ethyl-2,7-dimethyl octane and 3-amino-s-triazole with the MUPs.

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