Positive identification of the puberty-accelerating pheromone of the house mouse: the volatile ligands associating with the major urinary protein

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Five structurally diverse small ligands, all binding to the major urinary protein (MUP) of the male house mouse, show individually puberty-accelerating pheromonal activity in the recipient females. A recombinant MUP (identical structurally to the natural protein) has shown no biological activity. While four of these ligands were previously implicated in oestrus synchronization (Whitten effect), the same chemosignals now appear responsible for both sexual maturation and cycling in adult females.

Keywords: puberty-acceleration pheromone; urinary protein; mouse

1. INTRODUCTION

There is considerable evidence that chemical communication is involved in the regulation of sexual maturation in female rodents, with the house mouse (*Mus domesticus*) being the best physiologically documented case. Since the early 1960s, when the oestrus synchrony (Whitten 1958) of grouped female mice was observed (Marsden & Bronson 1964) following their exposure to male urine, numerous efforts were made to elucidate the nature of the male-originated pheromone(s) responsible for either the advancement of puberty in juvenile females or oestrus synchronization in the adult female colonies. With regard to the chemical nature of the 'male primer', literature controversies abound. For example, while the wind tunnel experiment of Whitten *et al.* (1968) on oestrus induction seems to favour the notion of the pheromone's volatility, Vandenberg and co-workers (Vandenberg 1969; Vandenberg *et al.* 1975, 1976), in their studies using the uterine weight assays (puberty acceleration), strongly advocate the idea that the pheromone is a large and/or non-volatile molecule, such as a protein or peptide. Although a rational argument has been made by Bronson & MacMillan (1983) that both the oestrus synchronization and puberty acceleration have the same endocrinological denominator, it has never been resolved whether the same chemosignal causes both effects.

In spite of a certain amount of progress in our understanding of the chemical nature of endocrinologically dependent urinary volatiles in the house mouse (Novotny *et al.* 1984, 1990a; Schwende *et al.* 1986) and the endocrinological and behavioural effects caused by the synthetic analogues of these compounds (Novotny *et al.* 1985, 1990a,b; Jemiolo *et al.* 1986)—all apparently small molecules—a relatively recent report (Mucignant-Caretta

et al. 1995) attributes the male-to-female puberty acceleration activity to a urinary protein and/or its N-terminal hexapeptide. This so-called major urinary protein (MUP) is, in fact, a conglomerate of polypeptide isoforms (Finlayson *et al.* 1965), all having molecular mass of less than 20 kD. MUP is apparently under testosterone control, being expressed predominantly in adult males. The points relative to the question of pheromones' volatility are that (i) in spite of repeated purifications and lyophilization steps, the fractions biologically active in the uterine weight assays (Vandenberg *et al.* 1975; Novotny *et al.* 1980) continue to possess an odour reminiscent of mouse urine; and (ii) even after extensive purging of the unfractionated urine for many hours, the samples retain their odour, their capability of 'yielding' numerous volatile organic molecules and their biological activity (Novotny *et al.* 1980, 1999).

Here, we provide evidence that five out of the six volatile organic molecules with a strong affinity to MUP are individually active in the uterine weight bioassays. Moreover, neither a recombinant MUP, nor the hexapeptide with the alleged (Mucignant-Caretta *et al.* 1995) biological activity, can themselves promote uterine growth in juvenile female mice. Interestingly, the synthetic analogues of certain mouse volatile chemosignals (namely, 3,4-dehydro-*exo*-brevicommin (DB), 2-*sec*-butyl-4, 5-dihydrothiazole (BT), and α - and β -farnesene), all exhibiting this biological activity and a strong affinity to MUP, were previously implicated in oestrus synchronization (Jemiolo *et al.* 1986; Ma *et al.* 1999). This communication thus supports Bronson's opinion (Bronson & MacMillan 1983) that puberty acceleration in juvenile females and oestrus synchronization in an adult female colony are likely to be caused by the same male-originated chemosignal. As discussed below, there are several possible advantages of a system where the active pheromone is ligated to a larger and less volatile carrier molecule, such as MUP of the house mouse.

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2. MATERIALS AND METHODS

(a) *Experimental animals and testing procedures*

Adult ICR (albino) mice, originated from Harlan/Sprague-Dawley, Inc. (Indianapolis, IN), were bred to produce all litters. The pregnant females were isolated in individual cages one week prior to parturition. After birth, each litter was counted and sexed, adjusting the litter size to ten, with a sex ratio of 7:3 (female:male). After weaning at day 21, juvenile females were weighed and assigned randomly to different experimental groups, with each group receiving a different olfactory stimulus or an appropriate control solution. Each experimental group was placed in a separate room. Animals were singly or doubly caged (every group had three to four cages each occupied by two mice). All animals were housed in polypropylene cages with opaque sides and fitted wire-lids (12 cm × 27 cm × 17 cm). A wood-shaving bedding was changed once per week. Food and water were provided *ad libitum*. All rooms were maintained at 21–22 °C, 70% relative humidity and 12 L:12 D lighting regime, with lights on at 06.00.

Each experimental treatment of the juvenile females was begun at day 21 and continued for six consecutive days. Each experimental mouse was treated twice daily (09.00–10.00; 16.00–17.00) on the nasal groove and external nares with a 30 µl solution of an appropriate experimental substance, through a small plastic tube adapted to a microsyringe. Separate microsyringes were used for different treatment groups. At day 27, the mice were weighed again and sacrificed by cervical dislocation. Their uteri were removed, trimmed from surrounding tissue and weighed on the analytical balance. The uterine weight was used as an index for the pheromonal (puberty acceleration) activity. The data were evaluated through the one-way analysis of variance (ANOVA) (Zar 1984). To overcome the distribution problem, the data were log transformed prior to their statistical analysis (Vandenbergh *et al.* 1975).

To accommodate a large number of experimental groups into our animal facility (each experimental group occupying a separate room), two separate trials were conducted at different times (experiment 1: June 1996; experiment 2: October 1996). In experiment 1, 46 adult females were mated to produce progeny. Only the pups with a body weight ranging from 12.5–14.8 g (at weaning) were chosen. Eight treatment groups (on average, 20 animals for each treatment, with the exact numbers specified below) were set to test an effect of the following stimuli: (i) a mixture of DB and BT (at 1.3 ppm concentration in water), 20 animals; (ii) a mixture of the same compounds, dissolved at 1.3 ppm in the urine of castrated adult males, 19 animals; (iii) a newly found, unique male urinary component, 6-hydroxy-6-methyl-3-heptanone (Novotny *et al.* 1999), dissolved in water at 2000 ppm level, 19 animals; (iv) the MUP complex (19 animals), isolated from the urine of adult male mice through a Sephadex G50 column and reconstituted in water (after lyophilization) to its natural concentration (4 mg ml⁻¹, as assayed by UV absorbance at 280 nm); (v) a hexapeptide N-Glu-Glu-Ala-Arg-Ser-Met (dissolved at 500 µg ml⁻¹), 23 animals; (vi) adult male urine, used as a positive control (20 animals); and (vii) water used as a conventional control (20 animals). In experiment 2, 63 adult females were mated to produce progeny. Only the pups with a body weight from 12.0–14.3 g (at the time of weaning) were selected. They were assigned randomly to ten treatment groups (13–20 animals per treatment), followed by the uterine weight assays: (i) the male MUP, prepared as before (20 animals); (ii) MUP from testosterone-treated females

(0.3 mg d⁻¹ dose for ten days prior to urine collection)—this protein was concentrated to the level of male MUP, as judged by UV absorbance (280 nm) of the protein solution (20 animals); (iii) untreated female MUP (17 animals), concentrated to a comparable protein ultraviolet absorbance (280 nm); (iv) geraniol, dissolved at 10 ppm in water (13 animals); (v) β-farnesene, dissolved in castrate urine at 250 ppm (13 animals); (vi) a mixture of α- and β-farnesene, dissolved in castrate urine at 250 ppm (14 animals); (vii) DB, dissolved in castrate urine at 1.3 ppm (13 animals); (viii) BT, dissolved in castrate urine at 1.3 ppm (13 animals); (ix) water, used as a control (20 animals) for (i), (ii), (iii) and (iv); (x) castrate urine (13 animals), used as a control for (v), (vi), (vii) and (viii).

During September–October 1997, when the recombinant MUP (see below) was prepared for our experiments (Židek *et al.* 1999), 60 additional young females were tested, using the identical procedures as described above, with three different stimuli: (i) recombinant MUP (4 mg ml⁻¹, 20 animals); (ii) natural MUP (positive control, 20 animals); and (iii) water (conventional control, 20 animals).

Adult male and female ICR mice were used as urine donors. The urine samples were collected by holding the animals over a clean glass plate and gently pressing the flank areas. Upon collection, samples were frozen (–20 °C) until used for either MUP isolation or biological testing.

(b) *Reagents and analytical methods*

Synthetic analogues of the male mouse pheromones (DB, BT, and α- and β-farnesenes) were prepared using the previously described syntheses (Wiesler *et al.* 1984; North & Pattenden 1990; Novotny *et al.* 1999). Geraniol was purchased from Sigma (St Louis, MO). The synthesis and properties of a recently identified 6-hydroxy-6-methyl-3-heptanone are reported elsewhere (Novotny *et al.* 1999).

MUP fractions from the normal male and female urinary samples, as well as from testosterone-treated females, were prepared through size-exclusion chromatography on a Sephadex G50 column (60 cm × 8 mm, internal diameter (i.d.)) and assayed through UV absorbance monitoring. The MUP profiles were further checked through a standard polyacrylamide gel electrophoresis (Laemmli 1970).

The isolated protein fractions (3 ml) were extracted with 0.5 ml aliquots of ether and the extract was further analysed by separating its components on a Rtx-5 (Restek, Bellefonte, PA) column (60 m × 0.25 mm, i.d.) attached to the electron impact ion source of a Hewlett-Packard 5890 series II mass spectrometer. The separated compounds were structurally identified from their mass spectra and chromatographic retention coinciding with the data from authentic compounds.

Recombinant MUP-I was expressed in *Escherichia coli* as described in detail elsewhere (Židek *et al.* 1999). Briefly, the part of the MUP-I gene (cLivl clone, provided by J. Bishop, Centre for Genome Research, University of Edinburgh, UK) that encodes the mature protein was cloned into pET-28b(+) plasmid. A factor Xa protease cleavage site incorporated in the N-terminal region provided an easy way to produce a native-like protein. The identity of this protein was verified through the use of matrix-assisted laser desorption/ionization mass spectrometry (Voyager DETM RP BioSpectrometryTM Work Station, PerSeptive Biosystems, Framingham, MA), yielding the correct mass of 18692 ± 4, an identical tryptic map and an isoelectric point of 4.6 (indistinguishable from the native protein when run in parallel).

Figure 1. Total ion chromatogram of the ether extract of MUP from male mice. L, 6-hydroxy-6-methyl-3-heptanone or its equilibrium 'lactol' form (Novotny *et al.* 1999) shows as the large peak, the small peaks designated with the same letter are its degradation products, dihydrofurans (Novotny *et al.* 1999); DB, 3,4-dehydro-*exo*-brevicomine; BT, 2-*sec*-butyl-4,5-dihydrothiazole; G, geraniol; β, β-farnesene; α, α-farnesene. The unlabelled trace peaks are contaminants from distilled water and buffers.

in a gel system. Measurements of the binding capacity for BT and its total characterization by NMR spectroscopy (Židek *et al.* 1999) showed that the protein was prepared in its native form. Moreover, the routine Edman sequencing procedure verified its terminal residues as N-Glu-Glu-Ala-Ser-SerBThr. N-Glu-Glu-Ala-Arg-Ser-Met was synthesized using Rainin PS3 (Rainin, Woburn, MA) equipment and common chemicals. Its purity was verified by the reversed-phase high-performance liquid chromatography and determination of its molecular mass (721.78 Da).

3. RESULTS

The MUP complex isolated from the urine of normal males retained its 'mousy' odour in spite of its clear separation from the free low-molecular urinary components on the Sephadex column. However, from the many previously identified (Schwende *et al.* 1986; Novotny *et al.* 1990*a,b*) urinary volatile constituents, only a few seem selectively retained by MUP (figure 1), implicating the structures shown in figure 2 as potential chemosignals.

It has now been well established that the production of MUP in mice is strongly dependent on the androgen levels (Knopf *et al.* 1983). Females and castrated males in our experiments produced only a small fraction of the urinary protein isolated from normal, adult males (results not shown); this is in agreement with the earlier observations (Knopf *et al.* 1983) and a corresponding substantial decrease of the above volatile constituents. However, a repeated treatment of female mice with testosterone increased the MUP fraction to the level comparable to normal males (results not shown). The corresponding gas

Figure 2. Chemical structures of (a) (*S*)-2-*sec*-butyl-4,5-dihydrothiazole; (b) (*R,R*)-3,4-dehydro-*exo*-brevicomine; (c) *E,E*-α-farnesene; (d) *E*-β-farnesene; (e) 6-hydroxy-6-methyl-3-heptanone and its equilibrium cyclic form; and (f) geraniol.

chromatogram indicated that the hydroxy ketone-lactol and DB urinary concentrations were also brought to the range associated with the normal male. Interestingly, only a trace of geraniol and no detectable amount of BT were observed in the androgenized females or testosterone-treated castrates. The MUP isolated from testosterone-treated females had a strong odour reminiscent of the male mouse urine.

Two independent trials, employing both the natural stimuli and treatments with synthetic compounds (applied individually or in certain mixtures), demonstrate statistically significant increases in the uterine weight for several cases (figure 3*a,b*): MUP isolated from the normal males and testosterone-treated females were both effective, as was the aqueous solution of 6-hydroxy-6-methyl-3-heptanone (Novotny *et al.* 1999). To our surprise, DB and BT (applied individually or in a mixture dissolved in either the castrate urine or plain water), and both farnesene isomers were also found to be effective. Geraniol was the only component binding to the MUP complex that was found inactive in the uterine weight assays.

The tested levels of synthetic ligands is justified by the fact that (i) lactol concentrations between 630 and 1820 ppm were observed in the randomly collected male urine pools (Novotny *et al.* 1999); (ii) the farnesenes are non-polar compounds with a relatively poor solubility in water, and their urinary and preputial gland concentrations are far greater than what has been seen in figure 1; (iii) geraniol, a structural 'relative' of the farnesenes with an alcohol functionality, is more soluble in water, making the losses through manipulation less likely than for the farnesenes; and (iv) the levels of BT and DB chosen were similar to those potentiating oestrus synchronization in our earlier work (Jemiolo *et al.* 1986).

Table 1 shows the results comparing the effect of natural MUP with r-MUP I, a protein overexpressed in *E. coli* which is structurally identical to one of the major components of the natural MUP complex in terms of its exact mass, sequence and the binding behaviour towards BT (Židek *et al.* 1999). Unlike the MUP isolated from the male mouse urine, the recombinant protein had no characteristic odour and was devoid of any volatiles during a GC-MS analysis. These results indicate the lack of

Figure 3. Uterine weights for (a) experiment 1 and (b) experiment 2. BT, 2-sec-butyl-4,5-dihydrothiazole; DB, 3,4-dehydro-*exo*-brevicomin; DB/BT1, a 1:1 mixture of DB and BT in water; DB/BT2, a 1:1 mixture of DB and BT in castrate urine; CU, castrate urine; L, lactol; MUP, major urinary protein; P, hexapeptide; U, adult male urine; FTM, MUP from testosterone-treated females; MM, male MUP; FM, female MUP; W, water; G, geraniol; β, β-farnesene; αβ, a 1:1 mixture of α- and β-farnesene. Only statistical comparisons between the water group and the experimental groups are shown in the graph (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

biological activity for the recombinant protein. Additionally, the hexapeptide, alleged to be biologically active in the report from a different group (Mucignant-Caretta *et al.* 1995), had no effect on the uterine weight (figure 3).

At the beginning of treatments (day 21), the body weights were recorded for all experimental animals, and once again, on day 27. Statistical analyses indicated that there were no differences among treatments within each experimental set either for the body weight at day 21 or day 27 ($p > 0.05$). Due to a seasonal influence, the animals in experiment 1 had slightly higher body weights on both day 21 and day 27 than those in the other experiments. This may have contributed to the tendency for slightly larger uterine weights in experiment 1. Due to the limitation of space, the treatment experiments in the second set had to be conducted with two female mice per cage. We have, however, verified independently (results not shown) that there was no statistically significant difference between the singly and doubly caged mice in the times for vaginal opening, oestrus induction and uterine weights under the described conditions.

Table 1. Effect of the natural and recombinant MUPs on sexual maturation in mice

group	<i>n</i>	uterine weight on day 27 (mg, mean $\pm$ s.e.m.)
natural MUP	20	90.10 $\pm$ 8.24
recombinant MUP	20	40.25 $\pm$ 3.62
water	20	41.75 $\pm$ 3.24

4. DISCUSSION

The urinary proteins excreted by various rodents represent an intriguing class of biomolecules. They belong to the general class of lipocalins, small proteins with the capability of binding a variety of relatively hydrophobic ligands (Flower 1996). The MUP of the house mouse is a complex of protein isoforms of approximately 18 kD of mass with the proven capacity (Novotny *et al.* 1980, 1999; Robertson *et al.* 1993) for binding low-molecular weight entities, including two volatile substances with a proven primer effect of oestrus synchronization (Jemiolo *et al.* 1986). The advantages of such a ligand–protein complexation may include a slow release (Hurst *et al.* 1998; Robertson *et al.* 1998) of volatile chemosignals into the environment, protection of a labile pheromone and perhaps even a proper delivery of pheromones to the site of reception (if the mice engage in a direct physical contact with the main source of chemosignal, such as urine). These are all equilibrium-driven situations.

The widespread notion that MUPs are the pheromones themselves can be challenged on several grounds: (i) even though the protein fraction from the mouse urine is active in the uterine weight bioassays (Mucignant-Caretta *et al.* 1995; Novotny *et al.* 1980, 1999), its strong odour and instrumentally measurable content of volatiles cannot be ignored; (ii) BT and DB, the molecules implicated in oestrus synchronization (Jemiolo *et al.* 1986), attractiveness to females (Jemiolo *et al.* 1985) and intermale aggression (Novotny *et al.* 1985), bind strongly to MUP; and (iii) DB was recently found as a ligand facilitating transmembrane signalling (Moss *et al.* 1997) in a receptive neuron (from vomeronasal organ). The results shown in this communication clarify further the relationship between MUP and its volatile ligands.

As seen in figure 1, several biochemically and physiologically relevant ligands bind to the male-originated MUP: BT and DB, two components with a previously shown chemosignalling function (Novotny *et al.* 1985, 1990a; Jemiolo *et al.* 1985, 1986); α- and β-farnesene, two terpenoid isomers related to an intermediate in the steroid biosynthetic pathway (Stryer 1995), which are further known to signal dominance among males (Novotny *et al.* 1990b), be attractive to females (Jemiolo *et al.* 1991) and promote oestrus synchronization (Ma *et al.* 1999); and 6-hydroxy-6-methyl-3-heptanone, a compound with a recently proven puberty-accelerating activity (Novotny *et al.* 1999). Geraniol is an odoriferous, terpenic substance (Budavari 1996) whose chemosignalling function, if any, is presently unknown. In a comparative GC–MS analysis of the volatiles from MUP of androgen-treated females and castrated males (results not shown), while DB reappeared,

BT was absent, suggesting a testicular control of biosynthesis or excretion of the latter.

The individually tested synthetic analogues of the ligands binding to MUP brought a most surprising result: in spite of their very different chemical structures, they were all capable of enhancing the uterine weight (while tested individually or in mixtures) to the level statistically comparable to the effect of the whole urine or the natural (isolated) MUP. In fact, the only substance lacking this activity was geraniol. The overall biological activity is attributable to the combined effects of these ligands, whether bound to MUPs or in their dissociated forms.

It is noteworthy that the farnesenes have a different glandular source from the rest of the urinary components binding to MUP. They are the most conspicuous components of the mouse preputial gland, while the remaining substances originate from the bladder urine (Novotny *et al.* 1990b). Whether or not the farnesene-MUP complex is assembled already in the preputial secretion or in the voided urine is currently unknown. Importantly, the fact that both the farnesenes and the bladder-originated volatile ligands are active in puberty acceleration could help resolve yet another long-term controversy in the literature pertaining to a source of the oestrus-inducing chemosignal (Chipman & Albrecht 1974; Bronson & Whitten 1968). We have previously found that a mixture of BT with DB in water (Jemiolo *et al.* 1986) and a mixture of α - and β -farnesene (Ma *et al.* 1999) are both active in oestrus induction. This fact, combined with their activity in the uterine weight assays shown here, supports the argument made earlier by Bronson & MacMillan (1983) that puberty acceleration in juvenile females and oestrus synchronization in adult animals should have the same endocrinological denominator and thus, similar chemosignal(s).

The recombinant MUP, devoid of the volatile ligands, has no biological activity (table 1). The same was found to be true for the MUP-associated (N-terminal) hexapeptide, claimed previously (by Mucignant-Caretta *et al.* 1995) as active. It should also be noted that this terminal sequence is atypical for the naturally found MUPs; it might have been intentionally chosen as a possible (pseudo) gene product. The absence of biological response to recombinant MUP-1 is the most compelling argument for the key role of volatile ligands. The results of Vandenberg *et al.* (1975) and perhaps those of Mucignant-Caretta *et al.* (1995) (using different methodologies and animal strains) can be explained by the complexation of pheromones with MUPs and persistence of the complexed form even after exhaustive extraction of the urinary material with organic solvent. The active pheromones seem to occur in at least three urinary fractions: (i) as bound to MUP; (ii) in the peptide-containing fractions following MUP (Novotny *et al.* 1999); and (iii) as free ligands. The work towards understanding of the physical causes of complexation for all individually identified chemosignals is in progress.

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