A unique urinary constituent, 6-hydroxy-6-methyl-3-heptanone, is a pheromone that accelerates puberty in female mice

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Background: Olfactorily mediated puberty acceleration in female mice (measured by an increase in uterine weight) has been observed since the 1960s without the active chemosignal being structurally identified. There are many controversies in the literature as to whether this male-originated pheromone is a volatile substance. We investigated the chemical nature of the urinary fractions that are responsible for the characteristic uterine weight increases.

Results: The active pheromone was identified as 5,5-dimethyl-2-ethyltetrahydrofuran-2-ol and/or its open-chain tautomer (6-hydroxy-6-methyl-3-heptanone). A series of cyclic vinyl ethers were isolated from chromatographically active fractions of the urine. Because these compounds did not accelerate puberty, we postulated that these ethers were degradation products of a lactol (5,5-dimethyl-2-ethyltetrahydrofuran-2-ol). The lactol was then detected directly in the mouse urine extract using a silylation agent. Synthetic 6-hydroxy-6-methyl-3-heptanone had strong biological activity, whereas its close structural analogs did not.

Conclusions: The male house mouse excretes into its urine a large quantity of a volatile substance that has a unique lactol/hydroxyketone structure. This substance is capable of binding to the less volatile urinary constituents, such as proteins or peptides, and is active in puberty-acceleration bioassays. The controversies regarding the volatility of the puberty-accelerating pheromones can now be explained by considering a complex of volatile lactol/hydroxyketone and urinary proteins.

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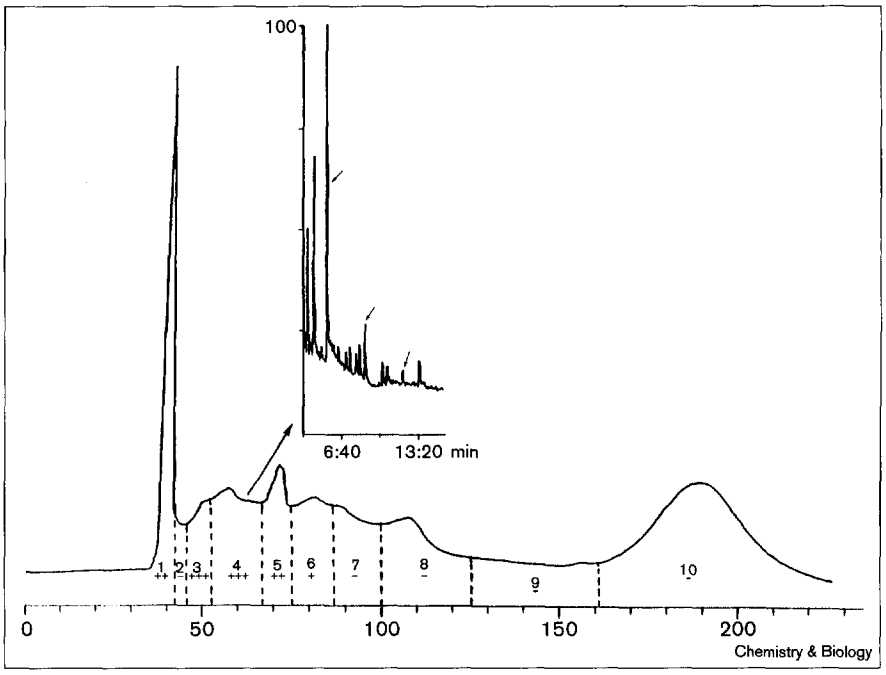
Introduction

In 1969, Vandenbergh [1] first reported that early puberty in the female house mouse (*Mus domesticus*) can be induced by exposure to male urine of the same species (the puberty acceleration can be measured by an increase in uterine weight). Subsequent chromatographic studies [2–4] aimed at characterizing the pheromone responsible for the puberty-accelerating effect provided evidence that biological activity resides with either the protein fraction of urine [4] (a conglomerate known as the major urinary protein, MUP [5]), which has a molecular weight of less than 20 kDa, and/or a nonprotein fraction consisting of molecules smaller than 1,000 Da [3,4]. Further exacting fractionation using chromatographic techniques such as ion-exchange [4,6] or reverse-phase high-performance liquid chromatography [7] produces numerous fractions of peptide-like materials, with the biological activity located in more than one fraction (or chromatographic peak). It has been suggested that the pheromone is either a biologically active peptide [3], or a small (presumably volatile) substance [4] capable of binding to certain peptides or proteins contained in urine.

Relevant to the question of the pheromone's volatility is that, in spite of repeated purifications and lyophilizations, the biologically active fractions continue to possess an odor reminiscent of mouse urine; and even after extensive purging of the unfractionated urine for many hours, the samples retain their odor and continue to yield numerous volatile organic molecules [4,8]. Possible advantages of a system in which the active chemical is bound to a larger and less volatile carrier molecule include slow and consistent release of the pheromone for its primer action, control of its accumulation and excretion, as well as protection of a labile substance against degradation.

We provide here structural and biological evidence that implicates 6-hydroxy-6-methyl-3-heptanone (and/or its tautomeric lactol, 5,5-dimethyl-2-ethyltetrahydrofuran-2-ol) as a potent chemosignal (pheromone) that accelerates puberty in the female house mouse. This male-originated, biologically active compound was traced through its characteristic dehydration products from the urinary chromatographic fractions, and synthesis and testing of the synthetic compound in juvenile females resulted in

Figure 1



A chromatographic search for the active substance(s). A size-exclusion chromatogram of male mouse urine (from wild-type strain BALB/C) as detected by UV absorbance at 280 nm. Sephadex G-10 was used, packed into a 60 cm × 1.6 cm, i.d., column; flow-rate: 0.5 ml/min of 0.1 M ammonium bicarbonate in water. Collection of fractions 1–10 was followed by the analysis of 1.0 ml fraction aliquots by a headspace preconcentration technique [24] and GC/MS analysis. A typical chromatogram of fraction 4 is included (inset). The peaks indicated by arrows were spectrally identified as the cyclic vinyl ethers, the characteristic decomposition products of the lactol pheromone. The remaining peaks in fraction 4 have been identified as 3-hexanone, hexanal, 4-heptanone and 2-heptanone together with several trace contaminants from water. Carbonyl compounds were ubiquitous throughout the entire elution profile, and the previously identified estrus synchronization pheromones [11] were eluted in fraction 1. The abundance (+) of the cyclic vinyl ethers and their absence (–) are marked in the lower chromatogram with the respective fractions.

enhanced uterine weights that were statistically comparable to the natural stimulus. Similar results were obtained using two different strains of mice, (SJL/J × SWR/J) F_1 and ICR/Alb. Structural 'relatives' of the natural pheromone were found to be inactive.

Results

Pheromone structural identification

Size-exclusion chromatography on a Sephadex G-10 column, under conditions comparable to the previously reported work on isolating the pheromonally active factors [3,4], yielded a number of fractions (Figure 1). The first circumstantial evidence on the presence of certain volatile compounds was obtained through gas chromatography/mass spectrometric (GC/MS) profiling of the 'active fractions' [3,4] obtained after the early liquid chromatography fractionation. The biologically active fractions invariably contained a series of cyclic vinyl ethers with the formula $C_8H_{14}O$ [9] (see structures **1** in Figure 2). These ethers were present in fraction 1 (which corresponds to the MUP [5]), were undetectable in fraction 2, but were quite abundant in fractions 3 and 4 (which roughly correspond to the previously reported biological activity [3,4]), and were much less abundant in fractions 5 and 6. No cyclic ethers were detected in the remaining fractions. For the sake of brevity, only one volatile component profile is shown in Figure 1 (fraction 4).

Because the synthesized authentic cyclic vinyl ethers failed to accelerate puberty (using the uterine weight assays; data not shown), we postulated that these compounds (**1**) are simply dehydration products (analysis artifacts) of 5,5-dimethyl-2-ethyltetrahydrofuran-2-ol, a lactol that is in equilibrium with its open chain (hydroxyketone) tautomer (Figure 2).

Presence of the postulated ketoalcohol in mouse urine was directly verified by extracting male mouse urine with diethyl ether and using a silylation agent effective for tertiary alcohols [10]. Its chromatographic retention was identical to that of a synthesized, authentic compound (see below), as was its mass spectrum (m.w. 216 plus characteristic fragments at m/z 201 and 131). Subsequent synthesis of the hydroxyketone/lactol, which dehydrated spontaneously to the full set of vinyl ethers in a hot injection port of a capillary gas chromatograph, verified our hypothesis. Although the thermal lability of the lactol complicates an easy direct analysis, GC determination of the cyclic vinyl ethers can be used semiquantitatively to monitor the lactol in biological samples.

Biological responses to the chemosignal

Uterine weights, along with the overall animal weights, are known to vary in an absolute sense with the season and with animal strains. Nevertheless, by testing the pheromone-exposed females along with appropriate controls (water as the negative stimulus and the intact male urine as the positive stimulus) we have been able to arrive at statistically meaningful comparisons in this work and other studies pertaining to the mouse pheromones [11,12].

Three different sets of animal tests are reported below: results with SJL/J × SWR/J)F_1 hybrid females (which provides continuity with our previous work [4]) that implicate 6-hydroxy-6-methyl-3-heptanone as the active pheromone-accelerating compound for the first time; results with ICR/Alb mice, verifying the biological activity of this compound in comparison with its structural 'relative', 7-hydroxy-6-methyl-3-heptanone; and an experiment that compares additional hydroxyketone/lactol structures to the natural pheromone in terms of biological activity.

The results from the bioassays of the first and second experimental sets are shown in Table 1. In agreement with our previous work [4,6,7], the DEAE-2 fraction, but not the chromatographically preceding DEAE-1 fraction, induced a significant increase in uterine weight. Exposing (SJL/J × SWR/J)F_1 hybrid females [13] to the synthetic analog of the naturally occurring lactol resulted in a qualitatively similar increase in uterine weight as does the male urine and DEAE-2 fraction, when compared with the control (water). Only 3% of F_1 females showed elevated uterine weight under control conditions, compared with 71%, 64% and 57% for the male urine, DEAE-2 and the hydroxyketone/lactol, respectively. (We categorized animals whose uterine weights fell into one S.D. range of the weights in the control group as 'nonresponding females')

Although the bioassay had to be modified somewhat for the ICR/Alb females [14], to take into account the genetic variation between the different *Mus domesticus* strains, a similar trend with the uterine weights was observed (see Table 1b). 27-day old ICR/Alb females exhibited significantly increased uterine weights as a result of exposure to male urine or the synthetic analog of the naturally occurring lactol.

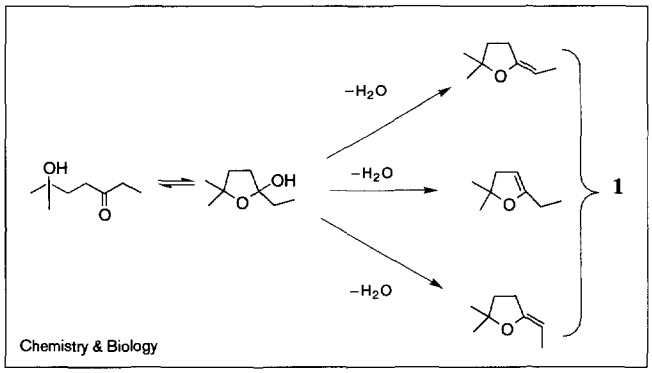
Structural analogs of the natural lactol

A relatively small variation in the compound's structure (position of a hydroxy group in the 7-hydroxy compound) rendered it biologically inactive (Table 1b). The percentage of females that responded to the various stimulants was as follows: male urine (75%), 6-hydroxy-6-methyl-3-heptanone (59%), water (22%), 7-hydroxy-6-methyl-3-heptanone (16%). In the third set of experiments, which aimed to further probe the structure–activity relationship between pheromone and biological activity, only the natural hydroxyketone/lactol was capable of eliciting the puberty-accelerating response (Table 2).

Discussion

The uterine assays described above demonstrate that the effect of the synthesized urinary constituent is qualitatively similar to the previously described puberty-acceleration effect of male urine in females. It should be noted that the effect of the whole urine is almost always greater than that of either the active urinary fractions ([4] and this work)

Figure 2



Structure of the pheromone in its two equilibrium forms and its dehydration products (a series of cyclic vinyl ethers).

or the synthetic analogs. This is not hard to explain — although the dehydration products (cyclic vinyl ethers) were ubiquitous in the Sephadex and other liquid-chromatographic fractions, only a limited number of fractions with an above-threshold quantity of the active pheromone will trigger a measurable biological response. Moreover, the lactol exhibits a strong affinity for the urinary protein material and other nonvolatile urinary constituents: even after the urinary fractions have been extensively purged with helium, the cyclic ethers remain detectable. In structural terms, the lactol structure is potentially capable of forming a bond with the primary amine [15] (reportedly, some lactol structures can react with a variety of aliphatic amines to yield 2-alkyl-amino-tetrahydrofurans), sulfhydryl or hydroxy groups of a protein or peptide. From the above-mentioned silylation experiment, we estimated that the (extracted) lactol concentrations in the intact male urine were at least 200 ppm. Although this figure hardly makes the lactol a trace urinary component (other volatile mouse pheromones [11,12,16] are much lower in concentration), the silylation experiment might have grossly underestimated the concentration of the lactol in the urine because it is susceptible to degradation during manipulation and has a strong affinity for the MUP.

In order to more quantitatively assess the urinary lactol concentration, we subjected the urine samples to headspace analysis and analyzed the combined peak areas for the three major degradation products (cyclic vinyl ethers, **1**) using gas chromatography. These values were, in turn, compared with the series of synthetic lactol samples prepared in the range of 20–2,000 ppm in saline solution. A linear relationship between the combined peak areas and lactol concentration was obtained (which has a correlation coefficient of 0.9927). Eight male mouse urinary pools collected randomly during the previous year (kept frozen before analysis) had lactol values ranging from 613–1819 ppm, indicating that the solvent extraction procedure greatly underestimated its

Table 1

Uterine weight response (± SEM) of female mice to natural and synthetic urinary chemosignals.

Stimulus	Uterine weight (mg)	Responding females (%)	Number of females
(a) SJL/J × SWR/J hybrids (F_1)			
Control samples:			
Male urine	80.0 ± 6.8*	71	31
Water	31.2 ± 3.2†	3	34
Urine fractions:			
DEAE 1	36.0 ± 5.6†	18	11
DEAE 2	65.5 ± 4.7‡	64	44
Synthetic compound:			
6-hydroxy-6-methyl-3-heptanone	64.1 ± 4.9‡	57	44
(b) IRC/Alb			
Control samples:			
Male urine	102.9 ± 12.0*	76	25
Water	37.0 ± 2.9‡	22	40
Synthetic compounds:			
6-hydroxy-6-methyl-3-heptanone	70.3 ± 5.8†	59	42
7-hydroxy-6-methyl-3-heptanone	44.8 ± 4.7‡	16	50

Statistical comparisons were made using a single-factor of analysis of variance with Duncan's new multiple range test [26]. In (a) $F(d.f = 4,159) = 13.19$, $p < 0.0005$; the means in the column not marked with the same superscript symbol are significantly different at the level of 0.002. In (b) $F(d.f. = 3,153) = 19.76$, $p < 0.0005$; the means in the column not marked with the same superscript symbols are significantly different at the level of 0.002.

urinary content. In addition, residual levels greater than 300 ppm were consistently recovered after the first headspace extraction (which presumably corresponds to material held strongly by the urinary protein/peptides that re-equilibrates into the free solution).

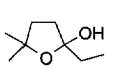
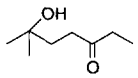
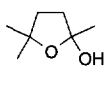
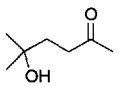
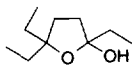
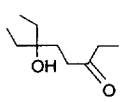
Just as with the other previously identified mouse pheromones [16], the abundance of the urinary lactol/hydroxyketone shows endocrinological dependence (data not shown). After castration of the mature male mice, urinary levels of the cyclic vinyl ethers were decreased 3–6-fold, which was roughly comparable to the levels in normal females outside the estrus period. During estrus, the levels of these compounds increase [17], whereas pregnant and lactating females have depressed levels of cyclic ethers [18]. At first glance, this finding might appear to contradict the biological experiments of Drickamer [19] who found that urine from pregnant and lactating female mice accelerates puberty in other females. A strong biological effect of enhanced ketone levels [14,18] that might override a 'deficiency' in lactol's excretion should also be considered, however. Determination of the urinary levels of 6-hydroxy-6-methyl-3-heptanone in three different strains of *Mus domesticus* (males and females of wild-type BALB/C, CF-1 and ICR/Alb) with the same endocrine status revealed only slight variations (F.X., T.-M.X. and M.V.N., unpublished observations), which can be explained by the different genetic backgrounds.

The puberty-accelerating activity of 6-hydroxy-6-methyl-3-heptanone (synthetic lactol) demonstrated in this work is another example of an isolated substance (without a biological matrix) that can act as a primer chemosignal (pheromone) in a mammalian system [11,12]. This pheromone is unique because it can bind strongly to proteins or peptides, and possibly to other polar urinary constituents. This 'pheromone-carrier system' might be involved in eliciting the full biological response — application of an active volatile material to the nasal opening of a female may be less effective than a longer-term exposure (such as the one obtained through application of the carrier-pheromone complex with a subsequent slow release of the active substance).

The initial observations [3,4] of the puberty-acceleration activity being associated with the urinary factors of limited volatility have been important in both identifying the actual chemosignal and elucidating the general role of the major urinary protein as a slow-release agent for the active pheromones, as postulated previously [4,20]. The recent claim [21] that the puberty-acceleration activity is due to MUP itself, or its terminal hexapeptide fragment (N-Glu–Glu–Ala–Arg–Ser–Met), has not been corroborated in our laboratory: neither the hexapeptide, nor a recombinant MUP has biological activity in the uterine weight assays (M.V.N., W.M., D.W. and L. Zidek, unpublished observations). In contrast, 6-hydroxy-6-methyl-3-heptanone and the other male-originated pheromones [16] bind strongly to the MUP, which might contribute to their overall biological activity. In view of the fact that large molecules can find access to the receptive tissue of the vomeronasal organ [22], presumably the site of action

Table 2

Biological responses to various lactols.

Lactol	Open structure	N	Body weights (g) Day 21	Body weights (g) Day 27	Uterine weight (mg)	Comparison
		10	13.18 ± 0.26	17.84 ± 0.32	56.56 ± 2.27	a,d
		11	12.96 ± 0.22	17.72 ± 0.20	37.82 ± 1.79	b,a
		11	13.10 ± 0.25	17.95 ± 0.34	43.82 ± 4.88	c
Water		9	13.01 ± 0.22	18.05 ± 0.53	40.33 ± 7.09	d

All values were expressed as $\bar{x} \pm$SEM. The mean uterine weights of the four experimental groups (from top to bottom) were represented by a,b,c and d, respectively. When a group is marked with a letter from another group, the difference between the two groups is significant ($p < 0.05$).

for the reproduction-related chemosignals, the MUP/pheromone complex could be utilized to participate in the 'perireceptor events' [23] when the receptive animals come into direct contact with the urinary stimulus. A chemically labile substance such as 6-hydroxy-6-methyl-3-heptanone is a good candidate to be 'protected' through its association with such a lipocalin protein serving a transport function. Alternatively, an active airborne chemosignal could be preconcentrated and 'encapsulated' by one or more mucosal proteins and transported to its reception site at the membrane. Under different ecological circumstances, either option could have distinct advantages for mammalian communication.

Significance

Puberty in the female house mouse is accelerated by exposure to urine from the male of the same species. This phenomenon has been observed since the 1960s without the active pheromone being identified. It has proved very difficult to purify the active compound from urine samples, as the chemosignal is found in both protein and nonprotein chromatographic fractions. For this reason, controversies regarding the nature of the pheromone (whether or not it is a volatile substance) have persisted.

We have deduced the structure of the pheromone as the lactol 5,5-dimethyl-2-ethyltetrahydrofuran and/or its hydroxyketone tautomer, 6-hydroxy-6-methyl-3-heptanone. Both are quite volatile, but differ from similar compounds because they are capable of binding to less volatile urinary constituents such as proteins and peptides. The decades-old controversy of whether or not the male pheromone is volatile can therefore be explained by considering pheromone/major urinary protein complexation.

Complexation of the pheromone to a carrier protein might, in fact, contribute to its biological activity. Because 6-hydroxy-6-methyl-3-heptanone is chemically labile, it is probably protected from degradation by association with a more stable protein. In addition, a carrier protein complex that slowly releases the pheromone might be much more effective than transient exposure to the pheromone alone.

Materials and methods

Chromatographic separations and mass spectrometry

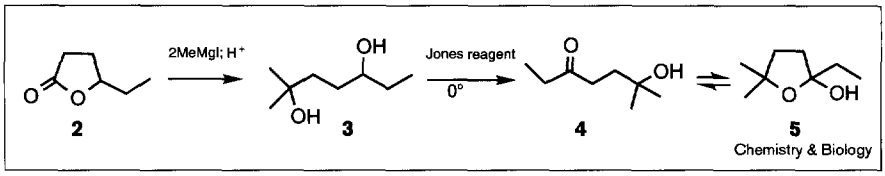
Aliquots (10 ml) of wild-type BALB/C male mouse urine were filtered, lyophilized and redissolved in 1 ml of bicarbonate buffer before application to a 60 cm × 1.6 cm, i.d., Sephadex G-10 column. Ammonium bicarbonate (0.1 M) was used as the mobile phase at 0.1 ml/min flow-rate. The collected 1 ml fractions were subjected to a previously described [24] headspace preconcentration on the Tenax GC adsorbent packed as a 4.0 mg cartridge into a glass liner of the injection part of a Hewlett Packard 5982A GC/MS. The collected volatile components of the urine were cryogenically focused at the column inlet [24] before the development of a chromatographic profile with a 60 m × 0.25 mm, i.d., glass capillary column (coated with UCON 50-HB-2000 stationary phase) and mass-spectral acquisition. From the numerous volatile urinary metabolites of the house mouse [8], certain cyclic vinyl ethers (observed already in a previous, unrelated study [9]) became a specific target of this GC/MS analysis.

A 20 ml aliquot of male mouse urine was extracted with diethyl ether and subjected to the silylation agent effective for tertiary alcohols [10]. The ether extract was dried over anhydrous Na_2SO_4 for 12 h and further concentrated using a Kuderna–Danish apparatus to ~1 ml, with a subsequent derivatization with 300 µl of a 1:1 mixture of hexamethyldisilazane and trimethylchlorosilane (Tri-Sil from Pierce, Rockford, IL, USA) in 1.0 ml dimethylsulfoxide. After evaporation of residual ether from the reaction mixture, the product was extracted with pentane and concentrated to 200 µl with a stream of dry nitrogen and analyzed by capillary GC/MS, using a 30 m × 0.25 mm, i.d., glass capillary column coated with SE-30 silicone elastomer.

Chemical synthesis of hydroxyketone/lactols

Synthesis of 6-hydroxy-6-methyl-3-heptanone was effected in two steps, starting from commercially available γ-caprolactone (4-hydroxyhexanoic

Figure 3



Synthesis of the active pheromone.

acid lactone) as shown in Figure 3. During the second step, it was essential to keep the reaction mixture at 0°C to prevent a cleavage due to the Jones oxidation (details of the synthesis are available as Supplementary Material).

The structural analogs in addition to **4**, namely 5-hydroxy-5-methyl-2-hexanone and 6- hydroxy-6-ethyl-3-octanone were prepared by varying reagents **2** and **3** (using ethylmagnesium bromide and 4-hydroxypentanoic acid, respectively; Figure 3). 7-Hydroxy-6-methyl-3-heptanone (in equilibrium with its lactol form) was synthesized as shown in Figure 4.

The disposition of functionality in this structural analog suggested a Michael addition as the key synthetic step. Ethyl propionate **6** was first converted to its ketene ethyl silyl acetal **7**, which underwent acid-catalyzed addition to 1-penten-3-one [25]. Conversion of the delta-keto ester **8** to the corresponding ethylene ketal **9**, reduction of the ester group to a primary alcohol group, and deketalization of the structure **10** yielded the final products **11** and **12**.

The identities of all products were verified using NMR spectroscopy and combined GC/MS.

Uterine weight determinations

Due to the limitations of animal housing capacity, availability of animals and effective interplay between biological and chemical tasks of this research project, the testing of puberty acceleration in juvenile females took place over a period of many months. Two different strains of *Mus domesticus* were used: hybrid (SJL/J × SWR/J) F_1 females, regarded originally as a most highly sensitive strain in this bioassay [13], and ICR/Alb females. The F_1 hybrids were bred at the Laboratory Animal Resource Facility of Indiana University from SJL/J females and SWR/J males purchased from the Jackson Laboratory, Bar Harbor, Maine. The founders of our ICR/Alb colony came from Ward's Natural Science Establishment, Rochester, New York, and, somewhat later, from Sprague Dawley, Inc., Indianapolis, Indiana. The limited availability of the hybrid mice was the principal reason for switching to the readily available ICR/Alb mice.

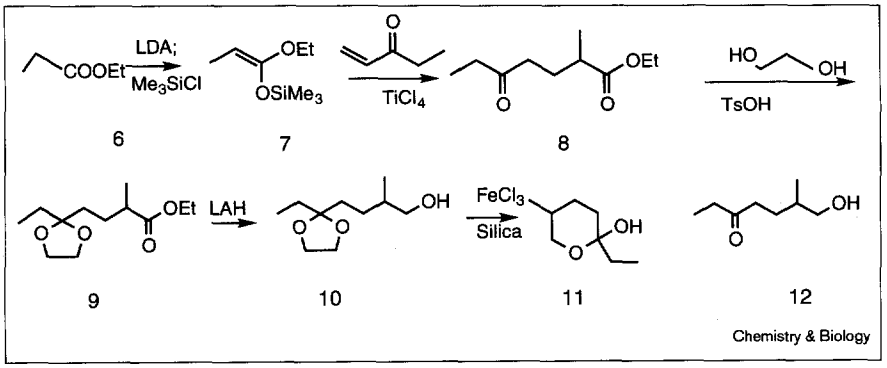
The requirements for age, body weight and time of treatment for F_1 (SJL/J × SWR/J) females were based on the data obtained by Wilson *et al.* [13]. The maximum response of F_1 females to male urine occurred between 25 and 29 days of age when their body weight was 14–16 g. Young females were weighed and weaned at 21 days. They were singly-caged after weaning and throughout the following treatment period. At 25 days, they were weighed again to select only the females that weighed between 14 g and 15 g for exposure to the chemical stimuli. The exposure lasted 3 days (days 25–27). At 29 days, the exposed animals were sacrificed and their uteri were removed and weighed on an analytical balance.

The hybrid F_1 females were treated with the following stimuli: diluted (1:10) wild-type BALB/C male urine; water (control); an active chromatographic fraction of the male urine [4] designated DEAE 2; or a chromatographically preceding, biologically inactive fraction designated DEAE 1; and synthetic lactol.

In the second set of experiments, young female ICR/Alb mice were weaned and weighed after 21 days. Only females that weighed between 15.5 g and 17.0 g were assigned to the pheromone tests. Age, body weight and time requirements for ICR/Alb mice were based on the data by Jemiolo *et al.* [14]). After weaning, the test animals were housed (one per cage) and exposed to the chemical stimuli from 21–26 days of age. On day 27, the animals were sacrificed and their uteri removed and weighed. The stimulus samples used in this case were diluted (1:10) ICR/Alb male urine; water (control); synthetic lactol; and 7-hydroxy-6-methyl 3-heptanone, a synthetic compound structurally similar to the identified urinary substance ('synthetic lactol'). The third set of experiments followed the same protocol as the second set. The additional tested compounds were 5-hydroxy-5-methyl-2-hexanone and 6-hydroxy-6-ethyl-3-octanone.

The volumes of all stimulus samples were 30 µl when they were applied to the upper lip of a tested female with a small soft brush. Concentrations used for the two initial synthetic substances (Table 1) were 2,000 ppm, and levels of 200 ppm were used subsequently (Table 2). A separate 'bioassay room' was used for the animals exposed to different

Figure 4



Synthesis of 7-hydroxy-6-methyl-3-heptanone.

samples. Testing was carried out at different (nonoverlapping) periods; only one type of a stimulus sample was tested in the bioassay room, at one time. All animals were housed in plastic cages (12 × 28 × 17 cm) and maintained at 21°C, 50–70% humidity and 12 h light/12 h dark daily regime.

Supplementary material

Experimental details of the synthesis of 6-hydroxy-6-methyl-3-heptanone is available with the online version of this paper.

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