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Absolute Configuration of 2-*sec*-Butyl-4,5-dihydrothiazole in Male Mouse Urine

Andrea Cavaggioni[1], Carla Mucignat-Caretta[1] and Giuseppe Zagotto[2]

[1]Dipartimento di Anatomia e Fisiologia Umana, Università di Padova, 35100 Padova, Italy and [2]Dipartimento di Chimica Farmaceutica, Università di Padova, 35100 Padova, Italy

Correspondence to be sent to: Andrea Cavaggioni, Dipartimento di Anatomia e Fisiologia Umana, Università di Padova, 35100 Padova, Italy. e-mail: andrea.cavaggioni@unipd.it

Abstract

The absolute configuration of 2-*sec*-butyl-4,5-dihydrothiazole (DHT) in urine of adult male mice was determined through chiral trifluoroacetyl derivative capillary chromatography by comparing the retention time with synthetic standards. (*S*)-DHT was extracted from fresh urine, while neither (*R*)-DHT nor the racemization of (*S*)-DHT were detected. We can conclude that DHT in urine possesses the *S* configuration, although we cannot exclude a minor component in the *R* configuration. (*S*)-DHT was then characterized for binding to the complex of major urinary proteins of male mouse urine (MUP) and for a behavioral response, the competitive scent marking behavior (countermarking). The binding constant of (*S*)-DHT to MUP (determined by competitive displacement) was 8.2 ± 0.6 μM (mean ± SD) and was 10.5 ± 0.6 μM for *R*-DHT, thus excluding a relevant difference in binding. (*S*)-DHT modified countermarking in a peculiar way. Male mice were slow in countermarking urinary spots streaked 2 days earlier and on top of which (*S*)-DHT was added shortly before the test. This response was not seen when adding (*S*)-DHT to freshly streaked urinary spots or to clean paper. Unlike (*S*)-DHT, (*R*)-DHT prompted countermarking rather than delaying it. We can further conclude that (*S*)-DHT in male mouse urine is an aversive chemosignal for countermarking.

Key words: chemosignal, enantiomer, mouse, pheromone, 2-*sec*-butyl-4,5-dihydrothiazole

Introduction

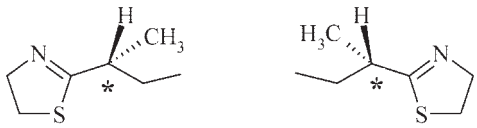
Chemical signalling among mice by means of urine is paramount for their social relationships, survival and reproduction (Johnston, 1998). This chemical signaling system, however, is still largely unraveled. The urine of adult male mouse contains a variety of odorant compounds (Novotny *et al.*, 1984; Andreolini *et al.*, 1987; Novotny *et al.*, 1995), as well as a complex of proteins (MUP) which bind odorants (Bacchini *et al.*, 1992; Robertson *et al.*, 1993; Cavaggioni and Mucignat-Caretta, 2000; Beynon and Hurst, 2003). An odorant compound present in a sizeable amount in male mouse urine is 2-*sec*-butyl-4,5-dihydrothiazole (DHT). The molecule possesses an asymmetric carbon atom in the 2-*sec*-butyl system. Thus, DHT can exist in a pair of enantiomers (*S*)-DHT and (*R*)-DHT, i.e. mirror-symmetric, non-superimposable molecules that differ only in their interaction with other chiral substrates such as chiral molecules or polarized electromagnetic fields. The chemical structure of the two enantiomers is shown in Figure 1, where the asymmetric carbon is indicated by an asterisk.

The absolute configuration of DHT in mouse urine has not yet been resolved (Novotny *et al.*, 1995). The laboratory synthesis of the two enantiomers has been recently achieved (Tashiro and Mori, 1999). The main objective of this work was to determine the absolute configuration of DHT, taking advantage of the new laboratory standards. Earlier studies with racemic DHT had characterized the binding to MUP (Bacchini *et al.*, 1992; Zidek *et al.*, 1999) and some behavioral effects on mice (Novotny *et al.*, 1985, 1990). We wanted to extend these studies to DHT enantiomers. As a behavioral test of the substances, competitive scent marking was used, namely, the tendency displayed by a male mouse to countermark with his own urine, spots of urine released by other mice (Hurst, 1990; Humphries *et al.*, 1999).

Materials and methods

Animals

Male Albino Swiss mice, ~7 months old, were maintained at 23 ± 1°C, with 60 ± 5% relative humidity, on a 12 h light:12 h dark cycle with lights on at 6 a.m. The mice had free access to a dry mouse diet (16% protein, 2% fat) and water. The mice were raised in plastic cages $42 \times 26 \times 15$ cm^3 at a density of 5–6 mice per cage. The bedding of wood sawdust was changed twice a week. Fifteen days before the experiment the male mice were isolated in cages of the same



R isomer **S** isomer

Figure 1 The chemical structure of the two enantiomers, (*S*)-DHT and (*R*)-DHT.

size. The experiments conformed to the Italian law on animal experimentation and handling.

Urine collection and organic extraction

Isolated male mice were placed over a grid in clean cages for 2–3 h in the morning and the voidings were collected from the floor of the cage with a pipette. Voidings of >0.1 ml were not collected. Urine (1 ml) was extracted with one volume of dichloromethane shortly after collection. The phases were separated by sedimentation (2600 *g*, 1 min in a refrigerated CS-15R Beckman centrifuge) and the organic phase was recovered. Before chromatography, the volume was reduced to ~1/10 by flushing a gentle stream of nitrogen on the surface through a Pasteur pipette. To test racemization, urine (with NaN_3 0.02% as a bacteriostatic) was left overnight at room temperature, 37°C or 60°C before extracting. To test volatility, fresh urine (1 ml) was spotted on 3MM chromatography paper (Whatman International, Ltd, Maidstone, UK) and dried in a sterile airstream (0.37 m/s) for 2, 5 or 8 h. The paper was then extracted with 2 ml of dichloromethane and the extract was reduced to ~1/20 before chromatography.

Chiral chromatography

Samples of (*S*)-DHT [enantiomeric excess (e.e.) 97.2%] and (*R*)-DHT (e.e. 96.7%), 5% in hexane solution, were kindly provided by Prof. Kenji Mori (University of Tokyo). Upon arrival, the samples were diluted in absolute ethanol to make a 0.5% solution and stored at –20°C. Eighteen months later, at the end of the experiments, chiral chromatography showed that (*S*)-DHT had not racemized appreciably and the (*R*)-DHT solution contained 6.5% (*S*)-DHT.

All gas chromatographic (GC) analyses were performed using a Varian CP-3800 gas chromatograph equipped with a split capillary inlet system and a flame ionization detector interfaced to the Star Chromatography Workstation. The injector and detector temperatures were 200 and 300°C, respectively. Helium was used as the carrier gas with an inlet pressure of 8.0 kPa, linear velocity of 1.3 ml/min and split ratio of 20:1. The capillary GC column, Chiraldex-TA (Trifluoroacetyl Derivative), was obtained from Advanced Separation Technologies, Inc. Astec (Whippany, NJ). Trifluoroacetylation of the 3-position hydroxyl groups after pentylation of the 2,6-hydroxyl groups creates a phase with high selectivity for oxygen containing analytes in the form of alcohol, ketones, acids, aldehydes and lactones. The oven temperature was kept for 5 min at 70°C and then raised to 140°C at 1°C/min rate. With these experimental conditions we obtained a relative retention factor α of 1.02 in five control runs. An ~3% contamination of one enantiomer over the antipode could be detected. Mass spectrometry was performed on a HPLC-MS Mariner API-TOF (PerSeptive Biosystem, Framingham, MA). The mass range was 100–1000 Th. The column flow was 16 µl/min of acetonitrile:water (1:1) and 1% formic acid. Mass spectrometry confirmed that the two enantiomers had not decomposed during transport and storage.

DHT–MUP binding isotherms

MUP was isolated from adult male mouse urine by ammonium sulfate precipitation (50–70% saturation at 0°C), extensive dialysis against 9% NaCl and concentration with polyethylene glycol (17 000–20 000 mol. wt). Storage was at –20°C. Binding equilibrium at room temperature between MUP and DHT isomers was obtained by the diffusion of the ligands in air (Ferrari *et al.*, 1997). Briefly, a 50 µl drop of MUP (0.7 mg/ml) in 10 mM phosphate buffer solution, pH 7.0, was left hanging from the cover of a glass Petri dish whose surface had been lightly made hydrophobic by silanization. The dish contained phosphate buffer to which an excess of the tracer ligand [^{3}H]2-isobutyl-3-methoxypyrazine and DHT had been added. Forty-eight hours later, the drop was absorbed on paper and the radioactivity measured with 2% precision. Five binding isotherms were determined with 1.0 or 0.5 µM tracer and 0, 37.5 or 75 µM DHT isomer. The inhibition constant, K_I, was determined assuming competitive inhibition according to the relationship

$$\frac{1}{B} = \frac{K_m}{B_{max}} \frac{\left(1 + \frac{I}{K_I}\right)}{S} + \frac{1}{B_{max}}$$

where B and S are the bound and free tracer concentrations respectively, K_m/B_{max} is the ratio of the binding constant of the tracer to the maximal binding, I is the free DHT concentration and K_I the inhibition constant of DHT. It was finally assumed that $K_I = K_m$(DHT), the binding constant of DHT. The parameters of the equation were derived from the experimental data by least squares approximation.

Behavioral test

Twelve male mice, ~7 months old, were tested in the morning in a period ranging from January to April. Each mouse was tested in a cage of the same dimensions as the home cage. The bottom of the cage was covered with a rectangular sheet of polythene-backed absorbing paper (BenchGould, Whatman International), with the polythene side down. The sheet was divided in three regions with two faint pencil lines, the central region being twice as large as the lateral regions: 10.5/21/10.5 cm respectively. The test

substance was spotted in the center of one lateral region and the control in the opposite region. The central region was left clean. Under dim light, a mouse was placed in the central region and left free to move for 20 min under inspection. The voidings, the drops, the time to the first voiding and the fecal pellets were counted, both in the test and control region. The cage was half turned after every third test to balance any effect of orienting cues external to the cage. The test stimuli were (i) either 75 or 2.5 µg of (*S*)-DHT or (*R*)-DHT in 15 µl of ethanol; (ii) 15 µl of unfamiliar male mouse urine, collected either shortly before the test or 2 days before the test and left over 3MM chromatography paper in a chemical hood until used; and finally (iii) 0.8 mg of MUP, with natural urinary ligands bound, in 15 µl of 10 mM phosphate buffer solution. In a number of trials the stimuli were combined. Controls were the carrier solutions.

Statistical analysis

The behavioral data were analyzed with the Wilcoxon matched-pairs signed-ranks test for experimental and control data, and with the Wilcoxon test for data that were not matched.

Results

The absolute configuration of DHT

A chiral capillary column Chiraldex TA and mild chromatographic conditions were found which allowed the separation of the DHT isomers, with approximate retention times of 35 min 20 s for (*S*)-DHT and 35 min 58 s for (*R*)-DHT. In these conditions, a 3% contamination of one isomer over the other could be detected. When dichloromethane extracts of adult male mouse urine were analyzed, DHT was always detected. The comparison of the retention times of urinary DHT with the synthetic standards of (*S*)-DHT and (*R*)-DHT identified urinary DHT as (*S*)-DHT while (*R*)-DHT, if present, was below the detection limit, as seen in Figure 2.

The racemization of DHT in urine was also studied. Only (*S*)-DHT was extracted in sizable amounts from samples of urine left either at room temperature or at 37°C overnight before extracting, whereas no DHT was recovered from a sample left at 60°C. The evaporation of (*S*)-DHT of urine absorbed on paper and dried in air flux for 2 h or longer was also investigated. No appreciable (*S*)-DHT was extracted after drying, suggesting that a substantial amount had diffused in air in a two h lapse of time.

Binding constant of (*S*)-DHT and (*R*)-DHT to MUP

The binding of DHT isomers to the MUP complex (isolated from male mouse urine) was studied by diffusion equilibrium (48 h) of the isomers through air, using the competitive displacement of [^{3}H]2-isobutyl-3-methoxypyrazine (a high-affinity ligand). The binding isotherms fitted a competitive displacement and the binding constant for (*S*)-DHT was estimated at 8.2 ± 0.6 µM, and at 10.5 ± 0.6 µM for (*R*)-DHT (mean ± SD, $n = 5$) (Figure 3).

(*S*)-DHT and the competitive scent marking (countermarking) response

Twelve male mice were tested individually in a cage with 15 µl of the test isomer spotted on one side of the cage (test region) and the control in the other side (control region), see the Methods section. The observation lasted 20 min and the

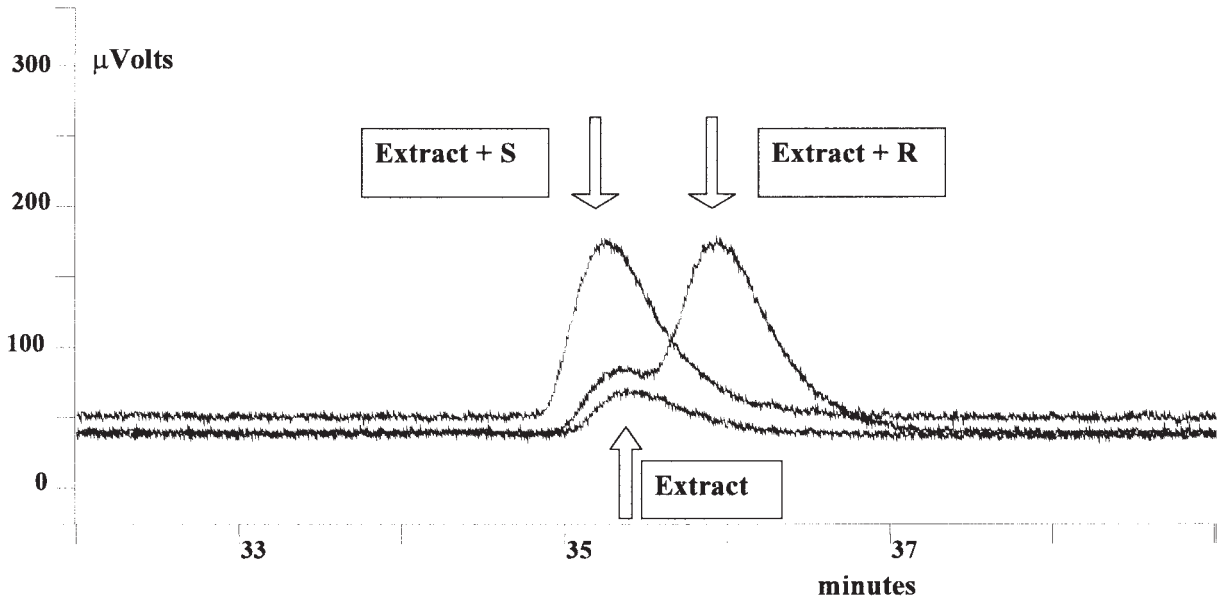


Figure 2 Chiral gas chromatography of urine extract without and with DHT standards added. Chiraldex TA 30 m capillary column, He carrier gas at 1.3 ml/min and split ratio 20:1. Oven temperature 70–140°C at 1°C/ min rate. Relative retention factor $\alpha = 1.02$. Enantiomeric resolution 3% of one isomer over its antipode. S, (*S*)-DHT; R, (*R*)-DHT.

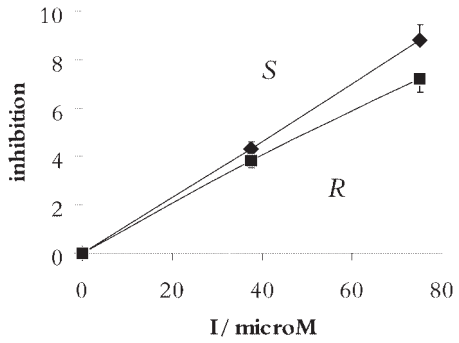


Figure 3 Inhibition of [^{3}H]2-isobutyl-3-methoxypyrazine binding to the MUP complex by (*S*)-DHT and (*R*)-DHT (mean ± SD, $n = 5$). On the ordinate I/K_I (see Materials and methods) as a function of DHT isomer concentration, I. S, (*S*)-DHT; R,for (*R*)-DHT. Inhibition derived from five binding isotherms at room temperature after 48 h of equilibration through air. The MUP complex was isolated from adult male mouse urine by ammonium sulfate precipitation followed by dialysis.

number of voidings, the drops voided, the time to the first voiding and the fecal pellets released were counted. (*S*)-DHT modified this response in a peculiar way. Table 1, which represents the data from five experiments and whose stimuli are written on the left, reports the voidings, the drops, the time for the first voiding (T_1) and the fecal pellets counted during the test, both in the test (first line) and control region (second line). Significant differences between data are shown using the same superscripts.

In experiment 1, male mice were slow in countermarking spots of unfamiliar male mouse urine, that had been streaked 2 days earlier and on top of which (*S*)-DHT (75 or 2.5 μM) had been added shortly before the test, whereas they promptly countermarked the spots with (*R*)-DHT added. In experiments 2 and 3, DHT isomers (75 μM) did not modify the response when presented on a spot of fresh urine (experiment 2) or on clean paper (experiment 3). Experiments 4 and 5 are controls without added DHT. In experiment 4, male mice significantly countermarked 15 μl spots of fresh and 2-day-old unfamiliar male mouse urine, though to an extent that did not reach statistical significance. In experiment 5, male mice promptly countermarked spots of MUP, in agreement with Humphries *et al.* (1999).

Discussion

The data show that male mice excrete (*S*)-DHT in urine whereas its antipode, if present, is <3% of (*S*)-DHT. This observation should be compared with the racemic composition obtained out of a nickel complexation GC column by Novotny *et al.* (1995). The reason of the discrepancy is not clear. In the present work, however, the samples were analyzed in mild chromatographic conditions, a trifluoroacetylated stationary phase with weak molecular interactions and a low temperature, and this could have contributed to avoiding racemization.

The absolute configuration of various natural products with a $C(CH_3)C_2H_5$ system has been determined in a variety of organisms. Insect pheromones such as trogodermal, the dermestid beetle (*Trogoderma inclusum*) pheromone (Mori, 1973; Mori *et al.*, 1978), the pine sawfly (*Microdiprion pallipes*) pheromone (Nakamura and Mori, 1999) and alkaloids isolated from the Caribbean marine sponge *Axinella* sp. (Seki and Mori, 2001), all possess an *R* configuration. On the other hand, (+)-isoleucine and optically active (–)-2-methylbutan-1-ol in fusel oil possess the *S* configuration (Barton, 1999). Hence, while insects and sponges possess chemosignals in the *R* configuration, mice possess the *S* configuration.

(*S*)-DHT produced by mice seems to be stable in urine over time for hours and the standards diluted in ethanol proved to be stable over months. This observation makes it unlikely that (*S*)-DHT racemization is a significant time signal for the mouse (Carman, 1993). The stability of (*S*)-DHT is surprising because its stereospecific laboratory synthesis was reported to be plagued by a high rate of racemization (Novotny *et al.*, 1995), neat (*S*)-DHT racemizing 6% at room temperature over 5 days (Tashiro and Mori, 1999) and a 10% racemization in 1 h was estimated in methanol-d_4 by deuterium exchange experiments monitored spectroscopically by Novotny *et al.* (1995). The reason of the discrepancy remains unknown but the present observation made in urine would seem more physiological. On the other hand, no appreciable amount of (*S*)-DHT could be extracted from dry spots of urine. This observation is in agreement with an ~60% loss from urine spots left over glass at room temperature for 1 h, as reported by Hurst *et al.* (1998). It is conceivable that most of the (*S*)-DHT diffuses gradually in air as a spot dries in the field. To this point, it has been shown that binding to MUP may prolong the life of volatile chemosignals (Hurst *et al.*, 1998). It cannot be excluded that a fraction of (*S*)-DHT is oxidized by the atmospheric oxygen perhaps to the sulfoxide derivative.

Binding experiments of DHT enantiomers to the MUP complex confirm computer assisted predictions of DHT docking to the binding site that tended to exclude relevant differences between the two isomers (Zidek *et al.*, 1999). These present binding constants agree with that determined earlier for racemic DHT by competitive displacement using the MUP complex (Bacchini *et al.*, 1992) and a recombinant isoform (Ferrari *et al.*, 1997), but are ~10 times greater than that determined by gas chromatography, 1.3 ± 0.1 μM, and by isothermal calorimetry, 0.74 ± 0.04 μM (Zidek *et al.*, 1999; Sharrow *et al.*, 2003), still using racemic DHT. The difference may be due to the different isolation procedures of MUP or, more interestingly, to a structural change induced by the substitution of the tracer ligand in the MUP hydrophobic pocket. The affinity determined here does not refer to any particular MUP isoform since the entire complex of MUP isolated from male mouse urine was used without fractionating the different isoforms. Different

Table 1 Competitive scent marking (mean and SEM, $n = 12$) in 20 min tests with S- or R-DHT isomer (first line) and without (second line)

Stimulus	Voidings		Drops		T1 /s		Pellets	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Experiment 1								
S-DHT[1] + old urine	0.54	0.37	1.72	1.16	1005[ab]	113	1.54	0.43
Old urine	1.09	0.31	3.00	1.02	439[b]	158	1.54	0.31
R-DHT[1] + old urine	1.91[c]	0.69	5.18[d]	1.78	464[ae]	161	1.42	0.53
Old urine	0.72[c]	0.38	1.72[d]	0.87	852[e]	157	1.82	0.44
S-DHT[2] + old urine	1.50[f]	0.10	3.41	2.02	566[g]	871	2.83	1.75
Old urine	2.50[f]	0.79	4.58	2.50	205[g]	217	3.75	1.66
R-DHT[2] + old urine	4.27	0.87	9.18	2.33	372	160	2.91	0.68
Old urine	4.55	0.80	9.72	2.21	236	119	2.54	0.66
Experiment 2								
S-DHT[1] + fresh urine	1.12	0.42	3.33	1.22	561	164	0.83	0.30
Fresh urine	1.08	0.29	1.91	0.54	535	161	0.66	0.22
R-DHT[1] + fresh urine	1.08	0.45	3.25	1.22	650	157	0.83	0.21
Fresh urine	1.01	0.27	1.75	0.66	486	157	1.66	0.30
Experiment 3								
S-DHT[1]	1.01	0.33	1.41	0.65	706	133	2.17	0.32
Control	0.50	0.15	1.01	0.42	879	105	1.91	0.36
R-DHT[1]	0.66	0.14	1.75	0.70	750	136	2.83	0.41
Control	1.01	0.33	1.41	0.45	730	143	2.58	0.61
Experiment 4								
Fresh urine	1.25[h]	0.35	2.41[i]	0.79	552[j]	144	0.67	0.28
Control	0.17[h]	0.11	0.17[i]	0.11	1090[j]	75	1.16	0.38
Old urine	1.17	0.30	2.50	0.99	553	153	1.33	0.59
Control	0.58	0.23	1.33	0.50	903	123	0.58	0.23
Experiment 5								
MUP[3]	3.17	0.66	6.42	1.70	223[k]	95	2.82[l]	0.53
Control	1.50	0.44	2.75	0.93	593[k]	123	2.00[l]	0.39
Blank								
—	3.42	2.71	7.08	5.42	358	491	2.25	1.36
—	4.08	3.35	8.58	7.46	438	513	3.58	2.09

[a,h,i,j]$2P \leq 0.01$; [b,c,d,e,f,g,k,l]$2P \leq 0.05$.
[1]75 µg, [2]2.5 µg; [3]0.8 mg.

isoforms may bind different isomers with different affinity and one isoform may bind one isomer and another to its antipode with different affinity (Hurst *et al.*, 1998). The present experiments show, however, that the affinity for the two isomers, determined using the MUP complex as isolated from male mouse urine, does not explain a selective excretion of (*S*)-DHT starting from a racemic composition of DHT. It is more likely that (*S*)-DHT is the metabolic product, although the metabolic pathway is not known. Novotny proposed that (*S*)-DHT could derive from the reaction of (+)-isoleucine with cysteine (Novotny *et al.*, 1995). The site of synthesis is unknown although thiazoline derivatives are also found in feces, probably as products of microbial metabolism.

The present behavioral result that (*S*)-DHT at a physiological concentration delays countermarking is in agreement with earlier observations that DHT is a chemosignal associated with the aggressive status of the mouse (Novotny *et al.*, 1985, 1990). Another thiazoline, 2,4,5-trimethylthiazoline, a component of the fox feces, is an unconditioned fear chemosignal which induces the freezing reaction and primes endocrine modifications in rodents (Wallace and Rosen, 2000; Tanapat *et al.*, 2001). We do not know whether the aversive properties of dominant mouse urine (Jones and Nowell, 1989) are due exclusively to (*S*)-DHT and farnesenes, another class of MUP ligands found in male mouse urine (Jemiolo *et al.*, 1992) since mice are likely to respond to a variety of odorants of male mouse urine rather than to a single odorant. Unlike (*S*)-DHT, synthetic (*R*)-DHT attracts countermarking instead of delaying it, confirming that rats discriminate members of a wide range of enantiomeric pairs (Rubin and Katz, 2001). In countermarking experiments, attraction had been observed earlier when adding menadione, a MUP ligand that is a product of microbial metabolism in the intestine as well as a component of a mouse's diet (Humphries *et al.*, 1999). We do not know, aside from (*R*)-DHT and menadione, whether any MUP ligand which is not normally present in appreciable amounts in urine becomes a novelty stimulus which attracts countermarking when added to urine (Matthews, 1980).

It had been shown earlier that DHT modifies male mouse aggression provided it is given in a urinary context rather than as a pure compound (Novotny *et al.*, 1985). The present data show, moreover, that adding (*S*)-DHT delays countermarking of 2-day-old urine spots, but not of fresh ones. What makes the difference is not clear but it is conceivable that a balance of attracting and aversive odorants determines the response. The chemical identification, the behavioral characterization and the gradual release in time of all urinary components have not yet been carried out, to our knowledge. Once more, this shows how little we know about chemosignaling among mice through urinary cues.

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