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Stereoselectivity in mammalian chemical communication: male mouse pheromones

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Abstract. Two male mouse pheromones, 3,4-dehydro-*exo*-brevicomin (DHB) and 2-*sec*-butyldihydrothiazole (SBT), are chiral molecules which were previously tested in their respective bioassays as racemic mixtures. The focus of this study has been to determine the absolute configuration of their natural forms and its relation to stereospecific biological action. DHB was established as the R,R-enantiomer possessing biological activity. Due to an extremely easy racemization of SBT under very mild conditions, enantioselectivity of its transmission and its action at the receptor site appear to be of secondary importance.
Key words. Male mouse pheromones; stereospecific response; chromatographic resolution of optical isomers; estrus synchronization.

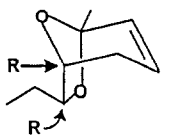
Chemical communication through active urinary substances (pheromones) regulates much of the social behavior and reproductive physiology of *Mus domesticus*, the common house mouse[1,2]. Mouse urine contains numerous volatile substances[3], some of which are utilized as important chemosignals. We have previously implicated two structurally unique compounds, 3,4-dehydro-*exo*-brevicomin (DHB)[4,5], and 2-*sec*-butylthiazoline (SBT), as the male mouse pheromones: DHB and SBT act, synergistically, in promoting intermale aggression[6], sexual attraction[7], and estrus synchronization (Whitten effect) in female mice[8].

The structural uniqueness of both DHB and SBT resides in their optical activity (fig. 1). While these compounds were originally synthesized[4,5] for biological tests[6–8] as racemic mixtures, it would appear reasonable that only one enantiomer for each molecule is formed during their biosynthesis. Stereospecificity of odor perception[9,10], in general, further underscores the well-known importance of enantioselectivity in biological processes. In fact, studies on insect pheromones stress importance of stereoselectivity: sex attractant of the gypsy moth[11], aggregation pheromone of the ambrosia beetle[12,13] and the alarm pheromone of the leaf cutting ant[14] are among the examples of such specificity. A modern, comprehensive theory of olfaction takes into account a three-point interaction between odor stimuli and olfactory receptors[15].

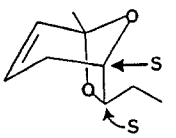
In this communication, we wish to report our findings on the optical activities of DHB and SBT excreted in the male mouse urine and their relation to biological activity. Utilizing the recent advances in the enantiomeric separation, we were able to resolve both pheromone components into their optical antipodes and compare the natural material with the synthesized[5,16,17] materials. While the natural DHB was positively identified as having the R,R absolute configuration, SBT possesses a more 'flexible' structure. Racemization of SBT occurs so readily that such a structural arrangement, as seen by mass-spectral (MS) and nuclear magnetic resonance (NMR) experiments, can easily take place under physiological conditions. The estrus synchronization tests[6], carried out at two different dilutions of the pheromones in a testing solution, provide evidence that, at least for DHB, a correct stereochemistry is important for biological activity.

Materials and methods

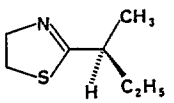
Isolation of the pheromones from mouse urine. Mouse urine for the isolation of pheromones and bioassays was collected from 12 adult (3–4 months old) C57BL/6J male mice. The urine collection took place in metal metabolism cages for 24-h periods. During the entire collection period the urine containers were kept in dry



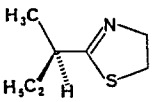
(R,R)-3,4-dehydro-*exo*-brevicomin



(S,S)-3,4-dehydro-*exo*-brevicomin



(S)-2-*sec*-butyl-4,5-dihydrothiazole



(R)-2-*sec*-butyl-4,5-dihydrothiazole

Figure 1. Different optical activities of DHB (above) and SBT (below).

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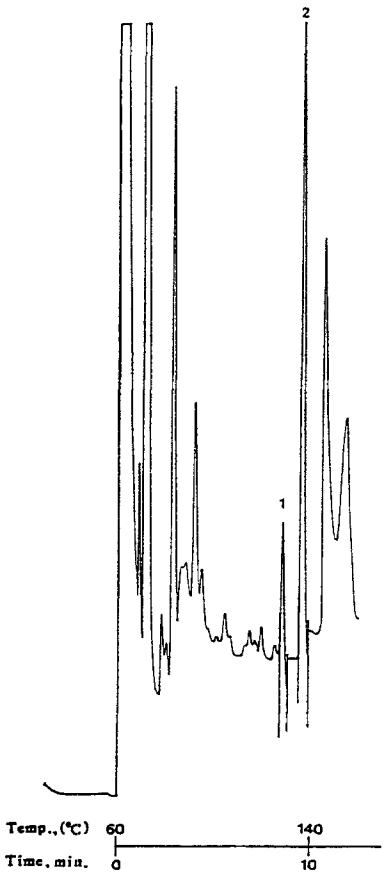


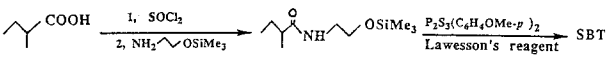
Figure 2. Gas chromatogram of the ether extract of the male mouse urine obtained with a preparative-scale Carbowax 20M column. Peak 1: SBT; peak 2: DHB.

ice. Immediately after collection, the urine specimens were stored at −20 °C until their use for chemical or biological investigations.

80 mL volume of collected urine was filtered and extracted with two 80 mL-aliquots of diethyl ether. The organic layers were combined, dried over Na_2SO_4, and concentrated approximately 100-fold by the use of a Kuderna-Danish apparatus. This solution was further concentrated to about 100 µL under a stream of dry nitrogen. The isolation of DHB and SBT was done through a preparative-scale gas chromatography (Varian 3700 gas chromatograph equipped with a thermal conductivity detector, 3 m × 2 mm, i.d., stainless steel column, packed with 10% Carbowax 20M on Supelcoport solid support, 100/120 mesh). The materials eluted from the gas-chromatographic column (fig. 2) were collected on a small cartridge of Tenax GC adsorbent (Applied Science Laboratories, State College, PA), packed in 3 mm, i.d., injection liners, and then desorbed in an injector of a gas chromatograph, and condensed in a U-shaped tube, immersed in liquid nitrogen.

Syntheses of DHB and SBT. The racemic 3,4-dehydro-*exo*-brevicomin was synthesized as described in our earlier report[5]. The R,R- and S,S-3,4-dehydro-*exo*-brevicomin were synthesized through the carbonyl-epoxide rearrangement in conjunction with the Sharpless asymmetric epoxidation procedure, as reported by Wasserman and Oku[17], and purified by near-critical fluid chromatography[18]. The structure of these products was confirmed by infrared spectra (IR Perkin-Elmer Model 298 spectrometer), proton magnetic resonance spectra (^{1}H NMR, Varian Enl-390 spectrometer), and mass spectra (Hewlett Packard 5992A gas chromatograph/mass spectrometer).

The synthesis of SBT is described in the following scheme:



(1)

A solution of 4.57 mL (41.9 mmol) 2-methylbutanoic acid and 3.06 mL (42.6 mmol) thionyl chloride in 17 mL chloroform was heated under reflux for 1 h, cooled, and added per drop at 5 °C to a solution of 5.58 g 2-(trimethylsilyloxy)ethylamine[19] and 5.83 mL (41.9 mmol) triethylamine in 8 mL chloroform. Removal of chloroform by rotary evaporation, substitution of ether, filtration, drying of the filtrate (by $MgSO_4$), and reconcentration yielded 7.51 g acceptably pure N-(2-trimethylsilyloxyethyl)-2-methylbutanamide (**1**), 34.6 mmol, 83%.

To a solution of 6.42 g (29.6 mmol) (**1**) in 15 mL toluene was added 5.98 g (14.8 mmol) Lawesson's reagent (Aldrich Chemical Co., Milwaukee, WI). The mixture was heated under nitrogen at 100 °C for 3.5 h, cooled, and divided between ether and aqueous sodium bicarbonate. The dried organic phase was subjected to rotary evaporation, followed by fractional distillation. 2.40 g (16.8 mmol, 58%) SBT (identity determined by NMR) was collected, b.p. 74–77 °C at 10 torr.

A search of the literature for a suitable general synthesis of 2-alkyl-4,5-dihydrothiazoles revealed a one-step method, attributable to Ohta et al.[20], which involves heating the corresponding nitrile with 2-aminoethanethiol in refluxing ethanol. We have adapted this method to the synthesis of SBT, but no equally attractive method based on the more readily available corresponding carboxylic acids was revealed. We, therefore, determined to convert intermediate (**1**) (readily prepared from 2-methylbutanoic acid by standard methods) to the corresponding thioamide using Lawesson's thionation reagent[21] and proceed to SBT by desilylation and conversion of the resulting hydroxyl group to a suitable leaving group. SBT appeared as the principal product of the thionation, in ca. 60% yield as judged by GC, with no indication of thioamide.

The synthesis of 4,5-dihydrothiazole used by us require several hours' heating in the key step. The thionation step of our synthesis is done under acidic conditions,

while Ohta's reaction is carried out at near-neutral pH. However, each of the two methods, when applied to chiral substrates (S-2-methylbutanoic acid or 2-methylbutanenitrile) resulted in total racemization of the product.

Gas-chromatographic separation of DHB enantiomers. The columns used were a 30 m × 250 µm, i.d., Cyclodex-B fused silica column provided by J & W Scientific (Folsom, CA), and a 50 m × 250 µm, i.d., Pyrex column statically coated with OV-101 containing 0.06 M manganese(II)-bis(heptafluorobutyrylcamphor) which was synthesized according to Schurig and Weber's method[22]. The columns were installed in a Varian 3700 gas chromatograph or a Carlo Erba 4160-02 gas chromatograph. Both gas chromatographs were equipped with a flame ionization detector. The oven temperature was kept at 60 °C for the Pyrex column and programmed from 30 °C to 120 °C at 2 °C/min for the Cyclodex column. Injector temperature was maintained at 240 °C and detector temperature at 260 °C. The sample injected was 0.1 µL, at a split ratio of 1:50. The natural DHB was also analyzed using a headspace sampling technique[23]. DHB was sparged from a 1-mL mouse urine sample together with other volatiles at room temperature with purified helium gas at a flow rate of 100 mL/min and adsorbed onto a precolumn packed with 4 mg of Tenax GC. The sample was subsequently desorbed in the heated injection port of a gas chromatograph. The chiral resolution of DHB was then observed with the combined gas chromatograph/mass spectrometer.

Gas-chromatographic separation of SBT isomers. The column used was a 25 m × 250 µm, i.d., fused silica column coated with a 0.2 µm film of the stationary phase containing Ni-"s"-V-Cam provided by CC&CC Service (Kirchentellinsfurt, Germany). The column was installed in a Carlo Erba Fractovap model 4160-02 gas chromatograph. The column was wrapped in aluminum foil in order to minimize thermal peak splitting. The oven temperature was 120 °C. Nitrogen carrier gas pressure was 3.3 bar. 0.2 µL sample was injected, at a split ratio of 1:22.

Biological testing. Females used in this study were 3–4 months old C57/6J mice. Initially, all animals were housed under crowded conditions (10 animals per cage), without disturbance, for 3 weeks. Following this adaptation period, the females were randomly divided into seven groups. Each female was checked daily for the presence of vaginal estrus. This examination (without exposure to an olfactory stimulus) lasted 10 days ('control conditions'). During the following 10-day period ('experimental conditions'), different olfactory stimulus samples were introduced to the individual groups of females: group 1: water (n = 30); group 2: male urine (n = 20); group 3: racemic DHB (n = 20); group 4: S,S-DHB at 1.3 p.p.m. (n = 20); group 5: R,R-DHB at 1.3 p.p.m. (n = 30); group 6: S,S-DHB at 0.65 p.p.m. (n = 20); and, group 7: R,R-DHB at 0.65 p.p.m. (n = 20). All females continued to be examined for the presence of vaginal estrus. Vaginal smears were taken daily between 15.00 and 16.00 h.

A Petri dish (5 cm, i.d.) containing a 0.5 mL sample aliquot was placed in the center of the cage housing the females. During the exposure period, the Petri dish with test sample or water (control condition) was placed in a wire-mesh hemicycle (5 cm i.d. and 2 cm height) that prevented a physical contact with the sample but allowed exposure to the airborne olfactory cues. The animals were exposed to various olfactory stimuli daily for 2 h (16.00–18.00 h) for 10 days. The stimulus samples were aqueous solutions of the pheromone synthetic analogs provided at the concentration of a) 0.65 p.p.m. (low concentration) and b) 1.3 p.p.m. (natural urinary concentration), or a natural stimulus (male mouse urine).

Deuterium exchange in SBT. a) With deuteriated water: 4 µL 1% SBT (in hexane) was spiked into 2 mL D_2O. At predetermined time intervals and temperatures, 1-mL samples were analyzed by GC-MS using the headspace sampling procedure[23]. b) With deuteriated methanol: 5 µL 1% SBT was spiked into 1 mL CD_3OD. Simultaneously, 0.2 µL samples were directly analyzed by GC/MS. The exchange ratios were also determined through comparison of the relative peak intensities of the characteristic fragments. For confirmation, the exchange ratios were also determined by nuclear magnetic resonance. The proton NMR spectra were recorded with a 300-MHz instrument. The deuterium exchange ratios were calculated by comparison of the resonance peak areas of characteristic protons. A pH dependence of the deuterium exchange processes was followed.

Results and discussion

The separation of DHB enantiomers could be accomplished on either the manganese-chelate column or the Cyclodex capillary. These column types operate on different selectivity principles: while the complexation chromatography[22] is involved in resolving the optical isomers on the column containing a small percentage of the heptafluorobutyrylcamphor complex of manganese, the cavity size of a cyclodextrin derivative attached to the stationary phase backbone is primarily responsible for the differential migration of the isomers. The cyclodextrin-based column yielded greater resolution (relative retention, $\alpha = 1.06$) than the manganese complex column ($\alpha = 1.03$), but both could easily establish that the natural DHB is of the R,R configuration. The individually synthesized optical isomers were used to determine the order of elution. Figure 3 compares the chromatograms of the synthesized[5] DHB racemic mixture (A) with the urinary volatile profile (B) and the

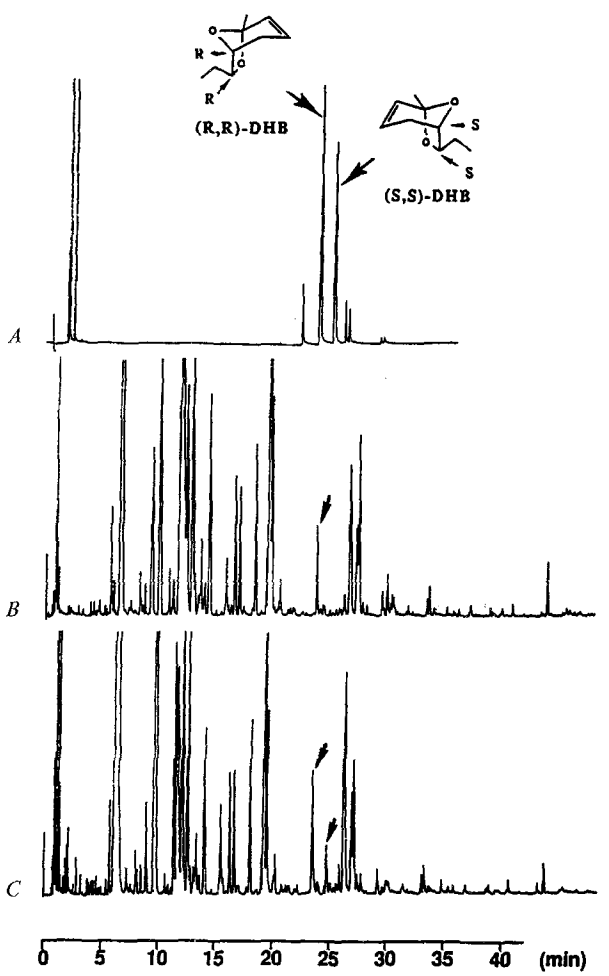


Figure 3. Capillary gas-chromatographic profiles of *A* synthetic DHB enantiomeric mixture, *B* male mouse urinary volatiles, and *C* male mouse urinary volatiles spiked with racemic DHB.

same urinary sample spiked with the racemic DHB (C). The GC/MS data confirmed the identity of urinary DHB and the two major components of the racemic synthetic mixture (the two minor peaks eluting later (part A) represent the enantiomers of the brevicomin byproduct). We could also verify that no appreciable racemization of the natural R,R-DHB occurred due to the micropreparative recovery of DHB after gas chromatography.

Working with SBT proved considerably more difficult. First, under a variety of synthetic conditions, either freshly prepared SBT enantiomers or their precursors quickly turned into a racemic mixture (as judged by the optical rotation measurements). Second, we encountered difficulties resolving the SBT enantiomers chromatographically; neither the cyclodextrin-based stationary phase nor the manganese complex column could resolve the racemate under any set of experimental conditions. Procedures leading to the formation of diastereoisomers (introduction of a second chiral center into the molecule) were equally unproductive. A custom-made (nickel complexation), specialty capillary

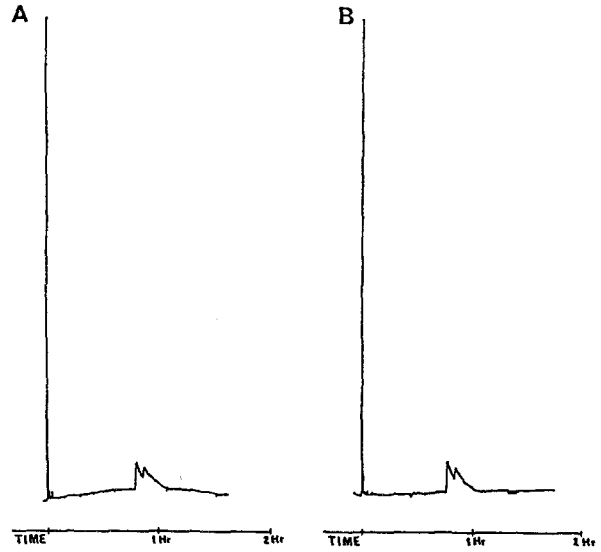
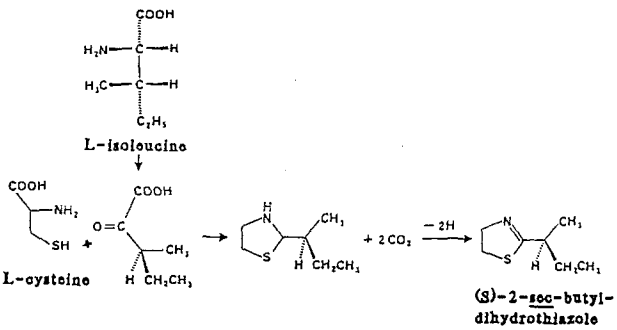


Figure 4. Capillary gas chromatograms of *A* synthetic SBT, and *B* natural SBT after recovery from a GC packed column.

column provided a partial resolution of the synthetic racemic SBT and the natural material trapped from a gas-chromatographic column (fig. 4A and B). The evidence that both resolved peaks are SBT was further secured through their mass spectra using the GC/MS combination.

Comparing (A) and (B) parts of figure 4, however, suggests that SBT isolated from the natural source is racemic. It is reasonable to expect that the natural compound is configured as S-SBT if it is metabolically derived from the natural forms of the amino acids isoleucine and cysteine, in which the oxidative deamination of isoleucine can yield 2-oxo-3-methylpentanoic acid, forming a Schiff base with cysteine and, along with two decarboxylations and ring closure, provide the thiazoline ring structure:

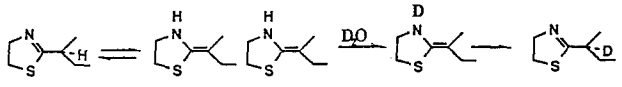


(2)

Consequently, the racemization process is likely to occur either during the isolation (extraction) procedure or due to the relatively high (140 °C) temperature of the packed GC column. The alternative ways of isolation that we tried, such as liquid chromatography, solid-

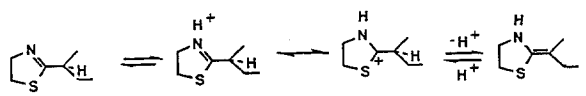
phase extraction, and supercritical fluid chromatography, always produced a racemic SBT.

If the natural isoleucine is the logical precursor of SBT, its chirality is expected to be transferred to the pheromone (SBT). However, racemization at C-3 becomes increasingly possible due to a tautomerization process affecting the adjacent carbon atom on the ring. To investigate this possibility, a brief deuterium exchange experiment was carried out and monitored spectroscopically:



(3)

The results indicated that deuterium exchange on SBT occurs with either D_2O or methanol-d_4. At 60 °C, exposure to methanol-d_4 for 2.5, 11.5, and 30 h caused the degree of deuteriation to be 8, 10, and 16%, respectively, while the exposure to D_2O gave similar figures up to 200 h in the solution. At 26 °C, only 10% exchange was observed after 10 h in methanol-d_4. Importantly, the pH of the medium greatly influenced the deuterium exchange process: at pH = 1.0 for 3 h, 73.4% conversion was observed, while pH = 11.0 produced only 10.6% deuteriated product during the same period. Obviously, the acidic medium may promote racemization more readily than the alkaline medium. Protonation of the nitrogen atom is expected to enhance the lability of the *alpha* proton:



(4)

It appears that the usual acidity of the mouse urine (pH ≈ 5.0) may promote racemization of a biosynthetically derived SBT. Likewise, however, relatively low enantiomer conversion could also occur at the site of olfactory reception. Given the racemization difficulties with SBT, the efforts to solve this problem appear counterproductive.

The proposition of enantiomeric selectivity of the pheromone reception could still be tested biologically with the individual optical isomers of DHB (in presence of racemic SBT). We have shown previously[8] that the two compounds act, synergistically, as the primer pheromone of male mice to restore the normal activity of ovaries and normal length of estrous cycle in the females. We have established in this work that the natural mouse urinary DHB is the R,R-enantiomer. Is the female mouse olfactory system responsive to this isomer alone?

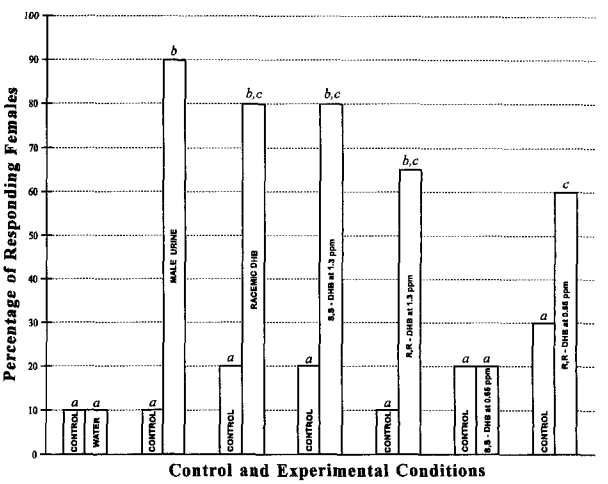
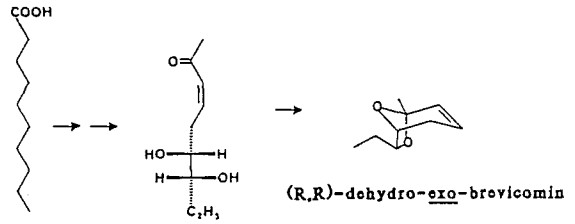


Figure 5. Percentage of female mice exhibiting a regular estrous cycle after treatment with different chemosignals. The bars marked with different letters (*a*, *b*, *c*) are statistically significant at $p < 0.02$.

An obvious limitation to studies involving the biological activity of enantiomeric compounds is their optical purity. With the present means of ensuring such optical purity (stereospecific synthesis or highly effective preparative separation of the enantiomers from each other), it has been nearly impossible to work with pure compounds. Chromatographic analysis of the stereospecifically synthesized DHB enantiomers[17], under the conditions of figure 3, has shown approximately 80% purity of each isomer. While the racemic mixture[5] tested for its effect on the estrous cycle of female mice reported by us earlier[8] showed biological activity, similar results would be expected from a mixture where the active enantiomer is roughly a 20% 'impurity'. However, a dilution experiment involving both isomers, tested separately, is likely to answer the question of perception stereospecificity.

Figure 5 shows that the majority of females exposed to racemic DHB (80%), S,S-DHB (80%) and R,R-DHB (65%) (all added at the natural urinary concentration together with the racemic SBT) responded to these stimuli in a qualitatively similar fashion as the females treated with male urine (90%). All females started to have a regular estrous cycle. At the low stimulus concentration, R,R-DHB had, once again, a similar effect on the tested females (60%) as the natural stimulus (male urine), while the S,S-DHB appeared less active (20%). Difference between the females responding to a treatment by having a regular estrous cycle restored (60–90%) and the females not responding (control animals and the females exposed to S,S-DHB at low concentration) was statistically significant. (Level of significance for the multiple comparison using the Tukey test was set at $p < 0.02$.) A lack of intensive investigatory behavior[7] by the tested females toward the low-concentration samples supports the notion that the positive behavioral response to a more concentrated

S,S-DHB is probably due to 'impurity', the R,R-isomer. The biological activity of the R,R-DHB is consistent with its occurrence in the natural stimulus sample. Biosynthetically, DHB of this configuration could arise from the metabolism of decanoic acid:



(5)

An initial step could be β-oxidation of decanoic acid, giving a corresponding β-ketoacid. This intermediate could then lose the terminal carboxyl group as CO_2. Subsequent dehydrogenation of this intermediate at carbon 3 and 4 to give a *cis* double bond, and oxidation of carbon 6 and 7 to give the *trans*-glycol might give rise to a precursor of DHB with the R,R absolute configuration. Further research is needed to verify this pathway.

Acknowledgements. During the initial stages of this study, Professors H. Wasserman and S. Danishefsky, of the Department of Chemistry, Yale University, kindly supplied us with the small samples of dehydro-*exo*-brevicomin enantiomers. Their help has been greatly appreciated.

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