Tissue distribution and substrate specificity of an epoxide hydrase in the gypsy moth, *Lymantria dispar*

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Abstract. The stereoselectivity of the enzymatic hydration of disparlure, the pheromone for the gypsy moth *Lymantria dispar*, and for two *meso* analogues was determined. A single epoxide hydrase (EH), present in various male and female moth tissues, converted disparlure and the analogues to their respective *threo*-(*R,R*)-diols with high stereoselectivity as determined by analysis of the diols by chiral phase capillary gas chromatography. This EH recognizes the *cis*-nature of the dialkyl oxirane, but shows poor discrimination of the two alkyl chains.
Key words. Pheromone analogue; substrate analogue; disparlure; *Lymantria dispar*; gypsy moth; epoxide hydrase; pheromone metabolism; lipophilic site; epoxide; oxirane; stereoselective hydration.

The gypsy moth (*Lymantria dispar*) is a major forest pest in Europe and the United States. Although most moth species use mixtures of compounds as their sex pheromone[2], *L. dispar* apparently uses a single compound, *cis*-7,8-epoxy-2-methyloctadecane (disparlure)[3], as its sex pheromone. Once the pure (+)-(7*R*,8*S*)- and (−)-(7*S*,8*R*)-enantiomers of disparlure[4] (fig. 1) became available, studies showed that 1) the (+)-enantiomer is attractive to *L. dispar* males while a combination of (+)- and (−)-disparlure inhibits attraction of males to females[4–7], and 2) individual sensory hairs of the male antennae possess two separate sensory neurons; one responsive to (+)-disparlure and the other responsive to (−)-disparlure[4,5]. Only (+)-responsive cells are stimulated by pheromone extracts of female *L. dispar*, and this has been interpreted to mean that female *L. dispar* emit only (+)-disparlure[5].

Insects possess substrate-selective catabolic enzymes that clear pheromone from their sensory tissues[8,9], but the precise role of these enzymes in pheromonal perception is poorly understood. An epoxide hydrase (EH) is present in male and female antennae and legs of *L. dispar* that converts disparlure to the corresponding *threo*-7,8-diol (**4**)[10]. Moreover, the male antennal EH produced the *threo*-(7*R*,8*R*)-diol enantiomer stereoselectively; that is (+)-(7*R*,8*S*)-, (−)-(7*S*,8*R*)-, and racemic disparlure were all converted to the (7*R*,8*R*)-diol with enantiomeric excesses of ≥92% as determined by analysis of the derivatized diols by chiral phase capillary gas chromatography (CP-CGC)[11].

In this study, we determined the stereoselectivity of the hydration of racemic disparlure by the EH derived from male antennae (MA), male legs (ML), female antennae (FA), and female legs (FL). The stereoselectivity of the male antennal EH-mediated reaction on two *meso*-analogues of disparlure (**2** and **3**, fig. 1) was also examined. In all cases, the *threo*-(*R,R*)-diol was the major product. These results suggested that same EH was present in the four *L. dispar* tissues studied. Furthermore, it appears that the orientation of the oxirane ring in the active site of the EH, rather than a particular arrangement of alkyl side-chains, is what determines the stereochemical outcome of the hydration.

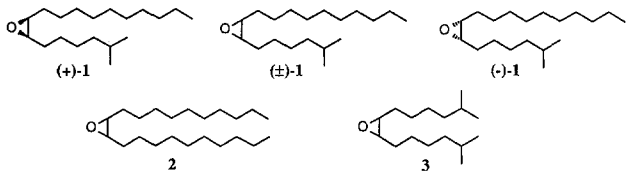
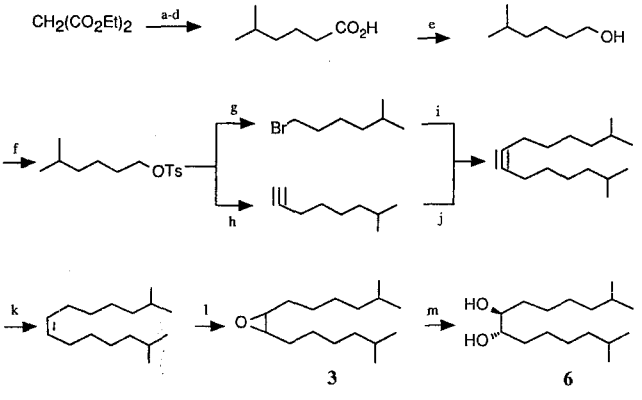


Figure 1. Structures of disparlure (**1**) and the disparlure analogues *meso*-**2** and *meso*-**3**.



Scheme. Synthesis of *meso*-2,13-dimethyl-7,8-epoxytetradecane (**3**). Reagents: (a) NaOEt, then $(CH_3)_2CHCH_2CH_2Br$; (b) KOH; (c) HCl; (d) heat; (e) $LiAlH_4$, Et_2O; (f) TsCl, pyridine, $CHCl_3$; (g) LiBr, acetone; (h) lithium acetylide-ethylene diamine complex, DMSO; (i) HMPA; (j) *n*-BuLi, THF, 10 °C; (k) H_2-Pd/$BaSO_4$-pyridine; (l) *m*-CPBA, CH_2Cl_2; (m) aq. $HClO_4$, *p*-dioxane.

The synthesis of disparlure stereoisomers (+)-**1**, (−)-**1**, (±)-**1**, and the *meso*-straight-chain oxirane **2** was described previously[10]. The synthesis of *meso*-branched-chain oxirane **3** is shown in scheme 1. The *meso*-oxirane **3** was obtained after eleven steps in 15% overall yield[12]. All intermediates and *meso*-oxirane **3** were characterized by 300 MHz ^1H and 75 MHz ^{13}C NMR spectroscopy, low-resolution gas chromatography/mass spectrometry, and were either homogeneous by thin-layer chromatography or were ≥95% pure by CGC. In addition, *meso*-oxirane **3** also gave a satisfactory high-resolution

mass spectra (M^+ for $C_{16}H_{32}O$: calc. 240.2454; obs. 240.2450). For use as synthetic diol standards, oxiranes **1, 2,** and **3** also were hydrated non-enzymatically (aq. $HClO_4$/*p*-dioxane) to their corresponding *threo*-diols (**4, 5,** and **6**, respectively) as described[10].

Oxiranes **1, 2,** and **3** were enzymatically hydrated to *threo*-diols **4, 5,** and **6** as follows. To a homogenate[11] of 100 tissue equivalents (MA, FA, ML, or FL) in 5 ml of 10 mM Tris·HCl buffer (pH = 7.0) was added 125 µl (2 mg) of a 16 mg/ml solution of the appropriate substrate in ethanol. Samples were incubated for 72 h at room temperature and extracted with 80:20 hexane:ethyl acetate. The combined organic layers were washed with water, dried over $MgSO_4$, and chromatographed on silica gel to give the purified diol. A typical isolated yield was 0.5 mg (ca 25%).

Finally, the enantiomeric composition of the EH-produced diols was determined by CP-CGC. These analyses were performed on a Varian 3500 GC equipped with a 30 m × 0.25 mm XE-60-L-valine-(*R*)-α-phenylethylamide (XE-60-L-Val-(*R*)-α-PEA) column. The XE-60-L-Val-(*R*)-α-PEA liquid phase was synthesized in our laboratory as described for the closely related XE-60-L-Val-(*S*)-α-PEA phase[13]. The column film thickness was approximately 0.2 µm (T_{max} = 175 °C). A splitless injector (250 °C) and flame ionization detector (280 °C) were used. Prior to CP-CGC analysis, all diols were derivatized as their bis(trifluoroacetates). Thus, to ca 20 µg of each diol was added 100 µl of methylene chloride and 50 µl of trifluoroacetic anhydride. After 1 h, the volatiles were removed under a stream of nitrogen, and the residue was dissolved in 50–100 µl of methylene chloride and analyzed by CP-CGC. Initial GC oven conditions were 50 °C (1 min) followed by programming to the final temperature at 20 °C per minute; the final oven temperature was optimized for each derivatized diol. The data are summarized in the table and representative CP-CGC traces are shown in figure 2. That the (7*S*,8*S*) enantiomer was the first eluting enantiomer was determined by co-injection of the bis-(trifluoroacetate) of known *threo*-(7*S*,8*S*)-**4** with the bis-(trifluoroacetate) of known racemic *threo*-**4** (data not shown). By analogy with *threo*-**4**, the (*S*,*S*) configuration was assigned to the first-eluting enantiomer of **5** and **6**.

Both *meso*-analogues **2** and **3** were converted to their respective (7*R*,8*R*) and (11*R*,12*R*) diols by the male antennal EH, as was the case for the disparlure enantiomers and racemic disparlure (fig. 3). This is explained best by invoking a preferred epoxide-binding orientation in the EH active site, with either disparlure alkyl chain occupying either of two putative lipophilic binding regions of the EH. Thus it appears that the EH recognizes disparlure as though it were a *meso*-compound. While both (+)-**1** and (−)-**1** gave the same (7*R*,8*R*)-diol (as determined by CP CGC), there is some preference for a particular arrangement of the side chains, as demonstrated in our kinetic studies using tritium-labeled (+)-**1** and

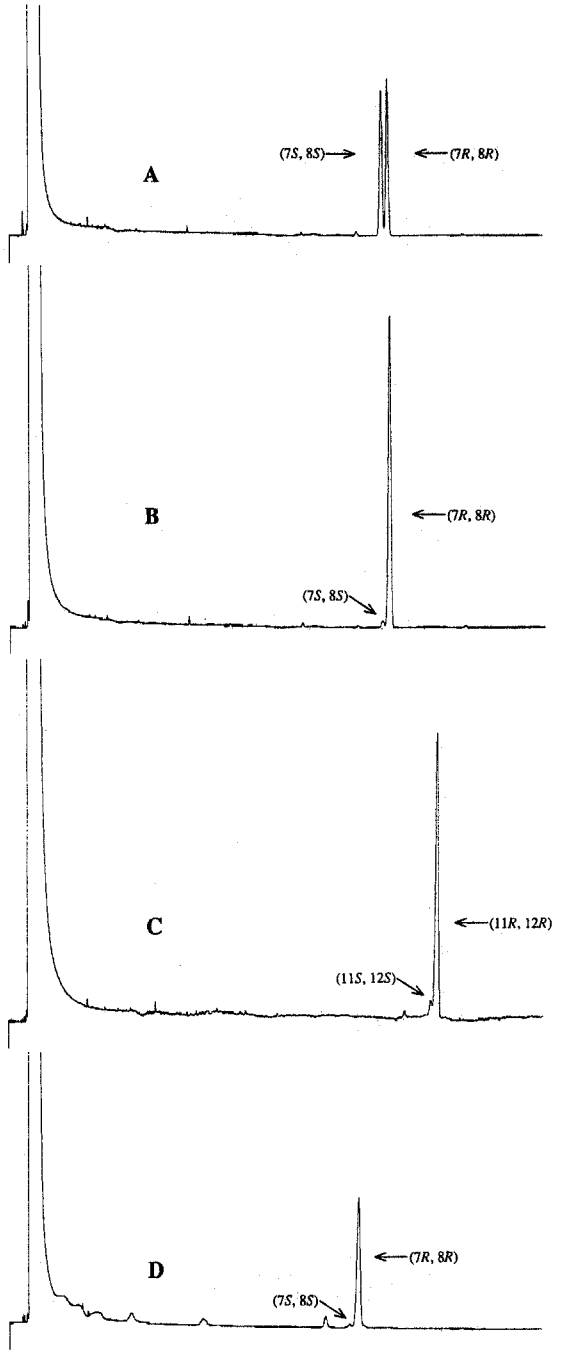


Figure 2. Separation of enantiomeric bis(trifluoroacetate) derivatives of diols **4, 5** and **6** produced by the *L. dispar* EH. These diols were produced by the male antennal EH; diols produced by other *L. dispar* EHs gave similar CP-CGC traces. The column was the 30 m × 0.25 mm XE-60-L-Val-(*R*)-α-PEA described in the text. In all cases the GC temperature program was 50 °C initial temperature (1-min hold), followed by programming to the final temperature at 20 °C per min. The final temperature was optimized to give the best resolution for each derivatized diol. *A* Non-enzymatically hydrated (±)-**1**; final temperature = 150 °C. *B* EH-produced diol from (±)-**1**; final temperature = 150 °C; 7*S*,8*S* enantiomer = 22.3 min, 7*R*,8*R* enantiomer = 22.8 min. *C* EH-produced diol from *meso*-**2**; final temperature = 175 °C; 11*S*,12*S* enantiomer = 25.3 min, 11*R*,12*R* enantiomer = 25.7 min. *D* EH-produced diol from *meso*-**3**; final temperature = 120 °C; 7*S*,8*S* enantiomer = 20.4 min, 7*R*,8*R* enantiomer = 21.0 min.

Separation of the bis(trifluoroacetates) of disparlure diols and disparlure analogue diols on the XE-60-L-Val-(*R*)-α-PEA column

Entry	Disparlure	Reagent[1]	7*S*,8*S*(%)	7*R*,8*R*(%)
1	(±)-**1**	$HClO_4$	49.7	50.3
2	(±)-**1**	MA	1.5	98.5
3	(+)-**1**	MA	1.9	98.1
4	(−)-**1**	MA	3.5	96.5
5	(±)-**1**	FA	6.7	93.3
6	(±)-**1**	ML	6.1	93.9
7	(±)-**1**	FL	13.1	86.9
8	*meso*-**2**	$HClO_4$	49.8	50.2
9	*meso*-**2**	MA	2.2	97.8
10	*meso*-**3**	$HClO_4$	50.1	49.9
11	*meso*-**3**	MA	3.0	97.0
12	known 7*S*,8*S*-diol		100	0
13	known 7*S*,8*S*-diol[2]		100	0

[1] MA = male antennae; FA = female antennae; ML = male legs; FL = female legs. [2] After 7 days in the trifluoroacetic anhydride/CH_2Cl_2 derivatization reagent.

(−)-**1**[10]. By virtue of their symmetry, the *meso* analogues eliminate this side-chain preference, and the hydration of these analogues is further evidence that there is a single EH in male antennae which can use either disparlure enantiomer (or *meso*-analogue) as a substrate.

The data also indicate that the same EH is found in all four of the tissues studied. Regardless of the substrate or of the source of the EH, the major product is the (*R*,*R*)-diol. The data suggest that the male antennal EH produces (*R*,*R*)-diol of the highest enantiomeric purity, with compositions of ca 97–99% (*R*,*R*) for diols **4**, **5**, and **6** (table, entries 2, 3, 4, 9, and 11). Diols produced from (±)-**1** by the female antennal and the male leg EH had lower enantiomeric purities (ca 93–94% (7*R*,8*R*); entries 5 and 6). Diol produced from (±)-**1** by the female leg EH was of the lowest enantiomeric purity (ca 87% (7*R*,8*R*); entry 7). Because the (7*R*,8*R*) diol was produced in high enantiomeric excess in each tissue examined and for each substrate examined, it appears the 'antennal' EH is the predominant EH in other moth tissues as well.

Several explanations are possible for the observed variation in enantiomeric purity of the diol produced by the various tissues. One possibility, racemization under the derivatization conditions, can be ruled out. After 7 days in trifluoroacetic anhydride/trifluoroacetic acid, the authentic *threo*-(7*S*,8*S*)-diol showed no loss of stereochemical integrity (table, entries 12 and 13). Another possibility is that the stereoselectivity of hydration is related to the relative rates of hydration of the disparlure enantiomers. The natural pheromone, (+)-**1**, was hydrated by the male antennal EH most rapidly, followed by (±)-**1**, with (−)-**1** being hydrated the slowest[10]. Despite this kinetic difference, (+)-**1**, (−)-**1**, and (±)-**1** all gave the (7*R*,8*R*)-diol of approximately the same enantiomeric purity (table, entries 2, 3, and 4). Also, since both enantiomers gave the same *threo*-(7*R*,8*R*)-diol, the potential complication of the presence of ca 2–3 mol% of the undesired antipode in each disparlure enantiomer can be eliminated. A third possibility for the decreasing stereoselectivity is that male antennal pheromone binding proteins (PBPs)[9, 14] solubilize the oxirane substrates and alter the delivery of the substrate to the EH. Fourth, the long incubation times (72 h) required to generate sufficient quantities of diol for GC analysis may allow low-abundance, non-specific EHs to act[15], thus decreasing the enantiomeric purity. Finally, the EH titers vary in different tissues, making absolute comparisons difficult[16]. Apparently EH activity alone cannot provide *L. dispar* males with the means to distinguish the disparlure enantiomers. Moreover, we previously showed that the male antennal PBP binds both disparlure enantiomers with equal affinity[14]. Recognition of disparlure enantiomers must therefore occur at the level of the dendritic membrane protein receptor, and efforts to demonstrate this are in progress.

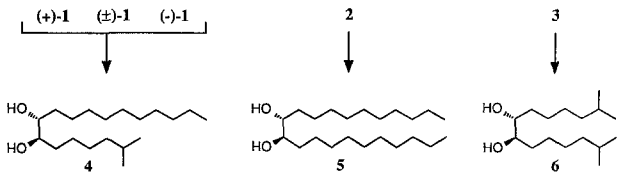


Figure 3. Hydration of any enantiomer of disparlure or of the disparlure analogues by the *L. dispar* EH gives only the *threo*-(*R*,*R*)-diol.

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1 S.M.G. and G.D.P thank the United States Department of Agriculture for financial support and Dr Y. Masaki (Gifu University, Japan) for a generous gift of authentic *threo*-(7*S*,8*S*)-2-methyloctadecanediol.

2 Baker, T. C., Experientia *45* (1989) 248.
3 Bierl, B. A., Beroza, M., and Collier, C. W., Science *170* (1970) 87.
4 Mori, K., and Ebata, T., Tetrahedron *42* (1986) 3471, and references cited therein.
5 Hansen, K., Physiol. Ent. *9* (1984) 9, and references cited therein.
6 Cardé, R. T., in: Behavior Modifying Chemicals for Insect Pest Management, p. 51. Eds R. L. Ridgway, R. M. Silverstein and M. N. Inscoe. Dekker, New York 1990.
7 Kolodny-Hirsch, D. M., and Schwalbe, C. P., in: Behavior Modifying Chemicals for Insect Pest Management, p. 363. Eds R. L. Ridgway, R. M. Silverstein and M. N. Inscoe. Dekker, New York 1990.
8 Prestwich, G. D., in: Pheromone Biochemistry, p. 474. Eds G. D. Prestwich and G. J. Blomquist. Academic Press, New York 1987.
9 Vogt, R. G., in: Pheromone Biochemistry, p. 385. Eds G. D. Prestwich and G. J. Blomquist. Academic Press, New York 1987.
10 Prestwich, G. D., Graham, S. M., Kuo, J.-W., and Vogt, R. G., J. Am. chem. Soc. *111* (1989) 636.
11 Prestwich, G. D., Graham, S. M., and König, W. A., J. chem. Soc., chem. Commun. (1989) 575.
12 Graham, S. M., Ph.D. thesis. State University of New York at Stony Brook, 1990.
13 König, W. A., in: The Practice of Enantiomer Separation by Capillary Gas Chromatography, p. 12. Hüthig, Heidelberg 1987.
14 Vogt, R. G., Köhne, A. C., Dubnau, J. T., and Prestwich, G. D., J. Neurosci. *9* (1989) 3332.
15 Wixtrom, R. N., and Hammock, B. D., in: Biochemical Pharmacology and Toxicology, vol. 1, p. 1. Eds D. Zakim and D. A. Vessey. Wiley, New York 1985.
16 Graham, S. M., and Prestwich, G. D., unpublished results.

0014-4754/92/010019-03$1.50 + 0.20/0