Pergamon

Tetrahedron: *Asymmetry* 11 (2000) 4451–4462

TETRAHEDRON: *ASYMMETRY*

# Regio- and enantioselective hydrolysis of phenyloxiranes catalyzed by soluble epoxide hydrolase

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Received 20 June 2000; revised 11 October 2000; accepted 14 October 2000

**Abstract**

The regio- and enantioselective hydrolysis of several phenyloxiranes catalyzed by soluble epoxide hydrolase (sEH) was investigated using recombinant human, mouse or cress sEH. Results indicate that human and mouse sEH enantioselectively hydrolyze (*S*,*S*)-alkyl-phenyloxiranes faster than the (*R*,*R*)-alkyl-phenyloxiranes investigated in this study, while cress sEH displayed opposite enantioselectivity. Preparation of pure (2*R*,3*R*)-3-phenylglycidol from the racemic mixture was achieved with a 31% yield using human sEH as catalyst. The sEH enzymes were found to be regioselective at the benzylic carbon of the phenyloxiranes, supporting the proposed mechanism in which one or more tyrosine residues in the active site of the enzyme act as a general acid catalyst in the alkylation half reaction. © 2000 Published by Elsevier Science Ltd.

## 1. Introduction

Epoxide hydrolases (E.C. 3.3.2.3) are enzymes that catalyze the hydrolysis of epoxides into *vicinal*-diols, and have been found in yeasts,[1] fungi,[2] plants,[3] mammals,[4] insects,[5] and bacteria.[6] The soluble epoxide hydrolase (sEH) is one of several known epoxide hydrolases and is a member of the α,β-fold hydrolase family of enzymes.[4] Mammalian sEHs are important detoxification enzymes involved in xenobiotic transformation of exogenous epoxides,[7,8] and they have also been found to play a regulatory role in the biosynthesis and degradation of physiological homeostasis mediators,[9–12] including the putative natural substrates of epoxy linoleates (leukotoxin and isoleukotoxin)[11] and epoxy arachidonates.[9] Plant sEHs are hypothesized to aid in the biosynthesis of cutin, and the cress and potato sEHs have been found to be selective towards various substrates.[13] Therefore, investigating the regio- and enantioselectivity of these sEH enzymes is important to better understand (1) xenobiotic detoxification and

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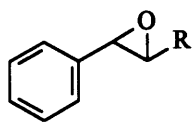
PII: S0957-4166(00)00437-7

endogenous metabolism processes catalyzed by sEH, (2) the positioning of substrate and topographical structure in the sEH active site and (3) the fundamental selective hydrolysis inherent to sEH that makes these enzymes interesting biocatalysts.

Catalytic hydrolysis by sEH occurs by a two-step mechanism.[4,14] In this mechanism for sEH, the first step involves the attack of an aspartate carboxylic anion to produce an enzyme linked β-hydroxylalkyl ester intermediate. During the second step, an activated water molecule hydrolyzes the ester intermediate to release the *vicinal*-diol product. Thus, for this mechanism, sEH effectively adds a water molecule to one of the epoxide carbons via an $S_N2$-type mechanism, thereby inverting the stereochemistry of that carbon. Through this mechanism, *cis*-epoxides are transformed into *threo*-diols, while *trans*-epoxides result in *erythro*-diols. Although this stereochemical mechanism of hydrolysis is known, less is understood about the regio- and enantioselectivity of sEH enzymes.

The goal of this study is to determine the regio- and enantioselectivity of mammalian and plant sEH enzymes on phenyloxiranes. Phenyloxiranes are commonly-used substrates to measure the specific activity of sEH and are readily available as either separate enantiomers or as a racemic mixture easily separated by chiral chromatography.[4] *trans*-Phenyloxiranes of (*R*,*R*)- and (*S*,*S*)-configurations were chosen as substrates since *cis*-phenyloxiranes have been found to be very bad substrates for sEH. Phenyloxiranes are important precursors to chiral drugs such as arylethanolamines, which are used as anti-inflammatories,[15] and arylpropionic acids, which are used as adrenergic drugs.[16] Hence, sEH enzymes could be used as biocatalysts to produce enantiomerically enriched phenyloxiranes via enzymatic resolution. Herein, we report the enantioselective hydrolysis of the phenyloxiranes illustrated in Scheme 1, including styrene oxide **1**, phenylpropylene oxide **2**, phenylglycidol **3**, *trans*-stilbene oxide **4** and *para*-chloro-*trans*-stilbene oxide **5** catalyzed by recombinant human, mouse, and cress sEH enzymes, and the regioselective hydrolysis of compounds **2** and **3** with mouse sEH and compound **3** with cress sEH.




R = **1**: H
**2**: $CH_3$
**3**: $CH_2OH$
**4**: $C_6H_5$
**5**: $p$-$C_6H_4Cl$

Scheme 1. Epoxides used as substrates for human sEH, mouse sEH, and cress sEH

## 2. Results and discussion

Compounds **1**–**5** were assayed at 30°C with purified recombinant sEH enzyme and the reactions were stopped before 30% enzymatic hydrolysis occurred to insure excess substrate. Control experiments without enzyme were performed in parallel to account for any background hydrolysis, which was negligible (0–1%). Compounds **1**, **2** and **3** were assayed as separate enantiomers and analyzed by GC/FID, while compounds **4** and **5** were tested as racemates and analyzed by HPLC using a chiral column to resolve the enantiomers. All five of the phenyloxirane compounds included in this investigation were found to be substrates for the sEH enzymes, however the turnover rates varied dramatically. The catalytic turnover rate for compounds **1**–**5** are shown in Table 1.

Table 1
Catalytic turnover rate for the respective enantiomers of compounds **1**–**5** (nmol substrate/min/mg protein; mean ± standard deviation)

| Substrate | Human sEH | Mouse sEH | Cress sEH |
|---|---|---|---|
| **1** (*R*) | 30.9 ± 4.8 | 41.7 ± 2.2 | 266 ± 63 |
| (*S*) | 78.9 ± 4.7 | 53.1 ± 4.6 | 392 ± 25 |
| **2** (*R*,*R*) | 8.39 ± 2.75 | 88.7 ± 18.5 | 713 ± 156 |
| (*S*,*S*) | 58.5 ± 18.4 | 271 ± 11 | 166 ± 33 |
| **3** (*R*,*R*) | 25.4 ± 9.4 | 81.7 ± 11.0 | 783 ± 83 |
| (*S*,*S*) | 177 ± 4 | 427 ± 2 | 179 ± 69 |
| **4** | 98.3 ± 1.0 | 325 ± 12 | 1820 ± 160 |
| **5** | 965 ± 185 | 332 ± 100 | 5520 ± 760 |
| *t*-DPPO[a] | 4500 ± 200 | 17000 ± 300 | 2490 ± 90 |

[a] *trans*-1,3-Diphenylpropene oxide data from Morisseau et al.[13]

The enantioselectivity ($E$) for the sEH enzymes was calculated using the equation described by Chen et al.[17]

$$E = \frac{V_a}{V_b} = \frac{\ln(A/A_0)}{\ln(B/B_0)} \quad (1)$$

In this equation, $V_a$ and $V_b$ are the velocities of transformation for each enantiomer, $A$ and $B$ represent the amount of each enantiomer remaining after the reaction, while $A_0$ and $B_0$ are the amount of each enantiomer before the reaction begins. The enantioselectivity for the sEH enzymes with compounds **1**–**5** are reported in Table 2. Enantioselectivity by sEH, while low, was found with compounds **1**–**4** for human sEH, **2**–**4** for mouse sEH, and **2** and **3** with cress sEH. There was no enantioselectivity found for compound **5** with any of the sEH enzymes. Furthermore, no enantioselectivity was seen for compound **1** with mouse sEH, nor for compounds **1** and **4** with cress sEH. Overall, all three sEH enzymes showed the greatest enantioselectivity with compounds **2** and **3**, and human sEH exhibited higher enantioselectivity than mouse or cress sEH. In order to confirm results obtained at analytical scale, preparation of (*R*,*R*)-**3** was conducted at a millimolar scale. Following the method described below, pure

Table 2
Enantioselectivity for compounds **1**–**5** (mean ± standard deviation)

| Substrate | Human sEH | Mouse sEH | Cress sEH |
|---|---|---|---|
| **1**[a] | 2.6 ± 0.2 (*S*)[b] | 1.3 ± 0.1 | 1.5 ± 0.2 |
| **2**[a] | 7.0 ± 0.3 (*S*,*S*) | 3.1 ± 0.2 (*S*,*S*) | 4.3 ± 0.2 (*R*,*R*) |
| **3**[a] | 7.0 ± 0.4 (*S*,*S*) | 5.2 ± 0.1 (*S*,*S*) | 4.4 ± 0.4 (*R*,*R*) |
| **4**[c,d] | 3.2 ± 0.8 (*S*,*S*) | 2.7 ± 0.5 (*S*,*S*) | 1.4 ± 0.4 |
| **5**[c,d] | 1.06 ± 0.04 | 1.19 ± 0.04 | 1.03 ± 0.01 |

[a] Calculated using $E = (V_a/V_b)$.
[b] Indicates configuration of the preferred enantiomer hydrolyzed by sEH.
[c] Calculated using $E = \ln(A/A_0)/\ln(B/B_0)$.
[d] Enantioselectivities were also determined for potato sEH and found to be 1.9 ± 0.7 for compound **4**, and 1.002 ± 0.002 for compound **5**.

(*R*,*R*)-**3** (e.e. = 94%) was obtained with a 31% yield using HsEH as catalyst. This corresponds to an *E* value of 7.8. This is close to the value (7.0) found at analytical scale, confirming the accuracy of the *E* determination.

To our knowledge, there are no other studies that have investigated the enantioselective hydrolysis by sEH with compounds **3**, **4** or **5**. However, there have been some studies concerning the enantioselective hydrolysis of compounds **1** and **2** by sEH (Table 3). With compound **1**, no enantioselectivity was found with mouse nor cress sEH. This agrees with findings with sEH from rabbit liver and the fungus *Cuninghamella elegans*.[18,19] However, human sEH in this study was found to be enantioselective with **1**. This is similar to the results found with sEH from the fungus *Beauveria sulferescens*.[2] Interestingly, sEH from the fungi *Syncephalastrum racemosum*,[20] and *Aspergillus niger*,[2] and the bacterium *Agrobacterium radiobacter*[21] were also found to be enantioselective with compound **1**, yet these enzymes preferred the opposite configuration. For compound **2**, all three sEH enzymes in this study were found to be enantioselective. This agrees with the findings from the sEH of two fungi including *Aspergillus terreus* and *B. sulferscens*,[19,22] yet differs from rabbit liver sEH, where no enantioselectivity was detected.[18] It is interesting to note that of the six sEH enzymes found to biocatalyze compound **2**, only cress sEH from this study was found to be enantioselective for the (*R*,*R*)-configuration. Overall, the results for compounds **1** and **2** from this study and others discussed above suggest that there is no relationship between the enantioselective hydrolysis of these compounds by sEH within the four kingdoms including mammals, plants, fungi, and bacteria. Therefore, minor changes in the catalytic site may yield major changes in substrate selectivity, including enantioselectivity.

Table 3
Enantioselective hydrolysis of sEH enzymes from various species

| Compound | sEH species enantioselective for | | |
|---|---|---|---|
| | None | (*S*)- or (*S*,*S*)-enantiomer | (*R*)- or (*R*,*R*)-enantiomer |
| | | (*E* is indicated between parentheses) | |
| **1** | Mouse<br>Cress<br>Rabbit[a]<br>*C. elegans*[b] | Human (2.6)<br>*B. sulferescens*[c] (49)[d] | *S. racemosum*[e] (8)<br>*A. radiobacter*[f] (16)<br>*A. niger*[c] (11)[d] |
| **2** | Rabbit[a] | Human (7.0)<br>Mouse (3.1)<br>*B. sulferescens*[c] (87)[d]<br>*A. terreus*[b] (70) | Cress (4.3) |

[a] Data from Bellucci et al.[18]
[b] Data from Moussou et al.[19]
[c] Data from Pedragosa-Moreau et al.[22]
[d] Calculated using the equation for *E*.[35]
[e] Data from Moussou et al.[20]
[f] Data from Spelberg et al.[21]

One interesting result was the finding that human and mouse sEH have the opposite enantioselectivity of cress sEH (Table 2). Within each sEH enzyme, the enantioselectivity for the (*R*,*R*)- or (*S*,*S*)-configuration is conserved and not affected by the R group for the phenyloxirane compounds tested. The enantioselectivity of human sEH was plotted against mouse and

cress sEH (data not shown). Over the series of compounds tested, no good correlations were found ($r^2$ <0.74). These findings, quite surprising because the percent identity between the protein sequence of the human and mouse sEH is 92%,[23] underline that minor changes in the enzyme structure may result in major changes in substrate selectivity and enantioselectivity. This suggests that enantioselectivity could be tailored by slight alterations in enzyme structure.

Molar refractivity ($M_R$) is a common parameter used to evaluate the size of a part of a molecule. Because $M_R$ is an important factor to determine the preference of sEH for its substrates,[24] a correlation between this factor and enantioselectivity was studied. As shown in Fig. 1, the $M_R$ of the phenyloxirane R group[25] was plotted against the enantioselectivity for human, mouse, and cress sEH. For all three enzymes, the enantioselectivity is small for compound **1** ($M_R = 0.1$), rises with compound **2** ($M_R = 0.56$), has a maximum for compound **3** ($M_R = 0.72$), and then falls with compounds **4** ($M_R = 2.54$) and **5** ($M_R = 3.14$). A similar trend is found when the enantioselectivity is plotted against steric parameters, but there is no trend with hydrophobicity (data not shown). These data suggest that there may be an optimum size for the R group that corresponds to higher enantioselectivity by the sEH enzymes. However, this maximum spans a large range of $M_R$ values, indicating that the sEH enzymes could probably interact with a large variety of substrates as one would expect for an enzyme involved in xenobiotic degradation and lipid metabolism.

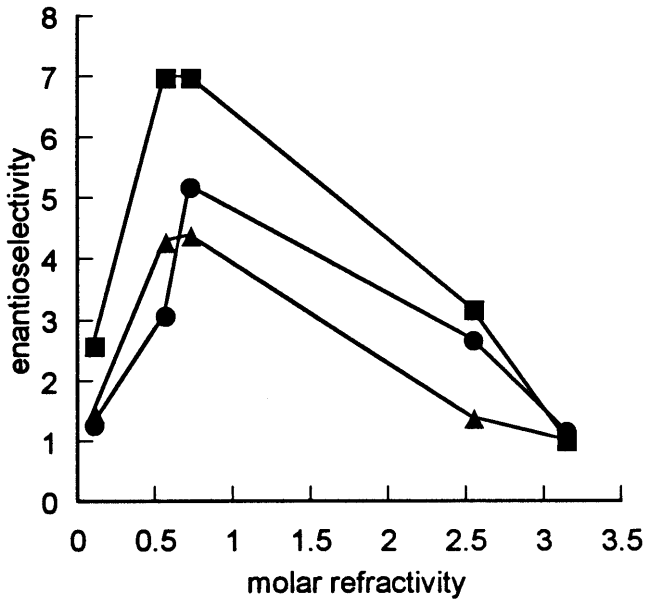



Figure 1. Correlation between the enantioselectivity of mouse sEH (●), human sEH (■), and cress sEH (▲) versus molar refractivity. The molar refractivity coefficients were determined using the R groups that are bonded to the basic phenyloxirane structure. Values are given as the mean±standard deviation

In order to investigate the selectivity of the sEH enzymes further, the regioselectivity of mouse sEH was evaluated for compounds **2** and **3** with incorporation of $H_2{}^{18}O$. Due to the opposite enantioselectivity between the mouse and cress sEH, the regioselectivity of cress sEH was also explored with compound **3**. Results indicate that the sEH enzymes are regioselective at the benzylic carbon for compounds **2** and **3** (Table 4). Mouse sEH shows almost complete regioselectivity at the benzylic carbon for the four compounds tested, (*R*,*R*)-**2**, (*S*,*S*)-**2**, (*R*,*R*)-**3**,

and (*S*,*S*)-**3**, of 98.3, 100, 100, and 99.2%, respectively. While cress sEH is highly regioselective at the benzylic carbon of (*R*,*R*)-**3** (99.8%), the same enzyme is less regioselective (62.7%) for the same carbon of (*S*,*S*)-**3**. Table 4 also shows other studies that investigated the regioselectivity of sEH enzymes with various phenyloxirane substrates.[14,18,19] With the exception of sEH from *A. terreus* and (*R*,*R*)-**2**,[19] all the sEH enzymes are regioselective at the benzylic carbon of the unsubstituted phenyloxiranes.

Table 4
Regioselective hydrolysis of sEH enzymes various species

| Substrate | sEH from | Percent incorporation at | |
|---|---|---|---|
| | | Benzylic carbon | Homobenzylic carbon |
| (*R*,*R*)-**2** | Mouse | 98.3 | 1.7 |
| | Rabbit[a] | 98 | 2 |
| | *A. terreus*[b] | 50 | 50 |
| (*S*,*S*)-**2** | Mouse | 100 | 0 |
| | Rabbit[a] | 98 | 2 |
| | *A. terreus*[b] | 95 | 5 |
| (*R*,*R*)-**3** | Mouse | 100 | 0 |
| (*S*,*S*)-**3** | Mouse | 99.2 | 0.8 |
| (*R*,*R*)-**3** | Cress | 99.8 | 0.2 |
| (*S*,*S*)-**3** | Cress | 62.7 | 37.3 |
| *t*-DPPO | Mouse[c] | 97.1 | 2.9 |

[a] Data from Bellucci et al.[18]
[b] Data from Moussou et al.[19]
[c] *trans*-1,3-Diphenylpropene oxide data from Borhan et al.[14]

The active site of mouse sEH contains a catalytic triad with a nucleophilic Asp residue, a His residue and an orienting Asp residue. Opposite of these residues are a pair of Tyr residues that can activate an epoxide towards formation of the enzyme linked β-hydroxylalkyl ester intermediate and/or can stabilize that intermediate in the first step of the two-step mechanism,[26,27] while, in a second step, deacylation occurs via attacks of a water molecule, activated by Asp495 and His525, upon the Asp carbonyl group of the ester intermediate. Therefore, epoxide hydrolysis by sEH occurs via a push–pull mechanism where the electrophilic Tyr residues can pull on the oxirane ring and the nucleophilic Asp residue can push. The regioselective attack at the benzylic carbon found in this study, thus, suggests a general acid-catalyzed-like activation of the epoxide oxygen by one or both tyrosines during the first step of hydrolysis and is consistent with the published mechanism of mouse sEH.[24,26]

Fig. 2 shows the positioning of compound (*R*,*R*)-**3** in the active site of mouse sEH. In this figure, compound **3** is in its ground state and positioning of the substrate near Asp 333 with the epoxide oriented toward Tyr 374 and Tyr 465 was assumed from previous studies.[26,27] The substrate was then manually docked using *x*-, *y*- and *z*-translation and rotation movements to maximize the van der Waals' interactions between the substrate and the enzyme, by minimizing the global interactions energy. The best positioning found (Fig. 2) corresponded to an energy of 26 kcal/mol. In this model, the oxygen atom of the epoxide moiety of the substrate is 2.86 Å from Tyr 465, a distance consistent with hydrogen bonding, while the benzylic carbon of the

substrate is 1.80 Å from Asp 333, a reasonable distance for covalent bond formation. Tyr 374 was found to be 4.75 Å from the epoxide moiety of (*R*,*R*)-**3** in this model. This distance is too long for hydrogen bonding, but does not rule out possible hydrogen bonding in other conformations or stabilization of this or other substrates. Hydrogen bonding from the tyrosine residues can pull the oxygen of the epoxide, causing the adjacent carbons to be more electropositive in character. Therefore, nucleophilic attack by Asp 333 occurring at the benzylic position is not surprising due to the stabilization of a partial positive charge by the aromatic π electrons.

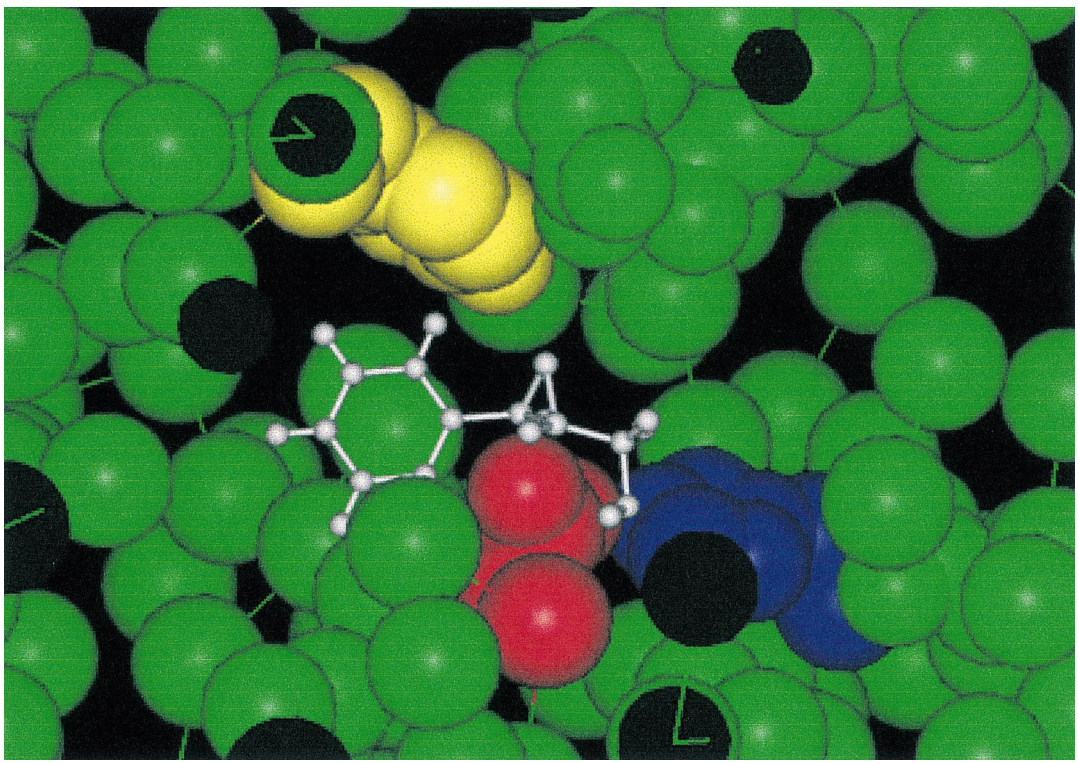


Figure 2. Manual docking of (*R*,*R*)-3 (colored white) in the active site of mouse sEH. The Asp 333 residue is red, the His 523 is blue and the Tyr 465 residue is yellow

(*R*,*R*)-**3** was also manually docked with the phenyl moiety rotated to the opposite side of the catalytic pocket, closer to the His 523 residue (Figure not shown). The distances between the benzylic carbon and Asp 333 and between the epoxide oxygen and Tyr 465 and Tyr 374 are similar to the values found as seen in Fig. 2 (1.87, 2.85 and 5.32 Å, respectively), yet the maximum van der Waals' interactions accomplished between the substrate and the enzyme were almost twice that of the opposite positioning of the substrate (47 kcal/mol). This may suggest that the substrate positions itself similarly as in Fig. 2, however, with a less favorable positioning for hydrolysis than the other enantiomer, (*S*,*S*)-**3**.

## 3. Conclusions

In this study, the regio- and enantioselective hydrolysis by sEH enzymes of phenyloxirane compounds is evaluated. For compounds **3**, **4**, and **5**, the selectivity by sEH enzymes is investigated for the first time. The sEH enzymes were most enantioselective for compounds **2** and **3**, and human sEH exhibited the highest enantioselectivity ($E=7$) of the three enzymes. Preparation of pure (2*R*,3*R*)-**3** was obtained with a 31% yield using human sEH as catalyst. Interestingly, human and mouse sEH were both enantioselective for (*S*,*S*)-alkyl-phenyloxiranes, while cress sEH was enantioselective for (*R*,*R*)-alkyl-phenyloxiranes. The mouse sEH was nearly completely regioselective for attack at the benzylic carbon for both enantiomers of compounds **2** and **3** (98.3–100%), while cress sEH is almost completely regioselective for the benzylic carbon of (*R*,*R*)-**3** (99.8%) and primarily regioselective at the same carbon for (*S*,*S*)-**3** (62.7%).

Collectively, the data presented here indicate that sEH enzymes have high regioselectivity, yet low enantioselectivity, toward phenyloxirane substrates. The regioselective hydrolysis of these enzymes at the benzylic carbon contributes further to the proposed push–pull mechanism, in which one or more tyrosines polarize the epoxide oxygen in the formation of the enzyme linked β-hydroxylalkyl ester intermediate during the first step of hydrolysis. Overall, the selectivities of the sEH enzymes, outlined in Tables 3 and 4, suggest primarily regioselective hydrolysis at the benzylic carbon of these phenyloxiranes, yet there is no general enantioselectivity trend with these substrates. With a racemic mixture, both regio- and enantioselectivity contribute to the absolute configuration of the produced diol and remaining epoxide. Therefore, these selectivities need to be considered when choosing an sEH enzyme to produce chiral phenyloxirane compounds.

## 4. Experimental

### 4.1. Chemicals

The substrates of (*R*)- and (*S*)-styrene oxide **1**, (*R*,*R*)- and (*S*,*S*)-phenylpropylene oxide **2**, (*R*,*R*)- and (*S*,*S*)-phenylglycidol **3**, *trans*-stilbene oxide **4** and *para*-chloro-*trans*-stilbene oxide **5**, as well as *trans*-stilbene, 1-undecanol, 1-methyl-3-phenylpropene, and phenylethanediol were all purchased from Aldrich Chemical Co. All solvents and reagents were obtained from Fisher Scientific. $^{3}$H-*trans*-1,3-Diphenylpropane oxide was prepared previously in the laboratory.[28] The derivatizing agents used for GC were *n*-butyl boronic acid from the Aldrich Chemical Co. and bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Supelco (Bellefont, PA). Bovine serum albumin (BSA) was purchased from Pierce, Inc. (Rockford, IL).

### 4.2. Synthesis of diols

Diol standards were synthesized from the epoxide substrates of **2** and **3** via acid hydrolysis as adapted by Moussou et al.[29] Briefly, approximately 100 μL of epoxide was dissolved in 50 mL MeCN:water (4:1) in a round-bottomed flask equipped with a stirring bar. One drop of concentrated sulfuric acid was added and the reaction mixture was stirred. Product formation was monitored by TLC (silica gel plates). After 1–6 h, the reaction mixture was neutralized with 20 mL of deionized water saturated with sodium bicarbonate. The MeCN was evaporated and

the solution was extracted with 40 mL ether (3×). The organic layers were pooled and dried over sodium sulfate, with subsequent solvent evaporation to yield the *vicinal*-diol product. The compounds were purified by silica gel chromatography and recrystallization. Final products were tested for purity by TLC and GC and structural identity was verified by MS and $^{1}$H NMR.[30,31]

### 4.3. Enzyme preparation and purification

The recombinant soluble epoxide hydrolase enzymes of human, mouse, and cress were prepared and purified as previously described.[32,33] Briefly, recombinant cDNA of each enzyme was cloned into the baculovirus expression system. Insect cells from *Trichoplusia ni* were transfected with prepared baculovirus in order to express the desired enzyme and subsequently were purified from cell lysate via affinity chromatography.[34] Initial activity was assayed using $^{3}$H-*trans*-1,3-diphenylpropane oxide as previously described,[28] and the protein concentration was determined using the Bradford BCA assay (Pierce, Inc., Rockford, IL) with BSA as the control enzyme.

### 4.4. Enantioselectivity assays

Hydrolysis experiments were performed in a 30°C shaking water bath at 120 rpm. Recombinant human, mouse, or cress sEH were diluted in 0.1 M sodium phosphate buffer at pH 7.4 containing 0.01% BSA. Incubations with the substrate were performed in order to determine the turnover rate and, thus, the appropriate enzyme dilution to be used for subsequent assays. Diluted enzyme (100 μL) was added to culture tubes (10×75 mm) on ice until ready for use. Control experiments to account for non-enzymatic hydrolysis were performed using buffer containing only BSA. The tubes were preincubated without substrate for 1 min. Substrate was then added (1 μL of 10 mM substrate in EtOH) for a final concentration of 0.1 mM substrate in each tube. The tubes were removed at predetermined timepoints, and the reactions were stopped by the addition of sodium chloride and solvent, followed by vigorous vortexing for 20 s. Samples were centrifuged at 4000–7000 rpm for 5 min and put into a dry ice–acetone bath to freeze the aqueous layer. The solvent was removed with Pasteur pipettes and transferred to 400 μL inserts in 1.5 mL amber sample vials.

Enantiomers of **1**, **2**, and **3** were assayed separately. Samples were extracted once with ether (250 μL), which was evaporated to apparent dryness and subsequently brought up in 50 μL of ethyl acetate. Extraction efficiency for recovered compounds **1**, **2**, and **3** were 73±3, 89±7 and 92±3%, respectively, although partitioning into the organic phase was quantitative. Samples were analyzed using GC on a J & W Scientific DB-5 column (15 m×0.32 mm i.d.×0.25 μm film).

For compound **1**, diol appearance was monitored on a Hewlett–Packard (HP) 5890A gas chromatograph equipped with a flame-ionization detector and an HP 3396A integrator. The phenylethanediol product was analyzed as the *n*-butyl boronic acid derivative using *trans*-stilbene as the internal standard (column conditions: 60°C for 2 min, 20°C/min to 110°C, 50°C/min to 200°C for 1 min; injector at 250°C, detector at 280°C; head pressure at 30 psi).

For compound **2**, diol appearance was monitored on an HP 6890 gas chromatograph equipped with a 5973 mass spectral detector. The phenylpropanediol product of compound **2** was analyzed using 1-undecanol as an internal standard (column conditions: 60°C for 2 min, 20°C/min to 110°C, 50°C/min to 210°C for 1 min; injector at 200°C, detector at 280°C; head pressure at 30 psi).

For compound **3**, substrate disappearance was monitored by GC/FID as described above. Compound **3** was derivatized with BSTFA to form the trimethylsilyl-derivative and *trans*-stilbene was used as an internal standard (column conditions: 80°C for 2 min, 20°C/min to 140°C, 50°C/min to 200°C for 1 min; injector at 250°C, detector at 280°C; head pressure at 30 psi).

Racemic **4** and **5** were used in the enzymatic assays. Hexane (200 μL), containing an internal standard of 1-methyl-3-propene, was used to extract the substrate from each sample. Extraction efficiency for compounds **4** and **5** were 101±3 and 95±3%, respectively. Samples (100 μL) were directly injected for analysis. Substrate disappearance was monitored using an HP 1100 HPLC equipped with a normal phase chiral column (Chiralcel-OB from J. T. Baker, Inc.; 25×0.46 cm i.d., 9:1 hexane:isopropanol at 0.5 mL/min). To assign absolute configuration to the enantiomers of compound **4**, peaks were separated via chiral HPLC and similar fractions were pooled. The solvent was evaporated and each enantiomer was dissolved in EtOH. The specific rotations were measured as +328 (*c* 2.09, EtOH), indicating (*R*,*R*)-**4** and −223 (*c* 1.96, EtOH), indicating (*S*,*S*)-**4**.[36] The specific rotations for enantiomers of compound **5** were not measured because no enantioselectivity was found.

### 4.5. Preparation of (2R,3R)-3-phenylglycidol *3*

Purified human sEH (15 mg) was dissolved in 99 mL of sodium phosphate buffer (0.1 M pH 7.4) containing 1 mM of EDTA and 0.2 mg/mL of BSA. The mixture was agitated at 100 rpm at 30°C. After 5 min, 1 mmol (150 mg) of racemic 3-phenylglycidol **3** dissolved in 1 mL of ethanol was added ($[S]_{final}$: 10 mM). The mixture was agitated at 30°C for an additional 8 h (until around 70% of conversion was obtained). The reaction was then stopped by saturation with NaCl. The remaining epoxide **3** was extracted with diethylether (3×100 mL). The organic phases were pooled, washed with brine, dried over $MgSO_4$ and evaporated, yielding 46 mg (31% yield) of white crystal (mp 51–52°C). This compound give one spot on TLC, which migrated similarly to the commercial epoxide **3**. Additionally, it has a similar mass spectra as the racemic **3**. The measurement of its specific rotation $\{[\alpha]_D^{20}$ +47 (*c* 2, $CHCl_3)\}$ indicates that it is the (2*R*,3*R*) enantiomer of **3** with an enantiomeric excess (e.e.) of 94%.

### 4.6. Regioselectivity assays

Undiluted mouse and cress sEH in sodium phosphate buffer (50 μL, 0.1 M, pH 7.4) were added into 6×50 mm glass culture tubes. The enzymes were frozen in a dry ice–acetone bath and lyophilized. Samples were reconstituted in 50 μL $H_2{}^{18}O$ (99%) and kept on ice. Control experiments were performed using $H_2{}^{16}O$. Compound **2** or **3** was added (0.5 μL of 10 mM in EtOH) for a final concentration of 0.1 mM of substrate. Samples were then vortexed and incubated in a shaking water bath at 30°C for 30 min. The water was evaporated using a vacuum pump and the residue resuspended in MeCN (250 μL). Samples were centrifuged at 3000 rpm for 5 min and the solvent was transferred to 400 μL inserts in vials. After the MeCN was evaporated, the samples were derivatized in 50 μL of 1:1 BSTFA:pyridine at 60°C for 15 min. Samples were analyzed using GC/MS, as described above, equipped with a DB-XLB column (30 m×0.25 mm×0.25 μm; oven program: 60°C for 1 min, 25°C/min to 320°C for 3 min). Fragments were monitored for $^{18}O$-incorporation (*m*/*z* 181:179 and 119:117 for the diol of compound $^{18:16}O$-**2** and *m*/*z* 181:179 and 207:205 for the diol of compound $^{18:16}O$-**3**).

### 4.7. Substrate docking of (R,R)-3 in mouse sEH

A crystal structure coordinate data set was unavailable for compound **3** in the Cambridge Structural Database. It was therefore modeled from a 2D sketch and converted to a 3D object in the Biosym's Builders Module. The 3D structure was subsequently optimized with the Gaussian Package using an ab initio approach and an RHF/6-31G basis set. The optimized structure was manually docked in the active site of mouse sEH while monitoring the van der Waals' interaction energy for its lowest energy state. This was accomplished in Biosymn's Docking Module.

## Acknowledgements

This work was supported in part by NIEHS Grant ES02710 and NIEHS Center for Agrochemical Research ES05707. K.C.W. was supported in part by the Society of Toxicology Graduate Fellowship sponsored by Proctor & Gamble and by the Superfund Basic Research Program ES04699. We would like to gratefully thank John W. Newman for his help with instrumentation.

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