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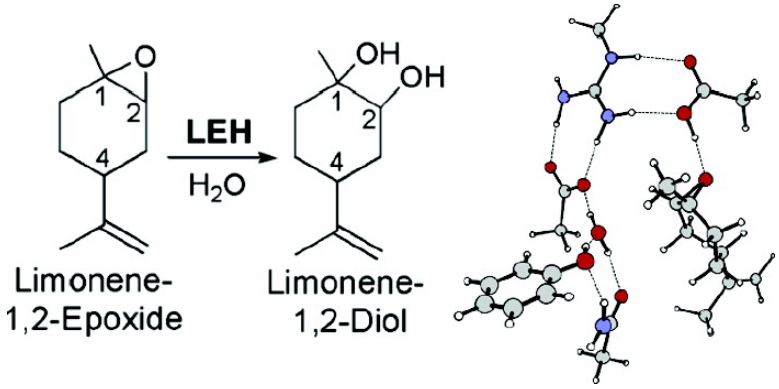

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Catalytic Mechanism of Limonene Epoxide Hydrolase, a Theoretical Study

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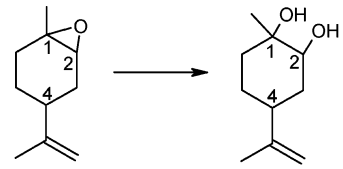
Abstract: The catalytic mechanism of limonene epoxide hydrolase (LEH) was investigated theoretically using the density functional theory method B3LYP. LEH is part of a novel limonene degradation pathway found in *Rhodococcus erythropolis* DCL14, where it catalyzes the hydrolysis of limonene-1,2-epoxide to give limonene-1,2-diol. The recent crystal structure of LEH was used to build a model of the LEH active site composed of five amino acids and a crystallographically observed water molecule. With this model, hydrolysis of different substrates was investigated. It is concluded that LEH employs a concerted general acid/general base-catalyzed reaction mechanism involving protonation of the substrate by Asp101, nucleophilic attack by water on the epoxide, and abstraction of a proton from water by Asp132. Furthermore, we provide an explanation for the experimentally observed regioselective hydrolysis of the four stereoisomers of limonene-1,2-epoxide.

Introduction

Epoxide hydrolases (EHs) are ubiquitous in nature and are found in a variety of organisms including mammals, bacteria, plants, and insects. EHs' main roles include detoxification, catabolism, and regulation of signaling molecules.[1,2] During recent years, EHs have acquired special interest because of their potential to be used as biocatalysts for asymmetric hydrolysis of epoxides.[3,4] A number of EHs from various organisms have been discovered, most of which belong to a group with relatively high internal sequence similarity. The structures of several members in this group have been determined, and they all exhibit an α/β-hydrolase fold.[5−7] However, a few epoxide hydrolases have been isolated that do not exhibit any sequence similarity to the α/β-hydrolase fold family; among these is the limonene-1,2-epoxide hydrolase (LEH) from *Rhodococcus erythropolis* DCL14.[8] LEH is part of the limonene degradation pathway in *R.erythropolis* DCL14, a pathway that allows the bacterium to grow on limonene as the sole source of carbon and energy. LEH catalyzes the hydrolysis of limonene-1,2-epoxide to give the vicinal diol limonene-1,2-diol (Scheme 1).[9]

Scheme 1. The Epoxide Hydrolysis Reaction Catalyzed by LEH[a]



Limonene-1,2-epoxide Limonene-1,2-diol

[a] There exist four stereoisomers of limonene-1,2-epoxide, all of which are substrates for LEH.

The mechanism of EHs belonging to the α/β-hydrolase fold family was for a long time thought to be general base-catalyzed.[1,10] However, mechanistic studies as well as a number of crystal structures have shown that the reaction occurs via a two-step mechanism, which involves attack of an aspartate residue on the epoxide, resulting in a stable enzyme−substrate intermediate, followed by hydrolysis in the second step.[11,12] The nucleophilic aspartate is part of a catalytic triad composed of two aspartates and a histidine residue.[13] It has been shown that two tyrosines are likely to act as acid catalysts and activate the epoxide ring during the reaction, thus facilitating attack by the nucleophilic aspartate.[7,14,15]

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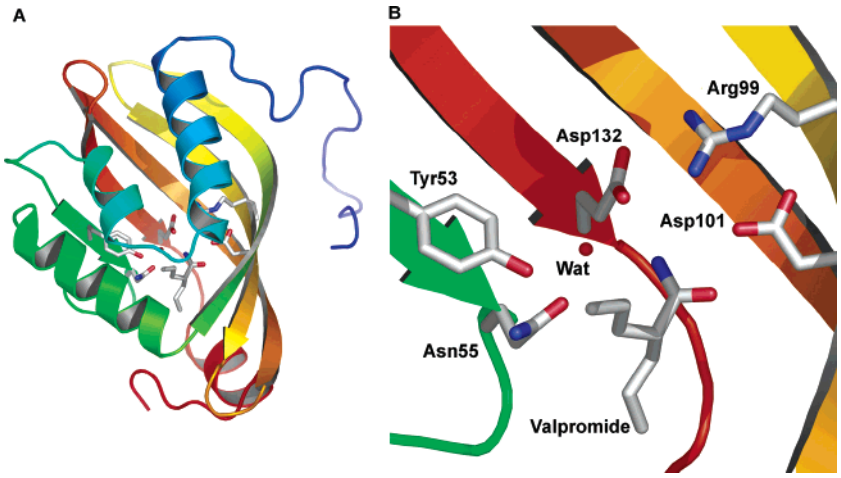
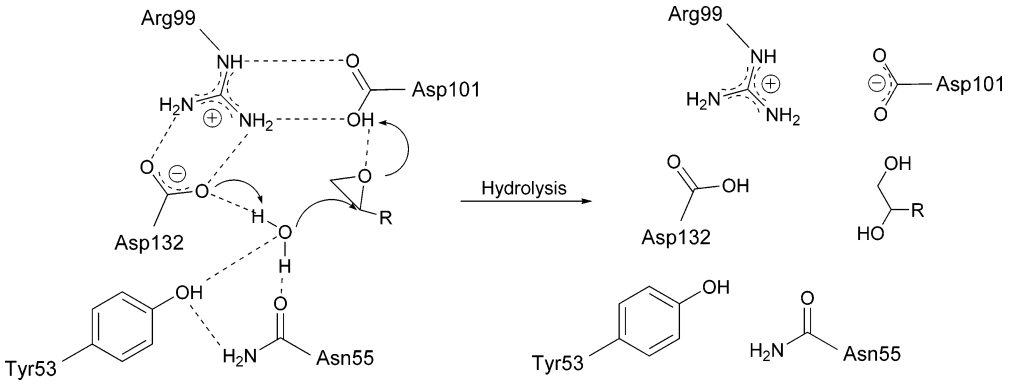


Figure 1. (A) Overall view of the structure of LEH with the inhibitor valpromide bound in the active site. The cartoon is colored from blue at the N-terminus to red at the C-terminus. (B) Close-up view of the active site. The experimentally observed water molecule that is putatively important in the mechanism is denoted Wat. Coordinates from PDB deposition 1NU3 have been used to generate the figure.[17]

Scheme 2. Proposed LEH Mechanism (adapted from Arand et al.[17])



LEH differs in both structural and mechanistic aspects from the α/β-hydrolase fold EHs. For example, LEH is much smaller than the α/β-hydrolase fold EHs and does not contain any of the highly conserved motifs of the catalytic triad found in α/β-hydrolase fold EHs.[16] Also, the recent LEH crystal structure revealed that LEH does not have an α/β-hydrolase fold and exhibits a novel active site structure.[17] Finally, various experiments have shown that the LEH mechanism is substantially different from that of the α/β-hydrolase fold EHs.[8,17,18] For LEH, epoxide hydrolysis seems to occur without the formation of a covalent enzyme−substrate intermediate.[17,18] LEH-mediated hydrolysis of 1-methylcyclohexene oxide showed preference for attack at the most substituted epoxide carbon, and it was therefore concluded that the LEH mechanism is acid-catalyzed.[18] This is based on the observation that epoxide opening under acidic conditions usually leads to attack on the most substituted carbon, because this carbon holds a larger partial positive charge.[19]

The crystal structure of LEH revealed a curved β-sheet with three α-helices packed on top (Figure 1A). A cluster of five charged and polar residues constitutes the active site (Figure 1B).[17] Thus, LEH has a totally different fold and catalytic machinery than the α/β-hydrolase fold family of epoxide hydrolases. In fact, LEH is assumed to be the founding member of a new protein family.[16] Several proteins with significant structural and sequence similarities have been identified very recently.[20] Crystallization of the epoxide hydrolase Rv2740 from *Mycobacterium tuberculosis* revealed an active site that is extremely similar to the active site of LEH.[20] Both proteins share the same putative catalytic residues and are suggested to employ the same mechanism for epoxide hydrolysis.[20] Mutagenesis studies confirmed the importance of the proposed catalytic residues found in the active site of LEH.[17] Based on these results as well as structural data, a putative reaction mechanism for LEH was put forward (Scheme 2).[17] While Tyr53 and Asn55 mainly seem to function in positioning a water molecule in a favorable position for epoxide attack, Arg99, Asp101, and Asp132 are suggested to be actively involved in proton donation and abstraction during the reaction and have hence been described as an Asp−Arg−Asp triad.[17] Asp101 is proposed to donate a proton to the oxirane ring of the substrate, while Asp132 abstracts a proton from the water molecule, facilitating nucleophilic attack on the epoxide carbon. Arg99 positions the

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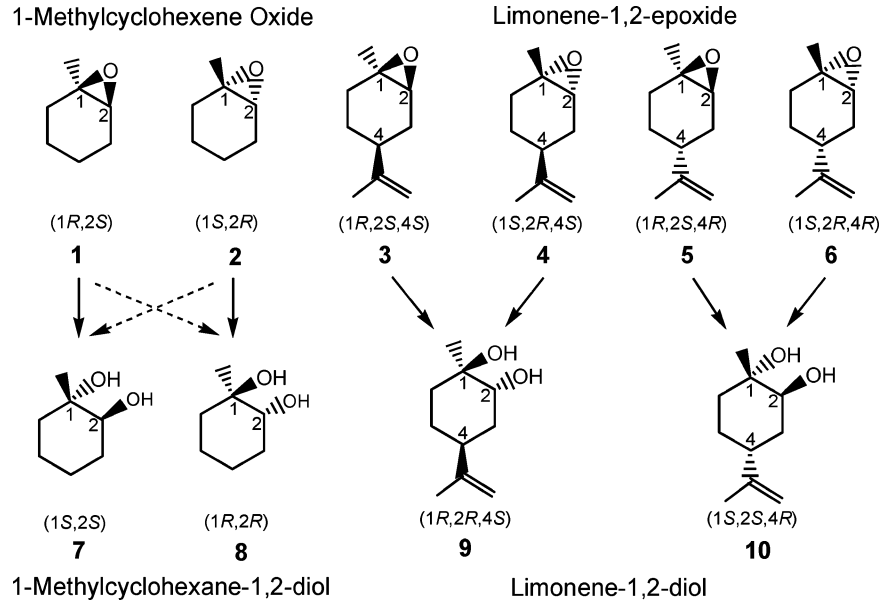
Scheme 3. The Substrates Investigated in This Article[a]



[a] Limonene-1,2-epoxide is the natural LEH substrate.

carboxylate groups of the two aspartates and assists also in charge stabilization.[17]

Although quite appealing, the proposed catalytic mechanism of LEH leaves a number of interesting questions unanswered. For instance, although experiments with 1-methylcyclohexene oxide showed a preferred attack on the most substituted carbon,[18] hydrolysis of the four stereoisomers of limonene-1,2-epoxide results in attack on the most substituted epoxide carbon for the stereoisomers **4** and **5** ((1*S*,2*R*,4*S*)- and (1*R*,2*S*,4*R*)-limonene-1,2-epoxide, Scheme 3) and attack on the less substituted carbon for the two others, **3** and **6** ((1*R*,2*S*,4*S*)- and (1*S*,2*R*,4*R*)-limonene-1,2-epoxide, Scheme 3).[9,21] An intriguing question is thus what governs the regioselectivity of epoxide opening. For example, why are the two isomers **3** and **5** attacked differently, although they display the same (1*R*,2*S*) stereochemistry at the oxirane carbons, where attack occurs? The only difference between **3** and **5** is the orientation of the isopropenyl group, indicating that this chiral center, although situated on a carbon atom far from the reacting epoxide, plays a crucial role for the regioselectivity of limonene-1,2-epoxide hydrolysis. Yet how does the isopropenyl group affect epoxide hydrolysis? Other interesting questions are of more general nature. For instance, can the LEH mechanism be referred to as acid-catalyzed? If yes, does it occur in a stepwise fashion, that is, protonation of the epoxide as a distinct first step, or is it a one-step reaction mechanism, where proton transfer and epoxide attack by water occur concertedly?

In this paper, we report quantum chemical calculations on the LEH-mediated reaction. We probe the energetics of the reaction mechanism with a model of the LEH active site consisting of the five residues proposed to be implicated in the reaction mechanism. The aim of this paper is to obtain a deeper understanding of LEH-mediated catalysis, in particular to provide an explanation for the observed regioselectivity of limonene-1,2-epoxide hydrolysis.

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Computational Details

All calculations presented here were performed using the density functional theory method B3LYP[22] as implemented in the Gaussian 03 program package.[23] Geometry optimizations were performed with the double-ξ plus polarization basis set 6-31G(d,p). To obtain more accurate energies, single point calculations on the optimized geometries were performed with the larger basis set 6-311+G(2d,2p), which includes diffuse functions and double polarization functions on each atom. Solvent effects were calculated at the 6-31G(d,p) level by performing single point calculations on the optimized structures using the CPCM model.[24] In this model, the solvent is represented by a constant dielectric medium surrounding a cavity containing the solute. The dielectric constant was chosen as $\epsilon = 4$, which is the standard value used in modeling protein surroundings. In a few additional calculations performed on substrate molecules alone (without protein surroundings), $\epsilon = 80$ was used. Frequency calculations were performed with the 6-31G(d,p) basis set to obtain zero-point vibrational energies and to confirm the nature of the various stationary points. The latter implies no negative eigenvalues for minima and one imaginary frequency for transition states. Freezing some atoms to their crystallographic positions gives rise to a few small negative eigenvalues for the optimized structures; however, these are only in the order of 10 cm^{-1}. All reported energies are corrected for solvation and zero-point vibrational effects.

Results and Discussion

Chemical Model. A model of the active site of LEH was made on the basis of the crystal structure of LEH in complex with the valpromide inhibitor (PDB code 1NU3).[17] Coordinates for the five amino acids proposed to be important for catalysis were extracted from the PDB file as well as those for a water molecule, which in the crystal structure was observed to be

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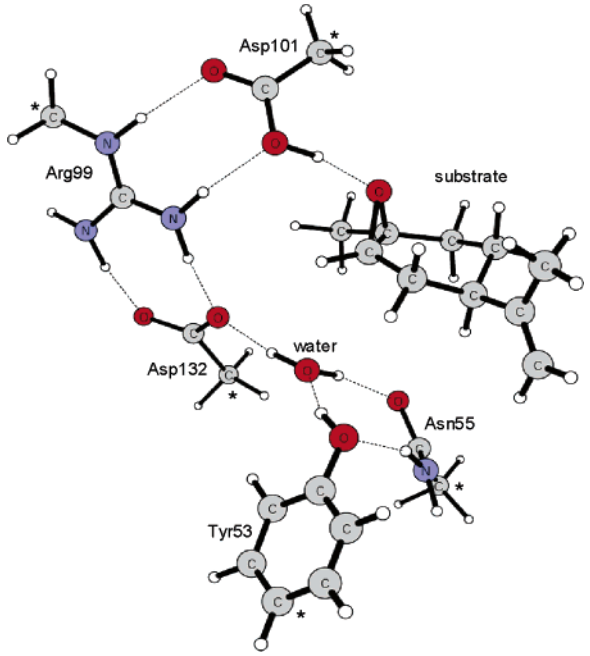
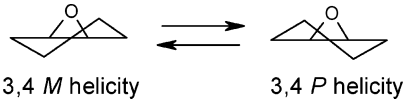


Figure 2. LEH-active site model including a substrate molecule ((1*S*,2*R*,4*R*)-limonene-1,2-epoxide). Asterisks (*) show atoms that are kept frozen to their crystallographically observed positions in calculations.

located close to the putative catalytic residues. Hydrogen atoms were added manually, and the five amino acids were truncated so that in principle only side chains were part of the active site model. The two aspartates were thus represented by acetic acid, tyrosine by phenol, asparagine by acetamide, and arginine by *N*-methyl-guanidine. The points of truncation were kept frozen to preserve the spatial arrangement of the active site residues. The model had a total size of 53 atoms without added substrate. Depending on which substrate was added, the model increased to either 73 (with 1-methylcyclohexene oxide) or 80 atoms (with limonene-1,2-epoxide). A picture of the LEH active site model with (1*S*,2*R*,4*R*)-limonene-1,2-epoxide as substrate is shown in Figure 2.

Substrates. Various substrates were modeled into the LEH active site model, followed by geometry optimizations. The six chosen substrates that were investigated theoretically are the two enantiomers (1*R*,2*S*) and (1*S*,2*R*) of 1-methylcyclohexene oxide (**1** and **2**, respectively) and the four stereoisomers (1*R*,2*S*,4*S*), (1*S*,2*R*,4*S*), (1*R*,2*S*,4*R*), and (1*S*,2*R*,4*R*) of the natural LEH substrate limonene-1,2-epoxide (**3**, **4**, **5**, and **6**, respectively, see Scheme 3). Because there does not exist any crystal structure of LEH in complex with a substrate or substrate analogue, the crystal structure of LEH in complex with the competitive inhibitor valpromide (dipropylacetamide)[17] was used as a guideline for positioning of the substrates in the active site model. In the crystal structure, the valpromide carbonyl is found in hydrogen-bonding distance to Asp101, and it is assumed that valpromide binds in a position similar to that of the LEH substrates.[17] The substrates were modeled into the active site model with the epoxide carbon pointing in the same direction as the valpromide carbonyl, thus allowing for hydrogen bonding to Asp101. This position of the substrates is also supported by the fact that nucleophilic attack by the catalytic water molecule has to occur from the opposite side of the oxirane ring to allow for optimal orbital overlap and epoxide cleavage to occur.

Scheme 4. The Two Possible Helicities of the Half-Chair Conformation of Cyclohexene Oxide



1-Methylcyclohexene Oxide. Although 1-methylcyclohexene oxide is not a natural substrate of LEH, it can still be hydrolyzed by this enzyme with a relative activity of 47% as compared to (4*R*)-limonene-1,2-epoxide.[21] However, one of the two enantiomers of 1-methylcyclohexene oxide, the (1*R*,2*S*) stereoisomer (**1**), is the preferred substrate, while (1*S*,2*R*)-1-methylcyclohexene oxide (**2**) is hydrolyzed at a slower rate.[18] Regioselectivity for 1-methylcyclohexene oxide is reported to be 85:15 (C1:C2); that is, attack at the most substituted carbon is favored. Hydrolysis of **1** thus mainly yields (1*S*,2*S*)-1-methylcyclohexane-1,2-diol (**7**), while conversion of **2** mainly gives (1*R*,2*R*)-1-methylcyclohexane-1,2-diol (**8**);[18] see Scheme 3.

In the quantum chemical calculations performed on this substrate, it must be taken into account that the epoxide is a substituted cyclohexane. While cyclohexane normally adopts a chair conformation, presence of the oxirane ring only allows for a half-chair conformation, in which four of the atoms are in the same plane. This conformation can exist in two different forms, which are best described by their helicities, *M* or *P* (see Scheme 4). The helicities play a crucial role for the understanding of the regioselectivity of epoxide opening of cyclohexene oxides. The two different conformers of the various substituted cyclohexene oxides described in this text will be referred to as 3,4 *M* or 3,4 *P*, meaning an *M* or *P* helicity around the 3,4 bond.[25,26] It should be noted that the two forms can interconvert and are rapidly equilibrating with ratios depending on the energy difference between the two conformers. For unsubstituted cyclohexene oxide, there is no energy difference between the two helicities and they are expected to exist in equal amounts. For 1-methylcyclohexene oxide, there will be a very small energy difference between the 3,4 *M* and the 3,4 *P* form due to the methyl group. Calculations on **2** alone (without LEH active site model) show a difference of 0.2 kcal/mol in favor of the 3,4 *P* helicity.[27] This difference is so small that it can be assumed that a mixture of 1-methylcyclohexene oxide is composed of 3,4 *M* and 3,4 *P* in approximately equal amounts. However, often one helicity will be preferred over the other during enzymatic hydrolysis, usually because it is better accommodated by the active site. For LEH, it is not known which helicity is preferred, if any, and calculations on both helicities have therefore been performed. For each of them, attack on either C1 or C2 was investigated. Thus, for (1*R*,2*S*)-1-methylcyclohexene oxide, four different reactions have been investigated, attack of water on either C1 or C2 for 3,4 *M* and attack on either C1 or C2 for 3,4 *P*.

LEH-Mediated Hydrolysis of (1*R*,2*S*)-1-Methylcyclohexene Oxide. The substrate was modeled into the active site model presented above in either 3,4 *M* or 3,4 *P* helicity, and geometry optimizations were performed. The resulting reactant (Re) geometries will be referred to as Re-1*M* and Re-1*P*, respectively,

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(26) This helicity assignment is independent of starting numbering from the *R* or the *S* carbon of cyclohexene oxide.[25]
(27) Solvent correction performed with $\epsilon = 80$.

Table 1. Calculated Barriers and Reaction Energies (in kcal/mol) for LEH-Mediated Conversion of 1-Methylcyclohexene Oxide to 1-Methylcyclohexane-1,2-diol

reaction	substrate[a]	attack on carbon	TS[b]	product[c]	barrier	reaction energy
Re-1*M* → Pr-1*M*C1	(1*R*,2*S*), 3,4 *M*	C1	twist-boat	(1*S*,2*S*)	17.5	−3.4
Re-1*M* → Pr-1*M*C2	(1*R*,2*S*), 3,4 *M*	C2	chair	(1*R*,2*R*)	15.9	−9.9
Re-1*P* → Pr-1*P*C1	(1*R*,2*S*), 3,4 *P*	C1	chair	(1*S*,2*S*)	14.9	−9.5
Re-1*P* → Pr-1*P*C2	(1*R*,2*S*), 3,4 *P*	C2	twist-boat	(1*R*,2*R*)	19.2	−4.0
Re-2*M* → Pr-2*M*C1	(1*S*,2*R*), 3,4 *M*	C1	chair	(1*R*,2*R*)	16.0	−9.0
Re-2*M* → Pr-2*M*C2	(1*S*,2*R*), 3,4 *M*	C2	twist-boat	(1*S*,2*S*)	19.1	−3.2
Re-2*P* → Pr-2*P*C1	(1*S*,2*R*), 3,4 *P*	C1	twist-boat	(1*R*,2*R*)	19.0	−2.8
Re-2*P* → Pr-2*P*C2	(1*S*,2*R*), 3,4 *P*	C2	chair	(1*S*,2*S*)	15.7	−9.5

[a] Epoxide stereochemistry and helicity around the 3,4 bond. [b] Conformation of substrate in the transition state. [c] Stereochemistry of the resulting diol.

Table 2. Important Distances (Å) of the Eight Transition States Optimized for 1-Methylcyclohexene Oxide

distance[a]	TS-1*M*C1	TS-1*M*C2	TS-1*P*C1	TS-1*P*C2	TS-2*M*C1	TS-2*M*C2	TS-2*P*C1	TS-2*P*C2
*r*1 Asp132-O to water-H	1.55	1.54	1.57	1.51	1.57	1.48	1.56	1.51
*r*2 water-H to water-O	1.02	1.03	1.02	1.03	1.02	1.04	1.02	1.03
*r*3 water-O to epoxide-C[b]	2.28	2.18	2.32	2.14	2.37	2.14	2.29	2.18
*r*4 epoxide-C[b] to epoxide-O	2.04	1.92	1.98	1.95	1.97	1.94	2.04	1.91
*r*5 epoxide-O to Asp101-H	1.06	1.07	1.08	1.06	1.06	1.06	1.04	1.08
*r*6 Asp101-H to Asp101-O	1.43	1.39	1.38	1.44	1.41	1.42	1.47	1.39

[a] See Figure 3 for definitions of *r*1−*r*6. [b] The epoxide carbon that is attacked by water, that is, C1 or C2.

where 1 indicates the substrate in Scheme 3, and *M* and *P* indicate the helicity around bond 3,4. Transition state (TS) structures for attack on either carbon were optimized and will be referred to as TS-1*M*C1, TS-1*M*C2, TS-1*P*C1, and TS-1*P*C2, where C1 and C2 denote the carbon on which attack occurs. The vicinal diols that are the resulting products (Pr) from the above reactions were also optimized and are referred to as Pr-1*M*C1, Pr-1*M*C2, Pr-1*P*C1, and Pr-1*P*C2. Calculated barriers and reaction energies are listed in Table 1. Figure 3 shows Re-1*P*, TS-1*P*C1, TS-1*P*C2, Pr-1*P*C1, and Pr-1*P*C2. The geometries obtained for calculations on Re-1*M* are analogous to those obtained for Re-1*P* and are not shown. Distances *r*1−*r*6 corresponding to bonds that are broken or formed during the reaction are shown in Table 2 for all four transition states TS-1*M*C1, TS-1*M*C2, TS-1*P*C1, and TS-1*P*C2 (see Figure 3 for a definition of *r*1−*r*6).

Our results support the proposed reaction mechanism (Scheme 2) involving protonation of the substrate by Asp101, nucleophilic attack of the water molecule on one of the epoxide carbons, and proton abstraction of a proton from water by Asp132. Tyr53, Asn55, and Asp132 form hydrogen bonds with the water molecule and hold it in a perfect position for nucleophilic attack on the oxirane ring of the substrate. Hydrogen bonds between Arg99 and the two aspartates stabilize the negative charge found on Asp132 in the reactant and on Asp101 in the product.

From our results, it can be concluded that LEH employs a general acid/general base-catalyzed mechanism, with Asp132 acting as general base and Asp101 as general acid. The observed mechanism of proton abstraction−proton donation is a common theme in enzyme catalysis and is sometimes referred to as a push−pull mechanism. It might be noted that epoxide cleavage involving an acid catalyst is often understood as implying carbocation formation and an S_N1 reaction mechanism. We find, however, that protonation, epoxide cleavage, and formation of the bond between the water oxygen and the epoxide carbon occur concertedly through a one-step S_N2-like reaction mechanism. It was also tried to geometry optimize the protonated intermediate that would be expected in a stepwise mechanism. This, however, was not possible, further supporting that the reaction is concerted and not stepwise.

It should be noted that, although LEH-mediated epoxide cleavage is shown to involve epoxide protonation, it cannot immediately be concluded that attack on the most substituted carbon (C1) is preferred. The obtained energies (see Table 1) show that for Re-1*M*, attack is actually preferred on C2 (a barrier of 15.9 kcal/mol as compared to 17.5 kcal/mol for attack on C1). For Re-1*P*, on the other hand, attack is preferred on C1 (a barrier of 14.9 kcal/mol as compared to 19.2 kcal/mol for attack on C2). If the resulting transition state structures are examined in detail, the explanation for this becomes obvious. For both TS-1*M*C2 and TS-*1P*C1, the conformation of the substrate in the transition state is close to that of a chair, while for TS-*1M*C1 and TS-1*P*C2 a twist-boat conformation is observed (see Figure 3). A twist-boat conformation lies several kcal/mol higher in energy than a chair conformation, and a reaction that proceeds through a chair conformation will therefore be preferred. For the individual conformers, 3,4 *M* or 3,4 *P*, attack is thus not determined by electronic factors but by the conformation of the resulting transition state. It should also be noted from Figure 3 that the diol products still exhibit the same chair or twist-boat conformations as the transition state. This explains why the products resulting from a reaction proceeding through a twist-boat transition state lie higher in energy than the ones formed by proceeding through a chair conformation (see Table 1).

Having established that attack occurs on the carbon that leads to a chair conformation, it should be remembered that the 3,4 *M* and the 3,4 *P* forms are expected to be in rapid equilibrium; that is, both conformers are present during LEH-mediated hydrolysis of **1**. The question is thus on which of the two helicities attack is preferred. The calculated energies reveal that for the two chair TSs the barrier is 14.9 for TS-1*P*C1 and 15.9 for TS-1*M*C2, showing that TS-*1P*C1 will be preferred over TS-1*M*C2. The difference of 1.0 kcal/mol corresponds very well to the experimentally observed regioselectivity of 85:15 (C1:C2) for 1-methylcyclohexene oxide.[18] The observation that TS-1*P*C1 will be preferred over TS-*1M*C2 can have two origins

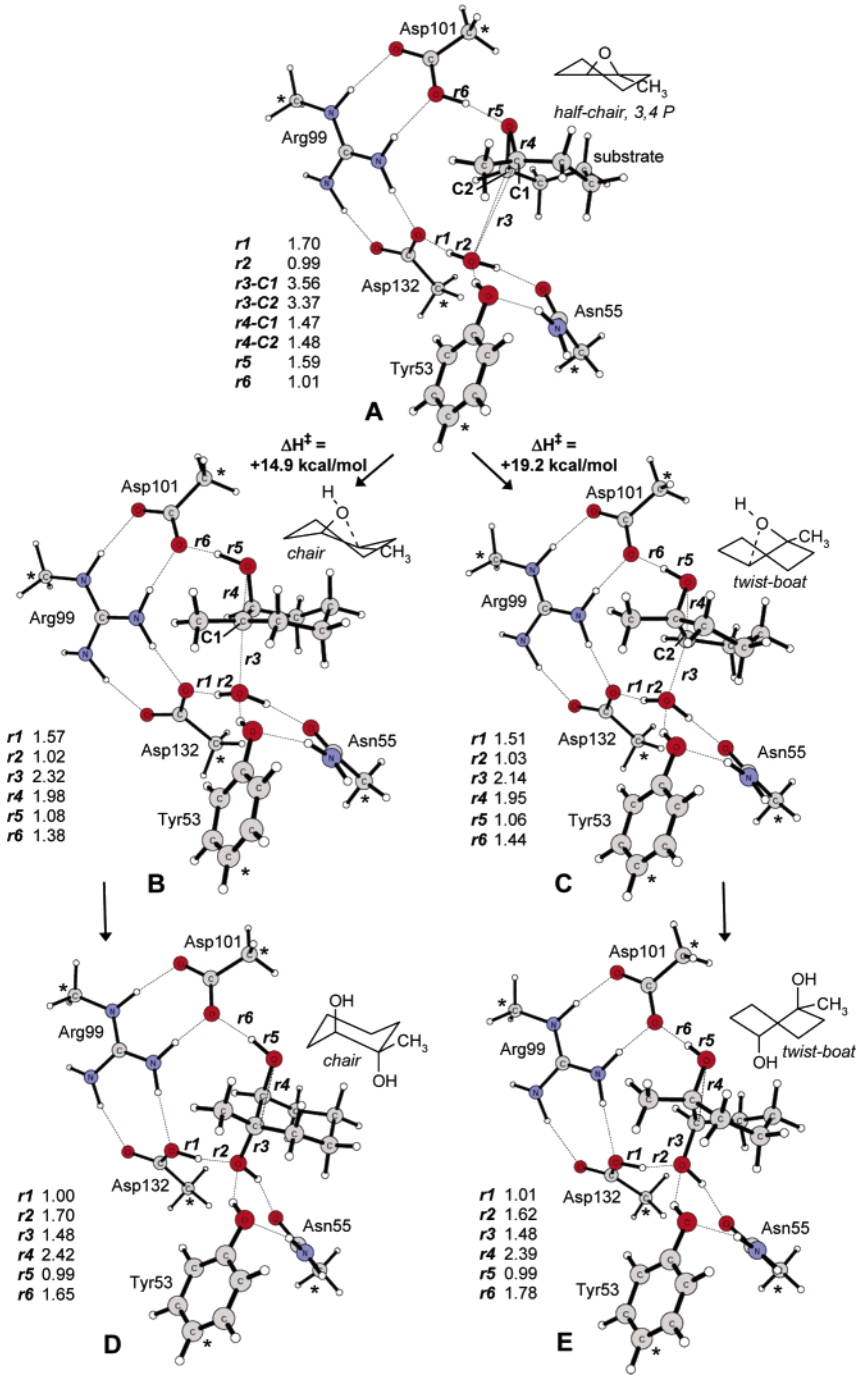

Figure 3. LEH-mediated hydrolysis of (1*R*,2*S*)-1-methylcyclohexene oxide (3,4 *P*). (A) Re-1*P*, (B) TS-1*PC*1, (C) TS-1*PC*2, (D) Pr-1*PC*1, (E) Pr-1*PC*2. Insets show the substrate conformation. Distances *r*1−*r*6 are in angstroms.

− either that attack at C1 is preferred over attack at C2 or that attack on helicity 3,4 *P* is preferred over helicity 3,4 *M*. The latter is expected if one of the two helicities fits better into the active site. However, our active site model does not have a size with which such a preference could be observed. The most likely explanation is that LEH preferably catalyzes attack on the most substituted carbon, at least for this particular substrate. The preference for the most substituted carbon is easily understandable, because the observed protonation of the epoxide results in a polarization of the epoxide C−O bond. The partial positive charge developed will preferentially be situated on the most substituted carbon (C1), because of the possibility of charge stabilization, and attack on C1 will therefore be facilitated. Comparing the water to epoxide distances (r3) for the four transition states, it can be seen that for attack on C1 they are 2.28 and 2.32 Å, while for attack on C2 they are 2.18 and 2.14 Å (see Table 2). The transition state for attack on C1 hence lies earlier with respect to this degree of freedom.

It might be added that for all products the two hydroxyls are observed in diaxial positions (Figure 3D and E). It is generally known that ring opening of cyclohexene oxides always leads to diaxial products (sometimes referred to as the Fürst Plattner rule).[19] The diaxial products can in principle subsequently ring-flip to give diequatorial products, which lie lower in energy. This would be expected to occur for 1-methylcyclohexane-1,2-diol, because there is no large substituent present that would

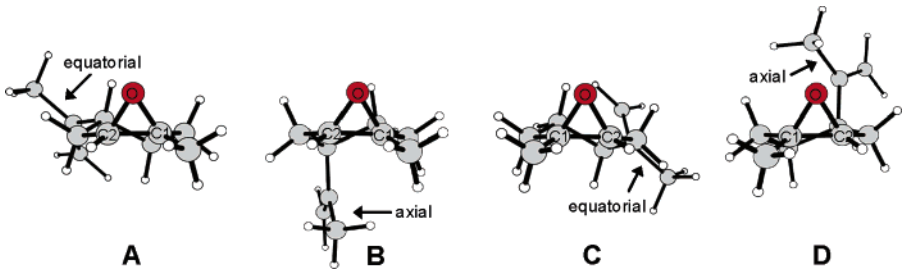

Figure 4. Different positions of the isopropenyl substituent dependent on the helicity of the substrate. (1*R*,2*S*,4*R*)-limonene-1,2-epoxide **5** with (A) 3,4 *P* helicity and (B) 3,4 *M* helicity, (1*S*,2*R*,4*R*)-limonene-1,2-epoxide **6** with (C) 3,4 *P* helicity and (D) 3,4 *M* helicity. Arrows point toward the isopropenyl group, while axial and equatorial refer to the position of the group. The energy difference of (B) relative to (A) is 3.0 kcal/mol and of (D) relative to (C) is 5.7 kcal/mol.

impair ring-flipping. However, this flipping is not expected to be catalyzed by LEH and was therefore not the subject of our investigation.

LEH-Mediated Hydrolysis of (1*S*,2*R*)-1-Methylcyclohexene Oxide. (1*S*,2*R*)-1-Methylcyclohexene oxide (**2**) was also modeled into the LEH active site model in either 3,4 *M* or 3,4 *P* helicity and geometry optimized. Resulting reactants are Re-2*P* and Re-2*M*. Transition states for attack on either C1 or C2 were optimized and are referred to as TS-2*P*C1, TS-2*P*C2, TS-2*M*C1, and TS-2*M*C2. The diaxial products are Pr-2*P*C1, Pr-2*P*C2, Pr-2*M*C1, and Pr-2*M*C2. Because all geometries are analogous to those obtained with (1*R*,2*S*)-1-methylcyclohexene oxide, these are not shown here. However, calculated barriers and reaction energies are shown in Table 1, and important distances of the transition state geometries are given in Table 2.

For this substrate, we observe the same one-step mechanism as for the other enantiomer. Regarding the regioselectivity, the situation is opposite to that for the (1*R*,2*S*)-isomer, because reactions resulting in a chair conformation are obtained by attack on C2 of the 3,4 *P* conformer and C1 of the 3,4 *M* conformer. However, the obtained energies of 16.0 kcal/mol for TS-2*M*C1 and 15.7 kcal/mol for TS-2*P*C2 are so close that we cannot conclude that one of them will be preferred over the other. This is in slight disagreement with experimental results, because these data do not support the reported preference for attack on C1 of (1*S*,2*R*)-1-methylcyclohexene oxide.[18] However, it might be noted that the experimental data on which the reported regioselectivity of (1*S*,2*R*)-1-methylcyclohexene oxide is based harbor large uncertainties.[18]

Limonene-1,2-epoxide. The experimental hydrolysis of limonene-1,2-epoxide has shown interesting results.[9,21] For all four substrates, hydrolysis is completely regioselective with attack on the most substituted carbon (C1) for **4** and **5** and the less substituted carbon (C2) for **3** and **6**. The only product of conversion of **3** and **4** is thus (1*R*,2*R*,4*S*)-limonene-1,2-diol (**9**), while (1*S*,2*S*,4*R*)-limonene-1,2-diol (**10**) is the only product for conversion of **5** and **6**; that is, the stereoisomers are hydrolyzed in an enantioconvergent manner. It has been implied that the regioselectivity of substrate hydrolysis can be explained by active-site structure imposed variation of the relative orientations of the stereoisomers.[17] However, as shall be shown below, our quantum chemical calculations indicate that the observed regioselectivity of limonene-1,2-epoxide hydrolysis is not governed by the orientation of the substrate in the active site but is rather due to the half-chair conformation of limonene-1,2-epoxide. The chair and twist-boat conformations of the transition states will determine where attack is likely to occur, as presented for 1-methylcyclohexene oxide above. However, while 1-methylcyclohexene oxide gives a mixture of products,[18] because the two possible helicities will favor attack on different carbons (see above), limonene-1,2-epoxide hydrolysis is absolutely regioselective.[9,21] This can be explained by the presence of the isopropenyl substituent in limonene-1,2-epoxide. In principle, limonene-1,2-epoxide can exist in two different helicities just as 1-methylcyclohexene oxide. However, in this case, one helicity will be considerably lower in energy than the other due to the position of the isopropenyl group, as shown below.

It is generally known that a substituent on a six-membered ring will be preferred to be in a position referred to as equatorial, because steric interactions are minimized in this way. The alternative axial position is normally avoided. If **6** is taken as an example, the 3,4 *M* form would have the isopropenyl group in an axial position, while the 3,4 *P* form has the isopropenyl group positioned equatorially (Figure 4). Calculations show that the 3,4 *M* conformer of **6** lies 5.7 kcal/mol higher in energy than the 3,4 *P* form.[27] These calculations were performed without LEH, and the energy difference between the two forms is thus solely due to the orientation of the isopropenyl group. For **5**, the situation is in principle the same. The energy difference is somewhat smaller, because in this case the axial isopropenyl group points in the opposite direction of the oxirane ring, which is not as sterically unfavorable as having an axial isopropenyl pointing in the same direction as the oxirane (Figure 4). The energy difference between the axial and the equatorial forms of **5** is calculated to be 3.0 kcal/mol, which, however, still can be considered fairly large. It can thus be assumed that for each limonene-1,2-epoxide stereoisomer, only the helicity with the isopropenyl in an equatorial position will be observed. LEH-mediated hydrolysis of limonene-1,2-epoxide has hence only been modeled for one helicity for each stereoisomer. It should be noted that the above explains how the stereochemistry of the isopropenyl substituent affects the regioselectivity of limonene-1,2-epoxide hydrolysis, because the isopropenyl group locks the substrate in one helicity, in which attack will occur only on the carbon that results in formation of a chair transition state, as shown below.

LEH-Mediated Hydrolysis of Limonene-1,2-epoxide. The different stereosiomers of limonene-1,2-epoxide **3**, **4**, **5**, or **6** were modeled into the active site model and geometry optimized. For all stereosiomers, the isopropenyl substituent was placed equatorially, implying 3,4 *M* helicity for stereosiomers **3** and **4** and 3,4 *P* helicity for **5** and **6**. The optimized reactants

Table 3. Calculated Barriers and Reaction Energies (in kcal/mol) for LEH-Mediated Conversion of Limonene-1,2-epoxide to Limonene-1,2-diol

reaction	substrate[a]	attack on carbon	TS[b]	product[c]	barrier	reaction energy
Re-3 → Pr-3C1	(1*R*,2*S*,4*S*), 3,4 *M*	C1	twist-boat	(1*S*,2*S*,4*S*)	17.6	−3.5
Re-3 → Pr-3C2	(1*R*,2*S*,4*S*), 3,4 *M*	C2	chair	(1*R*,2*R*,4*S*)	16.5	−9.7
Re-4 → Pr-4C1	(1*S*,2*R*,4*S*), 3,4 *M*	C1	chair	(1*R*,2*R*,4*S*)	16.1	−9.5
Re-4 → Pr-4C2	(1*S*,2*R*,4*S*), 3,4 *M*	C2	twist-boat	(1*S*,2*S*,4*S*)	19.0	−3.6
Re-5 → Pr-5C1	(1*R*,2*S*,4*R*), 3,4 *P*	C1	chair	(1*S*,2*S*,4*R*)	14.9	−9.7
Re-5 → Pr-5C2	(1*R*,2*S*,4*R*), 3,4 *P*	C2	twist-boat	(1*R*,2*R*,4*R*)	19.5	−4.1
Re-6 → Pr-6C1	(1*S*,2*R*,4*R*), 3,4 *P*	C1	twist-boat	(1*R*,2*R*,4*R*)	19.0	−2.8
Re-6 → Pr-6C2	(1*S*,2*R*,4*R*), 3,4 *P*	C2	chair	(1*S*,2*S*,4*R*)	16.3	−9.4

[a] Epoxide stereochemistry and helicity around the 3,4 bond. [b] Conformation of the substrate in the transition state. [c] Stereochemistry of the resulting diol.

Table 4. Important Distances (Å) of the Eight Transition States Optimized for the Four Stereosiomers of Limonene-1,2-epoxide

distance[a]	TS-3C1	TS-3C2	TS-4C1	TS-4C2	TS-5C1	TS-5C2	TS-6C1	TS-6C2
*r*1 Asp132-O to water-H	1.54	1.53	1.58	1.48	1.56	1.51	1.56	1.51
*r*2 water-H to water-O	1.02	1.03	1.02	1.04	1.02	1.03	1.02	1.03
*r*3 water-O to epoxide-C[b]	2.26	2.12	2.37	2.14	2.31	2.14	2.28	2.18
*r*4 epoxide-C[b] to epoxide-O	2.04	1.93	1.98	1.94	1.98	1.96	2.04	1.92
*r*5 epoxide-O to Asp101-H	1.06	1.07	1.06	1.06	1.08	1.06	1.04	1.08
*r*6 Asp101-H to Asp101-O	1.42	1.39	1.41	1.42	1.37	1.42	1.47	1.38

[a] See Figure 5 for definitions of *r*1−*r*6. [b] The epoxide carbon that is attacked by water, that is, C1 or C2.

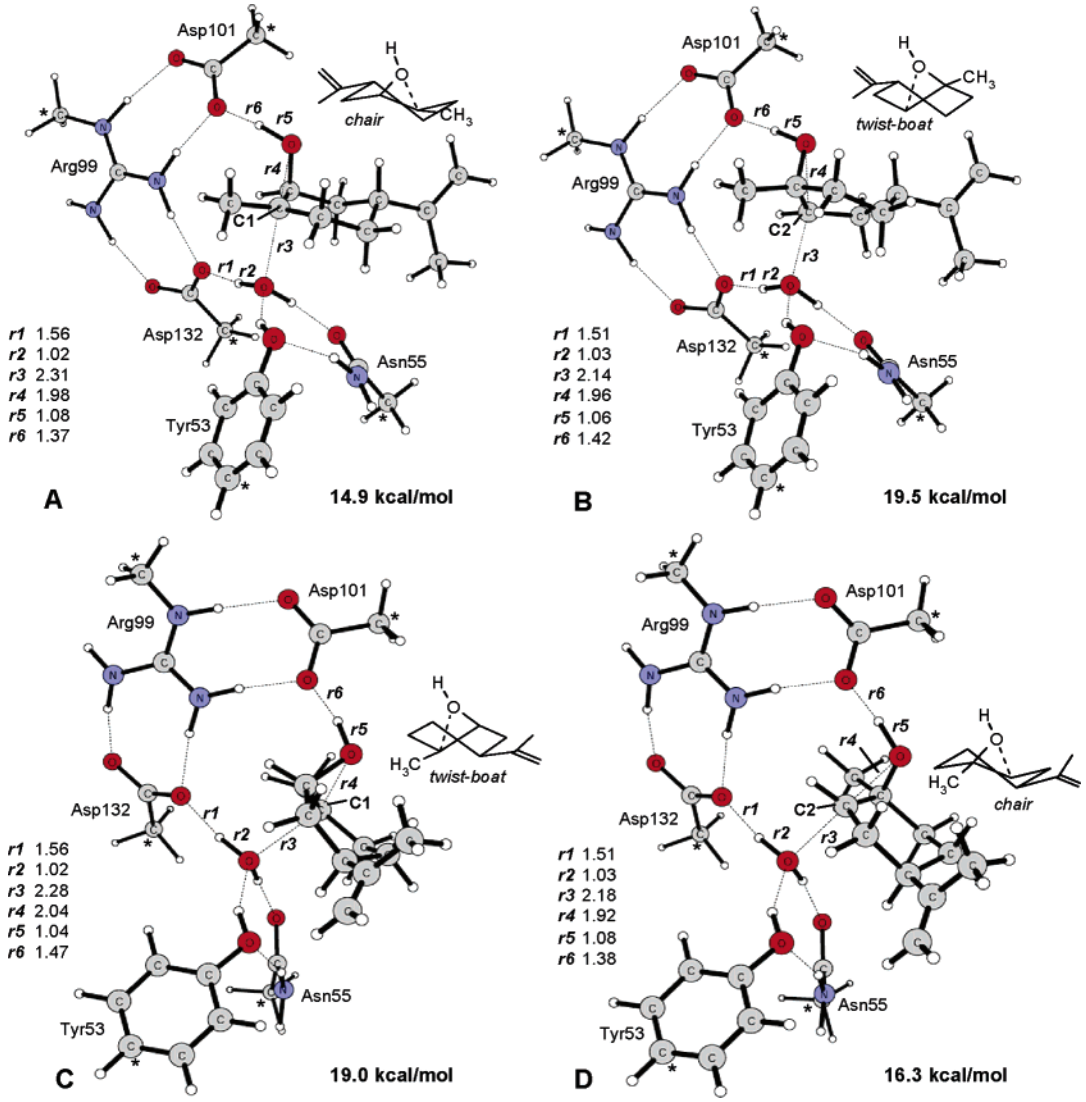

Figure 5. Optimized transition state structures for LEH-mediated hydrolysis of **5** (1*R*,2*S*,4*R*)-limonene-1,2-epoxide (3,4 *P*) and of **6** (1*S*,2*R*,4*R*)-limonene-1,2-epoxide (3,4 *P*). (A) TS-5C1, (B) TS-5C2, (C) TS-6C1, (D) TS-6C2. Insets show the substrate conformation. Distances *r*1−*r*6 are in angstroms. Calculated barriers for the different transition states are also shown.

are referred to as Re-3, Re-4, Re-5, and Re-6, respectively. For all reactants, attack on either C1 or C2 was investigated, and the resulting transition states are accordingly referred to as TS-3C1, TS-3C2, TS-4C1, TS-4C2, TS-5C1, TS-5C2, TS-6C1, and TS-6C2. The products are called Pr-3C1, Pr-3C2, Pr-4C1, Pr-4C2, Pr-5C1, Pr-5C2, Pr-6C1, and Pr-6C2. Barriers and reaction energies are listed in Table 3, and important distances are shown in Table 4. The geometries for TS-5C1, TS-5C2, TS-6C1, and TS-6C2 are shown in Figure 5.

We observe the same general acid/general base-catalyzed one-step mechanism for LEH-mediated hydrolysis of the natural substrate as for 1-methylcyclohexene oxide. The different stereoisomers of limonene-1,2-epoxide also exhibit an equivalent transition from half-chair conformation in the reactant geometries to chair or twist-boat conformation in the transition states and finally to diaxial chair or twist-boat products. The barrier for LEH-mediated hydrolysis of **5** is calculated as 14.9 kcal/mol for the reaction proceeding through a chair transition state (TS-5C1), while the barrier for attack on C2 (TS-5C2) is found to be as large as 19.5 kcal/mol. It can be concluded from the energy difference that attack will only occur on C1, which is in agreement with the experimentally observed regioselectivity.[9,21] It can also be noted that the experimentally determined activation energy of LEH for hydrolysis of **5** has been reported to be 12.4 kcal/mol.[8,28] The calculated barrier of 14.9 kcal/mol is thus reasonably close to the experimental value. The barriers observed for the other stereoisomers of limonene-1,2-epoxide indicate that, also for these, transition states with the substrate in a chairlike conformation will be preferred (see Table 3). In each case, our results reproduce the experimentally observed regioselectivity, that is, attack on C2 for **3**, C1 for **4**, and C2 for **6**.[9,21] The regioselectivity of limonene-1,2-epoxide hydrolysis is thus not determined by electronic factors, but by conformational factors.

On the Protonation State of Asp101. The above results clearly support the proposed LEH mechanism. However, one feature of this mechanism that might seem questionable is the protonation state of Asp101. We have investigated if LEH-mediated epoxide hydrolysis is possible without the assistance of the Asp101 proton, that is, employing a general base-catalyzed mechanism only. Epoxide opening at C1 of (1*R*,2*S*)-1-methylcyclohexene oxide (3,4 *P*) **1** was modeled without a proton present on Asp101. The optimized transition state structure is displayed in Figure 6. The barrier for the general base-catalyzed reaction was found to be as large as 44.1 kcal/mol. This should be compared to the barrier of 14.9 kcal/mol found for the general acid/general base-catalyzed mechanism (Figure 3, Table 1). The high barrier can be explained by the lack of stabilization of the formed oxyanion. From the crystal structure of LEH, it was not possible to identify any residues that could aid in oxyanion stabilization, except Asp101 in its protonated form. The obtained results are consistent with observations that chemical hydrolysis of limonene-1,2-epoxide and 1-methylcyclohexene oxide only occurs under acidic conditions, while the substrates are stable under basic conditions.[16,18] It can thus be excluded that LEH employs a general base-catalyzed reaction mechanism only. One can, furthermore, envision that in case Asp101 is not protonated in the pre-catalytic state of LEH, protonation could be a distinct first step in the catalytic mechanism, probably with bulk water as the proton donor. This would only be associated with a small energetic cost, in which case the energies presented in the previous subsections would be slightly higher, but no more than a few kcal/mol.

(28) It might be noted that the experimental activation energy of LEH was determined with a diastereomeric mixture of (1*R*,2*S*,4*R*)-limonene-1,2-epoxide and (1*S*,2*R*,4*R*)-limonene-1,2-epoxide. However, because their hydrolysis occurs sequentially, it can be assumed that the determined value only is based on the substrate that is converted first, that is, (1*R*,2*S*,4*R*)-limonene-1,2-epoxide.[8,21]

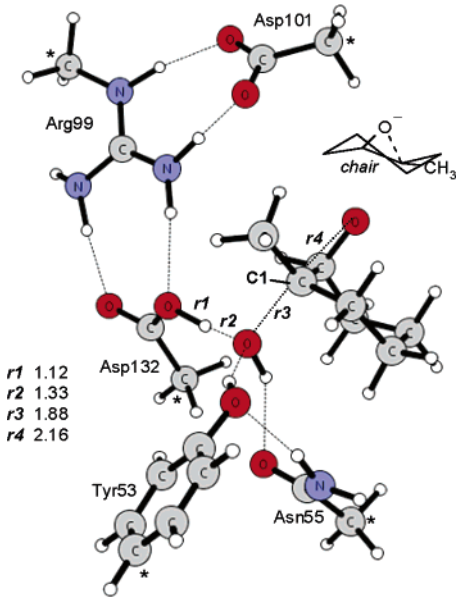

Figure 6. Optimized transition state structure for general base-catalyzed hydrolysis of (1*R*,2*S*)-1-methylcyclohexene oxide (3,4 *P*). Distances *r*1−*r*4 are in angstroms.

Conclusions

In this paper, we have reported a theoretical examination of the catalytic mechanism of limonene epoxide hydrolase (LEH). Hydrolysis of different substrates was investigated with an LEH active site model consisting of five residues and a crystallographically observed water molecule. From our calculations, we conclude that LEH employs a general acid/general base-catalyzed concerted reaction mechanism, which involves epoxide protonation by Asp101, nucleophilic attack by water, and abstraction of a proton from water by Asp132. We were also able provide an explanation for the experimentally observed regioselectivity of limonene-1,2-epoxide hydrolysis. The isopropenyl group of limonene-1,2-epoxide was shown to play a crucial role, because it restricts the half-chair conformation of limonene-1,2-epoxide to one of two possible helicities. In this conformation, attack on the different epoxide carbons will lead to either a chairlike or a twist-boat transition state structure, the latter, however, resulting in a higher barrier. The regioselectivity of limonene-1,2-epoxide is thus governed by conformational and not electronic factors.

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Supporting Information Available: Complete list of authors for ref 23 and coordinates for all LEH structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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