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Efficient Epoxide Hydrolase Catalyzed Resolutions of (+)- and (−)-*cis*/*trans*-Limonene Oxides

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This manuscript is dedicated to Wolf-Dieter "Woody" Fessner on the occasion of his 60th birthday

The synthesis of enantiomerically pure *cis*- and *trans*-limonene oxides and their corresponding diols from easily accessible raw materials has been of much interest for a long time. A straightforward one-step biocatalytic resolution of the (+)-*cis*/*trans* limonene oxide and the (−)-*cis*/*trans*-limonene oxide has been investigated. Epoxide hydrolases showing complementary stereoselectivity were recombinantly expressed in *Escherichia coli*, which allowed easy purification. The conditions for the selective epoxide hydrolase catalyzed ring-opening reactions have been optimized and enabled the preparation of all limonene oxide enantiomers. The described utilization of recombinant epoxide hydrolases for the synthesis of all limonene oxide enantiomers was superior to chemical routes and represents a highly resource-efficient one-step preparation.

Introduction

Chiral epoxides and vicinal diols are of much interest as building blocks in organic synthesis, as well as in biosynthetic pathways used by nature, for which the exact stereochemical configuration is important.[1–4] Specifically, limonene oxides and their corresponding limonene diols have been found in a variety of metabolic pathways,[5,6] and a number of different approaches have been described for the chemical synthesis of these compounds in enantiomerically pure form. The direct catalytic asymmetric oxidation of limonenes has been extensively explored, but the optical purity of the obtained chiral limonene oxides was generally not high enough. For example, although the catalytic stereoselective epoxidation of (*S*)-(−)-limonene and (*R*)-(+)-limonene with the Jacobsen chiral (*R*,*R*)- and (*S*,*S*)- or even achiral [Mn(salen)] complexes was achieved at high conversion, the selectivity and diastereomeric excess (*de*) values were low.[7–9] Also, the exploitation of heterogeneously immobilized chiral molybdenum(II) complexes led to mixtures of monoepoxide and diepoxide as products with *de* values between 40 and 60%.[10] A chiral bishydroxamic acid complex of molybdenum selectively oxidized the most electron-rich double bond in limonene, but the product showed only a 61% *de* value.[11] Alternatively, the commercially available *cis*/*trans* mixture of (+)-limonene oxide has been explored for direct separation methods of the diastereomers,[12] by which it was difficult to obtain pure products, as well as for the preparation of the *cis*-(*R*)-(+)-limonene oxide by selective epoxide ring opening of the *trans* isomer by nucleophilic amines, such as pyrrolidine and piperidine, and for the preparation of the *trans*-(*R*)-(+)-limonene oxide by selective hydrolysis of the *cis*-(*R*)-(+)-limonene oxide by less nucleophilic amines, such as triazole or pyrazole.[13] The *trans*-(+)-limonene oxide has also been prepared by hydrolytic kinetic separation of the (+)-limonene oxide *cis*/*trans* mixture without additional catalyst, but the reaction required heating to reflux for over 120 h.[14]

Therefore, there is a need to develop innovative and industrially viable manufacturing methods that combine excellent selectivity with sustainability and improved safety, health, and environmental aspects. The strategy that is used by nature to open selectively the epoxide ring is based on catalysis by epoxide hydrolases (EHs, EC 3.3.2.*x*)[15] and has attracted interest for preparing bioactive compounds, agrochemicals, flavors, fragrances, and metabolites.[16] Most of the EHs that have been investigated over the last few decades as biocatalysts for enantioselective kinetic resolutions of racemic epoxide mixtures and for the stereoselective hydrolysis of *meso*-epoxides[4,17] belong to the α/β-hydrolase superfamily and show the typical fold of other hydrolytic enzymes, such as lipases and proteases.

More recently, a family of atypical EHs, the limonene-1,2-epoxide hydrolases (LEHs, EC 3.3.2.8), has been discovered and characterized.[18,19] The first example was isolated from a *Rhodococcus erythropolis* strain that is capable of using either (+)- or (−)-limonene as the sole carbon and energy source. Further studies have shown that the *R. erythropolis* LEH (*Re*-LEH) has features that are distinct from those of the previously described EHs, such as a much smaller molecular mass, no se-

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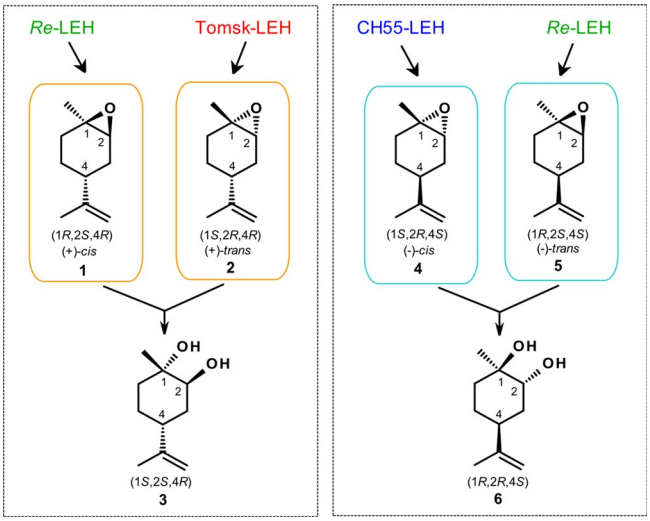
quence similarity, and a completely different protein fold.[20] Moreover, the proposed catalytic mechanism differs from that used by EHs of the α/β-hydrolase superfamily by proceeding in a concerted fashion through the activation of a water molecule by three charged residues in the active site.[20,21] Another EH showing similarity to *Re*-LEH has been identified from *Mycobacterium tuberculosis*,[22] but its functional properties and possible synthetic application have not been deeply studied so far. As far as the substrate specificity of *Re*-LEH is concerned, this enzyme shows a rather narrow substrate scope, with a marked preference for limonene-1,2-oxide and some related compounds, for example, cyclic *meso*-epoxides.[23] This fact, together with the low enantioselectivity shown in the kinetic resolution of typical EH substrates, for example, styrene oxide,[23] has partly limited the interest toward its synthetic exploitation. Only a few examples of practical applications of *Re*-LEH in synthetic organic chemistry have been reported to date.[24,25] In contrast, *Re*-LEH has been recently the subject of different protein engineering studies aimed at the improvement of useful applicative features, such as the stereoselectivity[26] and thermostability.[27]

Interestingly, *Re*-LEH, as well as other EHs,[28] is capable of performing enantioconvergent processes, thanks to complementary regioselectivity with respect to which oxirane carbon atom is attacked on each substrate enantiomer. This ability was first observed during studies into the *Re*-LEH-catalyzed hydrolysis of the natural substrate limonene-1,2-oxide.[23] Specifically, the enzymatic epoxide ring-opening of *cis*/*trans* mixtures of (+)- and (−)-limonene oxide resulted in, respectively, (1*S*,2*S*,4*R*)- and (1*R*,2*R*,4*S*)-limonene-1,2-diol as the sole products (Scheme 1, compounds **3** and **6**, respectively). Additionally, *Re*-LEH has shown a strong preference for the *cis* form of (+)-limonene oxide (**1**) and for the *trans* form of (−)-limonene oxide (**5**). Under the used experimental conditions, this fact resulted in a "sequential" hydrolysis behavior, with the hydrolysis of the slow-reacting diastereomers starting only after complete consumption of the preferred ones.



Scheme 1. Enantioconvergent hydrolysis of *cis*/*trans* mixtures of (+)-limonene oxide (left) and (−)-limonene oxide (right) catalyzed by selected LEHs (see the Supporting Information for more details).

Recently, we have discovered and characterized two novel LEHs (Tomsk-LEH and CH55-LEH) from hot-springs metagenomic libraries.[29] In agreement with their natural sources, both enzymes, which have been cloned and overexpressed in *Escherichia coli*, have higher optimal temperatures and apparent melting temperatures than *Re*-LEH. Moreover, they are also active within a broad pH range (5.0–9.5) and thus show attractive features for synthetic application.

If tested on *cis*/*trans* mixtures of (+)- and (−)-limonene oxide, both Tomsk-LEH and CH55-LEH have shown appreciable activity toward these substrates. The same enantioconvergent processes previously described for *Re*-LEH have been observed and led to the quantitative conversion of the (+)- and (−)-limonene oxide *cis*/*trans* mixtures into the **3** and **6** diols, respectively.

Unexpectedly, the novel LEHs have shown a markedly different stereopreference for the limonene-1,2-epoxide isomers than *Re*-LEH. In particular, Tomsk-LEH has shown the opposite stereospecificity in the hydrolysis of *cis*/*trans* mixtures of (+)-limonene oxide by preferring the *trans* isomer (Scheme 1, **2**), and CH55-LEH prefers the *cis* form of (−)-limonene oxide (**4**). The reasons underlying this behavior have not been completely clarified yet. However, analysis of the crystal structures of the novel LEHs in comparison with that of *Re*-LEH suggests the possible role of different active-site residues in controlling the access and recognition of limonene oxide isomers,[29] and site-directed mutagenesis experiments are currently ongoing in our labs to understand the specific amino acid contributions.

With, for the first time, LEHs with complementary stereopreference for the limonene oxide isomers in hand, we wondered whether this finding could be practically exploited to perform the biocatalytic resolution of *cis*/*trans* mixtures of (+)- and (−)-limonene oxide, which would thus allow the simple and straightforward preparation of enantiomerically pure limonene oxides; the results are reported herein.

Results and Discussion

To establish whether the proposed resolution processes would be suitable to application on a preparative scale, the performances of the selected LEHs were investigated at high substrate concentrations. The preliminary information about the activity and stereospecificity of the three LEHs on the hydrolysis of limonene oxide mixtures had been obtained for small-scale reactions (1 mL total volume) and with quite diluted substrate solutions (10 mmol L^{-1}),[29] conditions that are not suitable for large-scale applications.

Gram-scale resolutions of (+)- and (−)-limonene oxide mixtures catalyzed by LEHs

After some preliminary investigations on an analytical scale at increasing substrate concentrations (data not shown), a first set of preparative-scale reactions (20 mL total volume, reac-

Table 1. Preparative resolution of (+)- and (−)-limonene oxide mixtures catalyzed by LEHs under non-optimized conditions.[a]

Process parameter	(+)-Limonene oxide		(−)-Limonene oxide	
	A: *Re*-LEH	B: Tomsk-LEH	C: CH55-LEH	D: *Re*-LEH
enzyme [mg mL^{-1}]	0.2	1.5	1.5	0.4
reaction time [h]	3	22	8	2
epoxide yield [%][b]	45 (**2**)	38 (**1**)	33 (**5**)	18 (**4**)
diol yield [%][c]	49 (**3**)	53 (**3**)	67 (**6**)	77 (**6**)
STY[d,e] [mmol L^{-1} h^{-1}]	74.2	8.7	20.7	44.3
specific productivity [μmol mg^{-1} h^{-1}][e]	371	5.8	14	111
ECN[e,f] [mg mmol^{-1}]	0.9	7.9	9.1	4.5

[a] Reactions (20 mL) performed in KP_i buffer (pH 8.0) and 10% (v/v) CH_3CN containing 5 mol L^{-1} substrate (0.5 mol L^{-1} final concentration, 1.52 g, 1.64 mL) at $T=20$ °C. [b] Recovery yield estimated based on the unreacted epoxide isomer indicated in brackets. [c] Recovery yield estimated based on the formed diol indicated in brackets. [d] STY: space-time yield. [e] Calculated based on epoxide recovery. [f] ECN: enzyme consumption number.

tions A–D, Table 1) was set up at a 50 times higher initial substrate concentration (0.5 mol L^{-1}) than in our previous studies.

Commercially available *cis*/*trans* mixtures of (+)-limonene oxide (59:41 mixture of **1** and **2**, Figure 1a) and (−)-limonene oxide (55:45 mixture of **4** and **5**, Figure 1d) were dissolved in CH_3CN (5 mol L^{-1}) and diluted with 9 volumes of the appropriate LEH-containing buffer solution. During our characterization studies, the novel enzymes Tomsk-LEH and CH55-LEH showed a lower specific activity toward the limonene oxide mixtures than *Re*-LEH did,[29] so a higher amount of these biocatalysts (1.5 mg mL^{-1}) was employed in reactions B and C, whereas *Re*-LEH was used at concentrations of 0.2 and 0.4 mg mL^{-1} in reactions A and D, respectively. All of the reactions were performed at $T=20$ °C and pH 8.0, and samples were withdrawn at scheduled times and extracted with ethyl acetate.

After different reaction times ($t=2$–22 h, Table 1), chiral GC analysis of the bioconversion samples showed the complete hydrolysis of the preferred isomer (**1**, **2**, **4**, and **5** in reactions A, B, C, and D, respectively) and the corresponding formation of diols **3** (reactions A and B) and **6** (reactions C and D). The residual limonene oxide stereoisomers were recovered by extraction with ethyl acetate and were easily separated from the diols by flash chromatography.

All reaction products (unreacted limonene oxides and diols) were recovered with good to excellent yields and in enantiopure form. Figure 1 shows the chiral GC analysis of the crude limonene oxide mixtures (a and d) and the four pure limonene oxide isomers after the enzymatic resolutions (b, c, e, and f). No formation of byproducts was observed (see the Supporting Information for the complete GC analysis of reactions A–D showing the formation of diols **3** and **6**).

It is worth mentioning that the exploitation of LEHs at comparably high substrate concentrations (76 g L^{-1}) has never been reported before; the closest example was the application of *Re*-LEH to the resolution of *trans*-spiroepoxide,[24] which, after process optimization, was performed at a 10 g L^{-1} substrate concentration.

Different behaviors of the biocatalysts can be deduced from a careful analysis of the process parameters of the four preparative reactions shown in Table 1. Although satisfactory yields (38%) were obtained in reaction B catalyzed by Tomsk-LEH in the resolution of the (+)-limonene oxide mixture, this reaction achieved the lowest space-time yields (STY) and specific-productivity values as a result of the very long reaction time ($t=22$ h). Very good STY and specific-productivity values were obtained in the very fast reactions A and D catalyzed by *Re*-LEH on the (+)- and (−)-limonene oxide mixtures, respectively. However, the relatively lower recovery yield (18%) obtained in reaction D suggests a concomitant hydrolysis of both stereoisomers **4** and **5** that could possibly be controlled, for example, by using a suitable enzyme/substrate ratio and changing the reaction conditions.

Optimization of the biocatalyzed processes

Several reaction parameters were considered to optimize the described resolution processes.

First of all, to avoid the undesired chemical hydrolysis of the substrates, the stability of the commercially available limonene epoxide mixtures was investigated by incubation at a wide range of pH (6.5–9.0) and temperature (20–50 °C) values. The assays were performed at a high substrate loading (152 g L^{-1}, 1 mol L^{-1}), in the absence of organic cosolvents, and with vigorous shaking. With consideration of the very small amount of organic cosolvent used in reactions A–D (acetonitrile, 18 μL mL^{-1}), we envisaged that the direct addition of neat substrates to the aqueous phase providing the biphasic system could possibly be applied during the enzymatic resolutions. Interestingly, the substrates showed a remarkable stability; negligible hydrolysis (<2%) was observed after 24 h in the whole range of tested conditions. The concentration of limonene oxides in the aqueous phase was experimentally determined to be about 0.24 mol L^{-1} (for details, see the Experimental Section).

The choice of the most suitable pH and temperature values among the mentioned ranges for each resolution reaction was therefore made on the basis of the already available information on the influence of these parameters on the activity and stability of the three LEHs.[29] As far as the pH value is concerned, we decided to continue our studies at pH 8.0, because all of the LEHs showed good performances under this condition. However, Tomsk-LEH and CH55-LEH, both from thermophilic sources, have higher optimal temperatures and apparent melting temperatures than *Re*-LEH. Specifically, the novel LEHs showed optimal activity, under analytical assay conditions, at $T=40$ and 60 °C, respectively, whereas the *Re*-LEH optimal activity was at $T=30$ °C.[29] To avoid undesired loss of activity of

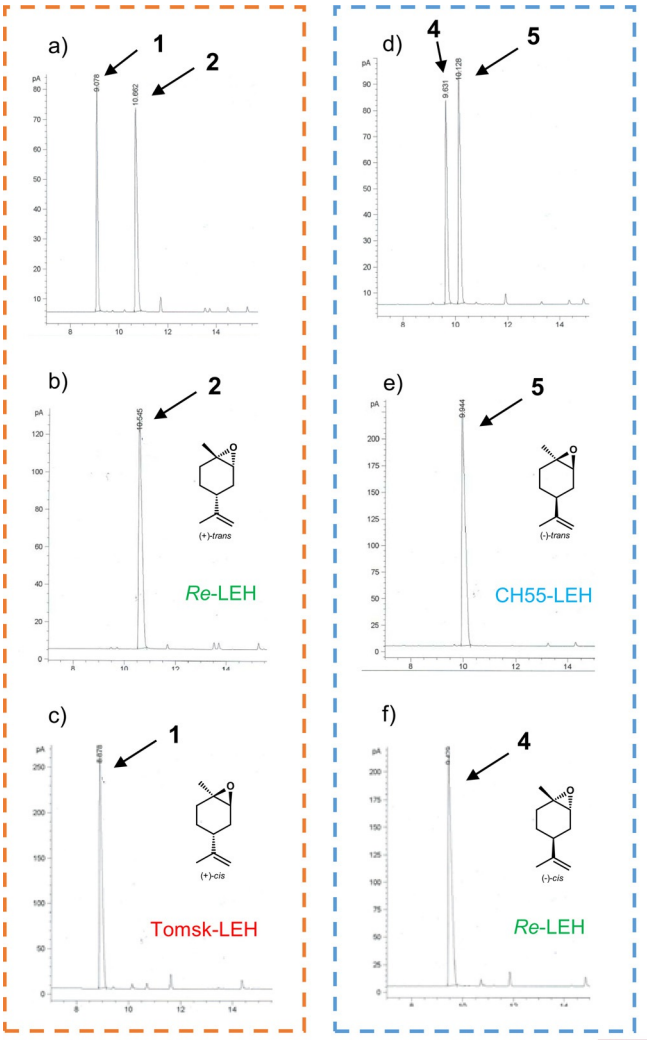


Figure 1. Chiral GC analysis of commercially available *cis*/*trans* mixtures of limonene oxide and enantiomerically pure limonene oxides obtained by enzymatic resolution: a) (+)-limonene oxide; b) **2** recovered from *Re*-LEH-catalyzed resolution (Table 1, reaction A); c) **1** recovered from Tomsk-LEH-catalyzed resolution (reaction B); d) (−)-limonene oxide; e) **5** recovered from CH55-LEH-catalyzed resolution (reaction C); f) **4** recovered from *Re*-LEH-catalyzed resolution (reaction D). Under the same GC conditions, the diols **3** and **6** eluted at $t_R = 21.44$ and 21.28 min, respectively (see the Supporting Information for more details).

the enzymes during the reactions, we took into account the additional stress conditions faced by the biocatalysts during preparative bioconversions, especially in the application of biphasic systems, for example, high substrate concentration and mechanical stress as a result of the shaking or stirring of the mixtures. Therefore, we decided to test the preparative reactions at temperatures that were 10 °C lower than the optimal temperatures estimated under analytical conditions, that is, $T =$ 20, 30, and 50 °C, for *Re*-LEH, Tomsk-LEH, and CH55-LEH, respectively.

Under these pH and temperature conditions, we investigated the effect of different initial substrate loadings, from 0.5 to 2 mol L^{-1}. A limonene oxide loading of 2 mol L^{-1} corresponds to about 330 mL L^{-1}, that is, a biphasic system with a neat substrate/enzymatic aqueous solution ratio of about 1:2. A set of small-scale (5 mL) reactions was set up with suitable amounts of the respective biocatalysts, and samples were withdrawn at scheduled times and submitted to chiral GC analysis to estimate the hydrolysis of the limonene oxide isomers.

Figure 2 shows the results of the resolution of (+)-limonene oxides catalyzed by *Re*-LEH (a) or Tomsk-LEH (c) and of (−)-limonene oxides catalyzed by CH55-LEH (b) or *Re*-LEH (d). In each graph, the time-course experiments of the hydrolysis of the *cis* isomer (continuous lines) and the *trans* isomer (dashed lines) at increasing substrate loadings (0.5, 1, and 2 mol L^{-1} of the *cis*/*trans* mixtures) are shown.

The obtained results suggest that the use of organic cosolvents to help the solubilization of the substrates is not necessary, because all of the tested LEHs were capable of performing the hydrolysis on neat substrate-containing biphasic systems. Moreover, the stereospecific behavior of the LEHs was not significantly altered at increased substrate loadings.

In a more detailed analysis of each enzymatic process, excellent performances were observed in the resolution of the (+)-limonene oxide mixture catalyzed by *Re*-LEH (Figure 2a). In fact, the reaction was performed at the 2 mol L^{-1} concentration with perfectly conserved stereospecificity, and the reaction time with the same biocatalyst loading was only doubled in the respect of that applied for the reaction performed at 0.5 mol L^{-1}. The obtainment of the *cis* isomer from the same (+)-limonene oxide mixture by Tomsk-LEH-catalyzed resolution (Figure 2c) was not significantly improved. The lower activity shown by this LEH towards the target substrate in comparison with the other two enzymes was indeed confirmed, and, although the selective hydrolysis of the *trans* isomer could be clearly observed even at concentrations higher than 0.5 mol L^{-1}, our data suggest that an additional substrate loading could require a reaction time that is too long or an increase in the enzyme/substrate ratio.

Interesting outcomes were obtained in our studies of the resolution of the (−)-limonene oxide mixture (Figure 2b and d). In the case of the reaction catalyzed by CH55-LEH and aimed at the obtainment of the enantiomerically pure *trans* isomer (Figure 2b), the increase of the reaction temperature to 50 °C led to an excellent resolution even at a substrate loading of 2 mol L^{-1} in reasonable reaction times. As previously observed with the resolution of (+)-limonene oxides catalyzed by *Re*-LEH (Figure 2a), the so-called "sequential hydrolysis" phenomenon, that is, the hydrolysis of the slowest reacting substrate only after complete consumption of the preferred one, was also conserved at very high substrate loading. The same effect was less pronounced in the process shown in Figure 2d, that is, the resolution of (−)-limonene oxides catalyzed by *Re*-LEH to obtain the enantiomerically pure *cis* isomer. This fact is consistent with the previously discussed outcome of reaction D (Table 1) and with our hypothesis that the lower yields obtained in that process could be related to the concomitant hydrolysis of both isomers. Anyway, these time-course experiments suggest that this last reaction could be conveniently performed at a 1 mol L^{-1} concentration without significant loss of stereospecificity.

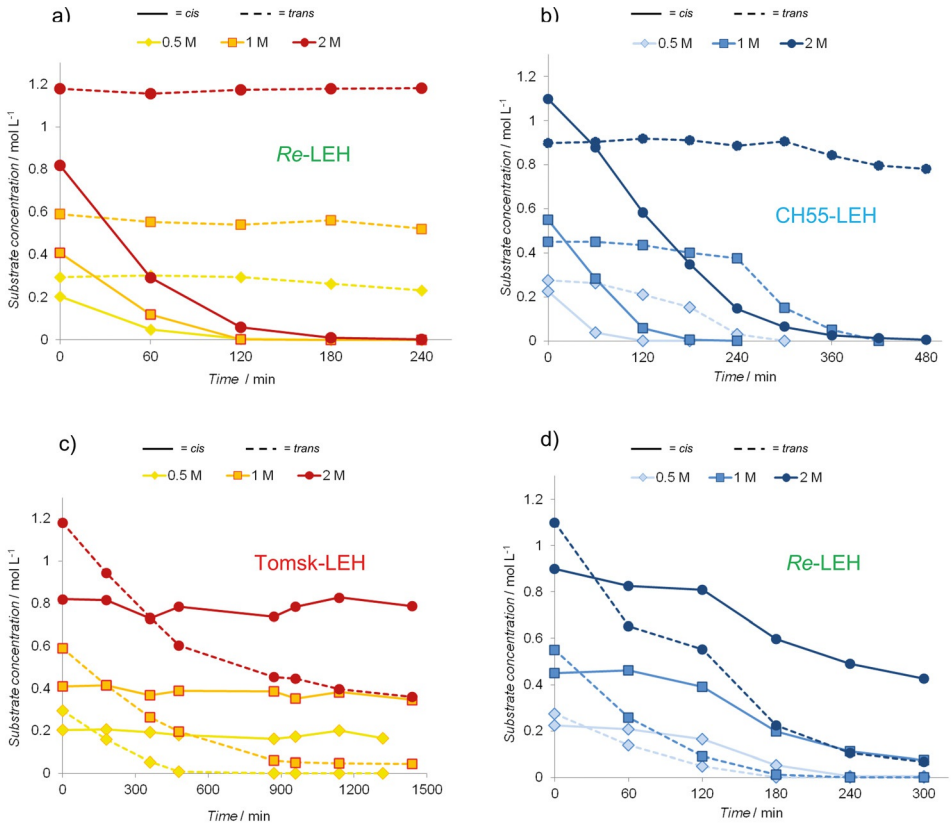


Figure 2. Effect of substrate concentration on the enzymatic resolution of limonene oxide mixtures (*cis* isomers: continuous lines; *trans* isomers: dashed lines): a) (+)-limonene oxide (**1** + **2** mixture) with *Re*-LEH at $T = 20\,^{\circ}C$; b) (−)-limonene oxide (**4** + **5** mixture) with CH55-LEH at $T = 50\,^{\circ}C$; c) (+)-limonene oxide (**1** + **2** mixture) with Tomsk-LEH at $T = 30\,^{\circ}C$; d) (−)-limonene oxide (**4** + **5** mixture) with *Re*-LEH at $T = 20\,^{\circ}C$.

Solvent-free resolutions of (+)- and (−)-limonene oxide mixtures catalyzed by LEHs under optimized conditions

A novel set of preparative-scale reactions (20 mL total volume, reactions E–H, Table 2) was set up with the information from our optimization studies.

Specifically, all of the reactions were performed by addition of neat substrates to the respective enzymatic aqueous solutions, that is, without addition of organic cosolvents, and the mixtures were shaken and monitored by chiral GC analysis at scheduled times. The resolution of (+)-limonene oxides catalyzed by *Re*-LEH (reaction E) and that of (−)-limonene oxides catalyzed by CH55-LEH (reaction G) were performed at 2 mol L^{-1} substrate loading, whereas reaction F (Tomsk-LEH and (+)-limonene oxides) was performed at 0.5 mol L^{-1} and reaction H (*Re*-LEH and (−)-limonene oxides) was performed at 1 mol L^{-1}. The *Re*-LEH-catalyzed resolutions were run at $T = 20\,^{\circ}C$, the one with Tomsk-LEH at $T = 30\,^{\circ}C$, and that with CH55-LEH at $T = 50\,^{\circ}C$.

After different reaction times (Table 2), the enantiomerically pure limonene oxide stereoisomers (**2**, **1**, **5**, and **4** in reactions E, F, G, and H, respectively) and the formed diols **3** (reactions E and F) and **6** (reactions G and H) were recovered and purified as previously described for reactions A–D. As expected, the outcome of reaction F was quite similar to that of the corresponding reaction in the first trial, that is, reaction B (Table 1). The only remarkable difference was the use of a lower amount of biocatalyst (0.7 instead of 1.5 mg mL^{-1}) that was made possible by performing the reaction at a higher temperature (30 instead of 20 °C). As far as the other three resolution processes are concerned (reactions E, G, and H), we were pleased to see that the optimization of the reaction conditions allowed a significant improvement of the process parameters.

In particular, the STY and specific productivity of reactions E and H, both catalyzed by *Re*-LEH, had approximately doubled with respect to those estimated in reactions A and D, respectively. In the case of reaction G, catalyzed by CH55-LEH, both parameters increased by about six times with respect to the values reported for the corresponding reaction C; this outcome is related both to the higher substrate loading and the higher reaction rate at $T = 50\,^{\circ}C$. For all four reactions, the enzyme consump-

Table 2. Solvent-free preparative resolution of (+)- and (−)-limonene oxide mixtures catalyzed by LEHs after process optimization.[a]

Process parameter	(+)-Limonene oxide		(−)-Limonene oxide	
	E: *Re*-LEH	F: Tomsk-LEH	G: CH55-LEH	H: *Re*-LEH
substrate volume [mL]	6.55	1.64	6.55	3.28
substrate total amount [g]	6.09	1.52	6.09	3.04
substrate loading [mol L^{-1}]	2	0.5	2	1
enzyme [mg mL^{-1}]	0.2	0.7	1.5	0.4
reaction temperature [°C]	20	30	50	20
reaction time [h]	4.5	24	6	4.5
epoxide yield [%][b]	44 (**2**)	33 (**1**)	36 (**5**)	34 (**4**)
diol yield [%][c]	40 (**3**)	59 (**3**)	64 (**6**)	66 (**6**)
STY[d,e] [mmol L^{-1} h^{-1}]	167.3	6.6	120.5	76.0
specific productivity [μmol mg^{-1} h^{-1}][e]	837	9.4	80	190
ECN[e,f] [mg $mmol^{-1}$]	0.26	4.4	2.1	1.1

[a] Reactions (20 mL) performed in KP_i buffer (pH 8.0). [b] Recovery yield estimated based on the unreacted epoxide isomer indicated in brackets. [c] Recovery yield estimated based on the formed diol indicated in brackets. [d] STY: space-time yield. [e] Calculated based on epoxide recovery. [f] ECN: enzyme consumption number.

tion numbers (ECNs) were lower than those estimated in the first set of reactions. The optimized space-time yields, the product yields per unit amount of enzyme, and the straightforward reaction make this process design attractive for scaling up, which has already been successfully demonstrated for the preparative resolution of (+)-*cis*/*trans*-limonene oxides catalyzed by *Re*-LEH.

Conclusions

The epoxide hydrolase catalyzed resolution of mixtures of (+)-*cis*/*trans*-limonene oxide and (−)-*cis*/*trans*-limonene oxide represents a straightforward, economical, and sustainable route to all limonene oxide enantiomers. The selected toolbox of recombinant epoxide hydrolases has turned out to be superior to the best direct separation methods and chemical routes for the synthesis of all limonene oxide enantiomers. In addition, the (1*S*,2*S*,4*R*)-limonene-1,2-diol and the (1*R*,2*R*,4*S*)-limonene-1,2-diol are easily accessible. The solvent-free process design and the achieved product yields make this highly resource-efficient one-step reaction not only attractive for large-scale production but also for further resolutions of *cis*/*trans*-epoxide mixtures to obtain valuable enantiopure epoxides and 1,2-diols.

Experimental Section

General

(+)-*cis/trans*-Limonene oxide (purity 97%, *ee* 98%) and (−)-*cis/trans*-limonene oxide (purity 99%, *ee* 99%) were from Sigma–Aldrich (catalog numbers 218324 and 218332, respectively).

Thin-layer chromatography (TLC) analyses were performed on precoated silica gel 60 F_{254} plates (Merck) and treated with molybdate reagent ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (42 g), $Ce(SO_4)$ (2 g), concentrated H_2SO_4 (62 mL), made up to 1 L volume with deionized water).

Product recovery was performed by extraction of the reaction mixtures with AcOEt (3 volumes) and purification by flash chromatography (silica, petroleum ether/AcOEt, 95:5) on silica gel LC60A (40–63 mesh, Grace).

Unless otherwise stated, all chemicals were of analytical grade and were purchased from Sigma–Aldrich.

Analytical methods

GC analysis: GC analyses were performed on a AGILENT 6850 (Network GC System) gas chromatograph equipped with a chiral capillary column (MEGA DEX DAC-BETA, Legnano, Italy; 0.25 mm diameter, 25 m length, and 0.25 μm thickness) and a flame ionization detector. At scheduled time points, reaction samples (100 μL) were extracted with an equal volume of a 0.025 mg mL^{-1} benzophenone solution in AcOEt in the presence of saturated NaCl and injected into the GC system. The column temperature was initially raised from 80 to 110 °C at a rate of 2 °C min^{-1} and then was raised from 110 to 200 °C at a rate of 10 °C min^{-1} at 2 mL min^{-1} flow rate. Retention times were: **1**: 9.07 min; **2**: 10.66 min; **3**: 21.44 min; **4**: 9.63 min; **5**: 10.13 min; **6**: 21.28 min; internal standard benzophenone: 22.45 min. The substrate and product peak areas were normalized to benzophenone, and concentrations were calculated with calibration curves obtained with authentic substrate/product standards (2.5–20 mmol L^{-1}). One unit of activity (U) is defined as the enzyme activity that hydrolyzes 1 μmol of substrate per min under the assay conditions described above. Stereochemical configuration was determined based on commercially available authentic standards of **1** and **2** and on *Re*-LEH reference data.[26]

NMR spectroscopy: NMR spectra were measured in D_2O at RT on a Bruker Avance III 600 MHz spectrometer equipped with a Broadband Observe probe head with *z* gradient at 600.2 MHz for ^1H NMR analysis and 150.9 MHz for ^{13}C NMR analysis.

Culture conditions, protein expression, and purification

Cultures of *E. coli* 10 G harboring plasmids pRhamReLEH, pRhamTomskLEH, and pRhamCH55 LEH,[29] respectively, were cultivated in Luria–Bertani (LB) medium containing 30 μg mL^{-1} kanamycin, at $T=37$ °C and with shaking at 220 rpm overnight. These cultures were subsequently used for the inoculation of LB medium (500 mL) supplemented with 30 μg mL^{-1} kanamycin. Cells were grown at $T=37$ °C and with shaking at 220 rpm until they reached an optical density at $\lambda=600$ nm of 0.6–0.8; they were then induced by adding rhamnose (0.2% (w/v) final volume) and kept for 24 h at $T=30$ °C with shaking at 220 rpm. The cells were harvested by centrifugation (3000 *g* for 30 min at $T=4$ °C), resuspended in wash buffer (10 mL, 20 mM KP_i (potassium phosphate) buffer, pH 7.0, 500 mM NaCl, 20 mM imidazole), and disrupted by sonication. Protein purification was performed by Ni-NTA (GE Healthcare) chromatography as previously described.[29]

Preparative resolutions under non-optimized conditions (reactions A–D)

Preparative-scale resolutions under non-optimized conditions were performed by adding a 5 M solution in CH_3CN (2 mL) of (+)-limonene oxide (59:41 mixture of **1** and **2**) or (−)-limonene oxide (55:45 mixture of **4** and **5**) (1.522 g, 10 mmol) to 25 mM KP_i buffer (pH 8.0, 18 mL) containing the purified LEHs (4 mg of *Re*-LEH for reaction A, 30 mg of Tomsk-LEH and CH55-LEH for reactions B and C, respectively, and 8 mg of *Re*-LEH for reaction D). Reactions were performed in plastic tubes (50 mL) and incubated at $T=20$ °C with shaking (180 rpm). GC monitoring and product recovery and purification were performed as described above. Resolved epoxide yields and diol yields are determined according to the total amount of the initial *cis*/*trans*-limonene oxide mixture.

Reaction A: Epoxide **2** was recovered as a transparent oil (0.678 g, 45%) and analyzed by ^1H, ^{13}C, and HSQC NMR spectroscopy (see Figure S3 in the Supporting Information). Diol **3** was recovered as a transparent oil (0.847 g, 49%) and analyzed by ^1H, ^{13}C, and HSQC NMR spectroscopy (see Figure S4 in the Supporting Information).

Reaction B: Epoxide **1** was recovered as a transparent oil (0.583 g, 38%) and analyzed by ^1H, ^{13}C, and HSQC NMR spectroscopy (see Figure S5 in the Supporting Information). Diol **3** was recovered as a transparent oil (0.911 g, 53%) and analyzed by ^1H, ^{13}C, and HSQC NMR spectroscopy (see Figure S4 in the Supporting Information).

Reaction C: Epoxide **5** was recovered as transparent oil (0.503 g, 33%) and analyzed by ^1H, ^{13}C, and HSQC NMR spectroscopy (see Figure S6 in the Supporting Information). Diol **6** was recovered as a transparent oil (1.148 g, 67%) and analyzed by ^1H, ^{13}C, and

HSQC NMR spectroscopy (see Figure S7 in the Supporting Information).

Reaction D: Epoxide **4** was recovered as a transparent oil (0.270 g, 18%) and analyzed by ^{1}H, ^{13}C, and HSQC NMR spectroscopy (see Figure S8 in the Supporting Information). Diol **6** was recovered as a transparent oil (1.310 g, 77%) and analyzed by ^{1}H, ^{13}C, and HSQC NMR spectroscopy (see Figure S7 in the Supporting Information).

Investigations on substrate stability and solubility

The stabilities of (+)-limonene oxide and (−)-limonene oxide were determined at 1 M substrate concentration (0.1522 g, 164 μL, 1 mmol) in 25 mM KP_i buffer (1 mL total volume) at pH values ranging from 6.5 to 9.0 and at temperatures ranging from 20 to 50 °C, in plastic tubes (2 mL) with vigorous shaking (1400 rpm). Substrate hydrolysis was monitored after 24 h by GC analysis as previously described.

To estimate the solubility of the limonene oxides in the aqueous phase under the previously described biphasic system conditions, a 10 mL neat substrate:buffer mixture was vigorously shaken for 5 min and then centrifuged to separate the undissolved substrate from the aqueous phase. The latter was then extracted with AcOEt (3 volumes) and the solvent was evaporated.

Process optimization

The optimizations of the reaction conditions for the A–D resolution processes were performed at different substrate concentrations (0.5 M (381 mg, 410 μL, 2.5 mmol), 1 M (761 mg, 819 μL, 5 mmol), and 2 M (1.522 g, 1.639 μL, 10 mmol)) in 25 mM KP_i buffer (pH 8.0, 5 mL total volume) in the presence of the purified LEHs (1 mg of *Re*-LEH for reaction A, 7.5 mg of Tomsk-LEH and CH55-LEH for reactions B and C, respectively, and 1 mg of *Re*-LEH for reaction D at the 0.5 M substrate concentration or 2 mg of *Re*-LEH for reaction D at the 1 M and 2 M substrate concentrations). Reactions were performed in plastic tubes (15 mL) and incubated at different temperatures (reactions A and D: $T=20$ °C; reaction B: $T=30$ °C; reaction C: $T=50$ °C) with shaking (180 rpm). At scheduled times, the reactions were monitored by GC analysis as described above.

Preparative resolutions under optimized conditions (reactions E–H)

Reaction E: The preparative resolution of (+)-limonene oxide catalyzed by *Re*-LEH was performed by adding (+)-limonene oxide (6.55 mL, 6.09 g, 40 mmol) to 25 mM KP_i buffer (pH 8.0, 13.45 mL) containing the purified *Re*-LEH (4 mg). The reaction was performed in a plastic tube (50 mL), incubated at $T=20$ °C with shaking (180 rpm), and monitored by GC analysis at scheduled times. After 4.5 h, product recovery was performed as described above, to afford epoxide **2** (2.69 g, 75%) and diol **3** (3.19 g, 40%), both as transparent oils.

Reaction F: The preparative resolution of (+)-limonene oxide catalyzed by Tomsk-LEH was performed by adding (+)-limonene oxide (1.64 mL, 1.522 g, 10 mmol) to 25 mM KP_i buffer (pH 8.0, 18.36 mL) containing purified Tomsk-LEH (14 mg). The reaction was performed in a plastic tube (50 mL), incubated at $T=30$ °C with shaking (180 rpm), and monitored by GC analysis at scheduled times. After 24 h, product recovery was performed as described above, to afford epoxide **1** (0.506 g, 81%) and diol **3** (1.05 g, 59%), both as transparent oils.

Reaction G: The preparative resolution of (−)-limonene oxide catalyzed by CH55-LEH was performed by adding (−)-limonene oxide (6.55 mL, 6.09 g, 40 mmol) to 25 mM KP_i buffer (pH 8.0, 13.45 mL) containing purified CH55-LEH (30 mg). The reaction was performed in a plastic tube (50 mL), incubated at $T=50$ °C with shaking (180 rpm), and monitored by GC analysis at scheduled times. After 6 h, product recovery was performed as described above, to afford epoxide **5** (2.24 g, 67%) and diol **6** (4.45 g, 64%), both as transparent oils.

Reaction H: The preparative resolution of (−)-limonene oxide catalyzed by *Re*-LEH was performed by adding (−)-limonene oxide (3.28 mL, 3.04 g, 20 mmol) to 25 mM KP_i buffer (pH 8.0, 16.72 mL) containing purified *Re*-LEH (8 mg). The reaction was performed in a plastic tube (50 mL), incubated at $T=20$ °C with shaking (180 rpm), and monitored by GC analysis at scheduled times. After 4.5 h, product recovery was performed as described above, to afford epoxide **4** (1.04 g, 76%) and diol **6** (2.24 g, 65.7%), both as transparent oils.

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