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Access to enantiopure aromatic epoxides and diols using epoxide hydrolases derived from total biofilter DNA

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ABSTRACT

Metagenomic DNA is a rich source of genes encoding novel epoxide hydrolases (EHs). We retrieved two genes encoding functional EHs from total DNA isolated from biofilter-derived biomass, using PCR with EH-specific degenerate primers followed by genome-walking PCR. The degenerate primers were based on two EH-specific consensus sequences: HGWP and GHDWG. The resulting recombinant EHs, Kau2 and Kau8, were expressed in *Escherichia coli*, and their enantioselectivity and regioselectivity were determined using 13 different epoxide substrates. The EH Kau2 had broad substrate specificity and preferentially hydrolyzed epoxides with *S*-configuration. It showed high enantioselectivity towards aromatic epoxides such as styrene oxide, *p*-nitrostyrene oxide, and *trans*-1-phenyl-1,2-epoxypropane. In addition, Kau2 showed enantioconvergent hydrolysis activity. The EH Kau8 also showed broad substrate specificity and preferentially hydrolyzed epoxides with *R*-configuration. High enantioselectivity was observed for *p*-nitrostyrene oxide, and the hydrolysis activity of Kau8 was less enantioconvergent than that of Kau2. To determine the usefulness of Kau2 for synthetic applications, preparative-scale biohydrolysis reactions were performed. Specifically, two kinetic resolutions were carried out with 80 g/L of racemic *trans*-1-phenyl-1,2-epoxypropane, affording both (1*R*,2*R*)-epoxide and the corresponding (1*R*,2*S*)-diol in high enantiomeric excess (>99%) and good yield (>45%). In addition, a process based on enantioconvergent hydrolysis by the EH Kau2 was established for racemic *cis*-1-phenyl-1,2-epoxypropane at a concentration of 13 g/L, resulting in the formation of the corresponding (1*R*,2*R*)-diol with a 97% yield and an enantiomeric excess exceeding 98%.

1. Introduction

Enantiopure epoxides and vicinal diols are valuable chiral building blocks for the synthesis of optically active pharmaceuticals [1–3]. These chiral building blocks can be prepared from available racemic epoxides using enantioselective or enantioconvergent epoxide hydrolases (EHs). Examples of enantiopure epoxides that are valuable for the synthesis of pharmacologically active compounds include *p*-nitrostyrene oxide (**6**), *p*-chlorostyrene oxide (**7**), *m*-chlorostyrene oxide (**8**), and *trans*- and *cis*-1-phenylpropene oxide (**10**, **11**) (Fig. 1) [4–9]. Enantioselective EHs preferentially react with one epoxide enantiomer, leaving the other enantiomer unreacted. In a kinetic resolution experiment with an enantioselective EH, the maximum yield of enantiopure epoxide is 50%. In contrast, enantioconvergent EHs lead to the formation of only one diol enantiomer during the entire biotransformation reaction, and thus have a theoretical yield of 100% [10]. In many cases, enantiopure diols can then be transformed chemically to the corresponding epoxides without affecting enantiomeric excess [11,12].

A literature search showed that traditional screening approaches have identified about thirty highly enantioselective EHs (with *E*-values > 50) from microbes and plants. EH–substrate pairs with high enantioconvergence are rare [13–18], and it appears that no single EH is likely to show high enantioselectivity with many types of epoxides. As a consequence, libraries of EHs have to be created.

In this report, we used PCR techniques and EH-specific consensus primers to isolate two novel EH-encoding genes, termed *eph-k2* and *eph-k8*, directly from total biofilter DNA. These EHs were subsequently expressed in the surrogate host organism *Escherichia coli* using standard molecular biology tools. The biocatalytic potential of these enzymes was investigated using thirteen epoxide substrates, and one of the EHs was also used for preparative-scale

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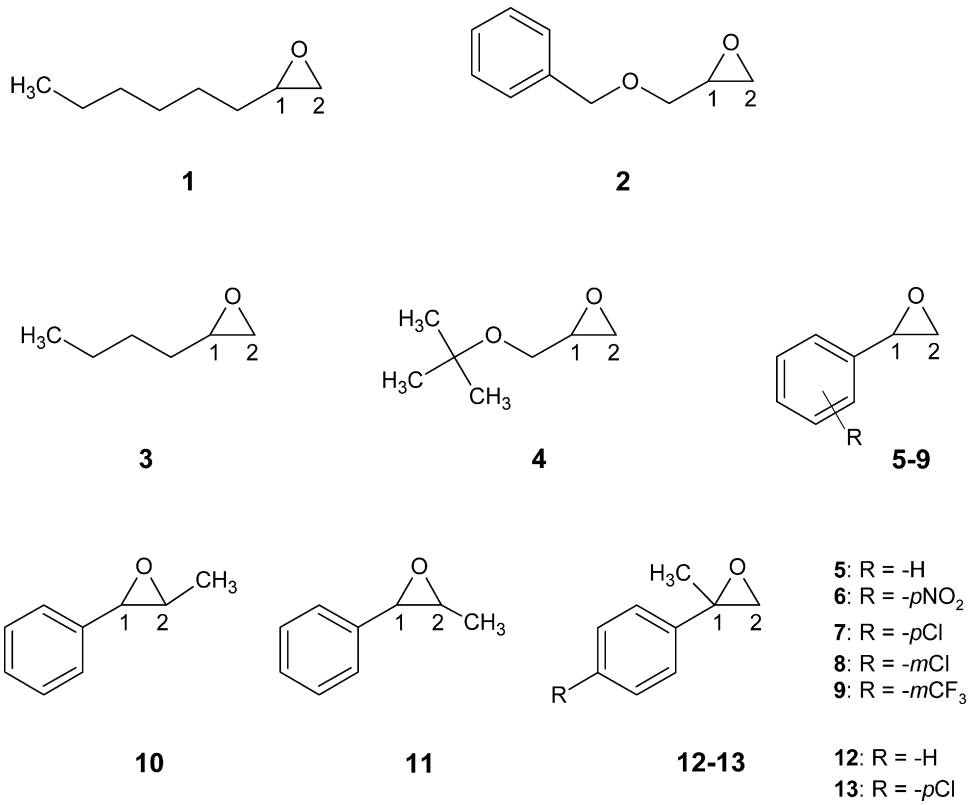

Fig. 1. Epoxides used as substrates in this study. **1**, 1,2-epoxyoctane; **2**, benzyl glycidyl ether; **3**, 1,2-epoxyhexane; **4**, *tert*-butyl glycidyl ether; **5**, styrene oxide; **6**, *para*-nitrostyrene oxide; **7**, *para*-chlorostyrene oxide; **8**, *meta*-chlorostyrene oxide; **9**, *meta*-trifluoromethylstyrene oxide; **10**, *trans*-1-phenylpropene oxide; **11**, *cis*-1-phenylpropene oxide; **12**, 1-phenyl-1-methyloxirane; **13**, 1-(*para*-chlorophenyl)-1-methyloxirane.

biotransformation experiments to assess its usefulness in synthetic processes.

2. Experimental

2.1. Chemicals

The racemic epoxides **1**–**5** and the enantiopure compounds (*R*)-**1**, (*S*)-**1**, (*R*)-**2**, (*S*)-**2**, (*R*)-**5**, (*S*)-**5**, (1*S*,2*S*)-**10**, and (1*R*,2*R*)-**10** were purchased from Aldrich or Fluka and were used as received. All other epoxides were synthesized as previously described [19–23]. ^{1}H NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 300. Chemical shifts are reported in p.p.m.

2.2. Retrieving EH-encoding genes from metagenomic DNA

An alignment of 31 prokaryotic gene sequences encoding known and putative EHs revealed 3 highly conserved sequences which had been described previously [24]. Two of the conserved sequences, H-G-W/F-P and G-G/H-D-W-G, were used to design EH-specific degenerate primers using a design strategy described previously [25,26]. The blending method of Gabor et al. [27] was used to extract the bacterial biomass and its total DNA from a biofilter installed for purification of styrene-containing off-gas. The biofilter was composed primarily of activated charcoal as an adsorbent for the microorganisms. The isolated DNA was purified using a High Pure PCR Product Purification Kit (Roche) followed by size-exclusion chromatography with a Chroma Spin-1000 column (Clontech Laboratories, Inc.) and phenol–chloroform extraction. The purified total DNA was used as template for amplification of EH gene fragments using degenerate EH-consensus primers Def170 and Der250 (Table 1). Amplified gene fragments of approx-

Table 1
PCR primer sequences.

Primer[a]	Sequence	Core region[b]				
		H	G	W	P	
Def170	5′-CCTGTTCTACTNHYN	CAY	GGN	TGG	CCN	GA-3′

Primer[a]	Sequence	Core region[b]					
		G	H	D	W	G	
	5′-	GGN	CAY	GAY	TGG	GGN	GCGGCTGTTGCTGC-3′
Der250	3′-	CCN	GTR	CTR	ACC	CCN	CGCCGACAACGACG-5′

K2Af	5′-TGGCGGCACCAGATCAGGG-3′
K2Bf	5′-GCCTATGACATCGAGAACCTGACGG-3′
K2Ar	5′-CCGTCAGGTTCTCGATGTCATAGGC-3′
K2Br	5′-TGGTGCCGCCATGAAAACG-3′
K2Cf	5′-AAGGTTTTCGTCGATATGTTCAGGG-3′
K2Df	5′-ATCAACTGGTATCGCAACATGACC-3′
EH2f	5′-GCGCCTGCAATGATGGACAT-3′
EH2r	5′-TTTGGTCTAGAGCTACTGTTCCGACGAATGTTT-3′
K8Af	5′-ATGCGGGGCTACGGTCGC-3′
K8Bf	5′-TACAATCATTGGGAAGGGACAAAGC-3′
K8Ar	5′-TGTCCCTTCCCAATGATTGTAAGAGC-3′
K8Br	5′-AGCCCAACGCTGCGAATGC-3′
EH8f	5′-CGAAACAACTACGAATCACCTATGTT-3′
EH8r	5′-TATTTTCTAGATGGTTTGACATGTGAATGATTCTC-3′

[a] Primers designated De are degenerate EH-consensus primers used to amplify internal EH gene fragments. Primer names with the letter "f" are forward primers, those containing the letter "r" are reverse primers. Primers EH2f, EH2r and EH8f, EH8r were used to amplify the full-length genes from metagenomic DNA. Primers EH2r, and EH8r introduced an XbaI restriction site (underlined) downstream of the gene.

[b] The amino acid consensus sequences encoded at the core positions of the EH-consensus primers are shown.

imately 240 bp were isolated from an agarose gel and ligated into pGEM-T Easy (Promega) for sequencing. Then, the total DNA was partially digested with restriction enzyme Sau3AI and linker DNA was ligated to the cohesive ends [26]. The modified total DNA was used for genome-walking PCR [26,28] using the following pairs of nested (Table 1) and linker-specific primers. For *eph-k2*: K2Af-C1, K2Bf-C2 (1st genome-walking step); K2Ar-C1, K2Br-C2 (2nd genome-walking step); and K2Cf-C1, K2Df-C2 (3rd genome-walking step). For *eph-k8*: K8Af-C1, K8Bf-C2 (1st genome-walking step); and K8Ar-C1, K8Br-C2 (2nd genome-walking step). The linker-specific primers C1 and C2 were obtained from TaKaRa. After 2–3 steps of genome walking, the sequences of the full-length EH genes could be predicted. Primers specific for the flanking regions of the putative genes (Table 1) allowed amplification of the full-length genes using the original purified metagenomic DNA as template. The resulting PCR products were restricted with XbaI and ligated into the expression vector pSE420 (Invitrogen). After transformation of *E. coli* TOP10 cells (Invitrogen), plasmid DNA was isolated from selected colonies, and the EH-encoding inserts were sequenced using an automated DNA sequencer ABI PRISM 3130xl (Applied Biosystems). The complete nucleotide sequences of the novel EH-encoding genes *eph-k2* and *eph-k8* are available from GenBank (accession numbers FJ848342 and FJ848343, respectively).

2.3. Enzyme preparation

Recombinant *E. coli* RE3 cells (CCM 4228) [29,30] harbouring the plasmids pSEKau2 or pSEKau8 were used for expression of the EHs Kau2 or Kau8, respectively. Cells were cultivated at 28 °C in 10-L bioreactors (Biostat MD; Sartorius BBI Systems) using a minimal medium of the following composition (in $g\,L^{-1}$): sucrose (10.0), KH_2PO_4 (13.6), $(NH_4)_2SO_4$ (4.0), NaOH (3.0), $MgSO_4{\cdot}7H_2O$ (2.0), $CaCl_2{\cdot}2H_2O$ (0.05), and $FeSO_4{\cdot}7H_2O$ (0.01). The pH was not controlled. The dissolved oxygen tension was kept constant at 20% of air saturation by controlling the stirrer speed using a pO_2-controller. After induction with IPTG (final concentration of 0.45 mM) at a biomass concentration of ~1 $g\,L^{-1}$ (cdw), the cultivation temperature was reduced to 25 °C. Luria–Bertani (LB) broth supplemented with 100 $\mu g\,mL^{-1}$ of ampicillin was used as growth medium for the seed culture. The cells were harvested 13 h after IPTG addition, washed with 100 mM phosphate buffer (pH 7.5) containing 2 mM 2-mercaptoethanol and 1 mM EDTA, resuspended in the same buffer, and disrupted by high pressure (27 kpsi) using a Cell Disruptor from Constant Systems Ltd. (Daventry, UK). Cell debris was removed by centrifugation (11 000 × *g* for 60 min), and the EHs were enriched by fractionated ammonium sulphate precipitation using saturation levels of 35% and 75%.

2.4. Protein methods

SDS polyacrylamide gels (10%) were stained with Coomassie brilliant blue R250. Protein gels were scanned, and the level of expression/enrichment was analyzed using a Bio Image Intelligent Quantifier program (B.I. Systems Corp.). Protein concentrations were determined by bicinchoninic acid (BCA protein assay kit, Pierce Chemical Company), using BSA as a protein standard.

2.5. Determination of enzyme activities and kinetic parameters

EH activities were determined by achiral GC (see the Supplementary material). Using epoxide **5** (2.75 mM) as substrate, pH optima of the enriched enzymes were determined at 28 °C by measuring enzyme activity in the presence of 8% ethanol (v/v) after incubation for 30 min in either Tris–HCl (0.15 M) or wide range buffer [31]. Temperature optima were assessed in 40 mM phosphate buffer (pH 7.5) containing 2 mM 2-mercaptoethanol (buffer A), 8% ethanol, and 2.75 mM epoxide **5** for 45 min. Thermal stabilities were tested in buffer A at different temperatures at a constant incubation time of 30 min. Residual enzyme activities were then determined at 28 °C. Kinetic parameters were calculated using a nonlinear regression program (Enzfitter, Elsevier Biosoft). Experimental data were fitted to the equations of Michaelis–Menten or Hill (allosteric kinetics) [32].

2.6. Determination of enantioselectivity and regioselectivity

Small-scale conversions were carried out with ammonium sulphate-precipitated enzyme at 28 °C in a total volume of 7 mL using 3.5 mM of substrate, 5% ethanol, and buffer A. GC conditions for analysis of epoxides, diols or acetonides are listed in the Supplementary material. Absolute configurations were determined based upon elution order using established GC conditions [19,21–23,26]. During enzymatic hydrolysis, 12–14 aliquots were withdrawn from the reaction mixture at different time points to follow the conversion from epoxide to diol. Procedures for extraction and derivatization are listed in the Supplementary material. Enantioselectivities (*E*-values) were determined as described elsewhere [33]. Eq. (1) allowed calculation of the regioselectivity coefficients α_R and α_S from the enantiomeric excess of the residual epoxide, ee_s, the enantiomeric excess of the formed diol, ee_p, and the extent of conversion *c* by linear regression [16]:

$$ee_p = \alpha_S - \alpha_R + (1 - \alpha_S - \alpha_R) \cdot ee_s \cdot (1 - c) \cdot c^{-1} \quad (1)$$

Plotting ee_p against $ee_s{\cdot}(1-c){\cdot}c^{-1}$ gives an intercept *q* of $\alpha_S - \alpha_R$. Using the values for the intercept *q* and the slope *m* from the linear regression, α_R can be calculated according to Eq. (2):

$$\alpha_R = \frac{1 - m - q}{2} \quad (2)$$

2.7. Preparative-scale biohydrolysis of rac-**10** using the EH Kau2

The harvested cells (see above) were resuspended in 20 mM ammonium carbonate buffer (pH 7.6) prior to freezing. The reactions were performed at 26 °C in a propelled reactor in buffer A with 5% (v/v) glycerol using either freeze-dried (0.2 g) or thawed whole cells (5 mL, 0.15 g dry weight). *rac*-**10** (2.4 g, corresponding to 17.9 mmol) was added to the mixture to give a total reaction volume of 30 mL. The biphasic reaction medium was vigorously stirred (350 rpm) to form an emulsion. During enzymatic hydrolysis, samples were withdrawn to follow the enzymatic resolution by chiral GC analysis. The reactions were stopped by centrifugation to pellet the cells. Cold hexane was used for extraction (3× 30 mL). The combined hexane fractions were dried over anhydrous sodium sulphate and filtered. The filtrate was concentrated under reduced pressure. Bulb-to-bulb distillation (77 °C, 4 mbar) afforded the epoxide (1*R*,2*R*)-**10**. The formed diol was extracted with ethyl acetate (3× 30 mL) after saturation with NaCl. After drying over sodium sulphate, bulb-to-bulb distillation (150 °C, 0.1 mbar) afforded the diol (1*R*,2*S*)-**10a** as a white solid (melting point: 43–44 °C). ^{1}H NMR: δ = 1.08 (d, 3H, CH_3, J = 6.4 Hz); 2.25 (s large, 1H, OH); 2.80 (s large, 1H, OH); 4.05 (m, 1H, H-2); 4.67 (d, 1H, H-1, J = 4.2 Hz); 7.25–7.40 (m, 5H, C_6H_5).

2.8. Preparative-scale biohydrolysis of rac-**11** using the EH Kau2

The reaction was performed in a propelled reactor with whole cells (0.3 g dry weight) using the same reaction conditions as described in Section 2.7. *rac*-**11** (1.0 g, corresponding to 7.46 mmol) was added to give a total reaction volume of 77 mL. The biphasic reaction medium was stirred vigorously to form an emulsion, and the reaction was stopped after 7 h by centrifugation. The

supernatant was saturated with NaCl, and the diol was extracted with ethyl acetate (3× 40 mL). After drying over magnesium sulphate, bulb-to-bulb distillation (135 °C, 0.04 mbar) afforded the diol (1*R*,2*R*)-**11a** as a white solid (melting point: 58–59 °C). ^{1}H NMR: δ = 1.07 (d, 3H, CH_3, J = 6.3 Hz); 2.70 (s large, 1H, OH); 2.83 (s large, 1H, OH); 3.87 (m, 1H, H-2); 4.37 (d, 1H, H-1, J = 7.3 Hz); 7.25–7.45 (m, 5H, C_6H_5).

3. Results and discussion

Many types of cultivable microorganisms, including bacteria, fungi, and yeast, exhibit EH activities. However, it is thought that many more EH-encoding genes exist in unknown or uncultivable microorganisms. Their genomes are collectively referred to as the metagenome, and this metagenome is accessible by extracting total DNA from a particular sample. EH-encoding genes from the metagenome can then be obtained by high-throughput activity screening of expression libraries [34]. Hybridization of EH-specific probes with metagenomic DNA can also be used to identify putative EH genes [34]. We recently developed a PCR-based approach to identify EH genes in metagenomic DNA. Specifically, we used degenerate consensus primers specific for EHs followed by genome-walking PCR to directly amplify putative EH-encoding genes from isolated total DNA [26]. The use of PCR primers based on conserved regions plus metagenomic DNA as a template has been used to identify genes encoding novel members of a specific enzyme class from unknown or uncultivable microorganisms [28]; this was reported in seminal papers describing the recovery of lipase and 2,5-diketo-D-gluconic acid reductase genes from environmental DNA [35,36].

3.1. Cloning of two novel EH genes from metagenomic DNA

After amino acid sequence alignment, three highly conserved regions were identified in EH sequences: (1) the consensus sequence G-Y-G-X-S/T; (2) the active-site consensus sequence G-G/H-D-W-G; and (3) the H-G-W/F-P motif [24]. Regions (2) and (3) were used to design the degenerate EH-specific primers Der250 and Def170 (Table 1). With these degenerate consensus primers, fragments of putative EH genes were amplified directly from metagenomic DNA. The amplification products were cloned and four clones were randomly selected for sequence analysis. A BLASTP search at NCBI found three unique translated sequences with homology to known or putative EHs. All three sequences appeared to be fragments of EHs, because all contained the EH-specific consensus region G-Y-G-X-S/T positioned between the primer annealing sites (2) and (3). Two clones (Kau2 and Kau8) were selected for further cloning experiments. The insert sequences of these clones were used to design nested genome-walking primers. The entire putative EH sequences corresponding to the insert sequences of clones Kau2 and Kau8 were obtained by genome-walking PCR with linker-coupled metagenomic DNA as template using nested primers (Table 1) in combination with linker-specific primers. The regions adjacent to the original insert sequence of clone Kau8 were assessed by one upstream and one downstream genome-walking step, allowing assembly (via overlapping sequences) of the entire putative EH gene. Three genome-walking steps were needed to obtain the entire sequence of the putative EH gene represented by clone Kau2. Each potential full-length gene was recovered from the original metagenomic DNA by a final PCR using primer pairs specific for the 3′ and 5′ ends of the two genes. The sequences of the final PCR products, represented by clones Kau2 and Kau8, differed from the expected sequences by 9 and 0 nucleotides, respectively. The EH gene derived from clone Kau2 (*eph-k2*) encoded a 339-amino-acid protein of 38.2 kDa, which is hereafter referred to as Kau2. The second EH gene (*eph-k8*) encoded a 357-amino-acid protein of 39.5 kDa, which is hereafter referred to as Kau8. A BLASTP search revealed that Kau2 most resembled putative bacterial EHs from *Bradyrhizobium* sp. ORS278 and *Bradyrhizobium* sp. BTAi1, with 73% sequence identity with both. BLASTP searching also showed that the protein encoded by *eph-k8* shared 51% identity with a bacterial EH from *Stigmatella aurantiaca* DW4/3-1, and 43% identity with a hypothetical EH from the fungal strain *Magnaporthe grisea* 70-15.

The novel EHs contained the amino acid sequences HGWP, GHDWG, and GYGAT (for Kau2) or GYGRS (for Kau8), which are all EH-specific consensus sequences. At the amino acid level, there was only 22% identity between the Kau2 and Kau8 protein sequences. Both EH-encoding genes contained no introns and are thus probably of bacterial origin. However, they are related to EHs of higher organisms. Comparing the predicted amino acid sequences of the Kau2- and Kau8-derived EHs with the five sequences of EHs with known structures [37–41] revealed that the highest similarity was found with mouse liver EH (amino acids 239–540) and human EH (amino acids 258–541). The novel EHs therefore belong to the bacterial family of EHs that is related to EHs of higher organisms. This family was previously identified by phylogenetic analysis of 95 EHs along with homology modelling [42].

As noted, our method for amplifying EH-specific gene fragments used primers based on the two consensus sequences H-G-W/F-P and G-G/H-D-W-G. The first sequence is located at a tight bend of the polypeptide backbone, involves a peptide bond in *cis* conformation between the aromatic residue and proline, and is highly EH-specific [24]. The second consensus sequence contains the active-site nucleophile, aspartate. We previously used a third EH-specific consensus sequence, GYGFS, together with GHDWG to amplify EH-specific gene fragments [26]. However, this approach amplified short sequences, ~140 bp in length, making subsequent primer design more difficult than with the primer pairs used in the present report.

Using EH-specific primers in conjunction with PCR and genome-walking techniques, we were able to retrieve two EH-encoding genes from total DNA isolated from biofilter-derived biomass. In order to characterize Kau2 and Kau8, the genes were expressed in *E. coli* using an expression vector that had the strong IPTG-controlled *trc* promoter. After ammonium sulphate precipitation of expressed Kau2 and Kau8, selected biochemical characteristics and hydrolytic activities towards 11 terminal and 2 non-terminal epoxides were determined, as were their enantioselectivities and regioselectivities.

3.2. Characterization of the novel EHs Kau2 and Kau8

Both recombinant enzymes were overexpressed in *E. coli* RE3 at levels of ~20% of the total soluble protein (Fig. 2), as judged by SDS-PAGE analysis. The same analytical procedure revealed that ammonium sulphate precipitation of Kau2 and Kau8 resulted in enzyme samples with ~35% of the protein being heterologous. Both enzymes showed highest activities at pH 7.5 (Table 2). Further, both enzymes turned out to be only modestly thermostable; no loss of enzyme activity was observed after 30 min at incubation temperatures of ≤35 °C for both enzymes. The presence of substrate improved the thermal stability of the enzymes; optimum temperatures of 36 and 40 °C were determined for Kau8 and Kau2, respectively. Ethanol, which is often used as a co-solvent, affected enzyme activities at concentrations exceeding 15% and 11% (v/v) for Kau2 and Kau8, respectively.

Rate dependence on substrate concentrations followed Michaelis–Menten kinetics for epoxides **1** and **2** in the presence of Kau8 (Table 3). A significant deviation from Michaelis–Menten kinetics was observed for Kau2 when changing the substrate from

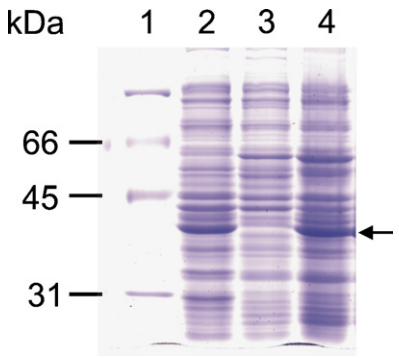

Fig. 2. SDS-PAGE analysis of Kau2 and Kau8 overexpressed in *E. coli* RE3. Lane 1: molecular weight marker; lanes 2–4: clarified crude extracts; lane 2: *E. coli* RE3(pSEKau8); lane 3: *E. coli* RE3; lane 4: *E. coli* RE3(pSEKau2).

epoxide **1** to epoxide **2** (Fig. 3 and Table 3). There are two possible explanations for such a sigmoid substrate saturation curve: (1) true cooperativity in substrate binding of a multi-subunit enzyme, or (2) a slow equilibrium between two enzyme forms with substantially different enzyme activities [43,44]. This so-called hysteretic behavior has been observed or postulated with an increasing number of enzymes, including the *Solanum tuberosum* EH [45]. Further detailed research will be necessary to fully explain the observed kinetic cooperativity of Kau2 with epoxide **2** as substrate.

Table 2
Selected biochemical properties of Kau2 and Kau8.

Parameter	Kau2	Kau8
pH optimum	7.5	7.5
pH of half-maximum activity	4.8; 9.5	4.5; 9.7
Temperature optimum (°C)	40	36
Temperature of half-maximum activity (°C)	18; 44	19; 42
Thermostability		
Temperature with no loss of activity (°C)	≤35	≤35
Temperature with 50% of activity loss (°C)	37	37
Resistance to ethanol		
Optimal ethanol conc. (%, v/v)	14	10
Ethanol conc. of half-maximum activity (%, v/v)	18	19

Table 3
Steady-state kinetic parameters for Kau2 and Kau8 reacting with each enantiomer of epoxides **1** and **2**[a].

Substrate and parameter	Kau2	Kau8
Epoxide **1**		
Abs. configuration (residual epoxide)	*S*	*S*
Abs. configuration (main diol)	*R*	*R*
v_{max} (*R*-epoxide, μmol min^{-1} mg^{-1})	13.4	21.8
K_m (*R*-epoxide, mM)	6.9	3.8
v_{max} (*S*-epoxide, μmol min^{-1} mg^{-1})	7.9	2.5
K_m (*S*-epoxide, mM)	4.4	3.5
α_R (%)	3.5	0
α_S (%)	6.9	6.3
E-value[b]	1.1 (1.3)	8.0 (8.5)
Epoxide **2**		
Abs. configuration (residual epoxide)	*S*	*R*
Abs. configuration (main diol)	*S*	*S*
v_{max} (*R*-epoxide, μmol min^{-1} mg^{-1})	0.44	4.5
K_m (*R*-epoxide, mM)	n.a.	12.0
n (*R*-epoxide, Hill coefficient)	2.29	1.0
v_{max} (*S*-epoxide, μmol min^{-1} mg^{-1})	0.33	3.8
K_m (*S*-epoxide, mM)	n.a.	10.0
n (*S*-epoxide, Hill coefficient)	2.22	1.0
α_R (%)	2.8	4.7
α_S (%)	0.5	1.0
E-value[b]	n.a. (1.1)	1.8 (1.9)

n.a.: not applicable.
[a] Enriched enzymes were used.
[b] Calculated from the v_{max}/K_m ratios of the two enantiomers. *E*-values calculated from kinetic resolution experiments are given in parenthesis.

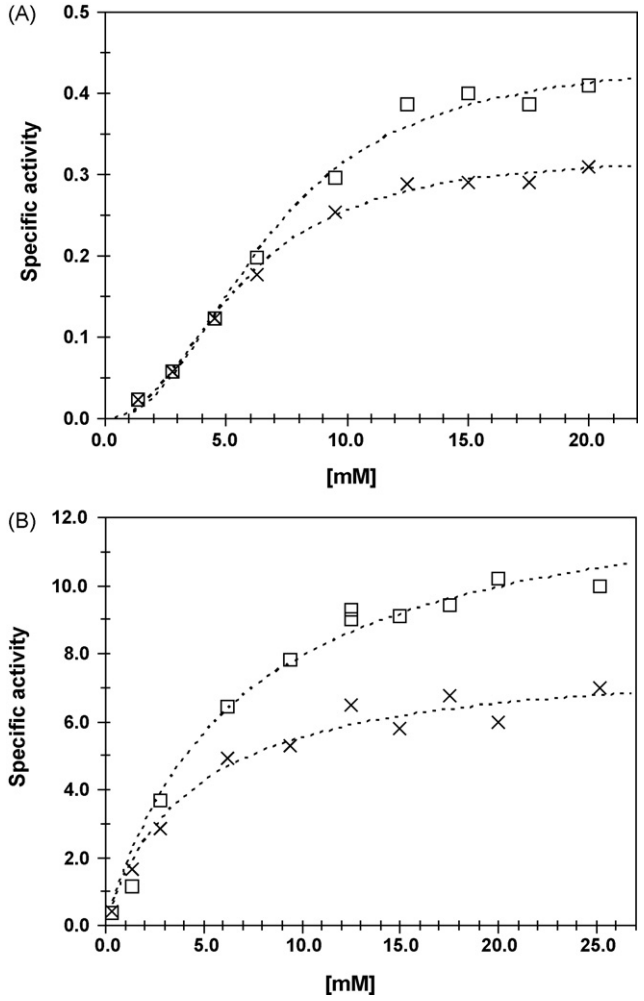

Fig. 3. Substrate saturation curves using EH Kau2 and enantiopure epoxide **2** (A) and enantiopure epoxide **1** (B) as substrates. The reactions were performed at 28 °C with enriched enzyme. The specific activities are given in μmol (mg of protein)$^{-1}$ min^{-1}. Squares represent data obtained with (*R*)-enantiomers, crosses represent data obtained with (*S*)-enantiomers. Data were fitted using the Hill equation (A) or the Michaelis–Menten equation (B).

The EHs Kau2 and Kau8 had broad substrate spectra, reacting with both aliphatic and aromatic epoxides. With three exceptions, epoxides **2**, **3** and **13** (Fig. 1), Kau2 showed an opposite enantiopreference compared to Kau8 (Tables 3 and 4). Enantioselectivities were determined to range broadly from very low to high for some aromatic compounds such as epoxides **5**, **6**, and **10** (Table 4). The EH Kau2 exhibited higher enantioselectivity than Kau8 for almost all aromatic epoxides (**5**–**8** and **10**–**13**; Fig. 1); the reverse was true for the aliphatic epoxides **1** and **3**, and the glycidyl ether **4**. Substitutions at the *meta* or *para* positions of the aromatic ring can have a pronounced effect on activity and enantioselectivity, with the *para*-nitro group being the most favourable one for high enantioselectivity. Interestingly, the *trans*-epoxide **10** gave the highest specific activity (22.1 μmol mg^{-1} min^{-1}) and enantioselectivity (*E* exceeding 200) of all tested Kau2–substrate combinations. In addition, its diastereomeric counterpart, epoxide **11**, was hydrolyzed by Kau2 in a fully enantioconvergent reaction, affording the diol (1*R*,2*R*)-**11a** with an excellent enantiomeric excess (*ee*) value of more than 99% at 100% conversion. In general, the EH Kau2 had

Table 4
Parameters of the enzymatic hydrolysis of *rac*-**3** to *rac*-**13** using the EHs Kau2 and Kau8[a].

Substrate	Epoxide hydrolase	Specific activity[b]	Absolute configuration		*E*-value	α_R (%)[c]	α_S (%)[c]	ee_{diol} (%)[d]
			Residual epoxide	Diol				
rac-**3**	Kau2	0.95	*S*	*R*	1.3 ± 0.1	0	51 ± 4	50 (51)
	Kau8	1.3	*S*	*R*	6 ± 1	0	70 ± 5	70 (70)
rac-**4**	Kau2	0.24	*S*	*S*	1.03 ± 0.02	54 ± 5	39 ± 4	14 (14)
	Kau8	0.10	*R*	*S*	10 ± 1	1 ± 1	1 ± 1	1 (0)
rac-**5**	Kau2	1.8	*R*	*R*	65 ± 5	20 ± 9 (10)	98 ± 2 (98)	77 (78)
	Kau8	0.60	*S*	*R*	12 ± 2	11 ± 4 (17)	83 ± 6 (86)	71 (71)
rac-**6**	Kau2	9.1	*R*	*R*	80 ± 7	0	88 ± 5	87 (88)
	Kau8	0.96	*S*	*R*	65 ± 6	4 ± 3	24 ± 4	22 (20)
rac-**7**	Kau2	12.5	*R*	*R*	35 ± 4	10 ± 3	95 ± 3	85 (85)
	Kau8	1.1	*S*	*R*	14 ± 2	20 ± 5	34 ± 7	13 (15)
rac-**8**	Kau2	1.9	*R*	*R*	17 ± 3	0	92 ± 2	91 (92)
	Kau8	0.53	*S*	*R*	9 ± 1	4 ± 2	48 ± 5	45 (45)
rac-**9**	Kau2	0.22	*R*	*R*	2.4 ± 0.4	8 ± 2	89 ± 5	82 (81)
	Kau8	0.89	*S*	*R*	8 ± 1	1 ± 1	83 ± 6	75 (82)
rac-**10**	Kau2	22.1	1*R*,2*R*	1*R*,2*S*	>200	(35)	(100)	79
	Kau8	0.38	1*S*,2*S*	1*S*,2*R*	4 ± 1	90 ± 8 (97)	95 ± 5 (99)	7 (2)
rac-**11**	Kau2	1.0	1*R*,2*S*	1*R*,2*R*	10 ± 1	0	100	>99 (100)
	Kau8	0.13	1*S*,2*R*	1*R*,2*R*	8 ± 1	39 ± 6	97 ± 3	59 (58)
rac-**12**	Kau2	1.0	*R*	*S*	2.3 ± 0.3	60 ± 6	18 ± 5	41 (41)
	Kau8	0.66	*S*	*S*	1.02 ± 0.2	100	100	3 (0)
rac-**13**	Kau2	1.1	*R*	*S*	6 ± 1	56 ± 6 (38)	7 ± 4 (6)	50 (50)
	Kau8	0.16	*R*	*S*	1.1 ± 0.07	29 ± 4 (35)	26 ± 4 (25)	4 (3)

[a] Enriched enzymes were used.
[b] Specific activities were determined at 28 °C and are expressed as μmol (mg of protein)$^{-1}$ min^{-1}.
[c] The numbers in parentheses indicate regioselectivity coefficients as determined using enantiopure epoxides as substrates.
[d] *ee* of the diol was determined at 100% conversion of the reaction. The theoretical *ee*-values of the diols at 100% conversion are indicated in parentheses. The theoretical *ee* is based on the regioselectivity coefficients, which were obtained using Eqs. (1) and (2).

a much higher potential for enantioconvergence than Kau8, as exemplified by the biotransformation reactions with epoxides **6**–**8** and **10**–**13** (see the ee_p values at 100% conversion in Table 4). Calculation of the regioselectivity coefficients revealed that with Kau2 as the biocatalyst, most epoxides with *R*-configuration at position C_1 were attacked predominantly at position C_2 ($\alpha_R < \beta_R$; see Fig. 4 and Tables 3 and 4), leading to retention of the configuration at position C_1. In several cases, the attack at position C_2 of the *R*-epoxide was exclusive (epoxides **3**, **6**, **8**, and **11**). On the other hand, most epoxides with *S*-configuration at position C_1 were attacked predominantly at this position when using the Kau2 biocatalyst ($\alpha_S > \beta_S$; see Fig. 4 and Table 4), leading to inversion of the configuration at this position. In many cases, the interplay of the Kau2 regioselectivity coefficients was such that the main

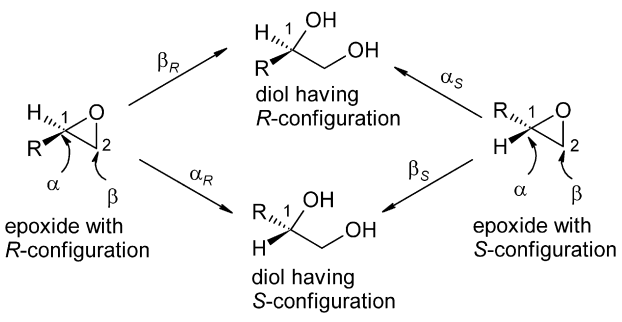

Fig. 4. Attack of the oxirane ring can occur at either carbon atom C_1, resulting in a diol with an inverted configuration (α-attack), or at carbon atom C_2, resulting in a diol with a retained configuration (β-attack) [10,16,48]. The following relationships hold: $\alpha_R + \beta_R = 100\%$, and $\alpha_S + \beta_S = 100\%$.

diol product showed a high *ee*-value during the entire biotransformation reaction. The high degree of enantioconvergence and the high enantioselectivity towards epoxides **5**, **6**, and **10** makes Kau2 a potentially attractive biocatalyst for future preparative-scale reactions. Future genetic engineering work may lead to a fully enantioconvergent EH for substrates other than epoxide **11**. Moreover, higher activities may be achievable through amino acid substitutions in the substrate tunnel region, as shown for the EH from *Aspergillus niger* M200 [46]. Further, combined use of both Kau2 and Kau8 may result in an enantioconvergent process for production of enantioenriched diols **5a** and **9a**.

The active sites of Kau2 and Kau8 are such that non-terminal epoxides are also accepted as substrates. In this respect, the active-site cavities of the EHs Kau2 and Kau8 are likely to be similar to the known active-site structures of the EHs from mouse, man and potato, which contain large cavities that are optimal for binding non-terminal epoxides [37–39]. This hypothesis is supported by the high amino acid sequence similarities between Kau2 and Kau8 and the mouse and human EH sequences noted above.

3.3. Preparative-scale experiments

To determine whether the Kau2 biocatalyst could be useful for synthetic applications, three preparative-scale biotransformations were performed. Freeze-dried or thawed whole cells expressing Kau2 were used for the reactions with epoxides **10** and **11** as substrates. A biphasic system with 2.4 g of epoxide **10** as the organic phase and freeze-dried whole cells in a total reaction volume of 30 mL afforded 1.15 g of epoxide (1*R*,2*R*)-**10** with an *ee* of 99.3% and a yield of 48%, and 1.25 g of diol (1*R*,2*S*)-**10a** with an *ee* of 99.5% and a yield of 46% (Fig. 5). Using a similar biphasic biotransformation

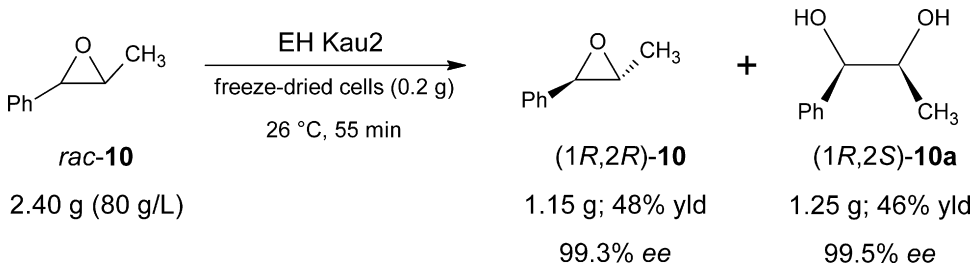

Fig. 5. Preparative resolution of racemic epoxide **10** using the EH Kau2 at high substrate concentration.

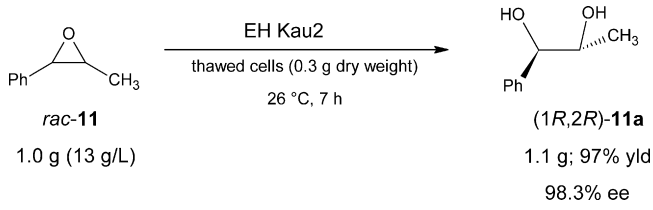

Fig. 6. Preparative enantioconvergent biohydrolysis of racemic epoxide **11** using the EH Kau2.

system with 2.4 g of epoxide **10** and thawed cells, 1.15 g of (1*R*,2*R*)-**10** with an *ee* of 99.0%, and 1.22 g of (1*R*,2*S*)-**10a** with an *ee* of 99.8% were isolated with yields of 48% and 45%, respectively. These experiments clearly showed that the Kau2 biocatalyst can be applied in biphasic biotransformation reactions with high epoxide concentrations of 80 g/L (600 mM). Three reaction cycles with recycled Kau2 biocatalyst were also performed, using the same preparative-scale biotransformation conditions as described above. A decrease of 30% in enzymatic activity was observed for the second cycle. Consequently, a longer reaction time of 95 min was needed to obtain *ee*-values of ≥99.0% for (1*R*,2*R*)-**10** and (1*R*,2*S*)-**10a**. The enzymatic activity in the third cycle was determined to be 45% of the original activity, and the reaction time increased to 160 min. Future experiments will show if a better operational stability for repeated batch reactions may be achieved through enzyme immobilization. Using the same biocatalyst, a preparative-scale enantioconvergent process was carried out with 1.0 g of substrate **11** using an epoxide concentration of 13 g/L (97 mM) in the reaction mixture (Fig. 6). The biotransformation afforded an excellent yield (1.1 g, 97%) of the diol (1*R*,2*R*)-**11a** in nearly enantiopure form (*ee* 98.3%). The ^{1}H NMR spectra of the isolated epoxides and diols were identical to those described previously [19,47]. Thus, Kau2 showed no significant product inhibition, making it a useful biocatalyst for preparative-scale resolution and enantioconvergent biotransformation reactions.

4. Conclusion

We retrieved two EH-encoding genes directly from metagenomic DNA using PCR with EH-specific consensus primers and genome walking. We showed that the two enzymes, termed Kau2 and Kau8, were functional EHs with promising biocatalytic characteristics for the hydrolytic kinetic resolution of several aromatic epoxides. The Kau2 and Kau8 enzymes generally showed opposite enantioselectivity and in several cases had a high potential for enantioconvergence. Kau2 was successfully used in preparative-scale kinetic resolution reactions with epoxide **10** at high substrate concentrations as well as in an enantioconvergent process, allowing quantitative preparative access to enantiopure diol (1*R*,2*R*)-**11a** from epoxide **11**. We are currently exploring the synthetic potential of these new EHs in greater depth. In particular, additional non-terminal epoxides will be tested. We expect that metagenomic DNA contains many more EHs with industrial value that await discovery. The use of PCR with degenerate consensus primers and genome walking may help us mine this untapped resource.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.01.016.

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