

## Enantioselective Epoxidation of Terminal Alkenes to (*R*)- and (*S*)-Epoxides by Engineered Cytochromes P450 BM-3

Takafumi Kubo, Matthew W. Peters, Peter Meinhold, and Frances H. Arnold\*<sup>[a]</sup>

**Abstract:** Cytochrome P450 BM-3 from *Bacillus megaterium* was engineered for enantioselective epoxidation of simple terminal alkenes. Screening saturation mutagenesis libraries, in which mutations were introduced in the active site of an engineered P450, followed by recombination of beneficial mutations generated two P450

BM-3 variants that convert a range of terminal alkenes to either (*R*)- or (*S*)-epoxide (up to 83% *ee*) with high catalytic turnovers (up to 1370) and high

epoxidation selectivities (up to 95%). A biocatalytic system using *E. coli* lysates containing P450 variants as the epoxidation catalysts and in vitro NADPH regeneration by the alcohol dehydrogenase from *Thermoanaerobium brockii* generates each of the epoxide enantiomers, without additional cofactor.

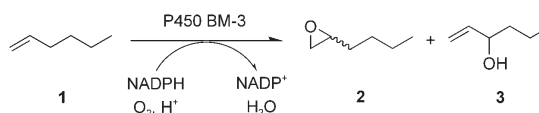
**Keywords:** alkenes • biocatalysis • cofactors • cytochrome p450 • enantioselectivity • epoxidation

### Introduction

Enantioselective epoxidation of terminal alkenes remains a synthetic challenge. Catalytic systems such as titanium–tartrate,<sup>[1]</sup> manganese–salen,<sup>[2]</sup> and iron–porphyrin<sup>[3]</sup> complexes have been developed for asymmetric alkene epoxidation; however, a chemical catalyst effective on simple linear terminal alkenes has not yet been reported.<sup>[4]</sup> Several enzymes, including cytochromes P450,<sup>[5]</sup> methane monooxygenases,<sup>[6]</sup> toluene monooxygenases,<sup>[7]</sup> styrene monooxygenases,<sup>[8]</sup> and chloroperoxidases,<sup>[9]</sup> as well as microbial whole-cell catalysts such as *Rhodococcus rhodochrous*,<sup>[10]</sup> catalyze the enantioselective epoxidation of terminal alkenes. High optical purity is achieved in some cases, but these biocatalytic systems generally only produce a single enantiomer and only accept a limited range of alkene substrates.<sup>[11]</sup> While chiral terminal epoxides can be prepared by kinetic resolution of racemic epoxides with cobalt–salen catalysts<sup>[12]</sup> or epoxide hydrolases,<sup>[11]</sup> direct enantioselective epoxidation remains an attractive goal for its simplicity and higher potential yield.

The fatty acid hydroxylase cytochrome P450 BM-3 from *Bacillus megaterium* has proven to be a versatile platform for engineering oxidation catalysts for a variety of nonnatur-

al substrates.<sup>[13]</sup> We previously demonstrated that a combination of directed evolution by random mutagenesis over the whole gene and modification of active-site residues increased alkane hydroxylation activity of BM-3 and altered its regio- and enantioselectivity.<sup>[14]</sup> Various BM-3 mutants also exhibited respectable activity on several alkenes, but generated mainly the allyl-hydroxylated product **3** rather than epoxide **2** from 1-hexene (**1**, Scheme 1).<sup>[15]</sup> Here we report further engineering of BM-3 variants that produce enantio-enriched epoxides from simple terminal alkenes. Two engineered cytochromes P450 show good catalytic turnovers and high epoxidation selectivities. One variant produces (*R*)-epoxides, while the other gives (*S*)-epoxides with up to 83% *ee*.



Scheme 1. Oxidation of 1-hexene by P450 BM-3.

### Results and Discussion

**Creation and screening of enzyme libraries:** Starting from previously isolated BM-3 variant 9-10 A,<sup>[14]</sup> which exhibits high (hydroxylation) activity on small alkanes and some epoxidation activity towards terminal alkenes, we targeted key residues in the active site for further modification. Using the

[a] T. Kubo, Dr. M. W. Peters, P. Meinhold, Prof. F. H. Arnold  
Division of Chemistry and Chemical Engineering  
California Institute of Technology  
1200 E. California Blvd. MC210-41, Pasadena, CA 91125 (USA)  
Fax: (+1) 626-568-8743  
E-mail: frances@cheme.caltech.edu

crystal structure of the heme domain of wild-type BM-3 with a bound substrate,<sup>[16]</sup> we selected 11 residues within 5 Å of the ten carbon atoms of the (fatty acid) substrate closest to the heme: A74, L75, V78, F81, A82, F87, T88, T260, I263, A264, A328. Saturation mutagenesis was used to generate 11 libraries, each containing all possible amino acid substitutions at one of these positions. These enzyme libraries were screened for epoxidation activity towards 1-hexene by using 4-(*p*-nitrobenzyl)pyridine, which reacts with terminal epoxides to form a blue adduct that can be quantified spectroscopically.<sup>[17,18]</sup> The assay conditions reported previously by us<sup>[18]</sup> were modified to greatly improve the sensitivity (10 µM for detection of 1,2-epoxyhexane). Five of the libraries—at positions L75, A82, F87, I263, A328—yielded enzymes with improved activity relative to 9-10A. From these, eight variants of 9-10A containing amino acid substitutions L75S, A82F, A82L, F87I, F87L, F87V, I263A, A328V were selected and subsequently shown to have not only increased total catalytic turnover number (TTN) for epoxide production from 1-hexene, but also increased (*R*)- or (*S*)-enantioselectivity (Table 1).

A library containing different combinations of these eight active-site mutations in 9-10A was created and screened for epoxidation activity on 1-hexene. Two variants isolated from this second-generation library, SH-44 and RH-47, are particularly efficient 1-hexene epoxidation catalysts; they also exhibit opposite enantioselectivities (Table 2). SH-44 contains

two active-site substitutions, F87V and I263A, in addition to the 14 that distinguish 9-10A from wild-type BM-3. RH-47 also contains the I263A substitution, but in combination with A82F and A328V (as well as the 9-10A mutations). Residues A82, F87, and A328 are known determinants of regio- and enantioselective oxidation in BM-3.<sup>[14,19]</sup>

**Catalytic properties of cytochrome P450 BM-3 variants SH-44 and RH-47:** The catalytic properties of wild-type BM-3, parent 9-10A, and the SH-44 and RH-47 variants reported here are detailed in Table 2. The total turnover numbers (TTNs) of SH-44 and RH-47 for 1-hexene epoxidation are eight- and fivefold higher than parent 9-10A, respectively, and more than 30-fold higher than wild-type BM-3. Epoxidation selectivities are also greatly improved: SH-44 and RH-47 produce ~90% epoxide, while wild-type and 9-10A mainly produce the allylic hydroxylation product. Of particular note, SH-44 and RH-47 catalyze epoxidation with opposite enantioselectivities: SH-44 selectively produces the (*S*)-epoxide, while RH-47 makes the (*R*)-epoxide with moderate enantiomeric excesses (71 and 60% *ee*, respectively).

Activities towards linear terminal alkenes of length C5 to C8 were also determined (Table 3). Variant SH-44 supports more turnovers on shorter alkenes (1370 on 1-pentene vs 200 on 1-octene), and the epoxide accounted for at least 80% of the product for all the alkenes tested. Variant RH-47 shows similar, moderate TTNs and high regioselectivities on all the alkenes. The highest enantioselectivity is 83% *ee* (for (*R*)-1,2-epoxyoctane) by RH-47. The favored 1,2-epoxide remained the *S* enantiomer with SH-44 and the *R* enantiomer with RH-47 for all the substrates. No chemical catalysts are known to epoxidize the terminal linear alkenes used here with more than 50% *ee*.<sup>[20]</sup> Furthermore, no biocatalyst has been engineered to epoxidize these alkenes to produce both enantiomers prior to this report. The TTNs of SH-44 and RH-47 towards linear terminal alkenes are comparable to those reported for nonasymmetric chemical catalysts. To our knowledge, the best reported TTN is 2200 on 1-octene, by a polyoxometalate with molecular oxygen as the oxidant.<sup>[21]</sup> This catalyst, however, requires a much longer reaction time and higher temperature (over 300 h at 80°C) than P450 BM-3, which catalyzes the same reaction with hundreds of turnovers at room temperature in less than 3 h.

We also determined the activities of SH-44 and RH-47 for hexane hydroxylation (Table 4). The selectivities were consistent with the relative product distributions on 1-hexene: both SH-44 and RH-47 convert hexane primarily to 1- and 2-hexanol (cf. 9-10A, which mostly produces 3-hexanol from hexane and 1-hexen-3-ol from 1-hexene), indicating a shift in the carbon atoms activated by the iron–oxygen species towards terminal positions.

Table 1. Effects of beneficial mutations observed in cytochrome P450 variant 9-10A.

Mutation in 9-10A	TTN <sup>[a]</sup>	Epoxidation selectivity [%] <sup>[b]</sup>	Epoxide optical purity [% <i>ee</i> ] <sup>[c]</sup>
none	140	25	4 ( <i>R</i> )
L75S	220	45	19 ( <i>S</i> )
A82F	210	64	2 ( <i>R</i> )
A82L	220	49	16 ( <i>R</i> )
F87I	450	67	45 ( <i>S</i> )
F87L	350	76	79 ( <i>S</i> )
F87V	670	73	73 ( <i>S</i> )
I263A	300	68	11 ( <i>R</i> )
A328V	280	81	63 ( <i>R</i> )

[a] Total catalytic turnover number for epoxidation of 1-hexene. Epoxide was quantified by the NBP method. Reaction conditions: 0.1 µM P450 (*E. coli* cell lysate), 4 mM substrate (1-hexene), 0.5 mM NADPH, 1 hour at room temperature. [b] Selectivity for 1,2-epoxyhexane formation. Remainder is mostly 1-hexen-3-ol. These data were obtained by GC analysis. [c] Favored enantiomer is listed in parentheses. Data obtained by GC analysis.

Table 2. Epoxidation of 1-hexene by wild-type cytochrome P450 BM-3 and its variants.<sup>[a]</sup>

Enzyme	TTN <sup>[b]</sup>		Product distribution [%] <sup>[c]</sup>			Epoxide optical purity [% <i>ee</i> ] <sup>[c]</sup>
	epoxide 2	all products	epoxide 2	3-ol 3	other products	
Wt	20	200	10	85	5	17 ( <i>R</i> )
9-10A	130	530	25	65	10	4 ( <i>R</i> )
SH-44	1090	1240	88	6	6	71 ( <i>S</i> )
RH-47	610	660	93	2	5	60 ( <i>R</i> )

[a] Reaction conditions: 0.1 µM P450, 4 mM substrate (1-hexene), 0.5 mM NADPH, 3 h at 20°C. Data were obtained by GC on a chiral column. [b] Total catalytic turnover number. Errors are at most 6%. [c] Errors are less than 1%.

Table 3. Epoxidation of terminal alkenes by cytochrome P450 BM-3 variants SH-44 and RH-47.<sup>[a]</sup>

Variant	Substrate	TTN <sup>[b]</sup>	Epoxidation selectivity [%] <sup>[c]</sup>	Epoxide optical purity [% ee] <sup>[c]</sup>
SH-44	1-pentene	1370	93	73 (S)
SH-44	1-hexene	1090	88	71 (S)
SH-44	1-heptene	500	85	65 (S)
SH-44	1-octene	200	84	55 (S)
RH-47	1-pentene	570	94	60 (R)
RH-47	1-hexene	610	93	60 (R)
RH-47	1-heptene	550	95	76 (R)
RH-47	1-octene	560	92	83 (R)

[a] Experimental conditions are those for Table 2. [b] Total catalytic turnover number for epoxidation. Errors are at most 12%. [c] Errors are less than 1%.

Table 4. Hydroxylation of hexane by engineered cytochrome P450 BM-3 variants.<sup>[a]</sup>

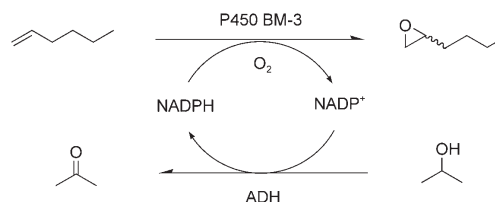
Variant	TTN <sup>[b]</sup>	Product distribution [%] <sup>[c]</sup>			
		1-hexanol	2-hexanol	3-hexanol	other
9-10 A	3960	0.6	22	77	0.4
SH-44	2300	6	56	34	4
RH-47	4070	22	73	3	2

[a] Experimental conditions are those for Table 2. [b] Errors are at most 11%. [c] Errors are less than 1%.

TTNs on hexane were significantly higher than on 1-hexene, which suggests that epoxidation of linear terminal alkenes leads to more rapid enzyme inactivation.<sup>[22]</sup> Possible inactivation mechanisms include heme alkylation and peptide modification, as reported for other P450s.<sup>[23,24]</sup> Styrene is a terminal alkene that is known not to cause P450 inactivation.<sup>[23]</sup> Variants SH-44, RH-47, and even 9-10 A exhibit very high TTNs for styrene epoxidation (14000 for SH-44, 10000 for RH-47, 4000 for 9-10 A).

**Epoxidation with NADPH regeneration:** Cytochrome P450 BM-3-catalyzed epoxidation requires an equivalent of NADPH to convert an equivalent of alkene substrate. Thus efficient use of the enzyme requires cofactor recycling, which can be accomplished *in vitro*<sup>[25]</sup> or *in vivo*, in whole cells. The whole-cell reaction is simple, but sometimes difficult when the substrate is hydrophobic, as are the alkenes investigated here. Therefore, we tested *in vitro* NADPH regeneration to perform enantioselective epoxidation with the two BM-3 variants. Dehydrogenases such as alcohol dehydrogenase, glucose dehydrogenase, isocitric dehydrogenase and formate dehydrogenase can be used as the cofactor-regenerating enzyme.<sup>[26]</sup> We chose alcohol dehydrogenase (ADH), because the alcohol can serve not only as the regeneration driving force, but also as the cosolvent for hydrophobic substrates. We employed the NADPH-dependent alcohol dehydrogenase from *Thermoanaerobium brockii*, which is highly thermo- and solvent-stable, and specific to shorter and secondary alcohols.<sup>[27]</sup> NADPH was recycled by the ADH-catalyzed oxidation of 2-propanol to acetone coupled to the reduction of NADP<sup>+</sup> (Scheme 2).

An *E. coli* cell lysate containing the variant P450s was used for epoxidation so that the initial cofactor is supplied



Scheme 2. NADPH is regenerated by coupling to the oxidation of 2-propanol catalyzed by ADH.

from the lysate. As shown in Table 5, this system converted 1-hexene to the 1,2-epoxyhexanes with good yields in 7 h, by using NADPH regenerated by ADH. The enantioselectivities and epoxidation selectivities of SH-44 and RH-47 observed earlier were preserved in this system. That no additional cofactor was necessary makes the synthesis more economical. We believe that this is the first example of a monooxygenase-catalyzed epoxidation with *in vitro* NADPH regeneration, although epoxidation with regeneration of NADH by NADH-dependent formate dehydrogenase has been reported.<sup>[28]</sup>

Table 5. Epoxidation of 1-hexene by cytochrome P450 BM-3 variants SH-44 and RH-47, using the NADPH regeneration system.<sup>[a]</sup>

Variant	SH-44	RH-47
added substrate [mM]	10	10
remaining substrate [mM]	0.1	1.2
1,2-epoxyhexane [mM]	7.6	7.2
total products [mM]	8.0	7.4
mass balance [%] <sup>[b]</sup>	81	86
epoxidation selectivity [%] <sup>[c]</sup>	95	98
epoxide optical purity [% ee]	71 (S)	57 (R)

[a] Reactions were performed under the following conditions: 10  $\mu$ M P450 (*E. coli* cell lysate), 1 Unit mL<sup>-1</sup> ADH, 1 vol% 2-propanol, and 7 h reaction at room temperature. Oxygen was initially pressurized to 0.15 MPa (gauge) and added at 2 h and 4 h (1–2 mL at ambient pressure). 10 mM substrate (1-hexene) was added over 3 h. [b] Mass balance =  $\{c(\text{remaining substrate}) + c(\text{total products})\} / c(\text{added substrate}) \times 100$ . 19% and 14% of substrate could not be recovered; this seems to be caused by the high volatility of the substrate and the product epoxide. No significant by-products were detected. [c] Epoxidation selectivity =  $c(1,2\text{-epoxyhexane}) / c(\text{total products}) \times 100$ .

## Conclusion

We have engineered P450 BM-3 for enantioselective epoxidation of terminal alkenes by using saturation mutagenesis and recombination coupled with a colorimetric high-throughput screen for epoxide formation to identify efficient epoxidation catalysts. P450 variants isolated in this way convert a range of terminal alkenes to the (R)- or (S)-epoxides with high catalytic turnovers and high epoxidation selectivities. We also showed that P450-catalyzed epoxidation could be performed by using cell lysate containing the P450 and alcohol dehydrogenase for regeneration of the expensive NADPH cofactor, without additional NADPH. We anticipate that improvements in catalytic properties including

enantioselectivity on desired target substrates can be achieved using the methods of mutagenesis and screening outlined here. This and our recent report describing enantioselective hydroxylation of protected carboxylic acids by other BM-3 variants<sup>[29]</sup> demonstrate that the BM-3 active site can be readily molded for enantio- and regioselective oxidation of a range of substrates.

## Experimental Section

**Materials:** 1-Pentene (99%), 1-hexene (99%), 1-octene (98%), styrene (99%), product standards such as racemic epoxides (except 1,2-epoxyheptane), (*R*)-1,2-epoxyhexane, (*R*)-1,2-epoxyoctane, 1-hexen-3-ol, hexanols and solvents were purchased from Sigma-Aldrich. 1-Heptene (98%) and 1,2-epoxyheptane were purchased from TCI America. *n*-Hexane (99%) was purchased from Fluka. NADPH was obtained from Biocatalytics, Inc.

**Plasmids and expression of P450 BM-3:** P450 BM-3 was expressed as described.<sup>[30]</sup> The P450 BM-3 gene or mutants thereof, which include a silent mutation to introduce a *SacI* site 130 bp upstream of the end of the heme domain, were cloned behind the double *tac* promoter of the expression vector pCWori<sup>[31]</sup> (pBM3 WT18-6). *E. coli* DH5 $\alpha$ , transformed with these plasmids, was used for expression of P450 BM-3 on a 500 mL scale as well as for expression in 96-well plates.

**Purification of P450 BM-3:** For protein production, supplemented terrific broth (TB) medium (500 mL, 100  $\mu\text{g mL}^{-1}$  ampicillin) in a 2.8 L flask was inoculated with an overnight culture (3 mL) and incubated at 30°C and 180–200 rpm shaking. After 24 h of incubation,  $\delta$ -aminolevulinic acid hydrochloride (ALA; 0.5 mM) was added, and expression was induced by addition of isopropyl- $\beta$ -D-thiogalactoside (IPTG; 1 mM). Cells were harvested by centrifugation 24 h after induction. The enzymes were purified following published procedures.<sup>[31]</sup> Enzyme concentration was measured in triplicate from the CO difference spectra.<sup>[32]</sup>

**Preparation of cell lysates for high-throughput screening:** Single colonies were picked and inoculated into 1 mL deep-well plates containing Luria-Bertani (LB) medium (350  $\mu\text{L}$ , supplemented with 100  $\mu\text{g mL}^{-1}$  ampicillin). The plates were incubated at 30°C, 250 rpm, and 80% relative humidity. After 24 h, clones from this preculture were inoculated using a 96-pin replicator into 2 mL deep-well plates containing TB medium (500  $\mu\text{L}$ , supplemented with 100  $\mu\text{g mL}^{-1}$  ampicillin, 10  $\mu\text{M}$  IPTG and 0.5 mM ALA). The cultures were grown at 30°C, 250 rpm, and 80% relative humidity for another 24 h. Cells were then pelleted and stored frozen at –20°C until they were resuspended in 500  $\mu\text{L}$  100 mM phosphate buffer (pH 8, containing 0.5  $\text{mg mL}^{-1}$  lysozyme, 2 Units  $\text{mL}^{-1}$  DNase I, and 10 mM  $\text{MgCl}_2$ ). After 30 min at 37°C, the lysates were centrifuged and the supernatant was used for activity measurements in 96-well microtiter plates.

**High-throughput epoxide formation assay:** For high-throughput screening of mutants based on epoxide formation, the published method<sup>[18]</sup> using 4-(*p*-nitrobenzyl)pyridine (NBP) was modified as follows: to *E. coli* supernatant (30–40  $\mu\text{L}$ ), phosphate buffer (100 mM, pH 8) was added to 100  $\mu\text{L}$  of total volume on the 96-well plate. After addition of a solution 0.4 mL 1-hexene in ethanol (1.5  $\mu\text{L}$ ) as substrate, oxidation was started by addition of 50  $\mu\text{L}$  of 1.5 mM NADPH solution (in buffer). The plate was sealed with a plastic film (GeneMate) immediately so that epoxide does not evaporate. After 30 minutes incubation at room temperature, a solution of NBP (100  $\mu\text{L}$ ; 6 wt/vol % NBP, 19.5 vol % 1-butanol, 80 vol % propylene glycol, 0.5 vol % acetic acid) was added, and the plate was sealed again with a film. The plate was placed in an oven at 80°C for 2.5 h and chilled on ice for 10 minutes. Subsequently, solution of triethylamine in acetone (100  $\mu\text{L}$ , 50 vol %) was added to develop color, followed by measurement of absorbance at 580 nm on a plate-reader.

**Construction and screening of saturation mutagenesis libraries:** Mutations were introduced into mutant 9-10A by PCR overlap extension mutagenesis<sup>[33]</sup> using Pfu turbo DNA polymerase (Stratagene). The primers

for each saturation library contained all possible combinations of bases, NNN (N=A, T, G, or C), at the codon for a particular residue. The primers in the forward direction for each library were: 74NNNfor (5'-GTCAANNCTTAAATTTGCACG-3'), 75NNNfor (5'-GTCAAGCGNNNAATTTGCACG-3'), 78NNNfor (5'-GCTTAAATTTNNNCGTGATTTTGCACG-3'), 81NNNfor (5'-CGTGATNNNGCAGGAGAC-3'), 82NNNfor (5'-CGTGATTTNNNGGAGAC-3'), 87NNNfor (5'-GAGACGGGTANNNACAAGCTGGAC-3'), 88NNNfor (5'-GGAGACGGGTATTNNNAGCTGGACG-3'), 260NNNfor (5'-CAAATTATTNNNTTCTTAATTGCGGGAC-3'), 263NNNfor (5'-ACATTCCTANNNGCGGGACACGAAAC-3'), 264NNNfor (5'-ACATTCCTAATTNNNGACACGAAAC-3'), and 328NNNfor (5'-CCAACTNNNCTGCGTTTCC-3'). The reverse primers for each of these libraries complement their corresponding forward primers. For each mutation, two separate PCRs were performed, each using a perfectly complementary primer, BamHIfor (5'-GGAAACAGGATCCATCGATGC-3') and SacIrev (5'-GTGAAGGAATACCGCAAGC-3'), at the end of the sequence and a mutagenic primer. The resulting two overlapping fragments that contain the mutations were then annealed in a second PCR to amplify the complete mutated gene. The full gene was then cut with *Bam*HI and *Sac*I restriction enzymes and ligated with T4 ligase (Invitrogen) into pBM3\_WT18-6, previously cut with *Bam*HI and *Sac*I to remove the wild-type gene. The ligation mixtures were then transformed into *E. coli* DH5 $\alpha$  electrocompetent cells and plated onto LB agar plates to form single colonies for picking. 176 clones were screened for each library in the first screening. Positive clones that showed higher epoxide formation than parent 9-10A were re-assayed in the second screening, in which each clone was inoculated in one column (8 wells). P450 concentration was also measured in the second screening by CO difference spectroscopy to correct for the enzyme expression level. Positive clones were grown on TB medium (25 mL) to prepare cell lysates under the same conditions that are described in the "Purification of P450 BM-3" section. Cell lysates were used for characterization of mutants.

**Recombination of P450 BM-3 mutants:** Recombination of BM-3 mutations identified in the saturation mutagenesis libraries was performed by repeating PCR overlap extension mutagenesis with mutagenic primers and mixed DNA templates as described below. Step 1 (recombination of L and S at residue 75 and of A, F, and L at residue 82): a PCR was performed by using a mutagenic primer 75LSfor (5'-CTTAAGTCAAGCGTYRAAATTTGCACG-3'), SacIrev, and a DNA plasmid mixture of mutant A82F, mutant A82L, and 9-10A as PCR template. These plasmids were mixed at a same concentration (estimated from ABS<sub>260</sub>). Another PCR was also performed using the complementary reverse primer of 75LSfor, BamHIfor, and the plasmid of 9-10A as a template. The resulting two overlapping fragments were then assembled in a second PCR to give the whole gene recombined at residue 75 and 82. Step 2 (recombination of F, V, I, and L at residue 87): a PCR with a mutagenic primer 87FVILfor (5'-GAGACGGGTTANTYACAAGCTGGAC-3'), SacIrev, and the plasmid DNA of 9-10A as a template was performed. Another PCR using the complementary reverse primer of 87FVILfor, BamHIfor, and the assembled DNA fragment from step 1 as a template was also performed. The resulting two fragments were then assembled in a second PCR to give the whole gene recombined at 75, 82, and 87. Step 3 (recombination of I and A at residue 263 and of A and V at residue 328): a PCR with a mutagenic primer 263IAfor (5'-CAAATTATTACATTCTARYHGCGGGACACG-3'), SacIrev, and a DNA plasmid mixture of mutant A328V and 9-10A as a template was performed. Another PCR with the complementary reverse primer of 263IAfor and BamHIfor with the assembled DNA fragment from step 2 as a template was also performed. The resulting two fragments were then assembled in a second PCR to give the whole gene recombined at residues 75, 82, 87, 263, and 328. The recombined gene was then cut with *Bam*HI and *Sac*I restriction enzymes, ligated into vector DNA, and transformed into *E. coli* DH5 $\alpha$ . The library size was 192, and about 500 clones were screened for epoxide formation in the first screening. 22 clones, which showed same or higher epoxide formation than the mutant F87V, were re-assayed in the second screening. Positives from this were then grown on TB medium (25 mL) for preparation of cell lysates and characterization. Two mutants (SH-44

and RH-47) that showed the highest turnovers with different enantioselectivity were grown on a 500 mL scale and purified as described above.

**Quantification of epoxides by the NBP method:** Epoxides were quantified using NBP under conditions described in the "High-throughput epoxide formation assay" section. Enzymatic reactions were performed on a 0.6 mL scale in 1.7 mL tubes. Calibration of the epoxide was conducted using a standard reagent of 1,2-epoxyhexane.

**Chiral gas chromatography:** A CycloSil-B chiral capillary column (Agilent Technologies, 30 m length, 0.32 mm ID, 0.25  $\mu$ m film thickness) and a Flame Ionization Detector (FID) were used. The reaction products were determined and quantified by using standard racemic epoxides and alcohols. The absolute configurations of epoxides were determined by comparing the reaction product with a standard (*R*)-enantiomer-enriched epoxide in the case of 1,2-epoxyhexane and 1,2-epoxyoctane. For 1,2-epoxypentane and 1,2-epoxyheptane, the standard (*S*)-enantiomer-enriched epoxides were prepared using the yeast strain *Rhodospiridium toruloides* (ATCC 10657) expressing an epoxide hydrolase that is known to hydrolyze (*R*)-epoxides preferentially.<sup>[34]</sup> Conditions for GC analysis for 1,2-epoxyhexane were: isothermal at 70 °C for 2.5 min, linear gradient to 200 °C in 13 min and isothermal at 200 °C for 5 min. The conditions were slightly modified for different epoxides. The separation of the *R* and *S* enantiomers of the epoxides gave the following retention times: 5.04 and 5.14 min (1,2-epoxypentane), 5.21 and 5.29 min (1,2-epoxyhexane), 14.17 and 14.35 min (1,2-epoxyheptane) and 19.39 and 19.46 min (1,2-epoxyoctane).

**Analysis of alkene epoxidation and alkane hydroxylation:** Oxidations of 1-pentene, 1-hexene, 1-heptene, 1-octene, styrene, and *n*-hexane were performed on 1 mL scale in phosphate buffer (pH 8, 100 mM) containing P450, substrate, and 1% ethanol in 1.7 mL tubes. Reactions were started by adding NADPH (dissolved in buffer). After reaction, a solution of 1-octanol in ethanol (10  $\mu$ L; 10 mM) was added as the internal standard and chloroform (300  $\mu$ L) was also added to extract products. The tubes were vortexed vigorously for 1 min, and subsequently centrifuged for 1 min at 14000 rpm. The lower organic layer was collected and analyzed by gas chromatography to determine total catalytic turnover numbers, product distributions, and enantiomeric excess. Reactions were performed in triplicate.

**Epoxidation with NADPH regeneration:** *E. coli* cells were grown in a TB culture (500 mL), and P450 expression was induced as described above. Cells were collected, suspended in phosphate buffer (20 mL, 100 mM, pH 8), and disrupted by sonication. The cell lysate was recovered by centrifugation, and P450 concentration was measured by CO binding (SH-44, 63  $\mu$ M, RH-47, 30  $\mu$ M). Epoxidation reactions were performed in a vial (12 mL), with phosphate buffer (100 mM, pH 8), *E. coli* cell lysate containing P450 and the alcohol dehydrogenase from *Thermoanaerobium brockii* (purchased from Sigma). The contents were mixed and the vial sealed with a crimp cap (Agilent Technologies). Oxygen was initially pressurized from the top and added every few hours using a syringe. 1-Hexene was dissolved in 2-propanol (1 M), and the solution was slowly added to the vial with a syringe over a few hours with stirring. A portion of the reaction mixture was sampled from the vial and extracted with chloroform, and the extract was analyzed by gas chromatography. Total reaction volume was 9 mL.

## Acknowledgements

The authors thank Dr. Nathan Dalleska for assistance with gas chromatography. This work is supported by the National Science Foundation (BES-0313567), the Donors of the American Chemical Society Petroleum Research Fund (ACS PRF Alternative Energy Postdoctoral Fellowship for MWP) and the Nippon Shokubai Co., Ltd.

- [1] T. Katsuki, K. B. Sharpless, *J. Am. Chem. Soc.* **1980**, *102*, 5974–5976.
- [2] W. Zhang, J. L. Loebach, S. R. Wilson, E. N. Jacobsen, *J. Am. Chem. Soc.* **1990**, *112*, 2801–2803.
- [3] E. Rose, Q. Z. Ren, B. Andrioletti, *Chem. Eur. J.* **2004**, *10*, 224–230.

- [4] V. B. Valodkar, G. L. Tembe, R. N. Ram, H. S. Rama, *Catal. Lett.* **2003**, *90*, 91–94.
- [5] C. A. Martinez, J. D. Stewart, *Curr. Org. Chem.* **2000**, *4*, 263–282.
- [6] S. J. Elliott, M. Zhu, L. Tso, H. H. T. Nguyen, J. H. K. Yip, S. I. Chan, *J. Am. Chem. Soc.* **1997**, *119*, 9949–9955.
- [7] K. McClay, B. G. Fox, R. J. Steffan, *Appl. Environ. Microbiol.* **2000**, *66*, 1877–1882.
- [8] A. Schmid, K. Hofstetter, H. J. Feiten, F. Hollmann, B. Witholt, *Adv. Synth. Catal.* **2001**, *343*, 732–737.
- [9] A. F. Dexter, F. J. Lakner, R. A. Campbell, L. P. Hager, *J. Am. Chem. Soc.* **1995**, *117*, 6412–6413.
- [10] K. Furuhashi, *Biological Routes to Optically Active Epoxides*, Wiley, New York, **1992**, pp. 167–186.
- [11] A. Archelas, R. Furstoss, *Annu. Rev. Microbiol.* **1997**, *51*, 491–525.
- [12] M. Tokunaga, J. F. Larrow, F. Kakiuchi, E. N. Jacobsen, *Science* **1997**, *277*, 936–938.
- [13] A. W. Munro, D. G. Leys, K. J. McLean, K. R. Marshall, T. W. B. Ost, S. Daff, C. S. Miles, S. K. Chapman, D. A. Lysek, C. C. Moser, C. C. Page, P. L. Dutton, *Trends Biochem. Sci.* **2002**, *27*, 250–257; V. B. Urlacher, S. Lutz-Wahl, R. D. Schmid, *Appl. Microbiol. Biotechnol.* **2004**, *64*, 317–325.
- [14] M. W. Peters, P. Meinhold, A. Glieder, F. H. Arnold, *J. Am. Chem. Soc.* **2003**, *125*, 13442–13450.
- [15] E. T. Farinas, M. Alcalde, F. Arnold, *Tetrahedron* **2004**, *60*, 525–528.
- [16] D. C. Haines, D. R. Tomchick, M. Machius, J. A. Peterson, *Biochemistry* **2001**, *40*, 13456–13465.
- [17] F. Zoicher, M. M. Enzelberger, U. T. Bornscheuer, B. Hauer, R. D. Schmid, *Anal. Chim. Acta* **1999**, *391*, 345–351.
- [18] M. Alcalde, E. T. Farinas, F. H. Arnold, *J. Biomol. Screening* **2004**, *9*, 141–146.
- [19] S. Graham-Lorence, G. Truan, J. A. Peterson, J. R. Falck, S. Z. Wei, C. Helvig, J. H. Capdevila, *J. Biol. Chem.* **1997**, *272*, 1127–1135; Q. S. Li, J. Ogawa, R. D. Schmid, S. Shimizu, *FEBS Lett.* **2001**, *508*, 249–252.
- [20] R. Sinigalia, R. A. Michelin, F. Pinna, G. Strukul, *Organometallics* **1987**, *6*, 728–734; R. I. Kureshy, N. H. Khan, S. H. R. Abdi, S. T. Patel, P. Iyer, E. Suresh, P. Dastidar, *J. Mol. Catal. A* **2000**, *160*, 217–227.
- [21] Y. Nishiyama, Y. Nakagawa, N. Mizuno, *Angew. Chem.* **2001**, *113*, 3751–3753; *Angew. Chem. Int. Ed.* **2001**, *40*, 3639–3641.
- [22] Enzymes are rapidly inactivated. Initial rates on 1-hexene epoxidation are very high (80 turnovers  $\text{min}^{-1}$  for S-44 and 160 turnovers  $\text{min}^{-1}$  for R-47).
- [23] P. R. Ortiz de Montellano, M. A. Correia, *Annu. Rev. Pharmacol. Toxicol.* **1983**, *23*, 481–503.
- [24] K. He, Y. A. He, G. D. Szklarz, J. R. Halpert, M. A. Correia, *J. Biol. Chem.* **1996**, *271*, 25864–25872.
- [25] W. A. van der Donk, H. M. Zhao, *Curr. Opin. Biotechnol.* **2003**, *14*, 421–426.
- [26] R. Wichmann, D. Vasic-Racki, *Adv. Biochem. Eng./Biotechnol.* **2005**, *92*, 225–260.
- [27] L. S. al-Kassim, C. S. Tsai, *Biochem. Cell Biol.* **1990**, *68*, 907–913.
- [28] K. Hofstetter, J. Lutz, I. Lang, B. Witholt, A. Schmid, *Angew. Chem.* **2004**, *116*, 2215–2218; *Angew. Chem. Int. Ed.* **2004**, *43*, 2163–2166.
- [29] D. Munzer, P. Meinhold, M. W. Peters, S. Fiechtenhofer, H. Griengl, A. Glieder, F. H. Arnold, A. de Raadt, *Chem. Commun.* **2005**, *20*, 2597–2599.
- [30] A. Glieder, E. T. Farinas, F. H. Arnold, *Nat. Biotechnol.* **2002**, *20*, 1135–1139.
- [31] E. T. Farinas, U. Schwaneberg, A. Glieder, F. H. Arnold, *Adv. Synth. Catal.* **2001**, *343*, 601–606.
- [32] T. Omura, R. Sato, *J. Biol. Chem.* **1964**, *239*, 2370–2378.
- [33] R. Higuchi, B. Krummel, R. K. Saiki, *Nucleic Acids Res.* **1988**, *16*, 7351–7367.
- [34] A. L. Botes, C. A. G. M. Weijers, P. J. Botes, M. S. van Dyk, *Tetrahedron: Asymmetry* **1999**, *10*, 3327–3336.

Received: May 26, 2005

Published online: October 20, 2005