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Asymmetric epoxidation of alkenes and benzylic hydroxylation with P450tol monooxygenase from *Rhodococcus coprophilus* TC-2†

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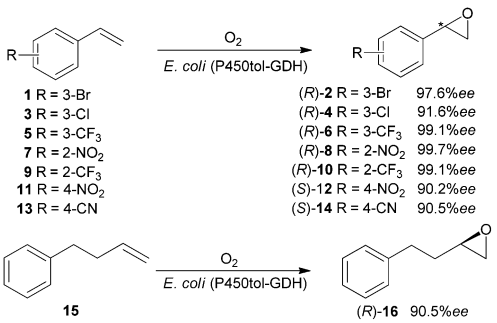
P450tol monooxygenase was discovered as a unique and highly enantioselective enzyme for asymmetric epoxidation of some terminal alkenes containing electron-withdrawing groups and benzylic hydroxylation of several ethylbenzenes giving the corresponding useful and valuable products, such as (*R*)-2- and 3-substituted styrene oxides, (*S*)-4-substituted styrene oxides, and (*S*)-benzylic alcohols, in high ee.

Enzymatic oxidation is a useful tool for green and selective oxidation that is challenging but important for sustainable manufacturing of chemicals and pharmaceuticals, due to the non-toxicity, high chemo-, regio-, and enantio-selectivity, mild reaction conditions, and the use of molecular oxygen in aqueous media as a green and cheap oxidant. Many enzymes have been discovered and developed for asymmetric oxidations,[1] but they often show high substrate specificity. Thus, it is necessary to develop new enzymes with novel substrate specificity and high enantioselectivity for green oxidations.

Asymmetric epoxidation of alkenes is an important class of oxidation reactions and provides ready access to enantiopure epoxides that are useful and valuable fine chemicals and pharmaceutical intermediates.[2] Asymmetric epoxidation by using Sharpless[3] and Jacobsen[4] methods are well known to produce enantiopure epoxides from allylic alcohols and isolated alkenes, respectively. Enzymes such as styrene monooxygenase (SMO),[5] xylene monooxygenase (XMO),[6] chloroperoxidase (CPO),[7] and cytochrome P450 enzymes[8] have been reported for asymmetric epoxidation, with their own substrate specificity and enantioselectivity. We are interested in developing new enzymes for highly enantioselective epoxidations of alkenes that are difficult to perform using current enzymatic and chemical catalysts, to prepare enantiopure epoxides. A group of asymmetric epoxidations of terminal alkenes containing



Scheme 1 Asymmetric epoxidation of 2-, 3-, or 4-substitued styrenes, and 4-phenyl-1-butene with *E. coli* (P450tol-GDH) to produce the corresponding enantiopure epoxides.

electron-withdrawing group are selected as the target reactions (Scheme 1). Products (*R*)-**2**, **4**, **6**, **8**, **10**, **16** and (*S*)-**12**, **14** are all useful and valuable pharmaceutical intermediates (ESI,† Table S3). The corresponding chemical epoxidations are either not reported or unsatisfactory,[9] and no enzymes have been reported for these targeted enantioselective epoxidations except for the P450BM3 F87G mutant which catalyses the epoxidation of **3** to give (*R*)-**4** in 94.6% ee.[10]

To discover a new enzyme for the targeted reactions, we started with the screening of 100 toluene- or ethylbenzene-degrading bacterial strains for the asymmetric epoxidation of 3-bromostyrene **1**. *Rhodococcus coprophilus* TC-2 was found to give (*R*)-**2** in 97.6% ee. The enzyme responsible for the epoxidation was found to be a toluene inducible monooxygenase with a molecular weight of around 50 KDa (ESI,† Fig. S1b) which catalysed the initial oxidation of toluene to benzyl alcohol during degradation. To clone the gene encoding for this enzyme, the whole genome of this bacterium was sequenced, and the two gene clusters containing a group of genes responsible for the oxidations of toluene to benzyl alcohol, benzaldehyde, and then benzoic acid were identified. The gene clusters were found to carry two putative class I P450 monooxygenases. The two P450s with the corresponding ferredoxin and ferredoxin reductase were cloned and heterologously expressed in *E. coli*. One of them, P450 (P450tol), has a molecular weight of 49 kDa and

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catalyses the epoxidation of **1** to give (*R*)-**2** in 97.6% ee, and thus it was identified as the monooxygenase responsible for epoxidation in the TC-2 strain. P450tol shares 46% sequence identity with the P450terp from *Pseudomonas aeruginosa*.[11]

E. coli (P450tol-1) co-expressing P450tol and its ferredoxin (Fdx) and ferredoxin reductase (FdR) showed an activity of 0.52 U g^{-1} cell dry weight (CDW) for the epoxidation of 3-bromo-styrene **1**. To improve the activity, another three pairs of Fdx and FdR from different bacteria were co-expressed with P450tol (ESI,† Table S7). *E. coli* (P450tol-4) expressing P450tol and the Fdx and FdR from *Sphingomonas* sp. HXN-200 gave 6-fold higher activity (3.0 U g^{-1} CDW). The activity was further improved to 5.8 U g^{-1} CDW by engineering of *E. coli* (P450tol-GDH) co-expressing glucose dehydrogenase (GDH) for NAD(P)H regeneration (Fig. 1). The cells were grown easily to a high density (7.0–7.8 g CDW per L) in a shaking flask. For comparison, *E. coli* (P450terp-GDH) co-expressing the three-components of P450terp and GDH was constructed using the same plasmids.

Epoxidation of the selected alkenes with the resting cells of *E. coli* (P450tol-GDH) and *E. coli* (P450terp-GDH) are listed in Table 1. P450tol showed unique and excellent enantioselectivity for (*R*)-epoxidation of 2- or 3-substituted styrenes **1**, **5**, **7**, and **9** containing strong electron-withdrawing groups, affording the corresponding epoxides (*R*)-**2**, **6**, **8**, and **10** in 97.6–99.7% ee. P450tol also showed high (*R*)-enantioselectivity for the epoxidation of **3** to give (*R*)-**4** in 91.6% ee (Table 1, entries 1–5). These reactions also achieved high conversions as well as high yields of the desired epoxides. In comparison, P450terp gave lower (*R*)-enantioselectivity for the same epoxidations (entries 9–13). No enzymes were reported before for these epoxidations except for the epoxidation of **3**: SMO is (*S*)-enantioselective for the epoxidation of **3**,[5c] and the P450 BM-3 F87G mutant gave (*R*)-**4** in 94.6% ee with unknown conversion.[10]

Surprisingly, P450tol showed unique and high (*S*)-enantioselectivity for the epoxidation of 4-nitrostyrene **11** and 4-cyanostyrene **13**, affording (*S*)-epoxides **12** and **14** in 90.0% and 90.2% ee, respectively (Table 1, entries 6 and 7). The conversions and yields were also excellent. In comparison, P450terp gave (*R*)-enantioselectivity for these epoxidations with a product ee of 65.2 and 79.1%, respectively; P450pyrTM, a P450pyr triple mutant, also demonstrated (*R*)-enantioselectivity;[8e] and SMO showed no epoxidation of **13**.[5c]

P450tol also gave unique and high (*R*)-enantioselectivity for the epoxidation of the unconjugated terminal alkene **15**, producing

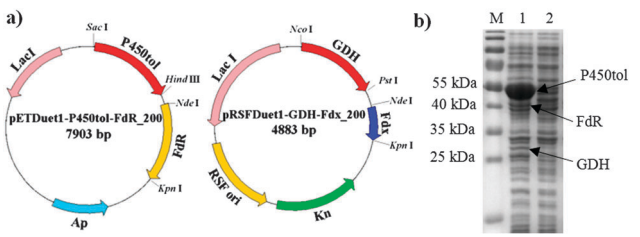


Fig. 1 (a) The dual plasmid configurations for expression of P450tol, ferredoxin reductase (FdR) and ferredoxin (Fdx) from *Sphingomonas* sp. HXN-200, and GDH in *E. coli* T7 as *E. coli* (P450tol-GDH). (b) SDS-PAGE of cell-free extracts of *E. coli* recombinants. Lane M: marker; lane 1: *E. coli* (P450tol-GDH); lane 2: *E. coli* containing dual plasmids pETDuet/pRSFDuet as a negative control.

Table 1 Epoxidation of 2-, 3-, or 4-substituted styrenes and 4-phenyl-1-butene with resting cells of *E. coli* (P450tol-GDH) or *E. coli* (P450terp-GDH)

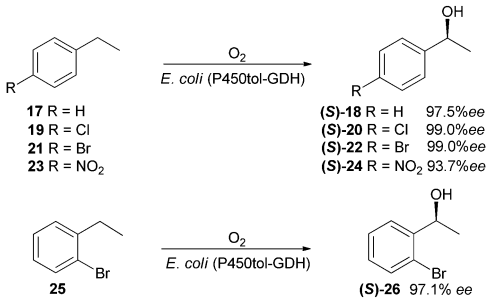
Entry	Sub.[a]	Enzyme	Prod.	Conv.[b] (%)	Yield[c] (%)	Prod. ee[d] (%)
1	**1**	P450tol	(*R*)-**2**	>99	98	97.6
2	**3**	P450tol	(*R*)-**4**	>99	98	91.6
3	**5**	P450tol	(*R*)-**6**	>99	99	99.1
4	**7**	P450tol	(*R*)-**8**	78	77	99.7
5	**9**	P450tol	(*R*)-**10**	75	74	99.1
6	**11**	P450tol	(*S*)-**12**	>99[e]	97	90.0
7	**13**	P450tol	(*S*)-**14**	>99	99	90.2
8	**15**	P450tol	(*R*)-**16**	75	74	90.5
9	**1**	P450terp	(*R*)-**2**	98	97	82.3
10	**3**	P450terp	(*R*)-**4**	66	65	79.2
11	**5**	P450terp	(*R*)-**6**	>99	42	75.2
12	**7**	P450terp	(*R*)-**8**	80	80	83.5
13	**9**	P450terp	(*R*)-**10**	>99	98	88.2
14	**11**	P450terp	(*R*)-**12**	>99[e]	98	65.2
15	**13**	P450terp	(*R*)-**14**	>99	98	79.1
16	**15**	P450terp	(*R*)-**16**	80	78	96.3

[a] Reactions were conducted with 2 mM substrate in 4 mL of cell suspension (10 g CDW per L) of *E. coli* (P450tol-GDH) or *E. coli* (P450terp-GDH) in 100 mM potassium phosphate (KP) buffer (pH 8.0) containing 1 wt% glucose at 30 °C and 250 rpm for 5 h. [b] Determined by HPLC analysis (based on the decrease of substrate). [c] Determined by HPLC analysis (based on the formation of epoxide product). [d] Determined by chiral HPLC analysis. [e] Reaction was conducted for 0.5 h.

(*R*)-**16** in 90.5% ee with high conversion and yield. Interestingly, P450terp showed a slightly higher (*R*)-enantioselectivity for the epoxidation (96.3% product ee) than P450tol. No other enzymes or chemical catalysts have been reported for this epoxidation.

Asymmetric hydroxylation is another important class of oxidation reactions. For instance, enantioselective benzylic hydroxylation could give the corresponding enantiopure benzylic alcohols that are useful and valuable building blocks for the production of fine chemicals and pharmaceuticals.[12] Some metalloporphyrins were developed for benzylic hydroxylations, but with very low product ee.[13] Many enzymes have been reported for benzylic hydroxylations,[14] with their own substrate specificity and enantioselectivity. As many P450 monooxygenases are known to catalyse both epoxidation and hydroxylation reactions, we decided to explore P450tol for several unique and highly enantioselective benzylic hydroxylations which do not proceed well in the presence of either chemical or bio-catalysts. Benzylic hydroxylations of ethylbenzene **17** and 4- or 2-substituted ethylbenzenes **19**, **21**, **23** and **25** to the corresponding (*S*)-benzyl alcohols are selected as the target reactions (Scheme 2). Enantiopure (*S*)-benzyl alcohols **18**, **20**, **22**, **24**, and **26** are useful pharmaceutical intermediates (ESI,† Table S4) and no enzymes are reported for these hydroxylations with high enantioselectivity.

P450tol showed unique and high (*S*)-enantioselectivity for the targeted hydroxylations of ethylbenzenes. As shown in Table 2, hydroxylation of ethylbenzenes **17**, **19**, **21**, and **25** with P450tol gave (*S*)-benzyl alcohols **18**, **20**, **22**, and **26** in 97.1–99.0% ee (Table 2, entries 1–3, 5). Hydroxylation of **23** gave (*S*)-**24** in 93.7% ee (Table 2, entry 4). In comparison, P450CYP154H1, P450cam and peroxygenase (AaeAPO) have all been shown to catalyse the hydroxylation of **17** with (*R*)-enantioselectivity.[15] Naphthalene dioxygenase (NDO) has demonstrated low enantioselectivity for the hydroxylation of **17**, giving (*S*)-**18** in only 77% ee.[16]



Scheme 2 Asymmetric hydroxylation of ethylbenzene and its derivatives with *E. coli* (P450tol-GDH) to prepare the corresponding enantiopure (*S*)-benzylic alcohols.

Table 2 Hydroxylation of ethylbenzene and its derivatives with resting cells of *E. coli* (P450tol-GDH)

Entry	Sub.[a]	Prod.	Time (h)	Conv.[b] (%)	Selectivity[c]	ee[d] (%)
1	**17**	(*S*)-**18**	8	90	85 : 15	97.5
2	**19**	(*S*)-**20**	5	98	95 : 5	99.0
3	**21**	(*S*)-**22**	5	97	95 : 5	99.0
4	**23**	(*S*)-**24**	5	50	95 : 5	93.7
5	**25**	(*S*)-**26**	10	25	>99 : 1	97.1

[a] Reactions were conducted with different substrate concentrations (5 mM for **19**, 3 mM for **21** and **23**, 2 mM for **23** and **25**) in 4 mL of cell suspension (10 g CDW per L) of *E. coli* (P450tol-GDH) in 100 mM KP buffer (pH 8.0) containing 1 wt% glucose at 30 °C and 250 rpm. [b] Determined by HPLC analysis. [c] Selectivity is defined as the molar ratio of the chiral alcohol product to the corresponding ketone. [d] Determined by chiral HPLC analysis.

While the chemical selectivity to (*S*)-**26** is >99%, the selectivities to (*S*)-**20**, (*S*)-**22**, (*S*)-**24** are 95% with the formation of 5% of α-ketone. 85% selectivity was observed for the hydroxylation to (*S*)-**18**. The conversions for ethylbenzene **17** and its 4-substituted derivatives **19** and **21** were much higher than that for 2-substituted ethylbenzene **25**.

The epoxidation was then investigated with resting cells of *E. coli* (P450tol-GDH) in an *n*-hexadecane–water biphasic system, to avoid substrate and product inhibition. The results are listed in Table 3. Epoxidation of **1**, **3**, and **5** reached the specific activity of 5.7–6.9 U g^{-1} CDW and produced (*R*)-**2**, **4**, **6** in 17–18 mM. The hydroxylation of **21** was performed in another system, a resin–water biphasic system, to avoid the substrate and product inhibition as well. It gave a specific activity of 8.0 U g^{-1} CDW and produced (*S*)-**22** in 21 mM.

In conclusion, P450tol monooxygenase from *Rhodococcus coprophilus* TC-2 was discovered as a unique and highly enantioselective enzyme for asymmetric epoxidation of alkenes and benzylic hydroxylations. P450tol was the first enzyme with excellent enantioselectivity and high conversion for the (*R*)-epoxidation of 2- and 3-substituted styrenes **1**, **5**, **7**, and **9**, (*S*)-epoxidation of 4-substituted styrenes **11** and **13**, and (*R*)-epoxidation of unconjugated terminal alkene **15**. The epoxidations provide with simple syntheses of the corresponding (*R*)-styrene oxides **2**, **6**, **8** and **10** in 97.5–99.7% ee, (*S*)-styrene oxides **11** and **13** in 90.0–90.2% ee, and (*R*)-epoxide **16** in 90.5% ee, respectively. P450tol shares 46% sequence identity with P450terp, but it is much more enantioselective for the epoxidations and also shows the opposite enantioselectivity for the epoxidation of

Table 3 Epoxidation of alkenes and hydroxylation of 1-bromo-4-ethylbenzene with resting cells of *E. coli* (P450tol-GDH) in an *n*-hexadecane–water biphasic system

Entry	Sub.[a]	Time (h)	Prod.	Activity[b] (U g^{-1} CDW)	Prod. Conc.[c] (mM)	ee[d] (%)
1	**1**	12	(*R*)-**2**	6.9	17	97.6
2	**3**	12	(*R*)-**4**	5.7	18	91.6
3	**5**	12	(*R*)-**6**	5.7	17	99.7
4	**7**	12	(*R*)-**8**	2.6	6.4	99.1
5	**13**	12	(*S*)-**14**	3.2	6.5	90.2
6	**21**[e]	12	(*S*)-**22**	8.0	21	99.0

[a] Reactions were conducted in a mixture of 2 mL of cell suspension (10 g CDW per L) of *E. coli* (P450tol-GDH) in 100 mM KP buffer (pH 8.0) containing 1 wt% glucose with 1 mL *n*-hexadecane containing 40 mM substrate (refers to aqueous volume) at 30 °C and 250 rpm. [b] Determined for the first 1 h. [c] Determined by HPLC analysis (refers to aqueous volume). [d] Determined by chiral HPLC analysis. [e] Reaction was conducted using 70 mM substrate in 5 mL of cell suspension (10 g CDW per L) of *E. coli* (P450tol-GDH) in 100 mM KP buffer (pH 8.0) containing 0.21 g resin XAD16 and 1 wt% glucose at 30 °C and 250 rpm.

4-substituted styrenes. P450tol was also the first enzyme with high (*S*)-enantioselectivity for the hydroxylations of ethylbenzenes **17**, **19**, **21**, **23**, and **25**, producing the corresponding (*S*)-benzylic alcohols **18**, **20**, **22**, and **26** in 97.1–99.0% ee and (*S*)-benzylic alcohols **24** in 93.7% ee. Preliminary work on the epoxidation and hydroxylation with resting cells of *E. coli* (P450tol-GDH) in an *n*-hexadecane–water or resin–water biphasic system showed the potential of using this enzyme to prepare the epoxides and benzylic alcohols that are useful pharmaceutical intermediates.

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