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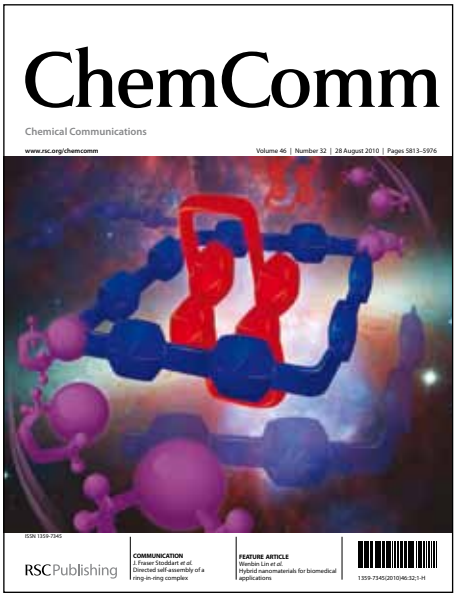

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Engineered P450pyr Monooxygenase for Asymmetric Epoxidation of Alkenes with Unique and High Enantioselectivity

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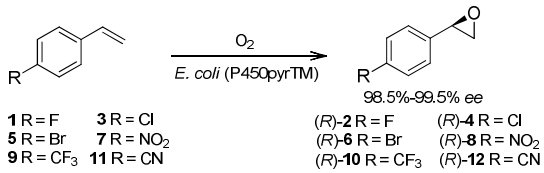
A triple mutant of P450pyr monooxygenase (P450pyrTM) catalysed the epoxidation of several *para*-substituted styrenes as the first enzyme showing high (*R*)-enantioselectivity and high conversion, demonstrated broad substrate range, and showed high enantioselectivity for the epoxidation of an unconjugated 1,1-disubstituted alkene, 2-methyl-3-phenyl-1-propene, and a cyclic alkene, *N*-phenoxycarbonyl-1, 2, 5, 6-tetrahydropyridine, respectively.

Enantiopure epoxides are important and versatile intermediates for the syntheses of pharmaceuticals, fine chemicals and biologically active compounds.[1] Asymmetric epoxidation of alkenes presents a simple access to enantiopure epoxides. Remarkable progress has been made for the asymmetric epoxidation using Sharpless and Jacobsen's methods.[2,3] Nevertheless, asymmetric epoxidation of many types of alkenes such as terminal alkenes still remains as a significant challenge. On the other hand, many enzymes have been reported for asymmetric epoxidation, providing with green and useful alternatives.[4] However, there are also limitations regarding substrate acceptance and enantioselectivity preference. For example, styrene monooxygenase (SMO)[5] and xylene monooxygenase (XMO)[6] show only (*S*)-selectivity for the epoxidation of styrene and its derivatives. Chloroperoxidase (CPO) is able to convert several aromatic and aliphatic alkenes to (*R*)-epoxides, but with low to moderate enantioselectivtiy.[7] Several native and engineered cytochrome P450 monooxygenases are known for asymmetric epoxidation,[8] but their enantio-selectivities are often not very high. F87G mutant of P450 BM-3 remains as the only good example, giving product *ee* of 92-94% (*R*) for the epoxidation of styrene and 3-chlorostyrene.[9]

We are interested in developing new enzymes for asymmetric epoxidations of terminal alkenes with high and unique enantioselectivity to prepare useful and valuable enantiopure epoxides. A group of (*R*)-*para*-substituted styrene oxides are selected as the target products (Scheme 1). (*R*)-2-(4-fluoro-phenyl)oxirane **2** is an intermediate for synthesizing nonane derivatives for the treatment of cocaine addiction.[10] (*R*)-2-(4-nitrophenyl)oxirane **8** is useful for synthesizing *β*-Adrenergic Blocker (*R*)-Nifenalol for the treatment of cardiovascular diseases.[11] (*R*)-2-(chlorophenyl)oxirane **4**, (*R*)-2-[(4-(trifluoro-methyl)] phenyloxirane **10**, and (*R*)-2-(4-cyanophenyl)oxirane **12** are the intermediates for synthesizing potent and selective human β_3 adrenergic receptor agonists.[12] (*R*)-2-(4-bromophenyl)oxirane **6** is useful for synthesizing H3 receptor agonist.[13] Asymmetric epoxidation of the corresponding *para*-substituted styrenes to produce these (*R*)-epoxides remains as a significant challenge: no enzymes are reported for the highly (*R*)-selective epoxidation; no enzymes even show activity towards the substrates **7**, **9** and **11** containing strong electron-withdrawing groups; Jacobsen catalysts encounter difficulty with these terminal alkenes and gave only 85% product *ee* for the epoxidation of 4-fluorostyrene **1**.[14] Biomimetic system such as chiral dichlororuthenium(IV) porphyrin and Iron twin-coronet porphyrins catalysed the epoxidations with low to moderate enantioselectivity, giving only 65 % and 54% *ee* of (*R*)-**4** and (*R*)-**8**, respectively.[15]



Scheme 1. Asymmetric epoxidation of *para*-substituted styrenes with *E. coli* (P450pyrTM) to produce (*R*)-*para*-substituted styrene oxides

To discover the appropriate enzyme, we started with the screening of the variants of P450pyr monooxygenase[16] generated by directed evolution[16c] for the asymmetric epoxidation of 4-fluorostyrene **1**. The triple mutant I83H/M305Q/A77S (P450pyrTM) was found to show excellent (*R*)-enantioselectivity, giving the corresponding epoxide (*R*)-**2** in 98.8% *ee*. This mutant was then used for the epoxidation of other *para*-substituted styrenes **3**, **5**, **7**, **9**, and **11** to afford the corresponding epoxides (*R*)-**4**, **6**, **8**, **10**, and **12** in 97.3-99.4% *ee* (Table 1, entries 1-6). These epoxidations gave also high conversion (82-97%) to the desired products. Although wild type P450pyr catalysed also the same reactions (entries 7-12), P450pyrTM showed much higher (*R*)-enantioselectivity and conversion, and it also inverted the enantioselectivity from (*S*) to (*R*) for the epoxidation of substrates **1** and **3**. Compared with all reported enzymes, P450pyrTM showed unique and high enantioselectivity for (*R*)-

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Table 1. Epoxidation of *para*-substituted styrenes with resting cells of *E. coli* (P450pyrTM) or *E. coli* (P450pyr) [a]

Entry	Sub.	Enzyme	Prod.	Conv. (%) [b]	*ee* (%) [c]
1	**1**	P450pyrTM	(*R*)-**2**	97	98.8
2	**3**	P450pyrTM	(*R*)-**4**	95	97.3
3	**5**	P450pyrTM	(*R*)-**6**	93	98.6
4	**7**	P450pyrTM	(*R*)-**8**	82	97.3
5	**9**	P450pyrTM	(*R*)-**10**	94	99.4
6	**11**	P450pyrTM	(*R*)-**12**	97	98.5
7	**1**	P450pyr	(*S*)-**2**	35	82.2
8	**3**	P450pyr	(*S*)-**4**	64	4.9
9	**5**	P450pyr	(*R*)-**6**	80	47.1
10	**7**	P450pyr	(*R*)-**8**	48	45.2
11	**9**	P450pyr	(*R*)-**10**	57	65.2
12	**11**	P450pyr	(*R*)-**12**	30	52.9

[a] Reactions were conducted with 2 mM substrate in 4 mL cell suspension (10 g cdw/L) of *E. coli* (P450pyrTM) or *E. coli* (P450pyr) in 100 mM KP buffer (pH 8.0) containing 1 wt% glucose at 30°C and 250 rpm for 5 h. [b] Determined by HPLC analysis. [c] Determined by chiral HPLC analysis.

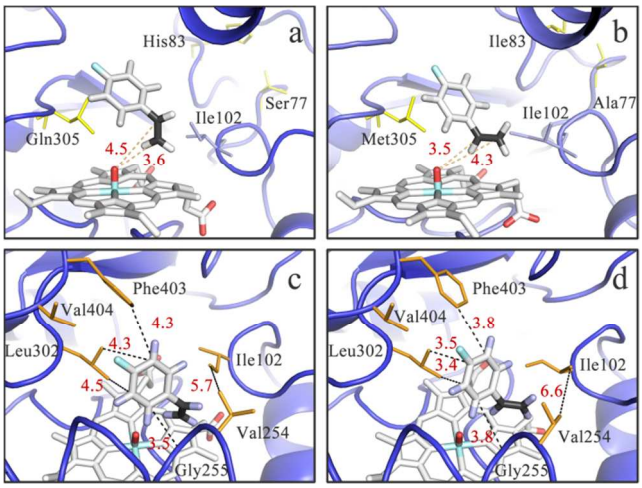
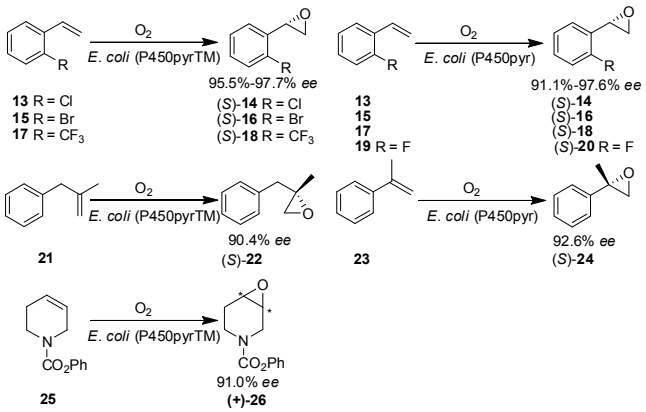


Figure 1. (a, b) Catalytically active pose of 4-fluorostyrene **1** in P450pyrTM (a) and P450pyr (b). (c, d) Hydrophobic binding pocket towards 4-fluorostyrene **1** in P450pyrTM (c) and P450pyr (d).

epoxidation of *para*-substituted styrenes. It also accepted the substrates containing strong electron-withdrawing groups. P450pyrTM is the first reported enzyme for this type of epoxidations to produce the (*R*)-epoxides in high *ee* (97.3-99.4%). In comparison, SMO catalysed the epoxidation of **1**, **3** and **5** with (*S*)-enantioselectivity, and showed no activity for the epoxidation of 4-cyanostyrene **11** containing a strong electron-withdrawing group;[5d] P450BM3 and P450terp catalysed the epoxidation of 4-chlorostyrene **3** to give (*R*)-**4** in 46% and 82% *ee*, respectively.[8a] To the best of our knowledge, the epoxidation with P450pyrTM represents also the first example of a P450 monooxygenase-catalysed epoxidation with very high product *ee*.

To understand the molecular basis of the enantioselectivity of P450pyrTM, the binding of the substrates were predicted using molecular docking method. As shown in Fig. 1a, the active pose of 4-fluorostyrene **1** towards the heme in the catalytic pocket of P450pyrTM demonstrates clearly the (*R*)-selective epoxidation. Further investigation revealed that the phenyl ring of substrate **1** fits very well into the catalytic pocket, due to the hydrophobic interaction with Leu302, Phe403, Val404, and an α-helix (Fig.1c). Meanwhile, the narrow space between Val254 and



Scheme 2. Asymmetric epoxidation of *ortho*-substituted styrenes, 1,1-disubstituted alkenes, and cyclic alkene with *E. coli* (P450pyrTM) or *E. coli* (P450pyr)

Table 2. Epoxidation of *ortho*-substituted styrenes, 1,1-disubstituted alkenes, and cyclic alkene with the resting cells of *E. coli* (P450pyrTM) or *E. coli* (P450pyr) [a]

Entry	Sub.	Enzyme	Prod.	Conv. (%) [b]	*ee* (%) [c]
1	**13**	P450pyrTM	(*S*)-**14**	51	95.5
2	**15**	P450pyrTM	(*S*)-**16**	40	97.1
3	**17**	P450pyrTM	(*S*)-**18**	7.6	97.7
4	**19**	P450pyrTM	(*R*)-**20**	38	43.3
5	**21**	P450pyrTM	(*S*)-**22**	96 [d]	90.4
6	**23**	P450pyrTM	(*S*)-**24**	40	58.1
7	**25**	P450pyrTM	(+)-**26**	>99 [e]	91.0
8	**13**	P450pyr	(*S*)-**14**	61	97.6
9	**15**	P450pyr	(*S*)-**16**	54	95.2
10	**17**	P450pyr	(*S*)-**18**	7.7	96.3
11	**19**	P450pyr	(*S*)-**20**	31	91.1
12	**21**	P450pyr	(*S*)-**22**	96 [d]	61.7
13	**23**	P450pyr	(*S*)-**24**	43	92.6
14	**25**	P450pyr	(+)-**26**	>99 [e]	65.3

[a] Reactions were conducted with 2 mM substrate in 4 mL cell suspension (10 g cdw/L) of *E. coli* (P450pyrTM) or *E. coli* (P450pyr) in 100 mM KP buffer (pH 8.0) containing 1 wt% glucose at 30°C and 250 rpm for 5 h. [b] Determined by HPLC analysis. [c] Determined by chiral HPLC analysis. [d] Reaction was conducted for 1 h. [e] Reaction was conducted with 5 mM substrate for 3 h.

Ile102 (5.7Å) directs the orientation of the terminal C-C double bond of the substrate vertical to the porphyrin plane of heme. In comparison, the active pose of **1** in P450pyr shows a different orientation of the terminal C-C double bond towards the heme (Fig. 1b), giving rise to the (*S*)-selective epoxidation. A 6.6Å of gap was predicted between Val254 and Ile102, providing a moderate space for the C-C double bond to take the position parallel to the porphine plane (Fig. 1d). Similar binding patterns for other *para*-substituted styrenes were also observed (Table S4 and Fig. S4), explaining the inversion of (*S*)- to (*R*)-selectivity for substrate **3** and the increase of (*R*)-selectivity for substrate **5** by switching the enzyme from P450pyr to P450pyrTM.

P450pyrTM was also found to catalyse the epoxidation of *ortho*-substituted styrenes with high (*S*)-enantioselectivity (Scheme 2). Epoxidation of substrates **13**, **15** and **17** gave (*S*)-epoxides **16**, **14**, and **18** in 95.5-97.7% *ee* (Table 2, entries 1-3). P450pyr showed the same excellent enantioselectivity in such oxidations, including the epoxidation of substrate **19** to give (*S*)-**20** in 91.1% *ee* (Table 2, entries 8-11). SMO is known to catalyse this type of (*S*)-epoxidations, but no other P450 enzymes were reported for these reactions with high enantioselectivity. Also no

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Table 3. Epoxidation of alkenes with the resting cells of *E. coli* (P450pyrTM) in a resin/water biphasic system [a]

Entry	Sub.	Time (h)	Prod.	Activity (U/g cdw) [b]	Conc. (mM) [c]	*ee* (%) [d]
1	**1**	12	(*R*)-**2**	18	24	98.5
2	**3**	12	(*R*)-**4**	13	18	99.1
3	**5**	12	(*R*)-**6**	15	22	99.1
4	**7**	12	(*R*)-**8**	12	18	98.7
5	**9**	12	(*R*)-**10**	5.4	11	99.5
6	**21**	12	(*S*)-**22**	11	17	90.2

[a] Reactions were conducted with 70 mM substrate in 5 mL cell suspension (10 g cdw/L) of *E. coli* (P450pyrTM) in 100 mM KP buffer (pH 8.0) containing 0.21 g resin XAD16 and 1 wt% glucose at 30°C and 250 rpm for 12 h. [b] Determined over the first 1 h. [c] Determined by HPLC analysis. [d] Determined by chiral HPLC analysis.

enzyme was reported for the epoxidation of **17**. Enantiopure (*S*)-*ortho*-substituted styrene oxides are useful intermediates as well. For example, (*S*)-**20** is useful for synthesizing a NMDA receptor antagonist for the treatment of Parkinson's disease.[17]

The epoxidation of 1,1-disubstituted unconjugated alkenes **21** with P450pyrTM gave (*S*)-**22** in 90.4% *ee* and 96% conversion (Table 2, entry 5). (*S*)-**22** is useful for the synthesis of (*R*)-mevalonolactone, a precursor of steroids, terpenoids, carotenoids, and opentanoids.[18] For this epoxidation, P450pyrTM is much better than other known enzymes such as SMO [13% *ee* (*S*), 6% yield][5c] and CPO [70% *ee* (*R*), 41% yield].[7] There is also no chemical catalysts reported for this reaction. P450pyr catalysed also the epoxidation of **21**, but with lower product *ee*. On the other hand, P450pyr showed higher enantioselectivity than P450pyrTM for the epoxidation of 1, 1-disubstituted conjugated alkene **23**, giving (*S*)-**24** in 92.6% *ee* (Table 2, entry 13).

Finally, P450pyrTM was examined for the epoxidation of cyclic alkene **25**. It gave the corresponding epoxide (+)-**26** in 91.0% *ee* with >99% conversion (Table 2, entry 7). No other enzyme or chemical catalyst was reported for this reaction. (+)-**26** can be easily transformed to (3*R*,4*R*)-3,4-dihydroxy-piperidine, an useful intermediate for synthesizing xylanase inhibitor isofagomine for the treatment of diabetes and gaucher disease.[19]

The epoxidations with resting cells of *E. coli* (P450pyrTM) was then performed in a resin/water biphasic system to avoid the substrate and product inhibition by keeping a low concentration of substrate and product in aqueous phase (for details, see ESI). The results are listed in Table 3. Better productivity was achieved than that with single aqueous system: 22 mM vs 1.9 mM of (*R*)-**6** (70 mM substrate **5**, Fig. S29 & S31). (*R*)-**2**, **4**, **6**, **8**, and **10** were all obtained in >98% *ee*, meeting the *ee* requirement for chiral intermediates in pharmaceutical manufacturing.

In conclusion, P450pyrTM was discovered as the first enzyme with excellent (*R*)-selectivity and high conversion (82-97%) for the epoxidation of several *para*-substituted styrenes. It worked well also with the substrates containing electron-withdrawing groups. These epoxidations provides with a simple access to the corresponding (*R*)-*para*-substituted styrene oxides **2**, **4**, **6**, **8**, **10**, and **12** in 98.5-99.5% *ee* that are useful pharmaceutical intermediates and cannot be prepared by using other enzymatic or chemical epoxidation systems. For the first time, a P450 monooxygenase-catalysed epoxidation with very high product *ee* was demonstrated. P450pyrTM showed also a broad substrate range, catalysing the epoxidation of unconjugated 1,1-disubstituted terminal alkene **21** and cyclic alkene **25** with high enantioselectivity as the best catalyst for these reactions. Molecular docking provides with insight into the understanding of the structural basis for the enantioselectivity of P450pyrTM and P450pyr, being useful for further engineering of this type of enzymes for other enantioselective transformations. The epoxidations with resting cells in a resin/water biphasic system enhanced the productivity. Further optimization of the biotransformations and processes is underway.

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