Catalysis Science & Technology

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: H. Lin, Y. Tang, S. Dong, R. Lang and H. Chen, *Catal. Sci. Technol.*, 2020, DOI: 10.1039/D0CY00081G.

ROYAL SOCIETY OF CHEMISTRY

rsc.li/catalysis

View Article Online
DOI: 10.1039/D0CY00081G

ARTICLE

A new monooxygenase from *Herbaspirillum huttiense* catalyzed highly enantioselective epoxidation of allylbenzenes and allylic alcohols

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Hui Lin, Yanhong Tang, Shuang Dong, Ruibo Lang and Hongge Chen *

Asymmetric epoxidation is a green route to enantiopure epoxides, but often suffers low enantioselectivity toward unconjugated terminal alkenes. Mining of the NCBI non-redundant protein sequences with a reconstructed ancestral sequence based on six styrene monooxygenases identified a monooxygenase (HhMo) from *Herbaspirillum huttiense* with 29.6-32.3% sequence identity to styrene monooxygenases, which was previously annotated as an alanine phosphoribitol ligase. HhMo catalyzed the epoxidation of allylbenzenes with moderate to excellent enantioselectivity yielding the corresponding epoxides in up to 99% ee. The HhMo-catalyzed epoxidation could also achieve the kinetic resolution of racemic secondary allylic alcohols, yielding the corresponding epoxides with up to 50% yields, as well as excellent enantio- and diastereo-selectivity (up to >99% ee and >99% de) within 20-60 min, making a greener strategy for the production of valuable enantiopure glycidol derivatives in fine chemicals and pharmaceuticals.

Introduction

Chiral epoxides are versatile intermediates for the synthesis of biologically active molecules and natural products [1-3]. The asymmetric epoxidation presents one of the most powerful strategies for the synthesis of enantiopure epoxides, since it supplies straightforward access to diverse chiral epoxides [4, 5]. Much progress has been achieved in the field of asymmetric epoxidation of alkenes, including many chemical and enzymatic protocols that have been developed during the last few decades [2, 4].

Although the chemo-catalyzed asymmetric epoxidations have been extensively developed, the asymmetric epoxidation of unconjugated terminal olefins, such as allylbenzene, usually suffers insufficient enantioselectivities [4]. The Sharpless epoxidation is the most efficient chemo-process for the epoxidation of α-hydroxyl substituted terminal olefins, which offered the chiral epoxides with a high enantiomeric excess (ee) of 90-98% [6, 7]. However, the Sharpless epoxidation of secondary allylic alcohols with phenyl substituents suffers low activity and poor enantioselectivity. For example, the kinetic resolution of 1-phenylallyl alcohol only yielded the corresponding oxide with 90% and 46% yield at -20 ºC after several days [8, 9].

Several monooxygenases have been used to develop eco-friendly enzymatic epoxidations of unconjugated terminal olefins, while they suffer either low activity or poor enantioselectivity. *Pseudomonas oleovorans* monooxygenase catalyzed the asymmetric epoxidation of allyl phenyl ethers and allyl benzyl ethers, but most of the epoxides from the process only with good enantioselectivity (70-90% ee) [10]. Styrene monooxygenase (SMO) is a two-component flavoprotein composed of a FAD-dependent monooxygenase (StyA) and NADH-dependent flavin oxidoreductase (StyB) that regenerates the reduced FAD [11]. SMOs are excellent biocatalysts for the epoxidation of many aromatic conjugated and unconjugated alkenes [4, 12, 13]. SMO from *Rhodococcus* sp. ST-5 and ST-10 catalyzed the epoxidation of allylbenzene yielding the corresponding (*S*)-epoxide with only moderate enantioselectivities of 76% and 65% ee, respectively [14]. SMO from *Rhodococcus opacus* 1CP catalyzed the epoxidation of allylbenzene giving enantiopure (*S*)-allylbenzene oxide (99% ee), but producing only 54 mg/L epoxide [15]. SMOs from marine microbes *Paraglaciecola agarilytica* NO2 and *Marinobacterium litorale* DSM 23545 were also reported to have the activity for the asymmetric epoxidation of allylbenzene, while the enantiomeric excess of the epoxide was only moderate (67% and 83% ee, respectively) [16]. Previously, we used SMO from *Pseudomonas* sp. LQ26 (StyAB2) to catalyze the epoxidation of allylbenzene and produce the corresponding epoxide with only 36% ee [17]. Moreover, a triple mutant of P450pyr monooxygenase (P450pyr I83H/M305Q/A77S) could also catalyze the epoxidation of allylbenzene but only yielded the (*R*)-epoxide with 90% ee [18]. Overall, six styrene monooxygenases have been proved to have the catalytic activity on the epoxidation of unconjugated terminal alkenes. However, they showed either low enantioselectivity (36-83% ee) or poor activity.

Typically, one of the known enzymes' sequence was used as the reference to mine potential enzymes from a database. In some cases, some of the known enzymes may have high activity, some of them may have high stability, and the others may have high enantioselectivity. So it is difficult to identify new excellent

College of Life Sciences, Henan Agricultural University, No. 63 Nongye Road, 450002, Henan, People's Republic of China. Email: honggeyz@henau.edu.cn
Electronic Supplementary Information (ESI) available. See DOI: 10.1039/x0xx00000x

enzymes from a database by using only one of the known enzymes as the reference. Here, we reconstructed an ancestral protein from the 6 known SMOs and used it as the reference to identify new enzymes for the epoxidation of unconjugated terminal alkenes with high activity and excellent enantioselectivity. We identified one monooxygenase from *Herbaspirillum huttiense*, which was previously annotated as alanine phosphoribitol ligase, and the enzyme-catalyzed the epoxidation of allylbenzene derivatives and secondary allylic alcohols with high activity and yielded the epoxides with up to >99% ee and >99% de.

Results and discussion

Ancestral protein guided enzyme mining

Since no suitable protein reference sequence was available for mining new enzymes, we reconstructed an ancestral protein from the 6 styrene monooxygenases and used it as the reference. The FASTML Server was used to reconstruct the ancestral protein (designated as AnStyA, **Fig. S1**) of the 6 styrene monooxygenases based on the maximum likelihood [19]. The AnStyA has 62-85% identities with the 6 SMOs.

To analyze the structural motifs in styrene monooxygenase, MEME was used to discover the motifs in the 6 known styrene monooxygenases [20]. Three main motifs were identified with motif 1 including site 1-69, motif 2 including site 92-180, and motif 3 including site 193-405 (**Fig. S2**). Based on the structure of styrene monooxygenase [21], it showed that motif 1 contained 2 amino acid residues in the FAD-binding site, motif 3 contained 3 residues in the FAD-binding site and the most of residues in the substrate-binding site, while motif 2 did not contain any residues either in the substrate-binding site or in the FAD-binding site.

The AnStyA-catalyzed epoxidation of allylbenzene was first investigated and it yielded the epoxide with only 44% ee (data not shown). As we expected, AnStyA catalyzed the epoxidation of unconjugated terminal alkene producing the epoxide in poor enantioselectivity because of the high identity of AnStyA to known SMOs (62-85%). We hypothesized that distantly related monooxygenases might show higher enantioselectivity and high catalytic activity. Here, AnStyA was submitted to a BLASTp search against the entire NCBI nr protein database, returning the 500 sequences with E value < 4e-53. The returned sequences had 31-85% identities with AnStyA. Since the 6 known styrene monooxygenases, having 62-85% identities with AnStyA, have either low enantioselectivity or poor activity. Those sequences having 30-50% identities with the AnStyA were considered as candidates and were subjected to structural motifs analysis to match the pattern in styrene monooxygenases. Finally, two enzymes were selected to be studied. One was a putative monooxygenase from *Amycolatopsis sacchari* (GenBank No. WP_091504755) containing motif 1 and motif 2 and with 50% identity with AnStyA (**Table S1, Fig. S3**), and the other one was a flavin-containing putative alaninephosphoribitol ligase from *Herbaspirillum huttiense* (GenBank No. WP_039783212) only containing motif 2 and with 31% identity with AnStyA (**Table S1, Fig. S4**).

Enzymatic epoxidation screening

To create a functional catalytic system, the codon-optimized genes of *A. sacchari* monooxygenase (designated as AsMO) and the putative alaninephosphoribitol ligase from *H. huttiense* (designated as HhL) were synthesized and cloned into plasmid pETB, which carried the NADH-dependent flavin oxidoreductase (PsStyB, GenBank No. ADE62391.1) to reduce the enzyme-bound FAD to $FADH_2$ [17]. The resultant plasmids were used to express AsMO or HhL including a C-terminal 6x His tag, and PsStyB.

The *E. coli* cells expressing the candidate enzyme and PsStyB were used as the catalyst for the epoxidation of allylbenzene, and the results showed that both enzymes could catalyze the epoxidation of allylbenzene **1a**. AsMo catalyzed the epoxidation of allylbenzene and yielded the corresponding epoxide with 48% yield and 50% ee, and HhL catalyzed the epoxidation of allybenzene and yielded the corresponding epoxide with 30% yield and >99% ee. It showed that AsMo containing motif 1 and motif 2 had a higher catalytic epoxidation activity but moderate enantioselectivity, while the HhL only containing motif 2 gave excellent enantioselectivity. Here, the putative alaninephosphoribitol ligase from *Herbaspirillum huttiense* (HhL) was renamed as monooxygenase (designated as HhMo) because its functions as a monooxygenase.

HhMO was further tested for the epoxidation of a secondary allylic alcohol, 1-phenylallyl alcohol, resulting in 100% conversion of (*S*)-1-phenylallyl alcohol and yielding the epoxide with >99% ee and >99% de. HhMo-catalyzed epoxidation had a very high catalytic activity, excellent enantio- and diastereo-selectivity on the secondary allylic alcohol compared to those of Sharpless epoxidation and StyAB2-catalyzed epoxidation [9, 22]. HhMo was identified as an excellent enzyme for the epoxidation of unconjugated terminal alkenes, and it was used for further analysis.

Optimization of the reaction conditions and enzyme kinetic analysis

Here, the effects of temperature, co-solvent, and pH on the HhMo-catalyzed epoxidation were carefully conducted. HhMo exhibited high catalytic activities at the temperature between 35-39 ºC and gave the highest yield of epoxide at 37 ºC (**Fig. 1A**). The bi-phase system has been proved as an efficient condition for the enzymatic epoxidation [23]. When octane, cyclohexane, toluene, or bis(2-ethylhexyl) phthalate (BEHP) was used as the co-solvent, the results showed the system containing 10% octane achieved the highest yield (**Fig. 1B**). BEHP, being the most efficient co-solvent in bioepoxidation of styrene catalyzed with other styrene monooxygenases [17, 23], resulted in a much lower yield (**Fig. 1B**). As for the optimal pH, the HhMo-catalyzed epoxidation of allylbenzene achieved the highest yield at pH 6.0 (**Fig. 1C**).

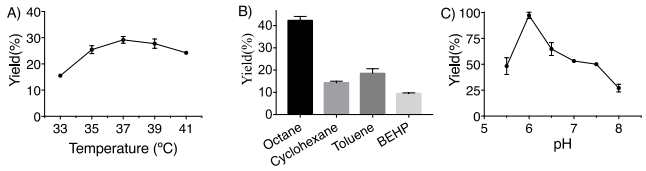


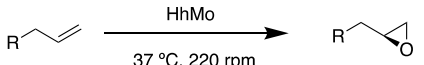
Fig. 1. The effects of temperature, co-solvent, and pH on the HhMo-catalyzed epoxidation. Whole *E. coli* cells expressing HhMo and PsStyB were used to convert allylbenzene, and the yields of the product were measured by GC. Standard deviations of triplicates are represented by error bars.

The HhMo protein was purified by Ni-resin, and the purity of the sample was confirmed by SDS-PAGE (**Fig. S5**). The steady-state kinetics of the epoxidation of 1-phenylallyl alcohol at 37 ºC and pH 6.0 were measured (**Fig. S6**). The reactions were carried out in a reconstituted system containing purified HhMo, PsStyB, catalase, and formate dehydrogenase [18]. Kinetic parameters of HhMo were investigated at varying concentrations of 1-phenylallyl alcohol. The K_m value determined by non-linear regression was 2.273 ± 0.679 μM, which was lower than that of SMOs catalyzing the epoxidation of styrene [11, 17]. The turnover number k_{cat} of the enzymatic reaction was 0.0182 ± 0.006 min^{-1}, which was significantly lower than that of SMOA from *Pseudomonas* sp. VLB120 and StyA from *Pseudomonas* sp. LQ26 catalyzing the epoxidation of styrene [11, 17]. In the activity assay system, the purified HhMo aggregated in 2-3 min, which might result in the lower k_{cat} of the purified HhMo.

HhMo-catalyzed asymmetric epoxidation of allylbenzene derivatives

Encouraged by the excellent enantioselectivity epoxidation of allylbenzene **1a**, more non-conjugated terminal alkenes were investigated for the HhMo-catalyzed epoxidation. All the tested allylbenzene derivatives could be epoxidized, while the yield and enantioselectivity of epoxides varied greatly with the substituents (**Table 1**). The HhMo catalyzed the epoxidation of allylbenzene derivatives (**2a**-8**a**) producing the corresponding epoxides with 16-92% yields, which were lower than that of allylbenzene. HhMo may have a relatively small substrate binding site, which led the enzyme to decrease the catalytic activity to the substituted allylbenzenes. The epoxidation of allylbenzene derivatives by HhMo yielded the epoxides in moderate to excellent enantioselectivity. The epoxidation of the substrates with fluoro- or methyl- on the phenyl moiety (**2a-6a**) yielded the epoxides in good to excellent enantioselectivities (71-95% ee), while the substrates harboring methyloxyl substituent on the phenyl moiety (**7a** and **8a**) decreased the enantioselectivity of the epoxidation and produced the epoxides only in 35% and 70% ee. The HhMo-catalyzed epoxidation of the substrates with *meta*-substituent on the phenyl group (**2a** and **5a**) had higher enantioselectivity and activity than those of the substrates with *ortho*- or *para*-substituent on the phenyl group (**3a**, **4a**, and **6a**-**8a**). HhMo had varied enantioselectivities of substituent allylbenzenes, which was different from that of the SMOs catalyzing the epoxidation of styrene derivatives. Normally, SMOs always achieve excellent enantioselectivities (99% ee) on different styrene derivatives [4, 12].

Table 1. HhMo catalyzed epoxidation of unconjugated terminal alkenes. [a]



Entry	Substrate	Product	yield (%)	ee (%)
1	R = C_6H_5-, **1a**	**1b**	100	99
2	R = *m*-F-C_6H_5-, **2a**	**2b**	68	91
3	R = *p*-F-C_6H_5-, **3a**	**3b**	92	87
4	R = *o*-CH_3-C_6H_5-, **4a**	**4b**	16	71
5	R = *m*-CH_3-C_6H_5-, **5a**	**5b**	67	96
6	R = *p*-CH_3-C_6H_5-, **6a**	**6b**	27	95
7	R = *o*-CH_3O-C_6H_5-, **7a**	**7b**	33	35
8	R = *p*-CH_3O-C_6H_5-, **8a**	**8b**	42	70
9	R = $C_6H_5CH_2$-, **9a**	**9b**	13	81
10	R = C_6H_5-O-, **10a**	**10b**	11	46
11	 , **11a**	- [b]		
12	 , **12a**	-		

[a] Reaction performed in 20 mL potassium phosphate buffer (pH 6.0, 100 mM), 8 mg alkene, 2.0 g wet cells, and *n*-octane (5% (v/v) at 37 °C, 220 rpm, 24 h. The ee of the epoxide was determined *via* chiral HPLC. [b] no epoxide was detected.

Moreover, 4-phenyl-1-butene (**9a**) and allyl phenyl ether (**10a**) also could be epoxidized by HhMo yielding the corresponding epoxide with 81% ee (**9b**) and 46% ee (**10b**), respectively (**Table 1**, Entries 9-10). However, the enantioselectivity of the HhMo-catalyzed epoxidation of allyl phenyl ether was lower than that of *P. oleovorans* monooxygenase-catalyzed epoxidation [9]. HhMo was further used in the epoxidation of two alkenes (**11a** and **12a**) with an electron-withdrawing group. However, no epoxide products were detected. The result showed that the electron-deficient alkenes appeared unfavorable for the HhMo-catalyzed epoxidation, which was consistent with those of other SMOs [24, 25].

HhMo-catalyzed asymmetric epoxidation of secondary allylic alcohols

First, the kinetic resolution of the racemic 1-phenylallyl alcohol **13a** by HhMo was investigated (**Fig. 2**). About 50% (*S*)-**13a** (25% conversion of total *rac*-**13a**) was converted into the (1*R*, 2*R*)-phenyl glycidol **13b** after 6 min, and then about 80% (*S*)-**13a** (40% conversion of total *rac*-**13a**) was converted after 10 min. The (*S*)-**13a** was totally epoxidized into **13b** with excellent enantioselectivity (ee >99%) and excellent diastereoselectivity (de >99%) in 20 min, leaving the (*R*)-**13a** unreacted. The (*R*)-**13a** could not be converted into epoxide by extending the reaction time, which showed the enzyme had a specific activity on the epoxidation of (*S*)-alcohol. Moreover, the (*R*)-**13a** was used as the substrate for the freshly prepared resting *E. coli* cells expressing HhMo, which resulted in no epoxide was detected by HPLC. All the results indicated that HhMo had very high stereospecificity of (*S*)-**13a** (*E* >200).

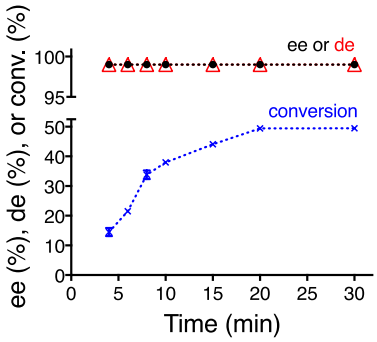
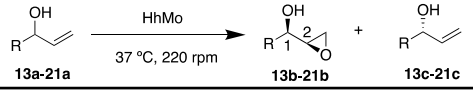


Fig. 2. Kinetic resolution of *rac*-1-phenylallyl alcohol by HhMo. The conversion (cross, ×), ee (filled circle, ●), and de (blank triangle, △) were determined by chiral HPLC. Standard deviations of triplicates are represented by error bars.

HhMo-catalyzed epoxidation gave the kinetic resolution of *rac*-**13a**, resulting in much higher activity and diastereoselectivity than those of chemo-catalytical epoxidation [8]. For example, the famous Sharpless epoxidation and the vanadium-based epoxidation needed up to 12 days for 50% conversion but yielded the epoxide with lower ee (90-93% ee) [6, 9]. The favored enantiomer of the epoxidation by HhMo is the same as that of StyAB2 from *Pseudomonas* sp. LQ26, while the activity and diastereoselectivity of the kinetic resolution of **13a** by HhMo were higher than that by StyAB2 [22], which required 180 min to finish the reaction, and yielded the epoxide with 96-98% de. The results showed that HhMo might be a strong candidate for the preparation of enantiopure glycidol derivatives More secondary allylic alcohols were investigated as the substrate for the epoxidation by HhMo (**Table 2**). In most cases, HhMo epoxidized (*S*)-allylic alcohols and yielded the corresponding (*R*)-glycidols in excellent enantio- and diastereo-selectivity (up to >99% ee, >99% de), but varied yield. Substrates **14a** and **15a** are derived from **13a** with fluoro-substituent on the phenyl moiety. For both the *mate*-fluoro- and *para*-fluoro-substituted substrates, HhMO-catalyzed epoxidation had very high activity and excellent enantioselectivity, producing the (*R*, *R*)-glycidol in 35 min and 30 min, respectively. For the substrate **16a** and **17a** containing naphthyl group, HhMo had excellent enantio- and diastereo-selectivity of the epoxidation of both 1-naphthyl and 2-naphthyl substituted substrates, giving the corresponding epoxide with >99% ee and >99% de. However, HhMo exhibited lower activity in both **16a** and **17a** than that of **13a**-**15a**, which showed the naphthyl-substituent substrates might not easy to access the substrate-binding site. Again, the results showed that HhMo might have a relatively small substrate binding site, which led the enzyme to have lower activity toward the larger substrates.

Table 2. HhMo-catalyzed epoxidation of racemic secondary allylic alcohols **13a–21a**.[a]



13a-21a 13b-21b 13c-21c

Entry	R =	*t*/min	epoxide **13b**-**21b**				
			dr[b]	ee[c] (%)	de[c] (%)	yield (%)	*E* [d]
1	Ph, **13a**	20	>99 :1	>99	>99	50	>200
2	*m*-FC_6H_4, **14a**	35	>99 :1	>99	96	50	197
3	*p*-FC_6H_4, **15a**	30	>99 :1	>99	>99	50	>200
4	1-Naphthyl, **16a**	60	>99 :1	>99	>99	8	>200
5	2-Naphthyl, **17a**	60	>99 :1	>99	>99	5	>200
6	2-Thienyl, **18a**	20	>99 :1	>99	>99	50	>200
7	3-Thienyl, **19a**	20	>99 :1	>99	>99	50	>200
8	Benzyl, **20a**	60	65:35	>99	31	12	2
9[e]	Cyclohexyl, **21a**	60	>99 :1	>99	>99	50	>200

[a] Reaction performed in 20 mL phosphate buffer (pH 6.0, 100 mM), 10 mg alkene, 2.0 g wet cells, and *n*-octane (5% (v/v) at 37 °C, 220 rpm. The absolute configuration of **13b** was established by comparing the ^{1}H-NMR spectrum and [α]^{25} with that in the literature, and the others based on the analogous chromatographic behavior of racemic mixtures. [b] Diasteroisomeric ratios (dr) determined *via* ^{1}H NMR. [c] Determined *via* chiral HPLC analysis. [d] Enantiomeric ratio E = ln[1-Yield(1 + de)]/ln[1-Yield(1 - de)]. [e] Determined by chiral GC.

The substrate range was extended to substrate **18a**-**20a** with 2-thienyl, benzyl, and cyclohexyl group, which have been proved to be the poor substrate for the StyAB2-catalyzed epoxidation [22]. StyAB2-catalyzed epoxidation of **18a** and **19a** yielded the epoxide only with 31% de and 86% de, respectively. However, HhMo catalyzed the epoxidation of **18a** and **19a** with very high activity, excellent enantio- and diastereo-selectivity, converting all (*S*)-**18a** and (*S*)-**19a** to the corresponding epoxide with 99% ee and 99% de in 20 min. But the epoxidation of benzyl-substituted substrate **20a** by HhMo resulted in low yield and fair diastereoselectivity (31% de) of the epoxide **18b**, with the *E* value of 2, which was similar to the StyAB2-catalyzed epoxidation of **20a**. Moreover, the HhMo-catalyzed epoxidation of aliphatic substrate **21a** yielded the corresponding epoxide **21b** with >99% ee and >99% de. This is the first example of monooxygenase-catalyzed epoxidation of the aliphatic substrate with excellent enantio- and diastereo-selectivity.

Conclusions

In summary, we reconstructed an ancestral protein from all the 6 known styrene monooxygenases able to catalyze the allylbenzene epoxidation and then used it as the reference for mining new enzymes from the database. We successfully identified a novel monooxygenase from *H. huttiense* (HhMo), which was previously annotated as an alanine phosphoribitol

ligase, for asymmetric epoxidation of allylbenzene derivatives with excellent enantioselectivity (up to 99% ee) and secondary allylic alcohols with excellent enantio-and diastereo-selectivity (99% ee and 99% de). HhMo-catalyzed epoxidation could give the kinetic resolution of secondary allylic alcohols, which only took 20-60 minutes to finish the reaction, yielding the glycidol derivatives with contiguous stereogenic centers in excellent enantioselectivity. The highly efficient, enantio- and diastereo-selective HhMo-catalyzed epoxidation system provides a green alternative to classic chemo-catalyzed synthesis and has been shown its great potential in chiral synthesis.

Experimental

Chemicals and general methods

Commercially available reagents were used without further purification. Allylbenzene derivatives were purchased from Sigma-Aldrich (St Louis, MO, USA) or Aladdin (Shanghai, China). ^{1}H NMR spectra were recorded on a Brucker-400 spectrometer in $CDCl_3$, all signals were expressed as ppm down field from tetramethylsilane. Optical rotations were measured with a Perkin Elmer 341 polarimeter. Protein concentrations were measured using the Bradford assay (Coomassie Brilliant Blue G-250) and bovine serum albumin as the protein standard. Protein purity was analyzed using SDS-PAGE using 4-12% polyacrylamide gradient gel (SurePAGE, Genscript, Nanjing, China) running in 50 mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7 and stained with Coomassie Blue.

Reconstruction of ancestral protein and searching motifs in styrene monooxygenases

Styrene monooxygenase from *Pseudomonas* sp. LQ26 (PsLQ26StyA, GenBank No. ADE62390), *R. opacus* 1CP (RoStyA, GenBank No. AII82583), *Rhodococcus* sp. ST-5 (RsST5StyA, GenBank No. BAL04132), *Rhodococcus* sp. ST-10 (RsST10StyA, GenBank No. BAL04129), *P. agarilytica* (PaStyA, GenBank No. WP_008305084.1), and *M. litorale* (MlStyA, GenBank No. WP_027855270.1) were downloaded from Genbank. To reconstruct the ancestral protein of the 6 known styrene monooxygenases, MAFFT online server (https://mafft.cbrc.jp/alignment/server/index.html) was used to align the sequences and create the phylogenetic tree [26], and then the ancestral protein (AnStyA, **Fig. S1**) was reconstructed in the FASTML Server (http://fastml.tau.ac.il) based on the maximum likelihood [19]. To identify the motif of the six known styrene monooxygenases, a structural motif search was conducted using MEME (http://meme-suite.org/tools/meme) (**Fig. S2**) [20].

Mining enzymes from the database

The reconstructed ancestral protein AnStyA (SI) was submitted to a BLASTp search against the entire NCBI nr protein database. The returned 500 sequences had 31-85% identities with AnStyA. The proteins had 30-50% identities with AnStyA were subjected to structural motifs analysis with the 6 known SMOs using MEME. A putative monooxygenase from *Amycolatopsis sacchari* (GenBank No. WP_091504755) containing motif 1 and motif 2 and with 50% identity with AnStyA (**Table S1, Fig. S3**), and a flavin-containing putative alaninephosphoribitol ligase from *Herbaspirillum huttiense* (GenBank No. WP_039783212) only containing motif 2 and with 31% identity with AnStyA were selected (**Table S1, Fig. S4**).

Plasmids construction

The gene sequences of those two proteins (WP_091504755.1, named as AsMo and WP_039783212.1, named as HhL) were codon-optimized for the expression in *E. coli*, and synthesized by Genewiz (Shenggong, Shanghai, China). These genes were amplified by using the primers (HhMo-F and HhMo-R, AsMo-F and AsMo-R) in **Table S2**, and the pET24a (Invitrogen, USA) was amplified by the primers (24a-F and 24a-R) in **Table S2**. The genes were inserted in the pET24a plasmid using the SLIC method [27]. The constructed plasmids (pET24a-HhL and pET24a-AsMO) were transferred into the *E. coli* DH5a competent cells. Plasmids were extracted by Miniprep kit (Vezyme, Suzhou, China), and the sequence was confirmed by sequencing (Shenggong, Shanghai, China). To insert the StyB from *Pseudomonas* sp. LQ26 into pET24a-AsMO and pET24a-HhL, the StyB sequence was amplified from pETB using primers pETB-F and pETB-R, and the pET24a-AsMO and pET24a-HhMo plasmid were amplified with primers 24AsMo-F and 24AsMo-R, and 24HhMo-F and 24HhMo-R, respectively. The resultant plasmid pET24a-HhL-StyB or pET24a-HhL-StyB was constructed using the SLIC method, and the sequence was confirmed by sequencing (Shenggong, Shanghai, China).

Protein expression and purification

The constructed plasmids (pET24a-HhMo, pET24a-AsMO-StyB, pET24a-HhL-StyB) were transferred into *E. coli* BL21 (DE3) competent cells. LB medium (10 mL) containing kanamycin (50 ug/mL) in 100 mL flask was inoculated with a single colony of *E. coli* BL21 (DE3) (pET24a-HhMo), *E. coli* BL21 (DE3) (pET24a-AsMO-StyB), or *E. coli* BL21 (DE3) (pET24a-HhL-StyB), cultured at 37 ºC and 220 rpm for overnight. The overnight cultures were used as seeds to inoculate LB medium (150 mL) containing kanamycin (50 ug/mL) in 500 mL flasks, cultured at 37 ºC and 220 rpm for about 3 h (OD_{600} = 1.0). IPTG was added into the culture to the final concentration of 0.05 mM, the cultures were incubated at 16 ºC and 220 rpm for 24 h. Cells were harvested by centrifugation (5 min, at 4700 g) at 4 ºC, the cells were washed by phosphate buffer (0.1 M, pH 7.02) twice.

The collected cells (2.5 g) were resuspended in phosphate buffer (10 mL, 0.1 M, pH 7.02) containing 20% glycerol, potassium chloride (0.5 M), DTT (0.1 mM), 1mM phenylmethyl sulfonylfluoride (PMSF), and imidazole (5 mM), and then the cells were lysed by low-temperature ultra high-pressure homogenizer JN-mini (JNBio, China) at 1000 bar and 4 ºC for 3 times. The lysis was centrifuged at 8000 rpm and 4 ºC for 30 min to remove the cell debris. The supernatant was collected, and the protein was purified by Ni^{2+}-NTA agarose resin (Qiagen, Germany), and the purity of the sample was checked by SDS-PAGE (SurePAGE, Genscript, Nanjing, China) (**Fig. S5**), pooled, concentrated and removed the salts by Amicon® Ultra-15 Centrifugal Filter Unit (10 K, Merck Millipore) and used freshly.

Kinetic parameters assay

The kinetic parameters k_{cat} and K_m of HhMo were determined by measuring the production of (*S*)-epoxide **13b** by HPLC. The reaction mixture containing 3 μM of purified HhMo, 3 μM of purified PsStyB, 0.5 U formate dehydrogenase (Sigma-Aldrich, USA), 650 U catalase (Sigma-Aldrich, USA), 150 mM sodium formate (Sigma-Aldrich, USA), 50 mM NADH (Sigma-Aldrich, USA), 75 μM FAD (Sigma-Aldrich, USA), 30% glycerol, bovine serum albumin (BSA, 10 mg/mL) (NEB, USA) and varying concentrations of 1-phenylallyl alcohol (from 100 uM in DMSO), in 100 mM potassium phosphate buffer (5 mL, pH 6.0). The mixture was incubated at 37 ºC and 220 rpm for 15 min. The mixture was extracted by ether, and then the organic phase was analyzed by Thermo Scientific TRACE 1300 gas chromatograph equipped with TraceGOLD TG-5MS column (Thermo Scientific, USA). The parameters were calculated by the Prism program (Graphpad, San Diego, CA, USA) (**Fig. S6**).

Preparation of enantiopure epoxides by asymmetric epoxidations with recombinant *E. coli* and product assay

The harvested *E. coli* whole cells (2.0 g) were resuspended in potassium phosphate buffer (20 mL, 0.1 M, pH 6.0), and were transferred into a flask (100 mL), and then *n*-octane (5% (v/v)) and alkene (10 mg) were added in the mixture. The reaction was carried out at 37 ºC for 20 min with gyratory shaking at 220 rpm. The mixture was extracted by ether (20 mL x 3). The organic phase was combined, dried by anhydrous Na_2SO_4, and then the solvents were removed under a certain vacuum. The crude products were analyzed by HPLC or GC for analyzing the conversion, or were further purified by silica gel chromatography. The conversions of the reactions were performed on a Thermo Scientific UltiMate 3000 UHPLC system equipped with diode array detector (DAD) and Kinetic C18 column (Phenomenex, USA). The Optical purities were determined by chiral HPLC using CHIRALPAK AS-H column (Daicel, Japan), or chiral GC using CHIRASIL-DEX CB column (Agilent Technologies, USA).

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We are grateful to the National Natural Science Foundation of China (31571775), Key Laboratory of Environmental and Applied Microbiology of the Chinese Academy of Sciences (KLCAS-2018-1), and Major Science and Technology Projects in Henan Province (192102110163) for financial support, Prof. Zhong-Liu Wu (Chengdu Institute of Biology, CAS) for providing the plasmid pETAB2, pETB and helpful discussions, and Prof. Romas Kazlauskas (UMN) for comments during the writing of the manuscript.

Notes and references

1. N. Wan, J. Tian, X. Zhou, H. Wang, B. Cui, W. Han and Y. Chen, *Adv. Synth. Catal.*, 2019, **361**, 4651-4655.
2. A. Fingerhut, O. V. Serdyuk and S. B. Tsogoeva, *Green Chem.*, 2015, **17**, 2042-2058.
3. H.-B. Cui, L.-Z. Xie, N.-W. Wan, Q. He, Z. Li and Y.-Z. Chen, *Green Chem.*, 2019, **21**, 4324-4328.
4. H. Lin, J.-Y. Liu, H.-B. Wang, A. A. Q. Ahmed and Z.-L. Wu, *J. Mol. Catal. B Enzym.*, 2011, **72**, 77-89.
5. S. Hwang, C. Y. Choi and E. Y. Lee, *J. Ind. Eng. Chem.*, 2010, **16**, 1-6.
6. T. Katsuki and K. B. Sharpless, *J. Am. Chem. Soc.*, 1980, **102**, 5974-5976.
7. V. S. Martin, S. S. Woodard, T. Katsuki, Y. Yamada, M. Ikeda and K. B. Sharpless, *J. Am. Chem. Soc.*, 1981, **103**, 6237-6240.
8. M. M. Heravi, T. B. Lashaki and N. Poorahmad, *Tetrahedron Asymmetry*, 2015, **26**, 405-495.
9. M. E. Jung and Y. H. Jung, *Tetrahedron Lett.*, 1989, **30**, 6637-6640.
10. H. Fu, M. Newcomb and C. H. Wong, *J. Am. Chem. Soc.*, 1991, **113**, 5878-5880.
11. K. Otto, K. Hofstetter, M. Röthlisberger, B. Witholt and A. Schmid, *J. Bacteriol.*, 2004, **186**, 5292-5302.
12. T. Heine, A. Scholtissek, S. Hofmann, R. Koch and D. Tischler, *ChemCatChem*, 2020, **12**, 199-209.
13. S. Wu, Y. Zhou and Z. Li, *Chem. Commun.*, 2019, **55**, 883-896.
14. H. Toda, R. Imae, T. Komio and N. Itoh, *Appl. Microbiol. Biotechnol.*, 2012, **96**, 407-418.
15. C. E. Paul, D. Tischler, A. Riedel, T. Heine, N. Itoh and F. Hollmann, *ACS Catal.*, 2015, **5**, 2961-2965.
16. C. Cui, C. Guo, H. Lin, Z.-Y. Ding, Y. Liu and Z.-L. Wu, *Enzyme Microb. Technol.*, 2020, **132**, 109391.
17. H. Lin, J. Qiao, Y. Liu and Z.-L. Wu, *J. Mol. Catal. B Enzym.*, 2010, **67**, 236-241.
18. A. Li, J. Liu, S. Q. Pham and Z. Li, *Chem. Commun.*, 2013, **49**, 11572-11574.
19. H. Ashkenazy, O. Penn, A. Doron-Faigenboim, O. Cohen, G. Cannarozzi, O. Zomer and T. Pupko, *Nucleic Acids Res.*, 2012, **40**, W580-W584.
20. T. L. Bailey, N. Williams, C. Misleh and W. W. Li, *Nucleic Acids Res.*, 2006, **34**, W369-W373.
21. U. E. Ukaegbu, A. Kantz, M. Beaton, G. T. Gassner and A. C. Rosenzweig, *Biochem.*, 2010, **49**, 1678-1688.
22. H. Lin, Y. Liu and Z.-L. Wu, *Chem. Commun.*, 2011, **47**, 2610-2612.
23. S. Panke, M. Held, M. G. Wubbolts, B. Witholt and A. Schmid, *Biotechnol. Bioeng.*, 2002, **80**, 33-41.
24. Y.-C. Liu and Z.-L. Wu, *Chem. Commun.*, 2016, **52**, 1158-1161.
25. S. Bernasconi, F. Orsini, G. Sello, A. Colmegna, E. Galli and G. Bestetti, *Tetrahedron Lett.*, 2000, **41**, 9157-9161.
26. K. Katoh, J. Rozewicki and K. D. Yamada, *Brief. Bioinform.*, 2019, **20**, 1160-1166.
27. M. Z. Li and S. J. Elledge, *Methods Mol. Biol.*, 2012, **852**, 51-59.

View Article Online
DOI: 10.1039/D0CY00081G

A novel monooxygenase (HhMo) from *Herbaspirillum huttiense* catalyzed the epoxidation of allylbenzenes and secondary allylic alcohols yielding the epoxides with excellent enantio- and diastereo-selectivity (up to >99% ee and >99% de). HhMo also catalyzed the epoxidation of aliphatic alkene yielding the epoxide with >99% ee and >99% de.