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Unusually Broad Substrate Profile of Self-Sufficient Cytochrome P450 Monooxygenase CYP116B4 from *Labrenzia aggregata*

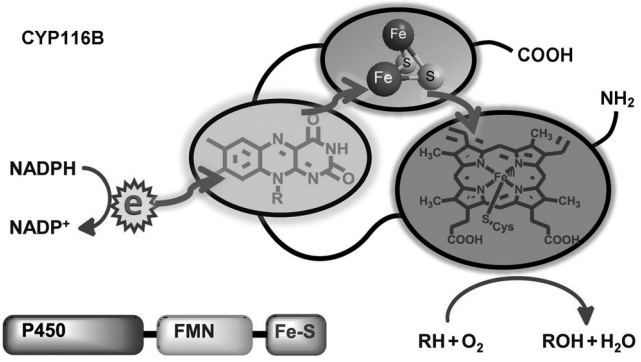
Yue-Cai Yin,[a] Hui-Lei Yu,*[a, b] Zheng-Jiao Luan,[a] Ren-Jie Li,[a] Peng-Fei Ouyang,[a] Jing Liu,[a] and Jian-He Xu[a, b]

A new member of the CYP116B subfamily—P450$_{LaMO}$—was discovered in *Labrenzia aggregata* by genomic data mining. It was successfully overexpressed in *Escherichia coli*, purified, and subsequently characterized spectroscopically, and its catalytic properties were assessed. Substrate profiling of the P450$_{LaMO}$ revealed that it was a versatile catalyst, exhibiting hydroxylation and epoxidation activities as well as *O*-dealkylation and asymmetric sulfoxidation activities. Diverse compounds, including alkylbenzenes, aromatic bicyclic molecules, and terpenoids, were shown to be hydroxylated by P450$_{LaMO}$. Such diverse catalytic activities are uncommon for the bacterial P450s, and the P450$_{LaMO}$ -mediated stereoselective hydroxylation of inactivated C–H bonds—ubiquitous and relatively unreactive in organic molecules—is particularly unusual. The self-sufficient nature of P450$_{LaMO}$, coupled with its broad substrate range, highlights it as an ideal template for directed evolution towards various applications.

Introduction

The cytochrome P450 monooxygenases (P450s or CYPs) are a superfamily of heme-containing enzymes capable of catalyzing a wide range of synthetically challenging oxidation reactions in a regio- and stereospecific manner.[1] In particular, stereoselective hydroxylation, epoxidation, and asymmetric sulfoxidation reactions make P450s very attractive in the organic synthesis and pharmaceutical industries, especially in comparison with chemical catalysts.[2] The use of many P450s in organic synthesis is, however, severely hampered by their reliance on compatible electron-transport chains, relatively low enzyme activities, poor stabilities, and low efficiencies of electron utilization.[3] One of the strategies for improving electron transfer and enhancing the coupling efficiencies of certain P450s is to use a redox-self-sufficient P450 system, in which the redox partners are fused to the P450 heme domain. This unified structural organization confers many inherent advantages, such as an improved electron turnover rate, as well as the facility to express, purify, and use a single protein rather than multiple, separate components for in vitro applications.[4]

The discovery of P450$_{BM3}$ (CYP102A1) in *Bacillus megaterium* presented the first example of single-polypeptide CYP-redox system in which the catalytic heme domain is naturally C-terminally fused to an FAD/FMN reductase (cytochrome P450 reductase; CPR), displaying nearly 100% electron coupling efficiency and a high catalytic turnover rate.[5] The high activity of this enzyme has inspired a number of research groups to construct artificial self-sufficient P450 systems through genetic fusion of the P450 monooxygenase of interest to a cognate or noncognate reductase. In 2002, a distinctive CYP-redox system, classified as the CYP116B subfamily (Scheme 1), was discovered in *Ralstonia metallidurans* CH34 (CYP116B1) and *Rhodococcus* sp. NCIMB 9784 (CYP116B2 or P450$_{RhF}$).[6] In this subfamily, the reducing equivalents are supplied by a reductase domain containing an unusual FMN- and [2Fe–2S] cluster resembling phthalate dioxygenase reductase (PDOR). Furthermore, another two members—CYP116B3 from *Rhodococcus ruber* DSM 44319



Scheme 1. Domain organization and electron flow in CYP116B enzymes.

[a] *Y.-C. Yin, Dr. H.-L. Yu, Z.-J. Luan, R.-J. Li, P.-F. Ouyang, J. Liu, Prof. J.-H. Xu*
Laboratory of Biocatalysis and Synthetic Biotechnology
State Key Laboratory of Bioreactor Engineering
East China University of Science and Technology
130 Meilong Road, Shanghai 200237 (China)
E-mail: huileiyu@ecust.edu.cn

[b] *Dr. H.-L. Yu, Prof. J.-H. Xu*
Shanghai Collaborative Innovation Center for Biomanufacturing Technology
130 Meilong Road, Shanghai 200237 (China)

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and $P450_{SMO}$ from *Rhodococcus* sp. ECU0066—have since been cloned and characterized.[7] Of these isoforms from the CYP116B subfamily, $P450_{RhF}$ shows obvious dealkylation activities, whereas $P450_{SMO}$, identified by our group, exhibits asymmetric sulfoxidation activities with excellent enantioselectivity.[8]

In contrast with those of the extensively studied $P450_{BM3}$, the structure and substrate ranges of these natural CYP-PDOR-like fusion proteins have yet to be explored in detail, and there have been relatively few studies on the evolution and application of enzymes of this class. However, the redox constitution of the CYP116B subfamily with the PDOR-like domain is similar to those of the auxiliary redox domains of most prokaryotic P450s (class I), making the evolution of CYP116B of much research interest.

Benefiting from modern rapid sequencing technology, genomic data mining of various organisms has contributed to an increasing pool of P450s of as yet unknown function, providing an effective and promising approach for the discovery of new oxidation biocatalysts.[9] Here we present the first example of discovery of a new P450-PDOR-like fusion enzyme—$P450_{LaMO}$—from *Labrenzia aggregata* IAM 12614 through genome data mining. This enzyme was identified as a member of the CYP116B subfamily, with 60% homology to $P450_{RhF}$ from *Rhodococcus* sp. NCIMB 9784 and 62% homology to $P450_{SMO}$ from *Rhodococcus* sp. ECU0066. After cloning and expression, $P450_{LaMO}$ was purified and characterized spectroscopically, and its catalytic properties were assessed. The broad substrate profile of $P450_{LaMO}$ was then systematically studied on the basis of difference kinds of oxidation reactions.

Results and Discussion

Cloning, overexpression, and screening

A genome mining approach based on sequence homology was adopted to find new P450 monooxygenases belonging to the CYP116B subfamily. Homologous sequences of two known CYP116B subfamily members—$P450_{SMO}$ (SwissProt C7SFP5) and $P450_{RhF}$ (SwissProt Q8KU27)—were used as the template sequences. Twenty putative monooxygenase candidates sharing 30–91% amino acid identities with $P450_{SMO}$ or $P450_{RhF}$ were selected from the UniProt database (Table S1 in the Supporting Information), and 14 of these were successfully overexpressed in *E. coli* (Figure S2). The indicator substrates 7-ethoxycoumarin and *p*-chlorothioanisole were used to identify monooxygenases with higher activities and enantioselectivities.[8] As shown in Table S3, $P450_{RpMO}$ from *Rhodococcus pyridinivorans* showed the highest dealkylation activity towards 7-ethoxycoumarin, whereas $P450_{LaMO}$ from *L. aggregata* IAM 12614 showed the highest activity towards *p*-chlorothioanisole, with excellent enantioselectivity ($\geq$96% *ee*). $P450_{LaMO}$ was thus chosen as the potential biocatalyst for further studies.

Sequence alignment of the CYP116B subfamily

The multiple amino acid sequence alignment of $P450_{LaMO}$ and known members of the CYP116B subfamily is shown in Figure 1. The results showed that these enzymes share almost identical amino acid motifs for the coordination of O_2, FMN, NADPH, and [2Fe–2S][6a] and display a high level of homology overall. This suggests that the selected putative monooxygenase is a member of the CYP116B subfamily and includes a ferredoxin domain, a flavin-containing reductase domain, and a P450 monooxygenase domain.

Protein purification and characterization

$P450_{LaMO}$ (gene 4932; UniProt A0P0F6) was expressed in *E. coli*, purified to homogeneity with a nickel-nitrilotriacetic acid (Ni-NTA) column, and then concentrated to 20 μM (P450 concentration was determined by CO difference spectroscopy[10]). The specific activity of the purified enzyme towards *p*-chlorothioanisole was 7.0 $U\,g^{-1}$, a 3.3-fold improvement over the crude extract. SDS-PAGE analysis clearly showed a protein band corresponding to a size of approximately 87 kDa (Figure S2).

UV–visible spectroscopic characterization of $P450_{LaMO}$ revealed the typical signature of a P450-CPR fusion protein (Figure 2). The absorption spectrum of the oxidized $P450_{LaMO}$ showed a Soret band at 420 nm, typical for heme-containing enzymes, as well as a shoulder between 450 and 500 nm, corresponding to the flavin cofactors of the reductase domain. The characteristic α and β absorption bands of the ferroheme could be seen at 533 and 564 nm, respectively. On aerobic reduction with sodium dithionite, the Soret band decreased in intensity and was slightly blue-shifted to 414 nm. Addition of CO to the dithionite-reduced $P450_{LaMO}$ resulted in a shift of the Soret peak to 450 nm, with the appearance of a much smaller peak at 422 nm resulting from the inactive fraction of the enzyme. The difference spectrum between the reduced form and the reduced CO-bound form showed the typical maximum at 450 nm. This assay was also employed to determine the concentration of functional P450 enzyme, with use of a extinction coefficient of 91 000 $M^{-1}\,cm^{-1}$.[10]

The cofactor preference of the PDOR-like reductase domain of $P450_{LaMO}$ was determined by reduction of cytochrome *c* as an electron acceptor. Both NADPH and NADH were tested as possible electron donors, and a slightly higher affinity for NADPH was observed ($K_{m,NADPH} = 9.3 \pm 0.6$ μM whereas $K_{m,NADH} = 24.9 \pm 2.9$ μM, Table 1). Similarly, kinetic studies of NAD(P)H-dependent sulfoxidation of *p*-chlorothioanisole confirmed $P450_{LaMO}$ preference for NADPH. Therefore, in all further experiments, NADPH was used as the electron donor.

Table 1. Steady-state kinetic parameters of $P450_{LaMO}$ with NAD(P)H.

Substrate	NADPH		NADH	
	K_M [μM]	k_{cat} [s^{-1}]	K_M [μM]	k_{cat} [s^{-1}]
cytochrome *c*	9.3 ± 0.6	20.6 ± 0.4	24.9 ± 2.9	12.9 ± 0.4
p-chlorothioanisole	6.9 ± 0.9	1.26 ± 0.03	26.1 ± 6.7	0.83 ± 0.06

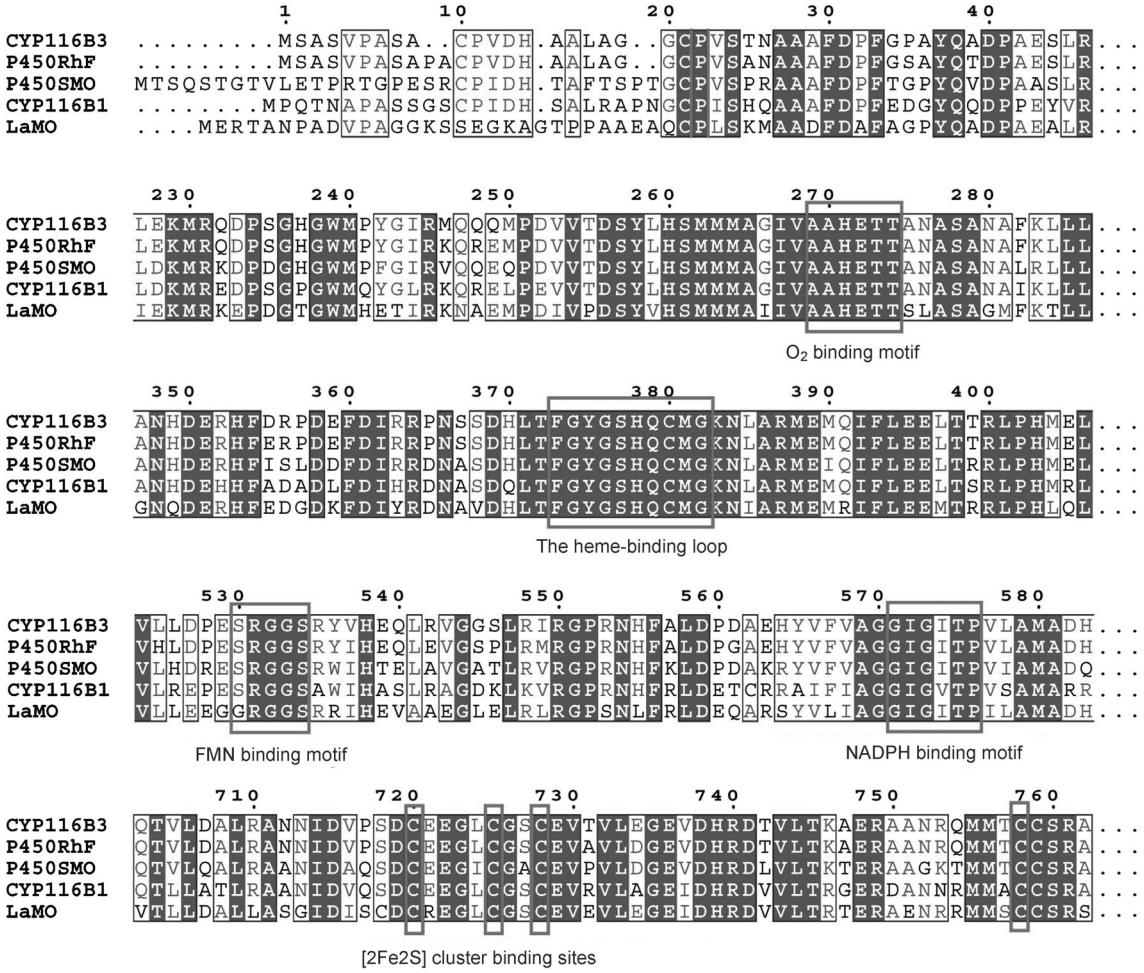


Figure 1. Sequence alignment of important catalytic domains of the P450-PDOR-like fusion enzymes of the CYP116B subfamily by use of the program ClustalX. Relevant amino acid motifs for the coordination of O_2, FMN, NADPH, and [2Fe–2S] are enclosed in dark gray boxes.

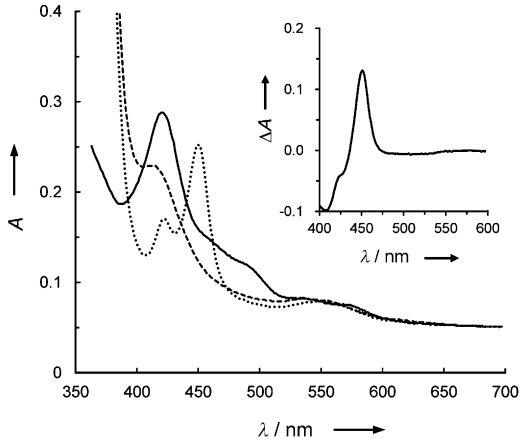


Figure 2. UV/Vis absorption spectra for purified $P450_{LaMO}$ (≈2.2 μM) showing the purified oxidized enzyme (——), the dithionite-reduced enzyme (-----), and the reduced CO-bound complex (····). The CO-bound reduced difference spectrum is shown in the inset.

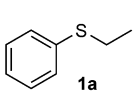
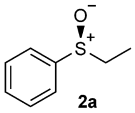
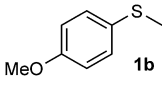
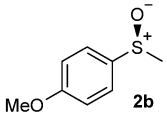
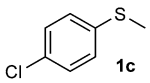
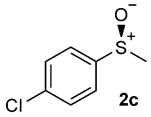
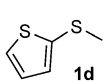
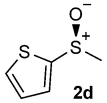
The effects of temperature and pH on $P450_{LaMO}$ were then investigated with use of a temperature range of 20–50 °C (Figure S3 A) and a pH range of 6.0–10.0 (Figure S3 B). The activity of $P450_{LaMO}$ was sensitive to buffer pH, with optimal activity at pH 8.5 in Tris·HCl buffer. The enzyme showed optimal activity at 40 °C within the temperature range tested, despite the stability being dramatically deceased at this temperature. The thermostability of the purified $P450_{LaMO}$ was further examined at temperatures of 20, 30, and 40 °C (Figure S4). The half-lives of the enzyme measured at these temperatures were 23, 15, and 2 h, respectively.

Substrate spectrum of $P450_{LaMO}$

To explore the range of substrates that can be converted by $P450_{LaMO}$, different alkanes, aromatic compounds, and terpenes were tested in different types of oxidation reactions: sulfoxidation, *O*-dealkylation, epoxidation, and hydroxylation.

Sulfoxidation: In continuation of our previous study, which revealed that $P450_{SMO}$ mediates the sulfoxidation of substituted alkyl–aryl sulfides, we initially assessed the activity of $P450_{LaMO}$ against a panel of prochiral sulfide derivatives (**1a**–**d**, Table 2), three of which had different substituents at the *para*-position. Prochiral sulfide **1c** proved to be an excellent substrate (Table 2), thus indicating that $P450_{LaMO}$ has higher activity to-

Table 2. Conversion of different sulfide substrates catalyzed by $P450_{LaMO}$.

Substrate	Product	Substrate loading [mM]	Conv. [%][a]	*ee* [%][b]
 1a	 2a	2.0	89.5	>99
 1b	 2b	2.0	81.9	70.0
 1c	 2c	2.0	98.2	96.1
 1d	 2d	1.0	58.0	90.9

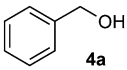
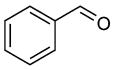
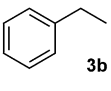
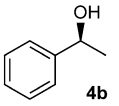
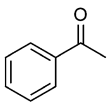
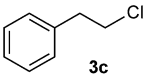
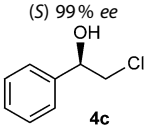
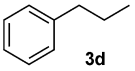
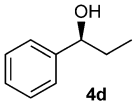
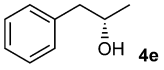
[a] Determined by GC analysis. [b] Determined by HPLC (Chiralcel OD-H column). Absolute configurations were assessed from *Rhodococcus* sp. ECU0066 data.[7b]

wards substrates with an electron-withdrawing group. For *p*-chlorothioanisole (**1c**), and ethyl phenyl sulfide (**1a**), high *ee* values (≥90%) were achieved. Although the level of conversion of *p*-methoxythioanisole (**1b**) was 81.9%, the *ee* value of the product was much lower than those of the other sulfides. Chiral sulfoxides represent an important structural motif in medicinal chemistry and in the pharmaceutical industry: structure **2b**, for example, is an important intermediate in the synthesis of calcium-channel antagonist dihydropyridine analogues,[11] so more efficient routes for its preparation are always desirable.

Dealkylation: For dealkylation, $P450_{LaMO}$ tends to accept small-molecule *p*-nitroanisole as an excellent substrate, and 100% conversion to the corresponding dealkylated product was achieved (Table 3). $P450_{LaMO}$ also showed higher de-ethylation activity than demethylation activity towards 7-(m)ethoxycoumarin. Much lower activity was displayed by $P450_{LaMO}$ in the conversion of 6-methoxyquinoline.

Hydroxylation: Regio- and stereoselective hydroxylation at nonactivated carbon atoms is a very useful reaction in organic chemistry for the functionalization of alkanes.[12] Up to now, there have been no reports on stereoselective hydroxylation catalyzed by P450s of the CYP116B subfamily. In this part of our study, $P450_{LaMO}$ was first tested for activity towards several alkyl aromatic hydrocarbons including toluene (**3a**), ethylbenzene (**3b**), (2-chloroethyl)benzene (**3c**), and propylbenzene (**3d**). The results demonstrated that the side chains of the alkylbenzenes were oxidized selectively at the α-position, providing easy access to the corresponding conjugated secondary benzyl alcohols, with high *ee* values: a maximum of 99% *ee* was obtained for (*S*)-1-phenylethanol (**4b**), 92.1% *ee* for (*R*)-2-chloro-1-phenylethanol (**4c**), 90.3% *ee* for (*S*)-1-phenylpropan-1-ol (**4d**), and 88.4% *ee* for (*S*)-1-phenylpropan-2-ol (**4e**, Table 4). Hydroxylation of toluene and ethylbenzene also yielded small amounts of α-ketones. In the conversion of propylbenzene (**3d**) by $P450_{LaMO}$, the main product was (*S*)-1-phenylpropan-1-ol (**4c**), but a small amount of (*S*)-1-phenylpropan-2-ol was also detected; the latter is a more useful intermediate for the preparation of amphetamines and amphetaminil,[13] (*R*)-selegiline,[14] and cathepsin K inhibitors.[15] Butylbenzene was also accepted as a substrate by $P450_{LaMO}$, but only trace amounts of product (conv. 10.8%) were detected and the hydroxylation occurred at different sites along the alkyl chain, except for the terminal sites (the ratio of the three products was 32.6:8.8:58.6). Because of the low degree of conversion, the enantiopurities of the three hydroxylation products have not yet been investigated.

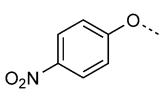
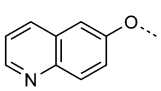
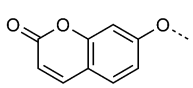
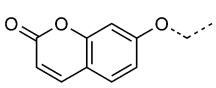
Table 4. Hydroxylation of alkyl aromatic hydrocarbons by $P450_{LaMO}$.[a]

Substrate	Product	By-product	Product ratio	Conv. [%]
 3a	 4a		88:12	24.4
 3b	 4b (*S*) 99% *ee*		82:18	39.3
 3c	 4c (*R*) 92.1% *ee*	–	–	24.6
 3d	 4d (*S*) 90.3% *ee*	 4e (*S*) 88.4% *ee*	78:22	30.9

[a] Product ratios and levels of conversion were determined by GC/MS analysis; the *ee* values were determined by chiral GC analysis (CP-Chirasil column).

Bicyclic molecules are frequently found in nature and are attracting increasing attention from organic and pharmaceutical chemists because of their broad pharmacological and biological activities. Indenols, for example,[16] are of particular value,

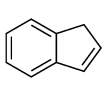
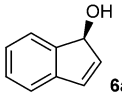
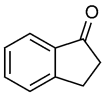
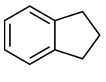
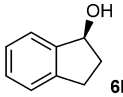
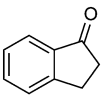
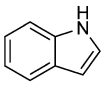
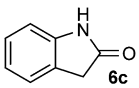
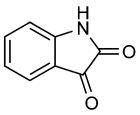
Table 3. *O*-Dealkylation catalyzed by $P450_{LaMO}$.

Substrate				
Conv.[%]	100[a]	15.4[a]	47.8[b]	75.6[b]

[a] Determined by GC analysis. [b] Determined by HPLC (C18 column).

due both to their broad spectrum of biological activity and to their wide-ranging utility as synthetic tools in the design of various bioactive molecules. Enantiomerically pure (1*S*,2*R*)-1-aminoindan-2-ol is an important intermediate in HIV protease inhibitor synthesis and possesses significant antiviral activity.[17] The bicyclic hydrocarbons indene (**5a**), indane (**5b**), and indole (**5c**) were therefore also chosen to investigate the substrate spectrum of P450$_{LaMO}$ (Table 5). Indene (**5a**) was oxidized stereospecifically to (*S*)-inden-1-ol (**6a**) with an *ee* of 33.0%. The bicyclic hydrocarbon with a saturated fused ring, indane (**5b**), was hydroxylated in a similar manner to yield (*S*)-indan-1-ol (90% of the total product) with 75.3% *ee*. P450$_{LaMO}$ also converted indole (**5c**), forming a mixture of 1,3-dihydroindol-2-one and 1*H*-indole-2,3-dione (with a ratio of 1:3).

Table 5. Hydroxylation of bicyclic hydrocarbons by P450$_{LaMO}$.[a]

Substrate	Product	By-product	Product ratio	*ee* [%]	Conv. [%]
 5a	 6a		70:30	33.0	71.4
 5b	 6b		90:10	75.3	57.2
 5c	 6c		25:75	–	17.4

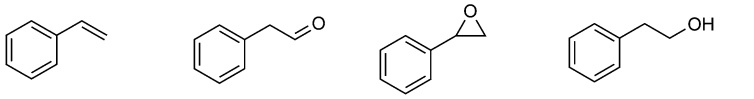
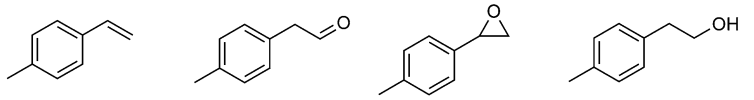
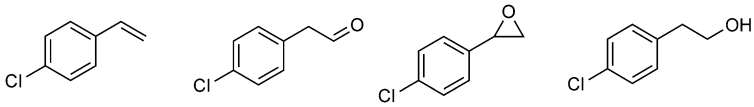
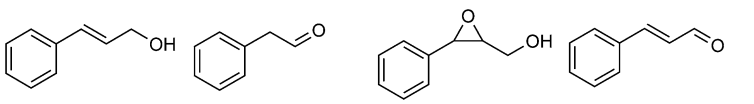
[a] Product ratios and levels of conversion were determined by GC/MS analysis; the *ee* values were determined by chiral HPLC analysis (OD-H column).

To investigate selectivity towards terpenoid substrates, we tested α-ionone, β-ionone, α-pinene, β-pinene, limonene, and linalool. P450$_{LaMO}$ was shown to oxidize the sesquiterpenoid analogues α-ionone, β-ionone, and linalool, but no activity was observed with α-pinene, β-pinene and limonene. In the conversion of β-ionone, the high regioselectivity of P450$_{LaMO}$ was particularly remarkable, and the product was observed to be 4-hydroxy-β-ionone, an important intermediate in the synthesis of carotenoids and the plant hormone abscisic acid.[18] In contrast, P450$_{LaMO}$ was also found to convert α-ionone, but gave rise to multiple oxidized products as revealed by GC/MS. It is interesting to note that linalool could also be converted by P450$_{LaMO}$, forming the furanoid linalool oxide, an important constituent of oolong, black, and green tea and also found in many essential oils and fruit aromas (e.g., in papaya).

Epoxidation: Styrene oxide and its derivatives are versatile intermediates in the synthesis of valuable chemicals such as antidiabetic agents and dopamine agonists.[19] Interestingly, when investigating the activity of P450$_{LaMO}$ towards olefin compounds, we found that P450$_{LaMO}$ could catalyze the addition of water to nonactivated alkenes, another type of hydroxy-functionalization of nonactivated C–H bonds.[20] All four of the substrates tested were partially converted into their corresponding epoxides and aldehydes (Table 6). The main products obtained from 4-chlorostyrene and 4-methylstyrene were not styrene oxides but alcohols, whereas the main product obtained from cinnamyl alcohol was cinnamaldehyde. The aldehyde products probably originated from the initial formation of the intermediate epoxides. A possible mechanism for this reaction was proposed in a previous study.[21]

P450$_{LaMO}$ is a catalytically self-sufficient P450 enzyme that only requires the NAD(P)H cofactor for substrate turnover. In the presence of NADPH, P450$_{LaMO}$ catalyzes the *O*-dealkylation, asymmetric sulfoxidation, and epoxidation of a range of substituted aromatics. More importantly, it can also catalyze the hydroxylation of C–H bonds (which are ubiquitous and rather unreactive in organic molecules) with considerable regioselectivity and enantioselectivity. In the case of ethylbenzene, CYP116B3 exhibited hydroxylation activity yielding two products (1-phenylethanol and 2-phenylethanol), but no regioselectivity or product *ee* values were reported.[7a] Wild-type P450$_{BM3}$ hydroxylated propylbenzene mainly to (*R*)-1-phenylpropan-1-ol with 90% *ee* and 99% regioselectivity,[22] whereas the engineered P450pyrSM2 exhibited the best result for β-hydroxylation of propylbenzene with 95% *ee* (*S*), and 98% regioselectivity.[23] Recently, another member of the CYP116B subfamily,

Table 6. Olefin epoxidation mediated by P450$_{LaMO}$.[a]

Substrate	Products	Product ratio	Conv. [%]
		19:47:34	59.1
		11:35:54	40.2
		19:8:73	77.0
		9:38:53	68.3

[a] Product ratios and levels of conversion were determined by GC/MS analysis. The *ee* values were not determined.

$P450_{RhF}$ was also reported to display a high degree of substrate promiscuity.[24] Nevertheless, no highly regio- and enantioselective hydroxylation of alkanes by $P450_{RhF}$ has been reported so far.

Enzymes with more promiscuous substrate selectivity are more likely to prove useful as templates for directed protein evolution than less promiscuous enzymes with greater activity, as long as the desired activities are already present.[25] The potential for diverse applications of the P450s has prompted extensive protein engineering research directed towards enhancing their activity or coupling efficiencies and improving their selectivities. The best example of this is $P450_{BM3}$; numerous variants for the conversion of a diverse range of substrates have been produced by random mutagenesis or by rational design based on the crystal structure of this enzyme.[26] Although no crystal structures for the P450-PDOR-like fusion enzymes of the CYP116B subfamily are yet available, the self-sufficient nature of $P450_{LaMO}$ coupled with its newly established broad substrate promiscuity highlight it as an ideal template for directed evolution. Our future work will therefore focus on the crystallization and evolution of $P450_{LaMO}$ to provide a more robust biocatalyst with higher activity and selectivity.

Conclusion

We have presented the discovery of a new self-sufficient P450 by genome mining with the purpose of finding a monooxygenase with new substrate specificities. This strategy yielded a versatile monooxygenase, $P450_{LaMO}$, which exhibits an unusually broad substrate range for different oxidation reactions including hydroxylation, alkene epoxidation, *O*-dealkylation, and sulfoxidation. $P450_{LaMO}$ thus possesses great potential as a biocatalyst for further directed evolution and diverse applications.

Experimental Section

General: δ-Aminolevulinic acid and sodium dithionite were purchased from Aladdin Chemicals Co., Ltd. (Shanghai, China). Cytochrome *c* was purchased from Sigma–Aldrich. 7-Ethoxycoumarin and 7-hydroxycoumarin were obtained from TCI (Shanghai, China). All other chemicals used are widely commercially available and were of analytical grade purity or higher.

Kits for plasmid extraction and DNA purification were purchased from Qiagen (Shanghai, China). Restriction endonucleases and plasmid pMD18-T for the cloning of PCR products were obtained from Takara (Dalian, China). Plasmid pET-28a(+) for heterogeneous expression was obtained from Novagen (Shanghai, China). The microbial strains used for genome mining were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), the National Collections of Industrial, Food and Marine Bacteria (NCIMB Ltd; Aberdeen, UK), or from our own collection. *E. coli* DH5α and *E. coli* BL21(DE3) used for cloning and expression of P450 monooxygenases were from our own laboratory stocks.

Genome data mining for CYP116B monooxygenases: A library of putative monooxygenases was constructed by genome mining. A total of 20 monooxygenases, each with 33–97% amino acid sequence homology to the templates $P450_{SMO}$ and $P450_{RhF}$, were selected from the UniProt/Swiss-Prot database. After heterogenous overexpression in *E. coli* BL21(DE3), enzyme activity and enantioselectivity towards *p*-chlorothioanisole were determined (as listed in Table S1).

Cloning, expression, and purification: For the expression and purification of the P450s, *E. coli* BL21(DE3) cells harboring plasmid pET28a-His-P450 (with a His_6-tag at the N terminus) were used. The primers used for each gene target encoding a P450 are listed in Table S2. The resulting pET28-P450 plasmids were transformed into *E. coli* BL21(DE3) cells. The cells were grown in Terrific Broth medium [500 mL, containing kanamycin (50 $\mu g\,mL^{-1}$)] at 37 °C to an optical density of 0.8 at 600 nm. IPTG (0.2 mM) and δ-aminolevulinic acid (δ-ALA, 0.5 mM) were then added to induce protein expression, and the culture was incubated for another 24 h at 16 °C. The cells were harvested by centrifugation at 5000 *g* for 20 min, washed twice with saline, resuspended in buffer A [sodium phosphate buffer (pH 8.0, 50 mM) containing glycerol (10%, *v/v*), NaCl (500 mM), imidazole (10 mM)] and lysed on ice with an ultrasonic oscillator (JY92-II, Scientz Biotech. Co., Ltd.). After centrifugation (8000 *g* for 20 min), the cell lysate supernatant was filtered and loaded onto a His trap Ni-NTA FF column (5 mL, GE Healthcare), pre-equilibrated with buffer A. Proteins were eluted with an increasing gradient of imidazole (10 to 250 mM) in buffer A at a flow rate of 1 $mL\,min^{-1}$. Fraction purities were determined by SDS-PAGE, and all fractions containing target protein were collected and dialyzed against sodium phosphate buffer (pH 8.0, 50 mM) for desalting. The concentration of P450 protein was determined by CO difference spectroscopy as described elsewhere.[10] Finally, the sample was concentrated and stored at −80 °C in glycerol (20%) until further use.

Spectral characterization, activity detection, and kinetics assays: UV absorption spectra (400–500 nm) of the CO-bound recombinant CYP proteins were measured after sodium dithionite reduction. P450 monooxygenase concentrations were estimated from difference spectra between the reduced, CO-bound form and the reduced form, as described by Omura and Sato.[10] P450 monooxygenase concentrations were then determined by use of an extinction coefficient of 91 $mM^{-1}cm^{-1}$ at 450 nm.

NADPH oxidation activities and kinetics assays were recorded at 30 °C with a Beckman DU 730 spectrophotometer (Beckman). Activities were determined by monitoring the decreases in absorbance of NADPH at 340 nm ($\varepsilon_{NADPH} = 6.22\ mM^{-1}cm^{-1}$) at 30 °C. The standard assay mixture (1 mL) consisted of *p*-chlorothioanisole (0.2 mM) and $P450_{LaMO}$ (0.1–0.2 μM) in Tris·HCl buffer (pH 8.5, 50 mM). The reaction was initiated by the addition of NADPH (0.1 mM). One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADPH per minute. For the reference sample, NADPH consumption rates were measured in the absence of substrate.

Reductase activities were determined by a standard cytochrome *c* assay. Each reaction was performed in Tris·HCl buffer (pH 8.5, 50 mM) containing $P450_{LaMO}$ (2 nM) and cytochrome *c* (0.1–0.4 mM). The reaction was initiated by addition of NADPH (0.1 mM). Kinetic constants, K_M and k_{cat}, were calculated by fitting the reaction rates measured across a range of substrate concentrations to the Michaelis–Menten kinetic model.

Biotransformation mediated by $P450_{LaMO}$: Reactions were performed with NADPH cofactor regeneration by glucose dehydrogenase (GDH) from *Bacillus subtilis*. The reaction mixtures (1 mL) each contained substrate (500 μM, 1.0–2.0 mM for sulfide substrates), $NADP^+$ (200 μM), glucose (10 mM), and GDH (5 U) in Tris·HCl buffer (pH 8.5, 100 mM). Each reaction was initiated by the

addition of pure enzyme (3.0 μM) with subsequent shaking for 5 h at 800 rpm and 30 °C. The reaction was quenched by addition of ethyl acetate (750 μL) to the reaction plate, followed by vigorous vortexing. The mixture was centrifuged at 10000 *g* for 10 min, after which the upper layer containing any remaining substrate and products (i.e., the organic layer) was extracted. The sample was subsequently dried and applied to GC/MS for structural and quantitative analyses.

Product identification and chiral analysis: Product analysis was carried out by GC/MS (Shimadzu GCMS-QP2010, FS-Supreme-5, column length 30 m, internal diameter 0.25 mm, film thickness 0.25 μm, detection limits ppm) with helium as the carrier gas. Mass spectra were collected after 4.5 min (solvent cut time) by electron ionization. The column oven was programmed as follows: 50/60 °C for 5 min, rising by 10 °C min^{-1} to 250/280 °C. One microliter of the biotransformation extract was injected. The products were identified by comparing the experimentally observed MS fragment ion peaks with standard MS (*m/z*, relative intensity) extracted from the mass spectrum database by similarity searching, or by comparison with the GC retention times of authenticated reference samples. Product distributions and levels of conversion were calculated from the peak area ratios.

To determine the levels of conversion in sulfoxidation reactions, achiral GC analysis was conducted with a Rxi-5Sil column (30 m × 0.25 mm × 0.25 μm). The temperature program was 120 °C for 5 min, ramp at 10 °C min^{-1} to 200 °C, hold at 200 °C for 1 min. Reversed-phase HPLC (C18 250 × 4.6 mm, 5 μm) with UV detection at 320 nm was used for analyzing the dealkylation of 7-methoxy- and 7-ethoxycoumarines, with methanol/water (65:35, *v/v*) as the mobile phase (flow rate, 1.0 mL min^{-1}).

The absolute configurations and enantiomeric excesses (*ee*) of the resulting chiral oxidates were identified by comparing their retention times by chiral GC/HPLC with those of authenticated reference samples or with literature data. Chiral GC analysis was performed with a Shimadzu GC-17A gas chromatograph equipped with a flame ionization detector and a split injection system and a capillary column DB-17 (30 m × 0.554 mm i.d.; J&W Scientific, Folsom, CA, USA). The temperature programs for the various chemicals were:

1-phenylethanol: 100 °C for 5 min, 100–160 °C at 10 °C min^{-1}, 160 °C for 5 min,

1-phenylpropan-1-ol and 1-phenylpropan-2-ol: 110 °C for 15 min, 110–120 °C at 10 °C min^{-1}, 120 °C for 10 min, and

2-chloro-1-phenylethanol: 140 °C for 15 °C min.

The enantiomeric excesses of the inden-1-ol, indan-1-ol, and sulfoxide products were determined by HPLC (LC-10AT; Shimadzu, Japan) with a chiral column (Chiralcel OD-H, 250 × 4.6 mm, Daicel Co., Japan), and elution with hexane/isopropanol (95:5, *v/v*, 1.0 mL min^{-1}) and detection at 254 nm. Authenticated standards for each enantiomer of each sulfoxide were prepared by previously established methods.[7b,8a]

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Keywords: cytochromes · data mining · hydroxylation · oxidoreductases · protein engineering · substrate promiscuity

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Y.-C. Yin, H.-L. Yu, Z.-J. Luan, R.-J. Li, P.-F. Ouyang, J. Liu, J.-H. Xu*

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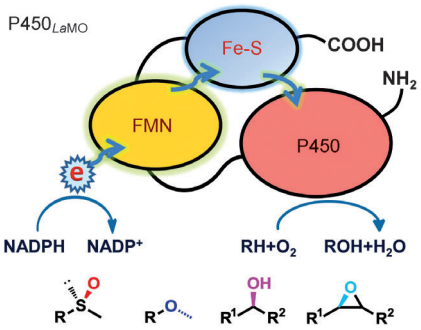
Unusually Broad Substrate Profile of Self-Sufficient Cytochrome P450 Monooxygenase CYP116B4 from *Labrenzia aggregata*



Catalytic versatility of P450$_{LaMO}$: A new redox-self-sufficient P450 of the CYP116B subfamily was discovered by data mining. A substrate spectrum study showed that it was a catalytic versatile monooxygenase, mediating oxidation reactions as diverse as hydroxylation, alkene epoxidation, *O*-dealkylation, sulfoxidation, and even hydration.