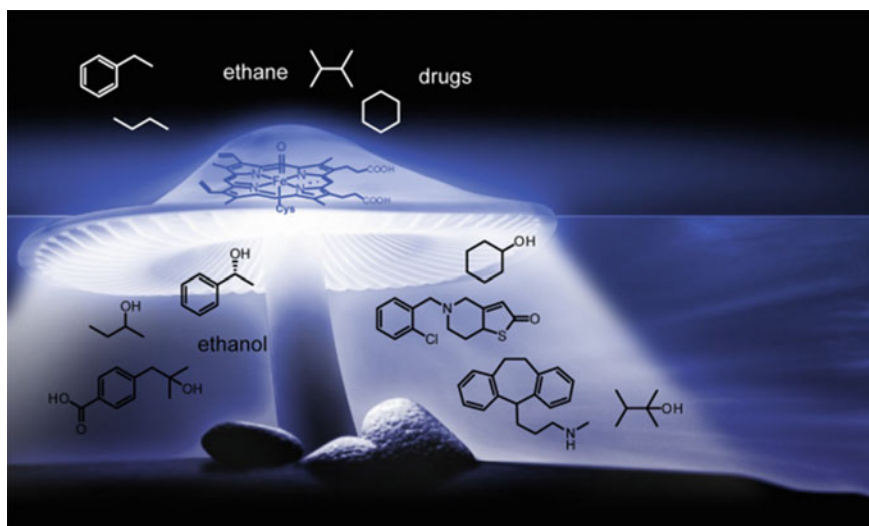


Chapter 2

Efficient and Selective Alkane Hydroxylation Reactions Catalyzed by the Fungal Peroxygenase *AaeAPO*



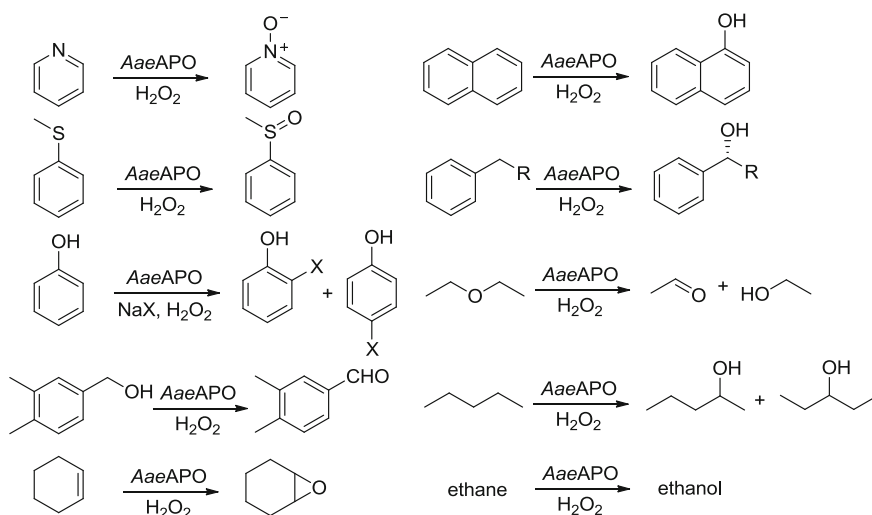
Abstract In this chapter, we report *AaeAPO* catalyzed alkane hydroxylations with H_2O_2 as the sole oxidant. High selectivity for alcohols, high efficiency of H_2O_2 utilization, high regioselectivity and stereospecificity were observed. The scope of the alkane substrates includes linear, branched and cyclic hydrocarbons, further expanding to gaseous ethane, propane and neopentane. Metabolites of several drug molecules were also analyzed and compared with P450s metabolites. Combining with coenzymes or cofactors, *AaeAPO* can also utilize the environmentally desirable oxidant O_2 to perform C–H oxidation. All these various reactions suggest that *AaeAPO* has potential practical application as an industrial biocatalyst.

2.1 Introduction

The fungal peroxygenase *Aae*APO has become a promising biocatalyst in biotechnological applications. It has a proximal cysteine ligating to the heme iron, the same as chloroperoxidase (CPO) and cytochrome P450s. It also fills the gap between peroxidases and monooxygenases, catalyzing various kinds of reactions [1], such as nitrogen oxidation, sulfoxidation, halogenations [2], olefin epoxidation, ether cleavage [3], aromatic [4], benzylic [5] and alkyl [6] hydroxylation by using peroxide as oxidants (Scheme 2.1). More importantly, *Aae*APO catalyzes reactions with high efficiency and selectivity, making them have more potential industrial applications such as producing pharmaceutical drugs, use as antimicrobial agents or detergents and so on.

Since *Aae*APO is a highly glycosylated, extracellular enzyme, it has high stability with wide tolerant temperature range and even tolerates in organic solvents [6]. For example, its stability has been tested in different nonpolar organic solvents over time. Data showed that the peroxygenase maintained at least 50 % of its activity after 30 min of incubation in all solvents tested and almost no loss of activity after 2 h of incubation in acetone/water mixture (60 % v/v) (Table 2.1). There are numerous advantages to use an enzyme in organic or organic-water mixed solvents system [7, 8]. For example, the possibility of carrying out new reactions that are impossible in water because of kinetic, thermodynamic or solubility restrictions. It might also prevent side reactions caused by the high concentration of water molecules. So, this high stability of *Aae*APO in organic solvents broadens its technological utility.

The C–H bonds strength of alkanes are quite strong, for example 99 kcal/mol for C–H bonds in cyclohexane. Sometimes, alkanes are even ideal solvents for very



Scheme 2.1 Different types of reactions catalyzed by *Aae*APO

Table 2.1 Stability of peroxxygenase *AaeAPO* in different organic solvents

Solvent	Relative enzyme activity (%)		
	30 min	60 min	120 min
Acetone ^a	98.5 ± 5.6	101.4 ± 2.8	108.5 ± 1.4
HMN ^b	102.7 ± 9.2	106.7 ± 13.1	99.9 ± 9.4
<i>n</i> -Hexane	96.4 ± 2.8	101.4 ± 4.7	99.6 ± 13.5
Tetra ^b	87.6 ± 0.3	54.4 ± 0.5	65.5 ± 0.1
Tridecane	105.7 ± 23.6	86.1 ± 11.8	55.3 ± 18.5
2,3-DMB	85.8 ± 13.6	76.6 ± 5.1	32.8 ± 17.7
DCM ^b	51.9 ± 0.2	43.0 ± 0.2	–

Experiments were performed by Dr. Sebastian Peter and data were from Ref. [6]

^a(60 %); *HMN* Heptamethylnonane, *DMB* Dimethylbutane, *DCM* Dichloromethane

^bAlthough the enzyme was still active after aqueous extraction, the conversion of *n*-hexane in this solvent was not efficient

reactive oxidation catalysts. So they are difficult to activate. The functionalization of inert C–H bonds has been a challenge in the field of organic chemistry for a long time. Biomimetic transition-metal complexes are designed as catalysts to hydroxylate alkanes, but these complexes support very few total turnovers. Nature has design several biological systems to perform this function. For example, methane monooxygenase (MMO) from *Methylococcus capsulatus* (Bath) can hydroxylate C–H bonds of methane as well as other alkanes to yield alcohols [9]. Alkane hydroxylase (AlkB) from *Pseudomonas putida* GPo1 hydroxylates propane and butane and supports cell growth [10, 11]. AlkB selectively oxidizes at the terminal carbon of *n*-alkanes to produce the 1-alkanols. Cytochrome P450s are also capable of hydroxylating alkanes, but most of the time the selectivity is not obvious. Most of the biological systems require cofactors, such as NADPH and partner enzymes, such as reductase, to transfer electrons. So, the discovery of a simple and high efficiency biocatalyst is quite desirable for the potential industrial applications.

2.2 Results and Discussion

2.2.1 Hydroxylation of Alkanes with High Efficiency and Selectivity

Peroxygenase *AaeAPO* was found to efficiently hydroxylate a variety of alkanes, including linear, branched and cyclic saturated hydrocarbons using H₂O₂ as the terminal oxidant [6] (Scheme 2.2). The alkane hydroxylation reaction catalyzed by *AaeAPO* showed both regioselectivity and stereoselectivity. For example, *AaeAPO* prefers tertiary and secondary carbons but not primary carbons. Most linear alkanes (C₂–C₁₆) hydroxylations happened at the 2-position and 3-position. Experiments with *n*-heptane and *n*-octane showed that the hydroxylation proceeded with

Scheme 2.2 Alkane hydroxylation catalyzed by *AaeAPO*

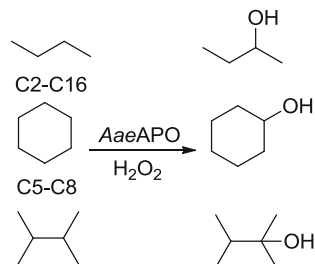


Table 2.2 Product distributions (% total alcohols) and % *ee* of selected products

Substrate	2-alcohol		3-alcohol		Ketones (%) ^b
	(%) ^a	<i>ee</i> %	(%) ^a	<i>ee</i> (%)	
Propane	100				Trace
<i>n</i> -Butane	100	(<i>S</i>) 30.8 ± 4.7			Trace
<i>n</i> -Pentane	34.1 ± 0.1	(<i>S</i>) 36.3 ± 4.7	65.9 ± 0.1		0.13 ± 0.18
<i>n</i> -Hexane	52.6 ± 0.7	(<i>R</i>) 62.5 ± 2.5	47.4 ± 0.7	(<i>R</i>) 79.5 ± 5.1	1.3 ± 0.1
<i>n</i> -Heptane	60.5 ± 0.1	(<i>R</i>) 62.2 ± 2.9	39.5 ± 0.1	(<i>R</i>) 99.9 ± 0.1	3.0 ± 0.2
<i>n</i> -Octane	55.0 ± 0.3	(<i>R</i>) 50.6 ± 1.5	45.0 ± 0.3	(<i>R</i>) 99.9 ± 0.1	1.1 ± 0.1

Experiments were performed by Dr. Sebastian Peter and data were from Ref. [6]

^aProduct distribution determined as ratio of a specific alcohol product in relation to the total amount of all alcohol products

^bProduct distribution for ketones was similar to that of alcohol product distribution. The numbers reported here are the total of all ketones (%) relative to total products (alcohols and ketones)

complete stereoselectivity for the (*R*)-enantiomer of the corresponding 3-alcohol. However, with short linear alkanes, such as *n*-butane and *n*-pentane, the major alcohol products were (*S*)-enantiomers (Table 2.2). Branched alkanes were oxidized regioselectively as well and cyclic alkanes (C₅–C₈) also yielded monohydroxylated products (Fig. 2.1). For example, 2,3-dimethylbutane was oxidized to 2,3-dimethylbutan-2-ol with 2,2,3,3-tetramethyloxirane as a minor product. Cyclohexane hydroxylation resulted cyclohexanol with only trace amounts of cyclohexanone as an over-oxidized product. However, multihydroxylated reaction products, such as diols, were not detected.

The high stereoselectivity of *AaeAPO* catalyzed linear alkane hydroxylation reactions are worthy of further discussion. It is interesting to compare the values obtained with those of engineered P450s [12], which were optimized for the selective oxidation of alkanes. In general, the *ee*-values of the (*R*)-enantiomers and (*S*)-enantiomers obtained are somewhat higher than those of engineered P450s; in particular, this applies to the *ee*-value of more than 99 % for the 3-alcohols obtained after the reaction of peroxxygenase with *n*-heptane or *n*-octane.

Our results demonstrate that *AaeAPO* catalyzes the hydroxylation of diverse alkanes. This type of reaction suggests that *AaeAPO* may have a role in the

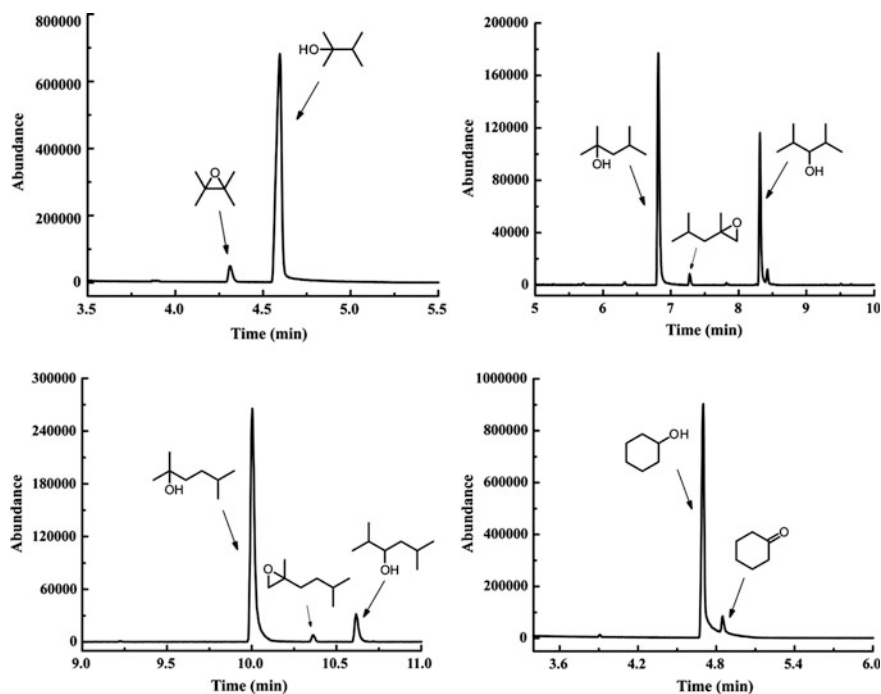
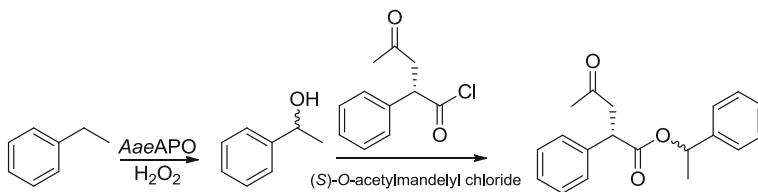


Fig. 2.1 GC/MS total ion chromatogram of alkane hydroxylation product mixtures catalyzed by AaeAPO

biodegradation of lignin or cutin fragments, or in the detoxification of aliphatic compounds that show fungicidal activity, such as terpenes and oxylipins in soils, plant litter, and wood [13].

2.2.2 Hydroxylation at Benzyl Position with a High Degree of Stereoselectivity

Enantiomeric analysis of the *R* and *S* stereoisomers of 1-phenylethanol was done by GC of the diastereomeric esters formed by the reaction of enzymatic mixture with (*S*)-*O*-acetylmandelyl chloride [14] (Scheme 2.3). Our result showed that the benzylic hydroxylation results in (*R*)-1-phenylethanol as the major product (Fig. 2.2). The highest *ee* could be 99 % [15]. The ability of producing pure *R*-isomer at the benzylic position is of high importance and has potential industrial application because they are important building blocks used in organic synthesis. The yield of (*R*)-1-phenylethanol per enzyme has been optimized in a fed-batch reactor, TTN of 43000 related to (*R*)-1-phenylethanol and a space-time yield of



Scheme 2.3 The determination of enantiomers

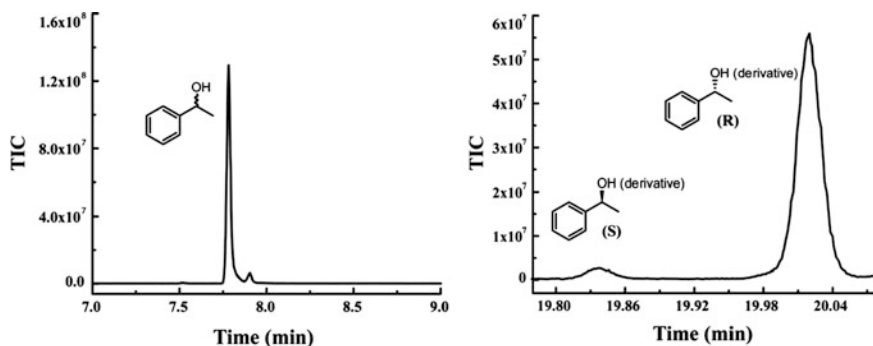


Fig. 2.2 Left GC trace of reaction mixture from the hydroxylation of ethylbenzene catalyzed by *AaeAPO*. Right GC trace of diastereomeric (*S*)-*O*-acetylmandelyl esters of 1-phenylethanol mixtures

approximately $60 \text{ g L}^{-1} \text{ d}^{-1}$ [5]. It proves the application of *AaeAPO* in a process-oriented approach.

The range of acceptable substrates for *AaeAPO* is also wider than that of CPO. The benzylic oxidation of ethylbenzene catalyzed by CPO gave 2-phenethyl alcohol in the *R* configuration with *ee* of 97 %. The oxidation of propylbenzene resulted in *S*-1-phenyl-1-propanol in the *S* configuration, *ee* of 88 % [16]. However, *AaeAPO* oxidized propylbenzene producing the *R* isomer as well. Unlike CPO, cumene, tetralin and indane are all good substrates for *AaeAPO* [5].

The *ee* of benzylic hydroxylation by *AaeAPO* is also higher than some P450s. For example, P450cam hydroxylate ethylbenzene with *ee* of about 46 % [17]. Ethylbenzene hydroxylation by a newly discovered P450_{BS_P} resulted the *ee* from 35 to 68 % with different carboxylic acids as decoy molecules [18].

2.2.3 Hydroxylation of Neopentane and Ethane

Methane (CH_4) is the simplest hydrocarbon and is the principal component of natural gas. It is abundant throughout the world. Methane is not used very efficiently because it is a combustible gas that requires impractical and expensive gas pipelines and liquefaction stations for transportation and handling. Lots of oil wells combust methane instead of storing it for future use. Methods of converting methane into more useful chemicals and fuels are challenging. Some costly and inefficient methods require high temperatures and pressures. A direct method for conversion of methane into methanol would be ideal because methanol is useful energy that is easy to handle. The oxidation of methane is difficult because the C–H bond strength in methane is as high as 104 kcal/mol.

The biological oxidation of methane is catalyzed by MMOs. But MMOs are complex enzymes and many of them are membrane proteins. There are many problems with using it as a biocatalyst for industrial application. P450s or engineered P450s have also been shown to accept methane. By using the enzyme P450 BM3 (CYP102A1) from *Bacillus megaterium* as the catalyst, a maximum TON of 2472 were achieved with perfluoro octanic acids as additives [19]. The additives are chemical inert to fill the binding pocket and reduce the freedom of small substrates. Another enzyme, P450153A6 has also shown the ability of methane oxidation by using iodosylbenzene as the oxidant [20]. These results confirmed that methane C–H bond can be activated by P450s. Engineered P450s have also shown the ability to oxidize ethane to ethanol [21]. We want to know about the ability of *Aae*APO for the oxidation of small gaseous molecules.

The reaction was set up with saturated neopentane or ethane in buffer under 1 atm pressure and at room temperature. The reaction mixtures were subjected to ^1H NMR analysis directly after the reactions were done. Then, neopentanol was extracted to organic solvent and can be analyzed by GC-MS. Figure 2.3 shows GC

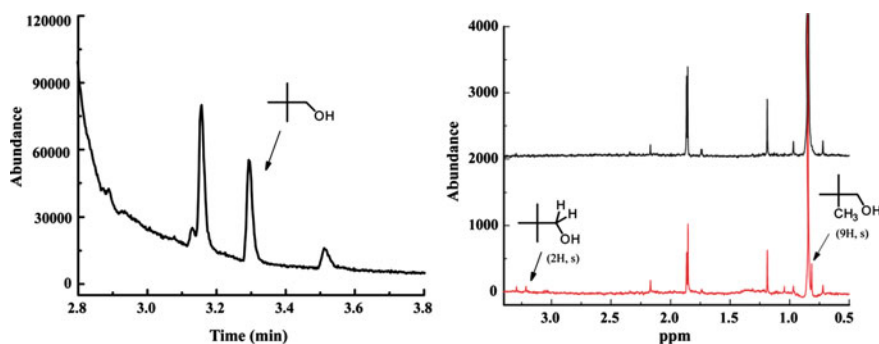
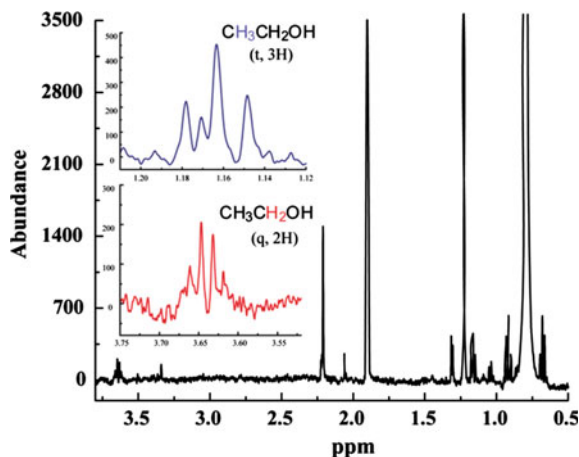


Fig. 2.3 Left GC trace of product mixture after the reaction of neopentane with H_2O_2 catalyzed by *Aae*APO. Right ^1H NMR of product mixture and compare it with control. There are new peaks corresponding to neopentanol. The total turnover was about 30. The final concentration of ethanol was 13 μM calculated from an internal standard which was added before doing NMR

Fig. 2.4 ^1H NMR of ethane oxidation catalyzed by *Aae*APO. New peaks are formed and corresponding to ethanol. The total turnover was about 10. The final concentration of ethanol was $6\ \mu\text{M}$ calculated from an internal standard which was added when doing NMR



trace and water suppressed ^1H NMR of mixtures after neopentane reactions. In the right figure, the arrows pointed to two new peaks corresponding to neopentanol (2H, s and 9H, s). No neopentanol was formed in the controls without adding *Aae*APO. The total turnover was about 30. In Fig. 2.4, the enlarged two panels show ethanol peaks (2H, q and 3H, t). No over-oxidized products were detected. The total turnover was about 10. Our results showed that without the help of additives, *Aae*APO is better than wild-type P450 BM3 for small alkanes oxidation. The result is competitive with some engineered P450s.

Methane oxidation was also carried out under the same conditions. But no detection of methanol was found. Maybe the TTN is so small that ^1H NMR is not sensitive enough for the detection of trace amount of products. But the successful conversion of ethane to ethanol is a good starting point. It is worth trying more reaction conditions on methane oxidation, such as the use of decoy molecules and run the reaction under high pressure.

2.2.4 Drug Metabolites

About one-third of the P450s within mammals are found in the liver and their major functions are involved in the degradation of xenobiotics. And it has been recognized that most of the pharmaceutical compounds are metabolized by P450s. So far the most intensively studied route of drug metabolism is the P450-catalysed oxidation reactions [23, 24]. Metabolite characterization has become one of the key drivers of the drug discovery process. Some of the drug metabolites kept active or even become more active. Some of the metabolites are toxic. So studying the metabolites of drugs helps the design of new drugs and makes important decisions on drug candidates.

Similar to P450s, *Aae*APO has showed powerful oxidization capability to selectivity functionalize inert C–H bonds to hydroxyl groups. So, here we expand

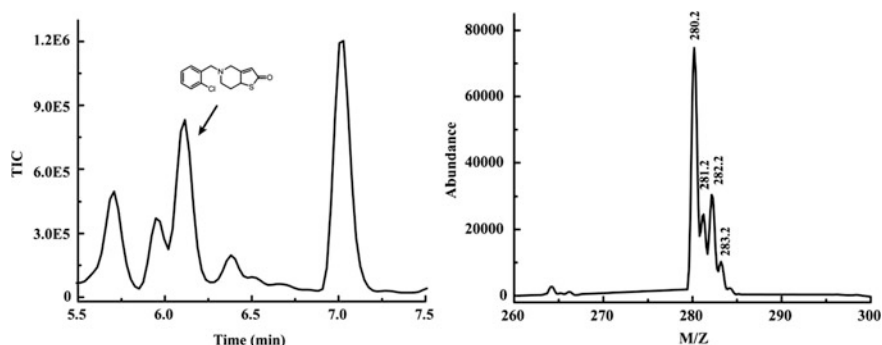
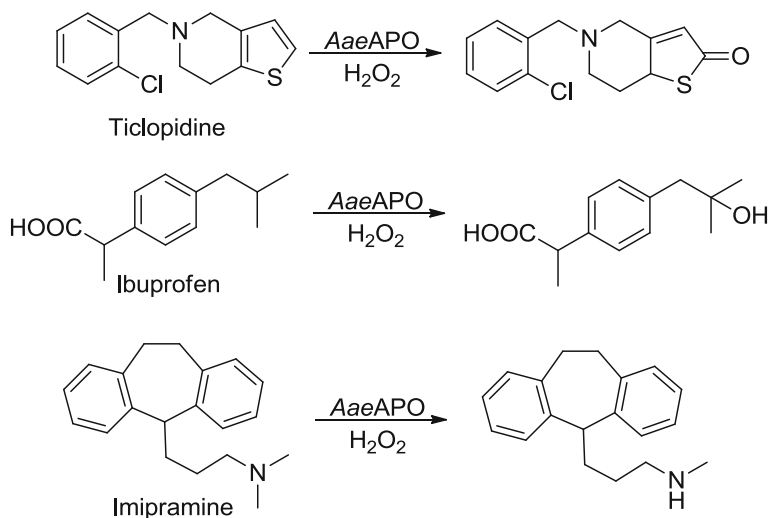


Fig. 2.5 HPLC trace and ESI-MS of ticlopidine metabolites

its substrates to pharmaceutical drugs and study the metabolites (Scheme 2.4). Our results suggest that *AaeAPO* could be a useful biocatalyst to prepare pharmacologically relevant drug metabolites and for the discovery of new commercial products including drugs.

Clopidogrel (Plavix) is a prodrug metabolized by cytochrome P450 to an active form that inhibits ADP-induced platelet aggregation. Before clopidogrel, its analog ticlopidine was used in patients. Early studies have showed that among all of metabolites of ticlopidine, 2-oxo-ticlopidine was the only metabolite that had anti-platelet activity in vivo [24]. Our HPLC-MS analysis showed that 40 % of the total metabolites by *AaeAPO* in vitro was 2-oxo-ticlopidine (Fig. 2.5). The retention



Scheme 2.4 Several examples of drug metabolites by *AaeAPO*

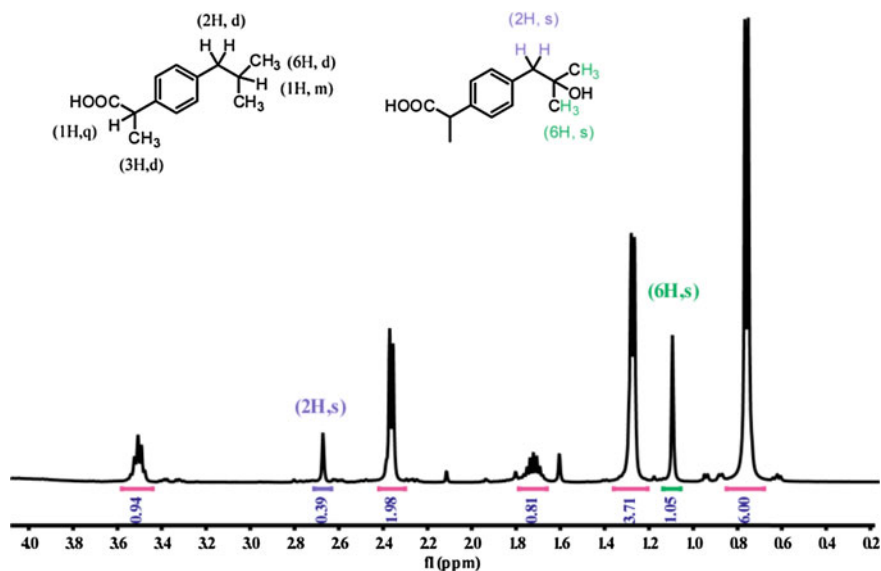


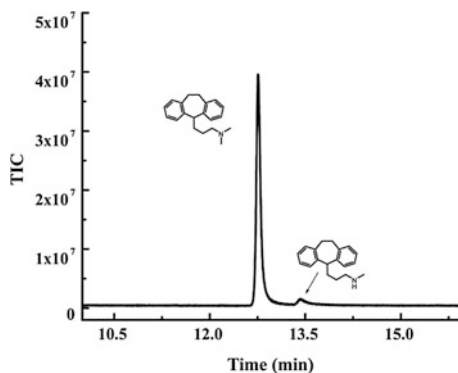
Fig. 2.6 Water suppressed ^1H NMR of ibuprofen metabolites

time and ESI-MS was confirmed by the authentic standard. So *Aae*APO might have the same function as CYP2C19 in the liver [25].

Ibuprofen is a drug to relieve pain, tenderness, swelling, and stiffness caused by osteoarthritis. Analyzed by ^1H NMR of the reaction mixture, the major metabolite of ibuprofen by *Aae*APO is 2-hydroxyibuprofen (80 % of total metabolites) (Fig. 2.6). Similar to CYPs, 2-hydroxyibuprofen is the major metabolite. Whereas 3-hydroxyibuprofen is almost exclusively produced by CYP2C9 [26].

Imipramine is an antidepressant medicine. The metabolite of imipramine by *Aae*APO is the demethylated product desipramine, the same as CYP1A2/2C19/3A4 [27]. With human liver microsomes in vitro, the major metabolites formed are desipramine and 2-hydroxyimipramine as well (Fig. 2.7).

Fig. 2.7 GC trace of the imipramine metabolites



2.2.5 Flavin Cofactors and Glucose Oxidase Coenzymes

2.2.5.1 Too Much H_2O_2 Can Kill the Activity of the Enzyme

High concentration of H_2O_2 could inactivate enzyme by oxidative degradation of the heme prosthetic group. The Michaelis-Menten profile of saturated veratryl alcohol or benzyl alcohol conversion catalyzed by *Aae*APO with different concentration of H_2O_2 showed that 2.5 mM of H_2O_2 decreased the formation rates dramatically (Fig. 2.8). Though portionwise addition of oxidants by the syringe pump can maintain the total H_2O_2 concentration at acceptable levels and it was shown to increase the total turnover number (TTN) of *Aae*APO. There are still contacts between highly concentrated H_2O_2 at the needle top with enzyme solution. The heterogeneous external addition speeds up the enzyme inactivation. So a method of generating H_2O_2 homogeneously by the reduction of O_2 is desirable.

2.2.5.2 Glucose Oxidase Coenzymes Catalytic Cycle

Glucose oxidase catalyzes the oxidation of glucose to produce H_2O_2 and gluconic acid. In the GOx-catalyzed redox reaction, its cofactor FAD works as the initial electron acceptor and is reduced to FADH_2 . Then FADH_2 is oxidized by the final electron acceptor, molecular oxygen. O_2 is then reduced to hydrogen peroxide (H_2O_2) (Fig. 2.9). This enzymatic method solves the problem of generating H_2O_2 in situ. But in order to make a specific amount of product, equal amount of glucose has to be used. In the end, the reaction generates equal amount of gluconic acid as the by-product [16] (Scheme 2.5).

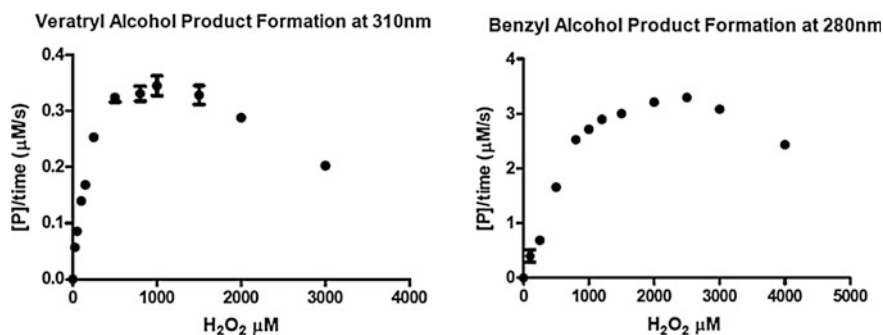


Fig. 2.8 The product formation rates change catalyzed by *Aae*APO with different concentration of H_2O_2

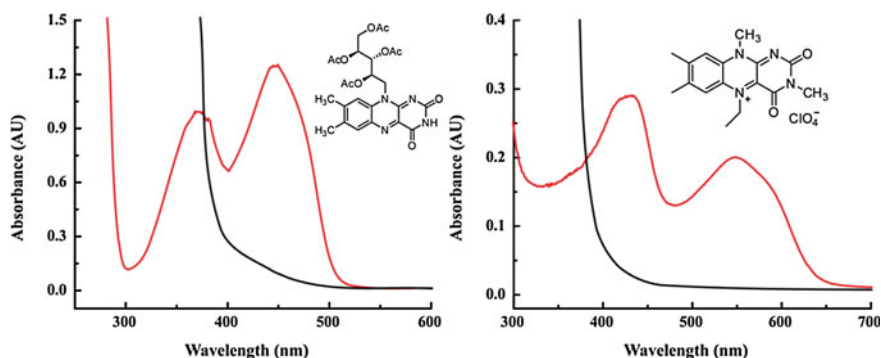
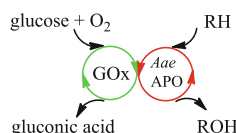


Fig. 2.9 The UV-vis spectrum of TARF and LumiFlEt at KP 50 mM pH 6. The red lines are oxidized forms and the black lines are reduced forms by excess DTT

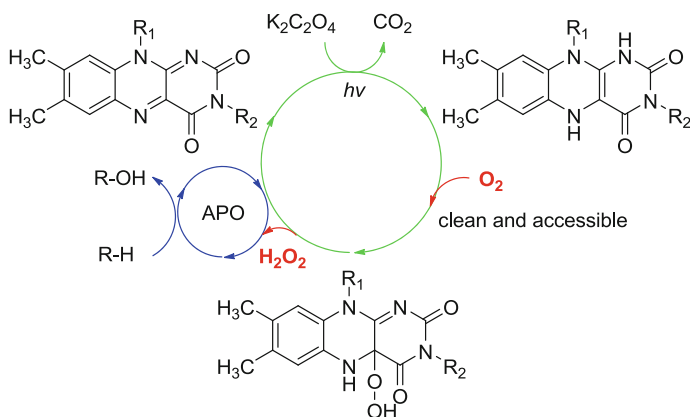


Scheme 2.5 The couple of *AaeAPO* and glucose oxidase catalytic cycles

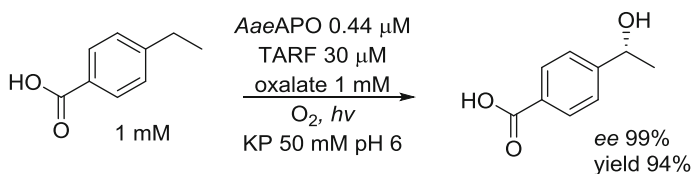
2.2.5.3 Flavin Cofactors Catalytic Cycle

Instead of using glucose oxidase as coenzyme, FAD or flavin moiety might serve the same function as the electron transport system in heme enzymes [28, 29]. A catalytic amount of flavin analogs were added into the system. Upon initiation by light, with the presence of electron sources such as EDTA or oxalate, flavin got reduced. The reduced $\text{Fl}_{\text{red}}\text{H}_2$ reacts with O_2 in the solution, produces H_2O_2 and regenerates the oxidative form of Fl_{ox} . When the flavin catalytic cycle is coupled with *AaeAPO* cycle, typical *AaeAPO* substrates are converted (Scheme 2.6). Control experiments without flavin, electron sources or under the dark results in no products formation. Without *AaeAPO*, there is also no products formation, flavin only catalyzed by itself. The region- and stereo-selectivity are the same as adding external oxidants. For example, when using ethylbenzoic acid as the substrate, almost 100 % conversion to (*R*)-1-phenylethanol was obtained (Scheme 2.7). The byproduct of the reaction using EDTA as the electron donors are formaldehyde and ethylene diamine. However, the byproduct of using oxalate is CO_2 which is a clean gas generated out of the reaction mixture (Scheme 2.8).

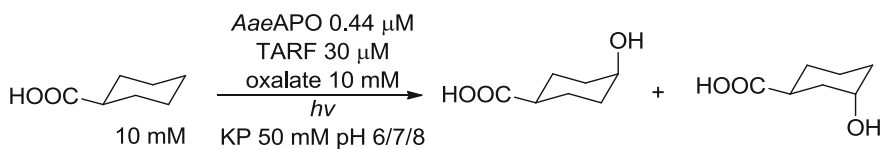
The percentage of conversion was confirmed by ^1H NMR (Fig. 2.10), the spectrum of products is distinct from the starting material. The triplet at 1.1 ppm is the methyl group of benzoic acid. The yield was calculated from the integration ratio between the new doublet at 1.36 ppm and the peak at 1.1 ppm. It turned out



Scheme 2.6 The catalytic cycles of *AaeAPO* reaction coupled with flavins



Scheme 2.7 Ethylbenzene hydroxylation by *AaeAPO* coupled with Flavin



Scheme 2.8 Cyclohexanecarboxylic acid hydroxylation by *AaeAPO* coupled with Flavin

the protein was still active when the reaction was stopped. We also optimized this clean photo-driven reaction by changing the concentration of flavin, using different flavins, different light sources, various reaction pHs and so on. The concentration of flavin concentration was optimized at 30 μM . The TTN could not be improved with more concentrated flavin solution. N_2H_4 is also a good electron source. But the reaction did not work when EDTA and oxalate were replaced by N_2H_4 . The hydroxylation reaction at pH 6 was better than neutral and slightly basic conditions (Fig. 2.11) Four different flavins were tried (Table 2.3). The TARF gave the maximum yield. Lumiflavin and Phlumiflavin did not work well. This is possibly due to the positive charge on the catalysts.

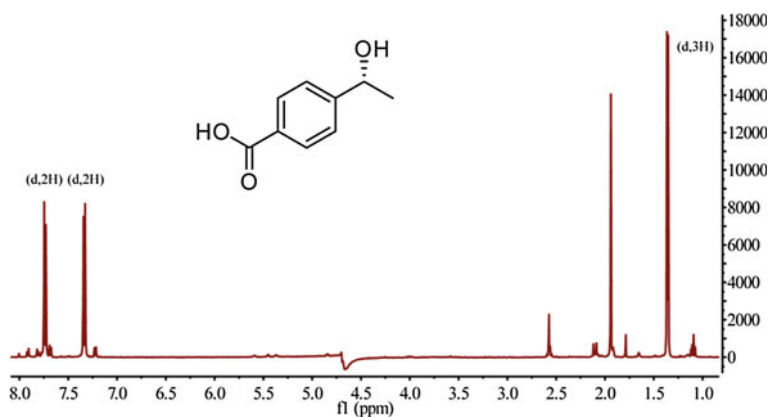


Fig. 2.10 Water suppressed ^1H NMR of ethylbenzoic acid hydroxylation products (94 % conversion)

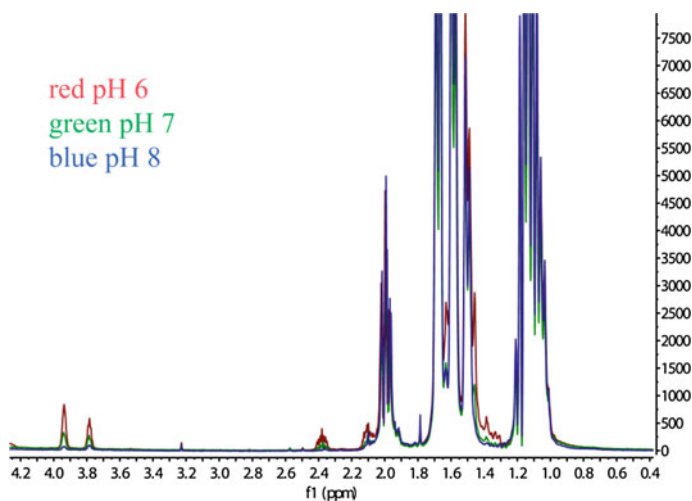
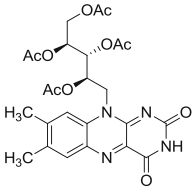
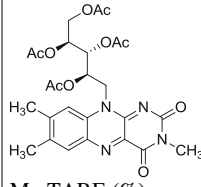
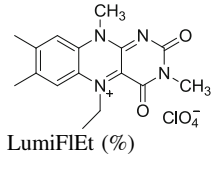
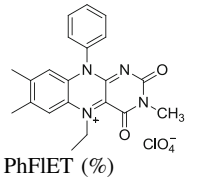


Fig. 2.11 Water suppressed ^1H NMR of cyclohexanecarboxylic acid hydroxylation product mixtures at pH 6, 7 or 8. The sharp two triplets at 3.78 and 3.95 ppm indicated that the hydroxylation happened on two axial sites

Like ethylbenzoic acid and cyclohexanecarboxylic acid, ethane and neopentane were also converted to their corresponding alcohol products with this new method. We are trying to carefully tune the active site of *AaeAPO* by using different flavins in order to oxidize methane.

Table 2.3 Conversion of ethylbenzoic acid hydroxylation catalyzed by *Aae*APO and different flavins

 TARF (%)	 Me-TARF (%)	 LumiFIET (%)	 PhFIET (%)
45	36	4	0

2.3 Conclusions

In this chapter, we have demonstrated that *Aae*APO catalyzes the hydroxylation of diverse alkanes with high selectivity and efficiency. H_2O_2 was used as the sole friendly oxidant. But the flavin light-driven catalytic cycle coupled with *Aae*APO couple increases the stability of *Aae*APO and takes advantage of molecular oxygen as the green oxidant. The wide substrate scope and various kinds of reactions catalyzed by *Aae*APO prove its potential role as a good biocatalyst.

2.4 Experimental

Reagents: wild-type peroxygenase from *A. aegerita* (isoform II, pI 5.6, 46 kDa) was produced in bioreactors with a soybean-flour suspension as growth substrate, and purified as described previously [30]. The enzyme preparation was homogeneous by SDS/PAGE, and exhibited an $A_{418 \text{ nm}}/A_{280 \text{ nm}}$ ratio of 1.7. The specific activity of the peroxygenase was 59 U mg^{-1} , where 1 U represents the oxidation of 1 μmol of 3,4-dimethoxybenzyl alcohol to 3,4-dimethoxybenzaldehyde in 1 min at RT. All chemicals and glucose oxidase were of the best available purity from Aldrich. Different flavins were kindly provided by Dr. Erika Milczek. 2-oxo-ticlopidine authentic standard was kindly provided by Nick Boaz. (*S*)-*O*-acetylmandelyl chloride was made from thionyl chloride and (*S*)-*O*-acetylmandelic acid. D_2O was obtained from Cambridge Isotope Laboratories, Inc. Water used in all experiments was de-ionized (Millipore, Milli-Q). Buffers were prepared fresh daily using either citric acid/sodium citrate (pH 3–5), $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (pH 6–8).

Instruments: UV-vis spectral measurements were made with a Hewlett Packed 8453 diode array spectrophotometer at room temperature. GC-MS analysis were run using an Agilent 7890A GC coupled to a 5975 Inert MSD with a Rtx-5Sil MS column using the following temperature profile: 50°C hold 2 min, $10^\circ\text{C min}^{-1}$ to 230°C . HPLC-MS analysis were done with HP 1100 A MS equipped with an auto-sampler. Separation was performed with a Luna C_{18} $50 \text{ mm} \times 4.6 \text{ mm}$ column

and gradient separation with acetonitrile and water. Water suppressed ^1H NMR and ^{13}C NMR spectra were recorded on a 500 MHz Bruker Avance II spectrometer.

Reaction conditions for alkane hydroxylation and drug metabolites: The reaction mixtures (0.20 ml, stirred at room temperature) contained 0.4 U of *Aae*APO, desired buffer pH (100 mM) and 2 μl of neat liquid alkanes or continuously bubbled with gaseous alkanes or 50 mM of drugs. The solution was mixed well by a stirred bar and then the reaction was started by syringe pumping 20 μl of 50 mM H_2O_2 with a rate of 1 $\mu\text{l}/\text{min}$. The mixture was extracted with ethyl acetate or DCM. The organic layers were combined, dried by Na_2SO_4 and then analyzed by GC/MS. Or when the reaction was done, the mixture was directly analyzed by HPLC/MS. Or when the reaction was done, several drops of D_2O were added into the solution then the mixture was subjected to water suppressed ^1H NMR and ^{13}C NMR.

Diastereomeric derivatization of 1-phenylethanol: The dried and concentrated DCM solution of 1-phenylethanol from the enzyme reaction was kept on ice in a septum-sealed vial. Dry pyridine (2 μL) was added, followed by excess amount of (S)-*O*-propionylmandelyl chloride (100 mM solution in DCM). The solution was stirred and allowed to stand at room temperature for at least 1 h. Sufficient reaction time was always used to ensure quantitative conversion of alcohol to ester. The resulting solution went through a short silicone gel column and then the solution was directly analyzed by GC-MS.

Coenzyme and cofactor reaction condition: A typical flavin light-driven reaction was performed by well mixing *Aae*APO (1U) with flavin (100 μM), EDTA or oxalate (10 mM) and substrates (10 mM) in a buffered solution. The open vial was placed in front of a lamp and illuminated for 30 min under aerobic conditions. If a closed vial was used, its cap was connected with a balloon filling with pure oxygen.

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