Use of Chemical Auxiliaries to Control P450 Enzymes for Predictable Oxidations at Unactivated C-H Bonds of Substrates

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Abstract

Cytochrome P450 enzymes (P450s) have the ability to oxidize unactivated C-H bonds of substrates with remarkable regio- and stereoselectivity. Comparable selectivity for chemical oxidizing agents is typically difficult to achieve. Hence, there is an interest in exploiting P450s as potential biocatalysts. Despite their impressive attributes, the current use of P450s as biocatalysts is limited. While bacterial P450 enzymes typically show higher activity, they tend to be highly selective for one or a few substrates. On the other hand, mammalian P450s, especially the drug-metabolizing enzymes, display astonishing substrate promiscuity. However, product prediction continues to be challenging. This review discusses the use of small molecules for controlling P450 substrate specificity and product selectivity. The focus will be on two approaches in the area: (1) the use of decoy molecules, and (2) the application of substrate engineering to control oxidation by the enzyme.

Keywords

Biocatalysis • Decoy molecule • Fatty acid • Perfluorinated carboxylic acid • Carbolide • Chemical auxiliary • Theobromine • Molecularly imprinted polymer

8.1 Introduction

Cytochrome P450 enzymes, herein referred to as P450s, form a large family of heme-dependent monooxygenases. Ubiquitous in nature, they are found in bacteria, fungi, mammals and other organisms. In bacteria, P450s are involved typically in the biosynthesis of secondary metabolites, while in mammals they are responsible largely for the metabolism of xenobiotics (foreign compounds) such as drugs, carcinogens and environmental pollutants, as well as the biosynthesis and metabolism of endogenous or naturally-occurring bioactive compounds such as eicosanoids (eicosanoic acids, leukotrienes, prostaglandins), fatty acids, steroids and vitamins

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[1]. One attribute that allows P450s to perform such diverse roles is their ability to oxidize unactivated C-H bonds of substrates with remarkable regio- and stereoselectivity [1–4]. It is this feature that has caught the attention of synthetic chemists. In this respect, there has been huge interest in potentially harnessing the biocatalytic prowess of P450s. Unfortunately, these enzymes are not without limitations. They often suffer from low stability in vitro, require expensive cofactors and demonstrate difficult product predictability.

8.2 Biocatalysis

Biocatalysis is defined as the use of biomolecules, whether naturally-occurring or engineered, for the purpose of facilitating a desired reaction. It can provide many of the benefits of chemical catalysis, adhering at the same time to many principles of green chemistry [5–8]. Typically, an ideal catalyst should be able to predictably catalyze chemo-, regio- and stereoselective reactions with high efficiency while maintaining a large substrate scope. Today, further economical and environmental constraints must also be considered [5, 8]. Although the field of biocatalysis has greatly expanded over the last few decades, there are still many obstacles to overcome, which vary on an enzyme-to-enzyme basis. Many enzymes typically suffer from low stability, narrow substrate scope, need for expensive cofactors and high production costs. Significant efforts by the research community to overcome these problems have led, over the past century, to major advances, yet more challenges lie ahead. The recent popularity of "green chemistry" is definitely positioning biocatalysis as a top research priority.

8.2.1 Chemical Catalysts for Oxidations at Unactivated C-H Bonds of Substrates

A variety of chemical catalysts are known for substrate oxidation at unactivated C-H bonds [9–11]. These catalysts can be separated into two general classes, either containing or lacking an active-site metal. Metal-containing catalysts typically have complex coordinating ligands responsible for tuning the reduction-oxidation (redox) potential of the metal, as well as imparting a degree of chemo-, regio- and stereoselectivity. For example, the metalloporphyrin catalysts were modeled after the prosthetic heme-iron group found in the active site of P450s [10]. In contrast to P450s, metalloporphyrin-based chemical catalysts can contain different metals such as cobalt, copper, iron, manganese, ruthenium and vanadium [10]. Although selectivity can be achieved with these catalysts, it is typically poor and unpredictable and must be determined empirically. In many cases, numerous oxidation products are obtained ranging from aliphatic and aromatic alcohols to carbonyls [12].

Reasonable predictability and good yields for oxidations at unactivated C-H bonds have been observed with non-porphyrin, metal-based catalysts [9–11]. The regio- and stereoselectivity of this second class of metal-catalysts is generally dependent on the steric and electronic properties of the substrate. Furthermore, the catalysts tend to favor oxidation at tertiary C-H bonds over secondary C-H bonds, or else over-oxidation and loss of chirality is the norm [13, 14]. Although excellent diastereoselectivity has been achieved with some chemical catalysts, to date there have been no reports of *enantioselective* metal catalysts performing hydroxylations of unactivated CH_2 groups. Chiral oxaziridine catalysts developed by Du Bois and coworkers [15] can favor enantioselective hydroxylations. However, these catalysts show a high preference for tertiary C-H bonds over less energetically favorable secondary C-H bonds. Moreover, they do not distinguish between multiple sterically and electronically similar tertiary C-H bonds. Finally, metal-free catalysts such as dioxiranes and oxaziridines have also been shown to oxidize unactivated C-H bonds and favoring reactions at tertiary carbons [15, 16].

Although there are a variety of chemical catalysts available for oxidation of unactivated C-H bonds, there are still important needs in this area. Overall, chemical catalysts suffer from poor selectivity and product predictability, and they

often yield over-oxidation products. Furthermore, their selectivity is based on electronic and steric properties of the substrates, which often limits them to oxidation of more electronically favorable tertiary bonds and prevents discrimination between C-H bonds similar in these aspects.

8.2.2 P450 Biocatalysts

In parallel, interest has been invested into the design and use of P450s as biocatalysts in hopes of providing complementary reactivity to the available chemical toolbox. As mentioned previously, P450s are well known for their exceptional ability to selectively oxidize a large variety of substrates [2, 3, 17, 18]. This provides them with two attributes that are highly sought in a catalyst: scope and selectivity. As an example, human P450s can be credited for being involved in the metabolism of approximately 75 % of all clinical drugs [17]. Their impressive ability to selectively discern unactivated methylene bonds among many electronically and sterically similar groups is obvious when looking at the structure of drug metabolites [2, 3, 17, 18]. In addition, P450s have the ability to oxidize C-H bonds of varying strength, from methine to methyl groups, often without over-oxidation to the ketone. Despite these impressive attributes, a number of drawbacks still limit the applications of P450s as biocatalysts [19]. Poor stability and low turnover rates, as well as the need for expensive cofactors, are among their greater limitations. To overcome some of these issues, industrial processes, for example, typically use fermentation reactors [20].

In research settings, P450s of microbial origin are usually preferred because they are more easily expressed in high yields and in a soluble form, and have higher activities and coupling efficiencies. In contrast, mammalian P450s have a wide scope of reactivity and convoluted chemo-, regio- and stereoselectivity. Their ability to accept various substrates provides them with a clear advantage in a research context or with non-natural substrates.

8.2.3 The Promiscuity Paradox

While mammalian P450s may be attractive biocatalysts because of their high substrate promiscuity, predicting the structures of their products remains challenging. This promiscuity paradox is an important obstacle hindering the use of mammalian P450s as biocatalysts. Although a large diversity of substrates is recognized, particularly by xenobiotic-metabolizing P450s, many of these substrates are suboptimal and/or have multiple binding modes. As a result, increased uncoupling rates are often observed due to uncontrolled water access to the active site [21]. One strategy to overcome these issues involves protein engineering, which has been used to improve enzymatic activity, change substrate specificity and alter product distributions [22, 23]. As reviewed below, other research groups have attempted to control P450 selectivity and improve product predictability by using small molecules. There are generally two strategies by which this has been achieved. The first approach uses decoy molecules to control the site of oxidation by blocking a part of the active site. In contrast, the second strategy applies substrate engineering to control oxidation by the enzymes.

8.3 Decoy Molecules

Decoy molecules are inert dummy molecules that are structurally similar to the natural substrate of a specific enzyme [24]. The term was first coined by Watanabe and coworkers in 2007 [24] and was considerably explored with respect to $P450_{BS\beta}$. The general mode of action for decoy molecules involves partial filling up of the active-site space and consequent decrease of the degree of translational freedom allowed for the substrate while in the enzyme. In this respect, the decoys have the ability to increase the selectivity of the enzymatic reaction by prompting the substrate to bind in a consistent orientation. Careful consideration must be used when selecting a decoy molecule. It must either be inert and not be transformed by the enzyme, or not be in

competition with the desired substrate. To this end, two types of decoy molecules have been explored for their P450-directing capabilities: (1) short-chain fatty acids, and (2) perfluorinated fatty acids.

8.3.1 Short-Chain Fatty Acids

In 2007, experiments reported by the Watanabe group enabled the recognition of unnatural substrates by P450s without the use of mutagenesis [24]. At the time, this strategy was unique in taking advantage of short-chain fatty acids to act as ligands of $P450_{BS\beta}$, mimicking part of the substrate and leaving a small portion of the substrate binding pocket available near the heme iron (Fig. 8.1). This allowed the transformation of the unnatural substrates guaiacol, styrene, ethylbenzene and thioanisole (Fig. 8.2) [24, 25]. In these studies, $P450_{BS\beta}$ was chosen as the biocatalytic system because it is soluble, does not require a redox partner and can efficiently use hydrogen peroxide instead of oxygen and the expensive NADPH cofactor. Isolated from *Bacillus subtilis*, $P450_{BS\beta}$ (also known as P450 152A1) normally catalyzes the hydroxylation of long-chain fatty acid substrates such as myristic acid to form α- and β-hydroxymyristic acid [26].

As part of their initial studies, the Watanabe group evaluated a series of decoy molecules for facilitating guaiacol oxidation by $P450_{BS\beta}$ [24]. Thus, carboxylic acids varying in length from two to ten carbons were added to the reaction mixtures and the products were quantified. Many of the acids allowed for guaiacol oxidation to occur, with the highest reaction rate being observed in the presence of heptanoic acid (3,750 turnovers min^{-1}). This rate is tenfold higher than that reported for the natural substrate, myristic acid, suggesting activation of the enzyme by the decoy molecule. This enhancement was attributed to the decoy molecules keeping the catalytic cycle "on", which long-chain fatty acid substrates normally do when they bind. The catalytic cycle is turned "off"

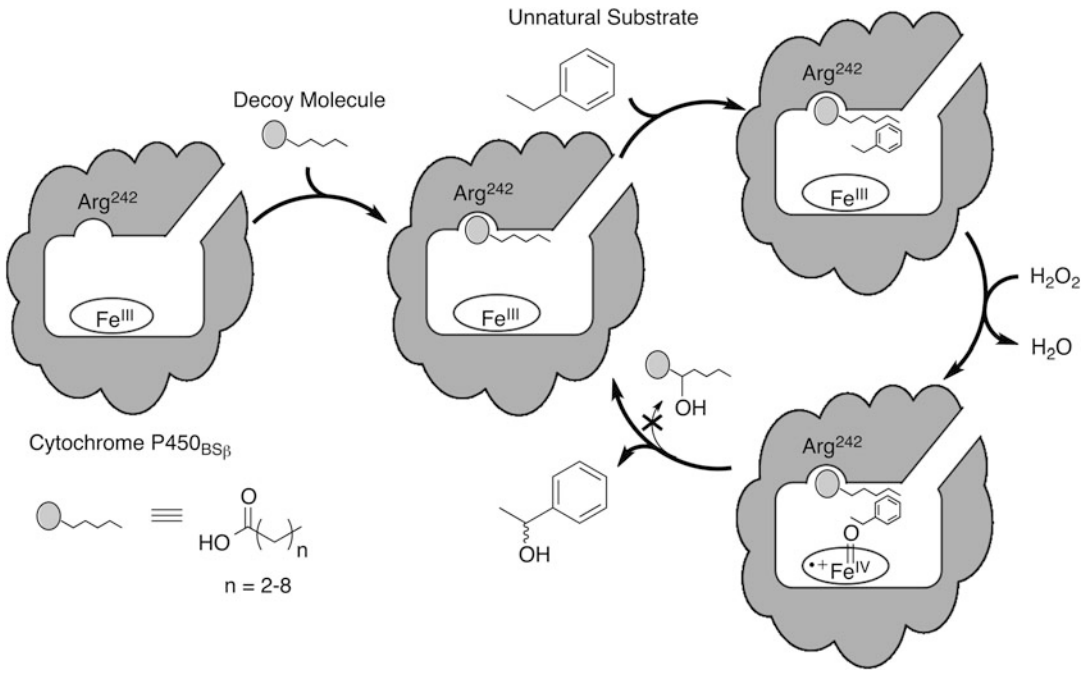


Fig. 8.1 Suggested catalytic cycle for the oxidation of unnatural substrates by $P450_{BS\beta}$ in the presence of decoy molecules [24]

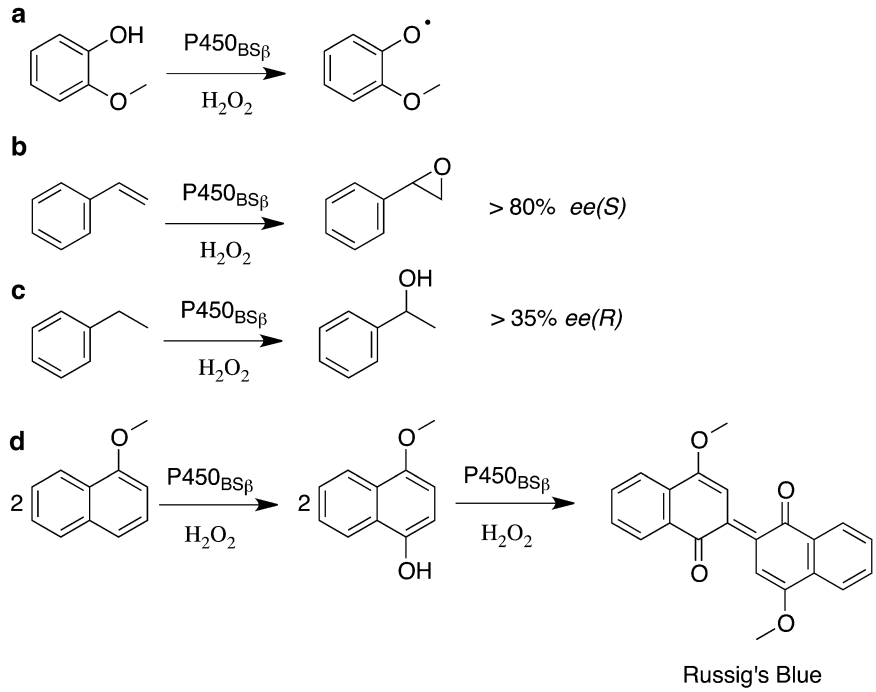


Fig. 8.2 Substrate scope for the oxidation of unnatural substrates by $P450_{BS\beta}$ in the presence of the decoy molecules, heptanoic or hexanoic acid. Substrates shown are **a** guaiacol, **b** styrene, **c** ethylbenzene and **d** 1-methoxynaphthalene, along with the major product of their oxidation by $P450_{BS\beta}$ [24, 28]

when the fatty acid dissociates [24]. As expected, in the presence of myristic acid, no oxidation of guaiacol occurred.

The concept was further investigated with styrene epoxidation by $P450_{BS\beta}$ in the presence of carboxylic acids with 4–8 carbon chain lengths [24]. Styrene epoxidation occurred in the presence of any decoy molecule examined, with both regioselectivity and enantioselectivity (*ee*) greater than 80 %. The maximum reaction rate of 334 turnovers min^{-1} was observed in the presence of hexanoic acid. Furthermore, under the same conditions, hydroxylation of ethylbenzene afforded the corresponding 2° alcohol [24]. Oxidation of the methylene carbon occurred with the highest enantioselectivity (68 % *ee* (*R*)) and highest reaction rate (28 turnovers min^{-1}) in the presence of heptanoic acid. Taken together, these results demonstrate that $P450_{BS\beta}$ can catalyze the oxidation of non-fatty acid substrates when tricked into misrecognizing short-chain fatty acids instead of the natural myristic acid substrate.

To confirm the proposed mechanism, the authors obtained crystal structures of $P450_{BS\beta}$ complexed with heptanoic acid [27]. Using crystal structure analysis, they showed that fatty acids with carbon chain lengths less than ten carbon atoms are too short to extend into the hydrophobic substrate access channel [27]. This results in loose fixation of the hydrophobic tail, preventing hydroxylation of the decoy molecules and allowing for small molecules to enter and act as substrates. Comparison of the decoy molecule-bound crystal structure to the substrate-free crystal structure also demonstrated that binding of the decoy molecules did not induce large structural changes.

Following their initial success with decoy molecules, Watanabe and coworkers further investigated the scope of molecules that can be oxidized using $P450_{BS\beta}$. In 2010, they reported that $P450_{BS\beta}$ can catalyze the conversion of 1-methoxynaphthalene to 4,4′-dimethoxy-[2,2′]-binaphthalenylidene-1,1′-dione, also called Russig's blue dye (Fig. 8.2d) [28]. This study

demonstrated that hydroxylation of an aromatic C-H bond (turnover rate of 112 min^{-1}) can be catalyzed by this P450, adding to the scope of the system. This was the first example of an efficient aromatic C-H bond oxidation by a hydrogen peroxide-dependent P450 enzyme. The expected *O*-demethylation product was not observed, yet the reaction was not as regioselective as with styrene or ethylbenzene. Additionally, by comparing the conversion rate of the intermediate 4-methoxy-1-naphthol to the conversion rate of 1-methoxynaphthalene, it was determined that the rate-limiting step is the first step, i.e., hydroxylation of the aromatic C-H bond of 1-methoxynaphthalene. With this in mind, the authors investigated the use of 1-methoxynaphthalene as the substrate in a colorimetric assay for the indirect observation of $P450_{BS\beta}$ catalytic activity. To test this idea, they compared the effects of carboxylic acids with lengths of 4–10 carbons on the turnover of 1-methoxynaphthalene by $P450_{BS\beta}$. Since the effect of the decoy molecules was similar for 1-methoxynaphthalene, ethylbenzene and styrene, they used the formation of Russig's blue to estimate activities of a series of $P450_{BS\beta}$ mutants generated in search of higher aromatic C-H bond oxidation activity [28]. Their colorimetric assay allowed them to set up high-throughput screening for the identification of many other types of decoy molecules. From this screen, they found that wild-type $P450_{BS\beta}$ in combination with pentanoic acid shows the best activity for aromatic C-H bond oxidation. Mutant V170F in combination with octanoic acid had comparable activity. Unexpectedly, they identified mono-*tert*-butyl succinate as an additional useful potential decoy molecule. However, its binding mode remains unclear.

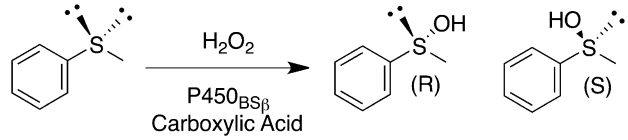
Because the observed catalytic activity and selectivity depended on the length of the carboxylic acid carbon chain used as a decoy molecule, the authors hypothesized that structural changes to the decoy molecule might affect the stereochemical outcome of the reaction. Thus, sulfoxidation of thioanisole was examined in the presence of various decoy molecules (Table 8.1) [25]. The majority of the decoy molecules examined allowed for the sulfoxidation of thioanisole to occur without over-oxidation to the sulfone product, generally favoring *R*-stereoselectivity with modest *ee*. The highest turnover (514 min^{-1}) and stereoselectivity (29 % *ee*) was obtained once again with heptanoic acid. After testing a large scope of potential decoy molecules, it was concluded that branched carboxylic acids both with and without a chiral center could serve as decoy molecules.

Interestingly, *p*-substituted methylphenylacetic acid effectively inverted the stereoselectivity to favor the *S* enantiomer (11 % ee) while *m*- and *o*-substitutions maintained *R*-stereoselectivity. Molecular modeling simulations with phenylacetic acid (PAA), *p*-methylphenylacetic acid (*p*-MPAA), *o*-methylphenylacetic acid (*o*-MPAA) and *m*-methylphenylacetic acid (*m*-MPAA) showed that for *p*-MPAA, the methyl group is placed over the heme porphyrin ring resulting in a steric clash with thioanisole, while for *m*- and *o*-MPAA the methyl group is positioned away from the heme plane avoiding steric hindrance. After modeling thioanisole in the active site with minimized steric repulsion, it was clear that with PAA, *m*-MPAA and *o*-MPAA, the *pro-R* side of the sulfur lone pairs is closer to the heme iron, allowing for oxidation. For *p*-MPAA however, due to steric repulsion of the methyl group with the thioanisole phenyl ring, the *pro-S* lone pair is closer to the heme (Fig. 8.3). Although the *S*-enantioselectivity observed in the presence of the decoy molecules was not particularly high, this was the first example of a non-mutagenic method for inversion of stereochemistry in a P450.

8.3.2 Perfluorinated Fatty Acids

After it was established that $P450_{BS\beta}$ can be tricked into oxidizing unnatural substrates using decoy molecules, Watanabe and others directed their focus on another fatty acid-metabolizing P450. In 2011, groups led by Reetz and by Watanabe reported independently on the oxidation capabilities of $P450_{BM3}$ (P450 102A1) toward short alkanes with the aid of decoy molecules [29, 30]. Isolated from *Bacillus megaterium*, $P450_{BM3}$ has the highest catalytic

Table 8.1 Sulfoxidation of thioanisole catalyzed by $P450_{BS\beta}$ in the presence of carboxylic acid decoy molecules [25]



Decoy molecule	Turnover rate[a] (min^{-1})	% ee (*R* or *S*)
None	24 ± 2	4 ± 0 (*R*)
$CH_3(CH_2)_6COOH$	251 ± 20	21 ± 4 (*R*)
$CH_3(CH_2)_5COOH$	514 ± 72	29 ± 1 (*R*)
$CH_3(CH_2)_4COOH$	390 ± 53	21 ± 2 (*R*)
$CH_3(CH_2)_3COOH$	274 ± 48	16 ± 1 (*R*)
$CH_3(CH_2)_2COOH$	261 ± 75	18 ± 0 (*R*)
CH_3CH_2COOH	270 ± 21	20 ± 1 (*R*)
(S)-(+)-2-Methylbutyric acid	214 ± 27	19 ± 1 (*R*)
(±)-2-Methylbutyric acid	146 ± 22	13 ± 1 (*R*)
$PhCH_2CH_2CH_2COOH$	370 ± 39	19 ± 0 (*R*)
$PhCH_2CH_2COOH$	462 ± 37	21 ± 0 (*R*)
$PhCH_2COOH$ (PAA)	71 ± 10	14 ± 0 (*R*)
o-Me-$PhCH_2COOH$ (*o*-MPAA)	34 ± 9	9 ± 1 (*R*)
m-Me-$PhCH_2COOH$ (*m*-MPAA)	32 ± 8	12 ± 2 (*R*)
p-Me-$PhCH_2COOH$ (*p*-MPAA)	89 ± 14	11 ± 2 (*S*)
p-Isopropyl-$PhCH_2COOH$	55 ± 2	10 ± 1 (*S*)
PhCOOH	41 ± 5	12 ± 1 (*R*)

[a]The unit for catalytic activity is nmol product min^{-1} nmol $P450^{-1}$

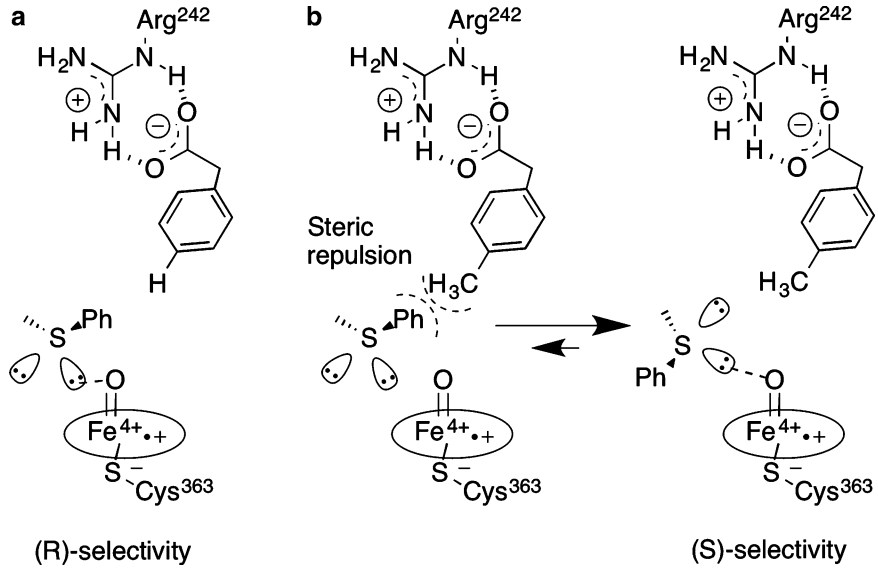


Fig. 8.3 Suggested transition states for stereochemical inversion during the sulfoxidation of thioanisole by $P450_{BS\beta}$ with **a** PAA and **b** *p*-MPAA [25]

rate reported for a P450, >15,000 turnovers min^{-1}, observed for the oxidation of arachidonic acid [30]. Its natural substrates include myristic and palmitic acid, which consist of 14 and 16 carbon chains, respectively [29, 30]. The high catalytic rate of $P450_{BM3}$ is attributed largely to the fact that its heme and reductase domains are located on the same polypeptide chain, resulting in efficient electron coupling [29, 30]. As with many other P450s, $P450_{BM3}$ is known for its large active site that is able to incorporate two molecules at a time [31–33]. Although this is ideal for achieving the cooperative effects desired with decoy molecules, problems can arise with small substrates due to a large number of binding orientations [29]. $P450_{BM3}$ catalyzes the hydroxylation of long-chain fatty acids at the ω−1, ω−2, or ω−3 positions. Here again, molecules that are not fatty acids are typically not accepted as substrates because the carboxylate group is required for catalytic activity. Although many mutagenic studies have been performed with $P450_{BM3}$ with respect to broadening the substrate scope [34–44], this study demonstrated that perfluorocarboxylic acids (PFCs) can be used as decoy molecules to expand the substrate scope of $P450_{BM3}$ without resorting to mutagenesis. PFCs are a great alternative to fatty acids because the greater bond energy of C-F (116 kcal mol^{-1}) compared to that of C-H (95–99 kcal mol^{-1}) renders them inert toward oxidation [30]. Moreover, PFCs are considerably larger in size than fatty acids. For example, the CF_3 moiety is comparable in size to an ethyl group [45]. Overall, PFCs take up more space in the active site while maintaining the hydrophobic character of standard fatty acids [29].

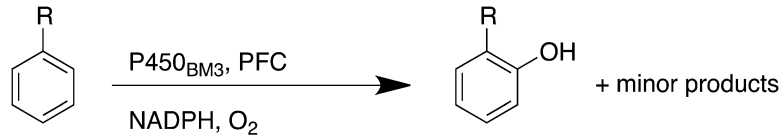
The ability of PFCs to bind to $P450_{BM3}$ was first confirmed by monitoring changes in the UV/Vis spectrum [29, 30] and the consumption of NADPH in the presence of PFCs that were 8–14 carbons in length [30]. Reetz and coworkers conducted further spectral studies to characterize the effect of the PFCs in the active site. They concluded that PFCs not only reduce the space in the active site as do the original fatty acid molecules, but also activate the enzyme by displacing the distal water molecule and converting the heme iron from low-spin to high-spin [29]. Next, the directing effects of the PFCs on hydroxylation were examined with a scope of small alkane substrates ranging from methane to octane and including some constitutional isomers of hexane (Table 8.2) [29, 30]. Although the majority of the PFCs allowed for hydroxylation of the small molecules to occur, the length of the PFC chain had a significant effect on the rate of oxidation. Smaller molecules typically showed better turnover with longer PFCs. Moreover, in the absence of PFCs, no transformation occurred [29, 30]. In all cases, PFCs had a positive effect on the enzymatic turnover of the alkanes but little effect on regioselectivity and stereoselectivity [29]. This was attributed to the lack of functional groups on the alkanes, preventing consistent orientation. Interestingly, Watanabe demonstrated that $P450_{BM3}$ can successfully hydroxylate propane, butane and cyclohexane to 2-propanol, 2-butanol and cyclohexanol respectively in the presence of a PFC but did not oxidize methane or ethane [30]. As an example, propane was converted at a rate of 67 turnovers min^{-1} in the presence of PFC-C10, while cyclohexane was converted at a rate of 110 turnovers min^{-1} in the presence of PFC-C9 [30]. In contrast, Reetz and coworkers observed that *n*-butane had higher turnover with PFC-C7 (3,632 min^{-1}) while propane had the highest turnover with PFC-C11 (1,021 min^{-1}). The oxidation of methane to methanol is notoriously more challenging. Initial studies with $P450_{BM3}$ and PFCs by Reetz and coworkers suggested that methane might be converted to methanol [29]. However, further analysis of the data revealed that methane was not oxidized to any appreciable extent [46]. Although the turnover of propane reported by both groups is still lower than the turnover reported for $P450_{BM3}$ mutants, the PFC method is much simpler to apply than generation of mutant enzymes [29]. Finally, it is important to note that substitution of the hydrogen atoms for fluorines in the decoy molecule was crucial for the reactions to occur. Indeed, $P450_{BM3}$ did not convert propane to propanol in the presence of decanoic acid [30].

Table 8.2 Conversion rates of gaseous molecules by $P450_{BM3}$ in the presence of PFCs [29, 30]

Substrate	PFC[a]	Rate[b] (min^{-1})	Regioselectivity	Refs.
Methane	C10	2,472		[29]
Propane	C11	1,021		[29]
	C10	67		[30]
Butane	C7	3,632		[29]
	C10	113		[30]
Hexane	C11	525	2-/3-Hexanol = 77:23	[29]
Octane	C9	1,184	2-/3-/4-Octanol = 10:42:48	[29]
Cyclohexane	C9	110		[30]
3-Methylpentane	C9	476	2-/3-Hydroxy = 88:12	[29]
3,3-Dimethylbutane	C9	891	Only 2-Hydroxy	[29]
2,3-Dimethylbutane	C11	3,241	Only 2-Hydroxy	[29]

[a]Optimal perfluorinated carboxylic acid reported
[b]Observed turnover rates

Table 8.3 Hydroxylation of aromatic rings by $P450_{BM3}$ [47]



Substrate	PFC[a]	Rate (min^{-1})[b]	Major product
Benzene	C9	120 ± 9	Phenol
Toluene	C9	220 ± 7	*Ortho*
Anisole	C9	260 ± 4	*Ortho*
Chlorobenzene	C9	120 ± 4	*Ortho*
Nitrobenzene	C9	0.9 ± 0.05	*Ortho*
Acetophenone	C9	2.9 ± 0.1	*Ortho*

[a]The chain length of PFC that elicited the greatest effect
[b]Rates are given in units of min^{-1} per P450. Uncertainty is given as the standard deviation from at least three measurements

More recently, Watanabe, Shoji and colleagues published another study that utilizes $P450_{BM3}$ for the hydroxylation of aromatic rings such as those of benzene, toluene, anisole, chlorobenzene, nitrobenzene and acetophenone (Table 8.3) [47]. The reaction was successfully catalyzed using $P450_{BM3}$ and various PFCs with exclusive formation of phenols. The transformation of benzene proceeds most efficiently in the presence of PFC-C9, with a turnover rate of 120 min^{-1}. For toluene hydroxylation, the turnover rate was higher (220 min^{-1}) but diminished when electron withdrawing groups decorated the ring. Although PFCs had a positive effect on the rate, they did not affect regioselectivity. For all aromatic substrates examined, the major hydroxylation site was *ortho* to the existing substituent, even when the *meta* position is electronically favored. This suggests that the regioselectivity is controlled mainly by the residues in the active site and not by the PFC or the substrate [47].

In summary, the use of decoy molecules provides a simple and quick way for increasing the substrate scope of these biocatalysts without the need for protein mutagenesis. They can also be used to influence the regio- and stereoselectivity of P450-catalyzed reactions. This is attributed to the fact that $P450_{BS\beta}$ and $P450_{BM3}$

can accept multiple substrates into their relatively large active sites leading to positive cooperative effects [31–33]. Such methodology leaves room for further investigation of decoy molecule design and their effect on reaction selectivity and turnover. Finally, this elegant strategy remains to be expanded to P450s other than $P450_{BS\beta}$ and $P450_{BM3}$, and especially to drug-metabolizing P450s, many of which show cooperative behavior.

8.4 Substrate Engineering

While many research groups in the P450 area have focused on engineering the proteins for biocatalytic applications, other groups have instead explored the idea of engineering the substrates. There are many instances in the literature suggesting that the presence of specific functional groups can improve substrate acceptance by biocatalysts [48–53]. This concept is often referred to as substrate engineering and is typically applied to non-natural substrates that are unlikely to show high affinity for the enzyme and for which products may be difficult to predict [54, 55]. To apply the substrate-engineering concept, the desired substrate is first covalently linked to another molecule, termed the chemical auxiliary, which has specific recognition motifs (Fig. 8.4). The auxiliary serves to improve recognition by the enzyme and encourage productive binding, as well as control the binding orientation for selective transformation [54, 56]. Once the transformation is achieved, the auxiliary is cleaved off and the desired product isolated.

Weber and coworkers investigated the use of chemical auxiliaries that they termed docking/protecting (d/p) groups for improving the scope of hydroxylation by various monooxygenases [54, 55]. They evaluated various microorganisms as catalysts for the hydroxylation of non-natural substrates (Fig. 8.5). They first demonstrated that the selected substrates were not hydroxylated, or did not undergo side reactions, in the absence of the docking/protecting groups. However, covalent addition of a chemical auxiliary to the substrates

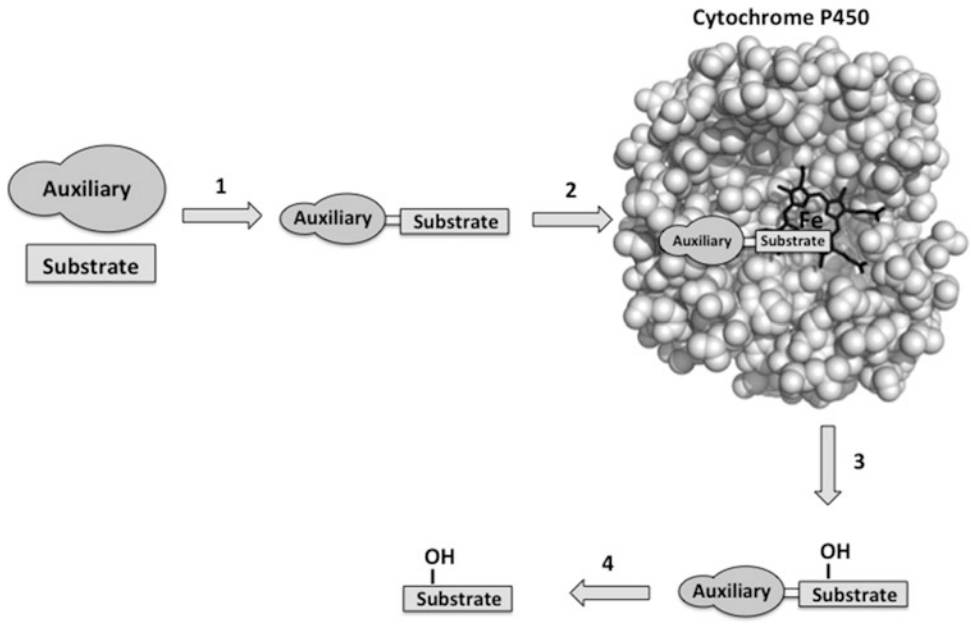


Fig. 8.4 A simplified depiction of the use of chemical auxiliaries to aid in transformation of unnatural substrates by P450s. Typical steps include *1* chemical coupling of the auxiliary to the substrate of interest, *2* incubation of the auxiliary-substrate with the enzyme, *3* oxidation of the non-natural substrate by the P450 enzyme and product release, and *4* chemical cleavage of the auxiliary

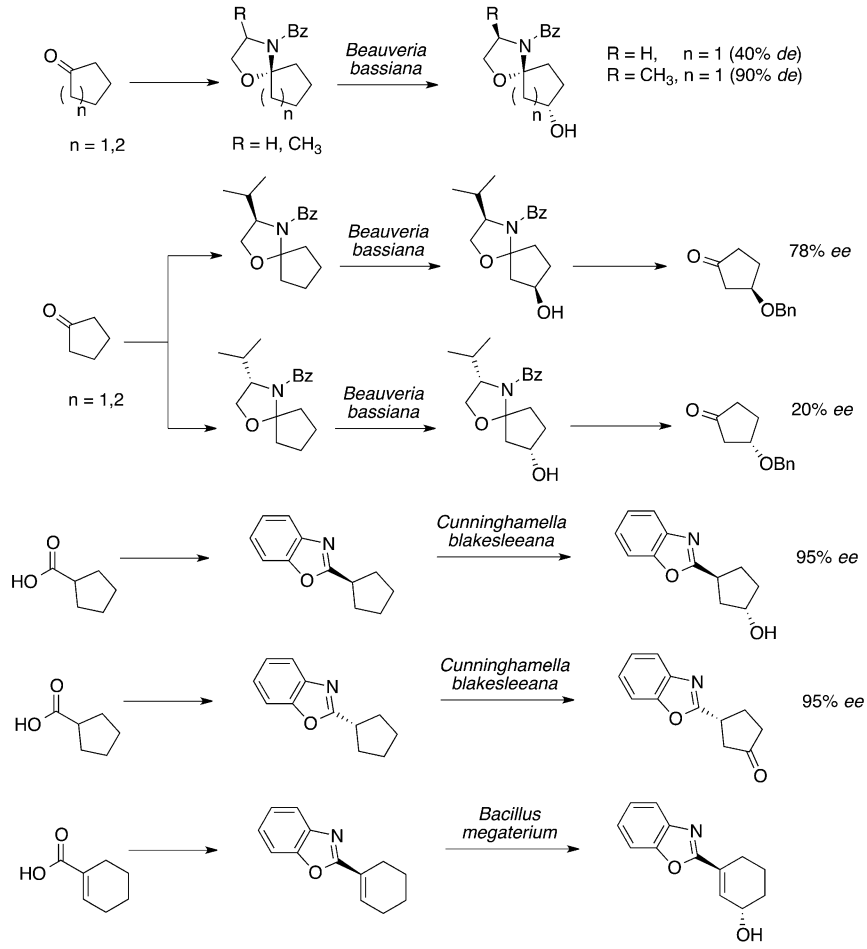


Fig. 8.5 Selected examples from the substrate scope explored by Griengl and coworkers using substrate engineering for biohydroxylation of cyclic alkanes [54, 55]

resulted in more selective hydroxylation by the microorganisms. Reduction, oxidation and other side reactions became minor [54]. A subsequent study demonstrated that regio- and enantioselectivity can be significantly altered by varying the nature of the auxiliary [55]. Although these studies were successful at narrowing down the number of products and favoring hydroxylation, whole-cell biocatalysts frequently yield multiple products. Purified enzymes offer a more defined system, often allowing structure-activity relationships to be drawn. Enzymes remain a popular choice for biocatalysts [57–59].

8.4.1 Bacterial P450s

Building on the work of Weber and coworkers, Munzer et al. [60] applied the 2-aminophenol chemical auxiliary for the expansion of the substrate scope of $P450_{BM3}$. Their work combined the use of substrate engineering and protein mutagenesis. Thus, 2-aminophenol was used as a chemical auxiliary for establishing cyclopentanoic acid as a substrate of various $P450_{BM3}$ mutants. Cyclopentanoic acid was not turned over in the absence of the auxiliary, and only two out of the four possible

diastereoisomers were produced when the carboxylate was cyclized into a benzoxazole.

In a separate study by the same group, protecting-group manipulations at the anomeric carbon of globally-protected monosaccharides were used to control the regioselectivity of $P450_{BM3}$-catalyzed deprotection elsewhere on the ring (Fig. 8.6) [38]. For example, their work showed that benzylation at the anomeric position turns globally-methylated galactose and glucose into $P450_{BM3}$ substrates that are regioselectively deprotected at O-4 by $P450_{BM3}$ F87A or F87I mutants.

Sherman and coworkers investigated the potential of another P450 enzyme for hydroxylation of engineered substrates [56]. Their studies focused on the oxidation of carbocyclic rings covalently linked to a desosamine glycoside auxiliary. A mutant of the macrolide P450 monooxygenase PikC was used [56]. PikC is involved in the hydroxylation of the 12- and 14-membered ring macrolides, YC-17 and narbomycin, respectively, in the pikromycin biosynthetic pathway of *Streptomyces venezuelae* [61, 62]. Although PikC is not naturally self-sufficient, the authors created a self-sufficient fusion mutant, $PikC_{D50N}$-RhFRED, which is ~13 times more active than the wild-type enzyme, making it more attractive as a biocatalyst [63]. This self-sufficient P450 was used in the transformation of engineered substrates. Based on the X-ray crystal structure of PikC complexed with different macrolides, the authors reasoned that the desosamine moiety might position the macrolide substrates in the active site. This reasoning stemmed from the observation that the majority of hydrogen bonds and electrostatic interactions within the active site are formed by the desosamine group of the natural substrates, while the macrolactones are held in place merely through more general hydrophobic interactions [64, 65]. In support of their hypothesis, they first synthesized a series of carbocyclic rings ranging in size from 12 to 15 carbons and linked to a desosamine glycoside, altogether referred to as carbolides. Following reaction with PikC, the conversion yields ranged from 35 % to 65 %, decreasing as the ring size increased (Table 8.4). In each case, however, multiple hydroxylation products were detected. The authors next obtained X-ray crystal structures for two of their carbolides complexed with $PikC_{D50N}$. Based on structure analysis, synthetic standards of the most likely major products were synthesized and the LC-MS retention times were compared to those of the reaction products. This allowed the authors to assign the regioselectivity for the majority of the reaction products, showing that hydroxylation by PikC occurs primarily at sites most distant from the auxiliary. Interestingly, no over-oxidation was observed and no hydroxylation was detected on the auxiliary. Following this, other types of desosamine derivatives were synthesized, both cyclic and linear, and reacted with $PikC_{D50N}$-RhFRED. Transformation of smaller and more rigid cyclic derivatives was not observed, possibly due to their inability to reach the heme-iron oxidized species. The linear substrates produced multiple monohydroxylation products in modest

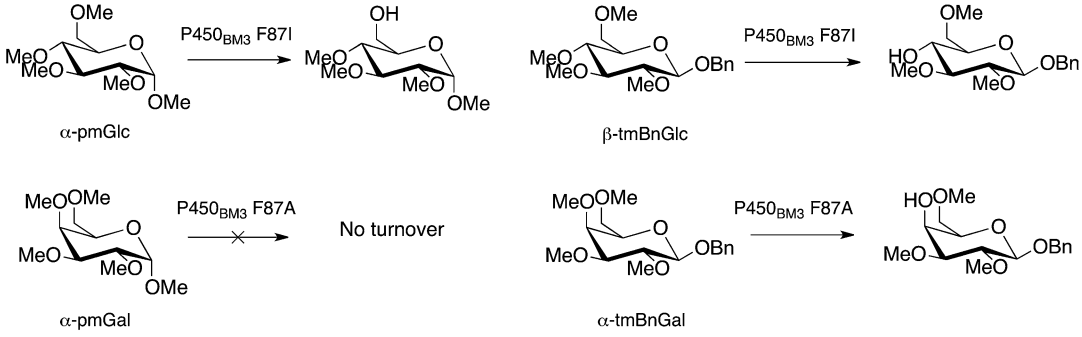
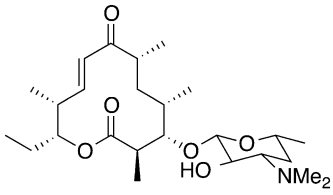
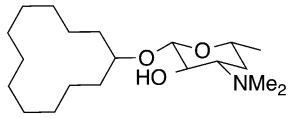
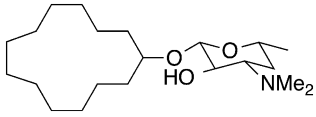
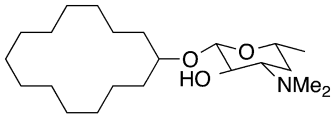
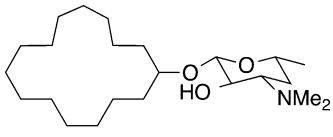
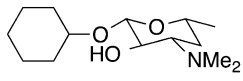
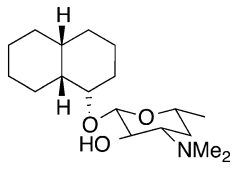
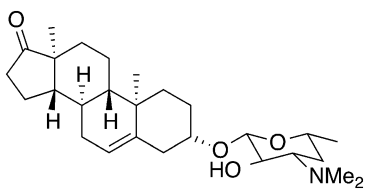


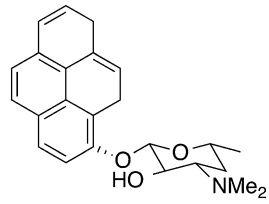
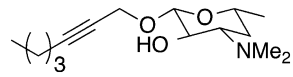
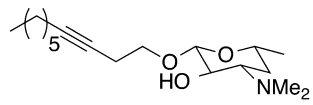
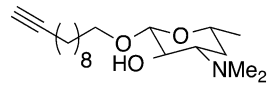
Fig. 8.6 Deprotection of globally-methylated glucose (glc) and galactose (gal) by $P450_{BM3}$ mutants [38]

Table 8.4 The scope and turnover of unnatural substrates by $PikC_{D50N}$-RhFRED [56]

Substrate	K_d (μM)	Yield (%)	No. of products
	19	>99	2
	309	47	7
	218	65	6
	289	63	6
	243	35	9
	NB[a]	0	
	NB	0	0
	NB	<1	0

(continued)

Table 8.4 (continued)

Substrate	K_d (μM)	Yield (%)	No. of products
	>5,000	4	1
	NB	0	0
	2,900	8	5
	2,300	14	7

[a]*NB* no binding

yields, and an aromatic derivative was the only substrate to yield a single oxidation product albeit in fairly low yield. The crystal structures suggest that the inherent flexibility of the carbocycles can lead to multiple binding modes. Moreover, it was established that the desosamine moiety adopts two different binding modes that vary significantly, resulting in the flipping or rotating of the whole substrate, which can further explain the observed poor regio- and stereoselectivities.

8.4.2 Mammalian P450s

Most mammalian P450 enzymes show unequalled substrate promiscuity among P450s [2, 20, 66, 67]. This is an attractive quality for a useful and versatile biocatalyst, and can eliminate the need for protein mutagenesis to expand the substrate scope. While the studies reviewed above aimed at expanding the substrate scope of bacterial enzymes, more recent studies use substrate engineering to control the regio- and stereoselectivity of mammalian P450s in a predictable manner. This was successfully achieved with two human P450 enzymes, P450 3A4 and P450 2E1 [68, 69].

Human P450 3A4 is found mainly in the liver and accounts for the metabolism of about 50 % of all xenobiotics [70]. Highly promiscuous by nature due to its large and flexible active site, substrate prediction with this enzyme has been very challenging. The rules that govern substrate binding and oxidation selectivity have still not been fully defined. Auclair and coworkers have successfully applied substrate engineering using theobromine as the chemical auxiliary for controlling and predicting the regio- and stereoselectivity of P450 3A4-catalyzed oxidations [68].

Drawing inspiration from the known substrate lisofylline, it was envisaged that the theobromine moiety could serve as a chemical auxiliary for

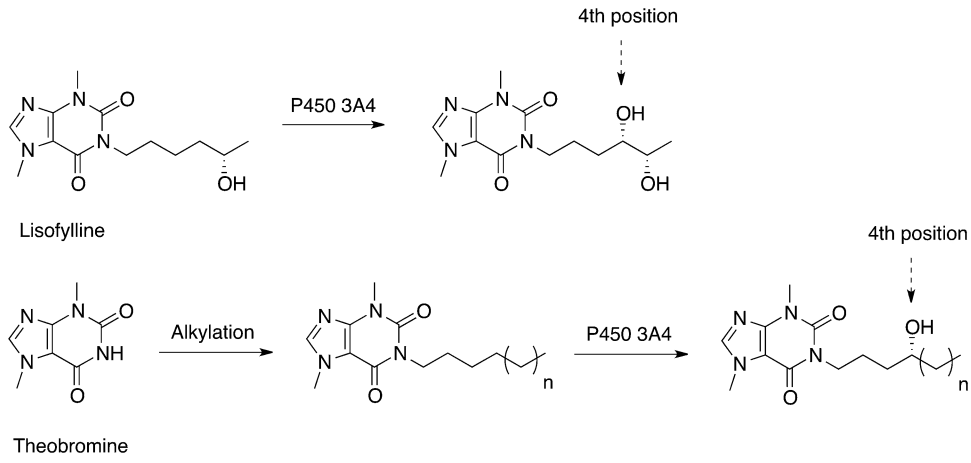


Fig. 8.7 Oxidation of lisofylline and theobromine derivatives by P450 3A4

the transformation of other substrates (Fig. 8.7). Lisofylline is metabolized in part by P450 3A4 via hydroxylation at the fourth carbon from the theobromine moiety [71]. It was therefore hypothesized that substrates covalently bound to theobromine would be hydroxylated at the fourth atom from theobromine.

To investigate the directing potential of theobromine, various small alkanes and alkenes were covalently linked to theobromine and the resulting substrate-auxiliaries were reacted with P450 3A4 in the presence of either CHP alone or NADPH, O_2 and the NADPH-P450 oxidoreductase (CPR) redox partner. CHP was used to bypass the consumption of the expensive NADPH cofactor and CPR, as previously reported [66]. Product distribution and stereoselectivity for the P450 3A4-catalyzed hydroxylation of theobromine-substrates were confirmed to be indistinguishable for both the NADPH/O_2/CPR and the CHP systems. CHP also has the advantage of improving the P450 3A4 initial catalytic rate by 30 % [66]. The conversion yields of theobromine derivatives and product structures were determined using LC-MS and MS fragmentation patterns, by comparison with authentic synthetic standards. As predicted, theobromine successfully directed hydroxylation at the fourth atom away from the auxiliary for all the compounds that had a fourth methylene carbon, or directed epoxidation where a double bond was present at the fourth carbon (Fig. 8.8). Additionally, there was a definite preference for *pro-R* facial selectivity during oxygen atom insertion.

Transformation proceeded with excellent regioselectivity for most substrates while enantiomeric ratios reached 75:25 for hydroxylations and >99:1 for epoxide formation. In all cases, no over-oxidation to the ketone and no oxidation of the auxiliary were observed. After optimization of the conditions, the conversion yields reached 70 %. This was the first example where products were reliably predicted for complex substrates reacted with a promiscuous P450. Further investigations examined functional group tolerance and showed that with the theobromine auxiliary, P450 3A4 was able to selectively oxidize methylene C-H bonds at the fourth position in the presence of nearby 3° C-H bonds, double bonds and heteroatoms (Fig. 8.8). Again, product distribution and stereoselectivity for theobromine-substrates were indistinguishable for both the NADPH/O_2/CPR and CHP systems. Although crystal structures of the theobromine-containing substrates complexed with P450 3A4 have yet to be obtained, in silico docking studies support the idea that theobromine may bind P450 3A4 in a mode conducive to reaction at the fourth carbon from the auxiliary. In contrast to previous studies, this work reports a chemical auxiliary that functions to position the substrates in the active

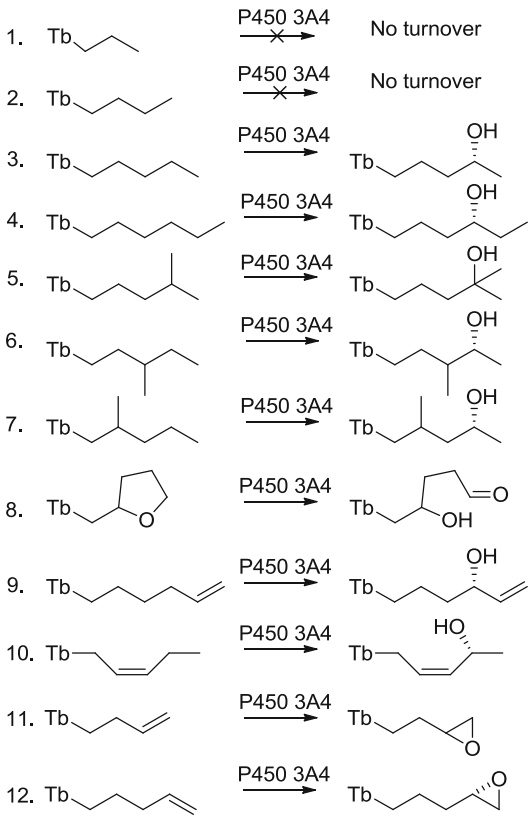


Fig. 8.8 The scope of functionalized theobromine substrates and their conversion products by P450 3A4. Oxidations were performed in the presence of either CHP or NADPH, O_2 and CPR; *Tb* theobromine [68]

site such that, like a ruler, the site of oxidation can be predicted based on the distance from the auxiliary.

While the use of theobromine as a chemical auxiliary shows promise for controlling the site of oxidation by P450 3A4, this system offers a number of other advantages. Theobromine is not only achiral, inexpensive and easily functionalized in high yields, but also contains a chromophore enabling easier purification and isolation of the starting material and products. Additionally, theobromine can penetrate cells allowing for the scale up of this method for fermentation purposes. With this in mind, Larsen et al. investigated yet another potential role for the theobromine auxiliary: facilitating product recovery from whole-cell reaction mixtures [72]. Due to the complex nature of the medium in whole-cell reactions, isolation of the desired product typically accounts for ~80 % of the cost. Extraction of the product often requires large volumes of organic solvents, followed by extensive purification, which is not only costly but also time consuming and generates large volumes of waste. To circumvent these issues, the authors turned to the aid of molecularly imprinted polymers (MIPs) designed to specifically recognize theobromine for isolation of starting materials and products of P450 3A4 reactions [72]. Imprinted polymers are generated by polymerization of monomers and cross-linkers in the presence of a template [73]. The MIPs are expected to have high affinity for molecules resembling the template and can serve to extract such molecules (referred to as target molecules) from a complex mixture [74–81]. The interactions between the polymer and the target molecules are non-covalent interactions, and the affinity is largely based on the complementarity of the size, shape, electrostatics and hydrogen bonding of the MIP with the target molecule. Thus, MIPs were synthesized using various theobromine derivatives as templates. In each case, the MIPs performed very well, with >85 % recovery of the theobromine derivatives and high reusability. The efficacy was maintained whether the target molecules were extracted from dilute, concentrated, aqueous or complex (LB broth) mixtures, and resulted in >90 % purity of the isolated product. In contrast, when the molecule used as the template to generate the MIP was structurally different from theobromine, as with isopropyl-β-D-1-thiogalactopyranoside (IPTG), the recovery of theobromine derivatives dropped below detection. In combination, these results show that chemical auxiliaries such as theobromine can find use not only in controlling the selectivity of P450 transformations but also in facilitating product recovery. This strategy should apply to other systems.

In a similar approach, the Auclair group used type II ligands as chemical auxiliaries to target unnatural substrates to the P450 2E1 active site [69]. Although type II ligands are typically viewed as P450 inhibitors due to stabilization of

the low spin-state via coordination of the heme-iron to an aromatic nitrogen [69, 82, 83], studies have shown that in general, type II binding ligands have a higher affinity for the active site than similar but nitrogen-lacking substrates [84–86]. Moreover, a number of reports have demonstrated that P450s such as P450 3A4 can metabolize type II ligands, and in some cases to a greater extent than related type I ligand analogues [84, 87–91]. In hopes of uncovering complementary selectivity to that observed with the theobromine auxiliary, the authors investigated a variety of type II ligands as chemical auxiliaries for transformation of substrates by P450 2E1 [69]. After screening various pyridines, quinolone and imidazole derivatives, they focused further studies on nicotinate as the chemical auxiliary. Nicotinate looked promising because not only is methyl nicotinate a known type II ligand of P450 2E1 [85], but the naturally occurring P450 2E1 substrate, nicotine-derived nitrosamine ketone (NKK), contains a nicotinate group, positioned a few carbons away from the site of oxidation [92]. In addition, nicotinate is inexpensive, has a chromophore and is easily functionalized, reversibly, at the carboxylic acid group. A series of nicotinate esters were thus generated and reacted with P450 2E1 in the presence of the natural NADPH cofactor and CPR, or CHP [66]. From the results obtained, the authors confirmed the preference of P450 2E1 to oxidize aliphatic CH_2 or alkenyl CH groups furthest from the auxiliary ($\omega-1$). This selectivity has previously been observed for the hydroxylation of fatty acids such as lauric, myristic, palmitic and stearic acids by P450 2E1 [93]. In the absence of the auxiliary, however, none of the substrates tested by the authors were significantly transformed by the enzyme. These results show that type II binding can be used to favor and direct biocatalysis.

Although the use of chemical auxiliaries in biocatalysis requires two extra steps, this is often offset by a number of advantages, the key one being that the desired products might otherwise require several synthetic steps to be prepared. Moreover, the chemical auxiliaries can be used to facilitate detection and recovery of the products.

8.5 Conclusion

Overall, substrate engineering is largely unexplored in the area of P450 biocatalysis. However, the successful stories summarized here demonstrate the high potential of this strategy.

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