# Towards the use of P450 enzymes in synthesis: cofactor replacement and activity of CYP3A4 in non-aqueous media

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## ABSTRACT

Enantioselective synthesis is one of the most important challenges of today's synthetic chemists. In particular, the hydroxylation of non-activated C-H bonds remains a significant challenge that few chemical catalysts have succeeded to overcome. The P450 enzymes, a family of heme-containing monooxygenases including more than 5000 known isoforms, are gaining considerable attention due to their ability to catalyze the very difficult regio- and stereo-selective oxidation of inactivated C-H bonds. The use of such enzyme is however limited by their functional complexity, low activity, need for cofactors, and poor stability. In this thesis, we elected to study the human P450 CYP3A4, because of its high substrate promiscuity. The first part of the project involved the replacement of the required cofactors (NADPH and cytochrome P450 reductase) by some cheap hydrogen peroxide donors or organic peroxides. Several surrogates, such as sodium percarbonate and cumene hydroperoxide, were found to be efficient at replacing the natural cofactor, without a significant loss of stability and activity. The second part of this thesis deals with optimization of the lyophilization conditions. Among the numerous additives tested, some sugars led to significant lyoprotection during the freeze-drying process. Finally, in the third part, the effect of the presence of organic solvents and ionic liquids on CYP3A4 activity was evaluated.

# RÉSUMÉ

La synthèse enantiosélective demeure l'un des plus importants défis en chimie organique. L'hydroxylation de liaisons C-H non activées est tout particulièrement problématique, et rares sont les catalyseurs chimiques capables de surmonter ce problème. Les enzymes P450 forment une superfamille de mono-oxygénases, incluant plus de 5000 isoformes, et impliquées notamment dans le métabolisme des xénobiotiques. Leur faculté à catalyser l'oxidation régio- et stéreo-sélective de liaisons C-H non activées leur a valu de susciter un intérêt grandissant au cours des dernières années. L'utilisation des enzymes P450 se heurte cependant à de nombreux obstacles, tels que leur faible activité et stabilité, et leur dépendance vis-à-vis de cofacteurs et de partenaires redox. Nous avons choisi d'étudier l'isoforme humain CYP3A4, en raison de son abilité à transformer une multitude de substrats. La première partie du projet avait pour objectif d'identifier des péroxides susceptibles de remplacer efficacement les cofacteurs naturels de l'enzyme. Puis les conditions de lyophilisation ont été optimisées. Finalement, l'activité de CYP3A4 a été evaluée en présence de solvants organiques et de liquides ioniques.

## ACKNOWLEGEMENTS

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Last but not least, I would like to give my special thanks to Mathieu whose patient love enabled me to complete this work.

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## ABBREVIATIONS

| | | |
|---|---|---|
| 6α-OHT | ..... | 6α-hydroxytestosterone |
| 6β-OHT | ..... | 6β-hydroxytestosterone |
| Å | ..... | armstrong |
| ACN | ..... | acetonitrile |
| BFC | ..... | 7-benzyloxy-4-trifluoromethylcoumarin |
| BME | ..... | β-mercaptoethanol |
| BSA | ..... | bovine serum albumin |
| CHAPS | ..... | 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate |
| CHP | ..... | cumene hydroperoxide |
| CPR | ..... | cytochrome P450 reductase |
| CYP | ..... | cytochrome P450 enzyme |
| CYP3A4 | ..... | human cytochrome P450 3A4 |
| Cyt c | ..... | cytochrome c |
| DAD | ..... | diode array detector |
| DCM | ..... | dichloromethane |
| DMF | ..... | *N,N*-dimethylformamide |
| DMSO | ..... | dimethyl sulfoxide |
| DTT | ..... | dithiothreitol |
| EDTA | ..... | ethylenediaminetetraacetic acid |
| FAD | ..... | flavin adenine dinucleotide |
| FMN | ..... | flavin mononucleotide |
| FTIR | ..... | Fourrier transform infrared spectroscopy |
| g | ..... | gram |
| h | ..... | hour |

| | | |
|---|---|---|
| $H_2O_2$ | ..... | hydrogen peroxide |
| HFC | ..... | 7-hydroxy-4-trifluoromethylcoumarin |
| HPLC | ..... | high performance liquid chromatography |
| hrs | ..... | hours |
| ILs | ..... | ionic liquids |
| IPTG | ..... | isopropyl-β-D-thiogalactoside |
| kD | ..... | kilo dalton |
| LB | ..... | Luria-Bertani broth |
| LC-MS | ..... | liquid chromatography mass spectroscopy |
| M | ..... | molar |
| MeOH | ..... | methanol |
| MFC | ..... | 7-methoxy-4-trifluorocoumarin |
| mg | ..... | miligram |
| min | ..... | minute |
| ml | ..... | mililiter |
| mol | ..... | mole |
| MOPS | ..... | 3-[*N*-morpholino]propanesulfonic acid |
| MW | ..... | molecular weight |
| NADP | ..... | nicotinamide adenine dinucleotide phosphate, oxidized form |
| NADPH | ..... | nicotinamide adenine dinucleotide phosphate, reduced form |
| Ni-NTA | ..... | nickel nitrilotriacetic acid resin |
| nm | ..... | nanometer |
| NMR | ..... | nuclear magnetic resonnance |
| $OD_{600}$ | ..... | optical density at 600 nm |
| P450 | ..... | cytochrome P450 enzyme |
| PMSF | ..... | phenylmethanesulfonyl fluoride |

| | | |
|---|---|---|
| r.t. | ..... | room temperature |
| RH | ..... | typical substrate of P450 enzymes |
| ROH | ..... | typical product of the action of P450 enzymes on RH |
| rpm | ..... | rotation per minute |
| SDS-PAGE | ..... | sodium dodecyl sulfate- polyacrylamide gel electrophoresis |
| sec | ..... | second |
| SPB | ..... | sodium perborate |
| SPC | ..... | sodium percarbonate |
| std. dev. | ..... | standard deviation |
| TB | ..... | terrific broth |
| tBHP | ..... | *tert*-butylhydroperoxide |
| TFA | ..... | trifluoroacetic acid |
| THF | ..... | tetrahydrofuran |
| Tst | ..... | testosterone |
| UHP | ..... | urea-hydrogen peroxide adduct |
| UV | ..... | ultraviolet |
| v/v | ..... | volume/volume |
| w/v | ..... | weight/volume |
| λ | ..... | wavelength |
| λem | ..... | emission wavelength |
| λex | ..... | excitation wavelength |
| δ-ALA | ..... | δ-aminolevulinic acid |

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## PREAMBLE TO THESIS

The work related to cofactor replacement presented in this thesis (section 2.2.3) has been published in a communication (A. Chefson, J. Zhao, and K. Auclair, Replacement of natural cofactors by selected hydrogen peroxide donors or organic peroxides results in improved activity for CYP3A4 and CYP2D6, *ChemBioChem* 2006, 7, 916–919). Jin Zhao was responsible for the work with CYP2D6 and I carried out the experiments with CYP3A4.

The section 1.3 from the introduction has also been partially published as a Highlight (A. Chefson, K. Auclair, Progress towards easier use of P450 enzymes, *Mol. Biosys.*, 2006, 2, 462-469).

# 1. INTRODUCTION

This introduction will describe first the use of enzymes in organic solvents, followed by a quick overview of P450 enzymes, and finally the recent advances towards the use of P450 enzymes in synthesis.

## 1.1. BIOCATALYSIS IN NON AQUEOUS SOLVENTS

Enzymes are Nature's catalysts. They can carry out chemical reactions with an efficacy that exceeds that of synthetic industrial catalysts by far. These biocatalysts are most often highly regio- and stereo-selective, sometimes catalyze reactions inaccessible by chemical means, and work under mild conditions. These qualities make them the ideal "green" catalysts and have motivated their use in synthesis. The need for enantiomerically pure chemicals is also responsible for the growing interest in enzymatic asymmetric synthesis.

Many biocatalytic processes have been optimized and are useful to chemists and industrials, in particular to carry out enzymatic resolutions.[1] The examples of such applications are far too numerous to be cited here exhaustively, but some of the most widespread applications are enzyme-based resolution procedures for amino acids via the hydrolysis of their esters, using α-chymotrypsin, subtilisin, lipases, or proteases.[2] Among the frequently used hydrolases, lipases have received the more attention, and have been successfully used for the resolution of lactones,[3] β-lactams,[4] bicyclic 1-heteroaryl amines,[5] (-)-ormeloxifene intermediates,[6] and cyclopropane derivatives.[7]

Enzymes have evolved to be active under aqueous conditions; however, the use of enzymes in organic solvents is of great interest to organic chemists. Indeed, most substrates and products used in organic synthesis are poorly soluble or even unstable under aqueous conditions, and water can also give rise to unwanted side-reactions, or shift the thermodynamic equilibrium away from the desired reaction. Finally, due to the high boiling point of water, product recovery may be difficult. All these reasons have stimulated the search for systems based on organic solvents, despite the early beliefs that enzymes could only be active in aqueous environments. Studies over the past 15 years have firmly established not only that some enzymes can perform perfectly well in non-aqueous media, but also that they can catalyze new reactions, be more stable, and exhibit new behavior such as enzyme memory. Since the early work by Klibanov,[8] Buttler,[9] and Wong[10] in the early 80s, the number of publications on enzymes in organic synthesis has constantly increased over the years, and a number of excellent reviews have been published in the recent years.[11,12,13,14,15,16,17,18,19,20]

### 1.1.1. Enzyme activity in organic media

Several approaches have been successful to use enzymes in non-aqueous conditions. The addition of water miscible co-solvents has been studied,[9] most often yielding a sharp decrease of activity with increasing amounts of solvents. Biphasic aqueous-organic systems have shown more promising results with enzymes. The mass transfer can be facilitated by the formation of reverse micelles, whose stabilization is achieved using surfactants.[21,22,23] Finally, nearly anhydrous organic solvents (< 0.02% water) have also been used with success. This alternative has received a considerable amount of attention among synthetic chemists during the last two decades.[24]

At first sight, the substitution of bulk water with organic solvents seems detrimental in the light of the conventional idea that enzymes are denatured in organic solvents. The initial observation that enzymes may lose their activity in aqueous-organic mixtures yet retain most activity in pure organic solvents was astonishing. By investigating the secondary structure of lysozyme and subtilisin, Klibanov and coworkers have shown that although proteins are denatured in aqueous-organic mixtures (acetonitrile, tetrahydrofuran and 1-propanol), many enzymes remain essentially intact in the corresponding anhydrous solvents.[25] This is explained by the enzyme rigidity in the absence of water, which normally acts like a molecular lubricant.[26] With such a rigid conformation, the enzyme capacity to undergo denaturation is severely impaired. A number of other studies confirm that various enzymes retain their native structure in selected anhydrous solvents.[27,28,29,30,31]

Even though the activity is usually lower in neat organic solvents than in water, effective strategies to improve the activity in solvents are emerging. In particular, it has been shown that the nature of the organic solvent is very important. The most hydrophobic solvents are superior to maintain the enzymatic activity. This can be explained by the ability of hydrophilic solvents to strip the essential water molecules away from the enzyme molecule.[32] Laane et al.[33] found a direct correlation between activity and solvent hydrophobicity, expressed as the *log P*, where P is the partition coefficient of the solvent in an octanol/water biphasic system. They established this correlation for yeast and mold lipases, 20β-hydroxysteroid dehydrogenase and xanthine oxidase. This study led to the rule that hydrophilic solvents with $\log P < 2$ often lead to enzyme deactivation, while apolar solvents with $\log P > 4$ seems to be more compatible with enzymes. No clear correlation is observed for the region $1 < \log P < 4$. This trend

has been confirmed by many others; however it should be added that it does not apply to all enzymes.

### 1.1.2. How much water do enzymes need?

It was soon realized that the amount of water around the enzyme was critical for activity in organic solvents, and the real question became how much of it is needed.[34] The enzyme cannot "see" more than a monolayer or so of water at its surface. As long as the hydration shell is maintained around the enzyme, the majority of bulk water can be replaced with organic solvents without loss of the active conformation. The optimum amount of water often lies within a narrow range, and is very specific for each enzyme. Proteases and lipases are similar in their water requirement,[32] while other enzymes might require various amounts of water. The thermodynamic water activity ($a_w$) is the preferred measure to quantify the amount of water present in a reaction system. It can be determined by a simple laboratory instrument which uses an electrolytic measurement cell, and is calibrated with saturated salt solutions of known water activity. Several methods have been used to fix the water activity of reaction mixtures.[35] The first obvious one is to directly add a known amount of water into the anhydrous solvent. Alternatively, both the organic solvent and the substrate solution can be equilibrated in atmospheres of known water activity, typically using a saturated solution of an appropriate salt (**Figure 1**). A wide range of water activities can be obtained using this method (**Table 1**).

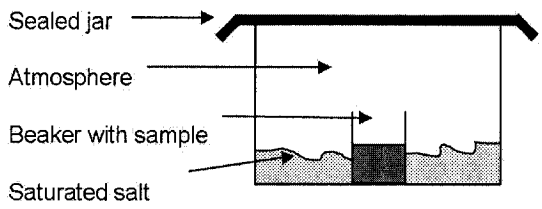



**Figure 1.** Equilibration of samples using saturated salt solution.

**Table 1.** Saturated salt water activities at 25°C

| | |
|---|---|
| LiCl | 0.113 |
| KAc | 0.225 |
| $MgCl_2$ | 0.328 |
| $K_2CO_3$ | 0.432 |
| $Mg(NO_3)_2$ | 0.529 |
| NaBr | 0.576 |
| KI | 0.689 |
| NaCl | 0.753 |
| KCl | 0.843 |
| $KNO_3$ | 0.936 |
| $K_2SO_4$ | 0.973 |

Another method is to pre-equilibrate the organic solvent and substrate solution by addition of salt hydrates pairs. As long as both forms of the salt remain present at equilibrium, the water activity will be fixed to a value characteristic of the pair chosen. Here again, different water activities can be obtained by choosing different salt pairs, as shown in **Table 2**.

**Table 2.** Selected salt pairs useful for water activity control in biocatalysis

| Salt pair | Equilibrium $a_w$ at 25°C |
|---|---|
| NaI.2/0[a] | 0.12 |
| $Na_2HPO_4$.2/0 | 0.16 |
| $LiSO_4$.1/0 | 0.17 |
| NaAc.3/0 | 0.28 |
| NaBr.2/0 | 0.35 |
| $Na_2S_2O_3$.5/2 | 0.37 |
| $Na_2P_2O_7$.10/0 | 0.49 |
| $CaHPO_4$.2/0 | 0.50 |
| $Na_2HPO_4$.7/2 | 0.61 |
| $Na_2HPO_4$.12/7 | 0.80 |
| $Na_2SO_4$.10/0 | 0.80 |

[a]The pairs are identified by a shorthand notation: NaI.2/0 means a combination of $NaI.2H_2O$ and anhydrous NaI.

### 1.1.3. New properties in organic solvents

As mentioned above, enzymes have a very restricted conformational mobility in nonpolar solvents. Interestingly, this remarkable feature of enzymes in organic solvents makes it possible to alter the selectivity and increase the activity by using a memory effect. For example by lyophilizing (freeze-drying) the enzyme in the presence of a specific inhibitor or substrate,[36,37] a given conformation of the enzyme can be trapped or literally imprinted onto the enzyme (**Figure 2**). This unique property, called molecular imprinting, is limited to nearly anhydrous media where enzymes are sufficiently rigid to maintain the imprint-induced activated conformation. The addition of more than 0.1 % water in the organic solvent was shown to cancel the imprinting effect, due to an increase in enzyme flexibility upon rehydration. [37,37,38]

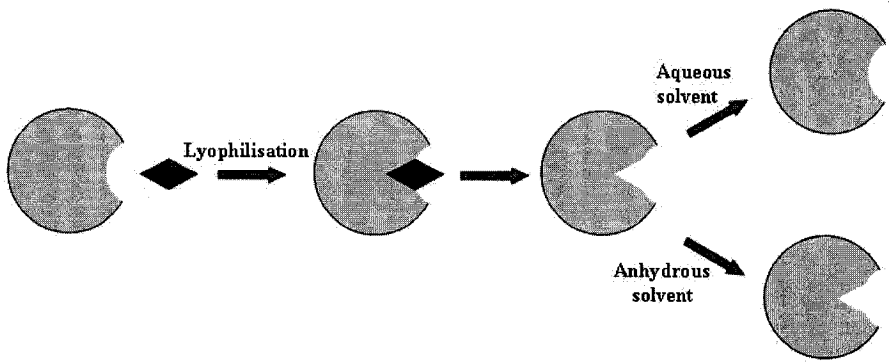



**Figure 2**. Principle of molecular imprinting

The pH is one of the key factors for enzyme activity, yet it has a different meaning in organic solvent. Under non-aqueous conditions, enzymes exhibit what has been termed a "pH memory effect". Enzyme powders suspended in organic solvents are maximally active if lyophilized from a buffer corresponding to optimum pH determined in aqueous solutions.[32,39] This yields a protein in the ionization state necessary for catalysis.

### 1.1.4. Applications

Of all enzymes, hydrolases are the most employed for industrial biotransformations. It is estimated that approximately 80% of all industrially used enzymes are hydrolases. Other enzyme families will probably gain more importance in the near future. Lyases and isomerases have attracted interests for industrial applications because of their unique properties. Oxido-reductases are cofactor-dependent, thus requiring an efficient regenerating system. Because they catalyze very interesting reactions, they are currently gaining immense attention. The P450 enzymes presented in the following chapters are an example of monooxygenases with a large potential for industrial applications.

## 1.2. P450 ENZYMES

### 1.2.1. Generalities and nomenclature

Cytochrome P450 enzymes (P450s or CYPs) are heme monooxygenases found in all five kingdoms of life. The P450s were named in 1962 by Omura and Sato[40] after the observation of a unique absorption band at 450 nm for these enzymes following reduction and binding to carbon monoxide. These ubiquitous enzymes form a large superfamily, and are categorized into families and subfamilies according to amino acid sequence similarity. A general nomenclature based on sequence homology was proposed by Nelson *et al.*[41]. In this system, the enzymes are considered to belong to the same family when the homology is greater than 40%, and this is designated by an Arabic numeral. Greater homology ($\geq$55%) classifies them in the same subfamily, designated by a capital letter. Finally a number denotes the individual enzyme. For instance, CYP3A4 is

a P450 isoform belonging to family 3 and sub-family 3A. According to the P450 homepage regularly updated by David Nelson,[42] the number of cytochrome P450s as of Januray 8th 2005 included 1581 animal, 1740 plant, 203 lower eukaryote, 508 fungi and 472 bacterial sequences, for a total of 4504 different natural P450 sequences known.

P450s are involved in the biosynthesis of secondary metabolites such as antibiotics, immunosuppressants and antitumors in bacteria and fungi, and hormones, steroids and vitamins in mammals. A key role of p450s in mammals is the metabolism of exogenous substances, including most of the drugs and pollutants. Human P450s are involved in the metabolism of more than 90% of the current pharmaceuticals.[43] CYP3A4 itself contributes to approximately 30% of the total CYP content in the liver,[44] and is estimated to be responsible for the metabolism of more than 60% of drugs currently available on the market.[45] Many P450s add a hydroxyl group to molecules during Phase I of drug metabolism. The more hydrophilic compound produced is then more easily excreted, or modified by conjugation (Phase 2 of drug metabolism). Some metabolites can also be toxic or carcinogenic. For example aflatoxin B1 is oxidated to a carcinogenic compound by CYP3A4 in the liver, which is one of the major causes of liver cancer in the world.[46] P450 catalysis is also required to activate some prodrugs. For example, encainide is actually devoid of pharmacological activity and needs to be *O*-demethylated by CYP2D6 to become an effective anti-arrhythmic.[47] **Table 3** lists the main function of some human P450 subfamilies.

**Table 3.** Human P450 families and their main functions

| Isoform | Function |
|---|---|
| CYP1 | Drug metabolism |
| CYP2 | Drug metabolism and steroid biosynthesis |
| CYP3 | Drug metabolism |
| CYP4 | Arachidonic acid and fatty acid biosynthesis |
| CYP5 | Thromboxane A2 synthase |
| CYP7A | Bile acid biosynthesis |
| CYP7B | Brain specific form of 7-α hydroxylase |
| CYP8A | Prostacyclin synthase |
| CYP8B | Bile acid biosynthesis |
| CYP11 | Steroid biosynthesis |
| CYP17 | Steroid biosynthesis (17-α hydroxylase) |
| CYP19 | Steroid biosynthesis |
| CYP20 | Unknown |
| CYP21 | Steroid biosynthesis |
| CYP24 | Vitamin D degradation |
| CYP26A | Retinoic acid hydoxylase |
| CYP26B | Probable retinoic acid hydroxylase |
| CYP26C | Probable retinoic acid hydroxylase |
| CYP27A | Bile acid biosynthesis |
| CYP27B | Vitamin D3 1-α hydroxylase, activation of vitamin D3 |
| CYP27C | Unknown |
| CYP39 | 7-α hydroxylation of 24-hydroxycholesterol |
| CYP46 | Cholesterol 24-hydroxylase |
| CYP51 | Cholesterol biosynthesis, lanosterol 14-α demethylase |

### 1.2.2. Classes of P450 enzymes

P450s can be divided into four classes depending on the associated reductase components used by the enzymes to accept electrons from NADPH. The molecular organization for each of these classes is shown on **Figure 3**. Mitochondrial and most bacterial P450s are three component systems, comprising a P450, a ferredoxin and a

NADH-dependent, FAD-containing ferredoxin reductase (**Class I**). Eukaryotic class I P450s are associated with the mitochondrial membrane. Bacterial P450s on the other hand are soluble enzymes. The microsomal P450s (**Class II**), form a membrane-bound, two component system, including a NADPH-dependent diflavin reductase (FAD and FMN) and the P450. **Class III** P450s, such as P450 BM3 from *Bacillus megaterium*, contain the same cofactors as the class II P450s but are soluble and fused into one continuous polypeptide. **Class IV** P450s, recently discovered in *Rhodococcus,*[48] are also soluble, one component enzymes but contain NADPH-dependent, FMN-containing reductase and ferredoxin fused to the heme domain.

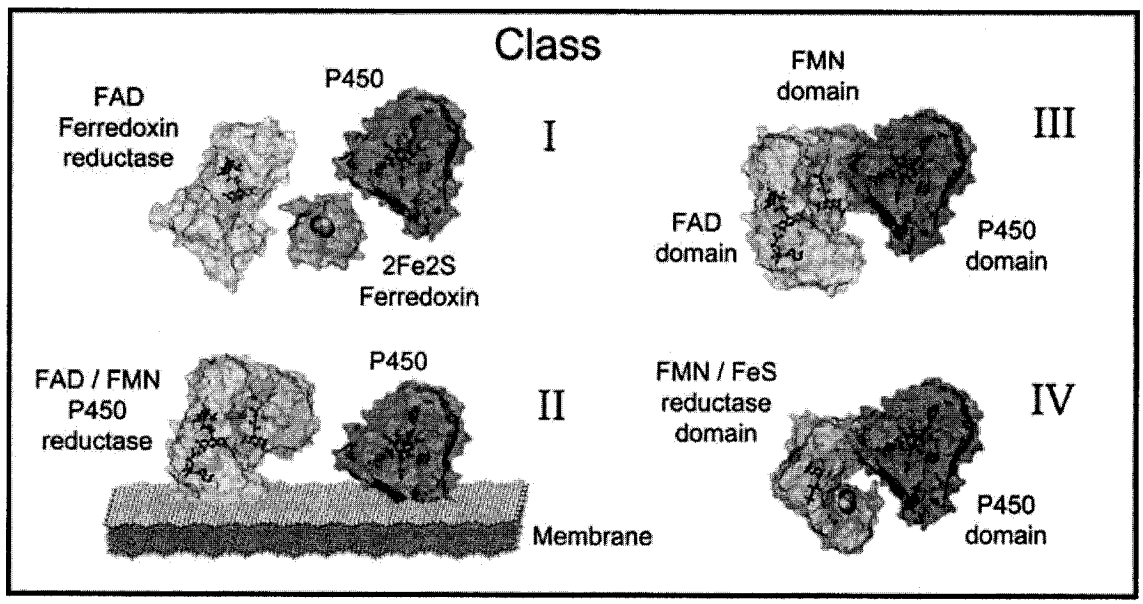



**Figure 3.** Schematic representation of the different classes of cytochrome P450 systems. Class I systems comprise a FAD-containing flavodoxin reductase, an iron-sulfur protein (ferredoxin), and the P450. In a class II system, the P450 is partnered with a diflavin reductase, whereas in the class III system, the diflavin reductase is fused to the P450. The new class IV system is made up of an FMN-containing reductase with a ferredoxin-like center linked to a P450 in a single polypeptide.

### 1.2.3. Structure of P450 enzymes

P450cam was the first P450 for which a crystal structure was reported in 1987,[49] and remained the paradigm for P450 structure-function studies until the 3D structure of P450BM3 was solved in 1993.[50] Since then the number of published P450 crystal structures has dramatically increased, and at present there are at least 155 such structures deposited in the Protein Data Bank. Crystal structures of mammalian P450s have been somewhat difficult to obtain because of their membrane embedment. CYP2C5[51] and CYP2B4[52] were the first mammalian P450s for which structures were determined. CYP2C8[53], CYP2C9[54], CYP3A4[55,56], CYP2A6[57] and CYP2D6[58] are the only human P450s crystallized so far. P450 enzymes share a common overall fold and topology, as shown for CYP3A4 in **Figure 4**.

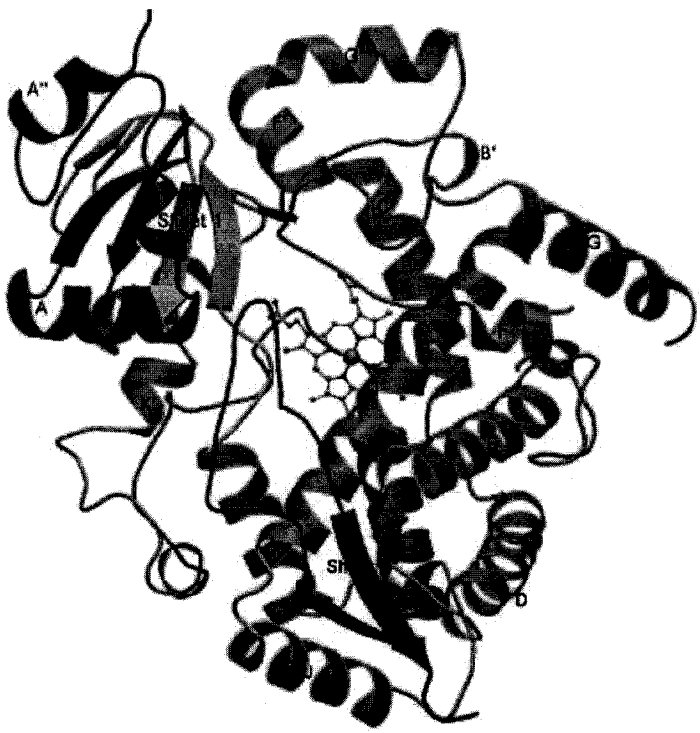



**Figure 4.** Overall fold of CYP3A4. The heme is depicted as a ball-and-stick model in the center of the molecule, flanked by helix I. Helix A", not present in other P450 structures, is shown at the top left of the structure.[55]

The conserved P450 core is a four-helix bundle composed of three parallel helices (D, L and I) and one antiparallel (E). The F and G helices form both sides of the substrate access channel and the ceiling of the active site. The prosthetic heme group is located between the proximal helix L and the distal helix I. The heme iron is bound to a conserved cysteine thiolate. The porphyrin is bound via salt bridges between the propionates and lysine (Lys), arginine (Arg) or histidine (His) side chains[59], and hydrophobic interactions to the pyrroles. The active sites are very similar for all P450 structures reported so far[60] (**Figure 5**). In the resting state, a molecule of water is coordinated to the iron on the distal side of the heme. This species is referred to as a 6-coordinated low spin (6cLS) enzyme. The residues lining the interior of the substrate access channel are believed to play an important role in determining specificity and orientation of the substrate as it enters the active site.[61]

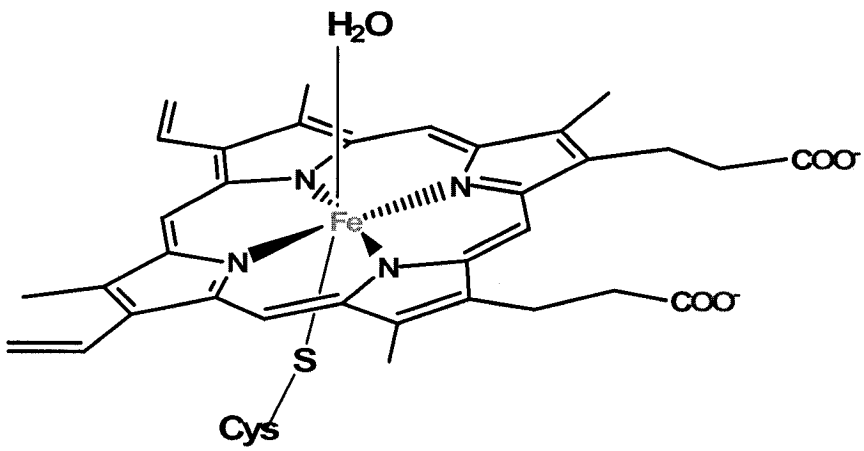



**Figure 5.** P450 enzymes active site (heme) in the resting state.

### 1.2.4. P450 mechanism

P450s are believed to share a common mechanism,[62] summarized in **Figure 6**. Mechanistic information on this cycle has come largely from studies of P450cam, yet

assumed to hold for all P450s. As described in the previous paragraph, the P450 heme iron is proximally ligated by a cysteine-thiolate, and in the resting state, the ferric iron is distally ligated by water. In the first step of the catalytic cycle, the molecule of water is released from the active site pocket upon binding of the substrate. A 5-coordinate high spin (5cHS) species results. The 6cLS and 5cHS can be differentiated by UV/Vis spectroscopy since they absorb near 416 nm and 390 nm respectively. This change in absorbance is often referred to as a type I shift of the Soret band. The second step of P450 catalysis involves the reduction of iron from ferric to ferrous, via transfer of one electron from NADPH to the enzyme. This step requires the participation of a redox partner (see next section for details). The reduced system easily binds oxygen at the iron to form an oxyferrous complex. By addition of a proton and transfer of a second electron from NADPH, a hydroperoxide complex is produced. This intermediate is rapidly transformed to an oxo-ferryl complex, the most reactive species of this cycle. This iron oxo intermediate abstracts a hydrogen atom from the substrate (RH) to generate a radical (R·) which recombines with the hydroxyl radical to yield the product (ROH). As the hydroxylated product is released, a water molecule binds the iron to regenerate the resting state of the enzyme.

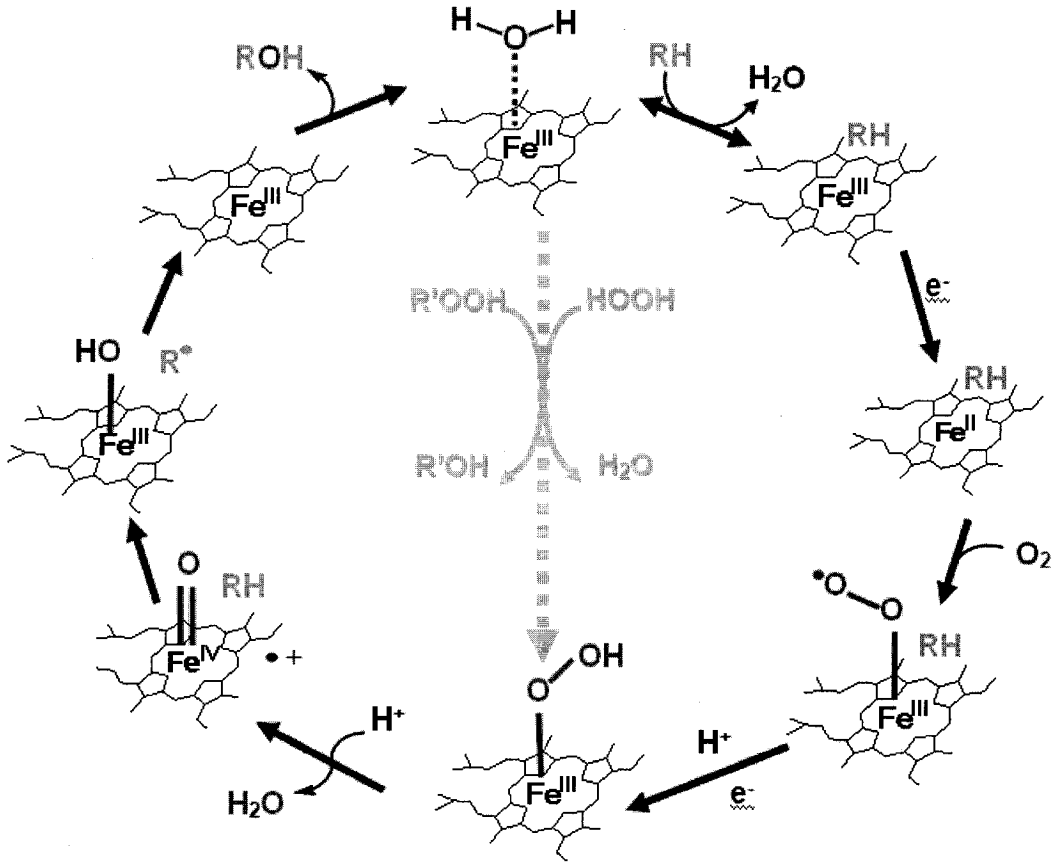



**Figure 6.** Currently accepted catalytic cycle of P450 enzymes. The cycle is initiated by binding of the substrate to the ferric P450, allowing the first electron transfer to iron and binding of oxygen. A second electron transfer results in the formation of the iron-peroxo species which loses water to form the oxyferryl intermediate. This intermediate reacts with the substrate to yield the hydroxylated product which diffuses from the active. The dashed arrow shows the "peroxide shunt" pathway.

There is however a "peroxide shunt" pathway that bypasses the electron transfer steps and leads to oxidative activity independent of NAD(P)H and the redox partner. In this pathway (dashed arrow in **Figure 6**), hydrogen peroxide or organic peroxides react with the low spin ferric enzyme to directly form the $Fe^{III}$-OOH species. This pathway is not believed to be significant *in vivo* nevertheless it may be advantageous in enzymatic synthesis. Potential use of this alternative pathway will be discussed further in section 1.3 of this introduction.

### 1.2.5. P450 reactions and substrate characteristics

The general reaction catalyzed by P450s can be summarized by **Equation 1** below. Overall, P450 enzymes are monooxygenases that require NAD(P)H as an electron donor and a redox partner to transfer these electrons between NAD(P)H and the P450. P450s reduce one molecule of dioxygen to introduce an oxygen atom into one of the C-H bonds of the substrate (RH), and recycle the second oxygen atom to water.

$$\mathbf{RH + O_2 + NADPH + H^+ \xrightarrow{P450 + CPR} ROH + NADP^+ + H_2O} \qquad \textbf{(Eq 1)}$$

Hydroxylations at inactivated C-H bonds are only one example of the numerous reactions catalyzed by P450 enzymes. Other reactions such as *N*-, *S*-, and *O*-dealkylation, *N*-oxidation, sulfoxidation, and epoxidation are also catalyzed by these enzymes.[63,64] The most common classes of reactions are listed in **Table 4** below, and illustrated on **Table 5**. Among these reactions, enantio- and regio-selective hydroxylation of inactivated C-H bonds is undoubtedly of major interest to organist chemists. Many research groups have attempted to develop chemical catalysts for this transformation, yet it remains a difficult task. P450 enzymes represent ideal candidates for the development of such catalysts.

The substrate promiscuity of P450 enzymes is another advantage that motivates studies to optimize their use as biocatalysts. Some P450s are highly specific for one or two substrates whereas others tolerate an exceptionally large number of substrates. In particular, the human P450s CYP2D6 and CYP3A4 show very high promiscuity in substrate specificity, and have more than 200 known substrates each. The lack of crystal structures for many mammalian P450 isoforms led researchers to carry numerous modeling experiments and structure-activity relationships (SARs) studies.[65,66,67,68] These helped understand the chemical and physical characteristics required by each isoform for

a compound to be its substrate, but predictions remain highly inaccurate. Most of the known P450 substrates are large hydrophobic molecules.[69] SARs studies by Lewis *et al.* revealed that CYP1A2 usually accommodates planar molecules, neutral or weakly basic, whereas CYP3A4 is more specific for large compounds, with neutral or basic character. The substrates of CYP2E1 are generally small neutral molecules. Those of CYP2C9 have the characteristic of being weakly acidic whereas CYP2D6 substrates tend to be very diverse and weakly basic.[70] Some general characteristics of human P450 substrates are summarized in **Table 6**.

**Table 4.** Main classes of reactions catalyzed by cytochrome P450 enzymes[71,72,73]

| **Reaction type** |
|---|
| Aliphatic and aromatic hydroxylation |
| *N-O*-dealkylation |
| Heteroatom oxidation |
| Epoxidation from alkenes and aromatic rings |
| 1,2-Group migration |
| Carbon–carbon bond cleavage |
| Oxidation of unsaturated ring systems |
| Isomerization |
| Reduction |
| Desaturation |
| Oxidation of alcohols |
| Oxidative cleavage of esters |
| Ring expansion |
| Ring formation |
| Ring opening |
| One electron oxidation |
| Aldehyde scission |
| Dehydration |
| Dehydrogenation |
| Coupling reaction |

**Table 5.** Selected reactions catalyzed by P450 enzymes

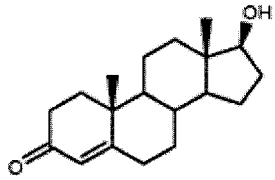
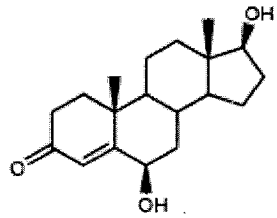
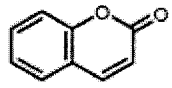
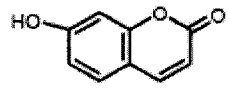
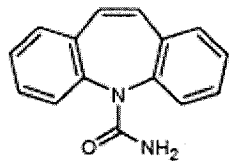
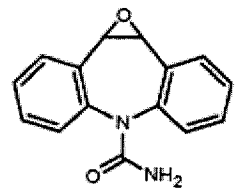
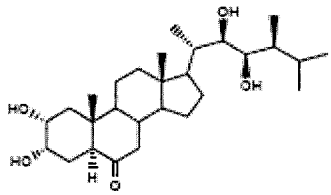
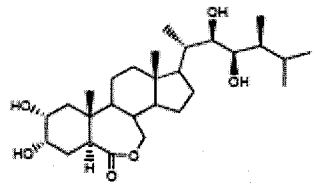
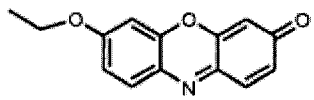
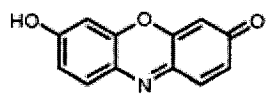
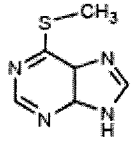
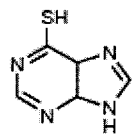
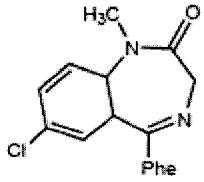
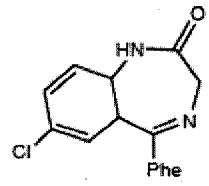
| Type of reaction | Substrate | Isoform | Product |
|---|---|---|---|
| **Hydroxylation at aliphatic carbon** |  Testosterone | CYP3A4 |  6β-hydroxytestosterone |
| **Hydroxylation at aromatic carbon** |  Coumarin | CYP2A6 |  7-hydroxycoumarin |
| **Epoxidation** |  Carbamazepine | CYP3A4 |  Carbamazepine-10,11-epoxide |
| **Baeyer-Villiger** |  Castasterone | CYP85A2 |  Brassinolide |
| **O-dealkylation** |  7-Ethoxyresorufin | CYP1A2 |  Resorufin |
| **S-dealkylation** |  6-Methylmercaptopurine | CYP1A2 |  6-Mercaptopurine |
| **N-dealkylation** |  Diazepam | CYP2C19<br>CYP3A4 |  Nordiazepam |

**Table 6.** Characteristics of human P450 substrates

| CYP | General structural and physicochemical characteristics |
|---|---|
| 1A2 | Planar (poly)aromatic/heterocyclic amines and amides |
| 2A6 | Compounds usually containing ketonic or nitroso groups, generally polar |
| 2B6 | Non-planar molecules, usually lipophilic with H-bond donor/acceptor |
| 2C9 | Generally weakly acidic with H-bond donor/acceptor |
| 2C19 | Generally neutral or basic with H-bond donor/acceptor |
| 2D6 | Nitrogenous bases with sites of metabolism 4-7Å from the basic nitrogen |
| 2E1 | Structurally diverse, generally neutral compounds of low molecular weight |
| 3A4 | Structurally diverse compounds of relatively high molecular weight |

## 1.3. P450 ENZYMES AS POTENTIAL CATALYSTS

P450s are of considerable interest in synthetic organic chemistry because of their impressive ability to catalyze the insertion of oxygen into non-activated C-H bonds. This useful reaction in organic chemistry has received much attention over several decades, but still remains a significant challenge. For example, Vaulted binaphthyl metalloporphyrins,[74] chiral Salen Mn-based complexes,[75] the complex $Cp^*Rh(\eta^4\text{-}C_6Me_6)$,[76] microporous aluminophosphates with metal ions such as $Fe^{III}$, $Co^{III}$ or $Mn^{III}$,[77] and $Cu(OAc)_2$[78] can catalyze C-H activation towards "O" insertion but show low stereoselectivity and/or low yield. Biocatalysts such as P450 enzymes represent a promising alternative.[79] Their use in industrial processes is currently very limited, due to their functional complexity, low activity, need for cofactors and redox partner, and poor stability[80] (**Figure 7**). The different methods used by several research groups to address some of these drawbacks will be discussed in the following sections.

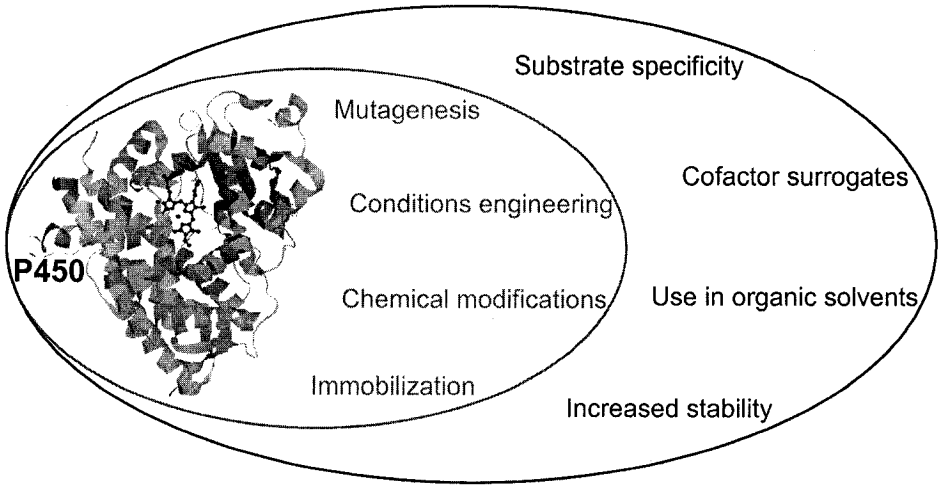



**Figure 7.** Strategies towards a better use of P450 enzymes

### 1.3.1. Current applications using P450 enzymes

The applications of P450s in research mainly involve the generation of drug metabolites. The identification of active metabolites early on in the drug development process requires large amounts of pure metabolites, and the use of P450s is considered an advantageous alternative to traditional chemical synthesis. Human P450s expressed in *E. coli* or insect cells have been successfully used to prepare various metabolites,[81,82,83] and engineered bacterial P450 BM3 has recently been used to prepare human metabolites of propanolol.[84]

Industrial applications of P450s have so far been restricted to whole-cell systems which mostly solve the cofactor regeneration problem. One of the highly successful examples is the production of the steroid hydrocortisone in engineered yeast co-expressing four P450s.[85] Before the development of this technology by Aventis-Pharma and the CNRS, hydrocortisone was prepared by semi-synthesis in a sophisticated process involving a multi-step chemical procedure and a microbial bioconversion. Pregnenolone, another steroid, can be produced from ergosterol using engineered

*Saccharomyces cerevisiae* co-expressing P450scc, adrenodoxin and adrenodoxin reductase.[86] These two biocatalytic processes represent a decisive achievement for both the industrial competitiveness and the development of green industrial processes. Although not purely synthetic in nature, another interesting industrial application of P450s exploits their involvement in anthocyanins biosynthesis which produces the delphinidin-derived pigments found in blue or violet flowers. This is of particular interest for the production of transgenic blue roses, impossible to obtain by hybridization methods. Suntory and Calgene Pacific (now Florigene), have capitalized on this natural pathway to develop genetically engineered "blue roses". The genes coding for P450s, flavonoid 3'-hydroxylase and flavonoid 3',5'-hydroxylase were successfully isolated from petunia and orchids, and cloned into roses.[87,88,89] The color of delphinidin is blue at high pH and rose under acidic conditions. As a result of the pH in their vacuoles, rose varieties marketed as 'Blue Boy' or 'Blue Bell' are invariably lavender or purple. Blue carnations have been more successfully produced with this technique,[90] thanks to their more alkaline vacuolar environment.

The development of industrial processes using P450s faces numerous challenges not encountered with enzymes such as hydrolases, lyases or transferases. The great potential of P450 enzymes in applications such as the production of steroids and drug metabolites or bioremediation has motivated researchers to find methods to overcome these drawbacks. Significant progress has been made to improve their activity, stability and cofactor regeneration during the past few decades.

### 1.3.2. P450 mutagenesis for improved activity, substrate tailoring, and tolerance to organic solvents

Most of the studies with purified enzymes have focused on the bacterial enzymes CYP102 (P450 BM3) from *Bacillus megaterium* and CYP101 (P450 CAM) from *Pseudomonas putida* because of their solubility, high expression level in *E. coli* and higher activity.[91] These enzymes are however very specific and must be mutated to accept new substrates. To create variants with modified enzymatic properties, both rational design (site-directed mutagenesis)[92] and directed evolution have been used.[93]

#### 1.3.2.1. P450 BM3

The native P450 BM3 catalyzes the hydroxylation of long-chain saturated fatty acids (C12-C22) preferentially at the ω-1, ω-2 and ω-3 positions.[94,95] It exhibits no catalytic activity towards fatty acids with a chain length of less than 12 carbon atoms, except for *p*-nitrophenoxycarboxylic acids with a chain length of 10 or 11 (10-pNCA or 11-pNCAs), but not 8-pNCA.[96] The substitution of phenylalanine 87 by alanine (F87A) created a mutant able to hydroxylate lauric acid and myristic acid almost exclusively at the ω-position.[97] This mutant also achieved complete conversion of 12-pNCA.[96] The F87A mutant was further subjected to rational evolution, which combines rational design and directed evolution, to create mutants with activity on shorter chain length substrates.[98] Site-specific randomization mutagenesis at position 87 of this mutant led to further improvement of its activity towards shorter fatty acid derivatives.[99] In the course of these investigations, Schmid and coworkers observed that one triple mutant efficiently hydroxylated indole to indigo and indirubin.[100] Further studies of this mutant revealed that it was also able to hydroxylate several other substrates such as octanoic acid, *n*-octane, α- and β-ionone, naphthalene, anthracene, quinoline, 2-, 6-, and 8-methylquinoline, all of which bear little or no resemblance to the fatty acid substrates of the native enzyme.[101] Arnold and coworkers also applied directed evolution to engineer

P450 BM3 for alkane hydroxylation. Several variants, including mutant 139-3, were found much superior to the wild-type enzyme towards hydroxylation of *n*-octane, hexane, cyclohexane, pentane,[102,] and even two-fold more active towards the natural substrates lauric acid and palmitic acid.[103] It was later reported that P450 BM3 139-3, is a good epoxidation catalyst for benzene, styrene, cyclohexene, 1-hexene and propylene.[104] Arnold and coworkers explored a combination of directed evolution and site-directed mutagenesis to produce the P450 BM3 mutant 9-10A, which showed improved activity towards propane compared to variant 139-3.[105] To obtain an ethane-hydroxylating P450, they next carried directed evolution on the P450 BM3 9-10A mutant, this time targeting the active site rather than the entire heme domain.[106] The mutant with the highest activity, P450 BM3 53-5H, catalyzes the conversion of propane to propanol at a rate of 370 $min^{-1}$ and that of ethane to ethanol at a rate of 0.4 $min^{-1}$. Labrou and coworkers have also used directed evolution to engineer the substrate specificity of P450 BM3. The mutant P15S showed approximately 6- to 9-fold increased activity towards SDS, lauric acid and 1,4-naphthoquinone, and enhanced activity for ethacrynic acid and $\epsilon$-amino-*n*-caproic acid.[107] To extend the spectrum of P450 BM3-catalyzed reactions, Arnold and coworkers have used saturation mutagenesis at selected active site residues of P450 BM3 mutant 9-10A, and produced variant 77-9H which exhibited 52% selectivity for the $\omega$-position of *n*-octane.[108] The same method generated two other mutants able to convert a range of terminal alkenes to either (R)- or (S)- epoxides with up to 83% ee.[109] More recently, the same group has shown that P450 BM3 mutant 9-10A-F87A catalyzes the enantioselective $\alpha$-hydroxylation of 2-arylacetic acid derivatives and buspirone with up to 99.5% ee.[110]

In addition, directed evolution has been used to create P450 BM3 mutants more tolerant towards organic co-solvents.[111] Random mutations on P450 BM3 F87A yielded

variants showing 10-fold increases in activity in the presence of 2% THF, and 6-fold in 25% DMSO. The engineered mutants were also significantly more resistant to acetone, acetonitrile, DMF and ethanol. The P450 BM3 variants and their substrate selectivity are summarized in **Table 7**.

**Table 7.** Substrate selectivity of P450 mutants from P450-BM3 obtained by directed evolution, site-directed (rational) mutagenesis or a combination of both (rational evolution)

| Mutant | Mode of preparation | New substrates and transformations |
|---|---|---|
| WT | | Long chain saturated fatty acids (ω–1, ω–2, ω–3) [94,95] |
| F87A | Site-directed mutagenesis | Laurate, myristate (ω position)[97], 12-pNCA[96] |
| F87A(LARV) | Rational evolution | Shorter chain length substrates, 8-pNCA[98] |
| | Site-specific randomization mutagenesis of F87A(LARV) | Shorter-chain fatty acid derivatives[99] |
| F87A5F5 | Random mutations on F87A | Increased resistance to organic co-solvents[111] |
| F87A/L188Q/ A74G | Rational evolution | Indole to indigo, broad range of non-natural substrates[100] |
| 139-3 | Five generations of mutagenesis | Octane, hexane, cyclohexane, pentane (hydroxylation), benzene, styrene, cyclohexene, 1-hexene (epoxidation)[104] |
| 9-10A | Rational evolution | Propane[105] |
| 53-5H | Directed evolution of 9-10A | Propane, ethane[106] |
| 77-9H | Saturation mutagenesis of 9-10A | Terminal alkane hydroxylation and epoxidation[108,109] |
| P15S | Directed evolution | SDS, lauric acid, 1,4-naphthoquinone, ethacrynic acid[107] |
| 9-10A-F87A | Site-directed mutagenesis | 2-arylacetic acid derivatives (α), buspirone formation[110] |

#### 1.3.2.2. P450cam

P450cam catalyzes the hydroxylation of camphor to 5-*exo*-hydroxycamphor. As described for P450 BM3, most of the engineering work on P450cam has focused on creating mutants for efficient oxidation of alkanes. Wong and coworkers have used site-

directed mutagenesis to create mutants that hydroxylate adamantane with nearly 5-fold increased activity compared to the wild-type enzyme,[112] and other mutants with improved activity towards (+)-α-pinene and (S)-limonene.[113,114] The variant Y96A was found to hydroxylate benzylcyclohexane, benzoylcyclohexanol and benzyloxycarbonylpiperidines at C-4.[115] Styrene oxide formation rates were also enhanced up to 25-fold with some single mutants.[116] Moreover, some variants were ≥9 times as active as the wild-type P450cam towards linear alkanes such as pentane, hexane and heptane.[117] More modest improvements in activity were observed with branched alkanes such as 2-methylpentane, 3-methylpentane and 2-methylhexane.[118] Use of bulky amino acid substitutions to reduce the volume of the substrate binding pocket of P450cam yielded mutants able to oxidize the smaller alkanes butane and propane more efficiently.[119,120] As reported for P450 BM3 by the group of Arnold,[106] Wong and coworkers have created a P450cam mutant able to catalyze the transformation of ethane to ethanol.[121]

As an alternative strategy to improve the stability of P450cam, Ortiz de Montellano and coworkers envisaged to covalently attach the heme cofactors to the enzyme by an autocatalytic mechanism resembling that of CYP4 enzymes.[122] Thus, based on sequence alignments with CYP4 isoforms, the P450cam G248D and G248E mutants were designed and characterized.[123] Only the G248E variant showed significant ligation of the heme to the enzyme; however, this mutant displayed reduced camphor hydroxylase activity and its relative stability has not been reported. The P450cam variants and their substrate selectivity are summarized in **Table 8**.

**Table 8.** Substrate selectivity of P450 mutants from P450-BM3 obtained by directed evolution or site-directed mutagenesis.

| Mutant | Mode of preparation | New substrates and transformations |
|---|---|---|
| WT | | Camphor (5-exo) |
| Y96F,Y96F/F193L | Site-directed mutagenesis | Adamantane[112] |
| Y96F/V247L | Site-directed mutagenesis | (+)-α-pinene and (S)-limonene[113,114] |
| Y96A | Site-directed mutagenesis | Benzylcyclohexane, benzoylcyclohexanol (C-4)[115] |
| Y96A and Y96F | Site-directed mutagenesis | Styrene, linear alkanes (pentane, hexane and heptane)[117] |
| F87W/Y96F/T101L /V247L (EB) | Site-directed mutagenesis | Alkanes (butane, propane)[119,120] |
| EB/L294M/T185M/ L1358P/G248A | Directed evolution | Ethane[121] |

### 1.3.2.3. Other P450s

Directed evolution of mammalian P450s has recently been reviewed.[124] CYP1A2 mutants obtained by random mutagenesis displayed up to 4-fold enhanced $k_{cat}/K_m$ towards the oxidation of 7-ethoxyresorufin[125] and phenacetin.[126] Further mutagenesis afforded mutants showing enhanced activity with 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline[127] and 7-methoxyresorufin.[128] Guengerich and coworkers have engineered CYP2A6 for the production of indigo,[129] and for the conversion of 4- and 5-benzyloxyindole to colored products.[130] Kumar and coworkers have used a rational approach based on the crystal structure of CYP2C5 to produce a CYP2B1 variant able to catalyze the hydrogen peroxide-supported transformation of progesterone.[131] They later reported the directed evolution of CYP2B1 to generate mutants with enhance catalytic efficiency towards 7-benzyloxyresorufin, benzphetamine, testosterone, cyclophosphamide and ifosfamide.[132] The mammalian P450 variants and their substrate selectivity are summarized in **Table 9**.

**Table 9.** Substrate selectivity of P450 mutants from mammalian P450s obtained by directed evolution, random mutagenesis or rational evolution.

| Mutant | Mode of preparation | New substrates and transformations |
|---|---|---|
| **CYP1A2**: E225I, E225N, F226Y | Random mutagenesis | 7-ethoxyresorufin, phenacetin[125,126] |
| E225N/Q258H/ G437D | Directed evolution | 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline[127] |
| E163K/V193M/ K170Q | Directed evolution | 7-methoxyresorufin[128] |
| **CYP2D6**: L240C/N297Q | Rational evolution | 4- and 5-benzyloxyindole, indigo production[129,130] |
| **CYP2B1:** various mutants | Directed evolution | $H_2O_2$ supported hydroxylation of progesterone[131] 7-benzyloxyresorufin, benzphetamine, testosterone, cyclophosphamide and ifosfamide[132] |

### 1.3.3. Improved stability by immobilization

Although considerable progress has been made towards the tailoring of P450 enzymes for new substrates, their poor stability remains a major obstacle to applications in synthesis. Immobilization on various supports has proven very useful to enhance the stability of numerous enzymes such as lipases,[133] but fewer success stories of P450 immobilization have been reported. The first example dates back to 1988 when Wiseman and coworkers[134] immobilized purified P450s from *S. cerevisiae* along with their reductase by entrapment in calcium alginate or in polyacrylamide, or by adsorption on cyanogen bromide-activated Sepharose 4B. The three methods showed good retention of the enzymes onto the support, and the remaining activity after 4 weeks was 15, 40 and 58% respectively, compared to 7% without immobilization. A decade later, the plant CYP71B1 fused to a P450 reductase was immobilized onto colloidal liquid aphrons.[135] This system displayed a 10-fold higher turnover for the demethylation of erythromycin, and retained enzymatic activity for more than 24 hours. Kelly and coworkers reported the immobilization of prokaryotic CYP105D1 with a ferredoxin onto the ionic exchange

resin DE52.[136] No significant loss of activity was detected after 24 hours of reaction, but $k_{cat}$ was slightly reduced compared to that of the free enzyme. Ionic exchange resins were also investigated as solid supports for P450 BM3.[137] Although the enzyme bound firmly to DEAE and SuperQ, neither of these systems was suitable for biotechnological applications because of product adsorption on the resins. Among the other types of matrices investigated, Celite, polypropylene derivatives and alkylsepharoses were ineffective, but more promising results were achieved by encapsulation of P450 BM3 in a sol-gel matrix derived from tetraethoxyorthosilicate. This approach resulted in a biocatalyst with a half-life of 29 days at 25°C, compared to 2 days for the free enzyme. A variant of P450 BM3 was also efficiently immobilized on epoxy-activated Sepharose. At 4°C, no loss of activity was observed for 40 days.[107]

### 1.3.4. Cofactor regeneration or replacement

The need for the expensive cofactor NADPH and a redox partner is undoubtedly a key issue in the use of isolated P450s in biotechnology. Attempts to overcome this issue include in-situ NADPH regeneration by enzymatic, chemical, and electrochemical methods, as well as the replacement of the natural cofactors by various methods (**Figure 8**).

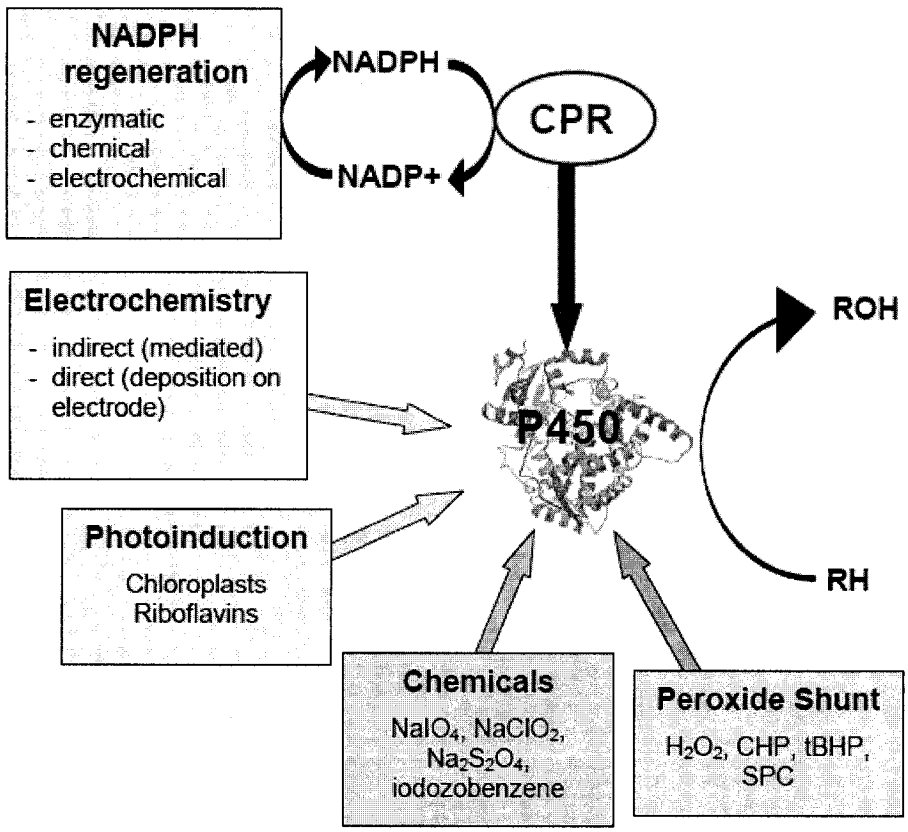



**Figure 8.** Methods used to regenerate or replace the natural cofactors of P450 enzymes.

One of the most common approaches to overcome this problem involves co-regeneration systems. Thus a second enzyme such as a dehydrogenase, and a second substrate can be added to convert $NAD(P)^+$ back to NAD(P)H *in situ*. Glucose-6-phosphate dehydrogenase has been widely used to recycle $NADP^+$;[138] however, this method complicates the isolation of the desired product. An alternative is to use formate dehydrogenase (FDH), which catalyzes the $NAD^+$-mediated oxidation of formic acid to the easily removed carbon dioxide. Unfortunately, most P450s are NADPH-dependent. The group of Kragl has recently engineered a FDH mutant with higher activity towards $NADP^+$.[139] This mutant has successfully been used to recycle NADPH in a system where both FDH and P450 BM3 are entrapped in a sol-gel matrix. Other attempts have been made to regenerate NADPH by non-enzymatic methods, and are summarized in a recent

review.[140] So far, the organometallic complex $[Cp^*Rh(bpy)(H_2O)]^{2+}$ is the only chemical catalyst for NADH and NADPH regeneration that has been successfully coupled to a monooxygenase reaction.[141,142]

Replacement of the cofactors by electrochemical methods has also had some success. Estabrook and coworkers have reported an alternative cofactor system based on the mediator cobalt(III)sepulchrate ($Co^{III}$sep) and a platinum electrode to support hydroxylation by a recombinant P450 4A1-P450 reductase fusion protein.[143,144] The platinum electrode was later successfully replaced by zinc dust for use with some P450 BM3 mutants.[145,146] Limitations of this method include the production of reactive oxygen species[143] and $Co^{III}$sep aggregation. Udit *et al.* have designed 1,1'-dicarboxycobaltocene to overcome these drawbacks; however this reductant displays acute sensitivity to dioxygen.[147]

The methods described above involve indirect or mediated electron transfers, via a low molecular weight redox compound which shuttles electrons between the enzyme and the electrode. Direct or unmediated electron transfers, also referred to as electrochemical reduction, have also been investigated, and recently reviewed.[148] The first example used a bare edge-plane graphite (EPG) electrode to reduce P450cam.[149] Direct electrochemistry with P450s on electrodes is generally difficult owing to the deeply buried cofactor and instability of the enzyme upon interaction with the electrode. Recent improvements include the modification of the electrode surface and the use of attached mediators, which assist in the catalytic cycle.[150,151,152] Rusling and coworkers have reported a reversible electron transfer between pyrolitic graphite electrodes and P450cam incorporated in thin films.[153] They also explored layer-by-layer polyion adsorption of P450cam onto gold electrodes coated with mercaptopropanesulfonic

acid.[154,155,156] Sheller and colleagues immobilized P450cam on a glassy carbon electrode modified with sodium montmorillonite, and observed a rate of electron transfer similar to that observed with the natural P450cam-putidaredoxin system.[157] Other bacterial P450s studied electrochemically include P450cin (CYP176A),[158] and P450 BM3,[159] both immobilized within a didodecyldimethylammonium bromide (DDAB) surfactant film cast onto an EPG working electrode. A detailed electrochemical and spectroscopic study of P450 BM3-DDAB films has recently been published.[160] The first electrochemical investigations of mammalian P450s was reported in 2000. P450s 2B4 and 1A2 covalently linked to riboflavin and reduced on rhodium-graphite electrodes, hydroxylated their substrates at rates comparable to those obtained with NADPH.[161,162] CYP2B4 has also been reduced on clay-modified glassy electrodes.[163] Interestingly, human CYP1A2 deposited on carbon cloth electrodes catalyzed styrene oxidation faster than P450cam.[164] Reduction of CYP3A4 embedded in a polyelectrolyte was also successful.[165] Electrochemical reduction of CYP2E1 on glassy carbon or gold electrodes supported the formation of *p*-nitrocatechol from *p*-nitrophenol.[166] The human P450s 2C9, 2C18 and 2C19 confined within a DDAB surfactant film on the working electrode surface were successfully reduced electrochemically.[167] Preliminary studies with P450scc (CYP11A1) have employed screen-printed rhodium–graphite electrodes and riboflavin.[168,169]

An alternative non-enzymatic method for cytochrome P450 reduction uses light-driven catalytic systems. They can be designed to regenerate NADPH or directly reduce the heme. For example, photosystems I and II from spinach or cactus chloroplasts have been used to regenerate NADPH. This system supported *O*-deethylation of 7-ethoxycoumarin by a CYP1A1-reductase fusion protein.[170,171,172] More recently, Shumyantseva and coworkers took advantage of the high photosensitivity of riboflavins

to photoinduce electron transfers to CYP2B4,[173,174] ultimately producing a photo-activated enzyme.[175]

The use of chemicals to directly replace one or more cofactors is yet another alternative to facilitate the use of P450 enzymes in synthesis. Strong oxidants that directly oxidize the iron of P450s to an iron-oxo species (e.g. sodium periodate, sodium chlorite, iodozobenzene and peracids) have proven useful to hydroxylate various substrates, yet have a high potential to generate side reactions.[176,177,178,179] Dithionite was also shown to support the P450 BM3 catalyzed hydroxylation of palmitate, but with a rate of reduction of the heme iron much slower than with NADPH.[180]

In a process termed the peroxide shunt pathway, hydrogen peroxide may be employed as a source of both electrons and oxygen for heme proteins (Figure 1). Although many P450s are known to accept peroxides or aqueous hydrogen peroxide as cofactors surrogates,[181,182,183,184,185] this pathway is generally not efficient. This interesting property has however attracted interest to create versatile single-enzyme hydroxylation biocatalysts. Laboratory evolution of P450cam by Arnold and coworkers suggested that one mutant had an enhanced rate of hydrogen peroxide-driven naphthalene hydroxylation.[186] Isolation and characterization of this mutant by Ishimori and coworkers has however revealed that it generates only trace amounts of hydroxylated naphthalene.[187] Arnold and coworkers were successful at engineering P450 BM3 to improve its peroxygenase activity.[188] The initial reaction rates were however significantly lower than those observed with the wild type enzyme with its natural cofactors. They further engineered the P450 BM3 heme domain as a peroxygenase to prepare authentic human metabolites of propanolol.[84]

## 1.4. RESEARCH GOALS

Cytochrome P450 enzymes have attracted a lot of interest since their discovery by Oruma and Sato in 1962.[40] This attention stems not only from the importance of these enzymes in drug metabolism and drug interactions, but also in their unequalled catalytic properties. In particular, their impressive ability to catalyze the regio- and stereo-selective hydroxylation of non-activated C-H bonds is of great interest for synthetic chemists, who still strive to carry out efficiently these types of reactions with chemical catalysts. Additionally, many mammalian P450 isoforms accept a large variety of substrates which makes them ideal catalysts for asymmetric organic synthesis. Their need for cofactors is however a major drawback. Moreover, P450 enzymes have only been used in aqueous media, and their application in organic media still faces numerous challenges. These enzymes are also known to exhibit low activity and stability. Our goal was to address some of these issues, and to take one step further toward making P450 enzymes useful tools to synthetic chemists.

Our research focused on the human P450 CYP3A4, selected for its remarkable substrate promiscuity. The main objectives of this project were to find an efficient and convenient way to bypass the need for the natural cofactors (CPR, NADPH), to optimize the catalysis in aqueous medium, to minimize the harmful effects of lyophilization on this enzyme, and to evaluate the CYP3A4 ability to perform reactions under non-aqueous conditions. Several chemicals were tested as cofactor surrogates. A survey of the effects of water miscible co-solvents, pure anhydrous organic solvents, and ionic liquids is included. These studies also included the optimization of the freeze-drying conditions, an essential process for the last part where the enzyme is tested in organic solvents.

# 2. RESULTS AND DISCUSSION

## 2.1. PREPARATION AND CHARACTERIZATION OF ENZYMES

### 2.1.1. Cytochrome P450 reductase (CPR) expression, purification and characterization

As discussed in the introduction, the cytochrome P450 reductase (CPR) is a flavoprotein containing both FAD and FMN, needed to transfer electrons from NADPH to the P450. CPR was expressed and purified after modification of a published procedure.[189] The concentration of CPR was calculated to be 8 μM using the peak at 455 nm after oxidation of the flavin with potassium ferricyanide (**Figure 9**), and the expression yield was around 0.7 mg/liter of culture. The capacity of the purified CPR protein to reduce cytochrome c in the presence of NADPH was tested by UV, and the peaks at 520 nm and 550 nm on the UV spectrum confirmed that the protein was active (**Figure 10**). The enzyme purity was confirmed by SDS-PAGE analysis. The expected single band at ~75 kD was observed (**Figure 11**).

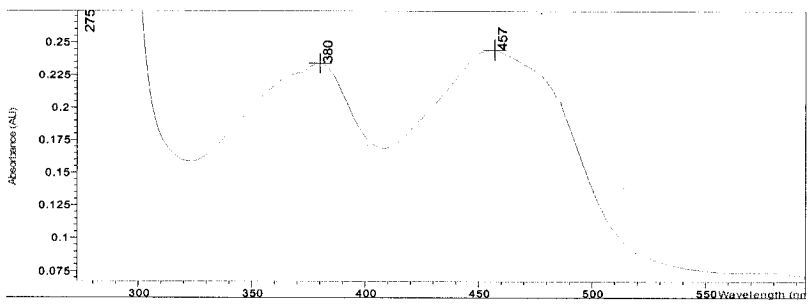



**Figure 9.** CPR concentration determination. After oxidation of the flavin with potassium ferricyanide ($K_3Fe(CN)_6$), small and broad peaks appear at 380 and 455 nm, and the concentration is determined using an extinction coefficient of 21.2 $mM^{-1}$ $cm^{-1}$ at 455 nm.

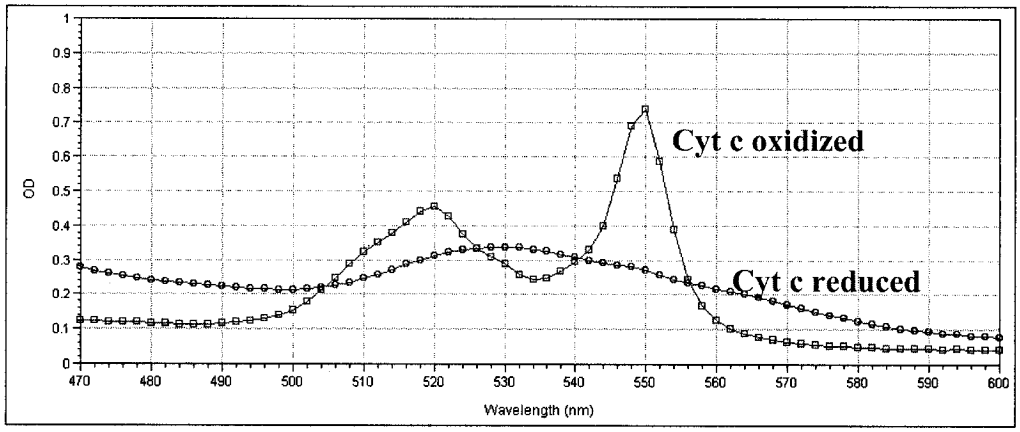



**Figure 10.** CPR activity determination. Cytochrome c reduction by CPR/NADPH is demonstrated by the appearance of peaks at 520 and 550 nm.

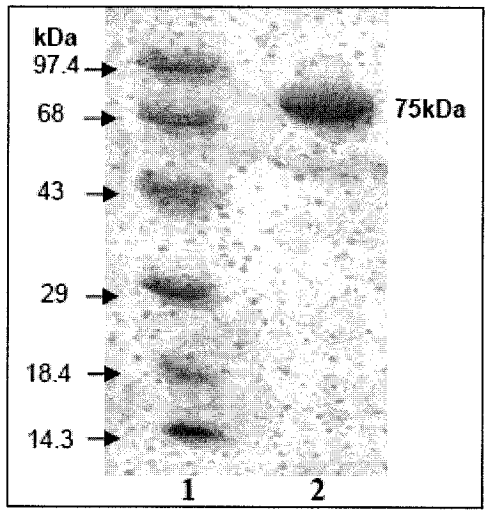



**Figure 11.** SDS-PAGE analysis of purified CPR with Phastgel 12.5 % homogenous gel. The gel was stained with Coomassie blue. Lane 1: molecular weight markers. Protein sizes are indicated on the left. Lane 2: CPR sample after purification.

### 2.1.2. CYP3A4 expression, purification and characteriation

CYP3A4 was expressed with a N-terminal histidine tag in *E. coli* DH5α cells, after modification of a previously reported procedure.[190] The enzyme purity was confirmed by SDS-PAGE analysis (**Figure 12**) which revealed a strong band at ~52 kDa, the approximate calculated molecular weight of this protein.

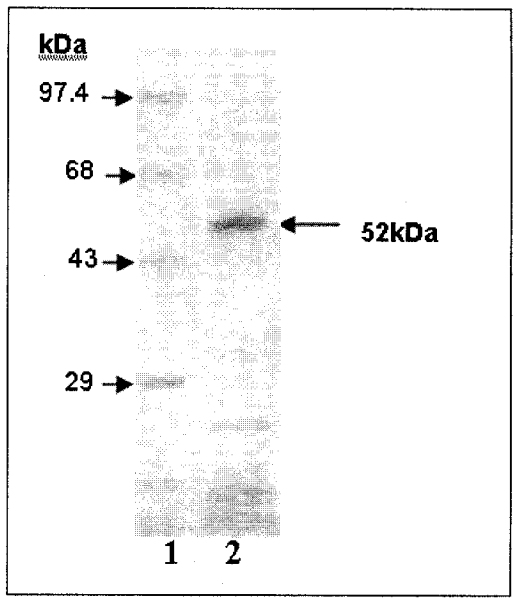



**Figure 12.** SDS-PAGE analysis of purified CYP3A4 with Phastgel 12.5 % homogeneous gel. The gel was stained with Coomassie blue. Lane 1: molecular weight markers. Protein sizes are indicated on the left. Lane 2: CYP3A4 sample after purification.

The reported procedure had to be optimized by trial and error to obtain good purity. Key changes to the protocol included the use of low imidazole concentrations in the equilibration buffer (10 mM), as well as in the washing buffer (up to 30 mM) to minimize non-specific binding during purification on the nickel column.

The concentration of the final enzyme solution was typically between 5 and 8 μM (300 μg/ml) as determined by the CO difference spectrum method (**Figure 13**) using $\varepsilon_{450}$ = 91 $mM^{-1}$ $cm^{-1}$. Binding of carbon monoxide to the reduced P450 results in the formation of $Fe^{2+}$-CO complex with heme Soret absorption maxima shifted to 450 nm. The typical yield obtained was 0.7 mg of CYP3A4 per liter of culture. The Rz (ratio $A_{Soret}/A_{280}$) was around 1 (the Rz ratio of pure P450s is around 1.5). Some of the impurities that could account for the low Rz are decomposed P450 (as shown by a small peak at 420 nm on **Figure 13**, as well as various low molecular weight proteins as

suggested by bands on the SDS gel (**Figure 12**). Gel permeation chromatography could be used if purer enzyme was needed. The total protein content as determined by the Lowry method was about 1000 μg/ml.

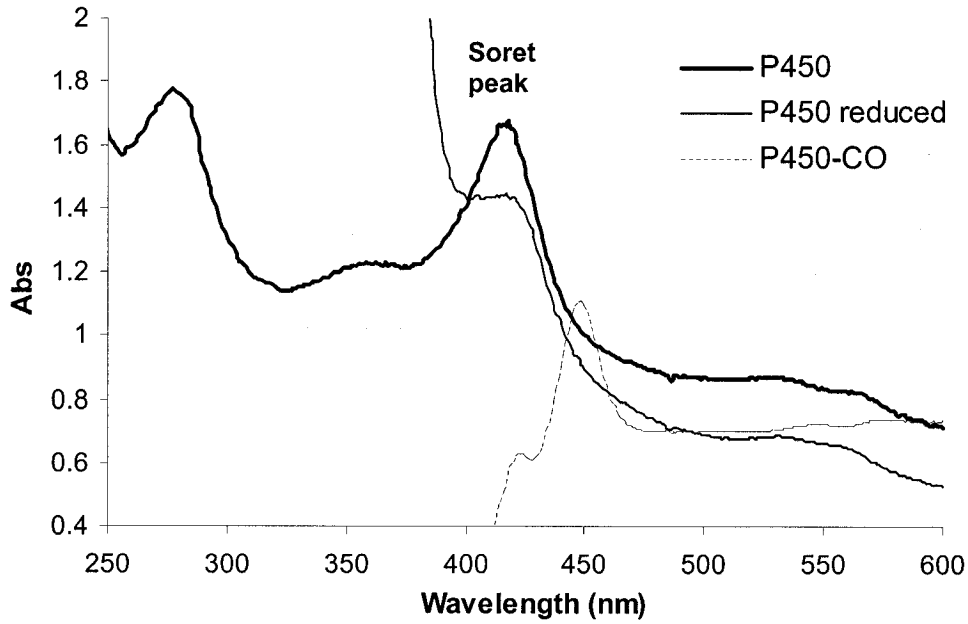



**Figure 13.** Spectral determination of P450 concentration. Spectra are shown for the oxidized (bold line), sodium dithionite-reduced (thin line) and CO-difference spectrum of the reduced form (dashed line) forms of the enzyme.

### 2.1.3. Activity assays of CYP3A4

A simple and rapid assay was used to assess CYP3A4 activity. The test reaction involved the debenzylation of 7-benzyloxy-4-trifluoromethylcoumarin (BFC) to the fluorescent product 7-hydroxy-4-trifluoromethylcoumarin (HFC), directly followed by fluorescence. The reaction mixture contained the purified CYP3A4, CPR, and the substrate BFC. NADPH was added to initiate the reaction. The P450 activity is revealed by an increase in fluorescence over time (**Figure 14**).

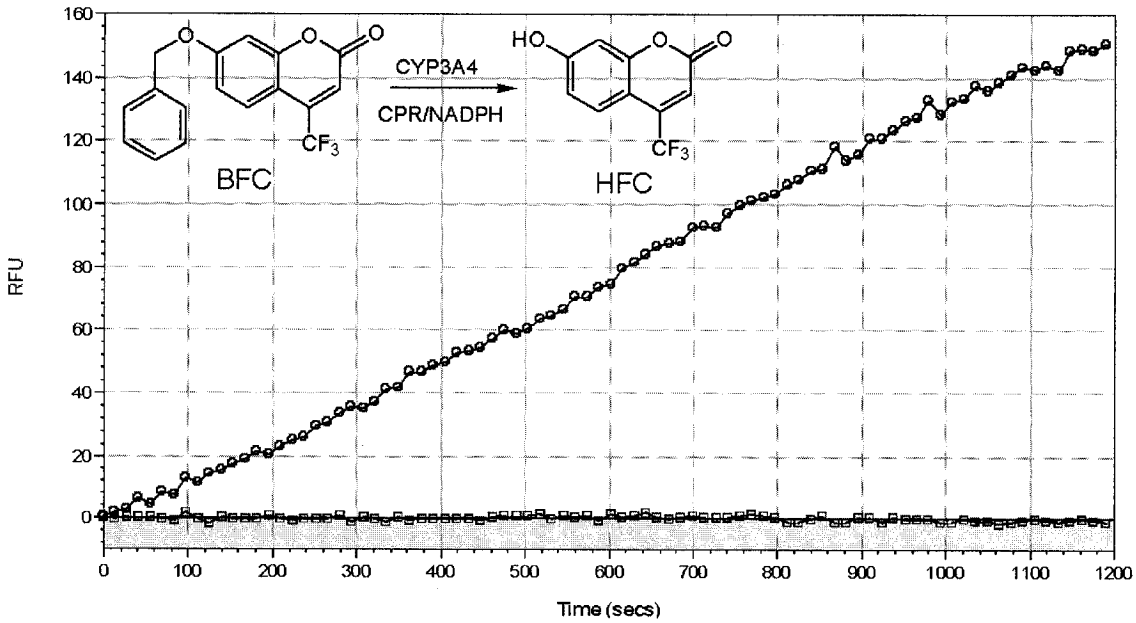
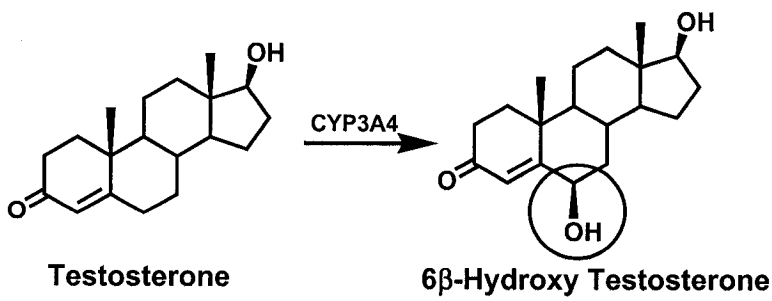



**Figure 14.** Debenzylation of BFC to HFC by CYP3A4 monitored by fluorescence (○). The control reaction without enzyme did not show any fluorescence (□). λex = 409 nm, λem = 530 nm.

Another assay was also developed using HPLC to allow quantification of all products. To this end, the 6β-hydroxylation of testosterone (**Scheme 1**) was selected because of its wide use as a characteristic assay for CYP3A4 activity. The major product is 6β-hydroxytestosterone (or 6β-androstenedione)[191,192] and minor products include 2β-hydroxy, 15β-hydroxy, and 1β-hydroxytestosterone as reported by Guengerich *et. al.*[193]




**Scheme 1.** 6β-hydroxylation of testosterone by CYP3A4

Before testing CYP3A4 activity under non-natural environments, the reaction

had to be first optimized under standard conditions. The first part of this study consisted in setting up the methods for product analysis and quantification by HPLC, while the second part focused on the replacement of the natural cofactors and conditions optimization.

## 2.2. OPTIMIZATION OF CYP3A4 ACTIVITY IN AQUEOUS CONDITIONS

### 2.2.1. Product extraction

After incubation of the reaction mixture, the substrate (testosterone) and product (6β-hydroxytestosterone) must be separated from the enzyme and the salts for analysis. Extraction in an organic solvent is a convenient choice. Several solvents were tested for their extraction efficiency (3 extractions in a row). The HPLC peak area of testosterone and 6β-hydroxytestosterone of extracted and non-extracted standards were compared, at different concentrations. The results are summarized in **Table 10**. Dichloromethane yielded the best extraction efficiencies (> 92% for all the concentrations tested), and was therefore selected as the solvent of choice in further studies.

**Table 10.** Extraction efficiency of the substrate (testosterone) and the product (6β-hydroxytestosterone) with 3 organic solvents

| | Concentration | Ethyl acetate | Ethyl ether | Dichloromethane |
|---|---|---|---|---|
| Testosterone | 50 μM | 84.5 | 86.9 | 98.21 |
| | 100 μM | 85.9 | 91.1 | 97.4 |
| | 150 μM | 86.40 | 89.4 | 97.9 |
| 6β-OHT | 5 μM | 73.5 | 91.2 | 95.2 |
| | 10 μM | 93.2 | 89.7 | 93.1 |
| | 20 μM | 80.9 | 88.4 | 92.8 |

### 2.2.2. Quantification of 6β-hydroxytestosterone

Amongst internal standards commonly used for quantification of testosterone metabolites (see **Figure 15**), cortexolone, a hydroxylated progesterone analog, was chosen because it is an inexpensive, nonregulated steroid. It is chemically similar to the hydroxytestosterone metabolites and can therefore reliably count on extraction recovery.

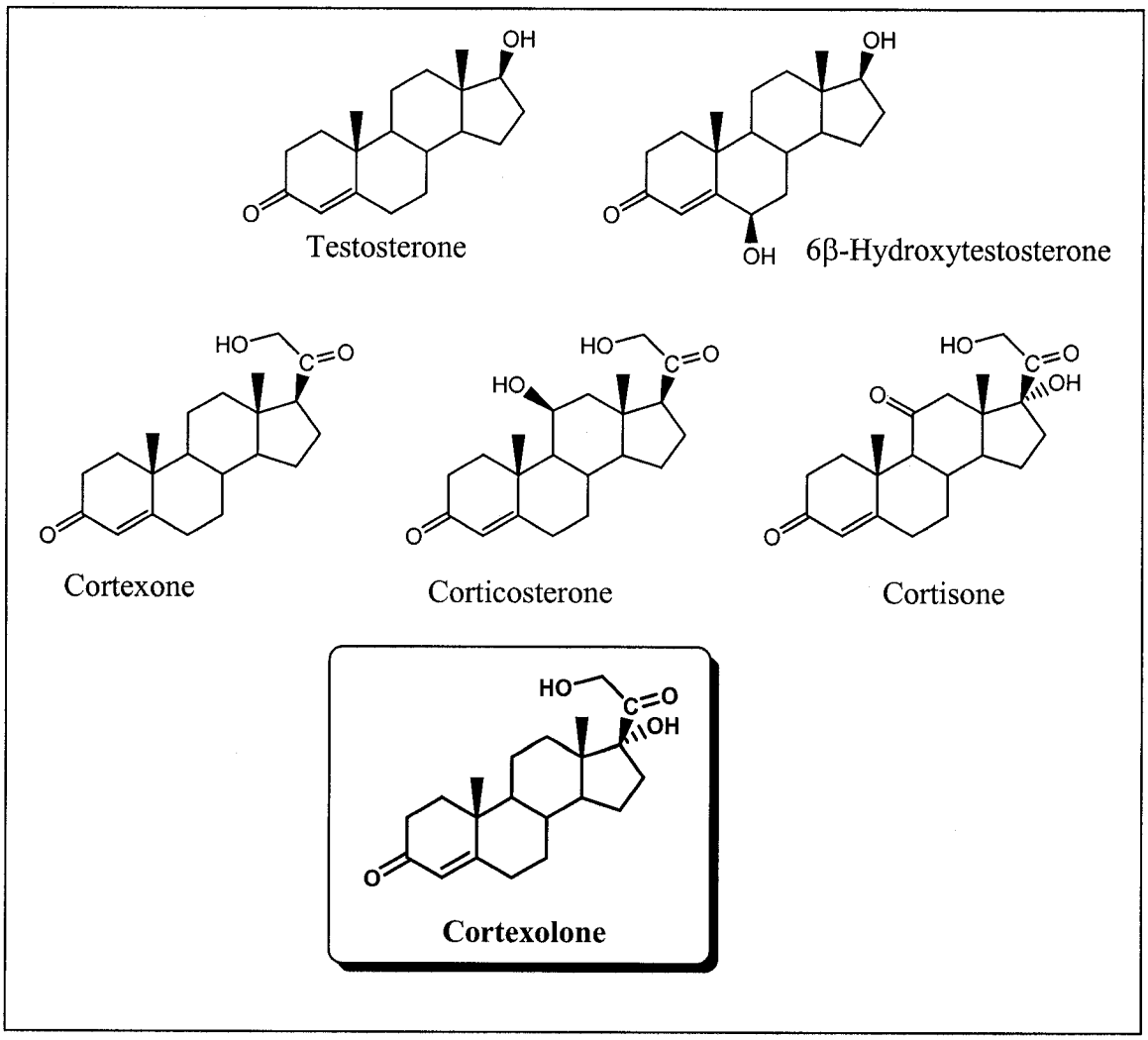



**Figure 15.** Structures of testosterone, the metabolite 6β-hydroxytestosterone, and the commonly used internal standards for quantification of testosterone metabolites.

Quantification of the 6β-hydroxytestosterone formed during the enzymatic

reactions was achieved from a calibration curve obtained after spiking the incubation mixture (no CYP) with 6β-OHT (various concentrations) and cortexolone (fixed concentration). A linear regression of peak-area ratios versus concentration of 6β-OHT was constructed (**Figure 16**) and used to quantify the product formation.

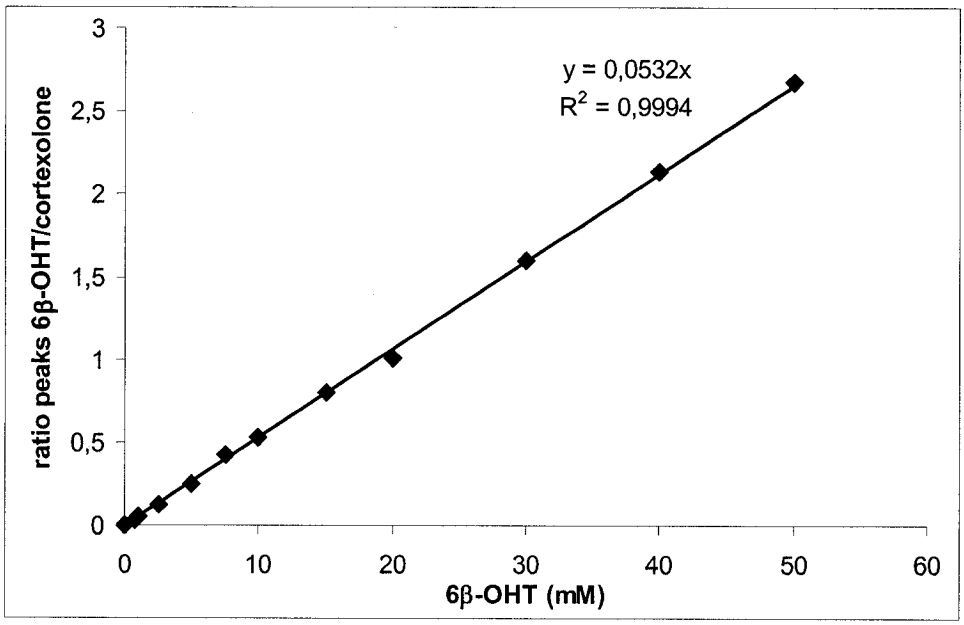



**Figure 16.** Calibration curve of 6β-hydroxytestosterone with 20 μM cortexolone

### 2.2.3. Replacement of the natural cofactors by hydrogen peroxide donors and organic peroxides

#### 2.2.3.1. Product formation with peroxide surrogates

As discussed in the introduction, the need for expensive cofactors is one of the major drawbacks related to the use of P450s in synthesis. Although many P450 enzymes are also known to accept peroxides or aqueous hydrogen peroxide as a source of oxygen and electrons (shunt pathway), this pathway is generally not efficient. When we first attempted to replace the natural cofactors of CYP3A4, CPR and NADPH, with peroxides in the BFC debenzylation assay, the formation of foam in the reaction wells prevented an

accurate and reproducible fluorescence measurement. The 6β-hydroxylation of testosterone was then used. Several concentrations of aqueous $H_2O_2$ were tested ranging from 30 mM to 1 M. HPLC analysis of the reaction mixtures revealed that the activity was negligible. It was therefore concluded that for CYP3A4, aqueous hydrogen peroxide was not an effective surrogate for the natural cofactors. Non-aqueous sources of hydrogen peroxide and organic peroxides were evaluated next: sodium percarbonate (SPC, $Na_2CO_3 \cdot 1.5\ H_2O_2$), sodium perborate (SPB, $NaBO_3 \cdot H_2O_2$), urea-hydrogen peroxide adduct (UHP), cumene hydroperoxide (CHP) and *t*-butylhydroperoxide (tBHP). The product formation after 1 hr was compared to that of a control reaction with the natural cofactors. Several concentrations were tested for each peroxide. The HPLC traces for the best conditions (SPC and CHP) are shown on **Figure 17**. The results for all the peroxides tested are summarized in **Table 11**. As expected, the enzyme regio- and stereoselectivity are unchanged. The best activity was obtained with CHP and reached 132% of the activity promoted by the natural cofactors (CPR and NADPH). Relatively low concentrations of CHP were sufficient. SPC was also found to efficiently support the reaction, initially yielding 77% of the natural activity. Considering that the addition of carbonates to the reaction mixture would considerably affect the pH and thus the enzyme activity, the reaction with SPC was further optimized by varying the buffer concentration and pH, and reached 119% of the control activity (CPR/NADPH) when 1 M of potassium phosphate at pH 7.0 instead of 7.4 was used (**Table 12**).

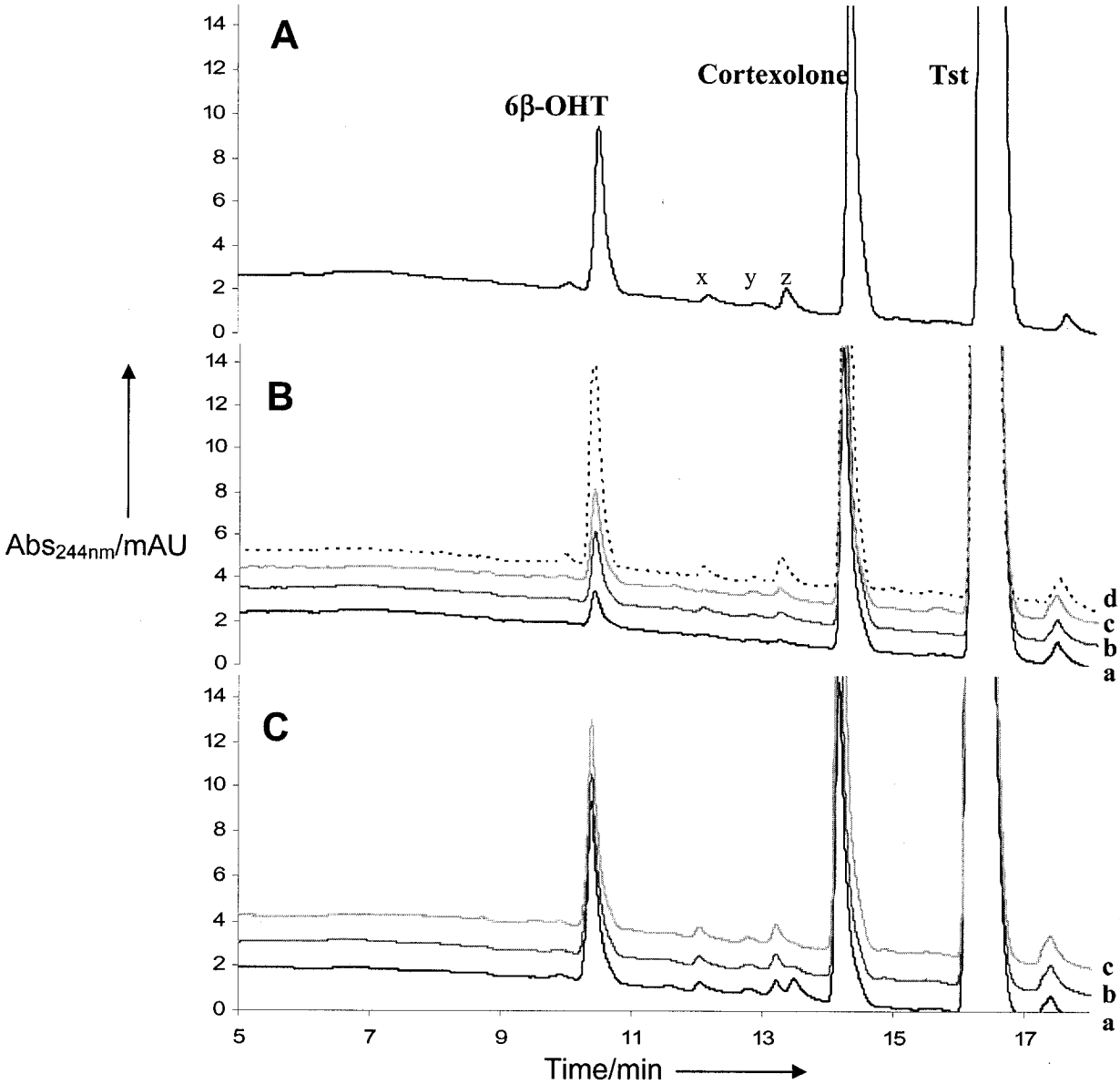



**Figure 17**. 6β-hydroxylation of testosterone by CYP3A4 with peroxide surrogates. HPLC chromatograms with detection at 244 nm following incubation at 37°C for 1 hr of CYP3A4 (0.6 μM), testosterone (115 μM) and cofactor in 0.1 M potassium phosphate at pH 7.4, unless otherwise stated. The reaction mixtures were spiked with the internal standard cortexolone and extracted with $CH_2Cl_2$. After evaporation, the residue was redissolved in methanol (150 μl) before injection into the HPLC. **A**: reference reaction initiated with CPR (2.4 μM) and NADPH (1 mM). **B**: reaction with sodium percarbonate at 50 mM (a), 100 mM (b), 500 mM (c), in 1 M potassium phosphate buffer at pH 7.4, and 500 mM in 1 M potassium phosphate pH 7.0 (d). **C**: reaction with cumene hydroperoxide

at 0.1 mM (a), 0.5 mM (b), and 1 mM (c). The retention times of 6β-hydroxytestosterone, cortexolone and testosterone were 10.4, 14.2 and 16.2 min respectively. The peaks x, y and z are other metabolites formed during the reaction, unidentified but very likely to be the 1β, 15β and 2β hydroxylated products according to Guengerich and coworkers.[193]

**Table 11**: Formation of 6β-hydroxytestosterone by CYP3A4 with hydrogen peroxide donors or organic peroxides expressed as a percentage of the reaction with the natural cofactors (CPR and NADPH).

| Peroxide equiv. (mM) | **SPC**[a] | **SPB** | **UHP** | **CHP** | **tBHP** | **$H_2O_2$aq** |
|---|---|---|---|---|---|---|
| 0.1 | - | - | - | 83 ± 8 | 6 ± 2 | N.D.[c] |
| 0.5 | - | - | - | 114 ± 12 | 13 ± 4 | N.D. |
| 1 | - | - | - | **132 ± 17** | 21 ± 2 | N.D. |
| 2 | - | - | - | 92 ± 10 | **35 ± 3** | N.D. |
| 5 | - | - | - | 61 ± 6 | 16 ± 1 | N.D. |
| 50 | 28 ± 3 | 2 ± 2 | 2 ± 1 | - | - | N.D. |
| 100 | 47 ± 5 | 4 ± 3 | 3 ± 3 | - | - | N.D. |
| 500 | **119**[b] **± 12** | **10 ± 3** | **7 ± 4** | - | - | N.D. |
| 1000 | 28 ± 4 | 6 ± 2 | 4 ± 3 | - | - | N.D. |

[a]Analysis of 6β-hydroxytestosterone formation by HPLC after 1 h incubation at 37°C of CYP3A4 (0.9 μM) and testosterone (115 μM), initiated with various concentrations of hydrogen peroxide donors or organic peroxides. The buffer was 0.1 M potassium phosphate at pH 7.4, and 1 M potassium phosphate at pH 7.4 for the reaction with SPC. The control reaction was with the natural cofactors CPR (2.4 μM) and NADPH (1 mM); [b]Several potassium phosphate buffer concentrations and pHs were tested with 500 mM SPC, 1 M potassium phosphate at pH 7.4 gave 77% activity, while the highest activity of 119% was obtained at pH 7.0. [c]N.D.: below the detection limit of 0.1 μM (1%).

**Table 12.** Effect of the buffer in CYP3A4 reactions with sodium percarbonate

| Potassium phosphate concentration | pH | % of the activity with CPR/NADPH |
|---|---|---|
| 500 mM | 6.0 | 86 ± 2.2 |
| | 6.5 | 78 ± 4.3 |
| | 7.0 | 86 ± 2.8 |
| | 7.5 | 15 ± 1.5 |
| 750 mM | 6.0 | 61 ± 6.6 |
| | 6.5 | 82 ± 8.1 |
| | 7.0 | 99 ± 4.7 |
| | 7.5 | 63 ± 3.7 |
| 1 M | 6.0 | 54 ± 4.1 |
| | 6.5 | 81 ± 5.8 |
| | **7.0** | **119** ± 3.4 |
| | 7.5 | 77 ± 2.4 |

Thus after optimization, CHP and SPC yield comparable activities, although CHP is effective at lower total concentrations. CYP3A4 produces 1.3 times more 6β-hydroxytestosterone in the presence of CHP and 1.2 times more with SPC than with its natural cofactors. The other hydrogen peroxide donors and organic peroxides produced less than 50% of the natural activity. In all cases no product formation was detected in the control reactions without CYP3A4.

SPC, SPB and UHP are powders with an active oxygen content equivalent to 27.5%, 32% and 35% $H_2O_2$ respectively (by mass). These hydrogen peroxide donors present the advantage of releasing $H_2O_2$ slowly and locally on application, creating milder oxidation conditions around the enzyme than aqueous hydrogen peroxide. This was verified by comparing the activity of CYP3A4 with SPC when the reaction mixture was shaken after addition, which led to complete dissolution of the salt, or when the dissolution occurred slowly overtime without shaking. In the former case no product formation was detected, confirming the need for a slow release of hydrogen peroxide in the reaction mixture. The possibility of direct peroxide transfer between SPC and the

P450 was discarded based on the knowledge that SPC is an adduct of carbonate and hydrogen peroxide, known to begin releasing hydrogen peroxide as soon as it starts dissolving. The release can be observed as bubbles, obvious when looking at the reaction tube immediately after addition of SPC to a liquid.

#### 2.2.3.2. Initial rate and turnover number

Once it was established that some peroxides were able to promote a significant activity, the initial reaction rates and total turnover numbers were determined for the best two peroxides, SPC and CHP. The reaction rates were obtained using the initial slope (<5 min) of the product formation curve. Interestingly, the increased product formation observed in the presence of CHP or SPC (**Table 11**) can be explained by a proportional increase in the initial rates of product formation (**Table 13**). Thus the initial rates are ~30% higher with optimal amounts of SPC or CHP than with CPR/NADPH.

**Table 13:** Initial rates of product formation and maximum turnover numbers for the CYP3A4-catalyzed 6β-hydroxylation of testosterone in the presence of CPR/NADPH or peroxide equivalents.

| Cofactor | CPR/NADPH | CHP | SPC |
|---|---|---|---|
| Initial rate (μmol μmol$^{-1}$ min$^{-1}$)[a] | 5.9 ± 0.9 | 9.1 ± 1.5 | 7.1 ± 1.2 |
| Turnover[a] | 46 ± 4 | 42 ± 5<br>50 ± 4[b] | 26 ± 2 |

[a]6β-OHT formation after incubation at 37°C of CYP3A4 (0.9 μM) and testosterone (115 μM). The turnover number was calculated after a 4 hr reaction. The buffer was 0.1 M potassium phosphate at pH 7.4, and 1 M potassium phosphate at pH 7.0 for the reaction with SPC. The cofactors tested were either CPR (3.6 μM) and NADPH (1 mM + 1 mM added every 30 min ), CHP (1 mM + 1 mM added every 30 min, or [b]0.1 mM + 0.1 mM added every 30 min) or SPC (500 mM).

### 2.2.3.3. CYP3A4 stability with the cofactor surrogates

Because peroxides have been reported to react with the heme moiety of P450s and lead to enzyme inactivation,[194] the stability of CYP3A4 in the presence of surrogate and natural cofactors were compared. A first attempt was made to evaluate the P450 stability by UV measurement. The decrease of the Soret peak at 416 nm upon addition of the cofactor or peroxide was used as an indicator of the enzyme denaturation. Unfortunately this method was not convenient because of the strong interfering absorption of NADPH at 340 nm, and because of the bubbles formation accompanying the dissolution of SPC. This assay could hence only be used with CHP. At 37°C and in the presence of the optimum amount of CHP (1 mM), CYP3A4 lost about 90% of its activity in 1 hr, and was completely inactive after 2 hrs (**Figure 18**).

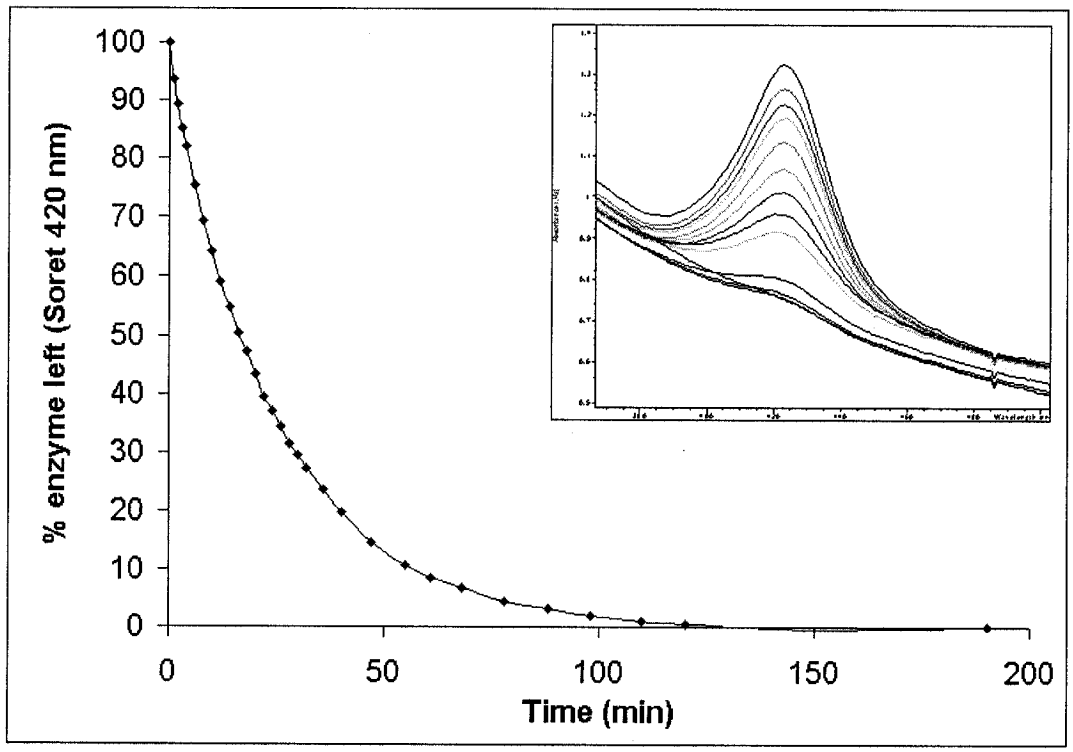



**Figure 18.** Stability of CYP3A4 at 37°C in the presence of 1 mM CHP, evaluated by the decrease of the Soret peak at 416 nm (as shown in the inset).

In order to compare the stability of CYP3A4 with CPR/NADPH and with the cofactor surrogates, an alternative method had to be used. A large excess of substrate and cofactor(s) were used (fresh cofactor was added every 15 or 30 min) and product formation was monitored over time. The enzyme was considered inactive when product formation reached a plateau. Under these conditions, CYP3A4 shows comparable stability whether CPR/NADPH or CHP are used (~3 hrs, **Figure 19**). This was not expected based on previous literature,[194] and is most likely explained by the small amount of CHP used.

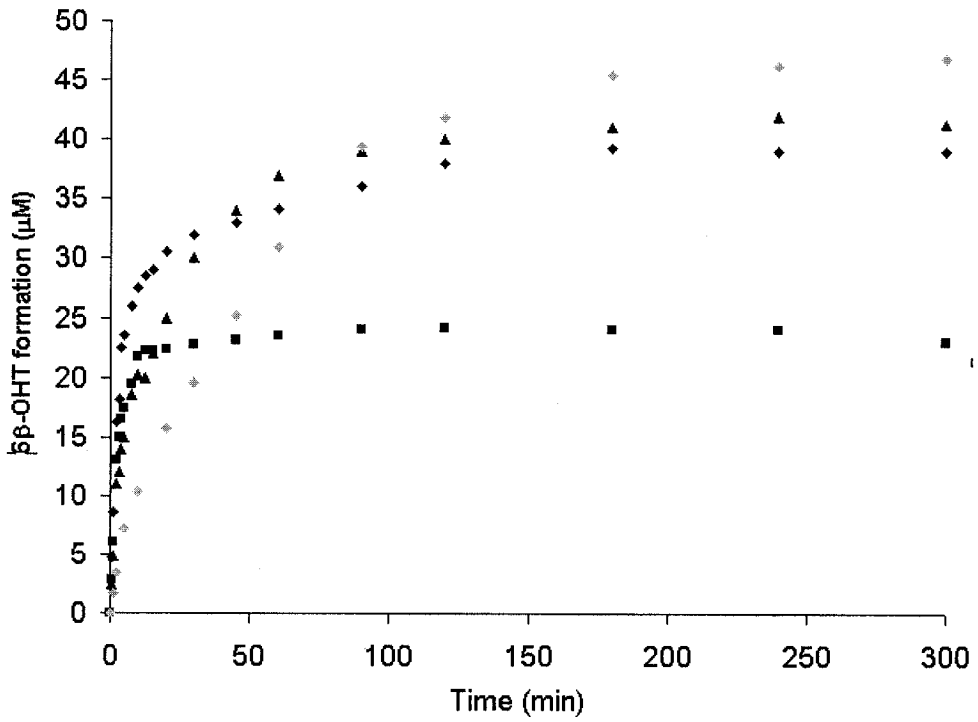



**Figure 19**. 6β-hydroxytestosterone formation as measured by HPLC (244 nm) after incubation at 37°C for different times of CYP3A4 (0.9 μM) and testosterone (115 μM) in 0.1 M potassium phosphate buffer at pH 7.4, or 1 M at pH 7.0 with SPC. Each point on the plot represents an average of two measurements, and for each, a total reaction volume of 300 μl was used. The reaction was initiated with CHP (1 mM + 1 mM added every 30 min (♦), 0.1 mM + 0.1 mM added every 30 min (♦)), 500 mM SPC (■), or CPR (3.6 μM ) and NADPH (1 mM + 1 mM after 15 min then every 30 min) (▲).

When the quantity of CHP added is further reduced (multiple additions of 0.1 mM instead of 1 mM, see **Table 13** and **Figure 19**) the initial rate decreases; however, the enzyme is active for ~4 hours and the maximum turnover number rises (still comparable to CPR/NADPH). It should be noted that the results of **Table 11** can only be compared to **Figure 19** for the first 15 min. Indeed, **Figure 19** was obtained after multiple additions of CPR/NADPH or CHP, whereas the results of **Table 11** were collected after optimization with a single addition of the cofactors.

CYP3A4 represents an ideal biocatalyst because of its high substrate promiscuity and its ability to catalyze chemically challenging hydroxylations at inactivated C-H bonds. In summary, we report an improvement of almost two fold in reaction rates when the natural cofactors of CYP3A4 are replaced with SPC or CHP. This is the first time that such an improvement compared to the use of natural cofactors is reported for P450 enzymes. This is accomplished with amounts of cofactor surrogates much lower than previously reported, which likely allowed minimization of side reactions. These results suggest that the need for expensive cofactors can be circumvented without significant decreases in yields by replacement with very cheap commercial compounds, directly with the wild type enzymes. We anticipate that this alternative approach may apply to other P450 enzymes and will be of considerable use for future applications of P450s in synthesis.

## 2.3. OPTIMIZATION OF THE CYP3A4 LYOPHILIZATION CONDITIONS

The next step of the project was to test the enzyme activity in the presence of various organic solvents. This study required the freeze-drying or lyophilization of the enzyme, in order to obtain a powdered enzyme that could be resuspended in the organic

solvent. Because lyophilization often leads to decreased enzymatic activity, optimization of the conditions was next undertaken. In all the experiments described in this section, CYP3A4 was lyophilized under different conditions and redissolved in buffer to measure the hydroxylation of testosterone under standard conditions with CHP. The resulting activity was compared with the activity of non-lyophilized enzyme.

### 2.3.1. Presence of glycerol and effect of lyophilization

The presence of 10% glycerol in the enzyme storage buffer appeared to be a problem during the lyophilization, causing most of the enzyme sample to foam out of the container. The remaining enzyme mixture was sticky, suggesting that the removal of the glycerol was necessary to obtain a dry enzyme powder. This was confirmed after removal of the glycerol from the enzyme solution by dialysis prior to lyophilization, yielding to nice powdered enzyme.

Giving the large amount of samples needed to carry out a set of experiments, dialysis of the enzyme samples immediately before freeze-drying was not always convenient, and we envisaged removing the glycerol from the storage buffer directly after the purification. It was obviously necessary to first evaluate its importance for the long-term enzyme stability at -80°C. The enzyme activity was then tested with or without glycerol, after 0, 2, 4, 8 or 12 weeks of storage at -80°C, with or without lyophilization, and was compared to the activity in standard conditions with 10% glycerol and no lyophilization. The results are shown in **Figure 20**.

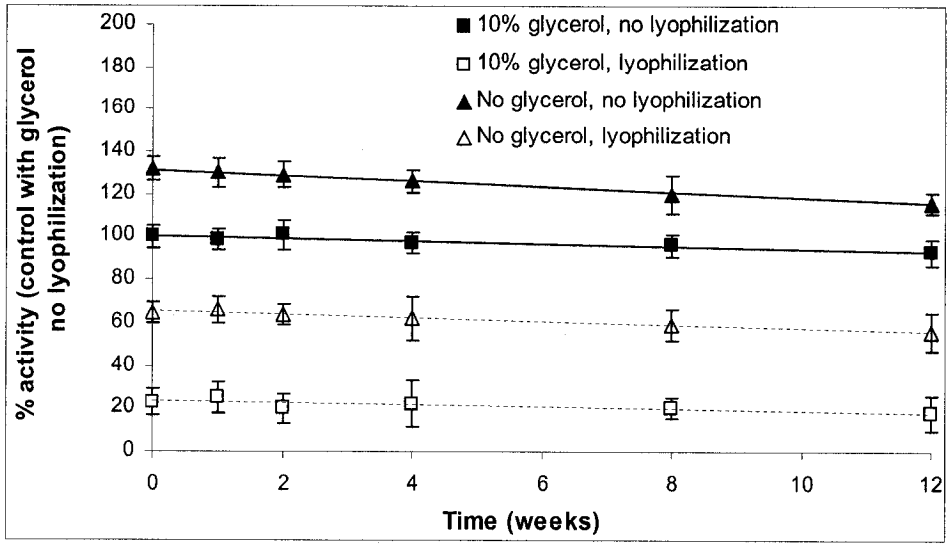



**Figure 20.** Activity of CYP3A4 over several weeks of storage at -80°C, with or without glycerol in the storage buffer, with or without lyophilization. The activity is normalized at 100% for the reaction at t = 0 in the presence of 10% glycerol without lyophilization. Each data point is an average of 3 measurements.

The first significant result is that without lyophilization, the enzyme is 30% more active when glycerol is removed. Upon lyophilization, the enzyme activity is decreased by as much as 5 fold when glycerol is present in the mixture, but only 2 fold without glycerol. In terms of storage at -80°C, glycerol doesn't affect much the stability over the period of time tested. After 4 months, the activity decreases only by 7% when glycerol is present and 13% when glycerol is removed. Following this finding, glycerol was always removed from the storage buffer directly at the end of the purification procedure (no glycerol in the dialysis buffer).

### 2.3.2. Freezing method

Another factor affecting lyophilization conditions is the method of freezing the enzyme. The samples have to be frozen at -80°C before starting the process. The most obvious method is the standard dry ice/acetone bath. However, when a very large number

of samples (>20) are treated at the same time, some samples usually start to thaw before the last samples are frozen. As an alternative method, the samples were left in a -80°C freezer for 2 hours. This freezing method is however slower than the previous one, and results in a larger loss of activity after lyophilization compared to the dry ice/acetone method (**Figure 21**). The remaining activity after lyophilization was 43.9% when using the freezer compared to 51.3 % with the dry ice/acetone bath. A good compromise was obtained when the samples were first quickly frozen in a dry ice/acetone bath, leading to the formation of a frozen shell, before spending another hour in the freezer to make sure the core was frozen as well. The activity after lyophilization was 53.1% in this case. This method was then selected as the method of choice when a large number of samples were treated at the same time.

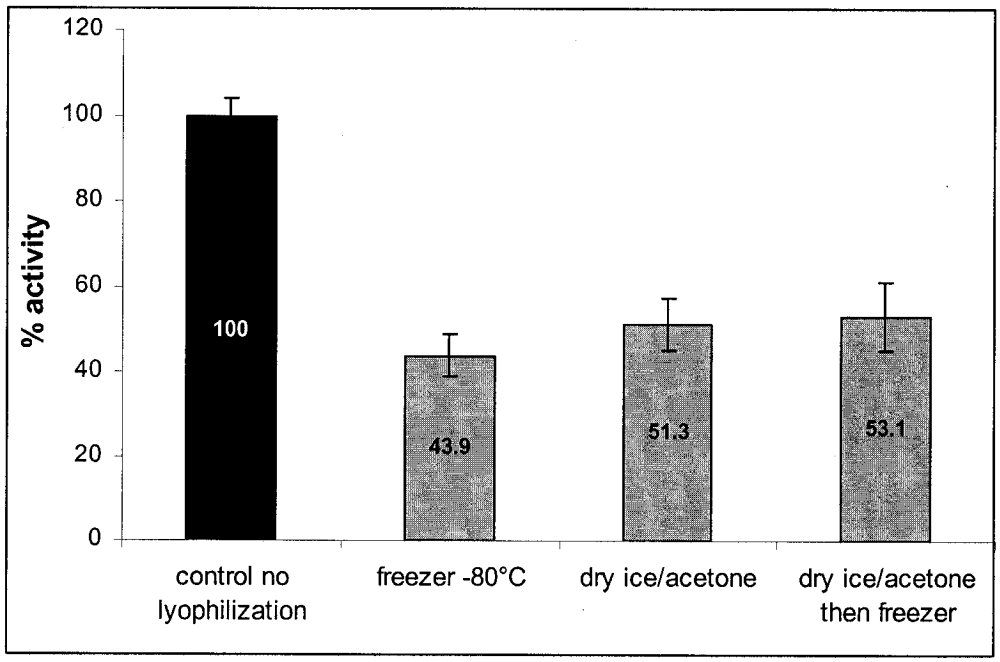



**Figure 21.** Effect of the freezing method applied to the sample prior to lyophilization.

### 2.3.3. Lyoprotection

Lyophilization of enzymes is useful not only for storage but also to prepare

them for use in non-aqueous environments. As demonstrated in the previous paragraphs, the freeze-dried CYP3A4 powder redissolved in buffer is ~50% less active than the initial enzyme solution. This belies the common belief that lyophilization-induced denaturation is reversible upon rehydration, and that it is a critical factor for nonaqueous enzymology only.[195,196,197] In fact, it is believed that rehydration does not lead to the recovery of active proteins unless the native structure is preserved during the freeze-drying process.[198] Numerous studies have demonstrated that excipients can help preserving the structure and activity of enzymes,[199,200,201,202] although the precise mechanism by which these additives function has not been fully elucidated yet. Most of these studies focused on the activity of enzymes suspended in organic solvents after lyophilization, rather than in aqueous medium. Among the commonly used lyoprotectants, the glucose oligomers cyclodextrins have emerged as very effective additives to stabilize some proteins during lyophilization. α-Chymotrypsin lyophilized in the presence of 2,3,6-tri-*O*-methyl β-cyclodextrin displayed activity 40 fold higher than free α-chymotrypsin in acetonitrile.[203] When added before lyophilization methyl-β-cyclodextrin (MβCD) was shown to increase by up to 160-fold the serine protease substilisin Carlsberg activity in THF and 1,4-dioxane.[204,195] The main mechanism involved is the restoration of the H-bonds with the protein after water is removed by dehydration, the cyclodextrin thus acting as a water-mimicking agent.[205] Crown-ethers have also had some success as lyoprotectants,[206] and are believed to protect the enzymes by forming non-covalent macrocyclic complexes with lysine ammonium groups of the enzyme.[207] Addition of 18-crown-6 (18C6) during lyophilization of α-chymotrypsin increased its activity 640-fold when tested in cyclohexane, but the effect was less pronounced with 15-crown-5 (15C5) and 12-crown-4 (12C4). The activity of subtilisin Carlsberg and trypsin was also significantly increased

by addition of 18-C-6 before lyophilization.[208] Lipases have been successfully activated by co-lyophilization with thiacrown ethers.[209,210,211,212,213] Finally, the use of sugars has been widely adopted to preserve the biological activity of enzymes from freeze-drying-induced stress. Sorbitol, sucrose, xylitol, trehalose and mannitol at concentrations of 2% (w/v) before lyophilization improve the activity of *Aspergillus oryzae* protease by up to 60-fold in anhydrous pyridine or carbon tetrachloride, and sorbitol activates α-chymotrypsin and subtilisin Carlsberg 20- and 45-fold respectively.[196] Co-lyophilization with sucrose was reported to have a beneficial effect on the activity of several lipases in hexane.[214,215] *Burkhollderia cepacia* lipase co-lyophilized with sucrose, trehalose and mannitol showed an activity up to 4.7-fold higher in toluene than that without sugars.[216] In addition to protecting enzymes for function in organic solvents, the effect of sugars during lyophilization was also studied upon rehydration. L-Asparaginase co-lyophilized with disaccharides (trehalose, lactose, maltose, or sucrose) or monosaccharides (glucose or mannitol) retained up to 82% of its activity in buffer compared to only 37% for the enzyme freeze-dried alone.[217] Ascorbate oxidase co-lyophilized with sucrose, mannitol or glucose retained most of its activity upon rehydration.[218] Similar results were obtained with smaller proteins, such as the plasma membrane $H^+$-ATPase.[219] Several carbohydrates were found to preserve enzyme activity during freeze-drying and rehydration, particularly trehalose which led to a complete protection at 20 mg/mg protein.

The fact that the stress sustained by CYP3A4 during the freezing and dehydration processes yields to a 48% loss in activity led us to evaluate the use of protecting additives. The molecules tested for their potential lyoprotecting effect on CYP3A4 are shown in **Figure 22**. Two cyclodextrins (methyl-β-cyclodextrin (MβCD)

and hydroxypropyl-β-cyclodextrin (HPβCD)), two crown ethers (18C6 and 15C5), the two monosaccharides sorbitol and mannitol, and the disaccharides trehalose and sucrose, were tested at different concentrations. As shown in **Figure 23** and **Figure 24**, the co-lyophilization of CYP3A4 with the cyclodextrins and the crown ethers did not significantly help protecting the enzyme upon rehydration. Only low concentrations of 18C6 and 15C5 (molar ratio to enzyme of ~50) increased the remaining activity (from 52 to 60%) after lyophilization.

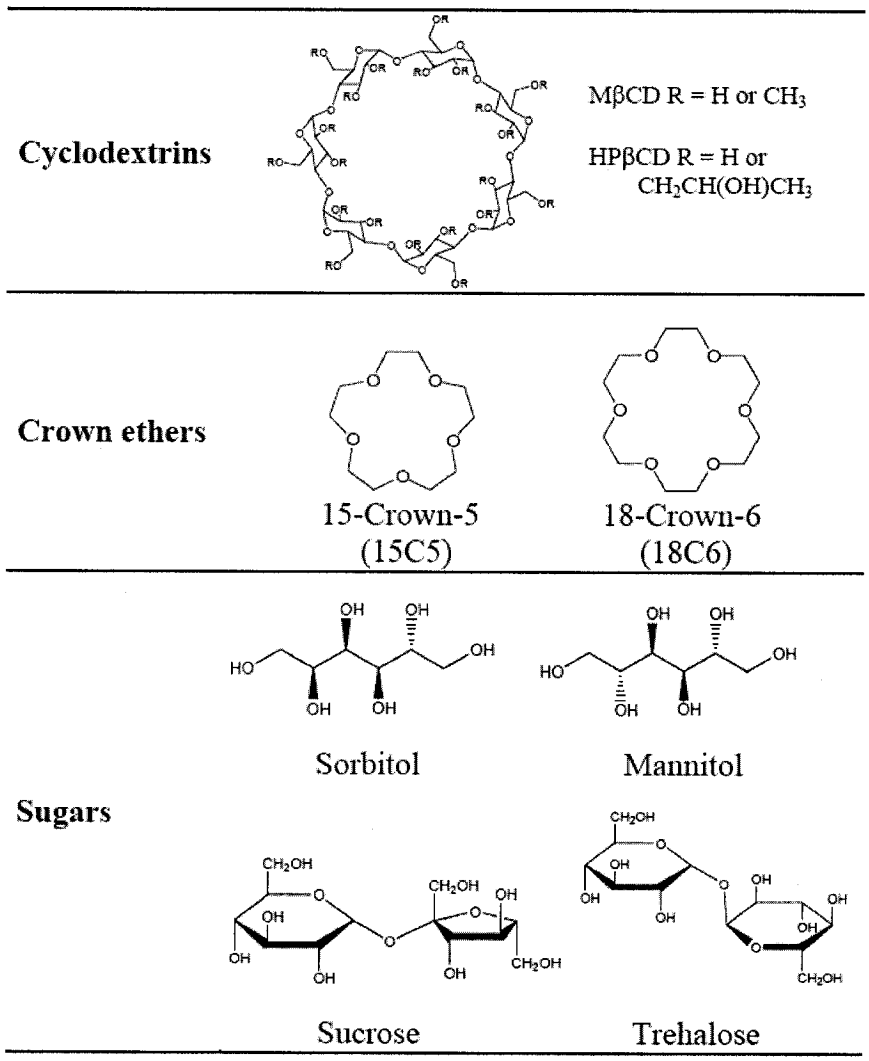



**Figure 22.** Structures of the molecules tested as potential lyoprotectants during the lyophilization of CYP3A4.

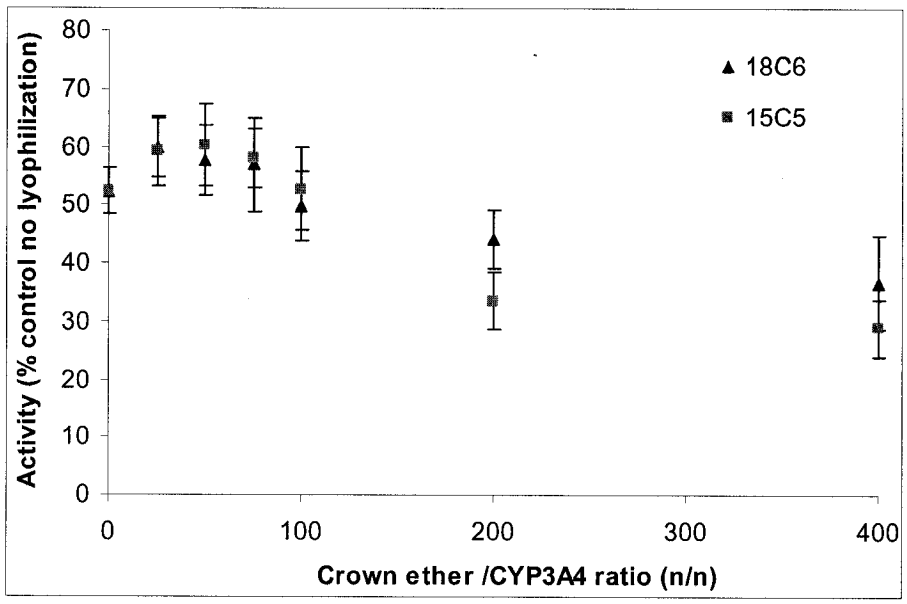



**Figure 23.** Effect of co-lyophilization (24 hrs) with different amounts of 18C6 and 15C5 on the 6β-hydroxylation of testosterone (115 μM) catalyzed by CYP3A4 (0.4 μM) in 0.1 M potassium phosphate buffer at pH 7.4, initiated with cumene hydroperoxide (1 mM), and incubated for 1 h at 37°C.

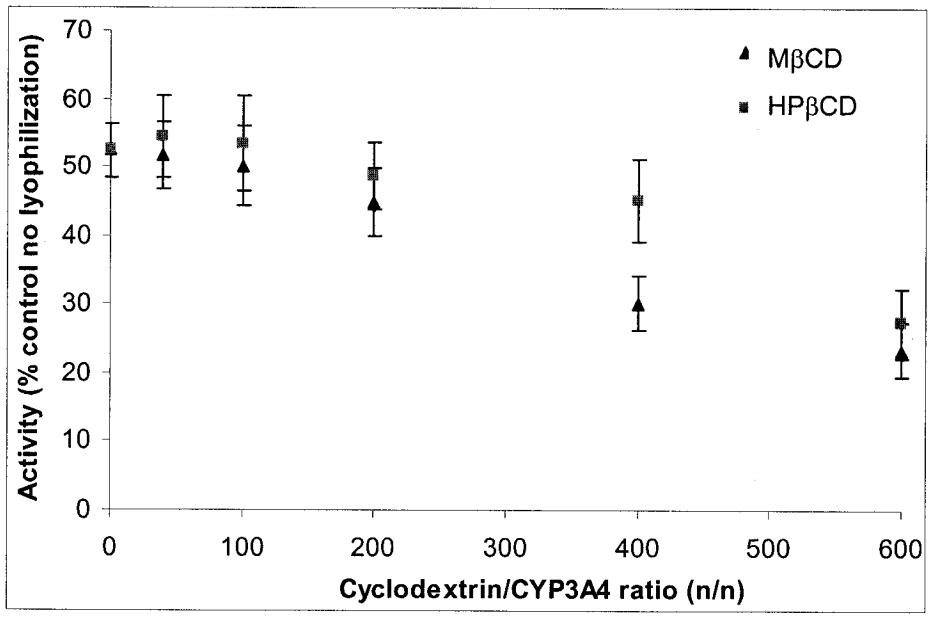



**Figure 24.** Effect of co-lyophilization (24 hrs) with different amounts of methyl-β-cyclodextrin (MβCD) and hydropropyl-β-cyclodextrin (HPβCD) on the 6β-hydroxylation of testosterone (115 μM) catalyzed by CYP3A4 (0.4 μM) in 0.1 M potassium phosphate buffer at pH 7.4, initiated with cumene hydroperoxide (1 mM), and incubated for 1 h at 37°C.

The sugars were much more effective for the retention of CYP3A4 enzymatic activity after lyophilization (**Figure 25**). Both dissacharides (sucrose and sorbitol) appeared to confer similar levels of protection to the enzyme. The level of activity was dependent on the amount of sugar used. Very high ratios of sugar over enzyme (between 5000 and 10000 w/w) allowed the retention of ≥90% of the activity, compared to 52% for the enzyme freeze-dried alone. Sorbitol was almost as effective at lower concentrations (1000-2000 w/w), helping retain about 85% of the activity. The protecting effect quickly dropped when the amount of sorbitol increased further, and no activation remained at concentrations of 10000 w/w and higher. At concentrations ≤1000 w/w, sorbitol, sucrose and trehalose had similar effects. Mannitol was the least effective sugar, barely increasing the residual activity to 60% at concentrations of 2000-5000 w/w. In all cases, no improvement of the activity was observed in the controls without lyophilization, suggesting that the sugar protects against the harmful effects of lyophilization, as opposed to activating the enzyme.

Even though the process of lyophilization was found harmful to enzyme activity, we demonstrated that the use of additives during lyophilization can help preserving most of the enzymatic activity upon rehydration. Sugars were the most efficient excipients, especially sucrose and trehalose which lead to complete protection at high concentrations. The cyclodextrins and crown ethers tested did not significantly affect the enzyme activity.

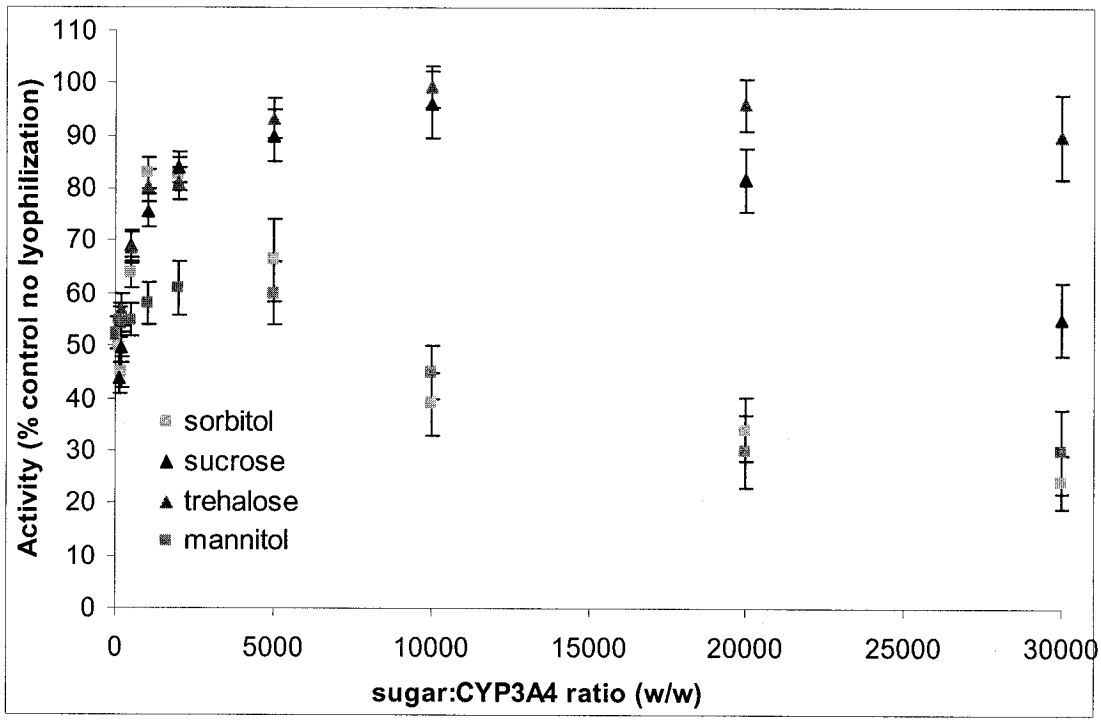



**Figure 25.** Effect of co-lyophilization (24 hrs) with different amounts of sorbitol, sucrose, trehalose and mannitol on the 6β-hydroxylation of testosterone (115 μM) catalyzed by CYP3A4 (0.4 μM) in 0.1 M potassium phosphate buffer at pH 7.4, initiated with cumene hydroperoxide (1 mM), and incubated for 1 h at 37°C.

## 2.4. P450 CYP3A4 ACTIVITY IN THE PRESENCE OF ORGANIC SOLVENTS AND IONIC LIQUIDS

### 2.4.1. Addition of water-miscible co-solvents to the enzymatic reaction

For optimum activity of enzymes in buffer, high concentrations of substrates are often required. This is not always possible with hydrophobic substrates. One approach to overcome this issue is to increase the substrate solubility by adding water-miscible co-solvents to the reaction mixture. For some enzymes a small increase in activity was observed upon addition of solvents; however, the presence of organic solvents usually has severe negative effects on the catalytic activity of enzymes, including P450 enzymes.

Polyphenol oxidase and trypsin were up to 2 fold more active in the presence of 10 to 20% DMF or THF, but acid phosphatase and peroxidase gradually lost their activity with increasing concentrations of acetonitrile, dioxane, DMF and THF. The four enzymes lost at least 90% of their activity with concentrations of solvents above 50%.[220] A few studies evaluating the effect of common organic solvents on the enzymatic activity of P450 enzymes have been published. Most studies were carried out with human liver microsomes[221,222,223] or human hepatocytes.[224] The extent of the effects observed was very variable for the seven P450 isoforms tested. Acetone and acetonitrile at 1% were found to activate the CYP1A2-catalyzed caffeine *N-3*-demethylation by more than 2-fold, and dextromethorphan *O*-demethylation by CYP2D6 was moderately increased in the presence of isopropanol and DMSO (1%).[221] In all other cases, the addition of organic solvents had inhibitory effects on the P450s activities, at concentrations as low as 1%. In the case of CYP3A4, Hickman *et al.* [221] reported ~ 20% inhibition of the enzymatic activity in microsomes with 1% acetone, acetonitrile, DMSO or methanol. Both Chauret *et al.*[222] and Busby *et al.*[223] reported at least 25% inhibition of CYP3A4 by acetonitrile (5%), methanol (3%), and DMSO (0.1%) in microsomes. Easterbrook *et al.*[224] also observed 60% reduced activity of CYP3A4 in the presence of 2% DMSO, and less than 5% inhibition with 2% acetonitrile or methanol in hepatocytes. More recently, the group of Arnold has created variants of the bacterial P450 BM3 with improved resistance to organic co-solvents.[111] The isolated wild-type P450 BM3 shows high-sensibility to various solvents. Addition of THF yields by far to the most drastic decrease of activity (35% remaining activity in 2% THF). DMSO has the least effect with 45% of the activity remaining in 25% solvent. The other solvents (acetone, DMF, acetonitrile and ethanol) showed similar results, about 10 to 12% solvent was necessary to decrease the activity by

90%. The variant W5F5 was 2.5-fold more active than the wild type P450 BM3 in the absence of organic solvent, and showed a 6-fold higher specific activity in 25% DMSO (3.4-fold in 2% THF). This mutant could also tolerate higher concentrations of all four other cosolvents.

No studies of P450 activity in the presence of water-immiscible solvents have been reported. Moreover, no such studies with water-miscible solvents have been published for *purified* human P450s. First, we wanted to evaluate the effect of common organic co-solvents (acetone, acetonitrile, DMSO, THF and methanol) on the 6β-hydroxylase activity of CYP3A4, promoted by the two cofactor surrogates SPC and CHP. The results are summarized in **Figure 26** and **Figure 27**. For all organic solvents tested, the results were very similar for both cofactor surrogates. The addition of a very small amount of organic solvent (1%) led to a significant decrease in the activity of CYP3A4, from 10% with DMSO to ~50% with THF. Similarly to the results reported with P450 BM3,[111] THF led to the sharpest decrease in activity. Less than 2% of the activity remained when 2.5% THF was present. No product formation was detected for THF concentrations ≥5%. The presence of acetonitrile affected the activity to a lesser extent, and was slightly less detrimental with SPC than with CHP. Upon addition of 1% acetonitrile in the reaction mixture, the activity decreased by only 10% with SPC and 35% with CHP, but the remaining activity with 5% acetonitrile was 20% with SPC and only 7% with CHP. The three other solvents, acetone, DMSO and methanol, gave very similar results. At a concentration of 5 %, the solvents reduced the activity to about 35%. In all cases, only a residual activity was left in the presence of 15% solvent, the highest being 4% with DMSO.

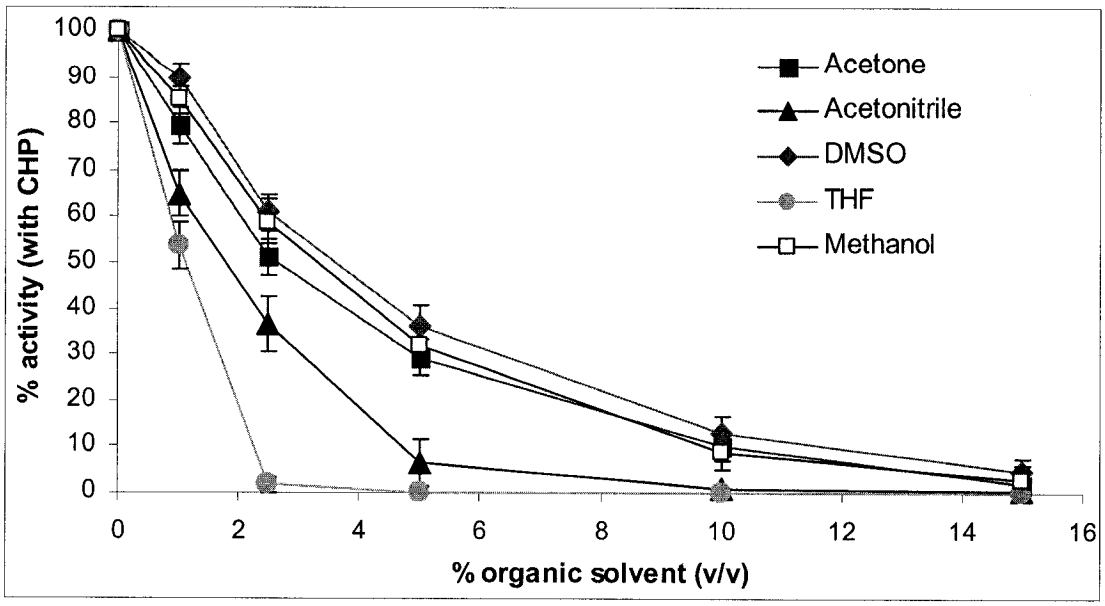



**Figure 26.** Effect of five water-miscible organic co-solvents on the 6β-hydroxylation of testosterone (115 μM) catalyzed by CYP3A4 (0.9 μM) in 0.1 M potassium phosphate buffer at pH 7.4, initiated with cumene hydroperoxide (1 mM). The reaction mixtures were incubated for 1 h at 37°C. ($n$=2, mean ± std. dev.)

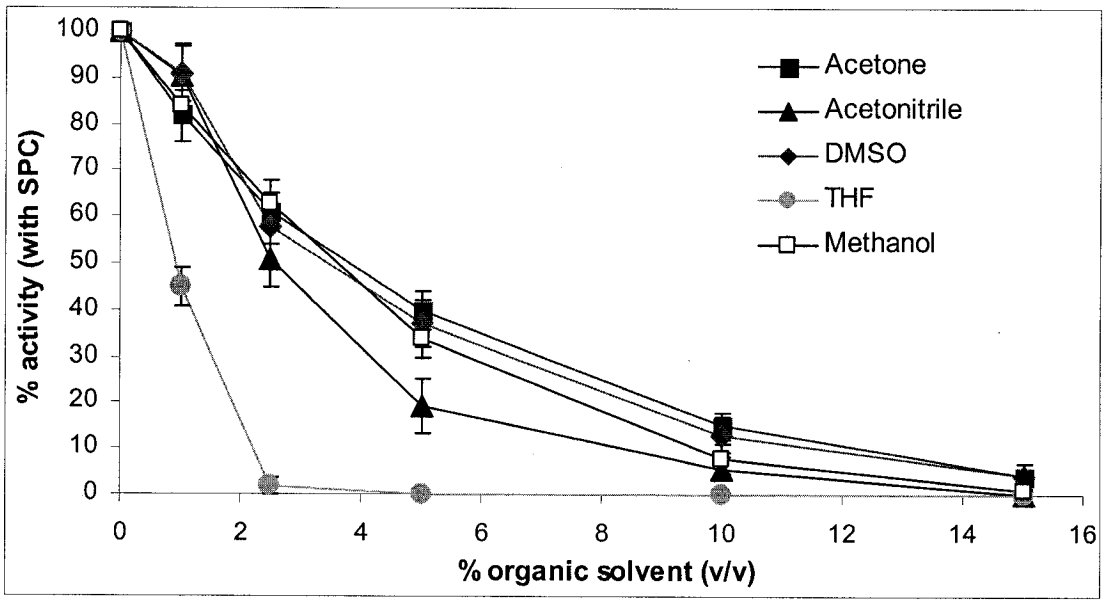



**Figure 27.** Effect of five water-miscible organic co-solvents on the 6β-hydroxylation of testosterone (115 μM) catalyzed by CYP3A4 (0.9 μM) in 0.1 M potassium phosphate buffer at pH 7.4, initiated with sodium percarbonate (12.5 mg, equivalent to 500 mM $H_2O_2$). The reaction mixtures were incubated for 1 h at 37°C. ($n$ = 2, mean ± std. dev.)

As expected, our study confirms that the presence of organic solvents can greatly affect the enzymatic activity of CYP3A4. THF is the most detrimental to CYP3A4 activity, with less than 10% activity remaining when 2.5% THF is present. In the same amount of acetone, DMSO or methanol, more than 50% activity remains.

### 2.4.2. Addition of ionic liquids to the enzymatic reaction

Ionic liquids (ILs) are organic salts with melting points under 100°C, usually lower than room temperature. They exhibit interesting physical properties, including the ability to dissolve a wide range of polar and non-polar organic, inorganic and polymeric compounds. Another important feature of ionic liquids is their designability. Indeed, their chemical and physical properties, such as miscibility with water or organic solvents can be tuned by varying the sidechains lengths on the cation and the nature of the anion. For example, the ionic liquids containing the tetrafluoroborate anion ($BF_4^-$) are water-miscible, while those with the hexafluorophosphate anion ($PF_6^-$) are immiscible. In recent years, ionic liquids have emerged as possible "green" solvents largely because they have no measurable vapor pressure. These remarkable features explain why the use of ionic liquids to replace organic solvents has recently attracted so much attention, for both chemical and biocatalytic processes. The most widely studied applications of ionic liquids with enzymes involve biphasic systems with water-immiscible ionic liquids. In particular, 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM] [$PF_6$]) has been used in biphasic systems for the formation of 3-cyanobenzamide and 3-cyanobenzoic acid by nitrile hydratase,[225] and the synthesis of Z-aspartame with thermolysin.[226] The oxidation of syringaldazine catalyzed by laccase C was shown to tolerate only moderate concentrations of the water-miscible 4-methyl-*N*-butylpyridinium tetrafluoroborate ([4-

MBP][$BF_4$]). The initial rate of the reaction dropped more than 2-fold when 10% [4-MBP][$BF_4$] was present, and by 25 fold if the ionic liquid concentration reached 25%.[227]

No studies have been reported about the effect of ionic liquids on P450 activity. We wanted to investigate the effect of several common ionic liquids on the activity of CYP3A4. The ionic liquids chosen for this study included 3 water-miscible ones (1-methyl-3-methylimidazolium tetrafluoroborate ([MMIM] [$BF_4$]), 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM] [$BF_4$]), and *N*-butylpyridinium tetrafluoroborate ([BPyr] [$BF_4$]), as well as the commonly used water-immiscible [BMIM] [$PF_6$]. The water-miscible ionic liquids were used exactly as described above for the organic co-solvents, at concentrations ranging from 1 to 50% (v/v). The water-immiscible [BMIM] [$PF_6$] was used in a biphasic system with the potassium phosphate buffer, at concentrations ranging from 1 to 75% (v/v). The reactions were initiated with the cofactor surrogate cumene hydroperoxide. As expected, the activity drops upon addition of small percentages of ionic liquids (**Figure 28**). The water-miscible ionic liquids all have a very similar effect, and show a decrease in activity of CYP3A4 comparable to that observed in the presence of organic co-solvents. At concentrations of 2.5%, these ionic liquids reduced the activity to about 60%, and only 10% activity remains when the concentration is increased to 10%. With the three systems, no product formation was detected when the concentration of ionic liquid was 15% or more, and the enzyme precipitated. Slightly better results were obtained with the biphasic system [BMIM] [$PF_6$]/buffer. More than 50% of the activity remains with 5% [BMIM] [$PF_6$], and a five-fold reduction in activity is obtained with 15%. The presence of 50% [BMIM] [$PF_6$] is necessary to decrease the activity below 10%.

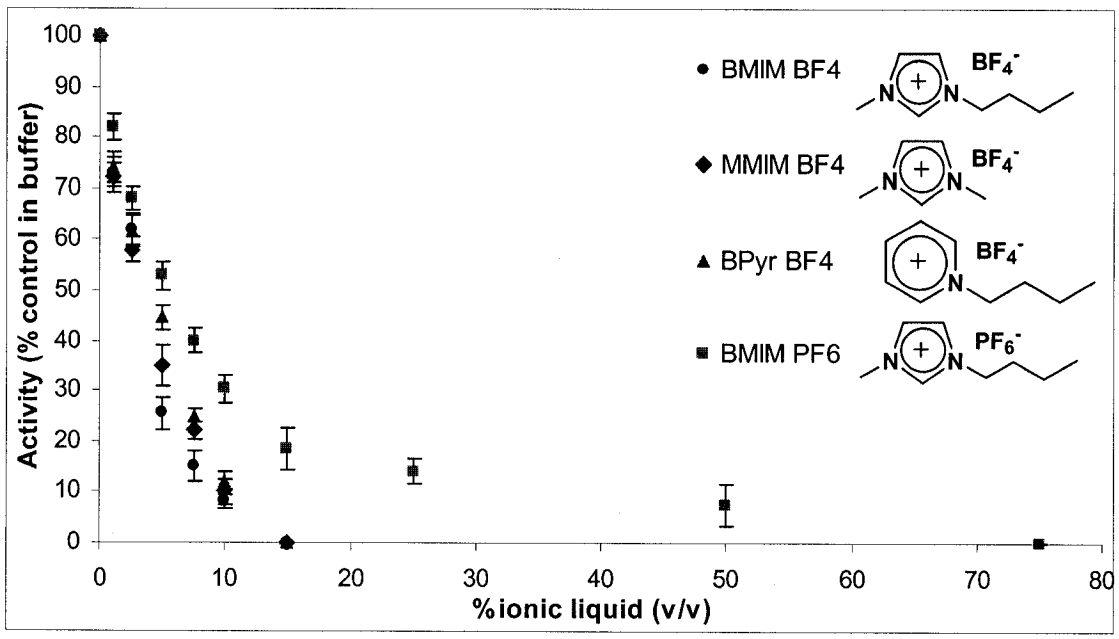



**Figure 28.** Effect of different ionic liquids (3 water-miscible and 1 water-immiscible ionic liquids) on the 6β-hydroxylation of testosterone (115 μM) catalyzed by CYP3A4 (0.9 μM) in 0.1 M potassium phosphate buffer at pH 7.4, initiated with cumene hydroperoxide (1 mM), and incubated for 1 h at 37°C. ($n$ = 2, mean ± standard deviation)

As with organic co-solvents, a low content of water-miscible ionic liquids is tolerated by CYP3A4 without major effect on activity. Such homogeneous solvent systems may help solubilize non-polar substrates. The use of a biphasic system seems however more promising since higher content of ionic liquid can be used without significant harm to the enzyme. The amounts tolerated are however not sufficient to dissolve hydrophobic substrates in large amounts.

### 2.4.3. CYP3A4 activity in water-immiscible organic solvents

The final step in this project was to assay the activity of the lyophilized CYP3A4 enzyme as a suspension in water-immiscible organic solvents. This would be useful for the transformation of very hydrophobic substrates by this enzyme. Fifteen solvents were first screened: n-pentane, hexane, cyclohexane, heptane, iso-octane, ethyl acetate, ethyl

ether, butyl ether, isopropylether, n-butanol, toluene, benzene, dichloromethane, 1,2-dichloroethane and chloroform. The enzyme was lyophilized in the presence of sucrose (2000 w:w). This condition was selected using a compromise between lyoprotecting effect (as reported in the paragraph 2.2.3.) and technical practicability. Even though higher amounts of sucrose or trehalose resulted in better protection, when the amount of enzyme is increased, the reaction vessel is filled entirely with sugar powder, thus preventing resuspension in organic medium. Sucrose was selected over trehalose because in addition to being a hundred times cheaper, the resulting lyophilized sample is more homogeneous and easier to handle.

After lyophilization, the enzyme was resuspended in organic solvent with substrate, and the reaction was initiated with cumene hydroperoxide. At the end of the incubation, the organic solvent was transferred to a glass vial, and evaporated in vacuo. The residue was redissolved in methanol before analysis by HPLC. The possibility that some product may have been "trapped" in the remaining wet mixture of enzyme, buffer salts and sucrose was taken into account. This was envisaged knowing that a non-negligible amount of water (0.85%) was present in the reaction mixture, introduced by the cumene hydroxide solution in water:methanol (90:10). If some 6β–OHT was formed during the reaction in organic solvent, it may partition in the thin aqueous layer. Thus the aqueous layer was homogeneously redissolved in acidified water (a condition preventing any reaction from occurring), and the products extracted as usual in dichloromethane. Overall, for each solvent tested, two samples were thus analyzed, the organic fraction (except for decane and dodecane which could not be evaporated) and the aqueous fraction extracted in DCM. None of the organic fractions contained 6β–OHT. However, some product was detected in the aqueous fractions for reactions in pentane, hexane,

cyclohexane, heptane and iso-octane. With those solvents, cortexolone was found predominantly in the aqueous fraction, but both peaks (in the aqueous and organic fractions) were taken into account when quantifying the 6β-OHT formation. Testosterone on the other hand was more important in the organic fraction. This difference of solubility was expected based on structure (one extra hydroxyl group in the product 6β-OHT) and confirmed as decribed below. Product formation in the organic solvents (alkanes) was about 10% that in the control reaction (**Figure 29**). **Figure 30** shows the HPLC traces of both the aqueous and organic fractions for each of the five alkanes as well as the control reaction in buffer (after lyophilization with sucrose).

To verify if this partitioning of the substrate and product corresponds to solubility, the actual partition coefficient of testosterone, 6β-hydroxytestosterone and cortexolone between buffer and solvent (hexane, cyclohexane, n-heptane, n-pentane and iso-octane) were measured. The partition coefficients were determined after equilibration at 37°C of a biphasic system (buffer/alkane) containing the three compounds, followed by analysis of each phase by HPLC. The alkane-buffer partition coefficients were calculated for each compound as the ratio of its peak area in the organic phase to its peak area in the aqueous phase. As expected, 6β-hydroxytestosterone and cortexolone do not partition well in the organic solvents. The partition coefficient of 6β-hydroxytestosterone could not be calculated in any of the solvents tested since it was below our detection limit by HPLC. Cortexolone was not found in either of the organic phases except for hexane, but the partition coefficient was very low (0.08). On the contrary, testosterone partitions slightly better in all organic solvents, except for pentane (**Table 14**).

These results corroborate the previous findings, and explain why the product formation could only be detected in the aqueous fraction. This experiment also confirmed that halogenated solvents (dichloromethane, chloroform and 1,2-dichloroethane) are the solvents of choice for extraction of the reaction substrates and products (as determined for dichloromethane in paragraph 2.2.1.), since 99.4, 99.6 and 98.1% of the testosterone was found in the organic fractions respectively (and cortexolone was only detected in the organic fractions).

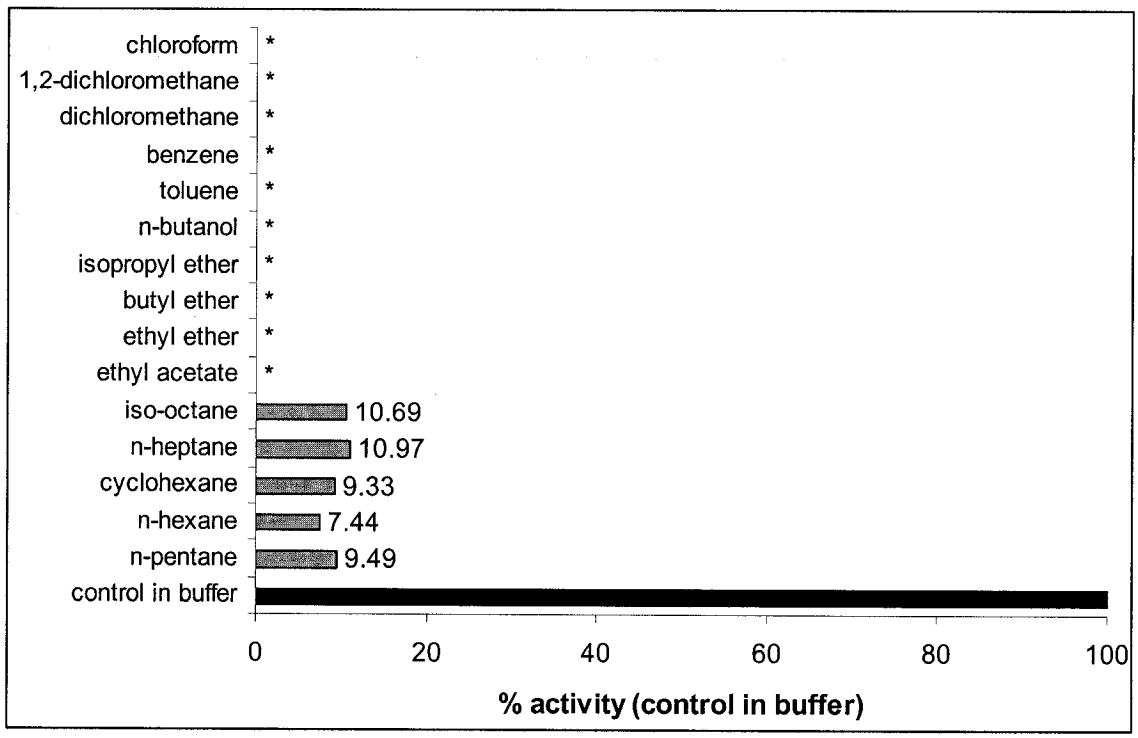



**Figure 29.** Activity of CYP3A4 (1.30 μM) lyophilized with sucrose (2000 w:w) incubated for 2 hrs with testosterone (40 μM) and cumene hydroperoxide (1 mM) in various organic solvents. The activity is measured as the % of 6β-OHT formed in the presence of organic solvents compared to that formed in the control reaction in buffer (both after lyophilization with sucrose). *: below detection limit.

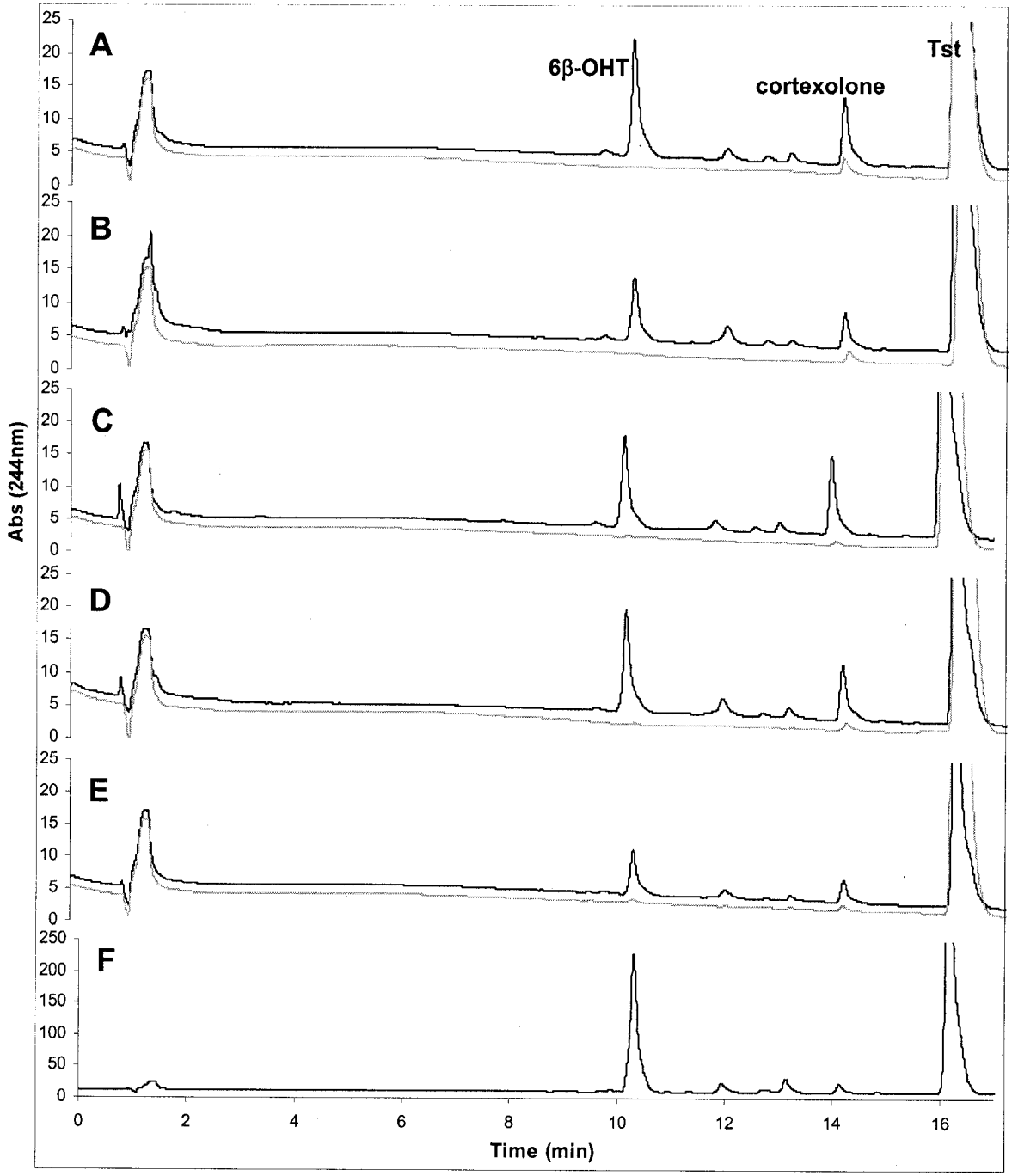



**Figure 30.** HPLC chromatograms with detection at 244 nm of the aqueous (black line) and organic (gray line) fractions following incubation at 37°C for 2 hrs of CYP3A4 (1.3 μM, lyophilized for 24 hrs with sucrose 2000 w:w), testosterone (40 μM) and CHP (1 mM, 9 μl from a 0.1 M solution in water:methanol 90:10) in 900 μl of n-pentane (**A**), n-hexane (**B**), cyclohexane (**C**), n-heptane (**D**), iso-octane (**E**), or 0.1 M potassium phosphate buffer at pH 7.4 (**F**, this is the control reaction, note that y-axis is different)

**Table 14.** Partition coefficients of testosterone between different organic solvents and buffer at 37°C

| Solvent | Partition coefficient alkane/buffer at 37°C[a] |
|---|---|
| n-Hexane | 1.38 ± 0.4 |
| Cyclohexane | 2.34 ± 0.9 |
| n-Heptane | 1.38 ± 0.3 |
| n-Pentane | 0.66 ± 0.3 |
| Iso-octane | 1.13 ± 0.1 |

[a] The partition coefficient was calculated as the ratio of the HPLC peak area of testosterone in the organic phase to its peak area in the aqueous phase.($n$ = 3, mean ± standard deviation)

Since enzymatic activity was observed only in hydrophobic solvents, a broader range of alkanes were selected for further studies (n-pentane, iso-pentane, n-hexane, cyclohexane, methylcyclohexane, n-heptane, n-octane, and iso-octane). Experiments were carried out as described above, however enzyme samples were lyophilized with or without sucrose. For the samples lyophilized in the presence of sucrose, some product was once again detected in the presence of the ten solvent tested. **Figure 31** shows the product formation in the organic solvents expressed as a percentage of that in the control reaction in buffer.

Surprisingly, no product formation was detected when no sucrose was present during lyophilization. The enzyme lyophilized without sucrose was however active in buffer as shown by the control reaction (**Figure 32**). This result suggests that the lyophilization-induced denaturation is irreversible in organic solvent, but partially reversed by buffer. Although sucrose is not able to fully preserve the native secondary structure during lyophilization, it contributes to reduce the conformational changes occurring during the process. This effect has been reported before with other enzymes.[214,215,216]

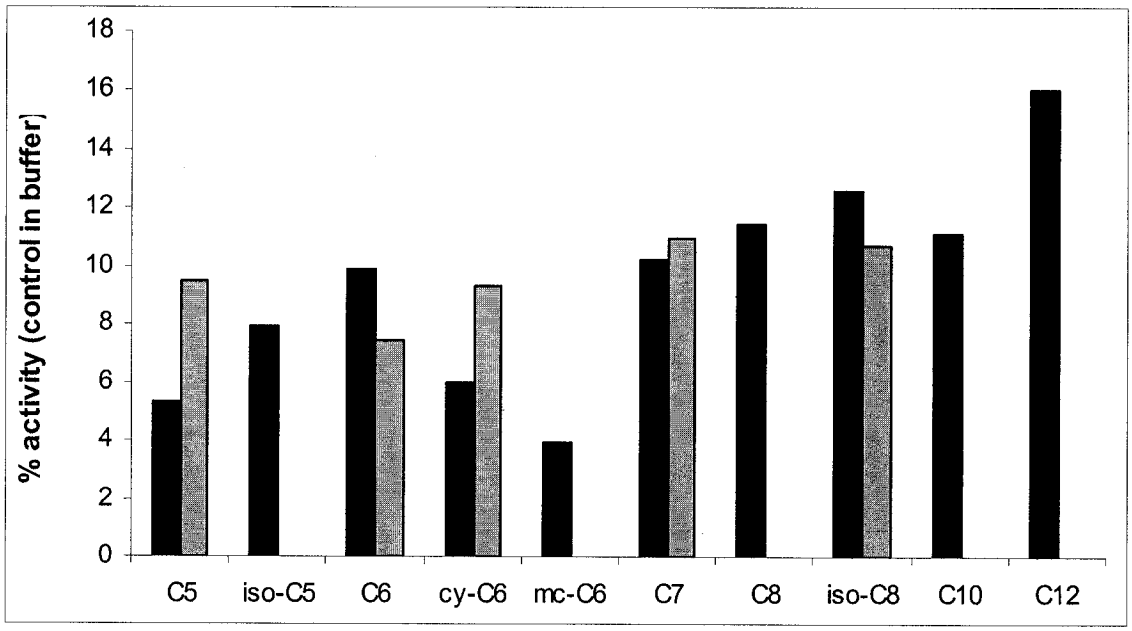



**Figure 31.** Activity of CYP3A4 (1.30 μM) lyophilized with sucrose (2000 w:w) incubated for 2 hrs with testosterone (40 μM) and cumene hydroperoxide (1 mM) in different organic solvents (C5: n-pentane, iso-C5: iso-pentane, C6: n-hexane, cy-C6: cyclohexane, mc-C6: methylcyclohexane, C7: n-heptane, C8: n-octane, iso-C8: iso-octane, C10: n-decane, C12: n-dodecane). The activity is measured as the % of 6β-OHT formed in organic solvents compared to that formed in the control reaction in buffer (after lyophilization with sucrose). Gray bars represent results obtained during the initial screening of 15 organic solvents, and black bars represent the results obtained during the subsequent screening of 10 alkanes.

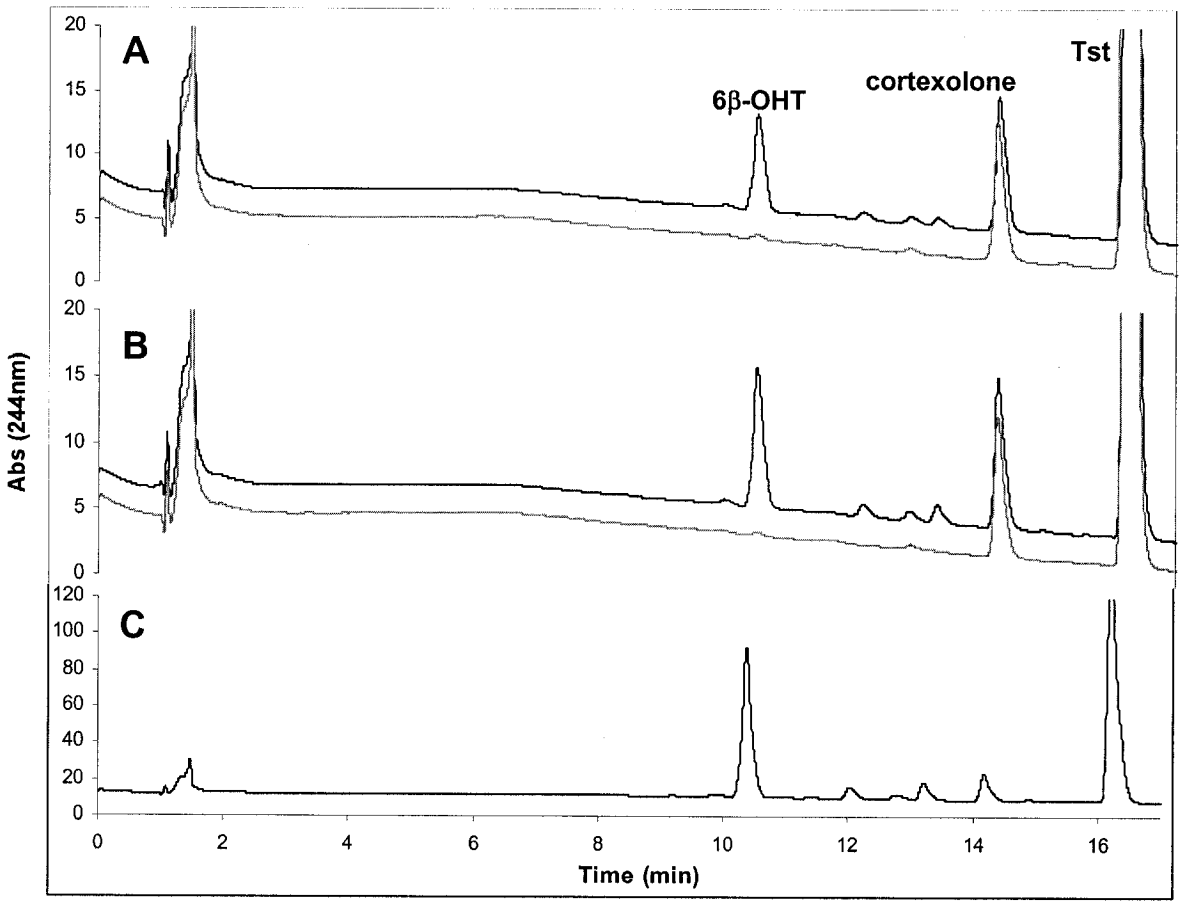



**Figure 32.** HPLC chromatograms with detection at 244 nm of the aqueous fraction following incubation at 37°C for 2 hrs of CYP3A4 (1.3 μM), testosterone (40 μM) and CHP (1 mM, 9 μl from a 0.1 M solution in water:methanol 90:10) in n-hexane (**A**) or iso-octane **(B).** The enzyme was lyophilized alone (gray line) or in the presence of sucrose (black line). The control reaction was carried out in 0.1 M potassium phosphate buffer at pH 7.4 with enzyme lyophilized without sucrose (**C**).

Another aspect of enzymatic reactions in organic solvent that we wanted to examine is the molecular imprinting effect. As discussed in the introduction, the presence of substrate during lyophilization has been shown to "imprint" the enzyme with a conformation favorable for reaction. This property is however restricted to nearly anhydrous media, more than 0.1% water in the solvent increases the enzyme flexibility upon rehydration and cancels the imprinting effect. Eight alkanes were once again tested (all but n-pentane and iso-pentane which boil at 37°C, making them inconvenient to use),

with enzyme lyophilized with sucrose and with or without testosterone. To ensure that minimum water was present in the system, cumene hydroxide was this time added from a solution in the corresponding organic solvent. As described before, the organic solvent was transferred into a glass vial after incubation. The remaining enzyme/sucrose suspension, although supposedly containing less water than in the previous experiment, still formed a sticky aggregate. As it was again envisaged that some product might be trapped into this matrix of sucrose, the sticky aggregates were treated as previously described for the thin aqueous layer (dissolved in acidified water and extracted with dichloromethane). Again, 6β-OHT was only detected in the aqueous fractions, and the product formation was as much as 75-fold higher (for n-octane) with the "imprinted" samples than with the non-imprinted ones, which represents almost 20% of the product formation in the control reaction in buffer without lyophilization. **Figure 33** shows the HPLC chromatographs for the reactions in n-octane, with both the imprinted and non-imprinted enzyme, as well as the chromatograph of the control reaction in buffer. It is important to note that even though the product peak in n-octane seems to represent much less than the $1/5^{th}$ of the peak in buffer, the internal standard peak is also much smaller, simply revealing that some sample may have been lost at some point during the handling. The ratio 6β-OHT/cortexolone is what is taken into account when quantifying the product formation. The turnover numbers for the reactions in organic solvents are $\leq 10$ except for the reaction in n-octane which exhibits a turnover of 26. This represents about half the maximum turnover (50) found earlier in aqueous buffer (**Table 13**). All the results are summarized in **Table 15.**

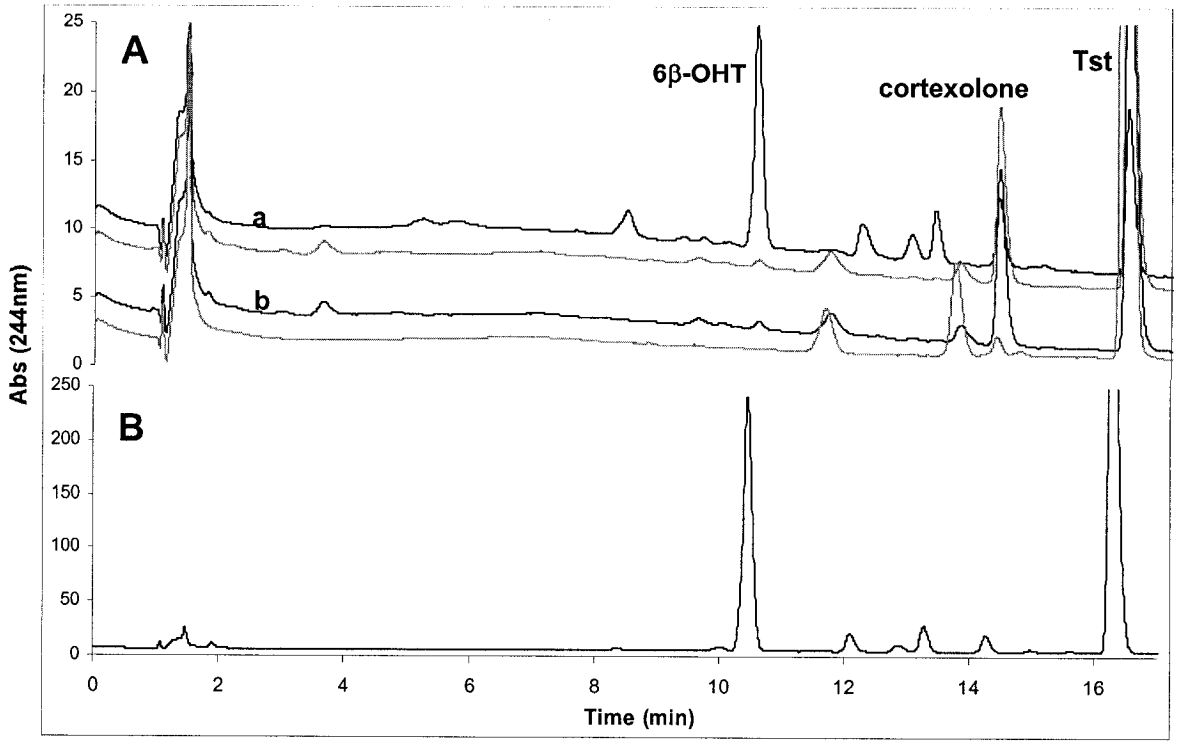



**Figure 33.** HPLC chromatograms with detection at 244 nm of the aqueous (black line) and organic (gray line) fractions following incubation at 37°C for 2 hrs of CYP3A4 (2 μM), testosterone (40 μM) and CHP (1 mM, 9 μl from a 0.1 M solution in n-octane) in n-octane (**A**). Testosterone was added before (**a**) or after lyophilization (**b**). The control reaction was carried out in 0.1 M potassium phosphate buffer at pH 7.4 with lyophilized enzyme (**B**). Note that y-axis is different.

**Table 15.** Effect of molecular imprinting (testosterone present during lyophilization) on the CYP3A4 activity in organic solvents

| | **Imprinting activation factor in organic solvents[a]** | **% control in buffer[b]** | **Turnover number[c]** |
|---|---|---|---|
| n-Hexane | 9.5 | 4.1 | 5.8 |
| Cyclohexane | 5.8 | 2.2 | 3.1 |
| Methylcyclohexane | 4.7 | 1.6 | 2.3 |
| n-Heptane | 9.5 | 5.3 | 7.5 |
| n-Octane | **74.6** | **18.8** | **26.5** |
| iso-Octane | 8.9 | 6.7 | 9.4 |
| n-Decane | 7.8 | 7.4 | 10.4 |
| n-Dodecane | 6.9 | 2.5 | 3.6 |

[a] The imprinting activation factor was calculated as the ratio of product formed when testosterone was present during lyophilization to that when it was not. [b] Product formation with the imprinted samples expressed as the percentage of the product formation in the control reaction in buffer. [c] calculated after incubation for 2hrs of CYP3A4 (2 μM), testosterone (40 μM) and CHP (1 mM) in organic solvents.

Another important result obtained in this experiment is that the product formation by the non-imprinted enzyme in organic solvents is much lower (about 10-fold) than in the previous assays. This dramatic loss of activity might be explained by the difference in water content. In the previous experiments, almost 1% water was present in the reaction system, introduced by the aqueous solution of CHP. In this assay however, CHP was prepared in a solution of anhydrous organic solvents. The results show that CYP3A4 is more active in organic solvents when some water is present. This was not expected based on literature. Indeed, previous work with other enzymes suggests that a water content below 0.1 % is needed to observe activity in organic solvents.

In summary, our experiments with CYP3A4 in the presence of organic solvents revealed that the enzymatic reaction can tolerate low amounts of water-miscible co-solvents. The activity drops by about 60% when 5% acetone, acetonitrile, DMSO or methanol is present. Small amounts of water-miscible ionic liquids have similar effects, decreasing the activity by 40% at concentrations around 2.5%. The presence of the water-immiscible ionic liquid [BMIM] [$PF_6$] is slightly less detrimental, 25% ionic liquid is necessary to decrease the activity to 15%. CYP3A4, once lyophilized, is also active when suspended in hydrophobic solvents (n-pentane, n-hexane, cyclohexane, methylcyclohexane, n-heptane, n-octane, iso-octane, n-decane, n-dodecane). The product formation in n-octane is almost 20% that in buffer after lyophilization. The addition of sucrose during lyophilization is necessary to preserve the activity in organic solvents. When the water content drops from 0.85% to less than 0.1%, the activity in organic solvents is greatly reduced. The addition of the substrate testosterone during lyophilization helps activating the enzyme in nearly anhydrous media.

# 3. CONCLUSIONS AND FUTURE DIRECTION

Asymmetric synthesis is undoubtedly the most important challenge of today's synthetic chemists. In particular, the regio- and stereo-selective oxidation of inactivated C-H bonds remains a significant issue that few chemical catalysts have succeeded to overcome. Nature has evolved biocatalysts which are able to efficiently carry out these difficult reactions. Examples of such remarkable catalysts include the P450 enzymes, found in all kingdoms of life. This family of heme-containing monooxygenases includes more than 5000 known isoforms. They have gained significant attention over the last decades, because of their potential as powerful biocatalysts in chemical synthesis.

Our research aims to develop conditions that would facilitate the use of P450 enzymes in synthesis. CYP3A4 was selected for its exceptional substrate promiscuity. The enzyme was expressed in *E. coli* competent cells and purified by nickel affinity. The first step was to find an effective way to replace the natural cofactors, NADPH and cytochrome P450 reductase, to ease CYP3A4 use in synthesis. We have demonstrated that CYP3A4-catalyzed 6β-hydroxylation of testosterone can be efficiently supported by some cheap hydrogen peroxide donors or organic peroxides. In particular, sodium percarbonate (a solid source of hydrogen peroxide) and cumene hydroperoxide led to higher enzymatic activity than with the natural cofactors. Moreover, this is achieved without a significant cost to the enzyme stability. The presence of glycerol in the storage buffer was found not essential for the enzyme stability overtime, and the enzyme was more active in buffer in the absence of glycerol. Moreover, the loss of activity after lyophilization was more important with glycerol (80%) than without (50%), so glycerol could be safely removed from the storage buffer for all the following studies.

The next step was to survey the effect of various water-miscible co-solvents and ionic liquids on CYP3A4 activity in buffer. It was found that CYP3A4 could tolerate small amounts of such additives ($\leq$5%) in the reaction medium without significant loss of activity. The results were even more promising with the water-immiscible ionic-liquid 1-butyl-3-methylimidazolium hexafluorophosphate (30% activity remaining when 10% ionic liquid is present). These conditions were however not sufficient to dissolve large amounts of most hydrophobic substrates.

Finally, the enzyme was tested in water-immiscible organic solvents. First, the lyophilization process had to be optimized. It was found that some sugars, when added in large amounts, could help protect the enzyme against the harmful effects of lyophilization (50% loss of activity). Among the various organic solvents tested, only hydrophobic ones were found to maintain activity of the lyophilized enzyme. Further studies revealed that the addition of sucrose during lyophilization was essential for activity of CYP3A4 in organic solvents. Finally, "imprinting" the enzyme with the substrate testosterone during lyophilization was an efficient method to increase the enzymatic activity in non-aqueous media.

These studies reveal that many of the drawbacks associated with the use of enzymes in synthesis can be overcome with CYP3A4. The substrate specificity issue is partially addressed by the choice of this promiscuous isoform. Cofactors can be replaced by cheap hydrogen peroxide donors or organic peroxides, such as sodium percarbonate or cumene hydroperoxide. The drastic loss of activity after lyophilization can be prevented by addition of protective excipients, such as sucrose, before lyophilization. Finally, CYP3A4 lyophilized with sucrose and imprinted with the substrate testosterone is active

in hydrophobic solvents, especially n-octane in which the product formation is almost 20% that in aqueous conditions. Future directions towards facilitating the use of CYP3A4 in synthesis may include immobilization of the enzyme on solid supports, a strategy which has been successfully used with other enzymes. We could also consider the use of mutagenesis to create mutants more stable and active in organic solvents. Finally, more substrates will need to be tested, in order to broaden the applicability of CYP3A4 in synthesis. "Sexy" substrates remain to be found, which will firmly establish the great usefulness of P450 enzymes in organic synthesis.

# 4. MATERIALS AND METHODS

## 4.1. CHEMICALS

The CYP3A4 pSE3A4His expression plasmid was a gift from Dr. J. R. Halpert from the University of Arizona, and the cytochrome P450 reductase OR263 plasmid was kindly donated to us by Dr. Charles B. Kasper from the University of Wisconsin. The culture media ingredients yeast extract, tryptone and peptone were purchased from BD Biosciences (San Jose, CA). DH5α supercompetent cells and agarose were from Invitrogen (Carlsbad, CA). The 2',5'-ADP Sepharose 4B and DEAE Sepharose resins were from Amersham Bioscience (Baie d'Urfé, QC, Canada). The Ni-NTA Nickel Affinity Gel was purchased from Qiagen. The CYP3A4 substrate testosterone (4-androsten-17β-ol-3-one) was a kind gift from Dr. Eisenberg in our department and the metabolites 6β-hydroxytestosterone (4-androsten-6β,17β-diol-3-one) and 6α-hydroxytestosterone (4-androsten-6α,17β-diol-3-one) were purchased from Steraloids (Newport, RI). The ionic liquids [MMIM] [$BF_4$] (1-methyl-3-methylimidazolium tetrafluoroborate), [BMIM] [$BF_4$] (1-butyl-3-methylimidazolium tetrafluoroborate), [BMIM] [$PF_6$] (1-butyl-3-methylimidazolium hexafluorophosphate) and [BPyr] [$BF_4$] (*N*-butylpyridinium tetrafluoroborate) were a king gift from Dr. Chan. All solvents were purchased from Fisher and were of HPLC grade.

## 4.2. INSTRUMENTS

UV absorption spectra were recorded on a Cary 5000 UV spectrophotometer (Varian, Mississauga, ON, Canada). Fluorescence measurements were obtained on a Spectramax GeminiXS (Molecular Devices Corp., Sunnyvale, CA) using 96-well flat

bottom assay plate (Corning Incorporated Life Sciences, New York, NY). Analytical HPLC analyses were performed on an Agilent 1100 modular system consisting of an auto-sampler, a quaternary pump system, a photodiode-array detector, a fluorescence detector, and a thermostated column compartment. The Agilent Chemstation software version A.10.02 was used to control the operation and data acquisition. Analysis of testosterone 6β-hydroxylation by CYP3A4 used a 150 x 4.6 mm Zorbax Eclipse XDB-C8 5 μm column from Agilent protected with an analytical guard column. The elution consisted of a first isocratic step at 15% acetonitrile in water for 4 min, before a linear gradient to 50% acetonitrile over 12 min. The flow rate was 1.5 ml/min, and the column temperature was set to 30°C. Detection was at 244 nm, and the injection volume was 25 μl. Under these conditions, the retention times of testosterone, 6β-hydroxytestosterone, 6α-hydroxytestosterone and cortexolone were 16.3, 9.9, 10.5 and 14.2 min respectively. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with an Amersham Phastsystem on 12.5 % homogeneous gel with staining in coomassie blue. The lyophilization of enzyme solutions was achieved using a Labconco lyophilizer.

## 4.3. MEDIA FOR BACTERIAL GROWTH

**LB medium.** Dissolve bacto-tryptone (10 g/l), bacto–yeast extract (5 g/l), and NaCl (5 g/l) in water, and adjust the pH to 7.5 with NaOH. Autoclave 20 min at 120°C to sterilize the medium. Allow the autoclaved medium to cool to 55°C and add desired amount of ampicillin (filtered sterilized).

**LB agar plates**. Add bacto-agar (15 g/l) to LB medium prior to autoclaving, and add ampicillin to the cooled medium before pouring the plates.

**TB medium.** Dissolve tryptone (12 g), yeast extract (24 g), and glycerol (4 ml) in milliQ water (900 ml) and autoclave. Prepare and autoclave separately the $TB_{salts}$ solution, containing $KH_2PO_4$ (2.31 g/l) and $K_2HPO_4$ (12.54 g /l), then add 100 ml of $TB_{salts}$ to 900 ml of TB medium. Allow the medium to cool down before adding ampicillin (50 μg/ml).

**SOC medium**. Dissolve tryptone (20 g), yeast extract (5 g), NaCl (0.5 g), KCl (2.5 ml of 1 M) in milliQ water (1 l), adjust the pH to 7.0 with 10 N NaOH, and autoclave to sterilize. Add sterile 1 M glucose (20 ml) immediately before use.

## 4.4. PREPARATION OF ENZYMES

### 4.4.1. CPR expression and purification

*E. coli* DH5α supercompetent cells were transformed with the cytochrome P450 reductase OR263 plasmid and spread onto LB medium agar plate containing ampicillin (100 μg/l) and incubated overnight at 37°C. Four separate colonies were added to LB medium (6 ml) containing ampicilin (100 μg/l). The culture was incubated overnight (225 rpm, 37°C, 17 hrs) before dilution in TB (3 ml in 1 l) supplemented with ampicillin (100 μg/l) and riboflavin (1 μg/l). CPR expression was induced with IPTG (0.5 mm) at OD600 ~ 0.8 (~3 h at 37°C and 225 rpm). The cultures were further grown at 37°C for 20 hrs. Unless mentioned otherwise, all the steps in the protein purification were carried at 4°C and storage was at -80°C. Cells were collected by centrifugation (4614 ×g, 25 min) and the harvested cells (46.8 g) were resuspended in TSE buffer (60 ml). Lysozyme (1.2 mg) was added and the mixture was incubated for 20 min. After centrifugation (3000 × g, 30 min), the pellet was lysed for 20 min in lysis buffer (60 ml, containing 50 mm Tris base at pH 8.0, 0.5 M EDTA, 10 mg/l aprotinin, and 1 mm

PMSF). The cells were further sonicated 7 times at 60% duty cycle and power 8 for 30 sec each time with salt-ice bath cooling. Centrifugation (12,000 × g, 10 min, 4°C) was used to remove the supernatant and the pellet was sonicated again in the same matter. After the second centrifugation, the two batches of supernatant were combined and suspended in affinity buffer (120 ml of: 50 mm Tris base at pH 8.0 containing 10% glycerol (v/v), 0.1% Triton X100 (v/v), 0.1 mm EDTA, and 0.05 mm DTT). After another centrifugation (41,000 × g, 45 min), PMSF (1 mm) was added. A homogenous solution was obtained after 3 hrs of gentle stirring. The protein solution was applied to a 2',5' ADP Sepharose 4B column (16 ml) equilibrated with affinity buffer (60 ml). The column was first washed with buffer A (40 ml of affinity buffer containing 0.5 mm adenosine), followed by elution of the protein with buffer B (Affinity buffer containing 2 mm adenosine 2'-monophosphate (2'-AMP)). The fractions containing the 70 kDa protein were pooled. The sample was loaded onto a fast flow DEAE Sepharose column (8 ml) equilibrated with DEAE-EQ buffer (100 ml of: 0.2 m potassium phosphate buffer at pH 7.4 containing 20% glycerol (v/v) and 1 g/l CHAPS), and washed with buffer C (50 ml of: DEAE-EQ buffer containing 10 mm $\beta$-mercaptoethanol). The protein was eluted with a linear gradient increasing from 0.1 to 0.5 m potassium chloride in buffer C. The fractions containing the 70 kDa protein were identified by SDS-PAGE (homogeneous 12.5%) and dialyzed against buffer D (1 l of 0.1 m potassium phosphate buffer at pH 7.4 containing 20% glycerol (v/v). The overall yield was calculated to be 0.6 mg/l.

### 4.4.2. CPR concentration and activity determination

The concentration of CPR was determined by oxidation with potassium ferricyanide. An aliquot of CPR (130 μL) was diluted in 100 mM potassium phosphate

buffer at pH 7.6 (266 μL) before addition of potassium ferricyanide (4 μL of a 700 μM solution). The concentration was calculated using an extinction coefficient of 21.2 $mM^{-1}$ $cm^{-1}$ at 455 nm for the oxidized form of CPR. The activity of CPR was evaluated by cytochrome c reduction in the presence of NADPH. The assay mixture contained CPR (0.5 μM), cytochrome c (50 μM), and NADPH (100 μM) in 300 mM phosphate buffer at pH 7.6. The blank did not contain NADPH. Cytochrome c reduction by CPR is revealed by absorption peaks at 520 and 550 nm.

### 4.4.3. Expression and purification of His-tagged CYP3A4

CYP3A4 was expressed using a modification of the procedure used by Domanski *et al.*[190] The plasmid was transformed into *Escherichia coli* DH5α supercompetent cells, and the transformed cells were grown in 1 ml of SOC medium (used as a cell growth medium to ensure maximum transformation efficiency) for 1 h at 37°C. The cells were then grown overnight on LB agar plates with ampicillin (50 μg/ml). Culture tubes containing LB media (7 ml) supplemented with ampicillin (50 μg/ml) were inoculated next. After 24 hrs of growth at 37°C with shaking at 250 rpm, 10 flasks of 750 ml TB medium containing ampicillin (50 μg/ml) were inoculated each with 3 intermediate culture tubes (total of 21 ml). The flasks were placed at 37°C with shaking at 250 rpm for 3 hrs (i.e. until $OD_{600}$ = 0.6), after which protein expression was induced with IPTG (1 mM) followed by addition of δ-ALA (80 mg/mL). The incubation temperature was reduced to 30°C, and shaking to 190 rpm for 48 hrs. All following steps were carried out at 4°C. The cells were collected by centrifugation at 4000 × g for 15 min. The pellets were resuspended in buffer E (50 mL of: 100 mM 3-[*N*-morpholino]propanesulfonic acid] (MOPS) at pH 8 containing 10% glycerol, 1 mM

EDTA, 2 mM phenylmethanesulfonyl fluoride (PMSF)). Lysozyme (10 mg/g of cells) was added, and the suspension was stirred slowly for 15 min, after which the suspension was supplemented with protease inhibitors (1.6 μg/ml leupeptin, 1 μg/ml aprotinin, 0.8 μg/ml bestatin, and 0.7 μg/ml pepstatin A). The suspension was then sonicated on an ice-salt bath at 60% and power 8. After 2 sonication cycles of 20 sec each, the suspension was centrifuged at 100,000 × g for 60 min. The supernatant was removed and the pellets resuspended in buffer F (10 mL of: 100 mM MOPS at pH 7.4, containing 10% glycerol and 2 mM PMSF). CHAPS (0.5%) and potassium chloride (0.5 M) were then added. Cells were gently stirred for 2 hrs before centrifugation (1 h at 100,000 × g). The pellet is discarded and the CHAPS-solubilized P450 preparation is stored at -80°C until the next step.

The His-tagged protein was purified by affinity column chromatography using the Ni-NTA metal affinity resin (1.5 ml), pre-equilibrated with 5 column volumes of EQ-buffer (buffer F containing 0.5% CHAPS, 0.5 M KCl, and 5 mM imidazole) at a flow rate of 0.5 ml/min. The P450 preparation was then loaded onto the column at the same flow rate. The column was washed with 10 column volumes of EQ-buffer, then with 10 column volumes of 100 mM MOPS buffer at pH 7.4 containing 10% glycerol and 10 mM imidazole. The last washing buffer contained 20 mM imidazole. The P450 was eluted in EL-buffer (100 mM MOPS at pH 7.4 containing 10% glycerol and 200 mM imidazole). The orange-colored fractions were evaluated spectroscopically for protein content and by SDS-PAGE (12.5% homogeneous gels) for purity assessment. The fractions containing the CYP3A4 protein were pooled and dialyzed (twice 4 l of: 100 mM MOPS buffer at pH 7.4 containing 10% or 0%glycerol, 0.2 mM dithiothreitol and 1 mM EDTA) at 4°C. The solution was aliquoted for storage at -80°C. The overall yield was 0.6 mg/liter of culture.

### 4.4.4. Quantification of P450 enzymes and total protein

The P450 content was measured from the spectra of the enzyme reduced in the presence of reduced carbon monoxide as described by Omura and Sato[40]. Thus the CYP3A4 heme iron was reduced with addition of a few solid grains of sodium dithionite ($Na_2S_2O_4$) in quartz cuvettes containing the buffered enzyme solution. Formation of the enzyme-carbon monoxide complex was achieved by slow bubbling of CO gas into the reduced enzyme solution for about 20 sec. The UV absorption spectra was recorded immediately, and the concentration of P450 was determined using the extinction coefficient $\varepsilon_{450}$ = 91 $mM^{-1}$ $cm^{-1}$. The total protein content was determined using a commercially available Protein Assay Kit Procedure No. P 5656 (Sigma Diagnostics, St. Louis, MO), using the Lowry assay with precipitation. Bovine serum albumin was used for calibration.

## 4.5. STOCK SOLUTIONS FOR ENZYME ACTIVITY ASSAYS

The stock solution of BFC (5.5 μM, used as substrate for CYP3A4 in the fluorescence assay) was prepared from an aqueous dilution of a 300 μM solution in acetonitrile. The cofactor NADPH was dissolved to give a final concentration of 25 mM in the same buffer used to dilute the enzyme tested (100 mM potassium phosphate pH 7.4). Stock solutions of testosterone and the two metabolites 6α–hydroxytestosterone and 6β-hydroxytestosterone (1 mg/ml) were prepared in methanol and stored at 4°C. The stock solutions of protease inhibitors (leupeptin, aprotinin, pepstatin A and bestatin) were prepared in milliQ water and stored at -20°C, and the solution of PMSF was prepared fresh in acetone.

## 4.6. DEBENZYLATION OF BFC IN AQUEOUS CONDITIONS ASSAYED BY FLUORESCENCE

This assay was used to rapidly monitor the activity of the purified CYP3A4, and was conducted in a total volume of 300 µl in 96-wells microplates. CYP3A4 (0.66 µM), CPR (2.66 µM) and the substrate BFC (15 µM) were incubated at 37°C in 0.1M potassium phosphate at buffer pH 7.4 for 5 minutes. The reaction was then initiated by addition of NADPH (1 mM), and the fluorescence of the product HFC was monitored at 37°C during 20 minutes, with an excitation wavelength of 410 nm and an emission wavelength of 530 nm. A control reaction without CYP was monitored using the same conditions.

## 4.7. PRODUCT EXTRACTION

Potassium phosphate buffer (100 mM, 300 µl) was spiked with testosterone (10, 100, and 500 ng/ml) and 6β-OHT (10, 100, and 500 ng/ml). The mixtures were extracted with dichloromethane, ethyl acetate or ethyl ether (3 x 500 µl). The organic layer was transferred into a glass vial and the solvent evaporated to dryness in an evaporator. The samples were redissolved in MeOH (300 µl) and injected in the HPLC. Unextracted standards consisted of solutions of testosterone and 6β-OHT prepared at the same concentrations in MeOH, and injected into the HPLC system. The percentage recovery was measured by comparing the peak areas obtained from the extracted samples to those obtained from the corresponding unextracted reference solutions, and calculated as follows:

*Recovery (%) = (peak area of extracted plasma/peak area of unextracted*

*standard)*100*

Quantification of the 6β-hydroxytestosterone formed during the enzymatic reaction was achieved from a calibration curve obtained after spiking the incubation mixture (no CYP) with 6β-hydroxytestosterone (0 to 50 μM) and cortexolone (20 μM). A linear regression of peak-area ratio (6β-hydroxytestosterone/cortexolone) versus concentration of 6β-OHT was constructed and the concentration of 6β-hydroxytestosterone determined from the peak-area ratio relative to the calibration graph.

## 4.8. 6β-HYDROXYLATION OF TESTOSTERONE IN AQUEOUS CONDITIONS WITH DIFFERENT COFACTORS

### 4.8.1. Testosterone 6β-hydroxylation with hydrogen peroxide donors, organic peroxides, or CPR/NADPH

The reaction mixtures (300 μl) were prepared by mixing CYP3A4 (580 pmol, 50 μl of a 3.5 μM enzyme solution) and testosterone (115 μM) in potassium phosphate buffer (100 mM, pH 7.4). The reference reaction contained the same components plus CPR (1.5 nmol). The molar ratio of CYP3A4:CPR of 1:4 was selected with regard to near optimal activity as shown by the study of Wang *et. Al.*[228] After 5 min of preincubation at 37°C, the reaction was initiated using various concentrations of SPC, SPB, UHP, CHP, tBHP, aqueous $H_2O_2$, or NADPH (1 mM). After addition of the solid SPC, SPB or UHP, the reaction mixture was not immediately shaken, to allow the slow release of the hydrogen peroxide as the dissolution occurred. After 15 min the mixture was homogenized by gentle shaking. The reaction was terminated after 1 h of incubation at

37°C by addition of dichloromethane (500 µl) immediately followed by introduction of the internal standard cortexolone (15 µl of a 200 µM solution in methanol). The sample was vigorously vortexed, the layers separated by centrifugation (1500 × g for 2 min), and the organic layer was transferred to a dry vial. The extraction was repeated twice with $CH_2Cl_2$ (500 µl each), and the pooled organic extracts were evaporated to dryness using a roto-evaporator. The residue was redissolved in HPLC-grade methanol (150 µl), vigorously vortexed, sonicated and filtered through a polypropylene syringe filters (0.2 µm pore size, National Scientific Company) before injection in the HPLC. The identity of the products was established by co-elution with authentic standards and by mass spectroscopy. The blank incubation assays were lacking either CYP and NADPH, or the peroxide surrogates, and did not show any detectable products.

### 4.8.2. Effect of the buffer in CYP3A4 reactions with sodium percarbonate

The experiment was also repeated using the conditions described above but in 12 different buffers: 3 potassium phosphate concentrations (0.5 M, 0.75 M and 1 M), and 4 pHs for each (6.0, 6.5, 7.0 and 7.5).

### 4.8.3. CYP3A4 enzyme stability assays

The enzyme stability at 37°C was measured in the presence of CHP. The stability was evaluated spectrophotometrically by measuring the Soret absorbance at 416 nm. The enzyme sample (4.5 µM), containing testosterone (115 µM) and CHP (1 mM) was monitored every min over a 2 hrs period. Decreases in the intensity of the Soret peak ($A_{416nm} - A_{490nm}$) was used as an indicator of the enzyme denaturation. The stability of CYP3A4 (0.9 µM) in the presence of CPR/NADPH, CHP and SPC was also evaluated by measuring the product formation over time in the presence of excess substrate (115 µM)

and excess cofactor (fresh cofactor added every 15 or 30 min). The enzyme was considered denatured when product formation reached a plateau.

## 4.9. OPTIMIZATION OF LYOPHILIZATION CONDITIONS

### 4.9.1. Effect of glycerol on enzyme stability at -80°C and effect of lyophilization on enzyme activity

Purified CYP3A4 (7.5 μM, 750 μl) was dialyzed for 2 hrs in a Slide-A-Lyser® dialysis cassette 3.5K (Pierce, Rockford, IL) twice in 1 l of storage buffer without glycerol (80 mM MOPS pH 7.4, 1 mM EDTA, 0.2 mM DTT) at 4°C. Aliquots (30 μl) were transferred in 1.5 ml Eppendorf tubes, and kept at -80°C. After 0, 1, 2, 4, 8 and 12 weeks of storage, 2 samples containing glycerol and 2 samples without glycerol (duplicate analysis) were lyophilized, and the activity compared to the same samples without lyophilization. Lyophilization was performed at a condenser temperature of -52°C and a pressure $\leq 100 \times 10^{-3}$ mbars for 12 hrs. In each sample, buffer (300 μl or 260 μl of 1 M potassium phosphate buffer pH 7.0 for the lyophilized and non-lyophilized samples respectively) and testosterone (10 μl of 1 mg/ml) were added. After 5 min of pre-incubation at 37 °C, the reaction was initiated by addition of SPC (12.5 mg), and the mixture incubated for 1 h at 37 °C. The samples were treated with $CH_2Cl_2$ as described in section 3.8.1. before analysis by HPLC.

### 4.9.2. Effect of different additives during lyophilization

Samples containing glycerol-free enzyme solution (11.5 μM, 24 μl), 0.1 M potassium phosphate buffer at pH 7.4 (676 μl), and different amounts of additives (18-

crown-6, 15-crown-5, methyl-β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin, sucrose, sorbitol, mannitol and trehalose) were lyophilized for 24 hrs. Three holes were made in the Eppendorf's caps using a red-hot needle, and the pierced caps were replaced by new ones after freeze-drying. The dried samples were redissolved in 0.1 M potassium phosphate buffer at pH 7.4 (687 µl), and the substrate testosterone (50 µM, 10 µl of a 1mg/ml solution in methanol) was added (final CYP3A4 concentration of 0.4 µM). After 5 min of pre-incubation at 37°C, the reaction was initiated by addition of cumene hydroperoxide (1 mM , 7 µl of a 0.1 M stock solution in water:methanol 90:10), and the samples were shaken at 37°C, 250 rpm for 1 h. Controls were also prepared for each additive (at every concentration) with non-lyophilized enzyme. After the incubation, the samples were spiked with the internal standard cortexolone (15 µl from a 200 µM solution in methanol), extracted with $CH_2Cl_2$ (3 x 500 µl), evaporated, redissolved in MeOH (150 µM) and injected in the HPLC. The results were compared with the control reaction with non-lyophilized enzyme and no additive.

## 4.10. 6β-HYDROXYLATION OF TESTOSTERONE IN NON-AQUEOUS CONDITIONS

### 4.10.1. Effect of water miscible organic co-solvents on CYP3A4 activity

Samples (300 µl) containing CYP3A4 (50 µl, 0.9 µM), testosterone (115 µM, 10 µl of a 1 mg/ml solution in methanol), and 0, 1, 2.5, 5, 10 or 15 % v/v of co-solvent (acetone, acetonitrile, DMSO, methanol or THF) in buffer (0.1 M pH 7.4 for CHP or 1 M pH 7.0 for SPC) were pre-incubated at 37°C for 5 min before addition of SPC (12.5 mg,

equivalent to 500 mM $H_2O_2$) or CHP (1 mM, 3 µl from a 0.1 M stock solution prepared in the corresponding co-solvent). The volume of the CHP solution is taken into account when determining the final co-solvent concentration. For the samples containing 0% co-solvent, the CHP was added from a stock solution in water:methanol 95:5. After 1 h incubation at 37°C, the samples were treated and analyzed as previously described (section 4.8.1.).

### 4.10.2. Effect of ionic liquids on CYP3A4 activity

Samples (300 µl) containing CYP3A4 (30 µl, 0.9 µM), testosterone (115 µM, 10 µl of a 1 mg/ml solution in methanol), and 0, 1, 2.5, 5, 10, 15, 25, 50, 75 or 90 % v/v of ionic liquids (1-methyl-3-methylimidazolium tetrafluoroborate ([MMIM] [$BF_4$]), 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM] [$BF_4$]), or *N*-butylpyridinium tetrafluoroborate ([BPyr] [BF4])) in buffer (0.1 M pH 7.4) were pre-incubated at 37°C for 5 min before addition of CHP (1 mM, 3 µl from a 0.1 M stock solution in water:methanol 90:10). After 1 h incubation at 37°C, the samples were spiked with cortexolone (15 µl from a 200 µM solution in methanol) and extracted with $CH_2Cl_2$ (3 x 500 µl). The organic phase was transferred into a glass vial and evaporated. The ionic liquids did not evaporate (they have no vapour pressure). The volume was then completed to 300 µl with methanol, and the samples were filtered and injected in the HPLC (the fact that the final concentration of cortexolone in the injected samples was 10 µM instead of 20 µM was taken into account in the calculations).

### 4.10.3. Activity of CYP3A4 in water-immiscible organic solvents

**First enzymatic assay in 15 organic solvents (lyophilization with sucrose).** Samples

containing CYP3A4 (1.9 μM, 100 μl of a 11.5 μM solution), sucrose (120 mg, 2000 w:w sucrose:CYP3A4), and buffer (500 μl of 0.1 M potassium phosphate at pH 7.4) were lyophilized for 24 hrs. Water-saturated organic solvent (900 μl of n-pentane, hexane, cyclohexane, heptane, iso-octane, ethyl acetate, ethyl ether, butyl ether, isopropylether, n-butanol, toluene, benzene, dichloromethane, 1,2-dichloroethane or chloroform) and testosterone (40 μM, 10 μl of a 1mg/ml solution in methanol) were added to afford a suspension, and the reaction was initiated with addition of CHP (1 mM, 9 μl from a 0.1 M stock solution prepared in water:methanol 90:10) after 5 min of pre-incubation at 37°C (final CYP3A4 concentration 1.3 μM, final water content 0.85%, final methanol content 1.2%). Control reactions with lyophilized and non-lyophilized enzymes were also prepared. After 2 hrs of incubation at 37°C and shaking horizontally at 250 rpm, the samples were spiked with cortexolone (15 μl from a 200 μM solution in methanol), and centrifuged (2 min, 5000 rpm) to precipitate the enzyme. The organic solvent was transferred into a glass vial and evaporated in vacuo. Methanol (150 μl) was added before filtration and injection in the HPLC. The remaining wet mixture containing the enzyme, buffer salts and sucrose was redissolved in a concentrated aqueous solution of HCl (400 μl, 0.5 N), and the solution was extracted with $CH_2Cl_2$ (3 x 500 μl). The organic phase was transferred in a glass vial, and evaporated. Methanol was added (150 μl), and the samples were injected in the HPLC for analysis.

**Enzymatic assays in 10 hydrophobic solvents (lyophilization with or without sucrose).** Samples containing CYP3A4 (1.9 μM, 100 μl of a 11.5 μM solution), and buffer (500 μl of 0.1 M potassium phosphate at pH 7.4) were lyophilized for 24 hrs in the presence or not of sucrose (120 mg, 2000 w:w sucrose:CYP3A4). Water-saturated

organic solvent (900 µl of n-pentane, iso-octane, n-hexane, cyclohexane, methylcyclohexane, n-heptane, n-octane, iso-octane, n-decane, or n-dodecane) and testosterone (40 µM, 10 µl of a 1 mg/ml solution in methanol) were added to give a suspension, and the reaction was initiated with CHP (1 mM, 9 µl from a 0.1 M stock solution prepared in water:methanol 90:10) after 5 min of pre-incubation at 37°C (final CYP3A4 concentration of 1.3 µM, final water content 0.85%, final methanol content 1.2%). Control reactions with lyophilized (with or without sucrose) and non-lyophilized enzymes were also prepared. After 2 hrs of incubation at 37°C and shaking horizontally at 250 rpm, the samples were spiked with cortexolone (15 µl from a 200 µM solution in methanol), and treated exactly as described in the previous paragraph before injection in the HPLC. n-Pentane and iso-pentane boil at 37°C (boiling point of 36°C and 28°C respectively), so the reaction mixture must be cooled down before opening the cap to avoid projection. Once transferred in the vials, decane and dodecane could not be evaporated (boiling point of 174°C and 216°C respectively), so for these 2 solvents only the samples deriving from the $CH_2Cl_2$ extraction of the aqueous reconstitution were analyzed.

**Enzymatic assays in organic solvents after lyophilization with or without testosterone.** Samples containing CYP3A4 (3 µM, 100 µl of a 18 µM solution), and buffer (500 µl of 0.1 M potassium phosphate at pH 7.4) were lyophilized for 24 hrs in the presence of sucrose (120 mg, 2000 w:w sucrose:CYP3A4). For half of the samples, the substrate testosterone was added before lyophilization. The testosterone solution (10 µl of a 1 mg/ml solution in methanol was added in empty reaction tubes, and the tubes were left at room temperature until complete evaporation of the methanol before addition of

buffer and enzyme solution. The lyophilized powder was resuspended in organic solvent (900 µl of n-pentane, iso-octane, n-hexane, cyclohexane, methylcyclohexane, n-heptane, n-octane, iso-octane, n-decane, or n-dodecane) and testosterone (40 µM, 10 µl of a 1 mg/ml solution in methanol) was added (in the samples in which testosterone was not added before lyophilization). The reaction was initiated with CHP (1 mM, 9 µl from a 0.1 M stock solution prepared in the corresponding solvent) after 5 min of pre-incubation at 37°C (final CYP3A4 concentration of 2 µM, final water content ≤0.1%, final methanol content 1.0%). After 2 hrs of incubation at 37°C and shaking horizontally at 250 rpm, the samples were spiked with cortexolone (15 µl from a 200 µM solution in methanol), and centrifuged (2 min, 5000 rpm) to precipitate the enzyme. The organic solvent was transferred into a glass vial and evaporated in vacuo. Methanol (150 µl) was added before filtration and injection in the HPLC. The remaining wet mixture containing the enzyme, buffer salts and sucrose was redissolved in a concentrated aqueous solution of HCl (400 µl, 0.5 N), and the solution was extracted with $CH_2Cl_2$ (3 x 500 µl). The organic phase was transferred in a glass vial, and evaporated. Methanol was added (150 µl), and the samples were injected in the HPLC for analysis.

**Partition coefficients of testosterone, 6β-hydroxytestosterone and cortexolone between the different solvents and buffer at 37°C.** Testosterone (10 µl of a 1 mg/ml solution in methanol), 6β–hydroxytestosterone (30 µl of 100 µM solution in methanol) and cortexolone (15 µl of a 200 µM solution in methanol) were added to an emulsion of buffer (300 µl of 0.1 M potassium phosphate at pH 7.4) and organic solvents (300 µl of hexane, cyclohexane, heptane, pentane or iso-octane). The samples were vigorously vortexed during 2 min, kept at 37°C for 1 h, and vortexed again for 2 min before

centrifugation. The organic layer was transferred into a glass vial, evaporated, before addition of methanol (300 µl) and analysis by HPLC. The aqueous phase was directly injected in the HPLC. Experiments were run in triplicate. The partition coefficients were calculated using the HPLC peaks areas and the following equation:

$$\mathbf{P_{solvent/buffer} = C_{org}/C_{aq} = peak\ area_{[org]}/\ peak\ area_{[aq]}}$$

Where $C_{org}$ is the concentration of the compound in the organic phase and C[aq] is its concentration in the aqueous phase.

## REFERENCES


[1] J. A. Pesti, R. DiCosimo. Recent progress in enzymatic resolution and desymmetrization of pharmaceuticals and their intermediates. *Cur. Op. Drug Discov. Dev.* **2000**, 3, 764-782.

[2] T. Miyazawa. Enzymatic resolution of amino acids via ester hydrolysis. *Amino acids* **1999**, 16, 191-213.

[3] L. Blanco, E. Guibe-Jampel, G. Rousseau. Enzymatic resolution of racemic lactones. *Tet. Lett.* **1988**, 29, 1915-1918.

[4] J. Kaman, E. Forro, F. Fulop. Enzymatic resolution of alicyclic β-lactams. *Tet.: Asymm.* **2000**, 11, 1593-1600.

[5] K. A. Skupinska, E. J. McEachern, I. R. Baird, R. T. Skerlj, G. J. Bridger. Enzymatic resolution of bicyclic 1-heteroarylamines using *Candida antarctica* lipase B. *J. Org. Chem.* **2003**, 68, 3546-3551.

[6] S. V. Lehmann, J. Breinholt, P. S. Bury, T. E. Nielsen. Enzymatic resolution to (-)-ormeloxifene intermediates from their racemates using immobilized *Candida rugosa* lipase. *Chirality* **2000,** 12, 568-573.

[7] J. Pietruszka, T. Wilhelm, A. Witt. Kinetic enzymatic resolution of cyclopropane derivatives. *Synlett* **1999**, 12, 1981-1983.

[8] A. M. Klibanov. Enzymatic catalysis in anhydrous organic solvents. *Trends Biochem. Sci.* **1989**, 14, 141-144.

[9] L. G. Butler. Enzymes in non-aqueous solvents. *Enz. Microb. Technol.* **1979**, 1, 253-259.

[10] C.-H. Wong. Enzymatic Catalysts in Organic Synthesis. *Science* **1989,** 244, 1145-1152.

[11] T. Sugai. Application of enzyme- and microorganism-catalyzed reactions to organic synthesis. *Curr. Org. Chem.* **1999**, 3, 373-406.

[12] J. A. Pesti, R. Dicosimo. Recent progess in enzymatic resolution and desymmetrization of pharmaceuticals and their intermediates. *Curr. Opin. Drug Discov. Dev.* **2000**, 3,

764-782.

[13] V. Gotor. Pharmaceuticals through enzymatic transesterification and enzymatic aminolysis reactions. *Biocat. Biotrans.* **2000**, 18, 87-103.

[14] K. M. Koeller, C.-H. Wong. Enzymes for chemical synthesis. *Nature* **2001**, 409, 232-240.

[15] A. M. Klibanov. Improving enzymes by using them in organic solvents. *Nature* **2001**, 409, 241.

[16] S. M. Thomas, R. DiCosimo, V. Nagarajan. Biocatalysis: applications and potentials for the chemical industry. *Trends Biotechnol.* **2002**, 20, 238-242.

[17] K. Drauz, H. Waldmann, Enzymes Catalysis in Organic Synthesis: A Comprehensive Handbook. 2nd ed. Wiley-VCH. **2002**.

[18] E. N. Vulfson, P. J. Halling, H. L. Holland, Enzymes in Nonaqueous Solvents: Methods and Protocols. Methods in Biotechnology, Humana Press, **2001**.

[19] S. H. Krishna. Developments and trends in enzyme catalysis in nonconventional media. *Biotech. Adv.* **2002**, 20, 239-267.

[20] E. Garcia-Junceda, J. F. Garcia-Garcia, A. Bastida, A. Fernandez-Mayoralas, Enzymes in the synthesis of bioactive compounds : the prodigious decades. *Bioorg. Med. Chem.* **2004**, 12, 1817-1834.

[21] K. Martinek, A. V. Levashov, N. Llyachko, Y. L. Khmelnitski, I. V. Brezin. Micellar enzymology. *Eur. J. Biochem.* **1986**, 155, 453-468.

[22] J. Michizoe, H. Ichinose, N. Kamiya, T. Maruyama, M. Goto. Biodegradation of phenolic environmental polluants by a surfactant-laccase complex in organic media. *J. Biosci. Bioeng.* **2005,** 99, 642-647.

[23] K. Sawada, M. Ueda. Characteristics of aqueous microenvironments in non-ionic surfactant reverse micelles and their use for enzyme reactions in non-aqueous media. *J. Chem. Technol. Biotechnol.* **2004**, 79, 369-375.

[24] G. Carrea, S. Riva. Properties and Synthetic applications of Enzymes in Organic

Solvents. *Angew. Chem. Int. Ed.* **2000**, 39, 2226-2254.

[25] K. Griebenow, A. M. Klibanov. On protein denaturation in aqueous-organic mixtures but not in pure organic solvents. *J. Am. Chem. Soc.* **1996**, 118, 11695-11700.

[26] J. A. Rupley, G. Careri. Protein hydration and function. *Adv. Protein Chem.* **1991**, 41, 37-172.

[27] P. A. Fitzpatrick, A. C. U. Steinmetz, D. Ringe, A. M. Klibanov. Enzyme crystal structure in a neat organic solvent. *Proc. Natl. Acad. Sci. USA* **1993**, 90, 8653-8657.

[28] N. H. Yennawar, H. P. Yennawar, G. Farber. X-ray structure of γ-chymotrypsin in hexane. *Biochemistry* **1994**, 33, 7326-7337.

[29] J. L. Schmitke, L. Stern, A. M. Klibanov. Crystal structure of subtilisin Carlsberg in anhydrous dioxane and its comparison with those in water and acetonitrile. *Proc. Natl. Acad. Sci. USA* **1997**, 94, 4250-4255.

[30] G. Zhu., Q. Huang, Z. Wang, M. Qian, Y. Jia, Y. Tang. X-Ray studies on two forms of bovine β-crystals in neat cyclohexane. *Biochim. Biophys. Acta,* **1998**, 1429, 142-150.

[31] X.-G. Gao, E. Maldonado, R. Perez-Montfort, G. Garza-Ramos, M. Tuena de Gomez-Puyou, A. Gomez-Puyou, A. Rodriguez-Romero. Crystal structure of triosephosphate isomerase from Trypanosoma cruzii in hexane. *Proc. Natl. Acad. Sci. USA* **1999**, 96, 10062-10067.

[32] A. Zaks, A. M. Klibanov. Enzymatic catalysis in non-aqueous solvents. *J. Biol. Chem.* **1988**, 263, 3194-3201.

[33] C. Laane, S. Boeren, K. Vos, C. Veeger. Rules for optimization of biocatalysis in organic solvents. *Biotechnol. Bioeng.* **1987**, 30, 81-87.

[34] A. M. Klibanov. Improving enzymes by using them in organic solvents. *Nature* **2001**, 409, 241-246.

[35] E. N. Vulfson, P. J. Halling, H. L. Holland, Enzymes in Nonaqueous Solvents: Methods and Protocols. Methods in Biotechnology, Chap. 11. Humana Press, **2001.**

[36] K. Dabulis, A. M. Klibanov. Molecular imprinting of proteins and others

macrocolecules resulting in new absorbents, *Biotechnol. Bioeng.* **1992**, 39, 176-185.

[37] A. J. Russel, A. M. Klibanov. Inhibitor-induced enzyme activation in organic solvents. *J. Biol. Chem.* **1988**, 263, 11624-11626.

[38] J. O. Rich, J. S. Dordick. Controlling subtilisin activity and selectivity in organic media by imprinting with nucleophilic substrates. *J. Am. Chem. Soc.* **1997**, 119, 3245-3252.

[39] A. Zaks, A. M. Klibanov. Enzyme-catalyzed processes in organic solvents. *Proc. Natl. Acad. Sci. USA* **1985**, 82, 3192–3196.

[40] T. Omura, R. Sato. A new cytochrome in liver microsomes. *J. Biol. Chem.* **1964**, *239*, 2370-2378.

[41] D. R. Nelson, L. Koymans, T. Kamtaki, J. J. Stegeman, R. Feyereisen, D. J. Waxman, M. R. Waterman, O. Gotoh, M. J. Coon, R. W. Estabrook, I. C. Gunsalus, D. W. Nebert. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **1996**, 6, 1-42.

[42] David Nelson's homepage. http://drnelson.utmem.edu/CytochromeP450.html.

[43] P. R. Ortiz de Montellano, Cytochrome P450: Structure, Mechanism, and Biochemistry, third ed., Springer, New York, **2004**.

[44] T. Shimada, H. Yamazaki, M. Mimura, Y. Inui, F. P. Guengerish. Interindividual variations in human liver cytochrome P450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasiens. *J. Pharmacol. Exp. Ther.* **1994**, 270, 414-423.

[45] S. Cholerton, A. K. Daly, J. R. Idle. The role of individual human cytochromes P450 in drug metabolism and clinical response. *Trends Pharmacol. Sci.* **1992**, 13: 434-439.

[46] L. K. Kamdem, I. Meineke, U. Gödtel-Armbrust, J. Brockmöller, L. Wojnowski. Dominant Contribution of P450 3A4 to the hepatic carcinogenic activation of aflatoxin B1 *Chem. Res. Toxicol.* **2006,** 19, 577-586.

[47] D. M. Roden, H. J. Duff, D. Altenbern, R. L. Woosley. Antiarrhythmic activity of the O-demethyl metabolite of encainide. *J. Pharmacol. Exp. Ther.* **1982**, 221, 552-557.

[48] G. A. Roberts, G. Grogan, A. Greter, S. L. Flitsch, N. J. Turner. Identification of a new class of cytochrome P450 from a *Rhodococcus sp. J. Bacter.* **2002**, 184, 3898-3908.

[49] T. L. Poulos, B. C. Finzel, A. J. Howard. High-resolution crystal structure of cytcochrome P450cam. *J. Mol. Biol.* **1987**, 195, 687-700.

[50] K. G. Ravichandran, S.S. Boddupalli, C. A. Haserman, J. A. Peterson, J. Deisenhofer. Crystal structure of hemoprotein domain P450-BM3, a prototype for microsomal P450s. *Science* **1993**, 61, 731-736.

[51] P. A. Williams, J. Cosme, V. Sridhar, E. F. Johnson, D. E. McRee. Mammalian microsomal cytochrome P450 monooxygenase: structural adaptations for membrane binding and functional diversity. *Mol. Cell* **2000**, 5, 121-131.

[52] E. E. Scott, Y. A. He, M. R. Wester, M. A. White, C. C. Chin, J. R. Halpert, E. F. Johnson, C. D. Stout. An open conformation of mammalian cytochrome P450 2B4 at 1.6 A resolution. *Proc. Natl. Acad. Sci. USA* **2003**, 100, 13196-13201.

[53] G. A. Schoch, J. K Yano, M. R. Wester, K. J. Griffin, C. D. Stout, E. F. Johnson. Structure of human microsomal cytochrome P450 2C8. Evidence for a peripheral fatty acid binding site. *J. Biol. Chem.* **2004**, 279, 9497-9503.

[54] P. A. Williams, J. Cosme, A. Ward, H. C. Angove, D. M. Vinkovic, H. Jhoti. Crystal Structure of Human Cytochrome P450 2C9 with Bound Warfarin. *Nature* **2003**, 424-464.

[55] P. A. Williams, J. Cosme, D. M. Vinkovic, A. Ward, H. C. Angove, P. J. Day, C Vonrhein, I. J. Tickle, H. Jhoti. Crystal Structures of Human Cytochrome P450 3A4 Bound to Metyrapone and Progesterone. *Science* **2004**, 305, 683-686.

[56] J. K. Yano, M. R. Wester, G. A. Schoch, K. J. Griffin, C. D. Stout, E. F. Johnson. The Structure of Human Microsomal Cytochrome P450 3A4 Determined by X-ray Crystallography to 2.05-Å Resolution. *J. Biol. Chem.* **2004**, 279, 38091-38094.

[57] J. K. Yano, M. H. Hsu, K. J. Griffin, C. D. Stout, E. F. Johnson. Structures of human microsomal cytochrome P450 2A6 complexed with coumarin and methoxsalen. *Nat. Struct. Mol. Biol.* **2005**, 12, 822-823.

[58] P. Rowland, F. E. Blaney, M. G. Smyth, J. J. Jones, V. R. Leydon, A. K. Oxbrow, C. J. Lewis, M. G. Tennant, S. Modi, D. S. Eggleston, R. J. Chenery, A. M. Bridges. Crystal Structure of Human Cytochrome P450 2D6. *J. Biol. Chem.* **2006**, 281, 7614-7622.

[59] T. L. Poulos, B. C. Finzel, I. C, Gunsalus, G. C. Wagner, J. Kraut. The 2.6-Å crystal structure of *Pseudomonas putida* cytochrome P-450. *J. Biol. Chem.* **1985**, 260, 16122-16130.

[60] M. Sono, M. Roach, E. D. Coulter, J. H. Dawson. Heme-containing oxygenases. *Chem. Rev.* **1996**, 96, 2841-2887.

[61] H. Li, T. L. Poulos. Structural variation in heme enzymes: a comparative analysis of peroxidase and P450 crystal structures. *Structure* (Cambridge, MA, United States) **1994**, 2, 461-464.

[62] F. P. Guengerich. Rate-limiting steps in cytochrome P450 catalysis. *J. Biol.Chem.* **2002**, 383, 1553-1564.

[63] D. Mansuy. The great diversity of reactions catalyzed by cytochromes P450. *Comp. Biochem. Physiol. Part C* **1998**, 121, 5-14.

[64] R. E. White, M. J. Coon. Oxygen activation by P450. *Ann. Rev. Biochem.* **1980**, 49, 315-356

[65] D. F. V. Lewis, M. Dickins. Substrate SARs in human P450s. *DDT* **2002**, 7, 918-924.

[66] D. F. V. Lewis, M. N. Jacobs, M. Dickins. Compound lipophilicity for substrate binding to human P450s in drug metabolism. *DDT* **2004**, 9, 530-537.

[67] D. F. V. Lewis, S. Modi, M. Dickins. Quantitative structure-activity relationships (QSARs) within substrates of human cytochromes P450 involved in drug metabolism. *Drug Met. Drug Inter.* **2001**, 18, 221-242.

[68] S. B. Kirton, C. A. Baxter, M. J. Sutcliffe. Comparative modelling of cytochromes P450. *Adv. Drug Deliv. Rev.* **2002**, 54, 385-406.

[69] D. F. Lewis. Human cytochromes P450 associated with the phase 1 metabolism of drugs and other xenobiotics: a compilation of substrates and inhibitors of the CYP1,

CYP2 and CYP3 families. *Curr. Med. Chem.* **2003**, 10, 1955-1972.

[70] D. F. V. Lewis. Essential requirements for substrate binding affinity and selectivity toward human CYP2 family enzymes. *Arch. Biochem. Biophys.* **2002**, 409, 32-44.

[71] E. M. Gillam. Exploring the potential of xenobiotic-metabolizing enzymes as biocatalysts: evolving designer catalysts from polyfunctional cytochrome P450 enzymes. *Clin. Exp. Pharmacol Physiol.* **2005**, 32, 147-152.

[72] F. P. Guengerich. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem. Res. Toxicol.* **2001**, 14, 611–650.

[73] Z. P. Zhang, Y. Li, R. A. Stearns, P. R. Ortiz de Montellano, T. A. Baillie, W. Tang. Cytochrome P450 3A4-mediated oxidative conversion of a cyano to an amide group in the metabolism of pinacidil. *Biochemistry* **2002**, 41, 2712–2718.

[74] J. T. Groves, P. Viski. Asymmetric hydroxylation, epoxidation, and sulfoxidation catalyzed by Vaulted binaphthyl metalloporphyrins. *J. Org. Chem.* **1990**, 55, 3628-3634

[75] J. F. Larrow, E. N. Jacobsen. Kinetic resolution of 1,2-dihydronaphthalene oxide and related epoxides via asymmetric C–H Hydroxylation. *J. Am. Chem. Soc.* **1994**, 116, 12129-12130.

[76] H. Chen, S. Schlecht, T. C. Semple, J. F. Hartwig. Thermal, catalytic, regiospecific functionalization of alkanes. *Science* **2000**, 287, 1995-1997.

[77] J. M. Thomas, R. Raja, G. Sankar, R. G. Bell. Molecular sieve catalysts for the regioselective and shape-selective oxyfunctionalization of alkanes in air. *Acc. Chem. Res.* **2001**, 34, 191-200.

[78] X. Chen, X.-S. Hao, C. E. Goodhue, J.-Q. Yu. Cu(II)-catalyzed functionalizations of aryl C-H bonds using $O_2$ as an oxidant. *J. Am. Chem. Soc.* **2006**, 128, 6790-6791.

[79] Z. Li, D. Chang. Recent advances in regio- and stereolelective biohydroxylation of non-activated carbon atoms. *Curr. Org. Chem.* **2004**, 8, 1647-1658.

[80] V. B. Urlacher, S. Lutz-Wahl, R. D. Schmid. Microbial P450 enzymes in biotechnology. *Appl. Microbiol. Biotechnol.* **2004**, *64*, 317-325.

[81] A. Parikh, E. M. Gillam, F. P. Guengerich. Drug metabolism by Escherichia coli expressing human cytochromes P450. *Nat. Biotechnolol.* **1997**, 15, 784-788.

[82] T. H. Rushmore, P. J. Reider, D. Slaughter, C. Assang, M. Shou. Bioreactor systems in drug metabolism: synthesis of cytochrome P450-generated metabolites. *Metab. Eng.* **2000**, 2(2), 115-125.

[83] R. B. Vail, M. J. Homann, I. Hanna, A. Zaks. Preparative synthesis of drug metabolites using human cytochrome P450s 3A4, 2C9 and 1A2 with NADPH-P450 reductase expressed in Escherichia coli. *J. Ind. Microbiol. Biotechnol.* **2005**, 32, 67-74.

[84] C. R. Otey, G. Bandara, J. Lalonde, K. Takahashi, F. H. Arnold. Preparation of human metabolites of propanolol using laboratory-evolved bacterial cytochromes P450. *Biotechnol. Bioeng.* **2006**, 93, 494-499.

[85] F. M. Szczebara, C. Chandelier, C. Villeret, A. Masurel, S. Bourot, C. Duport, S. Blanchard, A. Groisillier, E. Testet, P. Costaglioli, G. Cauet, E. Degryse, D. Balbuena, J. Winter, T. Achstetter, R. Spagnoli, D. Pompon, B. Dumas. Total biosynthesis of hydrocortisone from a simple carbon source in yeast. *Nat. Biotechnol.* **2003**, 21, 143-149.

[86] C. Duport, R. Spagnoli, E. Degryse, D. Pompon. Self-sufficient biosynthesis of pregnenolone and progesterone in engineered yeast. *Nat. Biotechnol.* **1998**, 16, 186-189.

[87] F. Brugliera, G. Barri-Rewell, T. Holton, J. Mason. Isolation and characterization of a flavonoid 3'-hydroxylase cDNA clone corresponding to the Ht1 locus of Petunia hybrida. *Plant J.* **1999**, 19, 441-451.

[88] V. Su, B.-D. Hsu. Cloning and expression of a putative cytochrome P450 gene that influences the colour of *Phalaenopsis* flowers. *Biotechnol. Lett.* **2003**, 25, 1933-1939.

[89] T. A. Holton, F. Brugliera, D. R. Lester, Y. Tanaka, C. D. Hyland, J. G. T. Menting, C. Lu, E. Farcy, T. W. Stevenson, E. C. Cornish. Cloning and expression of cytochrome P450 genes controlling flower colour. *Nature* **1993**, 366, 276-279.

[90] Y. Fukui, Y. Tanaka, T. Kusumi, T. Iwashita, K. Nomoto. A rationale for the shift in

colour towards blue in transgenic carnation flowers expressing the flavonoid 3',5'-hydroxylase gene. *Phytochemistry* **2003**, 63, 15-23.

[91] R. Bernhardt. Cytochromes P450 as versatile biocatalysts. *J. Biotechnol.* **2006**, 124, 128-145.

[92] J. L. Cleland, C. S. Craik, *Protein Engineering: Principles and Practice*, Wiley-Liss, New York, **1996**.

[93] O. Kuchner, F. H. Arnold. Directed evolution of enzyme catalysts. *Trends Biotechnol.* **1997**, 15, 523-530.

[94] S. S. Boddupalli, R. W. Estabrook, J. A. Peterson. Fatty acid monooxygenation by cytochrome P-450 BM3. *J. Biol. Chem.* **1990**, 265, 4233-4239.

[95] J. H. Capdevila, S. Wei, C. Helvig, J. R. Falck, Y. Belosludtsev, G. Truan, S. E. Graham-Lorence, J. A. Peterson. The highly stereoselective oxidation of polyunsaturated fatty acids by cytochrome P450 BM3. *J. Biol. Chem.* **1996**, 271, 22663-22671.

[96] U. Schwaneberg, C. Schmidt-Dannert, J. Schmitt, R. D. Schmid. A continuous spectrophotometric assay for P450 BM3, a fatty acid hydroxylating enzyme, and its mutant F87A. *Anal. Biochem.* **1999**, 269, 359-366.

[97] C. F. Oliver, S. Modi, M. J. Sutcliffe, W. U. Primrose, L.-Y. Lian, G. C. K. Roberts. A Single Mutation in Cytochrome P450 BM3 Changes Substrate Orientation in a Catalytic Intermediate and the Regiospecificity of Hydroxylation. *Biochemistry* **1997**, 36, 1567-1572.

[98] Q.-S. Li, U. Schwaneberg, M. Fischer, J. Schmitt, J. Pleiss, S. Lutz-Wahl, R. D. Schmid. Rational evolution of a medium chain-specific cytochrome P450 BM3 variant. *Biochim. Biophys. Acta* **2001**, 1545, 114-121.

[99] O. Entz, Q.-S. Li, U. Schwaneberg, S. Lutz-Wahl, P. Fischer, R. D. Schmid. Modification of the fatty acid specificity of cytochrome P450 BM3 from Bacillus megaterium by directed evolution: a validated assay. *J. Mol. Catal. B: Enzym.* **2001**, 15, 123-133.

[100] Q.-S. Li; U. Schwaneberg, P. Fischer, R. D. Schmid. Directed evolution of the fatty-acid hydroxylase P450 BM3 into an indole-hydroxylating catalyst. *Chem. Eur. J.* **2000**, 6, 1531-1536.

[101] D. Appel, S. Lutz-Wahl, P. Fischer, U. Schwaneberg, R. D. Schmid. A P450 BM3 mutant hydroxylates alkanes, cycloalkanes, arenes and heteroarenes. *J. Biotechnol.* **2001**, 88, 167-171.

[102] E. T. Farinas, U. Schwaneberg, A. Glieder, F. H. Arnold. Directed evolution of a cytochrome P450 monooxygenase for alkane oxidation. *Adv. Synth. Catal.* **2001**, 343, 601-606.

[103] A. Glieder, E. T. Farinas, F. H. Arnold. Laboratory evolution of a soluble, self-sufficient, highly active alkane hydroxylase. *Nat. Biotechnol.* **2002**, 20, 1135-1139.

[104] E. T. Farinas, M. Alcalde, F. H. Arnold. Alkene epoxidation catalyzed by cytochrome P450 BM3 139-3. *Tetrahedron* **2004**, 60, 525-528.

[105] M. W. Peters, P. Meinhold, A. Glieder, F. H. Arnold. Regio- and Enantioselective Alkane Hydroxylation with Engineered Cytochromes P450 BM3. *J. Am. Chem. Soc.* **2003**, 125, 13442-13450.

[106] P. Meinhold, M. W. Peters, M. M. Y. Chen, K. Takahashi, F. H. Arnold. Direct conversion of ethane to ethanol by engineered cytochrome P450 BM3. *ChemBioChem* **2005**, 6, 1765-1768.

[107] I. Axarli, A. Prigipaki, N. E. Labrou. Engineering the substrate specificity of cytochrome P450 CYP102A2 by directed evolution: production of an efficient enzyme for bioconversion of fine chemicals. *Biomol. Eng.* **2005**, 22, 81-88.

[108] P. Meinhold, M. W. Peters, A. Hartwick, A. R. Hernandez, F. H. Arnold. Engineering cytochrome P450 BM3 for terminal alkane hydroxylation. *Adv. Synth. Catal.* **2006**, 348, 763-772.

[109] T. Kubo, M. W. Peters, P. Meinhold, F. H. Arnold. Enantioselective epoxidation of terminal alkenes to (R)- and (S)-epoxides by engineered cytochromes P450 BM3. *Chem. Eur. J.* **2006**, 12, 1216-1220.

[110] M. Landwehr, L. Hochrein, C. R. Otey, A. Kasrayan, J.-E. Backwall, F. H. Arnold. Enantioselective α-hydroxylation of 2-arylacetic acid derivatives and buspirone catalyzed by engineered cytochrome P450 BM3. *J. Am. Chem. Soc.* **2006**, 128, 6058-6059.

[111] T. S. Wong, F. H. Arnold, U. Schwaneberg. Laboratory evolution of cytochrome P450 BM3 monooxygenase for organic cosolvents. *Biotechnol. Bioeng.* **2004**, 85, 351-358.

[112] J.-A. Stevenson, J. P. Jones, L.-L. Wong. Mutations of phenylalanine-193 in the putative substrate access channel alter the substrate specificity of cytochrome P450cam. *Isr. J. Chem.* **2000**, 40, 55-62.

[113] S. G. Bell, R. J. Sowden, L.-L. Wong. Engineering the haem monooxygenase cytochrome P450cam for monoterpene oxidation. *Chem. Commun.* **2001**, 7, 635-636.

[114] S. G. Bell, X. Chen, R. J. Sowden, F. Xu, J. N. Williams, L.-L. Wong, Z. Rao. Molecular Recognition in (+)-α-Pinene Oxidation by Cytochrome P450cam. *J. Am. Chem. Soc.* **2003**, 125, 705-714.

[115] S. G. Bell, D. A. Rouch, L.-L. Wong. Selective aliphatic and aromatic carbon-hydrogen bond activation catalyzed by mutants of cytochrome P450cam. *J. Mol. Catal. B: Enzym.* **1997**, 3, 293-302.

[116] D. P. Nickerson, C. F. Harford-Cross, S. R. Fulcher, L.-L. Wong. The catalytic activity of cytochrome P450cam towards styrene oxidation is increased by site-specific mutagenesis. *FEBS Lett.* **1997**, 405, 153-156.

[117] J.-A. Stevenson, A. C. G. Westlake, C. Whittock, L.-L. Wong. The Catalytic Oxidation of Linear and Branched Alkanes by Cytochrome P450cam. *J. Am. Chem. Soc.* **1996**, 118, 12846-12847.

[118] J.-A. Stevenson, J. L. Bearpark, L.-L. Wong. Engineering molecular recognition in alkane oxidation catalyzed by cytochrome P450cam. *New J. Chem.* **1998**, 22, 551-552.

[119] S. G. Bell, J.-A. Stevenson, H. D. Boyd, S. Campbell, A. D. Riddle, E. L. Orton, L.-L. Wong. Butane and propane oxidation by engineered cytochrome P450cam. *Chem.*

*Commun.* **2002**, 5, 490-491.

[120] S. G. Bell, E. Orton, H. Boyd, J.-A. Stevenson, A. Riddle, S. Campbell, L.-L Wong. Engineering cytochrome P450cam into an alkane hydroxylase. *J. Chem. Soc. Dalton Trans,* **2003**, 11, 2133-2140.

[121] F. Xu, S. G. Bell, J. Lednik, A. Insley, Z. Rao, L.-L. Wong. The heme monooxygenase cytochrome P450cam can be engineered to oxidize ethane to ethanol. *Angew. Chem. Intl. Ed.* **2005**, 44, 4029-4032.

[122] C. Colas, P. R. Ortiz de Montellano. Autocatalytic radical reactions in physiological prosthetic heme mofification. *Chem. Rev.* **2003**, 103, 2305-2332.

[123] J. Limburg, L. A. Lebrun, P. R. Ortiz de Montellano. The P450cam G248E mutant covalently binds its prosthetic heme group. *Biochemistry* **2005**, 44, 4091-4099.

[124] S. Kumar, J. R. Halpert. Use of directed evolution of mammalian cytochromes P450 for investigating the molecular basis of enzyme function and generating novel biocatalysts. *Biochem. Biophys. Res. Commun.* **2005**, 338, 456-464.

[125] A. Parikh, P. D. Josephy, F. P. Guengerich. Selection and Characterization of Human Cytochrome P450 1A2 Mutants with Altered Catalytic Properties. *Biochemistry* **1999**, 38, 5283-5289.

[126] C.-H. Yun, G. P. Miller, F. P. Guengerich. Rate-Determining Steps in Phenacetin Oxidations by Human Cytochrome P450 1A2 and Selected Mutants. *Biochemistry* **2000**, 39, 11319-11329.

[127] D. Kim, F. P. Guengerich. Selection of Human Cytochrome P450 1A2 Mutants with Enhanced Catalytic Activity for Heterocyclic Amine N-Hydroxylation. *Biochemistry* **2004**, 43, 981-988.

[128] D. Kim, F. P. Guengerich. Enhancement of 7-methoxyresorufin O-demethylation activity of human cytochrome P450 1A2 by molecular breeding. *Arch. Biochem. Biophys.* **2004**, 432, 102-108.

[129] K. Nakamura, M. V. Martin, F. P. Guengerich. Random Mutagenesis of Human Cytochrome P450 2A6 and Screening with Indole Oxidation Products. *Arch.*

*Biochem. Biophys.* **2001**, 395, 25-31.

[130] Z.-L. Wu, L. M. Podust, F. P. Guengerich. Expansion of Substrate Specificity of Cytochrome P450 2A6 by Random and Site-directed Mutagenesis. *J. Biol. Chem.* **2005**, 280, 41090-41100.

[131] S. Kumar, E. E. Scott, H. Liu, J. R. Halpert. A rational approach to re-engineer cytochrome P450 2B1 regioselectivity based on the crystal structure of cytochrome P450 2C5. *J. Biol. Chem.* **2003**, 278, 17178-17184.

[132] S. Kumar, C. S. Chen, D. J. Waxman, J. R. Halpert. Directed evolution of mammalian cytochrome P450 2B1. *J. Biol. Chem.* **2005**, 280, 19569-19575.

[133] W. Tischer, F. Wedekind. Immobilized enzymes: methods and applications. *Top. Curr. Chem.* **1999**, 200, 95-126.

[134] D. L. King, M. R. Azari, A. Wiseman. Immobilization of cytochrome P450 enzymes from *Saccharomyces cerevisiae*. *Methods Enzymol.* **1988**, 137, 675-686.

[135] S. B. Lamb, D. C. Lamb, S. L. Kelly, D. C. Stuckey. Cytochrome P450 immobilization as a route to bioremediation/biocatalysis. *FEBS Lett.*, **1998**, 431, 343-346.

[136] M. Taylor, D. C. Lamb, R. J. Cannell, M. J. Dawson, S. L. Kelly. Cofactor recycling with immobilized heterologous cytochrome P450 105D1. *Biochem. Biophys. Res. Commun.* **2000**, 279, 708-711.

[137] S. C. Maurer, H. Schulze, R. D. Schmid, V. Urlacher. Immobilization of P450 BM3 and an NADP+ Cofactor Recycling System: Towards a Technical application of Heme-Containing Monooxygenases in Fine Chemical Synthesis. *Adv. Synth. Catal.* **2003**, 345, 802-810.

[138] E. I. Karaseva, A. N. Eremin, D. I. Metelitsa. Optimization of the use of glucose-6-phosphate dehydrogenase under chemical regeneration of cofactor. *Vestsi Akademii Navuk BSSR, Seryya Khimichnykh Navuk* **1986**, 4, 76-80.

[139] K. Seelbach, B. Riebel, W. Hummel, M.-R. Kula, V. I. Tishkov, A. M. Egorov, C. Wandrey, U. Kragl. A novel, efficient regenerating method of NADPH using a new

formate dehydrogenase. *Tetrahedron Lett.* **1996**, 37, 1377-1380.

[140] F. Hollmann, K. Hofstetter, A. Schmid. Non-enzymatic regeneration of nicotinamide and flavin cofactors for monooxygenase catalysis. *Trends Biotechnol.* **2006**, 24, 163-171.

[141] F. Hollmann, B.Witholt, A. Schmid. Non-enzymatic regeneration of nicotinamide and flavin cofactors for monooxygenase catalysis. *J. Mol. Catal. B: Enzym.* **2002**, 19-20, 167-176.

[142] K. Vuorilehto, S. Lutz, C. Wandrey. Indirect electrochemical reduction of nicotinamide coenzymes. *Bioelectrochem.* **2004**, 65, 1-7.

[143] K. M. Faulkner, M. S. Shet, C. W. Fisher, R. W. Estabrook. Electrocatalytically driven hydroxylation of fatty acids using cytochrome P450 4A1. *Proc. Natl Acad. Sci.* **1995**, 92, 7705-7709.

[144] R. W. Estabrook, K. M. Faulkner, M. S. Shet, C. W. Fisher. Application of electrochemistry for P450-catalyzed reactions. *Methods Enzymol.* **1996**, 272, 44-51.

[145] U. Schwaneberg, D. Appel, J. Schmitt, R. D. Schmid. P450 in biotechnology: zinc driven hydroxylation of p-nitrophenoxydodecanoic acid using P450 BM-3 F87A as a catalyst. *J. Biotechnol.* **2000**, 84, 249-257.

[146] J. Nazor, U. Schwaneberg. Laboratory evolution of P450 BM-3 for mediated electron transfer. *ChemBioChem* **2006**, 7, 638-644

[147] A. K. Udit, F. H. Arnold, H. B. Gray. Cobaltocene-mediated catalytic monooxygenation using holo and heme domain cytochrome P450 BM3. *J. Inorg. Biochem.* **2004**, 98, 1547-1550.

[148] V. V. Shumyantseva, T. V. Bulko, A. I. Archakov. Electrochemical reduction of cytochrome P450 as an approach to the construction of biosensors and bioreactors. *J. Inorg. Biochem.* **2005**, 99, 1051-1063.

[149] J. Kazlauskaite, A. C. G. Westlake, L.-L. Wong, H. Hill, O. Allen. Direct electrochemistry of cytochrome P450cam .*Chem. Commun.* **1996**, 18, 2189-2190.

[150] K. Di Gleria, H. Hill, O. Allen, L.-L. Wong. *N*-(2-ferrocene-ethyl)maleimide: a new

electroactive sulphydryl-specific reagent for cysteine-containing peptides and proteins. *FEBS Lett.* **1996**, 390, 142-144.

[151] K. Di Gleria, D. P. Nickerson, H. Hill, O. Allen, L.-L. Wong, V. Fueloep. Covalent Attachment of an Electroactive Sulfydryl Reagent in the Active Site of Cytochrome P450cam as Revealed by the Crystal Structure of the Modified Protein. *J. Am. Chem. Soc.* **1998**, 120, 46-52.

[152] K. K.-W. Lo, L.-L. Wong, H. Hill, O. Allen. Surface-modified mutants of cytochrome P450cam: enzymatic properties and electrochemistry. *FEBS Lett.* **1999**, 451, 342-346.

[153] Z. Zhang, A.-E. F. Nassar, Z. Lu, J. B. Schenkman, J. F. Rusling. Direct electron injection from electrodes to cytochrome P450cam in biomembrane-like films. *J. Chem Soc. Faraday Trans.* **1997**, 93, 1769-1774.

[154] Y. M. Lvov, Z. Lu, J. B. Schenkman, X. Zu, J. F. Rusling. Direct Electrochemistry of Myoglobin and Cytochrome p450cam in Alternate Polyion Layer-by-Layer Films with DNA and Poly(styrenesulfonate). *J. Am. Chem. Soc.* **1998**, 120, 4073-4080.

[155] X. Zu, Z. Lu, Z. Zhang, J. B. Schenkman, J. F. Rusling. Electroenzyme-Catalyzed Oxidation of Styrene and cisMethylstyrene Using Thin Films of Cytochrome P450cam and Myoglobin. *Langmuir* **1999**, 15, 7372-7377.

[156] B. Munge, C. Estavillo, J. B. Schenkman, J. F. Rusling. Optimization of electrochemical and peroxide-driven oxidation of styrene with ultrathin polyion films containing cytochrome P450cam and myoglobin. *ChemBioChem* **2003**, 4, 82-89.

[157] C. Lei, U. Wollenberger, C. Jung, F. W. Scheller. Clay-Bridged Electron Transfer between Cytochrome P450cam and Electrode. *Biochem. Biophys. Res. Commun.* **2000**, 268, 740-744.

[158] K.-F. Aguey-Zinsou, P. V. Bernhardt, J. J. De Voss, K. E. Slessor. Electrochemistry of P450cin: new insights into P450 electron transfer. *Chem. Commun.* **2003**, 3, 418-419.

[159] B. D. Fleming, Y. Tian, S. G. Bell, L.-L. Wong, V. Urlacher, H. Hill, O. Allen. Redox properties of cytochrome P 450BM3 measured by direct methods. *Eur. J. Biochem.* **2003**, 270, 4082-4088.

[160] A. K. Udit, K. D. Hagen, P. J. Goldman, A. Star, J. M. Gillan, H. B. Gray, M. G. Hill. Spectroscopy and lectrochemistry of cytochrome P450 BM3-surfactant film assemblies. *J. Am. Chem. Soc.* **2006**, 128, 10320-10325.

[161] V. V. Shumyantseva, T. V. Bulko, T. T. Bachmann, U. Bilitewski, R. D. Schmid, A. I. Archakov. Electrochemical reduction of flavocytochromes 2B4 and 1A2 and their catalytic activity. *Arch. Biochem. Biophys.* **2000**, 377, 43-48.

[162] V. V. Shumyantseva, T. V. Bulko, S. A. Usanov, R. D. Schmid, C. Nicolini, A. I. Archakov. Construction and characterization of bioelectrocatalytic sensors based on cytochromes P450. *J. Inorg. Biochem.* **2001**, 87, 185-190.

[163] V. V. Shumyantseva, Y. D. Ivanov, N. Bistolas, F. W. Scheller, A. I. Archakov, U. Wollenberger. Direct Electron Transfer of Cytochrome P450 2B4 at Electrodes Modified with Nonionic Detergent and Colloidal Clay Nanoparticles. *Anal. Chem.* **2004**, 76, 6046-6052.

[164] C. Estavillo, Z. Lu, I. Jansson, J. B. Schenkman, J. F. Rusling. Epoxidation of styrene by human cyt P450 1A2 by thin film electrolysis and peroxide activation compared to solution reactions. *Biophys. Chem.* **2003**, 104, 291-296.

[165] S. Joseph, J. F. Rusling, Y. M. Lvov, T. Friedberg, U. Fuhr. An amperometric biosensor with human CYP3A4 as a novel drug screening tool. *Biochem. Pharmacol.* **2003**, 65, 1817-1826.

[166] A. Fantuzzi, M. Fairhead, G. Gilardi. Direct Electrochemistry of Immobilized Human Cytochrome P450 2E1. *J. Am. Chem. Soc.* **2004**, 126, 5040-5041.

[167] A. Shukla, E. M. Gillam, D. J. Mitchell, P. V. Bernhardt. Direct electrochemistry of enzymes from the cytochrome P450 2C family. *Electrochem. Commun.* **2005**, 7, 437-442.

[168] V. V. Shumyantseva, G. Deluca, T. Bulko, S. Carrara, C. Nicolini, S. A. Usanov, A. I. Archakov. Cholesterol amperometric biosensor based on cytochrome P450scc. *Biosen. Bioelectron.* **2004**, 19, 971-976.

[169] V. V. Shumyantseva, S. Carrara, V. Bavastrello, D. Jason Riley, T. V. Bulko, K. G.

Skryabin, A. I. Archakov, C. Nicolini. Direct electron transfer between cytochrome P450scc and gold nanoparticles on screen-printed rhodium-graphite electrodes. *Biosens. Bioelectron.* **2005**, 21, 217-222.

[170] Y.-S. Kim, M. Hara, K. Ikebukuro, J. Miyake, H. Ohkawa, I. Karube. Photo-induced activation of cytochrome P450/reductase fusion enzyme coupled with spinach chloroplasts. *Biotechnol. Tech.* **1996**, 10, 717-720.

[171] M. Hara, H. Ohkawa, M. Narato, M. Shirai, Y. Asada, I. Karube, J. Miyake. Regeneration of NADPH by cactus chloroplasts: coupling reaction with P450 monooxygenase. *J. Ferment. Bioeng.* **1997**, 84, 324-329.

[172] M. Hara, S. Iazvovskaia, H. Ohkawa, Y. Asada, J. Miyake. Immobilization of P450 monooxygenase and chloroplast for use in light-driven bioreactors. *J. Biosci. Bioeng.* **1999**, 87, 793-797.

[173] V. V. Shumyantseva, V. Uvarov, V.Yu., O. E. Byakova, A. I. Archakov. Reductase and oxygenase activities of semisynthetic flavocytochromes based on cytochrome P450 2B4. *Biochem. Mol. Biol. Int.* **1996**, 38, 829-838.

[174] V. V. Shumyantseva, V. Uvarov, V.Yu., O. E. Byakova, A. I. Archakov. Semisynthetic flavocytochromes based on cytochrome P450 2B4: reductase and oxyganase activities. *Arch. Biochem. Biophys.* **1998**, 354, 133-138.

[175] V. V. Shumyantseva, T. V. Bulko, R. D. Schmid, A. I. Archakov. Photochemical properties of a riboflavins/cytochrome P450 2B4 complex. *Biosens. Bioelectron.* **2002**, 17, 233-238.

[176] E. G. Hrycay, J. A. Gustafsson, M. Ingelman-Sundberg, L. Ernster. Sodium periodate, sodium chlorite, and organic hydroperoxides as hydroxylating agents in hepatic microsomal steroid hydroxylation reactions catalyzed by cytochrome P450. *FEBS Lett.* **1975**, 56, 161-165.

[177] J. A. Gustafsson, E. G. Hrycay, L. Ernster. Sodium periodate, sodium chlorite, and organic hydroperoxides as hydroxylating agents in steroid hydroxylation reactions catalyzed by adrenocortical microsomal and mitochondrial cytochrome P450. *Arch. Biochem. Biophys.* **1976**, 174, 438-451.

[178] E. G. Hrycay, J. A. Gustafsson, M. Ingelman-Sundberg, L. Ernster. The involvement of cytochrome P-450 in hepatic microsomal steroid hydroxylation reactions supported by sodium periodate, sodium chlorite, and organic hydroperoxides. *Eur. J. Biochem.* **1976**, 61, 43-52.

[179] G. D. Nordblom, R. E. White, M. J. Coon. Studies on hydroperoxide-dependent substrate hydroxylation by purified liver microsomal cytochrome P450. *Arch. Biochem. Biophys.* **1976**, 175, 524-533.

[180] X. Fang, J. R. Halpert. Dithionite supported hydroxylation of palmitic acid by cytochrome P450-BM3. *Drug Metab. Dispos.* **1996**, 24, 1282-1285.

[181] V. Ullrich, H. J. Staudinger, *Model systems in studies of the chemistry and the enzymatic activation of oxygen*, B. B. Brodie, J. R. Gilette, H. S. Ackermann (Eds.), *Handbuch der Experimentellen Pharmakologie* Vol. 28/2, Springer-Verlag, Berlin, New York, **1971**, pp. 251-263.

[182] F. F. Kadlubar, K. C. Morton, D. M. Ziegler. Microsomal-catalyzed hydroperoxide-dependent C-oxidation of amines. *Biochem. Biophys. Res. Commun.* **1973**, 54, 1255-1261.

[183] A. D. Rahimtula, P. J. O'Brien. Hydroperoxide catalyzed liver microsomal aromatic hydroxylation reactions involving cytochrome P450. *Biochem. Biophys. Res. Commun.* **1974**, 60, 440-447.

[184] A. D. Rahimtula, P. J. O'Brien. Hydroperoxide-dependent O-dealkylation reactions catalyzed by liver microsomal cytochrome P450. *Biochem. Biophys. Res. Commun.* **1975**, 62, 268-275.

[185] A. Ellin, S. Orrenius. Hydroperoxide-supported cytochrome P460-linked fatty-acid hydroxylation in liver microsomes. *FEBS Lett.* **1975**, 50, 378-381.

[186] H. Joo, Z. Lin, F. H. Arnold. Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation. *Nature* **1999**, 399, 670-673.

[187] K. Matsuura, T. Tosha, S. Yoshioka, S. Takahashi, K. Ishimori, I. Morishima. Structural and functional characterization of laboratory evolved cytochrome P450cam

mutants showing enhanced naphthalene oxygenation activity. *Biochem. Biophys. Res. Commun.* **2004**, 323, 1209-1215.

[188] P. C. Cirino, F. H. Arnold. A self-sufficient peroxide-driven hydroxylation biocatalyst. *Angew. Chem. Intl. Ed.* **2003**, 42, 3299-3301.

[189] A. L. Shen, T. D. Porter, T. E. Wilson, C. B. Kasper. Structural analysis of the FMN binding domain of NADPH-cytochrome P450 oxidoreductase by site-directed mutagenesis. *J. Biol. Chem.* **1989**, 264, 7584-7589.

[190] T. L. Domanski, J. Liu, G. R. Harlow, J. R. Halpert. Analysis of Four Residues within Substrate Recognition Site 4 of Human Cytochrome P450 3A4: Role in Steroid Hydroxylase Activity and α-Naphthoflavone Stimulation. *Arch. Biochem. Biophys.* **1998**, 350, 223-232.

[191] F. P. Guengerich, M. V. Martin, P. H. Beaune, P. Kremers, T. Wolff, D. J. Waxman. Characterization of rat and human liver microsomal cytochrome p450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J. Biol. Chem.* **1986**, 261, 5051-5060.

[192] D. J. Waxman, C. Attisano, F. P. Guengerich, D. P. Lapenson. Cytochrome P450 steroid hormone metabolism catalyzed by human liver microsomes. *Arch. Biochem. Biophys.* **1988**, 263, 424-436.

[193] J. A. Krauser, M. Voehler, L. Tseng, A. B. Schefer, M. Godejohann, F. P. Guengerich. Testosterone 1β-hydroxylation by human cytochrome P450 3A4. *Eur. J. Biochem.* **2004**, 271, 3962-3969

[194] K. He, L. M. Bornheim, A. M. Falick, D. Maltby, H. Yin, M. A. Correia. Identification of the heme-modified peptides from cumene hydroperoxide-inactivated cytochrome P450 3A4. *Biochemistry* **1998**, 37, 17448-17457.

[195] I. Montanez-Clemente, E. Alvira, M. Macias, A. Ferrer, M. Fonceca, J. Rodriguez, A. Gonzales, G. Barletta. Enzyme activation in organic solvents: Co-lyophilization of subtilisin carlsberg with methyl-β-cyclodextrin renders an enzyme catalyst more active than the cross-linked enzyme crystals. *Biotech. Bioeng.* **2002**, 78, 53-59.

[196] K. Dabulis, A. M. Klibanov. Dramatic enhancement of enzymatic activity in organic solvents by lyoprotectants. *Biotech. Bioeng.* **1993**, 41, 566-571.

[197] K. Griebenow, A. M. Klibanov. Lyophilization-induced reversible changes in the secondary structure of proteins. *Proc. Nat. Acad. Sc. USA* **1995**, 92, 10969-10976.

[198] S. J. Prestrelski, T. Arakawa, J. F. Carpenter. Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. II. Structural studies using infrared spectroscopy. *Arch. Biochem. Biophys.* **1993**, 303, 465-473.

[199] T. Arakawa, S. J. Prestrelski, W. C. Kenney, J. F. Carpenter. Factors affecting short-term and long-term stabilities of proteins. *Adv. Drug Del. Rev.* **1993**, 10, 1-28.

[200] J. F. Carpenter, M. J. Pikal, B. S. Chang, T. W. Randolph. Rational design of stable lyophilized protein formulations: some practical advice. *Pharm. Res.* **1997**, 14, 969-975.

[201] K. Izutsu, S. Yoshioka. Stabilization of protein pharmaceuticals in freeze-dried formulations. *Drug Stab.* **1995**, 1, 11-21.

[202] I. Roy, M. N. Gupta. Freeze-drying of proteins: some emerging concerns. *Biotechnol. Appl. Biochem.* **2004,** 39, 165-177.

[203] Y. Ooe, S. Yamamoto, M. Kobayashi, H. Kise. Increase of catalytic activity of a-chymotrypsin in organic solvent by co-lyophilization with cyclodextrins. *Biotech. Lett.* **1999**, 21, 385-389.

[204] K. Griebenow, Y. Laureano, A. M. Santos, I. Montanez-Clemente, I. Rodriguez, M. W. Vidal, G. Barletta. Improved enzyme activity and enantioselectivity in organic solvents by methyl-β-cyclodextrins. *J. Am. Chem. Soc.* **1999**, 121, 8157-8163.

[205] A. M. Santos, I. Montanez-Clemente, G. Barlett, K. Griebnow. Activation of serine protease subtilisin Carlsberg in organic solvents: combined effect of methyl-β-cyclodextrin and water. *Biotech. Lett.* **1999**, 21, 1113-1118.

[206] J. Broos, W. Verboom, J. F. J. Engbersen, D. N. Reinhoudt. Crown ethers can enhance enzyme activity in organic solvents. *Prog. Biotech.* Biocatalysis in Non-Conventional Media, **1992**, 8, 691-695.

[207] D.-J. van Unen, J. F. J. Engbersen, D. N. Reinhoudt. Why do crown ethers activate enzymes in organic solvents? *Biotech. Bioeng.* **2002**, 77, 248-255.

[208] J. Broos, I. K. Sakodinskaya, J. F. J. Engbersen, W. Verboom, D. N. Reinhoudt. Large activation of serine proteases by pretreatment with crown ethers. *J. Chem. Soc., Chem. Comm.* **1995**, 2, 255-256.

[209] T. Itoh, Y. Takagi, T. Murakami, Y. Hiyama, H. Tsukube. Crown ethers as regulators of enzymatic reactions: enhanced reaction rate and enatioselectivity in lipase-catalyzed hydrolysis of 2-cyano-1-methylethyl acetate. *J. Org. Chem.* **1996**, 61, 2158-2163.

[210] T. Itoh, K. Mitsukura, K. Kaihatsu, H. Hamada, Y. Takagi, H. Tsukube. Remarkable acceleration of a lipase-catalyzed reaction by a thiacrown ether additive: buffer-free higly regioselective partial hydrolysis of 4-acetoxy-2-methylbut-2-enylacetate. *J. Chem. Soc. Perkin Trans.* **1997**, 1, 2275-2278.

[211] T. Itoh, K. Mitsukura, W. Kanphai, Y. Takagi, H. Kihara, H. Tsukube. Thiacrown ether technology in lipase-catalyzed reaction: scope and limitation for preparating optically active 3-hydroxyalkanenitriles and applications to insect pheromone synthesis. *J. Org. Chem.* **1997**, 62, 9165-9172.

[212] Y. Takagi, J. Teramoto, H. Kihara, T. Itoh, H. Tsukube. Thiacrown ethers as regulator of lipase-catalyzed transesterification in organic media: practical optical resolution of ally alcohols. *Tetrahedron Lett.* **1996**, 37, 4991-4992.

[213] Y. Mine, K. Fukunaga, K. Itoh, M. Yoshimoto, K. Nakao, Y. Sugimura. Enhanced enzyme activity and enantioselectivity of lipases in organic solvents by crown-ethers and cyclodextrins. *J. Biosci. Bioeng.* **2003**, 5, 441-447.

[214] L. T. Kanerva, O. Sundholm. Enzymatic acylation in the resolution of methyl threo-2-hydroxy-3-(4-methoxyphenyl)-3-(2-X-phenylthio)propionates in organic solvents. *J. Chem. Soc. Perkin. Trans.* **1993**, 1, 2407-2410.

[215] W. Pu, Y. Li-Rong, W. Jian-Ping. Immobilization of lipase by salts and the transesterification activity in hexane. *Biotechnol. Lett.* **2001**, 23, 1429-1433.

[216] F. Secundo, G. Carrea. Mono- and disaccharides enhance the activity and enantioselectivity of *Burkholderia cepacia* lipase in organic solvent but do not significantly affect its conformation. *Biotechnol. Bioeng.* **2005**, 92, 438-446.

[217] K. R. Ward, G. D. J. Adams, H. O. Alpar, W. J. Irwin. Protection of the enzyme L-asparaginase during lyophilization-a molecular modeling approach to predict required level of lyoprotectants. *Inter. J. Pharmaceutics* **1999**, 187, 153-162.

[218] G. D'Andrea, M. L. Salucci, L. Avigliano. Effect of lyoprotectants on ascorbate oxidase activity after freeze-drying and storage. *Process Biochem.* **1996**, 31, 173-178.

[219] J. G. Sampedro, G. Guerra, J.-P. Pardo, S. Uribe. Trehalose-mediated protection of the plasma membrabe $H^+$-ATPase from *Kluyveromyces lactis* during freeze-drying and rehydration. *Crybiology* **1998**, 37, 131-138.

[220] R. Batra, M. N. Gupta. Enhancement of enzyme activity in aqueous-organic solvent mixtures. *Biotech. Lett.* **1994**, 16, 1059-1064.

[221] D. Hickman, J.-P. Wang, Y. Wang, J. D. Unadrat. Evaluation of the selectivity of *in vitro* probes and suitability of organic solvents for the measurement of human cytochrome P450 monooxygenase activities. *Drug Met. Dispos.* **1997**, 26, 207-215.

[222] N. Chauret, A. Gauthier, D. A. Nicoll-Griffith. Effect of common organic solvents on in vitro cytochrome P450-mediated metabolic activities in human liver microsomes. *Drug Met. Dispos.* **1998**, 26, 1-4.

[223] W. F. Busby, J. M. Ackermann, C. L. Crespi. Effect of methanol, ethanol, dimethylsulfoxide and acetonitrile on in vitro activities of cDNA-expressed human cytochromes P450. *Drug Met. Dispos.* **1999**, 27, 246-249.

[224] J. Easterbrook, C. Lu, Y. Sakai, A. P. Li. Effects of organic solvents on the activities of cytochrome P450 isoforms, UDO-dependent glucoronyl transferase, and phenol sulfotransferase in human hepatocytes. *Drug Met. Dispos.* **2001**, 29, 141-144.

[225] S. G. Cull, J. D. Holbrey, V. Vargas-Mora, K. R. Seddon, G. J. Lye. Room-temprature

ionic liquids as replacements for organic solvents in multiphase bioprocess operations. *Biotech. Bioeng.* **2000**, 69, 227-233.

[226] M. Erbeldinger, A. J. Mesiano, A. J. Russel. Enzymatic catalysis of formation of Z-apartame in ionic liquid – an alternative to enzymatic catalysis in organic solvents. *Biotechnol. Prog.* **2000**, 16, 1129-1131.

[227] G. Hinckley, V. V. Mozhaev, C. Budde, Y. L. Khmelnitski. Oxidative enzymes possess catalytic activity in systems with ionic liquids. *Biotech. Lett.* **2002**, 24, 2083-2087.

[228] H. Wang, R. Dick, H. Yin, E. Licad-Coles, D. L. Kroetz, G. Szklarz, G. Harlow, J. R. Halpert, M. A. Correia. Structure-function relationships of human liver cytochromes P450 3A: aflatoxin B1 metabolism as a probe. *Biochemistry* **1998**, *37*, 12536-12545.