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# Regioselective C-H oxidation of fatty acids with biocatalysts

**MASTER`S THESIS**

To achieve the University degree of

Diplom-Ingenieur

Master`s degree programme: Technical Chemistry

submitted to

**Graz University of Technology**

Supervisor

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Institute of Chemistry, University of Graz

Graz, September 2019

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# 1 Introduction

Traditional organic synthesis is mainly based on the reactivities of functional groups,[1] which means that the starting materials of classic chemical conversions need to be functionalized in the appropriate position. Avoiding this requirement in organic synthesis could therefore have the potential of shortening various synthetic strategies. However, to circumvent classic, functional group based organic reactions, $sp^3$ hybridized C-H bonds need to be activated in a selective way.[1] This functionalization of C-H bonds in a regio- and stereoselective way represents a huge challenge for chemical methodology.[2]

Besides organometallic chemistry, biocatalysis represents an important strategy for selective C-H functionalization, because in nature various enzymes catalyze direct and selective C-H bond functionalization reactions. The enzyme methane monooxygenase (MMO) for example catalyzes the hydroxylation of methane.[3] Cytochrome P450 monooxygenases form a whole superfamily of C-H bond activating enzymes. A very abundant reaction catalyzed by these P450 enzymes is the hydroxylation of unactivated C-H bonds. Furthermore, epoxidations,[4] sulfoxidations,[5] reductive dehalogenations,[6] decarboxylations[7] and many other reactions are performed by P450 enzymes.[8] But P450 enzymes do not only perform a broad range of reactions, they also accept a huge variety of substrates ranging from simple alkanes to complex steroid molecules. This diversification is due to the fact that P450 enzymes are involved in many metabolic pathways, which are often associated with defending strategies of organisms like the detoxification of xenobiotics. Since xenobiotics can have quite different structures, a certain flexibility of P450 enzymes in terms of substrate scope is required.[9] Among the broad range of substrates which are converted by P450 enzymes, fatty acids are quite important from a technical point of view, due to their availability from renewable resources and because the hydroxylation products of fatty acids (hydroxy-fatty acids), find application in food-, cosmetic- and pharmaceutical industry.[10]

P450 enzymes which perform hydroxylation either in close vicinity of the fatty acid carboxy terminus or the ω-position are already known (chapter 2.3). However, they generally lack high regio-selectivity. To employ these enzymes on large scale in industrial transformations, their natural selectivities need to be changed and/or increased. Furthermore, also the typically low activities of P450 enzymes should be optimized for such applications. Both required optimizations of natural P450 enzymes can be achieved *via* protein engineering.[10] This thesis

deals with the optimization of P450 enzymes in their application as biocatalysts for fatty acid hydroxylation.

# 2 Theoretical Background

## 2.1 Classification of P450 enzymes

More than 35000 different gene sequences can be assigned to the P450 superfamily.[11] Thus, within this superfamily of enzymes considerable variations of primary sequences are observed. Sequence identities of lower than 20% can be found and only very few amino acids are conserved within P450 sequences overall.[9] In contrast to the strongly varying primary sequences, the structural fold of P450 enzymes (example shown in Figure 1) is highly conserved.[12] A central part of this P450 structure is a bundle of four α-helices, from which three appear in parallel form. Embedded between two of the mentioned α-helices, a heme group occurs in all P450 enzymes as catalytically active site. The central iron ion of this heme group is coordinated by the thiolate group of a conserved cysteine, which acts as a fifth ligand and connects the heme group with its protein environment. This thiolate ligand is an important factor for the characteristic absorbance of these enzymes at 450 nm, which is observed when the ferrous state of the heme iron is complexed by CO as sixth ligand. The described absorption is such a fundamental property of the active site structure of P450 enzymes, that it became the name giving property of this enzyme superfamily.[13, 14]

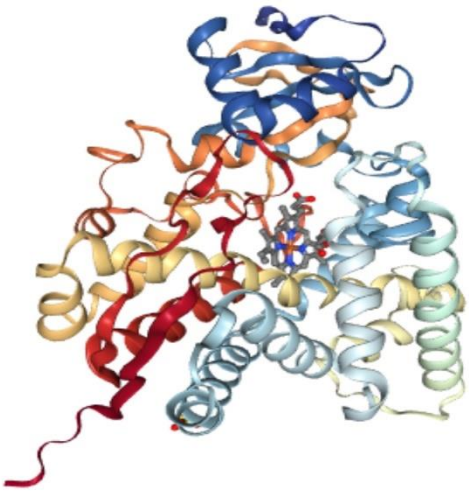


**Figure 1:** Representative P450 crystal structure [structure of $P450_{OleT}$, heme group is depicted as grey unit with orange centre (heme iron)].

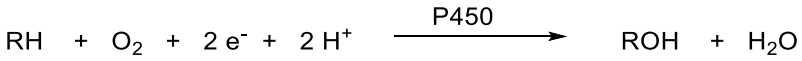
Contrary to the thiolate bound heme group and the overall structural fold, the substrate recognition sites of various P450 enzymes can be subject of considerable variations, enabling the P450 superfamily to accept a broad substrate scope.[9] Based on their sequence identities, P450 enzymes can be divided in families (≥ 40% sequence identity) and subfamilies (≥ 55% sequence identity). This sequence based classification is also found in the common nomenclature of P450 enzymes.[15] The common name for the gene 1 enzyme of the subfamily A within the CYP102 family is therefore CYP102A1.[10]

Another common way of P450 classification depends on the utilized electron transfer system and will be discussed in chapter 2.2.3.

## 2.2 Mechanism of P450 mediated C-H hydroxylation

### 2.2.1 Basic catalytic cycle

P450 mediated C-H hydroxylation uses molecular oxygen as oxidizing agent. As a result of the mechanism, one of the oxygen atoms of $O_2$ is inserted into a C-H bond, whereas the second oxygen atom is converted to water getting electrons from NADH or NADPH. Due to the total reaction (Scheme 1), P450 enzymes are classified as monooxygenases.



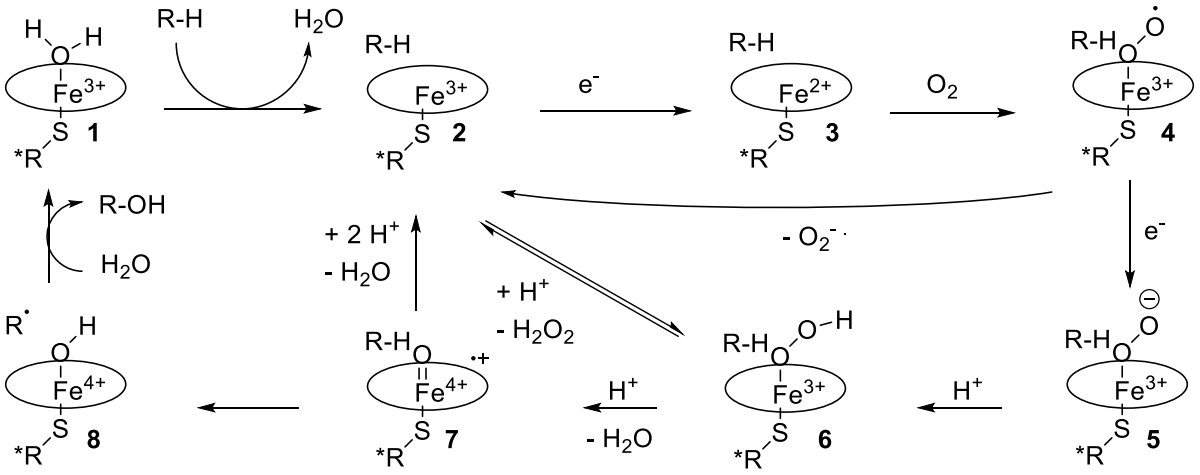

**Scheme 1:** Overall reaction of P450 monooxygenases.

Since molecular oxygen occurs in a triplet state, its direct reactions with substrates at ambient conditions are spin-forbidden processes. Thus the P450 enzymes are required to activate molecular oxygen and convert it into an iron oxygen species, which is capable of oxidizing a certain C-H bonds at physiological temperature and pressure.[16]

Most investigations dealing with the catalytic mechanism of P450 enzymes were based on bacterial P450 enzymes, since these P450 enzymes are, in contrast to the membrane bound mammalian, plant and fungal P450 enzymes, easily expressed in soluble form.[17] Especially the camphor hydroxylating enzyme CYP101 from *Pseudomonas pudita* served as prototype enzyme for mechanistic investigations.[18]

Responsible for the catalytic activity of P450 enzymes is the thiolate coordinated heme group in the active centre of the enzyme.

At beginning of the catalytic cycle, the central iron ion of the heme group appears in a low spin, ferric resting state. In this resting state a molecule of water coordinates to the ferric heme species as sixth ligand. The catalytic cycle of P450 enzymes (Scheme 2) is started by the binding of a hydrophobic substrate to the low spin ferric-heme resting state (**1**) of the P450 enzyme. This binding of the substrate molecule is an entropy driven process, since it causes the release of water molecules from the enzyme binding pocket. Thus the substrate binding is mainly due to hydrophobic interactions.[16, 19] Due to the binding of the substrate the water molecule which is coordinated to the ferric iron (sixth ligand) is commonly displaced, causing a spin shift of the ferric iron from low spin to a high spin ferric state (**2**). Furthermore also the redox potential of the ferric iron is increased, caused by the destabilizing effect of the water departure.[13] Because of its increased redox potential the high spin ferric intermediate (**2**) can now be reduced by an appropriate electron transfer system, yielding a ferrous iron-heme complex (**3**). Oxygen as well as CO (giving a complex with absorption at 450 nm) are good ligands for iron in ferrous state. When oxygen coordinates to the ferrous iron intermediate, it forms a ferric superoxido species (**4**), which can be reduced with another external electron source to a ferric peroxido species (**5**). This electron transfer process appears to be the rate determining step of the catalytic cycle. Based on the protonation of the ferric peroxide anion (**5**) a ferric hydroperoxido species (**6)** is formed, which is commonly known as compound 0.[10] Protonation of compound 0 leads to a heterolytic cleavage of the O-O bond forming compound I (**7**), which can be described as a ferryl oxido species. Because of its high oxidation sate iron ion, compound I is highly reactive and abstracts a hydrogen radical from the substrate, yielding compound 2 (**8**), which donates its hydroxy ligand to the previously formed substrate radical and gets thereby reduced. This reaction finally closes the catalytic cycle. Besides the main catalytic cycle there are also some shortcut pathways known, which are commonly termed as uncoupling reactions (chapter 2.2.4).[10, 20] The described mechanism is often referred to as abstraction-rebound pathway.[21]




**Scheme 2:** Accepted catalytic cycle of a P450 mediated hydroxylation (R* represents the enzyme).[10]

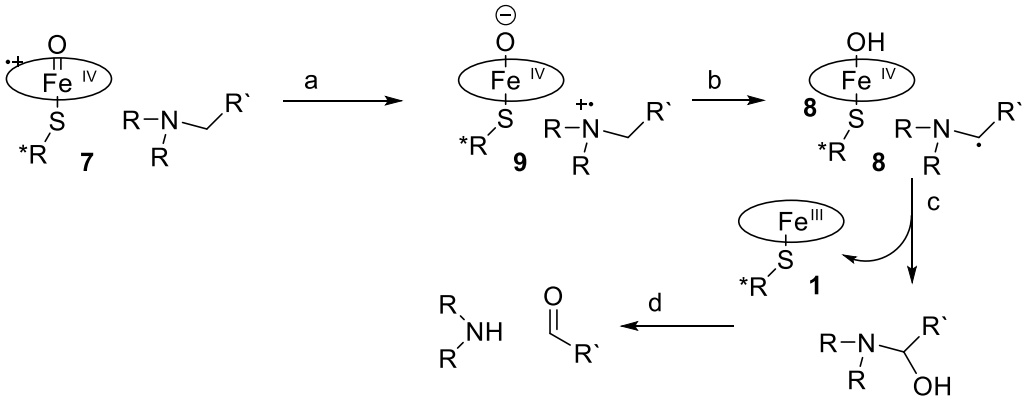
From a metabolic point of view, it is important to avoid that the ferric-heme resting state (**1**) is reduced in absence of a substrate molecule in the active site. Because this would lead to the formation of ferric superoxide species *via* the ferrous state. The ferric superoxide would then follow the described P450 cycle, generating activated iron-oxide species like compound I. If no substrate is in the close vicinity to the heme centre in such a case, the formation of these activated iron oxygen species would not only be a waste of redox equivalents, but it would also be a risk of damaging the overall enzyme structure.[22]

It was reported that bacterial P450 enzymes avoid such a situation by a substrate binding induced change of the redox potential of the ferric resting state. This change of redox potential is concomitant with a change of the iron spin state from low to high spin, caused by displacement of the water ligand in the resting state due to substrate binding. Thus the electron transfer system can only reduce the ferric heme resting state, when a substrate molecule is bound to the active site of the enzyme.[23] In contrast to bacterial P450 enzymes, this substrate controlled change of redox potential was observed to be absent in many membrane bound P450 enzymes.[24] Furthermore it was observed that some P450 enzymes have their ferric resting species already in high spin state, which means that substrate binding cannot change the spin state in these cases anymore.[25] Overall the relation between change of spin state, substrate binding and variation of redox potential seems to be quite delicate and varying within the P450 superfamily.

### 2.2.2 Catalytically active species

The central intermediate of the P450 mechanism (Scheme 2) is compound I, which is envisioned as an iron (IV) oxo species with a radical-cation delocalized over its porphyrin ligand (Por·$^{+}$Fe(IV)=O).[17] Due to its high reactivity compound I could for long times not actually be observed in the catalytic cycle of P450 enzymes. Therefore the proposed structure of compound I was mainly based on biomimetic models, calculations and the structure of chloroperoxidase (CPO) compound I. CPO compound I served as model system for compound I derived from P450 systems because it has on the one hand a thiolate ligated heme group like in P450 enzymes, but is on the other hand stable enough to be spectroscopically characterized.[25,26] Further support for the structural assignment of compound I as Por·$^{+}$Fe(IV)=O comes from a high primary isotope effect (11.5), observed in the hydroxylation of 2,3,5,6 tetradeuteronorbornane.[27] In terms of spectroscopic characterization only the UV-VIS spectrum of compound I was known until 2010. This UV-VIS spectrum (absortion maxima: 370, 610 and 690 nm) is based on transiently formed compound I of thermostable P450 enzymes originating from *Sulfolobus sulfactaricus*.[28,29] Green and coworkers found that the yield of transiently formed compound I can be increased to approximately 75% by using highly purified enzymes.[30] These improvements led to Mössbauer and EPR spectra of P450 compound I, which supported the structural assignment of it as Por·$^{+}$Fe(IV)=O, partly upon comparison to the corresponding spectra of CPO compound I.[26, 31]

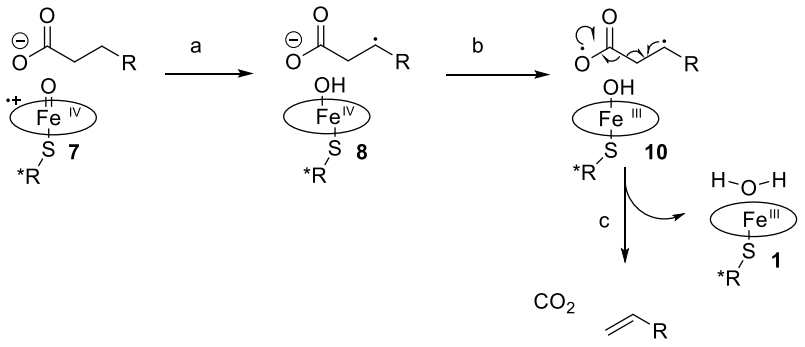
Apart from hydroxylation reactions, P450`s compound I is also viewed to be responsible for other reactions.[25] One of these reactions is the dealkylation of amines. The originally assumed mechanism for this reaction started with a single electron transfer (SET) from the nitrogen lone pair of the amine to compound I (**7**), yielding a reduced form of compound I (**9**) and an aminium radical (Scheme 3). Due to acidification of the hydrogen atoms in α position to the aminium radical, one of them could be abstracted by compound **9** (by proton abstraction **9** is converted to **8**). This step also includes electronic rearrangement and leads to the formation of the carbon centred radical that can react with **8** *via* the already mentioned radical rebound mechanism to produce a hemiaminal, which finally collapses and releases a dealkylated amine.[32]




**Scheme 3:** Mechanism of P450 catalyzed dealkylation of amines; a: SET, b: abstraction of α proton and electronic rearrangement, c: rebound step forming hemiaminal, d: collapse of hemiaminal.[32]

The amine dealkylation mechanism in Scheme 3 seems rather complicated and it is indeed argued. Radical clock experiments with CYP2B1 for example suggested that the hemiaminal in Scheme 3 is directly formed *via* hydroxylation of the methylene group, bound to the nitrogen atom of the investigated amine. However because this hydroxylation was assumed to be promoted by compound I as well (*via* the hydrogen abstraction-rebound mechanism), it can be stated that amine dealkylations depend on P450 compound I, no matter which proposed mechanism for the hemiaminal formation is actually true.[33]

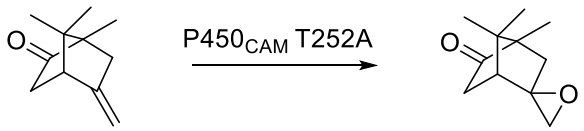
Another P450 catalyzed reaction is the decarboxylation of fatty acids performed by the P450 enzyme $OleT_{JE.}$ One proposed mechanism starts with the abstraction of a hydrogen atom by compound I (**7**), yielding a radical centre on the fatty acid in ß-position to the carboxy moiety (Scheme 4). Electron transfer from the carboxy moiety of the fatty acid to species **8** reduces its iron, forming species **10**. Resulting from this electron transfer, a second radical centre is formed on the fatty acid substrate. Intramolecular radical recombination, leads to elimination of $CO_2$ and formation of an olefin.[34]




**Scheme 4:** Proposed mechanism for P450 catalyzed decarboxylation; R represents an alkyl residue; a: hydrogen abstraction by compound I, b: Reduction of **8** *via* SET, c: radical recombination, resting state of P450 enzyme (**1**) is formed by protonation of **10**.[34]

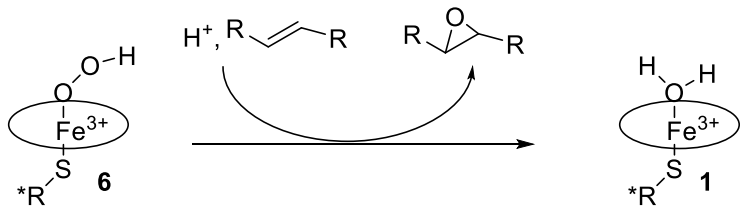
For the P450 catalyzed decarboxylation, also an alternative mechanism is discussed in literature. This alternative mechanism assumes that a carbocation (centre of positive charge in ß-position to the carboxy moiety) acts as central intermediate in the corresponding catalytic cycle.[34] Besides the depicted examples, P450 compound I is also assumed to be responsible for further reactions like nitrations, rearrangements and desaturations.[35, 36] Further flexibility of the P450 catalytic cycle arises from the fact that not only compound I can act as the actual catalytically active species. Radical clock experiments for example led to the idea that compound 0 (**8**) could also act as oxidizing agent, parallel to compound I in C-H hydroxylation reactions.[21]

Based on mutated P450 variants it was postulated that compound 0 is responsible for epoxidation reactions. The $P450_{CAM}$ variant T252A for instance epoxidized olefins like the example in Scheme 5 without hydroxylation.[37]




**Scheme 5:** Conversion performed by Dawson *et. al.* based on $P450_{CAM}$ variant T252A.[37]

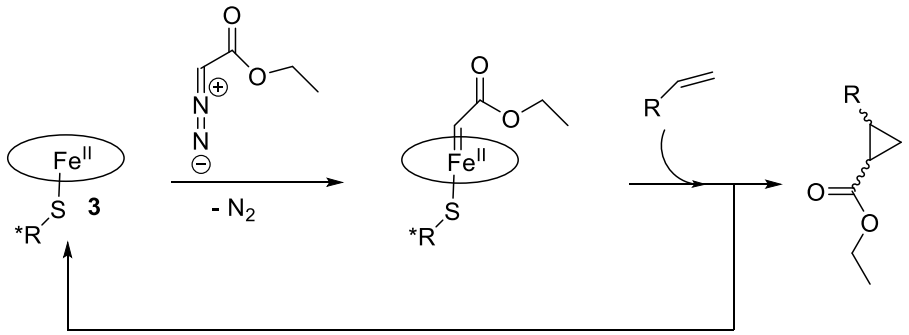
This shift from hydroxylating to epoxidizing activity was explained by the substitution of the T252 amino acid, which is responsible for the protonation of compound 0. Since this protonation is not possible in the T252A variant, the formation of compound I is impeded in this enzyme variant. Thus, the epoxidizing activity of this enzyme variant proofed that compound 0 can act as catalytically active species. It was proposed that the catalytic cycle of the epoxidation performed by $P450_{CAM}$ T252A is closed by the reaction shown in Scheme 6, bypassing compounds I and II.[37]




**Scheme 6:** Conversion of compound 0 (**6**) with a proton and an olefin yields an epoxide and directly forms the resting state of the usual P450 catalytic cycle.[37]

Due to the flexibility of the mentioned P450 catalytic cycle, variants of P450 enzymes are even able to perform reactions, which do not occur in nature. An example of such reactions was investigated by Frances Arnold. In these studies, variants of CYP102A1 ($P450_{BM3}$) from

*Bacillus megaterium* were used to catalyze the cyclopropanation of styrene. This reaction is initiated by formation of an iron-carbenoid species from P450 catalyst in its ferrous form (**3**) and a diazo compound as carbene precursor. The dependence on a non-natural diazo compound as reagent is the reason why P450 enzymes do not perform cyclopropanations in nature. However, the iron-carbenoid species in this reaction converts styrene in the respective cyclopropane. Enzyme engineering of $P450_{BM3}$ led to significantly increased turnover numbers, disatereo-, and enantioselectivities. Furthermore, the reaction required a reducing agent ($Na_2S_2O_3$) for formation of **3**. Anaerobic conditions also turned out to be advantageous for this conversion. The mechanism of P450 mediated cyclopropanation is shown in Scheme 7.[38]




**Scheme 7:** Principle of $P450_{BM3}$ catalyzed cyclopropanation of olefins.[38]

The shown examples are intended to demonstrate how flexible the P450 catalytic cycle is. Beside the variable reaction possibilities of compound I, the central intermediate in the P450 catalytic cycle, also intermediates prior to compound I can be the origin of certain reactions. This flexibility is the reason for the diverse array of reactions, catalyzed by the P450 superfamily.[21]

### 2.2.3 Electron transfer system

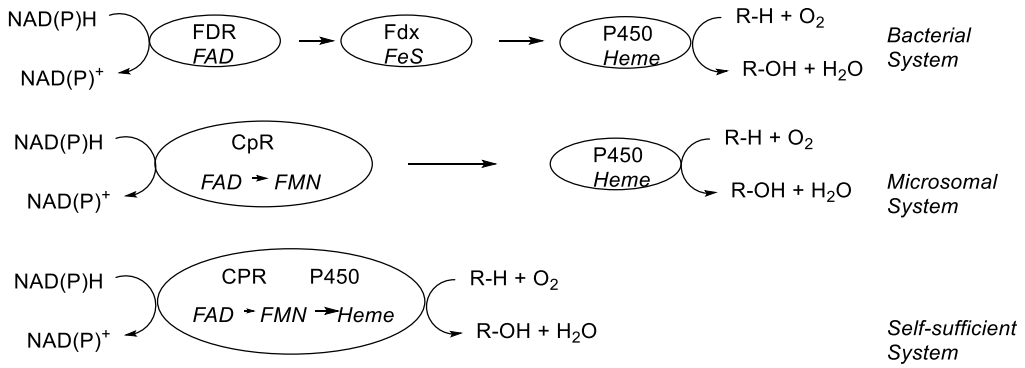
During the P450 catalytic cycle (Scheme 1) two electrons are transferred to the heme cofactor in the P450 enzyme. The first electron reduces the high spin ferric state (**2**) to a ferrous state, while the second electron converts the ferric superoxide (**4**) to a ferric peroxido species (**5**). Several different electron transfer systems, based on various enzymes and cofactors, are known today. Three basic electron transport systems employed by P450 enzymes are the bacterial system, the mitochondrial system and the self-sufficient system.[39]

The bacterial system employs three separate enzymes, one of which being the P450 enzyme. In this system a ferrodoxin reductase enzyme collects reduction equivalents from NAD(P)H by

reducing its FAD cofactor.[40] The resulting $FADH_2$ containing enzyme transfers the reduction equivalents then to a ferredoxin enzyme, which is able to store an electron in its iron sulfur cluster.[41] Reduced ferredoxin enzymes are finally responsible for reducing the actual P450 enzyme by two consecutive single electron transfers. The basic principle of the bacterial system can also be found in the mitochondrial electron transfer system, but with the difference that only the ferredoxin is soluble, while the other two enzymes of the system are membrane bound.

The microsomal system consists just of two enzymes, including the P450 enzyme. In this system reducing equivalents are transferred from a NAD(P)H species on a cytochrome P reductase (CpR) enzyme. The CpR enzyme is equipped with a FAD and a flavin mononucleotide (FMN) cofactor to deliver the redox equivalents to the P450 enzyme.[42]

The self-sufficient system is represented by P450 enzymes, where the heme domain is attached to the electron transfer system. $P450_{BM3}$ is an example for such a self-sufficient system.




**Scheme 8:** Representation of basic electron transfer systems; lines represent flow of redox equivalents; cofactors are written in italic form.

The described electron transfer systems are shown in Scheme 8. Beside these electron transfer systems also other electron transfer systems are known, which arise from variations of cofactors for example. Hannemann *et. al.* described the different electron transfer systems in detail.[43]

### 2.2.4 P450 enzymes acting as peroxygenases

The requirement of an electron transfer system is regarded as a disadvantage of P450 catalyzed conversions, because of the cost of the cofactors employed for the electron transfer system. Additionally, the alternative utilization of whole cell preparations for such conversions suffers

from difficult downstream processing, required to isolate the intended products.[44] A promising solution to overcome these problems is the operation of P450 enzymes in the peroxygenase mode. In this operational mode of P450 enzymes $H_2O_2$ is used to generate hydroperoxo species compound 0 (**6**) directly from the high spin ferric state (**2**), without the requirement for redox equivalents. Thus, when P450 enzymes are operated in the peroxygenase mode, no electron transfer system is required.[14]

The mechanistic basis for the peroxygenase mode is the so called peroxide shunt. This short cut of the P450 catalytic cycle is usually an unwanted side reaction. The $H_2O_2$ shunt converts compound 0 (**6**) *via* protonation of its proximal oxygen atom (Figure 2) directly to the high spin ferric species **2**. Protonation of the distal oxygen atom leads to the loss of a water molecule and formation of compound I (**7**), following the usual P450 catalytic path.[16]

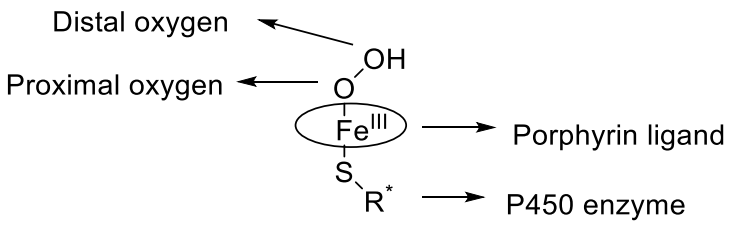
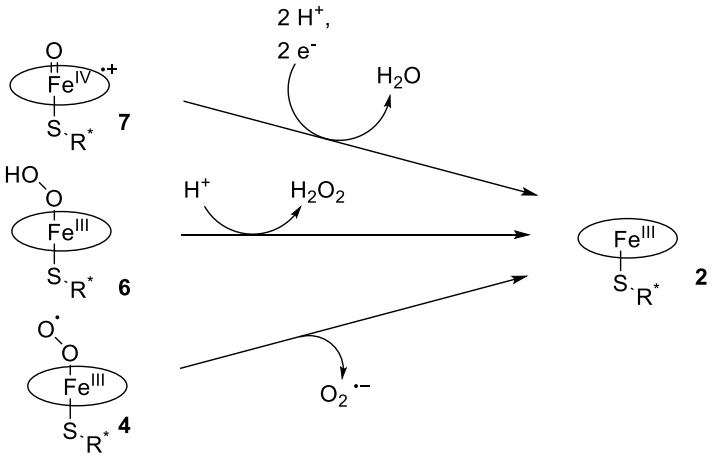



**Figure 2:** Representation of P450 compound 0 (**6**); protonation of distal oxygen leads to compound I, while protonation of proximal oxygen initiates hydrogen peroxide shunt.[16]

The $H_2O_2$ shunt pathway represents a waste of redox equivalents, because two electrons are needed to produce compound 0 (**6**). Direct conversion of compound 0 to species **2** permits useful oxidation of a substrate molecule. The fraction of redox equivalents [ultimately originating from NAD(P)H], that does not lead to product formation because of shunt pathways, is described as uncoupling.[45] Beside the peroxide shunt pathway, there are also two other uncoupling reactions occurring in the P450 catalytic cycle. In one of them the ferric superoxido species **4** loses a superoxide anion radical, yielding high spin ferric species **2**. The superoxide anion radical is then converted to oxygen and $H_2O_2$ by a dismutation reaction.[46] Loss of water from compound I (**7**) *via* protonation and concomitant reduction with two additional redox equivalents also leads to species **2**. The described shunt pathways are summarized in Scheme 9.




**Scheme 9:** Uncoupling reactions occurring in P450 catalytic cycle.

In situations when a substrate is not properly bound to the active centre of a P450 enzyme, the shunt pathways help to avoid damaging of the enzyme, by degrading the formed active iron-oxygen species.[47]

Due to the reversibility of the peroxide shunt, compound 0 can be formed by oxidation of high spin ferric species **2** with hydrogen peroxide. However, with the majority of known P450 enzymes this peroxygenase mode appears to be rather inefficient because of enzyme degradation processes, induced by $H_2O_2$. Nevertheless, it was found that the P450 family CYP 152 performs well under peroxygenase conditions. Members of the CYP152 family, like $P450_{SP\alpha}$ (CYP152B1), $P450_{Bs\beta}$ (CYP152A1) or $P450_{OleT}$ (CYP152L1) are involved in the fatty acid metabolism. The crystal structure of CYP152A1 showed that the Thr252 amino acid, which appears in most P450 enzymes, is replaced by an arginine amino acid. Furthermore, it was found that a phenylalanine amino acid in close vicinity of the heme cofactor, which is a conserved element in ordinary P450 monooxygenases, is missing in CYP152A1. So, the ability of CYP152 enzymes to efficiently use $H_2O_2$ as oxidant can be related to certain structural features of these enzymes.[14]

## 2.3 Regioselective hydroxylation of fatty acids

Saturated fatty acids are substrates with multiple, chemically similar methylene groups. Thus C-H hydroxylation of fatty acids not only represents a mechanistically interesting challenge, but also demands high standards of regioselectivity. Within a fatty acid alkyl chain three different regions can be roughly defined. One of these regions is defined by methylene groups that are close to the carboxy terminus of the fatty acid. If hydroxylation happens in this region, it is described as carboxy terminal hydroxylation. Hydroxylation at the opposite side of the fatty acid alkyl chain thus represents the hydroxylation of a methyl group, which is thermodynamically more challenging and defined as terminal hydroxylation. If hydroxylation occurs in the middle of the carbon chain, it is defined as in-chain hydroxylation. The nowadays known P450 enzymes cover all three described regioselectivities. Table 1 summarizes the regioselectivities of different P450 enzyme families.[10]

**Table 1:** Regioselectivities of P450 enzyme families.[10]

| Regioselectivity | P450 enzyme family |
|---|---|
| carboxy terminal | CYP152 |
| in-chain | CYP1, CYP2, CYP77, CYP96, CYP102, CYP107, CYP119, CYP267, CYP505, |
| terminal | CYP102, CYP153, CYP52, CYP76 |

### 2.3.1 Carboxy terminal hydroxylation

Selective hydroxylation of fatty acids at the carboxy terminus is known to be catalyzed by P450 enzymes belonging to the CYP152 family. The enzymes in this family act as peroxygenases (Chapter 2.2.4).[10] An example of this enzyme family is $P450_{Bsß}$ (CYP152A1) from *Bacillus subtilis.* This enzyme hydroxylates myristic acid either in α or in ß position (utilizing $H_2O_2$ as oxidant), with a slight preference for the ß-position.[48] It was also shown that $P450_{Bsß}$ is able to hydroxylate polyaromatic compounds. 9-methyl-anthracen for example was hydroxylated with > 99% conversion.[49] Based on its crystal structure the α/ß selectivity of $P450_{Bsß}$ can be explained. The enzyme has a trigonal overall shape. Two separate channels are leading to the heme containing active site of the enzyme. The entrance of one of these channels is surrounded

by a cluster of positively charged amino acid residues, which is thought to attract the negatively polarized carboxy group of fatty acid substrates. The interior of this channel is equipped with hydrophobic side chains to stabilize the alkyl residues of fatty acids. Fatty acid carboxylate groups undergo electrostatic interactions with the guanidinium moiety of an arginine residue (Arg242). This arginine residue, which is conserved in the CYP152 family (see also chapter 2.2.4) is located in close vicinity to the active heme centre of the enzyme. Because of this spatial arrangement, the methylene groups in α and ß positions are hydroxylated by $P450_{Bs\beta}$. In Figure 3 the crystal structure of $P450_{Bs\beta}$ is depicted.[50]

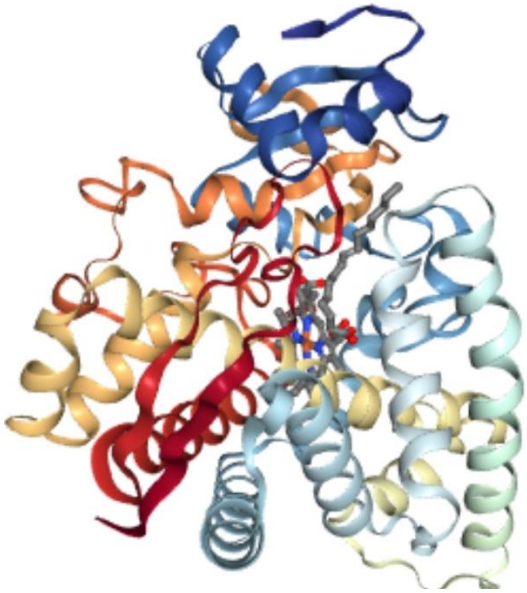


**Figure 3:** Crystal structure of $P450_{Bs\beta}$ with bound fatty acid. C-α and C-ß of the fatty acid are in close vicinity of the heme iron ion (shown as orange dot).[50]

While $P450_{Bs\beta}$ produces a mixture of α- and ß-hydroxylated fatty acids, another member of the CYP152 peroxygenases, namely $P450_{SP\alpha}$ (CYP152B1) from *Sphingomonas paucimobilis*, catalyzes exclusively α-hydroxylation of fatty acids. $P450_{SP\alpha}$ shares 44% sequence identity with $P450_{Bs\beta}$. The X-ray structure of $P450_{SP\alpha}$ is similar to the structure of its family member $P450_{Bs\beta}$. It has also a trigonal basic structure and possesses two channels to the active centre, one of which is used by the substrate, whereas the other channel is supposed to be used by the oxidizing agent ($H_2O_2$). Furthermore, $P450_{SP\alpha}$ also binds fatty acid carboxylate moieties with an arginine residue. However, in $P450_{SP\alpha}$ this arginine residue is located at position 241 (Arg241). Figure 4 shows a crystal structure of $P450_{SP\alpha}$.[51]

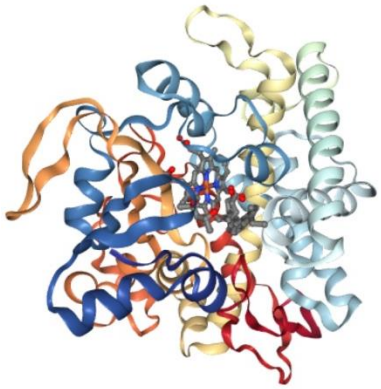


**Figure 4:** Crystal structure of $P450_{SP\alpha}$. Central heme iron ion depicted in orange.[12]

Another P450 peroxygenase with high α-selectivity in fatty acid hydroxylations is $P450_{CLA}$ (CYP152A2) from *Clostridium acetobutylicium*. But in contrast to $P450_{SP\alpha}$, $P450_{CLA}$ also gives ß-hydroxylated fatty acids as minor products. The α/ß selectivity in hydroxylation reactions depends on the identity of the converted fatty acid but is always in favour of the α-hydroxylated product. $P450_{CLA}$ shows 57% sequence identity with $P450_{Bs\beta}$. It also has an Arg242 residue to bind the substrate.[52]

$P450_{MP}$ (CYP152MP) from *Methylobacterium polpuli* hydroxylates long chain fatty acids (C18, C16) mainly in ß-position to the carboxy moiety. Alongside hydroxylation also an oxidative decarboxylation is catalyzed by this peroxygenase. Conversion of C18:0 for instance gave 48.3% ß-hydroxylated product and 38.3% heptadecene (decarboxylation product of C18:0). The remaining fraction of products was formed by the corresponding α-, γ-, δ- and ε-hydroxylated fatty acids. In conversions of smaller fatty acids (C14, C12) decarboxylated products could not be detected anymore. Furthermore, a larger fraction of the observed hydroxy-fatty acids was formed by in chain hydroxylation. Overall $P450_{MP}$ appears to give a rather wide product distribution.[53]

When it comes to decarboxylation of fatty acids $P450_{OleT}$ (CYP152L1) from *Jeotgalicoccus species* is especially efficient. Long chain fatty acids are the natural substrates of this peroxygenase, but $P450_{OleT}$ also accepts shorter fatty acids like C8, C10 or C12. Conversion of these fatty acids with $P450_{OleT}$ gives mainly the corresponding n-1 olefins (60-90% share of substrate distribution). As minor products α-, ß- and γ-hydroxylated fatty acids are formed, with ß-hydroxylated fatty acids representing the largest fraction within the hydroxylated fatty acids (70-100% of total hydroxy-fatty acids depending on the substrate).[54]

### 2.3.2 Terminal hydroxylation

$P450_{BM3}$ (CYP102A1) from *Bacillus megaterium* is a self-sufficient (chapter 2.2.3) monooxygenase with a size of 119 kDa. Due to the efficient electron transfer enabled by the fused redox domain, $P450_{BM3}$ is considered as the most active P450 monooxygenase known so far. Similar to the CYP152 family, $P450_{BM3}$ is assumed to bind the carboxy terminus of a fatty acid substrate with an arginine residue (R47). However, this arginine residue is located on the surface of the enzyme, so that the ω-terminus of a fatty acid points towards the active heme centre of the enzyme. For most fatty acids it was observed that the ω-2 methylene group is predominantly hydroxylated. Hydroxylation of the ω-methyl group can be achieved by employing the Q55P/N70S/F87I/M185T/A197V/K202R/V216A/M237L/N239T/I263F/ A328V variant of $P450_{BM3}$.[55] Besides $P450_{BM3}$ there are also other P450 enzymes, like CYP153 or CYP52, that are able to catalyze terminal hydroxylations. A detailed summary of these enzymes can be found in the review of Hammerer *et. al.*[10]

### 2.3.3 In-chain hydroxylation

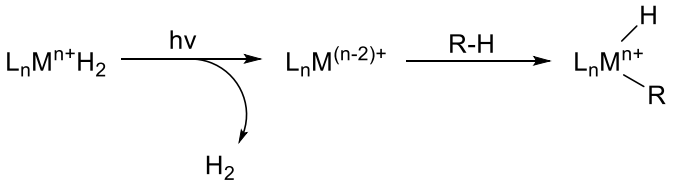
P450 enzymes that catalyze in-chain hydroxylation are usually not as selective as enzymes that promote carboxy terminal hydroxylation. This is due to the fact that the enzyme can only discriminate between the in chain methylene groups by bringing the fatty acid substrate into an appropriate conformation.[10] $P450_{BM3}$ hydroxylates fatty acids in subterminal positions ω-1, ω-2 and ω-3. Introduction of mutations into $P450_{BM3}$ not only led to ω-selective variants (Chapter 2.3.2) but also to variants which catalyze in-chain hydroxylation. Variant V78A/F87A/S72Y for example produced significant amounts of δ-hydroxy lauric acid (16% share of products) from lauric acid. Employing the wild type enzyme this product could not be detected. This example shows that the regioselectivity of $P450_{BM3}$ can be efficiently shifted by the introduction of certain mutations, but it remains difficult to achieve a narrow product distribution, which would be desirable from a technical point of view.[56]

Selective in chain hydroxylation was observed in biotransformations with the self-sufficient enzyme $P450_{TT}$ (CYP116B46) from *Tepidiphilus thermophilus.* Conversion of decanoic acid with this enzyme gave predominantly δ-hydroxy-decanoic acid (90% share of products). For other substrates (octanoic and dodecanoic acid) somewhat lower regioselectivities were

observed with this enzyme. However, $P450_{TT}$ showed that also in-chain hydroxylations can be performed in a selective way by P450 enzymes.[57]

## 2.4 Chemical C-H hydroxylation

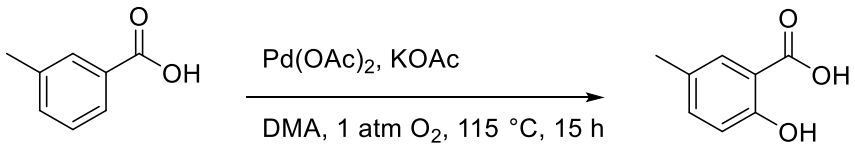
The activation and concomitant conversion of unactivated C-H bonds, like the ones occurring in the backbone of fatty acids or alkanes, represents a major challenge in modern organometallic chemistry. Low lying HOMO`s and high energy LUMO`s are responsible for the low reactivity of such C-H bonds. Nevertheless, it was shown that insertion of an organometallic complex into a C-H bond is in principle possible by oxidative addition. For this type of oxidative additions, coordinatively unsaturated precursor complexes are formed by flash photolysis in the presence of alkane substrates. Such a C-H activation, which must be carried out in the absence of other functional groups, is shown in Scheme 10.




**Scheme 10:** Metal-catalyzed activation of a C-H bond *via* oxidative addition. An example for the $L_nM^{n+}H_2$ precursor is $(\eta^5C_5Me_5)$-$(PMe_3)Ir^{III}H_2$.

As soon as the C-H bond is broken *via* formation of an organometallic species, further reactions can take place, leading to the overall conversion of an unactivated C-H bond.[58]

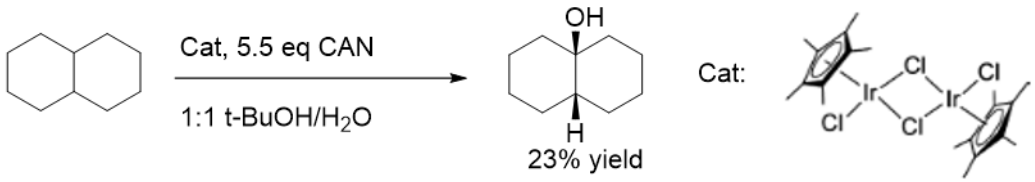
An example were such a C-H activation leads to transformation of a C-H bond into a hydroxy moiety is given by the conversion of a benzoic acid derivative and molecular oxygen in the presence of $Pd(OAc)_2$ and a potassium salt (Scheme 11).




**Scheme 11:** Transition metal catalyzed hydroxylation of 2-methyl-benzylic acid (DMA = N,N-dimethyl acetamide).[59]

Because of the required carboxy group in α position of the reacting C-H bond and the aromatic nature of the substrate, the reaction shown above, seems to be quite far off a fatty acid hydroxylation.[59]

Iridium-catalyzed hydroxylation of decalin is in contrast more similar to fatty acid hydroxylation. This reaction uses cerium (IV) ammonium nitrate (CAN) as oxidant. The reaction is depicted in Scheme 12.[60]




**Scheme 12:** Irridium catalyzed hydroxylation of decalin.[57]

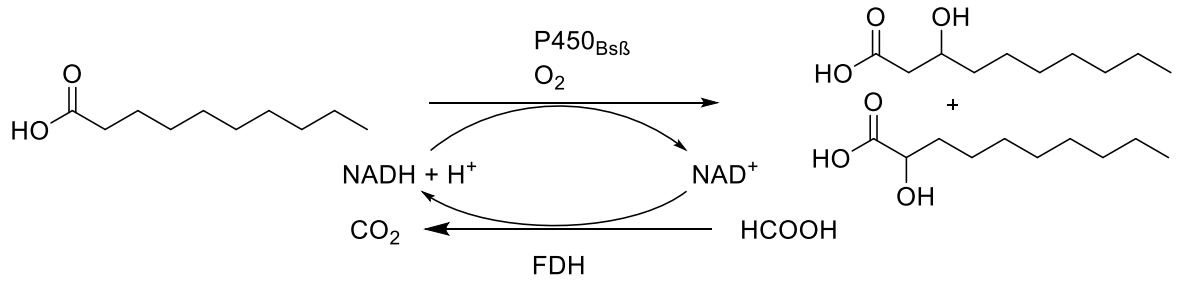
Although the reaction in Scheme 12 represents already a hydroxylation of an aliphatic C-H group, its regioselectivity is restricted to the thermodynamically weakest C-H bond at the tertiary carbon. Transition metal catalyzed regioselective hydroxylations of fatty acids or other molecules, would be still more demanding and are not reported yet. Even if a regioselective, transition metal catalyzed method for fatty acid hydroxylation was developed, such a method would work under harsh conditions or with toxic reagents. Therefore biocatalytic hydroxylation of fatty acids with P450 enzymes seems to be the most efficient method for the selective production of hydroxy-fatty acids or selective C-H activation overall.

# 3 Objectives of the Thesis

The aim of this project was to achieve regioselective hydroxylation of decanoic acid at various positions by employing P450 enzymes as biocatalysts. These studies can be seen as a first step towards an efficient and environmentally friendly biotransformation of various fatty acids to different hydroxy fatty acids, which find application in cosmetic-, food-, and pharmaceutical industry.

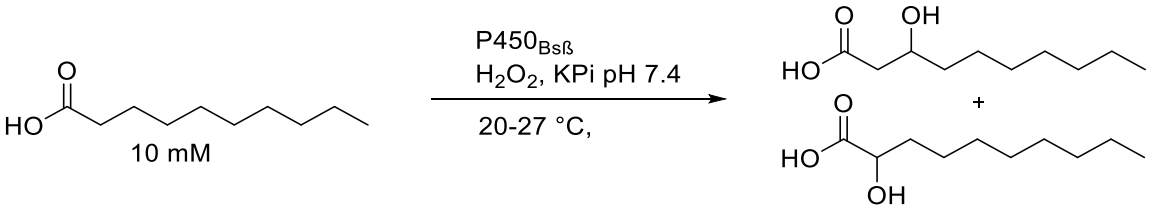
The P450 enzymes Bsβ from *Bacillus subtilis* (CYP152A1) and MP from *Methylobacterium populi* (CYP152MP) were investigated for fatty acid hydroxylation. 51 different Bsß variants were expressed and characterized, while 9 $P450_{MP}$ variants were designed using the PROSS server.[61] These variants were cloned, expressed and investigated in biotransformations employing decanoic acid (C10) as substrate. The $P450_{MP}$ wildtype enzyme is known to catalyze the decarboxylation of long chain fatty acids starting from myristic acid. Besides decarboxylation, this enzyme also performs hydroxylation reactions over a variety of fatty acid positions (α-, β-, γ-, δ- and ε-position), but with low regioselectivity (chapter 2.3.1). A limitation of $P450_{MP}$ is its tedious expression in soluble form. By consulting the PROSS server, mutations which should lead to increased expression levels and enhanced enzyme activities, were predicted.

In contrast to the $P450_{MP}$ enzyme, $P450_{Bsß}$ can be expressed easily in soluble form. The wild type enzyme hydroxylates fatty acids in α-and ß-positions respectively, with a slight preference towards the α-position (ß/α = 0.78 in case of C10:0 as substrate).[62] The $P450_{Bsß}$ variants were generated by site directed mutagenesis, furnishing mutations which increase or decrease the cavity of the active site. 11 amino acids located in the hydrophobic channel of the active site were targeted (L41, L42, L70, V74, L78, V170, F173, F289, F292 and L293). These amino acids were substituted by either alanine (A) or phenylalanine (F). After introduction of the desired mutations into the $P450_{Bsß}$ gene, the corresponding enzyme variations were expressed in *E. coli* cells. For the first assessment of the resulting enzyme activities, the cell free extracts were directly used to convert decanoic acid. The investigated enzyme variants were tested with a NADH dependent electron transfer system (CamAB) and $O_2$ as oxygen source (monooxygenase mode). Formiate dehydrogenase (FDH) was used as cofactor recycling system for the screening (Scheme 13).




**Scheme 13:** Hydroxylation employing $P450_{Bsß}$ in the monooxygenase mode (FDH = formiate dehydrogenase).

The enzyme variants which gave the highest ß/α ratio in terms of hydroxy group introduction, were then further tested in the peroxygenase mode. In the peroxygenase mode the $P450_{Bsß}$ enzymes use $H_2O_2$ as oxygen source (Scheme 14). No cofactor recycling system is required when the P450 enzyme is operated in the peroxygenase mode, which simplifies the reaction. To test the enzyme variants in the peroxygenase mode they needed to be purified in order to exclude catalases from the corresponding cell free extracts (CFE).




**Scheme 14:** Reaction mechanism of $P450_{Bsß}$ in the peroxygenase mode.

The overall target of the investigations with $P450_{Bsß}$ variants was to identify enzyme variants, which hydroxylate decanoic acid with high regioselectivity in ß-position. Besides this regioselectivity (defined by high ß/α-ratio), the targeted $P450_{Bsß}$ variants should also show high activity in terms of substrate conversion. In the context of this thesis the $P450_{Bsß}$ screening started with double variants of the $P450_{Bsß}$ wild type enzyme. The mutations employed in these double mutated variants were chosen based on previous screenings with single variants. So the most regioselective (in terms of ß-hydroxylation) and/or active (in terms of substrate conversion) single variants which were found in these previous studies were combined to give the double variants which were investigated during this master thesis. Based on the screening results of the double variants, triple variants were also produced and tested in the described biotransformations as part of this master thesis.

# 4 Results and Discussion

## 4.1 Investigation of $P450_{MP}$ variants

### 4.1.1 Cloning of $P450_{MP}$ variants

Ten different $P450_{MP}$ variants with multiple mutations were designed based on prediction by the PROSS server.[61] These mutated genes were then purchased from the company Gene Art. One of the ten ordered variants could however not be delivered. Variants (hp1, hp2, hp3, hp4, h1, h3, h4, h5 and h6) were cloned into pET28(a)+ as well as pDB-HisGST vectors. Variants with the designation `h` had a fixed heme, meaning that the active site (heme containing) of these mutated variants were identical to the active site of the wild type enzyme, contrary to other parts of the enzyme which were subjected to various mutations based on calculations by the PROSS server. In variants with the designation `hp` the heme group and the surrounding of a bound palmitic acid substrate were fixed. For the digestion of the vectors and the purchased DNA fragments the restriction enzymes *Xho I* and *Nde I* were used. The resulting agarose gels are depicted in Figure 5.

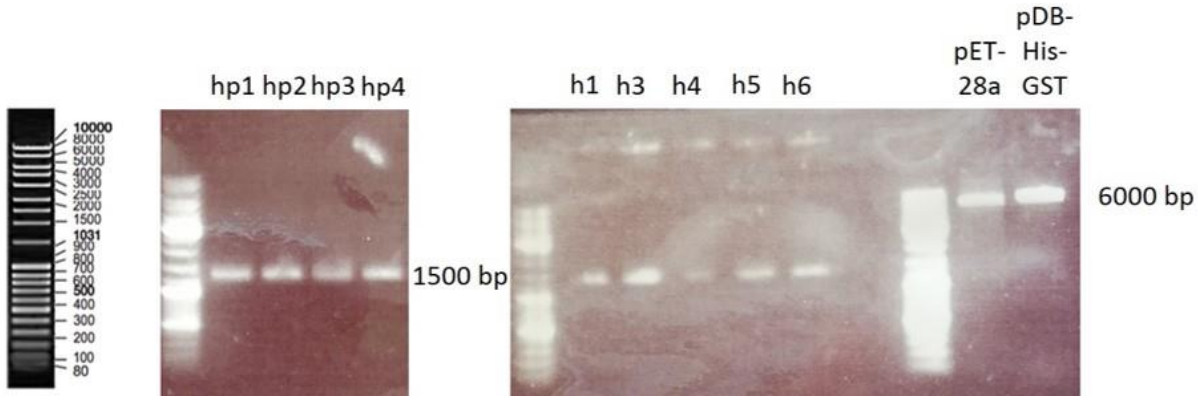



**Figure 5:** Agarose gels of digested $P450_{MP}$ PROSS genes and vectors; mutated MP genes (hp1-hp4; h1, h3-h6): 1280 bp, digested pET28a vector: 5289 bp, digested pDB-HISGST vector: 5950 bp.

### 4.1.2 Expression of MP PROSS variants

After cloning, amplification in *E. coli NEB 5α* and transformation of the $P450_{MP}$ variants into expression host *E. coli BL21 (DE3)* cells, the enzymes were expressed in modified TB medium (chapter 6.9). For both employed vector systems kanamycin was used as antibiotic. Expression of the $P450_{MP}$ variants was checked with SDS gels, which are shown in Figure 6.

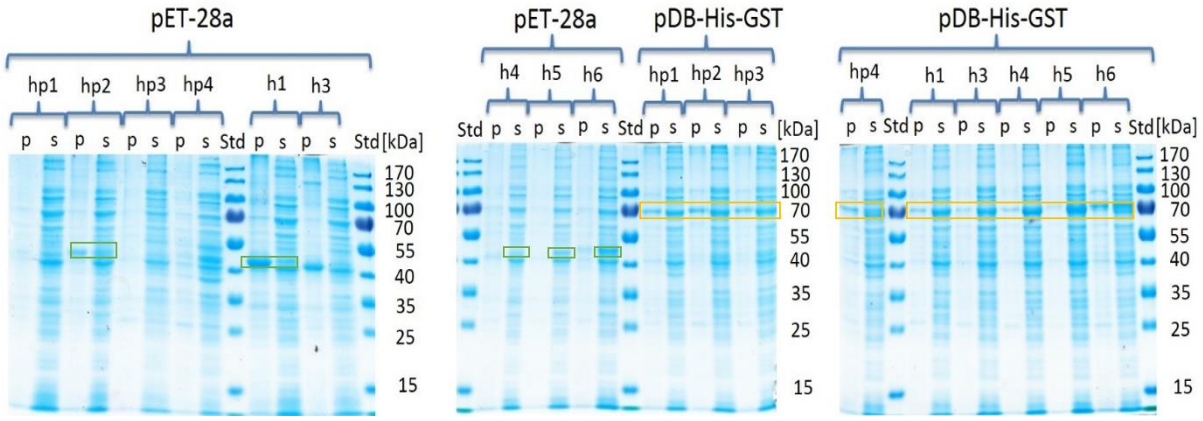



**Figure 6:** SDS gels of the expressed MP variants [p: pellet fraction, s: soluble fraction, Std: protein ladder mix, $P450_{MP}$ variants: 50 kDa (highlighted with green rectangles), $P450_{MP}$ variants with GST tag: 70 kDa (highlighted with yellow rectangles)].

The obtained SDS gels showed that the $P450_{MP}$ variants were expressed at low levels. In the soluble fractions of $P450_{MP}$ variants in pET28(a)+ vectors, a small band at 50 kDa could be observed for $P450_{MP}$ variants h4, h5 and h6. The bands at 40 kDa below the bands, representing $P450_{MP}$ variants h4, h5 and h6, represent natural proteins of the *E. coli* host, since they could be observed in all soluble fractions, also in those with $P450_{MP}$ variants cloned into pDB-HisGST vectors. For MP-hp2 in pET28(a)+ a band at 50 kDa could be observed in the pellet fraction. The variants that were cloned into pDB-HisGST vectors were expressed at 70 kDa, due to the additional weight caused by the addition of the GST-tag, which was added to increase the solubility. This increased solubility of $P450_{MP}$ variants, expressed in pDB-HisGST vectors, was also confirmed by the SDS gels. $P450_{MP}$ variants with GST-tag gave more intensive bands (70 kDa) in the soluble fractions than in the pellet fractions.

The enzymes for the SDS gels shown above were expressed in 100 mL modified TB medium (chapter 6.10). To produce enough enzyme for the intended screening of the MP variants (chapter 6.16), each enzyme variant was expressed in 900 mL modified TB medium (3 x 300 mL). Because the thiamine stock got exhausted and the delivery time of new thiamine exceeded this thesis, it was decided to express the last three enzyme variants (hp4, h1 and h3, all cloned into pET28a vectors), which still needed to be screened, in TB medium. Besides the utilized expression medium and the missing addition of thiamine, everything else was carried out analogous to the expression of the other $P450_{MP}$ variants. To assess the effect of this varied expression, a SDS gel was prepared (Figure 7).

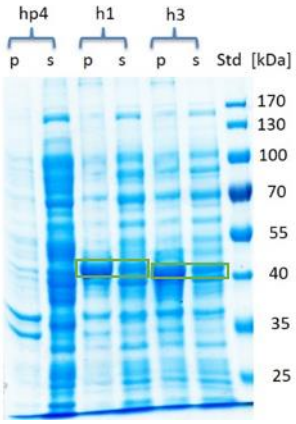



**Figure 7:** SDS gel of $P450_{MP}$ variants expressed in TB media [all of them were cloned into pET28a vectors; p: pellet fraction, s: soluble fraction, $P450_{MP}$ variants: 50 kDa (highlighted in green rectangles)].

The SDS gel in Figure 7 shows that the pellet fraction of variants h1 and h3 showed nice expression of the targeted enzymes (50 kDa). This was also found when variants h1 and h3 were expressed in modified TB medium (with thiamine) as part of the first expression study (Figure 6). Furthermore, the mentioned variants gave also bands at approximately 50 kDa in the corresponding soluble fractions. Another observation, which can be drawn from Figure 7 is, that the bands associated to variant h3 (~ 50 kDa) are shifted to slightly lower sizes compared to variant h1. In contrast to variants h1 and h3, variant hp4 showed no notable overexpression, which is also in agreement with the SDS gel obtained during the expression studies (Figure 6).

### 4.1.3 Screening of MP variants

The $P450_{MP}$ enzyme variants were purified as described in chapter 6.16. The cell free extract which was the basis of each purification and was generated by sonication of three cell pellets (each pellet originating from 300 mL mod. TB medium or TB medium). The resulting purified enzymes were rebuffered to KPi buffer (0.1 M, pH = 7.4). Since the screening of decanoic acid (C10) with the $P450_{MP}$ variants, was planned at enzyme concentrations of 4 µmol/L, the produced enzyme solutions were concentrated with spin tubes. Table 2 reports the amounts of enzymes which could be produced by the described purification process.

**Table 2:** Amount of purified enzymes. Each enzyme solution resulted from cells cultivated in 900 mL (3 x 300 mL) of mod. TB medium (no mark) or TB medium (marked with an Asterisk).

| Variants in pDB-HISGST | n – pur. enz. [nmol] | Variants in pET28a(+) | n – pur. enz. [nmol] |
|---|---|---|---|
| hp1 | / | hp1 | 11.0 |
| hp2 | 8.0 | hp2 | 26.5 |
| hp3 | 11.2 | hp3 | 10.0 |
| hp4 | 20.3 | hp4* | 0 |
| h1 | 22.3 | h1* | 6.7 |
| h3 | 7.2 | h3* | 0.8 |
| h4 | 23.4 | h4 | 8.3 |
| h5 | 53.8 | h5 | 1.5 |
| h6 | 0 | h6 | 0.5 |
| wt | 31.1 | | |

The data in Table 2 did not show an effect of the utilized vector system on the produced amounts of purified enzyme. Variant h5 cloned into pDB-HISGST was expressed significantly better than most other variants. Contrary to variant h5, P450 expression was not observed and very limited with variant h6 in pET28a and pDB-HISGST, respectively. Variant h1-pDB-HisGST was expressed much better than variant h3-pDB-HISGST, although both variants gave intensive bands at ~50 kDa in their SDS gel (Figure 6). Probably this finding is connected to the observation that the bands representing variant h3-pDB-HISGST are slightly shifted compared to the analogous bands of variant h1-pDB-HISGST (chapter 4.1.1). Due to the obtained P450 concentrations in the produced solutions of the purified $P450_{MP}$ variants, the intended enzyme concentration of 4 µmol/L in the conversions of decanoic acid could not be reached with all variants. Table 3 summarizes the initial enzyme concentrations in conversions with variants that were not expressed good enough to reach a 4 µM initial enzyme concentration.

**Table 3:** Initial P450 concentrations (Concentration in 1 mL sample prior to addition of diluted $H_2O_2$) in decanoic acid conversions, lower than 4 µmol/L.

| Variant | c-P450 (µmol/L) |
|---|---|
| h5-pET28a | 1.4 |
| h3-pET28a | 0.7 |
| h4-pET28a | 0.4 |

The generated product mixtures in this screening were analyzed *via* GC-FID. In Figure 8 the chromatogram, resulting from the conversion of decanoic acid (C10) with variant h5-pDB-HISGST is shown.

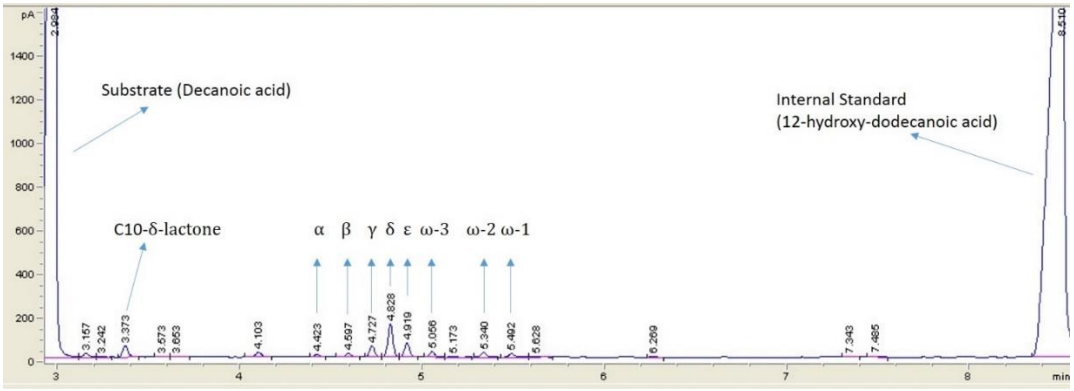



**Figure 8:** GC-FID chromatogram of product mixture resulting from conversion of decanoic acid with MP-h5-pDB-HisGST. Peaks indicated with Greek letters (α - ω-1) represent hydroxylated decanoic acids, with the position of the hydroxy group indicated by the Greek letter itself.

Identification of the different product peaks in GC-FID was accomplished by comparison with GC-MS spectra. In Figure 9 the GC-MS chromatogram of the conversion with h5-pDB-HisGST is shown.

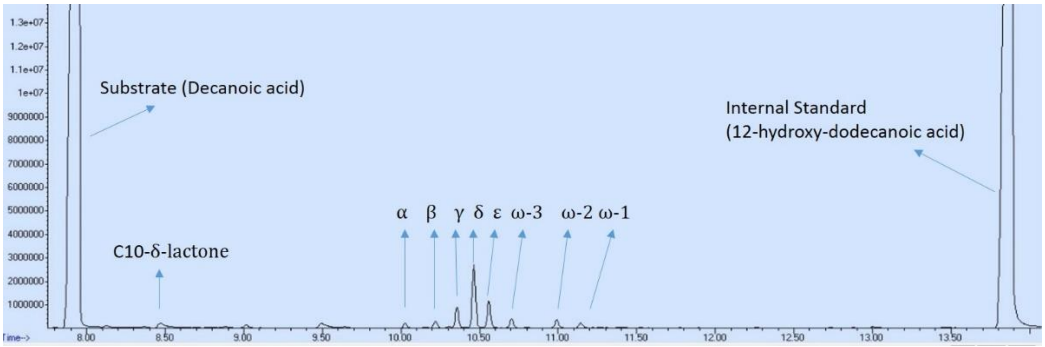



**Figure 9:** GC-MS chromatogram of product mixture resulting from conversion of decanoic acid with MP-h5-pDB-HisGST. Peaks indicated with Greek letters (α - ω-1) represent hydroxylated decanoic acids, with the position of the hydroxy group indicated by the Greek letter itself.

The mass spectra belonging to the peaks in the shown GC-MS chromatogram can be found in the appendix. Generally, the $P450_{MP}$ variants produced a broad distribution of hydroxy-decanoic acid regioisomers. All regioisomers of hydroxy-decanoic acid besides ω-hydroxy-decanoic acid could be observed.

The detailed results of the $P450_{MP}$-variant screening are shown in Tables 4 and 5.

**Table 4:** Decanoic acid (C10) hydroxylation results of MP variants, with product mixtures, produced in biotransformations, that could not be quantified properly (due to low GC-FID intensities) (pet: pET28a(+), pdb: pDB-HisGST, Subs: substrate (decanoic acid), Lac: δ-decalactone, α – ω-1: Hydroxylated decanoic acid with hydroxy position indicated by the Greek letter, n.d.: not detected).

| Variant | Subs [%] | Lac [%] | α [%] | β [%] | γ [%] | δ [%] | ε [%] | ω-3 [%] | ω-2 [%] | ω-1 [%] |
|---|---|---|---|---|---|---|---|---|---|---|
| hp4pdb | 91 ± 0 | < 1 | < 1 | < 1 | < 1 | < 1 | < 1 | < 1 | < 1 | < 1 |
| h1pdb | 98 ± 1 | < 1 | < 1 | n.d. | < 1 | < 1 | < 1 | < 1 | < 1 | n.d. |
| h4pdb | 91 ± 3 | 1 ± 0 | < 1 | < 1 | < 1 | 3 ± 0 | < 1 | < 1 | < 1 | < 1 |

Reaction conditions: 1 mL sample with decanoic acid (C10, 10 mM), DMSO (5% v/v), 4 µM purified enzyme added as solution in buffer (KPi 0.1 M, pH 7.4). To this 1 mL sample with described initial concentrations, 860 µL $H_2O_2$ (0.04 M in KPi) were added over 8.6 h *via* a syringe pump. Reactions were carried out at room temperature 21-27 °C and analyzed on GC-FID [Quantification based on external calibration, amounts of quantified substances are given relative to the introduced amount of substrate (C10), <1 means that substances could be detected but quantification was not possible due to the operation limits of the GC-FID].

In contrast to the variants shown in Table 4, the products of the variants in Table 5 could be quantified.

**Table 5:** Decanoic acid (C10) hydroxylation results of MP variants, [pet: pET28a(+), pdb: pDB-HisGST, Subs: recovered substrate (recovered decanoic acid), Lac: δ-decalactone, α – ω-1: Hydroxylated decanoic acid with hydroxy position indicated by the Greek letter, variants marked with an Asterisk had lower than 4 µM initial enzyme concentration (Table 3)].

| | | **Product distribution** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Variant | Subs [%] | Lac [%] | α [%] | β [%] | γ [%] | δ [%] | ε [%] | ω-3 [%] | ω-2 [%] | ω-1 [%] |
| wtpdb | 94 | 9.2 | 6.0 | 12.9 | 8.6 | 27.0 | 16.2 | 8.2 | 6.9 | 5.0 |
| hp1pet | 99 | 6.0 | 7.2 | 14.9 | 10.6 | 23.4 | 12.4 | 7.5 | 5.5 | 12.5 |
| hp2pet | 100 | 8.9 | 4.7 | 3.4 | 12.9 | 31.1 | 21.5 | 7.7 | 5.6 | 4.2 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| hp3pet | 99 | 6.9 | 0 | 14.4 | 12.5 | 25.1 | 14.1 | 7.3 | 6.0 | 13.7 |
| h1pet | 100 | 8.2 | 2.3 | 15.8 | 12.2 | 33.3 | 11.0 | 3.0 | 7.1 | 7.1 |
| h3pet* | 97 | 0 | 0 | 30.9 | 8.1 | 9.0 | 5.8 | 6.3 | 35.4 | 4.5 |
| h4pet* | 93 | 9.4 | 7.5 | 6.6 | 15.9 | 33.9 | 9.3 | 5.2 | 4.2 | 8.0 |
| h5pet* | 92 | 0 | 0 | 34.2 | 15.7 | 15.2 | 7.3 | 0 | 0 | 27.5 |
| h6pet | 95 | 0 | 19.4 | 7.9 | 11.8 | 0 | 0 | 15.6 | 36.3 | 9.0 |
| hp2pdb | 88 | 5.7 | 43.4 | 1.7 | 7.3 | 17.9 | 13.4 | 4.9 | 3.6 | 2.1 |
| hp3pdb | 94 | 7.8 | 13.3 | 2.8 | 12.4 | 28.3 | 19.2 | 7.4 | 5.2 | 3.5 |
| h3pdb | 99 | 6.5 | 2.9 | 17.1 | 13.7 | 27.7 | 7.8 | 7.8 | 3.1 | 13.4 |
| h5pdb | 92 | 13.8 | 2.9 | 4.9 | 12.2 | 34.7 | 15.8 | 6.0 | 5.5 | 4.2 |

Reaction conditions: 1 mL sample with decanoic acid (C10, 10 mM), DMSO (5% v/v), 4 µM purified enzyme added as solution in buffer (KPi 0.1 M, pH 7.4). To this 1 mL sample with described initial concentrations, 860 µL $H_2O_2$ (0.04 M in KPi) were added over 8.6 h *via* a syringe pump. Reactions were carried out at room temperature 21-27 °C and analyzed on GC-FID [Quantification based on external calibration, amount of recovered substrate is given relative to the introduced amount of substrate (C10)].

The data in Tables 4 and 5 show that the investigated $P450_{MP}$ variants hydroxylate decanoic acids in nearly all possible positions, except the terminal carbon. In addition to the broad product distribution, the formed amounts of the different hydroxy-decanoic acids were low. The most active variant was variant hp2-pDB-HisGST with a conversion of 12% (TTN = 300). In terms of product distribution, δ-hydroxy-decanoic acid appeared to be the most abundant product, formed in conversions of decanoic acid with most $P450_{MP}$ variants. Whenever δ-hydroxy-decanoic acid was found in the product mixtures, also C10-δ-lactone was detected. Variant hp2-pDB-HisGST was not only the most active variant, but showed also a somewhat different product distribution. This variant predominantly produced α-hydroxylated decanoic acid with a product share of 43.4%, which was the highest selectivity for a certain product achieved within all $P450_{MP}$ enzyme variants. The biotransformations of variants h3-pET28a and h6-pET28a gave 7-hydroxy-decanoic acid as main product (product shares of 35.4% and 36.3%). It can be expected that also the variants that did not show the full portfolio of possible hydroxylation products, share this broad activity but the produced concentrations were too low for quantification due to the low enzyme concentrations (Table 3).

## 4.2 Investigation of $P450_{Bs\beta}$ enzyme variants

### 4.2.1 Results of single $P450_{Bs\beta}$ variants

To alter the regioselectivity of hydroxylations catalyzed by $P450_{Bs\beta}$, this enzyme was modified by site directed mutagenesis. The introduced mutations were intended to change the cavity size of the active site. In Figure 10 the amino acids in the active site of $P450_{Bs\beta}$, which were subjected to mutations, are highlighted.

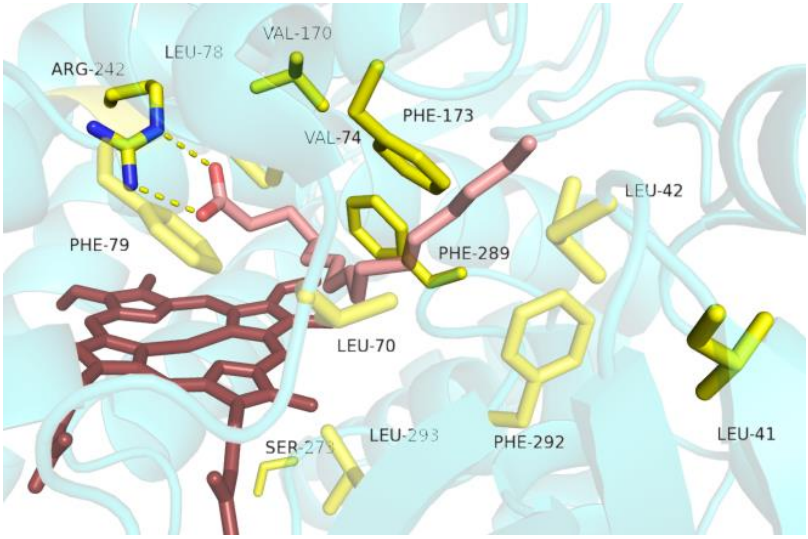



**Figure 10:** Active site of $P450_{Bs\beta}$ (PDB code: 1IZO) with C16 bound as substrate. Amino acids subjected to mutagenesis are highlighted in yellow. The heme cofactor is shown in red-brown.[62]

Some interesting results of the C10 based screening of $P450_{Bs\beta}$ singlevariants are shown in table 6.

**Table 6:** Decanoic acid hydroxylation results of $P450_{Bs\beta}$ single variants, **α**: α-hydroxy-decanoic acid, **ß**: ß-hydroxy-decanoic acid.[62]

| Mutation | Conv. [%] | **α + ß** [%] | β/α-OH |
|---|---|---|---|
| wt | 98 | 59 | 0.78 |
| L41A | 93 | 55 | 0.89 |
| L42A | >99 | 61 | 0.90 |
| L70A | 97 | 65 | 0.33 |
| L78A | 61 | 27 | 2.00 |
| F79A | 74 | 41 | 0.37 |
| F292A | 98 | 60 | 1.13 |
| L41F | 99 | 63 | 0.95 |
| L42F | 95 | 67 | 0.91 |
| L78F | 64 | 36 | 1.72 |
| L78V | 30 | 6 | 1.61 |
| L78I | >99 | 54 | 1.83 |

| | | | |
|---|---|---|---|
| L78W | 2 | 6 | 1.01 |
| F79V | 86 | 57 | 0.21 |
| F79L | 80 | 58 | 0.21 |
| F79I | 57 | 37 | 0.20 |
| F79W | 5 | 9 | 0.52 |
| V170F | 32 | 13 | 2.23 |
| S273- | 98 | 63 | 0.76 |

Reaction conditions: 10 mM C10, 5% v/v EtOH, CFE (10 µM P450 enzyme), KPi buffer (0.1 M, pH = 7.4), 0.05 U CamAB, 0.2 mM NADH, ≥ 2 U FDH, 0.1 M ammonium-formiate, 20-27 °C, 20 h, α- and ß-hydroxy decanoic acid were quantified *via* external calibration, Conversion was based on substrate recovery.

Variants L78A, L78F, L78I and V170F gave the highest ß/α selectivities (2.00, 1.72, 1.83 and 2.23 respectively) in terms of decanoic acid hydroxylation within this previous screening stage. However these somewhat more regioselective variants suffer from low decanoic acid conversions, except variant L78I, for which a conversion of 99 % could be observed. Variant V170F in contrast gave a conversion of only 32 %. Screening of the $P450_{Bs\beta}$ single variants was performed by Lucas Hammerer prior to this thesis.

### 4.2.2 Cloning of double and triple $P450_{Bs\beta}$ variants

Based on the screening results of $P450_{Bs\beta}$ single variants, $P450_{Bs\beta}$ double variants were prepared. For these double variants, mutations which turned out to be advantageous in the first screening round, were combined. The double variants were generated by site directed mutagenesis, as it is described in chapter 6.6. For the production of double variants, the already existing single variants of the $P450_{Bs\beta}$ enzyme were used as templates. Besides the mainly targeted double variants, also triple variants were produced. The exact mutation patterns of these triple variants were chosen based on the screening results of double and single (chapter 4.2.1) variants. All generated variants are summarized in Table 7.

**Table 7:** $P450_{Bs\beta}$ variants for the regioselective hydroxylation of decanoic acid ($C_{10}$).

| Variant | AA Exchange | Variant | AA Exchange |
|---|---|---|---|
| RS | F292A, L293A | IIK | L42F, V170F |
| RK | F292A, V170F | ER | L78A, F292A |
| AII | L41A, L42F | ES | L78A, L293A |
| AG | L41A, L78F | EK | L78A, V170F |
| A6 | L41A, L78I | 6R | L78I, F292A |
| AS | L41A, L293A | 6S | L78I, L293A |
| AK | L41A, V170F | 6K | L78I, V170F |
| IR | L41F, F292A | ABR | L41A, L42A, F292A |
| IB | L41F, L42A | ABZ | L41A, L42A, S273- |
| I II | L41F, L42F | AGZ | L41A, L78F, S273- |
| IE | L41F, L78A | BGZ | L42A, L78F, S273- |
| IG | L41F, L78F | B6S | L42A, L78I, L293A |
| I6 | L41F, L78I | BZR | L42A, S273-, F292A |
| IS | L41F, L293A | I II6 | L41F, L42F, L78I |
| IZ | L41F, S273- | I6B | L41F, L78I, L42A |
| IK | L41F, V170F | I6R | L41F, L78I, F292A |
| BE | L42A, L78A | IIEK | L42F, L78A, V170F |
| B6 | L42A, L78I | II6R | L42F, L78I, F292A |
| BS | L42A, L293A | II6A | L42F, L78I, L41A |
| BK | L42A, V170F | II6K | L42F, L78I, V170F |
| IIR | L42F, F292A | GZR | L78F, S273-, F292A |
| IIE | L42F, L78A | 6RB | L78I, F292A, L42A |
| IIG | L42F, L78F | 6RS | L78I, F292A, L293A |
| II6 | L42F, L78I | ZB6 | S273-, L42A, L78I |
| IIS | L42F, L293A | ZGI | S273-, L78F, L41F |
| IIZ | L42F, S273- | ZGII | S273-, L78F, L42F |

The mutations were introduced by Quickchange PCR using the $P450_{Bs\beta}$ coding gene cloned into a pET28a vector as template. The produced variants were amplified in XL10 Gold cells and then transformed into *E. coli* BL21(DE3) cells, which acted as host cells for the enzyme expression.

### 4.2.3 Expression of P450$_{Bsß}$ variants

The *E. coli* cells containing the P450$_{Bsß}$ coding genes were cultivated in LB media. SDS gels were prepared to check for successful soluble expression (Figure 11).

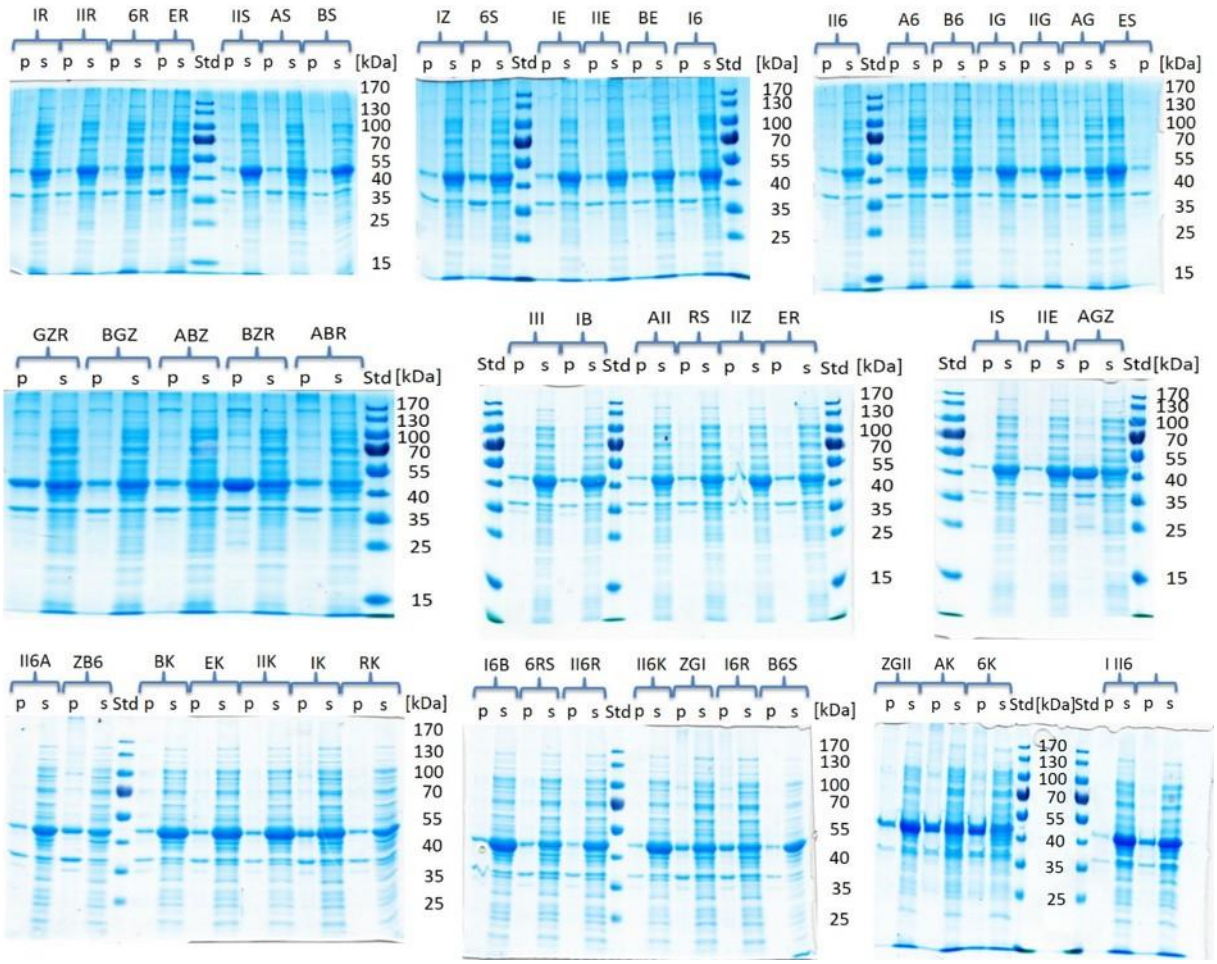



**Figure 11:** SDS gels of expressed Bsß variants (p: pellet fraction, s: soluble fraction, Std: protein ladder, P450$_{Bsß}$ variants: 50 kDa).

SDS PAGE results confirmed that all generated P450$_{Bsß}$ variants were expressed successfully in soluble form (size: 50 kDa).

### 4.2.4 Screening of P450$_{Bsß}$ double variants

The generated P450$_{Bsß}$ double variants were screened for the hydroxylation of decanoic acid (C10) in the monooxygenase mode, as described in chapter 6.14. The results of this screening are depicted in Figure 12.

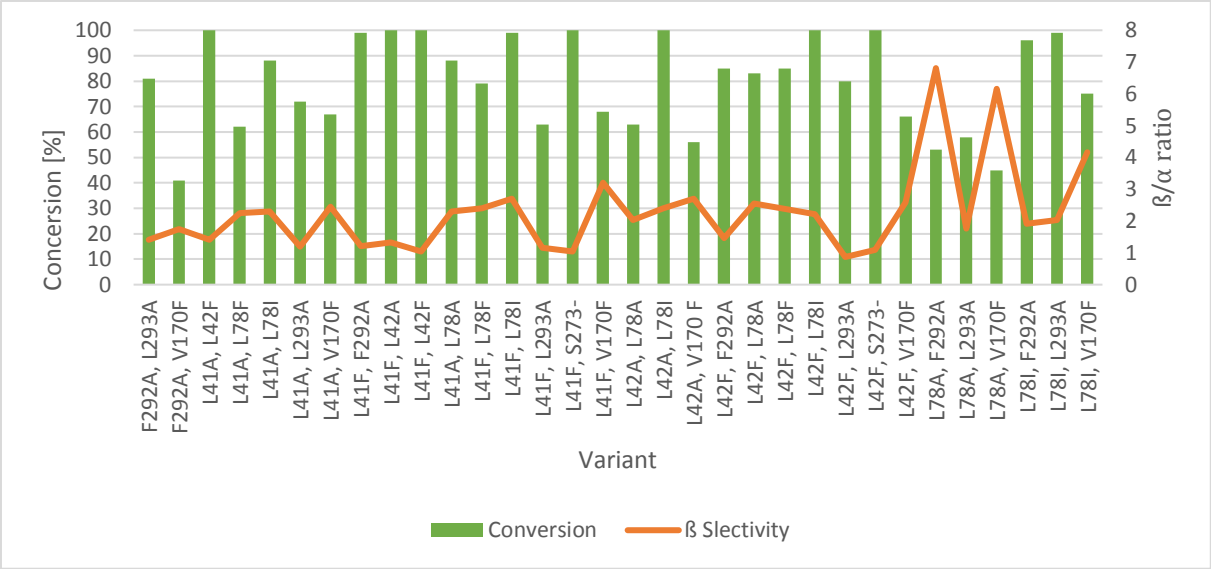



**Figure 12:** Screening results of $P450_{Bs\beta}$ double variants (orange line: ß/α ratio; green columns: conversion based on substrate recovery).

Figure 12 shows that variants with high selectivities gave low conversions, whereas variants with > 99% conversion gave relatively low ß/α ratios. The detailed results of the $P450_{Bs\beta}$ double variant screening are summarized Table 8.

**Table 8:** Decanoic acid (C10) hydroxylation results of $P450_{Bs\beta}$ double variants.

| Variant | Substrate [%] | α-OH-product [%] | ß-OH-product [%] | ß/α ratio |
|---|---|---|---|---|
| F292A, L293A | 19 ± 4 | 9 ± 1 | 13 ± 1 | 1.41 |
| F292A, V170F | 59 ± 3 | 5 ± 0 | 9 ± 1 | 1.74 |
| L41A, L42F | 0 ± 0 | 18 ± 2 | 26 ± 4 | 1.42 |
| L41A, L78F | 38 ± 1 | 8 ± 0 | 18 ± 2 | 2.25 |
| L41A, L78I | 12 ± 2 | 6 ± 0 | 14 ± 1 | 2.30 |
| L41A, L293A | 28 ± 8 | 3 ± 1 | 4 ± 1 | 1.19 |
| L41A, V170F | 33 ± 3 | 8 ± 0 | 21 ± 0 | 2.46 |
| L41F, F292A | 1 ± 2 | 25 ± 1 | 31 ± 0 | 1.21 |
| L41F, L42A | 0 ± 0 | 18 ± 0 | 24 ± 0 | 1.33 |
| L41F, L42F | 0 ± 0 | 25 ± 1 | 26 ± 0 | 1.04 |
| L41F, L78A | 55 ± 16 | 4 ± 1 | 9 ± 1 | 2.34 |
| L41F, L78F | 21 ± 1 | 10 ± 0 | 23 ± 0 | 2.40 |
| L41F, L78I | 1 ± 0 | 11 ± 0 | 31 ± 3 | 2.70 |
| L41F, L293A | 37 ± 2 | 5 ± 2 | 5 ± 2 | 1.16 |
| L41F, S273- | 0 ± 0 | 24 ± 0 | 25 ± 1 | 1.04 |

| | | | | |
|---|---|---|---|---|
| L41F, V170F | 32 ± 1 | 8 ± 0 | 25 ± 1 | 3.20 |
| L42A, L78A | 37 ± 1 | 6 ± 0 | 11 ± 0 | 2.03 |
| L42A, L78I | 0 ± 0 | 15 ± 1 | 36 ± 1 | 2.40 |
| L42A, V170F | 44 ± 2 | 5 ± 0 | 13 ± 0 | 2.71 |
| L42F, F292A | 15 ± 2 | 15 ± 1 | 22 ± 1 | 1.47 |
| L42F, L78A | 17 ± 1 | 11 ± 0 | 28 ± 1 | 2.56 |
| L42F, L78F | 15 ± 1 | 12 ± 0 | 30 ± 0 | 2.38 |
| L42F, L78I | 0 ± 0 | 15 ± 0 | 32 ± 0 | 2.21 |
| L42F, L293A | 20 ± 17 | 20 ± 7 | 18 ± 7 | 0.87 |
| L42F, S273- | 0 ± 0 | 23 ± 1 | 25 ± 1 | 1.10 |
| L42F, V170F | 34 ± 4 | 8 ± 1 | 20 ± 1 | 2.58 |
| L78A, F292A | 47 ± 5 | 1 ± 0 | 8 ± 0 | 6.81 |
| L78A, L293A | 42 ± 1 | 5 ± 1 | 10 ± 1 | 1.77 |
| L78A, V170F | 55 ± 1 | 1 ± 0 | 8 ± 0 | 6.16 |
| L78I, F292A | 4 ± 5 | 20 ± 1 | 39 ± 1 | 1.92 |
| L78I, L293A | 1 ± 0 | 13 ± 1 | 27 ± 1 | 2.04 |
| L78I, V170F | 25 ± 0 | 8 ± 0 | 34 ± 0 | 4.16 |

Reaction conditions: decanoic acid (C10, 10 mM), 10 µM P450 enzyme added as CFE in buffer (KPi 0.1 M, pH = 7.4), EtOH (5% v/v), CamAB (0.05 U), $NH_4HCO_2$ (100 mM), FDH (≥ 2U), NADH (200 µM), 1 mL reaction volume, pH = 7.4, 21-27 °C, 20 h, reactions performed in triplicates, substances were quantified *via* external calibration, amounts of quantified substances are given relative to the amount of introduced substrate.

Within the $P450_{Bs\beta}$ double variants, variants L78A/F292A and L78A/V170F gave the highest ß-selectivity for the hydroxylation of decanoic acid (ß/α ratio of 6.81 and 6.16 respectively). However, the disadvantage of these regioselective variants was their low activity, because both variants produced only 9% (relative to introduced substrate) of hydroxylated decanoic acid (α- plus ß-regioisomer).

Variants L41F/L78F, L41F/V170F, L42A/L78I, L42A/V170F, L42F/V170F and L78I/V170F showed a good compromise between regioselectivity and activity. ß/α ratios of 2.40, 3.20, 2.40, 2.71, 2.58 and 4.26 were found in decanoic acid conversion, catalyzed by these $P450_{Bs\beta}$ variants. The respective activities of these variants (indicated by combined amount of produced α- and ß-hydroxy-decanoic acid relative to introduced substrate) are 33%, 33%, 51%, 18%, 28% and 42%. With 51% of substrate converted to hydroxy-decanoic acids (α- and ß-regioisomers) variant L42A/L78I belonged to the most active $P450_{Bs\beta}$ double variants, only matched by variants L41F/F292A, L42F/S273- and L78I/F292A. But these three variants gave

significantly lower ß/α ratios (1.21, 1.10 and 1.92 respectively) than variant L42A/L78I. From a technical point of view variant L78I/V170F is especially interesting because it converted 42% of introduced substrate to α- and ß-hydroxy-decanoic acids with a ß/α ratio of 4.16, which represents the best compromise between activity and regioselectivity.

Generally, most produced double variants gave a higher ß regioselectivity in decanoic acid hydroxylation than the $P450_{Bs\beta}$-wt enzyme (ß/α = 0.78). Only the double mutated variant L42F/L293A gave a ß/α ratio lower than 1.

Because some of the $P450_{Bs\beta}$ double variants were far more regioselective than the most regioselective single variant, it was decided to produce $P450_{Bs\beta}$ triple variants. For their preparation the already produced double mutants served as templates.

### 4.2.5 Screening of $P450_{Bs\beta}$ triple variants

The generated triple variants of $P450_{Bs\beta}$ were screened in analogy to the double variants. The corresponding results are depicted in Figure 13.

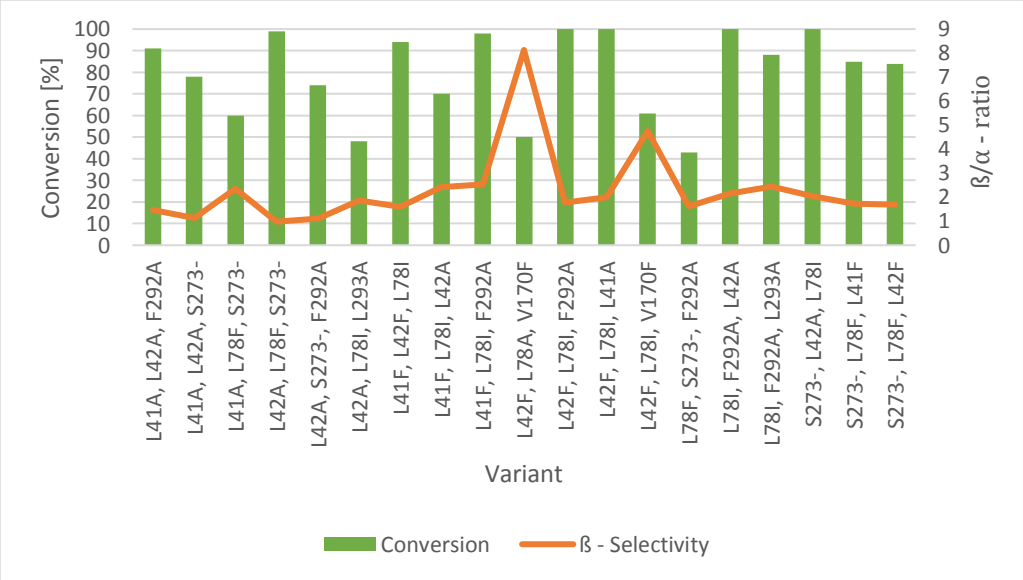



**Figure 13:** Screening results of $P450_{Bs\beta}$ triple variants (orange line: ß/α ratio; green columns: conversion based on substrate recovery).

Figure 13 shows the same basic trend as Figure 12. This means that variants with higher regioselectivity, were less active. The detailed results of the $P450_{Bs\beta}$ triple variant screening are summarized in Table 9.

**Table 9:** Decanoic acid (C10) hydroxylation results of $P450_{Bsß}$ variants.

| Variant | Substrate [%] | α-OH-product [%] | ß-OH-product [%] | ß/α ratio |
|---|---|---|---|---|
| L41A, L42A, F292A | 9 ± 1 | 20 ± 1 | 29 ± 1 | 1.47 |
| L41A, L42A, S273- | 22 ± 2 | 10 ± 1 | 11 ± 1 | 1.13 |
| L41A, L78F, S273- | 40 ± 2 | 5 ± 0 | 12 ± 0 | 2.36 |
| L42A, L78F, S273- | 1 ± 1 | 25 ± 0 | 24 ± 0 | 0.98 |
| L42A, L78I, L293A | 50 ± 1 | 3 ± 1 | 5 ± 2 | 1.80 |
| L42A, S273-, F292A | 26 ± 1 | 19 ± 1 | 21 ± 1 | 1.11 |
| L41F, L42F, L78I | 6 ± 10 | 20 ± 6 | 31 ± 8 | 1.59 |
| L41F, L78I, L42A | 30 ± 0 | 2 ± 0 | 4 ± 0 | 2.43 |
| L41F, L78I, F292A | 2 ± 0 | 13 ± 0 | 33 ± 1 | 2.53 |
| L42F, L78A, V170F | 50 ± 3 | 1 ± 0 | 11 ± 0 | 8.13 |
| L42F, L78I, F292A | 0 ± 0 | 20 ± 0 | 35 ± 0 | 1.77 |
| L42F, L78I, L41A | 0 ± 0 | 17 ± 0 | 34 ± 0 | 2.00 |
| L42F, L78I, V170F | 39 ± 1 | 5 ± 0 | 25 ± 1 | 4.75 |
| L78F, S273-, F292A | 57 ± 2 | 4 ± 0 | 7 ± 0 | 1.61 |
| L78I, F292A, L42A | 0 ± 0 | 17 ± 1 | 36 ± 1 | 2.16 |
| L78I, F292A, L293A | 43 ± 4 | 4 ± 1 | 10 ± 2 | 2.47 |
| S273-, L42A, L78I | 0 ± 0 | 17 ± 1 | 34 ± 2 | 2.05 |
| S273-, L78F, L41F | 15 ± 1 | 18 ± 0 | 31 ± 0 | 1.73 |
| S273-, L78F, L42F | 16 ± 1 | 18 ± 1 | 31 ± 2 | 1.68 |

Reaction conditions: decanoic acid (C10, 10 mM), 10 µM P450 enzyme added as CFE in buffer (KPi 0.1 M, pH = 7.4), EtOH (5% v/v), CamAB (0.05 U), $NH_4HCO_2$ (100 mM), FDH (≥ 2U), NADH (200 µM), 1 mL reaction volume, pH = 7.4, 21-27 °C, 20 h, reactions performed in triplicates, substances were quantified *via* external calibration, amounts of quantified substances are given relative to the amount of introduced substrate.

The $P450_{Bsß}$ triple variant L42F/L78A/V170F was found to be the most selective enzyme variant (ß/α = 8.13). But as most other regioselective variants, variant L42F/L78A/V170F showed low activity [12% of decanoic acid (C10) substrate converted to α- and ß-hydroxy-decanoic acid].

Variant L42F/L78I/V170F showed also a good regioselectivity for decanoic acid hydroxylations (ß/α = 4.75). Since this variant was also relatively active (30% of introduced decanoic acid were converted to α- and ß- hydroxy-decanoic acid), this variant also represents a good compromise between activity and regioselectivity. Another interesting finding is, that

except variant L78A/F292A, all variants with ß/α ratios higher than 4, contained the V170F mutation.

Within the group of t $P450_{Bsß}$ triple variants, variant L42A/S273-/F292A was the least regioselective (ß/α = 1.11). All other triple variants gave ß/α ratios between 1.5 and 2.5.

### 4.2.6 Biotransformations in the peroxygenase mode

Due to their high regioselectivity and/or activity 7 $P450_{Bsß}$ variants (L41F/V170F, L42A/L78I, L78A/V170F, L78A/F292A, L78I/F292A, L41F/L78I/F292A and L42F/L78A/V170F) were chosen to be purified and tested in the peroxygenase mode as described in chapter 6.16. To obtain reasonable amounts of enzyme, each $P450_{Bsß}$ variant was expressed in 3 x 300 mL LB medium. With the purification and buffer exchange processes, deeply red solutions of the mentioned Bsß variants in KPi buffer (0.1 M, pH = 7.4) were produced. The amounts of purified enzymes are summarized in Table 10.

**Table 10:** Obtained amounts of purified $P450_{Bsß}$ variants resulting from 3 x 300 mL LB medium.

| **BSß variant** | **m-$P450_{Bsß}$ [mg]** |
|---|---|
| L41F/V170F | 27.3 |
| L42A/L78I | 24.1 |
| L78A/F292A | 31.2 |
| L78A/V170F | 7.8 |
| L78I/F292A | 33.1 |
| L41F/L78I/F292A | 33.3 |
| L42F/L78A/V170F | 11.7 |

As shown in Table 10, very good expression was observed with variants L78A/F292A, L78I/F292A and L41F/L78I/F292A. In case of these enzyme variants, approximately 33 mg of purified enzyme could be generated. Also, variants L41F/V170F and L42A/L78I (27.3 and 24.1 mg respectively) were expressed rather well. However, in case of variants L78A/V170F and L42F/L78A/V170F a significant drop of the expression level (7.8 and 11.7 mg) compared to the previous mentioned $P450_{Bsß}$ variants could be recognized. It is also interesting that both low expressed variants included the mutations L78A and V170F in their sequences. Overall it can

be said that the $P450_{Bs\beta}$ variants were expressed much better in soluble form than the $P450_{MP}$ variants (4.1.2).

The results of the decanoic acid (C10) screenings in the peroxygenase mode, based on the mentioned purified $P450_{Bs\beta}$ variants, are summarized in Table 11.

**Table 11:** Decanoic acid (C10) hydroxylation results of $P450_{Bs\beta}$ variants in the peroxygenase mode.

| **Variant** | **Substrate [%]** | **α OH– product [%]** | **ß OH– product [%]** | **ß/α ratio** |
|---|---|---|---|---|
| L41F/V170F | 42 ± 7 | 10 ± 1 | 23 ± 1 | 2.20 |
| L42A/L78I | 1 ± 0 | 13 ± 1 | 29 ± 2 | 2.19 |
| L78A/F292A | 24 ± 6 | 14 ± 2 | 34 ± 4 | 2.35 |
| L78A/V170F | 63 ± 8 | 2 ± 0 | 12 ± 2 | 5.05 |
| L78I/F292A | 0 ± 1 | 23 ± 1 | 35 ± 1 | 1.52 |
| L41F/L78I/F292A | 0 ± 0 | 20 ± 1 | 38 ± 9 | 1.87 |
| L42F/L78A/V170F | 45 ± 4 | 4 ± 0 | 23 ± 1 | 5.39 |

Reaction conditions: decanoic acid (10 mM), 10 µM P450 enzyme added as purified enzyme in buffer (KPi 0.1 M, pH = 7.4), EtOH (5% v/v), 40 µL of 0.5 M $H_2O_2$ added over 8 h (2 equ. $H_2O_2$), 1 mL reaction volume, pH = 7.4, 21-23 °C, 20 h, reactions performed in triplicates, substances were quantified *via* external calibration, amount of quantified substance is given relative to the amount of introduced substrate (C10).

The observed regioselectivities and conversions of the enzymes, which were tested in the monooxygenase and the peroxygenase mode are summarized in Table 12. In this Table the activities of the tested enzymes are expressed by the combined yield of α and ß-hydroxy decanoic acid.

**Table 12:** Comparison of Bsß variants in monooxygenase and the peroxygenase mode.

| | Monooxygenase mode | | Peroxygenase mode | |
|---|---|---|---|---|
| **Variant** | **ß/α-ratio** | **Combined yield [%]** | **ß/α-ratio** | **Combined yield [%]** |
| L41F/V170F | 3.20 | 33 ± 1 | 2.20 | 33 ± 2 |
| L42A/L78I | 2.40 | 51 ± 2 | 2.19 | 42 ± 3 |
| L78A/F292A | 6.81 | 9 ± 0 | 2.35 | 48 ± 6 |
| L78A/V170F | 6.16 | 9 ± 0 | 5.05 | 14 ± 2 |
| L78I/F292A | 1.92 | 42 ± 0 | 1.52 | 58 ± 2 |
| L41F/L78I/F292A | 2.53 | 46 ± 1 | 1.80 | 58 ± 10 |
| L42F/L78A/V170F | 8.13 | 12 ± 0 | 5.39 | 27 ± 1 |

As shown in Table 12, regioselectivity towards ß-position of the decanoic acid hydroxylation was decreased by operating the enzymes in the peroxygenase mode. For instance variant L42F/L78A/V170F gave a ß/α ratio of 5.39 in the peroxygenase mode, which is 33% lower than the observed ß/α ratio of this enzyme variant in the monooxygenase mode (ß/α: 8.13). Variants L78A/V170F and L42A/L78I showed low reduction of their ß-selectivity by switching from monooxygenase to the peroxygenase mode and gave ß/α ratios in the peroxygenase mode of 5.05 and 2.19 respectively. In contrast variant L78A/F292A showed the lowest ß/α ratio comparing the monooxygenase to the peroxygenase mode (reduction of 65%). However with the exception of variant L78A/F292A the relative order of regioselectivities of the investigated variants in the peroxygenase mode was the same as in the monooxygenase mode. Contrary to the ß-hydroxylating-selectivities, the hydroxylating activities of the $P450_{Bsß}$ variants were generally higher in the peroxygenase mode than in the monooxygenase mode. With variant L42F/L78A/V170F the produced amount of α and ß-hydroxy decanoic acids in the peroxygenase mode is more than 2 fold higher than in the monooxygenase mode. The only variant which produced less hydroxylated decanoic acid (either in α or ß position) in the peroxygenase mode compared to the monooxygenase mode was variant L42A/L78I. Except variant L42A/L78I it can be stated that operating $P450_{Bsß}$ variants in the peroxygenase mode increases their hydroxylation activity, but decreases the regioselectivity of this hydroxylation.

### 4.2.7 NMR characterization α- and ß-hydroxy-decanoic acids

To confirm the formation of α- and ß-hydroxy-decanoic acids *via* biotransformation of decanoic acid with different $P450_{Bs\beta}$ variants, the corresponding product mixtures were characterized *via* NMR measurements. To generate the required amount of product, 20 biotransformations (20 x 1 mL) of decanoic acid were carried out in the peroxygenase mode. Each of these biotransformations was carried out in the same way as the biotransformations described in chapter 6.16. As biocatalyst $P450_{Bs\beta}$ variant L78I/F292A was chosen, due to its high conversion and a relatively good ß/α selectivity. The work up procedure of these conversions is described in chapter 6.18. Since it turned out that preparative separation of α- and ß-hydroxy-decanoic acid is hard to achieve (preparative HPLC failed), it was decided to simply record a NMR spectrum of the corresponding mixture (Figure 14).

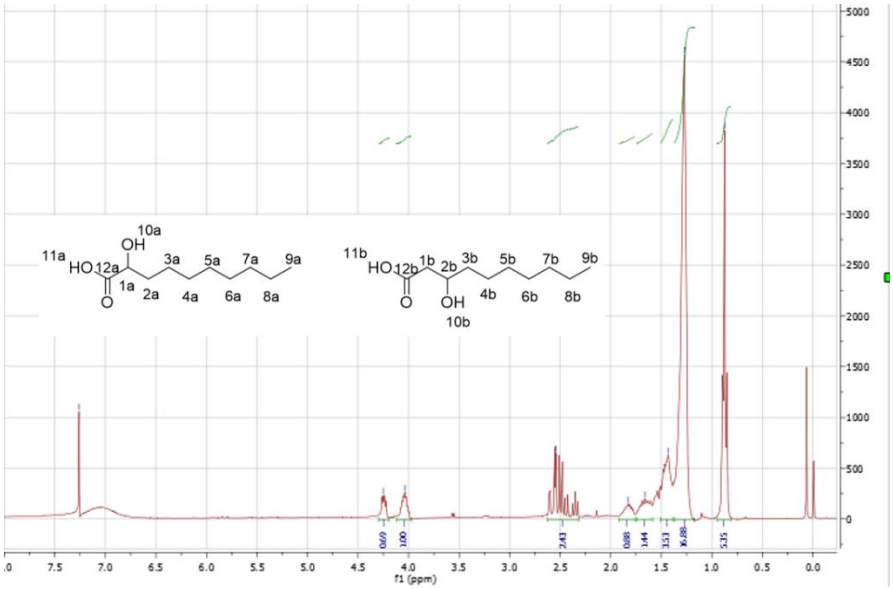



**Figure 14:** $^{1}$H-NMR of product mixture resulting from the hydroxylation of decanoic acid (C10) by $P450_{Bs\beta}$ variant L78I/F292A in the peroxygenase mode; Interpretation: 300.36 MHz, $CDCl_3$, δ = 0.87 (t, 9a, 9b), 1.26 (m, 4a, 5a, 6a, 7a, 8a, 4b, 5b, 6b, 7b, 8b), 1.44 (m, 3a, 3b), 1.64 (m, 2a), 1.81 (m, 2a), 2.47 (dd, 1b), 2.58 (dd, 1b), 4.03 (m, 2b), 4.25 (t, 1a).

The signals at 4.04 ppm and 4.25 ppm represent the hydroxymethylene protons (2b and 1a respectively) occurring in the two observed hydroxy-decanoic acid regioisomers. Thus the integral ratio of these signals delivers the ratio of the hydroxy-decanoic acid regioisomers in

the produced mixture. In fact the product ratio (ß-hydroxy-decanoic acid/α-hydroxy decanoic acid) obtained from $^{1}$H-NMR spectroscopy gave a value of 1.43, which is in good agreement with the ß/α ratio of $P450_{Bsß}$ variant L78I/F292A in the peroxygenase mode, obtained by GC-FID (ß/α = 1.52, Table 10). To better envision the parts of the $^{1}$H-NMR spectrum in Figure 16 which originate from ß-hydroxydecanoic acid, a NMR of almost pure α-hydroxy-decanoic acid was recorded.

The required α-hydroxy-decanoic acid sample was prepared in the same way as the α-hydroxy-decanoic acid – ß-hydroxy-decanoic acid mixture was prepared, except the utilized P450 enzyme. Because for the selective generation of α-hydroxy-decanoic acid from decanoic acid $P450_{CLA}$ was used as biocatalyst. This enzyme is known to perform α-hydroxylation of fatty acids in a highly regioselective fashion.[63] $P450_{CLA}$ was purified and used in biotransformations of decanoic acid analogous to $P450_{Bsß}$ variant L78I/F292A. The resulting NMR spectrum (Figure 15) originating from the $P450_{CLA}$ mediated hydroxylation is much less crowded than the NMR spectrum of the mixture and is in accordance with spectra of α-hydroxy-decanoic acid. So with the spectrum of α-hydroxy decanoic acid and literature references the formation of a α-hydroxy and ß-hydroxy-decanoic acid mixture by $P450_{Bsß}$ variants was further confirmed *via* NMR spectroscopy. The recorded $^{13}$C-NMR spectra can be found in the appendix (9.7).

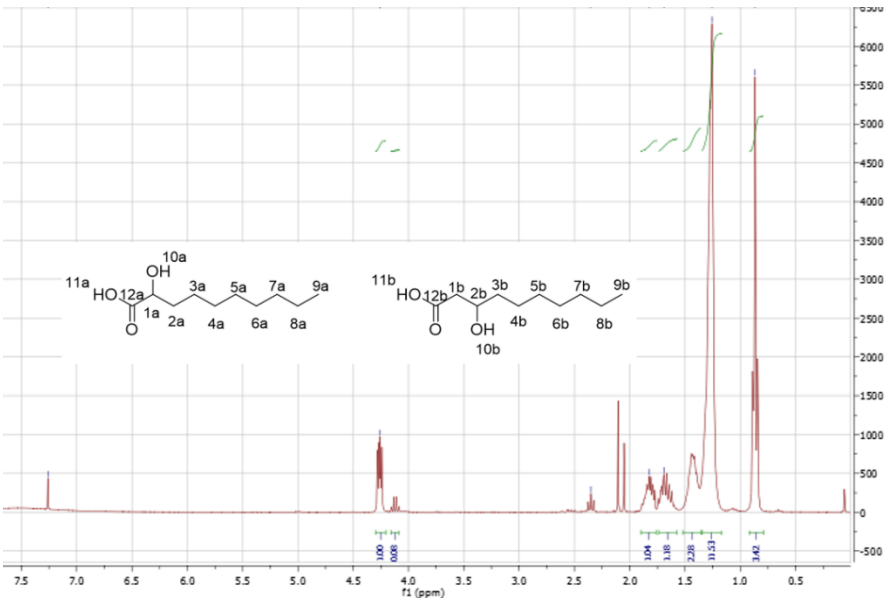



**Figure 15:** $^{1}$H-NMR spectrum of product mixture resulting from biotransformation of decanoic acid catalysed by $P450_{CLA}$ in the peroxygenase mode; Interpretation: 300.36 MHz, $CDCl_3$, δ = 0.87 (t, 9a), 1.25 (m, 4a, 5a, 6a, 7a, 8a), 1.41 (m, 3a), 1.64 (m, 2a), 1.81 (m, 2a), 4.25 (t, 1a).

# 5 Summary and Outlook

The aim of this thesis was to work on regioselective hydroxylations of decanoic acid (C10) with different P450 enzymes as biocatalysts. Initially, different $P450_{MP}$ variants generated by the PROSS server, were screened with decanoic acid (C10) as substrate for better stability and activity. All investigated $P450_{MP}$ variants gave a broad distribution of hydroxy-decanoic acid regioisomers. In addition to this low regioselectivity, the products were formed only in low amounts [probably due to the low initial enzyme concentrations (4 µM)]. Furthermore, the observed expression levels of the $P450_{MP}$ variants were low. Thus, generation of relevant amounts of purified $P450_{MP}$ was hard to achieve and required pellets obtained from 900 mL cultivation medium, as wells as large amounts of kanamycin and other additives. Therefore, the investigated $P450_{MP}$ variants are still far from application for the regioselective hydroxylation of decanoic acid.

In contrast $P450_{Bsß}$ variants were expressed successfully in soluble form at high expression levels and could be easily purified. Since α-hydroxylating peroxygenases are already known ($P450_{CLA}$, $P450_{SPα}$) the target was to shift the ß/α ratio of $P450_{Bsß}$-wt from 0.78 as far to ß-hydroxylation as possible. This should be achieved *via* targeted introduction of point mutations into the $P450_{Bsß}$-wt sequence. In this regard, an alanine scanning, and phenylalanine scanning strategy was followed and the beneficial mutations were combined to double and triple variants. The most selective $P450_{Bsß}$ variant (L42F/L78A/V170F) in terms of ß-hydroxylation gave a ß/α ratio of 8.13, which is more than 10 times higher than the ß/α ratio of the wild type enzyme, but at the cost of activity. Some variants, like L78I/V170 showed a good compromise between regioselectivity and activity (ß/α = 4.16, 42% yield). The achieved results with $P450_{Bsß}$ variants are thus already close to the goal of clean ß-hydroxylation of decanoic acid with good conversion.

Another advantage of the $P450_{Bsß}$ system for technical applications is that these enzymes can be run with cheap $H_2O_2$ as oxidant in the absence of any further redox enzymes and co-factors [NAD(P)H]. Therefore, the corresponding reaction mixtures are relatively clean, which simplifies product isolation and purification dramatically. This aspect was shown by the fact that simple extraction of the reaction mixtures was sufficient, to produce hydroxy-decanoic acids, that gave reasonable NMR-spectra. Additionally, it was found that utilization of $P450_{Bsß}$ variants in the peroxygenase mode increases the formed product yield. However, in concert with better yields, the ß/α ratio of the decanoic acid hydroxylation is decreased up to 33%.

However, to really employ $P450_{Bs\beta}$ enzyme variants in the peroxygenase mode as part of large-scale technical processes another problem has to be overcome, as in large scale enzyme purification *via* the His-tag method is not desirable from an economic point of view. A solution to this problem could be to knock out catalase coding genes in the DNA of the *E. coli* host cells used for enzyme expression.[64] In such a hypothetical case simple CFE could be used for fatty acid biotransformations in the peroxygenase mode, as no $H_2O_2$ degrading catalases would be present in the CFE. Alternatively, the enzyme may be expressed in *Pichia pastoris*, which allow excretion of the enzyme into the medium, thereby avoiding purification. So overall running $P450_{Bs\beta}$ variants in the peroxygenase mode represents a highly promising system for regioselective fatty acid hydroxylation, but some further optimizations of this system are still required.

# 6 Experimental Section

## 6.1.1 General aspects and materials

All chemicals were purchased from Sigma Aldrich, Alfa Aesar, Acros Organics, Fluka, TCI chemicals and abcr in the highest available purity. The reagents were used without further purification, unless stated otherwise.

The gel extraction kit and miniprep kit were purchased from QIAGEN. Restriction enzymes, ligase and ligase buffer were obtained from Thermo Scientific. Fast Digest buffer was purchased from Fermentas. Quickchange PCR kit came from Agilent Technologies. $P450_{MP}$ gene sequences and PCR primer were ordered from Gene Art and Eurofins Genomics.

Procedures which afforded sterile conditions were carried out either in the laminar flow station or besides a Bunsen burner flame. Pyridine for derivatization reactions was stored under argon atmosphere.

## 6.1.2 Analytical instruments

### *6.1.2.1 Nuclear Magnetic Resonance Spectroscopy*

All NMR spectra ($^{1}H$, $^{13}C$, H,H-COSY, HSQC) were recorded on a Bruker NMR unit [300 MHz ($^{1}H$), 75 MHz ($^{13}C$)] with autosampler. Chemical shifts δ [ppm] are referenced to residual protonated solvent signals as internal standard [DMSO-$d_6$: δ = 2.50 ppm ($^{1}H$), 39.52 ppm ($^{13}C$), $CDCl_3$: δ = 7.26 ppm ($^{1}H$), 77.16 ppm ($^{13}C$)]. Signal multiplicities are abbreviated as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quadruplet), m (multiplet). 2-D experiments H,H-COSY and HSQC were used for resonance assignment. Chemical shifts are given in ppm (parts per million) and coupling constants in Hz (Hertz). All $^{13}C$-experiments were $^{1}H$ decoupled for enhancing sensitivity and clarity of the obtained spectra.

### *6.1.2.2 Gas Chromatography with flame ionization detection (GC-FID)*

GC-FID measurements were performed on an Agilent Technologies 7890 (G3440A) GC system with an Agilent Technologies J&W GC-column HP-5MS [(5%-phenyl) methylpolysiloxane; length: 30 m, inner diameter: 0.250 mm; film: 0.25 µm] at a constant hydrogen flow rate with $H_2$ 5.0 as carrier gas. The sample was injected in split mode using an

Agilent Technologies 7683 Series autosampler and an Agilent Technologies 7683B Series injector. The GC was coupled to a FID detector. For all GC-FID measurements in this thesis the following temperature program was used.

**Temperature program:** 170 °C hold 3 min, ramp 1: 10 °C/min – 220 °C – hold 1 min, ramp 2: 5 °C/min - 280 °C hold 1 min, postrun 300 °C, 5 µL injection volume.

#### *6.1.2.3 Gas Chromatography with mass selective detection*

GC-MS measurements were performed on an Agilent Technologies 7890 (G3440A) GC system with an Agilent Technologies J&W GC-capillary column [(5%-phenyl) methylpolysiloxane; length: 30 m, inner diameter: 0.250 mm ; film: 0.25 µm] at a constant helium flow rate of 0.7 mL/min. The sample was injected in split mode using an Agilent Technologies 7683 Series autosampler and an Agilent Technologies 7683B Series injector (injection temperature: 250 °C, split ratio: 99/1). The GC was coupled to a MS detector (70 eV electron impact ionization, m/z 33-400 was scanned). For all GC-MS measurements in this thesis the following temperature program was used.

**Temperature program (Achiral MSD):** 100 °C hold 0.5 min, ramp 1: 10 °C/min – 300 °C.

## 6.2 DNA digestion

Synthetic gene fragments were ordered from Gene Art. All gene fragments and the vectors were digested with *NdeI* and *XhoI*. The digestion mixtures were prepared under sterile conditions. While the digestion mixtures for ordered genes were incubated 1 hour 30 minutes, digestion mixtures for the digestion of vectors were incubated for 30 minutes. Both reaction mixtures were incubated at 37 °C without mixing. The digestion mixtures were prepared according to Table 13.

**Table 13:** Digestion mixtures for gene fragments and vectors.

| Component | Digestion of vectors Volume [µL] | Digestion of gene fragments Volume [µL] |
|---|---|---|
| $H_2O$ (nuclease free) | x | x |
| Fast Digest Buffer 10x | 2 | 2 |
| 1`st restriction enzyme | 1 | 1 |
| 2`nd restriction enzyme | 1 | 1 |
| DNA solution | y (500 ng DNA) | y (200 ng DNA) |
| Total | 20 | 20 |

In the case of gene fragments, a volume containing 200 ng DNA was taken, while for vector digestions volumes containing 500 ng DNA were employed. The digestion mixture was filled up with nuclease free water to a total volume of 20 µL.

## 6.3 Agarose gel electrophoresis

After digestion, the targeted DNA fragments were separated from other components of the digestion mixtures. Therefore, agarose gel electrophoresis was performed. The agarose gel (1%) was prepared by dissolving agarose (400 mg) in TAE-buffer (40 mL) by heating in a microwave oven. After cooling the solution to about 40 °C, SYBRsafe-dye (4 µL) was added. Then the agarose solution was poured into a moult, where it cooled and solidified. For the preparation of the samples, which should be separated by the agarose gel electrophoresis, the corresponding digestion mixture (20 µL) was mixed with of MassRuler 6x loading dye (4 µL). The resulting mixture was then loaded onto the agarose gel. As DNA ladder GeneRuler$^{TM}$ DNA ladder mix

was loaded (5µL). The agarose gels were run in TAE buffer (100 V, 50 min). The separated gene fragments could be visualized under UV light. To get the pure DNA fragments, parts of the agarose gel containing the desired DNA fragments were excised, yielding gel slices which were then subjected to a gel extraction.

## 6.4 Agarose gel extraction

For the gel extraction, the gel slices were weighed and three-fold volume of buffer QG was added. The resulting mixture was incubated (10 min, 50 °C), which led to the formation of a yellow solution. Then one volume (V equals mass of gel slice; 100 mg = 100 µL) of isopropanol was added. The resulting mixture was transferred onto a spin column and centrifuged (1 min, 14600 rpm). The targeted DNA fragments were then bound to the membrane of the spin column. In order to wash the DNA fragments, PE buffer (750 µL) was added and spinned down (1 min, 14600 rpm). The membrane in the spin column was dried by centrifugation (14600 rpm, 1 min). To elute the DNA fragments nuclease free water (50 µL) was utilized and spinned down into a sterile eppendorf tube (1 min, 14600 rpm). The DNA concentrations of the resulting solutions were then determined *via* UV-VIS spectroscopy.

## 6.5 Ligation

The digested DNA fragments were ligated, furnishing a recombinant plasmid, containing the intended gene fragment, which is termed as insert DNA fragment. The ligation mixtures were prepared according to Table 14 and incubated without shaking (25 °C, 3 h). To inactivate the employed ligase, the ligation mixtures were incubated at elevated temperature (10 min, 70 °C).

**Table 14:** Composition of ligation mixtures.

| Components | Volume [µL] |
|---|---|
| $H_2O$ (nuclease free) | x |
| T4 DNA ligase Buffer 10x | 2 |
| Vector DNA solution (30 ng) | y |
| Insert DNA solution | z |
| T4 DNA ligase | 1 |
| Total | 20 |

The volume of the insert DNA solution (z) was calculated according to equation 1.

$$z = \frac{y \cdot B_{insert} \cdot f \cdot c_{plasmid}}{B_{plasmid} \cdot c_{insert}} \quad \text{equ. 1}$$

Z: Volume of insert DNA solution [µL]

B: Size of gene fragment [bp]

f: Dilution factor (5, ratio of gene and vector 5:1)

c: Concentration of DNA solutions (ng/µL)

y: Volume of vector solution [µL]

Ligated vectors were transformed into *E. coli NEB 5α* competent cells for amplification.

## 6.6 QuickChange PCR

Mutations were introduced to the $P450_{Bsß}$ wt gene fragment *via* site directed mutagenesis, which was carried out based on the QuickChange II XL Site-Directed Mutagenesis Kit from Agilent Technologies. As template a vector containing the gene fragment, which should be mutated was used. The mutagenesis reaction mixtures were prepared according to Table 15.

**Table 15:** Composition of mutagenesis reaction mixtures.

| Component | Volume [µL] |
|---|---|
| $H_2O$ (nuclease free) | x |
| Reaction Buffer 10x | 5 |
| QuickSolution | 3 |
| Primer sense (10 pmol/µL) | 1 |
| Primer antisense (10 pmol/µL) | 1 |
| dNTP mix | 1 |
| DNA solution | 0.2 |
| Pfu Ultra DNA polymerase | 1 |
| Total | 50 |

The water volume was chosen in a way to get a total reaction volume of 50 µL. In theory, a volume of DNA solution containing 20 ng DNA should be taken. After their preparation, the mixtures were mixed by pipetting and subsequently centrifuged (1 min, 14600 rpm). The samples were subjected to a temperature program in a PCR machine, which is given in Table 16.

**Table 16:** Temperature program for site directed mutagenesis of $P450_{Bs\beta}$ variants.

| Step | Temperature [°C] | Time | Cycles |
|---|---|---|---|
| Initial Denaturation | 95 | 1 min | 1 |
| Denaturation | 95 | 50 sec | 18 |
| Annealing | 60 | 50 sec | 18 |
| Extension | 68 | 6 min 40 sec | 18 |
| Final Extension | 68 | 7 min | 1 |

After the PCR was finished, unmodified templates were digested by the addition of *Dpn*I (1 µL) followed by incubation (1 h, 37 °C). To amplify DNA samples originating from site directed mutagenesis, they were transformed into XL 10- Gold cells. For this purpose XL 10-Gold cells (45 µL) were pipetted into precooled eppis. Then ß-mercaptoethanol (2 µL) was added to each sample. The resulting samples were then incubated on ice for (10 min). After this

incubation *Dpn* I treated DNA samples (2 µL) were added to each sample. Starting from this point the transformation was carried on identically to the transformation of DNA into *NEB 5α* cells (chapter 6.7).

## 6.7 Transformation of DNA fragments into *E. coli NEB 5α* cells

Vectors containing the desired DNA fragments, which were produced by basic cloning techniques (digestion followed by ligation), were transformed into *E. coli NEB 5α* cells, to amplify the targeted DNA sequences. For the transformation, commercially available *NEB 5α* cells (50 µL) were put on ice. When the competent cells were thawed, DNA solution (10 µL, deduced from ligation) was added to the *NEB 5α* cells. This mixture was then incubated for 30 minutes on ice. Afterwards the mixture was subjected to a heat shock (30 sec, 42 °C). After the samples have been cooled down on ice, S.O.C. growth medium (250 µL) was added to each sample and the cells were incubated (1 h, 37 °C, 300 rpm). Thereafter 150 µL of the samples were plated on LB agar plates (containing the corresponding antibiotics) and incubated (30 °C, over night).

## 6.8 ONC`s and Minipreps

When the *NEB 5α* or *XL 10-Gold* cells have formed colonies on the LB agar plates, overnight cultures (ONC`s) were prepared. Therefore LB medium (10 mL) was given into a falcon tube. After addition of the respective antibiotic [10 µL, kanamycin in case of pDB-HisGST and pET28(a)+ vectors], one colony of the agar plate was picked and transformed to the LB medium. The resulting ONC was incubated overnight (37 °C, 120 rpm).

To extract the amplified plasmid, Miniprep (QIAGEN) was carried out. For this purpose, cells contained in 5 mL LB medium were spinned down with a centrifuge (5 min, 14600 rpm). The resulting pellets were resuspended in P1 buffer and P2 buffer (250 µL each) were added. After inverting the samples 6 times, buffer N3 (350 µL) was added and the mixtures were again inverted for 6 times before they were spinned down in a centrifuge (10 min, 14600 rpm). The resulting supernatant was transferred into a spin column and then spinned down in a centrifuge (1 min, 14600 rpm), which bound the targeted DNA fragments to the membrane of the spin column. The DNA was then washed with PE buffer (750 µL), followed by centrifugation (1

min, 14600 rpm) to remove the PE buffer. To finally elute the targeted DNA fragments, nuclease free water (50 µL) was put into the spin columns and spinned down in the centrifuge (5 min, 14600 rpm). The DNA concentrations of the resulting solutions were determined *via* UV-VIS spectroscopy. To verify the mutation, a sample for sequencing (Microsynth) was taken (30 µL with a DNA concentration between 50 and 100 ng/µL).

## 6.9 Expression of targeted enzymes

The obtained miniprep samples were transformed into *E. coli BL21(DE3)* cells for expression. Therefore *BL21(DE3)* (100 µL) were cooled on ice. Then DNA (200-300 ng) was added. From this step the transformation procedure was analogous to the transformation of DNA into *NEB 5α* cells.

ONC`s were prepared and used for inoculation on the next day. For the expression of $P450_{MP}$ variants a modified TB medium (12 g/L yeast extract, 6 g/L tryptone and 1 g/L bactopeptone) was used. After its preparation, the modified TB medium was autoclaved overnight. To the autoclaved medium kanamycin [1‰ v/v (50 mg/mL), for variants in pDB-HisGST as well as in pET 28a vectors], P450 trace element solution[63] (1‰ v/v and a thiamine solution (1‰ v/v, 125 mg/mL) were added. Then the corresponding ONC (0.5 % v/v) was added to the cultivation media. The inoculated media were then incubated (37 °C, 120 rpm) until an OD value of 0.8 was reached (2-3 h). When the mentioned OD value was reached, 0.5‰ v/v of a 1 M 5-aminolevulinic acid (5-ALA) solution was added to each medium. Then the cell cultures were incubated (1 h, 20 °C, 120 rpm) to cool to the induction temperature. To induce protein expression 0.1‰ v/v of a 1 M IPTG solution was added. Expression was carried out overnight (20 °C, 120 rpm).

For the expression of the $P450_{Bsß}$ variants, autoclaved LB medium was used. For cell growth, kanamycin [1‰ v/v (50 mg/mL)], P450 trace element solution (1‰ v/v) and the corresponding ONC (0.5% v/v) were added to the LB media. Incubation (37 °C, 120 rpm) was used to reach an OD value of 0.8. Then a 1M 5-ALA solution (0.5‰ v/v) was added, followed by incubation (1h, 25 °C, 120 rpm). To initiate the $P450_{Bsß}$ overexpression 0.1‰ v/v of a 1 M IPTG solution were added to the medium. The expression was then carried out over night (25 °C, 120 rpm).

## 6.10 Cell Harvest and Sonication

After expression, the *E. coli BL21(DE3)* cells were harvested. The samples were centrifuged (20 min, 4000 rpm, 4 °C), furnishing a cell pellet. The remaining cultivation medium was removed and KPi buffer (0.1 M, 10 mL/g pellet) was added. The cells were resuspended in the KPi buffer by shaking for about 2 h. From the resulting suspensions samples (1 mL) were taken for SDS gels. The remaining suspension was transferred into falcon tubes, which were than subjected to centrifugation (20 min, 4600 rpm, 4 °C). Remaining buffer was finally removed from the cell pellets. The produced cell pellets were stored at -80 °C until further use.

To access the expressed enzymes, the cells were broken by sonication. For the sonication KPi buffer (0.1 M, 5 mL/g pellet) was added to each pellet. In Table 17 the used sonication program is summarized.

**Table 17:** Utilized sonication programs.

| Parameter | Program for cell pellets | Program for 1 mL sample |
|---|---|---|
| Net sonication time | 2:30 min | 15 sec |
| Amplitude | 30 % | 30 % |
| Time ON | 2 sec | 0.1 sec |
| Time OFF | 4 sec | 0.5 sec |

During the sonication process the samples were cooled with an ice water mixture. Each sample was sonicated twice, with a pause in between (2 min). After sonication the resulting suspensions were transferred into small centrifugation tubes and centrifuged (20 min, 16000 rpm, 4 °C). The obtained cell free extracts (CFE) showed a red colour (heme in P450 enzymes) and were used for reactions on the same day.

## 6.11 SDS PAGE

To analyze expression levels, SDS PAGE was performed. The SDS gels consisted of a separation and a collection part. For the production of these gels, plastic molds were set up. 80% of such a mold was filled with a precursor mixture for the separation gel [5 mL acrylamide

(30%), 5.625 mL separation buffer, 4.093 mL water, 150 µL SDS solution (10%), 120 µL of ammonium persulfate solution (APS; 20 mg/200 µL), 12 µL tetramethylethylendiamine (TEMED); volume of described mixture was intended for 4 gels]. The separation gel mixture was then covered with a layer of isopropanol. When the separation gel was formed due to radical polymerization (10 – 15 min), the isopropanol was removed and the last part of the mold was filled with a precursor mixture for the collection gel [0.833 mL acrylamide (30%), 0.625 mL separation buffer, 3.462 mL water, 50 µL SDS solution (10%), 25 µL of APS (20 mg/200 µL), 5 µL TEMED; volume of described mixture was intended for 4 gels)]. To generate slots for loading samples, a plastic comb was pressed into the collection gel before polymerization started. After full polymerization of the collection gel, the whole SDS gel was kept inside the plastic mold and stored at 4 °C until use.

As samples for the SDS gels, samples (1 mL) deduced from cell harvesting, were taken. The 1 mL samples were sonicated with the sonication program described in Table 16. After sonication the samples were centrifuged (5 min, 14600 rpm), separating cell residues in form of a pellet from the supernatant. The supernatants were transferred into extra Eppendorf tubes. To make the remaining pellets also accessible for the SDS gel, they were dissolved in an urea solution (6 M, 800 µL). Protein concentration of the supernatant was measured to identify the volume, needed for 15 µg protein. This was done *via* the Bradford method.

Based on the protein concentration, determined by the Bradford assay, the volume of supernatant, which needed to be loaded, was calculated. Usually 2 to 6 µL of supernatant were loaded per SDS pocket. Since the dissolved pellet (in 6 M urea) was assumed to have the same concentration as the supernatant, the same volume was taken for the SDS gel as well. The calculated volumes of supernatant and pellet solution were prepared in eppendorf tubes. Then the same amount of Lammeli Loading Buffer was added. The resulting mixture was spinned down in a centrifuge, followed by incubation (5 min, 90 °C) to denaturate the proteins. After this denaturation step the samples were loaded to the SDS gel. To be able to identify the masses of the expected protein bands, a protein standard (Page Ruler Prestained protein ladder, 5 µL) was loaded to the SDS gel. The SDS gel was finally run in SDS buffer (90 V). When the samples have reached the separation part of the gel, the voltage was increased to 120 V. To make the bands on the SDS gel visible they were stained overnight.

## 6.12 Bradford assay

For the Bradford assay a 1:5 diluted Bradford solution (980 µl) was prepared in a UV-VIS cuvette. Then a 1:10 dilution of the supernatant (20 µL) was added to this Bradford solution, giving a slight blue colour. The resulting mixture was measured in a UV-VIS spectrometer, giving the total protein concentration.

## 6.13 CO titration

To determine the P450 concentration in the CFE, CO titration was performed. This was done by taking advantage of the CO affinity of the iron-porphyrin complex (heme) contained in P450 enzymes. The principle of this CO titration is that binding of CO to the Fe-porphyrin complex changes the absorption spectrum of the investigated samples. Therefore UV-VIS spectroscopy could be used for deducing the P450 concentration. KPi buffer (900 µL, 0.1 M) was mixed with the CFE sample (100 µL) in a cuvette. *Via* a syringe CO was bubbled into the resulting mixture. At this moment the CO has not yet bound to the Fe-porphyrin complex. Therefore a baseline could be measured at the UV-VIS spectrometer. When this baseline was measured a spatula tip of sodium dithionite was added to the sample, which made the CO bind to the iron-porphyrin complex. Then the UV-VIS spectrum of the sample was measured. If active P450 enzyme was present in the sample, a peak at approximately 450 nm was observed. Based on the intensity of this peak the concentration of P450 in the CFE could be calculated by equation 2.

$$c = \frac{A_{450} - A_{490}}{\varepsilon} \cdot f \cdot 1000 \qquad \text{equ. 2}$$

c = P450 concentration [µmol/L]

A = absorption

ε = extinction coefficient (91 used for calculations) [L/mmol]

f = dilution factor (10)

Sometimes the measured UV-VIS spectra also showed peaks at 420 nm, representing inactive forms of the P450 enzymes.

## 6.14 Cytochrome C assay

In the monooxygenase mode, P450 enzymes require additional enzymes responsible for electron transfer processes (CamAB was used in this study). Since the CamAB activity in the screening reactions needed to stay constant, it was necessary to measure the activity of CamAB samples, which was done with the Cytochrome C assay. The assay began with the preparation of KPi buffer (0.1 M, 920 µL) in a UV-VIS cuvette. Then a Cytochrome C solution (18.4 mg/mL, 50 µL) and the investigated CamAB solution (10 µL) were added. The resulting mixture was subjected to a blank measurement in the UV-VIS spectrometer. After this blank measurement, a 10 mM NADH solution (20 µL) was added to the investigated sample. Then a kinetic measurement at the UV-VIS spectrometer was started, measuring the absorbance at 450 nm (3 min). Based on the slope of the resulting curve the activity of the CamAB solution could be calculated (equation 3).

$$a = \frac{\Delta abs}{V \cdot \varepsilon \cdot d} \qquad \text{equ. 3}$$

a = activity of CamAB [U/mL]

Δabs = Difference of absorption between time points x and x + 1 min (x: time starting from addition of NADH)

V = volume of CamAB (0.01 mL)

ε = extinction coefficient of cytochrome C [28 $(mM*cm)^{-1}$]

d = path length (1 cm)

## 6.15 Conversion of decanoic acid by $P450_{Bs\beta}$ variants

To screen the generated $P450_{Bs\beta}$ variants for activity and regioselectivity, 1 mL scale conversions of decanoic acid were performed. In these conversions the P450 enzymes were operated in the monooxygenase mode, employing a formiate based cofactor recycling system and CamAB as electron transfer enzyme (Scheme 1). The reactions were carried out in KPi buffer (0.1 M, pH 7.4). The reaction samples were prepared in 4 mL glass vials. In first hand an amount of CFE was given into the glass vial that would lead to a P450 concentration of 10

µM in the final reaction mixture (1 mL). Then the master mix (MM) was added to the samples. For the preparation of the MM, ammonium formate (6.3 mg/sample) and FDH (5 mg/sample) were dissolved in a certain amount of the mentioned KPi buffer, followed by the addition of CamAB. After addition of the MM it was necessary to add certain amounts of KPi buffer to the reaction mixtures in order to equalize differences of added enzyme volumes (due to different enzyme concentrations of the supernatants of different $P450_{BSß}$ variants). Then a solution of decanoic acid in ethanol (200 mM, 50 µL) was added to each sample. To finally start the reactions NADH solution (10 mM, 20 µL) in KPi buffer (0.1 M, pH 7.4) was added to each sample. The reaction mixtures were shaken at room temperature (20 h, 21-27 °C). Reactions were always performed in triplicates (exact reaction conditions are stated in chapter 4.2.3).

## 6.16 Purification of the $P450_{Bsß}$ variants

The $P450_{Bsß}$ variants which showed good selectivity for ß - hydroxylation were tested in the peroxygenase mode with $H_2O_2$ as oxygen source (Scheme 2). CFE could not be used because *E. coli* produce catalase, which catalyses the disproportionation of $H_2O_2$ in $H_2O$ and $O_2$. Therefore the $P450_{Bsß}$ variants were purified *via* His-tag purification.

The enzyme purification started with sonication of the cell pellets. For the sonication lysis buffer (0.1 M Kpi, 300 mM KCl, 20 mM imidazole, pH 7.4, degassed) was added to each cell pellet (10 mL buffer/ g pellet). The sonication procedure was the same as described in chapter 6.10. After sonication, the samples were centrifuged (20 min, 16000 rpm, 4 °C). The obtained supernatant was then filtrated through a syringe filter (0.45 µm), before it was subjected to the actual purification process, which started with equilibration of the purification column (5 mL, His Trap) by pumping lysis buffer (50 mL) through it. The utilized pump was operated at a velocity of 5 mL/min. After the column was cleaned, the supernatant was loaded, which changed the colour of the purification column from blue to red (heme of binding protein). The loaded column was then washed with lysis buffer (50 mL). Then the targeted enzyme was eluted with elution buffer (0.1 M KPi, 300 mM KCl, 300 mM imidazole, pH 7.4, degassed). Therefore all red droplets leaving the column were collected. When the whole P450 enzyme was eluted (column was blue again), the column was cleaned with 50 mL of cleaning buffer (0.1 M Kpi, 300 mM KCl, 500 mM imidazole, pH 7.4, degassed). If the column was used for the purification of another enzyme variant, it was washed with lysis buffer (50 mL), before the described purification sequence was started again. When the purifications were finished the column was

washed with 50 mL water (degassed) and stored in EtOH (20% v/v, degassed). Since the purified enzyme variants were dissolved in elution buffer they needed to be transferred into the reaction buffer (0.1 M KPi, pH 7.4). Therefore a buffer exchange was carried out immediately after the enzyme purification. For the buffer exchange, buffer exchange columns (PD10 Desalting Columns, GE Healthcare) were washed four times with the KPi buffer (3 mL, 0.1 M, pH 7.4), into which the enzymes should be transferred. Then the purified enzyme (dissolved in elution buffer, 2.5 mL) were loaded onto the column and finally eluted in the reaction buffer (3.5 mL). After each buffer exchange the columns were washed again four times, before they were washed and stored in 20% v/v EtOH.

## 6.17 $P450_{Bs\beta}$ catalyzed conversions of decanoic acid with $H_2O_2$ as oxidant

Reactions were carried out in glass vials (4 mL) in 1 mL scale. Preparation of the reaction mixtures started with transformation of purified enzyme to the glass vial (volume was chosen in a way, that a 10 µM P450 concentration was achieved in the final reaction mixture). Differences of the used enzyme volumes were equalized with KPi buffer (0.1 M, pH = 7.4). Finally a solution of decanoic acid in ethanol (200 mM, 50 µL) was added, to achieve a substrate concentration of 10 mM in the reaction mixture, with 5 % v/v ethanol as co solvent. To start the reactions, a $H_2O_2$ solution (500 mM, 40 µL) was added to each sample over 8 hours (5 µL/h) *via* a syringe pump. In total 2 equivalents of $H_2O_2$ were added to the reaction mixtures. The samples were shaken for a total time of 20 hours at room temperature (21-27 °C) and each reaction was carried out in triplicates.

## 6.18 Purification of $P450_{MP}$ variants

Since the hydroxylating activity of the expressed $P450_{MP}$ variants towards decanoic acid should be tested in the peroxygenase mode, these enzymes were purified. This was again done by His-tag purification. Depending on the host vector (pET28a or pDB-HisGST) into which the different MP gene sequences were cloned, different buffer systems were employed for purification. In case of $P450_{MP}$ sequences cloned into pET28a vectors, the whole purification, including buffer exchange, was carried out analogously to the purification of $P450_{Bs\beta}$ variants (chapter 6.15).

The $P450_{MP}$ sequences cloned into pDB-HisGST vectors, expressed $P450_{MP}$ enzyme variants which were equipped with a GST tag. This GST tag increases the solubility of the $P450_{MP}$-variants, when a detergent like sodium-cholate is present in the solvent. Thus the buffers for the purification of $P450_{MP}$ variants with GST tag contained sodium cholate (0.8% w/v). Beside this difference to the usual purification buffers, the $P450_{MP}$ purification buffers also contained glycerol and NaCl (substituting KCl). The purification of $P450_{MP}$ variants with GST tag itself started similar to the already described purification processes. Thus MP-lysis buffer (0.05 M Kpi, 100 mM NaCl, 0.8% (w/v) sodium-cholate, 15% (v/v) glycerol, 10 mM imidazole, pH 7.4, deagassed/ 10 mL/g) was added to the corresponding cell pellets in first hand (3 cell pellets were used per variant). After sonication the suspensions were centrifuged (16000 rpm, 20 min, 4 °C). Then the resulting supernatants (~ 60 mL/enzyme variant) were filtrated. The His-tag column was washed with MP-lysis buffer (50 mL), followed by loading the supernatant fraction. Thereafter the loaded enzyme was washed with MP-washing buffer [0.05 M Kpi, 100 mM NaCl, 0.8% (w/v) sodium-cholate, 15% (v/v) glycerol, 30 mM imidazole, pH 7.4, deagassed /50 mL]. For elution of the targeted enzyme approximately 10 mL of MP-elution buffer [0.05 M Kpi, 100 mM NaCl, 0.8% (w/v) sodium-cholate, 15% (v/v) glycerol, 250 mM imidazole, pH = 7.4, deagassed / 50 mL] were used. Cleaning of the column was done with the usual cleaning buffer. Buffer exchange was performed as described in chapter 6.16. It is important to note that the final buffer into which the purified enzymes with GST tag were brought was simple KPi buffer (0.1 M, pH = 7.4) without any additives like sodium-cholate. This was done in order to reduce the complexity of the investigated system. And in fact only the enzyme variant resulting from MP-hp1-pDB-HisGST precipitated in the described buffer system. All other MP variants with GST tag appeared to be soluble in KPi buffer.

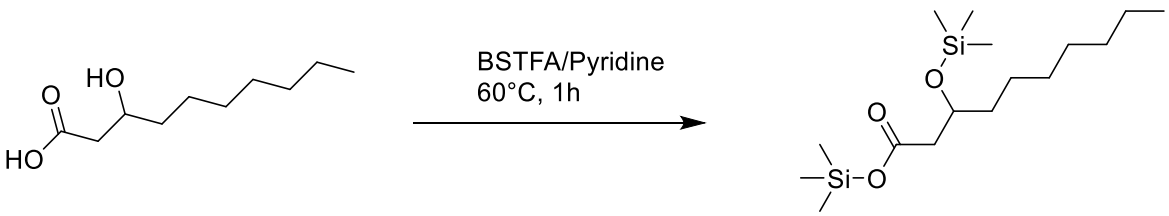
## 6.19 Conversions of decanoic acid with MP variants

$P450_{MP}$ variants were tested in the peroxygenase mode. $H_2O_2$ (3.44 eq) was therefore used as oxidizing agent, and was added in form of a 0.04 M solution *via* a syringe pump (860 µL with 100 µL/h) to a solution (1 mL) containing the investigated MP variant plus substrate. For the production of this `initial` solution an amount of purified enzyme was given into a 4 mL glass vial, which would lead to a P450 concentration of 4 µM in the initial solution (solution prior to addition of the $H_2O_2$ solution, 1 mL). Then the KPi buffer (0.1 M, pH 7.4) was added to give a volume of 950 µL, before a decanoic acid solution (200 mM in DMSO, 50 µL) was added. This

led to a final substrate concentration of 10 mM with 5% v/v DMSO in the initial samples. After completion of the initial samples the described amount of $H_2O_2$ solution was added to the sample *via* a syringe pump. Reactions were carried out at room temperature (21-27 °C) under constant shaking for a total time of 15 h.

## 6.20 GC analysis

Regioselectivity and activity of the investigated enzyme variants were analyzed by gas chromatography (GC). To perform GC measurements, unconverted substrate as well as its hydroxylated forms needed to be extracted from the reaction mixture and derivatized. Conversions with $P450_{Bs\beta}$ variants were stopped after 20 hours by the addition of hydrochloric acid (5 M, 100 µL). Then the extraction was performed by adding ethyl acetate (400 µL) to the samples. The utilized ethyl acetate contained 12-hydroxydodecanoic acid as internal standard (3.45 mM). The resulting mixtures were mixed thoroughly before they were subjected to centrifugation (5 min, 14600 rpm) to separate the phases, followed by separation of the organic phase with a pipette. The remaining aqueous phase was extracted again with ethyl acetate (400 µL, containing internal standard). The combined organic phases were dried over $Na_2SO_4$, before 100 µL of each organic phase were transferred into new eppis. In these eppis a 1:1 pyridine-BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide) mixture (200 µL) was added to the samples. Incubation of the resulting mixtures (1 h, 60 °C, 650 rpm) furnished the derivatization of hydroxyl groups and carboxyl groups to the respective trimethylsilyl-ethers and -esters. In Scheme 15 this derivatization is shown for ß-hydroxy-decanoic acid.




**Scheme 15:** Derivatization of ß-hydroxy-decanoic acid.

Due to the low yields observed in $P450_{MP}$ catalyzed hydroxylations, these conversions were subjected to a different work up procedure. For this work up the completed, $P450_{MP}$ mediated conversions, (1860 µl after $H_2O_2$ addition), were divided into two parts with 850 µL each. Both

parts originating from one sample were quenched *via* addition of HCl (5 M, 100 µL). Then both parts were extracted with ethyl acetate [2 x 400 µL, containing 12-hydroxy-dodecanoic acid (3.45 mM)]. The combined organic phases of the two parts originating from one sample were combined and dried over $Na_2SO_4$. In order to increase the signal intensities in GC, the solvent of the combined and dried organic phases was removed under a pressurized air stream. The resulting residues were then dissolved in a 1:1 BSTFA/pyridine mixture (100 µL) and incubated (1 h, 60 °C, 650 rpm).

After completed derivatization the samples were transferred to GC vials and analyzed on GC-FID. For data analysis, the peak areas of unconverted substrate and hydroxylated decanoic acids (mostly α and ß hydroxydecanoic acids) were normalized by using the peak area of the internal standard. The measurements were made on the HP5 column with the temperature program described in chapter 6.1.2.2. External calibration was used for quantification (appendix). Calibration for hydroxy-decanoic acid was made with ω-hydroxy decanoic acid, which was commercially available and assumed to give the same response factor on GC-FID as the other hydroxy-decanoic acid regioisomers. The observed retention times of all analyzed products are shown in Table 18.

**Table 18:** Retention times of investigated substance on GC-FID with system parameters described in chapter 6.1.2.

| Compound | Retention time [min] | Compound | Retention time [min] |
|---|---|---|---|
| Decanoic acid | 3.0 | ε-OH-decanoic acid | 4.9 |
| 12-OH-dodecanoic acid | 8.5 | ω-3-OH-decanoic acid | 5.1 |
| α-OH-decanoic acid | 4.4 | ω-2-OH-decanoic acid | 5.3 |
| ß-OH-decanoic acid | 4.6 | ω-1-OH-decanoic acid | 5.5 |
| γ-OH-decanoic acid | 4.7 | C10-δ-lactone | 3.4 |
| δ-OH-decanoic acid | 4.8 | | |

## 6.21 Isolation of α- and ß-hydroxy decanoic acid for NMR characterization

For the isolation of α- and ß-hydroxy-decanoic acid mixture or α-hydroxy-decanoic acid (depending on the utilized enzyme) in relevant amounts for NMR-characterization, 20 samples

were run in parallel as described in chapter 6.17. For the work up all samples were united in a falcon tube. Then HCl (5 M, 2 mL) was added to quench the reaction mixtures. The targeted products were extracted with ethyl acetate (2 x 14 mL). The united organic phases were dried over $Na_2SO_4$, followed by removal of the solvent on a rotary evaporator, yielding clear oils, that crystallized over time. The hydroxy-decanoic acids generated in this way were dissolved in $CDCl_3$ with 1% v/v tetrammethylsilsane (TMS) and subjected to NMR measurements.

# 7 Acknowledgement

Nach gut einem Jahr, in dem ich mal mehr, mal weniger intensiv an meiner Masterarbeit gearbeitet habe, habe ich nun den Punkt erreicht, an dem ich all denjenigen die mich während und vor dieser Zeit unterstützt haben, danken kann. Dazu möchte ich mich zu allererst bei Prof. Wolfgang Kroutil bedanken, der mir die Möglichkeit gegeben hat meine Masterarbeit in seiner Arbeitsgruppe zu schreiben. Auch für die rasche und unkomplizierte Korrektur meiner Arbeit möchte ich mich an dieser Stelle gleich bedanken.

Ein besonderes Dankeschön gilt meinem Betreuer Lucas Hammerer. Lieber Lucas, ich bin der Meinung, dass ich von dir sehr vieles lernen konnte, das mir bei meinen zukünftigen Aufgaben helfen wird. Du warst stets bemüht, mich so gut wie möglich während meiner Masterarbeit zu unterstützen. Gleichzeitig hast du es mir aber auch immer mitgeteilt, wenn ich etwas falsch gemacht habe. Dies geschah aus meiner Sicht aber immer auf einer korrekten Art und Weise, weshalb ich aus meinen Fehlern immer auch lernen konnte. Bedanken möchte ich mich weiters auch für deine aufmunternden Worte in den besonders arbeitsintensiven Phasen meiner Arbeit.

Natürlich gab es neben Lucas auch viele andere Personen in der Arbeitsgruppe denen ich hiermit danken möchte. Zu aller erst wäre da Tamara, die mir bei vielen Problemen im Laboralltag rasch und unkompliziert weiterhelfen konnte. Auch bei Christoph Winkler möchte ich mich hiermit explizit bedanken. Er stand mir nicht nur bei wissenschaftlichen Fragen zur Verfügung, sondern nahm sich auch die Zeit um über meine Arbeit zu sehen. An dieser Stelle könnte ich noch weit mehr Personen aus der Arbeitsgruppe persönlich nennen, aber letztendlich möchte ich mich so und so bei allen in der Gruppe recht herzlich bedanken. Und dies nicht nur für die rasche und unkomplizierte Hilfe bei Fragen meinerseits, sondern auch für die tolle Arbeitsatmosphäre. Ich glaube, dass der Zusammenhalt und die positive Umgangsweise in dieser Arbeitsgruppe etwas ganz Besonderes ist.

Abschließend möchte ich noch meinen Eltern danken, die mich bis jetzt in jeder Situation bedingungslos unterstützt haben.

# 8 Abbreviations

| | |
|---|---|
| $^{13}$C-NMR | carbon NMR |
| $^{1}$H-NMR | proton NMR |
| $C_q$ | quartonary carbon |
| d | doublet |
| dd | double doublet |
| eV | electron Volt |
| GC | gas chromatography |
| GC-MS | gas chromatography with mass spectrometry |
| H,H-COSY | H,H correlated spectroscopy |
| HSQC | hetero single quantum coherence |
| H | Hertz |
| *J* | coupling constant |
| m | multiplet |
| m/z | mass to charge ratio |
| MHz | mega Hertz |
| NMR | nuclear magnetic resonance |
| ppm | chemical shift |
| PCR | polymerase chain reaction |
| s | singlet |
| SDS | sodium dodecyl sulfate |
| $t_r$ | retention time |
| UV | ultra violet |
| δ | chemical shift or indication of regiochemistry |
| % | percentage |
| ° C | degree Celsius |
| µL | microliter |
| cm | centimetre |
| eq | equivalent |
| g | gram |
| h | hours |
| M | molar |

| | |
|---|---|
| mM | milli molar |
| mg | milligram |
| µg | micro gram |
| min | minute |
| mL | millilitre |
| mmol | millimol |
| nm | nanometre |
| % w/w | percentage per weight |

# 9 Appendix

## 9.1 Sequences – $P450_{Bs\beta}$

### 9.1.1 Proteine Sequence – $P450_{Bs\beta}$

MNEQIPHDKSLDNSLTLLKEGYLFIKNRTERYNSDLFQARLLGKNFICMTGAEAAKVFYDTDRFQRQN
ALPKRVQKSLFGVNAIQGMDGSAHIHRKMLFLSLMTPPHQKRLAELMTEEWKAAVTRWEKADEVVLFE
EAKEILCRVACYWAGVPLKETEVKERADDFIDMVDAFGAVGPRHWKGRRARPRAEEWIEVMIEDARAG
LLKTTSGTALHEMAFHTQEDGSQLDSRMAAIELINVLRPIVAISYFLVFSALALHEHPKYKEWLRSGN
SREREMFVQEVRRYYPFGPFLGALVKKDFVWNNCEFKKGTSVLLDLYGTNHDPRLWDHPDEFRPERFA
EREENLFDMIPQGGGHAEKGHRCPGEGITIEVMKASLDFLVHQIEYDVPEQSLHYSLARMPSLPESGF
VMSGIRRKS

### 9.1.2 Nucleotide Sequence – $P450_{Bs\beta}$

ATGAATGAGCAGATCCCGCATGATAAAAGCCTGGATAATAGCCTGACCCTGCTGAAAGAAGGTTACCT
GTTTATCAAAAATCGCACCGAACGTTATAACAGCGACCTGTTTCAGGCACGTCTGCTGGGTAAAAACT
TTATTTGTATGACCGGTGCAGAAGCAGCCAAAGTTTTTTATGATACCGATCGTTTTCAGCGTCAGAAT
GCACTGCCGAAACGTGTTCAGAAAAGCCTGTTTGGTGTTAATGCAATTCAGGGTATGGATGGTAGCGC
ACATATTCATCGTAAAATGCTGTTTCTGAGCCTGATGACCCCTCCGCATCAGAAACGTCTGGCAGAAC
TGATGACCGAAGAATGGAAAGCAGCAGTTACCCGTTGGGAAAAAGCAGATGAAGTTGTTCTGTTTGAA
GAGGCCAAAGAAATTCTGTGTCGTGTTGCATGTTATTGGGCAGGCGTTCCTCTGAAAGAAACCGAAGT
TAAAGAACGTGCCGATGATTTTATCGATATGGTTGATGCATTTGGTGCAGTTGGTCCGCGTCATTGGA
AAGGTCGTCGTGCACGTCCGCGTGCAGAAGAGTGGATTGAAGTTATGATTGAAGATGCACGTGCCGGT
CTGCTGAAAACCACCAGTGGCACCGCACTGCATGAAATGGCGTTTCATACCCAAGAAGATGGTAGCCA
GCTGGATAGCCGTATGGCAGCAATTGAACTGATTAATGTTCTGCGTCCGATTGTGGCCATTAGCTATT
TTCTGGTTTTTAGCGCACTGGCCCTGCATGAACATCCGAAATACAAAGAATGGCTGCGTAGCGGTAAT
AGCCGTGAACGTGAAATGTTTGTTCAAGAAGTGCGTCGTTATTATCCGTTTGGTCCGTTTCTGGGTGC
ACTGGTTAAAAAAGATTTTGTGTGGAACAACTGCGAATTCAAAAAAGGCACCAGCGTTCTGCTGGATC
TGTATGGCACCAATCATGATCCGCGTCTGTGGGATCATCCTGATGAATTTCGTCCGGAACGTTTTGCA
GAACGTGAAGAAAACCTGTTCGATATGATTCCGCAGGGTGGTGGTCATGCAGAAAAAGGTCATCGTTG
TCCGGGTGAAGGTATTACCATTGAAGTGATGAAAGCCAGCCTGGATTTCCTGGTTCATCAGATTGAAT
ATGATGTGCCGGAACAGAGCCTGCATTATAGTCTGGCACGTATGCCGAGCCTGCCGGAAAGCGGTTTT
GTTATGAGCGGTATTCGTCGCAAAAGCTGA

## 9.2 Sequences – $P450_{MP}$

### 9.2.1 Proteine Sequence – $P450_{MP}$

MPAAIATHRFRKARTLPREPAPDSTLALLREGYGFIRNRCRRHDSDLFAARLLLSPVICMSGAEAARH
FYDGHRFTRRHALPPTSFALIQDHGSVMVLDGAAHLARKAMFLSLVGEEALQRLAGLAERHWREAVSG
WARKDTVVLLDEAHRVLTAAVCEWVGLPLGPTEVDARAREFAAMIDGTGAVGPRNWRGHLYRARTERW
VRKVIDEIRSGRRDVPPGAARTIAEHQDADGQRLDRTVAGVELINVLRPTVANARYIVFAAMALHDHP
HQRAALADGGEAAERFTDEVRRFYPFIPFIGGRVRAPFHFGGHDFREGEWVLMDLYGTNRDPRLWHEP
ERFDPDRFARETIDPFNMVSHGAGSARDGHRCPGEGITRILLRTLSRQLAATRYTVPPQDLTLDLAHV
PARPRSGFVMRAVHAP

### 9.2.2 Nucleotide Sequence – $P450_{MP}$

gtgccgcgcggcagccatATGCCTGCAGCAATTGCAACCCATCGTTTTCGTAAAGCACGTACCCTGCC
TCGTGAACCGGCACCGGATAGCACCCTGGCACTGCTGCGTGAAGGTTATGGTTTTATTCGTAATCGTT
GTCGTCGTCATGATAGCGACCTGTTTGCAGCACGTCTGCTGCTGAGTCCGGTTATTTGTATGAGCGGT
GCAGAAGCAGCCCGTCATTTTTATGATGGTCATCGTTTTACCCGTCGCCATGCACTGCCTCCGACCAG
CTTTGCACTGATTCAGGATCATGGTAGCGTTATGGTTCTGGATGGTGCAGCACATCTGGCACGTAAAG
CAATGTTTCTGAGCCTGGTTGGTGAAGAAGCACTGCAGCGCCTGGCAGGTCTGGCAGAACGTCATTGG
CGTGAAGCAGTTAGCGGTTGGGCACGCAAAGATACCGTTGTTCTGCTGGATGAAGCACATCGTGTTCT
GACCGCAGCAGTTTGTGAATGGGTTGGTCTGCCGCTGGGTCCGACCGAAGTTGATGCACGTGCCCGTG
AATTTGCAGCAATGATTGATGGTACAGGTGCAGTTGGTCCGCGTAATTGGCGTGGTCATCTGTATCGT
GCACGTACCGAACGTTGGGTTCGTAAAGTTATTGATGAAATTCGTAGCGGTCGTCGTGATGTTCCTCC
GGGTGCAGCCCGTACCATTGCAGAACATCAGGATGCAGATGGTCAGCGTCTGGATCGTACCGTTGCCG
GTGTTGAACTGATTAATGTTCTGCGTCCGACCGTTGCAAATGCACGTTATATTGTTTTTGCAGCCATG
GCACTGCATGATCATCCGCATCAGCGTGCAGCACTGGCAGATGGTGGTGAAGCAGCAGAACGTTTTAC
CGATGAAGTTCGTCGTTTTTATCCGTTTATTCCGTTCATTGGTGGTCGTGTTCGTGCACCGTTTCATT
TTGGTGGTCATGATTTTCGTGAAGGCGAATGGGTTCTGATGGATCTGTATGGCACCAATCGTGATCCG
CGTCTGTGGCATGAACCGGAACGTTTTGATCCTGATCGTTTTGCACGTGAAACCATTGATCCGTTTAA
TATGGTTAGCCATGGTGCAGGTAGCGCACGTGATGGTCACCGCTGTCCTGGTGAAGGTATTACCCGTA
TTCTGCTGCGTACCCTGAGCCGTCAGCTGGCAGCAACCCGTTATACCGTTCCGCCTCAGGATCTGACC
CTGGATCTGGCACATGTTCCGGCACGTCCGCGTAGCGGTTTTGTTATGCGTGCAGTTCATGCACCGTA
Actcgagccactgagatccggc

### 9.2.3 Nucleotide Sequences of $P450_{MP}$ variants.

$P450_{MP}$ hp1 nucleotide sequence

GTGCCGCGCGGCAGCCATATGCCTGCAGCAATTGCAACCCATCGTTTTCGTAAAGCACGTACCCT
GCCTCGTGAACCGGCACCGGATAGCACCCTGGCACTGCTGCGTGAAGGTTATGGTTTTATTCGTA
ATCGTTGTCGTCGTCATGATAGCGACCTGTTTCAGGCACGTCTGCTGGGTCGTCCGGTTATTTGT
ATGAGCGGTGCAGAAGCAGCACGTTTTTTCTATGATGGTGATCGTTTTACCCGTCGTGGTGCACT
GCCTCCGACCAGCTTTGCACTGATTCAGGATCATGGTAGCGTTATGGTTCTGGATGGTGCAGCAC
ATCTGCATCGTAAAGCCATGTTTCTGAGCCTGGTTGGTCCGGAAGCACTGCAGCGTCTGGCAGAA
CTGGCCGAACGTCATTGGCGTGAAGCAGTTAGCGGTTGGGCACGTAAAGATACCGTTGTTCTGCT
GGATGAAGCACATCGTGTTCTGACCCGTGCAGTTTGTGAATGGGTTGGTCTGCCGCTGGGTCCGA
CCGAAGTTGATGCACGTGCCCGTGAATTTGCAGCAATGATTGATGGTACAGGTGCAGTTGGTCCG
CGTAATTGGCGTGGTCGTCTGTATCGTGCACGTACCGAACGTTGGATTCGTAAAGTTATTGATGA
AATTCGTAGCGGTCGTCGTGATGTTCCTCCGGGTGCAGCCCGTACCATTGCAGAACATCGTGATG
CCGATGGTCAGCTGCTGGATCCGACAGTTGCAGCAGTTGAACTGATTAATGTTCTGCGTCCGACC
GTTGCAAATGCACGTTATATTGTTTTTGCAGCACTGGCCCTGCATGATCATCCGCACTGGCGTGC
ACGTCTGGCCGATGGTGATGAAGCAGCAGAACGTTTTGTTGATGAAGTGCGTCGTTTTTATCCGT
TTATTCCGTTCATTGGTGGTCGTGTTCGTCGTCCGTTTGAATTTGGTGGTCATGATTTTCGTGAA
GGCACCTGGGTTCTGATGGATCTGTATGGCACCAATCATGATCCGCGTCTGTGGCATGAACCGGA
ACGCTTTGATCCAGATCGTTTTGCACGTGAAACCATTGATCCGTTTAATATGGTTAGCCATGGTG
CAGGTAGCGCACGTGATGGTCACCGTTGTCCTGGTGAAGGTATTACCCGTATTCTGCTGAAAACC
CTGAGCCGTCAGCTGGCAGCAACCCGTTATGAAGTTCCGCCTCAGGATCTGACCCTGGATCTGGC
ACATGTTCCGGCACGTCCGCGTAGCGGTTTTGTTATGCGTAATGTTCGTGCACCGTAACTCGAGC
CACTGAGATCCGGC

P450$_{MP}$ hp2 nucleotide sequence

GTGCCGCGCGGCAGCCATATGCCTGCAGCAATTGCAACCCATCGTTTTCGTAAAGCACGTACCCT
GCCTCGTGAACCGGCACCGGATAGCACCCTGGCACTGCTGCGTGAAGGTTATGGTTTTATTCGTA
ATCGTTGTCGTCGTCATGATAGCGACCTGTTTCAGGCACGTCTGCTGGGTCGTCCGGTTATTTGT
ATGAGCGGTGCAGAAGCAGCACGTTTTTTCTATGATCCTGATCGTTTTACCCGTCGTGGTGCACT
GCCTCCGACCAGCTTTGCACTGATCCAGGATCATGGTAGCGTTATGGTTCTGGATGGTGCAGCAC
ATCTGCATCGTAAAGCCATGTTTCTGAGCCTGGTTACACCGGAAGCACTGCAGCGTCTGGCAGAT
CTGGCCGAACGTCATTGGCGTGAAGCAGTTAGCGAATGGGCACGTAAAGATACCGTTGTTCTGCA
TGATGAAGCCCATCGTGTTCTGACCCGTGCAGTTTGTGAATGGGTTGGTGTTCCGCTGACACCGA
CCGAAGTTGATGCACGTGCCCGTGAATTTGCAGCAATGATTGATGGTACAGGTGCAGTTGGTCCG
CGTAATTGGCGTGGTCGTCTGGCACGTGCGCGTACCGAACGTTGGGCACGCAAACTGATTGATGA
AATTCGTAGCGGTCGTCGTGATGTTCCTCCGGGTGCAGCCCGTACCATTGCAGAACATCGTGATG
CCGATGGTCAGCTGCTGGATCCGACCGTTGCAGCAGTTGAACTGATTAATGTTCTGCGTCCGACC
GTGGCAAATGCACGTTATATTGTTTTTGCAGCACTGGCCCTGCATGATCATCCGCACTGGCGTGC
CCGTCTGCGTGATGGTGATGAAGCAGCAGAACGTTTTGTTGATGAAGTGCGTCGTTTTTATCCGT
TTATTCCGTTCATTGGTGGTCGTGTTCGTAAACCGTTTGAGTTTAATGGTCATGATTTTCGTGAA
GGCACCTGGGTTCTGATGGATCTGTATGGCACCAATCATGATCCGCGTCTGTGGCATGAACCGGA
ACGCTTTGATCCGGACCGTTTTGCAGATGAAACCATTGATCCGTTTAATATGGTTAGCCATGGTG
CAGGTAGTGCACGTGATGGTCATCGTTGTCCTGGTGAAGGTATTACCCGTATTCTGCTGAAAACA
CTGGCTCGTCAGCTGGCAGCAACCCGTTATACCGTTCCGCCTCAGGATCTGACCCTGGATCTGGC
ACATGTTCCGGCACGTCCGCGTTCAGGTTTTGTTATGCGTAATGTTCGTGCACCGTAACTCGAGC
CACTGAGATCCGGC

P450$_{MP}$ hp3 nucleotide sequence

GTGCCGCGCGGCAGCCATATGCCTGCAGCAATTGCAACCCATCGTTTTCGTAAAGCACGTACCCT
GCCTCGTGAACCGGCACCGGATAGCACCCTGGCACTGCTGCGTGAAGGTTATGGTTTTATTCGTA
ATCGTTGTCGTCGTCATGATAGCGACCTGTTTCAGGCACGTCTGCTGGGTCGTCCGGTTATTTGT
ATGAGCGGTGCAGAAGCAGCACGTTTTTTCTATGATCCTGATCGTTTTACCCGTCGTGGTGCACT
GCCTCCGACCAGCTTTGCACTGATCCAGGATCATGGTAGCGTTATGGTTCTGGATGGTGCAGCAC
ATCTGCATCGTAAAGCCATGTTTCTGAGCCTGGTTACACCGGAAGCACTGCAGCGTCTGGCAGAA
CTGGCCGAACGTCATTGGCGTGAAGCAGTTAGCGAATGGGCACGTAAAGATACCGTTGTTCTGCA
TGATGAAGCCCATCGTGTTCTGACCCGTGCAGTTTGTGAATGGGTTGGTGTTCCGCTGACACCGA
CCGAAGTTGATCAGCGTGCACGTGAATTTGCAGCAATGATTGATGGTACAGGTGCAGTTGGTCCG
CGTAATTGGCGTGGTCGTCTGGCACGTGCGCGTACCGAACGTTGGGCACGCAAACTGATTGAAGA
AATTCGTAGCGGTCGTCGTGATGTTCCTCCGGGTGCAGCCCGTACCATTGCAGAACATCGTGATG
CCGATGGTCAGCTGCTGGATCCGACCGTTGCAGCAGTTGAACTGATTAATGTTCTGCGTCCGACC
GTGGCAAATGCACGTTATATTGTTTTTGCAGCACTGGCCCTGCATGATCATCCGGAATGGCGTCA
GCGTCTGCGTGATGGTGATGAATATGCAGAACGTTTTGCAGATGAAGTGCGTCGCTTTTATCCGT
TTATTCCTTTTATTGGTGGTCGTGTGCGTAAACCGTTTGAGTTTAATGGTCATGATTTTCGTGAA
GGCACCTGGGTTCTGATGGATCTGTATGGCACCAATCATGATCCGCGTCTGTGGCATGAACCGGA
ACGCTTTGATCCGGACCGCTTTGCCGATGAAACCATTGATCCGTTTAATATGGTTAGCCATGGTG
CAGGTTCAGCACGTGATGGTCATCGTTGTCCTGGTGAAGGTATTACCCGTATTCTGCTGAAAACA
CTGGCTCGTCAGCTGGCAGCAACCCGTTATGAAGTTCCGCCTCAGGATCTGACCCTGGATCTGGC
ACATGTTCCGGCACGTCCGCGTTCAGGTTTTGTTATGCGTAATGTTCGTGCACCGTAACTCGAGC
CACTGAGATCCGGC

P450$_{MP}$ hp4 nucleotide sequence

GTGCCGCGCGGCAGCCATATGCCTGCAGCAATTGCAACCCATCGTTTTCGTAAAGCACGTACCCT
GCCTCGTGAACCGGCACCGGATAGCACCCTGGCACTGCTGCGTGAAGGTTATGGTTTTATTCGTA
ATCGTTGTCGTCGTCATGATAGCGACCTGTTTCAGGCACGTCTGCTGGGTCGTCCGGTTATTTGT
ATGAGCGGTGCAGAAGCAGCACGTTTTTTCTATGATCCTGATCGTTTTACCCGTCGTGGTGCACT
GCCTCCGACCAGCTTTGCACTGATCCAGGATCATGGTAGCGTTATGGTTCTGGATGGTGCAGCAC
ATCTGCATCGTAAAGCCATGTTTCTGAGCCTGGTTAGTCCGGAAGCACTGCAGCGTCTGGCAGAT
CTGGCCGAACGTCATTGGCGTGAAGCAGTTAGCGAATGGGCACGTAAAGATACCGTTGTTCTGTA
TGATGAAGCACAGCGTGTTCTGACCCGTGCAGTTTGTGAATGGGTTGGTGTTCCGCTGACACCGG
ATGAAGTTGATCAGCGTGCACGTGAATTTGCAGCAATGATTGATGGTACAGGTGCAGTTGGTCCG
CGTAATTGGCGTGGTCGTCTGGCACGTGCGCGTACCGAACGTTGGGCACGCAAAGTTATTGAAGA
AATTCGTAGCGGTCGTCGTGATGTTCCTCCGGGTGCAGCCCGTGTTATTGCAGAACATCGTGATG
CCGATGGTCAGCTGCTGGATCCGCATGTTGCAGCAGTTGAACTGATTAATGTTCTGCGTCCGACC
GTTGCAAATGCACGTTATATTGTTTTTGCAGCACTGGCCCTGCATGATCATCCGGAATGGCGTCA
GCGTCTGCGTGATGGTGATGAATATGCAGAACGTTTTGCAGATGAAGTGCGTCGCTTTTATCCGT
TTATTCCTTTTATTGGTGGTCGTGTGCGTAAACCGTTTGAGTTTAATGGTCATGATTTTCGCAAA
GGCACCTGGGTTCTGATGGATCTGTATGGCACCAATCATGATCCGCGTCTGTGGGAAGAACCGGA
ACGCTTTGATCCGGACCGCTTTGCCGATGAAACCATTGATCCGTTTAATATGGTTAGCCATGGTG
CAGGTTCAGCACGTGATGGTCATCGTTGTCCTGGTGAAGGTATTACCCGTATTCTGCTGAAAACA
CTGGCTCGTCAGCTGGCAGATACCCGTTATGAAGTTCCGCCTCAGGATCTGACCTATGATCTGGC
ACATGTTCCGGCACGTCCGCGTTCAGGTTTTGTTATGCGTAATGTTCGTGCACCGTAACTCGAGC
CACTGAGATCCGGC

P450$_{MP}$ h1 nucleotide sequence

GTGCCGCGCGGCAGCCATATGCCTGCAGCAATTGCAACCCATCGTTTTCGTAAAGCACGTACCCT
GCCTCGTGAACCGGCACCGGATAGCACCCTGGCACTGCTGCGTGAAGGTTATGGTTTTATTCGTA
ATCGTTGTCGTCGTCATGATAGCGACCTGTTTCAGGCACGTCTGCTGGGTCGTCCGGTTATTTGT
ATGAGCGGTGCAGAAGCAGCACGTCATTTTTATGATCCTGATCGTTTTACCCGTCGTGGTGCACT
GCCTCCGACCGTTTTTGCACTGATCCAGGATCATGGTAGCGTTATGGTTCTGGATGGTGCAGCAC
ATCTGCATCGTAAAGCCATGTTTCTGAGCCTGGTTACACCGGAAGCACTGCAGCGTCTGGCAGAT
CTGGCCGAACGTCATTGGCGTGAAGCAGTTAGCAAATGGGCACGTAAAGATACCGTTGTTCTGTA
TGATGAAGCACAGCGTGTTCTGACCCGTGCAGTTTGTGAATGGGTTGGTGTTCCGCTGGGTCCTG
AAGAGGTTGATGCACGTGCCCGTGAATTTGCAGCAATGATTGATGGTGCCGGTGCAGTTGGTCCG
CGTCACTGGCGTGGTCGTCTGGCACGTGCGCGTACCGAACGTTGGGTTCGTAAACTGATTGATGA
AATTCGTAGCGGTCGTCGTGATGTTCCTCCGGGTGCAGCCCGTACCATTGCAGAACATCAGGATG
CCGATGGTCAGCGCCTGGATCGTACCGTTGCAGCAGTTGAACTGATTAATGTTCTGCGTCCGACA
GTTGCAAATGCCCGTTATATTGTTTTTGCAGCACTGGCCCTGCATGATCATCCGCATTGGAGAGC
ACGTCTGGCCGATGGTGATGAAGCCGCAGAACGTTTTACCGATGAAGTTCGTCGTTTTTATCCGT
TTATTCCGTTCATTGGTGGTCGTGTTCGTCGTCCGTTTGAATTTGGTGGTCATGATTTTCGTGAA
GGCACCTGGGTTCTGATGGATCTGTATGGCACCAATCATGATCCGCGTCTGTGGCATGAACCGGA
ACGTTTTGATCCGGACCGTTTTGCGCGTGAAACCATTGATCCGTTTAATATGGTTAGCCATGGTG
CAGGTAGTGCACGTGATGGTCATCGTTGTCCTGGTGAAGGTATTACCCGTATTCTGCTGCGTACC
CTGAGCCGTCAGCTGGCAGCAACCCGTTATACCGTTCCGCCTCAGGATCTGACCCTGGATCTGGC
ACATGTTCCGGCACGTCCGCGTTCAGGTTTTGTTATGCGTAATGTTCGTGCACCGTAACTCGAGC
CACTGAGATCCGGC

P450$_{MP}$ h3 nucleotide sequence

GTGCCGCGCGGCAGCCATATGCCTGCAGCAATTGCAACCCATCGTTTTCGTAAAGCACGTACCCT
GCCTCGTGAACCGGCACCGGATAGCACCCTGGCACTGCTGCGTGAAGGTTATGGTTTTATTCGTA
ATCGTTGCCGTCGTTTTGATAGCGACCTGTTTCAGGCACGTCTGCTGGGTCGTCCGGTTATTTGT
ATGAGCGGTGCAGAAGCAGCACGTTTTTTCTATGATCCTGATCGTTTTACCCGTCGTGGTGCACT
GCCTCCGACCGTTTTTAAACTGATTCAGGATCACGGTAGCGTTATGGTTCTGGATGGTGCAGCAC
ATCTGCATCGTAAAGCCATGTTTCTGAGCCTGGTTAGTCCGGAAGCACTGCAGCGTCTGGCAGAT
CTGGCCGAACGTCATTGGCGTGAAGCAGTTAGCAAATGGGCACGTAAAGATACCGTTGTTCTGTA
TGATGAAGCAGCCCGTGTTCTGACCCGTGCAGTTTGTGAATGGGTTGGTGTTCCGCTGAGTCCGG
ATGAAGTTGATCAGCGTGCACGTGAATTTGCAGCAATGATTGATGGTGCCGGTGCAGTTGGTCCG
CGTCACTGGCGTGGTCGTCTGGCACGTGCGCGTCTGGAACGTTGGATGCGCAAACTGATTGAAGA
AATTCGTAGCGGTCGTCGTGATGTTCCTCCGGGTGCAGCCCGTACCATTGCAGAACATCGTGATG
CCGATGGTCAGCTGCTGGATCGTACCGTTGCAGCAGTTGAACTGATTAATGTTCTGCGTCCGACA
GTTGCAAATGCCCGTTATATTGTTTTTGCAGCACTGGCCCTGCATGATCATCCGCATTGGAGAGC
ACGTCTGGCCGATGGTGATGAATATGCAGAACGTTTTGCAGATGAAGTGCGTCGCTTTTATCCGT
TTATTCCTTTTATTGGTGGTCGTGTTCGTCGTCCGTTTGAATTTGGTGGTCATGATTTTCGTGAA
GGCACCTGGGTTCTGATGGATCTGTATGGCACCAATCGTGATCCGCGTCTGTGGCATGAACCGGA
ACGCTTTGATCCGGACCGTTTTGCGCGTGAAACCATTGATCCGTTTAATATGGTTAGCCATGGTG
CAGGTAGCGCACGTGATGGTCATCGTTGTCCTGGTGAAGGTATTACCCGTATTCTGCTGAAAACC
GCAGCACGTCAGCTGGCAGCAACCCGTTATACCGTTCCGCCTCAGGATCTGACCTATGATCTGGC
ACATGTTCCGGCACGTCCGCGTAGCGGTTTTGTTATGCGTAATGTTCGTGCACCGTAACTCGAGC
CACTGAGATCCGGC

P450$_{MP}$ h4 nucleotide sequence

GTGCCGCGCGGCAGCCATATGCCTGCAGCAATTGCAACCCATCGTTTTCGTAAAGCACGTACCCT
GCCTCGTGAACCGGCACCGGATAGCACCCTGGCACTGCTGCGTGAAGGTTATGGTTTTATTCGTA
ATCGTTGCCGTCGTTTTGATAGCGACCTGTTTCAGGCACGTCTGCTGGGTCGTCCGGTTATTTGT
ATGAGCGGTGCCGAAGCAGCACGTATGTTTTATGATCCTGATCGTTTTACCCGTCGTGGTGCACT
GCCTCCGCGTGTTTTTAAACTGATTCAGGATCACGGTAGCGTTATGGTTCTGGATGGTGCAGCAC
ATCTGCATCGTAAAGCCATGTTTCTGAGCCTGGTTACACCGGAAGCACTGCAGCGTCTGGCAGAT
CTGGCCGAACGTCATTGGCGTGAAGCAGTTAGCGAATGGGCACGTAAAGATGAAGTTGTTCTGTA
TGATGAAGCAGCCCGTGTTCTGACCCGTGCAGTTTGTGAATGGGTTGGTGTTCCGCTGAGTCCGG
ATGAAGTGGATCAGCGTGCACGTGAATTTGCAGCAATGATTGATGGTGCCGGTGCAGTTGGTCCG
CGTCACTGGCGTGGTCGTCTGGCACGTGCGCGTCTGGAACGTTGGATGCGCAAACTGATTGAAGA
AATTCGTAGCGGTCGTCGTGATGTTCCTCCGGGTGCAGCCCGTACCATTGCAGAACATCGTGATG
CCGATGGTCAGCTGCTGGATCGTACCGTTGCAGCAGTTGAACTGATTAATGTTCTGCGTCCGACC
GTTGCAAATGCACGTTATATTGTTTTTGCAGCACTGGCCCTGCATGATCATCCGCACTGGCGAGA
ACGTCTGCGTGATGGTGATGAATATGCAGAACGTTTTGCCGATGAAGTGCGTCGTTTTTATCCGT
TTATTCCGTTCATTGGTGGTCGTGTTCGTCGTCCGTTTGAATGGCATGGTCATGATTTTCGCAAA
GGCACCTGGGTTCTGATGGATCTGTATGGCACCAATCATGATCCGCGTCTGTGGCATGAACCGGA
ACGCTTTGATCCGGACCGTTTTGCGCGTGAAACCATTGATCCGTTTAATATGGTTAGCCATGGTG
CAGGTAGCGCACGTGATGGTCATCGTTGTCCTGGTGAAGGTATTACCCGTATTCTGCTGAAAACC
GCAGCACGTCAGCTGGCAGCAACCCGTTATACCGTTCCGCCTCAGGATCTGACCTATGATCTGGC
ACATGTTCCGGCACGTCCGCGTAGCGGTTTTGTTATGCGTAATGTTCGTGCACCGTAACTCGAGC
CACTGAGATCCGGC

P450$_{MP}$ h5 nucleotide sequence

```
GTGCCGCGCGGCAGCCATATGCCTGCAGCAATTGCAACCCATCGTTTTCGTAAAGCACGTACCCT
GCCTCGTGAACCGGCACCGGATAGCACCCTGGCACTGCTGCGTGAAGGTTATGGTTTTATTCGTA
ATCGTTGCCGTCGTTTTGATAGCGACCTGTTTCAGGCACGTCTGCTGGGTCGTCCGGTTATTTGT
ATGAGCGGTGCCGAAGCAGCACGTATGTTTTATGATCCTGATCGTTTTACCCGTCGTGGTGCACT
GCCTCCGCGTGCATTTAAACTGATTCAGGATCACGGTAGCGTTATGGTTCTGGATGGTGAAGCAC
ATCTGCATCGTAAAGCCATGTTTCTGAGCCTGGTTACACCGGAAGCACTGCAGCGTCTGGCAGAA
CTGGCCGAACGTCATTGGCGTGAAGCAGTTGAAGATTGGGCACGTAAAGATGAAGTTGTGCTGTA
TGATGAAGCAGCCCGTGTTCTGACCCGTGCAGTTTGTGAATGGGTTGGTGTTCCGCTGGATCCGG
ATGAAGTGGATCGTCGTGCACGTGAATTTGCAGCAATGATTGATGGTGCCGGTGCAGTTGGTCCG
CGTCACTGGCGTGGTCGTCTGGCACGTCGTCGTCTGGAACGCTGGATGCGCAAACTGATTGAAGA
AATTCGTAGCGGTCGTCGTGATGTTCCGCCTGGTGCAGCCCGTACCATTGCAGAACATCGTGATG
CCGATGGTCGCCTGCTGGATCGTACCGTTGCAGCCGTTGAACTGATTAATGTTCTGCGTCCGACC
GTTGCAAATGCACGTTATATTGTTTTTGCAGCACTGGCCCTGCATGATCATCCGGAATGGCGTGA
ACGTCTGCGTGATGGTGATGAATATGCAGAACGTTTTGCCGATGAAGTGCGTCGTTTTTATCCGT
TTATTCCGTTCATTGGTGGTCGTGTTCGTAAACCGTTTGAATGGCATGGTCATGATTTTCGCAAA
GGCACCTGGGTTCTGATGGATCTGTATGGCACCAATCATGATCCGCGTCTGTGGCATGAACCGGA
ACGCTTTGATCCGGACCGCTTTGCAGATGAAACCATTGATCCGTTTAATATGGTTAGCCATGGTG
CAGGTAGCGCACGTGATGGTCATCGTTGTCCTGGTGAAGGTATTACCCGTATTCTGCTGAAAACC
GCAGCACGTCAGCTGGCAGCAACCCGTTATACCGTTCCGCCTCAGGATCTGACCTATGATCTGGC
ACATGTTCCGGCACGTCCGCGTAGCGGTTTTGTTATGCGTAATGTTCGTGCACCGTAACTCGAGC
CACTGAGATCCGGC
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P450$_{MP}$ h6 nucleotide sequence

```
GTGCCGCGCGGCAGCCATATGCCTGCAGCAATTGCAACCCATCGTTTTCGTAAAGCACGTACCCT
GCCTCGTGAACCGGCACCGGATAGCACCCTGGCACTGCTGCGTGAAGGTTATGGTTTTATTCGTA
ATCGTTGCCGTCGTTTTGATAGCGACCTGTTTCAGGCACGTCTGCTGGGTCGTCCGGTTATTTGT
ATGAGCGGTGCCGAAGCAGCAAAAATGTTTTATGATCCTGATCGTTTTACCCGTCGTGGTGCACT
GCCTCCGCGTGCATTTAAACTGATTCAGGATCACGGTAGCGTTATGGTTCTGGATGGTGAAGCAC
ATCTGCATCGTAAAGCCATGTTTCTGAGCCTGGTTAGTCCGGAAGCACTGCAGCGTCTGGCAGAT
CTGGCCGAACGTCATTGGCGTGAAGCAGTTGAAGAATGGGCACGTAAAGATGAAGTTGTGCTGTA
TGATGAAGCAGCACGTGTTCTGACCCGTGCAGTTTGTGAATGGGTTGGTGTTCCGCTGGATCCGG
ATGAAGTGGATCAGCGTGCACGTGAATTTGCAGCAATGATTGATGGTGCCGGTGCAGTTGGTCCG
CGTCACTGGCGTGGTCGTCTGGCACGTCGTCGTCTGGAACGCTGGATTCGCAAACTGATTGAAGA
AATTCGTCGTGGTCGCCGTGATGTTCCGCCTGGTGCAGCCCGTACCATTGCAGAACATCGTGATG
CCGATGGTCGCCTGCTGGATCGTACCGTTGCAGCCGTTGAACTGATTAATGTTCTGCGTCCGACC
GTTGCAAATGCACGTTATATTGTTTTTGCAGCACTGGCCCTGCATGATCATCCGGAATGGCGTGA
ACGTCTGCGTGATGGTGATGAATATGCAGAATGGTTTGCCGATGAAGTGCGTCGTTTTTATCCGT
TTATTCCGTTCATTGGTGGTCGTGTTCGTAAACCGTTTGAATGGCATGGTCATGATTTTCGCAAA
GGCACCTGGGTTCTGATGGATCTGTATGGCACCAATCATGATCCGCGTCTGTGGCATGAACCGGA
ACGTTTTGATCCGGACCGTTTTGCAGATGAAACCATTGATCCGTTTAATATGGTTAGCCATGGTG
CAGGTAGCGCACGTGATGGTCATCGTTGTCCTGGTGAAGGTATTACCCGTATTCTGCTGAAAACC
GCAGCACGTCAGCTGGCAGATACCCGTTATACCGTTCCGCCTCAGGATCTGACCTATGATCTGAG
CCAGGTTCCGGCACGTCCGCGTAGCGGTTTTGTTATGCGTAATGTTCGTGCACCGTAACTCGAGC
CACTGAGATCCGGC
```

## 9.3 Sequences of utilized Primers

| Mutation | Mutagenic Primers |
|---|---|
| L41A | 5'-gcgacctgtttcaggcacgtGCgctgggtaaaaactttat-3' (sense)<br>5'-ataaagtttttacccagcGCacgtgcctgaaacaggtcgc-3' (antisense) |
| L42A | 5'-gacctgtttcaggcacgtctgGCgggtaaaaactttatttgtatg-3' (sense)<br>5'-catacaaataaagtttttaccGCcagacgtgcctgaaacaggtc-3' (antisense) |
| L70A | 5'-cgttttcagcgtcagaatgcaGCgccgaaacgtgttca-3' (sense)<br>5'-tgaacacgtttcggcGCtgcattctgacgctgaaaacg-3' (antisense) |
| V74A | 5'-tgcactgccgaaacgtgCtcagaaaagcctgtttg-3' (sense)<br>5'-caaacaggcttttctgaGcacgtttcggcagtgca-3' (antisense) |
| L78A | 5'-ccgaaacgtgttcagaaaagcGCgtttggtgttaatgcaattca-3' (sense)<br>5'-tgaattgcattaacaccaaacGCgcttttctgaacacgtttcgg-3' (antisense) |
| L78V | 5'-cgaaacgtgttcagaaaagcGtgtttggtgttaatgcaatt-3' (sense)<br>5'-aattgcattaacaccaaacaCgcttttctgaacacgtttcg-3' (antisense) |
| L78F | 5'-ccgaaacgtgttcagaaaagcTtCtttggtgttaatgcaattcag-3' (sense)<br>5'-ctgaattgcattaacaccaaaGaAgcttttctgaacacgtttcgg-3' (antisense) |
| F79A | 5'-aaacgtgttcagaaaagcctgGCtggtgttaatgcaattcaggg-3' (sense)<br>5'-ccctgaattgcattaacaccaGCcaggcttttctgaacacgttt-3' (antisense) |
| F79V | 5'-aacgtgttcagaaaagcctgGttggtgttaatgcaattcag-3' (sense)<br>5'-ctgaattgcattaacaccaaCcaggcttttctgaacacgtt-3' (antisense) |
| V170A | 5'-cgatgattttatcgatatggCtgatgcatttggtgcagttg-3' (sense)<br>5'-caactgcaccaaatgcatcaGccatatcgataaaatcatcg-3' (antisense) |
| V170F | 5'-gccgatgattttatcgatatgTttgatgcatttggtgcagttg-3' (sense)<br>5'-caactgcaccaaatgcatcaaAcatatcgataaaatcatcggc-3' (antisense) |
| V170W | 5'-agaacgtgccgatgattttatcgatatgTGGgatgcatttggtgcagttg-3' (sense)<br>5'-caactgcaccaaatgcatcCCAcatatcgataaaatcatcggcacgttct-3' (antisense) |
| V170R | 5'-gccgatgattttatcgatatgCGtgatgcatttggtgcagttgg-3' (sense)<br>5'-ccaactgcaccaaatgcatcaCGcatatcgataaaatcatcggc-3' (antisense) |
| F173A | 5'-ccgatgattttatcgatatggttgatgcaGCtggtgcagttggtcc-3' (sense)<br>5'-ggaccaactgcaccaGCtgcatcaaccatatcgataaaatcatcgg-3' (antisense) |
| F173W | 5'-gatgattttatcgatatggttgatgcatGGggtgcagttggtccg-3' (sense) |

| | |
|---|---|
| F289A | 5'-cggaccaactgcaccCCatgcatcaaccatatcgataaaatcatc-3' (antisense) |
| | 5'-gtgcgtcgttattatccgGCtggtccgtttctgggtgc-3' (sense) |
| | 5'-gcacccagaaacggaccaGCcggataataacgacgcac-3' (antisense) |
| F289W | 5'-tcaagaagtgcgtcgttattatccgtGGggtccgtttctggg-3' (sense) |
| | 5'-cccagaaacggaccCCacggataataacgacgcacttcttga-3' (antisense) |
| F292A | 5'-gttattatccgtttggtccgGCtctgggtgcactggttaaaa-3' (sense) |
| | 5'-ttttaaccagtgcacccagaGCcggaccaaacggataataac-3' (antisense) |
| L293A | 5'-tcgttattatccgtttggtccgtttGCgggtgcactggtt-3' (sense) |
| | 5'-aaccagtgcacccGCaaacggaccaaacggataataacga-3' (antisense) |
| K78I | 5'-ccgaaacgtgttcagaaaagcAtTtttggtgttaatgcaattcagg-3' (sense) |
| | 5'-cctgaattgcattaacaccaaaAaTgcttttctgaacacgtttcgg-3' (antisense) |
| F79Q | 5'-gaaacgtgttcagaaaagcctgCAGggtgttaatgcaattcagg-3' (sense) |
| | 5'-cctgaattgcattaacaccCTGcaggcttttctgaacacgtttc-3' (antisense) |
| Q85M | 5'-gaaaagcctgtttggtgttaatgcaattATgggtatggatggtag-3' (sense) |
| | 5'-ctaccatccataccATaattgcattaacaccaaacaggcttttc-3' (antisense) |
| Q85R | 5'-gtttggtgttaatgcaattcGTggtatggatggtagcgcac-3' (sense) |
| | 5'-gtgcgctaccatccataccACgaattgcattaacaccaaac-3' (antisense) |
| R242T | 5'-caattgaactgattaatgttctgACtccgattgtggccattagc-3' (sense) |
| | 5'-gctaatggccacaatcggaGTcagaacattaatcagttcaattg-3' (antisense) |
| R242S | 5'-ttgaactgattaatgttctgAgtccgattgtggccattag-3' (sense) |
| | 5'-ctaatggccacaatcggacTcagaacattaatcagttcaa-3' (antisense) |
| R242K | 5'-cagcaattgaactgattaatgttctgAAAccgattgtggccattagctattttc-3' (sense) |
| | 5'-gaaaatagctaatggccacaatcggTTTcagaacattaatcagttcaattgctg-3' (antisense) |
| R242H | 5'-gaactgattaatgttctgcAtccgattgtggccattag-3' (sense) |
| | 5'-ctaatggccacaatcggaTgcagaacattaatcagttc-3' (antisense) |
| R242Q | 5'-caattgaactgattaatgttctgcAGccgattgtggccattag-3' (sense) |
| | 5'-ctaatggccacaatcggCTgcagaacattaatcagttcaattg-3' (antisense) |
| R242N | 5'-gcaattgaactgattaatgttctgAAtccgattgtggccattagc-3' (sense) |
| | 5'-gctaatggccacaatcggaTTcagaacattaatcagttcaattgc-3' (antisense) |
| F79Q (Q85M) | 5'-gaaaagcctgCAGggtgttaatgcaattATgggtatggatggtag-3' (sense) |
| | 5'-ctaccatccataccATaattgcattaacaccCTGcaggcttttc-3' (antisense) |
| N239R (R242T) | 5'-gcagcaattgaactgattaGAgttctgACtccgattgtggcca-3' (sense) |

| | |
|---|---|
| N239R (R242S) | 5'-tggccacaatcggaGTcagaacTCtaatcagttcaattgctgc -3' (antisense) |
| | 5'-gtatggcagcaattgaactgattaGAgttctgAgtccgattg-3' (sense) |
| | 5'-caatcggacTcagaacTCtaatcagttcaattgctgccatac-3' (antisense) |
| N239R (R242Q) | 5'-ggcagcaattgaactgattaGAgttctgcAGccgattgtg-3' (sense) |
| | 5'-cacaatcggCTgcagaacTCtaatcagttcaattgctgcc-3' (antisense) |
| N239R (R242N) | 5'-gtatggcagcaattgaactgattaGAgttctgAAtccgattgtg-3' (sense) |
| | 5'-cacaatcggaTTcagaacTCtaatcagttcaattgctgccatac-3' (antisense) |
| L41F | 5'-gcgacctgtttcaggcacgtTtCctgggtaaaaactttatttgt-3' (sense) |
| | 5'-acaaataaagtttttacccagGaAacgtgcctgaaacaggtcgc-3' (antisense) |
| L42F | 5'-gacctgtttcaggcacgtctgTtCggtaaaaactttatttgtatga-3' (sense) |
| | 5'-tcatacaaataaagtttttaccGaAcagacgtgcctgaaacaggtc-3' (antisense) |
| L78W | 5'-gccgaaacgtgttcagaaaagcTGgtttggtgttaatgcaattcag-3' (sense) |
| | 5'-ctgaattgcattaacaccaaacCAgcttttctgaacacgtttcggc-3' (antisense) |
| F79L | 5'-cgtgttcagaaaagcctgttAggtgttaatgcaattcag-3' (sense) |
| | 5'-ctgaattgcattaacaccTaacaggcttttctgaacacg-3' (antisense) |
| F79I | 5'-aacgtgttcagaaaagcctgAttggtgttaatgcaattcag-3' (sense) |
| | 5'-ctgaattgcattaacaccaaTcaggcttttctgaacacgtt-3' (antisense) |
| F79W | 5'-ccgaaacgtgttcagaaaagcctgtGGggtgttaatgcaattca-3' (sense) |
| | 5'-tgaattgcattaacaccCCacaggcttttctgaacacgtttcgg-3' (antisense) |
| F173W | 5'-gatgattttatcgatatggttgatgcatGGggtgcagttggtccg-3' (sense) |
| | 5'-cggaccaactgcaccCCatgcatcaaccatatcgataaaatcatc-3' (antisense) |
| L42A (L41A) | 5'-cctgtttcaggcacgtGCgGCgggtaaaaactttatttgta-3' (sense) |
| | 5'-tacaaataaagtttttaccGCcGCacgtgcctgaaacagg-3' (antisense) |
| F292A (L293A) | 5'-ttattatccgtttggtccgGCtgcgggtgcactggttaaa-3' (sense) |
| | 5'-tttaaccagtgcacccgcaGCcggaccaaacggataataa-3' (antisense) |
| L42F (L41F) | 5'-gcgacctgtttcaggcacgtTtCTtCggtaaaaactttatttgtatg-3' (sense) |
| | 5'-catacaaataaagtttttaccGaAGaAacgtgcctgaaacaggtcgc-3' (antisense) |
| L42A (L41F) | 5'-cgacctgtttcaggcacgtTtCGCggtaaaaactttatttg-3'(sense) |
| | 5'-caaataaagtttttacccGCGaAacgtgcctgaaacaggtcg-3' (antisense) |
| L42F (L41A) | 5'-acctgtttcaggcacgtGCgTtCggtaaaaactttatttgtatg-3' (sense) |
| | 5'-catacaaataaagtttttaccGaAcGCacgtgcctgaaacaggt-3' (antisense) |
| L214- | 5'-caccagtggcaccgcacatgaaatggcgtttca-3' (sense) |

| | |
|---|---|
| I370- | 5'-tgaaacgccatttcatgtgcggtgccactggtg-3' (antisense)<br>5'-cgggtgaaggtattaccgaagtgatgaaagccag-3' (sense)<br>5'-ctggctttcatcacttcggtaataccttcacccg-3' (antisense) |
| S273- | 5'-aatggctgcgtagcggtaatcgtgaacgtgaaatg-3' (sense)<br>5'-catttcacgttcacgattaccgctacgcagccatt-3' (antisense) |

## 9.4 Mutations – $P450_{MP}$ genes (h – series)

**Table 19:** Mutations of MP-h variants.

| h1 | h3 | h4 | h5 | h6 |
|---|---|---|---|---|
| ALA 49 GLU | HIS 43 PHE | HIS 43 PHE | HIS 43 PHE | HIS 43 PHE |
| LEU 54 GLY | ALA 49 GLU | ALA 49 GLU | ALA 49 GLU | ALA 49 GLU |
| SER 55 ARG | LEU 54 GLY | LEU 54 GLY | LEU 54 GLY | LEU 54 GLY |
| GLY 72 PRO | SER 55 ARG | SER 55 ARG | SER 55 ARG | SER 55 ARG |
| HIS 73 ASP | HIS 68 PHE | HIS 68 MET | HIS 68 MET | ARG 67 LYS |
| HIS 79 GLY | GLY 72 PRO | GLY 72 PRO | GLY 72 PRO | HIS 68 MET |
| SER 85 VAL | HIS 73 ASP | HIS 73 ASP | HIS 73 ASP | GLY 72 PRO |
| ALA 105 HIS | HIS 79 GLY | HIS 79 GLY | HIS 79 GLY | HIS 73 ASP |
| GLY 115 THR | SER 85 VAL | THR 84 ARG | THR 84 ARG | HIS 79 GLY |
| GLU 116 PRO | ALA 87 LYS | SER 85 VAL | SER 85 ALA | THR 84 ARG |
| GLY 124 ASP | ALA 105 HIS | ALA 87 LYS | ALA 87 LYS | SER 85 ALA |
| GLY 136 LYS | GLY 115 SER | ALA 105 HIS | ALA 101 GLU | ALA 87 LYS |
| LEU 146 TYR | GLU 116 PRO | GLY 115 THR | ALA 105 HIS | ALA 101 GLU |
| HIS 150 GLN | GLY 124 ASP | GLU 116 PRO | GLY 115 THR | ALA 105 HIS |
| ALA 155 ARG | GLY 136 LYS | GLY 124 ASP | GLU 116 PRO | GLY 115 SER |
| LEU 163 VAL | LEU 146 TYR | GLY 136 GLU | GLY 124 GLU | GLU 116 PRO |
| THR 168 GLU | HIS 150 ALA | THR 142 GLU | SER 135 GLU | GLY 124 ASP |
| THR 184 ALA | ALA 155 ARG | LEU 146 TYR | GLY 136 ASP | SER 135 GLU |
| ASN 191 HIS | LEU 163 VAL | HIS 150 ALA | THR 142 GLU | GLY 136 GLU |
| HIS 195 ARG | GLY 166 SER | ALA 155 ARG | LEU 146 TYR | THR 142 GLU |
| TYR 197 ALA | THR 168 ASP | LEU 163 VAL | HIS 150 ALA | LEU 146 TYR |
| VAL 208 LEU | ALA 172 GLN | GLY 166 SER | ALA 155 ARG | HIS 150 ALA |
| GLY 244 ALA | THR 184 ALA | THR 168 ASP | LEU 163 VAL | ALA 155 ARG |
| MET 266 LEU | ASN 191 HIS | ALA 172 GLN | GLY 166 ASP | LEU 163 VAL |

| | | | | |
|---|---|---|---|---|
| GLN 274 TRP | HIS 195 ARG | THR 184 ALA | THR 168 ASP | GLY 166 ASP |
| ALA 277 ARG | TYR 197 ALA | ASN 191 HIS | ALA 172 ARG | THR 168 ASP |
| GLY 282 ASP | THR 201 LEU | HIS 195 ARG | THR 184 ALA | ALA 172 GLN |
| ALA 308 ARG | VAL 205 MET | TYR 197 ALA | ASN 191 HIS | THR 184 ALA |
| HIS 311 GLU | VAL 208 LEU | THR 201 LEU | HIS 195 ARG | ASN 191 HIS |
| ARG 332 HIS | ASP 210 GLU | VAL 205 MET | TYR 197 ALA | HIS 195 ARG |
| ALA 420 ASN | GLN 231 ARG | VAL 208 LEU | ALA 199 ARG | TYR 197 ALA |
| HIS 422 ARG | ARG 237 LEU | ASP 210 GLU | THR 201 LEU | ALA 199 ARG |
| | GLY 244 ALA | GLN 231 ARG | VAL 205 MET | THR 201 LEU |
| | MET 266 LEU | ARG 237 LEU | VAL 208 LEU | VAL 205 ILE |
| | GLN 274 TRP | GLY 244 ALA | ASP 210 GLU | VAL 208 LEU |
| | ALA 277 ARG | MET 266 LEU | GLN 231 ARG | ASP 210 GLU |
| | GLY 282 ASP | GLN 274 TRP | GLN 236 ARG | SER 214 ARG |
| | ALA 284 TYR | ALA 276 GLU | ARG 237 LEU | GLN 231 ARG |
| | THR 289 ALA | ALA 277 ARG | GLY 244 ALA | GLN 236 ARG |
| | ALA 308 ARG | ALA 279 ARG | MET 266 LEU | ARG 237 LEU |
| | HIS 311 GLU | GLY 282 ASP | HIS 273 GLU | GLY 244 ALA |
| | ARG 383 LYS | ALA 284 TYR | GLN 274 TRP | MET 266 LEU |
| | LEU 385 ALA | THR 289 ALA | ALA 276 GLU | HIS 273 GLU |
| | SER 386 ALA | ALA 308 ARG | ALA 277 ARG | GLN 274 TRP |
| | LEU 403 TYR | HIS 311 GLU | ALA 279 ARG | ALA 276 GLU |
| | ALA 420 ASN | PHE 312 TRP | GLY 282 ASP | ALA 277 ARG |
| | HIS 422 ARG | GLY 313 HIS | ALA 284 TYR | ALA 279 ARG |
| | | GLU 319 LYS | THR 289 ALA | GLY 282 ASP |
| | | ARG 332 HIS | ALA 308 LYS | ALA 284 TYR |
| | | ARG 383 LYS | HIS 311 GLU | ARG 287 TRP |
| | | LEU 385 ALA | PHE 312 TRP | THR 289 ALA |
| | | SER 386 ALA | GLY 313 HIS | ALA 308 LYS |
| | | LEU 403 TYR | GLU 319 LYS | HIS 311 GLU |
| | | ALA 420 ASN | ARG 332 HIS | PHE 312 TRP |
| | | HIS 422 ARG | ARG 350 ASP | GLY 313 HIS |
| | | | ARG 383 LYS | GLU 319 LYS |
| | | | LEU 385 ALA | ARG 332 HIS |
| | | | SER 386 ALA | ARG 350 ASP |
| | | | LEU 403 TYR | ARG 383 LYS |
| | | | ALA 420 ASN | LEU 385 ALA |

| | | | | |
|---|---|---|---|---|
| | | | HIS 422 ARG | SER 386 ALA<br>ALA 391 ASP<br>LEU 403 TYR<br>ALA 406 SER<br>HIS 407 GLN<br>ALA 420 ASN<br>HIS 422 ARG |

## 9.5 Mutations – P450$_{MP}$ genes (hp – series)

**Table 19:** Mutations of MP-hp variants.

| hp1 | hp2 | hp3 | hp4 |
|---|---|---|---|
| ALA 49 GLU | ALA 49 GLU | ALA 49 GLU | ALA 49 GLU |
| LEU 54 GLY | LEU 54 GLY | LEU 54 GLY | LEU 54 GLY |
| SER 55 ARG | SER 55 ARG | SER 55 ARG | SER 55 ARG |
| HIS 68 PHE | HIS 68 PHE | HIS 68 PHE | HIS 68 PHE |
| HIS 73 ASP | GLY 72 PRO | GLY 72 PRO | GLY 72 PRO |
| HIS 79 GLY | HIS 73 ASP | HIS 73 ASP | HIS 73 ASP |
| ALA 105 HIS | HIS 79 GLY | HIS 79 GLY | HIS 79 GLY |
| GLU 116 PRO | ALA 105 HIS | ALA 105 HIS | ALA 105 HIS |
| GLY 124 GLU | GLY 115 THR | GLY 115 THR | GLY 115 SER |
| ALA 155 ARG | GLU 116 PRO | GLU 116 PRO | GLU 116 PRO |
| HIS 195 ARG | GLY 124 ASP | GLY 124 GLU | GLY 124 ASP |
| VAL 205 ILE | GLY 136 GLU | GLY 136 GLU | GLY 136 GLU |
| GLN 231 ARG | LEU 146 HIS | LEU 146 HIS | LEU 146 TYR |
| ARG 237 LEU | ALA 155 ARG | ALA 155 ARG | HIS 150 GLN |
| ARG 240 PRO | LEU 163 VAL | LEU 163 VAL | ALA 155 ARG |
| GLY 244 ALA | GLY 166 THR | GLY 166 THR | LEU 163 VAL |
| MET 266 LEU | HIS 195 ARG | ALA 172 GLN | GLY 166 THR |
| GLN 274 TRP | TYR 197 ALA | HIS 195 ARG | THR 168 ASP |
| ALA 277 ARG | VAL 205 ALA | TYR 197 ALA | ALA 172 GLN |
| GLY 282 ASP | VAL 208 LEU | VAL 205 ALA | HIS 195 ARG |
| THR 289 VAL | GLN 231 ARG | VAL 208 LEU | TYR 197 ALA |

| | | | |
|---|---|---|---|
| ALA 308 ARG | ARG 237 LEU | ASP 210 GLU | VAL 205 ALA |
| HIS 311 GLU | ARG 240 PRO | GLN 231 ARG | ASP 210 GLU |
| GLU 321 THR | GLY 244 ALA | ARG 237 LEU | THR 226 VAL |
| ARG 332 HIS | MET 266 LEU | ARG 240 PRO | GLN 231 ARG |
| ARG 383 LYS | GLN 274 TRP | GLY 244 ALA | ARG 237 LEU |
| THR 395 GLU | ALA 277 ARG | MET 266 LEU | ARG 240 PRO |
| ALA 420 ASN | ALA 279 ARG | HIS 273 GLU | THR 241 HIS |
| HIS 422 ARG | GLY 282 ASP | GLN 274 TRP | GLY 244 ALA |
| | THR 289 VAL | ALA 276 GLN | MET 266 LEU |
| | ALA 308 LYS | ALA 277 ARG | HIS 273 GLU |
| | HIS 311 GLU | ALA 279 ARG | GLN 274 TRP |
| | GLY 313 ASN | GLY 282 ASP | ALA 276 GLN |
| | GLU 321 THR | ALA 284 TYR | ALA 277 ARG |
| | ARG 332 HIS | THR 289 ALA | ALA 279 ARG |
| | ARG 350 ASP | ALA 308 LYS | GLY 282 ASP |
| | ARG 383 LYS | HIS 311 GLU | ALA 284 TYR |
| | SER 386 ALA | GLY 313 ASN | THR 289 ALA |
| | ALA 420 ASN | GLU 321 THR | ALA 308 LYS |
| | HIS 422 ARG | ARG 332 HIS | HIS 311 GLU |
| | | ARG 350 ASP | GLY 313 ASN |
| | | ARG 383 LYS | GLU 319 LYS |
| | | SER 386 ALA | GLU 321 THR |
| | | THR 395 GLU | ARG 332 HIS |
| | | ALA 420 ASN | HIS 338 GLU |
| | | HIS 422 ARG | ARG 350 ASP |
| | | | ARG 383 LYS |
| | | | SER 386 ALA |
| | | | ALA 391 ASP |
| | | | THR 395 GLU |
| | | | LEU 403 TYR |
| | | | ALA 420 ASN |
| | | | HIS 422 ARG |

## 9.6 Calibrations

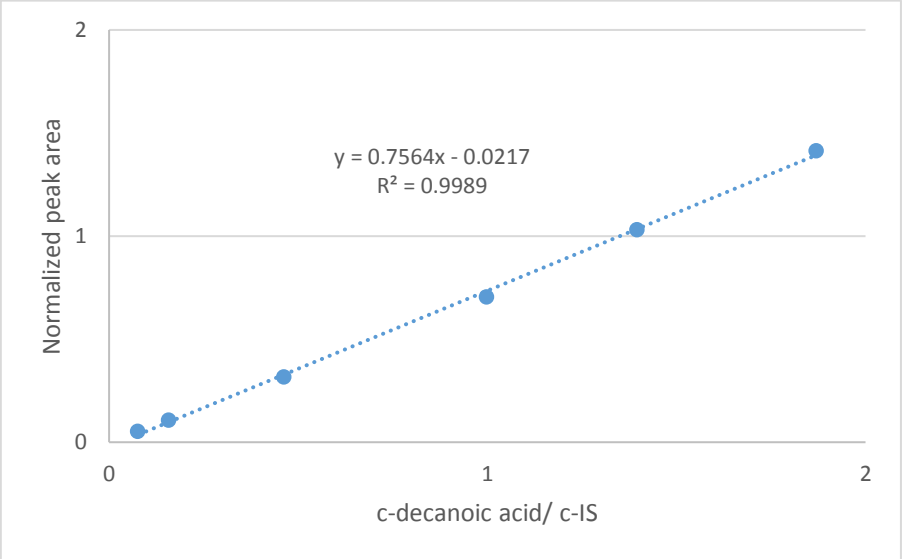


**Figure 16:** Calibration of decanoic acid; used for substrate quantification in $P450_{MP}$ samples ; IS: 12-Hydroxy-decanoic acid.

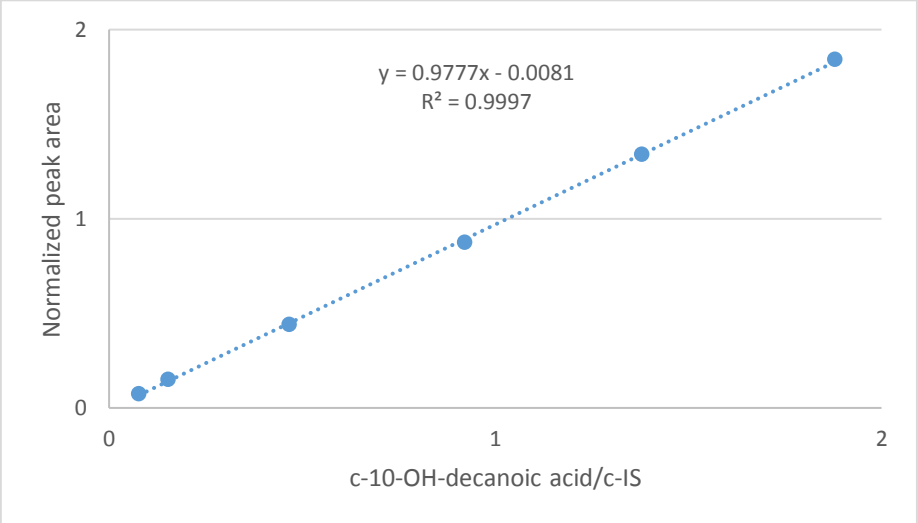


**Figure 17:** Calibration of 10-OH-decanoic acid; used for quantification of all regioisomers of hydroxy-decanoic acid in $P450_{MP}$ samples; IS: 12-Hydroxy-decanoic acid.

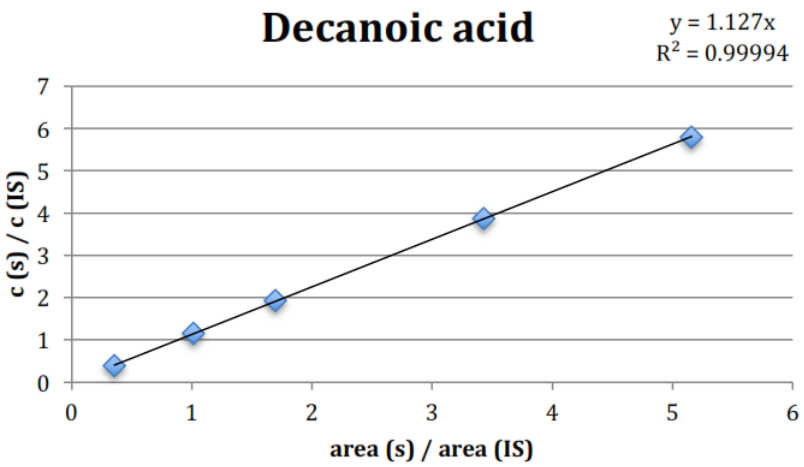


**Figure 18:** Calibration of decanoic acid; used for substrate quantification in $P450_{Bs\beta}$ samples; IS: 12-Hydroxy-decanoic acid.[62]

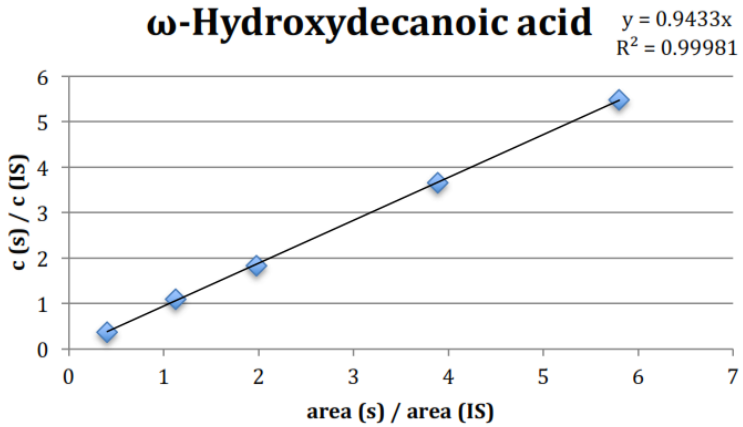


**Figure 19:** Calibration of 10-OH-decanoic acid; used for quantification of all regioisomers of hydroxy-decanoic acid in $P450_{Bs\beta}$ samples; IS: 12-Hydroxy-decanoic acid.[62]

## 9.7 $^{13}$C-NMR spectra

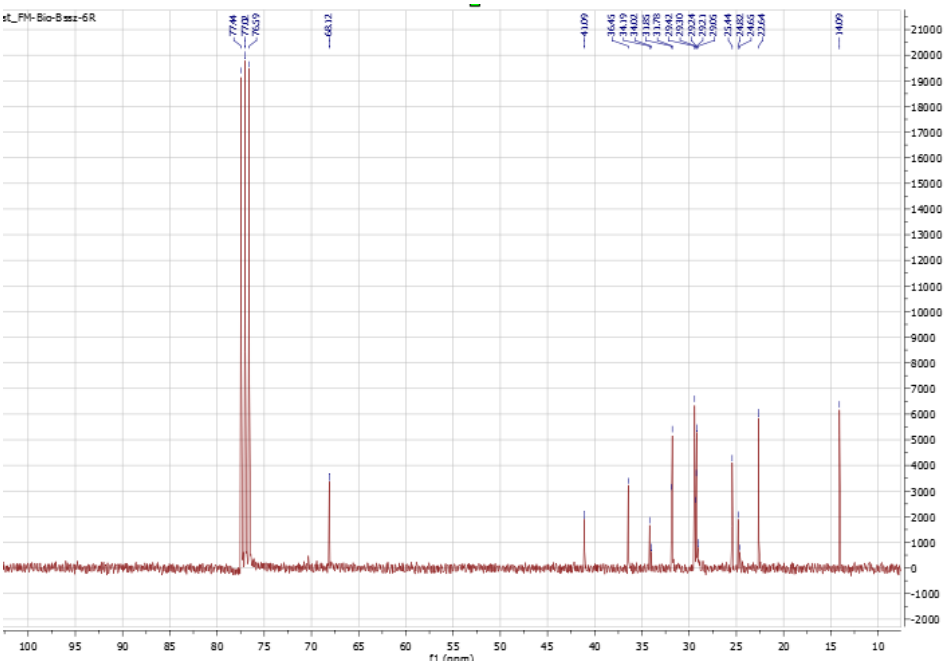

**Figure 20:** $^{13}$C-NMR of product mixture resulting from biotransformation of decanoic acid with $P450_{Bs\beta}$ variant L78I/F292A.

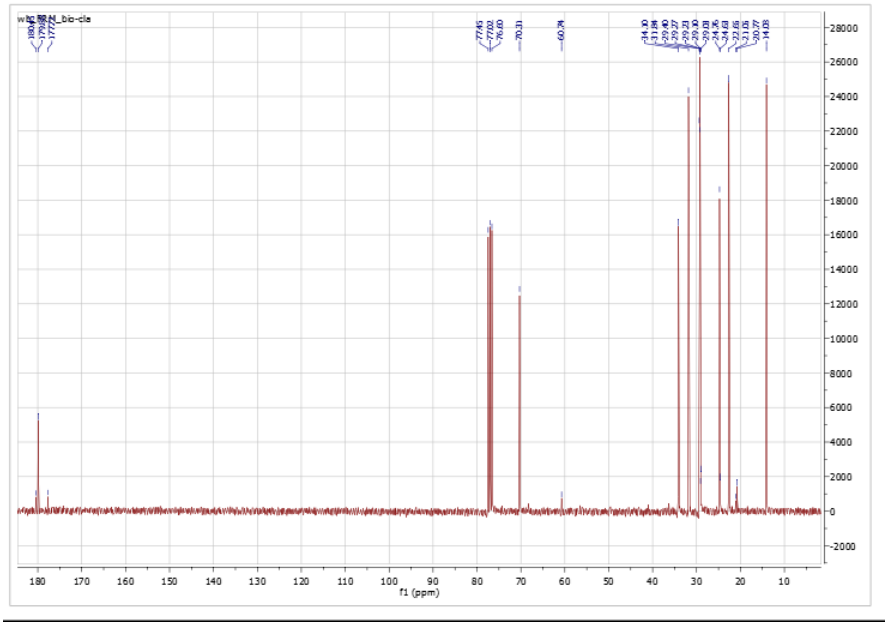

**Figure 21:** $^{13}$C-NMR of product mixture resulting from biotransformation of decanoic acid with $P450_{CLA}$..

# 10 Mass spectra

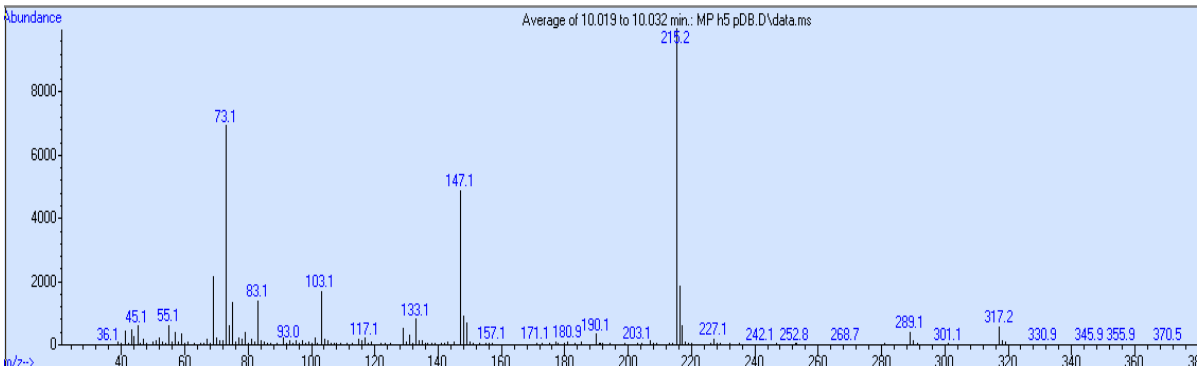


**Figure 22:** Mass spectrum of trimethylsilyl-((2-trimethylsilyl)oxy)-decanoate.

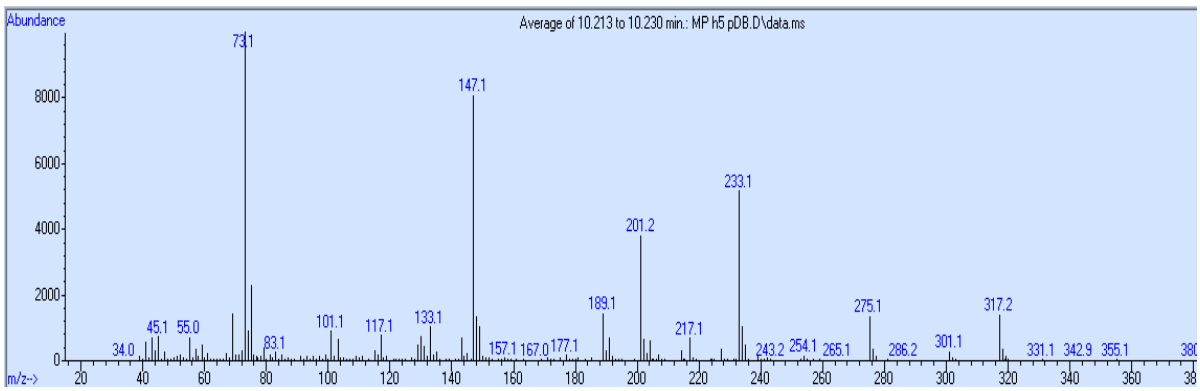


**Figure 23:** Mass spectrum of trimethylsilyl-((3-trimethylsilyl)oxy)-decanoate.

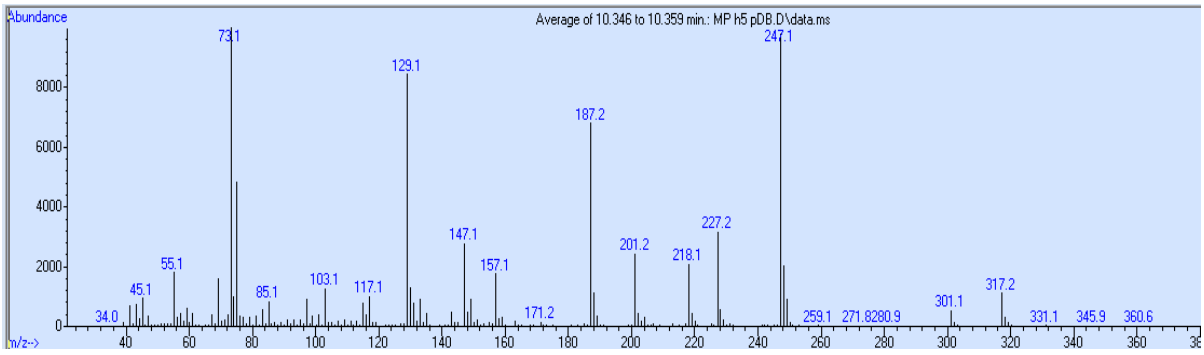


**Figure 24:** Mass spectrum of trimethylsilyl-((4-trimethylsilyl)oxy)-decanoate.

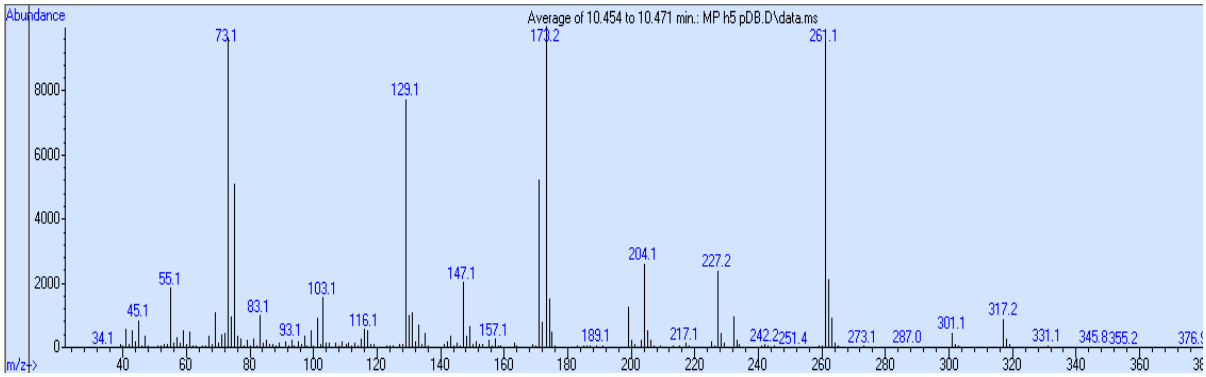


**Figure 25:** Mass spectrum of trimethylsilyl-((5-trimethylsilyl)oxy)-decanoate.

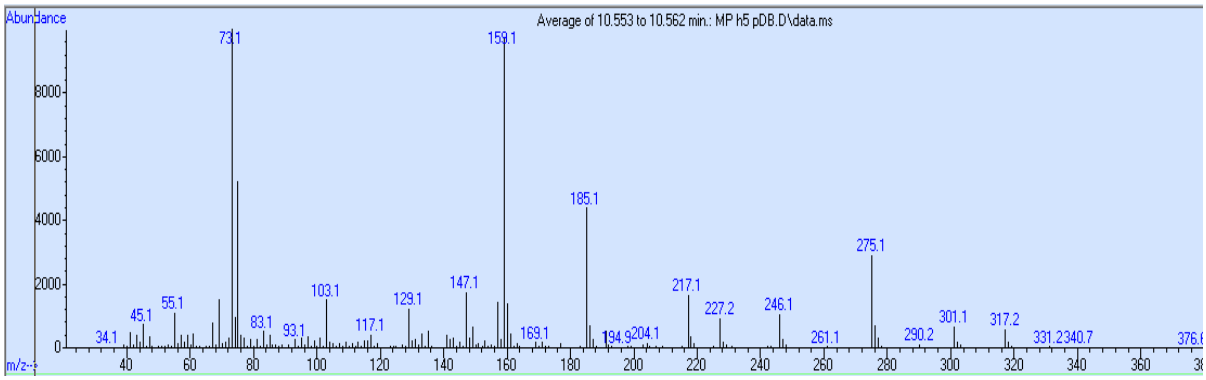


**Figure 26:** Mass spectrum of trimethylsilyl-((6-trimethylsilyl)oxy)-decanoate.

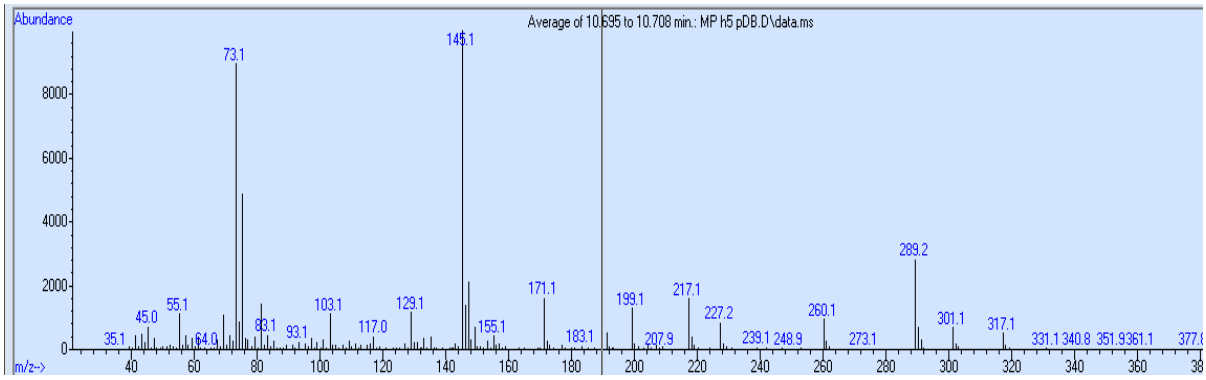


**Figure 27:** Mass spectrum of trimethylsilyl-((7-trimethylsilyl)oxy)-decanoate.

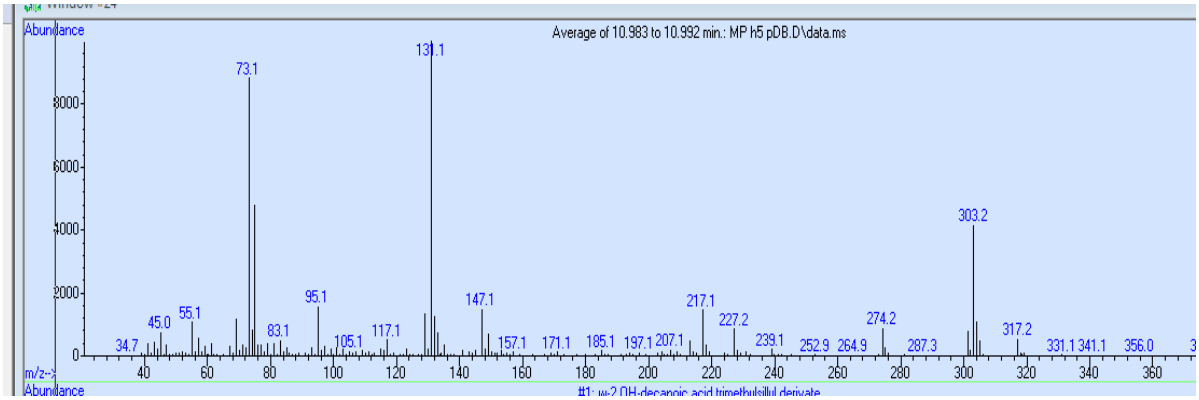


**Figure 28:** Mass spectrum of trimethylsilyl-((8-trimethylsilyl)oxy)-decanoate.

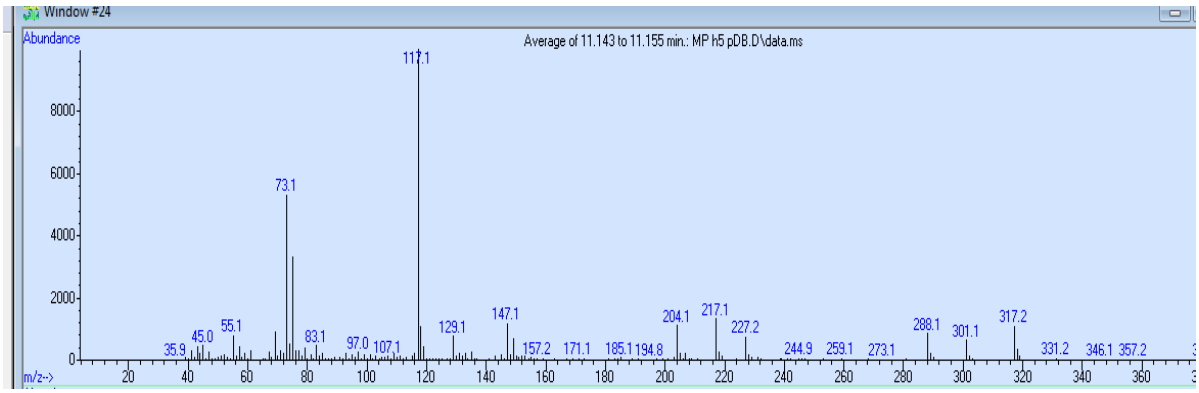


**Figure 29:** Mass spectrum of trimethylsilyl-((9-trimethylsilyl)oxy)-decanoate.

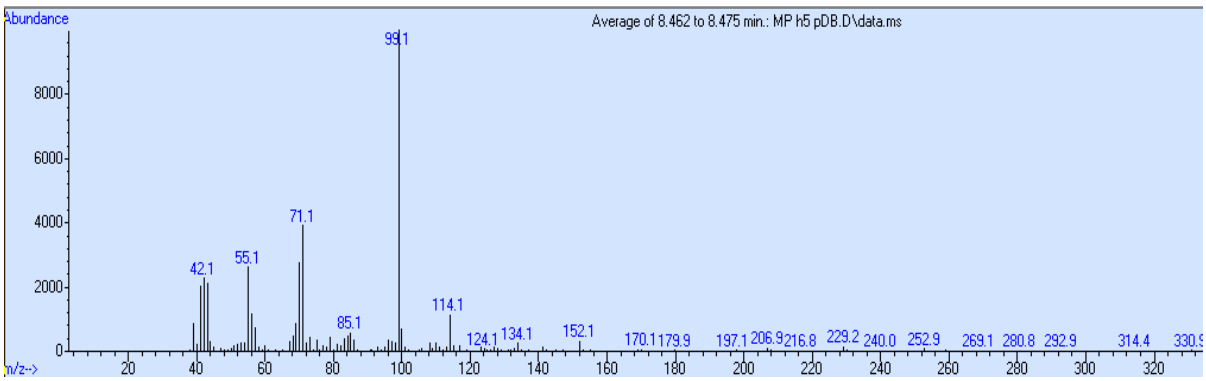


**Figure 30:** Mass spectrum of C-10-δ-lactone.

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# 12 Curriculum Vitae

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|---|---|---|
| **Personal Details** | Address: | Unterer Rosenberg 8<br>8472 Strass in Stmk.; Austria |
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| | E-Mail address: | michael.friess@student.tugraz.at |
| | Date of birth: | 31.08.1995 |
| | Citizenship: | Austria |
| **Education** | Currently: | Master study of Technical Chemistry at the TU Graz.<br>Started on 11th of November 2017.<br>Graduation expected within summer 2019.<br>Focus of elective subjects on organic chemistry and Biocatalysis.<br>Master thesis in the group of Prof. Kroutil.<br>Practical part (8 months) of master thesis already finished. |
| | September 2013-October 2017 | Bachelor study of Chemistry (NAWI Graz).<br>Interrupted by military service (September 2014-March 2015).<br>Bachelor degree passed with distinction on 6th of October 2017.<br>Bachelor thesis in organic chemistry with the title `Stereoselective Synthesis of Vicinal Amino Alcohols` under supervision of Prof. Breinbauer. |
| | September 2005-June 2013 | School of secondary education (BG/BRG Leibnitz)<br>Final examination passed with distinction. |
| **Further Information** | Scientific Interests | Main interests concerned with synthetic organic chemistry and biocatalysis. In the field of biocatalysis I am most interested in the mechanistic principle of various enzymes. |
| | Languages | German and English |
| | Non-academic skills and hobbies | Driving licence for classes A, B, C and E (Austrian system).<br>Voluntary member in the fire brigade of Spielfeld.<br>Sports |