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Terpene Hydroxylation with Microbial Cytochrome P450 Monooxygenases

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Abstract Terpenoids comprise a highly diverse group of natural products. In addition to their basic carbon skeleton, they differ from one another in their functional groups. Functional groups attached to the carbon skeleton are the basis of the terpenoids' diverse properties. Further modifications of terpene olefins include the introduction of acyl-, aryl-, or sugar moieties and usually start with oxidations catalyzed by cytochrome P450 monooxygenases (P450s, CYPs). P450s are ubiquitously distributed throughout nature, involved in essential biological pathways such as terpenoid biosynthesis as well as the tailoring of terpenoids and other natural products. Their ability to introduce oxygen into nonactivated C–H bonds is unique and makes P450s very attractive for applications in biotechnology. Especially in the field of terpene oxidation, biotransformation methods emerge as an attractive alternative to classical chemical synthesis. For this reason, microbial P450s depict a highly interesting target for protein engineering approaches in order to increase selectivity and activity, respectively. Microbial P450s have been described to convert industrial and pharmaceutically interesting terpenoids such as ionones, limone, valencene, resin acids, and triterpenes (including steroids) as well as vitamin D_3. Highly selective and active mutants have been evolved by applying classical site-directed mutagenesis as well as directed evolution of proteins. As P450s usually depend on electron transfer proteins, mutagenesis has also been applied to improve the interactions between P450s and their respective redox partners. This chapter provides an overview of terpenoid hydroxylation reactions catalyzed by bacterial P450s and highlights the achievements made by protein engineering to establish productive hydroxylation processes.

Keywords Terpenes · Terpenoids · P450 · Monooxygenase · CYP106A1 · CYP106A2 · Adrenodoxin · Adrenodoxin reductase · Abietic acid · Boswellic acid · Dammarane · Steroid · Vitamin D_3 · Ionone · Valencene · Ferredoxin · Ferredoxin reductase · Protein engineering

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Abbreviations

Adx	Adrenodoxin
P450s	Cytochrome P450 enzymes
BM3	Cytochrome P450 102A1
$P450_{cam}$	Cytochrome P450 101A1
CPR	Cytochrome P450 reductase
DMSO	Dimethylsulfoxide
epPCR	Error-prone polymerase chain reaction
Fdx	Ferredoxin
FdR	Ferredoxin reductase
FMN	Flavine mononucleotide
FAD	Flavine adenine dinucleotide
NAD(P)H	Nicotine amide adenine dinucleotide (phosphate)
Pdx	Putidaredoxin
THF	Tetrahydrofuran
VD_3	Vitamin D_3
$1\alpha,25(OH)_2VD_3$	1α,25-dihydroxyvitamin D_3
CYP	cytochrome P450
KBA	11-keto-β-boswellic acid
PDB	Protein Data Bank
VDR	Vitamin D receptor

Contents

1 Introduction

Although the biosynthesis of terpenoids[1] in plants is broadly studied, biosynthesis and modifications of the terpenoid skeleton in microorganisms has been much less investigated. Therefore, microorganism reaction pathways of terpenoid biosynthesis as well as involved enzymes are much less identified and characterized as compared with the plant systems. An essential step in the biosynthesis of terpenes is the cyclization of isoprene subunit chains by different enzymes belonging to the family of terpene synthases. Depending on the number of isoprene units, the resulting structures can be classified into hemiterpenoids (one isoprene unit), monoterpenoids (two isoprene units), sesquiterpenoids (three isoprene units), diterpenoids (four isoprene units), sesterterpenoids (five isoprene units), triterpenoids (six isoprene units), tetraterpenoids (eight isoprene units), and polyterpenoids with a larger number of isoprene units. Terpene synthases have also been found in microorganisms, indicating the pivotal role of terpenoids in this kingdom of life [1]. In addition to their basic carbon skeleton, terpenoids also differ from one another in their functional groups, which form the basis for their diverse properties. Further modifications of terpene olefins include the introduction of acyl-, aryl-, or sugar moieties and usually start with oxidations catalyzed by cytochrome P450 monooxygenases (P450s, CYPs) [2].

P450s are heme-containing enzymes that are ubiquitously distributed in all biological kingdoms and exhibit activity towards a diverse range of substrates [3]. Members of this enzyme family are not only involved in the biosynthesis of physiologically important compounds such as terpenes, steroids, vitamins, and bile acids, but also play a major role in the detoxification of xenobiotics, herbicides, and insecticides. The central step in the P450-catalyzed oxidation is the activation of oxygen by the reduced heme iron of the enzyme and the incorporation of one atom of molecular oxygen into the substrate while the other oxygen atom is reduced to water [4]. The reaction is usually a hydroxylation reaction, but P450s are also able to catalyze reactions such as heteroatom oxygenation, dealkylation, epoxidation, aromatic hydroxylation, reduction, dehalogenation, and even C–C bond formation or cleavage [5, 6]. P450 enzymes are categorized into families and subfamilies depending on the sequence identity, with members of the same family being defined as usually having ≥40 % sequence identity. Representatives of the same subfamily are always >55 % identical [7]. As external monooxygenases, P450s depend on NAD(P)H and corresponding electron transfer proteins in order to perform the enzymatic reaction. According to the composition of their electron transport chain, P450 enzymes can be grouped into different classes [8]. The most common classes

[1] The difference between terpenes and terpenoids is that compounds belonging to the latter category contain additional functional groups whereas terpenes are solely composed of carbon and hydrogen. Cytochromes P450 are often involved in the further functionalization of terpenes into terpenoids, but terpenoids themselves can also be substrates for P450 enzymes. For this reason the terms terpenes and terpenoids are used synonymously throughout the chapter.

are shown in Fig. 1. For example, plant P450s are localized in the endoplasmic reticulum, belong to class II of the P450 group, and need a NADPH-dependent FAD and FMN-containing cytochrome P450 reductase (CPR) for their activity, whereas many of the bacterial P450s get the necessary electrons via a FAD-containing reductase and an iron–sulfur protein (ferredoxin). A special case is CYP102A1,

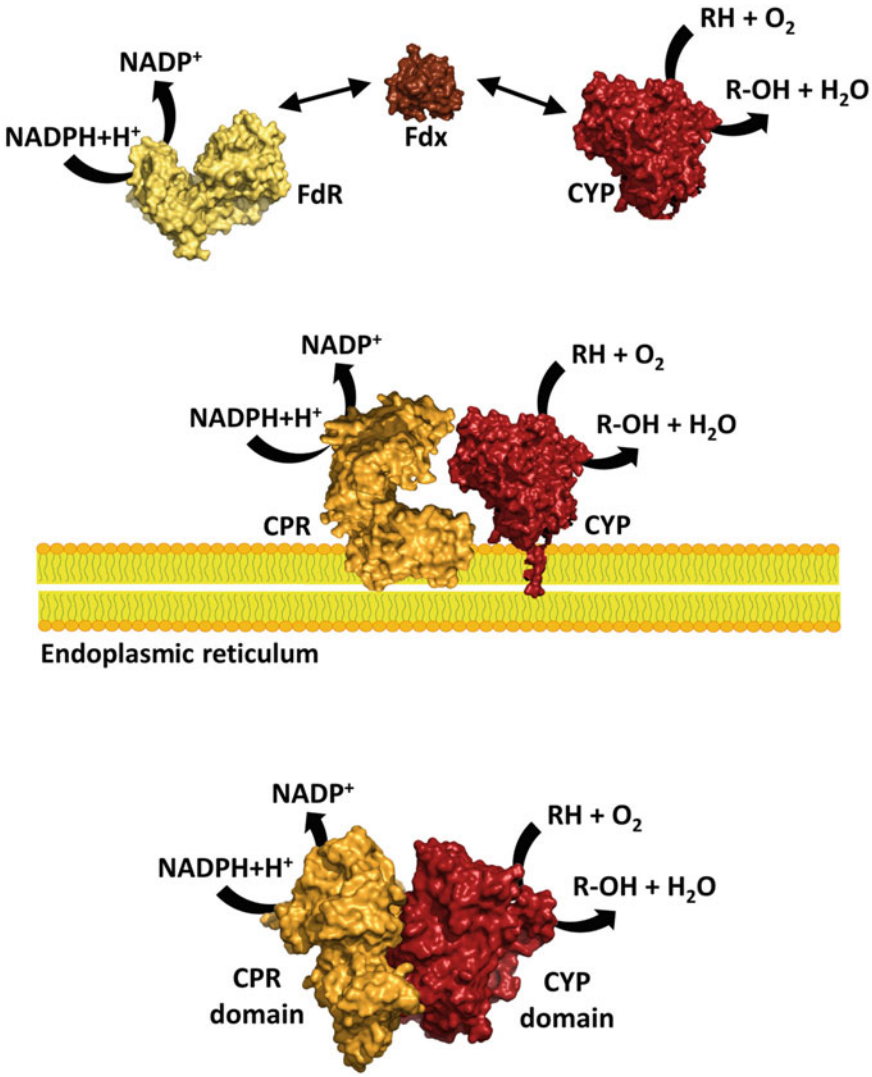


Fig. 1 Schematic organization of different cytochrome P450 systems and their classification according to [8]. *Top* class I, soluble bacterial system (e.g., CYP101A1). *Middle* class II, microsomal system (e.g., plant P450s). *Bottom* class VIII, bacterial CPR-P450 fusion system (e.g., CYP102A1)

where a FAD- and FMN-containing reductase domain is fused within one gene with a corresponding heme domain (Fig. 1).

In addition to their central role in essential biological pathways such as terpenoid biosynthesis, their ability to introduce oxygen into nonactivated C–H bonds under normal pressure and at room temperature makes P450s very attractive for applications in the field of biotechnology. Their tremendous potential is supported by the diverse spectrum of catalyzed reactions on a vast range of complex molecules and the possibility of applying protein engineering techniques to improve the characteristics of P450s further for biotechnological purposes [3, 9]. Although at present only a few microbial P450s have been described as being involved in the modification of terpenoids, their importance will certainly increase in the following years. In this review we summarize present knowledge on the identification, characterization, and engineering of microbial P450s for terpene modification.

2 Terpene Hydroxylation and Cytochromes P450

Terpenoids are the largest group of natural products and conservative estimates suggest that there are tens of thousands of terpenoids present in plants and fungi. Additionally, it has become increasingly clear during the last decade that many bacteria also produce terpenes. A major boost in this research area was given by newly developed techniques of genome mining, in which bioinformatic tools are used to screen bacterial genome sequences for conserved functional domains typical of terpene synthases [1]. The combination of genome mining and biochemical characterization leads to the simultaneous discovery of new natural products and the responsible biosynthetic enzymes. In fact, this approach has revealed a plethora of previously unknown enzymes, among them many cytochromes P450. In many cases, however, the physiological role of the biosynthetic pathways and their products, respectively, still need to be elucidated. Because the involved enzymes catalyze a variety of unusual biochemical reactions, they provide a wide range of possible applications, not least for sustainable production processes in the field of biotechnology.

Terpenes and their derivatives possess a broad spectrum of applications in varying areas and are therefore very interesting for different sectors of industry. Prominent examples include the anticancer agent taxol and the antimalaria drug artemisinin for the pharmaceutical industry or volatile monoterpenoids such as geraniol, citral, and menthol for the flavor and fragrance industries. In general terms, terpenoids possess a vast number of biological properties that can often be influenced by modification of their base structure. Especially the introduction of a hydroxyl group usually shows an influence on the volatility, efficacy, solubility, and toxicity of the substance. However, regio- and enantioselective hydroxylation through chemical synthesis is difficult to achieve due to side reactions, low yields, and the need of expensive catalysts. In contrast, the application of P450 enzymes for bioconversion processes allows the regio- and stereoselective introduction of

oxygen under mild reaction conditions [10]. As a result, P450s are very attractive for the production and modification of terpenoids. Despite their impressive synthetic potential, practical application of P450s in industrial bioconversion processes is still limited [9]. Expression of typically membrane-bound plant P450s in microbial hosts tends to pose problems and results in low solubility and protein yields. Further challenges including their cofactor dependency and their overall low activity make these enzymes a difficult system with which to work. In view of these problems, soluble bacterial P450 systems are often considered as a substitute [11]. They usually exhibit higher catalytic turnover rates and their solubility and high expression level in heterologous hosts make them easier to handle in the laboratory. Due to these properties, bacterial P450s constitute ideal targets for protein engineering and are, therefore, not limited to the reactions that they naturally catalyze.

3 Origin and Identification of Microbial Terpene-Hydroxylating P450s

The number of described cytochrome P450 sequences is rapidly growing, with more than 21,000 representatives being named roughly about 50 years after the discovery of this enzyme family (http://drnelson.uthsc.edu/CytochromeP450.html) [7, 12]. Nonetheless, the examples for microbial P450 enzymes capable of converting substrates of terpene origin are still limited. Table 1 provides an overview of the best investigated microbial P450s that are known to act on terpenoids. It can be expected that this number will grow very quickly in the coming years due to industrial interest in developing processes that are biomimics of natural pathways and more sustainable, for example, using less energy, water, and hazardous compounds. Especially in the field of terpene oxidation, biotransformation methods (i.e., methods that utilize microorganisms or enzyme systems) emerge as an attractive alternative [13]. Some of their most prominent advantages are the ability to proceed under mild conditions and the lack of toxic waste production compared with many traditional chemical methods [14]. The use of microorganisms in terpene biotransformation started in the 1950s and was originally based on the discovery of microbial terpene metabolism. In pioneering studies, a pseudomonad isolated from sewage sludge was found to decompose (+)-camphor [15]. The responsible enzyme for the initial hydroxylation of camphor was later identified as CYP101A1 ($P450_{cam}$) [16, 17], the first described P450s of bacterial origin. Similarly, the oxidation of terpenes by fungi was reported in the same period with the discovery of α-pinene conversion into several oxygenated products by *Aspergillus niger* [18]. Further monoterpenoid substrates of different *A. niger* strains include, for instance, linalool and limonene [19, 20]. Although there are indications that P450s are not involved in the transformation of limonene by *A. niger*, most microorganism-mediated conversions of terpenes seem to proceed via cytochrome P450 monooxygenases.

Table 1 Microbial Cytochromes P450 with activities towards terpenoids

Cytochrome P450	Organism	Catalyzed reaction	Reference
P450cam (CYP101A1)	*Pseudomonas putida*	Camphor to 5-hydroxycamphor Diverse terpenoid hydroxylations by engineered enzyme variants	[117]
P450 BM-3 (CYP102A1)	*Bacillus megaterium* ATCC 14581	Diverse terpenoid hydroxylations by engineered enzyme variants	[118–120]
P450moxA (CYP105)	*Nonomuraea recticatena* IFO 14525	Oleanoic acid to queretaroic acid	[121]
CYP105A1	*Streptomyces griseolus*	Vitamin D_3 to 1α,25-dihydroxyvitamin D_3 Hydroxylation of abietane-type diterpene resin acids Epoxidation of pimarane-type diterpene resin acids	[11, 76]
CYP105A2	*Pseudonocardia autotrophica*	Vitamin D_3 to 25-hydroxyvitamin D_3	[122]
CYP105A3	*Streptomyces carbophilus*	Compacting to pravastatin	[123]
CYP106A1	*Bacillus megaterium* DSM319	11-Keto-β-boswellic acid (KBA) to 7β-hydroxy-KBA, 15α-hydroxy-KBA and 7β,15α-dihydroxy-KBA	[81]
CYP106A2	*Bacillus megaterium* ATCC 13368	Hydroxylation of several di- and triterpenoids	[73, 80, 83]
CYP107 (Vdh)	*Pseudonocardia autotrophica*	Vitamin D_3 to 1α-hydroxy- and 25-hydroxyvitamin D_3	[124]
CYP107 family member	*Sebekia benihana*	Vitamin D_3 1α- and 25-hydroxylation	[125]
P450terp (CYP108)	*Pseudomonas* sp.	α-Terpinol to 7-hydroxyterpineol	[126]
CYP109B1	*Bacillus subtilis*	(+)-Valencene to (+)-nootkatone via cis- or trans-nootkatol α-Ionone to 3-hydroxy-α-ionone β-Ionone to 4-hydroxy-β-ionone	[69]
CYP109D1	*Sorangium cellulosum* So ce56	α-Ionone to 3-hydroxy-α-ionone β-Ionone to 4-hydroxy-β-ionone	[45]
CYP110C1	*Nostoc* sp. PCC 7120	Germacrene A hydroxylase	[127, 128]
P450lin (CYP111)	*Pseudomonas incognita*	Linalool to 8-hydroxylinalool	[129]
CYP120A1	*Synechocystis* sp. PCC 6803	Various hydroxylations of retinoic acid	[130]
CYP153A6	*Mycobacterium* sp. HXN-1500	(−)-Limonene to perrilyl alcohol	[58]

(continued)

Table 1 (continued)

Cytochrome P450	Organism	Catalyzed reaction	Reference
CYP170A1	*Streptomyces coelicolor* A3(2) and *Streptomyces avermitilis*	Epi-isozizaene to (4S)- and (4R)-albaflavenols to albaflavenone Moonlighting terpene synthase	[131, 132]
CYP175A1	*Thermus thermophilus* HB27	β-Carotene to β-cryptoxanthin	[133]
P450cin (CYP176A1)	*Citrobacter braakii*	Cineole to (S)-6β-hydroxycineole	[107]
P450-cin	*Bacillus cereus* UI-1477	1,4-Cineole hydroxylations	[134]
CYP226A family members	*Burkholderia xenovorans* LB400	Abietane diterpenoid hydroxylation	[71]
CYP238A1	*Pseudomonas putida* KT2440	*cis*- and *trans*-Nerolidol to the 9-hydroxy product Binding of acyclic and cyclic terpene alcohols such as farnesol, nerolidol, linalool, and terpineol	[135]
CYP264B1	*Sorangium cellulosum* So ce56	α-Ionone to 3-hydroxy-α-ionone β-Ionone to 3-hydroxy-β-ionone	[53]
P450(camr)	*Rhodococcus* sp. NCIMB 9784	(+)-Camphor to 6-endo-hydroxycamphor	[136]
Putative CYPs CotB3 +CotB4	*Streptomyces melanosporofaciens* MI614-43F2	Cyclooctat-9-en-7-ol to cyclooctat-9-en-5,7-diol to cyclooctatin	[137]

The number of microorganisms described to convert terpenes is continuously growing. A major driving force behind this is the extensive research for the discovery of novel microbial production routes towards flavors and fragrances and the rising interest and demand of bioactive terpenoid compounds for pharmaceutical applications. A straightforward approach in identifying an adequate biocatalyst is the screening of microorganisms that can use the substrate as the sole carbon source, thereby indicating the existence of a substrate-degrading metabolic pathway. The first step is the isolation and characterization of organisms that come into close contact with terpenoids in their natural habitat. Examples include the isolation of the α- and β-pinene degrading *Bacillus pallidus* BR425 from pine trees [21] and the identification of limonene-degrading microorganisms from orange peel [22]. Other interesting targets for screening approaches are organisms that are known to have a well-developed xenobiotic metabolism, which in turn indicates the presence of interesting P450s. Both approaches are extensively applied and contribute to the description of newly identified terpene biotransformations by microorganisms, whereas in many cases the P450 enzymes that catalyze the reactions are not yet identified. The identification and isolation of the genes and enzymes, however,

opens up the possibility of applying protein engineering techniques to overcome the problem of low transformation rates, which are still the main obstacle for large-scale applications [9]. Furthermore, the cloning of P450s allows their expression in heterologous whole-cell hosts that can enable a more effective terpene oxidation process. For several examples given in Table 1, the terpene-hydroxylating activity of the cytochrome P450 was not described until years after the first isolation and characterization of the enzyme. This is often the case with so-called orphan P450s, for which the natural substrate is not known, or xenobiotic-degrading P450s with a broad substrate range [23]. In such circumstances, screening of compound libraries is a common method to examine the substrate range and often results in the identification of new terpenoid substrates. On the other hand, the examples of CYP102A1 (P450 BM-3) and CYP101A1 ($P450_{cam}$) have proven that protein engineering is a powerful method to develop tailored enzymes for the oxidation of a given terpene substrate.

Taken together, the rapidly growing number of P450 structures, the continuous identification of microorganisms capable of converting terpenes, triggered by the trend of classical chemical oxidation methods for terpenes gradually being replaced by biotechnological methods, as well as recent progress in the development of effective protein engineering methods, will surely lead to a steady increase in the identification and importance of microbial cytochromes P450 for terpene hydroxylation.

4 Engineering of Microbial Cytochromes P450 for Terpene Hydroxylation

Numerous protein engineering approaches have been applied to improve the performance of P450s further, and most work has been focused on CYP101A1 from *Pseudomonas putida* and CYP102A1 from *Bacillus megaterium*. Both are well-characterized, representative enzymes that can be easily expressed in *E. coli*. They are also the first two P450 enzymes for which crystal structures could be solved [24, 25]. CYP101A1 naturally oxidizes the terpenoid (1R)-camphor to 5-exo-(1R)-hydroxycamphor, the first step in the camphor metabolism pathway of *P. putida*. For the completion of the electron transfer reaction, the enzyme depends on two additional electron transfer proteins: putidaredoxin, a ferredoxin of the [2Fe–2S] type, and putidaredoxin reductase, a FAD-containing enzyme. It can therefore be assigned to class I of the P450 group. In contrast, the CYP102A1 is a self-sufficient fusion enzyme that contains both a P450 and a reductase domain and therefore only requires NADPH and oxygen to function (class VIII) [8]. As a result, the catalytic activity of this fatty acid hydroxylase is comparatively high, with a k_{cat} value of more than 5,000 min^{-1} for the hydroxylation of arachidonic acid [26]. In comparison, most other P450 reactions are very slow, with k_{cat} values of less than 100 min^{-1}.

The different protein engineering methods that have been applied to alter P450 enzymes can be grouped into different categories and are illustrated in the following sections using CYP101A1 and CYP102A1 as examples.

4.1 Engineering by Directed Evolution

Directed evolution is a method that mimics natural selection in order to generate proteins with new or improved activities. This approach starts with the generation of a library of mutated P450 enzymes using techniques that randomly introduce mutations into the protein sequence, for example, error-prone polymerase chain reaction (*ep*PCR). After screening for new or improved activities, the respective mutants are isolated and then used to generate new libraries by random mutagenesis or recombination technologies. These iterative cycles continue until the desired mutant is obtained. The possibilities of this technique are manifold and span not only the improvement of catalytic activity and change of regio- or stereoselectivity but also the conversion of nonnatural substrates. Many studies have been performed to engineer the heme domain-coding region of CYP102A1 using directed evolution. Examples include the improvement of activity: the wild-type CYP102A1 typically hydroxylates saturated and nonsaturated fatty acids, but also has a low hydroxylation activity for β-ionone of less than 1 min^{-1}. Two rounds of *ep*PCR resulted in the triple mutant A74E F87V P386S, which exhibited an increase in activity of up to 300-fold compared to the wild-type enzyme [27]. Furthermore, there are many examples demonstrating that directed evolution is a powerful tool for the generation of P450 libraries with completely new activities. However, because these activities are often accompanied by low enzyme activity and stability, the most promising candidates usually need additional optimization by rational protein design methods. Other strategies to enhance the performance of CYP102A1 as catalyst in chemical synthesis using laboratory evolution methods aim towards an improvement of enzyme stability [28]. Among the broad range of substrates that are hydroxylated by CYP102A1 are also water-insoluble compounds, for example, the sesquiterpene valencene. In these cases, polar organic cosolvents are added to increase substrate solubility and to achieve high catalytic efficiency. Applying directed evolution, the tolerance of CYP102A1 towards the cosolvents dimethylsulfoxide (DMSO), tetrahydrofuran (THF), acetone, acetonitrile, dimethylformamide, and ethanol could be significantly increased [29].

4.2 Engineering by Rational Protein Design

In contrast to directed evolution, rational protein design relies on methods such as site-directed mutagenesis with the aim of rationally altering the properties of an enzyme. A fundamental prerequisite for this method is a solid structural basis in

terms of profound knowledge of the key amino acid residues that are involved in the characteristics to be influenced. The analysis of protein structural models, especially in their substrate-bound state, provides us with some insight into the mechanism of substrate binding and furnishes ideas for rational engineering. The role of CYP101A1 and CYP102A1 as model enzymes for the rational protein design of cytochromes P450 is highlighted by the fact that there are crystal structure data available for several dozens of mutants of both enzymes (see Protein Data Bank (PDB) at: http://www.rcsb.org/pdb/). Most studies of these enzymes focus on altering the specificity and regioselectivity of the catalyzed hydroxylation reactions. Analysis of the active site of CYP101A1 and comparisons of the structure of target substrates with that of camphor led to the design of active-site mutants with greatly enhanced activity for the oxidation of (+)-α-pinene [30]. Based on the enzyme/substrate contacts revealed by the structure of the triple mutant F87W/Y96F/V247L with (+)-α-pinene bound within the active site, additional mutations were carried out and resulted in an improved selectivity for the formation of the natural fragrances and flavors (+)-*cis*-verbenol and (+)-verbenone [31]. In addition, results from studies of other mutants suggest that CYP101A1 can be engineered to oxidize compounds substantially larger than camphor by manipulating the active site volume and topology to maneuver the substrate binding orientation [32].

A central starting point for the rational protein design of CYP102A1 was the identification of a hydrophobic channel between the surface of the enzyme and the proximal side of the heme. The hydrophobic residues in this channel, especially amino acid F87, have been shown to affect both the substrate specificity and regioselectivity [33]. Replacements of this residue were used in many studies as the starting point for the creation of new site-directed mutants with specificities towards nonnatural substrates. Another CYP102A1 mutant created by rational mutagenesis for the hydroxylation of shorter-chain fatty acids was unexpectedly capable of hydroxylating various alkanes and cycloalkanes, indicating some promise for engineering the enzyme for isoprenoid substrates [34].

4.3 Engineering by Chimeragenesis

Chimeragenesis imitates the natural principle of gene recombination by using techniques such as DNA shuffling and SteP recombination to produce a variety of chimeric genes [35, 36]. This method is often more efficient than random mutagenesis for generating mutants with new properties, inasmuch as the amino acid substitutions have already proven to be successful in one of the parents. However, genes normally need to exhibit a sequence identity of 70 % or higher in order to be successfully recombined. Problems may arise due to the generally low sequence identity of P450s of less than 20 % and recombination at crossover points that produce unstable and inactive mutants. An effective tool to overcome this shortcoming in designing P450 chimeras is the structure-based computation algorithm SCHEMA, which was designed to identify optimal crossover points for generating

protein fusions [37]. Chimeragenesis can be applied using P450 enzymes with a high sequence homology to change the substrate selectivity and activity. Additionally, the construction of chimeras between soluble and membrane-bound P450s may provide a means of generating soluble P450s that are functionally active in microbial hosts. The creation of diverse libraries of chimeric P450s therefore opens exciting opportunities for the engineering of P450s.

In the case of CYP102A1, a "scanning chimeragenesis" approach was used to study the roles of individual structural elements that determine the substrate specificity towards terpenoids [38]. For this, several amino acid residues of the CYP102A1 substrate binding site were replaced with a homologous fragment from CYP4C7, an insect P450 catalyzing the regio- and stereospecific hydroxylation of the sesquiterpenoid farnesol to (10E)-12-hydroxyfarnesol [39]. The regiospecificity of monooxygenation was shifted and resulted in the formation of new products not seen with wild-type CYP102A1. Further studies eventually led to a chimeric enzyme that produces 12-hydroxyfarnesol as the major product, with an approximately twofold increase in turnover number towards farnesol as compared with wild-type CYP102A1 [40]. Another promising application for chimeragenesis is the production of chimeric oxygenases in which the P450 domain is fused to the reductase domain of a self-sufficient P450. A good example for this is the fusion of CYP101A1 to the reductase domain of P450RhF, comprising flavin mononucleotide- and NADH-binding motifs and a [2Fe-2S] ferredoxin-like center [41]. The resulting catalytically self-sufficient chimera can achieve high conversion rates and has a high potential for applications in biocatalysis, especially of the natural terpene substrates. Additionally, the production of a library of self-sufficient chimeric P450 enzymes and mutants can also be a useful tool for high-throughput screening protocols for the identification of novel biooxidation activities [42].

4.4 Engineering Improved Redox Partner Interaction

The above-described generation of self-sufficient cytochrome P450 variants addresses an important issue in P450-catalyzed reaction. As external monooxygenases, most P450s have to interact with partner proteins to receive electrons for oxygen activation and substrate conversion, and in most cases, the rate-limiting step is the transfer of the second electron to the hydroxylase component. Improving redox partner interaction and electron transfer is, therefore, an important tool to enhance product formation and thereby improves the potential of P450 systems for biotechnological applications [3, 9, 43].

Investigations of the native CYP101A1 system, consisting of the monooxygenase, the electron transport protein putidaredoxin, and putidaredoxin reductase expressed in *E. coli*, showed a marked enhancement in the conversion of camphor when utilizing C73S/C85S-Pdx, a stable putidaredoxin mutant instead of the wild-type protein [44]. Aside from homologous redox systems, choosing heterologous redox partners and their further tuning by protein design can also increase the

activities of CYP systems. A good example of this is the mammalian electron transfer protein adrenodoxin (Adx) that turned out to be three- to fivefold more efficient in supporting the substrate conversion by a myxobacterial CYP than the autologous redox systems [45]. Because the bacterial Pdx transfers electrons much faster to its redox partner CYP101A1, an "evolutionary" approach was used to produce a truncated Adx-mutant with an additional amino acid exchange (S112W) that exhibits higher similarity to Pdx than the wild-type. This mutant showed a 75-fold increase in the homologous CYP11A1-dependent cholesterol conversion system [46]. Its usefulness for the conversion of terpenoids by bacterial CYPs needs to be investigated. However, the truncated Adx variant proved to be a very efficient heterologous redox partner in many bacterial P450 bioconversion systems, also in those converting terpenoids [47].

These results demonstrate that protein engineering methods are not limited to the hydroxylase component of P450 systems and further improvement of redox partner interaction can surely lead to increased substrate conversion.

5 Examples for Terpenoid Conversions Using Microbial Cytochromes P450

The following sections provide selected examples for the conversion of important terpenoids by microbial cytochromes P450 and thereby show different approaches in exploiting the biotechnological potential of these enzymes. Aside from monoterpenes (limonene), sesquiterpenes (valencene), diterpenes (resin acids), and triterpenes, additional examples are given for terpene derivatives such as norisoprenoids (ionone), steroids, and vitamin D_3.

5.1 Ionones

Ionones (α-, β-, and γ-) are norisoprenoids that are derived from the degradation of carotenoids. They are aroma components of floral scents and can be found in a variety of essential oils. The organoleptic properties of ionones and especially their oxygenated derivatives have attracted the attention of the fragrance and flavor industries. Additionally, α-ionone and its derivatives form important components of insect lures and can favor insect pollination [48], whereas hydroxy-β-ionone is an important intermediate for the synthesis of carotenoids and deoxyabscisic acid, a synthetic analogue of the phytohormone abscisic acid [49]. Because of the potential industrial applications, several microbial transformation studies of α- and β-ionone were performed using various fungal strains including *Aspergillus* sp. [50] and bacterial strains including *Streptomyces* sp. [51].

Cytochrome P450 monooxygenases are very useful for the oxygenation of ionones and several P450s from actinomycetes that are able to hydroxylate α- and β-ionone have been identified. Among those are CYP105A1 and CYP105B1 are from *Streptomyces griseolus* and CYP105D1 is from *Streptomyces griseus*. Using a recombinant expression system in *E. coli*, all three enzymes were shown to convert both α- and β-ionone into hydroxylated products. Although the activity of CYP105A1 and CYP105D1 towards α-ionone was rather low, CYP105B1 was able to produce 3-hydroxy-α-ionone with a conversion rate of more than 50 % [52]. Comparable results could be obtained for the conversion of β-ionone, with 4-hydroxy-β-ionone being the main product. Regarding the moderate enantioselectivity of CYP105B1 towards β-ionone, this enzyme provides an ideal target for biocatalyst improvement, either by rational protein design or directed evolution.

In contrast to the hydroxylation of α- and β-ionone with members of the CYP105 family where a mixture of different hydroxylated products has been observed in addition to the main product, two P450s from the soil-dwelling myxobacterium *Sorangium cellulosum* So ce 56 were found to hydroxylate both α- and β-ionone in a very regioselective manner. For CYP109D1, the exclusive production of the main products of the CYP105 catalyzed reactions, namely 3-hydroxy-α-ionone from α-ionone and 4-hydroxy-β-ionone from β-ionone, could be shown using a reconstituted P450 system [45]. The reason for the high regioselectivity of the enzymatic hydroxylation lies in the stereochemistry of ionones. The presence of two methyl groups at C-1 directs any oxidative attack towards C-3. Likewise, the electronic activation of the allylic hydrogens by the double bond of the cyclohexane ring governs the regioselective hydroxylation to position C-4 of β-ionone. Nevertheless, another P450 from this organism, namely CYP264B1, was shown to hydroxylate both α- and β-ionone in a highly regioselective manner at the C-3 position, giving 3-hydroxy-α-ionone and 3-hydroxy-β-ionone, respectively [53]. For the elucidation of the structural basis for the selectivity of ionone hydroxylation, both α- and β-ionone were docked into the active sites of CYP109D1 and CYP264B1 (see Fig. 2). The computational data justified the experimental observations and form the structural basis for advanced studies using rational protein design.

Concerning the activity, the most promising P450 for the hydroxylation of ionones is still CYP102A1. Based on earlier studies on the expansion of its substrate range [34], several P450 CYP102A1 mutants have been constructed using rational protein design as well as directed evolution that are able to hydroxylate β-ionone at C-4 regioselectively with high activity and rather high coupling efficiency [27].

As for most terpenoid substrates, the number of identified P450s that are able to accept ionones as substrates is constantly increasing. With CYP101C1 from *Novosphingobium aromaticivorans* DSM12444, a homologue of CYP101A1 has been described very recently that does not bind camphor but is capable of binding and hydroxylating ionone derivatives including α- and β-ionone and β-damascone [54]. Scheme 1 gives an overview of P450 catalyzed ionone conversions.

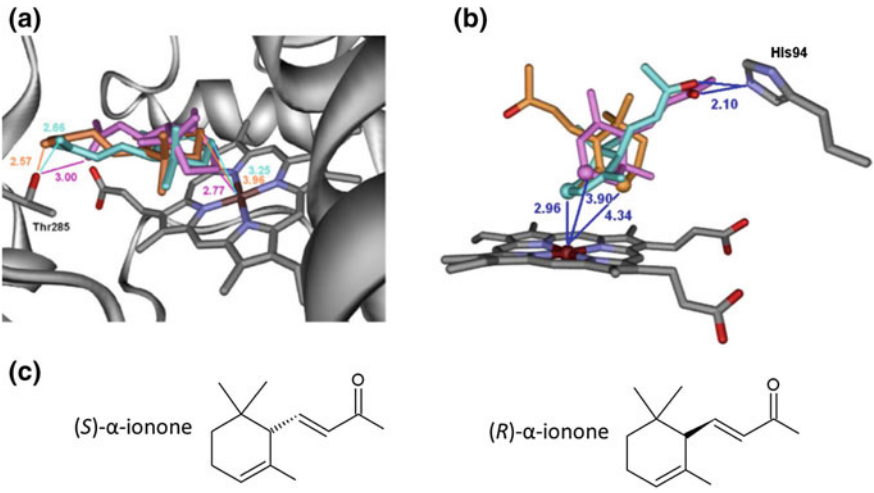
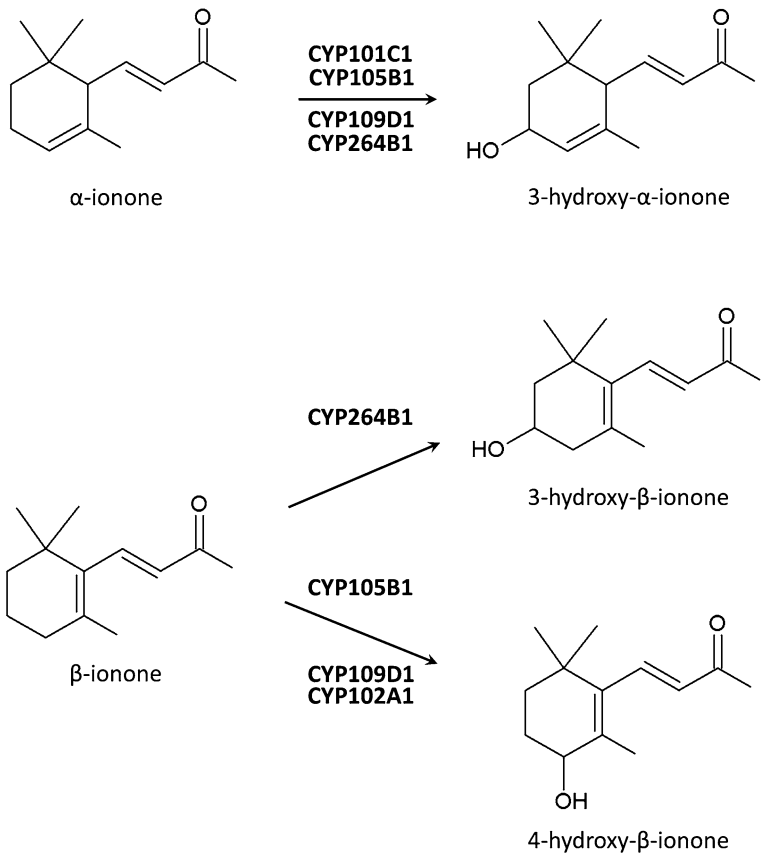


Fig. 2 Docking positions of ionone in the homology model of CYP264B1 (**a**) and CYP109D1 (**b**). The *R*-isomer of α-ionone is shown in pink, the *S*-isomer in cyan, and β-ionone in orange. The distances between the position where hydroxylation occurs and the heme iron are given in Ångstroms. (**c**) *R*- and *S*-isomer of α-ionone. Figure (**a**) taken from [53] and Figure (**b**) taken from [45]

5.2 Limonene

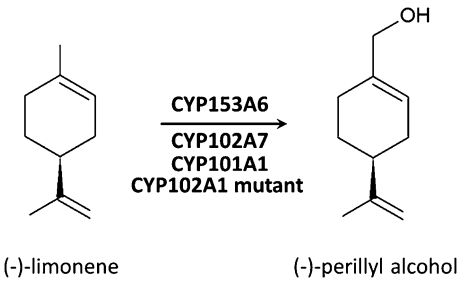
Limonene is a chiral cyclic monoterpene, the D-enantiomer of which constitutes the main component of many citrus peel oils, such as that from oranges. D-Limonene is usually obtained via centrifugal separation or steam distillation of citrus fruits and areas of application include cosmetics and perfumery as well as food manufacturing where it is used as a flavoring. It is also added to cleaning products due to its lemon/orange odor. In contrast, the enantiomer L-limonene (e.g., found in noble fir and peppermint plants) has a piney/turpentine-like odor. The main metabolites of limonene in the human body are (+)- and (−)-perillyl alcohol, which are products of 7-hydroxylation by CYP2C9 and CYP2C19 in the liver [55]. The CYP-catalyzed reaction of (−)-limonene to (−)-perillyl alcohol is shown in Scheme 2. Aside from its antibacterial and antifungal effects, (−)-perillyl alcohol is of considerable pharmaceutical interest due to its anticarcinogenic properties [56]. As with many other terpenoids, the extraction of perillyl alcohol from plant tissues yields only small amounts of this compound and the chemical oxidation of readily accessible limonene results in a complex mixture of products. The production of significant quantities of side products, namely carveol, carvone, and terpineol, also occurred in the case of the first microbial enzyme system described to transform limonene to perillyl alcohol in *Bacillus stearothermophilus* BR388 [57]. In a study with the aim of identifying bacteria capable of regiospecific hydroxylation of limonene, 1,800 bacterial strains were grown on a range of relatively reduced substrates such as



Scheme 1 CYP-catalyzed reactions of α- and β-ionone

toluene, naphthalene, and various alkanes. Because previous results demonstrated that many catabolic oxygenases accept a wide range of unnatural substrates, this procedure was chosen to identify oxygenases involved in catabolic pathways that would probably also accept L-limonene as a substrate. Using this approach, CYP153A6 from *Mycobacterium* sp. strain HXN-1500 was found to oxidize limonene exclusively at the carbon atom in position 7 (see Scheme 2) [58]. In recent studies, metabolic engineering was applied with the aim of utilizing this enzyme for limonene and perillyl alcohol production from simple carbon sources in *E. coli* [59]. To this end, the genes of a heterologous mevalonate pathway, a GPP synthase, a limonene synthase, and CYP153A6 were expressed in the bacterial host. After several gene modifications to improve the enzyme availability and activity, L-limonene titers reached 435 mg/l and perillyl alcohol was produced with 105 mg/l from 1 % glucose. These results mark an important step in the production of valuable terpenoids from simple sugars using microbial enzymes and hosts.

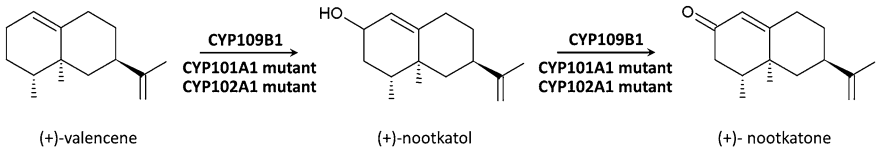
Scheme 2 CYP-catalyzed reaction of (−)-limonene to (−)-perillyl alcohol



Another P450 that has been tested for perillyl alcohol production in *E. coli* is CYP101A1. After coexpression with its homologous redox partners from *P. putida*, the whole-cell 7-hydroxylation of L-limonene to (−)-perillyl alcohol could be confirmed [60]. CYP102A1, the second major model P450, is also able to produce perillyl alcohol from limonene. In an approach to optimize the regiospecificity through rational protein design, this enzyme has been engineered to increase the selectivity of limonene hydroxylation at the terminal allylic C-7 position by up to 97 % [61]. The applied methods could also be useful in the engineering of other limonene hydroxylases such as CYP102A7 from *Bacillus licheniformis* [62] and CYP147F1 from *Streptomyces peucetius* [63].

5.3 Valencene

The bicyclic sesquiterpenoid (+)-nootkatone was first isolated from the Nootka cypress tree *Callitropsis nootkatensis*, but it can also be found in the fruits of citrus plants including grapefruit and pomelo. It exhibits unique odor characteristics and is, therefore, a highly valued and highly demanded aroma compound for the food and flavor industry. In addition, (+)-nootkatone is also an effective and environmentally friendly repellent of insects such as mosquitos, ticks, and bedbugs [64, 65]. Limitations in application are dictated by the costly extraction of this compound from natural sources in connection with inadequate yields. Access to (+)-nootkatone in industrial production processes is, therefore, often provided by oxidation of the cheap and abundantly available sesquiterpene (+)-valencene. However, chemical oxidation methods often rely on unsafe agents such as *tert*-butyl peracetate or *tert*-butyl hydroperoxide in combination with surface-functionalized silica-supported metal catalysts [66]. As an alternative to chemical oxidation methods, a huge number of biotechnological processes using whole-cell biotransformations in bacteria and fungi, as well as plants have been described [67]. In addition to laccases and peroxidases, many of these biotransformation reactions are catalyzed by enzymes of the cytochrome P450 superfamily. The involvement of a P450 enzyme is often detected indirectly as in the case of (+)-valencene bioconversion in the ascomycete *Chaetomium globosum*, where a decrease in product concentrations could be observed



Scheme 3 CYP-catalyzed reaction of (+)-valencene to (+)-nootkantone via (+)-nootkatol

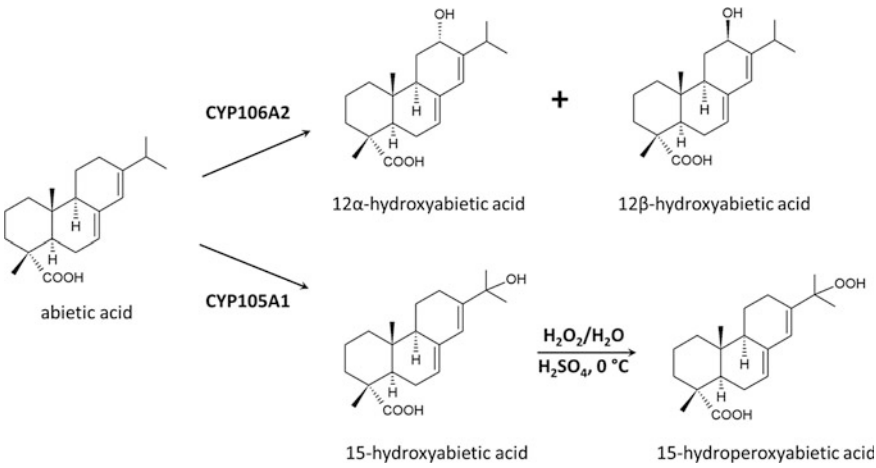
after addition of the P450 inhibitor proadifen [68]. The direct identification of efficient and regioselective P450 enzymes matching commercial requirements still remains a challenge. In a study testing 125 bacterial P450 enzymes, CYP109B1 from *Bacillus subtilis* was found to catalyze the oxidation of (+)-valencene at position C-2 to nootkatol and (+)-nootkatone with the production of 120 mg l^{-1} of the desired products after optimizing the reaction [69] (see Scheme 3). However, the biooxidation suffered from the formation of undesired multioxygenated products that can appear when the active site of the enzyme allows different binding orientations for an unnatural substrate. A similar observation could be made in the case of (+)-valencene conversion with CYP102A1, where the selectivity pattern of the reaction suggests multiple orientations of the substrate within the active site [70]. In contrast, different mutants of CYP101A1 could oxidize (+)-valencene with a regioselectivity for C-2 oxidation of more than 85 % yield, whereas the wild-type enzyme is not able to accept (+)-valencene as a substrate. The protein engineering challenges therefore differ for these two P450s: both substrate specificity and selectivity of the CYP102A1 require improvement whereas the CYP101A1 exhibits the desired selectivity but the activity has to be increased.

5.4 Resin Acid Diterpenoids

Diterpenes are derived from geranylgeranyl pyrophosphate and usually have the molecular formula $C_{20}H_{32}$. They form the basis for important compounds such as the vitamin precursors retinol and phytol. In addition, many diterpenes and their derivatives exhibit antibacterial, antifungal, and anti-inflammatory actions. Prominent representatives are the tricyclic diterpenoids of the abietane- and pimarane-type which are gathered under the name resin acids and can be found in tree resins. Other examples include labdane, a bicyclic diterpene that forms the core for a wide range of natural products, and taxadiene, an important intermediate in the synthesis of the mitotic inhibitor taxol used in cancer chemotherapy. The number of described microbial P450 enzymes that can be used for the biotransformation of diterpenoid substrates is still limited. Recently, two members of the CYP226 family able to hydroxylate different abietane-type diterpenoids could be identified in *Burkholderia xenovorans* LB400 [71]. This proteobacterium is well known for its noncatabolic physiological adaptation to organic compounds and both CYP226 enzymes have distinct roles in the abietane catabolism. Many other organisms that are capable of

abietane diterpenoid biodegradation have been described and the involvement of P450s has been proven in several cases, such as the *Pseudomonas abietaniphila* BKME-9 [72]. It can, therefore, be speculated that the number of cytochromes P450 described as diterpenoid hydroxylases will sharply grow in the future. In addition to those enzymes involved in catabolic pathways, orphan P450s also play an important role when it comes to the description of hydroxylating activity towards new substrates. For example, the CYP106A2 from *B. megaterium* ATCC 13368, whose natural substrate is still unknown but which is known to hydroxylate different steroids, was recently also described as a regioselective allylic bacterial diterpene hydroxylase [73]. By screening of a compound library consisting of more than 16,000 potential substrates, the diterpene derivative dihydrochinopimaric acid could be identified as binding in the CYP106A2 active site. Further studies with structurally related compounds showed the conversion of abietic acid to 12α- and 12β-hydroxyabietic acid, respectively (see Scheme 4). The existence of many mutants of CYP106A2, created by directed evolution as well as rational protein design [74, 75], provides an excellent source to exploit the potential of this P450 for further biotechnological applications. Due to their diverse biological properties, the production of hydroxylated diterpene derivatives could be especially useful for the pharmaceutical industry. A vivid example is the conversion of abietic acid into 15-hydroxyabietic acid catalyzed by CYP105A1 from *S. griseolus* [14, 76] (see Scheme 4). This substance is the precursor of the strong contact allergen 15-hydroperoxyabietic acid and only hard to obtain from natural sources or by chemical means. The availability via the recently established biotechnological process gives a new and promising basis for the production of this compound for test developments against allergic compounds.

Much effort has been undertaken to engineer microbial hosts for the production of functionalized diterpenoids, for example, in the production of important drugs



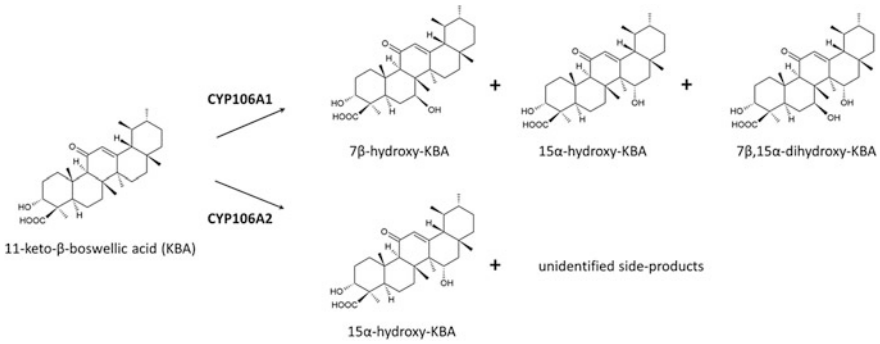
Scheme 4 CYP-catalyzed hydroxylations of abietic acid

such as taxol. In most cases, researchers focus on the plant P450s of the homologous biosynthetic pathways. However, because their use is accompanied with problems and challenges of the membrane localization of these enzymes, the identification and engineering of bacterial P450s for these reactions could prove to be very beneficial.

5.5 Triterpenoids

Triterpenoids consist of six isoprene units and the simplest triterpene regarding the complexity of the carbon skeleton is squalene. This compound is produced via head-to-head condensation of two C15 units of farnesyl diphosphate and constitutes the precursor of polycyclic triterpenes. From a biological perspective, the most important triterpenoid structures are the oleanane, ursane, lupane, and dammarane/euphane carbon skeletons [77]. In contrast to mono- and sesquiterpenes, which are mostly found in essential oils of plants, triterpenes are predominantly found in resin and balsams. Their biological effects are diverse and comprise anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory, and tonic effects [77]. However, the use of many triterpenoids for pharmaceutical applications is restricted due to hemolytic and cytostatic properties. In order to overcome these limitations and to expand the range of usable triterpenes, a modification of these compounds by means of chemical or biotechnological techniques is possible [78]. For this purpose, microbial transformation is particularly suitable because it often results in a modification with a high regio- and enantioselectivity and many microorganisms that are able to transform triterpenoids have been described [78]. An example is *B. megaterium* ATCC 13368, which was shown to perform an hydroxylation at the C-1 or C-11 site of the antimelanoma agent betulinic acid [79]. The aforementioned CYP106A2 from this organism belongs to the few described microbial P450s that can transform triterpenes. Recent studies showed that this P450 is able to hydroxylate the pentacyclic triterpene 11-keto-β-boswellic acid (KBA) to 15α-hydroxy-KBA, depicting an interesting reaction because this position is not accessible via synthetic chemical methods [80].

Comprehensive studies with CYP106A1, another member of the CYP106 family, revealed that this P450 mainly hydroxylates KBA at position 7β. Additional products, namely 15α-hydroxy-KBA and 7β,15α-dihydroxy-KBA, were formed in a lower amount [81]. These novel KBA derivatives can possibly show an enhanced bioavailability or improved pharmaceutical activities compared to the substrate KBA or can be the basis for additional modifications by chemical methods [81]. This is especially interesting inasmuch as pentacyclic triterpenes have gained significant importance because their chemical structures resemble those of steroids and there is an increasing demand especially for boswellic acids for pharmaceutical and medicinal studies [82]. Scheme 5 shows the hydroxylation of 11-keto-β-boswellic acid by CYP106 family members. Moreover, the substrate range of CYP106A2 is not limited to KBA. The screening of a natural product library revealed the binding



Scheme 5 Hydroxylation of 11-keto-β-boswellic acid by CYP106 family members

of additional triterpenes and conversion of the tetracyclic dammarane-type triterpenoid dipterocarpol to 7β,11α-dihydroxydipterocarpol could be shown [83]. These are very recent research results, therefore thus far no protein engineering studies have been performed to exploit fully the potential of P450s for triterpenoid functionalization. Considering the background of two closely related P450 enzymes, CYP106A1 and CYP106A2, that are both capable of hydroxylating the same substrate with a different regioselectivity, the starting point for the elucidation of the structural basis that determines this selectivity, and subsequent approaches to influence it, seems to be very promising.

5.6 Steroids

Like triterpenoids, steroids are derived from squalene and constitute a class of very important molecules that are involved in the regulation of essential processes in mammals. They are synthesized from cholesterol, which is biologically produced from lanosterol in animals. The fact that steroidal drugs belong to the second most marketed drugs next to antibiotics [84] highlights the important role of steroids in biotechnology [3, 85]. Because the chemical synthesis of steroid compounds requires multiple reaction steps, harsh reaction conditions, and cost-intense catalysts that are often toxic to organisms and harmful to the environment, microbial biotransformations have been used for the generation of novel steroidal drugs for decades [86]. This carries the advantage that low-cost precursors such as cholesterol or diosgenin can be exploited for further hydroxylations in positions that are interesting for the production of novel hydroxysteroids (7α, 9α, 11α, 11β, 16α, and 17α). Although the history of microbial steroid production started in the early 1950s, the steroid-modifying enzymes have not yet been characterized in most cases. An exception is CYP154C5 from *Nocardia farcinica*, which was identified as potent 16α-hydroxylase for testosterone and similar steroids [87]. Moreover, CYP106A2, which was identified in the 1970s to be responsible for the ability of *B. megaterium*

ATCC 13368 to convert progesterone into 15β-hydroxyprogesterone [88–90], is also able of catalyzing a 15β-hydroxylation of other 3-oxo-Δ4-steroids such as 11-deoxycorticosterone, testosterone, and 11-deoxycortisol [74, 75, 80, 91]. Later on it was found that di- and triterpenes can also be highly selectively hydroxylated by CYP106A2 [73, 80, 83, see above].

As a soluble prokaryotic P450, CYP106A2 depicts an excellent candidate to establish a steroid bioconversion process. It can be expressed with high yields in *E. coli* and *B. megaterium* and has already been applied for the preparative hydroxylation of several steroids, including dehydroepiandrosterone, pregnenolone, testosterone, deoxycorticosterone, deoxycortisol, and progesterone [92–94]. Recent studies showed that the main hydroxylation position of CYP106A2 is influenced by the character of groups attached to C-3 in the steroid core: 3-oxo-Δ4-steroids are preferentially hydroxylated at the 15β position whereas the 7β-position is favored when converting 3-hydroxy-Δ5-steroids [92]. However, the hydroxylation reaction is not strictly regioselective and the formation of different side products occurs in many CYP106A2-dependent bioconversions as, for instance, in the case of progesterone where 11α-, 6β-, and 9α-hydroxyprogesterone are formed. Albeit these hydroxylations occur to a much lower extent, their products are of higher interest from an industrial point of view. Rational protein design and directed evolution approaches have, therefore, been performed to shift the selectivity of CYP106A2 in favor of progesterone 11α-hydroxylation [28, 75, 95]. To this end, the primary structures of CYP106A2 and the mammalian steroid-11β-hydroxylase CYP11B1 were aligned in order to identify amino acids that could be involved in the determination of stereoselectivity in the steroid hydroxylation. The greatest differences were found in the substrate recognition sites of both enzymes, and using site-directed mutagenesis, five amino acid substitutions (S394I, A395L, T396R, G397P, and Q398S) were performed to change the respective amino acids in CYP106A2 into those of CYP11B1. Two mutants, A395L and G397P, increased the 11α-hydroxylation efficiency of CYP106A2 by a factor of four [75]. To improve the 11α-hydroxylation further, saturation mutagenesis at positions A395 and G397 was performed and more than 13,000 transformants were screened. The mutants A395I and A395I/G397K have 8.9-fold and 11.5-fold higher 11α-hydroxylation activity compared with the wild-type. However, the total activity of the mutants was reduced by approximately 50 % compared with the wild-type enzyme. To increase the activity of these mutants, site-directed mutagenesis was applied to introduce previously identified mutations that result in higher active enzyme variants. This approach eventually led to the identification of three mutants (A106T/A395I/R409L, M155I/F165L/A395I, and D217V/A395I) with significantly increased k_{cat}/K_m values and 41–55 % 11α-hydroxylating selectivity. These mutants as well as the highly selective T89N/A395I and A106T/A395I mutants were tested for in vivo 11α-hydroxylation of progesterone using a whole-cell bioconversion system established for CYP106A2 in *E. coli*. The highest in vivo selectivity (80.9 % 11α-hydroxyprogesterone) was achieved with the double mutant T89N/A395I, demonstrating an impressive alteration in regioselectivity [28].

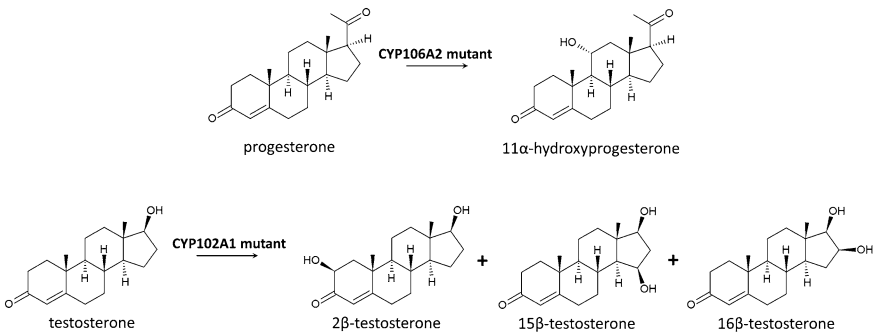
Due to the high interest in hydroxylated steroids, numerous studies also examined the potential of the well-characterized CYP102A1 as steroid hydroxylase and many mutants of this enzyme that catalyze the conversion of steroids were engineered over the past years. The first CYP102A1 variant described as steroid hydroxylase is the triple mutant R47L/F87V/L88Q. This mutant converts testosterone into 2β-hydroxy-, 15β-hydroxy-, and 16β-hydroxytestosterone. To identify amino acids crucial for testosterone biotransformation, the respective single and double mutants were also investigated. However, none of the single mutants was able to hydroxylate testosterone, whereas the double mutants F87V/L188Q and R47L/F87V converted testosterone into the same products as the triple mutant, although with a four- to tenfold lower conversion rate. Furthermore, positive homotropic cooperativity was observed with testosterone as substrate, suggesting that multiple substrate molecules can bind simultaneously into the binding pocket of CYP102A1 R47L/F87V/L188Q. Due to the lack of activity of the mutant R47L/L188Q, it was assumed that the mutation F87V is crucial for the testosterone hydroxylation activity of CYP102A1 [96]. Analysis of the ligand-free crystal structure revealed that phenylalanine 87 lies perpendicular to the heme at the end of the substrate-access channel. The central role of this residue in substrate selectivity has been studied before by replacing the large nonpolar residue with smaller ones such as alanine, resulting in the acceptance of a number of nonnatural substrates, due to the reduction of steric hindrance in the active site. The role of F87 in substrate selectivity and regioselectivity during testosterone conversion was studied using the highly active testosterone-hydroxylating mutant CYP102A1 M11, containing eight mutations in total [97]. Saturation mutagenesis was performed at position 87 to introduce all 20 canonical amino acids and testosterone was used to characterize the regioselectivity of the mutants. It was found that all mutants were able to catalyze testosterone hydroxylation, although the activities and regioselectivities differ [56]. It is noteworthy that these CYP102A1 mutants are thus far the only known microbial enzymes that catalyze the 16β-hydroxylation of steroids with F87I being the mutation with the highest 16β-hydroxylating activity [56].

Another recent study showed that CYP102A1 F87A hydroxylates testosterone to a mixture of 2β- and 15β-hydroxytestosterone (51 and 46 %), and 3 % 6β- and 16β-hydroxytestosterone. Applying laboratory-controlled evolution with the iterative combinatorial active-site saturation test (CAST) approach, a mutant with >95 % regioselectivity in favor of 2β-hydroxytestosterone as well as a mutant with opposite regioselectivity leading to >95 % 15β-hydroxytestosterone could be evolved [98]. The investigation of the ability of these mutants to hydroxylate other steroids in addition to testosterone showed that mutant F87A was also active towards progesterone. Interestingly, the substrate change shifted the selectivity from C-15 to C-16, with a low selectivity resulting in a mixture of 2β- and 16β-products of 18:82. By testing the mutants created with testosterone as substrate, a CYP102A1 variant with 100 % 2β-hydroxylating activity and a highly selective 16β-hydroxylating mutant producing 91 % 16β-hydroxyprogesterone were identified [98]. Among other amino acids that play important roles in the selectivity and activity of substrate conversion is alanine 82. Mutations at this position altered the

selectivity towards 16β-hydroxylation for both testosterone and norethisterone and led to a 42-fold increase in V_{max} for 16β-hydroxylation [98]. Mutations in this protein region can also increase the coupling efficiency of steroid hydroxylation, most probably because of a more efficient water exclusion from the active site due to a stronger substrate–enzyme interaction. Other studies concerning the hydroxylation of testosterone by different CYP102A1 mutants revealed a shift in the stereoselectivity from the 16β- to the 16α product resulting from a single amino acid substitution from serine to isoleucine at position 72 [99]. These examples highlight the power of protein engineering in the generation of highly specific steroid-hydroxylases based on the knowledge about important regions that determine substrate specificity and reaction selectivity. Scheme 6 highlights selected steroid hydroxylations catalyzed by bacterial cytochrome P450s.

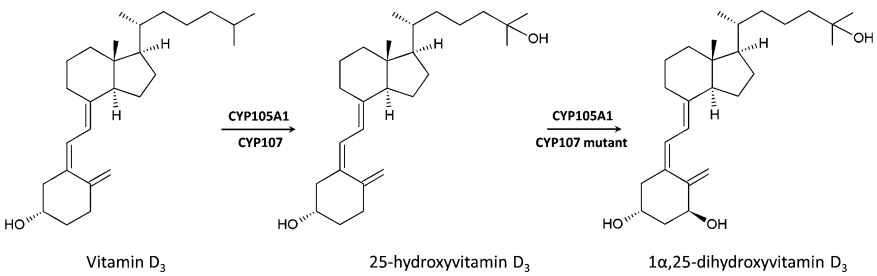
5.7 *Vitamin D_3*

Similar to steroids, Vitamin D_3 (VD_3) is derived from lanosterol, a tetracyclic triterpenoid compound. VD_3 is an important circulating secosteroid that regulates bone and calcium metabolism, cellular proliferation, differentiation, and immune response. In addition to these classical roles, a variety of nonclassical activities is described including effects on muscle function, cardiovascular homeostasis, and neuronal function [100]. In animals, it is produced in the skin by UV-light activation of 7-dehydrocholesterol and then transported into the liver by a vitamin D binding protein where it is hydroxylated at C-25 by CYP2R1. The following 1α-hydroxylation of 25(OH)D_3 into the active form 1α,25-dihydroxyvitamin D_3 is catalyzed by CYP27B1. Although this mitochondrial P450 is expressed in many tissues, only the kidneys are considered as a production site for circulating 1α,25$(OH)_2VD_3$. The active VD_3 mediates a variety of endocrine effects via binding to the vitamin D receptor (VDR), and regulation of VDR-dependent gene



Scheme 6 Exemplary steroid hydroxylations catalyzed by P450s

expression [101, 102]. VD_3 is essential for the human body and deficiency is associated with a variety of diseases and overall mortality. Pharmaceutical applications of VD_3, for instance, in the treatment of hypothyroidism, osteoporosis, and chronic renal failure, rely on the use of the active compound and result in a high demand for $1\alpha,25(OH)_2D_3$. Chemical synthesis of hydroxylated vitamin D requires approximately 20 steps and achieves only low yields, therefore there is great interest in a simple biotechnological route towards the dihydroxylated VD_3. One approach that has been realized for the biotechnological production of $1\alpha,25(OH)_2D_3$ is the expression of CYP27B1 for the hydroxylation of $25(OH)D_3$ at position 1α in recombinant *E. coli* cells. However, the 25-hydroxylated derivative is an expensive precursor for biotechnological approaches and a system capable of hydroxylating vitamin D_3 at both the 1α- and 25-positions would be much more efficient from an economical point of view. The first prokaryotic production of $1\alpha,25(OH)_2D_3$ was described in 1992 after screening several hundred *Streptomyces* strains towards their ability to hydroxylate VD_3. In this way, *Streptomyces sclerotialus* FERM BP-1370 and *Streptomyces roseosporus* FERM BP-1574 were found to convert $25(OH)D_3$ and $1\alpha(OH)D_3$, respectively, to $1\alpha,25(OH)_2D_3$ [103]. Given that none of the strains converted VD_3 directly into $1\alpha,25(OH)_2D_3$, an additional 500 bacterial and 400 fungal strains were investigated in further studies, and eventually led to the identification of 12 strains converting vitamin D_3 directly into the dihydroxylated derivative. Among these 12 strains, the fungus *Amycolata autotrophica* FERM BP-1573 exhibited the highest activity. Bioconversions with *A. autotrophica* (now renamed *Pseudonocardia autotrophica*) yielded 8.3 mg $25(OH)D_3$/l culture and 0.17 mg $1\alpha,25(OH)_2D_3$/l culture from cultivations in a 200-l fermenter after 120 h. Mapping the chromosome of *A. autotrophica* led to the identification of P450VD25, which was classified as CYP105A2 and cloned into *Streptomyces lividans*, where it provided 25-hydroxylation activity towards vitamin D_3. The VD_3 1α-hydroxylase (Vdh) from *P. autotrophica* could be identified as a member of the CYP107 family and conversion of VD_3 to $1\alpha,25(OH)_2D_3$ via $25(OH)D_3$ (see Scheme 7) was shown with this enzyme after purification from recombinant *E. coli*. It can, therefore, be assumed that CYP107 has both VD_3 25- and $25(OH)D_3$ 1α-hydroxylation activity [104]. This P450 is now applied in the bioconversion of vitamin D_3 into $1\alpha,25(OH)_2D_3$ [105]. To increase the hydroxylation efficiency of CYP107, random mutagenesis was performed and 1,000



Scheme 7 CYP-catalyzed hydroxylations of vitamin D_3

transformants were tested towards increased vitamin D_3 biotransformation. The results indicated eight hotspots associated with higher activity. Based on these findings, site-directed mutagenesis studies revealed four mutations with high vitamin D hydroxylating activity: T70R, V156S, E216A, and E384R. Each of these mutants showed a two- to threefold elevated biotransformation activity compared with the wild-type enzyme expressed in *E. coli*. Furthermore, the combination of the mutants showed a synergistic effect on enzymatic activity and the mutant combining all four amino acid substitutions showed a 7.9 times higher 1α,25$(OH)_2D_3$ product formation rate. Because the described amino acid residues are all located in the loop regions of the protein that undergo dramatic conformational changes upon substrate binding, the mutations may increase the flexibility of substrate binding sites or other domains involved in catalysis [104]. These results show that the hotspots for protein engineering of cytochromes P450 in order to achieve higher activities are not restricted to the active site of the enzyme. Another example of this is the highly active CYP107 mutant T107A. This residue is located in the ferredoxin binding site and crystallographic and kinetic analysis revealed that the mutation changes the protein conformation from an open to a closed state which increases the binding affinity with ferredoxin [106].

Another P450 that is capable of hydroxylating VD_3 is CYP105A1 from *S. griseolus*, an enzyme with a broad substrate range that was first identified in connection with its characteristic of metabolizing sulfonylurea herbicides. Sharing a sequence similarity of >55 % with CYP105A2, its ability to accept VD_3 as a substrate was further investigated. Cloning and functional expression in *E. coli* confirmed that CYP105A1 hydroxylates VD_3 [21] (see Scheme 7). In contrast to CYP105A2, which lacks the 1α-hydroxylase activity, CYP105A1 even converts VD_3 into 1α,25$(OH)_2D_3$ via 25$(OH)D_3$. However, the dihydroxylated VD_3 is formed in much lower amounts than the main metabolite 25$(OH)D_3$. Further drawbacks are the additional production of a trihydroxylated VD_3 derivative and the overall low activity towards these substrates. With the objective of increasing enzyme activity by structure-based protein design, the crystal structure of CYP105A1 was solved and the amino acid residues Arg73 and Arg84, located within the active site, were identified as key residues in vitamin D_3 catalysis [107]. Based on this finding, a series of single and double mutants was produced by site-directed mutagenesis leading to the identification of the double mutant R73V/R84A with a 435-fold higher specificity constant compared with the wild-type CYP105A1. The vitamin D_3 25-hydroxylation activity of the optimized CYP105A1 is comparable to the wild-type CYP107, whereas its 1α-hydroxylation activity towards 25$(OH)D_3$ is approximately one order of magnitude higher. Co-crystallization of the mutants with the reaction product revealed that the mutation R84A most probably increases the coupling efficiency of CYP105A1 by stabilizing the oxygenated form of the enzyme, whereas R73V (or R73A) alters the hydrogen-bond network resulting in a rearrangement in the water surrounding of the 1α-hydroxygroup [108]. The double mutant was used to establish a whole-cell VD_3 conversion system with *S. lividans* TK23 as host strain and VD_3 was hydroxylated into 1α,25$(OH)_2D_3$ to a notable amount [109]. These studies depict an excellent

example for the straightforward route from identification of an enzyme that is capable of biotransforming a given substrate, its subsequent optimization using structure-guided protein design, and the following development of a biotechnological process.

6 Use of Whole-Cell Systems

Although cytochromes P450 might seem the enzyme species of choice for biooxidation, their application faces more challenges compared to enzymes such as hydrolases, lyases, or transferases. As mentioned before, the low activity and stability of P450s as well as their need for auxiliary electron transfer proteins and expensive cofactors pose challenges in the application of these enzymes [9]. Certain progress has been made regarding the use of isolated P450 enzymes, among them different techniques of cofactor regeneration and immobilization of the protein in order to achieve higher stability. Nevertheless, the isolation of proteins is a time-consuming and expensive task and the applications of P450s in industrial processes are mostly restricted to fermentation. This carries the advantage of increased protein stability and efficient cofactor supply by the host cell metabolism. A major hurdle that remains, however, is the low productivity and yield of these biotransformations. Most P450-catalyzed processes are close to the minimum requirements and almost all examples of implemented industrial P450 processes concentrate on the production of compounds with high pharmaceutical interest for which the final product concentrations do not need to be as high as for the production of fine chemicals [110]. The use of whole cells also delivers new adjusting screws for optimization. The most efficient process can only be ensured when all necessary components are available at the right concentration, time, and location in the cell for optimal reaction conditions. The first question that needs to be addressed is, therefore, the selection of the appropriate host for the biotransformation. In the majority of cases, *E. coli* is the host organism of choice as the techniques of molecular biology are well developed and most P450s can be expressed as stable proteins displaying high activity. There are, however, cases where other organisms lead to better results. An example is the CYP153A6-catalyzed bioconversion of (−)-limonene to (−)-perillyl alcohol which reached 3.0 U/g in *P. putida* Gpo12 compared with 0.1 U/g in *E. coli* based on dry cell weight [58]. Because the transfer of large molecules across the cell membrane can become a limiting factor, the substrate availability within the host cell is an important point to be considered. Whole-cell bioconversion of abietic acid using different host organisms revealed that the transport of the diterpene resin acid into the gram-negative *E. coli* cell is much lower compared to the gram-positive *B. megaterium* [14]. In such cases, the use of permeabilizing agents or host engineering, for example, through cloning of additional transporter proteins, might be a promising approach to circumvent this problem. Another important issue in bioconversion processes is the formation of unwanted side products. In the case of the CYP153A6-catalyzed (−)-limonene

hydroxylation in *P. putida* KT2440, the side products perillyl aldehyde and perillic acid constituted up to 26 % of the total amount of oxidized terpenes. In contrast, no perillic acid and only minor amounts of perillyl aldehyde were formed when *E. coli* W3110 was used as a host [111]. A good way to achieve a higher concentration of the desired product is the setup of aqueous-organic two-liquid–phase systems. Using this approach, by-product formation in the biooxidation of (+)-valencene by recombinant *E. coli* cells expressing CYP109B1 could be significantly reduced [69].

A particularly exciting and challenging task in the use of whole cells is the construction of complete biosynthetic pathways. The metabolic engineering of several enzymatic steps allows the biosynthesis of complex products from simple carbon sources. An impressive example is the production of the antimalaria drug artemisinin in yeast and *E. coli*, where the overexpression of genes from the mevalonate biosynthetic pathway from *S. cerevisiae* resulted in an increased availability of endogenous precursors for terpenoid biosynthesis and thus artemisinic acid levels that are suitable for an industrial production process [112, 113]. In further studies that propose a novel semibiosynthetic route, the native CYP71AV1 from *Artemisia annua* that catalyzes the conversion of amorpha-4,11-diene to artemisinic acid was replaced by the bacterial CYP102A1. Saturation mutagenesis of key residues in the active site of this substrate-promiscuous P450 led to a mutant that was able to perform an epoxidation of amorpha-4,11-diene which could then be transformed by high-yielding chemistry to artemisinin [114]. This example illustrates the high potential of whole-cell systems that express terpenoid synthases together with P450 genes as interesting platforms for the easy and cheap production of valuable terpenoid compounds from simple carbon sources.

7 Concluding Remarks

Members of the cytochrome P450 superfamily significantly contribute to the diversification of natural compounds [115]. They usually depict the first enzymes to modify an initial terpene synthase product in plants. Although major advances concerning the engineering of plant P450s towards easier use for isoprenoid functionalization have been made during the last two decades, these enzymes still pose great challenges, especially in terms of microbial expression. Therefore, microbial, especially soluble bacterial, P450s catalyzing terpene hydroxylation became the focus of interest for biotechnological production of terpenoid compounds because they are easier to express in bacteria. As shown in this review, various bacterial P450s have been described catalyzing an efficient conversion of terpenoids with significant yields of up to several hundred mg/l/d, thus demonstrating the potential of these families of P450 enzymes for the production of terpenoids for the fragrance, flavor, and pharmaceutical industries. The discovery and characterization of further genome sequences as well as focused screening for

terpenoid conversion of known bacteria will further expand the source of P450s displaying activity towards terpenoids.

As P450s depend on redox partners, the identification, cloning, and coexpression of these electron transfer proteins might, however, comprise a significant barrier. While plant P450s use only one kind of electron transfer protein (CPR, Fig. 1), the redox partners of bacterial P450s might differ significantly [8]. However, it was shown that heterologous reconstitution of enzymatic activity using redox partners from other organisms, especially using bovine adrenodoxin can be very efficient [47]. In addition, strategies using fusion proteins with known redox domains may be a promising tool to overcome this obstacle [9]. The applicability of P450s for biotechnological purposes (e.g., terpenoid production) is further underlined by the major advantage of P450s in terms of their robustness against mutations, particularly in their substrate binding pocket. Although the basis for this ability is not yet fully elucidated, the high conformational variability and nonpolar nature of the active site seem to make an important contribution [116]. This high evolvability in terms of maintaining a folded structure and the catalytic activity, therefore, provides an excellent starting point for both rational and evolutionary approaches to improve substrate scope, selectivity, and activity. Such engineering methods can be used especially effectively with bacterial P450s, which are soluble and under normal conditions can be easily expressed with very high yields in bacteria. Successful examples have been described demonstrating the change in the selectivities of investigated P450s as well as improvements concerning activities and stabilities (see Sect. 5). With the decreasing costs associated with the redesign and engineering of these proteins in conjunction with higher chances of a successful outcome, P450-catalyzed syntheses become increasingly attractive for biotechnological applications. This is particularly true for the industrial production of terpenoid compounds in fields such as pharmaceuticals or flavors and fragrances.

Taken together, the examples described in this chapter show that microbial P450s are promising tools for the production of modified terpenes and illustrate how enzyme engineering can be used to redesign P450s in order to solve challenging chemical problems. The vastly growing number of P450 crystal structures deposited in the protein databank creates a valuable foundation for future studies using rational protein design to create enzymes that accommodate a wider range of unnatural substrates and exhibit improved or altered activity. Moreover, coexpression of engineered plant and bacterial P450s and combination of their different selectivities for the production of completely novel terpenoid structures will open new horizons for the production of flavor and fragrance as well as pharmaceutical compounds.

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