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Expanding the Toolbox of Organic Chemists: Directed Evolution of P450 Monooxygenases as Catalysts in Regio- and Stereoselective Oxidative Hydroxylation

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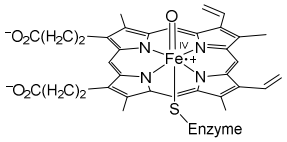
Cytochrome P450 enzymes (CYPs) have been used for more than six decades as catalysts for the CH-activating oxidative hydroxylation of organic compounds with formation of added-value products. However, it has not been possible to control regio- and stereoselectivity in a general manner, which is necessary for wide (industrial) applications. Especially directed evolution as a Darwinian approach to protein engineering has changed the situation for the better during the last 3-4 years leading to extensive progress, which is summarized and analysed in this Feature Article.

Introduction

The development of catalysts for the CH-activating oxidative hydroxylation RH→ROH of simple and complex organic compounds with high levels of regio- and stereoselectivity constitutes an area of intense current interest, synthetic transition metal catalysts[1] and biocatalysts of the type cytochrome P450 monooxygenases (CYPs)[2] being the main options. No catalyst can ever be truly general, and therefore synthetic organic chemists welcome a toolbox which features a variety of different man-made catalysts as well as biocatalysts,[3] the two often being complementary. More than six decades ago chemists at Upjohn, Merck, Pfizer and Schering utilized various organisms harboring CYPs in order to oxidatively hydroxylate steroids regio- and stereoselectively, the reaction of readily available progesterone with formation of the 11α-product being a prime example in which a fungus strain served as the catalyst.[4] The 11α-alcohol was easily converted by a few chemical steps into cortisone or hydrocortisone. This is a seminal example of a commercial process that combines the virtues of biocatalysis and synthetic methods, and a viable alternative to Woodward's impressive 40-step total synthesis of hydrocortisone.[5]

In the years that followed, further fungal and bacterial strains were screened for the hydroxylation of many other compounds. In parallel research, CYPs were isolated and characterized structurally and mechanistically.[2] The basic feature of the mechanism involves abstraction of an H-atom by the catalytically active high-spin heme-Fe=O intermediate (Compound I, Scheme 1) with formation of the respective radical, followed by rapid C-O bond formation.[2]



Scheme 1 Compound I [Ferryl (Fe^{IV})-oxo-π porphorin cation radical].[2]

Currently, whenever a given compound needs to be oxidatively hydroxylated in a biocatalytic manner, it is best to first test the available wild-type (WT) CYPs.[2,6] Unfortunately, this is not routinely successful. Either so-called rational design utilizing site-specific mutagenesis,[7] or directed evolution can then be invoked.[8] In rational design, structural and computational data is utilized in order to make reasonable choices for amino acid exchanges around the enzyme's binding pocket. This may require a trial and error strategy involving not just the generation of a single mutant, but also the formation of several likewise "designed" variants. The method has been applied to CYPs for a long time, especially by the groups of Negishi,[9] Poulos,[10] L.-L. Wong,[11] Munro[12] Vermeulen,[13] Commandeur,[13] Pleiss,[7,14] Urlacher,[15] Schmid,[15] Schwaneberg,[16] Bernhardt[17] and Hauer,[18] among others.[2] For example, on the basis of structural comparisons of different CYPs, Negishi showed 20 years ago that mutation L209N in mouse P450-(2a-4) induces androstenedione hydroxylase activity with high selectivity for 15α.[9] A more recent contribution is due to Wong et al, who designed the P450cam variant F87A/Y96F/L244A/V247L, which is an excellent catalyst for the regio- and diastereoselective hydroxylation of (+)-valencene with formation of (+)-*trans*-nootkatol (86%

selectivity).[19] This type of research has led to the accumulation of a great deal of information regarding the correlation of mutant structures and catalytic properties,[2f] which is useful in the expanding area of protein engineering of CYPs.

Directed evolution is a widely applied protein engineering method comprising iterative steps of gene mutagenesis, expression and screening (or selection) of mutant enzyme libraries, each cycle exerting "evolutionary pressure".[8] Due to the Darwinian character of the method, it is currently the most reliable approach to protein engineering. Random mutagenesis was first applied by Sligar to a CYP in 1997 using a mutator strain which led to a mutant showing altered regioselectivity in the oxidative hydroxylation of fatty acids, but enantioselectivity was not reported.[20] In a more systematic approach, Arnold et al applied directed evolution to a CYP as the catalyst in the oxidation of long-chain (protected) alcohols regioselectively at terminal positions,[21a] which was followed by an investigation using linear alkanes such as octane or nonane.[21b] The latter study resulted in some improvement of regioselectivity, but enantioselectivity at non-terminal positions was low to moderate. Later, some of the mutants were screened for structurally very different substrates without performing any further mutagenesis, the oxidative hydroxylation of phenyl acetic acid esters being an example (up to 93% ee).[21c]

Until 2011 directed evolution had not been exploited for obtaining CYP mutants showing both high regioselectivity *and* high stereoselectivity (each >95%) which is needed for meaningful applications. An additional challenge is to evolve both (*R*)- and (*S*)-selective mutants. This Article features recent highlights in the field of directed evolution of selective CYPs with emphasis on the increasingly important role of such selective biocatalysts in synthetic organic chemistry. In order to illuminate the molecular biological problems in this endeavor, sufficient attention is paid to methodology development in directed evolution as analyzed in selected case studies; for details the interested reader is referred to recent reviews.[8]

Short introduction to methods and strategies in laboratory evolution

The most commonly used gene mutagenesis techniques are error-prone polymerase chain reaction (epPCR) (a shotgun method which addresses the whole gene/protein), saturation mutagenesis (randomization of canonical amino acids at predetermined sites), and DNA shuffling (mimic of sexual evolution in nature).[8] Since screening is the bottleneck of directed evolution, much work has gone into the establishment of medium- and high-throughput assays,[22] in addition to the development of efficient mutagenesis strategies which enable the production of high-quality libraries requiring less screening.[8] Iterative saturation mutagenesis (ISM) has emerged as a particularly effective approach which delivers small and "smart" mutant libraries.[8a,23] Sites labeled A, B, C, D, etc. around the binding pocket of an enzyme according to the Combinatorial Active-Site Saturation Test (CAST) are first identified using X-ray data or homology models, each comprising one or more amino acid positions (Fig. 1a).[23,24] Following screening of the initial saturation mutagenesis libraries, the best mutants serve as templates for randomization at the other sites, and the evolutionary process is continued until the desired degree of enzyme improvement has been achieved. Fig. 1b shows 2-, 3- and 4-site ISM systems. It is not necessary to explore all pathways, but some may be more proficient than others. Details and guidelines for applying ISM have been published elsewhere.[23] In order to minimize the screening effort so that automated GC or HPLC can be considered, reduced amino acid alphabets have been used, e.g., NDT codon degeneracy encoding 12 instead of the normal 20 canonical amino acids as building blocks at the randomization sites. Keeping Emil Fischer's lock-and-key hypothesis in mind, this Darwinian approach is perhaps the most logical way to reshape the binding pocket of an enzyme in the quest to manipulate regio- and stereoselectivity. Prerequisite is an X-ray structure or a homology model. In the absence of structural data, epPCR or older methods such as mutator strains[20,25] constitute alternatives.

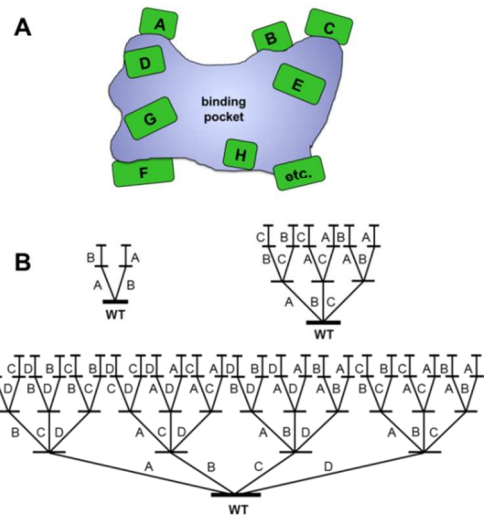


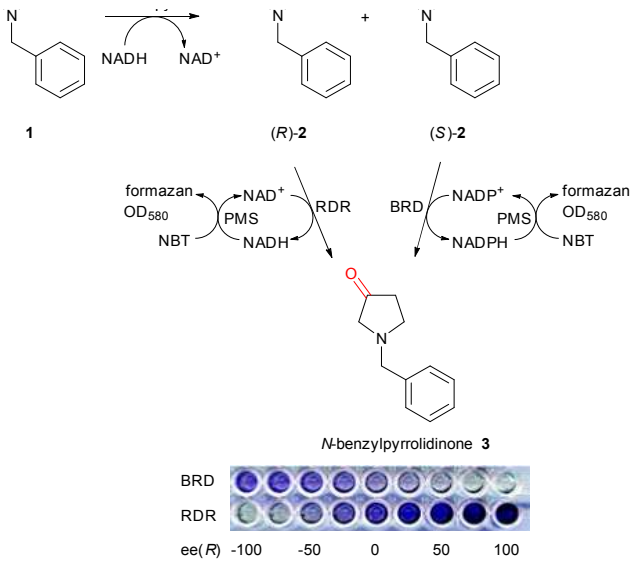
Fig. 1 a) Sites A, B, C, D, etc. surrounding the binding pocket of an enzyme chosen for iterative saturation mutagenesis (ISM); b) 2-, 3- and 4-site ISM systems.[23]

In addition to the creation of "smarter" mutant libraries, efficient screening systems have also been developed.[22] Whereas some methods such as multiplexing mass spectrometry (MS) in combination with isotopically labelled substrates require expensive instrumentation,[26a] automated GC and HPLC robotically taking samples directly from the wells of 96-format (or larger) microtiter plates have emerged as the preferred analytical techniques.[26b-c] They allow the screening of about 300-600 transformants per day. On-plate UV/Vis pretests assessing CYP activity are also recommended, monitoring NADPH-consumption being one option.[22] Along a different line, the known concept of enzyme activity profiling using multiple substrates for "fingerprinting" in order to identify and classify enzymes has been adapted by Fasan and coworkers for protein engineering of CYPs.[27] Accordingly, five methoxy-bearing chromogenic probes of different structure and

size are subjected to P450-catalysis, oxidation of the methyl group at the [-OCH_3] moiety leading to formaldehyde which is rapidly detected by the Purpald-based colorimetric assay.

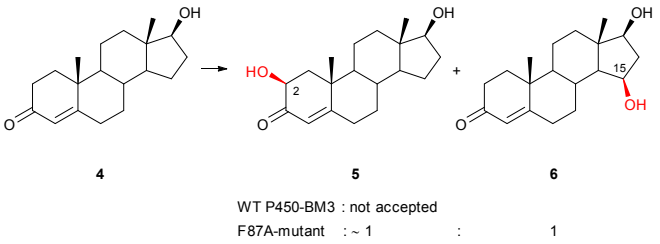
Initial examples of CYP mutants with notable regio- and stereoselectivity generated by directed evolution

In 2010/2011 two studies appeared describing directed evolution for generating P450 mutants with notable degrees of regio- *and* stereoselectivity. Zhao et al reported protein engineering of P450pyr in the oxidative hydroxylation of *N*-benzylpyrrollidine (**1**), WT leading to the regioselective formation of (*S*)-**2** with poor enantioselectivity (43% ee) (Scheme 2).[28] A colorimetric high-throughput plate-test using two complementary alcohol dehydrogenases (ADHs) was developed for screening several thousand transformants within a short time. The goal was inversion of enantioselectivity, which was achieved by applying saturation mutagenesis at 17 amino acid positions within 5 Å of the heme-docked substrate. Following the identification of an improved (*S*)-selective variant (65% ee) and one showing reversed enantioselectivity (42% ee in favor of (*R*)-**2**), ISM was applied at the respective remaining 1-residue sites. This provided the best (*R*)-selective mutant N100S/T186I (83% ee). Improved (*S*)-selective mutants were not found. For further improvements, randomization at multiple residue sites employing reduced amino acid alphabets and techniques for escaping from local minima (if encountered) need to be applied.[23,29]



Scheme 2 Screening system used in the P450pyr catalyzed model reaction **1→2** utilizing two complementary ADHs.[28]

Based on our long-standing interest in developing directed evolution not just for thermostabilization but also for stereoselectivity as the catalytic parameter,[30] we likewise had an interest in P450 enzymes.[31] We chose P450-BM3 as the catalyst, a well-known self-sufficient CYP from *Bacillus megaterium*,[2,21,32] and testosterone (**4**) as the substrate which is not accepted by WT (Scheme 3).[33] In previous work it had been demonstrated that due to steric reasons the single mutant F87A accepts a larger range of compounds than WT P450-BM3.[2,32] In the reaction of **4**, this mutant proved to be active, but unselective with formation of a 1:1 mixture of the 2ß- and the 15ß-isomer in addition to small amounts of other alcohols.[33]



Scheme 3 P450-BM3(F87A) catalysed oxidative hydroxylation of testosterone (**4**).[33]

The demanding problem of evolving 2ß- and 15ß-selective mutants separately without any erosion of diastereoselectivity was solved by applying ISM. CAST residues were chosen, which can be treated as single residue sites for saturation mutagenesis, or they can be grouped into a variety of different multiple-residue sites for randomization. The latter option entails higher genetic diversity, but also increased screening effort. In several recent ISM studies involving other types of enzymes, the use of multiple residue randomization sites has proven to be more productive than saturation mutagenesis at 1-residue sites,[23] but in the case of CYPs this issue has not been resolved with certainty (and indeed both strategies can be successful). Guided by the crystal structure of P450-BM3 reported by Peterson,[32d-e] we grouped 20 single sites into 9 multi-residue randomization sites as shown in Fig. 2.[33]

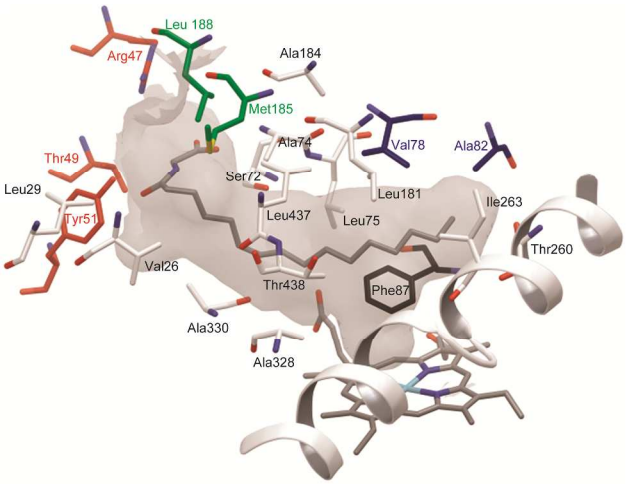


Fig. 2 Overview of the 9 defined CAST sites[33] A (red), B (dark blue), C (orange), D, E, F, G, H and I (all in white) in *N*-palmitoylglycine (dark grey) bound P450-BM3 WT (PDB ID: 1JPZ).[32d-e] The heme cofactor is shown in dark grey and helix I is given as a point of reference. The inner surface of the active site cavity is represented in light grey and the potential substrate/exit lies south west, close to site A.

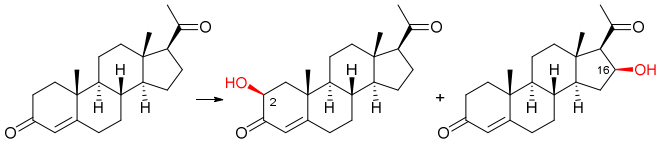
It was not necessary to test all 9 randomization sites, because A, B and C sufficed. In order to reduce the screening effort of libraries A and C, a reduced amino acid alphabet[23] based on NCD codon degeneracy was chosen which encodes 12 amino acids (Gly, Val, Leu, Ile, Phe, Ser, Cys, Tyr, His, Asp, Asn and Arg).[33] In the case of library B, NNK codon degeneracy encoding all 20 canonical amino acids was employed. In the initial round of mutagenesis, 8,700 transformants were screened by automated HPLC-analysis (400 transformants/day). Table 1 features the best mutants arising from these initial libraries, which shows that the synthetic problem was essentially solved, up to 97% 2ß-selectivity favoring **5** and 94% 15ß-selectivity with formation of **6** being achieved with complete ß-diastereoselectivity. In order to boost 15ß-selectivity, one round of ISM was applied, leading to an improved variant R47Y/T49F/V78L/A82M/F87A (96% regioselectivity and 100% diastereoselectivity). A total of only 9,300 transformants were screened in this study. Some of the point mutations had been described in earlier studies utilizing other types of substrates.[2]

Table 1 Typical P450-BM3 mutants as catalysts in the oxidative hydroxylation of testosterone (**4**)[33]

Mutant	Library	Mutations	%-Conv.[a]	**5**	:	**6**	:	Others[c]
Starting Enzyme	-	F87A	21	52		45		3
KSA-1	Lib 330NNK	A330W/F87A	79	97		3		0
KSA-2	Lib A	R47I/T49I/Y51I/F87A	67	94		6		0
KSA-3	Lib A	R47I/T49I/Y51V/F87A	53	94		6		0
KSA-4	Lib A	R47Y/T49F/F87A[d]	84	37		62		1
KSA-5	Lib B	V78L/A82F/F87A	86	3		91		6
KSA-6	Lib B	V78T/A82F/F87A	58	4		91		6
KSA-7	Lib B	V78I/A82F/F87A	78	3		89		8
KSA-8	Lib B	V78I/A82D/F87A	69	84		16		0
KSA-9	Lib B	V78V/A82N/F87A[d]	32	58		40		2
KSA-10	Lib B	V78C/A82G/F87A	55	44		55		1
KSA-11	Lib C	M185N/L188N/F87A	65	28		68		4
KSA-12	Lib C	M185S/L188C/F87A	74	49		50		1
KSA-13	Lib C	M185G/L188G/F87A	73	47		52		1
KSA-14	Lib AB	R47Y/T49F/V78L/A82M/F87A[b]	85	3		96		1
KSA-15	Lib AB	R47Y/T49F/V78I/A82M/F87A[b]	91	3		94		3
KSA-16	Lib AB	R47Y/T49F/V78T/A82F/F87A[b]	60	2		90		8

[a] Conditions: 1 mM testosterone, 24 h, 25 °C, resting cells containing expressed P450 mutants. For detailed expression and reaction conditions, see Supplementary Information. [b] Mutant KSA-4 was used as a parent and site B was addressed. Mean values are given, standard deviation for conversion is ± 5% and for selectivity ± 1%. [c] Minor side products were identified by HPLC as 16ß-hydroxytestosterone and 6ß-hydroxytestosterone. [d] Wild-type amino acid was retained in at least one position; mutant KSA-9 contains a silent mutation V78V (GTA→GTG).

Some of the best mutants were then used in the oxidative hydroxylation of other steroids such as progesterone (**7**) without performing additional mutagenesis experiments. In this case the starting enzyme P450-BM3 (F87A) shifts the point of attack from position 15 to position 16, the 2ß:16ß ratio amounting to 18:82. It was found that variant A82N/F87A is >99% 2ß-selective in favor of **8**, while F87A/A330W results in reversal of regioselectivity (9:91 in favor of **9**) (Scheme 4).[33]



Scheme 4 P450-BM3 mutants evolved for testosterone (**4**) as catalysts in the oxidation of progesterone (**7**).[33]

We have discovered that these libraries constitute valuable collections of mutants which are excellent catalysts not just for steroids, but also for structurally very different substrates. For industrial purposes, we have pooled the most active hits from several studies with formation of a commercially available library of about 350 variants which can be used as a starting point for hydroxylating any new compound.

The experimental data resulting from the reactions of testosterone (**4**) called for a theoretical analysis of the origin of 2ß- and 15ß-selectivity. Since CYP-catalyzed oxidative hydroxylations constitute high-energy radical abstraction reactions by the high-spin catalytically active intermediate heme-Fe=O (Compound I) in the rate-determining step,[2] it is unlikely that the protein environment stabilizes the transition state of these enzymatic processes to a significant extent. The crucial feature of a selective mutant must involve the optimal positioning of the substrate in the binding pocket so that the respective H-atom of the methylene moiety points toward catalytically active heme-Fe=O. Mulholland has estimated that the optimal O-H-C angle is about 130°.[34] Using molecular dynamics (MD) simulations and induced fit docking as provided by the Schrödinger software package[35a] and the QM-derived force fields for Compound I,[35b] we were able to propose a reasonable model.[33] In the case of the non-selective starting mutant F87A, two energetically similar poses for substrate **1** in close vicinity to heme-Fe=O were identified, one poised for 2ß-hydrogen abstraction and the other for reaction at the 15ß-hydrogen atom. When considering the 2ß- and 15ß-selective mutants R47I/T49I/F87A and R47Y/T49F/V78L/A82M/F87A, respectively, the situation is very different because the binding pocket has been reshaped considerably. Testosterone is now forced to adopt quite different poses in the two mutants, one leading to the 2ß- and the other to the 15ß-product (Fig. 3).[33] The induced fit Schrödinger software readily allows the

visualization of amino acid side-chain movements at the binding pocket upon interaction of the substrate with the X-ray structure, which appears to be more cumbersome using other programs.

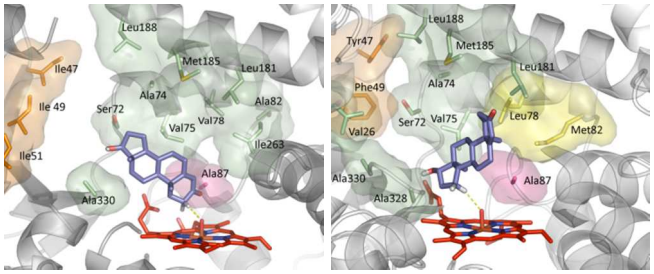
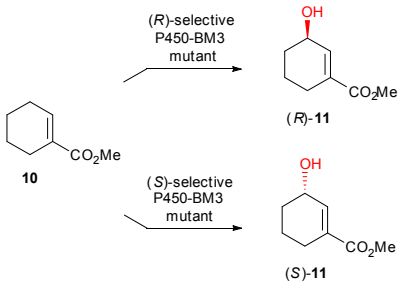


Fig. 3 Computed pose of testosterone (**4**) explaining 2ß-selectivity (mutant R47I/T49I/F87A) and the respective pose leading to 15ß-selectivity (mutant R47Y/T49F/V78L/A82M/F87A).[33]

Commandeur, Vermeulen et al also obtained a 2ß-selective mutant F87N by generating all 19 mutants at this hot spot.[36a] Although an explanation for the origin of selectivity was not presented, the result shows once more that 19 mutants at position 87 of P450-BM3 should be considered as potential candidates when new substrates are tested. Yun et al have also combined rational design and random mutagenesis successfully for steroid hydroxylation.[36b] Combinatorial alanine substitution at selected hot spots of P450-BM3 has been used to generate variants that demethylate methyl-ether and methyl-amine functions of large substrates, but this approach has not worked well in regio- and stereoselective hydroxylation of steroids.[36c]

Generalizing the process of directed evolution of P450 enzymes with high regio- and stereoselectivity

Following the above studies,[28,33] it remained to be demonstrated that directed evolution can be applied to generate CYP mutants displaying >95% regio- and >95% enantioselectivity for both (*R*)- and (*S*)-products in the oxidative hydroxylation of a given substrate. Using once again structure-guided ISM as the mutagenesis strategy, ester **10** as the substrate and P450-BM3 as the catalyst, this goal was reached (Scheme 5).[37] WT P450-BM3 is only 84% regioselective (16% other oxidation products) with poor enantioselectivity in slight favor of (*R*)-**11** (34% ee).



Scheme 5 Model reactions used in evolving regio- and enantioselective P450-BM3 mutants with the goal of achieving selectivities of at least 95%.[37]

In this study a different CAST/ISM strategy was followed. Based on the crystal structure of WT P450-BM3,[32d-e] 24 single residues were chosen for potential saturation mutagenesis, which can be assigned to three structural categories[37]: 1) residues closest to the catalytically active heme-Fe=O; 2) residues still around the binding pocket but significantly farther away from heme-Fe=O; 3) residues at the entrance to the binding pocket but fairly far (15 Å) from heme-Fe=O (Fig. 4).

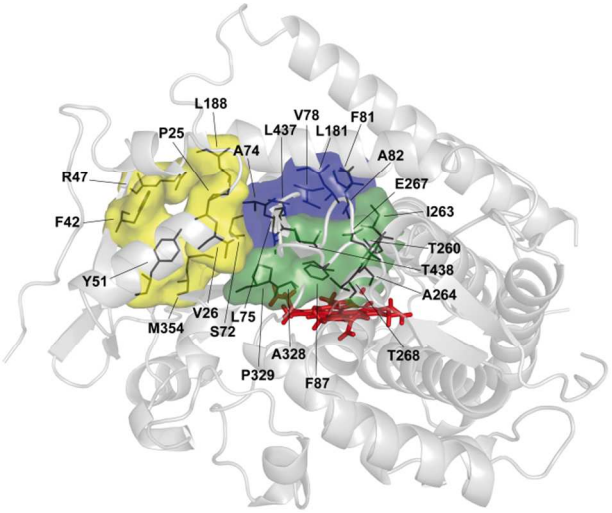


Fig. 4 The 24 residues in P450-BM3 considered for saturation mutagenesis,[37] guided by the X-ray structure of the heme domain (PDB ID: 1JPZ).[32d-e] These residues were assigned to three categories marked green (residues closest to heme-Fe=O), blue (residues relatively far from the heme-Fe=O but still next to the binding pocket), and yellow (residues at entrance to the large binding pocket); the heme cofactor is marked in red.

Saturation mutagenesis was first performed individually at 23 of the 24 single residues (position 329 was not considered at this point), and at selected 2-residue sites F87/A328, A328/F329 and V78/L437. In the case of single-residue sites, NNK codon degeneracy was applied requiring the screening of only 100 transformants for 95% library coverage. This number increases to 3,000 when randomizing a 2-residue site, which is the reason why we chose NDT codon degeneracy encoding only 12 amino acids (Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser and Gly), but requiring much less screening for 95% library coverage (430 transformants)[23]. Pre-screening for activity was performed by the traditional UV/Vis assay monitoring NADPH consumption (which is somewhat crude but fast), followed by GC-based ee-analysis of active hits. In this case taking samples robotically directly from microtiter plates for automated GC-based ee-analysis was not employed which would be most efficient, because at the time we had not optimized the analytical setup (today we can screen one sample of this reaction mixture in less than 7 minutes). Mutants showing ee-values ≥40% are listed in Table 2, which reveals some remarkable trends. Double mutants originating from randomization at 2-residue sites are generally more selective than the single mutants generated by saturation mutagenesis at single-residue sites. An exception is the (*R*)-selective variant A82M (80% ee). All of the double mutants are (*R*)-selective, the three best ones leading to 96% ee with more than 95% conversion in the model reaction (10 mM, 0.1 mmol scale, 8 h).

Mutations closer to the heme-Fe=O seem to have a higher impact on enantioselectivity.

Table 2 P450-BM3 mutants obtained in the initial saturation mutagenesis libraries as catalysts in the model reaction **10**→**11** with ≥ 40% ee.[37]

Entry	Site	Mutations	%-Enantio.[a]
1	-	WT	34, (*R*)
2	47	R47Y	56, (*R*)
3	47	R47G	52, (*R*)
4	78	V78T	54, (*R*)
5	78	V78S	45, (*R*)
6	82	A82M	80, (*R*)
7	82	A82L	57, (*R*)
8	87	F87D	64, (*S*)
9	87	F87I	48, (*R*)
10	87	F87G	46, (*S*)
11	87	F87P	42, (*R*)
12	260	T260L	47, (*R*)
13	260	T260V	44, (*R*)
14	263	I263G	66, (*S*)
15	263	I263C	55, (*S*)
16	328	A328V	60, (*R*)
18	78/181	V78C/L181I	60, (*R*)
19	87/328	F87V/A328V	96, (*R*) (94–99)[b]
20	87/328	F87V/A328N	96, (*R*) (94–96)[b]
21	87/328	F87I/A328V	96, (*R*) (86–99)[b]
22	87/328	F87I/A328N	86, (*R*)
23	328/329	A328V/P329Y	85, (*R*)
24	328/329	A328N/P329Y	84, (*R*)
25	328/329	A328V/P329V	84, (*R*)
26	328/329	A328I/P329C	81, (*R*)
27	328/329	A328V/P329H	81, (*R*)
28	328/329	A328I/P329I	80, (*R*)
29	328/329	A328V/P329G	77, (*R*)
30	328/329	A328V/P329C	77, (*R*)
31	328/329	A328V/P329S	73, (*R*)
32	328/329	A328V/P329I	72, (*R*)

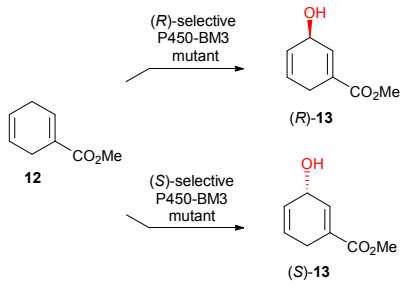
[a] Average of at least two different time points. [b] Values observed following several scaled up experiments under different conditions.

In an initial attempt to enhance (*S*)-selectivity,[37] the single mutations of some of the moderately (*S*)-selective variants were combined to form double mutants, a strategy that has been used in the evolution of stereoselective lipase mutants.[38] In the present case such a traditional approach failed to provide notably improved variants because 70% were essentially inactive, and only one improved variant was identified (I63C/A328L; 79% ee). While these results may appear odd, recent studies regarding the question of additive versus non-additive mutational effects in protein engineering show that combining positive mutations need not lead to improved variants.[39] For example, when combining two (*S*)-selective single mutations of an enzyme to the respective double mutant, the result may be reversed (*R*)-selectivity.[39] At this point we opted for the previously engineered *Escherichia coli* strain BOU730 capable of NADPH regeneration, which has the advantage that the usual requirement of adding glucose dehydrogenase for cofactor regeneration is not necessary.[40] With this host, ISM was invoked employing the best variant I263G as the template for randomization at a limited number of single sites F81, F87 and A328 using NNK, NDT and NNK codon degeneracies, respectively.

Robotic GC with samples being taken directly from the wells of 96-format microtiter plates was employed for these mini-libraries. This provided notably improved (*S*)-selective mutants, namely F87Y/I263G (90% ee) from library at residue 87 and I263G/A328S (96% ee; >95% conversion at 2.5 mM final substrate concentration; 3 μmol scale, 20 h) from library at position 328. Upon performing scale-up to 1.1 mmol, the (*R*)-selective mutant F87V/A328N led to 94-96% ee favoring (S)-**11** (7% other oxidation side-products at 99% conversion). Under similar conditions the (*S*)-selective variant I263G/A328S led to slightly reduced enantioselectivity (94% ee; 76% conversion; 3% other oxidation products). Since selectivity may vary somewhat depending upon the substrate concentration, a syringe pump for gradual addition was used in further scale-up. Bioprocess optimization[41] was not performed here nor in most of the other protein engineering studies cited in this Feature Article, which would be necessary for large-scale (industrial) applications.

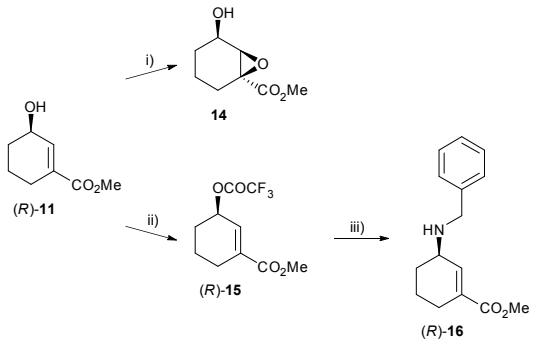
Since the initial libraries were (crudely) pre-screened for activity, some highly (*S*)- and/or (*R*)-selective variants may have been missed. Therefore, the F87/A238 library was generated once more and screened directly by chiral GC. This led to the re-discovery of most of the previous variants, but also to several new ones: F87L/A328V (98% ee/*R*), A328S (97% ee/*S*), F87N/A328I (96% ee/*R*) and F87L/A328I (95% ee/*R*). All of these variants ensure >95% conversion (at 2.5 mM; 3 μmol scale; 20 h). The lessons learned from this study are twofold: 1) Use the engineered *E. coli* strain BOU730 with built-in NADPH regeneration; 2) Vary and control the ratio of substrate to P450 enzyme, and if this influences stereoselectivity, employ a syringe pump for continuous addition of substrate in scale-up experiments. A total of 4,600 transformants were tested in this study, most by the UV-Vis-based pre-screen followed by GC-based ee-analysis.

Upon subjecting structurally related **12** (readily accessible by Diels-Alder reaction) to oxidative hydroxylation catalyzed by P450-BM3 variants F87V/A328N and I263G/A328S, enantioselectivities of 84% ee in favor of (*R*)-**13** and 93% ee in favor of (*S*)-**13** were observed at conversions of 99% and 89%, respectively, (Scheme 6).[37] Of particular interest is (*S*)-**13**, because it had been prepared as the O-silyl protected form in 11 synthetic steps and used as a pivotal intermediate in the preparation of a number of biologically active molecules.[42] Thus, the present two-step sequence is a viable alternative.



Scheme 6 Selective oxidative hydroxylations catalyzed by P450-BM3 mutants.[37]

Many P450-catalysed hydroxylation products are biologically interesting compounds, as in the case of numerous metabolites,[2] but they can also serve as synthetic intermediates in subsequent transformations with formation of value-added products.[4] Recent examples include deoxyfluorination reactions (R-OH→R-F) reported by Arnold et al.[43] In the present study, (*R*)-**11** was exploited as an intermediate enroute to structurally quite different products. Hydroxy-controlled diastereoselective epoxidation[44] with formation of **14** characterized by three contiguous centers of chirality, and Pd-catalysed regioselective allylic nucleophilic substitution[45] of **15** leading to the GABA-analog **16** are illustrative examples (Scheme 7).[37]



Scheme 7 Chemical modification of compound (*R*)-**11**[37]. Reagents and conditions: a) *m*-CPBA, RT, 19 h, CH_2Cl_2, 82%; b) $(CF_3CO)_2O$, NEt_3, 30 min, 87%; c) $BnNH_2$, $Pd(PPh_3)_4$, PPh_3, RT, 1 h, PhMe, Ar, 85%.

In another study, Li et al considered once more the already discussed P450pyr-catalysed hydroxylation of *N*-benzylpyrrolidine (**1**) with formation of (*R*)- and (*S*)-**2** (Scheme 2).[46a] In this case an MS-based high-throughput screening system[26a,d] was applied, deuterium-labeled (*R*)-D-**1** and (*S*)-D-**1** serving as substrates (Fig. 5). In the previous ISM-based study,[28] the best (*S*)-selective was only moderately enantioselective (53% ee). In order to enhance (*S*)-selectivity, 20 residues in the vicinity of the active site were chosen for saturation mutagenesis guided by the crystal structure, namely A77, I82, I83, L98, P99, N100, I102, A103, S182, D183, T185, T186, L251, V254, G255, D258, T259, L302, M305 and F403, each library requiring the screening of only 188 transformants.[46a] Some of the best mutants were used as templates for saturation mutagenesis at selected single sites, and the ISM process was continued in selected upward pathways (total of 6204 transformants screened). The best variant favoring (*S*)-**2** with 98% ee proved to be I83H/M305Q/A77S having a respectable specific activity of 5.2 U g^{-1} cdw (81% activity of WT P450pyr (Fig. 5).[46a] (*R*)-selective mutants for improving the moderate 83% ee previously evolved[28] were not mentioned in this paper.

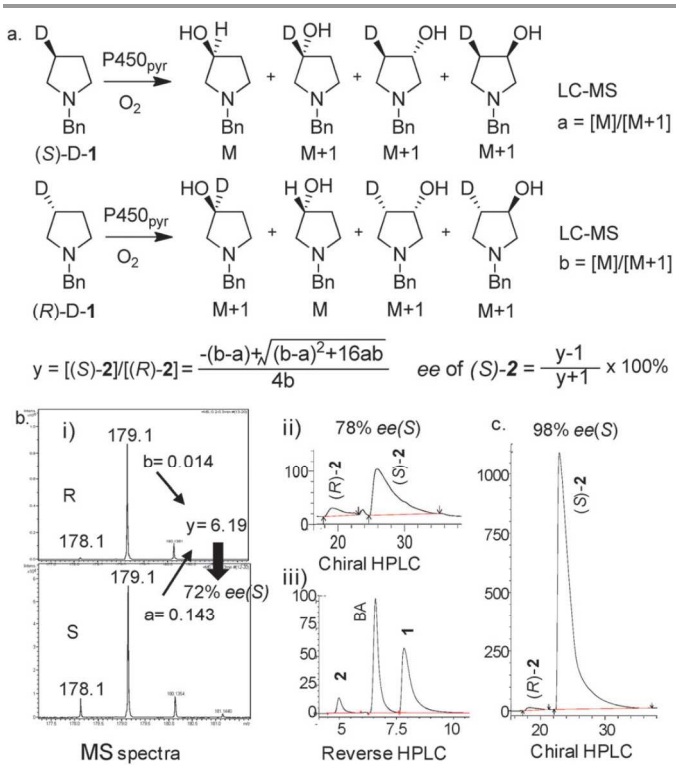
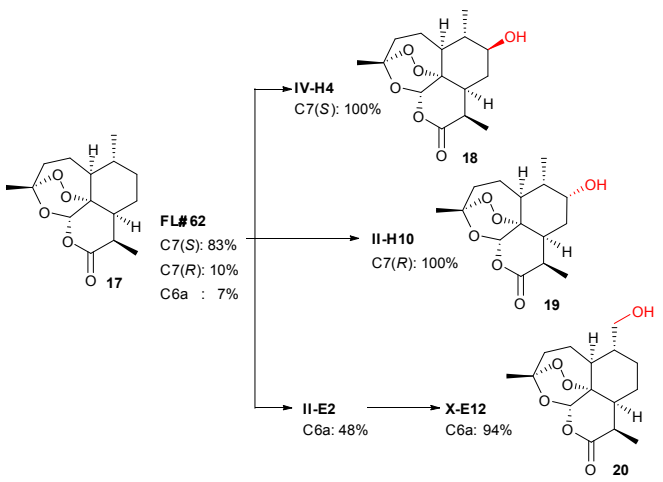


Fig. 5 (a) High-throughput ee assay based on the use of a deuterated substrate and MS detection;[46a] (b) example of screening an enantioselective mutant (I83H). (i) MS spectra for fast ee determination, (ii) chiral HPLC chromatogram for accurate ee analysis, (iii) reversed phase HPLC chromatogram for conversion analysis; (c) chiral HPLC chromatogram of the purified product 2 from biohydroxylation of *N*-benzyl pyrrolidine 1 with best mutant I83H/M305Q/A77S.

When faced with the daunting problem of controlling non-terminal hydroxylation of alkanes lacking functional groups with high regio- and enantioselectivity, the group of Li relied again on ISM.[46b] Using P450pyr as catalyst and *n*-octane as substrate, six rounds of ISM provided variant A77Q/I83F/N100S/F403I/T186I/L302V leading to (*S*)-2-octanol (98% ee) and complete regioselectivity. Other compounds such as propylbenzene also reacted selectively.[46b]

In a noteworthy report by Fasan and coworkers, P450-BM3 was subjected to directed evolution in order to generate mutants which enable late-stage hydroxylation of the anti-malaria therapeutic drug artemisinin (**17**), the site of attack not being defined at the start of the study (Scheme 8).[47] Since WT P450-BM3 does not accept **17**, the most active mutant generated earlier by the fingerprinting method, FL#62, was used as the starting gene. It is characterized by 16 point mutations: V78A, F81S, A82V, F87A, P142S, T175I, A180T, A184V, A197V, F205C, S226R, H236Q, E252G, R255S, A290V, and L353V.[27] This variant accepts substrate **17** with formation of three main products **18**, **19** and **20** in a ratio of 83:10:7. Further improvements were therefore necessary.[47]



Scheme 8 P450-BM3 mutants as catalysts in the oxidative hydroxylation of artemisinin (**17**).[47]

As in previous studies,[33,37,46] saturation mutagenesis was applied at sites surrounding or near the binding pocket. Using NNK codon degeneracy encoding all 20 canonical amino acids, eight randomization libraries were constructed at the following sites: 78/87, 78/81/87, 78/87/181, 78/87/184, 78/81/82/87, 81/82/87/184, 78/81/82/87/181/184 and 74/81/82/87/181/184. The authors estimate that this diversity includes more than 10^5 library members,[47] which would entail huge screening efforts if 95% library coverage were to be strived for. In fact, based on the Patrick/Firth algorithm[48] for calculating the number of transformants needed to be screened for 95% library coverage, NNK-based randomization at a 6-residue site would require the screening of $>3x10^9$ transformants as calculated by the CASTER computer aid[49], which is obviously unrealistic. Instead of employing reduced amino acid alphabets as shown to be successful previous P450-BM3 studies[33,37] and in directed evolution of other enzyme types as well,[23] a different strategy was followed based on the fingerprinting method[27] (see Introduction above). In a first step, 12,500 transformants were screened for activity in 96-well plates using five semisynthetic chromogenic probes, the liberated formaldehyde being detected by the UV/Vis Purpald-based assay at 550 nm (Fig.6a).[47]

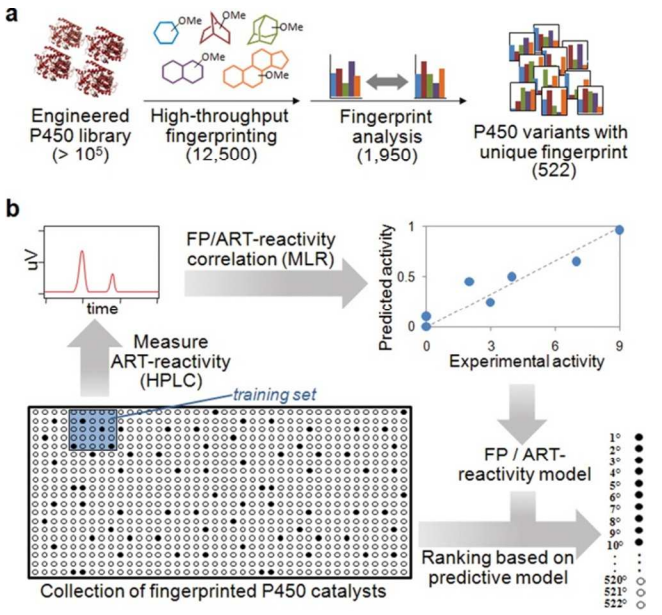


Fig. 6 Fingerprint-based strategy to guide the discovery of regio-and stereoselective artemisinin-hydroxylating P450 catalysts.[47] (a) Schematic summary of the process of identification of the functionally diverse P450 variants from the active site libraries via high-throughput fingerprinting followed by fingerprint comparative analysis (tier 2). (c) Outline of the method applied to predict ART reactivity via multivariate fingerprint analysis (tier 3). FP: fingerprint. MLR: multiple linear regression.

This screening step delivered about 1,950 active variants (criterion: "> 10% of parent enzyme activity on at least one of the fingerprint probes"), and 522 variants functionally unique (criterion: "larger than 20% variation on at least one of the 5 fingerprint components compared to the parent enzyme and any other member of the library").[47] Instead of testing all 522 variants by automated HPLC (which may not have been available), the authors developed a guide for searching for mutants that react with the actual substrate of interest (**17**). They realized that this compound had little similarity with the chromogenic fingerprint probes, and therefore developed a further guide by correlating the P450-BM3 fingerprints with experimental **17**-reactivities across a small subset of the enzyme's mutants (Fig. 6b). The reader is referred to the original study for details,[47] suffice it to say that at the end of the overall process, 75 variants were inspected by HPLC analysis, one (IV-H4) showing complete selectivity for **18**, and the other (II-H10) being fully selective for its diastereomer **19** (Table 3).

From the 75-membered pool, another variant (II-E2) was identified which leads to 48%-selectivity in favor of attack at the C6 methyl group with formation of **20** (Scheme 8). In order to improve this variant, ISM was invoked using II-E2 as the template and performing saturation mutagenesis at positions 74/181/184.[47] For 95% library coverage, this would require the screening of about 98,000 transformants. The authors randomly picked 3,000 transformants for screening not using the actual substrate **17**, but employing the same fingerprinting procedure involving the five chromogenic probes used earlier.[27] This finally provided variant X-E12 showing 94% selectivity for product **20**. The essential results together with kinetic data are

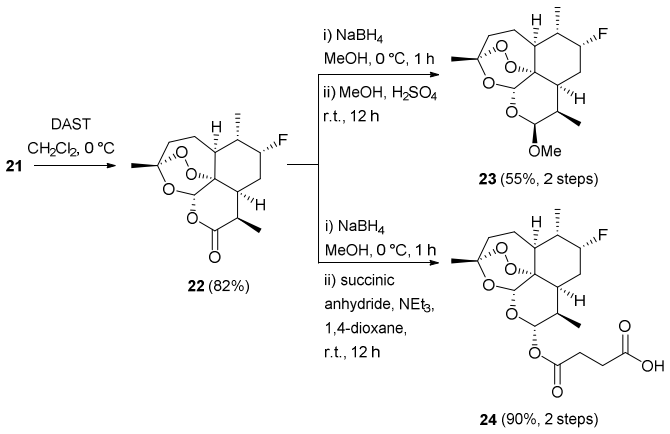
summarized in Table 3. Since FL#62 was used as the starting enzyme, the final three variants are characterized by 19 to 22 point mutations, which are relatively high numbers in directed. Thermostability data was not reported.

Table 3 Best P450-BM3 mutants as catalysts in the oxidative hydroxylation of artemisinin[47] (**17**).[a]

	Amino acid substitutions[b]							%-Prod. distribution						
Variant	74	78	81	82	87	181	184	18	19	20	TTN	*KD* (μM)	prod. formation rate[c]	%-CE[d]
FL#62	A	A	S	V	A	L	V	83	10	7	339 ± 12	29 ± 5	316 ± 20	41.9
IV-H4		S	V	A				100	0	0	362 ± 15	53 ± 11	100 ± 4	71.4
V-H2		S	I	A				96	0	4	434 ± 21	45 ± 10	41 ± 2	23.5
II-H10		N	F	T	F	F	T	0	100	0	270 ± 08	61 ±2	32 ± 2	12.8
III-B1		F	F	A				19	81	0	403 ± 17	38 ±2	72 ± 2	23.3
II-E2		N	F	A				22	30	48	393 ± 25	164 ± 18	148 ± 3	38.2
X-E12	V	N	F	A		A	T	4	2	94	113 ± 12	300 ± 24	72 ± 3	45.1
X-F11	T	N	F	A			S	0	8	92	376 ± 19	234 ± 29	56 ± 2	56.3

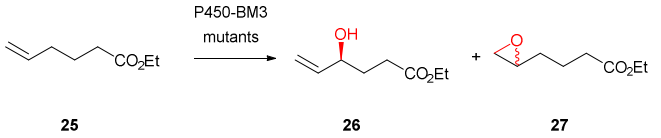
[a] Mean values and standard deviations are calculated from triplicate experiments. [b] Mutations in FL#62 vs P450-BM3 are V78A, F81S, A82 V, F87A, P142S, T175I, A180T, A184V, A197V, F205C, S226R, H236Q, E252G, R255S, A290V, L353V. [c] Moles of product per mole of P450 per minute. Rates are measured over initial 30 s. [d] CE: coupling efficiency; Ratio between product formation rate and NADPH oxidation rate in the presence of artemisinin.

Large-scale reactions (400 mg of **17**) utilizing the best variants led to ≈90% yields of the three products **18**, **19** and **20**, respectively. Compound **18** was deoxofluorinated to provide **21** in stereochemically pure form, which was subsequently transformed into **23** and **24** (Scheme 9).[47] The transformations starting from **17** nicely demonstrate the complementary nature of biocatalysis and modern synthetic organic methods.



Scheme 9 Added-value reactions of compound **21**.[47]

In another recent study, Pietruszka and coworkers took a different approach in the successful attempt to evolve P450-BM3 mutants for regio- and enantioselective oxidative hydroxylation of ω-alkene carboxylic acid esters.[50] Rather than applying saturation mutagenesis at known hot spots around the binding pocket of the enzyme using NNK codon degeneracy, a 65-membered mutant library was created as follows: A P450-BM3 library was constructed by specific exchanges of the amino acid codons for R47, Y51 and L188, in addition to the generation of single and double mutant combinations A74G, F87V, A74G/F87V (GV), A74G/L188Q (GQ), F87V/L188Q (VQ) and A74G/F87V/L188Q (GVQ). All of the 65 members of this library had been described previously in the literature. The library was screened by GC analysis using 6-heptene carboxylic acid ethyl ester (**25**) as the model compound (Scheme 10).[50]



Scheme 10 P450-BM3 catalysed oxidation of ester **25**.[50]

Table 4 shows that especially the double mutant A74G/L188Q is an excellent catalyst, leading to 90% regioselectivity in favor of allylic hydroxylation (**26**) versus epoxidation (**27**), and 95% ee favoring the (*S*)-alcohol **26**. Several homologs of **25** led to similar results. Although MD or docking computations were not performed, the authors speculate that the ester function can serve as an anchor point in the large binding pocket of P450-BM3, since similar alkenes lacking this functional moiety react with significantly lower degrees of selectivity. The synthetic results are significant, because the chiral allylic alcohols serve as intermediates in the synthesis of a variety of different biologically active products.

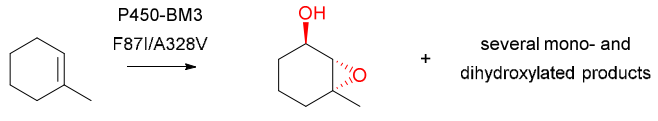
Table 4 Screening and characterization of P450-BM3 monooxygenase mutants for the stereoselective allylic hydroxylation of ethyl 6-heptenoate (**25**).[50]

Entry	P450-BM3 mutant	%-Conv.	**26**:**27**	%-Enantio.	%-CE
1	enzyme library	0–59	65:35–97:3	87–98	-
2	A74G	47±3	89(±0):11	96±1	36±1
3	L188Q	37±2	85(±0):15	93±1	51±1
4	A74G/L188Q	49±1	90(±0):10	95±1	52±1

The approach taken in this study does not mean that such small collections of previously generated mutants will routinely lead to success when targeting other substrates. But it does show that it is not always necessary to generate new saturation mutagenesis libraries. Indeed, depending upon the structure of a new substrate, our group either starts by screening small previously generated CAST libraries, or design new small libraries guided by structural biology.

In the attempt to generate selective variants for the oxidation of 1-methylcyclohexene (**28**) (Scheme 11),[51] we first tested WT P450-BM3 as well as some of the best hits that had

been obtained in the earlier ISM-based study using ester **10** as substrate.[37] Since olefin **28** lacks functional groups with lone electron pairs that can anchor in the enzyme's binding pocket by means of H-bonds, we did not expect high selectivities. Indeed, fairly indiscriminate oxidation reactions occurred which include hydroxylation and epoxidation with formation of about a dozen different products of known structure. Upon testing the double mutant F87I/A328V, which in the reaction of the ester **10** ensured 95% regioselectivity and 96% ee in favor of (*R*)-**11**,[37] the fingerprint profile of the product mixture changed significantly. The only product showing high enantiopurity is the hydroxy-epoxide **29** with the *trans*-(1*S*,2*R*,6*R*) configuration (Scheme 11).[51] It is the product of a synthetically interesting tandem reaction, which unfortunately occurs only to a small extent, and which needs to be optimized by directed evolution. Protein engineering of CYPs enabling sequential oxidations with creation of multiple centers of chirality is a challenging research area for the coming years.



28 *trans*-(1*S*,2*R*,6*R*)-**29** 98% ee, (4%)

Scheme 11 P450-BM3 F87I/A328V catalysed oxidation of 1-methylcyclohexene **28**.[51]

In a more recent project, we considered CYP-catalysed regioselective hydroxylation of tetralones **30a-c** because the 4-hydroxy derivatives **31a-c** are known to be valuable components and/or building blocks of a variety of biologically active natural products and pharmaceuticals (Scheme 12).[52] These include Asian and European folk medicines such as glucosides from *Juglans mandshuria* containing (*S*)-**31a** which have been used in China to treat certain cancers, dermatosis and pain, as well as the fresh pericarps of *Juglans sigillata* used in the treatment of various illnesses.[53a-b] Industrially developed 8MAPK inhibitors which act as anti-inflammatory agents for treating respiratory diseases likewise deserve mention.[53c]

30a R^1=R^2=H (*S*)-**31a**
30b R^1=H; R^2=CH_3O (*R*)-**31b**
30c R^1=CH_3O; R^2=H (*S*)-**31c**

Scheme 12 P450-BM3 catalysed hydroxylation of tetralones **30a-c** with regioselective formation of the 4-hydroxy derivatives **31a-c**.[52]

Using the parent compound **30a** as the model substrate, WT P450-BM3 was found to be surprisingly regioselective with sole formation of the desired 4-hydroxy derivative **31a**, but enantioselectivity proved to be poor (33% ee) in slight favor of the (*S*)-enantiomer. We first tested 40 previously generated variants which had shown activity toward methylcyclohexane in another study.[54] As summarized in Table 5, several mutants were discovered that display >98% regioselectivity and 96-99% ee in favor of (*S*)-**31a**, all of them being single mutants at position 328, a hot spot reported in other studies.[2] (*R*)-selective variants were not found in this small collection. In the case of substrate **30b**, the best mutant is A328F which is (*R*)-selective but at significantly lower regioselectivity (48%) (Table 5, entry 7). Therefore, saturation mutagenesis at position 328 was performed using NNK codon degeneracy, requiring the screening of only ≈100 transformants for 95% library coverage. This resulted in the identification of variant A328P which shows 94% ee in favor of (*R*)-**31b** at slight expense of regioselectivity (85%) (Table 5, entry 8). This small library was then screened in the hydroxylation of substrate **30c**, because WT P450-BM3 is essentially stereorandom (1% ee in minute favor of (*S*)-**31c**). Two improved variants were identified (Table 5, entries 10 and 11). Structurally related substrates such as indanone, indane and tetralin were also tested using some of the already generated variants, which resulted in high selectivities. All in all, less than 200 transformants had to be screened in this study.

Table 5 P450-BM3 catalysed oxidative hydroxylation of tetralones **30a-c**.[52]

Entry	Substrate	P450-BM3	Product	%-Regio.	%-Enantio.	TOF[b] [min^{-1}]	%-Conv.[b,c]
1	**30a**	WT	**31a**	99	33, (*S*)	1.9	86
2	**30a**	A328F	**31a**	98	99, (*S*)	3.8	>99
3	**30a**	A328K	**31a**	99	96, (*S*)	-[d]	56
4	**30a**	A328R	**31a**	99	88, (*S*)	-	59
5	**30a**	A328Y	**31a**	99	97, (*S*)	-	39
6	**30b**	WT	**31b**	97	82, (*R*)	2.2	86
7	**30b**	A328F	**31b**	48	95, (*R*)	1.3	64
8	**30b**	A328P	**31b**	85	94, (*R*)	-	71
9	**30c**	WT	**31c**	91	1, (*S*)	6.2	88
10	**30c**	A328F	**31c**	50	99, (*S*)	3.0	75
11	**30c**	A328I	**31c**	84	86, (*S*)	-	92

[a] Values were obtained by averaging at least three independent experiments performed with resting cells at 5 mM. [b] TOF and conversions were calculated for WT and the best mutants. [c] Conversion calculated after 20 h. [d] Not determined.

In order to understand the origin of regio- and enantioselectivity of the hydroxylation of the parent compound tetralone (**30a**), docking experiments were performed. Several docking poses of similar energy were found, but only one of

them fulfilled the requirement of close proximity to the high spin catalytically active heme-Fe=O with the H-atom abstraction occurring at an angle of about 130° as predicted to be ideal for such a reaction. This pose clearly suggests that the pro-*S* atom of the benzylic CH_2-group should react preferentially, in accord with the observed absolute configuration of product **31a**.[52] The conformational dynamics of 1-tetralone was then investigated by performing two unrestrained MD simulations on the docked structure. Although the substrate rarely gets close enough to heme-Fe=O, when it does, it is positioned in a way that causes the pro-*S* hydrogen-atom to point toward the catalytically active species (Fig. 7).[52] The question why 7-methoxytetralone **30b** causes reversal of enantioselectivity was also answered.

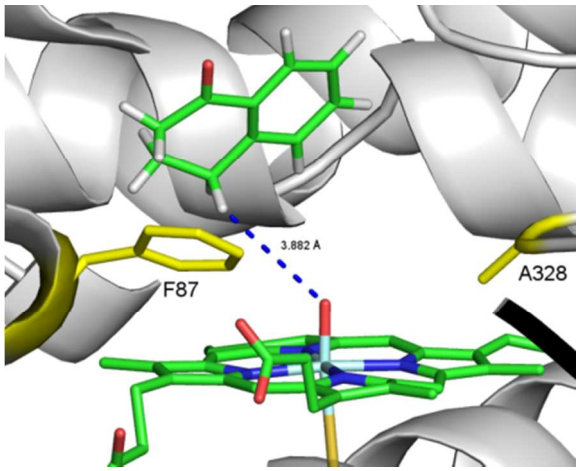
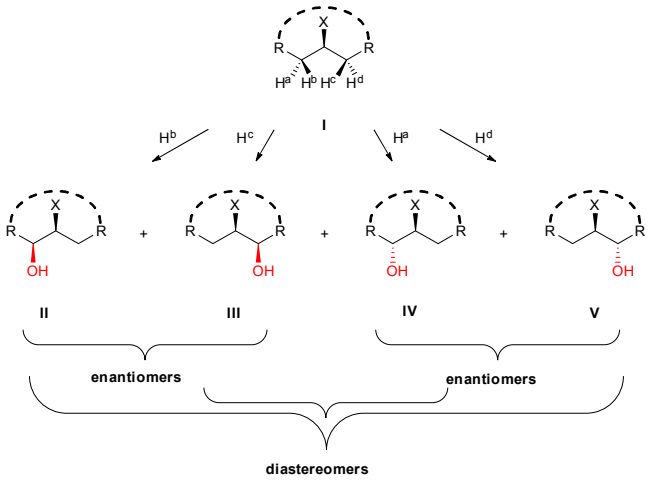
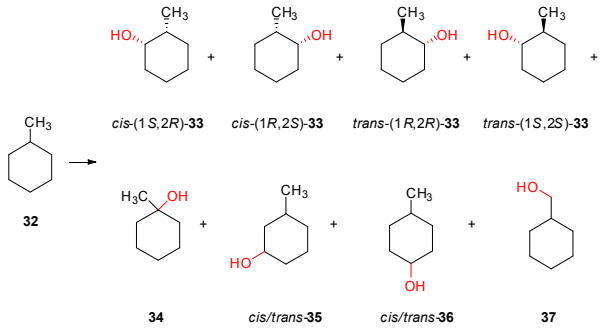


Fig. 7 Structure obtained from an unrestrained molecular dynamics simulation of 1-tetralone (**30a**) in WT P450-BM3 (after 34,940 ps).[52] The O-H distance between the ferryl oxygen of Compound I and the pro-*S* hydrogen attached to C4 of 1-tetralone is highlighted by the blue dashed line. The F87 and A328 residues are also highlighted in yellow stick form.

Recently, directed evolution of P450-BM3 was used to generate variants that induce enantioselective α-hydroxylation of ketones,[55] a biocatalytic route to chiral acyloins which are useful intermediates in the synthesis of various biologically active products. In a final study to be highlighted here, we discuss our attempts to devise systems starting from achiral substrates which upon a single oxidative hydroxylation lead to the creation of two or more new centers of chirality.[54a] If achiral substrates of the type I shown in Scheme 13 are considered in which regioselectivity operates in favor of the two methylene groups next to the X-carrying C-atom, then hydroxylation can occur at four different stereotopic H-atoms, each leading to a different stereoisomer (two diastereomers and their two mirror images). Finding catalysts that enable regio-, diastereo- and enantioselectivity is the challenge in this endeavor. We chose methylcyclohexane (**32**) as the model compound, a "difficult" substrate lacking functional groups (Scheme 14).[54a]



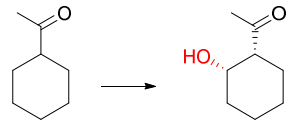
Scheme 13 Stereochemical consequences of oxidative hydroxylation of prochiral compounds of the type **I**.[54a]



Scheme 14 Possible products of P450-BM3 catalyzed oxidative hydroxylation of methylcyclohexane (**32**).[54a]

WT P450-BM3 proved to be surprisingly regio-(78%), diastereo- (>97%) and enantioselective (82% ee) in favor of *cis*-(1*S*,2*R*)-**33**, in addition to **34** (17%), **35** (4%) and **36** (1%).[54a] In order to boost selectivity, a limited number of saturation mutagenesis experiments were carried out, specifically randomization at CAST sites V78/T88, V78/L181, and T268/A328 using a highly reduced amino acid alphabet encoding only six building blocks (Phe, Tyr, Trp, Lys, Arg and His) and the corresponding WT amino acids, and site F87 using NNK codon degeneracy. Upon screening a total of 760 transformants by automated GC, the known variant F87A was identified which led to an undesired shift of regioselectivity in preference of hydroxylation at the weakest CH-bond with formation of **34** (71%), while variant A328F increased enantioselectivity at slight expense of regioselectivity (**33**/71%; d.r. = 97:3 (*cis*); 92% ee).[54a]

Seven mono-substituted cyclohexane derivatives were also tested with WT and the two variants F87A and A328F, which led to similar selectivities. An example is the oxidation of cyclohexyl methyl ketone (**38**) with preferential formation of *cis*-(1*R*,2*S*)-**39** (78% regioselectivity; d.r. = 98:2 and 96% ee at 85% conversion) (Scheme 15).[54a] WT is only 58% regioselective and moderately enantioselective (54% ee).



38 *cis*-(1*R*,2*S*)-**39**

Best mutant:	Regioselectivity:	78%
	Diastereoselectivity:	98 : 2
	Enantioselectivity:	96%
	Conversion:	>85%

Scheme 15 Regio-, diastereo- and enantioselective oxidative hydroxylation of methyl cyclohexyl ketone (**38**).[54a]

This strategy for desymmetrizing prochiral compounds leading to the simultaneous creation of two chirality centers in a single hydroxylation event constitutes a unique way to transform simple achiral starting materials into value-added products. To date, chiral synthetic catalysts have not been developed for such transformations. By choosing appropriate substrates, it is possible to perform similar single hydroxylations with simultaneous creation of three or more chirality centers with high regioselectivity, but thus far it has not been possible to control enantioselectivity.[54b]

In addition to the substrates featured thus far in this Feature Article, other types of compounds have also been subjected to CYP-catalysed hydroxylation, generally (but not always) utilizing protein engineered mutants to control selectivity. Recent examples are summarized in Table 6. Included are selected studies of epoxidation, although this type of CYP-catalysed reaction is not systematically covered here. The question when a CYP induces epoxidation rather than hydroxylation has not been answered satisfactorily.[2]

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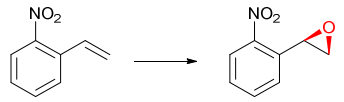
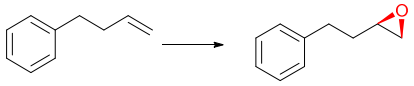
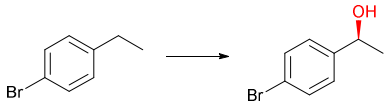
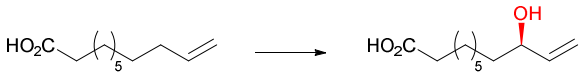
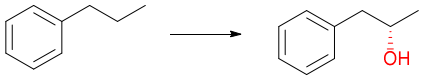
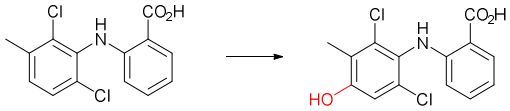
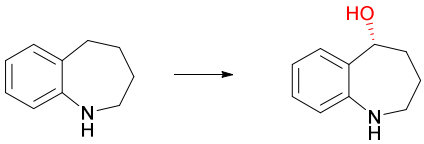
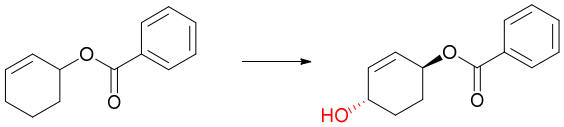
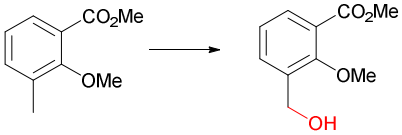
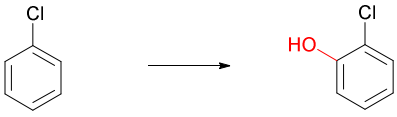
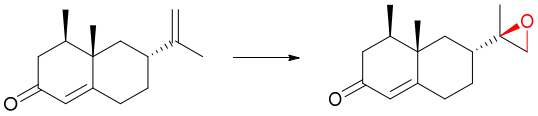
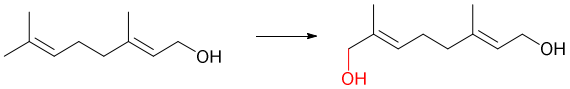
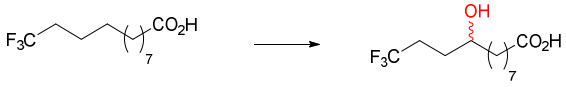
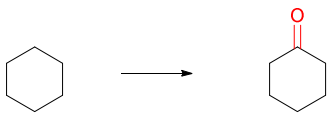
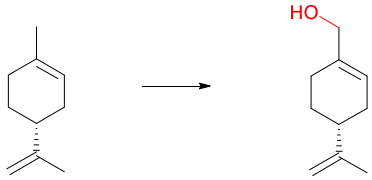
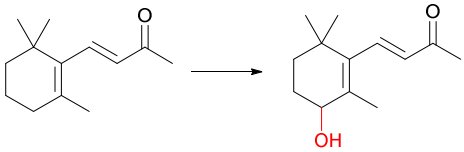
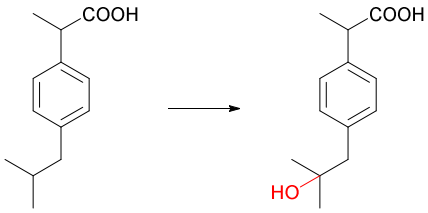
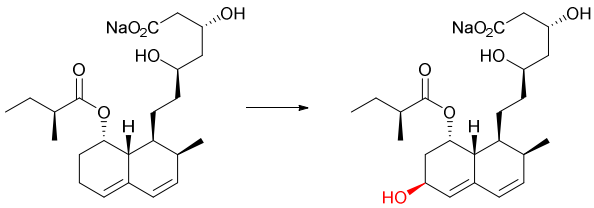
Table 6 Selected recent examples of selective hydroxylation and epoxidation reactions catalysed by P450s.

Entry	P450	Mutations	Reaction	%-Regio.	%-Enantio.	Ref.
1	P450tol	WT		99	(*R*)-99	56
2	P450terp	WT		98	(*R*)-96	56
3	P450tol	WT		95	(*S*)-99	56
4	P450-BM3	WT/L407C-Ru1		100	(*R*)-85	57
5	P450pyr	A77Q/I83F/N100S/ T186I/L302V/F403I		>99	(*S*)-98	58
6	P450-BM3	R47L, E64G, F81I, F87 V, E143G, L188Q, E267 V, G415S V87F		89	-	59
7	P450 *Pseudomonas plecoglossicida*	WT		99	(*R*)-98	60
8	P450cam	F87A/ Y96F		>90		61
9	P450-BM3	F87A		100		62
10	P450-BM3	R47S/Y51W/I401M		>99		63
11	P450-BM3	V78A, H138Y, T175I, V178I, A184V, H236Q, E252G, R255S, A290V, A295T, L353V		96		64
12	P450 from *Thermobifida fusca* YX	WT		100		65
13	P450-BM3	WT		100	-[a]	66

14	P450-BM3 & ADH from *L. kefir*	R47C, V78F, A82S, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, A328F, L353V, E464G, I710T L52I, L188P, I366V		-[b]		67
15	P450-BM3	264V/A238V/L437F		97		18
16	P450 from *Sorangium cellulosum*	WT		100	-[c]	68
17	P450-BM3	D251G/G307H		100		69
18	P450sca-2/Pdx/Pdr	G52S/F89I/ P159A/D269E/T323A/E370V/T85F/T119S/V194N/N363Y		100		70

[a] Racemic product detected. [b] 0.41 g L^{-1}, TON = 6531 (referring to the P450 monooxygenase). [c] Enantiomeric excess not given.

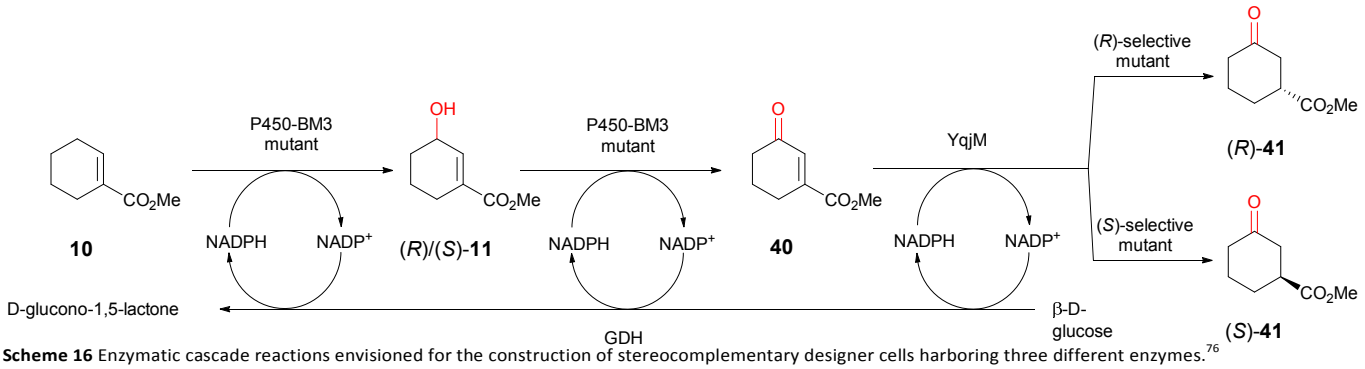
Example 18 in Table 6 deserves special attention because it involves an elegant strategy for converting mevastatin into pravastatin (a cholesterol-lowering drug). Lin et al applied ISM to the novel hybrid system P450sca-2/Pdx/Pdr in order to increase activity and enhance electron transfer.[70] Upon screening a total of only 1,100 transformants, a mutant was identified which is about one order of magnitude more active than non-engineered enzyme (whole-cell biotransformation activity of 378 mg/L).

Other contributions relating to P450 enzymes as catalysts in organic chemistry

It has already been noted that rational design using site-specific mutagenesis continues to contribute notably to the general field of CYPs as catalysts in organic chemistry. In yet another study, Commandeur and collaborators generated a minimal library of 25 combinatorial single and double mutants starting from a template (R47L/F87V/L188Q/E267V) which they had previously produced by rational design, leading to two additional point mutations A82W/S72I.[71] This novel mutant led to regioselective hydroxylation of testosterone at the 16-position with 81% α-selectivity, the first case of inversion of diastereoselectivity at this position. Merging rational design and directed evolution constitutes an attractive strategy. In a fundamentally different approach, unnatural amino acids have been used in protein engineering of P450-BM3.[72]

Rational design was also practiced by Keasling, Baker and coworkers in their attempt to produce P450-BM3 mutants as catalysts in the oxidation of amorpha-4,11-diene.[73] One of the mutants (F87A/A328L) led to the formation of artemisinic-11S,12-epoxide at titers >250 mg/L. In polyketide metabolic pathway engineering, Schulz, et al developed minimally invasive mutagenesis as a unique way to generate biosynthetic polyketide compound libraries, but thus far CYPs have not been targeted.[74a] Brück et al have reviewed the field of microbial factory engineering which includes CYP-catalysis in the production of cyclic sesqui- and diterpenes.[74b] CYPs have also been employed in cascade reactions, as in the conversion of a methylene group into the respective ketone function.[67,75-76] Sieber et al converted the unactivated C5-methylene group of camphor into 2,5-diketobornane by employing a mixture of P450cam enzymes and the dehydrogenase FdeH on a preparative scale (followed by transition metal catalyzed reductive amination with formation of the diamine).[75]

Our group was interested in creating designer cells for stereocomplementary *de novo* enzymatic cascade reactions in which cells harboring P450-BM3 mutant genes and mutant genes of the enoate reductase YqjM form the catalytic system. The goal was to construct two different strain systems, one providing (*R*)-**41**, the other the enantiomeric product (*S*)-**41** (Scheme 16).[76] The difficulty in this cascade reaction scheme is the necessity of the P450-BM3 mutants not only to be regioselective, but also substrate-selective (e.g., neither intermediate **40** nor product **41** should be accepted!). This was achieved with three different versions, the best delivering (*R*)- or (*S*)-**41** (both 99% ee) with yields of 72-75%.



Scheme 16 Enzymatic cascade reactions envisioned for the construction of stereocomplementary designer cells harboring three different enzymes.[76]

An under-explored "substrate engineering" strategy in CYP-catalyzed transformations, originally demonstrated by Griengl,[77a] has been elegantly extended by Sherman et al.[77b] In order to hydroxylate certain carbocyclic compounds regioselectively, the substrates were attached to a desosamine glycoside via an acetal linkage with formation of a compound that binds specifically to the CYP PikC ($PikC_{D50N}$-RhFRED). The aminosugar-derived anchoring group allowed for regioselective oxidation reactions.[77b]

The use of chemically inert activating additives such as per-fluoro fatty acids which enter the large binding pocket of P450-BM3 allows small alkanes such as propane (but not methane) to be oxidatively hydroxylated with high activity and pronounced coupling efficiency.[78a-b] Other additives have also been reported.[78c] In another contribution, light-driven stereoselective CYP-catalyzed hydroxylations have been described, a novel technique which replaces standard NADPH-regeneration systems.[79]

Along a different line, Drone et al developed a reliable expression system for one of the members of the CYP153 (AH153) family, CYP153A13a, in *E. coli* by fusing it to RhFred and to a histidine affinity tag.[80] This self-sufficient soluble CYP is an excellent catalyst for terminal oxidation of linear alkanes, and also accepts cyclic alkanes such as cyclohexane. Finally, protein engineering of CYPS has been applied for enhancing stability,[81] while immobilization[82] studies likewise contribute to the applicability of these enzymes.

Conclusions and perspectives

More than six decades after the original industrial work based on various fungal and microbial strains for the regioselective hydroxylation of steroids,[4a] the use of cytochrome P450 monooxygenases (CYPs) has been extended to include a wide variety of substrates. However, regio- and stereoselectivity of WT CYPs is not routinely satisfactory. During the last 3-4 years, the application of directed evolution[8] as an alternative to rational design[7] has emerged as a powerful tool in protein engineering of CYPs. The most reliable strategy in directed evolution is structure-based iterative saturation mutagenesis (ISM) at sites surrounding the binding pocket, because it delivers high-quality libraries which can be screened on-plate using automated GC or HPLC.[8a,23] We expect this trend to continue with inclusion of numerous synthetically useful substrates, including those in which a single hydroxylation event creates two or more chirality centers with formation of value-added products.[54] The regio- and stereochemical control of sequential hydroxylations in one-pot reactions likewise constitutes a challenging goal for enriching the toolbox of organic chemists.[51]

Biocatalytic retrosynthesis will play a greater role in the future,[83] which can be expected to include catalysis by CYPs. In terms of large-scale industrial applications of these enzymes, bioprocess engineering remains essential, as in any commercial enzymatic reaction.[41] Combining the benefits of additive effects[78] with directed evolution also needs to be considered.

The ultimate goal would be the development of reliable mutagenesis methods and strategies with which CYP-catalysed oxidative hydroxylation can be directed regio- and stereoselectively to *any* CH-position of a given compound that the researcher may be interested in. Presently, natural product chemists hesitate to design synthetic sequences which rely on late-stage CH-activating hydroxylation. As more mutational and structural information emerges from laboratory evolution of CYPs and from the catalytic profiles of new P450 enzymes, the situation can be expected to change. Such data serve as a basis for enhancing the quality of new mutant libraries requiring less screening. For example, ISM can be focused on two or more hot spots identified in earlier studies. Some CYP mutant libraries are commercially available. If a mutant produces at least some of the desired product in a reaction mixture resulting from the hydroxylation of a particular compound, then it can be used as the template for tuning the

catalytic profile in subsequent structure-guided directed evolution.

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Notes and references

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