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Achieving Regio- and Enantioselectivity of P450-Catalyzed Oxidative CH Activation of Small Functionalized Molecules by Structure-Guided Directed Evolution

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Directed evolution of the monooxygenase P450-BM3 utilizing iterative saturation mutagenesis at and near the binding site enables a high degree of both regio- and enantioselectivity in the oxidative hydroxylation of cyclohexene-1-carboxylic acid methyl ester. Wild-type P450-BM3 is 84% regioselective for the allylic 3-position with 34% enantioselectivity in favor of the *R* alcohol. Mutants enabling *R* selectivity (>95% *ee*) or *S* selectivity (>95% *ee*) were evolved, while reducing other oxidation products and thus maximizing regioselectivity to >93%. Control of the substrate-to-enzyme ratio is necessary for obtaining optimal and reproducible enantioselectivities, an observation which is important in future protein engineering of these mono-oxygenases. An *E. coli* strain capable of NADPH regeneration was also engineered, simplifying directed evolution of P450 enzymes in general. These synthetic results set the stage for subsequent stereoselective and stereospecific chemical transformations to form more complex compounds, thereby illustrating the viability of combining genetically altered enzymes as catalysts in organic chemistry with traditional chemical methods.

Introduction

Directed evolution[1] of stereoselective enzymes[1,2] is now part of the ever-expanding toolbox serving synthetic organic chemistry, generally complementing methods based on chiral synthetic catalysts. This Darwinian approach to asymmetric catalysis has been applied successfully to a variety of different enzyme types, including lipases, esterases, epoxide hydrolases, nitrilases, oxynitrilases, aldolases, aminotransferases, monoamino-oxidases and Baeyer–Villiger monooxygenases.[2] P450 enzymes[3] have also been subjected to protein engineering, which includes laboratory evolution as well as traditional site-specific mutagenesis, mainly in the quest to influence the regioselectivity of CH-activating oxidative hydroxylation (C–H → C–OH), which has led to notable results.[3,4] However, the ability to control both regio- and stereoselectivity at synthetically useful levels (≥95%) by directed evolution, ideally at different positions on an optional basis, has remained elusive until recently.[5] Such a task is different from systematically screening different P450 enzymes or mutants generated for other purposes, a strategy which may be successful in some cases, but is not generally applicable.[4j,n] The challenge stems from the unique mechanism of P450-catalyzed reactions, a high-energy process involving radical hydrogen abstraction by a high-spin heme-$Fe^{V}=O$ species, followed by C–O bond formation.[3] Many factors contribute to the absence or presence of regio- and stereoselectivity in a given case,[3,4] especially the mode of binding as determined by hydrophobic effects, hydrogen bonding to the substrate's functional groups, differences in strength of the various C–H bonds in a substrate, and possible electrostatic effects.[6] Recently, we reported the first example of achieving this goal by evolving mutants of P450-BM3 (CYP102A1)[7] from *Bacillus megaterium* as catalysts for the selective oxidative hydroxylation of testosterone and other steroids.[5] By using iterative saturation mutagenesis (ISM) in the form of a combinatorial active-site saturation test (CAST), which focuses on randomization sites around the binding pocket,[2,8] it was possible to generate mutants that were either 2β- or, optionally, 15β-regioselective (96–97%), with ≥99% diastereoselectivity (oxidation on the β-face of the steroid).[5] The starting monooxygenase P450-BM3 is regiorandom but fully β-selective in these particular reactions. Although the results constitute an important advance, it can be speculated that this approach works this well in the P450-BM3-catalyzed reactions only for large and rigid molecules such as steroids. Smaller non-natural compounds may have a higher propensity to bind at different sites and in different poses within the large binding pocket, depending upon the extent of the various types of interactions. Here we show that structure-guided laboratory evolution based on CASTing/ISM[2,8] is, in fact, successful in the quest to control both regio- and enantioselectivity of P450-catalyzed oxidative hydroxylation in such cases. The goal was to evolve mutants that were highly regioselective as well as highly enantioselective in a given model reaction, striving for *R* and *S* selectivity on an optional basis. Selectivities well above 90% would approach an ideal oxidative conversion in organic chemistry, thereby complementing cur-

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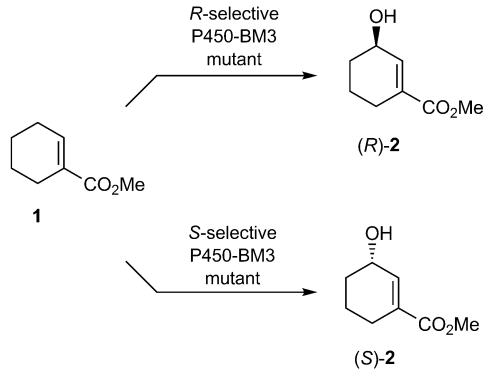
rent synthetic organic approaches based on selective reagents or catalysts which likewise address the challenging problem of selective CH activation.[9]

Results and Discussion

Experimental platform

We chose cyclohexene-1-carboxylic acid methyl ester (**1**)[10] as the molecule to be regio- and enantioselectively hydroxylated. Wild-type (WT) P450-BM3 provides alcohol **2** with a regioselectivity of 84% (16% other oxidation products) and *R* selectivity of 34% *ee*. One of the factors leading to preferred reaction at the allylic 3-position might be the activating influence of the olefinic double bond. Our goal was to evolve both *R* and *S* mutants of P450-BM3 to obtain (*R*)- and (*S*)-**2**, respectively, while maximizing regioselectivity to a synthetically useful level (Scheme 1).



Scheme 1. P450 hydroxylation of substrate **1**.

P450-BM3 consists of a heme-dependent monooxygenase and a fused electron-delivering NADPH-dependent diflavin-reductase, with these structural properties leading to unusually high turnover numbers in P450-catalyzed reactions.[3,7] X-ray structures of the heme domain in the unbound[11] and inhibitor (*N*-palmitoylglycine)-bound[12] forms have been reported, the latter serving in the previous[5] and present studies as a guide for designing focused libraries by using saturation mutagenesis. On the basis of this structural information, 24 residues were considered for saturation mutagenesis (Figure 1). These can be assigned to three categories, namely 1) residues closest to the catalytically active heme, 2) residues significantly farther away from the heme but still near the binding pocket, and 3) residues at the entrance to the binding pocket and relatively far (>15 Å) from the heme. Such positions have been considered in previous protein engineering studies for other purposes using different substrates.[4,5,13]

In principle, the single positions can be subjected to saturation mutagenesis individually, or they can be grouped into randomization sites comprising two or more residues, which would allow for cooperative epistatic interactions (more than additivity) between the mutations within a site.[14] We applied

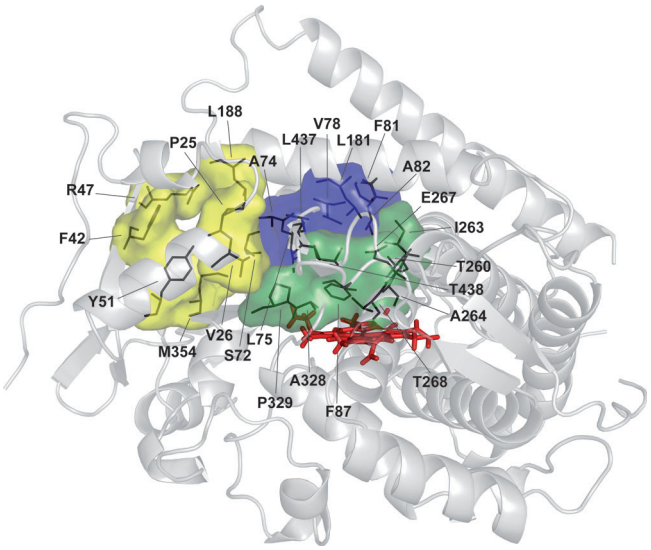


Figure 1. The 24 residues in P450-BM3 considered for saturation mutagenesis, guided by the X-ray structure of the heme domain (PDB ID: 1JPZ).[12] These residues were assigned to three categories marked green (residues closest to the heme), blue (residues relatively far from the heme-Fe but still next to the binding pocket), and yellow (residues at entrance to the large binding pocket); the heme cofactor is marked in red.

both strategies by performing saturation mutagenesis at 23 out of 24 single-residue sites (position 329 was not considered at this point), as well as at selected two-residue sites (Phe87/Ala328, Ala328/Phe329, Val78/Leu181 and Val78/Leu437). In the case of single-residue sites, the use of NNK codon degeneracy encoding all 20 canonical amino acids requires the screening of about 100 transformants for 95% library coverage (assuming the absence of amino acid bias); this number increases to about 3000 when considering two-residue sites.[8a,14b] In the latter case, we therefore utilized NDT codon degeneracy encoding 12 amino acids (Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, and Gly) and requiring only 430 transformants for 95% library coverage.[8a,14b] Saturation mutagenesis was carried out by two different cloning methods (either QuikChange[15] or the Gibson–Venter in vitro isothermal assembly[16]), with prescreening for activity performed by traditional UV/Vis-based measurement of NADPH consumption in the initial reaction phase followed by GC-based assessment of regioselectivity and *ee* determination of the best mutants (Supporting Information).

Mutants generated in initial saturation mutagenesis libraries

The results of screening the initial libraries are shown in Table 1, which lists mutants with ≥40% *ee*. We noticed that the overall hit-identification procedure leads to *ee* values which, in a given case, can vary by up to 8%. Other less stereoselective mutants are documented in Table S3 of the Supporting Information. Table 1 reveals some remarkable results. The best mutants in the initial single position libraries are variants A82M with 80% *ee*/*R* (entry 6), and I263G with 66% *ee*/*S*

Table 1. *R*- and *S*-selective P450-BM3 mutants obtained in the initial saturation mutagenesis libraries as catalysts in the model reaction **1**→**2** characterized by ≥ 40% *ee*, with screening being performed by UV/Vis-based measurement of NADPH consumption in the initial reaction phase followed by chiral GC.[a]

	Site	Mutation	% *ee*[b] (config.)
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)[c]
20	87/328	F87V/A328N	96 (*R*) (94–96)[c]
21	87/328	F87I/A328V	96 (*R*) (86–99)[c]
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] Reaction conditions are described in the Supporting Information. [b] Average of at least two different time points. [c] Values observed following several scaled up experiments under different conditions.

(entry 14). Interestingly, the library obtained by randomization at position 87 contains single mutants displaying either *R*- or *S*-selectivities ranging between 48% *ee*/*R* (mutant F87I; entry 9) and 64% *ee*/*S* (mutant F87D; entry 8). Other mutants, such as F87P (42% *ee*/*R*; entry 11) and F87G (46% *ee*/*S*; entry 10), likewise show that position 87 is indeed a hot spot, as indicated in previous studies on other substrates.[4,13] However, none of the single mutants reach enantioselectivities even close to 90% *ee*. In contrast, when randomizing two-residue sites by using the reduced amino acid alphabet encoded by NDT codon degeneracy, several highly *R*-selective double mutants were discovered, the three best variants (entries 19–21) each leading to 96% *ee* with conversions ≥95% (10 mM, 0.1 mmol scale, 8 h) in the model reaction **1**→**2**. Improved *S*-selective mutants were not found in these saturation mutagenesis experiments. This set of data suggests that: 1) amino acid residues closer to the heme (green area in Figure 1) have a higher impact on enantioselectivity, and 2) access to the *S* enantiomer is more difficult. For this reason, a direct *ee*-based screening appeared necessary for further improvements.

In an attempt to boost the reversed *S* enantioselectivity, we initially generated a small number of double mutants by combining some of the mutations of the *S*-selective single variants. This approach led to limited success, since 70% of the mutants were inactive and only one (I263C/A328L) showed improved *S* enantioselectivity (79% *ee*). Therefore, we invoked iterative saturation mutagenesis (ISM)[2,8,14] by utilizing the best variant, I263G, as a template and randomizing a limited number of single sites (F81, F87, and A328) using NNK, NDT, and NNK codon degeneracy, respectively. This choice was based on the results of the initial rounds of mutagenesis at these positions, which led to reversal of enantioselectivity in favor of (*S*)-**1**. At this point, direct chiral GC analysis was applied. Moreover, an engineered *Escherichia coli* strain, BL21-Gold(DE3) Δ*dkgA*:: T7-*gdh* (BOU730 strain) capable of NADPH regeneration was used as a host (see the Experimental Section), with the advantage that the usual requirement of adding glucose dehydrogenase for the cofactor regeneration no longer holds. This experimental setup simplified the search for mutants displaying enhanced enantioselectivity. The ISM experiments, with I263G as the template, provided notably improved *S*-selective mutants in the case of saturation mutagenesis at positions 87 and 328, namely F87Y/I263G (90% *ee*) and I263G/A328S (96% *ee*, conversion ≥95% at 2.5 mM final substrate concentration, 3 μmol scale, 20 h), respectively. The library at position 81 failed to provide any mutants displaying enhanced enantioselectivity.

Careful analysis of the reaction of compound **1** with WT P450-BM3 as the catalyst revealed, in addition to (*R*)-**2** (34% *ee*), the presence of 16% side products comprising a regioisomeric alcohol and other oxidation products (98% conversion at 2.5 mM, 4 μmol scale, 20 h). In large-scale experiments on the *R*-selective mutant (F87V/A328N), observed *ee* values were in the range of 94–96%, and the formation of side products was reduced (7% of other oxidation products at ≥99% conversion, 23 mM final substrate concentration, 1.1 mmol scale, 8 h). In similar scale-up experiments on the *S*-selective mutant I263G/A328S (15 mM, 0.7 mmol scale, 23 h), enantioselectivity was slightly reduced to 94% *ee* with a conversion of 76% (3% of other oxidation products). The ratio of substrate to enzyme was found to influence stereoselectivity to some degree, with slow addition of substrate ensuring optimal results,[17] which is particularly important in large-scale experiments. This was best performed by using a syringe pump (see the Experimental Section). Whereas enantioselectivity of the best *R*- and *S*-selective mutants is similarly high, the latter was less active. A literature search showed that these mutants (F87V/A328N and I263G/A328S) have not been reported previously. Interestingly, selected point mutations at positions 87 and 328 have been combined previously for other purposes.[13e] However, the present results show that randomization at a two-residue site with a reduced amino acid alphabet is a more reliable strategy.

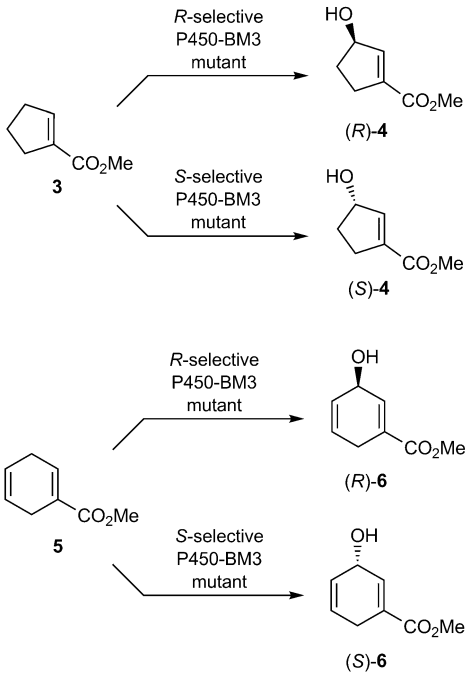
As the initial screening system was based on activity, followed by GC-determination of enantioselectivity of the apparently most active variants, some highly *S*- or *R*-selective mutants may well have been missed. Therefore, the F87/A328 library was generated once more, this time with screening being performed directly by chiral GC. In these experiments,

an alternative method of saturation mutagenesis based on the use of a megaprimer was used,[18] which is an improvement over previous related approaches originally based on the MEGAWHOP protocol.[19] Remarkably, six *R*-selective variants (Table 2, entries 2, 4–8) and one *S*-selective variant (entry 3) were found, all showing ≥90% *ee*. Five of the mutants (Table 2, entries 2, 3, 4, 6, and 7) were not identified in the previous F87/A328 library. All mutants shown in Table 2 led to ≥95% conversion (at 2.5 mM, 3 μM substrate scale, 20 h; Supporting Information).

Table 2. *R*- and *S*-selective P450-BM3 mutants as catalysts in the model reaction **1**→**2** obtained from the F87/A328 library, which was constructed by using the megaprimer saturation mutagenesis method;[18] screening was performed directly by chiral GC analysis.[a] Only mutants characterized as having ≥75% *ee* are listed here.

	Mutation	% *ee*[b] (config.)		Mutation	% *ee*[b] (config.)
1	WT	34 (*R*)	7	F87D/A328I	93 (*R*)
2	F87L/A328V	98 (*R*)	8	F87V/A328V[d]	93 (*R*)
3	A328S	97 (*S*)	9	F87N/A328V	87 (*R*)
4	F87N/A328I	96 (*R*)	10	F87S/A328I	86 (*R*)
5	F87V/A328N[c]	96 (*R*)	11	F87I/A328F	85 (*R*)
6	F87L/A328I	95 (*R*)	12	F87S/A328V	75 (*R*)

[a] Reaction conditions are described in the Supporting Information. [b] Average of at least two independent experiments. [c] Entry 5 corresponds to entry 20 in Table 1 (same mutant formed by different methods). [d] Entry 8 corresponds to entry 19 in Table 1 (same mutant formed by different methods).



Scheme 2. P450 hydroxylation of substrates **3**.

Other compounds as substrates

In order to explore how some of the hits, specifically evolved for compound **1**, perform as catalysts in regio- and stereoselective CH activation of structurally related other small molecules without rescreening the libraries or performing additional mutagenesis experiments, compounds **3** and **5** were subjected to oxidation (Scheme 2). Table 3 shows that in both cases, the *R* and *S* enantiomers of **4** and **6**, respectively, are accessible with respectable stereoselectivities in the range of 84–94% *ee*. Racemic **6** was previously prepared by Danishefsky and coworkers by Diels–Alder reaction of trans-methyl β-nitroacrylate with trans-1-trimethylsilyloxy-1,3-butadiene, followed by elimination and desilylation.[20a] In other work by Berchtold et al., the *tert*-butyldimethylsilylated form of (*S*)-**6** was obtained in a multistep sequence and used as a chiral intermediate for preparing chorismate-type compounds.[20b] In the present study, a two-step synthesis is involved in which the starting compound **5** is accessible by Diels–Alder reaction of butadiene and propiolic acid methyl ester in high yield[21] (Supporting Information). In spite of the observation that about one-third of the reaction products in the second step, catalyzed by P450-BM3 mutant I263G/A328S comprise undesired oxidation products, the overall approach described herein appears attractive.

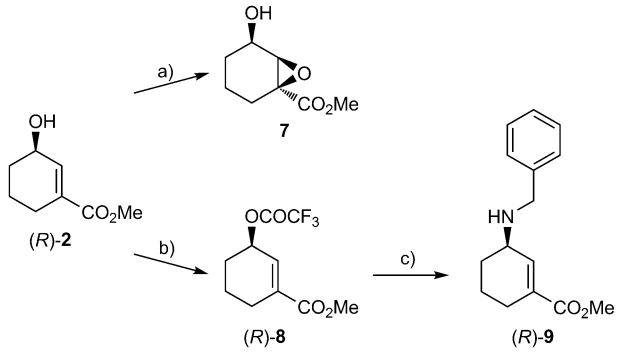
Selective modification of reaction products

Oxidative CH activation of the type described here can be exploited for transforming simple organic compounds into more complex products by designing appropriate stereoselective or stereospecific cascade reactions using synthetic reagents/catalysts or enzymes. For example, reaction of compound (*R*)-**2** with *m*-CPBA resulted in essentially one compound, with diastereoselectivity resulting from the directing effect of the alcohol moiety[22] (Scheme 3). We also wanted to introduce amino functionalities, and therefore turned to Pd-catalyzed stereospecific and regioselective allylic substitution, which can be ex-

Table 3. Performance of three mutants specifically evolved for the reaction of **1**, which served as catalysts in the oxidative hydroxylation of substrates **3** and **5** (2–5 mM, 3–8 μmol scale; Supporting Information).

Substrate	Mutant	Product	% *ee* (config.)[a]	Conversion [%][b]	*t* [h]	Other products [%][b]
3	WT	**4**	7 (*S*)	77	20	58
3	F87I/A328V	**4**	94 (*R*)	≥99	8	13
3	I263G/A328S	**4**	84 (*S*)	81	20	17
5	WT	**6**	7 (*S*)	90	24	26
5	F87V/A328N	**6**	84 (*R*)	≥99	8	30
5	I263G/A328S	**6**	93 (*S*)	89	24	36

[a] Average of at least two independent experiments. [b] From GC analysis of the crude products.



Scheme 3. Chemical modification of compound (*R*)-**2**. 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%.

pected to proceed with retention of configuration, although not always with predictable regioselectivity.[23] To this end, (*R*)-**2** was acylated with trifluoroacetic acid anhydride[23e] to form (*R*)-**8**, which in turn was subjected to Pd-catalyzed allylic substitution with benzylamine as a nucleophile. The only substitution product that was detected proved to be (*R*)-**9**, showing that complete regioselectivity in favor of attack at the γ-position had occurred (Scheme 1). GABA-analogues of this type are of potential pharmaceutical interest as neurological agents[24] and, in the case of product **6**, as possible chiral precursors of influenza neuraminidase inhibitors such as Tamiflu or analogues thereof.[25]

Conclusions

We have demonstrated that rational structure-guided directed evolution, as exemplified by the CAST/ISM approach,[2,8,14] is a viable tool for evolving mutants of P450-BM3 that allows essentially complete control of regio- and enantioselective oxidative hydroxylation of small cyclic compounds bearing an ester functionality. Scaling up to the 1.1 mmol level has been demonstrated in a model reaction involving cyclohexene-1-carboxylic acid methyl ester (**1**) for both the *R*- and *S*-configured oxidation products. Two other related substrates likewise undergo regio- and enantioselective oxidative hydroxylation with the mutants evolved for model compound **1** without the necessity of performing additional mutagenesis experiments or screening previous libraries. These substrates contain ester groups, but the present approach is likely to be just as successful when targeting the selective hydroxylation of other small compounds bearing different types of functional moieties. Indeed, as this paper was being completed, a report by Li et al. appeared in which the CAST/ISM strategy was applied successfully to P450pyr from *Sphingomonas* sp. HXN-200, used as a catalyst in the oxidative hydroxylation of *N*-benzyl pyrrolidine and resulting in a mutant that exhibits 98% *S* selectivity at the unactivated 3 position.[26] The authors reported that this is the first instance of directed evolution of a P450 enzyme leading to truly high enantioselectivity in a model reaction. It should be noted that WT P450pyr was known to be completely regioselective at the inactivated 3 position (53% *ee* (*S*)), with no reaction occurring at the activated N-benzylic position where the C–H bond strength is expected to be considerably lower.[26,27] Specific positioning of a substrate in the binding pocket of P450 enzymes, whatever the underlying factors may be, determines regioselectivity. In the case of P450pyr acting as a catalyst in the 3-selective hydroxylation of *N*-benzyl pyrrolidine, reversal of enantioselectivity of up to 83% *ee* had previously been achieved by utilizing iterative saturation mutagenesis (ISM).[4c] Combining present and previous results gives the potential user of structure-guided directed evolution[2,8,14] confidence that P450 enzymes can be engineered successfully in the quest to control regio- and stereoselectivity in the oxidative hydroxylation of a variety of structurally different functionalized molecules. Such selective enzymatic oxidative CH activation, combined with subsequent stereoselective or stereospecific transformations by reagents or synthetic catalysts, constitutes a strategy in synthetic organic chemistry that complements other approaches.[9]

Based on the experience gained in our latest investigation, we make two recommendations for future protein engineering studies of P450 enzymes: 1) utilization of the engineered *E. coli* strain as a host with "built-in" NADPH regeneration, thereby making the use of glucose dehydrogenase unnecessary (Experimental Section), and 2) careful control of the ratio of substrate to P450 enzyme, as this parameter can influence stereoselectivity, with slow addition of substrate by a syringe pump being a viable strategy for obtaining optimal results in scale-up experiments.

Future work will concentrate on the biophysical characterization of the mutants described herein, including kinetics, spectroscopic studies, molecular-dynamics simulations, and possibly crystal structures. A remaining challenge for directed evolution would be complete control of regio- and enantioselectivity in the oxidative hydroxylation of small alkanes devoid of any functional groups,[4k,28] although this is less interesting from a synthetic viewpoint.

Experimental Section

Molecular biology

Reagents: E. coli strain BL21-Gold(DE3) was obtained from Stratagene. Restriction enzymes (NcoI, HindIII, DpnI), Taq DNA ligase, and stock solutions of dATP, dTTP, dGTP, and dCTP (100 mM) were purchased from New England Biolabs. T5 exonuclease was provided by Epicenter. NADPH was obtained from Calbiochem or from Codexis. Glucose dehydrogenase was also purchased from Codexis. $NADP^+$ and NAD^+ were purchased from Sigma. KOD hot start polymerase, dNTPs, $MgSO_4$, and DNA vectors pACYCDuet-1 and pRSFDuet-1 were obtained from Novagen. Phusion polymerase was provided by Finnzymes. An *E. coli* gene deletion kit was provided by Gene Bridges. Oligonucleotides were purchased from Invitrogen in standard, desalted form and used without further purification (Table S1). DNAse I, lysozyme, and DTT were obtained from AppliChem. LB medium, in a premixed powder form, and kanamycin (kan) were obtained from ROTH. IPTG and PEG-4000 were obtained from Fermentas. TB medium contained yeast extract (24 g L^{-1}), peptone (12 g L^{-1}), glycerol (4 mL L^{-1}), KH_2PO_4 (0.017 M), and K_2HPO_4 (0.072 M). SOC medium contained yeast extract

($20\,g\,L^{-1}$), peptone ($5\,g\,L^{-1}$), glucose (20 mM), NaCl (10 mM), KCl (2.5 mM), $MgCl_2$ (10 mM), and $MgSO_4$ (10 mM).

General procedures: Plasmids and PCR products were purified with Qiagen spin columns. Electrocompetent cells were prepared in house according to standard protocols.[29] All cloning manipulations were based on the in vitro ligation isothermal assembly protocol described by Gibson et al.[16] Briefly, purified vector (ca. 100 ng) and insert (ca. 100 ng) in a final volume of 5 μL distilled water were mixed with 15 μL of assembly master mixture and incubated at 50 °C for 1 h in a PCR thermocycler. The ligation reaction mixture was kept on ice until an aliquot (1–3 μL) was used to transform electrocompetent cells. The assembly master mixture was prepared by adding the following reagents into 218 μL of distilled water: 98.2 μL isothermal reaction buffer (5×), 6.8 μL Phusion polymerase, 2 μL T5 exonuclease (10× diluted in T5 exonuclease buffer), and 50 μL Taq DNA ligase. Isothermal reaction buffer (6 mL total volume; 5×) was prepared by mixing the following reagents (final concentrations indicated in parenthesis): 3 mL of 1 M Tris·HCl (500 mM; pH 7.5), 1.5 g PEG-4000 (25%, *w/v*), 300 μL of 1 M DTT (50 mM), 300 μL of 1 M $MgCl_2$ (50 mM), 300 μL of 100 mM NAD^+ (5 mM), and 60 μL of 100 mM stock solution of each of the four dNTPs (800 μM) in the appropriate amount of distilled water. Isothermal reaction buffer, in 500 μL aliquots and assembly master mixture in 15 μL aliquots were stored at −20 °C. Saturation mutagenesis was carried out by using either: 1) QuikChange protocol with KOD hot start polymerase,[15] 2) an isothermal ligation assembly of degenerate insert and vector, prepared by two standard PCR reactions with appropriate degenerate primers,[30] and/or 3) an improved version of the megaprimer method.[18]

Subcloning of gene encoding $P450_{BM3}$ (CYP102A1) into pRSFDuet-1 vector: A plasmid (pETM11-BM3; 8490 bp) harboring the gene encoding $P450_{BM3}$ was kindly provided by Dr. Sabrina Kille.[18] To enhance the efficiency of the QuikChange reaction, the gene of interest was subcloned into a smaller vector (pRSFDuet-1; 3829 bp). The backbone of pRSF-Duet-1 was amplified by using primers pRSF-NcoI (ggt ata tct cct tat taa agt taa aca aaa tta ttt cta cag g) and pRSF-AvrII (taa cct agg ctg ctg cca ccg ctg agc aat aac), and the template plasmid was eliminated after restriction digestion with HindIII. The gene encoding $P450_{BM3}$ was amplified by using pETM11-BM3 as a template and primers pRSF-BM3-NcoI (ttt tgt tta act tta ata agg aga tat acc atg gca att aaa gaa atg cct cag cca aaa acg) and pRSF-BM3-AvrII (gtt att gct cag cgg tgg cag cag cct agg tta tta ccc agc cca cac gtc ttt tgc gta tcg). The oligonucleotides contained a sequence complementary to the vector sequence at the 5′-end (complementary regions are underlined). The template was eliminated by treating the PCR reaction mixture with DpnI (2× 1 μL for 16 h). The PCR reactions contained 5 μL of 10×KOD hot start polymerase buffer, 5 μL dNTPs (2 mM each), 10 μL of the appropriate forward and reverse primer (2.5 μM each), 2 μL $MgSO_4$ (25 mM), template (25 ng), and 0.5 μL of KOD polymerase in a final volume of 50 μL distilled water. The two PCR products were purified with a QIAquick PCR purification spin column (Qiagen) and ligated in vitro as described above (Figure S1). *E. coli* BL21-Gold(DE3) transformants were selected from LB-agar plates containing kan ($40\,\mu g\,mL^{-1}$). Plasmids from four colonies were isolated, and the desired construct (pRSF-$P450_{BM3}$, Sequence S1) was confirmed by NcoI restriction digestion analysis and sequencing of relevant regions. Mutant $P450_{BM3}$ F87A was subcloned from pETM11 into a pRSFDuet-1 vector following the same procedure.

Construction of E. coli *BL21-Gold(DE3) ΔdkgA:: FRT-T7-gdh strain (BOU730):* Regeneration of NADPH was necessary for the $P450_{BM3}$-catalyzed biohydroxylation reactions to proceed efficiently. This was accomplished by three means: 1) by providing an exogenous NAPDH regeneration system (i.e. glucose/glucose dehydrogenase) in a cell lysate, 2) by relying on the metabolic machinery of *E. coli* when whole cells were employed (i.e., only glucose was added), or 3) by introducing a single copy of a gene encoding glucose dehydrogenase from *Bacillus megaterium* (GDH_{BM3}) into the chromosomal DNA of *E. coli* cells. We relied on the Quick and Easy *E. coli* Gene Deletion Kit from Gene Bridges[31,32] for introduction of *gdh* into the genomic DNA of *E. coli* BL21-Gold(DE3) strain with simultaneous replacement of an endogenous gene, *dkgA*, encoding the aldo-keto reductase DkgA. The latter is not necessary for the survival of *E. coli* cells.[33,34] Briefly, the FRT-flanked resistant cassette provided by the kit was cloned into the pACYC-Duet vector upstream of T7 promoter 1. The *gdh* gene was subcloned into the latter vector downstream of T7 promoter 1 to afford pACYC-FRT-KAN-FRT-GDH (Figure S2). The entire fused construct, FRT-resistant marker-T7 promoter-*gdh*, was amplified by using primers containing a sequence homologous to the target gene, *dkgA*, at the 5′-end. Upon disruption via homologous recombination, with the help of a Red/ET plasmid, the resistant marker was removed by a FLP-recombinase step (Figure S2). The kanamycin resistant marker was placed upstream of the T7 promoter of pBOU67804 (a derivative of pACYCDuet-1 harboring the YqjM gene) by in vitro ligation as described above. Specifically, the antibiotic resistant region of the disruption cassette was amplified by PCR by using the primers pACYC-frt-kan-fw (tgt ccg gga tct cga cgc tct ccc tta tgc aat taa ccc tca cta aag ggc ggc cgc) and pACYC-frt-kan-rc (agt gag tcg tat taa ttt cct aat gca gga gtc taa tac gac tca cta tag ggc tcg), with FRT-PGK-gb2-neo-FRT as template DNA (Gene Bridges). The oligonucleotides contained a sequence complementary to the vector sequence at the 5′-end (complementary regions are underlined). The PCR reaction contained 5 μL of 10×KOD hot start polymerase buffer, 5 μL dNTPs (2 mM each), 10 μL of the appropriate forward and reverse primer (2.5 μM each), 2 μL $MgSO_4$ (25 mM), template (50 ng), and 0.5 μL of KOD polymerase in a final volume of 50 μL distilled water. The backbone of pBOU67804 was PCR amplified by using the primers pACYC-T7-fw (gac tcc tgc att agg aaa tta ata cga ctc ac) and pACYC-*Pfo*I-rc (gca taa ggg aga gcg tcg aga tcc cgg ac). The PCR reaction contained 5 μL of 10×KOD hot start polymerase buffer, 5 μL dNTPs (2 mM each), 10 μL of the appropriate forward and reverse primer (2.5 μM each), 2 μL $MgSO_4$ (25 mM), template (20 ng), and 0.5 μL of KOD polymerase in a final volume of 50 μL distilled water. The template was eliminated by treating the PCR reaction mixture with DpnI (2×1 μL) for 16 h. *E. coli* BL21-Gold(DE3) transformants were selected from LB-agar plates containing kan ($40\,\mu g\,mL^{-1}$) and chloramphenicol ($20\,\mu g\,mL^{-1}$). Plasmids from eight colonies were isolated, and the desired construct (pBOU68408) was confirmed by sequencing of relevant regions. The YqjM gene in pBOU68408 was replaced by the *gdh* gene from *Bacillus megaterium* by in vitro ligation to afford pBOU71106, designated as pACYC-FRT-KAN-FRT-GDH (Figure S2). Specifically *gdh* (Sequence S3) was PCR amplified from pRSF-GDH (plasmid kindly provided by Dr. Huabao Zheng) by using the pair of primers lib1-pRSF-DGH-NcoI (ttt tgt tta act tta ata agg aga tat acc atg tat aca gat tta aaa gat aaa gta gta) and Rlib1-pRSF-DGH-AvrII (gtt att gct cag cgg tgg cag cag cct agg tta tta tcc ccg tgc tgc ttcg aat cat gg). The backbone of pBOU68408 was amplified by using the pair of primers pRSF-NcoI (ggt ata tct cct tat taa agt taa aca aaa tta ttt cta cag g) and pRSF-AvrII (taa cct agg ctg ctg cca ccg ctg ag caa taa c). The oligonucleotides contained a sequence complementary to the vector sequence at the 5′-end (complementary regions are underlined). The template was eliminated by treating the PCR reaction mixture with DpnI (2×1 μL) for 16 h. *E. coli* BL21-Gold(DE3) transformants were selected from LB-agar plates containing kan

(40 μg mL^{-1}) and chloramphenicol (20 μg mL^{-1}). Plasmids from eight colonies were isolated, and the desired construct (pBOU71106) was confirmed by sequencing of relevant regions.

A disruption cassette flanked by short regions homologous to *dkgA* was amplified from pBOU71106 by using primers Up-dkgA-FRT-Kan (atg gct aat cca acc gtt att aag cta cag gat ggc aat gtc atg ccc cag caa tta acc ctc act aaa ggg cgg ccg) and Down-GDH-dkgA (tta gcc gcc gaa ctg gtc agg atc ggg acc gag acg ctt gcc ctg atc gag ttt tgt tat tat ccg cgt cct gct tgg aat gat ggg tac). The homologous regions are underlined. *E. coli* BL21-Gold(DE3) strain harboring pRedET$^{amp(R)}$ (Gene Bridges) was transformed with the aforementioned disruption cassette. Expression of redα, β, and γ genes of the λ phage red recombinase with the *recA* gene under the control of an arabinose-inducible promoter facilitated recombination among the homologous regions of the disruption cassette and the *dkgA* locus of *E. coli* chromosomal DNA (Figure S2). Cells were grown at 37 °C to eliminate pRedET$^{amp(R)}$ (due to its temperature-sensitive replicon) with selection for kanamycin resistance. Fourteen kan-resistant colonies were re-streaked on LB-agar plates containing kan (20 μg mL^{-1}) and screened by colony PCR for the disruption of *dkgA*. A single colony, BOU72304, contained the desired disruption. The expression of GDH under the T7 promoter was confirmed by SDS-PAGE analysis of boiled cells induced with IPTG (Figure S3). An aliquot of clear lysate from the same culture was used to monitor the increment of absorbance at 340 nm due to GDH-dependent formation of NADPH in presence of glucose/NADP$^+$. Uninduced BOU72304 cells were used as a negative control (Figure S3). The resulting strain, *E. coli* BL21-Gold(DE3) ΔdkgA:: FRT-KAN-FRT-T7-GDH, was rendered electrocompetent and transformed with pCP20 (Coli Genetic Stock Center, CGSC) and/or with 707-FLPe plasmid (Gene Bridges). In our hands, transformation of 707-FLPe into BL21(DE3) proved inefficient. The kanamycin resistance gene was removed by FLP-mediated site-specific recombination to leave a single FRT site, along with the T7 promoter–GDH cassette, at the original *dkgA* locus. The elimination of the marker in colony BOU73009 was confirmed by absence of resistance to kanamycin and by colony PCR. The resulting strain, *E. coli* BL21-Gold(DE3) Δ*dkgA*:: FRT-T7-gdh, was designated BOU730.

Chemistry

General remarks: Starting compounds **1** and **3** were purchased from Sigma–Aldrich and Acros and were used without further purification. Racemic standards **2**[35] and **4**[36] were prepared by reduction of the corresponding six-membered[37] and five-membered[36] ring ketones with $NaBH_4$ in MeOH as described in the Supporting Information. Compound **5**[38] was prepared according to similar literature protocols.[39] All other reagents, including dry solvents, were purchased from Acros, Sigma–Aldrich, and Alfa and were used without further purification. NMR spectra were recorded on a Bruker Avance 300 or DRX 400 (^{1}H: 300 MHz or 400 MHz, ^{13}C: 75 MHz or 101 MHz) spectrometer with TMS as internal standard ($d=0$) unless otherwise noted. High-resolution EI mass spectra were measured on a Finnigan MAT 95S spectrometer. High-resolution mass spectra recorded in ESI and APCI mode were performed on a ThermoScientific LTQ-FT spectrometer. Conversion and enantiomeric excess were determined by achiral and chiral gas chromatography as described. Alternatively, the enantiomeric excess of product **9** could be measured by HPLC. Optical rotation measurements were performed on a Rudolph Research Analytical, Autopol IV at 25 °C. Analytical thin layer chromatography was performed on Merck silica gel 60 F254q, while Merck silica gel 60 (230–400 mesh ASTM) was used for column chromatography. Reactions that required inert atmosphere (nitrogen or argon) were carried out by using standard Schlenk techniques.

Procedure for scaled-up biohydroxylation reactions with P450 mutants:

Preparation of (*R*)-methyl 3-hydroxycyclohex-1-enecarboxylate (2): For scaling up the biohydroxylation reactions, an Erlenmeyer flask (50 mL) containing LB (10 mL) and kan (50 μg mL^{-1}), was inoculated with a colony from BOU730 cells expressing (*R*)-P450 mutant (F87V/A328N), and incubated overnight at 37 °C with shaking. An aliquot of this pre-culture was inoculated into TB (500 mL) containing kan (50 μg mL^{-1}) (initial OD of 0.1 at 600 nm). Culture was grown at 30 °C until an OD_{600} of 0.6–0.8 was reached, then IPTG was added to a final concentration of 0.2 mM, and the culture was grown at 30 °C over 12 h with agitation. Cells were pelleted by centrifugation (4000 rpm, Fiberlite F10-6x500y, Sorvall, 15 min at 4 °C). The pellet [4.5–5 g wet mass (ca. 20 μM P450)[40] was resuspended in M9 minimal salts medium without a nitrogen source [50 mL, pH 7.0 containing Na_2HPO_4 (12.8 g L^{-1}), KH_2PO_4 (3.0 g L^{-1}), and NaCl (0.5 g L^{-1})] containing glucose (100 mM) and NADP$^+$ (0.5 mM). Resuspended cells were transferred to a 250 mL three-necked, round-bottomed flask, and carboxy-methyl-cyclohexene (**1**) was added via a Hamilton syringe [154 μL total volume, 14× 11 μL (0.08 mmol) every 45 min, 1.13 mmol in total]. The reaction was carried out at 25 °C for 8 h at 150 rpm (Heidolph magnetic stirrer). A pH meter was also mounted to the installation in order for the pH to be monitored continuously and maintained in the 7–7.5 range by the addition of NaOH (5 M). During the reaction time, several aliquots were withdrawn to evaluate conversion and (*R*)-**2** *ee*%. After completion, the reaction mixture was extracted with EtOAc (4×100 mL), the organic phase was dried with Na_2SO_4, the solvent was evaporated, and the residue was subjected to column chromatography to give (*R*)-methyl 3-hydroxycyclohex-1-enecarboxylate (**2**) as a colorless oil. GC analyses of the crude reaction extract indicated final conversion ≥99% and, in addition to (*R*)-**2** (155 mg, 88%), 7% of other oxidation products were detected: ($R_f=0.25$ EtOAc/petroleum ether 1:4); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=6.87$ (s, 1H), 4.35 (s, 1H), 3.75 (s, 3H), 2.25 (m, 2H), 1.98–1.50 ppm (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=167.84$, 139.87, 132.58, 66.10, 51.93, 31.29, 24.36, 19.22 ppm; HRMS (APCI+) calcd for $C_8H_{13}O_3$ $[M+H]^+$: 157.0859, found: 157.0857; 94–96% *ee*/*R*.

Preparation of (*S*)-methyl 3-hydroxycyclohex-1-enecarboxylate (2): For scaling up the production of (*S*)-**2**, BOU730 cells expressing (*S*)-P450 mutant (I263G/A328S) were grown in LB as described above. Although we scaled up the production of (*S*)-**2** using the whole-cell strategy as described for (*R*)-**2**, the best results were observed with lysed cells. Briefly, an aliquot of the LB pre-culture was inoculated into TB (500 mL) containing kan (50 μg mL^{-1}; initial OD of 0.1 at 600 nm). The culture was grown until an OD_{600} of 0.6–0.8 was reached, then IPTG was added to a final concentration of 0.2 mM, and the culture was grown at 30 °C for 20–24 h with agitation. Cells were harvested by centrifugation (4000 rpm, Fiberlite F10-6x500y, Sorvall, 15 min at 4 °C), and the pellet (4.5–5 g wet mass, ca. 23 μM P450) was resuspended in lysis buffer (40 mL) [phosphate buffer (pH 7.4, 100 mM), lysozyme (14 mg mL^{-1}), and DNAse I (6 U mL^{-1})]. The suspension was incubated for 30 min with agitation in an ice bath, then sonicated for four cycles of 30 s, alternating with four cycles of 30 s on ice. Cellular debris was pelleted by centrifugation (5000 rpm, fixed rotor F34-6-38, Eppendorf, 20 min at 4 °C), and the supernatant was diluted to 50 mL with reaction buffer [phosphate buffer (pH 7.4, 100 mM), glucose (100 mM final concentration), and NADP$^+$ (500 μM final concentration)]. Lysed cells were transferred to a 250 mL round bottomed flask

with three necks, and carboxy-methyl-cyclohexene (**1**; diluted 1:1 in CH_3CN) was added via a Hamilton syringe by using an automatic syringe pump (200 μL total volume, 10 $\mu L\,h^{-1}$, 0.73 mmol in total). The reaction was carried out at 25 °C for 20 h at 150 rpm (Heidolph magnetic stirrer). A pH meter was mounted to the installation in order for the pH to be monitored continuously and maintained in the 7–7.5 range by the addition of NaOH (5 M). The pH dropped to 6.35 overnight but was then raised to 7–7.5. After all of the starting material was injected, the mixture was allowed to stir for an additional 3 h, during which time the conversion did not change significantly according to GC measurements. Therefore, the reaction was stopped [based on the fact that small amounts of (*S*)-**2** alcohol are consumed, as we noticed in previous optimization trials (data not shown)]. The mixture was then extracted with EtOAc (4×100 mL), the organic phase was dried with Na_2SO_4, the solvent was evaporated, and the residue was subjected to column chromatography to give (*S*)-methyl 3-hydroxycyclohex-1-enecarboxylate (**2**) as a colorless oil. GC analyses of the reaction crude extract indicated a final conversion of 76% and, in addition to (*S*)-**2** (51 mg, 44% yield, 94% *ee*), 1.5% of other oxidation products were detected.

Cloning of the gene encoding Re-ADH into the pACYCDuet-1 vector, mutant libraries, screening protocols, reaction conditions for determination of relative activity and enantioselectivity of mutants obtained from the UV/Vis-based protocol, selection of suitable $P450_{BM3}$ mutants for production of (*R*)- and (*S*)-**2** alcohol, preparation of racemic standards, compound syntheses and characterization, absolute configuration determination, conditions for GC and HPLC analyses, achiral and chiral GC chromatograms, supporting figures, tables, and sequences can be found in the Supporting Information.

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