ACS Catalysis

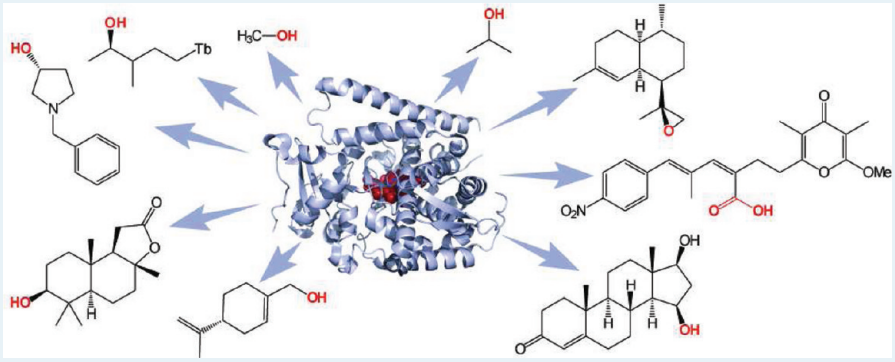
Review

pubs.acs.org/acscatalysis

Tuning P450 Enzymes as Oxidation Catalysts

Rudi Fasan*

Department of Chemistry, Hutchison Hall, University of Rochester, Rochester, New York 14627, United States



ABSTRACT: The development of catalytic systems for the controlled oxidation of C−H bonds remains a highly sought-after goal in chemistry owing to the great utility of such transformation toward expediting the synthesis and functionalization of organic molecules. Cytochrome P450 monooxygenases are the catalysts of choice in the biological world for mediating the oxidation of sp^3 and sp^2 C−H bonds with a high degree of chemo-, regio-, and stereoselectivity and in a wide array of compounds of varying complexity. The efficiency of these enzymes, compared with chemical methods, to catalyze the insertion of oxygen into unactivated C−H bonds under mild reaction conditions has sparked interest among researchers toward investigating and exploiting P450s for a variety of synthetic applications. Realizing the synthetic potential of these enzymes, however, depends upon the availability of effective strategies to tune the reactivity of natural P450s to obtain viable oxidation catalysts for the desired transformation. This review describes recent efforts in this area involving the use of protein engineering, substrate engineering, guest/host activation, and functional screening strategies. The development of engineered P450s for drug metabolite production and emerging methodologies involving the integration of P450-catalyzed transformations in preparative-scale chemoenzymatic syntheses are also presented. Key challenges that need to be addressed to capitalize on P450 oxidation catalysis for chemical synthesis are discussed.

KEYWORDS: *cytochrome P450, C−H bond oxidation, regioselective and stereoselective hydroxylation, biocatalysis, enzyme engineering*

1. INTRODUCTION

Catalytic methods for the controlled oxidation of carbon−hydrogen (C−H) bonds can provide most concise, atom-economical, and convenient solutions to the preparation and functionalization of organic molecules.[1−4] Performing this transformation with high efficiency and selectivity, however, constitutes a formidable challenge owing to the strength of C−H bonds; the occurrence of several C−H bonds of similar energy in organic compounds, especially in complex molecules; and the higher reactivity of the oxidized products compared to the reagents, which can lead to undesired overoxidation reactions.

Chemical Strategies for C−H Oxidation. The past decade has witnessed remarkable progress in the development of chemical strategies for oxygenation of C−H bonds. The combined use of "directing groups" and transition metal catalysts, for example, has provided a way to favor the oxidation of a sp^3 or sp^2 C−H bond located in proximity of a directing functionality.[5−8] Other approaches have capitalized on the difference in reactivity among the various C−H bonds in the molecule to afford the oxidation of electronically activated C−H bonds, typically tertiary or heteroatom-bearing C−H bonds, with high selectivity over inherently less reactive secondary and primary sites.[9−15] Alternatively, "biomimetic" supramolecular assemblies have been implemented that integrate structural elements to bind and position the substrate in a specific orientation above a metal center, thus allowing for C−H bond oxidation with high regioselectivity.[16−20]

Despite this progress, current chemical strategies for C−H oxidation are not devoid of important limitations. The need for directing groups, for example, inherently restricts the scope of this approach to settings in which such functional groups preexist or can be readily installed and removed. Whereas catalytic systems are clearly superior to stoichiometric oxidizing reagents from an atom-economy standpoint, limited catalytic efficiencies are often observed with the former (2−50 turnovers), in particular in the context of complex molecules. The most limiting aspect, however, remains the unsolved

Received: January 2, 2012
Revised: February 13, 2012
Published: February 22, 2012

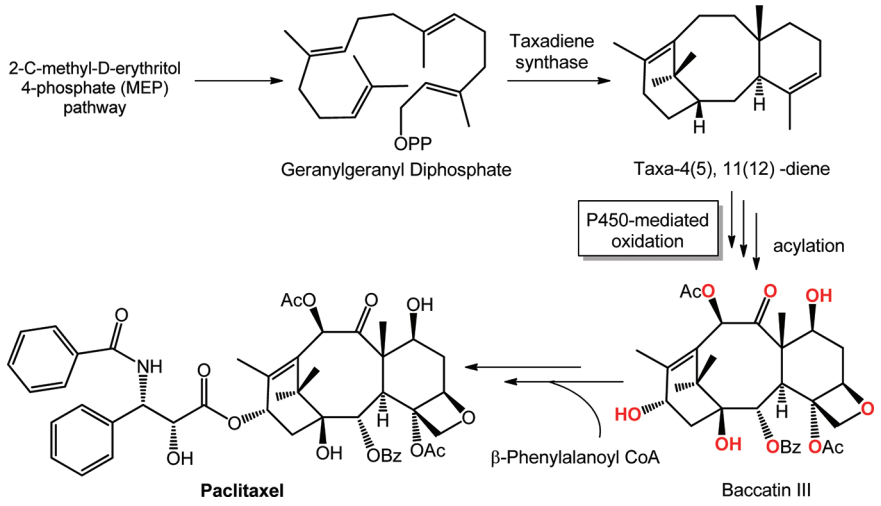


Figure 1. Biosynthesis of paclitaxel (Taxol).

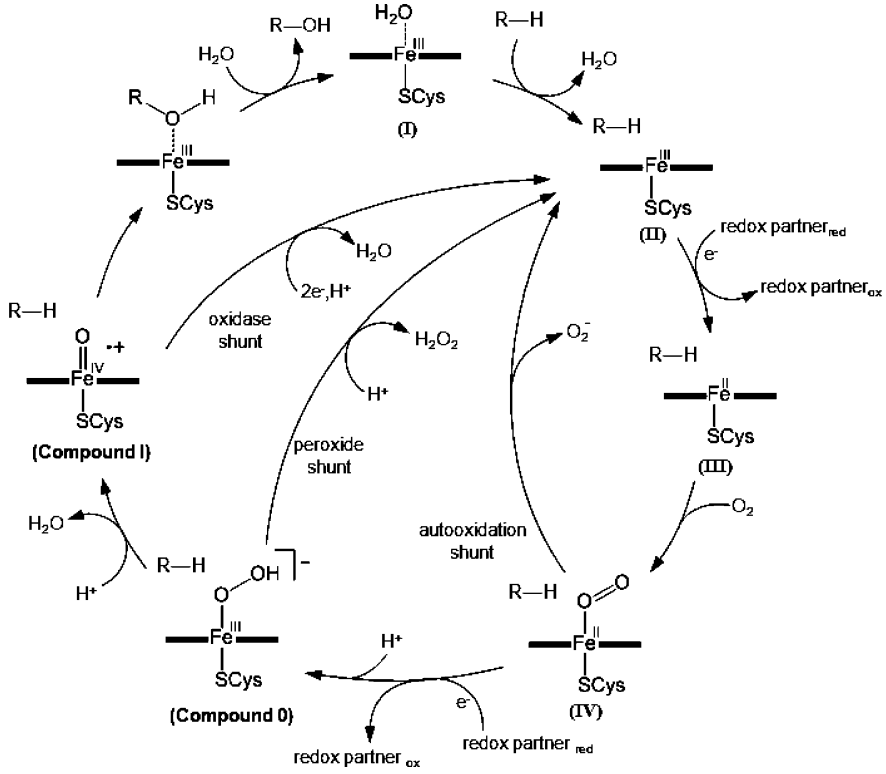


Figure 2. The catalytic cycle of P450 enzymes.

problem of tuning the selectivity of chemical reagents/catalysts to access C−H sites other than the inherently most reactive C−H bond in a given molecule because of electronic or stereoelectronic effects. As a result, the vast majority of C−H bonds occurring in substrates of high but also moderate complexity remain currently inaccessible via chemical methods.[21] In this context, the exploitation of biological oxidation catalysts such as cytochrome P450 enzymes (P450s or CYPs) has emerged as an attractive, alternative strategy toward the oxyfunctionalization of strong, unactivated C−H bonds.

P450s as Oxidation Catalysts. Cytochromes P450 (P450s or CYPs) constitute a vast superfamily of enzymes (>12 000[22]) involved in a wide range of oxidative processes as part of the biosynthesis of natural products and signaling molecules (e.g., steroids, terpenes, alkaloids, eicosanoids), the metabolic breakdown of drugs in humans, and the biodegradation of xenobiotics and pollutants. Most prominent reactions catalyzed by P450s are the monooxygenation of aliphatic (sp^3) and aryl (sp^2) C−H bonds, C−C double bonds (epoxidation), and heteroatoms (N-, S-oxidation).[23−25] A number of other interesting, albeit less common transformations, such as C−C bond formation,[26,27] C−C bond cleavage,[28−30] and Baeyer−Villiger oxidation[31] have also been reported for this versatile class of biological catalysts.[32−34]

Studies with microsomal P450s and small aliphatic substrates[25] show that the site-selectivity of P450-catalyzed hydroxylation can be influenced by the reactivity of sp^3 C−H bonds following a tertiary > secondary > primary trend, as

dx.doi.org/10.1021/cs300001x | ACS Catal. 2012, 2, 647−666

observed with synthetic oxidizing reagents.[21] It is also clear, however, that such reactivity bias can be largely overridden via substrate−enzyme recognition, thereby enabling the oxidation of a single C−H bond with high chemo-, regio-, and stereoselectivity in the presence of multiple such bonds of similar or higher reactivity. A fascinating example of such capability is presented by the biosynthesis of the diterpenoid paclitaxel, in which a battery of different P450 monooxygenases catalyze the selective oxygenation of distinct aliphatic positions in the precursor molecule taxa-4(5),11(12)-diene, thus allowing for the functional decoration of this complex scaffold to give the well-known anticancer drug (Figure 1).[35] On the other hand, members of the CYP153 family, which catalyze the hydroxylation of medium- and long-chain alkanes selectively at their terminal position, showcase the ability of these enzymes to direct the oxidation toward the least reactive C−H bond in a given substrate.[36−38]

The potential ability of P450s to catalyze the oxidation of remote, unactivated C−H bonds under extremely mild reaction conditions and using molecular oxygen make these enzymes particularly attractive from a synthetic standpoint, especially in light of the limitations of chemical methods in this respect. Accordingly, CYPs have received increasing attention over the past decade as potential catalytic platforms for a variety of relevant applications, from the synthesis of fine chemicals to drug metabolite production and bioremediation. The complexity of P450 systems, however, poses nontrivial challenges to efforts aimed at adapting and exploiting these enzymes to execute non-native transformations. Undoubtedly, realizing the synthetic potential of P450s relies upon the availability of efficient strategies to tune the reactivity of these enzymes to expand their substrate scope as well as obtain catalytically efficient and highly selective P450-based catalysts to perform the desired transformation. Over the past years, important progress has been made, and a number of different approaches have been investigated toward this goal, which will be the focus of this review article.

2. P450 CATALYSIS

A number of excellent reviews have discussed the structural, functional, and mechanistic aspects of P450 enzymes[23−25,32,39−41] which will be thus only briefly summarized here, mainly to provide a basis for the remainder of the review.

A distinctive feature of P450 catalysis is the reductive activation of molecular oxygen at the level of the heme (iron protoporphyrin IX) prosthetic group, which is embedded in the protein core through iron coordination via the thiolate of a conserved cysteine (fifth ligand) and via ionic, van der Waals, and hydrogen bond interations.[39,42] This process ultimately leads to the formation of a highly electrophilic species corresponding to an oxoiron(IV)porphyrin π cation radical ("compound **I**"), which is considered to be the major species responsible for insertion of a single oxygen atom into the substrate.[23,25] Notably, trapping and characterization of this species, which has long remained elusive due to its very short-lived nature,[43−45] was recently accomplished.[46]

Figure 2 illustrates the currently accepted catalytic mechanism underlying P450-mediated monooxygenation reactions.[23,25,32] The cycle is initiated by binding of the substrate to the enzyme active site, which results in the displacement of the water ligand from the heme distal face (sixth ligand) and concomitant shift in the equilibrium of the ferric iron spin state from low-spin to high-spin. The heme spin shift is accompanied by a change in the heme redox potential, which enables transfer of a first electron from the redox partners to the heme with reduction of heme-Fe(III) to heme-Fe(II). Oxygen binding leads to a Fe-superoxo species (IV), which is subsequently reduced by a second electron, followed by protonation to give a ferric hydroperoxo form (compound **0**). Upon protonation, this species collapses, releasing one oxygen atom in the form of water and generating compound **I**, which oxygenates the substrate via a radical rebound or concerted mechanism.[23,25,47] After release of the oxygenated product and re-equilibration with water, the ferric heme iron is restored in its water-coordinated, resting low-spin state.

In most cases, the electrons required for oxygen reduction are derived from reduced pyridine nucleotides (NADH, NADPH), which are oxidized at the level of auxiliary redox proteins. The latter support P450 catalysis by transferring the two electrons from NAD(P)H to the heme iron of the monooxygenase in sequential steps during the catalytic cycle. Genomic and biochemical studies have revealed a great diversity in the molecular organization of P450s and their redox partners, which occur in nature as three-, two-, or single-component systems (Figure 3).[24,48] The large majority of

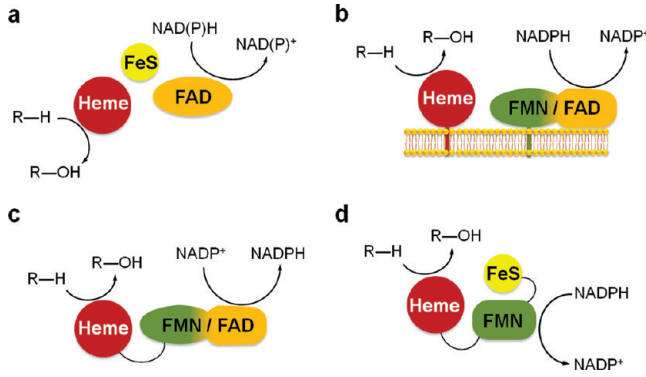


Figure 3. Different types of P450 systems: (a) bacterial, class I P450 system (e.g., $P450_{cam}$); (b) eukaryotic, membrane-bound class II P450 systems (e.g., human liver P450s); (c) P450-CPR fusion system (e.g., $P450_{BM3}$); (d) $P450_{RhF}$-type fusion system. FeS = iron−sulfur cluster, FMN = flavin mononucleotide, FAD = flavin adenine dinucleotide.

prokaryotic P450s are three-component systems, typically comprising a NAD(P)H-dependent FAD-containing reductase and a [2Fe-2S] ferredoxin (e.g., camphor hydroxylase $P450_{cam}$[49]) or a FMN-dependent flavodoxin (e.g., cineole hydroxylase $P450_{cin}$[50]) that shuttle electrons from the reductase to the monooxygenase protein (Figure 3a). Eukaryotic P450s such as drug-metabolizing human liver P450s and many plant and fungal P450s implicated in secondary metabolism are two-component systems, consisting of a membrane-bound P450 that relies on another membrane protein, namely a NAD(P)H-dependent diflavin [FAD/FMN] reductase (CPR), for supply of the electrons necessary to drive catalysis (Figure 3b). Albeit rarer, single-component P450 systems that can operate as redox self-sufficient P450s also exist (Figure 3c-d). Because of the advantages inherent in dealing with a single enzyme, as opposed to a multiprotein system, single-component P450s, such as $P450_{BM3}$, has represented a most attractive platform for the development of P450-based oxidation catalysts as well as served as inspiration model for the design of artificial redox self-sufficient P450 systems.

dx.doi.org/10.1021/cs300001x | ACS Catal. 2012, 2, 647−666

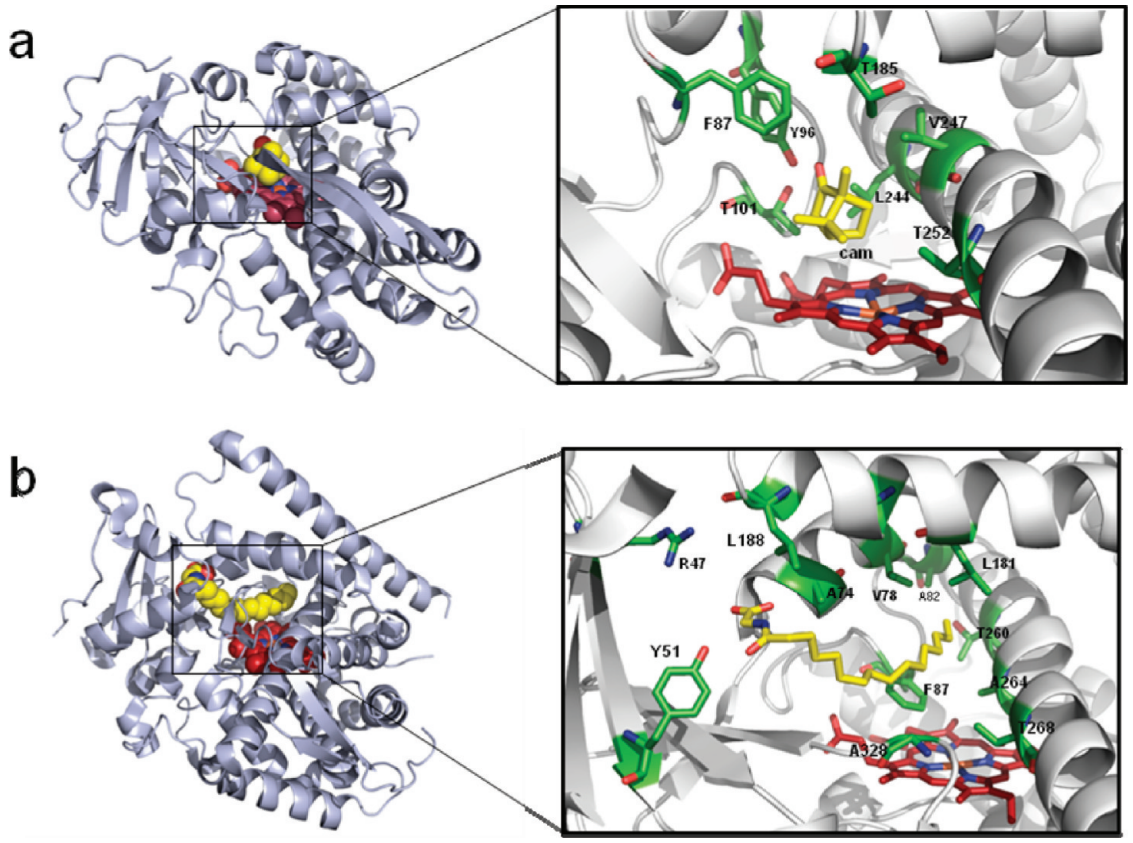


Figure 4. Crystal structures of (a) $P450_{cam}$ (PDB code 2CCP)[42] and (b) the heme domain of $P450_{BM3}$ (PDB code 1JPZ).[231] The heme and bound substrate (camphor in $P450_{cam}$, *N*-palmitoylglycine in $P450_{BM3}$) are colored in red and yellow (space-filling mode), respectively. Boxes: view of the enzyme active site highlighting the amino acid residues (green) that project their side chain in proximity of the bound substrate.

3. ALTERING P450 REACTIVITY VIA PROTEIN ENGINEERING

With a few notable exceptions (i.e., xenobiotic-metabolizing CYPs), naturally occurring P450s are typically characterized by a rather narrow substrate profile. Altering the substrate scope of these enzymes to accept non-native substrates is, thus, a first step toward converting them into catalysts of broader value. P450 engineering via rational, semirational, or evolutionary approaches has provided a major route toward this goal.[51−57] Among natural CYPs, $P450_{cam}$ (*Pseudomonas putida*) and $P450_{BM3}$ (*Bacillus megaterium*) have represented major targets for engineering, largely because of their high catalytic activity, high solubility and expression level in *Escherichia coli*, and the early availability of structural information about their monooxygenase component (Figure 4).[42,58]

$P450_{cam}$ is a "well-regulated" class I P450 (Figure 3a) that catalyzes the regio- and stereoselective oxidation of (+)-camphor to 5-*exo*-hydroxycamphor with a turnover rate of >2000 min^{-1} and a coupling efficiency of >95% under optimal conditions that is in the presence of saturating concentrations of the cognate redox partners putidaredoxin (Pdx) and putidaredoxin reductase (PdR).[59,60] $P450_{BM3}$ catalyzes the hydroxylation of linear and branched C_{12}−C_{20} fatty acids at subterminal (ω-1, ω-2, ω-3) positions with high turnover rates (1000−3500 min^{-1}) and high coupling of NADPH oxidation to product formation (88−98%).[61−63] $P450_{BM3}$ was the first identified example of a redox self-sufficient P450 system, featuring a heme-containing monooxygenase domain naturally fused to a diflavin (FAD/FMN) NADPH-dependent reductase (Figure 3c).[64]

Over the past decade, $P450_{cam}$ has been engineered, primarily via rational structure-based mutagenesis, to accept a variety of non-native substrates such as other terpenes (e.g., α-pinene, valencene),[65−67] alkanes,[68,69] styrene,[70] and other aromatic compounds.[71,72] These studies revealed that residues Y96, F87, L244, V247, whose side chains contour the heme pocket (Figure 4a), represent "hot spots" for modulating the substrate specificity of the enzyme. A single Y96F mutation, for example, was sufficient to enhance styrene epoxidation activity by 25-fold compared to the wild-type enzyme (rate: 4 → 100 min^{-1}; coupling: 7 → 32%).[70] In other studies, these positions were mutated with small apolar residues to enlarge the active site cavity, thus enabling the oxidation of large substrates, such as polycyclic aromatic hydrocarbons ($P450_{cam}$ F87A/Y96F; product: various hydroxylated regioisomers)[72] and valencene ($P450_{cam}$ F87A/Y96F/L244A/V247L; products: nootkatol and nootkatone),[65] albeit with only low (<3%) to moderate (30%) coupling efficiency, respectively. Site-directed and saturation mutagenesis of the aforementioned sites also enabled the identification of $P450_{cam}$ variants capable of hydroxylating polychlorinated benzenes[71] and diphenylmethane.[73] In efforts aimed at engineering $P450_{cam}$-based catalysts for short-chain alkane oxidation, the enzyme active site volume was reduced leading to variants such as $P450_{cam}$ F87W/Y96F/T101L/V247L, capable of converting butane to 2-butanol and propane to isopropyl alcohol.[68] Further structure-based mutagenesis led to the identification of a $P450_{cam}$ variant also able to convert

dx.doi.org/10.1021/cs300001x | *ACS Catal.* 2012, 2, 647−666

ethane to ethanol with a turnover rate of 78 min^{-1} and coupling efficiency of 10%.[69]

$P450_{BM3}$ features a long hydrophobic substrate channel connecting the protein surface to the heme pocket (Figure 4b).[58,74] More than a dozen amino acids extend their side chains toward this space, providing multiple targets for mutagenesis to modulate the substrate profile and regioselectivity of the enzyme. In this regard, the impact of modifications at F87, which is most proximal to the heme, was recognized in early investigations.[75,76] In subsequent studies, single mutations at F87 (e.g., to Val, Ala, or Gly) were found to result in greatly enhanced (10−100-fold) hydroxylation activities toward arenes and heteroarenes,[77,78] 2-arylacetic acid esters,[79] β-ionone,[80] and cycloalkanes[81] (Figure 5). Triple mutant variants containing alterations at the F87 site such as the rationally designed $P450_{BM3}$(R47L/Y51F/F87A)[82] or $P450_{BM3}$(A74G/F87V/L188Q), identified via an indole-based screen,[83] were reported to hydroxylate large polycyclic aromatic hydrocarbons at high rates (>100 min^{-1}) albeit with low coupling efficiency (3−10%).[84] These variants were later found to exhibit enhanced activity relative to the wild-type enzyme, also toward a variety of other non-native substrates.[80,85−88]

More recently, a minimal library of 24 $P450_{BM3}$ variants was created by mutating F87 and A328, which lie at opposite sides of the heme (Figure 4b), with five apolar residues.[89] Library screening against geranylacetone, nerylacetone, (4*R*)-limonene, and (+)-valencene revealed several variants (11/24) with significantly altered regioselectivity compared with the wild-type enzyme. Multiple oxidation products were observed in most cases, but two variants showed high selectivity (>97%) toward limonene 8,9-epoxidation. A variant identified in this study was further engineered, via successive rounds of molecular modeling, mutagenesis, and screening, to give a triple mutant (A264V/A328V/L437F) with much improved regioselectivity for limonene-to-perillyl alcohol conversion (Figure 5d).[90]

Random mutagenesis and directed evolution[91] has provided a particularly effective strategy toward identifying activity-enhancing mutations across the P450 fold whose impact on enzyme function could not be readily anticipated on the basis of the structure.[92−94] Using this approach and a high-throughput functional screen with an octane surrogate substrate (*p*-nitrophenyl octyl ether),[95] the Arnold group isolated a $P450_{BM3}$ variant (9−10A) with significantly enhanced oxidation activity on octane (30 → 3 000 total turnovers (TTN)) and other medium-chain alkanes.[92,93] Notably, out of the 13 heme domain mutations accumulated in 9−10A, only two are in the active site (V78A, A184 V). Active site mutagenesis of 9−10A has then enabled the development of variants with improved selectivity for the subterminal (ω-1) hydroxylation of C_6−C_{10} alkanes (75−85% regioselectivity; 40−50% ee),[93] the α-hydroxylation of 2-aryl-acetic acid esters (75−88% regioselectivity; 55−93% ee; 600−1600 TTN),[79] and the epoxidation of terminal C_5−C_8 alkenes (64−93% regioselectivity; 45−83% ee; 500−1300 TTN)[96] (Figure 5a−b). More recently, the same group subjected 9−10A (F87A) to combinatorial alanine mutagenesis to obtain variants with expanded active site cavities suitable for the oxidation of large substrates, such as steroids (Figure 5e), alkaloids, and MOM-protected sugars.[97]

Random mutagenesis has proved useful also to find mutations that enhance P450 thermostability.[98] In turn, thermostabilization prior to or in between mutagenesis rounds has enabled the accumulation of "demanding" amino acid

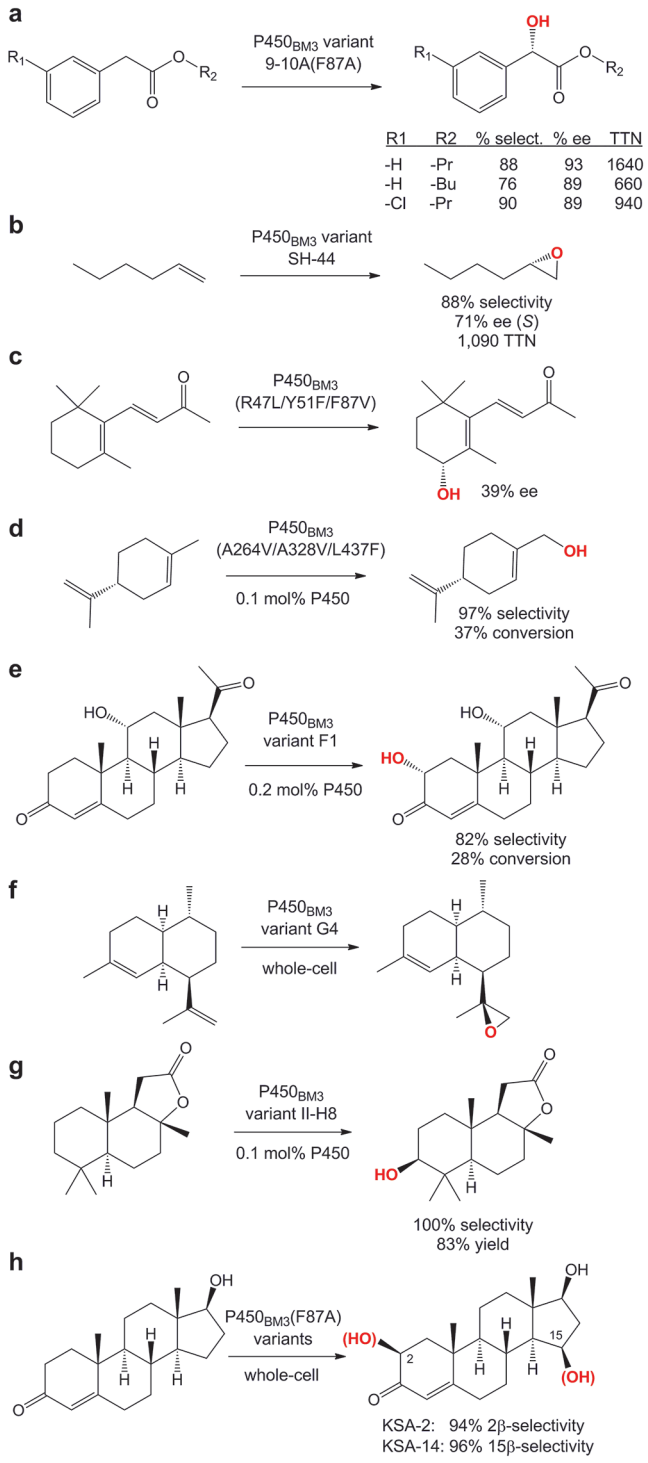


Figure 5. Oxyfunctionalization of non-native substrates by engineered variants of $P450_{BM3}$: (a) α-aryl-acetic acid esters;[79] (b) alkenes;[96] (c) β-ionone;[80] (d) limonene;[90] (e) steroid;[97] (f) amorphadiene;[126] (g) sclareolide;[148] (h) testosterone.[141] TTN = total turnovers.

substitutions required to achieve important shifts in function.[94,99,100] For example, Bloom et al. found that a highly destabilizing L75R mutation (ΔT_{50} = −8 °C) conferring naproxen-oxidation activity was tolerated by a thermostable $P450_{BM3}$ variant but not by a less stable counterpart.[99] During the re-engineering of $P450_{BM3}$ into an efficient P450 propane monooxygenase (vide infra), the largest improvement on a per-

dx.doi.org/10.1021/cs300001x | ACS Catal. 2012, 2, 647−666

residue basis in propane oxidation properties (i.e., 3-fold increase in TTN and coupling efficiency, 4-fold increase in k_{cat}/K_m) as well as largest shift in substrate profile resulted from a single, destabilizing L188P mutation within the F-helix (Figure 4b), which could be identified only after thermostabilization of the parent enzyme.[94,100] Since these studies, "prestabilization" of the parental P450 structure to enhance its robustness to mutagenesis has proved valuable in various other contexts.[97,101]

Another strategy to access P450s with new or altered functions has been through "chimeragenesis", which has been applied to both mammalian and bacterial P450s and using various approaches; namely, via DNA shuffling,[102−105] computationally guided recombination,[106−108] or by swapping substrate recognition regions (so-called "SRSs"[109]) across unrelated P450s.[110−113] Work in this area prior to 2006 has been recently reviewed, and therefore, it will not be discussed in details here.[51] In a more recent study, the SRS1 and SRS5 regions of insect CYP4C7, which hydroxylates farnesol at the terminal C12 position with high selectivity but low activity, were introduced into $P450_{BM3}$ to yield chimeras capable of producing 12-hydroxy-farnesol (>30% regioselectivity) or methyl 12-hydroxyfarnesoate (55% regioselectivity) not observed with wild-type $P450_{BM3}$.[113]

An interesting switch in function was recently observed upon active site mutagenesis of P450 AurH (*Streptomyces thioluteus*), which catalyzes a rather unusual tandem hydroxylation-tetrahydrofuranyl ring formation as part of aureothin biosynthesis (Figure 6).[114] Hertweck and co-workers found that

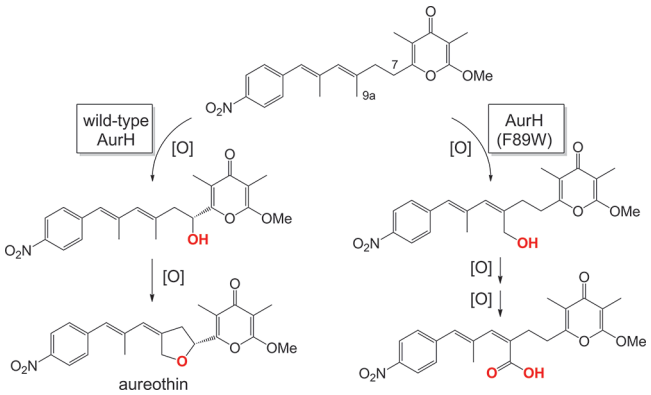


Figure 6. Active site mutation-induced shift from a four-electron hydroxylation-cyclization to a six-electron sp^3 C−H oxidation in P450 AurH.[114]

mutations of residues interacting with the pyrone ring of the substrate (F89W or T239F) strongly shifted the enzyme regioselectivity from C7 to the adjacent C9a site. Although important changes in site selectivity following mutagenesis were observed in many other cases (e.g., $P450_{cam}$ and $P450_{BM3}$ variants described above or other biosynthetic P450s such as PikC[115]), the AurH variants were peculiar in that they were able to catalyze sequential oxygenations at C9a, leading to the corresponding aldehyde and carboxylic acid (Figure 6). This finding is of particular interest because P450-catalyzed six-electron oxidation of sp^3 C−H bonds is rare[116−119] and participates in key steps of the biosynthesis of important natural products, such as the conversion of amorphadiene to artemisinic acid,[119] a precursor of the antimalarial artemisinin.[120,121]

Collectively, the results accumulated over several protein engineering experiments such as those cited above indicate that P450s possess a high degree of functional adaptability, which likely reflects the versatility of the conserved P450 fold to serve a wide range of functions in nature.[53] This feature makes these enzymes well suited to be exploited beyond their native function. At the same time, however, achieving high levels of regio- and stereoselectivity as well as elevated turnover numbers and coupling efficiency in the P450-catalyzed reaction is crucial for any practical consideration of these enzymes as oxidation catalysts. As discussed in the following sections, controlling and fine-tuning these properties have and continue to represent a considerable challenge because the demands associated with refining such features are considerably higher than simply altering the substrate profile of the P450 enzyme.

4. IMPROVING COUPLING EFFICIENCY IN P450 CATALYSTS

In many natural P450s, oxidation of the substrate proceeds highly coupled to NAD(P)H cofactor oxidation (e.g., 90−98% in $P450_{cam}$ and $P450_{BM3}$). Mechanisms controlling efficient coupling are almost invariably disrupted when P450s are challenged with non-native substrates or when amino acid substitutions are introduced in the enzyme as a result of a suboptimal enzyme−substrate interaction;[60] imperfect "sealing" of the active site to the solvent during catalysis;[122] or mutation-induced activation of the catalytic cycle, even in the absence of substrate (background or leak NAD(P)H oxidation rate).[123] Uncoupling can lead to the formation of reactive oxygen species (i.e., O_2^- or H_2O_2) via the autoxidation or peroxide shunt pathway (Figure 2), resulting in fast inactivation of the P450 and, thus, low turnover numbers. High levels of uncoupling are also particularly undesirable in the context of P450-catalyzed transformations in whole cells because reducing equivalents derived from the energy source (e.g., glucose, glycerol) are wasted without concomitant formation of the desired product.[124]

Achieving "native-like" coupling efficiency and turnover numbers on non-native substrates has proven challenging, with engineered P450s typically exhibiting coupling values from less than 1% to 30−40% and TTNs from a hundred to a few thousands. The recent re-engineering of $P450_{BM3}$ into a proficient P450 propane monooxygenase (called $P450_{PMO}$) has provided a first demonstration, however, that this hurdle can be overcome.[94,100] Using a domain engineering strategy, in which beneficial mutations within the heme, FMN, and FAD domain of the enzyme were first identified via random/structure-guided mutagenesis and then combined in a final step (Figure 7a), Fasan et al. showed that the propane hydroxylating properties of a poorly coupled $P450_{BM3}$ variant (coupling: 17.4%; TTN: 5650) could be improved to reach a catalytic efficiency (coupling: 98.2%; TTN: 45800) comparable with that of wild-type $P450_{BM3}$ with its preferred substrates (Figure 7b).[94] The improved TTN/coupling efficiency translated to a correspondingly large increase in the in vivo activity of the engineered P450s in whole-cell propane biohydroxylations (9 → 176 U g^{-1} cdw) to the point that host-related factors became limiting.[94] Further improvements in the propane-per-glucose yields (+230%) with the laboratory-evolved $P450_{PMO}$ were later achieved by removing competing NAD(P)H-utilizing processes in the *E. coli* strain via metabolic engineering.[125] Structural and modeling studies revealed that $P450_{PMO}$ featured a completely remodeled active site compared with $P450_{BM3}$, halved in

dx.doi.org/10.1021/cs300001x | ACS Catal. 2012, 2, 647−666

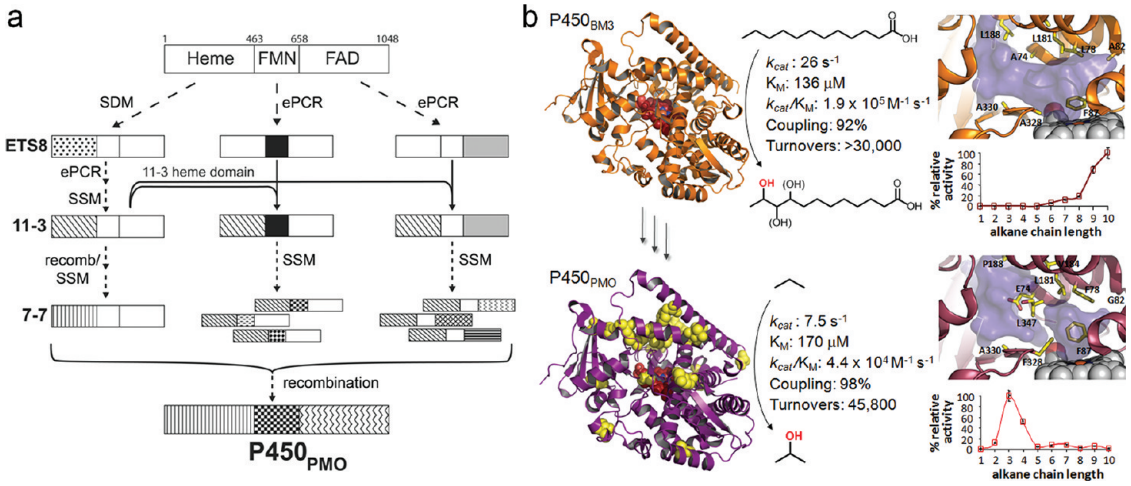


Figure 7. Laboratory evolution of a P450 propane monooxygenase.[94,100] (a) Domain engineering strategy. SSM = site-saturation mutagenesis; SDM = site-directed mutagenesis; ePCR = error-prone PCR. (b) Catalytic and kinetic properties, active site volume, and activity profile across C_1−C_{10} alkane series of wild-type $P450_{BM3}$ (orange) and $P450_{PMO}$ (purple). Mutated residues within the heme domain of the latter are displayed as sphere models (yellow). (Part of Figure 7B is reproduced with permission from ref 100. Copyright 2008 Elsevier).

volume (590 → 315 Å^3) and split into two "compartments", as a result of the multiple mutations (V78F, S74E, S82G, L188P, A328F) accumulated within this region (Figure 7b).[100] Such active-site reconfiguration was required to allow for a more productive interaction of the enzyme with the small-sized and apolar propane, as suggested by the progressive decrease in K_M and increase in propane-induced spin-shift across the "evolutionary lineage" of variants leading to $P450_{PMO}$.[100] Interestingly, progressive optimization of the enzyme for propane oxidation resulted in complete loss of fatty acid activity and respecialization of the variant for this function (vs oxidation of longer alkanes and terpenes). From a methodology standpoint, these papers highlighted the complementary advantages of random and structure-based mutagenesis as well as the importance of targeting the reductase component of the enzyme toward obtaining proficient P450 catalysts for a non-native transformation.

A recent contribution by Keasling and co-workers further illustrated the impact of coupling efficiency on the performance of P450-catalyzed transformations in whole-cell systems. The group reported the development of $P450_{BM3}$-based catalysts for the conversion of amorphadiene to artemisinic-11,12-epoxide (Figure 5f), a valuable intermediate for the preparation of the antimalarial drug artemisinin via semisynthesis.[126] The substrate-promiscuous $P450_{BM3}$(F87A) was found capable of supporting the desired reaction yielding artemisinic epoxide titers of 110 mg L^{-1} when expressed in an amorphodiene-producing *E. coli* strain. Further optimization of the biocatalyst by mutagenesis (R47L/Y51F/A328L) led to a variant with increased coupling efficiency (35 → 50%), which also enabled a 2.3-fold increase in the productivity of the strain (250 mg L^{-1}). Likely also contributing to the improved catalyst performance in vivo was the reduced activity of the P450 variant toward fatty acids, potentially competing for oxidation by the P450 in the cell, which was achieved by targeting the residues primarily responsible for fatty acid binding (R47/Y51).

Finally, Wong et al. recently described how single-site proline substitutions directed to structural elements adjacent to the heme group in $P450_{BM3}$ (A330P, I401P) could lead to an increase in both the catalytic rate and the coupling efficiency of the enzyme in the oxidation of various non-native substrates (fluorene, toluene, propylbenzene).[123] Interestingly, crystallographic analysis of the I401P mutant revealed structural similarities with the substrate-bound form of wild-type $P450_{BM3}$. These features were, however, not shared by the A330P variant, suggesting that different mechanisms are likely at the basis of the effects of these mutations on the enzyme catalytic properties.

5. HIGH-THROUGHPUT METHODS FOR CHARACTERIZATION OF P450 REGIO- AND STEREOSELECTIVITY

A most challenging problem currently related to the development of P450 catalysts for synthetic applications is concerned with the fine-tuning of the regio- and stereoselectivity of these enzymes. Several factors contribute to make this a particularly daunting task. First, there is the high degree of structural flexibility and active site plasticity exhibited by P450s, which often undergo important conformational changes upon substrate binding, as evidenced by crystallographic and NMR studies.[74,127−136] As a result, even when a high-resolution structure of the target enzyme is available, it is difficult to rationally design active site mutations that would steer the selectivity of the P450-catalyzed oxidation toward a specific site in the target molecule.[66,110,137,138] Second, mutations that are beneficial for expanding the substrate profile of a P450 typically result in expanded active sites, in which the substrate can dock via multiple orientations, thus translating in P450 catalysts with poor or only moderate regio-/stereoselectivity. Finally, whereas various methods are available for high-throughput screening of "generic" P450 oxygenase activity,[139,140] P450 regio- and stereoselectivity must be established on a case-by-case basis through labor- and time-consuming (chiral) HPLC/GC analyses, which inevitably limits the number of P450 catalyst-substrate combinations that can be evaluated within a reasonable time.

The challenge inherent to developing highly regio-/stereoselective P450 catalysts is well illustrated by a recent report on the directed evolution of $P450_{BM3}$ variants for regioselective steroid hydroxylation.[141] Starting from a parent enzyme, $P450_{BM3}$(F87A), with moderate regioselectivity for testosterone hydroxylation (2β and 15β regioisomers in a 51:46 ratio), the

dx.doi.org/10.1021/cs300001x | ACS Catal. 2012, 2, 647−666

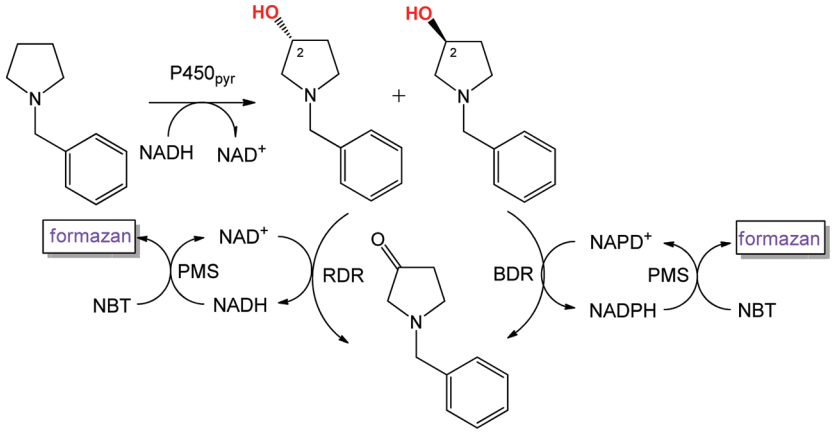


Figure 8. High-throughput colorimetric assay for screening for P450 variants with improved stereoselectivity toward *N*-benzyl-pyrrolidine C2-hydroxylation.[147]

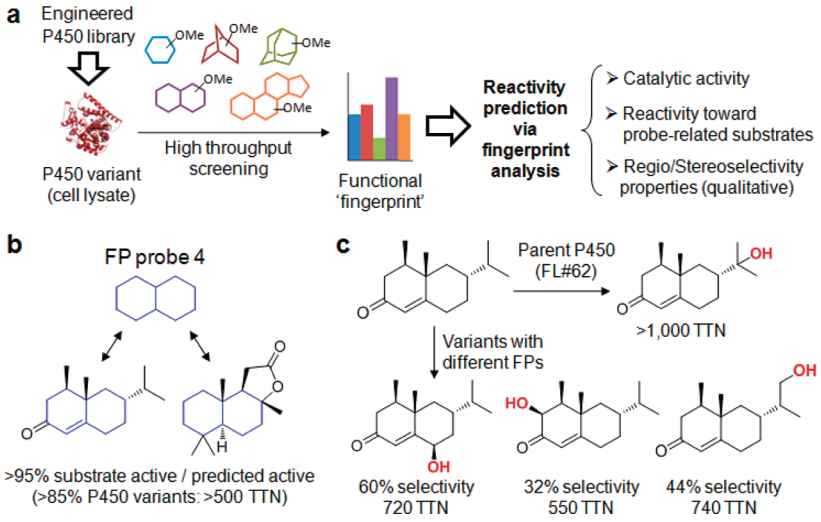


Figure 9. P450 fingerprinting method to rapidly explore the substrate profile and regio-/stereoselectivity properties of engineered P450 variants.[148]

group identified two variants, KSA-2 (R47I/T49I/Y51I/F87A) and KSA-5 (V78L/A82F/F87A) with 94% and 91% selectivity for 2β- and 15β-positions, respectively, after screening 8700 active-site variants in resting-cell format by HPLC (Figure 5h). A second round of directed evolution (600 recombinants) yielded KSA-14 (R47Y/T49F/V78L/A82M/F87A) with further improved 15β-selectivity (96%). Notably, both KSA-2 and KSA-5 exhibited improved coupling efficiency in steroid oxidation (30 and 46%, respectively) compared with the parent (6.5%). Characterization of these enzymes in purified form revealed, however, a lower level of regioselectivity than that observed in the whole-cell biotransformations (KSA-2, 88.4% vs 94% (2β-isomer); KSA-14, 81.8% vs 96% (15β-isomer)).

As an alternative to directed evolution, other researchers have opted for the screening of small libraries of preselected, functionally diverse P450 variants against a panel of target substrates.[142] The underlying idea in this case is to maximize the output of laborious (e.g., HPLC/GC-based) screens by focusing the screening efforts on high-quality, nonredundant P450 catalyst libraries and across a panel of different compounds. This method has provided a route to P450 catalysts that, in some cases, exhibited high regioselectivity for one or more of the compounds tested.[89,142−145]

Undoubtedly, high-throughput methods that can report on these subtle properties (i.e., regio- or stereoselectivity, or both) can be invaluable in aiding and guiding protein engineering/directed evolution efforts toward P450 catalysts with fine-tuned site-selectivity. Progress in this direction includes two assays designed ad hoc to identify P450s with improved regioselectivity toward terminal alkane (octane) hydroxylation. A first such assay makes use of hexylmethylether (HME) as a chromogenic octane mimic.[146] Terminal hydroxylation of HME (i.e., methoxy C−H) liberates formaldehyde, which can be detected colorimetrically using the reagent Purpald, whereas hydroxylation at other sites leads to either a less reactive aldehyde or unreactive alcohols. Using this assay, Meinhold et al. were able to identify a $P450_{BM3}$ variant (77-9H) with improved octane ω-hydroxylase activity (52% regioselectivity) starting from a parent enzyme (9-10A) with minimal selectivity toward this position (1% 1-octanol) upon screening a 10 000-member active site saturation library. The improvement in regioselectivity was found to be restricted to octane (across C_6−C_{10} *n*-alkanes) and to be accompanied by a decrease in TTN (3000 → 1300) and product formation rate (540 → 160 min^{-1}) relative to the parent enzyme. Urlacher and co-workers developed an alternative assay for the same purpose, this method involving coupling of P450-catalyzed alkane oxidation with an alcohol dehydrogenase (ADH)-based readout (NADH formation via ADH-dependent alcohol oxidation).[147] Specific detection of 1-octanol formed in the P450 reactions was

dx.doi.org/10.1021/cs300001x | *ACS Catal.* 2012, 2, 647−666

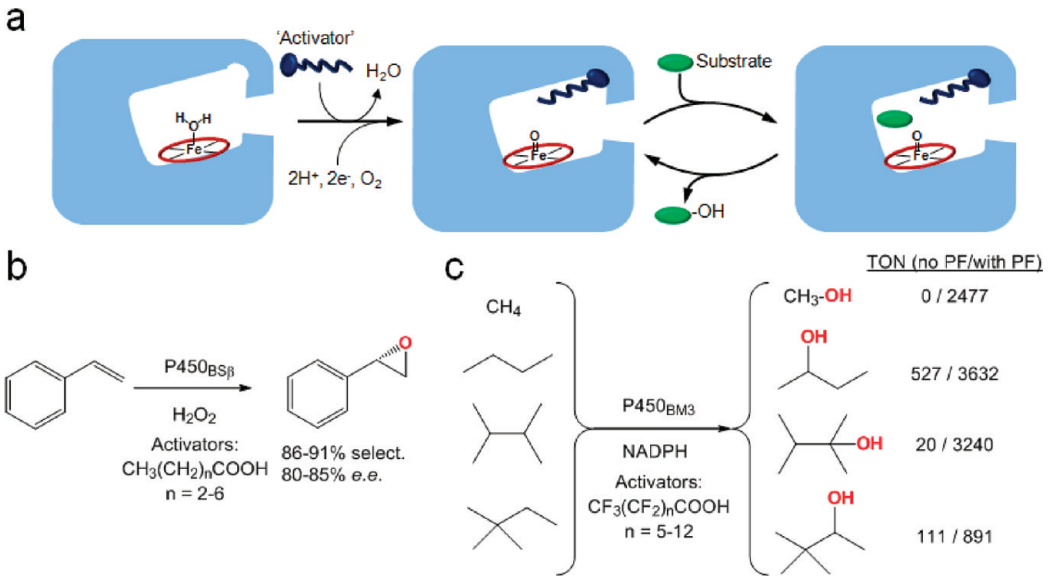


Figure 10. (a) Activator-based strategy for modulating P450 reactivity and its application in the context of (b) $P450_{BS\beta}$ and (c) $P450_{BM3}$.[149,153,154]

possible utilizing yeast ADH, which has increased activity on primary alcohols than secondary alcohols.[147] Upon screening a random mutagenesis library of 6500 CYP102A3 recombinants, the group succeeded in isolating a double-mutant derivative (S189Q/A330V) with improved (48%) regioselectivity for 1-octanol production.

Zhao and co-workers recently reported an enzyme-coupled assay for high-throughput screening of P450 variants with improved stereoselectivity toward *N*-benzyl-pyrrolydine hydroxylation (Figure 8).[148] This method involved coupling the P450 reaction with colorimetric detection of the activity of two different alcohol dehydrogenases specific for either the *S* or the *R* stereoisomer of the hydroxylation product (2-hydroxy-*N*-benzyl-pyrrolydine). Starting from P450pyr (*Sphingomonas* sp.) which has moderate *S* stereoselectivity for C2-hydroxylation (43% ee), the group succeeded in isolating a variant (F403L) with improved *S* stereoselectivity (65% ee) and one (N100S/T186I) with inverted stereoselectivity (83% ee (*R*)) after screening a series of active site mutagenesis libraries (3060 recombinants). Also in this case, the increase in selectivity led to a decrease in catalytic activity (55 → 23% conversion). Notably, this work validated a method for screening P450 stereoselectivity in high-throughput, albeit, as noted by the authors, its applicability in other context (i.e., other substrates and prochiral sites) remains contingent upon the availability of a suitable dehydrogenase pair with high selectivity toward both stereoisomers of the desired hydroxylation product.

Efforts toward developing a general, substrate-independent strategy for accelerating the discovery of P450 catalysts with diversified regio-/stereoselectivity have been undertaken by our group.[149] This strategy involves mapping the active site configuration of engineered P450 variants in high-throughput using a set of structurally diverse probes equipped with "reporter" methoxy groups (Figure 9a). This study showed that via analysis of the acquired "fingerprints", reliable predictions could be made regarding the substrate reactivity of the corresponding P450 variants toward a series of untested substrates structurally related to the fingerprint probes (i.e., pentylcyclohexanol, menthol, borneol, camphorsultam, 11,12-dehydronootkatone, sclareolide) (Figure 9b). In addition, the fingerprint profiles were found to relay qualitative information about the enzyme regioselectivity properties; that is, P450 variants with different fingerprints exhibited also different regioselectivity whereas variants sharing identical fingerprints displayed identical product profiles. The method was found useful to enable the rapid identification of P450 variants with diversified reactivity for oxyfunctionalization of distinct aliphatic positions in a complex substrate while requiring minimal analytical efforts. With 11,12-dehydronootkatone, for example, P450 catalysts with 32−60% regioselectivity toward three primary and secondary sp^3 C−H sites not targeted by the parental enzyme could be isolated after fingerprint-guided selection followed by GC screening of only 40 variants (Figure 9c).

6. MODULATION OF P450 REACTIVITY VIA NON-OXIDIZABLE ACTIVATORS

Recent papers described an interesting, alternative approach toward modulating the reactivity of cytochrome P450s, this involving the use of "activator" compounds to trigger P450 catalytic cycle and promote the concomitant oxidation of a target substrate (Figure 10a). A first implementation of this strategy was reported by Watanabe and co-workers in the context of $P450_{BS\beta}$ (CYP152A1) from *Bacillus subtilis*.[150] $P450_{BS\beta}$ catalyzes the H_2O_2-driven hydroxylation of myristic acid and other long-chain fatty acids at the α- and β-positions.[151] In this enzyme, an interaction between the substrate carboxylic group and the side chain of Arg242 residue located in proximity of the heme plays a key role both in substrate docking and catalysis.[151] On the basis of this mechanistic information, the group demonstrated that fatty acids carrying shorter (5- to 10-carbon-atom-long) alkyl chains, while not undergoing oxidization, could initiate $P450_{BS\beta}$ catalytic cycle and promote the oxidation of non-native substrates, such as guaiacol, ethylbenzene, and styrene.[150] Interestingly, the size of the fatty acid-based "decoys" was found to influence both the catalytic rate and stereoselectivity of the enzyme, with the activated $P450_{BS\beta}$ catalyzing the benzylic hydroxylation of ethylbenzene with rates of 11−28 turnovers/min and 35−68% ee and styrene epoxidation with rates of 136−334 turnovers/min and 80−85% ee, depending on the

dx.doi.org/10.1021/cs300001x | *ACS Catal.* 2012, 2, 647−666

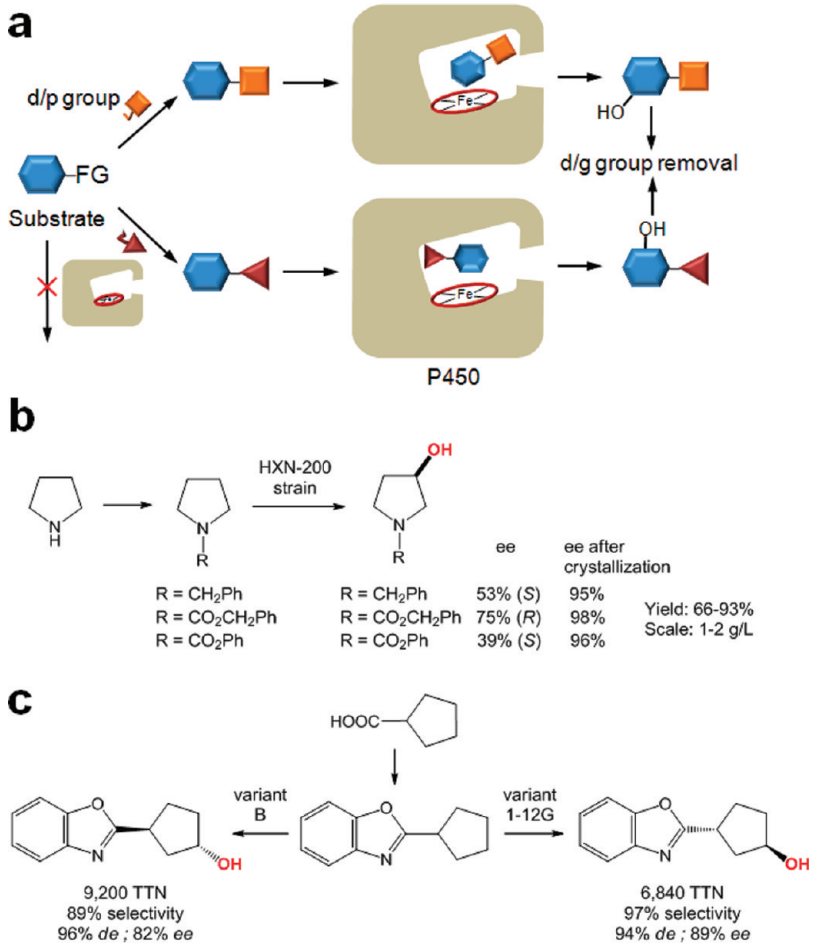


Figure 11. (a) Substrate engineering approach for improving the yield and selectivity of P450-catalyzed oxyfunctionalizations and representative examples of its application in the context of (b) pyrrolidine[161] and (c) cyclopentanecarboxylic acid.[163] FG = functional group; d/p = docking/protecting.

nature of the activator (Figure 10b). Subsequent structural analyses confirmed that the smaller size of the decoy molecules compared with myristic acid creates a cavity within the enzyme active site available for binding of the non-native substrate.[152] Further studies involving a larger substrate (4-methoxy-1-naphthol) also revealed that a number of different carboxylic acids other than *n*-alkyl carboxylic acids could be utilized as activators of $P450_{BS\beta}$.[153]

More recently, this activator-based strategy was extended to wild-type $P450_{BM3}$ (Figure 10c).[154,155] In independent contributions, the Watanabe and Reetz groups reported that long-chain perfluorocarboxylic acids (PFs), which mimic $P450_{BM3}$ preferred substrates but contain nonoxidizable C−F bonds in their side chains, could effectively trigger $P450_{BM3}$ catalytic cycle, enabling concomitant oxidation of various aliphatic substrates, such as cyclohexane, acyclic C_6 and gaseous (C_1−C_4) alkanes. As in the case of $P450_{BS\beta}$, the catalytic properties of the enzyme (product formation rate, turnover number, coupling efficiency) could be modulated using activators of varying structure. Testing PFs with chain lengths from 8 to 14 carbon atoms long, the Watanabe group observed optimal activity toward cyclohexane with C_8-PF (product formation rate: 60 min^{-1}; 45% coupling), toward butane with C_9-PF (product formation rate: 110 min^{-1}; 57% coupling), and toward propane with C_{10}-PF (product formation rate: 67 min^{-1}; 18% coupling). This trend was rationalized on the basis of a "match" between the size of the substrate and the size of the cavity created in proximity of the heme by the enzyme-bound PFs, with the assumption that the PF bind to the enzyme similarly to their nonfluorinated counterparts.[155]

Reetz and co-workers observed 4- to 10-fold increases in substrate turnover numbers with *n*-octane, linear and branched C_6 alkanes, and butane following the simple addition of the PF activators to the wild-type enzyme (Figure 10c). Most remarkably, PF-activated $P450_{BM3}$ was also found to hydroxylate methane to methanol, supporting up to 2470 turnovers in pressurized reaction vessels (10 atm) and with the optimal PF activator.[154] Engineered P450s were found earlier to be capable of oxidizing ethane (bond dissociation energy (BDE): 101 kcal/mol)[156] as well as the even stronger sp^3 C−H bond of iodomethane (103 kcal/mol).[100] Yet, the accessibility of methane (BDE: 105 kcal/mol) to P450-mediated oxidation was at question prior to this study, given that a different class of enzymes (i.e., nonheme iron monooxygenases) have evolved in nature for the hydroxylation of gaseous alkanes, including methane.[157−159] On the basis of molecular dynamics simulations, methane activity was proposed to stem from a restriction of the active site volume upon PF−$P450_{BM3}$ complex formation, which would favor the clustering of methane molecules above the Fe/heme. This hypothesis is in line with previous considerations on the laboratory-evolved $P450_{PMO}$, whose catalytic proficiency in propane oxidation was found to

dx.doi.org/10.1021/cs300001x | *ACS Catal.* 2012, 2, 647−666

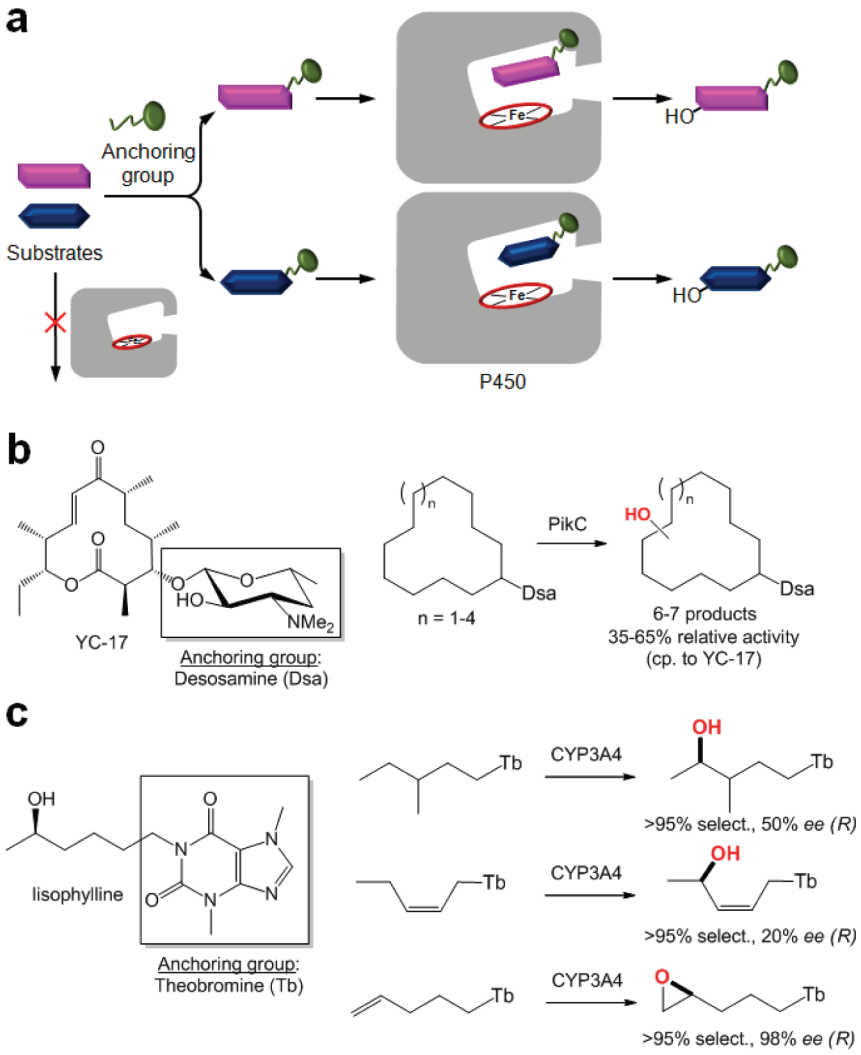


Figure 12. (a) Anchoring group-based substrate engineering strategy for P450-catalyzed oxidation of non-native substrates and examples of its application in the context of (b) P450 PikC[164] and (c) CYP3A4.[165]

derive, at least in part, from a significant reduction of the heme pocket volume to better accommodate the small-sized substrate (Figure 7b).[100] With wild-type $P450_{BM3}$, PF-mediated modulation of the enzyme activity has remained so far restricted to small-size substrates and had only a minor impact on the regio-/stereoselectivity of the enzyme.[154] In the future, it will be interesting to see whether via alternative activators or in conjunction with active site engineering, the substrate scope and enzyme site selectivity can be altered, as well. Altogether, these initial studies with $P450_{BS\beta}$ and $P450_{BM3}$ showed that the use of activators could provide a straightforward and technically simple approach to affect P450 reactivity. If proven viable for preparative-scale transformations, this method could furnish a valuable tool, complementary to or synergistic with protein engineering, to exploit P450s for synthetic transformations.

7. MODULATING P450 REACTIVITY VIA SUBSTRATE ENGINEERING

Substrate engineering has provided another strategy to enhance the utility of P450 enzymes for synthetic applications. The idea underlying this approach is that attributes of the P450-catalyzed transformation, such as substrate scope and regio- and stereoselectivity, can be improved via manipulation of the substrate structure rather than (or in combination with) variation of the P450 catalyst. Over the past years, two general types of substrate engineering strategies have been investigated: one directed at influencing the enzyme activity through the installation of variable functional groups into a specific position of the target substrate (Figure 11a) and another one involving the linkage of different substrates to a common chemical moiety that mediates anchoring to and thus recognition by the P450 enzyme (Figure 12a).

A first example of the former strategy is the so-called "docking/protecting" (d/p) group approach introduced by Griengl and co-workers to collectively address a number of issues associated with the microbial biohydroxylation of low-molecular-weight building blocks (e.g., low activity/selectivity in the oxidation reaction, side-reactions at the level of reactive functional groups in the substrate).[160] According to this approach, the target substrate is chemically modified with the two-fold purpose of facilitating specific recognition by the oxidizing P450(s) ("docking" role) and shielding a specific functional group in the molecule from undesired reactions ("protecting" role). Effective d/p strategies in the context of biohydroxylations with oxidizing strains (e.g., *Bauveria* spp., *Cunninghamella* spp., *Bacillus* spp.) were found to involve the formation of *N*-benzoyl-spirooxazolidine, benzoxazole, isosac-

dx.doi.org/10.1021/cs300001x | *ACS Catal.* 2012, 2, 647−666

charine/*N*-phenyl-carbamate, or *N*-benzoyl/*N*-benzyl derivatives via modification of carbonyl, carboxylic, hydroxyl, or amino groups, respectively, in the substrate.[161,162] In a number of cases, noticeable improvements in the yield and stereoselectivity of the biohydroxylation reaction were achieved (e.g., Figure 11b).[163] In a recent application of this strategy, a protected derivative of cyclopentanecarboxylic acid (2-cyclopentylbenzoxazole) was prepared and screened against four engineered alkane-hydroxylating $P450_{BM3}$ variants. Whereas the enzymes exhibited no activity on the unprotected substrate, two out of the four possible β-hydroxylated stereoisomers could be obtained with good to high stereoselectivity after substrate modification (Figure 11c).[164]

Recent work by the Arnold group on $P450_{BM3}$-based demethylases for selective deprotection/functionalization of permethylated sugars provides another notable demonstration of the potential of substrate engineering for modulating P450 reactivity.[144] In this study, the site selectivity of the P450-catalyzed reaction could be greatly enhanced by varying the nature of the anomeric substituent in the sugar substrate. For example, the C3-OMe selectivity of variant 9-10A on permethylated glucose was improved from 50% to >80% by switching from the β-*O*-benzyl-functionalized substrate to the α-*O*-methyl-protected one. Variant 9-10A(F87I), on the other hand, showed improved C4-OMe regioselectivity (>80%) with β-*O*-Bz-perGlc compared with α-*O*-Me-perGlc (30%). In the case of permethylated galactose, the selectivity of the $P450_{BM3}$ variant 2C6dr was shifted from producing a 1:1 mixture of the C2- and C3-demethylated products to giving only the C3-demethylated one by inverting the stereochemistry of the anomeric methoxy group (α-pmGal → β-pmGal).

A conceptually different substrate engineering strategy was recently presented in the context of the biosynthetic P450 PikC[165] (*Streptomyces venezuelae*) and the human drug-metabolizing CYP3A4,[166] this approach involving the use of an enzyme-anchoring moiety to direct P450-mediated oxidation toward a variable organic structure linked to such moiety (Figure 12a). PikC catalyzes the hydroxylation of the 14-membered ring macrolide narbomycin and the 12-membered ring macrolide YC-17 as part of the biosynthesis of the antibiotic pikromycin and methymycin, respectively.[167] Structural studies on PikC revealed that substrate recognition relies on multiple hydrogen bonds/ionic interactions engaging the common desosamine moiety of YC-17/narbomycin, whereas the macrolactone rings are contacted by the active site residues only via hydrophobic interactions.[115] On the basis of this information, Sherman and co-workers showed that the desosamine glycoside could be used as a PikC-anchoring group to drive the oxidation of alternative organic structures appended to this moiety (Figure 12b).[115] The group found that desosaminyl C_{12}- to C_{15}-cycloalkanes were recognized by PikC with reasonable affinity (K_D: 250−310 μM cp. to 19 μM for YC-17) and that the cycloalkyl rings were hydroxylated by the P450 with an activity 35−65% that observed with the native substrate, albeit with only limited regio- and stereoselectivity. Crystallographic data indicated that the poor regio- and stereocontrol of the oxidation reaction resulted from the ability of the carbocyclic moiety to adopt different conformations within the enzyme active site.

Most recently, Auclair and co-workers reported a remarkable example of the use of substrate engineering to afford the selective oxidation of a sp^3 C−H bond, or a C═C double bond, located at a defined distance from an enzyme-anchoring group.[166] Prior knowledge that lisofylline is hydroxylated by human CYP3A4 at the fourth carbon from the theobromine group[168] inspired these researchers to use the latter moiety as a chemical auxiliary to present various saturated and unsaturated C_4−C_6 alkyl substrates to the enzyme (Figure 12c). Notably, excellent regioselectivity (>95%) toward the fourth carbon atom was achieved for most of the substrates tested. For prochiral centers, (*R*)-stereoselectivity, with ee values ranging from 20 to 50%, was also observed. Docking studies suggested that the binding of the auxiliary theobromine to a subpocket of CYP3A4 active site enables the appended chain to be presented above the reactive heme center at a fixed distance and preferred orientation, thus leading to the observed C4-chemoselectivity and pro-*R* facial selectivity.

8. CREATION OF CATALYTICALLY SELF-SUFFICIENT P450S

The multicomponent and/or membrane-bound nature of most natural P450 systems is an important factor complicating the exploitation of these enzymes for synthetic purposes. As mentioned earlier, most prokaryotic P450s are three-component systems of the type depicted in Figure 3a, whereas many eukaryotic P450s of high interest (e.g., human P450s involved in drug metabolism or plant/fungal P450s implicated in natural product biosynthesis) are membrane-bound enzymes, requiring another membrane-associated redox protein for function (Figure 3b). Although polycistronic expression systems have provided a viable strategy to reconstitute either of these systems in catalytically competent form in heterologous hosts,[38,169−172] the application and optimization of multi-component P450s as oxidation catalysts remains problematic. These problems, together with the realization that $P450_{BM3}$ exceptional catalytic rates (>15 000 min^{-1} with arachidonic acid[62]) largely depend upon a most efficient cofactor → heme electron transfer[173] within this redox autonomous P450, have prompted the search for additional, natural catalytically self-sufficient P450 systems as well as inspired the creation of artificial ones.

After the discovery of $P450_{BM3}$ (CYP102A1) by Fulco,[64] other members of the CYP102A subfamily have been identified and isolated from *Bacillus* species, these exhibiting catalytic properties and substrate profiles similar to $P450_{BM3}$.[174−177] Eukaryotic fatty-acid hydroxylases related to $P450_{BM3}$ have also been isolated, these including CYP505A1 from *Fusarium oxysporium*[174] and other members of the CYP505 family.[178] More recently, genomic analyses have unveiled a new class of natural P450-redox partner fusions, which feature a FMN/[2Fe-2S] reductase linked to the C-terminus of the monooxygenase domain (Figure 3d) and have $P450_{RhF}$ (CYP116B2; *Rhodococcus* sp.) as a prototypical member.[179−181] Although the exact biological function of $P450_{RhF}$ remains unclear, this enzyme and homologous ones[182] were found to accept a variety of aryl compounds as substrates, thus displaying a markedly different substrate profile compared with the self-sufficient P450s identified before.[182,183]

With $P450_{BM3}$ serving as inspiration, artificial self-sufficient P450 systems have been created via genetic fusion of the P450 monooxygenase of interest to a cognate or noncognate reductase. Most of the early efforts in this area have focused on human drug-metabolizing P450s, with the goal of simplifying the isolation/characterization of these enzymes as well as improving their notoriously low activity, limited stability, and poor expression levels in heterologous hosts. A

dx.doi.org/10.1021/cs300001x | *ACS Catal.* 2012, 2, 647−666

number of studies have shown that genetic fusions of human liver P450s (CYP3A4, 1A1, 2D6, 4A1, or 2C9) with either human,[184,185] rat,[184,186,187] or yeast[188,189] CPR could be successfully expressed in yeast or *E. coli* while maintaining the drug metabolic activity of the original P450s. In most cases, however, these enzymes remained associated with membranes and/or required lipids/detergents for optimal function.

More recently, researchers have shown that fusion to the diflavin reductase domain of $P450_{BM3}$, which shares 35% sequence identity with human CPR, can furnish redox self-sufficient versions of human liver P450s (CYP2C9, 2C19, 3A4, 2E1) that can be expressed in *E. coli* as soluble proteins and in good yields (30−50 mg L^{-1} culture).[190−192] While improving their accessibility for functional studies, significant improvements with respect to the enzyme catalytic activity as result of CPR fusion have yet to be achieved, with the chimeric enzymes typically exhibiting turnover rates (1−50 min^{-1} range) and coupling efficiency (1−30%) comparable with those observed with reconstituted systems.[193] In addition to human P450s, genetic fusion to a CPR has proved to be a viable strategy for obtaining redox self-sufficient forms of other relevant eukaryotic P450s, such as mammalian P450s involved in steroid metabolism[194−199] and plant P450s involved in isoflavone[200] and taxol[201] biosynthesis.

In the context of bacterial class I P450s, several recent reports have demonstrated the ability of the reductase domain of $P450_{RhF}$ (RhFRed) to serve as a versatile redox partner to support catalysis of a noncognate monooxygenase in single polypeptide arrangements (Figure 13). Initial studies by

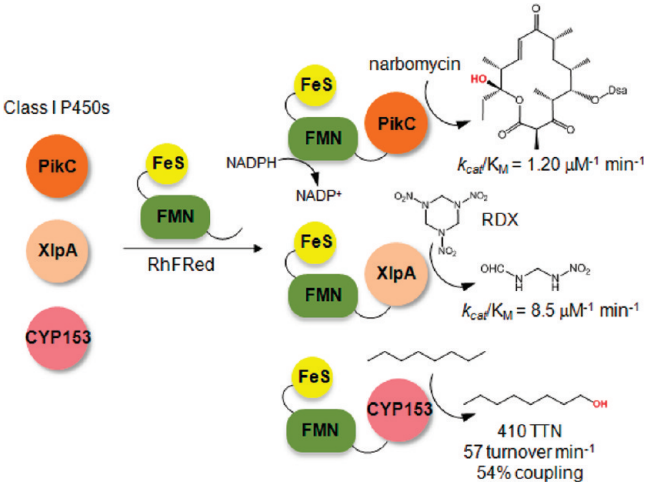


Figure 13. Examples of artificial redox self-sufficient P450 catalysts prepared via genetic fusion of class I P450 monooxygenases to the reductase domain of $P450_{RHF}$.[203−205]

Misawa and co-workers showed that chimeras of $P450_{cam}$, CYP203A, or CYP153A fused to the 16-amino acid linker region and reductase domain of $P450_{RhF}$ could catalyze the hydroxylation of their respective substrates (camphor, 4-hydroxybenzoate, or *n*-octane) in *E. coli* whole-cell reactions.[202] Later, other groups investigated analogous $P450_{cam}$-RhfRed and CYP153A13-RhfRed fusion constructs, confirming the redox self-sufficiency of these enzymes.[203,204] These studies also evidenced the importance of optimizing the P450-RhfRed linker region toward increasing catalytic function.[203]

This approach proved viable also in the context of two macrolide hydroxylases, PikC and $P450_{EryF}$,[205] and an explosive-degrading P450, called XlpA.[206] Interestingly, in some cases, fusion to RhfRed was found to lead to improvements in the catalytic properties of the P450 compared with a reconstituted system, while not imparting the substrate affinity (K_D) and regioselectivity of the monooxygenase.[204−206] For example, PikC-RhFRed was found to exhibit a 4-fold higher k_{cat}/K_m toward YC-17 hydroxylation compared with a PikC/Fdx/FdR mixture (0.96 vs 0.24 μM min^{-1}),[205] whereas CYP153A13-RhfRed displayed an increased decane hydroxylation rate (8 → 30 min^{-1}) compared with a CYP153A13/Fdx/FdR system.[204,207] Notably, the XlpA-RhFRed fusion enzyme was found to degrade the explosive RDX with a turnover rate of 46 min^{-1} and a coupling efficiency of 82%.[206] In contrast, $P450_{cam}$-RhFRed was found to hydroxylate (+)-camphor at about 1% the rate of an optimally reconstituted $P450_{cam}$/Pdx/PdR system (2.2 vs 2300 min^{-1}),[60,206] albeit this rate fell within an order of magnitude of that obtained with $P450_{cam}$ fused to its native redox partners (PdR−Pdx−$P450_{cam}$).[208]

Despite these somewhat variable outcomes, the RhfRed "stitching" approach appears to constitute a versatile strategy toward generating self-sufficient P450s, and its value toward accelerating the discovery and characterization of novel and/or 'orphan' class I P450s was demonstrated by Bruce and colleagues.[206] Future challenges in this area will include identifying ways to optimize the coupling efficiency of the P450-RhfRed chimeric constructs, which is often compromised,[204−206] resulting in low total turnover numbers.[204] Conceivably, the "domain engineering" strategy found effective toward identifying coupling-enhancing mutations in the reductase domains (FMN and FAD) of the enzyme during $P450_{PMO}$ evolution[94] (Figure 7a) could prove useful also in the context of the aforementioned artificial constructs, providing a means to identify mutations that could improve the interaction and electron transfer between the noncognate P450 and FMN/[2Fe2S] reductase. As shown,[203] manipulation of the P450-reductase linker region is also expected to offer opportunities toward enhancing the catalytic properties of these chimeric enzymes.

Alternative methods to create redox self-sufficient P450 systems other than via genetic fusion have also been investigated. Nagamune and co-workers recently generated a branched $P450_{cam}$/Pdx/PdR construct via linking Pdx to a glutamine-containing linker inserted between PdR−$P450_{cam}$ using a transglutaminase-mediated ligation.[209] The assembled P450 system was found to catalyze camphor oxidation with a 30-fold higher turnover rate (306 min^{-1}) compared with a stoichiometric mixture of $P450_{cam}$/Pdx/PdR (10 min^{-1}) and to display superior performance (10-fold) compared with a previously reported PdR/Pdx/$P450_{cam}$ fusion enzyme.[208] However, the observed substrate turnover rate remained ~15% that of an optimally reconstituted $P450_{cam}$/Pdx/PdR system,[60] and the need for the transglutaminase-catalyzed reaction is limiting.[60] Later, the same group described the preparation of a noncovalent assembly of $P450_{cam}$/Pdx/PdR via fusion of each of these components to PCNA proteins capable of forming heterotrimers.[210] This new design supported camphor oxidation at a rate of 500 min^{-1}, that is, 25% of the native $P450_{cam}$ activity observed under optimal conditions.

dx.doi.org/10.1021/cs300001x | *ACS Catal.* 2012, 2, 647−666

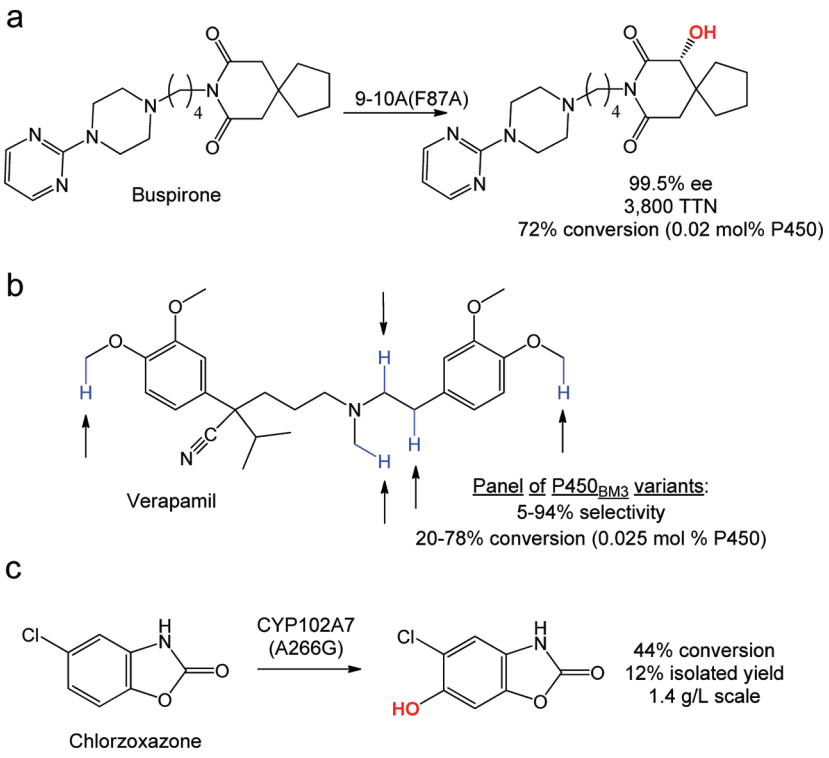


Figure 14. Representative examples of the application of engineered bacterial P450s for preparation of authentic human metabolites of marketed drugs such as (a) buspirone;[79] (b) verapamil,[143] and (c) chloroxazone.[178].

9. P450 CATALYSTS FOR DRUG METABOLITE PRODUCTION

P450s cover a prominent role in drug metabolic breakdown, participating in the oxidation of three-quarters of marketed drugs.[211] Characterization of the pharmacological properties, toxicity, and pharmacokinetics of human drug metabolites is crucial in the drug development process and mandatory for FDA approval of new therapeutics.[212] Yet, access to these compounds is often complicated by the need to devise a synthetic route for each metabolite and the stereochemical requirements associated with their preparation.

Whole-cell biotransformations with recombinant human liver P450s have provided a route to afford drug metabolites in multimilligram quantities (5−100 mg).[169,213−216] Over the past years, increasing attention has also focused on developing bacterial P450 variants with human-like metabolic activity to overcome limiting features of human P450s (i.e., low catalytic activity, instability, membrane-bound structure). Initial efforts in this area showed that $P450_{BM3}$ variants were capable of yielding authentic metabolites of propranolol[217] and a F87A mutant of the previously mentioned 9-10A,[93] was found to convert buspirone into its (*R*)-6-hydroxy derivative, a major product of human CYP3A4, in high regio- and stereoselectivity (Figure 14a).[79]

In a more recent study, the Arnold group also demonstrated that 12 of 13 mammalian metabolites of two marketed drugs (verapamil, astemizole) and a research compound (LY294002) could be produced, with varying selectivity and yields, by a panel of 120 engineered and chimeric $P450_{BM3}$ variants with broad substrate profile (Figure 14b).[143] Commandeur and co-workers reported that $P450_{BM3}$ triple mutant R47L/F87V/L188Q and improved derivatives thereof exhibited human CYP2D6-like metabolic activity on dextromethorphan and MDMA.[218,219] A small set of these variants were later found to oxidize, to a varying extent, 41 out of 43 marketed drugs.[220] The metabolic activity of $P450_{BM3}$ and variants thereof has been investigated also in the context of statins,[221] resveratrol,[222] phenacetin,[223] and chlorzoxazone.[224,225] Recently, Rentmeister et al. reported the directed evolution of $P450_{BM3}$ variants with improved activity (up to 1010 TTN) on acidic drugs (naproxen, ibuprofen), which are metabolized by human CYP2C9.[226] Glieder and co-workers described that variants of the redox self-sufficient CYP102A7 (*Bacillus* sp.) and CYP505X (*Aspergillus* sp.) could be successfully applied to produce authentic human metabolites of chlorzoxazone and diclofenac, respectively (Figure 14c).[178] While in most cases enzymatic reactions were performed only at an analytical scale, some of the aforementioned studies also demonstrated that, when variants with sufficient activity and regioselectivity could be found, multimilligram amounts (10−50 mg) of a given drug metabolite could be isolated from in vitro reactions,[143,178,226] providing first important demonstrations that engineered, nonhuman P450s could furnish a viable alternative to whole-cell transformations with heterologously expressed human P450s for drug metabolite production.

10. P450-MEDIATED CHEMOENZYMATIC SYNTHESIS

Although the potential impact of CYPs for organic synthesis has been often emphasized in the literature, until recently, there were no examples of the exploitation of these enzymes for the transformation of non-native substrates beyond oxyfunctionalization. A few recent reports have begun to illustrate the value of integrating P450-catalyzed reactions with chemical synthesis to enable transformations not readily accessible via conventional methods (Figure 15). A first example involved the implementation of a P450-based chemoenzymatic strategy for the selective, late-stage fluorination of unactivated sp^3 C−H bonds in organic molecules.[142]

dx.doi.org/10.1021/cs300001x | *ACS Catal.* 2012, 2, 647−666

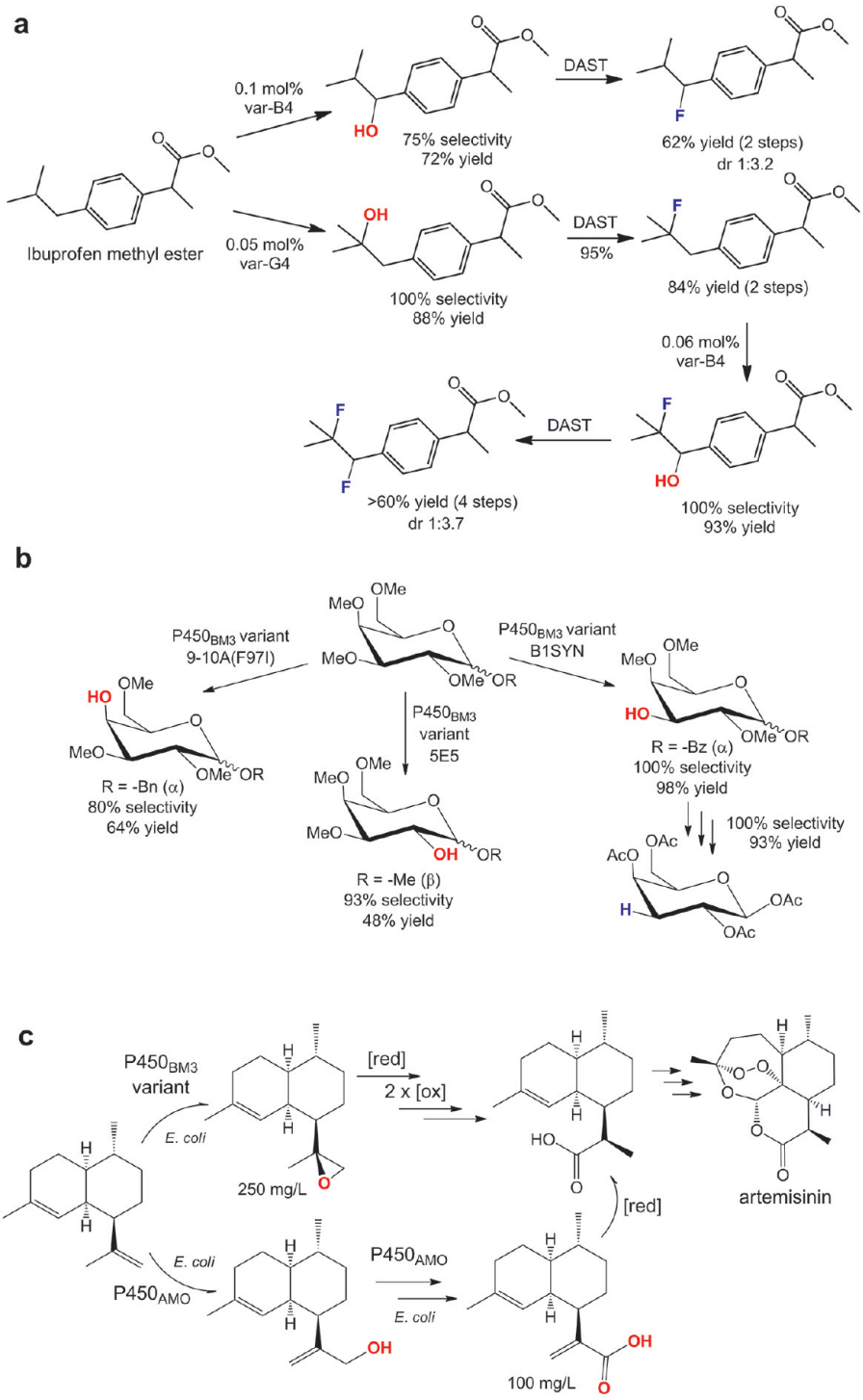


Figure 15. Chemoenzymatic strategies involving engineered P450 oxidation catalysts for (a) late-stage fluorination of drugs and druglike compounds,[141] (b) selective deprotection and functionalization of sugar building blocks,[143] and (c) preparation of a precursor of the antimalarial artemisinin.[126]

Because of the large impact of H → F substitutions on the membrane permeability, metabolic stability, and protein binding properties of bioactive molecules,[227−229] late-stage fluorination strategies are particularly valuable in medicinal chemistry and drug discovery campaigns. In this work, the authors showed how coupling P450-catalyzed sp^3 C−H hydroxylation (or $-OCH_3$ demethylation) to chemical deoxofluorination could provide concise and efficient routes to the regio- and stereoselective installation of fluorine substituents at nonactivated aliphatic sites of a drug or drug-like compound. Using a panel of ∼100 different $P450_{BM3}$ variants as a "reservoir" of diverse P450 catalysts, selective fluorination of a marketed drug and various cyclopentenones, Corey lactone, and arylacetic acid ester building blocks were achieved at a preparative scale (100−300 mg) and in high isolated yields (54−84%). In the case of the methyl ester prodrug of ibuprofen, this strategy was shown to provide rapid access to fluorinated derivatives modified at one or two remote

dx.doi.org/10.1021/cs300001x | ACS Catal. 2012, 2, 647−666

sites within the *iso*-butyl group (Figure 15a), which exhibited enhanced in vitro blood-brain barrier permeability. Because the sites of substrate oxidation via nonhuman P450s often overlap with those of human liver counterparts, an added benefit of the presented chemoenzymatic strategy was to enable fluorination and, thus, chemical protection of metabolically vulnerable sites in the drug.

A second example was provided in the context of the chemoenzymatic synthesis of functionalized mono- and disaccharides via selective P450-mediated demethylation.[144] The synthesis of oligosaccharides is often challenged by the need for numerous, sequential protection/deprotection steps. Lewis et al. showed how a whole set of singly demethylated regioisomers could be obtained from permethylated monosaccharides (glucose, galactose, mannose) using a set of engineered variants of the $P450_{BM3}$ derivative 9-10A in combination with substrate engineering. The P450-catalyzed demethylation reactions could be carried out at hundred-milligram scale, providing access to the desired products with high selectivity (77−100%) and isolated yields (44−98%) (Figure 15b). The ability to selectively deprotect the permetylated sugars via enzymatic means was then shown to enable the preparation of a novel 3-deoxygalactose and a 6-fluoro-6-deoxymannose derivative and as well as that of a β-1,4-linked disaccharide.

Finally, in the study of Dietrich et al.[126] mentioned earlier, the stereoselective epoxidation of amorphadiene by the engineered $P450_{BM3}$ variant enabled the implementation of an alternative and more convenient route for the chemo-biosynthetic production of dehydroartemisinic acid compared with that involving the use of the plant-derived $P450_{AMO}$ reported earlier by the same group (Figure 15c).[121] Albeit requiring two additional chemical steps, the new route was described to be more advantageous as a result of the higher titers of the biosynthetic precursor (amorphadiene epoxide vs artemisinic acid) as well as the shorter culture periods required for its production.

11. CONCLUSIONS

Enzyme-catalyzed transformations can offer key advantages for the synthesis of pharmaceuticals and other high-value compounds,[230] as demonstrated by the recent report on the use of an engineered transaminase for the large-scale manufacturing of the diabetes drug sitagliptin.[231] This is especially the case for P450s, given their potential utility toward functionalization of isolated, unactivated C−H bonds, which occur ubiquitously in organic molecules and remain largely inaccessible through chemical methods. Compared with other classes of enzymes, however, the exploitation of P450s for synthetic applications has remained very limited, largely due to the complexity of these multicofactor, multidomain/protein enzymatic systems. The research highlighted here illustrates the significant progress made over the past decade toward adapting and modulating the reactivity of natural P450s to execute non-native transformations. Protein engineering, both through rational design and directed evolution, has played a major role in these endeavors and it will continue to provide a major tool for this purpose. Other strategies, such as substrate engineering and modulation of P450 function via activators, are expected to provide alternative or synergistic tools for exploiting these enzymes for synthetic applications. A number of promising strategies for the creation of self-sufficient P450 systems have also emerged to overcome the limitations imposed by the multicomponent nature of several natural P450s. Importantly, a few papers have begun to demonstrate the utility of these enzymes to afford oxyfunctionalizations[87,94,126,166,178] and chemoenzymatic transformations of non-native substrates at relevant scales.[142,144]

This progress is encouraging, but it is clear that further advances are required to be able to capitalize on P450 catalysis for chemical synthesis. Whereas the possibility to exploit molecular recognition and different P450s to achieve the highly selective oxidation of multiple, stereoelectronically "unbiased" C−H bonds in a complex molecule (Figure 1) is a most attractive perspective, the development of P450 catalysts with fine-tuned regio-/stereoselectivity, high turnover efficiency, or both on a given compound remains a major challenge, which so far has been met with only sporadic success. More efficient and systematic methodologies, in terms of both P450 catalyst development and functional screening, for obtaining P450 catalysts with well-defined reactivity are required to increase the accessibility and scope of P450 catalysis for organic synthesis. Hopefully, future research will bring us closer to these important goals. Undoubtedly, addressing these challenges will bring about major benefits, including but not limited to that of empowering chemists with the ability to exploit P450-mediated C−H functionalization to construct and elaborate complex molecules.

AUTHOR INFORMATION

Corresponding Author

*Phone: +1 (585) 275-3504. Fax: +1 (585) 276-0205. E-mail: fasan@chem.rochester.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the University of Rochester Provost's Multidisciplinary Research Award.

REFERENCES

(1) Shilov, A. E.; Shul'pin, G. B. *Chem. Rev.* **1997**, *97* (8), 2879−2932.
(2) Godula, K.; Sames, D. *Science* **2006**, *312* (5770), 67−72.
(3) Labinger, J. A.; Bercaw, J. E. *Nature* **2002**, *417* (6888), 507−514.
(4) Dick, A. R.; Sanford, M. S. *Tetrahedron* **2006**, *62*, 2439−2463.
(5) Dangel, B. D.; Johnson, J. A.; Sames, D. *J. Am. Chem. Soc.* **2001**, *123* (33), 8149−8150.
(6) Maleczka, R. E. Jr.; Shi, F.; Holmes, D.; Smith, M. R. 3rd *J. Am. Chem. Soc.* **2003**, *125* (26), 7792−7793.
(7) Desai, L. V.; Hull, K. L.; Sanford, M. S. *J. Am. Chem. Soc.* **2004**, *126* (31), 9542−9543.
(8) Dick, A. R.; Hull, K. L.; Sanford, M. S. *J. Am. Chem. Soc.* **2004**, *126* (8), 2300−2301.
(9) Brodsky, B. H.; Du Bois, J. *J. Am. Chem. Soc.* **2005**, *127* (44), 15391−15393.
(10) Wender, P. A.; Hilinski, M. K.; Mayweg, A. V. *Org. Lett.* **2005**, *7* (1), 79−82.
(11) Lee, S.; Fuchs, P. L. *J. Am. Chem. Soc.* **2002**, *124* (47), 13978−13979.
(12) Chen, M. S.; White, M. C. *Science* **2007**, *318* (5851), 783−787.
(13) Chen, K.; Baran, P. S. *Nature* **2009**, *459* (7248), 824−828.
(14) Gomez, L.; Garcia-Bosch, I.; Company, A.; Benet-Buchholz, J.; Polo, A.; Sala, X.; Ribas, X.; Costas, M. *Angew. Chem., Int. Ed.* **2009**, *48* (31), 5720−5723.
(15) Chen, M. S.; White, M. C. *Science* **2010**, *327* (5965), 566−571.
(16) Cook, B. R.; Reinert, T. J.; Suslick, K. S. *J. Am. Chem. Soc.* **1986**, *108*, 7281−7286.

(17) Yang, J.; Gabriele, B.; Belvedere, S.; Huang, Y.; Breslow, R. *J. Org. Chem.* **2002**, *67* (15), 5057−5067.
(18) Das, S.; Incarvito, C. D.; Crabtree, R. H.; Brudvig, G. W. *Science* **2006**, *312* (5782), 1941−1943.
(19) Das, S.; Brudvig, G. W.; Crabtree, R. H. *Chem. Commun. (Camb.)* **2008**, *4*, 413−424.
(20) Das, S.; Brudvig, G. W.; Crabtree, R. H. *J. Am. Chem. Soc.* **2008**, *130* (5), 1628−1637.
(21) Newhouse, T.; Baran, P. S. *Angew. Chem., Int. Ed.* **2011**, *50* (15), 3362−3374.
(22) Nelson, D. R. *Hum. Genomics* **2009**, *4* (1), 59−65.
(23) Denisov, I. G.; Makris, T. M.; Sligar, S. G.; Schlichting, I. *Chem. Rev.* **2005**, *105* (6), 2253−2277.
(24) Munro, A. W.; Girvan, H. M.; McLean, K. J. *Nat. Prod. Rep.* **2007**, *24* (3), 585−609.
(25) Ortiz de Montellano, P. R. *Chem. Rev.* **2010**, *110* (2), 932−948.
(26) Zerbe, K.; Woithe, K.; Li, D. B.; Vitali, F.; Bigler, L.; Robinson, J. A. *Angew. Chem., Int. Ed.* **2004**, *43* (48), 6709−6713.
(27) Zenk, M. H.; Gerardy, R.; Stadler, R. *J. Chem. Soc.* **1989**, *22*, 1725−1727.
(28) Cryle, M. J.; De Voss, J. J. *Chem. Commun. (Camb.)* **2004**, *1*, 86−87.
(29) Pikuleva, I. A. *Drug Metab. Dispos.* **2006**, *34* (4), 513−520.
(30) Wilson, M. C.; Gulder, T. A.; Mahmud, T.; Moore, B. S. *J. Am. Chem. Soc.* **2010**, *132* (36), 12757−12765.
(31) Kim, T. W.; Hwang, J. Y.; Kim, Y. S.; Joo, S. H.; Chang, S. C.; Lee, J. S.; Takatsuto, S.; Kim, S. K. *Plant Cell* **2005**, *17* (8), 2397−2412.
(32) Sono, M.; Roach, M. P.; Coulter, E. D.; Dawson, J. H. *Chem. Rev.* **1996**, *96* (7), 2841−2888.
(33) Isin, E. M.; Guengerich, F. P. *Biochim. Biophys. Acta* **2007**, *1770* (3), 314−329.
(34) Ortiz de Montellano, P. R.; Nelson, S. D. *Arch. Biochem. Biophys.* **2011**, *507* (1), 95−110.
(35) Kaspera, R.; Croteau, R. *Phytochem. Rev.* **2006**, *5* (2−3), 433−444.
(36) Maier, T.; Forster, H. H.; Asperger, O.; Hahn, U. *Biochem. Biophys. Res. Commun.* **2001**, *286* (3), 652−658.
(37) Funhoff, E. G.; Bauer, U.; Garcia-Rubio, I.; Witholt, B.; van Beilen, J. B. *J. Bacteriol.* **2006**, *188* (14), 5220−5227.
(38) van Beilen, J. B.; Funhoff, E. G.; van Loon, A.; Just, A.; Kaysser, L.; Bouza, M.; Holtackers, R.; Rothlisberger, M.; Li, Z.; Witholt, B. *Appl. Environ. Microbiol.* **2006**, *72* (1), 59−65.
(39) Pylypenko, O.; Schlichting, I. *Annu. Rev. Biochem.* **2004**, *73*, 991−1018.
(40) Sevrioukova, I. F.; Poulos, T. L. *Arch. Biochem. Biophys.* **2011**, *507* (1), 66−74.
(41) Conner, K. P.; Woods, C. M.; Atkins, W. M. *Arch. Biochem. Biophys.* **2011**, *507* (1), 56−65.
(42) Poulos, T. L.; Finzel, B. C.; Howard, A. J. *J. Mol. Biol.* **1987**, *195* (3), 687−700.
(43) Egawa, T.; Shimada, H.; Ishimura, Y. *Biochem. Biophys. Res. Commun.* **1994**, *201* (3), 1464−1469.
(44) Kellner, D. G.; Hung, S. C.; Weiss, K. E.; Sligar, S. G. *J. Biol. Chem.* **2002**, *277* (12), 9641−9644.
(45) Spolitak, T.; Dawson, J. H.; Ballou, D. P. *J. Biol. Chem.* **2005**, *280* (21), 20300−20309.
(46) Rittle, J.; Green, M. T. *Science* **2010**, *330* (6006), 933−937.
(47) Groves, J. T. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (7), 3569−3574.
(48) Hannemann, F.; Bichet, A.; Ewen, K. M.; Bernhardt, R. *Biochim. Biophys. Acta* **2007**, *1770* (3), 330−344.
(49) Unger, B. P.; Gunsalus, I. C.; Sligar, S. G. *J. Biol. Chem.* **1986**, *261* (3), 1158−1163.
(50) Hawkes, D. B.; Adams, G. W.; Burlingame, A. L.; de Montellano, P. R. O.; De Voss, J. J. *J. Biol. Chem.* **2002**, *277* (31), 27725−27732.
(51) Bernhardt, R. *J. Biotechnol.* **2006**, *124* (1), 128−145.
(52) Grogan, G. *Curr. Opin. Biotechnol.* **2011**, *15* (2), 241−248.
(53) Jung, S. T.; Lauchli, R.; Arnold, F. H. *Curr. Opin. Biotechnol.* **2011**, *22* (6), 809−817.
(54) Lewis, J. C.; Arnold, F. H. *Chimia* **2009**, *63* (6), 309−312.
(55) Lewis, J. C.; Coelho, P. S.; Arnold, F. H. *Chem. Soc. Rev.* **2011**, *40* (4), 2003−2021.
(56) Monti, D.; Ottolina, G.; Carrea, G.; Riva, S. *Chem. Rev.* **2011**, *111* (7), 4111−4140.
(57) Whitehouse, C. J.; Bell, S. G.; Wong, L. L. *Chem. Soc. Rev.* **2012**, *41* (3), 1218−1260.
(58) Ravichandran, K. G.; Boddupalli, S. S.; Hasermann, C. A.; Peterson, J. A.; Deisenhofer, J. *Science* **1993**, *261* (5122), 731−736.
(59) Gelb, M. H.; Heimbrook, D. C.; Malkonen, P.; Sligar, S. G. *Biochemistry* **1982**, *21* (2), 370−377.
(60) Kadkhodayan, S.; Coulter, E. D.; Maryniak, D. M.; Bryson, T. A.; Dawson, J. H. *J. Biol. Chem.* **1995**, *270* (47), 28042−28048.
(61) Capdevila, J. H.; Wei, S.; Helvig, C.; Falck, J. R.; Belosludtsev, Y.; Truan, G.; Graham-Lorence, S. E.; Peterson, J. A. *J. Biol. Chem.* **1996**, *271* (37), 22663−22671.
(62) Noble, M. A.; Miles, C. S.; Chapman, S. K.; Lysek, D. A.; Mackay, A. C.; Reid, G. A.; Hanzlik, R. P.; Munro, A. W. *Biochem. J.* **1999**, *339*, 371−379.
(63) Cryle, M. J.; Espinoza, R. D.; Smith, S. J.; Matovic, N. J.; De Voss, J. J. *Chem. Commun. (Camb.)* **2006**, *22*, 2353−2355.
(64) Narhi, L. O.; Fulco, A. J. *J. Biol. Chem.* **1987**, *262* (14), 6683−6690.
(65) Sowden, R. J.; Yasmin, S.; Rees, N. H.; Bell, S. G.; Wong, L. L. *Org. Biomol. Chem.* **2005**, *3* (1), 57−64.
(66) Bell, S. G.; Chen, X. H.; Sowden, R. J.; Xu, F.; Williams, J. N.; Wong, L. L.; Rao, Z. H. *J. Am. Chem. Soc.* **2003**, *125* (3), 705−714.
(67) Bell, S. G.; Sowden, R. J.; Wong, L. L. *Chem. Commun. (Camb.)* **2001**, *7*, 635−636.
(68) Bell, S. G.; Stevenson, J. A.; Boyd, H. D.; Campbell, S.; Riddle, A. D.; Orton, E. L.; Wong, L. L. *Chem. Commun. (Camb.)* **2002**, *5*, 490−491.
(69) Xu, F.; Bell, S. G.; Lednik, J.; Insley, A.; Rao, Z.; Wong, L. L. *Angew. Chem., Int. Ed.* **2005**, *44* (26), 4029−4032.
(70) Nickerson, D. P.; Harford-Cross, C. F.; Fulcher, S. R.; Wong, L. L. *FEBS Lett.* **1997**, *405* (2), 153−156.
(71) Jones, J. P.; O'Hare, E. J.; Wong, L. L. *Eur. J. Biochem.* **2001**, *268* (5), 1460−1467.
(72) Harford-Cross, C. F.; Carmichael, A. B.; Allan, F. K.; England, P. A.; Rouch, D. A.; Wong, L. L. *Protein Eng.* **2000**, *13* (2), 121−128.
(73) Speight, R. E.; Hancock, F. E.; Winkel, C.; Bevinakatti, H. S.; Sarkar, M.; Flitsch, S. L.; Turner, N. J. *Tetrahedron: Asymmetry* **2004**, *15*, 2829−2831.
(74) Li, H.; Poulos, T. L. *Nat. Struct. Biol.* **1997**, *4* (2), 140−146.
(75) Oliver, C. F.; Modi, S.; Sutcliffe, M. J.; Primrose, W. U.; Lian, L. Y.; Roberts, G. C. *Biochemistry* **1997**, *36* (7), 1567−1572.
(76) Graham Lorence, S.; Truan, G.; Peterson, J. A.; Falck, J. R.; Wei, S. Z.; Helvig, C.; Capdevila, J. H. *J. Biol. Chem.* **1997**, *272* (2), 1127−1135.
(77) Li, Q. S.; Ogawa, J.; Schmid, R. D.; Shimizu, S. *FEBS Lett.* **2001**, *508* (2), 249−252.
(78) Sulistyaningdyah, W. T.; Ogawa, J.; Li, Q. S.; Maeda, C.; Yano, Y.; Schmid, R. D.; Shimizu, S. *Appl. Microbiol. Biotechnol.* **2005**, *67* (4), 556−562.
(79) Landwehr, M.; Hochrein, L.; Otey, C. R.; Kasrayan, A.; Backvall, J. E.; Arnold, F. H. *J. Am. Chem. Soc.* **2006**, *128* (18), 6058−6059.
(80) Urlacher, V. B.; Makhsumkhanov, A.; Schmid, R. D. *Appl. Microbiol. Biotechnol.* **2006**, *70* (1), 53−59.
(81) Weber, E.; Seifert, A.; Antonovici, M.; Geinitz, C.; Pleiss, J.; Urlacher, V. B. *Chem. Commun. (Camb.)* **2011**, *47* (3), 944−946.
(82) Carmichael, A. B.; Wong, L. L. *Eur. J. Biochem.* **2001**, *268* (10), 3117−3125.
(83) Li, Q. S.; Schwaneberg, U.; Fischer, P.; Schmid, R. D. *Chemistry* **2000**, *6* (9), 1531−1536.
(84) Li, Q. S.; Ogawa, J.; Schmid, R. D.; Shimizu, S. *Appl. Environ. Microbiol.* **2001**, *67* (12), 5735−5739.

(85) Appel, D.; Lutz-Wahl, S.; Fischer, P.; Schwaneberg, U.; Schmid, R. D. *J. Biotechnol.* **2001**, *88* (2), 167−171.
(86) Budde, M.; Morr, M.; Schmid, R. D.; Urlacher, V. B. *ChemBioChem* **2006**, *7*, 789−794.
(87) Maurer, S. C.; Kuhnel, K.; Kaysser, L. A.; Eiben, S.; Schmid, R. D.; Urlacher, V. B. *Adv. Synth. Catal.* **2005**, *347*, 802−810.
(88) Whitehouse, C. J.; Bell, S. G.; Tufton, H. G.; Kenny, R. J.; Ogilvie, L. C.; Wong, L. L. *Chem. Commun. (Camb.)* **2008**, *8*, 966−968.
(89) Seifert, A.; Vomund, S.; Grohmann, K.; Kriening, S.; Urlacher, V. B.; Laschat, S.; Pleiss, J. *ChemBioChem* **2009**, *10* (5), 853−861.
(90) Seifert, A.; Antonovici, M.; Hauer, B.; Pleiss, J. *ChemBioChem* **2011**, *12* (9), 1346−1351.
(91) Tracewell, C. A.; Arnold, F. H. *Curr. Opin. Biotechnol.* **2009**, *13* (1), 3−9.
(92) Glieder, A.; Farinas, E. T.; Arnold, F. H. *Nat. Biotechnol.* **2002**, *20* (11), 1135−1139.
(93) Peters, M. W.; Meinhold, P.; Glieder, A.; Arnold, F. H. *J. Am. Chem. Soc.* **2003**, *125* (44), 13442−13450.
(94) Fasan, R.; Chen, M. M.; Crook, N. C.; Arnold, F. H. *Angew. Chem., Int. Ed.* **2007**, *46* (44), 8414−8418.
(95) Farinas, E. T.; Schwaneberg, U.; Glieder, A.; Arnold, F. H. *Adv. Synth. Cat.* **2001**, *343* (6−7), 601−606.
(96) Kubo, T.; Peters, M. W.; Meinhold, P.; Arnold, F. H. *Chemistry* **2006**, *12* (4), 1216−1220.
(97) Lewis, J. C.; Mantovani, S. M.; Fu, Y.; Snow, C. D.; Komor, R. S.; Wong, C. H.; Arnold, F. H. *ChemBioChem* **2010**, *11* (18), 2502−2505.
(98) Salazar, O.; Cirino, P. C.; Arnold, F. H. *ChemBioChem* **2003**, *4* (9), 891−893.
(99) Bloom, J. D.; Labthavikul, S. T.; Otey, C. R.; Arnold, F. H. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103* (15), 5869−5874.
(100) Fasan, R.; Meharenna, Y. T.; Snow, C. D.; Poulos, T. L.; Arnold, F. H. *J. Mol. Biol.* **2008**, *383* (5), 1069−1080.
(101) Shapiro, M. G.; Westmeyer, G. G.; Romero, P. A.; Szablowski, J. O.; Kuster, B.; Shah, A.; Otey, C. R.; Langer, R.; Arnold, F. H.; Jasanoff, A. *Nat. Biotechnol.* **2010**, *28* (3), 264−270.
(102) Pikuleva, I. A.; Bjorkhem, I.; Waterman, M. R. *Arch. Biochem. Biophys.* **1996**, *334* (2), 183−192.
(103) Brock, B. J.; Waterman, M. R. *Arch. Biochem. Biophys.* **2000**, *373* (2), 401−408.
(104) Huang, W.; Johnston, W. A.; Hayes, M. A.; De Voss, J. J.; Gillam, E. M. *Arch. Biochem. Biophys.* **2007**, *467* (2), 193−205.
(105) Rosic, N. N.; Huang, W.; Johnston, W. A.; DeVoss, J. J.; Gillam, E. M. *Gene* **2007**, *395* (1−2), 40−48.
(106) Otey, C. R.; Silberg, J. J.; Voigt, C. A.; Endelman, J. B.; Bandara, G.; Arnold, F. H. *Chem. Biol.* **2004**, *11* (3), 309−318.
(107) Otey, C. R.; Landwehr, M.; Endelman, J. B.; Hiraga, K.; Bloom, J. D.; Arnold, F. H. *PLoS Biol.* **2006**, *4* (5), e112.
(108) Landwehr, M.; Carbone, M.; Otey, C. R.; Li, Y.; Arnold, F. H. *Chem. Biol.* **2007**, *14* (3), 269−278.
(109) Gotoh, O. *J. Biol. Chem.* **1992**, *267* (1), 83−90.
(110) Kumar, S.; Scott, E. E.; Liu, H.; Halpert, J. R. *J. Biol. Chem.* **2003**, *278* (19), 17178−17184.
(111) Murataliev, M. B.; Trinh, L. N.; Moser, L. V.; Bates, R. B.; Feyereisen, R.; Walker, F. A. *Biochemistry* **2004**, *43* (7), 1771−1780.
(112) Chen, C. K.; Shokhireva, T.; Berry, R. E.; Zhang, H.; Walker, F. A. *J. Biol. Inorg. Chem.* **2008**, *13* (5), 813−824.
(113) Chen, C. K.; Berry, R. E.; Shokhireva, T.; Murataliev, M. B.; Zhang, H.; Walker, F. A. *J. Biol. Inorg. Chem.* **2010**, *15* (2), 159−174.
(114) Zocher, G.; Richter, M. E.; Mueller, U.; Hertweck, C. *J. Am. Chem. Soc.* **2011**, *133* (7), 2292−2302.
(115) Sherman, D. H.; Li, S.; Yermalitskaya, L. V.; Kim, Y.; Smith, J. A.; Waterman, M. R.; Podust, L. M. *J. Biol. Chem.* **2006**, *281* (36), 26289−26297.
(116) Helliwell, C. A.; Poole, A.; Peacock, W. J.; Dennis, E. S. *Plant Physiol.* **1999**, *119* (2), 507−510.
(117) Rojas, M. C.; Hedden, P.; Gaskin, P.; Tudzynski, B. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98* (10), 5838−5843.
(118) Ro, D. K.; Arimura, G.; Lau, S. Y.; Piers, E.; Bohlmann, J. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102* (22), 8060−8065.
(119) Teoh, K. H.; Polichuk, D. R.; Reed, D. W.; Nowak, G.; Covello, P. S. *FEBS Lett.* **2006**, *580* (5), 1411−1416.
(120) Ro, D. K.; Paradise, E. M.; Ouellet, M.; Fisher, K. J.; Newman, K. L.; Ndungu, J. M.; Ho, K. A.; Eachus, R. A.; Ham, T. S.; Kirby, J.; Chang, M. C.; Withers, S. T.; Shiba, Y.; Sarpong, R.; Keasling, J. D. *Nature* **2006**, *440* (7086), 940−943.
(121) Chang, M. C.; Eachus, R. A.; Trieu, W.; Ro, D. K.; Keasling, J. D. *Nat. Chem. Biol.* **2007**, *3* (5), 274−277.
(122) Loida, P. J.; Sligar, S. G. *Biochemistry* **1993**, *32* (43), 11530−11538.
(123) Whitehouse, C. J.; Yang, W.; Yorke, J. A.; Rowlatt, B. C.; Strong, A. J.; Blanford, C. F.; Bell, S. G.; Bartlam, M.; Wong, L. L.; Rao, Z. *ChemBioChem* **2010**, *11* (18), 2549−2556.
(124) van Beilen, J. B.; Duetz, W. A.; Schmid, A.; Witholt, B. *Trends Biotechnol.* **2003**, *21* (4), 170−177.
(125) Fasan, R.; Crook, N. C.; Peters, M. W.; Meinhold, P.; Buelter, T.; Landwehr, M.; Cirino, P. C.; Arnold, F. H. *Biotechnol. Bioeng.* **2011**, *108* (3), 500−510.
(126) Dietrich, J. A.; Yoshikuni, Y.; Fisher, K. J.; Woolard, F. X.; Ockey, D.; McPhee, D. J.; Renninger, N. S.; Chang, M. C.; Baker, D.; Keasling, J. D. *ACS Chem. Biol.* **2009**, *4* (4), 261−267.
(127) Modi, S.; Sutcliffe, M. J.; Primrose, W. U.; Lian, L. Y.; Roberts, G. C. *Nat. Struct. Biol.* **1996**, *3* (5), 414−417.
(128) Yano, J. K.; Koo, L. S.; Schuller, D. J.; Li, H.; Ortiz de Montellano, P. R.; Poulos, T. L. *J. Biol. Chem.* **2000**, *275* (40), 31086−31092.
(129) Muralidhara, B. K.; Negi, S.; Chin, C. C.; Braun, W.; Halpert, J. R. *J. Biol. Chem.* **2006**, *281* (12), 8051−8061.
(130) Joyce, M. G.; Girvan, H. M.; Munro, A. W.; Leys, D. *J. Biol. Chem.* **2004**, *279* (22), 23287−23293.
(131) Li, Y. C.; Chiang, C. W.; Yeh, H. C.; Hsu, P. Y.; Whitby, F. G.; Wang, L. H.; Chan, N. L. *J. Biol. Chem.* **2008**, *283* (5), 2917−2926.
(132) Podust, L. M.; Poulos, T. L.; Waterman, M. R. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98* (6), 3068−3073.
(133) Jovanovic, T.; McDermott, A. E. *J. Am. Chem. Soc.* **2005**, *127* (40), 13816−13821.
(134) Schoch, G. A.; Yano, J. K.; Sansen, S.; Dansette, P. M.; Stout, C. D.; Johnson, E. F. *J. Biol. Chem.* **2008**, *283* (25), 17227−17237.
(135) Verras, A.; Alian, A.; de Montellano, P. R. *Protein Eng., Des. Sel.* **2006**, *19* (11), 491−496.
(136) Pochapsky, T. C.; Kazanis, S.; Dang, M. *Antioxid. Redox. Signaling* **2010**, *13* (8), 1273−1296.
(137) Dietrich, M.; Do, T. A.; Schmid, R. D.; Pleiss, J.; Urlacher, V. B. *J. Biotechnol.* **2009**, *139* (1), 115−117.
(138) Fujishiro, T.; Shoji, O.; Nagano, S.; Sugimoto, H.; Shiro, Y.; Watanabe, Y. *J. Biol. Chem.* **2011**, *286* (34), 29941−29950.
(139) Tee, K. L.; Schwaneberg, U. *Comb. Chem. High Throughput Screening* **2007**, *10* (3), 197−217.
(140) Rabe, K. S.; Gandubert, V. J.; Spengler, M.; Erkelenz, M.; Niemeyer, C. M. *Anal. Bioanal. Chem.* **2008**, *392* (6), 1059−1073.
(141) Kille, S.; Zilly, F. E.; Acevedo, J. P.; Reetz, M. T. *Nat. Chem.* **2011**, *3* (9), 738−743.
(142) Rentmeister, A.; Arnold, F. H.; Fasan, R. *Nat. Chem. Biol.* **2009**, *5* (1), 26−28.
(143) Sawayama, A. M.; Chen, M. M.; Kulanthaivel, P.; Kuo, M. S.; Hemmerle, H.; Arnold, F. H. *Chemistry* **2009**, *15* (43), 11723−11729.
(144) Lewis, J. C.; Bastian, S.; Bennett, C. S.; Fu, Y.; Mitsuda, Y.; Chen, M. M.; Greenberg, W. A.; Wong, C. H.; Arnold, F. H. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106* (39), 16550−16555.
(145) Vottero, E.; Rea, V.; Lastdrager, J.; Honing, M.; Vermeulen, N. P.; Commandeur, J. N. *J. Biol. Inorg. Chem.* **2011**, *16* (6), 899−912.
(146) Meinhold, P.; Peters, M. W.; Hartwick, A.; Hernandez, A. R.; Arnold, F. H. *Adv. Synth. Catal.* **2006**, *348* (6), 763−772.
(147) Lentz, O.; Feenstra, A.; Habicher, T.; Hauer, B.; Schmid, R. D.; Urlacher, V. B. *ChemBioChem* **2006**, *7* (2), 345−350.
(148) Tang, W. L.; Li, Z.; Zhao, H. *Chem. Commun. (Camb.)* **2010**, *46* (30), 5461−5463.

(149) Zhang, K.; El Damaty, S.; Fasan, R. *J. Am. Chem. Soc.* **2011**, *133* (10), 3242−3245.
(150) Shoji, O.; Fujishiro, T.; Nakajima, H.; Kim, M.; Nagano, S.; Shiro, Y.; Watanabe, Y. *Angew. Chem., Int. Ed.* **2007**, *46* (20), 3656−3659.
(151) Lee, D. S.; Yamada, A.; Sugimoto, H.; Matsunaga, I.; Ogura, H.; Ichihara, K.; Adachi, S.; Park, S. Y.; Shiro, Y. *J. Biol. Chem.* **2003**, *278* (11), 9761−9767.
(152) Shoji, O.; Fujishiro, T.; Nagano, S.; Tanaka, S.; Hirose, T.; Shiro, Y.; Watanabe, Y. *J. Biol. Inorg. Chem.* **2010**, *15* (8), 1331−1339.
(153) Shoji, O.; Wiese, C.; Fujishiro, T.; Shirataki, C.; Wunsch, B.; Watanabe, Y. *J. Biol. Inorg. Chem.* **2010**, *15* (7), 1109−1115.
(154) Zilly, F. E.; Acevedo, J. P.; Augustyniak, W.; Deege, A.; Hausig, U. W.; Reetz, M. T. *Angew. Chem., Int. Ed.* **2011**, *50* (12), 2720−2724.
(155) Kawakami, N.; Shoji, O.; Watanabe, Y. *Angew. Chem., Int. Ed.* **2011**, *50* (23), 5315−5318.
(156) Meinhold, P.; Peters, M. W.; Chen, M. M.; Takahashi, K.; Arnold, F. H. *ChemBioChem* **2005**, *6* (10), 1765−1768.
(157) Kotani, T.; Yamamoto, T.; Yurimoto, H.; Sakai, Y.; Kato, N. *J. Bacteriol.* **2003**, *185* (24), 7120−7128.
(158) Lieberman, R. L.; Rosenzweig, A. C. *Crit. Rev. Biochem. Mol. Biol.* **2004**, *39* (3), 147−164.
(159) Sluis, M. K.; Sayavedra-Soto, L. A.; Arp, D. J. *Microbiology* **2002**, *148* (Pt 11), 3617−3629.
(160) Braunegg, G.; de Raadt, A.; Feichtenhofer, S.; Griengl, H.; Kopper, I. I.; Lehmann, A.; Weber, H. J. *Angew. Chem., Int. Ed.* **1999**, *38* (18), 2763−2766.
(161) de Raadt, A.; Griengl, H.; Weber, H. *Chemistry* **2001**, *7* (1), 27−31.
(162) Li, Z.; Feiten, H. J.; Chang, D.; Duetz, W. A.; van Beilen, J. B.; Witholt, B. *J. Org. Chem.* **2001**, *66* (25), 8424−8430.
(163) de Raadt, A.; Griengl, H. *Curr. Opin. Biotechnol.* **2002**, *13* (6), 537−542.
(164) Munzer, D. F.; Meinhold, P.; Peters, M. W.; Feichtenhofer, S.; Griengl, H.; Arnold, F. H.; Glieder, A.; de Raadt, A. *Chem. Commun. (Camb.)* **2005**, *20*, 2597−2599.
(165) Li, S.; Chaulagain, M. R.; Knauff, A. R.; Podust, L. M.; Montgomery, J.; Sherman, D. H. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106* (44), 18463−18468.
(166) Larsen, A. T.; May, E. M.; Auclair, K. *J. Am. Chem. Soc.* **2011**, *133* (20), 7853−7858.
(167) Xue, Y.; Wilson, D.; Zhao, L.; Liu, H.; Sherman, D. H. *Chem. Biol.* **1998**, *5* (11), 661−667.
(168) Shin, H. S.; Slattery, J. T. *J. Pharm. Sci.* **1998**, *87* (3), 390−393.
(169) Parikh, A.; Gillam, E. M.; Guengerich, F. P. *Nat. Biotechnol.* **1997**, *15* (8), 784−788.
(170) Shet, M. S.; Fisher, C. W.; Estabrook, R. W. *Arch. Biochem. Biophys.* **1997**, *339* (1), 218−225.
(171) Bell, S. G.; Harford-Cross, C. F.; Wong, L. L. *Protein Eng.* **2001**, *14* (10), 797−802.
(172) Salamanca-Pinzon, S. G.; Guengerich, F. P. *Protein Expr. Purif.* **2011**, *79* (2), 231−236.
(173) Munro, A. W.; Daff, S.; Coggins, J. R.; Lindsay, J. G.; Chapman, S. K. *Eur. J. Biochem.* **1996**, *239* (2), 403−409.
(174) Nakayama, N.; Takemae, A.; Shoun, H. *J. Biochem.* **1996**, *119* (3), 435−440.
(175) Gustafsson, M. C.; Roitel, O.; Marshall, K. R.; Noble, M. A.; Chapman, S. K.; Pessegueiro, A.; Fulco, A. J.; Cheesman, M. R.; von Wachenfeldt, C.; Munro, A. W. *Biochemistry* **2004**, *43* (18), 5474−5487.
(176) Chowdhary, P. K.; Alemseghed, M.; Haines, D. C. *Arch. Biochem. Biophys.* **2007**, *468* (1), 32−43.
(177) Dietrich, M.; Eiben, S.; Asta, C.; Do, T. A.; Pleiss, J.; Urlacher, V. B. *Appl. Environ. Microbiol.* **2008**, *79* (6), 931−940.
(178) Weis, R.; Winkler, M.; Schittmayer, M.; Kambourakis, S.; Vink, M.; Rozzell, D.; Glieder, A. *Adv. Synth. Catal.* **2009**, *351*, 2140−2146.
(179) Roberts, G. A.; Grogan, G.; Greter, A.; Flitsch, S. L.; Turner, N. J. *J. Bacteriol.* **2002**, *184* (14), 3898−3908.
(180) De Mot, R.; Parret, A. H. *Trends Microbiol.* **2002**, *10* (11), 502−508.
(181) Roberts, G. A.; Celik, A.; Hunter, D. J.; Ost, T. W.; White, J. H.; Chapman, S. K.; Turner, N. J.; Flitsch, S. L. *J. Biol. Chem.* **2003**, *278* (49), 48914−48920.
(182) Liu, L.; Schmid, R. D.; Urlacher, V. B. *Appl. Environ. Microbiol.* **2006**, *72* (5), 876−882.
(183) Celik, A.; Roberts, G. A.; White, J. H.; Chapman, S. K.; Turner, N. J.; Flitsch, S. L. *Chem. Commun. (Camb.)* **2006**, *43*, 4492−4494.
(184) Shet, M. S.; Fisher, C. W.; Holmans, P. L.; Estabrook, R. W. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90* (24), 11748−11752.
(185) Deeni, Y. Y.; Paine, M. J. I.; Ayrton, A. D.; Clarke, S. E.; Chenery, R.; Wolf, C. R. *Arch. Biochem. Biophys.* **2001**, *396* (1), 16−24.
(186) Chun, Y. J.; Shimada, T.; Guengerich, F. P. *Arch. Biochem. Biophys.* **1996**, *330* (1), 48−58.
(187) Shet, M. S.; Fisher, C. W.; Holmans, P. L.; Estabrook, R. W. *Arch. Biochem. Biophys.* **1996**, *330* (1), 199−208.
(188) Hayashi, K.; Sakaki, T.; Kominami, S.; Inouye, K.; Yabusaki, Y. *Arch. Biochem. Biophys.* **2000**, *381* (1), 164−170.
(189) Inui, H.; Maeda, A.; Ohkawa, H. *Biochemistry* **2007**, *46* (35), 10213−10221.
(190) Helvig, C.; Capdevila, J. H. *Biochemistry* **2000**, *39* (17), 5196−5205.
(191) Dodhia, V. R.; Fantuzzi, A.; Gilardi, G. *J. Biol. Inorg. Chem.* **2006**, *11* (7), 903−916.
(192) Fairhead, M.; Giannini, S.; Gillam, E. M. J.; Gilardi, G. *J. Biol. Inorg. Chem.* **2005**, *10* (8), 842−853.
(193) Hlavica, P. *Biotechnol. Adv.* **2009**, *27* (2), 103−121.
(194) Sakaki, T.; Shibata, M.; Yabusaki, Y.; Murakami, H.; Ohkawa, H. *DNA Cell Biol.* **1990**, *9* (8), 603−614.
(195) Shet, M. S.; Fisher, C. W.; Arlotto, M. P.; Shackleton, C. H.; Holmans, P. L.; Martin-Wixtrom, C. A.; Saeki, Y.; Estabrook, R. W. *Arch. Biochem. Biophys.* **1994**, *311* (2), 402−417.
(196) Sakaki, T.; Kominami, S.; Hayashi, K.; Akiyoshi-Shibata, M.; Yabusaki, Y. *J. Biol. Chem.* **1996**, *271* (42), 26209−26213.
(197) Shet, M. S.; Fisher, C. W.; Estabrook, R. W. *Arch. Biochem. Biophys.* **1997**, *339* (1), 218−225.
(198) Duport, C.; Spagnoli, R.; Degryse, E.; Pompon, D. *Nat. Biotechnol.* **1998**, *16* (2), 186−189.
(199) Shet, M. S.; Fisher, C. W.; Tremblay, Y.; Belanger, A.; Conley, A. J.; Mason, J. I.; Estabrook, R. W. *Drug Metab. Dispos.* **2007**, *39* (2−3), 289−307.
(200) Leonard, E.; Lim, K. H.; Saw, P. N.; Koffas, M. A. *Appl. Environ. Microbiol.* **2007**, *73* (12), 3877−3886.
(201) Ajikumar, P. K.; Xiao, W. H.; Tyo, K. E.; Wang, Y.; Simeon, F.; Leonard, E.; Mucha, O.; Phon, T. H.; Pfeifer, B.; Stephanopoulos, G. *Science* **2010**, *330* (6000), 70−74.
(202) Nodate, M.; Kubota, M.; Misawa, N. *Appl. Microbiol. Biotechnol.* **2006**, *71* (4), 455−462.
(203) Robin, A.; Roberts, G. A.; Kisch, J.; Sabbadin, F.; Grogan, G.; Bruce, N.; Turner, N. J.; Flitsch, S. L. *Chem. Commun. (Camb.)* **2009**, *18*, 2478−2480.
(204) Bordeaux, M.; Galarneau, A.; Fajula, F.; Drone, J. *Angew. Chem., Int. Ed.* **2011**, *50* (9), 2075−2079.
(205) Li, S. Y.; Podust, L. M.; Sherman, D. H. *J. Am. Chem. Soc.* **2007**, *129* (43), 12940−12941.
(206) Sabbadin, F.; Hyde, R.; Robin, A.; Hilgarth, E. M.; Delenne, M.; Flitsch, S.; Turner, N.; Grogan, G.; Bruce, N. C. *ChemBioChem* **2010**, *11* (7), 987−994.
(207) Funhoff, E. G.; Salzmann, J.; Bauer, U.; Witholt, B.; van Beilen, J. B. *Enzym. Microb. Technol.* **2007**, *40* (4), 806−812.
(208) Sibbesen, O.; DeVoss, J. J.; deMontellano, P. R. O. *J. Biol. Chem.* **1996**, *271* (37), 22462−22469.
(209) Hirakawa, H.; Kamiya, N.; Tanaka, T.; Nagamune, T. *Protein Eng., Des. Sel.* **2007**, *20* (9), 453−459.
(210) Hirakawa, H.; Nagamune, T. *ChemBioChem* **2010**, *11* (11), 1517−1520.
(211) Guengerich, F. P. *Chem. Res. Toxicol.* **2008**, *21* (1), 70−83.
(212) Baillie, T. A. *Chem. Res. Toxicol.* **2008**, *21* (1), 129−137.

(213) Friedberg, T.; Pritchard, M. P.; Bandera, M.; Hanlon, S. P.; Yao, D.; McLaughlin, L. A.; Ding, S.; Burchell, B.; Wolf, C. R. *Drug Metab. Rev.* **1999**, *31* (2), 523−544.
(214) Rushmore, T. H.; Reider, P. J.; Slaughter, D.; Assang, C.; Shou, M. *Metab. Eng.* **2000**, *2* (2), 115−125.
(215) Vail, R. B.; Homann, M. J.; Hanna, I.; Zaks, A. *J. Ind. Microbiol. Biotechnol.* **2005**, *32* (2), 67−74.
(216) Schroer, K.; Kittelmann, M.; Lutz, S. *Biotechnol. Adv.* **2010**, *106* (5), 699−706.
(217) Otey, C. R.; Bandara, G.; Lalonde, J.; Takahashi, K.; Arnold, F. H. *Biotechnol. Adv.* **2006**, *93* (3), 494−499.
(218) van Vugt-Lussenburg, B. M. A.; Damsten, M. C.; Maasdijk, D. M.; Vermeulen, N. P. E; Commandeur, J. N. M. *Biochem. Biophys. Res. Commun.* **2006**, *346* (3), 810−818.
(219) van Vugt-Lussenburg, B. M. A.; Stjernschantz, E.; Lastdrager, J.; Oostenbrink, C.; Vermeulen, N. P. E; Commandeur, J. N. M. *J. Med. Chem.* **2007**, *50* (3), 455−461.
(220) Reinen, J.; van Leeuwen, J. S.; Li, Y. M.; Sun, L. F.; Grootenhuis, P. D. J.; Decker, C. J.; Saunders, J.; Vermeulen, N. P. E.; Commandeur, J. N. M. *Drug Metab. Dispos.* **2011**, *39* (9), 1568−1576.
(221) Kim, K. H.; Kang, J. Y.; Kim, D. H.; Park, S. H.; Park, S. H.; Kim, D.; Park, K. D.; Lee, Y. J.; Jung, H. C.; Pan, J. G.; Ahn, T.; Yun, C. H. *Drug Metab. Dispos.* **2011**, *39* (1), 140−150.
(222) Kim, D. H.; Ahn, T.; Jung, H. C.; Pan, J. G.; Yun, C. H. *Drug Metab. Dispos.* **2009**, *37* (5), 932−936.
(223) Kim, D. H.; Kim, K. H.; Kim, D.; Jung, H. C.; Pan, J. G.; Chi, Y. T.; Ahn, T.; Yun, C. H. *J. Mol. Catal. B-Enzym.* **2010**, *63* (3−4), 179−187.
(224) Park, S. H.; Kim, D. H.; Kim, D.; Kim, D. H.; Jung, H. C.; Pan, J. G.; Ahn, T.; Kim, D.; Yun, C. H. *Drug Metab. Dispos.* **2010**, *38* (5), 732−739.
(225) Di Nardo, G.; Fantuzzi, A.; Sideri, A.; Panicco, P.; Sassone, C.; Giunta, C.; Gilardi, G. *J. Biol. Inorg. Chem.* **2007**, *12* (3), 313−323.
(226) Rentmeister, A.; Brown, T. R.; Snow, C. D.; Carbone, M.; Arnold, F. H. *ChemCatChem* **2011**, *3*, 1065−1071.
(227) Muller, K.; Faeh, C.; Diederich, F. *Science* **2007**, *317* (5846), 1881−1886.
(228) Bohm, H. J.; Banner, D.; Bendels, S.; Kansy, M.; Kuhn, B.; Muller, K.; Obst-Sander, U.; Stahl, M. *ChemBioChem* **2004**, *5* (5), 637−643.
(229) Park, B. K.; Kitteringham, N. R.; O'Neill, P. M. *Annu. Rev. Pharmacol. Toxicol.* **2001**, *41*, 443−470.
(230) Patel, R. N. *ACS Catalysis* **2011**, *1* (9), 1056−1074.
(231) Savile, C. K.; Janey, J. M.; Mundorff, E. C.; Moore, J. C.; Tam, S.; Jarvis, W. R.; Colbeck, J. C.; Krebber, A.; Fleitz, F. J.; Brands, J.; Devine, P. N.; Huisman, G. W.; Hughes, G. J. *Science* **2010**, *329* (5989), 305−309.