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# Applications of microbial cytochrome P450 enzymes in biotechnology and synthetic biology

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Cytochrome P450 enzymes (P450s) are a superfamily of monooxygenase enzymes with enormous potential for synthetic biology applications. Across Nature, their substrate range is vast and exceeds that of other enzymes. The range of different chemical transformations performed by P450s is also substantial, and continues to expand through interrogation of the properties of novel P450s and by protein engineering studies. The ability of P450s to introduce oxygen atoms at specific positions on complex molecules makes these enzymes particularly valuable for applications in synthetic biology. This review focuses on the enzymatic properties and reaction mechanisms of P450 enzymes, and on recent studies that highlight their broad applications in the production of oxychemicals. For selected soluble bacterial P450s (notably the high-activity P450-cytochrome P450 reductase enzyme P450 BM3), variants with a multitude of diverse substrate selectivities have been generated both rationally and by random mutagenesis/directed evolution approaches. This highlights the robustness and malleability of the P450 fold, and the capacity of these biocatalysts to oxidise a wide range of chemical scaffolds. This article reviews recent research on the application of wild-type and engineered P450s in the production of important chemicals, including pharmaceuticals and drug metabolites, steroids and antibiotics. In addition, the properties of unusual members of the P450 superfamily that do not follow the canonical P450 catalytic pathway are described.

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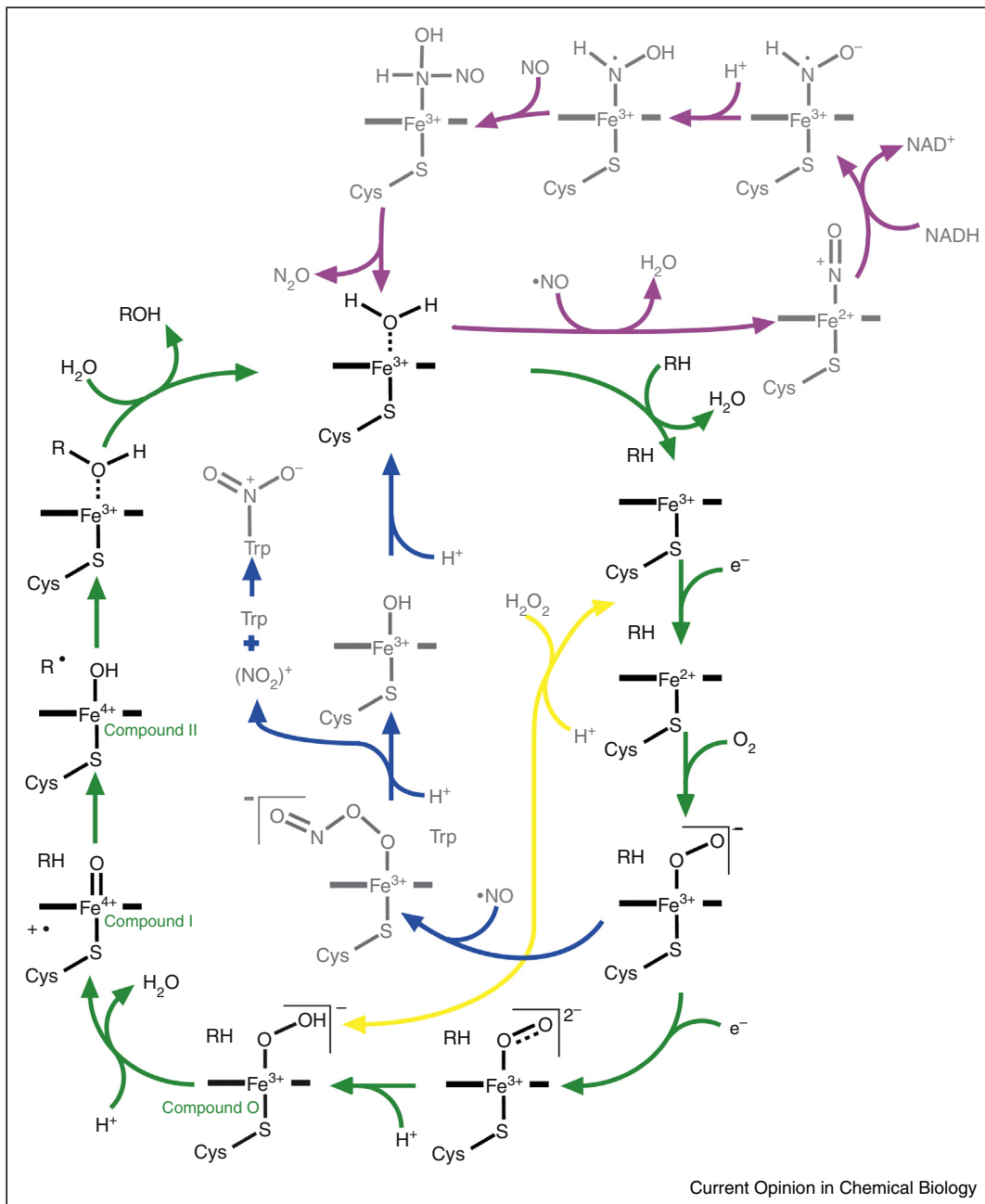
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## Introduction

The cytochromes P450 (P450s or CYPs) are an enzyme superfamily, members of which have a vast number of

physiological functions in organisms from archaea and bacteria through to man [1]. The majority of P450 enzymes are monooxygenases, catalysing the scission of dioxygen bound to their *b*-type heme iron, leading to the formation of a highly reactive ferryl iron-oxo intermediate (compound I) that catalyses the insertion of an oxygen atom into the substrate [2,3] (Figure 1). Oxidation of the P450 substrate can lead to several different chemical outcomes — including hydroxylation, epoxidation, demethylation or dealkylation, sulfoxidation, N-oxidation and decarboxylation [4]. Most P450s use NAD(P)H-driven redox partner systems for delivery of the two electrons required for reduction of the ferric heme iron (enabling dioxygen binding to ferrous heme) and for the subsequent reduction of the ferrous-oxo (formally ferric-superoxy) species to form a ferric-peroxo intermediate in the catalytic cycle (Figure 1). Eukaryotic P450s are bound to membranes with an N-terminal transmembrane helix, whereas prokaryotic P450s are soluble enzymes that lack the N-terminal membrane anchor. The majority of mammalian P450s are attached to the endoplasmic reticulum (microsomal P450s), although a subset involved in steroid biosynthesis are linked to the inner membrane of adrenal gland mitochondria [5]. The microsomal P450s are reduced by NADPH-cytochrome P450 reductase (CPR) — an FAD-binding and FMN-binding enzyme that is also attached to the ER membrane by an N-terminal anchor (a class II P450 redox system) [6]. The mitochondrial P450s interact instead with a soluble iron-sulfur protein (adrenodoxin), which sources electrons from the membrane-associated FAD-binding enzyme NADPH-adrenodoxin reductase (a class I system) [7]. Most of the characterized prokaryotic P450s also use a class I redox partner system (soluble ferredoxin reductase and ferredoxin; flavodoxins can surrogate in certain systems), though there is considerable further diversity in the redox partner apparatus that drives catalysis in prokaryotic P450 enzymes, as described further below [8,9]. The ability of P450s to perform regioselective and often stereoselective oxidation of their substrates makes them attractive catalysts for applications in synthetic biology and in the generation of high value oxychemicals that may not be economical to produce by synthetic chemistry. In the following sections, the diversity of P450 chemistry is described, and descriptions are given of how key P450 enzymes have been engineered for improved catalytic performance and for novel reactivity in the generation of useful chemical products.

Figure 1



The cytochrome P450 catalytic cycle. The canonical P450 catalytic cycle is shown with green arrows. In the resting state (the  $\text{Cys}-\text{Fe}^{3+}-\text{H}_2\text{O}$  species in black, common to all of the cycles shown) the heme iron is low-spin, ferric with a loosely coordinated water molecule in the distal position. Binding of substrate (RH) shifts the ferric heme iron equilibrium to the high-spin form, enabling the first electron transfer to reduce the iron to the ferrous state. Binding of dioxygen ( $\text{O}_2$ ), transfer of a second electron from a redox partner, two protonation steps (to the ferric-peroxy and ferric-hydroperoxy intermediates) involving active site amino acids, and the release of a water molecule results in the formation of the highly reactive ferryl-oxo porphyrin cation radical species known as compound I. Abstraction of a proton from the substrate forms a protonated ferryl intermediate (compound II) which hydroxylates the substrate radical according to the radical rebound mechanism [49]. Alternative P450 pathways exist for certain members of the P450 enzyme superfamily. Shown with blue arrows is the pathway to nitration of a tryptophan substrate using nitric oxide (NO) and  $\text{O}_2$ , as catalysed by TxtE (CYP1048A1) from *S. scabiei* and other *Streptomyces* species in a pathway to form the phytotoxin thaxtomin A [26\*]. This pathway deviates from the canonical cycle at the ferric-superoxo species; which reacts with NO rather than being further reduced to the ferric-peroxy form. While the mechanism of tryptophan nitration is not yet resolved, one model would be that the ferric-peroxynitrite species formed by reaction with NO could be protonated and undergo heterolytic scission to form a ferric-hydroxy ( $\text{Fe}^{3+}-\text{OH}$ ) heme species and a nitronium ( $\text{NO}_2^+$ ) ion. The nitronium ion could then react with tryptophan by electrophilic aromatic substitution to form the L-4-nitrotryptophan product, with further protonation of the  $\text{Fe}^{3+}-\text{OH}$  heme leading to restoration of a  $\text{H}_2\text{O}$ -coordinated resting state of the P450.

### P450 redox partner systems and other diverse routes to driving P450 catalysis

For the majority of P450 enzymes *in vivo*, redox partner systems are required for monooxygenase function. However, in recent years several different types of redox partner systems have been identified to expand the class I/class II paradigm [8<sup>\*\*</sup>,9]. Fulco's group discovered the first major outlier in *Bacillus megaterium* P450 BM3 (BM3, CYP102A1), a natural fusion of a soluble fatty acid hydroxylase P450 to a soluble CPR [10]. BM3 catalyses arachidonic acid oxidation with a reported  $k_{\text{cat}}$  of  $\sim 280 \text{ s}^{-1}$ ; substantially faster than any mammalian P450 [11]. BM3 naturally forms a dimer, and inter-monomer (CPR<sub>1</sub> to P450<sub>2</sub> and vice versa) electron transfer can support catalysis [12]. Attempts to mimic BM3's structural organization by fusion of its efficient CPR module to heterologous P450s have resulted in functional flavocytochrome enzymes, but these do not yet replicate the catalytic efficiency of BM3, likely due in part to their failure to generate dimers that facilitate domain-domain interactions as efficiently as in BM3, and/or to lower stability of the fusion enzymes [8<sup>\*\*</sup>,13,14].

More recently, a novel class of bacterial P450s fused to a NAD(P)H-dependent flavin-containing and 2Fe-2S cluster-containing reductase related to phthalate dioxygenase reductase (PDOR) was uncovered [15]. Members of this CYP116B family were shown to catalyse oxidation of, for example, thiocarbamate herbicides (CYP116B1) and alkanes (CYP116B5) [16,17]. Fusions of heterologous P450s to the CYP116B-type PDOR domain has proven successful in various instances, presumably in part due to these fusions being monomeric. Examples of reconstitution of catalytic activity in such fusions include a *Rhodococcus jostii* P450 linked to the CYP116B2 PDOR, producing an enzyme capable of N-demethylation of the drugs imipramine and diltiazem; and a fusion of the P450balk enzyme from the marine bacterium *Alcanivorax borkumensis* to the same reductase, generating a catalytically self-sufficient octane hydroxylase (producing 1-octanol) [18,19]. Recent studies also used fusion of a PDOR to an engineered mutant of *Amycolatopsis orientalis* CYP105AS1 to facilitate hydroxylation of the natural product compactin and formation of pravastatin for industrial scale production [20<sup>\*</sup>]. Other natural bacterial P450 fusions to flavodoxin and ferredoxin partners are

known; involved in catabolism of explosives and in sterol demethylation, respectively [21,22]. Such enzymes still require electron transfer from a separate NAD(P)H-dependent reductase, but do simplify this class I-like apparatus to a 2-component system. Fusions between P450s and native or heterologous ferredoxin/flavodoxin proteins have also been generated with a view to improving electron transfer efficiency to the P450, including a recent study in which the cineole oxidising P450cin (CYP176A1) from *Citrobacter braakii* was fused to its native flavodoxin (CinA) using peptides of different lengths. Variants were reconstituted with NADPH/*Escherichia coli* flavodoxin reductase; ultimately identifying a 10 amino acid linker sequence as optimal through studies of the conversion of cineole into its 2- $\beta$ -hydroxy-1,8-cineole product [23].

### Unusual microbial P450s and reactions

While most P450s adhere to the paradigm involving NADPH-dependent electron transfer from redox partners to the P450 heme iron, there are a number of P450 that have distinctive mechanisms. Indeed, some P450s function without requirement for redox partners. P450nor (CYP55A1) from the fungus *Fusarium oxysporum* (and related P450s in other fungi) catalyses the reduction of two molecules of nitric oxide (NO) to form dinitrogen monoxide (N<sub>2</sub>O) in the final step of a respiratory pathway in which nitrate/nitrite are converted into N<sub>2</sub>O [8<sup>\*\*</sup>,24]. NADH is used directly, and the crystal structure of a P450nor mutant in complex with a NADH analogue (NAAD — nicotinic acid pyridine dinucleotide) revealed the binding mode of the nicotinic acid ring moiety above the heme plane, in a position suitable (with reference to the NO-bound form) for reduction of the complex by hydride transfer [25] (Figure 1). In the case of TxtE (CYP1048A1) from *Streptomyces scabiei* (and orthologues from other plant pathogen *Streptomyces* spp.), the normal P450 catalytic cycle progresses as far as the ferric-superoxo intermediate, but thereafter there is binding of NO (produced by a *Streptomyces* NO synthase enzyme from the same gene cluster) and the formation of a reactive ferric peroxynitrite intermediate that catalyses the nitration of the P450 substrate L-tryptophan to produce L-4-nitrotryptophan in the first committed step in the production of the phytotoxin thaxtomin A [26<sup>\*</sup>]. More recent studies on TxtE have analysed the enzyme's ability to nitrate other molecules, and have included exploration of

(Figure 1 Legend Continued) An alternative mechanism might involve homolytic scission of the ferric-peroxynitrite intermediate to produce NO<sub>2</sub> and a reactive ferryl-oxo species. In this case the ferryl-oxo heme would abstract a hydrogen from the tryptophan substrate and enable a reaction with the NO<sub>2</sub>. However, there is no evidence to date for formation of a transient ferryl-oxo species in TxtE [26<sup>\*</sup>]. Shown with purple arrows is the pathway for conversion of NO to N<sub>2</sub>O catalysed by P450nor from *Fusarium oxysporum* in the final step of a fungal denitrification pathway. The reaction does not require additional redox partners, instead deriving its electrons directly from NADH [50]. The reaction is proposed to proceed via a heme iron-NO complex (presented here as a ferrous-NO<sup>+</sup> form, isoelectronic with the ferric-NO form), which is reduced (by NADH) and then protonated (using Asp393 in P450nor) to form a doubly protonated, singlet Fe<sup>3+</sup>-NHOH<sup>\*</sup> species. Further reaction with a second NO radical involves a spin-recoupling reaction in which a N-N bond is formed and a transient Fe<sup>3+</sup>-(N<sub>2</sub>O<sub>2</sub>H<sub>2</sub>) species occurs, which decomposes to release N<sub>2</sub>O, resulting in the restoration of the H<sub>2</sub>O-bound, ferric resting form of the P450 [51,52]. Shown with a yellow arrow is the peroxxygenase reaction catalysed by a number of P450s from the CYP152 family. Hydrogen peroxide is used directly to form the reactive ferric-hydroperoxo species compound 0, which then continues on the canonical P450 catalytic cycle [30<sup>\*</sup>,31,53,54].

the determinants of substrate binding in TxtE [27], as well as producing nitrated derivatives of 4-fluoro-L-tryptophan and 5-fluoro-L-tryptophan using TxtE fused to the BM3 CPR domain or to a CYP116B (PDOR)-type reductase [28].

A particularly interesting group of P450 enzymes (with potential in the biofuels area) are the P450 peroxygenases [29]. These are P450s that have evolved to use hydrogen peroxide ( $H_2O_2$ ) in place of redox partner systems, with  $H_2O_2$  converting the ferric P450 form directly to the reactive compound 0 species (Figure 1). This ‘peroxide shunt’ mechanism is a well known, but typically inefficient, method for driving P450 catalysis, due to competing oxidative damage to heme and protein by the peroxide [30]. The peroxygenases  $BS_\beta$  from *Bacillus subtilis* (CYP152A1) and  $SP_\alpha$  from *Sphingomonas paucimobilis* (CYP152B1) were the first P450 peroxygenases characterized — and catalyse predominantly  $\beta$ -hydroxylation and exclusively  $\alpha$ -hydroxylation of fatty acid substrates, respectively [8,29]. However, more recent studies showed that a novel member of this P450 class (OleT from a *Jeotgalicoccus* sp., classified as CYP152L1) catalyses mainly oxidative decarboxylation of long chain fatty acids to produce  $n-1$  terminal alkenes, while  $BS_\beta$  also produced a proportion of terminal alkene along with its major  $\beta$ -hydroxylated (and minor  $\alpha$ -hydroxylated) products. Evidence for peroxide-dependent 1-pentadecene production from palmitic acid was also produced using *E. coli* cells expressing CYP152 family P450s from other bacteria [30]. The structure of OleT in substrate-free and arachidic acid (C20:0)-bound forms demonstrated how the lipid carboxylate group binds close to the P450 heme and suggested a mechanism for substrate decarboxylation [31]. Other studies demonstrated that use of a heterologous class I redox partner system could also be used to drive OleT to produce a range of different alkenes [32]. OleT and other CYP152 P450s clearly have potential applications in production of alkenes for use as biofuels or in fine chemical applications.

### Engineering of bacterial P450 enzymes for novel oxychemical production

A large number of bacterial P450s have been expressed and characterized, and these enzymes have an enormous repertoire of substrates including fatty acids, steroids, polyketides and terpenes [33,34]. Recent studies have sought to expand this repertoire using protein engineering of P450s. This has typically been achieved using knowledge from P450 structure to guide rational redesign of the P450 active site, or by random mutagenesis or directed evolution approaches, or by a combination of these approaches. P450 BM3 has proven a popular model system, based on its catalytic self-sufficiency (with P450 and CPR units fused) and its high catalytic rate [1,11]. Recent examples include use of rational mutagenesis to facilitate the binding of the gastric proton pump inhibitor

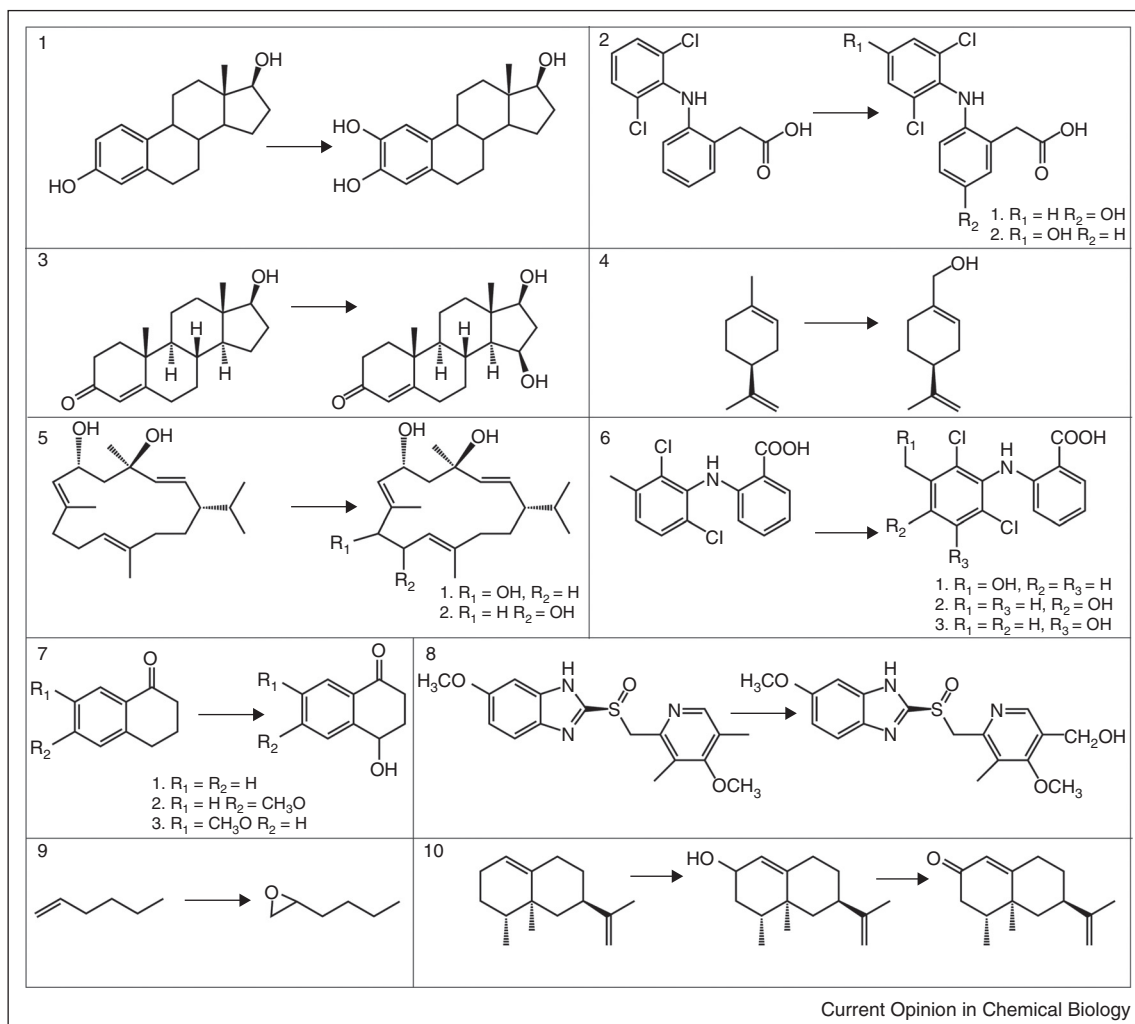
(PPI) drug omeprazole. The combined F87V and A82F mutations resulted in increased active site space and in a conformational change in the P450 to enable oxidation of omeprazole at the 5-methyl position (Figure 4a), mimicking the activity of the major human omeprazole-metabolising P450 CYP2C19 [35]. Subsequent work showed that other PPI drugs (e.g. lansoprazole) were also transformed to human P450-type metabolites by the same mutants [36]. The efficient production of human drug metabolites (for drug safety testing) is widely researched, and other examples include BM3 mutants that can make the human-type 4'-hydroxylated and 5-hydroxylated metabolites of the non-steroidal anti-inflammatory drug diclofenac [37]. BM3 mutants (generated using both site-directed and random mutagenesis) have also been used to produce metabolites of 17- $\beta$ -estradiol; including 2-hydroxy- $\beta$ -estradiol, a product formed by human CYP1 family P450s [38]. Further examples of the exploitation of BM3 mutants in oxychemical production are given in Figure 2.

Other recent examples of bacterial P450s engineered for synthetic biology applications include the fusion of several P450s from *Rhodococcus jostii* RHA1 with a PDOR module, leading to identification of catalytically self-sufficient enzymes generating drug metabolites, including CYP257A1 that produced *N*-demethylated products of the vasodilator diltiazem and the antidepressant imipramine [39]. In studies towards production of 1-butanol from *n*-butane, Nebel *et al.* used the *Polaromonas* sp. JS666 CYP153A for butane hydroxylation, and improved the 1-butanol product yield by introducing a G254A mutation in the P450. Further enhancement of product yield was achieved by fusing the mutant P450 to the P450 BM3 reductase to create a catalytically self-sufficient enzyme. Subsequent improvements were achieved by performing reactions at high pressure (15 bar) in efforts to overcome the limited aqueous solubility of *n*-butane [40]. P450sca-2 from *Streptomyces carbophilus* is an industrially important P450 that stereo-selectively hydroxylates compactin to form pravastatin. A functional triple fusion enzyme (P450sca-2/Pdx/Pdr) was created using putidaredoxin/putidaredoxin reductase partners. This construct was used to produce more active mutants by site-directed saturation mutagenesis focused on the substrate access site and substrate binding pocket regions, as well as on the proposed P450-Pdx interaction site, leading to substantial increases in product formation in whole cell transformations [41].

### Synthetic biology applications of P450 enzymes

The P450s have diverged into a vast number of biological catalysts that bind and oxidise substrates of widely varying size and chemical composition. As described above, P450 substrate selectivity can be further broadened by

Figure 2

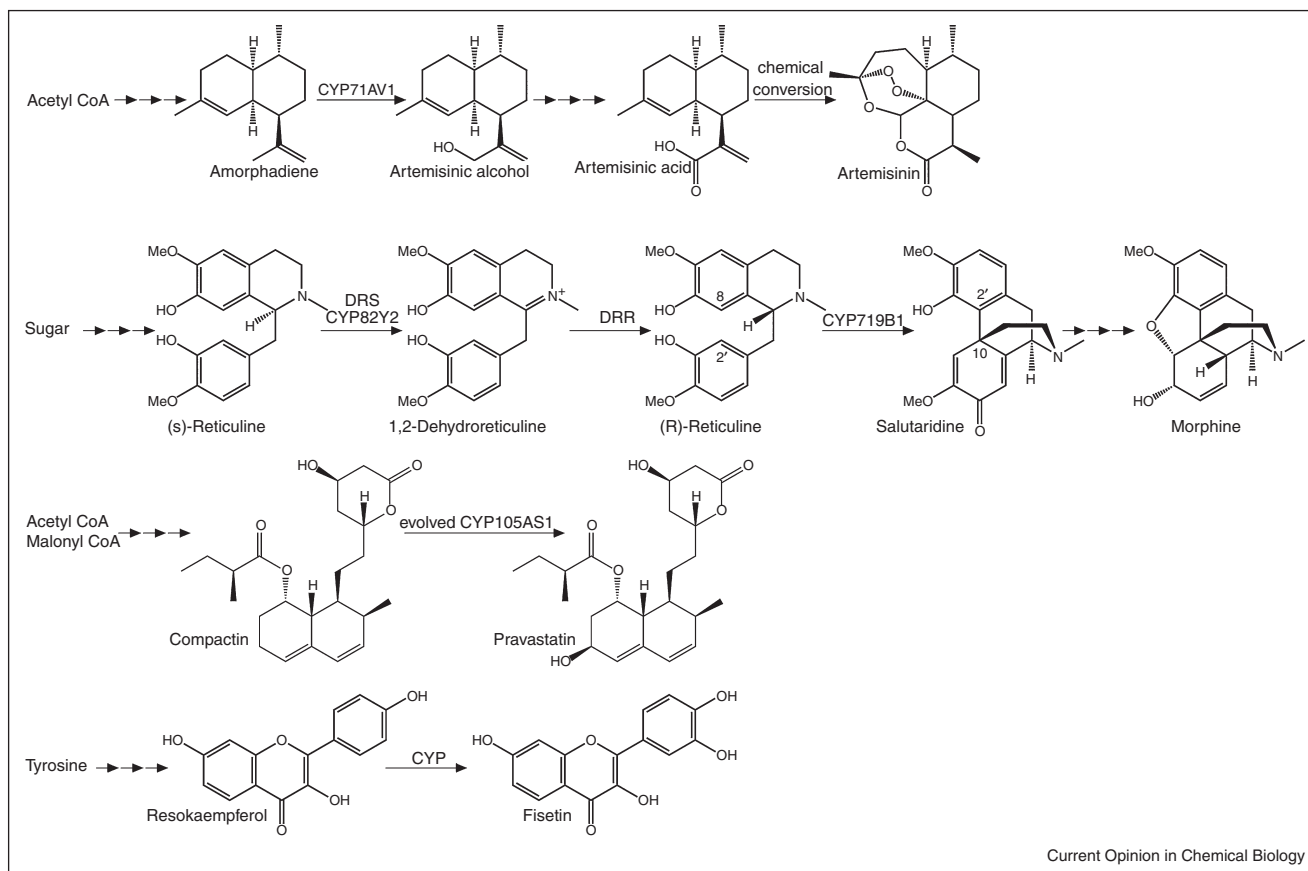


Diverse catalytic activities of mutant and wild-type forms of P450 BM3. P450 BM3 is a natural, soluble fusion of a fatty acid hydroxylase P450 to a cytochrome P450 reductase, and has the highest reported monooxygenase activity among the P450s [11]. Variants of P450 BM3 with novel substrate selectivity have been produced using both rational (structure-guided) and random/directed evolution mutagenesis procedures. The figure shows several examples of P450 BM3 mutants with novel substrate selectivity and applications in production of novel oxychemicals. **(1)** Conversion of 17-β-estradiol to 2-hydroxy-17β-estradiol by BM3 mutants made using both site-directed and random mutagenesis methods. The 2-hydroxy-β-estradiol product is also a metabolite made by human CYP1A1, CYP1A2 and CYP1B1 [38]. **(2)** Conversion of diclofenac to 4' and 5-hydroxy diclofenac products from members of a library of BM3 variants carrying multiple mutations. These are the same metabolites produced by human CYP2C9 and CYP3A4 [37]. **(3)** Conversion of testosterone to 15-β-hydroxytestosterone using other mutants from the same library as in (2) [37]. **(4)** Conversion of the terpene (-)-limonene to (-)-perillyl alcohol, a potential anti-tumour drug, using the A264V/A238V/L437F BM3 triple mutant [55]. **(5)** Production of 9-hydroxy-β-cembrene or 10-hydroxy-β-cembrene from β-cembrene using F78A/I263L and L75A/V78A/F87G mutants, respectively. The tobacco diterpene β-cembrene has antibacterial and antifungal properties [56]. **(6)** Conversion of the non-steroidal anti-inflammatory drug macrofenamic acid to 3-OH, 4-OH and 5-OH human-type metabolites using isolates from a BM3 mutant library [57]. **(7)** Hydroxylation of 1-tetralone derivatives using BM3 mutants from a directed evolution programme [58]. **(8)** Conversion of the gastric PPI drugs omeprazole and esomeprazole to their 5-hydroxylated, human CYP2C19-type metabolites by the BM3 F87V/A82F double mutant [35,36]. **(9)** Transformation of 1-hexene to 1,2-epoxyhexane by BM3 mutants produced by saturation mutagenesis of the active site and by recombination of beneficial mutations [59]. **(10)** Conversion of the sesquiterpene (+)-valencene to (+)-nootkatol and on to (+)-nootkatone using mutants of the camphor hydroxylase P450cam (CYP101A1, for oxidation of (+)-trans-nootkatol) and wild-type P450 BM3 for production of cis-(+)-nootkatol and trans-(+)-nootkatol, and (+)-nootkatone [60].

protein engineering. Several P450s and engineered variants have now been used in synthetic biology approaches for valuable chemical production by fermentation in microbial cells. Figure 3 shows recent important examples.

Artemisinin is a sesquiterpene lactone that is a standard antimalarial treatment worldwide. CYP71AV1 performs three successive oxidation reactions to convert amorpha-diene to artemisinic acid as a precursor to artemisinin in a

Figure 3

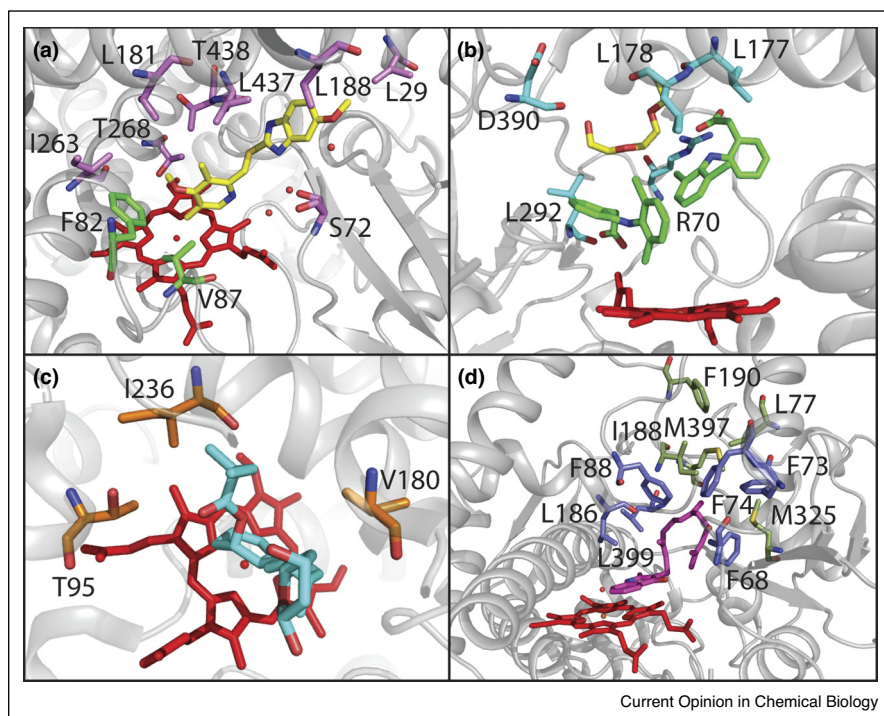


Biotechnological applications of cytochrome P450 enzymes. There are numerous examples of the exploitation of P450 enzymes in engineered pathways for the production of important biomolecules in microorganisms. **(a)** The antimalarial artemisinin, a sesquiterpene lactone, is generated chemically from artemisinic acid, which in turn is synthesised from acetyl CoA in an engineered pathway in *Saccharomyces cerevisiae*. The P450 CYP71AV1 from *Artemisia annua* is a multifunctional oxidase enzyme that catalyses key steps in hydroxylating amorphadiene to artemesinic alcohol, and then further to the aldehyde and on to artemisinic acid [42\*\*]. **(b)** Morphine has been synthesised successfully from sugar feedstock in an engineered pathway in *S. cerevisiae* [44\*\*]. The pathway exploits the reticuline epimerase from opium poppy, which is a multi-domain protein consisting of the P450 CYP82Y2 fused to an aldo-keto reductase (AKR). CYP82Y2 (1,2-dehydroreticuline synthase, DRS) catalyses the conversion of (*S*)-reticuline to 1,2-dehydroreticuline; and the AKR module (1,2-dehydroreticuline reductase, DRR) then converts the product to (*R*)-reticuline [43\*\*]. A second P450, CYP719B1, then converts (*R*)-reticuline to salutaridine [44\*\*,45]. This reaction involves reorientation and twisting of the (*R*)-reticuline, and an oxidative C–C bond coupling step catalysed by CYP719B1. Relevant carbon atoms are numbered according to Gesell *et al.* [45]. **(c)** The cholesterol-lowering drug pravastatin was synthesised in *Penicillium chrysogenum* by introduction of the genes encoding the production of the natural product compactin. The conversion of compactin to pravastatin was achieved by also introducing an engineered form of the *Amycolatopsis orientalis* P450 CYP105AS1. This form of the enzyme (P450Prava, containing five mutations) catalyses the stereo-selective hydroxylation of compactin to form the active pravastatin rather than its inactive epimer *epi*-pravastatin [20\*]. **(d)** A novel biosynthetic pathway for production of the plant flavonoid fisetin was engineered into *E. coli*. Fisetin has potential anti-cancer, anti-viral and other health benefits. Fisetin was synthesised from tyrosine in a nine-step process, the last step involving an unassigned P450 from *Petunia hybrida* that catalyses the conversion by hydroxylation of the flavonoid resokaempferol to fisetin [5].

*Saccharomyces cerevisiae* fermentation. Paddon *et al.* then used a singlet oxygen source to produce artemisinin from artemisinic acid (Figure 3a) [42\*\*]. *S. cerevisiae* fermentation has also been used to produce the analgesic morphine. The opium poppy reticuline epimerase is a fusion of a P450 (CYP82Y2) to an aldo-keto reductase (AKR). The CYP82Y2 transforms (*S*)-reticuline to 1,2-dehydroreticuline, and the AKR converts this intermediate to (*R*)-reticuline. Another P450 (CYP719B1)

then converts (*R*)-reticuline to salutaridine in a C–C phenol coupling reaction (Figure 3b) [43,44\*\*,45]. In a fermentation process using *Penicillium chrysogenum*, genes encoding compactin production were introduced into the strain and an esterase gene deleted to prevent product degradation. The introduction of an engineered *Amycolatopsis orientalis* P450 (P450Prava) facilitated stereoselective hydroxylation of compactin to form the cholesterol-lowering pravastatin (Figure 3c).

Figure 4



Structures of cytochrome P450 enzymes in complex with biotechnologically important substrates. **(a)** The active site of the omeprazole-bound F87V/A82F double mutant of P450 BM3 (PDB 4KEY). The omeprazole substrate is shown in yellow sticks, with the two mutated residues (A82F and F87V) labelled with green sticks. Other active site residues important in substrate binding are shown in purple sticks with labels, and the heme group is in red sticks. Three water molecules that interact with the substrate are seen as red spheres and the polypeptide backbone is shown as grey ribbon [35\*]. **(b)** Diclofenac-bound CYP105D7 from *Streptomyces avermitilis* (PDB 4UBS). Two molecules of diclofenac are bound and shown in green sticks. Five triethylene glycol molecules are also found bound to the protein, with one in the active site (shown in yellow sticks). Other residues important to substrate binding are shown in light blue sticks and are labelled. The heme group is shown as red sticks with the polypeptide backbone as grey ribbon [61]. **(c)** The compactin-bound mutant form of CYP105AS1 (P450Prava) from *Amycolatopsis orientalis* (PDB 4OQR). The bound compactin is shown in light blue sticks, with the mutated active site residues shown as orange sticks with labels (I95T, A180V and L236I). The heme is shown in red sticks and the polypeptide backbone as grey ribbon [20\*]. **(d)** The quinolone antibiotic aurachin RE-bound form of P450 RauA from *Rhodococcus erythropolis* (PDB 3WEC). The heme is shown in red sticks with the aurachin RE substrate shown as magenta sticks. Key residues for substrate binding are shown as blue sticks and are labelled. Other residues (shown in green and labelled) have hydrophobic side chains that close off the substrate-binding channel from solvent, and the polypeptide backbone is shown as grey ribbon [62].

X-ray crystallographic data demonstrated clearly how engineering enabled the productive binding of compactin to P450Prava (Figure 4c) [20\*]. A P450-dependent step was also important for the final hydroxylation step in production of the flavonol fisetin, which has reported anti-cancer properties. The unassigned P450 hydroxylates resokaempferol to form fisetin in an *E. coli* fermentation process (Figure 3d) [46].

Another successful example of the use of a P450 enzyme in the production of valuable compounds is the 2-step fermentation of tyrosine to caffeic acid using a tyrosine ammonia lyase to form *p*-coumaric acid. The *Rhodospseudomonas palustris* CYP199A2 proved more efficient for the subsequent conversion of *p*-coumaric acid to caffeic acid than did a bacterial flavoprotein 4-coumarate 3-hydroxylase. Caffeic acid exhibits anti-cancer

and antioxidant activities [47]. In a novel approach, Wlodarczyk *et al.* showed that two membrane-bound eukaryotic P450s (CYP79A1 and CYP71E1 from the synthetic pathway for the cyanogenic glucoside dhurrin in the grass species *Sorghum bicolor*) can be integrated into the thylakoid membrane of *Synechocystis* sp. PCC 6803 and that light-driven photosystem I and ferredoxin can replace a typical P450 redox partner system to produce substantial amounts of dhurrin in the cyanobacterium [48\*\*].

## Summary

Cytochrome P450 enzymes across Nature have an extraordinarily diverse substrate range. Mutagenesis strategies continue to expand this repertoire of substrates, particularly in the case of soluble microbial P450s for which structural data help to guide protein engineering.

In an era where synthetic biology is striving to replace synthetic chemistry for production of high-value chemicals, P450s have a crucial role. P450s can catalyse the specific addition of oxygen atoms at positions on chemical scaffolds, while this can be very challenging by traditional chemical methods. Many examples in this review highlight the crucial roles played by P450s in producing important molecules through microbial fermentation processes, and point to further applications for P450s in the synthesis of valuable chemicals such as antibiotics, drug metabolites, steroids and terpenes.

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- of special interest
- of outstanding interest

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