Trends in Biochemical Sciences

CellPress REVIEWS

Review

Natural Compounds as Pharmaceuticals: The Key Role of Cytochromes P450 Reactivity

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The design of drugs from natural products is a re-emerging area due to the need for bioactive compounds. The exploitation of natural products and their derivatives obtained by biocatalysis is in line with the higher attention given today to new sustainable technologies that better preserve the environment (green chemistry). The research field of cytochromes P450 (CYPs) is continuously providing new enzymes and mutants that produce metabolites suitable for late-stage functionalization for new potential drugs. This review provides an overview of the exploitation of CYPs as biocatalysts in drug synthesis. Additionally, recent progress in protein and metabolic engineering is provided to show how these enzymes offer a toolbox that can be combined with other biocatalytic or chemical processes to build new platforms for the green production of new drugs.

Highlights

Cytochromes P450 (CYPs) are enzymes physiologically involved in the biosynthesis of thousands of bioactive natural compounds in bacteria, fungi, plants, and mammals.

CYPs act on different molecular scaffolds and can be crucial for late-stage functionalization of bioactive compounds to produce new pharmaceuticals.

Some CYPs are already used at industrial level for the production of nature-inspired pharmaceuticals, for example, for the production of pravastatin, a cholesterol-lowering drug, by bacterial P450sca-2.

The use of CYPs in the synthesis of pharmaceuticals is expanding thanks to the progresses in protein, metabolic, and substrate engineering.

Natural Compounds: From Leads to New Drugs

The beneficial effects of natural compounds have been known for a long time and, today, approximately one-third of the drugs approved over the last 20 years are either **natural compounds** (see Glossary) or their derivatives [1]. For example, the plant-derived compound artemisinin was already used in traditional Chinese medicine for the treatment of malaria, but only in 2015 was the Nobel Prize in Physiology or Medicine co-awarded to Youyou Tu for the discovery of this antimalaria agent.

Nature provides an extraordinary number of **bioactive compounds**. The metabolic pathways leading to their synthesis often involve different enzymes and, as such, are difficult to be reproduced with standard chemical methods [2]. However, enzymes can act on complex substrates with high regio- and stereo-selectivity. Such properties have been exploited in industrial processes for the synthesis of bioactive compounds; consequently, the application of **biocatalysis** in the pharmaceutical field is expanding thanks to unprecedented scientific progress in this area [3]. Furthermore, enzymes have been successfully used to modify the **molecular scaffolds** provided by nature that can be used as **drug leads** to generate derivatives with improved pharmaceutical properties and/or lower toxicity. Thus, enzymes can be used either for the biosynthesis of natural compounds or to produce derivatives through the so-called **lead diversification (LD)**. This approach has been already successful in the case of the different generations of antibiotics that were derived from 'old' molecular scaffold used as drug leads [4].

Cytochromes P450 (CYPs) are optimal enzyme candidates for the biocatalytic synthesis of pharmaceuticals since they are involved in many biosynthetic pathways that produce natural and bioactive compounds in a wide range of organisms (described later). Nowadays, thanks to the advances in genomics, proteomics, and metabolomics, novel P450 enzymes, reactions, and metabolites are continuously discovered, making possible the exploitation of their wide natural variability in the area of pharmaceutical research. Moreover, protein and metabolic engineering enable the creation of new enzyme variants, allowing for the expansion of the natural chemical space with novel or improved activity and introduction of entire biosynthetic pathways in

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host organisms for the production of a molecule of interest. Here, examples of how CYPs are already exploited at industrial level and recent progress for their application are provided. Additionally, we describe how some classes of biologically active compounds can be considered as potential drugs or drug leads for functionalization by CYPs.

Why Are CYPs Optimal Candidates for Biocatalysis for Drug Production?

CYPs are a superfamily of heme-containing enzymes, mainly acting as monooxygenases, that possess common structural features (Box 1) and are classified according to their sequence

Box 1. Cytochromes P450 (CYPs) Structural Features [105]

CYPs share a common global fold and topology leading to a globular triangular shape, characterized by β-sheets (usually four, numbered 1–4) and α-helices (about 12, designated by letters A–L). The structural core consists of a four-helix bundle (composed of three parallel helices named D, L, and I and an antiparallel helix E), helices J and K, two sets of β-sheets, and a coil called the 'meander' that is a loop of around 14 amino acids amino-terminal to the loop containing the cysteine acting as heme ligand. The latter establishes multiple interactions with a conserved Glu-X-X-Arg sequence that is located on helix K on the heme proximal side (Figure I).

All P450s share a unique fingerprint sequence Phe-X-X-Gly-X-Arg-X-Cys-X-Gly in the heme-binding loop situated just before helix L. This motif constitutes the heme binding decapeptide loop carrying the cysteine fifth ligand of the heme iron. The heme cofactor is buried in the protein core flanked by helices L and I. Finally, another well-conserved motif is located on helix I with a consensus sequence Ala/Gly-Gly-X-Asp/Glu-Thr-Thr/Ser (Figure I). Such a motif forms the proton transfer groove important for catalysis and the terminal threonine is in the oxygen-binding pocket of the enzyme.

The so-called substrate-recognition sites (SRSs) define the substrate access channel and the active site of CYPs, as they are responsible for substrate binding and selectivity. Significant differences are therefore present in each individual enzyme, for example, in the position and conformation of the B-C loop that are important for their catalytic selectivity [105].

It has also to be considered that different open and closed conformations have been reported showing that CYPs undergo conformational changes upon ligand binding.

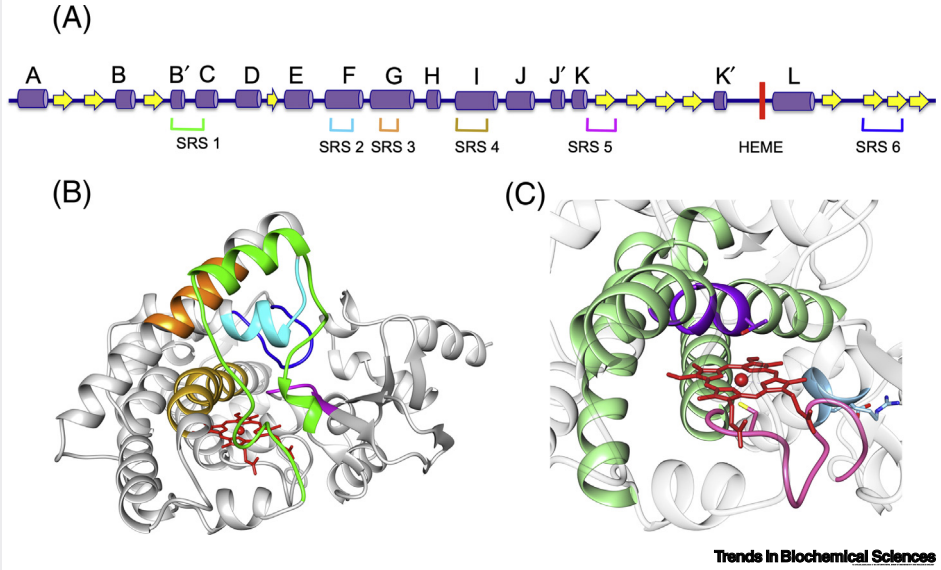

Figure I. The Structure of a Typical Cytochrome P450 (CYP). (A) Secondary structure elements typical of the P450 fold with the substrate recognition sites (SRS) shown. (B) The crystal structure of the heme domain of CYP116B5 (PDB ID: 6RO8) [52] with the SRS colored according to the scheme of panel (A). (C) The structurally conserved elements of CYPs. The four-helix bundle is shown in light green, the meander carrying the cysteine heme ligand in pink, the Glu-X-X-Arg motif in light blue, and the Ala/Gly-Gly-X-Asp/Glu-Thr-Thr/Ser consensus sequence is shown in violet. The heme cofactor is shown in red.

Glossary

Bioactive compound: a molecule that influences cellular or physiological activities in animals and humans. For example, health-promoting chemicals found in plants and in small quantities in certain food are considered bioactive compounds.
Biocatalysis: the use of natural catalysts to speed up chemical reactions. The natural catalysts are enzymes that can be isolated or used within a living cell. They can be used as single catalysts or in combination with other chemical or biochemical catalysts.
CYPome: cytochrome P450 complement, the genomic inventory of cytochrome P450 genes within a given organism, due to the possible coexistence of more P450-coding genes and pseudogenes.
Directed evolution: a protein engineering approach that uses random mutagenesis to mimic the process of Darwinian natural evolution at molecular level. By these means enzymes can be designed to have specific and new desired properties.
Drug lead: chemical entity that could potentially be developed into a novel drug by optimizing its beneficial effects and minimizing its side effects. This process requires chemistry optimization through chemical or enzymatic methods.
Green chemistry: the design of chemical products and processes that reduce or eliminate the generation of hazardous substances.
Late-stage functionalization: the introduction of small chemical groups in the last steps of the synthetic process. The C–H bonds activation of drug leads is one example of late-stage functionalization.
Lead diversification (LD): the functionalization of drug leads using chemical or biochemical catalysts to generate novel derivatives and potency or expand the biological activity.
Microsomes: vesicles (20–200 nm in diameter) prepared by fragmentation of membranes (mainly endoplasmic reticulum) by homogenization of hepatocytes.
Molecular scaffold: the core structure of bioactive compounds that can be used as a building block for new molecules.
Natural compound: molecules that are produced by living organisms and are found in nature.

similarities or on the basis of the redox partner required for catalysis [5,6]. They are widely distributed in nature, with around 350 000 sequences available from all kingdoms of life and in almost all living organisms, including viruses (Figure 1) [7]. The first crystal structure of viral CYPs was reported in 2019 [8,9]. One interesting point is that usually the study of the **CYPome** shows the presence of more than one CYP gene and pseudogene within the same organism and the resulting enzymes play different and mostly nonredundant metabolic roles.

Since the 1950s, it has been shown that more than 800 xenobiotics are substrates of liver oxidative enzymes, identified as CYPs in the 1960s [10,11]. Nowadays it is known that the evolutionary history of CYPs began with prokaryotes, where their role ranges from the degradation of different carbon sources (for example fatty acids, polyaromatics) to the synthesis of antibiotics and secondary metabolites. In eukaryotes, CYPs act on thousands of substrates, often on natural compounds with interesting properties for therapeutical applications (Figure 2). Fungal and plant CYPs are involved in the biosynthesis of primary and secondary metabolites with antibiotic, immunosuppressant, and mycotoxic activities and phytohormones [12–14]. In animals, they are involved in the biosynthesis of vitamins and steroid hormones. The 'natural' role of CYPs in the synthesis of bioactive compounds already offers a solid basis for their exploitation in biocatalysis for the production of pharmaceuticals.

Wide Range of Reactions and Substrates

The most common reaction carried out by CYPs is the hydroxylation of C–H bonds; the activation of unreactive C–H bonds is of particular interest since it is crucial for the biotransformation of compounds, especially when they lack functional groups useful for conjugation. Moreover, the C–H bond activation through hydroxylation is considered an important process in the so-called **late-stage functionalization** that is often a challenge in organic chemistry [15,16]. Hydroxylation can provide improved activity, selectivity, and solubility that can result in an increased and appropriate renal clearance. Indeed, hydroxyl groups are versatile functional groups widely used for the synthesis of drug candidates (Figure 3A, Key Figure) [16]. For example, late stage functionalization by liver **microsomes** carrying different CYPs in different mammalian species has already proven to be very useful for drug discovery, showing that the novel hydroxyl compounds can also minimize drug–drug interactions [17,18]. Indeed, this technology is already used by pharmaceutical companies for the synthesis of the phosphodiesterase 2A Inhibitor PF-06815189 that was obtained through late-stage functionalization by CYPs of monkey liver microsomes [17].

Many other P450 reactions have been reported, including C=C double bond epoxidation, decarboxylation, deaminations, desulfurations, peroxidations, N-, S-, and O-dealkylations, and sulfoxidations [19–21]. Interestingly, different oxidation reactions on the same substrate can be achieved by variants of the same enzyme. For example, variants of the bacterial CYP BM3 were used for the functionalization of quinoline scaffold, common in many natural compounds, and a variety of metabolites were produced through different reactions (Figure 3B) [22]. Unusual reactions carried out with new chemistry have been recently reviewed and they are essential to expand the chemical space of natural compounds [23,24]. Another important feature of CYPs is that complex reactions are catalyzed on a wide range of substrates [25]. Thanks to the development of high-throughput screening methods that allow the testing of libraries of candidate substrates, it is possible nowadays to contemporary test thousands of compounds in the so-called **substrate profiling** approach.

Protein Engineering Can Be Applied

CYPs share a similar and conserved molecular scaffold with defined regions [the so-called substrate recognition sites (SRS)] supporting their variability in the substrates recognized (Box 1).

Phytochemicals: bioactive compounds produced by plants.
Rational design: a protein engineering approach where mutants are created by site-directed mutagenesis on the basis of the prediction of how protein structure will affect protein function.
Site saturation mutagenesis: a method of protein engineering that allows study of the role of a specific amino acid by substituting it with all the other possible 19 residues.
Substrate profiling: the identification of the substrates (physiological and nonphysiological) that can be converted into one or more products by an enzyme though biochemical assays that can be in high-throughput format.
Substrate engineering: the development of substrate analogs carrying specific groups anchoring the molecule to the enzyme in a specific orientation, aimed at improving the substrate acceptance and reaction selectivity.

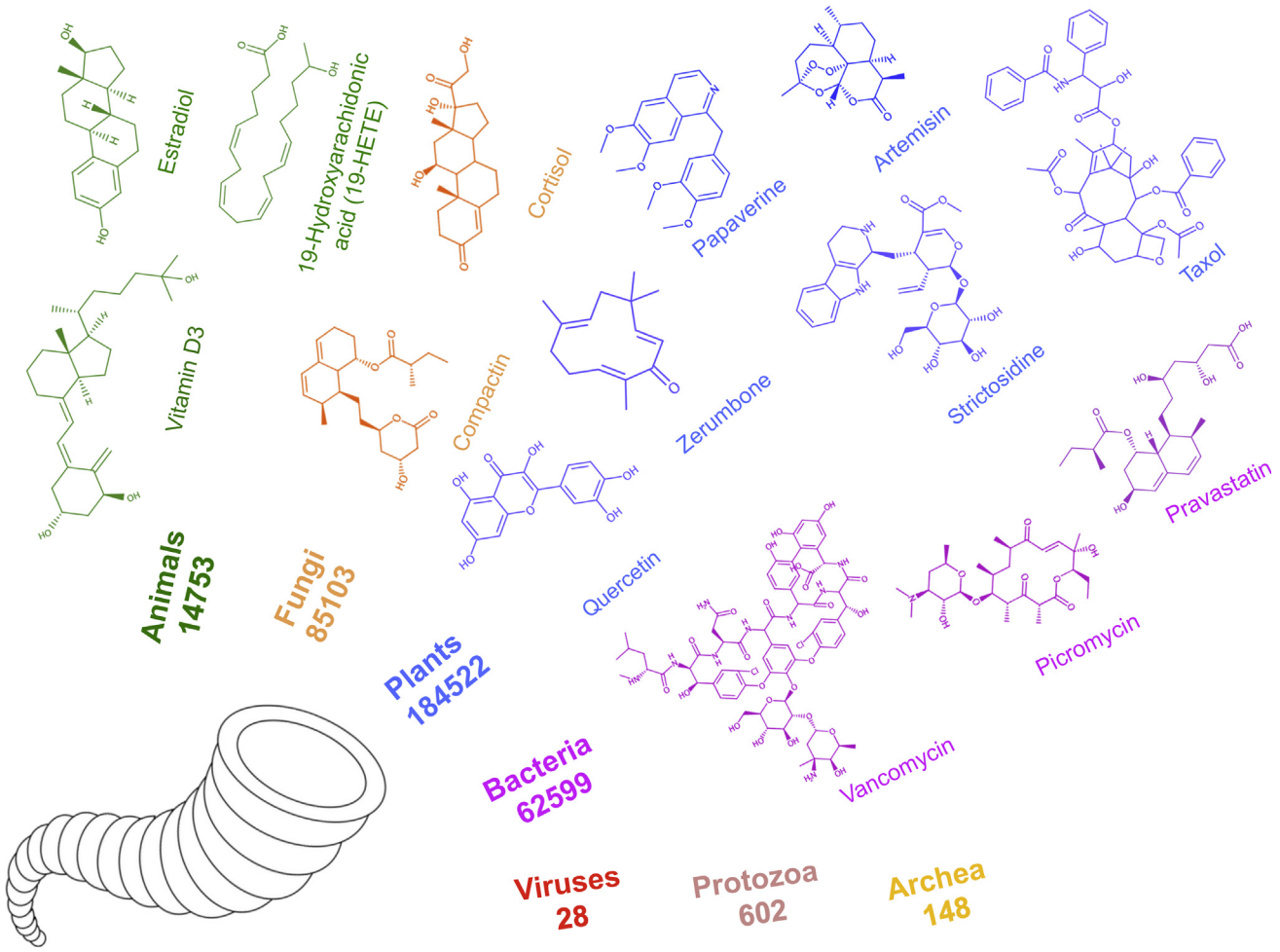

Figure 1. The Cornucopia of Compounds and Cytochromes P450 (CYPs) Offered by Nature. The figure shows the distribution of CYPs sequences in living organisms [10]; the number corresponds to the genes sequenced for each domain of life and some of the bioactive natural compounds requiring one or more CYPs are shown in colors according to the producing organism.

Thus, protein engineering has already been extensively applied to target the most variable regions with the aim of changing the substrate selectivity, as well as regio- and stereo-selectivity [26,27]. Both **rational design** and **directed evolution** approaches allowed the generation of mutants with improved features for biocatalytic applications in the pharmaceutical field (reviewed in [28–31]. Since the late 1990s, Frances Arnold has applied directed evolution to a range of CYPs for different purposes [32]. Since then, many other applications of this approach have been found and the 2018 Nobel Prize for Chemistry was awarded to Frances Arnold [33].

Protein engineering has also been applied to improve the solvent tolerability [34,35] and many thermostable P450 enzymes are now available from thermophile bacteria or mesophilic enzymes that have been engineered [36]. For example, through ancestor sequence reconstruction, it was possible to obtain the CYP3 ancestor enzyme with an improved thermostability and solvent tolerance compared with CYP3A4, while retaining the same activity and substrate specificity [37]. Thus, the P450 molecular scaffold supports the possibility of broadening the experimental conditions for their application in industrial biocatalytic processes.

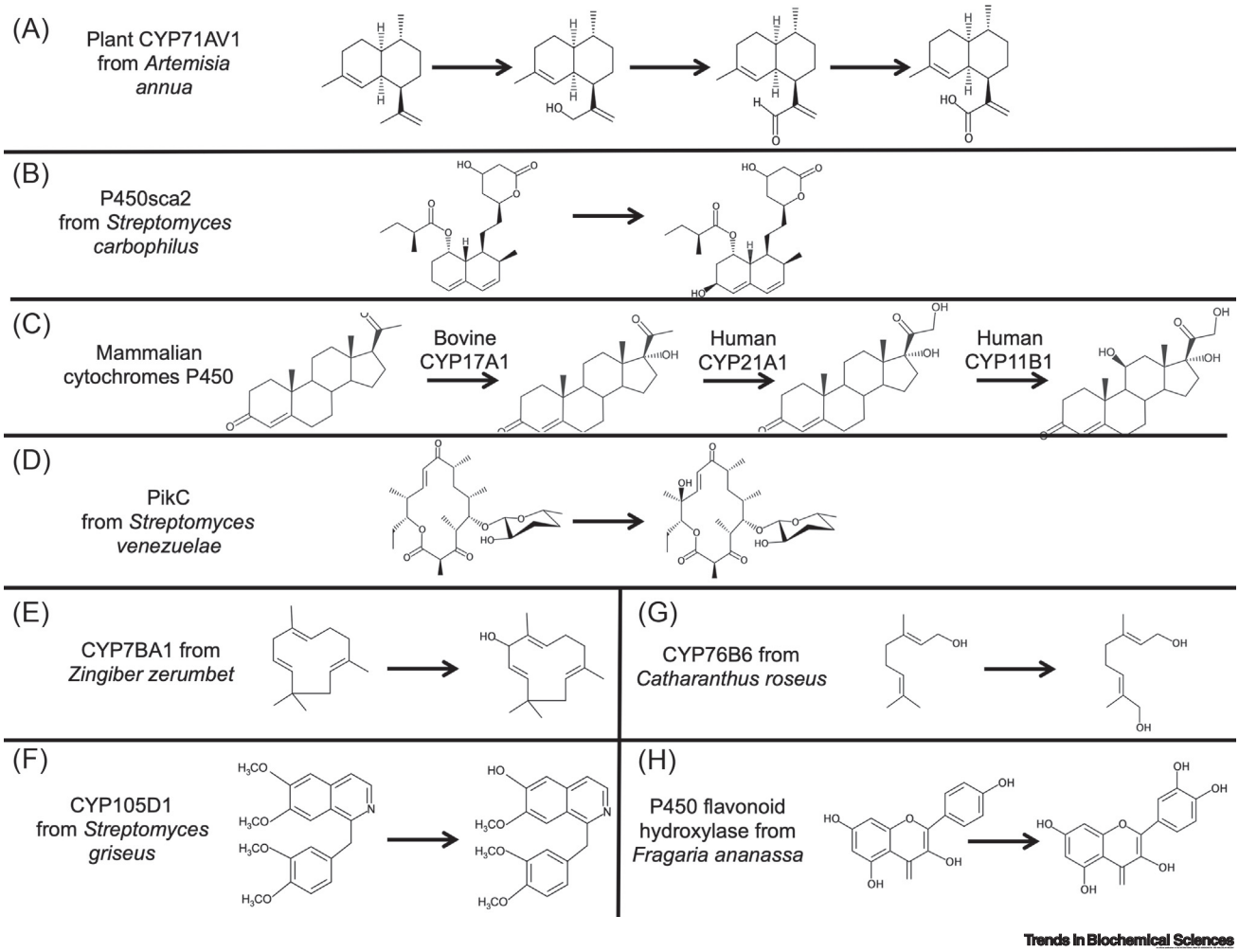

Figure 2. Cytochromes P450 (CYPs) Reactions on Natural Compounds of Pharmaceutical Interest. (A) The three-step conversion of amorphadiene into artemisic acid, a precursor of the antimalarial drug artemisinin, by CYP71AV1 from *Artemisia annua* [64,65]. (B) Biotransformation of compactin into pravastatin catalyzed by CYP105A3 or P450 sca-2 from *Streptomyces carbophilus* [107]. (C) Conversion of progesterone into cortisol by mammalian CYPs [71,72]. (D) Last step of picromycin biosynthesis catalyzed by CYP PikC [87]. (E) Plant CYP7BA1 from *Zingiber zerumbet* converts α-humulene into 8-hydroxy-α-humulene, which is the precursor of zerumbone [99]. (F) Papaverine is converted into 6-O-demethylpapaverine by CYP105D1 from *Streptomyces griseus* [97]. (G) Hydroxylation of geraniol into the 8-hydroxy metabolite by CYP76B6 from *Catharanthus roseus* [93]. (H) Conversion of kaempferol into quercetin, catalyzed by plant P450 flavonoid hydroxylase [101].

The Emerging Role of the P450 Redox Partner for Biocatalysis: A Limitation or an Opportunity?

Traditional Redox Partners

The catalytic mechanism of CYPs requires two electrons that are donated from reduced nicotinamide adenine dinucleotides (NADPH or NADH) to the heme iron via one or more redox partner protein(s). Such protein(s) are required since they can accept contemporarily the two electrons provided from NAD(P)H and deliver them sequentially, one by one, to the heme iron. The heme group cannot accept two electrons contemporarily and therefore cannot interact directly with NAD(P)H. The reduction partner can contain FAD, FMN, or iron-sulfur (FeS) centers bound to different domains of one protein or to separate proteins [5]. Different engineering approaches have been used to overcome problems related to the need of different proteins (i.e., reductase plus P450) and the costly NADPH. For example, in some cases, nonphysiological but highly efficient

Key Figure

The Important Role of Cytochromes P450 (CYPs) in Late-Stage Functionalization of Natural Compounds

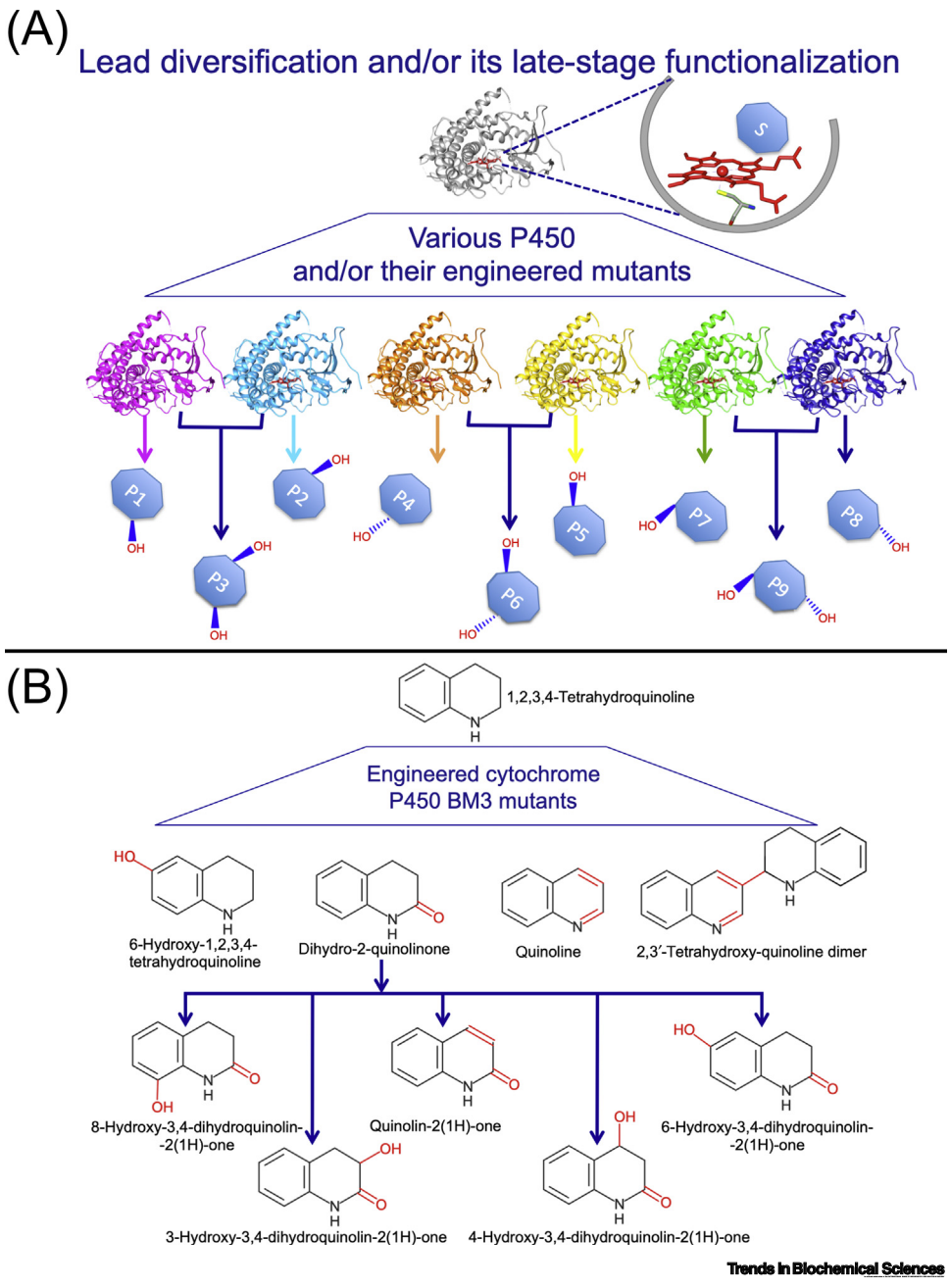

Figure 3. (A) CYPs activate C–H bonds mainly through the insertion of one or more hydroxyl moieties. This catalytic step is crucial in the late functionalization of molecules, since it can increase excretion, reduce toxicity, and sometimes avoids drug–drug interactions. In order to achieve functionalization in different positions, wild type CYPs (grey color) can be engineered,

(Figure legend continued at the bottom of the next page.)

reduction partners have been used for late-stage functionalization of natural compounds [38–40]; hybrid systems were developed to sustain the catalysis of CYP167A1 (EpoK) from *Sorangium cellulosum* for late-stage functionalization of epothilone, a compound with anticancer activity [40]. Also, fusions of reductases with CYPs at the genic level can produce self-sufficient enzymes, often retaining the same catalytic efficiency as the separate system [41–44].

When structural data about the interaction between CYPs and their reduction partners became available [45–48], a new picture emerged showing that the role of the reductase is not only limited to electron supply. On one hand, the crystal structure of bacterial P450cam in complex with its reduction partner showed that the latter affects the conformational state of the enzyme and catalysis. On the other hand, functional data show that alternative reduction partners can be used with the same P450 enzyme, resulting in different reactions and products formed from the same substrate [38,49]. Taken together, these experimental evidences indicate that the reduction partner has a structural effect on the P450 enzyme that, in turn, can alter the binding mode of substrates, resulting in different reaction products, which is the goal of late-stage functionalization of bioactive compounds.

Alternatives to Traditional Redox Partners

In addition, it has been demonstrated that catalysis can also be supported by alternative electron donors (Figure 4). For example, some P450 enzymes can use hydrogen peroxide as an electron source to drive catalysis in the so-called peroxide shunt [50]. The isolated P450 domain of some self-sufficient CYPs carrying a reduction domain fused to the P450 module has also been shown to use the peroxide shunt for the production of drug metabolites [51,52].

The use of light as a natural electron supplier of reducing equivalents to drive CYP catalysis has extensively been reviewed [53–56]. Its energy can be collected by a light-harvesting unit, named photosensitizer, that can produce hydrogen peroxide used by the CYP for catalysis through the peroxide shunt. Alternatively, the photosensitizer can be covalently attached to the P450 enzyme [57] or it can transfer the electrons to the P450 enzyme through a reductase module [58]. This is the case of photosystem I, which was used as a photosensitizer efficiently coupled to P450 catalysis for the light-driven production of bioactive natural compounds [59]. The electrons derived from photosystem I can be directed to the P450 enzyme via a ferredoxin (FdX) protein and catalysis can proceed with the canonical P450 catalytic cycle.

Careful engineering both at the electrode surface and on the P450 itself can enable the possibility of using electrode surfaces to supply electrons to the P450 catalytic unit. P450 enzymes can be immobilized through different methodologies, ranging from inclusion in polymeric films to covalent and oriented immobilization, for example, on gold surfaces. Different bacterial and human CYPs were demonstrated to be catalytically active also in the processing of pharmaceuticals [60–62].

Protein, Metabolic, and Substrate Engineering Involving CYPs for Drug Production

Protein and metabolic engineering have been widely shown to be very successful to improve the biocatalytic steps involving CYPs for the synthesis of many pharmaceuticals at the industrial

introducing one or more mutations producing variants (different colors) that can allow altered regio- and stereo-selectivity. Another option can be the use of CYPs from different sources (different colors) to achieve the desired reaction. Two or more enzymes/mutants can also be combined to introduce more functional groups. (B) Example of application of cytochrome P450 BM3 mutants carrying out different types of reactions for late-stage functionalization. Tetrahydroquinoline, a ubiquitous core motif found in natural products with important pharmaceutical properties, is converted into four metabolites. One of them is further functionalized into five different compounds by mutants of cytochrome P450 BM3 showing different oxidase activities, including hydroxylation in different positions, desaturation, C–C bond formation, and aromatization [22].

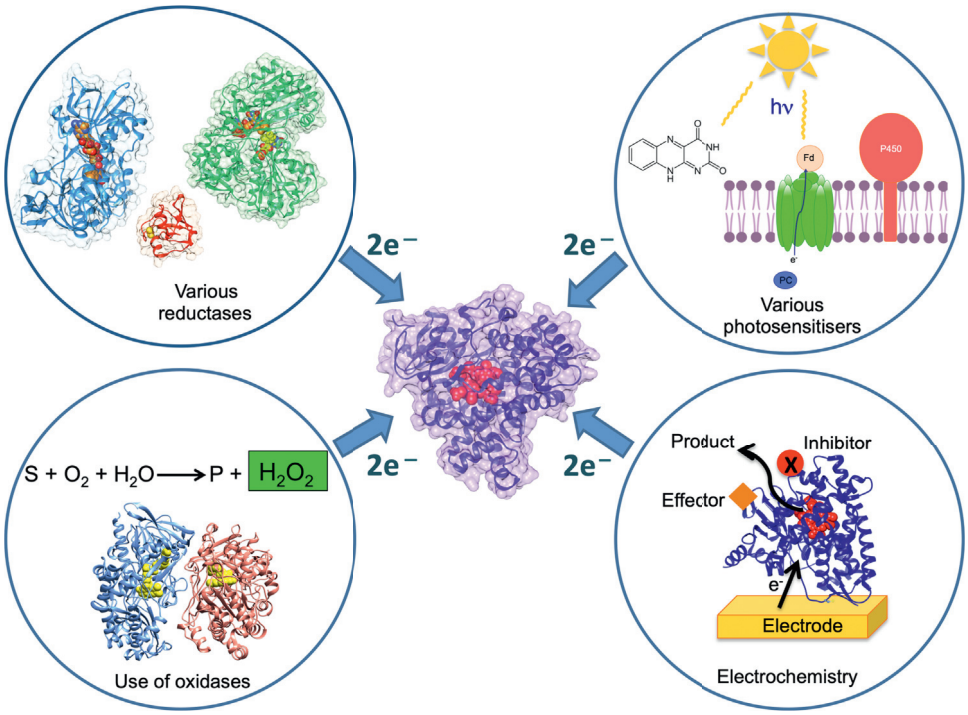

Figure 4. Natural and Artificial Electron Sources for Cytochromes P450 (CYPs). In nature, the two electrons required by CYPs for catalysis are provided by NAD(P)H via one or more reduction partners or by hydrogen peroxide. Artificial electron donors have been used, exploiting light through light harvesting units that can be photosystem I or organic molecules such as flavins. Moreover, CYPs have also been immobilized on electrode surfaces to receive the reducing equivalents necessary for catalysis directly from electrodes.

scale (Box 2). For example, protein engineering was applied to a member of the CYP105 family isolated from *Amycolatopsis orientalis* (CYP105S1 or P450Prava) to increase stereo-selectivity for the production of the cholesterol lowering drug pravastatin. The P450 enzyme was fused with its redox partner in a single polypeptide chain and the resulting self-sufficient protein was then introduced in the compactin-producing fungus *Penicillium chrysogenum*, enabling the production of 6 g l^{-1} of pravastatin in a single fermentation step [63].

Metabolic engineering has also been applied in the production of the antimalaria drug artemisinin. Genetically modified yeast has been used to produce farnesyl pyrophosphate through the mevalonate pathway and to decrease its use for sterol biosynthesis (downregulation of squalene synthase). Moreover, the gene coding for amorphadiene synthase and CYP71AV1 from *Artemisia annua* were introduced and a final concentration of 100 mg l^{-1} of artemisic acid was produced [64]. In 2013, the complete biosynthetic route was introduced in *Saccharomyces cerevisiae*, enabling the production of 25 g l^{-1} of artemisinic acid through fermentation [65]. In a more recent work, the expression levels of CYP725A4 in *Escherichia coli* were modulated and the interaction with the membrane and the redox partner optimized to obtain 570 mg l^{-1} of oxygenated taxanes in a bioreactor-scale fermentation [66].

Substrate engineering has been also successfully applied to CYPs for the late-stage functionalization. Using this approach, protecting groups can be designed and attached to the substrates to improve substrate acceptance and regio-selectivity [67]. In a recent work,

Box 2. Cytochromes P450 (CYPs) in Drug Production at Industrial Scale

The crucial role of CYPs in drug synthesis is well established and successful examples of the exploitation of these enzymes also at industrial level (reviewed in [106]) show that the gap between the lab and the industrial scale, due to the limitations that sometimes are difficult to address for these enzymes [21], has been filled.

One of the best examples of application of CYPs at industrial level is the synthesis of pravastatin, which is part of the statin family, a group of drugs developed to lower the levels of cholesterol, reducing the risk of cardiovascular diseases. They act as inhibitors of the 3β-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, that is the rate-controlling enzyme of the biosynthesis of cholesterol. Pravastatin derives from the fungal compound compactin through a stereo-selective hydroxylation, but it has better pharmacokinetics properties. The hydroxylation at the C6 position is carried out using CYP105A3 (P450sca2) from *Streptomyces carbophilus* [107] in a process developed by Sankyo (now Daiichi-Sankyo).

Another successful example for drug synthesis using CYPs at industrial scale is represented by the antimalaria drug artemisinin [108]. This compound is produced by the plant *Artemisia annua* and it was initially extracted from the plant for therapeutic purposes. Artemisinin is produced through a combination of chemical and biocatalytic methods that involve the plant cytochrome P450 CYP71AV1. The enzyme converts amorphadiene into artemisinic acid via three consecutive oxidation reactions and artemisinic acid is then chemically converted into artemisinin. This compound and its derivatives have been demonstrated to also possess anticancer activities *in vitro* and *in vivo* so that the interest in its industrial production is still increasing.

Other successful examples derive from the synthesis of bioactive steroids. They include the engineering of *S. cerevisiae* to produce hydrocortisone from simple carbon sources, thanks to the introduction of mammalian P450s and redox partners into the yeast [71,72] and the microbial steroid bioconversion used by Schering AG (Germany) that exploits immobilized *Curvularia lunata* mycelium for the production of cortisol from 11-deoxycortisol catalyzed by P450lun [109].

protecting groups were rationally designed on the compound vabicaserin, a serotonin (5-HT)2C receptor agonist, for the treatment of schizophrenia and bipolar disorder, to drive regio-selective monohydroxylation by P450 BM3 wild type and mutants [68], demonstrating how a combination of protein and substrate engineering can provide a rich toolbox for LD.

New Bioactive Compounds Synthesized by CYPs from Natural Products

Synthesis of Active Steroids

Steroid-based drugs are essential and of wide-spread use in the pharmaceutical field, where they are used for the production of antitumor, anti-inflammatory, antimicrobial, antiviral, antifungal, antiestrogenic, anticonvulsant, and antiallergy agents [69]. Nature already provides more than 250 steroids and, as their physiological biosynthetic pathways show, small differences in the steroid backbone alters their biological activity. Thus, the stereo- and regio-selectivity is crucial for the biological activity and often difficult to achieve by chemical synthetic methods. Nevertheless, in nature, bacterial, fungal, and mammalian CYPs provide a panel of natural biocatalysts that have successfully been introduced in industrial processes for biosynthetic purposes [69,70].

We feel one of the most fascinating examples is the exploitation by Sanofi-Aventis of the yeast *S. cerevisiae* as a drug-synthesizing cell factory for the production of cortisol [71,72]. An artificial biosynthetic pathway that includes four mammalian CYPs was introduced into the yeast through metabolic engineering, enabling a cascade reaction for the total biosynthesis of cortisol starting from glucose [71,72].

The discovery of new bacterial enzymes involved in steroid metabolism, together with protein engineering, is quite promising, especially for the active forms of vitamin D, 1α,25-dihydroxyvitamin D2 (1,25D2), and 1α,25-dihydroxyvitamin D3 (1,25D3). Indeed, analogs of these compounds have been developed for the clinical treatment of rickets, osteoporosis, psoriasis, secondary hyperparathyroidism, autoimmune diseases, and cancers. In particular, the VD2 analogs paricalcitol (19-nor-1,25D2) and doxercalciferol [1α(OH)D2] are now used as drugs for the treatment of secondary hyperparathyroidism [73]. However, since the chemical synthesis of the active

form of vitamin D requires many steps and results in low yields, the possibility of using biocatalytic processes is attractive and progress has been made in this direction. One example is provided by the enzyme CYP105A1, which was engineered to produce the biologically active form of vitamin D2 with a triple mutant that reached a 22-fold increase for the 1α-hydroxylation of 25-D2 [74]. In another work, two CYPs from *Bacillus megaterium* were found to hydroxylate vitamin D3; the enzyme CYP109E1 converts vitamin D3 into a mixture of 24(S)-hydroxyvitamin D3 [24S(OH)VD3], 25-hydroxyvitamin D3 [25(OH)VD3], and 24S,25-dihydroxyvitamin D3 [24S,25(OH)2VD3]. In a whole cell's biocatalytic process, 95% of conversion is achieved in 24 hours [75].

Recent advances through protein engineering supported by X-ray crystallography have overcome the possible poor selectivity in the reaction performed (i.e., different hydroxylation products) and the consequent shift of the reaction toward the product of interest. One example is the bacterial enzyme CYP106A2 from *B. megaterium* ATCC13368 that is physiologically involved in the conversion of progesterone to 15β-hydroxyprogesterone, with also minor 9α-, 11α-, and 6β-hydroxylase activity. The enzyme is also active toward different 3-oxo-Δ4, as well as 3-hydroxy-Δ5-steroids. Different works have shown that it is possible to engineer the enzyme to achieve 9α-, 11α-, and 6β-hydroxylation of different steroids [76]. The importance of these hydroxylated steroids has been discussed before [77,78]; briefly, the compound 9α-hydroxyprogesterone is an intermediate of the synthesis of the drugs fludrocortisone and fluoxymesterone, which show both glucocorticoidal and progestational activity. The 11α-hydroxyprogesterone has antiandrogenic and blood pressure-regulating activities and it can be used for the treatment of skin diseases in combination with other drugs [77,78].

The versatile enzyme CYP BM3 from *B. megaterium* has also recently been used for the application of a protein engineering approach using **site saturation mutagenesis** based on the information derived from mutability landscapes and molecular dynamics simulations [79]. In this work, efficient P450 BM3 mutants able to perform regio- and diastereo-selective hydroxylation of five different steroids at the C16-position were developed, producing C16 alcohols that are important as components of biologically active glucocorticoids [79].

Lastly, since mammalian CYPs are widely involved in steroid synthesis and processing, the possibility of using these enzymes to produce important compounds, such as the active form of vitamin D and pregnenolone, has also been explored. In the first case, high yields of conversion were reached using human CYP27A1, which was found to hydroxylate vitamin D3 into 25-hydroxyvitamin D3 (25-OH-D3) and cholesterol into 27-hydroxycholesterol. Moreover, this enzyme was found also to convert 7-dehydrocholesterol (7-DHC) into two metabolites, 26/27-hydroxy-7-dehydrocholesterol and 25-hydroxy-7-dehydrocholesterol, demonstrating that the flexibility of CYPs in substrates and reactions could sometimes be useful to generate new potentially bioactive molecules [80].

In the case of pregnenolone, a neuroactive steroid, a whole cell system where CYP11A1 with its redox partner were heterologously expressed in *B. megaterium* showed efficient conversion of cholesterol into pregnenolone due to the accumulation of the substrate into poly(3-hydroxybutyrate) (PHB) granules, where the catalyst and the substrate were found to colocalize [81]. The biosynthesis of pregnenolone from cholesterol would have a big impact since this reaction requires cleavage of the nonactivated side-chain of cholesterol, which is difficult to achieve. Moreover, pregnenolone is the precursor of about 300 approved steroid drugs and some derivatives have a potential in the treatment of neurological diseases [82].

Antibiotics

Macrocyclic antibiotics have been successfully used in clinical practice and they have been historically developed from few molecular scaffolds that have been modified by synthetic tailoring [4]. Indeed, more than two-thirds of clinically used antibiotics are natural products or their derivatives [83]. However, the available molecules are not sufficient to meet the fight against multidrug resistant pathogens; this is now a priority that requires the exploration of new scaffolds. Drug repurposing, as well as the use of old antibiotics as drug leads through late-stage functionalization, may offer new avenues not explored so far [4]. CYPs can be essential to meet this goal, as they are physiologically involved in complex biosynthetic pathways.

One good example of such complexity is the synthesis of glycopeptide antibiotics (GPAs) that are produced by some *Streptomyces*. GPAs are heptapeptides active toward Gram-positive bacteria, such as methicillin-resistant *Staphylococcus aureus* and penicillin-resistant *Staphylococcus pneumonia* [84]. They are synthesized by a complex protein machinery that includes a non-ribosomal peptide synthetase and three to four CYPs (Oxy) that catalyze oxidative crosslinking of aromatic side chains that is essential for their biological activity [85]. In a recent paper, the biosynthetic pathway of kistamicin, a divergent and highly crosslinked member of GPAs was elucidated and the crucial role of CYP OxyC in crosslinking phenolic amino acids was demonstrated [86], providing a new tool to create diversity in this important class of compounds.

Natural antibiotics have been also successfully exploited as drug leads for late-stage functionalization via activation of C–H bonds by CYPs. The enzyme PikC, belonging to the methymycin/pikromycin pathway in *Streptomyces venezuelae*, shows a unique substrate flexibility since it uses 12- and 14-membered ring macrolides as substrates and it is able to functionalize two positions on the macrolactone system [87]. Another CYP, P450 MycCI, was isolated from the mycinamicin biosynthetic pathway and demonstrated to be active on several 16- membered ring macrolactones [88].

CYPs can provide an important tool for the synthesis of novel macrolide- and ketolide-based antibiotics. The substrate of PikC is a glycosylated macrolactone and the sugar desosamine has a crucial role in anchoring the substrate in the active site and driving regio-selectivity [89]. This natural mechanism has been used as a strategy to drive regio-selective hydroxylation reactions in different positions to produce 11- and 12-membered macrolactones from a common linear substrate with PikC as the single biocatalyst [90]. The enzyme was also used in a chemoenzymatic platform that achieved the total synthesis of tylactone-based macrolide antibiotics and their late-stage diversification through *in vitro* cytochrome P450-mediated oxidation [91].

Phytopharmaceuticals and Derivatives

Phytochemicals represent a case in point example of the role of CYPs in generating an extraordinary metabolic diversity of potential drug candidates, including terpenes, alkaloids, and flavonoids.

Monoterpenes are valuable compounds with interesting pharmaceutical properties. Geraniol (3,7-dimethylocta-trans-2,6-dien-1-ol), an acyclic monoterpenic that exhibits various pharmacological properties [92], derives from geranyl diphosphate with hydroxylation at C-8 carried out by a CYP as part of the iridoid secologanin biosynthesis. Strictosidine, the precursor of different monoterpenoid alkaloids classes, including the terpenoid indole alkaloids (TIAs), is derived from iridoid secologanin. TIAs have important pharmacological properties, as demonstrated by the

therapeutical use of the anticancer molecule vinblastine [93]. Extensive metabolic engineering was applied on *S. cerevisiae* through selective deletions and introduction of 14 genes from *Catharanthus roseus* involved in monoterpene indole alkaloid biosynthesis, including four coding for CYPs. The product of one of them, CYP76B6, catalyzes the 8-hydroxylation of geraniol and it has been shown to be one of the bottlenecks of this biosynthetic pathway [94].

Other progress in the biocatalytic synthesis of alkaloids include a multi-enzyme cascade, where a CYP from the medicinal plant *C. roseus* was combined with an alcohol dehydrogenase to generate akuammicine, a *Strychnos* alkaloid, starting from 4,21-dehydrogeissoschizine (strictosidine aglycone). This compound is the deformylated form of preakuammicine, which is the precursor for vinblastine [95].

In another recent work, a new CYP, named NascB, was discovered from *Streptomyces* sp. (CMB-MQ030). This enzyme is involved in the biosynthetic pathway of a naturally occurring C^3-aryl hexahydropyrrolo[2,3-*b*]indole, also known as naseseazine C. The hexahydropyrrolo[2,3-*b*] indole (HPI) moiety of naseseazine C, usually referred to as pyrroloindoline, is a common motif found in different bioactive alkaloids, which showed anticancer and antibacterial activities as well as inhibition of cholinesterase [96]. The enzyme NascB was found to catalyze a radical cascade reaction that allows the formation of carbon–carbon bonds with high regio- and stereospecificity. By overexpressing the protein in *E. coli*, a whole-cell system was developed allowing the production of naseseazine C and 30 of its analogs, with some of them showing potent neuroprotective properties [96].

Another interesting case of how CYPs can transform natural compounds into drug leads is papaverine, an isoquinoline alkaloid from opium poppy. Papaverine showed potentially interesting anticancer properties and CYP105D1 from *Streptomyces griseus* ATCC 13273 was used for the regio-selective demethylation of papaverine to produce 6-O-demethylpapaverine with a yield of about 61% within 24 hours [97]. This metabolite is less toxic than papaverine and has a new hydroxyl group moiety that can be further derivatized.

The sesquiterpene zerumbone possesses anti-inflammatory, anti-HIV, and potent antitumoral properties [98]. In a recent work, an engineered yeast cell factory was created by introducing the three enzymes responsible for the biosynthesis into *S. cerevisiae*, including α-humulene 8-hydroxylase (CYP71BA1) from *Zingiber zerumbet* and a CYP-reductase from *Arabidopsis thaliana*. Other modifications, including the overexpression of the mevalonate pathway rate-limiting enzymes, were introduced and zerumbone production reached 40 mg l^{-1} [99]. Zerumbone, as well as other pharmaceutically interesting terpenes such as nootkatone and humulene, can be obtained also using microbial CYPs as an alternative to plant P450s, which are, in general, more difficult to manipulate [100].

CYPs are also involved in the biosynthesis of flavonoids. Metabolic engineering allowed the production of six different flavonoids (naringenin, liquiritigenin, kaempferol, resokaempferol, quercetin, and fisetin) *de novo* from glucose. To this end, two CYPs enzymes acting as flavonoid monoxygenases from *Fragaria ananassa* and *Petunia hybrid* were fused with the CYP reductase from *C. roseus* (CPR) and introduced in *S. cerevisiae* [101].

Concluding Remarks

Biocatalytic applications of CYPs to produce nature-inspired drugs is moving in two directions. The identification of new P450 enzymes and their biological functions is accompanied by the biotechnological progress made to use them for biocatalytic purposes [54]. These enzymes have a

large potential to synthetize complex compounds, especially if combined in cascade reactions with other enzymes or chemical methods, as demonstrated by several works [54,102]. However, there are some aspects that need to be addressed as they still limit their practical use in biocatalysis [21]. Nevertheless, there are cases in which these limitations have been overcome thanks to protein and metabolic engineering, as well as fusion of the redox partner leading to self-sufficient enzymes.

Other efforts in the CYP field involve the development of computational methods to predict the substrate profile of a specific CYP, as well as the possible metabolites [103,104]. This point is very important because, together with the possible creation of databases where substrates and reactions of these enzymes are annotated, they can allow the best P450 candidate for a specific biocatalytic conversion to be assigned (see Outstanding Questions).

In conclusion, CYPs provide a unique and powerful toolbox of enzymes that can be crucial to produce novel nature-inspired drugs through **green chemistry**; these are nowadays required to protect human health and environment.

Outstanding Questions

How many novel substrates and reactions carried out by cytochromes P450 are still unknown? How can we annotate them?

Will cytochromes P450 contribute more to the development of new generation molecules, such as antibiotics that will be able to overcome the problem of multidrug resistance?

How many cytochromes P450-driven biocatalytic steps will be introduced at industrial level in the next 10 years?

Will it be possible to harness our knowledge on P450 structures to make intelligent predictions on their function?

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