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# Redox Reactions Catalyzed by Isolated Enzymes

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## 1. INTRODUCTION

The class of oxidoreductases, to which belong all enzymes catalyzing oxidoreduction reactions, includes several groups of biocatalysts such as dehydrogenases, monooxygenases, dioxygenases, oxidases, peroxidases, etc. The reactions catalyzed by oxidoreductases are numerous and comprise oxidation of hydroxyl, aldehyde, and keto groups, oxidation of primary and secondary amines, hydroxylation of aromatic or nonactivated carbon atoms, dehydrogenation of carbon−carbon single bonds, heteroatom oxygenation, Baeyer−Villiger oxidation, and double bond epoxidation. Several oxidoreductases can also catalyze the reduction of such compounds as aldehydes, ketones, carboxylic acids, and double and triple carbon−carbon bonds. The electron acceptors, that is, the compounds that oxidize the substrate of interest, are as diverse as $NAD(P)^+$, cytochrome, molecular oxygen, hydrogen peroxide, disulfides, quinone or similar compounds, nitrogenous groups, iron−sulfur proteins, and flavin.

At the end of July 2010, almost 1200 different types of oxidoreductases have been classified by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). In the classical four digits code proposed in 1961 (EC a.b.c.d),[1] the number "1" has been assigned to indicate the class of oxidoreductases (first figure, "a"). The second figure ("b") indicates the functional group that is oxidized or reduced ("1" denotes a −CHOH− group, "2" a −CHO or −CO−COOH group or carbon monoxide, and so on). The third figure ("c") usually indicates the type of acceptor involved: "1" denotes $NAD(P)^+$, "2" a cytochrome, "3" molecular oxygen, "4" a disulfide, "5" a quinone or similar compound, "6" a nitrogenous group, "7" an iron−sulfur protein, "8" a flavin, "99" other acceptors. In subclasses EC 1.13 (oxidoreductases acting on single donors with incorporation of molecular oxygen) and EC 1.14 (oxidoreductases acting on paired donors, with incorporation or reduction of molecular oxygen), a different classification scheme is used, and subsubclasses (identified by the "c" figure) are numbered from 11 onward. They are also called "oxygenases" and, more specifically, "monooxygenases"' or "dioxygenases", based on the number of oxygen atoms that are incorporated in the oxidized substrate.

The peroxidases (oxidoreductases acting on a peroxide as acceptor) are also classified in a different way. The first three digits are always EC 1.11.1, while the different substrates that are oxidized are identified by the fourth figure ("d") of the code.

Figure 1 shows some of the oxidoreductases that have found significant applications in synthetic or analytical chemistry. As far as the exploitation of isolated enzymes is concerned, this has been mainly related to oxidoreductases involving three kinds of acceptors:

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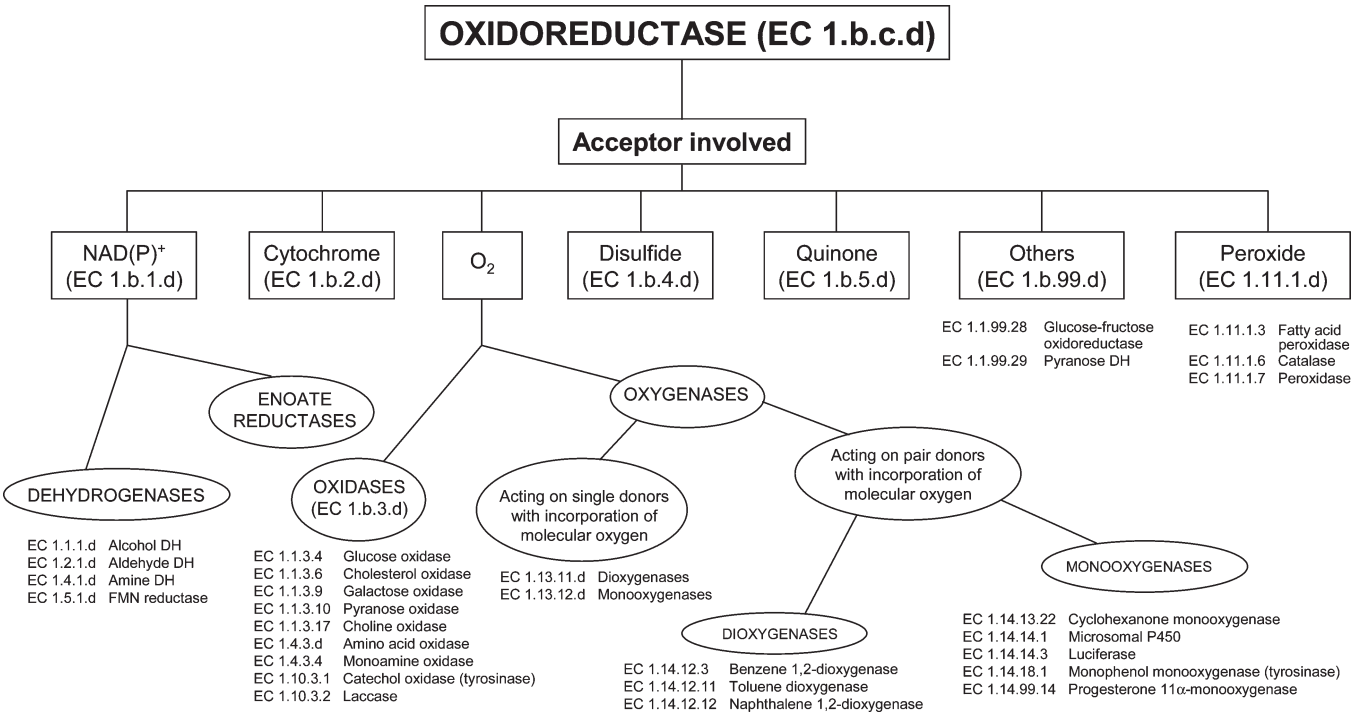


**Figure 1.** Classification of oxidoreductases that have found applications in synthetic or analytical chemistry.

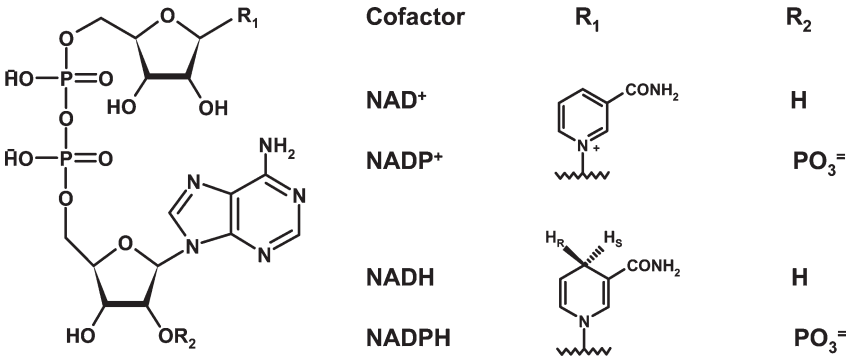


**Figure 2.** General formula of NAD(P)(H).

NAD(P)$^+$ (dehydrogenases and enoate reductases), molecular oxygen (oxidases, monooxygenases, dioxygenases), and hydrogen peroxide (peroxidases).

In this thematic issue of *Chemical Reviews* on "Enzymes in Synthesis", other Reviews describe in details either the performances of a specific group of oxidoreductases (Baeyer−Villiger monooxygenases,[2] alcohol and amine oxidases[3]) or their exploitation for the synthesis of enantiomerically enriched products (dehydrogenases and enoate reductases[4,5]).

To avoid undesired overlapping, these topics will not be discussed by us. Being an issue focused on the synthetic applications, the large number of reports on the exploitation of oxidoreductases in analytical assays will not be considered either. Finally, as a further "internal" limitation, this Review will not report in detail on biotransformations catalyzed by whole cells, which will be briefly treated only in the Special Techniques section (2.1.2 and 2.2.2).

Following a paragraph on general aspects related to the use of isolated oxidoreductases, the following groups of enzymes will be discussed in detail: dehydrogenases (hydroxysteroid dehydrogenases, UDP-glucose dehydrogenase, etc.), oxidases (sugar oxidases, tyrosinases, laccases), oxygenases (cytochromes P450, tyrosinases), and peroxidases.

## 2. SPECIAL TECHNIQUES

### 2.1. Regeneration of Nicotinamide Cofactors NAD(P)(H)

One of the first issues to consider in the synthetic application of isolated dehydrogenases (and monooxygenases) is the fact that the nicotinamide cofactors, that is, $\beta$-nicotinamide adenine dinucleotide (NAD$^+$), $\beta$-nicotinamide adenine dinucleotide phosphate (NADP$^+$), $\beta$-nicotinamide adenine dinucleotide reduced (NADH), and $\beta$-nicotinamide adenine dinucleotide phosphate reduced (NADPH) (Figure 2), are stoichiometrically consumed in the reactions catalyzed by these enzymes. Thus, as exemplified in Figure 3, in the oxidation of a generic alcohol to a ketone, there is NAD(P)$^+$ consumption, whereas in the reduction of a generic ketone to an alcohol, there is NAD(P)H consumption. The preparative-scale exploitation of dehydrogenases


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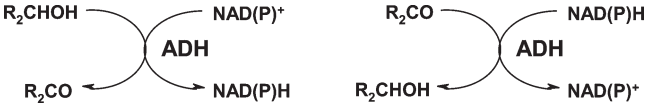


**Figure 3.** Oxidation of an alcohol to a ketone (left) and reduction of a ketone to an alcohol (right) catalyzed by an alcohol dehydrogenase (ADH).

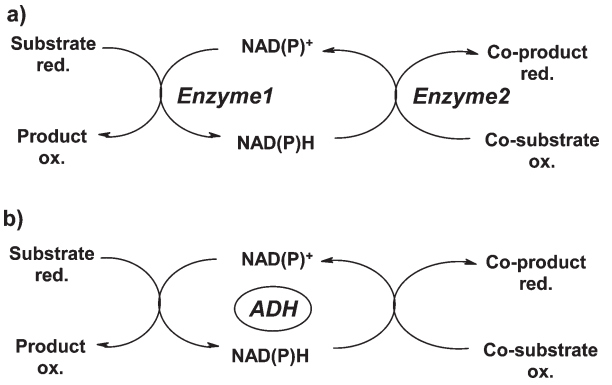


**Figure 4.** Enzymatic regeneration systems of nicotinamide cofactors illustrated in the direction of oxidized product formation: (a) enzyme-coupled system; (b) substrate-coupled system.

requires that cofactors are continuously regenerated in the reaction medium (in situ regeneration), so that they can be employed in catalytic amounts and no longer stoichiometrically. This is dictated by economic reasons because of the high cost of these cofactors, which is around 1500 euros per kilogram in the case of $NAD^+$ and around 6000 euros per kilogram in the case of $NADP^+$. The reduced forms NADH and NADPH are considerably more expensive than the oxidized ones.

The most significant strategies that have been developed for the in situ regeneration of NAD(P)(H) are the following: (a) enzymatic (either enzyme-coupled or substrate-coupled), (b) by engineered whole cells, (c) electrochemical, and (d) chemical. They will be briefly described and discussed in this Review.

**2.1.1. Enzymatic Strategies.** These strategies, which so far are those most developed and widely applied in biocatalysis, can be based on enzyme-coupled system or on substrate-coupled system, as illustrated in Figure 4, which schematizes the two systems in the direction of substrate oxidation. In the enzyme-coupled system (Figure 4a), the reduced substrate is oxidized by a specific dehydrogenase (enzyme 1) at the expense of $NAD^+$ or $NADP^+$, which is reduced to NADH or NADPH. NADH or NADPH, in turn, is reoxidized to $NAD^+$ or $NADP^+$ by an ancillary enzyme (enzyme 2) at the expense of a cheap cosubstrate. Therefore, the cofactor, which acts as a hydride shuttle between substrate and cosubstrate, can be employed in catalytic amounts. In the substrate-coupled system (Figure 4b), the enzyme that transforms the substrate of interest regenerates also the cofactor. This second approach is not of general applicability and is practically limited to alcohol dehydrogenase (ADH)-catalyzed reactions.

As suggested by Chenault and Whitesides,[6] an ideal enzymatic regeneration system should meet the following criteria: (a) the enzymes should be inexpensive and stable, (b) the enzymes should have high specific activity, (c) simple and inexpensive reagents that do not interfere with the isolation of the product of interest or with enzyme stability should be employed, (d) high turnover number (TN, moles of product formed per mole of cofactor per unit time) should be obtained, (e) high total turnover number (TTN, moles of product formed per mole of cofactor during the entire reaction period) should be achieved (economic considerations require that the TTN is at least between $10^2$ and $10^4$), and (f) an overall equilibrium for the coupled enzyme system favorable to product formation should be reached. In this way, the total amount of cofactor continuously present in the system can be reduced to a catalytic level, and the conversion of substrate to product can be completed.

The enzymatic regeneration systems most commonly employed for synthetic applications are depicted in Table 1, which indicates also the equilibrium constant values ($K_{eq}$) of the systems.[7] For NAD(P)H regeneration, both formate/formate dehydrogenase[8,9] and glucose/glucose dehydrogenase[9] appear to meet most of the criteria indicated by Chenault and Whitesides for an ideal regeneration system.[6] The enzymes, which can regenerate both NADH and NADPH, are stable and readily available, have a good specific activity and relatively low costs, and have a favorable or very favorable thermodynamic equilibrium. Moreover, the cosubstrates/coproducts do not interfere with the performance of the main enzyme and product recovery. A third very promising NAD(P)H regenerating system is that based on the oxidation of phosphite to phosphate catalyzed by phosphite dehydrogenase.[7a,10] However, the synthetic applications of this system are, at the moment, very limited[11,12] and do not permit one to draw any definite conclusions.

For $NAD(P)^+$ regeneration, two systems appear to meet fairly satisfactorily the criteria indicated by Chenault and Whitesides:[6] pyruvate/lactate dehydrogenase and 2-oxoglutarate/glutamate dehydrogenase (Table 1). The first system is superior to the second one regarding enzyme specific activity, stability, and cost, whereas the second one is superior regarding the equilibrium constant, whose high value will drive to near completion any NAD(P)H-dependent coupled enzymatic oxidation. Concerning acetone/*sec*-alcohol dehydrogenase, the main drawback is represented by the low value of the equilibrium constant, which prevents complete oxidation of the majority of the substrates of interest. However, this reaction can be made quasi-irreversible by using activated ketones such as chloroacetone, 1,1- and 1,3-dichloroacetone, 1,1,1-trichloroacetone, or ethyl acetoacetate.[13] The system based on NADH oxidation catalyzed by NADH oxidase possesses great potentialities for $NAD^+$ regeneration, because it makes use of molecular oxygen and because the reaction is irreversible. However, instability and product inhibition of the enzyme make the system unsuitable for preparative-scale synthetic applications.[14−16]

It should be mentioned that water-soluble macromolecular derivatives of NAD(P)(H) have also been employed, in conjunction with a continuous-flow membrane reactor, in dehydrogenase-catalyzed reactions. In this approach, both enzymes and macromolecular cofactors are confined, and practically 100% retained, in the reactor space that is closed off by an ultrafiltration membrane, through which low-molecular-weight substrates and products can freely pass.[7b,17,18] For example, by employing $NAD^+$ covalently linked to polyethylene glycol (MW 20 000), L-*tert*-leucine and other amino acids were produced at industrial scale with excellent results: substrate concentration up to 0.5 M, 95% conversion, space/time/yield 640 g/L/day, coenzyme total turnover number 125 000.[19,20] These results would suggest that macromolecular cofactors are the best choice for the industrial-scale application of NAD(P)H-dependent enzymes. However, there are at least two drawbacks that must be taken into consideration:

**Table 1. Ancillary Enzymatic Systems for in Situ Regeneration of NAD(P)(H)**

| system | $K_{eq}$ | ref |
|---|---|---|
| $NAD(P)^+ + \text{formate} \xrightarrow{\text{formateDH}} NAD(P)H + \text{carbon dioxide} + H^+$ | $7 \times 10^5$ M | 7a |
| $NAD(P)^+ + \text{glucose} \xrightarrow{\text{glucoseDH}} NAD(P)H + \text{glucono-}\delta\text{-lactone} + H^+ \xrightarrow{H_2O} \text{gluconate}$ | irreversible | |
| $NAD(P)^+ + \text{phosphite} + H_2O \xrightarrow{\text{phosphiteDH}} NAD(P)H + \text{phosphate} + H^+$ | $10^{11}$ | 7a |
| $NAD(P)H + \text{2-oxoglutarate} + NH_3 + H^+ \xrightarrow{\text{glutamateDH}} NAD(P)^+ + \text{glutamate} + H_2O$ | $6.5(11.6) \times 10^{14}$ $M^{-1}$ | 7b |
| $NADH + \text{pyruvate} + H^+ \xrightarrow{\text{lactateDH}} NAD^+ + \text{lactate}$ | $3.6 \times 10^{11}$ $M^{-1}$ | 7c |
| $NAD(P)H + \text{acetone} + H^+ \xrightarrow{\text{sec-alcoholDH}} NAD(P)^+ + \text{2-propanol}$ | $1.7 \times 10^9$ $M^{-1}$ | 7d |
| $NADH + O_2 + H^+ \xrightarrow{\text{NADH oxidase}} NAD^+ + H_2O$ | irreversible | |

(a) the synthesis of these derivatives is quite laborious, and the yields are between 40% and 70%, and (b) numerous enzymes do not work well with macromolecular cofactors (markedly decreased $V_{max}$ and/or markedly increased $K_m$ of the cofactor).[7b,21]

**2.1.2. Strategies Based on Engineered Whole Cells.** Overexpression, for synthetic applications, of an NAD(P)H-dependent enzyme in a cell might render internal cofactor regeneration not fast enough to cope with the high activity of the desired enzyme. The drawback can be overcome by overexpressing in the host cell also a suitable cofactor regeneration system. In the following, a few examples of this approach will be briefly described.

The asymmetric reduction of ethyl 4-chloro-3-oxobutanoate to ethyl (*R*)-4-chloro-3-hydroxybutanoate was carried out using *E. coli* cells, which coexpress the aldehyde reductase gene and the glucose dehydrogenase gene. In a water−organic solvent two-phase system, the product was obtained in high concentration (1.6 M), yield (94%), and optical purity (92%). The total turnover number (TTN) for the cofactor was very high (13 500).[22] Using the same approach, similar results were obtained in the synthesis of ethyl (*R*)-trifluoro-3-hydroxybutanoate.[23] The preparation of (*R*)-4-fluorophenyl-ethan-1-ol starting from 4-fluoroacetophenone was conducted by means of a tailor-made recombinant whole-cell biocatalyst, containing an alcohol dehydrogenase and a glucose dehydrogenase. In pure aqueous solvent media, the substrate (0.5 M) was transformed in the desired product with high conversion (95%) and excellent enantioselectivity (99%).[24] The enantioselective reduction of methyl acetoacetate was obtained by whole-cell biotransformation, combining an (*R*)-specific alcohol dehydrogenase and formate dehydrogenase coexpressed in *E. coli*. The substrate (40 mM) was completely transformed in methyl (*R*)-3-hydroxy butanoate.[25] Fraaije and co-workers have described NADPH regeneration by phosphite dehydrogenase, which was fused with several Baeyer−Villiger monooxygenases and expressed in *E. coli*.[11] The cofactor total turnover number ranged between 370 and 1750, depending on experimental conditions and nature of monooxygenases. One weakness of the system, that is, the instability of phosphite dehydrogenase, can be overcome by using a thermostable variant of phosphite dehydrogenase.[12] As for oxidation reactions, $NAD^+$ regeneration was accomplished by simultaneous overexpression in *E. coli* of an alcohol dehydrogenase, used to produce (3*R*)- and (3*S*)-acetoin and (2*S*,3*S*)-butanediol, and of an NADH oxidase.[26]

**2.1.3. Electrochemical Strategies.** Electrochemical regeneration of NAD(P)H appears appealing, and, indeed, numerous reports describing this approach have been published (for recent review articles, see refs 27−29). Because direct cathodic reduction of $NAD(P)^+$ suffers from low 1,4-regioselectivity for the nicotinamide ring and side reactions, and direct amperometric oxidation of NAD(P)H suffers from overpotentials and electrode fouling, organic and metal-containing electron shuttles for transfer of electrons between electrode and NAD(P)(H) were developed.[27−29] The productivity of enzymatic reactions making use of electrochemical cofactor regeneration was found to be between 0.005 and 3.04 g/L/h, whereas cofactor total turnover number was between 2.3 and 1034.[27,28] According to Kohlmann et al.,[29] electrochemical cofactor regeneration still suffers from several limitations: (a) the final product concentration is often too low for practical synthesis, (b) the productivity of the majority of electroenzymatic processes is rather low, and (c) it will take some time before electroenzymatic processes can be applied on industrial scale.

**2.1.4. Chemical Strategies.** Chemical regeneration is not so widely applied as the previously described methodologies for cofactor regeneration.[9,27] Simple ruthenium and rhodium complexes have been employed to catalyze in situ $NAD(P)^+$ reduction by dihydrogen. With this approach, the reduction of 2-heptanone to (*S*)-2-heptanol, catalyzed by alcohol dehydrogenase, was conducted with catalytic amounts of $NADP^+$ and a ruthenium complex. The total turnover number of both $NADP^+$ and ruthenium was, however, only 10.[30] The use of a platinum carbonyl cluster has also been described for the catalytic reduction of $NAD^+$ by dihydrogen; the reduction of pyruvate to lactate was catalyzed by lactate dehydrogenase.[31,32] Because both $NAD^+$ and lactate dehydrogenase are soluble only in water, and the carbonyl cluster is soluble only in organic solvents, a biphasic system consisting of water and dichloromethane was used. The total turnover number of $NAD^+$ in the process was 60.[31] As stated by Wichmann and Vasic-Racki,[27] however, chemical methods at present seem to suffer from cumbersome reaction conditions, expensive and/or toxic reagents, and/or unwanted side products, and therefore have not been preferred so far for commercial or preparative applications. Therefore, both chemical strategies and electrochemical strategies (treated above) appear at the moment less convenient for biocatalysis than do enzymatic strategies.

## 2.2. Substrate Supply and Product Removal

Biocatalysis in organic synthesis, and specifically the exploitation of oxidoreductases, presents, besides numerous advantages,


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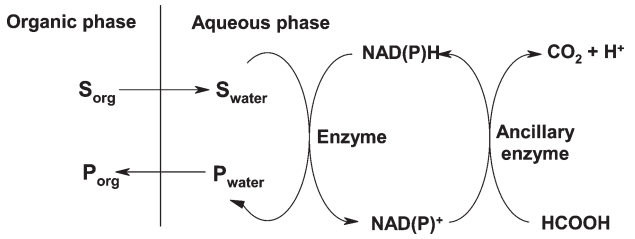


**Figure 5.** Scheme of a biocatalytic biphasic system involving cofactor regeneration.

some serious inconveniences. One of them is represented by the fact that a great number of organic compounds are poorly soluble in aqueous media, which leads to large reaction volumes and complicates product recovery. Moreover, substrates and/or products can be inhibitory or toxic for the biocatalyst. Further problems can also arise from the supply of oxygen and hydrogen peroxide, when they are needed as oxygenating agents. Several methodologies have been developed to overcome or minimize such limitations, and they will be briefly discussed in the following.

**2.2.1. Organic Solvents and Ionic Liquids.** The most obvious and simple way to increase the solubility of hydrophobic substrates (with simultaneous product removal) in biocatalyzed reactions is that based on the use of conventional organic solvents or of ionic liquids.[33−35] To this end, organic solvents and ionic liquids are mainly applied in three ways: (a) as cosolvents with water, (b) as a second phase in a water−organic solvent (or ionic liquid) biphasic system, and (c) alone as nonaqueous solvents. The first type of application is quite limited because ionic liquids, and even more so organic solvents, markedly inhibit or inactivate biocatalysts when utilized at the high concentrations necessary to substantially increase substrate solubility in the mainly aqueous reaction medium. One notable exception is represented by the recently reported conversion (92% yield) of prositagliptin ketone (200 g/L) to the enantiopure antidiabetic compound sitagliptin, catalyzed by an engineered transaminase in 50% DMSO.[36] The third type of application, that is, the use of organic solvents[34] and ionic liquids[35] as nonaqueous solvents, is very useful and common in the case of hydrolases, but shows no general advantage for oxidoreductases. In fact, only a few examples of this type of application have been reported for these enzymes. Among them, there are asymmetric oxidoreductions of alcohols and ketones catalyzed by alcohol dehydrogenase in several organic solvents,[37] asymmetric oxidations of organic sulfides catalyzed by horseradish peroxidase in iso-propyl alcohol and in methanol,[38] and oxidation of codeine to codienone catalyzed by morphine dehydrogenase in an ionic liquid.[39]

Instead, biphasic system has been widely applied with oxidoreductases.[33] The system is quite simple and consists of water and a water immiscible organic solvent (or ionic liquid) that form two phases (Figure 5). The organic (or ionic liquid) phase contains the hydrophobic substrates, whereas the aqueous phase contains the enzymes and the hydrophilic cofactors, when needed. On stirring or shaking, the substrate partitions in the aqueous phase where it is transformed into the product, which partitions back in the organic phase. The cofactor, that in the example depicted in Figure 5 is the NAD(P)H consumed in the reduction of a substrate of interest, is continuously regenerated in the reaction medium with an ancillary enzymatic system, at the expense of a cheap cosubstrate such as formate. Biphasic system based on conventional water-immiscible organic solvents will be discussed first. The system presents numerous advantages: (a) increased solubility of hydrophobic substrates, (b) smaller reaction volumes and increased volumetric productivity, (c) minimization of substrate/product inhibition of the biocatalyst, because of their low concentration in the aqueous phase where the reaction takes place, (d) minimization of enzyme inhibition/inactivation by the solvents, because of their low concentration in the aqueous phase, and (e) facilitated product recovery from the reaction medium. To be suitable for biocatalysis in biphasic system, solvents should meet as much as possible the following requisites: (a) to be compatible toward biocatalyst activity and/or stability, (b) to have high capacity to dissolve substrates and/or products, (c) to be inexpensive, biodegradable, and nontoxic, and (d) to have a relatively high boiling point. Several solvent physical-chemical parameters, which might be correlated with biocatalyst activity and/or stability, have been taken into consideration. Among these, there are log *P* (that is the logarithm of partition coefficient of a given solvent between 1-octanol and water), dielectric constant, Hildebrand solubility, three-dimensional solubility, and Dimroth−Reichardt $E_T$.[33b] Log *P* has been demonstrated to be the parameter more correlated with biocatalyst properties, in the sense that the higher is the log *P* value, the higher are the biocatalyst activity and stability.[33b,40] It should be emphasized that in biphasic system enzyme catalysis takes place in the aqueous phase and that it follows Michaelis−Menten kinetics.[33,41] As was said previously (see comments to Figure 5), a biphasic system has to be stirred or shaken to ensure fast transfer of substrates and products between phases. Vigorous shaking and stirring, however, can cause enzyme inactivation at the solvent−water interface.[33] This inconvenience can be overcome or drastically reduced by using properly immobilized enzymes,[42] or membrane reactors that prevent direct contact between the organic and aqueous phases.[33b,43]

Concerning ionic liquids and biphasic systems, it can be said that most of the considerations done for conventional organic solvents are valid also for ionic liquids. Room temperature ionic liquids (also called molten salts) are mixtures of anions and cations that do not crystallize at room temperature.[35] Changing the structure of anions or cations can tune their properties, for example, polarity. Among the advantages of ionic liquids, we can list the following: (a) enzyme activities, stabilities, and enantioselectivity in ionic liquids are sometimes higher than those observed in organic solvents, (b) ionic liquids permit one to carry out enzyme-catalyzed reactions in nonaqueous media on polar substrates such as peptides, sugars, nucleotides, and biochemical intermediates, and (c) ionic liquids are possible "green" replacements for organic solvents, because they have negligible vapor pressure and, therefore, may be easier to efficiently reuse than organic solvents. A serious drawback of ionic liquids is represented by the fact that product isolation is more complex, especially for nonvolatile materials.[35,44]

Besides ionic liquids, other neoteric solvents (which can be defined as new types of solvents or older materials that are finding new applications as solvents) such as supercritical fluids (especially supercritical carbon dioxide), fluorous solvents, and liquid polymers (eg, poly(ethylene glycol), poly(propylene glycol), and poly(tetrahydrofuran)) have been investigated as media for biocatalyzed reactions, mostly based on hydrolases.[45,46] Oxidoreductases, instead, have found only limited and small-scale applications with a few enzymes such as chloroperoxidase, alcohol dehydrogenase, glucose oxidase, cholesterol oxidase, and polyphenol oxidase.[45,46]

**2.2.2. Adsorbent Resins.** A valid alternative to the two-phase system is represented by hydrophobic adsorbent resins,

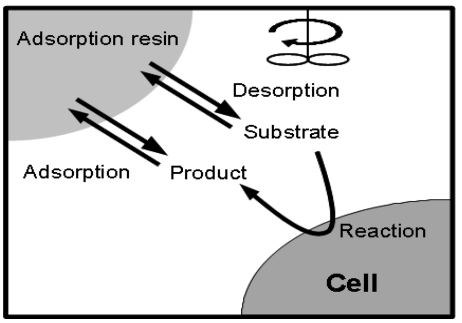


**Figure 6.** Scheme of in situ substrate supply and product removal by hydrophobic adsorbent resins.

which permit in situ substrate supply and product removal.[47−50] In this approach, high concentrations of a hydrophobic substrate are adsorbed onto a hydrophobic resin (e.g., XAD-7,[47] SP-207,[47] XAD 1180,[48] Dowex Optipore L-493[49,50]), which is then added to a bioreactor containing an aqueous buffer and a biocatalyst, generally in the form of whole cells. The aqueous slurry is kept stirring for the entire reaction period. The substrate partitions in the aqueous medium and diffuses inside the cells, where it is transformed in the product, which, in turn, diffuses back in the aqueous medium and is adsorbed by the resin (Figure 6). Because of the high affinity of hydrophobic substrates and products for the resin, their concentration in the aqueous reaction medium is very low, which minimizes possible toxicity effects of substrate-product on cells and/or inhibiting effects on cell enzymes.[47−50] It should be emphasized that adsorbent resins are not of general applicability. In fact, whereas they can be profitably used with whole cells, they do not work well with isolated enzymes, because isolated enzymes tend to adsorb onto the resins, which can negatively affect their performance. Furthermore, the process of product recovery from the resins, that is based on the use of pure organic solvents such as acetone and ethyl acetate, would inactivate the enzymes, preventing their reuse.

A notable example of application of resins refers to the stereoselective reduction of 3,4-methylene-dioxyphenyl acetone to the corresponding (*S*)-methylene-dioxyphenyl iso-propanol, catalyzed by *Zygosaccharomyces rouxii*.[47] The use of the resin XAD-7 permitted one to increase substrate concentration from 6 to 40 g/L (in a 300 L reaction volume); the overall reactor productivity was 75 g/L/day. Another example worth mentioning is the asymmetric Baeyer−Villiger oxidation of racemic bicyclo[3.2.0]hept-2-en-6-one, catalyzed by recombinant *E. coli* cells overexpressing cyclohexanone monooxygenase. The process was carried out to the kilogram scale using the resin DOWEX Optipore L-493, in a 50 L bioreactor.[49]

**2.2.3. Continuous Substrate Supply.** To eliminate or mitigate the problem of biocatalyst inhibition by substrate, continuous substrate feeding has also been adopted. The principle is to keep substrate concentration in the reactor low enough to avoid enzyme inhibition for the entire reaction period. This approach has been adopted, for example, in the reduction of acetophenone to (*S*)-phenylethanol by whole cells of *Rhodotorula glutinis*,[51] in transketolase-catalyzed synthesis of L-erythrulose,[52] and in cyclohexanone monooxygenase-catalyzed enantioselective oxidation of cyclic ketones to lactones and of organic sulfides to sulfoxides.[53] In the last case, continuous substrate feeding not only shortened reaction times but improved also the optical purity of the products.

**2.2.4. Supply of Oxygen and Hydrogen Peroxide.** In whole cell oxidations catalyzed by oxygen-dependent enzymes, oxygen supply to the cell might be a serious limitation to a highly productive process, because of the limited solubility of oxygen in water and because oxygen is consumed both as a substrate and for endogenous cell respiration. This is especially true, when using high cell density and/or cells with high specific enzymatic activity. Partial solution of this problem can be achieved by increasing oxygen pressure (which, however, might conflict with process safety) and by optimization of oxygen sparging.[54,55] An additional problem, especially evident with isolated enzymes, is represented by the fact that oxygen (or air) bubbles can cause significant hydrodynamic stress at the gas−liquid interface, which can be harmful or even lethal to enzymes and cells. To overcome this drawback, bubble-free oxygenation to dissolve oxygen in the liquid containing oxygen-dependent biocatalysts such as cyclohexanone monooxygenase[56] and laccase[57,58] has been adopted. With cyclohexanone monooxygenase, bubble-free aeration was obtained by using a thin silicone tube,[56] whereas with laccase, either silicone tube[57] or a composite membrane[58] was employed.

The inherent instability of heme peroxidases toward hydrogen peroxide, which oxidizes enzyme porphyrin ring, hampers the synthetic applications of these enzymes.[59,60] Attempts to solve this problem by protein engineering methods, such as directed evolution and site-directed mutagenesis, have been only partially successful, because increase of enzyme stability has been obtained at the expense of activity and affinity values (see Gil-Rodriguez et al., and references therein).[61] In preparative-scale applications, enzyme stability can be notably improved by maintaining a low concentration of hydrogen peroxide in the reaction medium, through stepwise or continuous addition of the oxidant.[59,60] Positive results have also been obtained by in situ generation of hydrogen peroxide by means of an oxidase such as, for example, glucose oxidase.[60]

## 3. DEHYDROGENASES

The NAD(P)H-dependent oxidoreductases have been attracting increasing interest from organic chemists because of their high specificity as catalysts for the oxidoreduction of a variety of hydroxyl and carbonyl compounds. Their main synthetic application is in the stereoselective asymmetric synthesis of enantiomerically enriched alcohols from their achiral carbonyl precursors, usually catalyzed by alcohol dehydrogenases. As this topic is discussed elsewhere in this issue,[4] in the following we will present examples related to the modification of natural compounds.

### 3.1. Hydroxysteroid Dehydrogenases

Nature is a tireless architect of complex chemical structures that, quite often, have been found useful to improve human health and well-being. Among them, steroids are probably the most widely investigated family of bioactive compounds. Their rigid tetracyclic structure decorated with sensitive functional groups has attracted synthetic organic chemists, who have regarded these molecules as ideal targets to develop and/or to apply new selective reactions.

In nature, the regio- and stereoselective interconversion of secondary alcohols to ketones on the steroid nucleus and side chain is catalyzed by a group of NAD(P)H-dependent oxidoreductases, the so-called hydroxysteroid dehydrogenases (HSDHs).[62]

In addition to their biochemical interest, these enzymes have initially found applications in analytical chemistry for the quantitative determination of steroids in serum or other physiological

fluids.[63] Evaluation of steroid concentration was performed by spectrophotometric, fluorometric, and bioluminescence assays, the latter method allowing the detection of as little as picomoles of steroids using enzymes coimmobilized on nylon tubes.[63d]

The synthetic exploitation of these enzymes has been related to the selective transformations of bile acids, that is, cholic acid (**1**, Figure 7), chenodeoxycholic acid (**2**), and ursodeoxycholic acid (**3**).[64] These compounds represent the major quantitative pathway by which cholesterol is metabolized in the human body and have important pharmaceutical applications related to their ability to solubilize cholesterol gallstones, particularly **3**. Thanks to their carboxylic group, bile acids are water-soluble at mild alkaline pH, and therefore enzymatic oxido-reductions on these compounds have been mainly performed in aqueous solutions with the in situ enzymatic regeneration of the nicotinamide cofactors NAD(P)(H).[6]

As an example, Figure 8 shows the regio- and stereospecific reductions of dehydrocholic acid (**4**) with different HSDHs.[65]

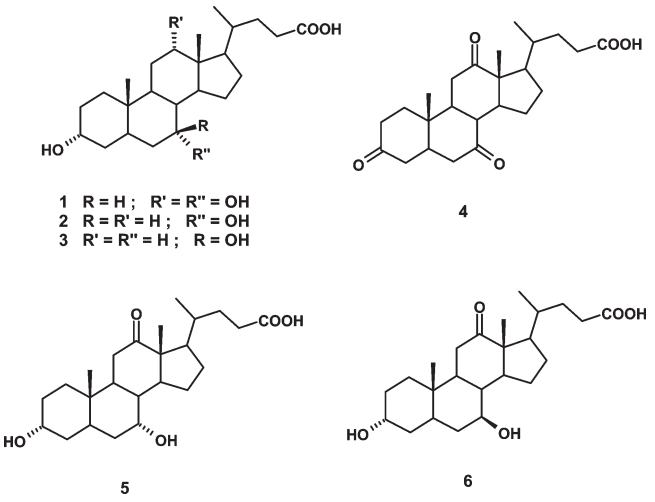


**Figure 7.** Compounds **1**–**6**.

The reduced products were obtained quantitatively, and, by using a suitable deuterated substrate donor for cofactor regeneration (i.e., 1-D-glucose and glucose dehydrogenase), it was also possible to synthesize selectively deuterated steroids.[66] Quantitative regioselective oxidation of cholic acid (**1**) at each of the three possible hydroxyls was similarly described.[65a] In more recent examples, new HSDHs have been isolated and shown to be able to catalyze the same regio- and stereospecific oxidoreductions.[67] A 12α-HSDH was also used for the preparative-scale oxidation of Gd(III) complexes of ligands containing derivatives of 7,12-dihydroxy or 12-hydroxy cholanoic acids.[68] Moreover, the hydroxyl α/β inversion at C-3 or C-7 of the steroid skeleton was obtained in a two-step enzymatic process (Figure 9).[69]

Compound **5**, 12-ketochenodeoxycholic acid, a key intermediate in the industrial chemical route used for the transformation of **1** into the bioactive derivative **3**, was enzymatically prepared from **1** in just one step by using 12α-HSDH.[65a,70] Following optimization of the reaction conditions, **5** was obtained on a multikilogram scale in a membrane reactor containing an initial 4% w/v solution of cholic acid (**1**). Free NADPH-dependent 12α-HSDH and its ancillary enzyme glutamate dehydrogenase were retained inside the reactor and reused for several cycles (up to 50). Later, a different $NADP^+$-regeneration system, based on the use of alcohol dehydrogenases together with a large excess of acetone, has been proposed.[71]

Alternative routes to ursodeoxycholic acid (**3**) have been investigated by combining multistep chemical and enzymatic transformations.[72] In the most recent example, the HSDHs-catalyzed one-pot synthesis of 12-ketoursodeoxycholic acid (**6**) from cholic acid (**1**) has been described, using five enzymes working together.[72d] As shown in Figure 10, the goal has been achieved by alternating oxidative and reductive steps in a one-pot system employing HSDHs with different cofactor specificity, NADH-dependent HSDHs in the oxidative step and a NADPH-dependent 7β-HSDH in the reductive one. Employing the enzymes both in free and in compartmentalized form, coupled in situ regeneration systems have been exploited not only to allow the

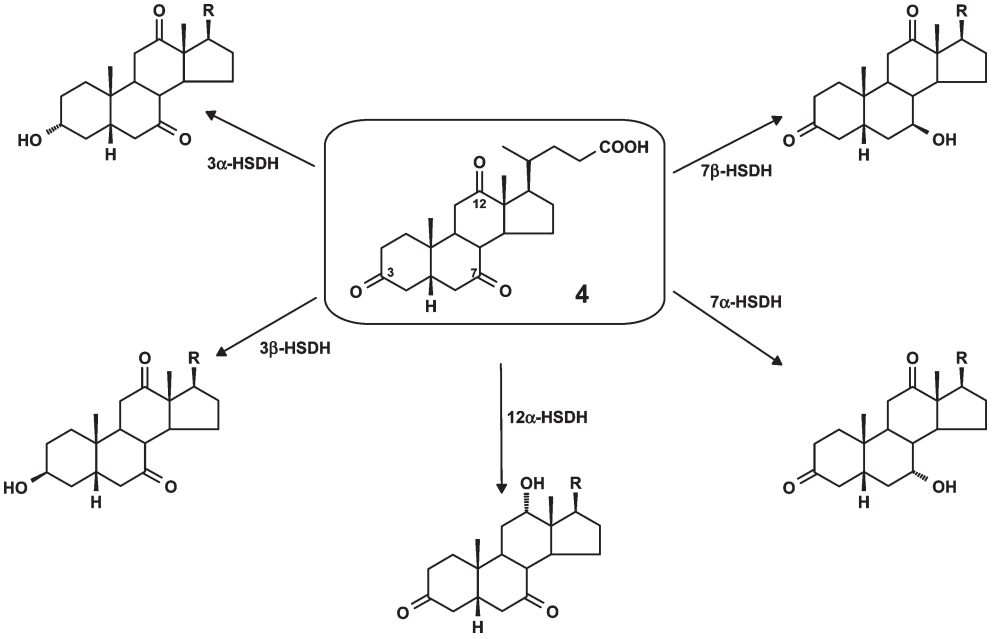


**Figure 8.** Regio- and stereospecific reduction of dehydrocholic acid (**4**) catalyzed by different HSDSs.


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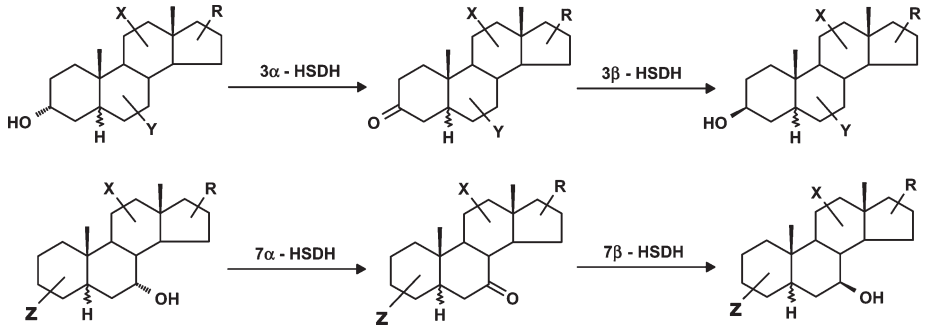


**Figure 9.** 3α/3β-OH and 7α/7β-OH inversions catalyzed by HSDHs.

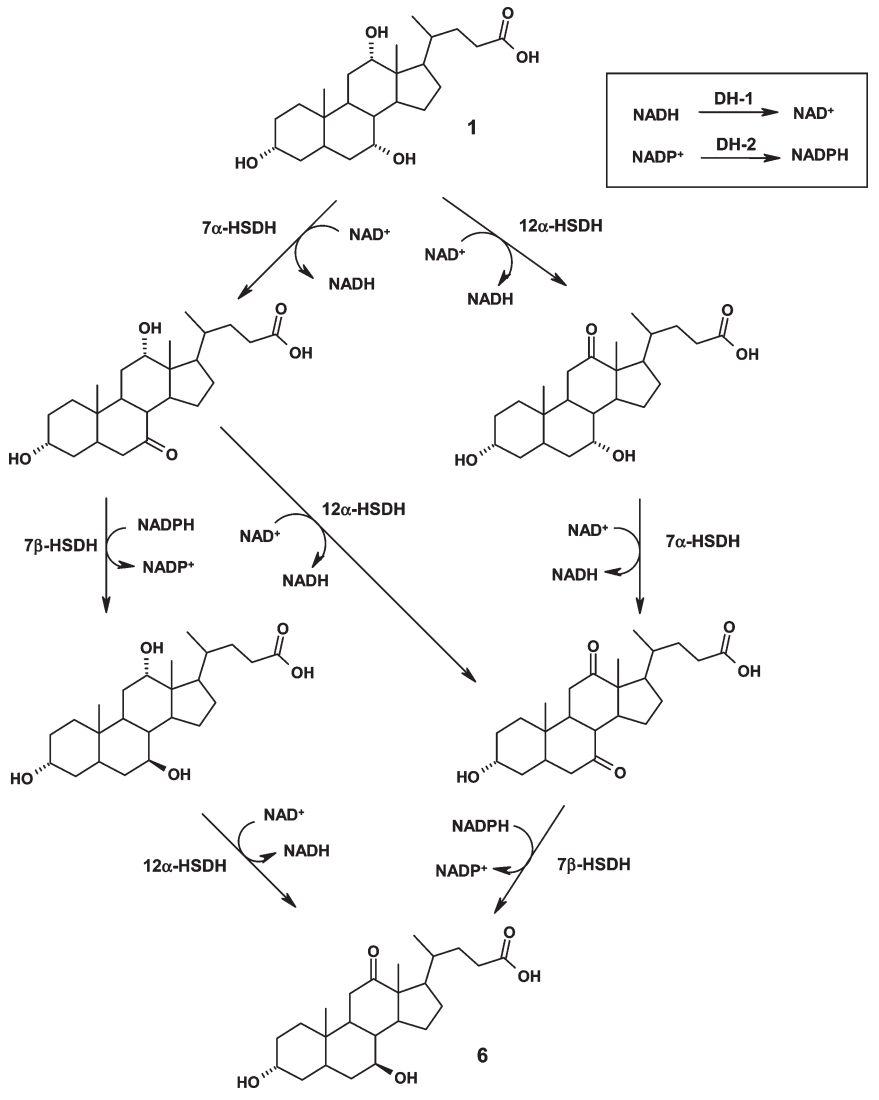


**Figure 10.** Alternative pathways and possible intermediates in the one-pot multienzymatic synthesis of 12-ketoursodeoxycholic acid (**6**) from cholic acid (**1**).

use of catalytic amounts of the cofactors, but also to provide the necessary driving force to opposite reactions (i.e., oxidation and reduction) acting on different sites of the substrate molecule.

As was previously discussed, the scaling up of dehydrogenase-catalyzed reactions can theoretically be performed in different reactors, using free or immobilized enzymes, neutral or charged membranes, and native or modified nicotinamide cofactors. In this respect, an interesting solution was given by the immobilization of native dehydrogenases into isoelectric traps, formed by pairs of isoelectric membranes encompassing their pI values. The enzyme (in the example a 3β-HSDH) was forced to perform its catalytic activity in an electric field coupled orthogonally to a


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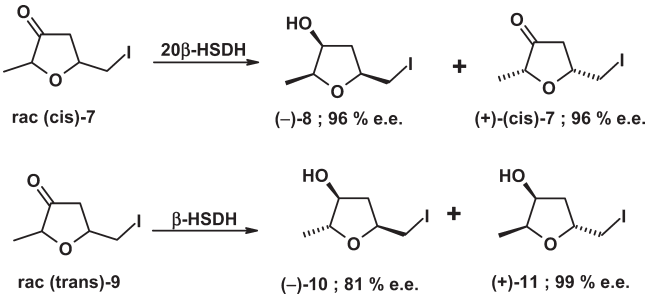


**Figure 11.** Stereoselective reduction of racemic muscarine precursors **7** and **9** by HSDHs.

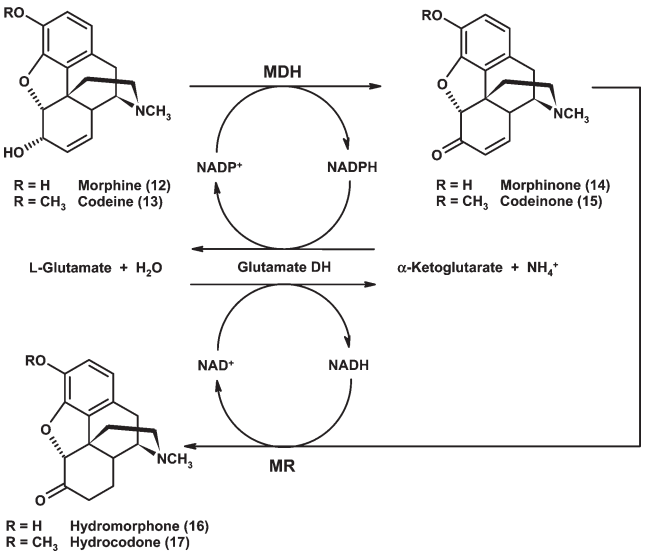


**Figure 12.** Enzymatic synthesis of hydromorphone (**16**) and hydrocodone (**17**). MDH, morphine-6-dehydrogenase; MR, morphinone reductase.

hydraulic flow, which allowed the continuous transportation and harvesting of the charged bile acid product, by the voltage gradient, into neighboring chambers.[73]

HSDHs are also active on neutral steroids. To overcome the very low water solubility of these compounds, innovative (by those times) two-phase systems were described in the late 1970s,[74] the reaction conditions being optimized with series of androstane and pregnane derivatives.[41,42,75]

Finally, it has to be pointed out the broad substrate specificity of this group of oxidoreductases when used in vitro. In fact, HSDHs, in addition to the previously described action on their natural steroidic substrates, work equally well on "unnatural" substrates. For instance, the preparation has been reported, in high enantiomeric excesses, of the chiral synthons shown in Figure 11, precursor of the eight stereoisomeric muscarines.[76] Moreover, previously these enzymes had been used for the enantioselective reduction of a series of bicycloheptenones.[77]

### 3.2. Miscellanea

As previously discussed (section 2.1.1), various dehydrogenases, belonging to different subclasses, are used for the efficient in situ enzymatic regeneration of the nicotinamide cofactors.

Other enzymes have found niche applications for the selective modification of natural compounds. For instance, this is the case

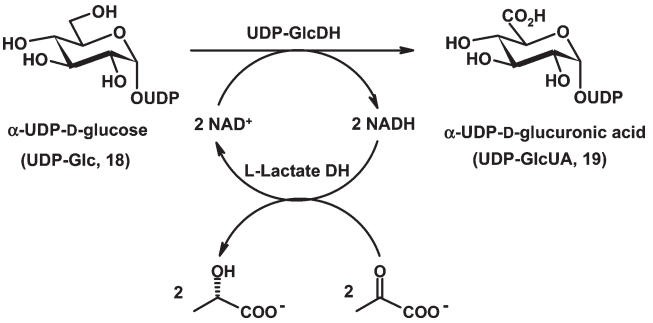


**Figure 13.** Preparative-scale synthesis of UDP-glucuronic acid (**19**).

of morphine-6-dehydrogenase (EC 1.1.1.218),[78] which has been proposed for the synthesis of semisynthetic analgesic opiates from the naturally occurring alkaloids morphine and codeine[79] and for the detections of the latter compounds using a specific biosensor.[80] Figure 12 shows the proposed approach to hydromorphone (**16**) and hydrocodone (**17**) exploiting the NADPH-dependent morphine-6-dehydrogenase (MDH), a NADH-dependent morphinone reductase (MR), and a cofactor aspecific (accepting both NADH and NADPH) glutamate dehydrogenase for the regeneration of both cofactors.[41,81]

Several dehydrogenases catalyze the selective oxidoreduction of different sugars. Information on the specific enzymes might be found in the Websites reported in ref 1. Quite interesting from a synthetic point of view is the uridinediphosphate-glucosedehydrogenase (UDP-GlcDH) (EC 1.1.1.22), which in vivo oxidizes UDP-glucose (UDP-Glc, **18**) to UDP-glucuronic acid (UDP-GlcUA, **19**),[82] and that has been used in vitro (coupled with lactate dehydrogenase for the in situ regeneration of $NAD^+$) to catalyze the same reaction on a multigram scale (Figure 13).[83] UDP-GlcUA is the activated sugar donor of glucuronyltransferases, the enzymes that in nature catalyze the glucuronidation of xenobiotics, making them soluble, and of hyaluronic synthase, which synthesizes the polysaccharide hyaluronic acid. UDP-GlcUA is quite expensive, and, moreover, to modulate the in vivo synthesis of glucuronates, it has a quite strong inhibitory effect on the enzymes that use it as a substrate. For this reason, in situ regeneration cycles of UDP-GlcUA in combination with glucuronyltransferases have been reported. Whereas a crude liver homogenate was used for the synthesis of $\beta$-glucuronides,[84] a well-defined enzyme combination was established for the synthesis of hyaluronic acid.[85] In the latter approach, exploiting the combined action of seven enzymes to regenerate in situ both of the sugar donors (UDP-GlcUA and UDP-*N*-acetyl-glucosamine, UDP-GlcNAc), a synthetic sample of hyaluronic acid was produced with an average molecular weight of $5.5 \times 10^5$ Da, corresponding to a degree of polymerization of 1.500.

## 4. OXIDASES

Oxidases are enzymes, which catalyze oxidation reactions using molecular oxygen as the electron acceptor and reducing it either to hydrogen peroxide or to water (without incorporation of oxygen atom(s) into the substrate).

To this subclass belong the alcohol oxidases, the amine oxidases, and the amino acid oxidases that will be discussed in detail elsewhere.[3] Our discussion will focus on two examples of sugar oxidases (among the many[1,86] that are classified as EC 1.1.3.d) and laccases (EC 1.10.3.2). Another interesting group


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acting on diphenols and related substances, the catechol oxidases (also known as polyphenol oxidase or tyrosinase, EC 1.10.3.1), will be presented in section 5.2.

### 4.1. Galactose Oxidases

Galactose oxidase (GAO) is a well-studied member of the family of extracellular radical monocopper oxidases of fungal origin that was first described in the late 1950s.[87] These enzymes perform two-electron redox chemistry,[88] coupling the oxidation of primary alcohols to aldehydes with the reduction of $O_2$ to $H_2O_2$. The apparent paradox, a two electron oxidation catalyzed by a single copper atom, is explained by the presence of a tyrosyl radical unit in the protein active site, which acts as a second redox center during the catalytic cycle. The physiological role of GAO is not clear, although it could be involved in the production of $H_2O_2$ for further use as a co-oxidant for lignin- and cellulose-degrading peroxidases and/or as an antibiotic defense within the rhizosphere. Several reports have been published detailing investigations into the enzyme's structure[89] and its mechanism,[90] and, consequently, into possible biomimetic metalloorganic catalysts.[91]

Figure 14 shows the GAO-catalyzed oxidation of D-galactose (**20**) at the C-6 position in the presence of oxygen to give D-galactohexodialdose (**21**) and hydrogen peroxide. The byproduct hydrogen peroxide usually must be destroyed with catalase to avoid inactivation of the enzyme. It is noteworthy the fact that the reaction stops at the oxidation stage of the aldehyde and does not proceed to the acid.

Galactose oxidase is an efficient oxidizing enzyme for substrates containing an accessible D-galactose moiety, as confirmed by the reported oxidation of galactosides, melibiose, raffinose, lactose, and lactitol. In addition, in the 1980s it was shown that the commercially available GAO from *Dactylium deudroides* could catalyze the stereospecific oxidation of glycerol, 3-halogenopropane-1,2-diols, and polyols to the corresponding aldehydes.[92] More recently, the scope and limitations of GAO for the enzymatic synthesis of aldehydes have been investigated in detail.[93] From the outset, it has been reported that the enzyme could not oxidize non-D-galactose-based carbohydrates, whereas it could work, although at widely varying velocities, with shorter polyol chains, which can be twisted to form a conformation similar to the D-galactose C4−C6 fragment (e.g., glycerol).[94] Beyond these substrates, its scope is severely limited, and it shows no reactivity at all for the more hydrophobic primary alcohols like 4-penten-1-ol.

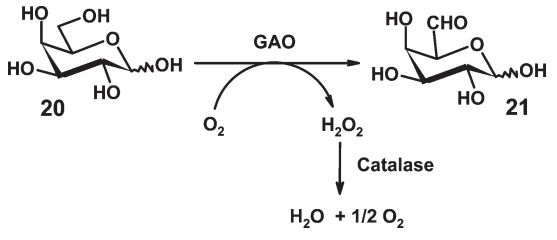


**Figure 14.** GAO-catalyzed oxidation of galactose (**20**).

As the C-6 OH oxidation products obtained from galactose and lactose are dialdehydes, their use as potential protein cross-linkers has been suggested, as a food-grade alternative to glutaraldehyde.[95]

In recent synthetic applications, GAO has been applied in chemoenzymatic one-pot cascade reactions without intermediate recovery steps. Examples are related to the preparation of galacturonates,[96] 4-deoxy-D-glucosides,[97] and, as shown in Figure 15, of biotinylated nucleotide sugars (**24**, **25**) to be used as labeled substrates for glycosyltransferases.[98a] In a subsequent paper, the same group reported on the combination of UDP-GlcNAc-4′-epimerase and GAO for the one-pot chemoenzymatic synthesis of the same biotinylated nucleotide sugars starting from the cheaper UDP-Glc and UDP-GlcNac.[98b] Other authors have described the tandem use of GAO and glycosidases.[99]

It deserves to be mentioned the exploitation of GAO for the selective modification or synthesis of polysaccharides containing galactopyranosidic residues. Back in the 1970s, it was described the combined use of GAO and tritiated potassium borohydride as a method for labeling pectic polysaccharides.[100] Recently, GAO was used in combination with catalase and horseradish peroxidase to selectively oxidize the C-6 hydroxyls of terminal galactose in a series of polysaccharides including spruce galactoglucomannan, guar galactomannan, larch arabinogalactan, corn fiber arabinoxylan, and tamarind seed xyloglucan.[101] New sugar-containing polymers were prepared by a chemoenzymatic strategy involving the GAO-catalyzed oxidation of galactose, followed by the in situ condensation of the formed dialdehyde with alkyl diamines and by the chemical reduction of the formed polyimines with $NaBH_3CN$

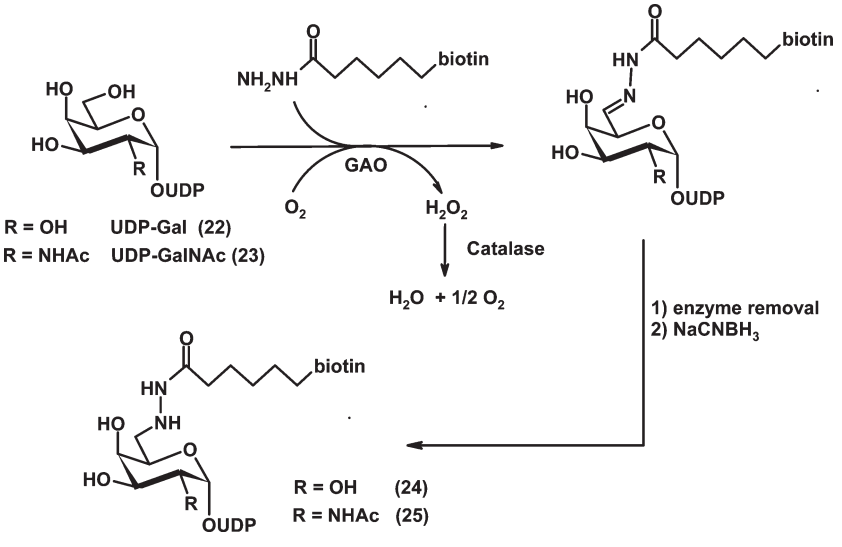


**Figure 15.** Chemoenzymatic synthesis of the biotinylated nucleotide sugars **24** and **25**.


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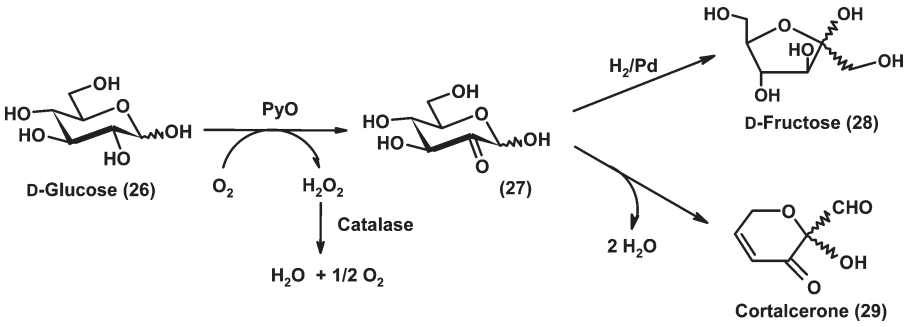


**Figure 16.** Examples of chemoenzymatic processes involving pyranose oxidase.

to give AA−BB-type polymer. Alternatively, GAO-catalyzed oxidation of galactosamine followed by chemical reduction gave an A−B-type polymerization.[102]

The main synthetic limitation of GAO is its lack of activity toward the C-6 OH oxidation of glucose and of its derivatives (the already mentioned glucose dehydrogenase (section 2.1.1) and its cognate glucose oxidase (EC 1.1.3.4) both act on position C-1). To overcome this drawback, several laboratories all around the world are applying the modern techniques of directed evolution to produce mutants with the desired activity toward unnatural substrates and improved performances toward the classical ones.[103] In this way, it has been possible to isolate and characterize mutants with improved kinetic performances (mainly lower $K_M$ values) for the natural substrates,[103b] with (still minor) activity toward glucose and glucose derivatives,[103c] and catalyzing the enantioselective oxidation of racemic secondary alcohols.[103a]

### 4.2. Pyranose Oxidases

Pyranose oxidases (PyO, EC 1.1.3.10) are flavoproteins, which are expressed in many lignin-degrading white rot fungi.[104] Unlike glucose oxidase, which acts on the anomeric carbon, they oxidize glucose and a number of common monosaccharides at their C-2 OH in the presence of oxygen to give the corresponding 2-keto sugars and hydrogen peroxide. It is believed that in these fungi this transformation is important to provide $H_2O_2$ for their lignin-decomposing peroxidases.

Since its first characterization in the late 1960s,[105] the importance of PyO for various biotechnological applications has been recognized, spanning from analytical to synthetic carbohydrate chemistry.[106] As an example of a patented process, Figure 16 shows the oxidation of glucose that, under optimal conditions, is quantitatively converted to the 2-keto derivative glucosone (**27**), which, in turn, can be transformed into fructose (**28**) by chemical reduction.[106] When applied to D-galactose, this process furnishes the rare sugar D-tagatose, whereas further examples have been described with other carbohydrates to produce sugar-based synthons.[107] On the other hand, when glucosone is treated under elimination conditions, the $\beta$-pyranone antibiotic cortalcerone (**29**) is produced.[108]

It has been shown that PyO can also use various quinones, complexed metal ions, and radicals as its electron acceptor.[109] Actually, some of these compounds are even better substrates for the enzyme than oxygen, suggesting that PyO can also play a direct role in the reduction of quinones during the process of ligninolysis.[110] It has been proposed that this excellent reactivity of PyO with alternative electron acceptors and a range of sugar substrates might be exploited in various attractive applications such as a biocomponent in biofuel cells. To this end, work is in progress to improve the catalytic performances of the wild-type enzymes from different sources.[111]

The promiscuity toward electron acceptors makes PyO similar to other FAD-containing sugar oxidoreductases, probably all fulfilling similar biological functions in lignocellulose breakdown, which have been classified as "dehydrogenases with other acceptors (EC 1.1.99.d)", such as cellobiose dehydrogenases (CDH, EC 1.1.99.18)[112] and pyranose dehydrogenases (PyDH, EC 1.1.99.29).[113]

The former enzymes oxidize the disaccharide cellobiose to cellobionolactone, but accept also other $\beta$-1,4-linked disaccharides (like lactose), oligosaccharides, and even cellulose.[114] CDH has presently only found technical use in highly selective amperometric biosensors with lower unspecific noise reactions for the measurement of lactose.[115]

As compared to pyranose oxidases, PyDHs display broader substrate specificity and a variable regioselectivity and are unable to utilize oxygen as electron acceptor using substituted benzoquinones and (organo) metallic ions instead.[113] Depending on the structure of the sugar in pyranose form (mono/di/oligosaccharide or glycoside) and the enzyme source, selective monooxidations at C-1, C-2, C-3, or dioxidations at C-2,3 or C-3,4 of the molecule to the corresponding aldonolactones (C-1) or (di)dehydrosugars (aldos(di)uloses) can be performed. Accordingly, new unusual sugar derivatives could be produced, isolated, and characterized.[116]

All of the described sugar oxidases are FAD-dependent enzymes. At the end of the sugar oxidation process, the reduced FADH moiety needs to be reoxidized to start a new catalytic cycle. This is not a problem when molecular oxygen is used as terminal electron acceptor, due to the simplicity of the aerobic reoxidation of the flavin cofactor. On the contrary, in processes employing a flavin dehydrogenase (like PyDH), the electron acceptor (quinones or other molecules) has to be added in stoichiometric amounts and separated from the reaction product, unless in situ regeneration systems are applied. Specifically, it has been shown that a wide range of redox mediators such as quinones and complex metal ions can be enzymatically reoxidized employing laccases, a group of oxidases that will be described in the following paragraph.

### 4.3. Laccases

Laccases (benzendiol:oxygen oxidoreductases, EC 1.10.3.2) belong to a group of polyphenol oxidases containing copper atoms in their catalytic center, which are called multicopper oxidases.[117] These enzymes have been widely described in plants and fungi (ascomycetes and basidiomycetes), where they are presumably involved in lignin synthesis and degradation processes, respectively. Additionally, laccases can play a role in fungal virulence by

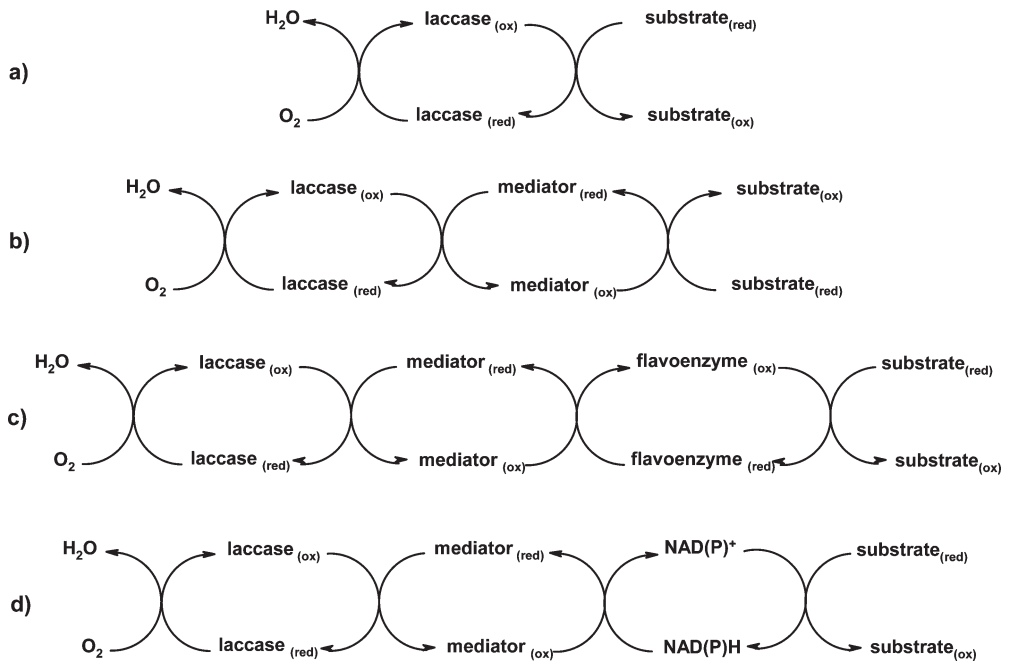


**Figure 17.** Laccase-catalyzed substrate oxidation in the absence (a) or in the presence (b−d) of chemical mediators.

detoxification from phytoalexins and tannins and in their morphogenesis. Several laccases and laccase-like proteins have also been recently found in bacteria, and their involvement in melanin production, spore coat resistance, morphogenesis, and copper homeostasis has been shown.

Laccases are able to catalyze the four-electron reduction of molecular oxygen to water, coupled with the concomitant oxidation of organic (usually aromatic) substrates (Figure 17a). The catalytic core of the enzyme, a four-copper atoms cluster, is oxidized by oxygen and is brought back to its reduced form by the oxidation of four substrate molecules to produce four radicals, which can subsequently react to give dimers, oligomers, and polymers.[118] The ability of laccases of directly oxidizing a broad range of substrates, including mono-, di-, and polyphenols, aminophenols, methoxyphenols, and aromatic amines, has been exploited in several technological applications, for example, in decontamination of industrial wastewaters containing phenol derivatives.[119] Moreover, mimicking nature, these enzymes can be used in the presence of low-molecular-weight redox mediators, whose enzymatic oxidation gives rise to stable high potential intermediates.[120] The so-called Laccase-Mediator Systems (LMS, Figure 17b) have found various technological applications, for example, in dyes removal processes in the textile, dye, and printing industries[121] and during the bleaching process in the pulp and paper industries.[122] The main advantages in using a LMS instead of the laccase alone are related to the fact that these reactive mediators can also nonenzymatically oxidize compounds with ionization potentials exceeding the redox potentials of laccases (usually in the range of 0.5−0.8 V for fungal enzymes) or bulky substrates, which could hardly penetrate into the enzyme active site. A noteworthy evolution of the LMS is shown in Figure 17c. In this case, the laccase-oxidized mediator in turn oxidizes the flavin cofactor of an oxidase in a coupled enzymatic system for the in situ regeneration of FAD.[123]

**4.3.1. Synthetic Exploitation of the Laccase-Mediator Systems (LMS).** The redox potential of fungal laccases is generally

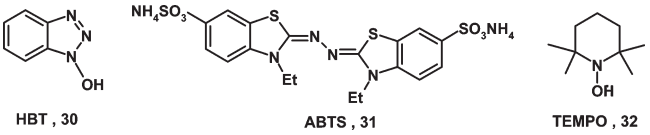


**Figure 18.** Chemical structure of three representative mediators used in LMS-catalyzed processes.

in the range 0.5−0.8 V. This makes fungal laccases unsuitable for the direct oxidation of various compounds with higher redox potential, a typical example being primary alcohols. Nonetheless, this limitation can be overcome by using the LMS: the target primary alcohol is oxidized by a redox mediator (used in catalytic amounts), which, in turn, is kept in its oxidized form by laccase catalysis (Figure 17b).[117d,124] As has been demonstrated by several authors, the different chemical structures of mediators dictate their respective mechanism of action, HBT (1-hydroxybenzotriazole, **30**, Figure 18) and ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), **31**) reacting via radical pathways, while TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy, **32**) and its analogues favor an ionic pathway.[125]

Investigations of the laccase-mediated delignification process have shown that redox mediators allow these enzymes to oxidize nonphenolic substituents in several lignin model compounds, particularly carrying benzyl alcohol groups. As a logical extension of these results, this biotransformation was applied to the oxidation of benzylic,[126] allylic,[126a] propargylic,[127] and aliphatic alcohols[126a] to the corresponding aldehydes and ketones. The evaluation of the performance of 12 different mediators in the oxidation of the model substrate 4-methoxybenzyl alcohol showed that TEMPO was the most effective compound for this kind of reaction.[128]

The laccase from *Trametes pubescens* and the chemical mediator TEMPO have been also used for the regioselective oxidation of the primary hydroxyl groups of various sugar derivatives. The efficiency of the system has been initially tested with simple


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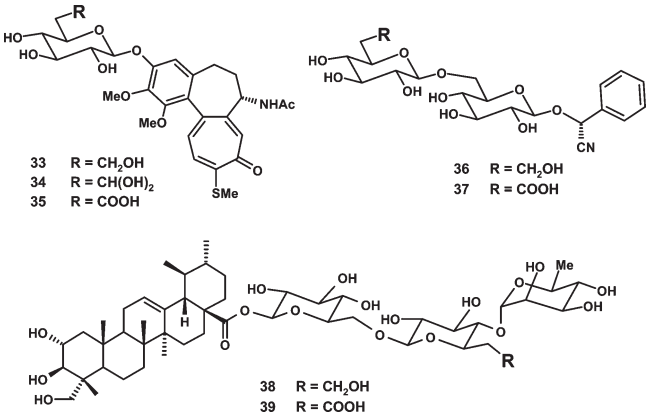


**Figure 19.** Natural glycosydes oxidized by laccases-TEMPO.

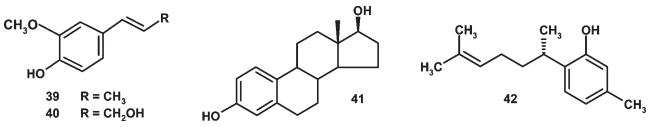


**Figure 20.** Natural phenolic derivatives oxidized by laccases.

mono- and disaccharides (i.e., methyl $\beta$-D-glucopyranoside), showing that the oxidation did not take place in the presence of only one of the two oxidative agents (TEMPO or laccase). Subsequently, this chemo-enzymatic approach has been exploited to achieve the partial oxidation of a water-soluble cellulose sample, as well as of various oligosaccharides.[129]

This methodology has been later extended to the regioselective oxidation of various natural glycosides. The influence of organic cosolvents on the stability and activity of the laccase from *T. pubescens* has been investigated with the model compound thiocolchicoside (**33**, Figure 19), only its sugar primary OH group being oxidized into the corresponding carboxylate **35** via the hydroxylated aldehydic intermediate (**34**).[130] The best reaction conditions have been then used for the selective modification of other target substrates such as amygdalin (**36**)[131] and asiaticoside (**38**).[132]

Besides alcohols, there are scant reports on the LMS-oxidations of others functional groups, such as ethers, alkenes, amides, aromatic methyl groups, and polycyclic aromatic hydrocarbons.[133] Recently, the oxidation of unsaturated lipids (fatty acids and sterols) by a laccase/HBT system has been studied.[134]

**4.3.2. Direct Oxidation of Organic Compounds Catalyzed by Laccases.** Reactive radical intermediates can be generated by laccases in the direct oxidation of phenols and further undergo self-coupling reactions leading to the formation of C−O and C−C dimers, oligomers, and, eventually, polymers. All of these compounds can be of synthetic relevance.

In most cases, a compromise between the degree of conversion of the substrate and the isolated yields is required, to avoid the extensive polymerization that may arise from the radical mechanism of the oxidative process. Moreover, due to the capability of electron-delocalized radicals to couple at various sites, different dimeric and oligomeric products can be isolated, depending on the structure of starting substrates.[135,136]

For instance, a mixture of dimeric and tetrameric derivatives has been isolated in approximately 30−40% yield from the oxidation reaction of isoeugenol (**39**, Figure 20) or coniferyl alcohol

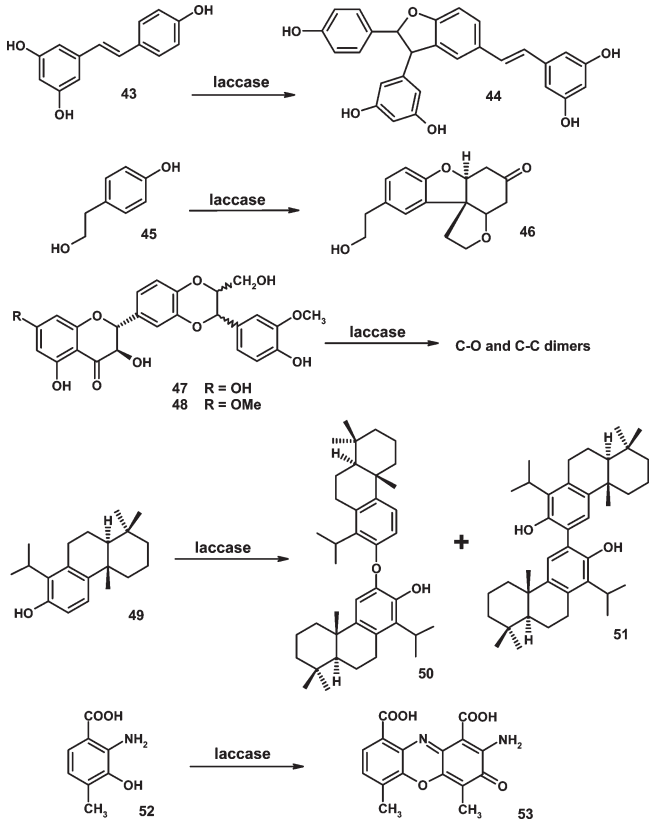


**Figure 21.** Main products isolated from the laccases-catalyzed oxidation of compounds **43**, **45**, **47**−**49**, **52**.

(**40**) in an acetone−water mixture catalyzed by *Rhus vernicifera* laccase.[137] Similarly, four C−C and C−O dimers could be isolated and fully characterized from the *T. pubescens* laccase-catalyzed oxidation of the steroid hormone 17$\beta$-estradiol (**41**) in a biphasic system with a 27% overall yield.[138] The latter transformation, catalyzed by a laccase from *Polyporus versicolor*, was also described more than 35 years ago and was one of the first examples, if not the very first, of the use of enzymes in biphasic systems.[139] The generation of products plurality can sometimes be advantageous as, for example, in the case of the biocatalyzed synthesis of the dimers of (*S*)-(+)-curcuphenol (**42**), which have been useful for the complete structural characterization of naturally occurring bicurcuphenol derivatives.[140]

In other cases, a more precise control of the oxidation process and, consequently, higher yield of the isolated dimers have been achieved. For example, the laccase-promoted dimerization of the stilbenic phytoalexin molecule *trans*-resveratrol (**43**, Figure 21) allowed the isolation of the racemic dehydrodimer **44** as a single product in 31% yield.[141] Later, a series of resveratrol-analogue hydroxystilbene derivatives have been considered, and a mechanism of laccase-catalyzed dimer-formation via radical−radical coupling has been suggested on the basis of the obtained results.[142] The accumulation of products with structures similar to the so-called "Pummerer's ketone" could be isolated in reasonable yields from *para*-alkyl phenols, such as tyrosol (**45**), to give the polycyclic dimers **46**.[143] The rate of the polymerization process can be also decreased by using selectively substituted derivatives of the target molecule. For instance, the reaction of the flavonolignan silybin (**47**) with *T. pubescens* laccase led to fast oligo- and polymerization, whereas oxidation of its 7-*O*-methyl derivative


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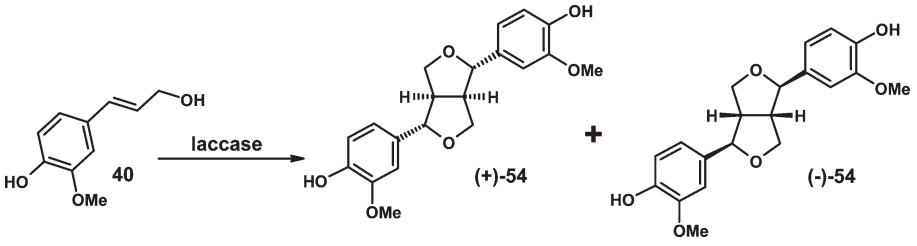


**Figure 22.** Laccase-catalyzed dimerization of coniferyl alcohol (**40**).

(**48**) provided good yields of only two dimers (C−O and C−C) in a 1:2.5 ratio.[144]

Recent works have also shown a significant and unexpected solvent influence on the nature and the relative ratio of the products obtained by laccase-catalyzed dimerizations. In a first report related to the oxidative coupling of tetrahydro-2-naphtol, the preferential formation of the symmetrical 1,1′ diphenolic product with respect to the 1,3′ dimer has been observed performing the reactions in the aromatic solvents benzene and toluene.[145] A further example is related to the laccase-mediated oxidation of (+)-totarol (**49**), a phenolic diterpenoid with interesting antibacterial activities. In this case, a "medium engineering" approach, including screening of different reaction conditions such as solvent, temperature, pH, and buffer concentration, has allowed the optimization of the conversion of **49** into the symmetrical C−C linked dimer **50**, which has been obtained with conversions up to 96% and in a 25.8-fold excess as compared to the C−O linked dimer coproduct **51**.[146] These results could be obtained by using a suitable water-miscible organic cosolvent and were obtained only in the laccase-catalyzed oxidation of **49** and not using chemical oxidants such as $FeCl_3$ or $MnO_2$.

Besides catalyzing the coupling of simple phenols, laccases have been shown to catalyze the biotransformation of other natural compounds[147] such as salicylic ester,[147a] flavonols,[147b] catechins,[147c] procyanidin (condensed tannin) type-B to type-A,[147d] penicillin X,[147e] alkaloids,[147f] 4-methyl-3-hydroxyanthranilic acid (**52**), and similar compounds, giving actinomycin-like products containing a phenoxazinone chromophore (i.e., **53**).[147g−i]

In nature, the above-described oxidative coupling of phenolic derivatives is a key step in the biosynthesis of several group of compounds like lignans, flavonolignans, and alkaloids. As previously discussed, these oxidative couplings can be reproduced in vitro. However, while in vivo the dimerization of propenylphenol derivatives to form lignans generally occurs with high regio-, diastereo-, and enantioselectivity, in vitro the enantioselectivity is negligible.[141,142] For example, the laccase-catalyzed oxidation of (*E*)-coniferyl alcohol (**40**) in vitro results in the formation of the dimer pinoresinol **54** as the main product, but in the racemic form (Figure 22).[148]

The rationale for the significant difference of the results obtained in vivo and in vitro remains an open question. However, in 1997, Lewis et al. showed that in the presence of a "dirigent" protein (DP) obtained from the plant *Forsythia intermedia* the oxidative coupling of **40** produced the enantiomerically pure (+)-pinoresinol ((+)-**54**).[149] This outstanding result has been recently reproduced by Beifuss and co-workers, who were also able to get the opposite enantiomer (−)-**54** using an enantiocomplementary dirigent protein from *Arapidopsis thaliana*.[148]

**4.3.3. Laccase-Catalyzed Cross-Coupling Domino Reactions.** Another possible reaction of laccase-generated radicals is their cross-coupling reaction with a different molecule in such a way that the reactive intermediates are trapped and do not give rise to the usual family of dimeric and oligomeric derivatives. More and more examples of these biocatalyzed domino reactions have appeared in the recent literature.

For instance, the efficient laccase-catalyzed coupling of the indolic alkaloids catharanthine (**55**) and vindoline (**56**) has been carried out. Following $NaBH_4$ reduction of the eiminium cationic intermediate, the synthetically useful dimer anhydrovinblastine (AVBL, **57**) has been isolated in 56% yield (Figure 23). The practicability of this bioconversion has been further confirmed through the condensation of catharanthine with the vindoline analogue 11-methoxydihydrotabersonine.[150]

Several other reports are based on the reactivity of substituted catechols or hydroquinones. Examples are related to the laccase-catalyzed cross-coupling of hydroquinone with mithramicine or (+)-catechin,[151] the *N*-coupling of dihydrocaffeic acid with various amines,[152] and the synthesis of Tinuvin, a benzotriazole-based UV-absorber, obtained by the coupling of 3-(3-*tert*-butyl-4-hydroxyphenyl)propionic acid methyl ester to 1*H*-benzotriazole.[153]

Laccases-generated quinones are also the key intermediates for several cascade reactions, such as diaminations,[154] Diels−Alder reactions,[155] and Michael additions.[156]

As an example of cascade diamination, Figure 24a shows the enzymatic cyclization of 2,5-dihydroxybenzoic acid (**58**) with 4,5-diaminoimidazole (**59**),[154a] whereas Figure 24b describes the reaction of methyl catechol (**61**) with a variety of dienes.[155] At variance, Figure 25 shows the laccase-initiated domino reaction of cyclohexane-1,3-diones (**62**) with catechol (**63**) for the synthesis of 3,4-dihydro-7,8-dihydroxy-2*H*-dibenzofuran-1-ones **64** (yields ranged from 70% to 97%).[156d]

**4.3.4. Miscellanea.** The use of laccases to catalyze selective oxidative deprotective reactions has been also proposed. In a first report, these enzymes have been used for the selective removal of phenylhydrazide-protecting groups in a mild process that caused neither oxidative modification nor destruction of methionine or tryptophan side chains.[157] Recently, it has been reported the oxidative deprotection (with or without mediators) of *p*-methoxyphenyl (PMP)-protected amines.[158]

As was previously anticipated, laccases can be used for the in situ regeneration of cofactors. In a recent example, the laccase from *Trametes pubescens* and the mediator 1,4-benzoquinone have been used for the oxidation of glucose to 2-ketoglucose catalyzed by pyranose-2-oxidase in a cascade process (in situ regeneration of FAD).[159] Moreover, a novel regeneration system for oxidized nicotinamide cofactors ($NAD^+$ and $NADP^+$) has been presented (Figure 17d). By combining ABTS-catalyzed oxidation of NAD(P)H with laccase-catalyzed utilization of molecular oxygen as terminal oxidant, a simple chemo-enzymatic $NAD(P)^+$ regeneration method has been achieved, to be added to the more classical system presented in section 2.1.[160]

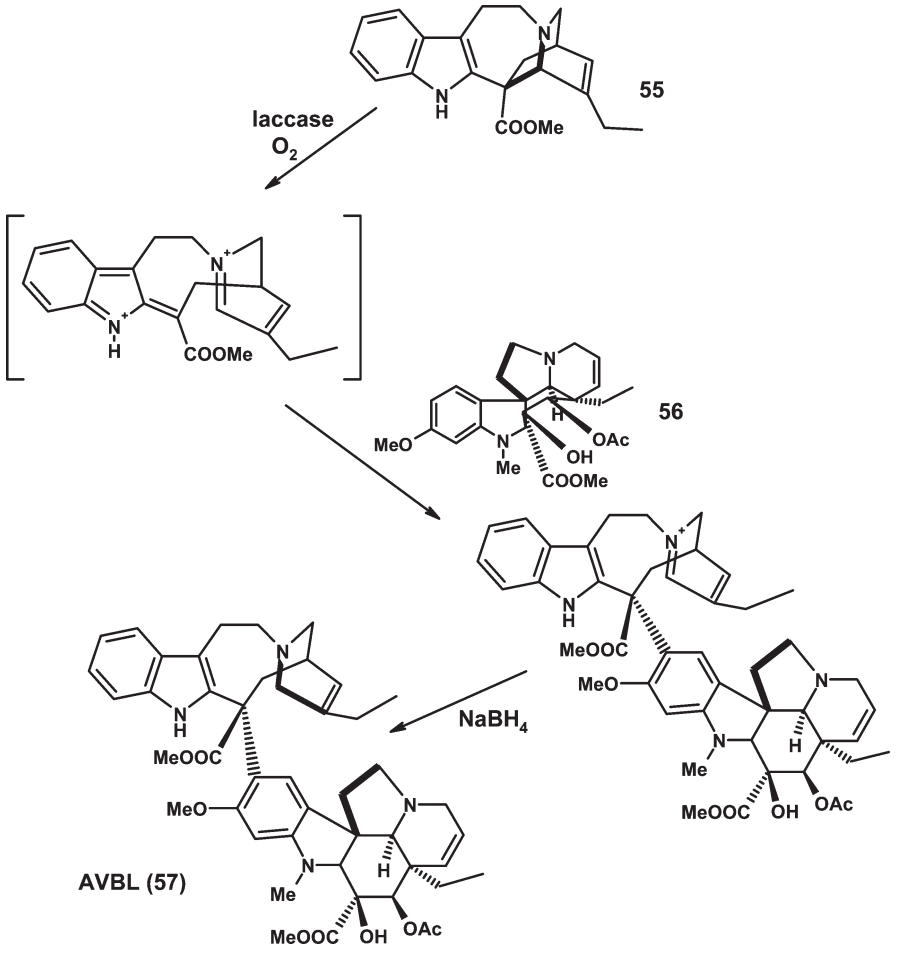


**Figure 23.** Chemo-enzymatic synthesis of the dimeric alkaloid anhydrovinblastine (57).

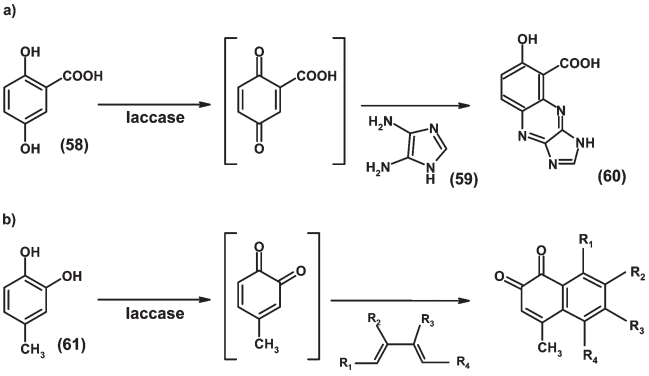


**Figure 24.** Examples of cascade diamination (a) and Diels–Alder reactions (b).

## 5. OXYGENASES

Oxygenases are oxidoreductases, which incorporate molecular oxygen. They are divided in two subclasses: EC 1.13, which incorporate molecular oxygen acting on single donors, and EC 1.14, acting on paired donors with incorporation of molecular oxygen into one or both of them. Accepted names for enzymes in both of these subclasses are also of the form "monooxygenase" and "dioxygenase", depending on the number of oxygen atoms that are transferred into the respective substrate molecules.

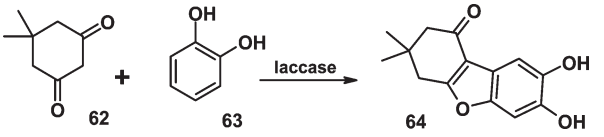


**Figure 25.** Example of cascade oxidation and Michael addition.

Reactions catalyzed by these enzymes are as diverse as hydroxylation of aliphatic and aromatic compounds, introduction of the hydroperoxid moiety, enantioselective Baeyer–Villiger reactions, sulfoxidations, amine oxidations, and epoxidations. Some of these biocatalyzed reactions have become real blockbusters in organic synthesis. Just to give an example, it is surely worth mentioning the hydroxylation at position C-11 of progesterone, catalyzed by a progesterone 11α-monooxygenase present in a strain of *Rhizopus nigricans*,[161] which in the 1950s opened the way to the industrial production of cortisone from the more available precursor progesterone.

The synthetic exploitation of several oxygenases can be only performed using whole cells systems that are not considered in this Review, whereas the Baeyer–Villiger monooxygenases are described in details in another work.[2] In the following, we will discuss two groups of enzymes, cytochromes P450 and tyrosinases.

### 5.1. Cytochromes P450

Cytochrome P450 enzymes (EC 1.14.c.d.) are a superfamily of heme-containing monooxygenases that are found widespread in


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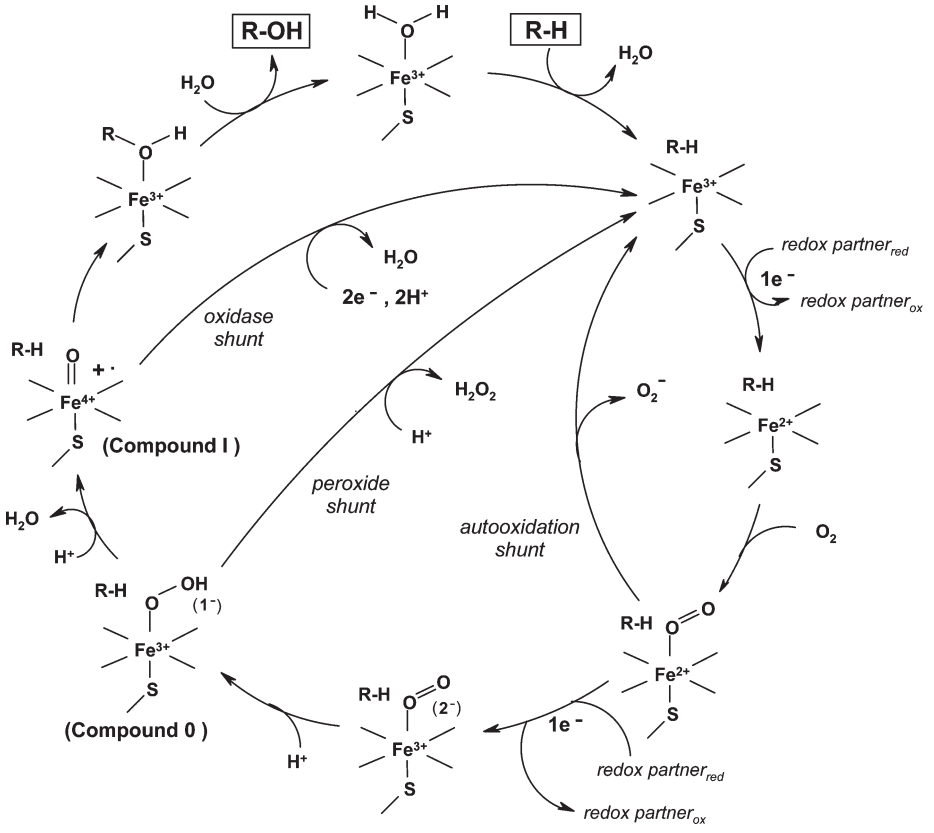


**Figure 26.** Cytochrome P450 catalytic cycle.

all kingdoms of life and play a fundamental role in the synthesis of primary and secondary metabolites, for example, steroids, antibiotics, and fatty acids, as well as in the detoxification and clearance of xenobiotics.[162−165] They catalyze a broad range of regio- and stereoselective oxidation reactions such as hydroxylations of nonactivated and aromatic carbon centers, epoxidations, dehalogenations, *N*- and *O*-dealkylations, sulfoxidations, and deaminations.

Substrate oxidation by P450 monooxygenases is started with the displacement of the distal water ligand from heme-thiolate group in its resting ferric state by substrate binding (Figure 26). The reduction of the heme iron to the ferrous state by a redox partner permits the formation of the ferrous-oxy intermediate, which is further reduced to the ferric peroxy state. This intermediate is then protonated to the ferric hydroperoxy compound (the so-called compound 0), which evolves to the ferryl-oxo state (compound I) by a second protonation, scission of the bound dioxygen, and release of a water molecule. Compound I is the reactive oxidant that allows substrate hydroxylation and the return of the heme iron to its ferric state. Finally, substrate dissociation is the result of the rebinding of a water molecule that completes the cycle.

Concerning the redox partners involved in cytochrome P450 catalytic activity, alternative types of redox systems have evolved in different organisms. In class I P450s, for example, in the *Pseudomonas putida* P450cam camphor hydroxylase system (CYP101A1), the redox partners are two distinct proteins, a FAD-containing reductase (putidaredoxin reductase) and a 2Fe-2S ferredoxin (putidaredoxin), which act in concert for the transferring of electrons from NADH to the P450. Most mammalian P450 enzymes, for example, liver hydroxylases, belong instead to the so-called class II and are bound to the endoplasmatic reticulum in close contiguity to their redox partner, a FAD- and FMN-containing enzyme (cytochrome P450 reductase), therefore forming a two-component system rather than a three-component system as in the bacterial enzymes. A similar electron transfer between FAD and FMN cofactors has been recognized in the so-called "self-sufficient" P450s, for example, in the *Bacillus megaterium* P450 BM-3 fatty acid hydroxylase (CYP102A1), which are consequently considered class II enzymes. In this case, the P450 system consists of a single soluble flavocytochrome with a multidomain structure affording an extremely rapid rate of internal electron transfer and, therefore, an efficient oxygenase activity. Other types of bacterial P450-redox partners multidomain enzymes where the N-terminal P450 is fused either to a FMN- and Fe/S-containing reductase have been recently identified.[166,167] A further different type of redox partner chain has been recently shown in *Sulfulobus* strains where electron transfer is not dependent on NAD(P)H oxidation, but rather on pyruvate dehydrogenation and is catalyzed by a 2-oxoacid-ferredoxin oxidoreductase coupled with a 7Fe ferredoxin.[168] Another peculiar P450 enzyme, P450nor (CYP55A1), produced by the filamentous fungus *Fusarium oxysporum*, catalyzes the reduction of nitric oxide by direct transfer of electrons from NADH to the heme group without participation of any NADH-linked reductase or reducing system.[169,170]

In addition to simple hydroxylation reactions, the oxidative activity of cytochromes P450 leads to a wide array of different chemical transformations (Figure 27). Epoxidations of terminal alkenes in, for example, unsaturated fatty acids are very common P450s-catalyzed reactions,[171] but they have been observed also in


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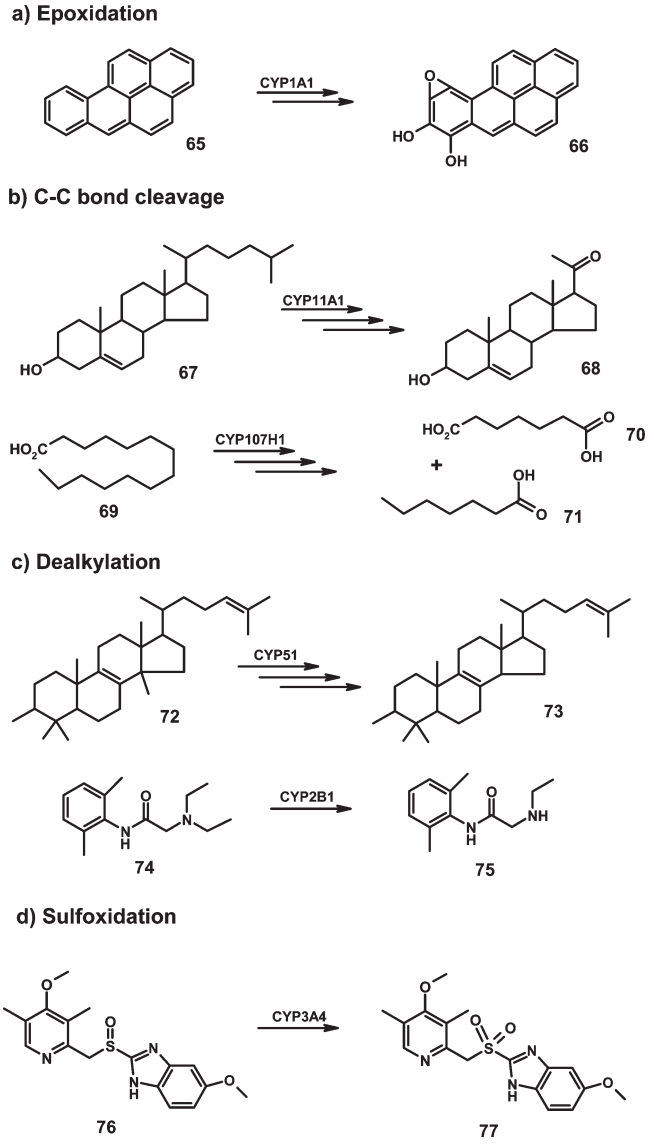


**Figure 27.** Different types of biotransformations catalyzed by cytochromes P450.

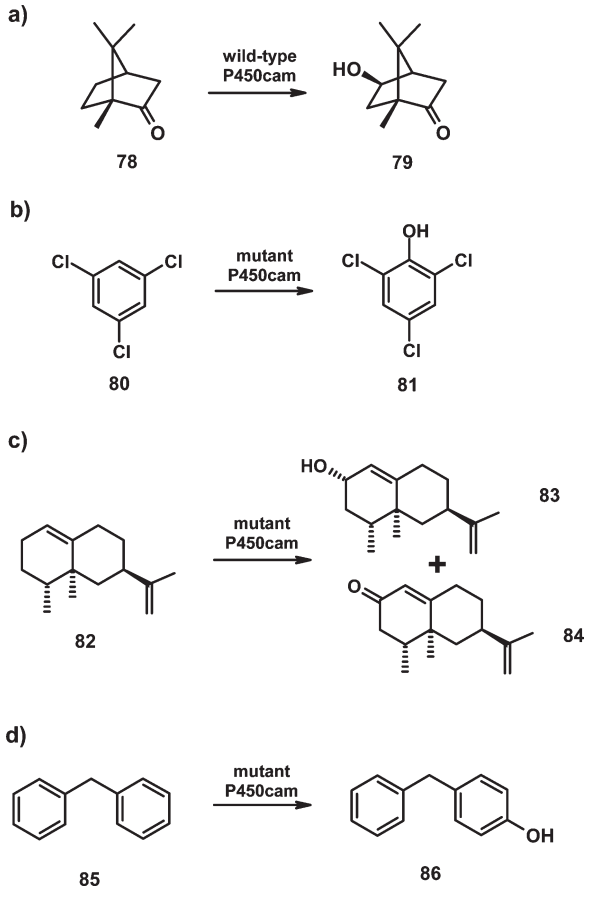


**Figure 28.** Reactions catalyzed by wild-type (a) and mutants (b–d) of cytochrome P450cam.

polycyclic aromatic compounds, as, for example, in the biotransformation of benzo[*a*]pyrene (**65**) to the carcinogenic epoxide derivative shown in Figure 27a.[172] The cleavage of C–C bonds can result as the outcome of a P450-catalyzed multistep oxidation: this has been observed in the case of the transformation of cholesterol (**67**) into pregnenolone (**68**), the first step in the biosynthesis of all steroid hormones, catalyzed by the mammalian enzyme P450scc (CYP11A1),[173] as well as in the cleavage of tetradecanoic acid (**69**) to pimelic acid (**70**), via consecutive alcohol and *threo*-diol intermediates formation, in the presence of the *B. subtilis* cytochrome P450Biol (Figure 27b).[174] Similar multistep processes have been hypothesized to occur in some dealkylation reactions catalyzed by this class of monooxygenases, for example, in the well-studied 14α-demethylation of lanosterol (**72**) to ergosterol (**73**) catalyzed by sterol demethylases (CYP51 subfamily).[175] Dealkylation reactions at different atoms, such as the *N*-de-ethylation of lidocaine (**74**) by two closely related P450s, 2B1 and 2B2,[176] have been reported as well (Figure 27c). Finally, sulfoxidation reactions have been described quite frequently in the metabolism of drugs by liver P450s, as in the case of the *S*-stereoselective oxidation of the proton pump inhibitor omeprazole (**76**) to the corresponding sulfone derivative shown in Figure 27d.[177]

During the last years, extensive effort has been devoted to protein engineering of wild-type P450 enzymes to explore and expand their substrate specificity and/or improve their stability. Most work has been carried out with two prokaryotic P450s, the *P. putida* camphor hydroxylase P450cam and the *B. megaterium* fatty acid hydroxylase P450 BM-3, both enzymes being soluble and easily overexpressed in heterologous hosts such as *E. coli*. Moreover, these P450s have been the first two members of the superfamily with an available crystal structure[178,179] and the best characterized in terms of catalytic mechanism and role of active site residues on substrate binding and catalysis.

A site-directed mutagenesis (rational) approach has been first used with P450cam, which naturally hydroxylates camphor (**78**) in the 5-exo position (Figure 28a) with a remarkable specificity. Enzyme variants with improved activity toward the oxidation of non-natural substrates have been obtained by increasing or decreasing the P450cam active-site volume and by suppressing the uncoupling pathways leading to hydrogen peroxide or water production at the expenses of reducing equivalents. Substitution of Tyr96 with more hydrophobic residues allowed the obtainment of mutants with activity toward different phenyl[180] and styrene derivatives,[181] while the double mutant engineered at both Tyr96 and Phe87 accepted polycyclic aromatic hydrocarbons such as


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phenantrene, fluoranthene, pyrene, and benzo[*a*]pyrene as substrates.[182] A further mutation at Val247 afforded a P450cam variant showing the ability of catalyzing the hydroxylation of 1,3,5-trichlorobenzene (**80**) and other polychlorinated aromatic compounds (Figure 28b).[183] The active-site cavity has been reduced in its volume by structure-guided mutations to make P450cam able to hydroxylate short-chained alkenes, such as butane and propane,[184] and even ethane to ethanol.[185] When mutations have been finalized to increase the space close to the heme group, P450cam mutants showing activity on the bulky sesquiterpene (+)-valencene (**82**, Figure 28c) were isolated.[186] The mutants were highly regioselective for C2 oxidation (>85%), the relative proportions of the main products nootkatol and nootkatone varying from 86% (+)-*trans*-nootkatol (**83**) and 4% nootkatone (**84**) for the F87A/Y96F/L244A/V247L mutant, to 38% nootkatol and 47% nootkatone for the F87 V/Y96F/L244A variant. Using a different approach, a library of 400 possible variants of P450cam has been produced by full randomization of two active-site amino acids (Y96 and F98) and coupled with GC/MS screening for product formation in the hydroxylation reaction of diphenylmethane to 4-hydroxydiphenylmethane (**85**, Figure 28d).[187]

Even more remarkable changes in substrate specificity have been achieved during the last years with the *B. megaterium* fatty acid hydroxylase P450 BM-3 by exploitation of both rational mutagenesis and directed evolution methods. Cytochrome P450 BM-3 is a self-sufficient monooxygenase that catalyzes the hydroxylation of long-chain saturated and unsaturated fatty acids ($\sim C_{12}$ to $C_{20}$) near the $\omega$ terminus (at $\omega$-1 to $\omega$-3 position) (Figure 29a). Structural analyses of enzyme crystals, both in the substrate-free and in the substrate-bound forms, showed the presence of a long hydrophobic channel and suggested important residues for substrate orientation in the catalytic intermediate and, consequently, for enzyme regioselectivity.[188] Accordingly, a single amino acidic mutation (Phe87Ala) afforded an enzyme variant able to catalyze fatty acid hydroxylation almost exclusively at the $\omega$ position, in marked contrast to the wild-type enzyme, for which no hydroxylation at this position has been observed. The importance of position 87 for substrate recognition has been further proved in epoxidation reactions of arachidonic acid[189] and 3-chlorostyrene[190] catalyzed by different P450 BM-3 mutants.

Because of the intrinsic complexity of the P450 systems, that is, the occurrence of multidomain catalysts or multienzymatic systems and the need for fine-tuning of redox coupling between hydroxylation and NAD(P)H consumption, directed evolution methods are particularly suited for the rapid obtainment of variants with new or improved activities. Moreover, self-sufficient P450s evolution can be carried out using a domain-based approach, in which the heme, FMN, and FAD domains are separately evolved and recombined in a second step. This can allow the introduction of thermostabilizing mutations in single domains that are not predictable by structural investigations and the selection of variants showing optimal intramolecular interactions among the domains.

One of the first tasks of P450 BM-3 directed evolution has been to generate variants able to efficiently hydroxylate short-chain linear alkanes such as those found in natural gas, for example, octane (wild-type P450 BM-3 displays no activity toward fatty acids smaller than $C_{12}$, but is slightly active on octane). To this aim, a screening assay for alkane oxidation activity was developed using the octane surrogate 1-nitro-4-(octyloxy)benzene as a substrate. The activity of about 2000 enzyme variants generated by error-prone PCR (ep-PCR) has been estimated on 96-well plates using a plate reader and confirmed by measuring the rate of NADPH oxidation and GC analysis of the alcohol products. A second round of error-prone PCR and screening of 3000 new clones yielded variants displaying up to 5 times the specific activity of wild-type P450 BM-3.[191] Further random mutagenesis rounds and recombination of beneficial mutations provided a mutant (53-5H) with very high activity on propane (5000 turnovers of propane to propanol at a rate of 370 $min^{-1}$) and another variant (35-E11) displaying a 5-fold increase in total turnover of ethane to ethanol.[192] Finally, individual optimization of the three domains (heme, FMN, and FAD domains) and recombination of active variants generated the so-called P450 BM-3 PMO (propane monooxygenase) variant showing activity on propane comparable to that of P450 BM-3 on its natural substrates and almost complete coupling of NADPH consumption to product hydroxylation.[193] Interestingly, the substrate specificity profile of PMO suggested that the strong selective pressure for activity toward this substrate somehow "specialized" the resulting biocatalyst for this particular reaction.[194] On the contrary, intermediate variants lying along the PMO evolution as well as BM-3 mutants from recombination experiments showed a broad substrate scope that could be exploited for the development of new P450s able to catalyze hydroxylation and/or epoxidation of various aliphatic and aromatic compounds, for example, benzene, styrene, cyclohexene, 1-hexene, and propylene.[195] A new BM-3 variant (9-10A) capable of efficient and highly enantioselective hydroxylation of different 2-aryl acetic acid derivatives (Figure 29b) was also selected from the clones produced during PMO evolution.[196] The most remarkable results were obtained in the synthesis of propyl mandelate (1640 TTN − total turnover number, 88% selectivity, 93% ee) and *m*-chloro butyl mandelate (700 TTN, 89% selectivity, 94% ee).

A panel of cytochrome BM-3 variants has been also screened for the regioselective hydroxylation of a series of cyclopentenone derivatives (Figure 29c). After flash chromatography purification, the hydroxylated products were subjected to deoxofluorination using the common nucleophilic fluorinating agent diethylaminosulfur trifluoride (DAST). Using this strategy, different fluorine sites were successfully introduced on these substrates with good to excellent regioselectivity (55−100%), and the corresponding fluorinated derivatives were recovered with 30−80% yields over the two steps.[197] In the same work, mono- and difluorination of the anti-inflammatory pro-drug ibuprofen methylester was achieved by two alternative chemo-enzymatic routes with high selectivity and efficiency at preparative scales. Moreover, as the oxidative activity of cytochromes P450 over methoxy groups resulted in a demethylation reaction and exposure of a free hydroxyl group, this chemo-enzymatic strategy has been further extended for the transformation of methoxy groups to fluorine and exploited for the preparation of fluorine-containing 5-phenyloxazoline derivatives and Corey lactones.

In another recent work, a collection of 120 different BM-3 mutants, generated by directed evolution and/or chimeragenesis and selected on the basis of their substrate specificity, has been tested for the preparation of mammalian (human) metabolites of the drugs verapamil (**87**, Figure 29d) and astemizole (**88**).[198] For each drug, hydroxylation at four different sites has been observed, leading to the corresponding hydroxylation or, in the case of activated carbon centers oxidations, dealkylation products. It has been shown that, thanks to the broad substrate specificity of the enzyme library, 12 of 13 known mammalian metabolites could be produced using the BM-3-derived collection, and seven new metabolites have been identified as well. Taking into account that

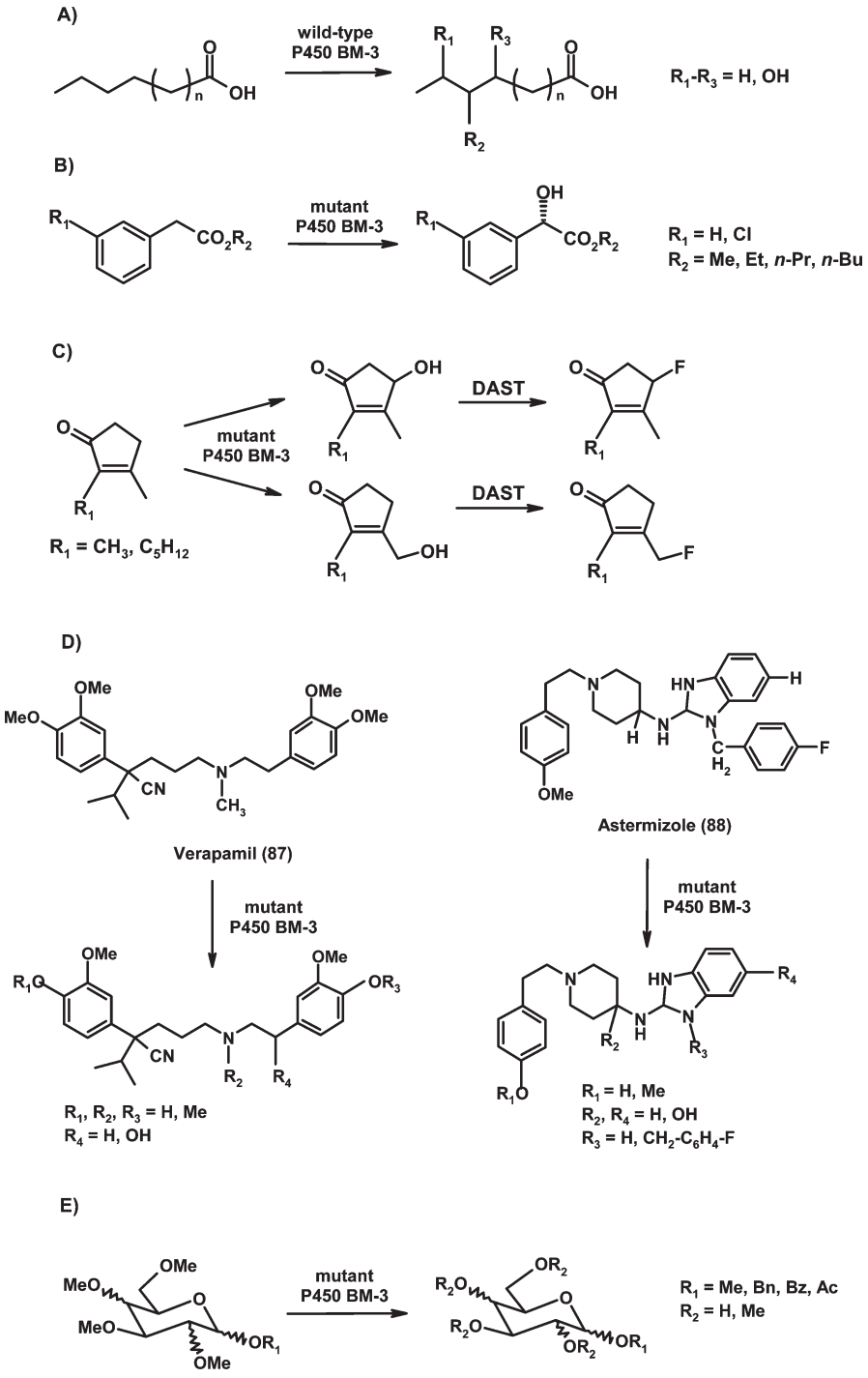


**Figure 29.** Reactions catalyzed by wild-type (a) and mutants (b−e) of cytochrome P450 BM-3.

the synthesis of drug metabolites at sufficient scale for pharmacological and toxicological evaluations in preclinical studies is not trivial and requires sometimes extensive synthetic work, it might be foreseen that exploitation of bacterial P450s for rapid identification and production of relevant quantities of the human metabolites of drug candidates will be further investigated in the future.

Cytochrome P450-catalyzed heteroatom demethylation has been also recently exploited for the regioselective deprotection of methylated monosaccharide derivatives (Figure 29e).[199] Starting from available libraries of BM-3 variants, a first screening of their reactivity toward a number of common pentamethyl hexoses (α-and β-glucose, α-galactose, and α-mannose derivatives) has been carried out using a high-throughput colorimetric assay for formaldehyde released during the reactions. The most promising reactions have been analyzed by GC to establish the regioselectivity of the deprotection reactions, and selected enzymes have been further submitted to random mutagenesis to provide total turnover numbers and regioselectivities sufficient for products recovery from preparative scale reactions. Highly selective demethylation reactions have been also achieved by substrate


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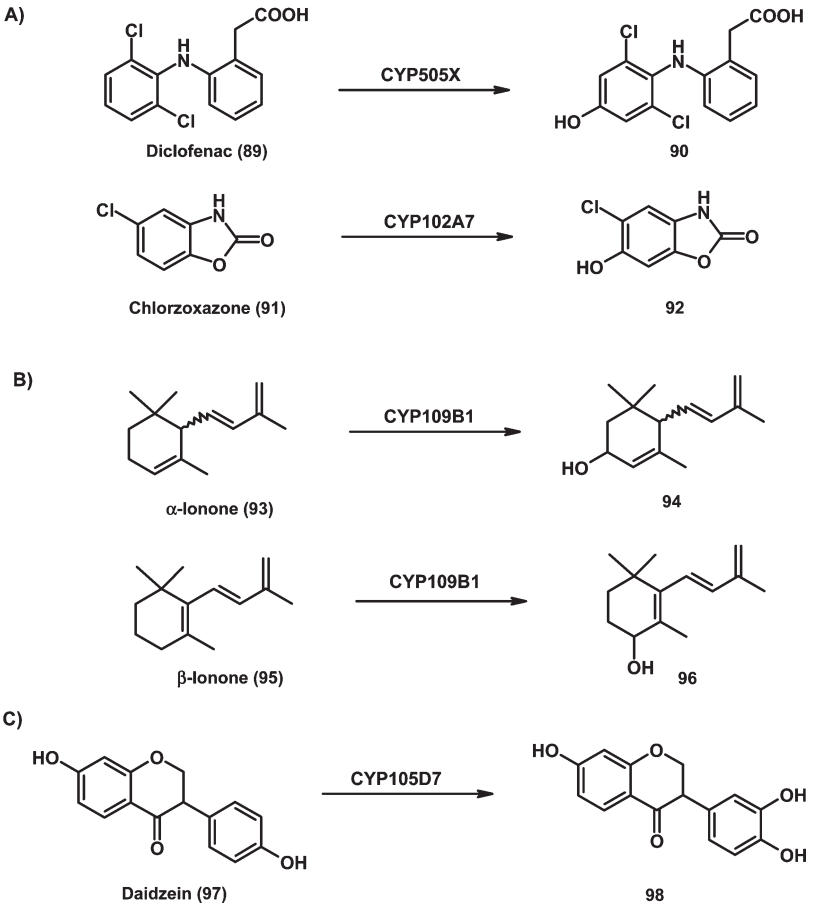


**Figure 30.** Regioselective hydroxylation reactions catalyzed by recently identified cytochromes P450.

engineering, specifically by testing a variety of anomeric substituents in the $\beta$ configuration, for example, methyl, benzyl, benzoyl, and acetyl groups.

Thanks to the deep knowledge of the contribution of single active-site residues to substrate binding and catalysis in P450 BM-3, it is now possible to generate minimal libraries by focusing on a reduced number of amino acids positions (the so-called "hotspots"), which are randomly mutated by saturation mutagenesis. This approach has been recently investigated in the search of P450 BM-3 variants able to selectively oxidize four terpene substrates, geranylacetone, nerylacetone, (4*R*)-limonene, and (+)-valencene.[200] A minimal P450 BM-3 library comprising 24 variants plus the wild-type enzyme has been constructed by combinatorial mutation of two hotspot positions (residues 87 and 328) with five hydrophobic amino acids (alanine, valine, phenylalanine, leucine, and isoleucine). The contribution of these hotspots to substrate recognition was expected to be relevant as they are located in the immediate vicinity of the heme, and their side chains strongly define the shape of the substrate binding site. Interestingly, not only have the regio- and stereoselectivity of the investigated reactions been altered by mutations, but also the chemoselectivity (hydroxylation vs epoxidation). Moreover, the biotransformations of the small acyclic terpenes neryl- and geranylacetone resulted in improved regio- and chemoselectivity with single mutants, for example, A328V, whereas a strong increase in regioselectivity in the oxidation of cyclic terpenes (4*R*)-limonene and (+)-valencene has been observed only in the presence of double mutated variants, this possibly suggesting the need of a more precise tuning of substrate−enzyme interactions for bulky substrates. Interestingly, in the case of (+)-valencene oxidation, the effects of mutations in position 87 and 328 have been shown to be cooperative instead of additive. In fact, some single mutants (F87L, A328V, and A328I) resulted completely inactive toward these substrates, while variants comprising two mutations (F87V/A328I, F87L/A328I, and F87V/A328V) showed not only activity, but also strongly increased regioselectivity.

New microbial cytochromes P450 have been recently identified and investigated as biooxidation catalysts. A sequence-based search of P450 BM-3 homologues provided 16 new self-sufficient P450s both of prokaryotic (*Bacilli*, *Ralstonia*) and eukaryotic (*Aspergilli*, *Fusarium*, *Gibberellae*, *Magnaporthe*, *Neurospora*) origin.[201] Recombinant expression in *E. coli* has been enhanced by coexpression of the GroEL/ES chaperone system; nine of these new P450s have been selected for further characterization and, according to available information for P450 BM-3, submitted to site-directed mutagenesis for activity improvement. Preparative-scale hydroxylation of diclofenac (**89**, Figure 30a) and chlorzoxazone (**91**) has been carried out using lyophilized preparations of P450s from *A. fumigatus* Af293 (CYP505X) and *B. licheniformis* ATTC 14580 (CYP102A7), respectively. Another new P450 from *B. subtilis* (CYP109B1) has been recently cloned in *E. coli* BL21-(DE3) and in vitro reconstituted with various redox system, including putidaredoxin reductase and putidaredoxin from *P. putida*, truncated bovine adrenodoxin reductase and adrenodoxin (AdR−Adx), flavodoxin reductase and flavodoxin from *E. coli*, and two flavodoxins from *B. subtilis* (YkuN and YkuP).[202,203]


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Substrate specificity of this new P450 has been investigated using a wide array of different compounds as substrates, for example, saturated and unsaturated fatty acids, *n*-alkanes and primary *n*-alcohols, and terpenes. Remarkable activity has been observed in the oxidation of the sesquiterpenoid analogues α-ionone (**93**, Figure 30b) and *β*-ionone (**95**) coupled with the AdR−Adx redox system, conversions of around 70% and 90% being achieved within 2 h, respectively. The same research group has recently cloned a new self-sufficient P450 from *Rhodococcus ruber*, consisting of a heme-containing P450 domain, a FMN-containing reductase, and a 2Fe-2S-ferredoxin. The enzyme has been submitted to directed evolution for activity improvement, and a quintuple mutant showing a 240-fold increased activity for 7-ethoxycoumarin deethylation and a 10-fold increased activity for 7-methoxycoumarin demethylation was selected by a newly developed high-throughput screening utilizing resting recombinant *E. coli* cells.[204]

Cytochromes P450-catalyzed steroid hydroxylation is a very common transformation for mammalian enzymes, but only few bacterial steroid hydroxylases have been reported up to now. Recently, a P450 from *B. megaterium* ATCC 13368 (CYP106A2) capable of hydroxylating 3-keto-4-ene steroids mainly at position C-15 has been cloned and characterized.[205] A whole-cell biocatalyst for the 15*β*-hydroxylation of various steroids has been developed by expressing simultaneously CYP106A2 and the bovine AdR−Adx redox system. ADH from *Lactobacillus brevis* has been used to implement the supply of reduced NADPH cofactor. Progesterone and testosterone conversion under optimized conditions have been carried out in batch experiments yielding overall productivity up to 5.5 g $L^{-1}$ $d^{-1}$. In a different approach, the same group has suggested the exploitation of human P450s, such as the microsomal enzyme CYP21, which catalyzes the regioselective 21-hydroxylation of progesterone, in recombinant form using the yeast *Schizosaccharomyces pombe* as a host.[206] Recombinant resting cells have proved to be efficient biocatalysts, and limitations related to substrate transport across the membranes have been overcome by cell permeabilization with detergents.

Actinomycetes P450s have not been deeply investigated up to now, but, thanks to the remarkable metabolic versatility of these microorganisms, it might be foreseen that new biocatalysts will become available soon from this source. For example, the hydroxylation of the isoflavone daidzein (**97**, Figure 30c) catalyzed by several actinomycetes strains has been investigated, and a strain of *Streptomyces avermitilis* has shown the best performances in terms of both activity and selectivity.[207] The isolation of the gene coding for the enzyme of interest was not trivial (*S. avermitilis* alone has 33 different P450s in its genome and various genes for alternative redox systems), but CYP105D7 has been finally identified as the target gene. Overexpression in *E. coli* and in *S. avermitilis* itself allowed the preparative conversion of daidzein (**97**) to the corresponding 7,3′,4′-trihydroxyflavone (**98**) with about 30% conversion yield.[208]

Recently, the high-throughput generation of libraries of redox-self-sufficient P450s has been achieved by the development of a ligation-independent cloning vector named "LICRED".[209] A range of P450 haem domains have been fused to the reductase of P450RhF (RhF-Red), previously identified in the soil bacterium *Rhodococcus* sp. NCIMB 9784, and successfully expressed in *E. coli*. A remarkable result has been obtained with the generation of a library of fusion constructs by using a family of P450s from a single prokaryotic genome, specifically the one from the soil actinomycete *Nocardia farcinica*, thus demonstrating the general applicability of this innovative platform.

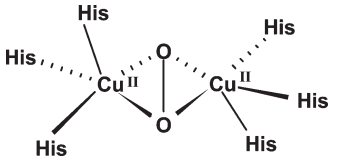


**Figure 31.** The copper cluster in the "oxy-tyrosinase".

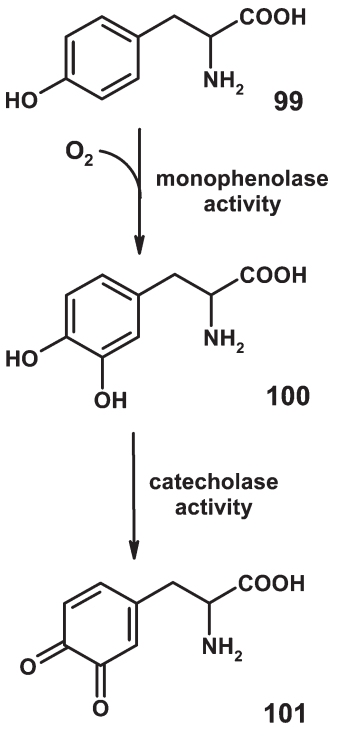


**Figure 32.** Tyrosinase-catalyzed oxidation of L-tyrosine (**99**).

## 5.2. Tyrosinases

Tyrosinases (also called several other names such as catechol oxidases, phenol, or polyphenol oxidases) are copper enzymes that are found in almost all domains of life and are responsible, for example, in the browning of vegetables and in the synthesis of melanins.[210] Because of their physiological importance, several reviews have been published, detailing what is known on their structure, catalytic mechanism, activation and inhibition, biotechnological, and synthetic applications.[211−215]

Tyrosinases bear two copper atoms in the active site, each of them coordinated by three histidine residues ("type-3 copper center"). Depending on the oxidation state of the copper atoms and the linking with molecular oxygen, the active site can exist in three different states. As an example, the copper cluster in the so-called "oxy-tyrosinase" is shown in Figure 31.

The oxidations catalyzed by these enzymes on L-tyrosine (**99**) via a complex reaction mechanism[216] are depicted in Figure 32. Tyrosinases first behave as monooxygenases on phenols, catalyzing their ortho-hydroxylations (monophenolase or cresolase activity). Subsequently, acting as oxidases, they transform the formed catechols into the corresponding quinones (diphenolase or catecholase activity). Because of these two functions, tyrosinases have


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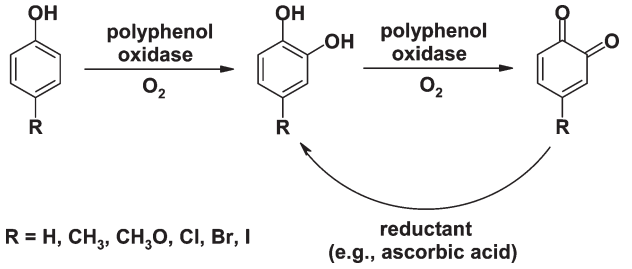


**Figure 33.** Chemo-enzymatic synthesis of substituted catechols.

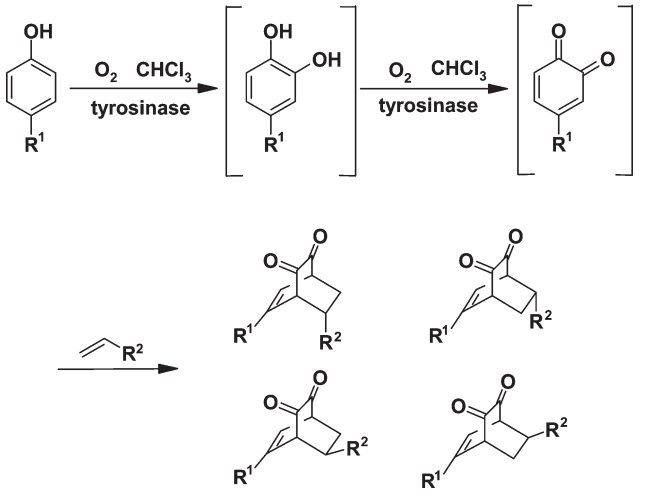


**Figure 34.** Tyrosinase-initiated Diels−Alder domino reactions.

been assigned both to subclass EC 1.14.18.1 (monophenol monooxygenases) and to subclass EC 1.10.3.1 (catechol oxidases).

The quinones formed are quite reactive molecules and, particularly in aqueous medium, generally undergo further nonenzymic reactions resulting in polymerization, leading to the formation of high-molecular mass melanins and related poly phenolic pigments. Quinones reactivity is more and more exploited for the modification of natural textile fibres, and several examples on the tyrosinase-catalyzed grafting of these materials in the presence of phenolic compounds have been published.[217−219] A recent report also deals on quinones that are able to induce DNA cross-linking.[220]

The reactivity of quinones plays also a pivotal role in the main applications of tyrosinases. In cases in which the synthetic target is the production of catechols from phenolic precursors (efficient *ortho*-hydroxylation of phenols is still not easily achieved by chemical synthesis), the quinones must be reduced in situ. This can be done by performing the reactions in the presence of a reductant, such as ascorbate or hydroxylamine, and of borate anions (which form complexes with catechols).[221−223] In the 1980s, Klibanov showed that it is possible to use a mushroom tyrosinase adsorbed on porous glass to catalyze the oxidation of a number of water insoluble phenols to *o*-quinones in chloroform.[224] While in water the enzymatic oxidation resulted in negligible yields due to the spontaneous polymerization of the quinones, a quantitative conversion was achieved in chloroform. The quinones produced were then nonenzymatically reduced to catechols by washing the organic phase with an ascorbate water solution (Figure 33). The results of further investigations on the behavior of tyrosinases in water-restricted media have been published more recently.[225−227]

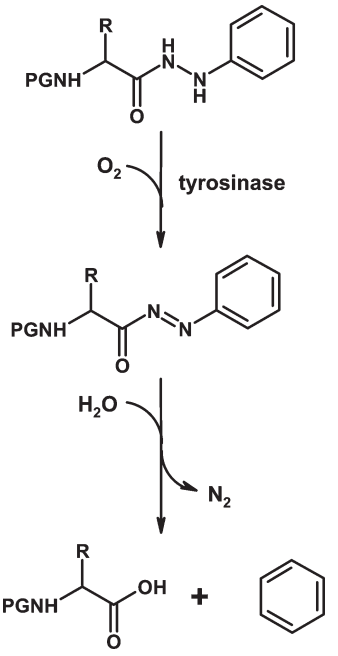


**Figure 35.** Enzymatic cleavage of amino acid phenylhydrazides.

In different applications, the reactivity of quinones is exploited for obtaining selective cascade reactions. For instance, as shown in Figure 34, the in situ-generated *ortho*-quinones underwent a Diels−Alder reaction in the presence of different dienophiles. This three-step reaction cascade allowed the preparation of functionalized bicyclo[2,2,2]octene-diones in a rapid and efficient manner.[228] A similar example has been more recently described using a crude extract of the mushroom *Agaricus bisporus*.[229]

Inter- or intramolecular autocondensations of reactive quinonic intermediates carrying nuclephilic moieties have been reported to give new polycyclic compounds, as exemplified by the oxidation of hydroxytyrosol[230] and of *N*-alkyl-4-aminobutylcatechols,[231] respectively.

Tyrosinase-catalyzed oxidation of natural phenolic derivatives is likely to occur in vivo. The behavior of some of these compounds (e.g., quercetin,[232] 17$\beta$-estradiol,[233] phloridzin,[234] chlorogenic acid[235]) has been investigated in vitro, and the corresponding products have been isolated and characterized. Worth noting is also the synthesis of specifically deuterated, tritiated, or $^{14}$C-labeled isotopomers of L-DOPA from the corresponding L-tyrosine derivatives.[236] Tyrosinases as well as other oxidoreductases have been exploited for the selective modification of solid-supported substrates, expanding the repertoire of biotransformations that can be carried out in the solid phase.[237]

Finally, it deserves to be mentioned the tyrosinase-catalyzed cleavage of amino acid phenylhydrazides, which has been proposed as a very mild and selective method for the C-terminal deprotection of peptides (Figure 35).[238]

## 6. PEROXIDASES

Peroxidases are a vast family of enzymes that catalyze the oxidation of a variety of substrates using hydrogen peroxide or alkyl hydroperoxides. These enzymes are classified in two groups depending on the presence or the absence of a heme group. Among the heme-containing proteins, the haloperoxidases


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**Table 2. Reactions Catalyzed by Peroxidases**

| | |
|---|---|
| 1 | oxidative dehydrogenation |
| | $2SH + H_2O_2 \rightarrow 2S^{\cdot} + 2H_2O$ |
| 2 | oxidative halogenation |
| | $SH + H_2O_2 + H^+ + X^- \rightarrow SX + 2H_2O$ |
| 3 | hydrogen peroxide dismutation |
| | $2H_2O_2 \rightarrow O_2 + 2H_2O$ |
| 4 | oxygen-transfer reaction |
| | $SH + H_2O_2 \rightarrow SOH + H_2O$ |

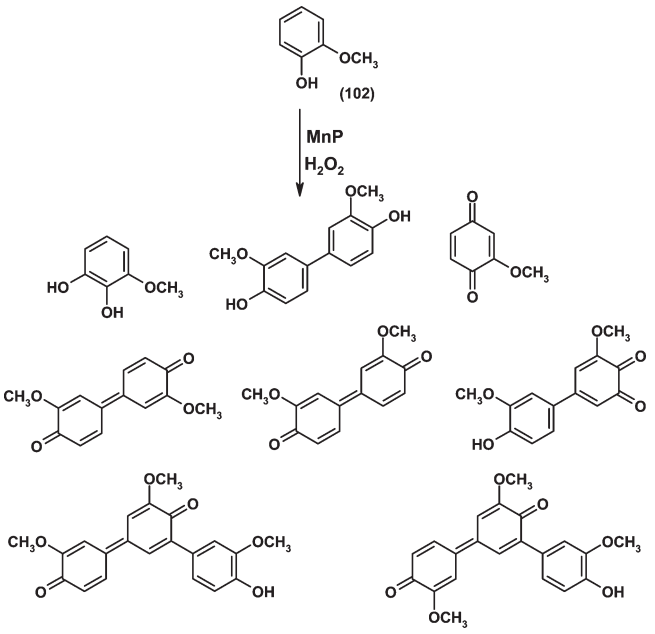


**Figure 36.** List of products obtained by MnP-catalyzed oxidation of guaiacol (**102**).

bromoperoxidase (BPO), chloroperoxidase (CPO), and iodoperoxidase (IPO) and the well-known horseradish peroxidase (HRP) have been investigated for synthetic purpose. Other interesting biocatalysts are the nonheme vanadium-peroxidases. The reactions catalyzed by peroxidases can be classified in four categories, as listed in Table 2.[59,239] While hydrogen peroxide dismutation is a side reaction that can be catalyzed by some heme peroxidases in the absence of the substrate, causing a progressive loss of activity of the enzyme,[240] the other transformations are of synthetic interest, as it will be shown in the following.

## 6.1. Oxidative Dehydrogenation

HRP is the peroxidase showing the widest specificity for oxidative dehydrogenation, particularly of phenolic derivatives,[241] which lead to the formation of biphenylic dimers and, over time, to mixtures of oligomeric oxidated products. Recently, an accurate study on the oxidation of guaiacol (**102**) by a manganese peroxidase (MnP) obtained from the lignin-degrading fungus *Phanerochaete chrysosporium* BKM-F-1767, revealed the structure of the entire set of dimeric and trimeric products (Figure 36).[242] HRP is not able to oxidize phenols carrying a bulky group in the meta position. To overcome this limitation, the same strategy adopted with laccases has been used, that is, the use of a mediator such as *N*-ethyl phenothiazine, phenothiazine-10-propionic acid, or veratryl alcohol. In this way, cardanol, an unsaturated *m*-$C_{15}$-phenol, has been polymerized (yield 64%, $M_n$ 3900, $M_w/M_n$ 1.5).[243]

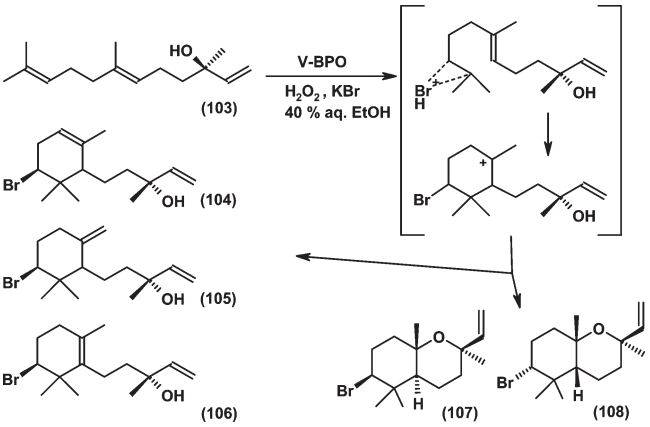


**Figure 37.** V-BPO-catalyzed formation of α-, *β*-, and *γ*-snyderol (**104**−**106**) and (+)-3*β*-bromo-8-epicaparrapioxides (**107** and **108**) from (+)-*trans*-nerolidol.

## 6.2. Oxidative Halogenation

Halogenated organic compounds play an important role in chemistry, and oxidative halogenation can be a sustainable way to produce them. While chemically these reactions can be catalyzed by metals in the presence of oxidants like oxygen and hydrogen peroxide,[244] enzymatically they are carried out exploiting peroxidases. The catalytic mechanism, which is obviously related to the nature of the enzymatic prosthetic group, is quite complex and is not fully understood yet.[245]

The CPO isolated from *Caldariomyces fumago* is the most studied halogenating enzyme.[246] It has shown a general lack of stereospecificity in the halogenation reaction, but with some noteworthy exceptions.[247] The same enzyme is also able to catalyze oxidative dehalogenation, as it has been shown by transforming, under acidic conditions, 2,4,6-trihalophenols or *p*-halophenols into the corresponding 2,6-dihalo-1,4-benzoquinones.[248]

Another interesting halogenating peroxidase is the vanadium bromoperoxidase (V-BPO) isolated from *Corallina pilulifera* and from *Corallina officinalis*. Several substrates have been brominated by this enzyme (anisole, 1-methoxynaphthalene, thiophene, styrene, cyclohexene, *trans*-cinnamic acid, *trans*-cinnamyl alcohol, and *cis*-propenylphosphonic acid), but the obtained products always resulted as mixtures of regio- and stereoisomers.[249] This apparent lack of selectivity has been initially explained as due to freely diffusible oxidized bromine species, produced by the peroxidase and acting as brominating intermediate.[250] However, at least two examples showed a direct brominating action of the peroxidase. The V-BPO from *C. officinalis* furnished two diastereomers of 2-bromo-1-phenylbutane-1,3-diol from *trans*-4-phenyl-3-buten-2-ol in a ratio (66/34) different from that obtained chemically with the brominating agent *N*-bromo-acetamide.[251] Later, it was shown that the bromination, followed by cyclization, of the terpene (+)-*trans*-nerolidol (**103**) catalyzed by V-BPO produced single diastereomers of the marine natural products α-, *β*-, and *γ*-snyderol (**104**−**106**), and a mixture of diastereomers of (+)-3*β*-bromo-8-epicaparrapi oxide (**107** and **108**, Figure 37).[252]


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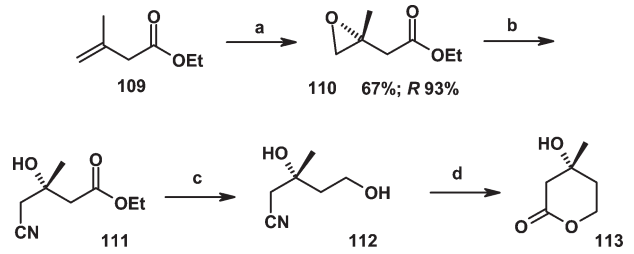



**Figure 38.** A concise route to (*R*)-mevalonolactone (**113**) via CPO oxidation of ethyl 3-methyl-3-butenoate (**109**). (a) *t*-BuOOH (2.0 equiv), CPO (0.014 equiv), 0.01 M Na citrate, pH = 5.5; (b) KCN (3.0 equiv), $F_3CCO_2H$ (2.0 equiv), EtOH; (c) $NaBH_4$, EtOH; (d) (i) NaOH, (ii) 10 % HCl, MeOH, (iii) $CH_3SO_3H$, THF.

### 6.3. Oxygen-Transfer Reactions

The mild and selective introduction of an oxygen atom in an organic compound is another attractive opportunity offered by peroxidases. As just one atom of oxygen is transferred from hydrogen peroxide to the substrate, in this respect these enzymes act as monooxygenases that can catalyze heteroatom oxidation (sulfur and nitrogen), epoxidation, and C−H oxidation (benzylic, allylic, alcohols oxidation, indoles, and benzofurans oxidation) reactions.

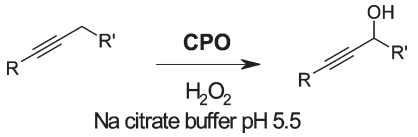
**6.3.1. Heteroatoms Oxidation.** Organic sulfoxides are useful synthons for the construction of various biologically active molecules, themselves often playing an important role as therapeutic drugs. Hundreds of different sulfides have been submitted to the action of several peroxidases (HRP, CPOs, and V-BPOs), giving sulfoxides with moderate/good enantiomeric excess, in some cases up to >99% ee with full conversion.[253] To decrease the spontaneous oxidation of sulfide by $H_2O_2$ and to improve the operational stability of the enzymes, the oxidant has been maintained at the lowest possible concentration by continuous addition or in situ production.[254]

The peroxidase-catalyzed *N*-oxidation reactions have received minor attention, and just a few examples are present in the literature. Arylamines such as *p*-toluidine, 4-chloroaniline, and 3,4-dichloroaniline, when subjected to CPO, afforded the corresponding nitroso derivative.[255] HRP is well-known to be able to oxidize aromatic amines, whereas usually aliphatic amines are not substrates for this enzyme. At variance, the tertiary amine center of certain indole alkaloids (isolated from *Vinca* species) could be oxidized to the corresponding iminium species.[256]

**6.3.2. Epoxidation.** CPO can catalyze the epoxidation of prochiral alkenes (short-chains *cis*-2-alkenes, isolated and conjugated (cyclic)-dienes, styrenes, indene) in the presence of hydrogen peroxide, the chemical and optical yields heavily depending on the structures of the substrates and of the formed products (sometimes the epoxydes are not stable under the reaction conditions and open to form the corresponding diols).[257]

(*R*)-Mevalonolactone (**113**) is a synthetic target of considerable interest, and, among the several described synthetic strategies, one of them exploits the CPO-catalyzed stereoselective epoxidation of ethyl 3-methyl-3-butenoate (**109**) as a key step (Figure 38).[258] Another interesting example is given by the CPO-catalyzed stereoselective oxidation of (*R*)-limonene to the corresponding epoxide, which is then followed by a spontaneous hydrolysis to give (*R*)-limonene-1,2-diol in excellent diastereomeric ratio.[259]

**Table 3. CPO-Catalyzed Enantioselective Propargylic Hydroxylation of R−C≡C−$CH_2$−R′**




| R | R′ | ee (%) | yield (%) | config |
|---|---|---|---|---|
| $CH_3$ | $CH_3$ | 57 | 7 | *R* |
| $CH_2CH_3$ | $CH_3$ | 91 | 26 | *R* |
| $CH_2CH_2CH_3$ | $CH_3$ | 87 | 30 | *R* |
| $CH_2(CH_2)_2CH_3$ | $CH_3$ | 78 | 8 | *R* |
| Ph | $CH_3$ | 86 | 15 | *R* |
| $CH_2OAc$ | $CH_3$ | 95 | 52 | *R* |
| $CH_2Br$ | $CH_3$ | 94 | 65 | *R* |
| $CH_2CH_2OAc$ | $CH_3$ | 83 | 26 | *R* |
| $CH_2CH_2Br$ | $CH_3$ | 94 | 25 | *R* |
| $CH_2OAc$ | $CH_2CH_3$ | 87 | 8 | N.D. |
| $COCH_3$ | $CH_3$ | no reaction | | |

**6.3.3. C−H Oxidation.** It has been shown that CPO can also catalyze the selective hydroxylation of C−H bond, a significant example being the oxidation of ethylbenzene to (*R*)-phenethyl alcohol (yield 20%, ee 97%).[257c] Another interesting example is related to the CPO-catalyzed enantioselective α-hydroxylation of triple bonds to give secondary propargylic alcohols, the results being summarized in Table 3.[260] Moreover, when primary propargylic alcohols have been subjected to the action of CPO and $H_2O_2$, they were rapidly oxidized to the corresponding aldehyde in good yields.[261] This finding suggested that, in general, CPO could oxidize primary alcohols to aldehydes, as confirmed by the preparative scale quantitative oxidation of *cis*-2-hexen-1-ol to the corresponding aldehyde by CPO and *tert*-butyl hydroperoxide.[262] In another example, the CPO-catalyzed oxidation of cyclopropylmethanols to cyclopropylaldehydes (using tert-butyl hydroperoxide as oxidant) has been described, the distal stereocenter being recognized with very low enantioselectivity with *trans*-cyclopropylmethanols and with good enantiomeric ratio with *cis*-derivative.[263]

Finally, the CPO-oxidation of benzothiophenes, benzofurans, and indoles has been the subject of detailed studies. Benzothiophenes gave sulfoxides and sulphones, indoles furnished the corresponding oxindoles in nearly quantitative yields, and benzofurans gave trans/cis diols or lactones, depending on the reaction conditions.[264]

## 7. CONCLUSIONS

This Review has offered an overview of the groups of oxidoreductases that are suitable for synthetic applications as isolated enzymes. Whereas some of these biocatalysts have been deeply investigated (i.e., dehydrogenases and peroxidases), others are currently receiving increasing interest as they have eventually become available, even commercially, via extensive exploitation of the modern techniques of molecular biology (i.e., laccases, cytochromes $P_{450}$). As the quest for "green" oxidative protocols is one of the main goals of sustainable chemistry, it is foreseeable that the exploitation of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oxidoreductases will remain one of the hot topics of biocatalysis in future years.

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## BIOGRAPHIES

Daniela Monti received her Laurea degree in Biological Science at the University of Milano in 1991 and her Specialization degree in Biotechnological Applications from the same institution in 1995 working on synthetic applications of glycosyltransferases. From 1996 to 2000, she worked on the development of new biocatalytic processes for the preparation of semisynthetic antibiotics as a research fellow at the Institute of Chemistry of Molecular Recognition (ICRM) of the Italian National Research Council in collaboration with Recordati SpA. Since 2001, she is a research scientist at ICRM. Her research interests focus on the identification and characterization of new biocatalysts and their application in organic synthesis.

Gianluca Ottolina graduated in Organic Chemistry at the University of Milan under the supervision of Prof. B. Danieli. After a fellowship with Prof. J. B. Jones at the University of Toronto, he joined the Institute of Chemistry of Molecular Recognition (ICRM) of the Italian National Research Council as a research scientist. In 1996, he received the Diploma di Specialità in Applied Biotechnology at the University of Milan. Since 2009, he is an adjunct professor at the University of Insubria. His main interests are biocatalysis and enzyme kinetics.

Giacomo Carrea graduated in chemistry at the University of Pavia. In 1969, he joined the Italian National Research Council (CNR) where he was the Director of the Institute of Chemistry of Molecular Recognition in Milan from 2002 until 2009, when he retired. He has worked in such fields as enzyme catalysis in organic solvents, synthesis of fine chemicals catalyzed by hydrolases, dehydrogenases, peroxidases, and monooxygenases, study of conformation of proteins and polypeptides, and analytical applications of bioluminescent enzymes. He is the author of about 230 publications in international peer reviewed journals.

Dr. Sergio Riva is the Director of the Institute of Chemistry of Molecular Recognition (ICRM) of the Italian National Research Council (CNR), where he has been working since 1984, and "Professore a Contratto" at the University of Modena and Reggio Emilia since 2000. He took his Laurea in Chemistry at Milano University in 1983 and his Diploma di Specialità in Organic Synthesis at Milano Politecnico in 1989. He spent one year (1987) at MIT (Cambridge, MA) working with Prof. A. Klibanov. In 1993, he was awarded the Ciamician medal by the Organic Chemical Division of the Italian Chemical Society for his research in Biocatalysis. His scientific activity is documented by more than 150 publications reporting on the isolation and characterization of different groups of enzymes and on the synthetic exploitation of these biocatalysts.

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