

## BIOCHEMICAL METHODS OF SYNTHESIS

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### Contents

1. Introduction
  2. Early Employment of Enzymes in Organic Synthesis
  3. Biotransformations in Organic Synthesis
    - 3.1. Hydrolytic Reactions
    - 3.2. Oxydation and Reduction Reactions
      - 3.2.1. Cofactor Recycling
      - 3.2.2. Reduction of Aldehydes, Ketones, and Derivatives
      - 3.2.3. Oxidation
  4. CarbonCarbon Bond-Forming Reactions
  5. Glycosyl-Transfer Reactions
  6. Halogenation Reactions
  7. Abzyme-Catalyzed Reactions
  8. Reactions Catalyzed by Artificial Enzymes
- Glossary  
Bibliography  
Biographical Sketches

### Summary

The utilization of enzymes in organic synthesis—either as pure compounds or as part of the whole cell, very often immobilized on solid supports—has made it possible to take advantage of their remarkable efficiency and selectivity to develop a great number of successful applications. The impact of biotransformations on synthetic organic chemistry is already relevant and will surely grow. The purpose of this chapter is to overview some of the fundamental aspects of this area, with a particular emphasis on synthetic applications. Examples of hydrolysis, oxidation, reduction, halogenation, and carbon–carbon bond-forming reactions are described, as well as recycling of cofactors with enzymes that require their presence. Mention is also made of catalytic antibodies (abzymes) and of a subject that appears to be one of the most exciting challenges of the modern catalysis—the design of synthetic models of biological catalysts.

### 1. Introduction

The catalytic properties of enzymes are far more impressive than those of synthetic catalysts that operate under more extreme conditions. Using them as catalysts to carry out organic transformations in organic synthesis has, therefore, attracted the interest of

many research groups and now represents one of the most promising and fast-growing areas of organic chemistry and biotechnology (see *Organic Synthesis*).

An enzyme is a polypeptide molecule or an ensemble of polypeptide molecules that catalyzes biochemical reactions with very high chemo-, regio-, and stereospecificity. In spite of its complex polymeric nature, however, molecular recognition between enzymes and substrates, as well the catalytic event, takes place in a very small, highly ordered portion of the biopolymer, which is referred to as the active center or catalytic cavity. A great deal of work has been directed towards the elucidation of the nature of the active center and to the understanding of the catalytic process in terms of the steric and electronic effects related to this limited region of the enzyme. These studies have shown that amino acid residues located in the active center may act as acids and bases or nucleophiles and electrophiles, and thus participate in the reaction mechanism. Most of the enzymes require the presence at the active center of cofactors, such as metal ions (e.g.,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ , or  $\text{Mn}^{2+}$ ), that can act as electrophiles and bind the substrate to generate activated derivatives or nonproteic, organic substances. When a cofactor is permanently bound to the enzyme it is called a prosthetic group, whereas the organic cofactor that is brought into play during the catalytic process is usually referred to as a coenzyme. Coenzymes generally act as acceptors or donors of chemical groups or atoms or electrons. It is worth mentioning that some coenzymes are derived from components that cannot be synthesized by mammals and are vitamins. In many cases, both metal ions and coenzymes may be required.

The first step of the enzyme-catalyzed reaction is the association of the substrate and the enzyme to give rise to the reversible formation of a substrate-enzyme complex. This substrate specificity is determined by the geometric alignment of the substrate in the active center, which in turn is directed by the structural pattern of the enzyme. Substrate specificity is explained on the basis of either lock and key or induced fit theory. The former assumes exact complementarity and rigidity in the formation of the substrate-enzyme complex—the substrate fits into the center like a key fits into a lock. However, the latter more modern theory proposes that there may be more flexibility in the formation of the substrate-enzyme complex, and the binding of substrate to enzyme may involve conformational changes of the active center so as to adapt it to some extent to the requirements of the substrate.

Usually, the formation of the substrate-enzyme complex does not involve covalent bonding, but only ionic forces (i.e., electrostatic, dipole–dipole, and hydrogen bonds) and nonpolar forces. When covalent bonding between the substrate and the enzyme is observed, a noncovalent substrate-enzyme complex precedes the covalent substrate-enzyme complex.

## 2. Early Employment of Enzymes in Organic Synthesis

The fermentation of sugar to ethanol by yeast has been known for a long time. Pasteur first observed that yeast produced glycerol as a minor component of sugar fermentation. Later research demonstrated that when *Saccharomyces cerevisiae* was grown in a medium buffered to alkaline pH, the yield of glycerol increased.

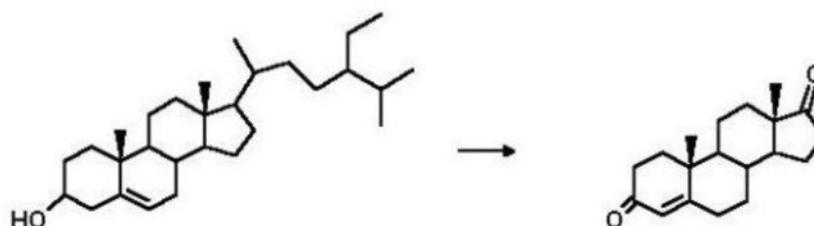
The degradation of sucrose by the micro-organism *Aspergillus niger* allowed, *c.* 1930, the production of citric acid. Today citric acid is used as a flavoring agent and antioxidant—for food and drink.

The extraction of amino acids from proteins is the oldest method for their production, and L-cysteine is still obtained in this way. However, at the present time, the manufacturing methods for L-glutamate, L-lysine, and L-arginine are all based on microbiological processes.

Steroids are derived from plants and mammals. In 1960, it was observed that progesterone could be converted into 11 $\alpha$ -hydroxyprogesterone using *Rhizopus arrhizus* (see Scheme 1). Another well-known transformation—performed by mycobacteria—is the oxidation of  $\beta$ -sytosterol to androstenedione (see Scheme 2).



Scheme 1



Scheme 2

### 3. Biotransformations in Organic Synthesis

Enzymes, either in the pure form or as part of the whole cell, have been used widely in synthetic organic chemistry to carry out a variety of transformations, and are expected to be used more extensively in the future. Immobilizing enzymes on solid supports has certainly provided significant advantages. Immobilized enzymes are usually more stable and efficient—where efficiency is expressed as the moles of substrate transformed per mole of enzyme in unit time. Their employment also simplifies the purification steps, as immobilized enzymes can be separated more easily from the products. Foreseeable developments will most probably deal with the utilization of DNA recombinant technology to prepare all the natural enzymes. Their production will be less expensive. In addition, it will also be possible to design new biocatalysts, unknown in nature. Clearly, further studies, new developments in biochemistry and molecular biology, and a better understanding of the structure–activity relationship are needed in order to achieve these ambitious targets.

In this chapter some of the most commonly used applications of the biocatalysis in organic synthesis, namely, hydrolytic reactions, oxidation and reduction reactions, carbon-carbon bond-forming reactions, glycosyl-transfer reactions, and halogenation reaction, are briefly reviewed.

### 3.1. Hydrolytic Reactions

Hydrolytic enzymes have been found particularly useful in organic synthesis:

- They couple a broad substrate specificity to a high stereoselectivity,
- Are easily available, and
- Do not require expensive and unstable coenzyme systems.

Most of the reactions studied deal with the hydrolytic transformation of amide and ester bonds, performed by proteases—enzymes that attack the peptide bonds of proteins and peptides—and esterases, usually lipases. Esterases catalyze reversibly the scission and synthesis of fatty acids and lower alcohols, and lipases—one of the main types of esterase—hydrolyze the ester bond of fats into fatty acids and glycerol. Lipases, in particular, exhibit high stability and activity in organic solvents. The crystal structures of many lipases reveal that the three-dimensional structures of hydrolases are more similar than expected from their amino-acid sequences.

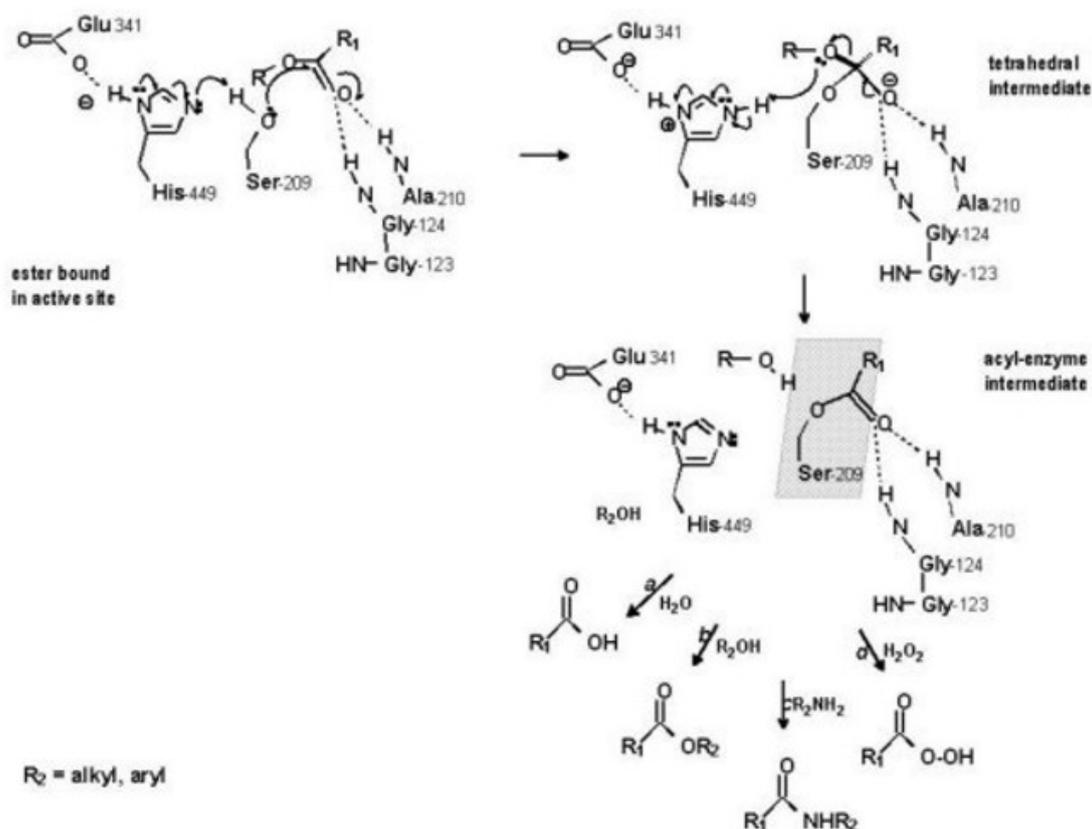
These hydrolases differ widely in size, and the only sequence motif common to all of them is a pentapeptide—Gly-X-Ser-X-Gly (X = amino acid)—that encompasses the active site serine. Nevertheless, all are serine esterases with a common arrangement of catalytic machinery and a protein chain that folds, at least in part, in an  $\alpha,\beta$ -hydrolase fold. The  $\alpha,\beta$ -hydrolase fold contains mostly parallel  $\beta$ -sheet in the core with  $\alpha$ -helices surrounding this core. This folding pattern arranges the residues of the catalytic triad—Ser, His, and either Glu or Asp—in an identical manner in all these enzymes. The nucleophilic serine rests at a hairpin turn between a  $\beta$ -strand and an  $\alpha$ -helix. The remaining two residues of the catalytic triad—His and either Glu or Asp—rest on one side of the serine, while the residues forming the oxyanion hole lie on the other.

The mechanisms of action for amide and ester hydrolyzing enzymes are very similar and involve a nucleophilic group in the active site, which can be either the hydroxyl group of a serine (e.g., pig-liver esterase, subtilisin, and the majority of microbial lipases), or a carboxyl group of an aspartic acid (e.g., pepsin) or a thiol function of a cysteine (e.g., papain).

The mechanism of the serine esterase has been elucidated in details (see Scheme 3).

Removal of the proton from the hydroxy group of the serine residue at position 209—Ser-209—by the imidazole nucleus of the His-449 residue increases the nucleophilicity of the oxygen during the formation of the tetrahedral intermediate in the first step. In the second step, an acyl-enzyme complex is generated via elimination of the alcoholic fragment from the tetrahedral intermediate, favored by protonation of the oxygen by the conjugate acid of the imidazole nucleus. The resultant acyl-enzyme intermediate is held in the oxyanion hole by the NH groups of Ala-210 and Gly-124—or Gly-123 for other

*Candida rugosa* lipases—through hydrogen bonding. Subsequently, a molecule of water attacks the acyl-enzyme intermediate, regenerating the enzyme and releasing the carboxylic-acid derivative ( $R^1\text{COOH}$ ) (see Scheme 1a).



When the enzyme is operating at low water concentrations, other nucleophiles can compete with water for the acyl-enzyme intermediate, thus leading to the following biotransformations:

- *Transesterification.* The nucleophilic attack of a different alcohol,  $R_2\text{OH}$ , leads to the corresponding ester  $R_1\text{COOR}_2$  (see Scheme 1b).
- *Synthesis of amides.* The nucleophilic attack of an amine,  $R_2\text{NH}_2$ , forms the amide  $R_1\text{CONHR}_2$  (see Scheme 1c).
- *Synthesis of peracids.* The nucleophilic attack of hydrogen peroxide leads to the formation of the peracid  $R_1\text{COOOH}$  (see Scheme 1d).

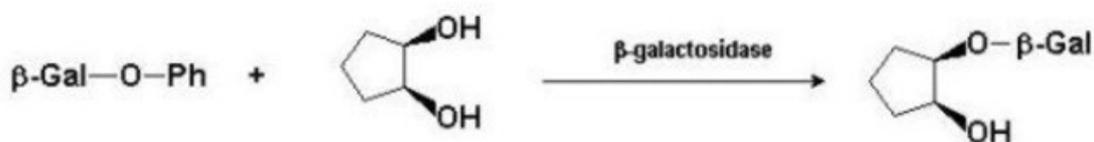
A detailed examination of the stereoselectivity of lipases towards secondary alcohols for hydrolysis reactions—where the substrate is an ester—and for esterification reactions—where the substrate is an alcohol—has established size-based empirical rules for the prediction of the stereopreference and the extent of stereoselectivity. These rules (see Figure 1) predict which enantiomer of a secondary alcohol reacts faster in lipase-

catalyzed reactions by comparing the sizes of the substituents at the stereogenic center. When the alcohol is drawn with the hydroxyl group pointing out of the page (see Figure 1a), the favored enantiomer bears the large substituent (e.g., a phenyl) on the right, and the medium substituent (e.g., a methyl) on the left. The importance of the substituent size has been confirmed by studies that established that lipases resolve poorly secondary alcohols with two similarly sized substituents. The resolution improves when the size of one substituent is increased. Similar rules are not valid for primary alcohols, especially when an oxygen atom is close to the stereogenic center. The lower stereoselectivity towards this class of compounds was related to the additional CH<sub>2</sub>-group, which introduces a kink between the stereogenic center and the hydroxy function in primary alcohols (see Figure 1b).



Figure 1. Empyric rules showing the enatiopreference of *Pseudomonas cepacia* lipase (PCL), toward (a) secondary alcohols and (b) primary alcohols  
Notes: M = medium-sized substituent; L = large substituent.

Glycosidases—belonging to the class hydrolytic enzymes—have also found application in biocatalytic synthesis. Glycosidases have a catabolic function *in vivo*, as they catalyze the cleavage of glycosidic linkages to form mono- or oligosaccharides from polysaccharides. It is customary to distinguish between exoglycosidases—they cleave terminal carbohydrate residues—and endoglycosidases, which can also split a carbohydrate chain in the middle. Substitution of water with other nucleophiles, such as a different carbohydrate or a primary or secondary nonnatural alcohol, allows for turning the degradative nature of these enzymes into more useful synthetic applications (e.g.,  $\beta$ -galactodidase has been employed in the asymmetric glycosylation of cyclic mesodiols) (see Scheme 4).



Scheme 4

### 3.2. Oxidation and Reduction Reactions

Enzymes concerned with oxidation-reduction processes (oxidoreductases) have been used successfully to perform a variety of oxidation and reduction reactions. These oxidation and reduction reactions have been carried out by using dehydrogenases (to

catalyze the transfer of hydrogen from one substrate to another), oxidases (to catalyze the transfer of hydrogen from a substrate to molecular oxygen), and oxygenases (to catalyze the insertion of oxygen into a substrate: mono-oxygenases incorporate only one atom of oxygen; di-oxygenases insert both oxygen atoms).

The oxidoreductive enzymes require cofactors that donate or accept the chemical equivalents for reduction or oxidation. Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) (see Figure 2) or adenine dinucleotide phosphate (NADP<sup>+</sup>) (see Figure 3) are the most commonly used coenzymes; they are involved in the oxidation and reduction processes through a mechanism that can be sketched as the transfer to and from the nicotinamide ring of an hydride anion (see Scheme 5).

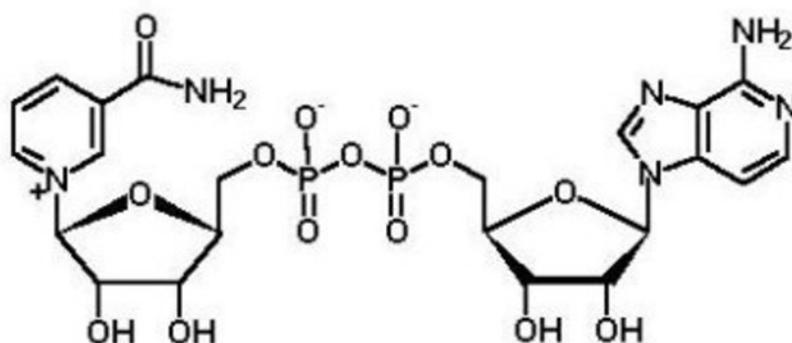


Figure 2. Structure of the nicotinamide adenine dinucleotide (NAD<sup>+</sup>) coenzyme

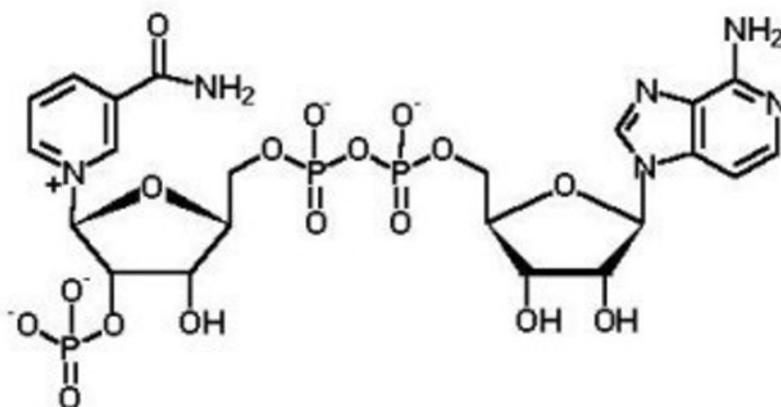
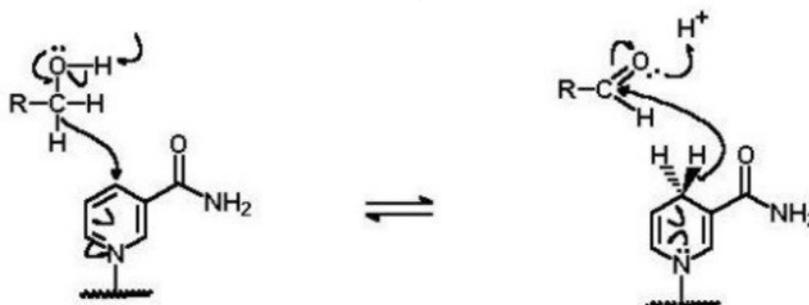


Figure 3. Structure of the nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>) coenzyme



Scheme 5

Flavine mononucleotide (FMN) (see Figure 4) and flavine adenine dinucleotide (FAD) (Figure 5) are encountered more rarely. Generally, they mediate hydrogenation–dehydrogenation processes via reversible reduction of the two conjugated double bonds of the iso-alloxazine ring (see Scheme 6). Flavine coenzymes are also required by a group of mono-oxygenases that catalyze the insertion of an oxygen atom into the substrate and that do not require metal ions. Not requiring metal ions means that the reaction with oxygen could have a free radical character at some stage of the mechanism. Since the substrate radicals are very unstable, it seems more likely that molecular oxygen reacts with the reduced form of flavin to give a flavine 4a-hydroperoxide (see Scheme 6), an oxidant stronger than hydrogen peroxide, which then reacts with the substrate through an ionic mechanism.

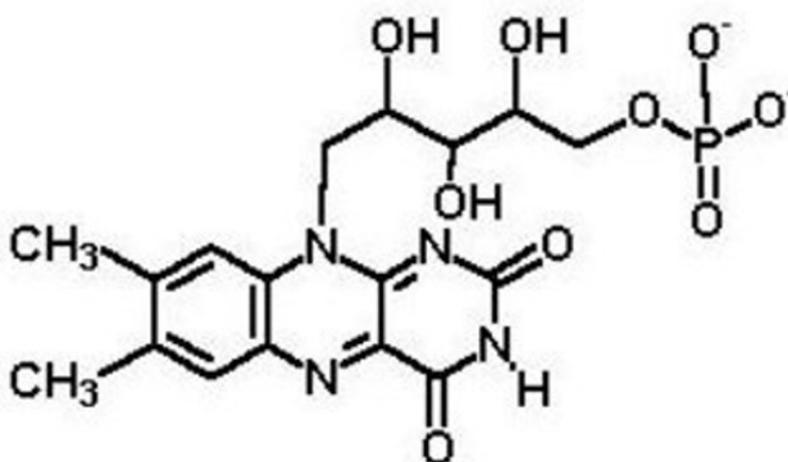


Figure 4. Structure of the flavine mononucleotide (FMN) coenzyme

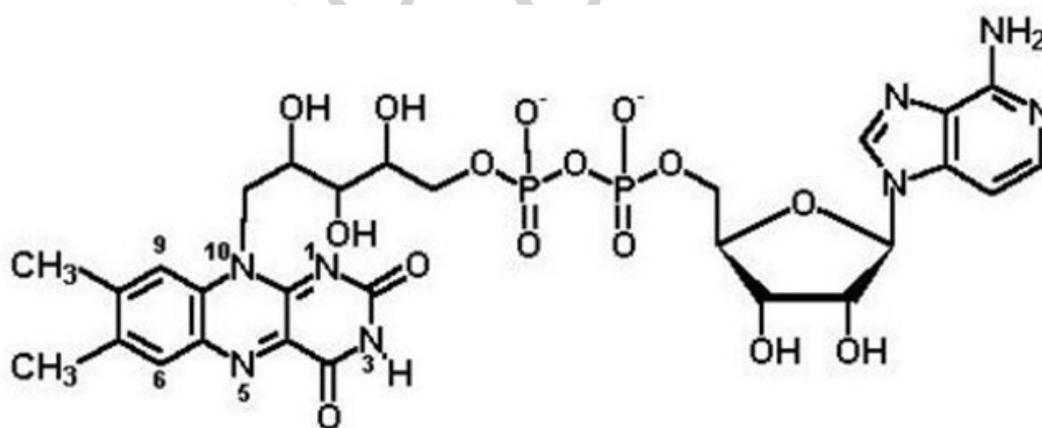
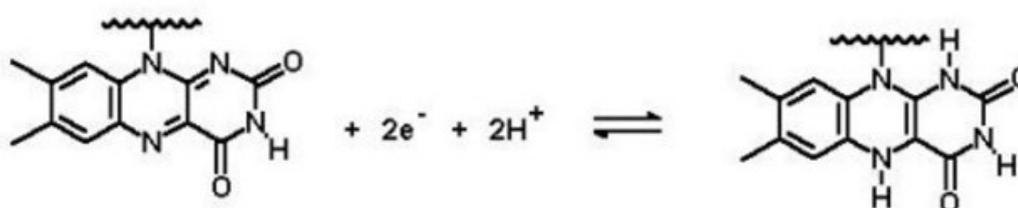
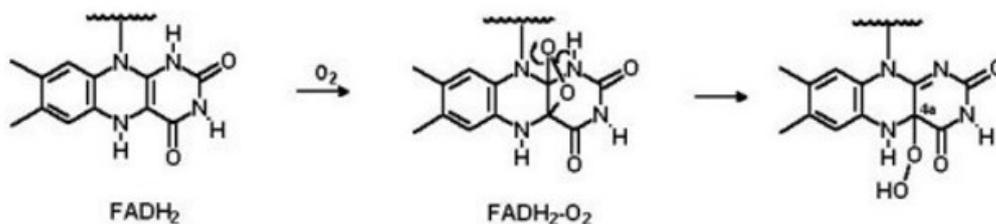


Figure 5. Structure of the flavine adenine dinucleotide (FAD) coenzyme



Scheme 6



Scheme 7

All these cofactors have two features in common: they are relatively unstable and they become really expensive if reactions have to be performed on a preparative scale. However, they may be regenerated *in situ* and re-enter the reaction cycle by using a second redox-reaction to reoxidize their reduced form or reduce their oxidized form. Thus, expensive cofactors are needed only in catalytic amounts decreasing the cost. The efficiency of such a recycling process is measured by the number of cycles which can be achieved before a coenzyme is destroyed. It is expressed as the total turnover number (TTN), which is the total number of moles of product formed per mole of cofactor during the course of the complete reaction.

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### Biographical Sketches

**Professor Bruno Botta** was born in 1954, and obtained a degree in Chemistry in 1977 at the University of Rome “La Sapienza,” Italy. He was a postdoctoral associate at the Department of Chemistry, UBC, Vancouver, Canada, during 1984–5, and researcher at Università Cattolica del S. Cuore, Rome, from 1981–92. He is now Professor of Organic Chemistry at the Faculty of Pharmacy, University of Rome “La Sapienza.” Professor Botta is the author of about 80 publications, including four patents on both antitumoral and hypotensive agents derived from living plants. His interest has been focused on the structural elucidation and synthesis of biological active molecules derived from living plants. Since 1984, he has been working in the field of plant tissue cultures in combination with chemistry directed toward the understanding of biosynthetic pathways of the compounds under investigation. During this time some plant enzymes, such as peroxidases, derived from the cell cultures, have been isolated and purified and some kinetic studies have been performed too. Since 1991, Professor Botta has focused his attention on both the synthesis and the host-guest studies of resorcinarenes.

**Professor Sandro Cacchi** was born in Macerata, Marche, Italy, in 1943. He began his university training at the University of Camerino, Italy, where he obtained a *Laurea* in Chemistry in 1967. Following this he moved directly to the University of Bologna, Italy, where he worked under the direction of Professor Caglioti. After doing his national service (1968–9), he went back to the University of Bologna and was promoted to Assistant Professor in 1970. In 1972 he joined the University of Rome “La Sapienza,” where he became Associate Professor of Organic Chemistry (1983) and full Professor of Organic Chemistry (1986). Professor Cacchi made a contribution early in his career with the development of new, selective synthetic procedures directed toward the functionalization or preparation of complex molecules. In that period he was also the co-author of a book (*Chimica delle Sostanze Naturali*, CEA., 1971) and wrote a monograph (*Principi di Bio-organica*, ISEDI, 1977) on the chemistry of natural substances. More recently, the utilization of palladium catalysis in organic synthesis has been a major goal that he has pursued in many ways, with the search for new and selective methodologies being a major thrust even in this area. He is the author of about 140 publications, four patents, and six books.