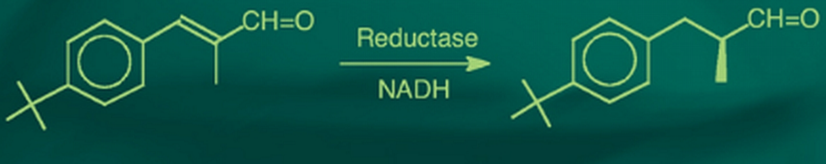
Kurt Faber

Biotransformations in Organic Chemistry

A Textbook

6th Edition



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Sixth revised
and corrected edition

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Preface

The use of natural catalysts – enzymes – for the transformation of nonnatural man-made organic compounds is not at all new: they have been used for more than 100 years, employed either as whole cells, cell organelles or isolated enzymes [1]. Certainly, the object of most of the early research was totally different from that of the present day. Thus the elucidation of biochemical pathways and enzyme mechanisms was the main reason for research several decades ago. It was mainly in the steep rise of asymmetric synthesis during the 1980s, that the enormous potential of applying natural catalysts to transform nonnatural organic compounds was recognized. What started as an academic curiosity in the late 1970s became a hot topic in synthetic organic chemistry in the 1990s. Although the early euphoria during the 'gold rush' in this field seems to have eased somewhat, there is still no limit to be seen for the future development of such methods, as indicated by the wave-like appearance of novel types of biocatalytic principles. As a result of this extensive research, there have been an estimated 15,000 papers published on the subject. To collate these data as a kind of 'super-review' would clearly be an impossible task and, furthermore, such a hypothetical book would be unpalatable for the non-expert [2–6].

The point of this textbook is to provide a *condensed* introduction to this field. It is written from an organic chemist's viewpoint in order to encourage more 'pure' organic chemists of any level to take a deep breath and leap over the gap between the 'biochemical' sciences and 'synthetic organic chemistry' by persuading them to consider biocatalytic methods as an equivalent tool when they are planning the synthesis of an important target molecule. At several academic institutions this book has served as a guide for updating a dusty organic chemistry curriculum into which biochemical methods had to be incorporated. The wide repertoire of classic synthetic methods has not changed but it has been significantly widened and enriched due to the appearance of biochemical methods. This is illustrated by the fact that the proportion of papers on the asymmetric synthesis of enantiopure compounds employing biocatalytic methods has constantly risen from zero in 1970 to about 8% in 1989 [7] and it was estimated that this value is now approaching a steady share of 15%. Certainly, biochemical methods are not superior in a general sense – they are no panacea – but they definitely represent a powerful synthetic tool to complement other methodology in modern synthetic organic chemistry.

In this book, the main stream of novel developments in biotransformations, which already had significant impact on organic chemistry, are put to the fore. Other cases, possessing great potential but still having to show their reliability, are mentioned more briefly. The literature covered by the sixth edition of this textbook extends to the end of 2010. Special credit, however, is given to some 'very old' papers as well as acknowledging the appearance of novel concepts. References are selected according to the philosophy that 'more is not always better'. Generally, I have attempted to sort out the most useful references from the pack, in order to avoid writing a book with the charm of a telephone directory! Thus, special emphasis is placed on reviews and books, which are often mentioned during the early paragraphs of each chapter to facilitate rapid access to a specific field if desired.

The first edition of this book appeared in September 1992 and was predominantly composed as a monograph. It was not only well received by researchers in the field but also served as a basis for courses in biotransformations worldwide. In the second, completely revised edition, emphasis was laid on didactic aspects in order to provide the first textbook on this topic in 1995. Its great success has led to the demand for updated versions with emphasis on new trends and developments. In this context, novel techniques – dynamic resolution, stereoinversion, and enantioconvergent processes – were incorporated, in addition to the basic rules for the handling of biocatalysts.

My growing experience of teaching the use of biotransformations at several universities and research institutions around the world has enabled me to modify the text of this sixth edition so as to facilitate a deeper understanding of the principles, not to mention the correction of errors, which escaped my attention during previous editions. I am grateful to numerous unnamed students for pointing them out and for raising questions and to my old Macintosh IIci, which reliably served for 14 years without crashing.

I wish to express my deep gratitude to Stanley M. Roberts (UK) for undergoing the laborious task of correcting the manuscripts of the early editions of this book, for raising numerous questions and for helpful comments. Special thanks also go to M. Müller, U. Bornscheuer, W.-D. Fessner, A. Liese (Germany), N.J. Turner (UK), J.-E. Bäckvall (Sweden), R. Kazlauskas (USA), B. Nidetzky, and W. Kroutil (Graz) for their helpful hints and discussions. This revised edition would not have been possible without the great assistance of A. Preisz and B. Mautner.

I shall certainly be pleased to receive comments, suggestions, and criticism from readers for incorporation in future editions.

Graz, Austria
Spring 2011

Kurt Faber

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Contents

Chapter 1
Introduction and Background Information

1.1 Introduction

Any exponents of classical organic chemistry might probably hesitate to consider a biochemical solution for one of their synthetic problems. This would be due to the fact, that biological systems would have to be handled. Where the growth and maintenance of whole microorganisms is concerned, such hesitation is probably justified. In order to save endless frustrations, close collaboration with a microbiologist or a biochemist is highly recommended to set up and use fermentation systems [1, 2]. On the other hand, isolated enzymes (which may be obtained increasingly easily from commercial sources either in a crude or partially purified form) can be handled like any other chemical catalyst.[1] Due to the enormous complexity of biochemical reactions compared to the repertoire of classical organic reactions, it follows that most of the methods described will have a strong empirical aspect. This 'black box' approach may not entirely satisfy the scientific purists, but as organic chemists are rather prone to be pragmatists, they may accept that the understanding of a biochemical reaction mechanism is not a conditio sine qua non for the success of a biotransformation.[2] In other words, a lack of detailed understanding of a biochemical reaction should never deter us from using it, if its usefulness has been established. Notwithstanding, it is undoubtedly an advantage to have an acquaintance with basic biochemistry and enzymology and with molecular biology, in particular.

Worldwide, about 80% of all chemical processes are performed catalytic leading to an annual product value of around 400 billion €. In this context, biocatalytic methods represent the main pillar of applied biotechnology, which has been coined

[1]The majority of commonly used enzyme preparations are available through chemical suppliers. Nevertheless, for economic reasons, it may be worth contacting an enzyme producer directly, in particular if bulk quantities are required. For a list of enzyme suppliers see the appendix (Chap. 5).

[2]After all, the exact structure of a Grignard-reagent is still unknown.

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as *White Biotechnology* by EuropaBio 2003, and which stands for the application of Nature's toolset to sustainable industrial production.[3]

1.2 Common Prejudices Against Enzymes

If one uses enzymes for the transformation of nonnatural organic compounds, the following prejudices are frequently encountered:

- *'Enzymes are sensitive'*.
  This is certainly true for most enzymes if one thinks of boiling them in water, but that also holds for most organic reagents, e.g., butyl lithium. When certain precautions are met, enzymes can be remarkably stable. Some candidates can even tolerate hostile environments such as temperatures greater than 100°C and pressures beyond several hundred bars (100 bar = 10 MPa) [3–5].
- *'Enzymes are expensive'*.
  Some are, but others can be very cheap if they are produced on a reasonable scale. Considering the higher catalytic power of enzymes compared to chemical catalysts, the overall efficiency of an enzymatic process may be better even if a rather expensive enzyme is required. Moreover, enzymes can be reused if they are immobilized. It should be emphasized that for most chemical reactions relatively crude and thus reasonably priced enzyme preparations are adequate. Due to the rapid advances in molecular biology, costs for enzyme production are constantly dropping.
- *'Enzymes are only active on their natural substrates'*.
  This statement is certainly true for some enzymes, but it is definitely false for the majority of them. Much of the early research on biotransformations was impeded by a tacitly accepted dogma of traditional biochemistry which stated that 'enzymes are nature's own catalysts developed during evolution for the regulation of metabolic pathways'. This narrow definition implied that man-made organic compounds cannot be regarded as substrates. Once this scholastic problem was surmounted [6], it turned out that the fact that nature has developed its own peculiar catalysts over 3×10^9 years does not necessarily imply that they are designed to work only on their natural target molecules. Research during the past two decades has shown that the substrate tolerance of many enzymes is much wider than previously believed and that numerous biocatalysts are capable of accepting nonnatural substrates of an unrelated structural type by often exhibiting

[3]Other sectors of biotechnology have been defined as 'Red' (biotechnology in medicine), 'Green' (biotechnology for agriculture and plant biotech) and 'Blue' (marine biotechnology), http://www.EuropaBio.org, http://www.bio.org

the same high specificities as for the natural counterparts. It seems to be a general trend, that, the more complex the enzyme's mechanism, the narrower the limit for the acceptability of 'foreign' substrates. It is a remarkable paradox that many enzymes display high specificities for a specific type of reaction while accepting a wide variety of substrate structures. After all, there are many enzymes whose natural substrates – if there are any – are unknown.

- *'Enzymes work only in their natural environment'.*
  It is generally true that an enzyme displays its highest catalytic power in water, which in turn represents something of a nightmare for the organic chemist if it is the solvent of choice. However, biocatalysts *can* function in nonaqueous media, such as organic solvents, ionic liquids, and supercritical fluids, as long as certain guidelines are followed. Only a decade ago, some key rules for conducting biotransformations in organic media were delineated. Although the catalytic activity is usually lower in nonaqueous environments, many other advantages can be accrued by enabling to catalyze reactions which are impossible in water and making many processes more effective (Sect. 3.1) [7–11].

1.3 Advantages and Disadvantages of Biocatalysts

1.3.1 Advantages of Biocatalysts

- *Enzymes are very efficient catalysts.*
  Typically the rates of enzyme-mediated processes are faster by a factor of 10^8–10^{10} than those of the corresponding noncatalyzed reactions, – in some cases even exceeding a factor of 10^{17}, and are thus far above the values that chemical catalysts are capable of achieving [12–14]. As a consequence, chemical catalysts are generally employed in concentrations of a mole percentage of 0.1–1%, whereas most enzymatic reactions can be performed at reasonable rates with a mole percentage of 10^{-3}–10^{-4}% of catalyst, which clearly makes them more effective by some orders of magnitude (Table 1.1).

Table 1.1 Catalytic efficiency of representative enzymes

Enzyme	Reaction catalyzed	TON
Carbonic anhydrase	Hydration of CO_2	600,000
Acetylcholine esterase	Ester hydrolysis	25,000
Penicillin acylase	Amide hydrolysis	2,000
Lactate dehydrogenase	Carbonyl reduction	1,000
Mandelate racemase	Racemisation	1,000
α-Chymotrypsin	Amide hydrolysis	100

TON = turnover number

- *Enzymes are environmentally acceptable.*
  Unlike heavy metals, for instance, biocatalysts are environmentally benign reagents since they are completely biodegradable.

- *Enzymes act under mild conditions.*
  Enzymes act within a range of about pH 5–8 (typically around pH 7) and in a temperature range of 20–40°C (preferably at around 30°C). This minimizes problems of undesired side-reactions such as decomposition, isomerization, racemization, and rearrangement, which often plague traditional methodology.

- *Enzymes are compatible with each other.*[4]
  Since enzymes generally function under the same or similar conditions, several biocatalytic reactions can be carried out in a reaction cascade in a single flask. Thus, sequential reactions are feasible by using multienzyme systems in order to simplify reaction processes, in particular if the isolation of an unstable intermediate can be omitted. Furthermore, an unfavorable equilibrium can be shifted towards the desired product by linking consecutive enzymatic steps. This unique potential of enzymes is increasingly being recognized as documented by the development of multienzyme systems, also denoted as 'artificial metabolism' [15].

- *Enzymes are not restricted to their natural role.*
  They exhibit a high substrate tolerance by accepting a large variety of man-made nonnatural substances and often they are not required to work in water. If advantageous for a process, the aqueous medium can often be replaced by an organic solvent (Sect. 3.1).

- *Enzymes can catalyze a broad spectrum of reactions.*
  Like catalysts in general, enzymes can only *accelerate* reactions but have no impact on the position of the thermodynamic equilibrium of the reaction. Thus, in principle, enzyme-catalyzed reactions can be run in both directions.

There is an enzyme-catalyzed process equivalent to almost every type of organic reaction [16], for example:

- Hydrolysis-synthesis of esters [17], amides [18], lactones [19], lactams [20], ethers [21], acid anhydrides [22], epoxides [23], and nitriles [24].
- Oxidation of alkanes [25], alcohols [26], aldehydes, sulfides, sulfoxides [27], epoxidation of alkenes [28], hydroxylation and dihydroxylation aromatics [29], and the Baeyer-Villiger oxidation of ketones [30, 31].
- Reduction of aldehydes/ketones, alkenes, and reductive amination [32].
- Addition-elimination of water [33], ammonia [34], hydrogen cyanide [35].
- Halogenation and dehalogenation [36], Friedel-Crafts-type alkylation [37], *O*-and *N*-dealkylation [38], carboxylation [39], and decarboxylation [40], isomerization [41], acyloin [42], and aldol reactions [43]. Even Michael

[4] Only proteases are exceptions to this rule for obvious reasons.

additions [44], Stetter reactions [45], Nef reactions [46], and Diels-Alder reactions [47–49] have been reported.

Some major exceptions, for which equivalent reaction types cannot be found in nature, is the Cope rearrangement – although [3,3]-sigmatropic rearrangements such as the Claisen rearrangement are known [50, 51]. On the other hand, some biocatalysts can accomplish reactions impossible to emulate in organic chemistry, e.g., the selective functionalization of ostensibly nonactivated positions in organic molecules, such as the hydroxylation of aliphatics.

This catalytic flexibility of enzymes is generally denoted as 'catalytic promiscuity' [52–58], which is divided into 'substrate promiscuity' (conversion of a nonnatural substrate), 'catalytic promiscuity' (a nonnatural reaction is catalyzed), and 'condition promiscuity' (catalysis occurring in a nonnatural environment).

Enzymes display three major types of selectivities:

– *Chemoselectivity*
  The purpose of an enzyme is to act on a single type of functional group, other sensitive functionalities, which would normally react to a certain extent under chemical catalysis, do survive unchanged. As a result, reactions generally tend to be 'cleaner' so that laborious removal of impurities, associated to side reactions, can largely be omitted.

– *Regioselectivity and Diastereoselectivity*
  Due to their complex three-dimensional structure, enzymes may distinguish between functional groups which are chemically identical but situated in different positions within the same substrate molecule [59, 60].

– *Enantioselectivity*
  Last but not least, all enzymes are made from L-amino acids and thus are chiral catalysts.[5] As a consequence, any type of chirality present in the substrate molecule is 'recognized' upon formation of the enzyme-substrate complex. Thus, a prochiral substrate may be transformed into an optically active product through a desymmetrization process and both enantiomers of a racemic substrate usually react at different rates, affording a kinetic resolution.

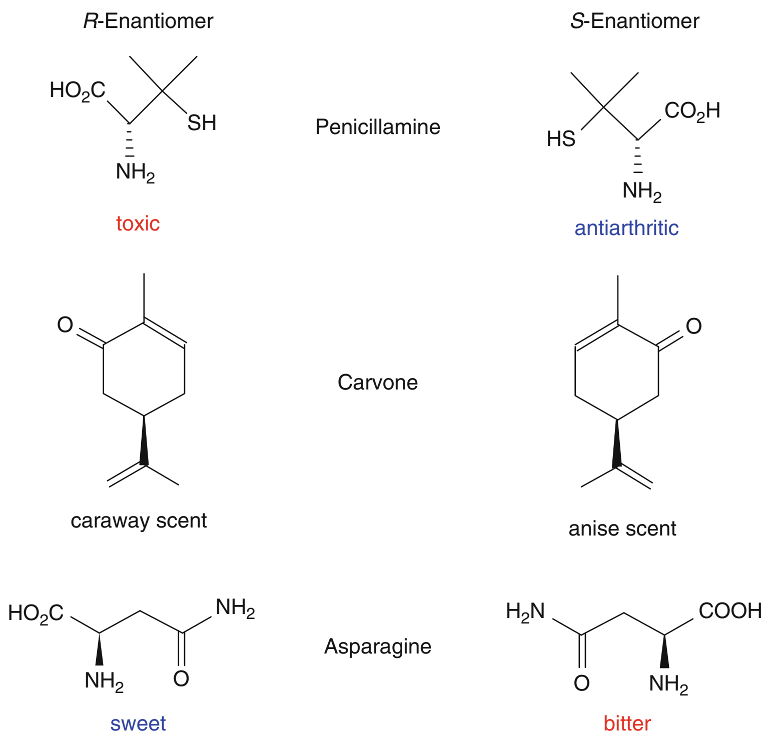
These latter properties collectively constitute the 'stereoselectivity' (in desymmetrizations) or 'enantioselectivity' (in kinetic resolutions) of an enzyme and represent its most important feature for asymmetric exploitation [62]. It is remarkable that this key feature was already recognized by E. Fischer back in 1898 [63].

All the major biochemical events taking place within an organism are governed by enzymes. Since the majority of them are highly selective with respect to the chirality of a substrate, it is obvious that the enantiomers of a given bioactive

[5] For exceptional D-chiral proteins see [61].

compound such as a pharmaceutical or an agrochemical will cause different biological effects [64]. Consequently, in a biological context, enantiomers must be regarded as two distinct species. The isomer with the highest activity is denoted as the 'eutomer', whereas its enantiomeric counterpart, possessing less or even undesired activities, is termed as the 'distomer'. The range of effects derived from the distomer can extend from lower (although positive) activity, no response or toxic events. The ratio of the activities of both enantiomers is defined as the 'eudismic ratio'. Some representative examples of different biological effects are given in Scheme 1.1.

Probably the most well-known and tragic example of a drug in which the distomer causes serious side effects is 'Thalidomide', which was administered as a racemate in the 1960s. At that time it was not known that the sedative effect resides in the (*R*)-enantiomer, but that the (*S*)-counterpart is highly teratogenic [65].[6]



Scheme 1.1 Biological effects of enantiomers

[6]According to a BBC-report, the sale of *rac*-thalidomide to third-world countries has been resumed in mid-1996!

As a consequence, racemates of pharmaceuticals and agrochemicals should be regarded with great caution. Quite astonishingly, 89% of the 537 chiral synthetic drugs on the market were sold in racemic form in 1990, while the respective situation in the field of pesticides was even worse (92% of 480 chiral agents were racemic) [66, 67]. Although at present many bioactive agents are still used as racemates for economic reasons, this situation is constantly changing due to increasing legislation pressure [68]. In 1992, the US Food and Drug Administration (FDA) adopted a long-awaited policy on the issue of whether pharmaceutical companies can market chiral compounds as racemic mixtures or whether they must develop them as single enantiomers [69–71]. According to these guidelines, the development of racemates is not prohibited a priori, but such drugs will have to undergo rigorous justification to obtain their approval based on the separate testing of individual enantiomers. Consequently, single enantiomers are preferred over racemates, which is indicated by the fact that the number of new active pharmaceutical ingredients (APIs) in racemic form remained almost constant from 1992–1999, but they almost disappeared from 2001 onwards, going in hand with the doubling of numbers for single enantiomers [72, 73]. For agrochemicals the writing is on the wall: the current climate of 'environmentality' is precipitating a dramatic move towards the enantiomeric purity of such agents. Overall this has caused an increased need for enantiopure compounds [74, 75].

Unfortunately, less than 10% of organic compounds crystallize as a conglomerate (the remainder form racemic crystals) largely denying the possibility of separating enantiomers by simple crystallization techniques – such as by seeding a supersaturated solution of the racemate with crystals of one pure enantiomer.

The principle of asymmetric synthesis [76] makes use of enantiomerically pure auxiliary reagents which are used in catalytic or sometimes in stoichiometric amounts. They are often expensive and cannot be recovered in many cases.

Likewise, starting a synthesis with an enantiomerically pure compound which has been selected from the large stock of enantiopure natural compounds [77] such as carbohydrates, amino acids, terpenes or steroids – the so-called 'chiral pool' – has its limitations. According to a survey from 1984 [78] only about 10–20% of compounds are available from the chiral pool at an affordable price in the range of US$ 100–250 per kg. Considering the above-mentioned problems with the alternative ways of obtaining enantiomerically pure compounds, it is obvious that enzymatic methods represent a valuable addition to the existing toolbox available for the asymmetric synthesis of fine chemicals [79].

1.3.2 Disadvantages of Biocatalysts

There are certainly some drawbacks worthy of mention for a chemist intent on using biocatalysts:

- *Enzymes are provided by nature in only one enantiomeric form.*
  Since there is no general way of creating mirror-image enzymes from D-amino acids, it is impossible to invert the chiral induction of a given enzymatic reaction

by choosing the 'other enantiomer' of the biocatalyst, a strategy which *is* possible if chiral chemical catalysts are involved. To gain access to the other enantiomeric product, one has to follow a long and uncertain path in searching for an enzyme with exactly the opposite stereochemical selectivity. However, this is sometimes possible, and some strategies how nature transforms mirror-image substrates using stereo-complementary enzymes have recently been analyzed [80].

- *Enzymes require narrow operation parameters.*
  The obvious advantage of working under mild reaction conditions can sometimes turn into a drawback. If a reaction proceeds too slow under given parameters of temperature or pH, there is only a narrow operational window for alteration. Elevated temperatures as well as extreme pH lead to deactivation of the protein, as do high salt concentrations. The usual technique to increase selectivity by lowering the reaction temperature is of limited use with enzymatic transformations. The narrow temperature range for the operation of enzymes prevents radical changes, although positive effects from certain small changes have been reported [81]. Quite astonishingly, some enzymes remain catalytically active even in ice [82, 83].

- *Enzymes display their highest catalytic activity in water.*
  Due to its high boiling point and high heat of vaporization, water is usually the least suitable solvent for most organic reactions. Furthermore, the majority of organic compounds are only poorly soluble in aqueous media. Thus, shifting enzymatic reactions from an aqueous to an organic medium would be highly desired, but the unavoidable price one has to pay is usually some loss of catalytic activity, which is often in the order of one magnitude [84].

- *Enzymes are bound to their natural cofactors.*
  It is a still unexplained paradox, that although enzymes are extremely flexible for accepting nonnatural substrates, they are almost exclusively bound to their natural cofactors which serve as molecular shuttles of redox equivalents [such as heme, flavin, or NAD(P)H] or as storage for chemical energy (ATP). The majority of these 'biological reagents' are relatively unstable molecules and are prohibitively expensive to be used in stoichiometric amounts. Unfortunately, they cannot be replaced by more economical man-made substitutes. Despite an impressive amount of progress, The recycling of cofactors is still not a trivial task (Sects. 2.1.4 and 2.2.1).

- *Enzymes are prone to inhibition phenomena.*
  Many enzymatic reactions are prone to substrate and/or product inhibition, which causes a drop in reaction rate at higher substrate and/or product concentrations, a factor which limits the efficiency of the process.[7] Whereas substrate inhibition

[7]For a convenient method for controlling the substrate concentration see [85].

can be circumvented comparatively easily by keeping the substrate concentration at a low level through continuous addition, product inhibition is a more complicated problem. The gradual removal of product by physical means is usually difficult as is the engagement of another consecutive step to the reaction sequence in order to effect in-situ chemical removal of the product.

- *Enzymes may cause allergies.*
  Enzymes may cause allergic reactions. However, this may be minimized if enzymes are regarded as chemicals and handled with the same care.

1.3.3 Isolated Enzymes vs. Whole Cell Systems

The physical state of biocatalysts which are used for biotransformations can be very diverse. The final decision as to whether one should use isolated, more-or-less purified enzymes or whole microorganisms – either in a free or immobilized form – depends on many factors, such as (*i*) the type of reaction, (*ii*) whether there are cofactors to be recycled, and (*iii*) the scale in which the biotransformation has to be performed. The general pros and cons of using isolated enzymes vs. whole (microbial) cells are outlined in Table 1.2.

A whole conglomeration of biochemistry, microbiology and biochemical engineering – biotechnology – has led to the development of routes to a lot of speciality chemicals (ranging from amino acids to penicillins), starting from cheap carbon sources (such as carbohydrates) and "cocktails" of salts, by using viable whole cells. Such syntheses requiring a multitude of biochemical steps are usually referred to as 'fermentation' processes since they constitute de novo syntheses in a biological sense. In contrast, the majority of microbially mediated biotransformations, often starting from relatively complex organic molecules, makes use of only a single (or a few) biochemical synthetic step(s) by using (or rather 'abusing'!) the microbe's enzymatic potential to convert a nonnatural organic compound into a desired product. The characteristics of processes using resting vs. fermenting whole cells are outlined in Table 1.3.

Facilitated by rapid advances in molecular biology, the use of wild-type microorganisms from natural environments possessing >4,000 genes[8] (which often show decreased yields and/or stereoselectivities due to competing enzyme activities) is constantly declining, while the application of recombinant cells (over)expressing the required protein(s) is rapidly increasing. Consequently, the catalytic protein becomes the dominant fraction in the cell's proteome and side reactions become negligible. If required, competing enzymes can be knocked

[8] *E. coli* has ~4,500 genes and *Saccharomyces cerevisiae* (baker's yeast) ~6,500 genes.

out completely, as long as they are not of vital importance for the primary metabolism. Such taylor-made genetically engineered organisms for biotransformations are often called ‘designer bugs’.

Table 1.2 Pros and cons of using isolated enzymes vs. whole cell systems

Biocatalyst	Form	Pros	Cons
Isolated enzymes	Any	Simple apparatus, simple workup, better productivity due to higher concentration tolerance	Cofactor recycling necessary, limited enzyme stabilities
	Dissolved in water	High enzyme activities	Side reactions possible, lipophilic substrates insoluble, workup requires extraction
	Suspended in organic solvents	Easy to perform, easy workup, lipophilic substrates soluble, enzyme recovery easy	Reduced activities
	Immobilized	Enzyme recovery easy	Loss of activity during immobilization
Whole cells	Any	No cofactor recycling necessary, no enzyme purification required	Expensive equipment, tedious workup due to large volumes, low productivity due to lower concentration tolerance, low tolerance of organic solvents, side reactions likely due to uncontrolled metabolism
	Growing culture	Higher activities	Large biomass, enhanced metabolism, more byproducts, process control difficult
	Resting cells	Workup easier, reduced metabolism, fewer byproducts	Lower activities
	Immobilized cells	Cell reuse possible	Lower activities

Table 1.3 Characteristics of resting vs. fermenting cells

	Resting cells	Fermenting cells
Microbial cells	Resting	Growing
Reaction type	Short, catalytic	Long, life process
Number of reaction steps	Few	Many
Number of enzymes active	Few	Many
Starting material	Substrate	C + N source
Product	Natural or nonnatural	Only natural
Concentration tolerance	High	Low
Product isolation	Easy	Tedious
Byproducts	Few	Many

1.4 Enzyme Properties and Nomenclature

1.4.1 Structural Biology in a Nutshell

The polyamide chain of an enzyme is kept in a three-dimensional structure – the one with the lowest ΔG [86] – which is predominantly determined by its primary sequence.[9] For an organic chemist, an enzyme may be compared with a ball of yarn: Due to the natural aqueous environment, the hydrophilic polar groups (such as $–COO^{-}$, –OH, $–NH_3^{+}$, –SH, and $–CONH_2$) are mainly located on the outer surface of the enzyme in order to become hydrated, with the lipophilic substituents – the aryl and alkyl chains – being buried inside. As a consequence, the surface of an enzyme is covered by a tightly bound layer of water, which cannot be removed by lyophilization. This residual water, or 'structural water' (see Fig. 1.1), accounts for about 5–10% of the total dry weight of a freeze-dried enzyme [87]. It is tighly bound to the protein's surface by hydrogen bonds, is a distinctive part of the enzyme, and is necessary to retain its three-dimensional structure and thus its catalytic activity. As a consequence, structural water differs significantly in its physical state from the 'bulk water' of the surrounding solution. There is very restricted rotation of the 'bound water' and it cannot freely reorientate upon

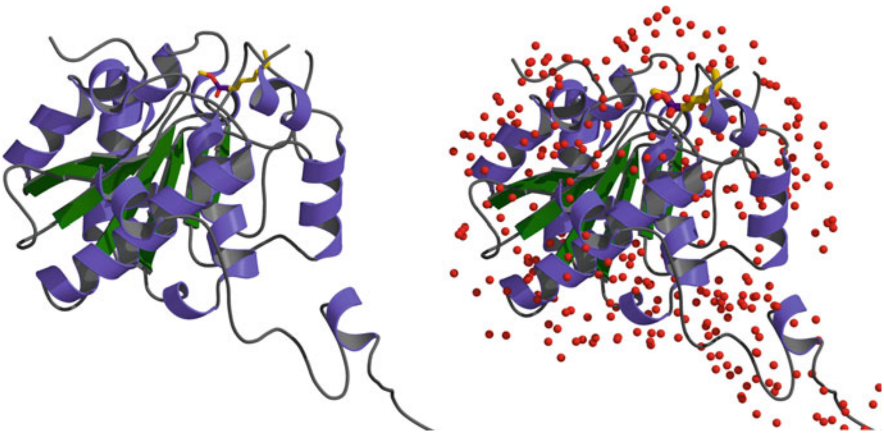


Fig. 1.1 Ribbon representation of the crystal structure of a *Candida antarctica* lipase B mutant bearing an inhibitor (*yellow*) bound to the active site (*left*). Structural water molecules are depicted as red dots (*right*).[10]

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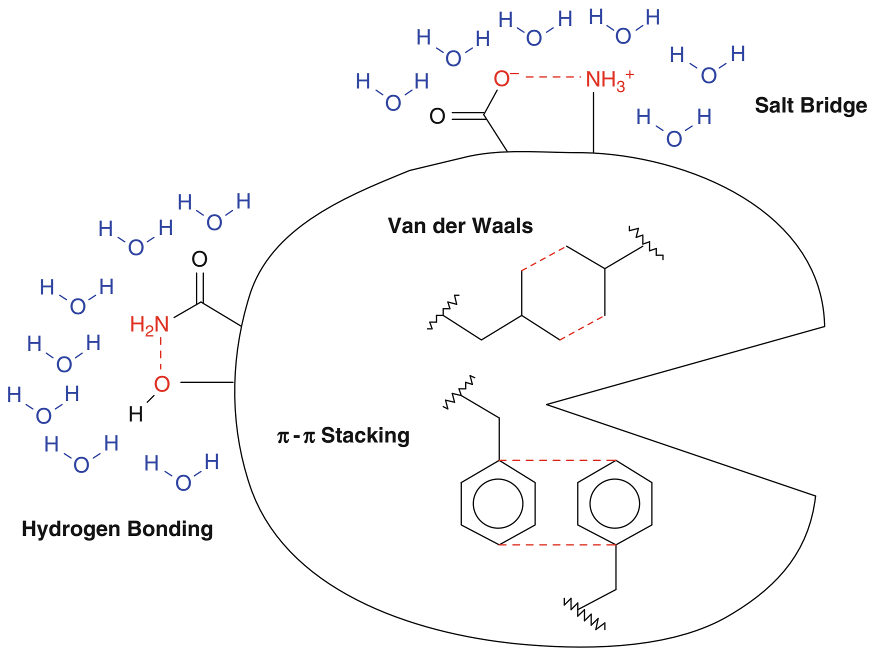
[9]The amino acid sequence of a protein is generally referred to as its 'primary structure', whereas the three-dimensional arrangement of the polyamide chain (the 'backbone') in space is called the 'secondary structure'. The 'tertiary structure' includes the arrangement of all atoms, i.e., the amino acid side chains are included, whereas the 'quarternary structure' describes the aggregation of several protein molecules to form oligomers.

[10]PDB entry 3icw, courtesy of U. Wagner.

freezing.[11] Exhaustive drying of an enzyme (e.g., by chemical means) would force the protein to change its conformation resulting in a loss of activity.

The whole protein structure is stabilized by a large number of relatively weak binding forces such as van der Waals interactions of aliphatic chains[12] and π-π stacking of aromatic amino acids, which are predominantly located inside the protein core (Scheme 1.2). In contrast, stronger hydrogen bonds and salt bridges[13] are often close to the surface. As a consequence of the weak binding forces inside and strong bonds at the surface, in a rough approximation, enzymes have a soft core but a hard shell and thus represent delicate and soft (jellyfish-like) structures.



Scheme 1.2 Schematic representation of binding forces within a protein structure

The latter facilitates conformational movements during catalysis (such as the 'induced fit', see below) thereby underlining the pronounced dynamic character of enzyme catalysis. Besides the main polyamide backbone, the only covalent bonds are –S–S– disulfide bridges. Enzymes are intrinsically unstable in solution and can be deactivated by denaturation, caused by increased temperature, extreme pH, or an unfavorable dielectric environment such as high salt concentrations.

[11]Water bound to an enzyme's surface exhibits a (formal) freezing point of about –20°C.

[12]Also called London forces.

[13]Also called Coulomb interactions.

The types of reaction leading to an enzyme's deactivation are as follows [88]:

– Rearrangement of peptide chains (due to partial unfolding) starts at around 40–50°C. Most of these rearrangements are reversible and therefore relatively harmless.
– Hydrolysis of peptide bonds in the backbone, in particular adjacent to asparagine units, occurs at more elevated temperatures. Functional groups of amino acids can be hydrolytically cleaved (again especially at sites adjacent to asparagine and glutamine residues) to furnish aspartic and glutamic acid, respectively. Both reaction mechanisms are favored by the presence of neighboring groups, such as glycine, which enable the formation of a cyclic intermediate. Thus, a negative charge (i.e., $–COO^-$) is created from a neutral group ($–CONH_2$). In order to become hydrated, this newly generated carboxylate moiety pushes towards the protein's surface causing irreversible deactivation.
– Thiol groups may interchange the –S–S– disulfide bridges, leading to a modification of covalent bonds within the enzyme.
– Elimination and oxidation reactions (often involving cysteine residues) cause the final destruction of the protein.

Thermostable enzymes from thermophilic microorganisms show an astonishing upper operation limit of 60–80°C and differ from their mesophilic counterparts by only small changes in primary structure [89]. The three-dimensional structure of such enzymes is often the same as those derived from mesophilics [90], but generally they possess fewer asparagine residues and more salt- or disulfide bridges. More recently, numerous (thermo)stable mutant enzymes have been obtained by genetic engineering. It is a common phenomenon, that an increased thermostability of proteins often goes in hand with an enhanced tolerance for organic solvents.

1.4.2 Mechanistic Aspects of Enzyme Catalysis

The unparalleled catalytic power of enzymes has sparked numerous studies on mechanistic theories to provide a molecular understanding of enzyme catalysis for almost a century. Among the numerous theories and rationales, the most illustrative models for the organic chemist are discussed here [91–93].

'Lock-and-Key' Mechanism

The first proposal for a general mechanism of enzymatic action was developed by E. Fischer in 1894[14] [94, 95]. It assumes that an enzyme and its substrate mechanistically interact like a lock-and-key fashion (Fig. 1.2). Although this assumption was quite sophisticated at that time, it assumes a completely rigid enzyme structure.

[14]'To use a picture I want to say that enzyme and glucoside must go together like key and lock in order to exert a chemical effect upon each other', see [94] p. 2992.

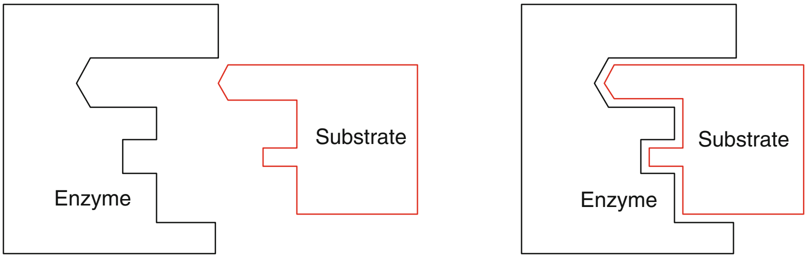


Fig. 1.2 Schematic representation of the 'lock-and-key' mechanism

Thus, it cannot explain why many enzymes do act on larger substrates, while they are inactive on smaller counterparts. Given Fischer's rationale, small substrates should be transformed at even higher rates than larger substrates since the access to the active site would be easier. Furthermore, the hypothesis cannot explain why many enzymes are able to convert not only their natural substrates but also numerous nonnatural compounds possessing different structural features. Consequently, a more sophisticated model had to be developed.

Induced-Fit Mechanism

This rationale, which takes into account that enzymes are not entirely rigid but rather represent delicate and soft structures, was developed by Koshland Jr. in the 1960s [96, 97].[15] It assumes that upon approach of a substrate during the formation of the enzyme-substrate complex, the enzyme can change its conformation under the influence of the substrate structure so as to wrap itself around its guest (Fig. 1.3). This phenomenon was denoted as the 'induced fit'. It can be illustrated by the interaction of a hand (the substrate) and a glove (the enzyme). This advanced model can indeed explain why in many cases several structural features on a substrate are required in addition to the reactive group. These structural features may be located at quite a distance from the actual site of the reaction. The most typical 'induced-fit' enzymes are the lipases. They can convert an amazingly large variety of artificial substrates which possess structures which do not have much in common with the natural substrates – triglycerides.

A schematic representation of the 'induced-fit' mechanism is given in Figure 1.3: Whereas A represents the reactive group of the substrate, X is the complementary reactive group(s) of the enzyme – the 'chemical operator'. Substrate part B forces the enzyme to adapt a different (active) conformation. Only then are the 'active'

[15]'A precise orientation of catalytic groups is required for enzyme action; the substrate may cause an appreciable change in the three-dimensional relationship of the amino acids at the active site, and the changes in protein structure caused by a substrate will bring the catalytic groups into proper orientation for reaction, whereas a non-substrate will not.' See [96].

groups X of the enzyme positioned in the right way to effect catalysis. If part B is missing, no conformational change (the 'induced fit')[16] takes place and thus the chemical operators stay in their inactive state.

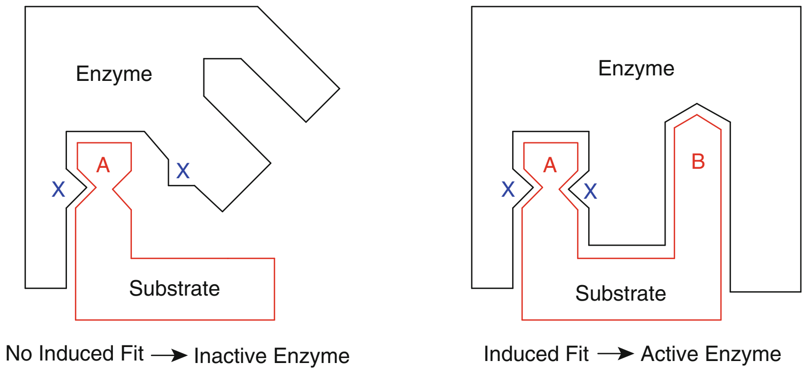


Fig. 1.3 Schematic representation of the 'induced-fit' mechanism

Desolvation and Solvation-Substitution Theory

More recently, M.J.S. Dewar developed a different rationale [99] in attempting to explain the high conversion rates of enzymatic reactions, which are generally substantially faster than the chemically catalyzed equivalent processes.[17] This so-called 'desolvation theory' assumes that the kinetics of enzyme reactions have much in common with those of gas-phase reactions. If a substrate enters the active site of the enzyme, it replaces *all* of the water molecules at the active site of the enzyme. Then, a formal gas-phase reaction can take place which mimics two reaction partners interacting without a 'disturbing' solvent. In solution, the water molecules impede the approach of the partners, hence the reaction rate is reduced. This theory would, inter alia, explain why small substrate molecules are often more slowly converted than larger analogues, since the former are unable to replace *all* the water molecules at the active site.

The 'desolvation' theory has recently been extended by a 'solvation-substitution' theory [101]. It is based on the assumption that the enzyme would not be able to strip off the water which is surrounding the substrate to effect a 'desolvation', because this would be energetically unfavorable. Instead, the solvent is *displaced* by the environment of the active site of the enzyme thereby affecting 'solvation substitution'. Thus, the (often) hydrophobic substrate replaces the water with the (often) hydrophobic site of the enzyme which favors the formation of the

[16]Conformational changes are differentiated into hinge- and shear-type movements [98].

[17]A 'record' of rate acceleration factor of 10^{14} has been reported. See [100].

enzyme-substrate complex. In addition, the replacement of water molecules within the active site during the substrate-approach decreases the dielectric constant within this area, which is comparable to that of a nonpolar (organic) solvent, which in turn enhances electrostatic enzyme-substrate interactions. The latter cause proper substrate-orientation thereby leading to an enhancement of catalytically productive events.[18]

Entropy Effects
A major reason for the exceptional catalytic efficiency of enzymes over small (chemical) catalysts is derived from the difference in size, which is roughly two orders of magnitude.[19] This allows the enzyme to enclose its substrate completely, with the catalytically active groups being positioned in close proximity, while non-productive movements are restricted. This rate-enhancing entropy effect is very similar to a (fast) intermolecular reaction, where the reacting groups are pre-arranged in close proximity. In contrast, (slow) intramolecular reactions in 'true' solution are impeded by unproductive movements caused by diffusion [102–105]. In any case, it is clear that a maximum change in entropy is only obtained upon a tight and close fit of a substrate into the pocket of an active site of an enzyme [106].

Electrostatic and Covalent Catalysis
Most transition states involve charged intermediates, which are stabilized within the active site of an enzyme via ionic bonds in 'pockets' or 'holes' bearing a matching opposite charge. Such charges are derived from acidic or basic amino acid side chains (such as Lys, Arg, Asp, or Glu)[20] or are provided by (Lewis acid-type) metal ions, typically Zn^{2+}. Computer simulations studies suggested that in enzymes electrostatic effects provide the largest contribution to catalysis [107]. As a prominent example, the tetrahedral intermediate of carboxyl ester hydrolysis is stabilized in serine hydrolases by the so-called 'oxyanion hole' (Scheme 2.1).

Many enzymes form covalent bonds with their substrates during catalysis, such as the acyl-enzyme intermediate in carboxyl ester hydrolysis (Scheme 2.1) or the glycol monoester intermediate in epoxide hydrolysis (Scheme 2.85). Despite the covalent enzyme-substrate bond, such species are metastable and should be regarded as 'activated intermediates'. Some enzymes utilize cofactors, such as

[18]This phenomenon is denoted as 'electrostatic catalysis' and was coined as 'Circe-effect' by WP Jencks.

[19]By average, enzymes are 100 times bigger than related chemical catalysts.

[20]It is important to note that the (modest) pK_a of typical amino acid side chains, such as $–NH_3^+$ or $–CO^{2-}$ can be substantially altered up to 2–3 pK_a-units through neighboring groups within the enzyme environment. As a consequence, the (approximately neutral) imidazole moiety of His can act as strong acid or base, depending on its molecular environment.

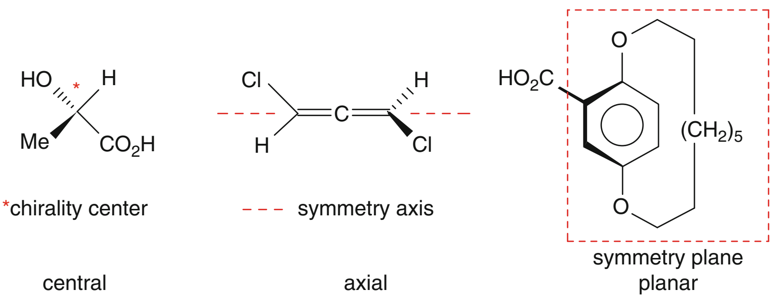
pyridoxal phosphate (PLP) or thiamine diphosphate (TPP), to form covalent intermediates during catalysis.

Since some enzymes can act faster than what would be predicted by the 'over-the-barrier' transition-state model ($\Delta\Delta G^{\neq}$), 'through-the-barrier' quantum tunneling of protons or electrons has been postulated [108, 109].

Three-Point Attachment Rule

This widely used rationale to explain the enantioselectivity of enzymes was suggested by A.G. Ogston [110]. Since chirality is a quality of space, a substrate must be positioned firmly in three dimensions within the active site of an enzyme in order to ensure spatial recognition and to achieve a high degree of enantioselection. As a consequence, at least three different points of attachment of the substrate onto the active site are required.[21]

This is exemplified for the discrimination of the enantiomers of a racemic substrate (A and B in Fig. 1.4) with its chirality located on a sp^3-carbon atom. For compounds possessing an axial or planar chirality involving sp^2- or sp-carbon atoms [112], respectively, analogous pictures can be created (Scheme 1.3). Although the majority of chiral molecules subjected to biotransformations possess central chirality located on an sp^3-carbon atom, all types of chiral molecules can be 'recognized' in principle, including those bearing a stereogenic sp^3-heteroatom such as phosphorus or sulfur [113].



Scheme 1.3 Examples for central, axial, and planar chirality

The use of the Three-Point Attachment Rule is described as follows (Figs. 1.4–1.6):

Case I: Enantiomer A is a good substrate because it allows an optimal interaction of its groups (A, B, C) with their complementary binding sites of the enzyme (A', B', C'). It ensures an optimal orientation of the reactive group

[21] The following rationale was adapted from [111].

(D) towards the chemical operator (↙) which is required for a successful transformation.

Cases II through IV: Regardless of its orientation in the active site, enantiomer B is a poor substrate because optimal binding and orientation of the reactive group D is not possible. Thus, poor catalysis will be observed (Fig. 1.4).

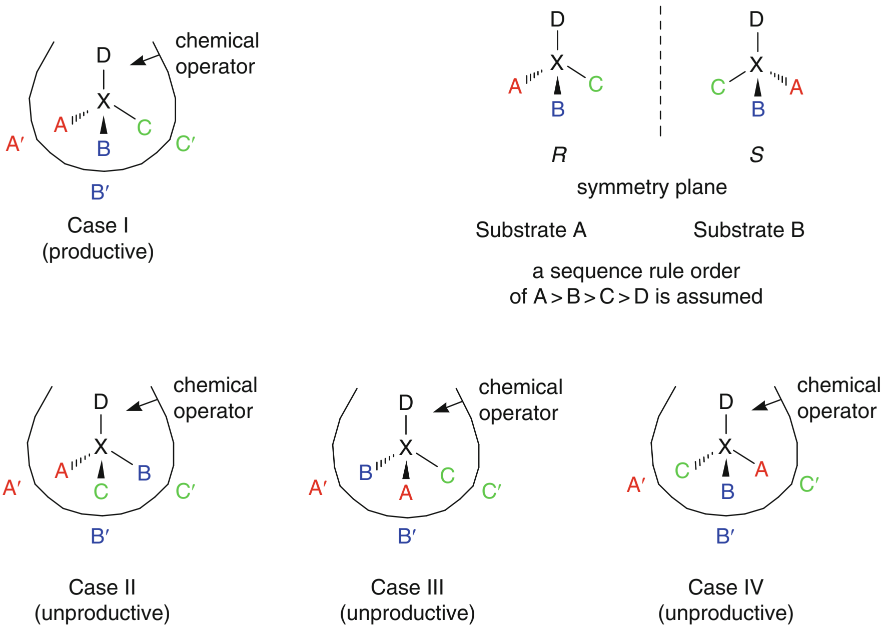
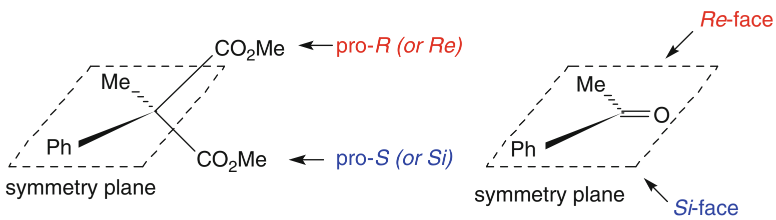


Fig. 1.4 Schematic representation of enzymatic enantiomer discrimination

If a prochiral substrate (C), bearing two chemically identical but stereochemically different enantiotopic groups (A), is involved, the same model can be applied to rationalize the favored transformation of one of the two groups A leading to an 'enantiotopos differentiation' (Scheme 1.4, Fig. 1.5).



Scheme 1.4 Enantiotopos and -face nomenclature

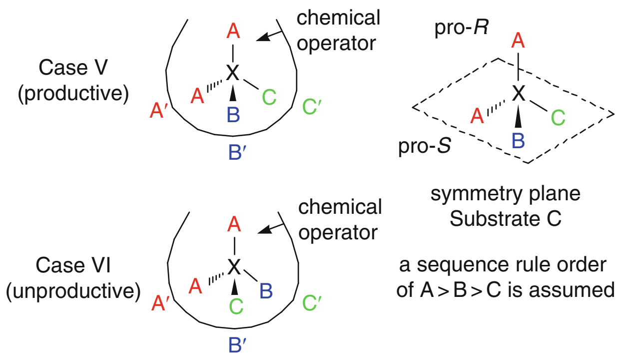


Fig. 1.5 Schematic representation of enzymatic enantiotopos discrimination

Case V: There is a good binding of a prochiral substrate (C) to the complementary enzyme's binding sites with the pro-(*R*) group out of the two reactive groups (A) being positioned towards the chemical operator.

Case VI: Positioning of the pro-(*S*) reactive group towards the chemical operator results in poor orientation of the other functions to their complementary sites, resulting in poor catalysis. As a consequence, the pro-(*R*) group is cleaved preferentially to its pro-(*S*) counterpart.

The ability of enzymes to distinguish between two enantiomeric faces of a prochiral substrate (D) – an 'enantioface differentiation' – is illustrated in Fig. 1.6.

Case VII: An optimal match between the functional groups of substrate D leads to an attack of the chemical operator on the central atom X from the (top) *Si*-side.

Case VIII: The mirror image orientation (also called 'alternative fit') of substrate D in the active site of the enzyme leads to a mismatch in the binding of the functional groups, thus an attack by the chemical operator, which would come from the (bottom) *Re*-side in this case, is disfavored.

Generally, many functional groups – and sometimes also coordinated metal ions – have to work together in the active site of the enzyme to effect catalysis. Individual enzyme mechanisms have been elucidated in certain cases where the exact three-dimensional structure is known. For most of the enzymes used for the biotransformation of nonnatural organic compounds, assumptions are made about their molecular action. However, the logic of organic reaction mechanisms, which is based on thinking in terms of polarities – nucleophile/electrophile and acid/base –, represents an excellent intellectual basis for explaining enzyme catalysis and organic chemists in particular will quickly see that there is nothing 'magic' about biocatalysts: they simply perform excellent organic chemistry.

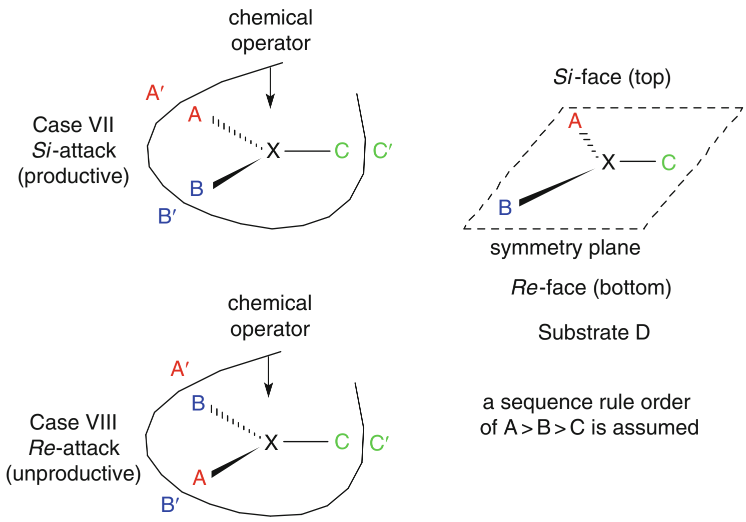
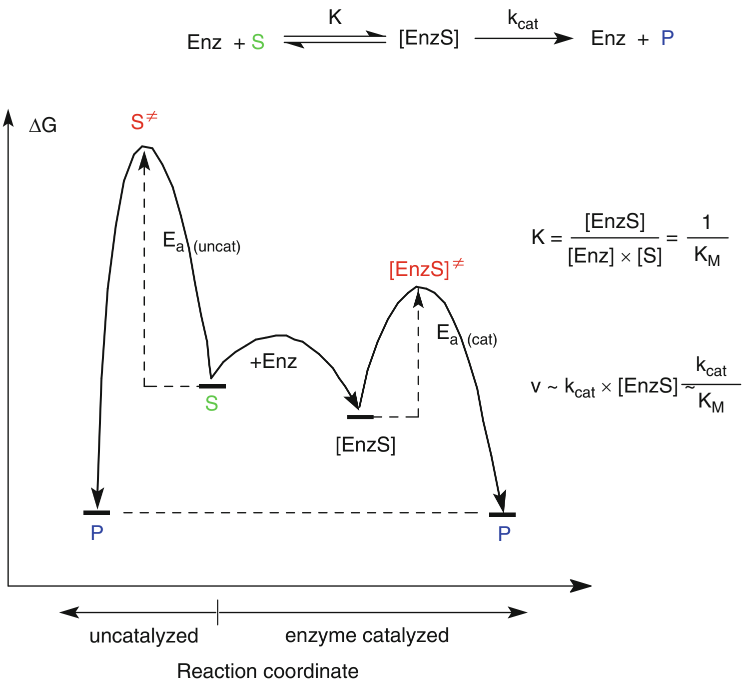


Fig. 1.6 Schematic representation of enzymatic enantioface discrimination

Kinetic Reasons for Selectivity

As in every other catalytic reaction, an enzyme (Enz) accelerates the reaction by lowering the energy barrier between substrate (S) and product (P) – the activation energy (E_a) [114]. The origin of this catalytic power – the rate acceleration – has generally been attributed to transition-state stabilization of the reaction by the enzyme [115], assuming that the catalyst binds more strongly to the transition state than to the ground state of the substrate, by a factor approximately equal to the acceleration rate [116] (Fig. 1.7). The dissociation constant for the enzyme-transition state complex $[EnzS]^{\neq}$ has been estimated to be in the range of 10^{-20} molar [117].

Virtually all stereoselectivities of enzymes originate from the energy difference in the enzyme-transition state complex $[EnzS]^{\neq}$ (Fig. 1.8). For instance, in an enantioselective reaction both of the enantiomeric substrates A and B (Fig. 1.4) or the two forms of mirror-image orientation of a prochiral substrate involving its enantiotopic groups or faces (Figs. 1.5 and 1.6) compete for the active site of the enzyme. Due to the chiral environment of the active site of the enzyme, *diastereomeric* enzyme-substrate complexes [EnzA] and [EnzB] are formed, which possess different values of free energy (ΔG) for their respective transition states $[EnzA]^{\neq}$ and $[EnzB]^{\neq}$. The result is a difference in activation energy ($\Delta\Delta G^{\neq}$) for both of the enantiomeric substrates or the ‘enantiomeric orientations’, respectively. As a consequence, one enantiomer (or orientation) will be transformed faster than the other. This process is generally referred to as ‘chiral recognition’.



Enz = enzyme, S = substrate, [EnzS] = enzyme-substrate complex, P = product, K = equilibrium constant for [EnzS] formation, k_{cat} = reaction rate constant for [EnzS] $\rightarrow$ Enz + P, E_a = activation energy, ≠ denotes a transition state, K_m = Michaelis-Menten constant, v = reaction velocity.

Fig. 1.7 Energy diagram of catalyzed vs. uncatalyzed reaction

The value of this difference in free energy, expressed as $\Delta\Delta G^{\neq}$, is a direct measure for the selectivity of the reaction which in turn determines the ratio of the individual reaction rates (v_A, v_B) of enantiomeric substrates A and B (or the two enantiotopic faces or groups competing for the active site of the enzyme, Fig. 1.8).[22] These values are of great importance since they determine the optical purity of the product. $\Delta\Delta G^{\neq}$ is composed of an enthalpy ($\Delta\Delta H^{\neq}$) and an entropy term ($\Delta\Delta S^{\neq}$). The enthalpy of activation is usually dominated by the breakage and formation of bonds when the substrate is transformed into the product. The entropy contribution includes the energy balance from the ‘order’ of the system, i.e., orienting the reactants, changes in conformational flexibility during the ‘induced-fit’, and various

[22]The individual reaction rates v_A and v_B correspond to $v_A = (k_{cat}/K_M)_A \cdot [\mathrm{Enz}] \cdot [\mathrm{A}]$ and $v_B = (k_{cat}/K_M)_B \cdot [\mathrm{Enz}] \cdot [\mathrm{B}]$, respectively, according to Michaelis-Menten kinetics. The ratio of the individual reaction rates of enantiomers is an important parameter for the description of the enantioselectivity of a reaction: $v_A/v_B = E$ (‘Enantiomeric Ratio’, see Sect. 2.1.1).

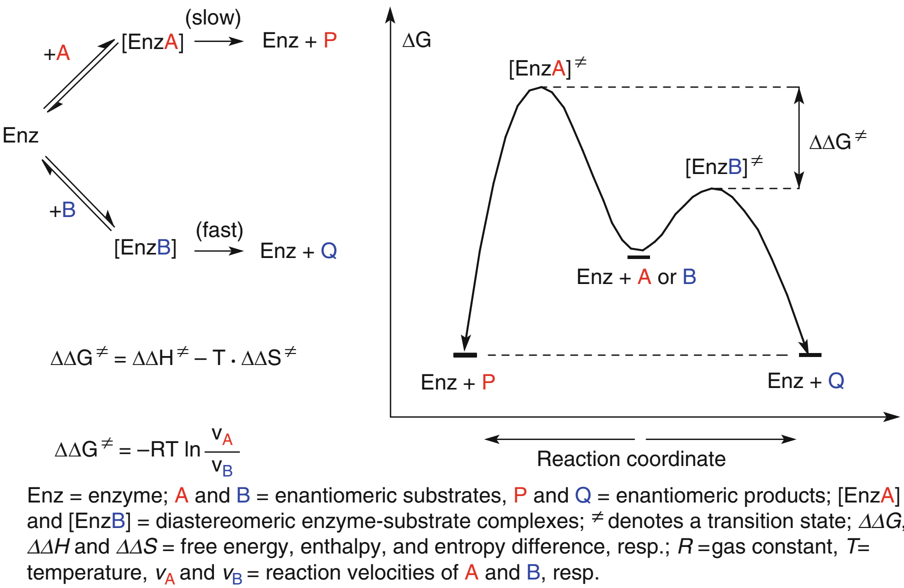


Fig. 1.8 Energy diagram for an enzyme-catalyzed enantioselective reaction

concentration and solvatation effects. Table 1.4 lists some representative values of enantiomeric excess of product (e.e.) corresponding to a given $\Delta\Delta G^{\neq}$ of the reaction.

Table 1.4 Free energy values $\Delta\Delta G^{\neq}$ for representative optical purities of product (e.e.) and the corresponding ratio of reaction rates of enantiomers (v_A, v_B)

$\Delta\Delta G^{\neq}$ [kcal/mol]	v_A/v_B	e.e. [%]
0.118	1.2	10
0.651	3	50
1.74	19	90
2.17	39	95
3.14	199	99
4.50	1,999	99.9

$$\text{e.e.}[\%] = \frac{P - Q}{P + Q} \times 100$$

Even a very small difference in free energy (e.g., 0.65 kcal/mol) can lead to a considerable enantiomeric excess of product (50%) (Table 1.4). Due to the logarithmic dependence between $\Delta\Delta G^{\neq}$ and e.e. of the product (Fig. 1.8), only a modest 1.75 kcal/mol is required to reach ~90% e.e., but the same amount of $\Delta\Delta G^{\neq}$ is roughly needed to push this value over the threshold of 99%! For virtually absolute selectivities, however, $\Delta\Delta G^{\neq}$ has to be considerably higher ($\geq$4.50 kcal/mol).

1.4.3 Classification and Nomenclature

At present about 3,700 enzymes have been recognized by the International Union of Biochemistry and Molecular Biology (IUBMB) [118–121] and if the prediction that there are about 25,000 enzymes existing in Nature is true [122], the bulk of this vast reservoir of biocatalysts still remains to be discovered and is waiting to be used. However, only a minor fraction of the enzymes already investigated (~10%) is commercially available. However, this number is steadily increasing.

For identification purposes, every enzyme has a four-digit number in the general form [EC A.B.C.D], where EC stands for 'Enzyme Commission'. The following properties are encoded:

A. denotes the main type of reaction (see Table 1.5);
B. stands for the subtype, indicating the substrate class or the type of transferred molecule;
C. indicates the nature of the co-substrate;
D. is the individual enzyme number.

As depicted in Table 1.5, enzymes have been classified into six categories according to the type of reaction they can catalyze. At first glance, it would seem advantageous to keep this classification throughout this book, since organic chemists are used to thinking in terms of reaction principles. Unfortunately, this does not work in practice for the following reasons: Due to the varying tolerance for

Table 1.5 Classification of enzymes

Enzyme class	Number		Reaction type	Utility[a]
	Classified	Available		
1. Oxidoreductases	~700	~100	Oxidation–reduction: oxygenation of C–H, C–C, C=C bonds, or overall removal or addition of hydrogen atom equivalents	+++
2. Transferases	~750	~100	Transfer of groups: aldehydic, ketonic, acyl, sugar, phosphoryl, methyl, NH_3	++
3. Hydrolases	~650	~180	Hydrolysis formation of esters, amides, lactones, lactams, epoxides, nitriles, anhydrides, glycosides, organohalides	+++
4. Lyases	~300	~40	Addition–elimination of small molecules on C=C, C=N, C=O bonds	++
5. Isomerases	~150	6	Isomerizations such as racemization, epimerization, rearrangement	+
6. Ligases	~80	5	Formation-cleavage of C–O, C–S, C–N, C–C bonds with concomitant triphosphate cleavage	±

[a] The estimated 'utility' of an enzyme class for the transformation of nonnatural substrates ranges from +++ (very useful) to ± (little use) [123].[23]

[23] Based on the biotransformation database of Kroutil and Faber (2010) ~14,000 entries.

nonnatural substrates, the importance for practical applications in organic synthesis is not at all evenly distributed amongst the different enzyme classes, as may be seen from the 'utility' column in Table 1.5 (compare Chap. 4). Furthermore, due to the widespread use of crude enzyme preparations (consisting of more than one active biocatalyst), one often does not know which enzyme is actually responsible for the biotransformation.

Last but not least, there are many useful reactions which are performed with whole microbial cells, for which it can only be speculated as to which of the numerous enzymes in the cell is actually involved in the transformation.

One particular warning should be given concerning catalytic activities which are measured in several different systems:

According to the SI system, catalytic activity is defined by the katal (1 kat = 1 mol s^{-1} of substrate transformed). Since its magnitude is far too big for practical application, it has not been widely accepted. The transformation of one mole of an organic compound within one second resembles an industrial-scale reaction and is thus not suited to describe enzyme kinetics. As a consequence, a more appropriate standard – The 'International Unit' (1 I.U. = 1 μmol of substrate transformed per min) – has been defined. Unfortunately, other units such as nmol/min or nmol/hour are also common, mainly to make the numbers of low catalytic activity look bigger. After all, it should be kept in mind that the activities using *nonnatural* substrates are often significantly below the values which were determined for *natural* substrates.

A comparison of the activity of different enzyme preparations is only possible if the assay procedure is performed exactly in the same way. Since most enzyme suppliers use their own experimental setup, an estimation of the cost/activity ratio of enzymes from various commercial sources is seldom possible by using published data and therefore activity data have to be determined independently.

The *catalytic power* of a (bio)catalyst can be conveniently described by the so-called 'turnover frequency' (TOF), which has the dimension of [time^{-1}]. It indicates the number of substrate molecules which are converted by a single (bio)catalyst molecule in a given period of time[24] [125].[25] For biochemical reactions, this unit is the second, for the slower chemical catalysts, the minute (or the hour, for very slow catalyst) is usually preferred. Since it is mass-independent, it allows to compare the performance of different (chemo- and bio-)catalytic systems. For the majority of enzymes used in biotransformations, TOFs are within the range of 10–1000 s^{-1}, whereas the respective values for chemical catalysts are one to two orders of magnitude lower.

[24]For a discussion of the pitfalls associated with TONs and TOFs see [124].

[25]Assuming that each catalyst molecule has a single active site. For enzymes obeying Michaelis-Menten kinetics the TON is equal to $1/k_{cat}$.

$$\text{Turnover Frequency (TOF)} = \frac{\text{Number of Substrates Converted}}{\text{Number of Catalyst Molecules} \times \text{Time}} \left[\frac{\text{Mol}}{\text{Mol} \times \text{Time}}\right]$$

The *productivity* of a (bio)catalyst is characterized by the dimensionless 'turnover number' (TON). It denotes the number of substrate molecules converted per number of catalyst molecules used within a given time span. TONs for enzymes typically range from 10^3 to 10^6 (Table 1.1) [126].

$$\text{Turnover Number (TON)} = \frac{\text{Number of Substrates Converted}}{\text{Number of Catalyst Molecules}} \left[\frac{\text{Mol}}{\text{Mol}}\right]$$

The comparison of TOFs and TONs of different (bio)catalysts should be exercised with great caution, since these numbers only indicate how fast the catalysts act at the onset of the reaction within a limited time span, but they do not tell whether the activity remains at a constant level or if it dropped due to catalyst/enzyme deactivation.

In every catalytic process, the *operational stability* of the catalyst under process conditions is a key parameter. It is described by the dimensionless 'total turnover number' (TTN), which is determined by the moles of product formed by the amount of catalyst spent and – in other words – it stands for the amount of product which is produced by a given amount of catalyst during its whole lifetime. If the TONs of repetitive batches of a reaction are measured until the catalyst is dead, the sum of all TONs would equal to the TTN. TTNs are also commonly used to describe the efficiency of cofactor recycling systems.

$$\text{Total Turnover Number (TTN)} = \frac{\text{Number of Substrates Converted}}{\text{Number of Catalyst Molecules}} \left[\frac{\text{Mol}}{\text{Mol}}\right] \text{(Lifetime)}$$

The efficiency of microbial transformations (where the catalytic activity of enzymes involved cannot be measured) is characterized by the so-called 'productivity number' (PN) [127], defined as:

$$\text{Productivity Number (PN)} = \frac{\text{Amount of Product Formed}}{\text{Biocatalyst (dry weight)} \times \text{Time}} \left[\frac{\text{Mol}}{\text{g} \times \text{Time}}\right]$$

which is the amount of product formed by a given amount of whole cells (dry weight) within a certain period of time. This number resembles the specific activity as defined for pure enzymes, but also includes several other important factors such as inhibition, transport phenomena, and concentration.

1.4.4 Coenzymes

A remarkable proportion of synthetically useful enzyme-catalyzed reactions require cofactors (coenzymes).[26] These compounds are relatively low in molecular weight compared to an enzyme (a few hundred Da, in contrast to the general range of 15,000 to 1,000,000 Da for enzymes) - which provide either ‘chemical reagents’ such as redox-equivalents, e.g., hydrogen, oxygen or electrons and carbon units. Alternatively, ‘chemical energy’ can be stored in energy-rich functional groups, such as acid anhydrides in ATP or PAPS. As a rule of thumb, enzymes are bound to their natural cofactors, which cannot be replaced by more economical man-made chemical substitutes.

Table 1.6 Common coenzymes required for biotransformations

Coenzyme	Reaction type	Recycling[a]
NAD^+/NADH	Carbonyl reduction &	(+) [++]
$NADP^+$/NADPH	Alcohol oxidation	(+) [+]
ATP[b]	Phosphorylation	(+) [+]
SAM	C_1-alkylation	(+) [±]
Acetyl-CoA	C_2-alkylation	(+) [±]
Flavins[c]	Baeyer-Villiger- & S-oxidation, C=C reduction	(–)
Pyridoxal-phosphate	Transamination, racemization	(–)
Biotin	Carboxylation	(–)
Thiamine diphosphate	C–C ligation	(–)
Metal-porphyrins[c]	Peroxidation, oxygenation	(–)

[a]Recycling of a cofactor is necessary (+) or not required (–), the feasibility of which is indicated in square brackets ranging from ‘feasible’ [++] to ‘complicated’ [±]
[b]For other triphosphates, such as GTP, CTP, and UTP, the situation is similar
[c]Many flavin- and metal porphyrin-dependent mono- or dioxygenases require additional NAD(P)H as an indirect reducing agent

Some cofactors are rather sensitive molecules and are gradually destroyed due to undesired side reactions occurring in the medium, in particular NAD(P)H and ATP. These cofactors are too expensive to be used in the stoichiometric amounts formally required. Accordingly, when coenzyme-dependent enzymes are employed, the corresponding coenzymes are used in catalytic amounts in conjunction with an efficient and inexpensive system for their regeneration in situ. Some methods for cofactor recycling are already well developed, but others are still problematic, as depicted in Table 1.6. Fortunately, some coenzymes are tightly bound to their respective enzymes so that external recycling is not required. Whereas the redox potential of NADH and NADPH is largely independent of the type of enzyme, the potential of flavin cofactors is significantly determined by the protein, to which it is covalently or noncovalently attached. Consequently, the redox capabilities of

[26]A ‘cofactor’ is tightly bound to an enzyme (e.g., FAD), whereas a ‘coenzyme’ can dissociate into the medium (e.g., NADH). In practice, however, this distinction is not always made in a consequent manner.

flavoproteins are widespread and encompass a broad range of oxidation and reduction reactions.

Many enzymes require coordinated metals such as Fe, Ni, Cu, Co, V, Zn, Ca, Mg, or Mn. In many cases, chemists do not have to worry about supplying these metals since they are already present, tightly bound to the enzyme. If this is not the case, they can easily be supplied by enrichment of the medium with the respective metal ions.

1.4.5 Enzyme Sources

The large majority of enzymes used for biotransformations in organic chemistry are employed in a crude form and are relatively inexpensive. The preparations typically contain only about 1–30% of actual enzyme, the remainder being inactive proteins, stabilizers, buffer salts, or carbohydrates from the fermentation broth from which they have been isolated. It should be kept in mind, that crude preparations are often more stable than purified enzymes.

The main sources of enzymes for biotransformations are as follows [128, 129]:

– The detergent industry produces many proteases and lipases in huge amounts. These are largely used as additives for detergents to effect the hydrolysis of proteinogenic and fatty impurities in the laundry process.
– The food industry uses proteases and lipases for meat and cheese processing and for the amelioration of fats and oils [130]. Glycosidases and decarboxylases are predominantly employed in the brewing and baking industries.
– Numerous enzymes can be isolated from slaughter waste or from cheap mammalian organs, such as kidney or liver.
– The richest and most convenient sources of enzymes are microorganisms. An impressive number of biocatalysts are derived from bacterial and fungal origin by cheap fermentation.
– Only a small proportion of enzymes used in biotransformations is obtained from plant sources, such as fruits (e.g., fig, papaya, pineapple) and vegetables (e.g., tomato, potato). Whereas sensitive plant cell cultures were used in the past, plant enzymes are nowadays cloned into a sturdy microorganism for production.
– Pure enzymes are usually very expensive and are thus mostly sold by the unit, while crude preparations are often shipped in kg amounts. Since the techniques for protein purification through His- or Strep-tagging are becoming easier and more economic, the use of (partially) purified enzymes in biotransformations is rapidly increasing.

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Chapter 2
Biocatalytic Applications

2.1 Hydrolytic Reactions

Of all the types of enzyme-catalyzed reactions, hydrolytic transformations involving amide and ester bonds are the easiest to perform using proteases, esterases, or lipases. The key features that have made hydrolases the favorite class of enzymes for organic chemists during the past two decades are their lack of sensitive cofactors (which otherwise would need to be recycled) and the large number of readily available enzymes possessing relaxed substrate specificities to choose from. About two-thirds of the total research in the field of biotransformations has been performed using hydrolytic enzymes of this type [1, 2]. The reversal of the reaction, giving rise to ester or amide *synthesis*, has been particularly well investigated using enzymes in solvent systems of low water activity. The special methodologies involved in this latter type of reaction are described in Sect. 3.1.

Other, more complex applications of hydrolases, such as those involving the formation and/or cleavage of phosphate esters, epoxides, nitriles, and organohalides, are described elsewhere in this book. In contrast to the group of proteases, esterases and lipases, they have had less impact on organic chemistry, although their synthetic potential should not be underestimated.

2.1.1 Mechanistic and Kinetic Aspects

The mechanism of amide- and ester-hydrolyzing enzymes is very similar to that observed in the chemical hydrolysis by a base. A nucleophilic group from the active site of the enzyme attacks the carbonyl group of the substrate ester or amide. This nucleophilic 'chemical operator' can be either the hydroxy group of a serine (e.g., pig liver esterase, subtilisin, and the majority of microbial lipases), a carboxyl group of an aspartic acid (e.g., pepsin) [3], or the thiol functionality of cysteine (e.g., papain) [4–6].

The mechanism, which has been elucidated in greater detail, is that of the serine hydrolases [7, 8] (see Scheme 2.1): Two additional groups (Asp and His) located

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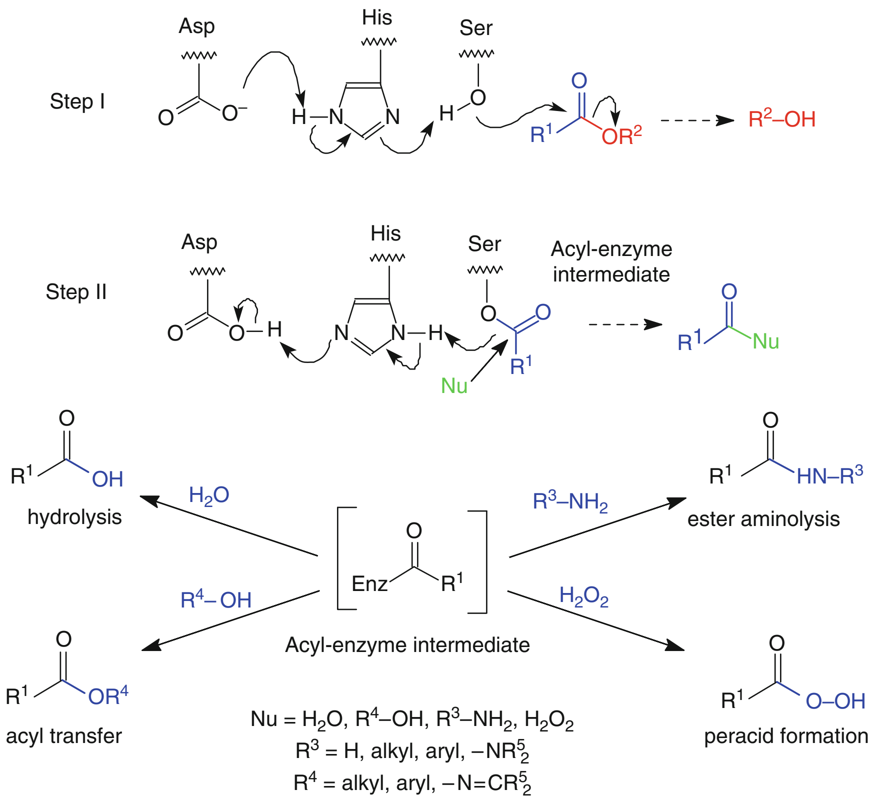


Scheme 2.1 The serine hydrolase mechanism

close to the serine residue (which is the actual reacting chemical operator at the active site) form the so-called catalytic triad [9–12].[1] The special arrangement of these three groups effects a decrease of the pK_a of the serine hydroxy group thus enabling it to perform a nucleophilic attack on the carbonyl group of the substrate R^1–CO–OR^2 (step I). Thus, the acyl moiety of the substrate becomes covalently linked to the enzyme, forming the 'acyl-enzyme intermediate' by liberating the leaving group (R^2–OH). Then a nucleophile (Nu), usually water, can in turn attack the acyl-enzyme intermediate, regenerating the enzyme and releasing a carboxylic acid R^1–COOH (step II).

When the enzyme is operating in an environment of low water activity – in other words, at low water concentrations – any other nucleophile can compete with the

[1]In acetylcholine esterase from electric eel and lipase from *Geotrichum candidum* Asp within the catalytic triad is replaced by Glu [11, 12].

water for the acyl-enzyme intermediate, thus leading to a number of synthetically useful transformations:

- Attack of another alcohol R^4–OH leads to a different ester R^1–CO–OR^4. This is an interesterification reaction, called enzymatic 'acyl transfer' [13, 14].
- The action of ammonia furnishes a carboxamide R^1–CO–NH_2 via an ammonolysis reaction [15, 16].
- An incoming amine R^3–NH_2 results in the formation of an *N*-substituted amide R^1–CO–NH–R^3, yielding an enzymatic aminolysis of esters [17, 18].
- Peracids of type R^1–CO–OOH are formed when hydrogen peroxide is acting as the nucleophile [19].
- Hydrazinolysis provides access to hydrazides [20, 21], and the action of hydroxylamine results in the formation of hydroxamic acid derivatives [22]. However, both of the latter transformations have not been used extensively.
- Thiols (which would lead to thioesters) are unreactive [23].

During the course of all of these reactions, any type of chirality in the substrate is 'recognized' by the enzyme, which causes a preference for one of the two possible stereochemical pathways for a reaction. The value of this discrimination is a crucial parameter since it stands for the 'selectivity' of the reaction. The latter is governed by the reaction kinetics. It should be noted, that the following chapter is not an elaboration on enzyme kinetics, but rather a compilation of the most important conclusions needed for obtaining optimal results from stereoselective enzymatic transformations.

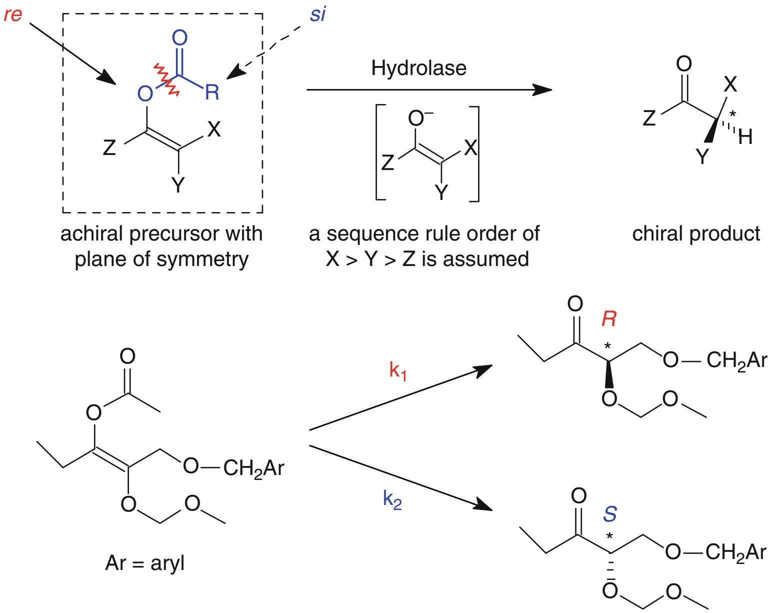
Since hydrolases nicely exemplify all different types of chiral recognition, we will discuss the underlying principles of these chiral recognition processes and the corresponding kinetic implications here [24]. Most of these types of transformations can be found within other groups of enzymes as well, and the corresponding rules can be applied accordingly.

Enantioface Differentiation

Hydrolases can distinguish between the two enantiomeric faces of achiral substrates such as enol esters possessing a plane of symmetry within the molecule [25]. The attack of the enzyme's nucleophilic chemical operator predominantly occurs from one side, leading to an unsymmetric enolization of the unstable free enol towards one preferred side within the chiral environment of the enzyme's active site [26]. During the course of the reaction a new center of chirality is created in the product (Scheme 2.2).

Enantiotopos Differentiation

If prochiral substrates possessing two chemically identical but enantiotopic reactive groups X (designated pro-*R* and pro-*S*) are subjected to an enzymatic transformation such as hydrolysis, a chiral discrimination between them occurs during the transformation of group X into Y, thus leading to a chiral product (Scheme 2.3). During the course of the reaction the plane of symmetry within the substrate is broken. The single-step asymmetric hydrolysis of a prochiral



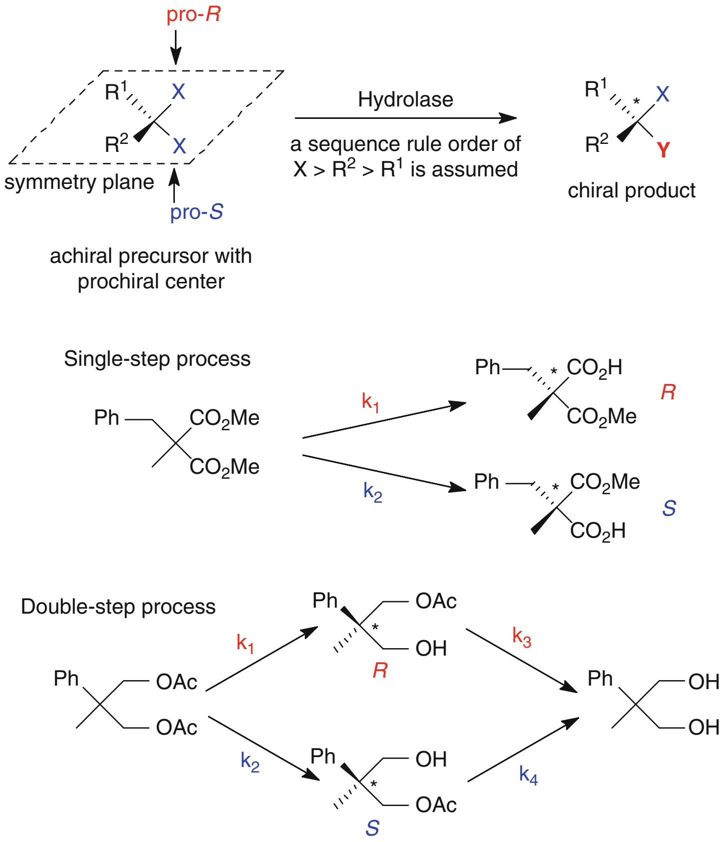
Scheme 2.2 Enantioface differentiation (achiral substrates)

α,α-disubstituted malonic diester by pig liver esterase or α-chymotrypsin is a representative example [27]. Here, the reaction terminates after a single reaction step at the carboxylate monoester stage since highly polar compounds of such type are heavily hydrated in an aqueous medium and are therefore generally not accepted by hydrolases [28].

On the other hand, when the substrate is a diacetate, the resulting monoester is less polar and thus usually undergoes further cleavage in a second step to yield an achiral diol [29]. However, since the second step is usually slower, the chiral monoester can be trapped in fair yield if the reaction is carefully monitored.

Similarly, the two chemically identical groups X, positioned on carbon atoms of opposite (*R*,*S*)-configuration in a *meso*-substrate, can react at different rates in a hydrolase-catalyzed reaction (Scheme 2.4). So, the optically inactive *meso*-substrate is transformed into an optically active product due to the transformation of one of the reactive groups from X into Y along with the destruction of the plane of symmetry within the substrate. Numerous open-chain or cyclic *cis*-*meso*-diesters have been transformed into chiral monoesters by this technique [30]. Again, for dicarboxylates the reaction usually stops after the first step at the carboxylate monoester stage, whereas two hydrolytic steps are usually observed with diacetoxy esters [31]. The theoretical yield of chiral product from single-step reactions based on an enantioface or enantiotopos differentiation or a desymmetrization of *meso*-compounds is always 100%.

If required, the interconversion of a given chiral hemiester product into its mirror-image enantiomer can be achieved by a simple two-step protection–deprotection

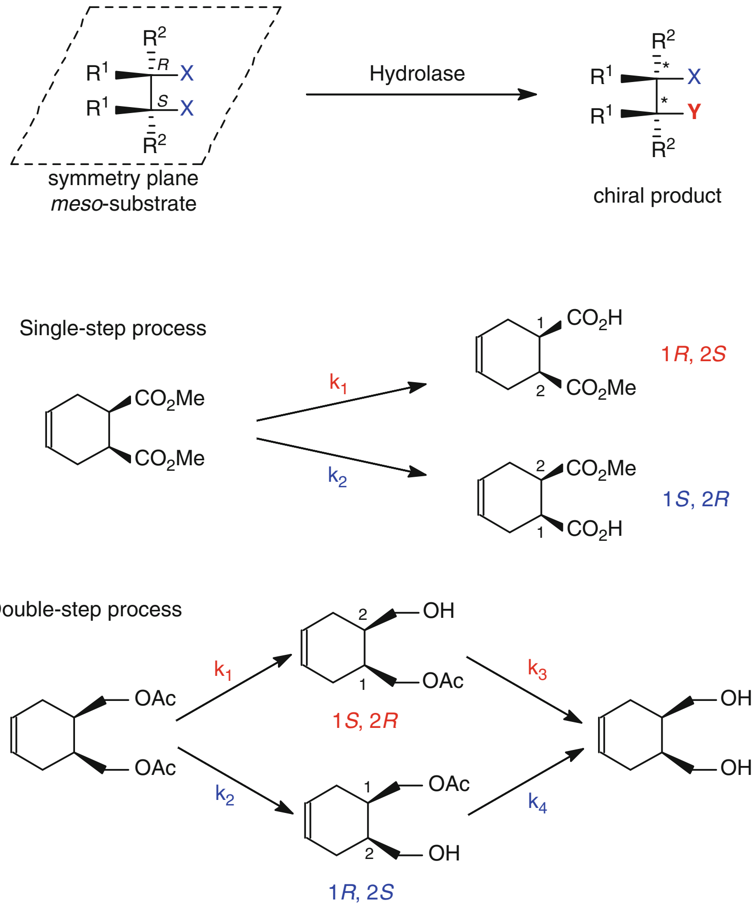


Scheme 2.3 Enantiotopos differentiation (prochiral substrates)

sequence. Thus, regardless of the stereopreference of the enzyme which is used to perform the desymmetrization of the bifunctional prochiral or *meso*-substrate, both enantiomers of the product are available and no ‘unwanted’ enantiomer is produced. This technique is often referred to as the ‘*meso*-trick’ [25].

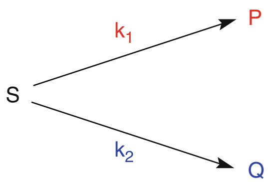
Since hydrolytic reactions are performed in an aqueous environment, they are virtually completely irreversible.[2] The kinetics of all of the single-step reactions described above is very simple (Fig. 2.1): a prochiral or a *meso*-substrate S is transformed into two enantiomeric products P and Q at different rates, determined by the apparent first-order rate constants k_1 and k_2, respectively (Schemes 2.2–2.4). The selectivity of the reaction (α [32]) is only governed by the ratio of k_1/k_2, which is *independent of the conversion* and therefore remains constant throughout

[2]Since the molar concentration of water in an aqueous solution is 55.5 mol/L, the equilibrium is shifted towards the hydrolysis-side to such an extent, that the reaction can be regarded as virtually irreversible.



Scheme 2.4 Desymmetrization of *meso*-substrates

the reaction. Thus, the optical purity of the product (e.e.$_P$) is *not* dependent on the extent of the conversion. Also, the selectivity observed in such a reaction can*not* be improved by stopping the reaction at different extents of conversion, but only by changing the 'environment' of the system (e.g., via substrate modification,



$$\alpha = \frac{k_1}{k_2}$$

$$\text{e.e.}_P = \frac{\alpha - 1}{\alpha + 1} = \frac{P - Q}{P + Q}$$

Fig. 2.1 Single-step kinetics

choice of another enzyme, the addition of organic cosolvents, and variations in temperature or pH). Different techniques for improving the selectivity of enzymatic reactions by variations in the ‘environment’ are presented on pp. 77–84 and 108–110.

As mentioned above, occasionally a second successive reaction step cannot be avoided with bifunctional prochiral or *meso*-diesters (Schemes 2.3 and 2.4). For such types of substrates the reaction does not terminate at the chiral carboxylate monoester stage to give the desired products P and Q (step 1), but rather proceeds via a second step (usually at a slower rate) to yield an achiral product (R). Here, the reaction kinetics become more complicated.

As depicted in Fig. 2.2, the ratio of P and Q – i.e., the optical purity of the product (e.e.) – depends now on four rate constants, k_1 through k_4, since the second hydrolytic step cannot be neglected. From the fact that enzymes usually show a continuous preference for reactive groups with the same chirality,[3] one may

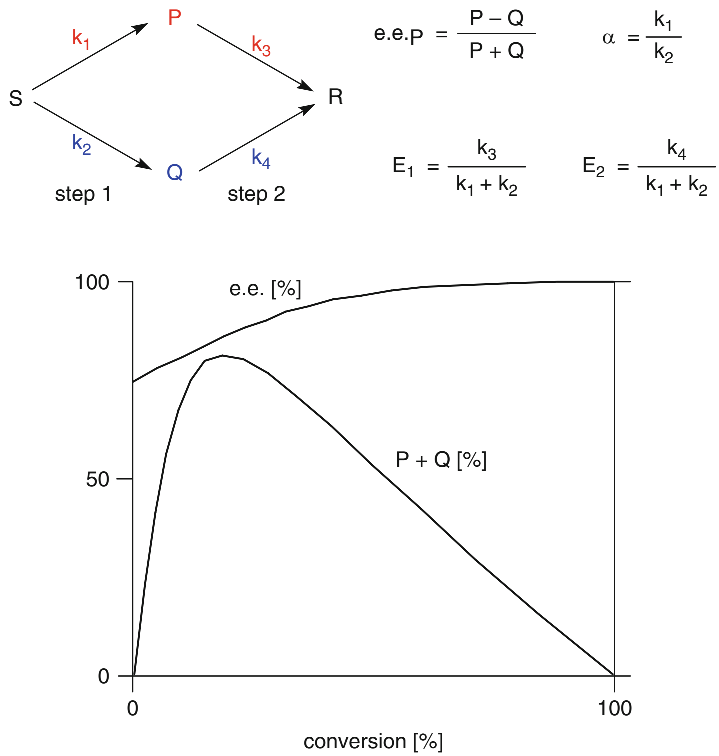


Fig. 2.2 Double-step kinetics

[3] These groups are called homochiral.

conclude that if S is transformed more quickly into P, Q will be hydrolyzed faster into diol R than P. Thus, the rate constants governing the selectivity of the reaction are often at an order of $k_1 > k_2$ and $k_4 > k_3$. Notably, the optical purity of the product monoester (e.e.) becomes a *function of the conversion* of the reaction, and generally follows the curve shown in Fig. 2.2.

Early stages of the reaction, the optical purity of the product is mainly determined by the selectivity (α) of the first reaction step, which constitutes an enantiotopos or enantioface differentiation, depending on the type of substrate.

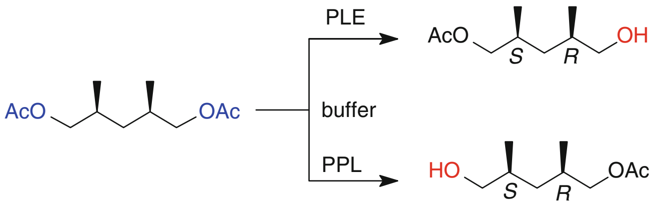
As the reaction proceeds, the second hydrolytic step, being a kinetic resolution, starts to take place to a more significant extent due to the increased formation of monoester P/Q, and its apparent 'opposite' selectivity compared to that of the first step (remember that $k_1 > k_2$, $k_4 > k_3$) leads to an enhancement of optical purity of the product (e.e.$_P$). In contrast, the product concentration [P + Q] follows a bell-shaped curve: After having reached a maximum at a certain conversion, the product concentration [P + Q] finally drops off again when most of the substrate S is consumed and the second hydrolytic step to form R at the expense of P + Q constitutes the main reaction. The same analogous considerations are pertinent for the reverse situation – an esterification reaction.

In general, it can be stated that the ratio of reaction rates of the first and the second step $[(k_1 + k_2)/(k_3 + k_4)]$ has a major impact on the *chemical yield* of P + Q, whereas the symmetry of the selectivities $[(k_1 > k_2, k_3 > k_4$ or $k_1 > k_2, k_4 > k_3)]$ determines the *optical purity* of the product. In order to obtain a high chemical yield, the first step should be considerably faster than the second to ensure that the chiral product can be accumulated, because then it is formed faster than it is further converted $[(k_1 + k_2) \gg (k_3 + k_4)]$. For a high e.e.$_P$, the selectivities of both steps should match each other ($k_1 > k_2, k_4 > k_3$), i.e., if P is formed predominantly in the first step from S, it should react at a slower rate than Q in the second step. Figure 2.2 shows a typical example of such a double-step process, where the first step is about ten times faster than the second, with selectivities matching ($k_1 = 100, k_2 = 10, k_3 = 1, k_4 = 10$).

In addition to trial-and-error experiments (i.e., by stopping such double-step reactions at various intervals and checking the yield and optical purity of the product), the e.e.-conversion dependence may also be calculated [33]. Data on the amounts of substrate S and monoester P and Q and its optical purity measured at various intervals can be used to determine the kinetic constants k_1 through k_4 for a given reaction by using the computer program 'SeKiRe' [34]. Thus, the enantiomeric excess of the monoester may be predicted as a function of its percentage present in the reaction mixture. The validity of this method has been verified by the desymmetrization of a prochiral *meso*-diacetate using pig liver esterase (PLE) and porcine pancreatic lipase (PPL) as shown in Scheme 2.5 [35].

Enantiomer Differentiation

When a racemic substrate is subject to enzymatic hydrolysis, chiral discrimination of the enantiomers occurs [36]. It should be noted that the chirality does not necessarily have to be of a central type, but can also be axial or planar to be 'recognized' by enzymes (Scheme 1.2). Due to the chirality of the active site of



Enzyme	Stereochemical preference	Kinetic constants		
		α	E_1	E_2
PLE	pro-*R*	2.47	0.22	0.60
PPL	pro-*S*	15.6	0.04	0.18

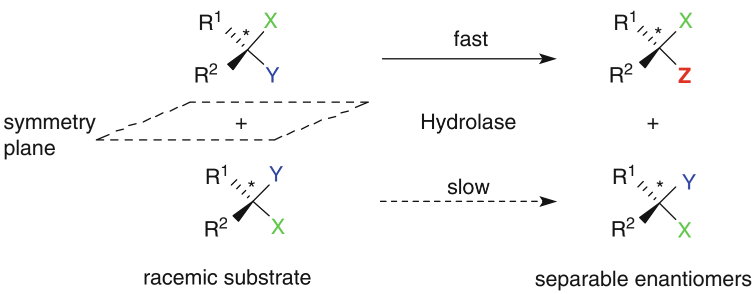
Scheme 2.5 Desymmetrization of a *meso*-diacetate

the enzyme, one enantiomer fits better into the active site than its mirror-image counterpart and is therefore converted at a higher rate, resulting in a kinetic resolution of the racemate. The vast majority of enzymatic transformations constitute kinetic resolutions and, interestingly, this potential of hydrolytic enzymes was realized as early as 1903 [37]! It is a remarkable observation that in biotransformations, kinetic resolutions outnumber desymmetrization reactions by about 1:4, which is presumably due to the fact that there are many more racemic compounds possible as opposed to prochiral and *meso*-analogs [38].

The most striking difference from the above-mentioned types of desymmetrization reactions, which show a theoretical yield of 100%, is that in kinetic resolution each of the enantiomers can be obtained in only 50% yield.

In some ideal cases, the difference in the reaction rates of both enantiomers is so extreme that the 'good' enantiomer is transformed quickly and the other is not converted at all. Then the enzymatic reaction will cease automatically at 50% conversion when there is nothing left of the more reactive enantiomer (Scheme 2.6) [39].

In practice, however, most cases of enzymatic resolution of racemic substrates are not as ideal, i.e., in which one enantiomer is rapidly converted and the other not at all. The difference in – or more precisely the ratio of – the reaction rates of the enantiomers



Scheme 2.6 Enantiomer differentiation

is not infinite, but measurable. The thermodynamic reasons for this have been discussed in Chap. 1. What one observes in these cases is not a complete standstill of the reaction at 50% conversion but a marked slowdown in reaction rate at around this point. In these numerous cases one encounters some crucial dependencies:

- The velocity of the transformation of each enantiomer varies with the degree of conversion, since the ratio of the two substrate enantiomers does not remain constant during the reaction.
- Therefore, the optical purity of both substrate (e.e.$_S$) and product (e.e.$_P$) becomes *a function of the conversion*.

A very useful treatment of the kinetics of enzymatic resolution, describing the dependency of the conversion (c) and the enantiomeric excess of substrate (e.e.$_S$) and product (e.e.$_P$), was developed by C.J. Sih in 1982 [40] on a theoretical basis described by K.B. Sharpless [41] and K. Fajans [42]. The parameter describing the selectivity of a resolution was introduced as the dimensionless 'enantiomeric ratio' (*E*), which remains constant throughout the reaction and is only determined by the 'environment' of the system [43–46].[4] *E* corresponds to the ratio of the relative second-order rate constants (v_A, v_B) of the individual substrate enantiomers (A, B) and is related to the k_{cat} and K_m values of enantiomers A and B according to Michaelis–Menten kinetics as follows (for the thermodynamic background see Fig. 1.7):

$$\text{Enantiomeric Ratio} \qquad E = \frac{v_B}{v_A} = \frac{\left[\frac{k_{cat}}{K_M}\right]_A}{\left[\frac{k_{cat}}{K_M}\right]_B} \qquad \Delta\Delta G^{\neq} = -RT \ln E$$

'Enantiomeric ratio' is not to be confused with the term 'enantiomer ratio' (e.r.), which is used to quantify the enantiomeric composition of a mixture of enantiomers (e.r. = [A]/[B]) [47].

Related alternative methods for the experimental determination of *E*-values have been proposed [48–50].

Irreversible Reaction. Hydrolytic reactions in aqueous solution can be regarded as completely irreversible due to the high 'concentration' of water present (55.5 mol/L). Assuming negligible enzyme inhibition, thus both enantiomers of the substrate are competing freely for the active site of the enzyme, Michaelis–Menten kinetics effectively describe the reaction in which two enantiomeric substrates (A and B) are transformed by an enzyme (Enz) into the corresponding enantiomeric products (P and Q, Fig. 2.3).

[4]The Enantiomeric Ratio (*E*) is a synonym for the so-called selectivity factor (*s*). Whereas the former term is used more often in biocatalyzed kinetic resolutions, the *s*-factor is more common in chemo-catalysis. In a mathematical sense, both are identical and describe the ratio of the relative (second-order) rate constants of enantiomers. For a comprehensive discussion see [46].

Instead of determining all individual rate constants (k_{cat}, K_M) for each of the enantiomers (a wearisome task for synthetic organic chemists, particularly when A and B are not available in enantiopure form), in order to gain access to the corresponding relative rates governing the selectivity of the reaction, the ratio of the initial reaction rates of the substrate enantiomers ($E = v_A/v_B$) can be mathematically linked to the conversion (c) of the reaction, and the optical purities of substrate (e.e.$_S$) and product (e.e.$_P$). In practice, these parameters are usually much easier to determine and do not require the availability of pure enantiomers.

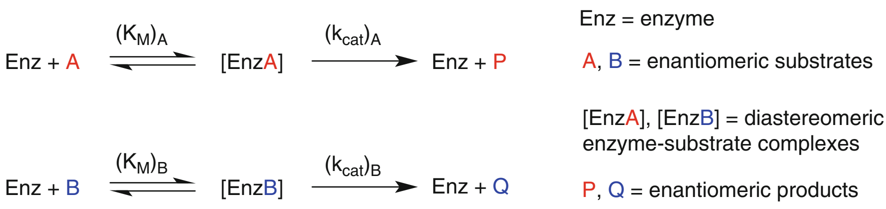


Fig. 2.3 Enzymatic kinetic resolution (irreversible reaction)

The dependence of the selectivity and the conversion of the reaction is:

For the product

$$E = \frac{\ln[1 - c(1 + e.e._P)]}{\ln[1 - c(1 - e.e._P)]}$$

For the substrate

$$E = \frac{\ln[(1 - c)(1 - e.e._S)]}{\ln[(1 - c)(1 + e.e._S)]}$$

c = conversion, e.e. = enantiomeric excess of substrate (S) or product (P), E = enantiomeric ratio

The above-mentioned equations give reliable results except for very low and very high levels of conversion, where accurate measurement is impeded by errors derived from sample manipulation. In such cases, the following equation is recommended instead, because here only values for the optical purities of substrate and product need to be measured. The latter are *relative* quantities in contrast to the conversion, which is an *absolute* quantity [51].

$$E = \frac{\ln \dfrac{[e.e._P(1 - e.e._S)]}{(e.e._P + e.e._S)}}{\ln \dfrac{[e.e._P(1 + e.e._S)]}{(e.e._P + e.e._S)}}$$

Two examples of enzymatic resolutions with selectivities of $E = 5$ and $E = 20$ are depicted in Fig. 2.4. The curves show that the product (P + Q) can be obtained in its highest optical purities before 50% conversion, where the enzyme can freely choose the 'well-fitting' enantiomer from the racemic mixture. So, the 'well-fitting' enantiomer is predominantly depleted from the reaction mixture during the course of the reaction, leaving behind the 'poor-fitting' counterpart. Beyond 50% conversion, the enhanced relative concentration of the 'poor-fitting' counterpart leads to its increased transformation by the enzyme. Thus, the e.e.$_P$ rapidly decreases beyond 50% conversion.

Analogous trends are seen for the optical purity of the residual slow-reacting enantiomer of the substrate (e.e.$_S$, as shown in the 'substrate' curve). Its optical purity remains low before 40%, then climbs significantly at around 50%, and reaches its maximum beyond the 60% conversion point.

High optical purity of substrate can easily be reached by extending the reaction to the appropriate point of conversion ($\geq$60%), but an attractive optical purity value for the product demands a very high selectivity for the enzyme-catalyzed reaction.

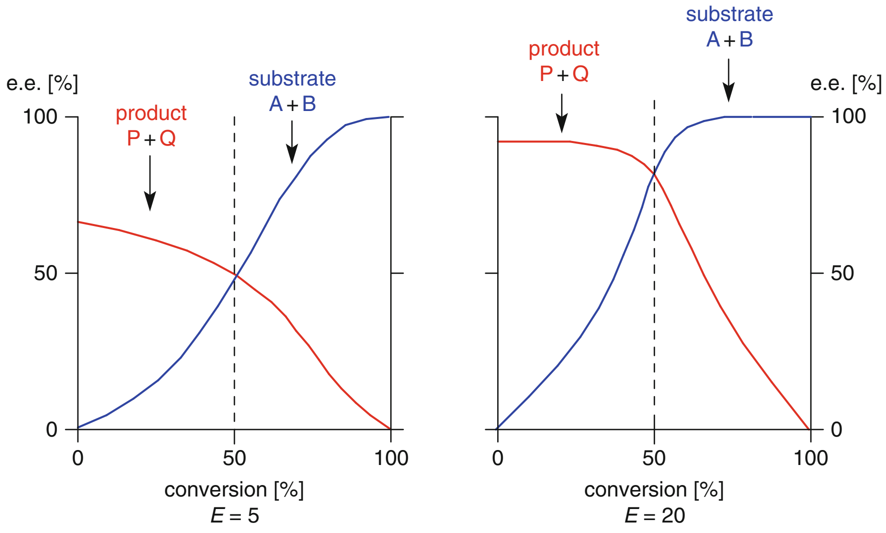


Fig. 2.4 Dependence of optical purities (e.e.$_S$/e.e.$_P$) on the conversion

Using the equations discussed above, the expected optical purity of substrate and product can be calculated for a chosen point of conversion and the enantiomeric ratio (E) can be determined as a convenient *conversion-independent* value for the 'enantioselectivity' of an enzymatic resolution. Free shareware programs for the calculation of the enantiomeric ratio for irreversible reactions can be obtained from the internet [52, 53]. As a rule of thumb, enantiomeric ratios below 15 are inacceptable for practical purposes. They can be regarded as being moderate to good in the range of 15–30, and above this value they are excellent. However, values of $E > 200$ cannot be accurately determined due to the inaccuracies emerging from the determination of the enantiomeric excess (e.g., by NMR, HPLC, or GC), because in this range even an extremely small variation of e.e.$_S$ or e.e.$_P$ causes a significant change in the numerical value of E.

In order to obtain optimal results from resolutions of numerous racemic substrates which exhibit moderate selectivities (E values ca. 20), one can proceed as follows (see Fig. 2.5): The reaction is terminated at a conversion of 40%, where the 'product' curve reaches its optimum in chemical and optical yield being closest to the 'ideal'

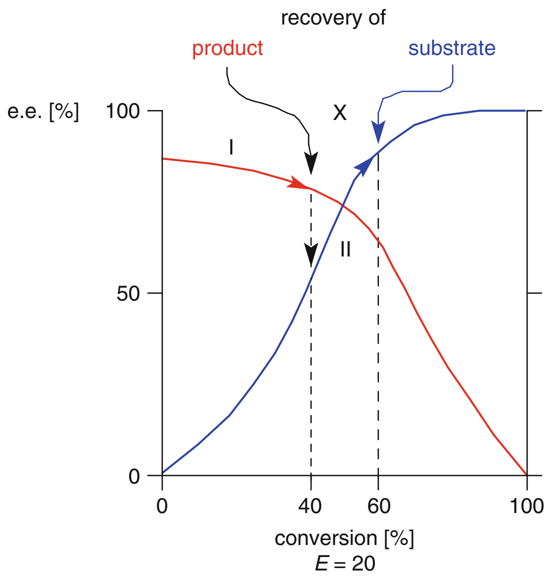


Fig. 2.5 Two-step enzymatic resolution

point X (step I). The product is isolated and the remaining substrate – showing a low optical purity at this stage of conversion – is subjected to a second hydrolytic step, until an overall conversion of about 60% is reached, where the 'substrate' curve is closest to X (step II). Now, the substrate is harvested with an optimal chemical and optical yield and the 20% of product from the second step is sacrificed or recycled. This two-step process [54] can be used to allow practical use of numerous enzyme-catalyzed kinetic resolutions which show incomplete selectivities.

Reversible Reaction. The situation becomes more complicated when the reaction is reversible [55].[5] This is the case if the concentration of the nucleophile which attacks the acyl-enzyme intermediate is limited and is not in excess (like water in a hydrolytic reaction). In this situation, the equilibrium constant (*K*) of the reaction – neglected in the irreversible type of reaction – plays an important role and therefore has to be determined.

The equations linking the enantioselectivity of the reaction (the enantiomeric ratio *E*), the conversion (c), the optical purities of substrate ($e.e._S$) and product ($e.e._P$), and the equilibrium constant *K* are as follows:

For the product

$$E = \frac{\ln[1 - (1 + K)c(1 + e.e._P)]}{\ln[1 - (1 + K)c(1 - e.e._P)]}$$

For the substrate

$$E = \frac{\ln[1 - (1 + K)(c + e.e._S\{1 - c\})]}{\ln[1 - (1 + K)(c - e.e._S\{1 - c\})]}$$

c = conversion, e.e. = enantiomeric excess of substrate (S) or product (P), *E* = enantiomeric ratio, K = equilibrium constant of the reaction

[5]For an alternative model see [56].

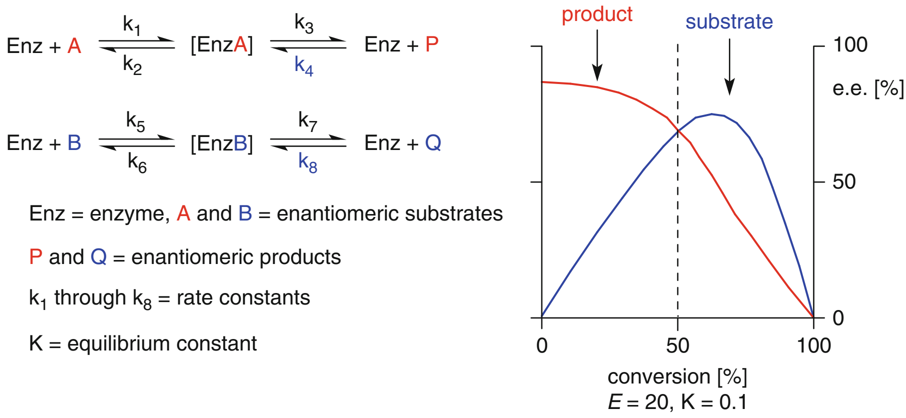


Fig. 2.6 Enzymatic kinetic resolution (reversible reaction)

As shown in Fig. 2.6, the product curve of an enzymatic resolution following a reversible reaction type remains almost the same. However, a significant difference compared to the irreversible case is found in the substrate curve: particularly at higher levels of conversion (beyond 70%) the reverse reaction (i.e., esterification instead of a hydrolysis) starts to predominate. Since the main steric requirements and hence the preferred chirality of the substrate stays the same, it is clear that the *same* enantiomer from the substrate and the product react preferentially in both the forward and the reverse reaction. Assuming that A is the better substrate than B, accumulation of product P and unreacted B will occur. For the reverse reaction, however, P is a better substrate than Q, because it is of the *same* chirality as A and therefore it will be transformed back into A at a faster rate than B into Q. As a result, the optical purity of the remaining substrate is depleted as the conversion increases. In other words, the reverse reaction, predominantly taking place at higher rates of conversion, constitutes a second – and in this case an undesired – selection of chirality which causes a depletion of e.e. of the remaining substrate.

All attempts of improving the optical purity of substrate and product of reversible enzymatic resolutions are geared at shifting the reaction out of the equilibrium to obtain an irreversible type. The easiest way to achieve this is to use an excess of cosubstrate: in order to obtain an equilibration constant of $K > 10$, about 20 M equivalents of nucleophile vs. substrate are considered to be sufficient to obtain a virtually irreversible type of reaction, in most cases. Other techniques, such as using special cosubstrates which cause an irreversible type of reaction, are discussed in Sect. 3.1.1.

Sequential Biocatalytic Resolutions. For a racemic substrate bearing *two* chemically and stereochemically identical reactive groups, an enzymatic resolution proceeds through two consecutive steps via an intermediate monoester stage. During the course of such a reaction the substrate is forced to enter the active site of the enzyme twice – it is therefore ‘double-selected’. Since each of the selectivities of both of the sequential steps determine the final optical purity of the product, exceptionally high selectivities can be achieved by using such a ‘double-sieving’ procedure.

As depicted in Fig. 2.7, a bifunctional racemic substrate consisting of its enantiomers A and B is enzymatically resolved via a first step to give the intermediate enantiomeric products P and Q. The selectivity of this step is governed by the constants k_1 and k_3. Then, both of the intermediate monoester products (P, Q) undergo a second reaction step, the selectivity of which is determined by k_2 and k_4, to form the enantiomeric final reaction products R and S. As a result, the optical purity of the substrate (A, B), the intermediate monoester (P, Q), and the final products (R, S) are a *function of the conversion* of the reaction, as shown by the curve in Fig. 2.7. The selectivities of each of the steps (E_1 and E_2) can be determined experimentally and the optical purities of the three species e.e.$_{A/B}$, e.e.$_{P/Q}$, and e.e.$_{R/S}$ can be calculated [57, 58]. Free shareware computer programs for the analysis, simulation, and optimization of such processes can be obtained over the internet [59].

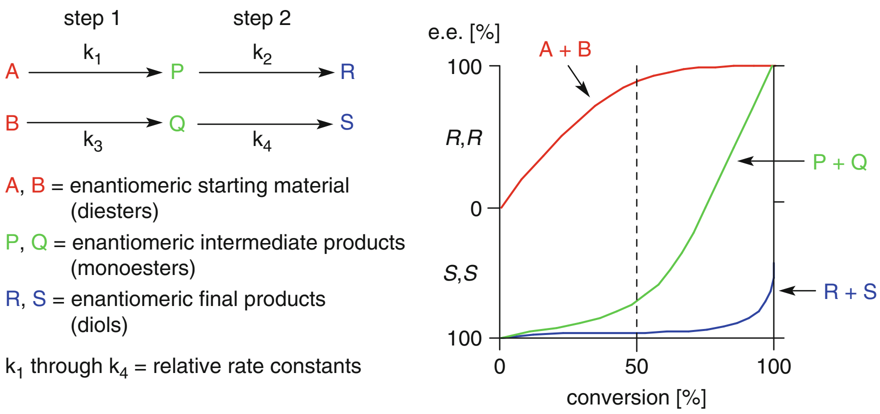
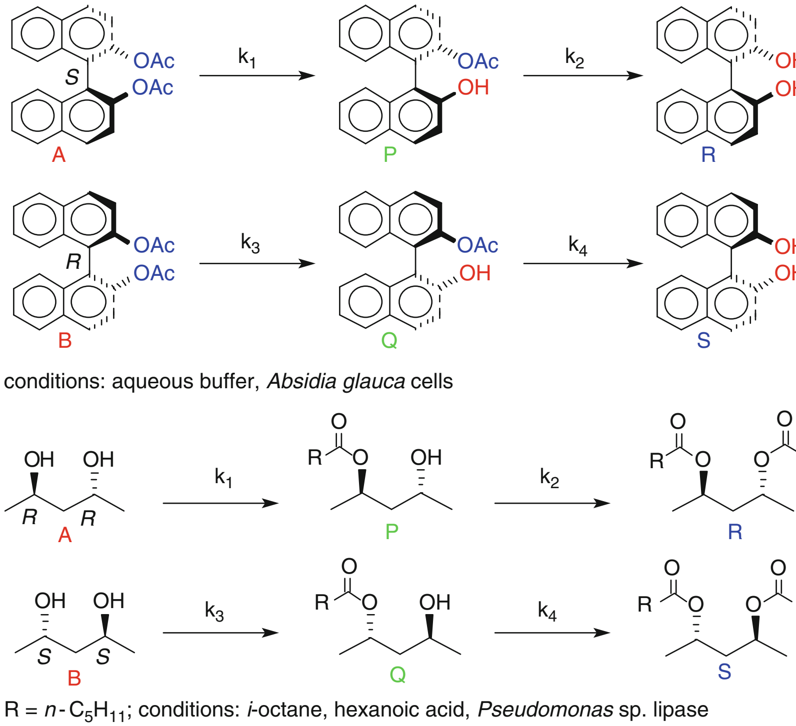


Fig. 2.7 Sequential enzymatic kinetic resolution via hydrolysis and esterification

It has been shown that the maximum overall selectivity (E_{tot}) of a sequential kinetic resolution can be related to the individual selectivities (E_1, E_2) of each of the steps [60]. E_{tot} represents the enantioselectivity that a hypothetical single-step resolution would need to yield the enantiomeric purity of the two-step resolution.

$$E_{tot} \sim \frac{E_1 \times E_2}{2}$$

This technique has been proven to be highly flexible. It was shown to work successfully not only in a hydrolytic reaction using cholesterol esterase [61] or microbial cells [62], but also in the reverse esterification direction in an organic solvent catalyzed by a *Pseudomonas* sp. lipase (Scheme 2.7). In a related fashion, a successful sequential resolution of a bifunctional 1,2-amine via ester aminolysis was reported [63].



Scheme 2.7 Sequential enzymatic resolution via hydrolysis and esterification

A special type of sequential enzymatic resolution involving a hydrolysis-esterification [64] or an alcoholysis-esterification sequence [65] is depicted in Fig. 2.8. In view of the mechanistic symmetry of enzymatic acyl transfer reactions

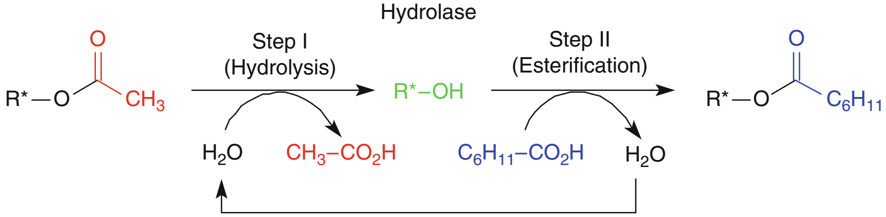
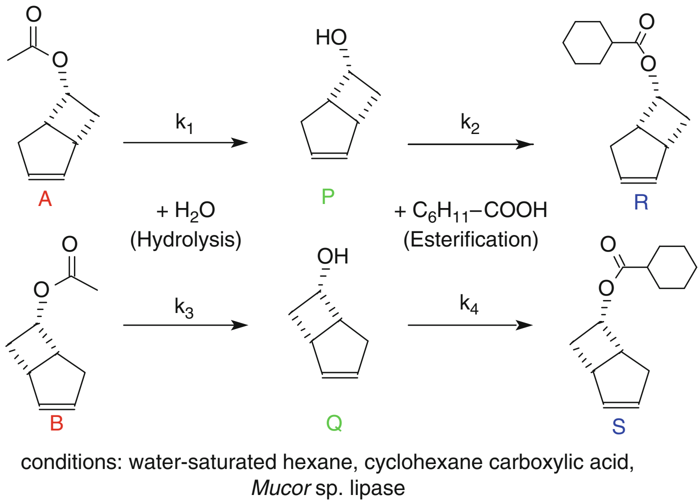


Fig. 2.8 Mechanism of concurrent sequential enzymatic kinetic resolution via hydrolysis-esterification in aqueous-organic solvent

(Scheme 3.6), the resolution of a racemic alcohol can be effected by enantioselective hydrolysis of the corresponding ester or by esterification of the alcohol. As the biocatalyst displays the same stereochemical preference in both reactions, the desired product can be obtained with higher optical yields, if the two steps are coupled sequentially. The basis of this approach parallels that of product recycling in hydrolytic reactions. However, tedious chromatographic separation of the intermediates and the accompanying re-esterification is omitted.

As shown in Scheme 2.8, the racemic starting acetate (A/B) is hydrolyzed to give alcohols (P/Q) in an organic medium containing a minimum amount of water, which in turn, by the action of the same lipase, are re-esterified with cyclohexanoic acid present in the mixture. Thus, the alcohol moiety of the substrate has to enter the active site of the lipase twice during the course of its transformation into the final product ester (R/S). An apparent selectivity of $E_{tot} = 400$ was achieved in this way, whereas the corresponding isolated single-step resolutions of this process were $E_1 = 8$ for the hydrolysis of acetate A/B, and $E_2 = 97$ for the esterification of alcohol P/Q with cyclohexanoic acid.



Scheme 2.8 Sequential enzymatic kinetic resolution via hydrolysis-esterification

Deracemization

Despite its widespread use, kinetic resolution has several disadvantages for preparative applications, particularly on an industrial scale. After all, an ideal process should lead to a single enantiomeric product in 100% chemical yield. The drawbacks of kinetic resolution are as follows:

- The theoretical yield of each enantiomer is limited to 50%. Furthermore, in general only one stereoisomer is desired and there is little or no use for the other.

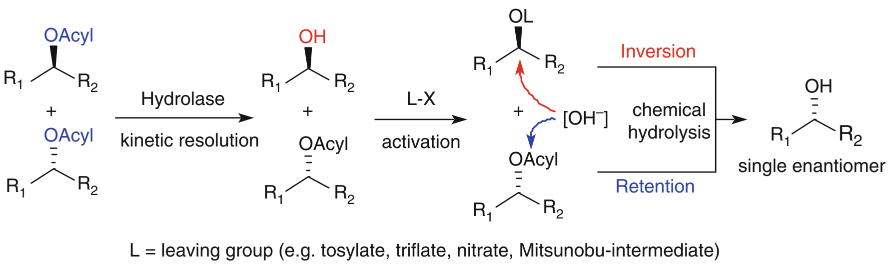
- Separation of the product from the remaining substrate may be laborious, in particular when simple extraction or distillation fails [66].
- As explained above, the optical purity of substrate and/or product is often less than perfect for kinetic reasons.

To overcome these disadvantages by avoiding the occurrence of the undesired 'wrong' enantiomer, several strategies are possible [67, 68]. All of these processes which lead to the formation of a single stereoisomeric product from a racemate are called 'deracemizations' [69].

Repeated Resolution. In order to avoid the loss of half of the material in kinetic resolution, it has been a common practice to racemize the unwanted enantiomer after separation from the desired product and to subject it again to kinetic resolution in a subsequent cycle, until virtually all of the racemic material has been converted into a single stereoisomeric product. For obvious reasons, this laborious procedure is not justified for laboratory-scale reactions, but it is a viable option for resolutions on an industrial scale, in particular for continuous processes, where the re-racemized material is simply fed back into the subsequent batch of the resolution process. At first sight, repeated resolution appears less than ideal and it certainly lacks synthetic elegance, bearing in mind that an infinite number of cycles are theoretically required to transform all of the racemic starting material into a single stereoisomer. Upon closer examination, though, re-racemization holds certain merits: a simple calculation shows that even if only 50% of the desired enantiomer is obtained after a single cycle, the overall (theoretical) yield increases to ~94% after only four cycles [70].

In practice, however, deracemization via repeated resolution is often plagued by low overall yields due to the harsh reaction conditions required for (chemical) racemization [71]. In view of the mild reaction conditions displayed by enzymes, there is a great potential for biocatalytic racemization based on the use of racemases of EC-class 5 [72, 73].

In-Situ Inversion. The final outcome of a kinetic resolution of a racemate is a mixture of enantiomeric product and substrate. Separating them by physical or chemical means is often tedious and might pose a serious drawback to commercial applications, especially if the mixture comprises an alcohol and an ester. However, if the molecule has only a single center of chirality, the alcohol may be chemically inverted into its enantiomer *before* separating the products (Scheme 2.9) [74, 75]. Introduction of a good leaving group, L (e.g., tosylate, triflate, nitrate, or Mitsunobu intermediate) yields an activated ester, which can be hydrolyzed with *inversion* of configuration, while the stereochemistry of the remaining carboxylic acid substrate ester is *retained* during hydrolysis. As a result, a *single* enantiomer is obtained as the final product. Since the $e.e._S$ and $e.e._P$ are a function of the conversion, it is obvious that the point where the kinetic resolution is terminated and the in-situ inversion is performed, has to be carefully chosen in order to obtain a maximum of the final $e.e._P$. The optimal value for the conversion can be



Scheme 2.9 Kinetic resolution with in-situ inversion

calculated as a function of the *E* value of the reaction, and it is usually at or slightly beyond a conversion of 50% [76, 77].

Dynamic resolution is a more elegant approach [78–80]. This comprises a classic resolution with an additional feature, i.e., the resolution is carried out using conditions under which the enantiomers of the substrate are in a rapid equilibrium (racemizing). Thus, as the well-accepted substrate-enantiomer is depleted by the enzyme, the equilibrium is constantly adjusted by racemization of the poorly accepted counterpart. To indicate the nonstatic character of such processes, the term 'dynamic resolution' has been coined [81, 82].[6]

In this case, several reactions have to occur simultaneously and their relative rates determine the stereochemical outcome of the whole process (Fig. 2.9):

- The enzyme should display high specificity for the enantiomeric substrates R/S ($k_R \gg k_S$ or $k_S \gg k_R$).
- Spontaneous hydrolysis (k_{spont}) should be a minimum since it would yield racemic product.
- Racemization of the substrate should occur at an equal or higher rate compared to the biocatalytic reaction in order to provide a sufficient amount of the 'well-fitting' substrate enantiomer from the 'poor-fitting' counterpart ($k_{rac}{}^{Sub} \geq k_R$ or k_S, resp.).
- Racemization of the product ($k_{rac}{}^{Prod}$) should be minimal.

Although the above-mentioned criteria are difficult to meet experimentally, the benefits are impressive. Examples of this type of biotransformation have increased recently [83–89]; several examples are given in subsequent chapters.

The kinetics of a dynamic resolution is outlined in the following example [79, 90]. Figure 2.9 shows the e.e.$_S$ and e.e.$_P$ plotted for an enantiomeric ratio of $E \sim 10$. In a classic resolution process, the product is formed in ~83% e.e. at the very beginning of the reaction, but this value rapidly decreases when the reaction is run towards ~50% conversion as indicated by the symbol '*'. In a dynamic process, this depletion *does not* occur, because the enzyme always encounters racemic

[6]Dynamic resolution is a type of second-order asymmetric transformation (see [80, 81]).

substrate throughout the reaction since the 'well-fitting' enantiomer is not depleted but constantly restored from the 'poor-fitting' counterpart via racemization. Thus, e.e.$_P$ remains constant throughout the reaction as indicated by the dashed arrow.

The e.e.$_P$ of dynamic processes is related to the enantioselectivity (E value) through the following formulas [91]:

$$\text{e.e.}_P = \frac{(E-1)}{(E+1)} \qquad\qquad E = \frac{(1+\text{e.e.}_P)}{(1-\text{e.e.}_P)}$$

In the case where the racemization (k_{rac}^{Sub}) is limited, the dynamic resolution gradually turns into a classic kinetic resolution pattern. Figure 2.9 shows the extent of the depletion of e.e.$_P$ depending on the conversion for several ratios of k_{rac}^{Sub}/k_R ($E \sim 10$). As can be expected, e.e.$_P$ decreases only slightly during the early stage of the reaction because the fast-reacting enantiomer is sufficiently available during this period. At higher levels of conversion, however, a serious drop in e.e.$_P$ will occur if the racemization cannot cope with the demand of the enzyme for the faster-reacting substrate enantiomer.

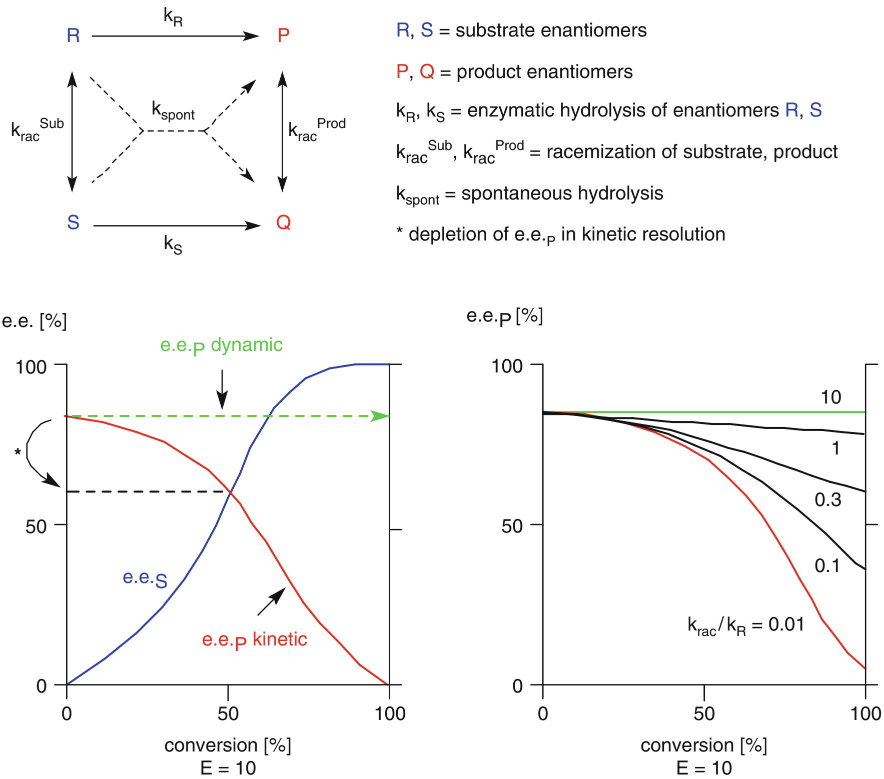


Fig. 2.9 Kinetic resolution with in-situ racemization

It is obvious that a high e.e.$_P$ for dynamic resolutions can only be achieved for reactions displaying excellent selectivities. For example, values for $E \sim 19$ and ~ 40 will lead to an e.e.$_P$ of 90% and 95%, respectively, but for an enantiomeric excess of 98% an enantiomeric ratio of ~100 is required.

2.1.2 Hydrolysis of the Amide Bond

The enzymatic hydrolysis of the carboxamide bond is associated to the biochemistry of amino acids and peptides [92]. The world production of enantiomerically pure amino acids accounted for more than 0.5 million tons of material and a market of ca. US $2 billion [93] per annum in 1992. The three amino acids dominating this area with respect to output and value (L-glutamic acid, L-lysine, and D,L-methionine) are produced by fermentation and by synthesis. However, a considerable number of optically pure D- and L-amino acids are prepared by using one of the enzymatic methods discussed below. L-Amino acids are increasingly used as additives for animal feed, for infusion solutions and as enantiopure starting materials for the synthesis of pharma- and agrochemicals or artificial sweeteners. Selected amino acids possessing the unnatural D-configuration have gained an increasing importance as bioactive compounds or components of such agents. For instance, D-phenylglycine and its *p*-hydroxy derivative are used for the synthesis of antibiotics such as ampicillin and amoxicillin, respectively, and D-valine is an essential component of the insecticidal synthetic pyrethroid fluvalinate (Table 2.1; Scheme 2.208).

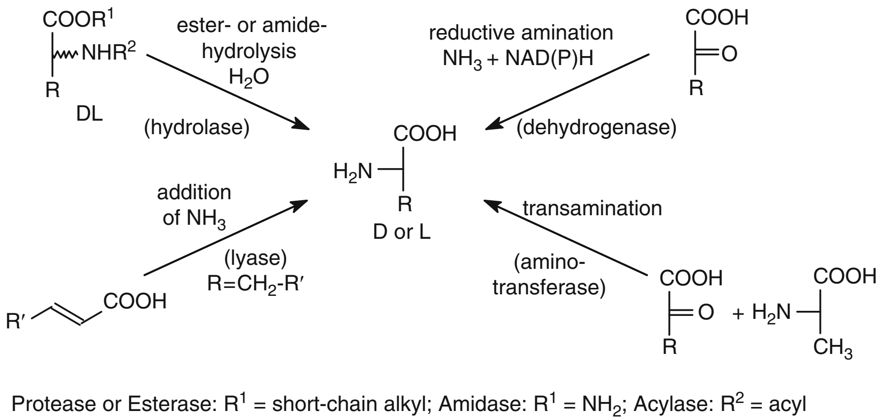
Table 2.1 World production of amino acids using biocatalytic processes (1980–2004)

Amino acid	Amount (t/year)
L-lysine	700,000
L-aspartic acid	13,000[a]
L-phenylalanine	10,000[a]
L-tryptophane	1,200
L-cysteine	500
L-alanine	500
L-methionine	400
L-2,4-dihydroxyphenylalanine	200
L-valine	150
D-phenylglycine	1,000
D-*p*-hydroxyphenylglycine	1,000

[a]Including the demand for the manufacture of the low-calorie sweetener aspartame

Among the principal methods for the enzymatic synthesis of enantiomerically pure amino acids depicted in Scheme 2.10, the most widely applied strategy is the resolution of racemic starting material (synthetically prepared from inexpensive bulk chemicals) employing easy-to-use hydrolytic enzymes such as proteases, esterases, and lipases. In contrast, more complex procedures requiring special expertise are the (1) reductive amination of α-keto acids using α-amino acid dehydrogenases (pp. 165–166), (2) asymmetric addition of ammonia onto α,β-unsaturated carboxylic acids catalyzed by lyases (Sect. 2.5.2), and (3) amino-group transfer using transaminases (Sect. 2.6.2) [94–96].

The generally appliable hydrolytic methods have been selected from the numerous strategies of enzymatic amino acid synthesis [92, 97–103]. They also are useful tools for the preparation of enantiomerically pure nonnatural amino acids [104], not only for industrial needs but also for research on a laboratory scale.



Scheme 2.10 Important enzymatic routes to enantiomerically pure α-amino acids

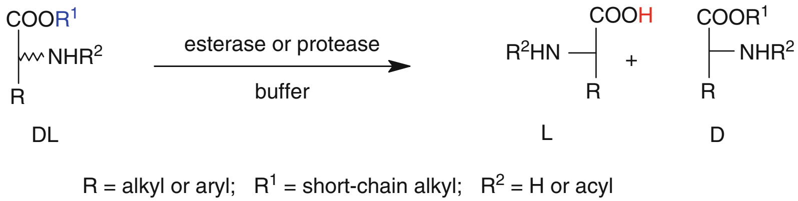
There is a common pattern to the majority of hydrolase reactions involving α-amino acid derivatives: the substrate enantiomer possessing the 'natural' L-configuration is accepted by the enzyme, while the 'unnatural' D-counterpart remains unchanged and thus can be recovered from the reaction medium. Different enzymes with the opposite enantiopreference are available for some selected processes such as the hydantoinase and acylase method (see below) to transform the desired enantiomer. Using strictly L-specific enzyme systems, additional synthetic protection and/or deprotection steps are required in those cases where the unnatural D-amino acid constitutes the desired product. The work-up procedure is usually easy: thus, the difference in solubility of the product and the remaining substrate at different pH in the aqueous medium facilitates their separation by conventional ion-exchange or extraction methods. Alternatively, highly insoluble Schiff bases can be prepared from aldehydes by condensation with *N*-unprotected amino acid derivatives in order to facilitate their isolation. The free amino acids are readily obtained from these derivatives by mild acid hydrolysis without loss of optical purity.

However, there is a limitation to the majority of these methods: the α-carbon atom bearing the amino group must not be fully substituted, since such bulky substrates are generally not accepted by hydrolases. Thus, optically pure α-methyl or α-ethyl amino acids are generally not accessible by these methods, although some exceptions are known [105, 106].

The recycling of the undesired enantiomer from the enzymatic resolution is of crucial importance particularly on an industrial scale [107]. The classical chemical method consists of the thermal racemization of an amino acid ester at about 150–170°C. Milder conditions can be employed for the racemization of the corresponding amides via intermediate formation of Schiff bases with aromatic aldehydes such as benzaldehyde or salicylaldehyde (Scheme 2.14). More recently, intense research has been devoted to the use of isomerase enzymes (such as amino acid racemases [108]) aiming at the development of dynamic resolution processes.

Esterase Method

A racemic amino acid ester can be enzymatically resolved by the action of a protease or (in selected cases) an esterase or a lipase. Remarkably, the first resolution of this type using a crude porcine pancreatic extract was reported in 1905 [109]! The catalytic activity of a protease on a carboxylic ester bond has frequently been denoted as 'esterase activity', although the mechanism of action involved does not differ in principle from that of an amide hydrolysis. Bearing in mind the greater stability of an amide bond as compared to that of an ester, it is reasonable that a protease, which is able to cleave a much stronger amide bond, is capable of hydrolyzing a carboxylic ester. Esterases, on the other hand, are generally unable to cleave amide bonds, although they can catalyze their formation via ester aminolysis (Sect. 3.1.3, Scheme 2.1). This does not apply to highly strained β-lactams, which can be hydrolyzed by some esterases (pig liver esterase) or lipases (Scheme 2.11) [110].

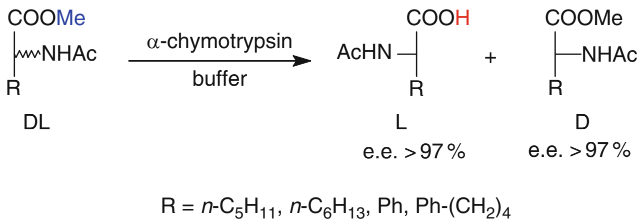


Scheme 2.11 Enzymatic resolution of amino acid esters via the esterase method

The amino group of the substrate may be either free or (better) protected by an acyl functionality, preferably an acetyl-, benzoyl-, or the *tert*-butyloxycarbonyl-(Boc)-group in order to avoid possible side reactions such as ring-closure going in hand with the formation of diketopiperazines. The ester moiety should be a short-chain aliphatic alcohol such as methyl or ethyl to ensure a reasonable reaction rate. When carboxyl ester hydrolases such as lipases are used in this process, it is recommended to use more lipophilic alcohol residues (e.g., *n*-butyl, *n*-hexyl, *n*-octyl) or activated analogs bearing electron-withdrawing substituents, such as chloroethyl [111] or trifluoroethyl [112], to ensure high reaction rates.

Numerous enzymes have been used to hydrolyze *N*-acyl amino acid esters, the most versatile and thus very popular catalyst being α-chymotrypsin isolated from bovine pancreas (Scheme 2.12) [113–115]. Since it is one of the early examples of a pure enzyme which became available for biotransformations, its mode of action is well understood. A useful and quite reliable model of its active site has been proposed in order to rationalize the stereochemical outcome of resolutions performed with α-chymotrypsin [116, 117]. Alternatively, other proteases, such as subtilisin [118, 119], thermolysin [120], and alkaline protease [121] are also commonly used for the resolution of amino acid esters. Even whole microorganisms such as lyophilized cells of baker's yeast, possessing unspecific proteases, can be used as biocatalysts for this type of transformation [122].

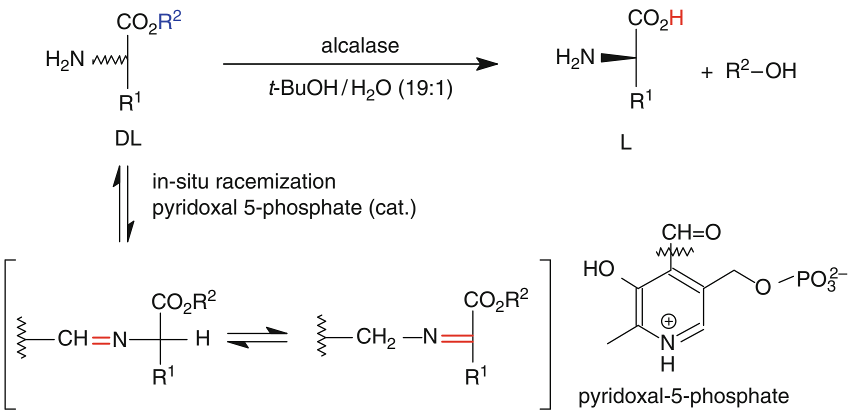
Carbonic anhydrase – an enzyme termed for its ability to catalyze the hydration of carbon dioxide forming hydrogen carbonate – can also be employed. In contrast to the above-mentioned biocatalytic systems, it exhibits the opposite enantiopreference by hydrolyzing the D-*N*-acylamino acid esters [123].



Scheme 2.12 Resolution of *N*-acetyl amino acid esters by α-chymotrypsin [123, 124]

An efficient dynamic resolution process for α-amino acid esters using a crude industrial protease preparation from *Bacillus licheniformis* ('alcalase')[7] has been developed (Scheme 2.13) [126]. The remaining unhydrolyzed D-enantiomer of the substrate was racemized in situ, catalyzed by pyridoxal-5-phosphate (PLP, vitamin B_6). Interestingly, this trick has been copied from nature, since pyridoxal-5-phosphate is an essential cofactor for biological amino-group transfer. PLP spontaneously forms a Schiff base with the amino acid ester (but not with the amino acid) which facilitates racemization through reversible proton migration. By using this method, a range of racemic amino acid esters were dynamically resolved in excellent chemical and optical yield. As a more economical substitute for pyridoxal 5-phosphate, its nonphosphorylated analog (pyridoxal) or salicylaldehyde are used in large-scale applications.

[7] 'Alcalase' is mainly used as additive in detergents for the degradation of proteinogenic impurities, its major enzyme component is subtilisin Carlsberg (alkaline protease A).



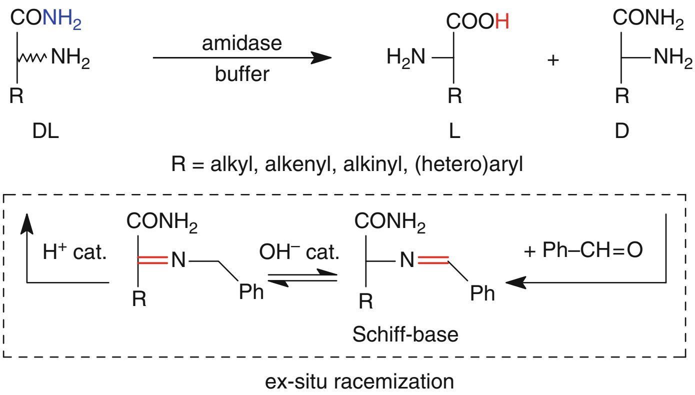
R^1	R^2		Product yield [%]	e.e.[%]
Ph–CH_2–	Ph–CH_2–	L-Phe	92	98
Ph–CH_2–	*n*-Bu–	L-Phe	92	98
4–Hydroxyphenyl–CH_2–	Ph–CH_2–	L-Tyr	95	97
4–Hydroxyphenyl–CH_2–	*n*-Pr–	L-Tyr	95	97
$(CH_3)_2$CH–CH_2–	Ph–CH_2–	L-Leu	87	93
n-Bu–	Ph–CH_2–	L-NorLeu	87	90
Et–	Ph–CH_2–	L-NorVal	87	91

Scheme 2.13 Dynamic resolution of amino acid esters

Amidase Method

α-Amino acid amides are hydrolyzed enantioselectively by amino acid amidases (occasionally also termed aminopeptidases) obtained from various sources, such as kidney and pancreas [127] and from different microorganisms, in particular *Pseudomonas*, *Aspergillus*, or *Rhodococcus* spp. [128]. For industrial applications, special amidases (e.g., from *Mycobacterium neoaurum* and *Ochrobactrum anthropi*) have been developed [129, 130], which, for instance, can be used to resolve α,α-disubstituted α-amino acid amides – which are otherwise not easily hydrolyzed, due to steric hindrance (Scheme 2.14) [105].

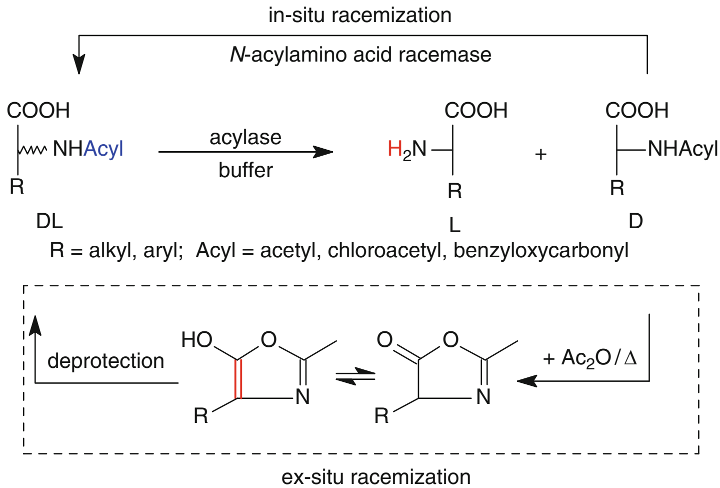
Again, the L-amino acids thus formed are separated from the unreacted D-amino acid amide by the difference in solubility in various solvents at various pH. After separation, unreacted D-amino acid amides can be recycled via base-catalyzed racemization of the corresponding Schiff-base intermediates in a separate step [131]. Since amino acid amides are less susceptible to spontaneous chemical hydrolysis in the aqueous environment than the corresponding esters, the products which are obtained by this method are often of higher optical purities.



Scheme 2.14 Enzymatic resolution of amino acid amides via the amidase method

Acylase Method

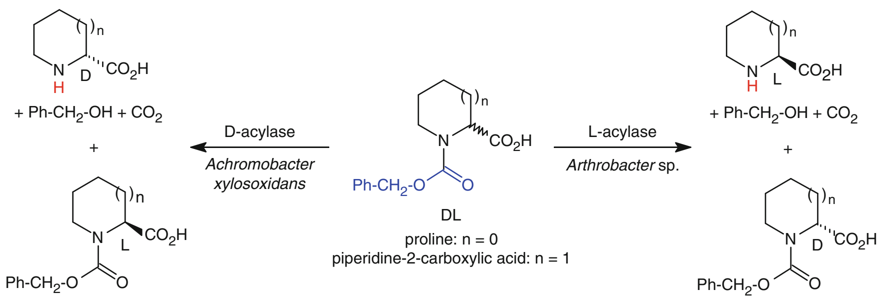
Aminoacylases catalyze the hydrolysis of *N*-acyl amino acid derivatives, with the acyl groups preferably being acetyl, chloroacetyl, or propionyl. Alternatively, the corresponding *N*-carbamoyl- and *N*-formyl derivatives can be used [132]. Enzymes of the amino acylase type have been isolated from hog kidney, and from *Aspergillus* or *Penicillium* spp. [133–135]. The versatility of this type of enzyme has been demonstrated by the resolution of racemic *N*-acetyl tryptophan, -phenylalanine, and -methionine on an industrial scale using column reactors (Scheme 2.15) [136, 137].



Scheme 2.15 Enzymatic resolution of *N*-acyl amino acids via the acylase method

On a laboratory scale, the readily available amino acylase from hog kidney is recommended [138]. It seems to be extremely substrate-tolerant, allowing variations of the alkyl- or aryl-moiety R within a wide structural range while retaining very high enantioselectivities. The latter enzyme has been frequently used as an aid in natural product synthesis (Scheme 2.17) [139, 140]. Unwanted enantiomers of *N*-acetyl amino acids can be conveniently racemized ex-situ by heating with acetic anhydride in a separate process. The mechanism of this racemization involves activation of the acid moiety via a mixed anhydride, which undergoes cyclization to form an oxazolinone (azlactone). The latter is subject to racemization through the intermediate achiral enol. Alternatively, the acylase method can be converted into a dynamic process if the nonreacting *N*-acylamino acid is racemized in-situ via an *N*-acylamino acid racemase [141, 142]. In contrast to the majority of amino acid racemases, which are cofactor-dependent (usually pyridoxal-5-phosphate), an enzyme which was isolated from *Amycolatopsis* sp. requires a divalent metal ion such as Co, Mn, or Mg for catalytic activity [143].

Cyclic amino acids, such as proline and piperidine-2-carboxylic acid are valuable building blocks for the synthesis of pharmaceuticals, such as the anti-migraine-agent eleptritan or the anticancer drug incel, respectively. In order to access both enantiomers by choice of the appropriate enzyme, enantiocomplementary acylases from microbial sources were developed using classic enrichment techniques. An L-acylase from *Arthrobacter* sp. furnishes the free L-amino acid plus the unreacted D-*N*-acyl-substrate enantiomer, while opposite enantiomers were obtained using a D-specific acylase from *Arthrobacter xylosoxidans* (Scheme 2.16) [144, 145].

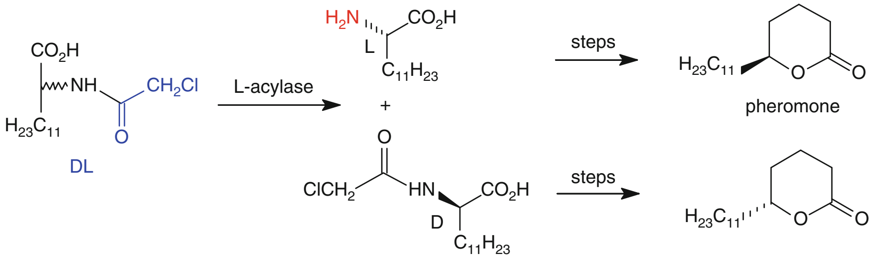


Scheme 2.16 Resolution of cyclic *N*-benzyloxycarbonyl amino acids using enantiocomplementary acylases

Interestingly, even *N*-acyl α-amino*phosphonic* acid derivatives have been resolved using penicillin acylase [146].

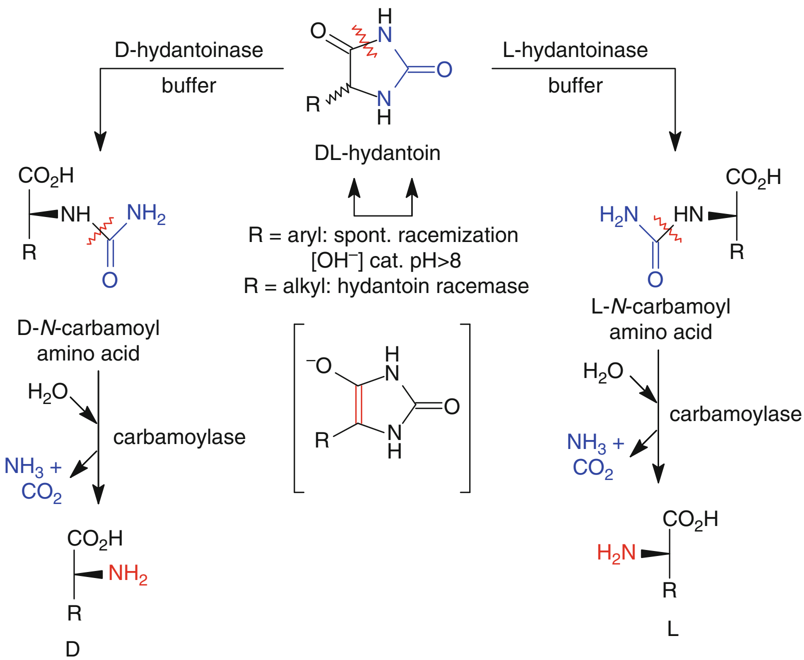
Hydantoinase Method

5-Substituted hydantoins may be obtained easily in racemic form from cheap starting materials such as aldehyde, hydrogen cyanide, and ammonium carbonate using the Bücherer–Bergs synthesis [147]. Hydantoinases from different microbial



Scheme 2.17 Enzymatic resolution of *N*-acyl amino acids for natural product synthesis

sources catalyze the hydrolytic ring-opening to form the corresponding *N*-carbamoyl-α-amino acids [148–150]. In nature, many (but not all) of these enzymes are responsible for the cleavage of dihydropyrimidines occurring in pyrimidine catabolism, therefore they are often also called 'dihydro-pyrimidinases' (Scheme 2.18) [151, 152].



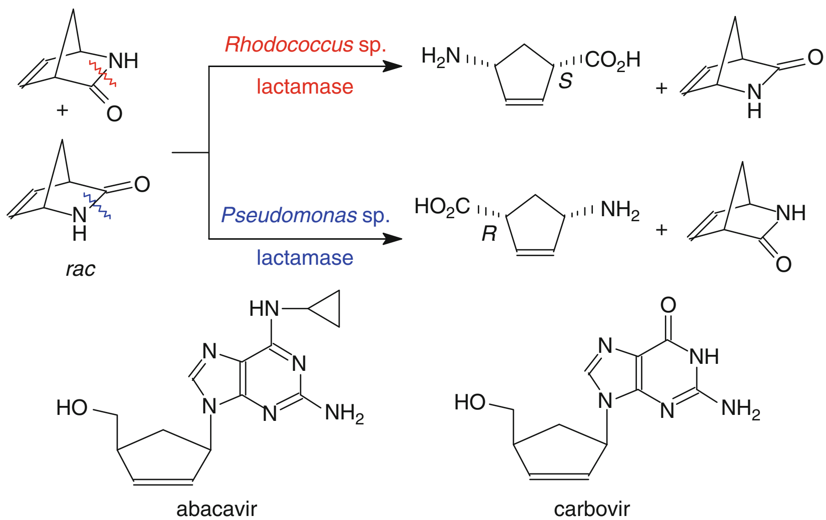
Scheme 2.18 Enzymatic resolution of hydantoins via the hydantoinase method

In contrast to the above-mentioned amino acid resolution methods involving amino acid esters, -amides, or *N*-acylamino acids where the natural L-enantiomer is preferably hydrolyzed from a racemic mixture, hydantoinases usually convert the opposite D-enantiomer [153–155], and L-hydantoinases are known to a lesser extent

[156–158]. In addition, D-hydantoinases usually possess a broader substrate spectrum than their L-counterparts. The corresponding *N*-carbamoyl derivatives thus obtained can be transformed into the corresponding amino acids by an *N*-carbamoyl amino acid amidohydrolase, which is often produced by the same microbial species. Alternatively, *N*-carbamoyl amino acids can be chemically hydrolyzed by treatment with nitrous acid or by exposure to an acidic pH (<4). One property of 5-substituted hydantoins, which makes them particularly attractive for large-scale resolutions is their ease of racemization. When R contains an aromatic group, the enantiomers of the starting hydantoins are readily equilibrated at slightly alkaline pH (>8), which is facilitated by resonance stabilization of the corresponding enolate. In contrast, aliphatic substituted hydantoins racemize very slowly under the reaction conditions compatible with hydantoinases due to the lack of enolate stabilization. For such substrates the use of hydantoin racemases is required to render a dynamic resolution process, which ensures a theoretical yield of 100% [159, 160].

Lactamase Method

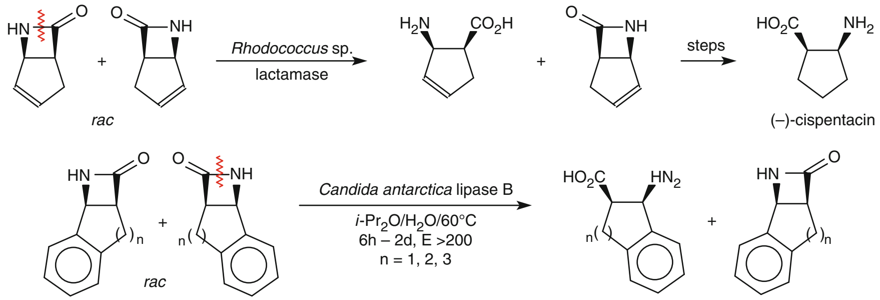
Due to their cyclic structure, cyclic amides (γ- and δ-lactams) are chemically considerably more stable and thus cannot be hydrolyzed by conventional proteases. However, they can be resolved using a special group of proteases acting on cyclic amide bonds – lactamases.



Scheme 2.19 Enzymatic resolution of bicyclic β- and γ-lactams via the lactamase method

The bicyclic γ-lactam shown in Scheme 2.19 is an important starting material for the production of antiviral agents, such as Carbovir and Abacavir. It can be efficiently resolved using enantiocomplementary γ-lactamases from microbial sources: an enzyme from *Rhodococcus equi* produced the (*S*)-configurated amino acid (plus enantiomeric non-converted lactam), and another lactamase isolated from *Pseudomonas solanacearum* acted in an enantiocomplementary fashion by providing the corresponding mirror-image products [161].

In contrast to γ- and δ-lactams, highly strained β-lactams are more easily susceptible to enzymatic hydrolysis and thus can be (slowly) hydrolyzed by carboxyl ester hydrolyses, such as esterases [162] and lipases [163] (Scheme 2.20).



Scheme 2.20 Enzymatic hydrolysis of strained β-lactams using lactamases and lipases

The bicyclic lactam shown in Scheme 2.20, which serves as starting material for the synthesis of the antifungal agent (–)-cispentacin, was efficiently resolved using *Rhodococcus equi* lactamase [164]. Remarkably, related benzo-fused β-lactam analogs of varying ring size were even accepted by lipase B from *Candida antarctica* [165]. Although the reactions were relatively slow (up to 2 days at 60°C), excellent enantioselectivities were obtained.

2.1.3 *Ester Hydrolysis*

2.1.3.1 Esterases and Proteases

In contrast to the large number of readily available microbial lipases, less than a dozen of true 'esterases' – such as pig and horse liver esterases (PLE [166,[8] 167] and HLE, respectively) – have been used to perform the bulk of the large number of highly selective hydrolyses of carboxylic esters. Thus, the use of a different esterase is not easy in cases where the reaction proceeds with insufficient selectivity with a standard enzyme such as PLE.

An esterase which has been shown to catalyze the hydrolysis of nonnatural esters with exceptionally high selectivities is acetylcholine esterase (ACE). It would certainly be a valuable enzyme to add to the limited number of available esterases but it has a significant disadvantage since it is isolated from *Electrophorus electricus* – the electric eel. Comparing the natural abundance of this species with the occurrence of

[8] It should be mentioned that some references and absolute configurations are wrong in this paper.

horses or pigs, its high price – which is prohibitive for most synthetic applications – is probably justified. Thus, the number of ACE applications is limited [168–171]. Additionally, cholesterol esterase is also of limited use, since it seems to work only on relatively bulky substrates which show some structural similarities to the natural substrates of cholesterol esterase, i.e., steroid esters [61, 172].

To overcome this narrow range of readily available esterases, whole microbial cells are sometimes used to perform the reactions instead of isolated enzyme preparations [173]. Although some highly selective conversions using whole-cell systems have been reported, it is clear that any optimization by controlling the reaction conditions is very complicated when viable whole cells are employed. Furthermore, for most cases the nature of the actual active enzyme system remains unknown.

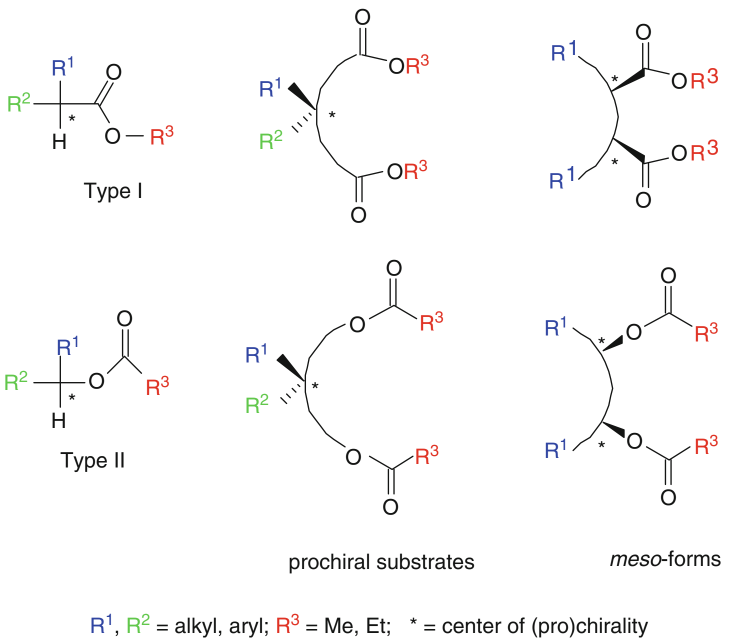
More recently, novel microbial esterases [174, 175] such as carboxyl-esterase NP [176] have been identified from an extensive screening in search for biocatalysts with high specificities for certain types of substrates. Since they have been made available in generous amounts by genetic engineering [177], they are now being used more widely. Despite numerous efforts directed towards the cloning and overexpression of microbial esterases, the number of synthetically useful enzymes – possessing a relaxed substrate specificity by retaining high enantioselectivity – are limited: many novel esterases showed disappointing selectivities [178, 179].

Fortunately, as mentioned in the foregoing chapter, a large number of proteases can also selectively hydrolyze carboxylic esters and this effectively compensates for the limited number of esterases [180]. The most frequently used members of this group are α-chymotrypsin [181], subtilisin [182] and, to a somewhat lesser extent, trypsin, pepsin [183], papain [184], and penicillin acylase [185, 186]. Since many of the studies on the ester-hydrolysis catalyzed by α-chymotrypsin and subtilisin have been performed together with PLE in the same investigation, representative examples are not singled out in a separate chapter but are incorporated into the following chapter dealing with studies on PLE. A more recently established member of this group is a protease from *Aspergillus oryzae* which seems to be particularly useful for the selective hydrolysis of bulky esters.

As a rule of thumb, when acting on nonnatural carboxylic esters, most proteases seem to retain a preference for the hydrolysis of that enantiomer which mimics the configuration of an L-amino acid more closely [187].

The structural features of more than 90% of the substrates which have been transformed by esterases and proteases can be reduced to the general formulas given in Scheme 2.21. The following general rules can be applied to the construction of substrates for esterases and proteases:

- For both esters of the general type I and II, the center of chirality (marked by an asterisk [*]) should be located as close as possible to the site of the reaction (that is, the carbonyl group of the ester) to ensure an optimal chiral recognition. Thus, α-substituted carboxylates and esters of secondary alcohols are usually more selectively hydrolyzed than their β-substituted counterparts and esters of chiral primary alcohols, respectively.



Scheme 2.21 Types of substrates for esterases and proteases

- Both substituents R^1 and R^2 can be alkyl or aryl groups, but they should differ in size and polarity to aid the chiral recognition process of the enzyme. They may also be joined together to form cyclic structures.
- Polar or charged functional groups located at R^1 and R^2, such as –OH, –COOH, –$CONH_2$, or –NH_2, which are heavily hydrated in an aqueous environment should be absent, since esterases (and in particular lipases) do not accept highly polar hydrophilic substrates. If such moieties are required, they should be masked with an appropriate lipophilic protective group.
- The alcohol moieties R^3 of type-I esters should be as short as possible, preferably methyl or ethyl. If neccessary, the reaction rate of ester hydrolysis may be enhanced by linking electron-withdrawing groups to the alcohol moiety to give methoxymethyl, cyanomethyl, or 2-haloethyl esters. In contrast, carboxylates bearing long-chain alcohols are usually hydrolyzed at reduced reaction rates with esterases and proteases.
- The same considerations are applicable to acylates of type II, where short-chain acetates or propionates are the preferred acyl moieties. Increasing the carbonyl reactivity of the substrate ester by adding electron-withdrawing substituents such as halogen (leading to α-haloacetates) is a frequently used method to enhance the reaction rate in enzyme-catalyzed ester hydrolysis [188].
- One limitation in substrate construction is common for both types of substrates: the remaining hydrogen atom at the chiral center must not be replaced, since α,α,α-trisubstituted carboxylates and esters of tertiary alcohols are usually too

bulky to be accepted by esterases and proteases, although there are some rare exceptions to this rule [189–192]. This limitation turns them into potential protective groups for carboxy- and alcoholic functionalities, such as *t*-butyl esters and pivalates, in case an enzymatic hydrolysis is not desired. For serine ester hydrolases, the rare ability to hydrolyze bulky esters was attributed to an atypical Gly-Gly-Gly-X-sequence motif (instead of the common Gly-X-motif) in the oxyanion cavity located within the active site, which was found in *Candida rugosa* and *Candida antarctica* lipase A [193–195].

- It is clear that both general substrate types (which themselves would constitute racemic substrates) may be further combined into suitable prochiral or *meso*-substrates (Scheme 2.21).

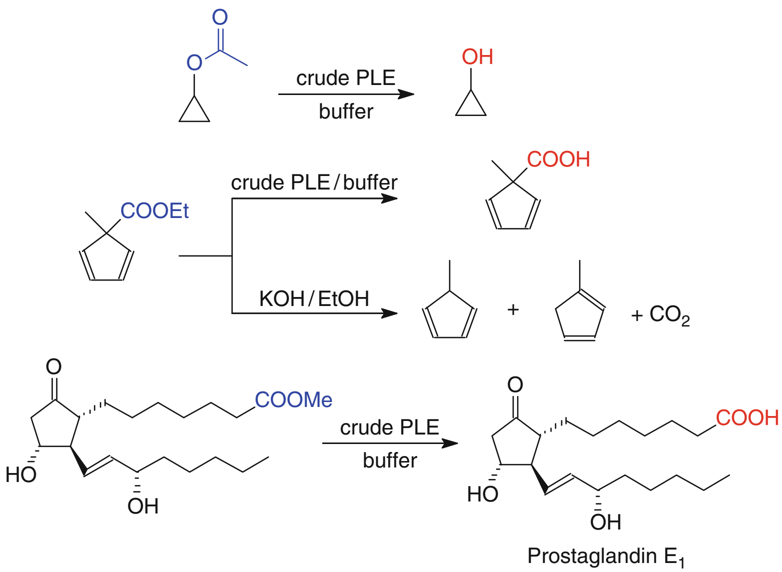
Pig Liver Esterase and α-Chymotrypsin

Amongst all the esterases, pig liver esterase (PLE) is clearly the champion considering its general versatility. This enzyme is constitutionally complex and consists of at least five so-called *iso*enzymes, which are associated as trimers of three individual proteins [196]. However, for many applications this crude mixture can be regarded as a single enzyme since the isoenzyme subunits often possess similar (but not identical [197]) stereospecificities [198]. Thus, the selectivity of crude PLE may vary, depending on the source and the pretreatment of the enzyme preparation [199]. The biological role of PLE is the hydrolysis of various esters occurring in the porcine diet, which would explain its exceptionally wide substrate tolerance. For preparative reactions it is not absolutely necessary to use the expensive commercially available enzyme preparation because a crude acetone powder which can easily be prepared from pig liver is a cheap and efficient alternative [200].

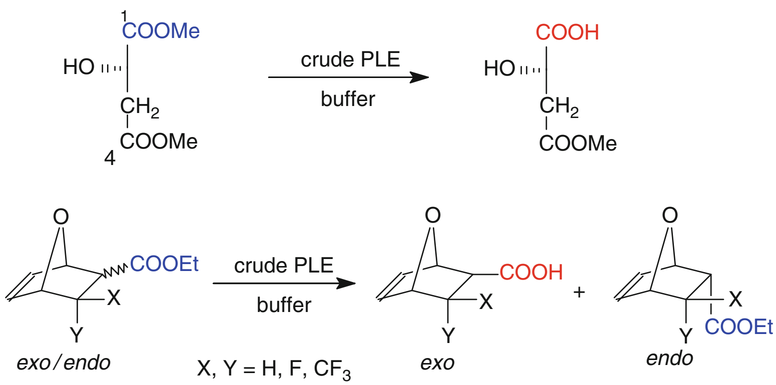
It seems to generally apply that the respective esterases from related sources such as liver of chickens, hamsters, guinea pigs, or rats were found to be less versatile when compared to PLE. In certain cases, however, esterases from rabbit [201, 202] and horse liver (HLE) [203, 204] proved to be useful substitutes for PLE.

Mild Hydrolysis. Acetates of primary and secondary alcohols such as cyclopropyl acetate [205] and methyl or ethyl carboxylates (such as the labile cyclopentadiene ester [206]) can be selectively hydrolyzed under mild conditions using PLE, avoiding decomposition reactions which would occur during a chemical hydrolysis under acid or base catalysis (Scheme 2.22). For example, this strategy has been used for the final deprotection of the carboxyl moiety of prostaglandin E_1 avoiding the destruction of the delicate molecule [207, 208].

Regio- and Diastereoselective Hydrolysis. Regiospecific hydrolysis of dimethyl malate at the 1-position could be effected with PLE as catalyst (Scheme 2.23) [209]. Similarly, hydrolysis of an *exo*/*endo*-mixture of diethyl dicarboxylates with a bicyclo[2.2.1]heptane framework occurred only on the less hindered *exo*-position [210] leaving the *endo*-ester untouched, thus allowing a facile separation of the two positional isomers in a diastereomeric mixture.



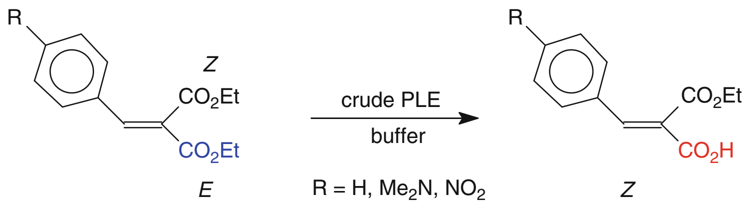
Scheme 2.22 Mild ester hydrolysis by porcine liver esterase



Scheme 2.23 Regio- and diastereoselective ester hydrolysis by porcine liver esterase

Separation of *E*/*Z*-Isomers. With *E*/*Z*-diastereotopic diesters bearing an aromatic side chain, PLE selectively hydrolyzed the ester group in the more accessible (*E*)-*trans*-position to the phenyl ring, regardless of the *p*-substituent [211] (Scheme 2.24). In analogy to the hydrolysis of dicarboxylates (Scheme 2.3) the reaction stopped at the (*Z*)-monoester stage with no diacid being formed. Other hydrolytic enzymes (proteases and lipases) were less selective in this case.

Desymmetrization of Prochiral Diesters. PLE has been used only relatively infrequently for the resolution of racemic esters, while α-chymotrypsin has played

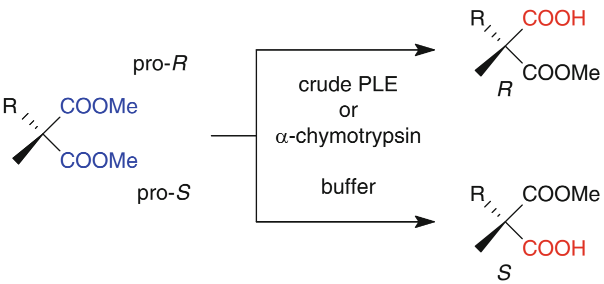


Scheme 2.24 Regioselective hydrolysis of *E*/*Z*-diastereotopic diesters by porcine liver esterase

a more important role. Instead, the '*meso*-trick' has been thoroughly used with the former enzyme.

As depicted in Scheme 2.25, α,α-disubstituted malonic diesters can be selectively transformed by PLE or α-chymotrypsin to give the corresponding chiral monoesters [212, 213].

These transformations demonstrate an illustrative example for an 'alternative fit' of substrates with different steric requirements. While PLE performs a selective

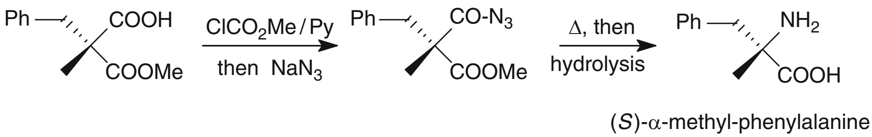


Enzyme	R	Configuration	e.e.[%]
PLE*	Ph–	*S*	86
PLE	C_2H_5–	*S*	73
PLE	*n*-C_3H_7–	*S*	52
PLE	*n*-C_4H_9–	*S*	58
PLE	*n*-C_5H_{11}–	*R*	46
PLE	*n*-C_6H_{13}–	*R*	87
PLE	*n*-C_7H_{15}–	*R*	88
PLE	*p*-MeO–C_6H_4–CH_2–	*R*	82
PLE	*t*-Bu–O–CH_2–	*R*	96
α-chymotrypsin	Ph–CH_2–	*R*	~100

* the ethyl ester was used

Scheme 2.25 Desymmetrization of prochiral malonates by porcine liver esterase and α-chymotrypsin hydrolysis of the pro-*S* ester group on all substrates possessing α-substituents (R) of a smaller size ranging from ethyl through *n*-butyl to phenyl, an increase of the steric bulkiness of R forces the substrate to enter the enzyme's active site in an opposite (flipped) orientation. Thus, with the more bulky substituents the pro-*R* ester is preferentially cleaved.

The synthetic utility of the chiral monoesters was demonstrated by the stereoselective degradation of the carboxyl group in the benzyl derivative using a Curtius rearrangement via an acyl azide intermediate [27] (Scheme 2.26). Finally, hydrolysis of the remaining ester group led to an optically pure α-methyl amino acid as exemplified for (*S*)-α-methylphenyl alanine. As mentioned in the foregoing chapter, such sterically demanding amino acids normally cannot be obtained by resolution of the appropriate amino acid derivative.

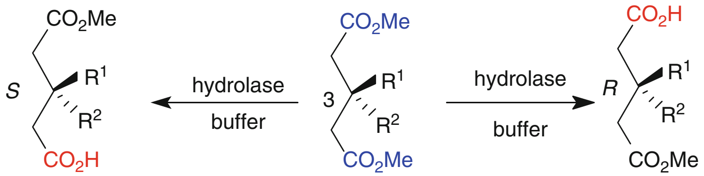


Scheme 2.26 Chemoenzymatic synthesis of α-methyl-L-amino acids

As shown in Scheme 2.27, the prochiral center may be moved from the site of the reaction into the β-position. Thus, chiral recognition by PLE [214–218] and α-chymotrypsin [219–222] is retained during the desymmetrization of prochiral 3-substituted glutaric diesters. Whole cells of *Acinetobacter lowfii* and *Arthrobacter* spp. have also been used as a source for esterase activity [223] and, once again, depending on the substitutional pattern on carbon-3, the desymmetrization can lead to both enantiomeric products.

Acyclic *meso*-dicarboxylic esters with a succinic and glutaric acid backbone were also good substrates for PLE [224] and α-chymotrypsin (Scheme 2.28) [225]. Interestingly, an additional hydroxy group in the substrate led to an enhancement of the chiral recognition process in both cases.

The full synthetic potential of the '*meso*-trick' has been exemplified by the desymmetrization of cyclic diesters possessing various kinds of structural patterns. A striking example of a 'reversal of best fit' for cyclic *meso*-1,2-dicarboxylates caused by variation of the ring size, is shown in Scheme 2.29 [226]: when the rings are small ($n = 1, 2$), the (*S*)-carboxyl ester is selectively cleaved, whereas the (*R*)-counterpart preferentially reacts when the rings are larger ($n = 4$). The highly flexible cyclopentane derivative of moderate ring size is somewhat in the middle of the range and its chirality is not very well recognized. The fact that the nature of the alcohol moiety of such esters can have a significant impact in both the reaction rate and stereochemical outcome of the hydrolysis was shown by the poor chiral recognition of the corresponding diethyl ester of the cyclohexane derivative, which was slowly hydrolyzed to give the monoethyl ester of poor optical purity [227].

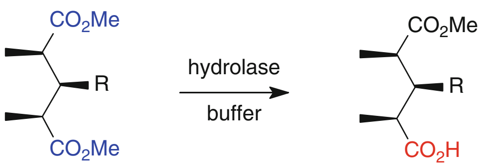


Hydrolase	R^1	R^2	Product	e.e. [%]
α-chymotrypsin*	AcNH–	H	*R*	79
α-chymotrypsin	Ph–CH_2–O–	H	*R*	84
α-chymotrypsin*	HO–	H	*R*	85
α-chymotrypsin	CH_3OCH_2O–	H	*R*	93
PLE	HO–	H	*S*	12
PLE	Ph–CH_2–CH=CH–CH_2–	H	*S*	88
PLE	CH_3–	H	*R*	90
PLE	AcNH–	H	*R*	93
PLE	*t*-Bu–CO–NH–	H	*S*	93
PLE	HO–	CH_3	*S*	99
Acinetobacter sp.*	HO–	H	*R*	>95
Arthrobacter sp.*	HO–	H	*S*	>95

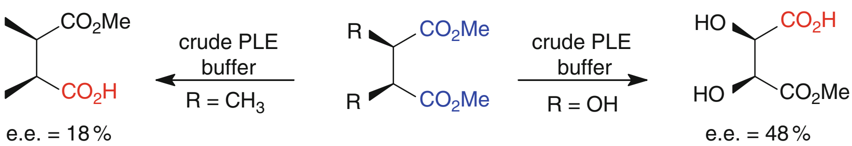
* the corresponding ethyl esters were used

Scheme 2.27 Desymmetrization of prochiral glutarates

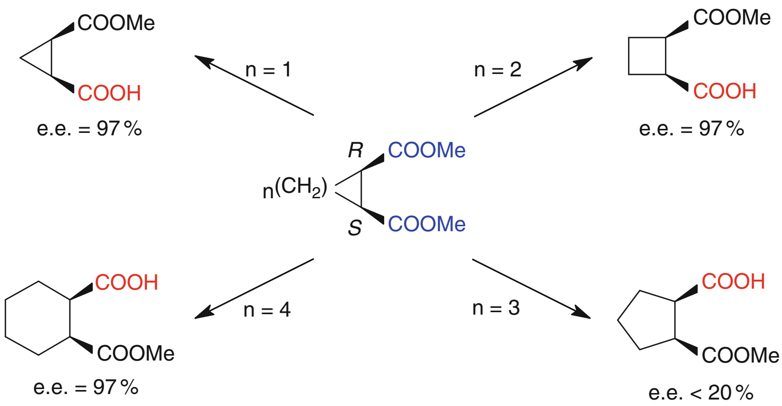
Bulky bicyclic *meso*-dicarboxylates, which were extensively used as optically pure starting materials for the synthesis of bioactive products, can be accepted by PLE [228]. Some selected examples are shown in Scheme 2.30. During these



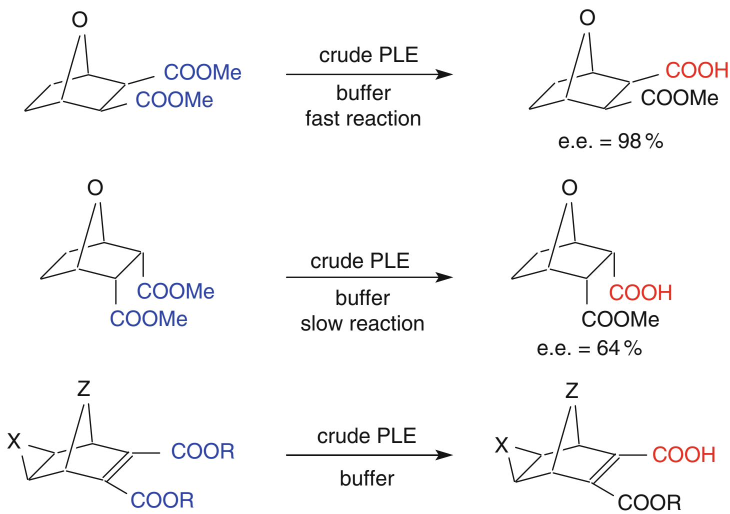
R	enzyme	e.e. [%]
H	crude PLE	64
OH	crude PLE	98
H	α-chymotrypsin	77



Scheme 2.28 Desymmetrization of acyclic *meso*-dicarboxylates by α-chymotrypsin and porcine liver esterase



Scheme 2.29 Desymmetrization of cyclic *meso*-1,2-dicarboxylates by porcine liver esterase

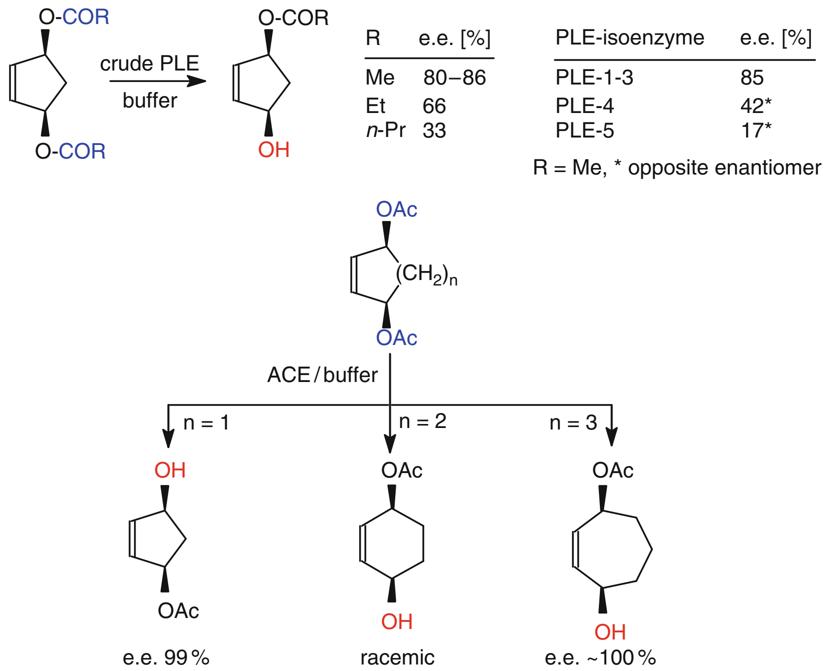


X	Z	R	e.e. [%]
bond	CH_2	Me	41
O	O	Me	77
O–C(CH_3)$_2$–O	O	Me	77
O–C(CH_3)$_2$–O	CH_2	Me	85
O–C(CH_3)$_2$–O	CH_2	Et	100
O–C(CH_3)$_2$–O	CH_2	*n*-Pr	45

Scheme 2.30 Desymmetrization of polycyclic *meso*-1,2-dicarboxylates by porcine liver esterase

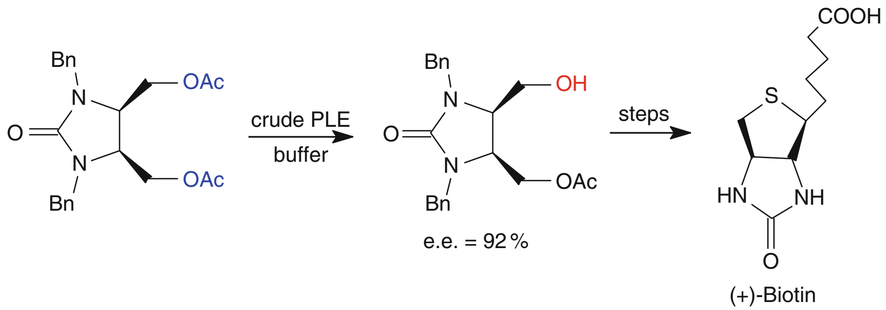
studies it was shown that the *exo*-configurated diesters were good substrates, whereas the corresponding more sterically hindered *endo*-counterparts were less selectively hydrolyzed at a significantly reduced reaction rate. Again, the importance of the appropriate choice of the alcohol moiety is exemplified [216]. Thus, while the short-chain methyl and ethyl esters were hydrolyzed with high selectivities, the propyl ester was not.

Cyclic *meso*-diacetates can be hydrolyzed in a similar fashion. As shown in Scheme 2.31, the cyclopentene *meso*-monoester [229], which constitutes one of the most important chiral synthons for prostaglandins and their derivatives [230], was obtained in an e.e. of 80–86% using crude PLE. In accordance with the above-mentioned hypotheses for the construction of esterase substrates, a significant influence of the acyl moiety of the ester was observed: the optical purity of the monoester gradually declined from 80–86% to 33% as the acyl chain of the starting substrate ester was extended from acetate to butanoate. A detailed study of the stereoselectivity of PLE isoenzymes revealed that isoenzymes PLE-1–3 gave almost identical results as the crude PLE preparation, whereas isoenzymes PLE-4 and PLE-5 showed lower stereoselectivities with a preference for the opposite enantiomer [231].



Scheme 2.31 Desymmetrization of cyclic *meso*-diacetates by porcine liver esterase and acetylcholine esterase

In order to avoid recrystallization of the optically enriched material (80–86% e.e.), which was obtained from the hydrolysis of *meso*-1,3-diacetoxy-cyclopentene with crude PLE, to enantiomeric purity, a search was conducted for another esterase, which would hydrolyze this substrate with an even better stereoselectivity. Acetylcholine esterase (ACE) was shown to be the best choice [232]. It hydrolyzed the cyclopentene diester with excellent stereoselectivity but with the *opposite* stereopreference as with PLE. Similar results were obtained by using lipases from porcine pancreas [233] and *Candida antarctica* [234]. When structural analogs of larger ring size were subjected to ACE hydrolysis, a dramatic effect on the stereochemical course was observed: while the six-membered *meso*-diester gave the racemic product, the seven-membered analog led to optically pure monoester of opposite configuration [235].



Scheme 2.32 Desymmetrization of *N*-containing cyclic *meso*-diester by porcine liver esterase

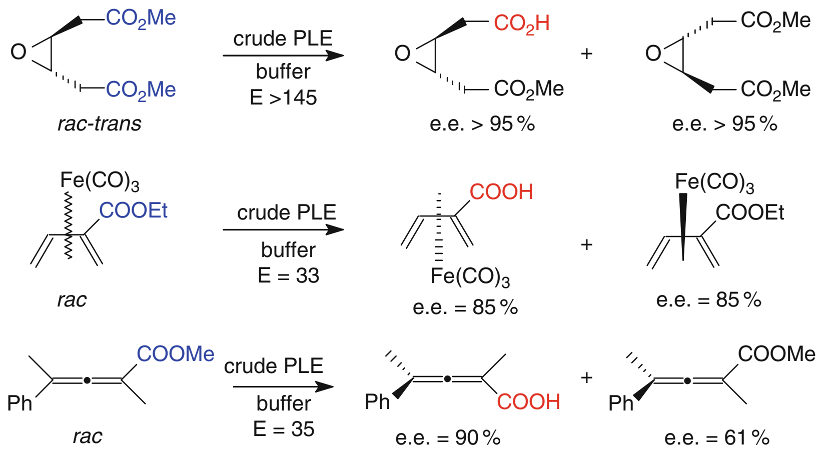
Cyclic *meso*-diacetates containing nitrogen functionalities proved to be excellent substrates for PLE (Scheme 2.32). In the benzyl-protected 1,3-imidazolin-2-one system – which serves as a starting material for the synthesis of the vitamin (+)-biotin – the optical yield of PLE-catalyzed hydrolysis of the *cis*-diacetate [236] was much superior to that of the corresponding *cis*-dicarboxylate [237].

Resolution of Racemic Esters. Although PLE-catalyzed resolution of racemic esters have been performed less often as compared to the desymmetrization of prochiral and *meso*-diesters, it can be a valuable technique.

It has been shown that chirality does not necessarily need to be located on a tetrahedral carbon atom, as in the case of the *trans*-epoxy dicarboxylates (Scheme 2.33) [238]. For example, the axial chirality of the racemic iron-tricarbonyl complex [239] and of the allenic carboxylic ester shown below [240], was well recognized by PLE.

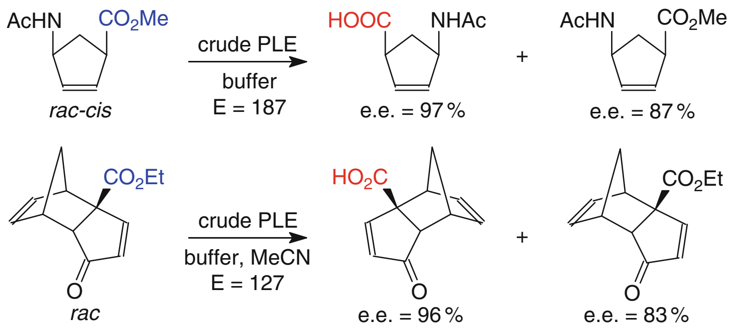
Resolution of an *N*-acetylaminocyclopentene carboxylate shown in Scheme 2.34 was used to access optically pure starting material for the synthesis of carbocyclic nucleoside analogs with promising antiviral activity [241]. The bulky tricyclic monoester was required for natural product synthesis [242].

An example demonstrating the high stereospecificity of PLE is the kinetic resolution of the cyclic *trans*-1,2-diacetate shown in Scheme 2.35 [243]. The (*R*,*R*)-diacetate enantiomer (possessing two ester groups showing the matching (*R*)-configuration) was hydrolyzed from the racemic mixture via the monoester



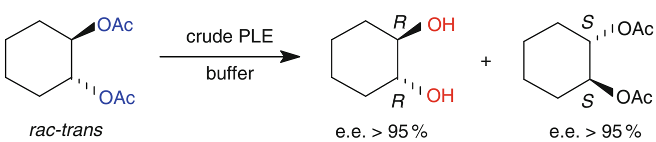
Scheme 2.33 Resolution of racemic carboxylic esters by porcine liver esterase

stage to yield the corresponding (*R*,*R*)-diol by leaving the (*S*,*S*)-diacetate untouched, since it possesses only nonmatching (*S*)-ester groups. Again, as observed in the desymmetrization of *cis*-*meso*-1,2-dicarboxylates, the enantioselectivity strongly



Scheme 2.34 Resolution of cyclic racemic carboxylic esters by porcine liver esterase

depended on the ring size: while the four- and six-membered substrates gave excellent results with opposite enantiopreference, the five-membered substrate analog was not suitable. It should be noted that a desymmetrization of the

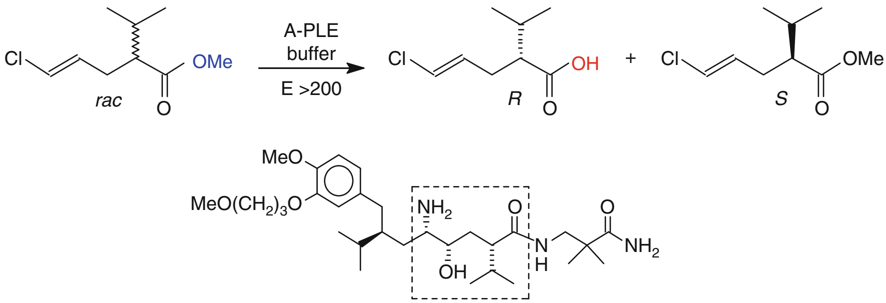


Scheme 2.35 Resolution of a cyclic *trans*-1,2-diacetate by porcine liver esterase

corresponding *cis*-*meso*-1,2-diacetates is impeded a priori by nonenzymic acyl migration which leads to facile racemization of any monoester that is formed.

Inspired by the broad substrate range of porcine liver esterase, cloning and overexpression of PLE isoenzymes was persued over the past few years in order to overcome imperfect stereoselectivities of crude PLE preparations and to provide a reliable enzyme source [244–246]. In addition, for industrial applications the use of enzymes from animal sources are often undesirable due to the risk of contaminations by viruses and prions and due to the fact that products derived from pigs are considered impure by several world religions.

Analysis of the amino acid sequences of PLE isoenzymes revealed that the remarkably small differences of ca. 20 amino acids are not distributed randomly but are located within distinct conserved areas. Among the different isoenzymes, PLE-1[9] and an isoenzyme termed A-PLE[10] [247] were shown to be most useful for stereoselective ester hydrolysis. The latter enzyme, which was expressed at a high level in *Pichia pastoris*, is remarkably stable and showed perfect enantioselectivity for the resolution of methyl (4*E*)-5-chloro-2-isopropyl-4-pentenoate, which is a key building block for the synthesis of the renin inhibitor aliskiren, which is used in the treatment of hypertension (Scheme 2.36) [248].



Aliskiren

Scheme 2.36 Resolution of α-chiral ester using the isoenzyme A-PLE

Microbial Esterases

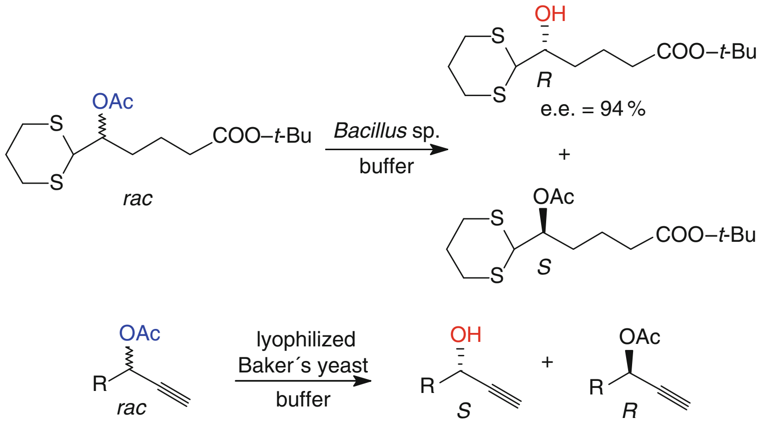
Complementary to the use of isolated enzymes, whole microbial cells have also been used to catalyze esterolytic reactions. Interesting cases are reported from bacteria, yeasts, and fungi, such as *Bacillus subtilis* [249], *Brevibacterium ammoniagenes* [250], *Bacillus coagulans* [251], *Pichia miso* [26], and *Rhizopus nigricans* [252]. Although the reaction control becomes more complex on using whole microbial cells, the selectivities achieved are sometimes impressive. This was shown by the successful resolution of a secondary alcohol via hydrolysis of its

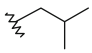
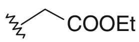
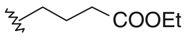
[9] PLE-1 is also named γ-PLE.

[10] 'Alternative pig liver esterase', this enzyme was also termed Pharma-PLE [246].

acetate by a *Bacillus* sp. (Scheme 2.37), while other biocatalytic methods to obtain the desired masked chiral hydroxyaldehyde failed [253]. In order to prevent an undesired ester hydrolysis at the terminal carboxyl functionality, it was efficiently blocked as its *tert*-butyl ester.

To overcome the problems of reaction control arising from the metabolism of fermenting microorganisms, resting cells of lyophilized baker's yeast have been proposed as a source of esterase activity. As shown in Scheme 2.34, 1-alkyn-3-yl acetates [254] were well resolved.



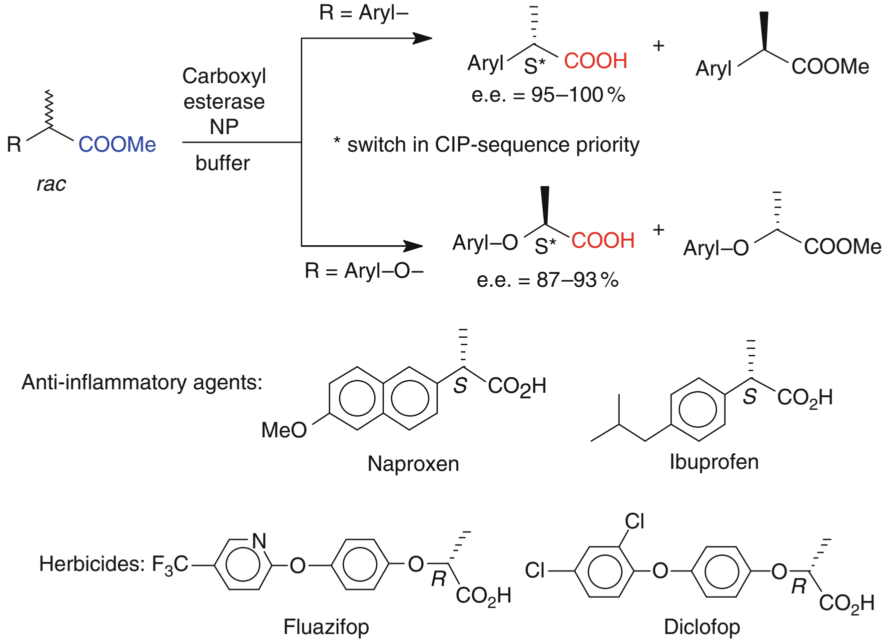
R	E	e.e. [%]	e.e. [%]
	46	91	72
	~100	91	>97
	89	96	59

Scheme 2.37 Hydrolytic resolution of *sec*-alcohols using whole microbial cells of *Bacillus* sp. and baker's yeast

Due to the importance of α-aryl- and α-aryloxy-substituted propionic acids as antiinflammatory agents (e.g., naproxen) and agrochemicals (e.g., the herbicide diclofop), respectively, where the majority of the biological activity resides in only one enantiomer (*S* for α-aryl- and *R* for α-aryloxy derivatives,[11] a convenient way for the separation of their enantiomers was sought by biocatalytic methods. Thus, an extensive screening program carried out by the industry has led to isolation of a novel esterase from *Bacillus subtilis* [255] (Scheme 2.38). The enzyme, termed 'carboxyl esterase NP', accepts a variety of substrates esters, including naproxen [256, 257]. It exhibits highest activity and selectivity when the substrate has an aromatic side chain, as with α-aryl- and α-aryloxypropionic acids. With α-aryl

[11]Be aware of the switch in the Cahn-Ingold-Prelog sequence priority.

derivatives the corresponding (*S*)-acids are obtained. α-Aryloxy analogs, however, are resolved with similar high specificities, but products have the opposite configuration (taking into account that a switch in CIP sequence priority occurs when going from aryl to aryloxy). This means that the stereochemical preference of carboxyl esterase NP is reversed when an extra oxygen atom is introduced between the chiral center and the aromatic moiety.

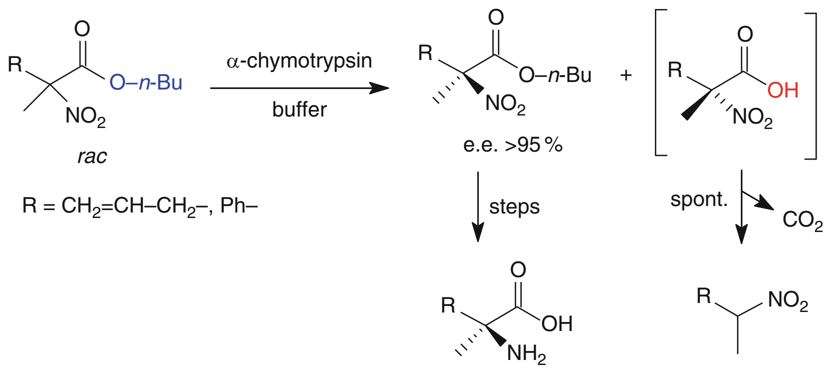


Scheme 2.38 Resolution of α-substituted propionates by carboxylesterase NP

Esterase Activity of Proteases

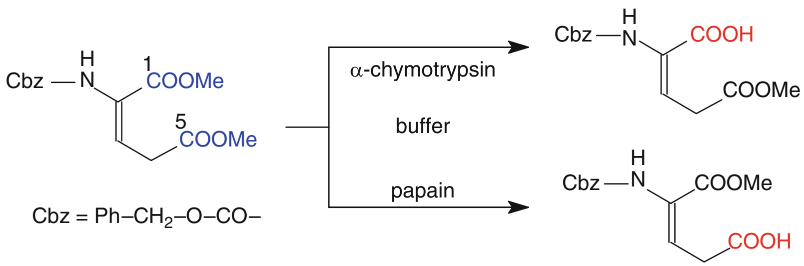
Numerous highly selective ester hydrolyses catalyzed by α-chymotrypsin [115] and papain have featured in excellent reviews [258] and the examples shown above should illustrate the synthetic potential, already fully established in 1976. The major requirements for substrates of type I (see Scheme 2.21) to be selectively hydrolyzed by α-chymotrypsin are the presence of a polar and a hydrophobic group on the α-center (R^1 and R^2, respectively) to mimic the natural substrates – amino acids.

An interesting example describing the asymmetric synthesis of α-methyl L-amino acids is shown in Scheme 2.39. Hydrolysis of the D-α-nitro esters from the D/L-mixture leads to the formation of the corresponding labile α-nitroacids, which readily decarboxylate to yield secondary nitro compounds. The L-configured enantiomers of the unhydrolyzed esters could be recovered in high optical purity and these were further transformed into α-methyl L-amino acid derivatives [259].



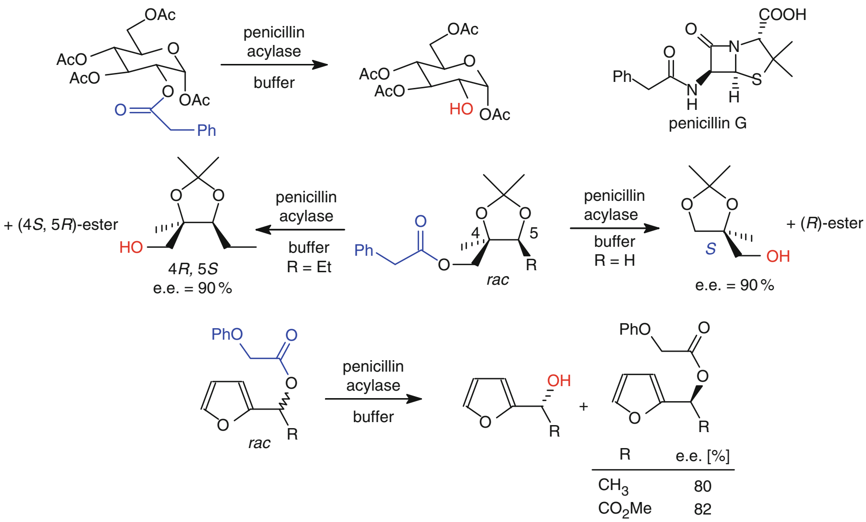
Scheme 2.39 Resolution of α-nitro-α-methyl carboxylates by α-chymotrypsin

Proteases such as α-chymotrypsin, papain, and subtilisin are also useful for regioselective hydrolytic transformations (Scheme 2.40). For example, while regioselective hydrolysis of a dehydroglutamate diester at the 1-position can be achieved using α-chymotrypsin, the 5-ester is attacked by the protease papain [260]. It is noteworthy that papain is one of the few enzymes used for organic synthetic transformations which originate from plant sources (papaya). Other related protease preparations are derived from fig (ficin) and pineapple stem (bromelain) [261].



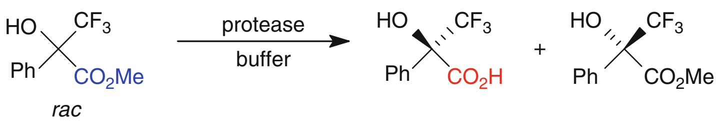
Scheme 2.40 Regioselective ester hydrolysis by proteases

More recently, two proteases have emerged as highly selective biocatalysts for specific purposes. Penicillin acylase is highly chemoselective for the cleavage of a phenylacetate group in its natural substrate, penicillin G. This enzyme now plays a major role in enzymatic protecting group chemistry [262, 263]. For instance, phenylacetyl groups can be removed in a highly chemoselective fashion in the presence of acetate esters (Scheme 2.41) [264, 265]. Furthermore, it can be used for the resolution of esters of primary [266] and secondary alcohols [267] as long as the acid moiety consists of a phenylacetyl group or a structurally closely related (heterocyclic) analog [268–270]. Some structural similarity of the alcohol moiety with that of the natural substrate penicillin G has been stated as being an advantage.



Scheme 2.41 Chemo- and enantioselective ester hydrolyses catalyzed by penicillin acylase

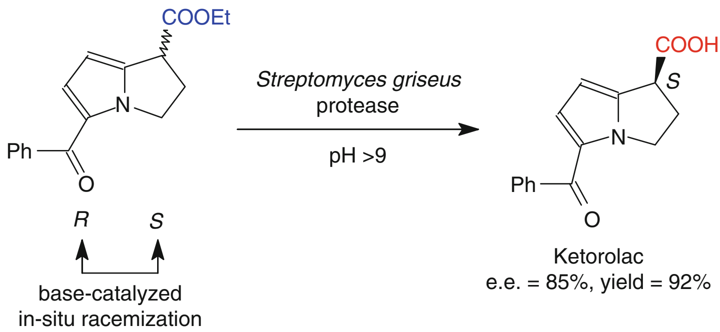
The use of subtilisin (a protease which is widely used in detergent formulations) as a biocatalyst for the stereospecific hydrolysis of esters is well established [271–273]. Along the same lines, a protease derived from *Aspergillus oryzae*, which has hitherto mainly been used for cheese processing, has been shown to be particularly useful for the resolution of sterically hindered substrates such as α,α,α-trisubstituted carboxylates [274] (Scheme 2.42). While 'traditional' proteases such as subtilisin were plagued by slow reaction rates and low selectivities, the α-trifluoromethyl mandelic ester (which constitutes a precursor of a widely used chiral derivatization agent, 'Mosher's acid' [275]) was successfully resolved by *Aspergillus oryzae* protease [276].



protease	E	e.e. [%]	e.e. [%]
subtilisin	2	25	25
Aspergillus oryzae protease	46	88	88

Scheme 2.42 Resolution of bulky esters by subtilisin and *Aspergillus oryzae* protease

An elegant example of a protease-catalyzed hydrolysis of a carboxylic ester was demonstrated by the dynamic resolution of the antiinflammatory agent 'ketorolac' via hydrolysis of its ethyl ester by an alkali-stable protease derived from



Scheme 2.43 Dynamic resolution with in-situ racemization by protease from *Streptomyces griseus*

Streptomyces griseus (Scheme 2.43) [83]. When the hydrolysis was carried out at pH > 9, base-catalyzed in-situ racemization of the substrate ester provided more of the enzymatically hydrolyzed (S)-enantiomer from its (R)-counterpart, thus raising the theoretical yield of the resolution of this racemate to 100%.

Optimization of Selectivity

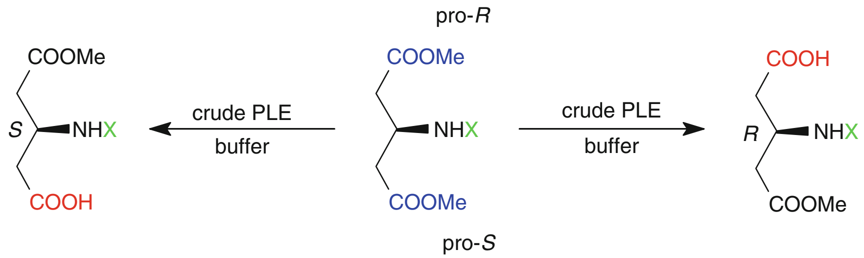
Many stereoselective enzymatic hydrolyses of nonnatural esters do not show a perfect selectivity, but are often in the range of 50–90% e.e., which corresponds to E values which are considered as 'moderate' to 'good' ($E = 3$–20). In order to avoid tedious and material-consuming processes to enhance the optical purity of the product, e.g., by crystallization techniques or via repeated kinetic resolution, several methods exist to improve the selectivity of an enzymatic transformation itself [24, 277]. Most of them can be applied to other types of enzymes.

Since every catalytic system consists of three main components – (bio)catalyst, substrate, and medium – there are three possibilities for the tuning of the selectivity:

- The ability to choose a different biocatalyst with a superior selectivity towards a given substrate mainly depends on the number of available options within the same enzyme class. This is certainly feasible for proteases and lipases, but not within the relatively small group of esterases. Alternatively, enzyme mutants possessing altered stereospecificities may be constructed.
- On the other hand, substrate modification (see below) is a widely employed strategy.
- Altering the properties of the medium – pH, temperature, cosolvents – is possible within certain limits and it can be a powerful technique to enhance enzyme selectivities.

Substrate engineering is one of the most promising techniques, which is applicable to all types of enzymatic transformations. As may be concluded from some of the foregoing examples, the ability of an enzyme to 'recognize' the chirality of a given substrate predominantly depends on its steric shape. Electronic effects are involved but usually less important [278–281]. Thus, by variation of the substrate structure (most easily performed by variation of protective groups of different size and/or polarity) an improved fit of the substrate may be achieved, leading to an enhanced selectivity of the enzyme.

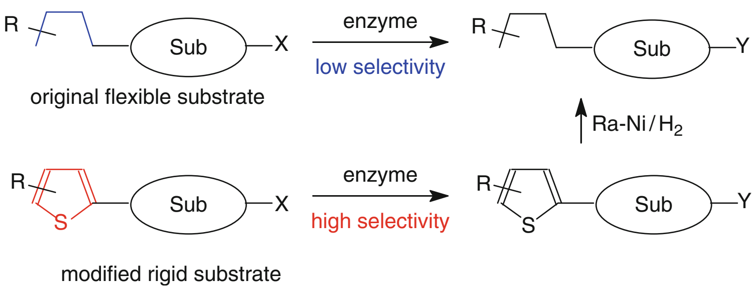
Scheme 2.44 shows the optimization of a PLE-catalyzed desymmetrization of dimethyl-3-aminoglutarate esters using the ‘substrate engineering’ approach [216]. By varying the *N*-protecting group in size and polarity, the optical purity of the monoester could be significantly enhanced as compared to the unprotected original substrate. In addition, a remarkable reversal in stereochemistry was achieved upon the stepwise increase of the size of the protective group X, which provides an elegant method for controlling the configuration of the product.



X	Configuration	e.e. [%]
H	*R*	41
CH_3–CO–	*R*	93
CH_2=CH–CO–	*R*	8
C_2H_5–CO–	*R*	6
n-C_4H_9–CO–	*S*	2
$(CH_3)_2$CH–CO–	*S*	54
c-C_6H_{11}–CO–	*S*	79
$(CH_3)_3$C–CO–	*S*	93
Ph–CH_2–O–CO–	*S*	93
(*E*)-CH_3–CH=CH–CO–	*S*	>97

Scheme 2.44 Optimization of porcine liver esterase-catalyzed hydrolysis by substrate modification

An interesting approach to substrate modification is based on the observation that enzyme selectivities are often enhanced with rigid substrate structures bearing π-electrons (Scheme 2.45). Thus, when a highly flexible aliphatic C-4 within a substrate (Sub) is not well recognized, it can be ‘chemically hidden’ in the corresponding thiophene derivative, which is often transformed more selectively. Then, the enantioenriched (hetero)aromatic product is desulfurized by catalytic hydrogenation using Raney-Ni to yield the saturated desired product in high e.e. [282, 283].



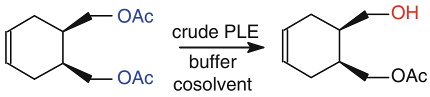
Scheme 2.45 Optimization of selectivity via introduction of a rigid thiophene unit

Medium Engineering. Variation of the aqueous solvent system by the addition of water-miscible organic cosolvents such as methanol, *tert*-butanol, acetone, dioxane, acetonitrile, dimethyl formamide (DMF), and dimethyl sulfoxide (DMSO) is a promising and quite frequently used method to improve the selectivity of hydrolytic enzymes, in particular with esterases (Scheme 2.45) [284–286]. Depending on the stability of the enzyme, the concentration of cosolvent may vary from ~10 to 50% of the total volume. At higher concentrations, however, enzyme deactivation is unavoidable. Most of these studies have been performed with PLE, for which a significant selectivity enhancement has often been obtained, especially by addition of dimethyl sulfoxide or low-molecular-weight alcohols such as methanol and *tert*-butanol. However, the price one usually has to pay on addition of water-miscible organic cosolvents to the aqueous reaction medium is a depletion in the reaction rate. The mechanistic action of such modified solvent systems on an enzyme is only poorly understood and predictions on the outcome of such a medium engineering cannot be made; however, the selectivity-enhancing effects are often dramatic.

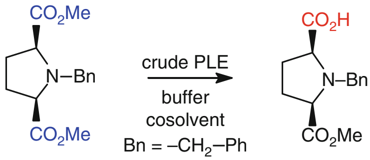
The selectivity enhancement of PLE-mediated hydrolyses upon the addition of methanol, *tert*-butanol, and dimethyl sulfoxide to the reaction medium is exemplified in Scheme 2.46. The optical purities of products were in a range of ~20–50% when a pure aqueous buffer system was used, but the addition of methanol and/or DMSO led to a significant improvement [287].

The ‘enantioselective inhibition’ of enzymes by the addition of chiral amines functioning as noncompetitive inhibitors has been reported for lipases [288]. This phenomenon is discussed in Sect. 2.1.3.2. For the selectivity enhancement of dehydrogenase reactions via addition of enzyme inhibitors see Sect. 2.2.3.

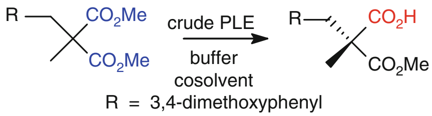
Variation of pH. Reactions catalyzed by hydrolases are usually performed in aqueous buffer systems with a pH closer to that of the pH optimum of the enzyme. Because the conformation of an enzyme depends on its ionization state (among others), it is reasonable to assume that a variation of the pH and the type of buffer may influence the selectivity of a given reaction. Such variations are facilitated by the fact that the pH activity profile of the more commonly used hydrolytic enzymes is rather broad and thus allows pH variations while maintaining an adequately high



Organic Cosolvent	Relative Rate [%]	e.e. [%]
None	100	55
DMSO (20 %)	70	59
DMSO (40 %)	28	72
DMF (20 %)	35	84
t-BuOH (5 %)	70	94
t-BuOH (10 %)	44	96



Organic Cosolvent	e.e. [%]
None	17
MeOH (10 %)	>97
DMSO (25 %)	>97



Organic Cosolvent	e.e. [%]
None	25
DMSO (50 %)	93

Scheme 2.46 Selectivity enhancement of porcine liver esterase by addition of organic cosolvents

activity. Interestingly, the effect of pH and buffer types on the selectivity of hydrolytic enzymes have been investigated to a lesser extent [289–292].

Variation of Temperature. Enzymes, like other catalysts, generally are considered to exhibit their highest selectivity at low temperatures – as supported by several experimental observations, not only with hydrolases [293] but also with dehydrogenases. It was only recently, however, that a rational understanding of temperature effects on enzyme stereoselectivity was proposed [294, 295]. It is based on the so-called 'racemic temperature' (T_{rac}) at which a given enzymatic reaction will proceed without stereochemical discrimination due to the fact that the activation energy is the same for both stereochemical forms participating in the reaction. Hence, there is no difference in free energy ($\Delta\Delta G^{\neq} = 0$, see Figs. 1.6 and 1.7).

$$\Delta\Delta G^{\neq} = \Delta\Delta H^{\neq} - T \cdot \Delta\Delta S^{\neq} \qquad T_{rac} = \text{'Racemic Temperature'}$$

$$\text{If } \Delta\Delta G^{\neq} = 0 \quad \text{then} \quad T = T_{rac} = \frac{\Delta\Delta H^{\neq}}{\Delta\Delta S^{\neq}}$$

From the Gibb's equation given above it follows that only the entropy term $\Delta\Delta S^{\neq}$ (but not the enthalpy $\Delta\Delta H^{\neq}$) is influenced by the temperature. Thus, the selectivity of an enzymatic reaction depends on the temperature as follows:

- At temperatures less than T_{rac} the contribution of entropy is minimal and the stereochemical outcome of the reaction is mainly dominated by the activation enthalpy difference ($\Delta\Delta H^{\neq}$). The optical purity of product(s) will thus *decrease* with *increasing* temperature.

- On the other hand, at temperatures greater than T_{rac}, the reaction is controlled mainly by the activation entropy difference ($\Delta\Delta S^{\neq}$) and enthalpy plays a minor role. Therefore, the optical purity of product(s) will *increase* with *increasing* trtemperature.

However, the major product obtained at a given temperature $T > T_{rac}$ will be the antipode to that at $T < T_{rac}$, thus a temperature-dependent *reversal* of stereochemistry is predicted. The validity of this rationale has been proven with the asymmetric reduction of ketones using a dehydrogenase from *Thermoanaerobium brockii* [296] (Sect. 2.2.2). In contrast to the above-mentioned dehydrogenases from thermophilic organisms, the majority of hydrolases used for biotransformations possess more restricted operational limits with respect to heating, thus narrowing the possibility of a significant selectivity enhancement by variation of temperature of the reaction. From the data available, it can be seen that upon lowering the temperature both an increase [297] or a decrease in the selectivity of hydrolase reactions may be observed [298]. The outcome of the latter experiments depends on whether the reaction has been performed above or below the racemic temperature of the enzyme used. The comparable low upper temperature of about 50°C for the majority of enzymes represents a serious limitation, while impressive effects have been observed upon cooling (–20 to –60°C) [299–301].

More recently, the application of microwave (MW) irradiation for the enhancement of reaction rates of organic-chemical reactions has become fashionable [302].[12] While conventional heating is due to polychromatic infrared radiation, microwaves are generated in a monochromatic manner. The benefit of MW heating has been proven in numerous types of organic reactions, but the detailed mechanistic principles of molecular action are still poorly understood and there is still much debate about the so-called hot-spot theory [303, 304]. However, for enzyme-catalyzed reactions, MW heating has been shown to be superior to conventional heating by leading to reduced enzyme deactivation and enhanced selectivities [305–307].

Monitoring of the reaction, bearing in mind the underlying kinetics (Sect. 2.1.1) can be very helpful, but only if the reaction is of a type where the extent of conversion of the reaction affects the optical purity of substrate and/or product. Re-esterification of the enantiomerically enriched (but not yet optically pure) product and subjecting the material to a repeated (second) enzymatic hydrolysis is certainly a tedious process but it may be a viable option.

Enzyme Engineering. Molecular biology has enabled the redesign of enzymes possessing improved performance in terms of enhanced stability at extreme temperatures and pH, and at high concentrations of reactants and organic (co)solvents, which is crucial for the construction of process-stable proteins for biotechnological applications. In addition to improved stability, enzymes also can be engineered for

[12] By definition, the range of microwave irradiation extends from 1 to 300 GHz; however, due to the resonance frequency of water (19.5 GHz), most of the applications are close to the latter range, i.e., 0.9 and 2.45 GHz.

enhanced (stereo)selectivities, which represents an equivalent to ligand tuning of homogeneous catalysts. Only some key issues are discussed below since this area requires special expertise in molecular biology – not necessarily a core specialty of synthetic organic chemists. For a deeper understanding, excellent introductory chapters can be found in recent books and reviews [308–312].

There are two distinct philosophies to enzyme engineering:

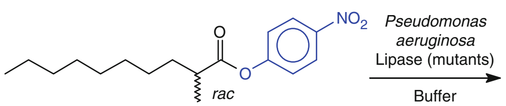
1. *Rational protein design* requires detailed knowledge of the three-dimensional structure of an enzyme, preferably from its high-resolution crystal structure or NMR measurements [313]. Alternatively, a computer-generated homology model may help, if the sequence identity is high enough.[13] In a first step, docking of the substrate to the active site allows to identify amino acid residues, which appear to interact closely with the structural features of the substrate during binding. Steric incompatibilities, such as collisional interference between residues, insufficient substrate binding in large pockets, or nonmatching polarities between hydrogen bonds or salt bridges can be identified and proposals for the replacement of (usually only few) amino acids can be made. The corresponding mutants are generated and tested for their catalytic properties. Sometimes, this rational approach yields impressive results, but (more often) mutant enzymes tell us that the rational analysis of the substrate binding based on a *static* (crystal) structure is insufficient to explain the *dynamic* process of protein (re)folding upon formation of the enzyme–substrate complex, which is a prerequisite for the successful *dynamics* of catalysis [314]. In addition, the tempting notion that mutations close to the active site are always better than distant ones is only a single aspect of a more complex story [315].
2. *Directed evolution* requires the availability of the gene(s) encoding the enzyme of interest, a suitable (microbial) expression system, a method to create mutant libraries, and an effective selection system – while structural information is irrelevant here. Mutant libraries are widely available, but the crucial aspect lies in the selection problem [316]: in order to identify the one (or the few) mutant protein(s) with improved properties amongst the vast number of variants (typically 10^4–10^6), which are (more or less) randomly generated,[14] an efficient screening method is required to find the tiny needle in the very big haystack. Adequate screening methods usually rely on spectral changes during catalysis. The drawback of this first-generation screening method is the requirement for a chromogenic or fluorogenic 'reporter group' in the substrate, which usually consists of a large (hetero)aromatic moiety which needs (at least) 10–14

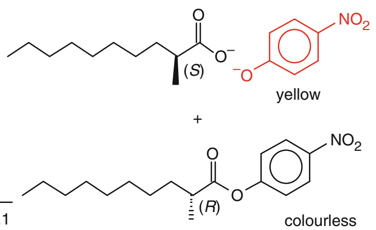
[13] A sequence identity of 70% translates into a reasonably well-defined model showing a root mean square deviation of 1–2 Å, which drops to a low value of 2–4 Å for proteins having only 25% identity.

[14] The possible number of mutants generated from a typical protein possessing 200 amino acids exponentially increases by the number of mutations: 3,800 variants are possible for a single mutation, 7,183,900 exist for 2 mutations, and 8,429,807,368,950 are possible for only 4 mutations.

π-electrons to be 'visible' by UV/VIS or fluorescence spectroscopy. Classic reporter groups are (colorless) *p*-nitrophenyl derivatives, liberating the (yellow) *p*-nitrophenolate anion upon enzyme catalysis, which in turn can be spectrophotometrically monitored at 410 nm (Scheme 2.47). Unfortunately, the original substrate (e.g., a methyl ester) is modified to a structurally very different *p*-nitrophenyl substrate ester analog. Since the mutants are screened for optimal activity/selectivity on the surrogate substrate, their performance with the 'real' (methyl ester) substrate will be less efficient. In order to create 'real' mutant enzymes for 'real' substrates, more sophisticated screening methods are recommended based either on a multienzyme assay for acetate (produced during ester hydrolysis, Scheme 2.47) [317], MS analysis of (deuterated) 'pseudo-enantiomeric' products, or time-resolved IR thermogravimetry [318–322]. After all, you always get what you screen for.

Scheme 2.47 shows the use of a surrogate ester substrate bearing a chromogenic (*p*-nitrophenyl) reporter group for the screening of *Pseudomonas aeruginosa* lipase mutants possessing improved enantioselectivities for the hydrolysis of an α-chiral long-chain fatty acid [323]. Pure substrate enantiomers were separately tested in 96-well microtiter plates using a plate reader for the readout of enantioselectivity. After four rounds of error-prone polymerase chain reaction (epPCR) at a low mutation rate (ensuring an average amino acid exchange rate of one per enzyme molecule), mutant A ($E = 11$) was obtained. The latter was improved via saturation mutagenesis through variation of all remaining 19 amino acids at position #155 yielding mutant B ($E = 20$). Another round of epPCR gave mutant C ($E = 25$), which could not be further improved. At high mutations rates, epPRC of the wild-type enzyme gave only slightly improved variants D and E ($E = 3.0$ and 6.5, respectively), which again could not be further improved. However, combinatorial multiple cassette mutagenesis (CMCM) of the wild-type enzyme in the 'hot region'



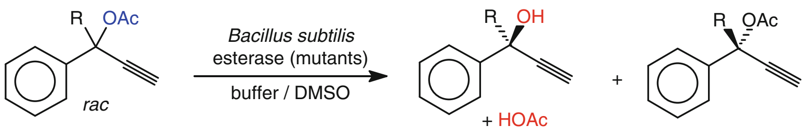


Lipase (mutant)	Method	*E*
wild type	—	1.1
mutant A	low rate epPCR (4x) at wild type	11
mutant B	saturation mutagenesis at #155 of mutant A	20
mutant C	low rate epPCR at mutant B	25
mutant D	high rate epPCR at wild type	3.0
mutant E	high rate epPCR at wild type	6.5
mutant G	CMCM #1160-163 at wild type	30
mutant J	CMCM with mutants D & E	>51

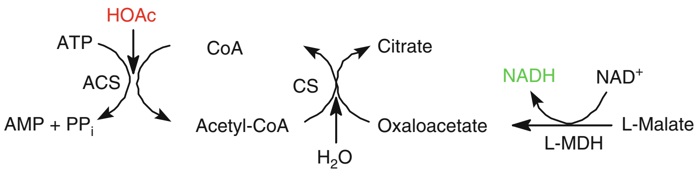
epPCR = error-prone polymerase chain reaction
CMCM = combinatorial multiple-cassette mutagenesis

Scheme 2.47 Screening for *Pseudomonas aeruginosa* lipase mutants showing enhanced enantioselectivities using a chromogenic surrogate substrate of amino acids 160–163 gave mutant G ($E = 30$). The latter could be further improved by DNA-shuffling with mutant genes D and E to finally yield mutant J, which exhibited a top value of $E > 51$.

An example for the successful generation of highly enantioselective esterase mutants capable of hydrolyzing acetate esters of *tert*-alcohols is shown in Scheme 2.48 [324, 325]. In order to improve the modest enantioselectivity of wild-type *Bacillus subtilis* esterase ($E = 5$ and 43), a library of ca. 5,000 mutants was constructed, which encompassed 2,800 active variants. Among the latter, the G105A and E188D mutants showed significantly enhanced enantioselectivities for both substrates ($E > 100$). An E188W/M193C double mutant even showed inverted enantiopreference ($E = 64$) for the trifluoromethyl substrate [326]. In order to avoid the undesired modification of the substrate by a chromogenic reporter group, a second-generation screening method was employed based on a commercial test kit: Thus, the acetate formed during ester hydrolysis was activated into acetyl-CoA catalyzed by acetyl-CoA synthase (at the expense of ATP). In a subsequent step, the acetate unit is transferred from acetyl-CoA onto oxaloacetate yielding citrate (catalyzed by citrate synthase). The oxaloacetate required for this reaction is formed by oxidation of L-malate (catalyzed by L-malate dehydrogenase) under consumption of NAD^+ yielding an equimolar amount of NADH, which can be spectrophotometrically monitored at 340 nm [327].



R	Enantioselectivity (E)		
	wild type	G105A	E188D
CH_3	5	56	45
CF_3	43	>100	>100



ACS = Acetyl CoA synthase; CS = Citrate synthase; L-MDH = L-Malate dehydrogenase
ATP = adenosine 5′-trimonophosphate; AMP = adenosine 5′-monophosphate; PP_i = inorganic diphosphate

Scheme 2.48 Enantioselectivities of wild-type *Bacillus subtilis* esterase and mutants acting on *tert*-alcohol esters using a multienzyme acetate assay

Model Concepts

Useful 'models' for the more commonly used enzymes have been developed to avoid trial-and-error modifications of substrate structures and to provide suitable tools for predicting the stereochemical outcome of enzymatic reactions on nonnatural substrates. These models should be able to provide the means to 'redesign' a substrate, when initial results are not satisfying with respect to reaction rate and/or selectivity. Since the application of such 'models' holds a couple of potential pitfalls, the most important principles underlying their construction are discussed here.

Molecular Modeling. The three-dimensional 'map' of the active site of an enzyme can be accurately determined by X-ray crystallography [328–330] or by NMR spectroscopy (provided that the enzyme is not too large). Since the tertiary structure of most enzymes is regarded as being closely related to the preferred form in a dissolved state [331], these methods allow the most accurate description of the structure of the enzyme. However, X-ray data for the prediction of the stereochemical outcome of an enzyme-catalyzed reaction can only represent a *static* protein structure. On the other hand, the chiral recognition process during formation of the enzyme–substrate complex is a highly complex *dynamic* process. Thus, any attempt of predicting the selectivity of an enzymatic reaction based on X-ray data is comparable to explaining the complex movements in a somersault from a single photographic snapshot.

Unfortunately, X-ray structures are available only for a few enzymes, such as α-chymotrypsin [116], subtilisin [181], and a number of lipases from *Mucor* spp. [9], *Geotrichum candidum* [332], *Candida rugosa* (formerly *cylindracea*) [333], *Candida antarctica* B [334], and *Pseudomonas glumae* [335] – while for a large number of synthetically useful enzymes such as pig liver esterase, relevant structural data are not available.

If the amino acid sequence of an enzyme is known either entirely or even in part, computer-assisted calculations can provide estimated three-dimensional structures of enzymes [336]. This is done by comparing the known parts of the enzyme in question with other enzymes whose amino acid sequence and three-dimensional structures are already known. Depending on the percentage of the homology, i.e., 'overlap', of the amino acid sequences, the results are more or less certain. In general, an overlap of about ~60% is sufficient for good results; less is considered too inaccurate.

Provided that the three-dimensional structure of an enzyme is available, several methods for predicting the selectivity and its stereochemical preference are possible:

- The enzyme–substrate complex is constructed in its transition state for both enantiomers and the energy value for both (diastereomeric) conformations within the active site of the enzyme are calculated via molecular dynamics (MD). The difference in free energy ($\Delta\Delta G^{\neq}$) – obtained via force field calculations – yields semiquantitative results for the expected selectivity [337].
- The difference in steric interactions during a (computer-generated) approach of two substrate enantiomers towards an acyl-enzyme intermediate can be used instead [338].
- If the transition state is not known with some certainty, the substrate can be electronically fitted into the active site of the enzyme. The orientation of

substrate enantiomers with respect to the chemical operator of the enzyme as well as possible substrate movements can be analyzed via MD [339]. This is achieved via (computer-generated) 'heating' of the substrate within the enzyme, followed by a slow electronic 'cooling process', which allows the substrate enantiomers to settle in their position representing the lowest energy minimum. Because selectivities are determined by differences in free energy of transition states, the first approach leads to the most accurate results.

Substrate Model. If neither X-ray data nor the amino acid sequence are available for an enzyme – which is unfortunately the case for many synthetically useful enzymes – one can proceed as follows: A set of artificial substrates having a broad variety of structures is subjected to an enzymatic reaction. The results, i.e., the reaction rates and enantioselectivities, then allow one to create a general structure of an imagined 'ideal' substrate, which an actual substrate structure should simulate as closely as possible to ensure rapid acceptance by the enzyme and a high enantioselection (Fig. 2.10). This idealized substrate structure is then called a 'substrate model'. Such models are quite popular; inter alia they have been developed for PLE [224] and *Candida rugosa* lipase [340, 341].

To ensure optimal selectivity by PLE, the α- and β-substituents of methyl carboxylates should be assigned according to their size (L = large, M = medium and S = small) with the preferably-accepted enantiomer being shown in Fig. 2.10.

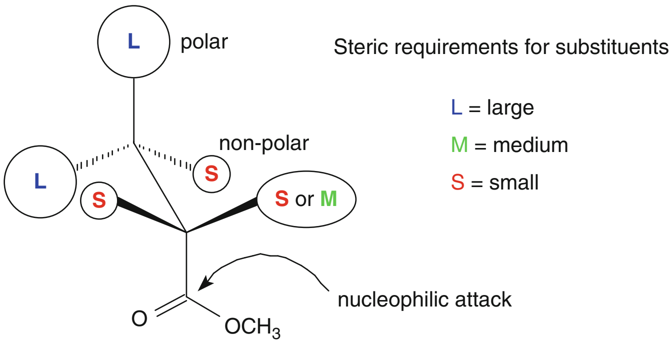


Fig. 2.10 Substrate model for porcine liver esterase

Active Site Model. Instead of developing an ideal *substrate structure* one also can try to picture the structure of the (unknown) *active site* of the enzyme by the method described above. Thus, substrates of varying size and polarity are used as probes to measure the dimensions of the active site. Therefore this approach has been denoted as 'substrate mapping' [342, 343]. Such *active site models* are frequently employed and they usually resemble an arrangement of assumed 'sites' or 'pockets' which are usually box- or cave-shaped. A relatively reliable active-site model for PLE [344]

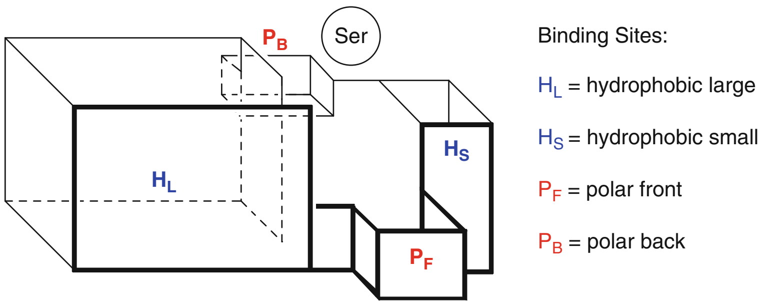


Fig. 2.11 Active-site model for porcine liver esterase

using cubic-space descriptors was based on the evaluation of the results obtained from over 100 substrates (Fig. 2.11).

The boundaries of the model represent the space available for the accommodation of the substrate. The important binding regions which determine the selectivity of the reaction are two hydrophobic pockets (H_L and H_S, with L = large and S = small) and two pockets of more polar character (P_F and P_B, with F = front and B = back). The best fit of a substrate is determined by positioning the ester group to be hydrolyzed close to the hydrolytically active serine residue and then arranging the remaining moieties in the H and P pockets.

Of course these crude models only yield reliable predictions if they are based on a substantial number of test substrates.

2.1.3.2 Lipases

Lipases are enzymes which hydrolyze triglycerides into fatty acids and glycerol [345, 346]. Apart from their biological significance, they play an important role in biotechnology, not only for food and oil processing [347–349] but also for the preparation of chiral intermediates [350, 351]. In fact, about 40% of all biotransformations reported to date have been performed with lipases. Thus, lipases constitute probably the most thoroughly investigated group of enzymes – to date numerous lipases have been cloned and more than a dozen crystal structures are available. Although they can hydrolyze and form carboxylic ester bonds like proteases and esterases, their molecular mechanism is different, which gives rise to some unique properties [352, 353].

The most important difference between lipases and esterases is the physico-chemical interaction with their substrates. In contrast to esterases, which show a 'normal' Michaelis-Menten activity depending on the substrate concentration [S] (i.e., a higher [S] leads to an increase in activity), lipases display almost no activity as long as the substrate is in a dissolved monomeric state (Fig. 2.12). However, when the substrate concentration is gradually enhanced beyond its solubility limit by forming a second (lipophilic) phase, a sharp increase in lipase activity takes

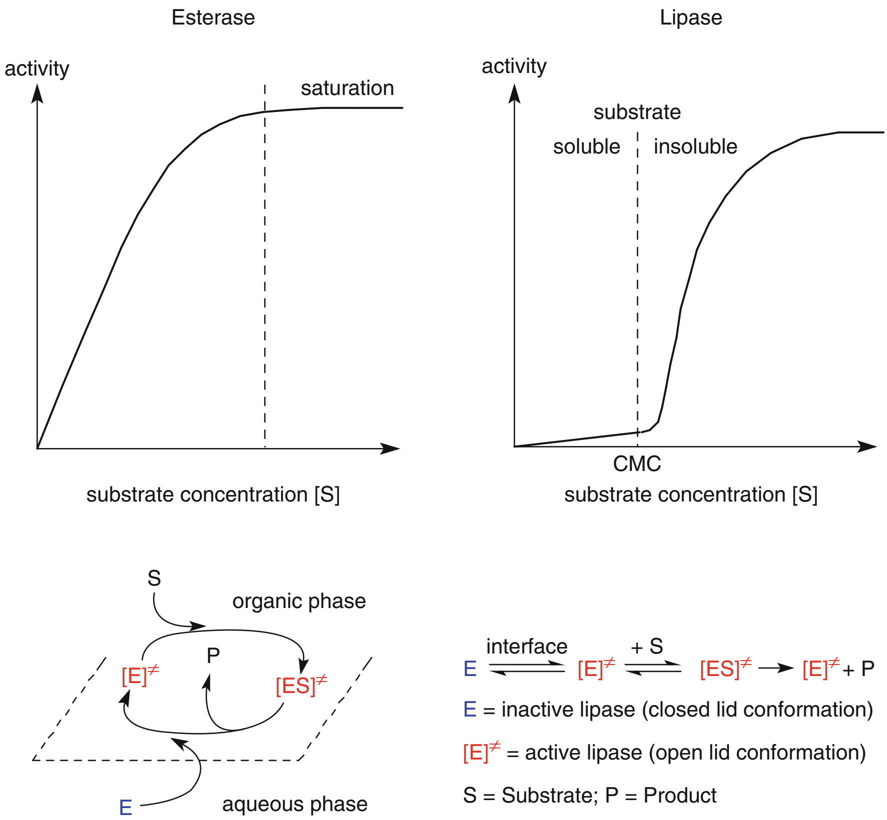


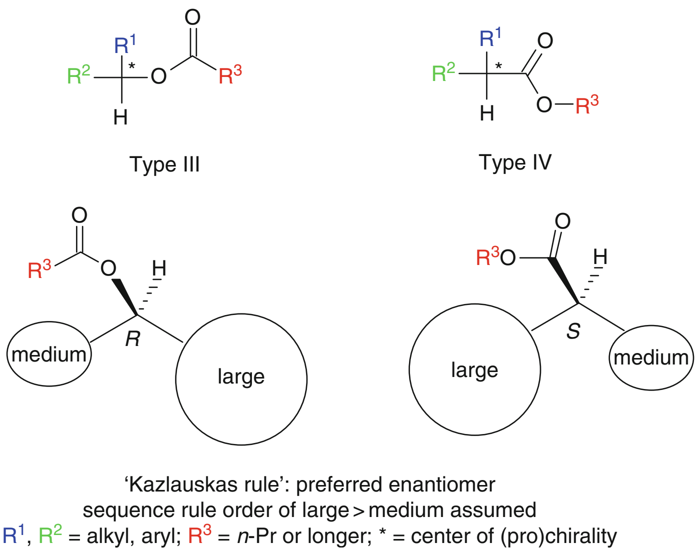
Fig. 2.12 Esterase and lipase kinetics

place [354, 355]. The fact that lipases do not hydrolyze substrates under a critical concentration (the 'critical micellar concentration', CMC), but display a high activity beyond it, has been called the 'interfacial activation' [356].

The molecular rationale for this phenomenon has been shown to be a rearrangement process within the enzyme [329]. A freely dissolved lipase in the absence of an aqueous/lipid interface resides in its inactive state [Enz], because a part of the enzyme molecule covers the active site. When the enzyme contacts the interface of a biphasic water-oil system, a short α-helix – the 'lid' – is folded back. Thus, by opening its active site the lipase is rearranged into its active state [Enz]$^{\neq}$.

Lipase-catalyzed hydrolyses thus should be conducted in a biphasic medium. It is sufficient to employ the substrate alone at elevated concentrations, such that it constitutes the second organic phase, or, alternatively, it may be dissolved in a water-immiscible organic solvent such as hexane, a dialkyl ether, or an aromatic solvent. Due to the presence of an interface, physical parameters influencing the mass-transfer of substrate and product between the aqueous and organic phase such as stirring or shaking speed have a marked influence on the reaction rate of lipases. Triacylglycerols such as triolein or -butyrin are used as standard substrates for the determination of lipase activity, whereas for esterases *p*-nitrophenyl acetate is the classic standard. For the above-mentioned reasons it is clear that the addition of water-immiscible organic solvents to lipase-catalyzed reactions is a useful technique to improve catalytic activities. In contrast, water-soluble organic cosolvents are more often used in conjunction with esterases, which operate in a 'true' solution.

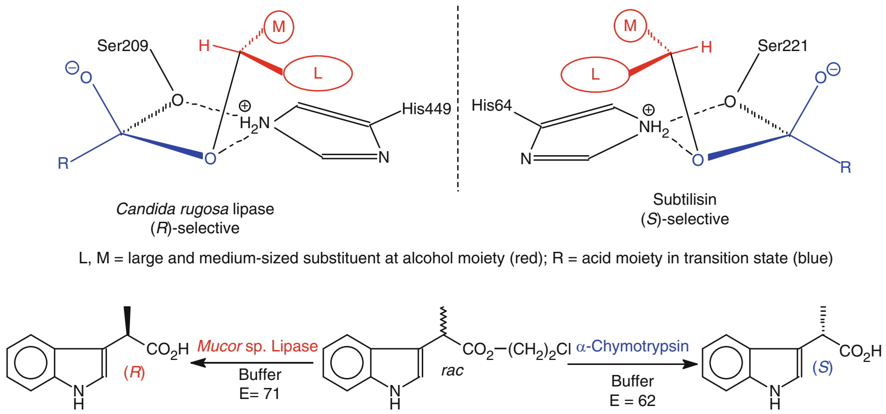
The fact that many lipases have the ability to hydrolyze esters other than glycerides makes them particularly useful for organic synthesis [357]. Furthermore, some lipases are also able to accept thioesters [358, 359]. In contrast to esterases, lipases have been used for the resolution of racemates much more than for effecting the '*meso*-trick'. Since the natural substrates are esters of the chiral alcohol, glycerol, with an achiral acid, it may be expected that lipases are most useful for hydrolyzing esters of chiral alcohols rather than esters of chiral acids. Although this expectation is true for the majority of substrates (see substrate type III, Scheme 2.49), a minor fraction of lipases also display high selectivity through recognizing the chirality of an acid moiety (substrate type IV).



Scheme 2.49 Substrate types for lipases

Some of the general rules for substrate-construction are the same as those for esterase-substrates (Scheme 2.21), such as the preferred close location of the chirality center and the necessity of having a hydrogen atom on the carbon atom bearing the chiral or prochiral center. However, other features are different:

- The acid moiety R^3 of lipase-substrate of type III should be of a straight-chain nature possessing at least three to four carbon units to ensure a high lipophilicity of the substrate. Despite that long-chain fatty acids such as oleates would be advantageous for a fast reaction rate, they do cause operational problems such as a high boiling point of the substrate and they tend to form foams and emulsions during extractive work-up. As a compromise between the two extremes – short chains for ease of handling and long ones for a high reaction rate – *n*-butanoates are often the esters of choice.
- Furthermore, the majority of lipases show the same stereochemical preference for esters of secondary alcohols (Scheme 2.49), which is known as the 'Kazlauskas' rule' [341]. Assuming that the Sequence Rule order of substituents R^1 and R^2 is large > medium, the preferably accepted enantiomer lipase-substrate of type III possesses the (*R*)-configuration at the alcoholic center. It should be noted that the Kazlauskas' rule for secondary alcohols (Type III) has an accuracy of ≥90%, whereas the predictability for the corresponding α-chiral acids (Type IV) is less reliable.
- Many proteases (such as α-chymotrypsin and subtilisin) and pig liver esterase exhibit a stereochemical preference opposite to that of lipases. This is because the catalytic triad of lipases and proteases – where the X-ray structure is known – has been found to be arranged in a mirror-image orientation [360]. Thus, the stereochemical outcome of an asymmetric hydrolysis can often be directed by choosing a hydrolase from a different class [361–364]. Scheme 2.50 depicts the quasi-enantiomeric oxy-anion transition-state intermediates during hydrolysis of a *sec*-alcohol ester catalyzed by *Candida rugosa* lipase (PDB entry 1crl) and the protease subtilisin (PDB entry 1sbn). While the nucleophilic Ser-residues approach from the back, both His are located at the inside, with the oxy-anions pointing outside. Both active sites have limited space for one of the large and



Scheme 2.50 Mirror-image orientation of the catalytic machinery of *Candida rugosa* lipase and the protease subtilisin and enantiocomplementary ester hydrolysis using *Mucor* sp. lipase and α-chymotrypsin

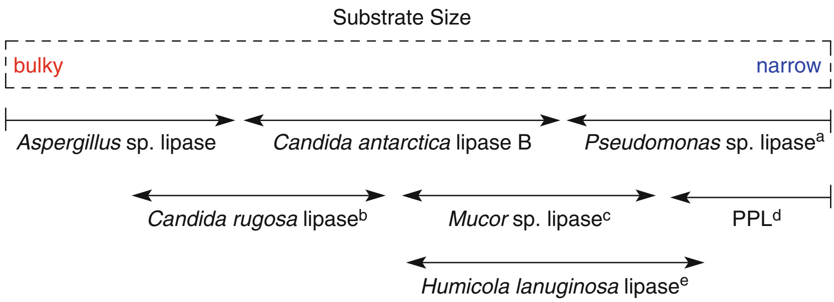
medium-sized substituents of the *sec*-alcohol moiety (red), but the mirror-image orientation of the catalytic center favors opposite enantiomers, which is exemplified by the hydrolytic kinetic resolution of an α-chiral indolpropionic ester using the (*R*)-selective *Mucor* sp. lipase and the (*S*)-selective protease α-chymotrypsin (Scheme 2.50) [363]. The activated 2-chloroethyl ester was used to ensure enhanced reaction rates.

- Substrate-type IV represents the general structure of a smaller number of esters which were hydrolyzed by lipases. When using lipases for type-IV substrates, the alcohol moiety R^3 should preferentially consist of a long straight-chain alcohol such as *n*-butanol. For esters of type IV the stereochemical preference is often (*S*) (Scheme 2.49) but the predictability is less accurate than with type-III substrates [343].

A large variety of different lipases are produced by bacteria or fungi and are excreted as extracellular enzymes, which makes their large-scale production particularly easy.[15] The majority of these enzymes are created by the organisms in two isoforms (isoenzymes), usually denoted as A and B. Both are closely related and usually show the same enantiopreference, but slight structural differences do exist, leading to certain differences in enantioselectivity. Crude technical-grade lipase preparations usually contain both isoforms; the only notable exception is *Candida antarctica* lipase, for which both pure isoforms A and B have been made available through genetic engineering. In contrast to esterases, only a minor fraction of lipases are isolated from mammalian sources such as porcine pancreas. Since some lipases from the same genus (for instance, from *Candida* or *Pseudomonas* sp.) are supplied by different commercial sources, one should be aware of differences in selectivity and activity among the different preparations, while these are usually not in the range of orders of magnitude [365]. The actual enzyme content of commercial lipase preparations may vary significantly, from less than 1% up to ~70% – and the selectivity of a lipase preparation from the same microbial source does not necessarily increase with its price! Among the ever increasing number of commercially available lipases only those which have been shown to be of a general applicability are discussed below. For a list of the more commonly used lipases see the Appendix (Chap. 5).

As a rule of thumb, the most widely used lipases may be characterized according to the steric requirements of their preferred substrate esters (Fig. 2.13). Whereas *Aspergillus* sp. lipases are capable of accepting relatively bulky substrates and therefore exhibit low selectivities on 'narrow' ones, *Candida* sp. lipases are more versatile in this regard. Both the *Pseudomonas* and *Mucor* sp. lipases have been found to be highly selective on substrates with limited steric requirements and hence are often unable to accept bulky compounds. Thus, substrates which are recognized with moderate selectivities by a *Candida* lipase, are usually more

[15]It seems to be a common phenomenon that microbiologists keep reclassifying microbial species every once in a while. Whether this is to confuse organic chemists, or for other reasons, is often not clear. However, neither the microorganism nor the lipase are changed by a new name.



[a]Lipases from *Pseudomonas cepacia* (syn. *Burkholderia cepacia*), *P. fluorescens*, *P. fragi*, *Chromobacterium viscosum* (syn. *Pseudomonas glumae* or *Burkholderia glumae*); [b]syn. *C. cylindracea*; [c]*Mucor miehei* (syn. *Rhizomucor miehei*), *M. javanicus* (syn. *Rh. javanicus*); [d]pure porcine pancreatic lipase; [e]identical to *Thermomyces lanuginosus*.

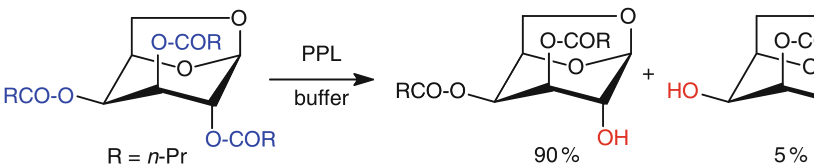
Fig. 2.13 Steric requirements of lipases.

selectively hydrolyzed by a *Pseudomonas* type. Porcine pancreatic lipase represents a crude mixture of different hydrolytic enzymes and is therefore difficult to predict. However, pure PPL prefers slim substrates.

Porcine Pancreatic Lipase

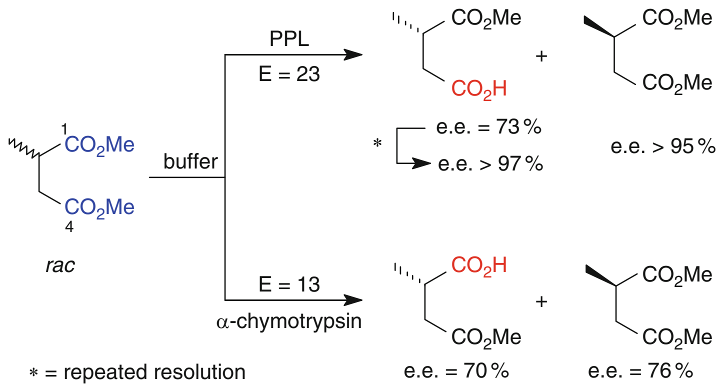
The cheapest and hence one of the most widely used lipases is isolated from porcine pancreas (PPL) [366–368]. The crude preparations contain a significant number of other hydrolases besides the 'true PPL'. The crude preparation mostly used for biotransformations is called 'pancreatin' or 'steapsin' and contains less than 5% protein. The main hydrolase impurities are α-chymotrypsin, cholesterol esterase, carboxypeptidase B, phospholipases, and other unknown hydrolases. Phospholipases can usually be neglected as undesired hydrolase impurities, because they prefer negatively charged substrate esters which mimic their natural substrates – phospholipids [369, 370]. On the other hand, α-chymotrypsin and cholesterol esterase can be serious competitors in ester hydrolysis. Both of the latter proteins – and also other unknown hydrolases – can impair the selectivity of a desired PPL-catalyzed ester hydrolysis by exhibiting a reaction of lower selectivity (or even of opposite stereochemistry). Thus, any models for PPL should be applied with great caution [371, 372]. Indeed, in some cases these hydrolase impurities have been shown to be responsible for the highly selective transformation of substrates which were not accepted by purified 'true PPL' (the latter being available at a high price in partially purified form). Cholesterol esterase and α-chymotrypsin are likely to act on esters of primary and secondary alcohols, whereas 'true PPL' is a highly selective catalyst for esters of primary alcohols. Despite the possible interference of different competing hydrolytic enzymes, numerous highly selective applications have been reported with crude PPL [373–375]. Unless otherwise stated, all of the examples shown below have been performed with steapsin.

Regioselective reactions are particularly important in the synthesis of biologically interesting carbohydrates, where selective protection and deprotection of hydroxyl groups is a central problem. Selective removal of acyl groups of peracylated carbohydrates from the anomeric center [376] or from primary hydroxyl groups [377, 378], leaving the secondary acyl groups intact, can be achieved with hydrolytic enzymes or chemical methods, but the regioselective discrimination between secondary acyl groups is a complicated task [379]. PPL can selectively hydrolyze the butanoate ester on position 2 of the 1,6-anhydro-2,3,4-tri-*O*-butanoyl-galactopyranose derivative shown in Scheme 2.51 [380]. Only a minor fraction of the 2,4-deacylated product was formed.



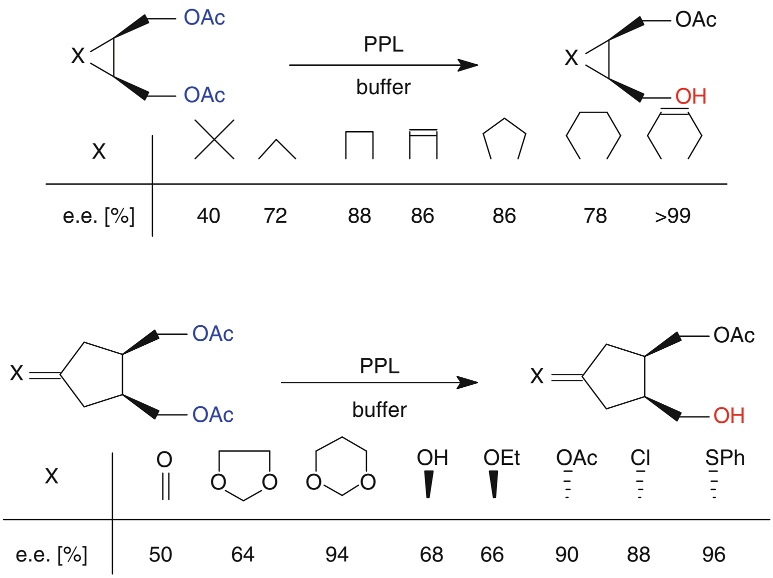
Scheme 2.51 Regioselective hydrolysis of carbohydrate esters by porcine panceatic lipase

A simultaneous regio- and enantioselective hydrolysis of dimethyl 2-methylsuccinate has been reported with PPL [381] with a preference for the (*S*)-ester and with the hydrolysis taking place at position 4 (Scheme 2.52). The residual unhydrolyzed ester was obtained with >95% e.e. but the monoacid formed (73% e.e.) had to be re-esterified and subjected to a second hydrolytic step in order to be obtained in an optically pure form. It is interesting to note that α-chymotrypsin exhibited the same enantio- but the opposite regioselectivity on this substrate, preferably hydrolyzing the ester at position 1 [382].



Scheme 2.52 Regio- and enantioselective hydrolysis of dimethyl α-methylsuccinate

The asymmetric hydrolysis of cyclic *meso*-diacetates by PPL proved to be complementary to the PLE-catalyzed hydrolysis of the corresponding *meso*-1,2-dicarboxylates (compare Schemes 2.29 and 2.53). The cyclopentane derivative,



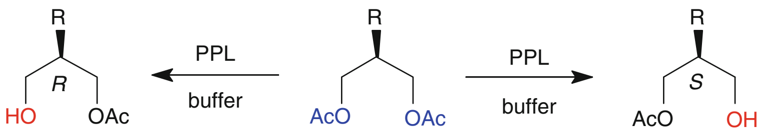
Scheme 2.53 Asymmetric hydrolysis of cyclic *meso*-diacetates by porcine pancreatic lipase

which gave low e.e. using the PLE method, was now obtained with 86% e.e. [31, 383]. This selectivity was later improved by substrate modification of the cyclopentane moiety [384], giving access to a number of chiral cyclopentanoid building blocks used for the synthesis of carbacyclic prostaglandin I_2 derivatives (therapeutic agents for the treatment of thrombotic diseases).

Chiral glycerols, optically active C_3-synthons, were obtained by asymmetric hydrolysis of prochiral 1,3-propanediol diesters using PPL (Scheme 2.54) [385]. A remarkable influence of a π-system located on substituents at position 2 on the optical purity of the products indicate that the selectivity of an enzyme does not depend on steric factors alone, but also on electronic issues [278, 281, 386]. Note that a rigid (*E*)-C=C bond or a bulky aromatic system [387] on the 2-substituent led to an enhanced selectivity of the enzyme as compared to the corresponding saturated analogs. When the configuration of the double bond was *Z*, a reversal in the stereochemical preference took place, associated with an overall loss of selectivity. Additionally, this study shows a positive influence of a biphasic system (using di-*iso*-propyl ether or toluene [388] as water-immiscible organic cosolvent) on the enantioselectivity of the enzyme.

The last two entries in Scheme 2.54 represent a 'chiral glycerol' derivative where a positive influence of the chain length of the fatty acid moiety on the selectivity of the enzyme was demonstrated [389, 390].

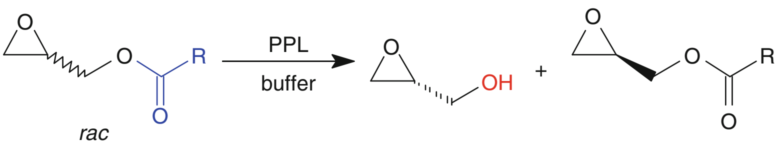
Chiral epoxy alcohols, which are not easily available via the Sharpless procedure [391], were successfully resolved with PPL (Scheme 2.55). Interestingly, the lipase is not deactivated by a possible reaction with the epoxide moiety and it is able to accept a large variety of structures [392, 393]. The significant influence of the



R	Cosolvent	Configuration	e.e. [%]
n-C_7H_{15}–	i-Pr_2O	*S*	70
$(CH_3)_2CH$–$(CH_2)_2$–	i-Pr_2O	*S*	72
(*E*)-n-C_5H_{11}–CH=CH–	none	*S*	84
(*E*)-n-C_5H_{11}–CH=CH–	i-Pr_2O	*S*	95
(*Z*)-n-C_5H_{11}–CH=CH–	i-Pr_2O	*R*	53
(*E*)-$(CH_3)_2CH$–CH=CH–	none	*S*	90
(*E*)-$(CH_3)_2CH$–CH=CH–	i-Pr_2O	*S*	97
(*Z*)-$(CH_3)_2CH$–CH=CH–	i-Pr_2O	*R*	15
Ph–	none	*S*	85–92
Ph–	toluene	*S*	99

Scheme 2.54 Desymmetrization of prochiral 1,3-propanediol diesters by porcine pancreatic lipase

nature of the acyl moiety on the selectivity of the resolution – again, the long-chain fatty acid esters gave better results than the corresponding acetate – may be attributed to the presence of different hydrolytic enzymes present in the crude PPL preparation [394, 395]. In particular α-chymotrypsin and cholesterol esterase are known to hydrolyze acetates of alcohols but not their long-chain counterparts.



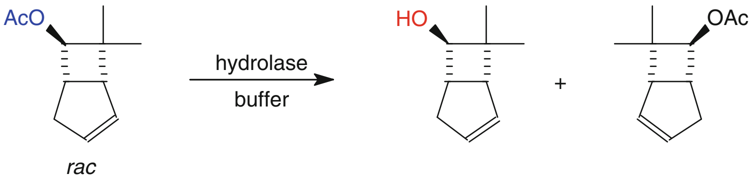
R	e.e. [%]	Selectivity (*E*)
CH_3	53	4
C_2H_5	88	11
n-C_3H_7	92	13
n-C_4H_9	96	16
n-C_3H_7	>99	>100*

*pure immobilized enzyme in the presence of 10 % dioxane

Scheme 2.55 Resolution of epoxy esters by porcine pancreatic lipase

Thus, they are more likely to be competitors of PPL on short-chain acetates. In order to improve the modest enantioselectivity of crude PPL for industrial applications, a pure hydrolase was isolated from crude pancreatin, which gave perfect enantioselectivity (E >100) in the presence of dioxane as cosolvent [396].

During a study on the resolution of the sterically demanding bicyclic acetate shown in Scheme 2.56 [397], which represents an important chiral building block for the synthesis of leukotrienes [398], it was found that crude steapsin is a highly selective catalyst for its resolution. On the other hand, pure PPL and α-chymotrypsin were unable to hydrolyze the substrate. Cholesterol esterase, another known hydrolase impurity in crude steapsin capable of accepting bulky substrates, was able to hydrolyze the ester but with low selectivity. Finally, a novel hydrolase which was isolated from crude PPL proved to be the enzyme responsible for the highly selective transformation.

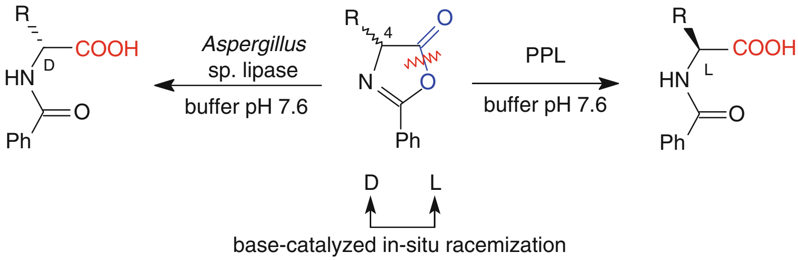


Enzyme	Reaction rate	Selectivity (*E*)
crude PPL	good	>300
pure PPL	no reaction	–
α-chymotrypsin	no reaction	–
cholesterol esterase	fast	17
novel ester hydrolase	good	210

Scheme 2.56 Resolution of bicyclic acetate by hydrolases present in crude porcine pancreatic lipase

Certain azlactones, such as oxazolin-5-ones, represent derivatives of activated esters and thus can be hydrolyzed by proteases, esterases, and lipases (Scheme 2.57) [399]. The products obtained are *N*-acyl α-amino acids. When proteases are employed, only products of modest optical purity were obtained due to the fact that the enzymatic reaction rate is in the same order of magnitude as the spontaneous ring opening in the aqueous medium ($k_{spont} \approx k_R$ or k_S).

On the other hand, lipases were found to be more efficient catalysts [400]. Thus, when the hydrolysis was carried out under carefully controlled reaction conditions at pH 7.6, *N*-benzoyl amino acids of moderate to excellent optical purities were obtained depending on the substituent on C-4. Whereas PPL led to the formation of L-amino acids, the D-counterparts were obtained with a lipase from *Aspergillus niger*. Furthermore, the rate of equilibration of the two configurationally unstable substrate antipodes under weakly basic conditions is sufficiently rapid to provide a



R	Lipase	Configuration	e.e. [%]
Ph–	PPL	L	76
CH_3–S–CH_2–CH_2–	PPL	L	80
Ph–CH_2–	PPL	L	>99
Ph–	*Aspergillus* sp.	D	80
CH_3–S–CH_2–CH_2–	*Aspergillus* sp.	D	83
Ph–CH_2–	*Aspergillus* sp.	D	>99

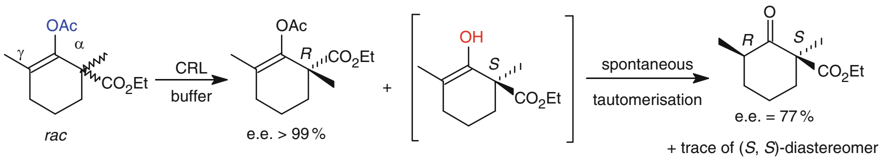
Scheme 2.57 Lipase-catalyzed dynamic resolution of oxazolin-5-ones

dynamic resolution via in-situ racemization with a theoretical yield of 100% ($k_{rac}^{Sub} \geq k_R$ or k_S, respectively), whereas the products are configurationally stable ($k_{rac}^{Prod} \approx 0$, compare Fig. 2.3).

Candida sp. Lipases

Several crude lipase preparations are available from the yeasts *Candida lipolytica*, *C. antarctica* (CAL), and *C. rugosa* (CRL, syn. *C. cylindracea*). The latter enzyme, the three-dimentional structure of which has been resolved by X-ray analysis [333], has been frequently used for the resolution of esters of secondary alcohols [401–406] and, to a lesser extent, for the resolution of α-substituted carboxylates [407, 408]. The CRL preparations from several commercial sources which contain up to 16% of protein [409] differ to some extent in their activity but their selectivity is very similar [410]. As CRL is able to accommodate relatively bulky esters in its active site, it is the lipase of choice for the selective hydrolysis of esters of cyclic secondary alcohols. To illustrate this point, some representative examples are given below.

The racemic cyclohexyl enol ester shown in Scheme 2.58 was enzymatically resolved by CRL to give a ketoester with an (*S*)-stereocenter on the α-position

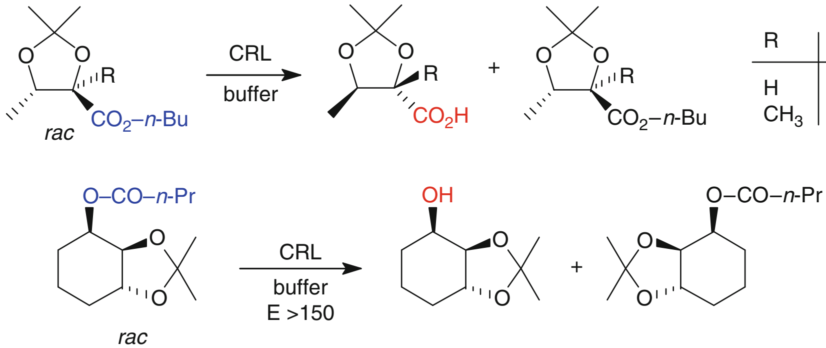


Scheme 2.58 Enzymatic resolution of a cyclic enol ester by *Candida rugosa* lipase

(77% e.e.) coupled with a diastereoselective protonation of the liberated enol, which led to an (*R*)-configuration on the newly generated center on the γ-carbon atom. Only a trace of the corresponding (*S*,*S*)-diastereomer was formed; the remaining (*R*)-enol ester was obtained in optically pure form [411]. In accordance with the concept that an acyloxy moiety is hydrolyzed preferentially to a carboxylate by 'typical' lipases, no significant hydrolysis on the ethyl carboxylate was observed.

Racemic 2,3-dihydroxy carboxylates, protected as their respective acetonides, were resolved by CRL [412] by using their lipophilic *n*-butyl esters (Scheme 2.59). It is particularly noteworthy that the bulky α-methyl derivatives could also be transformed, although compounds of this type are usually not accepted by hydrolases.

A number of cyclohexane 1,2,3-triols were obtained in optically active form *via* resolution of their esters using CRL as shown in Scheme 2.59 [413]. To prevent acyl migration which would lead to racemization of the product, two of the hydroxyl groups in the substrate molecule were protected as the corresponding acetal. In this case, a variation of the acyl chain from acetate to butanoate increased the reaction rate, but had no significant effect on the selectivity of the enzyme.



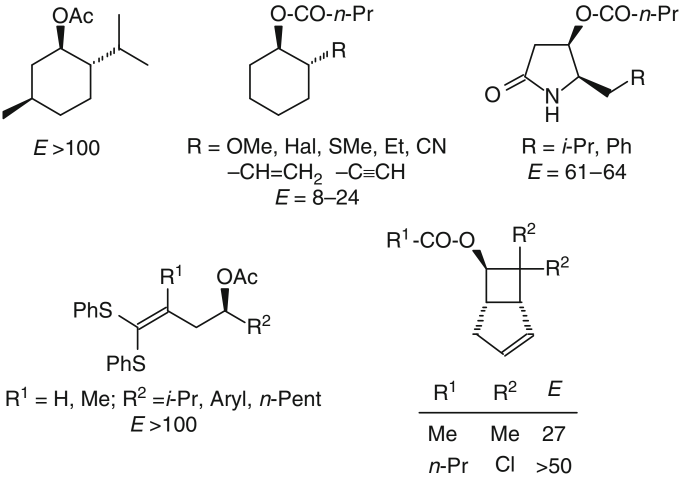
R	*E*
H	8
CH_3	91

Scheme 2.59 Enzymatic resolution of cyclic esters by *Candida rugosa* lipase

Typical substrates for CRL are esters of cyclic *sec*-alcohols, which usually give excellent enantioselectivities [397, 414–417]. In contrast, straight-chain substrates are only well resolved when sterically demanding substituents are present (Scheme 2.60) [418]. Esters of *prim*-alcohols usually yield modest stereoselectivities.

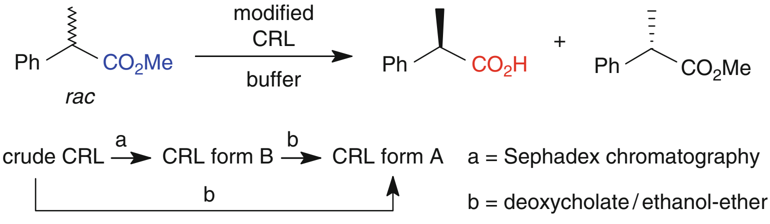
In order to provide a general tool which allows to predict the stereochemical outcome of CRL-catalyzed reactions, a substrate model for bicyclic *sec*-alcohols [54, 340, 419, 420] and an active site model [421] have been developed.

The fact that crude *Candida rugosa* lipase occasionally exhibits a moderate selectivity particularly on α-substituted carboxylic esters could be attributed to the presence of two isomeric forms of the enzyme present in the crude preparation [422, 423]. Both forms (denoted as fraction A and B), could be separated by Sephadex chromatography and were shown to possess a qualitatively identical (i.e., identical enantiopreference) but quantitatively different stereoselectivity



Scheme 2.60 Typical ester substrates for *Candida rugosa* lipase (reacting enantiomer shown)

(Scheme 2.61). Thus, racemic α-phenyl propionate was resolved with low selectivity ($E = 10$) using crude CRL, whereas enzyme-fraction A was highly selective ($E > 100$). The isomeric lipase fraction B showed almost the same moderate selectivity pattern as did the crude enzyme, although it possessed the same stereochemical preference. Treatment of form B with the surface-active agent deoxycholate and an organic solvent system (ethanol/ether) forced its transformation into the more stable conformer, form A, via an unfolding–refolding rearrangement. This noncovalent modification of the enzyme provided a method for the transformation of CRL of form B into its more stable and more selective isomer.



Lipase	Selectivity (*E*)
crude CRL	10
CRL form A	>100
CRL form B	21
CRL form B[c]	>50

[c] After treatment with deoxycholate and ethanol / ether.

Scheme 2.61 Selectivity enhancement of *Candida rugosa* lipase by noncovalent enzyme modification

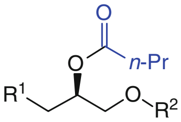
A related selectivity-enhancing noncovalent modification of *Candida rugosa* lipase, which does not require tedious protein chromatography and which is therefore applicable to large-scale reactions is based on the treatment of crude CRL with 50% aqueous *iso*-propanol, which led (after simple centrifugation and dialysis) to a modified lipase preparation, which was not only more active, but also showed considerably enhanced enantioselectivity [424].

The most versatile 'champion' lipase for preparative biotransformations is obtained from the basidomycetous yeast *Candida antarctica* (CAL) [425]. As indicated by its name, this yeast was isolated in Antarctica with the aim of finding enzymes with extreme properties to be used in detergent formulations. Like others, the organism produces two isoenzymes A and B, which differ to a significant extent [426]: whereas lipase A (CALA) is Ca^{2+}-dependent and more thermostable, the B-component is less thermotolerant and metal-independent. More important for preparative applications, the substrate-specificity varies a great deal, as the A-lipase is highly active in a nonspecific manner on triglycerides, showing a preference for the *sn*-2 ester group [427] and is not very useful for simple nonnatural esters. On the contrary, the B-component (CALB) is very active on a broad range of nonnatural esters. Both isoenzymes have been made available in pure form through cloning and overexpression in *Aspergillus oryzae* as the host organism [428] and various preparations of this enzyme are produced by NOVO (DK) in bulk quantities [429]. For the preparative applications discussed below, the B-component has been used more often.

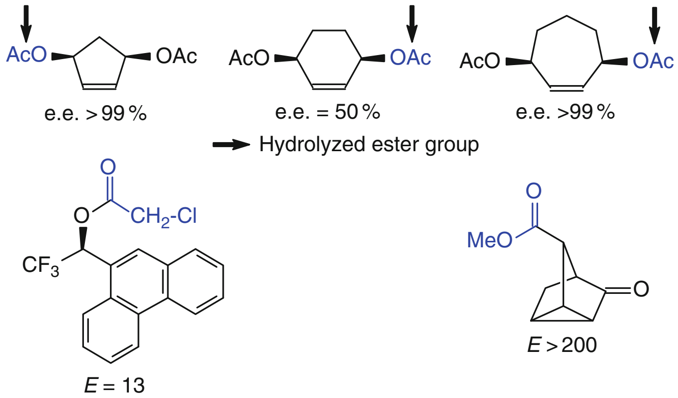
CALB is an exceptionally robust protein which is deactivated only at 50–60°C,[16] and thus also shows increased resistance towards organic solvents. In contrast to many other lipases, the enzyme appears to be rather 'rigid' and does not show a pronounced effect of interfacial activation [430], which makes it an intermediate between an esterase and a lipase. This latter property is probably the reason why its selectivity could be predicted through computer modeling to a fair extent [431], and for the majority of substrates the Kazlauskas' rule (Scheme 2.49) can be applied. In line with these properties of CALB, selectivity-enhancement by addition of water-miscible organic cosolvents such as *t*-butanol or acetone is possible – a technique which is rather common for esterases. All of these properties make CALB the most widely used lipase both in the hydrolysis [432–437] and synthesis of esters (Sect. 3.1.1).

A representative selection of ester substrates, which have been hydrolyzed in a highly selective fashion is depicted in Scheme 2.62 [234, 438–441]. The wide substrate tolerance of this enzyme is demonstrated by a variety of carboxyl esters bearing a chiral center in the alcohol- or the acid-moiety. In addition, desymmetrization of *meso*-forms was also achieved. In general, good substrates for CALB are somewhat smaller than those for *Candida rugosa* lipase and typically comprise (acetate or butyrate) esters of *sec*-alcohols in the (ω-1)- or (ω-2)-position with a straight-chain or monocyclic framework.

[16]In immobilized form, the upper operational limit increases to 60–80°C.



R^1	R^2	Cosolvent	*E*
Cl	CH_2–Ph	none	7
Cl	CH_2–Ph	acetone (30 %)	>200
Cl	$(CH_2)_2$–Ph	none	20
MeO	CH_2–Ph	none	16
MeO	CH_2–Ph	*t*-BuOH (20 %)	106
MeO	$(CH_2)_2$–Ph	none	>100



Scheme 2.62 Typical ester substrates for *Candida antarctica* lipase (reacting enantiomer shown)

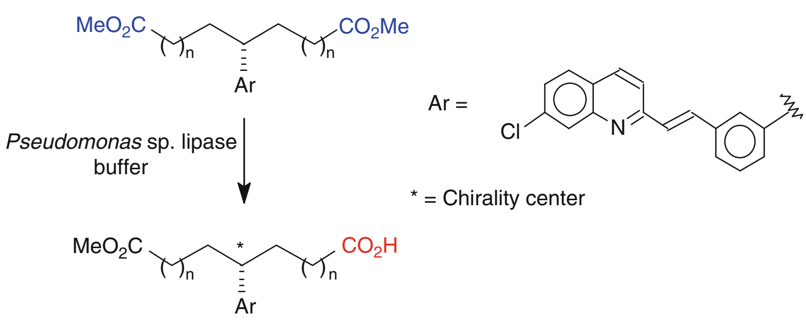
Pseudomonas **sp. Lipases**

Bacterial lipases isolated from *Pseudomonas fluorescens*, *P. aeruginosa, P. cepacia*, and *P. glumae* are highly selective catalysts [442].[17] The structures of both of the latter enzymes were elucidated by X-ray analysis [443, 444]. They seem to possess a 'narrower' active site than CRL, since they are often unable to accommodate bulky substrates, but they can be extremely selective on 'slim' counterparts [445–449]. Like the majority of the microbial lipases, the commercially available crude *Pseudomonas* sp. lipase preparations (PSL) all possess a stereochemical preference for the hydrolysis of the (*R*)-esters of secondary alcohols, but the selectivity among the different preparations may differ to some extent [450]. Various active-site models for PSL have been proposed [163, 451–453].

The exceptionally high selectivity of PSL on 'narrow' open-chain esters is demonstrated by the following examples (Scheme 2.63).

The desymmetrization of some prochiral dithioacetal esters possessing up to five bonds between the prochiral center and the ester carbonyl – the site of reaction – proceeded with high selectivity using PSL [454]. This example of a highly selective chiral recognition of a 'remote' chiral/prochiral center is not unusual amongst hydrolytic enzymes [455–457].

[17]Several *Pseudomonas* spp. were reclassified as *Burkholderia* spp.

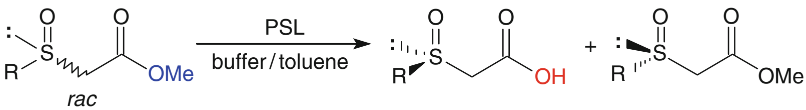


n	Distance[a]	e.e. [%]
1	3	48
2	4	>98
3	5	79

[a]Number of bonds between the prochiral center and the site of reaction.

Scheme 2.63 Desymmetrization of esters having a remote prochiral center by *Pseudomonas* sp. lipase

Chirality need not reside on a sp^3 carbon atom to be recognized by PSL but can be located on a sulfur atom (Scheme 2.64). Thus, optically pure aryl sulfoxides were obtained by lipase-catalyzed resolution of methyl sulfinyl acetates [458] in a biphasic medium containing toluene. The latter compounds are important starting materials for the synthesis of chiral allylic alcohols via the 'SPAC' reaction.

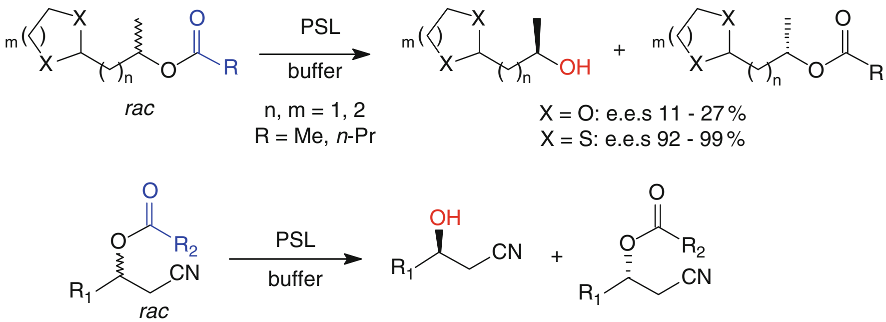


R	e.e. Acid [%]	e.e. Ester [%]	Selectivity (*E*)
p-Cl–C_6H_4–	91	>98	>97
Ph–	92	>98	>110
p-NO_2–C_6H_4–	97	>98	>200
c-C_6H_{11}–	>98	>98	>200

Scheme 2.64 Resolution of sulfoxide esters by *Pseudomonas* sp. lipase

Optically active α- and β-hydroxyaldehydes are useful chiral building blocks for the synthesis of bioactive natural products such as grahamimycin A_1 [459] and amino sugars [460]. PSL-catalyzed resolution of the corresponding dithioacetal esters gave both enantiomers in excellent optical purity (Scheme 2.65) [461]. A significant selectivity enhancement caused by the bulky sulfur atoms was demonstrated when thioacetals were employed instead of the corresponding *O*-acetal esters.

The selectivity of PSL-catalyzed hydrolyses may be significantly improved by substrate-modification through variation of the nonchiral acyl moiety (Scheme 2.65) [462]. Whereas alkyl- and chloroalkyl esters gave poor selectivities, again, the introduction of a sulfur atom to furnish the thioacetates proved to be advantageous. Thus, optically active β-hydroxynitriles, precursors of β-hydroxy acids and β-aminoalcohols, were conveniently resolved via the methyl- or phenyl-thioacetate derivatives.

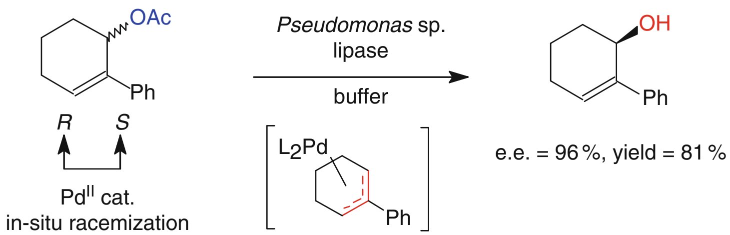


R^1	R^2	Selectivity (*E*)
Me–	Me	7
Me–	Cl–CH_2–	6
Me–	*n*-C_3H_7–	2
Me–	Me–O–CH_2–	14
Me–	Me–S–CH_2–	29
Ph–CH=CH–	Ph–S–CH_2–	55
Ph–	Ph–S–CH_2–	74

Scheme 2.65 Resolution of dithioacetal esters and β-acyloxynitriles by *Pseudomonas* sp. lipase

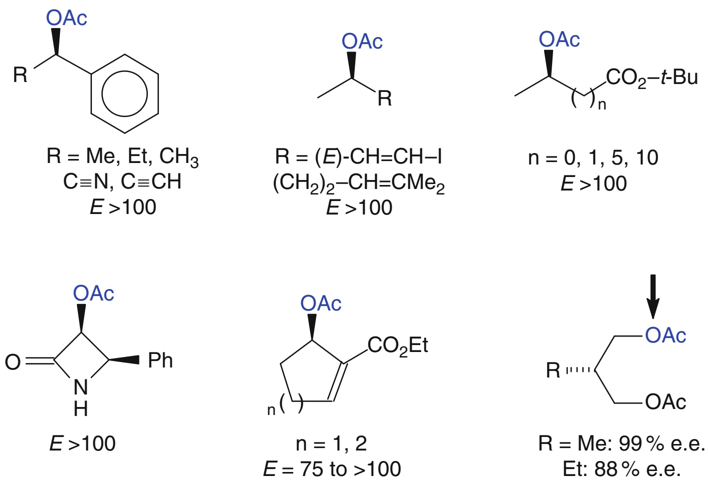
An elegant example for a dynamic resolution of an allylic alcohol via enantioselective ester hydrolysis is depicted in Scheme 2.66 [463]. Thus, *Pseudomonas* sp. lipase hydrolyzed the acetate ester with high specificity, while the in-situ racemization of the substrate enantiomers was effected by a catalytic amount of Pd^{II} leading to the product alcohol in 96% e.e. and 81% yield. However, the lipase has to be chosen with great care, since other hydrolytic enzymes such as acetylcholine esterase and lipases from *Penicillium roqueforti*, *Rhizopus niveus*, and *Chromobacterium viscosum* were incompatible with the metal catalyst.

The typical substrate for *Pseudomonas* sp. lipases is an (ω-1)-acetate ester bearing a rather small group on one side, whereas remarkable space is available for the large group on the opposite side (Scheme 2.67) [365, 464–467]. Esters of cyclic *sec*-alcohols are well accepted as long as the steric requirements are not too



Scheme 2.66 Dynamic resolution of an allylic alcohol ester using *Pseudomonas* sp. lipase and Pd^{II} catalysis

demanding [468–470]. A special feature of PSL is its high selectivity for racemic or prochiral *prim*-alcohols [471, 472], where other lipases often show insufficient stereorecognition.

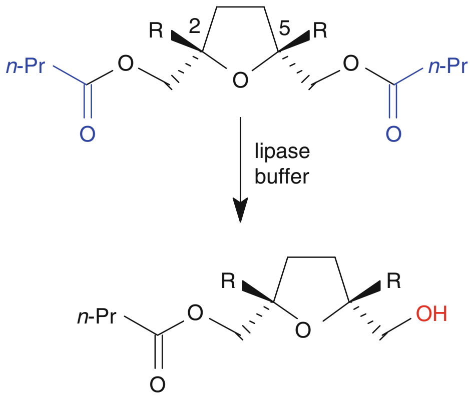


Scheme 2.67 Typical ester substrates for *Pseudomonas* sp. lipase (reacting enantiomer shown)

Mucor sp. Lipases

Lipases from *Mucor* species (MSL) [9, 473] such as *M. miehei* and *M. javanicus* (also denoted as *Rhizomucor*) have frequently been used for biotransformations [401, 474]. With respect to the steric requirements of substrates they seem to be related to the *Pseudomonas* sp. lipases. Similar to *Candida* and *Pseudomonas* sp. lipases, the different MSL preparations are related in their hydrolytic specificity [39].

A case where only MSL showed good selectivity is shown in Scheme 2.68 [475]. The desymmetrization of *meso*-dibutanoates of a tetrahydrofuran-2,5-dimethanol, which constitutes the central subunit of several naturally occurring polyether antibiotics [476] and platelet-activating-factor (PAF) antagonists, was investigated



Lipase	R	e.e. [%]
CRL	H	12
PPL	Me	20
PSL	H	81
MSL	H	>99
MSL	Me	>99

Scheme 2.68 Desymmetrization of bis(acyloxy-methyl)tetrahydrofurans by lipases

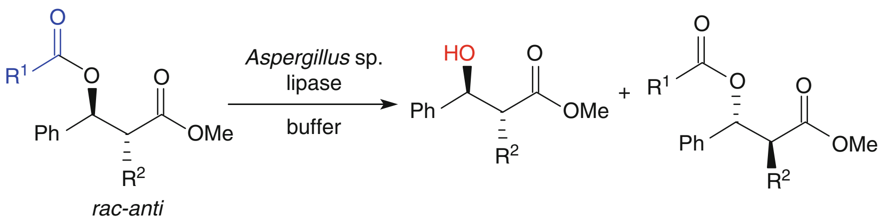
using different lipases. Whereas crude PPL and CRL showed low selectivity, PSL – as may be expected from its more narrow active site – was significantly better. *Mucor* sp. lipase, however, was completely selective leading to optically pure monoester products. It should be noted that the analogous reaction of the 2,5-unsubstituted acetate (R = H) with PLE at low temperature resulted in the formation of the opposite enantiomer [477].

The majority of lipase-catalyzed transformations have been performed using PPL, CRL, CAL, PSL, and MSL – the 'champion lipases' – and it may be assumed that most of the typical lipase substrates may be resolved by choosing one of this group. However, the largely untapped potential of other lipases which is just being recognized is illustrated by the following examples.

Sterically demanding α-substituted β-acyloxy esters were resolved using an *Aspergillus* sp. lipase (see Scheme 2.69).[18] Again, introduction of a thioacetate as the acyl moiety improved the selectivity dramatically [479]. The diastereomeric *syn*/*anti*-conformation of the substrate was of critical importance due to the fact that, in contrast to the *syn*-substrates, only the *anti*-derivatives were resolved with high selectivities.

Optically pure cyanohydrins are required for the preparation of synthetic pyrethroids, which are used as more environmentally acceptable agricultural pest-

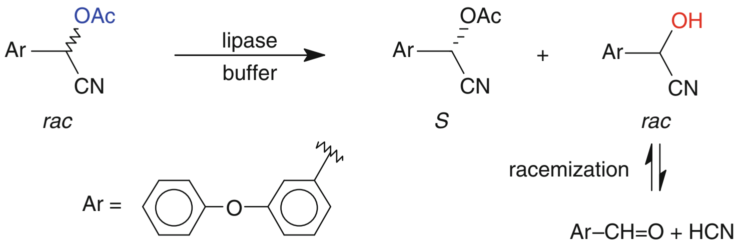
[18]For a predictive model for *Aspergillus* sp. lipase see [477].



R^1	R^2	Selectivity (*E*)
Me–	Me–	80
Me–	Et–	20
Me–	Me–S–	30
Me–S–CH_2–	Me–S–	180

Scheme 2.69 Resolution of α-substituted β-acyloxy esters by *Aspergillus* sp. lipase

control agents in contrast to the classic highly chlorinated phenol derivatives, such as DDT. Cyanohydrins also constitute important intermediates for the synthesis of chiral α-hydroxy acids, α-hydroxyaldehydes [480] and aminoalcohols [481, 482]. They may be obtained via asymmetric hydrolysis of their respective acetates by microbial lipases (Scheme 2.70) [483]. In the ester hydrolysis mode, only the remaining unaccepted substrate enantiomer can be obtained in high optical purity, because the formed cyanohydrin is spontaneously racemized since via its equilibrium with the corresponding aldehyde, liberating hydrocyanic acid at neutral pH values. However, it has recently been shown that the racemization of the



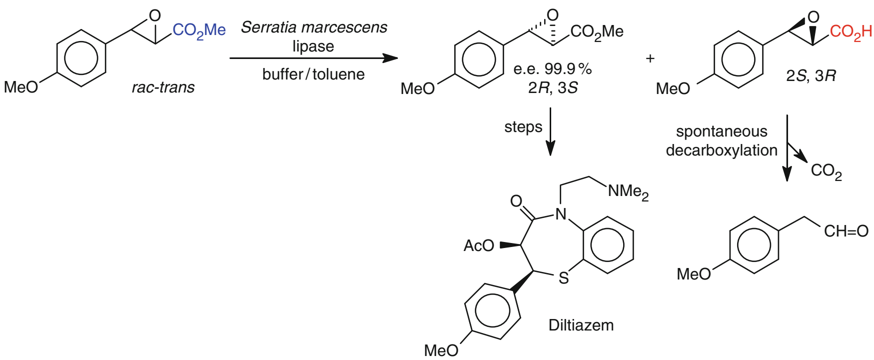
Lipase	e.e. Ester [%]	Configuration	Selectivity (*E*)
CRL	70	*R*	12
PSL	93	*S*	88
Alcaligenes sp.	93	*S*	88
Chromobacterium sp.	96	*S*	160
Arthrobacter sp.	>99	*S*	>200

Scheme 2.70 Hydrolysis of cyanohydrin esters using microbial lipases

cyanohydrin can be avoided when the hydrolysis is carried out at pH 4.5 [484] or in special nonaqueous solvent systems (see Sect. 3.1).

The resolution of the commercially important esters of (*S*)-α-cyano-3-phenoxybenzyl alcohol was only moderately efficient using lipases from *Candida rugosa, Pseudomonas*, and *Alcaligenes* sp. (Scheme 2.70). The best selectivities were obtained with lipases from *Chromobacterium* and *Arthrobacter* sp. [485], respectively.

The epoxy-ester shown in Scheme 2.71 is an important chiral building block for the synthesis of the Ca-channel blocker diltiazem, a potent agent for the treatment of angina pectoris, which is produced at >100 t/year worldwide. Resolution on industrial scale is performed via enantioselective hydrolysis of the corresponding methyl ester using lipases from *Rhizomucor miehei* ($E > 100$) or an extracellular lipase from *Serratia marcescens* ($E = 135$) in a membrane reactor. The (undesired) carboxylic acid enantiomer undergoes spontaneous decarboxylation yielding *p*-methoxyphenyl acetaldehyde, which is removed via extraction of the corresponding bisulfite adduct.



Scheme 2.71 Lipase-catalyzed resolution of an epoxy-ester on industrial scale

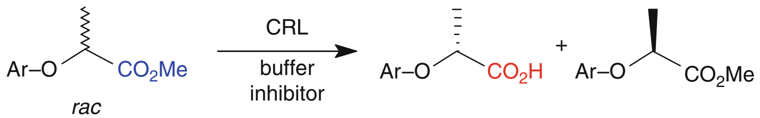
Another less known lipase is obtained from the mold *Geotrichum candidum* [486, 487]. The three-dimensional structure of this enzyme has been elucidated by X-ray crystallography [332] showing it to be a serine hydrolase (like MSL), with a catalytic triad consisting of an *Glu*-His-Ser sequence, in contrast to the more usual *Asp*-His-Ser counterpart. It has a high sequence homology to CRL (~40%) and shows a similar preference for more bulky substrates like *Candida rugosa* lipase.

Another extracellular lipase, called 'cutinase', is produced by the plant-pathogenic microorganism *Fusarium solani pisi* for the hydrolysis of cutin – a wax ester which is excreted by plants in order to protect their leaves against microbial attack [488]. The enzyme has been purified to homogeneity [489] and has been made readily available by genetic engineering [490]. Up to now it has not been widely used for biotransformations of nonnatural esters, but it certainly has a potential as a useful pure lipase for complementary purposes [491].

Optimization of Selectivity
Most of the general techniques for an enzymatic selectivity enhancement such as adjustment of temperature [492], pH [292], and the kinetic parameters of the reaction which were described for the hydrolysis of esters using esterases and proteases, are applicable to lipase-catalyzed reactions as well. Furthermore, the switch to another enzyme to obtain a better selectivity is relatively easy due to the large number of available lipases. Substrate modification involving not only the chiral alcohol moiety of an ester but also its acyl group [493], as described above, is a valuable technique for the selectivity improvement of lipase-catalyzed transformations. Bearing in mind that lipases are rather 'soft' enzymes, they are subject to a strong induced-fit and pronounced interfacial activation (Fig. 2.12). Thus, medium engineering with lipases has been shown to be more effective by applying biphasic systems (aqueous buffer plus a water-*immiscible* organic solvent) instead of monophasic solvents (buffer plus a water-*miscible* organic cosolvent).

Some special optimization techniques, which were developed with lipases in particular, are described below.

Enantioselective Inhibition of Lipases. The addition of weak chiral bases such as amines or aminoalcohols has been found to have a strong influence on the selectivity of *Candida rugosa* [288] and *Pseudomonas* sp. lipase [494]. The principle of this selectivity enhancement was elaborated as early as 1930! [495]. As shown in Scheme 2.72, the resolution of 2-aryloxypropionates by CRL proceeds with low to moderate selectivity in aqueous buffer alone. The addition of chiral bases of the morphinan-type to the medium led to a significant improvement of about one order of magnitude.



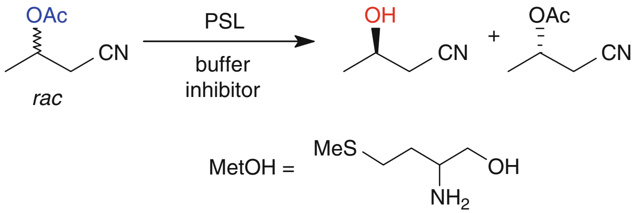
Ar–	Inhibitor	Selectivity (*E*)
2,4-dichlorophenyl–	none	1
2,4-dichlorophenyl–	dextroamethorphan[a]	20
2,4-dichlorophenyl–	levomethorphan	20
2,4-dichlorophenyl–	DMPA[b]	23
4-chlorophenyl–	none	17
4-chlorophenyl–	dextromethorphan	>100
4-chlorophenyl–	levomethorphan	>100

[a]Dextro- or levo-methorphan = D- or L-3-methoxy-*N*-methylmorphinane;
[b]*N,N*-dimethyl-4-methoxyphenethylamine.

Scheme 2.72 Selectivity enhancement of *Candida rugosa* lipase by enantioselective inhibition

Kinetic inhibition experiments revealed that the molecular action of the base on the lipase is a noncompetitive inhibition – i.e., the base attaches itself to the lipase at a site other than the active site – which inhibits the transformation of one enantiomer but not that of its mirror image. Moreover, the chirality of the base has only a marginal impact on the selectivity enhancement effect. The general applicability of this method – impeded by the high cost of morphinan alkaloids and their questionable use for large-scale synthesis – has been extended by the use of more simple amines such as *N, N*-dimethyl-4-methoxyphenethylamine (DMPA) [235].

Alternatively, simple aminoalcohols such as methioninol (MetOH) were shown to have a similar effect on PSL-catalyzed resolutions. As depicted in Scheme 2.73, the chirality of the base had a significant influence on the selectivity of the reaction in this case.



Inhibitor	Selectivity (*E*)
none	7
(*RS*)-MetOH	7
(*R*)-MetOH	8
(*S*)-MetOH	14
dextromethorphan	15

Scheme 2.73 Selectivity enhancement of *Pseudomonas* sp. lipase by enantioselective inhibition

Chemical Modification of Lipases. The chemical modification of enzymes involving the formation of covalent bonds are a major tool for elucidating the mechanisms of enzymatic catalysis [496–498]. These investigations were aimed primarily at defining those amino acids which participate in catalysis and those which are important in substrate binding. Furthermore, the properties of the enzyme such as solubility, pH optimum, inhibition patterns, and the relative reactivity towards different substrates – the specificity – can be varied by chemical modification. More recently, it was also shown that the enantioselectivity of a lipase may also be improved by covalent modification[19] [499–501] (compare Scheme 2.72 and Table 2.2).

[19] A report on the chemical modification of crude PPL using carbamates has to be taken with some caution, since in this case the selective (chemical) deactivation of competing enzymes in the crude steapsin-mixture may likewise be the case for selectivity enhancement, see [500].

Table 2.2 Selectivity enhancement of *Candida rugosa* lipase by covalent enzyme modification (for formulas see Scheme 2.72)

Modification	Selectivity (*E*)
None	1.5
Pyridoxal phosphate[a]	2.4
Tetranitromethane[b]	33
Tetranitromethane, then $Na_2S_2O_4$[c]	37

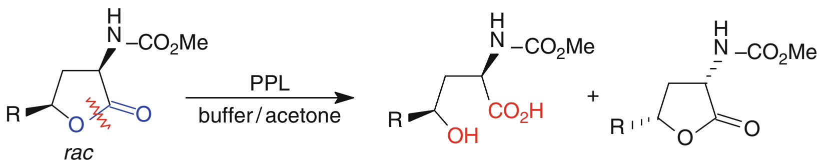
[a]Reductive alkylation of ε-amino groups of lysine
[b]Nitration of tyrosine
[c]Reduction of nitro-tyrosine to amino-tyrosine

The resolution of an α-2,4-dichlorophenoxypropionic acid ester shown in Scheme 2.72 proceeded with very low selectivity when *Candida rugosa* lipase was used in its native form (*E* = 1.5). Reductive alkylation of the ε-amino groups of lysine residues using pyridoxal phosphate led to only a small improvement. However, when tyrosine residues were nitrated with tetranitromethane, the lipase proved to be highly specific (*E* = 33). Reduction of the modified nitro-tyrosine lipase with sodium hydrosulfite (which transforms the nitro into an amino group) slightly enhanced the selectivity even further (*E* = 37).

2.1.3.3 Hydrolysis of Lactones

Owing to their cyclic structure, lactones are more stable than open-chain esters and thus are generally not hydrolyzed by ‘standard’ ester hydrolases. They can be hydrolyzed by lactonases [502], which are involved in the metabolism of aldoses [503] and the deactivation of bioactive lactones, such as *N*-acyl homoserine lactone [504]. In the hydrolytic kinetic resolution of lactones, the separation of the formed (water-soluble) hydroxycarboxylic acid from unreacted (lipophilic) lactone is particularly easy via extraction using an aqueous-organic system.

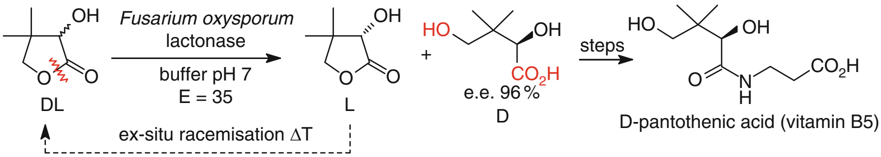
Crude PPL has been shown to hydrolyse γ-substituted α-amino lactones with moderate to good enantioselectivity, however, the identity of the enzyme responsible remains unknown (Scheme 2.74) [505].



R	e.e. Acid [%]	e.e. Lactone [%]	Selectivity (*E*)
H–	71	62	11
Ph–	86	32	18
CH_2=CH–	90	95	70

Scheme 2.74 Enantioselective hydrolysis of γ-lactones by porcine pancreatic lipase

Well-defined lactonases were identified in bacteria [506–508] and fungi. The most prominent example for the use of a lactonase comprises the resolution of DL-pantolactone, which is required for the synthesis of calcium pantothenate (vitamin B_5, Scheme 2.75). The latter is used as vitamin supplement, feed additive, and in cosmetics. An lactonase from *Fusarium oxysporium* cleaves the D-enantiomer from racemic pantolactone forming D-pantoate in 96% e.e. by leaving the L-enantiomer behind. After simple extractive separation, the unwanted L-lactone is thermally racemized and resubjected to the resolution process. In order to optimize the industrial-scale process, which is performed at ca. 3,500 t/year, the lactonase has been cloned and overexpressed into *Aspergillus oryzae* [509]. A corresponding enantiocomplementary L-specific lactonase was identified in *Agrobacterium tumefaciens* [510].



Scheme 2.75 Resolution of pantolactone using a lactonase.

2.1.4 Hydrolysis and Formation of Phosphate Esters

The hydrolysis of phosphate esters can be equally achieved by chemical methods and by phosphatases; the application of enzymes for this reaction is only advantageous if the substrate is susceptible to decomposition. Thus, the enzymatic hydrolysis of phosphates has found only a limited number of applications. The same is true concerning the enantiospecific hydrolyses of racemic phosphates affording a kinetic resolution.

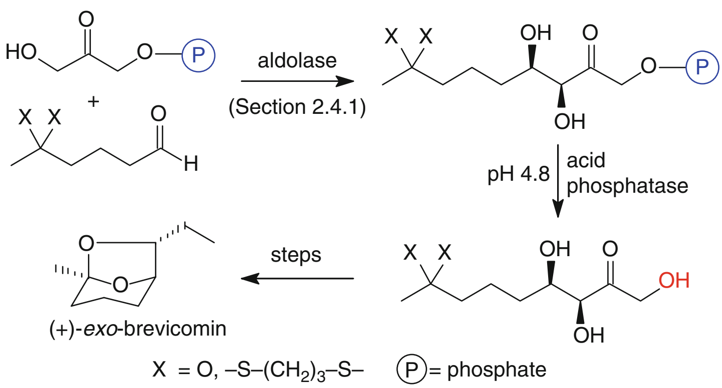
However, the *formation* of phosphate esters is of importance, particularly when regio- or enantioselective phosphorylation is required. Numerous bioactive agents display their highest activity only when they are transformed into phosphorylated analogs. Furthermore, a number of essential cofactors or cosubstrates for other enzyme-catalyzed reactions of significant synthetic importance involve phosphate esters. For instance, nicotinamide adenine dinucleotide phosphate ($NADP^+$) or glucose-6-phosphate (G6P) is an essential cofactor or cosubstrate, respectively, for some dehydrogenase-catalyzed reactions (Sect. 2.2.1). Dihydroxyacetone phosphate (DHAP) is needed for enzymatic aldol reactions (Sect. 2.4.1), and adenosine triphosphate (ATP) represents the energy-rich phosphate donor for most biological phosphorylation reactions. Glycosyl phosphates are essential for glycosyl transfer reactions catalyzed by carbohydrate phosphorylases (Sect. 2.6.1).

Hydrolysis of Phosphate Esters

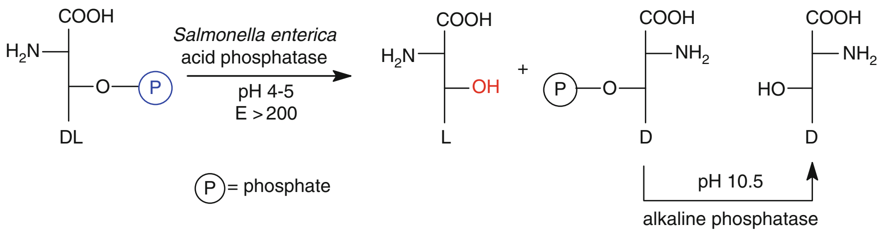
Chemoselective Hydrolysis of Phosphate Esters. Chemical hydrolysis of polyprenyl pyrophosphates is hampered by side reactions due to the lability of the molecule. Hydrolysis catalyzed by acid phosphatase – an enzyme named because it displays its pH-optimum in the acidic range – readily afforded the corresponding dephosphorylated products in acceptable yields [511].

Similarly, the product from a DHAP-depending aldolase-catalyzed reaction is a chemically labile 2-oxo-1,3,4-triol, which is phosphorylated at position 1. Dephosphorylation under mild conditions, without isolation of the intermediate phosphate species, by using acid phosphatase is a method frequently used to obtain the chiral polyol products [512–515]. As shown in Scheme 2.76, enzymatic dephosphorylation of the aldol product obtained from 5-substituted hexanal derivatives under mild conditions gave the sensitive chiral keto-triol in good yield. In this case the product could be transformed into (+)-*exo*-brevicomin, the sex pheromone of the pine bark beetle.

Enantioselective Hydrolysis of Phosphate Esters. In comparison with the hydrolysis of carboxyl esters, enantioselective hydrolyses of phosphate esters have been seldom reported. However, acid phosphatases were applied to the kinetic resolution of serine and threonine via hydrolysis of the corresponding *O*-phosphate esters (Scheme 2.77) [516]. As for the resolutions of amino acid derivatives using proteases, the natural L-enantiomer was hydrolyzed in the case of threonine *O*-phosphate, leaving the D-counterpart behind (E > 200). After separation of the D-phosphate from L-threonine, the D-enantiomer could be dephosphorylated using an unspecific alkaline phosphatase – an enzyme with the name derived from having its pH-optimum in the alkaline region. Interestingly, the N151D mutant exhibited an opposite enantiopreference for the D-enantiomer in case of DL-serine-*O*-phosphate (E = 18) [517].

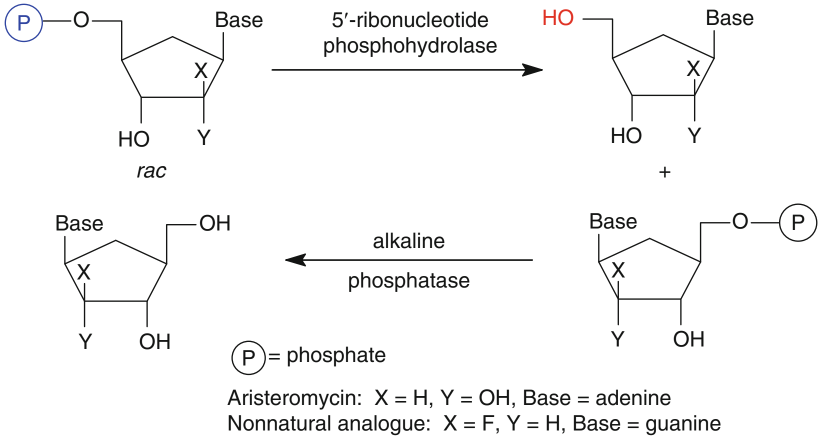


Scheme 2.76 Chemoselective enzymatic hydrolysis of phosphate esters



Scheme 2.77 Resolution of *rac*-threonine *O*-phosphate using acid phosphatase

Carbocyclic nucleoside analogs with potential antiviral activity, such as aristeromycin [518] and fluorinated analogs of guanosine [519], were resolved via their 5′-phosphates using a 5′-ribonucleotide phosphohydrolase from snake venom (see Scheme 2.78). Again, the nonaccepted enantiomer, possessing a configuration opposite to that of the natural ribose moiety, was dephosphorylated by unspecific alkaline phosphatase.



Scheme 2.78 Resolution of carbocyclic nucleoside analogs

Formation of Phosphate Esters

The introduction of a phosphate moiety into a polyhydroxy compound by classic chemical methods is tedious since it usually requires a number of protection and deprotection steps. Furthermore, oligophosphate esters as undesired byproducts arising from overphosphorylation are a common problem. Employing enzymes for the regioselective formation of phosphate esters can eliminate many of these disadvantages thus making these syntheses more efficient. Additionally, enantioselective transformations are also possible involving the desymmetrization of prochiral or *meso*-diols or the resolution of racemates.

Phosphate esters are usually synthesized by means of phosphorylating transferases called kinases, which catalyze the transfer of a phosphate moiety (or a di-[20] or triphosphate moiety) from an energy-rich phosphate donor, such as ATP. Due to the high price of these phosphate donors, they cannot be employed in stoichiometric amounts.[21] Since ATP cannot be replaced by less expensive man-made chemical equivalents, efficient in-situ regeneration (i.e., recycling) is necessary in order to reduce the cost of enzymatic phosphorylations. Fortunately, ATP recycling has become feasible on a molar scale [520, 521]. On the other hand, reversal of phosphate ester hydrolysis, i.e., the equivalent condensation reaction, has been performed in solvent systems with a reduced water content. Such systems would eliminate the use of expensive or chemically labile phosphate-donors but it is questionable if they will be of general use [522].

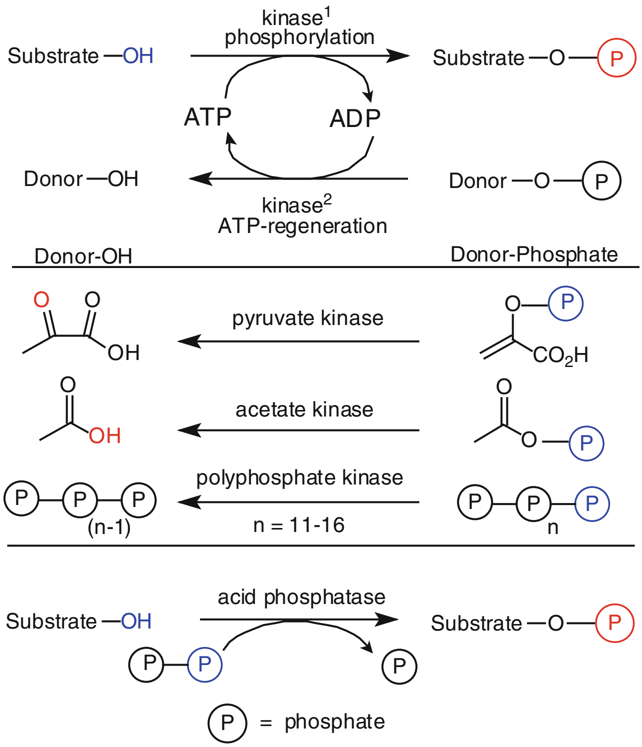
ATP Recycling. All phosphorylating enzymes (kinases) require nucleoside triphosphates (in most cases ATP) as a cofactor. In living organisms, these energy-rich phosphates are regenerated by metabolic processes, but for biocatalytic transformations, which are performed in vitro using purified enzymes, this does not occur. The (hypothetical) addition of stoichiometric amounts of these cofactors would not only be undesirable from a commercial standpoint but also for thermodynamic reasons. Quite often the accumulation of the inactive form of the consumed cofactor (most commonly the corresponding diphosphate, ADP) can tip the equilibrium of the reaction in the reverse direction. Thus, cofactors such as ATP are used only in catalytic amounts and are continuously regenerated during the course of the reaction by an auxiliary system which usually consists of a second enzyme and a stoichiometric quantity of an ultimate (cheap) phosphate donor (Scheme 2.79). As the nucleoside triphosphates are intrinsically unstable in solution, the XTP triphosphate species used in phosphorylation reactions is typically recycled ~100 times, with total turnover numbers (TTN) being in the range of about 10^6–10^8 mol of product per mol of enzyme.

The following ATP-regenerating systems have been proposed:

- The use of the phosphoenol pyruvate (PEP)/pyruvate kinase system is probably the most useful method for the regeneration of nucleoside triphosphates [523]. PEP is not only very stable towards spontaneous hydrolysis but it is also a stronger phosphorylating agent than ATP. Furthermore, nucleosides other than adenosine phosphates are also accepted by pyruvate kinase. The drawbacks of this system are the more complex synthesis of PEP [524, 525] and the fact that pyruvate kinase is inhibited by pyruvate at higher concentrations.
- Acetyl phosphate, which can be synthesized from acetic anhydride and phosphoric acid [526], is a commonly used regeneration system in conjunction with acetate kinase [527]. It is modestly stable in aqueous solution and while its phosphoryl donor potential is lower than that of PEP, it is considerably cheaper. As for pyruvate kinase, acetate kinase also can accept nucleoside phosphates other than adenosine, and it is inhibited by acetate.

[20] Also termed ‘pyro-phosphates’.

[21] The retail price for one mole of ATP is about US $4,500, bulk prices are about one tenth of that.

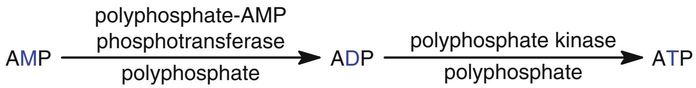


Scheme 2.79 Enzymatic recycling of ATP from ADP

- Promising ATP-recycling methods for large-scale applications use cheap inorganic polyphosphate as phosphate donor and polyphosphate kinase, respectively. Polyphosphate kinase from *E. coli* accepts also other nucleoside diphosphates and yields up to 40 regeneration cycles [528].
- An elegant (kinase- and XTP-independent) approach is to use of phosphatases in the reverse direction: Although these enzymes are phosphate ester hydrolases, some of them, such as the enzyme from *Shigella flexeri* is able to catalyse the backward (phosphorylation) reaction by accepting cheap pyrophosphate as phosphate donor [529].
- Two further ATP-recycling systems use carbamoyl phosphate and methoxycarbonyl phosphate as nonnatural phosphate donors together with carbamate kinase and acetate kinase, respectively [530, 531]. Both systems lead to the formation of highly unstable products – carbamic acid and methyl carbonate, respectively – which readily decompose forming NH_3/CO_2 or $MeOH/CO_2$ thereby driving the equilibrium towards completion. Unfortunately, both phosphate donors undergo spontaneous hydrolysis in in aqueous medium, which severely limits their applicability.
- Regeneration of other nucleoside triphosphates (GTP, UTP, and CTP) or the corresponding 2′-deoxynucleoside triphosphates – which are important substrates for enzyme-catalyzed glycosyl transfer reactions (Sect. 2.6.1)

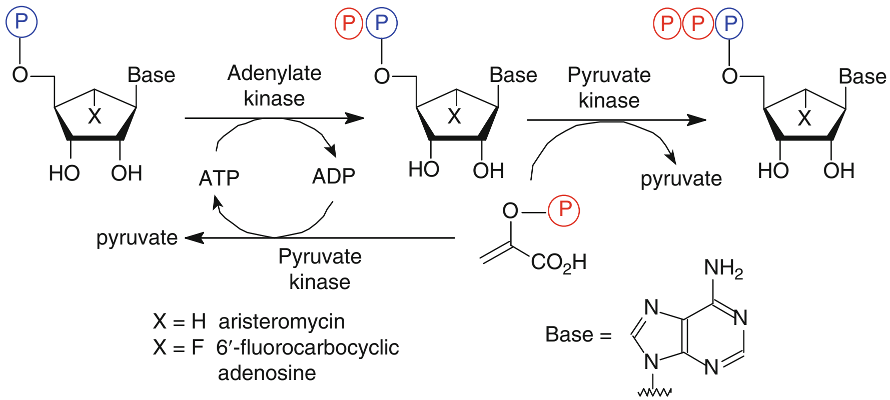
[523, 532, 533] – can be accomplished in the same manner using the acetate or pyruvate kinase systems.

- A number of reactions which consume ATP generate AMP rather than ADP as a product, only few produce adenosine [534]. ATP may be recycled from AMP using polyphosphate-AMP phosphotransferase and polyphosphate kinase in a tandem-process at the expense of inorganic polyphosphate as phosphate donor for both steps. Alternatively, the combination of adenosine kinase and adenylate kinase were used (Scheme 2.80) [535].



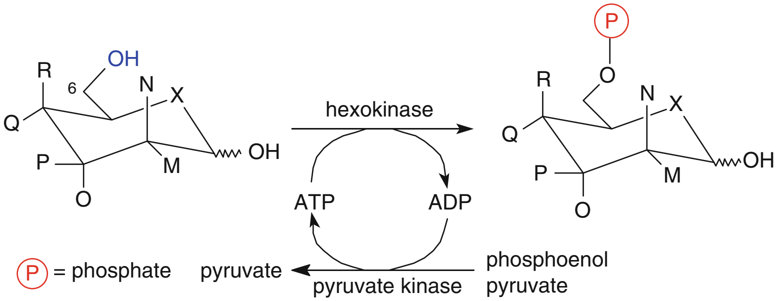
Scheme 2.80 Enzymatic recycling of ATP from AMP via ADP

A different approach to facilitate ATP-dependent phosphorylation reactions is based on a (chemically) modified nucleoside analog [536] (Scheme 2.81). Carbocyclic surrogates are often good mimics of the corresponding natural nucleoside by displaying a better stability due to their lack of the sensitive hemiaminal bond and are thus widely used in antiviral therapy. Carbocyclic ATP analogs, such as aristeromycin-triphosphate and 6′-fluorocarbocyclic adenosine-triphosphate, were synthesized using adenylate and pyruvate kinase using phosphoenol pyruvate as the ultimate phosphate donor. Both of the carba-ATPs were well accepted by glycerol kinase and hexokinase. The multi-step syntheses required for the preparation of the ATP-mimics may be justified by their enhanced stability and by the possibility of monitoring the phosphorylation reaction using the powerful ^{19}F-NMR in case of the fluoro-ATP analog.



Scheme 2.81 Enzymatic synthesis of carbocyclic ATP-mimics

Regioselective Phosphorylation. The selective phosphorylation of hexoses (and their thia- or aza-analogs) on the primary alcohol moiety located in position 6 can be achieved by a hexokinase (Scheme 2.82) [537, 538]. The other (secondary) hydroxyl groups can be either removed or they can be exchanged for a fluorine atom. Such modified hexose analogs represent potent enzyme inhibitors and are therefore of interest as potential pharmaceuticals or pharmacological probes. The most important compound in Scheme 2.80 is glucose-6-phosphate (G6P; N, O, R = H; M, P, Q = OH; X = O), which serves as a hydride source during the recycling of NAD(P)H when using glucose-6-phosphate dehydrogenase [539, 540] (Sect. 2.2.1).

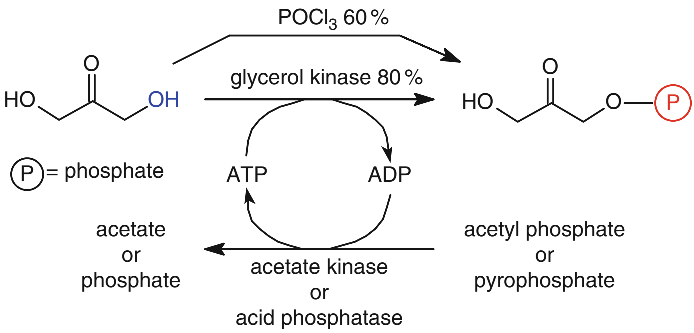


Substituents	Variations tolerated
M, P, Q	H, F, OH
N, O, R	H, F
X	O, S, NH

Scheme 2.82 Phosphorylation of hexose derivatives by hexokinase

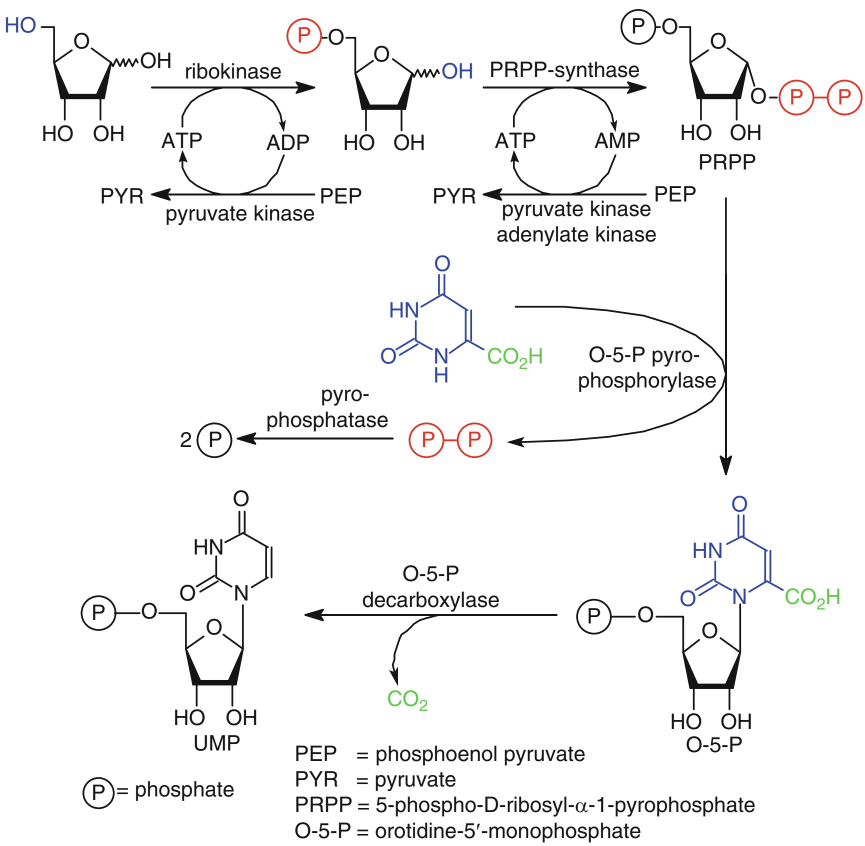
Another labile phosphate species, which is needed as a cosubstrate for DHAP-dependent aldolase reactions, is dihydroxyacetone phosphate (Scheme 2.83, also see Sect. 2.4.1). Its chemical synthesis using phosphorus oxychloride is hampered by moderate yields. Enzymatic phosphorylation, however, gives significantly enhanced yields of a product which is sufficiently pure so that it can be used directly in solution without isolation [541, 542].

5-Phospho-D-ribosyl-α-1-pyrophosphate (PRPP) serves as a key intermediate in the biosynthesis of purine, pyrimidine, and pyridine nucleotides, such as nucleotide cofactors [ATP, UTP, GTP, CTP, and NAD(P)H]. It was synthesized on a large scale from D-ribose using two consecutive phosphorylating steps [543] (Scheme 2.84). First, ribose-5-phosphate was obtained using ribokinase, subsequently a pyrophosphate moiety was transferred from ATP onto the anomeric center in the α-position by PRPP synthase. In this latter step, AMP (rather than ADP) was generated from ATP, which required adenylate kinase for ATP-recycling. Phosphoenol pyruvate (PEP)



Scheme 2.83 Phosphorylation of dihydroxyacetone by glycerol kinase

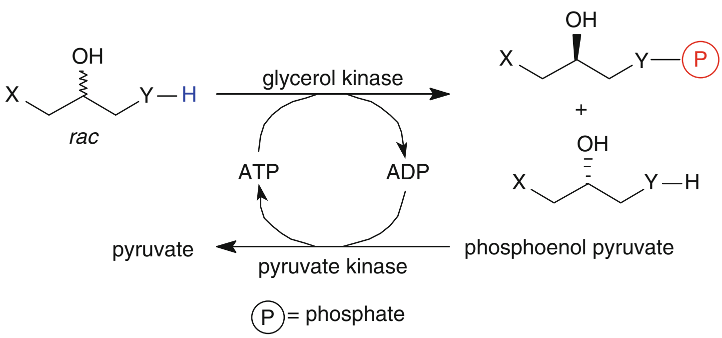
served as phosphate donor in all phosphorylation steps. The PRPP thus obtained was subsequently transformed into orotidine monophosphate (O-5-P) via enzymatic linkage of the nucleobase by orotidine-5′-pyrophosphorylase (a transferase) followed by



Scheme 2.84 Phosphorylation of D-ribose and enzymatic synthesis of UMP

decarboxylation of O-5-P by orotidine-5′-phosphate decarboxylase (a lyase) led to UMP in 73% overall yield.

Enantioselective Phosphorylation. Glycerol kinase [544] is not only able to accept its natural substrate, glycerol, to form *sn*-glycerol-3-phosphate [545], or close analogs of it such as dihydroxyacetone (see Scheme 2.83), but it is also able to transform a large variety of prochiral or racemic primary alcohols into chiral phosphates (Scheme 2.85) [546–548]. The latter compounds represent synthetic precursors to phospholipids [549] and their analogs [550].

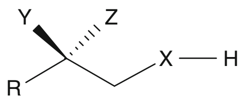


X	Y	e.e. Phosphate [%]	e.e. Alcohol [%]
Cl	O	>94	88
SH	O	94	n.d.
CH_3O	O	90	n.d.
Br	O	90	n.d.
OH	NH	>94	94

Scheme 2.85 Enantioselective phosphorylation of glycerol derivatives

As depicted in Scheme 2.85, the glycerol backbone of the substrates may be varied quite widely without affecting the high specificity of glycerol kinase. In resolutions of racemic substrates, both the phosphorylated species produced and the remaining substrate alcohols were obtained with moderate to good optical purities (88 to >94%). Interestingly, the phosphorylation of the aminoalcohol shown in the last entry occurred in an enantio- and chemoselective manner on the more nucleophilic nitrogen atom.

The evaluation of the data obtained from more than 50 substrates permitted the construction of a general model of a substrate that would be accepted by glycerol kinase (Fig. 2.14).



Position	Requirements
X	O, NH
Y	preferably OH, also H or F, but not NH_2
Z	H, OH (as hydrated ketone), small alkyl groups[a]
R	small groups, preferably polar, e.g. $–CH_2–OH$, $–CH_2–Cl$

[a]Depending on enzyme source

Fig. 2.14 Substrate model for glycerol kinase

2.1.5 Hydrolysis of Epoxides

Chiral epoxides and vicinal diols (employed as their corresponding cyclic sulfate or sulfite esters as reactive intermediates) are extensively employed high-value intermediates for the synthesis of enantiomerically pure bioactive compounds due to their ability to react with a broad variety of nucleophiles [551, 552]. As a consequence, extensive efforts have been devoted to the development of catalytic methods for their production. Although several chemical strategies are known for preparing them from optically active precursors, or via asymmetric syntheses involving desymmetrization or resolution methods [553], none of them is of general applicability and each of them has its merits and limits. Thus, the Sharpless epoxidation gives excellent stereoselectivities and predictable configurations of epoxides, but it is limited to allylic alcohols [554]. On the other hand, the Jacobsen epoxidation is applicable to nonfunctionalized alkenes [555]. The latter gives high selectivities for *cis*-alkenes, whereas the results obtained with *trans*- and terminal olefins were less satisfactory. As an alternative, a number of biocatalytic processes for the preparation of enantiopure epoxides via direct or indirect methods are available [556–559]. Among them, microbial epoxidation of alkenes would be particularly attractive by providing a direct access to optically pure epoxides, but this technique requires sophisticated fermentation and process engineering (Sect. 2.3.2.3) [560]. In contrast, the use of hydrolase enzymes for this purpose would be clearly advantageous. An analogous metal-based chemocatalyst for the asymmetric hydrolysis of epoxides is available.[22]

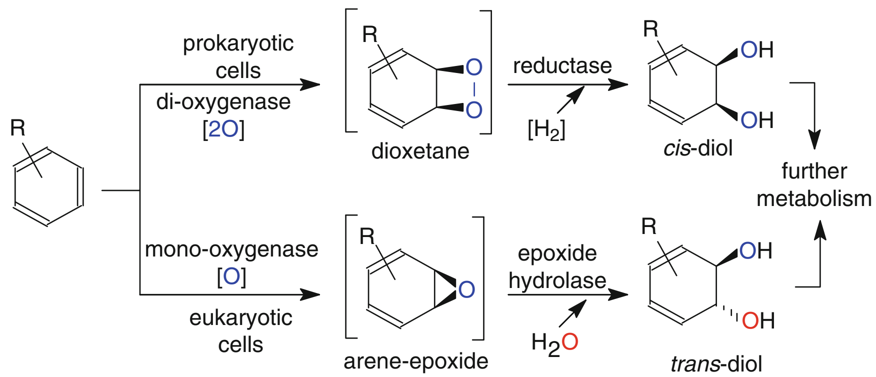
Enzymes catalyzing the regio- and enantiospecific hydrolysis of epoxides – epoxide hydrolases (EH)[23] [562] – play a key role in the metabolism of xenobiotics.

[22]For the corresponding asymmetric hydrolysis of epoxides using a chiral Co-salen complex see [560].

[23]Epoxide hydrolases are occasionally also called ‘epoxide hydratases’ or ‘epoxide hydrases’.

In living cells, aromatics and olefins can be metabolized via two different pathways (Scheme 2.86).

In prokaryotic cells of lower organisms such as bacteria, dioxygenases catalyze the cycloaddition of molecular oxygen onto the C=C double bond forming a dioxetane (Sect. 2.3.2.7). The latter species are reductively cleaved into *cis*-diols. In eukaryotic cells of higher organisms such as fungi and mammals, enzymatic epoxidation mediated by monooxygenases (Sect. 2.3.2.3) is the major degradation pathway. Due to the electrophilic character of epoxides, they represent powerful alkylating agents which makes them incompatible with living cells: they are toxic, cancerogenic, and teratogenic agents. In order to eliminate them from the cell, epoxide hydrolases catalyze their degradation into biologically more innocuous *trans*-1,2-diols,[24] which can be further metabolized or excreted due to their enhanced water solubility. As a consequence, most of the epoxide hydrolase activity found in higher organisms is located in organs, such as the liver, which are responsible for the detoxification of xenobiotics [563, 564].



Scheme 2.86 Biodegradation of aromatics

Enzyme Mechanism and Stereochemical Implications

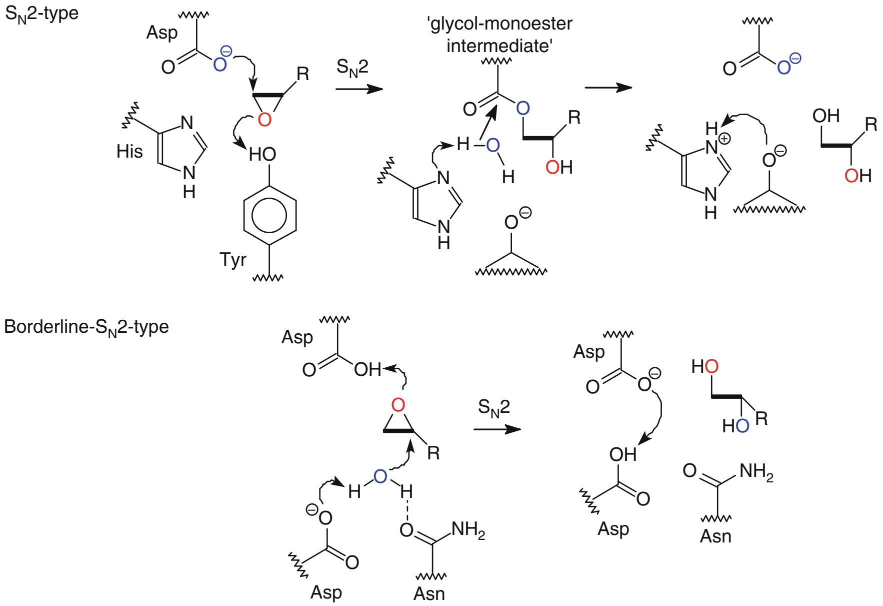
The mechanism of epoxide hydrolase-catalyzed hydrolysis has been elucidated from microsomal epoxide hydrolase (MEH) and bacterial enzymes to involve the *trans*-antiperiplanar addition of water to epoxides and arene oxides to give vicinal diol products. In general, the reaction occurs with *inversion* of configuration at the oxirane carbon atom to which the addition takes place and involves neither cofactors nor metal ions [565]. Two types of mechanism are known (Scheme 2.87).

S_N2-Type Mechanism. A carboxylate residue – aspartate – performs a nucleophilic attack on the (usually less hindered) epoxide carbon atom by forming a covalent glycol-monoester intermediate [566–568]. The latter species can be regarded as a

[24]For an unusual *cis*-hydration see [586].

'chemically inverted' acyl-enzyme intermediate in serine hydrolase reactions (Sect. 2.1.1). In order to avoid the occurrence of a charged oxy-anion, a proton from an adjacent Tyr-residue is simultaneously transferred. In a second step, the ester bond of the glycol monoester intermediate is hydrolyzed by a hydroxyl ion which is provided from water with the aid of a base – histidine [569] – thereby liberating the glycol. Finally, proton-migration from His to Tyr closes the catalytic cycle. This mechanism shows striking similarities to that of haloalkane dehalogenases, where a halide is displaced by an aspartate residue in a similar manner (Sect. 2.7.2) [570, 571]. In addition, a mechanistic relationship with β-glycosidases which act via formation of a covalent glycosyl-enzyme intermediate by retaining the configuration at the anomeric center is obvious (Sect. 2.6.2) [572].

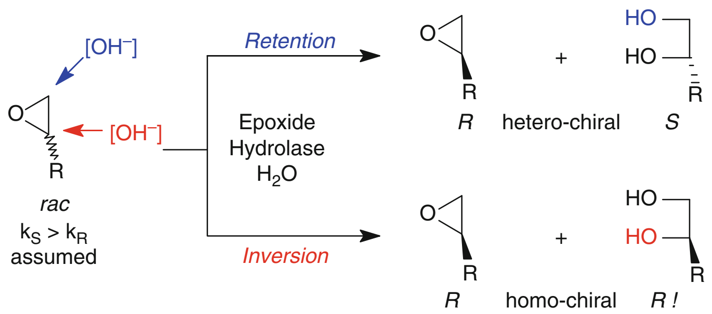
Borderline-S_N2-Type Mechanism. Some enzymes, such as limonene-1,2-epoxide hydrolase, have been shown to operate via a single-step push-pull mechanism [573]. General acid catalysis by a protonated aspartic acid weakens the oxirane to facilitate a simultaneous nucleophilic attack of hydroxyl ion, which is provided by deprotonation of H_2O via an aspartate anion. Due to the borderline-S_N2-character of this mechanism, the nucleophile preferentially attacks the higher substituted carbon atom bearing the more stabilized δ^+-charge. After liberation of the glycol, proton-transfer between both Asp-residues closes the cycle.



Scheme 2.87 S_N2- and borderline-S_N2-type mechanism of epoxide hydrolases

The above-mentioned facts have important consequences on the stereochemical course of the kinetic resolution of nonsymmetrically substituted epoxides. In contrast to the majority of kinetic resolutions of esters (e.g., by ester hydrolysis using proteases, esterases, and lipases) where the absolute configuration of the stereogenic center always remains the same throughout the reaction, the enzymatic hydrolysis of epoxides may take place via two different pathways (Scheme 2.88).

- Attack of the (formal) hydroxide ion on the less hindered (unsubstituted) oxirane carbon atom causes *retention* of configuration and leads to a hetero-chiral product mixture of enantiomeric diol and nonreacted epoxide.
- Attack on the stereogenic center leads to *inversion* and furnishes homochiral products possessing the same sense of chirality.



Scheme 2.88 Enzymatic hydrolysis of epoxides proceeding with retention or inversion of configuration

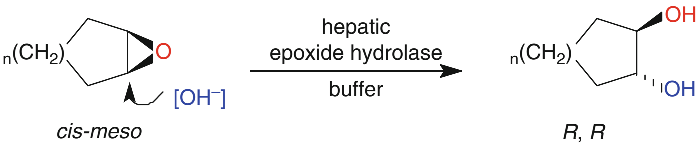
Although retention of configuration seems to be the more common pathway, inversion has been reported in some cases depending on the substrate structure and the type of enzyme [574, 575]. As a consequence, the absolute configuration of *both the product and the substrate* from a kinetic resolution of a racemic epoxide has to be determined separately in order to elucidate the stereochemical pathway. As may be deduced from Scheme 2.88, the use of the enantiomeric ratio is only appropriate to describe the enantioselectivity of an epoxide hydrolase as long as its regioselectivity is uniform, i.e., *only* inversion *or* retention is taking place, but *E*-values are inapplicable where mixed pathways, i.e., retention *and* inversion, are detected [576]. For the solution to this stereochemical problem, various methods were proposed [577].

Hepatic Epoxide Hydrolases

To date, two main types of epoxide hydrolases from liver tissue have been characterized, i.e., a microsomal (MEH) and a cytosolic enzyme (CEH), which are different in their substrate specificities. As a rule of thumb, with nonnatural epoxides, MEH has been shown to possess higher activities and selectivities when compared to its cytosolic counterpart.

Although pure MEH can be isolated from the liver of pigs, rabbits, mice, guinea pigs [578], or rats [579], a crude preparation of liver microsomes or even the 9,000 × g supernatant of homogenized liver was employed as a valuable source for EH activity with little difference from that of the purified enzyme being observed [580]. However, other enzyme-catalyzed side-reactions such as ester hydrolysis may occur with crude preparations.

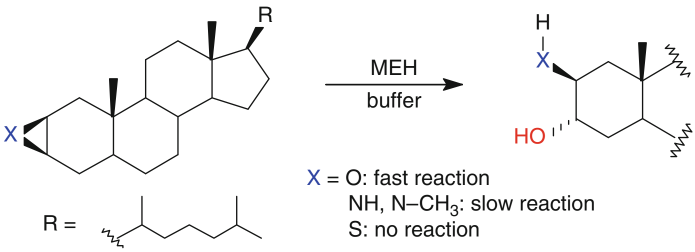
Cyclic *cis-meso*-epoxides can be asymmetrically hydrolyzed using hepatic epoxide hydrolases to give *trans*-diols. In this case, the (*S*)-configurated oxirane carbon atom is preferentially attacked and inverted to yield an (*R*,*R*)-diol (Scheme 2.89) [581, 582]. In comparison to the microsomal epoxide hydrolase, cytosolic EH exhibited a lower stereoselectivity.



n	e.e. of diol [%]	
	microsomal EH	cytosolic EH
1	90	60
2	94	22
3	40	30
4	70	no reaction

Scheme 2.89 Desymmetrization of cyclic *cis-meso*-epoxides by hepatic epoxide hydrolases

Utilizing steroid substrates, MEH was able to hydrolyze not only epoxides, but also the corresponding heteroatom derivatives such as aziridines to form *trans*-1,2-aminoalcohols albeit at slower rates (Scheme 2.90) [583]. The thiirane, however, was inert towards enzymatic hydrolysis. The enzyme responsible for this activity was assumed to be the same microsomal epoxide hydrolase, but this assumption was not proven.



Scheme 2.90 Enzymatic hydrolysis of steroid epoxides and aziridines by microsomal epoxide hydrolase

Although many studies have been undertaken with hepatic epoxide hydrolases, mainly aimed at the elucidation of detoxification mechanisms, it is unlikely that enzymes from these sources will be widely used as biocatalysts in preparative transformations, since they cannot be produced in reasonable amounts. In contrast, epoxide hydrolases from microbial sources have a great potential because scale-up of their production is considerably easier.

Microbial Epoxide Hydrolases

Although it was known for several years that microorganisms possess epoxide hydrolases, they were only scarcely applied to preparative organic transformations [584–588]. Thus, the hydrolysis of epoxides, which was occasionally observed during the microbial epoxidation of alkenes as an undesired side reaction causing product degradation, was usually neglected, and it was only recently that systematic studies were undertaken. It should be emphasized, that although a number of microbial epoxide hydrolases have been purified and characterized [589–593], the majority of preparative-scale reactions were performed by using whole-cell preparations or crude cell-free extracts with an unknown number of epoxide hydrolases being active.

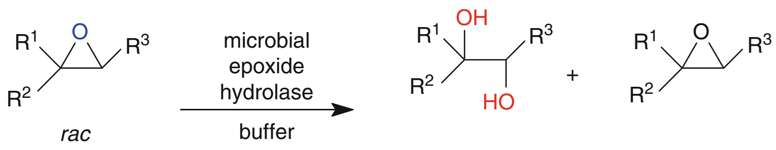
As a result, an impressive amount of knowledge on microbial epoxide hydrolases from various sources – such as bacteria, filamentous fungi, and yeasts – has been gathered and featured in several reviews [594–598]. The data available to date indicate that the enantioselectivities of enzymes from certain microbial sources can be correlated to the substitutional pattern of various types of substrates [599]:

- Red yeasts (e.g., *Rhodotorula* or *Rhodosporidium* sp.) give best enantioselectivities with monosubstituted oxiranes.
- Fungal cells (e.g., *Aspergillus* and *Beauveria* sp.) are best suited for styrene-oxide-type substrates.
- Bacterial enzymes (in particular derived from *Actinomycetes* such as *Rhodococcus* and *Nocardia* sp.) are the catalysts of choice for more highly substituted 2,2- and 2,3-disubstituted epoxides.

These trends are exemplified as follows (Scheme 2.91). Monosubstituted oxiranes represent highly flexible and rather 'slim' molecules, which make chiral recognition a difficult task [600][25], [601–603]. Thus, the majority of attempts to achieve highly selective transformations using epoxide hydrolases from bacterial and fungal origin failed for this class of substrates. The only notable exceptions were found among red yeasts, such as *Rhodotorula araucarae* CBS 6031, *Rhodosporidium toruloides* CBS 349, *Trichosporon* sp. UOFS Y-1118, and *Rhodotorula glutinis* CIMW 147. Regardless of the enzyme source, the enantiopreference for the (*R*)-enantiomer was predominant and the regioselectivity prevailed for the sterically less hindered carbon atom.

Styrene oxide-type epoxides have to be regarded as a special group of substrates, as they possess a benzylic carbon atom, which facilitates the formation of a carbenium ion through resonance stabilization by the adjacent aromatic moiety

[25]It must be emphasized that the microorganism used in this study (*Chryseomonas luteola*) causes inner ear infections in infants and belongs to safety Class II, see [599].



R^1	R^2	R^3	Enzyme source[a]	Selectivity[b]
CH_2Cl, $C(CH_3)_2O(CO)$*t*-Bu CH_2OCH_2Ph, *t*-C_4H_9	H	H	BEH	–
n-C_3H_7, *n*-C_4H_9, *n*-C_5H_{11}, *n*-C_6H_{13}, *n*-C_8H_{17}, *n*-$C_{10}H_{21}$	H	H	BEH	±
n-C_6H_{13}	H	H	FEH	–
CH_2OH, CH_2Cl, CH_2OCH_2Ph	H	H	YEH	– to ±
CH_3, Et	H	H	YEH	+
n-C_3H_7, *n*-C_4H_9, *n*-C_5H_{11}, *n*-C_6H_{13}	H	H	YEH	++

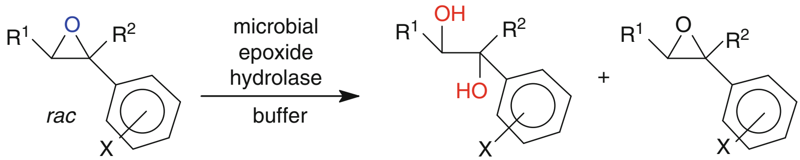
[a]BEH = bacterial, FEH = fungal and YEH = yeast epoxide hydrolase;
[b]enantioselectivities are denoted as (–) = low (*E* <4), (±) = moderate (*E* = 4–12), (+) = good (*E* = 13–50), (++) excellent (*E* >50).

Scheme 2.91 Microbial resolution of monosubstituted epoxides

(Scheme 2.92). Thus, attack at this position is electronically facilitated, although it is sterically hindered, and mixed regiochemical pathways (proceeding via retention *and* inversion) are particularly common within this group of substrates. As a consequence, any *E*-values reported have to be regarded with great caution, as long as the regioselectivity has not been clearly elucidated. The biocatalysts of choice were found among the fungal epoxide hydrolases, such as *Aspergillus niger* LCP 521 [604], *Beauveria densa* CMC 3240 and *Beauveria bassiana* ATCC 7159. Under certain circumstances, *Rhodotorula glutinis* CIMW 147 might serve as well [605–607].

Among the sterically more demanding substrates, 2,2-disubstituted oxiranes were hydrolyzed in virtually complete enantioselectivities using enzymes from bacterial sources ($E > 200$), in particular *Mycobacterium* NCIMB 10420, *Rhodococcus* (NCIMB 1216, DSM 43338, IFO 3730) and closely related *Nocardia* spp. (Scheme 2.93) [608, 609]. All bacterial epoxide hydrolases exhibited a preference for the (*S*)-enantiomer. In those cases where the regioselectivity was determined, attack was found to exclusively occur at the unsubstituted oxirane carbon atom.

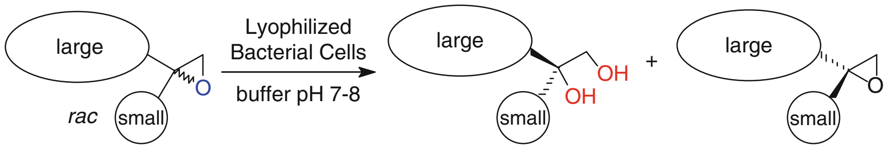
In contrast to 2,2-disubstituted epoxides, mixed regioselectivities are common for 2,3-disubstituted analogs and, as a consequence, *E*-values are not applicable (Table 2.3, Scheme 2.91) [610]. This is understandable, bearing in mind that the steric requirements at both oxirane positions are similar. Whereas fungal enzymes



R^1	R^2	X	Enzyme source[a]	Selectivity[b]
H	H	*p*-CH_3, *o*-Cl, *p*-Cl	BEH	±
H	CH_3	H	BEH	±
H	H	*o*-CH_3, *o*-Hal	YEH	–
H	H	H	YEH	±
H	H	*p*-F, *p*-Cl, *p*-Br, *p*-CH_3	YEH	+
CH_3	H	H	YEH	++
H	CH_3	H	FEH	–
	indene oxide		FEH	+
CH_3	H	H	FEH	++
H	H	H	FEH	++
H	H	*p*-NO_2	FEH	++

[a]BEH = bacterial, FEH = fungal and YEH = yeast epoxide hydrolase;
[b]enantioselectivities are denoted as (–) = low (*E* <4), (±) = moderate (*E* = 4–12), (+)=good (*E* = 13–50), (++) excellent (*E* >50).

Scheme 2.92 Microbial resolution of styrene oxide-type oxiranes



Small	Large	Enzyme source[a]	Selectivity[b]
CH_3	*n*-C_5H_{11}	FEH	±
CH_3	$(CH_2)_2Ph$, CH_2Ph	BEH	±
C_2H_5	*n*-C_5H_{11}	BEH	+
CH_3	*n*-C_4H_9, *n*-C_5H_{11}, *n*-C_7H_{15}, *n*-C_9H_{19}, $(CH_2)_4Br$, $(CH_2)_3CH{=}CH_2$	BEH	++

[a]BEH = bacterial, FEH = fungal and YEH = yeast epoxide hydrolase;
[b]enantioselectivities are denoted as (–) = low (*E* <4), (±) = moderate (*E* = 4–12), (+) = good (*E* = 13–50), (++) excellent (*E* >50).

Scheme 2.93 Enzymatic resolution of 2,2-disubstituted epoxides using microbial epoxide hydrolases

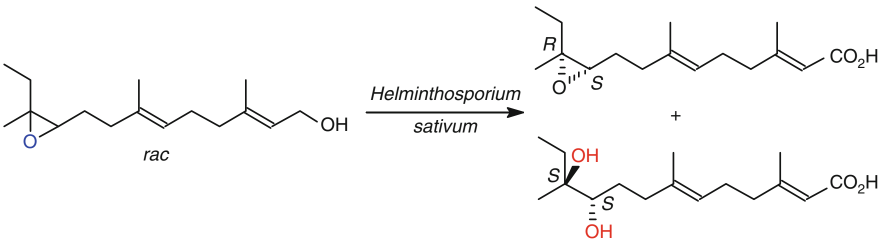
Table 2.3 Microbial resolution of 2,3-disubstituted epoxides (for substrate structures see Scheme 2.91)

R^1	R^2	R^3	Enzyme source[a]	Selectivity[b]
CH_3	H	n-C_5H_{11}	FEH	±
H	CH_3	n-C_5H_{11}	FEH	±
CH_3	H	CH_3	YEH	++
H	CH_3	CH_3	YEH	++
H	C_2H_5	n-C_3H_7	BEH	±
C_2H_5	H	n-C_4H_9	BEH	±
H	CH_3	n-C_4H_9, n-C_5H_{11}, n-C_9H_{19}	BEH	++
CH_3	H	n-C_4H_9	BEH[c]	++

[a]BEH = bacterial, FEH = fungal, YEH = yeast epoxide hydrolase
[b]enantio selectivities are denoted as (–) = low ($E < 4$), (±) = moderate (E = 4–12), (+) = good (E = 13–50), (++) excellent ($E > 50$)
[c]Enantioconvergent pathway, i.e., a sole stereoisomeric diol was formed

were less useful, yeast and bacterial epoxide hydrolases proved to be highly selective.

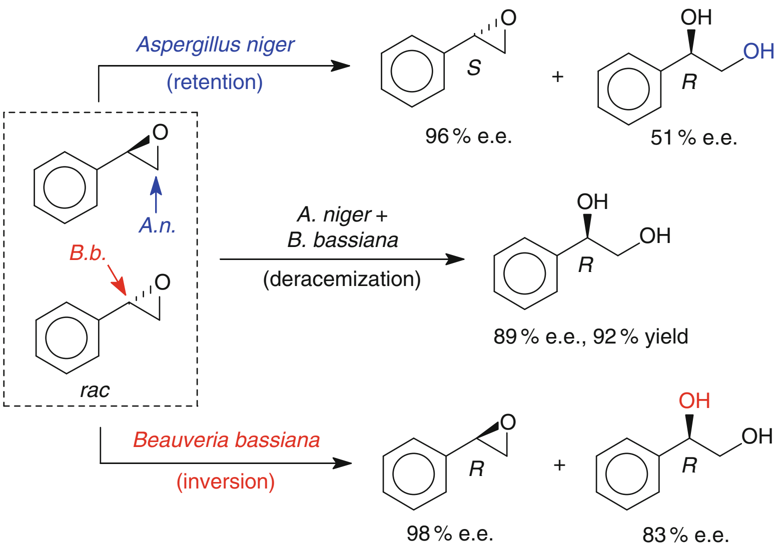
To date, only limited data are available on the enzymatic hydrolysis of trisubstituted epoxides [611–613]. For example, a racemic allylic terpene alcohol containing a *cis*-trisubstituted epoxide moiety was hydrolyzed by whole cells of *Helminthosporium sativum* to yield the (*S*,*S*)-diol with concomitant oxidation of the terminal alcoholic group (Scheme 2.94). The mirror image (*R*,*S*)-epoxide was not transformed. Both optically pure enantiomers were then chemically converted into a juvenile hormone [614].



Scheme 2.94 Microbial resolution of a trisubstituted epoxide

In order to circumvent the disadvantages of kinetic resolution, several protocols were developed towards the *enantioconvergent* hydrolysis of epoxides, which lead to a single enantiomeric vicinal diol as the sole product from the racemate.

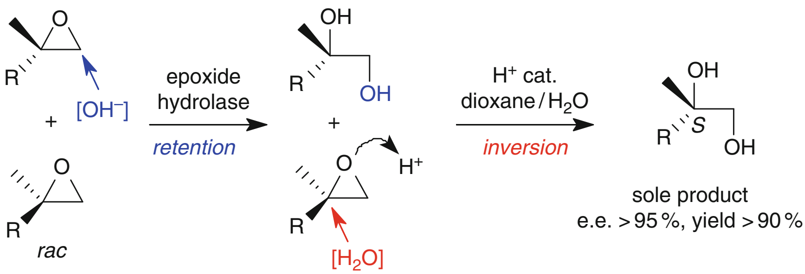
The first technique made use of two fungal epoxide hydrolases possessing matching opposite regio- and enantioselectivity for styrene oxide (Scheme 2.95) [615]. Resting cells of *Aspergillus niger* hydrolyzed the (*R*)-epoxide via attack at the less hindered carbon atom to yield the (*R*)-diol of moderate optical purity. The (*S*)-epoxide remained unchanged and was recovered in 96% e.e. In contrast, *Beauveria bassiana* exhibited the *opposite* enantio- and regioselectivity. It hydrolyzed the (*S*)-enantiomer but with an unusual *inversion of configuration* via attack at the



Scheme 2.95 Microbial resolution and deracemization of styrene oxide

more hindered benzylic position. As a result, the (*R*)-diol was obtained from the (*S*)-epoxide leaving the (*R*)-epoxide behind. By combining both microbes in a single reactor, an elegant deracemization technique was accomplished making use of both stereo-complementary pathways. Whereas *Aspergillus* hydrolyzed the (*R*)-epoxide with retention, *Beauveria* converted the (*S*)-counterpart with inversion. As a result, (*R*)-phenylethane-1,2-diol was obtained in 89% e.e. and 92% yield.

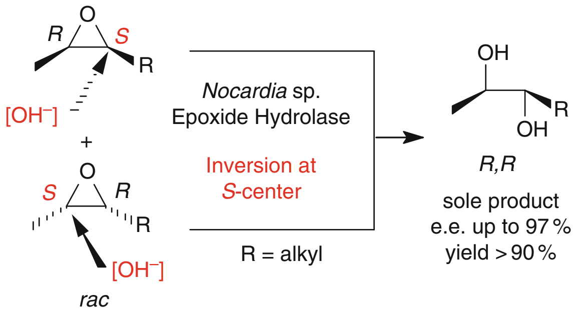
For 2,2-disubstituted oxiranes, this technique was not applicable because an enzyme to perform a highly unfavored nucleophilic attack on a fully substituted carbon atom would be required. In this case, a two-step sequence consisting of combined bio- and chemocatalysis was successful (Scheme 2.96) [616]. In the first step, 2,2-disubstituted oxiranes were kinetically resolved by using bacterial epoxide hydrolases in excellent selectivity. The biohydrolysis proceeds exclusively via attack at the unsubstituted carbon atom with complete *retention* at the stereogenic center. By



Scheme 2.96 Deracemization of 2,2-disubstituted oxiranes using combined bio- and chemo-catalysis

contrast, acid-catalyzed hydrolysis of the remaining nonconverted enantiomer under carefully controlled conditions proceeds with *inversion*. Thus, combination of both steps in a one-pot resolution-inversion sequence yields the corresponding (*S*)-1,2-diols in virtually enantiopure form and in excellent yields (>90%).

An exceptional case for an enantioconvergent biocatalytic hydrolysis of a (±)-*cis*-2,3-epoxyalkane is shown in Scheme 2.97 [617]. Based on ^{18}O-labeling experiments, the stereochemical pathway of this reaction was elucidated to proceed via attack of the (formal) hydroxyl ion at the (*S*)-configured oxirane carbon atom with concomitant *inversion* of configuration at both enantiomers with *opposite* regioselectivity. As a result, the (*R*,*R*)-diol was formed as the sole product in up to 97% e.e. in almost quantitative yield.



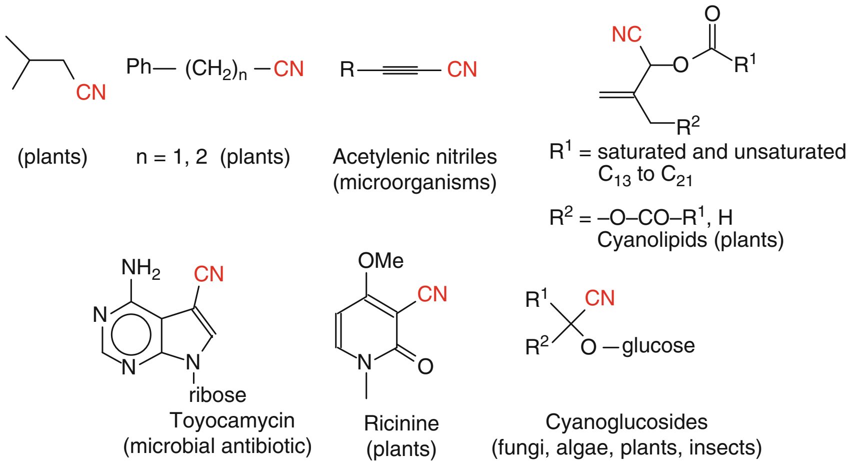
Scheme 2.97 Deracemization of 2,3-disubstituted oxiranes via enantioconvergent enzymatic hydrolysis

As an alternative to the enzymatic hydrolysis of epoxides, nonracemic vicinal diols may be obtained from epoxides via the nucleophilic ring-opening by nitrite catalyzed by halohydrin dehalogenase (a lyase). The corresponding nitrite-monoesters are spontaneously hydrolyzed to yield diols. For the application of this technique see Sect. 2.7.2.

2.1.6 Hydrolysis of Nitriles

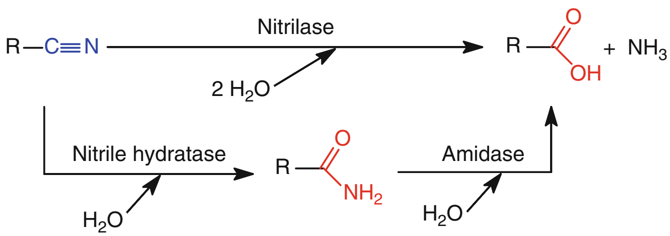
Organic compounds containing nitrile groups are found in the environment not only as natural products but also as a result of human activities [618]. Naturally occurring nitriles are synthesized by plants, fungi, bacteria, algae, sponges, and insects, but not by mammals. Cyanide is highly toxic to living cells and interferes with biochemical pathways by three major mechanisms:

- Tight chelation to di- and trivalent metal atoms in metalloenzymes such as cytochromes
- Addition onto aldehydes or ketones to form cyanohydrin derivatives
- Reaction with Schiff-base intermediates (e.g., in transamination reactions) to form stable nitrile derivatives [619]



Scheme 2.98 Naturally occurring organic nitriles

As shown in Scheme 2.98, natural nitriles include cyanogenic glucosides which are produced by a wide range of plants including major crops such as cassava [620] and sorghum (millet). Plants and microorganisms are also able of producing aliphatic or aromatic nitriles, such as cyanolipids, ricinine, and phenylacetonitrile [621]. These compounds can serve not only as a nitrogen storage, but also as protecting agents against attack by hungry predators, following the general philosophy that if one species has developed a defence mechanism, an invader will try to undermine it with a counterstrategy. As a consequence, it is not unexpected that there are several biochemical pathways for nitrile degradation, such as oxidation and – more important – by hydrolysis. Enzyme-catalyzed hydrolysis of nitriles may occur via two different pathways depending on steric and electronic factors of the substrate structure [622–625] (Scheme 2.99).



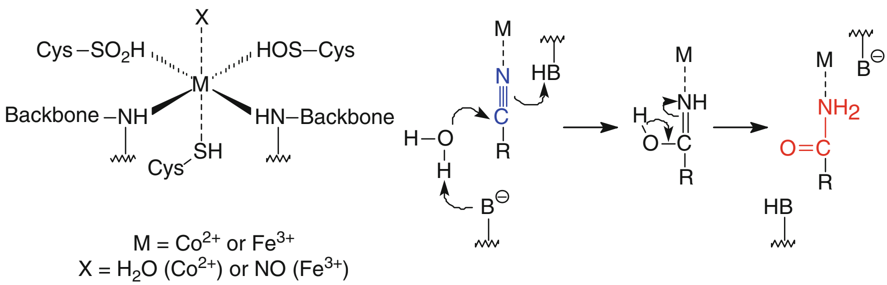
Scheme 2.99 General pathways of the enzymatic hydrolysis of nitriles

- Aliphatic nitriles are often metabolized in two stages. First they are converted to the corresponding carboxamide by a *nitrile hydratase* and then to the carboxylic acid by an *amidase* enzyme (a protease) [626].

- Aromatic, heterocyclic, and certain unsaturated aliphatic nitriles are often directly hydrolyzed to the corresponding acids without formation of the intermediate free amide by a so-called *nitrilase* enzyme. The nitrile hydratase and nitrilase enzyme use distinctively different mechanisms of action.

Nitrile hydratases are known to possess a tightly bound metal atom (Co^{2+} or Fe^{3+} [627]) which is required for catalysis [628–631]. Detailed studies revealed that the central metal is octahedrally coordinated to two NH-amide groups from the backbone and three Cys–SH residues, two of which are post-translationally modified into a Cys-sulfenic (–SOH) and a Cys-sulfinic (–SO_2H) moiety. This claw-like setting is required to firmly bind the non-heme iron or the non-corrinoid cobalt [632–634] (Scheme 2.100). The remaining axial ligand (X) is either a water molecule (Co^{2+}) [635] or nitric oxide (NO) which binds to Fe^{3+} [636, 637]. Quite remarkably, the activity of the latter protein is regulated by light: in the dark, the enzyme is inactive, because NO occupies the binding site for the substrate. Upon irradiation with visible light, NO dissociates and activity is switched on.

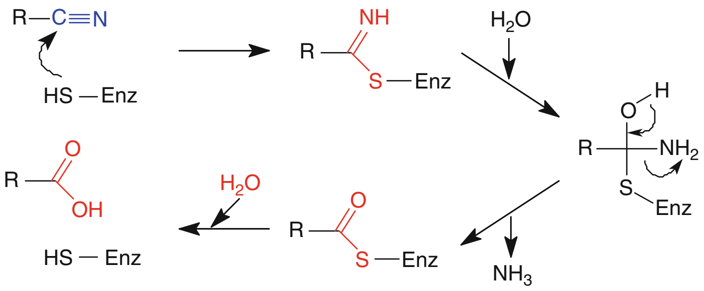
Three proposals for the mechanism of metal-depending nitrile hydratases have been suggested, the most plausible assumes direct corrdination of the nitrile to the metal, which (by acting as Lewis-acid) increases the electrophilicity of the carbon atom to allow attack of a water-molecule. The hydroxy-imino-species thus formed tautomerizes to form the carboxamide [638–640].



Scheme 2.100 Coordination sphere of the ferric ion in *Brevibacterium* sp. nitrile hydratase

On the other hand, nitrilases operate by a completely different mechanism (Scheme 2.101). They possess neither coordinated metal atoms, nor cofactors, but act through an essential nucleophilic sulfhydryl residue of a cysteine [641, 642], which is encoded in the nitrilase-sequence motif Glu–Lys–Cys [643]. The mechanism of nitrilases is similar to general base-catalyzed nitrile hydrolysis: Nucleophilic attack by the sulfhydryl residue on the nitrile carbon atom forms an enzyme-bound thioimidate intermediate, which is hydrated to give a tetrahedral intermediate. After the elimination of ammonia, an acyl-enzyme intermediate is formed, which (like in serine hydrolases) is hydrolyzed to yield a carboxylic acid [644].

Enzymatic hydrolysis of nitriles is not only interesting from an academic standpoint, but also from a biotechnological point of view [645–652]. Cyanide



Scheme 2.101 Mechanism of nitrilases

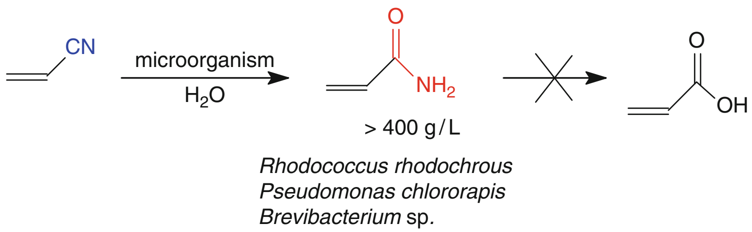
represents a widely applicable C_1-synthon – a ‘water-stable carbanion’ – but the conditions usually required for the chemical hydrolysis of nitriles present several disadvantages. The reactions usually require either strongly acidic or basic media incompatible with other hydrolyzable groups that may be present. Furthermore, energy consumption is high and unwanted side-products such as cyanide itself or considerable amounts of salts are formed during neutralization. Using enzymatic methods, conducted at physiological pH, most of these drawbacks can be avoided. Additionally, these transformations can often be achieved in a chemo-, regio-, and enantioselective manner. Due to the fact that isolated nitrile-hydrolyzing enzymes are often very sensitive [623], the majority of transformations have been performed using sturdy whole-cell systems.

Another important aspect is the enzymatic hydrolysis of cyanide for the detoxification of industrial effluents [653–656].

Chemoselective Hydrolysis of Nitriles

The microorganisms used as sources of nitrile-hydrolyzing enzymes usually belong to the genera *Bacillus*, *Brevibacterium*, *Micrococcus*, *Rhodococcus*, *Pseudomonas*, and *Bacteridium* and they generally show a broad metabolic diversity. Depending on the source of carbon and nitrogen – acting as ‘inducer’ – added to the culture medium, either nitrilases or nitrile hydratases are predominantly produced by the cell. Thus, the desired hydrolytic pathway leading to an amide or a carboxylic acid can often be biologically ‘switched on’ during the growth of the culture by using aliphatic or aromatic nitriles as inducers. In order to avoid substrate inhibition (which is a more common phenomenon with nitrile-hydrolyzing enzymes than product inhibition [657]) the substrates are fed continuously to the culture.

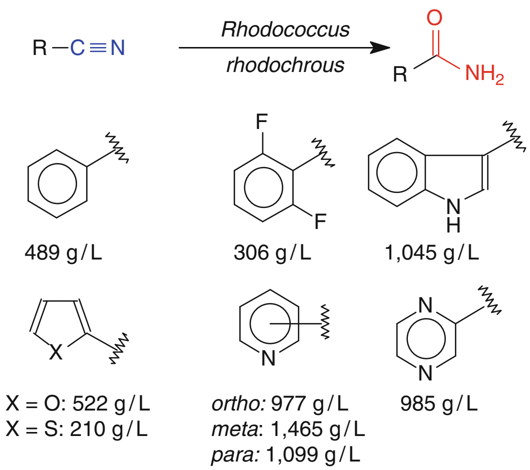
Acrylamide is one of the most important commodity chemicals for the synthesis of various polymers and is produced in an amount of about 200,000 t/year worldwide. In its conventional synthesis, the hydration of acrylonitrile is performed with copper catalysts. However, the preparative procedure for the catalyst, difficulties in its regeneration, problems associated with separation and purification of the formed acrylamide, undesired polymerization and over-hydrolysis are serious drawbacks. Using whole cells of *Brevibacterium* sp. [658, 659], *Pseudomonas chlororapis* [660, 661] or *Rhodococcus rhodochrous* [662] acrylonitrile can be converted into acrylamide in yields of >99%; the formation of byproducts such as acrylic acid is circumvented by blocking of the amidase activity. The scale of this biotransformation exceeds 30,000 t/year (Scheme 2.102).



Scheme 2.102 Chemoselective microbial hydrolysis of acrylonitrile

Aromatic and heteroaromatic nitriles were selectively transformed into the corresponding amides by a *Rhodococcus rhodochrous* strain [663]; the products accumulated in the culture medium in significant amounts (Scheme 2.103). In contrast to the hydrolysis performed by chemical means, the biochemical transformations were highly selective and occurred without the formation of the corresponding carboxylic acids.

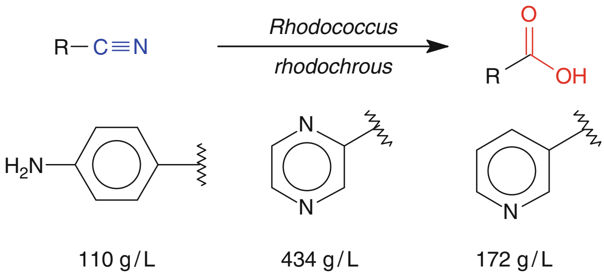
Even more important from a commercial standpoint was that *o*-, *m*-, and *p*-substituted cyanopyridines were accepted as substrates [664, 665] to give picolinamide (a pharmaceutical), nicotinamide (a vitamin), and isonicotinamide



Scheme 2.103 Chemoselective microbial hydrolysis of aromatic and heteroaromatic nitriles yielding carboxamides (product concentrations)

(a precursor for isonicotinic acid hydrazide, a tuberculostatic) (Scheme 2.103). Extremely high productivities were obtained due to the fact that the less soluble carboxamide product readily crystallized from the reaction medium in 100% purity. Nicotinamide – enzymatically produced on a scale of 6,000 t/year – is an important nutritional factor and is therefore widely used as a vitamin additive for food and feed supplies [666]. Pyrazinamide is used as a tuberculostatic.

By changing the biochemical pathway through using modified culture conditions, the enzymatic pathways of nitrile hydrolysis are switched and the corresponding carboxylic acids can be obtained (see Scheme 2.104). For instance, *p*-aminobenzoic acid, a member of the vitamin B group, was obtained from *p*-aminobenzonitrile using whole cells of *Rhodococcus rhodochrous* [667]. Similarly, the antimycobacterial agent pyrazinoic acid was prepared in excellent purity from cyanopyrazine [668]. Like nicotinamide, nicotinic acid is a vitamin used as an animal feed supplement, in medicine, and also as a biostimulator for the formation of activated sludge. Microbial hydrolysis of 3-cyanopyridine using *Rhodococcus rhodochrous* [669] or *Nocardia rhodochrous* [670] proceeds quantitatively, whereas chemical hydrolysis is hampered by moderate yields.

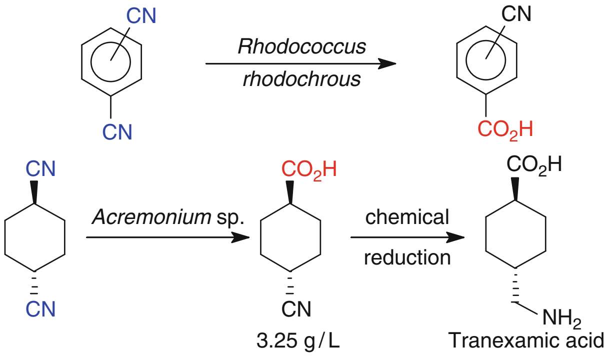


Scheme 2.104 Chemoselective microbial hydrolysis of aromatic and heteroaromatic nitriles yielding carboxylic acids (product concentrations)

Regioselective Hydrolysis of Dinitriles

The selective hydrolysis of one nitrile group out of several in a molecule is generally impossible using traditional chemical catalysis and the reactions usually result in the formation of complex product mixtures. In contrast, whole microbial cells can be very efficient for this purpose [671] (Scheme 2.105).

For instance, 1,3- and 1,4-dicyanobenzenes were selectively hydrolyzed by *Rhodococcus rhodochrous* to give the corresponding monoacids [672, 673]. In the aliphatic series, tranexamic acid (*trans*-4-aminomethyl-cyclohexane-1-carboxylic acid), which is a hemostatic agent, is synthesized from *trans*-1,4-dicyanocyclohexane. Complete regioselective hydrolysis was achieved by using an *Acremonium* sp. [674]. The outcome of regioselective nitrile hydrolysis is believed to depend on the distance of the nitrile moieties and the presence of other polar groups within the substrate [675, 676].

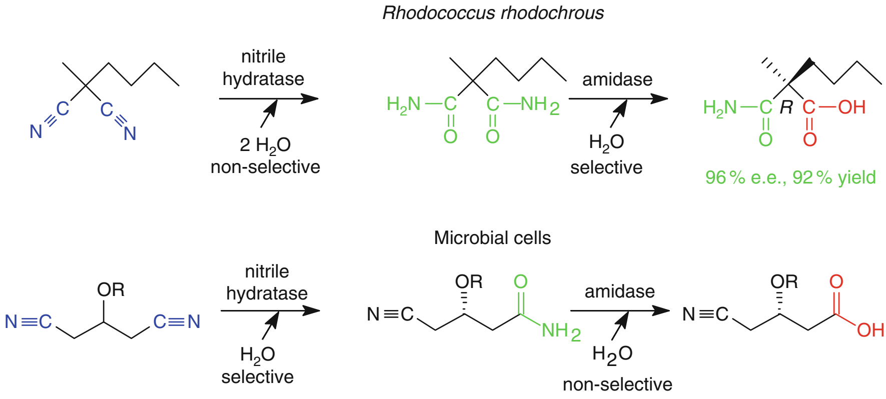


Scheme 2.105 Regioselective microbial hydrolysis of dinitriles

Enantioselective Hydrolysis of Nitriles
While most biocatalytic hydrolyses of nitriles make use of the mild reactions conditions and the chemo- and regioselectivity of nitrile-hydrolyzing enzymes, their stereoselectivity has been investigated more recently. It seems to be a common trend that both nitrilases and nitrile hydratases are often less specific with respect to the chirality of the substrate and that enantiodiscrimination often occurs during the hydrolysis of an intermediate carboxamide an amidase [677] (Scheme 2.99). As a rule, the 'natural' L-configured enantiomer is usually converted into the acid leaving the D-counterpart behind. This is not unexpected bearing in mind the high specificities of proteases on α-substituted carboxamides (see Sect. 2.1.2).

Desymmetrization of Prochiral Dinitriles. Prochiral α,α-disubstituted malononitriles can be hydrolyzed in an asymmetric manner by the aid of *Rhodococcus rhodochrous* [678] (Scheme 2.106). In accordance with the above-mentioned trend, the dinitrile was nonselectively hydrolyzed by the nitrile hydratase in the cells to give the dicarboxamide. In a second consecutive step, the latter was subsequently transformed by the amidase with high selectivity for the pro-(*R*) amide group to yield the (*R*)-amide-acid in 96% e.e. and 92% yield. This pathway was confirmed by the fact that identical results were obtained when the dicarboxamide was used as substrate. The nonracemic amide-acid product thus obtained serves as a starting material for the synthesis of nonnatural α-methyl-α-amino acids [679].

In contrast, prochiral glutarodinitriles were stereoselectively hydrolyzed via two steps using whole microbial cells: in a first step, a stereoselective nitrile hydratase furnished the monoamide, which was further hydrolyzed to the corresponding carboxylic acid by an amidase [646, 680]. The cyano-acids thus obtained served as buiding blocks for the synthesis of cholesterol-lowering drugs from the statin family. An impressive example for the development of stereoselective enzymes derived from the metagenome is the discovery of >130 novel nitrilases from biotope-specific environmental DNA libraries [681, 682]. Among these enzymes, 22 nitrilases showed (*S*)-selectivity for the desymmetrization of the unprotected

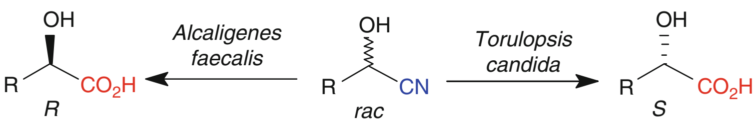


R	Microorganism	e.e. [%]
$-CH_2-Ph$	*Brevibacterium* R312	88
$-CH_2-Ph$	*Rhodococcus butanica* ATCC 21197	90
–CO–Ph	*Rhodococcus butanica* ATCC 21197	99

Scheme 2.106 Asymmetric microbial hydrolysis of a prochiral dinitrile

glutarodinitile (Scheme 2.106, R = H), while one produced the mirror-image (*R*)-enantiomer in 95–98% e.e. [683].

Kinetic and Dynamic Resolution of *rac*-Nitriles. α-Hydroxy and α-amino acids can be obtained from the corresponding α-hydroxynitriles (cyanohydrins) and α-aminonitriles [684], which are easily synthesized in racemic form from the corresponding aldehyde precursors by addition of hydrogen cyanide or a Strecker synthesis, respectively (Schemes 2.107 and 2.108). In aqueous systems, cyanohydrins are stereochemically labile and undergo spontaneous racemization via HCN elimination, which furnishes a dynamic resolution process. From aliphatic *rac*-cyanohydrins, whole cells of *Torulopsis candida* yielded the corresponding (*S*)-α-hydroxy acids [685], while (*R*)-mandelic acid is produced from *rac*-mandelonitrile

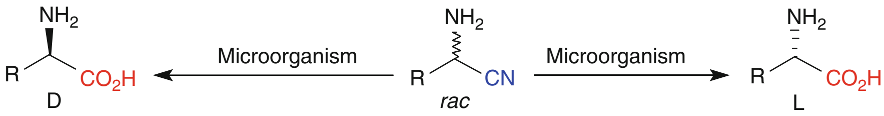


R	Microorganism	e.e. α-Hydroxy acid [%]
$(CH_3)_2CH-$	*Torulopsis candida*	>90
$(CH_3)_2CH-CH_2-$	*Torulopsis candida*	>95
Ph–	*Alcaligenes faecalis*	~100

Scheme 2.107 Stereocomplementary enantioselective hydrolysis of α-hydroxynitriles

on an industrial scale by employing resting cells of *Alcaligenes faecalis* [686] in almost >90 yield [687, 688].

In a related fashion, α-aminonitriles are enzymatically hydrolyzed to yield α-amino acids (Scheme 2.108). Whereas the enantiorecognition in *Brevibacterium imperiale* or *Pseudomonas putida* occurs through an amidase [689, 690], *Rhodococcus rhodochrous* PA-43, *Acinetobacter* sp. APN, and *Aspergillus fumigatus* possess enantiocomplementary nitrilases [689, 691, 692].



R	Microorganism	e.e. α-Amino acid [%]
Leu	*Rhodococcus rhodochrous*	90 (L)
Ala	*Rhodococcus rhodochrous*	57 (D)
Val	*Rhodococcus rhodochrous*	100 (L)
Met	*Rhodococcus rhodochrous*	96 (L)
PhGly	*Aspergillus fumigatus*	80 (L)
Ala	*Acinetobacter* sp.	74 (L)

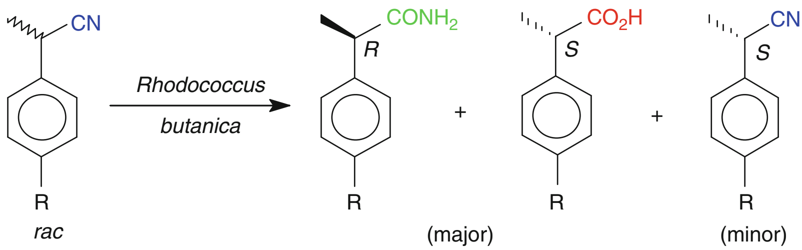
Scheme 2.108 Enantioselective hydrolysis of α-aminonitriles

Many kinetic resolutions of *rac*-nitriles were performed in search of a method to produce (*S*)-configurated α-arylpropionic acids, such as ketoprofen, ibuprofen, or naproxen, which are widely used as nonsteroidal antiinflammatory agents. Overall, enantioselectivities depended on the strain used, and whether a nitrilase- or nitrile hydratase-amidase pathway was dominant, which determines the nature of (enantiomeric) products consisting of a mixture of nitrile/carboxylic acid or amide/carboxylic acid, respectively [687, 693–696].

For organisms which express both pathways for nitrile hydrolysis, the stereochemical pathways can be very complex. The latter is illustrated by the microbial resolution of α-aryl-substituted propionitriles using a *Rhodococcus butanica* strain (Scheme 2.109) [697]. Formation of the 'natural' L-acid and the D-amide indicates the presence of an L-specific amidase and a nonspecific nitrile hydratase. However, the occurrence of the (*S*)-nitrile in case of Ibuprofen (R = *i*-Bu, e.e. 73%) proves the enantioselectivity of the nitrile hydratase [694]. In a related approach, *Brevibacterium imperiale* was used for the resolution of structurally related α-aryloxypropionic nitriles [698].

As a substitute for (expensive) commercial enzyme preparations for nitrile-hydrolysis, whole-cell preparations are recommended: *Rhodococcus* R312 [699][26]

[26]The strain was formerly denoted as *Brevibacterium* and is available as CBS 717.73.



R	e.e. Amide [%]	e.e. Acid [%]	e.e. Nitrile [%]
$(CH_3)_2CH–CH_2$–	99	87	73
Cl	76	>99	–
OCH_3	99	99	–

Scheme 2.109 Enantioselective hydrolysis of α-aryl propionitriles

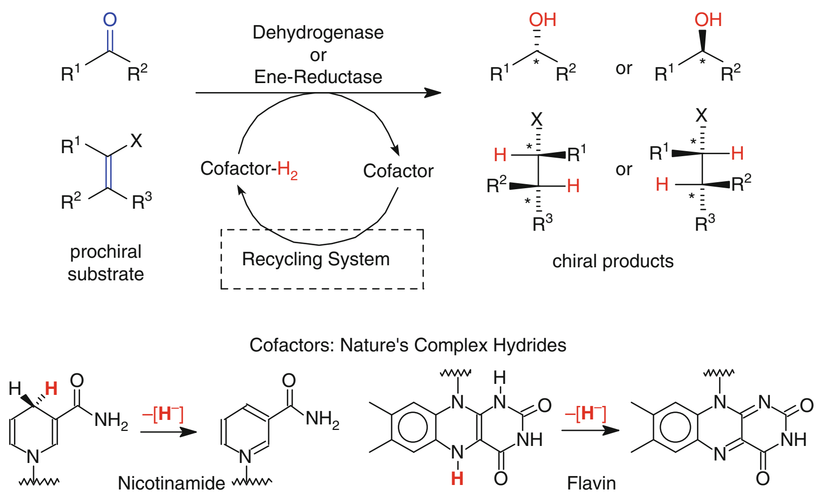
contains both nitrile-hydrolyzing metabolic pathways, whereas *Rhodococcus* DSM 11397 and *Pseudomonas* DSM 11387 contain only nitrile hydratase (no nitrilase) and nitrilase (no nitrile hydratase) activity, respectively [700].

2.2 Reduction Reactions

The enzymes employed in redox reactions are classified into three categories: dehydrogenases, oxygenases and oxidases [701–703]. Among them, alcohol dehydrogenases – also termed carbonyl reductases – have been widely used for the reduction of carbonyl groups (aldehydes, ketones) and ene-reductases are frequently employed for the bioreduction of (electronically activated) carbon-carbon double bonds. In contrast, the asymmetric bioreduction of C=N-bonds is only feasible for special types of substrates, such as in the reductive amination of α-keto acids yielding α-amino acids, but is generally not feasible for nonactivated Schiff-base type imines.

Since reduction usually implies the transformation of a planar sp^2-hybridized carbon into a tetrahedral sp^3-atom, it goes in hand with the generation of a stereogenic center and represents a desymmetrization reaction (Scheme 2.110). In contrast, the corresponding reverse process (e.g., alcohol oxidation or dehydrogenation) leads to the destruction of a chiral center, which is generally of limited use.

In contrast, oxygenases – named for using molecular oxygen as cosubstrate – have been shown to be particularly useful for oxidation reactions since they catalyze the functionalization of nonactivated C–H or C=C bonds, affording hydroxylation or epoxidation, respectively (Sect. 2.3). Oxidases, which are responsible for the transfer of electrons, have played a minor role in the biotransformation of nonnatural organic compounds, but they are increasingly used more recently (Sect. 2.3.3).



Scheme 2.110 Reduction reactions catalyzed by dehydrogenases

2.2.1 *Recycling of Cofactors*

The major and crucial distinction between redox enzymes and hydrolases described in the previous chapter, is that the former require redox cofactors, which donate or accept the chemical equivalents for reduction (or oxidation). For the majority of redox enzymes, nicotinamide adenine dinucleotide [NAD(H)] and its respective phosphate [NADP(H)] are required by about 80% and 10% of redox enzymes, respectively. Flavines (FMN, FAD) and pyrroloquinoline quinone (PQQ) are encountered more rarely. The nicotinamide cofactors – resembling 'Nature's complex hydrides' – have two features in common, i.e., they are relatively unstable molecules and they are prohibitively expensive if used in stoichiometric amounts.[27] In addition, they cannot be replaced by more economical man-made substitutes. Since it is only the *oxidation state* of the cofactor which changes during the reaction, while the remainder of the complex structure stays intact, it may be regenerated in-situ by using a second concurrent redox-reaction to allow it to re-enter the reaction cycle. Thus, the expensive cofactor is needed only in catalytic amounts, which leads to a drastic reduction in cost. The efficiency of such a recycling process is measured by the number of cycles which can be achieved before a cofactor molecule is finally destroyed. It is expressed as the 'total turnover number' (TTN, Sect. 1.4.2) – which is the total number of moles of product formed per mole of cofactor during its entire lifetime. As a rule of thumb, a few thousand cycles (10^3–10^4) are sufficient for redox reactions on a laboratory scale, whereas for technical purposes, total turnover

[27]The current bulk prices for one mole are: NAD^+ US $710, NADH US $3,000, $NADP^+$ US $5,000 and NADPH US $215,000; retail prices are about ten times higher.

numbers of at least 10^5 are highly desirable. The economic barrier to large-scale reactions posed by cofactor costs has been recognized for many years and a large part of the research effort concerning dehydrogenases has been expended in order to solve the problem of cofactor recycling [521, 704–707].

Cofactor recycling is no problem when whole microbial cells are used as biocatalysts for redox reactions. In this case, inexpensive sources of redox equivalents such as carbohydrates can be used since the microorganism possesses all the enzymes and cofactors which are required for metabolism. The advantages and disadvantages of using whole-cell systems are discussed in Sect. 2.2.3.

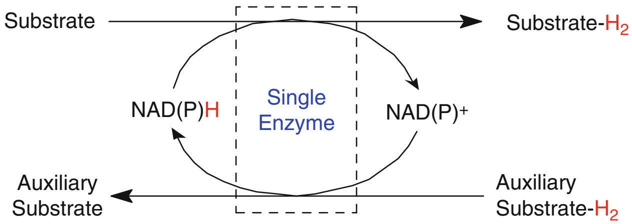
Recycling of Reduced Nicotinamide Cofactors

The easiest and least efficient method of regenerating NADH from NAD^+ is the nonenzymic reduction using a reducing agent such as sodium dithionite ($Na_2S_2O_4$) [708]. Since the corresponding turnover numbers of this process are very low (TTN $\leq$ 100), this method has only historical interest. Similarly, electrochemical [709–711] and photochemical regeneration methods [712–715] suffer from insufficient electron transport causing side-reactions and show low to moderate turnover numbers (TTN $\leq$ 1,000).[28] On the other hand, enzymic methods for NADH or NADPH recycling have been shown to be much more efficient and nowadays these represent the methods of choice. They may be conveniently subdivided into coupled-substrate and coupled-enzyme types.

Coupled-Substrate Process. Aiming at keeping things as simple as possible, the cofactor required for the transformation of the main substrate is constantly regenerated by addition of a second auxiliary substrate (H-donor) which is transformed by the *same* enzyme, but into the *opposite* direction (Scheme 2.111) [716–718]. To shift the equilibrium of the reaction in the desired direction, the donor must be applied in excess [719]. In principle, this approach is applicable to both directions of redox reactions [720]. Although the use of a single enzyme simultaneously catalyzing two reactions appears elegant, some significant disadvantages are often encountered in coupled-substrate cofactor recycling:

- The overall efficiency of the process is limited since the enzyme's activity is distributed between both the substrate and the hydrogen donor/acceptor.
- Enzyme deactivation is frequently encountered when highly reactive carbonyl species such as acetaldehyde or cyclohexenone are involved in the recycling process.
- Enzyme inhibition caused by the high concentrations of the auxiliary substrate – cosubstrate inhibition – is common.
- The product has to be purified from large amounts of auxiliary substrate used in excess.

[28] For example, if the reduction of $NAD(P)^+$ to NAD(P)H is 95% selective for hydride transfer onto the *p*-position of the nicotinamide ring, after 100 turnovers the residual activity of the cofactor would be 0.95^{100} being equivalent to only ~0.6%.

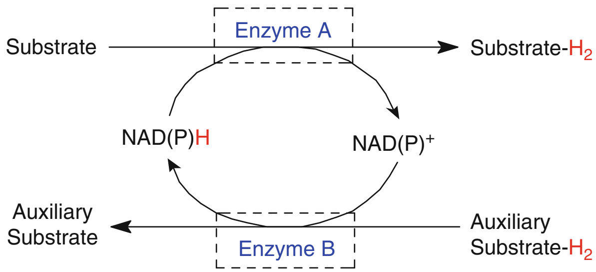


Scheme 2.111 Cofactor recycling by the coupled-substrate method

A special technique avoiding some of these drawbacks makes use of gas-membranes and is discussed in Sect. 3.2.

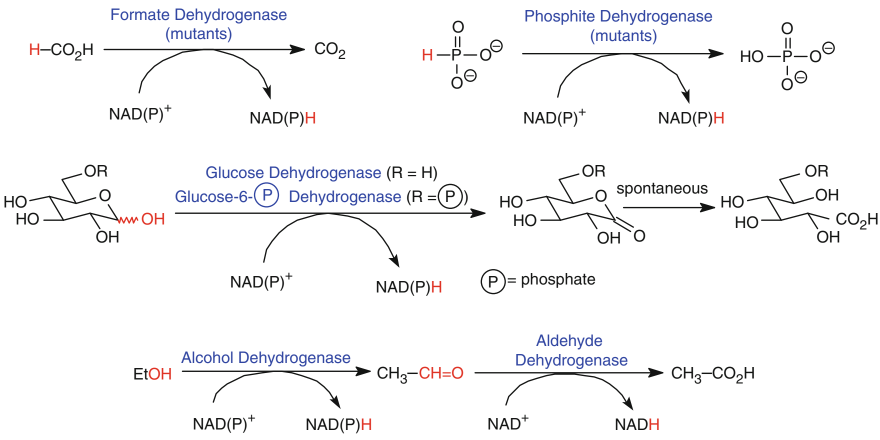
Coupled-Enzyme Approach. The use of two independent enzymes is more advantageous (Scheme 2.112). In this case, the two parallel redox reactions – i.e., conversion of the main substrate plus cofactor recycling – are catalyzed by *two different* enzymes [721]. To achieve optimal results, both of the enzymes should have sufficiently different specificities for their respective substrates whereupon the two enzymatic reactions can proceed independently from each other and, as a consequence, both the substrate and the auxiliary substrate do not have to compete for the active site of a single enzyme, but are efficiently converted by the two biocatalysts independently.

Several excellent methods, each having its own particular pros and cons, have been developed to regenerate NADH. On the other hand, NADPH may be regenerated sufficiently on a lab scale but a really inexpensive and reliable method is still needed for industrial-scale applications.



Scheme 2.112 Cofactor recycling by the coupled-enzyme method

The best and most widely used method for recycling NADH uses formate dehydrogenase (FDH), which is obtained from methanol-utilizing microorganisms, to catalyze the oxidation of formate to CO_2 (Scheme 2.113) [722, 723]. This method has the advantage that both the auxiliary substrate and the coproduct are



Scheme 2.113 Enzymatic regeneration of reduced nicotinamide cofactors

innocuous to enzymes and are easily removed from the reaction, which drives the reaction out of equilibrium. FDH is commercially available, readily immobilized and reasonably stable, if protected from autooxidation [724] and trace metals. The only disadvantage of this system is the high cost of FDH and its low specific activity (3 U/mg). However, both drawbacks can be readily circumvented by using an immobilized [725] or membrane-retained FDH system [726]. Overall, the formate/FDH system is the most convenient and most economical method for regenerating NADH, particularly for large-scale and repetitious applications, with TTNs (mol product/mol cofactor) approaching 600,000. The regeneration system based on FDH from *Candida boidinii* used as a technical-grade biocatalyst is limited by being specific for NADH [727]. This drawback has been circumvented by application of a genetically engineered formate dehydrogenase from *Pseudomonas* sp., which also accepts NADPH [728–730].

Another widely used method for recycling NAD(P)H makes use of the oxidation of glucose, catalyzed by glucose dehydrogenase (GDH, Scheme 2.113) [731, 732]. The equilibrium is shifted towards the product because the gluconolactone formed is spontaneously hydrolyzed to give gluconic acid. The glucose dehydrogenase from *Bacillus cereus* is highly stable [733] and accepts either NAD^+ or $NADP^+$ with high specific activity. Like FDH, however, GDH is expensive and product isolation from polar gluconate may complicate the workup. In the absence of purification problems, this method is attractive for laboratory use, and it is certainly a convenient way to regenerate NADPH.

Similarly, glucose-6-phosphate dehydrogenase (G6PDH) catalyzes the oxidation of glucose-6-phosphate (G6P) to 6-phosphoglucolactone, which spontaneously hydrolyzes to the corresponding phosphogluconate (Scheme 2.113). The enzyme from *Leuconostoc mesenteroides* is inexpensive, stable and accepts both NAD^+ and $NADP^+$ [540, 734], whereas yeast-G6PDH accepts only $NADP^+$. A major disadvantage of this system is the high cost of G6P. Thus, if used on a large scale, it must be enzymatically prepared from glucose using hexokinase and this involves the regeneration of ATP using kinases (see pp. 114–116). To avoid problems arising from multienzyme systems, glucose-6-sulfate and G6PDH from *Saccharomyces cerevisiae* may be used to regenerate NADPH [735]. The sulfate does not act as an acid catalyst for the hydrolysis of NADPH and is more easily prepared than the corresponding phosphate [736]. The G6P/G6PDH system complements glucose/GDH as an excellent method for regenerating NADPH and is a good method for regenerating NADH.

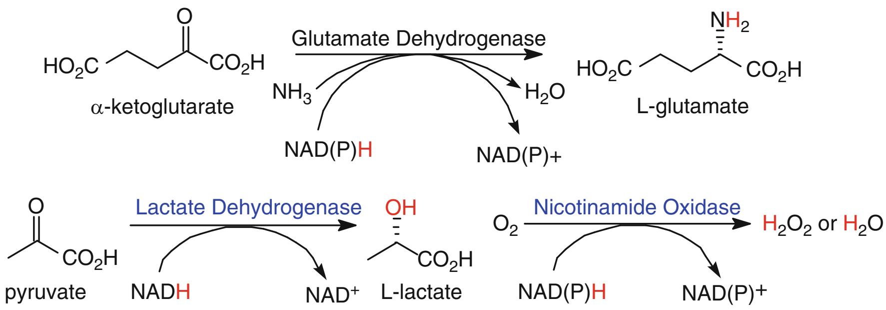
More recently, phosphite dehydrogenase has been shown to offer a promising alternative [737, 738]: The equilibrium is extremely favorable, both phosphite and phosphate are inoccuous to enzymes and act as buffer. The wild-type enzyme from *Pseudomonas stutzeri* accepts ony NAD^+ [739], but thermostable mutants were generated which are also able to reduce $NADP^+$ [740–742].

Ethanol and alcohol dehydrogenase (ADH) have been used extensively to regenerate NADH and NADPH [743, 744]. The low to moderate cost of ADH and the volatility of both ethanol and acetaldehyde make this system attractive for lab-scale reactions. An alcohol dehydrogenase from yeast reduces NAD^+, while an ADH from *Leuconostoc mesenteroides* is used to regenerate NADPH (Scheme 2.113). However, due to the low redox potential, only activated carbonyl substrates such as aldehydes and cyclic ketones are reduced in good yields. With other substrates, the equilibrium must be driven by using ethanol in excess or by removing acetaldehyde. The latter result may be achieved by sweeping with nitrogen [745] or by further oxidizing acetaldehyde to acetate [746], using aldehyde dehydrogenase thereby generating a second equivalent of reduced cofactor. All of these methods, however, give low TTNs or involve complex multi-enzyme systems. Furthermore, even low concentrations of ethanol or acetaldehyde inhibit or deactivate enzymes. Alternatively, a crude cell-free extract from baker's yeast has been recommended as an (unspecified) enzyme source for NADPH recycling by using glucose as the ultimate reductant [747].

A particularly attractive alternative for the regeneration of NADH makes use of hydrogenase enzymes, so called because they are able to accept molecular hydrogen directly as the hydrogen donor [748, 749]. The latter is strongly reducing, innocuous to enzymes and nicotinamide cofactors, and its consumption leaves no byproduct. For organic chemists, however, this method cannot be generally recommended because hydrogenase is usually isolated from strict anaerobic organisms. Thus, the enzyme is extremely sensitive to oxidation, is not commercially available and requires sophisticated fermentation procedures for its production. Furthermore, some of the organic dyes, which serve as mediators for the transport of redox equivalents from the donor onto the cofactor are relatively toxic (see below).

Recycling of Oxidized Nicotinamide Cofactors

For oxidation, reduction reactions can be run in reverse. The best and most widely applied method for the regeneration of nicotinamide cofactors in their oxidized form involves the use of glutamate dehydrogenase (GluDH) which catalyzes the reductive amination of α-ketoglutarate to give L-glutamate (Scheme 2.114) [750, 751]. Both NADH and NADPH are accepted as cofactors. In addition, α-keto-adipate can be used instead of the corresponding glutarate [752], leading to the formation of a high-value byproduct, L-α-aminoadipate.



Scheme 2.114 Enzymatic regeneration of oxidized nicotinamide cofactors

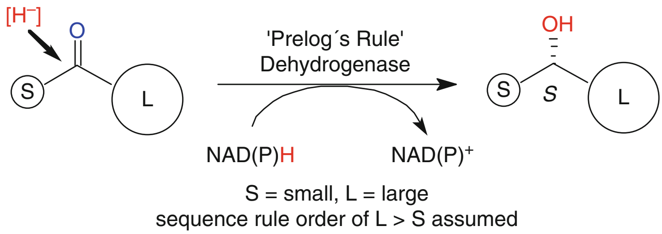
Using pyruvate together with lactate dehydrogenase (LDH) to regenerate NAD^+ offers the advantage that LDH is less expensive and exhibits a higher specific activity than GluDH [753]. However, the redox potential is less favorable and LDH does not accept $NADP^+$.

More recently, flavin-dependent nicotinamide oxidases, such as YcnD from *Bacillus subtilis* [754] or an enzyme from *Lactobacillus sanfranciscensis* [755] were employed for the oxidation of nicotinamide cofactors at the expense of molecular oxygen producing H_2O_2 or (more advantageous) H_2O via a two- or four-electron transfer reaction, respectively [756–758]. Hydrogen peroxide can be destroyed by addition of catalase and in general, both NADH and NADPH are accepted about equally well.

Acetaldehyde and yeast-ADH have also been used to regenerate NAD^+ from NADH [759]. Although reasonable total turnover numbers were achieved (10^3–10^4), the above-mentioned disadvantages of enzyme deactivation and self-condensation of acetaldehyde outweigh the merits of the low cost of yeast-ADH and the volatility of the reagents involved.

2.2.2 *Reduction of Aldehydes and Ketones Using Isolated Enzymes*

A broad range of ketones can be reduced stereoselectively using dehydrogenases to furnish chiral secondary alcohols [760–763]. During the course of the reaction, the enzyme delivers the hydride preferentially either from the *si*- or the *re*-side of the ketone to give (*R*)- or (*S*)-alcohols, respectively. The stereochemical course of the reaction, which is mainly dependent on the steric requirements of the substrate, may be predicted for most dehydrogenases from a simple model which is generally referred to as 'Prelog's rule' (Scheme 2.115) [764].



Dehydrogenase	Specificity	Cofactor	Commercially available
yeast-ADH	Prelog	NADH	+
horse liver-ADH	Prelog	NADH	+
Thermoanaerobium brockii-ADH	Prelog[a]	NADPH	+
Hydroxysteroid-DH	Prelog	NADH	+
Rhodococcus ruber ADH-A	Prelog	NADH	+
Rhodococcus erythropolis ADH	Prelog	NADH	+
Candida parapsilosis-ADH	Prelog	NADH	+
Lactobacillus brevis ADH	anti-Prelog	NADPH	+
Lactobacillus kefir-ADH	anti-Prelog	NADPH	+
Mucor javanicus-ADH	anti-Prelog	NADPH	–
Pseudomonas sp.-ADH	anti-Prelog	NADH	–

[a]anti-Prelog specificity on small ketones.

Scheme 2.115 Prelog's rule for the asymmetric reduction of ketones

It is based on the stereochemistry of microbial reductions using *Curvularia falcata* cells and it states that the dehydrogenase delivers the hydride from the *re*-face of a prochiral ketone to furnish the corresponding (*S*)-configured alcohol. The majority of the commercially available dehydrogenases used for the stereospecific reduction of ketones [such as yeast alcohol dehydrogenase (YADH), horse liver alcohol dehydrogenase (HLADH)] and the majority of microorganisms (for instance, baker's yeast) follow Prelog's rule [765]. *Thermoanaerobium brockii* alcohol dehydrogenase (TBADH) also obeys this rule, yielding (*S*)-alcohols when large ketones are used as substrates, but the stereopreference is reversed with small substrates. Microbial dehydrogenases which lead to the formation of anti-Prelog configurated

(*R*)-alcohols are known to a lesser extent, and even fewer are commercially available, e.g., from *Lactobacillus* sp. [766–768]. The substrate range of commercially available alcohol dehydrogenases has been mapped including aldehydes, (acyclic, aromatic, and unsaturated) ketones, diketones and various oxo-esters [769].

Yeast ADH has a very narrow substrate specificity and, in general, only accepts aldehydes and methyl ketones [770, 771]. Therefore, cyclic ketones and those bearing carbon chains larger than a methyl group are not accepted as substrates. Thus, YADH is only of limited use for the preparation of chiral secondary alcohols. Similarly, other ADHs from *Curvularia falcata* [772], *Mucor javanicus* and *Pseudomonas* sp. [773] are of limited use as long as they are not commercially available. The most commonly used dehydrogenases are shown in Fig. 2.15, with reference to their preferred size of their substrates [774].

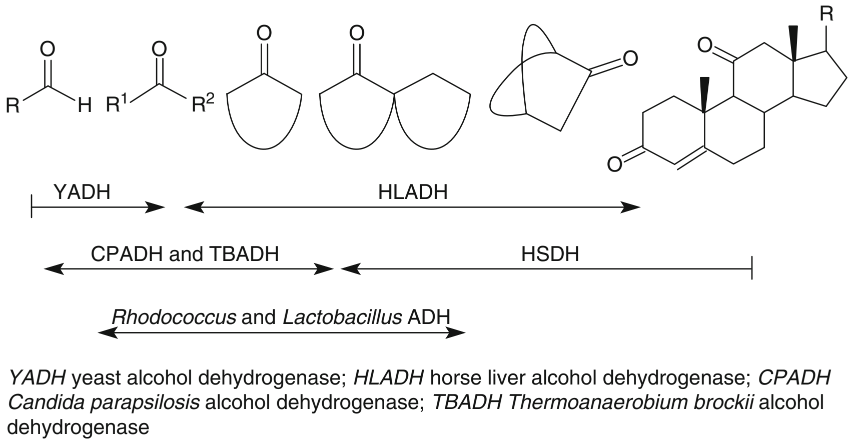
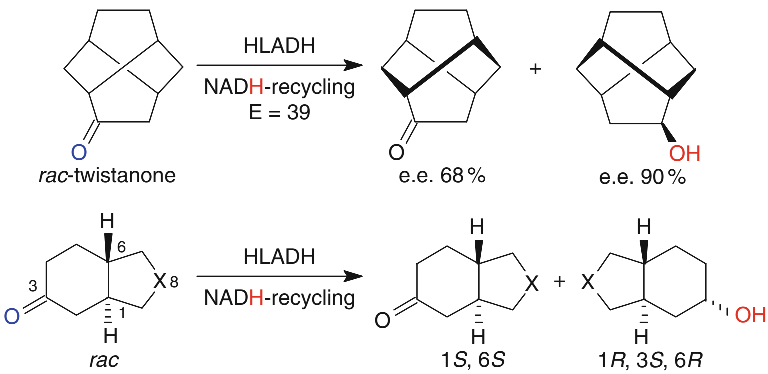


Fig. 2.15 Preferred substrate size for dehydrogenases.

Horse liver ADH is a very universal enzyme with a broad substrate specificity and excellent stereoselectivity. Historically, it is the most widely used dehydrogenase in biotransformations [775, 776].[29] The three-dimensional structure has been elucidated by X-ray diffraction [778]. Although the primary sequence is quite different, the tertiary structure of HLADH is similar to that of YADH [779]. The most useful applications of HLADH are found in the reduction of medium-ring monocyclic ketones (four- to nine-membered ring systems) and bicyclic ketones [780–782]. Sterically demanding molecules which are larger than decalines are not readily accepted and acyclic ketones are usually reduced with modest enantioselectivities [783, 784].

A considerable number of monocyclic and bicyclic racemic ketones have been resolved using HLADH with fair to excellent specificities [785–787]. Even

[29] For the catalytic mechanism see [776].



X	Selectivity (E)	e.e. Ketone [%]	e.e. Alcohol [%]
O	>120	60	>97
S	>110	53	>97

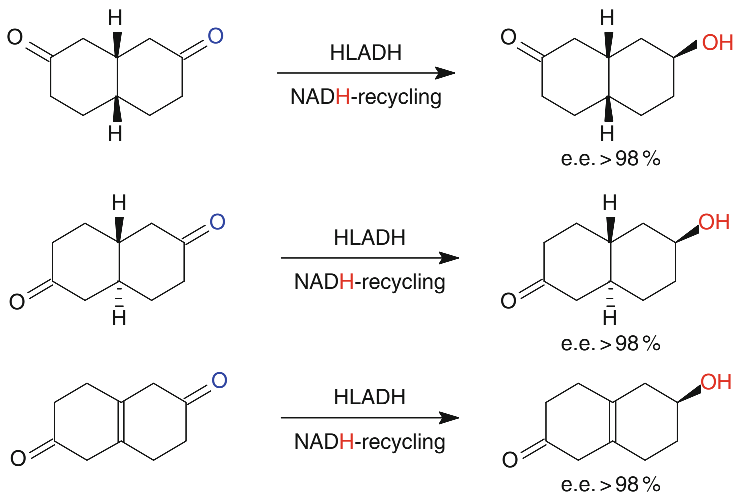
Scheme 2.116 Resolution of bi- and polycyclic ketones using horse liver alcohol dehydrogenase (HLADH)

sterically demanding cage-shaped polycyclic ketones were readily accepted [788, 789] (Scheme 2.116). For instance, *rac*-2-twistanone was reduced to give the *exo*-alcohol and the enantiomeric ketone in 90% and 68% e.e., respectively [790]. Also *O*- and *S*-heterocyclic ketones were shown to be good substrates (Scheme 2.116) [791–793]. Thus, (±)-bicyclo[4.3.0]nonan-3-ones bearing either an O or S atom in position 8 were resolved with excellent selectivities [784]. Attempted reduction of the corresponding *N*-heterocyclic ketones led to deactivation of the enzyme via complexation of the essential Zn^{2+} ion in the active site [794].

Every kinetic resolution of bi- and polycyclic ketones suffers from one particular drawback because the bridgehead carbon atoms make it impossible to recycle the undesired 'wrong' enantiomer via racemization. Hence the desymmetrization of prochiral diketones, making use of the enantioface- or enantiotopos-specificity of HLADH, is of advantage. For instance, both the *cis*- and *trans*-forms of the decalinediones shown in Scheme 2.117 were reduced to give (*S*)-alcohols with excellent optical purity. Similar results were obtained with unsaturated derivatives [743, 795].

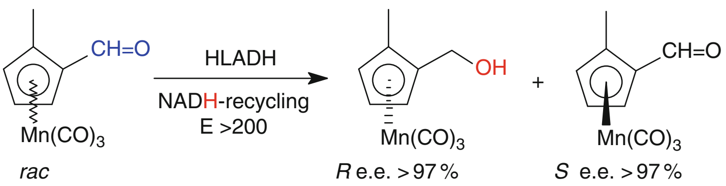
The wide substrate tolerance of HLADH encompassing nonnatural compounds is demonstrated by the resolution of organometallic derivatives possessing axial chirality [796]. For instance, the racemic tricarbonyl cyclopentadienyl manganese aldehyde shown in Scheme 2.118 was enantioselectively reduced to give the (*R*)-alcohol and the residual (*S*)-aldehyde with excellent optical purities [797].

In order to predict the stereochemical outcome of HLADH-catalyzed reductions, a number of models have been developed, each of which having its own merits. The first rationale emerged from the 'diamond lattice model' of V. Prelog, which was originally developed for *Curvularia falcata* [798]. A more recently developed



Scheme 2.117 Desymmetrization of prochiral diketones using HLADH

cubic-space descriptor is particularly useful for ketones bearing chirality center(s) remote from the location of the carbonyl group [799]. Alternatively, a quadrant rule may be applied [800].



Scheme 2.118 Enantioselective reduction of an organometallic aldehyde using HLADH

A useful substrate model based on a flattened cyclohexanone ring is shown in Fig. 2.16 [801]. It shows the Zn^{2+} in the catalytic site which coordinates to the

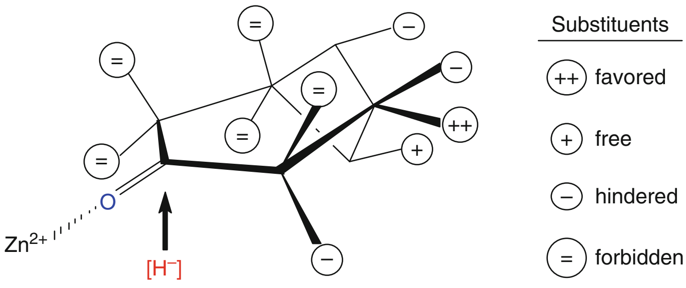
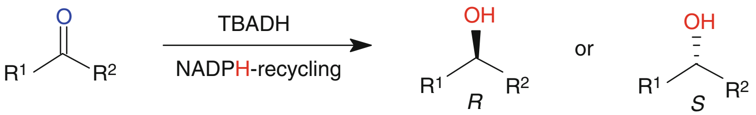


Fig. 2.16 Substrate model for HLADH

carbonyl oxygen atom and the nucleophilic attack of the hydride occurring from the bottom face. The preferred orientation of the substrate relative to the chemical operator – the hydride ion – can be estimated by placing the substituents into the ‘allowed’ and ‘forbidden’ zones.

YADH and HLADH are less useful for the asymmetric reduction of open-chain ketones, but this gap is efficiently covered by a range of alcohol dehydrogenases from mesophilic bacteria, such as *Rhodococcus* (ADH-A) and *Lactobacillus* (LBADH, LKADH), and thermophilic *Thermoanaerobacter* [802] and *Thermoanaerobium* (TBADH) strains (Scheme 2.119) [296, 803–806]. Some of these enzymes are remarkably thermostable (up to 85°C) and can tolerate the presence of organic solvents such as *iso*propanol, which serves as hydrogen-donor for NADP-recycling in a coupled-substrate approach [807–809].



R^1	R^2	Specificity	Configuration	e.e. [%]
CH_3	$CH(CH_3)_2$	anti-Prelog	*R*	86
CH_3	C_2H_5	anti-Prelog	*R*	48
CH_3	*cyclo*-C_3H_5	anti-Prelog	*R*	44
CH_3	*n*-C_3H_7	Prelog	*S*	79
CH_3	$C\equiv CH$	Prelog	*S*	86
Cl–CH_2–	CH_2–CO_2Et	Prelog	*R*[a]	90
CF_3	Ph	Prelog	*R*[a]	94
CH_3	CH_2–$CH(CH_3)_2$	Prelog	*S*	95
C_2H_5	*n*-C_3H_7	Prelog	*S*	97
C_2H_5	$(CH_2)_2$–CO_2Me	Prelog	*S*	98
CH_3	$(CH_2)_3$–Cl	Prelog	*S*	98
CH_3	*n*-C_5H_{11}	Prelog	*S*	99
CH_3	$(CH_2)_5$–Cl	Prelog	*S*	>99
C_2H_5	$(CH_2)_5$–Cl	Prelog	*S*	>99
n-C_3H_7	*n*-C_3H_7	no reaction		

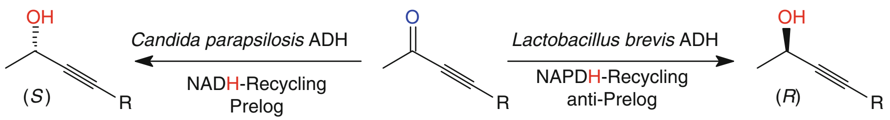
[a]Switch in CIP-sequence order

Scheme 2.119 Enantioselective reduction of ketones using *Thermoanaerobium brockii* alcohol dehydrogenase (TBADH)

Open-chain methyl- and ethyl-ketones are readily reduced by TBADH to furnish the corresponding secondary alcohols, generally with excellent specificities [810]. Similarly, ω-haloalkyl- [732, 811] and methyl- or trifluoromethyl ketones possessing heterocyclic substituents were converted into the corresponding secondary alcohols with excellent optical purities [812, 813]. However, α,β-unsaturated ketones and ketones where both substituents are larger than ethyl are not accepted. In general TBADH obeys Prelog's rule with 'normal-sized' ketones leading to (*S*)-alcohols, but the stereoselectivity was found to be reversed with small substrates. In order to predict the stereochemical outcome of TBADH reductions, an active site model based on a quadrant rule was proposed [814].

One serious drawback associated with TBADH and *Lactobacillus* ADHs in large-scale applications is their requirement for NADP(H). In an attempt to circumvent this problem, NAD(H)-vanadate, which is spontaneously formed in solution from catalytic amounts of the cheaper NAD(H) and inorganic vanadate (HVO_4^{2-}), was used as a substitute for NADPH [815]. However, undesired redox reactions associated with vanadate limited the practical applicability of this system.

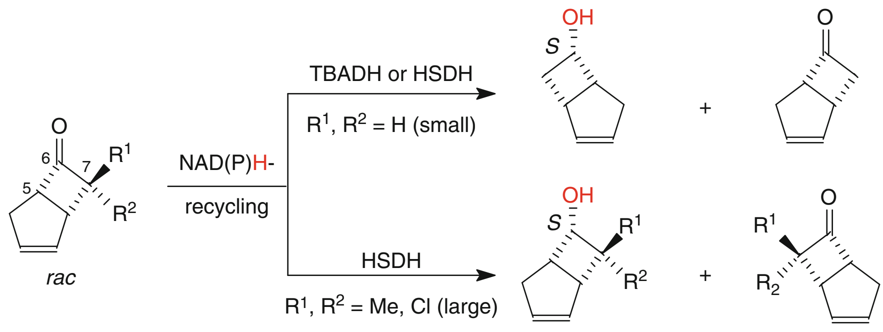
The key to access both stereoisomers of a *sec*-alcohol via asymmetric carbonyl reduction is the availability of stereocomplementary dehydrogenases. For open-chain ketones bearing a small and large substituent at each side, this is feasible by using an appropriate enzyme showing Prelog or anti-Prelog specificity. Whereas dehydrogenases from *Rhodococcus ruber*, *R. erythropolis*, and *Candida parapsilosis* produce the Prelog enantiomer, *Lactobacillus* ADHs furnish the corresponding mirror-image product, usually with high stereoselectivity (Scheme 2.120) [816]. In an analogous fashion, α-ketocarboxylic acids were reduced to the corresponding enantiomeric α-hydroxyacids using stereocomplementary lactate dehydrogenases (LDH) [817–820], or hydroxyisocaproate dehydrogenases (HicDHs) [821, 822].



R	*Candida parapsilosis* ADH Configuration	e.e.[%]	*Lactobacillus brevis* ADH Configuration	e.e.[%]
H	(*S*)	49	(*R*)	60
$SiMe_3$	(*S*)	57	(*R*)	>99
$SiMe_2Ph$	(*S*)	>99	(*R*)	>99
Ph	(*S*)	>99	(*R*)	>99
2-Pyridyl	(*S*)	>99	(*R*)	>99

Scheme 2.120 Stereocomplementary bioreduction using a Prelog and anti-Prelog dehydrogenase

Hydroxysteroid dehydrogenases (HSDH) are ideally suited enzymes for the reduction of bulky mono- [823] and bicyclic ketones (Scheme 2.121) [824]. This is not surprising if one thinks of the steric requirements of their natural substrates: steroids [825, 826].



R^1	R^2	Enzyme	e.e. Alcohol [%]
H	H	HSDH	≤10
H	H	TBADH	>95
H	Cl	HSDH	>90
Cl	Cl	HSDH	>95
Me	Cl	HSDH	>98
Me	Me[a]	HSDH	>95

[a] no reaction was observed with HLADH or TBADH

Scheme 2.121 Kinetic resolution of sterically demanding ketones using hydroxysteroid dehydrogenase (HSDH)

For instance, bicyclo[3.2.0]heptan-6-one systems were reduced with HSDH with very low selectivity when substituents in the adjacent 7-position were small (R^1, R^2 = H). On the other hand, TBADH showed an excellent enantioselectivity with this 'slim' ketone. When the steric requirements of the substrate were increased by additional methyl- or chloro-substituents adjacent to the carbonyl group, the situation changed. Then, HSDH became a very specific catalyst and TBADH (or HLADH) proved to be unable to accept the bulky substrates [827, 828]. The switch in the stereochemical preference is not surprising and can be explained by Prelog's rule: with the unsubstituted ketone, the position 5 is 'larger' than position 7. However, when the hydrogen atoms on carbon atom 7 are replaced by sterically demanding chlorine or methyl groups, the situation is reversed.

The majority of synthetically useful ketones can be transformed into the corresponding chiral secondary alcohols by choosing the appropriate dehydrogenase from the above-mentioned set of enzymes (Fig. 2.15). Other enzymes, which have been shown to be useful for specific types of substrates bearing a carbonyl group, are mentioned below.

The natural role of glycerol dehydrogenase is the interconversion of glycerol and dihydroxyacetone. The enzyme is commercially available from different sources and has been used for the stereoselective reduction of α-hydroxyketones [750]. Glycerol DH has been found to tolerate some structural variation of its natural substrate – dihydroxyacetone – including cyclic derivatives. An enzyme from *Geotrichum candidum* was shown to reduce not only α- but also β-ketoesters with high selectivity [829].

Enzymes from thermophilic organisms (which grow in the hostile environment of hot springs with temperatures ranging from 70 to 100°C) have recently received much attention [830–833]. Thermostable enzymes are not only stable to heat but, in general, also show enhanced stability in the presence of common protein denaturants and organic solvents. Since they are not restricted to working in the narrow temperature range which is set for mesophilic, 'normal' enzymes (20–40°C), an influence of the temperature on the selectivity can be studied over a wider range. For instance, the diastereoselectivity of the HLADH-catalyzed reduction of 3-cyano-4,4-dimethyl-cyclohexanone is diminished at 45°C (the upper operational limit for HLADH) when compared with that observed at 5°C [834]. On the other hand, a temperature-dependent *reversal* of the enantiospecificity of an alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* could be achieved when the temperature was raised to 65°C [835] (compare Sect. 2.1.3.1).

2.2.3 Reduction of Aldehydes and Ketones Using Whole Cells

Instead of isolated dehydrogenases, which require sophisticated cofactor recycling, whole microbial cells can be employed. They contain multiple dehydrogenases which are able to accept nonnatural substrates, all the necessary cofactors and the metabolic pathways for their regeneration. Thus, cofactor recycling can be omitted since it is automatically done by the living cell. Therefore, cheap carbon sources such as saccharose or glucose can be used as auxiliary substrates for asymmetric reduction reactions. Furthermore, all the enzymes and cofactors are well protected within their natural cellular environment.

However, these distinct advantages have to be taken into consideration alongside some significant drawbacks:

- The productivity of microbial conversions is usually low since the majority of nonnatural substrates are toxic to living organisms and are therefore only tolerated at low concentrations (~0.1–0.3% per volume).
- The large amount of biomass present in the reaction medium causes low overall yields and makes product recovery troublesome, particularly when the product is stored inside the cells and not excreted into the medium. Since only a minor fraction (typically 0.5–2%) of the auxiliary substrate is used for coenzyme recycling, the bulk of it is metabolized, forming polar byproducts which often impede product purification. Therefore, monitoring of the reaction becomes difficult.
- Finally, different strains of a microorganism most likely possess different specificities; thus it is important to use exactly the same culture to obtain comparable results with the literature [836].

- Stereoselectivities may vary to a great extent due to the following reasons:
  On the one hand, a substrate may be reduced by a *single* oxidoreductase via transition states for the two enantiomers (or the enantiotopic faces) which have similar values of free energy. In other words, inefficient chiral recognition takes place allowing an alternative fit of the substrate within a single enzyme.
  If *two* enzymes, each with high but *opposite* stereochemical preference, compete for the same substrate, the optical purity of the product is determined by the relative rates of the individual reactions. The latter, in turn, depend on the substrate concentration for the following reasons: At concentrations below saturation, the relative rates are determined by the ratio V_{max}/K_m for each enzyme. On the other hand, when saturation is reached using elevated substrate concentrations, the relative rates mainly depend on the ratio of k_{cat} of the two reactions. Consequently, when two (or more) enzymes are involved in the transformation of enantiomeric substrates, the optical purity of the product becomes a function of the substrate concentration, because the values of K_m and k_{cat} for the substrate enantiomers are different for both competing enzymes. With yeasts, it is a well-known phenomenon that lower substrate concentrations often give higher e.e.$_p$s [837].

The following general techniques can be applied to enhance the selectivity of microbial reduction reactions:

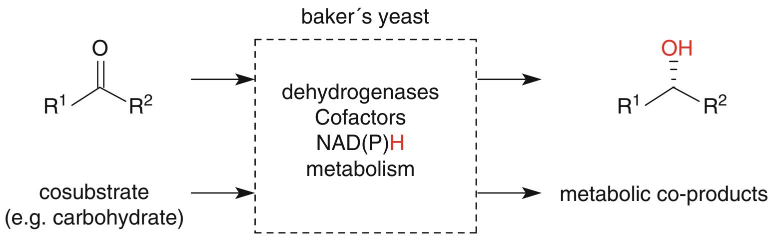
- Substrate modification, e.g., by variation of protecting groups which can be removed after the transformation [838–840]
- Variation of the metabolic parameters by immobilization [841–843]
- Using cells of different age [844]
- Variation of the fermentation conditions [845–847]
- Screening of microorganisms to obtain strains with the optimum properties (a hard task for nonmicrobiologists) [848, 849]
- Selective inhibition of one of the competing enzymes (see below)

Reduction of Aldehydes and Ketones by Baker's Yeast

Asymmetric Reduction of Ketones. Baker's yeast (*Saccharomyces cerevisiae*) is by far the most widely used microorganism for the asymmetric reduction of ketones [850–854]. It is ideal for nonmicrobiologists, since it is readily available at a very reasonable price. In addition, its use does not require sterile fermenters and it can therefore be handled using standard laboratory equipment. Thus, it is not surprising that yeast-catalyzed transformations of nonnatural compounds leading to chiral products have been reported from the beginning of the twentieth century [855] and the first comprehensive review which covers almost all the different strategies of yeast-reductions dates back to 1949! [856].

A wide range of functional groups within the ketone are tolerated, including heterocyclic- [857, 858], fluoro- [859–862], chloro- [863], bromo- [864], perfluoroalkyl- [865], cyano-, azido-, nitro- [866–868], hydroxyl- [869, 870], sulfur- [871–873], and dithianyl groups [874]. Even organometallic derivatives [875, 876], such as silyl- [877] and germyl groups [878] are accepted.

Simple aliphatic and aromatic ketones are reduced by fermenting yeast according to Prelog's rule to give the corresponding (*S*)-alcohols in good optical purities (Scheme 2.122) [771]. Long-chain ketones such as *n*-propyl-*n*-butylketone and several bulky phenyl ketones are not accepted; however, one long alkyl chain is tolerated if the other moiety is the methyl group [879, 880]. As might be expected, best stereoselectivities were achieved with groups of greatly different size.

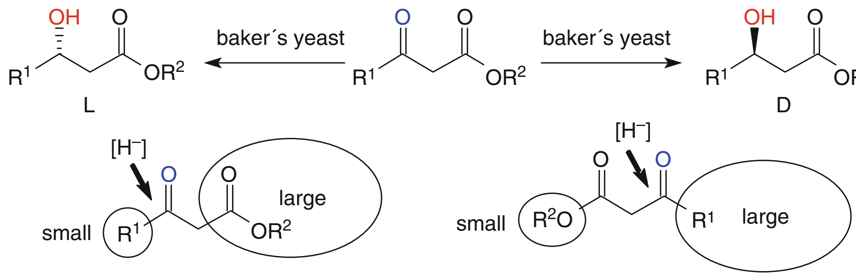


R^1	R^2	Configuration	e.e. [%]
Me	Et	*S*	67
Me	CF_3	*S*	>80
CF_3	CH_2–Br	*S*	>80
Me	*n*-Bu	*S*	82
Me	Ph	*S*	89
Me	CH_2–OH	*S*	91
Me	$(CH_2)_2$–CH=C$(CH_3)_2$	*S*	94
Me	*c*-C_6H_{11}	*S*	>95
Me	C$(CH_3)_2$–NO_2	*S*	>96

Scheme 2.122 Reduction of aliphatic and aromatic ketones using baker's yeast

Acyclic β-ketoesters (Scheme 2.123) are readily reduced by yeast to yield β-hydroxyesters [881, 882], which serve as chiral starting materials for the synthesis of β-lactams [883], insect pheromones [884], and carotenoids [885]. It is obvious that the enantioselectivity and the stereochemical preference for the *re*- or the *si*-side of the β-ketoester depends on the *relative size* of the alkoxy moiety and the ω-substituent of the ketone, which directs the nucleophilic attack of the hydride occurring according to Prelog's rule (Scheme 2.123). Therefore, the absolute configuration of the newly generated *sec*-alcoholic center may be directed by substrate modification using either the corresponding short- or long-chain alkyl ester, which switches the relative size of the substituents flanking the carbonyl group [886].

In baker's yeast, the reason for this divergent behavior is not due to an alternative fit of the substrates in a single enzyme, but rather due to the presence of a number of



R^1	R^2	Configuration	e.e. [%]
Cl–CH_2–	CH_3	D	64
Cl–CH_2–	C_2H_5	D	54
Cl–CH_2–	n-C_3H_7	D	27
Cl–CH_2–	n-C_5H_{11}	L	77
Cl–CH_2–	n-C_8H_{17}	L	97
$(CH_3)_2C$=CH–$(CH_2)_2$–	CH_3	D	92
–CCl_3	C_2H_5	D	85
–CH_3	C_2H_5	L	>96
N_3–CH_2–	C_2H_5	L	80
Br–CH_2–	n-C_8H_{17}	L	100
C_2H_5–	n-C_8H_{17}	L	95

Scheme 2.123 Reduction of acyclic β-ketoesters using baker's yeast

different dehydrogenases, possessing opposite stereochemical preferences, which compete for the substrate [837, 887]. A D-specific enzyme – belonging to the fatty acid synthetase complex – shows a higher activity towards β-ketoesters having a short-chain alcohol moiety, such as methyl esters. By contrast, an L-enzyme is more active on long-chain counterparts, e.g., octyl esters. Therefore, the stereochemical direction of the reduction may be controlled by careful design of the substrate, or by selective inhibition of one of the competing dehydrogenases.

Inhibition of the L-enzyme (which leads to the increased formation of D-β-hydroxyesters) was accomplished by addition of unsaturated compounds such as allyl alcohol [888] or methyl vinyl ketone (Table 2.4, Scheme 2.123) [889]. The

Table 2.4 Selectivity enhancement of yeast reductions via L-enzyme inhibition (for formulas see Scheme 2.123)

R^1	R^2	Conditions	Configuration	e.e. (%)
Cl–CH_2–	C_2H_5	Standard	D	43
Cl–CH_2–	C_2H_5	+ Allyl alcohol	D	85
C_2H_5	CH_3	Standard	D	37
C_2H_5	CH_3	+ CH_3–CO–CH=CH_2	D	89

same effect was observed when the yeast cells were immobilized by entrapment into a polyurethane gel [890, 891]. As expected, L-enzyme inhibitors led to a considerable increase in the optical purity of D-β-hydroxyesters.

Various haloacetates [892], thioethers [893], and allyl bromide [894] have been found to be selective inhibitors for the D-enzyme (Table 2.5, Scheme 2.123), which leads to an increased formation of the L-enantiomer. Depending on the substrate, the effect may even be pronounced enough to effect an *inversion* of the configuration of the resultant β-hydroxyester. In the absence of inhibitor, the D-enantiomer was obtained in moderate e.e., but the L-counterpart was formed upon addition of allyl bromide.

Table 2.5 Selectivity enhancement of yeast reductions via D-enzyme inhibition (for formulas see Scheme 2.123)

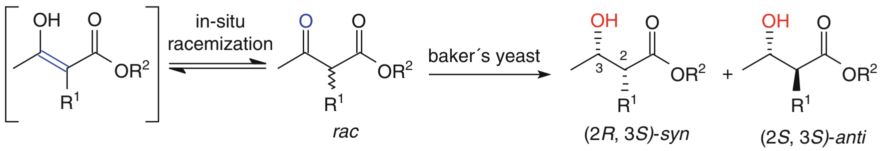
R^1	R^2	Conditions	Configuration	e.e. (%)
C_2H_5	CH_3	Standard	L	15
C_2H_5	CH_3	+Cl–CH_2–CO_2Et	L	91
Cl–CH_2–	*n*-C_6H_{13}	Standard	L	64
Cl–CH_2–	*n*-C_6H_{13}	+PhSCH=CH_2	L	97
C_2H_5	C_2H_5	Standard	D	40–50
C_2H_5	C_2H_5	+Allyl bromide	L	>98

Diastereoselective Reduction of Ketones by Baker's Yeast. Asymmetric microbial reduction of α-substituted ketones leads to the formation of diastereomeric *syn*- and *anti*-products. Because the chiral center on the α-position of the ketone is stereochemically labile, rapid in-situ racemization of the substrate enantiomers occurs via enolization[30] – leading to dynamic resolution [67, 895, 896]. Thus, the ratio between the diastereomeric *syn*- and *anti*-products is not 1:1, but is determined by the selectivities of the enzymes involved in the reduction process [897]. Under optimal conditions it can even be as high as 100:0 [898].

Diastereoselective yeast-reduction of ketones has been mainly applied to α-monosubstituted β-ketoesters leading to the formation of diastereomeric *syn*- and *anti*-β-hydroxyesters (Scheme 2.124) [899–902]. With small α-substituents, the formation of *syn*-diastereomers predominates, but the diaselectivity is reversed when the substituents are increased in size. In any case, the selectivity for the newly generated *sec*-alcohol center is always very high (indicated by the e.e.s) and its absolute configuration is determined by Prelog's rule.

These observations have led to the construction of a simple model which allows one to predict the diastereoselectivity (i.e., the *syn*/*anti*-ratio) of yeast-catalyzed reductions of α-substituted β-ketoesters (Fig. 2.17) [903]. Thus, when α-substituents are smaller than the carboxylate moiety, they fit well into the small pocket (S), with L being the carboxylate, but substrates bearing space-filling groups on the

[30]When the chiral center is moved to the β- or γ-position, in situ racemization is impossible and, as a consequence, *syn*/*anti*-diastereomers are always obtained in a 1:1 ratio.



R^1	R^2	e.e. [%]		Ratio
		syn	*anti*	*syn/anti*
CH_3	C_2H_5	100	100	83:17
CH_3	Ph–CH_2–	100	80	67:33
CH_2=CH–CH_2–	C_2H_5	100	100	25:75
Ph–CH_2–	C_2H_5	100	100	33:67
Ph–S–	CH_3	>96	>96	17:83

Scheme 2.124 Diastereoselective reduction of α-substituted β-ketoesters using baker's yeast

α-carbon occupy both of the pockets in an inverted orientation (i.e., the carboxylate group then occupies the pocket S). Again, the reason for the varying selectivity of baker's yeast reduction of α-substituted β-ketoesters is due to the presence of multiple dehydrogenases possessing different specificites for the substrate enantiomers [904].

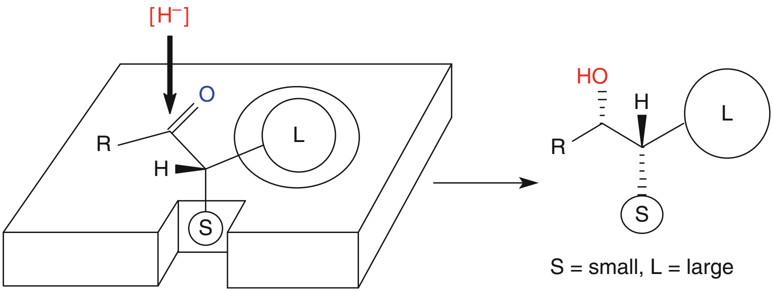
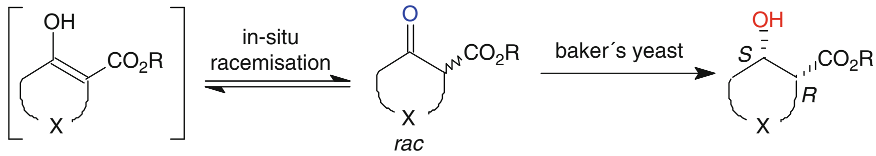


Fig. 2.17 Model for predicting the diastereoselectivity in yeast reductions

As may be predicted by this model, the yeast-reduction of cyclic ketones bearing an α-alkoxycarbonyl group exclusively leads to the corresponding *syn*-β-hydroxyesters (Scheme 2.125) [905–907]. The corresponding *anti*-diastereomers cannot be formed because rotation around the α,β-carbon–carbon bond is impossible with such cyclic structures. Furthermore, the reductions are generally more enantioselective than the corresponding acyclic substrate due to the enhanced rigidity of the system. Thus, it can be worthwhile to create a sulfur-containing ring in the substrate temporarily and to remove the heteroatom after the biotransformation to obtain the desired open-chain product (e.g., by Raney-Ni reduction) in order to benefit from enhanced selectivities (compare Scheme 2.45).



X	R	e.e. [%]
$–(CH_2)_2–$	C_2H_5	>98
$–(CH_2)_3–$	C_2H_5	86–99[a]
$–S–CH_2–$	CH_3	85
$–CH_2–S–$	CH_3	>95

[a]depending on the yeast strain used

Scheme 2.125 Yeast-reduction of cyclic β-ketoesters

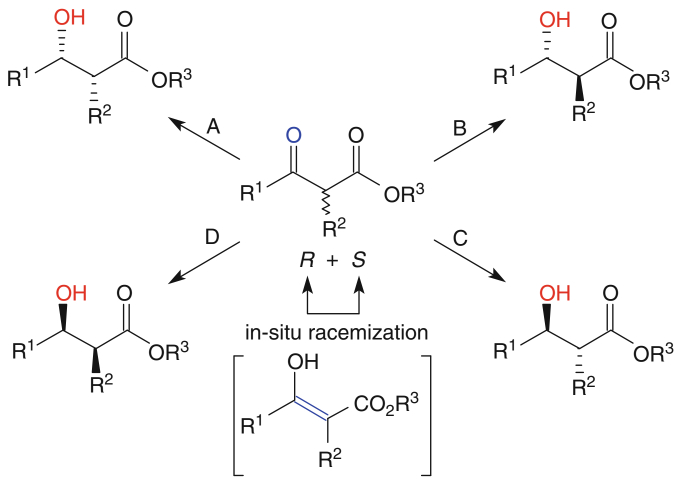
The biocatalytic reduction of α-substituted β-ketoesters with concomitant dynamic resolution has been proven to be extremely flexible (Scheme 2.126) [908–913]. Thus, by choosing the appropriate microorganism possessing the desired enantio- and diastereoselectivity, each of the four possible diastereomeric products were obtained in excellent enantiomeric and diastereomeric purity. As expected, the corresponding Prelog-configurated products (with respect to the newly generated *sec*-alcohol center, pathways A, B) were obtained by using baker's yeast and the mold *Geotrichum candidum*, respectively. For the diastereomers possessing the opposite configuration at the alcoholic center (anti-Prelog pathways C, D) other microorganisms had to be employed.

As long as the α-substituent consists of an alkyl- or aryl-group, dynamic resolution is readily achieved, leading to chemical yields far beyond the 50% which would be the maximum for a classic kinetic resolution. However, in-situ racemization is not possible due to electronic reasons for α-hydroxy- [914], α-alkylthio- [899], α-azido- [915], or α-acetylamino derivatives [916], which are subject to kinetic resolution. The same holds for substrates which are fully substituted at the α-position, due to the impossibility of form the corresponding enolate.

α-Ketoesters and α-ketoamides can be asymmetrically reduced to furnish the correponding α-hydroxy derivatives. Thus, following Prelog's rule, (*S*)-lactate [917] and (*R*)-mandelate esters [897] were obtained from pyruvate and α-ketophenylacetic esters by fermenting baker's yeast in excellent optical purity (e.e. 91–100%).

Cyclic β-diketones are selectively reduced to give β-hydroxyketones without the formation of dihydroxy products (Scheme 2.127) [918–921]. It is important, however, that the highly acidic protons on the α-carbon atom are fully replaced by substituents in order to avoid the (spontaneous) chemical condensation of the substrate with acetaldehyde, which is always present in yeast fermentations[31]

[31]Eichberger and Faber 1984, unpublished results.

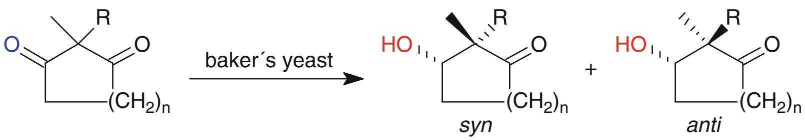


Pathway	R^1	R^2	R^3	Biocatalyst	Yield [%]	d.e. [%]	e.e. [%]	Ref.
A	Me	allyl	Et	baker's yeast	94	92	>99	[907]
A	Me	Me	*n*-Octyl	baker's yeast	82	90	>98	[908]
B	Me	Me	Et	*Geotrichum candidum*	80	>98	>98	[909]
B	Et	Me	Et	*Geotrichum candidum*	80	96	91	[910]
C	4–$MeOC_6H_4$–	Cl	Et	*Sporotrichum exile*	52	96	98	[911]
D	4–$MeOC_6H_4$–	Cl	Me	*Mucor ambiguus*	58	>98	>99	[912]

Scheme 2.126 Stereocomplementary microbial reduction of α-substituted β-ketoesters

and to avoid racemization of the β-hydroxyketone formed as product. Again, with small-size rings, the corresponding *syn*-products are formed predominantly, usually with excellent optical purity. However, the diastereoselectivity becomes less predictable and the yields drop when the rings are enlarged. Again, the stereochemistry at the newly formed secondary alcohol center can be predicted by Prelog's rule.

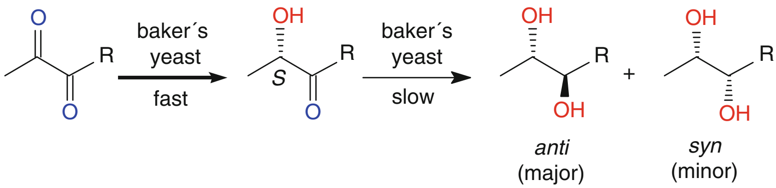
In contrast, the reduction of α-diketones does not stop at the keto-alcohol stage but leads to the formation of diols (Scheme 2.128). In general, the less hindered carbonyl group is quickly reduced in a first step to give the (*S*)-α-hydroxyketone according to Prelog's rule, but further reduction of the (usually more sterically hindered) remaining carbonyl group yields the corresponding diols predominantly in the *anti*-configuration as the final product [922, 923].



R	n	e.e. *syn* [%]	*syn*/*anti*
CH_2=CH–CH_2–	1	>98	90:10
HC≡C–CH_2–	1	>90	100:0
N≡C–$(CH_2)_2$–	1	>98	96:4
CH_2=CH–CH_2–	2	>98	45:55
HC≡C–CH_2–	2	>98	27:73
N≡C–$(CH_2)_2$–	2	>98	30:70
CH_2=CH–CH_2–	3	>98	100:0
CH_2=CH–CH_2–	4	>98	82:18
CH_2=CH–CH_2–	5	>98	no reaction

Scheme 2.127 Yeast reduction of cyclic β-diketones

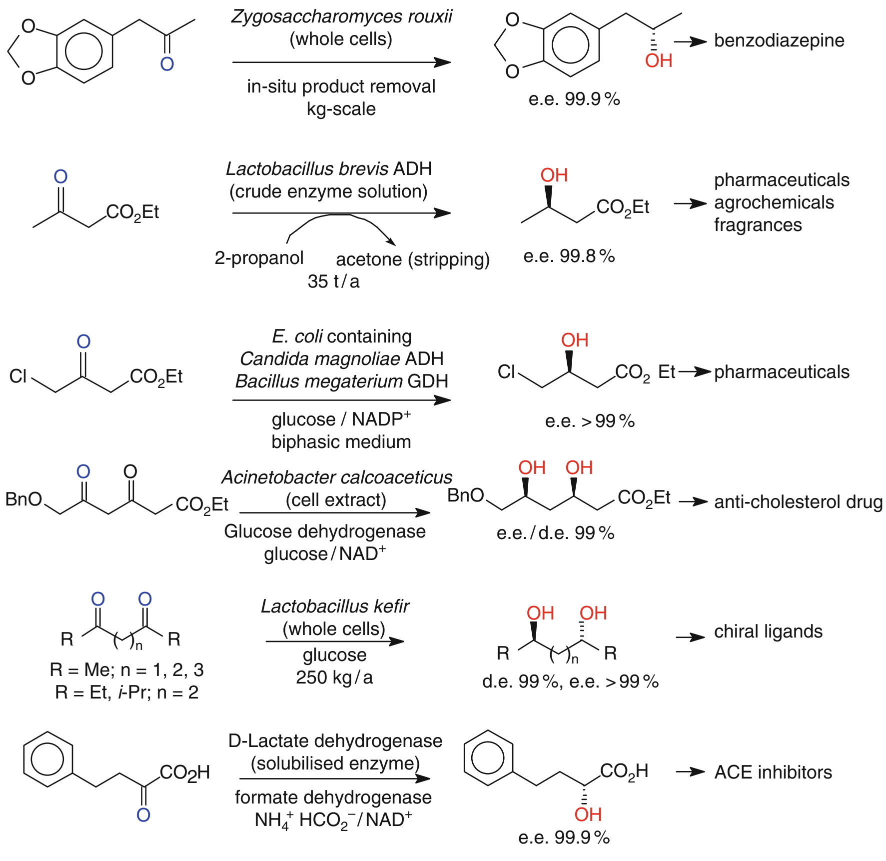
Secondary alcohols possessing the *anti-Prelog configuration* can be obtained from yeast reductions via substrate modification (Scheme 2.123) or through enzyme inhibition (Scheme 2.123, Tables 2.4 and 2.5). If these techniques are unsuccessful, the use of microorganisms other than yeast [767, 773, 924–928], such as *Pichia farinosa* [929], *Geotrichum candidum* [930, 931], and *Yarrowia lipolytica* [932] may be of an advantage. Even plant cell cultures such as *Gardenia* may be employed for this purpose [933, 934]. However, in this case the help of a microbiologist is recommended for organic chemists.



R	e.e. *anti*-diol [%]	*anti*/*syn*
Ph–	94	>95:<5
1,3-Dithian-2-yl–	97	95:5
Ph–S–CH_2–	>97	86:14

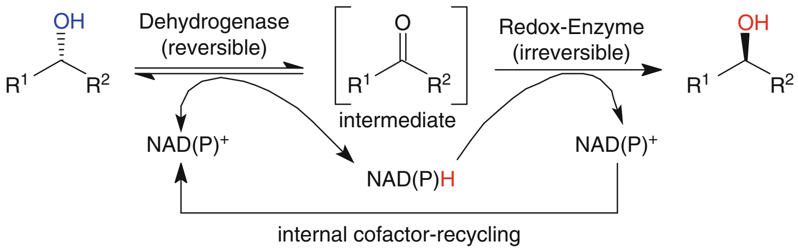
Scheme 2.128 Yeast reduction of α-diketones

The high level of technology available for the biocatalytic reduction of carbonyl compounds has allowed its implementation for the industrial-scale production of chiral building blocks containing *sec*-alcohol moieties. The system applied – either whole microbial (wild-type) cells, a designer bug containing a dehydrogenase plus cofactor-recycling enzyme, or the use of isolated enzymes – depends on the case and is mainly dependent on the economic and the patent situation. Representative examples are depicted in (Scheme 2.129) [935–940].



Scheme 2.129 Industrial-scale bioreduction of carbonyl compounds

Deracemization via Biocatalytic Stereoinversion. Racemic secondary alcohols may be converted into a single enantiomer via stereoinversion which proceeds through a two-step redox sequence (Scheme 2.130) [38, 941, 942]: In a first step, one enantiomer from the racemic mixture is selectively oxidized to the corresponding ketone while the other enantiomer remains unaffected. Then, the ketone is reduced in a second subsequent step by another redox-enzyme displaying *opposite stereochemical preference*. Overall, this process constitutes a deracemization technique, which leads to the formation of a single enantiomer in 100% theoretical yield



R^1	R^2	Microorganism(s)	Yield [%]	e.e. [%]	Ref.
Me	CH_2CO_2Et	*Geotrichum candidum*	67	96	[945]
Me	*p*-Cl–C_6H_4	*Geotrichum candidum*	97	96	[946]
Ph	CH_2OH	*Candida parapsilosis*	~100	~100[a]	[943]
Me	$(CH_2)_2CH{=}CMe_2$	*Bacillus stearothermophilus* + *Yarrowia lipolytica*	91	~100	[947]
Ph	CO_2H	*Pseudomonas polycolor* + *Micrococcus freudenreichii*	70	>99	[948]

[a]opposite configuration as shown

Scheme 2.130 Deracemization of *sec*-alcohols via microbial stereoinversion

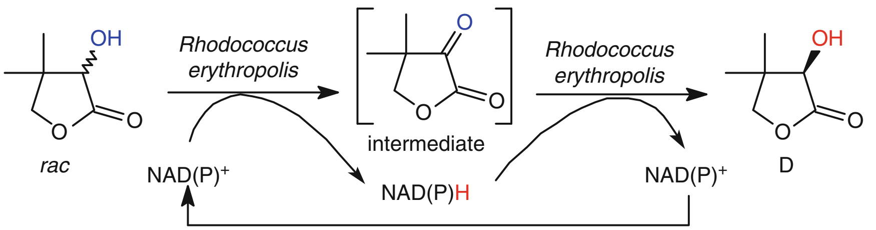
from the racemate [943]. Due to the presence of two consecutive oxidation–reduction reactions, the net redox balance of the process is zero and no external cofactor recycling is required since the redox equivalents are exchanged between both steps in a closed loop. In order to achieve a high optical purity of the product, at least one of the steps has to be irreversible for entropic reasons [944, 945].

The origin of the irreversibility of microbial/enzymatic deracemization of *sec*-alcohols proceeding through an oxidation–reduction sequence depends on the type of microorganism and is unknown in most cases. For instance, deracemization of various terminal (±)-1,2-diols by the yeast *Candida parapsilosis* has been claimed to operate via an (*R*)-specific NAD^+-linked dehydrogenase and an irreversible (*S*)-specific NADPH-dependent reductase [944]. Along these lines, the enzymatic stereoinversion of (biologically inactive) D-carnitine to furnish the desired bioactive L-enantiomer[32] was accomplished by using two stereocomplementary carnitine dehydrogenases. Due to the fact that both dehydrogenase enzymes are NAD(H)-dependent, the end point of the process was close to equilibrium (64%) [950]. By contrast, the stereoinversion of β-hydroxyesters using the fungus *Geotrichum candidum* required molecular oxygen, which would suggest the involvement of an alcohol oxidase rather than an alcohol dehydrogenase [951]. Recently, the

[32]L-Carnitine is an essential factor for the transport of long-chain fatty acids across mitochondrial membranes and is used in the treatment of certain dysfunctions of skeletal muscles, acute hypoglycemia, and heart disorders.

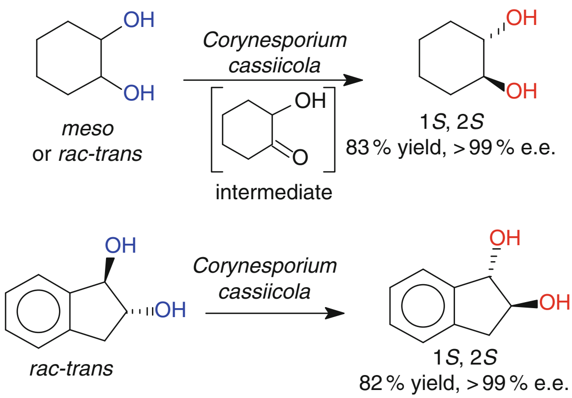
mechanism of enzymatic stereoinversion catalyzed by stereocomplementary dehydrogenases was elucidated to depend on the opposite cofactor-dependence for NADH and NADPH of the dehydrogenases involved [952, 953].

Microbial stereoinversion of *sec*-alcohols has become quite popular [954]. For instance, the deracemization of simple secondary alcohols proceeds with excellent results using the fungi *Geotrichum candidum* or *Candida parapsilosis*. In case the oxidation and reduction cannot be performed by a single species, two microorganisms may be used instead. For instance, *Bacillus stearothermophilus* and *Yarrowia lipolytica* or *Pseudomonas polycolor* and *Micrococcus freudenreichii* were coupled for the deracemization of the pheromone sulcatol and mandelic acid, respectively. In a similar fashion, (±)-pantoyl lactone – a key intermediate for the synthesis of pantothenic acid [955] – was deracemized by using washed cells of *Rhodococcus erythropolis* or *Candida* sp. (Scheme 2.131) [956, 957]. Thus, L-pantoyl lactone is oxidized to the α-ketolactone, which in turn is reduced by another dehydrogenase present in the organisms to yield the corresponding (*R*)-D-pantoyl lactone in 100% theoretical yield.



Scheme 2.131 Microbial deracemization of pantoyl lactone

Microbial stereoinversion has been shown to be extremely flexible, as it is also applicable to *sec*-diols possessing *two* stereocenters (Scheme 2.132) [958–960]. Thus, *meso*- or *rac-trans*-cyclohexane-1,2-diol was deracemized by

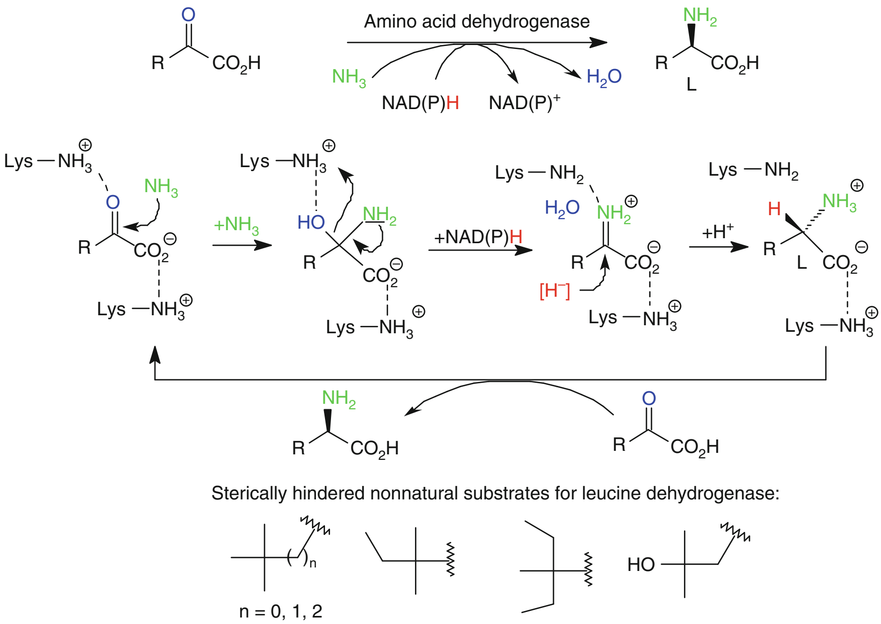


Scheme 2.132 Microbial stereoinversion of cyclic 1,2-diols

Corynesporium cassiicola DSM 62475 to give the (1*S*,2*S*)-enantiomer as the sole product in >99% e.e. and 83% yield. The process was shown to proceed in a stepwise fashion via the corresponding hydroxyketone as intermediate, which was detected in small amounts. More important is the deracemization of *rac*-*trans*-indane-1,2-diol, which was accomplished with excellent results in a similar fashion. The (1*S*,2*S*)-isomer is a central building block for the anti-HIV-agent indinavir [961].

Reductive Amination of α-Ketocarboxylic Acids

The (reversible) transformation of an α-ketocarboxylic acid in presence of ammonia and one equivalent of NAD(P)H furnishes the corresponding α-amino acid and is catalyzed by amino acid dehydrogenases [EC 1.4.1.X] [962]. Despite major differences in its mechanism, this reaction bears a strong resemblance to carbonyl group reduction and it formally respresents a reductive amination (Scheme 2.133). As deduced for L-Leu-dehydrogenase [963], the α-ketoacid substrate is positioned in the active site between two Lys-residues. Nucleophilic attack by NH_3 leads to a hemiaminal intermediate, which eliminates H_2O to form an iminium species. The latter is reduced by a hydride from nicotinamide forming the L-amino acid. Since this mechanism is highly tuned for α-keto/α-amino acids, it is clear that a neutral Schiff base cannot be accepted as substrate.



Scheme 2.133 Reductive amination of α-ketocarboxylic acids

Among the various amino acid dehydrogenases, Leu-DH has captured an important role for the synthesis of nonproteinogenic L-α-amino acids via asymmetric reductive amination of the corresponding α-ketoacids [964, 965]. A range of protease inhibitors used for the treatment of tumors and viral infections contain sterically hindered amino acids as key element for their biological action. The latter cannot be synthetized via the conventional (protease-dependent) methods (Sect. 2.1), but they are produced on industrial-scale making use of the relaxed substrate specificity of LeuDH in combination with NADH recycling using the formate dehydrogenase/formate system [966].

2.2.4 Reduction of C=C-Bonds

The asymmetric (bio)catalytic reduction of C=C-bonds goes in hand with the creation of (up to) two chiral centers and is thus one of the most widely employed strategies for the production of chiral compounds. Whereas *cis*-hydrogenation using transition-metal based homogeneous catalysts has been developed to an impressive standard,[33] stereocomplementary asymmetric *trans*-hydrogenation is still at the stage of development [967].

The biocatalytic counterpart for the stereoselective reduction of alkenes is catalyzed by flavin-dependent ene-reductases,[34] EC 1.3.1.31], which are members of the 'old yellow enzyme' family (OYE, Scheme 2.134) [968, 969], first described in the 1930s [970]. These enzymes are widely distributed in microorganisms and in plants. Some of them occur in well-defined pathways, e.g., in the biosynthesis of fatty acids and secondary metabolites, such as morphine [971] and jasmonic acid [972]. Others are involved in the detoxification of xenobiotics [973], such as nitro esters [974] and nitro-aromatics [975] like trinitrotoluene (TNT) [976].

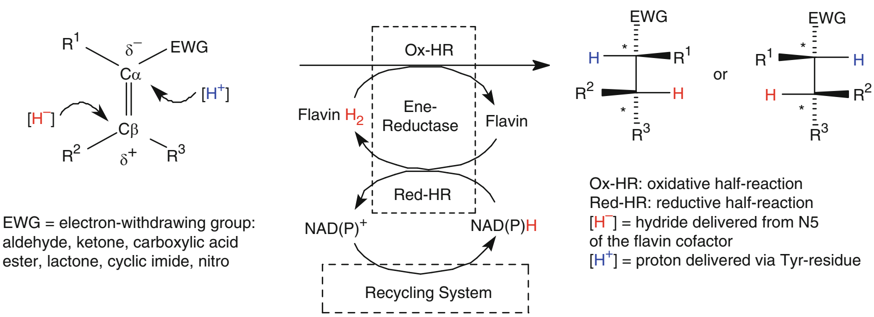
The catalytic mechanism of the asymmetric reduction of alkenes catalyzed by ene-reductases has been studied in great detail [977] and it has been shown that a hydride (derived from a reduced flavin cofactor) is stereoselectively transferred onto Cβ, while a Tyr-residue adds a proton (which is ultimately derived from the solvent) onto Cα from the opposite side (Scheme 2.134). As a consequence of the stereochemistry of this mechanism, the overall addition of [H_2] proceeds in a *trans*-fashion with absolute stereospecificity [978].[35] This reaction is generally denoted as the 'oxidative half reaction'. The catalytic cycle is completed by the so-called 'reductive half reaction' via reduction of the oxidized flavin cofactor at the expense of NAD(P)H, which is ultimately derived from an external H-source via another

[33] http://nobelprize.org/nobel_prizes/chemistry/laureates/2001/knowles-lecture.html and http://nobelprize.org/nobel_prizes/chemistry/laureates/2001/noyori-lecture.html.

[34] Ene-reductases are also denoted as enoate reductases.

[35] Rare cases for a reduction occurring in a *cis*-mode were observed with plant cell cultures and flavin-independent reductases: [977–978].

redox reaction. In contrast to alcohol dehydrogenases (carbonyl reductases), which show a rather pronounced preference for either NADH or NADPH [980], ene-reductases are more flexible in this respect: some enzymes are very specific [981], others are able to accept both cofactors equally well [982, 983]. Overall, the reaction resembles an asymmetric Michael-type addition of a chiral hydride onto an enone and, as a consequence of the mechanism, nonactivated C=C bonds are therefore completely unreactive [984]. Although the overall hydride pathway appears rather complex, practical problems are minimal since flavin cofactors are usually tighly bound to the enzyme and are thereby protected from environment.



Scheme 2.134 Asymmetric bioreduction of activated alkenes using flavin-dependent ene-reductases

Although the remarkable synthetic potential of enoate reductases has been recognized long ago, preparative-scale applications were severely impeded by two major problems: Simple to use whole-cell systems (most prominent baker's yeast [985], but also fungi and yeasts, such as *Geotrichum candidum*, *Rhodotorula rubra*, *Beauveria bassiana* [986]), *Aspergillus niger* are plagued by undesired side reactions, such as carbonyl reduction (catalyzed by competing alcohol dehydrogenases/carbonyl reductases) or ester hydrolysis (mediated by carboxyl ester hydrolases) [711]. On the other hand, the first generation of isolated (cloned) enoate reductases were obtained from (strict or facultative) anaerobes, such as *Clostridia* [987] or methanogenic *Proteus* sp. [988], which were inapplicable to preparative-scale transformations due to their sensitivity towards traces of molecular oxygen. It was only recently, that this bottleneck was resolved by providing oxygen-stable OYEs from bacteria, plants, and yeasts [989–996].

The following crude guidelines for the asymmetric bioreduction of activated alkenes using ene-reductases can be delineated:

- Only C=C-bonds which are 'activated' by electron-withdrawing substituents (EWG) are reduced (Scheme 2.135) [997], electronically 'isolated' double bonds are not accepted [998]. Acetylenic triple bonds yield the corresponding (*E*)-alkenes [999]. With activated, conjugated 1,3-dienes only the α,β-bond is selectively reduced, leaving the nonactivated γ,δ-bond behind (Scheme 2.135).

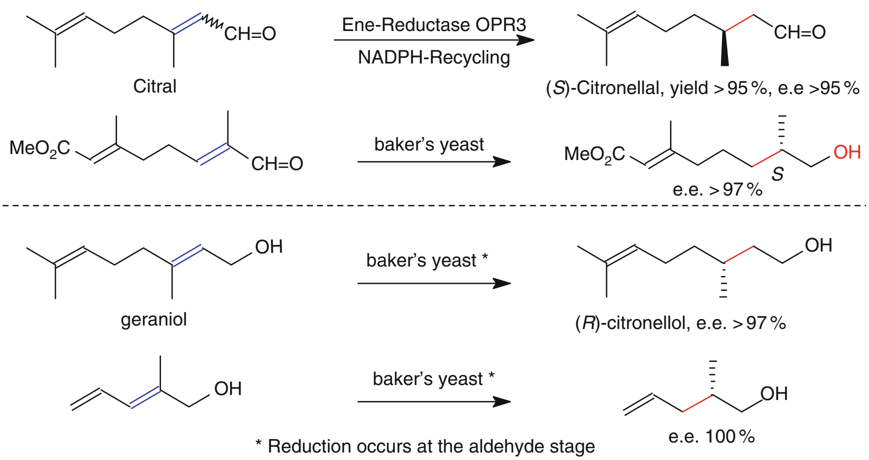
In a similar manner, cumulated 1,2-dienes (allenes) mainly give the corresponding 2-alkenes. A rearrangement of the allene to give an acetylene may be observed occasionally [1000].

The following functional groups may serve as 'activating' groups:

- α,β-Unsaturated carboxaldehydes (enals) are quickly reduced in a clean fashion yielding saturated aldehydes when pure ene-reductases are used. In contrast, whole-cell reductions are heavily plagued by competing carbonyl reduction, which often outcompetes the ene-reductase to furnish the corresonding allylic alcohol (thereby depleting the substrate) and/or the saturated *prim*-alcohol (via over-reduction of the desired product) [1001, 1002]. These undesired side-reactions sometimes allow to use an allylic alcohol as substrates, which is transformed via the corresponding enal by whole cells [1003] (Scheme 2.135).
- α,β-Unsaturated ketones are good substrates for ene-reductases. With whole cells, competing carbonyl-reduction is slower as compared to enals and the product distribution depends on the relative rates of competing carbonyl- and ene-reductases [1004, 1005] (Scheme 2.136).
- α,β-Unsaturated nitro compounds can be readily ransformed into chiral nitro-alkanes, depending on the type of OYE, reductive biodegradation may occur via the Nef-pathway [1006]. Due to the high acidity of nitroalkanes, any chiral center at Cα is racemized, wheras Cβ-analogs are perfectly stable [1007] (Scheme 2.137).
- Cyclic imides are readily reduced without competing side reactions.
- α,β-Unsaturated carboxylic acids or esters have to be regarded as 'borderline'-substrates:
  Simple α,β-unsaturated *mono*-carboxylic acids or -esters are not easily reduced by OYEs, but they are good substrates for 'enoate-reductases' from anaerobic organisms, which possess an additional (oxygen-sensitive) ferredoxin cofactor [1008]. However, the presence of an additional electron-withdrawing group (which alone would not be sufficient to act as activator), such as halogen, nitrile, etc., helps to overcome the low degree of activation [1009] (Scheme 2.138). Consequently, *di*-carboxylic acids and -esters are accepted by OYEs. Ester hydrolysis is a common side-reaction when using whole cells. Only few reports are available regarding α,β-unsaturated lactones. Due to their low carbonyl activitiy, carboxylic acids are less activated than the corresponding esters.
- Sometimes the absolute (*R*/*S*)-configuration of the product can be controlled by starting with (*E*)- or (*Z*)-alkenes. However, this is not always the case (Scheme 2.138).
- Steric hindrance at Cβ (where the hydride has to be delivered) seems to play an important role for OYEs. Consequently, sterically demanding substituents at the C=C-bond are more easily tolerated in the α-position.

The bioreduction of citral using the ene-reductase OPR3 (12-oxophytodienoic acid reductase) proceeds in a clean fashion yielding the fragrance compound

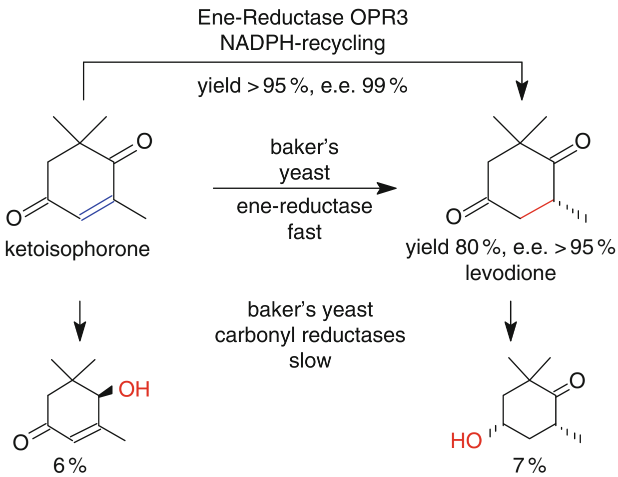
(*R*)-citronellal in excellent chemical and optical yields (Scheme 2.135). In contrast, baker's yeast reduction of a closely related enal bearing a carboxylic ester group yielded the saturated *prim*-alcohol as the major product cue to over-reduction of the aldehyde moiety. The less activated C=C bond adjacent to the ester remained unchanged [1010]. Instead of starting with an enal (whose aldehyde moiety would be reduced anyway) the corresponding allylic alcohol may serve as substrate in whole-cell bioreductions. Thus, geraniol gave (*R*)-citronellol in >97% e.e. [1011] and in a similar fashion only the α,β-bond was reduced in a conjugated 1,3-diene-1-ol [1012]. It is important to note that in whole-cell transformations the C=C-reduction of allylic alcohols always occurs at the aldehyde stage, which is reversibly formed as intermediate (Scheme 2.135).



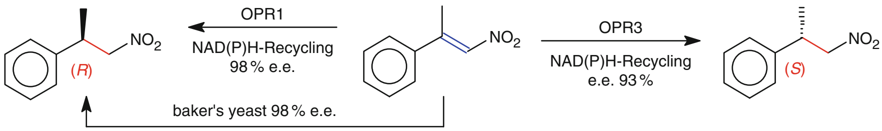
Scheme 2.135 Asymmetric bioreduction of enals and allylic alcohols using isolated ene-reductase and baker's yeast

In contrast to aldehydes, over-reduction is less pronounced on α,β-unsaturated ketones (Scheme 2.136). Nonracemic levodione, which is a precursor for the synthesis of carotenoids, such as astaxanthin and zeaxanthin, was obtained in 80% yield and >95% e.e. via yeast-mediated reduction of ketoisophorone. Two other products arising from over-reduction of the carbonyl moieties were formed in minor amounts [1013]. In contrast, no trace of carbonyl reduction was observed using ene-reductase OPR3 (Scheme 2.136).

Nitro-olefins are readily reduced by ene-reductases to form chiral nitro-alkanes (Scheme 2.137) [1014]. Using ene-reductase OPR1 or baker's yeast, the corresponding (*R*)-nitroalkanes were obtained in high e.e. Surprisingly, the mirror-image product was formed by using isoenzyme OPR2, which is highly homologous to OPR3 (53%).



Scheme 2.136 Asymmetric bioreduction of α,β-unsaturated diketone

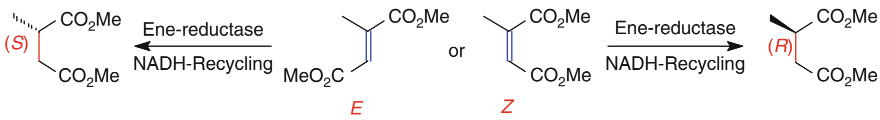


Scheme 2.137 Stereocomplementary bioreduction of nitro-olefins

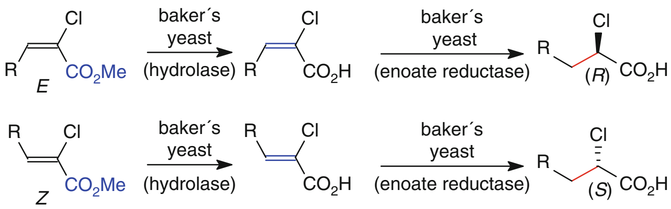
Simple *mono*carboxylic esters require the presence of an additional activating group, such as a halogen atom or a second ester, to be accepted by OYEs (Scheme 2.138). α-Substituted butenedioic esters were readily reduced by ene-reductases YqjM and OPR1 with excellent specificities, while the stereochemical outcome could be controlled by choice of the ene-reductase or by using an (*E*)- or (*Z*)-configurated substate: (*R*)-2-Methylsuccinate was obtained by using OPR1, regardless of the (*E*/*Z*)-configuration of the substrate. In contrast, with YqjM, the configuration of the product switched when an (*E*)-fumarate ester was used instead of a (*Z*)-maleate [1015].

A similar dependence of the configuration of the product on the (*E*/*Z*)-configuration of the substrate was observed during the yeast-mediated reduction of 2-chloro-acrylate esters [1016]. Whereas the chiral recognition of the (*Z*)-alkenes was perfect, the (*E*)-isomers gave products with lower e.e. In addition, it was shown that the microbial reduction took place on the carboxylic acid stage, which were formed enzymatically by hydrolysis of the starting esters prior to the reduction step [1017] (Scheme 2.138).

Only few reports are available on the asymmetric bioreduction of α,β-unsaturated lactones. For instance, β-substituted five-membered ring lactones were readily reduced by baker's yeast to give the (*R*)-configurated saturated analogs (Scheme 2.139) [1018]. The latter constitute versatile C_5-building blocks for terpenoid synthesis. The polarity of the sulfur-protecting group had a significant impact on



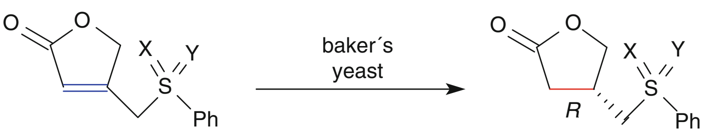
Configuration	YqjM		OPR1	
	c [%]	e.e. [%]	c [%]	e.e. [%]
Z	93	>99 (*R*)	91	>99 (*R*)
E	70	>99**(*S*!)**	99	>99 (*R*)



R	Configuration	e.e. [%]
C_2H_5–	(*E*)→(*R*)	47
$(CH_3)_2CH$–	(*E*)→(*R*)	68
$CHCl_2$–	(*E*)→(*R*)	92
C_2H_5–	(*Z*)→(*S*)	>98
$(CH_3)_2CH$–	(*Z*)→(*S*)	>98
$CHCl_2$–	(*Z*)→(*S*)	98

Scheme 2.138 Stereocontrol of ene-reduction via enzyme-type or substrate-configuration

the stereochemical course of the reaction. Whereas the thioether and the sulfoxide were readily converted with excellent selectivities, the corresponding more polar sulfone gave low chemical and optical yields.

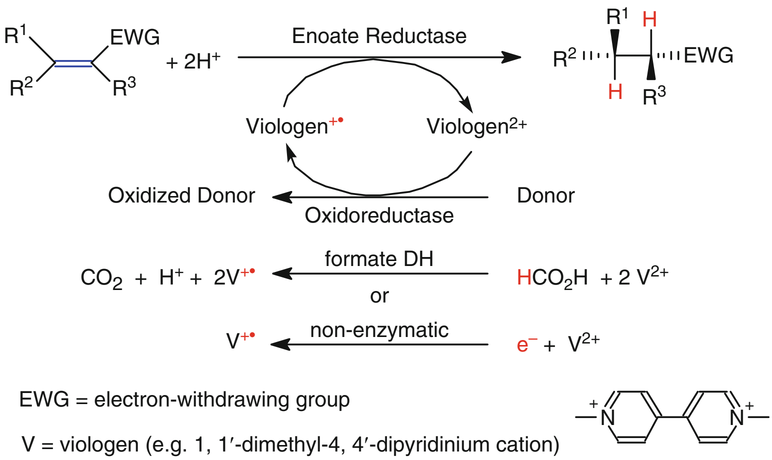


X	Y	e.e. [%]
–	–	99
O	–	97
O	O	77

Scheme 2.139 Asymmetric yeast reduction of α,β-unsaturated lactones

The ultimate source of redox equivalents in microbial reduction reactions is usually a carbohydrate. Since the majority of it is metabolized by the cells and only a minor fraction (typically 0.5–2%) is used for the delivery of redox equivalents onto the substrate, the productivity of such processes is usually low and side-reactions are common. In order to avoid the undesired metabolism of the auxiliary substrate, nondegradable organic dye molecules such as viologens have been used as shuttles ('mediators') for the electron-transport from the donor to the oxidized cofactor [1019]. Provided that the mediators are accepted by the ene-reductases and the recycling enzymes, the productivities were improved by one to three orders of magnitude.

The reduced form of the mediator (a radical cation) can regenerate NADH. The oxidized mediator (a dication) can be enzymatically recycled using an inexpensive reducing agent, such formate in combination with formate dehydrogenase, which is often present together with the enoate reductase in the same microorganism. Alternatively, electrons from the cathode of an electrochemical cell can be used in a nonenzymatic reaction. In this case, the course of the reaction can be easily monitored by following the current in the electrochemical cell. In addition, the equilibrium of the reaction can be shifted from reduction to oxidation by choosing a mediator with the appropriate redox potential. The disadvantage of this method is the considerable toxicity of the commonly used mediators, e.g., methyl- or benzyl-viologen, which have been used for some time as total herbicides, which are banned today (Scheme 2.140).[36]



Scheme 2.140 Cofactor recycling via a mediator

[36]Salts of methyl viologen (1,1′-dimethyl-4,4′-dipyridinium dication, 'Paraquat') have been used as a total herbicide.

2.3 Oxidation Reactions

Oxidation constitutes one of the key steps for the introduction of functional groups into the raw materials of organic synthesis which are almost invariably an alkane, an alkene, or an aromatic molecule.[37] Traditional methodology is plagued by several drawbacks, that is

- Many oxidants are based on toxic metal ions such as copper, nickel, or chromium, which are environmentally incompatible.
- Undesired side reactions are common due to a lack of chemoselective oxidation methods.
- The most inexpensive and innocuous oxidant, molecular oxygen, cannot be used efficiently.
- It is extremely difficult to perform oxidations in a regio- and stereoselective fashion.

Therefore, organohalogens have been widely used as intermediates for the synthesis of oxygenated compounds, which has led to severe problems in waste treatment due to recalcitrant halogenated organic compounds.

Many of the drawbacks mentioned above can be circumvented by using biological oxidation, in particular for those cases where stereoselectivity is required [1021, 1022].

The biooxidation reactions discussed in this chapter are grouped according to their requirement for the oxidant, i.e.:

- Dehydrogenation depending on a nicotinamide cofactor [NAD(P)H] (Sect. 2.3.1)
- Oxygenation at the expense of molecular oxygen (Sect. 2.3.2)
- Peroxidation reactions; requiring hydrogen peroxide or a chemical derivative thereof (Sect. 2.3.3)

For a classification of biooxidation reactions see Scheme 2.144.

2.3.1 Oxidation of Alcohols and Aldehydes

Oxidations of primary and secondary alcohols catalyzed by dehydrogenases to furnish aldehydes and ketones, respectively, are common chemical reactions that rarely present insurmountable problems to the synthetic organic chemist. In contrast to the corresponding reduction reactions, oxidation reactions using isolated dehydrogenase enzymes have been rather scarcely reported [1023]. The reasons for this situation are as follows.

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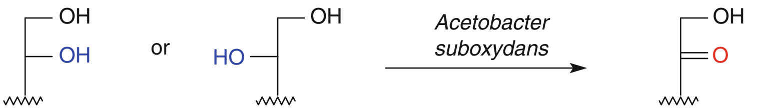
[37] It is an alarming fact that $\geq$90% of the hydrocarbons derived from drude oil are 'wasted' for energy-production (forming CO_2), the small remainder of $\leq$10% is used as raw material for the chemical industry to produce of (long-lasting) products [1019].

- Oxidations of alcohols using NAD(P)$^+$-dependent dehydrogenases are thermodynamically unfavorable. Thus, the recycling of the oxidized nicotinamide cofactor becomes a complicated issue (see p. 144).
- Due to the fact that the lipophilic aldehydic or ketonic products are often more tightly bound onto the hydrophobic active site of the enzyme than the more hydrophilic substrate alcohol, product inhibition is a common phenomenon in such reactions [759].
- Enzymatic oxidations usually work best at elevated pH (8–9) where nicotinamide cofactors and (particularly aldehydic) products are unstable.
- Oxidation of a secondary alcohol involves the *destruction* of an asymmetric center ($sp^3 \rightarrow sp^2$ hybrid) and is therefore of limited synthetic use.

Regioselective Oxidation of Polyols

The enzyme-catalyzed oxidation of alcohols is only of practical interest to the synthetic organic chemist if complex molecules such as polyols are involved (Scheme 2.141) [1024–1028]. Such compounds present problems of selectivity with conventional chemical oxidants. The selective oxidation of a single hydroxyl group in a polyol requires a number of protection–deprotection steps if performed by chemical means. In contrast, numerous sugars and related polyhydroxy compounds have been selectively oxidized in a single step into the corresponding keto-ols or ketoacids using a variety of microorganisms, for example *Acetobacter* or *Pseudomonas* sp. The regioselective microbial oxidation of D-sorbitol (obtained by catalytic hydrogenation of D-glucose) by *Acinetobacter suboxydans* yields L-sorbose, which represents the key step in the famous Reichstein–Grüssner process for the production of L-ascorbic acid (vitamin C).



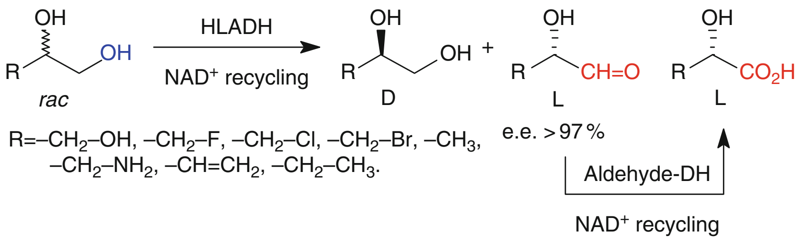
Substrate polyol	Product keto-alcohol	Reference
Adonitol	L-adonulose	[1024]
D-Sorbitol	L-sorbose	[1025]
L-Fucitol	4-keto-L-fucose	[1026]
D-Gluconic acid	5-keto-D-gluconic acid	[1027]
1-Deoxy-D-sorbitol	6-deoxy-L-sorbose	[1028]

Scheme 2.141 Regioselective oxidation of polyols by *Acetobacter suboxydans*

Kinetic Resolution of Alcohols by Oxidation

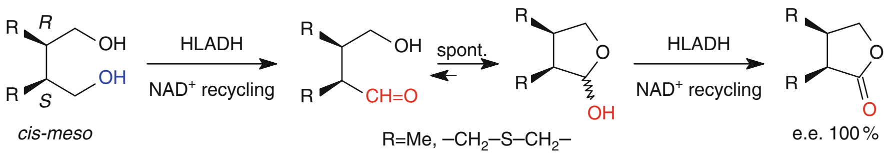
The major difficulty in the resolution of alcohols via selective oxidation of one enantiomer using isolated horse liver alcohol dehydrogenase (HLADH) is the regeneration of NAD$^+$. Besides the highly efficient enzymatic systems described in Sect. 2.2.1, a flavin mononucleotide (FMN) recycling system [1029] in which molecular oxygen is the ultimate oxidant was used to resolve numerous mono-, bi-, and polycyclic secondary alcohols by HLADH [786, 788, 1030, 1031]. To avoid enzyme deactivation, the hydrogen peroxide produced from O_2 during this process was removed using catalase [1032].

Glycols having a primary hydroxyl group were enantioselectively oxidized to yield L-α-hydroxyacids using a coimmobilized alcohol and aldehyde dehydrogenase system (Scheme 2.142). In the first step, kinetic resolution of the diol furnished a mixture of L-hydroxyaldehyde and the remaining D-diol. The former was oxidized in-situ by an aldehyde dehydrogenase to yield the L-hydroxyacid in high optical purity [791, 1033].



Scheme 2.142 Kinetic resolution of diols by a HLADH/aldehyde DH system

Desymmetrization of Prochiral or *meso*-Diols by Oxidation
In contrast to the resolution of secondary alcohols, where the more simple lipase technology is recommended instead of redox reactions, desymmetrization of primary diols of prochiral or *meso*-structure has been shown to be a valuable method for the synthesis of chiral lactones (Scheme 2.143) [1034].



Scheme 2.143 Desymmetrization of *meso*-diols by HLADH

As a rule of thumb, oxidation of the (*S*)- or pro-(*S*) hydroxyl group occurs selectively with HLADH (Scheme 2.143). In the case of 1,4- and 1,5-diols, the intermediate γ- and δ-hydroxyaldehydes spontaneously cyclize to form the more stable five- and six-membered hemiacetals (lactols). The latter are further oxidized in a subsequent step by HLADH to form γ- or δ-lactones following the same (*S*)- or pro-(*S*) specificity [1035]. Both steps – desymmetrization of the prochiral or *meso*-diol and kinetic resolution of the intermediate lactol – are often highly selective. By using this technique, enantiopure lactones were derived from *cis-meso*-2,3-dimethylbutane-1,4-diol and the cyclic thia-analog [1036], similar results were obtained with sterically demanding bicyclic *meso*-diols [1037].

2.3.2 Oxygenation Reactions

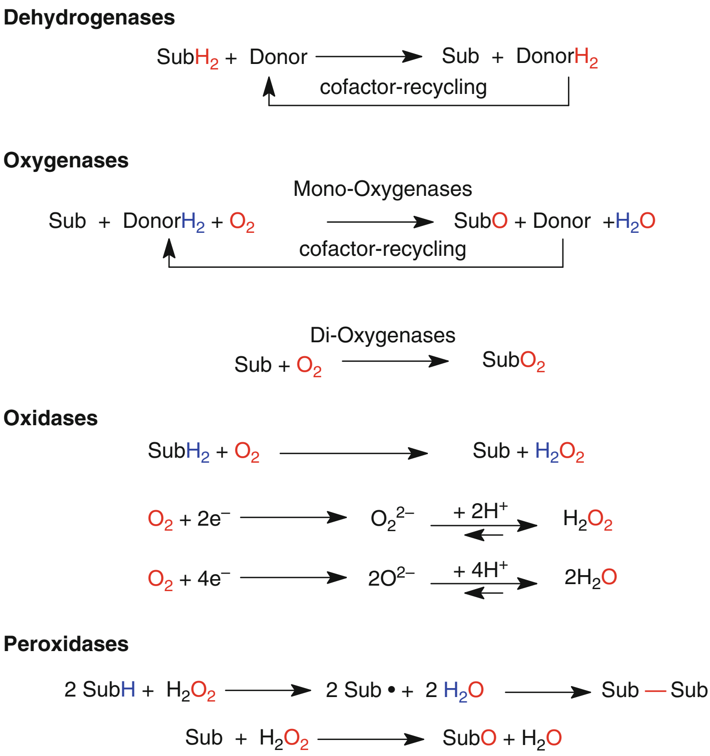
Enzymes which catalyze the direct incorporation of molecular oxygen into an organic molecule are called '*oxygen*ases' [1038–1041]. Enzymatic oxygenation reactions are particularly intriguing since direct oxyfunctionalization of nonactivated organic compounds remains a largely unresolved challenge to synthetic chemistry. On the one hand, there are numerous (catalytic) oxidation processes developed by industry to convert simple raw materials, such as alkanes, alkenes and aromatics into more valuable intermediate products (such as aldehydes, ketones, alcohols, carboxylic acids, etc.) at the expense of O_2.[38] However, the catalysts employed are highly sophisticated and thus show a very narrow substrate range, which limits their applicability to a single (or few) substrate(s) and they cannot be used for a wider range of organic compounds.[39] Unsurmountable problems persist where regio- or enantiospecificity is desired. On the other hand, highly selective oxygenation reactions may be achieved by means of biocatalysts.

Oxygen-transfer from molecular oxygen into organic acceptor molecules may proceed through three different mechanisms (Scheme 2.144).

- Monooxygenases incorporate *one* oxygen atom from molecular oxygen into the substrate, the other is reduced at the expense of a donor (usually NADH or NADPH) to form water [1042–1044].
- Dioxygenases simultaneously incorporate *both* oxygen atoms of O_2 into the substrate by forming a peroxy-species, thus they are sometimes misleadingly called oxygen transferases (although they are redox enzymes belonging to EC class 1).
- Oxidases [EC 1.1.3.X], on the other hand, mainly catalyze the electron-transfer onto molecular oxygen. This may proceed through a two- or four-electron transfer, involving either hydrogen peroxide or water as byproduct of the oxidation reaction, respectively. Oxidases include flavoprotein oxidases (amino acid oxidases, glucose oxidase, nicotinamide oxidase), metallo-flavin oxidases (aldehyde oxidase) and heme-protein oxidases (catalase, H_2O_2-specific peroxidases [1045]). Some of the enzymes have been found to be very useful. For instance, D-glucose oxidase is used on a large scale, in combination with catalase, as a food antioxidant [1046]; however, from a synthetic viewpoint, oxidases have not yet been utilized extensively [1047]. On the contrary, peroxidation reactions catalyzed by peroxidases at the expense of hydrogen peroxide have recently been found to be highly useful (Sect. 2.3.3).

[38] The most important processes with respect to scale are: *p*-xylene → terephthalic acid, ethylene → ethylene oxide, ethylene → acetaldehyde, ethylene/HOAc → vinyl acetate, methanol → formaldehyde, acetaldehyde → acetic acid.

[39] The only notable exception to this rule seems to be the Wacker-oxidation of alkenes.



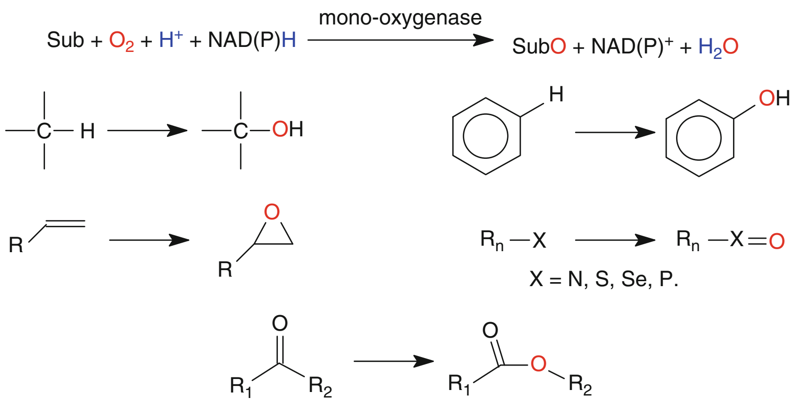
Scheme 2.144 Enzymatic oxidation reactions

Monooxygenases

Although the reaction mechanisms of various monooxygenases differ greatly depending on the subtype of enzyme, their mode of oxygen-activation is the same. Whereas one of the oxygen atoms from O_2 is transferred onto the substrate, the other is reduced to form a water molecule. The latter requires two electrons, which are derived from a cofactor, usually NADH or NADPH, serving as 'donor' (Scheme 2.144).

The net reaction and a number of synthetically useful monooxygenation reactions are shown in Scheme 2.145.

The generation of the activated oxygen-transferring species is mediated either by cofactors containing a transition metal (Fe or Cu) or by a heteroaromatic system (a pteridin [1048] or flavin [1049–1051]). The catalytic cycle of the iron-depending monooxygenases, the majority of which belong to the cytochrome P-450 type (Cyt P-450) [1052–1056], has been deduced largely from studies on the camphor hydroxylase of *Pseudomonas putida* [1057, 1058]. A summary of the catalytic cycle is depicted in Scheme 2.146.



Substrate	Product	Type of reaction	Type of cofactor
Alkane	alcohol	hydroxylation	metal-dependent
Aromatic	phenol	hydroxylation	metal-dependent
Alkene	epoxide	epoxidation	metal-dependent
Heteroatom[a]	heteroatom oxide	heteroatom oxidation	flavin-dependent
Ketone	ester/lactone	Baeyer-Villiger	flavin-dependent

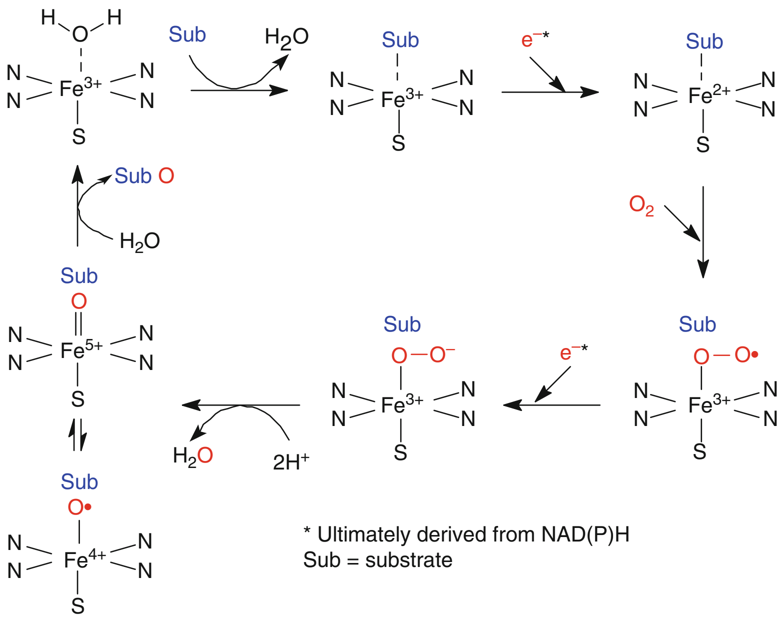
[a]N, S, Se, or P

Scheme 2.145 Monooxygenase catalyzed reactions

The iron species is coordinated equatorially by a heme moiety and axially by the sulfur atom of a cysteine residue. After binding of the substrate (Sub) by replacing a water molecule in a hydrophobic pocket adjacent to the porphine molecule [1059], the iron is reduced to the ferrous state. The single electron is delivered from NAD(P)H via another cofactor, which (depending on the enzyme) is a flavin-nucleotide, an iron-sulfur protein (ferredoxin) or a cytochrome b_5. Next, molecular oxygen is bound to give a Cyt P-450 dioxygen complex. Delivery of a second electron weakens the O–O bond and allows the cleavage of the oxygen molecule. One atom is expelled as water, the other forms the ultimate oxidizing Fe^{4+} or Fe^{5+} species, which – as a strong electrophile – attacks the substrate. Expulsion of the product (SubO) reforms the iron(III)-species and closes the catalytic cycle. Despite the fact that the mechanism of Cyt P-450 enzymes has been intensively investigated over several decades, many mechanistic details are still poorly understood.

Cyt P-450 enzymes got their name from their hemoprotein character: P stands for ‘pigment’ and 450 reflects the absorption of the CO-complex at 450 nm. To date, more than 6,000 different Cyt P-450 genes have been cloned[40] and these

[40]http://drnelson.utmem.edu/CytochromeP450.html.



Scheme 2.146 Catalytic cycle of cytochrome P-450-dependent monooxygenases

proteins are classified into four major groups (bacterial, mitochondrial, microsomal and self-sufficient Cyt) according to the mode of the electron-transport and the interaction between the subunits. A simplified schematic organization of Cyt P-450 systems is depicted in Fig. 2.18.

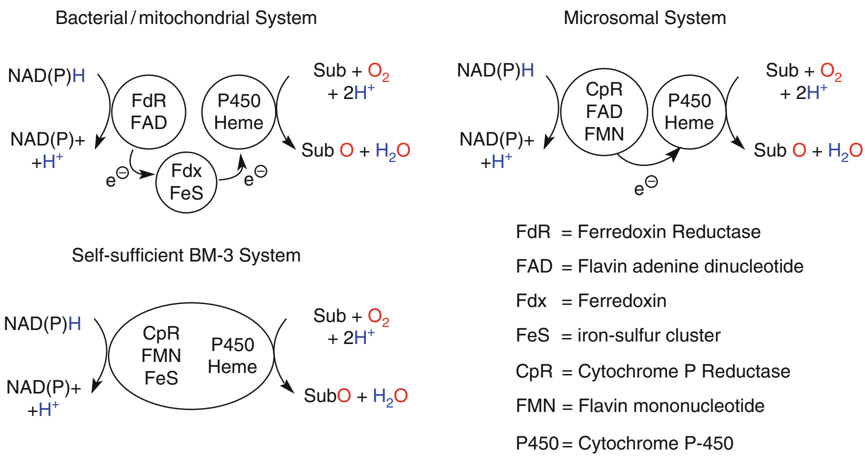
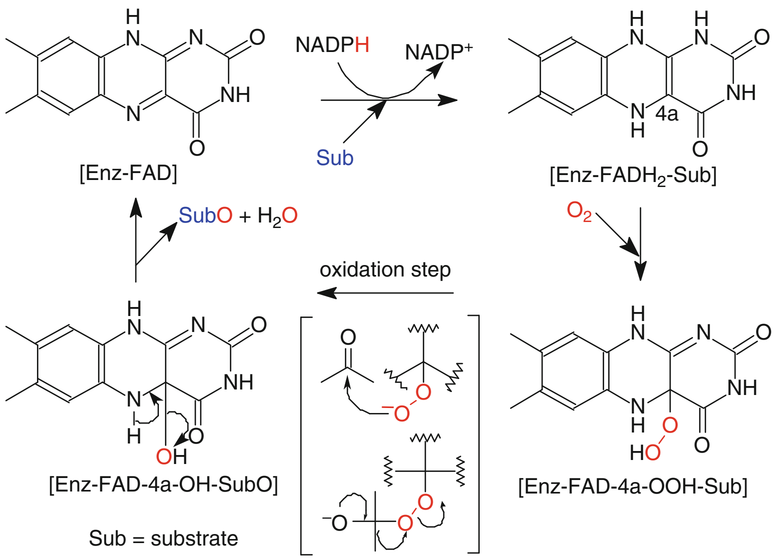


Fig. 2.18 Schematic organization and electron transport of cytochrome P-450 type monooxygenases

Bacterial and mitochondrial Cyt P-450 systems depend on three proteins: the P-450 monooxygenase with its heme unit, which performs the actual oxygenation of the substrate, a ferredoxin reductase, which accepts hydride equivalents from nicotinamide via an FAD cofactor and ferredoxin, which acts as electron shuttle between them using an iron-sulfur cluster as electron carrier [1060]. The microsomal system is somewhat simpler, as electron transfer occurs directly between the cytochrome P reductase (possessing an FMN and FAD cofactor) and the Cyt P-450 enzyme and thus does not require the ferredoxin. A minimal Cyt P-450 system is derived from *Bacillus megaterium* (BM-3) and it consists of a single (fusion) protein, which is made up of two domains, a cytochrome P reductase (containing FMN and the FeS cluster) and the P-450 enzyme. It is evident, that the latter system has been the prime target of studies directed towards the development of enzymatic oxygenation systems for preparative-scale applications [1061, 1062].

In contrast, flavin-dependent monooxygenases (see Scheme 2.147 and Table of Scheme 2.145) use a different mechanism which involves a flavin cofactor [1063–1065]. First, NADPH reduces the Enz-FAD complex thereby destroying aromaticity. The $FADH_2$ so formed is oxidized via Michael-type addition of molecular oxygen yielding a hydroperoxide species (FAD-4a-OOH). Deprotonation of the latter affords a peroxide anion, which undergoes a nucleophilic attack on the carbonyl group of the substrate, usually an aldehyde or a ketone. The tetrahedral species thus formed (corresponding to a Criegee-intermediate) collapses via rearrangement of the carbon-framework forming the product ester or lactone, respectively. Finally, water is eliminated from the FAD-4a-OH species to reform FAD. In addition to the Baeyer-Villiger oxidation, the flavin-4a-hydroperoxy species can



Scheme 2.147 Catalytic cycle of flavin-dependent monooxygenases

also mediate the hydroxylation of aromatics [1066–1070], alkene epoxidation [1071, 1072] and heteroatom-oxidation.

Whereas the Cyt P-450 mechanism resembles the chemical oxidation by hypervalent transition metal oxidants through an electrophilic attack (nature's permanganate), the FAD-dependent mechanism parallels the oxidation of organic compounds by peroxides or peracids, which act as nucleophiles (nature's *m*-chloroperbenzoic acid) [1073].

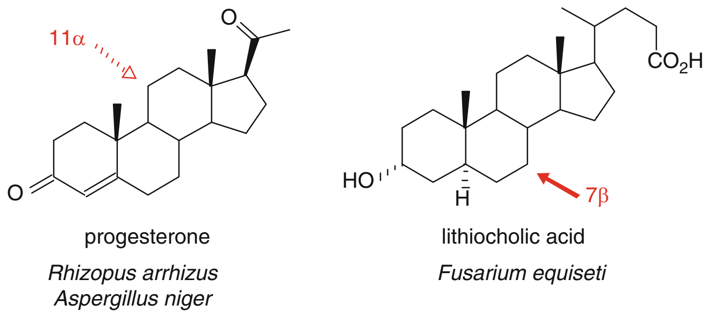
Many monooxygenases are closely linked to the respiratory chain and are therefore membrane-bound, which makes their isolation and (over)expression difficult. This fact and the need for recycling of NAD(P)H makes it clear that the majority of monooxygenase catalyzed reactions have been performed by using whole microbial cells, of by employing 'designer bug' host-cells co-expressing the required proteins. The disadvantage of this method lies in the fact that the desired product is often obtained in low yields due to further metabolism of the oxidation product by the cell. However, selective blocking of enzymes which are responsible for the degradation of the product or using enzyme-deficient mutants has been shown to make microbial oxygenation reactions feasible on a commercial scale.

2.3.2.1 Hydroxylation of Alkanes

The hydroxylation of nonactivated centers in hydrocarbons is one of the most useful biotransformations [1040, 1074–1079] due to the fact that this process has only very few counterparts in traditional organic synthesis [1080–1082]. In general, the relative reactivity of carbon atoms in bio-hydroxylation reactions declines in the order of secondary > tertiary > primary [1083], which is in contrast to radical reactions (tertiary > secondary > primary) [1084]. There are two main groups of hydrocarbon molecules, which have been thoroughly investigated with respect to microbial hydroxylation, i.e., *steroids* and *terpenoids*. Both have in common, that they possess a large main framework, which impedes the metabolic degradation of their hydroxylated products.

Intense research on the stereoselective hydroxylation of alkanes started in the late 1940s in the steroid field, driven by the demand for pharmaceuticals [1085–1090] In the meantime, some of the hydroxylation processes, e.g., 9α- and 16α-hydroxylation of the steroid framework [1091, 1092], have been developed to the scale of industrial production. Nowadays, vitually any center in a steroid can be selectively hydroxylated by choosing the appropriate microorganism.[41] For example, hydroxylation of progesterone in the 11α-position by *Rhizopus arrhizus* [1094] or *Aspergillus niger* [1095] made roughly half of the 37 steps of the conventional chemical synthesis redundant and made 11α-hydroxyprogesterone available for hormone therapy at a reasonable cost (Scheme 2.148). A highly selective hydroxylation of lithiocholic acid in position 7β was achieved by using

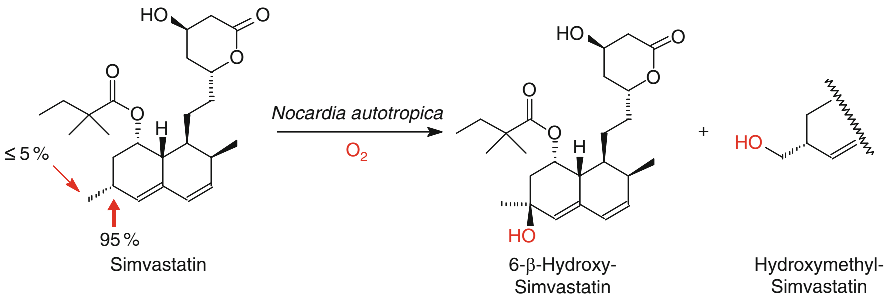
[41]For a complete list see [1092].



Scheme 2.148 Regio- and stereoselective microbial hydroxylation of steroids

Fusarium equiseti [1096]. The product (ursodeoxycholic acid) is capable of dissolving cholesterol and thus can be used in the therapy of gallstones.

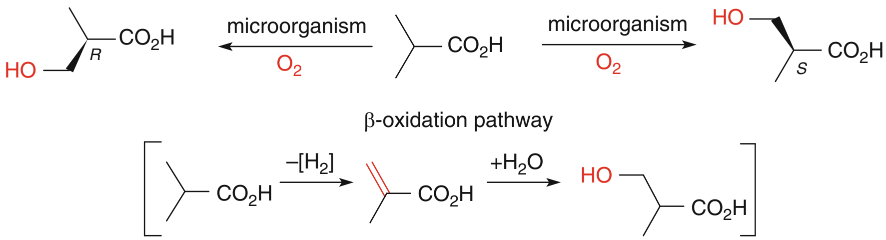
In a similar fashion, active pharmaceutical ingredients (APIs) are subjected to microbial hydroxylation. For instance, the regioselective allylic hydroxylation of the potent cholesterol-lowering drug simvastatin was achieved using *Nocardia autotrophica* to yield 6-β-hydroxy-simvastatin together with some minor side-products [1097]. An impressive amount of 15 kg of product was obtained from a 19 m^3 reactor (Scheme 2.149).



Scheme 2.149 Regioselective microbial hydroxylation of HMG-CoA reductase inhibitor Simvastatin

Optically active β-hydroxy-*iso*butyric acid has been used as a starting material for the synthesis of vitamins (α-tocopherol [1098]), fragrance components (muscone [1099]) and antibiotics (calcimycin [1100]). Both enantiomers may be obtained by asymmetric hydroxylation of *iso*butyric acid [1101, 1102] (Scheme 2.150). An intensive screening program using 725 strains of molds, yeasts and bacteria revealed that, depending on the microorganism, either the (*R*)- or the (*S*)-β-hydroxy-*iso*butyric acid was formed in varying optical purity. Best results were obtained using selected *Candida* and *Pseudomonas* strains.

Although the mechanism of this reaction was initially assumed to be a 'direct' hydroxylation at position β, more detailed studies showed that it proceeds via the

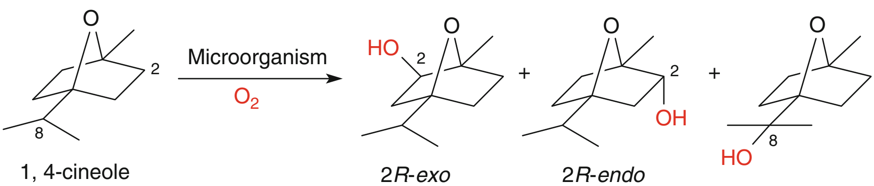


Microorganism	Configuration	e.e. [%]
Pseudomonas putida ATCC 21244	*S*	>95
Candida rugosa IFO 750	*S*	99
Candida rugosa IFO 1542	*R*	97

Scheme 2.150 Asymmetric microbial hydroxylation of *iso*butyric acid via the β-oxidation pathway

conventional β-oxidation pathway involved in fatty acid metabolism [1103]. The same sequence is followed during the transformation of 4-trimethylammonium butanoate to the β-hydroxy derivative (carnitine, see Scheme 2.212) [1104].

The production of new materials for the aroma and fragrance industry was the powerful driving force in the research on the hydroxylation of terpenes [924, 1105–1107]. For instance, 1,4-cineole, a major constituent of eucalyptus oil, was regioselectively hydroxylated by *Streptomyces griseus* to give 8-hydroxycineole as the major product along with minor amounts of *exo*- and *endo*-2-hydroxy derivatives, with low optical purity (Scheme 2.151) [1108]. On the other hand, when *Bacillus cereus* was used, (2*R*)-*exo*- and (2*R*)-*endo*-hydroxycineoles were exclusively formed in a ratio of 7:1, both in excellent enantiomeric excess [1109].



1, 4-cineole 2*R*-*exo* 2*R*-*endo*

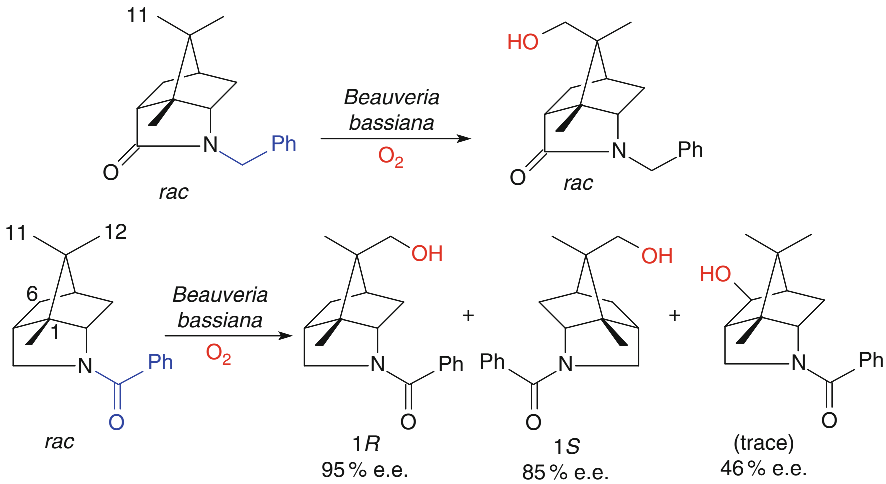
Microorganism	e.e. [%] *exo*	e.e. [%] *endo*	*exo*/*endo* ratio
Streptomyces griseus	46	74	1:1.7[a]
Bacillus cereus	94	94	7:1

[a]8-Hydroxycineole was the major product.

Scheme 2.151 Microbial hydroxylation of 1,4-cineole

Among the many hundreds of microorganisms tested for their capability to perform hydroxylation of nonnatural aliphatic compounds, fungi have usually been more often used than bacteria. Among them, the fungus *Beauveria bassiana* ATCC 7159 (formerly denoted as *B. sulfurescens*) has been studied most thoroughly [1110–1114]. In general the presence of a polar group in the substrate such as an acetamide, benzamide or *p*-toluene-sulfonamide moiety seems to be advantageous in order to facilitate the orientation of the substrate in the active site [1115]. Hydroxylation occurs at a distance of 3.3–6.2 Å from the polar anchor group.[42] In cases where a competition between cycloalkane rings of different size was possible, hydroxylation preferentially occurred in the order cycloheptyl > cyclohexyl > cyclopentyl.

In the majority of cases, hydroxylation by *Beauveria bassiana* occurs in a *regioselective* manner, but high *enantioselectivity* is not always observed. As shown in Scheme 2.152, both enantiomers of the *N*-benzyl-protected bicyclic lactam are hydroxylated with high regioselectivity in position 11, but the reaction showed very low enantioselectivity. On the other hand, when the lactam moiety was replaced by a more polar benzoyl-amide, which functions as polar anchor group, high enantiodifferentiation occurred. The (1*R*)-enantiomer was hydroxylated at carbon 12 and the (1*S*)-counterpart gave the 11-hydroxylated product [1116]. A minor amount of 6-*exo*-alcohol was formed with low enantiomeric excess.



Scheme 2.152 Regio- and enantioselective hydroxylation by *Beauveria bassiana*

In order to provide a tool to predict the stereochemical outcome of hydroxylations using *Beauveria bassiana*, an active site model was proposed [1117, 1118]. Alternatively, a substrate model containing a polar anchor group can be used [1119].

[42]Previously this value was believed to be 5.5 Å.

In summary, it is certainly feasible to achieve the (bio)hydroxylation of hydrocarbon compounds by using one of the many microorganisms used to date, but it is difficult to predict the likely site of oxidation for any novel substrate using monooxygenases. However, there are three strategies which can be employed to improve regio- and/or stereoselectivity in biocatalytic hydroxylation procedures:

- Variation of the culture by stressing the metabolism of the cells
- Broad screening of different strains[43]
- Substrate modification, particularly aiming at the variation of the (polar) anchor group [1120–1123]

2.3.2.2 Hydroxylation of Aromatic Compounds

Regiospecific hydroxylation of aromatic compounds by purely chemical methods is notoriously difficult. There are reagents for *o*- and *p*-hydroxylation available [1124, 1125], but some of them are explosive and byproducts are usually obtained [1126]. The selective bio-hydroxylation of aromatics in the *o*- and *p*-position to existing substituents can be achieved by using monooxygenases. In contrast, *m*-hydroxylation is rarely observed [1127]. Mechanistically, it has been proposed that in eukaryotic cells (fungi, yeasts and higher organisms) the reaction proceeds predominantly via epoxidation of the aromatic species which leads to an unstable arene-oxide (Scheme 2.86) [1128]. Rearrangement of the latter involving the migration of a hydride anion (NIH-shift) forms the phenolic product [1129]. An alternative explanation for the catalytic mechanism of flavin-dependent oxidases [which are independent of NAD(P)H] has been proposed which involves a hydroperoxide intermediate (FAD-4a-OOH, Scheme 2.147) [1130].

Phenolic components can be selectively oxidized by polyphenol oxidase – one of the few available isolated oxygenating enzymes[44] – to give *o*-hydroxylated products (catechols) in high yields [1131]. Unfortunately the reaction does not stop at this point but proceeds further to form unstable *o*-quinones, which are prone to polymerization, particularly in water (Scheme 2.153).

Two techniques have been developed to solve the problem of *o*-quinone instability:

- One way to prevent polymerization of the *o*-quinone is to remove it from the reaction mixture by chemical reduction (e.g., by ascorbate), which leads to catechols. Ascorbate, however, like many other reductants can act as an inhibitor of polyphenol oxidase. To circumvent the inhibition, the concentration of the reducing agent should be kept at a minimum. Furthermore, a borate buffer

[43] The following strains have been used more frequently: *Aspergillus niger*, *Cunninghamella blakesleeana*, *Bacillus megaterium*, *Bacillus cereus*, *Mucor plumbeus*, *Mortierella alpina*, *Curvularia lunata*, *Helmithosporium sativum*, *Pseudomonas putida*, *Rhizopus arrhizus*, *Rhizopus nigricans*, *Beauveria bassiana*.

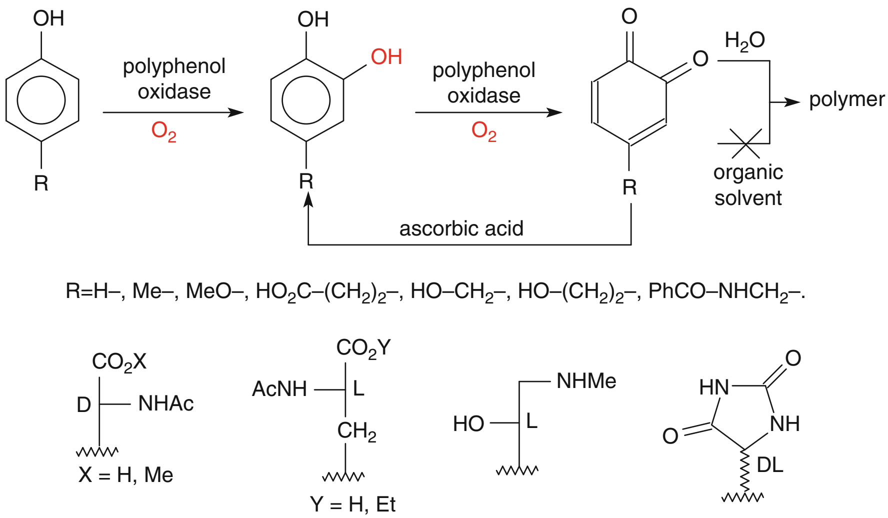
[44] Also called tyrosinase, catechol oxidase, cresolase.

which leads to the formation of a complex with catechols, thus preventing their oxidation, is advantageous [1132].

- Polymerization, which requires the presence of water, can also be avoided if the reaction is performed in a lipophilic organic solvent such as chloroform (Sect. 3.1) [1133].

The following rules for phenol hydroxylation have been deduced for polyphenol oxidase:

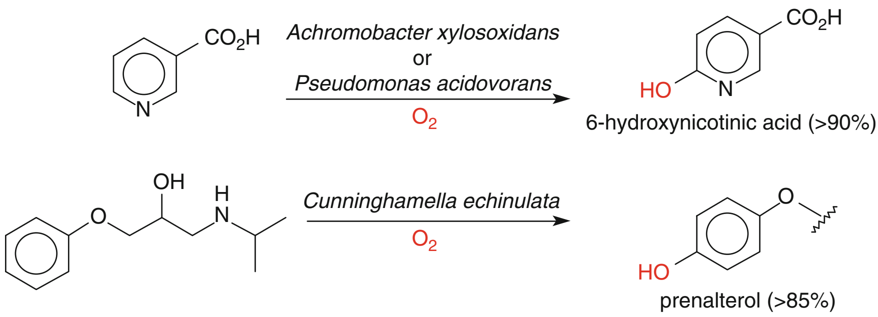
- A remarkable range of simple phenols are accepted, as long as the substituent R is in the *p*-position; *m*- and *o*-derivatives are unreactive.
- The reactivity decreases if the nature of the R group is changed from electron-donating to electron-withdrawing.
- Bulky phenols (*p*-*tert*-butylphenol and 1- or 2-naphthols) are not substrates; some electron-rich nonphenolic species such as *p*-toluidine are accepted.



Scheme 2.153 *o*-Hydroxylation of phenols by polyphenol oxidase

The synthetic utility of this reaction was demonstrated by the oxidation of amino acids and -alcohols containing an electron-rich *p*-hydroxyphenyl moiety (Scheme 2.153). Thus, L-DOPA (3,4-dihydroxyphenyl alanine) used for the treatment of Parkinson's disease, D-3,4-dihydroxy-phenylglycine and L-epinephrine (adrenaline) were synthesized from their *p*-monohydroxy precursors without racemization in good yield.

Regioselective hydroxylation of aromatic compounds can also be achieved by using whole cells [1134–1138]. For instance, 6-hydroxynicotinic acid is produced from nicotinic acid by *Pseudomonas acidovorans* or *Achromobacter xylosoxidans* on a ton-scale [1139]. Racemic prenalterol, a compound with important pharmacological



Scheme 2.154 Microbial hydroxylation of aromatics

activity as a β-blocker, was obtained by regioselective *p*-hydroxylation of a simple aromatic precursor using *Cunninghamella echinulata* (Scheme 2.154) [1140].

2.3.2.3 Epoxidation of Alkenes

Chiral epoxides are extensively employed high-value intermediates in the synthesis of chiral compounds due to their ability to react with a broad variety of nucleophiles. In recent years a lot of research has been devoted to the development of catalytic methods for their production [551, 1141]. The Katsuki-Sharpless method for the asymmetric epoxidation of allylic alcohols [1142, 1143] and the asymmetric dihydroxylation of alkenes are now widely applied and reliable procedures. Catalysts for the epoxidation of nonfunctionalized olefins have been developed more recently [555, 1144]. Although high selectivities have been achieved for the epoxidation of *cis*-alkenes, the selectivities achieved with *trans*- and terminal olefins were less satisfactory using the latter methods.

In contrast, the strength of enzymatic epoxidation, catalyzed by monooxygenases, is in the preparation of small and nonfunctionalized epoxides, where traditional methods are limited [557, 1145]. Despite the wide distribution of monooxygenases within all types of organisms, their capability to epoxidize alkenes seems to be associated mainly with alkane- and alkene-utilizing bacteria, whereas fungi are applicable to a lesser extent [1040, 1146–1150].

Biocatalytic asymmetric epoxidation of alkenes catalyzed by monooxygenases cannot be performed on a preparative scale with isolated enzymes due to their complex nature and their dependence on a redox cofactor, such as NAD(P)H. Thus, whole microbial cells are used instead. Although the toxic effects of the epoxide formed, and its further (undesired) metabolism by the cells catalyzed by epoxide hydrolases (Sect. 2.1.5), can be reduced by employing biphasic media, this method is not trivial and requires bioengineering skills [1151]. Alternatively, the alkene itself can constitute the organic phase into which the product is removed, away from the cells. However, the bulk apolar phase tends to damage the cell membranes, which reduces and eventually abolishes all enzyme activity [1152].

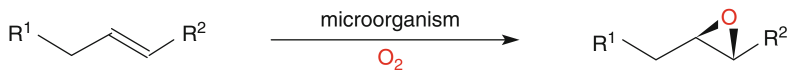
Once the problems of product toxicity were surmounted by sophisticated process engineering, microbial epoxidation of alkenes became also feasible on an industrial scale [1153, 1154]. The latter was achieved by using organic-aqueous two-phase systems or by evaporation for volatile epoxides. For instance, the epoxy-phosphonic acid derivative 'fosfomycin' [1155], whose enantiospecific synthesis by classical methods would have been extremely difficult, was obtained by a microbial epoxidation of the corresponding olefinic substrate using *Penicillium spinulosum*.

The most intensively studied microbial epoxidizing agent is the ω-hydroxylase system of *Pseudomonas oleovorans* [1156, 1157]. It consists of three protein components: rubredoxin, NADH-dependent rubredoxin reductase and an ω-hydroxylase (a sensitive nonheme iron protein). It catalyzes not only the hydroxylation of aliphatic C–H bonds, but also the epoxidation of alkenes [1158, 1159]. The following rules can be formulated for epoxidations using *Pseudomonas oleovorans* (Scheme 2.155).

- Terminal, acyclic alkenes are converted into (*R*)-1,2-epoxides of high enantiomeric excess along with varying amounts of ω-en-1-ols or 1-als [1160], the ratio of which depends on the chain length of the substrate [1161, 1162]. Alkene-epoxidation occurs mainly with substrates of 'moderate' chain length, such as 1-octene. However, alkane hydroxylation predominates over epoxidation for 'short' substrates (propene, 1-butene) and is a major pathway for 'long'-chain olefins.
- α,ω-Dienes are transformed into the corresponding terminal (*R*,*R*)-bis-epoxides.
- Cyclic, branched and internal olefins, aromatic compounds and alkene units which are conjugated to an aromatic system are not epoxidized [1163].
- To avoid problems arising from the toxicity of the epoxide [1164] (which accumulates in the cells and reacts with cellular enzymes) a water-immiscible organic cosolvent such as hexane can be added [1165, 1166].

Besides *Pseudomonas oleovorans* numerous bacteria have been shown to epoxidize alkenes [1167, 1168]. As shown in Scheme 2.155, the optical purity of epoxides depends on the strain used, although the absolute configuration is usually (*R*) [1169]. This concept has been recently applied to the synthesis of chiral alkyl and aryl gycidyl ethers [1170, 1171]. The latter are of interest for the preparation of enantiopure 3-substituted 1-alkylamino-2-propanols, which are widely used as β-adrenergic receptor-blocking agents [1172].

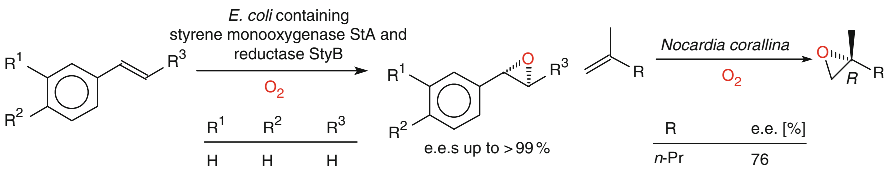
More recently, the structural restrictions for substrates which were elaborated by *Pseudomonas oleovorans* (see above) could be lifted by using different microorganisms. As can be seen from Scheme 2.155, nonterminal alkenes can be epoxidized by a *Mycobacterium* or *Xanthobacter* sp. [1173]. On the other hand, *Nocardia corallina* has been reported to convert branched alkenes into the corresponding (*R*)-epoxides in good optical purities (Scheme 2.156). Aiming at the improvement of the efficiency of microbial epoxidation protocols, a styrene monooxygenase (StyA) and reductase StyB required for electron-transport were co-expressed into *E. coli* to furnish a designer-bug for the asymmetric epoxidation of styrene-type substrates [1174, 1175].



Microorganism	R^1	R^2	Configuration	e.e. [%]
Pseudomonas oleovorans	*n*-C_5H_{11}	H	*R*	70-80
	H	H	*R*	86
	$NH_2CO–CH_2–C_6H_4–O$	H	*S*[a]	97
	$CH_3O(CH_2)_2–C_6H_4–O$	H	*S*[a]	98
Corynebacterium equi	CH_3	H	*R*	70
	n-$C_{13}H_{27}$	H	*R*	~100
Mycobacterium sp.	H	H	*R*	98
	Ph–O	H	*S*[a]	80
Xanthobacter Py2	Cl	H	*S*[a]	98
	CH_3	CH_3	*R, R*	78
Nocardia sp. IP1	Cl	H	*S*[a]	98
	CH_3	H	*R*	98

[a] CIP-sequence priority reversed

Scheme 2.155 Microbial epoxidation of alkenes



R^1	R^2	R^3
H	H	H
H	CH_3	H
H	H	CH_3
Cl	H	H

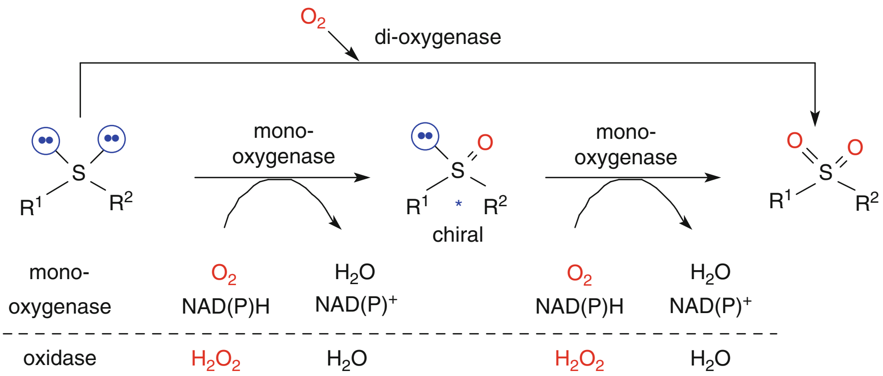
R	e.e. [%]
n-Pr	76
n-Bu	90
n-Pent	88

Scheme 2.156 Epoxidation of styrene derivatives and branched alkenes using cloned monooxygenase and *Nocardia corallina*

2.3.2.4 Sulfoxidation Reactions

Chiral sulfoxides have been extensively employed as asymmetric auxiliary group that assist stereoselective reactions. The sulfoxide functional group activates adjacent carbon–hydrogen bonds to allow proton abstraction by bases, and the corresponding anions can be alkylated [1176] or acylated [1177] with high diastereoselectivity. Similarly, thermal elimination [1178] and reduction of α-keto sulfoxides [1179] can proceed with transfer of chirality from sulfur to carbon. In spite of this great potential as valuable chiral relay reagents, with rare exceptions [1180], no general method is available for the synthesis of sulfoxides possessing high enantiomeric purities.

An alternative approach involves the use of enzymatic sulfur-oxygenation reactions catalyzed by monooxygenases [1181, 1182]. The main types of enzymatic sulfur oxygenation are shown in Scheme 2.157. The direct oxidation of a thioether by means of a dioxygenase, which directly affords the corresponding sulfone, is of no synthetic use since no generation of chirality is involved. On the other hand, the stepwise oxidation involving a chiral sulfoxide, which is catalyzed by monooxygenases or oxidases,[45] offers two possible ways of obtaining chiral sulfoxides.



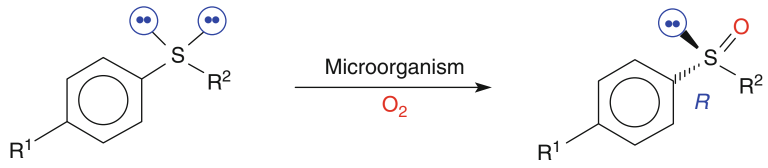
Scheme 2.157 Enzymatic sulfur oxygenation reactions

- The asymmetric monooxidation of a thioether leading to a chiral sulfoxide resembles a desymmetrization of a prochiral substrate and is therefore of high synthetic value.
- The kinetic resolution of a racemic sulfoxide during which one enantiomer is oxidized to yield an achiral sulfone is feasible but it has been shown to proceed with low selectivities.

The first asymmetric sulfur oxygenation using cells of *Aspergillus niger* was reported in the early 1960s [1184]. Since this time it was shown that the enantiomeric excess and the absolute configuration of the sulfoxide not only depend on the species but also on the strain of microorganism used [1185]. In general, the formation of (*R*)-sulfoxides predominates.

Thioethers can be asymmetrically oxidized both by bacteria (e.g., *Corynebacterium equi* [1186], *Rhodococcus equi* [1187]) and fungi (e.g., *Helminthosporium* sp. [1188] and *Mortierella isabellina* [1189]). Even baker's yeast has this capacity [1190, 1191]. As shown in Scheme 2.158, a large variety of aryl-alkyl thioethers were

[45]For the unusual microbial oxidation of a thioether catalyzed by a dioxygenase see [1182].



Microorganism	R^1	R^2	e.e. [%]
Mortierella isabellina	$(CH_3)_2CH$	CH_3	82
	H	$(CH_3)_2CH$	83
	H	C_2H_5	85
	C_2H_5	CH_3	90
	H	n-C_3H_7	~100
	Br	CH_3	~100[a]
Corynebacterium equi	H	CH_3	92
	CH_3	CH_3	97
	H	n-C_4H_9	~100
	H	CH_2–CH=CH_2	~100
baker´s yeast	CH_3	CH_3	92

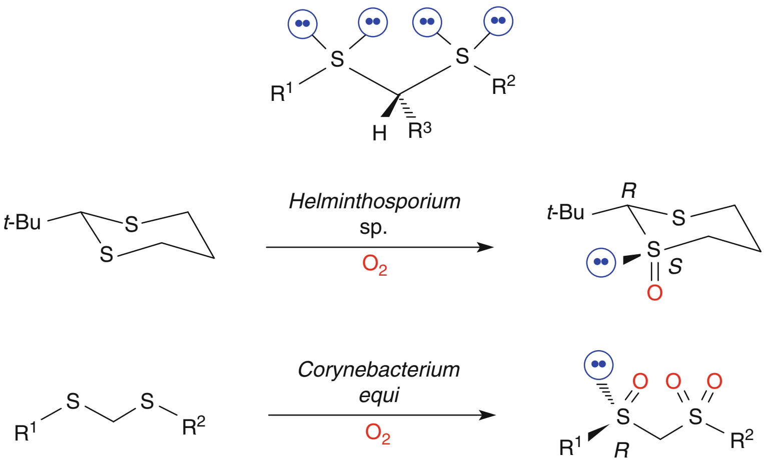
[a]Some sulfone was formed in this case.

Scheme 2.158 Microbial oxidation of aryl-alkyl thioethers

oxidized to yield sulfoxides with good to excellent optical purities [1192–1194]. The undesired second oxidation step was usually negligible, but with certain substrates the undesired formation of the corresponding sulfone was observed.

The transformation of thioacetals into mono- or bis-sulfoxides presents intriguing stereochemical possibilities. In a symmetric thioacetal of an aldehyde other than formaldehyde, the sulfur atoms are enantiotopic and each of them contains two diastereotopic nonbonded pairs of electrons (Scheme 2.159). Unfortunately, most of the products from asymmetric oxidation of thioacetals are of low to moderate optical purity [1195, 1196]. Two exceptions, however, are worth mentioning. Oxidation of 2-*tert*-butyl-1,3-dithiane by *Helminthosporium* sp. gave the (1*S*,2*R*)-monosulfoxide in 72% optical purity [1197] and formaldehyde thioacetals were oxidized by *Corynebacterium equi* to yield (*R*)-sulfoxide-sulfone products [1198] with excellent enantiomeric purity.

At present microbial sulfur oxidation reactions are certainly feasible on a preparative scale, but poor recoveries of the water-soluble products from the considerable amounts of biomass (a normal consequence of using whole cells as the biocatalyst) have to be taken into account. Whether the use of isolated oxidases will be an advantage [1199], future investigations will tell. For biocatalytic sulfur oxidation using peroxidase reactions, see Sect. 2.3.3.

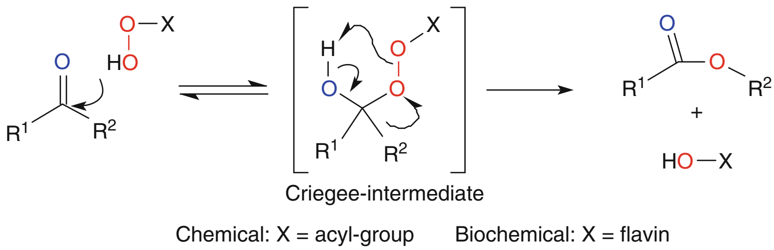


R^1	R^2	e.e. [%]
n-C_4H_9	*n*-C_4H_9	>95
CH_3	Ph	>95

Scheme 2.159 Microbial oxidation of dithioacetals

2.3.2.5 Baeyer-Villiger Reactions

Oxidation of ketones by peracids – the Baeyer-Villiger reaction [1200, 1201] – is a reliable and useful method for preparing esters or lactones (Scheme 2.160). The mechanism comprises a two-step process, in which the peracid attacks the carbonyl group of the ketone to form the so-called tetrahedral ‘Criegee-intermediate’ [1202]. The fragmentation of this unstable species, which proceeds via expulsion of a carboxylate ion going in hand with migration of a carbon–carbon bond, leads to the formation of an ester or a lactone. The regiochemistry of oxygen insertion of the chemical and the enzymatic Baeyer-Villiger reaction can usually be predicted by



Scheme 2.160 Mechanism of the chemical and biochemical Baeyer-Villiger oxidation

assuming that the carbon atom best able to support a positive charge will migrate preferentially [1203].

All mechanistic studies on enzymatic Baeyer-Villiger reactions support the hypothesis that conventional and enzymatic reactions are closely related [1063, 1204]. The oxidized flavin cofactor (FAD-4a-OOH, see Scheme 2.147) plays the role of a nucleophile similar to the peracid. The strength of enzyme-catalyzed Baeyer–Villiger reactions resides in the recognition of chirality [1205–1207], which has been accomplished by conventional means only recently, albeit in reactions exhibiting moderate selectivities [1208].

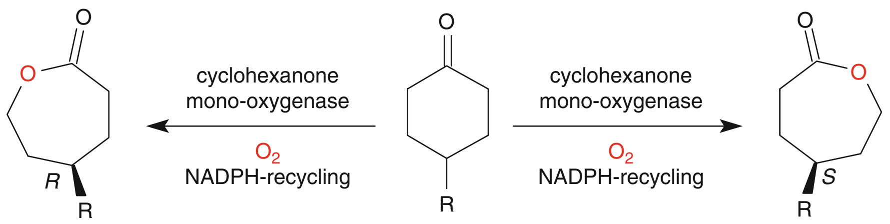
The enzymatic Baeyer-Villiger oxidation of ketones is catalyzed by flavin-dependent monooxygenases and plays an important role in the breakdown of carbon structures containing a ketone moiety. Whereas the early studies were performed by using whole microbial cells, particularly in view of avoiding the NAD(P)H-recycling problem [1209], an impressive number of bacterial 'Baeyer-Villigerases' were cloned, purified and characterized more recently [1210–1213]. In order to facilitate cofactor recycling, a selfsufficient fusion protein consisting of a Baeyer-Villigerase and a phosphite dehydrogenase unit for NADH-recycling were designed [1214]. The overall performance of the fusion-protein was comparable to that of the single (non-fused) proteins.

To avoid rapid further degradation of esters and lactones in microbial Baeyer-Villiger reactions catalyzed by hydrolytic enzymes and to maximize product accumulation in the culture medium, three approaches are possible:

- Blocking of the hydrolytic enzymes by selective hydrolase-inhibitors such as tetraethyl pyrophosphate (TEPP [1215]) or diethyl *p*-nitrophenylphosphate (paraoxon). However, all of these inhibitors are highly toxic and have to be handled with extreme caution.
- Development of mutant strains lacking lactone-hydrolases or
- Application of nonnatural ketones, whose lactone products are not substrates for the hydrolytic enzymes.

Prochiral (symmetric) ketones can be asymmetrically oxidized by a bacterial cyclohexanone monooxygenase from an *Acinetobacter* sp. to yield the corresponding lactones [1216, 1217]. As depicted in Scheme 2.161, oxygen insertion occurred on both sides of the ketone depending on the substituent in the 4-position. Whereas in the majority of cases products having the (*S*)-configuration were obtained, a switch to the (*R*)-lactone was observed with 4-*n*-butylcyclohexanone. Simple models are available, which allow the prediction of the stereochemical outcome of Baeyer-Villiger oxidations catalyzed by cyclohexanone monooxygenase of *Acinetobacter* and *Pseudomonas* sp. by determination of which group within the Criegee-intermediate is prone to migration [1218, 1219].

Racemic (nonsymmetric) ketones can be resolved via two pathways. The 'classic' form of a kinetic resolution involves a transformation in which one enantiomer reacts and its counterpart remains unchanged (Scheme 2.162) [1220]. For example, α-substituted cyclopentanones were stereospecifically oxidized by an

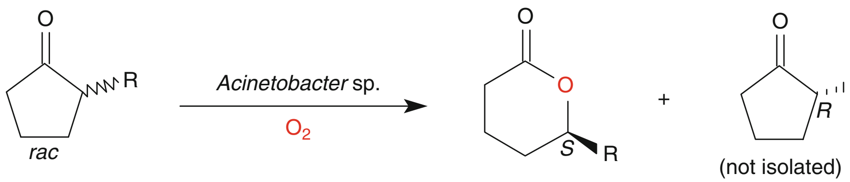


R	Configuration	e.e. [%]
CH_3–O–	*S*	75
Et–	*S*	>98
n-Pr–	*S*	>98
t-Bu–	*S*	>98
n-Bu–	*R*	52

Scheme 2.161 Desymmetrization of prochiral ketones via enzymatic Baeyer-Villiger oxidation

Acinetobacter sp. to form the corresponding (*S*)-configurated δ-lactones [1221], which constitute valuable components of various fruit flavors. The nonconverted (*R*)-ketones accumulated in the culture medium.

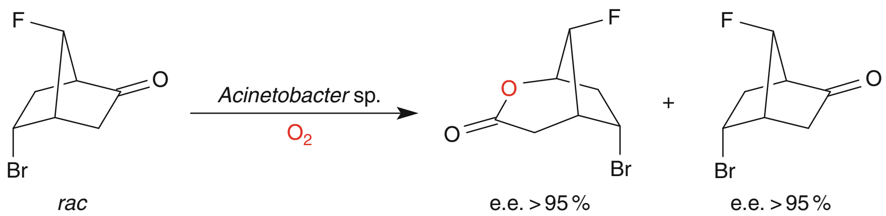
Bicyclic haloketones, which were used for the synthesis of antiviral 6′-fluoro-carbocyclic nucleoside analogs, were resolved by using the same technique [1222] (Scheme 2.163). Both enantiomers were obtained with >95% optical purity. The exquisite enantioselectivity of the microbial oxidation is due to the presence of the halogen atoms since the dehalogenated bicyclo[2.2.1]heptan-2-one was



R	e.e. of lactone [%]
n-C_5H_{11}	97
n-C_7H_{15}	95
n-C_9H_{19}	85
n-$C_{11}H_{23}$	73[a]

[a] the (*R*)-ketone showed an e.e. of 36 % in this case

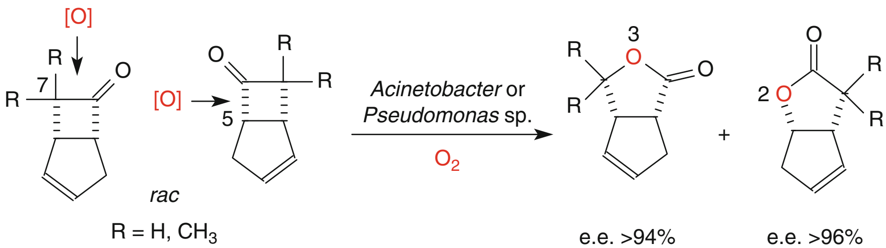
Scheme 2.162 Microbial Baeyer-Villiger oxidation of monocyclic ketones involving 'classic' resolution



Scheme 2.163 Microbial Baeyer-Villiger oxidation of a bicyclic ketone involving 'classic' resolution

transformed with low selectivity. On the other hand, replacement of the halogens by methoxy- or hydroxy groups gave rise to compounds which were not accepted as substrates.

The biological Baeyer-Villiger oxidation of a racemic ketone does not have to follow the 'classic' kinetic resolution format as described above, but can proceed via a 'nonclassic' route involving oxidation of *both* enantiomers with opposite regioselectivity. Thus, oxygen insertion occurs on the *two opposite sides* of the ketone at each of the enantiomers. As shown in Scheme 2.164, *both* enantiomers of the bicyclo[3.2.0]heptenones were microbially oxidized, but in an *enantiodivergent* manner [1223, 1224]. Oxygen insertion on the (5*R*)-ketone occurred as expected, adjacent to C7, forming the 3-oxabicyclic lactone. On the other hand, the (5*S*)-ketone underwent oxygen insertion in the 'wrong sense' towards C5, which led to the 2-oxabicyclic species. The synthetic utility of this system has been proven by the large-scale oxidation using an *E. coli* designer bug harboring cyclohexanone monooxygenase together with a suitabkle NADPH-recycling enzyme [1225, 1226]. In order to minimize product toxicity, in-situ substrate-feeding product removal (SFPR) was applied [1227, 1228].

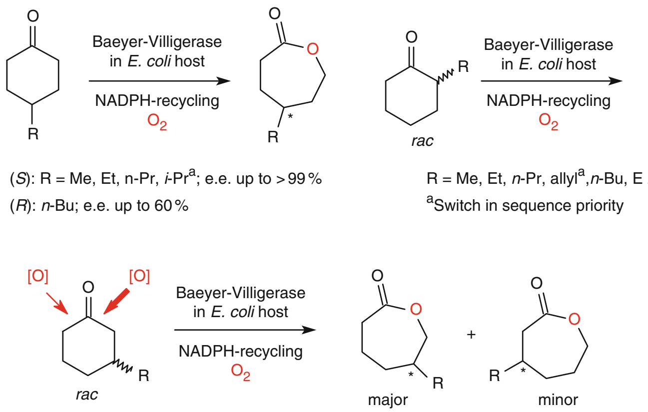


Scheme 2.164 Enantiodivergent microbial Baeyer-Villiger oxidation involving 'nonclassic' resolution

The molecular reasons of enantiodivergent Baeyer-Villiger reactions [1229, 1230] can either be the docking of the substrate in a single enzyme in two opposite modes or due to the presence of different monooxygenases present in the microbial cells [1231].

Whole-cell Baeyer–Villiger oxidations often suffer from low yields due to side reactions catalyzed by competing enzymes in the microorganisms. Furthermore, some of the most potent strains, such as *Acinetobacter calcoaceticus*, are potentially pathogenic and therefore have to be handled with extra care (see the Appendix, Chap. 5).[46] On the other hand, the use of isolated monooxygenases for Baeyer–Villiger oxidations is not trivial. On the one hand, there are enzymes available which show a desired opposite stereo- or enantiopreference [1232, 1233], on the contrary, the majority of these enzymes are linked to NADPH-recycling, which is notoriously difficult on a preparative scale. However, a monooxygenase is available from *Pseudomonas putida* which is dependent on *NADH*; this cofactor is much more readily recycled [1234].

In order to overcome these problems, a wide range of Baeyer-Villigerases from bacterial origin were cloned into a suitable (nonpathogenic) host, such as baker's yeast [1235–1239] or *E. coli* [1240]. Investigation of the regio- and enantioselectivity of Baeyer-Villigerases from bacteria isolated from an industrial wastewater treatment plant cloned into *E. coli* revealed the following trends (Scheme 2.165): (a) prochiral 4-substituted cyclohexanones underwent desymmetrization yielding (*R*)- (e.e.$_{max}$ 60%) or (*S*)-4-alkyl-ε-caprolactones (e.e.$_{max}$ > 99%), depending on the enzyme used and on the size of the substituent. (b) Racemic 2-substituted cyclohexanones underwent 'classic' kinetic resolution with absolute regioselectivity for oxygen-insertion at the predicted side to afford enantiomeric pairs of (*S*)-lactone and unreacted (*R*)-ketone with excellent enantioselectivities ($E \geq 200$). (c) In contrast, 3-substituted cyclohexanones furnished 'non-classic' kinetic resolution via oxygen-insertion at both sides with different regioselectivities to furnish



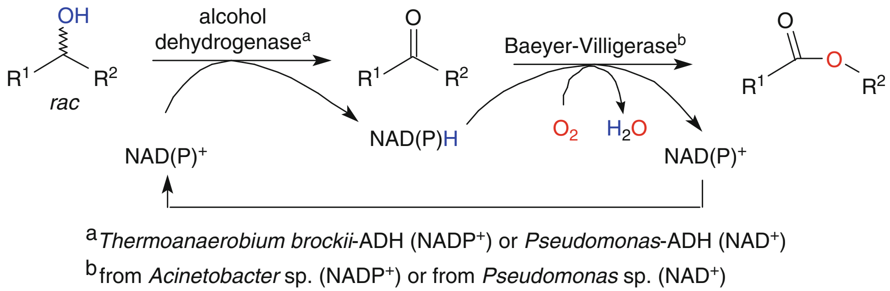
R	E
Me	60 (*S*)
Et	≥ 200 (*R*)
n-Pr	≥ 200 (*R*)
i-Pr	≥ 200 (*S*)[a]
n-Bu	20 (*R*) or (*S*)

Scheme 2.165 Regio- and enantioselective Baeyer-Villiger oxidation using cloned Baeyer-Villigerases exhibiting desymmetrization, and 'classic' and 'nonclassic' kinetic resolution

[46] *Acinetobacter calcoaceticus* NCIMB 9871 is a class-II pathogen.

regio-isomeric lactones. The enantioselectivites depended on the enzyme used and on size of the substituent [1241].

An alternative concept of cofactor recycling for isolated monooxygenases was developed using a coupled enzyme system (Scheme 2.166) [1242]. Thus, the substrate ketone is not used as such, but is rather produced by enzymatic oxidation of the corresponding alcohol (at the expense of $NADP^+$ or NAD^+, resp., Sect. 2.2.2) using a dehydrogenase from *Thermoanaerobium brockii* or from *Pseudomonas* sp. In a second step, the monooxygenase generates the lactone by consuming the reduced cofactor. Therefore, the NAD(P)H is concurrently recycled in a closed loop.



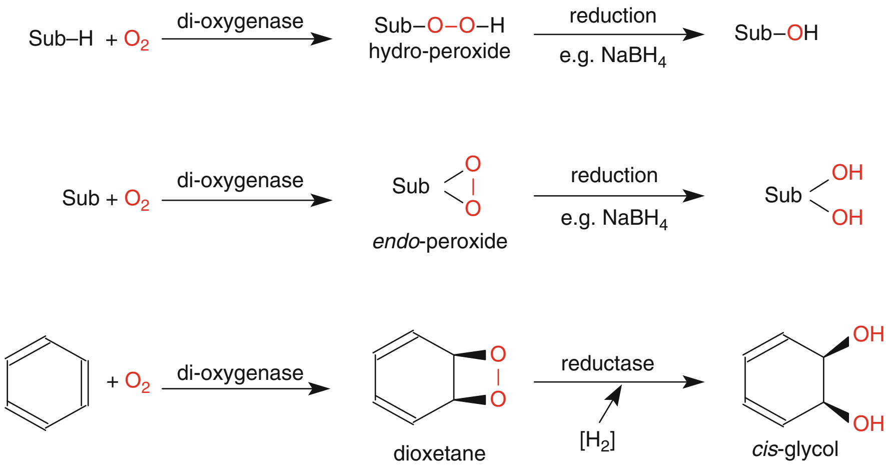
Scheme 2.166 Closed-loop cofactor recycling via a dehydrogenase-oxygenase system

Dioxygenases

Bacterial Rieske-type iron dioxygenases are nonheme enzymes, that contain an oxygenase component, an iron-sulfur flavoprotein reductase and FeS ferredoxin [1243, 1244]. Typical dioxygenase reactions, during which *two* oxygen atoms are simultaneously transferred onto the substrate, are shown in Scheme 2.167. In all cases, a highly reactive and unstable peroxo species is formed, e.g., a hydro- or endoperoxide. Both intermediates are highly reactive and may be subject to further transformations such as (enzymatic or nonenzymatic) reduction or rearrangement [1245]. In general, these latter intermediates cannot be isolated but are immediately reduced to yield the more stable corresponding (di)hydroxy derivatives.

- Alkenes may be oxidized, e.g., by a lipoxygenase, at the allylic position to furnish an allyl *hydro*peroxide which, upon reduction (e.g., by sodium borohydride) yields an allylic alcohol. In living systems, the formation of lipid peroxides is considered to be involved in some serious diseases and malfunctions including arteriosclerosis and cancer [1246].
- Alternatively, an *endo*-peroxide may be formed, whose reduction leads to a diol. The latter reaction resembles the cycloaddition of singlet-oxygen onto an unsaturated system and predominantly occurs in the biosynthesis of prostaglandins and leukotrienes. In prokaryotic cells such as bacteria the initial step of the metabolism of aromatic compounds consists of a (formal) cycloaddition of oxygen catalyzed by a dioxygenase (Scheme 2.86) [1247, 1248]. The resulting

intermediate *endo*-peroxide (dioxetane) is enzymatically reduced to yield synthetically useful *cis*-glycols (Scheme 2.167).



Scheme 2.167 Dioxygenase-catalyzed reactions

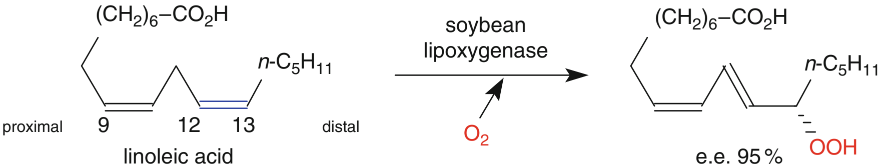
2.3.2.6 Formation of Peroxides

The biocatalytic formation of hydroperoxides seems to be mainly associated with dioxygenase activity found in plants, such as peas, peanuts, cucumbers, and potatoes as well as marine green algae. Thus, it is not surprising that the (nonnatural) compounds transformed so far have a strong structural resemblance to the natural substrates – fatty acids.

Allylic Hydroperoxidation. Lipoxygenase is a nonheme iron dioxygenase which catalyzes the incorporation of dioxygen into polyunsaturated fatty acids possessing a nonconjugated 1,4-diene unit by forming the corresponding conjugated allylic hydroperoxides [1249–1251]. The enzyme from soybean has received the most attention in terms of a detailed characterization because of its early discovery [1252], ease of isolation and acceptable stability [1253, 1254]. The following characteristics can be given for soybean lipoxygenase-catalyzed oxidations:

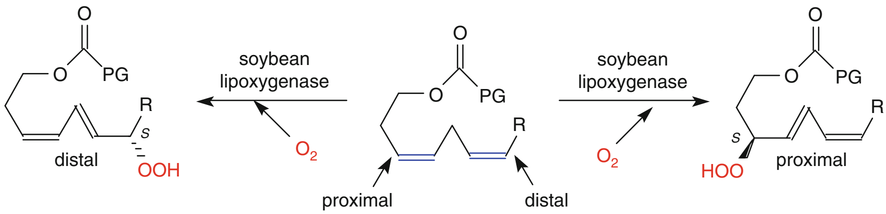
- The specificity of this enzyme has long been thought to be restricted to an all-(*Z*) configurated 1,4-diene unit at an appropriate location in the carbon chain of polyunsaturated fatty acids. However, it was shown that also (*E*,*Z*)- and (*Z*,*E*)-1,4-dienes are accepted as substrates [1255], albeit at slower rates.
- (*E*,*E*)-1,4-Dienes and conjugated 1,3-dienes are generally not oxidized.
- The configuration at the newly formed oxygenated chiral center is predominantly (*S*), although not exclusively [1256, 1257].
- In some cases, the oxidation of nonnatural substrates can be forced by increasing the oxygen pressure up to 50 bar [1258].

Oxidation of the natural substrate (*Z*,*Z*)-9,12-octadecadienoic acid (linoleic acid) proceeds highly selectively (95% e.e.) and leads to peroxide formation at carbon 13 (the 'distal' region) along with traces of 9-oxygenated product [1259] (the 'proximal' region, Scheme 2.168) [1260].



Scheme 2.168 Oxidation of linoleic acid by soybean lipoxygenase

In addition, it has been shown that soybean lipoxygenase can also be used for the oxidation of nonnatural 1,4-dienes, as long as the substrate is carefully designed to effectively mimic a fatty acid [1261]. Thus, the (*Z*,*Z*)-1,4-diene moiety of several long-chain alcohols could be oxidized by attachment of a prosthetic group (PG), which served as a surrogate of the carboxylate moiety (Scheme 2.169). This group can either consist of a polar $(CH_2)_n$–CO_2H or a CH_2–O–$(CH_2)_2$–OH unit [1262]. The oxidation occurred with high regioselectivity at the 'normal' (distal) site and the optical purity of the peroxides was >97%. After chemical reduction of the hydroperoxide (e.g., by Ph_3P [1263]) and removal



PG = prosthetic group

PG	R	distal / proximal	e.e. [%] distal
$(CH_2)_4CO_2H$	*n*-C_5H_{11}	95:5	98
$(CH_2)_4CO_2H$	CH_2Ph	89:11	98
$(CH_2)_4CO_2H$	$(CH_2)_3C(O)CH_3$	99:1	97
$CH_2O(CH_2)_2OH$	*n*-C_5H_{11}	99:1	98
$CH_2O(CH_2)_2OH$	*n*-C_8H_{17}	10:90	96
$CH_2O(CH_2)_2OH$	homogeranyl[a]	1:99	96

[a] = $(CH_2)_2CH{=}CCH_3(CH_2)_2CH{=}C(CH_3)_2$

Scheme 2.169 Oxidation of (*Z*,*Z*)-1,4-dienes by soybean lipoxygenase of the prosthetic group, the corresponding secondary alcohols were obtained with retention of configuration [1264].

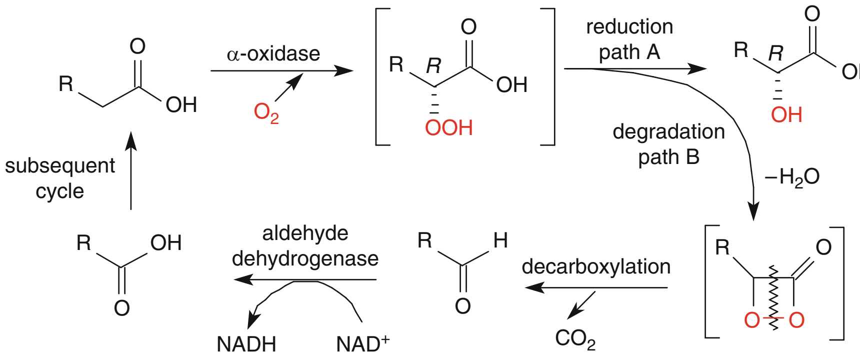
In addition, the regioselectivity of the oxidation – from 'normal' (distal) to 'abnormal' (proximal) – could be inverted by changing the lipophilicity of the modifying R groups and the spacer arm linking the prosthetic group PG (see Table 2.6 and Scheme 2.169). Increasing the lipophilicity of the distal R group from n-C_5 to n-C_{10} led to an increased reaction at the 'abnormal' site to form predominantly the proximal oxidation product. Consequently, when the lipophilicity of the proximal prosthetic group PG was increased by extending the spacer arm, the 'distal' product was formed in favor of the proximal.

Table 2.6 Variation of prosthetic groups (for formulas see Scheme 2.169)

PG	Variation	R	Distal/proximal
$(CH_2)_4CO_2H$	Distal	n-C_5H_{11}	95:5
$(CH_2)_4CO_2H$	Distal	n-C_8H_{17}	1:1
$(CH_2)_4CO_2H$	Distal	n-$C_{10}H_{21}$	27:73
$(CH_2)_2CO_2H$	Proximal	n-C_8H_{17}	20:80
$(CH_2)_4CO_2H$	Proximal	n-C_8H_{17}	1:1
$(CH_2)_6CO_2H$	Proximal	n-C_8H_{17}	85:15

α-Hydroperoxidation of Carboxylic Acids. The α-oxidation of fatty acids is known for higher plants such as pea leaves, germinating peanuts, cucumbers and potatoes as well for simple organisms such as marine green algae [1265]. The mechanism of this dioxygenase activity was elucidated in the mid-1970s (Scheme 2.170) [1266]. Occasionally, this activity has been denoted as 'α-oxidase', however, it should correctly be termed 'oxygenase', since an oxidase would produce H_2O_2 from O_2 rather than an α-hydroperoxy fatty acid going in hand with the direct incorporation of O_2 into the substrate. It is assumed that the flavoprotein-catalyzed oxidation of the fatty acid leads to an intermediary α-hydroperoxy acid, which can be further metabolized via two competing pathways: Whereas *reduction* furnishes an α-hydroxyacid (path A), *decarboxylation* leads to the corresponding aldehyde (path B). It has been speculated that the latter proceeds through an unstable intermediary α-peroxylactone. While the α-hydroxyacid is a final product and can thus be harvested, the aldehyde is further oxidized by an NAD^+-dependent aldehyde dehydrogenase to the next lower homologous fatty acid, which in turn can re-enter the α-oxidation in a subsequent cycle. The fact that α-oxidases are membrane-bound has impeded their isolation in pure form, and therefore structure elucidation is still pending. However, it has recently been shown that a crude enzyme extract from germinating pea leaves can be used for the α-hydroxylation of fatty acids and derivatives thereof via path A (Scheme 2.170) [1267, 1268]. The data available so far reveal the following picture:

- Substrates should be substantially lipophilic; more hydrophobic compounds, such as short-chain fatty acids and dicarboxylic acids, are not converted.
- Saturated fatty acids having 7–16 carbon atoms are well accepted, and



R	product ratio[a]	e.e. of α-hydroxyacid [%]
(E)-n-$C_8H_{17}CH{=}CH(CH_2)_6$	16:84	>99
$MeO_2C(CH_2)_7$	70:30	>99
n-$C_7H_{15}S(CH_2)_6$	99:1	>99
n-$C_{12}H_{25}$	99:1	>99
$C_2H_5S(CH_2)_9$	99:1	>99
(E)-n-$C_5H_{11}CH{=}CHCH_2$	~100:0	>99

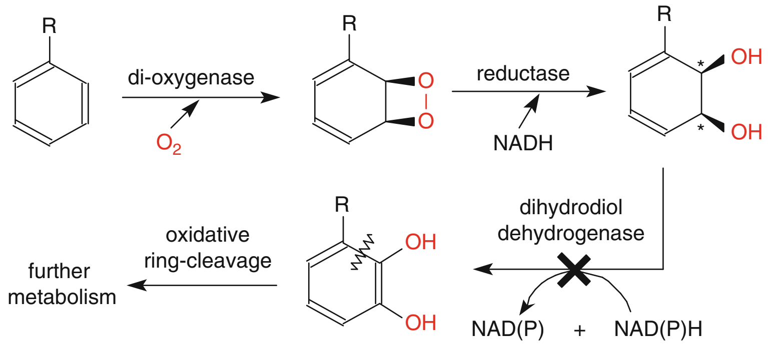
[a] α-hydroxyacid vs. aldehyde (via decarboxylation)

Scheme 2.170 α-Hydroperoxidation of fatty acid derivatives

- heteroatoms such as O and S are tolerated.
- Functional groups, such as carbon–carbon triple- and double-bonds, or heteroatoms have to be at a distance of at least three carbon atoms from the carboxylic acid moiety, otherwise α-hydroxylation does not take place.
- The ratio between substrate degradation (i.e., decarboxylation, path B) and the desired α-hydroxylation (path A) depends on the substrate structure, but it can be shifted by using optimized reaction conditions.
- The formation of the α-hydroperoxy acid is virtually absolute for the (*R*)-enantiomer.

2.3.2.7 Dihydroxylation of Aromatic Compounds

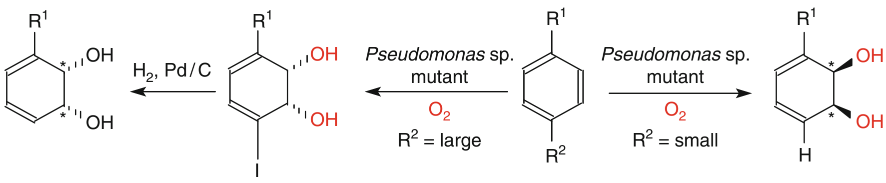
cis-Dihydroxylation by microbial dioxygenases constitutes the key step in the degradation pathway for aromatic compounds in lower organisms (Scheme 2.171) [1269, 1270], which is crucial for the removal of toxic pollutants from contaminated sites, such as oilspills. In 'wild-type' microorganisms, the chiral *cis*-glycols are rapidly further oxidized by dihydrodiol dehydrogenase(s), involving rearomatization of the diol intermediate with concomitant loss of chirality. The use of



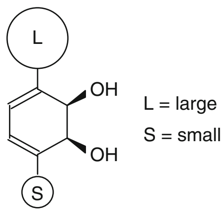
Scheme 2.171 Degradation of aromatics by microbial dioxygenases

mutant strains with blocked dehydrogenase activity [1271], however, allows the chiral glycols to accumulate in the medium, from which they can be isolated in good yield [1272, 1273]. The high standard of this technology allow to perform this useful biotransformation on ton-scale [1274].

For a number of mutant strains of *Pseudomonas putida*, the stereospecificity is high although the substrate specificity remains low with respect to the ring substituents R^1 and R^2 (Scheme 2.172), which allows their use in the asymmetric dihydroxylation of substituted aromatics [1275]. An impressive number of substituted aromatic compounds have been converted into the corresponding chiral *cis*-glycols, with excellent optical purities, on a commercial scale [1276–1278]. Even polysubstituted benzene derivatives can be converted into cyclohexadienediols and the regioselectivity of the oxygen addition can be predicted with some accuracy using a substrate model (Scheme 2.172) [1279–1281].



R^1	R^2
H, Me, Et, *n*-Pr, *i*-Pr, *n*-Bu, *t*-Bu, Et–O, *n*-Pr–O, Halogen, CF_3, Ph, Ph–CH_2, Ph–CO, CH_2=CH, CH_2=CH–CH_2, HC≡C	H
F, Me	I

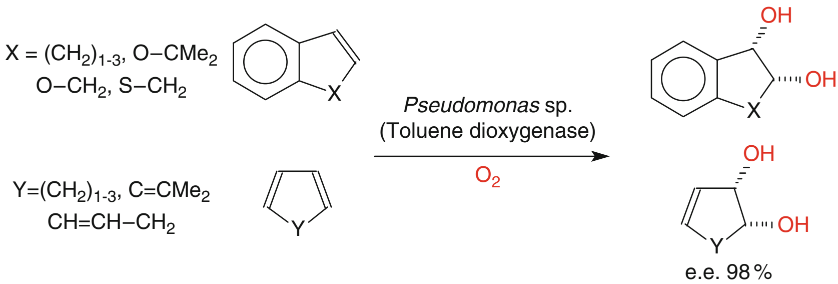


Scheme 2.172 Enantiocomplementary synthesis of *cis*-glycols

The substrates need not necessarily be monosubstituted aromatic compounds such as those shown in Scheme 2.172, but may also be extended to other species including fluoro- [1282], monocyclic- [1283], polycyclic- [1284], and heterocyclic derivatives [1285, 1286].

In order to gain access to products showing opposite configuration, a substrate modification approach using *p*-substituted benzene derivatives was developed. Thus, when *p*-iodo derivatives were used instead of the unsubstituted counterparts, the orientation of the oxygen addition was reversed, caused by the switch in relative size of substituents (I > F, I > CH_3). Subsequent removal of the iodine (which served as directing group) by catalytic hydrogenation led to mirror-image products [1287].

In a useful extension of the substrate pattern, it was shown that also nonaromatic C=C bonds can be *cis*-dihydroxylated to yield *erythro*-diols, as long as they are conjugated to an aromatic system (yielding styrene-derivatives) or to (at least) one additional alkene unit [1288–1290]. In contrast, isolated olefinic bonds are more readily metabolized via an epoxidation – epoxide hydrolysis sequence furnishing the corresponding *threo*-diols. Thus, *Pseudomonas putida* harboring toluene dioxygenase or naphthalene dioxygenase was able to oxidize a range of styrene-type alkenes and conjugated di- and -trienes (Scheme 2.173). The stereoselectivities were excellent for cyclic substrates but they dropped for open-chain derivatives (e.e.$_{max}$ 88%) [1291]. Depeding on the substrate and the type of enzyme, hydroxylation at benzylic or allylic positions were observed as side reactions.



Scheme 2.173 Dihydroxylation of conjugated alkenes using toluene dioxygenase

The synthetic potential of nonracemic *cis*-diols derived via microbial dihydroxylation has been exploited over recent years to synthesize a number of bioactive compounds. Cyclohexanoids have been prepared by making use of the possibility of functionalizing every carbon atom of the glycol in a stereocontrolled way. For instance, (+)-pinitol [1292] and D-*myo*-inositol derivatives [1293] were obtained using this approach. Cyclopentanoid synthons for the synthesis of prostaglandins and terpenes were prepared by a ring-opening/closure sequence [1294]. Rare carbohydrates such as D- and L-erythrose [1295] and L-ribonolactone [1296] were obtained from chlorobenzene as were pyrrolizidine alkaloids [1297]. Furthermore, a bio-inspired synthesis of the blue pigment indigo was developed on a commercial scale using the microbial dihydroxylation of indol [1298].

2.3.3 Peroxidation Reactions

Driven by the inability to use molecular oxygen as an oxidant efficiently for the transformation of organic compounds, chemists have used it in a partially reduced form – i.e., hydrogen peroxide [1299] or derivatives thereof.[47] H_2O_2 offers some significant advantages as it is cheap and environmentally benign – the only byproduct of oxidation being water. However, it is relatively stable and needs to be converted into a more active form in order to become an effective oxidant. This is generally accomplished either with organic or inorganic 'promoters' to furnish organic hydro- or endo-peroxides, peroxycarboxylic acids or hypervalent transition metal complexes based on V and Mo. Owing to these drawbacks, the number of industrial-scale oxidation processes using H_2O_2 as the oxidant is very limited.[48] On the other hand, biocatalytic activation of H_2O_2 by peroxidases allow a number of synthetically useful and often highly enantioselective peroxidation reactions, which offer a valuable alternative to traditional chemical methodology.

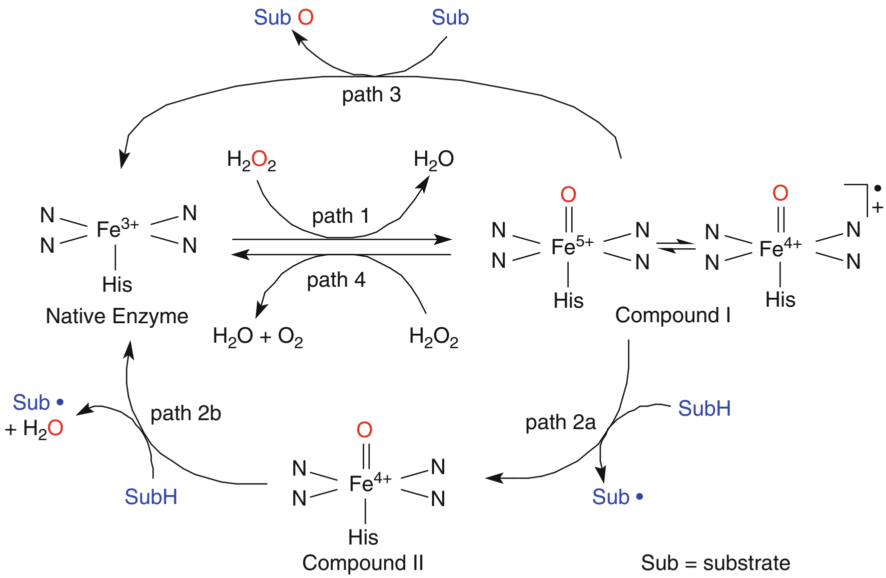
Peroxidases [EC 1.11.1.7] are a heterogeneous group of redox enzymes found ubiquitously in various sources [1300], such as plants [1301], microorganisms [1302] and animals. They are often named after their sources (e.g., horseradish peroxidase, lacto- and myeloperoxidase) or akin to their substrates (e.g., cytochrome c-, chloro- and lignin peroxidase). Although the biological role of these enzymes is quite diverse – ranging from (1) the scavenging of H_2O_2, (2) free radical oligomerization and polymerization of electron-rich aromatics to (3) the oxidation and halogenation of organic substrates – they have in common that they accept hydrogen peroxide or a derivative thereof (such as alkyl hydroperoxides) as oxidant. In line with these heterogeneous catalytic activities, the mechanism of action may be quite different and can involve a heme unit, selenium (glutathione peroxidase) [1303], vanadium (bromoperoxidase) [1304, 1305], manganese (manganese peroxidase) [1306] and flavin at the active site (flavoperoxidase) [1307]. The largest group of peroxidases studied so far are heme-enzymes with ferric protoporphyrin IX (protoheme) as the prosthetic group. Their catalytic cycle bears some similarities to that of heme-dependent monooxygenases (Sect. 2.3.2, Scheme 2.146), but owing to the diverse reactions they can catalyze, their pathways are more complex (Scheme 2.174). The mechanism of heme-dependent peroxidase catalysis has been largely deduced from horseradish peroxidase [1043, 1308–1310]. Its most important features are described as follows:

In its native state, the iron-III species is coordinated equatorially by a heme unit and axially by a histidine residue and is therefore very similar to cytochrome P 450 [1311]. The first step in the reaction involves oxidation of the Fe^{+3} to form an iron-oxo derivative called Compound I. The latter contains a $Fe^{+4}{=}O$ moiety and

[47] *tert*-Butylhydroperoxide and cumyl hydroperoxide.

[48] To date, the largest industrial-scale process is the oxidation of propene to propene oxide using *tert*-Bu–OOH.

a π-radical and is formally two oxidation equivalents above the Fe^{+3}-state.[49] In a peroxidase, this oxidation is achieved in a single step at the expense of H_2O_2 (path 1). In the monooxygenase pathway, the Fe^{3+}-species is oxidized by O_2, which requires two additional electrons (from a nicotinamide cofactor) to cover the net redox balance. Compound I represents the central hypervalent oxidizing species, which can react along several pathways. Path 2: Abstraction of a single electron from an electron-rich substrate such as an enol or phenol (forming a substrate radical) yields an Fe^{+4}=O species denoted Compound II (path 2a). Since the latter is still one oxidation equivalent above the Fe^{+3}-ground state, this process can occur a second time (forming another substrate radical, path 2b) to finally re-form the enzyme in its native state. Alternatively, incorporation of an O-atom onto a substrate (going hand in hand with a two-electron transfer) can occur in a single step (path 3). In the absence of any substrate, Compound I can re-form the native enzyme via disproportionation of H_2O_2, denoted as 'catalase-activity' (path 4).



Scheme 2.174 Catalytic cycles of heme-dependent peroxidases

Due to the fact that – in contrast to monooxygenases – no external nicotinamide cofactor is involved in any of the peroxidase cycles, peroxidases are highly attractive for preparative biotransformations. A number of synthetically useful reactions can be achieved (Scheme 2.175) [1312–1314].

[49]Compound I is comparable to the Fe^{+5} oxo-species in the mono-oxygenase cycle (see Scheme 2.146).

Oxidative dehydrogenation (path 2)

$$2\ SubH + H_2O_2 \longrightarrow 2\ Sub^{\bullet} + H_2O \longrightarrow Sub{-}Sub$$

Oxidative halogenation (path 3, Sub = Hal$^-$)

$$Sub + H_2O_2 + Hal^- + H^+ \longrightarrow Sub{-}Hal + 2\ H_2O$$

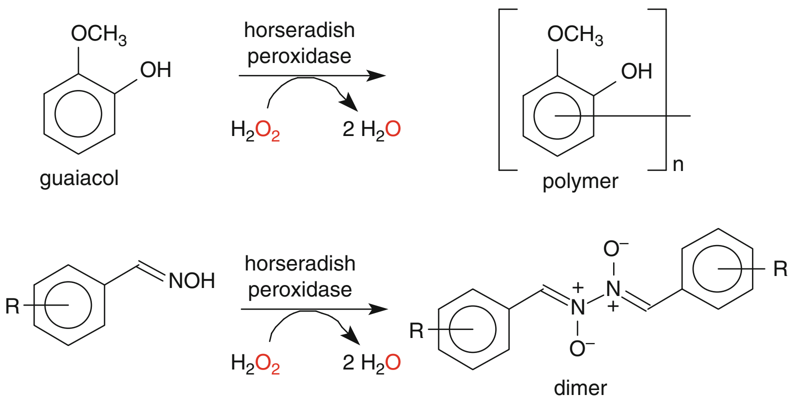
Oxygen transfer (path 3, Sub = organic compound)

$$Sub + H_2O_2 \longrightarrow SubO + H_2O$$

Scheme 2.175 Synthetically useful peroxidase reactions

Oxidative Dehydrogenation

This type of reaction is mainly restricted to heme peroxidases and it involves one-electron transfer processes with radical cations and radicals as intermediates (path 2). As a consequence, substrates are usually electron-rich (hetero)aromatics, which upon one-electron oxidation lead to resonance-stabilized radicals, which spontaneously undergo inter- or intramolecular coupling to form dimers or oligomers. This reaction is commonly denoted as the 'classical' peroxidase activity, since it was the first type of peroxidase-reaction discovered. Examples of such reactions are shown in Scheme 2.176. Oxidation of phenols (e.g., guaiacol, resorcin) and anilines (e.g., aniline, *o*-dianisidine) leads to the formation of oligomers and polymers under mild conditions [1315–1317]. In certain cases, dimers (e.g., aldoximes [1318], biaryls [1319]) have been obtained.



Scheme 2.176 Peroxidase-catalyzed oxidative dehydrogenation of aromatics

Oxidative Halogenation

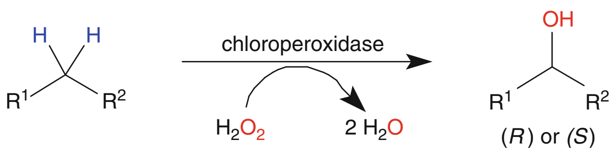
A class of peroxidases specializes in the (per)oxidation of halides (Cl$^-$, Br$^-$, I$^-$ but not F$^-$), thus creating reactive halogenating species (such as hypohalite), which in turn form haloorganic compounds [1320, 1321]. These reactions are described in Sect. 2.7.1.

Oxygen Transfer

From a synthetic viewpoint, selective oxygen transfer (path 3) is the most interesting peroxidation reaction. The transformations are comparable to those catalyzed by monooxygenases with one significant advantage – they are independent of redox cofactors, such as NAD(P)H.

Among the various types of reactions – C–H bond oxidation, epoxidation of alkenes and heteroatom oxidation – the most useful transformations are described below.

Hydroxylation of C–H Bonds. The only peroxidase known to effect the hydroxylation of C–H bonds is chloroperoxidase (CPO) from the marine fungus *Caldariomyces fumago*. Its large-scale production is facilitated by the fact that it is an extracellular enzyme, which is excreted into the fermentation medium [1322–1324].[50] In order to become susceptible towards hydroxylation by CPO, the C–H bonds have to be activated by a π-electron system. In the allylic position, hydroxylation is not very efficient and it is doubtful whether this procedure will be of practical use [1325]. By contrast, benzylic hydroxylation is readily effected and the corresponding *sec*-alcohols were isolated in high e.e. (Scheme 2.177)



R^1	R^2	Configuration	e.e. [%]
Ph	Me	(*R*)	97
Ph	Et	(*S*)	88
Et–C≡C–	Me	(*R*)	91
n-Pr–C≡C–	Me	(*R*)	87
AcO–CH_2–C≡C–	Me	(*R*)	95
Br–CH_2–C≡C–	Me	(*R*)	94
AcO–$(CH_2)_2$–C≡C–	Me	(*R*)	83
Br–$(CH_2)_2$–C≡C–	Me	(*R*)	94
AcO–CH_2–C≡C–	Et	(*R*)	98[a]

[a] very slow reaction

Scheme 2.177 Products from chloroperoxidase-catalyzed benzylic and propargylic C–H hydroxylations

[50] For an X-ray structure see [1323].

[1326, 1327]. Interestingly, in accordance with the above-described mechanism (Scheme 2.178, path 3) oxygen transfer was proven to proceed from hydrogen peroxide via Compound I onto the substrate. CPO is very sensitive with respect to the substrate structure as the stereochemistry of products was reversed from (*R*) to (*S*) when the alkyl chain was extended from ethyl to an *n*-propyl analog.

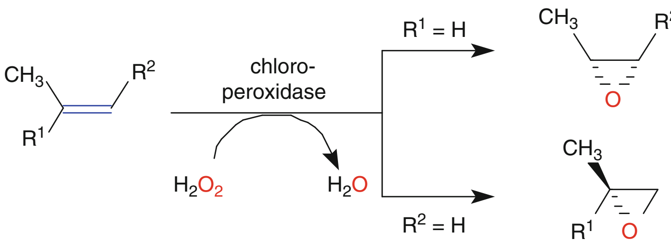
The selectivity of CPO-catalyzed propargylic hydroxylation was found to be sensitive with respect to the chain length of the alkyne substrate [1328]. Thus, whereas short alkyl groups (R^1) gave rise to poor enantioselectivity, longer alkyl chains diminished the reaction rate and only medium-chain compounds gave good results. The spatial requirements adjacent to the oxidation site are very strict: whereas compounds bearing methyl groups in position R^2 were oxidized with good yields, slow rates were reported for the corresponding ethyl analogs. Hydroxylation of aromatic C–H bonds seems to be possible, as long as electron-rich (hetero)aromatics, such as indol are used [1329, 1330].

Epoxidation of Alkenes. Due to the fact that the asymmetric epoxidation of alkenes using monooxygenase systems is impeded by the requirement for NADPH-recycling and the toxicity of epoxides to microbial cells, the use of H_2O_2-depending peroxidases represents a valuable alternative.

Unfortunately, direct epoxidation of alkenes by metal-free haloperoxidases led to racemic epoxides [1331, 1332]. Since the reaction only takes place in the presence of a short-chain carboxylic acid (e.g., acetate or propionate), it is believed to proceed via an enzymatically generated peroxycarboxylic acid, which subsequently oxidizes the alkene without the aid of the enzyme. This mechanism has a close analogy to the lipase-catalyzed epoxidation of alkenes (Sect. 3.1.5) and halogenation reactions catalyzed by haloperoxidases (Sect. 2.7.1), where enzyme catalysis is only involved in the formation of a reactive intermediate, which in turn converts the substrate in a spontaneous (nonenzymatic) followup reaction.

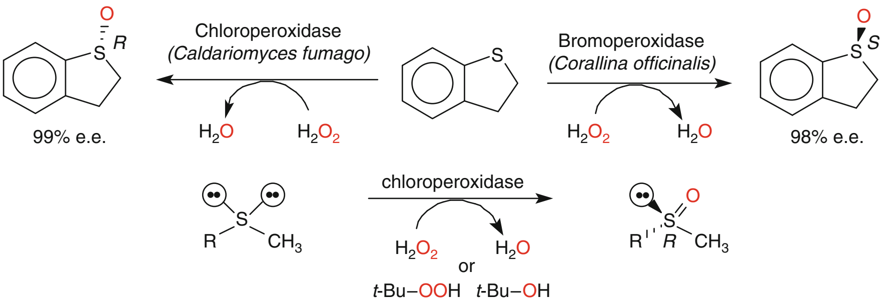
By contrast, chloroperoxidase-catalyzed epoxidation of alkenes proceeds with excellent enantioselectivites [1333, 1334]. For styrene oxide it was demonstrated that all the oxygen in the product is derived from hydrogen peroxide, which implies a true oxygen-transfer reaction (path 3, Scheme 2.174) [1335]. As depicted in Scheme 2.178, unfunctionalized *cis*-alkenes [1336] and 1,1-disubstituted olefins [1337, 1338] were epoxidized with excellent selectivities. On the other hand, aliphatic terminal and *trans*-1,2-disubstituted alkenes were epoxidized in low yields and moderate enantioselectivities [1339].

Sulfoxidation. Heteroatom oxidation catalyzed by (halo)peroxidases has been observed in a variety of organic compounds. *N*-Oxidation in amines, for instance, can lead to the formation of the corresponding aliphatic *N*-oxides or aromatic nitroso or nitro compounds. From a preparative standpoint, however, sulfoxidation of thioethers is of greater importance since it was shown to proceed in a highly stereo- and enantioselective fashion. Moreover, depending on the source of the haloperoxidase, chiral sulfoxides of opposite configuration could be obtained (Scheme 2.179).



R^1	R^2	e.e. [%]
H	n-C_4H_9–	96
H	$(CH_3)_2CH$–CH_2–	94
H	Ph–	96
Ph	H	89
CH_2–CO_2Et	H	93–94
$(CH_2)_2$–Br	H	85
n-C_5H_{11}	H	95

Scheme 2.178 Asymmetric epoxidation of alkenes using chloroperoxidase



R	Oxidant	e.e. [%]
p-CH_3–C_6H_4–	H_2O_2	98
	tert-Bu–OOH	70
p-CH_3–O–C_6H_4–	H_2O_2	90
	tert-Bu–OOH	61
Ph–	H_2O_2	98
p-Cl–C_6H_4–	H_2O_2	90
Ph–CH_2–	H_2O_2	90
2-pyridyl–	H_2O_2	99

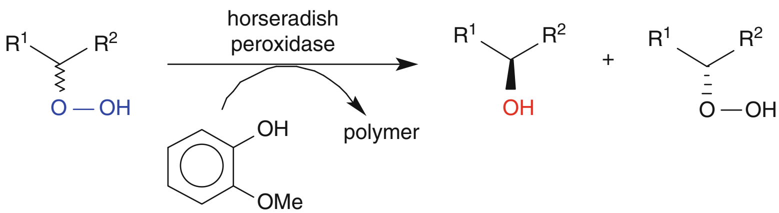
Scheme 2.179 Stereocomplementary oxidation of thioethers by haloperoxidases

Chloroperoxidase from *Caldariomyces fumago* is a selective catalyst for the oxidation of methylthioethers to furnish (*R*)-sulfoxides. Initial results were disappointing, as low e.e.'s were reported [1340]. The latter were caused by substantial nonenzymatic oxidation by hydrogen peroxide, which could be suppressed by optimization of the reaction conditions: whereas the use of *tert*-butylhydroperoxide was unsuccessful, the best results were obtained by keeping the concentration of H_2O_2 at a constant low level [1341, 1342].

Another vanadium-dependent haloperoxidase from the marine alga *Corallina officinalis* was shown to possess a matching opposite enantiopreference by forming (*S*)-sulfoxides [1343, 1344]. Although simple open-chain thioethers were not well transformed, cyclic analogs bearing a carboxylic acid moiety in a suitable position within the substrate were ideal candidates [1345].

Reduction of Peroxides. An intriguing alternative for the preparation of enantiopure hydroperoxides makes use of a peroxidase reaction performed in reverse, i.e., by enantioselective *reduction* (Scheme 2.180) [1346–1348].

Thus, when (chemically prepared) racemic hydroperoxides were subjected to the action of horseradish peroxidase, one enantiomer was reduced at the expense of *o*-methoxyphenol used as reductant, to give the corresponding secondary alcohol. The nonreacting enantiomer could be recovered in excellent e.e. The enzyme accepts sterically unencumbered hydroperoxides (with R^1 being the 'large' and R^2 the 'small' group), but those bearing sterically demanding branched substituents were unreactive. Of the various isoenzymes which are present in horseradish peroxidase, isoenzyme C is mostly responsible for the reduction [1349]. Similar activity was reported for a number of bacteria and fungi isolated from soil [1350] and for a peroxidase from the basidomycete *Coprinus cinereus* [1351].



R^1	R^2	Alcohol Config.	e.e. [%]	Hydroperoxide Config.	e.e. [%]
Et	CO_2Me	*S*	97	*R*	97
Ph	CH_2–OH	*S*	>95	*R*	>95
Ph	Me	*R*[a]	>99	*S*[a]	>99
Ph	Et	*R*[a]	95	*S*[a]	93

[a] switch in CIP-sequence order

Scheme 2.180 Enantioselective reduction of hydroperoxides using horseradish peroxidase

Although peroxidases are more easy to use than monooxygenases, several points concerning their practical application have to be considered:

- Given the tendency of peroxidases to undergo irreversible deactivation in the presence of substantial concentrations of H_2O_2 (which is common to many other enzymes) the oxidant has to be added continuously to the reaction in order to keep its concentration at a low level. This also suppresses its decomposition via the catalase activity of peroxidases (path 4, Scheme 2.174). In practice, this is conveniently achieved by using a H_2O_2-sensitive electrode coupled to an auto-titrator which adds the oxidant in a continuous fashion (peroxy-stat) [1352]. Furthermore, this minimizes the spontaneous (background) oxidation, which leads to the formation of racemic product.
- Depending on the enzyme–substrate combination, the replacement of hydrogen peroxide by *tert*-butyl hydroperoxide may be beneficial.
- If the reaction is incompatible with a peroxo species, molecular oxygen in presence of a chemical reductant (such as ascorbic or dihydroxyfumaric acid) may be used as oxidant [1353, 1354].

2.4 Formation of Carbon–Carbon Bonds

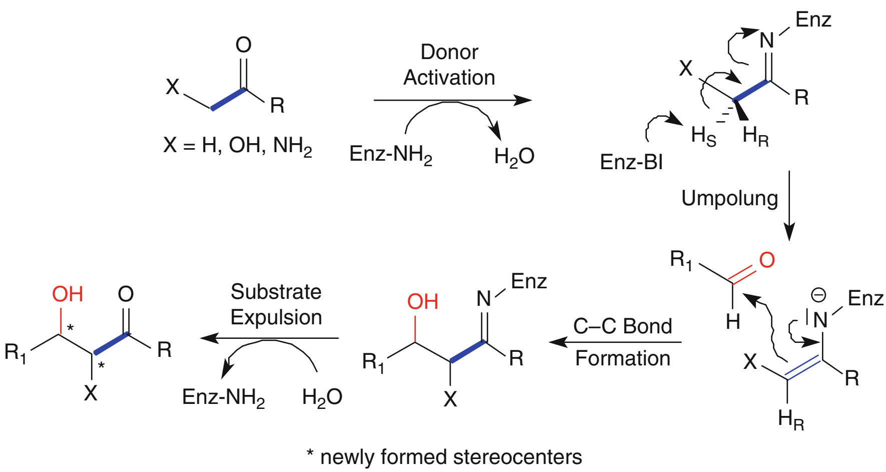
The majority of enzymatic reactions exploited to date involve degradation processes via *bond-breaking* reactions. The following enzymatic systems belonging to the class of lyases, which are capable of *forming* carbon–carbon bonds in a highly stereoselective manner, are known and are gaining increasing attention in view of their potential in *synthesis*.

- Aldol reactions catalyzed by aldolases are useful for the elongation of aldehydes by a two- or three-carbon unit.
- A C_2-fragment is transferred via transketolase reactions or via yeast-mediated acyloin or benzoin condensations.
- For the addition of cyanide (a C_1-synthon) to aldehydes by hydroxynitrile lyases see Sect. 2.5.1. For the sake of simplicity, the donor representing the umpolung reagent is drawn with bold C–C bonds throughout this chapter.

2.4.1 Aldol Reactions

Asymmetric C–C bond formation based on catalytic aldol addition reactions remains one of the most challenging subjects in synthetic organic chemistry. Although many successful nonbiological strategies have been developed [1355, 1356], most of them are not without drawbacks. They are often stoichiometric in auxiliary reagent and require the use of a metal or organocatalytic enolate complex to achieve stereoselectivity [1357–1360]. Due to the instability of such complexes in aqueous solutions, aldol reactions usually must be carried out in organic solvents at low temperature. Thus, for compounds containing polyfunctional polar groups, such as carbohydrates, the employment of conventional aldol reactions requires extensive protection protocols to be carried out in order to make them lipophilic and to avoid undesired cross-reactions. This requirement limits the application of conventional aldol reactions in aqueous solution. On the other hand, enzymatic aldol reactions catalyzed by aldolases, which are performed in aqueous solution at neutral pH, can be achieved without extensive protection methodology and have therefore attracted increasing interest [1361–1374].

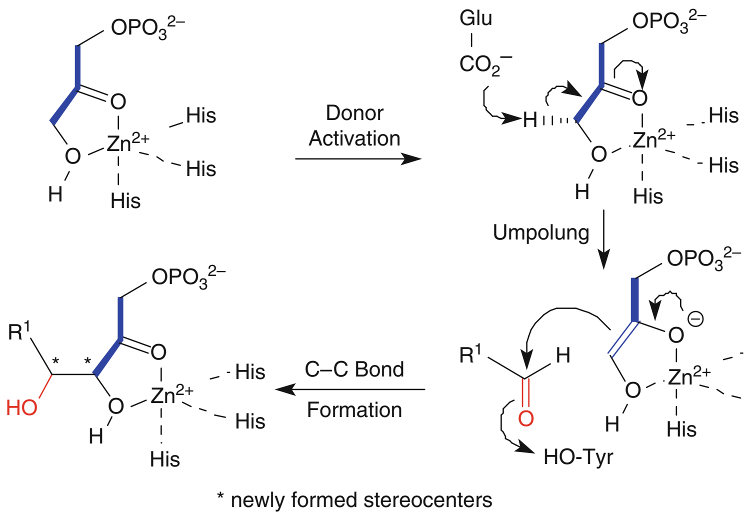
Aldolases were first recognized some 70 years ago. At that time, it was believed that they form an ubiquitous class of enzymes that catalyze the interconversion of hexoses into two three-carbon subunits [1375]. It is now known that aldolases operate on a wide range of substrates including carbohydrates, amino acids and hydroxy acids. A variety of enzymes has been described that add a one-, two-, or three-carbon (donor) fragment onto a carbonyl group of an aldehyde or a ketone with high stereospecificity. Since glycolysis and glyconeogenesis are a fundamental pillar of life, almost all organisms possess aldolase enzymes. Two distinct groups of aldolases, using different mechanisms, have been recognized [1376]. Regarding the formation of the (donor) carbanion, both of the mechanisms are closely related to conventional aldol reactions, i.e., carbanion stabilization is achieved via enolate- or enamine species (Schemes 2.181 and 2.182).



Scheme 2.181 Mechanism of type I aldolases

Type-I aldolases, found predominantly in higher plants and animals, require no metal cofactor, and catalyze the aldol reaction through a Schiff-base intermediate, which tautomerizes to an enamine species (Scheme 2.181) [1377]. First, the donor is covalently linked to the enzyme, presumably via the ε-amino group of a lysine to form a Schiff base. Next, base-catalyzed abstraction of H_s leads to the formation of an enamine species, which performs a nucleophilic attack on the carbonyl group of the aldehydic acceptor in an asymmetric fashion. Consequently, the two new chiral centers are formed stereospecifically in a *threo*- or *erythro*-configuration depending on the enzyme. Finally, hydrolysis of the Schiff base liberates the diol and regenerates the enzyme.

Type II aldolases are found predominantly in bacteria and fungi, and are Zn^{2+}-dependent enzymes (Scheme 2.182) [1378]. Their mechanism of action was recently affirmed to proceed through a metal-enolate [1379]: an essential Zn^{2+} atom in the active site (coordinated by three nitrogen atoms of histidine residues [1380]) binds the donor via the hydroxyl and carbonyl groups. This facilitates *pro*-(*R*)-proton abstraction from the donor (presumably by a glutamic acid residue acting as base), rendering an enolate, which launches a nucleophilic attack onto the aldehydic acceptor.

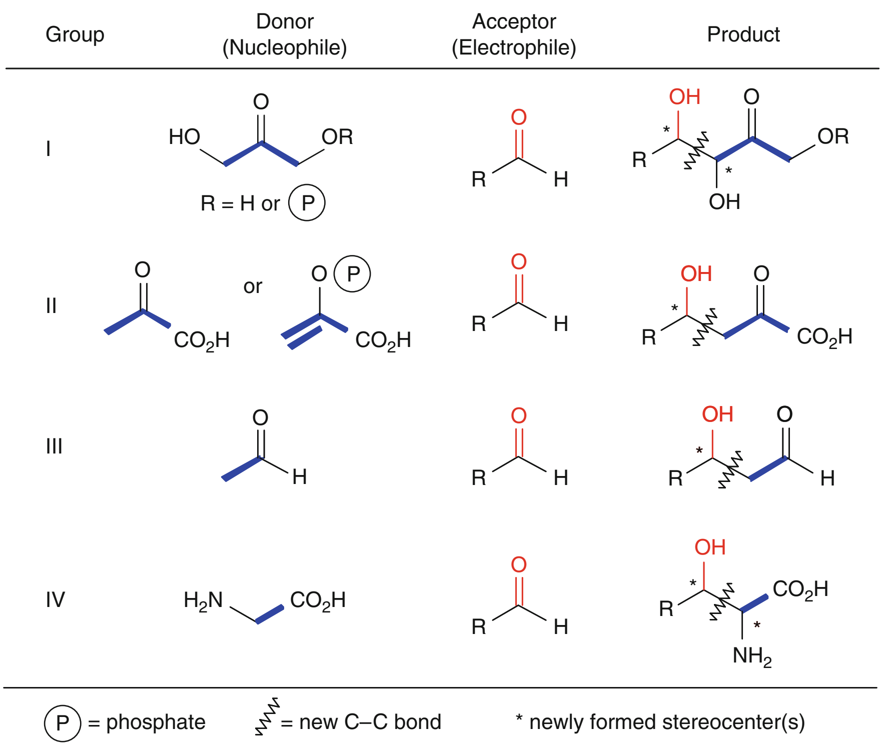


Scheme 2.182 Mechanism of metal-dependent type II aldolases

With few exceptions, the stereochemical outcome of the aldol reaction is controlled by the enzyme and does not depend on the substrate structure (or on its stereochemistry). Therefore, the configuration of the carbon atoms adjacent to the newly formed C–C bond is highly predictable. Furthermore, most aldolases are very restricted concerning their donor (the nucleophile), but possess relaxed substrate specificities with respect to the acceptor (the electrophile), which is the carbonyl group of an aldehyde or ketone. This is understandable, bearing in mind that the enzyme has to perform an umpolung on the donor, which is a sophisticated task in an aqueous environment!

To date more than 40 aldolases have been classified, the most useful and more readily available enzymes are described in this chapter. Bearing in mind that the natural substrates of aldolases are carbohydrates, most successful enzyme-catalyzed aldol reactions have been performed with carbohydrate-like (poly)hydroxy compounds as substrates. Depending on the donor, the carbon-chain elongation involves a two- or three-carbon unit (Scheme 2.183, donors are shown in bold).

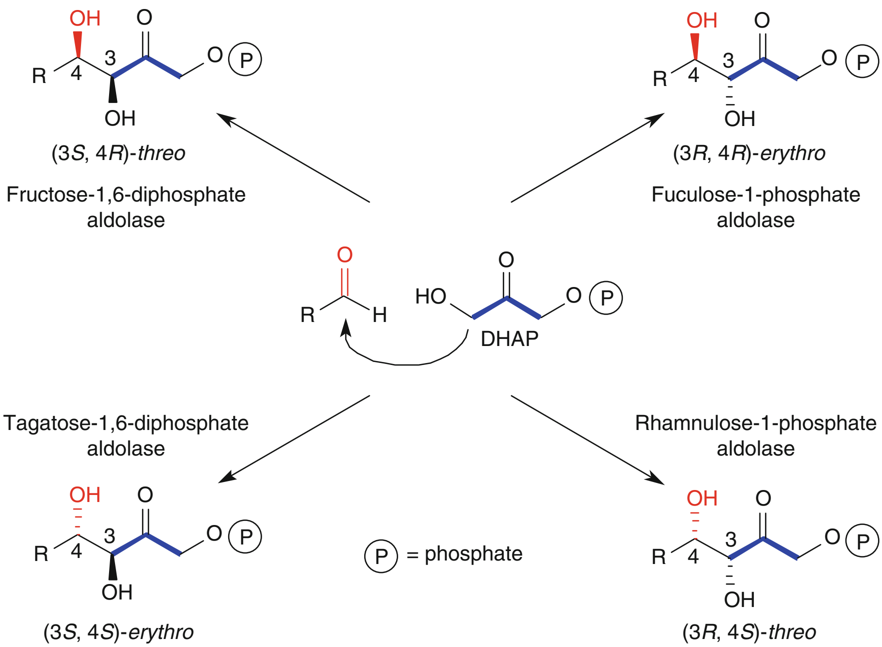
Aldolases are most conveniently classified into four groups according to their donor molecule. The best studied group I uses dihydroxyacetone or its phosphate (DHAP) as donor, resulting in the formation of a ketose 1-phosphate after reaction with an aldehyde acceptor. Within this group, enzymes capable of forming *all four possible stereoisomers* of the newly generated stereogenic centers in a complementary fashion are available (Scheme 2.184). Group II uses pyruvate (or phosphoenol pyruvate) as donor to yield 3-deoxy-2-keto acids as products (Scheme 2.193) [1381]. The third group consists of only one enzyme – 2-deoxyribose-5-phosphate aldolase (DERA) – which requires acetaldehyde (or close analogs) as donor to form 2-deoxy aldoses (Scheme 2.194). Finally, group IV aldolases couple glycine (as donor) with an acceptor aldehyde to yield α-amino-β-hydroxy acids (Scheme 2.196).



Scheme 2.183 Main groups of aldolases according to donor type

Group I: Dihydroxyacetone or DHA-Phosphate-Dependent Aldolases

The development of the full synthetic potential of DHA(P)-dependent aldolases into a general and efficient methodology for asymmetric aldol additions largely depends



Scheme 2.184 Stereocomplementary DHAP-dependent aldolases

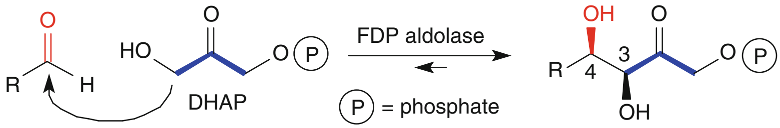
on the availability of the complete tetrad of enzymes, which allows to create all four possible stereoisomers at will, by simply selecting the correct biocatalyst.

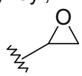
As shown in Scheme 2.184, all four stereocomplementary aldolases occurring in carbohydrate metabolism which generate the four possible stereoisomeric diol products emerging from the addition of DHAP onto an aldehyde have been made available by cloning and overexpression. The reaction proceeds with complete stereospecificity with respect to the configuration on carbon 3 and also (with slightly decreased specificity) on carbon 4.

Fructose-1,6-Diphosphate Aldolase. Fructose-1,6-diphosphate (FDP) aldolase from rabbit muscle, also commonly known as 'rabbit muscle aldolase' (RAMA), catalyzes the addition of dihydroxyacetone phosphate (DHAP) to D-glyceraldehyde-3-phosphate to form fructose-1,6-diphosphate (Scheme 2.185) [514, 1382].

The equilibrium of the reaction is predominantly on the product side and the specificity of substituent orientation at C-3 and C-4 (adjacent to the newly formed vicinal diol bond) is absolute – always *threo* (Scheme 2.185). However, if the α-carbon atom in the aldehyde component is chiral (C-5 in the product), only low chiral recognition of this remote stereocenter takes place. Consequently, if an α-substituted aldehyde is employed in racemic form, a pair of diastereomeric products will be obtained.

RAMA accepts a wide range of aldehydes in place of its natural substrate, allowing the synthesis of carbohydrates [1383–1386] and analogs such as



R (natural substrates)	R (nonnatural substrates)
D- or L-threose	H, Me, Et, *n*-Pr, *i*-Pr, CH=O, CO_2H, CH_2F, CH_2Cl,
D- or L-erythrose	CH_2OCH_2Ph, $(CH_2)_2OH$, $CH(OCH_3)CH_2OH$,
L-arabinose, D-ribose,	$(CH_2)_2OMe$, CHOHMe, $CHOHCH_2OMe$,
D-lyxose, D-xylose	CH_2Ph, COPh, $(CH_2)_3CH=O$,
D-glyceraldehyde-3-P	$CH_2P(O)(OEt)_2$, 2- and 3-pyridyl,
	4-cyclohexenyl, CH_2OP, 

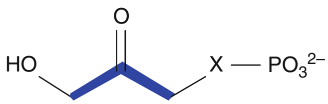
Scheme 2.185 Aldol reactions catalyzed by FDP aldolase from rabbit muscle

nitrogen-[513, 1387] and sulfur-containing sugars [1388], deoxysugars [1389], fluorosugars, and rare eight- and nine-carbon sugars [1390]. As depicted in Scheme 2.185, numerous aldehydes which are structurally quite unrelated to the natural acceptor substrate (D-glyceraldehyde-3-phosphate) are freely accepted [1391–1394].

For RAMA the following rules apply to the aldehyde component:

- In general, unhindered aliphatic, α-heterosubstituted, and protected alkoxy aldehydes are accepted as substrates.
- Sterically hindered aliphatic aldehydes such as pivaldehyde do not react with RAMA, nor do α,β-unsaturated aldehydes or compounds that can readily be eliminated to form α,β-unsaturated aldehydes.
- Aromatic aldehydes are either poor substrates or are unreactive.
- ω-Hydroxy acceptors that are phosphorylated at the terminal hydroxyl group are accepted at enhanced rates relative to the nonphosphorylated species.

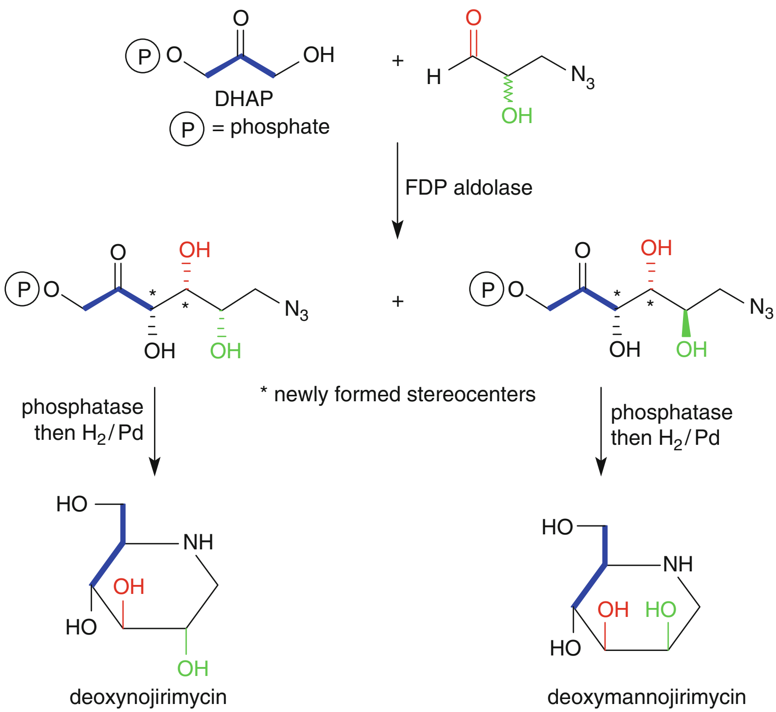
In contrast to the relaxed specificity for the acceptor, the requirement for DHAP as the donor is much more stringent. Several analogs which are more resistant towards spontaneous hydrolysis have been successfully tested as substitutes for DHAP (Scheme 2.186) [1395], however the reaction rates were reduced by about one order of magnitude [1396–1399].



X = O, NH, S, CH_2

Scheme 2.186 Nonnatural DHAP substitutes for fructose-1,6-diphosphate aldolase (RAMA)

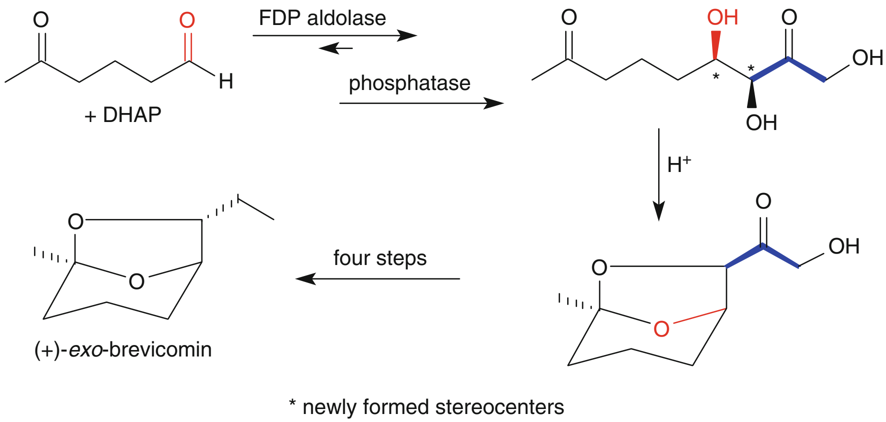
Within group-I aldolases, FDP aldolase from rabbit muscle has been extensively used for the synthesis of biologically active sugar analogs on a preparative scale (Scheme 2.187). For example, nojirimycin and derivatives thereof, which have been shown to be potent anti-AIDS agents with no cytotoxicity, have been obtained by a chemoenzymatic approach using RAMA in the key step. As expected, the recognition of the α-hydroxy stereocenter in the acceptor aldehyde was low [1400, 1401].



Scheme 2.187 Synthesis of aza-sugar analogs

An elegant synthesis of (+)-*exo*-brevicomin, the sex pheromone of the bark beetle made use of FDP-aldolase (Scheme 2.188) [1402]. RAMA-catalyzed condensation of DHAP to a δ-keto-aldehyde gave, after enzymatic dephosphorylation, a *threo*-keto-diol, which was cyclized to form a precursor of the pheromone. Finally, the side chain was modified in four subsequent steps to give (+)-*exo*-brevicomin.

Despite the fact that enzymatic aldol reactions are becoming useful in synthetic carbohydrate chemistry, the preparation of aldehyde substrates containing chiral centers remains a problem. Many α-substituted aldehydes racemize in aqueous solution, which would result in the production of a diastereomeric mixture, which is not always readily separable.

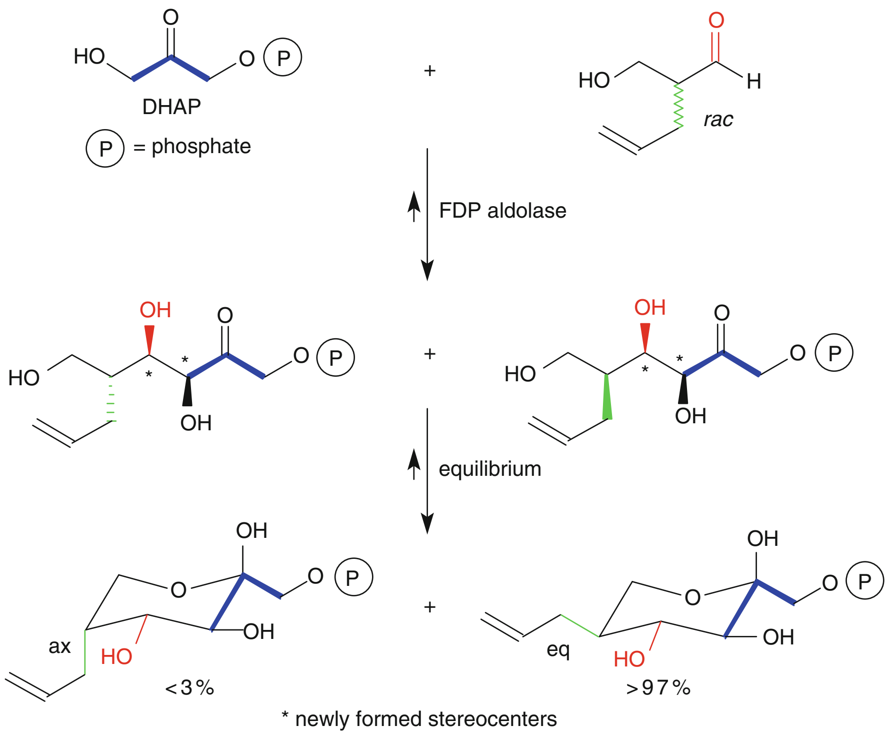


Scheme 2.188 Synthesis of (+)-*exo*-brevicomin

The following methods have been used to avoid the (often tedious) separation of diastereomeric products [512].

- In some cases, a stereoselective aldol reaction can be accomplished in a kinetically controlled process via kinetic resolution of the racemic α-substituted aldehyde. Thus, if the reaction is stopped before it reaches equilibrium, a single diastereomer is predominantly formed. However, as mentioned above, the selectivities of aldolases for such kinetic resolutions involving recognition of the (remote) chirality on the α-carbon atom of the aldehyde are usually low.
- Efficient kinetic resolution of α-hydroxyaldehydes can be achieved by inserting a negative charge (such as phosphate or carboxylate) at a distance of four to five atoms from the aldehydic center in order to enhance the binding of the acceptor substrate [1403].
- In cases wherein one diastereomer of the product is more stable than the other, one can utilize a thermodynamically controlled process (Scheme 2.189). For example, in the aldol reaction of *rac*-2-allyl-3-hydroxypropanal, two diastereomeric products are formed. Due to the hemiacetal ring-formation of the aldol product and because of the reversible nature of the aldol reaction, only the more stable product positioning the 5-allyl substituent in the favorable equatorial position is produced when the reaction reaches equilibrium.
- Another solution to the problem of formation of diastereomeric products is to subject the mixture to the action of glucose isomerase, whereby the D-ketose is converted into the corresponding D-aldose leaving the L-ketose component unchanged [1404].

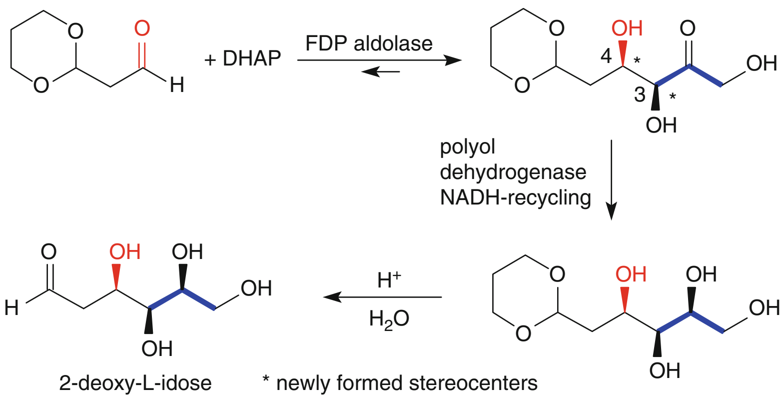
A potential limitation on the use of FDP aldolases for the synthesis of monosaccharides is that the products are always *ketoses* with fixed stereochemistry at the newly generated chiral centers on C-3 and C-4. There are, however, methods for establishing this stereochemistry at other centers and for obtaining aldoses instead of ketoses. This technique makes use of a monoprotected dialdehyde as the acceptor



Scheme 2.189 Thermodynamic control in aldolase reactions

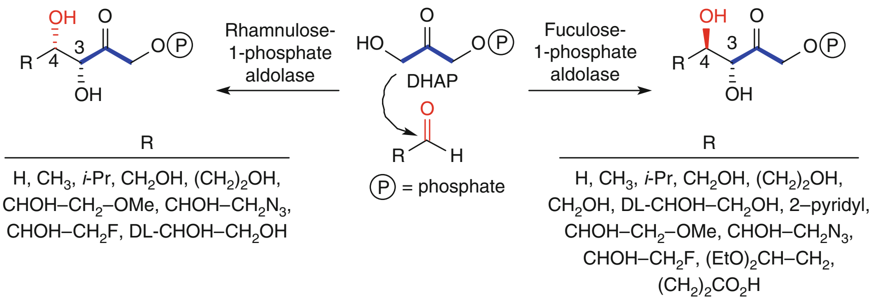
substrate (Scheme 2.190). After the RAMA-catalyzed aldol reaction, the resulting ketone is reduced in a diastereoselective fashion with polyol dehydrogenase. The remaining masked aldehyde is then deprotected to yield a new *aldose*.

Aldol additions catalyzed by two DHAP-dependent aldolases which exhibit a complementary stereospecificity to RAMA have been used to a lesser extent



Scheme 2.190 Synthesis of aldoses using FDP aldolase

(Schemes 2.184 and 2.191) [1405, 1406]. Although the selectivity with respect to the center on carbon 3 is absolute, in some cases the corresponding C-4 diastereomer was formed in minor amounts depending on the R substituent on the aldehyde. In those cases shown in Scheme 2.191, however, only a single diastereomer was obtained.



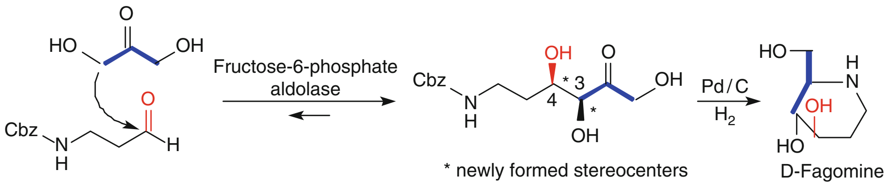
Scheme 2.191 Aldol reactions catalyzed by fuculose- and rhamnulose-1-phosphate aldolase

One drawback common to group I aldolase reactions is that most of them them require the expensive and sensitive phosphorylated donor dihydroxyacetone phosphate. This molecule is not very stable in solution (*t* ~ 20 h at pH 7), and its synthesis is not trivial [1407]. DHAP may be obtained from the hemiacetal dimer of dihydroxyacetone by chemical phosphorylation with $POCl_3$ [1408, 1409], or by enzymatic phosphorylation of dihydroxyacetone at the expense of ATP and glycerol kinase [546] (Sect. 2.1.4). Probably the most elegant and convenient method is the in situ generation of DHAP from fructose-1,6-diphosphate (FDP) using FDP aldolase, forming one molecule of DHAP as well as glyceraldehyde-3-phosphate. The latter can be rearranged by triosephosphate isomerase to give a second DHAP molecule [541]. This protocol has been further extended into a highly integrated ‘artificial metabolism’ to obtain DHAP from inexpensive feedstocks, such as glucose or fructose (yielding two equivalents of DHAP) and sucrose (four equivalents) via an enzymatic cascade consisting of up to seven enzymes [1410].

The presence of the phosphate group in the aldol adducts facilitates their purification by ion-exchange chromatography or by precipitation as the corresponding barium salts. Cleavage of phosphate esters is usually accomplished by enzymatic hydrolysis using acid or alkaline phosphatase (Sect. 2.1.4).

Attempts to use a mixture of dihydroxyacetone and a small amount of inorganic arsenate, which spontaneously forms dihydroxyacetone arsenate which is a mimic of DHAP and is accepted by FDP aldolase as a substrate, were impeded by the toxicity of arsemate [1411, 1412]. However, the use of borate ester mimics of DHAP offers a valuable nontoxic alternative for preparative-scale reactions [1413].

An elegant solution to avoid the neccessity for DHAP is the use of a recently discovered bacterial transaldolase, which catalyzes the aldol rection between glyceraldehyde-3-phosphate and (nonphosphorylated) dihydroxyacetone forming fructose-6-phosphate. Since its stereospecificity is identical to that of fructose-1,6-diphosphate aldolase, it represents a useful extension of the aldolase toolbox (Scheme 2.184) [1414–1417]. As depicted in Scheme 2.192, coupling of dihydroxyacetone with an *N*-protected 3-aminopropanal gave the (3*S*,4*R*)-*threo*-diol, which was reductively cyclized to furnish the rare aza-sugar D-fagomine, which acts as glycosidase inhibitor and shows antifungal and antibacterial activity [1418].



Scheme 2.192 Aldol reaction catalyzed by dihydroxyacetone-dependent fructose-6-phosphate aldolase

Group II: Pyruvate-Dependent Aldolases

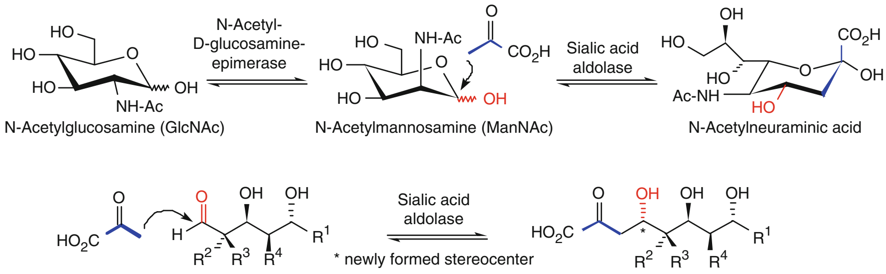
For thermodynamic reasons, pyruvate-dependent aldolases have catabolic functions in vivo, whereas their counterparts employing (energy-rich) phosphoenol pyruvate as the donor are involved in the biosynthesis of keto-acids. However, both types of enzymes can be used to synthesize α-keto-β-hydroxy acids in vitro.

Sialic Acid Aldolase. *N*-Acetylneuraminic acid (NeuAc, also termed sialic acid) aldolase catalyzes the reversible addition of pyruvate onto *N*-acetylmannosamine to form *N*-acetylneuraminic acid (Scheme 2.193) [1419, 1420]. Since the equilibrium for this reaction is near unity, an excess of pyruvate must be used in synthetic reactions to drive the reaction towards completion. NeuAc was previously isolated from natural sources such as cow's milk, but increasing demand prompted the development of a two-step synthesis from *N*-acetylglucosamine using chemical or enzymatic epimerization to *N*-acetylmannosamine, followed by coupling of pyruvate catalyzed by sialic acid aldolase on a multi-ton scale [1421–1424]. Besides NeuAc, the production of structural analogs is of significance since neuraminic acid derivatives play an important role in cell adhesion and biochemical recognition processes [1425]. The cloning of the enzyme has reduced its cost [1426].

In line with the substrate requirements of FDP aldolase, the specificity of sialic acid aldolase appears to be absolute for pyruvate (the donor), but relaxed for the aldehydic acceptor. As may be seen from Scheme 2.193, a range of mannosamine derivatives have been used to synthesize derivatives of NeuAc [1427–1432]. Substitution at C-2 of *N*-acetylmannosamine is tolerated, and the enzyme exhibits only a slight preference for defined stereochemistry at other centers.

Other group II aldolases of preparative value are 3-deoxy-D-manno-octulosonate (KDO[51]) aldolase [1433, 1434], 2-keto-3-deoxy-6-phosphogalactonate aldolase

[51]Also named 2-keto-3-deoxyoctanoate (KDO).



R^1	R^2	R^3	R^4
CH_2OH	NH–Ac	H	OH[a]
CH_2N_3	NH–Ac	H	OH
CH_2OCH_3	NH–Ac	H	OH
CH_2OH	NH–Ac	H	OCH_3
CH_2OH	H	H	OH
CH_2OH	OH	H	OH
CH_2OH	OH	H	H
CH_2OH	N_3	H	OH
H	OH	H	OH
H	H	OH	OH

[a]the natural substrate Neu5Ac

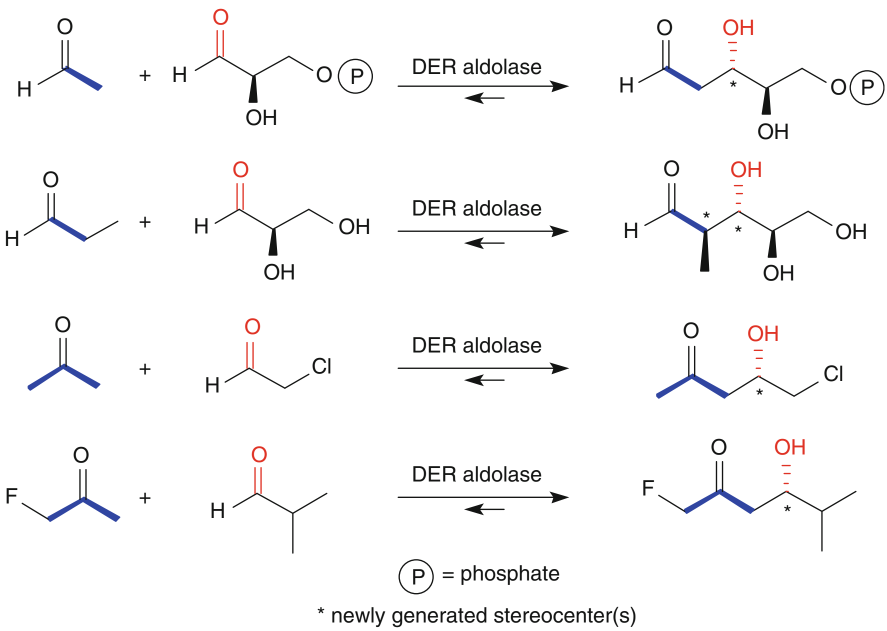
Scheme 2.193 Industrial-scale synthesis of *N*-acetylneuraminic acid using a two-enzyme system and aldol reactions catalyzed by sialic acid aldolase

[1381, 1435] and 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthetase [1436].

Group III: Acetaldehyde-Dependent Aldolases

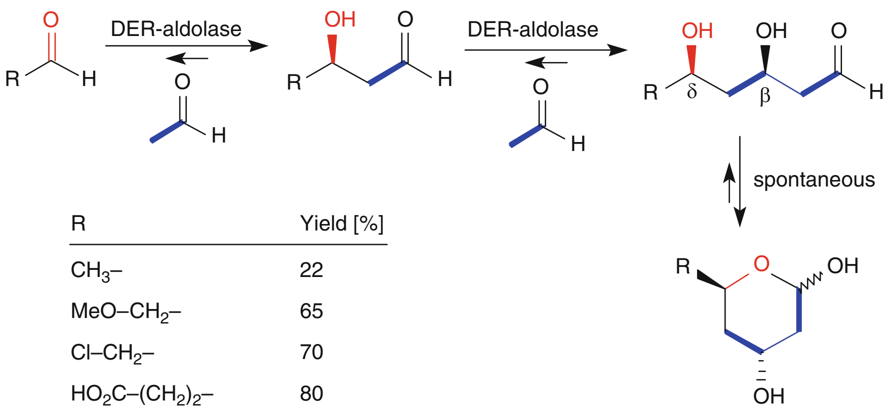
2-Deoxyribose-5-Phosphate Aldolase. The only aldolase known so far which accepts acetaldehyde as donor is 2-deoxyribose-5-phosphate (DER) aldolase. In vivo, DER aldolase catalyzes the reversible aldol reaction of acetaldehyde and D-glyceraldehyde-3-phosphate to form 2-deoxyribose-5-phosphate. This aldolase is unique in that it is the only aldolase that condenses *two aldehydes* (instead of a ketone and an aldehyde) to form aldoses (Schemes 2.183 and 2.194).[52] Interestingly, the enzyme (which has been overproduced [1438]) shows a relaxed substrate specificity not only on the acceptor side, but also on the donor side. Thus, besides acetaldehyde it accepts also acetone, fluoroacetone and propionaldehyde as donors, albeit at a much slower rate. Like other aldolases, it transforms a variety of aldehydic acceptors in addition to D-glyceraldehyde-3-phosphate.

[52]For a related aldolase see [1436].



Scheme 2.194 Aldol reactions catalyzed by 2-deoxyribose-5-phosphate aldolase

An elegant method for sequential aldol reactions performed in a one-pot reaction has been discovered for 2-deoxyribose-5-phosphate aldolase (Scheme 2.195) [1439]. When a (substituted) aldehyde was used as acceptor, condensation of acetaldehyde (as donor) led to the corresponding β-hydroxy aldehyde as intermediate product. The latter, however, can undergo a second aldol reaction with another acetaldehyde donor, forming a β,δ-dihydroxy aldehyde. At this stage, this aldol



R	Yield [%]
CH_3–	22
MeO–CH_2–	65
Cl–CH_2–	70
$HO_2C–(CH_2)_2$–	80

Scheme 2.195 Sequential aldol reactions catalyzed by DER aldolase

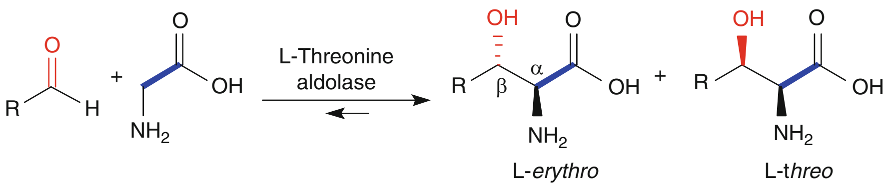
cascade (which would lead to the formation of a polymeric product if uninterrupted) is terminated by the (spontaneous) formation of a stable hemiacetal. The latter does not possess a free aldehydic group and therefore cannot serve as acceptor any more.

The dihydroxylactols thus obtained can be oxidized by NaOCl to the corresponding lactones, which represent the chiral side chains of several cholesterol-lowering 3-hydroxy-3-methylglutaryl-(HMG)-CoA reductase inhibitors, which are collectively denoted as 'statins' [1440].[53] Several derivaties thereof are produced on industrial scale using DER-aldolase mutants at product concentrations exceeding 100g/L [1441, 1442].

This concept provides rapid access to polyfunctional complex products from cheap starting materials in a one-pot reaction. It has recently been extended by combining various types of aldolases together to perform three- and four-substrate cascade reactions [1443, 1444].

Group IV: Glycine-Dependent Aldolases

One remarkable feature of group IV aldolases is their requirement for an amino acid as donor – glycine (Scheme 2.196) [1445, 1446]. Thus, α-amino-β-hydroxy acids are formed during the course of the reaction.



R	*erythro* [%]	*threo* [%]
Me–	93	7
Ph–CH_2–O–CH_2–	92	8
Ph–CH_2–O–$(CH_2)_2$–	54	47
Ph–$(CH_2)_3$–O–CH_2–	92	8

Scheme 2.196 Aldol reactions catalyzed by L-threonine aldolase

D- and L-Threonine Aldolase. These enzymes are involved in the biosynthesis/degradation of α-amino-β-hydroxyamino acids, such as threonine and they usually show absolute specificity for the α-amino-configuration, but have only low specificities for the β-hydroxy-center, thereby leading to diastereomeric *threo*/*erythro*-mixtures of products [1447]. For biocatalytic applications, several threonine

[53] For instance, atorvastatin (Lipitor™) or rosuvastatin (Crestor™).

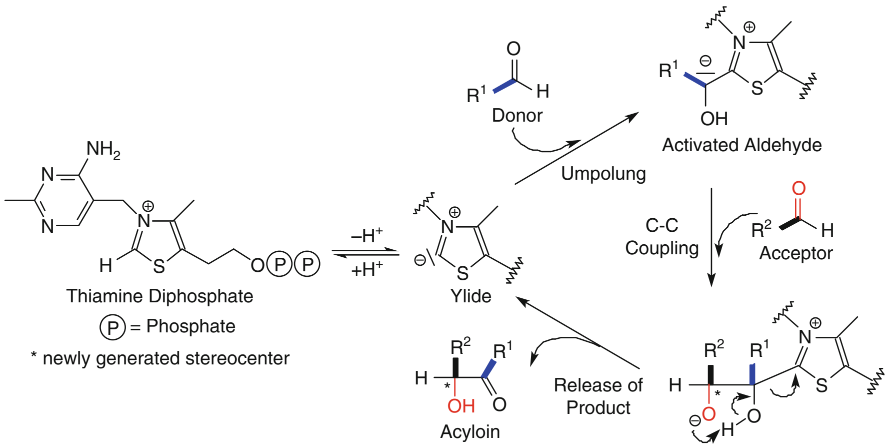
aldolases show broad substrate tolerance for various acceptor aldehydes [1448, 1449], including aromatic aldehydes; however, α,β-unsaturated aldehydes were not accepted. β-Hydroxyamino acids constitute an important class of natural products of their own right and are components of complex structures, such as the β-hydroxytyrosine moiety in vancomycin. It is thus not surprising, that threonine aldolases have been frequently used for their synthesis.

For instance, the L-enzyme from *Candida humicola* was used in the synthesis of multifunctional α-amino-β-hydroxy acids, which possess interesting biological properties (Scheme 2.196) [1449]. A number of benzyloxy- and alkyloxy aldehydes were found to be good acceptors. Although the stereoselectivity of the newly generated α-center was absolute (providing only L-amino acids), the selectivity for the β-position bearing the hydroxyl group was less pronounced, leading to *threo*- and *erythro*-configurated products. More recently, the use of a recombinant D-threonine aldolase was reported [1450, 1451].

Unfortunately, the position of the equilibrium does not favor synthesis, which requires to push the reaction by employing either an excess of the donor glycine (which is difficult to separate from the product) or the acceptor aldehyde (which at high concentrations may deactive the enzyme). A recently developed protocol relies on pulling of the equilibrium by (irreversible) decarboxylation of the formed α-amino-β-hydroxycarboxylic acid catalyzed by a decarboxylase to yield the corresponding aminoalcohols as final products [1452].

2.4.2 Thiamine-Dependent Acyloin and Benzoin Reactions

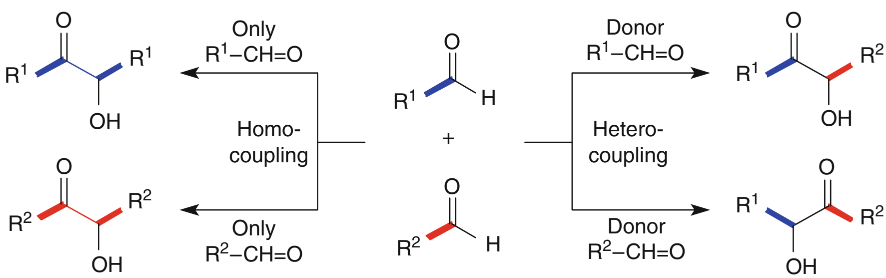
In the aldol reaction, C–C coupling always takes place in a head-to-tail fashion between the umpoled Cα atom of an enolate- or enamine-species (acting as donor) and the carbonyl C of an acceptor forming a β-hydroxycarbonyl product. In contrast, head-to-head coupling of two aldehydic species involving both carbonyl C atoms would lead to α-hydroxycarbonyl compounds, such as acyloins or benzoins. For this reaction, one aldehyde has to undergo umpolung at the carbonyl C, which is accomplished with the aid of an intriguing cofactor: thiamine diphosphate (ThDP, Scheme 2.197) [1453–1456]. This cofactor is an essential element for the formation/cleavage of C–C, C–N, C–O, C–P, and C–S bonds and plays a vital role as vitamin B_1 [1457–1459]. A schematic representation of the mechanism of enzymatic carboligation by ThDP-dependent enzymes is depicted in Scheme 2.197. In a first step, ThDP is deprotonated at the iminium carbon, leading to a resonance-stabilized carbanion. The latter performs a nucleophilic attack on an aldehyde (R^1–CH=O), which is converted into the donor by forming a covalently bound carbinol species bearing a negative charge. This umpoled species attacks the second (acceptor) aldehyde (R^2–CH=O) going in hand with C–C bond formation. Tautomerization of the diolate intermediate goes in hand with release of the α-ketol (acyloin/benzoin) product and regeneration of the cofactor.



Scheme 2.197 Thiamine diphosphate-dependent carboligation of aldehydes

In the enzymatic aldol reaction, the role of the donor and acceptor is strictly determined by the specificity of the enzyme and only one coupling product can be obtained. In contrast, the possible product range is more complex in acyloin and benzoin reactions: If only a single aldehyde species is used as substrate, only one product can be obtained via homocoupling; however, a pair of regioisomeric α-hydroxyketones can be obtained via heterocoupling, when two different aldehydes are used, the ratio of which is determined by the choice of substrates (e.g., benzoyl formate vs. benzaldehyde, pyruvate vs. acetaldehyde), and the specificites of enzymes, respectively (Scheme 2.198).

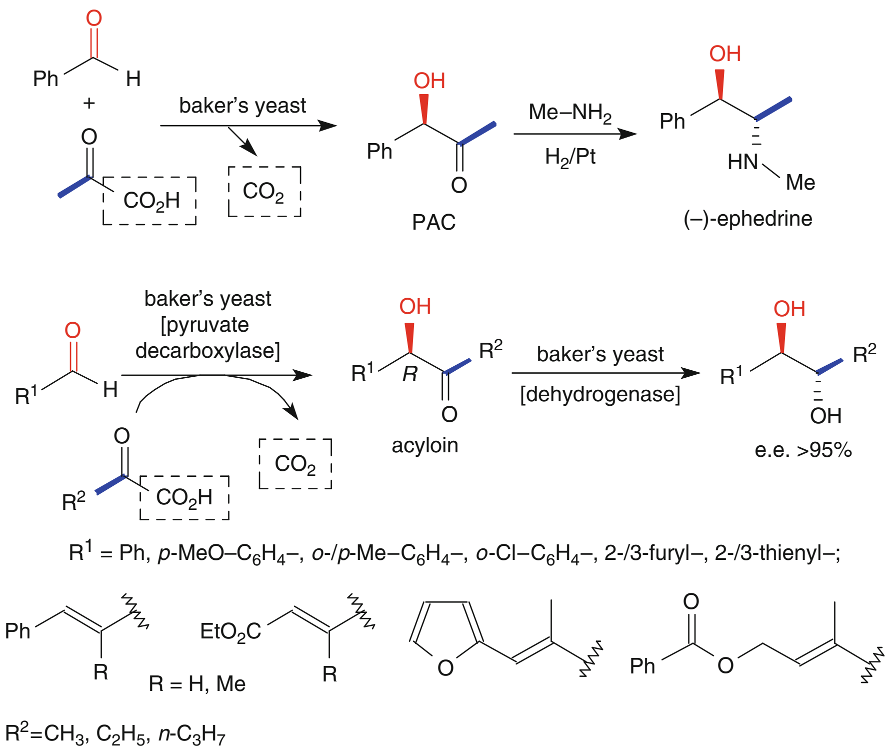
Stereocontrol in mixed acyloin and benzoin reactions is high only if the carboligation encompasses at least one (large) aromatic aldehyde, whereas with two (small) aliphatic aldehydes only moderate e.e.s are generally obtained.



Scheme 2.198 Regioisomeric α-hydroxyketones obtained from homo- and heterocoupling of aldehydes

Acyloin and Benzoin Reactions

Historically, the biocatalytic acyloin condensation was first observed by Liebig in 1913 during studies on baker's yeast [1460]. A few years later, Neuberg and Hirsch reported the formation of 3-hydroxy-3-phenylpropan-2-one (phenyl acetyl carbinol, PAC) from benzaldehyde by fermenting baker's yeast [1461]. Without knowledge on the actual enzyme(s) involved, this biotransformation assumed early industrial importance when it was shown that the acyloin thus obtained could be converted into (–)-ephedrine by diastereoselective reductive amination, a process which is still utilized in almost unchanged form at a capacity of 120 t/year [1462, 1463] (Scheme 2.199). Subsequent studies revealed that this yeast-based protocol can be extended to a broad range of aldehydes [1464, 1465].



Scheme 2.199 Synthesis of (–)-ephedrine via baker's yeast catalyzed acyloin reaction and acyloin formation catalyzed by pyruvate decarboxylase

Despite its important history, it was during the early 1990s, that the reaction pathway was elucidated in detail [1466] and it turned out that the enzyme responsible for this reaction is pyruvate decarboxylase (PDC) [1467]. The C_2-unit (equivalent to acetaldehyde) originates from the decarboxylation of pyruvate and is transferred to the *si*-face of the aldehydic substrate to form an (*R*)-α-hydroxyketone (acyloin) with the aid of the cofactor TDP [1468]. Since pyruvate decarboxylase accepts α-ketoacids other than pyruvate, C_2- through C_4-equivalents can be transferred onto a large variety of aldehydes [1469–1471]. In whole-cell (yeast) transformations, the resulting acyloin is often reduced in a subsequent step by yeast alcohol dehydrogenase to give the *erythro*-diol. The latter reaction is a common feature of baker's yeast whose stereochemistry is guided by Prelog's rule (see Sect. 2.2.3, Scheme 2.118). The optical purity of the diols is usually better than 90% [1472–1476].

It must be mentioned, however, that for baker's yeast-catalyzed acyloin reactions the yields of chiral diols are usually in the range of 10–35%, but this is offset by the ease of the reaction and the low price of the reagents used. Depending on the substrate structure, the reduction of the aldehyde to give the corresponding primary alcohol (catalyzed by yeast alcohol dehydrogenases, see Sect. 2.2.3) and saturation of the α,β-double bond (catalyzed by ene-reductases, see Sect. 2.2.4) are the major competing reactions. To avoid low yields associated with yeast-catalyzed transformations, acyloin- [1477–1479] and benzoin-reactions [1480] are nowadays performed using isolated enzymes [1481].

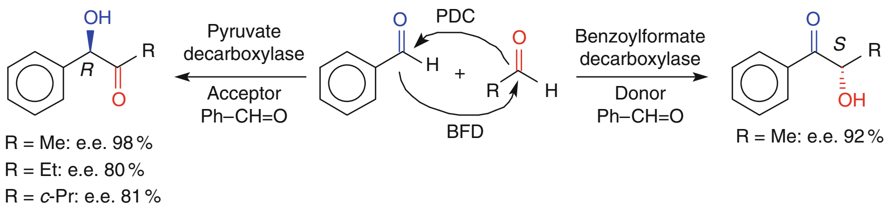
Pyruvate Decarboxylase

In vivo, pyruvate decarboxylase [EC 4.1.1.1] catalyzes the nonoxidative decarboxylation of pyruvate to acetaldehyde and is thus a key enzyme in the fermentative production of ethanol. The most well-studied PDCs are obtained from baker's yeast [1477, 1482, 1483] and from *Zymomonas mobilis* [1484].

From a synthetic viewpoint, however, its carboligation activity is more important [1485–1487]: All PDCs investigated so far prefer small aliphatic aldehydes as donors, used either directly or applied in the form of the respective α-ketocarboxylic acids. The latter are decarboxylated during the course of the reaction, which drives the equilibrium towards carboligation. Straight-chain α-ketoacids up to C-6 are good donors, whereas branched and aryl-aliphatic analogs are less suitable. On the acceptor side, aromatic aldehydes are preferred, leading to PAC-type regioisomeric acyloins (Schemes 2.199 and 2.200). Self-condensation of small aldehydes yielding acetoin-type products may occur.

Benzoylformate Decarboxylase

BFD [EC 4.1.1.7] is derived from mandelate catabolism, where it catalyzes the nonoxidative decarboxylation of benzoyl formate to yield benzaldehyde. Again, the reverse carboligation reaction is more important [1488–1490]. As may be deduced from its natural substrate, is exhibits a strong preference for large aldehydes as donor substrates encompassing a broad range of aromatic, heteroaromatic, cyclic aliphatic and olefinic aldehydes [1480]. With acetaldehyde as acceptor, it yields the complementary regio-isomeric product to PDC (Scheme 2.200).

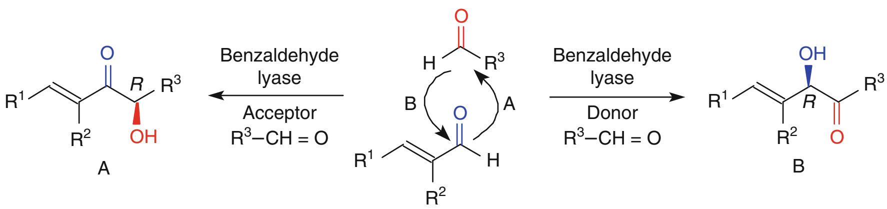


Scheme 2.200 Regiocomplementary carboligation of aldehydes catalyzed by pyruvate and benzoylformate decarboxylase

Benzaldehyde Lyase

Up to now, the only biochemically characterized benzaldehyde lyase (BAL) [EC 4.1.2.38] was derived from a *Pseudomonas fluorescens* strain, which was isolated from wood, showing the ability to grow on lignin-degradation products, such as anisoin (4,4′-dimethoxybenzoin) and benzoin. BAL cleaves these latter compounds to furnish more simple aromatic aldehydes [1491]. In contrast to PDC and BFD, BAL shows only negligible decarboxylation activity and C–C lyase- and carboligation are dominant [1492–1494]. Especially the self-ligation of benzaldehyde yields benzoin with high activity and stereoselectivity (e.e. >99), making this enzyme very interesting for industrial processes [1495]. For benzoin formation, *o*-, *m*-, and *p*-substituted aromatic aldehydes are widely accepted as donors [1496]. Hetero-coupling of aromatic and aliphatic aldehydes (acting as acceptor) result in the formation of (*R*)-2-hydroxypropiophenone derivatives in analogy to BFD. On the acceptor side, formaldehyde, acetaldehyde and close derivatives, such as phenyl-, mono-, or dimethoxyacetaldehyde are tolerated.

The remarkable synthetic potential of BAL is demonstrated by the regiocomplementary benzoin condensation of α,β-unsaturated aldehydes acting as donor or acceptor, respectively. While large aldehydes acted as donors (product type A), small counterparts served as acceptors leading to isomeric olefinic acyloins B in high e.e.s [1497] (Scheme 2.201).



R^1	R^2	R^3	Product	e.e. [%]
Ph	H	CH_3	A	80
Ph	CH_3	CH_3	A	>96
$-(CH_2)_4-$		$CH(OMe)_2$	A	93
H	CH_3	3,5-di–MeO–C_6H_3	B	>98
H	CH_3	Ph	B	>98

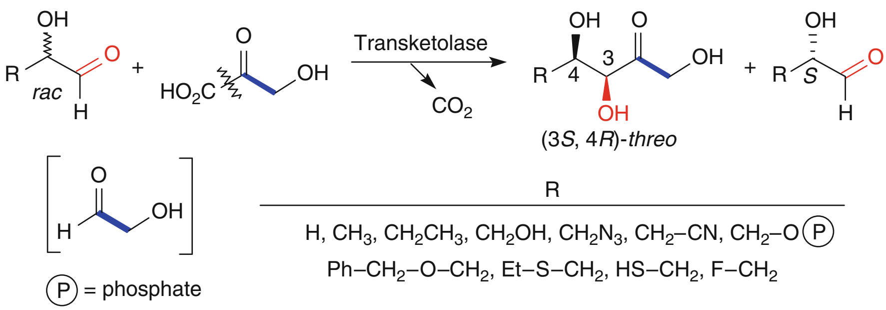
Scheme 2.201 Regiocomplementary carboligation of aldehydes catalyzed by benzaldehyde lyase

Transketolase

In the oxidative pentose phosphate pathway, ThDP-dependent transketolase[54] catalyzes the reversible transfer of a 2-carbon hydroxyacetaldehyde-unit from a ketose phosphate (D-xylulose-5-phosphate) to an aldose (D-ribose-5-phosphate) (Scheme 2.202) [1498]. Its mechanism resembles a classical acyloin condensation mediated by ThDP. Although the substrate specificity of transketolase has not been fully explored, it appears to be a promising catalyst for use in synthesis [1499, 1500]. Fortunately, the natural phosphorylated substrate(s) can be replaced by hydroxypyruvate [1501], which is decarboxyled to furnish a hydroxyacetaldehyde unit thereby driving the reaction towards completion. The C-2 fragment is transferred onto an aldehyde acceptor yielding an acyloin possessing a *threo*-diol configuration. This method has allowed the synthesis of a number of monosaccharides on a preparative scale [1502–1504].

Transketolases from various sources have been shown to possess a broad acceptor spectrum yielding products with complete (*S*)-stereospecificity for the newly formed stereocenter [1505]. Generic aldehydes are usually converted with full stereocontrol and even α,β-unsaturated aldehydes are accepted to some degree. However, hydroxylated aldehydes show enhanced rates by mimicking the natural substrate [1499]. Transketolases can be obtained from yeast [1506] and spinach [1507] and their overexpression has opened the way for large-scale production [1508, 1509].

Interestingly, transketolase recognizes chirality in the aldehydic acceptor moiety to a greater extent than the aldolases. Thus, when (stereochemically stable) racemic α-hydroxyaldehydes are employed as acceptors, an efficient kinetic resolution of the α-center is achieved (Scheme 2.202). Only the (α*R*)-enantiomer is transformed into the corresponding keto-triol leaving the (α*S*)-counterpart behind [1510]. In a related manner, when (±)-3-azido-2-hydroxypropionaldehyde was chosen as

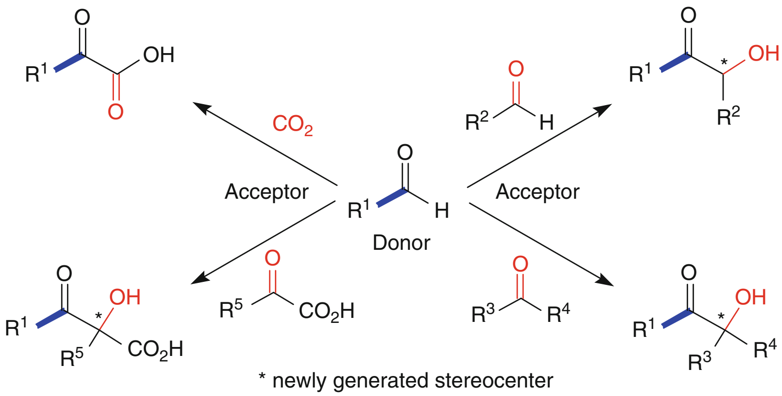


Scheme 2.202 Acyloin reactions catalyzed by transketolase

[54]Correctly, this enzyme has the charming name ‘D-seduheptulose-7-phosphate: D-glyceraldehyde-3-phosphate glycoaldehyde transferase’.

acceptor, only the D-(R)-isomer reacted and the L-(S)-enantiomer remained unchanged [1401].

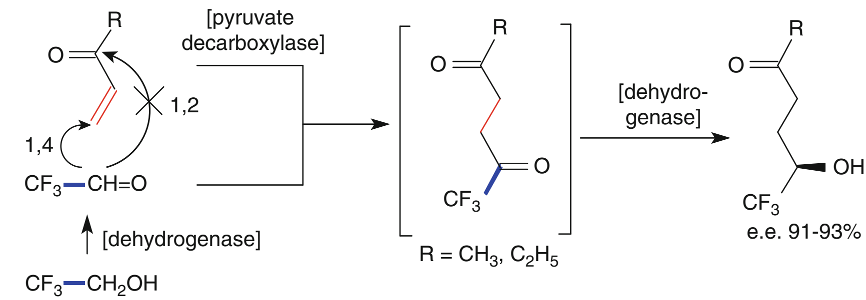
The broad synthetic potential ThDP-dependent enzymes for asymmetric C–C bond formation is by far not fully exploited with the acyloin- and benzoin-condensations discussed above. On the one hand, novel branched-chain α-keto-acid decarboxylases favorably extend the limited substrate tolerance of traditional enzymes, such as PDC, by accepting sterically hindered α-ketoacids as donors [1511]. On the other hand, the acceptor range may be significantly widened by using carbonyl compounds other than aldehydes: Thus, ketones, α-ketoacids and even CO_2 lead to novel types of products (Scheme 2.203).



Scheme 2.203 Future potential of thiamine-dependent C–C bond formation

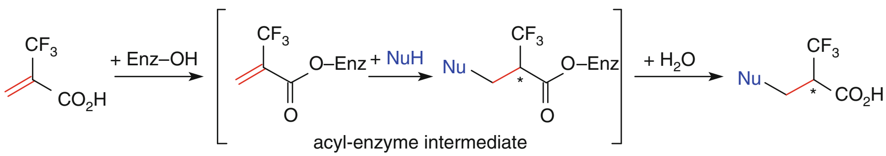
2.4.3 *Michael-Type Additions*

During an acyloin condensation mediated by baker's yeast, a side reaction involving a Michael-type addition was observed when trifluoroethanol was added to the medium (Scheme 2.204) [1512]. Although the exact mechanism of this reaction has not yet been elucidated, it presumably proceeds via the following sequence: In a first step, trifluoroethanol is enzymatically oxidized (by yeast alcohol dehydrogenase) to trifluoroacetaldehyde, which is converted into its anion equivalent by ThDP. Instead of undergoing an acyloin reaction (resembling a 1,2-addition), the latter is added in a 1,4-addition mode across the C=C double bond of the α,β-unsaturated carbonyl compound to form trifluoromethyl ketones as intermediates. In baker's yeast, these are stereoselectively reduced by dehydrogenase(s) to form chiral trifluoromethyl carbinols or the corresponding lactones, respectively. Absolute configurations were not specified, but are probably (R) as predicted by Prelog's rule.



Scheme 2.204 Michael addition catalyzed by baker's yeast

Stereospecific Michael addition reactions also may be catalyzed by hydrolytic enzymes (Scheme 2.205). When α-trifluoromethyl propenoic acid was subjected to the action of various proteases, lipases and esterases in the presence of a nucleophile (NuH), such as water, amines, and thiols, chiral propanoic acids were obtained in moderate optical purity [1513]. The reaction mechanism probably involves the formation of an acyl enzyme intermediate (Sect. 2.1.1, Scheme 2.1). Being an activated derivative, the latter is more electrophilic than the 'free' carboxylate and undergoes an asymmetric Michael addition by the nucleophile, directed by the chiral environment of the enzyme. In contrast to these observations made with crude hydrolase preparations, the rational design of a 'Michaelase' from a lipase-scaffold gave disappointingly low stereoselectivities [1514–1517].



Nucleophile	Enzyme	e.e. [%]
H_2O	*Candida rugosa* lipase	70
Et_2NH	*Candida rugosa* lipase	71
H_2O	pig liver esterase	60
Et_2NH	pig liver esterase	69
PhSH	pig liver esterase	50

Scheme 2.205 Asymmetric Michael addition catalyzed by hydrolytic enzymes

2.5 Addition and Elimination Reactions

Among the various types of transformations used in organic synthesis, addition reactions are the 'cleanest' since two components are combined into a single product with 100% atom efficiency [1518, 1519].

The asymmetric addition of small molecules such as hydrogen cyanide onto C=O bonds or water and ammonia across C=C bonds is typically catalyzed by lyases. During such a reaction one or (depending on the substitution pattern of the substrate) two chiral centers are created from a prochiral substrate.

2.5.1 Cyanohydrin Formation

Hydroxynitrile lyase enzymes catalyze the asymmetric addition of hydrogen cyanide onto a carbonyl group of an aldehyde or a ketone thus forming a chiral cyanohydrin [1520–1524],[55] a reaction which was used for the first time as long ago as 1908 [1525]. Cyanohydrins are rarely used as products per se, but they represent versatile starting materials for the synthesis of several types of compounds [1526]:

Chiral cyanohydrins serve as the alcohol moieties of several commercial pyrethroid insecticides (see below) [1527]. Hydrolysis or alcoholysis of the nitrile group affords chiral α-hydroxyacids or -esters and Grignard reactions provide acyloins [1528], which in turn can be reduced to give vicinal diols [1529]. Alternatively, the cyanohydrins can be subjected to reductive amination to afford chiral ethanolamines [1530]. α-Aminonitriles as well as aziridines are obtained via the corresponding α-sulfonyloxy nitriles [1531].

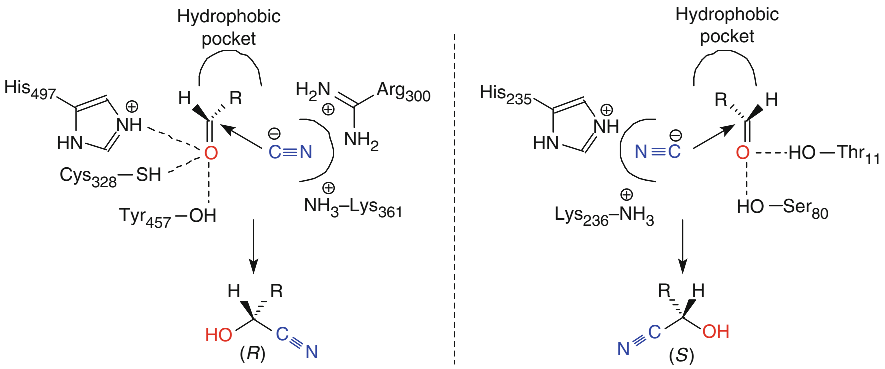
Since only a single enantiomer is produced during the reaction – representing a desymmetrization of a prochiral substrate – the availability of different enzymes of opposite stereochemical preference is of importance to gain access to both (*R*)- and (*S*)-cyanohydrins (Scheme 2.207). Fortunately, an impressive number of hydroxynitrile lyases of opposite stereochemical preference can be isolated from cyanogenic plants [1532–1536].

(*R*)-Specific enzymes are obtained predominantly from the *Rosacea* family (almond, plum, cherry, apricot) and they have been thoroughly investigated [1537–1540]. They contain FAD in its oxidized form as a prosthetic group located near (but not in) the active site, but this moiety does not participate in catalysis and seems to be an evolutionary relict.

On the other hand, (*S*)-hydroxynitrile lyases [1541–1544] were found in *Sorghum bicolor* [1545] (millet), *Hevea brasiliensis* [1546, 1547] (rubber tree),

[55] Hydroxynitrile lyases were often also called 'oxynitrilases'. However, this term should be abandoned.

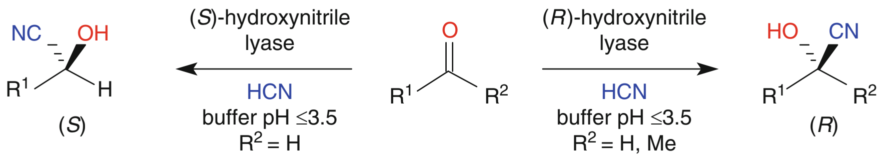
Ximenia americana [1548] (sandalwood), *Sambucus niger* [1549] (elder), *Manihot esculenta* [1543, 1550] (cassava), flax, and clover. They do not contain FAD and they exhibit a more narrow substrate tolerance, as aliphatic aldehydes are not always accepted. Furthermore, the reaction rates and optical purities are sometimes lower than those which are obtained when the (*R*)-enzyme is used. Based on X-ray structures [1551, 1552], the mechanism of enzymatic cyanohydrin formation has been elucidated as follows (Scheme 2.206) [1553]: The substrate is positioned in the active site with its carbonyl group bound through network of hydrogen bonds involving His/Cys/Tyr or Ser/Thr-moieties, while the lipophilic residue is accommodated in a hydrophobic pocket. Nucleophilic addition of cyanide anion occurs from opposide sides from cyanide-binding pockets, which are made of positively charged Arg/Lys- or His/Lys-residues [364].



Scheme 2.206 Schematic representation of (*R*)- and (*S*)-hydroxynitrile formation by HNLs from almond and *Hevea brasiliensis*, respectively

The following set of rules for the substrate-acceptance of (*R*)-hydroxynitrile lyase was delineated [1554].

- The best substrates are aromatic aldehydes, which may be substituted in the *meta*- or *para*-position; also heteroaromatics such as furan and thiophene derivatives are well accepted [1555–1558].
- Straight-chain aliphatic aldehydes and α,β-unsaturated aldehydes are transformed as long as they are not longer than six carbon atoms; the α-position may be substituted with a methyl group.
- Methyl ketones are transformed into cyanohydrins [1559], while ethyl ketones are impeded by low yields [1560].
- For large or sterically demanding aldehydes, such as *o*-chlorobenzaldehyde, *(*R)-HNL mutants possessing a more spacious active site were constructed [1561, 1562]. The (*R*)-*o*-chloromandelonitrile thus obtained represents the chiral core of the blockbuster clopidogrel (Plavix) (Scheme 2.207).



Scheme 2.207 Stereocomplenentary asymmetric cyanohydrin formation

Table 2.7 Synthesis of (*R*)-cyanohydrins from aldehydes and ketones

R^1	R^2	e.e. (%)
Ph–	H	94
p-MeO–C_6H_4–	H	93
2-furyl–	H	98
n-C_3H_7–	H	92–96
t-Bu–	H	73
(*E*)-CH_3–CH=CH–	H	69
n-C_3H_7–	Me	95
n-C_4H_9–	Me	98
$(CH_3)_2$CH–$(CH_2)_2$–	Me	98
CH_2=CH(CH_3)–	Me	94
Cl–$(CH_2)_3$–	Me	84

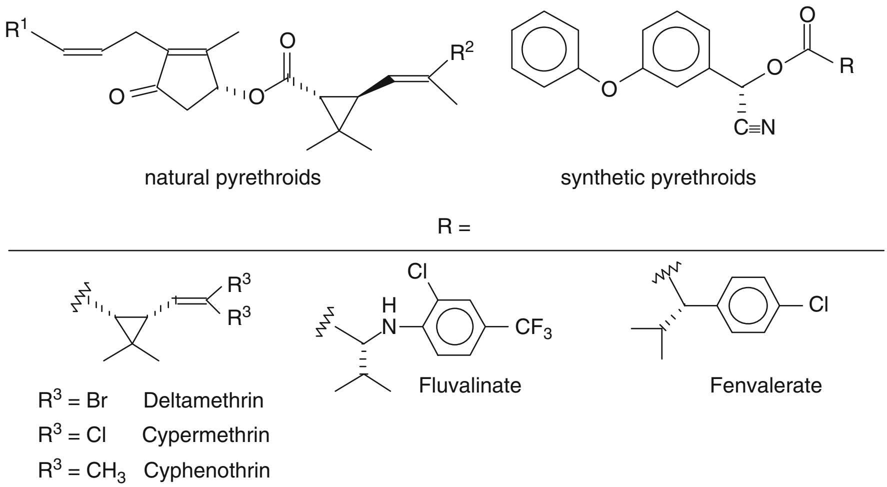
The (*S*)-hydroxynitrile lyase from *Hevea brasiliensis* has been made available in sufficient quantities by cloning and overexpression to allow industrial-scale applications [1563]. It should be noted that also α,β-unsaturated aliphatic aldehydes were transformed into the corresponding cyanohydrins in a clean reaction. No formation of saturated β-cyano aldehydes through Michael-type addition of hydrogen cyanide across the C=C double bond occurred. The latter is a common side reaction using traditional methodology.

Of particular interest is the industrial-scale synthesis of the (*S*)-configured cyanohydrin from *m*-phenoxybenzaldehyde (Table 2.8), which is an important intermediate for synthetic pyrethroids.

Table 2.8 Synthesis of (*S*)-cyanohydrins from aldehydes

R^1	R^2	e.e. (%)
Ph–	H	96–98
p-HO–C_6H_4–	H	94–99
m-C_6H_5O–C_6H_4–	H	96
3-thienyl–	H	98
n-C_5H_{11}–	H	84
n-C_8H_{17}–	H	85
CH_2=CH–	H	84
(*E*)-CH_3–CH=CH–	H	92
(*E*)-*n*-C_3H_7–CH=CH–	H	97
(*Z*)-*n*-C_3H_7–CH=CH–	H	92
n-C_5H_{11}–	CH_3	92
$(CH_3)_2$CH–CH_2–	CH_3	91

Synthetic pyrethroids comprise a class of potent insecticides with structural similarities to a number of naturally occurring chrysanthemic acid esters found in the extract of pyrethrum flowers (*Chrysanthemum cinerariaefolium*). These natural products constitute highly potent insecticides, but their instability (inherent to the cyclopentenone moiety) precludes their broad application in agriculture. This fact has led to the development of a range of closely related analogs, which retain the high insecticidal activity of their natural ancestors but are more stable (Scheme 2.208). All of these synthetic pyrethroids contain asymmetric carbon atoms and it is well established that their insecticidal activity resides predominantly in one particular isomer. In order to reduce the environmental burden during pest control, single isomers are marketed [1564].



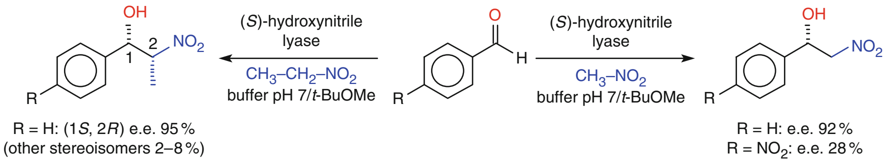
Scheme 2.208 Natural and synthetic pyrethroids

Two particular problems which are often encountered in hydroxynitrile lyase-catalyzed reactions are the spontaneous nonenzymatic formation of racemic cyanohydrin and racemization of the product due to equilibration of the reaction. As a result, the optical purity of the product is decreased. Bearing in mind that both the chemical formation and the racemization of cyanohydrins are pH-dependent and require water, three different techniques have been developed in order to suppress the reduction of the optical purity of the product.

- Adjusting the pH of the medium to a value below 3.5, which is the lower operational pH-limit for most hydroxynitrile lyases.
- Lowering the water-activity of the medium [1565] by using water-miscible organic cosolvents such as ethanol or methanol. Alternatively, the reaction can be carried out in a biphasic aqueous-organic system or in a monophasic organic solvent (e.g., ethyl acetate, di-*i*-propyl, or methyl *t*-butyl ether) which contains only traces of water to preserve the enzyme's activity.

- In order to avoid the use of hazardous hydrogen cyanide, *trans*-cyanation reactions were developed using either acetone cyanohydrin [1566] or (±)-2-pentanone cyanohydrin [1567] as donor for hydrogen cyanide. The latter are considerably more easy to handle. Using this technique, the competing chemical cyanohydrin formation is negligible due to the low concentration of free hydrogen cyanide and the use of free hydrogen cyanide is avoided.

A fascinating variant of the enzymatic cyanohydrin formation consists in the use of nitroalkanes (as nonnatural nucleophiles) instead of cyanide (Scheme 2.209) [1568, 1569]. Overall, this constitutes a biocatalytic equivalent to the Henry-reaction producing vicinal nitro-alcohols, which are valuable precursors for amino alcohols. Using (*S*)-HNL, the asymmetric addition of nitromethane to benzaldehyde gave the nitroalcohol in 92% e.e., while for *p*-nitrobenzaldehyde the stereoselectivity dropped sharply. With nitroethane, two stereocenters are created: Whereas the stereoselectivity for the alcoholic center was high (e.e. 95%), the recognition for the adjacent center bearing the nitro moiety was modest and other (dia)stereomers were formed in up to 8%.



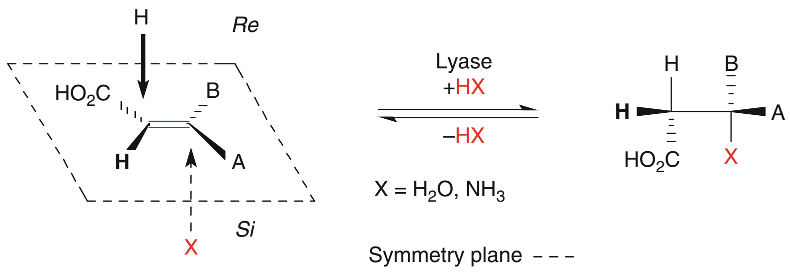
Scheme 2.209 Asymmetric Henry-reaction catalyzed by (*S*)-hydroxynitrile lyase

2.5.2 Addition of Water

The asymmetric addition of water or ammonia onto olefins is one of the 'dream-reactions' on organic synthesis and represents one of the (largely unsolved) problems of catalysis. Enzymes called hydratases or ammonia lyases can catalyze this reaction. However, they only act on activated alkenes, such as α,β-unsaturated carboxylic acids, and their substrate tolerance is rather narrow and only allow minor structural variations of their natural substrate(s), which severely limits their application in organic synthesis.

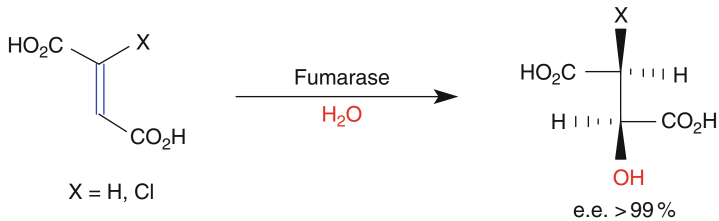
Fumarase [EC 4.2.1.2] and malease [EC 4.2.1.31] catalyze the stereospecific addition of water onto carbon–carbon double bonds conjugated with a carboxylic acid [1570]. The analogous addition of ammonia is catalyzed by aspartase [EC 4.3.1.1], 3-methylaspartase [1571], and phenylalamine ammonia lyase [EC 4.3.1.5].

Both reactions are mechanistically related and take place in a *trans*/*anti*-manner [1572, 1573], with protonation occurring from the *re*-side (Scheme 2.210). These close mechanistic similarities may be explained by the fact that some of these enzymes show a remarkable degree of amino acid homology [1574]. Within this group of enzymes, substrate tolerance is rather narrow, but the stereoselectivities observed are exceptionally high.



Scheme 2.210 Lyase-catalyzed addition of water and ammonia onto activated C=C bonds

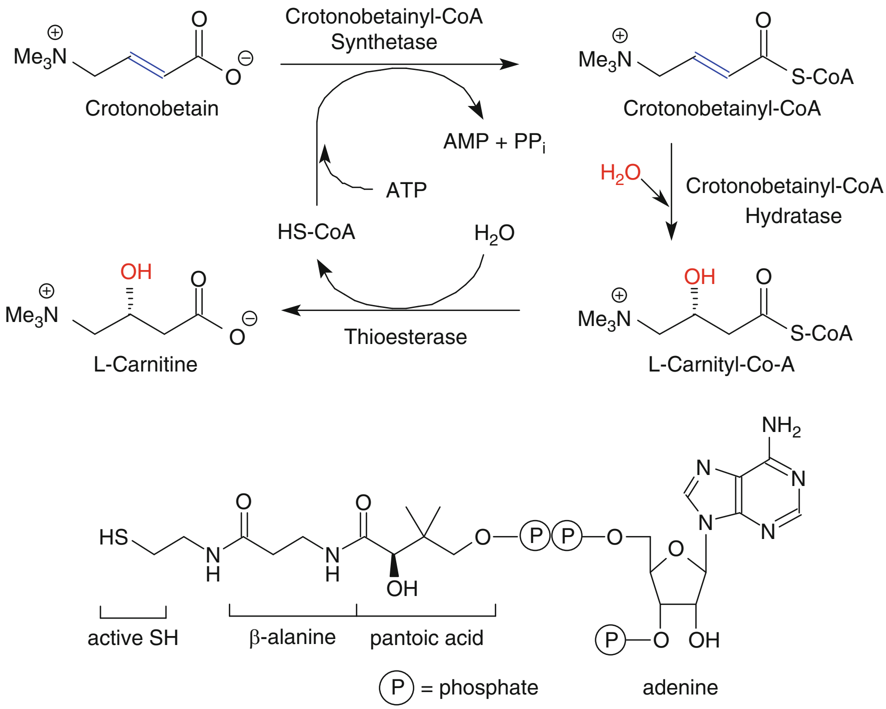
The addition of water onto fumaric acid leads to malic acid derivatives (Scheme 2.211). Only fumarate and chlorofumaric acid are well accepted; the corresponding (sterically more encumbered) bromo-, iodo-, and methyl derivatives are transformed at exceedingly low rates, albeit with excellent stereoselectivities. Fluoro- and 2,3-difluorofumaric acid are accepted but their transformation suffers from decomposition reactions of the first-formed fluoromalic acid. Replacement of one of the carboxylic groups or changing the stereochemistry of the double bond from (*E*) to (*Z*) is not tolerated by the enzyme [1575]. The analogous hydration of the stereoisomeric (*Z*)-isomer (maleic acid) produces the mirror-image (*R*)-malate and is catalyed by malease (maleate hydratase) [1576, 1577]. The latter enzyme also accepts 2-methylmaleate (citraconate) to form (*R*)-2-hydroxy-2-methylsuccinate (2-methylmaleate) [1578]. The industrial-scale production of (*S*)-malate, which is used as an acidulant in fruit juices, carbonated soft drinks, and candies, is performed using the above described hydratases at a capacity of up to 2,000 t/year [1579, 1580].



Scheme 2.211 Fumarase-catalyzed addition of water

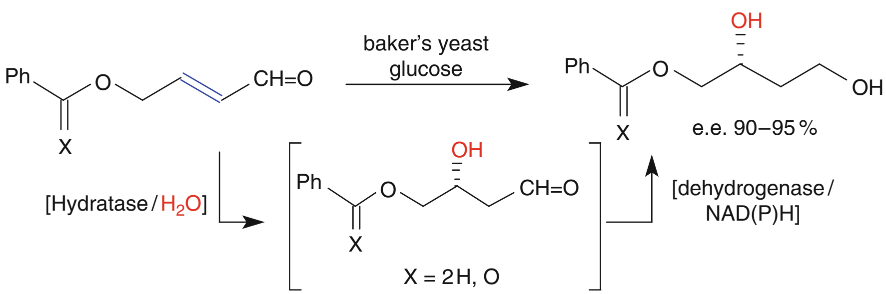
In contrast to the hydration of highly activated olefinic *di*acids, α,β-unsaturated *mono*carboxylic acids have to be activated via a thioester linkage onto the cofactor Coenzyme A. The latter is catalyzed by an enoyl-CoA synthetase and requires ATP as energy source. The enoyl-CoA intermediate is hydrated by an enoyl-CoA hydratase yielding the corresponding β-hydroxyacyl-CoA as product, which is finally hydrolyzed by a thioesterase to liberate the β-hydroxycarboxylic acid and CoA, which re-enters the catalytic cycle. Due to the complexity of this multienzyme-system requiring ATP and CoA, hydration of acrylic acid derivatives is always performed using whole cells [1274, 1581, 1582].

An elegant example for this biotransformation is the asymmetric hydration of crotonobetaine yielding the 'nutraceutical' (*R*)-carnitine (Scheme 2.212), which is used as an additive in baby food, geriatric nutrition and health sport. In order to avoid the undesired degradation of the product, mutant strains lacking carnitine dehydrogenase have been developed at a capacity of >100 t/year [1583–1585].



Scheme 2.212 Asymmetric hydration of crotonobetaine to carnitine via a multienzyme-system

An unexpected reaction catalyzed by baker's yeast with potential synthetic utility was observed as a side reaction during an attempt to asymmetrically reduce substituted crotonaldehyde derivatives (Scheme 2.213). Thus, a lyase-catalyzed addition of water occurred in presence of a 4-benzyloxy- (X = 2 H) or benzoyloxy substituent (X = O) in the substrate [1474, 1586].

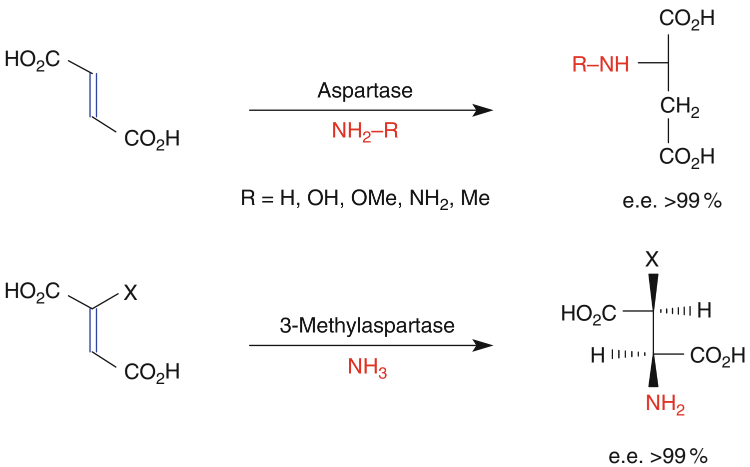


Scheme 2.213 Addition of water by fermenting baker's yeast

The capacity of microbial cells of different origin to perform an asymmetric hydration of C=C bonds has only been poorly investigated but they show a promising synthetic potential. For instance, *Fusarium solani* cells are capable of hydrating the 'inner' (*E*)-double bond of terpene alcohols (e.g., nerolidol) or -ketones (e.g., geranyl acetone) in a highly selective manner [1587]. However, side reactions such as hydroxylation, ketone-reduction, or degradation of the carbon skeleton represent a major drawback. On the other hand, resting cells of *Rhodococcus rhodochrous* catalyzed the asymmetric addition of water onto the C=C bond of α,β-unsaturated butyrolactones with high enantioselectivity, furnishing β-hydroxy-lactones in moderate yields [1588].

2.5.3 Addition of Ammonia

Enzymic amination of fumaric acid using aspartase leads to the formation of L-aspartic acid, which is performed at a capacity of 1,200 t/year (Scheme 2.214)



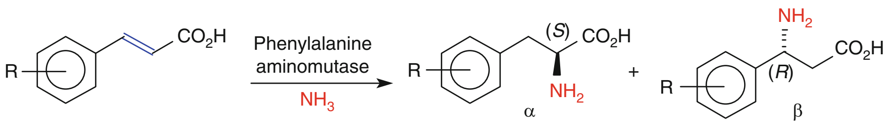
X	Yield [%]
H	90
Cl–	60
CH_3–	61
C_2H_5–	60
$(CH_3)_2CH$–	54
n-C_3H_7–	49
n-C_4H_9–	0

Scheme 2.214 Amination of fumarate derivatives by aspartase and 3-methylaspartase

[1589–1592]. Although aspartase is one of the most specific enzymes known by accepting only its natural substrate [1593–1595], some variations concerning the nucleophile are tolerated: Hydroxylamine, hydrazine, methoxyamine, and methylamine are accepted and furnish the corresponding L-aspartate *N*-derivatives [1596–1598].

In contrast to aspartase, some structural variations are tolerated by the related 3-methylaspartase (Scheme 2.214). For instance, the methyl group in the natural substrate may be replaced by a chlorine atom or by small alkyl moieties [1599], but the fluoro- and the iodo-analog (X = F, I) are not good substrates. Although the bromo-derivative is accepted, it irreversibly inhibits the enzyme [1600].

In a related fashion, asymmetric amination of (*E*)-cinnamic acid yields L-phenylalanine using L-phenylalanine ammonia lyase [EC 4.3.1.5] at a capacity of 10,000 t/year [1274, 1601]. A fascinating variant of this biotransformation consists in the use of phenylalanine aminomutase from *Taxus chinensis* (yew tree), which interconverts α- to β-phenylalanine in the biochemical route leading to the side chain of taxol [1602]. In contrast to the majority of the cofactor-independent C–O and C–N lyases discussed above, its activity depends on the protein-derived internal cofactor 5-methylene-3,5-dihydroimidazol-4-one (MIO) [1603]. Since the reversible α,β-isomerization proceeds via (*E*)-cinnamic acid as achiral intermediate, the latter can be used as substrate for the amination reaction. Most remarkably, the ratio of α- vs. β-amino acid produced (which is 1:1 for the natural substrate, R = H) strongly depends on the type and the position of substituents on the aryl moiety: While *o*-substituents favor the formation of α-phenylalanine derivatives, *p*-substituted substrates predominantly lead to β-amino analogs. A gradual switch between both pathways occurred with *m*-substituted compounds. With few exceptions, the stereoselectivity remained excellent (Scheme 2.215) [1604, 1605].



R	Ratio α/β	α e.e. [%]	β e.e. [%]
H	1 : 1	>99	>99
o-F, *o*-Cl, *o*-Br, *o*-Me	>98 : 2	>99	n.d.
m-F	86 : 14	92	n.d.
m-Me	20 : 80	>99	>99
p-*n*-Pr	12 : 88	n.d.	>99
p-Et	9 : 91	n.d.	>99
p-Me	4 : 96	>99	>99

Scheme 2.215 Formation of α- and β-phenylalanine derivatives using phenylalanine ammonia mutase

2.6 Transfer Reactions

2.6.1 *Glycosyl Transfer Reactions*

Oligosaccharides and polysaccharides are important classes of naturally occurring compounds [1606]. They play a vital role in intracellular migration and secretion of glycoproteins, cell–cell interactions, oncogenesis, and interaction of cell surfaces with pathogens [1607–1609]. The building blocks are monosaccharides which (theoretically) occur in an enormous number of stereoisomers, which results in a structural diversity far greater than that possible with peptides of comparabale size [1610].[56] Fortunately, Nature is using almost exclusively pentoses and hexoses for in vivo synthesis.

The ready availability of such oligosaccharides of well-defined structure is critical for the synthesis of drug candidates. Isolation of these materials from natural sources is a complex task and is not economical on a large scale due to the low concentration of the structures of interest in the complex mixtures of carbohydrates obtained from natural sources. Chemical synthesis of complex oligosaccharides is one of the greatest challenges facing synthetic organic chemistry since it requires many protection and deprotection steps which result in low overall yields [1611]. Moreover, stereospecific chemical synthesis of oligosaccharides, in particular of the important α-sialylated structures, is difficult. In this context, biocatalysts are attractive as they allow the regio- and stereospecific synthesis of oligosaccharides with a minimum of protection and deprotection steps [1363–1365, 1612–1618]. There are two groups of enzymes which can be used for the synthesis of oligosaccharides.

Glycosyl transferases are the biocatalysts which are responsible for the *biosynthesis* of oligosaccharides in vivo. They require that the sugar monomer which is to be linked to the growing oligosaccharide chain is activated by phosphorylation prior to the condensation step. The activating group on the anomeric center, a leaving group (LG, Scheme 2.216), is either a nucleoside phosphate (usually a *di*phosphate in the Leloir pathway [1619]) or a simple phosphate (in non-Leloir pathway enzymes [1620]). Glycosyl transferases are highly specific with respect to their substrate(s) and the nature of the glycosidic bond to be formed.

Glycosidases belong to the class of hydrolytic enzymes and have a *catabolic* function in vivo as they hydrolyze glycosidic linkages to form mono- or oligosaccharides from polysugars. Consequently, they are generally less specific when compared to glycosyl transferases. Both of these types of enzymes can be used for the synthesis of oligosaccharides and related compounds.

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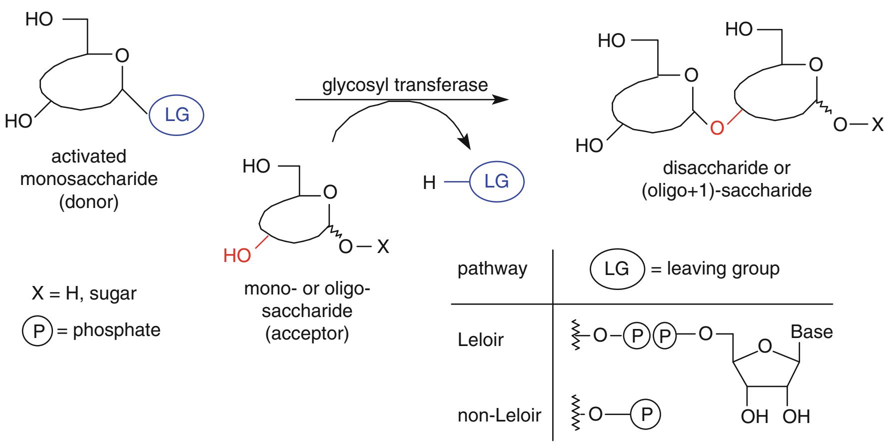
[56]The possible number of linear and branched oligosaccharide isomers for a reducing hexasaccharide was calculated to encompass 1.05×10^{12} structures, see [1609].

2.6.1.1 Glycosyl Transferases

Three fundamental steps constitute the glycoside-bond formation in the Leloir pathway: activation, transfer and modification [1621]. These steps represent biological solutions to the problems also faced by chemists, i.e., chemical activation of sugars, regio- and stereospecific formation of glycosidic linkages and final elaboration of the products. In the first step, a sugar is phosphorylated by a kinase to give a sugar-1-phosphate. This activated sugar subsequently reacts with a nucleoside triphosphate (usually uridine triphosphate, UTP) under catalysis of a nucleoside transferase and forms a chemically activated nucleoside diphosphate sugar (NDP, Scheme 2.216). These key nucleoside diphosphate sugars constitute the activated 'donors' in the subsequent condensation with the 'acceptors' (the hydroxyl group of a mono- or oligosaccharide, a protein or a lipid). The latter step is catalyzed by a glycosyl transferase. To ensure proper functioning of the cell, a large number of highly specific glycosyl transferases seem to be neccessary, since each NDP-sugar requires a distinct group of glycosyl transferases.



Scheme 2.216 Synthesis of oligosaccharides by glycosyl transferases

More than one hundred glycosyl transferases have been identified to date and each one appears to specifically catalyze the formation of a unique glycosidic linkage [1622].

Chemists apply enzymes of the Leloir pathway to the synthesis of oligosaccharides [1623–1627]. Two requirements are critical for the success of this approach, namely the availability of the sugar nucleoside phosphates at reasonable costs and the availability of the matching glycosyl transferases. Although the first issue is being resolved for most of the common NDP-sugars utilizing phosphorylating enzymes (Sect. 2.1.4), the supply of cheap glycosyl transferases remains a problem. Only a few of these enzymes are commercially available; because isolation of these membrane-bound (unstable) proteins is difficult, since they are present only in low concentrations [1628]. In this context, genetic engineering has a major impact by making glycosyl transferases more stable and readily available. Since the membrane-unbound (soluble) portion of glycosyl transferases alone (containing the catalytic domain) is stable and fully active, one promising possibility is to express only this part in engineered microorganisms [1629]. Another disadvantage associated with glycosyl transferase reactions is that the reactions often exhibit coproduct inhibition caused by the released nucleoside phosphate (LG-OH). A simple solution to these problems is to keep their concentration at a low level by in-situ regeneration of the sugar nucleotide from the released nucleoside phosphate (Scheme 2.217).

Enzymatic methods [1630] of preparing the NDP-sugars have several advantages over the chemical methods [1631]. For example, the NDP-sugar may be generated in situ (e.g., by epimerization of UDP-Glc to UDP-Gal), making it possible to drive unfavorable equilibria in the required direction. Purification steps may be eliminated because the byproducts of enzyme-catalyzed methods do not interfere with further enzymatic steps.

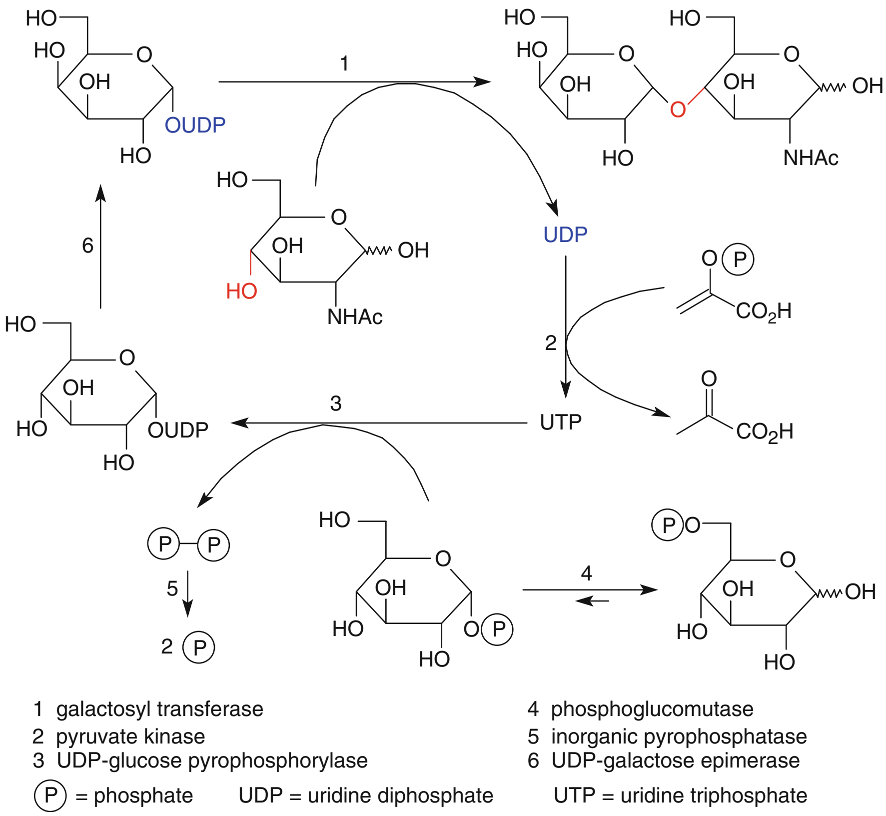
Table 2.9 Glycoside synthesis using β-galactosyl transferase from the Leloir pathway (donor = UDP-Gal, Scheme 2.216)

Acceptor	Product
Glc–OH	β-Gal–(1→4)-Glc–OH
GlcNAc–OH	β-Gal–(1→4)-GlcNAc–OH
β-GlcNAc–(1→4)-Gal–OH	β-Gal–(1→4)-β-GlcNAc–(1→4)-Gal–OH
β-GlcNAc–(1→6)-Gal–OH	β-Gal–(1→4)-β-GlcNAc–(1→6)-Gal–OH
β-GlcNAc–(1→3)-Gal–OH	β-Gal–(1→4)-β-GlcNAc–(1→3)-Gal–OH

The point of interest to synthetic chemists is the range of acceptors and donors that can be used in glycosyl transferase-catalyzed reactions. Fortunately, the specificity of glycosyl transferases is high but not absolute.

UDP-galactosyl (UDP-Gal) transferase is the best-studied transferase in terms of specificity for the acceptor sugar. It has been demonstrated that this enzyme catalyzes the transfer of UDP-Gal to a remarkable range of acceptor substrates of the carbohydrate-type [1622, 1632–1635]. Other glycosyl transferases, although less well-studied than UDP-Gal transferase, also appear to tolerate various acceptors as substrates [1636–1639] (Table 2.9).

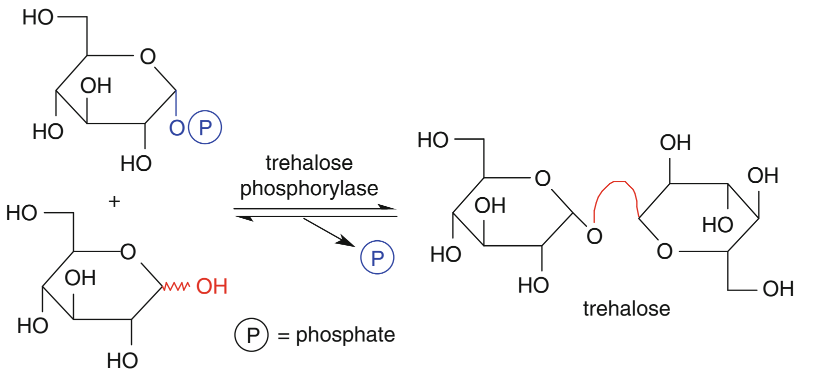
The use of the multienzyme systems, which arise due to the need to prepare the activated UDP-sugar in situ, is exemplified with the synthesis of *N*-acetyllactosamine [1640] (Scheme 2.217). Glucose-6-phosphate is isomerized to its 1-phosphate by phosphoglucomutase. Transfer of the activating group (UDP) from UTP is catalyzed by UDP-glucose pyrophosphorylase liberating pyrophosphate, which is destroyed by inorganic pyrophosphatase. Then, the center at carbon 4 is epimerized by UDP-galactose epimerase in order to drive the process out of the equilibrium. Finally, using galactosyl transferase, UDP-galactose is linked to *N*-acetylglucosamine to yield *N*-acetyllactosamine.



Scheme 2.217 Synthesis of *N*-acetyllactosamine using a six-enzyme system (Leloir pathway)

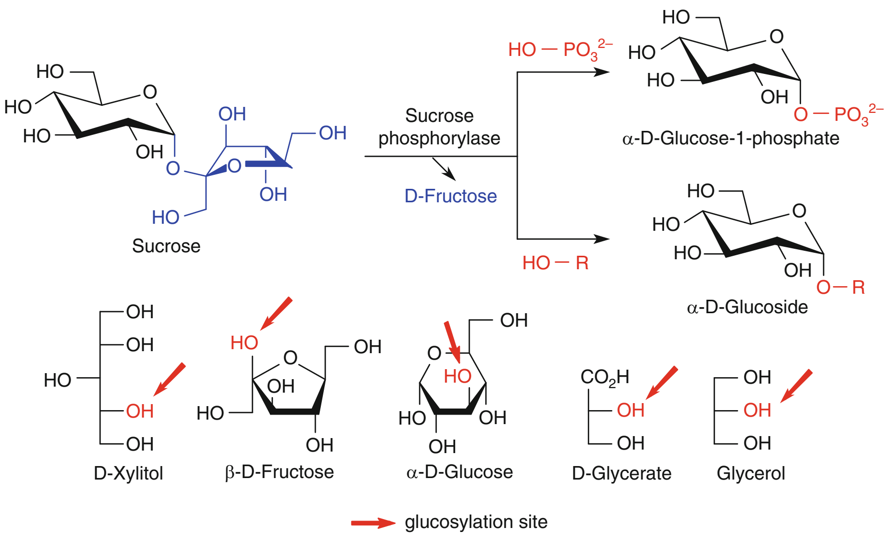
The liberated UDP is recycled back to the respective triphosphate by pyruvate kinase at the expense of phosphoenol pyruvate. The overall yield of this sequence was in the range of 70% when performed on a scale greater than 10 g.

More recently, attention has been drawn to oligosaccharide synthesis via non-Leloir routes (Scheme 2.218). In this case, the activated donor is a more simple sugar-1-phosphate, which can be transferred by a single sugar phosphorylase. The latter catalyze the reversible cleavage/formation of a glycosyl bond using phosphate as nucleophile/leaving group, respectively [1641, 1642]. Depending on their mode of action, they belong to the group of glycosyl transferases or glycosidases [1643]. For example, trehalose, one of the major storage carbohydrates in plants, fungi, and insects, was synthesized from glucose and its 1-phosphate using trehalose phosphorylase as the catalyst [1644].



Scheme 2.218 Synthesis of trehalose via the non-Leloir pathway

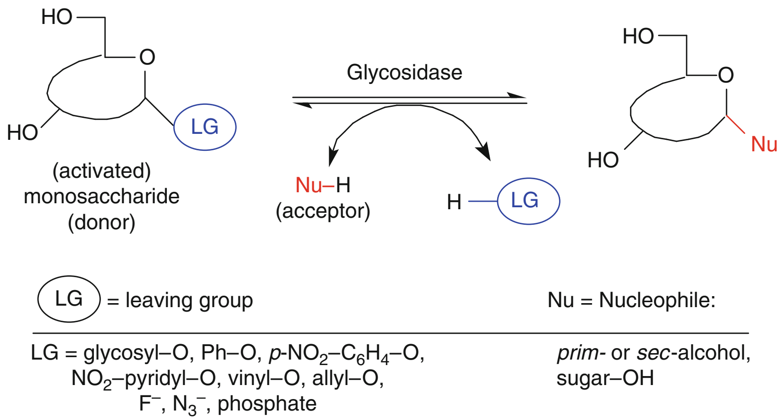
Glycoside synthesis becomes even more facile using sucrose phosphorylase. This bacterial transglucosidase catalyzes the cleavage of the disaccharide sucrose using phosphate as nucleophile to yield α-D-glucose-1-phosphate and D-fructose (Scheme 2.219). In the absence of phosphate, the enzyme-glucosyl intermediate can be intercepted by various nucleophiles bearing an alcoholic group to yield the corresponding α-D-glucosides in high yields [1645, 1646]. Aryl alcohols and polyhydroxylated compounds, such as sugars and sugar alcohols are often glycosylated in a highly selective fashion. The major advantage of this system is the weak hydrolase activity of sucrose phosphorylase and the high-energy content of the cheap glucosyl donor sucrose. Several of these products constitute biocompatible solutes, which regulate the water-balance of the cell, prevent protein denaturation and stabilize membranes and are thus used as natural osmolytes and moisturising agents [1647].



Scheme 2.219 Synthesis of α-D-glycosides using sucrose phosphorylase

2.6.1.2 Glycosidases

In the breakdown of carbohydrates, glycosidases (also termed 'glycohydrolases' [1648]) cleave glycosidic bonds. Since they are true hydrolases they are independent of any cofactor. Two groups of them exist: *exo*-glycosidases only cleave terminal sugar residues, whereas *endo*-glycosidases are also able to split a carbohydrate chain in the middle. In general, glycosidases show high (but not absolute) specificity for both the glycosyl moiety and the nature of the glycosidic linkage, but little if any specificity for the aglycone component which acts as a leaving group ([LG-H], Scheme 2.220) [1649]. It has long been recognized that the nucleophile (NuH, which is water in the 'normal' hydrolytic pathway) can be replaced by other nucleophiles, such as another sugar or a primary or secondary (nonnatural) acceptor alcohol. This allows to turn the degradative nature of glycosyl hydrolysis towards the more useful *synthetic* direction [1612, 1650–1654]. Interestingly, this potential was already recognized as early as 1913! [1655].



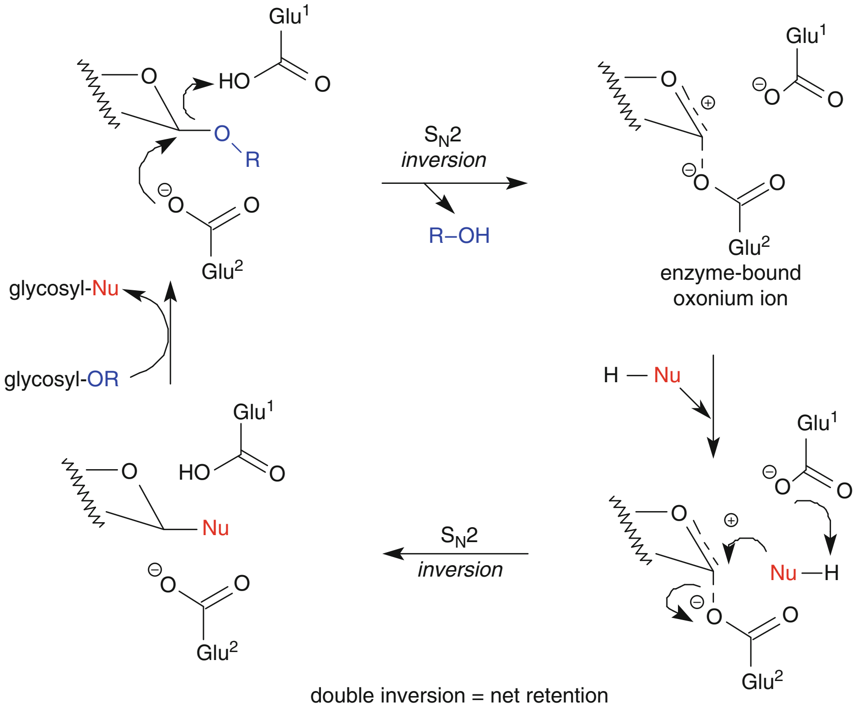
Scheme 2.220 Glycoside synthesis using glycosidases

Major advantages of glycosidase-catalyzed glycosyl transfer are that there is minimal (or zero) need for protection and that the stereochemistry at the newly formed anomeric center can be completely controlled through the choice of the appropriate enzyme, i.e., an α- or β-glucosidase. However, regiocontrol with respect to the acceptor remains a problem, particularly when mono- or oligosaccharides carrying multiple hydroxy groups are involved.

Depending on the stereochemical course of glycoside formation, i.e., whether *retention* or *inversion* of the configuration at the anomeric center is observed, glycosidases operate via two separate and distinct mechanisms (Schemes 2.221, 2.222)

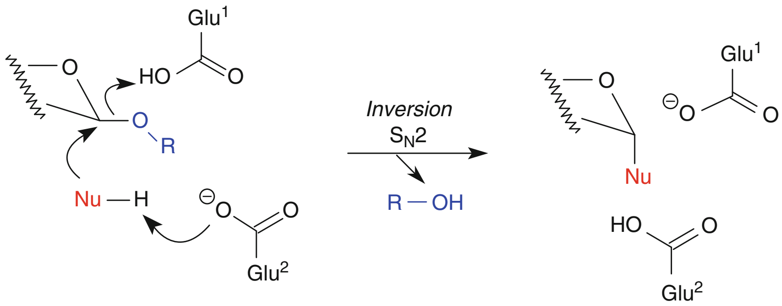
[1656–1660]. Examples of the retaining enzymes are β-galactosidase, invertase and lysozyme. Inverting glycosidases, such as trehalase and β-amylase, have been used for the synthesis of alkyl glycosides to a lesser extent.

Although the first proposal for the mechanism of retaining glycosidases in 1953 has undergone some refinements, it is still valid in its sense (Scheme 2.221) [572, 1661, 1662]: The active site contains two glutamic acid residues (Glu^1 and Glu^2), which can act as an acid or a base, respectively. In the first step, Glu^1 acts as an acid by protonation of the anomeric oxygen, making the (oligo)saccharide moiety [RO] a good leaving group, while the glycosyl residue is bound to the enzyme via Glu^2 as oxonium ion [1663, 1664]. Then, the leaving group ROH is displaced by the incoming nucleophile NuH (usually water) via diffusion. In the second step, the nucleophile is deprotonated by Glu^1 and the glycosyl-enzyme intermediate from attacks from same face from which the leaving group R-OH was expelled. Since both steps constitute an S_N2-reaction, *double inversion* results in *net retention* of configuration.



Scheme 2.221 Mechanism of retaining glycosidases

In contrast, *inverting* glycosidases act via a single step: Direct nucleophilic displacement of the aglycone moiety (ROH) by a nucleophile (NuH) via S_N2 leads to *inversion* of anomeric configuration (Scheme 2.222).



Scheme 2.222 Mechanism of inverting glycosidases

Reverse Hydrolysis

Glycosidases can be used for the synthesis of glycosides in two modes. The *thermodynamic* approach is the reversal of glycoside hydrolysis by shifting the equilibrium of the reaction from hydrolysis to synthesis. This procedure uses a free (nonactivated) monosaccharide as substrate and it has been referred to as 'direct glycosylation' or 'reverse hydrolysis' (Fig. 2.19, pathway A) [1665–1668]. Since in an aqueous environment the equilibrium constant for this reaction lies strongly in favor of hydrolysis, high concentrations of both the monosaccharide and the nucleophilic component (carbohydrate or alcohol) must be used. As a consequence, yields in these reactions are generally low and reaction mixtures comprised of thick syrups up to 75% by weight are not amenable to scale-up.

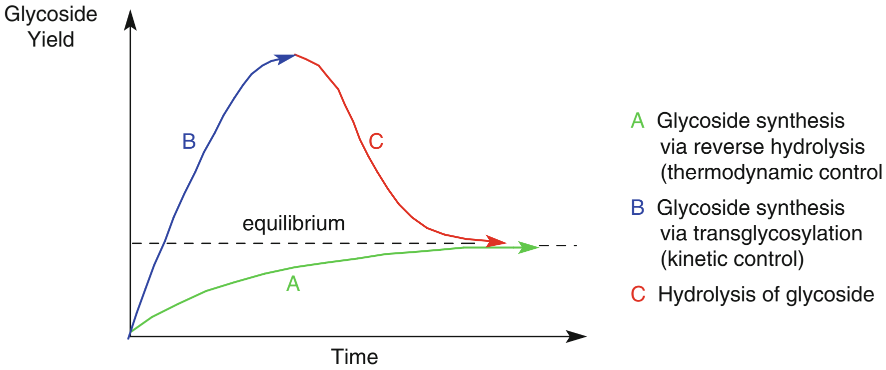


Fig. 2.19 Glycosylation via thermodynamic and kinetic control

Other methods to improve such procedures make use of aqueous-organic two-phase systems [1669, 1670] and polyethylene-glycol-modified glycosidases [1671]. However, the direct enzymatic synthesis of alkyl glycosides is generally hampered by the low solubility of carbohydrates in organic media. More polar solvents, such as DMF, DMSO or pyridine, would be self-defeating, considering that the products are often intended for use in food and personal care products. Alternatively, the reaction can be performed at temperatures below 0°C or the glycoside formed can be removed from the reaction medium by selective adsorption [1672]. In summary, glycoside synthesis via the reverse hydrolysis approach is less than ideal.

Transglycosylation

The second route – the *kinetic* approach – utilizes a preformed activated glycoside, which is coupled onto the nucleophile by an appropriate glycosidase and is referred to as 'transglycosylation' (Fig. 2.19, pathway B) [1673, 1674]. The enzyme-glycoside intermediate is then trapped by a nucleophile other than water to yield a new glycoside. In this case, activated glycosyl donors which possess an aglycone moiety with good leaving group properties, i.e., poor nucleophilicity, are used [1675, 1676]. Good donors are, for instance, glycosyl fluorides [537, 1677, 1678], -azides [1679, 1680], (hetero)aryl- (usually *p*-nitrophenyl- or nitropyridyl- [1681]), vinyl- and allyl-glycosides [1682, 1683]. Transglycosylation gives higher yields as compared to reverse hydrolysis and is generally the method of choice [1684, 1685]. Since the glycoside formed during the reaction is also a substrate for the enzyme in hydrolysis causing its degradation (Fig. 2.19, pathway C), the success of this procedure as a preparative method depends on the following crucial parameters:

- Transglycosylation must be faster than glycoside hydrolysis
- The rate of hydrolysis of the product being slower than that of the glycosyl donor

In practice these conditions can be attained readily. It should be emphasized that an analogous situation can be found in enzymatic peptide synthesis using proteases (Sect. 3.1.4). The primary advantages of using glycosidases in comparison to glycosyl transferases is that expensive activated sugar nucleosides are not required and glycosidases generally are more readily available than glycosyl transferases. Furthermore, there is total control over the configuration at the newly generated anomeric center.

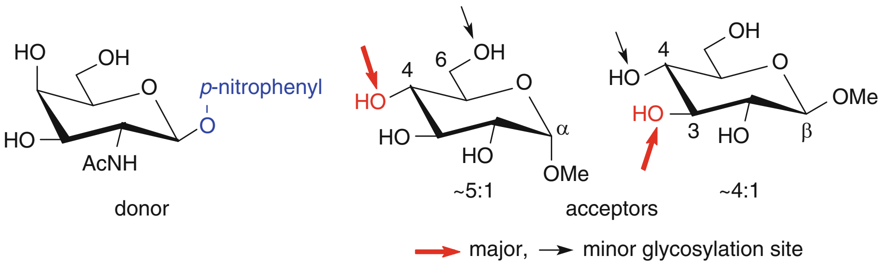
The major drawbacks, however, are low yields and the frequent formation of product mixtures due to the limited selectivity of glycosidases with respect to the glycosidic acceptor, in particular due to the formation of undesired 1,6-linkages. The regio- and stereoselectivity of transglycosylation reactions is influenced by a number of parameters such as reaction temperature [1686], concentration of organic cosolvent, the reactivity of the activated donor [1687], the nature of the aglycone [1688, 1689], and the anomeric configuration of the acceptor glycoside [1690] (Table 2.10).

Table 2.10 Transglycosylation catalyzed by glycosidases (Scheme 2.220)

Enzyme	Donor/glycoside	Acceptor/nucleophile	Product(s)
α-Galactosidase	α-Gal-*O*-*p*-Ph-NO_2	α-Gal-*O*-allyl	α-Gal-(1→3)-α-Gal-*O*-allyl
α-Galactosidase	α-Gal-*O*-*p*-Ph-NO_2	α-Gal-*O*-Me	α-Gal-(1→3)-α-Gal-*O*-Me[a]
α-Galactosidase	α-Gal-*O*-*p*-Ph-NO_2	β-Gal-*O*-Me	α-Gal-(1→6)-β-Gal-*O*-Me[b]
β-Galactosidase	β-Gal-*O*-*o*-Ph-NO_2	α-Gal-*O*-Me	β-Gal-(1→6)-α-Gal-*O*-Me
β-Galactosidase	β-Gal-*O*-*o*-Ph-NO_2	β-Gal-*O*-Me	β-Gal-(1→3)-β-Gal-*O*-Me[c]

[a]α-Gal-(1→6)-α-Gal-*O*-Me
[b]α-Gal-(1→3)-β-Gal-*O*-Me
[c]β-Gal-(1→6)-β-Gal-*O*-Me are formed in minor amounts

This latter fact has been used as a convenient tool to modulate the regioselectivity of glycosylation by switching the configuration at the anomeric center of the glycosidic acceptor. This technique has been commonly denoted as 'anomeric control' (Scheme 2.223).



Scheme 2.223 Anomeric control in *N*-acetylglucosaminyl transfer onto α- and β-D-methylglucosides by β-galactosidase

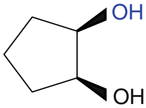
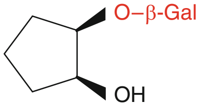
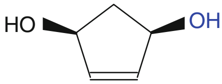
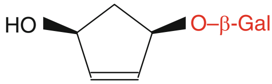
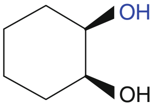
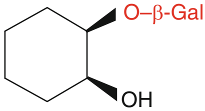
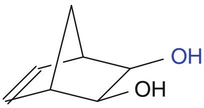
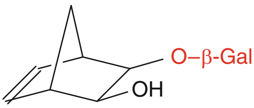
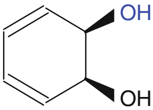
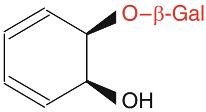
For instance, when the α-anomer of methyl-D-glucoside was used as acceptor and *p*-nitrophenyl-β-*N*-acetyl-D-galactosaminide as donor in a *trans*-glycosylation reaction catalyzed by β-galactosidase from *Aspergillus oryzae*, two transfer products possessing a 1,4- and 1,6-linkage were formed in a ratio of ~5:1, respectively. On the other hand, when using the β-anomer of the acceptor, the corresponding 1,3- and 1,4-glucosides were formed instead (ratio ~4:1) [1691].

Besides the synthesis of natural glycosides, a considerable number of nonnatural alcohols have been employed as nucleophiles in transglycosylation reactions (Tables 2.11 and 2.12) [1692, 1693]. The types of transformation include the desymmetrization of *meso*-diols and the kinetic resolution of racemic primary and secondary alcohols. In discussing enantioselection towards a (chiral) nonnatural acceptor, it should be kept in mind that the donor carbohydrate moiety is chiral and, as a consequence, the glycosylation products are *diastereomers* rather than enantiomers. In general, the stereocontrol during desymmetrization of prochiral or the kinetic resolution of racemic alcohols by glycosidases performs much worse than, e.g., lipases and alcohol dehydrogenases.

(Bi)cyclic *meso*-1,2- or 1,3-diols have been transformed into the corresponding monoglycosides in moderate to good diastereoselectivity using β-galactosidase from *Escherichia coli*, which is readily available from the dairy industry. As may be seen from Table 2.11, the selectivity strongly depends on the structure of the aglycone component [1694].

In some cases, the kinetic resolution of racemic primary and secondary alcohols was feasible. On the one hand, the enantioselectivity of glycosidases involving the glycosylation of primary alcohol moieties in 1,2-propanediol, glycerol or glycidol was negligible [1695, 1696], however, better results were obtained for *sec*-alcohols (Table 2.12). This fact is understandable if one considers the rules for chiral recognition for carboxyl ester hydrolases (Sects. 2.1.3.1 and 2.1.3.2),

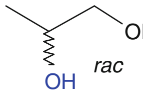
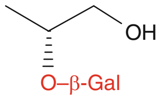
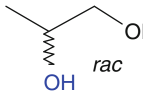
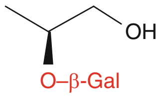
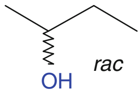
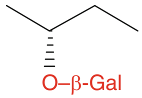
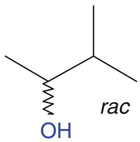
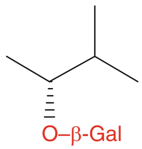
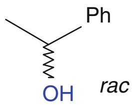
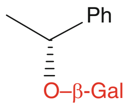
Table 2.11 Desymmetrization of cyclic *meso*-diols by glycosylation using β-galactosidase from *Escherichia coli*

Donor/glycoside	Acceptor/nucleophile	Product	d.e. (%)
β-Gal–O–Ph			96
β-Gal–O–Ph			50
β-Gal–O–Ph			90
β-Gal–O–Ph			75
Lactose			80

where the distance of the center of chirality to the point of reaction should be a minimum. Since the preferentially formed (diastereomeric) product is also the better substrate for (undesired) hydrolysis, the exact determination of the enantioselection in kinetic resolutions catalyzed by glycosidases is a complex task. As may be deduced from Table 2.12, the enantioselective glycosylation of short-chain *sec*-alcohols gave low to moderate d.e.'s. On the other hand, better results were obtained when both of the stereogenic groups significantly differed in size [1697–1699]. In recent years, a number of thermostable glycosidases have been identified and characterized. The most remarkable among them are the β-glucosidase [1700] and the β-galactosidase from the hyperthermophilic archean *Pyrococcus furiosus*.

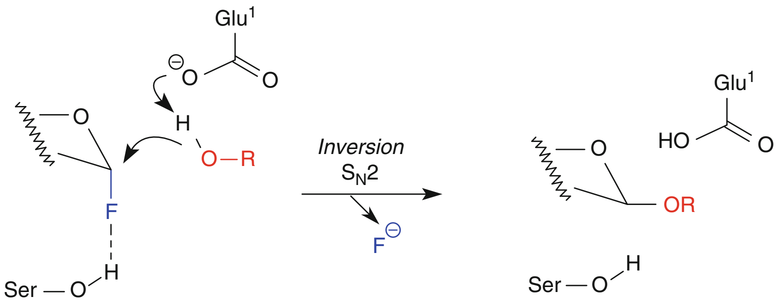
A major improvement in the use of glycosidases for glycoside synthesis was the rational re-design of the catalytic site to disable the undesired hydrolysis of the glycoside product, while maintaining glycoside synthesis activity (Scheme 2.224).

Table 2.12 Kinetic resolution of (±)-alcohols by glycosylation using β-galactosidase from *Escherichia coli*

Donor/glycoside	Acceptor/nucleophile	Product	d.e. (%)
Lactose			33
β-Gal–O–Ph			95[a]
β-Gal–O–C_6H_4–*o*-NO_2			64
β-Gal–O–C_6H_4–*o*-NO_2			80
β-Gal–O–C_6H_4–*o*-NO_2			98

[a]The β-galactosidase from *Sulfolobus solfataricus* was used

Replacement of the Glu[2]-residue acting as base in the native enzyme by a Ser residue allowed to bind an activated glycosyl fluoride as donor. The latter is attacked by the acceptor nucleophile, which is deprotonated by Glu[1], forming

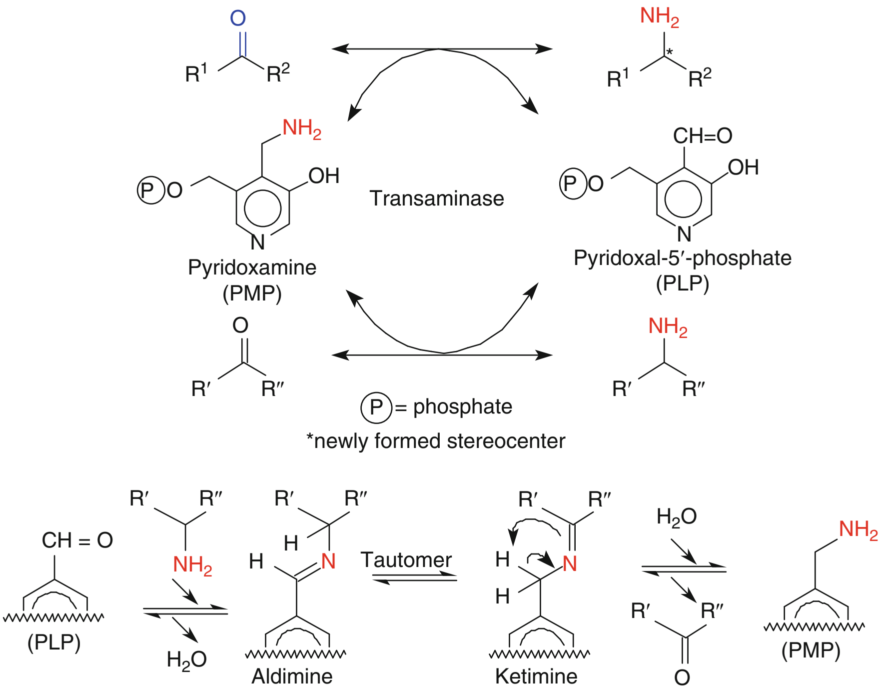


Scheme 2.224 Mechanistic principle of an inverting glycos*ynthase*

the glycoside product. In the native enzyme, the latter would undergo subsequent hydrolysis by a water molecule activated by Glu[2] but this is impossible in the Ser-mutant. Such active-site mutants of glycosidases (aptly denoted as 'glycosynthases' [1701–1706]) show greatly enhanced yields of glycosides due to the elimination of their undesired hydrolysis.

2.6.2 Amino Transfer Reactions

Transaminases (also termed amino transferases [EC 2.6.1.X]) catalyze the redox-neutral amino-transfer reaction between an amine donor and a carbonyl group serving as acceptor (Scheme 2.225) [94, 1707–1712]. These enzymes require an 'activated benzaldehyde' (pyridoxal-5′-phosphate, PLP, vitamin B_6) as cofactor, which functions as a molecular shuttle for the transfer of the NH_3-moiety. In a first step, PLP forms a ketimine Schiff base with the amine-donor. Tautomerization of the C=N bond yields an aldimine, which is hydrolyzed to yield the cofactor in its aminated form (pyridoxamine, PMP). The latter reacts through the same order of events with the carbonyl group of the substrate to form the amine product and



Scheme 2.225 Transaminase-catalyzed amino-transfer

regeneration of PLP. Since the tightly bound cofactor needs no external regeneration system, asymmetric transamination has the potential for large-scale synthesis of nonracemic amines.

Although transaminases were discovered already half a century ago [1713, 1714], their use for the biotransformation of (nonnatural) substrates was mainly impeded by two obstacles: The majority of transaminases available were only active on α-amino/α-ketoacids as substrates and techniques to shift the equilibrium towards amine formation had to be developed. The first significant advances in transamination for organic synthesis were achieved by Celgene Co., who employed transaminases for the preparation of nonracemic amines, preferentially via (less efficient) kinetic resolution of *rac*-amines via enantioselective de-amination [1715, 1716]. Within the last decade, several breakthroughs with respect to the (commercial) availability of stereo-complementary transaminases possessing a broad substrate spectrum and a set of techniques to shift the equilibrium of transamination in favor of amine synthesis were accomplished, which make enzymatic transamination nowadays a reliable technique for the asymmetric synthesis of amines.

On a genomic level, transaminases are classified into (up to six) subgroups [1717, 1718], among which group II comprises the most useful transaminases, which are able to accept non-α-amino acid type substrates. Since their natural substrates typically encompass ω-aminocarboxylic acids, such as ornithine, lysine, β-alanine, and ω-aminobutyrate, they are commonly denoted as ω-transaminases (ω-TA), in contrast α-transaminases, which only act on α-amino acid-type substrates.

In view to access both stereoisomers of a chiral amine via transamination by choice of an appropriate (*R*)- or (*S*)-selective ω-TA, screening studies were undertaken which revealed an impressive number of (*S*)-ω-TAs and some more rarely occurring (*R*)-selective enzymes [1719–1722]. The most widely used enzymes are obtained from *Vibrio fluvialis* [1723], *Chromobacterium violaceum* [1724, 1725], *Pseudomonas aeruginosa* [1726], *Bacillus megaterium* [1727], and *Alcaligenes denitrificans* [1728]. Thermostable mutants were derived from an ω-TA from *Arthrobacter citreus* [1729].

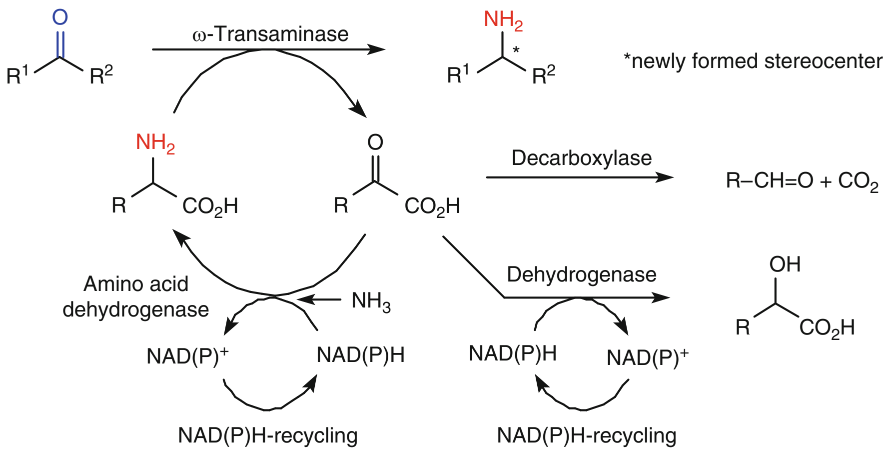
Depending on the substrate preference of the employed transaminase, the following couples of sacrificial amine donor/keto acceptors were used, which are often derived from the α-aminoacid pool (such as alanine/pyruvate, phenylalanine/phenylpyruvate, glutamic acid/α-ketoglutarate, aspartic acid/α-ketosuccinate) or constitute simple amines/ketones, such as 2-propylamine/acetone and 2-butylamine/2-butanone. It should be kept in mind that the absolute configuration of a chiral amine-donor has to match the stereospecificity of the ω-TA in order to be accepted.

In transamination, equilibrium constants are close to unity at best and the amino transfer from an α-amino acid to a ketone is strongly disfavored.[57] To even worsen

[57]The equilibrium constant between acetophenone and alanine was reported to be 8.8×10^{-4}, see [1729].

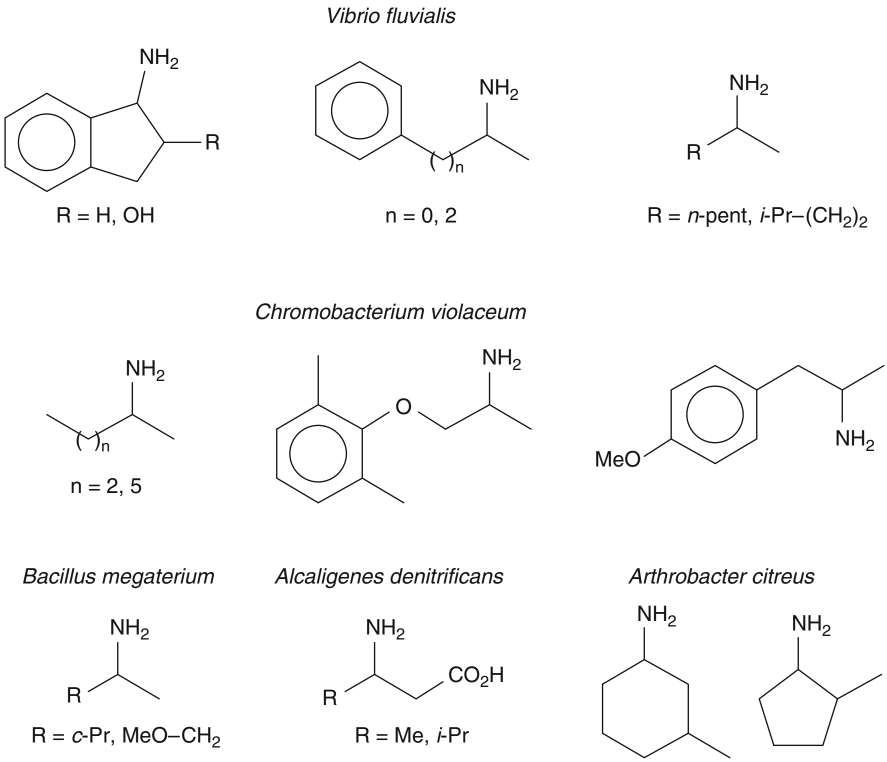
the situation, ω-TAs often show cosubstrate and/or coproduct inhibition at elevated concentrations, which prevents to push amine formation by employing an excess of amine donor. In order to solve this problem, several strategies have been developed (Scheme 2.226):

- The most simple approach is the physical removal of the coproduct ketone by evaporation. However, this is only feasible for simple amine-donors, such as 2-propylamine or 2-butylamine, which lead to highly volatile ketones [1730, 1731].
- The use of an amine donor, which forms an unstable keto coproduct. For instance, cysteinesulfinic acid was used in transamination to furnish the β-sulfinic acid analog of pyruvate, which spontaneously decomposes into SO_2 and pyruvate [1732]. In a related fashion, α,ω-diamino acids, such as ornithine or leucine yield amino-ketoacids, which (nonenzymatically) cyclize to the corresponding Δ^2-pyrroline-5-carboxylate and Δ^1-piperidine-2-carboxylate, respectively, as dead-end products [1733, 1734].
- Removal of the coproduct via an additional enzymatic step is usually more effective: For instance, decarboxylation of an α-ketoacid (e.g., pyruvate or phenylpyruvate, formed from alanine or phenylalanine, respectively) using pyruvate or phenylpyruvate decarboxylase, yields an aldehyde and CO_2 [1735, 1736]. In a similar fashion, pyruvate may be removed by condensation to acetoin going in hand with decarboxylation catalyzed by acetolactate synthase [1737].
- Carbonyl-reduction of the keto-coproduct by a suitable dehydrogenase, such as alcohol dehydrogenase or lactate dehydrogenase, yields the corresponding alcohol or α-hydroxyacid and requires NAD(P)H-recycling [1738].
- The most efficient approach is probably the direct recycling of alanine from pyruvate via NADH-dependent reduction in presence of ammonia catalyzed by alanine dehydrogenase. Overall, this sequence resembles a metal-free asymmetric reductive amination, which only requires ammonia and a low-cost reducing agent for NAD(P)H-recycling in molar amounts [1739].



Scheme 2.226 Shifting the equilibrium in transamination

The availability of a broad variety of ω-TAs together with efficient techniques to shift the equilibrium allows the biocatalytic synthesis of amines from the corresponding ketones via amino-group transfer. The potential of this protocol is demonstrated by a selection of amines, which can be obtained in nonracemic form by using the most prominent ω-transaminases (Scheme 2.227).



Scheme 2.227 Representative substrate spectrum of selected ω-transaminases

2.7 Halogenation and Dehalogenation Reactions

Halogen-containing compounds are not only produced by man, but also by Nature [1740–1742]. A brominated indole derivative – Tyrian purple dye – was isolated from the mollusc *Murex brandaris* by the Phoenicians. Since that time, more than 1,000 halogenated natural products of various structural types have been isolated from sources such as bacteria, fungi, algae, higher plants, marine molluscs, insects, and mammals [1743]. Whereas fluorinated and iodinated species are rather rare, chloro and bromo derivatives are found more often. The former are predominantly produced by terrestrial species [1744] and the latter in marine organisms [1745]. For instance, about 10^7 tons of bromoalkanes such as bromoform and methylene bromide are released from coastal brown algae *Ascophyllum nodosum* into the atmosphere worldwide [1746, 1747]. Although the natural function of halogenating enzymes is not yet known, they do seem to be involved in the defence mechanism of their hosts. For instance, some algae produce halometabolites, which makes them inedible to animals [1748]. In contrast to hydrolytic or redox enzymes, which have been investigated since about a century, halogen-converting biocatalysts have been a subject of research for only the last 30 years [1749–1754].

2.7.1 Halogenation

Although an impressive number of halometabolites have been identified, only a few type of halogenating enzymes have been characterized to date [1755–1758]: Flavin-dependent halogenases [1759], α-ketoglutarate-dependent nonheme iron-halogenases [1760] and haloperoxidases. Among them, the latter group showed the broadest substrate scope and thus had a dominant impact in biotransformations [1300, 1320, 1761–1763]. This type of redox enzyme is widely distributed in nature and is capable of carrying out a multitude of halogenation reactions following the general equation shown below, where X stands for halide (Cl^-, Br^- and I^-, but not F^-). For redox reactions catalyzed by haloperoxidases which do not involve a halide (such as hydroxylation, epoxidation, or sulfoxidation) see Sect. 2.3.3.

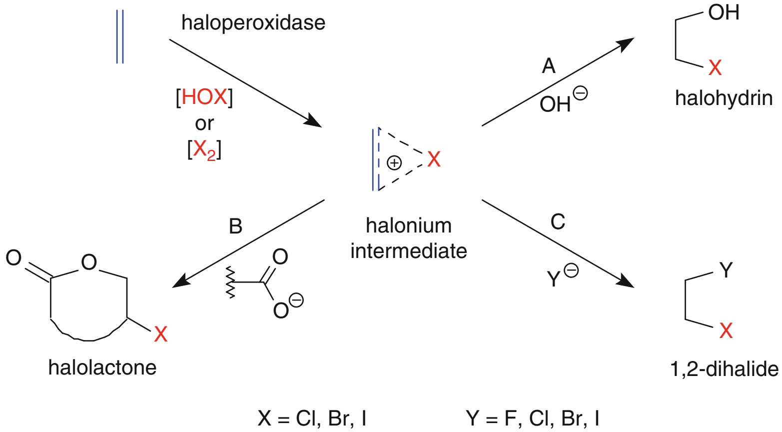
$$\text{Sub}{-}\text{H} + \text{H}_2\text{O}_2 + \text{X}^- + \text{H}^+ \xrightarrow[\text{X = Cl, Br, I}]{\text{Haloperoxidase}} \text{Sub}{-}\text{X} + 2\,\text{H}_2\text{O}$$

The individual enzymes are called chloro-, bromo-, and iodoperoxidase. The name reflects the smallest halide ion that they can oxidize, in correlation to the corresponding redox potential. Bearing in mind their unique position as halogenating enzymes and the large variety of structurally different halometabolites produced by them, it is not surprising that the majority of haloperoxidases are characterized by a low product selectivity and wide substrate tolerance. As a consequence, any asymmetric induction observed in haloperoxidase-catalyzed reactions is usually low.

The most intensively studied haloperoxidases are the chloroperoxidase from the mold *Caldariomyces fumago* [1322] and bromoperoxidases from algae [1764] and bacteria such as *Pseudomonas aureofaciens* [1765], *Ps. pyrrocinia* [1766], and *Streptomyces* sp. [1767]. The only iodoperoxidase of preparative use is isolated from horseradish root [1768]. A haloperoxidase isolated from milk has been reported to be useful for the formation of halohydrins [1769].

Halogenation of Alkenes

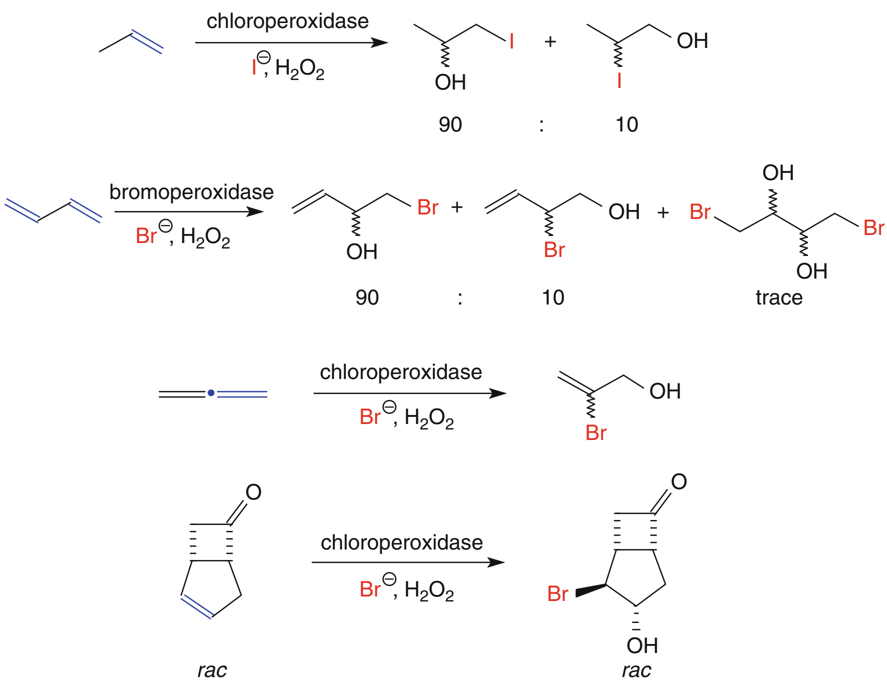
Haloperoxidases have been shown to transform alkenes by a formal addition of hypohalous acid to produce halohydrins. The reaction mechanism of enzymatic halogenation has been debated for some time and it is now accepted that it proceeds via a halonium intermediate [1770, 1771], similar to the chemical formation of halohydrins (Scheme 2.228). The former species is derived from hypohalous acid or molecular halogen, which is in turn produced by the enzyme via oxidation of halide [1772]. In support of this, a HOCl-adduct of Fe^{3+}-protoporphyrin IX was identified as a 'direct' enzyme-halogen intermediate involved in chloroperoxidase-catalyzed halogenation [1773].



Scheme 2.228 Haloperoxidase-catalyzed transformation of alkenes

Functional groups present in the alkene can lead to products other than the expected halohydrin by competing with hydroxyl anion (pathway A) for the halonium intermediate. Unsaturated carboxylic acids, for instance, are transformed into the corresponding halolactones due to the nucleophilicity of the carboxylate group (pathway B) affording a halolactonization [1774, 1775]. Similarly, if the concentration of halide ion in the reaction mixture is increased, 1,2-dihalides are formed (pathway C) [1776]. Although this latter transformation may primarily be regarded as an undesired side-reaction, it offers the unique possibility of introducing fluorine, which is not oxidized by haloperoxidases, into the substrate via an enzyme-catalyzed process. Furthermore, migration of functional groups such as halogen [1777] and loss of carbon-containing units such as acetate and formaldehyde may occur, particularly when an oxygen substituent is attached to the C=C bond [1778, 1779].

All types of carbon–carbon double bonds – isolated (e.g., propene), conjugated (e.g., butadiene) and cumulative (e.g., allene) – are reactive (Scheme 2.229) [1780]. The size of the substrate seems to be of little importance since steroids [1781] and

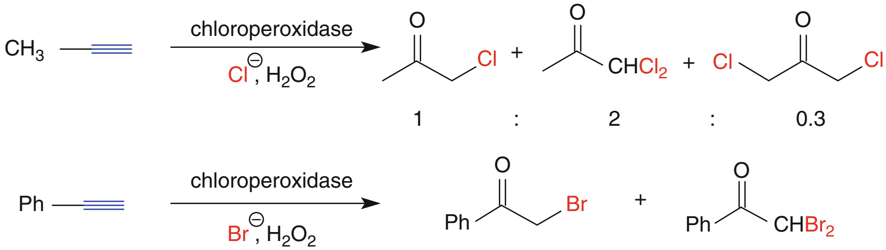


Scheme 2.229 Formation of halohydrins from alkenes

sterically demanding bicyclic alkenes [1782] are accepted equally well. Any regioselectivity observed reflects the (predominant) chemical and nonenzymatic nature of halohydrin formation.

Halogenation of Alkynes

With alkyne substrates, haloperoxidase-catalyzed reactions yield α-haloketones (Scheme 2.230) [1783]. As with alkenes, the product distribution in the reaction with alkynes is dependent on the halide ion concentration. Both homogeneous and

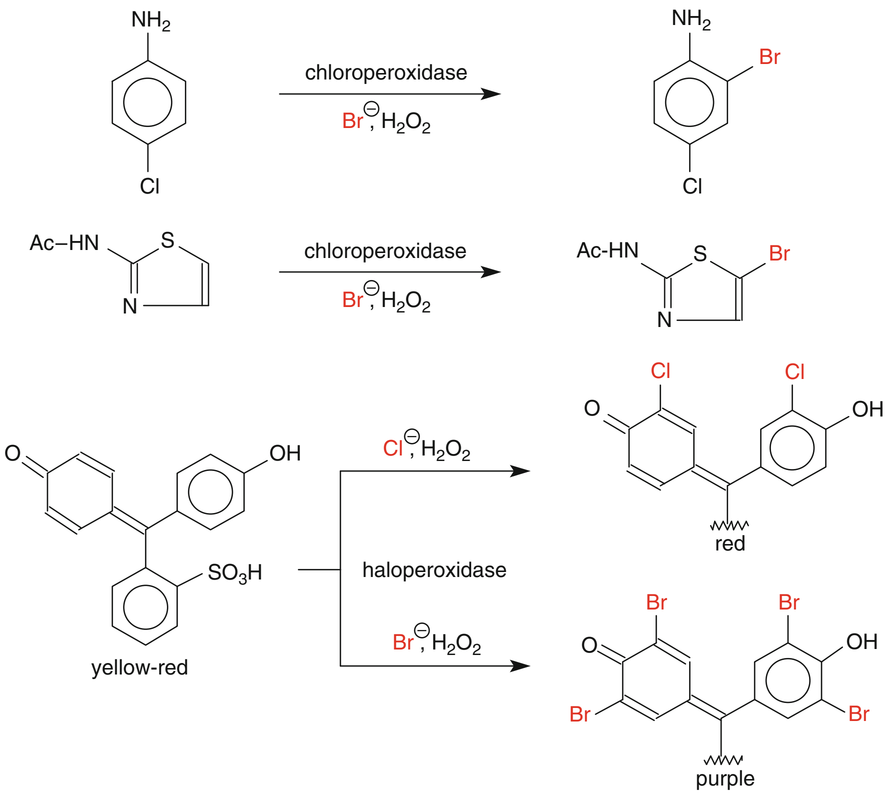


Scheme 2.230 Haloperoxidase-catalyzed reactions of alkynes

heterogeneous dihalides can be formed, dependent upon whether a single halide species or a mixture of halide ions are present.

Halogenation of Aromatic Compounds

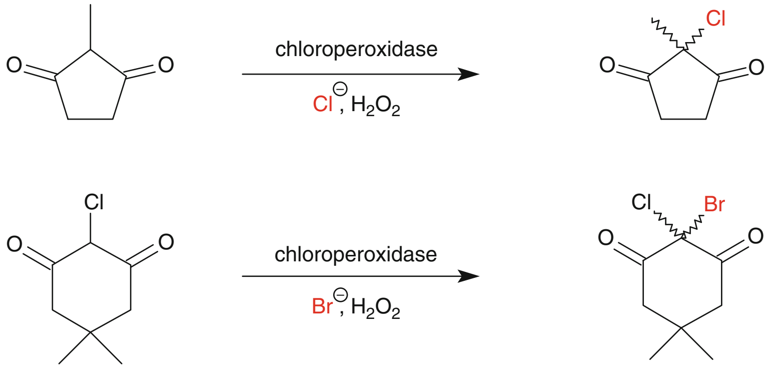
A wide range of electron-rich aromatic and heteroaromatic compounds are readily halogenated by haloperoxidases (Scheme 2.231) [1784–1786]. Bearing in mind the electrophilic character of the halogenating species, electron-rich phenols [1787, 1788] and anilines [1789] as well as their respective *O*- and *N*-alkyl derivatives are particularly well accepted. The color change of phenolic dyes such as phenol red or fluorescein upon halogenation serves as a simple assay for haloperoxidases [1790]. Since haloperoxidases are also peroxidases, they also can catalyze halide-independent peroxidation reactions of aromatics (see Sect. 2.3.3). Thus, dimerization, polymerization, oxygen insertion and de-alkylation reactions are encountered as undesired side-reactions, particularly whenever the halide ion is omitted or depleted from the reaction mixture.



Scheme 2.231 Halogenation of aromatic compounds

Halogenation of C–H Groups

Similar to the chemical process, halogenation of C–H groups is only possible if they are appropriately activated by adjacent electron-withdrawing substituents, for example carbonyl groups, which facilitate enolization. Since the reactivity seems to be a function of the enol content of the substrate, simple ketones like 2-heptanone are unreactive [1791], but highly enolized 1,3-diketones are readily halogenated to give the corresponding 2-mono- or 2,2-dihalo derivatives (Scheme 2.232) [1792]. As with the formation of halohydrins from alkenes, selectivities are low and the reactivity of the substrate is independent of its size. For example, monocyclic compounds such as barbituric acid [1793] and sterically demanding polycyclic steroids are equally well accepted [1794]. β-Ketoacids are also halogenated, but the spontaneous decarboxylation of the intermediate α-halo-β-ketoacid affords the corresponding α-haloketones [1795]. The chloroperoxidase-catalyzed halogenation of oximes was shown to proceed via a two-step sequence via a halonitroso intermediate which is further oxidized to furnish a α-halonitro product [1796].



Scheme 2.232 Halogenation of electronically activated C–H groups

Halogenation of N- and S-Atoms

Amines are halogenated by haloperoxidases to form unstable haloamines, which readily deaminate or decarboxylate, liberating the halogen [1797]. This pathway constitutes a part of the natural mammalian defence system against microorganisms, parasites and, perhaps, tumor cells. However, it is of no synthetic use. In an analogous fashion, thiols are oxidized to yield the corresponding sulfenyl halides. These highly reactive species are prone to undergo nucleophilic attack by hydroxyl ion or by excess thiol [1798, 1799]. As a result, sulfenic acids or disulfides are formed, respectively.

In view of the predominant chemical nature of biohalogenation, it seems that enzymatic halogenation reactions involving haloperoxidase enzymes do not show any significant advantage over the usual chemical reactions due to their lack of regio- and stereoselectivity. A benefit, however, lies in the mild reaction conditions employed.

2.7.2 Dehalogenation

The concentrations of haloorganic compounds in the ecosphere has remained reasonably constant due to the establishment of an equilibrium between biosynthesis and biodegradation. Due to man's recent activities, a large number of halogen-containing compounds – most of which are recalcitrant – are liberated either by intent (e.g., insecticides), or because of poor practice (lead scavengers in gasoline) or through abuse (dumping of waste) into the ecosystem. These halogenated compounds would rapidly pollute the earth if there were no microbial dehalogenation pathways [1800, 1801]. Five major pathways for enzymatic degradation of halogenated compounds have been discovered (Table 2.13) [1802–1805].

Table 2.13 Major biodegradation pathways of halogenated compounds

Reaction type	Starting material		Products
Reductive dehalogenation	C–X	→	C–H + X^-
Oxidative degradation	H–C–X	→	C=O + HX
Dehydrohalogenation	H–C–C–X	→	C=C + HX
Epoxide formation	HO–C–C–X	→	epoxide + HX
Hydrolysis	C–X + H_2O	→	C–OH + HX

X = Cl, Br, I

Redox enzymes are responsible for the replacement of the halogen by a hydrogen atom via reductive dehalogenation [1806, 1807] and for oxidative degradation [1808]. Elimination of hydrogen halide leads to the formation of an alkene [1809], which is further degraded by oxidation. Since all of these pathways proceed either with a loss of a functional group or through removal of a chirality center, they are of little use for the biocatalytic synthesis of organic compounds. On the other hand, the enzyme-catalyzed formation of an epoxide from a halohydrin and the hydrolytic replacement of a halide by a hydroxyl functionality take place in a stereocontrolled fashion and are therefore of synthetic interest.

Dehalogenases

Hydrolytic dehalogenation catalyzed by dehalogenases (or 'halido-hydrolases') proceeds by formal nucleophilic substitution of the halogen atom with a hydroxyl ion [1810, 1811]. Neither cofactors nor metal ions are required for the enzymatic activity. Depending on the enzyme source, the reaction may either proceed with *retention* or *inversion* of configuration.[58] It is this stereospecificity which makes

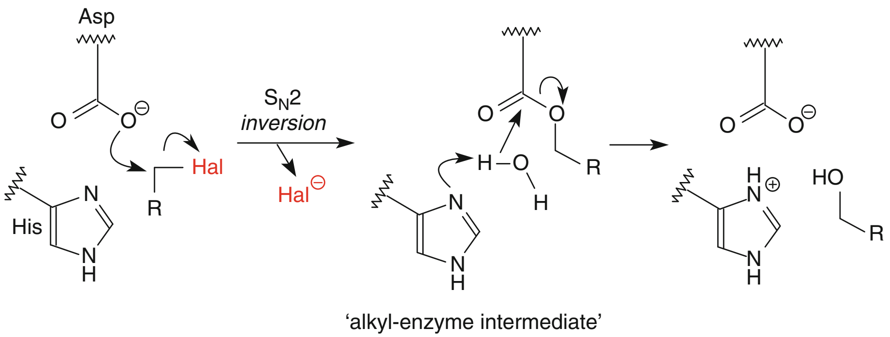
[58]For an example exhibiting a retention of configuration see [1811].

them attractive for synthetic purposes. To date, the only types of dehalogenases being of importance for preparative biotransformations due to their stereospecificity are those acting on α-haloacids (Table 2.14).

Table 2.14 Specificities of dehalogenases

Substrate	Enzyme subtype	Specificity
Alkyl-halide	Haloalkane dehalogenase	Low
Aryl-halide	Haloaromatic dehalogenase	Low
α-Haloacid	α-Haloacid dehalogenase	High

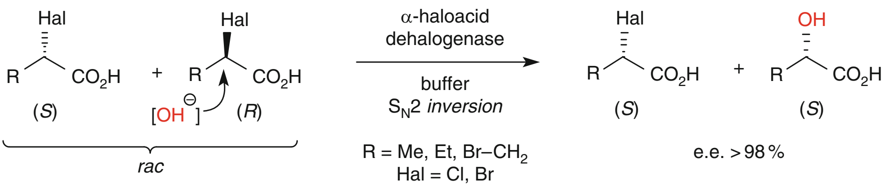
The mechanism of haloalkane dehalogenase acting with *inversion* of configuration was shown to have close similarities to that of epoxide hydrolases (Sect. 2.1.5) and retaining glycosidases (Sect. 2.6.2). Thus, the carboxyl moiety of an aspartate residue attacks the halide by forming an 'alkyl-enzyme intermediate' (Scheme 2.233). Being a carboxyl ester, the latter is hydrolyzed by a hydroxyl ion which is provided from water by the aid of a histidine [570, 1813]. On the other hand, α-haloacid dehalogenases acting with *retention* of configuration were shown to possess a catalytically active sulfhydryl residue.



Scheme 2.233 Mechanism of inverting haloalkane dehalogenases

Two sub-types of α-haloacid dehalogenases have been characterized which are classified according to their preferred type of substrate: *2-Haloacid dehalogenase* is able to accept all kinds of short-chain 2-haloacids [1814, 1815], while *haloacetate dehalogenase* exclusively acts on haloacetates [1816, 1817]. Both of them are inactive on nonactivated isolated halides. Interestingly, the reactivity of organic halides (fluoride through iodide derivatives) in dehalogenase reactions depends on the source of enzyme. In some cases it increases from iodine to fluorine derivatives, which is in sharp contrast to the corresponding chemical reactivity with nucleophiles such as hydroxyl ion [1818]. The most intriguing aspect of dehalogenases is their enantiospecificity [1819]. Depending on the growth conditions, the microbial production of (*R*)- or (*S*)-specific enzymes may be induced [1820, 1821]. This makes the stereoselective hydrolysis of α-haloacids to give the corresponding α-hydroxyacids possible [1822].

(*S*)-2-Chloropropionic acid is a key chiral synthon required for the synthesis of a range of important α-aryl- and α-aryloxypropionic acids used as anti-inflammatory agents and herbicides (Scheme 2.38). Several attempts to resolve racemic 2-chloropropionic acid via enzymatic methods using 'classic' hydrolases have been reported to proceed with varying degrees of selectivity [1823]. An elegant approach makes use of an (*R*)-specific dehalogenase enzyme from *Pseudomonas putida* NCIMB 12018 (Scheme 2.234) [1824–1826]. Thus, from a racemic mixture of α-haloacid, the (*R*)-enantiomer is converted into the (*S*)-hydroxyacid product via *inversion* of configuration leaving the (*S*)-α-haloacid behind. Some minor structural variations of the substrate are tolerated. This process has been scaled-up to industrial production at a capacity of 2,000 t/year [1827].



Scheme 2.234 Resolution of 2-chloropropanoic acid derivatives by 2-haloacid dehalogenase

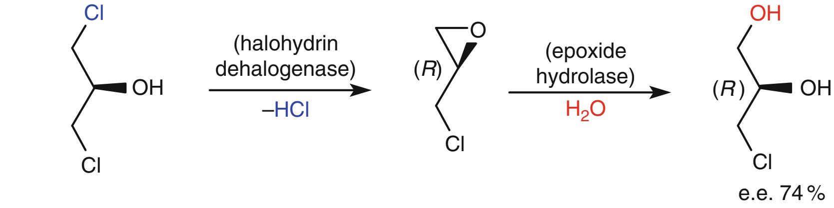
Two drawbacks of α-haloacid dehalogenation – i.e., the hydrolytic instability of α-bromoacids in aqueous solvent systems and the limited substrate tolerance of α-haloacid dehalogenases – can be overcome by using anhydrous organic solvents [1828]. Thus, long-chain α-haloacids (which were not accepted as substrates in water) were successfully transformed with good specificity in toluene, acetone or even in dimethyl sulfoxide.

Halohydrin Dehalogenases

The biodegradation of halohydrins proceeds through a two-step mechanism involving epoxide-formation catalysed by halohydrin dehalogenases [EC 3.8.1.5],[59] followed by epoxide hydrolase-mediated formation of *vic*-diols (Sect. 2.1.5), which are oxidatively degraded. A number of organisms possessing halohydrin dehalogenase and epoxide hydrolase activity, respectively, were found among bacteria (*Flavo-* [1830, 1831], *Corynebacteria* [1832], *Arthrobacter erithrii* [1833, 1834], *Pseudomonas* sp. [1835]), fungi (*Caldariomyces fumago*), and algae (*Laurencia pacifica*).

[59] Previously, halohydrin dehalogenases were also termed 'haloalcohol dehalogenases' or 'halohydrin epoxidases', see [1828].

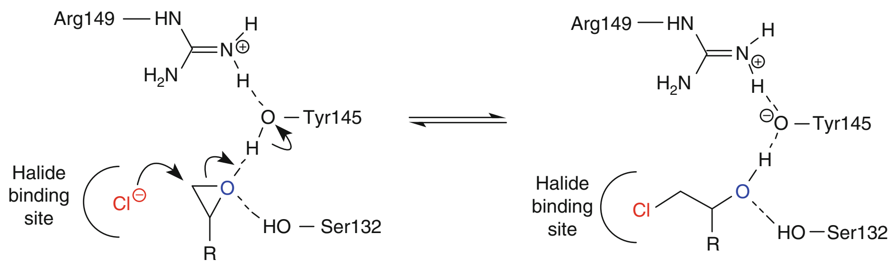
First hints on the stereoselectivity of halohydrin dehalogenases were obtained from studies on the desymmetrization of prochiral 1,3-dichloropropan-2-ol yielding epichlorohydrin using resting cells of *Corynebacterium* sp. (Scheme 2.235) [1836]. In two-step sequence, (*R*)-3-chloropropane-1,2-diol was formed in 74% e.e. via epichlorohydrin through the sequential action of an (unspecified) halohydrin dehalogenase and an epoxide hydrolase [1837]. Further studies revealed that these activities are widespread among bacteria [1838–1842].



Scheme 2.235 Asymmetric microbial degradation of prochiral halohydrin by a *Corynebacterium* sp.

A breakthrough was achieved by cloning and overexpression of halohydrin dehalogenases from *Agrobacterium radiobacter*, which allowed the preparative-scale application of these enzymes under well-defined conditions [1843].

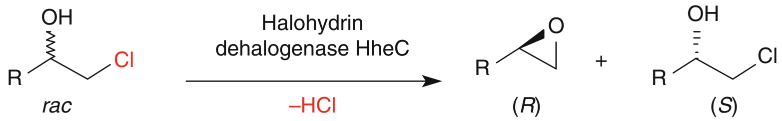
The mechanism of halohydrin dehalogenase was shown to proceed in a reversible fashion via a push-pull-like nucleophilic attack of halide (provided by a lipophilic halide binding site) with simultaneous activation of the epoxide through protonation by a Tyr residue (Scheme 2.236) [1844, 1845].



Scheme 2.236 Catalytic mechanism of halohydrin dehalogenase from *Agrobacterium radiobacter*

Using pure halohydrin dehalogenase (HheC), competing activities observed in whole-cell preparations were eliminated and halohydrins could be resolved via enantioselective ring-closure with excellent enantioselectivities yielding enantiomeric epoxides and nonreacted halohydrins (Scheme 2.237) [1846].

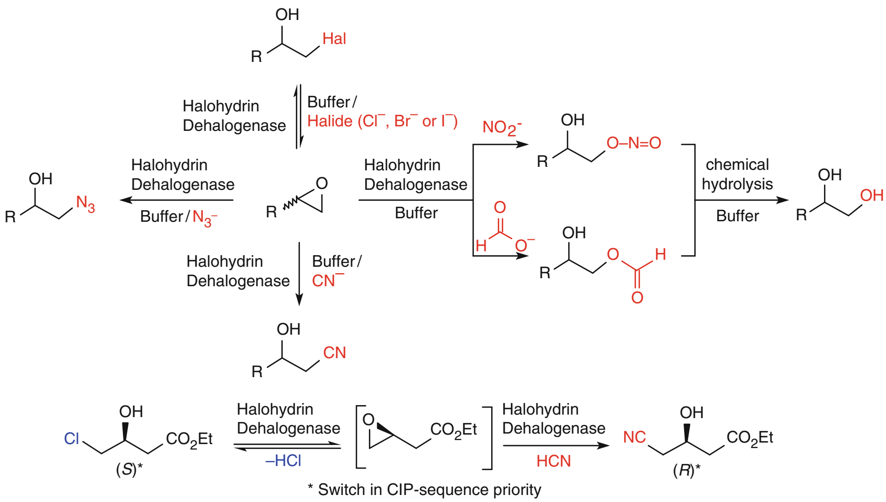
Subsequent studies revealed that the natural nucleophile halide (Cl, Br, I) could be replaced by nonnatural analogs, such as azide [1847], nitrite [1848], cyanide [1849],



R	Enantioselectivity (*E*)
(*E*)–Me–CH=CH–	>200
(*E*)-Et–CH=CH–	177
(*E*)-*n*-C_4H_9–CH=CH–	>200
(*E*)-Ph–CH=CH–	>200
2-Furyl	>200
2-Thiophenyl	65

Scheme 2.237 Kinetic resolution of halohydrins using halohydrin dehalogenase

(thio)cyanate and formate by maintining the exquisite regioselectivity of nucleophilic attack at the less hindered oxirane carbon atom. Whereas the reaction rates observed with cyanide, (thio)cyanate, and formate were comparable to those using halide, azide and nitrite proved to be much better nucleophiles [1850]. Nonlinear and nonanionic nucleophiles, such as H_2S, acetate, PO_4^{3-}, CO_3^{2-}, BO_3^{3-}, and F^- were unreactive. The use of *N*-nucleophiles opened the way to prepare 1,2- and 1,3-aminoalcohols using azide or cyanide via the corresponding 1-azido-2-ols and 1-cyano-2-ols, respectively (Scheme 2.238).



Scheme 2.238 Regio- and enantioselective ring-opening of epoxides using nonnatural nucleophiles catalysed by halohydrin dehalogenase

The *mono*-nitrite (or formate) esters of *vic*-diols obtained via enzymatic ring-opening of epoxides in presence of nitrite (or formate) are unstable and undergo spontaneous (nonenzymatic) hydrolysis to furnish the corresponding diols. This protocol offers a useful complement to the asymmetric hydrolysis of epoxides. Depending on the type of substrate and the enzymes used, enantio-complementary epoxide hydrolysis can be achieved [1851].

A one-pot two-step transformation of ethyl (*S*)-4-chloro-3-hydroxybutanoate (obtained via asymmetric bioreduction of the corresponding β-ketoester) via (reversible) epoxide-formation followed by ring-opening with cyanide was accomplished on a kg-scale using a halohydrin dehalogenase mutant. Ethyl (*R*)-4-cyano-4-butanoate was thus obtained in a highly chemoselective fashion without formation of byproducts, which plagued the chemical process. The latter product is a key intermediate for the synthesis of antihypocholesterolemic 'statin' agents [1852] (Scheme 2.238).

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Chapter 3
Special Techniques

Most biocatalysts can be used in a straightforward manner by regarding them as chiral catalysts and by applying standard methodology, i.e., in buffered aqueous solution. In order to broaden the applicability of enzymes, some special techniques have been developed. In particular, using biocatalysts in *nonaqueous media* rather than in water can lead to the gain of some significant advantages as long as some specific guidelines are followed [1]. Furthermore, 'fixation' of the enzyme by immobilization may be necessary, and the use of membrane technology may be advantageous as well. For both of the latter topics only the most simple techniques which can be adopted in an average organic chemistry laboratory are discussed (Sect. 3.2).

3.1 Enzymes in Organic Solvents

Water is a poor solvent for nearly all reactions in preparative organic chemistry because most organic compounds are insoluble in this medium. Furthermore, the removal of water is tedious and expensive due to its high boiling point and high heat of vaporization. Side-reactions such as hydrolysis, racemization, polymerization, and decomposition are often facilitated in the presence of water. These limitations were circumvented long ago by the introduction of organic solvents for the majority of organic chemical processes. On the other hand, conventional biocatalysis has mainly been performed in aqueous solutions due to the perceived notion that enzymes are most active in water and it has been tacitly assumed that organic solvents only serve to destroy their catalytic power. However, this commonly held opinion is certainly too simplistic, bearing in mind that in nature many enzymes or multienzyme complexes function in hydrophobic environments, for instance, in the presence of, or bound onto, a membrane [2]. Therefore it should not be surprising that enzymes can be catalytically active in the presence of organic solvents [3–12]. The role of water in functioning biological systems is contradictory. On the one hand, the enzyme depends on water for the majority of the noncovalent interactions that help to maintain its catalytically active conformation [13] but water also participates in most of the reactions which lead to denaturation. As a consequence,

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it may be anticipated that replacing *some* (but not *all*) of the water with an organic solvent would retain enzymatic activity. Equally, completely anhydrous solvents are incapable of supporting enzymatic activity because *some* water is always necessary for catalysis. The crucial answer to the question concerning *how much* water is required to retain catalytic activity is enzyme-dependent [14]. For example, α-chymotrypsin needs only 50 molecules of water per enzyme molecule to remain catalytically active [15], which is much less than is needed to form a monolayer of water around the enzyme. Other enzymes, like subtilisin and various lipases are similar in their need for trace quantities of water [16]. In other cases, however, much more water is required. Polyphenol oxidase, for instance, prefers a rather 'wet' environment and requires the presence of about 3.5×10^7 molecules of water [17].

The water present in a biological system can be separated into two physically distinct categories [18–20]. Whereas the majority of the water (>98%) serves as a true solvent ('bulk water'), a small fraction of it is tightly bound to the enzyme's surface ('bound water'). The physical state of bound water – as monitored by differences in melting point, heat capacity, EPR- and IR-spectroscopical properties – is clearly distinct from the bulk water and it should be regarded as a crucial integral part of the enzyme's structure rather than as adventitious residual solvent. Thus, bound water is also often referred to as 'structural water'. For a picture displaying the crystal structure of *Candida antarctica* lipase B with (and without) structural water molecules see Sect. 1.4, Fig. 1.1. If one extends this concept to enzymatic catalysis in organic media, it should be possible to replace the *bulk water* by an organic solvent without significant alteration of the enzyme's environment, as long as the *structural water* remains unaffected.

Biocatalytic transformations performed in organic media offer the following advantages:

- The overall yields of processes performed in organic media are usually better due to the omission of an extractive step during work-up. Thus, the loss-causing formation of emulsions can be avoided and the recovery of product(s) is facilitated by the use of low-boiling organic solvents.
- Nonpolar substrates are transformed at better rates due to their increased solubility [21].
- Since an organic medium is a hostile environment for living cells, microbial contamination is negligible. This is particularly important for reactions on an industrial scale, where maintaining sterility may be a serious problem.
- Deactivation and/or inhibition of the enzyme caused by lipophilic substrates and/or products is minimized since their solubility in the organic medium leads to a reduced local concentration at the enzyme's surface.
- Many side-reactions such as hydrolysis of labile groups (e.g., epoxides, acid anhydrides [22]), polymerization of quinones [17], racemization of cyanohydrins [23], or acyl migration [24] are water-dependent and are therefore largely suppressed in an organic medium.
- Immobilization of enzymes is not necessary because they may be recovered by simple filtration after the reaction due to their insolubility in organic solvents. Nevertheless, if it is desired, experimentally simple adsorption onto the surface

of a cheap macroscopic carrier such as diatomaceous earth (Celite), silica, or glass beads is possible. Desorption from the carrier into the medium – 'leaking' – is largely impeded in a lipophilic environment.

- Since many of the reactions which are responsible for the denaturation of enzymes (see Sect. 1.4) are hydrolytic reactions and therefore require water, it can be expected that enzymes should be more stable in an environment of low water content [25]. For instance, porcine pancreatic lipase is active for many hours at 100°C in a 99% organic medium but it is rapidly denaturated at this temperature when placed in pure water [26].
- Due to the conformational change (i.e., a partial unfolding and refolding) of the enzyme during the formation of the enzyme–substrate complex (the 'induced-fit'), numerous hydrogen bonds are reversibly broken and reformed. This process is greatly facilitated in an aqueous medium, which ensures that the broken bonds are rapidly replaced by hydrogen bonds to the surrounding water. Thus, it serves as a 'molecular lubricant' [27]. On the other hand, this process is impeded in an organic solvent and, as a consequence, enzymes appear to be there more 'rigid' [28]. Thus, it is often possible to control some of the enzyme's catalytic properties such as the substrate specificity [15, 29–31], the chemo- [32], regio- [33] and enantioselectivity [34–37] by variation of the solvent (Sect. 3.7.1).
- The most important advantage, however, is the possibility of shifting thermodynamic equilibria to favor *synthesis* over *hydrolysis*. Thus, by using hydrolase enzymes (mainly lipases and proteases) esters [38–40], polyesters [41, 42], lactones [43, 44], amides [35, 45], and peptides [46] can be *synthesized* in a chemo-, regio-, and enantioselective manner.

The solvent systems which have commonly been used for enzyme-catalyzed reactions containing organic media can be classified into three different categories.

Enzyme Dissolved in a Monophasic Aqueous-Organic Solution

The enzyme, the substrate and/or product are dissolved in a monophasic solution consisting of water and a *water-miscible* organic cosolvent, such as dimethyl sulfoxide, dimethyl formamide, tetrahydrofuran, dioxane, acetone or one of the lower alcohols, e.g., methanol or *tert*-butanol. Systems of this type are mainly used for the transformation of lipophilic substrates, which are sparingly soluble in an aqueous system alone and which would therefore react at low reaction rates. In some cases, selectivities of esterases and proteases may be enhanced by using water-miscible organic cosolvents (see Sect. 2.1.3.1). As a rule of thumb, most water-miscible solvents can be applied in concentrations up to ~10% of the total volume, only in some rare enzyme/solvent combinations even 50–70% of cosolvent may be used. If the proportion of the organic solvent exceeds a certain threshold, the essential structural water is stripped from the enzyme's surface leading to deactivation. Only rarely do enzymes remain catalytically active in water-miscible organic solvents with an extremely low water content; these cases are limited to unusually stable enzymes such as subtilisin and some lipases, e.g., from *Candida* *antarctica* [47–49]. Water-miscible organic solvents have also been successfully used to decrease the freezing temperature of aqueous systems when biocatalytic reactions were conducted at temperatures below 0°C ('cryoenzymology') [50–53].

Enzyme Dissolved in a Biphasic Aqueous-Organic Solution

Reaction systems consisting of two macroscopic phases, namely the aqueous phase containing the dissolved enzyme, and a second phase of a nonpolar organic solvent (preferably lipophilic and of high molecular weight) such as (chlorinated) hydrocarbons, aromatics or ethers, may be advantageous to achieve a spatial separation of the biocatalyst from the organic phase [54–56]. Thus, the biocatalyst is in a favorable aqueous environment and not in direct contact with the organic solvent, where most of the substrate/product is located. Therefore, the limited concentrations of organic material in the aqueous phase may circumvent inhibition phenomena. Furthermore, the removal of product from the enzyme surface drives the reaction towards completion. Due to the fact that in such biphasic systems the enzymatic reaction proceeds only in the aqueous phase, a sufficient mass transfer of the reactant(s) to and product(s) from the catalyst and between the two phases is necessary [57]. It is obvious that shaking or stirring represents a crucial parameter in such systems.

The number of phase distributions encountered in a given reaction depends on the number of reactants and products (A, B, C, D) which are involved in the transformation (Table 3.1). Each distribution, measured as the partition coefficient, is dependent on the solubilities of substrate(s) and product(s) in the two phases and this represents a potential rate-limiting factor.

Table 3.1 Partition coefficients involved in biphasic reactions

Type of reaction	Number of partition coefficients
$A \rightarrow B$	3
$A + B \rightarrow C$	4
$A \rightarrow B + C$	4
$A + B \rightarrow C + D$	7
Any type[a]	1

[a] for monophasic systems

Therefore, in biphasic systems the partition coefficient (a *thermodynamic* dimension) and the mass-transfer coefficient (a *kinetic* dimension) will dominate the k_{cat} of the enzyme. As a consequence, the overall reaction rate is mainly determined by the physical properties of the system (such as solubilities and stirring) and only to a lesser extent by the enzyme's catalytic power. In other words, the enzyme could work faster, but is unable to get enough substrate. Enhanced agitation (stirring, shaking) would improve the mass transfer but, on the other hand, it increasingly leads to deactivation of the enzyme due to mechanical shear and chemical stress.

Despite these problems, water-organic solvent two-phase systems have been successfully used to transform highly lipophilic substrates such as steroids [58], fats [59], and alkenes [60]. For instance, the use of biphasic solvent systems was shown to be crucial for the asymmetric epoxidation of alkenes (Sect. 2.3.2.3). In this way, the toxic effects of the epoxide produced by *Nocardia corallina* cells were minimized via extraction into a lipophilic organic solvent [61].

Enzyme Suspended in a Monophasic Organic Solution
Replacing all of the bulk water (which accounts for >98%) by a *water-immiscible* organic solvent leads to a suspension of the solid enzyme in a monophasic organic solution [62, 63]. Although the biocatalyst seems to be 'dry' on a macroscopic level, it must have the necessary residual structural water to remain catalytically active. Most of the research on such systems (which have proved to be extremely reliable, versatile and easy to use) has been performed during the 1980s, but it is striking that the first biotransformation of this kind was already reported in 1900! [64]. Due to the importance of this technique and its simplicity, all of the examples discussed below have been performed using solid 'dry' enzymes in organic solvents having a water content of $< 2\%$. However, it should be kept in mind that the catalytic activity of enzymes in nonconventional solvent systems generally is lower than the activity in water [65]. Overall, enzymes are insoluble in organic media and thus such reactions resemble a heterogeneous catalytic system.

The remarkable catalytic activity of solid proteins in organic solvents can be explained by their special properties [66]. In contrast to densely packed crystals of organic compounds of comparatively low molecular weight, which form rather dense and impenetrable structures, solid proteins represent soft and delicate aggregates. Due to the limited contact between the single protein molecules, there is sufficient mobility within the single enzyme unit to permit minor conformational changes consonant with formation of the enzyme–substrate complex [67]. The total surface of solid enzymes is within the range of $1–3 \times 10^6$ m^2/kg, which is close to that of silica or activated carbon. About one to two thirds of the total volume is hollow, with numerous solvent-filled cavities and channels running through a 'sponge-like' macroscopic aggregate. Thus, if sufficient agitation is provided, the substrate is not only transformed by the active sites exposed to the surface of the crystal but also at those buried inside. In order to tune a biocatalytic reaction in a monophasic organic solvent system, the following parameters should be considered [68, 69]:

pH-Memory. One particularly important aspect is the effect of the pH of the reaction medium. In organic solutions that lack a distinct aqueous phase, pH cannot be measured easily [70]. On the other hand, the ionization state of the enzyme which is a function of the pH, determines its conformation and hence its properties such as activity and selectivity. Since the ionization state of the charged groups of a protein does not change when placed in an organic solvent and thus remains 'frozen', it is important to employ solid enzymes that have been recovered by lyophilization or precipitation from a buffer at their pH optimum [71]. The latter fact has vividly been described as the 'pH-memory' of enzymes.

Enzyme State. The physical state of the enzyme may be crystalline, lyophilized or precipitated. Adsorption of enzymes onto the surface of a macroscopic (inorganic or organic) carrier material generates a better distribution of the biocatalyst and generally gives significantly enhanced reaction rates, in some cases up to one order of magnitude [72]. Any inorganic material such as Celite, silica gel or an organic nonionic support (e.g., XAD-8, Accurel [73]) may be used as the carrier.

Biocompatibility of Organic Solvent. In order to provide a measure for the 'compatibility' of an organic solvent with high enzyme activity, many parameters describing the hydrophobicity of the solvent, such as the Hildebrandt solubility parameter (δ), the Reichardt–Dimroth polarity parameter (ET), the dielectric constant (ε), and the dipole moment (μ), have been proposed [74, 75]. However, the most reliable results were obtained by using the logarithm of the partition coefficient (log *P*) of a given solvent between 1-octanol and water (Table 3.2) [76]. Although the effects of organic solvents on enzyme *stability* can be predicted with reasonably accuracy, the effects on enzyme (*stereo*)*selectivity* are only poorly understood and reliable predictions cannot be made [77].

Table 3.2 Biocompatibility of organic solvents

log *P*	Water-miscibility	Solvent effects on enzyme activity
−2.5 to 0	Completely miscible	May only be used to solubilize lipophilic substrates in concentrations of 10–20% v/v without deactivating the enzyme
0–1.5	Partially miscible	Causes serious enzyme distortion, may only be used with unusually stable enzymes[a] but deactivation is common for average proteins at elevated concentrations
1.5–2.0	Low miscibility	Causes some enzyme distortion, may be used with many enzymes but activities are often unpredictable
>2.0	Immiscible	Causes negligible enzyme distortion and ensures high retention of activity for almost all enzymes

[a]For instance, subtilisin and *Candida antarctica* lipase B

If the log *P* value is not available in the literature, it can be calculated from hydrophobic fragmental constants [78]. As may be deduced from the log *P* values of some selected common organic solvents (Table 3.3), *water-miscible* hydrophilic solvents such as DMF, DMSO, acetone, and lower alcohols cannot be used as 'neat' organic solvents, whereas *water-immiscible* lipophilic solvents such as (halo) alkanes, ethers and aromatics retain an enzyme's high catalytic activity. Only in certain cases, in which polar substrates such as polyhydroxy compounds and amino acid derivatives have to be dissolved, should water-miscible solvents such as dioxane, tetrahydrofuran, 3-methyl-3-pentanol, or pyridine be considered. However, in these solvents, most enzymes are deactivated and only exceptionally stable enzymes (for instance, subtilisin and *Candida antarctica* lipase B) can be used. It is an empirical phenomenon, that ball-shaped (round) organic solvents (e.g., *di-iso*propyl ether) often display higher biocompatibility than the corresponding straight-chain analog (e.g., *di-n*-propyl ether).

Table 3.3 log *P* values for common organic solvents

Solvent	log *P*	Solvent	log *P*
Dimethylsulfoxide	−1.3	Dipropylether[a]	1.9
Dioxane	−1.1	Chloroform	2.0
N,N-Dimethylformamide	−1.0	Benzene	2.0
Methanol	−0.76	Pentyl acetate[a]	2.2
Acetonitrile	−0.33	Toluene	2.5
Ethanol	−0.24	Octanol[a]	2.9
Acetone	−0.23	Dibutyl ether[a]	2.9
Tetrahydrofuran	0.49	Pentane[a]	3.0
Ethyl acetate	0.68	Carbon tetrachloride	3.0
Pyridine	0.71	Cyclohexane	3.2
Butanol[a]	0.80	Hexane[a]	3.5
Diethyl ether	0.85	Octane[a]	4.5
Propyl acetate[a]	1.2	Decane[a]	5.6
Butyl acetate[a]	1.7	Dodecane[a]	6.6

[a]Since the specific place of a molecular fragment is not significant for the log *P* value, only one solvent for every structural isomer is listed. Thus, the log *P* values of *n*-/*iso*propanol, *n*-/*tert*-butanol and di-*n*-/di-*iso*propyl ether are identical

Water Content (Water Activity). The ability of the solvent to strip off the bound water from the enzyme's surface depends not only on its polarity but also on its water content. Thus, the water content – more precisely the water activity (a_W) [79] – of an organic solvent has to be adjusted to the enzyme's requirements in order to ensure optimum activity [80–82]. The minimum amount of water required to maintain enzyme activity also depends on the enzyme type: Whereas lipases are able to operate at extremely low water activities of a_W 0.0–0.2, oxidoreductases and glycosidases require a_W of 0.1–0.7 and 0.5–0.8, respectively [83].

As a rule of thumb, acceptable activities are obtained in water-saturated organic solvents by using a buffer of low ionic strength. For large-scale applications, however, careful adjustment and maintenance of the water activity of the system is highly recommended to ensure optimal results. This can be conveniently achieved by a pair of salt hydrates added to the solvent by functioning as a 'water-buffer' (Table 3.4) [84–86]. Alternatively, a saturated salt solution (being in equilibrium with a sufficient amount of undissolved salt) is circulated through the

Table 3.4 Water activity (a_W) of saturated salt solutions and pairs of salt hydrates

Salt[a]	a_W	Salt-hydrate pair	a_W[b]
LiBr	0.06	$CaCl_2{\cdot}H_2O/2\ H_2O$	0.037
LiCl	0.11	NaI anh./2 H_2O	0.12
$MgCl_2$	0.33	Na_2HPO_4 anh./2 H_2O	0.16
K_2CO_3	0.43	NaOAc anh./3 H_2O	0.28
$Mg(NO_3)_2$	0.54	NaBr anh./2 H_2O	0.33
NaBr	0.58	$Na_4P_2O_7$ anh./7 H_2O	0.46
NaCl	0.75	$Na_2HPO_4{\cdot}2\ H_2O/7\ H_2O$	0.57
KCl	0.84	Na_2SO_4 anh./10 H_2O	0.76
K_2SO_4	0.97	$Na_2HPO_4{\cdot}7\ H_2O/12\ H_2O$	0.80

[a]In equilibrium with a saturated salt solution
[b]At 20°C

reaction compartment via a silicone tubing that is submerged in the reaction medium. Any water produced (or consumed) during the reaction is removed (or added) by diffusion through the tube walls, thus maintaining an equilibrium a_W set by the salt solution used [87].

Effects of Additives. The addition of enzyme-stabilizing agents – often denoted as 'activators' or 'enhancers' – at low concentration may be beneficial [88–90]. Although the effects of the stabilizers on the protein are only poorly understood on a molecular level making this technique therefore rather empirical, several groups of additives can be recommended [91, 92]:

- Polyalcohols such as carbohydrates, sugar alcohols, or glycerol are well known to stabilize proteins [93, 94] as well as inactive proteins (bovine serum albumin) and polymers which have a certain structural resemblance to that of water (e.g., polyethylene glycol, polyvinyl alcohol, and derivatives thereof).
- Small polar organic molecules (e.g., *N*,*N*-dimethyl formamide and formamide) are known to enhance reaction rates by acting as 'molecular lubricants' [95–97].
- The addition of salts (LiCl, NaCl, KCl [98]) or weak organic bases (e.g., triethylamine, pyridine [99, 100]) may improve reaction rates and selectivities via formation of salt-pairs of substrate and/or product, which shift equilibria into the desired direction.

Ionic Liquids. Salts that do not crystallize at (or close to) room temperature are called 'ionic liquids' (IL). Due to their exceptional properties, such as outstanding thermal stability (up to 300°C [101]), near-zero vapor pressure [102] and unconventional miscibility properties they are heralded as safe 'green' solvents and they are expected to replace some volatile and flammable organic solvents in the future [103, 104]. The most widely used components for the generation of ILs consist of peralkylated imidazolium, pyridinium, ammonium, and phosphonium cations and carboxylate, triflate, and triflic amidate anions (Fig. 3.1). Heavily fluorinated anions, such as BF_4^- and PF_6^- were recently replaced by more inocuous carboxylates for environmental reasons. Although tests using Reichardt's dye indicate that the polarity of ILs is similar to that of methanol, *N*-methylformamide, and 2-chloroethanol – which rapidly inactivate enzymes – the ILs surprisingly don't [105, 106] and it appears that enzymes that work in rather lipophilic organic solvents will also act in more polar ILs.

The polar nature of ILs increases the solubility of polar substrates, such as carbohydrates, which ensures enhanced reaction rates. Other potential advantages are increased enzyme stability [107] or stereoselectivity [108]. Furthermore, the properties of ILs can be easily tailored by simply choosing another combination of ions. On the down-side, ILs are considerably more expensive than organic solvents and are more viscous, which complicates their handling. Preliminary data indicate that some components of ILs are quite toxic and are not easily biodegradable [109].

Biocatalysis in ILs dates back to the year 2000 [110–112] and more recent studies indicate that (almost) all types of enzymes may act in such systems.

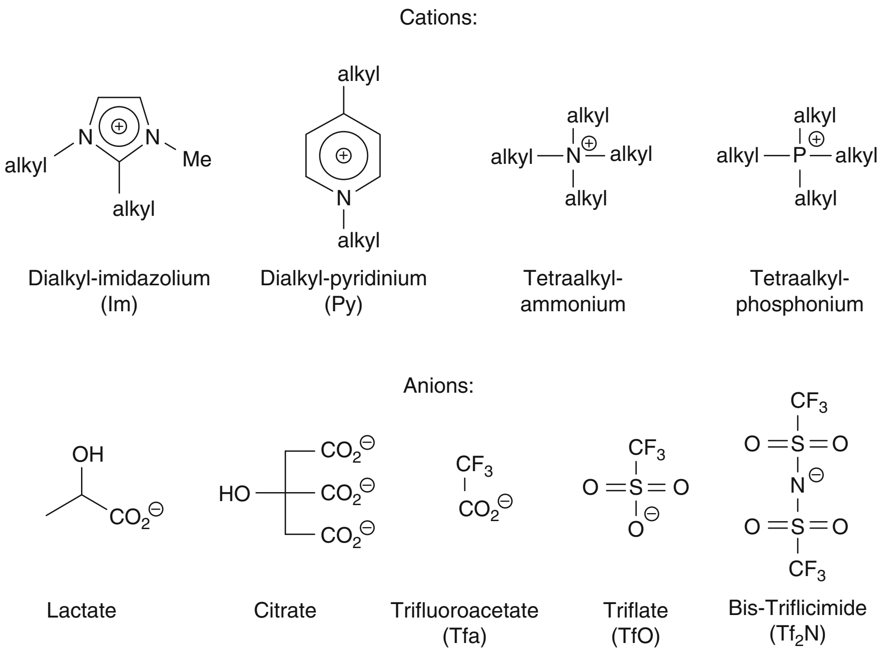


Fig. 3.1 Ionic components for the generation of ionic liquids

Successful examples were demonstrated for transesterification, perhydrolysis and ammonolysis (using lipases, esterases, and proteases), amide/peptide synthesis (using proteases), epoxide hydrolysis (using epoxide hydrolases), and glycoside synthesis (using glycosidases). Even redox-transformations, such as carbonyl reduction and sulfoxidation were possible [113–119].

Supercritical Gases. Instead of a lipophilic organic solvent, supercritical gases such as carbon dioxide,[1] freons (e.g., CHF_3), hydrocarbons (ethane, ethene, propane), or inorganic compounds (SF_6, N_2O) which exhibit solubility properties similar to that of a hydrocarbon such as hexane, can be used as solvent or cosolvent for the enzymatic transformation of lipophilic organic compounds [120–123]. Enzymes are as stable in these media as in lipophilic organic solvents. The use of supercritical gases is not restricted to a particular class of enzyme but, not surprisingly, the use of hydrolases is dominant. For instance, esterification [124], transesterification [125, 126], alcoholysis [127], and hydrolysis [128] are known as well as hydroxylation [129] and dehydrogenation reactions [130]. The most striking advantages of this type of solvent are a lack of toxicity, easy removal and the low viscosity, which is intermediate between those of gases and those of liquids. This latter property ensures high diffusion, being about one to two orders of magnitude

[1] T_{crit} 31°C and p_{crit} 73 bar.

higher than in common solvents. Furthermore, small variations in temperature or pressure may result in large solubility changes near the critical point, which allows to control an enzyme's catalytic properties such as reaction rate or stereoselectivity [131]. However, some disadvantages should be mentioned. The high-pressure equipment, that must withstand several hundred atmospheres pressure, requires a considerable initial investment and the depressurization step may cause enzyme denaturation due to mechanical stress [132, 133]. In addition, some supercritical fluids may react with sensitive groups located at the enzyme's surface causing a loss of activity. For instance, carbon dioxide is known to reversibly react with ε-amino groups of lysine residues by forming carbamates, going in hand with the removal/formation of a positive charge at the enzyme's surface [134]. The main use of supercritical gases as solvents is the production of 'natural' compounds used in cosmetics and food.

Alternatively, enzyme-catalyzed reactions may be performed in nonconventional media composed of microemulsions and liquid crystals [135]. The use of these systems, however, requires a great deal of knowledge of bioprocess engineering for the separation of the surfactant from substrate(s) and/or product(s).

The following basic rules should be considered for the application of solid ('dry') enzymes in organic media having a low water content:

- Hydrophobic solvents are more compatible than hydrophilic ones (log P of the organic solvent should be greater than ~1.5).
- The water layer bound to the enzyme must be maintained; this is accomplished by using water-saturated organic solvents or, alternatively, via control of the water activity.
- The 'micro-pH' must be that of the pH-optimum of the enzyme in water, a prerequisite that is fulfilled if the protein was isolated from an aqueous solution at the pH-optimum.
- Stirring, shaking, or sonication is necessary in order to maximize diffusion of substrate to the catalyst's surface.
- The addition of enzyme-stabilizing agents may improve the stability of the solid enzyme preparation significantly.

3.1.1 Ester Synthesis

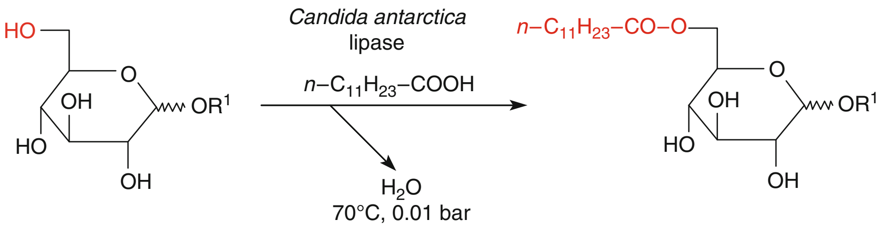
Esterification

In every synthetic reaction where a net amount of water is formed (such as an ester synthesis from an alcohol and a carboxylic acid [38–40]) physicochemical problems arise. Due to the fact that the lipophilic solvent (log $P > 1.5$) is unable to accommodate the water which is gradually produced during the course of the reaction, it is collected at the hydrophilic enzyme surface. As a consequence, the water gradually forms a discrete aqueous phase which encompasses the enzyme, finally separating substrate and enzyme from each other by a polar interface, which is difficult to penetrate for lipophilic substrate/product molecules. Thus, the rate slows down and the reaction may cease before reaching the desired extent of conversion. In order to solve this problem, two techniques have been developed.

- Removal of water from the system [136] (e.g., by evaporation [137], azeotropic distillation [138], or chemical drying [139, 140] via addition of molecular sieves or water-scavenging inorganic salts [141].
- Alternatively, the formation of water may be avoided by employing an *acyl-transfer* step rather than an esterification reaction.

Some of the methods for the removal of water have inherent disadvantages and are therefore not trivial. Evaporation of water from the reaction mixture can only be efficient if the alcohol and acid reactants have a low volatility (high boiling point). On the other hand, recovery of (solid) enzymes from organic solvents in the presence of solid inorganic water-scavengers such as salts or molecular sieves may be troublesome.

An example of a successful esterification reaction on industrial scale is shown in Scheme 3.1. 6-*O*-Acyl derivatives of alkyl glucopyranosides, which are used as fully biodegradable nonionic surfactants in cosmetics [142], were synthesized from fatty acids and the corresponding 1-*O*-alkyl glucopyranosides under catalysis of thermostable *Candida antarctica* lipase B in the absence of solvents [137]. In order to drive the reaction towards completion, the water produced during the reaction was evaporated at elevated temperature and reduced pressure (70°C, 0.01 bar).



R^1	Yield of 6-*O*-monoester [%]	Yield of diester(s) [%]
H	<5	0
Me	53	4
Et	93	5
i-Pr	93	4
n-Pr	96	17
n-Bu	94	22

Scheme 3.1 Biocatalytic synthesis of glucose esters used as biosurfactant

Acyl Transfer

Trans- or interesterifications, which do not form water during the course of the reaction, are usually easier to perform (Schemes 2.1 and 3.2) [143–145]. Furthermore, the water content of the reaction medium (more accurately the 'water activity', a_W), which is a crucial parameter for retaining the enzyme's activity, remains constant. As a consequence, it has only to be adjusted at the beginning of the reaction, but not constantly monitored. In acyl transfer reactions any trace of chemically available ‘bulk’ water, which may be present in the reaction medium, is quickly consumed at the expense of acyl donor, which is usually used in excess. The structural water, which is required to retain the enzyme’s activity is chemically ‘not available’ because it is too tightly bound onto the enzyme’s surface to be removed.

In contrast to hydrolytic reactions, where the nucleophile (water) is always in excess (55 mol/L), the concentration of the ‘foreign’ nucleophile in acyl transfer reactions (such as another alcohol) is always limited. As a result, trans- and interesterification reactions involving ‘normal’ esters are generally *reversible* in contrast to the *irreversible* nature of a hydrolytic reaction. This leads to a slow reaction rate and can cause a severe depletion of the selectivity of the reaction for kinetic reasons (Sect. 2.1.1).

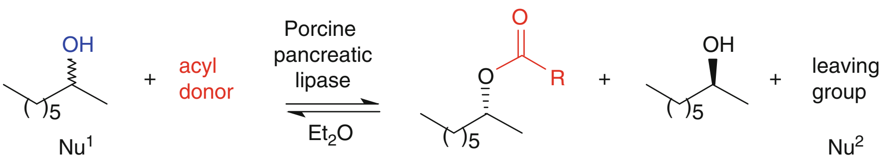
In order to avoid the undesired depletion of the optical purity of (predominantly) the remaining substrate during an enzymatic resolution under *reversible* reaction conditions, two tricks can be applied to shift the equilibrium of the reaction.

- Use of an excess of acyl donor; this may be expensive and not always compatible with the aim of maintaining high enzyme activity, but it may be helpful in some cases.
- A better solution, however, is the use of special acyl donors which ensure a more or less *irreversible* type of reaction.

The reversibility of transesterification reactions is caused by the comparable nucleophilicity of the incoming nucleophile (Nu^1) and the leaving group of the acyl donor (Nu^2), both of which compete for the acyl-enzyme intermediate in the forward and the reverse reaction (Scheme 3.2). If the nucleophilicity of the leaving group Nu^2 is decreased by the introduction of electron-withdrawing substituents, the reaction is shifted to the right, i.e., towards completion. This concept has been verified by the introduction of ‘activated’ esters [146], such as 2-haloethyl, cyano-methyl and oxime esters (Scheme 3.3). Although acyl transfer using activated esters is still reversible in principle, the equilibrium of the reaction is shifted so far to the product side that for preparative purposes it can be regarded as irreversible. To indicate this, the term ‘quasi-irreversible’ was proposed [147].

As shown in Scheme 3.2, the relative rate of the enantioselective acylation of (±)-2-octanol, catalyzed by porcine pancreatic lipase (PPL), was one to two orders of magnitude faster when ‘activated’ esters were used as acyl donors instead of ‘nonactivated’ methyl or ethyl alkanoates.

The following parameters should be considered before the ‘activated’ ester is chosen. Cyanomethyl esters have been used only rarely due to toxicity problems arising from formaldehyde cyanohydrin, which is liberated. 2-Haloethyl esters have been applied more widely. Among them, trifluoroethyl esters are the acyl donors of choice when the reactions are performed on a laboratory scale. Their degree of activation is high and trifluoroethanol can be evaporated easily during workup procedures. For larger batches, trichloroethyl esters are more economic but the removal of trichloroethanol during work-up can be troublesome due to its high

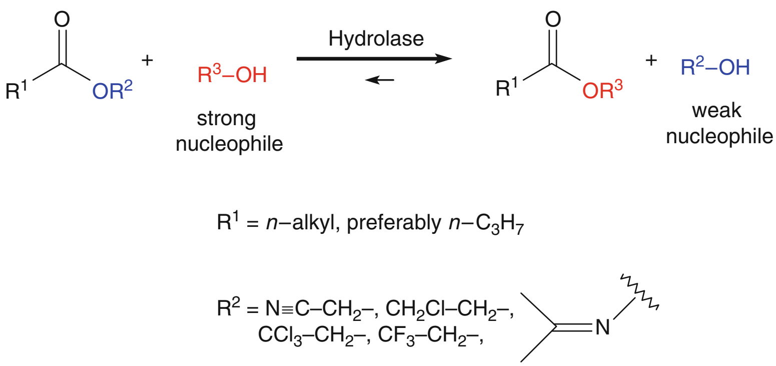


Acyl donor	R	Leaving group Nu²	Initial rate [%]
Ethyl acetate	Me	EtOH	0.3
2-Chloroethyl acetate	Me	$ClCH_2$–CH_2OH	1
Methyl butanoate	*n*-Pr	MeOH	5
Ethyl cyanoacetate	N≡CCH_2–	EtOH	6
Trichloroethyl trichloroacetate	Cl_3C–	Cl_3C–CH_2OH	7
Methyl bromoacetate	$BrCH_2$–	MeOH	14
Tributyrin	*n*-Pr	dibutyrin	34
Trichloroethyl butanoate	*n*-Pr	Cl_3C–CH_2OH	58
Trichloroethyl heptanoate	*n*-C_6H_{13}–	Cl_3C–CH_2OH	100

Scheme 3.2 Enzymatic acylation of (±)-2-octanol

boiling point (151°C). 2-Chloroethyl esters are cheap and the resulting 2-chloroethanol is easier to remove (bp 130°C), but their degree of activation is limited.

As an alternative, oxime esters have been proposed as acyl donors for acyl-transfer reactions [148]. During the reaction a weakly nucleophilic oxime is liberated which is unable to compete with the substrate alcohol for the acyl-enzyme intermediate. However, cosubstrate inhibition and problems in separating the nonvolatile oxime from the substrate alcohol during work-up may be encountered. Alternatively, thioesters have been used [149, 150]. The thiols liberated as byproducts are highly volatile



Scheme 3.3 Quasi-irreversible enzymatic acyl transfer using activated esters

and are easily removed by evaporation, thus driving the equilibrium. However, excellent ventilation is recommended in order to avoid a noxious laboratory atmosphere and complaints of labmates.

In contrast to the above-mentioned acyl donors which shift the equilibrium of the reaction to the product side by liberating a weakly nucleophilic co-product alcohol species, several concepts have been proposed for making the reaction completely irreversible (Scheme 3.4).

Enol esters such as vinyl or isopropenyl esters liberate unstable enols as coproducts, which tautomerize to give the corresponding aldehydes or ketones [151, 152] (Scheme 3.4). Thus, the reaction becomes *completely irreversible* and this ensures that all the benefits with regard to a rapid reaction rate and a high selectivity are accrued. Acyl transfer using enol esters has been shown to be about only ten times slower than hydrolysis (in aqueous solution) and about 10–100 times faster than acyl-transfer reactions using activated esters. In contrast, when nonactivated esters such as ethyl acetate were used, reaction rates of about 10^{-3}–10^{-4} of that of the hydrolytic reaction are observed (Table 3.5) [153].

Table 3.5 Relative rates of reactions catalyzed by hydrolases

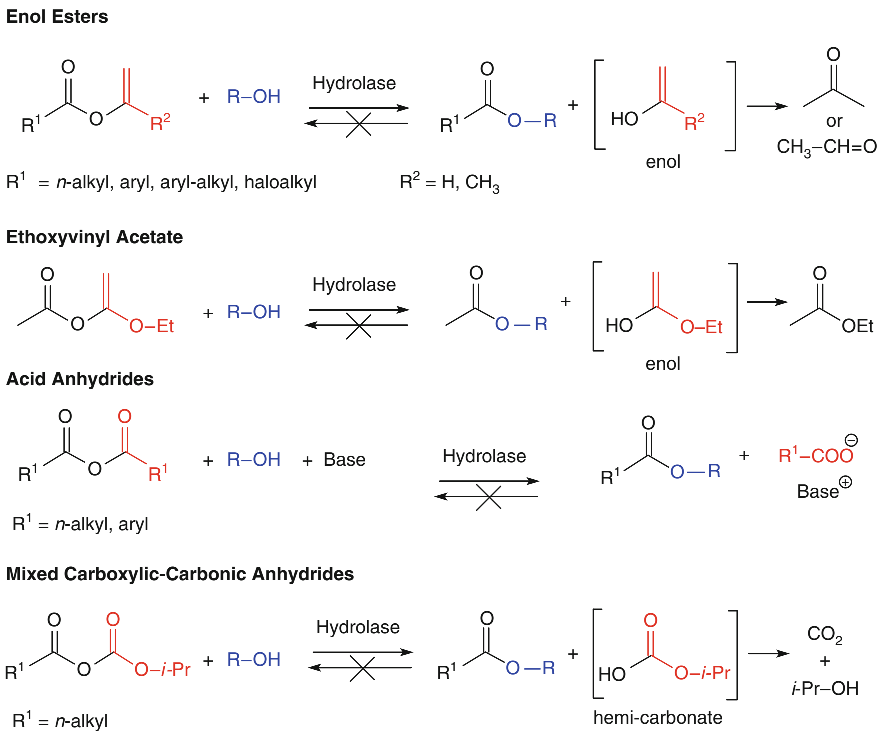
Reaction	Acyl donor	Relative rate
Ester hydrolysis	–	10,000
Acyl transfer	Enol esters	1,000
Acyl transfer	Acid anhydrides	1,000
Acyl transfer	Activated esters	10–100
Acyl transfer	Nonactivated esters	1–10

Due to steric reasons, vinyl esters give better reaction rates than isopropenyl esters and the former are therefore used most widely, but their use is not without drawbacks. Acetaldehyde, which is liberated during the reaction, is known to act as an alkylating agent by forming Schiff bases with the terminal amino group of lysine residues [154] in a Maillard-type of reaction [155]. Thus, a positive charge is removed from the enzyme's surface during the course of this reaction, which may cause enzyme deactivation. The extent of this depends on the nature of the enzyme [156, 157]. Whereas the majority of the more widely employed lipases seem to be quite stable, *Candida rugosa* (CRL) and *Geotrichum candidum* lipase are very sensitive.

Covalent immobilization of CRL onto an epoxy-activated macroscopic carrier leads to selective monoalkylation of the lysine amino residues which are involved in the deactivation reaction with retention of the positive charge. In contrast to the native enzyme, the immobilized enzyme is inert towards the formation of Schiff bases, which results not only in a greatly stabilized activity but also in a significant enhancement in selectivity [158]. The addition of a molecular sieve to the medium in order to trap acetaldehyde seems to have some benefit [159, 160]. Alternatively, the lipase may be stabilized by adsorption onto Celite [161].

The possibly harmful effects of acetaldehyde can be avoided by employing *i*-propenyl acetate, which yields (more innocuous) acetone as byproduct. Alternatively, ethoxyvinyl acetate can be used as acyl donor (Scheme 3.4) [162–164].

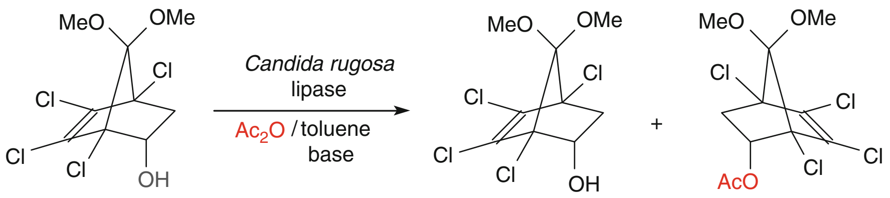
The latter renders ethyl acetate as byproduct, which is generally regarded as innocuous to ester-hydrolyzing enzymes. Unfortunately, ethoxyvinyl esters are rather expensive.



Scheme 3.4 Irreversible enzymatic acylation using enol esters and acid anhydrides

Acid Anhydrides. Another useful method of achieving completely irreversible acyl-transfer reactions is the use of *acid anhydrides* (Scheme 3.4) [165]. The selectivities achieved are usually high and the reaction rates are about the same as with enol esters. One of the advantages of this technique is that no aldehydic byproducts are formed and the enzyme is not acylated under the conditions employed, making its reuse possible.

For some enzymes, such as *Pseudomonas* sp. lipase (PSL), the liberated acid does not present any problems, but others like CRL are more sensitive and require more protection. For instance, when acetic anhydride is used, the liberated acetic acid may lead to a decrease of the pH in the micro-environment of the enzyme, thus leading to a depletion of activity and selectivity. The CRL-catalyzed resolution of the bicyclic tetrachloroalcohol shown in Scheme 3.5, using acetic anhydride as acyl donor, initially proceeded with only moderate selectivity ($E = 18$). Addition of a weak inorganic or (preferably) organic base such as 2,6-lutidine which functions



Base	Reaction rate	Selectivity (*E*)
none	good	18
$KHCO_3$	low	>200
2,6-Lutidine	good	>200

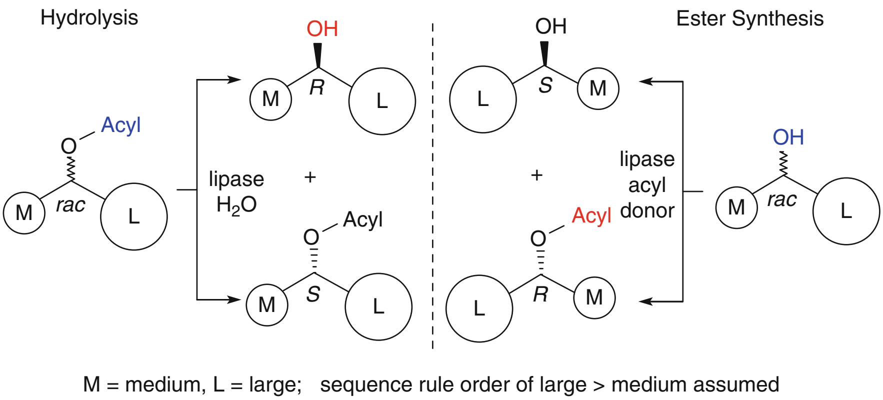
Scheme 3.5 Selectivity enhancement via addition of base

as an acid scavenger, led to a greater than tenfold increase in selectivity [166]. A similar acid-quenching effect could be observed by immobilization of CRL onto diatomaceous earth (Celite).

In contrast to symmetric acid anhydrides, which liberate one equivalent of carboxylic acid as byproduct, mixed anhydrides composed of a straight-chain carboxylic acid (R^1–CO_2H) and a carbonic ester bearing a branched secondary alcohol group (e.g., isopropyl) can be used instead. The hydrolase takes off the straight-chain carboxylic acid moiety from the acyl donor by liberating an unstable hemi-carbonate ester, which undergoes rapid decarboxylation, forming innocuous carbon dioxide and a *sec*-alcohol, thus rendering the reaction completely irreversible (Scheme 3.4) [167]. Cyclic acid anhydrides, such as succinic and glutaric acid anhydrides lead to the formation of a hemiester [168, 169]. Due to the presence of the carboxylic acid moiety, separation of the formed hemiester product from the nonreacted alcohol enantiomer is particularly easy using a (basic) aqueous-organic solvent system. Consequently, cyclic acid anhydrides are advantageous in large-scale applications.

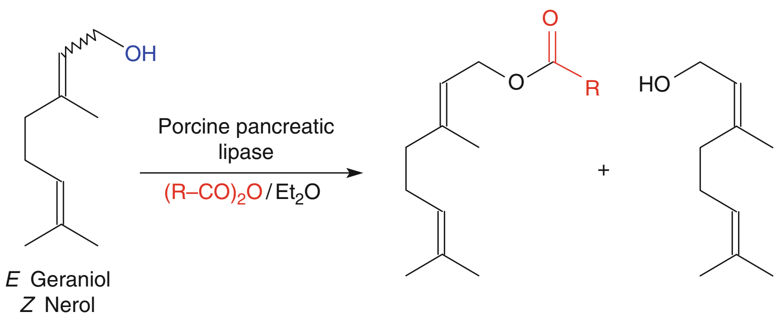
Besides the more often-used acyl donors mentioned above, others which would also ensure an irreversible type of reaction have been investigated [170]. Bearing in mind that most of the problems of irreversible enzymatic acyl transfer arise from the formation of unavoidable byproducts, emphasis has been put on finding acyl donors that possess cyclic structures, which would not liberate any byproducts at all. However, with candidates such as lactones, lactams, cyclic anhydrides (e.g., succinic acid anhydride [171]), enol lactones (e.g., diketene [172, 173]), and oxazolin-5-one derivatives [174], the drawbacks often outweighed their merits.

Enzyme-catalyzed acyl transfer can be applied to a number of different synthetic problems. The majority of applications that have been reported involve the desymmetrization of prochiral and *meso*-diols or the kinetic resolution of racemic primary and secondary alcohols. Since, as a rule, an enzyme's preference for a specific enantiomer is not reversed when water is replaced by an organic solvent, it is always the *same enantiomer* which is preferably accepted in hydrolysis and ester synthesis. Taking into consideration that hydrolysis and esterification represent reactions in *opposite directions*, products of *opposite configuration* are obtained (Scheme 3.6). In other words, if the (*R*)-enantiomer of an ester is *hydrolyzed* at a faster rate than its (*S*)-counterpart [yielding an (*R*)-alcohol and an (*S*)-ester], *esterification* of the racemic alcohol will lead to the formation of an (*S*)-alcohol and an (*R*)-ester.



Scheme 3.6 Symmetry in hydrolysis and ester synthesis reactions

Separation of *E*/*Z*-Stereoisomers. Stereoisomeric mixtures of the allylic terpene alcohols, geraniol and nerol, which are used in flavor and fragrance formulations, were separated by selective acylation with an acid anhydride using porcine pancreatic lipase (PPL) as catalyst (Scheme 3.7) [175]. Depending on the acyl donor employed, the slightly less hindered geraniol was more quickly acylated to give geranyl acetate leaving nerol unreacted. Acetic anhydride proved to be unsuitable,



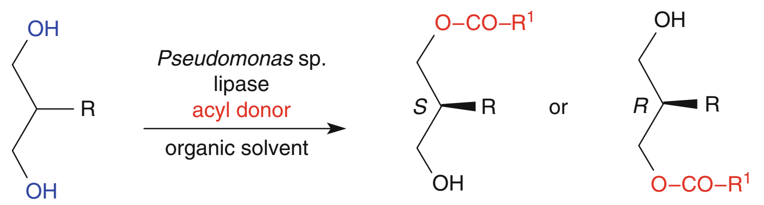
R	Geranyl ester [%]	Nerol [%]	Selectivity [k_E/k_Z]
n-C_3H_7–	85	16	11
n-C_5H_{11}–	66	7	13
n-C_7H_{15}–	72	7	15

Scheme 3.7 *E*/*Z*-Stereoselective enzymatic acylation of terpene alcohols

giving a low yield and poor selectivity, but longer-chain acid anhydrides were used successfully.

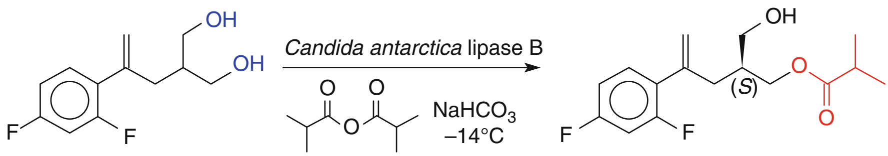
Desymmetrization of Prochiral and *meso*-Diols. Chiral 1,3-propanediol derivatives are useful building blocks for the preparation of enantiomerically pure bioactive compounds such as phospholipids [176], platelet activating factor (PAF), PAF-antagonists [177], and renin inhibitors [178]. A simple access to these synthons starts from 2-substituted 1,3-propanediols (Scheme 3.8). Depending on the substituent R in position 2, (*R*)- or (*S*)-monoesters were obtained in excellent optical purities using *Pseudomonas* sp. lipase (PSL) [179–182]. The last three entries demonstrate an enhancement in selectivity upon lowering the reaction temperature [183].

The desymmetrization of a prochiral 2-substituted 1,3-propanediol building block using *Candida antarctica* lipase B allowed the efficient synthesis of multi-ton quantities of the antifungal agent posaconazol (Scheme 3.8) [184]. Moderate chemical and optical yields of monoacetate were initially obtained using vinyl acetate as acyl donor, optimal results were obtained by using the sterically more demanding *i*-butyric anhydride in presence of $NaHCO_3$ at low temperatures to suppress undesired background acylation and acyl migration.



R	Acyl donor	R^1	Solvent	Configuration	e.e. [%]
Me	vinyl acetate	Me	$CHCl_3$	*S*	>98
CH_2–Ph	vinyl acetate[d]	Me	none	*R*	>94
O–CH_2–Ph	*i*-propenyl acetate	Me	$CHCl_3$	*S*	96
O–CH_2–Ph	vinyl acetate[d]	Me	none	*S*	90[a]
O–CH_2–Ph	vinyl acetate[d]	Me	none	*S*	92[b]
O–CH_2–Ph	vinyl acetate[d]	Me	none	*S*	94[c]

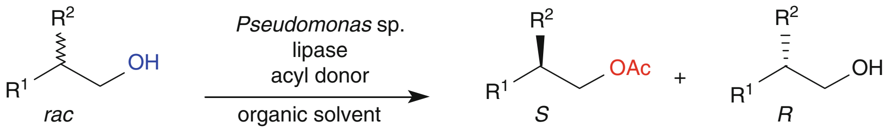
[a] performed at 25°C, [b] at 17°C, and [c] at 8°C, [d] used as acyl donor and as solvent



Scheme 3.8 Desymmetrization of 2-substituted propane-1,3-diols

Cyclic *meso-cis*-diols were asymmetrically acylated quite efficiently to give the respective chiral monoester by a PSL [185]. Whereas a slow reaction rate was observed in a reversible reaction using ethyl acetate as acyl donor, the reaction was about ten times faster when vinyl acetate was employed.

Kinetic Resolution of Alcohols. Primary alcohols may be resolved with moderate to good selectivities by *Pseudomonas* sp. lipase (PSL) using vinyl acetate [186] or acetic anhydride as the acyl donor (Scheme 3.9). Whereas the selectivities achieved were moderate with alkyl and aryl substituents, substrate modification via introduction of a bulky sulfur atom in R^2 helped considerably. In this way, chiral isoprenoid synthons having a C_5-backbone were obtained in >98% enantiomeric excess.



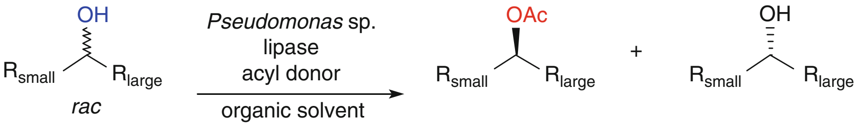
Acyl donor	Solvent	R^1	R^2	Selectivity (*E*)
Ac_2O	benzene	Me	Ph	12
Ac_2O	benzene	Et	*n*-Bu	2
Vinyl acetate	$CHCl_3$	Me	$(CH_2)_2SPh$	>100

Scheme 3.9 Kinetic resolution of primary alcohols

Numerous acyclic secondary alcohols have been separated into their enantiomers using lipase-catalyzed acyl-transfer [187]. As long as the substituents are different in size, excellent selectivities were obtained with lipases from *Candida antarctica* (CAL) and *Pseudomonas* sp. (PSL) [188].

The Katsuki–Sharpless epoxidation of allylic alcohols constitutes one of the most important developments in asymmetric synthesis [189, 190], but there are some limitations in this approach. For instance, they are not generally applicable to substrates other than allylic alcohols and the products have to be purified from significant amounts of catalyst residues. A useful alternative is in the lipase-catalyzed resolution (Scheme 3.10) [191, 192].

A generally applicable method for the preparation of optically active epoxides makes use of a lipase-catalyzed resolution of halohydrins bearing the halogen in the terminal position (Scheme 3.10). *Pseudomonas* sp. lipase-catalyzed acylation of racemic halohydrins affords a readily separable mixture of (*R*)-halohydrin and the corresponding (*S*)-ester in good to excellent optical purities [193, 194]. Treatment of the latter with base leads to the formation of epoxides with no loss of optical purity. A semiquantitative comparison of the reaction rate obtained with different acyl donors using substrates of this type revealed that they were in the order ethyl



R_{small}	R_{large}	Acyl donor	Selectivity (*E*)
Me	Ph–C(=CH_2)–	vinyl actate	>20
CH_2=CH–	(*E*)-Ph–CH=CH–	vinyl actate	>20
CH_2=CH–	Ph–C≡C–	vinyl actate	>20
HC≡C–	(*E*)-Ph–CH=CH–	vinyl actate	>20
Me	*n*-Bu–C≡C–	vinyl actate	>20
CH_2=C=CH–	Ph–CH_2–	vinyl actate	>20
Me	Me_3Si–C≡C–	vinyl actate	>20
CH_2–Cl	Ph–	*i*-propenyl acetate	100
CH_2–Br	2-Naphthyl–	*i*-propenyl acetate	95
CH_2–Cl	*p*-Tos–O–CH_2–	*i*-propenyl acetate	>100

Scheme 3.10 Kinetic resolution of unsaturated *sec*-alcohols and halohydrins

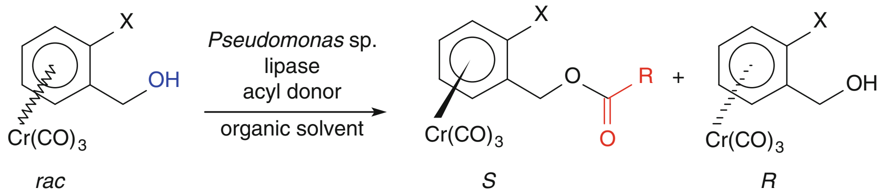
acetate ≪ trichloroethyl acetate < isopropenyl acetate < vinyl butanoate ~ vinyl octanoate ~ vinyl acetate [195].

Enantiomerically pure *trans*-cycloalkane-1,2-diols are of interest for the synthesis of optically active crown-ethers [196] or as chiral auxiliaries for the preparation of bidentate ligands [197]. A convenient method for their preparation consists in PSL-catalyzed enantioselective acylation (following a sequential resolution pattern, see Sect. 2.1.1), which yields varying amounts of diester, monoester, and remaining nonreacted diol in excellent optical purities [198]. The advantage of acyl-transfer in organic solvents lies in the suppression of undesired acyl migration, which plagues the hydrolysis of the corresponding diesters [24, 199, 200].

Along the same lines, the remarkable synthetic potential of enzyme-catalyzed irreversible acyl transfer in nearly anhydrous organic solvents can be demonstrated particularly well by the transformation of alcoholic substrates (such as organometallics or cyanohydrins) which are prone to decomposition reactions in an aqueous medium and thus cannot be transformed via enzyme-catalyzed hydrolysis reactions.

For instance, organometallic compounds such as hydrolytically labile chromium-tricarbonyl complexes, which are of interest as chiral auxiliary reagents for asymmetric synthesis [201], were easily resolved by PSL (Scheme 3.11) [202, 203]. A remarkable enhancement in selectivity was obtained when the acyl moiety of the vinyl ester used as acyl donor was varied. This concept was successfully employed for the resolution of 1-ferrocenylethanol, which cannot be well resolved via enzymatic hydrolysis due to the lability of 1-ferrocenyl acetate in aqueous systems [204, 205].

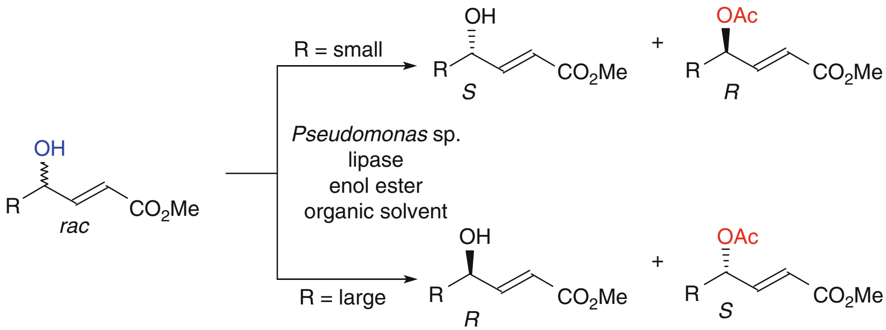
Chiral hydroxyesters would be accessible via enzymatic hydrolysis of their acyloxy esters, but a disadvantage which is commonly encountered in such resolutions is an undesired side reaction involving the hydrolysis of the carboxyl ester moiety which leads to the formation of hydroxyacids as byproducts. Thus, low



X	Acyl donor	R	Solvent	Selectivity (*E*)
$SiMe_3$	*i*-propenyl acetate	Me	none	30
Me	*i*-propenyl acetate	Me	none	>200
Me	vinyl acetate	Me	toluene	39
Me	vinyl octanoate	*n*-C_7H_{15}	toluene	67
Me	vinyl palmitate	*n*-$C_{15}H_{31}$	toluene	>200

Scheme 3.11 Kinetic resolution of organo-metallic hydroxy compounds

yields are often reported [206]. In contrast, if the resolution is carried out in an acyl transfer mode, the unwanted side reaction is completely suppressed because the hydroxyl functionality is the only nucleophile in the substrate molecule which can be acylated (Scheme 3.12), and no hydrolysis of the carboxylic acid ester can take



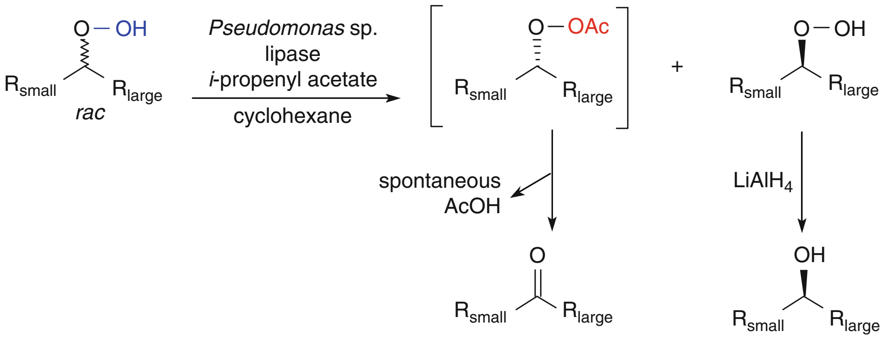
R	Acyl donor	Configuration Acetoxy ester	Hydroxy ester	Selectivity (*E*)
Me	*i*-propenyl acetate	*R*	*S*	>30
Et	*i*-propenyl acetate	*R*	*S*	>150
n-Pr	*i*-propenyl acetate	*R*	*S*	>20
i-Pr	vinyl acetate	*S*	*R*	1.6
i-Pr–CH_2–	vinyl acetate	*S*	*R*	2.5
c–C_6H_{11}–CH_2–	vinyl acetate	*S*	*R*	13
Me_2ThexSiO–$(CH_2)_2$–	vinyl acetate	*S*	*R*	>150

Thex = thexyl (1,1,2-trimethylpropyl).

Scheme 3.12 Kinetic resolution of γ-hydroxy-α,β-unsaturated esters place due to the absence of water [207]. This concept was successfully applied to the resolution of γ-hydroxy-α,β-unsaturated esters which are used for the synthesis of statin analogs [208, 209]. A reversal of the stereochemical outcome using PSL was observed when the size of the side-chain substituent R gradually increased.

This strategy has also been successfully applied to the preparation of optically active α-methylene-β-hydroxy esters and -ketones [210], which cannot be resolved using the Sharpless epoxidation technique because of the deactivating influence of the electron-withdrawing group on the alkene unit [211]. Similarly, optically active cyclopentanoids carrying a terminal carboxylate group useful for prostaglandin synthesis were obtained without the occurrence of undesired side reactions [212].

Racemic hydroperoxides may be resolved in organic solvents via lipase-catalyzed acyl transfer (Scheme 3.13). Although the so-formed acetylated (*R*)-peroxy-species is unstable and spontaneously decomposes to form the corresponding ketone via elimination of acetic acid, the remaining (*S*)-hydroperoxide was isolated in varying optical purity [213]. This concept was also applied to the resolution of a hydroperoxy derivative of an unsaturated fatty acid ester [214].



R_{small}	R_{large}	E.e. hydroperoxide [%]	Selectivity (*E*)
Me	*n*-Pr	10	1.2
Me	2-naphthyl	58	2.3
Et	Ph	62	3.7
Me	Ph	100	>20

Scheme 3.13 Kinetic resolution of hydroperoxides

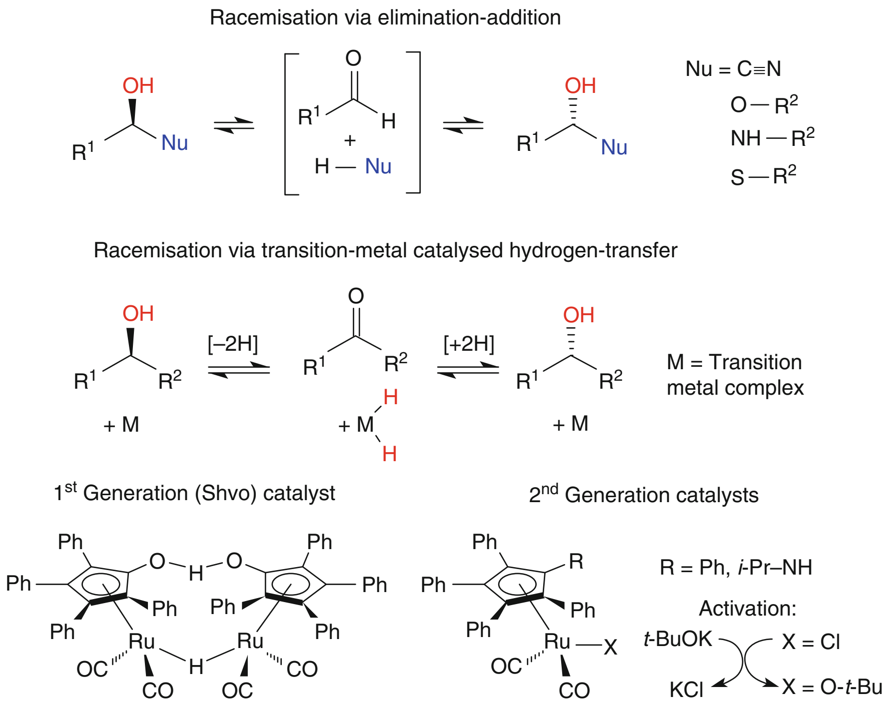
Dynamic Resolution. Lipase-catalyzed acyl transfer has become a well-established and popular method for the kinetic resolution of primary and secondary alcohols. In order to circumvent the limitations of kinetic resolution (i.e., a 50% theoretical yield of both enantiomers), several strategies have been developed, which achieve a more economic *dynamic* resolution process and allow the formation of a single stereoisomer as the sole product (for the theoretical background see Sect. 2.1.1). In contrast to compounds bearing a chiral center adjacent to an electron-withdrawing group (e.g., carboxylic acid esters), which facilitates in-situ racemization via an achiral enolate, *sec*-alcohols are more difficult to racemize (see Scheme 2.43).

Two techniques of general applicability are worth considering (Scheme 3.14):

- Several types of *sec*-alcohols bearing a leaving group (Nu) attached to the carbinol moiety are chemically unstable and therefore prone to decomposition via a reversible elimination-addition process of a nucleophile (HNu) onto an aldehyde or ketone, respectively. This applies to cyanohydrins (Nu = C≡N), and hemi(thio)acetals (Nu = OR^2, SR^2) or hemiaminals (Nu = NHR^2), respectively. It is obvious that the corresponding cyclic structures – (thio)lactols, etc. – behave in the same way [215–217].
- Stereochemically stable *sec*-alcohols can be racemized via an oxidation-reduction sequence catalyzed by (transition) metal complexes based on Al, Ru, Rh, and Ir [218, 219]. More labile allylic alcohols could be racemized using a vanadium-catalyst [220]. However, both racemization techniques have their potential pitfalls, since transition metal complexes may cause enzyme deactivation, whose biochemical mechanism is only poorly understood. Furthermore, some transition metal complexes are incompatible with the commonly used enol esters serving as acyl donors.

The first dynamic resolution making use of transition-metal catalyzed substrate-racemization was reported in 1996 [221]. Since then, rapid progress was made and this technology is nowadays used for industrial-scale applications using optimized protocols [222] (Scheme 3.14). First generation (Shvo-type) racemization catalysts were impeded by slow racemization rates, which required elevated temperatures (ca. 70°C), which could be tolerated by only very few thermostable lipases. In addition, popular enol-ester-type acyl donors, such as vinyl or *iso*propenyl acetate were incompatible with the transition metal complex, which required the use of *p*-chlorophenyl acetate liberating *p*-chlorophenol as toxic byproduct [223]. During recent years, most of these initial drawbacks were circumvented by the development of second-generation racemization catalysts, which do not react with enol esters and show high racemization rates already at room temperature. The pre-catalysts have to be activated by the displacement of a Cl atom by *t*-BuOK to render the catalytically active species [224–228].

Optically pure cyanohydrins are required for the synthesis of synthetic pyrethroids, which are more environmentally acceptable agents for agricultural pest control than the classic highly chlorinated phenol derivatives [229]. They are important intermediates for the synthesis of chiral α-hydroxyacids, α-hydroxyaldehydes [230], and aminoalcohols [231, 232]. By asymmetric hydrolysis of their respective acetates using microbial lipases [233], only the remaining nontransformed substrate enantiomer can be obtained in high optical purity because the cyanohydrin product is spontaneously racemized as it is in equilibrium with the corresponding aldehyde and hydrocyanic acid at values above pH ~ 4 (Sect. 2.1.3.2). In the absence of water, however, the cyanohydrins are stable and can be isolated in high optical and chemical yields (Scheme 3.15) [23, 234]. In this manner, *both* enantiomers are accessible.

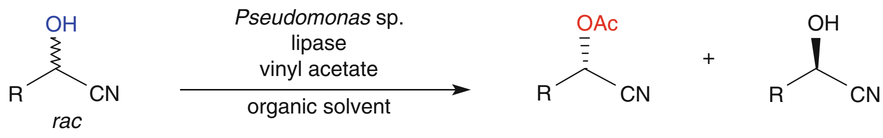


Scheme 3.14 Strategies for in-situ racemization of *sec*-alcohols in organic solvents

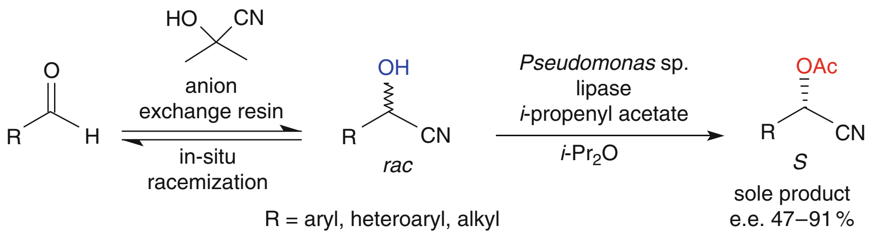
The kinetic resolution of cyanohydrins via enantioselective acylation may be converted into a dynamic process by making use of the chemical instability of cyanohydrins (Scheme 3.15) [235]. Thus, racemic cyanohydrins were generated from an aldehyde and acetone cyanohydrin (as a relatively safe source of hydrogen cyanide) under catalysis by an anion exchange resin. The latter also served as catalytic base for the in-situ racemization. Enantioselective acylation using PSL and *iso*propenyl acetate led to the exclusive formation of the corresponding (*S*)-cyanohydrin acetates in 47–91% optical purity.

An α-acetoxysulfide shown in Scheme 3.16 was used as central chiral building block for the synthesis of Lamivudine, a highly promising drug candidate for the treatment of HIV and HBV infections. Due to the different toxicities of the two enantiomers, an enantioselective route was required. Furthermore, applicability to large-scale synthesis and absence of any 'unwanted' enantiomers were important issues particularly in view of the possible drug application.

The solution was found by using an approach which is closely related to the dynamic resolution of cyanohydrins, i.e., a lipase-catalyzed enantioselective esterification employing vinyl acetate as acyl donor [236]. Thus, dynamic resolution was attempted by making use of the inherent instability of the racemic substrate, which – being a hemithioacetal – is in equilibrium with the

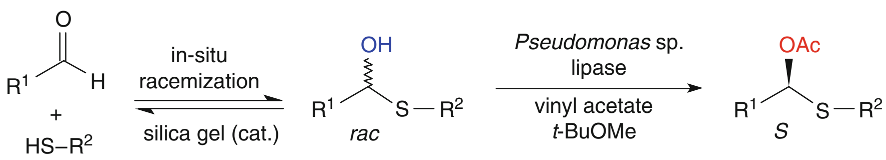


R	Solvent	e.e. [%] of		Selectivity
		Acetate	Cyanohydrin	(E)
n-Pr	CH_2Cl_2	55	33	5
Ph–CH_2–O–CH_2–	none	55	95	12
Ph–	CH_2Cl_2	68	80	13
4-HO–C_6H_4–	CH_2Cl_2	79	50	14
Ph–$(CH_2)_2$–	CH_2Cl_2	95	86	100



Scheme 3.15 Kinetic and dynamic resolution of cyanohydrins

corresponding aldehyde and thiol. Interestingly, the *Pseudomonas* sp. lipase catalyzed acyl transfer reaction spontaneously stopped at 50% conversion, indicating an insufficient in-situ racemization of the substrate. The latter problem was circumvented by adding silica gel to the mixture, which catalyzed the reversible



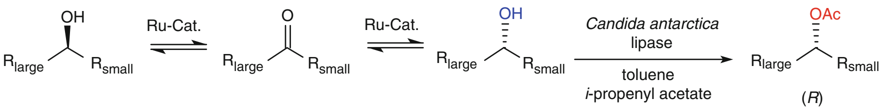
R^1	R^2	Product	
		yield [%]	e.e. [%]
–CO_2Me	*n*-Bu–	63	>95
–CO_2Me	Et_3Si–O–$(CH_2)_2$–	87	90
Ac–O–CH_2–	*n*-Bu–	87	87
Ac–O–CH_2–	*i*-Pr–	65	>95
Ac–O–CH_2–	*n*-Octyl–	85	>95

Scheme 3.16 Dynamic resolution of hemithioacetals

dissociation of the thioacetal into thiol and aldehyde. With the latter modification, a range of (*S*)-hemithioacetal esters were obtained in excellent optical purities with yields being considerably beyond the usual 50% limitation for classical kinetic resolutions.

Examples for the successful dynamic resolution of *sec*-alcohols using transition-metal-lipase/protease combo-catalysis are shown below.

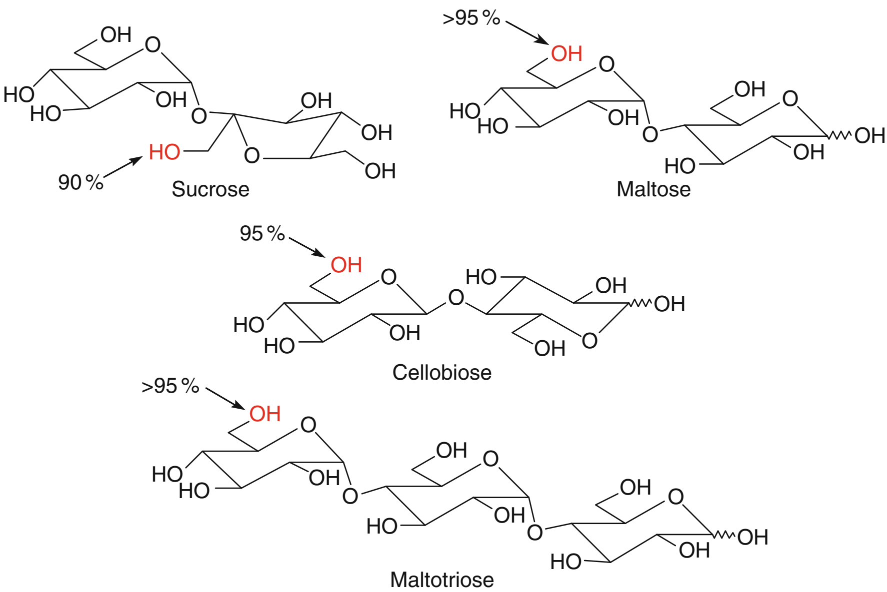
Dynamic resolution of various *sec*-alcohols was achieved by coupling a *Candida antarctica* lipase-catalyzed acyl transfer to in-situ racemization based on a second-generation transition metal complex (Scheme 3.17) [237]. In accordance with the Kazlauskas rule (Scheme 2.49) (*R*)-acetate esters were obtained in excellent optical purity and chemical yields were far beyond the 50% limit set for classical kinetic resolution. This strategy is highly flexible and is also applicable to mixtures of functional *sec*-alcohols [238–241] and *rac*- and *meso*-diols [242, 243]. In order to access products of opposite configuration, the protease subtilisin, which shows opposite enantiopreference to that of lipases (Fig. 2.12), was employed in a dynamic transition-metal-protease combo-catalysis [244, 245].



R_{large}	R_{small}	e.e. [%]	Yield [%]
Ph	Me	>99	95
c-C_6H_{11}	Me	>99	86
c-C_6H_{11}	$CH=CH_2$	>99	90
Ph–CH_2	Me	>99	90
n-Hexyl	Me	91	89
(*E*)-Ph–CH=CH	Me	98	93
Ph	$CH=CH_2$	81	62
t-Bu–O–CH_2	Me	99	97

Scheme 3.17 Dynamic resolution of *sec*-alcohols via Ru-catalyzed in-situ racemization

Regioselective Protection of Polyhydroxy Compounds. Selective protection and deprotection of compounds containing multiple hydroxyl groups such as carbohydrates and steroids is a current problem in organic synthesis [246]. By using standard methodology, a series of multiple steps is usually required to achieve the desired combination of protected and free hydroxyl functionalities. By contrast, enzymatic acyl transfer reactions in organic solvents have proven to be extremely powerful for such transformations by making use of the *regioselectivity* of hydrolytic enzymes [247]. Whereas the acylation of steroids in lipophilic organic solvents having a desired log *P* of greater than 2 is comparatively facile, carbohydrate



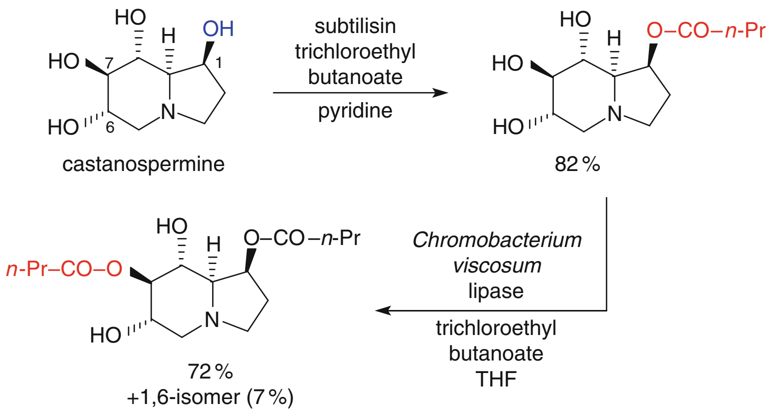
Scheme 3.18 Regioselective protection of primary hydroxy groups of carbohydrates (→ acylation site)

derivatives are only scarcely soluble in these media. Thus, more polar solvents such as dioxane, THF, 3-methyl-3-pentanol, DMF, or even pyridine have to be used. Thus, only the most stable enzymes such as PPL, *Candida antarctica* lipase, or subtilisin can be used.

Paralleling the difference in reactivity between primary and secondary hydroxyl groups, the former can be selectively acylated by using PPL in THF [248] or pyridine [47] as the solvent (Scheme 3.18). Unlike most of the other hydrolases, the protease subtilisin is stable enough to remain active even in anhydrous DMF [49]. In general, activated esters such as trihaloethyl esters have been used as acyl donors.

A greater challenge, however, is the regioselective discrimination of secondary hydroxyl groups due to the close similarity of their reactivity. This has been accomplished with steroids using anhydrous acetone [48] or benzene as solvent [249]. Due to the more lipophilic character of the solvents used, several lipases have also been employed in addition to subtilisin. For the regioselective acylation of secondary hydroxyl groups of sugar derivatives with blocked primary hydroxyl groups, pyridine [250] or mixed solvent systems (CH_2Cl_2/THF/acetone) have been used [251].

The plant alkaloid castanospermine is a potent glucosidase inhibitor and is currently being considered as an antineoplastic agent and for the treatment of AIDS [252]. Some of the corresponding *O*-acyl derivatives, which have been reported as being more active than castanospermine itself, were obtained by an enzyme-catalyzed regioselective acylation reaction (Scheme 3.19) [253]. Thus, the 1-OH group was selectively acylated using subtilisin as catalyst and *Chromobacterium*



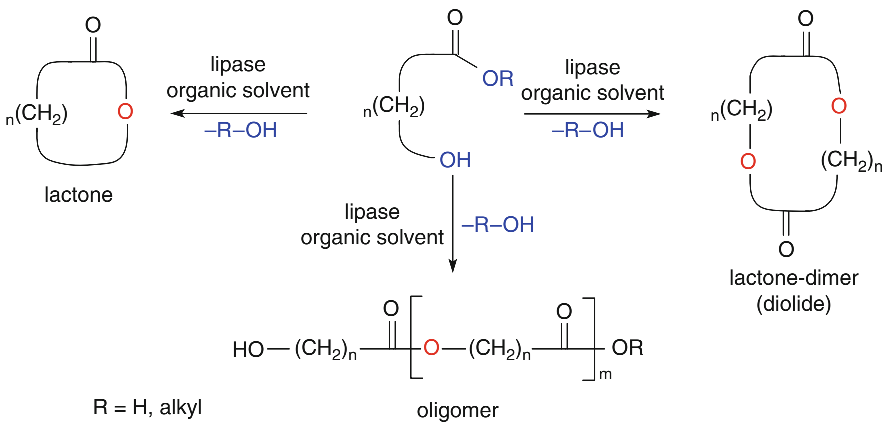
Scheme 3.19 Regioselective acylation of castanospermine

viscosum lipase was employed to esterify the hydroxyl group in position 7. Only minor amounts of the 1,6-isomer were detected.

3.1.2 Lactone Synthesis

Bearing in mind the enzyme-catalyzed esterification and transesterification described in the foregoing chapter, it is not surprising that lactones may be obtained from hydroxy acids or esters by cyclization via *intramolecular* esterification or acyl transfer reactions [254]. Under chemical catalysis, the course of the reaction is relatively simple and the formation of either lactones or open-chain oligomers mainly depends on the ring size of the product: Lactones with less than five or more than seven atoms in the ring are not favored and, thus, linear condensation products are formed predominantly. In contrast, five-membered lactones are easily formed, whereas the formation of six-membered structures is often accompanied by the formation of straight-chain oligomers. Usually the corresponding cyclic dimers – diolides – are not obtained by chemical methods.

In contrast, lipase-catalyzed lactonization often leads to a product pattern that is different from that obtained by chemical catalysis (Scheme 3.20). The outcome depends on several parameters, i.e., the length of the hydroxy acid, the type of lipase, the solvent, the dilution, and even the temperature [255, 256]. In addition, when racemic or prochiral hydroxyacids are employed as substrates, a kinetic resolution [257, 258] or desymmetrization may be accomplished with high selectivities [43]. It is obvious that enzymatic lactone formation is particularly easy with γ-hydroxy derivatives, which lead to the formation of (favored) five-membered ring lactones. The most important synthetic aspect of enzymatic lactone formation, however, is the possibility of directing the condensation reaction towards the formation of macrocyclic lactones and dilactones – i.e., macrolides and macrodiolides, respectively, which are difficult to obtain by chemical catalysis (Scheme 3.20) [44, 259]. This



Scheme 3.20 Lipase-catalyzed formation of lactones, diolides, and oligomers

strategy was employed as the key step in the synthesis of the naturally occurring antifungal agent (−)-pyrenophorin, a 16-membered ring macrocyclic diolide [260].

To ensure a highly desirable irreversible lactonization reaction, the use of hydroxy-substituted vinyl carboxylates has been suggested in analogy to the use of enol esters as acyl donors [261].

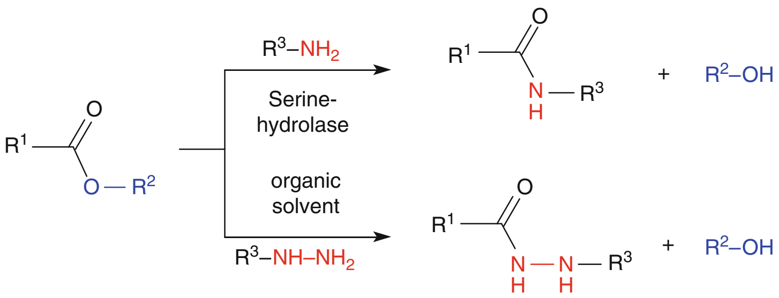
3.1.3 Amide Synthesis

When *N*-nucleophiles such as ammonia, amines or hydrazine are subjected to acyl-transfer reactions, the corresponding *N*-acyl derivatives – amides or hydrazides – are formed through interception of the acyl-enzyme intermediate by the *N*-nucleophile (Schemes 2.1 and 3.21) [262]. Due to the pronounced difference in nucleophilicity of the amine (or hydrazine) as compared to the leaving alcohol (R^2–OH) (Scheme 3.21), *aminolysis* reactions can be regarded as quasi-irreversible. Any type of serine hydrolase which forms an acyl-enzyme intermediate (esterases, lipases, and most proteases) is able to catalyze these reactions. Among them, proteases such as subtilisin and penicillin acylase and lipases from *Candida antarctica* and *Pseudomonas* sp. have been used most often.

Chemoselective Amide Synthesis. Enzyme-catalyzed chemoselective *ammonolysis* [263, 264] or *aminolysis* of esters [265] may be advantageous for the synthesis of carboxamides bearing an additional functional group, which is susceptible to nucleophilic attack, for instance, β-keto-, α,β-unsaturated, or propargylic amides [266]. The latter compounds cannot be obtained by using chemical catalysis due to competing side reactions which lead to enaminoesters and Michael adducts, respectively. The analogous *hydrazinolysis* of esters leading to the formation of hydrazides under mild reaction conditions, may be performed using enzymatic catalysis in a similar manner [267–269].

Enantioselective Amide Synthesis. More important, however, are transformations where chirality is involved. As may be deduced from Scheme 3.21, three different types of chiral recognition are possible, depending on the location of the chiral center in either R^1, R^2, or R^3.

- Esters of chiral acids (chiral R^1) can be resolved via acyl transfer using *N*-nucleophiles [45, 265].
- Chiral alcohols (center in R^2) may be separated via their esters through ammonolysis or aminolysis in a similar fashion [270].
- The most intriguing aspect, however, lies in the enantioselective *formation* of amides, where the center of chirality is located on the amine (chiral R^3) [271]. Thus, kinetic resolution of amines may be achieved [272–274].
- If both the ester and the amine are chiral, *diastereomeric* amides are formed, going in hand with recognition of both chiral entities [275].



Scheme 3.21 Ammonolysis, aminolysis, and hydrazinolysis of esters

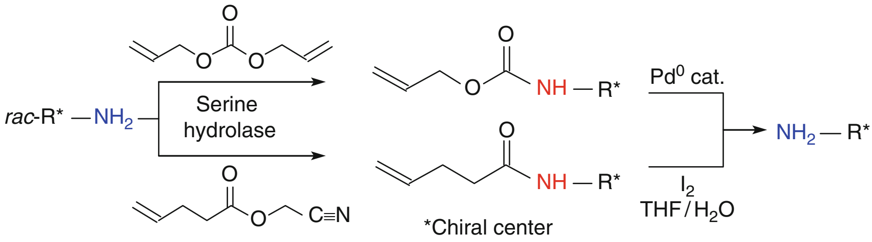
Because the nucleophilicity of an amine is significantly larger than that of an alcohol, the choice of the acyl donor is of crucial importance [276]. Many activated esters, such as ethyl trifluoroacetate and trifluoroethyl butanoate used as acyl donors for the acylation of alcohols are too reactive and lead to a certain amount of spontaneous (nonselective) background reaction, which causes a depeletion of selectivity. Less reactive acyl donors, such as benzyl *iso*-propenyl carbonate might be used in a solvent system (e.g., 3-methylpentan-3-ol), which suppresses the background reaction.[2] Simple carboxylic acid esters, such as ethyl acetate, may be used, but the (chemical) cleavage of the resulting carboxamides is rather difficult and requires harsh reaction conditions, which preclude the presence of other sensitive functional groups in the molecule. The acyl donors of choice for aminolysis reactions are as follows:

- Ethyl methoxyacetate yields a fast reaction rate in lipase-catalyzed aminolysis (i.e. about 100 times faster than ethyl butanoate) and the *N*-methoxyacetamides

[2] In principle, ethyl fluoroacetate would also fall into this category. However, its use is not recommended since fluoroacetic acid is a severe toxin by acting as inhibitor of the Krebs-cycle.

thus formed can be hydrolyzed under reasonably mild conditions. This technique is used for the resolution of amines on a multi-ton industrial scale [277].

- Diallyl carbonate leads to the formation of allyl carbamates (Scheme 3.22). The free amines can be selectively deprotected by using mild Pd(0)-catalysis [278].
- The use of cyanomethyl 4-pentenoate as acyl donor furnishes 4-pentenoyl carboxamides (Scheme 3.22) [279]. It is not only well accepted by serine-hydrolases, but can be cleaved using I_2 in THF/H_2O.

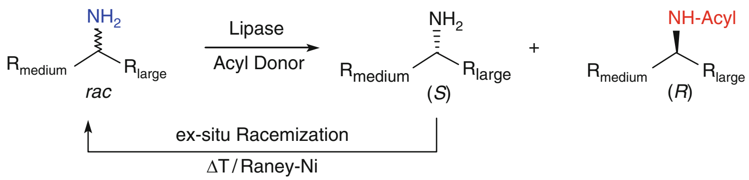


Scheme 3.22 Special acyl donors for the resolution of amines via aminolysis reactions

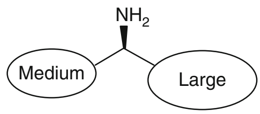
Representative examples for the resolution of amines via lipase-catalyzed aminolysis are given in Scheme 3.23. Among numerous enzymes, lipases from *Candida antarctica* and *Pseudomonas* sp. have been proven to be most useful [280]. Since primary amines of the type $R^1R^2CH–NH_2$ are isosteric with secondary alcohols, the rule predicting the faster reacting enantiomer in a lipase-catalyzed reaction (for *sec*-alcohols this rule is commonly referred to as ‘Kazlauskas rule’, Scheme 2.49) can be applied [281]. Thus, (*R*)-amines are preferentially acylated if the CIP-sequence priority of the substituents is large > medium. This process has been scaled up to a capacity of >1,000 t/year using ethyl methoxyacetate as acyl donor and produces a wide variety of α-chiral primary amines for pharma- and agro-applications, among them (*S*)-methoxy-isopropylamine, which represents the key building block for the herbicide Outlook™. The separation of formed amide from nonreacted amine can be achieved via extraction and undesired amine enantiomers are recycled via ex-situ racemization using Raney-Ni as catalyst [282].

In contrast to the facile in-situ racemization of *sec*-alcohols via Ru-catalysts (Schemes 3.14 and 3.17), which allows dynamic resolution, the isomerization of α-chiral amines requires more drastic conditions. Hydrogen transfer catalyzed by Pd [283, 284], Ru [285, 286] Ni, or Co [287] is slow and requires elevated temperatures close to 100°C, which still requires the spatial separation of (metal-catalyzed) racemization from the lipase aminolysis [288].

Interestingly, lipase-catalyzed acyl transfer onto SH-groups (corresponding to ‘ester thiolysis’ in analogy to aminolysis) does not take place [289]. As a result, the resolution of *sec*-thiols is not feasible by this method and has to be performed via hydrolysis or alcoholysis of the corresponding thioesters [290–292].



	R_{medium}	R_{large}
Aryl–alkyl	Me, Et, *c*-Pr	Ph, *p*-Cl–C_6H_4, *o,p*-di-Cl–C_6H_3, *m*- or *p*-MeO–C_6H_4, *p*-Me–C_6H_4, 1-naphthyl, 2-pyridyl.
Alkyl–alkyl	Me	Et, *n*-Pr, *i*-Pr, *c*-Pr, *t*-Bu Ph–$(CH_2)_{1,2}$–
Alkoxy–alkyl	Me	Ph–CH_2–O–$(CH_2)_{1,2}$– MeO–CH_2–



Reacting enantiomer

Scheme 3.23 Lipase-catalyzed dynamic resolution of amines via ester aminolysis

3.1.4 Peptide Synthesis

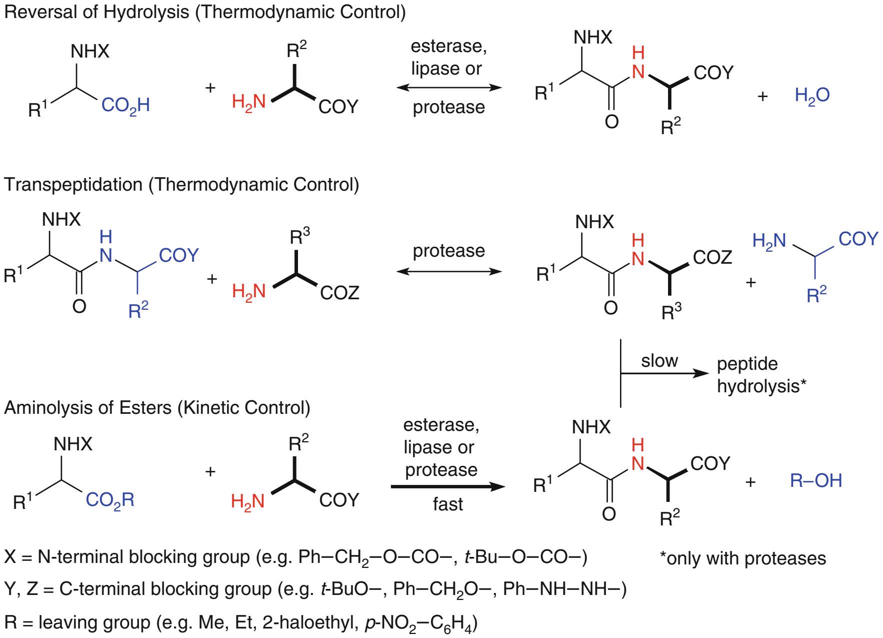
Peptides display a diverse range of biological activity. They may be used as sweeteners and toxins, antibiotics and chemotactic agents, as well as growth factors. They play an important role in hormone release either as stimulators or as inhibitors. The most recent application is their use as immunogens for the generation of specific antisera. At present, the most frequently used methods of peptide synthesis are purely chemical in nature, generally proceeding through a sequence of four steps. (a) First, all the functionalities of the educts, which are not to participate in the reaction, must be selectively protected. (b) Then, the carboxyl group must be activated in a second step to enable (c) the formation of the peptide bond. (d) Finally, the protective groups have to be removed in toto, if the synthesis is complete, or the amino- or carboxy-terminus must be selectively liberated if the synthesis is to be continued. Thus, an extensive protection and deprotection methodology is required. Two of the major problems associated with chemical peptide synthesis are a danger of racemization – particularly during the activation step – and the tedious (and sometimes impossible) purification of the final product from (diastereo)isomeric peptides with a closely related sequence. To circumvent these problems, peptide synthesis is increasingly carried out by making use of the specificities of enzymes, in particular proteases [46, 293–299]. Occasionally, the proteases used for peptide synthesis are also misleadingly called ‘peptide ligases’ [300, 301], they are, however, simple hydrolases and have nothing in common with class [EC 6.x.x.x].

Enzyme-catalyzed peptide synthesis has been intensely investigated during the last decades, although the first report on an enzyme-catalyzed peptide synthesis dates back to 1938 [302]. The pros and cons of conventional versus enzymatic peptide synthesis are summarized in Table 3.6.

Table 3.6 Pros and cons of chemical and enzymatic peptide synthesis

	Chemical	Enzymatic
Stereoselectivity	Low	High
Regioselectivity	Low	High
Amino acid range	Broad	Limited
Protective group requirements	High	Low
Purity requirements of starting materials	High	Moderate
Byproducts	Some	Negligible
Danger of racemization	Some	None

Enzyme-catalyzed peptide synthesis may be conducted via three basic ways: *Reversal of hydrolysis*, *transpeptidation* (which is used to a lesser extent), and *aminolysis of esters* (Scheme 3.24).



Scheme 3.24 Principles of enzymatic peptide synthesis

Thermodynamic Approach. Both of the former methods represent a reversible type of reaction and are therefore thermodynamically controlled.

Under physiological conditions, the equilibrium position in protease-catalyzed reactions is far over on the side of proteolysis. In order to create a driving force in the reverse direction towards peptide *synthesis*, the following constraints may be applied.

- One of the reactants is used in excess.
- Removal of product via formation of an insoluble derivative [303], by specific complex formation [304], or by extraction of the product into an organic phase by using a water-immiscible organic cosolvent.

- Lowering the water-activity (concentration) of the system by addition of water-miscible organic cosolvents. In this respect, polyhydroxy compounds such as glycerol or butane-1,4-diol have been shown to conserve enzyme activity better than the solvents which are more commonly employed, such as DMF, DMSO, ethanol, acetone, or acetonitrile [305]. Alternatively, peptide synthesis may also be performed with neat reactants – i.e., in the absence of solvents [306].

Kinetic Approach. The third method – aminolysis of esters – involves a kinetically controlled irreversible reaction, in which two nucleophiles (water and an amine) are competing for the acyl-enzyme intermediate [307]. As mentioned above this reaction can be regarded as irreversible. Thus, it is not surprising that besides proteases, other serine hydrolases which are capable of forming an acyl-enzyme intermediate (mainly lipases such as PPL [308], PLE, and CRL [309, 310]) may used for this reaction (Schemes 3.21 and 3.25). On the other hand, this method is not applicable to metallo- and carboxyproteases. Since the peptide formed during ester aminolysis may be hydrolytically cleaved in a subsequent reaction in the presence of water, these reactions have to be terminated before the equilibrium is reached (Fig. 3.2). Thus, the kinetics of enzymatic peptide synthesis has a strong resemblance to glycosyl transfer reactions (Sect. 2.6.2) [311]. Undesired peptide hydrolysis may be suppressed by performing the reaction in a frozen aqueous medium at –15°C [312]. Of course peptide hydrolysis may be neglected when nonproteolytic hydrolases, such as esterases and lipases, are used.

In general, naturally occurring (*N*-protected) L-amino acid esters of short-chain alcohols, such as methyl and ethyl esters are usually sufficiently reactive as ‘acceptors’ to achieve reasonable reaction rates in enzymatic peptide synthesis via aminolysis. For less reactive (nonnatural) analogs, such as α-substituted [313] or D-configured amino acids [314], activated esters are recommended, among them, 2-haloethyl (e.g., 2-chloroethyl, trifluoroethyl) [315], *p*-nitrophenyl [316], or guanidinophenyl esters [317]. For the use of ‘cyclic activated esters’ [5(4*H*)-oxazolones] see below.

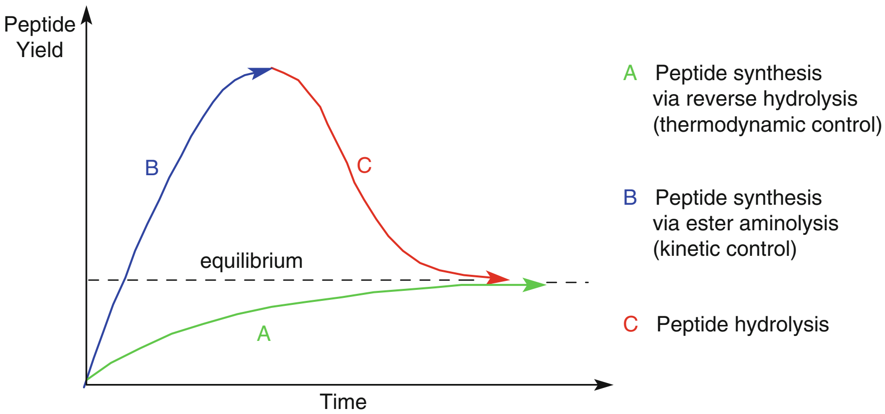


Fig. 3.2 Enzymatic peptide synthesis under thermodynamic and kinetic control

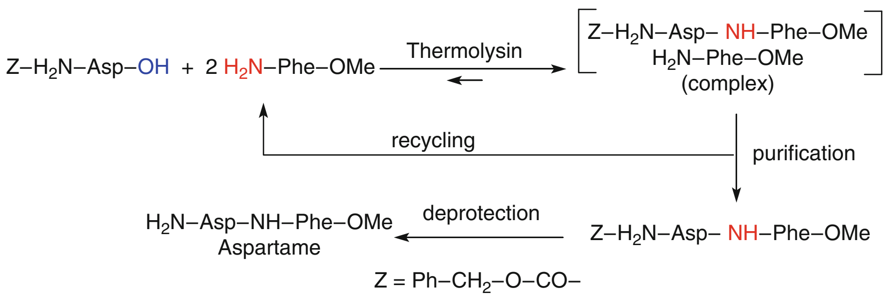
Because all hydrolases exhibit a certain substrate selectivity, the availability of a library of enzymes which can cover all possible types of peptide bonds is of crucial importance in order to make enzymatic peptide synthesis applicable to all possible amino acid combinations. Although the existing range of proteases is far from complete, it provides a reasonable coverage (Table 3.7). The most striking 'shortage' involves proline derivatives. The most commonly used proteases and their approximate selectivities are listed below, where X stands for an unspecified amino acid or peptide residue.

Table 3.7 Selectivities of proteases

Protease	Type	Specificity
Achromobacter protease	Serine protease	–Lys–X
α-Chymotrypsin, subtilisins	Serine protease	–Trp(Tyr,Phe,Leu,Met)–X
Carboxypeptidase Y	Serine protease	nonspecific
Elastase	Serine protease	–Ala(Ser,Met,Phe)–X
Trypsin, *Streptomyces griseus* protease	Serine protease	–Arg(Lys)–X
Staphylococcus aureus V8 protease	Serine protease	–Glu(Asp)–X
Papain, ficin	Thiol protease	–Phe(Val,Leu)–X
Clostripain, cathepsin B	Thiol protease	–Arg–X
Cathepsin C	Thiol protease	H–X–Phe(Tyr,Arg)–X
Thermolysin, *B. subtilis* protease	Metalloprotease	–Phe(Gly,Leu)–Leu(Phe)
Myxobacter protease II	Metalloprotease	X–Lys
Pepsin, cathepsin D	Carboxyl protease	–Phe(Tyr,Leu)–Trp(Phe,Tyr)

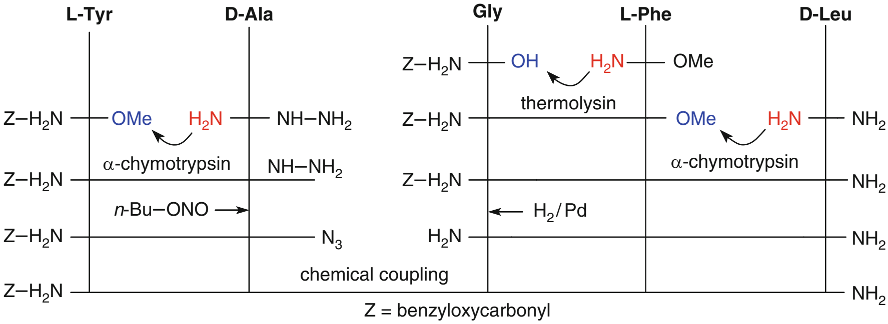
X = unspecified amino acid or peptide residue

The dipeptide ester L-Asp-L-Phe-OMe (Aspartame) is used in large amounts as a low-calorie sweetener. One of the most economical strategies for its synthesis involves an enzymatic step, which is run at a capacity of 2,000 t/year (Scheme 3.25). Benzyloxycarbonyl-(Z)-protected L-aspartic acid is linked with L-Phe–OMe in a thermodynamically controlled condensation reaction catalyzed by the protease thermolysin without formation of the undesired (bitter) β-isomer. Removal of the product via formation of an insoluble salt was used as driving force to shift the equilibrium of the reaction in the synthetic direction [318, 319].



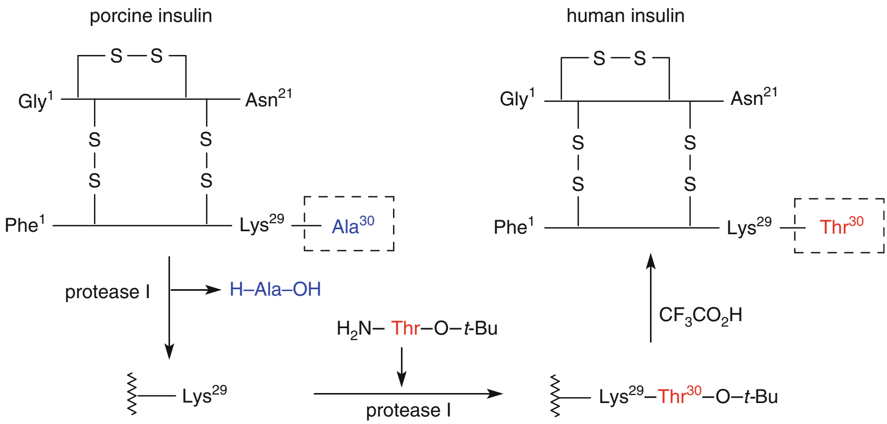
Scheme 3.25 Enzymatic synthesis of Aspartame

The wide potential of enzymatic peptide synthesis is illustrated with the chemoenzymatic synthesis of an enkephalin derivative – (D-Ala$_2$, D-Leu$_5$)-enkephalin amide (Scheme 3.26) [320]. Enkephalins are pain regulators and commonly undergo rapid enzymatic degradation in vivo, and therefore exert only limited biological activities. However, by substitution of a D-amino acid into the sequence, 'chirally muted' derivatives are obtained, which can elicit long-lasting pharmacological effects [321]. The Tyr-D-Ala and Gly-Phe subunits were obtained via an ester aminolysis and a condensation reaction using α-chymotrypsin and thermolysin, respectively. The latter dipeptide was extended by a D-Leu unit using the same methodology. After converting the C-terminal hydrazide protective group into an azide moiety and removal of the *N*-terminal Z-group by hydrogenolysis, the fragments were coupled by conventional methodology.



Scheme 3.26 Chemoenzymatic synthesis of an enkephalin derivative

Probably the most prominent example of an enzymatic peptide synthesis is the transformation of porcine insulin into its human counterpart (Scheme 3.27). As millions of diabetics suffer from insulin deficiency, and due to the fact that the demands cannot be satisfied by exploiting natural sources for obvious reasons,



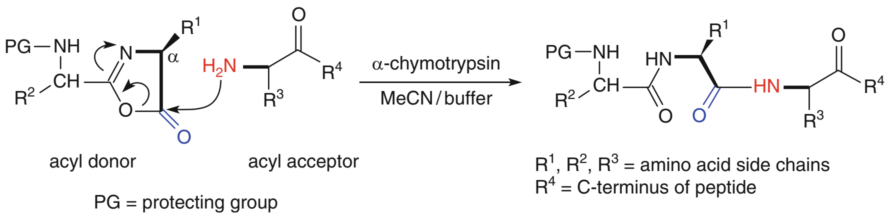
Scheme 3.27 Enzymatic conversion of porcine into human insulin

numerous attempts have been undertaken to convert porcine insulin into human insulin via exchange of a terminal alanine by a threonine residue [322–324]. A protease from *Achromobacter lyticus* which is completely specific for peptide bonds formed by a lysine residue [325] is used to selectively hydrolyze the terminal Ala^{30} residue from porcine insulin. The same enzyme is then used to catalyze the condensation reaction with threonine, protected as its *tert*-butyl ester. Finally, the *tert*-butyl group was removed by acid treatment to yield human insulin [326].

A special type of enzymatic peptide synthesis employs activated heterocyclic amino acid/peptide derivatives as acyl donors (Scheme 3.28) [327]. In a kinetically controlled approach, 5(4*H*)-oxazolones (which may be regarded as 'cyclic activated esters') are cleaved by α-chymotrypsin thereby generating an acyl enzyme intermediate. Then the amino acid/peptide segment is coupled onto the *N*-terminus of the acyl acceptor by forming a new peptide bond.

Some features of this technique are worthy of attention:

- Racemization of the activated acyl donor involving its α-center is largely suppressed.
- When racemic acyl donors are employed (with respect to the α-center), kinetic resolution proceeds with incomplete selectivity and with moderate preference for the L-enantiomer.
- Protection of the C-terminal carboxyl moiety (R^4) is not required since the oxazolone is a much better acyl donor than the (competing) carboxyl group on the acyl acceptor.
- The chemical yields may be diminished by undesired enzymatic hydrolysis of the acyl donor, the extent of which depends on the nature of the acyl donor.

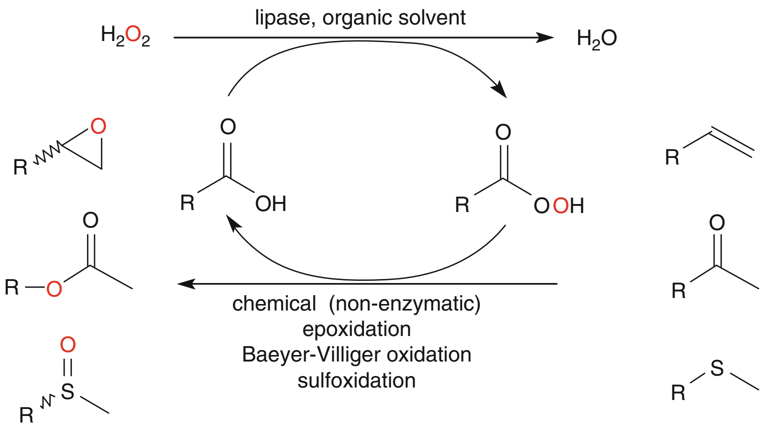


Scheme 3.28 Peptide synthesis using 5(4*H*)-oxazolones as acyl donors

3.1.5 Peracid Synthesis

In contrast to the acidic conditions usually applied for in situ generation of peroxycarboxylic acids, they may be generated under virtually neutral conditions in a suitable organic solvent directly from the parent carboxylic acid and hydrogen peroxide via lipase catalysis (Scheme 3.29).

The mechanism involves a perhydrolysis of the acyl-enzyme intermediate by the nucleophile hydrogen peroxide (Scheme 2.1) [328]. The peroxy acids thus formed



Alkene	Yield of epoxide [%]
1-Octene	~5
3-Ethylpent-2-ene	~80
Tetramethylethylene	>95
Cyclohexene	>95

Scheme 3.29 Lipase-catalyzed peracid formation and catalytic epoxidation

can be used in situ for the epoxidation of alkenes [329], the Baeyer-Villiger oxidation of carbonyl compounds [330] and the sulfoxidation of thioethers, while the liberated fatty acid re-enters the cyclic process [331, 332]. It should be noted that the epoxidation reaction itself takes place without involvement of the enzyme; therefore no significant enzyme-induced selectivities were detected.

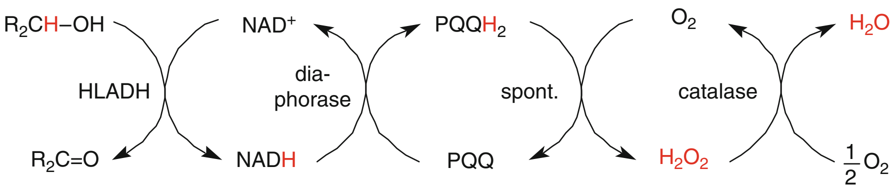
The main advantages of this method are the mild conditions employed and a higher safety margin due to the fact that only catalytic concentrations of peracid are involved. This aspect is particularly important for oxidation reactions on an industrial scale, such as the sulfur-oxidation of penicillin G into its 1-(*s*)-oxide, which is a key intermediate en route to cephalosporins [333]. Medium-chain alkanoic acids (C_8–C_{16}) and a biphasic aqueous-organic solvent system containing toluene or *tert*-butanol give the best yields. Among various lipases tested, an immobilized lipase from *Candida antarctica* was shown to be superior to lipases from *Candida rugosa* and *Pseudomonas* sp.

3.1.6 Redox Reactions

In contrast to hydrolases, redox enzymes such as dehydrogenases and oxidases have been used less often in organic solvents because they require cofactors, e.g., nicotinamide adenine dinucleotide species. The latter are highly polar (charged) compounds and are therefore completely insoluble in a lipophilic medium. As a consequence, the cofactor is irreversibly bound to a protein molecule and cannot freely be exchanged between enzymes, which is necessary for its recycling. These limitations can be circumvented to some extent by co-precipitation of enzyme and cofactor onto a macroscopic carrier provided that a minimum amount of water is present [334, 335]. Thus, the cofactor is able to freely enter and exit the active site of the enzyme but it cannot disaggregate from the carrier into the medium because it is trapped together with the enzyme in the hydration layer on the carrier surface. As a result, acceptable turnover numbers are achieved due to an improved cofactor stability [336–338].

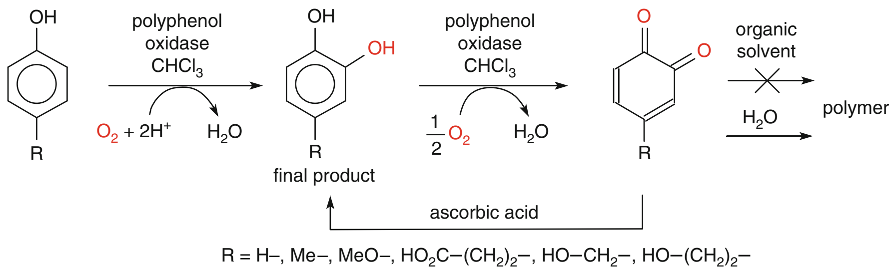
Redox reactions catalyzed by alcohol dehydrogenases (e.g., from horse liver, HLADH) may be performed in organic solvents in both the reduction and oxidation mode, if the recycling system is appropriately modified (Sect. 2.2.1). Reduction of aldehydes/ketones and oxidation of alcohols is effected by NADH- or NAD^+-recycling, using ethanol or *iso*butyraldehyde respectively.

An alternative way of NAD^+ recycling makes use of a three-enzyme cascade with molecular oxygen as the ultimate oxidant (Scheme 3.30) [339]. As in the methods described above, all the enzymes and cofactors have to be precipitated together. Thus, NADH which is produced by HLADH-catalyzed oxidation of a secondary alcohol is re-oxidized by diaphorase at the expense of pyrroloquinoline quinone (PQQ) [340]. The reduced form of the latter ($PQQH_2$) is spontaneously oxidized by molecular oxygen producing hydrogen peroxide, which, in turn, is destroyed by catalase.



Scheme 3.30 NAD^+-Recycling via a diaphorase-catalase system

Polyphenol oxidase catalyzes the hydroxylation of phenols to catechols and subsequent dehydrogenation to *o*-quinones (Sect. 2.3.2.2, Scheme 2.153) [341]. The preparative use of this enzyme for the regioselective hydroxylation of phenols is impeded by the instability of *o*-quinones in aqueous media, which rapidly polymerize to form polyaromatic pigments leading to enzyme deactivation [342]. Since water is an essential component of the polymerization reaction, the *o*-quinones formed are stable when the enzymatic reaction is performed in an organic solvent (Scheme 3.31). Subsequent nonenzymatic chemical reduction of the *o*-quinones (e.g., by ascorbic acid) to form stable catechols leads to a net regioselective hydroxylation of phenols [343]. Depending on the substituent R in the *p*-position, cresols were obtained in good yields. Electron-withdrawing and bulky substituents decreased the reactivity, and *o*-, and *m*-cresols were unreactive. The preparative use of this method was demonstrated by the conversion of *N*-acetyl-L-tyrosine ethyl ester into the corresponding L-DOPA-derivative. An



Scheme 3.31 Polyphenol-oxidase-catalyzed regioselective hydroxylation of phenols

incidential observation that the related enzyme horseradish peroxidase remains active in nearly anhydrous organic solvent was reported in the late 1960s [344].

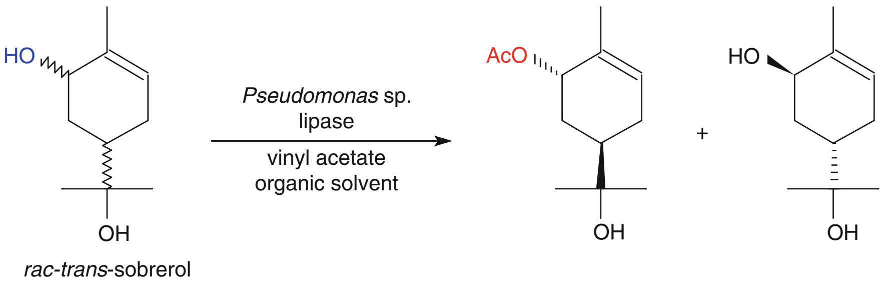
3.1.7 Medium Engineering

As may be deduced from the introductory chapter, any solvent exerts a significant influence on the conformation of an enzyme, which in turn governs its catalytic efficiency and its chemo-, regio- and stereoselectivity. Thus, it is reasonable to expect that an enzyme's specificity may be controlled by varying the solvent's properties. For reactions performed in water, however, this is hardly possible, because its physicochemical properties are fixed by Nature and can only be altered within a very narrow margin, e.g., by addition of water-miscible (polar) organic cosolvents at low concentrations (p. 79). On the other hand, when a reaction is performed in an organic solvent, the latter can be chosen from a large repertoire having different physicochemical parameters, such as dipole moment, polarity, solubility, boiling point, straight-chain or cyclic structure, etc. Therefore, the outcome of an enzyme-catalyzed reaction may be controlled by choosing the appropriate organic solvent [345]. The modulation of enzyme specificity by variation of the solvent properties has been commonly denoted as 'medium engineering' [77].

For instance, the almost exclusive specificity of proteases for L-configurated amino acid derivatives may be 'destroyed' when reactions are carried out in organic solvents [346]. This makes them useful for the synthesis of peptides containing nonnatural D-amino acids, which are usually not substrates for proteases.

The influence of organic solvents on enzyme enantioselectivity is not limited to the group of proteases, but has also been observed with lipases, and is a general phenomenon [347–351]. As a rule of thumb, the stereochemical preference of an enzyme for one specific enantiomer usually remains the same, although its selectivity may vary significantly depending on the solvent. In rare cases, however, it was possible to even invert an enzyme's enantioselectivity [352–354].

As shown in Scheme 3.32, resolution of the mycolytic drug *trans*-sobrerol was achieved by acyl transfer using vinyl acetate and PSL as the catalyst. The selectivity



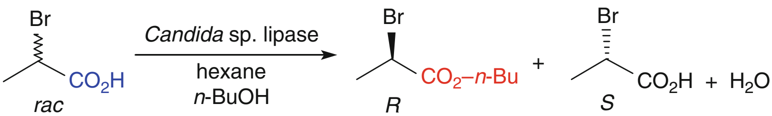
Solvent	log P	Dielectric constant (ε)	Selectivity (*E*)
Vinyl acetate	0.31	–	89
THF	0.49	7.6	69
Acetone	−0.23	20.6	142
Dioxane	−1.14	2.2	178
3-Pentanone	0.80	17.0	212
t-Amyl alcohol	1.45	5.8	518

Scheme 3.32 Optimization of selectivity by solvent variation

of the reaction markedly depended on the solvent used, with *tert*-amyl alcohol being best. As may be deduced from the physicochemical data given, any attempts to link the observed change in selectivity to the lipophilicity (expressed as the log *P*) or the dielectric constant (ε) were unsuccessful.

Besides the nature of the solvent itself, it is also the intrinsic water content (more precisely, the water activity, a_W), which has an influence on the enzyme selectivity (Scheme 3.33). For instance, the resolution of 2-bromopropionic acid by esterification using *Candida rugosa* lipase proceeded with a significantly enhanced selectivity when the water content of the solvent was gradually increased [355].

The whole set of data available so far demonstrates that the nature of the organic solvent and its water content exerts a strong influence on the catalytic properties of an enzyme. However, no general rationale, which would allow the prediction of



Added water [%]	Initial rate [%]	Selectivity [*E*]
0	11	17
0.05	21	29
0.075	66	39
0.125	100	81

Scheme 3.33 Optimization of selectivity by adjusting the water content

the selectivity enhancement mediated by medium engineering has been presented so far [356].

3.2 Immobilization

In practice, three significant drawbacks are often encountered in enzyme-catalyzed reactions.

- Many enzymes are not sufficiently stable under the operational conditions and they may lose catalytic activity due to auto-oxidation, self-digestion and/or denaturation by the solvent, the solutes or due to mechanical shear forces.
- Since enzymes are water-soluble molecules, their repeated use, which is important to ensure their economic application [357], is problematic due to the fact that they are difficult to recover from aqueous systems and to separate from substrates and products.
- The productivity of industrial processes, measured as the space-time yield is often low due to the limited tolerance of enzymes to high concentrations of substrate(s) and product(s).

These problems may be overcome by 'immobilization' of the enzyme [358–369]. This technique involves either the attachment of an enzyme onto a solid support via coupling onto a carrier or linkage of the enzyme molecules to each other via cross-linking. Alternatively, the biocatalyst may be confined to a restricted area from which it cannot leave but where it remains catalytically active by entrapment into a solid matrix or a membrane-restricted compartment. Thus, *homogeneous* catalysis using a native enzyme turns into *heterogeneous* catalysis when immobilized biocatalysts are employed. Depending on the immobilization technique, the properties of the biocatalyst such as stability, selectivity [370, 371], k_{cat} and K_m value, pH and temperature characteristics, may be significantly altered [372] – sometimes for the better, sometimes for the worse. At present, predictions about the effects of immobilization are very difficult to make. The following immobilization strategies are most widely employed (Fig. 3.3) [373, 374].

Adsorption
Adsorption of a biocatalyst onto a water-insoluble macroscopic carrier is the easiest and oldest method of immobilization. It may be equally well applied to isolated enzymes as well as to whole viable cells. For example, adsorption of whole cells of *Acetobacter* onto wood chips for the fermentation of vinegar from ethanol was first used in 1815! Adsorbing forces are of different types, such as van der Waals (London) forces, ionic interactions, and hydrogen bonding, and are all relatively weak. The appealing feature of immobilization by adsorption is the simplicity of the procedure. As a result of the weak binding forces, losses in enzyme activity are usually low, but desorption (leakage) from the carrier may be caused by even minor

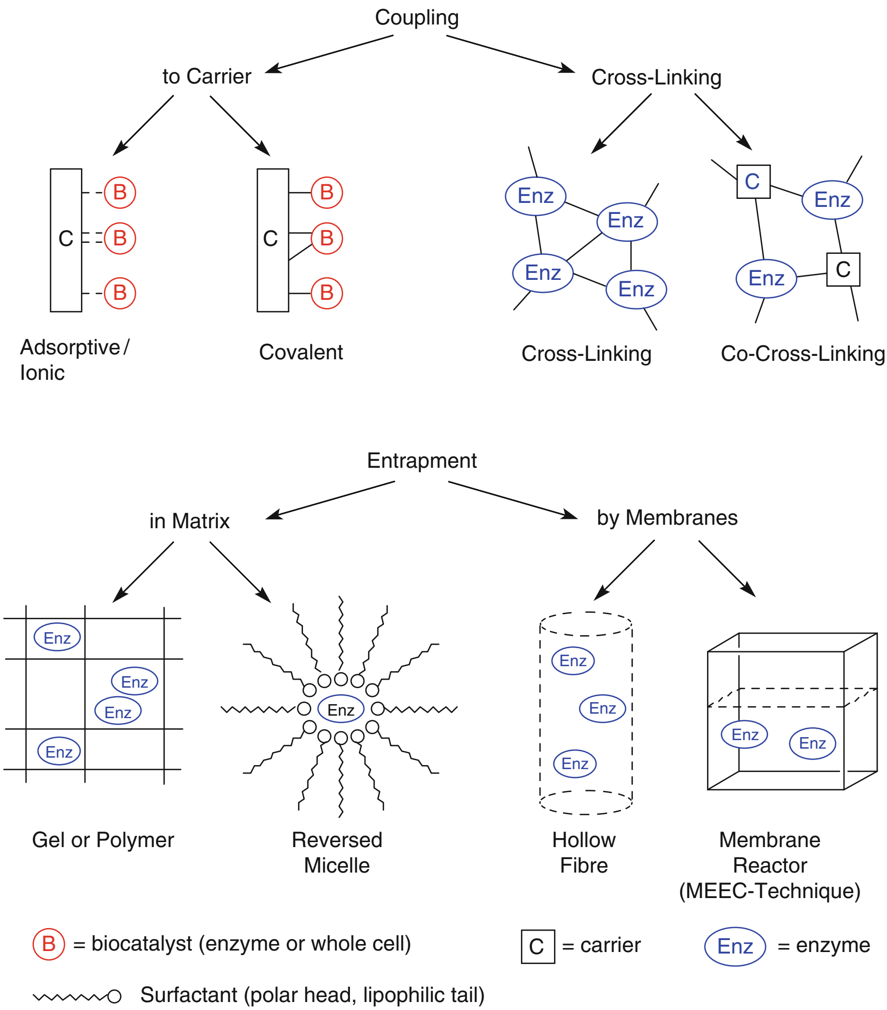


Fig. 3.3 Principles of immobilization techniques

changes in the reaction parameters, such as a variation of substrate concentration, the solvent, temperature, or pH.

Numerous inorganic and organic materials have been used as carriers: activated charcoal [375], alumina [376], silica [377, 378], diatomaceous earth (Celite) [165, 379], cellulose [380], controlled-pore glass, and synthetic resins [381]. In contrast to the majority of enzymes, which preferably adsorb to materials having a polar surface, lipases are better adsorbed onto lipophilic carriers due to their peculiar physicochemical character (Sect. 2.1.3.2) [72, 382–385]. Adsorption is the method of choice when enzymes are used in lipophilic organic solvents, where desorption cannot occur due to their insolubility in these media.

Ionic Binding

Due to their polar surfaces, ion exchange resins readily adsorb proteins. Thus, they have been widely employed for enzyme immobilization. Both cation exchange resins such as carboxymethyl cellulose or Amberlite IRA [386], and anion exchange resins, e.g., *N*,*N*-diethyl-aminoethylcellulose (DEAE cellulose) [387] or sephadex [388], are used industrially. Although the binding forces are stronger than the forces involved in simple physical adsorption, ionic binding is particularly susceptible to the presence of other ions. As a consequence, proper maintenance of ion concentrations and pH is important for continued immobilization by ionic binding and for prevention of desorption of the enzyme. When the biocatalytic activity is exhausted, the carrier may easily be reused by reloading it with fresh biocatalyst.

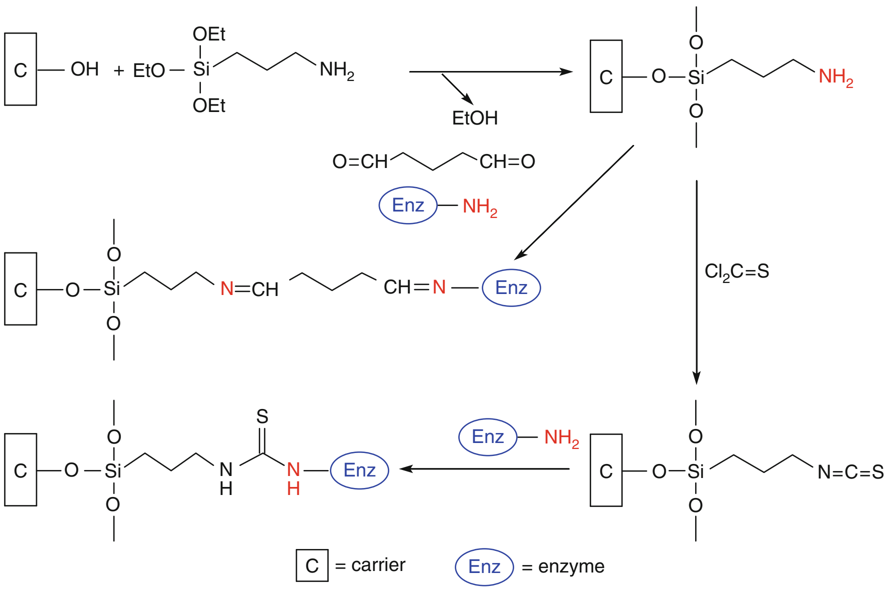
Covalent Attachment

Covalent binding of an enzyme onto a macroscopic carrier leads to the irreversible formation of stable chemical bonds, thus inhibiting leakage completely. A disadvantage of this method is that rather harsh conditions are required since the biocatalyst must undergo a chemical reaction. Consequently, some loss of activity is always observed [389, 390]. As a rule of thumb, each bond attached to an enzyme decreases its native activity by about one fifth. Consequently, residual activities generally do not exceed 60–80% of the activity of the native enzyme, and values of around 50% are normal. The functional groups of the enzyme which are commonly involved in covalent binding are nucleophilic, i.e., mainly *N*-terminal and ε-amino groups of lysine, but also carboxy-, sulfhydryl-, hydroxyl-, and phenolic functions. In general, covalent immobilization involves two steps, i.e., (a) activation of the carrier with a reactive 'spacer' group and (b) enzyme attachment. Since viable cells usually do not survive the drastic reaction conditions required for the formation of covalent bonds, this type of immobilization is only recommended for isolated enzymes.

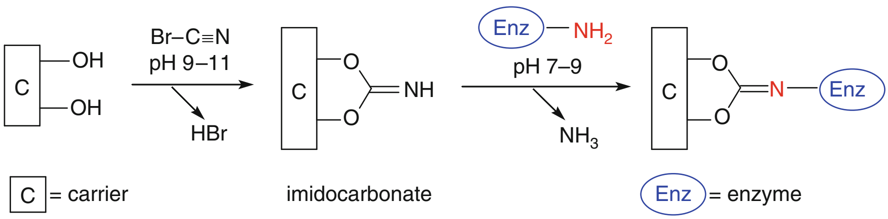
Porous glass is a popular inorganic carrier for covalent immobilization [391]. Activation is achieved by silylation of the hydroxy groups using aminoalkylethoxy- or aminoalkyl chlorosilanes as shown in Scheme 3.34. In a subsequent step, the aminoalkyl groups attached to the glass surface are either transformed into reactive isothiocyanates or into Schiff bases by treatment with thiophosgene or glutardialdehyde, respectively. Both of the latter species are able to covalently bind an enzyme through its amino groups.

Carriers based on natural polymers of the polysaccharide type (such as cellulose [392], dextran [393], starch [394], chitin, or agarose [395]) can be useful alternatives to inorganic material due to their well-defined pore size. Activation is achieved by reaction of adjacent hydroxyl groups with cyanogen bromide leading to the formation of reactive imidocarbonates (Scheme 3.35). Again, coupling of the enzyme involves its amino groups. To avoid the use of hazardous cyanogen bromide, some of these pre-activated polysaccharide-type carriers may be obtained from commercial sources.

During recent years, synthetic copolymers (e.g., based on polyvinyl acetate) have become popular (Scheme 3.36) [396–398]. Partial hydrolysis of some of the acetate



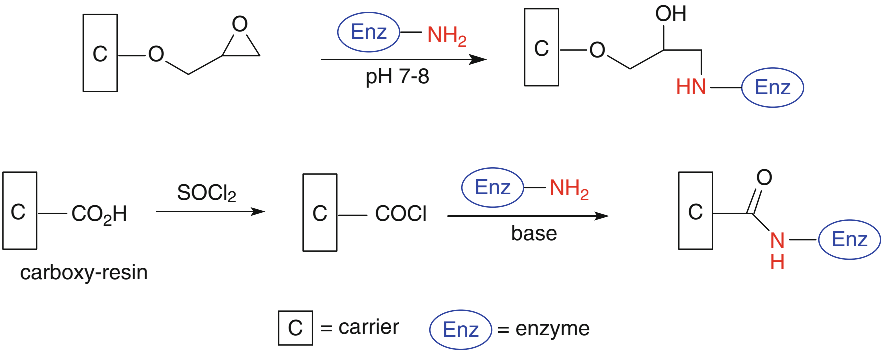
Scheme 3.34 Covalent immobilization of enzymes onto inorganic carriers



Scheme 3.35 Covalent immobilization of enzymes onto natural polymers

groups liberates a hydroxyl functionality, which is activated by epichlorohydrin [399]. A number of such epoxy-preactivated resins are commercially available. Among them, Eupergit C™ is widely used. It is a macroporous copolymer of *N*,*N′*-methylene-bi-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide with an average particle size of 170 µm and a pore diameter of 25 nm [400]. Enzyme attachment occurs with the formation of a stable C–N bond via nucleophilic opening of the epoxide groups by amino groups of the enzyme under mild conditions. In contrast to the majority of the above-mentioned covalent immobilization reactions, which remove a positive charge from the enzyme's surface (due to the formation of Schiff bases or acylation involving an amino group), this method preserves the charge distribution of the enzyme since it constitutes an *N*-alkylation process.

Alternatively, cation exchange resins can be activated by transforming their carboxyl groups into acid chlorides, which then form stable amide bonds with the amino groups of an enzyme.

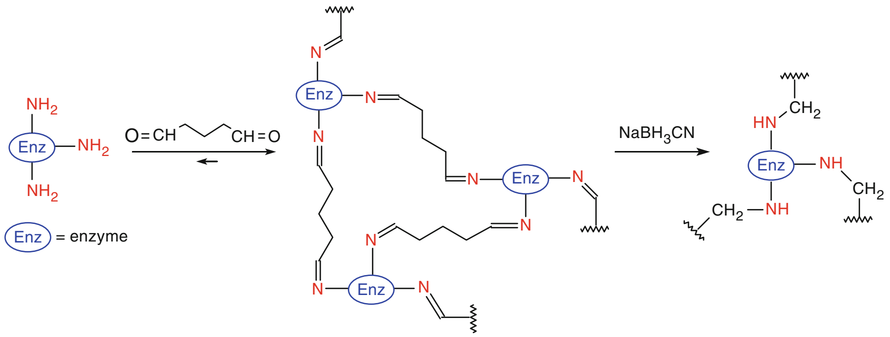


Scheme 3.36 Covalent immobilization of enzymes onto synthetic polymers

A novel approach to immobilization of enzymes via covalent attachment is the use of stimulus-responsive 'smart' polymers, which undergo dramatic conformational changes in response to small alterations in the environment, such as temperature, pH, and ionic strength [401–403]. The most prominent example is a thermo-responsive and biocompatible polymer (poly-*N*-isopropyl-acrylamide), which exhibits a critical solution temperature around 32°C, below which it readily dissolves in water, while it precipitates at elevated temperatures due to the expulsion of water molecules from its polymeric matrix. Hence, the biotransformation is performed under conditions, where the enzyme is soluble. Raising the temperature leads to precipitation of the immobilized protein, which allows its recovery and reuse. In addition, runaway reactions are avoided because in case the reaction temperature exceeds the critical solution temperature, the catalyst precipitates and the reaction shuts down.

Cross-Linking

The use of a carrier inevitably leads to 'dilution' of catalytic activity owing to the introduction of a large proportion of inactive ballast, ranging from 90 to $>$ 99%, which leads to reduced productivities. This can be avoided via attachment of enzymes *onto each other* via 'cross-linking' through covalent bonds [404, 405]. By this means, insoluble high-molecular aggregates are obtained. The enzyme molecules may be crosslinked either with themselves or may be co-crosslinked with other inactive 'filler' proteins such as albumins. The most widely used bifunctional reagents used for this type of immobilization are α,ω-glutardialdehyde (Scheme 3.37) [406, 407], dextran polyaldehyde [408] dimethyl adipimidate, dimethyl suberimidate, and hexamethylenediisocyanate or -isothiocyanate. The advantage of this method is its simplicity, but it is not without drawbacks. The soft aggregates are often of a gelatine-like nature, which prevents their use in packed-bed reactors. Furthermore, the activities achieved are often limited due to diffusional problems, since many of the biocatalyst molecules are buried inside the complex structure which impedes their access by the substrate. The reactive groups



Scheme 3.37 Crosslinking of enzymes by glutaraldehyde

involved in the crosslinking of an enzyme are not only free amino functions but also sulfhydryl- and hydroxyl groups.

Crosslinking can be performed with dissolved (monomeric) proteins, and also by using them in microcrystalline or amorphous form. Thus, either 'cross-linked enzyme crystals' (CLECs) [409–414] or 'cross-linked enzyme aggregates' (CLEAs) are obtained [408, 415, 416]. The advantage of the solid aggregates thus obtained are increased stability against chemical and mechanical stress. Since they constitute a very firm solid matrix, they cannot be attacked by proteases [417] and are therefore inert towards (self)digestion. Due to the close vicinity of the individual enzymes molecules, cross-linking is particularly effective for oligomeric proteins.

However, some experimentation is usually necessary to find out the optimum conditions to obtain optimal results.

Entrapment into Gels

In case an enzyme does not tolerate direct binding, it may be physically 'encaged' in a macroscopic matrix. To ensure catalytic activity, it is necessary that substrate and product molecules can freely pass into and out of the macroscopic structure. Due to the lack of covalent binding, entrapment is a mild immobilization method which is also applicable to the immobilization of viable cells.

Entrapment into a biological matrix such as agar gels [418], alginate gels [419], or κ-carrageenan [420] is frequently used for viable cells. As depicted in Fig. 3.4, the gel-formation may be initiated either by variation of the temperature or by changing the ionotropic environment of the system. For instance, an agar gel is easily obtained by dropping a mixture of cells suspended in a warm (40°C) solution of agar into well-stirred ice-cold aqueous buffer. Alternatively, calcium alginate or κ-carrageenan gels are prepared in a similar fashion by adding a sodium alginate solution to a $CaCl_2$ or KCl solution, respectively. The main drawbacks of such biological matrices are their instability towards changes in temperature or the ionic environment and their low mechanical stability.

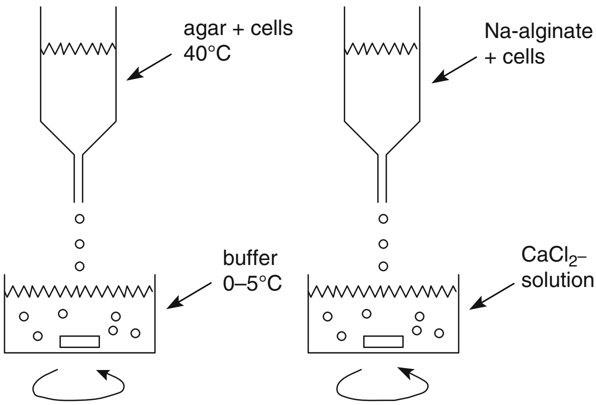


Fig. 3.4 Gel-entrapment of viable cells

In order to circumvent the disadvantages of gels based on biological materials, more stable *inorganic* silica matrices formed by hydrolytic polymerization of metal Si-alkoxides became popular. This so-called sol-gel process is initiated by hydrolysis of a tetraalkoxysilane of type $Si(OR)_4$, with R being a short-chain alkyl group such as *n*-propyl or *n*-butyl, in the presence of the enzyme [421, 422]. Hydrolysis and condensation of the $Si(OR)_4$ monomers, catalyzed by a weak acid or base, triggers the cross-linking and simultaneous formation of amorphous $(SiO_2)_n$ (Fig. 3.5).

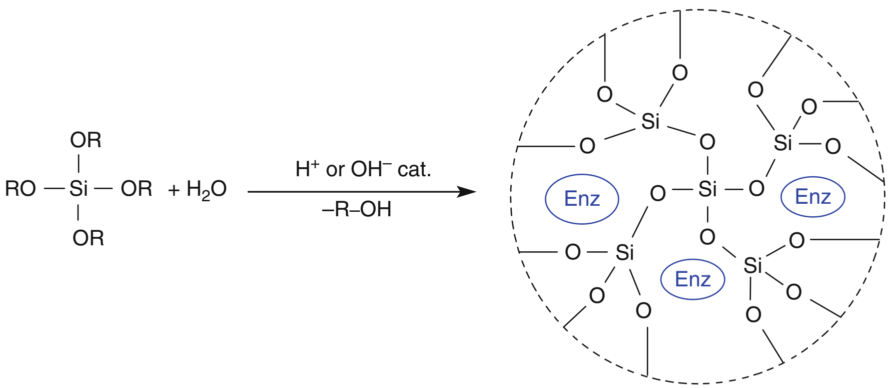


Fig. 3.5 Entrapment of enzymes in silica sol-gels

The latter constitutes a highly stable porous inorganic polymeric matrix that grows around the enzyme in a three-dimensional fashion. The sol-gel aggregates thus obtained ensure high enzyme activity and long operational stability. In addition, this process is well suited for large-scale applications since the materials are comparatively inexpensive [423–426].

A tight network which is able to contain isolated enzyme molecules (which are obviously much smaller than whole cells) may be obtained by polymerization of synthetic monomers such as polyacrylamide in the presence of the enzyme

[427–429]. It is obvious that the harsh conditions required for the polymerization makes this method inapplicable to whole cells.

Entrapment into Membrane Compartments

Enzymes may be enclosed in a restricted compartment bordered by a membrane. Although this does not lead to 'immobilization' per se, it provides a restricted space for the enzyme, which is separated from the rest of the reaction vessel. Depending on the type of reactor, various shapes, such as hollow fibers, and flat or cyclindric foils are used. Small substrate and/or product molecules can freely diffuse through the pores of the membrane, but the large enzyme cannot. The separation of a reaction volume into compartments by membranes is a close imitation of 'biological immobilization' within a living cell. Many enzymes are membrane-bound in order to provide a safe micro-environment for them within the cell. Two general methods exist for the entrapment of enzymes into membrane-restricted compartments.

Micelles and Vesicles. Mixtures of certain compositions containing water, an organic solvent and a detergent (Fig. 3.6, Scheme 3.38) give transparent solutions in which the organic solvent is the continuous phase [430]. The water is present in microscopic droplets which have a diameter of 6–40 nm [431] and are surrounded by the surfactant. The whole structure is embedded in the organic solvent and represents a micelle which is turned inside out. It is therefore termed a 'reverse micelle' (see Fig. 3.6). The latter are mimics for the micro-environment of the cell and can be regarded as artificial micro-cells. Thus, they provide high enzyme activity. On the other hand, when *water* constitutes the bulk phase, micelles may be formed by a symmetrical double layer of surfactant. The latter structure constitutes a 'vesicle' (liposome). The water trapped inside these micro-environments

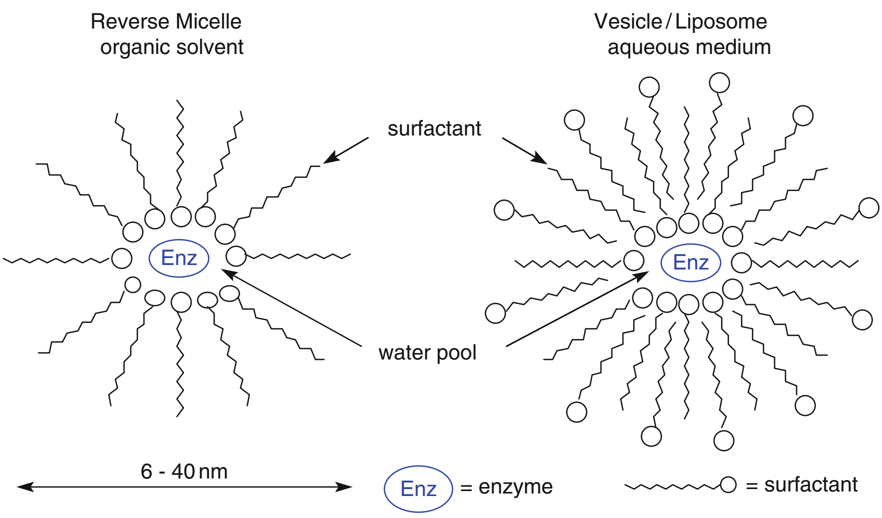
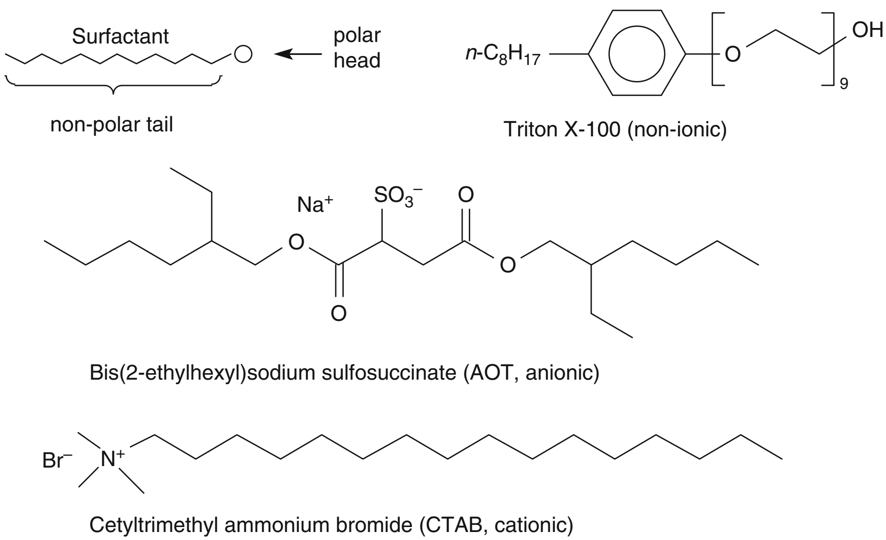


Fig. 3.6 Entrapment of enzymes in reversed micelles and vesicles



Scheme 3.38 Commonly employed surfactants

has several chemical and physical properties that deviate from 'normal' water, such as restricted molecular motion, decreased hydrogen bonding, increased viscosity, and a lower freezing point [432, 433]. Enzymes can be accommodated in these water-pools and can stay catalytically active [434–436]. The exchange of material from one micelle to another occurs by means of collisions and is a very fast process. Like in other immobilization methods, the catalytic activity of enzymes may be altered through entrapment in micelles. For instance, the activity [437–439] and the temperature stability [440] is often enhanced; in some cases also the specificity is changed [441, 442]. From a preparative point of view, it is important that compounds which are sparingly soluble in water (e.g., steroids) can be converted at much higher rates than would have been possible in aqueous media [443]. The disadvantages encountered when using micelles on a preparative scale are considerable operational problems during (extractive) workup, caused by the presence of a considerable amount of surfactant, which behaves like a soap.

Synthetic Membranes. A practical alternative to the use of sensitive biological matrices is the use of synthetic membranes [444, 445] based on polyamide or polyethersulfone (Fig. 3.7). They have long been employed for the purification of enzymes by ultrafiltration, which makes use of the large difference in size between high-molecular biocatalysts and small substrate/products molecules. Synthetic membranes of defined pore size, covering the range between 500 and 300,000 Da, are commercially available at reasonable cost. The biocatalyst is detained in the reaction compartment by the membrane, but small substrate/product molecules can freely diffuse through the pores of the barrier. This principle allows biocatalytic reactions to be performed in highly desirable *continuous* processes.

Furthermore, disadvantages caused by heterogeneous catalysis, such as mass-transfer limitations and alteration of catalytic properties, are largely avoided [446]. A variety of synthetic membranes are available in various shapes such as foils or hollow fibers.

A simplified form of a membrane reactor which does not require any special equipment may be obtained by using an enzyme solution enclosed in dialysis tubing like a tea bag (Fig. 3.7). This simple technique termed 'membrane-enclosed enzymatic catalysis' (MEEC) seems to be applicable to most types of enzymes except lipases [447–449]. It consists of a dialysis bag containing the enzyme solution, mounted on a gently rotating magnetic stirring bar.

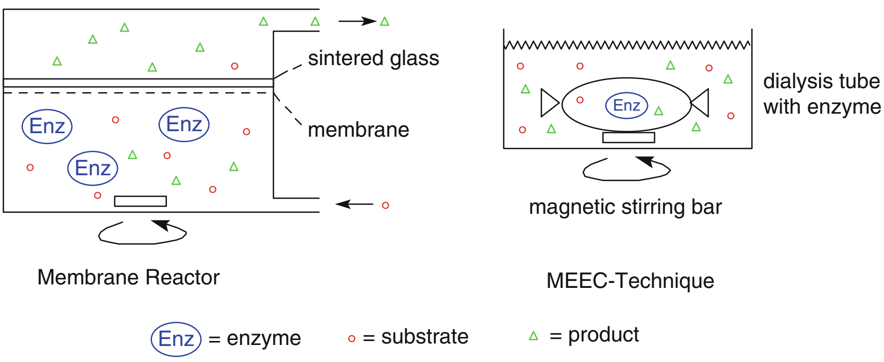
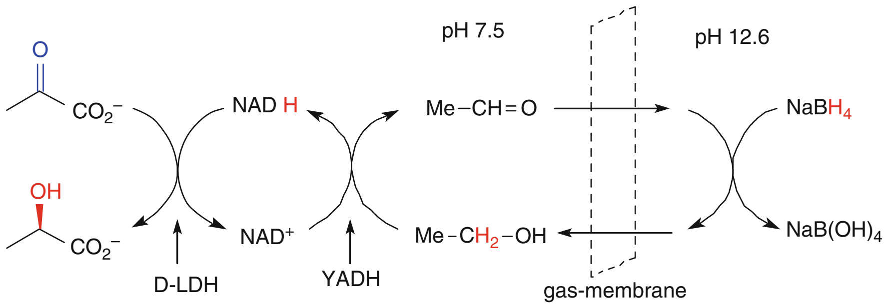


Fig. 3.7 Principle of a membrane reactor and the membrane-enclosed enzyme catalysis (MEEC)-technique

In some enzyme-catalyzed processes it is of an advantage to couple an additional (chemical) reaction onto the process in order to drive an unfavorable equilibrium in the desired direction. Very often, however, the harsh reaction conditions required for the auxiliary step are incompatible with the enzyme(s). In such cases, a membrane may be used to separate the enzyme-catalyzed reaction from the auxiliary process, while the chemical intermediates can pass freely through the barrier.

This concept has been applied to improve the cofactor recycling in a stereo-selective reduction catalyzed by lactate dehydrogenase (LDH) (Scheme 3.39) [450]. Thus, pyruvate was reduced to D-lactate at the expense of NADH using D-LDH. The cofactor was recycled via the yeast alcohol dehydrogenase (YADH)/ethanol system. In order to avoid enzyme deactivation by acetaldehyde and also to drive the reaction towards completion, acetaldehyde was reduced by sodium borohydride to yield ethanol, which in turn re-enters the process. To save the enzymes from being deactivated by borohydride and a strongly alkaline pH, the process was carried out in a reactor consisting of two compartments, which are separated by a supported gas-membrane, comparable to Gore-Tex. All volatile species (acetaldehyde and ethanol) can freely pass through the air-filled micropores, but



Scheme 3.39 Cofactor recycling using a gas-membrane reactor

the nonvolatiles (enzyme, cofactor, substrate, product, salts) cannot. Thus, a cycle number of >10,000 was achieved.

Immobilization of Cofactors

All of the above-mentioned immobilization techniques can readily be used for enzymes which are independent of cofactors and for those in which the cofactors are tightly bound (e.g., flavins, Sect. 1.4.3). For enzymes, which depend on charged cofactors, such as NAD(P)H or ATP, which readily dissociate into the medium, coimmobilization of the cofactor is often required to ensure a proper functioning of the overall system (Sects. 2.1.4 and 2.2.1).

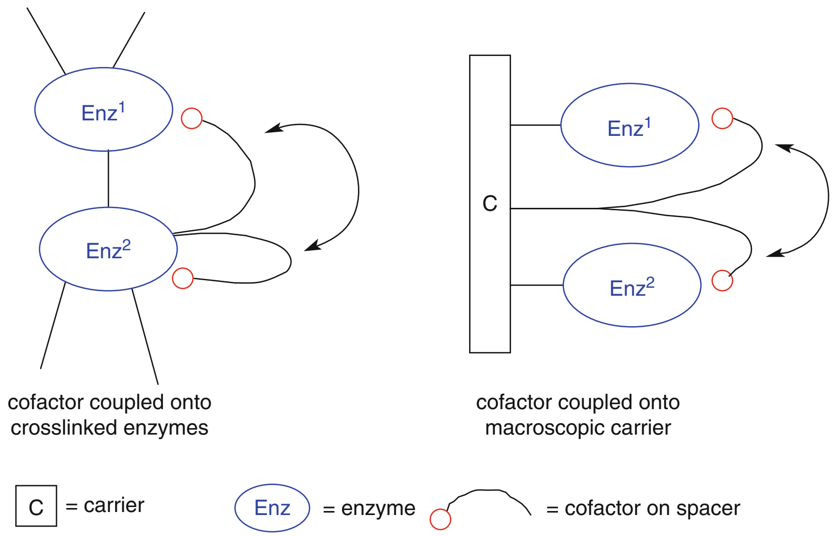


Fig. 3.8 Co-immobilization of cofactors

Two solutions to this problem have been put forward (Fig. 3.8).

- The cofactor may be bound onto the surface of a crosslinked enzyme or it may be attached to a macroscopic carrier. In either case it is essential that the spacer arm is long enough so that the cofactor can freely swing back and forth between both enzymes – Enz^1 to perform the reaction and Enz^2 to be regenerated. However, these requirements are very difficult to meet in practice.
- In membrane reactors a more promising approach has been developed in which the molecular weight of the cofactor is artificially increased by covalent attachment of large groups such as polyethylene glycol (MW 20,000) [451–453], polyethylenimine [454], or dextran moieties [455]. Although the cofactor is freely dissolved, it cannot pass the membrane barrier due to its high molecular weight.

It has to be emphasized that it is difficult to directly compare the different methodologies of enzyme immobilization since enzymes differ in their properties and details of the immobilization of industrial biocatalysts are often not disclosed.

3.3 Artificial and Modified Enzymes

Synthetic chemists have always admired the unparalleled catalytic efficiency and specificity of enzymes with some envy and have attempted to copy the catalytic principles which were developed by nature during evolution and to adopt them to the needs of chemical synthesis. Two strategies can be distinguished.

3.3.1 Artificial Enzyme Mimics

In the *biomimetic approach*, artificial enzymes (or better: enzyme models) are created de novo from a nonproteinogenic artificial scaffold, such a cyclodextrin, dendrimer or polymer, which is endowded with a rationally designed catalytic site [456]. The catalytic machinery represents a simplified chemical model of the chemical operators found in enzymes and is often derived from a cofactor (e.g., cytochrome, pyridoxal or pyridoxamin phosphate) or a catalytic metal acting as Lewis acid (e.g., Zn) or as central mediator in a redox reaction (e.g., Cu, Co, Mn, Fe). Although many of these so-called synzymes show astonishing catalytic activites, the catalytic efficiencies (expressed as rate accelerations and turnovers) and (stereo)selectivities are way too low to use them for synthetic transformations. What we gain from these studies, is a better understanding (and appreciation) of the numerous subtle contributions which add up in a synergistic fashion and thereby make enzymes as efficient as they are. After all, it appears that the structural complexity of the whole three-dimensional structure of a protein made up of several hundred amino acids is not out of mere biological luxury, but due to mechanistic necessity.

3.3.2 *Modified Enzymes*

Modified enzymes can be created by leaving their protein scaffold basically intact but altering some of their properties by chemical or genetic methods, which yields altered and/or improved enzymes for synthetic purposes. The following general strategies are discussed below [457]:

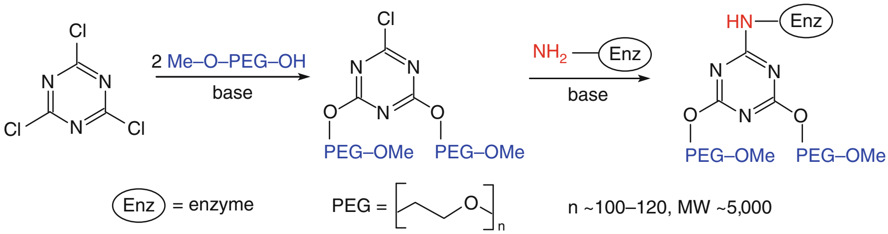
3.3.2.1 Chemically Modified Enzymes

Site-directed chemical modification of enzymes using group-specific reagents was established mainly during the 1960s aiming at the elucidation of enzyme structures and mechanisms [458, 459] rather than for the creation of biocatalysts with a better performance. In other words, enzyme modification has been developed more as an *analytical* rather than a *synthetic* tool.

Surface-Modified Enzymes. Enzymes acting in nearly anhydrous organic solvents always give rise to heterogeneous systems (Sect. 3.1). In order to turn them into homogeneous systems, which can be controlled more easily, proteins can be modified in order to make them soluble in lipophilic organic solvents. This can be readily achieved by covalent attachment of the amphipathic polymer polyethylene glycol (PEG) onto the surface of enzymes [460]. The pros and cons of PEG-modified enzymes are as follows [461, 462]:

- They dissolve in organic solvents such as toluene or chlorinated hydrocarbons, such as chloroform, 1,1,1-trichloroethane, or trichloroethylene [463].
- Their properties such as stability, activity [464, 465], and specificity [466, 467] may be altered.
- Due to their solubility in various organic solvents, spectroscopic studies on the conformation of enzymes are simplified [468].

The most widely used modifier is monomethyl PEG having a molecular weight of about 5,000 Da. Linkage of the polymer chains onto the enzyme's surface may be achieved by several methods, all of which involve reaction at the ε-amino groups of lysine residues. The latter are preferably located on the surface of the enzyme. A typical 'linker' is cyanuric chloride (Scheme 3.40). Nucleophilic displacement of



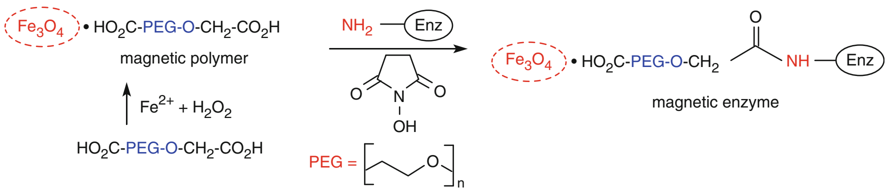
Scheme 3.40 Surface modification of enzymes by polyethylene glycol

two of the chlorine atoms of cyanuric chloride by monomethyl PEG yields a very popular modifier, which is attached to the enzyme via an alkylation reaction involving the remaining chlorine atom.

The cyanuric chloride/PEG method seems to work for all classes of enzymes, including hydrolases (lipases [469], proteases, glucosidases [470]) and redox enzymes (dehydrogenases, oxidases [471]). The residual activities are usually high (50–80%), and for most enzymes about five to ten PEG chains per enzyme molecule are sufficient to render them soluble in organic solvents. Care has to be taken to avoid extensive modification which leads to deactivation. PEG-modified enzymes may be recovered from a toluene solution by precipitation upon the addition of a hydrocarbon such as petroleum ether or hexane [472].

A special type of PEG modification, which allows the simple recovery of the enzyme from solution by using magnetic forces, is shown below (Scheme 3.41) [473]. When α,ω-dicarboxyl-PEG is exposed to a mixture of ferric ions and hydrogen peroxide, ferromagnetic magnetite particles (Fe_3O_4) are formed which are tightly adsorbed to the carboxylate. The remaining 'free end' of the magnetic modifier is then covalently linked to the enzyme via succinimide activation. When a magnetic field of moderate strength (5,000–6,000 Oe ≅ 53–75 A/m) is applied to the reaction vessel, the modified enzyme is removed from the solvent by being pulled to the walls of the container. When the magnet is switched off, the biocatalyst is 'released'.

The majority of applications using PEG-modified enzymes have involved the synthesis of esters [146, 474–478], polyesters [479], amides [480], and peptides [481, 482].



Scheme 3.41 Modification of enzymes by magnetite-polyethylene glycol

If an enzyme does not tolerate to be modified by covalent attachment of PEG residues, various lipids, such as simple long-chain fatty acids or amphiphilic compounds[3] can be attached onto its surface by mild adsorption. It has been estimated that about 150 lipid molecules are sufficient to cover an average protein with a lipophilic layer, which makes it soluble in organic solvents [62, 483]. This so-called 'lipid-coating' seems to be applicable to various types of enzymes, such as lipases [484], phospholipases [438], glycosidases [485], and catalase [486].

[3]For instance, glutamic acid dioleyl ester ribitol amide, didodecyl *N*-D-glucono-L-glutamate or Brij35.

Whereas the (enantio)selectivity was not significantly altered in most cases[4], lipid-coated enzymes showed significantly enhanced reaction rates in organic solvents (up >100-fold).

Bioimprinting. Bearing in mind that conformational changes are impeded in lipophilic organic solvents – in other words, the structure of the enzymes appears to be 'frozen' in such media but remains flexible in water – the specificity of biocatalysts may be altered in the following way (Fig. 3.9) [488, 489].

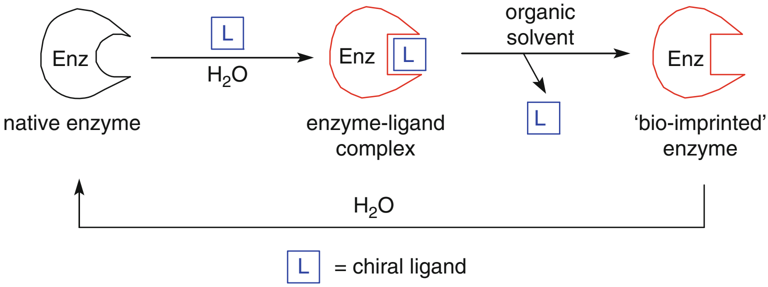


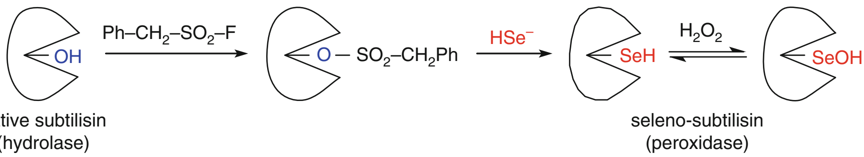
Fig. 3.9 Bioimprinting of enzymes

In an aqueous solution, the enzyme's active site is loaded with a (nonreactive) substrate mimic (a chiral ligand, L) by forming a complex which is similar to an enzyme–substrate complex. This process goes hand in hand with small conformational changes – the induced fit. When the complex is then placed into an anhydrous organic solvent, the ligand is washed away, but the enzyme is unable to adopt its former (native) conformation due to the low water content of the system. As a consequence, the three-dimensional structure of the enzyme remains 'frozen' in a modified form, as if 'remembering' the structure of the ligand. Thus, the selectivity of this 'bioimprinted' enzyme differs from that of the enzyme in water. Of course, bio-imprinting is restricted to anhydrous organic solvents, since the 'memory' is lost in aqueous systems. Although this technique has not been used widely for preparative biotransformations [490], it has provided valuable insight into the chiral recognition process [491].

Chemically Modified Enzymes. Besides varying the physicochemical properties of enzymes (such as their solubility), the catalytic properties of an enzyme can be fundamentally altered by chemical modification of the chemical operator in the active site. This technique leads to 'semisynthetic' enzymes, which often do not have much in common with their natural ancestors [492, 493]. Early efforts in this direction focussed on the modification of nucleophilic OH- or SH-residues in Ser- or Cys-hydrolases, such as subtilisin or papain, respectively. For example, the Ser-hydroxy group within the active site of subtilisin was converted to its selenium

[4]For a rare case of selectivity-enhancement see [487].

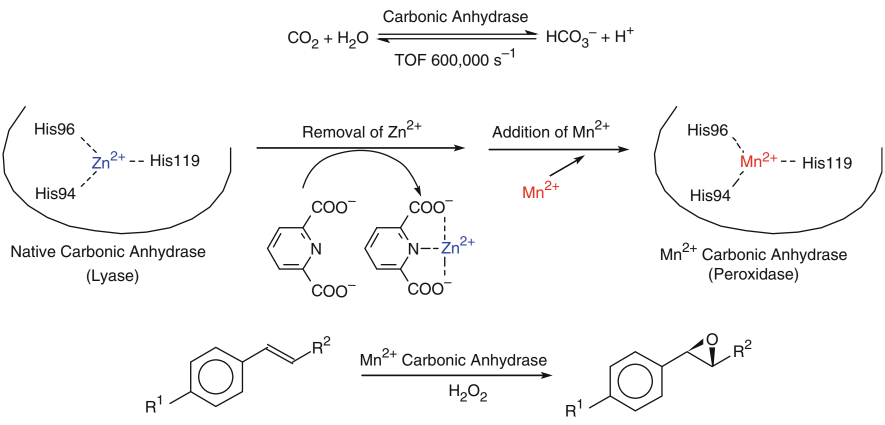
analog by a two-step procedure via chemical activation of the hydroxy group followed by nucleophilic displacement with HSe^- (Scheme 3.42) [494–496]. The seleno-subtilizing thus obtained showed a 700-fold enhanced rate for aminolysis versus hydrolysis in comparison to native subtilisin [497]. Furthermore, the Se–H group may be reversibly oxidized to its seleninic acid analog (subtilisin-Se–OH) by hydrogen peroxide or organic hydroperoxides giving rise to a semisynthetic peroxidase [498].



Scheme 3.42 Synthesis of seleno-subtilisin

In an analogous fashion, a flavin-type redox cofactor was attached to the nucleophilic thiol group in the active site of papain. By this means, a hydrolase was transformed into the artificial redox enzyme flavopapain [499–504].

A recent example for the mild chemical modification of an enzyme makes use of the replacement of the central metal ion by leaving the overall protein intact (Scheme 3.43) [505]. The lyase carbonic anhydrase is one of the fastest enzymes known and it catalyzes the equilibration of bicarbonate (derived from oxidative metabolism) into carbon dioxide to facilitate respiration. Removal of



R^1	R^2	Conversion [%]	e.e. [%]	TTN
H	H	12	58	21
Cl	H	13	67	22
H	CH_3	4	51	7.5
H	H	40*	49	1,500

* Heme-containing (native) horseradish peroxidase.

Scheme 3.43 Conversion of carbonic anhydrase (a lyase) into a peroxidase the central (redox-neutral) Zn^{2+}-ion in the active site by a strong chelating agent led to the metal-free apoenzyme, which was reconstituted by addition of Mn^{2+} to yield an artificial peroxidase, which was able to catalyze the asymmetric epoxidation of styrene-type alkenes at the expense of hydrogen peroxide as oxidant. Quite remarkably, its reaction rate and stereoselectivity was within the range of (heme-containing) native horseradish peroxidase, however, its stability was about 100 times lower and the artificial enzyme lost its activity after only 20 cycles due to autooxidation.

By reviewing the catalytic efficiencies and (stereo)selectivities of enzymes possessing a chemically modified active site, it becomes evident that studies on chemically modified enzymes have enhanced the understanding of the catalytic mechanism of enzymes rather than creating synthetic biocatalysts for preparative use [506].

3.3.2.2 Genetically Modified Enzymes

On the other hand, enzyme modification by 'natural' methods via site-directed mutagenesis using the power of molecular biology became a powerful and well-established technique for the generation of proteins possessing altered properties [507–510]. The most important strategies are briefly discussed with increasing order of difficulty.

Changing Substrate Specificity. The re-design of the three-dimensional structure of the active site of an enzyme by rational site-directed mutagenesis or via directed evolution (Sect. 2.1) is a state-of-the-art method to generate enzymes with altered substrate specificities. Thus, mutants possessing a more spacious active site can accommodate bulky substrates and enhanced stereoselectivities can be accrued by tightening the enzyme–substrate fit. This technology has been demonstrated to work for virtually all enzyme classes, including hydrolases [511–515], dehydrogenases [516, 517], lyases and transferases [518, 519].

Altering Cofactor Requirements. Although enzymes are generally very faithful concerning their cofactors, their specificites can be altered within a narrow frame. Replacement of NADPH by the cheaper and more easily recyclable NADH is possible using dehydrogenase mutants lacking the specific (often Arg-containing) phosphate binding site for NADPH [520–522].

Inverting Stereochemistry. Altering the stereochemical outcome of an enzyme reaction in order to access both stereoisomers is more challenging, because the main scaffold of the protein, which is invariably constructed from L-amino acids, remains the same. Nevertheless, successful examples were reported using rational [523–527] or directed evolution methods [528, 529].

Engineering Catalysis. The ‘holy grail’ of enzyme redesign is the engineering of entirely new catalytic activities, a property which is often denoted as catalytic promiscuity [530, 531]. The latter has been driven by the rapidly increasing number of crystal structures of proteins, which allow to understand the molecular details of their catalytic mechanism. In this context, it was possible to re-engineer the catalytic activities of well studied proteins to furnish switched activities or even completely novel functions, which are rarely found in Nature (Table 3.8).

Table 3.8 Examples for the catalytic promiscuity of rationally designed enzymes

Parent enzyme	Designer enzyme	Ref.
Protease	Nitrile hydratase	[532]
Esterase	Organophosphorous hydrolase	[533, 534]
Carbonyl reductase	Ene-reductase	[535]
Esterase	Epoxide hydrolase	[536]
Lipase	Aldolase	[537]
Lipase	Michael lyase	[538, 539]
Racemase	Amino transferase	[540]
Transaminase	β-Decarboxylase	[541]

Although the exchange of active site residues furnished novel catalytic functions, the overall efficiencies concerning catalytic rates or stereoselectivities were almost invariably very disappointing, indicating that the whole environment within the active site exerting numerous (energetically incremental) interactions with the substrate plays an important role in the catalytic mechanism, and not just the chemical operator.

3.3.3 Catalytic Antibodies

One of the most striking drawbacks of enzyme catalysis is that proteins cannot be rationally designed and then be prepared by a de novo synthesis for obvious reasons [542]. As a consequence, some synthetically useful transformations, which are not (or only rudimentary) found in nature, are largely excluded from enzymatic catalysis, in particular cycloadditions or rearrangement reactions. Furthermore, a search for biocatalysts possessing opposite stereochemical properties is usually a tedious empirical undertaking which is often unsuccessful. This gap may be filled by the development of synthetic enzymes [543–545]. Among them, catalytic antibodies constitute an intriguing intellectual concept [546–550]. To indicate their derivation, they are sometimes also called ‘abzymes’ – *a*nti*b*odies as en*zymes*.

The immune system produces a vast repertoire – in the range of 10^8–10^{10} [551] – of exquisitely specific proteins (antibodies) that protect vertebrates from ‘foreign’ invaders such as pathogenic bacteria and viruses, parasites and cancer cells. Antibodies have become invaluable tools in the detection, isolation and analysis of biological materials and are the key elements in many diagnostic procedures. Their potential, however, is not limited to biology and medicine, as their use as biocatalysis was investigated as well. Because they can also be elicited to a large array of synthetic molecules using hybridoma technology, they offer a unique approach for generating tailor-made enzyme-like catalysts. In a very simplified version they can be regarded as *enzymes possessing an active site, but no chemical operator*.

Antibodies are large proteins consisting of four peptide chains which have a molecular weight of about 150,000 Da (Fig. 3.10). There are two identical heavy (H, 50,000 Da) and light chains (L, 25,000 Da) which are crosslinked by disulfide bonds. The light chains are divided into two domains, the variable (V) and the constant (C) regions (V_L, C_L), while the heavy chains consist of V_H, C_H1, C_H2, and C_H3 domains. Both the variable V_H and V_L domains, which are located, to the first approximation, in the first 110 amino acids of the heavy and light chains, are highly polymorphic and change with each antigen (hapten) they are supposed to trap. Consequently, binding occurs in this region. On the other hand, the constant regions represent the basic framework of the antibody and are relatively invariant.

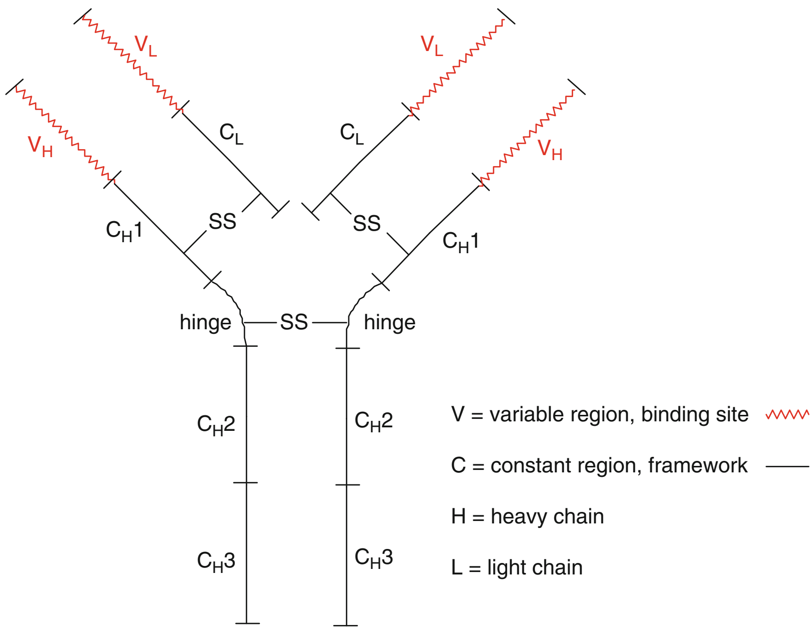
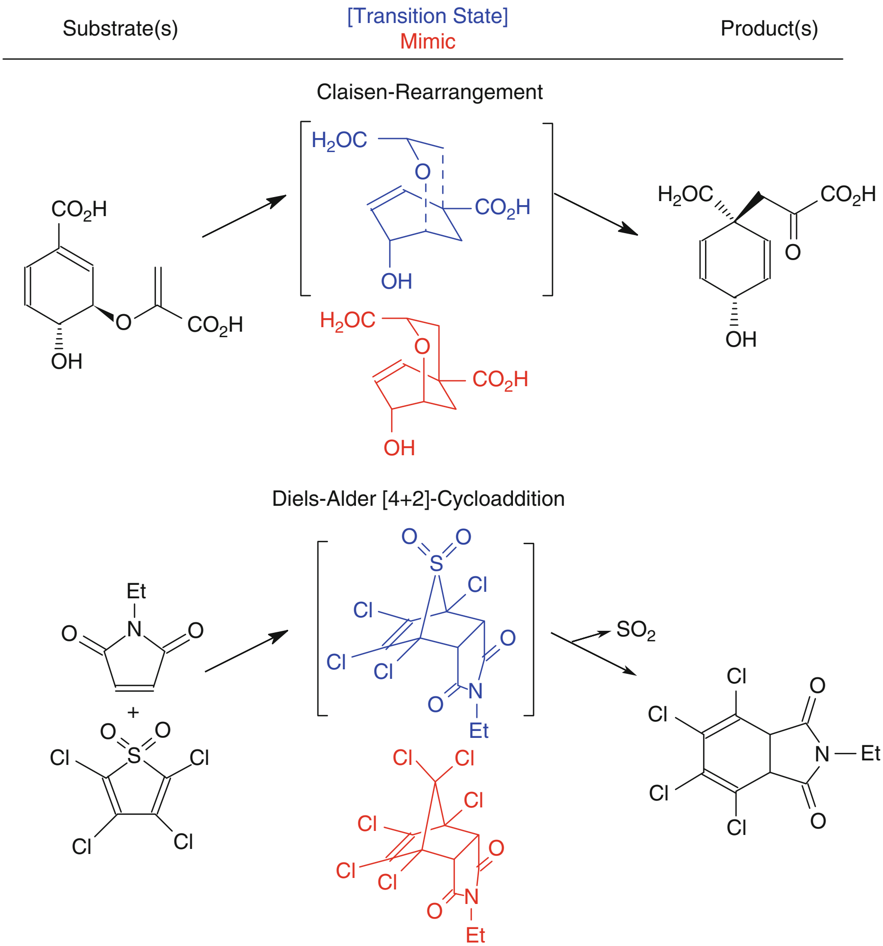


Fig. 3.10 Basic structure of a catalytic antibody

The general approach which has been used to generate catalytic antibodies is based on the complementariness of an antibody to its corresponding hapten, i.e., the ligand against which the antibody is raised. It may be compared with the 'negative' of a photographic picture. This fact has been exploited to generate artificial cavities (corresponding to the active site of an enzyme) that are complementary to the transition state of the reaction which is to be catalyzed. Thus, entropy requirements which are involved in the correct orientation of the reaction partners can be met.

If necessary, these combining sites may be equipped with an appropriately positioned catalytic amino acid side chain or a cofactor, which may be introduced either directly by site-directed mutagenesis or indirectly by chemical modification. Antibodies bind molecules ranging in size from about 6 to 34 Å with association constants of 10^4–10^{14} M^{-1}, in other words, binding is *extremely strong*. As with enzymes, binding occurs by Van der Waals (London), hydrophobic, electrostatic and hydrogen-bonding interactions.

The general protocol for obtaining catalytic antibodies is as follows (Scheme 3.44). After determination of the transition state of the reaction to be catalyzed, a chemically stable *transition state mimic* is synthesized. This 'model' is then used as an antigen to elicit antibodies. Then, the template is removed and the antibody is employed as catalyst. If required, the latter can then be genetically or chemically



Scheme 3.44 Antibody-catalyzed pericyclic reactions

modified by attachment of a reactive group [552], or a cofactor, in order to catalyze redox reactions [553]. Furthermore, like enzymes, catalytic antibodies can be immobilized and used in organic solvents [554].

Catalytic antibodies have been produced to catalyze an impressive variety of chemical reactions. For instance, stable phosphonic esters (or lactones, respectively) have been used as transition state mimics for the hydrolysis of esters and amides [555–557], acyl transfer [558] and lactonization [559] reactions, which all proceed via a tetrahedral carbanionic intermediate. In some instances, the reactions proved to be enantiospecific [560]. Elimination reactions [561, 562], reductions [563], formation and breakage of C–C bonds [564, 565] and even photochemical reactions [566] can be catalyzed. The cleavage of an ether [567] and *cis-trans* isomerization of alkenes have been reported [568].

More importantly from a preparative viewpoint, pericyclic reactions, such as the Claisen rearrangement [569, 570] or the Diels-Alder reaction [571, 572], have been catalyzed by antibodies, which were raised against bicyclic and tricyclic transition-state mimics (Scheme 3.44). The latter reactions normally cannot be catalyzed by enzymes. Also cationic cyclization reactions [573, 574] and an enantioselective Robinson annulation were achieved [575].

The catalytic power of abzymes is most conveniently measured as the rate acceleration compared to the uncatalyzed reaction. Although the early catalytic antibodies had only modest rate accelerations of a few hundred fold, more recent studies have produced artificial enzymes with catalytic activities almost approaching those of natural enzymes [576]. It must be noted, however, that in general, the rate accelerations of abzymes are in the range of 10^3–10^5, though in rare cases they may reach values of 10^8. As yet, the corresponding values for most enzymes are far better (10^8–10^{12}). Thus, in terms of catalytic efficiency, the most potent catalytic antibodies are just comparable to the slowest enzymes.

All the data presented so far indicate that catalytic antibodies will certainly help us to gain more insight into enzyme mechanisms rather than playing an important role in preparative biotransformations.

The following pros and cons for the utilization of abzymes can be summarized as follows:

- They offer a unique access to artificial tailor-made enzyme-like catalysts and are able to catalyze reactions which have no equivalent counterpart within the diverse natural enzymes.
- The construction of abzymes having an opposite stereochemical preference is possible.
- The catalytic efficiency is low in comparison to that of natural enzymes, mainly because the transition-state mimics which are used to elicit the antibody only act as a *template* for the true transition state.
- The production of abzymes on a substantial scale is tedious [577] and ‘preparative-scale’ reactions are still in the micromolar range [578, 579]. Furthermore, to avoid the occurrence of catalytically active enzyme impurities, the purity of the abzymes must be very high.

- The high binding energy for the substrate (up to 20 kcal/mol) which is required for efficient catalysis – remember, that catalytic antibodies do not have a chemical operator like enzymes – also results in tight binding of the product. Thus, product inhibition, which limits the overall performance, is an inherent disadvantage of catalytic antibodies [580].

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Chapter 4
State of the Art and Outlook

The biotransformations described in this book demonstrate that this area is in an active state of development and that enzymes have attained an important position in contemporary organic synthesis [1–3]. The increased interest of synthetic chemists in the biotransformation of nonnatural compounds has spurred the commercial availability of enzymes in various forms and grades of purity [4]. To date, almost 10% of all papers published in the area of synthetic organic chemistry contain elements of biotransformations [5] and >130 industrial-scale processes are documented worldwide [6–17]. As shown in Fig. 4.1, the frequency of use of a particular biocatalyst is not evenly distributed among the various types of enzymes and it is obvious that the more 'simple' hydrolytic reactions are dominant.[1] In this book, attention has been focused on those methods which are most useful for and accessible to synthetic chemists and the rating of methods according to their general applicability and reliability is intended!

In the following paragraphs a brief summary on the state of the art of biotransformations is given (Table 4.1). An outlook on future developments, however, should be taken with a 'grain of salt' as it bears a strong resemblance to an Austrian weather forecast.

Hydrolytic enzymes such as proteases, esterases and lipases are ready-to-use catalysts for the preparation of optically active carboxylic acids, amino acids, alcohols, and amines. The area is sufficiently well researched to be of general applicability for a wide range of synthetic problems. Consequently, about two thirds of the reported research on biotransformations involves these areas. This is facilitated by the fact that a considerable collection of commercially available proteases and lipases is available in conjunction with techniques for the improvement of their selectivities. The development of simple models aimed at the prediction of the stereochemical outcome of a given reaction is still a challenge and will be the subject of future studies. A search for novel esterases to enrich the limited number of available enzymes and for lipases showing 'anti-Kazlauskas' stereospecificities would be a worthwhile endeavor.

[1]Data from database Kroutil/Faber, ~15,000 entries, 2011.

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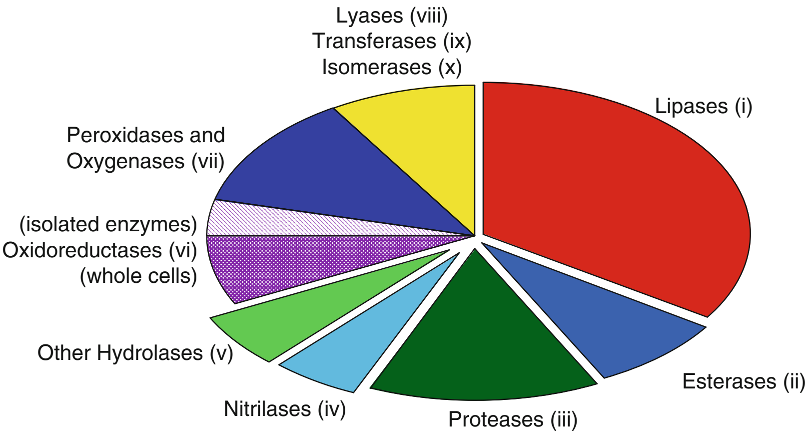

(i) Ester formation, -aminolysis, -hydrolysis; (ii) ester hydrolysis; (iii) ester and amide hydrolysis, peptide synthesis; (iv) nitrile hydrolysis; (v) hydrolysis of epoxides, halogens, phosphates, and glycosides; (vi) reduction of C=O and C=C bonds; (vii) hydroxylation or C-H bonds, sulfoxidation of thioethers, epoxidation of alkenes, Baeyer-Villiger oxidation of ketones, dihydroxylation of aromatics, peroxidation; (viii) cyanohydrin formation, acyloin and aldol reaction; (ix) glycosyl and amino-group transfer; (x) Claisen-type rearrangement, isomerization of carbohydrates, racemization.

Fig. 4.1 Frequency of use of particular biocatalysts in biotransformations

The asymmetric hydrolysis of epoxides, which was impeded by the lack of readily available sources of microbial enzymes, is now possible on a preparative scale. This method offers a valuable alternative to the asymmetric epoxidation of olefins, particularly for those substrates where chemical methods fail due to the absence of directing functional groups.

The microbial hydrolysis of nitriles to amides or carboxylic acids often does not proceed with high enantioselectivity, but it offers a valuable and mild alternative method to the harsh reaction conditions usually required for this conversion using traditional methodology. As a consequence, it has proven to be a very useful and competitive method in the chemoselective transformation of nitriles on an industrial scale.

The synthesis of optically active phosphate esters by phosphorylation is now possible which provides an important tool for pharmaceutical applications.

In contrast to the dominating hydrolases, about a quarter of the research in biotransformations involves reduction reactions. Isolated dehydrogenases and/or whole microbial (bacterial and fungal) cells can be used for the stereo- and enantioselective reduction of ketones to furnish the corresponding secondary alcohols on the industrial scale. The limitation that the majority of isolated dehydrogenases follow Prelog's rule, is currently being surmounted by the improved availability of enzymes possessing the opposite stereospecificity, and more of these enzymes are becoming commercially available.

Table 4.1 Pros and cons of biotransformations according to enzymes types

Enzyme type	Reaction catalyzed	Strength	Weakness	Solution
Lipase	Ester hydrolysis, -formation, -aminolysis	Many stable enzymes, organic solvents	Low predictability of stereoselectivity	State of the art anti-Kazlauskas lipases?
Esterase	Ester hydrolysis, -formation	Pig liver esterase proteases	Few esterases organic solvents	State of the art, novel esterases?
Protease	Ester and amide hydrolysis, ester aminolysis, peptide synthesis	Many stable proteases	No D-proteases	State of the art, engineered D-proteases?
Nitrilase, nitrile hydratase	Nitrile hydrolysis	Chemo- and regioselectivity	Enantioselectivity, enzyme stability	State of the art, stable (commercial) nitrilases?
Epoxide hydrolase phosphatase/ kinase haloalkane dehalogenase	Hydrolysis of epoxides, phosphate esters, haloalkanes	Chemocatalysis weak	Few enzymes, enzyme stability	New stable (commercial) enzymes?
Glycosidase	Oligosaccharide formation	Anomeric selectivity	Region- and diasteroselectivity, equilibrium	State of the art
Dehydrogenase	Reduction of aldehydes and ketones	Prelog-selectivity, NADH-enzymes, whole cells	Anti-Prelog-enzymes, NADPH-enzymes	State of the art, NADH-dependent anti-Prelog enzymes?
Ene-reductase	Reduction of enones, α,β-unsaturated esters	*trans*-reduction	Large-scale applications	State of the art
Mono-oxygenase	Hydroxylation, Baeyer-Villiger, epoxidation	Chemocatalysis fails	Sensitive multi-component NADPH-enzymes	Non-heme enzymes?
Di-oxygenase	Dihydroxylation of aromatics	Whole-cell systems	Isolated enzymes	State of the art non-aromatic substrates?
Peroxidase	Peroxidation, epoxidation	No cofactor	H_2O_2-sensitive	Stable enzymes?
Aldolase	Aldol reaction in H_2O	Stereo-complementary DHAP-enzymes	Dihydroxyacetone phosphate	State of the art, non-phosphorylated donors?
Transketolase	Transketolase reaction	Non-phosphorylated donors	Few enzymes	State of the art, more enzymes?
Hydroxynitrile lyase	Cyanohydrin formation	*R*- and *S*-enzymes	Few enzymes	State of the art, commercial enzymes?
Fumarase, aspartase	Addition of H_2O, NH_3	Chemocatalysis fails	Narrow substrate tolerance	New enzymes?
Glycosyl transferase	Oligosaccharide synthesis	High selectivity	Phosphorylated donors	State of the art?
Transaminase	Amine synthesis	High selectivity	Equilibrium	State of the art

Recent breakthroughs in the cloning of oxygen-stable ene-reductases enables the asymmetric bioreduction of activated carbon–carbon double bonds for preparative-scale applications. NADH recycling is performed on industrial scale, for the more sensitive NADPH analog improvements would be desirable.

Biocatalytic oxygenation processes are becoming increasingly important since traditional methodology is either not feasible or makes use of hypervalent metal oxides, which are ecologically undesirable when used on a large scale. As the use of isolated (heme-dependent) monooxygenases will presumably be always hampered by their multicomponent nature and their requirement for NAD(P)H recycling, many useful oxygenation reactions such as mono- and dihydroxylation, epoxidation, sulfoxidation, and Baeyer-Villiger reactions will continue to be performed using whole cell systems. It remains to be seen, whether nonheme oxygenases and oxidases will help to circumvent these limitations. Considering the lack of cofactor dependence, hydrogen-peroxide-dependent peroxidases offer promising alternative. The regio- and stereoselective oxidation of (poly)hydroxy compounds, which is notoriously difficult with nicotinamide-dependent dehydrogenases, will be presumably be possible in the near future by using (flavin-dependent) oxidases.

The formation of carbon–carbon bonds in an asymmetric fashion by means of aldolases and transketolases is a well-researched method, which is gaining prominence as a standard technique for the synthesis of carbohydrate-like target compounds. It is noteworthy that the full set of complementary aldolase enzymes are now available at a reasonable cost using genetic engineering. Along these lines, PLP-dependent lyases for acyloin and benzoin condensations are gaining importance due to their lack of sensitive phosphorylated cosubstrates. Alternatively, enzymatic (de)carboxylation reactions are currently being investigated for the selective cleavage and (more important) the formation of carbon–carbon bonds using CO_2 as a raw material for organic synthesis [18].

Glycosylation reactions using glycosidases and glycosyl transferases are gaining ground as more of these enzymes are made available by genetic engineering. Amino-group transfer and reductive amination recently became feasible through the use of (*R*)- and (*S*)-selective ω-transaminases. Furthermore, there is a significant potential of lesser-used enzymes from the class of isomerases, such as cyclases and racemases [19], to be exploited for the transformation of nonnatural substrates.

The synthesis of optically active (*R*)- and (*S*)-cyanohydrins by hydroxynitrile lyases is now well established. Several of these enzymes are commercially available and were successfully implemented in industry. The lyase-catalyzed addition of water and ammonia onto activated C=C bonds has little counterpart in traditional chemical catalysis and provides a highly desirable atom efficiency of 100%. Unfortunately, the presently used hydratases and ammonia lyases are impeded by a restricted substrate tolerance, which possibly might be circumvented by the design of suitable mutants.

Although halogenation and dehalogenation reactions can be catalyzed by enzymes, it is doubtful whether these reactions will be used widely, mainly because the corresponding conventional chemical methods are highly competitive.

The methodology concerning the employment of enzymes in nonaqueous solvents with respect to enzyme *activity* is well understood so as to be highly useful for organic chemists. Thus, the synthesis of esters, lactones, amides, peptides, and peracids by using enzymes is standard methodology in industry. On the other hand, the influence of the nature of organic solvents on an enzyme's *selectivity* is still poorly understood and we are far from being able to provide rules of general applicability.

Most recently, the combination of several (bio)catalytic steps onto each other in a cascade reaction [20] has resulted in the development of so-called deracemization techniques, which lead to the transformation of a racemate into a single stereoisomer as the sole product. In an economic sense, these methods are far superior to classic kinetic resolution, which provides two enantiomers each in 50% yield [21].

After all, enzymes have been optimizing their skills for more than 3×10^9 years so as to develop a lot of sophisticated chemistry, whereas organic chemists and genetic engineers have a track record of less than a century. There is strong evidence that the number of distinctly different enzyme mechanisms is finite, since we know examples of protein molecules that are unrelated by evolution but possess almost identically arranged functional groups. In many cases, Nature has obviously faced the same biochemical problem and has found the same optimum solution. In parallel to the development of novel enzymes by evolution through natural selection, tremendous advances in the field of genetic engineering provide biocatalysts at drastically decreased costs, and also allow the design of enzymes possessing enhanced stabilities and altered (stereo)specificities.

Finally, microorganisms can synthesize extremely complicated optically active molecules such as penicillins, polyketides, and steroids from inexpensive C-, N-, and S-sources. Although the initial investment in screening, selecting, and the development of mutant strains or cloning techniques is very high, many valuable compounds can be synthesized very easily where simple chemical methods will fail.

Most of the environmental contamination of greatest concern involves xenobiotic materials which have been produced with increasing diversity and volume in industrial and agricultural processes during the past century. These include hydrocarbons, heavy metals, neurotoxic pesticides, halogenated (aromatic) compounds, explosives, and carcinogens, which often do not have natural counterparts. Although their introduction into the ecosphere is lamentable, and the effects they can have on the environment is often devastating, numerous different types of microbial systems have already acquired the ability to act upon most of these xenobiotics. The likelihood that totally new enzymes with specific detoxification activities could evolve naturally in such a brief period of time is quite unrealistic. It is more likely that enzymes already present in microbial communities – possibly used for defense and maintenance functions – were adapted through chemical stress to address the new challenge. Whereas the destruction of nonnatural chemicals by microbial communities was generally performed with mixed populations (finally producing carbon dioxide, water, halides, and so forth), recent efforts are directed towards the selective transformation of toxins by specific enzymes into defined products, which eventually may be used again as raw material in further processes.

There is a general need for cleaner (‘green’) and sustainable chemistry, and although it is not the prerogative of biocatalysis, it is nevertheless an important feature of it. Social pressure forces the chemical industry to pay more attention to these issues, as the public becomes aware that the resources are limited and have to be used with utmost efficiency.

In summary, biocatalysts represent a new class of chiral catalysts that are useful for a broad range of highly selective organic transformations. Synthetic chemists capable of using this potential will have a clear advantage over those limited to nonbiological methods in their ability to tackle the new generation of synthetic problems at the interface between chemistry and biology arising from the necessity to use renewable feedstocks.

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Chapter 5
Appendix

5.1 Basic Rules for Handling Biocatalysts

Like with chemical catalysts, a few guidelines should be observed with biocatalysts to preserve activity and to handle them safely. Depending on the formulation – liquid, lyophilized, spray-dried, immobilized – different rules apply. Enzymes are often perceived as unstable and delicate entities, however, when treated in the right way, they can be as sturdy as almost any chemical catalyst. For optimal stability, enzyme preparations are usually stored best in their original commercial form, either as lyophilizate, spray-dried powder or (stabilized) liquid.

Safety
Direct contact with enzymes should be avoided. This is particularly important with respect to the inhalation of aerosols (from liquid formulations) or protein dust from lyophilized or spray-dried protein preparations. Depending on the person, the tendency to develop allergies (itching, red stains) can be very different. However, the development of serious health problems due to contact with cell-free protein formulations is extremely unlikely.

When working with whole (viable) cells, make sure you find out to which safety class the organism belongs *before* you start. Microorganisms are classified into four safety categories (Classes 1–4): Class 1 organisms, such as baker's yeast, and the vast majority of microorganisms described in this book are generally regarded as safe and special precautions are not required. On the other hand, cells belonging to Class 2 may cause infections (particularly if the immune system is damaged due to an already present infection) and should be handled with care in a laminar-flow cabinet. Every good culture collection provides reasonably reliable information on the safety classification of their microorganisms. When working with your own isolates, you should definitely obtain some information on the health hazards of your

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strains. For instance, this can be performed at reasonable cost at a culture collection, e.g., at DSMZ (see Sect. 5.5).

Occasionally, the use of an enzyme inhibitor may be necessary to suppress undesired side reactions due to further metabolism. You should be aware that the majority of them (in particular serine hydrolase inhibitors) are *extremely toxic* and must be handled with the utmost care, like (for example) osmium tetraoxide.

Preservation of Enzyme Stability

The following processes lead to rapid enzyme deactivation and thus should be avoided:

- Dilution of enzyme solutions: Enzymes are not happy in very dilute solutions and are therefore more stable at higher protein concentrations.
- Removal of additives, such as polyhydroxy compounds, carbohydrates, (in-active) filler proteins, etc.; these agents often serve as stabilizers.
- Enzyme purification: It is an unfortunate, but commonly encountered fact that pure proteins are generally less stable than crude preparations.
- Freezing and thawing: If unavoidable, freezing should be performed *fast* (e.g., via shock-freezing by dropping the protein solution or cell suspension into liquid nitrogen). Slow freezing leads to the formation of large ice crystals, which tend to damage proteins. The addition of polyhydroxy compounds (e.g., glycerol, sorbitol) serving as cryo-protectant is strongly recommended.
- Very high concentrations of substrate and/or product.
- Highly charged (inorganic) ions, such as Cr^{3+}, Cr^{6+}, Co^{2+}, Ni^{2+}, Fe^{3+}, Al^{3+}, $SO_4{}^{2-}$ should be avoided, alkali and halide ions are usually harmless if present at low to moderate concentrations.
- High ionic strength: Buffer concentrations should be low, i.e., 0.05–0.1 M.
- Extreme temperatures: The best temperature range for performing a biocatalytic reaction is between 20 and 30°C. Proteins and whole cells should be stored at 0 to +4°C. Most lyophilized enzyme preparations and whole cells can be stored at this temperature for many months without a significant loss of activity.
- Extreme pH: The optimal pH-range for the most commonly used enzymes is 7.0–7.5. If acid or base is emerging during the reaction, a proper pH must be maintained by, e.g., addition of an acid or base scavenger; if possible, this should occur in a continuous mode via an autotitrator.
- High shear forces, caused by sharp edges or rapid stirring, cause deactivation. Thus gentle shaking on a rotary shaker is recommended. If stirring is required, use an overhead stirrer with a glass paddle with round edges at low to moderate speed rather than a magnetic stirring bar, which acts as mortar and grinds your enzymes or cells to pieces.
- Metal surfaces: Some alloys can liberate traces of metal ions; reactors and components thereof made from glass or plastic are preferable.
- Precipitation of proteins (e.g., by addition of Na_2SO_4 or a water-miscible organic solvent, such as acetone or *i*-propanol) often leads to a significant degree of

deactivation. On the contrary, lyophilization is the milder method for the isolation of proteins from aqueous solutions.
- Complete dehydration causes irreversible deactivation. Proteins should therefore be able to keep their minimum of 'structural water' to retain their activity. In general, this is safely achieved via lyophilization.
- Large surfaces at the liquid–gas interface of bubbles and foams can lead to deactivation; foaming should therefore be avoided by, e.g., addition of an antifoam agent.
- Some agents, such as sodium azide and metal chelators (EDTA) are known to (irreversibly) deactivate enzymes.

Liquids. Liquid enzyme formulations usually contain a significant amount of stabilizing agents, such as carbohydrates, polyols and inactive (filler) proteins. In addition, side-products from the fermentation may be present. In general, they tend to stabilise enzymes and thus their removal (e.g., by dialysis) is not recommended. The same applies to dilution, as concentrated protein solutions are usually more stable. Liquid formulations should be stored in the cold (0 to +4°C), but avoid freezing! If long-term storage is required, two options are possible (if in doubt, test both methods on a sample first and check for any loss of activity):

- Split the liquid formulation into aliquots and shock-freeze them by dropping into liquid nitrogen. The frozen samples can be stored at –25°C for an (almost) unlimited period of time.
- Subject the liquid formulation to lyophilization. The latter can be conveniently stored at 0 to +4°C.

Lyophilizates and Powders. Most technical-grade enzyme preparations are shipped as solid lyophilizates or powders. In general, they are more stable than liquid formulations and they can be conveniently stored in the cold (0 to +4°C), but avoid freezing! Before you open an enzyme container (from the refrigerator) make sure that it has reached room temperature *before* you open it. Otherwise moisture will condense onto the (hygroscopic) enzyme powder and will gradually turn it into a sticky paste which goes in hand with a loss of activity. Be aware that protein dust is more susceptible to electrostatic interaction than solid organic compounds. Thus, enzymes tend to stick onto plastic surfaces and to spread as dust, particularly when you collect static electricity by shuffling along a plastic lab floor. Dissolve any solid enzymes in water before disposal.

Carrier-Fixed Immobilized Enzymes. The risk of formation of dust with immobilized enzymes is minimal. Storage at 0 to +4°C is recommended, but avoid freezing! Any vials from the refrigerator should be brought to room temperature before opening. Due to the presence of a macroscopic carrier particle, immobilized enzymes are particularly susceptible to mechanical disintegration. Thus, avoid rapid stirring and agitate only by gentle shaking. Soak any immobilized enzymes in water before disposal.

5.2 Abbreviations

ACE	Acetylcholine esterase	HSDH	Hydroxysteroid dehydrogenase
ADH	Alcohol dehydrogenase	HLE	Horse liver esterase
ADP	Adenosine diphosphate	KDO	3-Deoxy-D-*manno*-2-octulosonate-8-phosphate
AMP	Adenosine monophosphate		
Ar	Aryl	LDH	Lactate dehydrogenase
ATP	Adenosine triphosphate	LG	Leaving group
Bn	Benzyl	MEEC	Membrane-enclosed enzymatic catalysis
Boc	*tert*-Butyloxycarbonyl		
BPO	Bromoperoxidase	MEH	Microsomal epoxide hydrolase
CAL	*Candida antarctica* lipase	MOM	Methoxymethyl
Cbz	Benzyloxycarbonyl	MSL	*Mucor* sp. lipase
CEH	Cytosolic epoxide hydrolase	NAD^+/ NADH	Nicotinamide adenine dinucleotide
CLEA	Cross-linked enzyme aggregate		
CLEC	Crosslinked enzyme crystal	$NADP^+$/ NADPH	Nicotinamide adenine dinucleotide phosphate
CPO	Chloroperoxidase		
CRL	*Candida rugosa* lipase	NDP	Nucleoside diphosphate
CSL	*Candida* sp. lipase	NeuAc	*N*-acetylneuraminic acid
CTP	Cytosine triphosphate	Nu	Nucleophile
Cyt P-450	Cytochrome P-450	O-5-P	Orotidine-5-phosphate
DAHP	3-Deoxy-D-*arabino*-heptulosonate-7-phosphate	PEP	Phosphoenol pyruvate
		PEG	Polyethylene glycol
d.e.	Diastereomeric excess	PLE	Porcine liver esterase
DER	2-Deoxyribose-5-phosphate	PPL	Porcine pancreatic lipase
DH	Dehydrogenase	PQQ	Pyrroloquinoline quinone
DHAP	Dihydroxyacetone phosphate	PRPP	5-Phospho-D-ribosyl-α-1-pyrophosphate
DOPA	3,4-Dihydroxyphenyl alanine		
E	Enantiomeric ratio	PSL	*Pseudomonas* sp. lipase
e.e.	Enantiomeric excess	PYR	Pyruvate
EH	Epoxide hydrolase	RAMA	Rabbit muscle aldolase
Enz	Enzyme	Sub	Substrate
e.r.	Enantiomer ratio	TBADH	*Thermoanaerobium brockii* alcohol dehydrogenase
FAD	Flavine adenine dinucleotide		
FDH	Formate dehydrogenase	TEPP	Tetraethyl pyrophosphate
FDP	Fructose-1,6-diphosphate	Thex	Thexyl = 1,1,2-trimethylpropyl
FMN	Flavine mononucleotide	TPP	Thiamine pyrophosphate
Gal	Galactose	Tos	*p*-Toluenesulfonyl
GDH	Glucose dehydrogenase	TTN	Total turnover number
Glc	Glucose	UDP	Uridine diphosphate
GluDG	Glutamate dehydrogenase	UMP	Uridine monophosphate
G6P	Glucose-6-phosphate	UTP	Uridine triphosphate
G6PDH	Glucose-6-phosphate dehydrogenase	XDP	Nucleoside diphosphate
		XTP	Nucleoside triphosphate
GTP	Guanosine triphosphate	YADH	Yeast alcohol dehydrogenase
HLADH	Horse liver alcohol dehydrogenase	Z	Benzyloxycarbonyl

5.3 Suppliers of Enzymes

Almac	UK
Amano	Japan
American Biosystems	USA
ASA Spezialenzyme	Germany
Asahi Kasei	Japan
Bayer AG	USA
Biocatalysts	UK
Biozym	Germany
Biozyme	UK
Boehringer	Germany
C LEcta	USA
Calbiochem	USA
Calzyme	USA
Cambrex	USA
CHR Hansen	Denmark
ChrialVision	The Netherlands
Clea	The Netherlands
Codexis	USA
Daicel	Japan
Denka Saiken	Japan
DSM Gist (DSM Food Chemicals)	The Netherlands
Enz Bank	Korea
EnzySource	China
Evocatal	Germany
Evonik	Germany
Finnsugar	Finland
Fluka	Switzerland
Genzyme	UK
Gist Brocades	The Netherlands
InnoTech MSU	Russian Federation
Iris Biotech	Germany
Johnson Matthew	UK
Meito Sangyo	Japan
MP Biomedicals	UK
Nagase Sangyo	Japan
Novo Nordisk	Denmark
Oriental Yeast	Japan
Plant Genetic Systems	Belgium
Recordati	Italy
Sanofi	France
Sigma	USA
Takeda Yakuhin	Japan
Tanabe Seiyaku	Japan
Towa	Japan
UBC	Belgium
US Pharmaceuticals	USA
Verenium	USA
Wako Pure Chemical Industries	Japan
Worthington	USA

5.4 Commonly Used Enzyme Preparations

Esterases

Enzyme name	Biological source
Acetyl esterase	Orange flavedo
Acetylcholine esterase	Electric eel
Butyrylcholine esterase	Horse serum
Carboxyl esterase	Horse liver
Carboxyl esterase	Porcine liver
Cholesterol esterase	Porcine pancreas
Carboxyl esterase NP	*Bacillus subtilis*

Proteases

Enzyme name	Biological source
Amino acid acylase	Porcine kidney, *Aspergillus melleus*
Bromelain	Pineapple stem
α-Chymotrypsin	Bovine pancreas
Ficin	Fig latex
Papain	Papaya latex
Penicillin acylase	*Escherichia coli*
Pepsin	Porcine stomach
Protease	*Aspergillus oryzae*
Protease	*Aspergillus saitoi*
Protease	*Aspergillus sojae*
Protease	*Rhizopus* sp.
Protease	*Streptomyces griseus*
Proteinase K	*Tritirachium album*
Subtilisin Carlsberg	*Bacillus licheniformis*
Subtilisin BL	*Bacillus lentus*
Subtilisin BPN′	*Bacillus amyloliquefaciens*
Thermitase	*Thermoactinomycetes vulgaris*
Thermolysin	*Bacillus thermoproteolyticus*
Trypsin	Porcine pancreas
Trypsin	Bovine pancreas

Lipases

Biological source	Supplier[a]
Achromobacter sp.	Meito Sangyo
Alcaligenes sp.	Amano, Biocatalysts, Meito Sangyo (QL), Boehringer (Chirazyme L-10)
Aspergillus niger	Aldrich, Amano (A, AP6), Biocatalysts, Fluka, Novo (Palatase), Evonik
Bacillus subtilis	Towa Koso
Candida antarctica A	Boehringer (Chirazyme L-5), Fluka, Novo (SP526)
Candida antarctica B	Fluka, Novo (SP525 or SP435[1]), Boehringer (Chirazyme L-2)
Candida lipolytica	Amano (L), Biocatalysts, Fluka
Candida rugosa[b]	Aldrich, Amano (AY-30), Biocatalysts, Boehringer (Chirazyme L-3), Fluka, Meito Sangyo (MY, OF-360), Sigma (L 8525)
Chromobacterium viscosum[c]	Asahi, Biocatalysts, Finsugar (I), Fluka, Sigma, Toyo Yozo
Fusarium solani	DSM Gist (DSM Food Chemicals), Plant Genetic Systems
Geotrichum candidum	Amano (GC-20, GC-4), Biocatalysts, Sigma
Humicola lanuginosa[d]	Boehringer (Chirazyme L-8), Biocatalysts, Novo (SP524)
Mucor javanicus[e]	Amano (M), Biocatalysts, Fluka
Mucor miehei[f]	Amano (MAP), Boehringer (Chirazyme L-9), Biocatalysts, Fluka, Novo (SP524, Lipozyme)
Penicillium camembertii[g]	Amano (G), Sanofi
Penicillium cyclopium	Biocatalysts
Penicillium roquefortii	Amano (R), Biocatalysts, Fluka
Phycomyces nitens	Takeda Yakuhin
Porcine pancreas	Aldrich, Amano, Biocatalysts, Boehringer (Chirazyme L-7), Fluka, Röhm, Sigma (II, L 3126)
Pseudomonas aeruginosa	Amano
Pseudomonas cepacia[h]	Aldrich, Amano (P, P-30, LPL-80, LPL-200S, AH), Biocatalysts, Boehringer (Chirazyme L-1), Fluka, Sigma
Pseudomonas fluorescens	Amano (AK, YS), Biocatalysts
Pseudomonas fragi	Wako (B)
Pseudomonas sp.	Amano (AK, K-10), Boehringer (Chirazyme L-6), Fluka, Mitsubishi (SAM II), Evonik, Sigma
Rhizopus arrhizus	Biocatalysts, Boehringer, Fluka, Sigma
Rhizopus delemar[2]	Amano (D), Biocatalysts, Fluka, Sigma, Tanabe Seiyaku
Rhizopus japonicus	Amano, Biocatalysts, Nagase Sangyo
Rhizopus javanicus	Biocatalysts, Evonik
Rhizopus niveus	Amano (N), Biocatalysts, Fluka
Rhizopus oryzae	Amano (F-AP), Sigma
Rhizopus sp.	Amano
Staphylococcus sp.	Evonik
Thermomyces sp.	Novo (SP523), Boehringer (Chirazyme L-8)
Wheat germ	Fluka, Sigma

[a]Product code in brackets
[b]Formerly denoted as *Candida cylindracea*
[c]Also denoted as *Pseudomonas glumae*[3]
[d]Also denoted as *Thermomyces lanuginosus*
[e]Also denoted as *Rhizomucor javanicus*
[f]Also denoted as *Rhizomucor miehei*
[g]Also denoted as *Penicillium cyclopium*
[h]In early reports, this microorganism (ATCC 21808) was classified as *Pseudomonas fluorescens*, later as *P. cepacia*, most recently as *Burkholderia cepacia*. Neither the microorganism nor its lipase has changed by the reclassification
[1]SP525 is a powder containing about 40% of weight protein, while SP435 is the same enzyme immobilized on macroporous polypropylene and contains about 1% of weight protein. For some time, Novo supplied a lipase SP382, which was a mixture of lipases A and B.
[2]The amino acid sequences of lipases from *Rhizopus delemar, R. javanicus* and *R. niveus* are identical. The sequence of the lipase from *R. oryzae* differs by only two conservative substitutions.
[3]The amino acid sequence and biochemical properties of the lipases from *Pseudomonas glumae* and *Chromobacterium viscosum* are identical; see: Taipa MA, Liebeton K, Costa JV, Cabral JMS, Jaeger KE (1995) Biochim. Biophys. Acta 1256: 395; Lang D, Hofmann B, Haalck L, Hecht HJ, Spener F, Schmid RD, Schomburg D (1996) J. Mol. Biol. 259: 704.

5.5 Major Culture Collections

ARS (NRRL)	Agricultural Research Service Culture Collection USDA, 1815 N. University Street, Peoria, Illinois 61604, USA http://nrrl.ncaur.usda.gov
ATCC	American Type Culture Collection 12301 Parklawn Drive, Rockville, Maryland 20852, USA http://www.atcc.org
CBS	Centraalbureau voor Schimmelcultures, Julianalaan 67, NL-2628 Delft BC, The Netherlands http://www.cbs.knaw.nl
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen Mascheroder Weg 1b, D-38124 Braunschweig, Germany http://www.dsmz.de
IFO	Institute of Fermentation 17-85 Jusohomachi 2-chome, Yodogawaku, Osaka 532, Japan http://www.ifo.or.jp/db/f_abc_e.html
IMI (CMI)	International Mycological Institute Bakeham Lane, Kew, TW20 9TY Surrey, England http://herbariaunited.org/institution/IMI/
NCIMB (NCIB)	National Collections of Industrial and Marine Bacteria 23 St. Machar Drive, Aberdeen AB2 1RY, Scotland http://www.ncimb.co.uk
NCYC	National Collection of Yeast Cultures Food Research Institute, Colney Lane, Norwich, Norfolk NR4 7UA, UK http://www.ncyc.co.uk/
WDCM	World Data Centre for Microorganisms http://www.wfcc.info/datacenter.html

5.6 Pathogenic Bacteria and Fungi

Dangerous Pathogens

Bacteria	
Bacillus anthracis	*Mycobacterium bovis*
Bordetella pertussis	*Mycobacterium leprae*
Clostridium bifermentans	*Mycobacterium tuberculosis*
Clostridium botulinum	*Neisseria gonorrhoeae*
Clostridium fallax	*Neisseria meningitidis*
Clostridium histolyticum	*Pasteurella pestis*
Clostridium oedematiens	*Pseudomonas pseudomallei*
Clostridium septicum	*Salmonella typhi*
Clostridium welchii (perfringens)	*Streptococcus pneumoniae*
Corynebacterium diphtheriae	*Treponema pallidum*
Flavobacterium meningosepticum	*Treponema pertenue*
Leptospira icterohaemorrhagiae	*Vibrio cholerae*

Fungi	
Aspergillus fumigatus	*Histoplasma capsulatum*
Blastomyces dermatitidis	*Histoplasma farcimimosum*
Coccidioides immitis	*Paracoccidioides braziliensis*

Readily Infectious Pathogens

Bacteria	
Borrelis sp.	*Moraxella lacunata*
Brucella abortus	*Pasteurella tularensis*
Brucella melitensis	*Pseudomonas aeruginosa*
Brucella suis	*Pseudomonas pyocyanae*
Fusobacterium fusiforme	*Shigella dysenteriae*
Haemophilus aegypticus	*Shigella flexneri*
Haemophilus ducreyi	*Shigella sonnei*
Haemophilus influenzae	*Staphylococcus aureus*
Klebsiella pneumoniae	*Staphylococcus pyogenes*
Klebsiella rhinoscleromatis	

Fungi	
Candida albicans	*Sporotrichum schenkii*
Epidermophyton flossosum	*Trychophyton verrucosum*
Microsporum sp.	

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