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 half 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 organic solvent systems. The special methodologies involved in this latter type of reaction are described in Sect. 3.1.

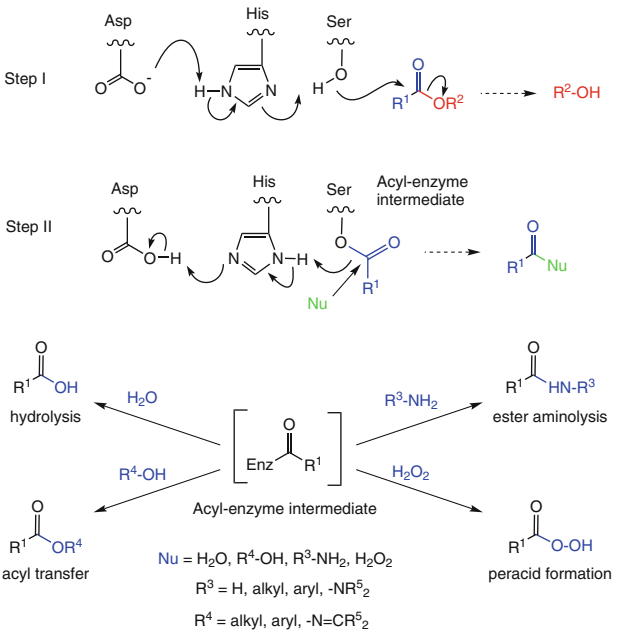
Other applications of hydrolases, such as those involving the formation and/or cleavage of phosphate esters, epoxides, nitriles, and organo-halides, are described in separate chapters.

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 carboxylate group of an aspartic acid (e.g., pepsin) [3], or the thiol functionality of cysteine (e.g., papain) [4–6].

K. Faber, *Biotransformations in Organic Chemistry*,
DOI 10.1007/978-3-319-61590-5_2

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



Scheme 2.1 The serine hydrolase mechanism

When the enzyme is operating in an organic solvent at low water concentrations – more precisely, at low water activity – any other nucleophile can compete with the 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 via an interesterification reaction, called 'acyl transfer' [13, 14].
- The action of ammonia furnishes a carboxamide R^1–CO–NH_2 via an ammonolysis reaction [15, 16].

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

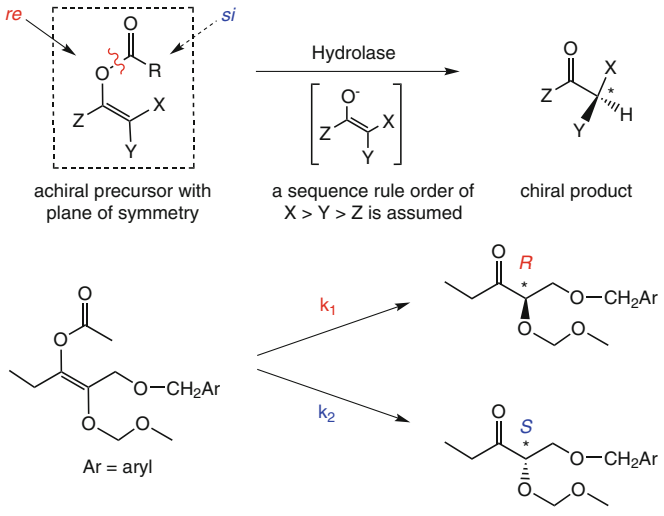
- An incoming amine R^3–NH_2 results in the formation of an *N*-substituted amide R^1–CO–NH–R^3, yielding an enzymatic aminolysis of esters [17, 18].
- Hydrazinolysis provides access to hydrazides [19, 20], and the action of hydroxylamine results in the formation of hydroxamic acid derivatives [21].
- Peracids of type R^1–CO–OOH are formed when hydrogen peroxide is acting as the nucleophile [22].
- 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. The magnitude of this discrimination is governed by the kinetics and is a crucial parameter since it stands for the 'selectivity' of the reaction. 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

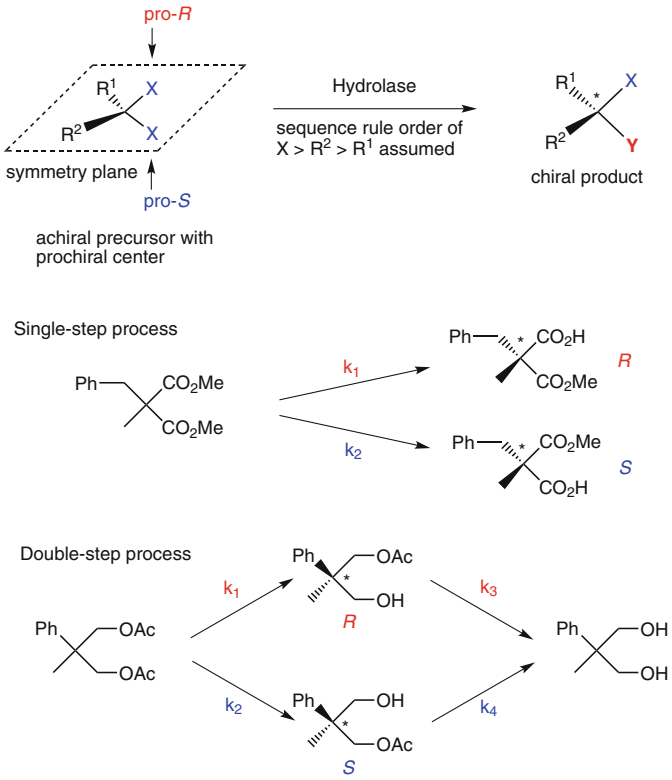
Hydrolases can distinguish between the two enantiomeric faces of achiral substrates such as enol esters possessing a plane of symmetry within the molecule [25]. The attack of the enzyme's nucleophilic chemical operator predominantly occurs from one side, leading to an unsymmetric enolization of the unstable 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).



Scheme 2.2 Enantioface differentiation (achiral substrates)

Enantiotopos Differentiation

If prochiral substrates possessing two chemically identical but enantiotopic reactive groups X (designated pro-*R* and pro-*S*) are subjected to enzymatic 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 α,α-disubstituted malonic diester by pig liver esterase or α-chymotrypsin is a representative example [27]. Here, the reaction terminates at the 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].



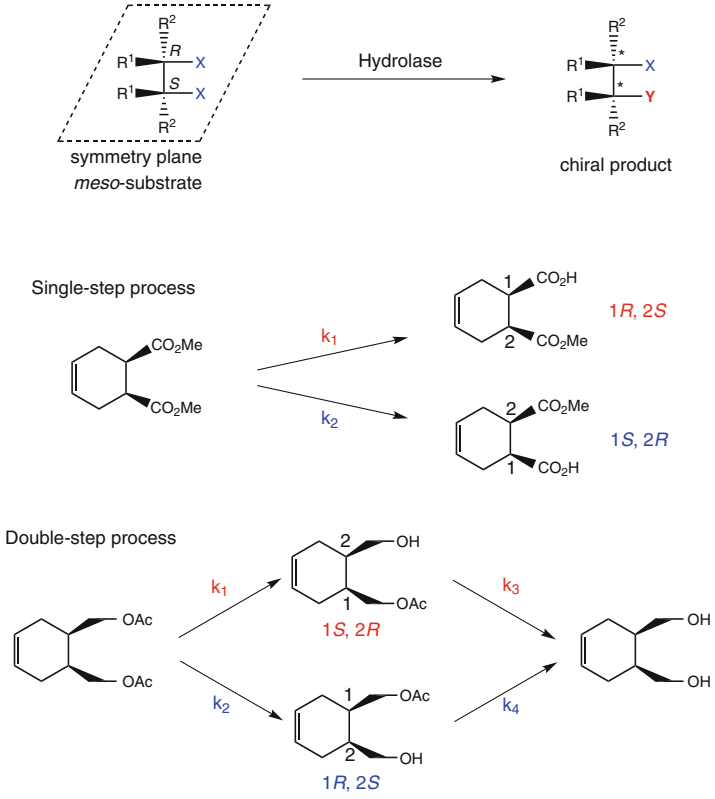
Scheme 2.3 Enantiotopos differentiation (prochiral substrates)

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, will react at different rates in a hydrolase-catalyzed reaction (Scheme 2.4). In this way, 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 diacetate 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 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].



Scheme 2.4 Desymmetrization of *meso*-substrates

Since hydrolytic reactions are performed in an aqueous environment, where the molar concentration of water is ~55.5 mol/L, they are virtually completely

irreversible. 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 (denoted α [32]) is only governed by the ratio of k_1/k_2, which is *independent of the conversion* and therefore remains constant throughout the reaction. Thus, the optical purity of the product (e.e.$_P$) is *not* dependent on the extent of the conversion. Consequently, 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, 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. 72–79 and 102–103.

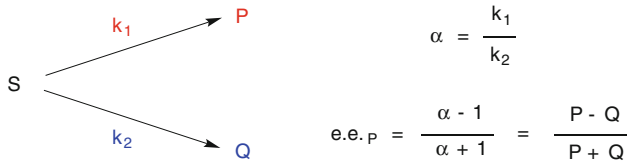


Fig. 2.1 Single-step kinetics

As mentioned above, occasionally a second successive reaction step cannot be avoided with diesters of prochiral or *meso*-diols (Schemes 2.3 and 2.4). For such types of substrates the reaction does not terminate at the chiral 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 desired product (e.e.$_P$) – depends now on four rate constants, k_1 through k_4, due to the presence of the second hydrolytic step. From the fact that enzymes usually show a continuous preference for reactive groups possessing the same chirality,[2] one can 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.$_P$) becomes a *function of the conversion* of the reaction, and generally follows the curve shown in Fig. 2.2.

[2]These groups are called homochiral.

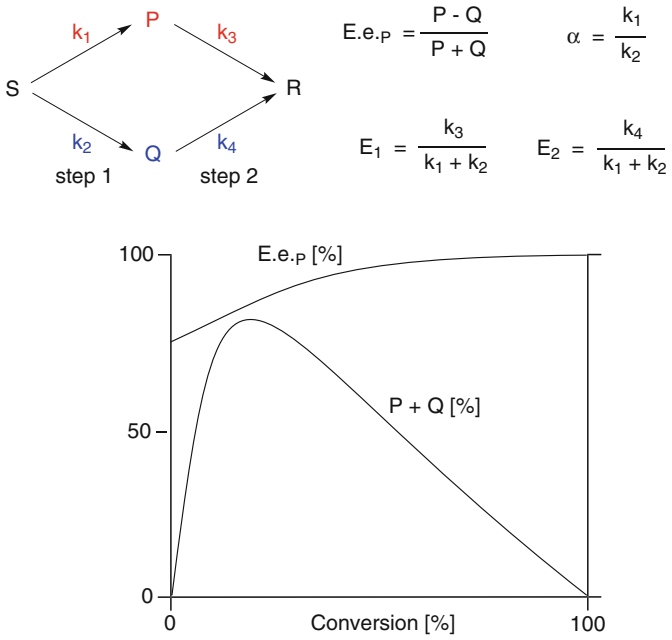


Fig. 2.2 Double-step kinetics

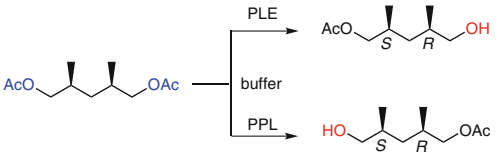
During early stages of the reaction, the optical purity of the product is mainly determined by the selectivity (α) of the first reaction step, which constitutes an enantiotopos or enantioface differentiation, depending on the type of substrate.

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. 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.$_P$), because Q is hydrolysed faster than P. In contrast, the product concentration [P + Q] follows a bell-shaped curve: After having reached a maximum at a certain conversion (as long as the first step is faster than the second), the product concentration finally drops off again when most of the substrate S is consumed and the second hydrolytic step (forming R at the expense of P + Q) begins to dominate. 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 versus the second step $(k_1 + k_2)/(k_3 + k_4)$ has a major impact on the *chemical yield* of P + Q, whereas the match or mismatch of the selectivities ($k_1 > k_2$, $k_3 < k_4$ or $k_1 > k_2$, $k_3 > k_4$, respectively) 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 is 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]. 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 [34].



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

Enantiomer Differentiation

When a racemic substrate is subject to enzymatic hydrolysis, chiral discrimination of the enantiomers occurs [35]. 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 1.3). Due to the chirality of the active site of 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 [36]! 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 more racemic compounds possible as opposed to prochiral and *meso*-analogs. After all, prochiral and *meso*-compounds have only two functional groups (R^1, R^2) available for variation, whereas racemates have three (R^1, R^2, R^3) [37].

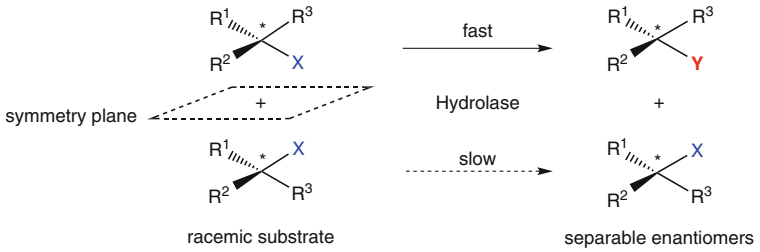
The most striking difference from the above-mentioned types of desymmetrization reactions, which show a theoretical yield of 100%, is that in kinetic resolution each of the enantiomers can be obtained in only 50% yield.

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) [38].

In practice, however, the enantioselectivity is not ideal, and the difference in – or more precisely the ratio of – the reaction rates of the enantiomers is not infinite, but measurable. The thermodynamic reasons for this have been discussed in Chap. 1 (Fig. 1.8). 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 substrate enantiomer varies with the degree of conversion, since their ratio 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*.



Scheme 2.6 Enantiomer differentiation

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 [39] on a theoretical basis described by K.B. Sharpless [40] and K. Fajans [41]. 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 [42–45].[3] 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. 1.8):

[3]The Enantiomeric Ratio (E) is a synonym for the so-called selectivity factor (s). Whereas E is used more often in biocatalyzed kinetic resolutions, the s-factor is more common in chemocatalysis. 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 [45].

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

The '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]) [46]. Related alternative methods for the experimental determination of *E*-values have been proposed [47–49].

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).

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), 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.

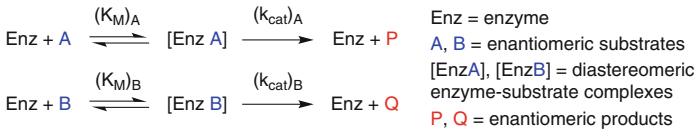


Fig. 2.3 Enzymatic kinetic resolution (irreversible reaction)

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

For the product	For the substrate
$E = \frac{\ln[1 - c(1 + e.e._P)]}{\ln[1 - c(1 - e.e._P)]}$	$E = \frac{\ln[(1 - c)(1 - e.e._S)]}{\ln[(1 - c)(1 + e.e._S)]}$

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, which are *relative* quantities, in contrast to the conversion, which is an *absolute* quantity [50].

$$E = \frac{\ln \dfrac{[\text{e.e.}_\text{P}(1 - \text{e.e.}_\text{S})]}{(\text{e.e.}_\text{P} + \text{e.e.}_\text{S})}}{\ln \dfrac{[\text{e.e.}_\text{P}(1 + \text{e.e.}_\text{S})]}{(\text{e.e.}_\text{P} + \text{e.e.}_\text{S})}}$$

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). Its optical purity remains low before 40%, then climbs significantly at around 50%, and reaches its maximum beyond the 60% conversion point.

Very high optical purity of substrate can be reached by extending the reaction beyond ~60% conversion, albeit at the price of reduced yield. Attractive optical purities for the substrate and product demand a very high enantioselectivity.

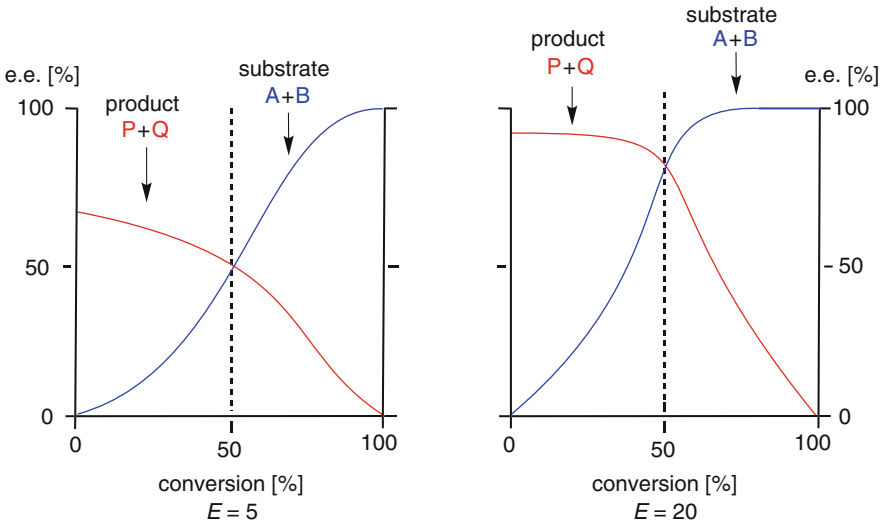


Fig. 2.4 Dependence of optical purities (e.e._S/e.e._P) on the conversion

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 [51].[4] 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 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' point X (step 1). 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 2). 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 [52] can be used to allow practical use of numerous enzyme-catalyzed kinetic resolutions which show incomplete selectivities.

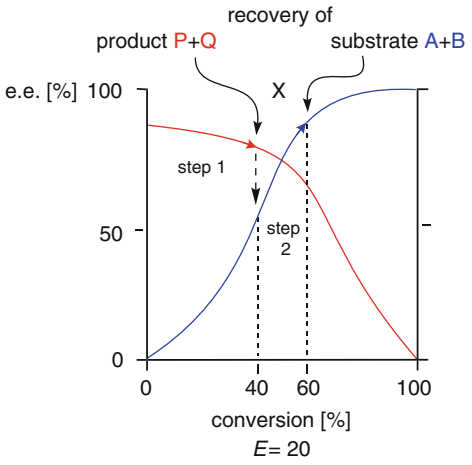


Fig. 2.5 Two-step enzymatic resolution

Reversible Reaction The situation becomes more complicated when the reaction is reversible [53, 54]. Then, 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.

[4] http://biocatalysis.uni-graz.at/enantio/

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

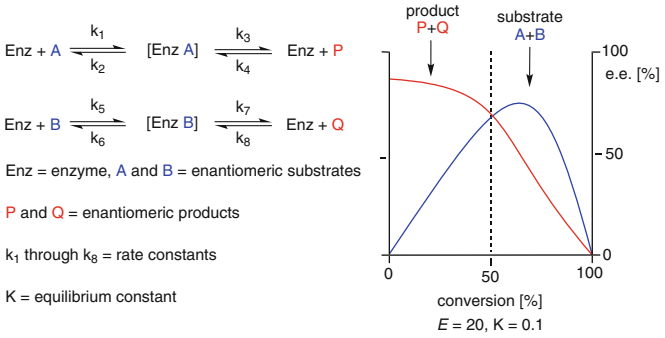


Fig. 2.6 Enzymatic kinetic resolution (reversible reaction)

As shown in Fig. 2.6, the product curve of an enzymatic resolution following a reversible reaction type remains almost the same as in the irreversible case. However, a significant difference 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 enantiopreference of the substrate stays the same in both directions, it follows 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 conversion levels, 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

nucleophile: in order to obtain an equilibration constant of $K > 10$, about 20 M equivalents of nucleophile versus substrate are sufficient to obtain a virtually irreversible type of reaction. 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 substrate e.e.$_{A/B}$, the intermediate e.e.$_{P/Q}$, and the final product e.e.$_{R/S}$ can be calculated [55, 56].

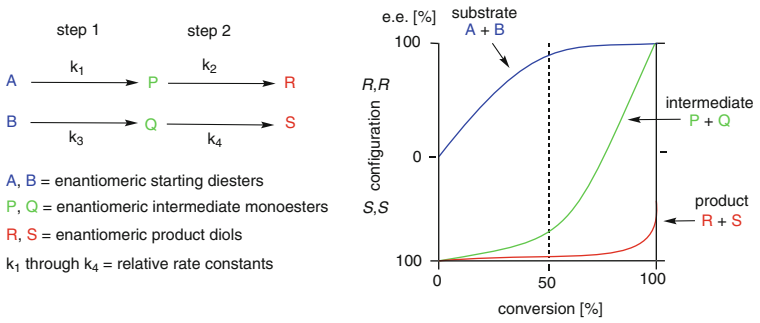
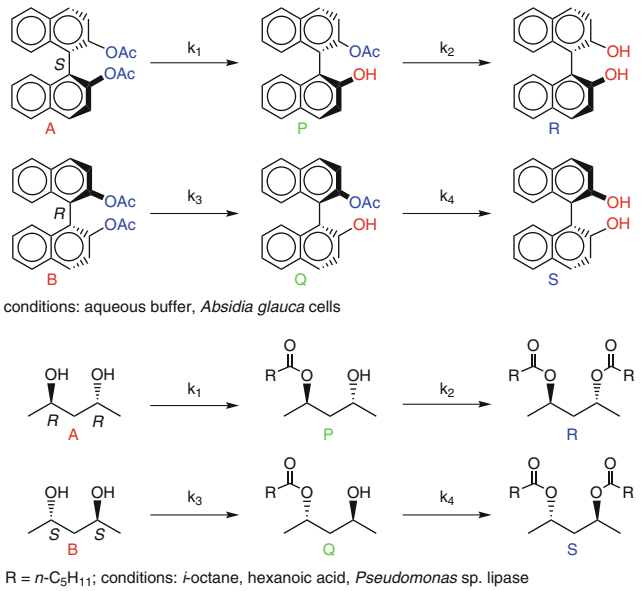


Fig. 2.7 Kinetis of sequential kinetic resolution of bifunctional substrates

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 [57]. 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 [58] or microbial cells [59], 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 [60].



R = *n*-C_5H_{11}; conditions: *i*-octane, hexanoic acid, *Pseudomonas* sp. lipase

Scheme 2.7 Sequential enzymatic resolution of bifunctional substrate via hydrolysis or esterification

A special type of sequential enzymatic resolution involving a hydrolysis-esterification [61] or an alcoholysis-esterification sequence [62] is depicted in Fig. 2.8. In view of the mechanistic symmetry of enzymatic acyl transfer reactions (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 accompanying re-esterification is omitted.

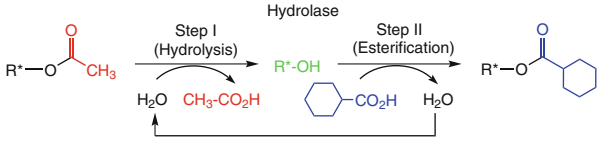
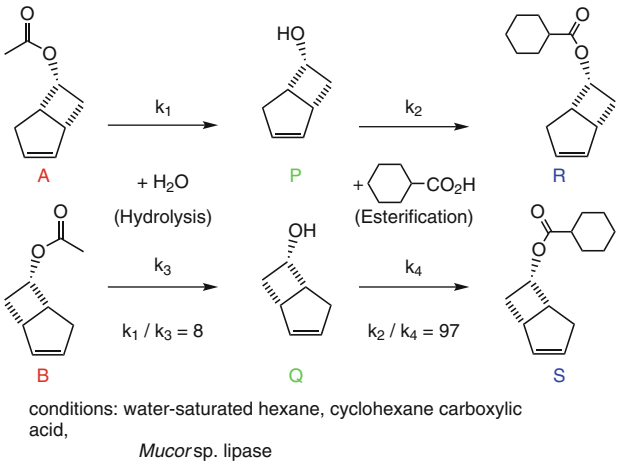


Fig. 2.8 Mechanism of sequential enzymatic kinetic resolution of monofunctional substrate via concurrent hydrolysis-esterification

As shown in Scheme 2.8, the racemic starting ester (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 of monofunctional substrate via concurrent hydrolysis-esterification in aqueous-organic solvent

Deracemization

Despite its widespread use, kinetic resolution has several disadvantages, 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:

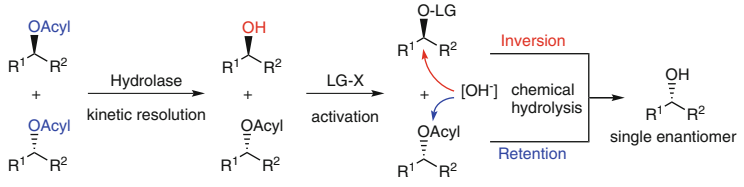
- The theoretical yield of each enantiomer is limited to 50%. Furthermore, in general only one stereoisomer is desired and there is little or no use for the other.
- Separation of the product from the remaining substrate may be laborious, in particular when simple extraction or distillation fails [63].
- 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 [64, 65]. All of these processes which lead to the formation of a single stereoisomeric product from a racemate are called 'deracemizations' [66–68].

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 continuously operated 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 although only 50% of the desired enantiomer is obtained after a single cycle, the overall (theoretical) yield increases to ~94% after only four cycles [69].

In practice, however, deracemization via repeated resolution is often plagued by low overall yields due to the harsh reaction conditions required for (chemical) racemization [70]. In view of the mild reaction conditions displayed by enzymes, racemases of EC-class 5 are increasingly being employed [71, 72].

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 can be chemically inverted into its enantiomer *before* separating the products (Scheme 2.9) [73, 74]. Introduction of a good leaving group, LG (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 calculated as a function of the E value of the reaction, and it is usually at or slightly beyond a conversion of 50% [75, 76].



Scheme 2.9 Kinetic resolution followed by in-situ inversion

Dynamic resolution is a more elegant approach [77–82] This comprises a classic resolution with an additional feature, i.e., the resolution is carried out using conditions under which the substrate enantiomers 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 [83, 84].[5]

In this case, several reactions 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 A/B ($k_A \gg k_B$ or $k_B \gg k_A$).
- 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 ($k_{rac}^{Sub} \geq k_A$ or k_B, 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 [85–91]; several examples are given in subsequent chapters.

The kinetics of a dynamic resolution is outlined in the following example [78, 92]. 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 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 [93]:

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

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_A ($E \sim 10$). 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

[5] Dynamic resolution is a type of second-order asymmetric transformation [79, 83]

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.

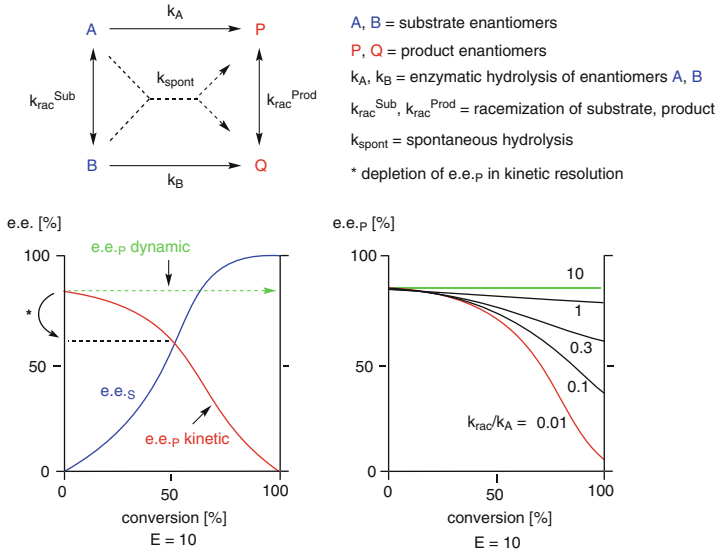


Fig. 2.9 Dynamic kinetic resolution with in-situ racemization

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.

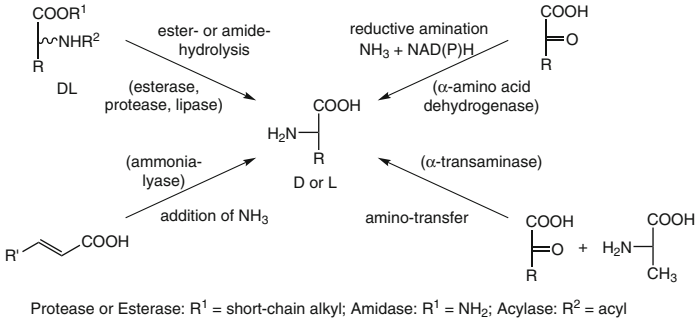
2.1.2 Hydrolysis of the Amide Bond

The enzymatic hydrolysis of the carboxamide bond is associated to the biochemistry of amino acids and peptides [94]. The world production of enantiomerically pure amino acids was estimated to comprise a market of ca. US $ 11 billion per annum in 2015 [95]. The amino acids dominating this area with respect to output and value are produced by fermentation (L-lysine, L-phenylalanine, L-tryptophan, L-threonine, L-arginine, L-histidine, L-isoleucine, L-serine, L-valine) [96] and by synthesis (D,L-methionine) on industrial scale. 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 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 (Scheme 2.208).

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 sophisticated procedures are the (1) reductive amination of α-keto acids using α-amino acid dehydrogenases (pp. 158–161), (2) asymmetric addition of ammonia onto α,β-unsaturated carboxylic acids catalyzed by ammonia lyases (Sect. 2.5.2), and (3) amino-group transfer using α-transaminases (Sect. 2.6.2) [97–99].

The hydrolytic methods discussed below were selected from the numerous strategies for amino acid synthesis [94, 100–106] for their flexibility, since they are not restricted to the 20 canonical amino acids, but also accept nonnatural analogs and give rise to D- or L-enantiomers. Several of these methods are employed on industrial scale [107].



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: In general, the substrate enantiomer possessing the 'natural' L-configuration is preferred by the enzyme, while the 'unnatural' D-counterpart remains unchanged and thus can be recovered from the reaction medium. 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. However, enzymes with complementary enantiopreference are available for some processes such as the amidase, hydantoinase and acylase method (see below) to directly obtain the desired enantiomer. The work-up procedure is usually easy, because the difference in solubility of the product and the remaining substrate at different pH medium facilitates their separation by extraction.

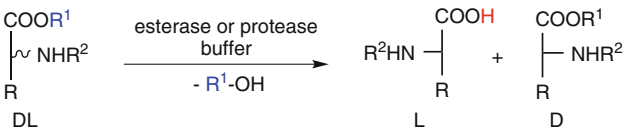
However, there is a limitation to the majority of these methods: the α-carbon atom bearing the amino group must not be fully substituted, since such bulky

substrates are generally not accepted by hydrolases. Thus, enantiopure α-methyl or α-ethyl amino acids are generally not accessible by these methods, although some exceptions are known [108, 109].

The recycling of the undesired enantiomer from the kinetic resolution is of crucial importance particularly on an industrial scale [110]. In the past, amino acid esters were thermally racemized at about 150–170 °C, milder conditions for the racemization of amino acid amides employed the formation of Schiff bases with aromatic aldehydes (such as benzaldehyde or salicylaldehyde) (Scheme 2.13). Nowadays, racemases [111] are used in 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 [112]! The catalytic activity of a protease on a carboxylic ester bond has frequently been denoted as 'esterase activity', although the mechanism of action does not differ 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 2.19) [113].



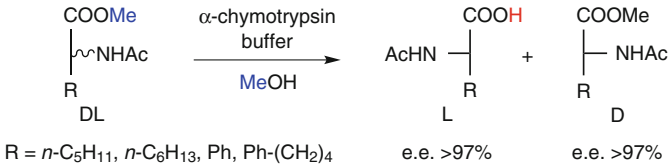
R = alkyl or aryl; R^1 = short-chain alkyl; R^2 = H or acyl

Scheme 2.11 Enzymatic resolution of α-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 with esterases or proteases. When lipases are used, 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 [114] or trifluoroethyl [115], 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) [118–120]. 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 [121, 122]. Alternatively, other proteases, such as subtilisin [123, 124], thermolysin [125], and alkaline protease [126] 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 employed [127].

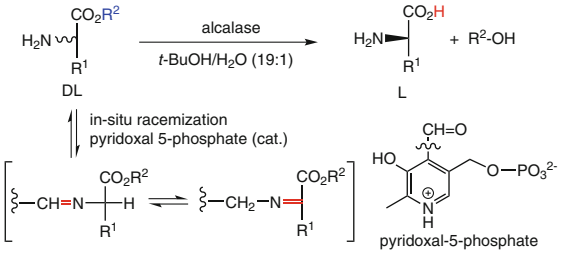
Carbonic anhydrase – an enzyme termed for its ability to catalyze the hydration of carbon dioxide forming hydrogen carbonate – can also be employed. In contrast to the above-mentioned enzymes, it exhibits the opposite enantiopreference by hydrolyzing the D-*N*-acylamino acid esters [116].



Scheme 2.12 Resolution of *N*-acetyl α-amino acid esters by α-chymotrypsin [116, 117]

An efficient dynamic resolution process for α-amino acid esters has been developed using a crude industrial protease preparation from *Bacillus licheniformis* ('alcalase')[6] (Scheme 2.13) [128]. 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. 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 preferable for large-scale applications.

[6] '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

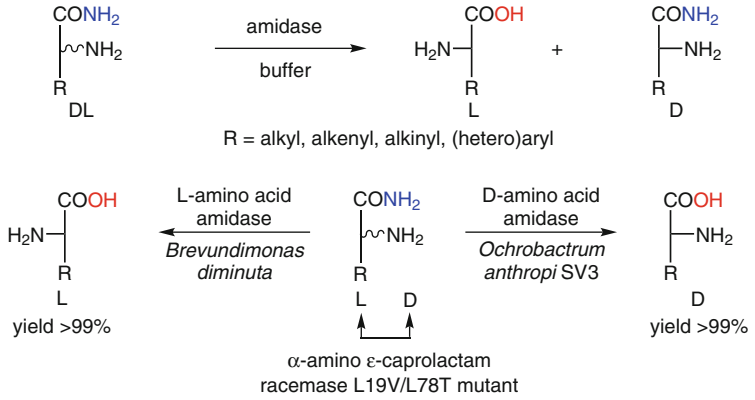
Scheme 2.13 Dynamic resolution of α-amino acid esters via the esterase method

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 [129] and from different microorganisms, in particular *Pseudomonas*, *Aspergillus*, or *Rhodococcus* spp. (Scheme 2.14, top) [130]. For industrial applications, special amidases (e.g., from *Mycobacterium neoaurum* and *Ochrobactrum anthropi*) have been developed [131, 132]. They are also accept α-substituted α-amino acid amides, which are otherwise not easily hydrolyzed due to steric hindrance [108]. Unreacted D-amino acid amides can be separated from the L-amino acids by extraction into organic solvents due to their different solubility at various pH. After separation, unreacted D-amino acid amides can be recycled ex-situ via base-catalyzed racemization of the corresponding Schiff-base intermediates in a separate step in analogy to the process depicted in Scheme 2.13 [133]. 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 compared to those obtained by the esterase method.

In order to avoid tedious separation and ex-situ racemization of the undesired enantiomer from kinetic resolution an elegant dynamic two-enzyme process was developed (Scheme 2.14, bottom) [134]. D-Amino acid amides were hydrolyzed enantioselectively using a thermostable mutant of D-amino acid amidase from *Ochrobactrum anthropi* SV3, while in-situ racemization of the racemic substrate was accomplished by a double mutant of α-amino-ε-caprolactam racemase (L19V/

L78T) from *Achromobacter obae*. Opposite L-amino acids were obtained by using an L-amino acid amidase from *Brevundimonas diminuta*. Both amidases and the racemase were co-expressed into a single *E. coli* host to facilitate handling.

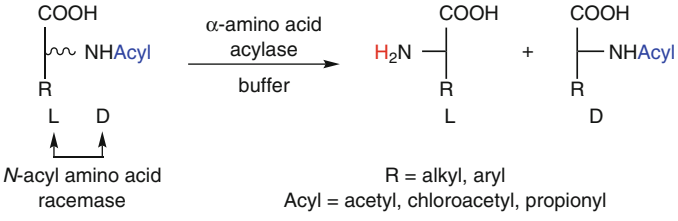


R	E.e. [%]	
	L	D
Ph-	>99	88
4-F-C_6H_4-	95	83
Ph-CH_2-	92	99
4-HO-C_6H_4-CH_2-	95	>99
2-, 3-, or 4-F-C_6H_4-CH_2-	91-98	93-97
4-Cl-C_6H_4-CH_2-	88	73
Ph-$(CH_2)_2$-	98	77

Scheme 2.14 Kinetic and dynamic 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, propionyl or benzoyl. Alternatively, the corresponding *N*-carbamoyl- and *N*-formyl derivatives can be used [135]. Enzymes of the amino acylase type have been isolated from hog kidney, and from *Aspergillus* or *Penicillium* spp. [136–138]. 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) [139, 140].

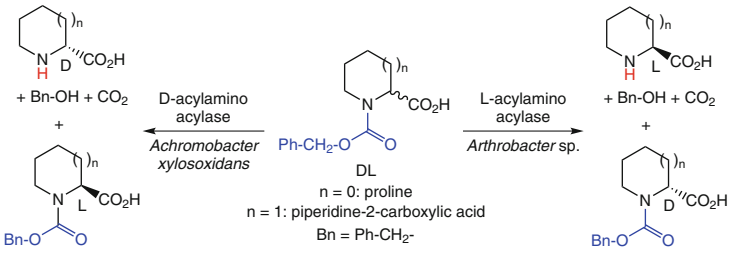


Scheme 2.15 Enzymatic resolution of *N*-acyl amino acids via the acylase method

On a laboratory scale, the readily available amino acylase from hog kidney is recommended [141]. It proved to be extremely substrate-tolerant, allowing variations of the alkyl- or aryl-moiety R within a wide structural range while retaining very high specificities for L-enantiomers, which made it a reliable tool for the synthesis of bioactive compounds [142–144]. Unwanted enantiomers of *N*-acetyl amino acids can be racemized ex-situ by heating with acetic anhydride, which involves activation of the acid moiety via a mixed anhydride, which undergoes cyclization to form an oxazolinone (azlactone). The latter is subject to racemization via an intermediate achiral enol. Like the amidase process, on large scale the acylase method was converted into a dynamic process by in-situ racemization of the nonreacting *N*-acylamino acid using an *N*-acylamino acid racemase [145–147]. 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 [148].

Although the majority of *N*-acylamino acid acylases are L-selective, several stereo-complementary D-acylases were identified [149–152], which allow to access D-amino acids. Cyclic amino acids, such as piperidine-2-carboxylic acid are valuable building blocks for the synthesis of pharmaceuticals, such as 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) [153–154].

Interestingly, even *N*-acyl α-amino*phosphonic* acid derivatives have been resolved using penicillin acylase [155].

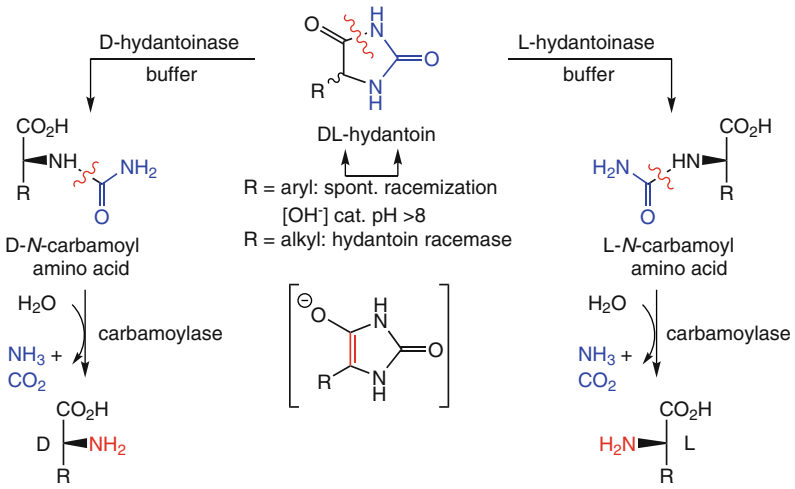


Scheme 2.16 Resolution of cyclic *N*-benzyloxycarbonyl amino acids using enantiocomplementary acylases

Hydantoinase Method

5-Substituted hydantoins are obtained in racemic form from cheap starting materials such as an aldehyde, hydrogen cyanide, and ammonium carbonate using the Bücherer–Bergs synthesis [156]. Hydantoinases from different microbial sources catalyze the hydrolytic ring-opening to form the corresponding *N*-carbamoyl–α-amino acids [157–159]. 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 2.17) [160–162].



Scheme 2.17 Enzymatic resolution of hydantoins via the hydantoinase method

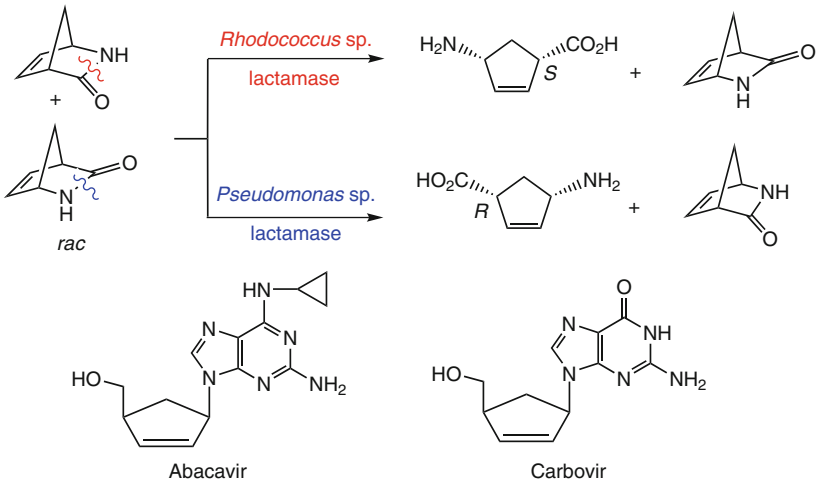
In contrast to the above-mentioned amino acid resolution methods involving amino acid esters, -amides, or *N*-acylamino acids where the natural L-enantiomer is preferably hydrolyzed, hydantoinases usually convert the opposite D-enantiomer [163–165], and L-hydantoinases are known to a lesser extent [166–168]. In addition, D-hydantoinases usually possess a broader substrate spectrum than their L-counterparts. Previously, *N*-carbamoyl amino acids thus obtained were chemically deprotected by treatment with nitrous acid or by exposure to an acidic pH (<4). Nowadays, they are enzymatically hydrolyzed to yield the corresponding amino acids by use of an *N*-carbamoyl amino acid amidohydrolase (carbamoylase) with matching enantiopreference, which is often produced by the same microbial species [169]. 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% [170, 171].

Lactamase Method

Due to their cyclic structure, cyclic amides (γ-, δ- and ε-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 [172].

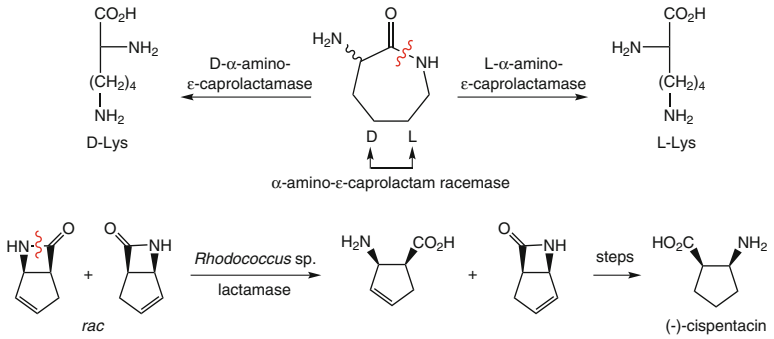
The bicyclic γ-lactam shown in Scheme 2.18 is an important starting material for the production of antiviral agents, such as Carbovir and Abacavir. It can be efficiently resolved using enantiocomplementary γ-lactamases from microbial sources: an enzyme from *Rhodococcus equi* produced the (*S*)-configurated 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 [173].



Scheme 2.18 Enzymatic resolution of bicyclic γ-lactams via the lactamase method

For the biocatalytic synthesis of lysine, an enantioselective lactamase is employed in the kinetic resolution of *rac*-α-amino-ε-caprolactam (Scheme 2.19, top) [174]. A suitable α-amino-ε-caprolactam racemase was found in several bacterial species, such as *Achromobacter*, *Alcaligenes* and *Flavobacterium*, detailed studies were performed with the enzyme from *Achromobacter obae* [175]. Quite remarkably, this racemase also accepts non-cyclic amino acid amides [176]. The racemase is used in combination with a suitable D- or L-α-amino-ε-caprolactamase in a dynamic process for the production of D- or L-lysine on an industrial scale in 100% yield at ~4000 t per annum from the racemic lactam [177].

In contrast to γ-, δ- and ε-lactams, highly strained β-lactams are more easily susceptible to enzymatic hydrolysis and thus can be (slowly) hydrolyzed by carboxyl ester hydrolases, such as esterases [178] and lipases [179, 180]. The bicyclic lactam shown in Scheme 2.19 (bottom), which serves as starting material for the synthesis of the antifungal agent (–)-cispentacin, was efficiently resolved using *Rhodococcus equi* lactamase [181].



Scheme 2.19 Enzymatic hydrolysis of strained β-lactams and α-amino-ε-caprolactam using lactamases

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 [182] 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 popular 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 horses or pigs, its high price – which is prohibitive for large-scale applications – is probably justified. Thus, the number of ACE applications is limited [183–186]. Additionally, also cholesterol esterase is of limited use, since it seems to prefer bulky substrates which show structural similarities to the natural substrates of cholesterol esterase, i.e., steroid esters [58, 187].

To overcome this narrow range of readily available esterases, whole microbial cells are sometimes used instead of isolated enzyme preparations [188]. 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 whole cells are employed, because in most cases the nature of the actual active enzyme system remains unknown.

More recently, novel microbial esterases [189, 190] such as carboxyl-esterase NP [191] 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 [192], 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 [193, 194].

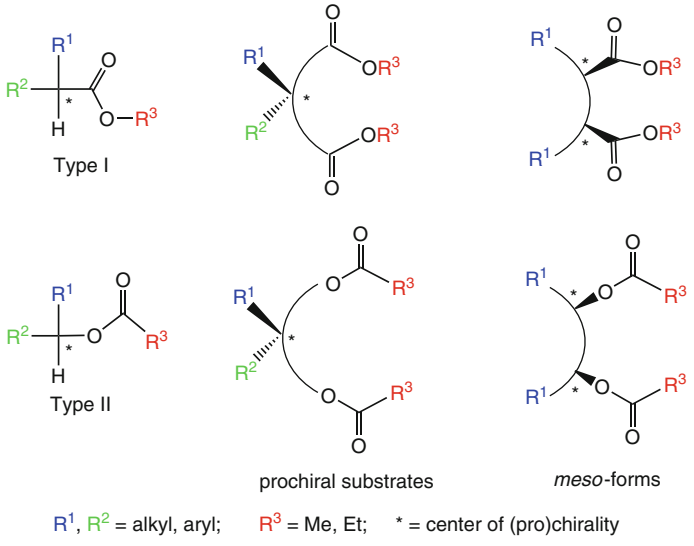
Fortunately, as mentioned in the foregoing chapter, a large number of proteases can also selectively hydrolyze carboxylic esters and this effectively compensates for the limited number of esterases [195]. The most frequently used members of this group are α-chymotrypsin [196], subtilisin [197] and, to a somewhat lesser extent, trypsin, pepsin [198], papain [199], penicillin acylase [200, 201] and a protease from *Aspergillus oryzae*. The latter enzyme 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 [202].

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 section but are incorporated into the following chapter.

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 2.20. 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.
- 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 attaching electron-withdrawing groups to the alcohol moiety to give methoxymethyl or 2-haloethyl esters, respectively. 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 or methoxy (leading to α-halo- or α-methoxyacetates) is a frequently used method to enhance the reaction rate in enzyme-catalyzed ester hydrolysis [203].
- 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 [204–207]. 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 [208–210].
- 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 2.20).



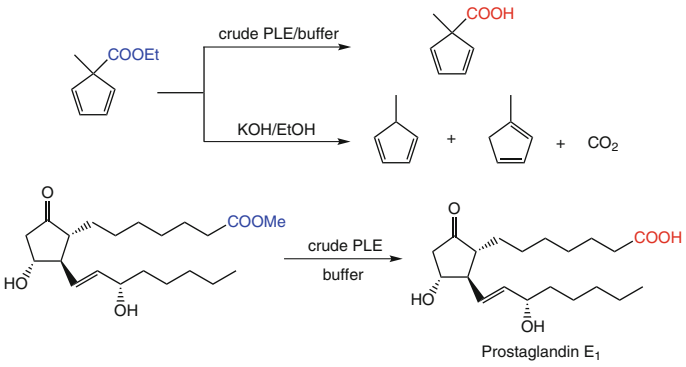
Scheme 2.20 Types of substrates for esterases and proteases

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 several so-called *iso*enzymes, which are associated as trimers of three individual proteins [211]. However, for many applications this crude mixture can be used without any problems although the isoenzyme subunits often possess similar (but not identical [212]) stereospecificities [213]. Thus, the selectivity of crude PLE may vary, depending on the source and the pretreatment of the enzyme preparation [214]. 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 [215].

In general, hepatic esterases from related sources such chickens, hamsters, guinea pigs, or rats were found to be less versatile when compared to PLE. In certain cases, however, esterases from rabbit [216, 217] and horse liver (HLE) [218, 219] proved to be useful substitutes for PLE.

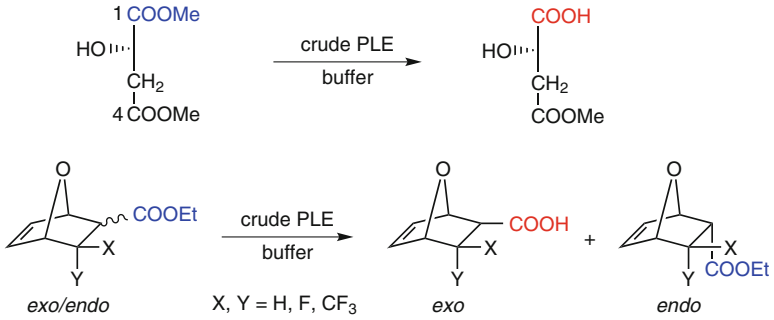
Mild Hydrolysis Acetates of primary and secondary alcohols such as cyclopropyl acetate [220] and methyl or ethyl carboxylates (such as the labile cyclopentadiene ester [221]) can be selectively hydrolyzed under mild conditions using PLE, avoiding decomposition reactions which would occur during a chemical hydrolysis under acid or base catalysis (Scheme 2.21). 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 [222, 223].



Scheme 2.21 Mild ester hydrolysis by porcine liver esterase

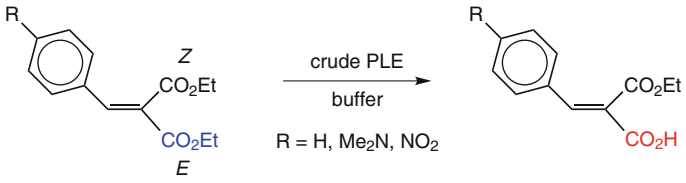
Regio- and Diastereoselective Hydrolysis Regiospecific hydrolysis of dimethyl malate at the 1-position could be effected with PLE as catalyst (Scheme 2.22) [224]. Similarly, hydrolysis of an *exo*/*endo*-mixture of diethyl dicarboxylates with a bicyclo[2.2.1]heptane framework occurred only on the less hindered *exo*-position

[225] leaving the *endo*-ester untouched, thus allowing a facile separation of the two positional isomers in a diastereomeric mixture.



Scheme 2.22 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 [226] (Scheme 2.23). 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.

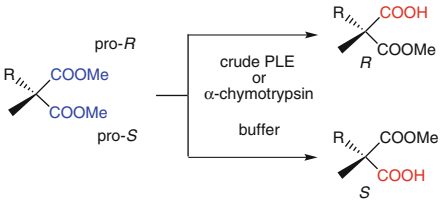


Scheme 2.23 Regioselective hydrolysis of *E*/*Z*-diastereotopic diesters by porcine liver esterase

Desymmetrization of Prochiral Diesters PLE has been used less frequently for the resolution of racemic esters (where α-chymotrypsin has played a more important role) but was employed more widely for the desymmetrization of prochiral diesters.

As depicted in Scheme 2.24, α,α-disubstituted malonic diesters can be selectively transformed by PLE or α-chymotrypsin to give the corresponding chiral monoesters [227, 228]. These transformations demonstrate an illustrative example for an 'alternative fit' of substrates with different steric requirements. While PLE

preferentially hydrolyses the pro-*S* ester group on substrates possessing small α-substituents (R) 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.

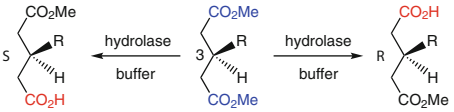


Enzyme	R	Configuration	e.e. [%]
PLE*	Ph-	*S*	86
PLE	C_2H_5-	*S*	73
PLE	*n*-C_3H_7-	*S*	52
PLE	*n*-C_4H_9-	*S*	58
PLE	*n*-C_5H_{11}-	*R*	46
PLE	*n*-C_6H_{13}-	*R*	87
PLE	*n*-C_7H_{15}-	*R*	88
PLE	*p*-MeO-C_6H_4-CH_2-	*R*	82
PLE	*t*-Bu-O-CH_2-	*R*	96
α-chymotrypsin	Ph-CH_2-	*R*	~100

* The ethyl ester was used.

Scheme 2.24 Desymmetrization of prochiral malonates by porcine liver esterase and α-chymotrypsin

As shown in Scheme 2.25, the prochiral center may be moved away from the ester moiety into the β-position. Thus, chiral recognition by PLE [229–233] and α-chymotrypsin [234–237] is retained during the desymmetrization of prochiral 3-substituted glutaric diesters. Whole cells of *Acinetobacter lowffii* and *Arthrobacter* spp. have also been used as a source for esterase activity [238] and, once again, depending on the substitutional pattern on carbon-3, the desymmetrization can lead to both enantiomeric products.

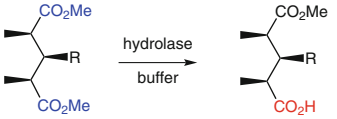


Hydrolase	R	Product	e.e. [%]
α-chymotrypsin*	AcNH-	*R*	79
α-chymotrypsin	Ph-CH_2-O-	*R*	84
α-chymotrypsin	CH_3OCH_2O-	*R*	93
PLE	AcNH-	*R*	93
PLE	CH_3-	*R*	90
PLE	Ph-CH_2-CH=CH-CH_2-	*S*	88
PLE	*t*-Bu-CO-NH-	*S*	93
PLE	HO-	*S*	12
α-chymotrypsin*	HO-	*R*	85
Acinetobacter sp.*	HO-	*R*	>95
Arthrobacter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

* The corresponding ethyl esters were used.

Scheme 2.25 Desymmetrization of prochiral glutarates

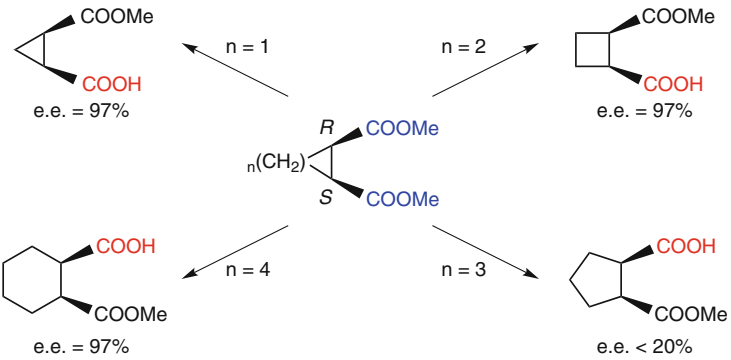
Acyclic *meso*-dicarboxylic esters with a glutaric acid backbone were also good substrates for PLE [239] and α-chymotrypsin (Scheme 2.26) [240]. Interestingly, an additional hydroxy group in the substrate led to an enhancement of the chiral recognition.



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

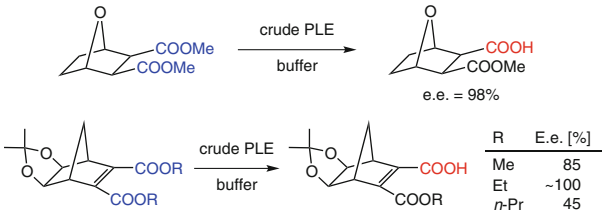
Scheme 2.26 Desymmetrization of acyclic *meso*-dicarboxylates by α-chymotrypsin and porcine liver esterase

The synthetic potential of the desymmetrization of cyclic *meso*-1,2-dicarboxylates by PLE is demonstrated in Scheme 2.27 [241]. A striking reversal of stereopreference was caused by variation of the ring size: 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 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 [242].



Scheme 2.27 Desymmetrization of cyclic *meso*-1,2-dicarboxylates by porcine liver esterase

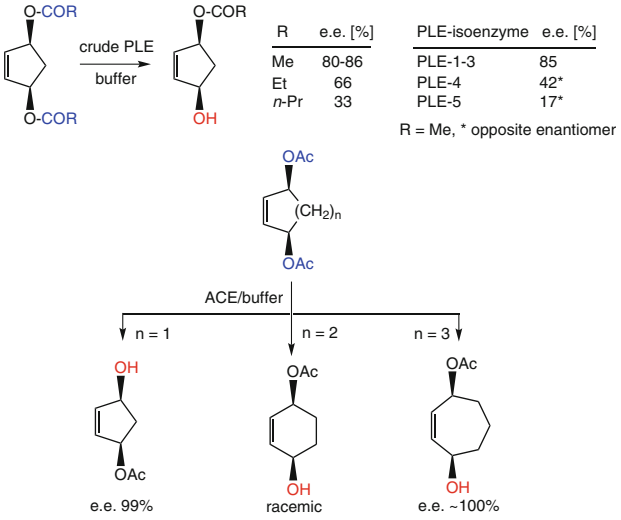
Bulky bicyclic *meso*-dicarboxylates, which were extensively used as optically pure building blocks for the synthesis of bioactive products, are well accepted by PLE [243]. While the *exo*-configurated diester was a good substrate (Scheme 2.28, top), the corresponding more sterically hindered *endo*-counterpart was hydrolyzed at a significantly reduced reaction rate and stereoselectivity (e.e. 64%). The importance of the appropriate choice of the alcohol moiety is exemplified with unsaturated analogs [231] (Scheme 2.28, bottom): While the short-chain methyl and ethyl esters were hydrolyzed with high selectivities, the propyl ester was not.



Scheme 2.28 Desymmetrization of bicyclic *meso*-1,2-dicarboxylates by porcine liver esterase

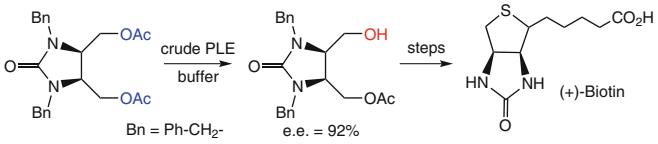
Cyclic *meso*-diacetates can be hydrolyzed in a similar fashion. As shown in Scheme 2.29 (top), the cyclopentene *meso*-monoester [244], which constitutes one of the most important chiral synthons for prostaglandin synthesis [245], was obtained in an e.e. of 80–86% using crude PLE. In accordance with 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 [246].

In order to avoid recrystallization of the optically enriched material (80–86% e.e.) obtained with crude PLE to enantiomeric purity, a search for a more selective esterase revealed that acetylcholine esterase (ACE) was the best choice [247]. It hydrolyzed the cyclopentene diester with excellent stereoselectivity but with the *opposite* stereopreference as with PLE (Scheme 2.29, bottom). Similar results were obtained by using lipases from porcine pancreas [248] and *Candida antarctica* [249]. 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 a racemic product, the seven-membered analog led to optically pure monoester of opposite configuration [250].



Scheme 2.29 Desymmetrization of cyclic *meso-sec*-diacetates by porcine liver esterase and acetylcholine esterase

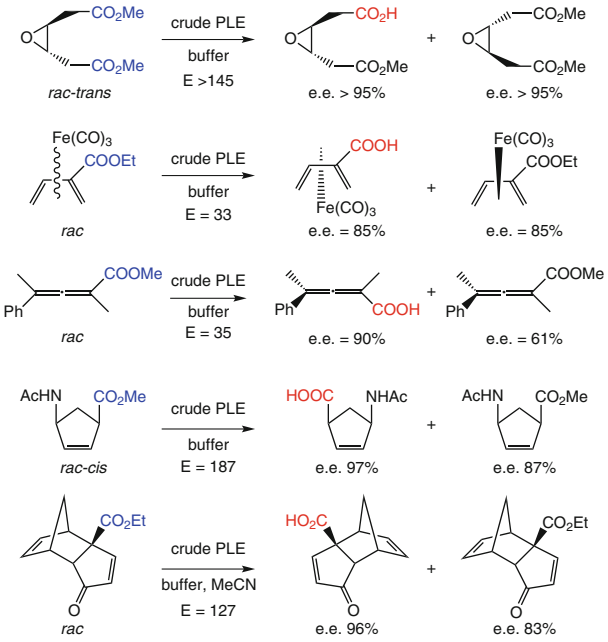
Cyclic *meso*-diacetates containing protected nitrogen functionalities proved to be excellent substrates for PLE, although the chiral recognition is more difficult due to the fact that diastereotopic *prim*-alcohols have to be differentiated (Scheme 2.30). In the benzyl-protected 1,3-imidazolin-2-one 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 [251] was much superior to that of the corresponding *cis*-dicarboxylate [252].



Scheme 2.30 Desymmetrization of *N*-protected cyclic *meso-prim*-diacetate by porcine liver esterase

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 has been proven to be a valuable technique for the resolution of non-natural esters. Interestingly, chirality does not necessarily need to be located on a tetrahedral carbon atom, as in the case of the *trans*-epoxy dicarboxylate [253], also axial chirality of an allenic carboxylic ester [254] and an iron-tricarbonyl complex [255] were well recognized by PLE (Scheme 2.31, top).

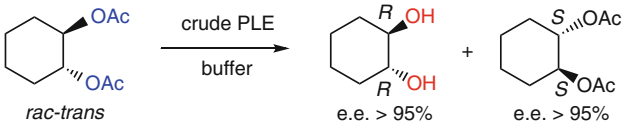
Resolution of an *N*-acetylaminocyclopentene carboxylate shown in Scheme 2.31 (bottom) was used to access optically pure starting material for the synthesis of carbocyclic nucleoside analogs with antiviral activity [256]. Also a very bulky tricyclic monoester (required for natural product synthesis) was resolved with remarkably good selectivity [257].



Scheme 2.31 Resolution of racemic carboxylic esters by porcine liver esterase

An example demonstrating the high stereospecificity of PLE is the kinetic resolution of the cyclic *trans*-1,2-diacetate shown in Scheme 2.32 [258]. The (*R*,*R*)-diacetate enantiomer – possessing two ester groups showing the matching (*R*)-configuration – was hydrolyzed from the racemic mixture via the monoester stage to yield the corresponding (*R*,*R*)-diol. The (*S*,*S*)-diacetate remained untouched, since it possesses only nonmatching (*S*)-ester groups. Again, as observed in the desymmetrization of *cis-meso*-1,2-dicarboxylates, the enantioselectivity strongly depended on the ring size: while the four- and six-membered substrates gave excellent results with opposite enantiopreference, the five-membered substrate

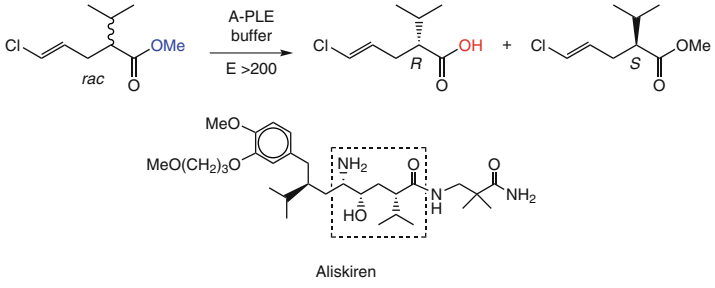
analog was not suitable. It should be noted that a desymmetrization of the corresponding *cis-meso*-1,2-diacetates is impeded by nonenzymic acyl migration which leads to facile racemization of any chiral monoester that is formed.



Scheme 2.32 Resolution of a cyclic *trans*-1,2-diacetate by porcine liver esterase

Inspired by the broad substrate range of porcine liver esterase, cloning and overexpression of PLE isoenzymes was persued over the past years in order to provide a reliable enzyme source and to overcome imperfect stereoselectivities of crude PLE preparations [259–261]. In addition, for the application of PLE-derived pharma products in humans, the use of enzymes from animal sources is 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.

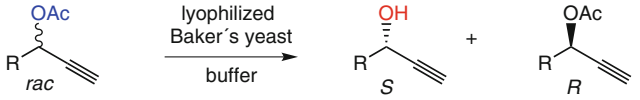
Analysis of the amino acid sequences of PLE isoenzymes revealed that the remarkably small differences of ca. 20 amino acids are not distributed randomly but are located within distinct conserved areas. Among the different isoenzymes, PLE-1 (also termed γ-PLE) and an isoenzyme termed A-PLE ('alternative pig liver esterase') [261, 262] 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 industrial-scale 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 2.33) [263].

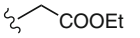
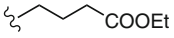


Scheme 2.33 Resolution of an α-chiral ester on industrial scale using the isoenzyme A-PLE

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* [264], *Brevibacterium ammoniagenes* [265], *Bacillus coagulans* [266], *Pichia miso* [26], and *Rhizopus nigricans* [267]. Although the reaction control becomes more complex on using whole microbial cells, the selectivities achieved are sometimes surprisingly high [268]. Since hydrolytic reactions do not require any cofactors, which are usually recycled by the metabolism of a 'living' fermenting organism, lyophilized 'resting' microbial cells can be used to minimize potential side reactions caused by competing enzymes. For instance, baker's yeast is a rich source of esterase activity, which was employed to resolve 1-alkyn-3-yl acetates with high selectivities [269] (Scheme 2.34).

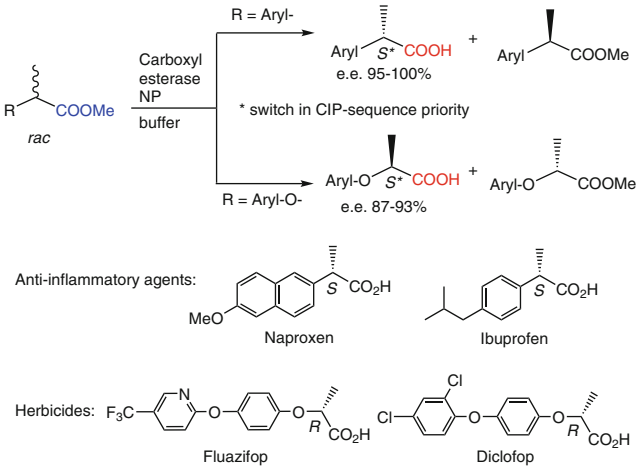


R	E	e.e. [%]	e.e. [%]
	46	91	72
 COOEt	~100	91	>97
 COOEt	89	96	59

Scheme 2.34 Hydrolytic resolution of *sec*-alcohols using whole (resting) cells of baker's yeast

Due to the importance of α-aryl- and α-aryloxy-substituted propionic acids as antiinflammatory agents (e.g., naproxen, ibuprofen) 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,[7] a convenient way for the separation of their enantiomers was sought by biocatalytic methods. An extensive screening program carried out by the industry has led to isolation of an esterase from *Bacillus subtilis* [270] (Scheme 2.35). The enzyme, termed 'carboxyl esterase NP', accepts a variety of substrates esters, including naproxen [271, 272]. 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 derivatives the corresponding (*S*)-acids are obtained. Also α-aryloxy analogs are resolved with similar high specificities, but products have the opposite spatial 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.

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



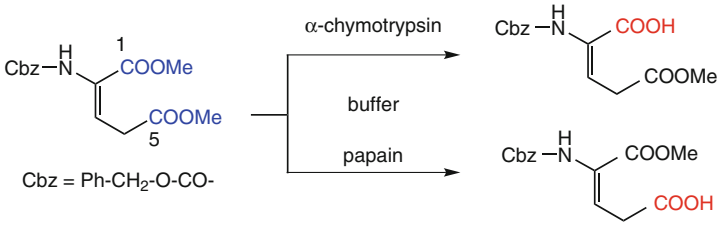
Scheme 2.35 Resolution of α-substituted propionates by carboxylesterase NP

Esterase Activity of Proteases

Numerous highly selective ester hydrolyses catalyzed by α-chymotrypsin [120] and papain have featured in excellent reviews [273] and the examples shown above illustrate their synthetic potential. On large scale, subtilisin (a protease which is widely used in detergent formulations) is a lost cost alternative [274–276].

The major requirements for substrates of type I (see Scheme 2.20) to be selectively hydrolyzed by proteases, such as α-chymotrypsin, pepsin, papain and subtilisin 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.

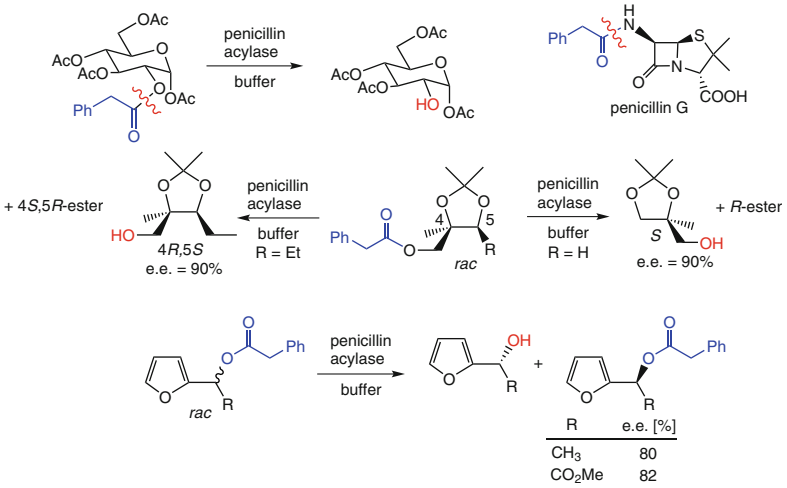
Proteases are also useful for regioselective hydrolytic transformations (Scheme 2.36). For example, while regio-selective hydrolysis of a dehydroglutamate diester at the 1-position was achieved using α-chymotrypsin, the 5-ester was attacked by papain [277]. The latter is one of the few enzymes used for organic synthetic transformations originating from plant sources (papaya). Related protease preparations are derived from fig (ficin) and pineapple stem (bromelain) [278].



Scheme 2.36 Regio-complementary ester hydrolysis by proteases

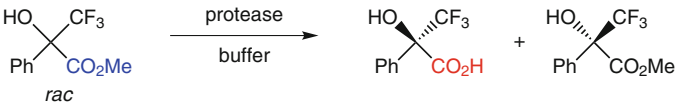
In addition to the above mentioned enzymes, two proteases have emerged as highly selective biocatalysts for hydrolysis. Penicillin acylase is highly

chemoselective for the cleavage of a phenylacetate group in its natural substrate, penicillin G, which is industrially used for the production of 6-aminopenicillanic acid (6-APA) (Scheme 2.37). This reaction has become a paradigm of how biocatalysis can contribute to make a chemical process more environmentally friendly. Chemical hydrolysis of 1 ton of Pen G requires ~10 tons of organic solvent (half of which is chlorinated) and problematic reagents, such as PCl_5 (600 kg) and amines (1 ton). The enzymatic hydrolysis using immobilized (reusable) Pen G acylase only requires a base to neutralize the acid formed [279]. Due to its specificity for a phenylacetate moiety, this enzyme can be employed in enzymatic protecting group chemistry [280, 281]. For instance, phenylacetyl groups can be removed in a highly chemoselective fashion in the presence of acetate esters (Scheme 2.37) [282, 283]. Furthermore, it can be used for the resolution of esters of primary [284] and secondary alcohols [285] as long as the acid moiety consists of a phenylacetyl group or a structurally closely related (heterocyclic) analog [286–288]. Some structural similarity of the alcohol moiety with that of the natural substrate penicillin G has been stated as being an advantage.



Scheme 2.37 Chemo- and enantioselective ester hydrolyses catalyzed by penicillin acylase

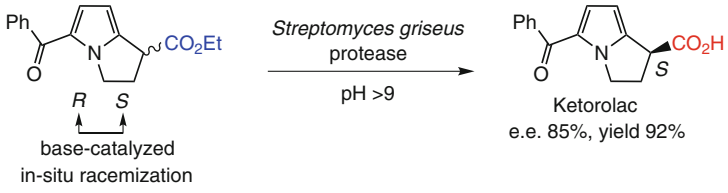
Along the same lines, a protease derived from *Aspergillus oryzae*, which has hitherto mainly been used for cheese processing, has proven useful for the resolution of sterically hindered substrates such as α,α,α-trisubstituted carboxylates [289] (Scheme 2.38). While 'traditional' proteases such as subtilisin were plagued by slow reaction rates and low selectivities, the α-trifluoromethyl mandelic ester (which constitutes a precursor of the chiral derivatization agent 'Mosher's acid' [290]) was successfully resolved by *Aspergillus oryzae* protease [291].



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

Scheme 2.38 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 *Streptomyces griseus* (Scheme 2.39) [85]. 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, thereby raising the theoretical yield of this racemate resolution to 100%.



Scheme 2.39 Dynamic resolution with in-situ racemization by protease from *Streptomyces griseus*

Optimization of Selectivity

Stereoselective enzymatic hydrolysis of nonnatural esters often shows imperfect selectivities with moderate to good Enantiomeric Ratios of about $E = 3$–20, which translates into $e.e._P$ values of 50–90%. In order to avoid tedious and material-consuming processes to enhance the optical purity of the product, e.g., by crystallization or via repeated kinetic resolution, several methods exist to improve the selectivity of an enzymatic transformation itself [24, 292]. In principle, they can be applied to all types of enzymes.

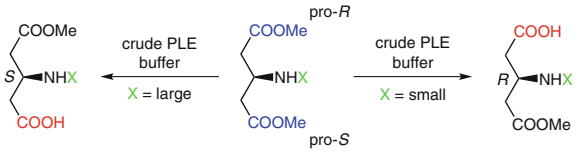
Since every catalytic system consists of three main components – (bio)catalyst, substrate, and medium – there are three possibilities for the tuning of the selectivity:

- Substrate modification is a straightforward and widely employed strategy.
- Altering the properties of the medium – pH, temperature, cosolvents – within certain limits is a simple and powerful technique to enhance enzyme selectivities.
- The ability to choose a different biocatalyst with a superior selectivity for a given substrate depends on the number of available candidates from the same

enzyme class. Enzyme screening is certainly a good option for proteases and lipases, but not within the relatively small group of esterases. The construction of enzyme mutants possessing altered stereospecificities by enzyme engineering is a laborious, but powerful strategy.

Substrate engineering is a promising technique, which is applicable to all types of enzymatic transformations. As may be concluded from the foregoing examples, the ability of an enzyme to 'recognize' the chirality of a given substrate predominantly depends on its steric shape. Although also electronic effects are involved, they are usually less important [293–296]. Thus, by variation of the substrate structure (most easily performed by chosing a protective group of different size and/or polarity) an improved fit of the substrate can be achieved, which leads to an enhanced selectivity of the enzyme.

Scheme 2.40 shows the optimization of a PLE-catalyzed desymmetrization of 3-aminoglutarate diesters using the 'substrate engineering' approach [231]. By varying the *N*-protecting group (X) 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 of stereopreference was achieved upon the stepwise increase of the size of group X, which allowed to control the absolute configuration of the product.

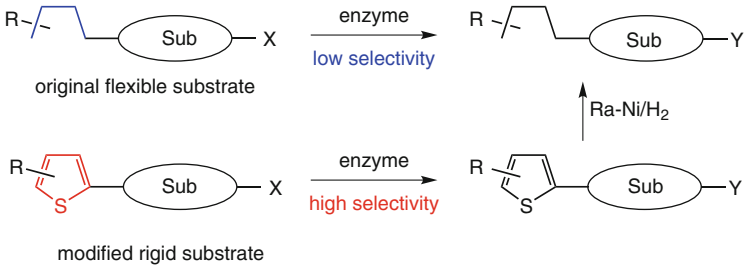


X	Configuration	e.e. [%]
H	*R*	41
CH_3-CO-	*R*	93
CH_2=CH-CO-	*R*	8
C_2H_5-CO-	*R*	6
n-C_4H_9-CO-	*S*	2
$(CH_3)_2$CH-CO-	*S*	54
c-C_6H_{11}-CO-	*S*	79
$(CH_3)_3$C-CO-	*S*	93
Ph-CH_2-O-CO-	*S*	93
(*E*)-CH_3-CH=CH-CO-	*S*	>97

Scheme 2.40 Optimization of porcine liver esterase-catalyzed hydrolysis by substrate modification

Another approach to substrate modification is based on the observation that enzyme selectivities are often enhanced with rigid substrate structures bearing π-electrons (Scheme 2.41). Thus, when a highly flexible aliphatic C-4 within a

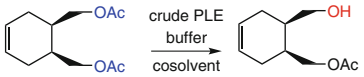
substrate (Sub) is stereochemically not well recognized, it can be 'chemically hidden' in the corresponding thiophene derivative, which is often transformed more selectively. Then, the enantioenriched heteroaromatic product is desulfurized by catalytic hydrogenation using Raney-Ni to yield the saturated desired product in high e.e. [297, 298].



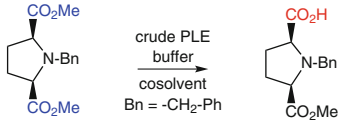
Scheme 2.41 Optimization of selectivity via introduction of a rigid thiophene unit

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 frequently used method to improve the selectivity of hydrolytic enzymes (Scheme 2.42) [299–301]. Depending on the stability of the enzyme, the concentration of cosolvent can be varied from ~10 to ~50% of the total volume. At higher concentrations, however, enzyme deactivation is unavoidable. Many studies have shown that a significant selectivity enhancement can be obtained, especially by addition of dimethyl sulfoxide or low-molecular-weight alcohols, such as *tert*-butanol. However, the price to pay on addition of water-miscible organic cosolvents to the aqueous reaction medium is a depletion in the reaction rate. The molecular reasons for enhanced enzyme selectivities in modified solvent systems is only partly understood and reliable predictions on the outcome of a medium engineering cannot be made (see Sect. 3.1). Consequently, this technique bears a strongly empirical character and requires trial and error experimentation, but in practice, however, the selectivity-enhancing effects are often dramatic.

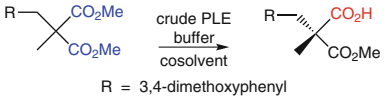
The selectivity enhancement of PLE-mediated hydrolyses upon the addition of methanol, *tert*-butanol, and dimethyl sulfoxide to the reaction medium is exemplified in Scheme 2.42. The optical purities of products were in a range of ~20–50% when a pure aqueous buffer system was used, but the addition of methanol and/or DMSO led to a significant improvement [302].



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 2.42 Selectivity enhancement of porcine liver esterase by addition of organic cosolvents

In biotransformations performed with crude enzyme preparations (e.g. lipases) or whole microbial cells (e.g. baker's yeast) stereoselectivities can be improved by addition of 'enhancers' (e.g. amines, alcohols), which act as noncompetitive inhibitors for competing (iso)enzymes possessing lower (or even opposite) selectivities [303]. This phenomenon is discussed on pp. 102–103 and p. 149, respectively.

Variation of pH Reactions catalyzed by hydrolases are usually performed in aqueous buffer systems with a pH close to that of the pH optimum of the enzyme. Because the conformation of an enzyme depends on its ionization state (among others), variation of the pH and the type of buffer will 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 activity [304–307].

Variation of Temperature Enzymes, like other catalysts, generally are considered to exhibit their highest selectivity at low temperatures, as supported by experimental observations with hydrolases [308]. A rational understanding of temperature effects on enzyme stereoselectivity was proposed using dehydrogenases [309, 310]. 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 of the reaction is the same for both stereochemical directions. In other words, there is no difference in free energy between [EnzA]$^{\neq}$ and [EnzB]$^{\neq}$, consequently $\Delta\Delta G^{\neq} = 0$ (Fig. 1.8).

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

T_{rac} = 'Racemic Temperature'

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 below 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* temperature.

However, the major product obtained at a temperature above T_{rac} will be the antipode to that below 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* [311] (Sect. 2.2.2). In contrast to the above-mentioned dehydrogenases from thermophilic organisms, the majority of hydrolases used for biotransformations (except *Candida antarctica* lipase B) possess more restricted thermal operational limits, which narrows the possibility of a significant selectivity enhancement by variation of the reaction temperature. From the data available, it can be seen that upon lowering the temperature both an increase [312] or a decrease in the selectivity of hydrolase reactions may be observed [313], depending on whether the reaction was performed above or below the racemic temperature (T_{rac}) of the enzyme used. The modest 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) [314–316]. In order to enhance reaction rates of organic-chemical reactions, microwave (MW) irradiation has become fashionable [317].[8]

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 existence of special microwave-effects (the so-called hot-spot theory) is still heavily debated [318–321]. For enzyme-catalyzed reactions, MW heating has been shown to be superior to conventional heating by leading to reduced enzyme deactivation and enhanced selectivities [322–324].

Enzyme Engineering Molecular biology has enabled the redesign of enzymes possessing improved performance in terms of enhanced stability at extreme

[8]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.

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 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 playground of synthetic organic chemists. For a deeper understanding, excellent introductory chapters can be found in recent books and reviews [325–332].

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 [333]. Alternatively, a computer-generated homology model may help, if the sequence identity is high enough. 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. 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 quite 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 to support the *dynamics* of protein catalysis [334]. 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 [335].
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.[9] Traditionally, mutant libraries are created by error-prone polymerase chain reaction (epPCR) with low mutation frequencies (1–3 mutations per 1000 base pairs). Since the possible number of mutants generated from a given protein exponentially increases by the number of mutations,[10] the crucial

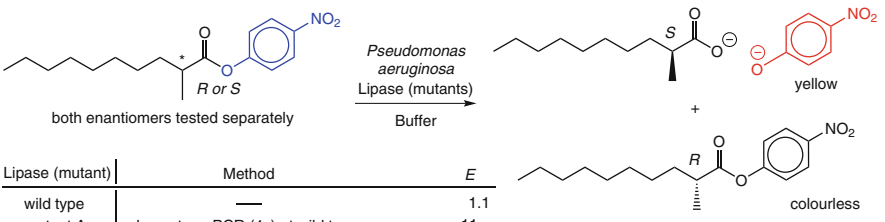
[9]The principle of directed evolution was first described by M. Eigen, see [336].

[10]The possible number of mutants generated from a protein possessing 200 amino acids are 3800 variants for a single mutation, 7,183,900 for two mutations, and 8,429,807,368,950 for only four mutations. Complete ramdomization would result in 20^{200} enzyme variants, which is more than the mass of the universe, even if only one molecule of each enzyme were to be produced.

aspect lies in the selection problem [337]: 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, 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 π-electrons to be 'visible' by UV/VIS or fluorescence spectroscopy. Classic reporter groups are (colorless) *p*-nitrophenyl derivatives, such as esters or glycosides, which liberate the (yellow) *p*-nitrophenolate anion upon enzyme catalysis. The latter can be spectrophotometrically monitored at 410 nm (Scheme 2.43). Unfortunately, by introduction of the chromogenic reporter group, 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 2.43) [338], MS analysis of (deuterated) 'pseudo-enantiomeric' products, or time-resolved IR thermogravimetry [339–343]. After all, 34% of random single amino acid replacements yield an inactive protein [344] and you always get what you screen for [345].

Scheme 2.43 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 kinetic resolution of an α-chiral long-chain fatty acid [346]. Pure substrate enantiomers were separately tested in 96-well microtiter plates using a plate reader for the readout of enantioselectivity. After four rounds of epPCR at a low mutation rate, mutant A ($E = 11$) was obtained. Sequence analysis of mutant A (and several other positive hits) revealed that position #155 was a 'hot spot' for beneficial variations. Hence, mutant A 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 on B gave mutant C ($E = 25$), which could not be further improved. At high mutations rates, epPRC of the wildtype enzyme gave only slightly improved variants D and E ($E = 3.0$ and 6.5, respectively) indicating further 'hot regions'. Combinatorial multiple cassette mutagenesis (CMCM) of the wild-type enzyme in the 'hot region' 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 $E > 51$ among ~40,000 mutants screened.

An example for the successful generation of highly enantioselective esterase mutants capable of hydrolyzing acetate esters of *tert*-alcohols is shown in Scheme 2.44 [347, 348]. In order to improve the modest enantioselectivity of wild-type *Bacillus subtilis* esterase ($E = 5$ and 43), a library of ca. 5000 mutants was constructed, which

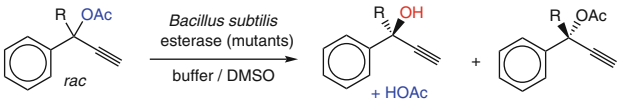


Lipase (mutant)	Method	*E*
wild type	—	1.1
mutant A	low rate epPCR (4x) at wild type	11
mutant B	saturation mutagenesis at #155 of mutant A	20
mutant C	low rate epPCR at mutant B	25
mutant D	high rate epPCR at wild type	3.0
mutant E	high rate epPCR at wild type	6.5
mutant G	CMCM #160-163 at wild type	30
mutant J	CMCM with mutants D & E	>51

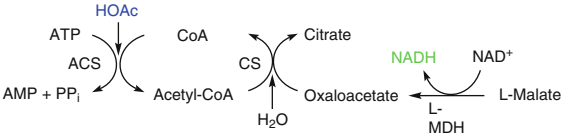
epPCR = error-prone polymerase chain reaction
CMCM = combinatorial multiple-cassette mutagenesis

Scheme 2.43 Screening for *Pseudomonas aeruginosa* lipase mutants showing enhanced enantioselectivities using pure enantiomers of a chromogenic surrogate substrate

encompassed 2800 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 [349]. 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 [350].



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'-triphosphate; AMP = adenosine 5'-monophosphate; PP_i = inorganic diphosphate

Scheme 2.44 Enantioselectivities of wild-type *Bacillus subtilis* esterase and mutants acting on *tert*-alcohol esters using a multienzyme acetate assay

Model Concepts

In order to avoid extensive enzyme engineering and trial-and-error modifications of substrate structures, several useful 'models' for the more commonly used enzymes have been developed to predict the stereochemical outcome of enzymatic reactions on nonnatural substrates. These models provide a rationale to 'redesign' a substrate or an enzyme, 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 and application are discussed here.

Molecular Modeling The structure of an enzyme in crystallized form can be accurately determined by X-ray crystallography [351–354]. Since the tertiary structure of most enzymes is closely related to the preferred form in a dissolved state [355], this method provides the most accurate 3D-description of an active site. However, X-ray data only represent a *static* protein structure, while the chiral recognition process during formation of the enzyme–substrate complex is a 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.

Although the rapidly increasing number of crystal structures of proteins,[11] which are available through the Protein Data Bank (PDB), encompass widely used enzymes, such as α-chymotrypsin [121], subtilisin [196], and lipases from *Mucor* spp. [9], *Geotrichum candidum* [356], *Candida rugosa* (formerly *cylindracea*) [357], *Candida antarctica* B [358], and *Pseudomonas glumae* [359], 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 a model for its three-dimensional structure [360]. This is done by comparing the amino acid sequence of the enzyme in question with that of other enzymes with known sequence and three-dimensional structure, which serve as blueprint. Depending on the percentage of the homology, i.e., 'overlap', of the amino acid sequences, the results are more or less accurate. In general, an overlap of about ~50–60% is sufficient for good results; less is considered too inaccurate. Tools for the construction of enzyme models, such as Modeller[12] or Phyre^2 [13] are available via Internet. The utility of various protein structure prediction methods was recently reviewed by Zhang [361].

[11] To date (2017), approx. 111.000 protein crystal structures are available.

[12] https://salilab.org/modeller/

[13] http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index

Provided that the three-dimensional structure of an enzyme is available, several methods for predicting the selectivity and its stereochemical preference are possible with various degrees of sophistication and effort [362–364]:

- 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 [365].
- The difference in steric interactions during a computer-generated approach of two substrate enantiomers towards an acyl-enzyme intermediate can be used instead [366].
- If the transition state is not known with some certainty, the substrate can be electronically fitted into the active site of the enzyme ('docking'). 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 [367]. 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. A simple free shareware program is AutoDock Vina [368]. However, it should be kept in mind, that errors of up to ±2.5 kcal/M in scoring energies are not uncommon, which renders the estimation of stereoselectivities an educated guess at best (compare Table 1.4).

A representative example for the prediction of the stereochemical outcome of the enzymatic hydrolysis of *rac*-1-phenylethyl acetate catalyzed by *Candida antarctica* lipase B based on its crystal structure (PDB: 5A71) is depicted in Fig. 2.10. The bottom of the active site shows the catalytic triad consisting of Asp-187 and His-224, which activate Ser-105 to perform a nucleophilic attack onto the carbonyl group of the ester moiety, forming a tetrahedral oxy-anion intermediate (see also Scheme 2.1). With the (*R*)-enantiomer (left), the bulky phenyl group is nicely accommodated in the large lipophilic binding site (pink), while the small methyl substituent is pointing upwards into the small pocket (yellow). In contrast, with the (*S*)-enantiomer (right), the phenyl group would clash into the α-helix to the right, while the methyl substituent would be inefficiently bound in the large pocket. Hence, the prediction for the preferred enantiomer is (*R*), also denoted as 'Kazlauskas-rule' (Scheme 2.45).

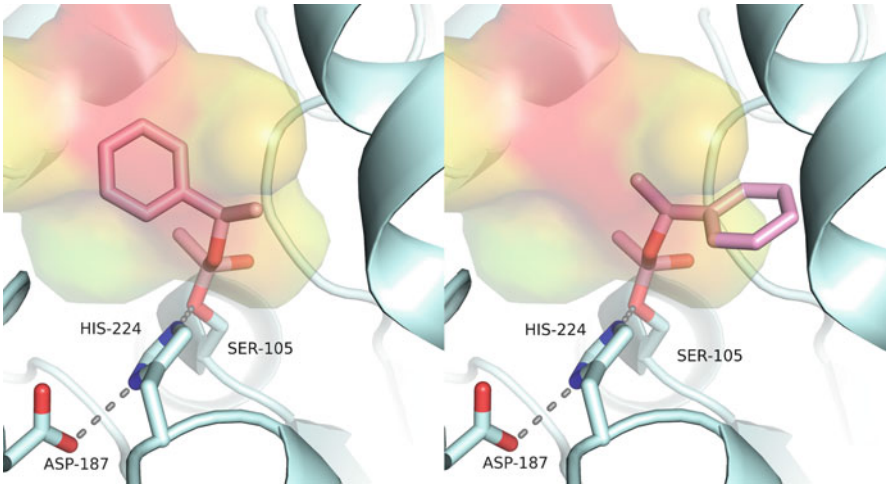


Fig. 2.10 Tetrahedral oxyanion-intermediates during hydrolysis of (*R*)- and (*S*)-1-phenylethyl acetate by *Candida antarctica* lipase B (Graphics prepared by PyMol v. 1.701, courtesy of Georg Steinkellner)

Substrate Model If neither X-ray data nor the amino acid sequence are available for an enzyme – which is not uncommon for 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 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 enantioselectivity. This idealized substrate structure is then called a 'substrate model' (Fig. 2.11, left). Such models have been developed for PLE [239] and *Candida rugosa* lipase [369, 370]. Of course these crude models only yield reliable predictions if they are based on a substantial number of test substrates.

To ensure optimal selectivity of PLE with methyl carboxylates, the α- and β-substituents should be assigned according to their size (L = large, M = medium and S = small) with the preferably-accepted enantiomer being shown in Fig. 2.11 (left).

Active Site Model Instead of developing an ideal *substrate structure* one also can delineate 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 in an approach denoted as 'substrate mapping' [371, 372]. 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 [373] using cubic-space descriptors was based on the evaluation of the results obtained from over 100 substrates (Fig. 2.11, right).

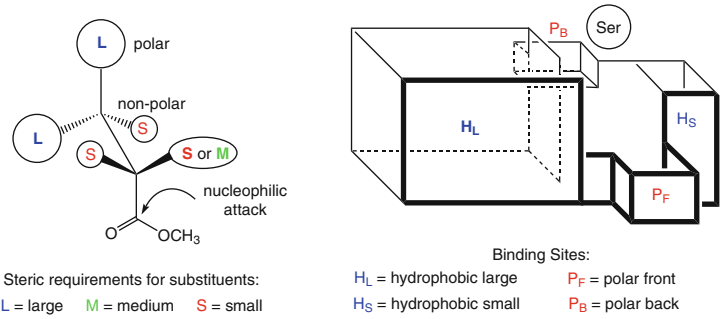


Fig. 2.11 Substrate model and active site model for porcine liver esterase

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.

2.1.3.2 Lipases

Lipases are enzymes which hydrolyze triglycerides into fatty acids and glycerol [374, 375]. Apart from their biological significance, they play an important role in biotechnology, not only for food and oil processing [376–378] but also for the preparation of chiral intermediates [379, 380]. In fact, about 30% of all biotransformations reported to date have been performed with lipases, which presumably constitute the most thoroughly investigated group of enzymes for biotransformations. To date, numerous lipases have been cloned and ~150 crystal structures are available. Although they can hydrolyze and form carboxylic ester bonds like proteases and esterases, their kinetic behaviour and substrate preference are different, which gives rise to some unique properties [381, 382].

The most important difference between lipases and esterases is the physicochemical 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 place [383, 384]. The fact that lipases do not hydrolyze substrates efficiently below a critical concentration (the 'critical micellar concentration', CMC), but display a high activity beyond it, has been called the 'interfacial activation' [385].

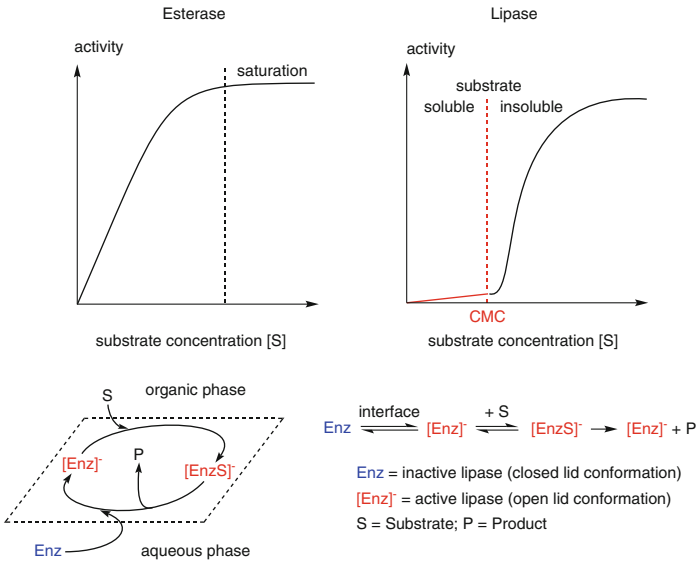
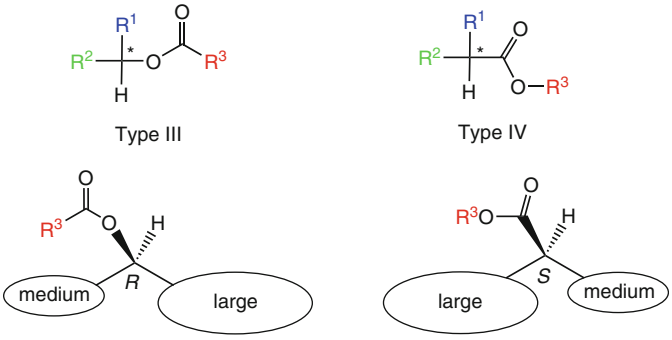


Fig. 2.12 Esterase and lipase kinetics

The molecular reason for this phenomenon is a conformational rearrangement within the enzyme [352]. 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 – the 'lid' – covers the active site. When the enzyme contacts the interface of a biphasic water-oil system, a short α-helix is folded back. Thus, by opening its active site the lipase is rearranged into its active state $[Enz]^{\neq}$.

To ensure optimal activity, 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.

The fact that many lipases have the ability to hydrolyze esters other than glycerides makes them particularly useful for organic synthesis [386, 387]. Furthermore, some lipases are also able to accept thioesters [388, 389]. In contrast to esterases, lipases have been used for the resolution of racemates more often than for the desymmetrization of *meso*-compounds. Since the natural substrates are esters of a 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 2.45), some lipases also display high selectivity through recognizing the chirality of an acid moiety (substrate type IV).



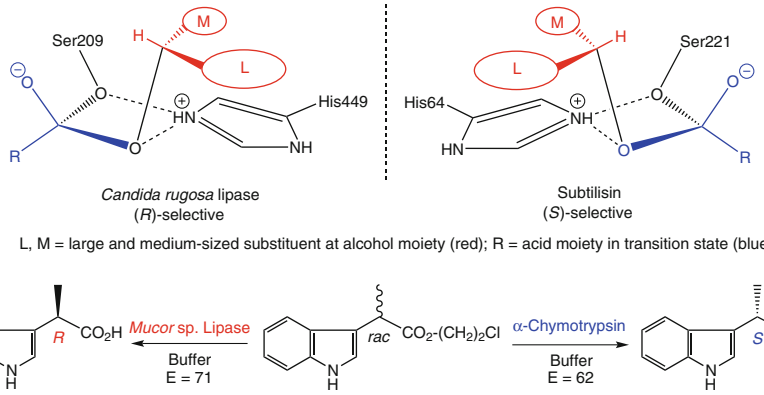
Scheme 2.45 Substrate types for lipases

Some of the general rules for substrate-construction are the same as those for esterase-substrates (Scheme 2.20), 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. Although 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 or *n*-butyl esters – are often the first choice.
- Furthermore, the majority of lipases show the same stereochemical preference for esters of secondary alcohols (Scheme 2.45), which is known as the 'Kazlauskas' rule' [370]. 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 an (*R*)-configuration at the alcoholic center. The rule for secondary alcohols (Type III) has an accuracy of $\geq$90%, whereas the predictability for the corresponding α-chiral acids (Type IV) is less reliable.
- Several 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 – as elucidated by their crystal structures – has been found to be arranged in a mirror-image orientation [390]. Thus, the stereochemical outcome of an asymmetric hydrolysis can often be directed by choosing a hydrolase from a different class [391–394]. Scheme 2.46 depicts the quasi-enantiomeric oxy-anion transition-state intermediates during hydrolysis of a *sec*-alcohol ester catalyzed by *Candida rugosa* lipase (PDB: 1crl) and the protease subtilisin (PDB: 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 suitable space for the large and medium-sized substituents of the *sec*-alcohol moiety (red). The mirror-image orientation of the catalytic center favors opposite enantiomers, which is exemplified by the hydrolytic kinetic resolution of an α-chiral indolyl propionic ester using the (*R*)-selective *Mucor* sp. lipase and the (*S*)-selective protease α-chymotrypsin (Scheme 2.46, bottom) [393]. The activated 2-chloroethyl ester was used to ensure enhanced reaction rates.

- Substrate-type IV represents the general structure of a lesser used ester type for 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 2.45) but the predictability is less accurate than with type-III substrates [372].



Scheme 2.46 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

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. The majority of these enzymes are created by the organisms in two isoforms (isoenzymes), usually denoted as type 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 [395].[14] 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.

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 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.

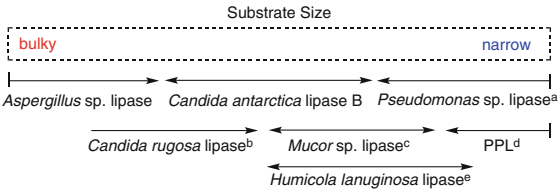


Fig. 2.13 Steric requirements of lipases. (**a**) Lipases from *Pseudomonas cepacia* (syn. *Burkholderia cepacia*), *P. fluorescens*, *P. fragi*, *Chromobacterium viscosum* (syn. *Pseudomonas glumae* or *Burkholderia glumae*); (**b**) syn. *C. cylindracea*; (**c**) *Mucor miehei* (syn. *Rhizomucor miehei), M. javanicus* (syn. *Rh. javanicus*); (**d**) pure porcine pancreatic lipase; (**e**) identical to *Thermomyces lanuginosus*.

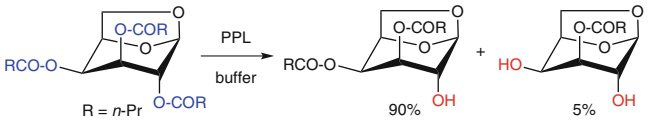
Porcine Pancreatic Lipase

The cheapest and hence one of the most widely used lipases is isolated from porcine pancreas (PPL) [396–398]. The crude preparations mostly used for biotransformations are called 'pancreatin' or 'steapsin' and contains less than 5% protein. Besides 'true PPL', which is available at a high price in partially purified form, they contain a significant number of other hydrolases. Interestingly enough, in some cases these

[14]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.

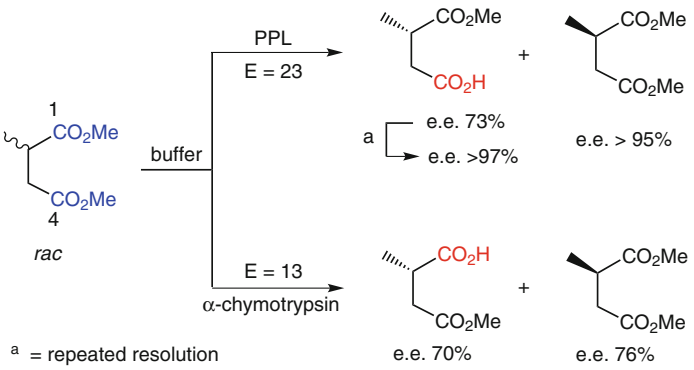
hydrolase impurities have been shown to be responsible for the highly selective transformation of substrates which were not accepted by purified 'true PPL'. The main hydrolase impurities are α-chymotrypsin, cholesterol esterase, carboxypeptidase B, phospholipases, and other unknown hydrolases. Phospholipases can usually be neglected as undesired hydrolase impurities, because they prefer negatively charged substrate esters which mimic their natural substrates – phospholipids [399, 400]. On the other hand, α-chymotrypsin and cholesterol esterase can be serious competitors in ester hydrolysis. Both of the latter proteins can impair the selectivity of a desired PPL-catalyzed ester hydrolysis by exhibiting a lower selectivity or even opposite stereopreference. Cholesterol esterase and α-chymotrypsin prefer esters of primary and secondary alcohols, whereas 'true PPL' is a highly selective catalyst for esters of primary alcohols only. Thus, any models for PPL should be applied with great caution [401, 402]. Despite the possible interference of different competing hydrolytic enzymes, numerous highly selective applications have been reported with crude PPL [403–405]. 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 [406] or from primary hydroxyl groups [407, 408], 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 [409]. 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 2.47 [410]. Only a minor fraction of the 2,4-deacylated product was formed.



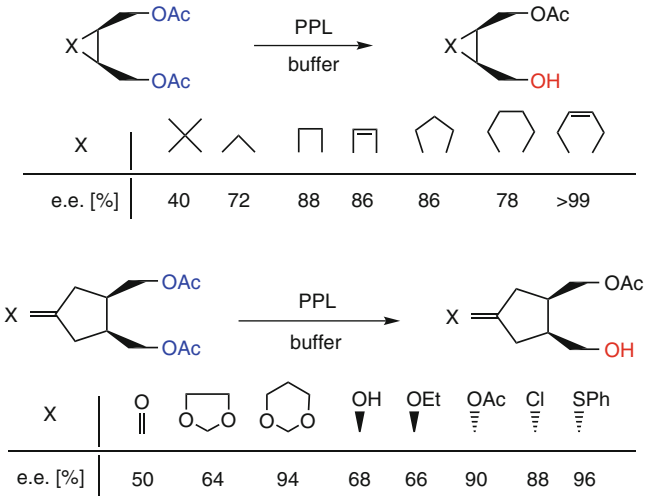
Scheme 2.47 Regioselective hydrolysis of carbohydrate esters by porcine panceatic lipase

A simultaneous regio- and enantioselective hydrolysis of dimethyl 2-methylsuccinate has been reported with PPL [411] with a preference for the (*S*)-ester and with the hydrolysis taking place at position 4 (Scheme 2.48). 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 [412].



Scheme 2.48 Regio- and enantioselective hydrolysis of dimethyl α-methylsuccinate

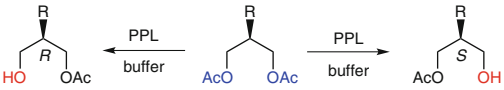
The asymmetric hydrolysis of cyclic *meso*-diacetates by PPL proved to be complementary to the PLE-catalyzed hydrolysis of the corresponding *meso*-1,2-dicarboxylates (compare Schemes 2.27 and 2.49). The cyclopentane derivative, which gave low e.e. using the PLE method, was now obtained with 86% e.e. [31, 413]. This selectivity was later improved by substrate modification of the cyclopentane moiety [414], giving access to a number of chiral cyclopentanoid building blocks for the synthesis of carbacyclic prostaglandin I_2 derivatives, which are therapeutic agents for the treatment of thrombotic diseases.



Scheme 2.49 Asymmetric hydrolysis of cyclic *meso*-diacetates by porcine pancreatic lipase

Chiral glycerols, optically active C_3-synthons, were obtained by asymmetric hydrolysis of prochiral 1,3-propanediol diesters using PPL (Scheme 2.50) [415]. 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

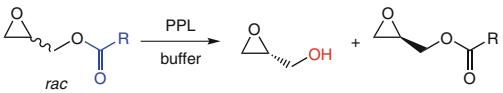
depend on steric factors alone, but also on electronic issues [293, 296, 416]. Note that a rigid (*E*)-C=C bond or a bulky aromatic system [417] on the 2-substituent led to an enhanced selectivity of the enzyme as compared to the corresponding saturated analogs. When the configuration of the double bond was *Z*, a reversal in the stereochemical preference took place, associated with an overall drop of selectivity. Additionally, this study shows a positive influence of a biphasic system (using di-*iso*-propyl ether or toluene [418] as water-immiscible organic cosolvent) on the enantioselectivity of the enzyme.



R	Cosolvent	Configuration	e.e. [%]
n-C_7H_{15}-	*i*-Pr_2O	*S*	70
$(CH_3)_2$CH-$(CH_2)_2$-	*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*)-$(CH_3)_2$CH-CH=CH-	none	*S*	90
(*E*)-$(CH_3)_2$CH-CH=CH-	*i*-Pr_2O	*S*	97
(*Z*)-$(CH_3)_2$CH-CH=CH-	*i*-Pr_2O	*R*	15

Scheme 2.50 Desymmetrization of prochiral 1,3-propanediol diesters by procine pancreatic lipase

Chiral epoxy alcohols, which are not easily available via the Sharpless procedure due to lack of a directing hydroxy moiety [419], were successfully resolved with PPL (Scheme 2.51). Interestingly, the lipase is not deactivated by a possible reaction with the epoxide moiety [420, 421]. The significant influence of the nature of the acyl moiety on the selectivity of the resolution – again, 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 [422, 423]. In particular α-chymotrypsin and cholesterol esterase are known to hydrolyze acetates of alcohols but not their long-chain counterparts. 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 [424].

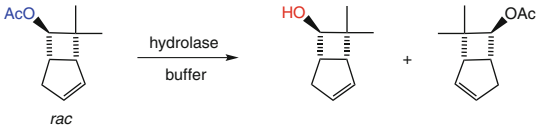


R	e.e. [%]	Selectivity (*E*)
CH_3	53	4
C_2H_5	88	11
n-C_3H_7	92	13
n-C_4H_9	96	16
n-C_3H_7	>99	>100 *

* Pure immobilised enzyme in presence of 10% dioxane.

Scheme 2.51 Resolution of epoxy esters by porcine pancreatic lipase

During a study on the resolution of the sterically demanding bicyclic acetate shown in Scheme 2.52 [425], which represents an important chiral building block for the synthesis of leukotrienes [426], it was found that crude steapsin is a highly selective catalyst for its resolution. In contrast, pure PPL and α-chymotrypsin were unable to hydrolyze the substrate and cholesterol esterase 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.



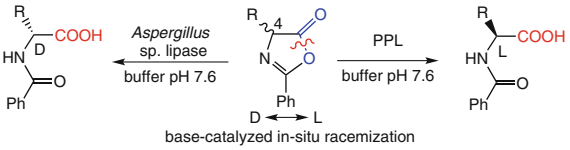
Enzyme	Reaction Rate	Selectivity (*E*)
crude PPL	good	>200
pure PPL	no reaction	-
α-chymotrypsin	no reaction	-
cholesterol esterase	fast	17
novel ester hydrolase	good	210

Scheme 2.52 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 2.53) [427] to yield *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 [428]. Thus, *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 racemization rate of the two configurationally unstable substrate antipodes under weakly basic conditions at pH 7.6 is sufficiently rapid to provide a dynamic resolution 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. 2.9).



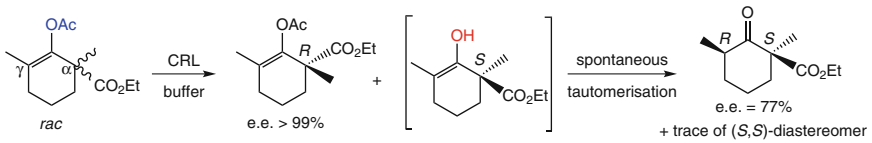
R	Lipase	Configuration	e.e. [%]
Ph-	PPL	L	76
CH_3-S-CH_2-CH_2-	PPL	L	80
Ph-CH_2-	PPL	L	>99
Ph-	*Aspergillus* sp.	D	80
CH_3-S-CH_2-CH_2-	*Aspergillus* sp.	D	83
Ph-CH_2-	*Aspergillus* sp.	D	>99

Scheme 2.53 Lipase-catalyzed dynamic resolution of oxazolin-5-ones

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 [357], has been frequently used for the resolution of esters of secondary alcohols [429–434] and, to a lesser extent, for the resolution of α-substituted carboxylates [435, 436]. The CRL preparations from several commercial sources which contain up to 16% of protein [437] differ to some extent in their activity but their selectivity is very similar [438]. 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.

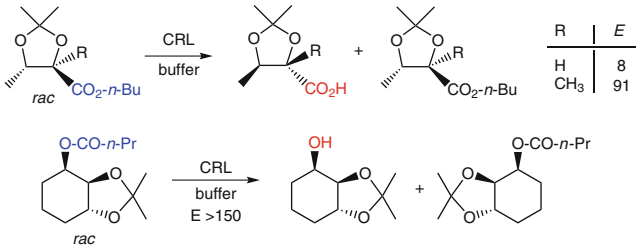
The racemic cyclohexyl enol ester shown in Scheme 2.54 was enzymatically resolved by CRL to give a ketoester with an (*S*)-stereocenter on the α-position (77% e.e.) coupled with a diastereoselective protonation of the liberated enol, which led to an (*R*)-configuration on the newly generated center on the γ-carbon atom, only a trace of the (*S*,*S*)-diastereomer was formed. The remaining (*R*)-enol ester was obtained in optically pure form [439]. In accordance with the substrate model Type IV (Scheme 2.45), no significant hydrolysis on the fully substituted ethyl carboxylate was observed.



Scheme 2.54 Enzymatic resolution of a cyclic enol ester by *Candida rugosa* lipase

Racemic 2,3-dihydroxy carboxylates, protected as their respective acetonides, were resolved by CRL [440] by using their lipophilic *n*-butyl esters (Scheme 2.55). 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 2.55 [441]. 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.

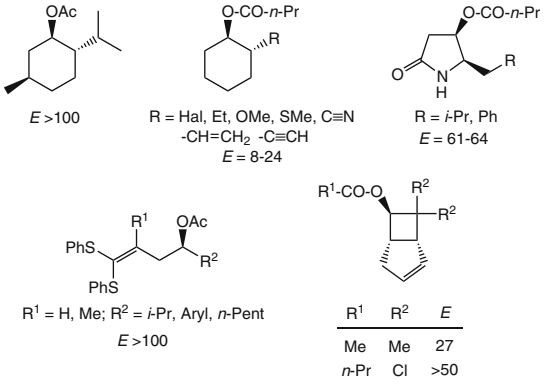


Scheme 2.55 Enzymatic resolution of cyclic esters by *Candida rugosa* lipase

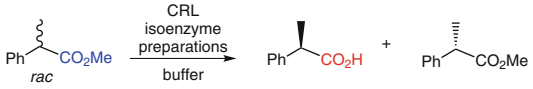
The ideal substrates for CRL are esters of cyclic *sec*-alcohols, which usually give excellent enantioselectivities [425, 442–445]. In contrast, straight-chain substrates are only well resolved when sterically demanding substituents are present (Scheme 2.56) [446]. Esters of *prim*-alcohols usually yield 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 [52, 369, 447, 448] and an active site model [449] 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 [450, 451]. Both forms—denoted as A and B—could be chromatographically separated and were shown to possess identical enantiopreference, but different enantioselectivity (Scheme 2.57). Thus, racemic α-phenyl propionate was resolved with low selectivity ($E = 10$) using crude CRL, whereas isoenzyme A was highly selective ($E > 100$). The isomeric lipase B showed almost the same moderate selectivity as the crude enzyme.



Scheme 2.56 Typical ester substrates for *Candida rugosa* lipase (reacting enantiomer shown)



Lipase	Selectivity (*E*)
crude CRL	10
CRL form A	>100
CRL form B	21

Scheme 2.57 Enantioselectivities of isoenzyme preparations of *Candida rugosa* lipase

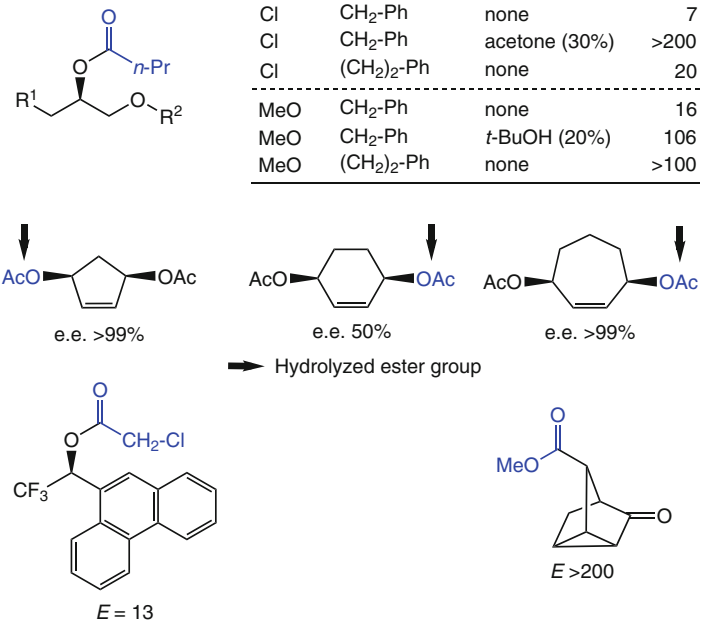
The most versatile 'champion' lipase for preparative biotransformations is obtained from the basidomycetous yeast *Candida antarctica* (CAL) [452]. As indicated by its name, this yeast was isolated in Antarctica with the aim of finding enzymes with extreme properties to be used in detergent formulations. Like others, the organism produces two isoenzymes A and B, which differ to a significant extent [453]: 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 [454] 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 [455] and various preparations of this enzyme are produced by Novozymes (DK) in bulk quantities [456]. 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,[15] and thus also shows increased resistance towards organic solvents. In contrast to many other lipases, the enzyme shows only weak interfacial activation

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

[457], which makes it an intermediate between an esterase and a lipase. Its selectivity could be predicted through computer modeling to a fair extent [458], and for the majority of substrates the Kazlauskas' rule (Scheme 2.45) 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 [459–464] 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 2.58 [249, 465–468]. 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.



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

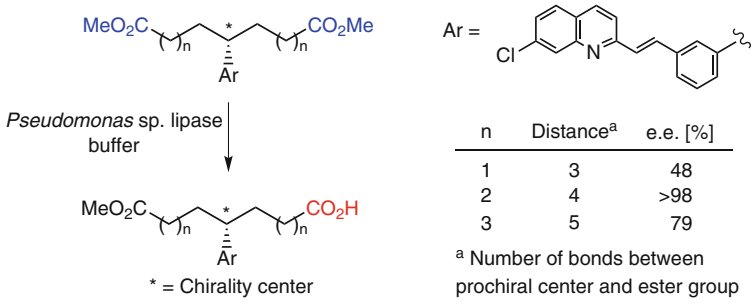
Scheme 2.58 Typical ester substrates for *Candida antarctica* lipase (reacting enantiomer shown)

***Pseudomonas* sp. Lipases**

Bacterial lipases isolated from *Pseudomonas fluorescens*, *P. aeruginosa*, *P. cepacia*, and *P. glumae* are highly selective catalysts [469].[16] 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 [470–474]. 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 [475]. Various active-site models for PSL have been proposed [179, 476–478] and the crystal structures of *P. cepacia* and *P. glumae* lipases were elucidated by X-ray analysis [479, 480].

The exceptionally high selectivity of PSL on 'narrow' open-chain esters is demonstrated by the following examples (Scheme 2.59).

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 [481]. This example of a highly selective chiral recognition of a 'remote' chiral/prochiral center is not unusual amongst hydrolytic enzymes [482–484].



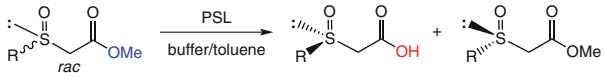
n	Distance[a]	e.e. [%]
1	3	48
2	4	>98
3	5	79

[a] Number of bonds between prochiral center and ester group

Scheme 2.59 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 2.60). Thus, optically pure aryl sulfoxides were obtained by lipase-catalyzed resolution of methyl sulfinyl acetates [485] in a biphasic medium containing toluene. The latter compounds are important starting materials for the synthesis of chiral allylic alcohols via the 'SPAC' reaction.

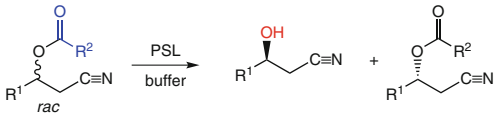
[16] Several *Pseudomonas* spp. were reclassified as *Burkholderia* spp.



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 2.60 Resolution of sulfoxide esters by *Pseudomonas* sp. lipase

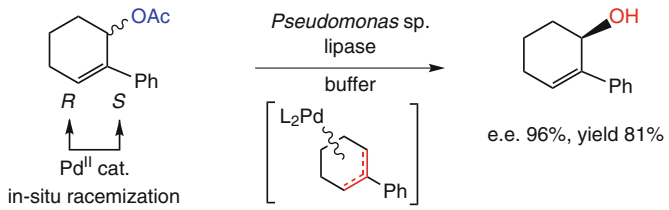
The selectivity of PSL-catalyzed hydrolyses may be significantly improved by substrate-modification through variation of the nonchiral acyl moiety (Scheme 2.61) [486]. Whereas alkyl- and chloroalkyl esters gave poor selectivities, the introduction of a sulfur atom to furnish the 2-thioacetates proved to be advantageous. Thus, optically active β-hydroxynitriles, precursors of β-hydroxy acids and β-aminoalcohols, were conveniently resolved via the methyl- or phenyl-2-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

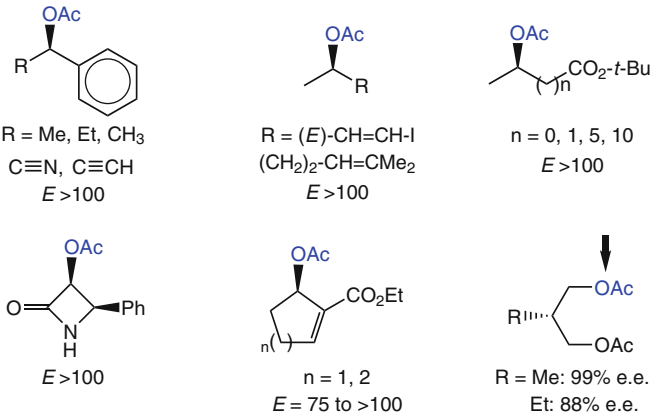
Scheme 2.61 Resolution of β-acyloxynitriles by *Pseudomonas* sp. lipase

An elegant example for a dynamic resolution of an allylic alcohol via enantioselective ester hydrolysis is depicted in Scheme 2.62 [487]. It is based on the combination of an enzyme with a (transition) metal catalyst in the same reactor, which has been termed 'enzyme-metal-combo-catalysis' [488], a technique which became very popular during recent years [489–491]. 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.



Scheme 2.62 Dynamic resolution of an allylic alcohol ester using *Pseudomonas* sp. lipase and Pd ^{II} catalysis

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 2.63) [395, 492–495]. Esters of cyclic *sec*-alcohols are well accepted as long as the steric requirements are not too demanding [496–498]. A special feature of PSL is its high selectivity for racemic or prochiral *prim*-alcohols [499, 500], where other lipases often show insufficient stereorecognition.



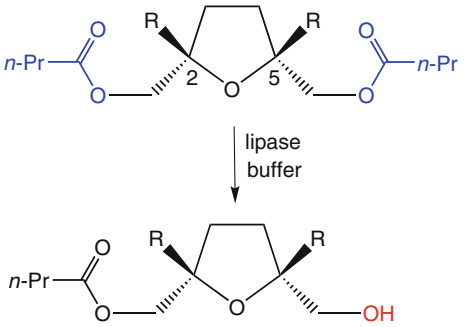
Scheme 2.63 Typical ester substrates for *Pseudomonas* sp. lipase (reacting enantiomer shown)

Mucor sp. Lipases

Lipases from *Mucor* species (MSL) [9, 501] such as *M. miehei* and *M. javanicus* (also denoted as *Rhizomucor*) have frequently been used for biotransformations [429, 502]. With respect to the steric requirements of substrates they seem to be related to the *Pseudomonas* sp. lipases. Like *Candida* and *Pseudomonas* sp. lipases, the different MSL preparations are related in their hydrolytic specificity [38].

A case where only MSL showed good selectivity is shown in Scheme 2.64 [503]. The desymmetrization of *meso*-dibutanoates of a tetrahydrofuran-2,5-dimethanol, which constitutes the central subunit of several naturally occurring polyether antibiotics [504] and platelet-activating-factor (PAF) antagonists, was investigated 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 [505].



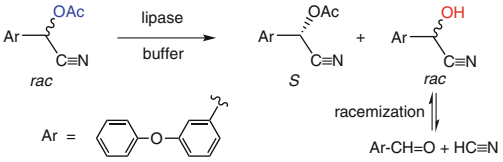
Lipase	R	e.e. [%]
CRL	H	12
PPL	Me	20
PSL	H	81
MSL	H	>99
MSL	Me	>99

Scheme 2.64 Desymmetrization of bis(acyloxy-methyl)tetrahydrofurans by lipases

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

Optically pure cyanohydrins are required for the preparation of synthetic pyrethroids, which are used as more environmentally acceptable insect pestcontrol 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 [506] and aminoalcohols [507, 508]. They may be obtained via asymmetric hydrolysis of their respective acetates by microbial lipases (Scheme 2.65) [509]. 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 cyanohydrin can be avoided when the hydrolysis is carried out at pH 4.5 [510] or in special nonaqueous solvent systems (see Sect. 3.1.1).

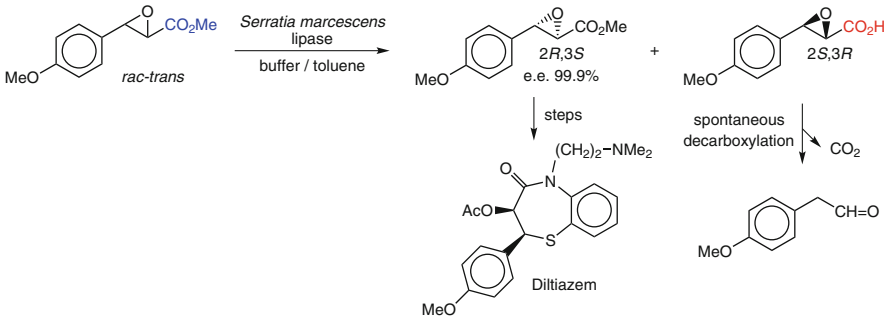
The resolution of the commercially important esters of (*S*)-α-cyano-3-phenoxybenzyl alcohol was only moderately efficient using lipases from *Candida rugosa*, *Pseudomonas*, and *Alcaligenes* sp. (Scheme 2.65). The best selectivities were obtained with lipases from *Chromobacterium* and *Arthrobacter* sp. [511], respectively.



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 2.65 Hydrolysis of cyanohydrin esters using microbial lipases

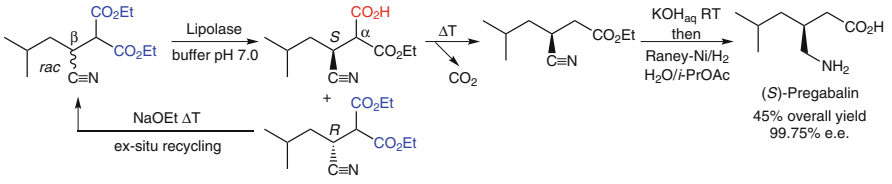
The epoxy-ester shown in Scheme 2.66 is an important chiral building block for the synthesis of the Ca-channel blocker diltiazem, a potent drug for the treatment of angina pectoris, which is produced at >100 t/year worldwide. Resolution on industrial scale is performed via enantioselective hydrolysis 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 [512, 513]. The (undesired) carboxylic acid enantiomer undergoes spontaneous decarboxylation yielding *p*-methoxyphenyl acetaldehyde, which is removed via extraction of the corresponding bisulfite adduct.



Scheme 2.66 Lipase-catalyzed resolution of an epoxy-ester on industrial scale

The power of lipases for the asymmetric synthesis of pharmaceuticals on industrial scale was demonstrated by the development of an improved process for the manufacture of pregabalin (Lyrica™), which is a widely employed γ-aminobutyric acid (GABA) analog used for the treatment of nervous disorders including epilepsy, anxiety and social phobia (Scheme 2.67). The initial process relied on tedious resolution of *rac*-pregabalin via diastereomer crystallization with (*S*)-mandelate. The second generation process introduces stereochemistry at a very early stage employing a hydrolytic kinetic resolution of a readily

accessible β-cyano-malonate diester as starting material [514]. Screening of several carboxyl ester hydrolases revealed that none of the obvious suspects (*Streptomyces griseus* protease, pig liver esterase), nor the 'champion-lipases' (from *Candida antarctica*, *Mucor* or *Pseudomonas* sp.) showed sufficient enantioselectivity for the chiral center at the remote β-position. Finally, two (*S*)-selective lipases were identified, of which *Thermomyces lanuginosus* lipase was selected for its perfect enantioselectivity and superior reaction rate. This enzyme was the first lipase produced by GMOs which is employed in detergent formulations since 1990 under the trademark Lipolase™ [515]. Enzymatic hydrolysis stops at the monoester stage and furnishes the hemiester with desired (*S*)-configuration at the β-center. Extractive separation of the non-reacted (*R*)-diester followed by base-catalyzed racemization allows its ex-situ recycling. Thermal decarboxylation of the (*S*)-hemiester yields the corresponding β-cyanoester without racemization. Alkaline ester hydrolysis and catalytic reduction of the nitrile in a one-pot procedure gave (*S*)-pregabalin in perfect e.e. After careful optimization, the lipase-mediated resolution process could be performed at 3M substrate concentration at a batch-size of 3.5 t in an 8 m^3 reactor with a TTN of ~10^5. The enzyme-based process allowed to reduce the usage of organic solvents (by 92%), Raney Ni (by 87%), and starting material (by 39%) and eliminated the need of mandelic acid. Overall, the E factor (i.e. the ratio of the mass of waste per mass of product [516]) was cut from 87 to a mere 17.



Hydrolase	Enantiopreference	Enantioselectivity (*E*)
Streptomyces griseus protease	*R*	20
porcine liver esterase	*S*	2
Candida antarctica lipase (A or B)	*S*	3-5
Mucor miehei lipase	*S*	41
Pseudomonas sp. lipase	*S*	51
Rhizopus delemar lipase	*S*	>200
Thermomyces lanuginosus lipase	*S*	>200

Scheme 2.67 Lipase-assisted chemoenzymatic synthesis of pregabalin on industrial scale

A lesser known lipase is obtained from the mold *Geotrichum candidum* [517, 518]. The three-dimensional structure of this enzyme has been elucidated by X-ray crystallography [356] 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.

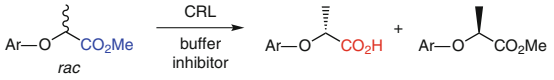
Another extracellular lipase, called 'cutinase', [519] is produced by the plant-pathogenic microorganism *Fusarium solani pisi* for the hydrolysis of cutin—a wax

ester which is excreted by plants in order to protect their leaves against microbial attack [520]. The enzyme has been purified to homogeneity [521] and has been made readily available by genetic engineering [522]. Due to its modest stereoselectivities it has not been used widely for asymmetric ester hydrolyses [523], but its unique ability to act on macroscopic polymer esters made it a prime candidate for the enzymatic modification of polyesters [524] and their biodegradation [525].

Optimization of Selectivity

Most of the general techniques for an enzymatic selectivity enhancement such as adjustment of temperature [526], buffer type and pH [307], 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 [527], as described above, is a valuable technique for the selectivity improvement of lipase-catalyzed transformations. Bearing in mind that lipases are subject to a strong induced-fit and pronounced interfacial activation (Fig. 2.12), medium engineering with lipases is generally 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).

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* [303] and *Pseudomonas* sp. lipase [528]. The principle of this selectivity enhancement was elaborated as early as 1930! [529]. As shown in Scheme 2.68, 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-	dextro- or levomethorphan[a]	20
2,4-dichlorophenyl-	DMPA[b]	23
4-chlorophenyl-	none	17
4-chlorophenyl-	dextro- or levomethorphan[a]	>100

[a] Dextro- or levo-methorphan = D- or L-3-methoxy-*N*-methylmorphinane.

[b] *N,N*-Dimethyl-4-methoxyphenethylamine.

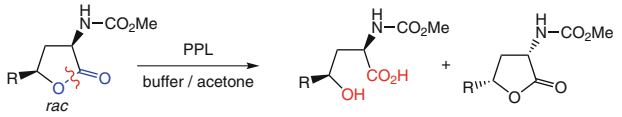
Scheme 2.68 Selectivity enhancement of *Candida rugosa* lipase by enantioselective inhibition

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, also denoted as 'allosteric effect' – which inhibits the transformation of one enantiomer but not that of its mirror image. Surprisingly, the chirality of the base has only a marginal impact on the selectivity enhancement effect. The 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) [250].

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 [530], which are involved in the metabolism of aldoses [531] and the deactivation of bioactive lactones, such as *N*-acyl homoserine lactone [532]. 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 2.69) [533].

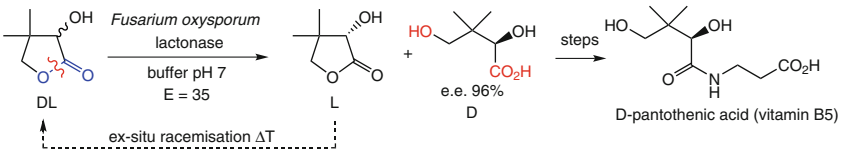


R	e.e. Acid [%]	e.e. Lactone [%]	Selectivity (*E*)
H-	71	62	11
Ph-	86	32	18
CH_2=CH-	90	95	70

Scheme 2.69 Enantioselective hydrolysis of γ-lactones by porcine pancreatic lipase

Well-defined lactonases were identified in bacteria [534–536] 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 2.70). 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. 3500 t/year, the lactonase has been cloned and overexpressed

into *Aspergillus oryzae* [537]. A corresponding enantiocomplementary L-specific lactonase was identified in *Agrobacterium tumefaciens* [538].



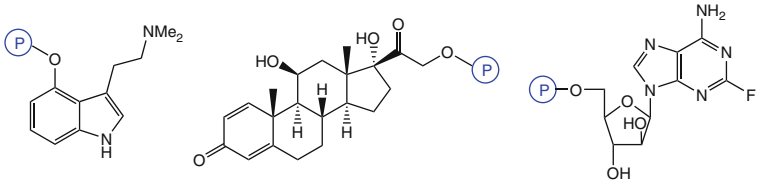
Scheme 2.70 Resolution of pantolactone using a lactonase

More recently, lactonases were employed in the biodegradation of mycotoxins, e.g. zearalenone [539]. Several lactonases were shown to possess also promiscuous phosphotriesterase-activities, which makes them valuable in the bioremediation of neurotoxic organophosphorus agents, which were widely employed as chemical warfare agents and as pesticides, such as parathion [540, 541].

2.1.4 Hydrolysis and Formation of Phosphate Esters

The hydrolysis of phosphate esters can be equally achieved by chemical methods and by phosphate ester hydrolases (phosphatases) and 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 enantioselective hydrolyses of racemic phosphates affording a kinetic resolution.

In contrast, the *formation* of phosphate esters is of importance, particularly when regio- or enantioselective phosphorylation is required. On the one hand, numerous bioactive agents display their highest activity only when phosphorylated, for instance the hallucinogen psilocibin found in 'magic mushrooms'. On the other hand, phosphorylation is a common strategy to increase the solubility of orally administered drugs [542, 543]. For instance, prednisolone phosphate is a water-soluble prodrug used as immunosuppressant and the non-natural nucleoside phosphate fludarabin is employed as cytostatic for the treatment of leukemia (Scheme 2.71).



Scheme 2.71 Phosphorylated bioactive compounds

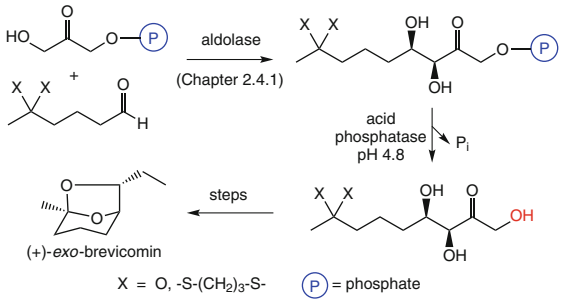
Furthermore, a significant proportion of essential cofactors (or cosubstrates) for other enzyme-catalyzed reactions involve phosphate esters. Adenosine triphosphate (ATP) represents the phosphate donor for most biological phosphorylation reactions and hence constitutes the universal 'energy-currency' in biological systems. For many redox-reactions, nicotinamide adenine dinucleotide phosphate ($NADP^+$) or glucose-6-phosphate (G6P) are an essential cofactor or cosubstrate (Sect. 2.2.1). Dihydroxyacetone phosphate (DHAP) is an important activated cosubstrate for enzymatic aldol reactions (Sect. 2.4.1), thiamine diphosphate (TDP) is an essential cofactor for enzymatic acyloin and benzoin condensations (Sect. 2.4.2) and pyridoxal-5′-phosphate (PLP) serves as molecular shuttle for transamination reactions (Sect. 2.6.2). Glycosyl phosphates are essential for glycosyl transfer reactions catalyzed by carbohydrate phosphorylases (Sect. 2.6.1). In addition, the emerging field of metabolic engineering creates a substantial market for phosphorylated metabolites [544].

Hydrolysis of Phosphate Esters

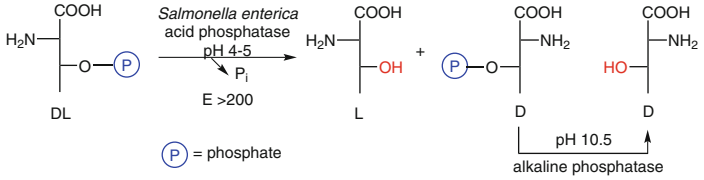
Chemoselective Hydrolysis of Phosphate Esters Chemical hydrolysis of polyprenyl pyrophosphates is hampered by side reactions due to the lability of the molecule. Hydrolysis catalyzed by acid phosphatase – an enzyme named because it displays its pH-optimum in the acidic range – readily afforded the corresponding dephosphorylated products in acceptable yields [545].

The product from a DHAP-depending aldolase-reaction is a sensitive 2-oxo-1,3,4-triol, which is phosphorylated at position 1 (Scheme 2.72). Mild dephosphorylation by using acid phosphatase without cumbersome isolation of the polar phosphorylated intermediate is a standard method to obtain the chiral polyol product [546–549] in good yield. In the latter example, it was transformed into the sex pheromone of the pine bark beetle (+)-*exo*-brevicomin.

Enantioselective Hydrolysis of Phosphate Esters In comparison with the hydrolysis of carboxyl esters, enantioselective hydrolyses of phosphate esters have been seldom reported due to problems to handle charged species. Acid phosphatases were applied to the kinetic resolution of serine and threonine via hydrolysis of the corresponding *O*-phosphate esters (Scheme 2.73) [550]. 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 ($E = 18$) [551].

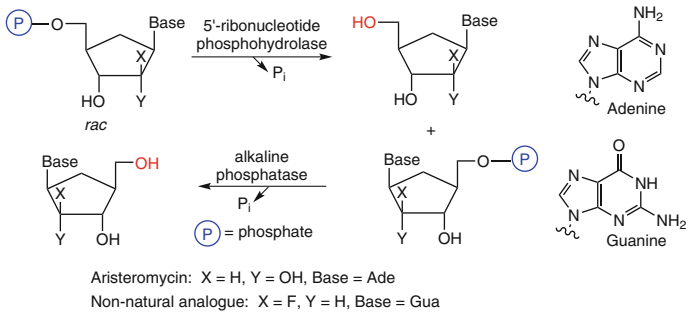


Scheme 2.72 Chemoselective enzymatic hydrolysis of phosphate esters



Scheme 2.73 Resolution of *rac*-threonine *O*-phosphate using acid phosphatase

Carbocyclic nucleoside analogs with potential antiviral activity, such as aristeromycin [552] and fluorinated analogs of guanosine [553], were resolved via their 5′-phosphates using a 5′-ribonucleotide phosphohydrolase from snake venom (see Scheme 2.74). After separation, the nonaccepted enantiomer, possessing a configuration opposite to that of the natural ribose moiety, was dephosphorylated by unspecific alkaline phosphatase.



Scheme 2.74 Resolution of carbocyclic nucleoside analogs

ATP-Dependent Phosphorylation Employing Kinases

The selective phosphorylation of a polyhydroxy compound by classic chemical methods using $POCl_3$ or phosphorochloridates is tedious since it usually requires a number of protection and deprotection steps. Furthermore, over-phosphorylation leading to undesired oligophosphate esters as byproducts is 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 via desymmetrization of prochiral or *meso*-diols or through racemate resolution is also possible.

In biological systems, phosphate esters are usually synthesized by means of phosphorylating transferases called kinases, which catalyze the transfer of a phosphate moiety (more rarely a di-[17] or triphosphate moiety) from an energy-rich phosphate donor, such as ATP, onto a nucleophile alcohol.[18] Due to the high price of these phosphate donors, they cannot be employed in stoichiometric amounts.[19] As with all cofactors in general, ATP cannot be replaced by less expensive man-made chemical equivalents, which requires efficient in-situ regeneration to render enzymatic phosphorylations more economic. Fortunately, ATP recycling has become feasible on a molar scale [554–557].

ATP Recycling In living organisms, ATP is regenerated by metabolic processes, but for biocatalytic transformations 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, because accumulation of the consumed cofactor (most commonly the corresponding diphosphate, ADP) can tip the equilibrium of the reaction in the reverse direction. Thus, nucleoside triphosphate 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 kinase enzyme and a stoichiometric quantity of a cheap high-energy phosphate donor (Scheme 2.75, Table 2.1). As nucleoside triphosphates are intrinsically unstable in solution, the triphosphate species is typically recycled a few 100 times [558, 559]. Sophisticated reaction engineering using a macromolecular ATP-PEG-construct in a membrane reactor has raised the ATP-cycle number to a solitary record number of ~20,000 mol product/mol ATP [560]. The total turnover numbers (TTN) concerning the enzyme performance are the range of ~10^6–10^8 mol of product per mol of enzyme.

The pro's and con's of the commonly used ATP-regenerating systems are as follows:

- The use of the phosphoenol pyruvate (PEP)/pyruvate kinase system is probably the most useful method for the regeneration of nucleoside triphosphates [561]. PEP is not only very stable towards spontaneous hydrolysis but it is also a very strong phosphorylating agent (Table 2.1). Furthermore, nucleosides other than adenosine phosphates are also accepted by pyruvate kinase. The drawbacks of this system is the considerable cost of PEP due to its more complex synthesis [562, 563] and the fact that pyruvate kinase is inhibited by pyruvate at higher concentrations.
- Acetyl phosphate can be easily synthesized from acetic anhydride and phosphoric acid and is therefore much cheaper than PEP [564]; together with acetate

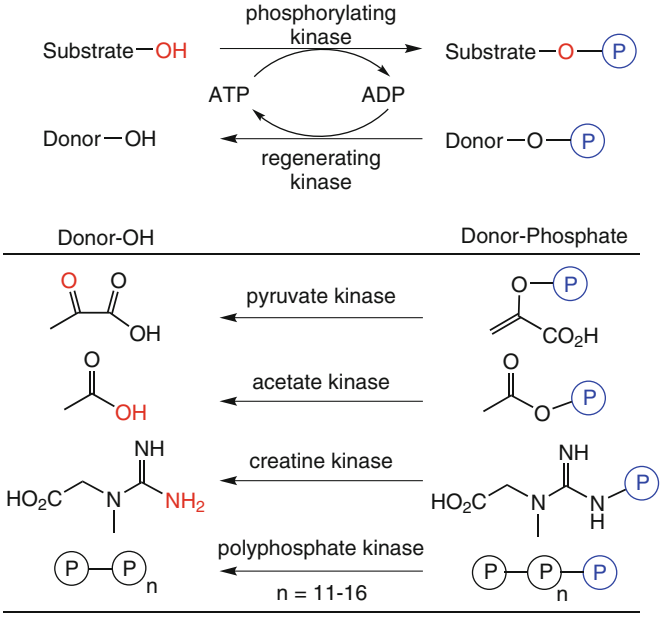
[17] Also termed 'pyro-phosphates'

[18] Phosphorylations involving C, N or S are very rare.

[19] The retail price for one mole of ATP is about US $4500, bulk prices are about one tenth of that.

kinase it is a commonly used regeneration system [565]. However, acetyl phosphate is modestly stable in aqueous solution ($t_{1/2}$ at pH 4.2–7.2 ca. 7–21 h) and its phosphoryl donor potential is lower than that of PEP. As for pyruvate kinase, acetate kinase also can accept nucleoside phosphates other than adenosine, and it is inhibited by acetate. 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) [561, 566, 567] – can be accomplished in the same manner using the PEP- or acetate kinase systems.

- As an alternative to expensive phosphenol pyruvate and hydrolytically unstable acetyl phosphate, the use of creatine kinase together with creatine phosphate has been proposed for ATP-recycling [568]. Due to the (controversially debated) role of creatine as food supplement for bodybuilding, creatine phosphate is relatively inexpensive and it is an equally strong phosphate donor as acetyl phosphate.
- Promising ATP-recycling methods for large-scale applications use cheap inorganic polyphosphate as phosphate donor and polyphosphate kinase, respectively [569, 570]. Polyphosphate is a ubiquitous natural polymer of tens to hundreds of orthophosphate residues linked by a high-energy phosphoanhydride bond, which is believed to be an ancient energy carrier preceding ATP in the prebiotic age [571]. It is widely used as an acidulant additive in soft drinks. Polyphosphate kinase from *E. coli* accepts also other nucleoside diphosphates and yields up to 40 regeneration cycles [572], but owing to the limited phosphate donor strength of poly/pyrophosphate, equilibrium yields for ATP are ≥85% [573].



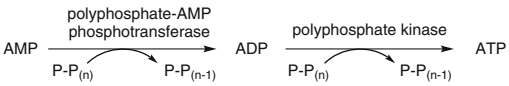
Scheme 2.75 Use of kinases for the enzymatic phosphorylation of alcohols and ATP recycling

Table 2.1 Standard free energy of phosphate donors upon hydrolysis

Phosphorylating agent	ΔG° [kcal mol^{-1}]
Phosphoenol pyruvate	−14.8
Acetyl phosphate	−10.3
Creatine phosphate	−10.3
Poly/pyrophosphate	−8.0
ATP → ADP + P_i	−7.3

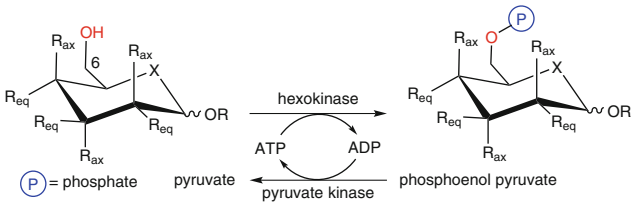
Two further ATP-recycling systems use carbamoyl phosphate (NH_2-CO-O-P) and methoxycarbonyl phosphate (MeO-CO-O-P) as nonnatural phosphate donors together with carbamate kinase and acetate kinase, respectively [574, 575]. Both systems lead to the formation of carbamic acid and methyl carbonate as unstable by-products, 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 aqueous media, which severely limits their applicability, hence these systems have not been widely employed.

A number of reactions which consume ATP generate AMP rather than ADP as a product, only few produce adenosine [576]. 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 (Scheme 2.76). Alternatively, the combination of adenosine kinase and adenylate kinase were used (Scheme 2.76) [577].



Scheme 2.76 Step-wise enzymatic recycling of ATP from AMP via ADP

Regioselective Phosphorylation The selective phosphorylation of hexoses and a few pentoses (e.g. D-arabinose) on the primary alcohol moiety can be achieved by hexokinase (Scheme 2.77) [578, 579]. The other (secondary) hydroxyl groups can be either removed or they can be exchanged for a fluorine atom, amino groups are tolerated on C2 [580], even thia- or aza-analogs or glucals are accepted. 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 2.77 is glucose-6-phosphate (R_{ax} = H; R_{eq} = OH; X = O), which serves as a hydride source during the recycling of NAD(P)H when using glucose-6-phosphate dehydrogenase [581, 582] (Sect. 2.2.1).

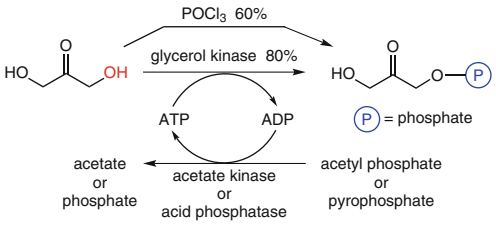


Substituents	Variations tolerated
R	H, Me
R_{eq}	H, F, OH, NH_2, AcNH
R_{ax}	H, F
X	O, S, NH

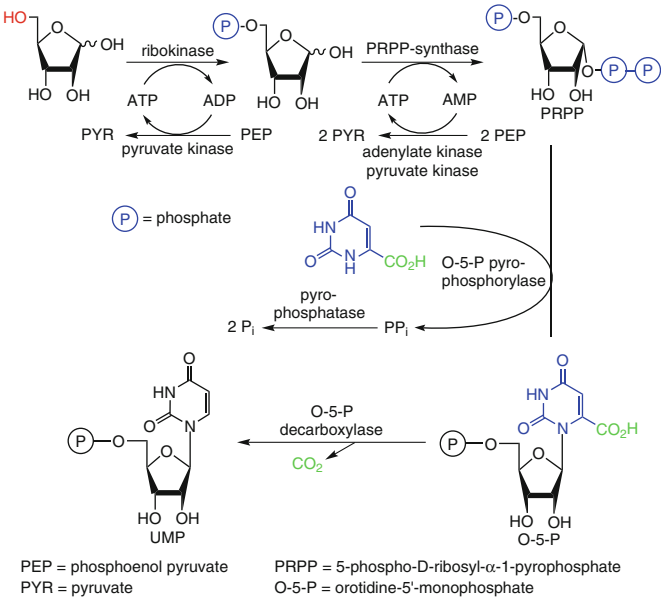
Scheme 2.77 Regioselective phosphorylation of hexose derivatives by hexokinase

Another important phosphate species, which is needed as a cosubstrate for DHAP-dependent aldolase reactions, is dihydroxyacetone phosphate (Scheme 2.78, also see Sect. 2.4.1). Its chemical synthesis using phosphorus oxychloride is hampered by moderate yields and the generation of side products. Enzymatic phosphorylation, however, gives significantly enhanced yields of a product which is sufficiently pure so that it can be used without isolation for the subsequent carboligation step [583, 584].

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 [585] (Scheme 2.79). First, D-ribose was phosphorylated at the *prim*-hydroxy group 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, which required adenylate kinase and pyruvate kinase for step-wise regeneration of ATP. Phosphoenol pyruvate (PEP) 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 decarboxylation of O-5-P by orotidine-5′-phosphate decarboxylase (a lyase) yielding UMP in 73% overall yield.

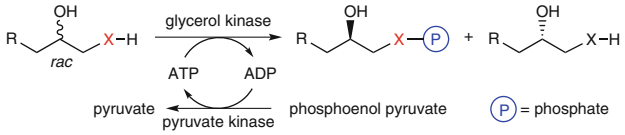


Scheme 2.78 Phosphorylation of dihydroxyacetone by glycerol kinase



Scheme 2.79 Phosphorylation of D-ribose and enzymatic synthesis of UMP

Enantioselective Phosphorylation Glycerol kinase [586] is not only able to accept its natural substrate, glycerol, to form *sn*-glycerol-3-phosphate [587], or close analogs of it such as dihydroxyacetone (see Scheme 2.78), but it is also able to transform a large variety of prochiral or racemic primary alcohols into chiral phosphates (Scheme 2.80) [588–590]. The latter compounds represent synthetic precursors to phospholipids [591] and their analogs [592].

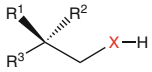


R	X	e.e. Phosphate [%]	e.e. Alcohol [%]
Cl	O	>94	88
SH	O	94	n.d.
CH_3O	O	90	n.d.
Br	O	90	n.d.
OH	NH	>94	94

Scheme 2.80 Enantioselective phosphorylation of glycerol derivatives

As depicted in Scheme 2.80, 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
R^1	preferably OH, also H or F, but not NH_2
R^2	H, OH (as hydrated ketone), small alkyl groups[a]
R^3	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

ATP-Independent Phosphorylation Employing Phosphatases

Enzymatic phosphorylation at the expense of ATP catalysed by kinases is predominantly involved in biological activation and messaging processes required for bio*synthesis*. Like most enzymes from primary metabolism, kinases posess a limited substrate spectrum, which – together with the requirement for ATP recycling – severely limits their applicability for the phosphorylation of non-natural substrates. In contrast, phosphate ester hydrolases (phosphatases) usually display a much broader substrate spectrum because they are found in bio*degradation* pathways. Although the ability of phosphatases to catalyse phosphate-transfer reactions yielding phosphate esters was already recognized in 1948 [593, 594], it was only recently, that the potential of ATP-independent phosphorylation was recognized [595, 596].

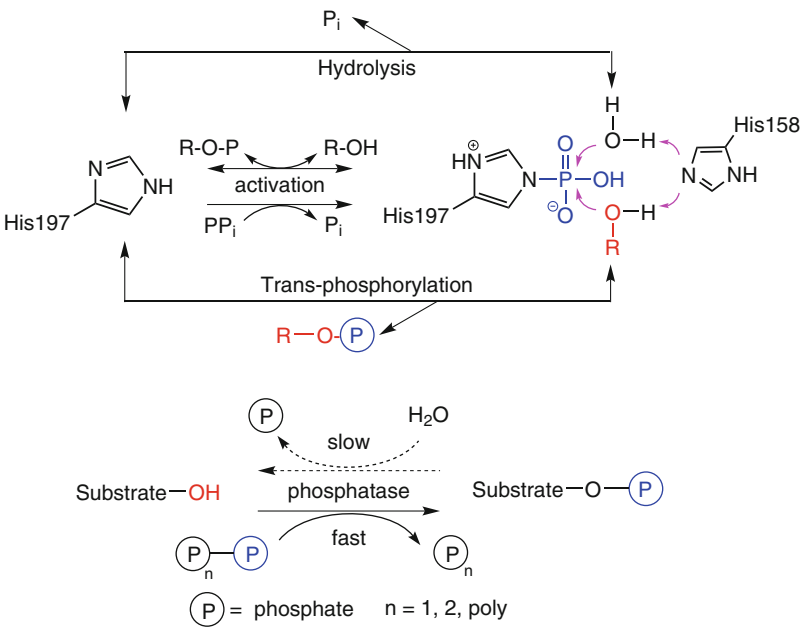
In order to enable phosphatases to catalyse phosphate transfer reactions, their mechanism of action must proceed through a covalent enzyme-phosphate intermediate in analogy to the acyl-enzyme intermediate in ester hydrolysis (Scheme 2.81, compare Scheme 2.1) [597]. In the hydrolysis mode, the phosphate ester is attacked by a nucleophilic His-residue[20] releasing ROH and forming a covalent enzyme-phosphate intermediate. The latter is attacked by water – through assistance of another His-residue – yielding phosphate and liberating His. In the trans-phosphorylation mode, the His-phosphate intermediate is preferably formed at the expense of an energy-rich di-, tri- or polyphosphate. Attack of the substrate alcohol R-OH yields the phosphate ester.

In practice, hydrolysis and trans-phosphorylation are taking place simultaneously and their relative rates depend on the reaction conditions, the type of

[20]In acid phosphatases, the active site nucleophile is usually a His (in AphA-St it is a carboxylate, Asp), in alkaline phosphatases it is a Ser or Thr residue.

substrates and the relative reactivity of the enzyme-phosphate intermediate with water or the alcohol substrate. In order to boost trans-phosphorylation over hydrolysis, the following strategies have been developed:

- In presence if a high-energy phosphate donor and elevated concentrations of the substrate alcohol, trans-phosphorylation is usually a fast process, which gives acceptable yields of the desired phosphate ester, which has to be recovered to prevent hydrolysis upon extended reaction times (kinetic control) [598–600].
- Kinetic control is facilitated by using a flow-system, where the phosphate donor and the substrate are continuously pumped through a column containing the immobilized phosphatase. By adjusting the flow rate, the residence (contact) time between enzyme and reactants can be tuned such that the maximum conversion is reached when the product leaves the column. Since the immobilized enzyme stays behind, no undesired hydrolysis can occur [601].
- Phosphatase mutants have been designed which show a greatly diminished hydrolysis rates but maintain good trans-phosphorylation activities [602].
- Reversal of hydrolysis is possible for selected substrates (e.g. glycerol), which are tolerated at very high concentrations (~70–95% v/v) at reduced water activities (thermodynamic control), where inorganic phosphate serves as phosphate donor [603, 604]. In practice, however, workup is problematic due to highly viscous reaction systems. A schematic representation of the time course of kinetic versus thermodynamic control is given in Fig. 2.19.

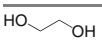
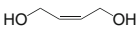
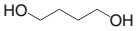
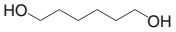
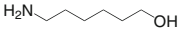


Scheme 2.81 Mechanism of phosphate ester hydrolysis and trans-phosphorylation catalyzed by phosphatase PhoN-Se via a covalent enzyme-phosphate intermediate

Acid phosphatases from *Shigella flexneri* (PhoN-Sf) [605] and *Salmonella enterica* (PhoN-Se) [606] phosphorylate not only simple alcohols, polyols and cyclic and aromatic alcohols, but also many simple carbohydrates using pyrophosphate as phosphate donor with a predominant regioselectivitity in favor of *prim*-hydroxy groups, only PhoN-Se converts *sec*-alcohols at low rates [607]. On the contrary, stereoselectivities observed so far were modest.

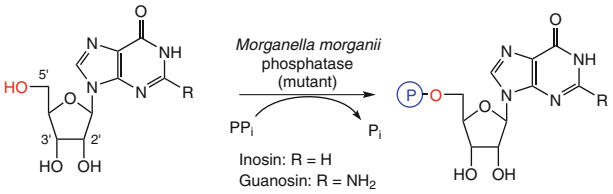
Regioselective monophosphorylation of diols using PhoN-Sf, PhoN-Se and PiACP (from *Prevotella intermedia*) using PP_i, PPP_i or polyP as phosphate donor revealed that on short-chain diols, only mono-phosphorylation occurred, bis-phosphates were formed to a small extent with long-chain analogs. Exclusive *O*-phosphorylation was observed with an aminoalcohol [608].

Table 2.2. Regioselective phosphorylation of diols and aminoalcohols using phosphatases

Substrate	Conc. [mM]	Enzyme	Product	Conc. [mM]
	500	PhoN-Sf	mono/bis-P >99/<1	176
	300	PhoN-Se	mono/bis-P 90/10	120
	500	PhoN-Sf	mono/bis-P 90/10	305
	500	PhoN-Sf	mono/bis-P 85/15	187
	500	PhoN-Se	O-P versus N-P >99/<1	85

Nucleotides are not only important intermediates for the synthesis of pharmaceuticals, but they also are widely used as flavor-enhancers and are thus produced on industrial scale (~16,000 t/a). Among them are inosine- (5-IMP) and guanosine 5′-monophosphate (5-GMP), whose biological activity depends on the position of the phosphate group: Whereas the 2′- and 3′-monophosphates are tasteless, the 5′-regioisomer is responsible for the 'umami'-taste.[21] As nucleosides, such as inosine and guanosine can be efficiently produced by fermentation, access to the corresponding 5′-nucleotides depends on regioselective phosphorylation. Non-specific acid phosphatases from *Morganella morganii* [609] and *Escherichia blattae* [610] were employed in trans-phosphorylation using PP_i as cheap phosphate donor. In order to meet the requirements for an industrial process, the native enzymes were mutated for enhanced binding of inosine by the phospho-enzyme intermediate, which favors trans-phosphorylation, while undesired hydrolysis is largely suppressed. The S72F/G74D/I153T-triple mutant was able to produce 140 g L^{-1} of inosine 5′-phosphate with a yield of 71% from inosine (Scheme 2.82).

[21]Together with sweetness, saltiness, bitterness and sourness, 'umami' or 'savory'-taste is one of the five basic tastes.



Scheme 2.82 Regioselective phosphorylation of nucleosides

The merits of enzymatic phosphorylation over chemical methods is the lack of side reactions owing to the mild reaction conditions. On lab-scale, the use of ATP-dependent phosphorylation is advantageous due to higher conversions and better selectivities of kinases despite the increased complexity connected with ATP recycling. On industrial scale, direct phosphate transfer from pyrophosphate mediated by phosphatases is preferable.

2.1.5 Hydrolysis of Epoxides

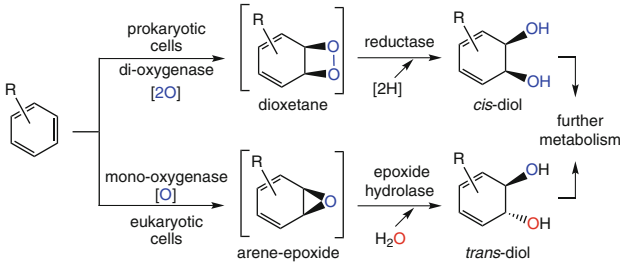
Chiral epoxides and vicinal diols (employed as their corresponding cyclic sulfate or sulfite esters as reactive intermediates) are extensively employed high-value intermediates for the synthesis of enantiomerically pure bioactive compounds due to their ability to react with a broad variety of nucleophiles [611, 612]. 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 [613], 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 [614]. On the other hand, the Jacobsen epoxidation is applicable to nonfunctionalized alkenes [615]. 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 [616–619]. 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. 2.3.3.3) [620]. 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 [621, 622].

Enzymes catalyzing the regio- and enantiospecific hydrolysis of epoxides – epoxide hydrolases (EH)[22] [623] – play a key role in the metabolism of xenobiotics.

[22]Epoxide hydrolases have been also called ‘epoxide hydratases’ or ‘epoxide hydrases’.

In living cells, aromatics and olefins can be metabolized via two different pathways (Scheme 2.83).

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. 2.3.3.7). The latter species are reductively cleaved into *cis*-diols. In eukaryotic cells of higher organisms such as fungi, yeasts and mammals, enzymatic epoxidation mediated by monooxygenases (Sect. 2.3.3.3) 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, 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 [624, 625].



Scheme 2.83 Oxidative biodegradation of aromatics

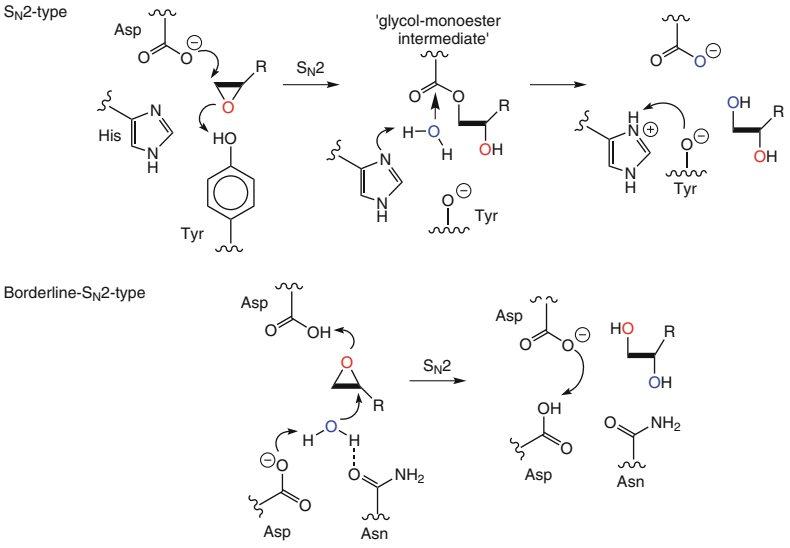
Enzyme Mechanism and Stereochemical Implications

The mechanism of epoxide hydrolase-catalyzed hydrolysis has been elucidated from microsomal epoxide hydrolase (MEH) and bacterial enzymes and involves the *trans*-antiperiplanar addition of water to epoxides 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 [626]. Two types of mechanism are known (Scheme 2.84).

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 [627–629]. The latter species can be regarded as a 'chemically inverted' acyl-enzyme intermediate in serine hydrolase reactions (Scheme 2.1). 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 [630] – 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 (Scheme 2.230)

[631, 632]. 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 (Scheme 2.217) [633].

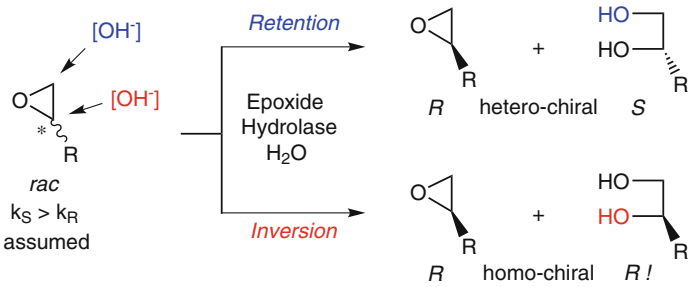
Borderline-S_N2-Type Mechanism Some enzymes, such as limonene-1,2-epoxide hydrolase, have been shown to operate via a single-step push-pull mechanism [634]. General acid catalysis by a protonated aspartic acid weakens the oxirane to facilitate a simultaneous nucleophilic attack of hydroxyl ion, which 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 2.84 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 2.85).

- 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 2.85 Enzymatic hydrolysis of epoxides proceeding with retention or inversion of configuration

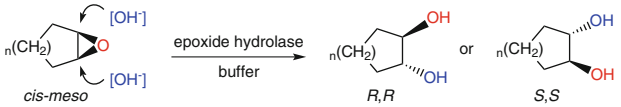
Although retention of configuration seems to be the more common pathway, inversion has been reported depending on the substrate structure and the type of enzyme [635, 636]. As a consequence, the absolute configuration of *both the product and the substrate* from a kinetic resolution of a racemic epoxide has to be determined separately in order to elucidate the stereochemical pathway. As may be deduced from Scheme 2.85, 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 [637]. For the solution to this stereochemical problem, various methods were proposed [638].

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. In general, MEH has been shown to possess higher activities and selectivities compared to its cytosolic counterpart.

Although pure MEH can be isolated from the liver of pigs, rabbits, mice, guinea pigs [639], or rats [640], a crude preparation of liver microsomes or even the 9000 × *g* supernatant of homogenized liver was employed as a source for EH activity with little difference from that of the purified enzyme being observed [641]. However, other enzyme-catalyzed side-reactions such as ester hydrolysis may occur with crude preparations.

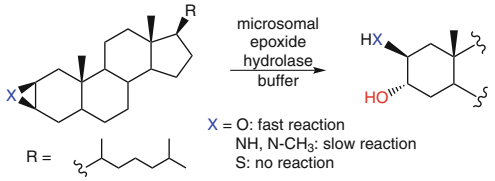
Cyclic *cis-meso*-epoxides can be asymmetrically hydrolyzed using hepatic epoxide hydrolases to give *trans*-diols. In this case, the (*S*)-configurated oxirane carbon atom is preferentially attacked and inverted to yield an (*R*,*R*)-diol (Scheme 2.86) [642, 643]. Among hepatic epoxide hydrolases, the microsomal enzyme was more selective than the cytosolic counterpart. In comparison, microbial epoxide hydrolases were considerably more stereoselective and showed stereo-complementary preferences [644–646].



n	Enzyme	Diol	
		Config.	E.e. [%]
1	microsomal EH	*R,R*	90
1	cytosolic EH	*R,R*	60
2	microsomal EH	*R,R*	94
2	cytosolic EH	*R,R*	22
1	*Rhodotorula glutinis* CIMW147	*R,R*	>98
2	*Rhodococcus erythropolis* DCL14 (mutant)	*S,S*	97
2	*Sphingomonas* sp. HXN-200	*R,R*	99
3	*Rhodococcus erythropolis* DCL14 (mutant)	*S,S*	98

Scheme 2.86 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 2.87) [647]. The thiirane, however, was inert towards enzymatic hydrolysis. The enzyme responsible for this activity was assumed to be the same microsomal epoxide hydrolase.



Scheme 2.87 Enzymatic hydrolysis of steroid epoxides and aziridines by microsomal epoxide hydrolase

Although many studies have been undertaken with hepatic epoxide hydrolases due to their importance in detoxification mechanisms [648], enzymes from these sources are unsuitable for preparative-scale transformations, since they cannot be obtained in reasonable amounts. In contrast, epoxide hydrolases from microbial sources are easy to produce by overexpression.

Microbial Epoxide Hydrolases

Although it was known for several years that microorganisms possess epoxide hydrolases, they were only scarcely applied to preparative organic transformations [649–652]. 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 systematic studies were undertaken later on. It should be emphasized, that for practical reasons, many preparative-scale reactions were performed by using whole-cell preparations or crude cell-free extracts containing an unknown number of epoxide hydrolases. Some microbial epoxide hydrolases have been purified and characterized [653–657].

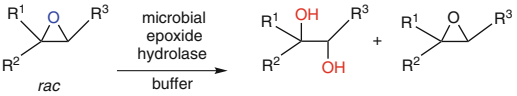
As a result, an impressive amount of knowledge on microbial epoxide hydrolases from various sources – such as bacteria, filamentous fungi, and yeasts – has

been gathered and featured in several reviews [658–666]. 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 [667]:

- 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, Nocardia* and *Sphingomonas* sp.) are the catalysts of choice for more highly substituted 2,2- and 2,3-disubstituted epoxides.

Monosubstituted oxiranes represent highly flexible and rather 'slim' molecules, which make chiral recognition a difficult task [668–671]. 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 (Scheme 2.88).

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 (Scheme 2.89). 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, *E*-values can only be applied to cases of single stereochemical pathways. The biocatalysts of choice were found among the fungal epoxide hydrolases, such as *Aspergillus niger* LCP 521 [672], *Beauveria densa* CMC 3240 and *Beauveria bassiana* ATCC 7159. Under certain circumstances, *Rhodotorula glutinis* CIMW 147 might serve as well [673–675].



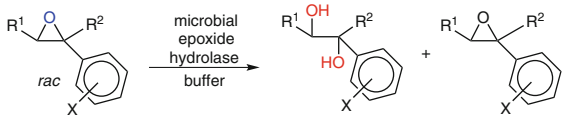
R^1	Enzyme Source	Selectivity[a]
CH_2Cl, $C(CH_3)_2O(CO)C(CH_3)_3$, CH_2OCH_2Ph, *t*-C_4H_9	bacterial	-
n-C_3H_7, *n*-C_4H_9, *n*-C_5H_{11}, *n*-C_6H_{13}, *n*-C_8H_{18}, *n*-$C_{10}H_{21}$	bacterial	±
n-C_6H_{13}	fungal	-
CH_2OH, C_2H_5, CH_2Cl, CH_2OCH_2Ph	yeast	- to ±
CH_3, *n*-C_2H_5	yeast	+
n-C_3H_7, *n*-C_4H_9, *n*-C_5H_{11}, *n*-C_6H_{13}	yeast	++

[a] Enantioselectivities are denoted as (-) = low (E <4), (±) = moderate (E = 4 - 12), (+) = good (E = 13 - 50), (++) excellent (E >50).

Scheme 2.88 Microbial resolution of monosubstituted epoxides (R^2, R^3 = H)

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 2.90) [676, 677]. 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.

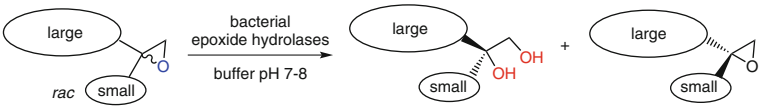
In contrast to 2,2-disubstituted epoxides, mixed regioselectivities are common for 2,3-disubstituted analogs and, as a consequence, *E*-values are not applicable (Table 2.3, Scheme 2.88) [678]. This is understandable, bearing in mind that the steric requirements at both oxirane positions are similar. Whereas fungal enzymes were less useful, yeast and bacterial epoxide hydrolases proved to be highly selective.



R^1	R^2	X	Enzyme Source	Selectivity[a]
H	H	*p*-CH_3, *o*-Cl, *p*-Cl	bacterial	±
H	CH_3	H	bacterial	±
H	H	*o*-CH_3, *o*-Hal	yeast	-
H	H	H	yeast	±
H	H	*p*-F, *p*-Cl, *p*-Br, *p*-CH_3	yeast	+
CH_3	H	H	yeast	++
H	CH_3	H	fungal	-
		indene oxide	fungal	+
CH_3	H	H	fungal	++
H	H	H	fungal	++
H	H	*p*-NO_2	fungal	++

[a] Enantioselectivities are denoted as (-) = low (E <4), (±) = moderate (E = 4 - 12), (+) = good (E = 13 - 50), (++) excellent (E >50).

Scheme 2.89 Microbial resolution of styrene oxide-type oxiranes



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

[a] Enantioselectivities are denoted as (-) = low (E <4), (±) = moderate (E = 4 - 12), (+) = good (E = 13 - 50), (++) excellent (E >50).

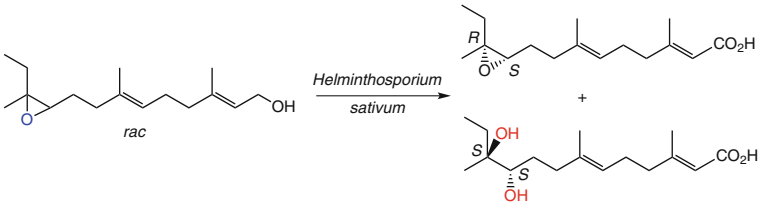
Scheme 2.90 Enzymatic resolution of 2,2-disubstituted epoxides using microbial epoxide hydrolases

Table 2.3 Microbial resolution of 2,3-disubstituted epoxides (for substrate structures see Scheme 2.88)

R^1	R^2	R^3	Enzyme source	Selectivity[a]
CH_3	H	n-C_5H_{11}	Fungal	±
H	CH_3	n-C_5H_{11}	Fungal	±
CH_3	H	CH_3	Yeast	++
H	CH_3	CH_3	Yeast	++
H	C_2H_5	n-C_3H_7	Bacterial	±
C_2H_5	H	n-C_4H_9	bacterial	±
H	CH_3	n-C_4H_9, n-C_5H_{11}, n-C_9H_{19}	Bacterial	++
CH_3	H	n-C_4H_9	Bacterial[b]	++

[a]Enantioselectivities are denoted as (−) = low ($E < 4$), (±) = moderate ($E = 4-12$), (+) = good ($E = 13-50$), (++) excellent ($E > 50$)
[b]Enantioconvergent pathway, i.e., a sole stereoisomeric diol was formed

To date, only limited data are available on the enzymatic hydrolysis of trisubstituted epoxides [679–684]. For example, a racemic allylic terpene alcohol containing a 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 2.91). The mirror image (*R*,*S*)-epoxide was not transformed. Both optically pure enantiomers were then chemically converted into a juvenile hormone [685].

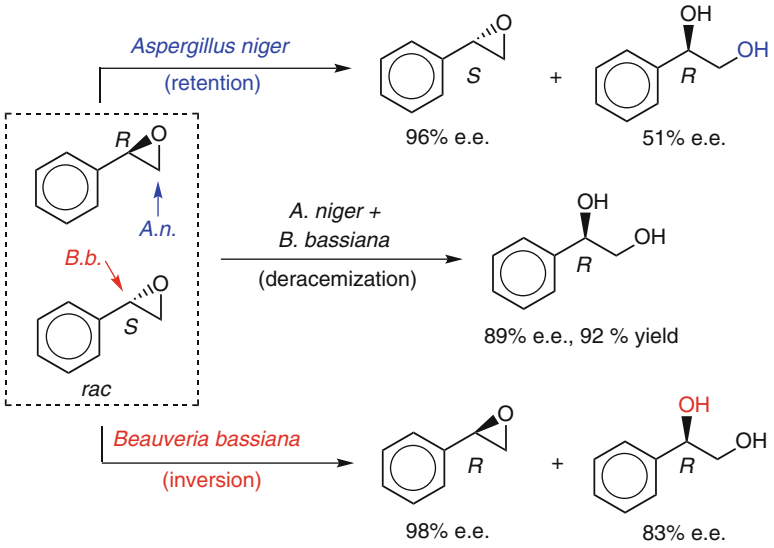


Scheme 2.91 Microbial resolution of a trisubstituted epoxide

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.

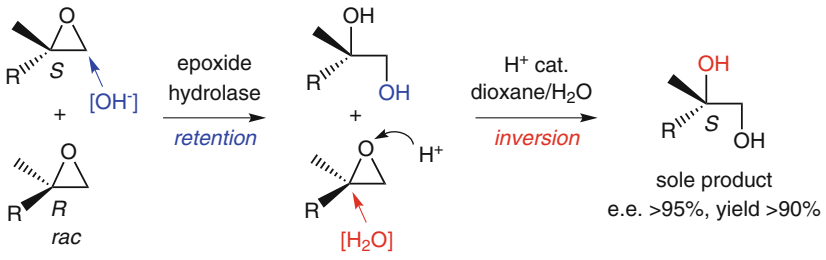
The first technique made use of two fungal epoxide hydrolases possessing matching opposite regio- and enantioselectivity for styrene oxide (Scheme 2.92) [686]. Resting cells of *Aspergillus niger* hydrolyzed the (*R*)-epoxide via attack at the less hindered carbon atom to yield the (*R*)-diol of moderate 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 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.



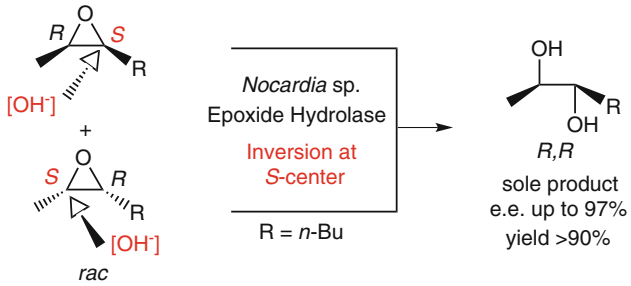
Scheme 2.92 Microbial resolution and deracemization of styrene oxide

For 2,2-disubstituted oxiranes, this technique was not applicable because it would require an enzyme performing a highly unfavored nucleophilic attack on a fully substituted carbon atom. In this case, a one-pot two-step sequence consisting of combined bio- and chemocatalysis was successful (Scheme 2.93) [687]. 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 contrast, acid-catalyzed hydrolysis of the remaining nonconverted enantiomer under carefully controlled conditions proceeds via an S_N2-borderline mechanism with *inversion* of configuration. 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%).



Scheme 2.93 Deracemization of 2,2-disubstituted oxiranes using combined bio- and chemo-catalysis

An exceptional case for an enantioconvergent biocatalytic hydrolysis of a (±)-*cis*-2,3-epoxyalkane is shown in Scheme 2.94 [688]. 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 2.94 Deracemization of 2,3-disubstituted oxiranes via enantioconvergent enzymatic hydrolysis

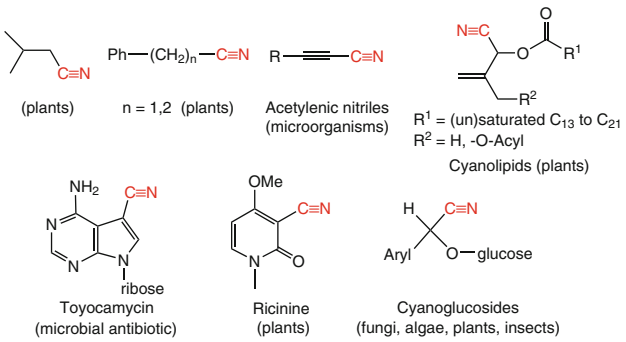
Enzymatic epoxide hydrolysis has been successfully upscaled to multigram batches using resting microbial cells containing (overexpressed) epoxide hydrolases. In order to avoid enzyme deactivation by the toxic substrate and to overcome solubility problems, aqueous-organic two-phase systems consisting of an alkane (hexane, *i*-octane) or an ether (MeO*t*Bu, iPr_2O) were employed [689, 690].

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

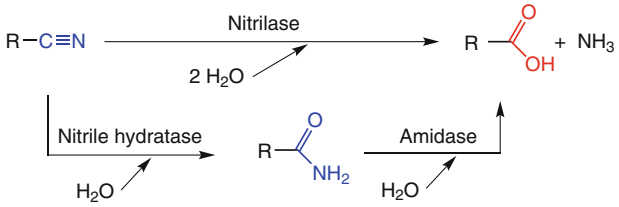
Organic compounds containing nitrile groups are found in the environment not only as a result of human activities, but also as natural products [691]. Naturally occurring nitriles are synthesized by plants, fungi, bacteria, algae, sponges, and insects, but not by mammals. This is puzzling, because cyanide 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 [692]



Scheme 2.95 Naturally occurring organic nitriles

As shown in Scheme 2.95, natural nitriles include cyanogenic glucosides which are produced by a wide range of plants including major crops such as almond, cassava [693] and sorghum (millet). Plants and microorganisms are also able of producing aliphatic or aromatic nitriles, such as cyanolipids, ricinine, and phenylacetonitrile [694]. These compounds can serve not only as a nitrogen storage, but also as protecting agents against attack by hungry predators. However, 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 [695–698] (Scheme 2.96).



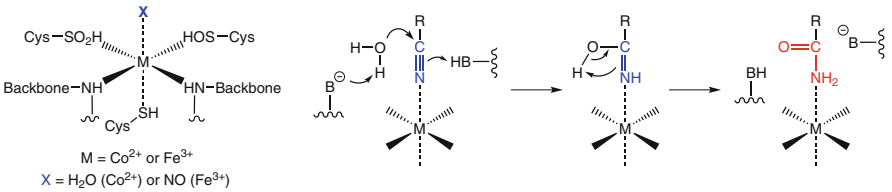
Scheme 2.96 General pathways of the enzymatic hydrolysis of nitriles

- Aliphatic nitriles are often metabolized in two stages. First they are converted to the corresponding carboxamide by a *nitrile hydratase* [699–701] and then to the carboxylic acid by an *amidase* enzyme (a protease) [702].
- 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 generally induced by amides and are known to possess a tightly bound metal atom (Co^{2+} or Fe^{3+} [703]) which is required for catalysis [704–

709]. In nitrile hydratase from *Bevibacterium* sp., 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 in a pseudo-porphyrin arrangement [710–712] (Scheme 2.97. The remaining axial ligand (X) is either a water molecule (Co^{2+}) [713] or nitric oxide (NO) which binds to Fe^{3+} [714, 715]. 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 coordination 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 [716–718].



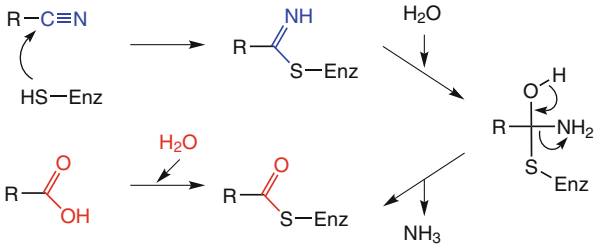
Scheme 2.97 Coordination sphere of Fe^{3+} and mechanism of *Brevibacterium* sp. nitrile hydratase

Nitrile hydratases from different sources are very similar to each other in terms of their substrate spectrum and accept a broad range of aliphatic, aromatic and arylaliphatic nitriles, generally with low or marginal stereoselectivities [719].

On the other hand, nitrilases operate by a completely different mechanism (Scheme 2.98). They possess neither coordinated metal atoms, nor cofactors, but act through an essential nucleophilic sulfhydryl residue of a cysteine [720, 721], which is encoded in the nitrilase-sequence motif Glu–Lys–Cys [722]. 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 [723]. According to their substrate specificities, nitrilases have been classified into three subtypes, aliphatic nitrilases, aromatic nitrilases and arylacetonitrilases, of which the latter are often enantioselective, which gives them the greatest potential for biotransformations [724].

Enzymatic hydrolysis of nitriles is not only interesting from an academic standpoint, but also from a biotechnological point of view [725–732]. Cyanide 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 extreme pH, which is incompatible with other hydrolyzable groups that may be present. Alternative methods

rely on metal catalysts, e.g. Raney-copper or manganese dioxide. Overall, energy consumption is high and unwanted side-products arising from over-hydrolysis or decomposition are common. 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 [696], the majority of transformations have been performed using sturdy whole-cell systems.



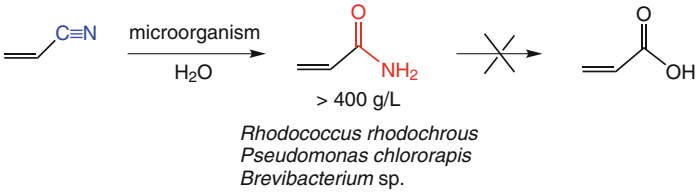
Scheme 2.98 Mechanism of nitrilases

Another important aspect is the enzymatic hydrolysis of cyanide and nitriles for the detoxification of industrial effluents [733–736].

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 [737]) the substrates are fed continuously to the culture.

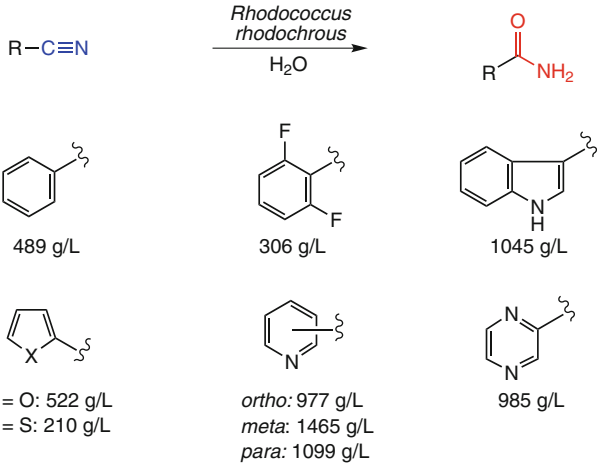
Acrylamide is one of the most important commodity chemicals for the synthesis of various polymers and is produced in an amount of about 2 Mt/year worldwide. In its conventional synthesis, the hydration of acrylonitrile is performed with copper catalysts. However, the preparative procedure for the catalyst, 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. [738, 739], *Pseudomonas chlororapis* [740, 741] or *Rhodococcus rhodochrous* [742] 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 600,000 t/year [743] (Scheme 2.99).



Scheme 2.99 Chemoselective microbial hydrolysis of acrylonitrile

Aromatic and heteroaromatic nitriles were selectively transformed into the corresponding amides by a *Rhodococcus rhodochrous* strain [744]; the products accumulated in the culture medium in significant amounts (Scheme 2.100). 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.

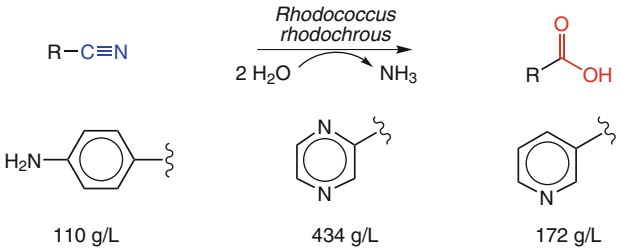
Important from a commercial standpoint was that *o*-, *m*-, and *p*-substituted cyanopyridines were accepted as substrates [745, 746] to give picolinamide (a pharmaceutical), nicotinamide (a vitamin), and isonicotinamide (a precursor for isonicotinic acid hydrazide, a tuberculostatic) (Scheme 2.100). 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 6000 t/year – is an important nutritional factor and is therefore widely used as a vitamin additive for food and feed supplies [747]. Pyrazinamide is used as a tuberculostatic.



Scheme 2.100 Chemoselective microbial hydrolysis of aromatic and heteroaromatic nitriles yielding carboxamides (product concentrations)

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 2.101). For instance,

p-aminobenzoic acid, a member of the vitamin B group, was obtained from *p*-aminobenzonitrile using whole cells of *Rhodococcus rhodochrous* [748]. Similarly, the antimycobacterial agent pyrazinoic acid was prepared in excellent purity from cyanopyrazine [749]. 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* [750] or *Nocardia rhodochrous* [751] proceeds quantitatively, whereas chemical hydrolysis is hampered by moderate yields.



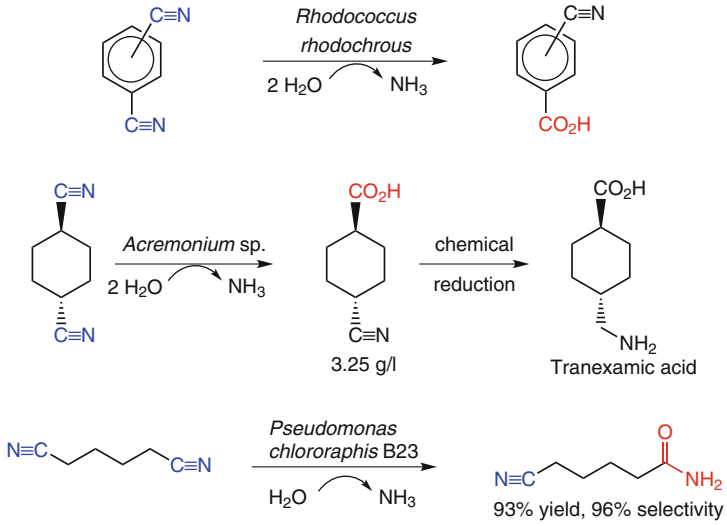
Scheme 2.101 Chemoselective microbial hydrolysis of aromatic and heteroaromatic nitriles yielding carboxylic acids (product concentrations)

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 [752] (Scheme 2.102).

For instance, 1,3- and 1,4-dicyanobenzenes were selectively hydrolyzed by *Rhodococcus rhodochrous* to give the corresponding monoacids [753, 754]. 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 mono-hydrolysis was achieved by using an *Acremonium* sp. [755]. 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 [756, 757].

5-Cyanovaleramide is required for the synthesis of the herbicide azafenidin. The chemical hydration of adiponitrile results in significant formation of the undesired di-amide and generates large amounts of waste products. In contrast, selective mono-hydration was achieved employing *Rhodococcus ruber* CGMCC3090 [758] or *Pseudomonas chlororaphis* B23. The latter process was upscaled using whole cells immobilized in Ca alginate beads to convert 12.7 t of adiponitrile with a selectivity of 96% [759].



Scheme 2.102 Regioselective microbial hydrolysis of dinitriles

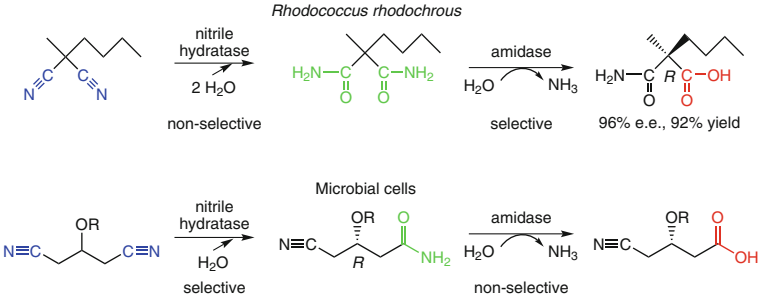
Enantioselective Hydrolysis of Nitriles

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 scarcely. 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 in nitrile hydratase-amidase pathways enantiodiscrimination often occurs during the hydrolysis of the intermediate carboxamide by the amidase [760] (Scheme 2.96). 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* [761] (Scheme 2.103). 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 [762].

In contrast, prochiral glutarodinitriles were stereoselectively hydrolyzed via two steps using whole microbial cells: in a first step, a stereoselective nitrile hydratase furnished the (*R*)-monoamide, which was further hydrolyzed to the corresponding carboxylic acid by an amidase [726, 763]. The cyano-acids thus obtained served as

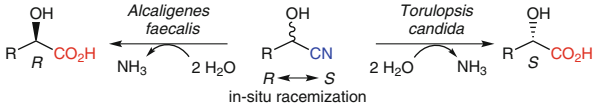
building blocks for the synthesis of cholesterol-lowering drugs from the statin family. An impressive example for the development of stereoselective enzymes derived from the metagenome is the discovery of >130 novel nitrilases from biotope-specific environmental DNA libraries [764, 765]. Among these enzymes, 22 nitrilases showed (*S*)-selectivity for the desymmetrization of the unprotcted 3-hydroxyglutarodinitrile (Scheme 2.103, R = H), while one produced the mirror-image (*R*)-enantiomer in 95–98% e.e. [766].



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 2.103 Asymmetric microbial hydrolysis of a prochiral dinitrile

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 [767], which are easily synthesized in racemic form from the corresponding aldehyde precursors by addition of hydrogen cyanide or a Strecker synthesis, respectively (Schemes 2.104 and 2.105). 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 [768], while (*R*)-mandelic acid is produced from *rac*-mandelonitrile on an industrial scale by employing resting cells of *Alcaligenes faecalis* [769] in >90 % yield [770, 771].



R	Microorganism	e.e. α-Hydroxyacid [%]
$(CH_3)_2$CH-	*Torulopsis candida*	>90
$(CH_3)_2$CH-CH_2-	*Torulopsis candida*	>95
Ph-	*Alcaligenes faecalis*	~100

Scheme 2.104 Stereocomplementary enantioselective hydrolysis of α-hydroxynitriles

In a related fashion, α-aminonitriles are enzymatically hydrolyzed to yield α-amino acids (Scheme 2.105). Whereas the enantiorecognition in *Brevibacterium imperiale* or *Pseudomonas putida* occurs through an amidase [772, 773], *Rhodococcus rhodochrous* PA-43, *Acinetobacter* sp. APN, and *Aspergillus fumigatus* possess enantiocomplementary nitrilases [772, 774, 775].

R	Microorganism	e.e. α-Aminoacid [%]
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)

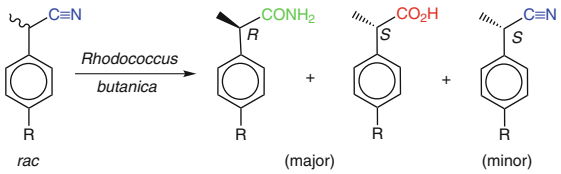
Scheme 2.105 Enantioselective hydrolysis of α-aminonitriles

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 [770, 776–779].

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 2.106) [780]. 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 [777]. In a related approach, *Brevibacterium imperiale* was used for the resolution of structurally related α-aryloxypropionic nitriles [781].

As a substitute for (expensive) commercial enzyme preparations for nitrile-hydrolysis, whole-cell preparations are recommended: *Rhodococcus* R312 [782][23] 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 [783].

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



R	e.e. Amide [%]	e.e. Acid [%]	e.e. Nitrile [%]
$(CH_3)_2CH\text{-}CH_2$-	99	87	73
Cl	76	>99	-
OCH_3	99	99	-

Scheme 2.106 Enantioselective hydrolysis of α-aryl propionitriles

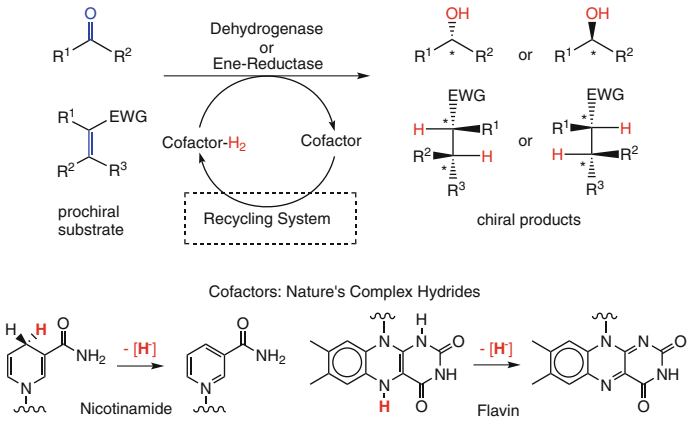
Enzymatic nitrile hydrolysis is a simple and convenient method to selectively obtain the corresponding carboxamides or carboxylic acids, depending on the type of enzyme(s) employed. Due to the sensitivity of nitrile-hydrolysing enzymes, whole microbial (resting) cells are used, in particular on industrial scale. Although excellent chemo- and regioselectivities are common, stereoselectivities may vary and are often incomplete.

2.2 Reduction Reactions

The enzymes employed for the majority of redox reactions are classified into three categories: dehydrogenases, oxygenases and oxidases (Scheme 2.144) [784–786]. Among them, alcohol dehydrogenases – also termed carbonyl reductases – have been widely used for the reduction of carbonyl groups (aldehydes, ketones) and ene-reductases are 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 (cyclic) Schiff-base type imines, or in the reductive amination of α-keto acids yielding α-amino acids.

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 2.107). 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, as well as electron-rich heteroatoms, affording C–H hydroxylation, C=C epoxidation, and thioether-oxidation, respectively (Sect. 2.3.3). Oxidases, which are responsible for the transfer of electrons, have gained increasing importance for the oxidation of alcohols (Sect. 2.3.1) and amines (Sect. 2.3.2) more recently.



Scheme 2.107 Reduction reactions catalyzed by dehydrogenases (*EWG* electron withdrawing group)

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.[24] 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, the cofactor 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. 1.4.3) – which is the total number of moles of product formed per mole of cofactor during its entire lifetime.[25] 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 numbers of at least 10^5 are highly desirable. The economic barrier to large-scale reactions posed by cofactor

[24]The current prices for 1 mole are: NAD^+ US $1400, NADH US $2600, $NADP^+$ US $18,000, NADPH US $70,000.

[25]In cofactor recycling the TTN is sometimes called 'cycle number'.

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 [555, 787–790].

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.

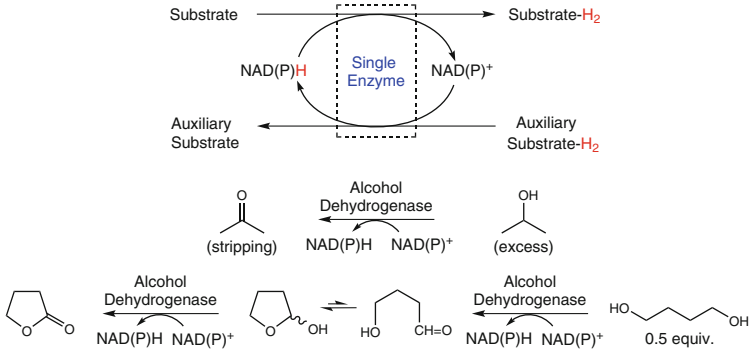
Recycling of Reduced Nicotinamide Cofactors

The easiest but least efficient method of regenerating NADH from NAD^+ is the nonenzymic reduction using a reducing agent such as sodium dithionite ($Na_2S_2O_4$) [791]. Since the corresponding turnover numbers of this process are very low (TTN $\leq$ 100), this method has only historical interest. Similarly, electrochemical [792–794] and photochemical regeneration methods [795–798] suffer from insufficient electron transport causing side-reactions and show low to moderate turnover numbers (TTN $\leq$ 1000).[26] 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 2.108) [799–801]. To shift the equilibrium of the reaction in the desired direction, the donor must be applied in excess [802]. In principle, this approach is applicable to both directions of redox reactions [803] and it constitutes a biological variant of a transfer-hydrogenation. 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 (hydrogen acceptor) and the auxiliary hydrogen donor.
- Enzyme inhibition caused by the high concentrations of the auxiliary substrate – cosubstrate inhibition – is common, in particular when highly reactive carbonyl species such as acetaldehyde or cyclohexenone are generated in the recycling process.
- The product has to be purified from large amounts of auxiliary substrate used in excess.

[26] 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%.

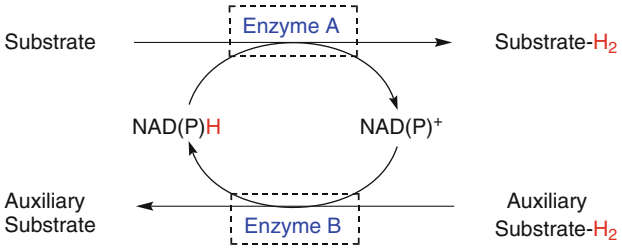


Scheme 2.108 Cofactor recycling by the coupled-substrate method

As a consequence, the coupled-substrate cofactor recycling only works with exceptionally sturdy dehydrogenases, which can tolerate high concentrations of a sacrificial *sec*-alcohol, such as 2-propanol, as hydride donor, but due to its simplicity it is the method of choice for industrial scale bioreductions. An elegant example to overcome some drawbacks of coupled-substrate nicotinamide recycling makes use of 1,4-butanediol as auxiliary substrate. Hydride abstraction yields 4-hydroxybutanal, which spontaneously cyclises to yield a lactol, which is irreversibly oxidised (by delivering a second hydride) to the corresponding butyrolactone. This drives the equilibrium without requirement for an excess of auxiliary substrate [804, 805]. A special technique avoiding some of these drawbacks makes use of gas-membranes and is discussed in Sect. 3.3.

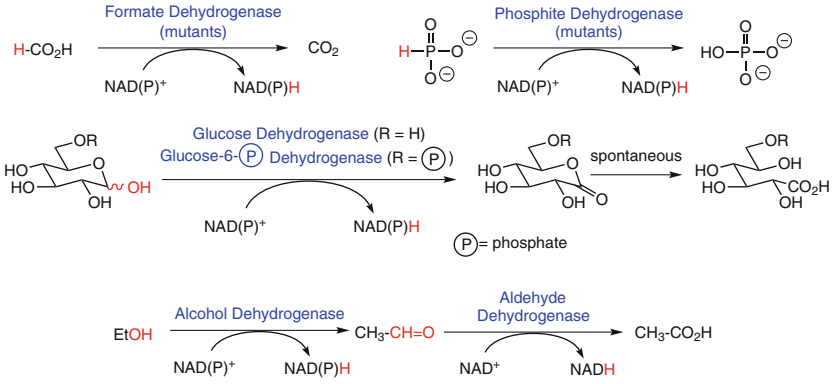
Coupled-Enzyme Approach The use of two independent enzymes is more advantageous (Scheme 2.109). In this case, the two parallel redox reactions – i.e., conversion of the main substrate plus cofactor recycling – are catalyzed by *two different* enzymes [806]. To achieve optimal results, both of the enzymes 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 independently converted by the two biocatalysts.

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 2.109 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 2.110) [807, 808]. This method has the advantage that both the auxiliary substrate and the coproduct are innocuous to enzymes and CO_2 is 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 [809] 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 [810] or membrane-retained FDH system [811]. 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 [812]. This drawback has been circumvented by application of a genetically engineered formate dehydrogenase from *Pseudomonas* sp., which also accepts NADPH [813–815].



Scheme 2.110 Enzymatic regeneration of reduced nicotinamide cofactors

Another widely used method for recycling NAD(P)H makes use of the oxidation of glucose, catalyzed by glucose dehydrogenase (GDH, Scheme 2.110)

[816, 817]. 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 [818] 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-phosphogluconolactone, which spontaneously hydrolyzes to the corresponding phosphogluconate (Scheme 2.110). The enzyme from *Leuconostoc mesenteroides* is inexpensive, stable and accepts both NAD^+ and $NADP^+$ [582, 819], 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 may be enzymatically prepared from glucose using hexokinase and this involves the regeneration of ATP using kinases (see pp. 107–109). Alternatively, glucose-6-sulfate and G6PDH from *Saccharomyces cerevisiae* may be used to regenerate NADPH [820]. The sulfate does not act as an acid catalyst for the hydrolysis of NADPH and is more easily prepared than the corresponding phosphate [821]. Overall, 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 [822, 823]: 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 only NAD^+ [824], but thermostable mutants were generated which are also able to reduce $NADP^+$ [825–827].

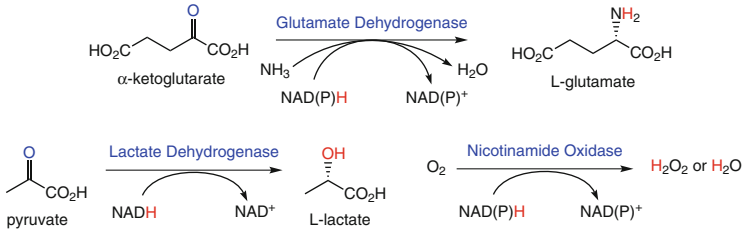
Ethanol and alcohol dehydrogenase (ADH) have been used in the past to regenerate NADH and NADPH [828, 829]. The low to moderate cost of ADH and the volatility of both ethanol and acetaldehyde make this system attractive for lab-scale reactions. An alcohol dehydrogenase from yeast reduces NAD^+, while an ADH from *Leuconostoc mesenteroides* is used to regenerate NADPH (Scheme 2.110). 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 may be achieved by sweeping with nitrogen [830] or by further oxidizing acetaldehyde to acetate [831], 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 [832].

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 [833, 834]. The latter is strongly reducing, innocuous to enzymes and nicotinamide cofactors, and its consumption leaves no

byproduct. For organic chemists, however, this method is of limited use because hydrogenase is usually isolated from strict anaerobic organisms. Thus, the enzyme is sensitive to oxidation, is not commercially available and requires sophisticated fermentation procedures for its production.[27] 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.

Recycling of Oxidized Nicotinamide Cofactors

For oxidation, reduction reactions can be run in reverse, although the equilibrium is strongly disfavoured. 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 2.111) [837, 838]. Both NADH and NADPH are accepted as cofactors. In addition, α-keto-adipate can be used instead of the corresponding glutarate [839], leading to the formation of a high-value byproduct, L-α-aminoadipate.



Scheme 2.111 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 [840]. However, the redox potential is less favorable and LDH does not accept $NADP^+$.

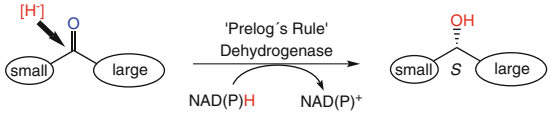
More recently, flavin-dependent nicotinamide oxidases, such as YcnD from *Bacillus subtilis* [841] or an enzyme from *Lactobacillus sanfranciscensis* [842] were employed for the (irreversible) oxidation of nicotinamide cofactors at the expense of molecular oxygen producing H_2O_2 or (more advantageous) H_2O via a two- or four-electron transfer reaction, respectively [843–845]. 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 [846]. 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.

[27] For an O_2-tolerant hydrogenase from *Ralstonia eutropha* see [835, 836].

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 [847–850]. 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 2.112) [851].



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 2.112 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 [852]. *Thermoanaerobium brockii* alcohol dehydrogenase (TBADH) also obeys this rule 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. [853–855]. Other ADHs from *Curvularia*

falcata [856], *Mucor javanicus* and *Pseudomonas* sp. [857] are of limited use as long as they are not commercially available.

The substrate range of commercially available alcohol dehydrogenases has been mapped including aldehydes, (acyclic, aromatic, and unsaturated) ketones, diketones and various oxo-esters [858]. The most commonly used dehydrogenases are shown in Fig. 2.15, with reference to their preferred size of their substrates [859].

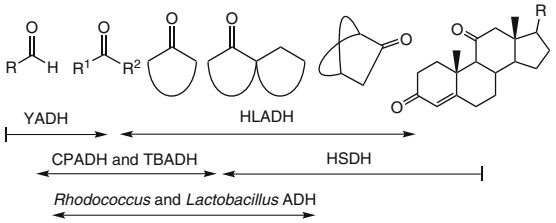


Fig. 2.15 Preferred substrate size for dehydrogenases. *YADH* yeast alcohol dehydrogenase, *HLADH* horse liver alcohol dehydrogenase, *CPADH Candida parapsilosis* alcohol dehydrogenase, *TBADH Thermoanaerobium brockii* alcohol dehydrogenase, *HSDH* hydroxysteroid dehydrogenase

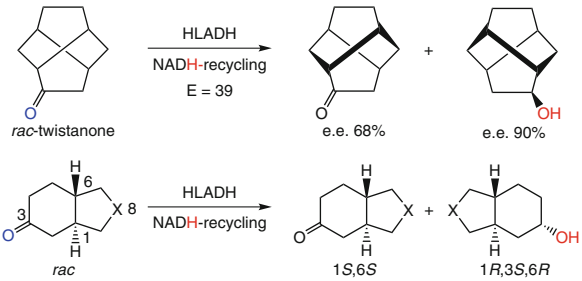
Yeast ADH has a very narrow substrate specificity and, in general, only accepts aldehydes and methyl ketones [860, 861]. Therefore, cyclic ketones and those bearing carbon chains larger than a methyl group are not accepted as substrates. Thus, YADH is only of limited use for the preparation of small chiral secondary alcohols.

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 [862, 863] and its mechanism was elucidated [863] on the basis of its crystal structure [864]. Although the primary sequence is quite different, the tertiary structure of HLADH is similar to that of YADH [865]. 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 [866–868]. Sterically demanding molecules which are larger than decalines are not readily accepted and acyclic ketones are usually reduced with modest enantioselectivities [869, 870]. HLADH consists of two isoenzymes (HLADH-E and HLADH-S[28]), which differ in their substrate preference [871].

A considerable number of monocyclic and bicyclic racemic ketones have been resolved using HLADH with fair to excellent specificities [872–874]. Even sterically demanding cage-shaped polycyclic ketones were readily accepted [875, 876] (Scheme 2.113). For instance, *rac*-2-twistanone was reduced to give the *exo*-alcohol and the enantiomeric ketone in 90% and 68% e.e., respectively [877]. Also *O*- and *S*-heterocyclic ketones were shown to be good substrates (Scheme 2.113) [878–880]. Thus, (±)-bicyclo[4.3.0]nonan-3-ones bearing either an O or S atom in position 8 were resolved with excellent selectivities

[28]The prefix 'S' stands for steroids, 'E' stands for ethanol.

[870]. 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 [881].



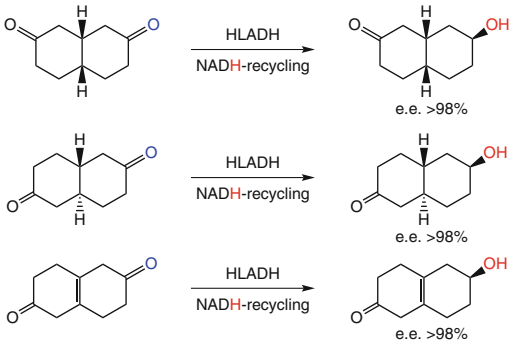
X	Selectivity (E)	e.e. Ketone [%]	e.e. Alcohol [%]
O	>120	60	>97
S	>110	53	>97

Scheme 2.113 Kinetic resolution of bi- and polycyclic ketones using horse liver alcohol dehydrogenase (HLADH)

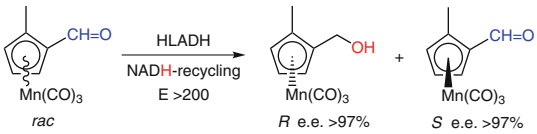
Every kinetic resolution of bi- and polycyclic ketones suffers from one particular drawback because the bridgehead carbon atoms make it impossible to recycle the undesired 'wrong' enantiomer via racemization. Hence the desymmetrization of prochiral diketones, making use of the enantioface- or enantiotopos-specificity of HLADH, is of advantage. For instance, both the *cis*- and *trans*-forms of the decalinediones shown in Scheme 2.114 were reduced to give (*S*)-alcohols with excellent optical purity. Similar results were obtained with unsaturated derivatives [828, 882].

The wide substrate tolerance of HLADH encompassing nonnatural compounds is demonstrated by the resolution of organometallic derivatives possessing axial chirality [883]. For instance, the racemic tricarbonyl cyclopentadienyl manganese aldehyde shown in Scheme 2.115 was enantioselectively reduced to give the (*R*)-alcohol and the residual (*S*)-aldehyde with excellent optical purities [884].

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 most useful substrate model based on a flattened cyclohexanone ring is shown in Fig. 2.16 [885]. It shows the Zn^{2+} in the catalytic site which coordinates to the carbonyl oxygen atom and the nucleophilic attack of the hydride occurring from the bottom face. The preferred orientation of the substrate relative to the hydride delivered from NADH can be estimated by placing the substituents into the 'allowed' and 'forbidden' zones.



Scheme 2.114 Desymmetrization of prochiral diketones using HLADH



Scheme 2.115 Enantioselective reduction of an organometallic aldehyde using HLADH

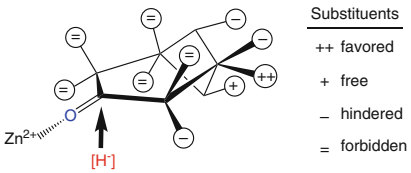
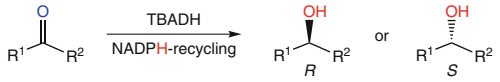


Fig. 2.16 Substrate model for HLADH for cyclic ketones

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* [886] and *Thermoanaerobium* (TBADH) strains (Scheme 2.116) [311, 887–890]. 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 [891–893].



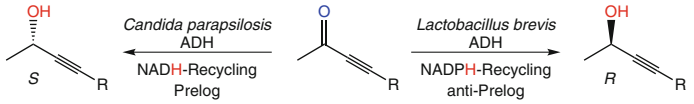
R^1	R^2	Specificity	Configuration	e.e. [%]
CH_3	$CH(CH_3)_2$	Anti-Prelog	*R*	86
CH_3	C_2H_5	Anti-Prelog	*R*	48
CH_3	*cyclo*-C_3H_5	Anti-Prelog	*R*	44
CH_3	*n*-C_3H_7	Prelog	*S*	79
CH_3	C≡CH	Prelog	*S*	86
Cl-CH_2-	CH_2-CO_2Et	Prelog	*R*[a]	90
CF_3	Ph	Prelog	*R*[a]	94
CH_3	CH_2-$CH(CH_3)_2$	Prelog	*S*	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	$(CH_2)_3$-Cl	Prelog	*S*	>99
n-C_3H_7	*n*-C_3H_7	no reaction		

[a] Switch in CIP-sequence order.

Scheme 2.116 Asymmetric 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 [894]. Similarly, ω-haloalkyl- [817, 895] and methyl- or trifluoromethyl ketones possessing heterocyclic substituents were converted into the corresponding secondary alcohols with excellent optical purities [896, 897]. 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 [898].

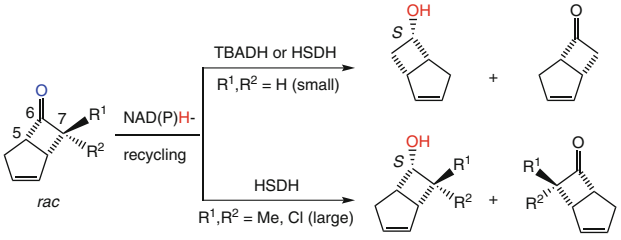
The key to access both stereoisomers of a *sec*-alcohol via asymmetric carbonyl reduction is the availability of stereocomplementary dehydrogenases. For open-chain ketones bearing a small and large substituent at each side, this is feasible by using an appropriate enzyme showing Prelog or anti-Prelog 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 2.117) [899]. In an analogous fashion, α-ketocarboxylic acids were reduced to the corresponding enantiomeric α-hydroxyacids using stereocomplementary lactate dehydrogenases (LDH) [900–903], or hydroxyisocaproate dehydrogenases (HicDHs) [904, 905].



R	*Candida parapsilosis* ADH		*Lactobacillus* brevis ADH	
	Configuration	E.e. [%]	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 2.117 Stereocomplementary bioreduction using a Prelog and anti-Prelog dehydrogenase

Hydroxysteroid dehydrogenases (HSDH) are ideally suited enzymes for the reduction of bulky mono- [906] and bicyclic ketones (Scheme 2.118) [907]. This is not surprising if one thinks of the steric requirements of their natural substrates: steroids [908, 909]. 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), but 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 [910, 911]. 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.



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

[a] No reaction was observed with HLADH or TBADH.

Scheme 2.118 Kinetic resolution of sterically demanding ketones using hydroxysteroid dehydrogenase (HSDH)

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 carbonyl substrates, are mentioned below.

One general limitation of alcohol dehydrogenases is their inability to convert sterically demanding ketones bearing bulky groups on both sides. This limitation was overcome by identification of two special ADHs from *Ralstonia* sp. DSM 6428 and *Sphingobium yanoikuyae* DSM 6900 [912]. The former enzyme reduced arylalkyl ketones bearing *n*-propyl- to *n*-pentyl chains with excellent Prelog-specificity [913].

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 [837]. 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 [914].

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 [915–918]. 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 [919]. 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 [920] (compare pp. 75–76).

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 recovery, particularly when the product is stored inside the cells and not excreted into the medium. Since only a small fraction (typically 0.5–2%) of the auxiliary cosubstrate is used for coenzyme recycling, while the bulk is metabolized forming polar byproducts, product purification is troublesome and 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 [921].
- Stereoselectivities may vary to a great extent due to the presence of multiple enzymes. 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. 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 [922].

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 [923–925]
- Variation of the metabolic parameters by immobilization [926–928]
- Using cells of different age [929]
- Variation of the fermentation conditions [930–932]
- Screening of microorganisms to obtain strains with the optimum properties (a hard task for nonmicrobiologists) [933, 934]
- Selective inhibition of one of the competing enzymes (see below)

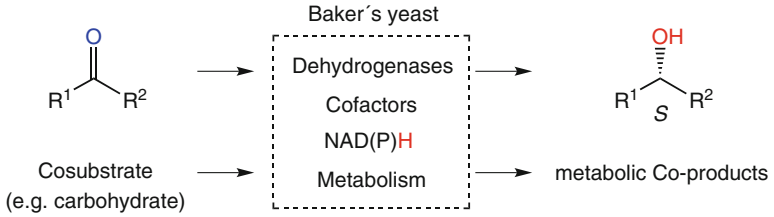
Reduction of Aldehydes and Ketones by Baker's Yeast

Asymmetric Reduction of Ketones Baker's yeast (*Saccharomyces cerevisiae*) is by far the most widely used microorganism for the asymmetric reduction of ketones [935–939]. It is ideal for nonmicrobiologists, since it is readily available at a very reasonable price and its use does not require sterile fermenters but can 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 [940] and the first comprehensive review which covers almost all the different strategies of yeast-reductions dates back to 1949! [941].

A wide range of functional groups within the ketone are tolerated, including heterocyclic- [942, 943], fluoro- [944–947], chloro- [948], bromo- [949], perfluoroalkyl- [950], cyano-, azido-, nitro- [951–953], hydroxyl- [954, 955], sulfur- [956–958], and dithianyl groups [959]. Even organometallic derivatives [960, 961], such as silyl- [962] and germyl groups [963] 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 2.119) [861]. 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 [964, 965]. As might be expected, best stereoselectivities were achieved with groups of greatly different size.



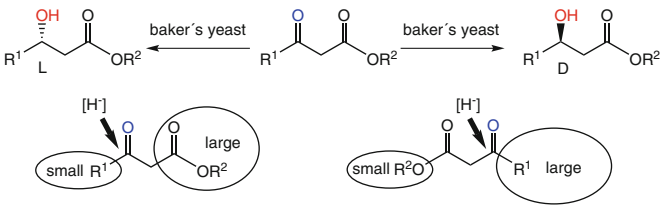
R^1	R^2	e.e. [%]
Me	Et	67
Me	CF_3	>80
CF_3	CH_2-Br	>80
Me	*n*-Bu	82
Me	Ph	89
Me	CH_2-OH	91
Me	$(CH_2)_2$-CH=C$(CH_3)_2$	94
Me	*c*-C_6H_{11}	>95
Me	C$(CH_3)_2$-NO_2	>96

Scheme 2.119 Reduction of aliphatic and aromatic ketones using baker's yeast

Acyclic β-ketoesters (Scheme 2.120) are readily reduced by yeast to yield β-hydroxyesters [966, 967], which serve as chiral starting materials for the synthesis of β-lactams [968], insect pheromones [969], and carotenoids [970]. 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 2.120). 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 [971].

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 different dehydrogenases, possessing opposite stereochemical preferences, which compete for the substrate [922, 972]. 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.



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

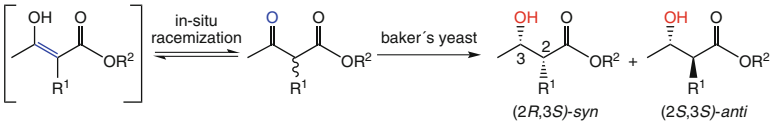
Scheme 2.120 Reduction of acyclic β-ketoesters using baker's yeast

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 [973] or methyl vinyl ketone [974]. The same effect was observed when the yeast cells were immobilized by entrapment into a polyurethane gel [975, 976]. As expected, L-enzyme inhibitors led to a considerable increase in the optical purity of D-β-hydroxyesters.

On the contrary, various haloacetates [977], thioethers [978], and allyl bromide [979] are inhibitors for the D-enzyme, which leads to an increased formation of the L-enantiomer.

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 – leading to dynamic resolution [64, 980, 981]. 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 [982]. Under optimal conditions it can even be as high as 100:0 [983]. 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.

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 2.121) [984–987]. With small α-substituents, the formation of *syn*-diastereomers predominates, but the diaselectivity is reversed when the substituents are increased in size. The diastereoselectivity (i.e., the *syn*/*anti*-ratio) of yeast-catalyzed reductions of α-substituted β-ketoesters can be predicted from the relative size of the α-substituent versus the carboxylate moiety using a simple model [988]. 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.

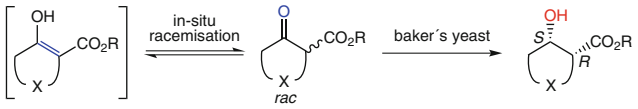


R^1	R^2	e.e. [%]		Ratio
rac		*syn*	*anti*	*syn/anti*
CH_3	C_2H_5	100	100	83:17
CH_3	Ph-CH_2-	100	80	67:33
CH_2=CH-CH_2-	C_2H_5	100	100	25:75
Ph-CH_2-	C_2H_5	100	100	33:67
Ph-S-	CH_3	>96	>96	17:83

Scheme 2.121 Diastereoselective reduction of α-substituted β-ketoesters using baker's yeast

The yeast-reduction of cyclic β-ketoesters exclusively leads to the corresponding *syn*-β-hydroxy-esters (Scheme 2.122) [989–991]. 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 stereoselective 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 2.41).



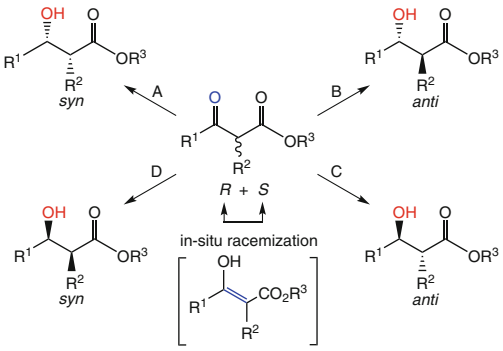
X	R	e.e. [%]
-$(CH_2)_2$-	C_2H_5	>98
-$(CH_2)_3$-	C_2H_5	86-99[a]
-S-CH_2-	CH_3	85
-CH_2-S-	CH_3	>95

[a] Depending on the yeast strain used.

Scheme 2.122 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 2.123) [992–997]. 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- [998], α-alkylthio- [984], α-azido- [999], or α-acetylamino derivatives [1000]. Consequently, they 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.

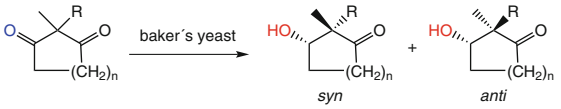


Path-way	R^1	R^2	R^3	Biocatalyst	yield [%]	d.e. [%]	e.e. [%]	Refer ences
A	Me	allyl	Et	baker´s yeast	94	92	>99	[988]
A	Me	Me	*n*-Octyl	baker´s yeast	82	90	>98	[989]
B	Me	Me	Et	*Geotrichum candidum*	80	>98	>98	[990]
B	Et	Me	Et	*Geotrichum candidum*	80	96	91	[991]
C	4-MeOC$_6$H$_4$-	Cl	Et	*Sporotrichum exile*	52	96	98	[992]
D	4-MeOC$_6$H$_4$-	Cl	Me	*Mucor ambiguus*	58	>98	>99	[993]

Scheme 2.123 Stereocomplementary microbial reduction of α-substituted β-ketoesters

α-Ketoesters and α-ketoamides can be asymmetrically reduced to furnish the corresponding α-hydroxy derivatives. Thus, following Prelog's rule, (*S*)-lactate [1001] and (*R*)-mandelate esters [982] were obtained from pyruvate and α-keto-phenylacetic esters by fermenting baker's yeast in excellent optical purity (e.e. 91–100%).

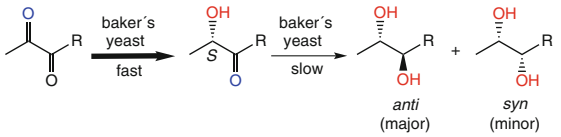
Cyclic β-diketones are selectively reduced to give β-hydroxyketones without the formation of dihydroxy products (Scheme 2.124) [1002–1005]. It is important, however, that the highly acidic protons on the α-carbon atom are fully replaced by substituents in order to avoid the (spontaneous) chemical condensation of the substrate with acetaldehyde, which is always present in yeast fermentations and to avoid racemization of the α-monosubstituted 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.



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 2.124 Yeast reduction of cyclic β-diketones

In contrast, the reduction of α-diketones does not stop at the α-hydroxyketone (acyloin) stage but leads to the formation of vicinal diols (Scheme 2.125). 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 [1006, 1007].

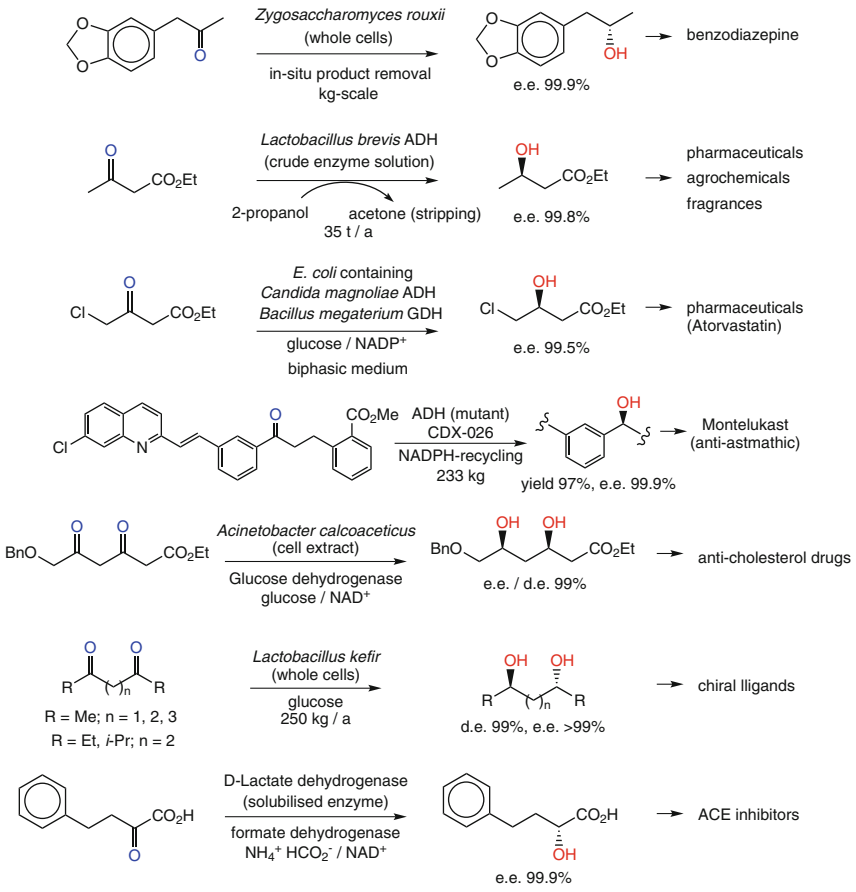


R	e.e. *anti*-diol [%]	*anti/syn*
Ph-	94	>95:<5
1,3-dithian-2-yl-	97	95:5
Ph-S-CH_2-	>97	86:14

Scheme 2.125 Yeast reduction of α-diketones

Secondary alcohols possessing the *anti-Prelog configuration* can be obtained from yeast reductions via substrate modification (Scheme 2.120) or through enzyme inhibition. If these techniques are unsuccessful, the use of microorganisms other than yeast [854, 857, 1008–1012], such as *Pichia farinosa* [1013], *Geotrichum candidum* [1014, 1015], and *Yarrowia lipolytica* [1016] may be of an advantage. Even plant cell cultures such as *Gardenia* may be employed for this purpose [1017, 1018]. However, in this case the help of a microbiologist is recommended for organic chemists.

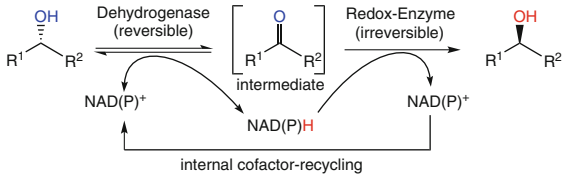
The high level of technology available for the biocatalytic reduction of carbonyl compounds has allowed its implementation for the industrial-scale production of chiral building blocks containing *sec*-alcohol moieties. The system applied – either whole microbial (wild-type) cells, a designer bug containing 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 2.126 [1019–1025].



Scheme 2.126 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 2.127) [37, 1031, 1032]: 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 from the racemate [1028]. 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 [1026, 1033].



R^1	R^2	Microorganism(s)	Yield [%]	e.e. [%]	References
Me	CH_2CO_2Et	*Geotrichum candidum*	67	96	[1023]
Me	*p*-Cl-C_6H_4	*Geotrichum candidum*	97	96	[1024]
CH_2OH	Ph	*Candida parapsilosis*	~100	~100	[1025]
Me	$(CH_2)_2CH{=}CMe_2$	*Bacillus stearothermophilus* + *Yarrowia lipolytica*	91	~100	[1026]
Ph	CO_2H	*Pseudomonas polycolor* + *Micrococcus freudenreichii*	70	>99	[1027]

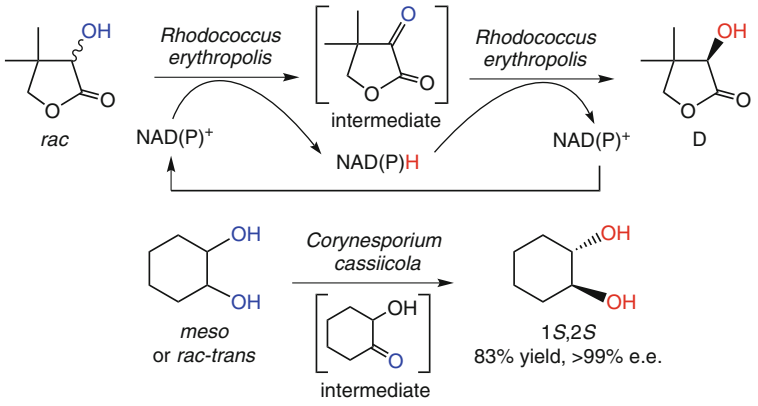
Scheme 2.127 Deracemization of *sec*-alcohols via microbial stereoinversion

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 [1033]. Along these lines, the enzymatic stereoinversion of (biologically inactive) D-carnitine to furnish the desired bioactive L-enantiomer[29] 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%) [1034]. 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 [1035]. Recently, the mechanism of enzymatic stereoinversion catalyzed by stereocomplementary

[29] 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

dehydrogenases was elucidated to depend on the opposite cofactor-dependence for NADH and NADPH of the dehydrogenases involved [1036, 1037].

Microbial stereoinversion of *sec*-alcohols has become quite popular [1038]. 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 [1039] – was deracemized by using resting cells of *Rhodococcus erythropolis* or *Candida* sp. (Scheme 2.128) [1040, 1041]. 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 2.128 Microbial deracemization of pantoyl lactone and 1,2-cyclohexanediol

Microbial stereoinversion has been shown to be extremely flexible, as it is also applicable to *sec*-diols possessing *two* stereocenters [1042–1044]. Thus, *meso*- or *rac-trans*-cyclohexane-1,2-diol was deracemized by *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 [1045].

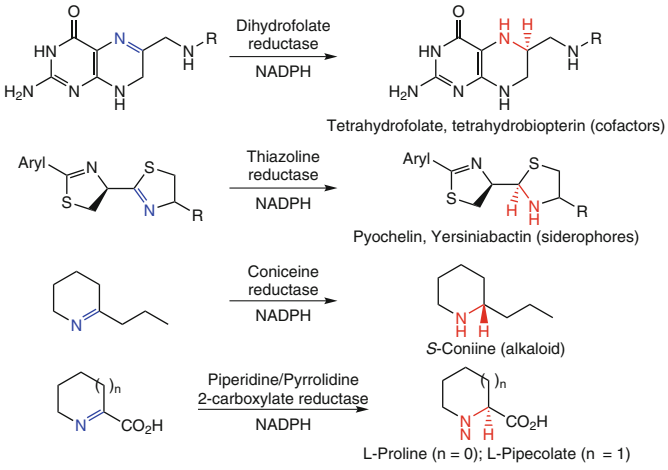
2.2.4 Reduction of C=N Bonds

According to recent estimates, chiral amine moieties are present in ~40% of active pharmaceutical ingredients and ~20% of agrochemicals [1046], and hence are

attractive targets for asymmetric synthesis. Established approaches for their preparation are the asymmetric addition of nucleophiles across imines (including the famous Mannich and Strecker reactions), asymmetric C-H amination and hydroamination and the asymmetric reduction of enamines and imines, which are either pre-formed or occur as intermediates in reductive amination [1047, 1048].

In this context, the asymmetric reduction of imines using NAD(P)H-dependent enzymes – imine reductases – represents an attractive option for biocatalysis [1049–1051]. This reaction can, in theory, give access to almost any *prim*-, *sec*- and *tert*-amine while the imine (or iminium) substrate is either pre-formed in a separate condensation step or is generated in-situ.

In Nature, C=N bond reduction is widespread and occurs in the biosynthesis of cofactors, most prominent 5,6,7,8-tetrahydrofolate and 5,6,7,8-dihydropterine (Scheme 2.129). The same holds for the reduction of a 2-thiazoline moiety in the biosynthesis of bacterial iron binding proteins (siderophores), for example Yersiniabactin. Furthermore, several alkaloids, such as coniine (from poisonous hemlock) and reticuline (from opium poppy) are derived via reduction of their imine precursors, as the cyclic amino acids L-pipecolate and L-proline are obtained from the corresponding Δ^1-imino-precursors. Although these NADPH-dependent C=N reductases are highly efficient, their substrate scope is very narrow and hence their importance lies in their physiological significance, rather than in their biocatalytic potential.

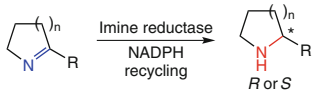


Scheme 2.129 Natural products derived via enzymatic C=N bond reduction

Consequently, the search for imine reductases of general applicability resorted to the use of whole microbial cells. In particular, yeasts [1052] (which have proven useful for carbonyl reduction) and bacteria [1053] were chosen in the early studies directed to the bioreduction of imines. Unfortunately, none of these proof-of-principle studies were investigated further, the responsible enzyme(s) were not identified, and some reports were not reproducible [1054].

Imines – and even more so iminium species – are rather reactive compounds due to their electrophilic character, which makes them susceptible to attack by a wide range of nucleophiles, including water. As a consequence, imines are notoriously unstable in aqueous systems at physiological conditions. The only notable exception are cyclic five- and six-membered imines and those bearing a carboxylic acid moiety on the imine carbon, which provides a stabilizing H-bond.

Imine reductases possessing a broad substrate tolerance were first identified from a large-scale screening encompassing 688 microbial strains including yeasts, bacteria, actinomycetes and fungi for their activity on 2-methyl-1-pyrroline as hydrolytically inert test substrate (Scheme 2.130, $n = 1$, R=CH_3) [1055]. Among all cultures tested, only five *Streptomyces* sp. turned out to be active, two of which showed satisfactory stereoselectivities, but with stereo-complementary behaviour: *Streptomyces* sp. GF3587 afforded (*R*)-2-methylpyrrolidine in 99% e.e. and strain GF3546 gave the (*S*)-enantiomer in 81% e.e. The responsible enzymes were purified, cloned and heterologously expressed [1056] and they constitute the first members of the now rapidly growing family of imine reductases. The substrate scope of both enzymes encompasses 5-, 6- and 7-membered cyclic imines, 3,4-dihydroisoquinolones and 3,4-dihydro-β-carbolines with excellent levels of stereoselectivity (Scheme 2.130). An α,β-unsaturated imine was chemoselectively reduced at the C=N bond and the alkene remained intact [1057, 1058]. In general, biotransformations using imine reductases have so far been carried out using resting cells of *E. coli* in which the respective imine reductase is heterologously expressed. This setup ensures NADPH recycling by the host cell through metabolism of glucose and overcomes the limited thermal stability of many imine reductases.



R	n	Imine reductase[a]	Conv. [%]	Config.	E.e. [%]
Me	1	GF3587	>98	*R*	>98
Me	1	GF3546	57	*S*	>95
Me	2	GF3587	>98	*R*	>98
Me	2	GF3546	>98	*S*	>98
Me	3	GF3587	>98	*R*	>98
Me	3	GF3546	>98	*S*	>98
n-Pr	2	GF3587	>98	*R*	>98
p-F-C_6H_4	2	GF3546	42	*R**	98
p-MeO-C_6H_4	2	GF3587	50	*S**	>98

[a] Whole cells of *E. coli* BL21 (DE3) containing (*R*)- or (*S*)-imine reductase from *Streptomyces* sp. GF3587 or GF3546, resp; * switch in CIP sequence priority.

Scheme 2.130 Asymmetric reduction of cyclic imines using stereocomplementary imine reductases

The discovery of the first imine reductases with a broad substrate scope resulted in identification of a broad range of additional enzymes via a sequence homology search. The hits have been used to generate an electronic library of several hundred putative imine reductases [1059]. Most imine reductases known to date originate from *Streptomyces*, but are also found in *Mycobacteria*, *Bacillus* and *Pseudonocardia* sp., and their physiological role and hence their natural substrate(s) are unknown. Although positively charged *N*-alkylated iminium species are reduced, electronically related carbonyl compounds are unreactive [1060]. Mechanistically, imine reductases do not possess a metal ion (such as Zn^{2+}) found in many alcohol dehydrogenases, but act through general acid-base catalysis. Imine reduction is assumed to proceed through hydride delivery from NAD(P)H onto the electrophilic imine carbon with concomitant protonation at N involving an Asp, His or Tyr residue acting as Brønsted acid to overcome the formation of an (energetically unfavourable) amide intermediate (R_2N^-). The large majority of imine reductases prefers NADPH as cofactor, only a few enzymes can use both nicotinamide species about equally well.

Reductive Amination of Ketones

In contrast to the reduction of hydrolytically stable cyclic imines which constitutes a viable protocol for the formation of cyclic *sec*-amines, open-chain imines derived from ketones and ammonia or short-chain *prim*-amines (e.g. methyl- or *n*-butylamine) are converted by imine reductases at low rates, which results in low to modest conversions (typically 50–70%) and requires high enzyme loadings [1061, 1062]. In addition, whole-cell preparations and crude cell lysates containing imine reductases are plagued with competing ketone reduction by alcohol dehydrogenases and the amine donor has to be employed in excess (typically ~50:1) to drive the equilibrium towards imine formation. Recently, imine reductases were identified which catalysed the reductive amination of cyclic, aliphatic and aromatic ketones (e.g. 2-hexanone or cyclohexanone) using ammonia and small aliphatic *prim*-amines (preferably methylamine) with encouraging results [1063].

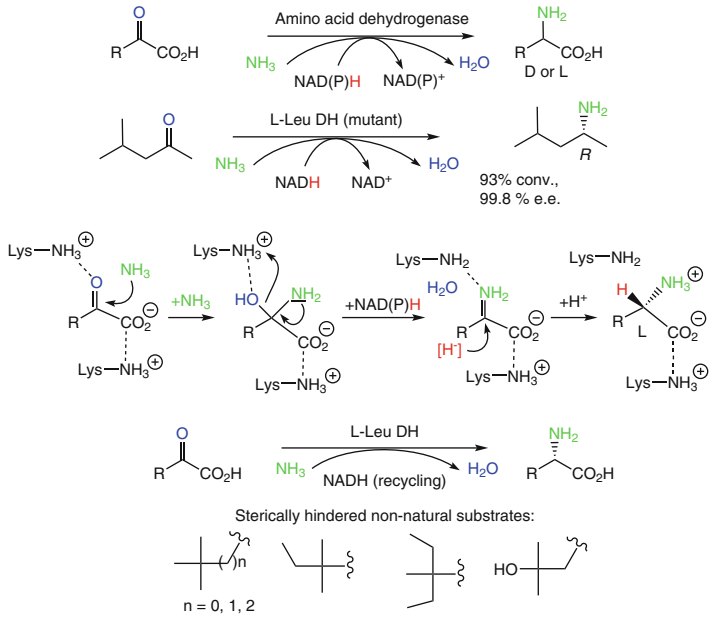
Reductive Amination of α-Ketocarboxylic Acids

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] [1064]. This reaction bears a strong resemblance to imine reduction and it formally represents a reductive amination (Scheme 2.131). A vast number of L-amino acid dehydrogenases from diverse organisms as well as variants engineered for industrial application has been described [1065–1068]. Stereo-complementary D-selective enzymes have been developed via protein engineering of *meso*-diaminopimelic acid D-dehydrogenases [1069, 1070] and they have been applied to the pilot-plant scale production of the non-natural amino acid D-5,5,5-trifluoromethylnorvaline [1071].

As deduced for L-Leu-dehydrogenase [1072], the α-ketoacid substrate is positioned in the active site between two Lys-residues (Scheme 2.131). 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. In contrast to imine reductases, amino acid dehydrogenases catalyse *both* imine formation and C=N bond reduction. Since this mechanism is highly tuned for α-keto/α-amino acids, it is clear that only ammonia is accepted as amine donor and a neutral imine (Schiff base) lacking the carboxylate moiety on the imine carbon atom cannot be accepted as substrate. Due to the importance of α-amino acids, both D- and L-amino acid dehydrogenases are important enzymes in industrial processes.

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 [1073, 1074]. 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. 2.1.2), 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 [1075]. In particular, L-*t*-leucine is a key intermediate for the synthesis of the HIV protease inhibitor Atazanavir.



Scheme 2.131 Reductive amination of α-ketocarboxylic acids using D- and L-amino acid dehydrogenases (*top*); reductive amination of a methyl ketone using a L-leucine dehydrogenase mutant (*center*); mechanism of L-leucine dehydrogenase

In analogy to α-amino acid dehydrogenases, nicotinamide-depending enzymes for the reductive amination of ketones lacking the carboxylate moiety would be termed 'amine dehydrogenases'. Since naturally occurring enzymes of this type are

unknown to date,[30] artificial amine dehydrogenases have been created by semi-rational protein design using existing α-amino acid dehydrogenase scaffolds. In a pioneering study, L-LeuDH from *Bacillus stearothermophilus* was chosen as starting point for directed evolution [1077]. A total of 19 amino acid residues was selected for mutagenesis including combinatorial active-site saturation (CAST). After several rounds of directed evolution, a quadruple variant (K68S, E114V, N261L, V291C) was obtained, which showed a reasonable specific activity of 0.69 U/mg in the reductive amination of 4-methyl-2-pentanone forming the corresponding (*R*)-amine in 99.8% e.e. Not surprisingly, two of the mutations introduced (K68S, N261L) involved those in binding of the natural substrate's carboxylate group. Their replacement by more unpolar amino acid residues completely abolished the restriction to α-amino/α-ketocarboxylic acids. This strategy was later successfully extended to L-phenylalanine dehydrogenase [1078].

2.2.5 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 [1079], stereocomplementary asymmetric *trans*-hydrogenation is less sophisticated [1080].

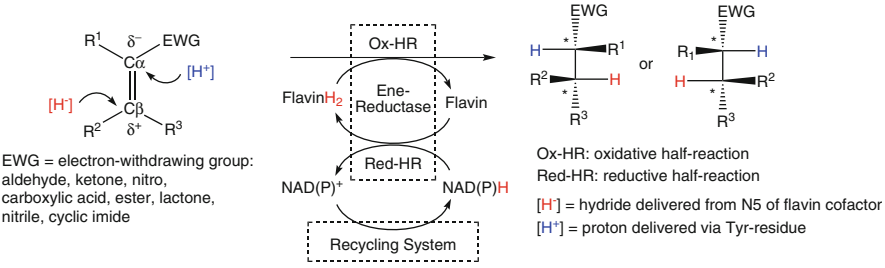
The biocatalytic counterpart for the stereoselective reduction of alkenes is catalyzed by flavin-dependent ene-reductases [EC 1.3.1.31], which are members of the 'old yellow enzyme' family (OYE, Scheme 2.132) [1081, 1082], first described in the 1930s by O. Warburg, who first demonstrated the requirement of a low molecular weight 'cofactor' for enzymatic catalysis [1083]. These enzymes are widely distributed in microorganisms and in plants. Some of them occur in well-defined pathways, e.g., in the biosynthesis of secondary metabolites, such as morphine [1084] and jasmonic acid [1085]. Others are involved in the detoxification of xenobiotics [1086], such as nitro esters [1087] and nitro-aromatics [1088] like trinitrotoluene (TNT) [1089]. *Ene*-reductases should not be confused with '*enoate* reductases',[31] which contain an Fe_4S_4-cluster in addition to flavin. These enzymes are found in strict anaerobic organisms and are very sensitive towards molecular oxygen, which makes them of limited use for preparative biotransformations [1090, 1091].

The catalytic mechanism of the asymmetric reduction of alkenes catalyzed by ene-reductases from the old yellow enzyme family has been studied in great detail

[30] For a rare exception see [1076].

[31] More precisely 2-enoate reductase. Since these enzymes belong to the same EC class 1.3.1.X, ene-reductases and enoate reductases are often confused.

[1092] 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 2.132). As a consequence of the stereochemistry of this mechanism, the overall addition of [H_2] proceeds in a *trans*-fashion with absolute stereospecificity [1093]. 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 redox reaction, which is employed for cofactor-recycling (Scheme 2.110). In contrast to alcohol dehydrogenases (carbonyl reductases), which show a rather pronounced preference for either NADH or NADPH [1094], ene-reductases are more flexible in this respect: some enzymes are very specific [1095], others are able to accept both cofactors equally well [1096, 1097]. 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 [1098]. Although the overall hydride pathway appears rather complex, practical problems are minimal since flavin cofactors are usually tightly bound to the enzyme and are thereby protected from the environment.



Scheme 2.132 Asymmetric bioreduction of activated alkenes using flavin-dependent ene-reductases

Although the remarkable synthetic potential of ene-reductases has been recognized long ago, preparative-scale applications were severely impeded by two major problems: Simple to use whole-cell systems, such as baker's yeasts [1099], and fungi, such as *Geotrichum candidum*, *Rhodotorula rubra*, *Beauveria bassiana* [1100] and *Aspergillus niger,* are plagued by undesired side reactions, particularly carbonyl reduction (catalyzed by alcohol dehydrogenases/carbonyl reductases) or ester hydrolysis (mediated by carboxyl ester hydrolases) [794]. On the other hand, the first generation of isolated (cloned) C=C bond reducing enzymes (enoate reductases) were obtained from (strict or facultative) anaerobes, such as *Clostridia* [1101] or methanogenic *Proteus* sp. [1102], 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 [1103–1111].

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 2.133) [1112], electronically 'isolated' double bonds are not accepted [1113]. In a rough approximation, the activating capability of an EWG goes in line with its electron-withdrawing strength. With activated, conjugated 1,3-dienes only the α,β-bond is selectively reduced, leaving the nonactivated γ,δ-bond behind (Scheme 2.133). 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 [1114].
- Acetylenic triple bonds yield the corresponding (*E*)-alkenes [1115]. The latter may be subject to further (slow) reduction.

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

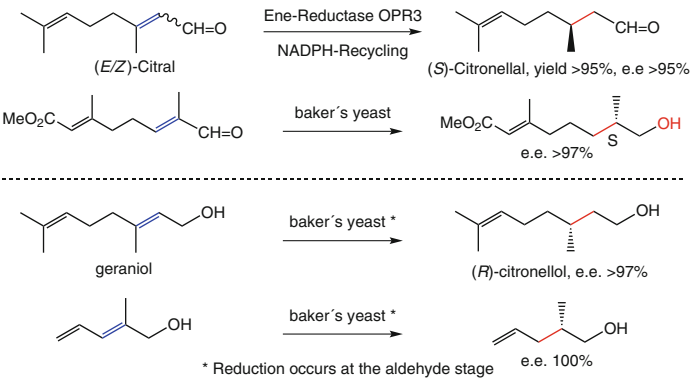
- α,β-Unsaturated carboxaldehydes (enals) are quickly reduced in a clean fashion yielding saturated aldehydes when pure ene-reductases are used. In contrast, whole-cell reductions are heavily plagued by competing carbonyl reduction, which often outcompetes the ene-reductase to furnish the corresponding allylic alcohol (thereby depleting the substrate) and/or the saturated *prim*-alcohol (via over-reduction of the desired product) [1116, 1117]. These undesired side-reactions sometimes allow to use an allylic alcohol as substrate, which is transformed via the corresponding enal by whole cells [1118] (Scheme 2.133).
- α,β-Unsaturated ketones (enones) 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 [1119, 1120] (Scheme 2.134).
- α,β-Unsaturated nitro compounds can be readily transformed into chiral nitroalkanes. Depending on the type of OYE, reductive biodegradation may occur via the Nef-pathway [1121]. Due to the high acidity of nitroalkanes, any chiral center at Cα is prone to racemization, whereas Cβ-analogs are perfectly stable [1122] (Scheme 2.135).
- Cyclic imides, such as maleimide, 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 *mono*-esters are not readily reduced by OYEs (they are substrates for 'enoate-reductases'). However, the presence of an additional electron-withdrawing group (which alone would not be sufficient to act as activator), such as halogen, helps to boost the degree of activation [1123] (Scheme 2.136). Consequently, *di*-carboxylic acids and *di*-esters are accepted by OYEs, although ester hydrolysis is a common side-reaction when using whole cells. Due to their reduced carbonyl activity, carboxylic acids are less activated than the corresponding esters.

- Only few reports are available regarding α,β-unsaturated lactones, their degree of activation parallels that of esters.
- α,β-Unsaturated nitriles may be used as substrates [1124].
- Sometimes the absolute (*R*/*S*)-configuration of the product can be controlled by starting with (*E*)- or (*Z*)-alkenes (Scheme 2.136) [1125].
- 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 2.133). The latter is a central intermediate for various terpenoid odorants, such as citronellyl nitrile and menthol.

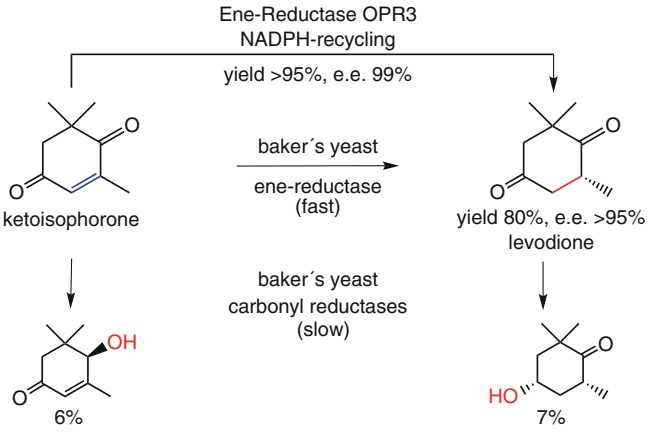
In contrast, baker's yeast reduction of a closely related enal bearing a carboxylic ester group yielded the saturated *prim*-alcohol as the major product due to over-reduction of the aldehyde moiety. The less activated C=C bond adjacent to the ester remained unchanged [1126]. Instead of starting with an enal (whose aldehyde moiety would be quickly reduced by baker's yeast) the corresponding allylic alcohol may serve as substrate in whole-cell bioreductions. Thus, geraniol gave (*R*)-citronellol in >97% e.e. [1127] and in a similar fashion only the α,β-bond was reduced in a conjugated 2,4-diene-1-ol [1128]. 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 2.133).



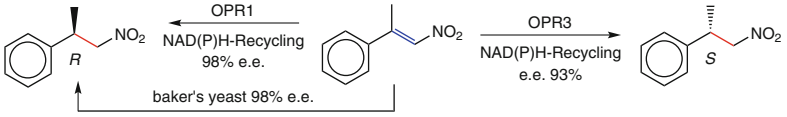
Scheme 2.133 Asymmetric bioreduction of enals and allylic alcohols using isolated ene-reductase and baker's yeast

In contrast to aldehydes, over-reduction is less pronounced on α,β-unsaturated ketones (Scheme 2.134). 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 reduction of the carbonyl moieties were formed in minor amounts [1129]. In contrast, no trace of carbonyl reduction was observed using ene-reductase OPR3 (Scheme 2.134). (3*R*,3′*R*)-Zeaxanthin is used in human nutrition and healthcare products and is considered for the treatment of age-related macula degeneration [1130].

Nitro-olefins are readily reduced by ene-reductases to form chiral nitro-alkanes (Scheme 2.135) [1131]. 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 2.134 Asymmetric bioreduction of α,β-unsaturated diketone



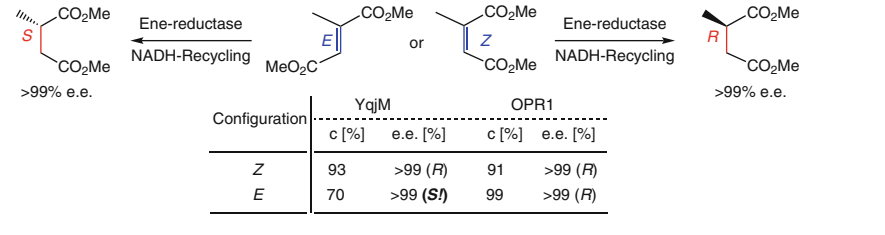
Scheme 2.135 Stereocomplementary bioreduction of nitro-olefins

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 2.136). 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 substrate: (*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

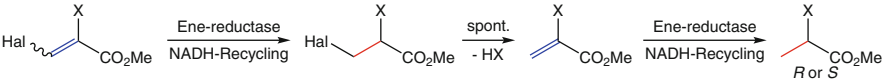
of a (*Z*)-maleate [1132]. This bioreduction was upscaled to 70g batch size with subsequent in situ ester hydrolysis [1133].

The suitability of β-substituted α-haloacrylate esters as substrates for ene-reductases was first proven using baker's yeast and the absolute configuration of the product was shown to depend on the (*E*/*Z*)-configuration of the substrate [1134]. While the chiral recognition of the (*Z*)-alkenes was perfect, the (*E*)-isomers gave products with lower e.e. and 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 [1135].

Undesired ester hydrolysis can be avoided when isolated ene-reductases are used. In this case, excellent results were achieved with methyl α-haloacrylates, which were reduced to (*R*)- or (*S*)-α-halopropionates in a stereo-divergent fashion depending on the ene-reductase employed [1136]. In contrast, halogen atoms in the β-position proved to be unsuitable. Although α,β-dihalo derivatives were rapidly reduced, the saturated 2,3-dihalo esters thus formed spontaneously underwent HX-elimination yielding an α-haloacrylate ester. Overall, this sequence consists of a (formal) reductive β-dehalogenation (Scheme 2.136) [1137].



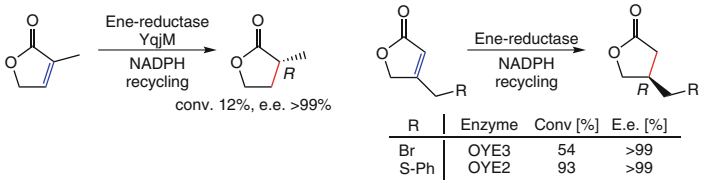
Configuration	YqjM		OPR1	
	c [%]	e.e. [%]	c [%]	e.e. [%]
Z	93	>99 (*R*)	91	>99 (*R*)
E	70	>99 (***S!***)	99	>99 (*R*)



X	Enzyme	Conv. [%]	E.e. [%]
Cl	OYE3	>99	89 (*S*)
	NCR	50	>99 (*R*)
Br	OYE3	>99	95 (*S*)
	NCR	>99	99 (*R*)
I	OYE1	54	90 (*S*)
	OPR1	5	40 (*R*)

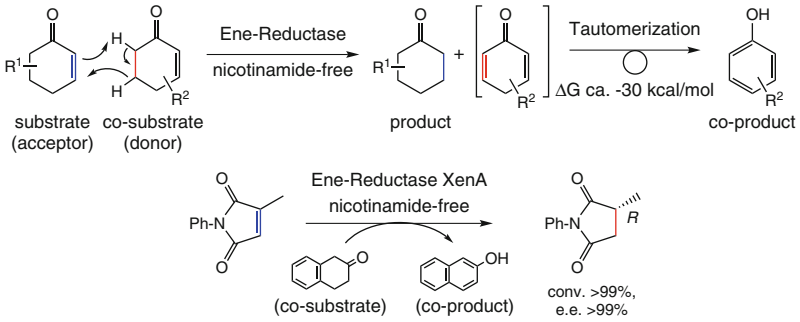
Scheme 2.136 Stereocontrol of ene-reduction via enzyme-type or substrate-configuration and reduction of haloacrylate esters

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 [1138]. More recent investigations using isolated ene-reductases revealed that butyrolactones bearing an α-substituent were reduced with excellent stereoselectivity, albeit at a slow rate. In contrast, β-substituted analogs were converted considerably faster. A chiral center in γ-substituted butyrolactones was nicely recognized and led to kinetic resolution with E-values of up to E = 49 (Scheme 2.137) [1139].



Scheme 2.137 Asymmetric bioreduction of α,β-unsaturated butyrolactones

The reverse reaction catalyzed by ene-reductases – α,β-desaturation of carbonyl compounds – is thermodynamically strongly disfavored because it involves the breakage of two C-H σ-bonds, which are not energetically compensated for by the newly formed C=C π-bond. Hence it is not feasible using $NAD(P)^+$ as driving force in a coupled-enzyme system (Scheme 2.132). However, it was shown to occur in the disproportionation of enones, which has been described as 'dismutase' or 'aromatase' activity of OYEs (Scheme 2.138) [1140]. While one enone molecule is reduced, the other is desaturated yielding the corresponding dienone, which quickly tautomerizes to form a phenol, thereby providing a large driving force of about −30 kcal/M, which drives the reaction to completion. Overall, this redox-neutral process is independent on nicotinamide cofactors, requires only a single ene-reductase and represents a coupled-substrate variant for C=C bioreduction. This method has been exploited using sacrificial hydrogen donors, such as cyclohex-2-enones, 2-tetralone and five-membered heterocyclic 3-ones as sacrificial hydrogen donors [1141].



Scheme 2.138 Disproportionation of enones and nicotinamide-independent hydrogen transfer

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.[32] Traditional methodology is plagued by several drawbacks, that is

[32]It is an alarming fact that ≥90% of the hydrocarbons derived from crude oil are 'wasted' for energy-production (forming CO_2), the small remainder of ≤10% is used as raw material for the chemical industry to produce (long-lasting) products [1142].

- Many oxidants are based on metal ions such as copper, manganese, iron, nickel, or chromium, which are often environmentally incompatible when used on large scale.
- 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 environmental problems 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 [1143–1145].

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)
- Oxidation and oxygenation at the expense of molecular oxygen (Sect. 2.3.1, 2.3.2 and 2.3.3)
- Peroxidation reactions requiring hydrogen peroxide or a derivative thereof (Sect. 2.3.4)

For a classification of biooxidation reactions see Scheme 2.144.

2.3.1 Oxidation of Alcohols and Aldehydes

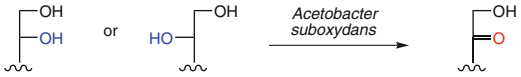
Oxidations of primary and secondary alcohols to furnish aldehydes and ketones, respectively, are common chemical reactions that rarely present insurmountable problems to the synthetic organic chemist. These reactions can be catalysed by alcohol dehydrogenases together with $NAD(P)^+$-recycling. However, in contrast to the corresponding (carbonyl) reduction reactions, alcohol oxidation using dehydrogenases have been reported to a lesser extent for the following reasons [1146]:

- Oxidations of alcohols using $NAD(P)^+$-dependent dehydrogenases are thermodynamically unfavorable. Thus, the recycling of the oxidized nicotinamide cofactor becomes a complicated issue (see Scheme 2.111).
- Enzymatic oxidations usually work best at elevated pH (8–9) where nicotinamide cofactors and (particularly aldehydic) products are unstable.
- Lipophilic aldehydes or ketones are often more tightly bound onto the hydrophobic active site of dehydrogenases than the more hydrophilic substrate alcohol. Hence, product inhibition is a common phenomenon, in particular when reactive aldehydes are involved [846].

- 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 2.139) [1147–1151]. Such compounds present selectivity-problems with conventional chemical oxidants, which requires protection–deprotection steps. In contrast, numerous sugars and related polyhydroxy compounds obtained from renewable resources have been selectively oxidized in a single step into the corresponding keto-ols or ketoacids using a variety of microorganisms, for example the vinegar-producing bacterium *Acetobacter* (Scheme 2.139). The regioselective microbial oxidation of D-sorbitol (obtained by catalytic hydrogenation of D-glucose) by *Acetobacter 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	References
adonitol	L-adonulose	[1144]
D-sorbitol	L-sorbose	[1145]
L-fucitol	4-keto-L-fucose	[1146]
D-gluconic acid	5-keto-D-gluconic acid	[1147]
1-deoxy-D-sorbitol	6-deoxy-L-sorbose	[1148]

Scheme 2.139 Regioselective oxidation of polyols by *Acetobacter suboxydans*

Kinetic Resolution and Desymmetrization of Alcohols by Oxidation

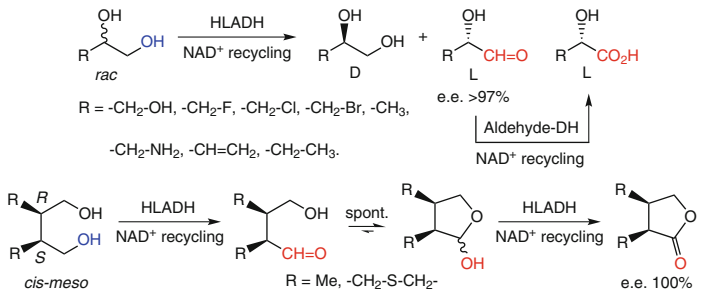
Among the enzymatic systems for $NAD(P)^+$-recycling described in Scheme 2.111, the use of a flavin mononucleotide (FMN) dependent nicotinamide oxidase is preferable, because it requires only molecular oxygen and is virtually irreversible [1152]. To avoid enzyme deactivation, the hydrogen peroxide produced during this two-electron transfer process is removed using catalase [1153]. In conjunction with HLADH, this system was employed for the kinetic resolution of mono-, bi-, and polycyclic secondary alcohols [873, 875, 1154, 1155]. Alcohols bearing an electron-withdrawing group (e.h. halogen, MeO, etc.) in the α-position form a strong internal H-bond and are difficult to oxidize by this method [1156].

Terminal glycols were regio- and enantioselectively oxidized at their *prim*-hydroxy group to yield L-α-hydroxyacids using a co-immobilized alcohol and aldehyde dehydrogenase system (Scheme 2.140). In the first step, kinetic resolution of the diol furnished a mixture of L-hydroxyaldehyde and the remaining D-diol. In order to avoid enzyme deactivation by the aldehyde species, it was oxidized in-situ by an aldehyde dehydrogenase to yield the more innocuous L-hydroxyacid in high

optical purity [878, 1157]. An analogous double-step oxidation of *prim*-alcohols yielding carboxylic acids using a single ADH variant was recently reported [1158].

In contrast to the resolution of secondary alcohols, where the more simple lipase technology is recommended instead of a redox reaction, desymmetrization of prochiral or *meso-prim*-diols is a valuable method for the synthesis of chiral lactones (Scheme 2.140) [1159].

As a rule of thumb, oxidation of the (*S*)- or pro-(*S*)-hydroxyl group occurs selectively with HLADH. 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 by maintaining (*S*)- or pro-(*S*) specificity [1160]. Both steps – desymmetrization of the prochiral or *meso*-diol and kinetic resolution of the intermediate lactol – are often highly stereoselective. Enantiopure lactones were derived from *cis-meso*-2,3-dimethylbutane-1,4-diol and the cyclic thia-analog [1161] and similar results were obtained with sterically demanding bicyclic *meso*-diols [1162].

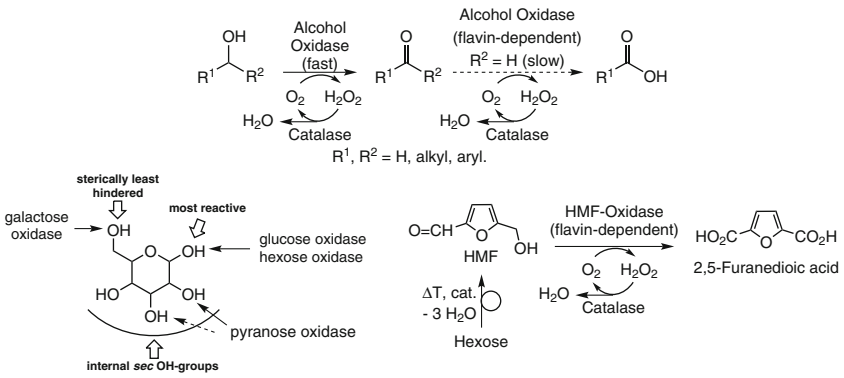


Scheme 2.140 Kinetic resolution of 1,2-diols and desymmetrization of *meso*-diols by a HLADH/aldehyde DH system

The issues of NAD(P)$^+$-recycling can be circumvented by using nicotinamide-independent alcohol oxidases [1163]. These enzymes are either metal- (Cu, Fe) or flavin-dependent and catalyse the oxidation of *prim*- and *sec*-alcohols at the expense of O_2 as oxidant by producing H_2O_2 as by-product, which is usually destroyed by catalase.[33] With *sec*-alcohols, the reaction stops at the ketone stage, but 'over-oxidation' of aldehydes obtained from *prim*-alcohols yields carboxylic acids (Scheme 2.141, top). This activity is often observed with flavin-depending alcohol oxidases. For synthetic applications, alcohol oxidation is a crucial step in the functionalization of carbohydrates and polyols, because it yields aldehydes or ketones, which are excellent acceptors for *C*-, *N*-, *O*- and *S*-nucleophiles. Thereby, a given carbon backbone derived from natural resources can be conveniently extended.

[33]Many *E. coli* strains, which are used as host for the heterologous overexpression of alcohol oxidases, contain a strong native catalase activity.

Fortunately, Nature provides a broad arsenal of alcohol oxidases acting on hexoses (Scheme 2.141 bottom) [1164]: Oxidation of the most reactive anomeric hydroxyl group by glucose or hexose oxidase yields the corresponding lactone, while galactose oxidase selectively oxidizes the *prim*-OH to the aldehyde moiety, pyranose oxidase predominantly forms 2-ketoses. Of particular interest is a recently discovered flavin-dependent 5-hydroxymethyl furfural oxidase (HMF oxidase), which converts HMF via a three-step sequence to furan-2,5-dioic acid (FDC) [1165]. HMF is obtained on multi-ton scale via acid-catalyzed thermal triple dehydration of hexoses and FDC is a promising replacement for (fossil-derived) terephthalic acid, ~50 mio t of which is annualy converted into PET-polymers.



Scheme 2.141 Oxidation of alcohols and aldehydes using alcohol oxidases

2.3.2 *Oxidation of Amines*

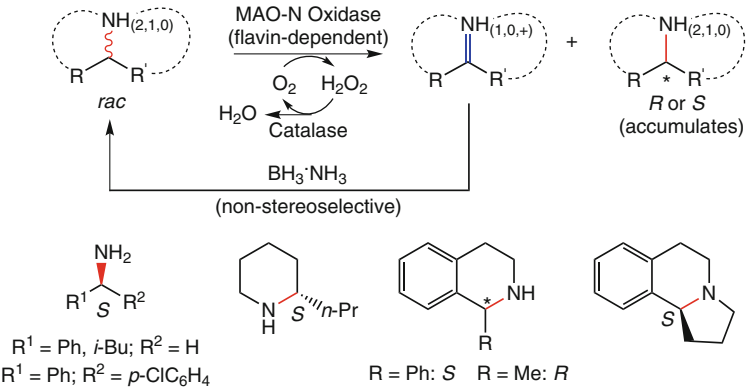
In close analogy to the oxidation of alcohols using alcohol oxidases, amines can be oxidized by imine oxidases at the expense of O_2 with concomitant production of H_2O_2. The main biochemical role of enzymes from microbial origin is the oxidative degradation of amines, which provides essential NH_3 for assimilation and growth. In particular, the flavin-dependent monoamine oxidase from *Aspergillus niger* (MAO-N) has served as platform for the development of numerous mutants, which oxidize *prim*-, *sec*- and *tert*-amines with high stereoselectivities [1166–1169].

In contrast to alcohol oxidation, which furnishes stable carbonyl compounds, amine oxidation yields unstable imines, which cannot be easily isolated and stored. However, two ingenious strategies have been deployed to render amine oxidation as a synthetically useful tool.

Cyclic Deracemization of Amines

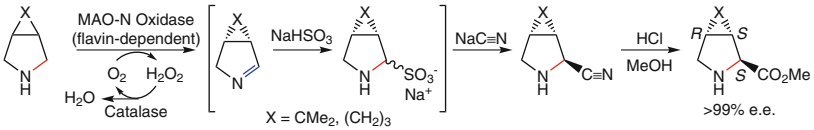
Enantioselective oxidation of an amine bearing an adjacent chiral C atom by an amine oxidase yields the corresponding achiral imine, while the non-converted enantiomer remains untouched. In order to overcome the 50% limit of kinetic resolution, the imine can be (non-stereoselectively) reduced in situ using a mild reducing agent, such as amine-borane complex, which yields an equimolar amount

of 25% amine enantiomers. Hence, after a single oxidation-reduction cycle, the entiomeric composition of the starting amine is 75:25, another cycle renders 87.5:12.5 and so forth (Scheme 2.142). Overall, the reacting enantiomer is gradually depleted while the non-reacting counterpart accumulates. Although this process – termed cyclic deracemization [1170] – lacks elegance at a quick glance, it furnishes an e.e. of 93.4 e.e. after only four cycles, whereas after seven cycles the e.e. is >99%. Starting from wild-type MAO-N, which is able to oxidize only simple primary amines, a panel of variants was developed through several rounds of random mutagenesis and directed evolution combined with rational design, which accept also *sec*- and *tert*-amines [1171].



Scheme 2.142 Cyclic deracemization of amines using monoamine oxidase (variants) combined with non-stereoselective imine reduction; accumulated product enantiomers are shown

Desymmetrization of cyclic *sec*-amines.
Asymmetric oxidation of (bi)cyclic *sec*-amines by MAO-N breaks the symmetry of these *meso*-structures and yields chiral imines. Being good electrophiles, the latter can be trapped in-situ as bisulfite adducts, which are directly converted into the corresponding α-aminonitriles with high *trans*-diastereoselectivity (30:1 to 100:1) by treatment with NaCN. Acid catalyzed methanolysis in a Strecker-like protocol yields the corresponding methyl carboxylate in 88% overall yield and >99% e.e. Non-natural mono-, bi- and tricyclic L-proline analogs of this type are key building blocks for the synthesis of peptidomimetic protease inhibitors, such as Boceprevir and Telaprevir, which are used for the treatment of chronic Hepatitis C (Scheme 2.143) [1172].



Scheme 2.143 Asymmetric oxidation of bicyclic *sec*-amines followed by in-situ trapping of imine and follow-up transformations

2.3.3 *Oxygenation Reactions*

Enzymes which catalyze the direct incorporation of molecular oxygen into an organic molecule are called '*oxygen*ases' [1173–1176]. 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 at the expense of O_2 into more valuable intermediate products, such as alcohols, aldehydes, ketones and carboxylic acids.[34] 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 on lab-scale for a wider range of organic compounds, except for the Wacker-oxidation of alkenes. Unsurmountable problems persist where regio- or enantiospecificity is desired.

The appealing use of O_2 as zero-priced oxidant comes with some drawbacks: Its triplet ground state makes it kinetically unreactive with organic molecules, which are in the singlet state, and it tends to form radical species, which are difficult to control and tend to cause side reactions. Furthermore, it is a four-electron oxidant, which makes it difficult to terminate an oxidation process at an intermediate stage, leading to 'over-oxidation'. However, nature has managed to tame O_2 to a remarkable extent and several highly selective oxygenation reactions may be achieved by means of biocatalysts.

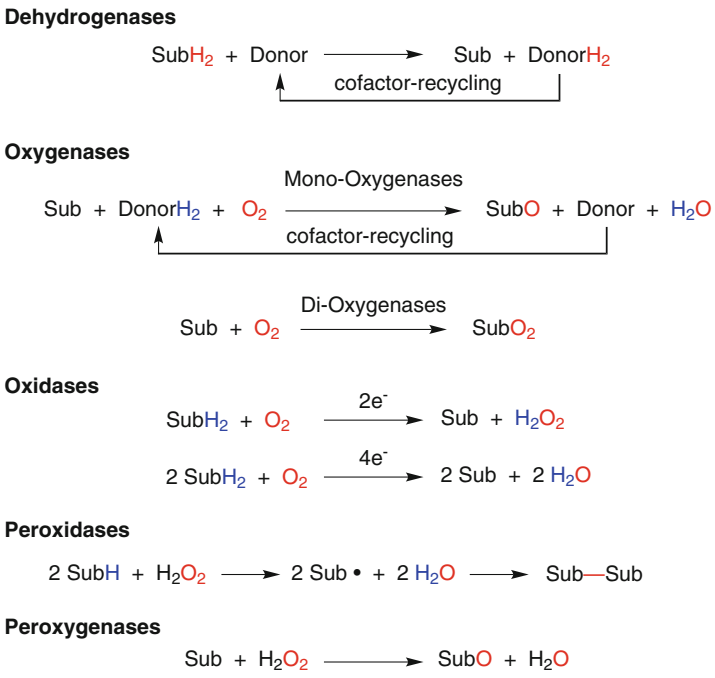
Oxygen-transfer from molecular oxygen into organic acceptor molecules may proceed through three different mechanisms (Scheme 2.144).

- 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 [1177–1179]. Overall this is a four-electron transfer, comprising two electrons each from the substrate and the cofactor.
- Dioxygenases simultaneously incorporate *both* oxygen atoms of O_2 into the substrate by forming a peroxy-species in a two-electron transfer.[35]
- Oxidases catalyze electron-transfer onto molecular oxygen, which proceeds via a two- or (more rarely) a four-electron transfer yielding either hydrogen peroxide or water, respectively, as byproduct. Incorporation of O into the substrate does

[34]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.

[35]Although they are redox enzymes belonging to EC class 1, occasionally they have been misleadingly called 'oxygen transferases'.

not occur. Oxidases include flavoprotein oxidases (such as alcohol, amine and nicotinamide oxidases), metallo-flavin oxidases (aldehyde oxidase) and heme-protein oxidases (catalase, H_2O_2-specific peroxidases [1180]).
- In a related fashion, per*oxidases* catalyze two-electron oxidations at the expense of hydrogen peroxide forming water.
- Per*oxygenases* incorporate O into a substrate from H_2O_2.

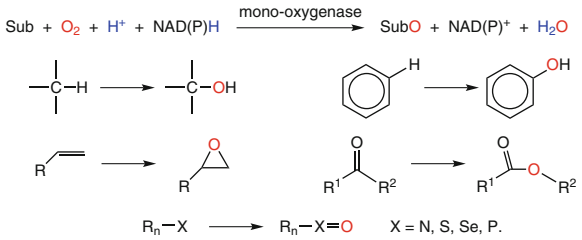


Scheme 2.144 Systematics of enzymatic oxidation reactions (donor = nicotinamide)

Monooxygenases

Although the reaction mechanisms of various monooxygenases differ greatly depending on the subtype of enzyme, their mode of oxygen-transfer 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.

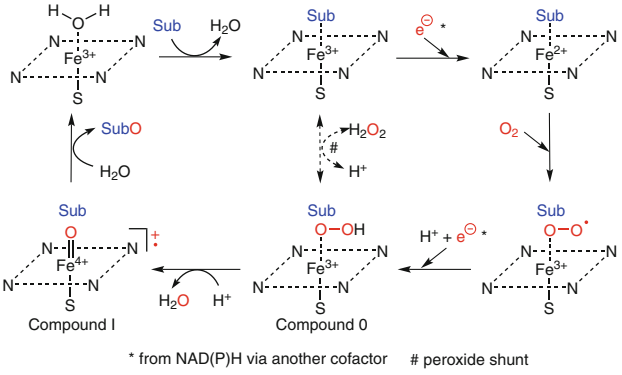


Substrate	Product	Type of Reaction	Type of Cofactor
alkane	alcohol	hydroxylation	metal-dependent
aromatic	phenol	hydroxylation	metal-dependent
alkene	epoxide	epoxidation	metal-dependent
heteroatom[a]	heteroatom-oxide	heteroatom oxidation	flavin-dependent
ketone	ester/lactone	Baeyer-Villiger	flavin-dependent

[a] N, S, Se or P.

Scheme 2.145 Monooxygenase catalyzed reactions and their typical (but not exclusive) cofactor-dependence

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 [1181] or flavin [1182–1184]). The catalytic cycle of the iron-depending monooxygenases, the majority of which belong to the cytochrome P-450 type (Cyt P-450) [1185–1189], has been deduced largely from studies on the camphor hydroxylase of *Pseudomonas putida* [1190, 1191]. A summary of the catalytic cycle is depicted in Scheme 2.146.



Scheme 2.146 Catalytic cycle of cytochrome P-450-dependent monooxygenases

The iron species is coordinated equatorially by a heme moiety and axially by the sulfur atom of a cysteine residue. Catalysis occurs in the remaining sixth coordination site. After binding of the substrate (Sub) by replacing a water molecule in a hydrophobic pocket adjacent to the porphine [1192], the iron is reduced to the

ferrous state ($Fe^{3+} \rightarrow Fe^{2+}$). The single electron is delivered from NAD(P)H via another cofactor, which (depending on the enzyme) is a flavin, 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 and protonation forms Compound 0. Protonation cleaves the O–OH bond with expulsion of water and forms the ultimate oxidizing Fe^{4+}=O species called Compound I, which – as a strong electrophile – attacks the substrate [1193]. Expulsion of the product (SubO) reforms the Fe^{3+} species and closes the catalytic cycle. Put simply, Cyt P-450 resembles an oxidation by a hypervalent transition metal oxidant (nature's permanganate).

Despite the fact that the mechanism of Cyt P-450 enzymes has been intensively investigated over half a century [1194], many mechanistic details are still poorly understood and it was only recently, that the existence of an Fe^{5+} species was ruled out [1195].

Aside from the productive cycle, Compound 0 can be formed directly by H_2O_2 through the so-called 'peroxide-shunt'. This obviates the necessity for additional electron-transport components described above, because no single-electron transfer occurs. However, so far, the use of P-450 enzymes in the peroxygenase-mode is impeded by limited enzyme stabilities in presence of H_2O_2 [1196]. Under certain conditions, Compound 0 may liberate H_2O_2, or Compound I may decompose forming H_2O, which wastes NAD(P)H in futile cycles, processes which are called 'uncoupling'. Hence, it is not surprising, that P-450 enzymes are comparatively slow catalysts with typical TOFs of ~1 s^{-1}.

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 200,000 distinct Cyt P-450 enzymes are known and these 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 [1197]. A simplified schematic organization of Cyt P-450 systems is depicted in Fig. 2.17.

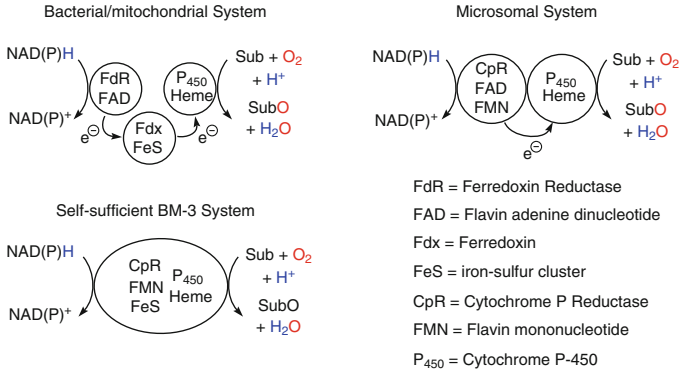
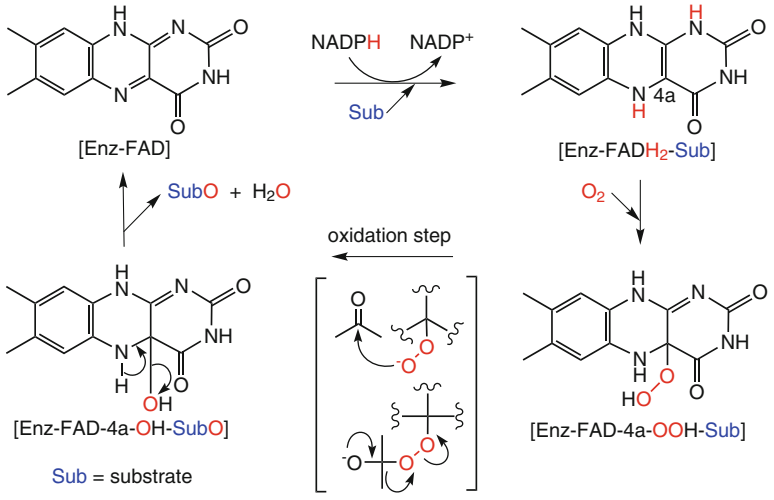


Fig. 2.17 Schematic organization and electron-transport of cytochrome P-450 monooxygenases

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 [1198]. 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. The minimal Cyt P-450 system BM-3 is derived from *Bacillus megaterium* 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 for its simplicity the latter system has been the prime target of studies directed towards the development of enzymatic oxygenation systems for preparative-scale applications [1199–1201].

Due to their inherent complexity of the electron-transport chain [1202], Cyt P450 monooxygenase-catalyzed systems are generally employed as whole microbial host cells ('designer bugs'), which co-express all the required proteins, including those required for NAD(P)H-recycling, in particular when applied to large-scale reactions [1203–1205].

In contrast, flavin-dependent monooxygenases (see Scheme 2.147 and Table of Scheme 2.145) use a different mechanism which involves a flavin cofactor [1206–1208]. First, NADPH reduces the Enz-FAD complex thereby breaking its aromaticity. The $FADH_2$ so formed is oxidized by molecular oxygen via Michael-type addition yielding a hydroperoxide (FAD-4a-OOH) or peroxyflavin-species (FAD-4a-OO^-), depending on its protonation state, which is determined by the molecular environment of the enzyme's active site. The latter can either perform an electrophilic or nucleophilic oxidation, such as alkene epoxidation or Baeyer-Villiger oxidations, respectively [1209]. In contrast to P-450 enzymes, flavin-dependent monoxygenases show negligible uncoupling.



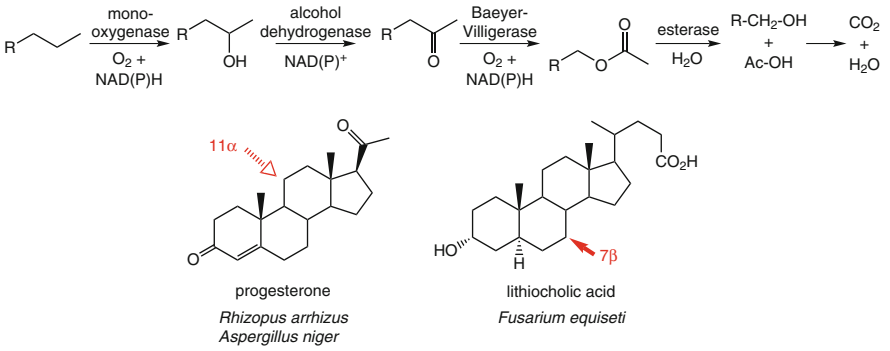
Scheme 2.147 Catalytic cycle of flavin-dependent monooxygenases

In Baeyer-Villiger oxidations, the peroxy-anion performs a nucleophilic attack on the carbonyl group of the aldehyde or ketone substrate. 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 also mediate the hydroxylation of aromatics [1210–1214], alkene epoxidation [1215, 1216] and heteroatom-oxidation, desaturation and oxidative C–C coupling [1217–1219]. Put simply, the FAD-OOH species resembles nature's *m*-chloroperbenzoic acid and the mechanism parallels the oxidation of organic compounds by peroxides or peracids [1220]. Flavin-dependent monooxygenases, such as Baeyer-Villigerases, can be used in isolated form together with a suitable nicotinamide-cofactor recycling system.

2.3.3.1 Hydroxylation of Alkanes

The hydroxylation of nonactivated centers in hydrocarbons is one of the most useful biotransformations [1175, 1221–1226] due to the fact that this process has only very few counterparts in traditional organic synthesis [1227–1229]. In general, the relative reactivity of carbon atoms in bio-hydroxylation reactions declines in the order of secondary > tertiary > primary [1230], which is in contrast to radical reactions (tertiary > secondary > primary) [1231].

Straight-chain hydrocarbons are preferably hydroxylated at the (ω-1)-position, and with whole cells, the corresponding 2-alkanol is quickly oxidized to the corresponding methyl ketone, which further undergoes Baeyer-Villiger oxidation forming the corresponding *prim*-acetate ester. The latter is hydrolysed to acetate and a *prim*-alcohol, both of which are channeled into the β-oxidation pathway yielding CO_2 and water (Scheme 2.148, top). Due to the inability to stop this metabolic pathway for *n*-alkanes at a certain intermediate stage, is of no practical value for synthetic biotransformations, but it takes care for the bioremediation of oil spills in the environment.

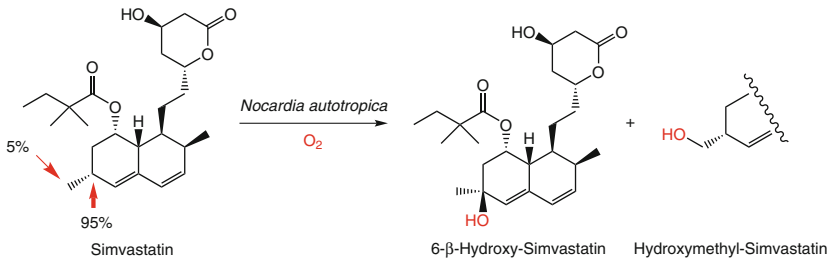


Scheme 2.148 Regio- and stereoselective microbial hydroxylation of steroids

In contrast, there are two main groups of hydrocarbon molecules, which can be efficiently transformed by microbial hydroxylation – *steroids* and *terpenoids*. Their common property is that they possess a large main framework, which impedes the metabolic degradation of their hydroxylated products.

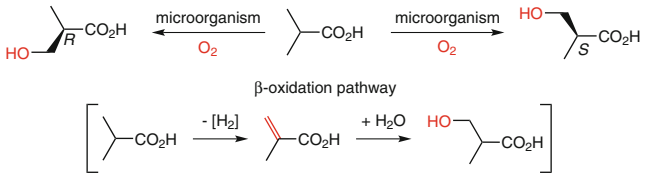
Intense research on the stereoselective hydroxylation of alkanes started in the late 1940s in the steroid field, driven by the demand for pharmaceuticals [1232–1237]. In the meantime, some of the hydroxylation processes, e.g., 9α- and 16α-hydroxylation of the steroid framework [1238, 1239], have been developed to the scale of industrial production. Nowadays, virtually any center in a steroid can be selectively hydroxylated by choosing the appropriate microorganism, for a comprehensive list see [1239]. For example, hydroxylation of progesterone in the 11α-position by *Rhizopus arrhizus* [1240] or *Aspergillus niger* [1241] 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, bottom). A highly selective hydroxylation of lithiocholic acid in position 7β was achieved by using *Fusarium equiseti* [1242]. The product (ursodeoxycholic acid) is capable of dissolving cholesterol and thus can be used in the therapy of gallstones.

In search for new drugs, active pharmaceutical ingredients (APIs) are often 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 [1243]. 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 [1244]), fragrance components (muscone [1245]) and antibiotics (calcimycin [1246]). Both enantiomers may be obtained by asymmetric hydroxylation of *iso*-butyric acid [1247, 1248] (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.

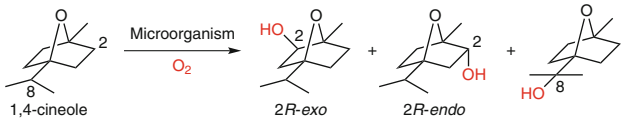


Microorganism	Configuration	e.e. [%]
Pseudomonas putida ATCC 21244	*S*	>95
Candida rugosa IFO 750	*S*	99
Candida rugosa IFO 1542	*R*	97

Scheme 2.150 Asymmetric microbial hydroxylation of *iso*butyric acid via the β-oxidation pathway

Although the mechanism of this reaction was initially assumed to be a 'direct' hydroxylation at position β, detailed studies showed that it proceeds via the conventional β-oxidation pathway involved in fatty acid metabolism [1249]. An analogous sequence is the basis for the transformation of 4-trimethylammonium butanoate to the β-hydroxy derivative carnitine (compare Scheme 2.203) [1250].

The production of new olfactory compounds for the aroma and fragrance industry was the powerful driving force in the research on the hydroxylation of terpenes [1008, 1251–1253]. 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) [1254]. 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 [1255].



Microorganism	e.e. [%] *exo*	e.e. [%] *endo*	*exo/endo* ratio
Streptomyces griseus	46	74	1:1.7[a]
Bacillus cereus	94	94	7:1

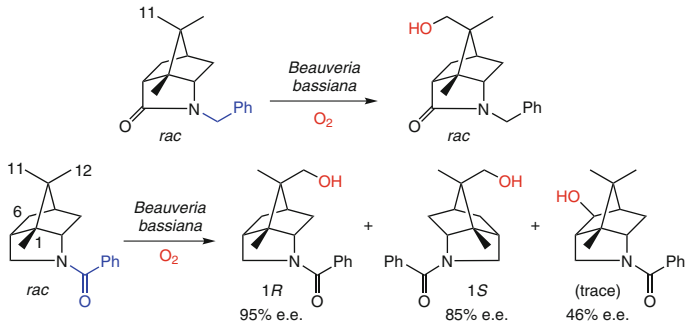
[a] 8-Hydroxycineole was the major product.

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 been more often used than bacteria. Among them, the fungus *Beauveria bassiana* ATCC 7159 (formerly denoted as *B. sulfurescens*) has been studied most thoroughly [1256–1260]. In general the presence of a polar group in the substrate such as an acetamide, benzamide or *p*-toluene-sulfonamide moiety proved to be advantageous

in order to firmly bind the substrate in the active site [1261]. Hydroxylation occurs at a distance of 3.3–6.2 Å from the polar anchor group. With cycloalkane rings of different size, 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 sterically more accessible 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 [1262]. 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 [1263, 1264] and a substrate model containing a polar anchor group were developed [1265].

In summary, (bio)hydroxylation of sterically demanding hydrocarbon compounds is feasible 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:

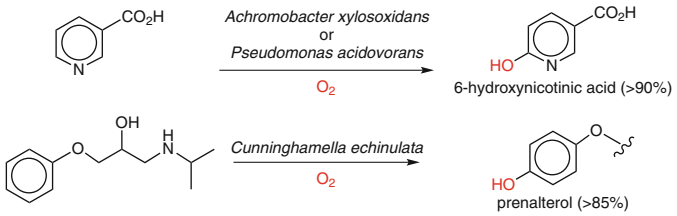
- Broad screening of different strains[36]
- Substrate modification, particularly by introduction of a polar anchor group [1266–1269]
- Variation of the culture by stressing the metabolism of the cells

[36]The following strains have been used more frequently: *Aspergillus niger*, *Cunninghamella blakesleeana*, *Bacillus megaterium*, *Bacillus cereus*, *Mucor plumbeus*, *Mortierella alpina*, *Curvularia lunata*, *Helminthosporium sativum*, *Pseudomonas putida*, *Rhizopus arrhizus*, *Rhizopus nigricans*, *Beauveria bassiana*.

2.3.3.2 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 [1270, 1271], but some of them are explosive and byproducts are usually obtained [1272]. 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 for electronic reasons [1273]. 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 2.83) [1274]. Rearrangement of the latter involving the migration of a hydride anion (NIH-shift) forms the phenolic product [1275].

Like steroids and terpenes, aromatic compounds are regioselectively hydroxylated by using whole cells [1276–1280]. For instance, 6-hydroxynicotinic acid is produced from nicotinic acid by *Pseudomonas acidovorans* or *Achromobacter xylosoxidans* on a scale of 20 t/a [1281]. Racemic prenalterol, a compound with important pharmacological activity as a β-blocker, was obtained by regioselective *p*-hydroxylation of a simple aromatic precursor using *Cunninghamella echinulata* (Scheme 2.153) [1282].



Scheme 2.153 Regioselective microbial hydroxylation of aromatics

Phenols can be selectively oxidized in the *o*-position by polyphenol oxidase[37] – one of the few oxygenating enzymes used in isolated form – to give catechols in high yields [1283]. 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 2.154).

Two techniques have been developed to solve the problem of *o*-quinone instability:

- One way to prevent *o*-quinone formation is by maintaining a reducing environment by addition of ascorbic acid, which prevents the over-oxidation and leads to the accumulation of catechols. Ascorbate, however, like many other reductants can act as an inhibitor of polyphenol oxidase and hence the concentration

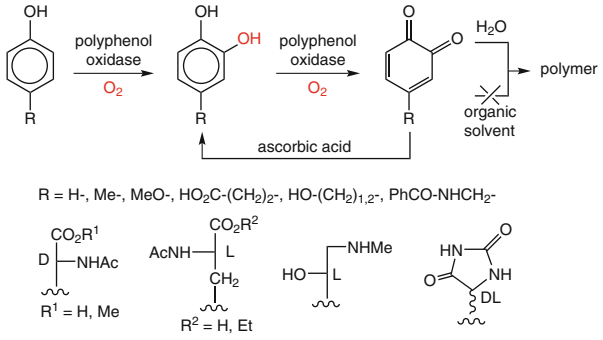
[37] Also called tyrosinase, catechol oxidase, cresolase.

of the reducing agent must be kept at a minimum. In addition, a borate buffer which leads to the formation of a catechol-borate complex is advantageous [1284].

- Polymerization, which requires the presence of water, can be avoided if the reaction is performed in a lipophilic organic solvent such as chloroform (Sect. 3.1.6) [1285].

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, some electron-rich nonphenolic species such as *p*-toluidine are accepted.
- For electronic reasons, 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.



Scheme 2.154 *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 2.154). 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.

2.3.3.3 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 [611, 1286]. The Katsuki-Sharpless method for the asymmetric epoxidation of allylic alcohols [1287, 1288] and the Jacobsen-catalysts for the epoxidation of nonfunctionalized olefins are now widely applied

and reliable procedures [615, 1289]. 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 [617, 1290]. 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 [1175, 1291–1295].

Like C-H hydroxylation, epoxidation of alkenes catalyzed by monooxygenases is preferably performed on a preparative scale with whole microbial cells. Toxic effects of the epoxide formed, which accumulates in the cells, where it reacts with cellular enzymes, and its further (undesired) metabolism catalyzed by epoxide hydrolases in whole cells (Sect. 2.1.5) can be minimized by employing biphasic media. 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 [1296]. In any case, these methods require bioengineering skills [1297].

Once the problems of product toxicity were surmounted by sophisticated process engineering, microbial epoxidation of alkenes became also feasible on an industrial scale [1298, 1299]. The latter was achieved by using organic-aqueous two-phase systems or by evaporation of volatile epoxides. For instance, the epoxy-phosphonic acid derivative 'fosfomycin' [1300], 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*.

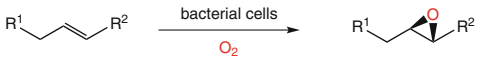
The most intensively studied microbial epoxidizing agent is the ω-hydroxylase system of *Pseudomonas oleovorans* [1301, 1302]. It consists of three protein components: the actual nonheme iron ω-hydroxylase and the electron-transport chain consisting of rubredoxin and NADH-dependent rubredoxin reductase. It catalyzes not only the hydroxylation of aliphatic C–H bonds, but also the epoxidation of alkenes [1303, 1304]. The following rules can be formulated for epoxidations using *Pseudomonas oleovorans* (Scheme 2.155).

- Terminal, acyclic alkenes of moderate chain length (e.g. 1-octene) are converted into (*R*)-1,2-epoxides of high enantiomeric excess along with varying amounts of ω-en-1-ols or 1-als [1305], the ratio of which depends on the chain length of the substrate [1306, 1307]. In contrast, alkane hydroxylation predominates over epoxidation for short (propene, 1-butene) and 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 [1308].
- To avoid problems arising from the toxicity of the epoxide [1309] a water-immiscible organic cosolvent such as hexane can be added [1310, 1311].

Besides *Pseudomonas oleovorans* numerous bacteria have been shown to epoxidize alkenes [1312, 1313]. As shown in Scheme 2.155, the optical purity of epoxides depends on the strain used, although the absolute configuration is usually (*R*) [1314]. This concept has been applied to the synthesis of chiral alkyl and aryl gycidyl ethers [1315, 1316]. 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 [1317].

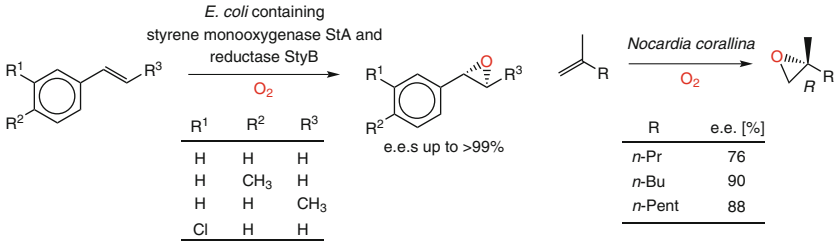
The structural restrictions for substrates elaborated for *Pseudomonas oleovorans* (see above) could be overcome by using different microorganisms. As can be seen from Scheme 2.155, nonterminal alkenes can be epoxidized by *Mycobacterium* or *Xanthobacter* spp. [1318]. On the other hand, *Nocardia corallina* converted 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 [1319, 1320].



Microorganism	R1	R2	Configuration	e.e. [%]
Pseudomonas	*n*-C_5H_{11}	H	*R*	70-80
oleovorans	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	CH_3	H	*R*	70
equi	*n*-$C_{13}H_{27}$	H	*R*	~100
Mycobacterium	H	H	*R*	98
sp.	Ph-O	H	*S*[a]	80
Xanthobacter	Cl	H	*S*[a]	98
Py2	CH_3	CH_3	*R,R*	78
Nocardia sp. IP1	Cl	H	*S*[a]	98
	CH_3	H	*R*	98

[a] Switch in CIP-sequence priority.

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

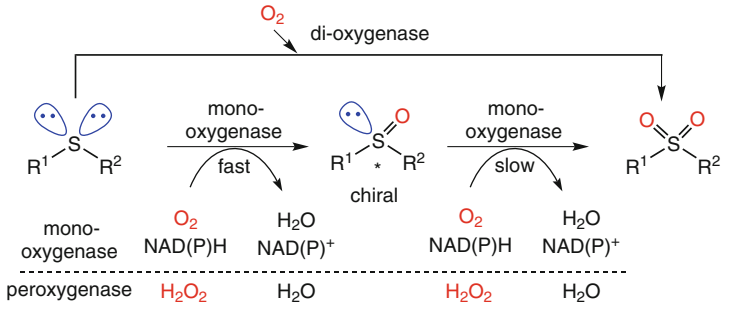
R	e.e. [%]
n-Pr	76
n-Bu	90
n-Pent	88

Scheme 2.156 Epoxidation of styrene derivatives and branched alkenes using cloned monooxygenase and *Nocardia corallina*

2.3.3.4 Sulfoxidation Reactions

Chiral sulfoxides are not only common pharmacophores in active pharmaceutical ingredients, but they have also 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 [1321] or acylated [1322] with high diastereoselectivity. Similarly, thermal elimination [1323] and reduction of α-keto sulfoxides [1324] can proceed with transfer of chirality from sulfur to carbon. In spite of this great potential as valuable chiral relay reagents, with rare exceptions [1325], 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 [1326, 1327]. 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 peroxidases,[38] offers two possible ways of obtaining chiral sulfoxides.



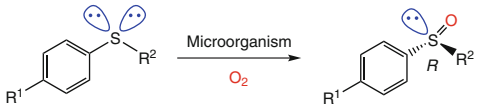
Scheme 2.157 Enzymatic sulfur oxygenation reactions

[38] Since O is incorporated into the substrate, peroxidases performing thioether oxidation should be correctly termed as 'peroxygenases', however, this distinction is often not made.

- 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 [1328]. 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 [1329]. In general, the formation of (*R*)-sulfoxides predominates.

Thioethers can be asymmetrically oxidized both by bacteria (e.g., *Corynebacterium equi* [1330], *Rhodococcus equi* [1331]) and fungi (e.g., *Helminthosporium* sp. [1332] and *Mortierella isabellina* [1333]). Even baker's yeast has this capacity [1334, 1335]. As shown in Scheme 2.158, a large variety of aryl-alkyl thioethers were oxidized to yield sulfoxides with good to excellent optical purities [1336–1338]. The second oxidation step was usually negligible, but with certain substrates the undesired formation of the corresponding sulfone was observed.

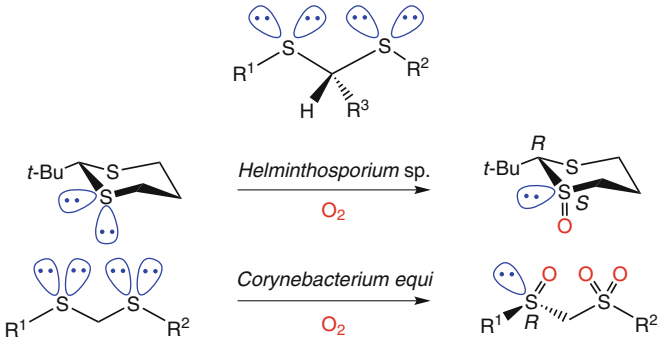


Microorganism	R^1	R^2	e.e. [%]
Mortierella isabellina	$(CH_3)_2CH$	CH_3	82
	H	$(CH_3)_2CH$	83
	H	C_2H_5	85
	C_2H_5	CH_3	90
	H	*n*-C_3H_7	~100
	Br	CH_3	~100[a]
Corynebacterium equi	H	CH_3	92
	CH_3	CH_3	97
	H	*n*-C_4H_9	~100
	H	CH_2-CH=CH_2	~100
baker´s yeast	CH_3	CH_3	92

[a] Some sulfone was formed in this case.

Scheme 2.158 Microbial oxidation of aryl-alkyl thioethers

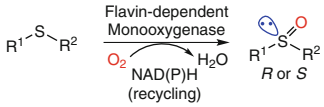
The transformation of thioacetals into mono- or bis-sulfoxides presents intriguing stereochemical possibilities. In a symmetric thioacetal of an aldehyde other than formaldehyde, the sulfur atoms are enantiotopic and each of them contains two diastereotopic nonbonded pairs of electrons (Scheme 2.159). Unfortunately, most of the products from asymmetric oxidation of thioacetals are of low to moderate optical purity [1339, 1340]. 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 [1341] and formaldehyde thioacetals were oxidized by *Corynebacterium equi* to yield (*R*)-sulfoxide-sulfone products [1342] with excellent e.e.



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

In order to avoid poor recoveries of the water-soluble sulfoxide from considerable amounts of biomass when using whole cells, isolated (flavin-dependent) monooxygenases have been successfully employed for thioether-oxidations together with NAD(P)H-recycling (for biocatalytic sulfur oxidation using peroxidases, see Sect. 2.3.4). In particular, cyclohexanone monooxygenase (CHMO) [1343–1345], phenylacetone monooxygenase (PAMO) [1346] and hydroxyacetone monooxygenase (HAPMO) [1347, 1348] were shown to be most useful. Overall, recoveries and stereoselectivities were high, and some enzymes exhibited stereo-complementary properties on selected substrates (Scheme 2.160).



R^1	R^2	Enzyme*	Config.	Conv. [%]	E.e. [%]
Me	*c*-Hexyl	CHMO	R	80	98
t-Bu	$CH=CH_2$	CHMO	R	78	98
Me	Ph	CHMO	R	88	99
Et	*p*-F-C_6H_4	CHMO	S	96	93
CH_2-CN	Ph	CHMO	R	90	92
CH_2-CN	*p*-Me-C_6H_4	CHMO	S	95	98
Me	*c*-Hexyl	HAPMO	S	>99	>99
Me	Ph	HAPMO	S	>97	>99
Me	2-Pyridyl	HAPMO	S	>99	>99
Me	4-Pyridyl	HAPMO	R	63	>99

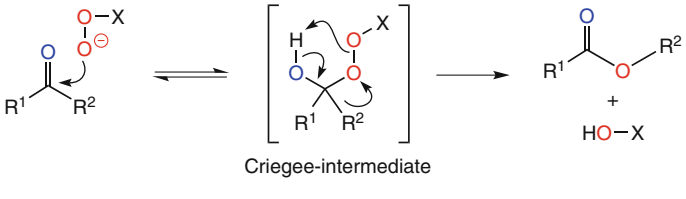
* CHMO = Cyclohexanone monooxygenase, HAPMO = hydroxyacetone monooxygenase.

Scheme 2.160 Enzymatic oxidation of thioethers using isolated monooxygenases

2.3.3.5 Baeyer-Villiger Reactions

Oxidation of ketones by peracids – the Baeyer-Villiger reaction [1349, 1350] – is a reliable and useful method for preparing esters or lactones (Scheme 2.161). 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' [1351]. 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 assuming that the carbon atom best able to support a positive charge will migrate preferentially, i. e. *tert*-alkyl > *sec*-alkyl ~ phenyl > *prim*-alkyl > methyl [1352].



Scheme 2.161 Mechanism of the chemical and biochemical Baeyer-Villiger oxidation

All mechanistic studies on enzymatic Baeyer-Villiger reactions support the hypothesis that conventional and enzymatic reactions are closely related [1206, 1353]. The oxidized flavin cofactor peroxy species (FAD-4a-OO$^-$, 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 [1354–1356], which has been accomplished by conventional means with moderate selectivities [1357].

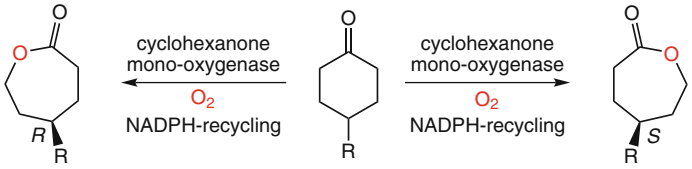
The enzymatic Baeyer-Villiger oxidation of ketones is catalyzed by flavin-dependent monooxygenases and plays an important role in the breakdown of carbon structures containing a ketone moiety (Scheme 2.148). Early studies were performed by using whole microbial cells, particularly in view of avoiding the necessity for NAD(P)H-recycling [1358]. However, whole-cell Baeyer–Villiger oxidations often suffer from low yields due to side reactions catalyzed by competing hydrolytic enzymes. 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).[39]

To avoid further degradation of esters and lactones in microbial Baeyer-Villiger reactions catalyzed by hydrolytic enzymes and to maximize product accumulation, the following approaches are possible:

- Blocking of the hydrolytic enzymes by selective hydrolase-inhibitors such as tetraethyl pyrophosphate (TEPP [1359]) 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.
- Application of nonnatural ketones, whose lactone products are not substrates for the hydrolytic enzymes.
- Use of isolated Baeyer-Villigerases together with NAD(P)H-recycling is nowadays the method of choice.

[39] *Acinetobacter calcoaceticus* NCIMB 9871 is a class-II pathogen.

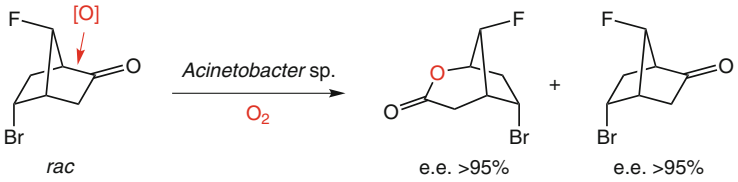
Prochiral (symmetric) ketones can be asymmetrically oxidized by a bacterial cyclohexanone monooxygenase from an *Acinetobacter* sp. to yield the corresponding lactones [1360, 1361]. As depicted in Scheme 2.162, oxygen insertion occurred on both sides of the ketone depending on the substituent in the 4-position. Whereas in the majority of cases products having the (*S*)-configuration were obtained, a switch to the (*R*)-lactone was observed with sterically demanding 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 [1362, 1363].



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 2.162 Desymmetrization of prochiral ketones via enzymatic Baeyer-Villiger oxidation

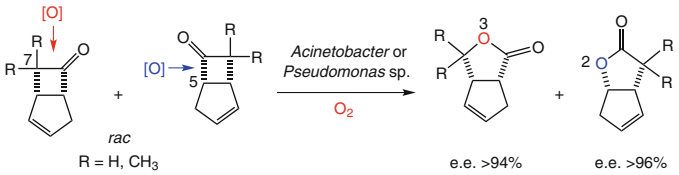
Racemic (nonsymmetric) ketones can be resolved via two pathways. The 'classic' form of a kinetic resolution involves a transformation in which one enantiomer reacts and its counterpart remains unchanged [1364]. For example, bicyclic haloketones, which were used for the synthesis of antiviral 6′-fluoro-carbocyclic nucleoside analogs, were resolved by using an *Acinetobacter* sp. [1365] (Scheme 2.163). Both enantiomers were obtained with >95% optical purity. Interestingly, the enantioselectivity of this microbial oxidation depends on the presence of the halogen atoms, since the dehalogenated bicyclo[2.2.1]heptan-2-one was 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.



Scheme 2.163 Microbial Baeyer-Villiger oxidation of a bicyclic ketone involving 'classic' resolution

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 [1366, 1367]. 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 suitable NADPH-recycling enzyme [1368, 1369]. In order to minimize product toxicity, in-situ substrate-feeding product removal (SFPR) was applied [1370, 1371].

It has been shown that the molecular reasons of enantiodivergent Baeyer-Villiger reactions [1372, 1373] 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 [1374].



Scheme 2.164 Enantiodivergent microbial Baeyer-Villiger oxidation involving 'nonclassic' resolution

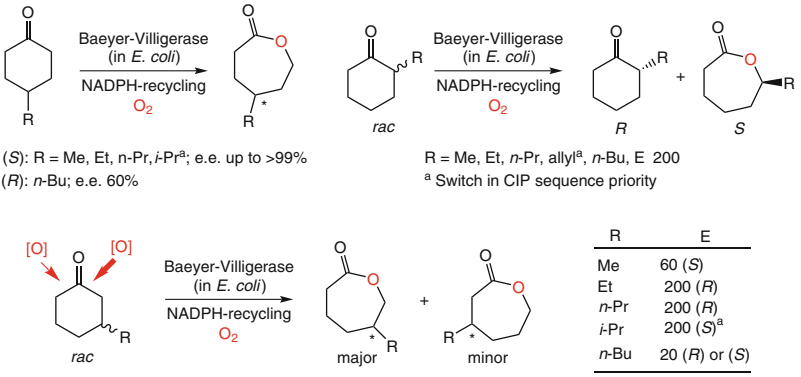
In order to overcome problems associated with whole-cell Baeyer-Villliger oxidations, an impressive number of bacterial 'Baeyer-Villigerases' possessing opposite stereopreference [1375, 1376] were purified, characterized [1377–1380] and cloned into a suitable (nonpathogenic) host, such as baker's yeast [1381–1385] or *E. coli* [1386].

The majority of Baeyer-Villigerases are NADPH-dependent, but several candidates (e. g. from *Pseudomonas putida*) accept NADH, which is more easily recycled [1387]. 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 [1388]. The overall performance of the fusion-protein was comparable to that of the single (non-fused) proteins. A clever concept of internal cofactor recycling for isolated Baeyer-Villigerases was developed using a coupled enzyme system [1389]. 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. 2.3.1) 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 via 'hydrogen-borrowing' (Scheme 3.42).

Investigation of the regio- and enantioselectivity of Baeyer-Villigerases from bacteria from an industrial wastewater treatment plant cloned into *E. coli* revealed the following trends (Scheme 2.165):

- 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.
- 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* ≥200).
- In contrast, 3-substituted cyclohexanones furnished 'non-classic' kinetic resolution via oxygen-insertion at both sides with different regioselectivities to furnish regio-isomeric lactones. The enantioselectivites depended on the enzyme used and on size of the substituent [1390].



Scheme 2.165 Regio- and enantioselective Baeyer-Villiger oxidation using cloned Baeyer-Villigerases via desymmetrization, 'classic' and 'nonclassic' kinetic resolution

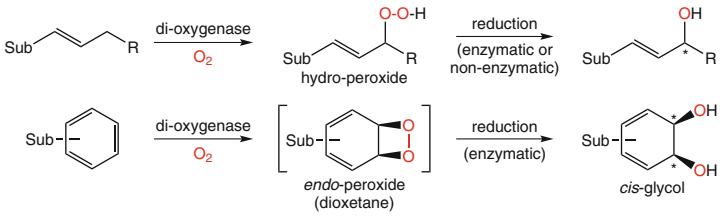
Dioxygenases

Typical dioxygenase reactions, during which *two* oxygen atoms are simultaneously transferred onto the substrate, are shown in Scheme 2.166. Insertion of O_2 into a C–H or C=C bond yields a highly reactive and unstable hydro- or endo-peroxide species, respectively, which is subject to (enzymatic or nonenzymatic) reduction or rearrangement to yield stable mono- or di-hydroxy products [1391].

- Non-conjugated 1,4-dienes may be oxidized by lipoxygenases at the allylic position to furnish an allyl *hydro*peroxide which, upon chemical 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 [1392].

- Alternatively, an *endo*-peroxide may be formed, whose enzymatic reduction leads to a vicinal diol (Scheme 2.166). The latter reaction resembles the cycloaddition of singlet-oxygen onto an unsaturated system. In mammals, it occurs in the biosynthesis of prostaglandins and leukotrienes, while in prokaryotic (bacterial) cells it constitutes the initial step in the oxidative biodegradation of aromatics (Scheme 2.83) [1393, 1394].



Scheme 2.166 Dioxygenase-catalyzed reactions

2.3.3.6 Formation of Hydroperoxides

The biocatalytic formation of hydroperoxides is 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 – (poly)unsaturated fatty acids.

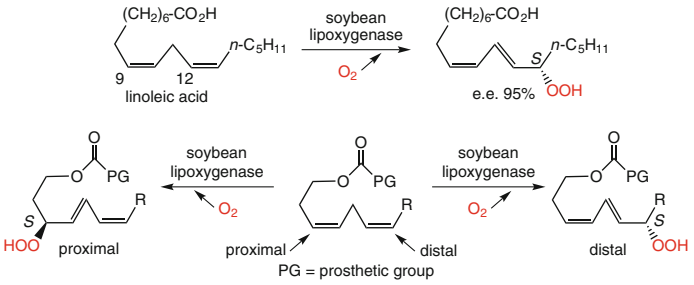
Allylic Hydroperoxidation Lipoxygenase is a nonheme iron dioxygenase which catalyzes the incorporation of dioxygen into an allylic C-H bond of polyunsaturated fatty acids possessing a nonconjugated 1,4-diene unit through a radical mechanism by forming the corresponding conjugated allylic hydroperoxides [1395–1397]. The enzyme from soybean has received the most attention in terms of a detailed characterization because of its early discovery [1398], ease of isolation and acceptable stability [1399, 1400]. The following characteristics can be given for soybean lipoxygenase-catalyzed oxidations:

- The enzyme has a preference for all-(*Z*) configurated 1,4-dienes at an appropriate location in the carbon chain of polyunsaturated fatty acids, (*E*,*Z*)- and (*Z*,*E*)-1,4-dienes are accepted at slower rates [1401].
- (*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 [1402, 1403].

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 [1404] (the 'proximal' region, Scheme 2.167) [1405].

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 [1406], insufficient reaction rates can be improved by an increased the oxygen pressure (up to 50 bar) [1407]. The (*Z*,*Z*)-1,4-diene moiety of several long-chain alcohols could be oxidized by attachment of a prosthetic group (PG), consisting of a polar $(CH_2)_n$–CO_2H or a CH_2–O–$(CH_2)_2$–OH unit, which serves as a surrogate of the carboxylate moiety (Scheme 2.167) [1408]. 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 [1409]) and removal of the prosthetic group, the corresponding secondary alcohols were obtained with retention of configuration [1410].



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 2.167 Natural and non-natural substrates of soybean lipoxygenase

In addition, the regioselectivity of the oxidation could be inverted from 'normal' (distal) to 'abnormal' (proximal) by changing the length of the distal substituent R and the spacer arm linking the prosthetic group PG (Table 2.4 and Scheme 2.167). Enhancing the lipophilicity of R from *n*-C_5 to *n*-C_{10} led to an increased reaction at the 'abnormal' site to form predominantly the proximal oxidation product. In contrast, extension of the spacer arm caused a switch to the 'distal' product.

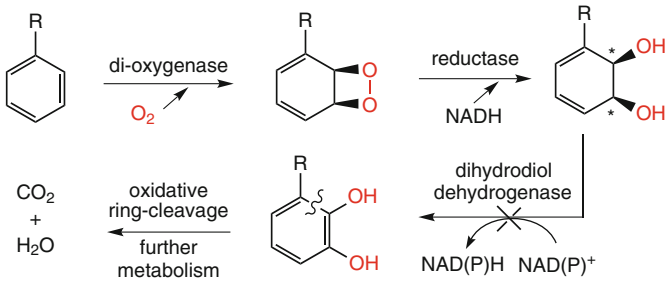
Table 2.4 Variation of prosthetic groups (for formulas see Scheme 2.167)

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

2.3.3.7 Dihydroxylation of Aromatic and Conjugated C=C Bonds

cis-Dihydroxylation by bacterial dioxygenases constitutes the initial key step in the oxidative degradation pathway for aromatic compounds (Scheme 2.168) [1411, 1412], which is crucial for the bioremediation of toxic pollutants from contaminated sites. In 'wild-type' microorganisms, the chiral *cis*-glycols initially formed are rapidly further oxidized by dihydrodiol dehydrogenase(s), involving rearomatization of the diol intermediate with concomitant loss of chirality [1413]. The use of mutant strains with blocked dehydrogenase activity [1414], however, allows the chiral glycols to accumulate in the medium, from which they can be isolated in good yield [1415, 1416].

Bacterial Rieske-type iron dioxygenases are multicomponent enzymes, that contain an a non-heme oxygenase component, which contains a 2Fe-2S Rieske cluster together with an adjacent catalytic Fe^{3+}-center (Fig. 2.18) [1417]. The latter forms a side-on complex with O_2, which performs a (formal) [2+2] cycloaddition with the C=C bond in the substrate, as deduced for naphthalene dioxygenase [1418]. The highly reactive (putative) dioxetane thus formed is immediately reduced to the corresponding *cis*-glycol by shuttling electrons from NAD(P)H through a sophisticated electron-transport system, via a flavin-dependent ferredoxin reductase and a ferredoxin [1419, 1420] onto the dioxygenase, like in Cyt P-450 monooxygenases (Fig. 2.17) [1421]. Hence, it is not surprising that Rieske-type dioxygenases are able to perform C-H hydroxylation, C=C epoxidation and thioether oxidation besides their main activity – *cis*-dihydroxylation of alkenes. In contrast, Cyt P-450 enzymes cannot catalyze dihydroxylations due to their Fe^{4+}=O center (Compound I, Scheme 2.146). Given the complexity of this mechanism, it is evident that C=C-dihydroxylations cannot be performed with cell-free systems.



Scheme 2.168 Oxidative degradation of aromatics by bacterial dioxygenases

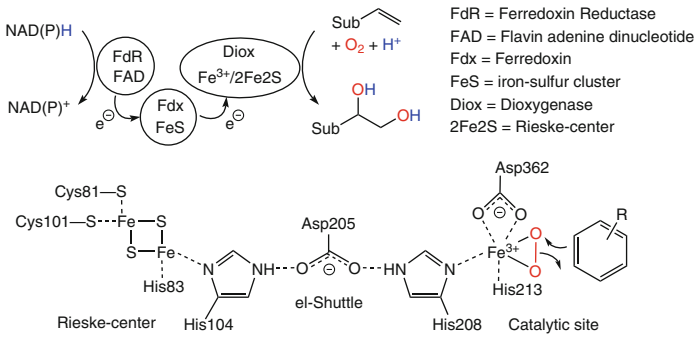
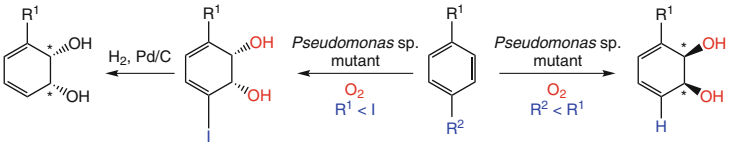


Fig. 2.18 Electron-transport chain and catalytic center of Rieske-type naphthalene dioxygenase

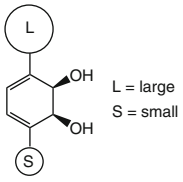
Mutant strains of *Pseudomonas putida* harboring toluene dioxygenase or naphthalene dioxygenase lacking the dihydrodiol dehydrogenase show broad substrate tolerance for ring substituents R^1 and R^2 by maintaining excellent stereospecificity (Scheme 2.169). Consequently, they have been widely employed for the asymmetric dihydroxylation of substituted aromatics [1422] and an impressive number of >300 arenes have been converted into the corresponding chiral *cis*-glycols with excellent optical purities, even on ton-scale with productivities of 20 g L^{-1} h^{-1} and product titers exceeding 80 g L^{-1} [1423–1427]. The stereoselectivity of the oxygen addition can be predicted with some accuracy using a substrate model (Scheme 2.169) [1428–1430].

The substrates need not necessarily be mono- or poly-substituted aromatic compounds such as those shown in Scheme 2.169, but may also be extended monocyclic- [1431], polycyclic- [1432], and heterocyclic derivatives [1433, 1434].

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 [1435].

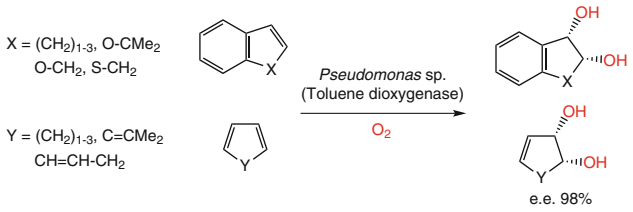


Relative size	R^1	R^2
$R^1 > R^2$	H, Me, Et, *n*-Pr, *i*-Pr, *n*-Bu, *t*-Bu, Et-O,*n*-Pr-O, Halogen, CF_3, Ph, Ph-CH_2,Ph-CO,CH_2=CH, CH_2=CH-CH_2, HC C	H
$R^2 > R^1$	F, Me	I



Scheme 2.169 Enantiocomplementary synthesis of *cis*-glycols

The substrate tolerance encompasses also nonaromatic C=C bonds, provided they are conjugated to aromatic systems (such as styrenes) or an additional alkene unit [1436–1438] yielding *ertho*-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 2.170). The stereoselectivities were excellent for cyclic substrates but they dropped for open-chain derivatives (e.e.$_{max}$ 88%) [1439]. Depending on the substrate and the type of enzyme, hydroxylation at benzylic or allylic positions were observed as side reactions. In contrast, isolated olefinic bonds react sluggishly and can only be dihydroxylated using dioxygenase mutants with varying success [1440].



Scheme 2.170 Dihydroxylation of conjugated alkenes using toluene dioxygenase

The synthetic potential of nonracemic *cis*-diols derived via microbial dihydroxylation has been exploited over the years for the synthesis 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 [1441] and D-*myo*-inositol derivatives [1442] were obtained using this approach. Cyclopentanoid synthons for the synthesis of prostaglandins and terpenes were prepared by a ring-opening/closure sequence [1443]. Rare carbohydrates such as D- and L-erythrose [1444] and L-ribonolactone [1445] were obtained from chlorobenzene as were pyrrolizidine alkaloids [1446]. Furthermore, a bio-inspired synthesis of the blue pigment indigo was developed on a commercial scale using the microbial dihydroxylation of indol [1447], and indene served as starting point for the synthesis of the antiviral agent Indinavir [1448, 1449].

2.3.4 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 [1450] or derivatives thereof, such as *t*-butyl and cumyl hydroperoxide. 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 activated. 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.[40] 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.

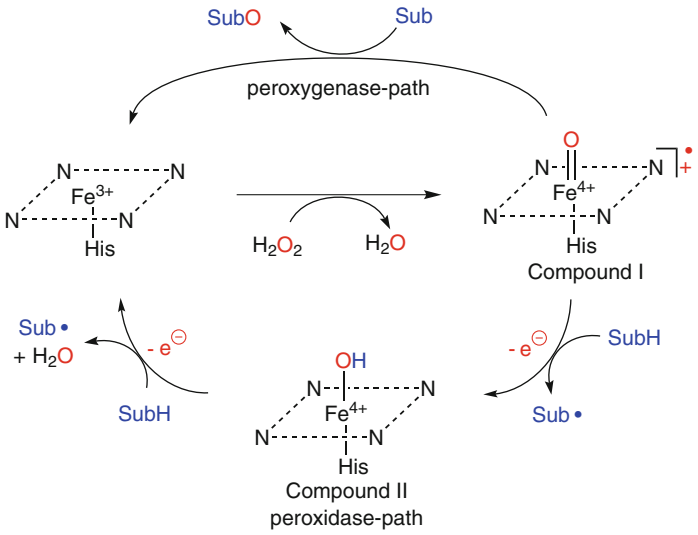
Peroxidases [EC 1.11.1.X] are a heterogeneous group of redox enzymes found ubiquitously in various sources [1451], such as plants [1452], microorganisms [1453] and animals. They are often named after their sources (e.g., horseradish peroxidase, lacto- and myeloperoxidase) or akin to their substrates (e.g., cytochrome c-, halo- and lignin peroxidase). Although the biological role of these enzymes is quite diverse—ranging from disproportionation of H_2O_2, free radical oligomerization and polymerization of electron-rich aromatics to the oxidation and halogenation of organic substrates—they have in common that they accept hydrogen peroxide or an alkyl hydroperoxide as oxidant. In line with these diverse catalytic activities, the mechanism of action may be quite different and can involve a heme unit, selenium (glutathione peroxidase) [1454], vanadium (bromoperoxidase) [1455, 1456], manganese (manganese peroxidase) [1457] and flavin at the active site (flavoperoxidase) [1458]. The largest group of peroxidases studied so far are heme-enzymes with ferric protoporphyrin IX (protoheme) as the prosthetic group. Their catalytic cycle bears strong similarities to that of heme-dependent monooxygenases (Sect. 2.3.3, Scheme 2.146), but their pathways are more complex (Scheme 2.171). The mechanism of heme-dependent peroxidase catalysis has been largely deduced from horseradish peroxidase [1178, 1459–1461] and its key features are described as follows:

In its native state, the Fe^{3+} species is coordinated equatorially by a heme unit and axially by a histidine residue and is therefore very similar to cytochrome P 450 [1462]. Activation occurs via a two-electron oxidation at the expense of H_2O_2 via the peroxide-shunt to form Compound I. The latter contains a Fe^{+4}=O π-radical moiety and is two oxidation steps above the Fe^{+3} ground state; (in the monooxygenase pathway, the Fe^{3+}-ground state 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 two major pathways:

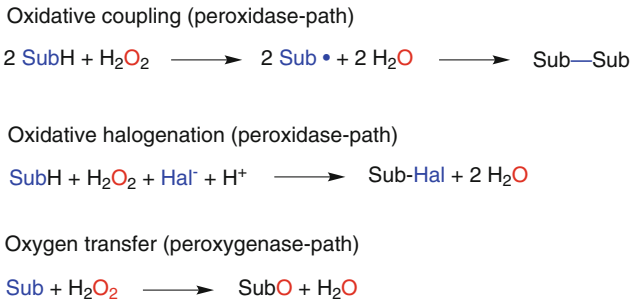
- **Peroxidase-path:** Abstraction of a single electron from an electron-rich substrate such as a phenol, an enol or halide forms a substrate radical and yields an Fe^{+4}=O species denoted Compound II. Since the latter is still one oxidation step above the Fe^{+3}-ground state, this process can occur a second time forming another substrate radical, to finally re-form the enzyme in its native state.
- **Peroxygenase-path:** Alternatively, Compound I can incorporate an O-atom onto a substrate via a two-electron transfer in a single step. Although formally this reaction should be denoted as 'per*oxygenase*'-activity, this distinction is not always made.

[40] A well known industrial-scale process is the oxidation of propene to propene oxide using *tert*-Bu–OOH.

Due to the fact that – in contrast to monooxygenases – no external nicotinamide cofactor is required in the peroxidase cycles, peroxidases are highly attractive for preparative biotransformations. A number of synthetically useful reactions can be achieved (Scheme 2.172) [1463–1465]. Depending on the enzyme–substrate combination, the replacement of hydrogen peroxide by *tert*-butyl hydroperoxide may be beneficial.



Scheme 2.171 Catalytic cycles of heme-dependent peroxidases



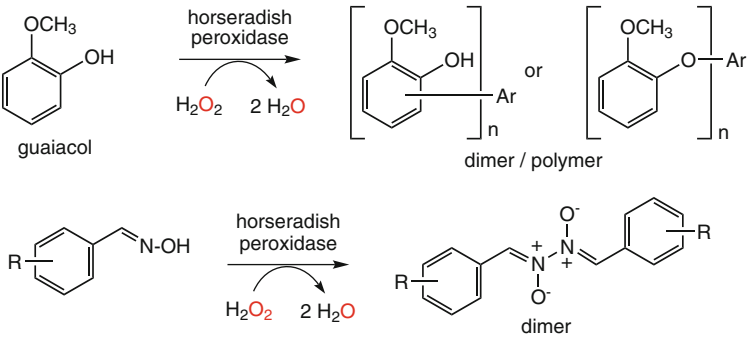
Scheme 2.172 Synthetically useful peroxidase and peroxygenase reactions

Oxidative Coupling

This reaction is commonly denoted as the 'classical' peroxidase activity, since it was the first type of peroxidase-reaction discovered.

It is mainly restricted to heme peroxidases and it involves the one-electron oxidation of phenols (e.g., guaiacol, resorcinol) and anilines (e.g., aniline, *o*-

dianisidine), forming resonance-stabilized radicals. The latter undergo spontaneous inter- or intramolecular C–C, C–O and/ or C–N coupling to yield dimers or polymers [1466–1468]. In certain cases, dimers, such as biaryls and aryl-ethers, have been obtained (Scheme 2.173) [1469, 1470].



Scheme 2.173 Peroxidase-catalyzed oxidative coupling of aromatics and arylether formation

Oxidative Halogenation

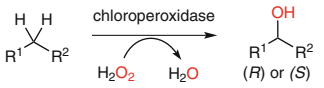
A class of peroxidases – *halo*peroxidases – specializes in the peroxidation of halides (Cl^-, Br^-, I^- but not F^-), thereby creating reactive halogenating species (such as hypohalite), which in turn form haloorganic compounds [1471, 1472]. These reactions are described in Sect. 2.7.1.

Oxygen Transfer

From a synthetic viewpoint, selective oxygen transfer via the peroxygenase-path is particularly intriguing, because it is comparable to those catalyzed by monooxygenases with one significant advantage – it is independent of redox cofactors, such as NAD(P)H. Among the various types of reactions – C–H bond oxidation, alkene epoxidation and heteroatom oxidation – the most useful transformations are described below.

Hydroxylation of C–H Bonds Heme-dependent chloroperoxidase (CPO) from the marine fungus *Caldariomyces fumago* has been found to effect the hydroxylation of C–H bonds. The large-scale production of CPO is facilitated by the fact that it is an extracellular enzyme, which is excreted into the fermentation medium [1473–1475] and its crystal structure has been solved [1474]. 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 [1476], but benzylic or propargylic hydroxylation is readily effected to furnish the corresponding *sec*-alcohols in high e.e. (Scheme 2.174) [1477, 1478]. 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 methyl to an

ethyl analog, albeit at a slow reaction rate. The selectivity of CPO-catalyzed propargylic hydroxylation was found to be sensitive with respect to the polarity and the alkyne chain length [1479]. In addition, hydroxylation of aromatic C–H bonds seems to be possible, as long as electron-rich (hetero)aromatics, such as indol are used [1480, 1481].



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
AcO-$(CH_2)_2$-C≡C-	Me	(*R*)	83
Br-CH_2-C≡C-	Me	(*R*)	94
Br-$(CH_2)_2$-C≡C-	Me	(*R*)	94

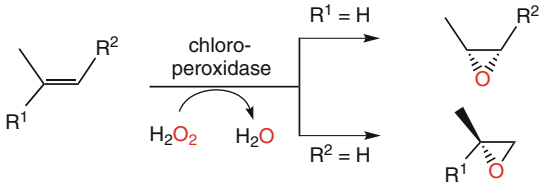
Scheme 2.174 Benzylic and propargylic C–H hydroxylations

Epoxidation of Alkenes Due to the fact that the asymmetric epoxidation of alkenes using monooxygenase systems is impeded by the toxicity of epoxides to microbial cells, the use of H_2O_2-depending peroxidases represents a valuable alternative.

Chloroperoxidase-catalyzed epoxidation of alkenes proceeds with excellent enantioselectivites (Scheme 2.175) [1482, 1483]. For styrene oxide it was demonstrated that all the oxygen in the product is derived from hydrogen peroxide, which proves the validity of direct oxygen-transfer via the peroxygenase-path (Scheme 2.171) [1484]. Unfunctionalized *cis*-alkenes [1485] and 1,1-disubstituted olefins [1486, 1487] 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 [1488].

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 2.176).

Chloroperoxidase from *Caldariomyces fumago* is a selective catalyst for the oxidation of methylthioethers to furnish (*R*)-sulfoxides. Initial results were

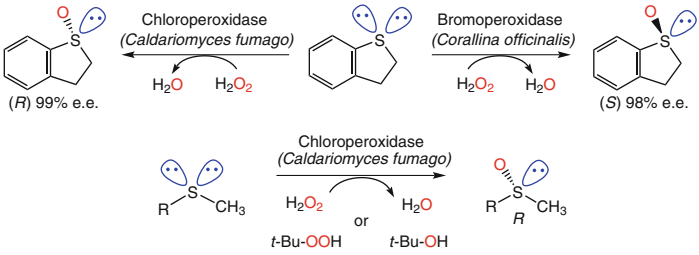


R^1	R^2	e.e. [%]
H	*n*-C_4H_9-	96
H	$(CH_3)_2CH$-CH_2-	94
H	Ph-	96
Ph	H	89
CH_2-CO_2Et	H	94
$(CH_2)_2$-Br	H	85
n-C_5H_{11}	H	95

Scheme 2.175 Asymmetric epoxidation of alkenes using chloroperoxidase

disappointing, as low e.e.'s were reported [1489]. 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 maintaining the concentration of H_2O_2 at a constant low level [1490, 1491].

A vanadium-dependent haloperoxidase from the marine alga *Corallina officinalis* was shown to possess a matching opposite enantiopreference by forming (*S*)-sulfoxides [1492, 1493]. Although simple open-chain thioethers were not well transformed, cyclic analogs were ideal candidates [1494].



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 2.176 Stereocomplementary oxidation of thioethers by haloperoxidases

Although on a superficial glimpse, peroxidases appear to be more easy to use than monooxygenases, several points limit their practical application considerably: Given the tendency of H_2O_2 to deactivate proteins in general, the operational stability of peroxidases in the presence of substantial concentrations of the oxidant is very limited and in order to achieve reasonable turnover numbers (usually only a few hundred) it has to be continuously added via autotitration using a H_2O_2-sensitive electrode ('peroxystat' [1495]) by maintaining a constant low level (~10 mM). Although this also minimizes spontaneous (non-selective) oxidation, reaction rates are modest too. Consequently, at this point, peroxygenases are no serious competitors for monooxygenases.

2.4 Formation of Carbon–Carbon Bonds

The majority of enzymatic reactions exploited for biotransformations involve functional group manipulations via *bond-breaking* reactions. The following enzymatic systems, which are capable of *forming* carbon–carbon bonds in a highly stereoselective manner, belong to the class of lyases and are gaining increasing attention in view of their potential in *synthesis*. Since these enzymes are involved in the biosynthesis and biodegradation of sugars, lyase-catalyzed reactions are generally equilibrium-controlled. The following strategies can be applied to drive C–C bond forming reactions towards completion:

- The primary hydroxycarbonyl products often spontaneously cyclize to yield a stable hemiacetal.
- C–C bond forming steps are often embedded in a reaction cascade, which pulls out the formed product from the equilibrium (Sect 3.2, cascade reactions).
- Some C-donor molecules, such as pyruvate, undergo decarboxylation, which provides a strong driving force.

For the sake of clarity, the donor representing the umpolung reagent is drawn with bold C–C bonds (blue) throughout this chapter.

- Aldol reactions catalyzed by aldolases are useful for the elongation of aldehydes by a two- or three-carbon unit yielding β-hydroxy compounds.
- Aldehydes of various size can be coupled in a head-to-head fashion to furnish α-hydroxycarbonyl compounds (acyloins, benzoins).
- A hydroxyacetyl C_2-fragment (equivalent to hydroxyacetaldehyde) is transferred via transketolase reactions.
- For the addition of the C_1-synthon cyanide to aldehydes by hydroxynitrile lyases see Sect. 2.5.3.

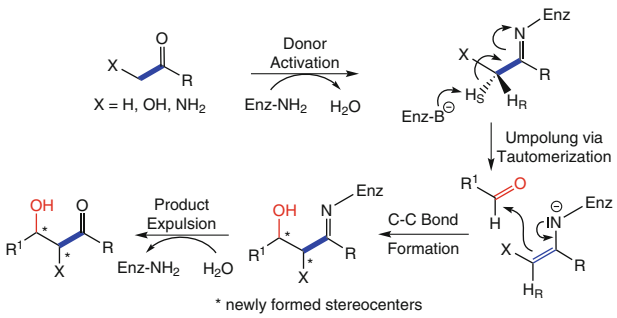
2.4.1 Aldol Reactions

Asymmetric C–C bond formation based on catalytic aldol addition reactions remains a challenging subject in synthetic organic chemistry. Although many

successful nonbiological strategies have been developed [1496, 1497], most of them are not without drawbacks. They are often stoichiometric in auxiliary reagent and require the use of a chiral metal or organocatalytic enolate complex to achieve stereoselectivity [1498–1501]. 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 polar functional groups, the employment of conventional aldol reactions requires extensive protection protocols in order to make them lipophilic and to avoid undesired cross-reactions, which 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 [1502–1519].

Aldolases were first recognized some 70 years ago. At that time, it was believed that they form an ubiquitous class of enzymes that catalyze a key step in glycolysis by interconversion of hexoses into two three-carbon subunits [1520]. 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 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, acting via different mechanisms during formation of the (donor) carbanion, have been recognized [1521]. Both of the mechanisms are closely related to conventional aldol reactions, i.e., carbanion formation (umpolung) is achieved via an enolate- or enamine species (Schemes 2.177 and 2.178).

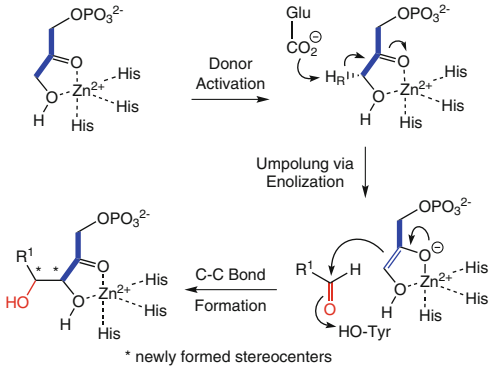


Scheme 2.177 Mechanism of type I aldolases

Type-I aldolases, found predominantly in higher plants and animals, require no metal cofactor. They catalyze the aldol reaction through a Schiff-base intermediate, which tautomerizes to an enamine species (Scheme 2.177) [1522]. First, the donor is covalently linked to the enzyme via the ε-amino group of a conserved lysine residue 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 aldol product and regenerates the enzyme.

Type II aldolases are found predominantly in bacteria and fungi, and are Zn^{2+}-dependent enzymes[41] (Scheme 2.178) [1523]. Their mechanism of action proceeds through a metal-enolate [1524]: an essential Zn^{2+} atom in the active site (coordinated by three nitrogen atoms of histidine residues [1525]) 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.



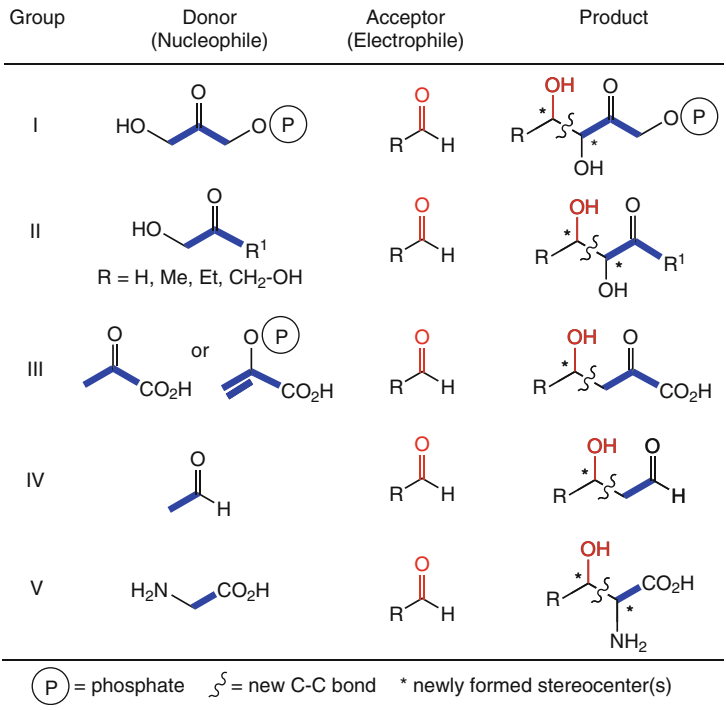
Scheme 2.178 Mechanism of metal-dependent type II aldolases

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 undergoing C–C bond formation is highly predictable. Furthermore, most aldolases are rather 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 approximately 50 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 generally involves a two- or three-carbon unit (Scheme 2.179, donors are shown in bold).

[41] Some enzymes use Mg^{2+} or Mn^{2+}.

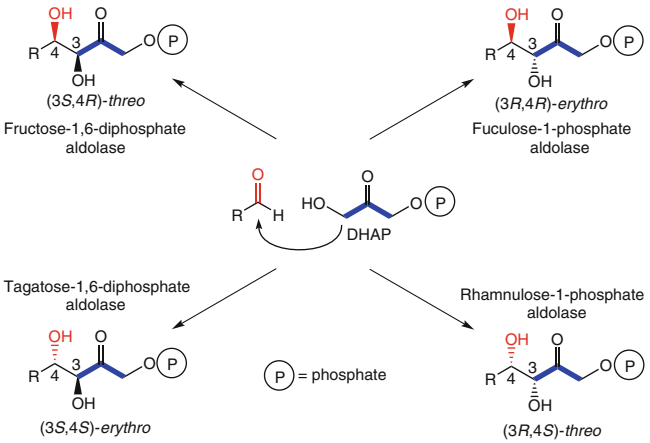
Aldolases are most conveniently classified into five groups according to their donor molecule. The best studied group I uses dihydroxyacetone phosphate (DHAP) as donor, resulting in the formation of a ketose 1-phosphate (Scheme 2.181). Within this group, enzymes capable of forming *all four possible stereoisomers* of the newly generated stereogenic centers in a complementary fashion are available (Scheme 2.180). Group II transfers (non-phosphorylated) short-chain hydroxycarbonyl donors, such as hydroxyacetaldehyde, (di)hydroxacetone (DHA) and 1-hydroxy-2-butanone (Scheme 2.188). Group III uses pyruvate (or phosphoenol pyruvate) as donor to yield 3-deoxy-2-keto acids (Scheme 2.189) [1526]. The fourth 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 2.190). Finally, group V aldolases couple glycine (as donor) with an acceptor aldehyde to yield α-amino-β-hydroxy acids (Scheme 2.192).



Scheme 2.179 Main groups of aldolases according to donor type

Group I: Dihydroxyacetone Phosphate-Dependent Aldolases

The exploitation of the full synthetic potential of DHAP-dependent aldolases into a general and efficient methodology for asymmetric aldol additions largely depends 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.



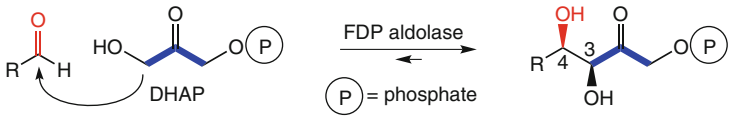
Scheme 2.180 Stereocomplementary DHAP-dependent aldolases

As shown in Scheme 2.180, 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 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 2.181) [548, 1527].

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 always *threo* (Scheme 2.181). 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 (D-glyceraldehyde 3-phosphate), allowing the synthesis of carbohydrates [1528–1531] and analogs such as nitrogen- [547, 1532] and sulfur-containing sugars [1533], deoxysugars [1534], fluoro-sugars, and rare eight- and nine-carbon sugars [1535]. As depicted in Scheme 2.181, numerous aldehydes which are structurally quite unrelated to the natural acceptor are freely accepted [1536–1539].



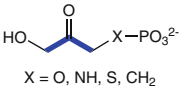
R (natural substrates)	R (non-natural substrates)
D- or L-threose	H, Me, Et, *n*-Pr, *i*-Pr, CH=O, CO_2H, CH_2F, CH_2Cl,
D- or L-erythrose	CH_2OCH_2Ph, $(CH_2)_2OH$, $CH(OCH_3)CH_2OH$,
L-arabinose, D-ribose,	$(CH_2)_2OMe$, CHOHMe, $CHOHCH_2OMe$,
D-lyxose, D-xylose	CH_2Ph, COPh, $(CH_2)_3CH=O$,
D-glyceraldehyde-3-(P)	$CH_2P(O)(OEt)_2$, 2- and 3-pyridyl,
	4-cyclohexenyl, CH_2O(P), 

Scheme 2.181 Aldol reactions catalyzed by FDP aldolase from rabbit muscle

For RAMA the following rules apply to the aldehyde component:

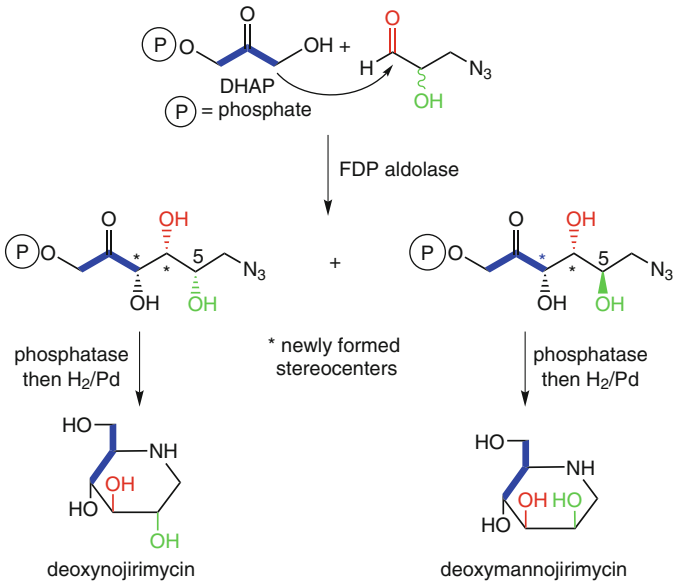
- In general, unhindered aliphatic, α-heterosubstituted, and protected alkoxy aldehydes are accepted as substrates.
- Sterically hindered aliphatic aldehydes such as pivaldehyde do not react with RAMA, nor do α,β-unsaturated aldehydes or compounds that can readily be eliminated to form α,β-unsaturated 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 isosteric analogs which are more resistant towards spontaneous hydrolysis have been successfully tested as substitutes for DHAP (Scheme 2.182) [1540], however the reaction rates were reduced by about one order of magnitude [1541–1544].



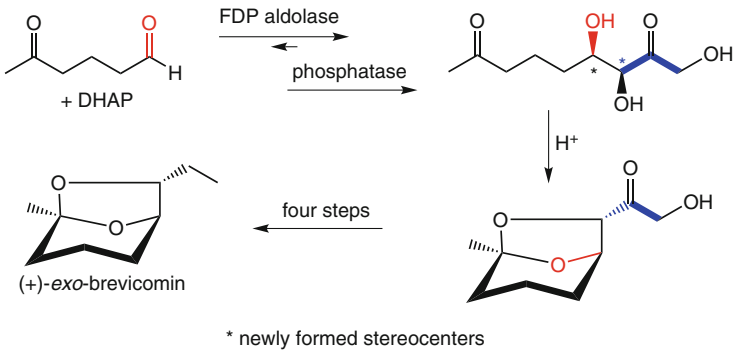
Scheme 2.182 Nonnatural DHAP substitutes for fructose-1,6-diphosphate aldolase (RAMA)

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 2.183). 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 and gave diastereomers with respect to C_5 [1545, 1546].



Scheme 2.183 Synthesis of aza-sugar analogs

An elegant synthesis of (+)-*exo*-brevicomin, the sex pheromone of bark beetles made use of FDP-aldolase (Scheme 2.184) [1547]. RAMA-catalyzed addition 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 de-functionalized in four subsequent steps to give (+)-*exo*-brevicomin.

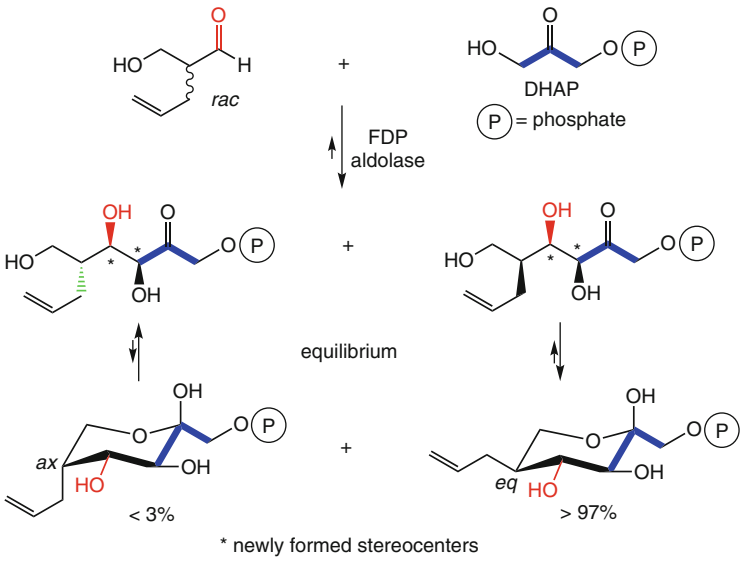


Scheme 2.184 Synthesis of (+)-*exo*-brevicomin

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.

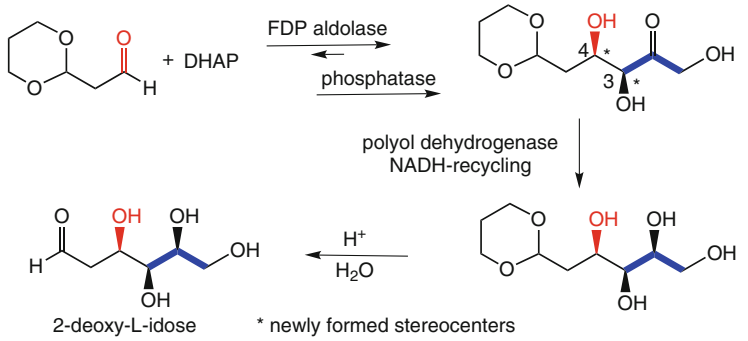
The following methods have been used to avoid the (often tedious) separation of diastereomeric products [546].

- 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 [1548].
- In some cases, a diastereoselective 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.
- In cases wherein one diastereomer of the product is more stable than the other, one can utilize a thermodynamically controlled process (Scheme 2.185). 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 [1549].



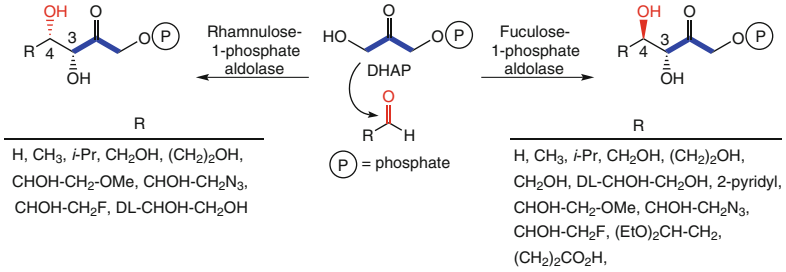
Scheme 2.185 Thermodynamic control in aldolase reactions

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. One way to overcome this limitation and to obtain aldoses instead of ketoses makes use of a monoprotected dialdehyde as the acceptor substrate (Scheme 2.186). 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*.



Scheme 2.186 Synthesis of aldoses using FDP aldolase

Aldol additions catalyzed by DHAP-dependent aldolases which exhibit a complementary stereospecificity to RAMA have been used to a lesser extent (Schemes 2.180 and 2.187) [1550, 1551]. 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 substitutent on the aldehyde. In those cases shown in Scheme 2.187, however, only a single diastereomer was obtained.



Scheme 2.187 Aldol reactions catalyzed by fuculose- and rhamnulose-1-phosphate aldolase

One drawback common to group I aldolases is that they require the expensive and sensitive phosphorylated donor dihydroxyacetone phosphate. This molecule is not very stable in solution ($t_{1/2}$ ~ 20 h at pH 7), and its synthesis is not trivial [1552]. DHAP may be obtained from the hemiacetal dimer of dihydroxyacetone by chemical phosphorylation with $POCl_3$ [1553, 1554], or by enzymatic

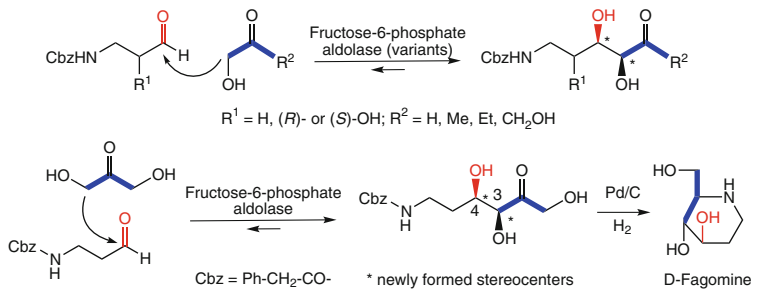
phosphorylation of dihydroxyacetone at the expense of ATP and glycerol kinase [588] (Sect. 2.1.4). Probably the most elegant and convenient method is the in situ generation of DHAP from fructose-1,6-diphosphate (FDP) using FDP aldolase, forming one molecule of DHAP as well as glyceraldehyde-3-phosphate. The latter can be rearranged by triosephosphate isomerase to give a second DHAP molecule [583]. This two-enzyme protocol has been further extended into a highly integrated 'artificial metabolism' derived from glycolysis 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 [1555]. The use of DHAP can be circumvented by in-situ formation of a DHA borate ester, which is able to mimic DHAP with rhamnulose 1,6-diphosphate aldolase [1556, 1557].

The presence of the phosphate group in the aldol adducts facilitates their purification by precipitation as the corresponding barium salts or via ion-exchange chromatography. Cleavage of phosphate esters is usually accomplished by enzymatic hydrolysis using acid or alkaline phosphatase (Sect. 2.1.4).

Group II: Dihydroxyacetone Dependent Aldolases

The recent discovery of D-fructose-6-phosphate aldolase from *E. coli* and the structurally related transaldolase B variant F178Y has opened an elegant solution to avoid the necessity for phosphorylated DHAP. Since the stereospecificity of these enzymes is identical to that of fructose-1,6-diphosphate aldolase, it represents a useful alternative to RAMA by avoiding the futile phosphorylation of the donor and the dephosphorylation of the aldol product.

Fructose-6-phosphate aldolase and variants thereof accept (non-phosphorylated) dihydroxyacetone and several structural analogs, such as hydroxyacetone, 1-hydroxy-2-butanone and hydroxyacetaldehyde (glycolaldehyde) (Scheme 2.188). In addition to this remarkable donor-flexibility, they also possess a broad tolerance for substituted acceptor aldehydes [1558–1563]. The synthetic applicability of this method was demonstrated by coupling of dihydroxy-acetone with *N*-protected 3-aminopropanal to yield the (3*S*,4*R*)-*threo*-diol, which was reductively deprotected and cyclized to furnish the rare aza-sugar D-fagomine. The latter acts as glycosidase inhibitor and shows antifungal and antibacterial activity [1564].



Scheme 2.188 Aldol reaction catalyzed by fructose-6-phosphate aldolase (variants) using non-phosphorylated 1-hydroxy-2-alkanones as donors

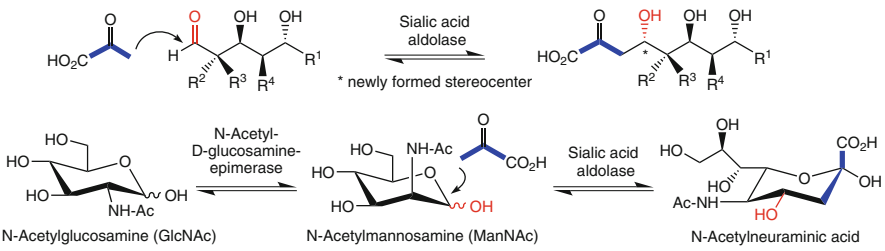
Group III: Pyruvate-Dependent Aldolases

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-γ-hydroxy acids in vitro. In these reactions, the equilibrium is less favorable and usually requires an excess of pyruvate to achieve a reasonable conversion. However, product isolation is facilitated by enzymatic decomposition of excess pyruvate by pyruvate decarboxylase to yield volatile CO_2 and acetaldehyde.

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 2.189) [1565, 1566]. 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 for the production of a precursor of the anti-viral drug Zanamivir [1567–1570]. 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 [1571]. The cloning of the enzyme has reduced its cost [1572].

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 2.189, a range of mannosamine derivatives have been used to synthesize derivatives of NeuAc [1573–1578]. Substitution at C-2 of *N*-acetylmannosamine is tolerated, and the enzyme exhibits only a slight preference for defined stereochemistry at other centers.

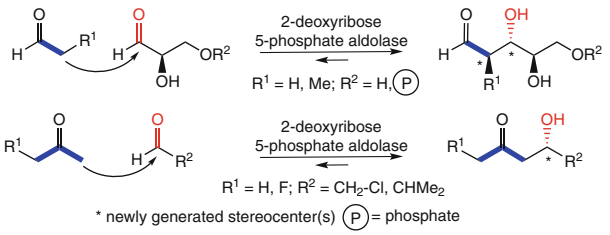
Other group III aldolases of preparative value are 3-deoxy-D-manno-octulosonate (KDO) aldolase [1579, 1580], macrophomate synthase [1581] as well as 2-keto-3-deoxy-6-phosphogluconate and -galactonate aldolases [1526, 1582].



Scheme 2.189 Aldol reactions catalyzed by sialic acid aldolase and industrial-scale synthesis of *N*-acetylneuraminic acid using a two-enzyme system

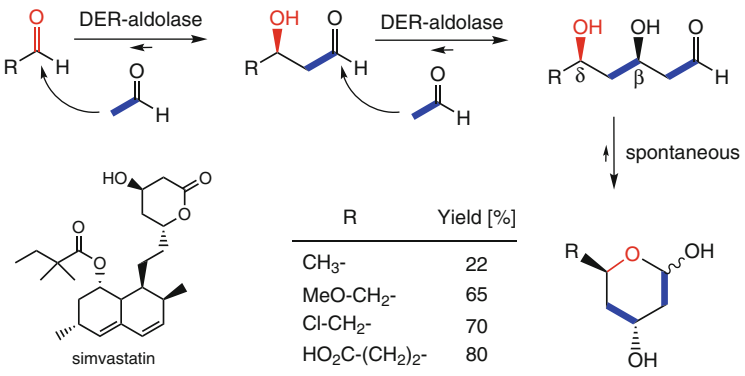
Group IV: Acetaldehyde-Dependent Aldolases

2-Deoxyribose-5-Phosphate Aldolase One of the rare aldolases, 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 (Scheme 2.190; $R^1 = H$, $R^2 =$ phosphate). This aldolase is unique in that it condenses *two aldehydes* (instead of a ketone and an aldehyde) in a self- or cross-aldol reaction to form aldoses (Schemes 2.179 and 2.190). Like fructose 6-phosphate aldolase, the enzyme (which has been overproduced [1583]) 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. DERA provides access to β-hydroxy aldehydes and -ketones with generation of a chiral center.



Scheme 2.190 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 2.191) [1584]. When a (substituted) aldehyde was used as acceptor, coupling of acetaldehyde (as donor) led to the corresponding β-hydroxy aldehyde as



Scheme 2.191 Sequential aldol reactions catalyzed by DER aldolase for synthesis of statin side chains

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 cascade (which would lead to the formation of a polymeric product if uninterrupted) is terminated by the (spontaneous) formation of a stable hemiacetal (lactol). 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, collectively denoted as 'statins' [1585].[42] Several derivatives thereof are produced on industrial scale using DER-aldolase mutants at product concentrations exceeding 100 g/L [1586, 1587].

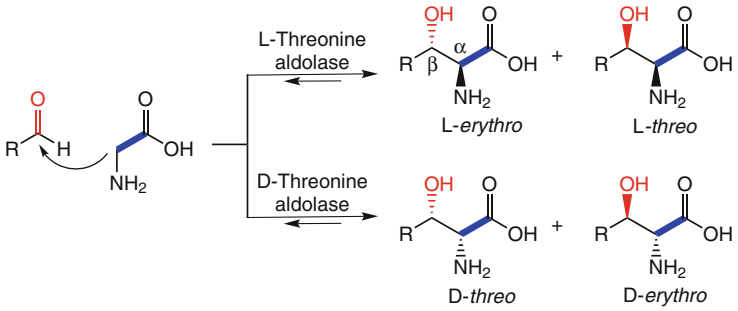
This concept provides rapid access to polyfunctional complex products from cheap starting materials in a one-pot reaction. It has recently been extended by combining various types of aldolases together to perform three- and four-substrate cascade reactions [1588, 1589].

Group V: Glycine-Dependent Aldolases

One remarkable feature of group V aldolases is their requirement for an amino acid as donor – glycine (Scheme 2.192) [1590, 1591]. Since the donor bears an amino group, their mechanism of action is related to Type I aldolases, with the difference that umpolung of the (glycine) donor to an enamine species is not effected by an ε-amino-moiety of lysine within the protein, but via Schiff-base formation with the aldehyde group of a pyridoxal-5′-phosphate cofactor (PLP, Sect. 2.6.2, Scheme 2.221). However, the nucleophilic attack of Cα onto an aldehyde acceptor forming an α-amino-β-hydroxy acid is essentially the same. Since two new stereocenters are formed, four possible stereoisomers can by formally obtained. However, in contrast to DHAP-dependent aldolases (Scheme 2.180), the complete set of stereo-complementary threonine aldolases has not yet been found [1592, 1593]. β-Hydroxyamino acids are multifunctional compounds with numerous applications in the synthesis of complex bioactive structures, such as peptide mimetics, protease inhibitors and antibiotics. It is thus not surprising, that threonine aldolases have been frequently used for their synthesis, also on industrial scale [1594].

D- and L-Threonine Aldolases These enzymes are involved in the biosynthesis/degradation of α-amino-β-hydroxyamino acids, such as threonine and they exquisitely control the stereochemistry of the α-amino configuration, which is either D or L, depending on the type of enzyme. However, they show only low-moderate specificities for the β-hydroxy-center, which leads to diastereomeric *threo*/*erythro* product mixtures [1595].

[42]For instance, atorvastatin (Lipitor™), rosuvastatin (Crestor™) or simvastatin.



R	Enzyme	D : L	*threo* : *erythro*
Me	L-ThrA	<1 : 99	1 : 99
Me	D-ThrA	>99 : 1	~50 : 50
i-Pr	L-ThrA	<1 : 99	6 : 94
i-Pr	D-ThrA	>99 : 1	93 : 7

Scheme 2.192 Aldol reactions catalyzed by L-threonine aldolase

For example, recombinant D- and L-threonine aldolases from *E. coli* and *Xanthomonas oryzae*, respectively, were very faithful with respect to the formation of D- or L-configurated centers at Cα, but their diastereoselectivity for the β-hydroxy group was less pronounced. In particular, the D-enzyme gave a 1:1 *threo-erythro* mixture of the natural amino acid threonine (R = Me), which was improved in case of the sterically more demanding non-canonical analog (R = *i*−Pr).

For biocatalytic applications, several threonine aldolases show broad substrate tolerance for various acceptors, including aromatic aldehydes [1596, 1597]; however, conjugated enals were not accepted. The L-enzyme from *Candida humicola* was used in the synthesis of multifunctional α-amino-β-hydroxy acids, which possess interesting biological properties (Scheme 2.192) [1597]. 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.

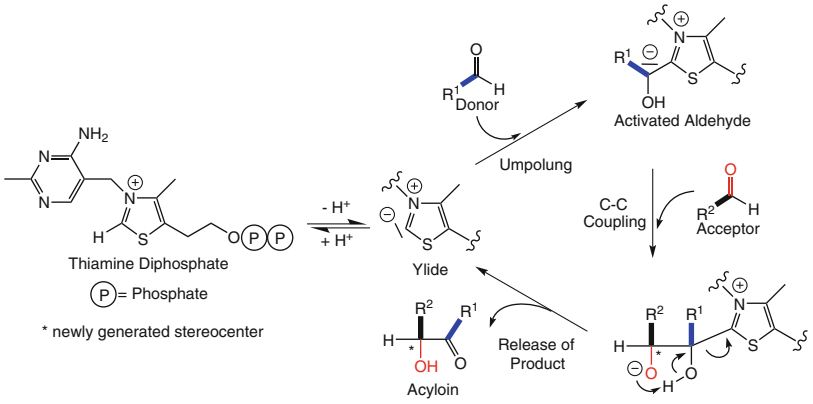
As with other aldolases, efforts were undertaken to overcome the limitation of threonine aldolases for their donor glycine. Screening uncovered novel aldolases, which were able to accept D- or L-alanine as donor, which opens the possibility to synthesize α-amino-α,α-dialkyl-β-hydroxy acids containing a quaternary center, which is difficult to obtain by conventional methods [1598, 1599]. Compounds of this type are important conformational modifiers of physiologically active peptides and building blocks for protease inhibitors.

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 [1600].

2.4.2 Thiamine-Dependent Acyloin and Benzoin Reactions

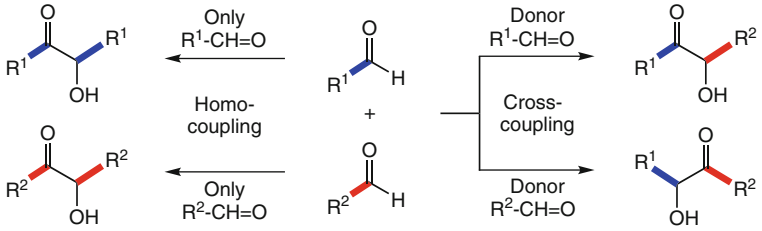
In the aldol reaction, C–C coupling always takes place in a head-to-tail fashion between the umpoled Cα atom of an enolate- or enamine-species (acting as donor) and the carbonyl C of an acceptor forming a β-hydroxycarbonyl product. In contrast, head-to-head coupling of two aldehydic species involving 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 2.193) [1601–1604]. 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 [1605–1607]. A schematic representation of the mechanism of enzymatic carboligation by ThDP-dependent enzymes is depicted in Scheme 2.193 [1608]. 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, equivalent to an enamine. This umpoled species attacks an acceptor aldehyde (R^2–CH=O) going in hand with C–C bond formation. Tautomerization of the diolate intermediate releases the α-ketol (acyloin/benzoin) product and regenerates the cofactor. Overall, the net reaction constitutes the transfer or an acyl anion equivalent to an aldehyde.



Scheme 2.193 Thiamine diphosphate-dependent carboligation of aldehydes (acyloin reaction)

In the enzymatic aldol reaction, the role of the donor and acceptor is strictly determined by the high specificity of the enzyme for the donor, hence only a single 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 is obtained via homocoupling; however, a pair of regioisomeric α-hydroxyketones can be obtained via cross-coupling, when two different aldehydes are used, the ratio of which is determined by the preference of the enzyme for the donor versus acceptor, e.g., acetaldehyde versus benzaldehyde or vice versa (Schemes 2.194 and 2.200).

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.

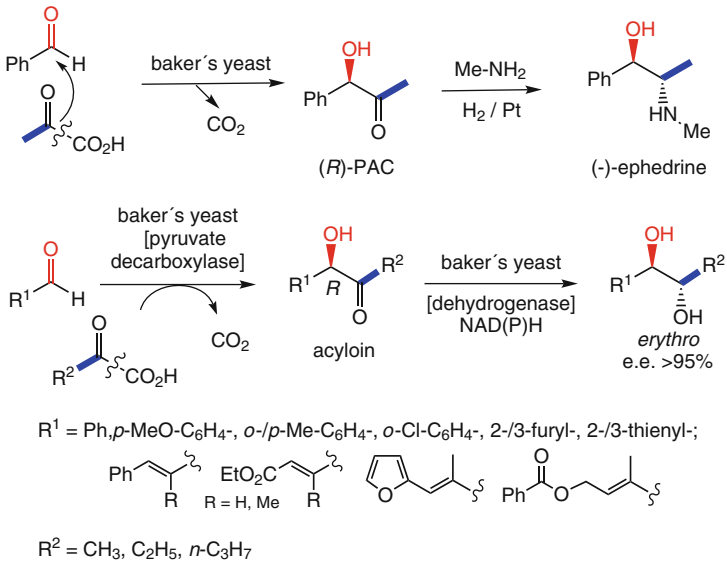


Scheme 2.194 Regioisomeric α-hydroxyketones obtained from homo- and cross-coupling of aldehydes

Acyloin and Benzoin Reactions[43]

Historically, the biocatalytic acyloin reaction was first observed by Liebig in 1913 during studies on baker's yeast [1609]. 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 [1610]. 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 operated on a scale of ~500 t/a [1611, 1612] (Scheme 2.195). Subsequent studies revealed that this yeast-based protocol can be extended to a broad range of aldehydes [1613, 1614].

[43]For the sake of clarity, the redox-neutral acyloin formation from two aldehydes is referred to as 'acyloin reaction', as opposed to the 'acyloin condensation', which constitutes the reductive condensation of two esters.



Scheme 2.195 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 [1615] and it turned out that the enzyme responsible for this reaction is pyruvate decarboxylase (PDC) [1616]. 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 [1617]. Since pyruvate decarboxylase accepts α-ketoacids other than pyruvate, C_2-through C_4-equivalents can be transferred onto a large variety of aldehydes [1618–1620]. 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. 2.2.2 and 2.2.3, Scheme 2.112). The optical purity of the diols is usually better than 90% [1621–1624].

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 acceptor aldehyde to give the corresponding primary alcohol (catalyzed by yeast alcohol dehydrogenases, see Sect. 2.2.2) and saturation of the α,β-double bond (catalyzed by ene-reductases, see Sect. 2.2.5) are the major competing reactions. To avoid low yields associated with yeast-catalyzed acyloin- [1625–1627] and benzoin-reactions [1625], isolated enzymes are nowadays used [1629–1631].

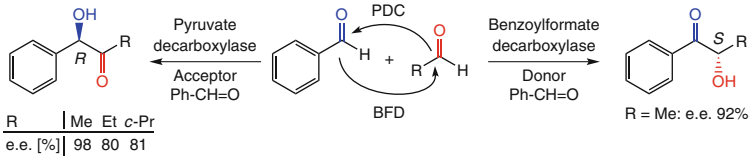
Pyruvate Decarboxylase

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 [1625, 1632, 1633] and from *Zymomonas mobilis* [1634].

From a synthetic viewpoint, however, its carboligation activity is more important [1635–1637]: 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 [1638]. 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 acyloins (Schemes 2.195 and 2.196). Self-coupling 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 [1639–1641]. 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 [1628]. With acetaldehyde as acceptor, it yields the complementary regio-isomeric product to PDC (Scheme 2.196).

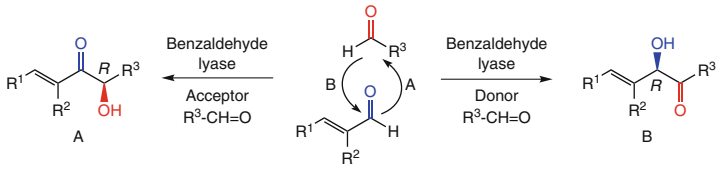


Scheme 2.196 Regiocomplementary carboligation of aldehydes catalyzed by pyruvate and benzoylformate decarboxylase

Benzaldehyde Lyase

Benzaldehyde lyase (BAL) [EC 4.1.2.38] from *Pseudomonas fluorescens*, which was able to grow on lignin-degradation products, such as benzoin, is a powerful biocatalyst for the homo- and cross-carboligation of various aromatic and aliphatic aldehydes. In contrast to PDC and BFD, BAL shows only negligible decarboxylation activity, while C–C lyase- and carboligation are dominant [1642–1644]. Especially the self-ligation of benzaldehyde yields benzoin with high activity and stereoselectivity (e.e. >99%), making this enzyme very interesting for industrial processes [1645]. For benzoin formation, *o*-, *m*-, and *p*-substituted aromatic aldehydes are widely accepted as donors [1646]. Cross-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.

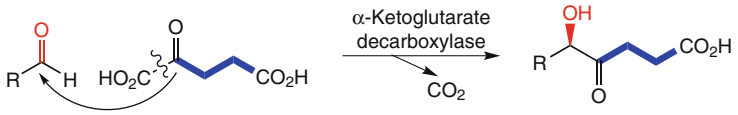
The remarkable synthetic potential of BAL is demonstrated by the regiocomplementary benzoin reaction of α,β-unsaturated aldehydes acting as donor or acceptor, respectively. While large aldehydes acted as donors (product type A), small counterparts served as acceptors leading to isomeric olefinic acyloins B in high e.e.s [1647] (Scheme 2.197).



Scheme 2.197 Regiocomplementary carboligation of aldehydes catalyzed by benzaldehyde lyase

α-Ketoglutarate decarboxylases

A useful extention to the set of acyloin-forming enzymes is the use of α-ketoglutarate decarboxylases [1648]. Like pyruvate decarboxylase, they catalyze the decarboxylative carboligation between an α-keto acid and aldehyde, but they use α-ketoglutarate as donor (Scheme 2.198). As a key molecule in the Krebs-cycle, the latter is abundantly available from glutamate. Among the enzymes tested, SucA from *E. coli* showed excellent stereoselectivities for aliphatic acceptors, whereas MenD (from *Mycobacterium tuberculosis*) was best for (substituted) benzaldehydes [1649]. This strategy allows acyloin formation from aldehydes going in hand with extension by a (succinoyl) C_4-unit. Concomitant decarboxylation provides a strong driving force and ensures quantitative conversions (Scheme 2.198).



R	Enzyme	E.e. [%]
Me, Et	SucA	94
n-Bu	SucA	90
n-Pent	SucA	82
o-F-, *m*-I-C_6H_4-, Ph	MenD	94-96

Scheme 2.198 Acyloin formation with C_4-extention using α-ketoglutarate decarboxylase SucA

Transketolase

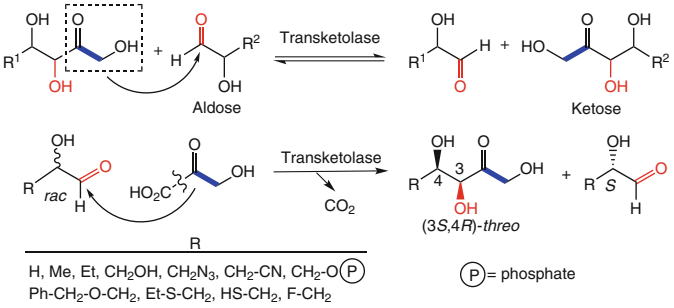
In the oxidative pentose phosphate pathway, ThDP-dependent transketolase[44] catalyzes the reversible interconversion of phosphorylated aldoses and ketoses via transfer of a terminal 2-carbon hydroxyacetyl-unit (Scheme 2.199) [1650]. Its

[44]Correctly, this enzyme has the charming name 'D-seduheptulose-7-phosphate: D-glyceraldehyde-3-phosphate glycoaldehyde transferase'.

mechanism resembles a classical acyloin reaction mediated by ThDP [1651, 1652]. Fortunately, the natural phosphorylated substrate(s) can be replaced by the donor hydroxypyruvate [1653], 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 monosaccharide-like acyloins on a preparative scale [1654–1657]. Transketolases were initially be obtained from yeast [1658] and spinach [1659], their overexpression has opened the way for large-scale production [1660, 1661].

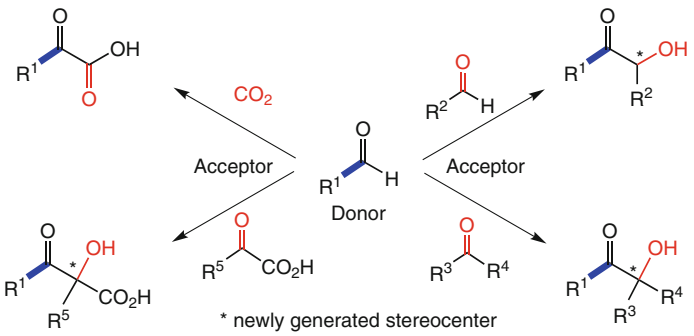
Transketolases from various sources have been shown to possess a broad acceptor spectrum yielding products with complete (*S*)-stereospecificity for the newly formed C-3 stereocenter [1662]. Generic aldehydes are usually converted with full stereocontrol and even α,β-unsaturated aldehydes are accepted to some degree.

α-Hydroxy aldehydes show enhanced rates by mimicking the natural substrate [1651]. Interestingly, transketolase recognizes chirality in the aldehydic acceptor moiety to a greater extent than aldolases. Thus, when (stereochemically stable) racemic α-hydroxyaldehydes are employed as acceptors, an efficient kinetic resolution of the α-center is achieved (Scheme 2.199). Only the (α*R*)-enantiomer is transformed into the corresponding keto-triol leaving the (α*S*)-counterpart behind [1663]. In a related manner, when (±)-3-azido-2-hydroxypropionaldehyde was chosen as acceptor, only the D-(*R*)-isomer reacted and the L-(*S*)-enantiomer remained unchanged [1546].



Scheme 2.199 Reversible interconversion of aldoses and ketoses and acyloin reaction catalyzed by transketolase

The broad synthetic potential ThDP-dependent enzymes for asymmetric C–C bond formation is by far not fully exploited with the acyloin- and benzoin-reactions 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 [1664]. On the other hand, the acceptor range may be significantly widened to encompass ketones, α-ketoacids and even CO_2, which leads to novel types of products (Scheme 2.200).



Scheme 2.200 Future potential of thiamine-dependent C–C bond formation

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 [1665, 1666].

The asymmetric addition of (small) molecules, such as water, ammonia and C-H acidic carbon nucleophiles (such as hydrogen cyanide, nitroalkanes, β-dicarbonyl compounds) onto C=C or C=O bonds is typically catalyzed by lyases. Depending on the substitution pattern of the substrate, up to two chiral centers are created from a prochiral substrate via desymmetrization.

2.5.1 Addition of Water

The asymmetric addition of water onto olefins is one of the 'dream-reactions' in organic synthesis and represents one of the (largely unsolved) problems of catalysis. Enzymes called hydratases [EC 4.2.1.X][45] can catalyze this reaction [1667].

In analogy to the rules of chemical catalysis, two different types of enzymatic hydration mechanisms exist:

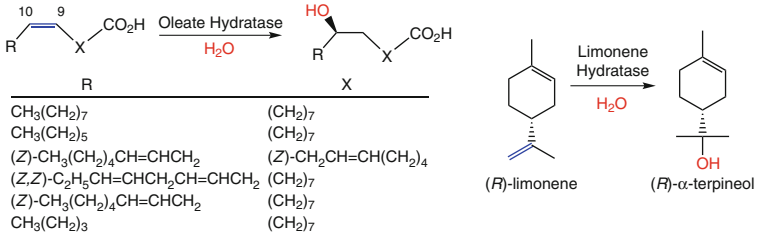
- Hydration of electron-rich (isolated) alkenes proceeds via acid-catalysis and obeys the Markovnikov rule, which dictates that the nucleophile [OH^-] is attached to the more highly substituted carbon [1668].
- Electron-deficient alkenes, which are polarized by an electron-withdrawing (carbonyl) group, are hydrated via Michael-type addition with nucleophilic attack at Cβ. If the corresponding proton to be added is in the α-position to a carboxyl group, hydration occurs in an *anti*-fashion (Scheme 2.202), in case of a (coenzyme A) thioester, the *syn*-product is preferred [1669, 1670].

[45] Occasionally also called 'hydro-lyases'.

For biotransformations, hydratases acting on isolated olefins are developed to a lesser extent, although a few have gained industrial importance.

Oleate hydratases from different sources are employed for the production of (*R*)-10-hydroxystearic acid starting from oleate [(9*Z*)-octadecenoic acid] with high volumetric productivities (12 g L^{-1} h^{-1}) (Scheme 2.201). Although regio- and stereoselectivities are very high, the substrate tolerance is somewhat limited to various long-chain unsaturated fatty acids possessing a (9*Z*)-olefinic bond, such as palmitoleic [(9*Z*)-hexadecenoic], γ-linoleic [(all-*Z*)-6,9,12-octadecatrienoic], linoleic [(all-*Z*)-9,12-octadecadienoic), myristoleic [(9*Z*)-tetradecenoic] and α-linoleic acid [(all-*Z*)-9,12,15-octadecatrienoic acid) in decreasing order of reactivity relative to oleic acid [1671].

In nature, limonene hydratase is involved in the biodegradation of the monoterpene limonene, which is available in large amounts as waste-product from citrus fruit processing. Regio- and enantioselective hydration of (*R*)-limonene yields (*R*)-α-terpineol, which is a popular olfactory component exerting a strong lilac-like smell [1672]. In contrast, the (*S*)-enantiomer displays a conifer-like odor.

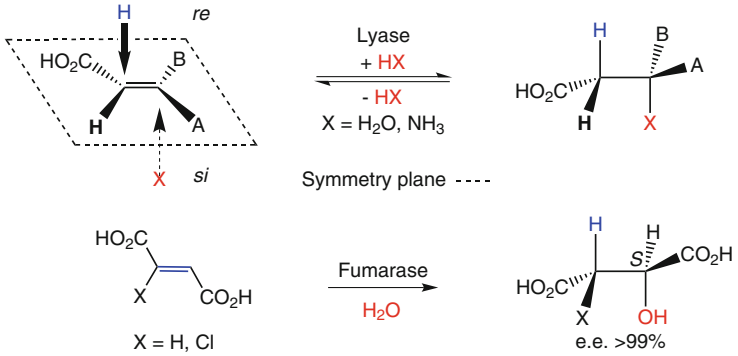


Scheme 2.201 Regio- and stereoselective hydration of non-activated isolated olefins

Fumarase and malease catalyze the stereospecific addition of water onto C=C bonds conjugated to a carboxylic acid [1673]. Both reactions are mechanistically related and proceed in a *anti*-fashion [1674, 1675], with protonation occurring from the *re*-side (Scheme 2.202). Both enzymes are complementary with respect to the (*E*)- or (*Z*)-configuration of their substrates and show exceptional stereoselectivities for the nucleophilic attack, but their substrate tolerance is rather narrow.

The addition of water onto fumaric acid by fumarase leads to (*S*)-malic acid (Scheme 2.202). The latter is used as an acidulant in fruit juices, carbonated soft drinks, and candies, and is performed at a capacity of ~2000 t/year [1676, 1677]. While 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. Replacement of one of the carboxylic groups or changing the stereochemistry of the double bond from (*E*) to (*Z*) is not tolerated by fumarase [1678].

The analogous hydration of the stereoisomeric (*Z*)-isomer (maleic acid) is catalysed by malease (maleate hydratase) and produces the mirror-image (*R*)-malate [1679, 1680]. The latter enzyme also accepts 2-methylmaleate (citraconate) to form (*R*)-2-hydroxy-2-methylsuccinate (2-methyl-maleate) [1681].

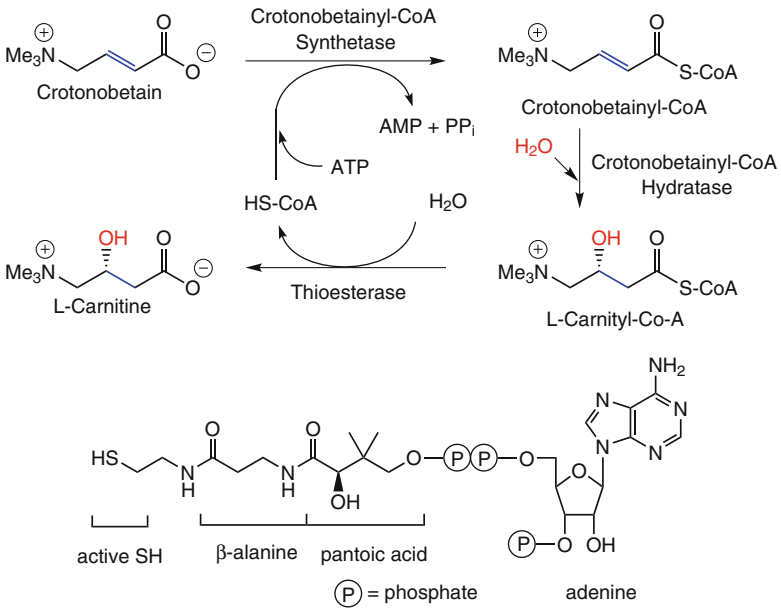


Scheme 2.202 *anti*-Selective asymmetric hydration and hydroamination of activated conjugated C=C bonds and fumarase-catalyzed formation of (*S*)-malate from fumaric acid

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 (Scheme 2.203). 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 in a *syn*-fashion 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 [1423, 1682, 1683].

An elegant example for this biotransformation is the asymmetric hydration of crotonobetaine yielding the 'nutraceutical' (*R*)-carnitine (Scheme 2.203), which is used as an additive in baby food, geriatric nutrition and health sport. In order to avoid the undesired degradation of the product by the whole-cell biocatalyst, mutant strains lacking carnitine dehydrogenase have been developed to produce (*R*)-carnitine at a capacity of >100 t/year [1684–1686].

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 non-activated 'inner' (*E*)-double bond of terpene alcohols (e.g., nerolidol) or –ketones (e.g., geranyl acetone) in a highly selective manner [1687]. 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



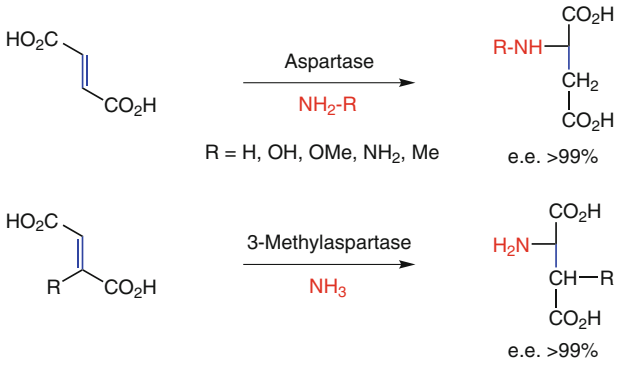
Scheme 2.203 Asymmetric hydration of crotonobetaine to carnitine via a multienzyme-system

of α,β-unsaturated butyrolactones with high enantioselectivity, furnishing β-hydroxylactones in moderate yields [1688]. Furthermore, C=C hydration reactions are often not far from equilibrium, which requires sophisticated process engineering, such as selective crystallization or the use of membrane technology.

2.5.2 *Addition of Ammonia*

The addition of ammonia across C=C bonds is equivalent to a 'hydroamination' and is catalyzed by ammonia lyases [1689], such as aspartase [EC 4.3.1.1], 3-methylaspartase [1690], and phenylalanine ammonia lyase [EC 4.3.1.5]. The equilibrium can be shifted by high concentrations of ammonia (~4–6 M) [1691].

Enzymatic amination of fumaric acid using aspartase is used for the production of L-aspartic acid at a capacity of ~10,000 t/year (Scheme 2.204) [1692–1695]. Although aspartase is one of the most specific enzymes known by accepting only its natural substrate [1696–1698], some variations concerning the *N*-nucleophile are tolerated: Hydroxylamine, hydrazine, methoxyamine, and methylamine are accepted and furnish the corresponding *N*-substituted L-aspartate derivatives [1699–1701].

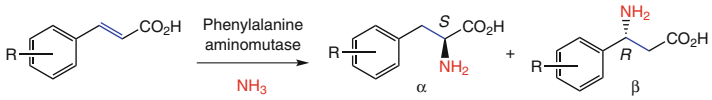


R	Yield [%]
H	90
Me, Et, Cl	60-61
i-Pr	54
n-Pr	49
n-Bu	0

Scheme 2.204 Amination of fumarate derivatives by aspartase and 3-methylaspartase

In contrast to aspartase, some structural variations are tolerated by the related 3-methylaspartase (Scheme 2.204). For instance, the methyl group in the natural substrate may be replaced by a chlorine atom or by small alkyl moieties [1702], but the fluoro- and the iodo-analog are not good substrates. Although the bromo-derivative is accepted, it irreversibly inhibits the enzyme [1703].

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 [1423, 1704]. The enzyme tolerates a wide variety of halogen substituents on the phenyl ring and also accepts heterocyclic analogs, such as pyridyl and thienyl derivatives [1705]. 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 [1706]. 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) [1707]. 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- versus 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 2.205) [1708, 1709].



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 2.205 Formation of α- and β-phenylalanine derivatives using phenylalanine ammonia mutase

2.5.3 Cyanohydrin Formation and Henry-Reaction

Hydroxynitrile lyase enzymes (HNLs) catalyze the asymmetric addition of hydrogen cyanide onto a carbonyl group of an aldehyde or a ketone thus forming a chiral cyanohydrin [1710–1715], [46] a reaction which was used for the first time as long ago as 1908 [1716]. In nature, HNLs catalyze the cleavage of cyanohydrins derived from cyanoglucosides and cyanolipids (Scheme 2.95) to liberate HCN as a defence mechanism against herbivores and microbial attack. This activity is not only widespread in plants, but is also found in bacteria, fungi, lichens and insects. Although they catalyze the same reaction, HNLs belong to (at least) four different structural folds, i.e. α/β-hydrolase proteins, oxidoreductases, cupins[47] and alcohol dehydrognases, which indicates a perfect example of convergent evolution [1717].

Cyanohydrins are rarely used as products per se, but they represent versatile starting materials for the synthesis of various types of compounds [1718]. Most prominent, chiral cyanohydrins constitute the alcohol moieties of several commercial pyrethroid insecticides (see Scheme 2.208) (see below) [1719].

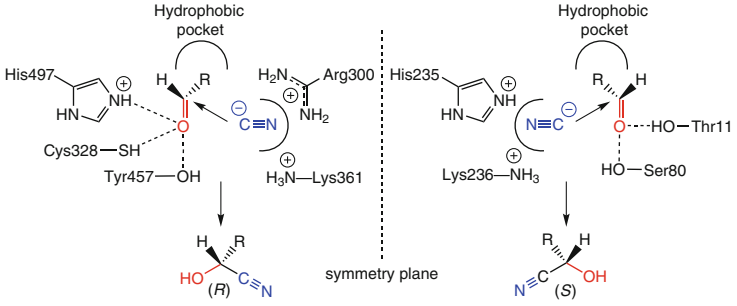
Since only a single enantiomer is produced during the reaction – through desymmetrization of a prochiral substrate – the availability of enzymes possessing opposite stereochemical preference is of importance to gain access to both (*R*)- and (*S*)-cyanohydrins (Scheme 2.207). Fortunately, an impressive number of hydroxy-nitrile lyases can be isolated from cyanogenic plants to meet this requirement [1720–1724].

(*R*)-Specific enzymes are obtained predominantly from the *Rosacea* family (almond, plum, cherry, apricot) and they have been thoroughly investigated [1725–1728]. 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.

[46] Outdated terms for hydroxynitrile lyases are 'oxynitrilases' or 'hydroxynitrilases'.

[47] A family of small barrel-shaped proteins, named after 'cupa' (Latin) = small barrel.

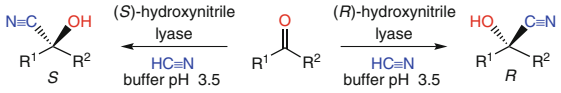
On the other hand, (*S*)-hydroxynitrile lyases [1729–1732] were found in *Sorghum bicolor* [1733] (millet), *Hevea brasiliensis* [1734, 1735] (rubber tree), *Ximenia americana* [1736] (sandalwood), *Sambucus niger* [1737] (elder), *Manihot esculenta* [1731, 1738] (cassava), flax, and clover. They do 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 [1739, 1740], the mechanism of enzymatic cyanohydrin formation has been elucidated as follows (Scheme 2.206) [1741]: The substrate is positioned in the active site with its carbonyl group bound through a 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 opposite sides from cyanide-binding pockets, which are made of positively charged Arg/Lys- or His/Lys-residues [394].



Scheme 2.206 Mechanism 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 [1742].

- 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 [1743–1746].
- Straight-chain aliphatic aldehydes are transformed as long as they are not longer than six carbon atoms; the α-position may be substituted with a methyl group. It is noteworthy, 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.
- Methyl ketones are transformed into cyanohydrins [1747], while ethyl ketones are impeded by low yields [1748].
- For large or sterically demanding aldehydes, such as *o*-chlorobenzaldehyde, (*R*)-HNL mutants possessing a more spacious active site were constructed [1749, 1750]. The (*R*)-*o*-chloromandelonitrile thus obtained represents the chiral core of the blockbuster clopidogrel (Plavix) to prevent heart attack or stroke (Scheme 2.207, Table 2.5).



Scheme 2.207 Stereocomplementary asymmetric cyanohydrin formation

Table 2.5 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

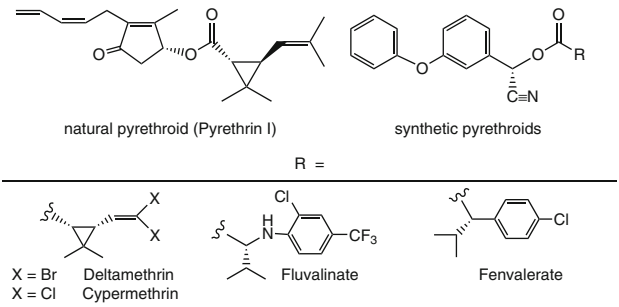
The (*S*)-hydroxynitrile lyase from *Hevea brasiliensis* has been made available in sufficient quantities by cloning and overexpression to allow industrial-scale applications [1751]. Of particular interest is the synthesis of the (*S*)-cyanohydrin from *m*-phenoxybenzaldehyde (Table 2.6), which is an important intermediate for synthetic pyrethroids.

Table 2.6 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-PhO–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*) (Scheme 2.208). 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. All of these synthetic pyrethroids contain asymmetric carbon atoms and their insecticidal activity resides predominantly in one particular isomer. In order to reduce the environmental burden during pest control, single isomers are marketed [1752].



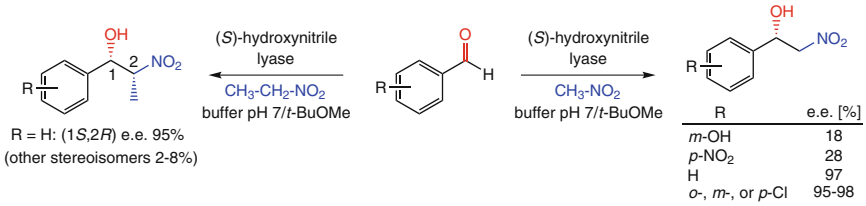
Scheme 2.208 Natural and synthetic pyrethroids

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 depletion 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 [1753] 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 acetone cyanohydrin [1754, 1755] as donor for hydrogen cyanide. The latter is considerably more easy to handle due to its higher boiling point (82 °C) compared to HCN (26 °C). Using this technique, the competing chemical cyanohydrin formation is negligible due to the low concentration of free hydrogen cyanide.

A fascinating variant of the enzymatic cyanohydrin formation consists in the use of nitroalkanes (as nonnatural nucleophiles) instead of cyanide (Scheme 2.209), which constitutes a biocatalytic equivalent to the Henry-reaction, which is not known in nature thus far. It produces vicinal nitro-alcohols, which are valuable precursors for amino alcohols. Using (*S*)-HNL from *Hevea brasiliensis* or *Arabidopsis thaliana*

[1756], the asymmetric addition of nitromethane to *p*-methyl- and halogen-substituted benzaldehydes gave the nitroalcohols in high e.e.s, while for *p*-nitro- and *m*-hydroxybenzaldehyde the stereoselectivity dropped sharply [1757, 1758]. With nitroethane, two stereocenters are created: Whereas the stereoselectivity for the alcoholic center at C_1 was high (e.e. 95%), the recognition for the adjacent center bearing the nitro moiety was modest and diastereomers were formed.



Scheme 2.209 Asymmetric Henry-reaction catalyzed by (*S*)-hydroxynitrile lyases

2.5.4 Michael-Type Additions

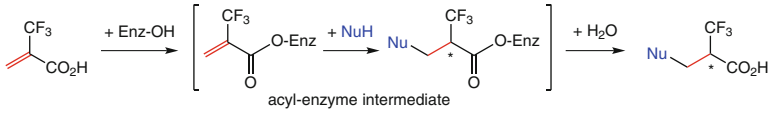
Since its serendipitous discovery in 1887 [1759], the nucleophilic addition of 1,3-dicarbonyl donors onto α,β-unsaturated carbonyl acceptors – known as the Michael-addition – became a central tool for the (stereoselective) C–C bond formation [1760, 1761]. Despite its simplicity based on base-catalysis, it is a puzzling fact that nature did not evolve an analogous enzyme and a 'Michael-lyase' as such does not exist.

Enzyme-catalyzed C–C bond forming Michael-type additions of umpoled carbonyl species onto α,β-unsaturated carbonyl acceptors are extremely rare and are found in polyketide pathways, e.g. in the biosynthesis of rhizoxin [1762]. An analogous reaction where a ThDP-bound carbanion performs a 1,4-addition onto an enal (instead of the usual 1,2-addition onto a carbonyl group to form an acyloin, Schemes 2.193 and 2.197) are catalyzed by PigD and MenD and are equivalent to the Stetter reaction [1763, 1764].

Early studies in search for enzyme-catalyzed Michael-type additions focused on the exploitation of the catalytic promiscuity of well-characterized hydrolytic enzymes, such as lipases, acylases and proteases using 1,3-dicarbonyl donors and enals or nitroalkenes as acceptors. Although encouraging catalytic activities translating into yields of up to 90% were found, stereoselectivities remained disappointingly low and e.e.s typically ranged within ~20–40% [1765–1767]. In the majority of cases, the product was (near) racemic [1768] or were not reported at all. Attempts to rationally design a 'Michael-lyase' from a lipase-scaffold gave disappointingly low stereoselectivities [1769–1772]. Consequently, these seminal studies are interesting from an evolutionary and mechanistic standpoint, but they are synthetically irrelevant.

As a typical example for a stereoselective Michael-type addition catalyzed by hydrolytic enzymes is depicted in Scheme 2.210. 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 [1773]. The

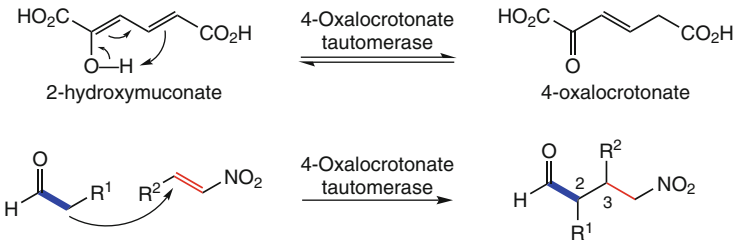
reaction mechanism probably involves the formation of an acyl enzyme intermediate (Sect. 1.1, Scheme 2.1). Being an activated derivative, the latter is more electrophilic than the 'free' carboxylate and undergoes an asymmetric Michael addition by the nucleophile, directed by the chiral environment of the enzyme.



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 2.210 Asymmetric Michael addition catalyzed by hydrolytic enzymes

The first successful attempt to unearth a highly stereoselective enzyme-catalyzed Michael-type addition employed 4-oxalocrotonate tautomerase (4-OT),[48] which catalyzes the tautomerization of 2-hydroxymuconate to 2-oxo-3-hexenedioate (4-oxalocrotonate), a step in the oxidative biodegradation of alkylbenzenes, such as toluene and xylene (Scheme 2.211). 4-OT contains a rare *N*-terminal proline residue (Pro-1) in its active site, which acts as acid/base in the 'natural' tautomerization. In contrast, the mechanism of the Michael-addition presumably involves a nucleophilic



R^1	R^2	e.e. [%]	d.e. [%]	yield [%]
H	Ph	89 (*S*)	—	46
H	*p*-Cl-C_6H_4	84 (*S*)	—	64
H	(*E*)-CH=CH-Ph	95 (*S*)	—	38
H	*i*-Bu	98 (*R*)	—	74
Me	Ph	50 (2*R*,3*S*)	86	64

Scheme 2.211 Tautomerization and Michael-type addition catalyzed by 4-oxalocrotonate tautomerase

[48] [EC 5.3.2.6] also called 2-hydroxymuconate tautomerase or 4-oxalocrotonate isomerase.

enamine intermediate (derived from a Schiff-base between Pro-1 and the aldehyde donor), a reminiscent to the organocatalytic analog. With acetaldehyde as donor, e.e.s of up to 98% were achieved, the stereoselectivities were lower with propanal [1774–1776].

2.6 Transfer Reactions

2.6.1 Glycosyl Transfer Reactions

Glycosidic molecules in the form of oligo- or polysaccharides represent about two-thirds of the carbon found in the biosphere, largely in the form of (hemi) cellulose and chitin.[49] While β-linked polysaccharides provide structural support, α-linked glycans, such as starch and glycogen, are more easily cleaved and serve as energy storage [1777]. As a consequence, glycosyl transfer is certainly one of the most important biochemical reactions [1778].

While polysaccharides are the basis for material sciences [1779], oligo-sugars play a vital role in intracellular migration and secretion of glycoproteins, cell–cell interactions, oncogenesis, and interaction of cell surfaces with pathogens [1780–1782]. 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 comparable size [1783].[50] 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 their low concentration in carbohydrate mixtures 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 [1784]. In this context, biocatalysts are attractive as they allow the regio- and stereospecific synthesis of oligosaccharides with a minimum of protection and deprotection steps [1504–1506, 1785–1793]. There are four groups of enzymes which can be used for the synthesis of oligosaccharides[51] (Scheme 2.212) [1794]. However, differences are not always clear-cut and mixed activities are sometimes observed.

Glycosyl transferases are responsible for the *biosynthesis* of oligosaccharides in vivo. They require that the sugar donor is activated on the anomeric center by

[49]Cellulose is the most abundant organic carbon in the ecosphere and its global standing crop has been estimated as 9.2×10^{11} tons, with an annual production of 0.85×10^{11} tons; the annual production of marine chitin was estimated as 2.3×10^{9} tons.

[50]The possible number of linear and branched oligosaccharide isomers for a reducing hexasaccharide was calculated to encompass 1.05×10^{12} structures, see [1782].

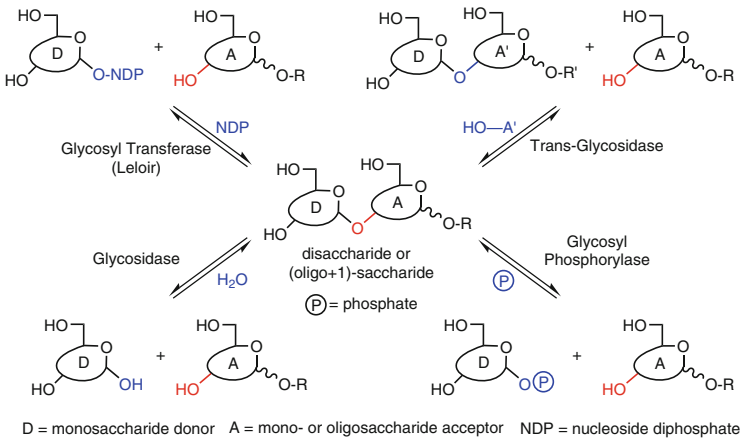
[51]http://www.cazy.org.

phosphorylation prior to the condensation step. The activating group acts as a leaving group (LG) and is either a nucleoside diphosphate (in the Leloir pathway [1795]) or a simple phosphate (in non-Leloir enzymes [1796]).

Trans-glycosidases interconvert carbohydrate chains by transferring one sugar unit from a (di)saccharide (acting as donor) onto an acceptor and they represent an alternative pathway for gluconeogenesis, which is independent on phosphorylated donors. Due to their role in biosynthesis, glycosyl transferases and trans-glycosidases are generally rather specific with respect to their substrate(s) and the nature of the glycosidic bond to be formed [1797].

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. Glycosidases can be used for glycoside synthesis in the reverse (condensation) direction.

Glycosyl phosphorylases use phosphate (instead of water) for the breakdown of a glycosidic bond, which yields a glycosyl phosphate rather than a non-activated monosugar. The phosphorolytic degradation of oligosaccharides is a more energy-efficient alternative to hydrolysis by glycosidases.



Scheme 2.212 Four different types of enzymatic glycosylation reactions

2.6.1.1 Glycosyl Transferases

In the Leloir-pathway, a sugar is phosphorylated in a first step by a kinase to give a sugar-1-phosphate. This activated sugar subsequently reacts with a nucleoside triphosphate under catalysis of a nucleoside transferase and forms a chemically activated nucleoside diphosphate sugar (NDP, Scheme 2.212) [1798]. These key nucleoside diphosphate sugars constitute the activated 'donors' in the subsequent condensation with the hydroxyl group 'acceptors' a mono- or oligosaccharide, a protein or a lipid,

which is catalyzed by a glycosyl transferase. The key building blocks serving as donors are UDP-Glc, UDP-Gal, UDP-GlcNAc, UDP-GalNAc, UDP-Xyl, GDP-Man, GDP-Fuc and CMP-sialic acid [1799]. Since each NDP-sugar requires a distinct group of glycosyl transferases, a large number of highly specific glycosyl transferases are neccessary. 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 [1800].

Chemists employ Leloir-enzymes to the synthesis of oligosaccharides and the majority of synthetic reactions are performed by UDP-glycosyl transferases [1801–1806]. Two requirements are currently limiting large-scale applications, namely the availability of the sugar nucleoside phosphates at reasonable costs and the matching glycosyl transferases. 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 [1807, 1808]. The availability of NDP-sugars is ensured by in-situ regeneration of the sugar nucleotide from the released nucleoside phosphate via utilizing phosphorylating enzymes, which avoids co-product inhibition caused by the released nucleoside diphosphate [1809] (Scheme 2.213).

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 [1800, 1810–1813] (Table 2.7). Other glycosyl transferases, although less well-studied than UDP-Gal transferase, also appear to tolerate various acceptors as substrates [1814–1817].

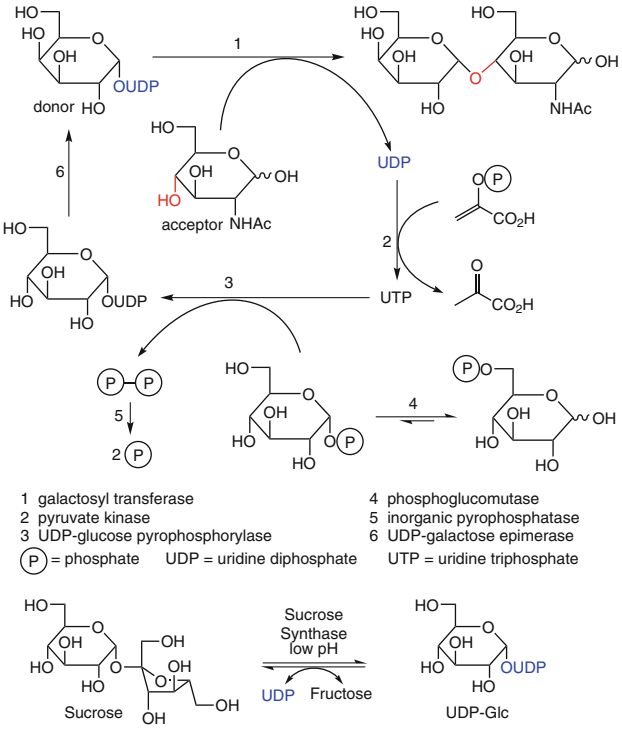
Table 2.7 Glycoside synthesis using β-galactosyl transferase from the Leloir pathway (donor = UDP-Gal, Scheme 2.212)

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 use of the multienzyme systems, which arise due to the need to prepare the activated UDP-donor sugar in situ, is exemplified with the synthesis of *N*-acetyllactosamine [1818] (Scheme 2.213). 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. 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.

An elegant method for the recycling of UDP-glucose from UDP employs sucrose synthase for the transfer of a glucose unit from (cheap) sucrose onto UDP. Process optimization allowed UDP-Glc synthesis at ~100 g scale [1819].

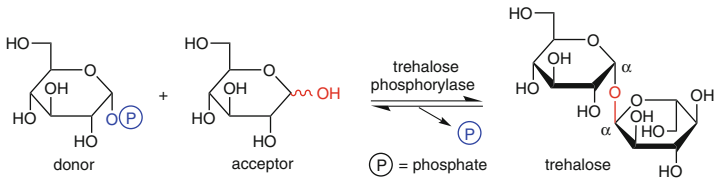


Scheme 2.213 Synthesis of *N*-acetyllactosamine using a six-enzyme system (Leloir pathway) and recycling of UDP-glucose at the expense of sucrose

2.6.1.2 Glycoside Phosphorylases and Trans-glycosidases

In order to avoid the need for activated nucleoside diphosphate sugar donors, attention has been drawn to oligosaccharide synthesis via non-Leloir transferases (Schemes 2.212 and 2.214). In this case, the activated donor is a more simple sugar-1-phosphate, which can be transferred by a single glycoside phosphorylase. The latter catalyzes the reversible cleavage/formation of a glycosidic bond using phosphate as nucleophile/leaving group, respectively [1820, 1821]. Retaining and inverting disaccharide phosphorylases for the conversion of sucrose, maltose, cellobiose and α,α-trehalose are known, which opens the way to use these naturally abundant disaccharides as cheap glycosyl donors [1822].

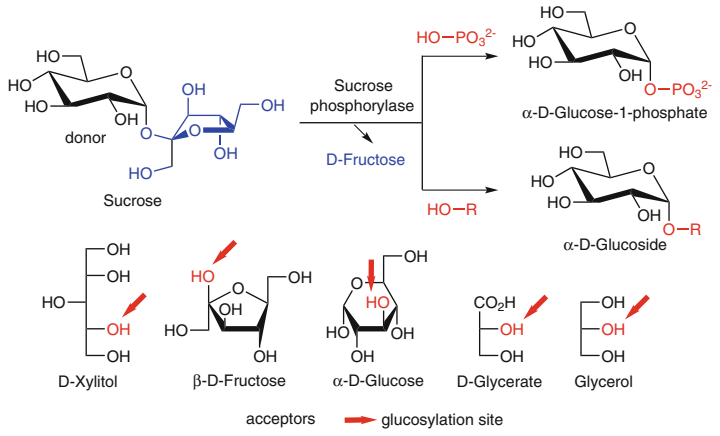
For example, trehalose, one of the major storage carbohydrates in plants, fungi, and insects, was synthesized from glucose and its 1-phosphate using trehalose phosphorylase as the catalyst in the reverse (condensation) reaction [1823].



Scheme 2.214 Synthesis of trehalose via the non-Leloir pathway

Glycoside synthesis is even more simple using trans-glycosidases, which use cheap non-phosphorylated (di)saccharides as donor substrates. Interestingly, the energy of the glycosidic bond in sucrose is of the same level as that of nucleotide-activated sugars, such as UDP-Glu. The major disadvantage is the small number of available specificities, which is mainly limited to the transfer of α-glycosyl- and β-fructosyl-groups [1824].

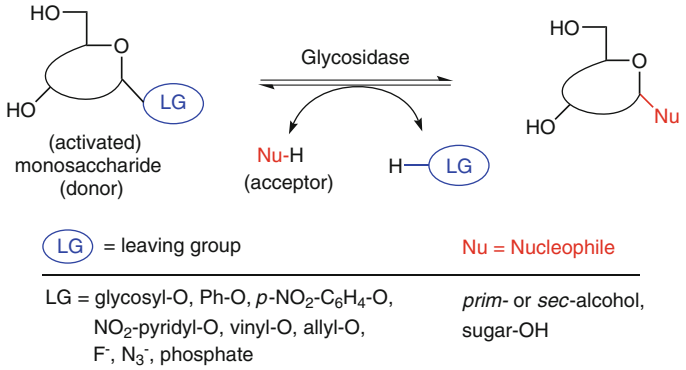
Sucrose phosphorylase is also capable of catalyzing glycosyl-transfer reactions. This bacterial transglucosidase catalyzes the cleavage of the disaccharide sucrose using phosphate as nucleophile to yield α-D-glucose-1-phosphate and D-fructose (Scheme 2.215). In the absence of phosphate, the enzyme-glucosyl intermediate can be intercepted by various nucleophiles bearing an alcoholic group to yield the corresponding α-D-glucosides in high yields [1825, 1826]. 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 for cosmetics [1827].



Scheme 2.215 Synthesis of α-D-glycosides using sucrose phosphorylase via trans-glycosylation

2.6.1.3 Glycosidases

A glycosidic bond is more stable than a phosphate ester or peptide bond, with a half-life of ~10^7 years at room temperature [1828]. Hence, its hydrolytic cleavage requires exceptionally proficient enzymes: Glycosidases (also termed 'glycohydrolases' [1829]) are independent of any cofactor and show k_{cat} values of ~10^2 s^{-1}, which translates into a rate acceleration of ~10^{17}. 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 2.216) [1830]. 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 [1785, 1831–1835]. Interestingly, this potential was already recognized as early as 1913! [1836].

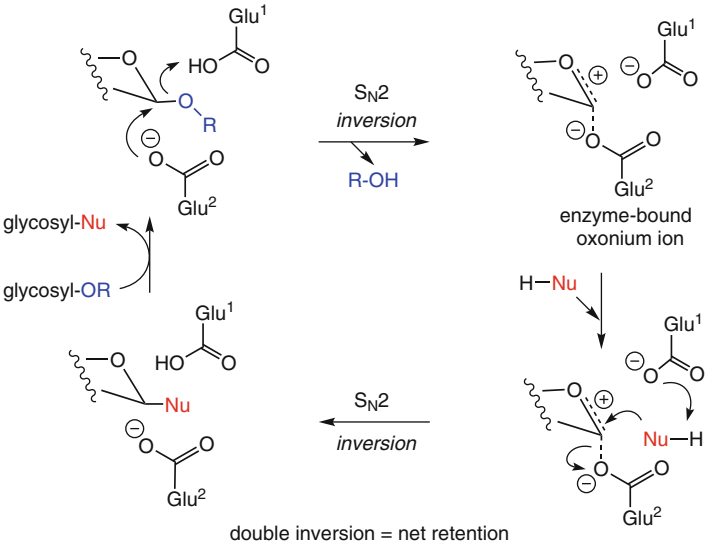


Scheme 2.216 Glycoside synthesis using glycosidases

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.

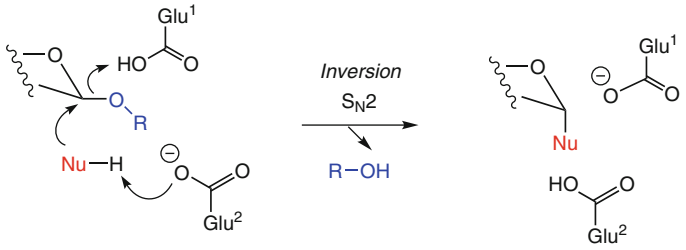
Depending on the stereochemical course of glycoside formation, i.e., whether *retention* or *inversion* of the configuration at the anomeric center is observed, glycosidases operate via two separate and distinct mechanisms (Schemes 2.217 and 2.218) [1837–1841]. Examples of the retaining enzymes are β-galactosidase, invertase and lysozyme. Inverting glycosidases, such as trehalase and β-amylase, have been used for the synthesis of alkyl glycosides to a lesser extent. In recent years, a number of thermostable glycosidases have been identified and characterized. The most remarkable among them are the β-glucosidase [1842] and the β-galactosidase from the hyperthermophilic archean *Pyrococcus furiosus*.

Although the first proposal for the mechanism of retaining glycosidases in 1953 has undergone some refinements, it is still valid in its sense (Scheme 2.217) [633, 1843, 1844]: 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 [1845, 1846]. 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 attacks the glycosyl-enzyme intermediate from the 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 2.217 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 2.218).



Scheme 2.218 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) [1847–1850]. 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.

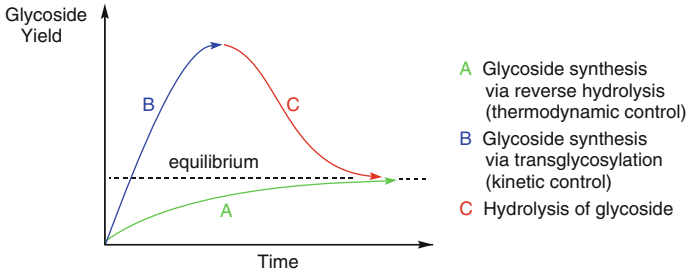


Fig. 2.19 Glycosylation via thermodynamic and kinetic control

Other methods to improve such procedures make use of aqueous-organic two-phase systems [1851, 1852] and polyethylene-glycol-modified glycosidases [1853]. 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, are inapplicable because 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 [1854]. In summary, glycoside synthesis via the reverse hydrolysis approach is less than ideal.

Transglycosylation

The second strategy – the *kinetic* approach – utilizes a preformed activated glycoside as donor, which is coupled onto the nucleophile acceptor by an appropriate glycosidase and is referred to as 'transglycosylation' (Fig. 2.19, pathway B) [1855, 1856]. 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 are used [1857, 1858]. Good donors are, for instance, glycosyl fluorides [578, 1859, 1860], -azides [1861, 1862], (hetero)aryl- (usually *p*-nitrophenyl- or nitropyridyl- [1863]), vinyl- and allyl-glycosides [1864, 1865]. Transglycosylation gives higher yields as compared to reverse hydrolysis and is generally the method of choice [1866, 1867]. 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 α/β-configuration at the newly generated anomeric center.

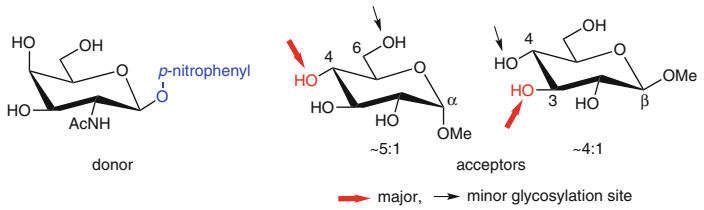
The major drawbacks, however, are incomplete yields and the frequent formation of product mixtures due to the limited selectivity of glycosidases with respect to the glycosidic acceptor, in particular due to the formation of undesired 1,6-linkages. The regio- and stereoselectivity of transglycosylation reactions is influenced by a number of parameters such as reaction temperature [1868], concentration of organic cosolvent, the reactivity of the activated donor [1869], the nature of the aglycone [1870, 1871], and the anomeric configuration of the acceptor glycoside [1872] (Table 2.8).

Table 2.8 Transglycosylation catalyzed by glycosidases (Scheme 2.216)

Enzyme	Donor/glycoside	Acceptor/nucleophile	Product(s)
α-Galactosidase	α-Gal-*O*-*p*-C_6H_4-NO_2	α-Gal-*O*-allyl	α-Gal-(1→3)-α-Gal-*O*-allyl
α-Galactosidase	α-Gal-*O*-*p*-C_6H_4-NO_2	α-Gal-*O*-Me	α-Gal-(1→3)-α-Gal-*O*-Me[a]
α-Galactosidase	α-Gal-*O*-*p*-C_6H_4-NO_2	β-Gal-*O*-Me	α-Gal-(1→6)-β-Gal-*O*-Me[b]
β-Galactosidase	β-Gal-*O*-*o*-C_6H_4-NO_2	α-Gal-*O*-Me	β-Gal-(1→6)-α-Gal-*O*-Me
β-Galactosidase	β-Gal-*O*-*o*-C_6H_4-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 as side products

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 denoted as 'anomeric control' (Scheme 2.219).



Scheme 2.219 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) [1873].

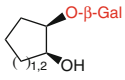
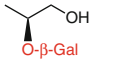
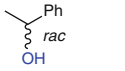
Besides the synthesis of natural glycosides, a considerable number of nonnatural alcohols have been employed as nucleophiles in transglycosylation reactions (Table 2.9) [1874, 1875]. The types of transformation include the desymmetrization of *meso*-diols and the kinetic resolution of racemic primary 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.

Cyclic *meso*-1,2-diols have been transformed into the corresponding monoglycosides in good diastereoselectivity using β-galactosidase from *Escherichia coli*, which is readily available from the dairy industry. As may be seen from Table 2.9, the selectivity strongly depends on the structure of the aglycone component [1876].

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 [1877, 1878], however, better results were obtained for *sec*-alcohols (Table 2.9) [1879–1881]. This fact is understandable if one considers the rules for chiral recognition for carboxyl ester hydrolases (see Schemes 2.20 and 2.45), where the distance of the center of chirality to the point of reaction should be a minimum. It is apparent that stereoselective glycosylation of alcohols is inferior compared to ester hydrolysis / esterification using standard hydrolases.

In contrast, regio-selective glycosylation of (poly)hydroxy compounds, such as steroids (digoxin, digitoxin), terpenoids (geraniol), hydroquinones (arbutin), vitamins (α-tocopherol), flavonoids (quercetin) and (poly)phenols (resveratrol) is a valuable technique to enhance the stability and water-solubility of these compounds or to modulate their (bio)activity.

Table 2.9 Desymmetrization of *meso*-diols and kinetic resolution of alcohols by glycosylation using β-galactosidase from *Escherichia coli*

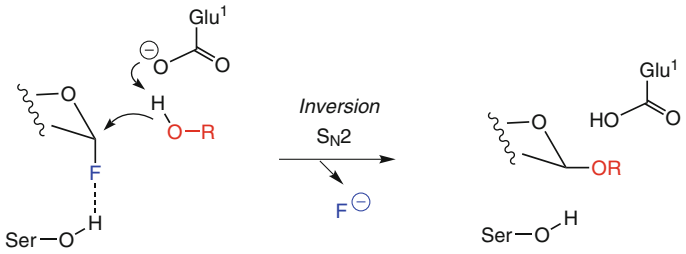
Donor/glycoside	Acceptor/nucleophile	Product	d.e. [%]
β-Gal-*O*-Ph			90–96
β-Gal-OPh			95[a]
β-Gal-OC_6H_4-*o*-NO_2			98

[a]The β-galactosidase from *Sulfolobus solfataricus* was used

Glyco-Synthases

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 2.220).

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 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' [1882–1887]) show greatly enhanced yields of glycosides due to the elimination of their undesired hydrolysis.

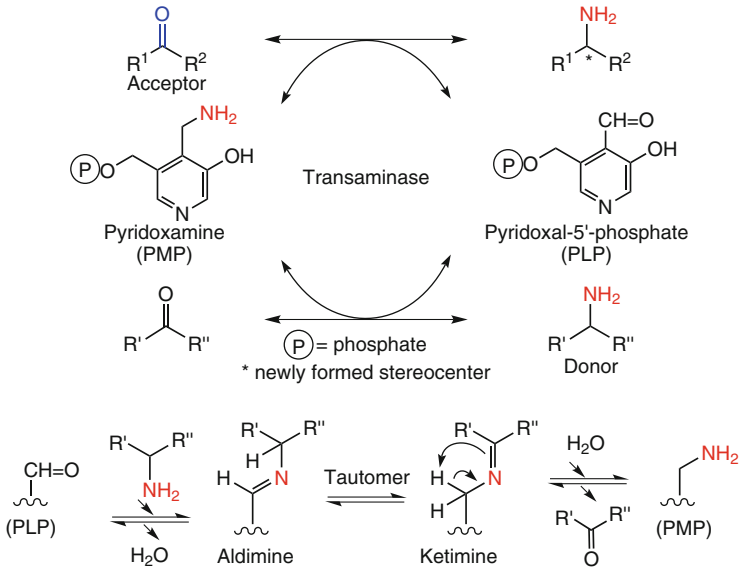


Scheme 2.220 Mechanistic principle of an inverting glycos*ynthase*

2.6.2 Amino Transfer Reactions

Transaminases [EC 2.6.1.X][52] catalyze the redox-neutral transfer of ammonia between an amine donor and a carbonyl acceptor group (Scheme 2.221) [97, 1888–1893]. Since free ammonia is highly toxic to living cells, this reaction is mediated via an 'activated benzaldehyde' (pyridoxal-5′-phosphate, PLP, vitamin B_6) as cofactor, which functions as a molecular shuttle for the [NH_3]-moiety. In a first step, PLP forms an aldimine Schiff base with the amine-donor. Tautomerization of the C=N bond catalyzed by a conserved Lys residue yields a ketimine, which is hydrolyzed to yield the cofactor in its aminated form (pyridoxamine, PMP). In the second step, the latter reacts (in reversed order) through the same events with the carbonyl group of the substrate to yield the amine product with regeneration of PLP [1894, 1895].

[52] Also denoted as amino transferases.



Scheme 2.221 Transaminase-catalyzed amino-transfer

Although transaminases were discovered already half a century ago [1896, 1897], their use for the biocatalytic synthesis of (nonnatural) amines was initially impeded by two obstacles: (i) The majority of transaminases available were only active on α-amino/α-ketoacids as substrates and (ii) 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 synthesis of nonracemic amines, preferentially via (less efficient) kinetic resolution of *rac*-amines by enantioselective de-amination [1898, 1899]. 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 industrial-scale asymmetric synthesis of amines [1900, 1901].

On a genomic level, transaminases are classified into subgroups [1902–1904]. α-Transaminases are very specific, as they only act on α-amino acids yielding the corresponding α-keto acids. In contrast, ω-transaminases (ω-TA) are more flexible and accept substrates bearing a distant carboxylate moiety (such as lysine, ornithine, β-alanine, and ω-aminobutyrate). Since they also accept *prim*-amines lacking a carboxylate group (e.g. *i*-Pr-NH_2, 2-Bu-NH_2), they are also referred to as 'amine transaminases [1905].

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 stereo-complementary ω-TAs [1906–1909]. The most widely used enzymes are obtained from *Vibrio fluvialis* [1910], *Chromobacterium violaceum* [1911, 1912], *Pseudomonas aeruginosa* [1913], *Bacillus megaterium* [1914], and *Alcaligenes denitrificans* [1915]. Thermostable mutants were derived from an ω-TA from *Arthrobacter citreus* [1916].

Because the transamination is reversible, the synthesis of nonracemic amines using ω-transaminases can be operated in two modes (Scheme 2.222):

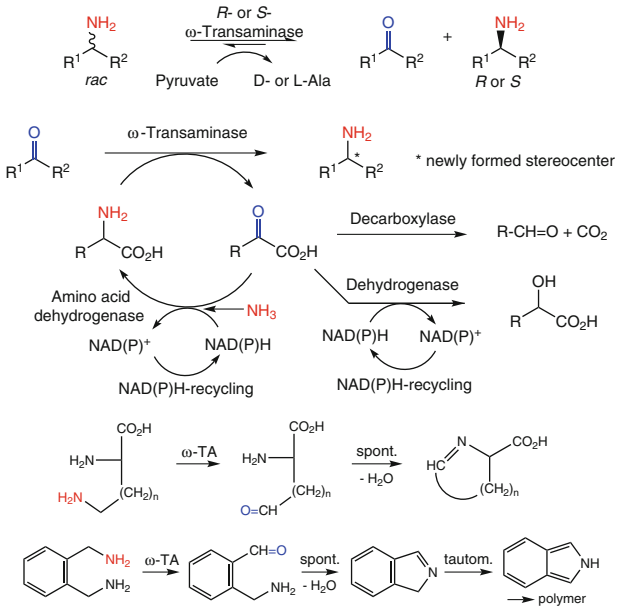
- Enantioselective deamination starts from a racemic amine via kinetic resolution, where one enantiomer is converted into the corresponding ketone, leaving the desired amine enantiomer untouched, which can be recovered in 50% theoretical yield. For thermodynamic reasons, pyruvate was employed as preferred amine acceptor yielding D- or L-alanine as by-product, depending on the stereopreference of the enzyme.
- Desymmetrization of prochiral ketones via asymmetric amination is preferred for its superior efficiency. Depending on the substrate preference of the employed transaminase, sacrificial amine donors derived from the α-aminoacid pool (e.g. Ala, Phe, Glu, Asp) or simple amines (*i*-Pr-NH_2, 2-Bu-NH_2) are commonly employed. 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.[53] To even worsen 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 [1917]. In contrast, *pulling* the equilibrium by co-product removal is much more effective. The following strategies have been developed (Scheme 2.222) [1918]:

- The most simple approach is to use *i*-Pr-NH_2 as amine donor and to remove the coproduct acetone at elevated temperature by evaporation [1919, 1920].
- Non-volatile coproducts are usually removed by an additional enzymatic step: 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 [1921, 1922]. Although this provides a strong driving force, the aldehyde thus formed is usually a good substrate and gets aminated.
- Carbonyl-reduction of the keto-coproduct by a suitable dehydrogenase in presence of NAD(P)H-recycling yields the corresponding alcohol. For instance, pyruvate can be conveniently reduced to lactate by lactate dehydrogenase [1923].

[53]The equilibrium constant between acetophenone and alanine was reported to be 8.8×10^{-4}, see [1916].

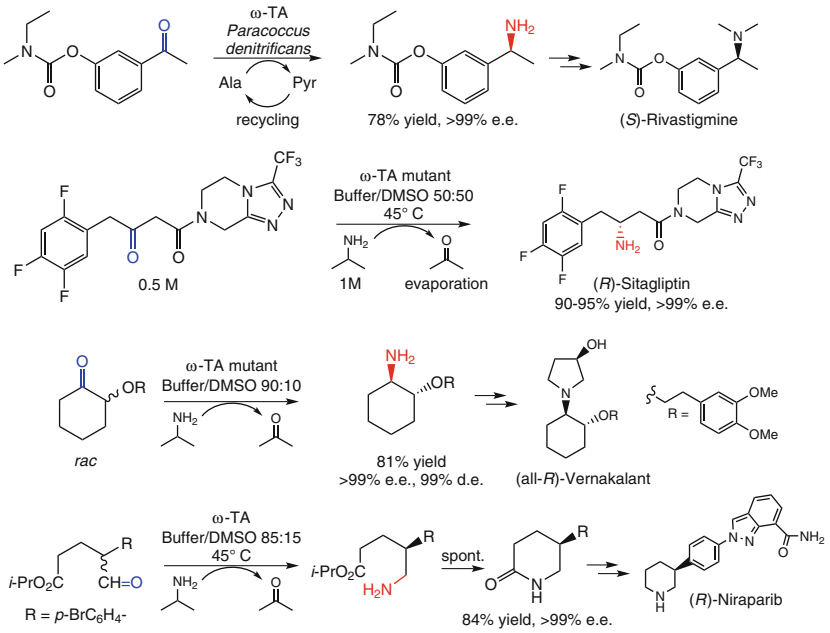
- The most efficient approach is probably the direct recycling of alanine from pyruvate via NADH-dependent reduction in presence of ammonia catalyzed by alanine dehydrogenase. Overall, this sequence is equivalent to a metal-free reductive amination [1924]. Using ammonium formate and formate dehydrogenase for NAD(P)H-recycling, this resembles a biocatalytic equivalent of the Leuckart-Wallach reaction.
- The use of amine donors, which form an unstable keto co-product [1925]. For instance, α,ω-diamino acids, such as ornithine (n = 2) or lysine (n = 3) yield α-amino-ω-ketoacids, which (nonenzymatically) cyclize to the corresponding Δ^2-pyrroline-5-carboxylate and Δ^1-piperidine-2-carboxylate, respectively, as dead-end products [1926, 1927]. In a related approach, *o*-xylylene diamine gives an amino aldehyde, which spontaneously undergoes 5-*exo-trig* cyclization, followed by tautomerization to yield *iso*-indol. The latter forms coloured polymers, which may serve as indicator for positive hits in mutant libraries, but complicates downstream-processing in prep-scale reactions [1928].



Scheme 2.222 Enantioselective de-amination of *rac*-amines (kinetic resolution) and asymmetric transamination of ketones (desymmetrization) with amine donors for equilibrium shift

To date, a broad range of wild-type (*R*)- and (*S*)-ω-transaminases are available, which accept ketones bearing a large and small group, while mutants accepting sterically demanding substrates bearing two bulky groups were obtained by directed evolution [1929]. Together with efficient techniques to shift the equilibrium, the stage was set for the large-scale synthesis of

nonracemic amines from the corresponding ketones. Considering the importance of amino groups in active pharmaceutical ingredients (APIs)[54] it is not surprising that the asymmetric enzymatic transamination on industrial scale was predominantly developed for pharma-applications, which is illustrated by the following examples (Scheme 2.223).



Scheme 2.223 Chemo-enzymatic synthesis of nonracemic amines for pharma-applications employing ω-transaminases

One of the first examples of a chemo-enzymatic route based on an enzymatic transamination step was the synthesis of the cholinesterase inhibitor (*S*)-Rivastigmine, which is used for the treatment of Alzheimer's or Parkinson's disease. The introduction of the (*S*)-amino moiety was accomplished by asymmetric amination of the aryl-methyl ketone precursor in 78% isolated yield and >99% e.e. using an ω-TA from *Paracoccus denitrificans* [1930]. The most prominent transamination-based process implemented on industrial scale – a benchmark in biocatalytic synthesis – was the production of the anti-diabetic drug (*R*)-Sitagliptin. Since wild-type ω-TAs were barely able to convert the sterically demanding ketone precursor (~4% conversion at best), elaborated directed evolution over 11 rounds was neccessary to provide a 27-mutant enzyme with improved catalytic performance and enhanced tolerance towards process conditions (~50% DMSO, 45 °C).

[54]The relative abundance of functional groups in APIs (in decreasing order) is hydroxy (~40%), carboxy (~22%), amino (~16%), sulfoxide (~3%), others (19%).

Eventually, (*R*)-sitagliptin was obtained in 90–95% yield and >99% e.e. at 200 g/L substrate concentration using *i*-Pr-NH_2 (1M) as amine donor [1931]. Due to its superior efficiency, the biocatalytic route replaced the Rh[Josiphos]-catalysed asymmetric enamine hydrogenation process.

An ω-TA was also engineered to adapt it to a process for the synthesis of the antiarrhythmic agent (all-*R*)-vernakalant. In this case, enzyme evolution was directed to provide a transaminase variant with inverted diastereoselectivity with respect to the chiral center adjacent to the carbonyl moiety. Careful choice of the reaction conditions allowed the in-situ racemization of the starting ketone, providing a single *trans*-diastereomer in 81% yield, 99% d.e. and >99% e.e. via dynamic resolution [1932]. In analogy, dynamic resolution is also feasible for aldehydes bearing a configurationally unstable center at Cα. This strategy was exploited for the preparation of the anti-cancer agent (*R*)-Niraparib, which relies on the enantioselective amination of a racemic aldehyde precursor as key step. The δ-amino-ester thus formed undergoes spontaneous ring-closure yielding a lactam, which efficiently pulls the equilibrium towards product formation [1933].

2.7 Halogenation and Dehalogenation Reactions

Halogen-containing compounds are not only produced by man, but also by Nature [1934–1936]. A brominated indole derivative – Tyrian purple dye[55] – was isolated from the mollusc *Murex brandaris* by the Phoenicians. Since that time, more than 5000 halogenated natural products of various structural types have been isolated from sources such as bacteria, fungi, algae, higher plants, marine molluscs, insects, and mammals [1937, 1938]. Whereas fluorinated and iodinated species are rather rare, chloro and bromo derivatives are found more often. The former are predominantly produced by terrestrial species [1939] and the latter in marine organisms [1940]. 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 [1941, 1942]. 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 [1943]. In contrast to hydrolytic or redox enzymes, which have been investigated since about a century, halogen-converting enzymes are a more recent subject of research after the first halogenase was reported in 1966 [1944–1950].

[55] 6,6'-Dibromoindigo.

2.7.1 Halogenation

Despite the impressive number of halometabolites identified so far, only a few types of halogenating enzymes have been characterized to date [1951–1954]: Whereas flavin- [1955] and α-ketoglutarate-dependent (nonheme) iron-halogenases [1956] are rather substrate specific, halo*peroxidases* show a broad substrate scope and thus had a dominant impact in biotransformations [1451, 1471, 1957–1960]. These enzymes are widely distributed in nature and enable a multitude of electrophilic halogenation reactions following the general equation shown in Scheme 2.224, where X stands for halide (Cl^-, Br^- and I^-, but not F^-,[56] [1961]). 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. For redox reactions catalyzed by haloperoxidases which do not involve a halide (such as hydroxylation, epoxidation, or sulfoxidation) see Sect. 2.3.4.

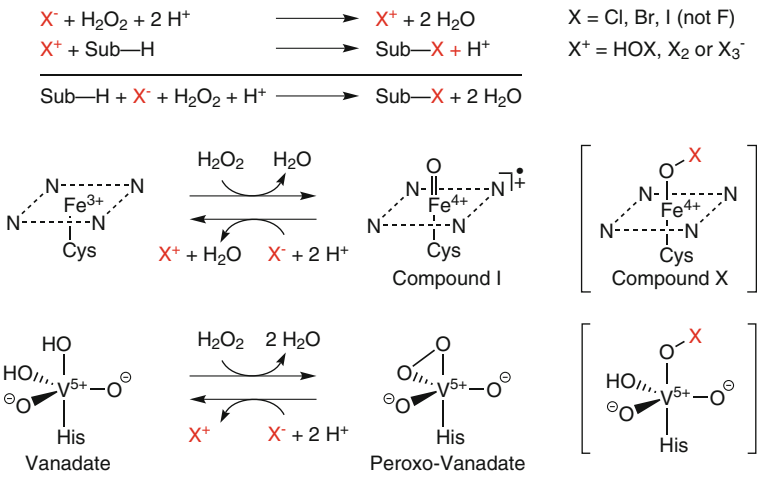
Despite their mechanistic differences, the overall net reaction of haloperoxidases consists of a two-electron oxidation of halide anion at the expense of H_2O_2 yielding [X^+] (Scheme 2.224).[57] Depending on the type of halide and the reaction conditions, such as pH, the electrophilic species may be halonium (X^+, X_3^+), halogen (X_2) or hypohalous acid/hypohalite (HOX/OX^-). Two major types of haloperoxidases depend on a catalytic metal:

- The mechanism of heme-iron-dependent enzymes is closely related to that of peroxygenases, i.e. H_2O_2-dependent oxidation of Fe^{3+} in the enzyme's resting state yields the Fe^{4+}-species Compound I (Scheme 2.171). The latter oxidizes halide in a two-electron transfer step (Scheme 2.224) [1962, 1963].
- In contrast, vanadium-depending haloperoxidases do not change the oxidation state of V^{5+} during the catalytic cycle, but switch between vanadate and peroxovanadate [1964, 1965].

For both enzymes, the fate of X^+ generated and the existence of a metal-bound hypohalite adduct is under debate, for heme-dependent haloperoxidases this elusive species is ironically denoted as 'Compound X' [1966]. The actual halogenation reaction is believed to take place outside of the active site and consequently, any asymmetric induction observed in haloperoxidase-catalyzed reactions is usually low.

[56]The high electronegativity of fluorine renders the formation of F^+ energetically prohibitive. Enzymatic (nucleophilic) fluorination is extremely rare and requires S-adenosylmethionine (SAM) as cofactor.

[57]In a side reaction, X^+ may react with H_2O_2 to form singlet oxygen ($X^+ + H_2O_2 \rightarrow {}^1O_2 + X^- + H^+$).



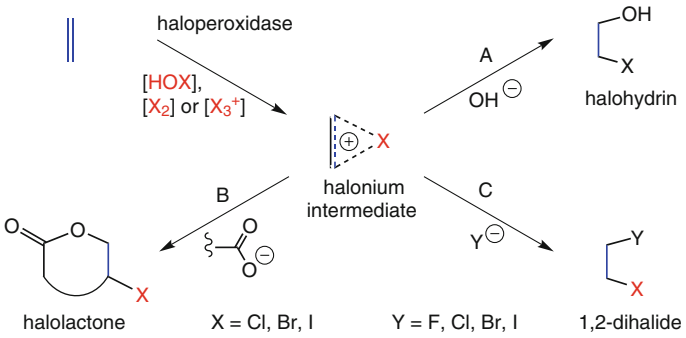
Scheme 2.224 Enzymatic halogenation catalyzed by haloperoxidases

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.

The most intensively studied haloperoxidases are the chloroperoxidase from the mold *Caldariomyces fumago* [1473] and bromoperoxidases from algae [1967] and bacteria such as *Pseudomonas aureofaciens* [1968], *Ps. pyrrocinia* [1969], and *Streptomyces* sp. [1970]. The only iodoperoxidase of preparative use is isolated from horseradish root [1971].

Halogenation of Alkenes

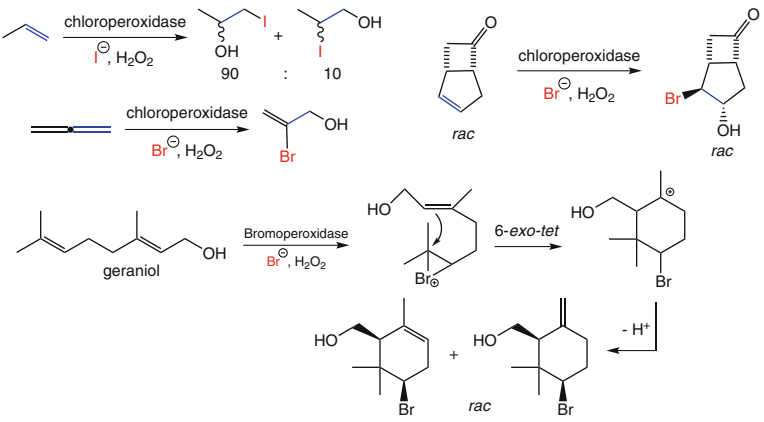
Haloperoxidases transform alkenes by a formal addition of hypohalous acid to produce halohydrins. The mechanism is identical to that of chemical halohydrin formation and proceeds via a halonium intermediate [1972–1974], (Scheme 2.225).



Scheme 2.225 Haloperoxidase-catalyzed transformation of alkenes

Functional groups present in the alkene can lead to products other than the expected halohydrin (pathway A) by competing with hydroxyl anion 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 [1975, 1976]. Similarly, in presence of elevated concentrations of halide, 1,2-dihalides are formed (pathway C) [1977]. This latter transformation offers the unique possibility of introducing fluorine, which is not oxidized by haloperoxidases, into the substrate. Furthermore, migration of functional groups such as halogen [1978] 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 [1979, 1980].

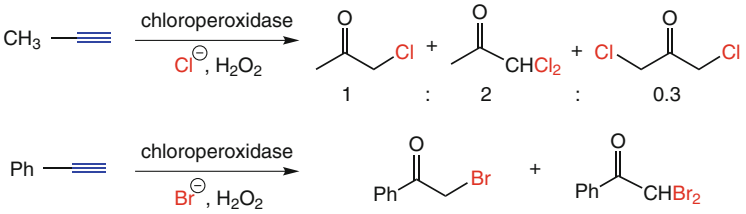
All types of carbon–carbon double bonds – isolated (e.g., propene), conjugated (e.g., butadiene) and cumulative (e.g., allene) – are reactive (Scheme 2.226) [1981]. The size of the substrate seems to be of little importance since steroids [1982] and sterically demanding bicyclic alkenes [1983] are accepted equally well. Any regioselectivity observed reflects the (predominant) chemical and nonenzymatic nature of halohydrin formation. The same holds for diastereoselectivity on (bi)cyclic structures, where attack of the halonium species preferably occurs from the less hindered *exo*-side, followed by nucleophilic ring opening in a *trans*-fashion. Geraniol was halogenated on the (electronically favored) terminal C=C bond, the corresponding bromonium ion underwent intramolecular 6-*exo-tet* cyclization yielding a cyclohexane carbenium ion, which upon deprotonation gave a mixture of regio-isomeric alkenes in racemic form bearing the Br and CH_2-OH substituents in the stereochemically preferred diaxial position (plus additional side products) [1984].



Scheme 2.226 Regio- and diastereoselective formation of halohydrins from alkenes

Halogenation of Alkynes

With alkyne substrates, haloperoxidase-catalyzed reactions yield α-haloketones (Scheme 2.227) [1985]. As with alkenes, the product distribution depends on the halide ion concentration. Both homogeneous and mixed dihalides can be formed, dependent upon whether a single halide species or a mixture of halide ions are present.

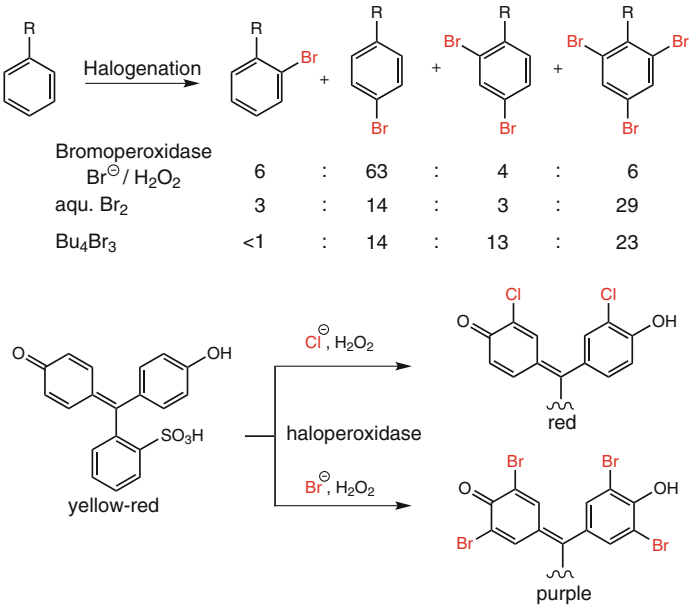


Scheme 2.227 Haloperoxidase-catalyzed reactions of alkynes

Halogenation of Aromatic Compounds

A wide range of electron-rich aromatic and heteroaromatic compounds are readily halogenated by haloperoxidases [1986–1988]. Bearing in mind the electrophilic character of the halogenating species, electron-rich phenols [1989, 1990] and anilines [1991] as well as their respective *O*- and *N*-alkyl derivatives are particularly well accepted. As in chemical electrophilic halogenation, the regioselectivity is dominated by the *ortho*- and *para*-directing effect of the substituent (Scheme 2.228) [1992]. In a comparative study, the bromination of phenol was performed with the V-depending bromoperoxidase from *Ascophyllum nodosum* and with different chemical brominating agents under identical reaction conditions. Two key points can be taken: Firstly, the ratio of *ortho*/*para* bromophenol is somewhat comparable in the chemical and enzymatic processes, and secondly, owing to the mild reaction conditions, the enzymatic reaction is more selective for *mono*-bromination than the chemical transformations [1993]. The color change of phenolic dyes such as phenol red or fluorescein upon halogenation serves as a simple assay for haloperoxidases [1994].

Since haloperoxidases are also peroxidases, they also can catalyze halide-independent peroxidation reactions of aromatics (Sect. 2.3.4). 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.

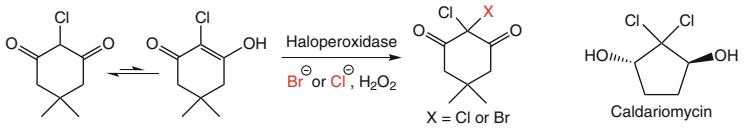


Scheme 2.228 Halogenation of aromatic compounds

Halogenation of C–H Groups

Similar to the chemical process, enzymatic halogenation of C–H groups is only possible if they are activated by adjacent electron-withdrawing substituents, for example carbonyl groups, which facilitate enolization. Since the reactivity depends on the enol content of the substrate, simple ketones like 2-heptanone are unreactive [1995], but highly enolized 1,3-diketones are readily halogenated to give the corresponding 2-*mono*- or 2,2-dihalo derivatives (Scheme 2.229) [1996]. For instance, monochloro dimedone has been used extensively to detect chlorinating and brominating haloperoxidases due to a hypsochromic shift of its absorbance maximum from 290 nm (owing to its enol content) to shorter wavelengths. In addition, it served as mimic in the elucidation of the biosynthesis of the intriguing highly chlorinated metabolite caldariomycin, which is formed by the (haloperoxidase-producing) fungus *Caldariomyces fumago*.

As with the formation of halohydrins from alkenes, stereoselectivities are low and the reactivity of the substrate is independent of its size. For example, monocyclic compounds such as barbituric acid derivatives [1997] and sterically demanding polycyclic steroids are equally well accepted [1998]. β-Ketoacids are also halogenated, but the spontaneous decarboxylation of the intermediate α-halo-β-ketoacid affords the corresponding α-haloketones [1999]. The chloroperoxidase-catalyzed halogenation of oximes was shown to proceed via a two-step sequence through a halonitroso intermediate which is further oxidized to furnish an α-halonitro product [2000].



Scheme 2.229 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 [2001]. This pathway constitutes a part of the natural mammalian defence system against microorganisms, parasites and, perhaps, tumor cells. 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 [2002, 2003]. As a result, sulfenic acids or disulfides are formed, respectively. Due to a lack of control, these reactions are of no synthetic use.

In view of the predominant chemical nature of biohalogenation, it seems that enzymatic halogenation reactions involving haloperoxidases do not show any significant advantage over the usual chemical reactions due to their lack of 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 [2004, 2005]. Five major pathways for enzymatic degradation of halogenated compounds have been discovered (Table 2.10) [2006–2009].

Table 2.10 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
Hydrolysis	C–X + H_2O	→	C–OH + HX
Epoxide formation	HO–C–C–X	→	epoxide + HX

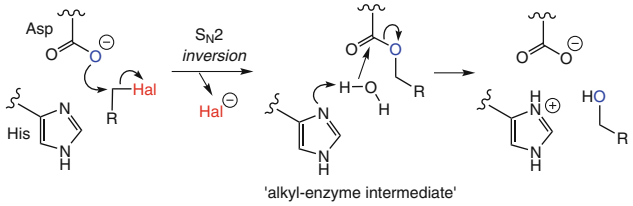
X = Cl, Br, I

Redox enzymes are responsible for the replacement of the halogen by a hydrogen atom via reductive dehalogenation [2010, 2011] and oxidative dehalogenation yields a carbonyl group [2012]. Elimination of hydrogen halide leads to the formation of an alkene [2013], 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 hydrolytic replacement of a halide by a hydroxy group and the formation of an epoxide from a halohydrin take place in a stereocontrolled fashion and are therefore of synthetic interest.

Dehalogenases

Hydrolytic dehalogenation catalyzed by dehalogenases [EC 3.8.1.X, formally hydrolases] proceeds by formal nucleophilic substitution of the halogen atom with a hydroxyl ion going in hand with *inversion* of configuration [2014]. The mechanism has close similarities to that of epoxide hydrolases (Sect. 2.1.5), i.e. the carboxyl moiety of an aspartate residue attacks the halide by forming an 'alkyl-enzyme intermediate' (Scheme 2.230). Being a carboxyl ester, the latter is hydrolyzed by a hydroxyl ion which is provided from water by the aid of a histidine [631, 2015].

To date, two types of dehalogenases have gained importance for preparative biotransformations due to their stereospecificities on haloalkanes and α-haloacids.

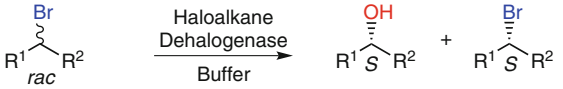


Scheme 2.230 Mechanism of inverting haloalkane and α-haloacid dehalogenases

Haloalkane Dehalogenases were intensely investigated for their crucial role in the biodegradation of halogenated pesticides, such as hexachlorocyclohexane (Lindane),[58] by soil bacteria [2016] and it was only recently, that their biocatalytic potential was recognized [2017, 2018].

Typical substrates for haloalkane dehalogenases DhaA, LinB and DbjA are *prim* or *sec* chloro-, bromo- and iodoalkanes. Halogens attached to olefinic or aromatic carbons are unreactive, as well as CX_2, CX_3 or C-F moieties. Enantioselectivities on 2-bromoalkanes with chain lengths of C_4 - C_7 ranged from poor to good [2019]. However, more polar substrates, such as α-bromo esters and -amides were resolved with excellent E-values with a strong preference for the (*R*)-enantiomer [2020, 2021]. Due to inversion of configuration during hydrolysis, the hydroxy product and remaining non-converted substrate are both (*S*)-configurated (homochiral) (Scheme 2.231).

[58]The use of Lindane was banned in 2009.



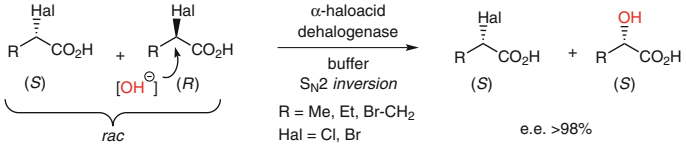
R^1	R^2	Enzyme	*E*
Me	Et	DhaA	2
Me	*n*-Pr	DbjA	145
Me	*n*-Bu	DbjA	68
Me	*n*-Pent	DbjA	28

R^1	R^2	Enzyme	*E*
Me	CO_2Me	DbjA	>200
Et	CO_2Me	DbjA	>200
Me	$CO\text{-}NHCH_2Ph$	DbjA	>200
Et	$CO\text{-}NHCH_2Ph$	LinB	>200

Scheme 2.231 Kinetic resolution of 2-bromoalkanes, α-bromoesters and amides using haloalkane dehalogenases

α-Haloacid Dehalogenases are specific for short-medium chain 2-halocarboxylic acids [2022, 2023]. In contratst to haloalkane dehalogenases, they can also convert α-fluoroalkanoates,[59] but they are inactive on (nonactivated) haloalkanes. Interestingly, the reactivity of halides with α-haloacid dehalogenases 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 [2024]. The most intriguing aspect of α-haloacid dehalogenases is their enantiospecificity [2025]. Depending on the growth conditions, the microbial production of stereo-complementary (*R*)- or (*S*)-specific enzymes may be induced [2026–2028].

(*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, respectively (Scheme 2.35). Several strategies to resolve racemic 2-chloropropionic acid via enzymatic ester hydrolysis using 'classic' hydrolases proceed with varying degrees of selectivity [2029]. An elegant approach makes use of an (*R*)-specific α-haloacid dehalogenase from *Pseudomonas putida* NCIMB 12018 (Scheme 2.232) [2030–2032]. 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 2000 t/year [2033].



Scheme 2.232 Resolution of 2-chloropropanoic acid derivatives by α-haloacid dehalogenase

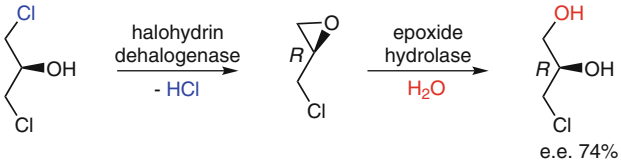
[59]Fluoroacetate is extremely toxic.

The hydrolytic instability of α-bromoacids in aqueous solvent systems and the limited solubility of long-chain analogs can be overcome by using organic solvents [2034]. Thus, long-chain α-haloacids (which were not accepted as substrates in water) were successfully transformed with good specificity in presence of toluene, acetone or DMSO.

Halohydrin Dehalogenases

The biodegradation of halohydrins proceeds through a two-step mechanism involving epoxide-formation catalysed by halohydrin dehalogenases [EC 4.5.1.X, formally lyases],[60] 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-* [2035, 2036], *Corynebacteria* [2037], *Arthrobacter erithrii* [2038, 2039], *Pseudomonas* sp. [2040]), fungi (*Caldariomyces fumago*), and algae (*Laurencia pacifica*).

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 2.233) [2041]. In a 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 [2042]. Further studies revealed that these activities are widespread among bacteria [2043–2047].

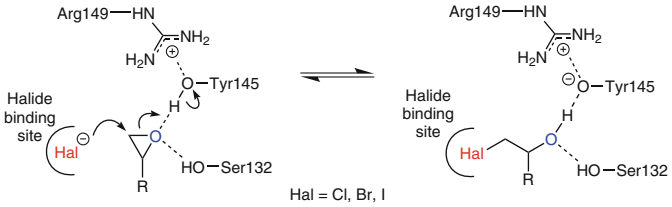


Scheme 2.233 Asymmetric microbial degradation of prochiral halohydrin by a *Corynebacterium* sp

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 [2048].

The mechanism of halohydrin dehalogenase was shown to proceed in a reversible fashion via nucleophilic attack of halide (provided by a lipophilic halide binding site) with simultaneous activation of the epoxide through protonation by a Tyr residue within a conserved catalytic triad of Ser-Tyr-Arg (Scheme 2.234) [2049, 2050].

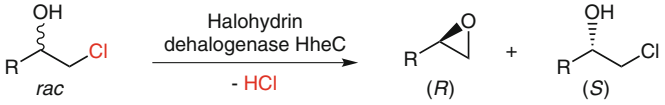
[60]Halohydrin dehalogenases were also (ambiguously) termed 'haloalcohol dehalogenases' or 'halohydrin epoxidases'.



Scheme 2.234 Catalytic mechanism of halohydrin dehalogenase from *Agrobacterium radiobacter*

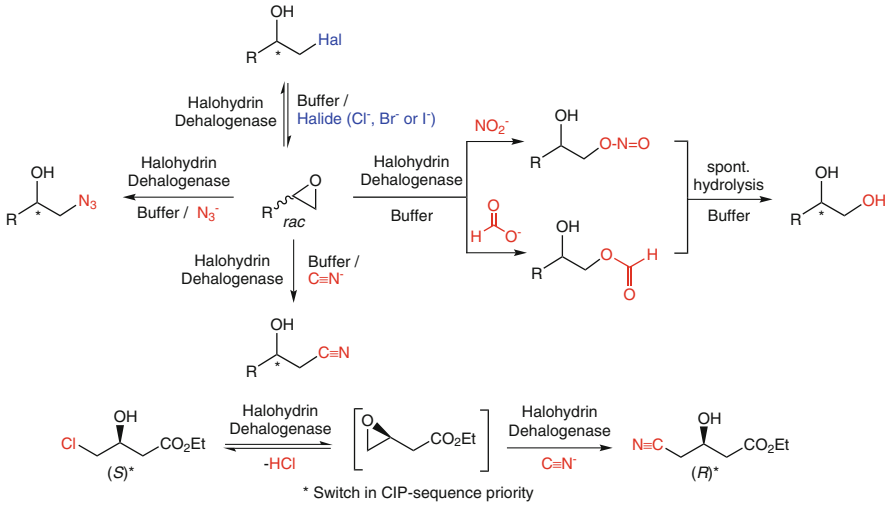
Using pure halohydrin dehalogenase (HheC), competing activities observed in whole-cell preparations were eliminated and halohydrins could be resolved via enantioselective ring-closure with excellent selectivities yielding (*R*)-epoxides and nonreacted (*S*)-halohydrins (Scheme 2.235) [2051, 2052]. A stereocomplementary enzyme (HheA) showing opposite stereoselectivity could be identified [2053].

Subsequent studies revealed that the natural nucleophile halide (Cl, Br, I) could be replaced by nonnatural analogs, such as azide [2054], nitrite [2055], cyanide [2056], (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 [2057]. 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 2.236).



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 2.235 Kinetic resolution of halohydrins using halohydrin dehalogenase



Scheme 2.236 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 [2058].

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 [2059] (Scheme 2.236).

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