Uwe T. Bornscheuer and Romas J. Kazlauskas

Hydrolases in Organic Synthesis

Regio- and Stereoselective Biotransformations

2nd edition

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R. Joseph Kazlauskas

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Xray structure showing a phosphonate transition-state analog bound in the active site of Pseudomonas cepacia lipase (PDB code: 1ys2)

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To

Aleli and Tanja

and to Jonas and Stasė

and also to

Lottie, Anna, Jonas and Annika

Preface for the 2nd edition

Over the decade, hydrolases have become key parts of the growing area of industrial biotechnology (Schmid et al., 2001; Shoemaker et al., 2003). We hope that the first edition of *Hydrolases in Organic Syntheses* contributed to this success. It was a key reference in our laboratories and an excellent starting point for new researchers in the field. For this success to continue, we felt that we had to keep it current with new developments in the area – directed evolution and protein design, dynamic kinetic resolutions, and the use of structures to explain selectivity.

Planning the 2nd edition presented a challenge – more than 900 publications in the area since 1999. We chose to focus on new areas, new insights, and new frontiers and not to focus on comprehensive lists of substrates. For this information, databases are more efficient than a book. We've added a new chapter on protein sources and optimization of biocatalyst performance for organic synthesis, an expanded chapter about directed evolution and a new chapter about catalytic promiscuity. In addition, we expanded sections dynamic kinetic resolution and immobilization and added new classes of hydrolases, such as haloalkane dehalogenases and organophosphorus hydrolases. In turn, we shortened some sections including a major shortening of the sections on lipid modification, which is of limited interest to organic chemists. These changes required a rearrangement of the book chapters. In addition, many sections contain minor updates.

We sincerely hope that these changes will extend and enhance the usefulness of this book.

Minneapolis/Greifswald, August 2005 Romas J. Kazlauskas, Uwe T. Bornscheuer

Preface for the 1st edition

Each traveller to a city seeks something different. One wants to see that special painting in the museum, another wants to drink the local beer, a third wants to meet a soulmate.

Each organic chemist also seeks something different from the field of biocatalysis. One wants high enantioselectivity, another wants reaction under mild conditions, a third wants to scale up to an industrial scale. We hope this book can be a guide to organic chemists exploring the field of biocatalysis. Enzyme-catalyzed reactions, especially hydrolase-catalyzed reactions, have already solved hundreds of synthetic problems usually because of their high stereoselectivity.

The organization is aimed at the chemist – by reaction type and by different functional groups. This information should help organic chemists identify the best hydrolase for their synthetic problem. In addition, we suggest how to choose an appropriate solvent, acyl donor, immobilization technique and other practical details. We hope that learning how others solved synthetic problems will generate ideas that solve the next generation of problems.

Although this book has more than 1 700 references, we might have missed important hydrolase-catalyzed reactions. The choices on what to include usually reflect our own research interests, but were sometimes arbitrary or even inadvertent. We will post corrections and additions on a web site: *http://pasteur.chem.mcgill.ca/hydrolases.html*.

Montreal/Stuttgart, February 1999 Romas J. Kazlauskas, Uwe T. Bornscheuer

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1 Introduction

Hydrolases are the group of enzymes that catalyze bond cleavage by reaction with water. The natural function of most hydrolases is digestive – to break down nutrients into smaller units for digestion. For example, proteases hydrolyze proteins to smaller peptides and then to amino acids and lipases hydrolyze lipids (triglycerides) to glycerol and fatty acids (Fig. 1). Because of the need to break down a wide range of nutrients, hydrolases usually have a broad substrate specificity.

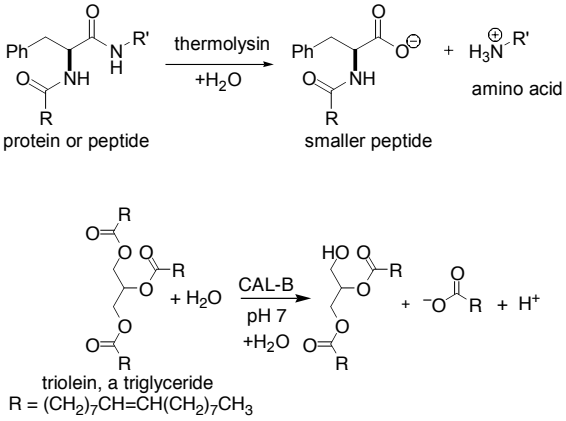


Fig. 1. The natural role of most hydrolases is digestive – to break down nutrients into smaller units. Thermolysin, a protease secreted by thermophilic bacteria, catalyzes the hydrolysis of proteins to peptides and then further to amino acids. Lipase B from *Candida antarctica* (CAL-B) catalyzes the stepwise hydrolysis of triglycerides (e.g., triolein) to fatty acids and glycerol. The reaction shows only the first step from a triglyceride to a diglyceride.

Several characteristics make hydrolases useful to the organic chemist. First, because of their broad substrate specificity, hydrolases often accept as substrates various synthetic intermediates. Second, hydrolases often show high stereoselectivity, even toward unnatural substrates. Third, besides hydrolysis, hydrolases also catalyze several related reactions – condensations (reversal of hydrolysis) and alcoholysis (a cleavage using an alcohol in place of water). Two examples from industry are shown in Fig. 2. Thermolysin catalyzes the condensation of two amino acid derivatives to make an aspartame derivative (Isowa et al., 1979). The reaction proceeds in the condensation direction because the product precipitates from solution. The high enantioselectivity permits using racemic starting materials and the high regioselectivity of thermolysin eliminates the need to protect the β-carboxyl group of the aspartic acid derivative. The second example is an alcoholysis reaction (Morgan et al., 1997a). The ester, vinyl acetate, is cleaved not by water, but by the substrate alcohol. The liberated vinyl alcohol (not shown) tautomerizes to acetaldehyde. These alcoholysis reactions are also called transesterification reactions.

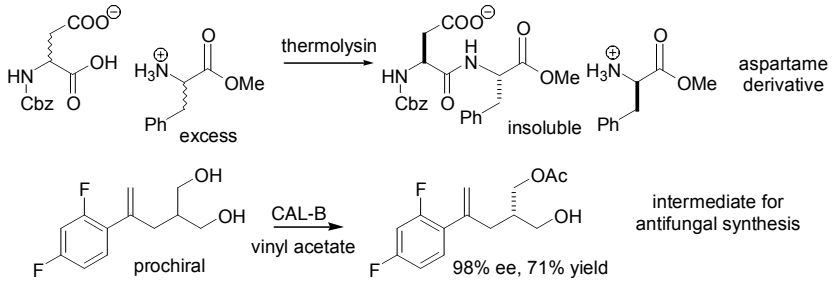


Fig. 2. Several unnatural, synthetically useful reactions catalyzed by hydrolases. Thermolysin catalyzes the regio- and enantioselective coupling of *N*-benzyloxycarbonyl-L-aspartate with L-phenylalanine methyl ester. Precipitation of the product drives this reaction in the condensation direction instead of the normal hydrolysis direction. This condensation is a key step in the manufacture of aspartame, a low-calorie sweetener. Because of the high enantioselectivity of thermolysin, racemic substrates may be used. Because of the high regioselectivity of thermolysin for the α-carboxyl group, the β-carboxyl group in the aspartic acid derivative needs no protection. Lipase B from *Candida antarctica* (CAL-B) catalyzes the enantioselective acetylation of a prochiral diol yielding an intermediate for the synthesis of antifungal agents. This example is an alcoholysis where the ester, vinyl acetate, is cleaved not by water, but by the substrate alcohol. This reaction is run in an organic solvent to avoid the competing hydrolysis.

Several other features make hydrolases convenient to use as synthetic reagents. Many hydrolases (approximately several hundred) are commercially available. They do not require cofactors and they tolerate the addition of water-miscible solvents (e.g., DMSO, DMF). Lipases, esterases and some proteases are also stable and active in neat organic solvents.

Enzymes are often classified according to the reaction catalyzed using an Enzyme Commission (EC) number. According to this classification, hydrolases form group 3 and are further classified according to the type of bond hydrolyzed. For example enzymes in the group 3.1 hydrolyze ester bonds (Tab. 1). Further classification into subcategories yields a four digit EC number. For example, lipases have the number EC 3.1.1.3. Classification of the more useful enzymes for organic synthesis is given in Tab. 1. A convenient web site to look up numbers and classification is at *http://www.expasy.ch/enzyme*. One disadvantage of this classification is that all enzymes catalyzing the same reaction have the same number, even though they may have very different structures, properties and other characteristics. For example, all lipases have the same number even though there are more than one hundred different lipases.

Tab. 1. Selected Hydrolases Useful in Organic Synthesis.

EC Number	Type of bond hydrolyzed	Examples
3.1	**Ester**	
3.1.1	in carboxylic acid esters	triacylglycerol lipase, acetylcholine esterase, phospholipase A_1, phospholipase A_2, glucon-olactonase, lipoprotein lipase
3.1.3–4	in phosphoric acid mono- or diesters	phospholipase C, phospholipase D
3.2	**Glycosidic**	
3.2.1	in *O*-glycosides	α-amylase, oligo-1,6-glucosidase, lysozyme, neuraminidase, α-glucosidase, β-galactosidase, α-mannosidase, N-acetyl-β-glucosaminidase, sucrose α-glucosidase, nucleosidases
3.3	**Ether**	
3.3.2	in epoxides	epoxide hydrolase
3.4	**Peptide**	
3.4.11	aminopeptidase	leucine aminopeptidase
3.4.16, 21	serine proteinase	subtilisin, chymotrypsin, thermitase
3.4.18, 22	cysteine proteinase	papain
3.4.17, 24	metalloproteinase	thermolysin
3.5	**Other amides**	
3.5.1	in linear amides	penicillin amidase (penicillin G acylase)
3.5.2	in cyclic amides	hydantoinase
3.5.5	in nitriles	nitrilase[a]
3.8	**Halide bonds**	
3.8.1	carbon-halide bonds	haloalkane dehalogenase

[a]Nitrile hydratase (EC 4.2.1.84), which catalyzes addition of water to a nitrile yielding an amide, is not a hydrolase, but a lyase.

This book describes the application of lipases and proteases in organic syntheses, but also surveys esterases, epoxide hydrolases, nitrile hydrolyzing enzymes and glycosidases. The emphasis is on examples that are synthetically useful, especially those that exploit the regio- and stereoselectivity of hydrolases.

2 Designing Enantioselective Reactions

2.1 Kinetic Resolutions

In a kinetic resolution, the enantiomeric purity of the product and starting material varies as the reaction proceeds (reviewed by Kagan and Fiaud, 1988). Thus, comparing enantiomeric purities for two kinetic resolutions is meaningful only at the same extent of conversion. To more conveniently compare kinetic resolutions, Charles Sih's group developed equations to calculate their inherent enantioselectivity (Chen et al., 1982; 1987; reviewed by Sih and Wu, 1989). This enantioselectivity, called the enantiomeric ratio, E, measures the ability of the enzyme to distinguish between enantiomers. A non-selective reaction has an E of 1, while resolutions with E's above 20 are useful for synthesis. To calculate E, one measures two of the three variables: enantiomeric purity of the starting material (ee_s), enantiomeric purity of the product (ee_p), and extent of conversion (c) and uses one of the three equations below (Eq. 1). Often enantiomeric purities are more accurately measured than conversion; in these cases, the third equation is more accurate.

$$E = \frac{\ln[1 - c(1 + ee_p)]}{\ln[1 - c(1 - ee_p)]}; \quad E = \frac{\ln[(1 - c)(1 - ee_s)]}{\ln[(1 - c)(1 + ee_s)]}; \quad E = \frac{\ln\left[\frac{1 - ee_s}{1 + (ee_s/ee_p)}\right]}{\ln\left[\frac{1 + ee_s}{1 + (ee_s/ee_p)}\right]} \tag{1}$$

High E values (≥ 100) are less accurately measured than low or moderate E values because the enantiomeric ratio is a logarithmic function of the enantiomeric purity. At $E \geq 100$, small changes in the measured enantiomeric purities give large changes in the enantiomeric ratio. Thus, the survey below avoids reporting E values above 100. In practice, we found that even E values near 50 were sometimes difficult to measure more precisely than ± 10. A simple program to calculate enantiomeric ratio using the above equations is freely available at *http://www.orgc.tugraz.at/* (Kroutil et al., 1997a). In spite of the fact that these equations include assumptions such as an irreversible reaction, one substrate and product, and no product inhibition, they are reliable in the vast majority of cases, especially for screening studies. Recently, faster spectrophotometric methods for measuring the enantiomeric ratio (Janes and Kazlauskas, 1997; Janes et al., 1998) using samples of pure enantiomers were developed. One method, called Quick E, is restricted to *p*-nitrophenyl derivatives of chiral carboxylic acids, but a more recent method can be used for any ester and identification of active and enantioselective hydrolases is based on a pH change using *p*-nitrophenol as pH indicator.

For careful optimization of reactions, three situations require a more careful approach. First, when the biocatalyst is a mixture of enzymes, for example, isozymes, which all act on the substrate, then the calculated E value reflects a weighted average of all the enzymes (Chen et al., 1982). When these enzymes differ significantly in their affinity for the substrate, then different enzymes will dominate the activity at different substrate

concentrations. Thus, the apparent enantioselectivity may vary as the reaction depletes the substrate or when the reaction is carried out with different initial substrate concentrations. When enzymes differ in their stability, apparent enantioselectivities for long vs. short reaction times may differ. To measure the true E-value, one must purify the enzymes and measure E separately.

Second, when product inhibits the reaction the apparent enantioselectivity can change (Rakels et al., 1994a; van Tol et al., 1995a; b). For example, addition of 4 v/v% ethanol to a carboxylesterase NP-catalyzed hydrolysis of ethyl 2-chloropropionate increased the enantioselectivity from 4.7 to 5.4 (see also Sect. 5.4.4.1). Rakels et al. (1994a) attributed this change not to changes in the inherent selectivity of the enzyme, but to selective inhibition of one of the enantiomers by ethanol. In another example (van Tol et al., 1995a; b), could not recover enantiomerically pure starting material in the PPL-catalyzed hydrolysis of glycidol butyrate even at high conversion. The enantiomeric purity of the remaining glycidol butyrate reached 95% ee at 70% conversion, but did not increase further even at 90% conversion. In other words, the apparent enantioselectivity dropped from 20 at 31% conversion to 2.7 at 95% conversion. van Tol et al. (1995a; b) attributed this plateau to product inhibition promoting the reverse reaction for the product enantiomer. To include product inhibition in the quantitative analysis, reseachers used more complex equations which take into account the mechanism of lipase-catalyzed reactions (ping-pong bi-bi). Until now few researchers included product inhibition in their analysis, but a readily available computer program (Anthonsen et al., 1995) simplifies this task.

Third, when the reaction is reversible, such as transesterification, one must include the equilibrium constant for the reaction (Chen et al., 1987). One can first measure the equilibrium constant in a separate experiment and then determine E from measurements of ee_s and ee_p. Anthonsen et al. (1995) developed a simpler approach where they determine both K and E by fitting a series of ee_s and ee_p measurements.

2.1.1 Recycling and Sequential Kinetic Resolutions

To enhance the enantiomeric purity, the enriched material can be isolated and resolved again. This double resolution is called recycling. Chen et al. (1982) derived an equation to predict the optimum degree of conversion in recycling reactions and many researchers have used this strategy (for an example see: Johnson et al., 1995). Brown et al. (1993) and Kanerva and Vänttinen (1997) reported several examples. A computer program for calculations is available at *http://www.orgc.tu-graz.ac.at* (Kroutil et al., 1997b). Guo (1993) reported plots to predict the maximum chemical yield in various situations. To minimize the work in recycling reactions, several groups used *in situ* recycling where the two resolutions are carried out stepwise, but without isolation of the intermediate products (Chen and Liu, 1991; Majeric and Sunjic, 1996; Sugai et al., 1996). Some authors called these reactions sequential kinetic resolutions, but we favor *in situ* recycling and reserve the term sequential kinetic resolution only for those reactions where both steps occur at the same time, such as the acylation of diols.

Like recycling reactions, sequential kinetic resolutions enhance the enantiomeric purity of the products (Caron and Kazlauskas, 1991; Guo et al., 1990; Kazlauskas, 1989). For example, hydrolysis of *trans*-1,2-diacetoxycyclohexane proceeds stepwise – first hy-

drolysis to the monoacetate, then to the diol (Fig. 3) (Caron and Kazlauskas, 1991). Both reactions favor the same enantiomer, thus, the two resolutions reinforce each other. Maximum reinforcement occurs when both reactions occur at comparable rates with an overall enantioselectivity of approximately (E_1 x E_2)/2 (Caron and Kazlauskas, 1991). In addition, sequential kinetic resolutions yield both the starting material and product in high enantiomeric purity at the same extent of conversion because the 'mistakes' remain in the intermediate product (monoacetate in the example in Fig. 3). In contrast, single step kinetic resolutions yield high enantiomeric purity for the product at < 50% conversion, but high enantiomeric purity for the starting material requires > 50% conversion.

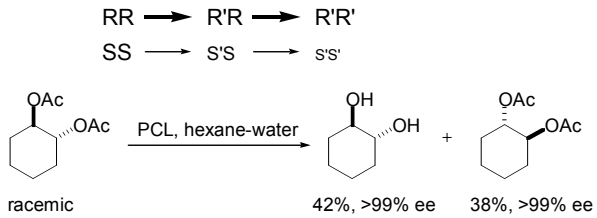


Fig. 3. Sequential kinetic resolution enhances the enantiomeric purity of the product through two enantioselective steps.

C_2-symmetric diols are especially well suited to sequential kinetic resolution because both steps are likely to have the same enantiopreference (Fig. 4).

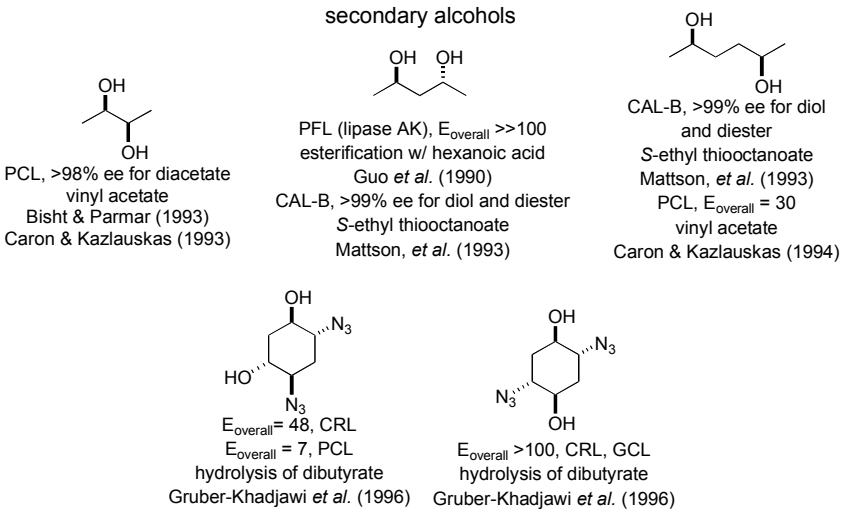


Fig. 4. Examples of C_2-symmetric diols resolved by sequential kinetic resolution include secondary and primary alcohols as well as diols with axial chirality.

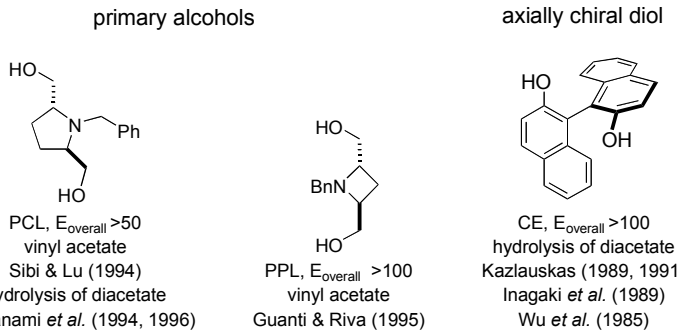


Fig. 4. Examples of C_2-symmetric diols resolved by sequential kinetic resolution include secondary and primary alcohols as well as diols with axial chirality (continued).

Unsymmetrical diols can also undergo a sequential kinetic resolution (Fig. 5).

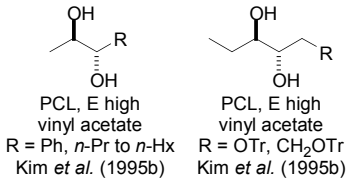


Fig. 5. Sequential kinetic resolution of non-C_2 symmetric diols.

Only one dicarboxylic acids was resolved by lipase-catalyzed sequential kinetic resolution and this was a special case. Node et al. (1995) hydrolyzed a racemic C_2-symmetric tetraester. The non-conjugated ester groups reacted selectively followed by spontaneous decarboxylation. Interestingly, CRL and RJL favored opposite enantiomers. Although Node et al. (1995) suggested possible racemization of the starting tetraester, which would allow a dynamic kinetic resolution (Sect. 2.1.2), they did not report yields over 50% . The lack of carboxylic acid examples may be due to more efficient resolution of alcohols by lipases, or to the slow hydrolysis of monoesters containing a charged carboxylate group by lipases (Fig. 6).

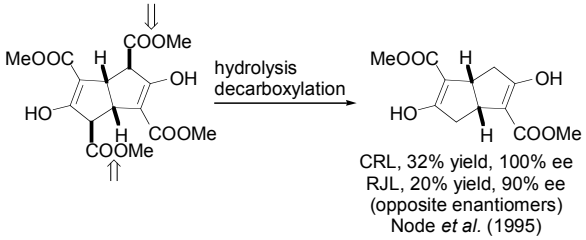
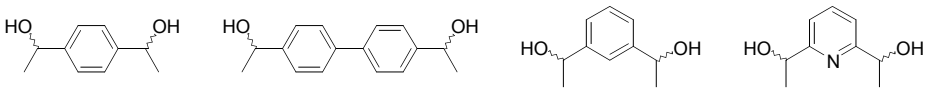


Fig. 6. Sequential kinetic resolution of a chiral diacid.

For substrates with a single functional group, researchers demonstrated a sequential kinetic resolution by *in situ* hydrolysis of an ester and reesterification to a new ester (Macfarlane et al., 1990). However, reversibility of these reactions limited the enhancement of enantioselectivity (Straathof et al., 1995). In these cases, an *in situ* recycling reaction (see above) is probably a better way to enhance the enantiomeric purity.

Enantioselective reactions can also separate diastereomers. For example, Wallace et al. (1992) used the (*R*)-enantioselectivity of PCL to separate a mixture of *meso* and racemic diols. The (*R,R*)-diol reacted to the diacetate, the (*R,S*)-diol to the monoacetate, and the (*S,S*)-diol did not react (Fig. 7).



PCL, $E_{overall}$ = high, acetylation or hydrolysis, Wallace *et al.* (1992)

Fig. 7. Enantioselective reactions separated diastereomers as well as enantiomers.

2.1.2 Dynamic Kinetic Resolutions

2.1.2.1 Introduction

Kinetic resolution limits the yield of each enantiomer to 50%. However, if the substrate racemizes quickly in the reaction mixture, then the yield of product enantiomer can be 100% (Fig. 8). This resolution with *in situ* racemization is called dynamic kinetic resolution or asymmetric transformation of the second kind (for reviews see: Stecher and Faber, 1997; Ward, 1995; Faber, 2001; Pellissier, 2003; Schnell et al., 2003). The requirements for a dynamic kinetic resolution are: (1), the substrate must racemize at least as fast as the subsequent enzymatic reaction, (2), the product must not racemize, and (3), as in any asymmetric synthesis, the enzymic reaction must be highly stereoselective. The equations relating product enantiomeric purity and enantioselectivity are the same as those for asymmetric syntheses (Sect. 2.2): $ee_P = (E - 1)/(E + 1)$ and $E = (1 + ee_P)/(1 - ee_P)$, where E is the enantiomeric ratio and ee_P is the enantiomeric purity of the product.

The key step in dynamic kinetic resolutions is the *in situ* racemization. A number of reactions can racemize organic substrates (review: Ebbers et al., 1997), but most conditions are too harsh to allow a simultaneous enzyme-catalyzed reaction. In the past, the difficulty of racemizing normal alcohols and carboxylate esters restricted these dynamic kinetic resolutions to special cases such as the one in Fig. 8b. However, recent discoveries of organometallic catalysts that can racemize a wide range of secondary alcohols have extended the range of these reactions. The sections below group the dynamic kinetic resolution examples according to the racemization mechanism.

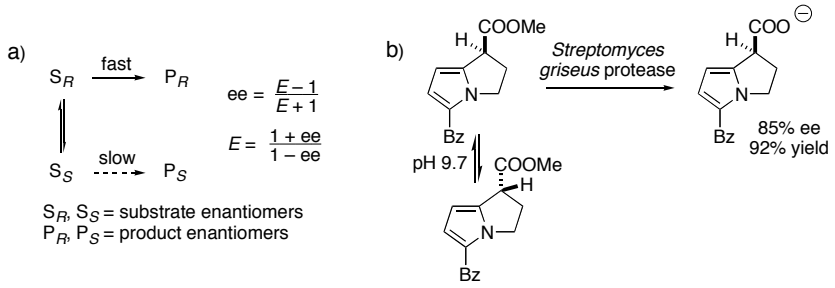


Fig. 8. Dynamic kinetic resolution. a) Dynamic kinetic resolution involves an *in situ* racemization of the substrate (S_R and S_S) combined with an enantioselective reaction of one substrate enantiomer (P_R favored in this scheme). b) An example of a dynamic kinetic resolution. The substrate ester contains a moderately acidic hydrogen α to the carbonyl and aromatic ring. Deprotonation to the achiral enolate and reprotonation racemized this ester. The protease catalyzed the enantioselective hydrolysis of one enantiomer. The product carboxylate did not racemize because the negative charge on the carboxylate made deprotonation less favorable (Fülling and Sih, 1987).

2.1.2.2 Racemization by Protonation/Deprotonation

The earliest dynamic kinetic resolutions involved 5-arylsubstituted hydantoins, which racemize spontaneously at pH > 8 via an enolate (Olivieri et al., 1981; Takahashi et al., 1979; Tsugawa et al., 1966). Hydantoinases catalyze the highly enantioselective hydrolysis of 5-monosubstituted hydantoins to *N*-carbamoyl-α-amino acids (Fig. 9).

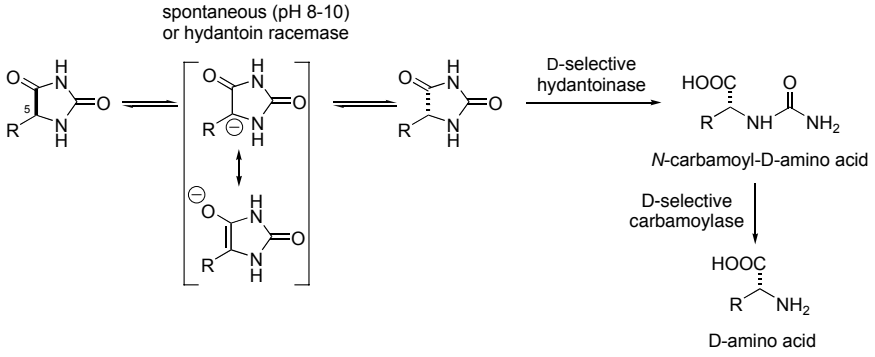
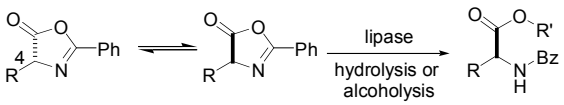


Fig. 9. Hydantoinases catalyze the hydrolysis of 5-monosubstituted hydantoins to *N*-carbamoyl α-amino acids. 5-Substituted hydantoins, especially 5-aryl hydantoins, racemize readily either enzymatically or chemically at pH 8–10 via the enolate. The figure shows a D-selective hydanoinase; L-selective hydanoinases are rare. The last step, removal of the *N*-carbamoyl group, may also contribute to the overall enantioselectivity.

The most important amino acids produced by this route are D-phenylglycine and D-4-hydroxyphenylglycine for production of the semisynthetic penicillins, ampicillin and amoxicillin, respectively (May et al., 2002). A similar process could also yield the L-series of α-amino acids, but the L-selective hydantoinases are rare. See also Sect. 4.2.1.3 for directed evolution and Sect. 6.4.2.1 for further information on hydantoinases.

Another dynamic kinetic resolution route to D-phenylglycine starts with (±)-phenylglycine methyl ester and yields the amide (Wegman et al., 1999). Phenylglycine methyl ester racemizes in the presence of aldehydes via a Schiff-base intermediate. Combining this racemization with a CAL-B-catalyzed ammonolysis at –20°C yielded D-phenylglycine amide in 85% ee and 85% yield.

The structurally similar 4-substituted-2-phenyloxazolin-5-ones also racemize readily, but finding enantioselective hydrolases has been more difficult (Eq. 2; Tab. 2). For R = Me, Bn, and several others, Bevinakatti et al. (1990; 1992) used a RML-catalyzed alcoholysis in organic solvents, but the enantioselectivity was only E = 3–5. Sih's group screened a dozen lipases for hydrolysis of the phenylalanine derivative (R = Bn) and found that PPL favored the natural (*R*)-enantiomer (E > 100), while ANL favored the unnatural (*S*)-enantiomer (E > 100) (Crich et al., 1993; Gu et al., 1992) (Tab. 2). However, these enzymes were less enantioselective toward other, similar derivatives. Several *Pseudomonas* lipases (PCL, Amano AK, Amano K-10) at 50°C in *t*-BuOMe catalyzed methanolysis of a variety of 4-substituted 2-phenyloxazolin-5-ones with enantioselectivities of 5–39, usually favoring the (*S*)-enantiomer.



(2)

Tab. 2. Lipase-Catalyzed Ring-Opening of 2-Phenyloxazolin-5-ones.

Lipase	Reaction	R =	E	References
RML	alcoholysis	Bn, Me, *n*-Pr, CH_2i-Pr	3–5 (*S*)	Bevinakatti et al. (1990; 1992)
PPL	hydrolysis	Bn	> 100 (*S*)	Gu et al. (1992)
ANL	hydrolysis	Bn	> 100 (*R*)	Gu et al. (1992)
ANL, PPL	hydrolysis	Ph, 4-OMePh, CH_2CH_2Ph, several CH_2Ar, CH_2i-Pr, CH_2CH_2SMe	2–12	Crich et al. (1993); Gu et al. (1992)
PXL[a]	alcoholysis	13 different examples	5–39	Crich et al. (1993)
RML	alcoholysis	*t*-Bu	> 100 (*S*)	Turner et al. (1995)
CAL-B	alcoholysis	Bn, CH_2i-Pr, *i*-Pr, indolemethylene	≥19	Brown et al. (2000)

[a] One of several *Pseudomonas* lipases: PCL, Amano AK, or Amano K-10. Most reactions favored the (*S*)-enantiomer, but in some cases the enantiopreference was either (*R*) or (*S*) depending on the amount of added water.

In several cases, the enantioselectivity reversed depending on whether the reaction mixture contained added water or not. The lipase usually hydrolyzed substrates with larger R groups (e.g., Ph, CH_2i-Pr) more selectively than small ones (e.g., Me). For preparative use, Crich et al. (1993) further resolved the enantiomerically-enriched methyl esters of *N*-benzoyl amino acids by protease-catalyzed cleavage of the ester. Turner et al. (1995) found that RML-catalyzed alcoholysis of the *t*-butyl derivative was highly enantioselective (99.5% ee, 94% yield), but only when the reaction mixture con-

tained a catalytic amount of triethylamine. The authors suggested that the triethylamine may increase enantioselectivity by forming an ion pair with a side product formed by hydrolysis instead of alcoholysis – *N*-benzoyl amino acid (Brown et al., 2000). Without ion pair formation, this carboxylic acid seems to lower the enantioselectivity of the reaction.

Simple esters of chiral carboxylic acids also undergo base-catalyzed racemization if the acid contains electron-withdrawing substituents. Fülling and Sih (1987) reported the first enzyme-catalyzed example using a *Streptomyces griseus* protease, which was described in Fig. 8 above. Acyloin derivatives also racemize quickly in the presence of a catalytic amount of triethylamine (Taniguchi et al., 1997; Taniguchi and Ogasawara, 1997), while butenolides racemize readily at room temperature and pyrrolinones racemize at 69°C, Fig. 10 (Thuring et al., 1996a; van der Deen et al., 1996). In each case, lipases catalyzed selective acetylation of one enantiomer in excellent yield.

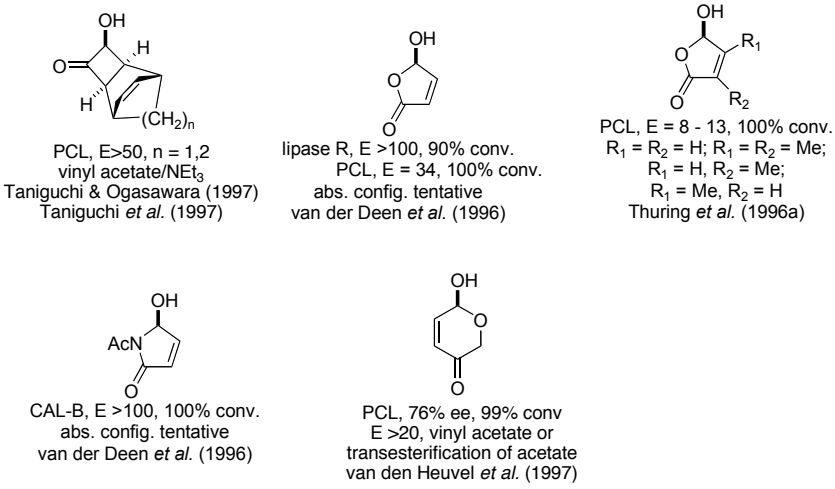
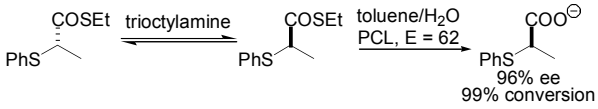


Fig. 10. Dynamic kinetic resolution of alcohols that racemize by deprotonation/protonation. The butenolides, acyloins, and pyrrolinones contain a carbonyl group or a vinylogous carbonyl group α to the stereocenter making deprotonations more facile. In each case, the product isolated was the corresponding acetate.

Tan et al. (1995) resolved 2-(phenylthio)propanoic acid by PCL-catalyzed hydrolysis of the thioester in the presence of trioctylamine (Eq. 3). Both the thioester and the trioctylamine promote racemization via an enolate mechanism. Similarly, Um and Drueckhammer (1998) resolved thioesters of 2-aryl and 2-aryloxypropanoic acids using subtilisin (E = 7-11) combined with *in situ* racemization promoted by trialkylamines. Chosing the type of thioester (e.g., 2,2,2-trifluoroethyl thiol ester) was the key to rapid racemization. Vörde et al. (1996) suggested that even simple esters may racemize in the presence of both CAL-B and α-phenylethylamine (Eq. 4). They did not detect racemization in the presence of only one of these.



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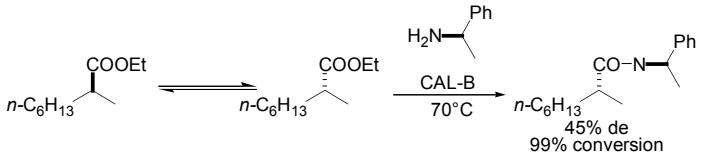


(4)

Racemases are an ideal way to convert kinetic resolutions to dynamic kinetic resolutions (review: Schnell et al., 2003). Research in this area accelerated recently and several examples have been reported. For example, an *N*-acetyl-α-amino acid racemase converts the acylase-catalyzed kinetic resolution of *N*-acetyl-α-amino acids (see Sect. 6.4.1.3) into a dynamic kinetic resolution (Tokuyama and Hatano, 1996; Tokuyama, 2001; Verseck et al., 2001; May et al., 2002). In the kinetic resolution approach, the unreacted D-*N*-acetyl-α-amino acid is separated, racemized chemically *ex-situ* and added to the next kinetic resolution. However, adding an *N*-acetyl-α-amino acid racemase eliminates the separation and racemization steps and allows use of a continuous bioreactor (Fig. 11).

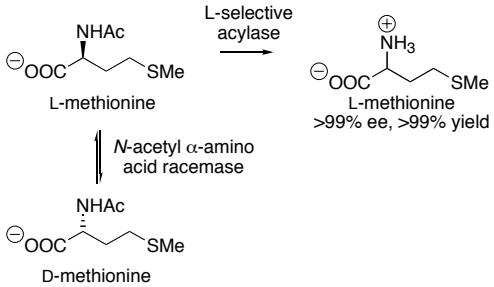


Fig. 11 Dynamic kinetic resolution of *N*-acetyl methionine using *N*-acetyl-α-amino acid racemase and an L-selective acylase (Tokuyama and Hatano, 1996). Replacing the L-selective acylase with a D-selective acylase yields D-methionine.

Interestingly, the *N*-acyl amino acid racemase from *Amycolaptosis* sp. discovered by Tokuyama and Hatano (1996) may be a case of mistaken identity due to catalytic promiscuity (see Sect. 1.1). This racemase is one-thousand times more efficient as a catalyst for a dehydration to form *o*-succinylbenzoate suggesting that succinylbenzoate formation is its true role, Fig. 12 (Palmer et al., 1999; Ringia et al., 2004). By changing the *N*-acyl amino acid from *N*-acetyl methionine (the previous best substrate for racemase activity) to *N*-succinyl phenylglycine, which better resembles the succinylbenzoate precursor, the efficiency of the racemization reaction increased one-thousand fold making it similar to the succinylbenzoate reaction.

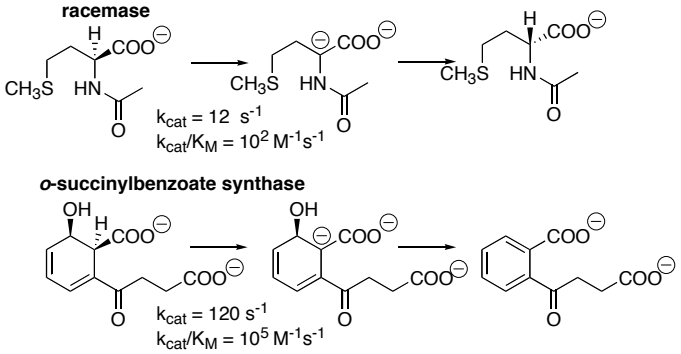


Fig. 12. An enzyme discovered as an *N*-acyl amino acid racemase is one thousand fold more efficient in the dehydration to form *o*-succinylbenzoate. Both reaction mechanisms involve a similar anion intermediate.

Several groups discovered an α-amino-ε-caprolactam racemase that also racemizes α-amino acid amides (Asano and Yamaguchi, 2005; Boesten et al., 2003). These amides come from the classic Strecker synthesis of amino acids and are therefore key precursors of α-amino acids. Asano and Yamaguchi (2005) used this racemase in combination with a D-selective amidases in a dynamic kinetic resolution of the unnatural D-α-amino acids.

Both mandelate racemase (Felfer et al., 2005) and lactate racemase (Glueck et al., 2005) accept a range of α-hydroxy carboxylic acid substrate analogs. Their application in a dynamic kinetic resolution with a hydrolase would require their use in nonaqueous solvents. The substrate α-hydroxy carboxylic acid must be enantioselectively esterified at the carboxylate or acylated on the hydroxy group. Unfortunately, mandelate racemase is inactive in nonaqueous solvents, but lactate racemase has not yet been tested.

2.1.2.3 Racemization by Addition/Elimination

Reversible base-catalyzed addition of HCN to aldehydes formed racemic cyanohydrins (Inagaki et al., 1991; 1992). Enantioselective acetylation of the (*S*)-cyanohydrin catalyzed by PCL yielded the acetate in good to moderate yields and enantiomeric purity. In general, PCL showed higher enantioselectivity toward cyanohydrins derived from aromatic aldehydes than from aliphatic aldehydes.

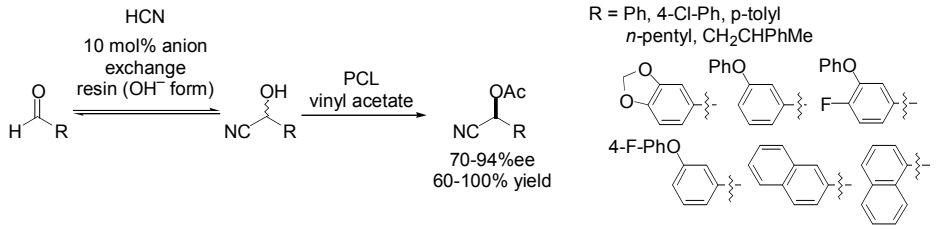


Fig. 13. Dynamic kinetic resolution of cyanohydrins via reversible addition of hydrogen cyanide to aldehydes.

CAL-B also shows high enantioselectivity toward cyanohydrins (Hanefeld et al., 2000). PCL did not catalyze acylation of the HCN donor, acetone cyanohydrin, a tertiary alcohol, presumably because it is too hindered (Fig. 13).

Veum et al. (2002), but dynamic kinetic resolutions with this lipase stopped at only 16% conversion. The culprit was water in the reaction mixture, which caused hydrolysis of the acyl donor (vinyl acetate) to form acetic acid. This acid neutralized the basic racemization catalyst and inactivated the enzyme. Adjusting the reaction conditions by using dry conditions, extra added base and a solid support that absorbs water strongly improved the reaction dramatically (Li et al., 2002; Veum et al., 2005). Under these optimized conditions, (*S*)-mandelonitrile acetate formed in 97% yield and 98% ee.

Similar reversible addition of thiols to aldehydes (catalyzed by silica gel) formed racemic hemithioacetals (Brand et al., 1995) (Fig. 14). Resolution using a PFL-catalyzed acetylation yielded precursors for nucleoside analogs.

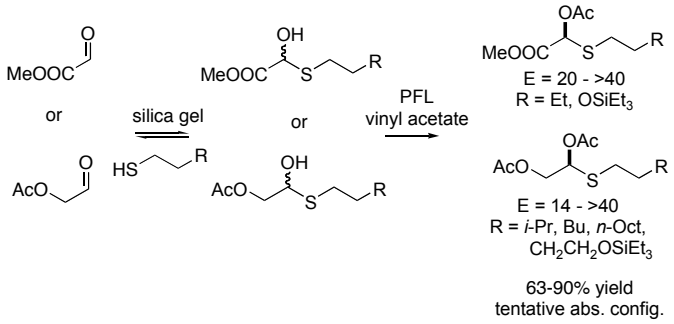


Fig. 14. Dynamic kinetic resolution of hemithioacetals via reversible addition of thiols to aldehydes.

Fig. 14 above included several examples of the related cyclic hemiacetals. These hemiacetals may racemize either via a protonation/deprotonation mechanism (most likely) or via an addition/elimination mechanism.

Another example of a dynamic kinetic resolution involving an addition/elimination mechanism is the reversible Michael addition to form the aryl isoxazoline combined with a PCL-catalyzed hydrolysis of the (*R*)-thioester, Fig. 15 (Pesti et al., 2001).

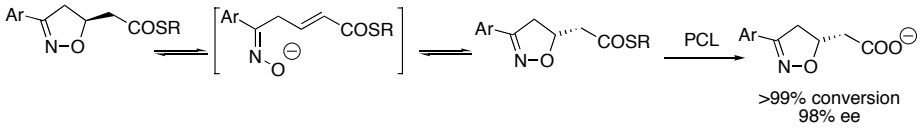
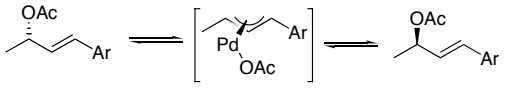


Fig. 15. Combination of a reversible Michael addition and lipase-catalyzed hydrolysis yields a DKR.

A major advance in dynamic kinetic resolutions was the discovery that lipases are compatible with some organometallic racemization catalysts. These catalysts can racemize a broad range of secondary alcohols so similar reaction conditions apply to a broad range of substrates. These organometallic racemization catalysts contain either palladium (for racemization of allylic acetates) or ruthenium (for racemization of secondary alcohols). The palladium catalysts follow an addition/elimination mechanism and

are discussed below. The ruthenium racemization catalysts involve addition/elimination of hydrogen, which will be discussed in Sect. 2.1.2.5 below.

Palladium(0) or palladium(II) complexes add reversibly to allylic acetates to form a π-allyl palladium complex. This addition can racemize allylic acetates (Eq. 5). The details of the mechanism may differ depending of the substrate and reaction conditions (Granberg and Bäckvall, 1992) and rearrangement reactions are a possible side reaction. Enzyme-catalyzed hydrolysis or transesterification of allylic acetates yields allylic alcohols, which do not undergo this racemization.



(5)

Allen and Williams (1996) used the Pd(II)-catalyzed racemization in water for a dynamic kinetic resolution of a cyclic allylic acetate: 1-acetoxy-2-phenyl-2-cyclohexene (Fig. 16). Although slow racemization limited the rate of the reaction, both the yield and enantioselectivity were good.

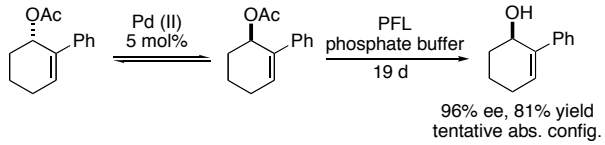


Fig. 16. Dynamic kinetic resolution of an allylic acetate via palladium-catalyzed racemization followed by an enantioselective lipase-catalyzed hydrolysis of the acetate.

Similar dynamic kinetic resolution of another cyclic allylic acetate – 5-acetoxy-cyclohex-3-enecarboxylic acid methyl ester – gave the cyclohexenol in Fig. 17. Choi et al. (1999) extended these reactions to acyclic allylic acetates (Fig. 17) and decreased the reaction times from 11-19 d to 1.5-6 d by using a palladium(0) complex in organic solvent. To minimize formation of undesired rearrangement products, Choi and coworkers added the racemization catalyst after 45-50% conversion to minimize side reactions (elimination to form the diene or replacement of acetate by isopropanol). As an alternative, the corresponding acyclic allylic alcohols can also be racemized by oxidation-reduction using ruthenium catalysts, see Sect. 2.1.2.5 below. The final product is the acetate in one case, the alcohol in the other, but in both cases it is the same enantiomer.

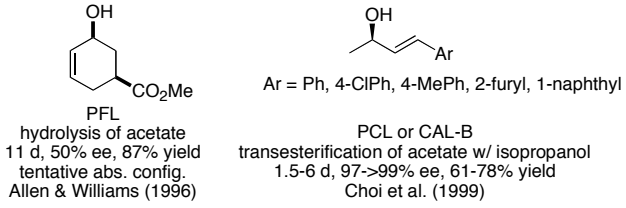


Fig. 17. Dynamic kinetic resolution of allylic acetates yielded allylic alcohols. Palladium complexes catalyzed the racemization of the starting allylic acetates.