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Review

Chemo-enzymatic deracemization methods for the preparation of enantiopure non-natural α-amino acids

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Contents

Abstract

The complete transformation of a racemate into one single enantiomer is defined as a deracemization. In amino acid chemistry, chemo-enzymatic deracemization is possible due to the ease of racemization of α-amino acids and the numerous enantioselective enzymatic systems operating on this class of compounds. Deracemization by dynamic kinetic resolution is a process in which the enantioselective catalyst is coupled with a second one promoting racemization of the reagent but not of the product. Deracemization by stereo-inversion is a convergent process in which the transformed isomer is finally converted into its enantiomer. These transformations can be applied for the preparation of enantiomerically pure α-amino acids of non-natural configuration or of L-α-amino acid of non-natural structure.

Keywords: Deracemization; Dynamic kinetic resolution; Stereo-inversion; Enzymatic catalysis; Biocatalysis; Amino acids

1. Introduction

Naturally occurring peptides based on proteinogenic amino acids find limited application as drugs due to rapid metabolism by proteolysis and interaction with multiple receptors [1]. Peptidomimetics have therefore found increased application as surrogates for their increased *in vivo* stability, enhanced potency, better oral absorption, improved distribution in tissue and increased selectivity in biological response. These compounds form the basis of important families of enzyme inhibitors [2]. The study and design of these structural scaffolds largely relies on α-amino acids possessing non-natural structures or non-natural absolute configurations [3].

Several non-proteinogenic amino acids are already important industrial targets: L-Dopa is used to alleviate some of the symptoms of Parkinson's disease, D-penicillamine is used for symptomatic treatment of arthritis. D-Phenylglycine and D-4-hydroxyphenylglycine are used in the semisynthetic

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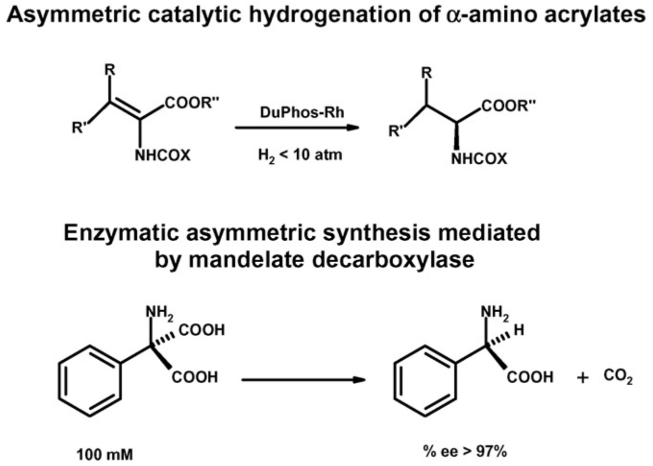
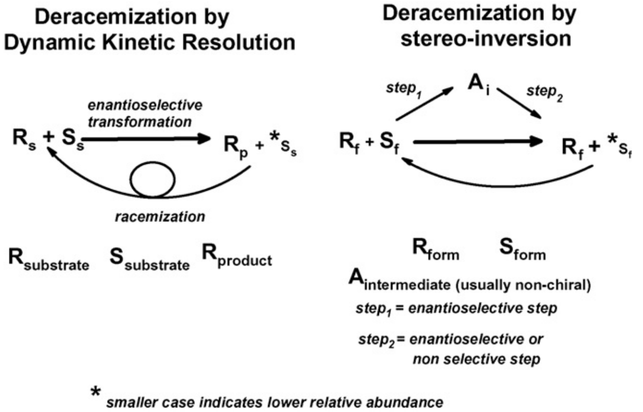


Scheme 1. Two examples of asymmetric synthesis applied to the preparation of non-racemic α-amino acids.



Scheme 2. Deracemization methods based on dynamic kinetic resolution or stereo-inversion.

broad-spectrum antibiotics like Ampicillin and Amoxicillin. D-2-Naphthylalanine is found in the peptide drug Nafarelin. A very important and successful drug for treating high-blood pressure, Enalapril (and analogues alike) contains L-homophenylalanine.

Moreover, due to their multifunctional structure non-natural amino acids are versatile building blocks in the synthesis of chiral compounds and are useful as templates in asymmetric catalysis [4].

While natural amino acids are obtained from fermentation or applying natural enzymatic systems, non-natural analogues can be prepared with methods based on asymmetric synthesis either via chemical catalysis [5] or biocatalysis [6].

The term asymmetric synthesis indicates a procedure by which a pro-chiral starting material is transformed into one single enantiomer in a reaction mediated by a chiral catalyst. The synthesis of phenylalanine through the catalytic hydrogenation of the corresponding amino-acrylate or the decarboxylation of phenylmalonates mediated by a mandelate decarboxylase are examples of a chemical and biocatalytic asymmetric synthesis, respectively (Scheme 1).

In recent times, the term *deracemization* has been used to describe techniques becoming increasingly important for the preparation of chiral compounds as single enantiomers. The term refers to a process in which starting from a racemate, one single enantiomer is obtained [7].

The two more common strategies to achieve such an objective are deracemization by dynamic kinetic resolution (Der-DKR) and deracemization by stereo-inversion (Der-SI) (Scheme 2).

Der-DKR is, in principle, a kinetic resolution process in which the non-transformed enantiomer is racemized *in situ*. The conditions are that a chiral catalyst promote the transformation of one enantiomer (R_s) into the product (R_p) while the other enantiomer is racemized at a comparable rate and the racemic mixture ($R_s + S_s$) is restored. The product (R_p) is not racemized under the same conditions. Unlike a simple kinetic resolution (KR) yielding a maximum of 50% of the product, with this technique 100% conversion can be reached. If compared with a DKR, the traditional KR seems to belong to an old chemistry era. However the majority of chiral molecules of industrial interest are still prepared by KR. This is due to the cost associated to the replacement of an established industrial process by a new one, even if intrinsically more convenient. In the last 10 years numerous chemo- or bio-catalytic systems for DKR have been reported in the literature and are operated at industrial scale [8].

Der-SI is a process in which one form (S_f) of the racemic starting material ($R_f + S_f$) is enantioselectively transformed into an intermediate (A_i, usually non-chiral) which can in turn react to give the form of opposite configuration (R_f) or the racemic mixture again ($R_f + S_f$). In the latter case, successive cycles allow the enrichment and eventually the complete transformation into one single enantiomer. An example of this method could be the selective oxidation of one enantiomer of a racemic secondary alcohol and the subsequent reduction with a catalyst of opposite stereo-preference [8b].

In this article recent methods of Der-DKR and Der-SI applied to the preparation of non-natural amino acids will be reviewed.

2. Deracemization methods for amino acid synthesis

Deracemization methods are particularly suited for non-racemic amino acid preparation since the synthesis of the racemic forms are well established. Although asymmetric variations of these methods (for asymmetric Strecker synthesis see Ref. [9]) have been reported in the literature, they are, at present, not mature enough for application on a larger scale. Yet, non-natural amino acids are required in preparative scale and high-enantiomeric excess. Enzymatic methods are particularly suited for these compounds for the large number of hydrolytic or oxidative enzymes for which amino acids are the favorite substrates. Acylase of type I and of type II have been isolated from hog kidney and used for the determination of absolute configuration of naturally occurring amino acids and for the kinetic resolution of synthetic ones. Amino peptidases are also naturally occurring animal enzymes hydrolysing amino acid amides. Long-established industrial methods based on racemate resolution with these enzymes are well known. Amino acid oxidases with complete D- and L-selectivity have been used for the assessment of amino acid absolute configuration [10].

The deracemization methods here described will be based on one enzymatic step coupled with a second enzymatic or chemical step.

2.1. Deracemization via dynamic kinetic resolution (DKR)

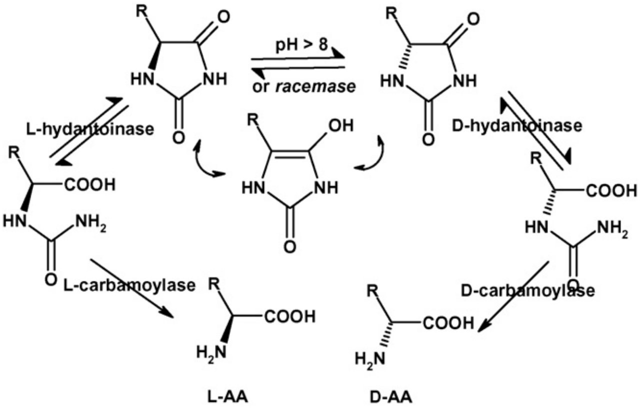
Requisite for a successful DKR are that the starting material is racemizable under the reaction conditions while the product is configurationally stable under the same conditions. Due to the industrial importance of amino acids, numerous studies have been devoted to the problem of effective racemization of the enantiomer remaining after a kinetic resolution [11].

However, racemization methods based on high temperature or extreme pH values are not suited for *in situ* racemization. Thus if enzymes are involved, the racemization reaction must be brought about in mild conditions, using the catalytic action of a second enzyme (racemase) or a base, exploiting the difference in the pK_a value of the methine on the α-carbon due to difference in structure between substrate and product.

2.1.1. Enzyme catalyzed kinetic resolution coupled with in situ racemization

Several effects can contribute to lowering the pK_a of the proton on the α-carbon of an amino acid derivative, the requisite for racemization. Both the transformation of the amine into a NH–CO bond either in a cyclic structure or in an open chain and the presence of the carboxylate in the form of an ester, amide or thio ester have the effect of a more prompt racemization of the amino acid derivative. The presence of a C=C bond in the position β–γ has a strong effect. A combination of more than one structural modification is often necessary in order to obtain an effective racemization under the conditions of pH and temperature at which the enzyme is active. Substrates on which a general method for an enzyme catalyzed kinetic resolution coupled with *in situ* racemization is successful are 5-monosubstituted hydantoins, containing both NH–CO and a CO–NH bonds (Sections 2.1.1.1 and 2.1.1.2), 4-substituted-2-phenyloxazolin-5-one (Sections 2.1.1.3 and 2.1.1.3.1) in which the amino acid nitrogen is in the form of an imino group and the carboxylate is present as a –CO–O bond and *N*-Boc-amino acid-thioesters in which the amino group is in the NH–CO– form, the carboxylate is in the form of a thio-ester and the R-group is aromatic (Section 2.1.1.4). The enzymatic kinetic resolution of amino acid esters coupled with aldehyde mediated *in situ* racemization (Section 2.1.1.5) and the enzymatic kinetic resolution of *N*-acyl amino acids coupled with *N*-acylase racemase assisted racemization (Section 2.1.1.6) complete the group of potentially effective Der-DKR for amino acids.

2.1.1.1. The hydantoinase–carbamoylase system for D*-amino acid synthesis.* 5-Monosubstituted hydantoins are α-amino acids cyclically protected at both the carboxyl- and the α-amino group. They can be easily prepared from an aldehyde and isocyanate, or by the Bucherer–Berg synthesis and similar methods. Indeed the hydantoin synthesis is also a practical method for the preparation of the racemic amino acid. Enzymes belonging to the dihydro-pyrimidinase family (having as natural substrate dihydrouracyl) hydrolyze the hydantoins to the carbamoyl amino acid. This can be hydrolyzed in turn to the amino acid by a second enzyme, a carbamoylase. Both enzymes can discriminate between enantiomers and if their action is cooperative, the L- or D-amino acid can be obtained (Scheme 3) [12]. The system is of special interest because the proton in the 5-position in the hydantoin ring (it will become the α-hydrogen in the α-amino acid) is quite more acidic than conventional protons in amino acid esters and amides and much more acidic than amino acid itself. Thus the hydantoin can often be racemized *in situ* at slightly basic pHs where the enzymes are still stable and active. If these conditions are met, the amino acid can be obtained in one single enantiomeric form in yields higher than 97%. Although the first observation of a D-hydantoin-hydrolyzing enzyme was due to Yamada et al. [13] the organism was lacking carbamoylase activity. Later, a strain of *Agrobacter radiobacter* was found to produce both D-hydantoinase and D-carbamoylase activity [14]. The bacteria were developed at the industrial level and applied to the large-scale production of D-*p*-hydroxy-phenylglycine and other D-amino acids.



Scheme 3. The hydantoinase–carbamoylase system for the deracemization of α-amino acids via DKR with *in situ* racemization.

The interconversion of the two enantiomeric hydantoins is crucial for the complete transformation of the racemate into one single enantiomer and the racemization step can become the rate limiting one. In fact the $t_{1/2}$ of racemization at pH 8.5 and 40 °C varies from 0.27 h for 5-phenyl-hydantoin to 55.9 h for the 5-isopropyl analogue. However convenient reaction rates can be obtained by regulating the biomass to substrate ratio [15a]. The use of the whole-cell system is necessary due to the limited stability of the carbamoyl-hydrolyzing enzyme and to the different requirements for optimal reactivity. The best carbamoylase activity is obtained by slightly lowering the reaction pH which is regulated from the development of carbon dioxide from the first reaction step [15b]. This requires that the reaction is performed in a pressurized reactor. The presence and the function of a racemizing enzyme in the cells have not been elucidated in the case of the D-selective microorganism. However the complete racemization of 5-isopropyl-hydantoin (the compound with a lower racemization rate) and the fact that the pH of 8.5 set at the start of the reaction is in fact immediately modified, strongly suggests the presence of a racemizing enzyme.

Table 1
Relative racemization rates and ee of D-amino acid obtained from five-substituted hydantoins with whole cells of *Agrobacterium radiobacter*

Five-substituted hydantoins	D-AA	$t_{1/2}$ rac (h)	ee (%)
5-Phenyl	D-Ph-Gly	0.27	>99
5-Hydroxymethyl	D-Ser	1.60	>99
5-Benzyl	D-Phe	5.00	>99
5-Methylthioethyl	D-Met	5.82	>99
5-1′-Hydroxyethyl	D-*allo*-Thr	6.41	>99
5-3′-Ureidopropyl	D-Cit[a]	14.26	>99
5-1′-Methylethyl	D-*allo*-Ile	15.84	>99
5-Imidazolylmethyl	D-His	16.09	>99
5-Isobutyl	D-Leu	21.42	>99
5-Methyl	D-Ala	33.98	>99
5-Isopropyl	D-Val	55.90	>99

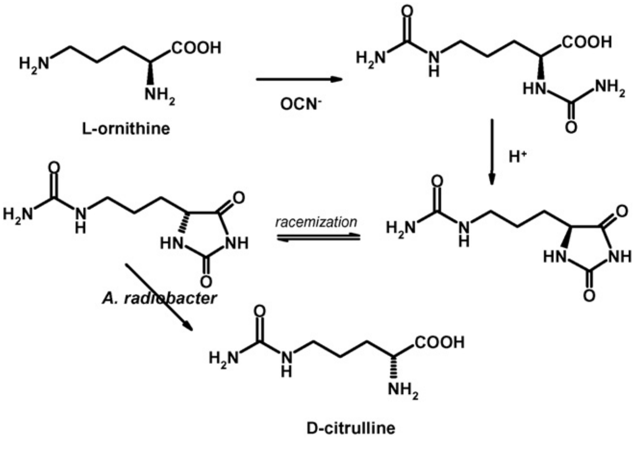
Source: Ref. [15a].
[a] D-Citrulline.

The D-hydantoinase–D-carbamoylase process has proved successful for the preparation of a large number of D-amino acids (Table 1) [14,15a].

Table 1 shows industrial data for the preparation of a number of D-amino acids from the corresponding 5-substituted hydantoins. Although the racemization rates can vary by two orders of magnitude, all the AAs in the table can be obtained on an industrial scale. Conversion can be complete in all cases. Isolated yields depend on the isolation method and the solubility of single compounds.

The application of the microorganism lacking carbamoylase activity can also been applied on an industrial scale, but it requires an additional chemical step for the removal of the carbamoyl function [13]. This method, using a D-hydantoinase from *B. brevis* followed by nitrous acid oxidation, is applied in the industrial preparation of D-*p*-hydroxy-phenyl glycine by Kaneka. The superiority of the microorganism containing D-hydantoinase + D-carbamoylase activity is shown in the application to the conversion L-ornithine ⇔ D-citrulline through the intermediate D,L-hydantoin depicted in Scheme 4 [12].

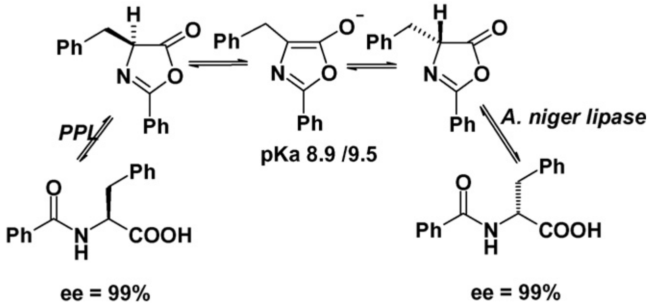
The enantioselectivity of the reaction has usually been attributed to the hydantoinase action. However in the case of fast transformed compounds, an accumulation of the carbamoyl derivative of L-configuration was observed. These suggest a non-complete enantioselectivity of the first enzyme. The carbamoyl derivative is still in equilibrium with the hydantoin and this can be responsible of the final complete conversion in one single enantiomer with high-enantiomeric excess. The D-hydantoinase–D-carbamoylase method is the most general and reliable method for the preparation of α-amino acids of D-configuration.



Scheme 4. The hydantoinase–carbamoylase system applied to the synthesis of D-citrulline.

2.1.1.2. The hydantoinase–carbamoylase system for L-amino acid synthesis. Despite a number of reports of strains with L-selective hydantoin-hydrolyzing enzymes [16] the commercial application of the hydantoinase process is still limited to the production of D-amino acids. Processes for the production of L-amino acids are limited by low space-time-yields and high-biocatalyst costs. Recently, a new generation of a L-hydantoinase process was developed based on a tailor-made recombinant whole-cell biocatalyst. Further reduction of biocatalyst cost by use of recombinant *Escherichia coli* cells overexpressing a hydantoinase, carbamoylase and hydantoin racemase from *Arthrobacter* sp. DSM 9771 were achieved. To improve the hydantoin-converting pathway, expression levels of the different genes were balanced on the basis of differences in the specific activities of the enzymes. This has been accomplished by using different gene dose coding for the respective enzymes. This highly active recombinant whole-cell biocatalyst could be produced in high-cell density fermentation at m^3 scale at concentrations above 50 g/L dry cell weight which further reduced the biocatalyst production cost compared to the original *Arthrobacter* strain. The system has been applied to the preparation of L-methionine. The L-hydantoinase has, in fact, a D-preference toward the racemic 5-thiomethyl hydantoin and the formation of L-methionine resides uniquely on the discrimination operated by the L-carbamoylase. The D-carbamoyl derivative equilibrates with the hydantoin thus restoring the possibility of having a cycle with 100% deracemization [17]. The space-time-yields are however limited [18]. Improvements of the deracemization process from rac-5-substituted hydantoins to L-amino acids still requires a more selective L-hydantoinase.

2.1.1.3. The enzymatic kinetic resolution of phenyl-oxazolinones coupled with in situ base catalyzed racemization (hydrolysis). Phenyl-oxazolinones are a protected form of amino acids which can be obtained by cyclization of *N*-benzoyl-amino acids. Their structure is similar to hydantoins with the relevant difference that the carboxylate is in a *lactone type* (–CO–O–) form while in the hydantoins it is in a *lactame type* form (–CO–N–). Lipases are known to be not well suited for amide bond transformation. Therefore oxazolinones were designed as alternative structures for a DKR similar to the one described for hydantoins involving lipases as resolving agents. Rapid enolization in slightly basic conditions should ensure the possibility for a DKR. The oxazolinone derived from phenylalanine proved to be an excellent substrate for porcine pancreatic lipase (PPL) and lipase from *Aspergillus niger*, although the transformation required rather long-reaction times (20 h) (Scheme 5). However this specific method is quite



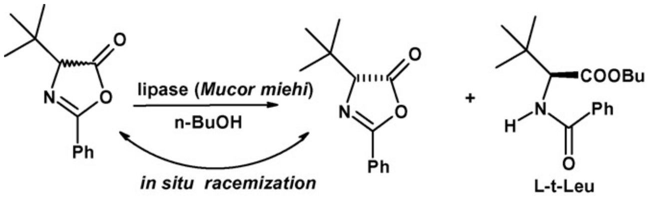
Scheme 5. 4-Substituted-2-phenyloxazolin-5-one as substrates for the DKR with *in situ* racemization catalyzed by lipases or esterases, with opposite selectivity.

interesting in that the two enzymes show, with this substrate, opposite enantiopreference. This possibility widens the scope of this transformation and it is an highly desirable feature for a DKR. Actually hydrolytic enzymes in the vast majority are specific for L-amino acid derivatives (with the notable exception of D-hydantoinases). While kinetic resolution has the limit of a maximum of 50% yield, in principle both enantiomers are available from the same biotransformation and only one enzyme can be used for obtaining both enantiomers [19]. In the case of a DKR the acquisition of both enantiomers requires the availability of two enzymes with opposite stereopreference. Lipases and esterases sometimes meet these conditions and this is one such case.

Altogether the hydrolysis has interesting characteristics only for the preparation of phenylalanine [20]. Other oxazolinones are transformed with much lower enantiomeric excess and longer reaction times.

2.1.1.3.1. The enzymatic kinetic resolution of phenyl-oxazolinones coupled with in situ base catalyzed racemization (transesterification). An alternative approach to the DKR of phenyl-oxazolinones is represented by the alcoholysis in organic solvents. The lipase from *Pseudomonas cepacia* was used for this purpose. In organic solvents with low-water activity, the non-enzymatic hydrolysis proceeds very slowly and the rate of enolization of the C-4 proton is sufficiently rapid so that 100% of the substrate is convertible into product. When water was used as the nucleophile in non-aqueous medium, the rate of ring fission was too slow to be useful. However, the chemoselectivity and reaction rate of the lipase catalyzed reaction in non-aqueous media can often be altered by the use of different nucleophiles. When methanol was used as the nucleophile, methanolysis of oxazolones proceeded at a useful rate to furnish methyl esters of *N*-benzoyl-L-α-amino acids. The optical purity of the products ranged from 66% to 98% ee [21].

While the substrate specificity of the *Pseudomonas* lipases is broad, the enantioselectivity, in most cases, needs to be improved. This may be achieved by the use of a second enzyme to affect a kinetic resolution of the enriched product. The Authors found two proteases capable of catalyzing the enantioselective hydrolysis of the methyl ester with *E* values of >100, leading to enantiopure L-*N*-benzoyl-α-amino acids. In turn, the *N*-benzoyl function was chemically cleaved without racemization under acidic conditions (6N HCl).



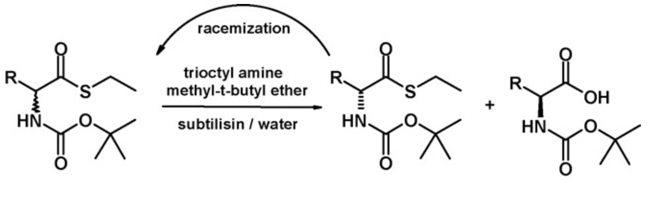
Scheme 6. 4-*t*-Butyl-2-phenyloxazolin-5-one as substrates for the DKR with *in situ* racemization catalyzed by lipase from *Mucor miehei*.

This type of synergistic coupling of an enantioselective reaction with a subsequent kinetic resolution leads to a marked enhancement in the enantiomeric purity. This concept has been employed in enzymatic and non-enzymatic asymmetric synthesis, although the successive enzymatic steps lowers the preparative significance of the procedure.

A method based on the DKR of 4-substituted-2-phenyloxazolin-5-one has been applied to the large-scale synthesis of *N*-benzoyl-L-*t*-leucine-butyl ester, an important non-natural amino acid intermediate of several peptide mimetics. The procedure is based on the alcoholysis of 4-*t*-butyl-2-phenyloxazolin-5-one with *n*-butanol catalyzed by the lipase from *Mucor mihei* (Scheme 6) [22]. The enantiomer which is not transformed is racemized *in situ* by a base catalyzed reaction. The reaction can be performed at a substrate concentration of 200 g/L in 24 h. Yields are 90%, and the ee greater than 95%. The method is considered as an industrial alternative to the established synthesis of L-*t*-leucine [23]. Results on the enzyme catalyzed deracemization of 4-substituted-2-phenyloxazolin-5-one are summarized in Table 2.

2.1.1.4. The enzymatic kinetic resolution of N-Boc-amino acid-thioesters coupled with base catalyzed racemization. Recently a new method leading to the preparation of a number of arylglycines of L-configuration has been published. The method is based on the hydrolysis of *N*-Boc-amino acid-thioesters catalyzed by an industrial preparation of the protease subtilisin (Scheme 7) [24].

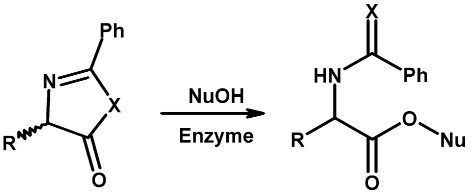
In thioesters, the acidity of the hydrogen in α-position is higher in comparison to the corresponding oxo-esters, amides or acids. The enzymatic transformation of a thioesters into the corresponding carboxylate with a higher pK_a of the α-proton, put the basis for a DKR, provided that the enzymatic systems withstand the basic conditions required for substrate racemization. This concept has been applied in the DKR of α-alkyl thioesters [25]. For application in amino acid chemistry racemic com-



R = Ph, 4-Cl-Ph, 4-F-Ph, 2-Cl-Ph, 2-F-Ph, 2-thienyl

Scheme 7. The enzymatic kinetic resolution of *N*-Boc-amino acid-thioesters coupled with base catalyzed racemization.

Table 2
Deracemization of 4-substituted-2-phenyloxazolin-5-one



R	*X*	Enzyme	NuOH	Solvent	Yield (%)	ee (%)	References
Ph–CH$_2$–	O	Novozyme	MeOH	MeOH	81	95 (S)	[22b]
Ph–CH$_2$–	O	Novozyme	EtOH	MeOH	82	97 (S)	[22b]
Ph–CH$_2$–	O	Novozyme	MeOH	CH$_3$CN	88	98 (S)	[22b]
i-Bu	O	Novozyme	MeOH	CH$_3$CN	96	97 (S)	[22b]
t-Bu	O	Lipozyme	*n*-BuOH	Toluene	67	99.5 (S)	[22a,23]
Ph	O	Lipase P-30	MeOH	MTBE	46	75 (S)	[22b]
Ph–CH$_2$–	O	Lipase P-30	MeOH	MTBE	80	78 (S)	[22b]
Ph–CH$_2$–	O	Lipase PL	H$_2$O	Buffer pH 7.6	>95	> 99 (S)	[20]
Ph–CH$_2$–	O	Lipase AP	H$_2$O	Buffer pH 7.6	>95	>99 (R)	[20]
CH$_3$SCH$_2$CH$_2$	S	Chymotrypsin	H$_2$O	Buffer pH 6.8	97	94	[22b]
n-Bu	S	Prozyme 6	H$_2$O	Buffer pH 6.8	86	99 (S)	[22b]

pounds were designed for this novel application. Racemization conditions were studied by observing the exchange rate of the α-proton of the thioester with deuterium from CD$_3$OD by ^{1}H NMR. All compounds tested showed fast exchange rates in conditions were the oxoesters and the acids were not racemized. Moreover subtilisin Carlsberg showed a good activity on these *N*-Boc amino acid derivatives with reaction rates comparable although inferior to the one observed with the oxo-esters. Fig. 1 shows the time course of the enzymatic hydrolysis of *N*-Boc-phenylglycine-ethylthio ester. The reaction was performed in a water-MTBE two-phase system in the presence of subtilisin. The reaction reached 50% conversion after 24 h. At this point the mixture was depleted of the L-enantiomer, substrate of the enzyme. When trioctyl-amine was added as a base to the reaction mixture, the racemizing conditions generated the L-enantiomer and the enzymatic hydrolysis started again reaching after 24 h complete conversion. The L-*N*-Boc-phenyl-glycine was isolated in 95% yield and >99% ee. The reaction diagram in Fig. 1, shows well the significance of a kinetic resolution with *in situ* racemization. The structural requirements are an aryl or vinyl group on the α-carbon, a variety of alkyl-groups on the sulphur atom, a carbonyl group on the nitrogen. Relative reaction rates, ease of racemization and ee of the L-amino acids isolated are reported in Table 3. Also in this case the application to the preparation of the D-enantiomers requires the catalysis of an enzyme with opposite selectivity.

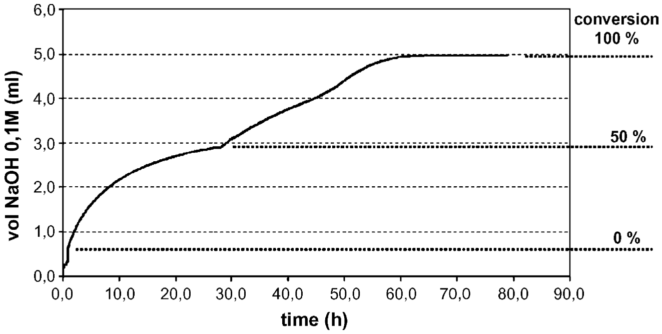
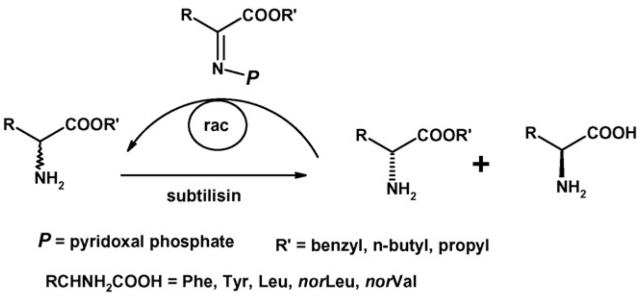


Fig. 1. Time course of the DKR of *N*-Boc-phenylglycine-ethylthio ester before and after TOA addition.

2.1.1.5. The enzymatic kinetic resolution of amino acid esters coupled with aldehyde mediated in situ racemization. All known amino acid racemases and epimerases ultimately employ a mechanism that involves the deprotonation of the amino acid at the α-carbon, followed by the reprotonation of the resulting carbanionic intermediate in the opposite stereochemical sense [26]. These enzymes face the difficult task of removing the α-protons of amino acids that are estimated to have pK_as as high as 21 (for the fully protonated form of the amino acid) [27]. Some racemizing enzymes like the well-studied alanine racemase overcomes this hurdle through the use of a pyridoxal phosphate cofactor [28]. The amino group of the substrate is first attached to the cofactor via an imine linkage. The cofactor serves as an electron sink that acidifies the proton on the α-carbon and allows the inversion to proceed via a deprotonation/reprotonation mechanism. Thus the imine formation with aldehydes has been used for the racemization of α-amino acids. If the racemization is combined with the preferential crystallization of one enantiomer, the process can become a DKR. The methods have been described

Table 3
Subtilisin catalyzed DKR of compounds *N*-Boc-amino acid-thioesters in the presence of trioctylamine for the deracemization to the corresponding L-amino acids

Substrate	Conversion (%)	Base/substrate	Time (h)	ee AA (%)
R = Ph	>95	0.5	48	>99
R = 4-Cl–Ph	>95	0.5	36	>99
R = 4-F–Ph	>95	0.5	20	>99
R = 2-Cl–Ph	>95	1	96	>99
R = 2-F–Ph	>95	1	12	>99
R = 2-Thienyl	>95	0.5	5	>99

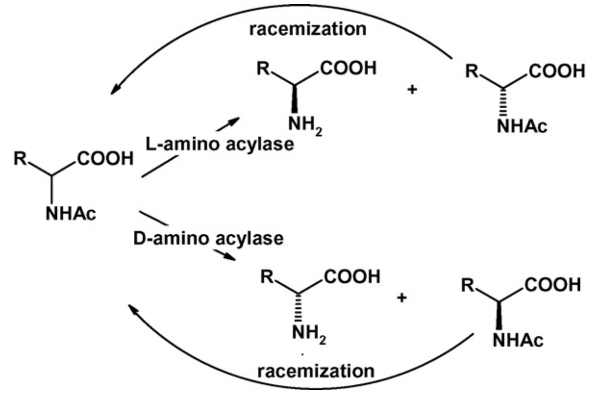


Scheme 8. Kinetic resolution of amino acid esters coupled with pyridoxal-phosphate mediated *in situ* racemization.

and applied on a all set of amino acids [29] or amino acid esters and amides [30] for industrial scopes. High temperatures were required for the aldehyde promoted racemization of the amino acid derivative. Recently the use of aldehydes-induced racemization combined with an enzyme catalyzed preferential hydrolysis of one enantiomers has been applied to the DKR of a series of amino acid esters using pyridoxal phosphate as aldehydes. The esters in a 19:1 mixture of 2-methyl-2-propanol and water were treated with an industrial preparation of subtilisin in the presence of 20 mol% of pyridoxal. During the reaction course the hydrolyzed amino acid is insoluble and precipitates in the reaction medium. The remaining ester is racemized by imine formation with pyridoxal phosphate. Rate of racemization of the amino acid is only marginal due to the higher pK_a of the α-proton and the low solubility. Yields exceeding 90% of the L-enantiomer are obtained with an ee ranging from 90% to 98%. With this method Phe, Tyr, Leu, NorVal and NorLeu of L-configuration have been obtained. The drawback of the method is the requirement of sensible amounts of the expensive racemization catalyst (Scheme 8) [31].

A similar concept was applied to the DKR of phenylglycines esters catalyzed by lipases in an amonolysis reaction [32]. D,L-Phenylglycine methyl esters were treated with ammonia in *t*-butyl alcohol/MTBE using lipase from *Candida antartica* B as a catalyst. At room temperature the difference in the racemization rate between the ester and the formed amide is not sufficient to allow the acquisition of compounds of high ee in yields above 50%. However running the reaction at −20 °C D-phenylglycine amide could be obtained in 85% yields and 88% ee. Both pyridoxal and salycilaldehyde were used as catalysts.

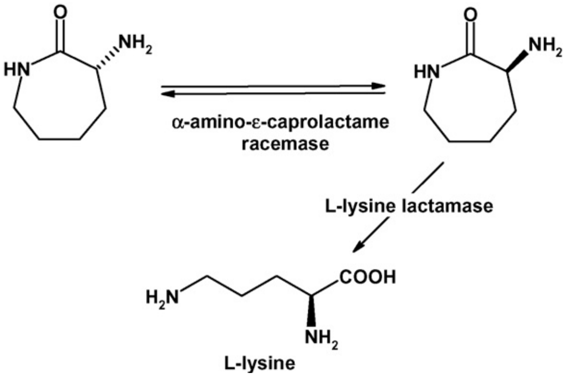
2.1.1.6. The enzymatic kinetic resolution of N-acyl amino acids coupled with N-acylase racemase racemization. Acylases are enzymes hydrolysing the *N*-acetyl derivatives of amino acids. They require free carboxylate for activity and have long been used for the kinetic resolution of amino acids. The unreacted enantiomer is usually racemized in a separate step by treatment with acetic anhydride. While acylase enzymes from hog kidney have an L-specificity, bacterial acylases with L- and D-specificity of bacterial origin have been isolated and used for the kinetic resolution of *N*-acetyl amino acids. An industrial process for the production of L-methionine and other proteinogenic and non-proteinogenic L-amino acids such as L-valine, L-phenylalanine, L-norvaline or L-aminobutyric acid has been established. Currently, several hundred tonnes per year of L-methionine are produced by this enzymatic conversion using an enzyme membrane reactor [33].



Scheme 9. The *N*-acylase catalyzed kinetic resolution of *N*-acyl amino acids coupled with racemase-induced racemization.

The starting material for the acylase process is a racemic mixture of *N*-acetyl-amino acids which are chemically synthesized by acetylation of D,L-amino acids with acetyl chloride or acetic anhydride in alkali in a Schotten–Baumann reaction [34]. The kinetic resolution of *N*-acetyl-D,L-amino acids is achieved by a specific L-acylase from *Aspergillus oryzae* which hydrolyzes only the L-enantiomer and produces a mixture of the corresponding L-amino acid, acetate, and *N*-acetyl-D-amino acid. After separation of the L-amino acid by a crystallization step, the remaining *N*-acetyl-D-amino acid is recycled by thermal racemization under drastic conditions (Scheme 9) [17]. In a similar process racemic amino acid amides are resolved with an L-specific amidase and the remained enantiomer racemized separately. Although in these multi-step processes final yields of the L-form can go beyond 50% of the starting material, the efficiency of the whole transformation is much lower than a DKR process with *in situ* racemization. On the other end the structural requirements for the free carboxylate do not allow the identification of derivatives racemizable *in situ*. Therefore the racemization requires the application of specific enzymes. Enzymes with such an activity have been found in various *Actinomycetes* strains [35]. From the wild-type strains, genetically modified organisms have been developed able to efficiently express an *N*-acylamino acid racemase, a suitable catalyst for the racemization of various industrially important aromatic as well as aliphatic *N*-acylamino acids [36]. D-Amino acids are also accessible by use of D-selective acylases. Selective *in situ* racemization of *N*-acetyl-amino acid could dramatically improve the acylase process by eliminating costly racemization and separation steps.

A whole-cell system has been developed expressing the genes of *N*-acylamino acid racemase and L-aminoacylase from *Deinococcus radiodurans* in *E. coli*. This system was used for the deracemization of D,L-*N*-Ac-homophenylalanine. The L-amino acid was obtained with quantitative yield when the expression of the two enzymes was regulated properly. The cells could be used several times in the biotransformation [34].



Scheme 10. Combination of a specific racemase with an hydrolytic enzyme.

The L-hydantoinase process or the combination of an L-acylase with and *N*-acylamino acid racemase are complementary methods and their application is strongly dependent on the specific case and will be influenced by the biocatalyst properties such as substrate specificity as well as the access to the cheapest substrate and the easiest product isolation. These two "100% optical and chemical yield approaches" provide a high degree of flexibility for the production of a large number of different L- or D-amino acids, which is especially important for fast changing targets and short development periods typical for the fine chemicals industry serving the needs of pharmaceutical industry.

2.1.1.7. Combination of specific racemases with an hydrolytic enzyme. The identification of specific racemases allows one to devise deracemization methods based on a racemase catalyzed step combined with other enzyme catalyzed reactions. Thus the racemic amino-ε-caprolactame obtained in a racemic form from cyclohexene through conventional synthetic steps, is selectively transformed into L-lysine. The D-enantiomer is continuously racemized in the same reactor with a specific racemase. The production is scaled to 4000 t/year by Toray Industries in Japan (Scheme 10) [46,47].

2.2. Deracemization via stereo-inversion (Der-S-I)

Deracemization by stereo-inversion is a multi-step chemo-enzymatic transformation which starts with a racemic amino acid derivative. While one of the enantiomers (R_f) is not affected by the enzymatic transformation, the other enantiomer (S_f) is transformed into a compound (A_i) which can, in turn, be transformed into the starting compound of opposite configuration (R_f) or into a racemate. In the first case the convergent process gives only the enantiomer (R_f), in the second one, in successive cycles the enantiomeric excess increases at each cycle, eventually reaching complete conversion into one single enantiomer. At the end of the process the enantiomer (S_f) is completely transformed into (R_f) [8b] (Scheme 2).

Deracemization by stereo-inversion in the amino acid field relies on the combination of

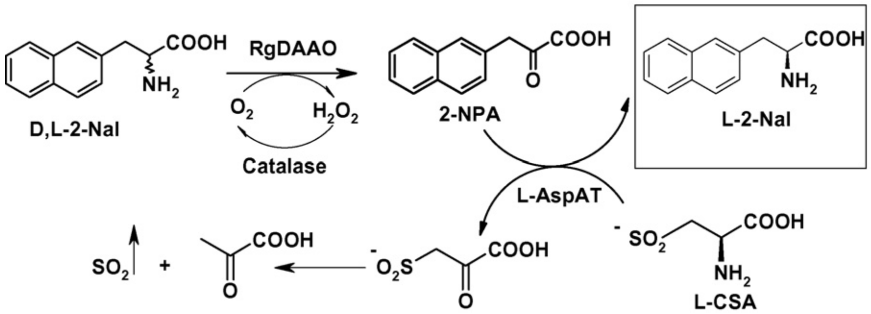
- amino acid oxidase and amino transferases (Section 2.2.1);
- amino acid oxidase, amino transferase and amino acid racemase (Section 2.2.2);
- *in vitro* and *in vivo* multiple-enzyme systems (Section 2.2.3);
- amino acid oxidase and chemical reduction (Section 2.2.4).

2.2.1. D-Amino acid oxidase (D-AAO) and L-amino transferase (L-AT)

D-Amino acid oxidase (D-AAO) is a highly stereoselective flavoenzyme which catalyzes the dehydrogenation of the D-isomer of amino acids to give the corresponding α-imino acids and, after subsequent hydrolysis, α-keto acids and ammonia.

D-AAO from the yeast *Rhodotorula gracilis* (RgD-AAO) exhibits a very high-turnover number, tight binding with the coenzyme FAD, a broad substrate specificity and has an active site large enough to accommodate even substrates of considerable size. It has been developed at an industrial level for the two-step conversion of cephalosporin C into 7-aminocephalosporanic acid, to detect and quantify D-amino acids, to produce α-keto acids from essential D-amino acids, and to resolve racemic mixtures of D,L-amino acids [37]. The enzymatic production of α-imino acids or α-keto acids from the corresponding amino acids is a convenient way of producing those compounds. The α-keto acids can be transformed into amino acids in a reaction catalyzed by an amino transferase (AT), in the presence of a stoichiometric amount of a second amino acid acting as an amino donor [38]. The α-imino acids instead can give an amino acid by means of an amino acid dehydrogenase (AADH). Both reactions can be considered overall as equivalent to reductive aminations.

A combination of D-AAO and L-AT is an example of a deracemization by SI. The product is an L-amino acid. The reaction catalyzed by AT has an equilibrium constant close to one, a very impractical situation leading to incomplete transformations and to the production of almost inseparable mixtures of amino acids; at least two, the amino acid product and the amino acid used as an amino donor). For preparative purposes it is therefore mandatory to shift the equilibrium to the right. This can be done by insolubilization or decomposition of one of the products. In one of the first examples in the literature reported on the deracemization of phenylalanine in a whole-cell system, Chibata showed that incubation of the racemic amino acid with a cell suspension of *Pseudomonas fluorescens* was able to deracemize the racemate yielding L-phenylalanine after 24 h of incubation [39]. Successive studies showed that the reaction proceeded via a D-selective oxidation to phenylpyruvate followed by L-selective transamination. The reaction was of general application with several microbial systems [40]. The reaction *in vitro* requires multiple enzyme catalysis and a system to shift the equilibrium [41]. A recent example of a deracemization procedure based on this coupled enzymatic system is the preparation of L-2-naphthylalanine (2-*N*-Ala) described in Scheme 11. The reaction occurs in one pot with initial oxidation of the D-amino acid catalyzed by D-AAO from *R. gracilis*. The hydrogen peroxide which is formed in stoichiometric amount is decomposed with catalase. The α-keto acid is the substrate for L-aspartate amino transferase (L-AspAT) which is able to use L-cysteine sulphinic acid (L-CSA) as an amino donor.



Scheme 11. Deracemization by stereo-inversion with the consecutive action of three different enzymes.

The reaction is driven towards the products by the decomposition of the sulphinyl-pyruvate to pyruvate and sulphur dioxide. The deracemization is complete yielding L-2-*N*-Ala in 95% yield and ee of 99.5%. The preparative significance of the transformation is lessened by the low space-time-yield due to the extremely low-water solubility of the naphthyl amino acid. However solid-to-solid bioconversion is in principle possible [42]. In a related approach, D,L-methionine can be efficiently deracemized to obtain the L-enantiomer using a multienzyme system consisting of a D-amino acid oxidase, a catalase, leucine dehydrogenase and formate dehydrogenase. The α-keto acid produced from the oxidation of the D-form is transformed into L-methionine in the presence of ammonia, leucine-DH and stoichiometric amount of NADH. The NAD^+ thus formed is recycled to NADH with ammonium formate and formate-DH [43].

2.2.2. L-*Amino acid oxydase (*L-*AAO) and* D-*amino transferase (*D-*AT) and generation of the* D-*amino donor with an amino acid racemase (AAR)*

The preparation of D-amino acids with the above two-enzyme system requires enzymes with opposite stereochemical selectivity and a suitable amino acid as a donor. While D-AAO is an enzymes whose function in nature is the elimination of amino acid with unnatural configuration (D-amino acids are components of some bacterial cell walls and occurs in peptides with special function like the ionofore valinomycin and the cyclic immuno suppressant peptide cyclosporine) arizing from racemization of L-amino acids for natural reasons (time, temperature), L-AAO are usually found in aggressive animals (snakes). Bacterial L-AAO often show too low a specific activity for preparative purpose [44]. Moreover D-amino transferases are less common than the L-ones and require D-amino acids as amino donors.

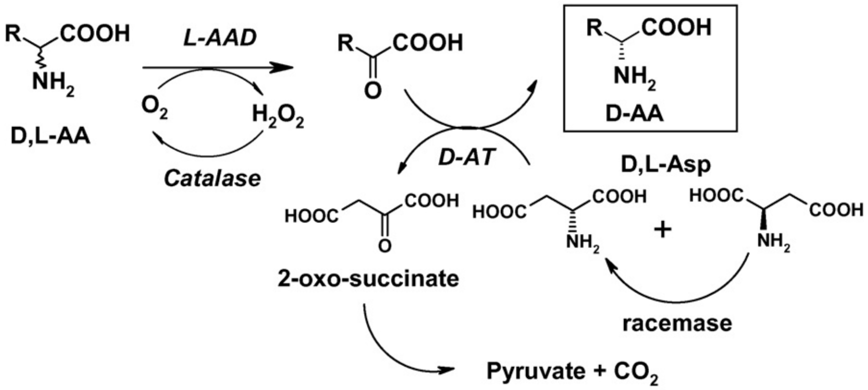
Several inventive procedures have been able to overcome these issues offering interesting multi-step enzyme catalyzed reactions for the deracemization of amino acids. The problem of the requirement of an amino donor of D-configuration can be solved by generating it *in situ* from the L-form with an amino acid racemase (AAR).

Scheme 12 describes the general procedure applied by Monsanto [45]. An L-amino acid deaminase, a flavoprotein from *Proteus myxofaciens* oxidizes the L-form of the racemic amino acid to the α-keto acid, leaving the D-form untouched. The keto-acid is the substrate of a D-AT which catalyzes the transamination to the D-AA, thus completing the deracemization. Since the D-AT requires a D-amino acid as an amino donor, D-aspartate is generated from the L-form with an aspartate racemase. The succinate formed spontaneously decarboxylates to pyruvate and carbon dioxide, thus shifting the equilibrium to the right. The method is reported for the industrial preparation of several D-AA (D-Ala, D-Phe, D-*t*-Leu, D-Tyr, D-Glu) [23].

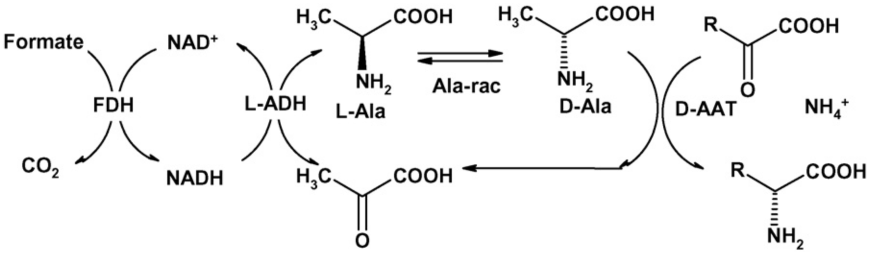
2.2.3. *In vitro and in vivo multiple-enzyme systems*

Enzymes requiring amino donors of D-configuration make use of specific racemases. Alanine racemase is a bacterial enzyme that catalyzes the racemization of L- and D-alanine.

Requires pyridoxal 5′-phosphate (PLP) as a cofactor. The enzyme plays an important role in the bacterial growth by providing D-alanine, a central molecule in the peptidoglycan



Scheme 12. Deracemization by stereo-inversion finalized to the preparation of D-amino acids.



Scheme 13. Deracemization by stereo-inversion in a combination of four enzymes.

assembly and cross-linking and has been purified from various sources.

The enzyme has been used for the production of various D-amino acids by a combination of L-alanine dehydrogenase, D-amino acid aminotransferase, and formate dehydrogenase. This method, although conceptually similar to the previously described deracemization system is in fact not a deracemization procedure because the substrate is already the α-keto acid itself. The generation of the α-keto acid from the racemic AA would require an amino acid oxidase of L-specificity. The reaction scheme for the preparation of a D-amino acid starts with the α-keto acid, readily transformed into the D-amino acid with a D-AT using D-Ala as an amino donor. Although pyruvate produced in the transamination reaction might not strongly compete with the α-keto acid substrate, the transformation into alanine with an alanine dehydrogenase of L-specificity generates L-alanine whose racemization affords the D-amino donor for the first reaction. Since the dehydrogenase is NADH dependent, the cofactor is recycled with the formate dehydrogenase/ammonium formate system (Scheme 13) [46,47].

In a related approach, D,L-methionine was efficiently deracemized to obtain the L-enantiomer using a multienzyme system consisting of a D-amino acid oxidase, a catalase, leucine dehydrogenase and formate dehydrogenase [42].

An alternative to a multienzymatic system is the construction of engineered microbial pathways to carry out the reactions in whole-cell system. The advantage of this approach is that all the necessary biocatalysts can be prepared by fermentation of a single recombinant strain without the need for enzyme isolation or purification. For instance a bacterial whole-cell catalytic system has been constructed in *E. coli* to convert L-amino acids into D-amino acid. An L-amino acid is first converted to its respective α-keto acid by the action of L-amino acid deaminase (L-AAD) and the keto acid is subsequently converted into the D-isomer by the action of D-amino acid transaminase (DAT) cloned from *Bacillus sphaericus* along with a cloned alanine racemase (AR) from *Salmonella typhimurium* used to recycle the amino donor, D-alanine [48].

2.2.4. *Amino acid oxidase and chemical in situ reduction of the initially formed imino compound*

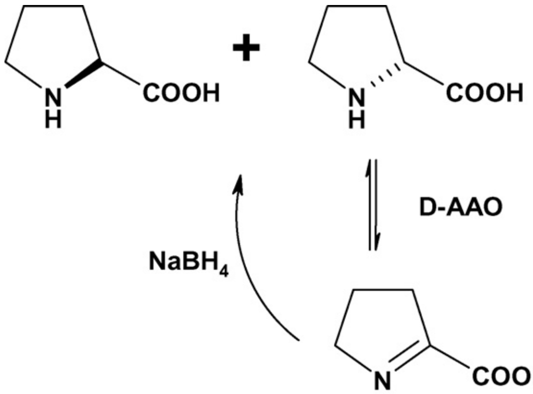
A simple and interesting procedure for the deracemization of α-AA has been introduced by Soda and co-workers [46,47] who combined the oxidation of the D-enantiomer of D,L-proline to dehydro-proline with a chemical reduction of the imine in one-pot, restoring the racemic mixture. If the reaction in the first step is completely enantioselective, the ee of the amino acid after 1 cycle is 50%. Repeating the reaction in successive cycles raises the ee near to 100%. (Scheme 14) [46].

In a similar way, D,L-pipecolic acid was converted to L-pipecolic acid [49].

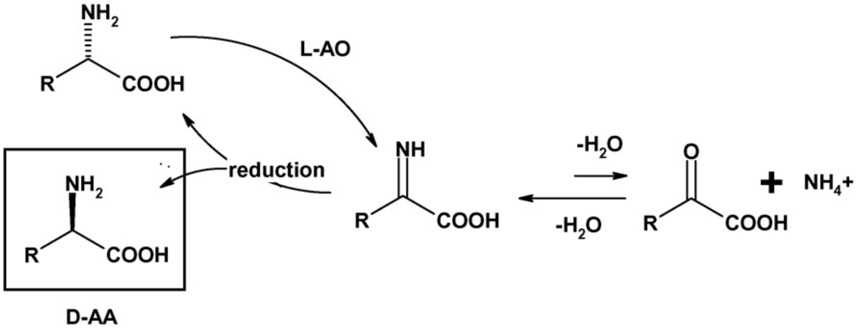
However, the use of sodium borohydride, which slowly decomposes in aqueous solution causes a significant rise in the pH value, which has to be controlled and interferes with the necessary repeated cycles. Turner and Fotheringham expanded the applicability of the method, using milder and water stable reducing agents [50] and applying amino acid oxidases with both D- and L-specificity.

Thus, using L-amino acid oxidase from *P. myxofaciens* and various amine–borane complexes, or D-amino acid oxidase from porcine kidney and sodium cyanoborohydride, the preparation of several natural and non-natural enantiopure D- and L-amino acids was achieved, respectively [51]. In a more recent report, several β- and γ-substituted-α-amino acids were deracemized using L-amino acid oxidase and sodium cyanoborohydride or sodium borohydride (Scheme 15) [52].

Aiming at the development of more efficient whole-cell biocatalysts, deracemization of 4-chlorophenylalanine was reported using *E. coli* cells expressing an L-amino acid aminotransferase from *Sinorhizobium meliloti* ATCC 51124. The enantiopure L-α-amino acid was obtained in high-optical yield *via* the tandem action of D-amino acid dehydrogenase from the *E. coli* host cell (induced by L-alanine in the medium), and the cloned L-amino acid aminotransferase from *S. meliloti* [6].



Scheme 14. Chemo-enzymatic deracemization with D-AAO and chemical reduction.



Scheme 15. Chemo-enzymatic deracemization with L-AAO and chemical reduction.

3. Conclusions

Deracemization methods are superior procedures aimed at the acquisition of a chiral compound in one enantiomeric form. They respect the principle of carbon economy, retaining most atoms of the starting material in the final product. They are multi-step methodology comprising at least one enantioselective step catalyzed by an asymmetric catalyst. Consecutive reaction steps can be carried out in the same pot, realizing a great simplification compared to procedures were the operation (resolution, separation, isolation, racemization) must occur in different reaction events. The preparation of both enantiomers in a deracemization process catalyzed by enzymes requires the availability of enzymes with opposite stereochemical preferences. The availability of these catalysts either as isolated proteins or in whole-cell systems is the crucial aspect of this technology. *In situ* racemization requires the design of substrates with suitable structural requirements or the availability of specific racemizing enzymes.

Among the methods reported, the microbial hydantoinase deracemization is the method of choice for the preparation of a variety of natural and non-natural amino acids of D-configuration. A corresponding system for the preparation of compounds of opposite configuration on a large scale is not available at present. The hydrolysis/alcoholysis of oxazolidinones derivatives can be applied on a larger scale but is limited to specific amino acids. The protease/base catalyzed deracemization of thioesters is promising and it is already run at rather larger scale. A drawback might be the reluctance to work with sulphur containing compounds on an industrial scale. Among the deracemization by stereo-inversion the multi enzymatic method DAO-AT has the limitation of the low-water solubility of some aromatic non-natural substrates. The requirement of recycling the cofactors when reducing enzymes are involved, is no longer considered an obstacle for the industrial scale up of such reactions. The methods based on selective enzymatic oxidation and non-selective chemical reduction reported in Schemes 14 and 15 require the development of substrate specific enzymes and the attainment of high-enantiomeric excess needs several successive oxidation–reduction cycles with the drawback of a discontinuous process.

In this review we have considered deracemization of α-amino acid with at least one enzyme catalyzed reaction. Continuous progress in protein and metabolic engineering will guarantee the applicability of deracemization methods to an increasing number of compounds from different chemical families.

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